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(54) Title: CRF CONJUGATES WITH EXTENDED HALF-LIVES

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Human/Rat CRF   SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII
Ovine CRF       SQEPPISLDLTFHLLREVLEMTKADQLAQQAHSNRKLLDIA
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FIG. 1

(57) Abstract: The present invention relates to conjugates of CRF that have been modified to include a moiety that protects CRF from degradation and prolongs the half-life of CRF. The CRF conjugates of the invention have an increased half-life which results in a dose-sparing effect and less frequent administration.

CRF CONJUGATES WITH EXTENDED HALF-LIVES

[0001] This application claims priority to U.S. Provisional Patent Application No. 61/116,260, filed November 19, 2008, incorporated herein by reference in its entirety.

1. FIELD OF INVENTION

[0002] The invention relates to conjugates of corticotropin-releasing factor (CRF) having an increased half-life and stability as compared to unmodified CRF.

2. BACKGROUND OF THE INVENTION

[0003] Corticotropin-Releasing Factor (CRF) is an endogenous 41 amino acid peptide first identified in 1981 as the major hypothalamic hormone responsible for stimulation of the pituitary-adrenal axis (Vale, W., et al., *Science* 213:1394-1397 (1981)). CRF can be obtained from natural sources, expressed recombinantly, or produced synthetically.

[0004] CRF has been shown to have a peripheral, non-endocrine function mediated biological activity as a potent inhibitor of edema and inflammation (Wei, E. T. et al., *Ciba Foundation Symposium* 172:258-276 (1993)). This has been confirmed in a series of experiments in which systemic administration of CRF has been shown to inhibit vascular leakage of plasma constituents and associated tissue swelling in response to injury or inflammatory mediators (Wei, E. T. et al., *European J. of Pharm.* 140:63-67 (1987), Serda, S. M. et al., *Pharm. Res.* 26:85-91 (1992) and Wei, E. T. et al., *Regulatory Peptides* 33:93-104 (1991)). CRF is also known in the art as corticotrop(h)in-releasing hormone (CRH), corticoliberin, corticorelin and CRF-41.

[0005] The CRF neuropeptide was first isolated from extracts of ovine hypothalami (OCRF; Vale, W., et al., *Science* 213:1394-1397 (1981)) and has subsequently been identified and isolated from the hypothalamus of numerous other mammals including rat (rCRF; Rivier, J., et al., *Proc. Natl. Acad. Sci. USA* 80:4851-4855 (1983)), porcine (PCRF; Schally, A., et al., *Proc. Natl. Acad. Sci. USA* 78:5197-5201 (1981) and human (hCRF; Shibahara, S., et al., *EMBO J.* 2:775-779 (1983)). Comparison of the amino acid sequences of CRF peptides from ovine, rat and human has shown that the rat and human peptides are identical, both differing at seven amino acid positions from the ovine peptide, the differences occurring largely in the C-terminal region of the peptides (Hermus, A., et al., *J. Clin. Endocrin. and Metabolism* 58:187-191 (1984) and Saphier, P., et al., *J. Endocrin.* 133:487-495 (1993)).

[0006] CRF has been shown to be a safe and useful pharmaceutical agent for a variety of different applications in humans. Specifically, *in vivo* administration of CRF has been extensively employed to help elucidate the cause of hyper- and hypo-cortisolemic conditions in humans and is an extremely useful diagnostic and investigative tool for various other disorders affecting the hypothalamic-pituitary-adrenal axis, including endogenous depression and Cushing's disease (Chrousos, G., et al., *N. Eng. J. Med.* 310:622 (1984) and Lytras, N., et al., *Clin. Endocrinol.* 20:71 (1984)). In fact, *in vivo* administration of CRF is useful to test corticotropic function of the anterior pituitary in all cases in which an impairment of the anterior pituitary function is suspected. This applies to patients with pituitary tumors or craniopharyngiomas, patients with suspected pituitary insufficiency, panhypopituitarism or empty sella syndrome, as well as patients with traumatic or post-operative injury to the pituitary region and patients who have undergone radiotherapy of the pituitary region. Thus, CRF clearly has utility for diagnostic analysis of the hypothalamus-pituitary-adrenal (HPA) axis.

[0007] For important peripheral applications, CRF also possesses *in vivo* anti-inflammatory activity. With regard to the anti-inflammatory activity of the CRF peptide, CRF prevents vascular leakage induced by a variety of inflammatory mediators that appear to act selectively on post-capillary venules in skin. CRF also inhibits injury- and inflammatory mediator-induced leakage from capillaries in muscle, cerebral micro-vessels, and lung alveolar capillaries. These observations suggest that CRF acts throughout the micro-circulation to preserve or restore endothelial cell integrity, thereby inhibiting fluid egress and white blood cell trafficking from the intravascular space and accumulation at sites of injury.

[0008] In light of the novel anti-inflammatory activity of the CRF peptide, numerous clinical indications are evident. For example, clinical indications for which the CRF peptide may find use include rheumatoid arthritis, edema secondary to brain tumors or irradiation for cancer, edema resulting from stroke, head trauma or spinal cord injury, post-surgical edema, asthma and other respiratory diseases and cystoid macular edema of the eye.

[0009] One of the challenges of many polypeptides used in disease treatment is that they have a relatively short half-life after administration. Proteins introduced into the blood are rapidly cleared from the mammalian subject by the kidneys. This is especially a problem in lower molecular weight polypeptides, such as CRF. Therefore, many polypeptide therapies require higher dosages or require shorter time periods between dosing to have their desired effect. Common approaches to extending the circulation half-life of therapeutic compounds is to encase them in liposomes, link proteins to human or bovine serum albumin, or

synthesize polymer conjugates of the active protein. Citation of any reference in Section 2 of this application is not an admission that the reference is prior art to the application.

3. SUMMARY OF THE INVENTION

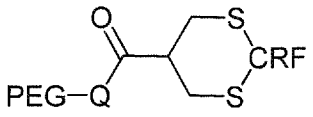
[0010] The present invention relates to conjugates of CRF that have been modified to include a moiety that protects CRF from degradation and prolongs the half-life of CRF. The CRF conjugates of the invention have an increased half-life which results in a dose-sparing effect and less frequent administration. An example of a CRF conjugate is CRF that has been modified to include moieties such as polyethylene glycol covalently bound to CRF.

[0011] In one embodiment, the invention provides for CRF conjugates comprising CRF, wherein the CRF is chemically modified with polyethylene glycol. In another embodiment, the CRF component of the CRF conjugate has the sequence identified as human CRF identified in Figure 1. Alternatively, the sequence of CRF may be modified or derivatized to include one or more changes in the amino acid sequence, including, but not limited to, insertions, deletions or substitutions. In yet another embodiment, the sequence of CRF has been modified to include one or more cysteine residues. The sequence of CRF may include cysteine as a substitution of one or more of the existing residues of CRF. Alternatively, the cysteine residue may be incorporated as an addition to the existing sequence of CRF. The cysteine residues may be inserted within the sequence of CRF, or the cysteine residue may be added to the amino or carboxy terminus of the sequence. In still another embodiment, cysteine residues are added to the amino and carboxy termini of the sequence. When two or more cysteine residues are present, one or more disulfide bonds may form between cysteine residues, thus forming a cyclic peptide.

[0012] In an embodiment of the invention wherein one or more cysteine residues have been incorporated into the sequence of CRF, the polyethylene glycol moiety may be covalently bound to CRF through one or more of the cysteine residues. Alternatively, the polyethylene glycol moiety may be covalently bound through one or more of the existing 41 amino acids of CRF, including, but not limited to, lysine, histidine, arginine, aspartic acid, glutamic acid, serine, as well as the N-terminus or C-terminus of the CRF polypeptide. In a particular embodiment of the invention, the CRF conjugate may have polyethylene glycol moieties attached via one or more lysine residues. The CRF conjugates of the invention include CRF which has been modified to include one or more polyethylene glycol polymers through a multitude of different sites in the CRF sequence. In one embodiment, the CRF conjugate comprises two PEG moieties bound to two cysteine residues. In one embodiment,

the CRF-PEG conjugate comprises one or more PEG groups simultaneously bound to two cysteine residues in a cysteine added variant of CRF. In another embodiment, the CRF-PEG conjugate comprises one or more PEG groups simultaneously bound to two cysteine residues that form a disulfide bond in a cysteine added variant of CRF. These conjugates may be produced via reductive cleavage of a disulfide bond, followed by a reaction in which the PEG moiety becomes bound to both thio groups. The resulting CRF conjugate contains a PEG moiety that bridges two sulfurs that had formed a disulfide bond. In a specific embodiment, the CRF conjugate contains a PEG bound to both the C-terminal and N-terminal cysteine residues of a cysteine added variant of CRF.

[0013] In a specific embodiment, a polyethylene glycol polymer is conjugated to a cysteine added variant of CRF according to general formula I:



I;

wherein both -S- are from cysteine residues in a cysteine added variant of CRF, which in certain embodiments form a disulfide bond, wherein Q represents a linking group, which is a direct bond, alkylene (preferably C_{1-10} alkylene), alkenylene, -O-alkylene-, -O-alkylene-O-, -O-alkylene-NR^1-, -NR^2-alkylene-, - NR^2-alkylene-O-, -NR^2-alkylene-NR^1-, -C(O)O-alkylene-, -C(O)O-alkylene-O-, -C(O)O-alkylene-NR^1-, -C(O)NR^2-alkylene-, -C(O)NR^2-alkylene-O-, -C(O)NR^2-alkylene-NR^1-, -C(NR^3R^4)NR^2-alkylene-, -C(NR^3R^4)NR^2-alkylene-O-, -C(NR^3R^4)NR^2-alkylene-NR^1-, -C(O)-O-, -C(O)-S-, -C(=NH)-NR^1-, -C(O)-N(R^1)-O-, -C(O)-N(R^1)-C(O)-, -C(O)-N(R^1)-, -C(O)-, -N(R^1)-, -O-C(O)-O-, -N=N-, -N=C(R1)-, -S-, -O-, an alpha amino acid derivative, a beta amino acid derivative, or an optionally-substituted aryl or heteroaryl group;

wherein the aryl group includes phenyl, benzoyl and naphthyl groups;

wherein the heteroaryl group includes pyridine, pyrrole, furan, pyran, imidazole, pyrazole, oxazole, pyridazine, primidine and purine;

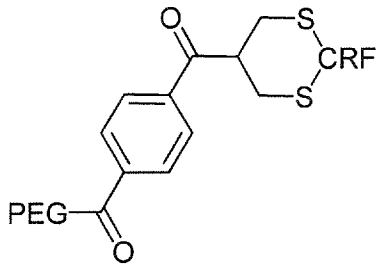
wherein each R^1, R^2, R^3, or R^4 is independently hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, aryl, aralkyl, heteroaryl, or heterocyclyl; and

wherein the linkage to the polymer may be by way of a hydrolytically labile bond, or by a non-labile bond.

[0014] Substituents which may be present on an optionally substituted aryl or heteroaryl group include, for example, one or more of the same or different substituents selected from -CN, -NO_2, -CO_2R, -COH, -CH_2OH, -COR, -OR, -OCOR, -OCO_2R, -SR, -SOR, -SO_2R,

-NHCOR, -NRCOR, -$NHCO_2R$, -$NR'CO_2R$, -NO, -NHOH, -NR'OH, -C=N-NHCOR, -C=N-NR'COR, -N^+R_3, -N^+H_3, -N^+HR_2, -N^+H_2R, halogen, for example fluorine or chlorine, -C≡CR, -$C=CR_2$ and ^{13}C=CHR, in which each R or R' independently represents hydrogen or an alkyl (preferably C_{1-6}) or an aryl (preferably phenyl) group. The presence of electron withdrawing substituents is especially preferred.

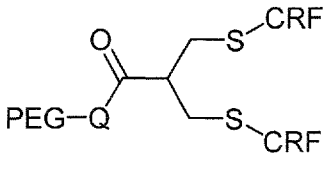
[0015] In one embodiment of formula I, PEG is conjugated to CRF according to formula II:



II.

[0016] Two cysteine added variants of CRF may be bound together via a disulfide bond, to form a CRF dimer. The CRF dimer may be conjugated to a polyethylene glycol containing moiety. In one embodiment, the CRF dimer conjugate is bound to PEG through the disulfide bond that binds the two CRF polypeptides together.

[0017] In a specific embodiment, a polyethylene glycol polymer is conjugated to two cysteine added variants of CRF according to general formula III:



III;

wherein both -S- are from cysteine residues in a cysteine added variant of CRF, which in certain embodiments form a disulfide bond, wherein Q represents a linking group, which is a direct bond, alkylene (preferably C_{1-10} alkylene), alkenylene, -O-alkylene-, -O-alkylene-O-, -O-alkylene-NR^1-, -NR^2-alkylene-, - NR^2-alkylene-O-, -NR^2-alkylene-NR^1-, -C(O)O-alkylene-, -C(O)O-alkylene-O-, -C(O)O-alkylene-NR^1-, -C(O)NR^2-alkylene-, -C(O)NR^2-alkylene-O-, -C(O)NR^2-alkylene-NR^1-, -C(NR^3R^4)NR^2-alkylene-, -C(NR^3R^4)NR^2-alkylene-O-, -C(NR^3R^4)NR^2-alkylene-NR^1-, -C(O)-O-, -C(O)-S-, -C(=NH)-NR^1-, -C(O)-N(R^1)-O-, -C(O)-N(R^1)-C(O)-, -C(O)-N(R^1)-, -C(O)-, -N(R^1)-, -O-C(O)-O-, -N=N-, -N=C(R1)-, -S-, -O-, an alpha amino acid derivative, a beta amino acid derivative, or an optionally-substituted aryl or heteroaryl group;

wherein the aryl group includes phenyl, benzoyl and naphthyl groups;

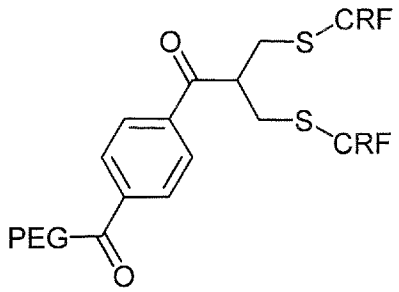
wherein the heteroaryl group includes pyridine, pyrrole, furan, pyran, imidazole, pyrazole, oxazole, pyridazine, primidine and purine;

wherein each R^1, R^2, R^3, or R^4 is independently hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, aryl, aralkyl, heteroaryl, or heterocyclyl; and

wherein the linkage to the polymer may be by way of a hydrolytically labile bond, or by a non-labile bond.

[0018] Substituents which may be present on an optionally substituted aryl or heteroaryl group include, for example, one or more of the same or different substituents selected from -CN, $-NO_2$, $-CO_2R$, -COH, $-CH_2OH$, -COR, -OR, -OCOR, $-OCO_2R$, -SR, -SOR, $-SO_2R$, -NHCOR, -NRCOR, $-NHCO_2R$, $-NR'CO_2R$, -NO, -NHOH, -NR'OH, -C=N-NHCOR, -C=N-NR'COR, $-N^+R_3$, $-N^+H_3$, $-N^+HR_2$, $-N^+H_2R$, halogen, for example fluorine or chlorine, -C≡CR, $-C=CR_2$ and $^{13}C=CHR$, in which each R or R' independently represents hydrogen or an alkyl (preferably C_{1-6}) or an aryl (preferably phenyl) group. The presence of electron withdrawing substituents is especially preferred.

[0019] In one embodiment of formula III, PEG is conjugated to CRF according to formula IV:

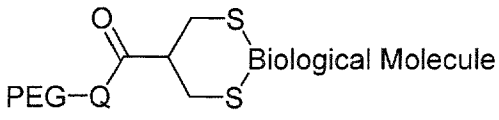


IV.

[0020] In one embodiment, a polyethylene glycol polymer is conjugated to a biological molecule to which one or more cysteine residues have been incorporated into the sequence of the biological molecule. In one embodiment, the biological molecule is a naturally occurring polypeptide or peptide which does not contain a cysteine residue, but in the context of the invention has been modified to incorporate a cysteine residue. Examples of such biological molecules include, but are not limited to, CRF, insulin, glucagon, and interferon. In one embodiment, the biological molecule is a synthetic polypeptide that does not contain a cysteine residue, but in the context of this invention has been modified to incorporate a cysteine residue. The sequence of the biological molecule may be modified or derivatized to include one or more cysteine residues, including, but not limited to, insertions, or substitutions. The sequence of the biological molecule may include cysteine as a substitution of one or more of the existing residues of the biological molecule. The cysteine residues may

be inserted within the sequence of the biological molecule, or the cysteine residue may be added to the amino and/or carboxy termini. In another embodiment, cysteine residues are added to both the amino and carboxy termini of the sequence of the biological molecule.

[0021] In still another embodiment, provided herein is a biological molecule conjugate having the structure of formula V;



V;

wherein both -S- are from cysteine residues in the biological molecule containing two or more cysteines, which in certain embodiments form a disulfide bond, wherein Q represents a linking group, which is a direct bond, alkylene (preferably C_{1-10} alkylene), alkenylene, -O-alkylene-, -O-alkylene-O-, -O-alkylene-NR^1-, -NR^2-alkylene-, - NR^2-alkylene-O-, -NR^2-alkylene-NR^1-, -C(O)O-alkylene-, -C(O)O-alkylene-O-, -C(O)O-alkylene-NR^1-, -C(O)NR^2-alkylene-, -C(O)NR^2-alkylene-O-, -C(O)NR^2-alkylene-NR^1-, -C(NR^3R^4)NR^2-alkylene-, -C(NR^3R^4)NR^2-alkylene-O-, -C(NR^3R^4)NR^2-alkylene-NR^1-, -C(O)-O-, -C(O)-S-, -C(=NH)-NR^1-, -C(O)-N(R^1)-O-, -C(O)-N(R^1)-C(O)-, -C(O)-N(R^1)-, -C(O)-, -N(R^1)-, -O-C(O)-O-, -N=N-, -N=C(R1)-, -S-, -O-, an alpha amino acid derivative, a beta amino acid derivative, or an optionally-substituted aryl or heteroaryl group;

wherein the aryl group includes phenyl, benzoyl and naphthyl groups;

wherein the heteroaryl group includes pyridine, pyrrole, furan, pyran, imidazole, pyrazole, oxazole, pyridazine, primidine and purine;

wherein each R^1, R^2, R^3, or R^4 is independently hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, aryl, aralkyl, heteroaryl, or heterocyclyl; and

wherein the linkage to the polymer may be by way of a hydrolytically labile bond, or by a non-labile bond.

[0022] The CRF conjugates of the invention have one or more of the biological activities of unmodified CRF. Such biological activities include, for example, the ability to stimulate the release of ACTH, the ability to treat tumor or to inhibit edema *in vivo*, and the ability to bind to CRF receptors. The biological activity of CRF conjugates may be determined using the assays described herein.

[0023] Compared to unmodified CRF (*i.e.*, CRF without a PEG attached), the conjugates of the present invention have an increased circulating half-life, plasma residence time and/or decreased clearance. In an embodiment of the invention, the CRF conjugates have increased clinical activity *in vivo* as compared to unmodified CRF. The conjugates of

the invention have improved potency, stability, area under the curve and circulating half-life. The CRF conjugates of the invention have an improved pharmacokinetic profile as compared to unmodified CRF. The CRF conjugates of the invention may show an improvement in one or more parameters of the pharmacokinetic profile, including AUC, C_{max}, clearance (CL), half-life, and bioavailability as compared to unmodified CRF.

[0024] In accordance with the present invention, the CRF conjugates are useful for the treatment of tumors in a patent. In accordance with the present invention, the CRF conjugates are useful for treatment or managing cancer in a patent. As used herein, the term "cancer" refers to a neoplasm or tumor resulting from abnormal uncontrolled growth of cells. The term "cancer" encompasses a disease involving both pre-malignant and malignant cancer cells. In accordance with the present invention, the CRF conjugates can be used to present tumor progression in a patient. As used herein, the term "tumor progression" means that the tumor growth is inhibited, stopped or reversed, that the size or Volume of the tumor remains the same or decreases, and/or no additional metastases of the tumor remains the same or decreases, and/or no additional metastases of the tumor are formed in other parts of the body.

[0025] In accordance with the present invention, the CRF conjugates are useful for treating brain edema in patients in need thereof. In accordance with the present invention, such brain edema may be the result of injury or disease to the brain. In particular, the present invention relates to methods of treating brain edema resulting from primary or metastasis brain tumors, comprising administering CRF conjugates to patients in need thereof. The CRF conjugates of the invention are useful in treating patients by reducing inflammation and edema in those patients, comprising administering a therapeutically effective amount of the CRF conjugates and formulations of the invention. The CRF conjugates of the invention are useful in providing vasoprotective effects which may be evidenced as a reduction in edema when administered to patients in need thereof. In particular, the methods of administering the CRF conjugates of the invention may be useful in reducing peritumoral brain edema.

[0026] The administration of CRF conjugates to a patient for the treatment of brain edema may be combined with other therapeutics for the treatment of edema. In particular, the CRF conjugates of the invention may be used in combination with steroidal therapeutics for the treatment of brain edema, including, but not limited to glucocorticoids. Glucocorticoid steroids include hydrocortisone, cortisone acetate, prednisone, prednisolone, methylprednisone, dexamethasone, betamethasone, triamcinolone, beclomethasone, fludrocortisone acetate, alderstone and deoxycorticosterone acetate. In accordance with the invention, when CRF conjugates are administered in combination with other therapeutics for

the treatment of brain edema, the other therapeutic may be administered concurrently, prior to or subsequently to the administration of the CRF conjugate.

[0027] In another aspect of the invention, the CRF conjugates may be administered to patients for the treatment of brain edema, wherein the conjugate is administered in a treatment regimen as a steroid sparing agent facilitating steroid taper. The invention also encompasses a method for managing brain edema in a patient in need thereof, comprising administering to the patient a therapeutically effective amount of a CRF conjugate and a steroid, wherein the method provides a steroid sparing effect. The present invention further provides a method for providing replacement therapy for steroid therapy in a subject receiving such therapy, which comprises administration of a steroid-sparing amount of a CRF conjugate. The invention also provides a method for treating brain edema, comprising a treatment regimen steroid in combination with a CRF conjugate, whereby total exposure to the steroid is reduced by the administration of the CRF conjugate.

[0028] The present invention relates to pharmaceutical compositions containing a CRF conjugate as the active ingredient. The CRF conjugate may be formulated with a pharmaceutically acceptable carrier. Due to the increased half-life of the CRF conjugate, the pharmaceutical compositions may contain a lower dose of CRF than typically administered to effectively treat edema. The pharmaceutical formulations of the invention may be formulated for parenteral administration, including, but not limited to, intradermal, subcutaneous, and intramuscular injections, and intravenous or intraosseous infusions. The pharmaceutical formulations of the present invention can take the form of solutions, suspensions, or emulsions that include a CRF conjugate, such as CRF chemically modified with polyethylene glycol, and a pharmaceutically acceptable diluent, adjuvant or carrier, depending on the route of administration.

[0029] The pharmaceutical compositions of the invention are formulated to deliver a therapeutic dose of the CRF conjugate of the invention. The dose of the CRF conjugates contained in pharmaceutical formulation can range from about 1 μg to about 10 mg. In certain embodiments the dose of the CRF conjugate can range from about 0.1 mg to about 5 mg, or from about 0.3 mg to about 2 mg. In certain embodiments, the dose of the CRF conjugate can be about 0.3 mg, about 0.5 mg, about 1 mg, about 2 mg, about 4 mg or about 5 mg.

[0030] The conjugates of the invention can be used in the same manner as unmodified CRF. However because of the improved properties of the CRF conjugates, the pharmaceutical formulations of the invention can be administered less frequently than the

unmodified CRF. For example, the CRF conjugates may be administered once weekly instead of the once daily for unmodified CRF. The present invention also encompasses dosing regimens, wherein the CRF derivatives may be administered once a day, once every two, three or four days, or once a week to effectively treat edema. Decreased frequency of administration is expected to result in improved patient compliance leading to improved treatment outcomes, as well as improved patient quality of life.

4. BRIEF DESCRIPTION OF THE DRAWINGS

[0031] **FIG. 1** shows the amino acid sequences of the human and rat CRF peptides as compared to that of the ovine CRF peptide. Amino acids are presented as their standard one-letter designations. Amino acids in the ovine sequence which are presented in bold font and are underlined are those that differ from the human/rat CRF sequence.

5. DETAILED DESCRIPTION OF THE INVENTION

[0032] The present invention is based on conjugates of CRF that have been modified to include a moiety that results in a form of CRF that has an increased circulating half-life or plasma residence time as compared to unmodified CRF. The present invention is also related to methods of preparing such conjugates. The present invention further relates to methods of using such conjugates for treating tumor or cancer in patients. The present invention also relates to methods of using such conjugates for reducing inflammation and edema in patients.

[0033] The CRF conjugates of the present invention have an improved pharmacokinetic profile as compared to unmodified CRF. The CRF conjugates of the invention may show an improvement in one or more parameters of the pharmacokinetic profile, including AUC, C_{max}, clearance (CL), half-life, and bioavailability as compared to unmodified CRF.

[0034] The CRF conjugates of the present invention include CRF with an unmodified amino acid sequence as is shown in Figure 1, wherein one or more residues are covalently bound to polyethylene glycol. CRF conjugates of the present invention also include cysteine added variants of CRF, where one or more cysteine residues have been inserted into one of the CRF amino acid sequences shown in Figure 1, or substituted for one or more residues of one of the CRF sequences shown in Figure 1. The conjugated cysteine added variants of CRF, include CRF sequences with cysteine residues added at the N-terminus, the C-terminus, or both the N-terminus and C-terminus of one of the amino acid sequences shown in Figure 1. When two or more cysteine residues are added to the sequence, two cysteine residues may together form a disulfide bond, thus forming a cyclic peptide. In a specific embodiment, a

cysteine residue at the C-terminus of the CRF sequence forms a disulfide bond with a cysteine residue at the N-terminus.

[0035] The CRF conjugates of the present invention can be used to treat cancer by administering to a patient in need thereof a therapeutically acceptable amount of a CFR conjugate. The CRF conjugates of the present invention can be used to prevent tumor progression of administering to a patient in need thereof a therapeutically acceptable amount of a CFR conjugate.

[0036] The CRF conjugates of the present invention can be used to treat edema by administering to a patient in need thereof a therapeutically acceptable amount of a CRF conjugate.

[0037] Another aspect of the invention is a method of treating edema comprising administering to a patient in need thereof a pharmaceutical composition comprising CRF chemically modified with polyethylene glycol and a pharmaceutically acceptable diluent, adjuvant or carrier.

[0038] Another aspect of the invention is a method for treating brain edema comprising administering CRF conjugate, wherein the conjugate is administered in a treatment regimen as a steroid sparing agent facilitating steroid taper.

[0039] Another aspect of the invention is a method for managing brain edema in a patient in need thereof comprising administering to the patient a therapeutically effective amount of a CRF conjugate and a steroid, wherein the method provides a steroid sparing effect.

[0040] Another aspect of the invention is a method for providing replacement therapy for steroid therapy in a subject receiving such therapy, the method comprising administration of a steroid-sparing amount of CRF conjugate.

[0041] Another aspect of the invention is a method for treating brain edema, comprising a treatment regimen steroid in combination with a CRF conjugate, whereby total exposure to the steroid is reduced by the administration of the CRF conjugate.

[0042] The term "area under the curve" or "AUC," as used herein in the context of administering a peptide drug to a patient, is defined as total area under the curve that describes the concentration of drug in systemic circulation in the patient as a function of time from zero to infinity.

[0043] As used herein, the term “clearance” or “renal clearance” is defined as the volume of plasma that contains the amount of drug excreted per minute.

[0044] As used herein, the terms "corticotropin releasing factor," "CRF," "corticotrop(h)in-releasing hormone," "CRH," "corticoliberin," "corticorelin," "CRF-41," and

grammatical equivalents thereof, have a functional definition and refer to peptides which share one or more of the biological activities of the native, intact CRF peptide. Such biological activities include, for example, the ability to stimulate the release of ACTH, the ability to inhibit edema *in vivo* and the ability to bind to CRF receptors. Each of the above terms is intended to denote the 41 amino acid human, rat, ovine, sheep, goat, porcine and fish corticotropin releasing factor peptides and CRF peptides from other mammals, whether isolated from natural source extraction and purification, from recombinant cell culture systems or synthesized using peptide synthesis technology. These terms are also intended to denote other CRF-related peptides which share one or more of the biological activities of the native CRF peptides, such as urocortin (Vaughan, J., et al., *Nature* 378:287-292 (1995); Donaldson, C. J., et al., *Endocrinology* 137(5):2167-2170 (1996); and Turnbull, A. V., et al., *Eur. J. Pharm.* 303:213-216 (1996)); urotensin I (Lederis, K., et al., *Science* 218:162-164 (1982)); and sauvagine (Montecucchi, P. C., et al., *Int. J. Pep. Prot. Res.* 16:191-199 (1980)).

[0045] The CRF peptides employed in the formulations of the present invention are preferably synthesized using solid- or solution-phase peptide synthesis techniques, however, other sources of the CRF peptide are readily available to the ordinarily skilled artisan. The amino acid sequences of the human, rat and ovine CRF peptides are presented in Figure 1. The terms "corticotropin releasing factor" and "CRF" likewise cover biologically active CRF equivalents; e.g., peptides differing in one or more amino acids in the overall amino acid sequence as well as substitutional, deletional, insertional and modified amino acid variants of CRF which substantially retain the biological activity normally associated with the intact CRF peptide.

[0046] As used herein, the term “CRF conjugate” refers to a CRF polypeptide that has been modified to include a moiety that results in an improved pharmacokinetic profile as compared to unmodified CRF. The improvement in the pharmacokinetic profile may be observed as an improvement in one or more of the following parameters: potency, stability, area under the curve and circulating half-life.

[0047] As used herein, the term “cysteine added variant of CRF” refers to CRF that has been modified by the insertion of one or more cysteine residues into the unmodified CRF sequence shown in Figure 1, or the substitution of one or more of the amino acid residues in the CRF polypeptide sequence shown in Figure 1, for cysteine residues.

[0048] As used herein, the term "half-life" or "$t_{1/2,}$" in the context of administering a peptide drug to a patient, is defined as the time required for plasma concentration of a drug in a patient to be reduced by one half. There may be more than one half-life associated with the

peptide drug depending on multiple clearance mechanisms, redistribution, and other mechanisms well known in the art. Usually, alpha and beta half-lives are defined such that the alpha phase is associated with redistribution, and the beta phase is associated with clearance. However, with protein drugs that are, for the most part, confined to the bloodstream, there can be at least two clearance half-lives. The precise impact of PEGylation on alpha phase and beta phase half-lives will vary depending upon the size and other parameters, as is well known in the art. Further explanation of "half-life" is found in Pharmaceutical Biotechnology (1997, DFA Crommelin and RD Sindelar, eds., Harwood Publishers, Amsterdam, pp 101 120).

[0049] As used herein, when referring to the administration of CRF conjugates of the invention, the term a "patient in need thereof," refers to a patient who has been diagnosed with a condition that may be treated by CRF, *e.g.*, tumor, cancer, or brain edema. In one embodiment, the patient is a human patient.

[0050] As used herein, the term "pharmaceutically acceptable" when used in reference to the formulations of the present invention denotes that a formulation does not result in an unacceptable level of irritation in the subject to whom the formulation is administered by any known administration regimen. What constitutes an unacceptable level of irritation will be readily determinable by those of ordinary skill in the art and will take into account erythema and eschar formation as well as the degree of edema associated with administration of the formulation.

[0051] As used herein the term "residence time," in the context of administering a peptide drug to a patient, is defined as the average time that drug stays in the body of the patient after dosing.

[0052] As used herein, the terms "treat," "treating" or "treatment of" mean that the severity of a subject's condition is reduced or at least partially improved or ameliorated and/or that some alleviation, mitigation or decrease in at least one clinical symptom is achieved and/or there is an inhibition or delay in the progression of the condition and/or delay in the progression of the onset of disease or illness. The terms "treat," "treating" or "treatment of" also means managing the disease state, *e.g.,* tumor, cancer, or brain edema.

[0053] As used herein, a "sufficient amount" or an "amount sufficient to" achieve a particular result refers to an amount of CRF conjugate that is effective to produce a desired effect, which is optionally a therapeutic effect (*i.e.,* by administration of a therapeutically effective amount). For example, a "sufficient amount" or "an amount sufficient to" can be an

amount that is effective to reduce the amount of steroid required to manage the tumor, cancer, or brain edema.

[0054] As used herein, a "therapeutically effective" amount is an amount that provides some improvement or benefit to the subject. Alternatively stated, a "therapeutically effective" amount is an amount that provides some alleviation, mitigation, and/or decrease in at least one clinical symptom. Clinical symptoms associated with the disorder that can be treated by the methods of the invention are well-known to those skilled in the art. Further, those skilled in the art will appreciate that the therapeutic effects need not be complete or curative, as long as some benefit is provided to the subject.

5.1 CRF Conjugates

[0055] The CRF conjugates of the invention have one or more of the biological activities of unmodified CRF. Such biological activities include, for example, the ability to stimulate the release of ACTH, the ability to treat tumor or cancer, the ability to inhibit edema *in vivo* and the ability to bind to CRF receptors. The biological activity of CRF conjugates may be determined using the assays described herein.

[0056] Compared to unmodified CRF (i.e., CRF without a PEG attached), the conjugates of the present invention have an increased circulating half-life, plasma residence time and/or decreased clearance. In an embodiment of the invention, the CRF conjugates have increased clinical activity *in vivo* as compared to unmodified CRF. The conjugates of the invention have improved potency, stability, area under the curve and circulating half-life. The CRF conjugates of the invention have an improved pharmacokinetic profile as compared to unmodified CRF. The CRF conjugates of the invention may show an improvement in one or more parameters of the pharmacokinetic profile, including AUC, C_{max}, clearance (CL), half-life, and bioavailability as compared to unmodified CRF.

[0057] CRF to be modified in accordance with the invention may be obtained and isolated from natural sources. CRF to be modified in accordance with the invention may be expressed recombinantly. CRF to be modified in accordance with the invention may be synthetically produced.

[0058] In one embodiment, the CRF component of the CRF conjugate has the sequence identified as human CRF identified in Figure 1. In one embodiment, the CRF component of the CRF conjugate has the sequence identified as rat or ovine CRF identified in Figure 1. Alternatively, the sequence of CRF may be modified or derivatized to include one or more changes in the amino acid sequence, including, but not limited to, insertions,

deletions or substitutions. In yet another embodiment the sequence of CRF has been modified to include one or more cysteine residues. The sequence of CRF may include cysteine as a substitution of one or more of the existing residues of CRF. Alternatively, the cysteine residue may be incorporated as an addition to the existing sequence of CRF. The cysteine residues may be inserted within the sequence of CRF, the cysteine residue may be added to the amino or carboxy terminus of the sequence, or a cysteine residue may be added at both the amino and carboxy termini.

[0059] A CRF-PEG conjugate containing a PEG bound to one or more functional groups of the naturally occurring CRF polypeptide leads to increased circulating half-life and plasma residence time, decreased clearance, and increased clinical activity *in vivo*. CRF may be modified by covalently binding a polyethylene glycol polymer through one or more of its 41-amino acids including, but not limited to, lysine, histidine, arginine, aspartic acid, glutamic acid, serine, as well as the N-terminal α-amino and C-terminal carboxylate groups of the protein. Polyethylene glycol polymer units can be linear or branched. The CRF-PEG conjugate may be delivered intravenously or subcutaneously via injection.

[0060] One aspect of the invention is a CRF-PEG conjugate, wherein PEG is bound to one or more amino groups of CRF. Another aspect of the invention is a CRF-PEG conjugate, wherein a polyethylene glycol polymer is bound to one or more carboxyl groups of CRF. Another aspect of the invention is a CRF-PEG conjugate where a polyethylene glycol polymer is bound to one or more hydroxyl groups of CRF.

[0061] Another aspect of the invention is a CRF-PEG conjugate where a polyethylene glycol polymer is bound to the lysine residue. The ε-amino group of lysine in CRF can be readily PEGylated by a variety of techniques, including, but not limited to, alkylation and acylation.

[0062] Another aspect of the invention is a CRF conjugate, where a polyethylene glycol polymer is bound to the N-terminal α-amino group. The N-terminal α-amino residue of CRF can form a PEG conjugate by a variety of techniques including, but not limited to, alkylation or acylation of the N-terminal α-amino group.

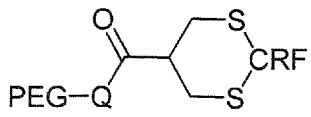
[0063] Another aspect of the invention is cysteine added variants of CRF that contain one or more PEG conjugated cysteine residues that have been substituted for naturally occurring residues in the CRF polypeptide sequence. Cysteine substituted CRF can be produced recombinantly by expressing DNA with point mutations that result in the substitution of a cysteine for a residue in naturally occurring CRF. For example the codon TCT, which codes for serine, can be mutated to TGC, which codes for cysteine, so in place of

one of the serine residues a cysteine will be expressed. If CRF is produced via synthetic means, in the course of the synthesis it is possible to substitute a cysteine residue in place of one or more residues that naturally occur in CRF. The cysteine can then be selectively conjugated to a polyethylene glycol polymer.

[0064] Another aspect of the invention is cysteine added variants of CRF that contain one or more PEG conjugated cysteine residues that have been inserted into the naturally occurring CRF sequence shown in Figure 1. If CRF is produced recombinantly, this can be done by inserting one or more cysteine codon(s) into the DNA sequence that codes for CRF. In solid phase protein synthesis, cysteines are added at any point of the protein synthesis by introducing an additional cysteine residue where desired. The cysteine can then be selectively bound to a polyethylene glycol polymer.

[0065] Another aspect of the invention is a cysteine added variant of CRF that contains a PEG conjugated cysteine residue inserted at the N-terminus. Another aspect of the invention is a CRF conjugate that contains a PEG bound to a cysteine residue inserted at the C-terminus. Another aspect of the invention is a CRF conjugate that contains PEG bound to cysteine residues inserted at both the N-terminus and the C-terminus. In a specific embodiment, a cysteine residue at the C-terminus of the CRF sequence forms a disulfide bond with a cysteine residue at the N-terminus. In one embodiment, the CRF-PEG conjugate comprises one or more PEG groups simultaneously bound to two cysteine residues in a cysteine added variant of CRF. In another embodiment, the CRF-PEG conjugate comprises one or more PEG groups simultaneously bound to two cysteine residues that form a disulfide bond in a cysteine added variant of CRF. These conjugates may be produced via reductive cleavage of a disulfide bond, followed by a reaction in which the PEG moiety becomes bound to both thio groups. The resulting CRF conjugate contains a PEG moiety that bridges two sulfurs that had formed a disulfide bond. In a specific embodiment, the CRF conjugate contains a PEG bound to both the C-terminal and N-terminal cysteine residue of a cysteine added variant of CRF.

[0066] In a specific embodiment, a polyethylene glycol polymer is conjugated to a cysteine added variant of CRF according to general formula I:



I;

wherein both -S- are from cysteine residues in a cysteine added variant of CRF, which in certain embodiments form a disulfide bond, wherein Q represents a linking group, which is a

direct bond, alkylene (preferably C_{1-10} alkylene), alkenylene, -O-alkylene-, -O-alkylene-O-, -O-alkylene-NR^1-, -NR^2-alkylene-, - NR^2-alkylene-O-, -NR^2-alkylene-NR^1-, -C(O)O-alkylene-, -C(O)O-alkylene-O-, -C(O)O-alkylene-NR^1-, -C(O)NR^2-alkylene-, -C(O)NR^2-alkylene-O-, -C(O)NR^2-alkylene-NR^1-, -C(NR^3R^4)NR^2-alkylene-, -C(NR^3R^4)NR^2-alkylene-O-, -C(NR^3R^4)NR^2-alkylene-NR^1-, -C(O)-O-, -C(O)-S-, -C(=NH)-NR^1-, -C(O)-N(R^1)-O-, -C(O)-N(R^1)-C(O)-, -C(O)-N(R^1)-, -C(O)-, -N(R^1)-, -O-C(O)-O-, -N=N-, -N=C(R1)-, -S-, -O-, an alpha amino acid derivative, a beta amino acid derivative, or an optionally-substituted aryl or heteroaryl group;

wherein the aryl group includes phenyl, benzoyl and naphthyl groups;

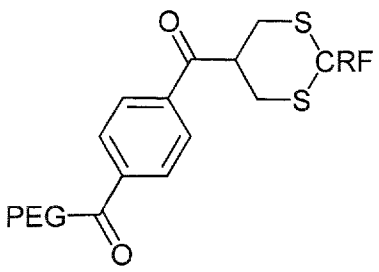
wherein the heteroaryl group includes pyridine, pyrrole, furan, pyran, imidazole, pyrazole, oxazole, pyridazine, primidine and purine;

wherein each R^1, R^2, R^3, or R^4 is independently hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, aryl, aralkyl, heteroaryl, or heterocyclyl; and

wherein the linkage to the polymer may be by way of a hydrolytically labile bond, or by a non-labile bond.

[0067] Substituents which may be present on an optionally substituted aryl or heteroaryl group include for example one or more of the same or different substituents selected from -CN, -NO_2, -CO_2R, -COH, -CH_2OH, -COR, -OR, -OCOR, -OCO_2R, -SR, -SOR, -SO_2R, -NHCOR, -NRCOR, -$NHCO_2R$, -NR'CO_2R, -NO, -NHOH, -NR'OH, -C=N-NHCOR, -C=N-NR'COR, -N^+R_3, -N^+H_3, -N^+HR_2, -N^+H_2R, halogen, for example fluorine or chlorine, -C≡CR, -C=CR_2 and ^{13}C=CHR, in which each R or R' independently represents hydrogen or an alkyl (preferably C_{1-6}) or an aryl (preferably phenyl) group. The presence of electron withdrawing substituents is especially preferred.

[0068] In one embodiment of formula I, PEG is conjugated to CRF according to formula II:

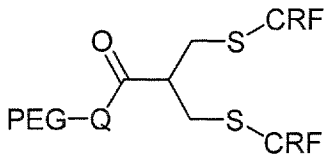


II.

[0069] Two cysteine added variants of CRF may be bound together via a disulfide bond, to form a CRF dimer. The CRF dimer may be conjugated to a polyethylene glycol containing

moiety. In one embodiment, the CRF dimer conjugate is bound to PEG through the disulfide bond that binds the two CRF polypeptides together.

[0070] In another specific embodiment, a polyethylene glycol polymer is conjugated to two cysteine added variants of CRF according to general formula III:



III;

wherein both -S- are from cysteine residues in a cysteine added variant of CRF, which in certain embodiments form a disulfide bond, wherein Q represents a linking group, which is a direct bond, alkylene (preferably C_{1-10} alkylene), alkenylene, -O-alkylene-, -O-alkylene-O-, -O-alkylene-NR^1-, -NR^2-alkylene-, - NR^2-alkylene-O-, -NR^2-alkylene-NR^1-, -C(O)O-alkylene-, -C(O)O-alkylene-O-, -C(O)O-alkylene-NR^1-, -C(O)NR^2-alkylene-, -C(O)NR^2-alkylene-O-, -C(O)NR^2-alkylene-NR^1-, -C(NR^3R^4)NR^2-alkylene-, -C(NR^3R^4)NR^2-alkylene-O-, -C(NR^3R^4)NR^2-alkylene-NR^1-, -C(O)-O-, -C(O)-S-, -C(=NH)-NR^1-, -C(O)-N(R^1)-O-, -C(O)-N(R^1)-C(O)-, -C(O)-N(R^1)-, -C(O)-, -N(R^1)-, -O-C(O)-O-, -N=N-, -N=C(R1)-, -S-, -O-, an alpha amino acid derivative, a beta amino acid derivative, or an optionally-substituted aryl or heteroaryl group;

wherein the aryl group includes phenyl, benzoyl and naphthyl groups;

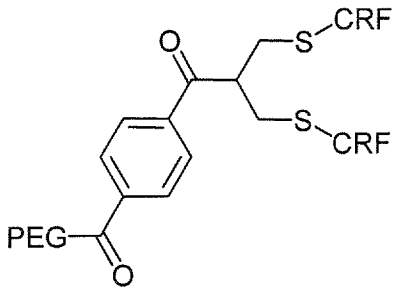
wherein the heteroaryl group includes pyridine, pyrrole, furan, pyran, imidazole, pyrazole, oxazole, pyridazine, pyrimidine and purine;

wherein each R^1, R^2, R^3, or R^4 is independently hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, aryl, aralkyl, heteroaryl, or heterocyclyl; and

wherein the linkage to the polymer may be by way of a hydrolytically labile bond, or by a non-labile bond.

[0071] Substituents which may be present on an optionally substituted aryl or heteroaryl group include, for example, one or more of the same or different substituents selected from -CN, -NO_2, -CO_2R, -COH, -CH_2OH, -COR, -OR, -OCOR, -OCO_2R, -SR, -SOR, -SO_2R, -NHCOR, -NRCOR, -$NHCO_2$R, -NR'CO_2R, -NO, -NHOH, -NR'OH, -C=N-NHCOR, -C=N-NR'COR, -N^+R_3, -N^+H_3, -N^+HR_2, -N^+H_2R, halogen, for example fluorine or chlorine, -C≡CR, -C=CR_2 and ^{13}C=CHR, in which each R or R' independently represents hydrogen or an alkyl (preferably C_{1-6}) or an aryl (preferably phenyl) group. The presence of electron withdrawing substituents is especially preferred.

[0072] In one embodiment of formula III, PEG is conjugated to CRF according to formula IV:



IV.

[0073] The groups, each R^1, R^2, R^3, R^4, and Q in formulae I and III are further defined herein. All combinations of the embodiments provided herein for such groups are within the scope of this disclosure.

[0074] In certain embodiments, R^1 is hydrogen or alkyl. In certain embodiments, R^1 is hydrogen. In certain embodiments, R^1 is alkyl.

[0075] In certain embodiments, R^2 is hydrogen or alkyl. In certain embodiments, R^2 is hydrogen. In certain embodiments, R^2 is alkyl.

[0076] In certain embodiments, R^3 is hydrogen or alkyl. In certain embodiments, R^3 is hydrogen. In certain embodiments, R^3 is alkyl.

[0077] In certain embodiments, R^4 is hydrogen or alkyl. In certain embodiments, R^4 is hydrogen. In certain embodiments, R^4 is alkyl.

[0078] In certain embodiments, Q is alkenylene, -O-alkylene-, -O-alkylene-O-, -O-alkylene-NR^1-, -NR^2-alkylene-, - NR^2-alkylene-O-, -NR^2-alkylene-NR^1-, -C(O)O-alkylene-, -C(O)O-alkylene-O-, -C(O)O-alkylene-NR^1-, -C(O)NR^2-alkylene-, -C(O)NR^2-alkylene-O-, -C(O)NR^2-alkylene-NR^1-, -C(NR^3R^4)NR^2-alkylene-, -C(NR^3R^4)NR^2-alkylene-O-, -C(NR^3R^4)NR^2-alkylene-NR^1-, -C(O)-O-, -C(O)-S-, -C(=NH)-NR^1-, -C(O)-N(R^1)-O-, -C(O)-N(R^1)-C(O)-, -C(O)-N(R^1)-, -C(O)-, -N(R^1)-, -O-C(O)-O-, -N=N-, -N=C(R1)-, -S-, -O-, an alpha amino acid derivative, a beta amino acid derivative, or an optionally-substituted aryl or heteroaryl group.

[0079] In certain embodiments, Q is -O-$(CH_2)_n$-, -O-$(CH_2)_n$-O-, -O-$(CH_2)_n$-NR^1-, -NR^2-$(CH_2)_n$-, - NR^2-$(CH_2)_n$-O-, -NR^2-$(CH_2)_n$-NR^1-, -C(O)O-$(CH_2)_n$-, -C(O)O-$(CH_2)_n$-O-, -C(O)O-$(CH_2)_n$-NR^1-, -C(O)NR^2-$(CH_2)_n$-, -C(O)NR^2-$(CH_2)_n$-O-, -C(O)NR^2-$(CH_2)_n$-NR^1-, -C(NR^3R^4)NR^2-$(CH_2)_n$-, -C(NR^3R^4)NR^2-$(CH_2)_n$-O-, or -C(NR^3R^4)NR^2-$(CH_2)_n$-NR^1-, wherein each R^1, R^2, R^3, or R^4 is as defined herein, and n is an integer of 1, 2, 3, 4, 5, or 6. In certain embodiments, n is 1. In certain embodiments, n is 2. In certain embodiments, n is 3. In certain embodiments, n is 4. In certain embodiments, n is 5. In certain embodiments, n is 6.

[0080] In certain embodiments, Q is an alpha amino acid derivate. Examples of suitable alpha amino acids include, but are not limited to, alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine.

[0081] In certain embodiments, Q is a beta amino acid derivative. An example of suitable beta amino acids is beta alanine.

[0082] The CRF conjugates of the invention have one or more of the biological activities of unmodified CRF. Such biological activities include, for example, the ability to stimulate the release of ACTH, the ability to treat tumor or cancer, the ability to inhibit edema *in vivo* and the ability to bind to CRF receptors. The biological activity of CRF conjugates may be determined using the assays described herein. In certain embodiments, the CRF conjugates provided herein have a molecule weight sufficient to avoid renal filtration. In certain embodiments, the CRF conjugates provided herein have a molecule weight of no less than about 60 kDa. In certain embodiments, the CRF conjugates provided herein have a molecule weight from about 60 to 62 kDa.

[0083] There are several different types of polyethylene glycol polymers that will form conjugates with CRF polypeptides. There are linear PEG polymers that contain a single polyethylene glycol chain, and there are branched or multi-arm PEG polymers. Branched polyethylene glycol contains 2 or more separate linear PEG chains bound together through a unifying group. For example, two PEG polymers may be bound together by a lysine residue. One linear PEG chain is bound to the α-amino group, while the other PEG chain is bound to the ε-amino group. The remaining carboxyl group of the lysine core is left available for covalent attachment to a protein. Both linear and branched polyethylene glycol polymers are commercially available in a range of molecular weights.

[0084] In one aspect of the invention, a CRF-PEG conjugate contains one or more linear polyethylene glycol polymers bound to CRF, wherein each PEG having a molecular weight between about 2 kDa to about 100 kDa. In another aspect of the invention, a CRF-PEG conjugate contains one or more linear polyethylene glycol polymers bound to CRF, wherein each branched PEG has a molecular weight between about 5 kDa to about 40 kDa. In yet another aspect of the invention, a CRF-PEG conjugate contains one or more linear polyethylene glycol polymers bound to CRF, wherein each linear PEG has a molecular weight between about 10 kDa. In certain embodiments, the CRF-PEG conjugate has a molecule weight sufficient to avoid renal filtration. In certain embodiments, the CRF-PEG

conjugate has a molecule weight of no less than about 60 kDa. In certain embodiments, he CRF-PEG conjugate has a molecule weight from about 60 to 62 kDa.

[0085] A CRF-PEG conjugate of this invention may contain one or more branched polyethylene glycol polymers bound to CRF, wherein each branched PEG has a molecular weight between about 2 kDa to about 100 kDa. In another aspect of the invention, a CRF-PEG conjugate contains one or more branched polyethylene glycol polymers bound to CRF, wherein each branched PEG has a molecular weight between about 5 kDa to about 40 kDa. In yet another aspect of the invention, a CRF-PEG conjugate contains one or more branched polyethylene glycol polymers bound to CRF, wherein each branched PEG has a molecular weight between about 10 kDa. In certain embodiments, the CRF-PEG conjugate has a molecule weight sufficient to avoid renal filtration. In certain embodiments, the CRF-PEG conjugate has a molecule weight of no less than about 60 kDa. In certain embodiments, he CRF-PEG conjugate has a molecule weight from about 60 to 62 kDa.

5.2 Methods of Producing CRF Derivatives

5.2.1. PEGylation of Amino Groups

[0086] CRF can be conjugated with polyethylene glycol, without the modification of the original 41 residue polypeptide chains. Both the lysine ε-amino group and the N-terminal α-amino group can be PEGylated by alkylation and acylation as demonstrated below.

[0087] The ε-amino group of lysine is a commonly used group for PEG conjugation of proteins, and CRF contains a single lysine residue. The PEG conjugation of lysine via its ε-amino group may be accomplished by methods including, but not limited to, acylation and alkylation. When a PEG-Alde Hyde reacts with an amino group, a Schiff base is formed. Harris and Herat (U.S. Pat. No. 5,252,714) incorporated herein by reference in its entirety, use polyethylene glycol propionaldehyde as the PEG aldehyde. The Schiff base is then reduced by sodium cyanoborohydride to produce a CRF-PEG conjugate. A drawback to this method is that Schiff base formation is slow, often requiring a day or more to occur. An alternative alkylation strategy is the use of PEG-tresyl chloride as the PEG alkylating reagent. The advantage of PEG-tresyl chloride is that it shows enhanced reactivity towards amino groups as demonstrated in Delgado (U.S. Pat. No. 5,349,052), incorporated herein by reference in its entirety. PEG conjugates of CRF can be further purified and isolated by techniques known in the art.

[0088] PEG conjugation of the ε-amino group of lysine via acylation is a technique known in the art for conjugating PEG polymers to the ε-amino group of lysine residues, such

as the lysine residue in CRF. Commonly employed PEG reagents are *N*-hydroxysuccinimidyl (NHS) esters of PEG as shown by Veronese, F.M. *Biomaterials.* 22(2001): 405-417. Other PEG acylation reagents are PEG-*p*-nitophenylcarbonate and PEG-trichlorophenylcarbonate in Veronese F.M. et. al. *Appl. Biochem. Biotechnol* 11(1985): 141-152, PEG oxycarbonylimidizole in Beauchamp, C.O. et al. *Anal. Biochem.* 131(1983): 25-33, and PEG-benzotriazole carbonate in Dolence et. al. (U.S. Pat. No. 5,650,234) incorporated herein by reference in its entirety. CRF-PEG conjugates synthesized by acylation can be purified and isolated by methods known in the art, including gel filtration or size exclusion chromatography.

[0089] The N-terminal α-amino group can be selectively bound to polyethylene glycol polymers, as taught in Kinstler (U.S. Pat. Nos. 6,586,398) incorporated herein by reference in its entirety.

[0090] One method of N-terminal PEGylation, is reductive alkylation with a PEG aldehyde, in a procedure similar to that described earlier. For example, a large excess methoxy PEG aldehyde can be mixed with the CRF protein in a buffered solution of pH 4-6. Sodium cyanoborohydride is added to the mixture, and the desired CRF-PEG conjugates can be isolated and purified by methods known in the art. The N-terminal amino group can also be modified by acylation with an activated NHS ester of PEG. To a slightly basic buffered solution of CRF, can be added a large excess of the PEG ester of NHS. After the reaction is complete, the CRF-PEG conjugate can be isolated and purified by methods known in the art.

5.2.2. Insertion and Substitution of Cysteine Residues

[0091] CRF derivatives where cysteines have been inserted or substituted can be produced by recombinant means using techniques known in the art. Expression of the desired cysteine substituted or inserted derivative may be done in either eukaryotic or bacterial cells by methods used by Shaw (U.S. Pat. No. 5,166,322) incorporated herein by reference in its entirety, for IL-3 cysteine added variants. Modifications to the naturally occurring CRF protein can be accomplished site directed PCR-based mutagenesis. Cox III (U.S. Pat. No. 7,214,779) incorporated herein by reference in its entirety, discloses cysteine added variants of granulocyte-macrophage colony stimulating factor (GCSF) that are produced by recombinant means. Cysteine added variants of CRF can also be made by synthetic methods. Cysteine residues can be substituted for another amino acid residue during the course of the synthesis. By adding an additional step to the solid phase synthesis of CRF, a cysteine residue can also be inserted where desired in the polypeptide sequence. In

solid phase synthesis, the cysteine may be added to the C-terminus of the CRF sequence at the first step of the synthesis. Alternatively, the cysteine may be added to the N-terminus of the CRF sequence, at the last step of solid phase synthesis. By adding a cysteine residue at the first and last steps of the solid phase synthesis, cysteine residues would be present at the C-terminus and N-terminus of the resulting cysteine added variant of CRF. A disulfide bond between the two cysteines may further result.

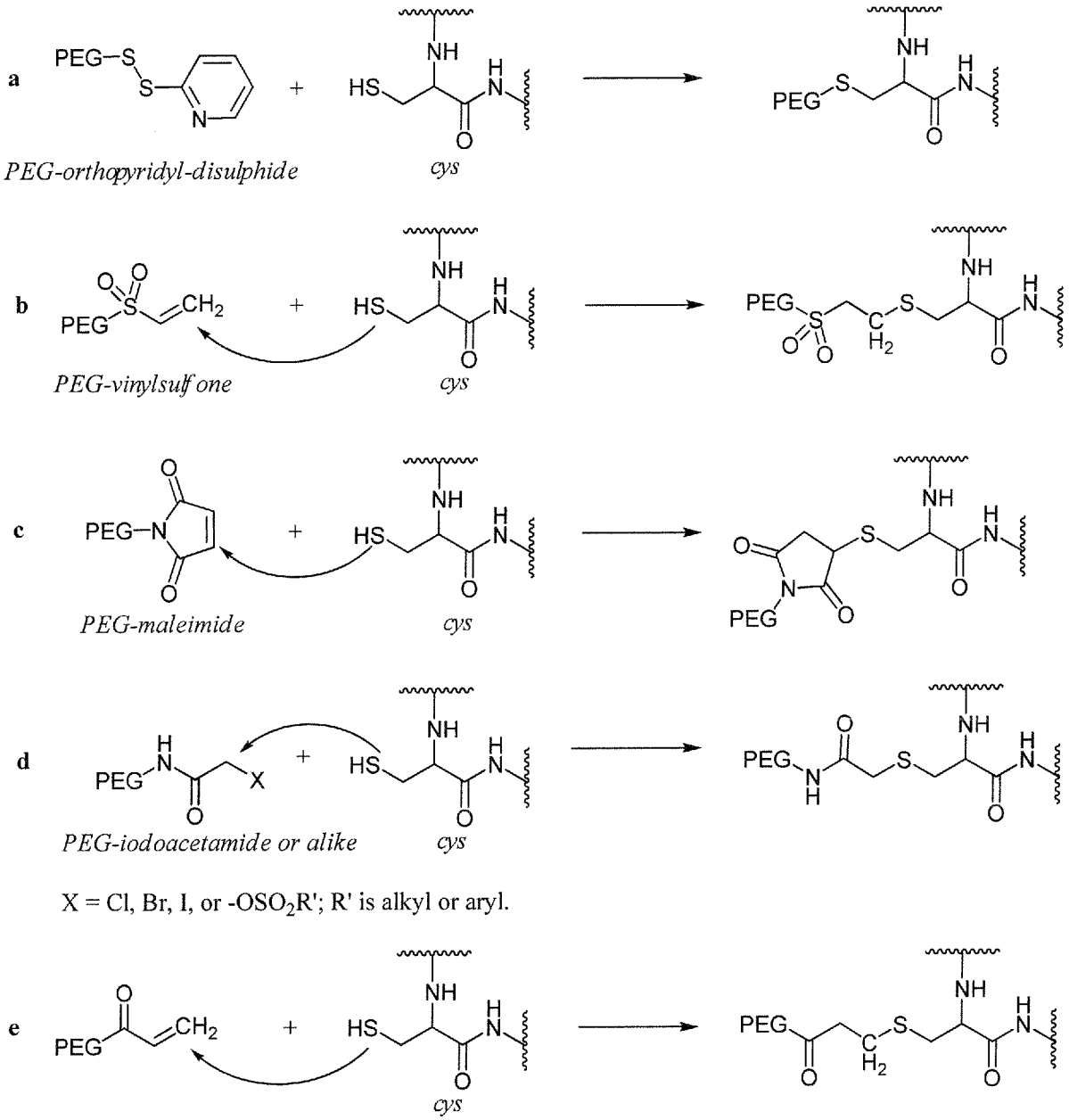
5.2.3. Techniques for the PEGylation of Cysteine Residues

[0092] A number of methods exist in the art for forming polyethylene glycol conjugated, or PEGylated cysteine residues. The advantage of these techniques are that they are selective for cysteine, which means that other amino acid residue side chains remain untouched by these methods. In **scheme 1a**, the activated disulphide, PEG ortho-pyridyl-disulphide, reacts with thiols to form the more stable symmetric disulphide. In **scheme 1b**, a cysteine residue reacts with PEG-maleamide, via a thiol addition to the activated double bond in a Michael addition reaction. In **scheme 1c** a conjugate attack by the thiol on the activated terminal vinyl group of PEG-vinylsulphone, yields the PEGylated cysteine residue. In **scheme 1d** the cysteine thiol displaces a leaving group, such as chloro, bromo, iodo, or tosyl, via a nucleophilic attack to yield the PEG conjugated cysteine residue. In **scheme 1e**, a cysteine residue reacts with PEG-α,β-unsaturated carbonylPEG, via a thiol addition to the activated double bond in a Michael addition reaction.

[0093] Two cysteine groups that together form a disulfide bond may also be PEGylated selectively by using the technique shown in **scheme 2**. The native disulfide bond is first reduced. One of the resulting thiols from this bond can nucleophilically attack an electrophilic group, such as a 1,4-addition to an enone. This is followed by the departure of a leaving group, such as, *e.g.* a sulfone. The subsequent elimination to a second enone, followed by 1,4-addition by the remaining thiol leads to the bridged disulfide with a PEG group attached.

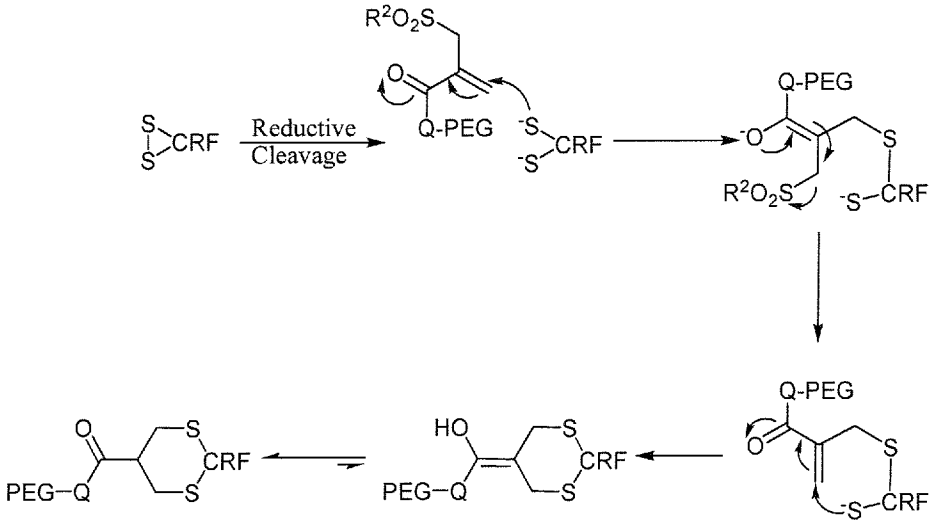
[0094] The chemistry as shown in **scheme 2** can also be used to couple with a CRF having a single reactive cysteine. After the coupling of the CRF to the bifunctional PEG, the second reactive site on the bifunctional PEG can be masked by the addition of a second thiol compound, such as methyl mercaptan or beta mercaptoethanol.

Scheme 1

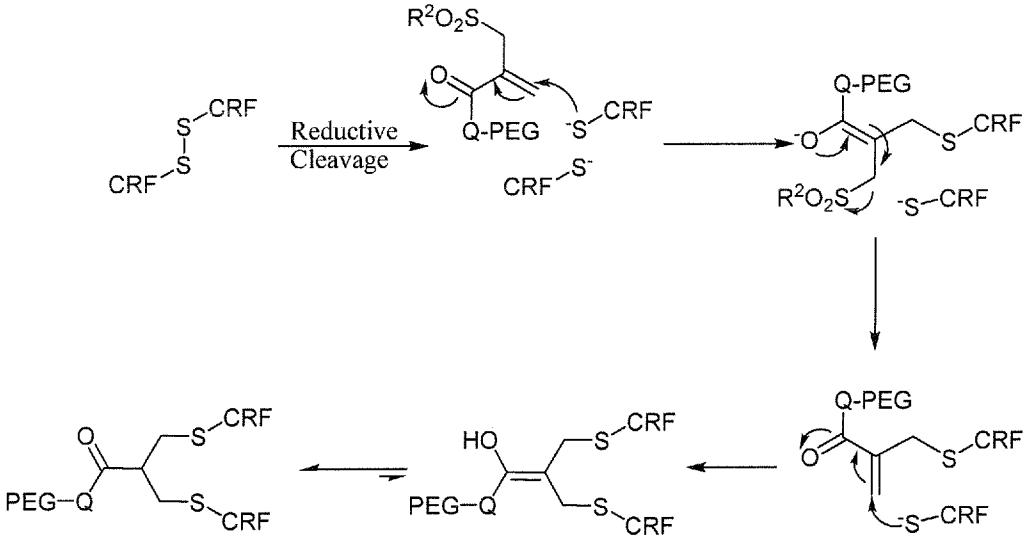


X = Cl, Br, I, or -OSO_2R'; R' is alkyl or aryl.

Scheme 2



[0095] For dimers of cysteine added variants of CRF, the PEGylation reaction proceeds via the scheme 2b.



5.3 Methods of Assaying Biological Activity

[0096] The CRF conjugates of the invention have one or more of the biological activities of unmodified CRF. Such biological activities include, for example, the ability to stimulate the release of ACTH, the ability to inhibit edema *in vivo* and the ability to bind to CRF receptors. The biological activity of CRF conjugates may be determined using biological assays known in the art, or the assay described in section 6.3.

5.4 Methods of Treating Tumors

[0097] The present invention is directed to methods for treating or managing cancer by administering a CRF conjugate to a human subject. In a related aspect, the methods of the disclosure can be used to prevent tumor progression.

[0098] In one aspect, provided herein is a method for preventing tumor progression in a human, by administering a composition comprising a CRF conjugate to a human diagnosed with or potentially having the tumor. In another aspect, provided herein is a method for preventing tumor progression in a human, by administering a composition comprising a CRF conjugate, wherein the CRF conjugate is administered at a dose effective to inhibit tumor progression, to a human diagnosed with or potentially having the tumor. As used herein and unless otherwise indicated the term "a human diagnosed with a tumor" refers to a human in which a neoplastic growth of a tissue which may be either benign or malignant exists and/or has been detected. In some embodiments, the term refers to a cancer patient. As used herein and unless otherwise indicated the term "a human that potentially has a tumor" refers to a human showing symptoms or abnormal tissue growth that are associated with a tumor, in such a human the tumor may have been detected or a physician has reasonable belief that the tumor exists based on clinical presentation.

[0099] Further, provided herein is a treatment regimen for prevention of tumor progression in a human, by administering a composition comprising a CRF conjugate to a human potentially having the tumor; and monitoring tumor progression in the human. As used herein and unless otherwise indicated, the term "monitoring" refers to methods that can be used to determine tumor growth, an increase in tumor size or volume, and/or formation of metastases. These methods comprise imaging technologies including X-ray radiography, computer tomography, and magnetic resonance imaging (MRI); the detection of biomarkers; biopsy procedures; and any other method known to a person of skill in the art, which may be used to determine tumor growth, an increase in tumor size or volume, and/or formation of metastases.

[0100] In accordance with any of the methods provided herein, the CRF conjugate can be administered over a period of time that exceeds three days, such as for five days or more, for seven to fourteen days or more, for two or three weeks or more, or for a year or more.

[0101] In accordance with the disclosure, the CRF conjugate can be administered continuously over that time or may be administered intermittently over that time. The administered dose of a CRF conjugate can be delivered as a single dose (*e.g.*, a single bolus injection) or intermittently by multiple injections or infusions. Alternatively, the

administered dose of a CRF conjugate is delivered over a period of time (*e.g.*, continuous infusion).

[0102] Administration of the CRF conjugate may be continued or repeated until the patient experiences stable disease or regression, or until the patient experiences disease progression or unacceptable toxicity.

[0103] The CRF conjugate can be administered either subcutaneously or intravenously. In one embodiment, the CRF conjugate is administered intravenously. In some embodiments, the CRF conjugate is administered by intravenous infusion at a rate of 4 μg/kg/hr to 100 μg/kg/hr. In other embodiments, the CRF conjugate is administered by subcutaneous injection. The amount of the CRF conjugate injected may vary. In certain embodiments, the amount of the CRF conjugate administered subcutaneously or intravenously may be in the range of 2 μg/kg to 1,000 μg/kg.

[0104] The total daily dose of the CRF conjugate administered to a patient diagnosed with a tumor may exceed 1 mg. In some embodiments, the total daily dose of the CRF conjugate is in the range of 0.5 mg to 20 mg. In some embodiments, the total daily dose of the CRF conjugate is in the range of 1 mg to 20 mg. In certain embodiments, the total daily dose of the CRF conjugate is in the range of 2.5 mg to 20 mg. In certain embodiments, the total daily dose of the CRF conjugate is in the range of 4 mg to 20 mg.

[0105] In a specific embodiment, the tumor is a brain tumor. The brain tumor may be a glioblastoma, glioma, ependymoma, astrocytoma, medulloblastoma, neuroglioma, oligodendroglioma or meningioma. Alternatively, the brain tumor may be a secondary brain tumor or a brain metastasis.

[0106] Other cancers and tumors that can be treated in accordance with the methods provided herein include, but are not limited to bladder cancer, breast cancer, cervical cancer, colon cancer (including colorectal cancer), esophageal cancer, head and neck cancer, liver cancer, lung cancer (both small cell and non-small cell), melanoma, myeloma, neuroblastoma, ovarian cancer, pancreatic cancer, prostate cancer, renal cancer, sarcoma (including osteosarcoma), skin cancer (including squamous cell carcinoma), stomach cancer, testicular cancer, thyroid cancer, and uterine cancer. In one embodiment, the methods encompass treating or managing colon, pancreas, breast, mesothelioma, cholangiocarcinoma, leiomyosarcoma, liposarcoma, melanoma, nasopharyngeal, neuroendocrine, ovarian, renal, salivary gland, small cell lung cancer, or spindle cell carcinoma.

[0107] In some embodiments, the tumor or cancer to be treated has relapsed or recurred. The term "relapsed" or "recurred" refers to a situation where patients who have had a remission of cancer after therapy have a return of cancer cells.

[0108] In other embodiments, the tumor or cancer to be treated has become refractory or resistant. The term "refractory" or "resistant" refers to a circumstance where patients, even after intensive treatment, have residual cancer cells in their body.

[0109] In the methods and compositions provided herein, the CRF conjugate can be used with or combined with other pharmacologically active compounds ("second active agents"). It is believed that certain combinations work synergistically in the treatment of particular types of cancers. The CRF conjugate can also work to alleviate adverse effects associated with certain second active agents, and some second active agents can be used to alleviate adverse effects associated with CRF.

[0110] In certain embodiments, the methods provided herein comprise administering CRF conjugates in combination with one or more second active agents, and optionally in combination with radiation therapy or surgery. The administration of CRF conjugates and the second active agents to a patient can occur simultaneously or sequentially by the same or different routes of administration. The suitability of a particular route of administration employed for a particular active agent will depend on the active agent itself (*e.g.*, whether it can be administered orally without decomposing prior to entering the blood stream) and the disease being treated. Recommended routes of administration for the second active agents are known to those of ordinary skill in the art. *See, e.g.*, *Physicians' Desk Reference*, 1755-1760 (56th ed., 2002).

[0111] In one embodiment, the second active agent is administered intravenously or subcutaneously and once or twice daily in an amount of from about 1 to about 1,000 mg, from about 5 to about 500 mg, from about 10 to about 375 mg, or from about 50 to about 200 mg. In certain embodiments, the second active agent is rituximab, oblimersen (GENASENSE®), GM-CSF, G-CSF, EPO, taxotere, irinotecan, dacarbazine, transretinoic acid, topotecan, pentoxifylline, ciprofloxacin, dexamethasone, vincristine, doxorubicin, COX-2 inhibitor, IL2, IL8, IL18, IFN, Ara-C, vinorelbine, or a combination thereof. In certain embodiments, the second active agent is etoposide, daunomycin, actinomycin D, mitomycin C, cisplatin, carboplatin, premetrexed, methotrexate, Ara-C, 5-Fu, wortmannin, geldanamycin, gemcitabin, or a combination thereof.

[0112] In another embodiment, provided herein are methods of treating or managing hematologic malignancies, which comprise administering the CRF conjugate in conjunction

with (*e.g.*, before, during or after) conventional therapy including, but not limited to, surgery, immunotherapy, biological therapy, radiation therapy, or other non-drug based therapy presently used to treat or manage cancer. Without being limited by theory, it is believed that CRF may provide additive or synergistic effects when given concurrently with conventional therapy.

[0113] In certain embodiments, the second active agent is co-administered with the CRF conjugate or administered with 1-50 hours delay. In certain embodiments, the CRF conjugate is administered first followed by administration with the second active agent with 1-50 hours delay. In certain embodiments, the second active agent is administered first followed by administration of the CRF conjugate with 1-50 hours delay.

[0114] In one embodiment, the CRF conjugate is administered in an amount from 1 μg/kg to 1,000 μg/kg, from 1 μg/kg to 100 μg/kg, from 2 μg/kg to 80 μg/kg, from 2 μg/kg to 50 μg/kg, from 4 μg/kg to 40 μg/kg, or from 5 μg/kg to 20 μg/kg alone or in combination with a second active agent disclosed herein, prior to, during, or after the use of conventional therapy.

[0115] In certain embodiments, the second active agent is temozolomide.

[0116] In certain embodiments, the daily dose of temozolomide is from about 1 to about 5,000 mg, from about 1 to about 1,000 mg, or from about 10 to 500 mg per day. In certain embodiments, the daily dose of temozolomide is about 10 mg, about 25 mg, about 50 mg, about 75 mg, about 83 mg, about 90 mg, about 98 mg, about 105 mg, about 112 mg, about 120 mg, about 128 mg, about 135 mg, about 143 mg, about 150 mg, about 158 mg, about 165 mg, about 173 mg, about 180 mg, about 188 mg, about 195 mg, about 200 mg, about 210 mg, about 220 mg, about 225 mg, about 240 mg, about 255 mg, about 260 mg, about 270 mg, about 280 mg, about 285 mg, about 300 mg, about 315 mg, about 320 mg, about 330 mg, about 340 mg, about 345 mg, about 360 mg, about 375 mg, about 380 mg, about 400 mg, about 420 mg, about 440 mg, about 460 mg, about 480 mg, or about 500 mg.

[0117] In certain embodiments, temozolomide is administered in an amount ranging from about 10 to about 500 mg/m^2/day, from about 50 to about 250 mg/m^2/day, or about 75 to about 200 mg/m^2/day. In certain embodiments, temozolomide is administered in an amount of about 10 mg/m^2/day, about 20 mg/m^2/day, about 30 mg/m^2/day, about 40 mg/m^2/day, about 50 mg/m^2/day, about 75 mg/m^2/day, about 100 mg/m^2/day, about 125 mg/m^2/day, about 150 mg/m^2/day, about 175 mg/m^2/day, or 200 about mg/m^2/day.

[0118] The administered dose can also be expressed in units other than as mg/m^2/day. For example, doses for parenteral administration can be expressed as mg/kg/day. One of ordinary skill in the art would readily know how to convert doses from mg/m^2/day to

mg/kg/day given either the height or weight of a subject or both (*see,* www.fda.gov/cder/cancer/animalframe.htm).

[0119] In certain embodiments, temozolomide is cyclically administered. In certain embodiments, temozolomide is administered daily in a single or divided doses for five days, one week, two weeks, three weeks, four weeks, five weeks, six weeks, eight weeks, ten weeks, fifteen weeks, or twenty weeks, followed by a rest period of about 1 day to about ten weeks. In certain embodiments, temozolomide is administered daily in a single or divided doses for five days, one week, two weeks, three weeks, four weeks, five weeks, six weeks, or eight weeks with a rest period of 1, 3, 5, 7, 9, 12, 14, 16, 18, 20, 22, 23, 24, 25, 26, 28, 29 or 30 days. In certain embodiments, the rest period is 7 days. In certain embodiments, the rest period is 14 days. In certain embodiments, the rest period is 23 days. In certain embodiments, the rest period is a period that is sufficient for bone marrow recovery. In certain embodiments, the rest period is a period that is sufficient for neutrophil recovery. In certain embodiments, the rest period is a period that is sufficient for platelet recovery. The frequency, number, and length of dosing cycles can be increased or decreased.

[0120] In certain embodiments, temozolomide is administered daily for four weeks, followed by six cycles of maintenance treatment. In certain embodiments, temozolomide in cycle 1 is administered once daily for five days, followed by a rest period of twenty-three (23) days. In certain embodiments, temozolomide in each of cycles 2 to 6 is administered once daily for five days, followed by a rest period that is sufficient for neutrophil and platelet recovery. In certain embodiments, each of cycles 2 to 6 starts when absolute neutrophil count (ANC) exceeds 1.5 x 10^9/L and the platelet count exceeds 100 x 10^9/L. In certain embodiments, the administration of temozolomide during cycles 1 to 6 may be discontinued if ANC is below 1 x 10^9/L or platelet count is below 50 x 10^9/L. The dosage in each cycle can be increased or decreased.

[0121] In certain embodiments, temozolomide is administered orally at 75 mg/m^2 daily for 42 days concomitant with 400 focal radiotherapy (60 Gy administered in 30 fractions) followed by maintenance treatment. Four weeks after completing the temozolomide and radiotherapy, temozolomide is administered for an additional 6 cycles of maintenance treatment. In cycle 1, temozolomide is administered at 150 mg/m^2 once daily for 5 days followed by 23 days without treatment. At the start of cycle 2, the dose is escalated to 200 mg/m^2, if the common toxicity criteria (CTC) non-hematologic toxicity for cycle 1 is no greater than Grade 2 (except for alopecia, nausea, and vomiting), absolute neutrophil count (ANC) is no less than 1.5 x 10^9/L, and the platelet count is no less than 100 x 10^9/L. The

dose remains at 200 mg/m^2 per day for the first 5 days of each subsequent cycle except if toxicity occurs. If the dose was not escalated at cycle 2, escalation should not be done in subsequent cycles.

[0122] In certain embodiments, the daily dose of temozolomide is adjusted according to neutrophil and platelet counts.

[0123] Other suitable second active agents include large molecules (*e.g.*, proteins) or small molecules (*e.g.*, synthetic inorganic, organometallic or organic molecules).

[0124] Examples of large molecule active agents include, but are not limited to, hematopoietic growth factors, cytokines, and monoclonal and polyclonal antibodies, particularly therapeutic antibodies to cancer antigens. Typical large molecule active agents are biological molecules, such as naturally occurring or artificially made proteins. Proteins that are particularly useful in the methods and compositions provided herein include proteins that stimulate the survival and/or proliferation of hematopoietic precursor cells and immunologically active poietic cells *in vitro* or *in vivo*. Others stimulate the division and differentiation of committed erythroid progenitors in cells *in vitro* or *in vivo*. Particular proteins include, but are not limited to: interleukins, such as IL-2 (including recombinant IL-II ("rIL2") and canarypox IL-2), IL-10, IL-12, and IL-18; interferons, such as interferon alfa-2a, interferon alfa-2b, interferon alfa-n1, interferon alfa-n3, interferon beta-I a, and interferon gamma-I b; GM-CF and GM-CSF; and EPO.

[0125] Particular proteins that can be used in the methods and compositions include, but are not limited to: filgrastim, which is sold in the United States under the trade name NEUPOGEN® (Amgen, Thousand Oaks, CA); sargramostim, which is sold in the United States under the trade name LEUKINE® (Immunex, Seattle, WA); and recombinant EPO, which is sold in the United States under the trade name EPOGEN® (Amgen, Thousand Oaks, CA).

[0126] Recombinant and mutated forms of GM-CSF can be prepared as described in U.S. patent nos. 5,391,485; 5,393,870; and 5,229,496; all of which are incorporated herein by reference. Recombinant and mutated forms of G-CSF can be prepared as described in U.S. patent nos. 4,810,643; 4,999,291; 5,528,823; and 5,580,755; all of which are incorporated herein by reference.

[0127] Also provided for use in combination with the CRF conjugate are native, naturally occurring, and recombinant proteins. Further encompassed are mutants and derivatives (*e.g.*, modified forms) of naturally occurring proteins that exhibit, *in vivo*, at least some of the pharmacological activity of the proteins upon which they are based. Examples of mutants

include, but are not limited to, proteins that have one or more amino acid residues that differ from the corresponding residues in the naturally occurring forms of the proteins. Also encompassed by the term "mutants" are proteins that lack carbohydrate moieties normally present in their naturally occurring forms (*e.g.*, nonglycosylated forms). Examples of derivatives include, but are not limited to, pegylated derivatives and fusion proteins, such as proteins formed by fusing IgG1 or IgG3 to the protein or active portion of the protein of interest. *See, e.g.*, Penichet, M.L. and Morrison, S.L., *J. Immunol. Methods* 248:91-101 (2001).

[0128] Antibodies that can be used in combination with the CRF conjugate include monoclonal and polyclonal antibodies. Examples of antibodies include, but are not limited to, trastuzumab (HERCEPTIN®), rituximab (RITUXAN®), bevacizumab (AVASTIN™), pertuzumab (OMNITARG™), tositumomab (BEXXAR®), edrecolomab (PANOREX®), and G250. CRF can also be combined with or used in combination with, anti-TNF-α antibodies.

[0129] Large molecule active agents may be administered in the form of anti-cancer vaccines. For example, vaccines that secrete or cause the secretion of, cytokines such as IL-2, G-CSF, and GM-CSF can be used in the methods and pharmaceutical compositions provided. *See, e.g.*, Emens, L.A., *et al.*, *Curr. Opinion Mol. Ther.* 3(1):77-84 (2001).

[0130] Second active agents that are small molecules can also be used to alleviate adverse effects associated with the administration of the CRF conjugate. However, like some large molecules, many are believed to be capable of providing a synergistic effect when administered with (*e.g.*, before, after or simultaneously) the CRF conjugate. Examples of small molecule second active agents include, but are not limited to, anti-cancer agents, antibiotics, immunosuppressive agents, and steroids.

[0131] Examples of anti-cancer agents include, but are not limited to: acivicin; aclarubicin; acodazole hydrochloride; acronine; adozelesin; aldesleukin; altretamine; ambomycin; ametantrone acetate; amsacrine; anastrozole; anthramycin; asparaginase; asperlin; azacitidine; azetepa; azotomycin; batimastat; benzodepa; bicalutamide; bisantrene hydrochloride; bisnafide dimesylate; bizelesin; bleomycin sulfate; brequinar sodium; bropirimine; busulfan; cactinomycin; calusterone; caracemide; carbetimer; carboplatin; carmustine; carubicin hydrochloride; carzelesin; cedefingol; celecoxib (COX-2 inhibitor); chlorambucil; cirolemycin; cisplatin; cladribine; crisnatol mesylate; cyclophosphamide; cytarabine; dacarbazine; dactinomycin; daunorubicin hydrochloride; decitabine; dexormaplatin; dezaguanine; dezaguanine mesylate; diaziquone; docetaxel; doxorubicin; doxorubicin hydrochloride; droloxifene; droloxifene citrate; dromostanolone propionate;

duazomycin; edatrexate; eflornithine hydrochloride; elsamitrucin; enloplatin; enpromate; epipropidine; epirubicin hydrochloride; erbulozole; esorubicin hydrochloride; estramustine; estramustine phosphate sodium; etanidazole; etoposide; etoposide phosphate; etoprine; fadrozole hydrochloride; fazarabine; fenretinide; floxuridine; fludarabine phosphate; fluorouracil; flurocitabine; fosquidone; fostriecin sodium; gemcitabine; gemcitabine hydrochloride; hydroxyurea; idarubicin hydrochloride; ifosfamide; ilmofosine; iproplatin; irinotecan; irinotecan hydrochloride; lanreotide acetate; letrozole; leuprolide acetate; liarozole hydrochloride; lometrexol sodium; lomustine; losoxantrone hydrochloride; masoprocol; maytansine; mechlorethamine hydrochloride; megestrol acetate; melengestrol acetate; melphalan; menogaril; mercaptopurine; methotrexate; methotrexate sodium; metoprine; meturedepa; mitindomide; mitocarcin; mitocromin; mitogillin; mitomalcin; mitomycin; mitosper; mitotane; mitoxantrone hydrochloride; mycophenolic acid; nocodazole; nogalamycin; ormaplatin; oxisuran; paclitaxel; pegaspargase; peliomycin; pentamustine; peplomycin sulfate; perfosfamide; pipobroman; piposulfan; piroxantrone hydrochloride; plicamycin; plomestane; porfimer sodium; porfiromycin; prednimustine; procarbazine hydrochloride; puromycin; puromycin hydrochloride; pyrazofurin; riboprine; safingol; safingol hydrochloride; semustine; simtrazene; sparfosate sodium; sparsomycin; spirogermanium hydrochloride; spiromustine; spiroplatin; streptonigrin; streptozocin; sulofenur; talisomycin; tecogalan sodium; taxotere; tegafur; teloxantrone hydrochloride; temoporfin; teniposide; teroxirone; testolactone; thiamiprine; thioguanine; thiotepa; tiazofurin; tirapazamine; toremifene citrate; trestolone acetate; triciribine phosphate; trimetrexate; trimetrexate glucuronate; triptorelin; tubulozole hydrochloride; uracil mustard; uredepa; vapreotide; verteporfin; vinblastine sulfate; vincristine sulfate; vindesine; vindesine sulfate; vinepidine sulfate; vinglycinate sulfate; vinleurosine sulfate; vinorelbine tartrate; vinrosidine sulfate; vinzolidine sulfate; vorozole; zeniplatin; zinostatin; and zorubicin hydrochloride.

[0132] Other anti-cancer drugs include, but are not limited to: 20-epi-1,25 dihydroxyvitamin D3; 5-ethynyluracil; abiraterone; aclarubicin; acylfulvene; adecypenol; adozelesin; aldesleukin; ALL-TK antagonists; altretamine; ambamustine; amidox; amifostine; aminolevulinic acid; amrubicin; amsacrine; anagrelide; anastrozole; andrographolide; angiogenesis inhibitors; antagonist D; antagonist G; antarelix; anti-dorsalizing morphogenetic protein-1; antiandrogen, prostatic carcinoma; antiestrogen; antineoplaston; antisense oligonucleotides; aphidicolin glycinate; apoptosis gene modulators; apoptosis regulators; apurinic acid; ara-CDP-DL-PTBA; arginine deaminase; asulacrine;

atamestane; atrimustine; axinastatin 1; axinastatin 2; axinastatin 3; azasetron; azatoxin; azatyrosine; baccatin III derivatives; balanol; batimastat; BCR/ABL antagonists; benzochlorins; benzoylstaurosporine; beta lactam derivatives; beta-alethine; betaclamycin B; betulinic acid; bFGF inhibitor; bicalutamide; bisantrene; bisaziridinylspermine; bisnafide; bistratene A; bizelesin; breflate; bropirimine; budotitane; buthionine sulfoximine; calcipotriol; calphostin C; camptothecin derivatives; capecitabine; carboxamide-amino-triazole; carboxyamidotriazole; CaRest M3; CARN 700; cartilage derived inhibitor; carzelesin; casein kinase inhibitors (ICOS); castanospermine; cecropin B; cetrorelix; chlorlns; chloroquinoxaline sulfonamide; cicaprost; cis-porphyrin; cladribine; clomifene analogues; clotrimazole; collismycin A; collismycin B; combretastatin A4; combretastatin analogue; conagenin; crambescidin 816; crisnatol; cryptophycin 8; cryptophycin A derivatives; curacin A; cyclopentanthraquinones; cycloplatam; cypemycin; cytarabine ocfosfate; cytolytic factor; cytostatin; dacliximab; decitabine; dehydrodidemnin B; deslorelin; dexamethasone; dexifosfamide; dexrazoxane; dexverapamil; diaziquone; didemnin B; didox; diethylnorspermine; dihydro-5-azacytidine; dihydrotaxol, 9-; dioxamycin; diphenyl spiromustine; docetaxel; docosanol; dolasetron; doxifluridine; doxorubicin; droloxifene; dronabinol; duocarmycin SA; ebselen; ecomustine; edelfosine; edrecolomab; eflornithine; elemene; emitefur; epirubicin; epristeride; estramustine analogue; estrogen agonists; estrogen antagonists; etanidazole; etoposide phosphate; exemestane; fadrozole; fazarabine; fenretinide; filgrastim; finasteride; flavopiridol; flezelastine; fluasterone; fludarabine; fluorodaunorunicin hydrochloride; forfenimex; formestane; fostriecin; fotemustine; gadolinium texaphyrin; gallium nitrate; galocitabine; ganirelix; gelatinase inhibitors; gemcitabine; glutathione inhibitors; hepsulfam; heregulin; hexamethylene bisacetamide; hypericin; ibandronic acid; idarubicin; idoxifene; idramantone; ilmofosine; ilomastat; imatinib (*e.g.*, GLEEVEC®); imiquimod; immunostimulant peptides; insulin-like growth factor-1 receptor inhibitor; interferon agonists; interferons; interleukins; iobenguane; iododoxorubicin; ipomeanol, 4-; iroplact; irsogladine; isobengazole; isohomohalicondrin B; itasetron; jasplakinolide; kahalalide F; lamellarin-N triacetate; lanreotide; leinamycin; lenograstim; lentinan sulfate; leptolstatin; letrozole; leukemia inhibiting factor; leukocyte alpha interferon; leuprolide+estrogen+progesterone; leuprorelin; levamisole; liarozole; linear polyamine analogue; lipophilic disaccharide peptide; lipophilic platinum compounds; lissoclinamide 7; lobaplatin; lombricine; lometrexol; lonidamine; losoxantrone; loxoribine; lurtotecan; lutetium texaphyrin; lysofylline; lytic peptides; maitansine; mannostatin A; marimastat; masoprocol; maspin; matrilysin inhibitors; matrix metalloproteinase inhibitors; menogaril; merbarone;

meterelin; methioninase; metoclopramide; MIF inhibitor; mifepristone; miltefosine; mirimostim; mitoguazone; mitolactol; mitomycin analogues; mitonafide; mitotoxin fibroblast growth factor-saporin; mitoxantrone; mofarotene; molgramostim;Erbitux, human chorionic gonadotrophin; monophosphoryl lipid A+myobacterium cell wall sk; mopidamol; mustard anticancer agent; mycaperoxide B; mycobacterial cell wall extract; myriaporone; N-acetyldinaline; N-substituted benzamides; nafarelin; nagrestip; naloxone+pentazocine; napavin; naphterpin; nartograstim; nedaplatin; nemorubicin; neridronic acid; nilutamide; nisamycin; nitric oxide modulators; nitroxide antioxidant; nitrullyn; oblimersen (GENASENSE®); O^6-benzylguanine; octreotide; okicenone; oligonucleotides; onapristone; ondansetron; ondansetron; oracin; oral cytokine inducer; ormaplatin; osaterone; oxaliplatin; oxaunomycin; paclitaxel; paclitaxel analogues; paclitaxel derivatives; palauamine; palmitoylrhizoxin; pamidronic acid; panaxytriol; panomifene; parabactin; pazelliptine; pegaspargase; peldesine; pentosan polysulfate sodium; pentostatin; pentrozole; perflubron; perfosfamide; perillyl alcohol; phenazinomycin; phenylacetate; phosphatase inhibitors; picibanil; pilocarpine hydrochloride; pirarubicin; piritrexim; placetin A; placetin B; plasminogen activator inhibitor; platinum complex; platinum compounds; platinum-triamine complex; porfimer sodium; porfiromycin; prednisone; propyl bis-acridone; prostaglandin J2; proteasome inhibitors; protein A-based immune modulator; protein kinase C inhibitor; protein kinase C inhibitors, microalgal; protein tyrosine phosphatase inhibitors; purine nucleoside phosphorylase inhibitors; purpurins; pyrazoloacridine; pyridoxylated hemoglobin polyoxyethylene conjugate; raf antagonists; raltitrexed; ramosetron; ras farnesyl protein transferase inhibitors; ras inhibitors; ras-GAP inhibitor; retelliptine demethylated; rhenium Re 186 etidronate; rhizoxin; ribozymes; RII retinamide; rohitukine; romurtide; roquinimex; rubiginone B1; ruboxyl; safingol; saintopin; SarCNU; sarcophytol A; sargramostim; Sdi 1 mimetics; semustine; senescence derived inhibitor 1; sense oligonucleotides; signal transduction inhibitors; sizofiran; sobuzoxane; sodium borocaptate; sodium phenylacetate; solverol; somatomedin binding protein; sonermin; sparfosic acid; spicamycin D; spiromustine; splenopentin; spongistatin 1; squalamine; stipiamide; stromelysin inhibitors; sulfinosine; superactive vasoactive intestinal peptide antagonist; suradista; suramin; swainsonine; tallimustine; tamoxifen methiodide; tauromustine; tazarotene; tecogalan sodium; tegafur; tellurapyrylium; telomerase inhibitors; temoporfin; teniposide; tetrachlorodecaoxide; tetrazomine; thaliblastine; thiocoraline; thrombopoietin; thrombopoietin mimetic; thymalfasin; thymopoietin receptor agonist; thymotrinan; thyroid stimulating hormone; tin ethyl etiopurpurin; tirapazamine; titanocene bichloride; topsentin;

toremifene; translation inhibitors; tretinoin; triacetyluridine; triciribine; trimetrexate; triptorelin; tropisetron; turosteride; tyrosine kinase inhibitors; tyrphostins; UBC inhibitors; ubenimex; urogenital sinus-derived growth inhibitory factor; urokinase receptor antagonists; vapreotide; variolin B; velaresol; veramine; verdins; verteporfin; vinorelbine; vinxaltine; vitaxin; vorozole; zanoterone; zeniplatin; zilascorb; and zinostatin stimalamer.

[0133] Specific second active agents include, but are not limited to, rituximab, oblimersen (GENASENSE®), remicade, docetaxel, celecoxib, melphalan, dexamethasone (DECADRON®), steroids, gemcitabine, cisplatinum, temozolomide, etoposide, cyclophosphamide, temozolomide (TEMODAR®), carboplatin, procarbazine, gliadel, tamoxifen, topotecan, methotrexate, ARISA®, taxol, taxotere, fluorouracil, leucovorin, irinotecan, xeloda, CPT-11, interferon alpha, pegylated interferon alpha (*e.g.*, PEG INTRON-A), capecitabine, cisplatin, thiotepa, fludarabine, carboplatin, liposomal daunorubicin, cytarabine, doxetaxol, pacilitaxel, vinblastine, IL-2, GM-CSF, dacarbazine, vinorelbine, zoledronic acid, palmitronate, biaxin, busulphan, prednisone, bisphosphonate, arsenic trioxide, vincristine, doxorubicin (DOXIL®), paclitaxel, ganciclovir, adriamycin, estramustine sodium phosphate (EMCYT®), sulindac, and etoposide.

[0134] In certain embodiments, the second active agent is etoposide, daunomycin, actinomycin D, mitomycin C, cisplatin, carboplatin, premetrexed, methotrexate, Ara-C, 5-Fu, wortmannin, gemcitabin, geldanamycin or a combination thereof.

[0135] In another embodiment, the methods provided herein comprise: a) administering to a patient in need thereof, a dose of about 1 mg to 20 mg of a CRF conjugate; and b) administering a therapeutically effective amount of a supportive care agent.

[0136] The term "the supportive care agent" refers to any substance that treats, prevents or manages an adverse effect from CRF treatment.

[0137] The supportive care agent is any substance that treats, prevents or manages an adverse effect from CRF treatment and is administered according to the appropriate dosing regimen for that substance. For example, different supportive care agents for treating nausea have different dosing regimen. While some are administered prophylactically, others are co-administered with a CRF conjugate while still others are administered after the administration of a CRF conjugate. Illustrative examples of supportive care agents their doses and dosing regimens are found in The Physician's Desk Reference.

5.5 Methods of Treating Edema

[0138] The present invention is also directed to methods of treating edema. The methods described herein include methods of treating edema comprising administering to a patient in need thereof a pharmaceutical composition comprising a CRF conjugate. In certain embodiments the CRF conjugate is CRF chemically modified with polyethylene glycol.

[0139] The present invention is also directed to methods of treating brain edema comprising administering CRF conjugate, wherein the conjugate is administered in a treatment regimen as a steroid sparing agent facilitating steroid taper.

[0140] In certain embodiments the methods described herein include methods for managing brain edema in a patient in need thereof comprising administering to the patient a therapeutically effective amount of a CRF conjugate and a steroid, wherein said method provides a steroid sparing effect. The CRF conjugates described here can be co-administered with any steroid including glucocorticoids, which are a class of steroid hormones characterized by an ability to bind with the cortisol receptor. Glucocorticoids steroids include hydrocortisone, cortisone acetate, prednisone, prednisolone, methylprednisolone, dexamethasone, betamethasone, triamcinolone, beclometasone, fludrocortisone acetate, aldosterone and deoxycorticosterone acetate.

[0141] In other embodiments, the methods described herein include methods for treating brain edema comprising a treatment regimen comprising administering to in a patient in need thereof a steroid in combination with a CRF conjugate, whereby total exposure to the steroid is reduced by the administration of the CRF conjugate.

[0142] The present invention also includes methods for providing replacement therapy for steroid therapy in a subject receiving such therapy, said method comprising administration of a steroid-sparing amount of CRF conjugate.

[0143] The total daily dose of the CRF conjugates described herein, such as CRF chemically modified with polyethylene glycol, can range from 1 μg to 10 mg. In certain embodiments the total daily dose of CRF conjugate can be 0.1 mg to 5 mg, or 0.3 mg to 2 mg. For example, the total daily dose of CRF chemically modified with polyethylene glycol can be about 0.3 mg, about 0.5 mg, about 1 mg, about 2 mg, about 4 mg or about 5 mg. The CRF conjugate can be administered once a day or multiple times a day until the desired daily dose of the CRF conjugate is reached. For example, 0.5 mg or 1.0 mg of a CRF conjugate can be administered 4 times a day to achieve a total daily dose of 2 mg or 4 mg of the CRF conjugate.

[0144] Examples of routes of administration of the CRF conjugate include parenteral routes such as, but not limited to, intradermal, subcutaneous, and intramuscular injections, and intravenous or intraosseous infusions. The compositions of the present invention can take the form of solutions, suspensions, emulsions that include a CRF conjugate, such as CRF chemically modified with polyethylene glycol, and a pharmaceutically acceptable diluent, adjuvant or carrier, depending on the route of administration.

[0145] In certain embodiments the CRF conjugates described herein can be administered by subcutaneous injection in an amount of 0.1 μg/kg to 1000 μg/kg. CRF conjugates can be administered subcutaneously in an amount of 1 μg/kg to 500 μg/kg, or 2 μg/kg to 100 μg/kg, or 2 μg/kg to 80 μg/kg, or 4 μg/kg to 40 μg/kg, or 5 μg/kg to 20 μg/kg. For example, CRF conjugates can be administered in 10 μg/kg, 30 μg/kg, 60 μg/kg, 100 μg/kg and 300 μg/kg doses.

[0146] In other embodiments, the CRF conjugates described herein can be administered by subcutaneous injection in an amount of 1 μg to 100 mg. CRF conjugates can be administered subcutaneously in an amount of 1 μg to 80 mg, 10 μg to 50 mg, 100 μg to 40 mg, 300 μg to 10 mg, 600 μg to 1 mg, and 800 μg to 1 mg. For example, CRF conjugates can be administered subcutaneously in 100 μg, 300 μg, 600 μg, 1 mg, 2 mg, 4 mg and 5 mg doses.

[0147] The CRF conjugates administered subcutaneously can be administered once a day or multiple times a day. For example, the dosages of CRF conjugates administered subcutaneously can be administered every hour, every two hours, every three hours, every four hours, every six hours, every eight hours or every 12 hours. Alternatively, the CRF conjugates can be administered once every two, three, four, five or six days. In certain embodiments the CRF conjugates can be administered once a week, once every two, three or four weeks or once a month. Dosages of CRF conjugates that are administered once a week or longer can be administered in the form of a depot.

[0148] In still other embodiments the CRF conjugates can be administered by intravenous infusion in an amount of 0.1 μg/kg/h to 100 μg/kg/h. For example, CRF conjugates can be administered intravenously in an amount of 1 μg/kg/h to 100 μg/kg/h, or 2 μg/kg/h to 80 μg/kg/h, or 2 μg/kg/h to 50 μg/kg/h, or 4 μg/kg/h to 40 μg/kg/h, or 5 μg/kg/h to 20 μg/kg/h.

[0149] In other embodiments the CRF conjugates can be administered intravenously in an amount of 1 μg/kg to 1000 μg/kg. For example CRF conjugates can be administered intravenously in an amount of 1 μg/kg to 100 μg/kg, or 2 μg/kg to 80 μg/kg, or 2 μg/kg to 50

μg/kg, or 4 μg/kg to 40 μg/kg, or 5 μg/kg to 20 μg/kg. For example, CRF conjugates can be administered in 0.5 μg/kg to 1 μg/kg, or 2 μg/kg to 8 μg/kg, or 4 μg/kg to 8 μg/kg, or 5 μg/kg doses.

[0150] The CRF conjugates described herein can be administered intravenously over a period of an hour or less than an hour. In certain embodiments the CRF conjugates can be administered intravenously over a period of one hour or more. For example, the dosages of CRF chemically modified with polyethylene glycol administered intravenously, discussed above can be administered over a period of 10 min., 30 min., 45 min., one hour, two hours, four hours, eight hours, 12 hours, 24 hours, 48 hours or 72 hours.

5.5.1. Dosing Regimens

[0151] Dosing regimens include administration of the CRF conjugates of the invention every other day or once weekly to a patent suffering from edema resulting from disease or injury to the brain or nervous system.

5.5.2. Pharmaceutical Compositions

[0152] The present invention relates to pharmaceutical compositions containing a CRF conjugate as the active ingredient. The CRF conjugate may be formulated with a pharmaceutically acceptable carrier. Due to the increased half-life of the CRF conjugate, the pharmaceutical compositions may contain a lower dose of CRF than typically administered to effectively treat edema. The pharmaceutical formulations of the invention may be formulated for parenteral administration, including, but not limited to, intradermal, subcutaneous, and intramuscular injections, and intravenous or intraosseous infusions. The pharmaceutical formulations of the present invention can take the form of solutions, suspensions, emulsions that include a CRF conjugate, such as CRF chemically modified with polyethylene glycol, and a pharmaceutically acceptable diluent, adjuvant or carrier, depending on the route of administration.

[0153] The pharmaceutical compositions of the invention are formulated to deliver a therapeutic dose of the CRF conjugate of the invention. The dose of the CRF conjugates contained in pharmaceutical formulation can range from 1 μg to 10 mg. In certain embodiments the dose of the CRF conjugate can range from 0.1 mg to 5 mg, or 0.3 mg to 2 mg. In certain embodiments, the dose of the CRF conjugate can be about 0.3 mg, about 0.5 mg, about 1 mg, about 2 mg, about 4 mg or about 5 mg.

[0154] The present invention is also directed to methods of treating edema by administering to a patient in need thereof a CRF conjugate and an additional therapeutic

agent. The additional therapeutic agent can be any agent that can alleviate edema or when in combination with the CRF conjugate, improve the conjugate's effect on edema or wherein the CRF conjugate can improve the effect of the additional therapeutic agent on the edema.

[0155] Suitable additional therapeutic agents include anti-inflammatory agents such as, but not limited to, corticosteroids. Corticosteroids include glucocorticoids and mineralocorticoids such as alclometasone, aldosterone, amcinonide, beclometasone, betamethasone, budesonide, ciclesonide, clobetasol, clobetasone, clocortolone, cloprednol, cortisone, cortivazol, deflazacort, deoxycorticosterone, desonide, desoximetasone, desoxycortone, dexamethasone, diflorasone, diflucortolone, difluprednate, fluclorolone, fludrocortisone, fludroxycortide, flumetasone, flunisolide, fluocinolone acetonide, fluocinonide, fluocortin, fluocortolone, fluorometholone, fluperolone, fluprednidene, fluticasone, formocortal, halcinonide, halometasone, hydrocortisone/cortisol, hydrocortisone aceponate, hydrocortisone buteprate, hydrocortisone butyrate, loteprednol, medrysone, meprednisone, methylprednisolone, methylprednisolone aceponate, mometasone furoate, paramethasone, prednicarbate, prednisone, prednisolone, prednylidene, rimexolone, tixocortol, triamcinolone, ulobetasol or combinations thereof.

[0156] Suitable additional agents also include diuretics such as loop diuretics, osmotic diuretics proximal diuretics, distal convoluted tubule diuretics and cortical collecting tubule diuretics. For example, suitable diuretics include, but are not limited to, glucose, mannitol, bumetanide, ethacrynic acid, furosemide, torsemide, amiloride, spironolactone, triamterene, bendroflumethiazide, hydrochlorothiazide, acetazolamide, dorzolamide, Phosphodiesterase, chlorthalidone, caffeine, metolazone or a combination thereof.

[0157] Additional agents that can be co-administered with the CRF conjugate include anti-neoplastic, anti-proliferative, anti-miotic agents such as paclitaxel, 5-fluorouracil, cisplatin, vinblastine, vincristine, epothilones, methotrexate, azathioprine, adriamycin and mutamycin; endostatin, angiostatin and thymidine kinase inhibitors, cladribine, taxol and its analogs or derivatives, paclitaxel as well as its derivatives, analogs or paclitaxel bound to proteins.

[0158] Additionally, the CRF conjugates described herein can be co-administered with other anti-cancer treatments such as, radiotherapy, chemotherapy, photodynamic therapy, surgery or other immunotherapy.

[0159] The CRF conjugate and the additional therapeutic agent can be administered sequentially or simultaneously. If administered sequentially, the order of administration is flexible. For instance, the CRF conjugate can be administered prior to administration of the

additional therapeutic agent. Alternatively, administration of the additional therapeutic agent can precede administration of the CRF conjugate.

[0160] Whether they are administered as separate compositions or in one composition, each composition is preferably pharmaceutically suitable for administration. Moreover, the CRF conjugate and the therapeutic agent, if administered separately, can be administered by the same or different modes of administration.

6. EXAMPLES

6.1 Syntheses of CRF Conjugates

[0161] The CRF conjugates of the invention can be readily synthesized using synthetic methods known in the art. The following synthetic examples demonstrate the syntheses of CRF-PEG conjugates, including CRF-PEG conjugates of cysteine added variants of CRF.

6.1.1. Example 1: PEGylation of the CRF Lysine Residue

[0162] The alkylation of the ε-amino group of the lysine residue in hCRF can be accomplished via reductive alkylation using PEG-propionaldehyde as the PEGylation agent. Human-CRF (1 mg) is stirred with an excess of PEG-propionaldehyde (3 mg) and a slight molar excess of sodium cyanoborohydride at room temperature in pH 9 borate buffer. High pH is used to avoid reduction of the aldehyde before Schiff base formation. In order to isolate the desired CRF-PEG conjugate, the mixture undergoes dialysis against phosphate buffered saline. In a system consisting of 8% dextran T-40, 6% PEG 8000, 0.15 M NaCl, and 0.010 M sodium phosphate pH 7.2, the CRF-PEG conjugate migrates to the top phase, while the unmodified CRF migrates to the bottom phase. The desired CRF-PEG conjugate may be further isolated by gel filtration chromatography.

[0163] Acylation of human-CRF with a polyethylene glycol group can be done using a PEG activated NHS ester. Human-CRF is solubilized (2-4 mg/mL) in 50 mM Bicine buffer. To the buffered hCRF solution is added to 10-20 molar equivalents of the PEG activated NHS ester. The reaction is stirred for 1 hour at room temperature. Upon completion of the reaction, the desired CRF-PEG conjugate may be isolated by gel filtration chromatography.

6.1.2. Example 2: PEGylation of Cysteine Added Variants of CRF

[0164] As discussed in section **5.2.3** there are a number of reagents that can be employed to covalently bind a cysteine residue to polyethylene glycol. This example employs PEG-maleimide or maleimido-PEG as the PEGylation reagent. A cysteine added

variant of CRF is diluted to 200 μg/mL in 20 mM Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) pH 6.75 buffer, 0.6 M NaCl, and 1% glycerol. Maleimido-PEG (1μl) is dissolved in a 10μl buffer composed of 20 mM Tris pH 7.4, 0.1M NaCl, and 0.01% Tween. The maleimdo-PEG may be diluted until the desired concentration is reached for reaction, and then it is added to the solution of CRF. Up to a 20-fold excess of maleimido-PEG may be used. The reaction is allowed to occur at room temperature for one hour, but the reaction may also occur at 4°C with longer reaction times. Upon completion the resulting cysteine added variant CRF-PEG conjugate may be purified by gel filtration chromatography.

6.1.3. Example 3: PEGylation of the cys-hCRF-cys via Disulfide Bond Bridging

[0165] To cys-hCRF-cys, which has cyclized via formation of a disulfide bond between the two cysteine residues, is added aqueous urea solution 2-mercaptoethanol. The pH of the resulting solution is adjusted to pH 8.5 using a 10% aqueous solution of methylamine. The reaction solution is then bubbled with nitrogen for approximately 30 min. Still purging with nitrogen the tube is heated at 37°C. The reaction mixture is then cooled in an ice-salt water bath and 10 mL of an argon purged chilled solution of 1N HCl:absolute ethanol is added to the reaction solution. A precipitation occurs and the precipitate is isolated by centrifugation and then washed three times with further portions of the HCl:absolute ethanol mixture and twice with nitrogen purged chilled diethyl ether. After each washing the precipitate is isolated by centrifugation. The washed precipitate is then dissolved in nitrogen purged deionized water and freeze-dried to afford a dry solid. Partial reduction of cys-hCRF-cys may be confirmed and quantitated using Ellman's Test, which gives the number free thiols per protein molecule.

[0166] Alternatively, the disulfide bond can be reduced using phosphine-containing reducing reagent, including tributylphosphine. When such a reducing reagent is used, the conjugation of the reduced cys-hCRF-cys and α-methoxy-ω-4-[2,2-bis[(p-tolylsulfonyl)-methyl]acetyl]benzamide derived from poly(ethylene)glycol can be carried out in the presence of the phosphine-containing reducing reagent.

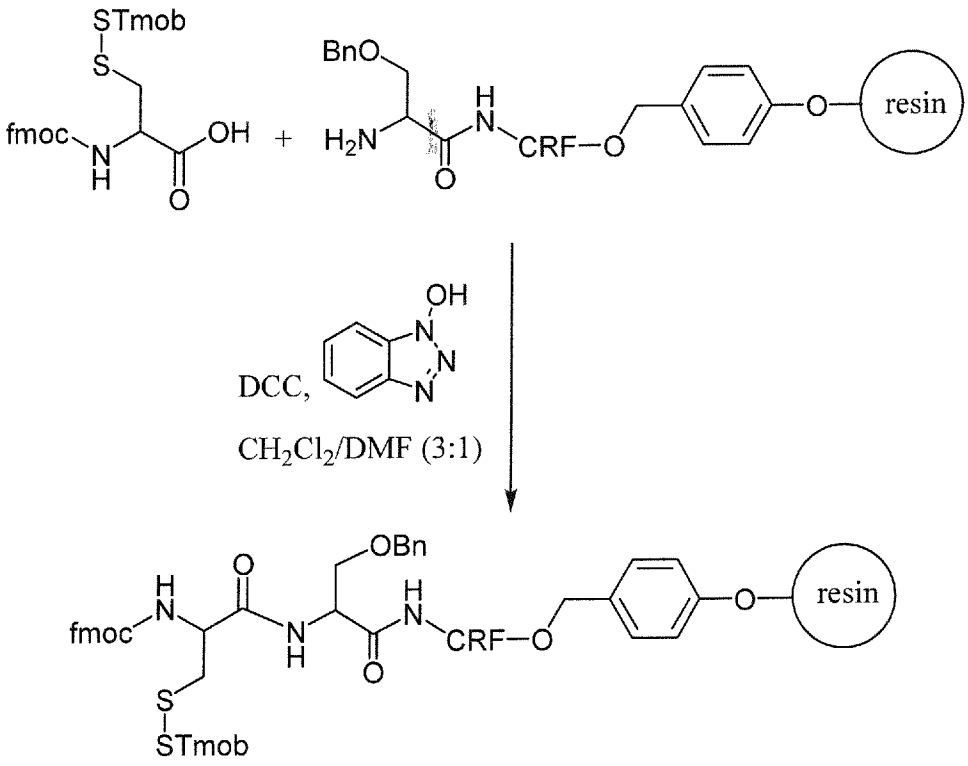
[0167] In an eppendorf, the partially reduced cys-hCRF-cys is dissolved in argon purged pH 8 ammonia solution. In a separate eppendorf, the polymer conjugating reagent, α-methoxy-ω-4-[2,2-bis[(p-tolylsulfonyl)-methyl]acetyl]benzamide derived from poly(ethylene)glycol is also dissolved in ammonia solution and the resulting solution is added to the Factor IX solution. The PEG eppendorf is washed with fresh ammonia solution and this

is also added to the main reaction eppendorf. The reaction eppendorf is then closed under argon and heated at 37°C for approximately 24 h and then allowed to cool to room temperature. The cooled reaction solution is then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

6.1.4. Example 4: Solid Phase Synthesis of a N-terminal Cysteine Added Variant of CRF

[0168] Synthesis of cysteine added variants of CRF can be made via solid phase peptide synthesis techniques. A cysteine residue may be inserted at the N-terminus of CRF at the last step of the synthesis as shown in scheme **3**.

Scheme 3



[0169] The *N*-terminus of unmodified hCRF is a serine residue, so it is to the α-amino group of serine that a cysteine residue is bound. A cysteine residue protected by S-2,4,6-trimethoxybenzyl (Tmob) is added to a solution of N-terminal deprotected CRF in a solution of dichloromethane/DMF in a ratio of 3:1. The coupling reaction can be monitored by the ninhydrin test for completion. Once complete, the solid phase is washed with

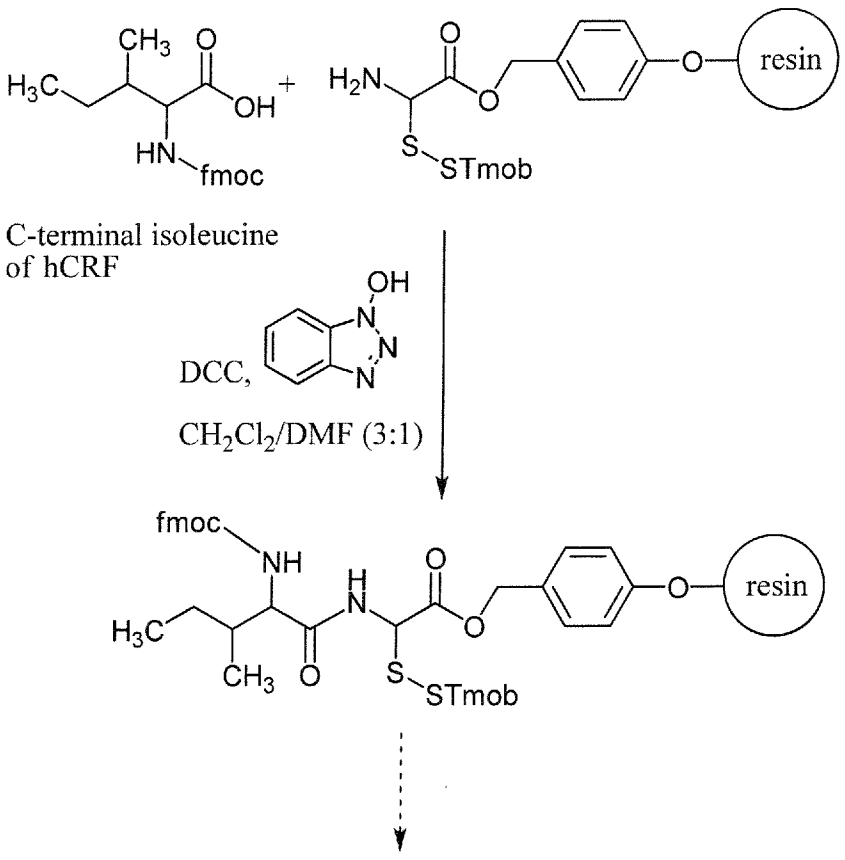
dichloromethane and methanol, and an additional wash with DMF can be performed after this coupling step, to yield the solid phase coupled intermediate above.

[0170] In this example, cysteine is the last amino acid added. Once coupled, removal from the solid support is accomplished with anhydrous trifluroacetic acid, followed by universal deprotection of all of the protecting groups on side chains, yielding an N-terminal cysteine added variant of CRF. The final polypeptide can be isolated by gel filtration chromatography. Insertions and substitutions of additional cysteine residues may be accomplished by similar preparations in the desired locations of the CRF polypeptide.

6.1.5. Example 5: Solid Phase Synthesis of a C-terminal Cysteine Added Variant of CRF

[0171] Synthesis of cysteine added variants of CRF can be made via solid phase peptide synthesis techniques. A cysteine residue may be inserted at the C-terminus of CRF at the first step of the synthesis as shown in scheme **4**.

Scheme 4



[0172] The C-terminus of unmodified hCRF is an isoleucine residue, so it is to the α-amino group of the C-terminal cysteine that an isoleucine residue is bound. A cysteine residue protected by S-2,4,6-trimethoxybenzyl (Tmob) is attached to the resin polymer. Isoleucine is added to the solution with DCC and 1H-benzo[d][1,2,3]triazol-1-ol in dichloromethane/DMF in a ratio of 3:1. The coupling reaction can be monitored by the ninhydrin test for completion. Once complete, the solid phase is washed with dichloromethane and methanol, and an additional wash with DMF can be performed after this coupling step, to yield the solid phase coupled intermediate above.

[0173] The sequential addition of amino acid residues is continued until the synthesis of the desired CRF variant is complete.

6.2 Human CRF Assay

[0174] The CRF conjugates of the invention have one or more of the biological activities of unmodified CRF. The biological activity of the CRF conjugates may be determined using the bioassays described herein. The CRF conjugates may have the same level of biologic activity as compared to unmodified CRF. Alternatively, the CRF conjugates may have lower levels of biologic activities when compared to unmodified CRF.

[0175] The following is a bioassay for CRF. The bioassay is to be based upon the binding of radio-labeled human CRF to its receptor on the cellular membrane of AtT-20 cells, a mouse pituitary cell line, or cells derived from the AtT-20 parental cell line. The assay is a competitive binding radio-receptor assay (RRA) that can discriminate between human CRF and closely related molecules. Whole cells or homogenized cell membrane preparations may be used in the assay. A competitive binding RRA was developed using 100 µl of membrane preparation, 100 µl of radio-labeled human CRF as tracer, and 100 µl of either buffer or competitor. The data obtained is expressed as Percent B/Bo, where B is the corrected CPM for the sample and Bo is the corrected CPM for the total binding tubes (i.e. no competitor).

[0176] This bioassay for CRF is based upon the ability of known membrane receptors for CRF to bind ^{125}I-Tyr^{0}-hCRF and to be displaced by unlabeled competitors. This type of assay is typically called a competitive binding radio-receptor assay (RRA). The unlabeled competitors that are of interest are different batches of hCRF (active drug substance), different lots of formulated drug product that contain hCRF, and CRF-related molecules, such as potential impurities in the active drug substance and known degradation products. Based upon the published literature, various cell lines have been found to express one or more of the

CRF receptor subtypes and have been used to measure the effects of CRF, CRF-related peptides, and various agonists and antagonists. For example, AtT-20 cells, a mouse anterior pituitary cell line, has been reported to express only CRF R1, and when CRF binds, an accumulation in intracellular cAMP and an increase in ACTH secretion are observed.

[0177] The physiologic effects of CRF are mediated by two G-protein coupled receptors which are the products of two different genes – CRF Receptor Type 1 (CRF R1) and CRF Receptor Type 2 (CRF R2). The two types of receptors share ~70% sequence homology and both are coupled to adenylate cyclase. However, the two types of receptors have different tissue distributions and bind ligands with different affinities. In addition to CRF, three CRF-related peptides have been discovered in mammals that bind to these receptors: urocortin (Ucn), Ucn II, and Ucn III which is also known as stresscopin. CRF plays a central role in the control of the hypothalamic-pituitary-adrenal axis under stress. Ucn is a 40-amino acid long peptide with 45% sequence homology to CRF that has been cloned from the Edinger-Westphal nucleus, and Ucn II (with 26% sequence homology to CRF) and III have been identified in human and mouse genomic data banks, and all have potent effects on appetite and on the cardiovascular system. All three Ucn's have approximately 10 fold higher affinity for CRF R2 than does CRF, and Ucn II and III are highly selective for CRF R2 since they have little affinity for the CRF R1 subtype. The CRF R2 has at least two and possibly three different splice variants – CRF R2α and CRF R2β and maybe CRF R2γ – which are expressed in different tissues and organs. In rats CRF R2α is predominately found in the brain including the hypothalamus, lateral septum, raphe nuclei of the mid-brain, olfactory bulb, and pituitary. In contrast, CRF R2β is predominately found in the heart, blood vessels, GI tract, and cardiac and skeletal muscle. In addition to the receptors, a CRF binding protein has been described that binds native CRF with a higher affinity than do any of the cellular receptors. The CRF binding protein is expressed in the brain and it might function as a regulator of CRF-mediated neurotransmission.

[0178] CRF and CRF-related peptides exert their effect through a cAMP-dependent protein kinase (PKA) pathway in the anterior pituitary and in AtT-20 cells. The connection between the changes in the intracellular cAMP concentration and the stimulation of ACTH secretion results from the interaction between cAMP and the concentration of free calcium ion in the cytosol. In these secretory cells, cAMP plays two major roles (1) to increase the influx of calcium ion into the cell which stimulates secretion and (2) to potentiate the effects of the increased intracellular calcium level on the secretory apparatus. CRF is reported to be

specific for activation through its interaction with CRF R1 type receptors: as reported in the literature, it does not activate cells through either the CRF R2α or CRF R2β receptor subtypes.

6.2.1. Materials Used and Methods Developed

[0179] 1. Cells used – AtT-20 and Att-20/D16v-F2 cells were purchased from ATCC (total cost = $493.00). During culture expansion, it became apparent that the AtT-20 cells grew not as single cells in suspension or attached but as "clumps of cells" in suspension. It also became apparent that dispensing these clumps evenly into assay tubes was very difficult. Therefore, AtT-20 cells were cloned by limiting dilution, and selected clones that grew as single cells (not as clumps) either in suspension or lightly-attached. The AtT-20 cells were successfully cloned, and 4 different clones were isolated (clones 1A10, 1G4, 2B8, and 2H1) that grew as single cells either lightly attached or in suspension

[0180] 2. Culture conditions – Initially all the cell lines and clones were grown in 90% DMEM with high glucose, 10% FCIII (HiClone Labs), containing penicillin and streptomycin, and pH adjusted to pH 7.2 with 4 M NaOH in a humidified atmosphere of 5% CO_2. After the first series of binding experiments were not successful, an alternative culture condition was investigated: 45% DMEM with high glucose, 45% Ham's F-12, 10% FCIII, containing penicillin and streptomycin, and pH adjusted to pH 7.2 with 4 M NaOH in a humidified atmosphere of 10% CO_2. When the binding of ^{125}I-Tyr^0-hCRF was assessed on cells grown under these modified conditions, binding was achieved and displacement with unlabeled competitor was also achieved with both ^{125}I-Tyr^0-human CRF and ^{125}I-Tyr^0-ovine CRF as tracer. All subsequent experiments were performed with cells grown under these conditions.

[0181] 3. Preparation of ^{125}I-Tyr^0-CRF – New lots of human Tyr^0-CRF (Tyr^0-hCRF) and ovine Tyr^0-CRF (Tyr^0-oCRF) were purchased from Bachem Bioscience (total cost = $842.82). The lyophilized powder was dissolved in 500 µL acetonitrile:water (1:1/v:v = 50% AcCN) and aliquoted in 2 µg, 10 µg, 50 µg, and 100 µg portions into tubes containing 5 µL, 10 µL, 50 µL, and 100 µL of 0.1 M sodium phosphate buffer pH 7.2, respectively. The samples were frozen on dry ice and re-lyophilized. Prior to radio-labeling, polypropylene microfuge tubes are coated with 20 µg of iodogen (Pierce Chem Co.) in 20 µL of dichloromethane and dried under vacuum. The radio-iodination reaction is performed in a chemical fume hood equipped with an activated charcoal filter system. Prior to starting the reaction, a 2 µg sample of Tyr^0-CRF is dissolved in 40 µl of acetonitrile:water (1:3/v:v =

25% AcCN), and 0.2 nmol of Tyr^0-CRF is transferred into an iodogen tube containing 20 µl of 0.1 M sodium phosphate buffer pH 7.2 and 500 µCi of carrier free $Na^{125}I$. The reaction is incubated at room temperature for 15 minutes with occasional mixing before the reaction mixture is transferred to the top of a 5 mL BioGel P-2 desalting column that has been washed and equilibrated with acetic acid:water (1:1/v:v = 50% AcOH). The ^{125}I-Tyr^0-CRF is eluted from the column with 50% AcOH and 0.5 mL fractions are collected. The radio-labeled peptide elutes immediately after the void volume of the column in fractions #4 and 5. The two fractions are pooled and the radio-labeled peptide is used without further purification or is purified by reverse phase HPLC if monoiodo-peptide is desired.

[0182] 4. RP-HPLC purification of ^{125}I-Tyr^0-hCRF – A C_8 or C_{18} RP-HPLC column is thoroughly equilibrated with 0.1% TFA in water; a 100 µl aliquot of the pooled radio-labeled peptide obtained from the desalting column is diluted to 1.0 mL with D. H_20 and immediately transferred to a 2.0 mL injection loop on a manual Rheodyne HPLC injector; and the flow thorough the injector is changed to inject the diluted ^{125}I-Tyr^0-hCRF onto the column. After the column is loaded, a linear gradient program of 0% to 80% acetonitrile in water over 40 minutes is started to elute the bound peptide; the radioactive flow counter is started; and fractions are collected for 0.5 min.

[0183] 5. Competitive binding radio-receptor assay for CRF using cell membrane preparations—Isolated membrane preparations, since the CRF R1 receptor is associated with the cellularcan, can be used in the assays. Cells are grown and isolated from T-75 flasks as described above, except after collection by centrifugation, they are resuspended in PBS with 1% BSA that contains 20 µg/mL aprotinin (Serologics) as a general protease inhibitor since when the cells are homogenized a number of intracellular proteases will be released.

[0184] The cell pellet is resuspended in a small volume (1.5 – 2.0 mL) of ice-clod PBS with 1%BSA and 20 µg/mL aprotinin, transferred to a 15 mL glass Dounce homogenizer fitted with a tight pestle on ice, and homogenized by 15 strokes with grinding. The lysed cells are transferred to microfuge tubes and centrifuged at 16,000 x g for 15 min at 4 °C to collect the particulate membrane fraction. The supernatant is discarded, the particulate fraction is washed by resuspending it in the same ice-cold buffer, and collecting the washed particulate membrane fraction by centrifugation. The membrane fraction is resuspended to a volume equal to 5 x 10^6 cells per mL based upon the number of cells originally isolated and homogenized.

[0185] The assay reaction is set up as described above (see 5.b) except 100 µl of suspended particulate fraction is used instead of whole cells and the buffer is PBS with 1% BSA and 20 µg/mL aprotinin. The protease inhibitor is included to protect the labeled tracer and competitors from degradation during the overnight incubation. The use of the particulate fraction has improved the reproducibility of the assay in which the displacement of trace by unlabeled CRF is measured in the particulate fraction obtained from the 4 different clones we isolated.

[0186] Once the RRA was performing as originally expected, it was tested using the same three competitors used in previous experiments on this project. A cell membrane preparation from clone 1A10 was prepared as described above and tested for its ability to discriminate between different molecules by displacement of the radio-labeled tracer. Concentrations ranging from 10 ng/tube to 3160 ng/tube of human CRF, ovine CRF and the unrelated peptide VIP were assayed for their ability to displace the ^{125}I-Tyr^{0}-human CRF tracer from its bound membrane association.

[0187] In accordance with the invention, the CRF conjugates of the invention have one or more of the biological activities of unmodified CRF, e.g. the ability to competitively bind to the CRF receptor. However, the CRF conjugates may demonstrate differing levels of activity to unmodified CRF.

6.3 Determination of the Pharmacokinetic Profile of CRF Conjugates

[0188] The CRF conjugates of the invention have an improved pharmacokinetic profile as compared to unmodified CRF. The CRF conjugates of the invention may show an improvement in one or more parameters of the pharmacokinetic profile, including AUC, C_{max}, clearance (CL), half-life, and bioavailability as compared to unmodified CRF. The following is an example of the determination of the pharmacokinetic profile of unmodified CRF when administered subcutaneously and intravenously.

[0189] The objective of this study was to determine the plasma concentration time profile of hCRF following a single intravenous and a single subcutaneous injection in three groups of Sprague-Dawley Crl:CD®BR rats. Concentrations of hCRF in the vehicle (5% mannitol/20mM pH 4.0 acetate buffer) were 10,100, and 1,000 µg/mL. A dosage volume of 1 mL/kg for all groups resulted in administered doses of 10,100, and 1,000 µg/kg of hCRF for all three dose groups. For the intravenous portion of the study, each of the three dose groups consisted of 72 males. Each of these groups was divided into three sets of replicates. Seven days after the intravenous portion of the study, 61 of the 72 animals from each of the

three dose groups were randomly selected for the subcutaneous portion of the study. Each dose group was divided into three sets of replicates. Blood samples were taken at multiple time points via orbital sinus bleeding. Following intravenous dosing, blood samples were collected at time points out to 24 hours post dose. Following subcutaneous dosing, blood samples were collected at time points out to 48 hours post dose. Blood samples were collected from three rats in each dose group for each time point. One animal in the 10 μg/kg group died during the blood collection following the intravenous administration of hCRF. All of the surviving animals were euthanized on the third day following subcutaneous dosing.

[0190] Plasma samples were prepared and hCRF concentrations in the plasma samples were determine by an ELISA method. The clearance of intravenously administered hCRF in the rat followed a single exponential pattern and the half-lives were determined to be 9.2, 20.7 and 26.7 minutes for doses of 10,100, and 1,000 μg/kg, respectively. The pharmacokinetics of hCRF administered either intravenously or subcutaneously is dose proportional between 100 and 1,000 μg/kg. At the 10 μg/kg intravenous dose level, the measured hCRF in plasma concentrations approached the detection limits of the ELISA assay. Pharmacokinetic analyses were conducted for this dose group using the limited data obtained. The pharmacokinetic values for the 10 μg/kg intravenous dose group differ from those for the 100 and 1,000 μg/kg groups. This may be a function of the limitations of the ELISA assay at these low levels, and/or may be due to the saturation of potential binding sites for hCRF at the higher doses.

[0191] The bioavailability of the subcutaneously administered hCRF at dose level of 100 and 1,000 μg/kg was calculated to be 41% and 37% respectively. In the 10 μg/kg subcutaneous dose group, the measured plasma concentrations were relatively low and approached the detection limit of the assay. A summary of several pharmacokinetic parameters is presented in Table 1 below.

Table 1

Dose (μg/kg)	AUC (μg/mL-min)	C_{max} (ng/mL)	CL (mL/min/kg)	$t_{1/2}^{\alpha}$ (min)	$t_{1/2}^{\beta}$ (terminal min)	Bioavail. %
10 (IV)	0.33 ± 0.03	25.1 ± 3.8	30.00 ± 3.06	9.22 ± 1.05	NA	NA
100 (IV)	30.97 ± 2.50	1036.6 ± 105.1	3.23 ± 0.26	20.71 ± 0.92	NA	NA
1,000 (IV)	292.80 ± 15.58	7604.2 ± 488.3	3.42 ± 0.18	26.69 ± 0.62	NA	NA
100 (SC)	12.86 ± 1.67	108.2 ± 16.7	7.78 ± 1.01	6.96 ± 3.44	62.6 ± 5.9	41 ± 6
1000 (SC)	107.57 ± 12.18	618.1 ± 78.9	9.30 ± 1.05	21.56 ± 8.15	72.1 ± 14.8	37 ± 5

CLAIMS

1. A method for inhibiting tumor progression in a human patient with cancer comprising administering a CRF conjugate comprising CRF, wherein the CRF is chemically modified with polyethylene glycol.

2. The method of claim 1, wherein the CRF has the sequence identified as human CRF.

3. The method of claim 2, wherein the sequence of CRF has been modified to include a cysteine residue.

4. The method of claim 3, wherein the one of the amino acids of CRF is substituted with a cysteine residue.

5. The method of claim 3, wherein the cysteine residue is added to the amino terminus of CRF.

6. The method of claim 3, wherein the cysteine residue is added to the carboxy terminus of CRF.

7. The method of claim 4, 5, or 6, wherein the polyethylene glycol is covalently bound via the cysteine residue.

8. The method of claim 5, wherein a second cysteine residue is added to the CRF.

9. The method of claim 8, wherein the cysteine residue is added to the carboxy terminus of CRF.

10. The method of claim 8 or 9, wherein polyethylene glycol is covalently bound to both cysteine residues.

11. The method of claim 2, wherein the polyethylene glycol is covalently bound to CRF via a lysine residue.

12. The method of claim 1, wherein the CRF conjugate has a longer half-life as compared to unmodified CRF.

13. The method of claim 1, wherein the CRF conjugate as a higher AUC as compared to unmodified CRF.

14. The method of claim 1, wherein the CRF conjugate has a higher bioavailability as compared to unmodified CRF.

15. The method of claim 1, wherein the CRF conjugate is administered subcutaneously.

16. The method of claim 1, wherein the CRF conjugate is administered intravenously.

17. The method of claim 1, wherein the CRF conjugate is administered once a day.

18. The method of claim 1, wherein the CRF conjugate is administered at a dose from 0.1 to 5 mg.

19. The method of claim 1, wherein the CRF conjugate is administered at a dose from 1 to 2 mg.

20. The method of claim 1, wherein the CRF conjugate is administered in combination with another anti-cancer agent.

Human/Rat CRF	SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII
Ovine CRF	SQEPPISLDLTFHLLREVLEMTKADQLAQQAHSNRKLLDIA

FIG. 1