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(57) Abrégé/Abstract:
The invention relates to peptides corresponding to regions of the amino acid sequence of TGF-β1 or TGF-β2 which retain, either in monomeric or polymeric forms, at least some of the biological activity of the respective full length TGF-β. The monomeric form of the peptide derived from TGF-β1 comprises the following amino acid sequence: CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP. The monomeric form of the peptide derived from TGF-β2 comprises the following amino acid sequence: CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA. Dimers may be formed via disulfide bonds between the amino-terminal cysteine residues, the carboxy-terminal cysteine residues, or amino- and carboxy-terminal cysteine residues of the monomer subunits.

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(54) Title: BIOLOGICALLY ACTIVE TGF-β1 AND TGF-β2 PEPTIDES

(57) Abstract

The invention relates to peptides corresponding to regions of the amino acid sequence of TGF-β1 or TGF-β2 which retain, either in monomeric or polymeric forms, at least some of the biological activity of the respective full length TGF-β. The monomeric form of the peptide derived from TGF-β1 comprises the following amino acid sequence: CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP. The monomeric form of the peptide derived from TGF-β2 comprises the following amino acid sequence: CLRPLYIDFKRDLGWKWIHEPKG-YNANFCAGA. Dimers may be formed via disulfide bonds between the amino-terminal cysteine residues, the carboxy-terminal cysteine residues, or amino- and carboxy-terminal cysteine residues of the monomer subunits.

BIOLOGICALLY ACTIVE TGF-β1 AND TGF-β2 PEPTIDES

Technical field

The present invention relates to biologically active, peptides corresponding to regions of the amino acid sequence of transforming growth factor β1 or β2 that are capable of mimicking the activity of the respective full length TGF-βs.

Background of the invention

PCT WO 84/001106, filed Sept. 23, 1983, describes transforming growth factor β1 (TGF-β1) and its use for the promotion of cell proliferation and tissue repair, wound healing and treatment of trauma.

U.S. Patent No. 4,843,063 describes two cartilage inducing factors, CIF-A and CIF-B, found in mammalian bone that (1) are cofactors for inducing cartilage formation *in vivo*; (2) promote connective tissue deposition *in vivo* in the absence of any added activating agent or cofactor; and (3) are active in the anchorage-independent cell growth assay used to characterize TGF-β. The assay is referred to as the TGF-β assay herein and is described in Methods for Preparation of Media, Supplements, and Substrate for Serum-free Animal Cell Culture (1984) pp. 181-194, Alan R. Liss, Inc.

U.S. Patent No. 4,806,523, filed March 6, 1986, discloses that CIF-A and CIF-B both possess anti-inflammatory activity and are inhibitors of mitogen stimulated T cell proliferation and B cell activation. It also reports that CIF is localized in centers of

hematopoiesis and lymphopoiesis and that CIF may, therefore, be useful for treating indications associated with malfunction or dysfunction of hematopoiesis or lymphopoiesis. CIF-A has since been shown to be identical to TGF-β1. CIF-B has since been recognized as a new form of β-type transforming growth factor and is now called TGF-β2.

U.S. Patent No. 4,822,606, filed April 7, 1986, describes novel peptides having immunosuppressive or immunoregulatory activity. These peptides are based on a 26 amino acid sequence which is highly conserved among retroviruses associated with immunosuppression.

European Patent Application 0 353 772 A2, filed August 4, 1988, discloses a method and compositions for inhibiting proliferation of epidermal cells with TGF-β1, TGF-β2, or a fragment thereof.

U.S. Patent No. 5,061,786, filed May 25, 1989, discloses a biologically active peptide corresponding to residues 16-31 of TGF-β1, with optional extensions of the amino terminus.

Disclosure of the Invention

The invention relates to biologically active peptides corresponding to regions of the amino acid sequence of TGF-β1 or of TGF-β2. The peptides retain and thus mimic the biological activities of mature, active TGF-βs. The peptides are thus suitable for use in any application for which TGF-βs are indicated. The peptides, either in monomeric or polymeric forms, retain at least some of the biological activity of the respective full length TGF-β. The monomeric form of the peptide derived from TGF-β1 comprises the following amino acid sequence: CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP. The monomeric form of the peptide derived from TGF-β2 comprises the following amino acid sequence:

CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA. Dimers may be formed via disulfide bonds between the amino-terminal, carboxy-terminal, or amino- and carboxy-terminal cysteine residues of the monomer subunits.

The present invention further provides compositions comprising the peptides of the invention and a pharmaceutically acceptable carrier therefor and methods of mimicking the effects of TGF-βs comprising administering to a patient an effective amount of such a composition.

Various embodiments of this invention provide a peptide comprising the amino acid sequence CLRPLYIDFKDLGWKWIHEPKGYNANFCAGA or an amino acid sequence substantially corresponding thereto, wherein a monomeric form of the peptide is less than the full length TGF-β2 sequence.

Various other embodiments of this invention provide a homodimeric peptide comprising a monomeric amino acid sequence of CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA or an amino acid sequence substantially corresponding thereto, wherein the monomers are linked via at least one interpeptide disulfide bond between at least one set of cysteine residues.

Various other embodiments of this invention provide a peptide comprising the amino acid sequence CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP or an amino acid sequence substantially corresponding thereto, wherein a monomeric form of the peptide is less than the full length TGF-β1 sequence and greater than about twenty amino acid residues.

The aforementioned peptides of this invention may be a dimeric form of a peptide of this invention.

Various other embodiments of this invention provide a homodimeric peptide comprising a monomeric amino acid sequence of CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP or an amino acid sequence substantially corresponding thereto, wherein the monomers are linked via at least one interpeptide disulfide bond between at least one set of cysteine residues.

Various other embodiments of this invention provide a composition comprising a peptide of this invention, including dimeric forms, and a pharmaceutically acceptable carrier therefor.

Various other embodiments of this invention provide the use of a peptide of this invention for preparation of a medicament to mimic the effects of TGF-βs.

Various other embodiments of this invention provide the use of a peptide or a composition of this invention for mimicking the effects of TGF-βs.

Various other embodiments of this invention provide a pharmaceutical product comprising a container containing a formulation comprising a peptide of this invention or a composition of this invention, and instructions associated with the container directing the use of the formulation for mimicking the effects of TGF-βs.

Various other embodiments of this invention provide a peptide of this invention or a composition of this invention for mimicking the effects of TGF-βs.

These and other embodiments of the present invention will be readily apparent to those of ordinary skill in view of the disclosure herein.

Brief Description of the Drawing

Figure 1 is a composite of line graphs depicting the results of the mink lung cell proliferation assay described in Example 2. Figure 1 consists of eight graphs labeled Fig. 1a-1h.

Detailed Description

Technical and scientific terms have been defined herein and this disclosure and the appended claims should be interpreted in view of those definitions. Unless specifically defined herein, all other technical and scientific terms have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although other methods and materials similar or equivalent to those described may be used in the practice or testing of the present invention, the preferred methods and materials are described herein.

It must be noted that as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural references unless the context clearly dictates otherwise. Thus, for example,

reference to "a peptide" or "an active peptide" includes mixtures of peptides of the general type described herein, and reference to "the method of administration" includes one or more methods of administration of the general type described herein and/or of the type which will be readily apparent to those of ordinary skill in the art.

The methods for making the peptides employ, unless otherwise indicated, conventional techniques of synthetic organic chemistry, protein chemistry, molecular biology, microbiology, and recombinant DNA technology, which are within the skill of one in the art. Such techniques are explained fully in the literature. See, e.g., Scopes, Protein Purification Principles and Practices, 2d ed. (Springer-Verlag, 1987), Methods in Enzymology (Colowick and Kaplan, eds., Academic Press, Inc.); Sambrook et al., Molecular Cloning: A Laboratory Manual, 2d ed., Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989, Handbook of Experimental Immunology, Vols. I-IV, (Weir and Blackwell, eds) (Blackwell Scientific Publications, 1986); House, Modern Synthetic Reactions, 2nd ed., Benjamin/Cummings, Menlo Park, Cal., 1972. Atherton and Sheppard, Solid Phase Peptide Synthesis: A Practical Approach (Oxford University Press, 1989); Steward and Young Solid Phase Peptide Synthesis, 2nd ed. (Pierce Chemical Co., 1984).

A. Definitions

In defining the present invention, the following terms will be employed, and are intended to be defined as indicated below.

As used herein, the amino acid sequence of TGF-β1 is that described by Derynck et al., Nucl. Acids Res., 15:3188-3189 (1987). The amino acid sequence of TGF-β2 is that described by Madisen et al., DNA, 7:1-8 (1988).

As used herein, the term "fibrinogenic" refers to factors that promote healing of damaged connective tissue resulting from injury inflicted by various types of trauma, inflammation, and immune reactions. These factors include but are not limited to those involved in fibroblast chemotaxis, synthesis of collagen, hyaluronate, and tissue inhibitor of metalloproteinase (TIMP).

As used herein, the phrase "biologically active" refers to the ability to mediate a biological function.

As used herein, the term "peptide" refers to an oligomer of at least two contiguous amino acid residues. Further, as used herein, the phrase "the peptides" refers to the peptides described herein unless otherwise indicated.

As used herein, the term "treat" is intended to mean prophylaxis or attenuation of an existing condition. Accordingly, in the case of inflammation, the invention method may be used to prevent inflammation or alleviate existing inflammation.

As used herein, the term "inflammation" is intended to encompass both acute responses (i.e. a response in which the inflammatory processes are active) and chronic responses (i.e. a response marked by slow progress and formation of new connective tissue). Chronic and acute inflammation may be distinguished by the cell types involved. Acute inflammation often involves polymorphonuclear neutrophils; whereas chronic inflammation is normally characterized by a lymphohistiocytic and/or granulomatous response.

Examples of specific types of inflammation are diffuse inflammation, focal inflammation, croupous inflammation, interstitial inflammation, obliterative inflammation, reactive inflammation, specific inflammation, toxic inflammation, and traumatic inflammation.

As used herein, an amino acid sequence "substantially corresponding" to TGF-β1 or TGF-β2 will have at least 70% sequence homology with the amino acid sequence of TGF-β1 or TGF-β2, respectively, and retain functional activity. Further, it is preferable that any differences in sequence homology possessed by a peptide or protein having a "substantially corresponding" sequence are differences which do not adversely affect a desired property of that peptide or protein.

As used herein, the term "septic shock" refers to the sequence of events triggered by bacteremia during which cell wall substances (endotoxin in Gram-negative organisms and peptidoglycan/teichoic acid complex in Gram-positive organisms) activate the complement, kinin, and ACTH/endorphin systems. This series of metabolic events ultimately progresses to a state of shock.

As used herein, the term "patient" refers to animals including humans suffering from a condition that can be cured or ameliorated by a TGF-β.

B. General Methods

TGF-βs exhibit activity in the TGF-β assay described in Methods for Preparation of Media, Supplements, and Substrate for Serum-free Animal Cell Culture (1984) pp. 181-194, Alan R. Liss, Inc. The assay determines the ability to induce anchorage-independent growth in non-neoplastic normal rat kidney fibroblasts by measuring the formation of cell colonies in soft agar.

TGF-βs isolated to date are nonspecies specific as regards TGF-β activity. TGF-βs derived from other

species are thus highly "homologous" in that they are similar in amino acid residue sequences and activity. These polypeptides are believed to have been highly conserved among animal species (i.e, wherein a given polypeptide from different mammalian species has an amino acid sequence that varies little, if at all, between species with respect to amino acid residue deletions, additives, or substitutions, and which variations do not adversely affect the non-species-specific activity of the molecule adversely) and have cross-species functionality. Accordingly, the peptides may be derived from cells or tissue of diverse animal origin or may be obtained by recombinant DNA technology. The peptides thus encompass the amino acid residue sequences of homologous TGF-βs. Correlatively, peptides from one vertebrate species may be used to treat another vertebrate species. The most common therapeutic uses of the peptides is in the treatment of patients such as humans, domestic animals such as cattle, sheep, and pigs, and sports or pet animals such as dogs, cats, and horses.

The peptides are useful in any application for which a TGF-β is indicated. The peptides mimic TGF-β activities including but not limited to inducing cartilage/bone formation and for repairing, replacing, or augmenting cartilage/bone tissue in animals including humans. Effective amounts of the peptides will normally be formulated with pharmacologically acceptable fluids or solid carriers. An effective amount of the peptides is an amount sufficient to evoke the biological response required to ameliorate the condition suffered by the patient.

The peptides may also be used in the same manner as other TGF-βs to promote (provoke and sustain) non-species specific cellular proliferation. Clinical applications of the cellular proliferation activity of

these compositions include topical administration, for instance, for burn or wound healing or tissue repair. In such uses an effective amount of the peptides are formulated with pharmacologically acceptable carriers. An effective amount of the peptides is an amount sufficient to induce soft tissue cell proliferation.

Topical dosage forms of the peptides can be formulated for instance as sprays, gels, ointments, or salves. Suitable dosage forms are those that are pharmacologically acceptable and into which an amount of the peptides can be dispersed so as to allow diffusion of an effective amount of the peptides to the treatment site. The peptides, alone or in combination, may be dispersed into or incorporated into a polymeric substance and coated onto implants. Such implants include, but are not limited to, collagenous soft and hard tissue implants, prostheses, sponges, wound dressings, and sutures. The peptides modulate local inflammatory responses to these foreign bodies and promote attachment of the implants, particularly prostheses. Since such implants are often made from permeable materials peptides incorporated into the implants can diffuse from the implant to exert their properties.

The peptides may also be useful systemically, for instance, for treating bone deficiencies, such as osteoporosis and osteopetrosis. For such treatment the peptides are formulated in therapeutically effective amounts with injectable carriers and administered parenterally to the patient. The dose is typically be in the range of about 0.001 μg/kg to 10 g/kg with a preferred range of about 100 μg/kg to 1 g/kg.

Systemic dosage forms may be formulated for gastrointestinal administration (i.e., liquids, pills, tablets, or suppositories) or for parenteral injection. The determination of dosages used in such applications

are within the skill of one in the art and depend on factors such as the nature of the condition being treated, the size of the patient, and responsiveness to the peptides administered, and are therefore determined by the caregiver.

The peptides may be used as oncostats in treating any type of cellular neoplasm, including, without limitation, carcinomas, myelomas, melanomas, and lymphomas. Particularly preferred targets are breast, lung, colon, and ovarian carcinomas. The peptides may be administered locally or systemically, depending on the nature and degree of the neoplasm being treated. For local administration an oncostatically effective amount of the peptides or mixtures formulated thereof are combined with a pharmacologically acceptable carrier and administered as a solid or semisolid implant which may or may not be of a sustained or a controlled release form. The peptides may also be formulated into an injectable for parenteral administration.

Alternatively, the peptides can be delivered to solid tumors in particular, including inoperable tumors, using current catheter technology for localized delivery via the arterial supply to the tumor. In this indication the peptides are mixed with a vasoocclusive agent, such as injectable collagen, which would provide a means to reduce perfusion of the tumor and at the same time provide for the localized delivery of the peptides. Clips may also be used to occlude venous drainage, and thus maintain high doses of the peptides in the tumor mass.

For systemic administration, oncostatically effective amounts of the peptides are formulated with pharmacologically acceptable carriers used for water soluble proteins including but not limited to physiological saline, sugar solutions and the like for

injection into circulation. Alternatively, the peptides may be formulated as a sustained release formulation to release the peptides into the circulation over a prolonged time period.

Specific targeting of the factor for tumor cells in systemic applications may be accomplished for instance by conjugation of a peptide to an antibody directed against tumor specific cell surface antigen(s). Enhanced tumor cell cytotoxicity may be accomplished by covalently radiolabeling the fibrinogenic peptide with ^{131}I, a cytotoxic agent. The peptides are readily iodinated and retain full biological activity. Monoclonal antibody preparations with specificity for particular tumor types, such as breast and ovarian tumors, are well known in the art. Other oncostats or chemotherapeutic drugs may be included in the formulation if desired.

The term "oncostatically effective" is intended to indicate a dose that effects a significant (>50%) inhibition of tumor cell proliferation. In *in vitro* assays, 50% inhibition is generally observed at TGF-β concentrations of the order of 0.2 ng/ml and saturation is achieved at 10 ng/ml. Inhibition may be monitored *in vivo* by monitoring the patient's tumor burden. The dose of peptide that is oncostatically effective in a given treatment depend upon the patient, the type and degree of cancer being treated and the mode of administration. In general, the amounts administered to adult humans are in the range of about 0.001 μg/kg to 10 g/kg. Corresponding systemic administration involves the higher segment of the range (100 μg/kg to 1 g/kg) due to clearance or other *in situ* inactivation of the polypeptide.

The peptides are useful in the treatment of both local and systemic inflammation. When used as a local anti-inflammatory agent, the peptides are usually

formulated in effective amounts with pharmacologically acceptable carriers in weight ratios to carrier in the range of 1:1,000 to 1:20,000.

When used to treat inflammation at internal sites locally, the peptides, alone or in combination, may be injected, inhaled, placed surgically, or otherwise administered locally, depending on the particular formulation and the site where inflammation control is desired.

For systemic administration, the peptides may be formulated with conventional carriers used with water soluble proteins for injection into circulation. Alternatively, they may be formulated as a sustained release implant formulation if the indication being treated so requires.

Examples of formulations for systemic, topical, or parental administration may be found in Remington's Pharmaceutical Sciences, Gernnaro, ed., Mack Publishing Co., Easton, PA, 1985.

The amount of the peptides administered to treat inflammation depends upon the patient, the inflammatory condition being treated, and the mode of administration. In general, amounts administered to adult humans are in the range of about 1 mg to 10 g. When the peptides are administered locally, amounts in the lower portion of the range are normally used, typically 1 μg to 100 mg. Correspondingly, systemic administration typically involves amounts in the 100 μg/kg to 1 g/kg range.

The peptides may be particularly effective in the treatment of inflammation involving the respiratory system. In this application, the peptides may be administered by inhalation with a suitable aerosol. In this form, the peptides would be useful for the treatment

of diffuse interstitial diseases of the lung including but not limited to, asbestosis, silicosis, or coal-miner's pneumoconiosis; the treatment of immunological diseases that involve the respiratory tract including, but not limited to, rheumatoid arthritis, lupus erythematosus, or Goodpasture's syndrome; and the treatment of granulomatosis and eosinophilic granulomatosis.

The peptides may be combined with carriers in the form of a salve, ointment, or other topical formulation and thereby be useful in the control of dermal inflammation by topical application. Such formulations would be particularly useful in the local treatment of skin conditions including, but not limited to, psoriasis vulgaris, contact dermatitis, dermal ulcers, and acute or chronic eczematous dermatitis.

The peptides may be used either alone or combined with a slow release carrier and injected into or around joints, bone, or muscle for wound healing or control of inflammation associated with various diseases. Such diseases include but are not limited to myositis (due to viral, bacterial, parasitic, fungal, or autoimmune processes); myasthenia gravis; osteomyelitis; osteoarthritis and rheumatoid arthritis.

Since TGF-β molecules have been shown to be stable at low pH and resistant to enzyme digestion, these factors may be delivered gastrointestinally. The peptides are thus particularly useful for the treatment of gastrointestinal disorders including but not limited to gastric and duodenal ulcers, granulomatous gastritis, esophagitis, enteritis and colitis.

TGF-βs have also been shown to be effective for the treatment of septic shock. International Publication Number WO 90/000903, filed July 21, 1989. The peptides may be administered prophylactically or therapeutically,

i.e., before, simultaneous with, or after an infection has set in. The peptides may be used to treat patients who are at-risk to bacterial infection, or who suffer from septicemia. Patients who are at risk include but are not limited to those receiving immunosuppressive therapy and those suffering from severe thermal burns or other serious injury, cystic fibrosis, renal failure, or cancer, or who are undergoing extensive surgical procedures or organ transplantation.

The peptides may further be used to treat a patient for an indication associated with a dysfunction or malfunction of hematopoiesis or lymphopoiesis. The peptides may be administered to these patients by any suitable technique, including systemic or local, as discussed above and will take into consideration the requirements of the individual patient, the method of administration, and other factors known to practitioners. Doses will typically be in the range of from about 100 μg/kg to 1 g/kg.

The peptides may also be used to protect hematopoietic stem cells from the myelotoxicity of chemotherapeutic drugs, such as cyclophosphamide and mephalan, or radiation therapy. In such applications a therapeutically effective amount of the peptides will be administered usually 3-72 hours prior to the administration of the chemotherapeutic drug or radiation therapy. The mode of administration is preferably interfemoral arterial, intraperitoneal, or subcutaneous, and is preferably by injection. Compositions and doses may be formulated as discussed for the above applications, taking into consideration the requirements of the individual patient, the nature of the drug or radiation therapy used, the method of administration of the composition, and other factors known to

practitioners. Doses will typically be in the range of 100 μg/kg to 1 g/kg.

TGF-β may also be used in the prevention of severe cardiac injury resulting from reperfusion of ischemic myocardium. Lefer et al., Science, 249:61 (1990). The peptides thus may be administered, preferably intravenously or intracardiac, prior to or after the onset of ischemia. Peptide compositions and doses may be formulated as discussed for above applications, taking into consideration the requirements of the individual patient and other factors known to practitioners. Doses will typically be in the range of 100 μg/kg to 1 g/kg.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make the peptides, formulate the compositions and use them in connection with the invention and are not intended to limit the scope of the invention.

Example 1 - Peptide Synthesis

The following peptides were synthesized:

TGF-$\beta 1_{16-31}$ corresponds to TGF-β1 residues 16 through 31, and has the amino acid sequence CVRQLYIDFRKDLGWK.

TGF-$\beta 2_{16-31}$ corresponds to TGF-β2 residues 16 through 31, and has the amino acid sequence CLRPLYIDFKRDLGWK.

TGF-$\beta 1_{16-47}$ corresponds to TGF-β1 residues 16 through 47, and has the amino acid sequence CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP.

TGF-$\beta 2_{16-47}$ corresponds to TGF-β2 residues 16 through 47, and has the amino acid sequence CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA.

Peptides were synthesized on an Applied Biosystems model 431A peptide synthesizer using Fmoc amino acids (Applied Biosystems). Fmoc amino acids were coupled through reactions mediated with N,N-dicyclohexylcarbodiimide (DCC) or [2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (HBTU) in 1-hydroxybenzotriazole (HOBt). The peptide synthesis protocol was modified to double couple certain residues and to shorten the coupling times of certain residues to minimize premature termination of peptide synthesis by diketopiperazine formation. The peptides were synthesized on 4-hydroxymethylphenoxymethyl copolystyrene-1% divinylbenzene resin (HMP-resin). Reactive sites on the HMP-resin were capped with acetic anhydride following addition of the first residue.

The crude peptide (approximately 250 μmol) was cleaved from the resin in 11.25 ml of 89% TFA, containing 0.75 g of crystalline phenol, 0.25 ml of ethylene dithiol, 0.5 ml of thioanisole, and water, at room temperature for over 1.5 hr. The resin was removed by filtration through a glass fritted disk and the filtrate was concentrated to 2 ml by using a rotoevaporator at 30°C. Crude peptide was precipitated by adding 50 ml of ice-cold diethyl ether and collected by filtration. The crude peptide was dissolved in 0.1% TFA, 30% acetonitrile and lyophilized. The dry peptide was stored at -20°C.

The dried crude peptide was dissolved in 0.1% TFA, 18% acetonitrile and chromatographed on a C18-reverse phase HPLC column (1 x 25 cm, Vydac*, C18TP510). Peptides were eluted with a linear acetonitrile gradient in 0.1% TFA. The peak containing the desired peptide was identified by N-terminal sequencing and was confirmed by amino acid analysis. Protein concentration was determined with a BCA assay (Pierce, Rockford, IL). Reduced forms of cysteine residues were assayed using

*Trademark

5,5'-dithio-bis-(2-nitrobenzoic acid). Ellman, Arch. Biochem. Biophys., 74:443 (1958).

To dimerize peptides, disulfide bonds were formed as follows. Purified peptide was dissolved at 5 mg/ml in 20% acetonitrile/80% dilute ammonium hydroxide (pH adjusted to 8.5 to 9.5 with 3% ammonium hydroxide). After stirring for 1 to 16 hr, precipitate that formed was dissolved by decreasing the pH to approximately 2 with 10% TFA. The reaction mixture was applied onto a C18-reverse phase HPLC column (1 x 25 cm, Vydac, C18TP510) and eluted with a linear acetonitrile gradient in 0.1% TFA. The dimeric forms of the peptides were identified by the shift in the elution position of the peak relative to the monomeric peptides. Monomeric and dimeric forms were confirmed by assaying for non-disulfide bonded cysteine residues and by mass spectroscopy.

Example 2 - Cell proliferation inhibition assay

TGF-β is known to inhibit proliferation of mink lung epithelial cells. The mink lung epithelial cell assay was thus used to test the ability of the peptides to mimic the biological activity of TGF-β.

Mink lung epithelial cells (ATCC, Rockville, MD, Mv1Lu CCL64) were plated at 0.5-1.0 x 10^6 cells/plate in 100 mm culture plates and grown in Eagle's minimal essential medium (MEM) supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin, nonessential amino acids, L-glutamine, and 10% fetal bovine serum (FBS). Cells were passaged while the cell density was subconfluent. Cells were detached with trypsin, collected by centrifugation at 800 x g for 2 min, and resuspended in the culture medium at 20,000 cells/ml. The cells were plated in 96-well microtiter plates at 1000 cells (50 μl)/well.

Peptides TGF-$\beta 1_{16-31}$, TGF-$\beta 1_{16-47}$, TGF-$\beta 2_{16-31}$, TGF-$\beta 2_{16-47}$ in monomeric and dimeric forms were lyophilized in the presence of sterile bovine serum albumin (BSA) carrier. Samples (50 μl) of the peptides were dissolved in the cell culture medium and added to the wells in triplicate. Recombinant human TGF-$\beta 2$ was used as the standard. The plates were incubated under a 5% CO_2/95% (V/V) air atmosphere at 37°C for 4 days.

The number of cells/well in this assay is proportional to the activity of the constitutively expressed enzyme, acid phosphatase. To measure acid phosphatase activity, the wells were rinsed with phosphate-buffered saline (PBS), filled with 100 μl of 0.1 M sodium acetate, pH 5.5/0.1% Triton X-100*/100 mM p-nitrophenyl phosphate, and the plates were incubated at 37°C for 2 hr. Ten μl of 1.0 N NaOH was added to each well, and after 20 min at room temperature, the absorbance at 405 nm was measured.

Some of the synthetic peptides and TGF-$\beta 2$ inhibited proliferation of the mink lung epithelial cells in a dose-dependant manner (Figure 1), as indicated by the decrease in absorbance values. The inhibition curve for TGF-$\beta 2$ has been included in each figure for comparison. Approximate concentrations at which a given peptide and TGF-$\beta 2$ inhibited cell proliferation half-maximally (ED_{50}) are summarized in Table 1.

*Trademark

Table 1

Peptide		ED_{50}
TGF-$\beta 1_{16-31}$	dimer	1-3 μM
	monomer	not active
TGF-$\beta 2_{16-31}$	dimer	not active
	monomer	not active
TGF-$\beta 1_{16-47}$	dimer	150 nM
	monomer	1 μM
TGF-$\beta 2_{16-47}$	dimer	4-14 nM
	monomer	not active
TGF-$\beta 2$		0.4-1 pM

TGF-$\beta 1_{16-31}$ dimer inhibited proliferation of mink lung epithelial cells with an ED_{50} of 1-3μM, comparable to levels of specific biological activity previously reported for this peptide. Chen et al., J. Bone Min. Res., 5: Abstract 26; and WO 90/14359. TGF-$\beta 2_{16-31}$, a peptide homologous to the TGF-$\beta 1_{16-31}$ peptide, was not active in either monomeric or dimeric form in the assay. The activity of the TGF-β1 synthetic peptide increased considerably when 16 additional carboxy terminal amino acid residues were added to form TGF-$\beta 1_{16-47}$. The dimer of TGF-$\beta 1_{16-47}$ exhibited an ED_{50} of about 150 nM. TGF-$\beta 1_{16-47}$ peptide monomer exhibited some activity. In contrast to all the peptides mentioned, TGF-$\beta 2_{16-47}$ peptide dimer exhibited an ED_{50} of about 4-14 nM. In comparison, bovine bone TGF-β2 inhibited proliferation of the cells with an ED_{50} of 0.4 to 1.0 pM. BSA alone was inactive in this assay. Thus, dimerized TGF-$\beta 2_{16-47}$ possesses about 11-38 fold more activity than dimerized TGF-$\beta 1_{16-47}$. None of the monomeric peptides were active below 1 μM.

It should be noted that activities of these peptides when assayed in the cell culture assay without prior lyophilization was comparable to the peptides co-lyophilized with BSA carrier prior to the assay.

Example 3 - Inhibition of murine thymocyte proliferation

In order to determine the effect of the peptides on proliferation of cell types other than mink lung epithelial cells, murine thymocytes were exposed to the peptides. Proliferation was measured by determining the amount of ^{3}H-thymidine incorporated into the thymocyte DNA in response to the peptides. The cell culture assay was performed as described by Ellingsworth et al., Cell. Immunol., 114:41-54 (1988). Single-cell suspensions of thymocytes were prepared from 4 to 8 week old C3H/HeJ mice and the cells were suspended in Eagle's MEM supplemented with 5% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 50 μM β-mercaptoethanol to 6.66×10^6 cells/ml. The thymocytes were plated into 96-well microtiter plates at 10^6 cells/well (150 μl). The cells were activated with 1.0 μg/ml of phytohemagglutinin and 8 units/ml of interleukin-1 (IL-1) (Boehringer Mannheim, Indianapolis, IN). 0.02 to 200 pmol of the peptides or 0.02 to 50 fmol of TGF-β2 were added and the total volume was adjusted to 250 μl with the culture medium. The cells were incubated at 37°C for 72 hr in a humidified incubator in 5% CO_2-95% air atmosphere. The cells were pulsed with 0.5 μCi/well of ^{3}H-thymidine 24 hours prior to cell harvest. The thymocytes were harvested onto glass fiber filters and dried. The amount of ^{3}H-thymidine incorporation was determined by liquid scintillation counting method.

The results obtained indicated that TGF-β2 inhibited incorporation of ^{3}H-thymidine into the DNA of murine thymocytes with an ED_{50} of approximately 4 pM. The TGF-$\beta 2_{16-47}$ dimer was also active as an inhibitor of thymocyte proliferation in this assay with an ED_{50} of 57 nM. However, both TGF-$\beta 1_{16-31}$ dimer and TGF-$\beta 2_{16-31}$

dimer did not affect ^{3}H-thymidine incorporation at concentrations up to 0.8 μM.

Example 4 - TGF-β receptor binding assay

Rat muscle myoblasts (L6, American Type Culture Collection) were plated in 6-well culture plates and cultured in DMEM supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10% FBS until the cells were 70-90% confluent. Medium was aspirated from the wells; and 1 ml of ice cold 20 mM glycine, 135 mM NaCl, pH 3.0 was added to each well for 2 min. The buffer was aspirated and each well was rinsed 2 times with 1 ml of DMEM, 25 mM Hepes, pH 7.4, 0.1% BSA. Samples of TGF-β1 and TGF-β2 (3 to 1600 pM) and of peptides (0.1 to 30 μM), each containing 25 pM ^{125}I-TGF-β1 or ^{125}I-TGF-β2 were prepared in DMEM, 25 mM Hepes, pH 7.4, 0.1% BSA and added in 500 μl aliquots to the wells. Radiolabeled TGF-β samples (500 μl) either without unlabeled TGF-β or with excess unlabeled TGF-β (20-25 nM) were prepared and added to other wells. After 1 hr incubation at room temperature, each well was rinsed 2 times with 1 ml of Hank's balanced saline solution, 0.1% BSA. To each well, 500 μl of 1% Triton X-100, 10% glycerol, 20 mM Hepes, pH 7.4, 0.01% BSA was added and incubated at 37°C with rotation for 30 min to lyse the cells. Samples were transferred into gamma-counting vials and counted for 1 min each. The results are presented in Table 2.

Table 2

Ability of the peptides to compete against binding of ^{125}I-TGF-β to L6 rat muscle myoblasts

Peptide		^{125}I-TGF-β	ED_{50}
TGF-β1		TGF-β1	10-30 pM
TGF-β2		TGF-β2	100-130 pM
TGF-$\beta1_{16-31}$	dimer	TGF-β1, TGF-β2	10-15 μM
	monomer	TGF-β1, TGF-β2	not active
TGF-$\beta1_{16-47}$	dimer	TGF-β1	not active
	monomer	TGF-β1	not active
TGF-$\beta2_{16-31}$	dimer	TGF-β2	30 μM
	monomer	TGF-β2	not active
TGF-$\beta2_{16-47}$	dimer	TGF-β2	3 μM
	monomer	TGF-β2	30 μM

The results obtained indicate that TGF-β1 competed against binding of ^{125}I-TGF-β1, and TGF-β2 competed against binding of ^{125}I-TGF-β2 for L6 rat muscle myoblasts, as previously reported by Segarini et al., J. Biol. Chem., 262:14655-14662 (1987). From the competitive binding data, the apparent dissociation constants were estimated to be 10-30 pM for TGF-β1 and 100-130 pM for TGF-β2. TGF-$\beta1_{16-31}$ and TGF-$\beta2_{16-47}$ peptide monomers, and TGF-$\beta1_{16-47}$ and TGF-$\beta2_{16-31}$ peptide monomers and dimers at concentrations of up to 30 μM were found to be either inactive or weakly active in competing against the binding of ^{125}I-TGF-β1 or ^{125}I-TGF-β2. However, TGF-$\beta2_{16-47}$ dimer competed against the binding of ^{125}I-TGF-β2 with an apparent dissociation constant of approximately 3 μM. TGF-$\beta1_{16-31}$ peptide dimer competed against the binding of ^{125}I-TGF-β1 with an apparent dissociation constant of 10-15 μM.

Example 5 - Localization of TGF-$\beta 2_{16-47}$ peptide activity and the disulfide bonds

To localize the regions of the TGF-$\beta 2_{16-47}$ peptide responsible for biological activity, TGF-$\beta 2_{32-47}$ was synthesized and compared to TGF-$\beta 2_{16-31}$. TGF-$\beta 2_{16-31}$ peptide, the N-terminal half of the TGF-$\beta 2_{16-47}$ peptide, was found to be inactive in the mink lung epithelial cell and thymocyte proliferation assays and in the receptor binding assay, performed as discussed above. The dimeric form of the TGF-β_{32-47} peptide was prepared by forming a disulfide bond at Cys_{44} between the two monomeric peptides.

Table 3

Competition against ^{125}I-TGF-$\beta 2$ in binding to L6 rat muscle myoblast TGF-β receptor

Peptide		ED_{50}
TGF-$\beta 2$		60 pM
TGF-$\beta 2_{32-47}$	dimer	not active
	monomer	not active

As shown in Table 3, the TGF-$\beta 2_{32-47}$ peptide as monomer and as dimer at concentrations up to 30 μM did not compete against the binding of ^{125}I-TGF-$\beta 2$ to the L6 rat muscle myoblasts. Furthermore, as shown in Table 4, the peptide as monomer and dimer at concentrations up to 10 μM did not inhibit the proliferation of mink lung epithelial cells. Since TGF-$\beta 2_{16-31}$ and TGF-$\beta 2_{32-47}$ peptides were not active in these assays, the activity of the TGF-$\beta 2_{16-47}$ peptide resides in the full length peptide.

The TGF-$\beta 2_{16-47}$ peptide contains two cysteine residues, Cys_{16} and Cys_{44}. It has been demonstrated by assaying for the reduced form of cysteine, that the two monomers are linked by a single disulfide bond, rather

than two disulfide bonds involving both Cys_{16} and Cys_{44}. Dimerization could thus occur by a disulfide bond between Cys_{16}-Cys_{16}, Cys_{44}-Cys_{44}, or Cys_{16}-Cys_{44}. Two peptides, TGF-$\beta 2_{16-47}Ser_{16}$ and TGF-$\beta 2_{16-47}Ser_{44}$, in which either Cys_{16} or Cys_{44} had been substituted with serine respectively, were synthesized as described in Example 1. Each of these peptides was able to dimerize to like peptides only by forming Cys_{16}-Cys_{16} or Cys_{44}-Cys_{44} disulfide bonds. The results are shown in Table 4.

Table 4

Activities of TGF-$\beta 2_{32-47}$ peptide and TGF-$\beta 2_{16-47}$ peptides with Cys to Ser substitutions. Inhibition of proliferation of mink lung epithelial cells.

Peptide		ED_{50}
TGF-$\beta 2$		1.6 pM
TGF-$\beta 2_{32-47}$	dimer	not active
	monomer	not active
TGF-$\beta 2_{16-47}Ser_{16}$	dimer	380 nM
	monomer	1.3 μM
TGF-$\beta 2_{16-47}Ser_{44}$	dimer	450 nM
	monomer	not active

The results obtained in Table 4 indicate that TGF-$\beta 2_{16-47}Ser_{16}$ peptide monomer and dimer inhibited the proliferation of mink lung epithelial cells with an ED_{50} of 1.3 μM and 380 nM, respectively. While TGF-$\beta 2_{16-47}Ser_{44}$ peptide dimer inhibited cell proliferation with an ED_{50} of 450 nM, the peptide monomer was inactive at concentrations of up to 10 μM.

Thus, these Ser-substituted peptides are significantly less active than TGF-$\beta 2_{16-47}$ peptide dimer in inhibiting proliferation of mink lung epithelial cells. These results suggest that the peptide dimer with

disulfide bond between Cys_{16} of one peptide and Cys_{44} of the other peptide is the form that is most active in inhibiting the proliferation of mink lung epithelial cells with ED_{50} in the range of 4-14 nM.

Thus, peptides with the biological properties of TGF-β have been disclosed. Although the preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

Claims

1. A peptide dimer wherein a monomer forming the peptide consists of the amino acid sequence CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA or a homolog thereof, wherein the homolog is at least 70% homologous to the amino acid sequence CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA, wherein the monomeric form of the peptide is less than the full length TGF-β2 sequence.

2. A composition comprising the peptide dimer of claim 1 and a pharmacologically acceptable carrier therefor.

3. A peptide dimer wherein the monomeric form of the peptide consists essentially of the amino acid sequence CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA.

4. A composition comprising the peptide dimer of claim 3 and a pharmacologically acceptable carrier therefor.

5. A homodimeric peptide wherein a monomeric form of the peptide consists of the amino acid sequence of CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA or a homolog thereof, wherein the homolog is at least 70% homologous to the amino acid sequence CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA, wherein the monomers are linked via at least one interpeptide disulfide bond between at least one set of cysteine residues.

6. A composition comprising the homodimeric peptide of claim 5 and a pharmacologically acceptable carrier therefor.

7. The homodimeric peptide according to claim 5, wherein the monomeric form of the peptide consists of the amino acid sequence

CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA
|
CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA

wherein the amino terminal cysteine residues are linked via a disulfide bond.

8. A composition comprising the homodimeric peptide of claim 7 and a pharmacologically acceptable carrier therefor.

9. The homodimeric peptide according to claim 5 consisting of the amino acid sequence

CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA
|
CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA

wherein the carboxy terminal cysteine residues are linked via a disulfide bond.

10. A composition comprising the homodimeric peptide of claim 9 and a pharmaceutically acceptable carrier therefor.

11. The homodimeric peptide according to claim 5 consisting of the amino acid sequence

CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA
┌──────────────────────────┘
CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA

wherein the amino terminal cysteine residue of one monomer is linked to the carboxy terminal cysteine residue of the other monomer.

12. A composition comprising the homodimeric peptide of claim 11 and a pharmaceutically acceptable carrier therefor.

13. A peptide consisting of the amino acid sequence CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP or a homolog thereof, wherein the homolog is at least 70% homologous to the amino acid sequence CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP, wherein a monomeric form of the peptide is less than the full length TGF-β1 sequence.

14. A peptide consisting of a dimeric form of a peptide according to claim 13.

15. A composition comprising the peptide of claim 13 or 14 and a pharmacologically acceptable carrier therefor.

16. A peptide consisting essentially of the amino acid sequence CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP.

17. A composition comprising the peptide of claim 16 and a pharmacologically acceptable carrier therefor.

18. A homodimeric peptide wherein a monomeric form of the peptide consists of the amino acid sequence of CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP or a homolog thereof, wherein the homolog is at least 70% homologous to the amino acid sequence CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP, wherein the monomers are linked via at least one interpeptide disulfide bond between at least one set of cysteine residues.

19. A composition comprising the homodimeric peptide of claim 18 and a pharmacologically acceptable carrier therefor.

20. The homodimeric peptide according to claim 18 consisting of the amino acid sequence

CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP
|
CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP

wherein the amino terminal cysteine residues are linked via a disulfide bond.

21. A composition comprising the homodimeric peptide of claim 20 and a pharmacologically acceptable carrier therefor.

22. The homodimeric peptide according to claim 18 consisting of the amino acid sequence

CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP

|

CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP

wherein the carboxy terminal cysteine residues are linked via a disulfide bond.

23. A composition comprising the homodimeric peptide of claim 22 and a pharmacologically acceptable carrier therefor.

24. The homodimeric peptide according to claim 18 consisting of the amino acid sequence

CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP

┌──────────────────────────┘

CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP

wherein the amino terminal cysteine residue of one monomer is linked to the carboxy terminal cysteine residue of the other monomer.

25. A composition comprising the homodimeric peptide of claim 24 and a pharmaceutically acceptable carrier therefor.

26. Use of a peptide or peptide dimer according to any one of claims 1, 3, 5, 7, 9, 11, 13, 14, 16, 18, 20, 22 and 24 for preparation of a medicament to mimic the effects of TGF-βs.

27. Use of a peptide or peptide dimer according to any one of claims 1, 3, 5, 7, 9, 11, 13, 14, 16, 18, 20, 22 and 24, or a composition according to any one of claims 2, 4, 6, 8, 10, 12, 15, 17, 19, 21, 23 and 25 for mimicking the effects of TGF-βs.

28. A pharmaceutical product comprising a container containing a formulation comprising a peptide or peptide dimer according to any one of claims 1, 3, 5, 7, 9, 11, 13, 14, 16, 18, 20, 22 and 24, or a composition according to any one of claims 2, 4, 6, 8, 10,

12, 15, 17, 19, 21, 23 and 25, and instructions associated with the container directing the use of the formulation for mimicking the effects of TGF-βs.

29. A peptide or peptide dimer according to any one of claims 1, 3, 5, 7, 9, 11, 13, 14, 16, 18, 20, 22 and 24, or a composition according to any one of claims 2, 4, 6, 8, 10, 12, 15, 17, 19, 21, 23 and 25, for mimicking the effects of TGF-βs.

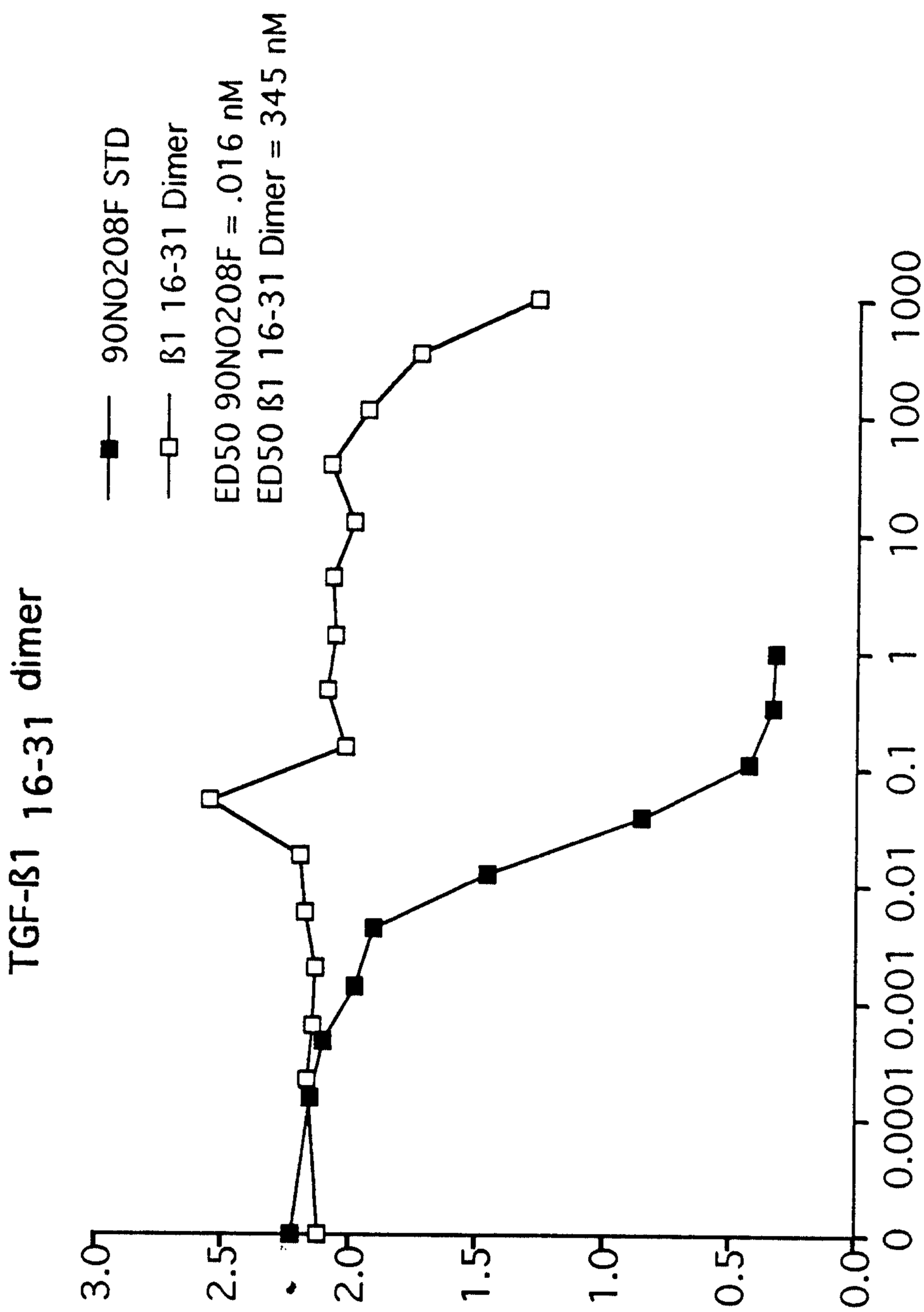
TGF-ß1 16-31 dimer
90NO208F STD
ß1 16-31 Dimer
ED50 90NO208F = .016 nM
ED50 ß1 16-31 Dimer = 345 nM
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
0.0001
0.001
0.01
0.1
1
10
100
1000
Concentration (nM)
Absorbance (405 nm)

Fig. 1a

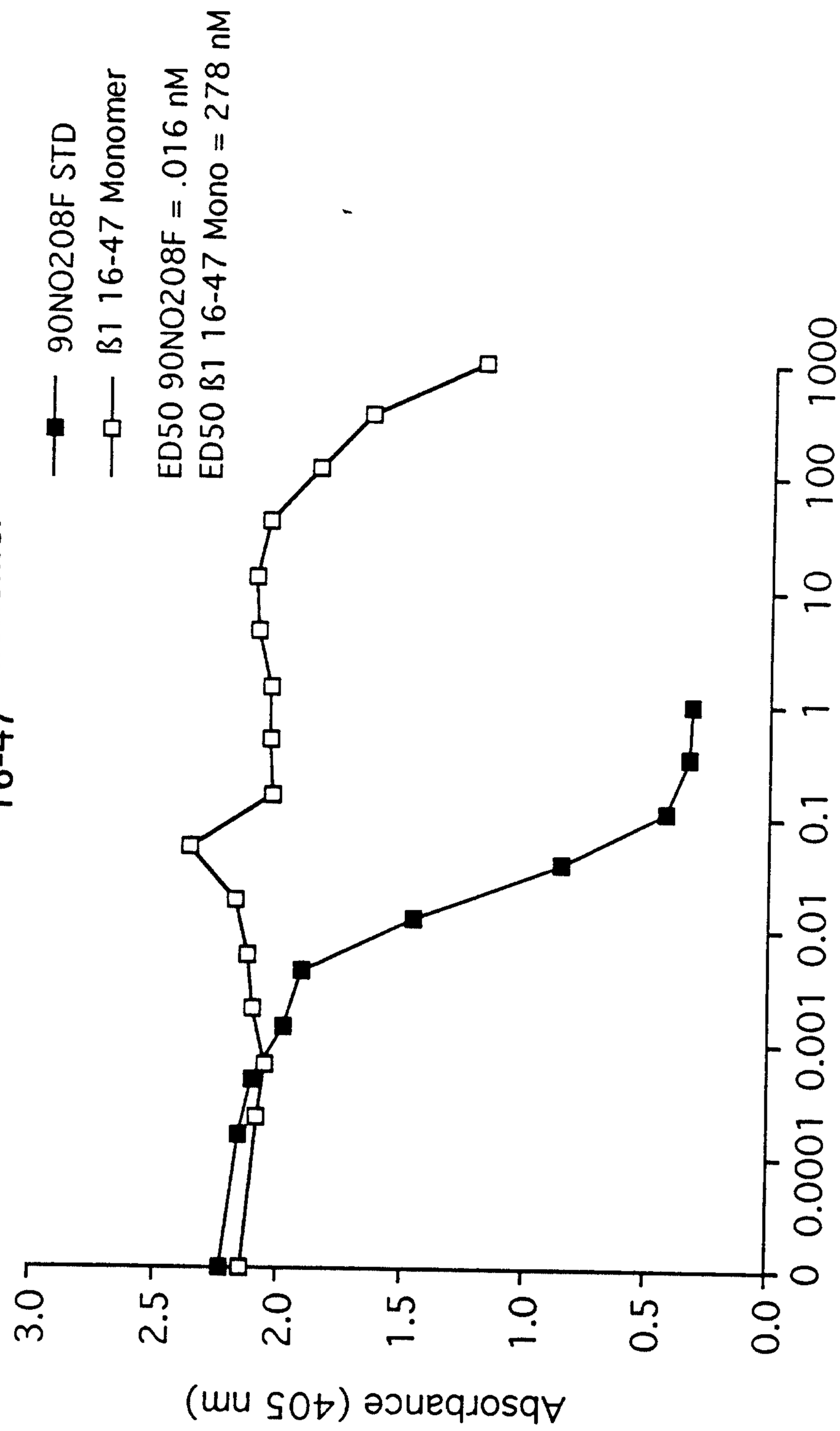
TGF-ß1 16-47 monomer
90NO208F STD
ß1 16-47 Monomer
ED50 90NO208F = .016 nM
ED50 ß1 16-47 Mono = 278 nM
Absorbance (405 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
0.0001
0.001
0.01
0.1
1
10
100
1000
Concentration (nM)

Fig. 1b

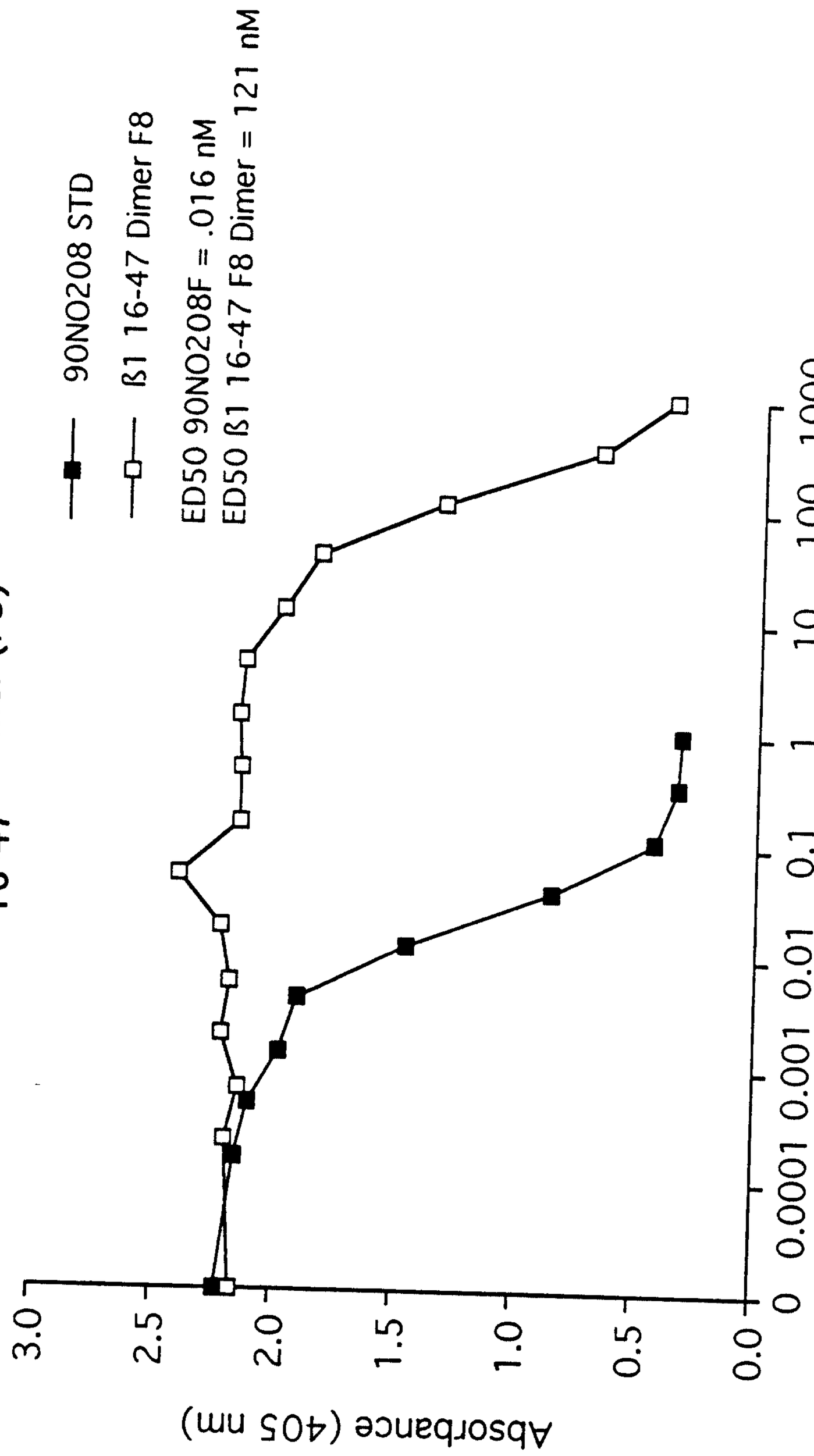
TGF-ß1 16-47 dimer (F8)
90NO208 STD
ß1 16-47 Dimer F8
ED50 90NO208F = .016 nM
ED50 ß1 16-47 F8 Dimer = 1.21 nM
Absorbance (405 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
0.0001
0.001
0.01
0.1
1
10
100
1000
Concentration (nM)

Fig. 1c

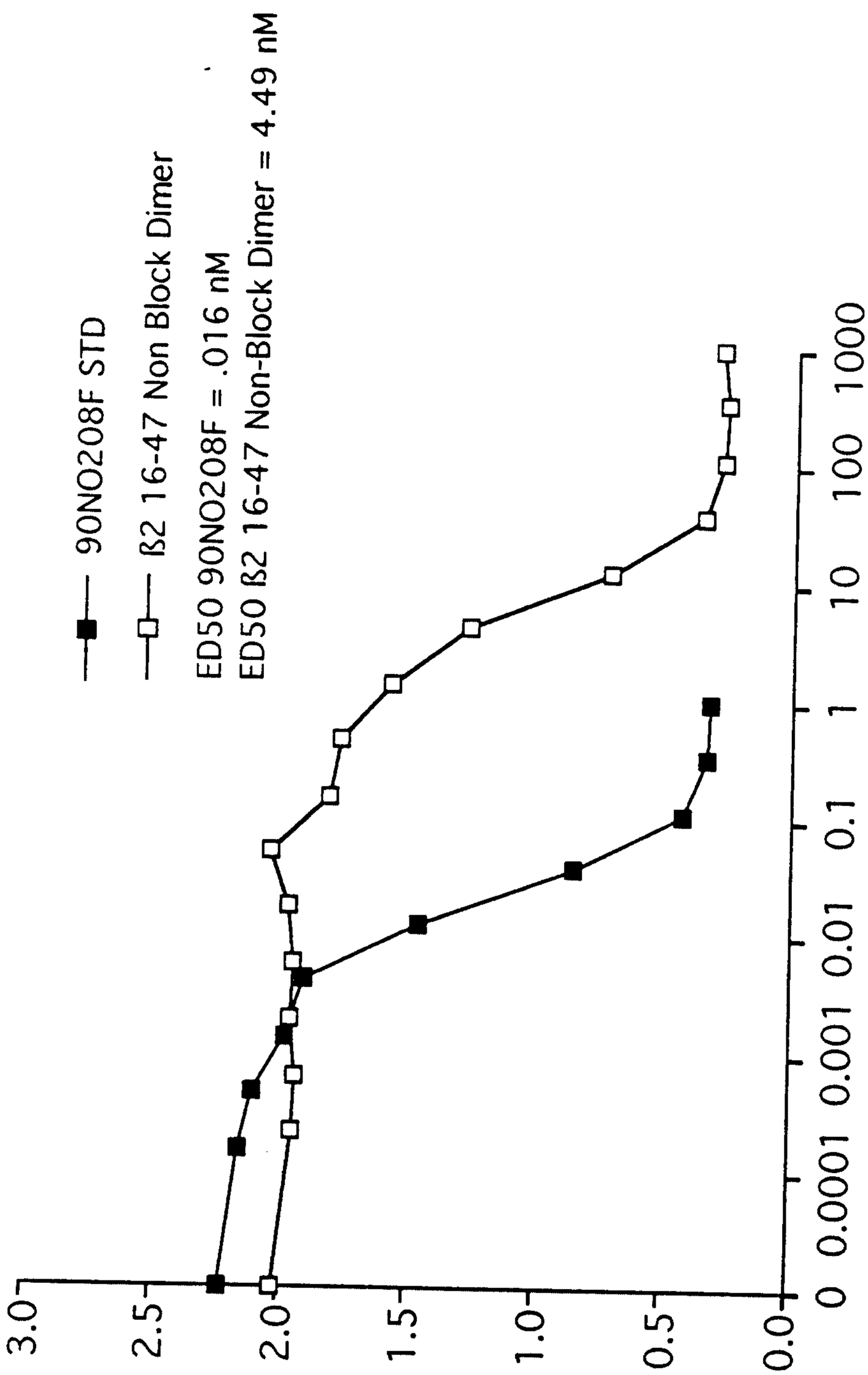
TGF-ß2 16-47 Non-Blocked Dimer
90NO208F STD
ß2 16-47 Non Block Dimer
ED50 90NO208F = .016 nM
ED50 ß2 16-47 Non-Block Dimer = 4.49 nM
Absorbance (405 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
0.0001
0.001
0.01
0.1
1
10
100
1000
Concentration (nM)

Fig. 1d

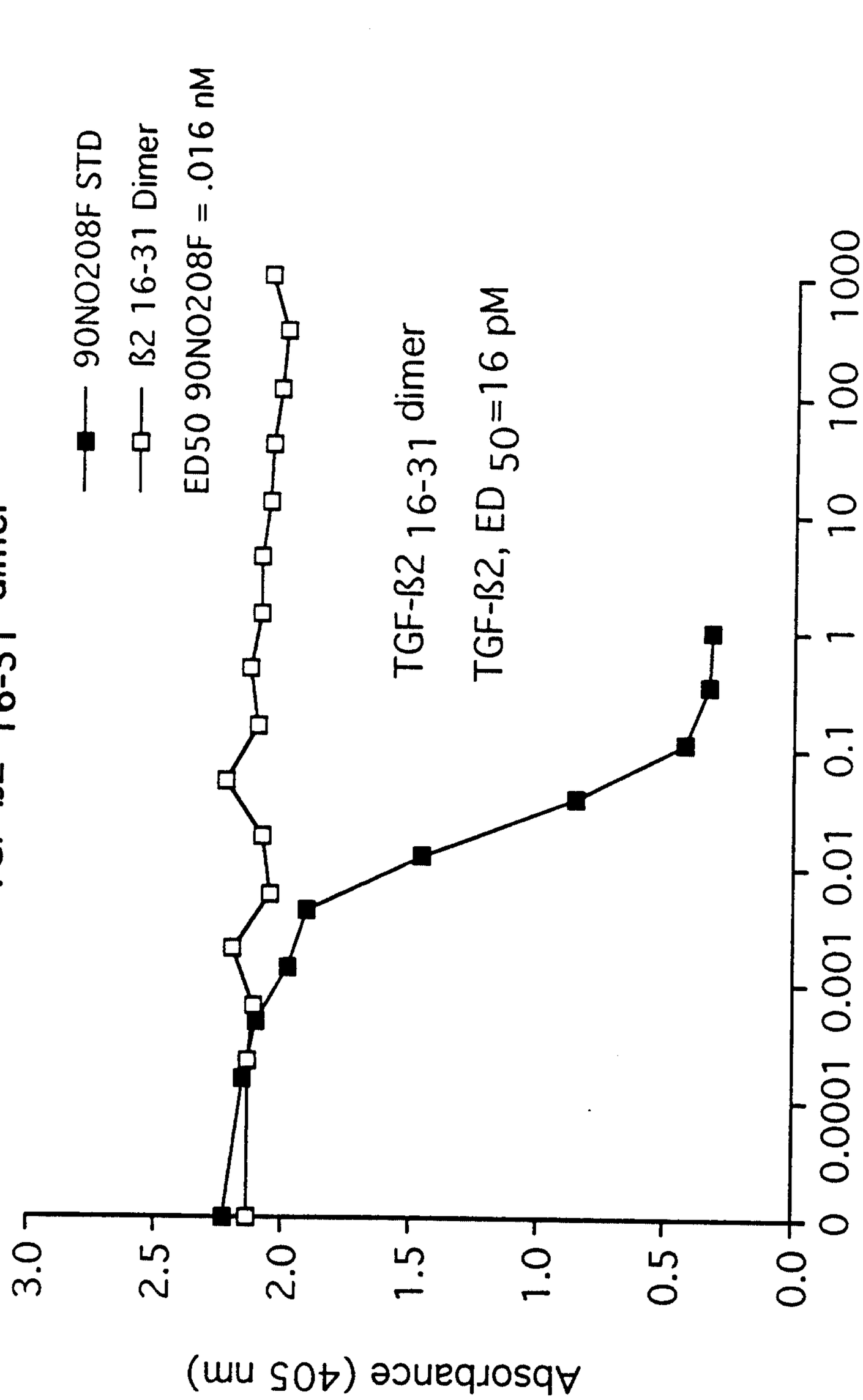
90NO208F STD
ß2 16-31 Dimer
ED50 90NO208F = .016 nM
TGF-ß2 16-31 dimer
TGF-ß2 16-31 dimer, ED 50=16 pM
Concentration (nM)
0 0.0001 0.001 0.01 0.1 1 10 100 1000
Absorbance (405 nm)
0.0 0.5 1.0 1.5 2.0 2.5 3.0

Fig. 1e

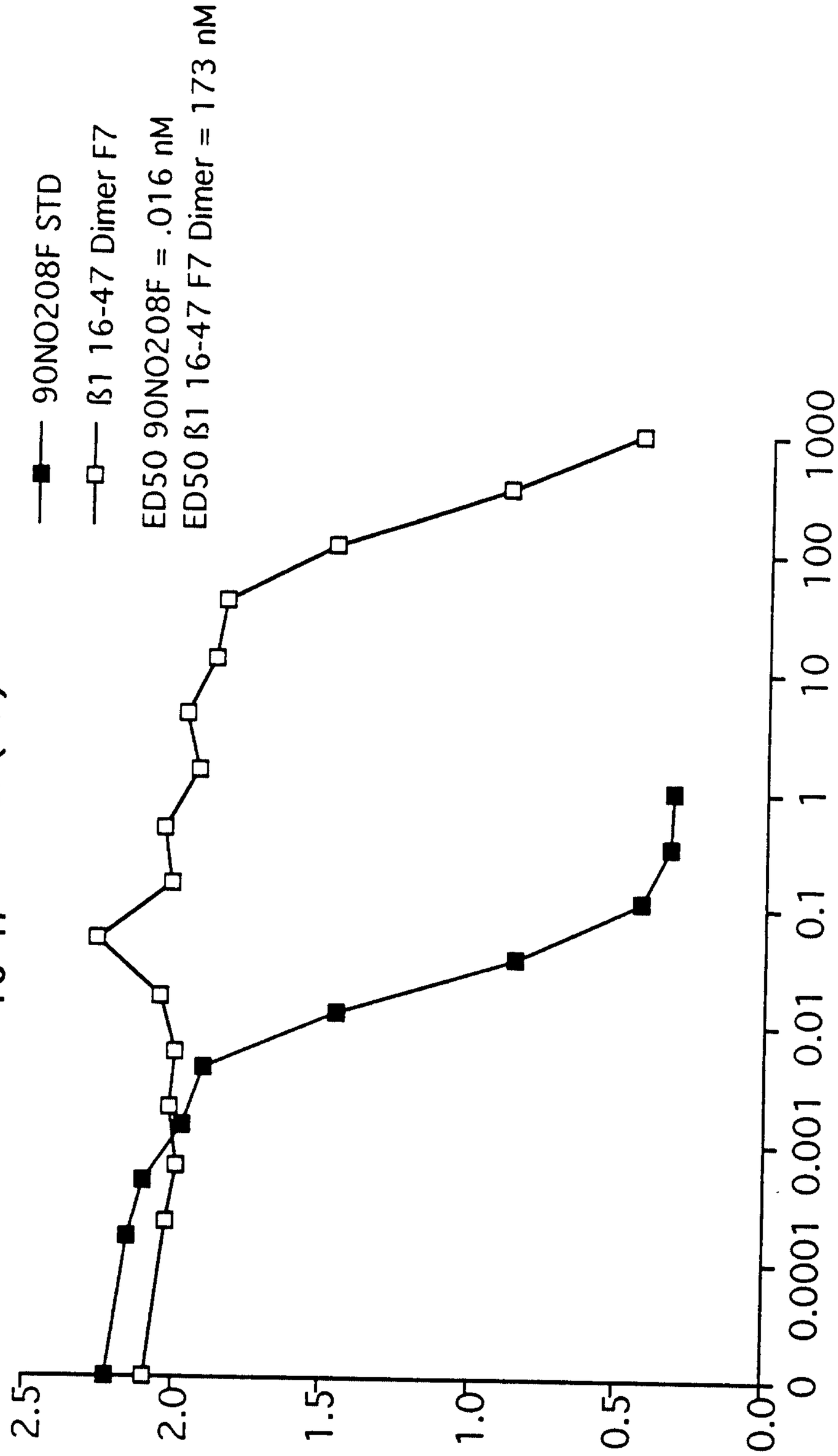
90NO208F STD
ß1 16-47 Dimer F7
ED50 90NO208F = .016 nM
ED50 ß1 16-47 F7 Dimer = .173 nM
TGF-ß1 16-47 dimer (F7)
Concentration (nM)
0.000 0.001 0.01 0.1 1 10 100 1000
0.0 0.5 1.0 1.5 2.0 2.5
Absorbance (405 nm)

Fig. 1f

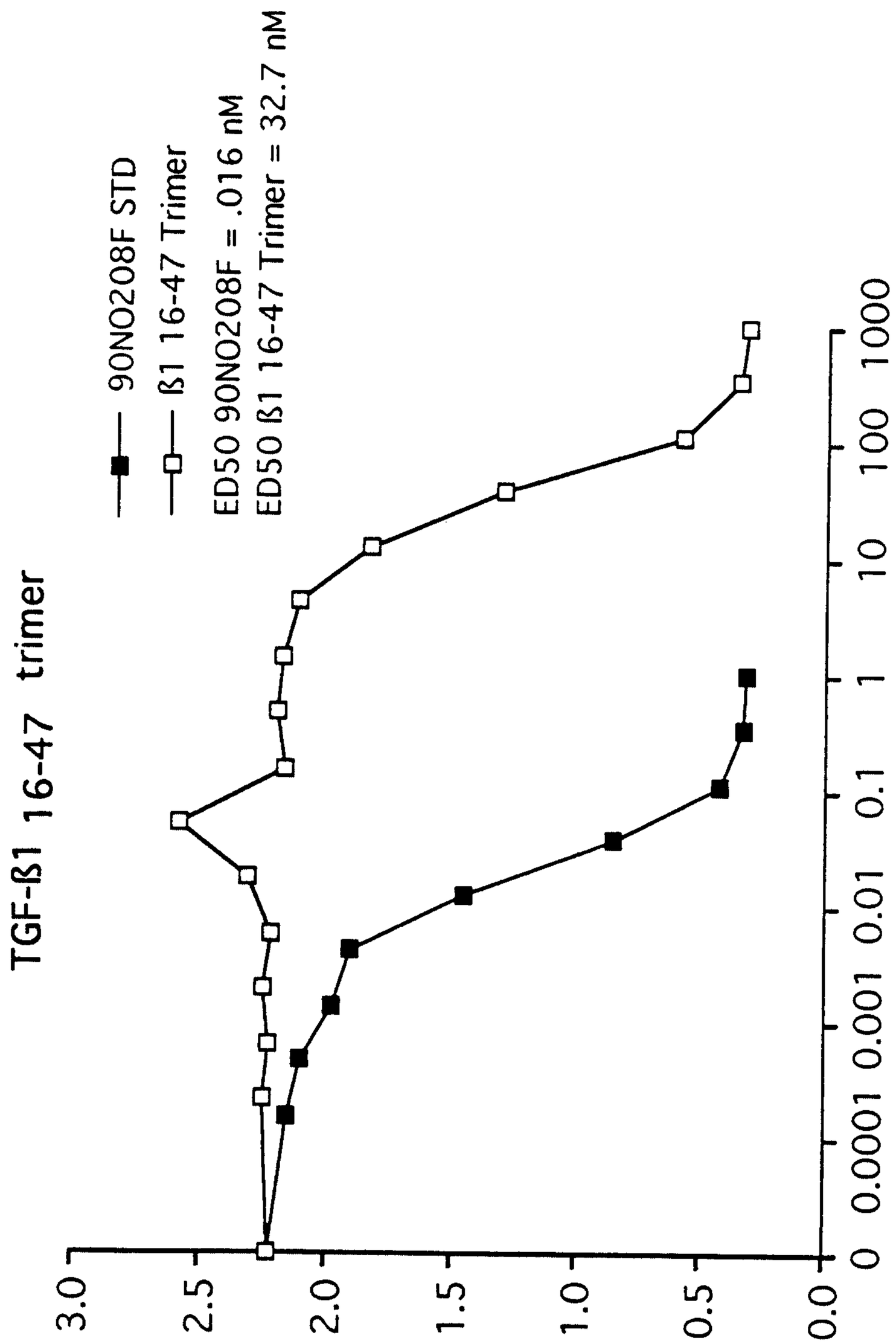
TGF-ß1 16-47 trimer
90NO208F STD
ß1 16-47 Trimer
ED50 90NO208F = .016 nM
ED50 ß1 16-47 Trimer = 32.7
Absorbance (405 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
0.0001
0.001
0.01
0.1
1
10
100
1000
Concentration (nM)

Fig 1g

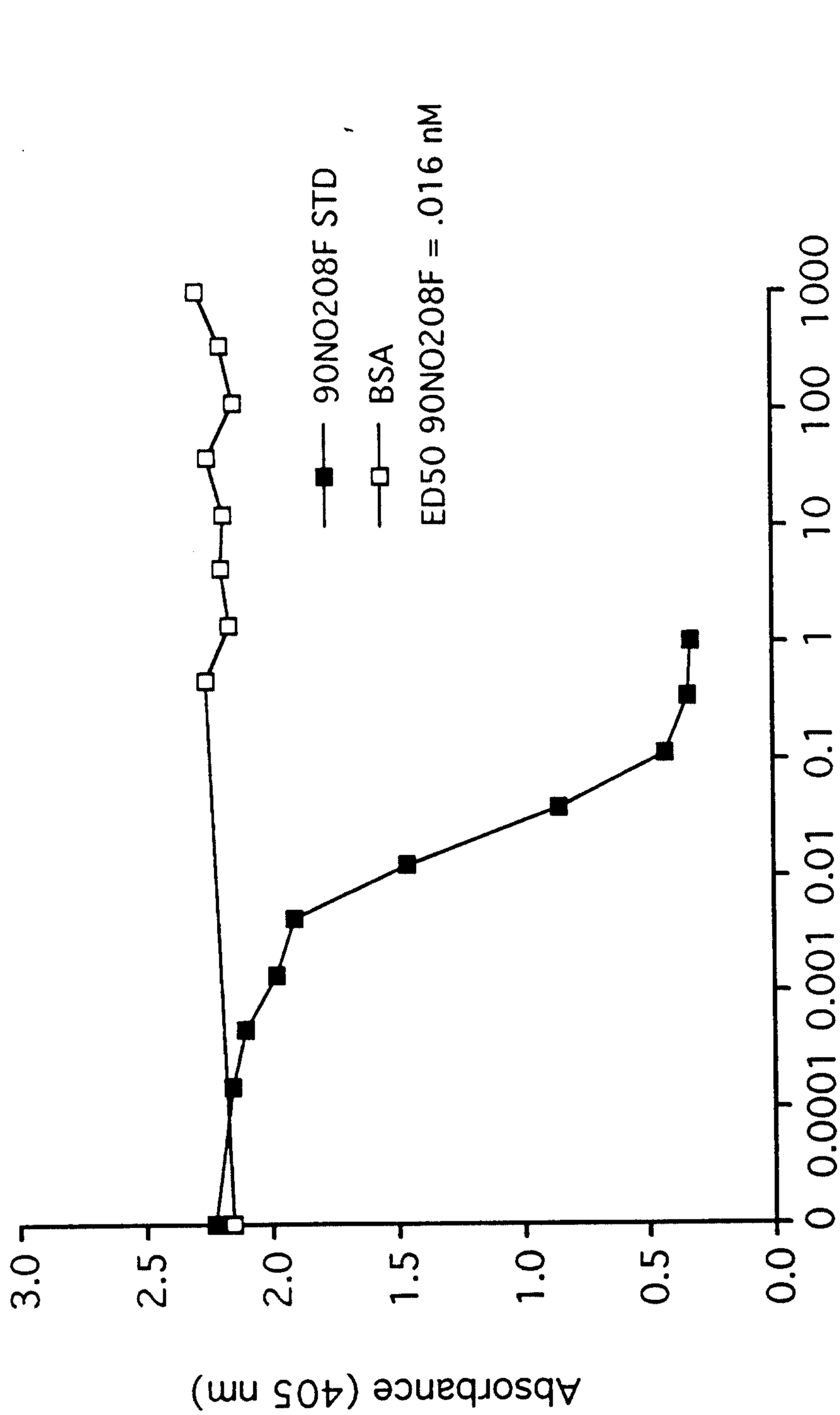
90NO208F STD
BSA
ED50 90NO208F = .016 nM
BSA Control
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
0.0001
0.001
0.01
0.1
1
10
100
1000
Concentration (nM)
Absorbance (405 nm)

Fig. 1h