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(54) **LIGANDS DOUBLES DU RECEPTEUR A_VB_3 ET DU RECEPTEUR ASSOCIE AUX METASTASES**

(54) **DUAL A_VB_3 AND METASTASIS-ASSOCIATED RECEPTOR LIGANDS**

(57) L'invention porte sur des composés pharmaceutiques ligands doubles du récepteur a_vb_3 et du récepteur associé aux métastases, sur l'utilisation de ces ligands doubles, seuls ou en association avec d'autres agents, dans des préparations pharmaceutiques, et sur des méthodes de traitement d'états induits par l'a_vb_3 et du cancer et d'autres maladies angiogènes telles que la rétinopathie diabétique, l'arthrite, les hémangiomes et le psoriasis.

(57) The present invention relates to pharmaceutical compounds which are dual a_vb_3 receptor/metastasis-associated receptor ligands. The use of these dual ligands alone or in conjunction with other agents in pharmaceutical compositions, and in methods for treating conditions mediated by a_vb_3 for the treatment of cancer and other angiogenic diseases, such as diabetic retinopathy, arthritis, hemangiomas, and psoriasis, are also disclosed.

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(54) Title: DUAL A_vb_3 AND METASTASIS–ASSOCIATED RECEPTOR LIGANDS

(57) Abstract

The present invention relates to pharmaceutical compounds which are dual a_vb_3 receptor/metastasis–associated receptor ligands. The use of these dual ligands alone or in conjunction with other agents in pharmaceutical compositions, and in methods for treating conditions mediated by a_vb_3 for the treatment of cancer and other angiogenic diseases, such as diabetic retinopathy, arthritis, hemangiomas, and psoriasis, are also disclosed.

Dual A_vb_3 and metastasis-associated receptor ligands

Priority

The present application claims priority under Title 35, United States Code, § 119 of United States Provisional Application Serial No. 60/081,074 filed April 8, 1998.

Field of the invention

The present invention relates to pharmaceutical compounds which are dual a_vb_3 receptor/metastasis-associated receptor ligands. The use of these dual ligands alone or in conjunction with other agents in pharmaceutical compositions, and in methods for treating conditions mediated by a_vb_3 for the treatment of cancer and other angiogenic diseases, such diabetic retinopathy, arthritis, hemangiomas, and psoriasis, are also disclosed.

Background of the invention

Cancer

Angiogenesis, the growth of new blood vessels, plays an important role in cancer growth and metastasis. In humans, the extent of vasculature in a tumor has been shown to correlate with the patient prognosis for a variety of cancers (Folkman, J., *Seminars in Medicine of the Beth Israel Hospital, Boston* **333**(26): 1757-1763, 1995; Gasparini, G., *European Journal of Cancer* **32A**(14): 2485-2493, 1996; Pluda, J. M., *Seminars in Oncology* **24**(2): 203-218, 1997). In normal adults, angiogenesis is limited to well controlled situations, such as wound healing and the female reproductive system (Battegay, E.J., *J Mol Med* **73**:-333-346, 1995; Dvorak, H.F, *New Engl J Med*, **315**: 1650-1659, 1986).

Animal studies suggest that tumors can exist in a dormant state, in which tumor growth is limited by a balance between high rates of proliferation and high rates of apoptosis (Holmgren, L. et al., *Nat. Med.* **1**(2): 149-153, 1995; Hanahan, D. et al., *Cell* **86**(3): 353-364, 1996). The switch to an angiogenic phenotype allows tumor cells to escape from dormancy and to grow rapidly, presumably as the result of a decrease in the apoptotic rate of the tumor cells (Bouck, *Cancer Cells*, **2**(6):

179-185, 1990; Dameron et al, *Cold Spring Harb Symp Quant Biol,* **59**: 483-489, 1994). The control of angiogenesis is thought to be a balance between factors that promote new vessel formation and anti-angiogenic factors that suppress the formation of a neovasculature (Bouck, N. et al., *Advances in Cancer Research* **69**: 135-173, 1996; O'Reilly et al., *Cell* **79**(2): 315-328, 1994).

The role of angiogenesis in tumor metastasis and methods by which malignant angiogenesis can be inhibited have been reviewed (Zetter, B.R., *Ann Rev. Med.* **49**: 407-424, 1998; Jekunen, A.P., and K.J.A. Kairemo, *Cancer Treat Rev.* **23**: 263-286, 1997). The role of growth factors in angiogenesis, particularly in ocular diseases, and the role of angiogenesis in ischemic diseases have also been reviewed (Miller, J.W. and P.A. D'Amore, *Textbook of Ocular Pharmacology,* (eds. Zimmerman, T.J. et al.), Lipincott-Raven Publishers, Philadelphia, PA, pp. 455-470, 1997; Breier, G. et al., *Thromb. Haemostasis* **78**(1): 678-683, 1997).

A_vb_3 antagonists

Adhesion forces are critical for many normal physiological functions. Disruptions in these forces, through alterations in cell adhesion factors, are implicated in a variety of disorders, including cancer, stroke, osteoporosis, restenosis, and rheumatoid arthritis (A. F. Horwitz, *Scientific American*, **276**(5): 68-75, 1997).

Integrins are a large family of cell surface glycoproteins which mediate cell adhesion and play central roles in many adhesion phenomena. Integrins are heterodimers composed of noncovalently linked α and β polypeptide subunits. Currently eleven different α subunits have been identified and six different β subunits have been identified. The various α subunits can combine with various β subunits to form distinct integrins.

One integrin known as a_vb_3 (or the vitronectin receptor) is normally associated with endothelial cells and smooth muscle cells. A_vb_3 integrins can promote the formation of blood vessels (angiogenesis) in tumors. These vessels nourish the tumors and provide access routes into the bloodstream for metastatic cells.

The a_vb_3 integrin is also known to play a role in various other disease states or conditions including tumor metastasis, solid tumor growth (neoplasia), osteoporosis, Paget's disease, humoral hypercalcemia of malignancy, angiogenesis, including tumor angiogenesis, retinopathy, arthritis, including rheumatoid

arthritis, periodontal disease, psoriasis, and smooth muscle cell migration (e.g. restenosis).

Tumor cell invasion occurs by a three step process: 1) tumor cell attachment to extracellular matrix; 2) proteolytic dissolution of the matrix; and 3) movement of the cells through the dissolved barrier. This process can occur repeatedly and can result in metastases at sites distant from the original tumor.

The a_vb_3 integrin and a variety of other αv-containing integrins bind to a number of Arg-Gly-Asp (RGD) containing matrix macromolecules. Compounds containing the RGD sequence mimic extracellular matrix ligands and bind to cell surface receptors. Fibronectin and vitronectin are among the major binding partners of a_vb_3 integrin. Other proteins and peptides have also bind the a_vb_3 ligand. These include the disintegrins (M. Pfaff et al., *Cell Adhes. Commun.* **2**(6): 491-501, 1994), peptides derived from phage display libraries (Healy, J.M. et al., *Protein Pept. Lett.* **3**(1): 23-30, 1996; Hart, S.L. et al., *J. Biol. Chem.* **269**(17): 12468-12474, 1994) and small cyclic RGD peptides (M. Pfaff et al., *J. Biol. Chem.*, **269**(32): 20233-20238, 1994). The monoclonal antibody LM609 is also an a_vb_3 integrin antagonist (D.A. Cheresh et al., *J. Biol. Chem.*, **262**(36): 17703-17711, 1987).

A_vb_3 inhibitors are being developed as potential anti-cancer agents. Compounds that impair endothelial cell adhesion via the a_vb_3 integrin induce improperly proliferating endothelial cells to die. A_vb_3 inhibitors can also help patients suffering from proliferative retinopathy, a complication of diabetes in which the retina sprouts weak and leaky blood vessels that can destroy the retina and causes blindness, or from restenosis, a process in which blood vessels in many patients can become occluded again over a period of time after the balloon angioplastic surgery.

Osteoporosis is a disorder involving loss of bone and increased risk of bone fracture, that occurs with age, particularly in women. This disease can result from the overactivity of osetoclast cells that bind to bone and degrade bone. The osteoclast binding occurs via the a_vb_3 integrin. Specific inhibitors that shield a_vb_3 integrins can prevent the destructive cells from adhering to bone. Additionally, it has been found that such agents can be useful as antivirals, antifungals and antimicrobials. Thus, compounds which selectively inhibit or antagonize a_vb_3 can be beneficial for treating such conditions.

The a_vb_3 integrin has been shown to play a role in melanoma cell invasion (Seftor et al., *Proc. Natl. Acad. Sci. USA*, **89**: 1557-1561, 1992). The a_vb_3 integrin

expressed on human melanoma cells has also been shown to promote a survival signal, protecting the cells from apoptosis (Montgomery et al., *Proc. Natl. Acad. Sci. USA*, **91**: 8856-8860, 1994).

Mediation of the tumor cell metastatic pathway by interference with the a_vb_3 integrin cell adhesion receptor to impede tumor metastasis would be beneficial. Antagonists of a_vb_3 have been shown to provide a therapeutic approach for the treatment of neoplasia (inhibition of solid tumor growth) because systemic administration of a_vb_3 antagonists causes dramatic regression of various histologically distinct human tumors (Brooks et al., *Cell*, **79**: 1157-1164, 1994).

The adhesion receptor identified as integrin a_vb_3 is a marker of angiogenic blood vessels in chick and man. This receptor plays a critical role in angiogenesis or neovascularization. Angiogenesis is characterized by the invasion, migration and proliferation of smooth muscle and endothelial cells by new blood vessels. Antagonists of a_vb_3 inhibit this process by selectively promoting apoptosis of cells in the neovasculature. The growth of new blood vessels, also contributes to pathological conditions such as diabetic retinopathy (Adonis et al., *Amer. J. Ophthal.*, **118**: 445-450, 1994) and rheumatoid arthritis (Peacock et al., *J. Exp. Med.*, **175**: 1135-1138, 1992). Therefore, a_vb_3 antagonists can be useful therapeutic targets for treating such conditions associated with neovascularization (Brooks et al., *Science*, **264**: 569-571, 1994).

The a_vb_3 cell surface receptor is also the major integrin on osteoclasts responsible for the attachment to the matrix of bone. Osteoclasts cause bone resorption and when such bone resorbing activity exceeds bone forming activity, osteoporosis (a loss of bone) results, which leads to an increased number of bone fractures, incapacitation and increased mortality. Antagonists of a_vb_3 have been shown to be potent inhibitors of osteoclastic activity both *in vitro* (Sato et al., *J. Cell. Biol.*, **111**: 1713-1723, 1990) and *in vivo* (Fisher et al., *Endocrinology*, **132**: 1411-1413, 1993). Antagonism of a_vb_3 leads to decreased bone resorption and therefore assists in restoring a normal balance of bone forming and resorbing activity. Thus it would be beneficial to provide antagonists of osteoclast a_vb_3 which are effective inhibitors of bone resorption and therefore are useful in the treatment or prevention of osteoporosis.

The role of the a_vb_3 integrin in smooth muscle cell migration also makes an a_vb_3 a therapeutic target for prevention or inhibition of neointimal hyperplasia which is a leading cause of restenosis after vascular procedures (Choi et al., *J. Vasc. Surg.* **19**(1): 125-134, 1994). Prevention or inhibition of neointimal

hyperplasia by pharmaceutical agents to prevent or inhibit restenosis would be beneficial.

White has reported that adenovirus uses a_vb_3 for entering host cells (*Current Biology*, **3**(9): 596-599, 1993). The integrin appears to be required for endocytosis of the virus particle and may be required for penetration of the viral genome into the host cell cytoplasm. Thus compounds which inhibit a_vb_3 would find usefulness as antiviral agents.

PCT Int. Appl. WO 97/08145 by Sikorski et al., discloses meta-guanidine, urea, thiourea or azacyclic amino benzoic acid derivatives as highly specific a_vb_3 integrin antagonists.

PCT Int. Appl. WO 96/00574 A1 960111 by Cousins, R.D. et al., describe preparation of 3-oxo-2,3,4,5-tetrahydro-1H-1,4-benzodiazepine and -2-benzazepine derivatives and analogs as vitronectin receptor antagonists.

PCT Int. Appl. WO 97/23480 A1 970703 by Jadhav, P.K. et al. describe annelated pyrazoles as novel integrin receptor antagonists. Novel heterocycles including 3-[1-[3-(imidazolin-2-ylamino)propyl]indazol-5-ylcarbonylamino]-2-(benzyl oxycarbonylamino)propionic acid, which are useful as antagonists of the αvβ3 integrin and related cell surface adhesive protein receptors.

PCT Int. Appl. WO 97/26250 A1 970724 by Hartman, G.D. et al., describe the preparation of arginine dipeptide mimics as integrin receptor antagonists. Selected compounds were shown to bind to human integin a_vb_3 with EIB <1000 nM and claimed as compds. useful for inhibiting the binding of fibrinogen to blood platelets and for inhibiting the aggregation of blood platelets.

PCT Int. Appl. WO 97/23451 by Diefenbach, B. et al. describe a series of tyrosine-derivatives used as alpha v-integrin inhibitors for treating tumors, osteoporoses, osteolytic disorder and for suppressing angiogenesis.

PCT Int. Appl. WO 96/16983 A1 960606. by Vuori, K. and Ruoslahti, E. describe cooperative combinations of a_vb_3 integrin ligand and second ligand contained within a matrix, and use in wound healing and tissue regeneration. The compounds contain a ligand for the a_vb_3 integrin and a ligand for the insulin receptor, the PDGF receptor, the IL-4 receptor, or the IGF receptor, combined in a biodegradable polymeric (e.g. hyaluronic acid) matrix.

PCT Int. Appl. WO 97/10507 A1 970320 by Ruoslahti, E; and Pasqualini, R.

describe peptides that home to a selected organ or tissue in vivo, and methods of identifying them. A brain-homing peptide, nine amino acid residues long, for example, directs red blood cells to the brain. Also described is use of *in vivo* panning to identify peptides homing to a breast tumor or a melanoma.

PCT Int. Appl. WO 96/01653 A1 960125 by Thorpe, Philip E.; Edgington, Thomas S. describes bifunctional ligands for specific tumor inhibition by blood coagulation in tumor vasculature. The disclosed bispecific binding ligands bind through a first binding region to a disease-related target cell, e.g. a tumor cell or tumor vasculature; the second region has coagulation-promoting activity or is a binding region for a coagulation factor. The disclosed bispecific binding ligand may be a bispecific (monoclonal) antibody, or the two ligands may be connected by a (selectively cleavable) covalent bond, a chemical linking agent, an avidin-biotin linkage, and the like. The target of the first binding region can be a cytokine-inducible component, and the cytokine can be released in response to a leukocyte-activating antibody; this may be a bispecific antibody which crosslinks activated leukocytes with tumor cells.

Metastasis-associated receptor ligands

Interferon alpha (IFN alpha) is a family of highly homologous, species-specific proteins that possess complex antiviral, antineoplastic and immunomodulating activities (Extensively reviewed in the monograph "Antineoplastic agents, interferon alfa," American Society of Hospital Pharmacists, Inc., 1996, LEXIS file GENMED [General Medicine], DIF [Drug Information file]). Interferon alpha also has anti-proliferative, and anti-angiogenic properties, and has specific effects on cellular differentiation (Sreevalsan, *in* "Biologic Therapy of Cancer", pp. 347-364, (eds. V.T. DeVita Jr., S. Hellman, and S.A. Rosenberg), J.B. Lippincott Co, Philadelphia, PA, 1995).

Interferon alpha is effective against a variety of cancers including hairy cell leukemia, chronic myelogenous leukemia, malignant melanoma, and Kaposi's sarcoma. The precise mechanism by which IFN alpha exerts its anti-tumor activity is not entirely clear, and may differ based on the tumor type or stage of disease. The anti-proliferative properties of IFN alpha, which may result from the modulation of the expression of oncogenes and/or proto-oncogenes, have been demonstrated on both tumor cell lines and human tumors growing in nude mice (Gutterman, J. U., *Proc. Natl. Acad. Sci., USA* **91**: 1198-1205, 1994).

The effectiveness of interferon in treating neoplastic diseases, particularly melanoma has been reviewed (Agarwala, S.S. and J.M. Kirkwood, *Curr. Opin. Oncol.*, **8**(2): 167-174, 1996). The treatment of metastatic melanoma with chemotherapeutic drugs, such as cisplatin, combined with biologic agents such as interferon alpha and interleukin-2, referred to as "biochemotherapy" or "chemoimmunotherapy" has also been reviewed (Anderson, C.M. et al., *Prog. Anti-Cancer Chemother.* (eds Khayat, D., and G.N. Hortobagyi), pp. 68-87, Blackwell, Malden, MA, 1997; Atkins, M.B., *Curr. Opin. Oncol.* **9**(2): 205-213, 1997). The status of single agent and combination cytotoxic chemotherapeutic regimens for melanoma have extensively reviewed (Lee, S.M. et al., *Br. Med. Bull.* **51**(3): 609-630, 1995).

Interferon is also considered an anti-angiogenic factor, as demonstrated through the successful treatment of hemangiomas in infants (Ezekowitz et al, *N. Engl. J. Med., May 28,* **326**(22) 1456-1463, 1992) and the effectiveness of IFN alpha against Kaposi's sarcoma (Krown, *Semin Oncol* **14**(2 Suppl 3): 27-33, 1987). The mechanism underlying these anti-angiogenic effects is not clear, and may be the result of IFN alpha action on the tumor (decreasing the secretion of pro-angiogenic factors) or on the neovasculature. IFN receptors have been identified on a variety of cell types (Navarro et al., *Modern Pathology* **9**(2): 150-156, 1996). A general review on the structure and function of type I and type II interferon receptors has been recently published (Pestka, S., *Semin. Oncol.* **24**(3, Suppl. 9), 18-40, 1997).

United States Patent 4,530,901 to Weissmann, describes the cloning and expression of IFN alpha-type molecules in transformed host strains. United States Patent 4,503,035, Pestka, describes an improved processes for purifying 10 species of human leukocyte interferon using preparative high performance liquid chromatography. United States Patent 5,231,176 to Goeddel, describes the cloning of a novel distinct family of human leukocyte interferons containing in their mature form greater than 166 and no more than 172 amino acids.

United States Patent 5,541,293 to Stabinsky, describes the synthesis, cloning, and expression of consensus human interferons. These are non-naturally occurring analogues of human (leukocyte) interferon alpha assembled from synthetic oligonucleotides. The sequence of the consensus interferon was determined by comparing the sequences of 13 members of the IFN alpha family of interferons and selecting the preferred amino acid at each position. These variants differ from naturally occurring forms in terms of the identity and/or location of one or more amino acids, and one or more biological and

pharmacological properties (e.g., antibody reactivity, potency, or duration effect) but retain other such properties.

The structure of the many human interferon alpha species that have been characterized and that of hybrid proteins that have been generated by recombinant means has been recently reviewed (Pestka, S., *Semin. Oncol.* **24**(3, Suppl. 9), 4-17, 1997). International standards and designations of all the interferon species that have been characterized has also been reviewed (Pestka, S.; Meager, A. *J. Interferon Cytokine Res.* **17**:(Suppl. 1), S9-S14, 1997).

Chemical ligation of peptides

Significant advances in the understanding of structure/function relationships are due in large part to the powerful genetic engineering techniques that allow the rapid mutagenesis, expression, and purification, protein variants. One significant limitation, however, is that the proteins purified from recombinant organisms are comprised of the 20 naturally-occurring L-amino acids, occasionally modified at certain positions by post-translational processing.

Chemical methods of peptide synthesis are not subject to such limitations, and may be used for the preparation of proteins or peptides containing D-amino acids or other synthetic amino acid analogs. Many peptide variants and small proteins have been synthesized using the stepwise solid-phase synthetic method (R.B. Merrifield, *J. Am Chem. Soc.* **85**: 2149, 1963). This approach may not be appropriate, however, for peptides longer than 40 to 50 amino acid residues due to problems with side reactions and purification that increase with peptide length. Fragment condensation, where two protected peptides are prepared, purified, and then coupled to form a longer peptide, is an alternative method (H. Kuroda et al. *Int. J. Pept. Protein Res.* **40**: 294, 1992). Enzymatic ligation of cloned or synthetic peptide segments has also been used (L. Abrahmsen et al., *Biochemistry* 30: 4151, 1991).

A new chemical ligation method, termed the chemoselective method, avoids many of problems associated with other total- or semi-synthetic methods (L.H. Huang et al., *Biochemistry*, **30**: 7402, 1991; C.J.A. Wallace et al., *J. Biol. Chem.* **267**: 3852, 1992). With this approach, an unprotected peptide with C-terminal carboxylthioester can be linked to the N-terminus of the N-terminal cysteine containing unprotected peptide selectively (M. Schnolzer, and S. B. H. Kent, *Science* **256**: 221, 1992; C. F Liu, and J. P. Tam, *J. Am. Chem. Soc.* **116**: 4149, 1994). Several biologically active peptides and proteins have been prepared (L. E.

Canne et al., *J. Am. Chem. Soc.* 117: 2998, 1995). This method is not limited to peptide ligation and has been used for preparation of cyclic proteins or peptides (L. Zhang and J. P. Tam, *J. Am. Chem. Soc.* **119**: 2363, 1997).

Bioconjugates - chemical groups conjugated to proteins

Chemicals have been conjugated onto proteins for a variety of applications including development of bioassays using conjugated antisera, therapeutic immunotoxins, extension of the *in vivo* half-life, and for tissue targeting. Enzymes have been used as a tool for the conjugation (T. Nakatsuka et al., *J. Am. Chem. Soc.* **109**: 3808, 1987; C. H. Wong et al., *J. Am. Chem. Soc.* **115**: 5893, 1993). Cross linking reagents, such as carbodiimdes, glutaldyhyde, and epoxides, have been broadly used for chemical conjugation (reviewed in Greg T. Hermanson, Bioconjugate Techniques, Academic Press, New York, NY, 1996).

Specificity is often a significant problem with both the enzymatic and chemical coupling methods due to difficulties in conjugating a chemical to specific amino acid residue(s) of a protein in a controlled manner. Recently, a chemoselective ligation method has been developed which can selectively link a compound with thioester functional group to the N-terminus of the N-terminal cysteine-containing peptides or proteins. The yield is high and the side reactions appears to be manageable (P. E. Dawson and S. B. H. Kent, *J. Am. Chem Soc.* **115**: 7263, 1993; P. E. Dawson et al., *Science* **266**: 778, 1994; L. E. Canne et al., *J. Am. Chem. Soc.* **117**: 2998, 1995; J. P. Tam et al, *Proc. Natl. Acad. Sci. USA* **92**: 12485, 1995).

Multifunctional anti-angiogenic bioconjugates

Compounds with multiple (e.g., dual) activities such as a_vb_3 antagonists conjugated to a metastasis-associated receptor ligand (e.g., interferon alpha which has an anti-angiogenic activity) can have improved biological properties by acting through several mechanisms. The two moieties can have distinct mechanisms, permitting synergy or increased efficacy, or both, as a result of blocking the angiogenic process at two separate points. Alternatively, the two moieties may act on the same type of cell, resulting in an increased binding, or other action, by virtue of increased avidity. Each moiety in this case participates in the targeting the other moiety to the appropriate site of action. Dimers or higher order multimers of these moieties with themselves or other chemical groups, including

proteins, can have increased efficacy or potency, or both, by virtue of either of these mechanisms.

A_vb_3 bioconjugates can have improved activities due to the ability to bind to two distinct receptors on the same cell type and thus demonstrate improved activity due to interactions with receptors on a single cell. These a_vb_3 bioconjugates can have improved therapeutic properties through a variety of mechanisms such as: (1) alterations in the overall on- or off-rates or K_a or K_d of the ligand(s) on the target cell, (2) activation or blockade of complementary receptor signaling pathways, and/or (3) more specific targeting of one or both of the components to the cell of interest. Further, the a_vb_3 bioconjugates are expected to possess a unique pharmacokinetic distribution and clearance profile (Dehmer et al., *Circulation*, **91**, 2188-2194, 1995; Tanaka et al., *Nature Medicine*, **3**, 437-442, 1997).

A_vb_3 bioconjugates can also have improved properties *in vivo,* compared to the two components individually, as a result of alterations in biodistribution or half-life. The improved properties can also result from the binding of the a_vb_3 bioconjugate to one or more of the receptors, pharmacokinetics, or uptake of the bioconjugate is altered in a favorable manner. These moieties are likely to act through complementary mechanisms. Therefore, bioconjugates containing anti-proliferative and anti-angiogenic activities can provide improved anti-tumor activity by using two distinct mechanisms to decrease tumor growth. The anti-proliferative moiety can act directly on the tumor to decrease its growth whereas the anti-angiogenic factor acts indirectly by preventing the growth of the neo-vasculature required for rapid tumor growth. A_vb_3 bioconjugates illustrate one example of a compound by this mechanism.

A chemoselective ligation method can be used to conjugate the a_vb_3 antagonists to the protein to improve the antitumor properties of interferon alpha. Small RGD-containing peptides or peptidomimetic a_vb_3 antagonists can also be conjugated to interferon by different linking methods (G.T. Hermanson, Bioconjugate Techniques, pp 169-286, Academic Press, 1996) including methods that use 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (WSC), Di-(N-succinimidyl) carbonate (DSC) and N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) (M. Brinkley, *Bioconjugate Chem.* **3**: 2, 1992).

Summary of the invention

The present invention relates to a_vb_3 bioconjugates, prepared by conjugating an a_vb_3 integrin antagonist with a polypeptide, processes for their preparation, pharmaceutical compositions containing them, and methods for their use. Methods of preparing highly-specific unconjugated a_vb_3 antagonists, the preparation of interferon alpha derivatives, and the conjugation of the a_vb_3 antagonists to an anti-angiogenic protein, such as interferon alpha and its derivatives, are disclosed in this application.

A compound contemplated by this invention is represented by the formula:

A^1-L^1-T^1, or a pharmaceutically acceptable salt thereof, wherein: **A^1** is an a_vb_3 antagonist, **T^1** is a metastasis-associated receptor ligand, and **L^1** is a covalent bond or a linker that covalently bonds **A^1** to **T^1**. Compounds of the formulas **A^1-T^1**, **T^1-L^1-A^1**, and **T^1-A^1**, are also contemplated.

Preferably, **L^1** is a peptide linker.

More preferably **L^1** is a peptide linker ranging in length from 2 through 20 amino acid residues.

Even more preferably, **L^1** is Gly-Asp-**L^2**, wherein; **L^2** is selected from the group consisting of Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Try, Val.

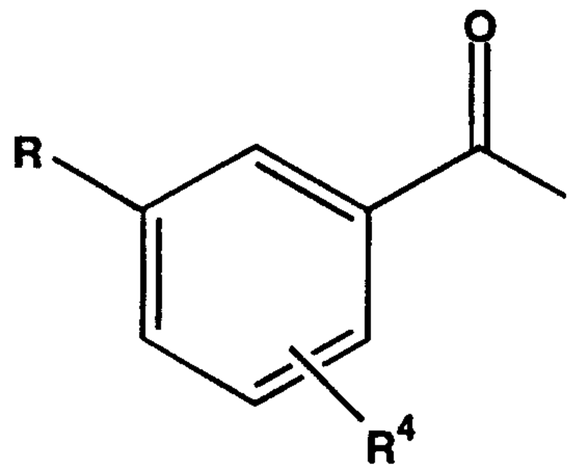
Preferably **T^1** is a polypeptide. More preferably, that polypeptide is selected from the group consisting of natural cytokines, synthetic cytokines, and anti-angiogenic proteins.

Preferably the cytokine is selected from the group consisting of interleukin 2, interleukin-7, interleukin-12, interleukin-15, interferons, progenepoietin-G, erythropoietin, erythropoietin receptor agonists, colony stimulating factors, and hematopoietic growth factors. Even more preferably, that cytokine is interferon.

Preferably, interferon is selected from the group consisting of type I interferon variants, interferon alpha 2a, interferon alpha 2b, interferon alpha hybrid A/D, consensus interferon. Even more preferably, the interferon is human.

Preferably, the anti-angiogenic protein is selected from the group consisting of angiostatin and endostatin.

Preferably A^1 is a compound of the formula I,



I

or a pharmaceutically acceptable salt thereof, wherein:

R^4 is one or more substituents independently selected from the group consisting of hydrogen, alkyl, hydroxy, alkoxy, aryloxy, halogen, haloalkyl, haloalkoxy, nitro, amino, alkylamino, acylamino, dialkylamino, cyano, alkylthio, alkylsulfonyl, carboxyl moieties, trihaloacetamide, acetamide, aryl, fused aryl, cycloalkyl, thio, monocyclic heterocycle, and fused monocyclic heterocycle;

R is R^1, R^2, or R^3, wherein

R^1 is NH_2-(C=NH)NH-, HO-NH-(C=NH)NH-, NH_2-(C=S)NH-, or HO-NH-(C=S)NH-;

R^2 is R^8-benzyl-NH-(C=**Y**)-NH-, wherein;

Y is O or S; and

R^8 is a substituent independently selected from the group consisting of the following substituents:

hydrido, halogen, haloalkyl, lower alkyl, alkoxy, methylenedioxy, ethylenedioxy, alkylthio, haloalkylthio, mercapto, hydroxy, cyano, nitro, carboxyl derivatives, aryloxy, amido, acylamino, amino, alkylamino, dialkylamino, trifluoroalkoxy, trifluoromethyl, sulfonyl, alkylsulfonyl, haloalkylsulfonyl, sulfonic acid, sulfonamide, unsubstituted aryl, fused aryl, monocyclic heterocycles, and fused monocyclic heterocycles;

unsubstituted aryl or aryl optionally substituted with one or more substituent selected from the group consisting of

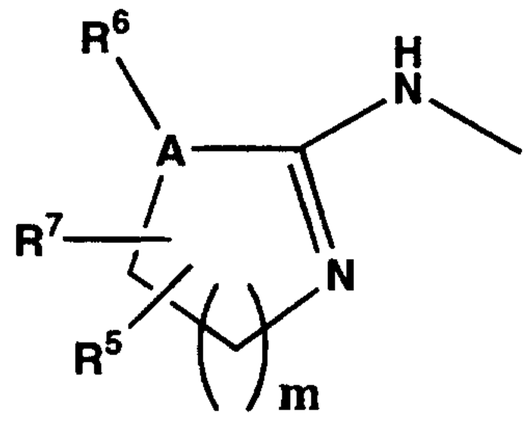
halogen, haloalkyl, lower alkyl, alkoxy, methylenedioxy, ethylenedioxy, alkylthio, haloalkylthio, mercapto, hydroxy, cyano, nitro, carboxyl derivatives, aryloxy, amido, acylamino, amino, alkylamino, dialkylamino, trifluoroalkoxy, trifluoromethylsulfonyl, alkylsulfonyl, sulfonic acid, sulfonamide, aryl, fused aryl, monocyclic heterocycles, and fused monocyclic heterocycles;

monocyclic heterocycles;

unsubstituted monocyclic heterocycle or monocyclic heterocycle optionally substituted with one or more substituent selected from the group consisting of

halogen, haloalkyl, lower alkyl, alkoxy, aryloxy, amino, nitro, hydroxy, carboxyl derivatives, cyano, alkylthio, and alkylsulfonyl;

R^3 is



wherein;

R^7 is hydrido or hydroxyl;

R^5 and R^6 are each substituents independently selected from the group consisting of hydrogen, lower alkyl, hydroxy, alkoxy, halogen, phenyl, amino, carboxyl or

carboxyl ester, and fused phenyl;

A is nitrogen or -CH=; and

m is an integer 1, 2, 3,or 4.

Additionally, the present invention relates to recombinant expression vectors comprising nucleotide sequences encoding the metastasis-associated receptor proteins, related microbial and eukaryotic expression systems, and processes for making the a_vb_3 precursors and the metastasis proteins. The invention also relates to pharmaceutical compositions containing the a_vb_3 bioconjugates, and methods for using the a_vb_3 bioconjugates.

Benefits of the invention are the provision of a pharmaceutical composition comprising a compound of the formula $\mathbf{A^1\text{-}L^1\text{-}T^1}$. Such compounds and compositions are useful in inhibiting or antagonizing the $\alpha_v\beta_3$ integrin and in targeting angiogenesis.

Another embodiment of the present invention relates to a method of selectively inhibiting or antagonizing the $\alpha_v\beta_3$ integrin and tumor angiogenesis. The invention further contemplates treating a disease or inhibiting pathological conditions associated selected from the group consisting of osteoporosis, humoral hypercalcemia of malignancy, Paget's disease, tumor metastasis, solid tumor growth (neoplasia), angiogenesis, including tumor angiogenesis, retinopathy including diabetic retinopathy, arthritis, including rheumatoid arthritis, periodontal disease, psoriasis, thrombosis, angina, atherosclerosis, smooth muscle cell migration and restenosis in a mammal in need of such treatment. Additionally, such pharmaceuticals are useful as antiviral, antifungal and antibacterial agents.

Definitions

The following is a list of abbreviations and the corresponding meanings as used interchangeably herein:

^{1}H-NMR = proton nuclear magnetic resonance

AcOH = acetic acid

BH_3-THF = borane-tetrahydrofuran complex

Bn = benzyl

BOC = *tert*-butoxycarbonyl

ButLi = butyl lithium

Bzl = benzyl ether

Cat. = catalytic amount

CH_2Cl_2 = dichloromethane

CH_3CN = acetonitrile

CH_3I = iodomethane

CHN analysis = carbon/hydrogen/nitrogen elemental analysis

CHNCl analysis = carbon/hydrogen/nitrogen/chlorine elemental analysis

CHNS analysis = carbon/hydrogen/nitrogen/sulfur elemental analysis

DCC = 1,3-dicyclohexylcarbodiimide

DIBAL = diisobutylaluminum hydride

DIEA = diisopropylethylamine

DMA = N,N-dimethylacetamide

DMAP = 4-(N,N-dimethylamino)pyridine

DMF = N,N-dimethylformamide

DSC = disuccinyl carbonate

EDCl = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride

Equiv = equivalence

Et = ethyl

Et_2O = diethyl ether

Et_3N = triethylamine

EtOAc = ethyl acetate

EtOH = ethanol

FAB MS = fast atom bombardment mass spectroscopy

g = gram(s)

GIHA = *meta*-guanidinohippuric acid

GIHA HCl = meta-guanidinohippuric acid hydrochloride

HBTU = 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

HPLC = high performance liquid chromatography

IBCF = isobutylchloroformate

i-Pr = iso propyl

i-Prop = iso propyl

K_2CO_3 = potassium carbonate

KOH = potassium hydroxide

KSCN = potassium thiocyanate

LiOH = lithium hydroxide

MBHA = methoxybenzhydrylamine

MCPBA = m-chloroperoxybenzoic acid or m-chloroperbenzoic acid

Me = methyl

MeOH = methanol

MesCl = methanesulfonylchloride

mg = milligram

$MgSO_4$ = magnesium sulfate

ml = milliliter

mL = milliliter

MS = mass spectroscopy

N_2 = nitrogen

$NaCNBH_3$ = sodium cyanoborohydride

NaH - sodium hydride

$NaHCO_3$ = sodium bicarbonate

NaOH = sodium hydroxide

Na_2PO_4 = sodium phosphate

Na_2SO_4 = sodium sulfate

NEt_3 = triethylamine

NH_4HC0_3 = ammonium bicarbonate

$NH_4^+HCO_2^-$ = ammonium formate

NMM = N-methylmorpholine

NMR = nuclear magnetic resonance

RPHPLC = reverse phase high performance liquid chromatography

RT = room temperature

OBzl = benzyl ester

Pd/C = palladium on carbon

Ph = phenyl

Pt/C = platinum on carbon

t-BOC = tert-butoxycarbonyl

TFA = trifluoroacetic acid

THF = tetrahydrofuran

TMEDA = trimethylethylenediamine

TMS = trimethylsilyl

Δ = heating the reaction mixture

The following is a list of definitions of various terms used herein:

The terms “chemical ligation” and “conjugation” mean a chemical reaction which covalently links two similar or dissimilar functional groups together intramolecularly or intermolecularly.

The term "peptide linker' means a compound which forms a carboxamide bond between two groups having one or more peptide linkages (CONH-) and serves as a connector for the propose of amelioration of the distance or space orientation between two molecules.

The term "multi-functional bioconjugate" means organic compounds consisting of two or more different types of biomolecules and at least one of the biomolecules has more than one copy of a specific structural or functional moiety.

The term "peptide" means organic compounds consisting of two or more aminoacyl residues covalently linked by carboxamide functional groups.

The term "polypeptide" means organic compounds consisting of more than two aminoacyl residues linked by carboxamide functional groups.

The terms "$\alpha_v\beta_3$", "a_vb_3", "avb3", and "alpha v beta 3" are used interchangeably.

The term "a_vb_3 precursor" means a compound, separately or in combination with a linker, is an a_vb_3 antagonist which can be conjugated to other small molecules, peptides, or polypeptides to form an a_vb_3 bioconjugate.

The term "a_vb_3 bioconjugate" means a molecule composed of an a_vb_3 antagonist fused directly, or indirectly through a linker to one or more other small molecules, peptides, or polypeptides, that retains a_vb_3 antagonist activity.

The term "multi-functional protein" means a single polypeptide which inherently possesses two distinct activities. In the context of the current invention, anti-angiogenic and/or anti-tumor activities are contemplated. The polypeptide can be formed by the covalent union of two distinct proteins or portions thereof, or two copies of the same protein, or portions thereof.

The term "anti-tumor" means possessing an activity which slows or abolishes the growth of, or which kills, or otherwise harms tumors *in vivo*.

The term "ligand" means a molecule that binds to a receptor to initiate (agonist) or block (antagonist) a response. Ligands may be small natural or synthetic molecules (e.g., neurotransmitters and their analogues) or may be large proteins or protein nucleic acid associates (e.g., viruses).

The term "receptor" means a component of a cell that interacts specifically with (receives) other molecules and, in appropriate combination, initiates a

biological response. Receptors may be protein, lipid, nucleic acid, or carbohydrate. The possess the fundamental property, when combined with the appropriate ligand, of expressing the information content of the receptor or the ligand.

The term "endogenous" means a cellular component that interacts with a specific receptor. The term "endogenous ligand" describes cellular molecules that interact with receptors that may have been defined earlier by synthetic or other approaches. A classic example is the discovery of endogenous opioid peptides for the opiate receptor.

The term "native sequence" refers to an amino acid or nucleic acid sequence which is identical to a wild-type or native form of a gene or protein.

The terms "mutant amino acid sequence," "mutant protein", "variant protein", "mutein", or "mutant polypeptide" refer to a polypeptide having an amino acid sequence which varies from a native sequence due to amino acid additions, deletions, substitutions, or all three, or is encoded by a nucleotide sequence from an intentionally-made variant derived from a native sequence.

The term "interferon" includes one of a group of species specific proteins which will induce antiviral and anti-proliferative responses in cells including type I interferon, type I interferon variants, interferon alpha 2a, interferon alpha 2b, interferon alpha hybrid A/D, consensus interferon, functional homologues thereof, and those encoded by a DNA sequences related to those in SEQ ID NO: 14, SEQ ID NO: 152, SEQ ID NO: 16, SEQ ID NO: 17, and SEQ ID NO: 18.

The term "composition" as used herein means a product which results from the mixing or combining of more than one element or ingredient.

The term "pharmaceutically-acceptable carrier", as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting a chemical agent.

The term "therapeutically-effective amount" means that amount of drug or pharmaceutical agent that elicits the biological or medical response of a tissue, system or animal that is being sought by a researcher or clinician.

As used herein, the terms "alkyl" and "lower alkyl", refer to a straight chain or branched chain hydrocarbon radical having 1 to about 10 carbon atoms, and 1 to about 6 carbon atoms, respectively. Examples of such alkyl radicals and

lower alkyl radicals are methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, t-butyl, pentyl, neopentyl, hexyl, isohexyl, octyl, nonyl, decyl, and the like.

As used herein the terms "alkenyl" or "lower alkenyl", refer to unsaturated acyclic hydrocarbon radicals containing at least one double bond and 2 to about 10 carbon atoms, and 2 to about 6 carbon atoms, respectively, which carbon-carbon double bond may have either *cis* or *trans* geometry within the alkenyl moiety, relative to groups substituted on the double bond carbons. Examples of such groups are ethenyl, propenyl, butenyl, isobutenyl, pentenyl, hexenyl, octenyl, nonenyl, decenyl, and the like.

As used herein the terms "alkynyl" or "lower alkynyl", refer to acyclic hydrocarbon radicals containing one or more triple bonds and 2 to about 10 carbon atoms, and 2 to about 6 carbon atoms, respectively. Examples of such groups are ethynyl, propynyl, butynyl, pentynyl, hexynyl, octynyl, nonynyl, decynyl, and the like.

The term "cycloalkyl" as used herein means saturated or partially unsaturated cyclic radicals containing 3 to about 8 carbon atoms and more preferably 4 to about 6 carbon atoms. Examples of such cycloalkyl radicals include cyclopropyl, cyclopropenyl, cyclobutyl, cyclopentyl, cyclohexyl, 2-cyclohexen-1-yl, and the like.

The term "aryl" as used herein denotes aromatic ring systems composed of one or more aromatic rings. Preferred aryl groups are those consisting of one, two or three aromatic rings. The term embraces aromatic radicals such as phenyl, pyridyl, naphthyl, thiophene, furan, biphenyl and the like.

As used herein, the term "cyano" is represented by a radical of the formula -CN.

The terms "hydroxy" and "hydroxyl" as used herein are synonymous and are represented by a radical of the formula -OH

The term "lower alkylene" or "alkylene" as used herein refers to divalent linear or branched saturated hydrocarbon radicals of 1 to about 6 carbon atoms.

As used herein the term "alkoxy" refers to straight or branched chain oxy containing radicals of the formula $-OR^{20}$, wherein R^{20} is an alkyl group as defined above. Examples of alkoxy groups encompassed include methoxy, ethoxy, n-propoxy, n-butoxy, isopropoxy, isobutoxy, sec-butoxy, t-butoxy, octyloxy, nonyloxy,

decyloxy, and the like.

As used herein the terms "arylalkyl" or "aralkyl" refer to a radical of the formula $-R^{22}-R^{21}$ wherein R^{21} is aryl as defined above and R^{22} is an alkylene as defined above. Examples of aralkyl groups include benzyl, pyridylmethyl, naphthylpropyl, phenethyl and the like.

As used herein the term "nitro" is represented by a radical of the formula $-NO_2$.

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

As used herein the term "haloalkyl" refers to alkyl groups as defined above substituted with one or more of the same or different halo groups at one or more carbon atom. Examples of haloalkyl groups include trifluoromethyl, dichloroethyl, fluoropropyl and the like.

As used herein the term "carboxyl" or "carboxy" refers to a radical of the formula -COOH.

As used herein the term "carboxyl ester" refers to a radical of the formula $-COOR^{23}$ wherein R^{23} is selected from the group consisting of H, alkyl, alkenyl, alkynyl, aralkyl or aryl as defined above.

As used herein the term "carboxyl derivative" refers to a radical of the formula $-C=Y^6-Y^7R^{23}$ wherein Y^6 and Y^7 are independently selected from the group consisting of O, N or S and R^{23} is selected from the group consisting of H, alkyl, alkenyl, alkynyl, aralkyl or aryl as defined above.

As used herein the term "amino" is represented by a radical of the formula $-NH_2$.

As used herein the terms "alkylsulfonyl," "alkylsulfone," "alkenylsulfonyl," "alkenylfone," "alkynylsulfonyl," "alkynylsulfone", refer to a radical of the formula $-SO_2-R^{24}$ wherein R^{24} is alkyl, alkenyl, alkynyl, as defined above.

As used herein the terms "alkylthio," "alkenylthio," "alkynylthio," refers to a radical of the formula $-SR^{24}$ wherein R^{24} is alkyl, alkenyl, alkynyl as defined above.

As used herein the term "sulfonic acid" refers to a radical of the formula $-SO_2-R^{25}$ wherein R^{25} is H, alkyl or aryl as defined above.

As used herein the term "sulfonamide" refers to a radical of the formula $-SO_2-NR^7R^8$ wherein R^7 and R^8 are as defined above.

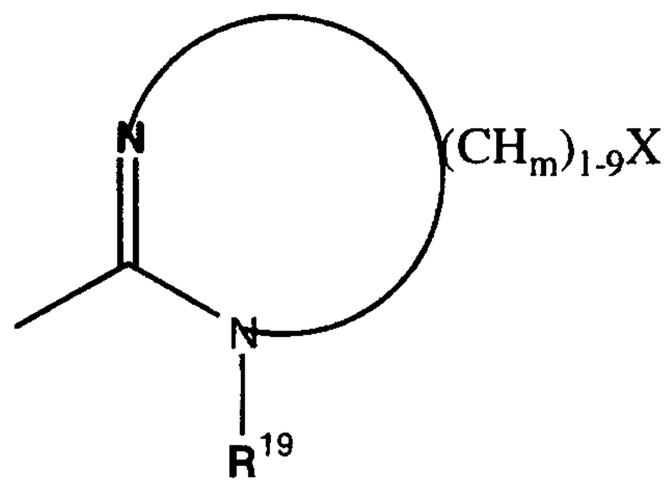
As used herein the term "fused aryl" refers to an aromatic ring such as the aryl groups defined above fused to one or more phenyl rings. Embraced by the term "fused aryl" is the radical naphthyl.

As used herein the terms "monocyclic heterocycle" or "monocyclic heterocyclic" refer to a monocyclic ring containing from 4 to about 12 atoms, and more preferably from 5 to about 10 atoms, wherein 1 to 3 of the atoms are heteroatoms selected from the group consisting of oxygen, nitrogen and sulfur with the understanding that if two or more different heteroatoms are present at least one of the heteroatoms must be nitrogen. Representative of such monocyclic heterocycles are imidazole, furan, pyridine, oxazole, pyran, triazole, thiophene, pyrazole, thiazole, thiadiazole, and the like.

As used herein the term "fused monocyclic heterocycle" refers to a monocyclic heterocycle as defined above with a benzene fused thereto. Examples of such fused monocyclic heterocycles include benzofuran, benzopyran, benzodioxole, benzothiazole, benzothiophene, benzimidazole and the like.

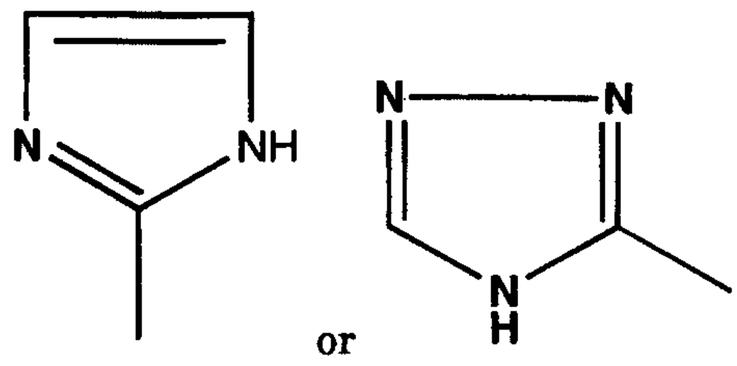
As used herein the term "methylenedioxy" refers to the radical $-OCH_2O-$ and the term "ethylenedioxy" refers to the radical $-OCH_2CH_2O-$.

As used herein the term "4-12 membered dinitrogen containing heterocycle refers to a radical of the formula



wherein m is 1 or 2 and R^{19} is H, alkyl, alkenyl, alkynyl, aryl, or aralkyl, and X is H or halide. More preferably the term refers to a 4-9 membered ring and includes rings such as imidazoline.

As used herein the term "5-membered optionally substituted heteroaromatic ring" includes for example a radical of the formula



and "5-membered heteroaromatic ring fused with a phenyl" refers to such a "5-membered heteroaromatic ring" with a phenyl fused thereto. Representative of such 5-membered heteroaromatic rings fused with a phenyl is benzimidazole.

As used herein the term "bicycloalkyl" refers to a bicyclic hydrocarbon radical containing 6 to about 12 carbon atoms which is saturated or partially unsaturated.

As used herein the term "acyl" refers to a radical of the formula $-COR^{26}$ wherein R^{26} is alkyl, alkenyl, alkynyl, aryl or aralkyl and optionally substituted thereon as defined above. Encompassed by such radical are the groups acetyl, benzoyl and the like.

As used herein the terms "thio" and "mercapto" refer to a radical of the formula -SH.

As used herein the term "sulfonyl" refers to a radical of the formula $-SO_2R^{27}$ wherein R^{27} is alkyl, alkenyl, alkynyl, aryl, or aralkyl as defined above.

As used herein the term "haloalkylthio" refers to a radical of the formula $-SR^{28}$ wherein R^{28} is haloalkyl as defined above.

As used herein the term "aryloxy" refers to a radical of the formula $-OR^{29}$ wherein R^{29} is aryl as defined above.

As used herein the term "acylamino" refers to a radical of the formula $R^{30}CONH-$ wherein R^{30} is alkyl, alkenyl, alkynyl, aralkyl or aryl as defined above.

As used herein the term "amido" refers to a radical of the formula $-CONH_2$.

As used herein the term "alkylamino" refers to a radical of the formula $-NHR^{32}$ wherein R^{32} is alkyl as defined above.

As used herein the term "dialkylamino" refers to a radical of the formula $-NR^{33}R^{34}$ wherein R^{33} and R^{34} are the same or different alkyl groups as defined above.

As used herein the term "trifluoromethyl" refers to a radical of the formula $-CF_3$.

As used herein the term "trifluoroalkoxy" refers to a radical of the formula $F_3C-R^{35}-O-$ wherein R^{35} is a bond or an alkylene group as defined above.

As used herein the term "alkylaminosulfonyl" refers to a radical of the formula $R^{36}-NH-SO_2-$ wherein R^{36} is alkyl as defined above.

As used herein the term "alkylsulfonylamino" refers to a radical of the formula $R^{36}-SO_2-NH-$ wherein R^{36} is alkyl as defined above.

As used herein the term "trifluoromethylthio" refers to a radical of the formula F_3C-S-.

As used herein the term "trifluoromethylsulfonyl" refers to a radical of the formula F_3C-SO_2-.

As used herein the term "4-12 membered mono-nitrogen-containing monocyclic or bicyclic ring" refers to a saturated or partially unsaturated monocyclic or bicyclic ring of 4-12 atoms and more preferably a ring of 4-9 atoms wherein one atom is nitrogen. Such rings may optionally contain additional heteroatoms selected from nitrogen, oxygen or sulfur. Included within this group are morpholine, piperidine, piperazine, thiomorpholine, pyrrolidine, proline, azacycloheptene and the like.

As used herein the term "benzyl" refers to the radical $-CH_2-Ph$.

As used herein the term "phenethyl" refers to the radical $-CH_2CH_2-Ph$.

As used herein the term "4-12 membered mono-nitrogen-containing monosulfur- or monooxygen-containing heterocyclic ring" refers to a ring of 4 to about 12 atoms, and more preferably 4 to about 9 atoms wherein at least one atom is a nitrogen and at least one atom is oxygen or sulfur. Encompassed within this definition are rings such as thiazoline and the like.

As used herein the term "arylsulfonyl" or "arylsulfone" refers to a radical of the formula $R^{37}-SO_2-$ wherein R^{37} is aryl as defined above.

As used herein the terms "alkylsulfoxide" or "arylsulfoxide" refer to radicals of the formula $R^{38}-SO-$ wherein R^{38} is, respectively, alkyl or aryl as defined above.

As used herein the term "phosphonic acid derivative" refers to a radical of the formula -P=O(-OR^{39})-OR^{40} wherein R^{39} and R^{40} are the same or different H, alkyl, aryl or aralkyl.

As used herein the term "phosphinic acid derivatives" refers to a radical of the formula -P=O(-H)-OR^{41} wherein R^{41} is H, alkyl, aryl or aralkyl as defined above.

As used herein the term "arylthio" refers to a radical of the formula -SR^{42} wherein R^{42} is aryl as defined above.

As used herein the term "monocyclic heterocycle thio" refers to a radical of the formula -SR^{43} wherein R^{43} is a monocyclic heterocycle radical as defined above.

As used herein the terms "monocyclic heterocycle sulfoxide" and "monocyclic heterocycle sulfone" refer, respectively, to radicals of the formula -SO-R^{43} and -SO_2-R^{43} wherein R^{43} is a monocyclic heterocycle radical as defined above.

As used herein the term "alkylcarbonyl" refers to a radical of the formula R^{50}-CO- wherein R^{50} is alkyl as defined above.

As used herein the term "arylcarbonyl" refers to a radical of the formula R^{51}-CO- wherein R^{51} is aryl as defined above.

As used herein the term "alkoxycarbonyl" refers to a radical of the formula R^{52}-CO- wherein R^{52} is alkoxy as defined above.

As used herein the term "aryloxycarbonyl" refers to a radical of the formula R^{51}-O-CO- wherein R^{51} is aryl as defined above.

As used herein the term "haloalkylcarbonyl" refers to a radical of the formula R^{53}-CO- wherein R^{53} is haloalkyl as defined above.

As used herein the term "haloalkoxycarbonyl" refers to a radical of the formula R^{53}-O-CO- wherein R^{53} is haloalkyl as defined above.

As used herein the term "alkylthiocarbonyl" refers to a radical of the formula R^{50}-S-CO- wherein R^{50} is alkyl as defined above.

As used herein the term "arylthiocarbonyl" refers to a radical of the formula R^{51}-S-CO- wherein R^{51} is aryl as defined above.

As used herein the term "acyloxymethoxycarbonyl" refers to a radical of the formula R^{54}-O-CH_2-CO- wherein R^{54} is acyl as defined above.

As used herein the term "arylamino" refers to a radical of the formula R^{51}-NH- wherein R^{51} is aryl as defined above.

As used herein the term "polyalkylether" refers to commonly used glycols such as triethyleneglycol, tetraethylene glycol, polyethylene glycol and the like.

As used herein the term "alkylamido" refers to a radical of the formula R^{50}-NH-CO- wherein R^{50} is alkyl as defined above.

As used herein the term "N,N-dialkylamido" refers to a radical of the formula $(R^{50})_2$-N-CO- wherein R^{50} is the same or different alkyl group as defined above.

As used herein the term "pivaloyloxymethyl" refers to a radical of the formula $(Me)_3C$-CO-O-CH2-.

As used herein the term "acyloxy" refers to a radical of the formula R^{55}-O- wherein R^{55} is acyl as defined above.

The compounds as shown in the present invention can exist in various isomeric forms and all such isomeric forms are meant to be included. Tautomeric forms are also included as well as pharmaceutically acceptable salts of such isomers and tautomers.

In the structures and formulas herein, a bond drawn across a bond of a ring can be to any available atom on the ring.

The term "pharmaceutically acceptable salt" refers to a salt prepared by contacting a compound of an a_vb_3 precursor or an a_vb_3 bioconjugate with an acid whose anion is generally considered suitable for human consumption. Examples of pharmacologically acceptable salts include the hydrochloride, hydrobromide, hydroiodide, sulfate, phosphate, acetate, propionate, lactate, maleate, malate, succinate, tartrate salts and the like. All of the pharmacologically acceptable salts may be prepared by conventional means. See Berge et al., (*J Pharm. Sci.*, **66**(1): 1-19, 1977) for additional examples of pharmaceutically acceptable salts.

For the selective inhibition or antagonism of a_vb_3 integrins, a compound of the present invention can be administered orally, parenterally, or by inhalation spray, or topically in unit dosage formulations containing conventional

pharmaceutically acceptable carriers, adjuvants and vehicles. The term parenteral as used herein includes, for example, subcutaneous, intravenous, intramuscular, intrasternal, infusion techniques or intraperitonally. See Wong and Parasrampuria (*Biopharm* **10**(11): 52-61 1997) for a current review of pharmaceutical excipients for the stabilization of proteins. See D.W. Osborne and J.J. Henke (*Pharmaceutical Technology* **21**(11): 58-67, 1997) for an extensive list of chemical compounds used as skin penetration enhancers.

A compound of the present invention is administered by any suitable route in the form of a pharmaceutical composition adapted to such a route, and in a dose effective for the treatment intended. Therapeutically effective doses of a compound required to prevent or arrest the progress of or to treat the medical condition are readily ascertained by one of ordinary skill in the art using preclinical and clinical approaches familiar to the medicinal arts.

Accordingly, the present invention provides a method of treating conditions mediated by selectively inhibiting or antagonizing the a_vb_3 cell surface receptor which method comprises administering a therapeutically effective amount of a compound selected from the class of compounds depicted herein as a_vb_3 precursors or a_vb_3 bioconjugates, wherein one or more these compounds is administered in association with one or more non-toxic, pharmaceutically acceptable carriers and/or diluents and/or adjuvants (collectively referred to herein as "carrier" materials) and if desired other active ingredients. The use of multiple administrations is particularly contemplated.

More specifically, the present invention provides a method for inhibition of the a_vb_3 cell surface receptor. Most preferably the present invention provides a method for one or more of the following: inhibiting bone resorption, treating osteoporosis, inhibiting humoral hypercalcemia of malignancy, treating Paget's disease, inhibiting tumor metastasis, inhibiting neoplasia (solid tumor growth), inhibiting angiogenesis including tumor angiogenesis, treating diabetic retinopathy, inhibiting arthritis, psoriasis and periodontal disease, and inhibiting smooth muscle cell migration including restenosis.

The general synthetic sequences for preparing the compounds useful in the present invention are outlined in Scheme I. Both an explanation of, and the actual procedures for, the various aspects of the present invention are described where appropriate. The following Schemes and Examples are intended to be merely illustrative of the present invention, and not limiting thereof in either scope or spirit. Those with skill in the art will readily understand that known variations of

the conditions and processes described in the Schemes and Examples can be used to synthesize the compounds of the present invention.

Unless otherwise indicated all starting materials and equipment employed were commercially available.

Scheme 1: Synthesis of dual a_vb_3-interferon bioconjugates

Peptides conjugated to a resin are covalently coupled to an a_vb_3 antagonist-linker and ligated to the reduced form of Cys-interferon alpha. The bioconjugate is then released from the resin, refolded, and purified to homogeneity.

Boc-NH-a.a.C=O-S-CH_2CH_2-CONH-Resin

⇓ TFA

NH_2-a.a.C=O-S-CH_2CH_2-CONH-Resin

⇓ linker, a_vb_3 antagonist precursor

a_vb_3 antagonist-linker-a.a.C=O-S-CH_2CH_2-CONH-Resin

⇓ HF

a_vb_3 antagonist-linker-a.a.-C=O-S-CH_2CH_2-$CONH_2$

↑

S-

|

$^+NH_3$ -CH-CO-interferon α

(Cys interferon α, reduced form)

⇓

a_vb_3 antagonist-linker-a.a.-C=O- NH-CH(SH)-CO-interferon α

(a_vb_3 antagonist-linker Cys interferon α, reduced form)

⇓ refolding, purification

a_vb_3 antagonist-linker Cys interferon α

Detailed Description of the Invention

The present invention encompasses bioconjugates which inherently possess two distinct activities. Bioconjugates with multi-functional (dual) a_vb_3 receptor antagonist/metastasis receptor-associated ligand activities are contemplated. In the context of this invention, anti-angiogenic or anti-tumor activities, or both, are contemplated. A contemplated bioconjugate can be formed by the covalent union of two distinct moieties, such as a small chemical and a polypeptide. Each of these distinct moieties may act through a different and specific cell receptor to initiate complementary biological activities.

Bioconjugates can also be formed by the covalent union of a small chemical and a peptide, a peptide and a polypeptide, two distinct polypeptides, two distinct peptides, and two distinct small molecules, provided that one of the two moieties alone or in combination with the other moiety possesses a_vb_3 antagonist activity, are also contemplated by this invention. Peptides in this sense are comprised of two to about 10 amino acid residues. Polypeptides are larger, comprised of from about 11 to about 500 amino acid residues.

Bioconjugates containing more than one copy of a moiety, achieved through the covalent attachment of more than one copy of that moiety to itself and then to the other moiety, or by covalent attachment of more than one copy of that moiety to the other moiety at multiple sites, are also contemplated by this invention.

An a_vb_3 bioconjugate composed of a small chemical moiety linked to a polypeptide moiety, for example, ($\mathbf{A^1}$-$\mathbf{L^1}$-$\mathbf{T^1}$) preferably has a polypeptide moiety ($\mathbf{T^1}$) with a different but complementary activity than the small chemical moiety ($\mathbf{A^1}$). Complementary activity is meant to be activity which enhances or changes the response to another cell modulator.

The polypeptide can be joined either directly or through a linker segment to the small chemical moiety. The term "directly" defines bioconjugates in which the polypeptide is joined without a linker. Thus $\mathbf{L^1}$ represents a chemical bond or a linker, preferably a polypeptide segment to which both $\mathbf{A^1}$ and $\mathbf{T^1}$ are joined. Most commonly $\mathbf{L^1}$ is a linear peptide in which $\mathbf{A^1}$ and $\mathbf{L^1}$ are joined by amide bonds, linking $\mathbf{A^1}$ to the amino-terminus of $\mathbf{L^1}$ and carboxy-terminus of $\mathbf{L^1}$ to the amino terminus of $\mathbf{T^1}$. Compounds of the formulas $\mathbf{A^1}$-$\mathbf{T^1}$, $\mathbf{T^1}$-$\mathbf{L^1}$-$\mathbf{A^1}$, and $\mathbf{T^1}$-$\mathbf{A^1}$ are also contemplated.

Additionally, this invention encompasses the use of modified T^1 molecules or mutated or modified DNA sequences encoding these molecules. The present invention also includes bioconjugates in which T^1 is a variant.

The linking group (L^1) is generally a polypeptide of between 1 and 500 amino acid residues in length. The linkers joining the two molecules are preferably designed to (1) permit the two molecules to fold and act independently of each other, (2) being free of ordered secondary structure which could interfere with the functional domains of the two proteins, (3) can exhibit minimal hydrophobic characteristics that could interact with the functional domains of the linked moieties and (4) provide steric separation of A^1 and T^1 such that A^1 and T^1 can interact simultaneously with their corresponding receptors on a single cell. Typically, surface amino acids in flexible protein regions include Gly, Asn and Ser. Virtually any permutation of amino acid residue sequences containing Gly, Asn and Ser satisfies the above criteria for a linker sequence. Other neutral amino acids, such as Thr and Ala, can also be used in the linker sequence. Additional amino acids may also be included in the linkers due to the addition of unique restriction sites in the linker sequence to facilitate construction of the multi-functional proteins.

Preferred L^1 linkers of the present invention include sequences selected from the group of formulas:

(SEQ ID NO: 24) -Gly-Asp-Ala-;

(SEQ ID NO: 25) -Gly-Asp-Ser-;

(SEQ ID NO: 26) -Gly-Gly-Gly-Gly-Ala-;

(SEQ ID NO: 27) -Gly-Gly-Gly-Gly-Ser-;

(SEQ ID NO: 28) -Gly-Asp-Ala-Gly-Gly-Gly-Gly-Ala-;

(SEQ ID NO: 29) -Gly-Asp-Ala-Gly-Gly-Gly-Gly-Ser-;

(SEQ ID NO: 30) -Gly-Asp-Ala-Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser-;

or

(SEQ ID NO: 31) -Gly-Asp-Ala-Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser-.

Other peptide, aliphatic, and alkyl linkers are also contemplated by the invention. These include linkers with the following formulas:

$-CO-(aa)_n-$, such as -CO-Gly-Gly-Gly-Gly-Ala-, and

$-CONH-(CH_2)_n-CO-Ala-$, such as $-CONH-(CH_2)_5-CO-Ala-$.

The present invention is, however, not limited by the form, size, or number of linker sequences employed. The only requirement of the linker is that it does not functionally interfere with the folding and function of the individual molecules of the multi-functional protein. Generally, however, each linker comprises four or more amino acid or carbon linking units.

Additional peptide sequences may also be added to facilitate purification or identification of bioconjugates (e.g., poly-His). A highly antigenic peptide may also be added that would enable rapid assay and facile purification of the bioconjugates by a specific monoclonal antibody.

Multi-functional bioconjugates of the present invention can exhibit useful properties such as having similar or greater biological activity when compared to a single factor or by having improved half-life or decreased adverse side effects, or a combination of these properties.

Multi-functional bioconjugates which have little or no activity are useful as antigens for the production of antibodies for use in immunology or immunotherapy, as genetic probes or as intermediates used to construct other useful bioconjugates.

Biological activity of the multi-functional bioconjugates of the present invention can be determined by tumor cell proliferation assays, endothelial cell migration assays, endothelial cell tube formation assays, mouse corneal micro-pocket angiogenesis assays, and tumor growth assays. Syngeneic models of mouse tumor growth, such as the Lewis Lung carcinoma assay (Sugiura and Stock, *Cancer Res.*, **15**: 38-51, 1955; O'Reilly et al., *Cell (Cambridge, Mass)* **79**(2): 315-328, 1994), and xenograft models of human tumors in nude or SCID mice using human breast cancer, prostate carcinoma, or melanoma cell lines are also used (Price, *Breast Cancer Research and Treatment*, **39**: 93-102, 1996; Sasaki et al., *Can. Res.* **55**: 3551-3557, 1995; Pretlow et al, *Can. Res.* **51**: 3814-3817, 1991 and Passaniti et al., *Int. J. Cancer*, **51**: 318-324, 1992; Felding-Habermann et al., *J. Clin. Invest.*, **89**: 2018-2022, 1992).

The biological activity of individual moieties can be performed using specific assays. The antiviral activity of interferon, for example, can be carried out by titering the potency of interferon preparations on Madin Darby bovine kidney cells infected with vesicular stomatitis virus (Rubinstein et al., *J. Virol.* **37**(2): 755-758, 1981). The a_vb_3 activity can be carried out using solid state binding assays.

The multi-functional bioconjugates of the present invention may have an improved therapeutic profile as compared to single-acting anti-angiogenic or anti-

tumor compounds or proteins. For example, some multi-functional bioconjugates of the present invention may have a similar or more potent anti-tumor activity relative to other anti-tumor compounds or proteins without having a similar or corresponding increase in side-effects.

Therapeutic targets

The multi-functional bioconjugates of the present invention are useful in the treatment of angiogenic-mediated diseases such as cancer, diabetic retinopathy, and macular degeneration. Among the cancers susceptible to treatment with the polypeptides of the present invention are lung cancer, breast cancer, ovarian cancer, prostate cancer, pancreatic cancer, gastric cancer, colon cancer, renal cancer, bladder cancer, melanoma, hepatoma, sarcoma, and lymphoma.

The present invention provides an improvement to the existing methods of treating solid tumors, in that it provides a process utilizing multi-functional bioconjugates that have improved biological activities. Therapeutic treatment of tumors with these multi-functional bioconjugates of the present invention can avoid undesirable side effects caused by treatment with presently available drugs. The treatment of solid tumors can include administration of a pharmaceutical composition containing the multi-functional bioconjugates to a patient.

Formulation and dosing

Other aspects of the present invention are methods and therapeutic compositions for treating the conditions referred to above. Such compositions comprise a therapeutically effective amount of one or more of the multi-functional bioconjugates of the present invention in a mixture with a pharmaceutically acceptable carrier. The compositions can also be admixtures containing adjunctive agents, such as chemotherapeutic or immunotherapeutic agents. This composition can be administered either parenterally, intravenously, or subcutaneously. Other routes of administration are also contemplated, including intranasal and transdermal routes, and by inhalation. When administered, the therapeutic composition for use in this invention is preferably in the form of a pyrogen-free, parenterally-acceptable aqueous solution. The preparation of such a parenterally-acceptable protein solution, having due regard to pH, isotonicity, stability and the like, is within the skill of the art.

Based upon standard laboratory techniques and procedures well known and appreciated by those skilled in the art, as well as comparisons with compounds of known usefulness, the compounds depicted herein as a_vb_3 precursors or a_vb_3 bioconjugates can be used in the treatment of patients suffering from the above pathological conditions. One skilled in the art will recognize that selection of the most appropriate compound of the invention is within the ability of one with ordinary skill in the art and depend on a variety of factors including assessment of results obtained in standard assay and animal models.

Treatment of a patient afflicted with one of the pathological conditions comprises administering to such a patient an amount of compound of the formula A^1-L^1-T^1 which is therapeutically effective in controlling the condition or in prolonging the survivability of the patient beyond that expected in the absence of such treatment. As used herein, the term "inhibition" of the condition refers to slowing, interrupting, arresting, or stopping the condition and does not necessarily indicate a total elimination of the condition. It is believed that prolonging the survivability of a patient, beyond being a significant advantageous effect in and of itself, also indicates that the condition is beneficially controlled to some extent.

As stated previously, a compound of the present invention can be used in a variety of biological, prophylactic, or therapeutic areas. It is contemplated that these compounds are useful in prevention or treatment of any disease state or condition wherein the a_vb_3 integrin plays a role.

The dosage regimen for the compounds and/or compositions containing the compounds is based on a variety of factors, including the type, age, weight, sex and medical condition of the patient; the severity of the condition; the route of administration; and the activity of the particular compound employed. Thus the dosage regimen may vary widely. Dosage levels of the order from about 0.01 mg to about 100 mg per kilogram of body weight per day are useful in the treatment of the above-indicated conditions.

The active ingredient administered by injection is formulated as a composition wherein, for example, saline, dextrose or water may be used as a suitable carrier. A suitable daily dose would typically be about 0.01 to 10 mg/kg body weight injected per day in multiple doses depending on the factors listed above.

For administration to a mammal in need of such treatment, the compounds in a therapeutically effective amount are ordinarily combined with one or more

excipients appropriate to the indicated route of administration. The compounds may be admixed with lactose, sucrose, starch powder, cellulose esters of alkanoic acids, cellulose alkyl esters, talc, stearic acid, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulphuric acids, gelatin, acacia, sodium alginate, polyvinylpyrrolidone, and/or polyvinyl alcohol, and tableted or encapsulated for convenient administration. Alternatively, a compound can be dissolved in water, polyethylene glycol, propylene glycol, ethanol, corn oil, cottonseed oil, peanut oil, sesame oil, benzyl alcohol, sodium chloride, and/or various buffers. Other excipients and modes of administration are well and widely known in the pharmaceutical art.

The pharmaceutical compositions useful in the present invention may be subjected to conventional pharmaceutical operations such as sterilization and/or may contain conventional pharmaceutical adjuvants such as preservatives, stabilizers, wetting agents, emulsifiers, buffers, and the like.

The dosage regimen involved in a method for treating the above-described conditions will be determined by the attending physician considering various factors which modify the action of drugs, e.g., the condition, body weight, sex, and diet of the patient, the severity of any infection, time of administration and other clinical factors. Generally, a daily regimen may be about 10 µg/kg of multi-functional proteins per kilogram of body weight. Dosages would be adjusted relative to the activity of a given multi-functional proteins and it would not be unreasonable to note that dosage regimens may include doses as low as 0.1 microgram and as high as 100 milligrams per kilogram of body weight per day.

In addition, there may exist specific circumstances where dosages of multi-functional bioconjugates would be adjusted higher or lower than the range of 0.2 - 100,000 micrograms per kilogram of body weight. These include co-administration with other anti-angiogenic or antitumor proteins or variants; co-administration with adjunctive agents such as chemotherapeutic or immunotherapeutic agents, co-administration with chemotherapeutic drugs and/or radiation; the use of glycosylated multi-functional bioconjugates; and various patient-related issues mentioned earlier in this section. As indicated above, the therapeutic method and compositions may also include co-administration with other human anti-tumor proteins, compounds, or bioconjugates.

A non-exclusive list of other appropriate anti-angiogenic or anti-tumor agents or treatments includes chemotherapy, radiation therapy, hormonal therapy, or interleukin-2, or combinations thereof. The dosage recited above would be

adjusted to compensate for such additional components in the therapeutic composition.

Cloning and expression of genes encoding multi-functional bioconjugates

The present invention also includes the DNA sequences which code for the protein portions of multi-functional bioconjugates, DNA sequences which are substantially similar and perform substantially the same function, and DNA sequences which differ from the DNAs encoding the protein portions of multi-functional bioconjugates of the invention only due to the degeneracy of the genetic code. Also included in the present invention are the oligonucleotide intermediates used to construct the mutant DNAs and the polypeptides coded for by these oligonucleotides.

Genetic engineering techniques now standard in the art (United States Patent 4,935,233 and Sambrook et al., "Molecular Cloning A Laboratory Manual", Cold Spring Harbor Laboratory, 1989) may be used in the construction of the DNA sequences of the present invention. One such method is cassette mutagenesis (Wells et al., *Gene* **34**:315-323, 1985) in which a portion of the coding sequence in a plasmid is replaced with synthetic oligonucleotides that encode the desired amino acid substitutions in a portion of the gene between two restriction sites.

Pairs of complementary synthetic oligonucleotides encoding the desired gene can be made and annealed to each other. The DNA sequence of the oligonucleotide would encode sequence for amino acids of desired gene with the exception of those substituted and/or deleted from the sequence.

Plasmid DNA can be treated with the chosen restriction endonucleases then ligated to the annealed oligonucleotides. The ligated mixtures can be used to transform competent *E. coli* cells to resistance to an appropriate antibiotic. Single colonies can be picked and the plasmid DNA examined by restriction analysis and/or DNA sequencing to identify plasmids with the desired genes.

Cloning of DNA sequences encoding the polypeptide component of these multi-functional bioconjugates may be accomplished by the use of intermediate vectors. Alternatively, one gene can be cloned directly into a vector containing the other gene. Linkers and adapters can be used for joining the DNA sequences, as well as replacing lost sequences, where a restriction site was internal to the region of interest. Thus genetic material (DNA) encoding one polypeptide, peptide linker,

and the other polypeptide is inserted into a suitable expression vector which is used to transform bacteria, yeast, insect cells or mammalian cells. The transformed organism or cell line is grown and the protein isolated by standard techniques. The resulting product is therefore a new protein which has all or a portion of one protein joined by a linker region to a all or a portion of second protein.

Another aspect of the present invention includes plasmid DNA vectors for use in the expression of the polypeptide component of these multi-functional bioconjugates. These vectors contain the novel DNA sequences described above which code for the novel polypeptides of the invention. Appropriate vectors which can transform microorganisms or cell lines capable of expressing the polypeptide component of these novel multi-functional bioconjugates include expression vectors comprising nucleotide sequences coding for the polypeptide component of these novel multi-functional bioconjugates joined to transcriptional and translational regulatory sequences which are selected according to the host cells used.

Vectors incorporating modified sequences as described above are included in the present invention and are useful in the production of the protein component of these multi-functional bioconjugates. The vector employed in the method also contains selected regulatory sequences in operative association with the DNA coding sequences of the invention and which are capable of directing the replication and expression thereof in selected host cells.

A method for producing the polypeptide component of these multi-functional bioconjugates is another aspect of the present invention. A method of the present invention comprises culturing suitable cells or cell lines, which have been transformed with a vector containing a DNA sequence coding for expression of a novel multi-functional protein. Suitable cells or cell lines can be bacterial cells. For example, the various strains of *E. coli* are well-known as host cells in the field of biotechnology. Examples of such strains include *E. coli* strains JM101 (Yanisch-Perron et al. *Gene* **33**: 103-119, 1985) and MON105 (Obukowicz et al., *Applied Environmental Microbiology* **58**: 1511-1523, 1992). Also included in the present invention is the expression of the multi-functional proteins utilizing a chromosomal expression vector for *E. coli* based on the bacteriophage Mu (Weinberg et al., *Gene* **126**: 25-33, 1993). Various strains of *B. subtilis* can also be employed in this method. Many strains of yeast cells known to those skilled in the art are also available as host cells for expression of the polypeptides of the present invention.

When expressed in the *E. coli* cytoplasm, the gene encoding the protein component of these multi-functional bioconjugates of the present invention can also be constructed such that at the 5' end of the gene codons are added to encode Met^{-2}-Ala^{-1}, Met^{-2}-Ser^{-1}, Met^{-2}-Cys^{-1}, or Met^{-1} at the N-terminus of the protein. The N-termini of proteins made in the cytoplasm of *E. coli* are affected by post-translational processing by methionine aminopeptidase (Ben Bassat et al., *J. Bacteriol.* **169:**751-757, 1987) and possibly by other peptidases so that upon expression the methionine is cleaved off the N-terminus. The polypeptide component of these multi-functional bioconjugates of the present invention may include polypeptides having Met^{-1}, Ala^{-1}, Ser^{-1}, Cys^{-1}, Met^{-2}-Ala^{-1}, Met^{-2}-Ser^{-1}, or Met^{-2}-Cys^{-1} at the N-terminus. These polypeptides may also be expressed in *E. coli* by fusing a secretion signal peptide to the N-terminus. This signal peptide is cleaved from the polypeptide as part of the secretion process.

The following examples illustrate the invention in greater detail although it is understood that the invention is not limited to these specific examples. Various other examples will be apparent to the person skilled in the art after reading the present disclosure without departing from the spirit and scope of the invention. It is intended that all such other examples be included within the scope of the appended claims.

Materials and Methods

Example 1: Preparation of free form of a_vb_3 antagonists

General method

Free forms of a_vb_3 antagonists (precursors) were prepared by the solid phase method using FMOC chemistry. When completed, a_vb_3 precursors were released from the solid support with trifluoroacetic acid treatment followed by reversed phase HPLC.

Compound 1

Fmoc-L-Ala (3 equiv) was coupled into Rink Amide MBHA resin, substitution 0.55 mmol/g (NovaBiochem, San Diego, CA) with HBTU (from Nova Biochem, San Diego, CA) as coupling agent. Deprotection was performed with 30% piperidine in DMF (1 X 2 min, 1 X 8 min). Fmoc Gly, Fmoc-L-Asp(OBu), and 3-[(1,4,5,6-tetrahydro-2-pyrimidinyl)amino] benzoic acid were coupled sequentially as described above. The crude product was released by treatment with Reagent K

(82.5% triflouroacetic acid, 5% phenol, 5% water, 5% thioanisole and 2.5% ethanedithiol) for 2 hours at 25 C and precipitated by addition of tert-butyl methyl ether. The product was dissolved in water and purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient was used as the eluted solvent). The structure was verified with mass spectrometer analyses (M + H = 576.5) and amino acid composition analyses.

Compound 2

Fmoc-L-benzylserine (3 equiv) was coupled into Rink Amide MBHA resin, substitution 0.55 mmol/g (NovaBiochem, San Diego, CA) with HBTU (from Nova Biochem, San Diego, CA) as coupling agent. Deprotection was performed with 30% piperidine in DMF (1 X 2 min, 1 X 8 min). Fmoc Gly, Fmoc-L-Asp(OBu), and 3-[(1,4,5,6-tetrahydro-2-pyrimidinyl)amino] benzoic acid were coupled sequentially as described above. The crude product was released by treatment with Reagent K (82.5% triflouroacetic acid, 5% phenol, 5% water, 5% thioanisole and 2.5% ethanedithiol) for 2 hours at 25 C and precipitated by addition of tert-butyl methyl ether. The product was dissolved in water and purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient was used as the eluted solvent). The structure was verified with mass spectrometer analyses (M + H = 592.5) and amino acid composition analyses.

Compound 3

Fmoc-L-Ala (3 equiv) was coupled into Rink Amide MBHA resin, substitution 0.55 mmol/g (NovaBiochem, San Diego, CA) with HBTU (from Nova Biochem, San Diego, CA) as coupling agent. Deprotection was performed with 30% piperidine in DMF (1 X 2 min, 1 X 8 min). Fmoc Gly, Fmoc-L-Asp(OBu), and 3-[(2-imidazolinyl)amino] benzoic acid were coupled sequentially as described above. The crude product was released by treatment with Reagent K (82.5% triflouroacetic acid, 5% phenol, 5% water, 5% thioanisole and 2.5% ethanedithiol) for 2 hours at 25 C and precipitated by addition of tert-butyl methyl ether. The product was dissolved in water and purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient was used as the eluted solvent). The structure was verified with mass spectrometer analyses (M + H = 562.5) and amino acid composition analyses.

Compound 4

Fmoc-O-benzyl-L-serine (3 equiv) was coupled into Rink Amide MBHA resin, substitution 0.55 mmol/g (NovaBiochem, San Diego, CA) with HBTU (from Nova Biochem, San Diego, CA) as coupling agent. Deprotection was performed with 30% piperidine in DMF (1 X 2 min, 1 X 8 min). Fmoc Gly, Fmoc-L-Asp(OBu), and 1-benzyl,3-(3-carboxyphenyl)-2-urea were coupled sequentially as described above. The crude product was released by treatment with Reagent K (82.5% triflouroacetic acid, 5% phenol, 5% water, 5% thioanisole and 2.5% ethanedithiol) for 2 hours at 25 C and precipitated by addition of tert-butyl methyl ether. The product was dissolved in water and purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient was used as the eluted solvent). The structure was verified with mass spectrometer analyses (M + H = 529.5) and amino acid composition analyses.

Compound 5

Fmoc-O-benzyl-L-serine (3 equiv) was coupled into Rink Amide MBHA resin, substitution 0.55 mmol/g (NovaBiochem, San Diego, CA) with HBTU (from Nova Biochem, San Diego, CA) as coupling agent. Deprotection was performed with 30% piperidine in DMF (1 X 2 min, 1 X 8 min). Fmoc Gly, Fmoc-L-Asp(OBu), and 3-[(1,4,5,6-tetrahydro, 5-hydroxyl, 2-pyrimidinyl)amino) benzoic acid were coupled sequentially as described above. The crude product was released by treatment with Reagent K (82.5% triflouroacetic acid, 5% phenol, 5% water, 5% thioanisole and 2.5% ethanedithiol) for 2 hours at 25 C and precipitated by addition of tert-butyl methyl ether. The product was dissolved in water and purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient was used as the eluted solvent). The structure was verified with mass spectrometer analyses (M + H = 608.5) and amino acid composition analyses.

Compound 6

Fmoc-O-benzyl-L-serine (3 equiv) was coupled into Rink Amide MBHA resin, substitution 0.55 mmol/g (NovaBiochem, San Diego, CA) with HBTU (from Nova Biochem, San Diego, CA) as coupling agent. Deprotection was performed with 30% piperidine in DMF (1 X 2 min, 1 X 8 min). Fmoc Gly, Fmoc-L-Asp(OBu), and 1-(3-carboxypheny)-2-thiourea (from Trans World Chemicals, Rockville, MD) were coupled sequentially as described above. The crude product was released by treatment with Reagent K (82.5% triflouroacetic acid, 5% phenol, 5% water, 5%

thioanisole and 2.5% ethanedithiol) for 2 hours at 25 C and precipitated by addition of tert-butyl methyl ether. The product was dissolved in water and purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient was used as the eluted solvent). The structure was verified with mass spectrometer analyses (M + H = 569.5) and amino acid composition analyses.

Compound 7

Fmoc-L-alanine (3 equiv) was coupled into Rink Amide MBHA resin, substitution 0.55 mmol/g (NovaBiochem, San Diego, CA) with HBTU (from Nova Biochem, San Diego, CA) as coupling agent. Deprotection was performed with 30% piperidine in DMF (1 X 2 min, 1 X 8 min). Fmoc Gly, Fmoc-L-Asp(OBu), and 3-[(2-imidazolinyl)amino) benzoic acid were coupled sequentially as described above. The crude product was released by treatment with Reagent K (82.5% triflouroacetic acid, 5% phenol, 5% water, 5% thioanisole and 2.5% ethanedithiol) for 2 hours at 25 C and precipitated by addition of tert-butyl methyl ether. The product was dissolved in water and purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient was used as the eluted solvent). The structure was verified with mass spectrometer analyses (M + H = 562.5) and amino acid composition analyses.

Compound 8

Fmoc-O-benzyl-L-serine (3 equiv) was coupled into Rink Amide MBHA resin, substitution 0.55 mmol/g (NovaBiochem, San Diego, CA) with HBTU (from Nova Biochem, San Diego, CA) as coupling agent. Deprotection was performed with 30% piperidine in DMF (1 X 2 min, 1 X 8 min). Fmoc Gly, Fmoc-L-Asp(OBu), and 3-[(1,4,5,6-tetrahydro-2-pyrimidinyl)amino] benzoic acid were coupled sequentially as described above. The crude product was released by treatment with Reagent K (82.5% triflouroacetic acid, 5% phenol, 5% water, 5% thioanisole and 2.5% ethanedithiol) for 2 hours at 25 C and precipitated by addition of tert-butyl methyl ether. The product was dissolved in water and purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient was used as the eluted solvent). The structure was verified with mass spectrometer analyses (M + H = 592.5) and amino acid composition analyses.

Compound 9

Fmoc-O-benzyl-L-serine (3 equiv) was coupled into Rink Amide MBHA resin, substitution 0.55 mmol/g (NovaBiochem, San Diego, CA) with HBTU (from Nova Biochem, San Diego, CA) as coupling agent. Deprotection was performed with 30% piperidine in DMF (1 X 2 min, 1 X 8 min). Fmoc Gly, Fmoc-L-Asp(OBu), and 3-[(1,4,5,6-tetrahydro-5-hydroxyl-2-pyrimidinyl)amino] benzoic acid were coupled sequentially as described above. The crude product was released by treatment with Reagent K (82.5% triflouroacetic acid, 5% phenol, 5% water, 5% thioanisole and 2.5% ethanedithiol) for 2 hours at 25 C and precipitated by addition of tert-butyl methyl ether. The product was dissolved in water and purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient was used as the eluted solvent). The structure was verified with mass spectrometer analyses (M + H = 608.5) and amino acid composition analyses.

Compound 10

Fmoc-O-benzyl-L-serine (3 equiv) was coupled into Rink Amide MBHA resin, substitution 0.55 mmol/g (NovaBiochem, San Diego, CA) with HBTU (from Nova Biochem, San Diego, CA) as coupling agent. Deprotection was performed with 30% piperidine in DMF (1 X 2 min, 1 X 8 min). Fmoc Gly, Fmoc-L-Asp(OBu), and were coupled 3-[(2-imidazolinyl)amino] benzoic acid sequentially as described above. The crude product was released by treatment with Reagent K (82.5% triflouroacetic acid, 5% phenol, 5% water, 5% thioanisole and 2.5% ethanedithiol) for 2 hours at 25 C and precipitated by addition of tert-butyl methyl ether. The product was dissolved in water and purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient was used as the eluted solvent). The structure was verified with mass spectrometer analyses (M + H = 569.5) and amino acid composition analyses.

Compound 11

Fmoc-O-benzyl-L-serine (3 equiv) was coupled into Rink Amide MBHA resin, substitution 0.55 mmol/g (NovaBiochem, San Diego, CA) with HBTU (from Nova Biochem, San Diego, CA) as coupling agent. Deprotection was performed with 30% piperidine in DMF (1 X 2 min, 1 X 8 min). Fmoc Gly, Fmoc-L-Asp(OBu), and 3-guanidino-5-triflouromethyl-benzoic acid were coupled sequentially as described above. The crude product was released by treatment with Reagent K (82.5% triflouroacetic acid, 5% phenol, 5% water, 5% thioanisole and 2.5%

ethanedithiol) for 2 hours at 25 C and precipitated by addition of tert-butyl methyl ether. The product was dissolved in water and purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient was used as the eluted solvent). The structure was verified with mass spectrometer analyses (M + H = 634.4) and amino acid composition analyses.

Compound 12

Fmoc-O-benzyl-L-serine (3 equiv) was coupled into Rink Amide MBHA resin, substitution 0.55 mmol/g (NovaBiochem, San Diego, CA) with HBTU (from Nova Biochem, San Diego, CA) as coupling agent. Deprotection was performed with 30% piperidine in DMF (1 X 2 min, 1 X 8 min). Fmoc Gly, Fmoc-L-Asp(OBu), and 3-guanidino-benzoic acid were coupled sequentially as described above. The crude product was released by treatment with Reagent K (82.5% triflouroacetic acid, 5% phenol, 5% water, 5% thioanisole and 2.5% ethanedithiol) for 2 hours at 25 C and precipitated by addition of tert-butyl methyl ether. The product was dissolved in water and purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient was used as the eluted solvent). The structure was verified with mass spectrometer analyses (M + H = 453.4) and amino acid composition analyses.

Compound 13

Fmoc -L- Ala (3 equiv) was coupled into Rink Amide MBHA resin, substitution 0.55 mmol/g (NovaBiochem, San Diego, CA) with HBTU (from Nova Biochem, San Diego, CA) as coupling agent. Deprotection was performed with 30% piperidine in DMF (1 X 2 min, 1 X 8 min). Fmoc Gly, Fmoc-L-Asp(OBu), and 3-[(5-hydroxyl-1,4,5,6-tetrahydro-2-pyrimidinyl)amino] benzoic acid were coupled sequentially as described above. The crude product was released by treatment with Reagent K (82.5% triflouroacetic acid, 5% phenol, 5% water, 5% thioanisole and 2.5% ethanedithiol) for 2 hours at 25 C and precipitated by addition of tert-butyl methyl ether. The product was dissolved in water and purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient was used as the eluted solvent). The structure was verified with mass spectrometer analyses (M + H = 592.5) and amino acid composition analyses.

Compound 14

Boc-Ala-S-CH_2CH_2COOH was prepared and coupled on methybenzhydrylamine resin (Peptide International, Louisville, KY) as described

(H. Hojo and S. Aimoto, *Bull Chem Soc. Jpn* **64**: 111, 1991). After deprotecting with 50% triflouroacetic acid in methylene chloride (2 X 1 min, 1 X 30 min) the remaining amino acid residue and the N-terminal arginine analog was attached on the solid support by N,N'-diisoproplycarbodiimide/1-hydroxybenzotriazole] as a coupling agent as described previously (L. Zhang & J.P. Tam, *J. Am. Chem. Soc.* **119**: 2363, 1997). After cleavage from the solid support with HF/anisole (9/1) at 0°C for 1 hour, the a_vb_3 thioester was purified by reversed phase HPLC. The purified a_vb_3 antagonist thioester was conjugated with interferon alpha (1-5) using the orthogonal coupling method described previously (J.P. Tam et al., *Proc. Natl. Acad. Sci. USA* **92**: 12485, 1995).

Example 2: Preparation of series 100 a_vb_3 antagonist-interferon alpha bioconjugates

General procedures

A_vb_3 antagonists were prepared as thioesters as described (H. Hojo and S. Aimoto, *Bull Chem Soc. Jpn* **64**: 111, 1991; L. E. Canne et al., *J. Am. Chem. Soc*: **118**: 5891, 1996). A_vb_3 thioester was reacted with protein or peptide with free N-terminal cysteine residue by using chemoselective method (P. E. Dawson et al., *Science* **266**: 776, 1994). The conjugate was purified by reversed phase HPLC or ionic exchange chromatography.

Compound 100 ('RGD"-Gly-Gly-Gly-Ala-interferon alpha)

'RGD'-Gly-Gly-Gly-Ala thioester was prepared as described (L.E. Canne et al., *Tetrahedron Lett.* **36**: 1217, 1995; L.E. Canne et al., *J. Am. Chem. Soc.* **118**: 5891, 1996). Interferon alpha was first reduced with a mixture of 6 mM cysteine and 1 mM cystine and then coupled with 'RGD'-Gly-Gly-Gly-Ala-thioester with native chemical ligation as described by S. Kent et al., in WO 96/34878.

Folding of the conjugated a_vb_3 antagonist interferon alpha was performed by cysteine/cystine disulfide exchange method (M.A. Siani et al., IBC 3rd Annual International Conference: Chemokines, Sept 1996, "Rapid modular synthesis of chemokines and analogues," available at http:// siani@gryphonsci.com/library/ chemokines/modsynthochemokines.htm).

Compound 101

Fmoc-L-Ala-S-CH_2-CH_2-COOH (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide International, Louisville, KY) with dicyclohexylcarbodiimde as coupling agent. Deprotection is performed with 50% TFA(1 X 2 min, 1 X 30 min)., Boc-L-Asp(OBzl), Boc-Gly, and 3-[(1,4,5,6-tetrahydro-2-pyrimidinyl)amino] benzoic acid are coupled sequentially as described in General Method. The crude product is released by treatment with hydrogen flouride/anisole (9/1) for 1 hours at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient is used as the eluted solvent). The structure is verified with mass spectrometer analyses (M + H = 536) and amino acid composition analyses. Interferon is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 102

Fmoc-L-Ala-S-CH_2-CH_2-COOH (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide International, Louisville, KY) with dicyclohexylcarbodiimde as coupling agent. Deprotection is performed with 50% TFA(1 X 2 min, 1 X 30 min)., Boc-L-Asp(OBzl), Boc-Gly, and 3-[(1,4,5,6-tetrahydro-5-hydroxyl, 2-pyrimidinyl)amino] benzoic acid are coupled sequentially as described in General Method. The crude product is released by treatment with hydrogen flouride/anisole (9/1) for 1 hours at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient is used as the eluted solvent). The structure is verified with mass spectrometer analyses (M + H = 552) and amino acid composition analyses. Interferon-alpha is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 103

Fmoc-L-Ser(Bzl)-S-CH_2-CH_2-COOH (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide International, Louisville, KY) with dicyclohexylcarbodiimde as coupling agent. Deprotection is performed with 50%

TFA(1 X 2 min, 1 X 30 min)., Boc-L-Asp(OBzl), Boc-Gly, and 3-[(1,4,5,6-tetrahydro-2-pyrimidinyl)amino] benzoic acid are coupled sequentially as described in General Method. The crude product is released by treatment with hydrogen flouride/anisole (9/1) for 1 hours at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient is used as the eluted solvent). The structure is verified with mass spectrometer analyses (M + H = 566) and amino acid composition analyses. Interferon is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reactedwith the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 104

Fmoc-L-Ser(Bzl)-S-CH_2-CH_2-COOH (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide International, Louisville, KY) with dicyclohexylcarbodiimde as coupling agent. Deprotection is performed with 50% TFA(1 X 2 min, 1 X 30 min)., Boc-L-Asp(OBzl), Boc-Gly, and 3-[(1,4,5,6-tetrahydro, 5-hydroxyl, 2-pyrimidinyl)amino] benzoic acid are coupled sequentially as described in General Method. The crude product is released by treatment with hydrogen flouride/anisole (9/1) for 1 hours at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient is used as the eluted solvent). The structure is verified with mass spectrometer analyses (M + H = 582) and amino acid composition analyses. Interferon is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reactedwith the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 105

Fmoc-L-Ala-S-CH_2-CH_2-COOH (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide International, Louisville, KY) with dicyclohexylcarbodiimde as coupling agent. Deprotection is performed with 50% TFA(1 X 2 min, 1 X 30 min)., Boc-L-Asp(OBzl), Boc-Gly, and 3-[(2-imidazolinylamino)] benzoic acid are coupled sequentially as described in General Method. The crude product is released by treatment with hydrogen flouride/anisole (9/1) for 1 hours at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient is used as the eluted solvent). The structure is

verified with mass spectrometer analyses (M + H = 566) and amino acid composition analyses. Interferon is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 106

Fmoc-L-Ser(Bzl)-S-CH_2-CH_2-COOH (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide International, Louisville, KY) with dicyclohexylcarbodiimde as coupling agent. Deprotection is performed with 50% TFA(1 X 2 min, 1 X 30 min)., Boc-L-Asp(OBzl), Boc-Gly, and 3-[(2-imidazolinylamino)] benzoic acid are coupled sequentially as described in General Method. The crude product is released by treatment with hydrogen flouride/anisole (9/1) for 1 hours at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient is used as the eluted solvent). The structure is verified with mass spectrometer analyses (M + H = 582) and amino acid composition analyses. Interferon is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 107

Fmoc-L-Ala-S-CH_2-CH_2-COOH (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide International, Louisville, KY) with dicyclohexylcarbodiimde as coupling agent. Deprotection is performed with 50% TFA(1 X 2 min, 1 X 30 min)., Boc-L-Asp(OBzl), Boc-Gly, and 1-benzy, 3-(3-carboxyphenyl)-2-urea are coupled sequentially as described in General Method. The crude product amino) benzoic is released by treatment with hydrogen flouride/anisole (9/1) for 1 hours at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient is used as the eluted solvent). The structure is verified with mass spectrometer analyses (M + H = 583) and amino acid composition analyses. Interferon is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 108

Fmoc-L-Ser(Bzl)-S-CH_2-CH_2-COOH (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide International, Louisville, KY) with dicyclohexylcarbodiimde as coupling agent. Deprotection is performed with 50% TFA(1 X 2 min, 1 X 30 min)., Boc-L-Asp(OBzl), Boc-Gly, and 3-[(1,4,5,6-tetrahydro-2-pyrimidinyl)amino] benzoic acid are coupled sequentially as described in General Method. The crude product is released by treatment with hydrogen flouride/anisole (9/1) for 1 hours at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient is used as the eluted solvent). The structure is verified with mass spectrometer analyses (M + H = 599) and amino acid composition analyses. Interferon is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 109

Fmoc-L-Ala-S-CH_2-CH_2-COOH (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide International, Louisville, KY) with dicyclohexylcarbodiimde as coupling agent. Deprotection is performed with 50% TFA(1 X 2 min, 1 X 30 min)., Boc-L-Asp(OBzl), Boc-Gly, and 3-guanidino-5-triflouromethyl-benzoic acid are coupled sequentially as described in General Method. The crude product amino) benzoic is released by treatment with hydrogen flouride/anisole (9/1) for 1 hours at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient is used as the eluted solvent). The structure is verified with mass spectrometer analyses (M + H =566) and amino acid composition analyses. Interferon is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 110

Fmoc-L-Ser(Bzl)-S-CH_2-CH_2-COOH (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide International, Louisville, KY) with dicyclohexylcarbodiimde as coupling agent. Deprotection is performed with 50% TFA(1 X 2 min, 1 X 30 min)., Boc-L-Asp(OBzl), Boc-Gly, and 3-guanidino-5-

triflouromethyl-benzoic acid are coupled sequentially as described in General Method. The crude product is released by treatment with hydrogen flouride/anisole (9/1) for 1 hours at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient is used as the eluted solvent). The structure is verified with mass spectrometer analyses (M + H = 582) and amino acid composition analyses. Interferon is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 111

Fmoc-L-Ala-S-CH_2-CH_2-COOH (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide International, Louisville, KY) with dicyclohexylcarbodiimde as coupling agent. Deprotection is performed with 50% TFA(1 X 2 min, 1 X 30 min)., Boc-L-Asp(OBzl), Boc-Gly, and 3-guanidino-benzoic acid are coupled sequentially as described in General Method. The crude product amino) benzoic is released by treatment with hydrogen flouride/anisole (9/1) for 1 hours at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient is used as the eluted solvent). The structure is verified with mass spectrometer analyses (M + H =498) and amino acid composition analyses. Interferon is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 112

Fmoc-L-Ser(Bzl)-S-CH_2-CH_2-COOH (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide International, Louisville, KY) with dicyclohexylcarbodiimde as coupling agent. Deprotection is performed with 50% TFA(1 X 2 min, 1 X 30 min)., Boc-L-Asp(OBzl), Boc-Gly, and 3-guanidino-benzoic acid are coupled sequentially as described in General Method. The crude product is released by treatment with hydrogen flouride/anisole (9/1) for 1 hours at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient is used as the eluted solvent). The structure is verified with mass spectrometer analyses (M + H = 514) and amino acid composition analyses. Interferon is first

reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 113

Fmoc-L-Ala-S-CH_2-CH_2-COOH (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide International, Louisville, KY) with dicyclohexylcarbodiimde as coupling agent. Deprotection is performed with 50% TFA(1 X 2 min, 1 X 30 min)., Boc-L-Asp(OBzl), Boc-Gly, and 1-(3-carboxy, -5-triflouomethylphenyl)-2-thiourea are coupled sequentially as described in General Method. The crude product amino) benzoic is released by treatment with hydrogen flouride/anisole (9/1) for 1 hours at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient is used as the eluted solvent). The structure is verified with mass spectrometer analyses (M + H =583) and amino acid composition analyses. Interferon is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 114

Fmoc-L-Ser(Bzl)-S-CH_2-CH_2-COOH (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide International, Louisville, KY) with dicyclohexylcarbodiimde as coupling agent. Deprotection is performed with 50% TFA(1 X 2 min, 1 X 30 min)., Boc-L-Asp(OBzl), Boc-Gly, and 1-(3-carboxy, 5-triflouromethylphenyl)-2-thiourea are coupled sequentially as described in General Method. The crude product is released by treatment with hydrogen flouride/anisole (9/1) for 1 hours at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient is used as the eluted solvent). The structure is verified with mass spectrometer analyses (M + H = 599) and amino acid composition analyses. Interferon is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 115

Fmoc-L-Ala-S-CH_2-CH_2-COOH (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide International, Louisville, KY) with dicyclohexylcarbodiimde as coupling agent. Deprotection is performed with 50% TFA(1 X 2 min, 1 X 30 min)., Boc-L-Asp(OBzl), Boc-Gly, and 1-(3-carboxyphenyl)-2-thiourea are coupled sequentially as described in General Method. The crude product amino) benzoic is released by treatment with hydrogen flouride/anisole (9/1) for 1 hours at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient is used as the eluted solvent). The structure is verified with mass spectrometer analyses (M + H =514) and amino acid composition analyses. Interferon is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 116

Fmoc-L-Ser(Bzl)-S-CH_2-CH_2-COOH (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide International, Louisville, KY) with dicyclohexylcarbodiimde as coupling agent. Deprotection is performed with 50% TFA(1 X 2 min, 1 X 30 min)., Boc-L-Asp(OBzl), Boc-Gly, and 1-(3-carboxyphenyl)-2-thiourea are coupled sequentially as described in General Method. The crude product is released by treatment with hydrogen flouride/anisole (9/1) for 1 hours at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient is used as the eluted solvent). The structure is verified with mass spectrometer analyses (M + H = 530) and amino acid composition analyses. Interferon is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 117

Fmoc-L-Ser(Bzl)-S-CH_2-CH_2-COOH (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide International, Louisville, KY) with dicyclohexylcarbodiimde as coupling agent. Deprotection is performed with 50% TFA(1 X 2 min, 1 X 30 min)., Boc-Gly, Boc-Ala, Boc-L-Asp(OBzl), Boc-Gly, and 3-

[(1,4,5,6-tetrahydro-2-pyrimidinyl)amino] benzoic acid are coupled sequentially as described in General Method. The crude product is released by treatment with hydrogen flouride/anisole (9/1) for 1 hours at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient is used as the eluted solvent). The structure is verified with mass spectrometer analyses (M + H = 1216) and amino acid composition analyses. Interferon is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 118

Fmoc-L-Ser(Bzl)-S-CH_2-CH_2-COOH (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide International, Louisville, KY) with dicyclohexylcarbodiimde as coupling agent. Deprotection is performed with 50% TFA(1 X 2 min, 1 X 30 min)., Boc-Gly, Boc-Ala, Boc-L-Asp(OBzl), Boc-Gly, and 3-[(1,4,5,6-tetrahydro-2-pyrimidinyl)amino] benzoic acid are coupled sequentially as described in General Method. The crude product is released by treatment with hydrogen flouride/anisole (9/1) for 1 hours at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient is used as the eluted solvent). The structure is verified with mass spectrometer analyses (M + H = 1549) and amino acid composition analyses. Interferon is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 119

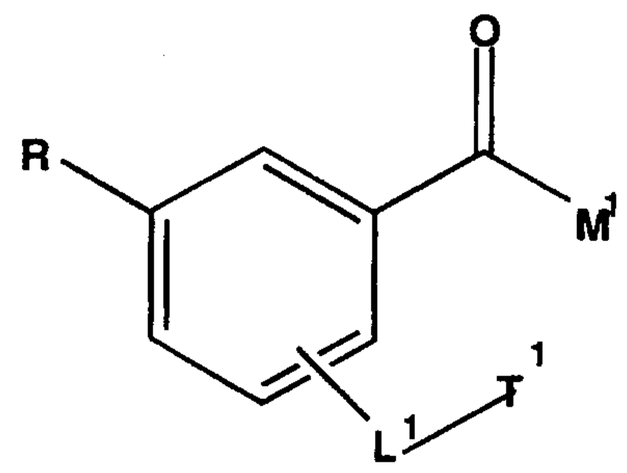
Fmoc-L-Ser(Bzl)-S-CH_2-CH_2-COOH (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide International, Louisville, KY) with dicyclohexylcarbodiimde as coupling agent. Deprotection is performed with 50% TFA(1 X 2 min, 1 X 30 min)., Boc-Gly, Boc-Ala, Boc-L-Asp(OBzl), Boc-Gly, and 3-[(1,4,5,6-tetrahydro-5-hydroxyl, 2-pyrimidinyl)amino] benzoic acid are coupled sequentially as described in General Method. The crude product is released by treatment with hydrogen flouride/anisole (9/1) for 1 hours at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05 % triflouroacetic acid (with 0 - 40% acetonitrile) linear gradient is used as the eluted solvent). The structure is verified with mass spectrometer analyses (M + H

= 1232) and amino acid composition analyses. Interferon is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Example 3: Preparation of series 200 a_vb_3 antagonist-interferon alpha bioconjugates

General procedures

A_vb_3 antagonist bioconjugates of the formula



can also be prepared by linking to the a_vb_3 moiety to a protein through a different linkage group than that described above for the Series 100 compounds. A_vb_3 thioester was reacted with protein or peptide with free N-terminal cysteine residue by using chemoselective method (P. E. Dawson et al., *Science* **266**: 776, 1994). The conjugate is purified by reversed phase HPLC or ionic exchange chromatography.

Compound 201

5-Aminoisophthanic acid is refluxed with ammonium isothiocynate (3 equiv) in 6N HCl for 18 hours. After being cooled to 25°C and filtered, the thiourea is extracted with ethyl acetate. After rotary evaporation the thiourea is solidified and filtered. 5-[(1,4,5,6-tetrahydro-2-pyrimidinyl] aminoisophthanic acid is prepared by reacting the thiourea with methyl iodide, and then with 1,3-diaminopropane.

Asp(OBzl)-Ala-NH_2 is prepared by coupling Boc-Asp(Bzl)-OH with Ala-NH_2 using the dicyclohexylcarbodiimde (DCC) method (M. Bodanszky and A. Bodanszky, The Practice of Peptide Synthesis, Springer-Verlag, New York, 1984, p22) and then deprotected with dioxine/HCl. Gly-Asp(Bzl)-Ala-NH_2 is prepared by

coupling Boc-Glycine with Asp(Bzl)-Ala-NH_2 using the DCC method and then deprotected with dioxine/HCl.

5-[(1,4,5,6-tetrahydro-2-pyrimidinyl]aminoisophthanyl Gly-Asp(Bzl)-Ala-NH_2 is prepared by coupling 5-[(1,4,5,6-tetrahydro-2-pyrimidinyl]aminoisophthanic acid with Gly-Asp(Bzl)-Ala-NH_2 (1 equiv) using BOP/DIEA method (L. Zhang and J.P. Tam, *J. Am. Chem. Soc.* **119**: 2363, 1997).

Fmoc-L-Ala-S-CH_2-CH_2-COOH prepared according to H. Horo and S. Aimoto (*Bull. Chem. Soc. Jpn.* **64**: 111, 1987) (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide international, Louisville, KY) with DCC as coupling agent. Deprotection is performed with 50% TFA (1 X 2 min., 1 X 30 min.).

Boc-Gly, and 5-[1,4,5,6-tetrahydro-2-pyrimidinyl] aminoisophthanyl Gly-Asp(Bzl)-Ala-NH_2, are coupled sequentially as described in General method. The crude product is released by treatment with hydrogen fluoride/anisole (9/1) for 1 hour at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05% triflouroacetic acid (0 - 40% acetonitrile) linear gradient is used as the eluted solvent). Interferon alpha is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cycstine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 202

5-Aminoisophthanic acid is refluxed with ammonium isothiocynate (3 equiv) in 6N HCl for 18 hours. After cooled to 25°C and filtered, the thiourea is extracted with ethyl acetate. After rotary evaporation the thiourea is solidified and filtered. 5-[(1,4,5,6-tetrahydro-2-pyrimidinyl] aminoisophthanic acid is prepared by reacting the thiourea with methyl iodide, and then with 1,3-diaminopropane.

Asp(OBzl)-Ser(Bzl)-NH_2 is prepared by coupling Boc-Asp(Bzl)-OH with Ser(Bzl)-NH_2 using the dicyclohexylcarbodiimde (DCC) method (M. Bodanszky and A. Bodanszky, The Practice of Peptide Synthesis, Springer-Verlag, New York, 1984, p22) and then deprotected with dioxine/HCl. Gly-Asp(Bzl)-Ser(Bzl)-NH_2 is prepared by coupling Boc-Glycine with Asp(Bzl)-Ser(Bzl)-NH2 using the DCC method and then deprotected with dioxine/HCl.

5-[(1,4,5,6-tetrahydro-2-pyrimidinyl]aminoisophthanyl Gly-Asp(Bzl)-Ser(Bzl)-NH_2 is prepared by coupling 5-[1,4,5,6-tetrahydro-2-pyrimidinyl]aminoisophthanic acid with Gly-Asp(Bzl)-Ser(Bzl)-NH_2 (1 equiv) using BOP/DIEA method (L. Zhang and J.P. Tam, *J. Am. Chem. Soc.* **119**: 2363, 1997).

Fmoc-L-Ala-S-CH_2-CH_2-COOH prepared according to H. Horo and S. Aimoto (*Bull. Chem. Soc. Jpn.* **64:** 111, 1987) (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide international, Louisville, KY) with DCC as coupling agent. Deprotection is performed with 50% TFA (1 X 2 min., 1 X 30 min.).

Boc-Gly, and 5-[(1,4,5,6-tetrahydro-2-pyrimidinyl]aminoisophthanyl Gly-Asp(Bzl)-Ser(Bzl)-NH_2, are coupled sequentially as described in General method. The crude product is released by treatment with hydrogen fluoride/anisole (9/1) for 1 hour at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05% triflouroacetic acid (0 - 40% acetonitrile) linear gradient is used as the eluted solvent). Interferon alpha is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 203

5-Aminoisophthanic acid is refluxed with ammonium isothiocynate (3 equiv) in 6N HCl for 18 hours. After cooled to 25°C and filtered, the thiourea is extracted with ethyl acetate. After rotary evaporation the thiourea is solidified and filtered. 5-[(1,4,5,6-tetrahydro-4-hydroxyl-2-pyrimidinyl]aminoisophthanic acid is prepared by reacting the thiourea with methyl iodide, and then with 1,3-diamino-2-hydroxylpropane.

Asp(OBzl)-Ala-NH_2 is prepared by coupling Boc-Asp(Bzl)-OH with Ala-NH_2 using the dicyclohexylcarbodiimde (DCC) method (M. Bodanszky and A. Bodanszky, The Practice of Peptide Synthesis, Springer-Verlag, New York, 1984, p22) and then deprotected with dioxine/HCl. Gly-Asp(Bzl)-Ala-NH_2 is prepared by coupling Boc-Glycine with Asp(Bzl)-Ala-NH2 using the DCC method and then deprotected with dioxine/HCl.

5-[(1,4,5,6-tetrahydro-2-pyrimidinyl]aminoisophthanyl Gly-Asp(Bzl)-Ala-NH_2 is prepared by coupling 5-[1,4,5,6-tetrahydro-2-pyrimidinyl]aminoisophthanic

acid with Gly-Asp(Bzl)-Ala-NH_2 (1 equiv) using BOP/DIEA method (L. Zhang and J.P. Tam, *J. Am. Chem. Soc.* **119**: 2363, 1997).

Fmoc-L-Ala-S-CH_2-CH_2-COOH prepared according to H. Horo and S. Aimoto (*Bull. Chem. Soc. Jpn.* **64:** 111, 1987) (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide international, Louisville, KY) with DCC as coupling agent. Deprotection is performed with 50% TFA (1 X 2 min., 1 X 30 min.).

Boc-Gly, and 5-[(1,4,5,6-tetrahydro-4-hydroxyl-2-pyrimidinyl]aminoisophthanyl Gly-Asp(Bzl)-Ala-NH_2, are coupled sequentially as described in General method. The crude product is released by treatment with hydrogen fluoride/anisole (9/1) for 1 hour at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05% triflouroacetic acid (0 - 40% acetonitrile) linear gradient is used as the eluted solvent). Interferon alpha is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 204

5-Aminoisophthanic acid is refluxed with ammonium isothiocynate (3 equiv) in 6N HCl for 18 hours. After cooled to 25°C and filtered, the thiourea is extracted with ethyl acetate. After rotary evaporation the thiourea is solidified and filtered. 5-[(1,4,5,6-tetrahydro-4-hydroxyl-2-pyrimidinyl] aminoisophthanic acid is prepared by reacting the thiourea with methyl iodide, and then with 1,3-diamino-2-hydroxylpropane.

Asp(OBzl)-Ala-NH_2 is prepared by coupling Boc-Asp(Bzl)-OH with Ser(Bzl)-NH_2 using the dicyclohexylcarbodiimde (DCC) method (M. Bodanszky and A. Bodanszky, The Practice of Peptide Synthesis, Springer-Verlag, New York, 1984, p22) and then deprotected with dioxine/HCl. Gly-Asp(Bzl)-Ser(Bzl)-NH2 is prepared by coupling Boc-Glycine with Asp(Bzl)-Ser(Bzl)-NH_2 using the DCC method and then deprotected with dioxine/HCl .

5-[(1,4,5,6-tetrahydro-2-pyrimidinyl] aminoisophthanyl Gly-Asp(Bzl)-Ser(Bzl)-NH_2 is prepared by coupling 5-[(1,4,5,6-tetrahydro-2-pyrimidinyl] aminoisophthanic acid with Gly-Asp(Bzl)-Ser(Bzl)-NH_2 (1 equiv) using BOP/DIEA method (L. Zhang and J.P. Tam, *J. Am. Chem. Soc.* **119**: 2363, 1997).

Fmoc-L-Ala-S-CH_2-CH_2-COOH prepared according to H. Horo and S. Aimoto (*Bull. Chem. Soc. Jpn.* **64:** 111, 1987) (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide international, Louisville, KY) with DCC as coupling agent. Deprotection is performed with 50% TFA (1 X 2 min., 1 X 30 min.).

Boc-Gly, and 5-[(1,4,5,6-tetrahydro-4-hydroxyl-2-pyrimidinyl]aminoisophthanyl Gly-Asp(Bzl)-Ser(Bzl)-NH_2, are coupled sequentially as described in General Method. The crude product is released by treatment with hydrogen fluoride/anisole (9/1) for 1 hour at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05% triflouroacetic acid (0 - 40% acetonitrile) linear gradient is used as the eluted solvent). Interferon alpha is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 205

5-Aminoisophthanic acid is refluxed with ammonium isothiocynate (3 equiv) in 6N HCl for 18 hours. After cooled to 25°C and filtered, the thiourea is extracted with ethyl acetate. After rotary evaporation the thiourea is solidified and filtered. 5-(2-imidazolinyl)aminoisophthanic acid is prepared by reacting the thiourea with methyl iodide, and then with 1,2-diaminoethane.

Asp(OBzl)-Ala-NH_2 is prepared by coupling Boc-Asp(Bzl)-OH with Ala-NH_2 using the dicyclohexylcarbodiimde (DCC) method (M. Bodanszky and A. Bodanszky, The Practice of Peptide Synthesis, Springer-Verlag, New York, 1984, p22) and then deprotected with dioxine/HCl. Gly-Asp(Bzl)-Ala-NH_2 is prepared by coupling Boc-Glycine with Asp(Bzl)-Ala-NH_2 using the DCC method and then deprotected with dioxine/HCl.

5-(2-Imidazolinyl)aminoisophthanyl Gly-Asp(Bzl)-Ala-NH_2 is prepared by coupling 5-(2-imidazonyl) aminoisophthanic acid with Gly-Asp(Bzl)-Ala-NH_2 (1 equiv) using BOP/DIEA method (L. Zhang and J.P. Tam, *J. Am. Chem. Soc.* **119**: 2363, 1997).

Fmoc-L-Ala-S-CH_2-CH_2-COOH prepared according to H. Horo and S. Aimoto (*Bull. Chem. Soc. Jpn.* **64**: 111, 1987) (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide international, Louisville, KY) with DCC as coupling agent. Deprotection is performed with 50% TFA (1 X 2 min., 1 X 30 min.).

Boc-Gly, and 5-(2-imidazolinyl) aminoisophthanyl Gly-Asp(Bzl)-Ala-NH_2, are coupled sequentially as described in General method. The crude product is released by treatment with hydrogen fluoride/anisole (9/1) for 1 hour at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05% triflouroacetic acid (0 - 40% acetonitrile) linear gradient is used as the eluted solvent). Interferon alpha is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cycstine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 206

5-Aminoisophthanic acid is refluxed with ammonium isothiocynate (3 equiv) in 6N HCl for 18 hours. After cooled to 25°C and filtered, the thiourea is extracted with ethyl acetate. After rotary evaporation the thiourea is solidified and filtered. 5-(2-Imidazolinyl)aminoisophthanic acid is prepared by reacting the thiourea with methyl iodide, and then with 1,3-diaminopropane.

Asp(OBzl)-Ser(Bzl)-NH_2 is prepared by coupling Boc-Asp(Bzl)-OH with Ser(Bzl)-NH_2 using the dicyclohexylcarbodiimde (DCC) method (M. Bodanszky and A. Bodanszky, The Practice of Peptide Synthesis, Springer-Verlag, New York, 1984, p22) and then deprotected with dioxine/HCl. Gly-Asp(Bzl)-Ser(Bzl)-NH_2 is prepared by coupling Boc-Glycine with Asp(Bzl)-Ser(Bzl)-NH_2 using the DCC method and then deprotected with dioxine/HCl.

5-(2-Imidazolinyl)aminoisophthanyl Gly-Asp(Bzl)-Ser(Bzl)-NH_2 is prepared by coupling 5-(2-imidazolinyl)aminoisophthanic acid with Gly-Asp(Bzl)-Ser(Bzl)-NH_2 (1 equiv) using BOP/DIEA method (L. Zhang and J.P. Tam, *J. Am. Chem. Soc.* **119**: 2363, 1997).

Fmoc-L-Ala-S-CH_2-CH_2-COOH prepared according to H. Horo and S. Aimoto (*Bull. Chem. Soc. Jpn.* **64:** 111, 1987) (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide international, Louisville, KY) with DCC as coupling agent. Deprotection is performed with 50% TFA (1 X 2 min., 1 X 30 min.).

Boc-Gly, and 5-(2-imidazolinyl)aminoisophthanyl Gly-Asp(Bzl)-Ser(Bzl)-NH_2, are coupled sequentially as described in General method. The crude product is released by treatment with hydrogen fluoride/anisole (9/1) for 1 hour at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05% triflouroacetic acid (0 - 40% acetonitrile) linear gradient is used as

the eluted solvent). Interferon alpha is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 207

5-Aminoisophthanic acid is refluxed with ammonium isothiocynate (3 equiv) in 6N HCl for 18 hours. After cooled to 25°C and filtered, the thiourea is extracted with ethyl acetate. After rotary evaporation the thiourea is solidified and filtered. 5-[(1,4,5,6-tetrahydro-2-pyrimidinyl] aminoisophthanic acid is prepared by reacting the thiourea with methyl iodide, and then with 1,3-diaminopropane.

Asp(OBzl)-Ala-NH_2 is prepared by coupling Boc-Asp(Bzl)-OH with Ala-NH_2 using the dicyclohexylcarbodiimde (DCC) method (M. Bodanszky and A. Bodanszky , The Practice of Peptide Synthesis, Springer-Verlag, New York, 1984, p22) and then deprotected with dioxine/HCl. Gly-Asp(Bzl)-Ala-NH_2 is prepared by coupling Boc-Glycine with Asp(Bzl)-Ala-NH_2 using the DCC method and then deprotected with dioxine/HCl.

5-[(1,4,5,6-tetrahydro-2-pyrimidinyl]aminoisophthanyl Gly-Asp(Bzl)-Ala-NH_2 is prepared by coupling 5-[(1,4,5,6-tetrahydro-2-pyrimidinyl]aminoisophthanic acid with Gly-Asp(Bzl)-Ala-NH_2 (1 equiv) using BOP/DIEA method (L. Zhang and J.P. Tam, *J. Am. Chem. Soc.* **119**: 2363, 1997).

Fmoc-L-Ala-S-CH_2-CH_2-COOH prepared according to H. Horo and S. Aimoto (*Bull. Chem. Soc. Jpn.* **64**: 111, 1987) (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide international, Louisville, KY) with DCC as coupling agent. Deprotection is performed with 50% TFA (1 X 2 min., 1 X 30 min.).

Boc-6-aminohexanoic acid, and 5-[(1,4,5,6-tetrahydro-2-pyrimidinyl]aminoiso-phthanyl Gly-Asp(Bzl)-Ala-NH_2, are coupled sequentially as described in General method. The crude product is released by treatment with hydrogen fluoride/anisole (9/1) for 1 hour at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05% triflouroacetic acid (0 - 40% acetonitrile) linear gradient is used as the eluted solvent). Interferon alpha is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general

method. The product is folded by cysteine/cycstine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Compound 208

5-Aminoisophthanic acid is refluxed with ammonium isothiocynate (3 equiv) in 6N HCl for 18 hours. After cooled to 25°C and filtered, the thiourea is extracted with ethyl acetate. After rotary evaporation the thiourea is solidified and filtered. 5-[(1,4,5,6-tetrahydro-2-pyrimidinyl] aminoisophthanic acid is prepared by reacting the thiourea with methyl iodide, and then with 1,3-diaminopropane.

Asp(OBzl)-Ser(Bzl)-NH_2 is prepared by coupling Boc-Asp(Bzl)-OH with Ser(Bzl)-NH_2 using the dicyclohexylcarbodiimde (DCC) method (M. Bodanszky and A. Bodanszky, The Practice of Peptide Synthesis, Springer-Verlag, New York, 1984, p22) and then deprotected with dioxine/HCl. Gly-Asp(Bzl)-Ser(Bzl)-NH_2 is prepared by coupling Boc-Glycine with Asp(Bzl)-Ser(Bzl)-NH_2 using the DCC method and then deprotected with dioxine/HCl .

5-(1,4,5,6-tetrahydro-2-pyrimidinyl)aminoisophthanyl Gly-Asp(Bzl)-Ser(Bzl)-NH_2 is prepared by coupling 5-[(1,4,5,6-tetrahydro-2-pyrimidinyl]aminoisophthanic acid with Gly-Asp(Bzl)-Ser(Bzl)-NH_2 (1 equiv) using BOP/DIEA method (L. Zhang and J.P. Tam, *J. Am. Chem. Soc.* **119**: 2363, 1997).

Fmoc-L-Ala-S-CH_2-CH_2-COOH prepared according to H. Horo and S. Aimoto (*Bull. Chem. Soc. Jpn.* **64:** 111, 1987) (3 equiv) is coupled into p-methylbenzhydrylamine resin (Peptide international, Louisville, KY) with DCC as coupling agent. Deprotection is performed with 50% TFA (1 X 2 min., 1 X 30 min.).

Boc-6-aminohexanoic acid, and 5-(1,4,5,6-tetrahydro-2-pyrimidinyl)aminoiso-phthanyl Gly-Asp(Bzl)-Ser(Bzl)-NH_2, are coupled sequentially as described in General method. The crude product is released by treatment with hydrogen fluoride/anisole (9/1) for 1 hour at 0°C and extracted with 10% acetic acid. The thioester is purified by C18 reversed phase HPLC. 0.05% triflouroacetic acid (0 - 40% acetonitrile) linear gradient is used as the eluted solvent). Interferon alpha is first reduced with dithiothreitol (Sigma, St. Louis, MO) and then reacted with the thioester using the chemoselective reaction as described in general method. The product is folded by cysteine/cystine disulfide bridge exchange method and purified by reversed phase C18 HPLC.

Example 4: Preparation of purified interferon for conjugation to a_vb_3 antagonists

General cloning methods

General methods of cloning, expressing, and characterizing proteins are found in T. Maniatis et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, 1982, and references cited therein, incorporated herein by reference; and in J. Sambrook et al., *Molecular Cloning, A Laboratory Manual,* 2nd edition, Cold Spring Harbor Laboratory, 1989, and references cited therein, incorporated herein by reference.

General methods describing the culture and biochemical analysis of cells, light microscopy and cell structure, and subcellular localization of genes and their products are found in D.L. Spector et al., *Cells: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1998, and references cited therein, incorporated herein by reference.

Unless noted otherwise, all specialty chemicals were obtained from Sigma, Co. (St. Louis, MO). Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA) or Boehringer Mannheim (Indianapolis, IN).

Transformation of E. coli strains

E. coli strains (Table 1), such as DH5α™ (Life Technologies, Gaithersburg, MD) and TG1 (Amersham Corp., Arlington Heights, IL) are used for transformation of ligation reactions and are the hosts used to prepare plasmid DNA for transfecting mammalian cells. *E. coli* strains, such as JM101 (Yanisch-Perron et al., *Gene*, 33: 103-119, 1985) and MON105 (Obukowicz et al., *Appl. and Envir. Micr.*, 58: 1511-1523, 1992) can be used for expressing the protein component of multi-functional bioconjugates of the present invention in the cytoplasm or periplasmic space.

Table of Strains

Designation	Description	Reference/Source

DH5α™	F-, *phi*80 d*lac*ZdeltaM15, delta(*lac*ZYA-*arg*F)U169, *deo*R, *rec*A1, *end*A1, *hsd*R17 (rk-,mk+), *pho*A, *sup*E44, lambda-, *thi*-1, *gyr*A96, relA1	Life Technologies, Rockville, Maryland
JM101 (ATCC#33876)	delta (*pro lac*), *sup*E, *thi*, F' (*tra*D36, *pro*A+B+, *lac*Iq, *lac*ZdeltaM15)	Yanisch-Perron et al., *Gene*, 33: 103-119, 1985
MON105 (ATCC#55204)	F-, lambda-,IN (rrnD, rrnE)1, rpoD+, *rpo*H358	Obukowicz et al., *Appl. and Envir. Micr.*, 58: 1511-1523, 1992
MON208	W3110 *rpo*H358, *lac*IQ, *omp*T::*kan*	Alan Easton
TG1	delta(lac-pro), *sup*E, *thi*-1, *hsd*D5/F'(*tra*D36, *pro*A+B+, *lac*Iq, *lac*ZdeltaM15)	Amersham Corp., Arlington Heights, Illinois

DH5α™ Subcloning efficiency cells are purchased as competent cells and are ready for transformation using the manufacturer's protocol, while both *E. coli* strains TG1 and MON105 are rendered competent to take up DNA using a $CaCl_2$ method. Typically, 20 to 50 mL of cells are grown in LB medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 150 mM NaCl) to a density of approximately 1.0 optical density unit at 600 nanometers (OD_{600}) as measured by a Baush & Lomb Spectronic spectrophotometer (Rochester, NY). The cells are collected by centrifugation and resuspended in one-fifth culture volume of $CaCl_2$ solution (50 mM $CaCl_2$, 10 mM Tris-Cl, pH7.4) and are held at 4°C for 30 minutes. The cells are again collected by centrifugation and resuspended in one-tenth culture volume of $CaCl_2$ solution. Ligated DNA is added to 0.2 mL of these cells, and the samples are held at 4°C for 30-60 minutes. The samples are shifted to 42°C for two minutes and 1.0 mL of LB is added prior to shaking the samples at 37°C for one hour. Cells from these samples are spread on plates (LB medium plus 1.5% Bacto-agar) containing either ampicillin (100 micrograms/mL, ug/mL) when selecting for ampicillin-resistant transformants, or spectinomycin (75 ug/mL) when selecting for

spectinomycin-resistant transformants. The plates are incubated overnight at 37°C.

Colonies are picked and inoculated into LB plus appropriate antibiotic (100 ug/mL ampicillin or 75 ug/mL spectinomycin) and are grown at 37°C while shaking.

Construction of plasmids encoding interferon alpha 2b

Construction of pMON30422 (encoding IFNa2b)

An interferon alpha 2b (IFNα-2b) gene was amplified from plasmid DNA from ATCC clone no. 67979 using the primer set, IFStart (SEQ ID NO. 1) and IFStop (SEQ ID NO. 2).

Oligo IFStart (SEQ ID NO:1)
GATCGACCAT GGCTTGTGAT CTGCCTCAAA CC 32

Oligo IFStop (SEQ ID NO:2)
CGATCGAAGC TTATTATTCC TTACTTCTTA AACTTT 36

The primers were designed to include the appropriate restriction enzyme recognition sites which allow cloning of the gene into expression plasmids. Conditions for polymerase chain reaction (PCR) amplification were 35 cycles 92°C degree. denaturation for one minute, 40°C annealing for one minute and 72°C extension for one minute. A 100 ul reaction contained 100 pmol of each primer and one ug of template DNA (isolated by Qiagen Miniprep); and 1X PCR reaction buffer, 200 uM dNTPs and 0.6 unit Taq DNA polymerase (Boehringer Mannheim). The PCR product was digested with restriction endonucleases *Nco*I and *Hin*dIII and gel-purified. The vector pMON6875, encoding a *Ptac* promoter, G10L ribosome binding site and P22 terminator, was digested with restriction endonucleases *Nco*I and *Hin*dIII. The digested PCR product and vector fragment were combined and ligated. A portion of the ligation reaction was used to transform *E. coli* strain MON208. Transformant bacteria were selected on spectinomycin-containing plates. Plasmid DNA was isolated and sequenced to confirm the correct insert. The resulting plasmid was designated pMON30422.

Construction of pMON30426 (encoding IFNa2b with optimized amino terminal codons)

To optimize expression of IFNα-2b in *E. coli*, a new gene with an optimized N-terminus was amplified from plasmid DNA from pMON30422 using the primer set, New IF-A (SEQ ID NO. 3) and IFStop (SEQ ID NO. 2).

Oligo IFstop (SEQ ID NO:2)

CGATCGAAGC TTATTATTCC TTACTTCTTA AACTTT 36

Oligo NewIF-A (SEQ ID NO:3)

GATCGACCAT GGCTTGTGAT CTGCCGCAAA CTCATAGCCT GGGTAGCCGT CGCACCCTGA 60

TGCTGCTGGC TCAGATGCGC CGTATCTCTC TTTTCTCCTG CTTGAAGGAC AGACA 115

The primers were designed to include the appropriate restriction enzyme recognition sites which allow cloning of the gene into expression plasmids. Conditions for polymerase chain reaction (PCR) amplification were 35 cycles 92°C denaturation for one minute, 40°C annealing for one minute and 72°C extension for one minute. A 100 ul reaction contained 100 pmol of each primer and one ug of template DNA; and 1X PCR reaction buffer, 200 uM dNTPs and 0.6 unit Taq DNA polymerase (Boehringer Mannheim). The PCR product was digested with restriction endonucleases *Nco*I and *Hin*dIII and gel-purified. Vector DNA encoding a *Ptac* promoter, G10L ribosome binding site and P22 terminator, pMON6875, was digested with restriction endonucleases *Nco*I and *Hind*III. The digested PCR product and vector fragment were combined and ligated. A portion of the ligation reaction was used to transform *E. coli* strain MON208. Transformed bacteria were selected on spectinomycin-containing plates. Plasmid DNA was isolated and sequenced to confirm the correct insert. The resulting plasmid was designated pMON30426. Both pMON30422 and pMON30426 encode the same peptide.

Construction of pMON20442 (encoding cysIFNa2b)

The DNA sequence encoding the N-terminus, Met-Ala-Cys-, of pMON30426 was changed to the DNA sequence encoding the N-terminus, Met-Cys-, of pMON20442 by mutagenesis. The QuikChange Site-Directed Mutagenesis Kit of Stratagene was used employing oligos IFcys.for (SEQ ID NO: 12) and IFcys.rev (SEQ ID NO: 13).

IFcys.for GAG ATA TAT cca tgT GTG ATC TGC CGC (SEQ ID NO: 12)

IFcys.rev GCG GCA GAT CAC Aca tgg ATA TAT CTC (SEQ ID NO: 13)

Construction of plasmids encoding interferon alpha A/D

Construction of pMON20405 (encoding IFN-A/D)

Plasmid, pMON30426, DNA was digested with the restriction enzymes *Nco*I and *Hind*III resulting in a 3207 bp vector fragment. Plasmid DNA from pMON30426 was digested with *Nco*I and *Bgl*II resulting in a 192 bp fragment. The 192 bp fragment along with a 315 bp *Bgl*II/*Hind*III fragment that was assembled from synthetic oligonucleotides IFND1 (SEQ ID NO. 4), IFND2 (SEQ ID NO. 5), IFND3X (SEQ ID NO. 6), IFND4X (SEQ ID NO. 7), IFND5 (SEQ ID NO. 8), IFND6 (SEQ ID NO. 9), IFND7 (SEQ ID NO. 10), and IFND8 (SEQ ID NO. 11) was ligated to the vector fragment. A portion of the ligation reaction was used to transform *E. coli* K-12 strain JM101. Transformant bacteria were selected on spectinomycin-containing plates. Plasmid DNA was isolated, analyzed by restriction analysis, and sequenced to confirm the correct insert. The genetic elements derived from plasmid pMON20405 are the pBR327 origin of replication, the *tac* promoter, the gene 10 leader (g10-L) ribosome binding site joined to human interferon (hIFN) alpha A/D hybrid, the P22 transcriptional terminator, and the streptomycin adenyltransferase gene.

Construction of pMON20433 (encoding cys IFN A/D)

The DNA sequence encoding the N-terminus, Met-Ala-Cys-, of pMON20405 was changed to the DNA sequence encoding the N-terminus, Met-Cys-, of pMON20433 by mutagenesis. The QuikChange Site-Directed Mutagenesis Kit of Stratagene was used employing oligos IFcys.for (SEQ ID NO: 12) and IFcys.rev (SEQ ID NO: 13). The vector pMON20433 (SEQ ID NO: 18) encodes interferon A/D (SEQ ID NO: 23).

Table of Plasmids

Plasmid	SEQ ID NO.	Marker	Description	Source
pMON30422	#14	spec	pMON6875 *Nco*I/*Hin*dIII + *Nco*I/*Hin*dIII-digested PCR fragment encoding interferon alpha 2b	This work

pMON30426	#15	spec	pMON6875 *NcoI/Hin*dIII + *NcoI/Hin*dIII-digested PCR fragment encoding interferon alpha 2b with optimized N-terminal codons	This work
pMON20442	#16	spec	pMON30426 modified by site directed mutagenesis to change the amino terminus from Met-Ala-Cys-IFN alpha 2b to Met-Cys-IFN alpha 2b	This work
pMON20405	#17	Spec	3207 bp *NcoI/Hind*III frag from pMON30426 + 192 bp frag from pMON30426 (A portion of IFN alpha) + 315 bp frag from 8 synthetic oligos with *Bgl*II/*Hind*III ends (D portion of IFN alpha). Creates IFN alpha A/D hybrid.	This work
pMON20433	#18	spec	pMON20405 modified by site directed mutagenesis to change the amino terminus from Met-Ala-Cys-IFN alpha A/D to Met-Cys-IFN alpha A/D	This work

Cloning and Expression

DNA isolation and characterization

Plasmid DNA can be isolated by a number of different methods and using commercially available kits known to those skilled in the art. Plasmid DNA is isolated using the Promega Wizard™ Miniprep kit (Madison, WI), the Qiagen QIAwell Plasmid isolation kits (Chatsworth, CA) or Qiagen Plasmid Midi or Mini kit. These kits follow the same general procedure for plasmid DNA isolation. Briefly, cells are pelleted by centrifugation (5000 x g), the plasmid DNA released with sequential NaOH/acid treatment, and cellular debris is removed by centrifugation (10000 x g). The supernatant (containing the plasmid DNA) is loaded onto a column containing a DNA-binding resin, the column is washed, and plasmid DNA eluted. After screening for the colonies with the plasmid of interest,

the *E. coli* cells are inoculated into 50-100 ml of LB plus appropriate antibiotic for overnight growth at 37°C in an air incubator while shaking. The purified plasmid DNA is used for DNA sequencing, further restriction enzyme digestion, additional subcloning of DNA fragments and transfection into *E. coli,* mammalian cells, or other cell types.

Sequence confirmation

Purified plasmid DNA is resuspended in dH_2O and its concentration is determined by measuring the absorbance at 260/280 nm in a Bausch and Lomb Spectronic 601 UV spectrometer. DNA samples are sequenced using ABI PRISM™ DyeDeoxy™ terminator sequencing chemistry (Applied Biosystems Division of Perkin Elmer Corporation, Lincoln City, CA) kits (Part Number 401388 or 402078) according to the manufacturer's suggested protocol usually modified by the addition of 5% DMSO to the sequencing mixture. Sequencing reactions are performed in a DNA thermal cycler (Perkin Elmer Corporation, Norwalk, CT) following the recommended amplification conditions. Samples are purified to remove excess dye terminators with Centri-Sep™ spin columns (Princeton Separations, Adelphia, NJ) and lyophilized. Fluorescent dye labeled sequencing reactions are resuspended in deionized formamide, and sequenced on denaturing 4.75% polyacrylamide-8M urea gels using ABI Model 373A and Model 377 automated DNA sequencers. Overlapping DNA sequence fragments are analyzed and assembled into master DNA contigs using Sequencher DNA analysis software (Gene Codes Corporation, Ann Arbor, MI).

Mammalian Cell Transfection / Production of Conditioned Media

The BHK-21 cell line can be obtained from the ATCC (Rockville, MD). The cells are cultured in Dulbecco's modified Eagle media (DMEM/high-glucose), supplemented to 2 mM (mM) L-glutamine and 10% fetal bovine serum (FBS). This formulation is designated BHK growth media. Selective media is BHK growth media supplemented with 453 units/mL hygromycin B (CalBiochem, San Diego, CA). The BHK-21 cell line was previously stably transfected with the HSV transactivating protein VP16, which transactivates the IE110 promoter found on the plasmid pMON3359 and pMON3633 and the IE175 promoter found in the plasmid pMON3360B (See Hippenmeyer et al., *Bio/Technology*, pp.1037-1041, 1993). The VP16 protein drives expression of genes inserted behind the IE110 or IE175 promoter. BHK-21 cells expressing the transactivating protein VP16 are designated BHK-VP16. The plasmid pMON1118 (See Highkin et al., *Poultry Sci.*,

70: 970-981, 1991) expresses the hygromycin resistance gene from the SV40 promoter. A similar plasmid, pSV2-hph, is available from ATCC.

BHK-VP16 cells are seeded into a 60 millimeter (mm) tissue culture dish at 3 x 10^5 cells per dish 24 hours prior to transfection. Cells are transfected for 16 hours in 3 mL of "OPTIMEM"™ (Gibco-BRL, Gaithersburg, MD) containing 10 ug of plasmid DNA containing the gene of interest, 3 ug hygromycin resistance plasmid, pMON1118, and 80 ug of Gibco-BRL "LIPOFECTAMINE"™ per dish. The media is subsequently aspirated and replaced with 3 mL of growth media. At 48 hours post-transfection, media from each dish is collected and assayed for activity (transient conditioned media). The cells are removed from the dish by trypsin-EDTA, diluted 1:10, and transferred to 100 mm tissue culture dishes containing 10 mL of selective media. After approximately 7 days in selective media, resistant cells grow into colonies several millimeters in diameter. The colonies are removed from the dish with filter paper (cut to approximately the same size as the colonies and soaked in trypsin/EDTA) and transferred to individual wells of a 24 well plate containing 1 mL of selective media. After the clones are grown to confluence, the conditioned media is re-assayed, and positive clones are expanded into growth media.

Expression of recombinant proteins in E. coli

E. coli strain MON105 or JM101 harboring the plasmid of interest are grown at 37°C in M9 plus casamino acids medium with shaking in an air incubator Model G25 from New Brunswick Scientific (Edison, NJ). Growth is monitored at OD_{600} until it reaches a value of 1.0 at which time nalidixic acid (10 mg/mL) in 0.1 N NaOH is added to a final concentration of 50 µg/mL. The cultures are then shaken at 37°C for three to four additional hours. A high degree of aeration is maintained throughout culture period in order to achieve maximal production of the desired gene product. The cells are examined under a light microscope for the presence of inclusion bodies (IB). One mL aliquots of the culture are removed for analysis of protein content by boiling the pelleted cells, treating them with reducing buffer and electrophoresis via SDS-PAGE (see Maniatis et al., "Molecular Cloning: A Laboratory Manual", 1982). The culture is centrifuged (5000 x g) to pellet the cells.

Purification

Isolation of Inclusion Bodies

The cell pellet from a 330 mL E. coli culture is resuspended in 15 mL of sonication buffer (10 mM 2-amino-2-(hydroxymethyl) 1,3-propanediol hydrochloride (Tris-HCl), pH 8.0 + 1 mM ethylenediaminetetraacetic acid (EDTA). These resuspended cells are sonicated using the microtip probe of a Sonicator Cell Disruptor (Model W-375, Heat Systems-Ultrasonics, Inc., Farmingdale, New York). Three rounds of sonication in sonication buffer followed by centrifugation are employed to disrupt the cells and wash the inclusion bodies (IB). The first round of sonication is a 3 minute burst followed by a 1 minute burst, and the final two rounds of sonication are for 1 minute each.

Purification

E. coli inclusion bodies were dissolved in 6 M Gn-HCl, 10 mM EDTA, pH 8.5-9; stirred at 4°C for 1 hr and then diluted 4-fold with 0.1M Tris/HCl pH 8.1. The solution is left to oxidize, while stirring, for 48-72 hrs at 4°C. The structure of refolded molecules is monitored by reverse-phase HPLC.

The sample was dialyzed against 20 volumes of 0.1 M NaCl at 4°C and the precipitate removed by centrifugation. The pH of the sample was adjusted to pH 4.5 by addition of NaP/phosphoric acid. The sample was dialyzed against 50 mM NaP, pH 4.5, 0.15 M NaCl. Precipitates are removed from the sample by centrifugation and the sample was applied to Mono S or S-sepharose and eluted with 1 M NaCl in 50 mM NaP, pH 4.5. The pH of the eluted sample was adjusted to pH 7 by the addition of dibasic NaP and then loaded onto a Pharmacia Cu-chelate resin, washed and eluted in 50 mM acidic acid, 0.25 M NaCl, pH 3.

If the sample was relatively pure at this point, no further processing was necessary. Occasionally a smaller molecular weight contaminant was present that was removed on a Q Sepharose column by adjusting the pH of the sample to 9.5 with Caps buffer and then loaded onto a Q Sepharose column. The protein was eluted with a 0.2 to 0.5M NaCl gradient at pH 9.5- 9.8.

In some cases the folded proteins can be affinity-purified using affinity reagents such as monoclonal antibodies or receptor subunits attached to a suitable matrix. Purification can also be accomplished using any of a variety of chromatographic methods such as: ion exchange, gel filtration or hydrophobic chromatography or reversed phase HPLC. These and other protein purification methods are described in detail in Methods in Enzymology, Volume 182 "Guide to Protein Purification" edited by Murray Deutscher, Academic Press, San Diego, California, 1990.

Protein Characterization

Purified proteins are analyzed by RP-HPLC, electrospray mass spectrometry, and SDS-PAGE. Protein quantitation is done by amino acid composition, RP-HPLC, and Bradford protein determination. In some cases tryptic peptide mapping is performed in conjunction with electrospray mass spectrometry to confirm the identity of the protein.

The structures of bioconjugates containing a_vb_3 inhibitors conjugated to interferon were characterized by electrospray mass spectrometry, SDS-PAGE, mapping of trypsin-digested fragments, and amino acid composition analysis. Endotoxin levels in recombinant protein samples were determined by standard assays.

Biological Assays

The activity of the compounds of the present invention was tested in the following assays. The results of testing in the assays are tabulated in Table 4.

Assay for Human Interferon-Induced Antiviral Activity

Antiviral activity induced by human interferon is measured spectrophotometrically as inhibition of the cytopathic effect that normally results from infection of Madin Darby bovine kidney cells, (ATCC CCL #22), with vesicular stomatitis virus (VSV) (ATCC VR-158) (Rubinstein et al., *J. Virol.* **37**(2): 755-758, 1981). VSV stocks are prepared on mouse L cells (L929) (ATCC CCL# 1). Samples of interferon are serially titrated in a 96-well plate format and incubated with 4 x 10^4 cells per well, 6 hours prior to addition of virus at a multiplicity of infection of 0.1 plaque forming units per cell. The cells are stained with crystal violet at 20-24 hours postinfection and staining is measured spectrophotometrically at 680 nm. The relative potencies of the samples are compared with Intron A, a recombinant interferon produced by Schering Plough and obtained by prescription.

Inhibitory effect on Proliferation of Daudi Cells

Human Burkitt's lymphoma Daudi cells (ATCC) are seeded at 2 x 10^4 cells/well into 96-well tissue culture plates and cells are cultured in the presence or absence of serial doses of rhIFN alpha 2b for 3 days. Cultures are pulsed with ^{3}H-thymidine for the last hour of the culture period, and the ^{3}H-thymidine uptake, counts per minute (cpm), measured on a Beta-plate reader. All samples are assayed in triplicate.

Endothelial cell proliferation assay

The endothelial cell proliferation assay was performed as described by Cao et al. (*J. Biol. Chem.* **271**: 29461-29467, 1996). Briefly, human dermal microvascular endothelial cells (HdMVEC, Clonetics) or bovine microvascular endothelial cells (BacEnd, Incell) were maintained in MCDB131 containing 5% heat-inactivated fetal bovine serum (FBS, Hyclone), antibiotics, 100 ug/ml heparin (Sigma) and 100 ug/ml endothelial mitogen (Biomedical Technologies). Confluent monolayers at passages 2-5 were dispersed in .05% trypsin and resuspended in complete medium. Five hundred ul of complete media containing 1.25 x 10^4 cells were seeded into wells of a 24-well tissue culture plate coated with 0.1% gelatin (Sigma). The cells were incubated overnight at 37°/5% CO_2 at which time the media was replaced with 250 ul of media containing 5% FBS and various concentrations of inhibitors. After 30 minutes of incubation, 250 ul of media containing 1 ng/ml bFGF (R&D Systems) was added and the cells were incubated for an additional 72 hours, at which time they were trypsinized and counted with a Coulter counter.

Endothelial cell migration assay

The endothelial cell migration assay is performed essentially as previous described (Gately et al., *Cancer Res.* **56**:4887-4890, 1996). To determine the ability of angiostatin to inhibit the migration of endothelial cells, migration assays were performed in a transwell chamber (Costar) containing 8 mm pore size polycarbonate membranes. The cells utilized in the assay were either human microvascular endothelial cells from Emory or bovine endothelial cells (kindly provided by Gately Northwestern University, Evanston, IL). The cells were starved overnight before use in MCDB131 + 0.1% BSA (human cells) or DMEM + 0.1% BSA (bovine cells), harvested, and resuspended in the same media at 10^6 cells/ml. The lower side of the transwells were coated with 0.1% gelatin for 30 minutes at 37°C before addition of 2 x 10^5 cells to the upper chamber. The transwell was moved to a well containing the chemoattractant (bFGF or VEGF) in the lower chamber. Migration was allowed to occur overnight at 37°C. The membranes were then fixed and stained, and the number of cells that migrated to the lower side of the membrane counted in 3 high powered fields.

Micro-pocket assay to evaluate anti-angiogenic activity

The corneal micropocket assay has been developed to evaluate the anti-angiogenic activity of test compounds in mice. BALBc or C57BL strains of mice are

anesthetized with avertin (tribromoethanol, 125 mpk, 0.3-.4 ml/mouse, i.p., 25 ga needle). The eyes are topically anesthetized with 0.5% proparacaine. Only one eye is used. The eye is proptosed with a small forceps and under an operating microscope, a central, intrastromal linear keratotomy is performed with a #15 blade parallel to the insertion of the lateral rectus muscle. A modified cataract knife (1 x 20 mm) is then inserted to dissect a lamellar micropocket to within 1 mm of the temporal limbus. A single Hydron pellet containing either basic fibroblastic growth factor or vascular endothelial growth factor (bFGF or VEGF) is placed on the eye and pushed into the pocket with one arm of the forceps. The flap is self-sealing. Antibiotic ointment (Neobacimyx) is applied once to prevent infection and to decrease irritation of the irregular ocular surface.

Compounds are administered immediately post-operatively. They can be administered either orally, intraperitoneally, subcutaneously or intravenously, depending on bioavailability and potency of the compound. Dosing is from one to three times daily for oral compounds, one or two per day for i.p. or s.q., and once per day via the tail vein for i.v. delivery. Volumes do not exceed 5 ml/kg orally, 10ml/kg i.p. or s.q. or 2.5 ml/kg i.v. All injections are done with a 25 guage needle.

On post-operative day 5 or 6 the mice are anesthetized with avertin (125 mpk, i.p.), the eyes proptosed, and the degree of neovascularization assessed by determining the maximum vessel length, and the contiguous circumferential zone involved. Using the formula for the area of an ellipse, the neovascular area is measured. The animals receive a thoracotomy while still anesthetized, to assure euthanasia. Some of the eyes are removed for histology. If blood samples are required for compound blood levels, the mice are bled by cardiac puncture immediately following the corneal neovascularization assessment. This is done via a substernal approach with a 1 inch, 23 guage needle, and the animal is subsequently euthanized.

Animals are monitored daily following surgery. Topical proparicaine is used as necessary to relieve irritation of the affected eye. The maximum number of bleeds per rat is four, every third day, although typically only two are required, one at day 4 or 6 and one at completion.

Mouse models for anti-tumor activity

Several mouse models can be used to evaluate the anti-tumor activity of the chimeric proteins; either direct effects on the growth of the primary tumor or effects on metastasis. These can be divided into two broad classes: syngeneic

models of mouse tumor in mice, such as the Lewis Lung Carcinoma (Sugiura and Stock, *Cancer Res.*, 15: 38-51, 1955; O'Reilly et al., *Cell* **79**(2): 315-328, 1994) and xenograft models of human tumors in nude or severe combined immunodeficiency (SCID) mice. Examples of the human tumor xenografts include: the breast cancer cell lines, MDA-MB-435 (Price, *Breast Cancer Research and Treatment*, **39**: 93-102, 1996) and MDA-231 (Sasaki et al., *Can. Res.* **55**: 3551-3557, 1995), the human prostate carcinoma cell line, PC-3 (Pretlow et al, *Can. Res.* **51**: 3814-3817, 1991; Passaniti et al., *Int. J. Cancer*, **51**: 318-324, 1992) and the human melanoma line M21 (Felding-Habermann et al., *J. Clin. Invest.*, **89**: 2018-2022, 1992).

Solid phase receptor assays - Vitronectin adhesion assay

Human vitronectin receptor ($\alpha_v\beta_3$) was purified from human placenta as previously described (Pytela et al., *Methods in Enzymology*, **144**: 475-489, 1987). Human vitronectin was purified from fresh frozen plasma as previously described (Yatohgo et al., *Cell Structure and Function*, **13**: 281-292, 1988)]. Biotinylated human vitronectin was prepared by coupling NHS-biotin from Pierce Chemical Company (Rockford, IL) to purified vitronectin as previously described (Charo et al., *J. Biol. Chem.*, **266**(3): 1415-1421 1991). Assay buffer, OPD substrate tablets, and RIA grade BSA were obtained from Sigma (St. Louis, MO). Anti-biotin antibody was obtained from CalBiochem (La Jolla, CA). Linbro microtiter plates were obtained from Flow Labs (McLean, VA). ADP reagent was obtained from Sigma (St. Louis, MO).

This assay was essentially the same as previously reported (Niiya et al., *Blood*, **70**: 475-483, 1987). The purified human vitronectin receptor ($\alpha_v\beta_3$) was diluted from stock solutions to 1.0 g/mL in Tris-buffered saline containing 1.0 mM Ca^{++}, Mg^{++}, and Mn^{++}, pH 7.4 (TBS^{+++}). The diluted receptor was immediately transferred to Linbro microtiter plates at 100 uL/well (100 ng receptor/well). The plates were sealed and incubated overnight at 4 C to allow the receptor to bind to the wells. All remaining steps were at room temperature. The assay plates were emptied and 200 uL of 1% RIA grade BSA in TBS^{+++} (TBS^{+++}/BSA) were added to block exposed plastic surfaces. Following a 2 hour incubation, the assay plates were washed with TBS^{+++} using a 96 well plate washer. Logarithmic serial dilution of the test compound and controls were made starting at a stock concentration of 2 mM and using 2 nM biotinylated vitronectin in TBS^{+++}/BSA as the diluent. This premixing of labeled ligand with test (or control) ligand, and subsequent transfer of 50 uL aliquots to the assay plate was carried out with a CETUS Propette robot; the final concentration of the labeled ligand was 1 nM and the highest concentration of test compound was 1.0×10^{-4} M. The competition occurred for two

hours after which all wells were washed with a plate washer as before. Affinity purified horseradish peroxidase labeled goat anti-biotin antibody was diluted 1:3000 in TBS^{+++}/BSA and 125 uL were added to each well. After 30 minutes, the plates were washed and incubated with OPD/H_2O_2 substrate in 100 mM/L Citrate buffer, pH 5.0. The plate was read with a microtiter plate reader at a wavelength of 450 nm and when the maximum-binding control wells reached an absorbance of about 1.0, the final A_{450} were recorded for analysis. The data were analyzed using a macro written for use with the EXCEL™ spreadsheet program. The mean, standard deviation, and %CV were determined for duplicate concentrations. The mean A_{450} values were normalized to the mean of four maximum-binding controls (no competitor added)(B-MAX). The normalized values were subjected to a four parameter curve fit algorithm (Rodbard et al., *Int. Atomic Energy Agency, Vienna*, pp 469, 1977), plotted on a semi-log scale, and the computed concentration corresponding to inhibition of 50% of the maximum binding of biotinylated vitronectin (IC_{50}) and corresponding R^2 was reported for those compounds exhibiting greater than 50% inhibition at the highest concentration tested; otherwise the IC_{50} is reported as being greater than the highest concentration tested. β-[[2-[[5-[(aminoiminomethyl)amino]-1-oxopentyl]amino]-1-oxoethyl]amino]-3-pyridinepropanoic acid which is a potent $\alpha_v\beta_3$ antagonist (IC_{50} in the range 3-10 nM) was included on each plate as a positive control.

Solid phase receptor assays - Purified IIb/IIIa receptor assay

Human fibrinogen receptor ($\alpha_{IIb}\beta_3$) was purified from outdated platelets. (Pytela, R., Pierschbacher, M.D., Argraves, S., Suzuki, S., and Rouslahti, E. "Arginine-Glycine-Aspartic acid adhesion receptors", *Methods in Enzymology* **144**: 475-489, 1987) Human vitronectin was purified from fresh frozen plasma as described (Yatohgo, T., Izumi, M., Kashiwagi, H., and Hayashi, M., "Novel purification of vitronectin from human plasma by heparin affinity chromatography," *Cell Structure and Function* **13**:281-292, 1988). Biotinylated human vitronectin was prepared by coupling NHS-biotin from Pierce Chemical Company (Rockford, IL) to purified vitronectin as previously described. (Charo, I.F., Nannizzi, L., Phillips, D.R., Hsu, M.A., Scarborough, R.M., "Inhibition of fibrinogen binding to GP IIb/IIIa by a GP IIIa peptide", *J. Biol. Chem.* **266**(3): 1415-1421, 1991). Assay buffer, OPD substrate tablets, and RIA grade BSA were obtained from Sigma (St. Louis, MO). Anti-biotin antibody was obtained from Calbiochem (La Jolla, CA). Linbro microtiter plates were obtained from Flow Labs (McLean, VA). ADP reagent was obtained from Sigma (St. Louis, MO).

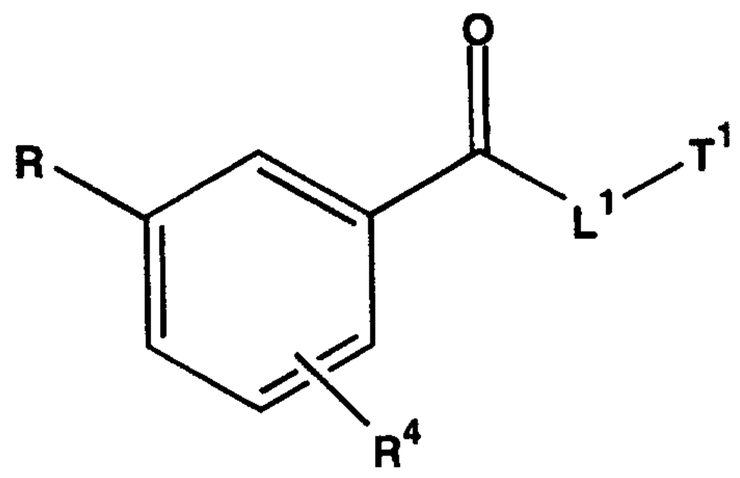
This assay is essentially the same reported in Niiya, K., Hodson, E., Bader, R., Byers-Ward, V. Koziol, J.A., Plow, E.F. and Ruggeri, Z.M., "Increased surface expression of the membrane glycoprotein IIb/IIIa complex induced by platelet activation: Relationships to the binding of fibrinogen and platelet aggregation", *Blood* **70**: 475-483, 1987). The purified human fibrinogen receptor (aIIbb3) was diluted from stock solutions to 1.0 µg/mL in Tris-buffered saline containing 1.0 mM Ca^{++}, Mg^{++}, and Mn^{++}, pH 7.4 (TBS^{+++}). The diluted receptor was immediately transferred to Linbro microtiter plates at 100 µL/well (100 ng receptor/well). The plates were sealed and incubated overnight at 4 C to allow the receptor to bind to the wells. All remaining steps were at room temperature. The assay plates were emptied and 200 µL of 1% RIA grade BSA in TBS^{+++} (TBS^{+++}/BSA) were added to block exposed plastic surfaces. Following a 2 hour incubation, the assay plates were washed with TBS^{+++} using a 96 well plate washer. Logarithmic serial dilution of the test compound and controls were made starting at a stock concentration of 2 mM and using 2 nM biotinylated vitronectin in TBS^{+++}/BSA as the diluent. This premixing of labeled ligand with test (or control) ligand, and subsequent transfer of 50 µL aliquots to the assay plate was carried out with a CETUS Propette robot; the final concentration of the labeled ligand was 1 nM and the highest concentration of test compound was 1.0×10^{-4} M. The competition occurred for two hours after which all welis were washed with a plate washer as before. Affinity purified horseradish peroxidase labeled goat anti-biotin antibody was diluted 1:3000 in TBS^{+++}/BSA and 125 µL were added to each well. After 30 minutes, the plates were washed and incubated with ODD/H_2O_2 substrate in 100 mM/L citrate buffer, pH 5.0. The plate was read with a microtiter plate reader at a wavelength of 450 nm and when the maximum-binding control wells reached an absorbance of about 1.0, the final A_{450} were recorded for analysis. The data were analyzed using a macro written for use with the EXCEL™ spreadsheet program. The mean, standard deviation, and %CV were determined for duplicate concentrations. The mean A_{450} values were normalized to the mean of four maximum-binding controls (no competitor added)(B-MAX). The normalized values were subjected to a four parameter curve fit algorithm, (Robard et al., *Int. Atomic Energy Agency, Vienna*, pp 469, 1977), plotted on a semi-log scale, and the computed concentration corresponding to inhibition of 50% of the maximum binding of biotinylated vitronectin (IC50) and corresponding R^2 was reported for those compounds exhibiting greater than 50% inhibition at the highest concentration tested; otherwise the IC50 is reported as being greater than the highest concentration tested. b-[[2-[[5-[(aminoiminomethyl)amino]-1-oxopentyl]amino]-1-

oxoethyl]amino]-3-pyridinepropanoic acid which is a potent a_vb_3 antagonist (IC50 in the range 3-10 nM) was included on each plate as a positive control.

All references, patents, or applications cited herein are incorporated by reference in their entirety.

Tables

Table 1: Unconjugated a_vb_3 antagonist precursors



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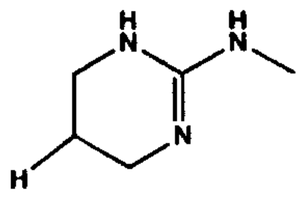
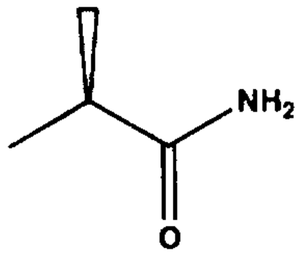
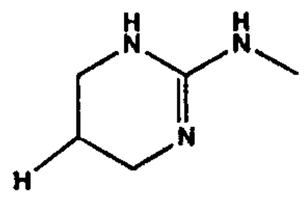
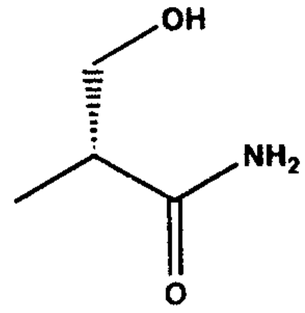
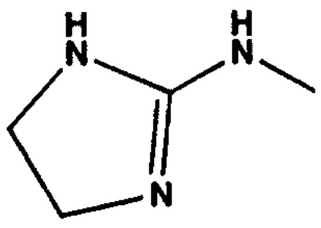
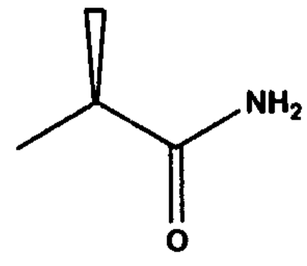
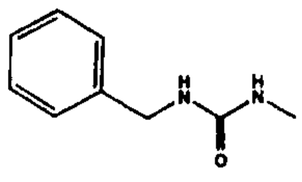
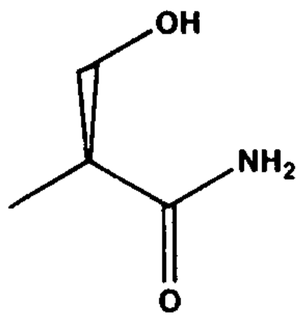
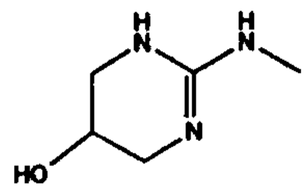
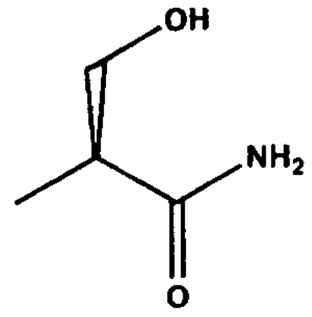
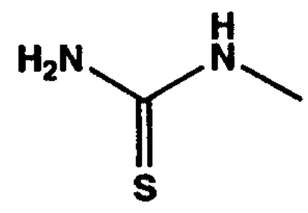
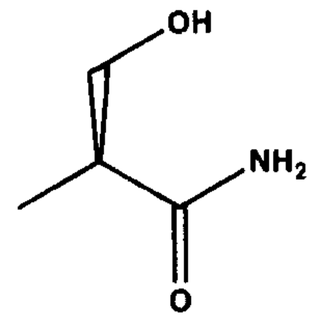
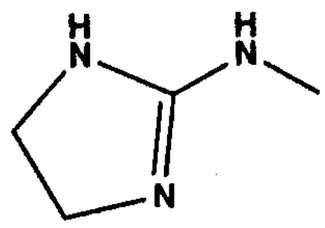
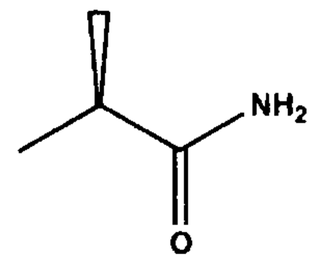
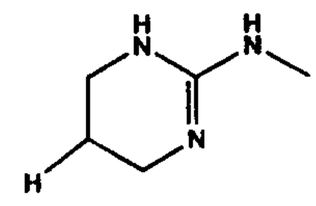
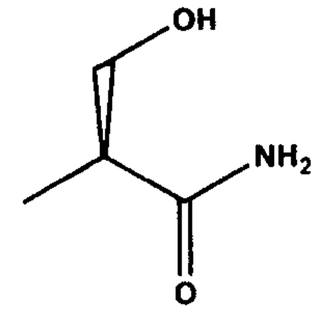
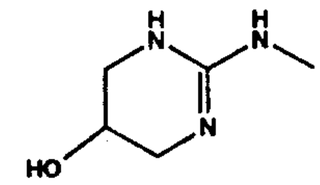
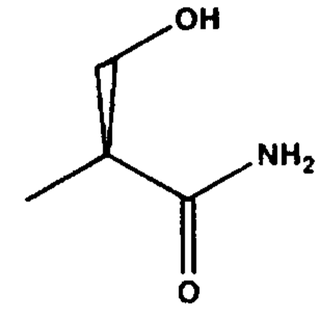
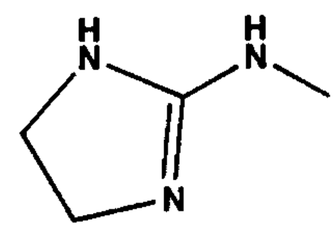
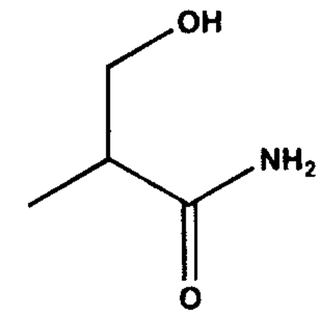
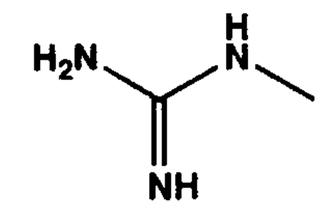
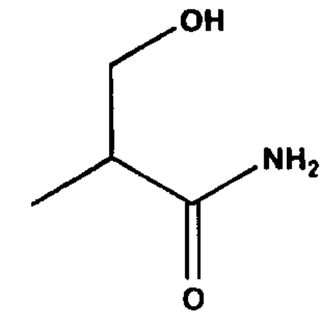
1		3, H	Gly Asp	
2		3, H	Gly Asp	
3		3, H	Gly Asp	
4		3, H	Gly Asp	

5		3, H	Gly Asp	
6		3, H	Gly Asp	
7		3, H	Gly Asp	
8		3, H	Gly Asp	
9		3, H	Gly Asp	
10		3, H	Gly Asp	
11		3, CF_3	Gly Asp	

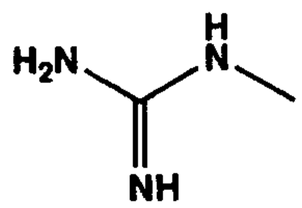
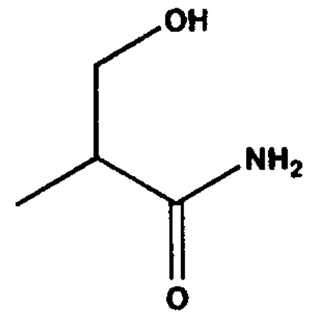
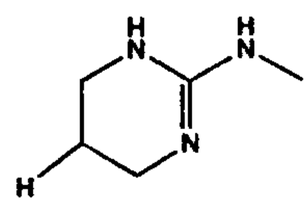
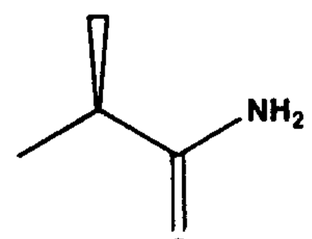
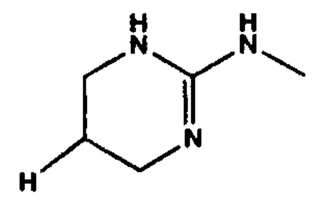
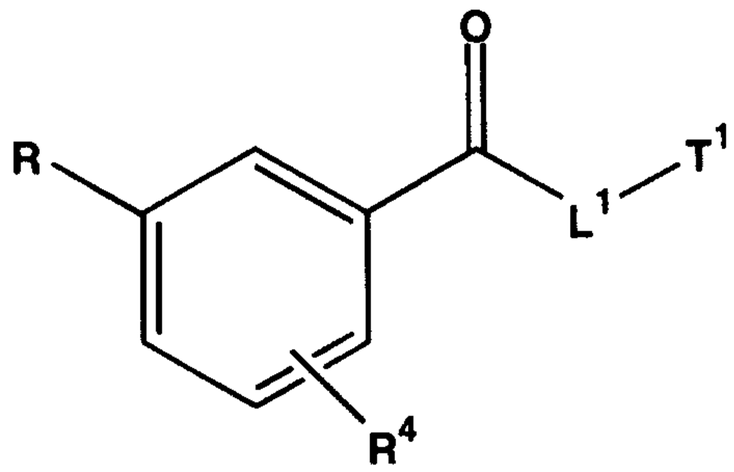
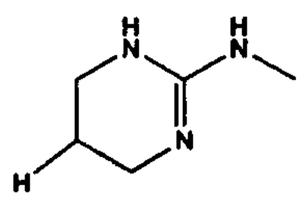
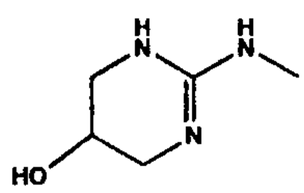
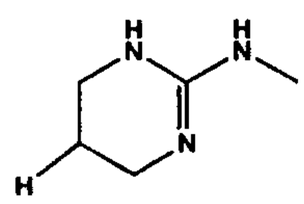
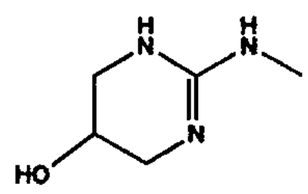
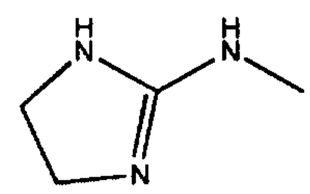
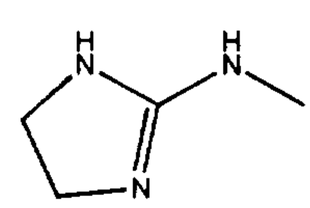
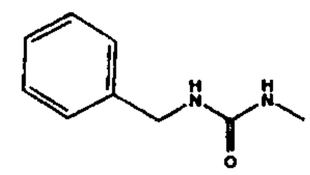
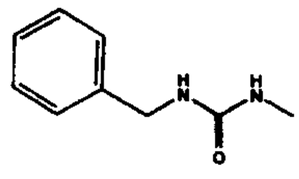
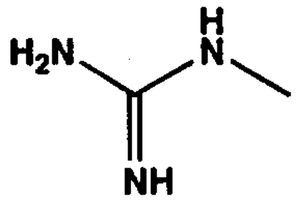
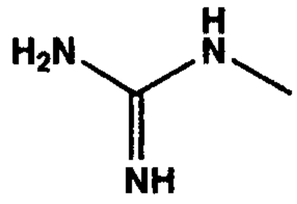
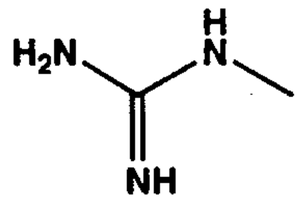
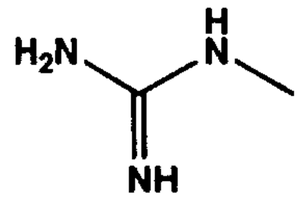
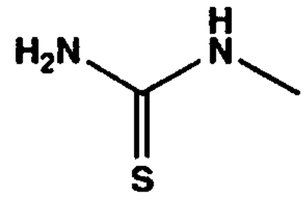
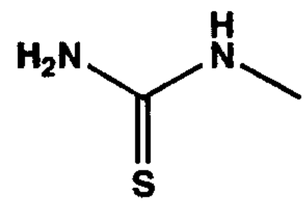
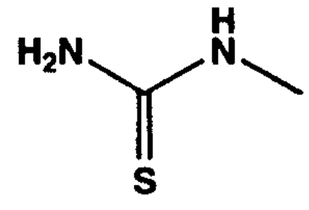
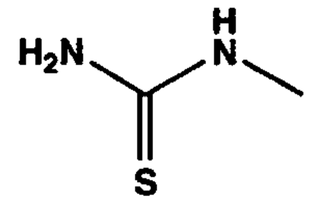
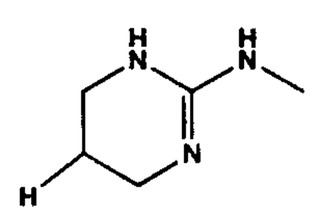
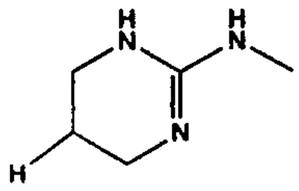
12		3, H	Gly Asp		
13		3, H	Gly Asp		
14		3, H	Gly Asp	ala-cys-asp-leu-pro-gln-NH_2	

Table 2: Series 100 a_vb_3 antagonist precursors conjugated to interferon



Compound #	R	R4	L1	T1
101		H	Gly Asp Ala	interferon α
102		H	Gly Asp Ala	interferon α
103		H	Gly Asp Ser	interferon α
104		H	Gly Asp Ser	interferon α
105		H	Gly Asp Ala	interferon α
106		H	Gly Asp Ser	interferon α
107		H	Gly Asp Ala	interferon α

108		H	Gly Asp Ser	interferon α
109		CF_3	Gly Asp Ala	interferon α
110		CF_3	Gly Asp Ser	interferon α
111		H	Gly Asp Ala	interferon α
112		H	Gly Asp Ser	interferon α
113		CF_3	Gly Asp Ala	interferon α
114		CF_3	Gly Asp Ser	interferon α
115		H	Gly Asp Ala	interferon α
116		H	Gly Asp Ser	interferon α
117		H	Gly-Asp-Ala-(Gly-Gly-Gly-Gly-Ser)$_2$-	interferon
118		H	Gly-Asp-Ala-(Gly-Gly-Gly-	interferon

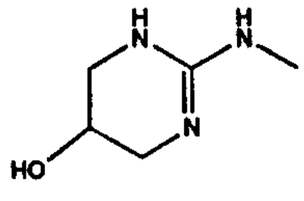
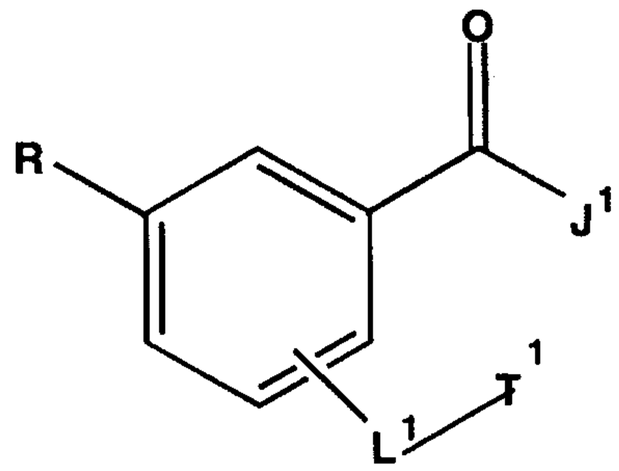
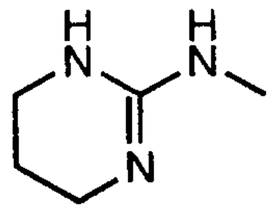
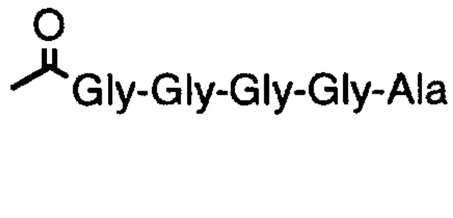
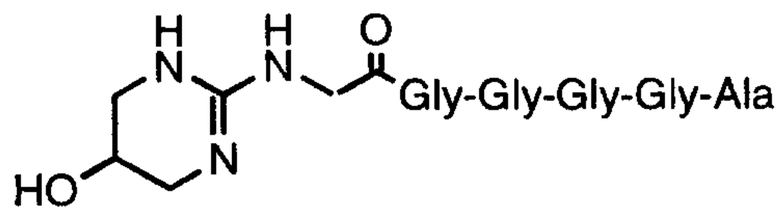
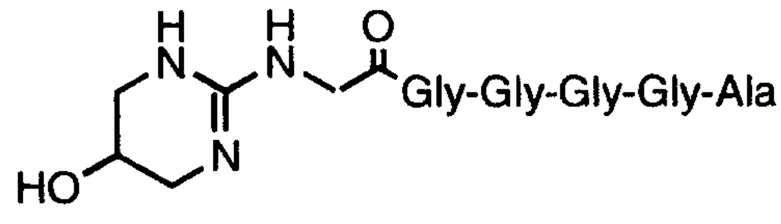
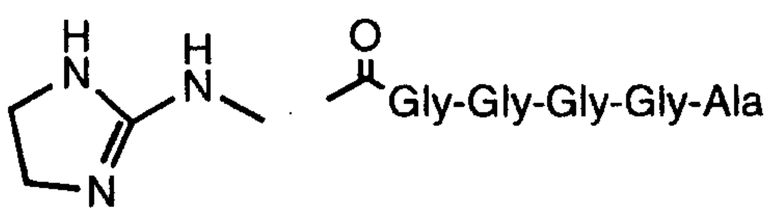
			Gly-Ser)$_3$-	
119		H	Gly-Asp-Ala-(Gly-Gly-Gly-Gly-Ser)$_2$-	interferon

Table 3: Series 200 a_vb_3 antagonist precursors conjugated to interferon



Cpd #	R	L¹	T¹	J¹
201			Interferon α	Gly-Asp-Ala-NH_2
202			Interferon α	Gly-Asp-Ser-NH_2
203			Interferon α	Gly-Asp-Ala-NH_2
204			Interferon α	Gly-Asp-Ser-NH_2
205			Interferon α	Gly-Asp-Ala-NH_2

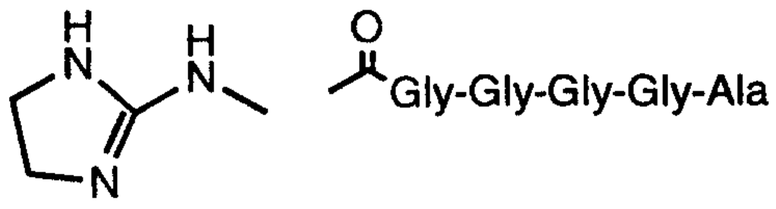
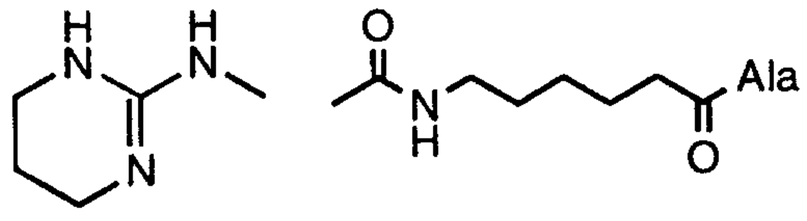
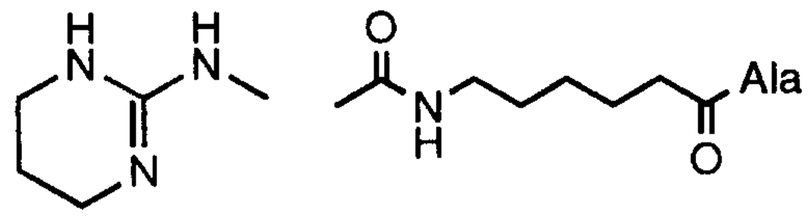
206		Interferon α	Gly-Asp-Ser-NH_2
207		Interferon α	Gly-Asp-Ala-NH_2
208		Interferon α	Gly-Asp-Ser-NH_2

Table 4: Solid-phase receptor binding assay of the free form a_vb_3 antagonists

Compound	IC 50 (nM) αvβ3	IC 50 (nM) IIb/IIIa
1	0.41	971
2	0.27	1000
3	0.53	393
4	1.33	28400
5	0.33	467
6	3.49	6140
7	0.53	393
8	0.26	705
9	0.33	467
10	0.36	235
11	0.62	172
12	1.31	245
13	0.27	557
14	0.56	901

SEQ ID Correlation table

SEQ ID NO.	SEQ ID Name
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2	oligo IFStop
3	oligo New IF-A
4	oligo IFND1
5	oligo IFND2
6	oligo IFND3X
7	oligo IFND4X
8	oligo IFND5
9	oligo IFND6
10	oligo IFND7
11	oligo IFND8
12	oligo IFcys.for
13	oligo IFcys.rev
14	36422.seq
15	30426.seq
16	20442.seq
17	20405.seq
18	20433.seq
19	36422.pep
20	30426.pep
21	20442.pep
22	20405.pep
23	20433.pep
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26	peptide linker
27	peptide linker
28	peptide linker
29	peptide linker
30	peptide linker
31	peptide linker

SEQUENCE LISTING

SEQUENCE LISTING

<110> Tjoeng, F.S.
Fok, K.F.

<120> Dual alpha v beta 3 and
metastasis-associated receptor ligands

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CLAIMS

We claim:

1. A compound of the formula A^1-L^1-T^1, or a pharmaceutically acceptable salt thereof, wherein:

A^1 separately or in combination with L^1 is an a_vb_3 antagonist,

T^1 is selected from the group consisting of metastasis-associated receptor ligands and amides.

L^1 is a covalent bond or a linker that covalently bonds A^1 to T^1.

2. A compound of claim 1, wherein said ligand is an agonist.

3. A compound of claim 1, wherein said ligand is an antagonist.

4. The compound as recited in claim 1 wherein T^1 is a polypeptide.

5. The compound as recited in claim 4 wherein said polypeptide is selected from the group consisting of natural cytokines, synthetic cytokines, and anti-angiogenic proteins.

6. A compound of claim 5, wherein said anti-angiogenic protein is selected from the group consisting of angiostatin and endostatin.

7. A compound of claim 5, wherein said polypeptide is selected from the group consisting of interleukin-2, interleukin-7, interleukin-12, interleukin-15, interferons and progenipoietin-G, erythropoietin, erythropoietin receptor agonists, colony stimulating factors, and hematopoietic growth factors.

8. A compound of claim 7, wherein said cytokine is human interferon.

9. A compound of claim 8, wherein said interferon is interferon alpha.

10. A compound of claim 9, wherein said interferon alpha is interferon alpha 2b.

11. A compound of claim 9, wherein said interferon alpha is interferon alpha A/D hybrid.

12. The compound of claim 9, wherein the amino acid sequence of the polypeptide is selected from the group consisting of

SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, and SEQ ID NO: 23.

13. A compound of claim 1, wherein said L^1 is a peptide linker.

14. The compound as recited in claim 13 wherein said peptide linker is a peptide ranging in length from 2 through 10 amino acids.

15. A compound according to claim 14 wherein

said peptide linker is Gly-Asp, and

said amide is selected from the group consisting of serine amide (-CH-(CH_3)-C=O-NH_2), alanine amide (-CH-(CH_2OH)-C=O-NH_2), and -ala-cys-asp-leu-pro-gln-NH_2.

16. A compound according to claim 14 wherein

said peptide linker is Gly-Asp-L^2,

wherein;

L^2 is selected from the group consisting of Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Try, Val.

17. A compound according to claim 16 wherein

L^2 is selected from the group consisting of Ala, Ser, Val.

18. The compound as recited in claim 16 wherein said peptide linker is one or more peptide sequences selected from the group consisting of;

(SEQ ID NO: 24) -Gly-Asp-Ala-;

(SEQ ID NO: 25) -Gly-Asp-Ser-;

(SEQ ID NO: 26) -Gly-Gly-Gly-Gly-Ala-;

(SEQ ID NO: 27) -Gly-Gly-Gly-Gly-Ser-;

(SEQ ID NO: 28) -Gly-Asp-Ala-Gly-Gly-Gly-Gly-Ala-;

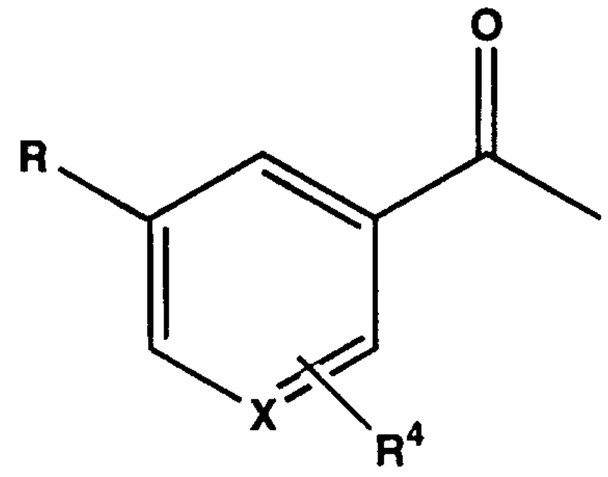
(SEQ ID NO: 29) -Gly-Asp-Ala-Gly-Gly-Gly-Gly-Ser-;

(SEQ ID NO: 30) -Gly-Asp-Ala-Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly- Ser-;

and

(SEQ ID NO: 31) -Gly-Asp-Ala-Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser-;

19. A compound of claim 1 wherein A^1 is a compound of the formula I,



I

or a pharmaceutically acceptable salt thereof, wherein:

X is C or N;

R^4 is one or more substituents independently selected from the group consisting of hydrogen, alkyl, hydroxy, alkoxy, aryloxy, halogen, haloalkyl, haloalkoxy, nitro, amino, alkylamino, acylamino, dialkylamino, cyano, alkylthio, alkylsulfonyl, carboxyl moieties, trihaloacetamide, acetamide, aryl, fused aryl, cycloalkyl, thio, monocyclic heterocycle, and fused monocyclic heterocycle;

R is R^1, R^2, or R^3, wherein

R^1 is NH_2-(C=NH)NH-, HO-NH-(C=NH)NH-, NH_2-(C=S)NH-, or HO-NH-(C=S)NH-; or

R^2 is R^8-(benzyl)-NH-(C=**Y**)-NH-, wherein;

Y is O or S; and

R^8 is a substituent independently selected from the group of the following substituents:

hydrogen, halogen, haloalkyl, lower alkyl, alkoxy, methylenedioxy, ethylenedioxy, alkylthio, haloalkylthio, mercapto, hydroxy, cyano, nitro, carboxyl derivatives, aryloxy, amido, acylamino, amino, alkylamino, dialkylamino, trifluoroalkoxy, trifluoromethyl, sulfonyl, alkylsulfonyl, haloalkylsulfonyl, sulfonic acid, sulfonamide, unsubstituted aryl, fused aryl, monocyclic heterocycles, and

fused monocyclic heterocycles;

unsubstituted aryl or aryl optionally substituted with one or more substituent selected from the group consisting of

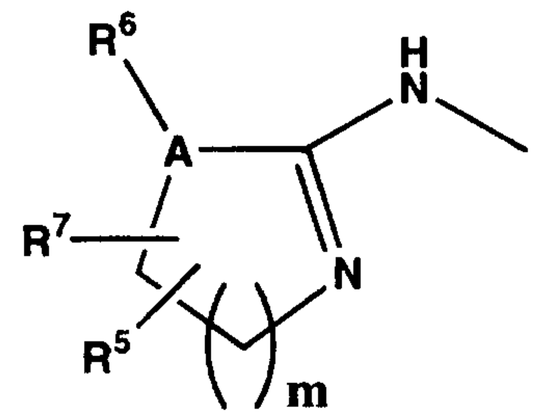
halogen, haloalkyl, lower alkyl, alkoxy, methylenedioxy, ethylenedioxy, alkylthio, haloalkylthio, mercapto, hydroxy, cyano, nitro, carboxyl derivatives, aryloxy, amido, acylamino, amino, alkylamino, dialkylamino, trifluoroalkoxy, trifluoromethylsulfonyl, alkylsulfonyl, sulfonic acid, sulfonamide, aryl, fused aryl, monocyclic heterocycles, and fused monocyclic heterocycles;

unsubstituted monocyclic heterocycle or monocyclic heterocycle optionally substituted with one or more substituent selected from the group consisting of

halogen, haloalkyl, lower alkyl, alkoxy, aryloxy, amino, nitro, hydroxy, carboxyl derivatives, cyano, alkylthio, and alkylsulfonyl;

or;

R³ is



wherein;

R⁷ is hydrogen or hydroxyl;

R⁵ and **R⁶** are each substituents independently selected from the group consisting of hydrogen, lower alkyl, hydroxy, alkoxy, halogen, phenyl, amino, carboxyl or carboxyl ester, and fused phenyl;

A is nitrogen or -CH=; and

m is an integer 1, 2, 3,or 4.

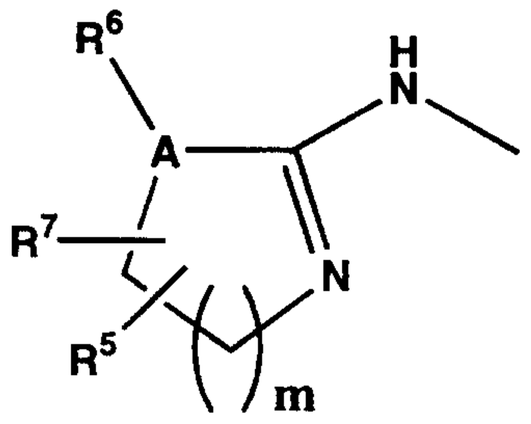
20. A compound of claim 19 wherein

X is C; and

R^4 is one or more substituents independently selected from the group consisting of hydrogen, hydroxy, halogen, and haloalkyl.

21. A compound of claim 19 wherein

R^3 is



wherein;

X is C;

R^4 is one or more substituents independently selected from the group consisting of hydrogen, hydroxy, halogen, and haloalkyl.

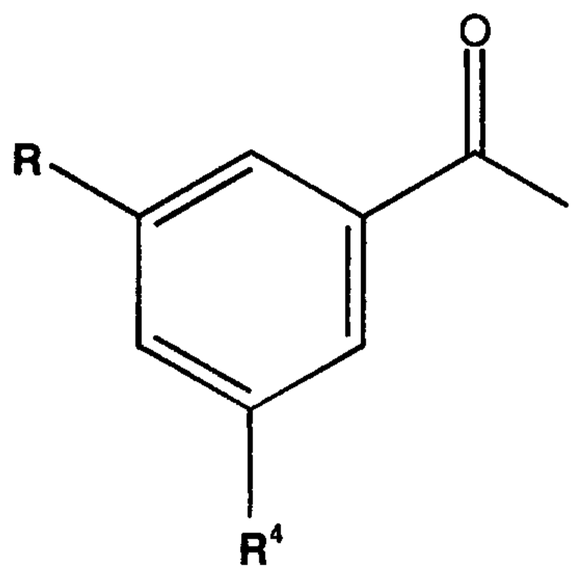
R^7 is hydrido or hydroxyl;

R^5 and R^6 are one or more substituents independently selected from the group consisting of hydrogen, lower alkyl, and hydroxy;

A is nitrogen; and

m is an integer 1 or 2.

22. A compound of claim 1, wherein A^1 is a compound of the formula III,



III

or a pharmaceutically acceptable salt thereof, wherein:

R^4 = H, CF_3, Cl;

R is selected from R^1 and R^2; wherein

R^1 is NH_2-(C=NH)-, HO-NH-(C=NH)-;

R^2 is benzyl-NH-(C=Y)- ; and

Y = O, S.

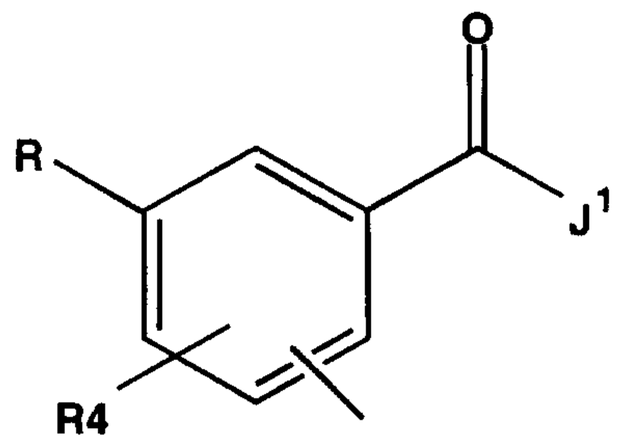
23. A compound selected from:

Compound 1, Compound 2, Compound 3, Compound 4, Compound 5, Compound 6, Compound 7, Compound 8, Compound 9, Compound 10, Compound 11, Compound 12, Compound 13, Compound 14, and Compound 15.

24. A compound selected from:

Compound 101, Compound 102, Compound 103, Compound 104, Compound 105, Compound 106, Compound 107, Compound 108, Compound 109, Compound 110, Compound 111, Compound 112, Compound 113, Compound 114, and Compound 115, Compound 116, Compound 117, Compound 118, and Compound 119.

25. A compound of claim 1 wherein; A^1 is a compound of the formula IV,



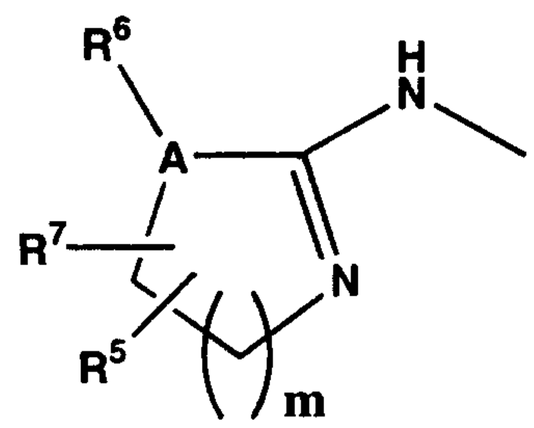
IV

or a pharmaceutically acceptable salt thereof, wherein:

J^1 is selected from the group consisting of Gly-Asp-Ala-NH_2, and Gly-Asp-Ser-NH_2.

R^4 is one or more substituents independently selected from the group consisting of hydrogen, hydroxy, halogen, and haloalkyl.

R is



wherein;

R^7 is hydrogen or hydroxyl;

R^5 and R^6 are one or more substituents independently selected from the group consisting of hydrogen, lower alkyl, hydroxy;

A is nitrogen; and

m is an integer 1 or 2.

26. A compound selected from:

Compound 201, Compound 202, Compound 203, Compound 204, Compound 205, Compound 206, Compound 207, and Compound 208.

27. Use of a compound of claim 1 for the manufacture of a medicament for therapeutic application to inhibit tumor growth.

28. A pharmaceutical compcsition comprising a therapeutically effective amount of the compound according to claim 1 in admixture with a pharmaceutically acceptable carrier.

29. The pharmaceutical composition of claim 28 further comprising an adjunctive agent.

30. The pharmaceutical composition of claim 29 further comprising a chemotherapeutic agent.

31. The pharmaceutical composition of claim 29 further comprising a immunotherapeutic agent.

32. A process of treating a human patient with an angiogenesis-mediated disease, comprising administering to said patient an effective amount of compound of claim 1.

33. The process of claim 32, wherein the angiogenesis-mediated disease is selected from the group consisting of cancer, arthritis, and macular degeneration.

34. A process of treating cancer comprising administering to a mammalian host suffering therefrom a therapeutically effective amount of a compound of claim 1 in unit dosage form.

35. A process of inhibiting elevated levels of tumor antigens comprising administering to a host in need thereof a therapeutically effective amount of a compound of claim 1 in unit dosage form.

36. A process of modulating tumors in a patient comprising administering an angiogenesis-inhibiting effective amount of the compound as recited in claim 1 to said patient.

37. A process of treating inhibiting the proliferation of tumor cells in a patient comprising administering an angiogenesis-inhibiting effective amount of the compound as recited in claim 1 to said patient.

38. The process of claim 37 wherein said tumor cells are selected from the group consisting of lung cancer, breast cancer, ovarian cancer, prostate cancer, pancreatic cancer, gastric cancer, colon cancer, renal cancer, bladder cancer, melanoma, hepatoma, sarcoma, and lymphoma.

39. A process for the treatment of a patient with a solid tumor, that comprises the steps of:

(a) administering an effective dose of angiogenesis-inhibiting effective amount of the compound as recited in claim 1 to a patient in a pharmaceutically acceptable vehicle; and

(b) maintaining said patient for a time period sufficient to cause a reduction in tumor size.

40. The process according to claim 39 wherein said steps (a) and (b) are repeated.