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(71) Demandeur/Applicant:
PHILOGEN S.P.A., IT

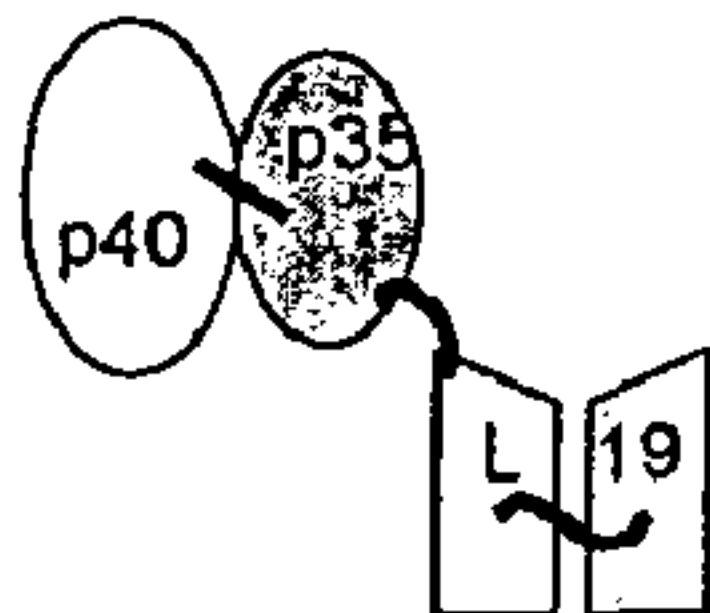
(72) Inventeurs/Inventors:
NERI, DARIO, CH;
GAFNER, VERENA, CH;
HALIN, CORNELIA, CH

(74) Agent: BERESKIN & PARR

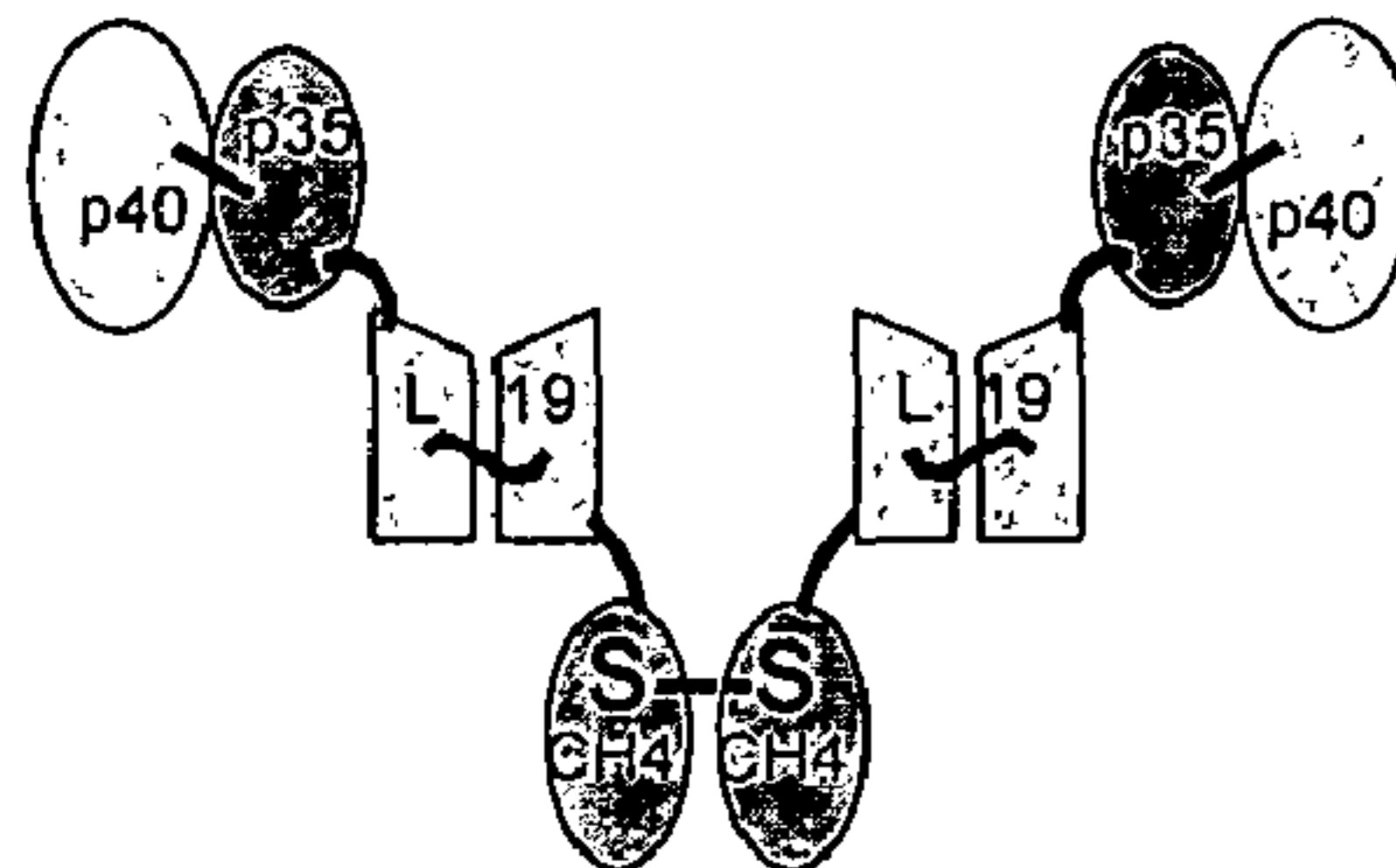
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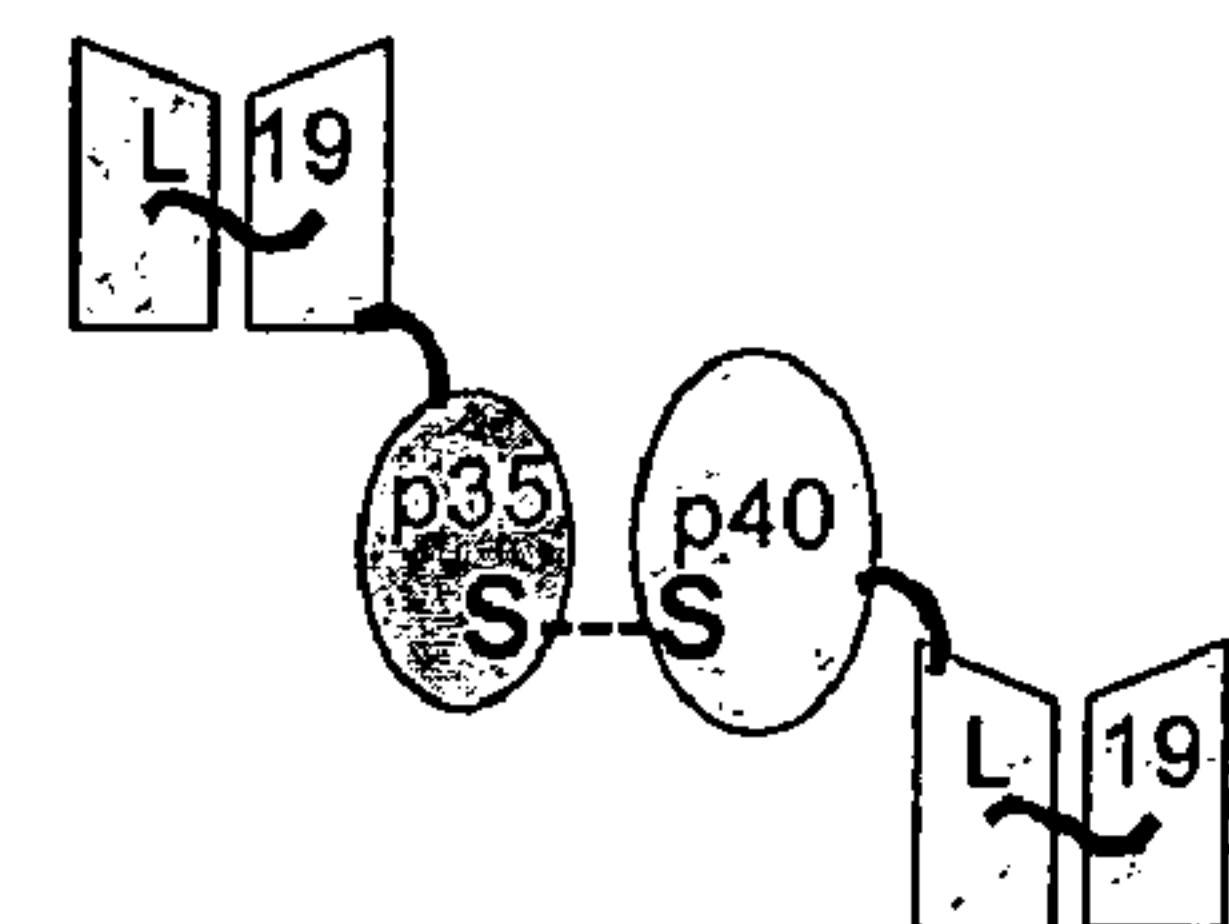
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(57) **Abrégé/Abstract:**

Conjugate for targeting therapeutic or diagnostic agent or drug to cells or tissues in a body, e.g. areas of neoplastic growth or angiogenesis. Use of conjugate for in vivo diagnosis or therapy e.g. inhibiting tumour growth or metastasis, inhibiting angiogenesis and/or treating cancer. Conjugate comprises therapeutic or diagnostic agent as oligomeric protein e.g. heterodimeric protein, wherein first and second subunits of protein are each conjugated to a specific binding member e.g. an antibody fragment such as scFv. Subunits of oligomer may be conjugated to specific binding members as fusion proteins. Conjugate may comprise IL-12 heterodimer having two subunits, each subunit fused to scFv L19 or TN11 for targeting IL-12 to extracellular matrix components associated with neoplastic growth and angiogenesis.



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(71) Applicant (for all designated States except US): **PHILO-GEN S.P.A** [IT/IT]; La Lizza 7, I-53100 Siena (IT).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **NERI, Dario** [IT/CH]; Zihlstrasse 35, CH-8107 Buchs (CH). **GAFNER, Verena** [CH/CH]; Forsthausweg 4, CH-3008 Bern (CH). **HALIN, Cornelia** [CH/CH]; Seestrasse 84, Staefa, CH-8712 Zurich (CH).

(74) Agents: **WALTON, Seán** et al.; Mewburn Ellis LLP, York House, 23 Kingsway, London Greater London WC2B 6HP (GB).

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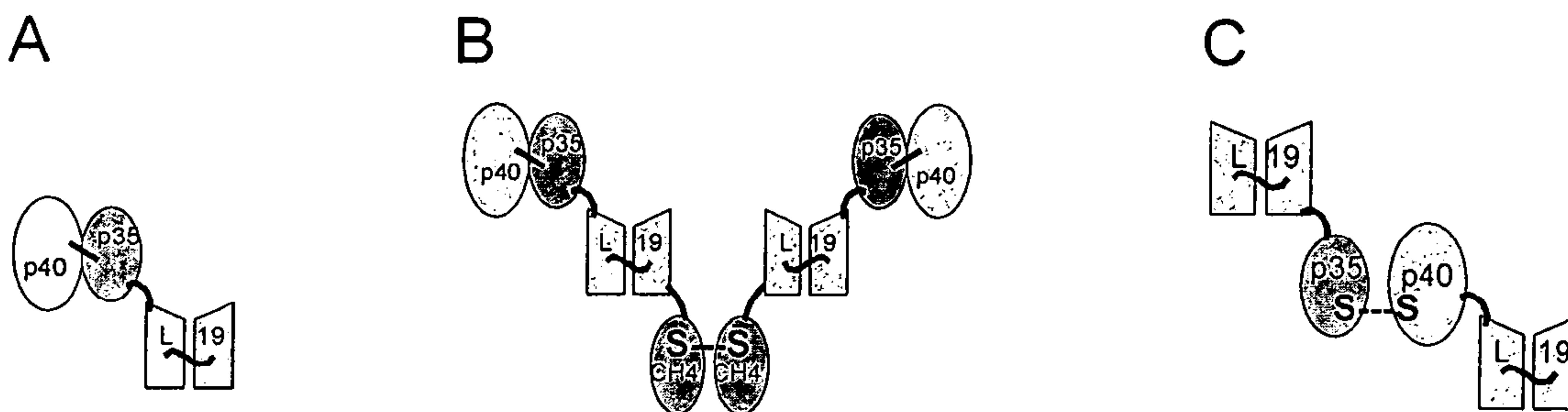
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(54) Title: FUSION PROTEIN OF ANTIBODY L19 AGAINST FIBRONECTIN ED-B AND INTERLEUKIN 12



(57) Abstract: Conjugate for targeting therapeutic or diagnostic agent or drug to cells or tissues in a body, e.g. areas of neoplastic growth or angiogenesis. Use of conjugate for in vivo diagnosis or therapy e.g. inhibiting tumour growth or metastasis, inhibiting angiogenesis and/or treating cancer. Conjugate comprises therapeutic or diagnostic agent as oligomeric protein e.g. heterodimeric protein, wherein first and second subunits of protein are each conjugated to a specific binding member e.g. an antibody fragment such as scFv. Subunits of oligomer may be conjugated to specific binding members as fusion proteins. Conjugate may comprise IL-12 heterodimer having two subunits, each subunit fused to scFv L19 or TN11 for targeting IL-12 to extracellular matrix components associated with neoplastic growth and angiogenesis.

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CONJUGATE FOR TARGETING OF DRUG

This invention relates to targeting of a drug to a desired *in vivo* site by conjugation to a target-specific specific binding member. The invention is especially directed to conjugates between IL12 and antibody molecules for therapeutic applications such as inhibition of pathological angiogenesis, including treatment of cancer and other tumours, rheumatoid arthritis, diabetic retinopathy, age-related muscular degeneration and angiomas.

The heterodimeric cytokine interleukin-12 (IL12) is a key mediator of innate and cellular immunity with potent antitumor and antimetastatic activity [4-6]. It is currently (Spring 2005) being tested in Phase II clinical trials for the treatment of cancer and infectious diseases. IL12 acts primarily on T and NK cells, stimulating their activity and the secretion of interferon- γ (IFN- γ) [7]. As with many other cytokines, however, the administration of recombinant human IL12 is associated with severe toxicity, even at doses as low as 1 μ g/kg/day [8, 9], hampering its development as an anti-cancer drug.

A targeted delivery of IL12 to the tumour environment may be used to increase the therapeutic index of the cytokine.

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Scientists at Lexigen have described the fusion of cytokines such as IL2 and IL12 to immunoglobulins in order to specifically target the cytokine [17, 49-51]. However, we, the present inventors, believe that there are limitations to such approaches. We recognise that IgG-cytokine fusions are in reality multi-functional proteins, and so in addition to antigen binding and cytokine activity, these IgG-based fusion proteins can activate complement and interact with Fc receptors. In our opinion this is an undesired property of IgG-cytokine fusions, as cytokines may be brought in proximity of cells (such as macrophage, neutrophils and natural-killer cells) carrying Fc receptors, thus hindering tumour targeting and causing non-specific cell activation. We consider it more desirable to use an antibody molecule lacking Fc, such as a single-chain Fv antibody fragment (scFv), instead of the full immunoglobulin.

30

We have previously demonstrated in rodent models of cancer that the therapeutic potential of IL12 can be considerably increased by fusing a single-chain polypeptide which sequentially encodes the subunits p40 and p35 of the murine cytokine IL12

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("scL12") with the human single-chain Fv antibody fragment L19 ("scFv(L19)"). ScFv(L19) has been shown to be capable of selective tumour targeting in patients with cancer [16]. L19 specifically binds the ED-B domain of fibronectin isoform B-FN, which is one of the best known markers angiogenesis [14, 25]. ED-B is an extra
5 domain of 91 amino acids found in the B-FN isoform and is identical in mouse, rat, rabbit, dog and man. B-FN accumulates around neovascular structures in aggressive tumours and other tissues undergoing angiogenesis, such as the endometrium in the proliferative phase and some ocular structures in pathological conditions, but is otherwise undetectable in normal adult tissues [1-3].

10

The fusion protein scL12-scFv(L19) exhibited a therapeutic index far superior to scL12 fused to a scFv of irrelevant specificity in mouse, and to recombinant murine IL12. These experiments clearly demonstrated the potential of antibody-based cytokine fusion proteins as an avenue for improving the therapeutic potential of IL12
15 [15]. The therapeutic potential of these results is considerable.

20

Targeting of IL12 at the level of tumour blood vessels, such as using L19, is therapeutically beneficial for a number of reasons. First, the tumour neovasculature is more accessible to intravenously-administered therapeutic agents than are tumour
20 cells, which helps avoid problems associated with the interstitial hypertension of solid tumours [10]. Second, angiogenesis (the growth of new capillaries from pre-existing blood vessels) is a characteristic feature of the majority of aggressive solid tumours [11]. Targeting IL12 to the neovasculature should allow the immunotherapy of a variety of different tumour types. Third, IL12 shows an anti-angiogenic activity,
25 conferred by its down-stream mediator IP-10 [12, 13].

30

We have already extensively studied the tumour targeting performance of scL12-scFv(L19), and also that of scFv(L19) fused to another anti-tumour cytokine, IL2 ("scFv(L19)-IL2") [18]. ScFv(L19)-IL2 displays an excellent tumour-targeting
30 performance in tumour-bearing mice, with tumour:blood and tumour:organ ratios as high as 30 : 1 twenty-four hours after intravenous injection. By contrast, the tumour-targeting ability of scL12-scFv(L19) in the same animal models is more modest, with tumour:blood and tumour:organ ratios generally worse than 10:1 at 24h, and with poor tumour:liver and tumour:spleen ratios [15]. These targeting results were
35 nevertheless superior compared to a fusion protein scL12-scFv(HyHEL10), specific to hen egg lysozyme but devoid of antigen-specific recognition in the mouse.

The tumour targeting properties of scFv(L19) have been shown to be improved when scFv(L19) was dimerised through a CH4 domain of human IgE, building a mini-antibody structure, also termed "small immune protein" or SIP. The tumour targeting properties of SIP(L19) have been previously described [21].

The present invention is based on work in which we, the inventors, compared tumour-targeting abilities of three conjugates of IL12 and scFv(L19) each having a different format of the cytokine and/or of the antibody, and found that the tumour-targeting ability of scIL12-scFv(L19) could be improved by changing the format of the conjugate in a particular way.

One format we tested was scIL12-scFv(L19), illustrated in Figure 1A. This conjugate showed modest tumour-targeting ability, consistent with the prior art findings.

Another format utilised the dimeric SIP(L19) construct mentioned above to create a homodimer of scIL12-SIP(L19), illustrated in Figure 1B. However, despite the prior art indication that tumour-targeting properties of L19 could be improved using the SIP format, we did not observe an increased tumour uptake of this conjugate.

Another format was a heterodimer of IL12 p40 and p35 subunits, in which each subunit was fused to scFv(L19), forming a scFv(L19)-p35/p40-scFv(L19) heterodimer as illustrated in Figure 1C. With this heterodimeric format we achieved a marked improvement in tumour uptake of the conjugate.

Thus, we have discovered that a new antibody-IL12 fusion protein format, consisting of two scFv fragments heterodimerised via the p40 and p35 subunits of IL12, retains full IL12 activity and displays an excellent tumour-targeting ability.

These results have significant therapeutic implications for improved targeting of IL12 to tumours and to other sites of pathological angiogenesis, e.g. for treating rheumatoid arthritis, diabetic retinopathy, age-related muscular degeneration and angiomas. The usefulness of this invention extends not only to the fusion of IL12 to scFv(L19), but also to conjugates between other specific binding members and other drugs and substances. For example, conjugates may be constructed with specific binding members other than scFv(L19) conjugated to IL12 subunits, such as other

antibody fragments specific for tumour-associated antigens e.g. isoforms of tenascin-C, and used for tumour targeting and cancer therapy. The wider implications also include a variety of other applications involving targeting of substances *in vivo*, including diagnostic methods as well as the prevention and treatment of diseases and other pathological conditions.

The invention in various aspects relates to the new conjugates, methods of producing them, nucleic acids encoding the conjugates or components thereof, pharmaceutical compositions containing the conjugates, and use of the conjugates in methods of treatment.

In one aspect, the invention is a conjugate comprising a protein having first and second subunits, wherein the first and second subunits are each conjugated to a specific binding member.

The protein is generally dimeric, preferably heterodimeric, and typically comprises a biologically active agent or drug, normally a therapeutic or diagnostic agent.

Thus, the conjugate normally has the following format:

[specific binding member] - [first subunit] - [second subunit] - [specific binding member]

The specific binding members are normally antibody molecules, preferably single chain Fv (scFv). Single chain Fv (scFv) antibody molecules are particularly preferred in the present invention owing to their small size, which provides physiological and therapeutic advantages for *in vivo* use of the conjugates. In addition, scFv lack an Fc region, potentially reducing anti-idiotypic reactions and also minimising undesirable properties relating to activation of complement and interaction with Fc receptors that may hinder tumour targeting and cause non-specific cell activation.

Thus, a preferred format of the conjugate is:

[ScFv] - [first subunit] - [second subunit] - [ScFv]

Alternatively, the specific binding member may be a single domain antibody, and/or an antibody fragment. Specific binding members and antibody molecules are described in more detail below.

- 5 The conjugate may be or may comprise a fusion protein, i.e. a polypeptide that is a translation product resulting from the fusion of two or more genes or nucleic acid coding sequences into one open reading frame (ORF). The fused expression products of the two genes or ORFs may be conjugated by a peptide linker (a short 2 - 20, preferably 2 - 15, residue stretch of amino acids). In one embodiment the peptide
10 linker is a 6-residue sequence GSADGG (SEQ ID NO: 15). The fusion protein may comprise signal peptide sequence, normally located upstream (5') of the specific binding member and subunit.

In conjugates of the invention, the first and second subunits are generally linked, for
15 instance they may be covalently linked, e.g. through one or more disulphide bonds. The protein may be oligomeric (e.g. dimeric, preferably heterodimeric), and may occur naturally in oligomeric form, and thus the first and second subunits in the conjugate may associate with each other in their natural way. The invention therefore allows use and maintenance of a natural format of the protein in the
20 conjugate. This avoids any need to construct or use a single-chain variant of the drug and maximises the potential for the drug to retain its full activity in the conjugate. In addition, conjugation between the subunits allows the conjugate to be conveniently constructed and assembled by allowing the first and second subunits to associate (e.g. dimerise) in the oligomer. Thus, typically the first and second subunits may
25 each be conjugated to a specific binding member, forming a pair of specific binding member-subunit constructs (e.g. heterodimers) that associate through the subunits. In a preferred embodiment, the first subunit is conjugated to a specific binding member as a first fusion protein, and the second subunit is conjugated to a specific binding member as a second fusion protein.

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Preferably, the conjugate has a molecular weight of 250 kDa or less, more preferably 200 kDa, 150 kDa, 125 kDa, 120 kDa or 115 kDa or less (i.e. an Mr of or less than 250 000, 200 000, 150 000, 125 000, 120 000 or 115 000). This may be the actual measured molecular weight (with or without glycosylation), or an estimated value
35 based on e.g. the expected molecular weight of the conjugate (with or, normally, without) glycosylation. A relatively small size of the conjugate increases its ability to

penetrate tissues and access the target site (e.g. site of angiogenesis, tumour or disease), thus increasing its therapeutic efficacy and reducing the dosage required, while still achieving a multivalent (generally bivalent) binding of the conjugate to the target.

5

In general, the protein and the specific binding member are selected according to the intended use of the conjugate. In a preferred embodiment, the protein is IL12. As discussed above, IL12 is suitable for treating cancer, inhibiting tumour growth and metastasis, and for treating other conditions associated with pathological angiogenesis. One or both specific binding members in a conjugate may specifically bind a marker associated with neoplastic (especially tumour) growth and/or angiogenesis, e.g. a marker located at the site of neoplastic growth and/or angiogenesis. Extracellular matrix components may be markers of neoplastic growth and/or angiogenesis, because the extracellular matrix is remodelled during these processes.

15

One example is the B-FN isoform of fibronectin which, as explained above, contains an extra domain ED-B. A specific binding member of the invention preferably binds specifically to ED-B of fibronectin isoform B-FN. The specific binding member may have an amino acid sequence comprising the VH CDR1 (SEQ ID NO: 25), VH CDR2 (SEQ ID NO: 26) and/or VH CDR3 (SEQ ID NO: 27) sequences of L19 and/or the VL CDR1 (SEQ ID NO: 28), VL CDR2 (SEQ ID NO: 29) and/or VL CDR 3 (SEQ ID NO: 30) sequences of L19. For example, the specific binding member may be an scFv having a VH domain with an amino acid sequence comprising VH CDR1, VH CDR2 and/or VH CDR3 of L19, and a VL domain with an amino acid sequence comprising VL CDR1, VL CDR2 and/or VL CDR3 of L19. A specific binding member may comprise a VH domain having an amino acid sequence with at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% sequence identity with the amino acid sequence of the L19 VH domain as set out in SEQ ID NO: 22, and/or comprises a VL domain having an amino acid sequence with at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% sequence identity with the amino acid sequence of the L19 VL domain as set out in SEQ ID NO: 23. Preferably the specific binding member is an scFv(L19) comprising an L19 VH domain (SEQ ID NO: 22) and an L19 VL domain (SEQ ID NO: 23). In a preferred embodiment, the specific binding member is scFv(L19) having the amino acid sequence SEQ ID NO: 5 (Figure 10).

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Another example is tenascin-C (TnC), which exists in various isoforms. In neoplastic tissues TnC containing additional domains are more widely expressed than in normal tissues, especially isoforms containing domain C (cTN-C) [33]. Thus, a specific binding member in conjugates of the invention may specifically bind tenascin-C isoforms associated with neoplastic tissues, especially cTN-C. The specific binding member may be TN11 scFv having a sequence as set out in SEQ ID NO: 21 (Figure 11) [33].

The specific binding member in conjugates of the invention may alternatively bind other tumour-associated antigens, i.e. antigens more prevalent in a tumour environment (such as on a tumour cell) than in a normal cellular environment (such as on non-tumour cells).

Normally, the two specific binding members in a conjugate are identical, or are at least both specific for the same target, antigen or epitope.

In a particularly preferred embodiment of the invention, the conjugate comprises a human IL12 heterodimer conjugated to and between two specific binding members, preferably scFv(L19); wherein

the heterodimer has a first (normally p40) subunit and a second (normally p35) subunit;

the first or p40 subunit is conjugated to a first specific binding member as a first fusion protein;

the second or p35 subunit is conjugated to a second specific binding member as a second fusion protein.

As noted above, the first and second subunits are typically covalently linked e.g. disulphide bonded.

Preferably, the first or p40 subunit is fused N-terminal to the specific binding member, i.e. the subunit is upstream of the specific binding member in the fusion protein and in the nucleic acid encoding it. In this form, the N-terminus of p40 can therefore be free (unfused), which is believed to maximise its activity.

Preferably, the second or p35 subunit is fused C-terminal to the specific binding member, i.e. the subunit is downstream of the specific binding member in the fusion

protein and in the nucleic acid encoding it. This may enhance expression of the fusion protein, because the specific binding member, especially with an upstream N-terminal signal peptide, may be easily expressed and thus allow efficient expression of the fusion protein.

5

Preferably, the first fusion protein has the amino acid sequence of p40-scFv(L19) as shown in SEQ ID NO: 1. Preferably, the second fusion protein has the amino acid of scFv(L19)-p35 as shown in SEQ ID NO: 2.

- 10 Conjugates of the invention may be produced in any available method, e.g. using recombinant techniques, for example by expressing all or part of the conjugate as a fusion protein.

For example, a conjugate may be produced in a method comprising:

- 15 expressing a first fusion protein comprising the first subunit and a specific binding member;
 expressing a second fusion protein comprising the second subunit and a specific binding member; and
 conjugating the first and second subunits together.

20

Normally the method comprises purifying the first and second fusion proteins after expression.

- 25 Normally the expression is performed in a host cell containing nucleic acid encoding the fusion protein, e.g. a cultured eukaryotic cell such as HEK or a CHO cell, or a bacterial cell such as *Escherichia coli*. Expression may therefore comprise culturing such a host cell. Where the first and second fusion proteins are expressed in the same cell (e.g. a cell co-transfected with or containing nucleic acids encoding the two fusion proteins), heterodimerisation or oligomerisation of the subunits may occur in
30 the cell or during purification of the fusion proteins from the cell. In other cases, the first and second fusion proteins may be expressed separately (e.g. in different cells) and then brought together (combined) such that the first and second subunits heterodimerise or otherwise associate.

- 35 Conjugating the subunits together may be an active or passive process. Conjugating may comprise exposing or subjecting the first and second fusion proteins to

conditions in which the first and second subunits are conjugated together (e.g. associate or oligomerise/heterodimerise). Conjugating may comprise disulphide bond formation between the subunits, or formation of another covalent linkage. Conjugation such as disulphide bond formation may occur under non-reducing
5 conditions, and therefore conjugating may comprise exposing the first and second fusion proteins to non-reducing conditions.

Suitable methods for expressing fusion proteins and producing conjugates according to the invention are given in detail in the Examples below.

10

As a further step, the method may comprise formulating the conjugate into a pharmaceutical composition. Generally this involves purifying the conjugate and combining it with a physiologically acceptable carrier. Pharmaceutical compositions are described in more detail below.

15

Nucleic acid molecules encoding the conjugates and parts thereof (e.g. encoding fusion proteins) also form part of the invention.

In one aspect the invention is a composition comprising

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a first nucleic acid molecule comprising a nucleotide sequence encoding a fusion protein, wherein the fusion protein comprises a specific binding member and a first protein subunit; and

a second nucleic acid molecule comprising a nucleotide sequence encoding a fusion protein, wherein the fusion protein comprises a specific binding member and a
25 second protein subunit.

30

The nucleic acid molecule may encode a peptide linker between the specific binding member and the subunit, such that in the fusion protein the specific binding member and subunit are joined by the peptide linker. Particular fusion proteins, subunits, specific binding member and linkers that may be encoded are described in more detail elsewhere herein. In a preferred embodiment the first and second subunits are IL12 heterodimeric subunits (typically p40 and p35 subunits), respectively, and preferably the specific binding member is an scFv, especially scFv(L19). In one embodiment the first nucleic acid molecule encodes the amino acid sequence of
35 IL12p40-scFv(L19) as set out in SEQ ID NO: 1 and/or the second nucleic acid

molecule encodes the amino acid sequence of scFv(L19)-IL12p35 as set out in SEQ ID NO: 2.

5 The nucleic acid molecule may be a vector, e.g. a plasmid suitable for expression of the nucleotide sequence. Thus, the first and second nucleic acid molecules may be first and second vectors. Normally the nucleotide sequence is operably linked to a regulatory element such as a promoter for transcription.

10 The first and second nucleic acid molecules may be contained in a host cell, which may be a cell co-transfected with the nucleic acid molecules or a daughter of such a cell. Cells, especially eukaryotic cells e.g. HEK and CHO cells, or bacterial cells e.g. *Escherichia coli*, containing the nucleic acid molecules also form part of the invention.

15 After expression from the nucleic acids, the fusion proteins may be conjugated through the subunits to form conjugates of the invention, as described elsewhere herein.

20 Conjugates according to the invention may be used in a method of treatment of the human or animal body, such as a method of treatment (which may include prophylactic treatment) of a disease or disorder in a patient (typically a human patient) comprising administering the conjugate to the patient.

25 Conditions treatable using the conjugates include cancer, other tumours and neoplastic conditions. The conjugates may be used to inhibit angiogenesis and thereby treat rheumatoid arthritis, diabetic retinopathy, age-related muscular degeneration, angiomas and tumours, including cancer. Treatment may include prophylactic treatment. The conjugates may also be administered in diagnostic methods, e.g. targeting and diagnosis of angiogenesis, which may be associated with
30 any of the above conditions. Other diseases and conditions may also be diagnosed and treated, according to the nature of the protein therapeutic or diagnostic agent contained in the conjugate, and the specificity of the specific binding member.

Accordingly, further aspects of the invention provide methods of treatment
35 comprising administering a conjugate of the invention, pharmaceutical compositions comprising such a conjugate, and use of such a conjugate in the manufacture of a

medicament for treatment of a condition or disease, for example in a method of making a medicament or pharmaceutical composition comprising formulating the conjugate with a physiologically acceptable carrier or excipient.

- 5 In accordance with the invention, compositions provided may be administered to a patient. Administration is preferably in a "therapeutically effective amount", this being sufficient to show benefit to the patient. Such benefit may be at least amelioration of at least one symptom. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated.
- 10 Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors. Appropriate doses of antibody are well known in the art [26, 27].

- 15 Conjugates of the invention, including those comprising an antibody antigen-binding domain, may be administered to a patient in need of treatment via any suitable route, usually by injection into the bloodstream and/or directly into the site to be treated, e.g. tumour or tumour vasculature. The precise dose and its frequency of administration will depend upon a number of factors, the route of treatment, the size and location of the area to be treated (e.g. tumour), the precise nature of the antibody
- 20 (e.g. scFv molecule), and the nature of any detectable label or other molecule contained in the conjugate.

- A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.
- 25 Other treatments may include the administration of suitable doses of pain relief drugs such as non-steroidal anti-inflammatory drugs (e.g. aspirin, paracetamol, ibuprofen or ketoprofen) or opiates such as morphine, or anti-emetics.

- Thus pharmaceutical compositions according to the present invention, and for use in
- 30 accordance with the present invention, may comprise, in addition to active ingredient (conjugate), a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration,
- 35 which may be oral, or by injection, e.g. intravenous. For intravenous injection, or injection at the site of affliction, the active ingredient will be in the form of a

parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability.

In conjugates of the invention, the protein normally comprises recombinantly produced first and second polypeptide subunits. The subunits may be glycosylated, and the degree and nature of such glycosylation may be controlled by selection of an appropriate host cell in which to express the subunits. The protein may comprise a biologically active agent, i.e. an agent that affects the structure or functioning of a subject organism to which it is administered. Normally the protein comprises a diagnostic or therapeutic agent. For example, it may comprise a substance used in diagnosis, prevention or treatment of a disease or pathological condition. The protein may comprise a marker or labelling agent e.g. for diagnosis. In the context of this invention the protein normally comprises a therapeutic substance for treating or preventing a pathological condition, especially angiogenesis, for example in treatment of cancer and other tumours, rheumatoid arthritis, diabetic retinopathy, age-related muscular degeneration and angiomas. It may for example comprise a toxin, enzyme or a mediator of immunity such as a cytokine.

The protein may be native or recombinant interleukin-12 (IL12). IL12 useful in the invention may be derived from any animal, e.g. human, rodent (e.g. rat, mouse), horse, cow, pig, sheep, dog, etc.. Human IL12 is preferred in conjugates for administration to humans. IL12 occurs naturally as a heterodimeric protein composed of a 40 kDa (p40) subunit and a 35 kDa (p35) subunit. The actual molecular weights of the subunits may vary, e.g. when expressed in different species and depending on whether the protein is glycosylated and on the glycosylation pattern. The terms "p40" and "p35" therefore do not imply that the subunits have molecular weights of exactly 40 and 35 kDa respectively. Instead, these terms are used to identify and distinguish the two heterodimeric subunits of IL12, which may more accurately be defined in terms of their amino acid sequences. Heterodimeric IL12 comprises a first and a second polypeptide subunit homologous or identical with or the p40 subunit and p35 subunit of human IL12, respectively. Typically, first subunit of IL12 comprises an amino acid sequence that has at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% sequence identity with the amino acid sequence of human IL12 subunit p40 as set out in SEQ ID NO: 3. Typically, the second subunit of IL12 comprises an amino acid sequence that has at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% sequence identity with the amino acid sequence of

human IL12 subunit p35 as set out in SEQ ID NO: 4. IL12 in conjugates of the invention retains a biological activity of IL12, e.g. an ability to act as a growth factor for activated T and NK cells, to enhance the lytic activity of NK/lymphokine-activated killer cells, to stimulate production of IFN- γ by resting PMBC, to inhibit angiogenesis (e.g. through the downstream mediator IP-10), and/or to inhibit tumour growth and/or metastasis.

A specific binding member is a member of a pair of molecules that have binding specificity for one another. The members of a specific binding pair may be naturally derived or wholly or partially synthetically produced. One member of the pair of molecules has an area on its surface, or a cavity, which specifically binds to and is therefore complementary to a particular spatial and polar organisation of the other member of the pair of molecules. Thus the members of the pair have the property of binding specifically to each other.

15

The specific binding member normally comprises a molecule having an antigen-binding site. For example, a specific binding member may be an antibody molecule or a non-antibody protein that comprises an antigen-binding site. An antigen binding site may be provided by means of arrangement of complementarity-determining regions (CDRs) on non-antibody protein scaffolds such as fibronectin or cytochrome B etc. [29, 30, 31], or by randomising or mutating amino acid residues of a loop within a protein scaffold to confer binding specificity for a desired target. Scaffolds for engineering novel binding sites in proteins have been reviewed in detail [31]. Protein scaffolds for antibody mimics have been disclosed [32], including proteins (antibody mimics) that include a fibronectin type III domain having at least one randomised loop. A suitable scaffold into which to graft one or more CDRs, e.g. a set of HCDRs, may be provided by any domain member of the immunoglobulin gene superfamily. The scaffold may be a human or non-human protein.

30 An advantage of a non-antibody protein scaffold is that it may provide an antigen-binding site in a scaffold molecule that is smaller and/or easier to manufacture than at least some antibody molecules. Small size of a specific binding member may confer useful physiological properties such as an ability to enter cells, penetrate deep into tissues or reach targets within other structures, or to bind within protein cavities of the target antigen.

35

Use of antigen binding sites in non-antibody protein scaffolds is reviewed in [34]. Typical are proteins having a stable backbone and one or more variable loops, in which the amino acid sequence of the loop or loops is specifically or randomly mutated to create an antigen-binding site having specificity for binding the target antigen. Such proteins include the IgG-binding domains of protein A from *S. aureus*, transferrin, tetranectin, fibronectin (e.g. 10th fibronectin type III domain) and lipocalins. Other approaches include synthetic "Microbodies" (Selecore GmbH), which are based on cyclotides - small proteins having intra-molecular disulphide bonds.

10

Although, as noted, CDRs can be carried by scaffolds such as fibronectin or cytochrome B [29, 30, 31], the structure for carrying a CDR or a set of CDRs of the invention will preferably be of an antibody heavy or light chain sequence or substantial portion thereof in which the CDR or set of CDRs is located at a location corresponding to the CDR or set of CDRs of naturally occurring VH and VL antibody variable domains encoded by rearranged immunoglobulin genes. The structures and locations of immunoglobulin variable domains may be determined by reference to Kabat et al. [35], and updates thereof, now available on the Internet (<http://immuno.bme.nwu.edu> or find "Kabat" using any search engine).

20

An antibody molecule is an immunoglobulin whether natural or partly or wholly synthetically produced. The term also covers any polypeptide or protein comprising an antibody antigen-binding site.

25

It is possible to take monoclonal and other antibodies and use techniques of recombinant DNA technology to produce other antibodies or chimeric molecules that retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the CDRs, of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin [37, 38, 39]. A hybridoma or other cell producing an antibody may be subject to genetic mutation or other changes, which may or may not alter the binding specificity of antibodies produced.

30

As antibodies can be modified in a number of ways, the term "antibody molecule" should be construed as covering any specific binding member or substance having an antibody antigen-binding site with the required specificity. Thus, this term covers

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antibody fragments and derivatives, including any polypeptide comprising an antibody antigen-binding site, whether natural or wholly or partially synthetic. Chimeric molecules comprising an antibody antigen-binding site, or equivalent, fused to another polypeptide are therefore included. Cloning and expression of chimeric
5 antibodies is well known [40, 41].

Further techniques available in the art of antibody engineering have made it possible to isolate human and humanised antibodies. For example, human hybridomas can be made as previously described [36]. Phage display, another established technique for
10 generating specific binding members has been described in detail [36, 42]. Transgenic mice in which the mouse antibody genes are inactivated and functionally replaced with human antibody genes while leaving intact other components of the mouse immune system, can be used for isolating human antibodies [43].

15 Synthetic antibody molecules may be created by expression from genes generated by means of oligonucleotides synthesized and assembled within suitable expression vectors [44, 45].

It has been shown that fragments of a whole antibody can perform the function of
20 binding antigens. Antibody fragments are preferred in conjugates of the invention owing to their small size and minimised interaction with other molecules and receptors (e.g. Fc receptor). Particularly preferred are single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site [46, 47]. scFv
25 may be stabilised by the incorporation of disulphide bridges linking the VH and VL domains [48].

Another small antigen-binding antibody fragment is a dAb (domain antibody), namely the variable region of an antibody heavy or light chain [28]. VH dAbs occur naturally
30 in camelids (e.g. camel, llama) and may be produced by immunising a camelid with a target antigen, isolating antigen-specific B cells and directly cloning dAb genes from individual B cells. dAbs are also producible in cell culture. Their small size, good solubility and temperature stability makes them particularly physiologically useful and suitable for selection and affinity maturation.

Single domain specific binding members, especially single domain antibodies such as dAbs, may be used in the invention, and are commercially available e.g. from Domantis, Phylos, Pieris and Affibody.

- 5 An antigen-binding site is the part of a molecule that binds to and is complementary to all or part of the target antigen. In an antibody molecule it is referred to as the antibody antigen-binding site, and comprises the part of the antibody that specifically binds to and is complementary to all or part of the target antigen. Where an antigen is large, an antibody may only bind to a particular part of the antigen, which part is
- 10 termed an epitope. An antibody antigen-binding site may be provided by one or more antibody variable domains. Preferably, an antibody antigen-binding site comprises an antibody light chain variable region (VL) and an antibody heavy chain variable region (VH).
- 15 The term "specific" may be used to refer to the situation in which one member of a specific binding pair will not show any significant binding to molecules other than its specific binding partner(s). The term is also applicable where e.g. an antigen-binding site is specific for a particular epitope that is carried by a number of antigens, in which case the specific binding member carrying the antigen-binding site will be able
- 20 to bind to the various antigens carrying the epitope.

L19 is a human recombinant antibody specific for the ED-B domain of fibronectin isoform B-FN. This antibody and its sequence have been previously described [20]. Single-chain Fv of L19 has also been described, and is preferentially employed in

25 conjugates of the invention. ScFv(L19) is an scFv comprising an L19 VH domain and an L19 VL domain, wherein the VH and VL are conjoined in a single polypeptide chain by a peptide linker sequence. The VH domain contains VH CDR1, CDR2 and CDR3 sequences, and the VL domain contains VL CDR1, CDR2 and CDR3 sequences, as shown in Figure 10. The L19 CDR sequences are:

30	VH CDR1	SFSMS	SEQ ID NO: 25
	VH CDR 2	SISGSSGTTYADSVKG	SEQ ID NO: 26
	VH CDR 3	PFPYFDY	SEQ ID NO: 27
	VL CDR 1	RASQSVSSSFLA	SEQ ID NO: 28
	VL CDR 2	YASSRAT	SEQ ID NO: 29
35	VL CDR 3	QQTGRIPPT	SEQ ID NO: 30

The VH domain may have an amino acid sequence as set out in SEQ ID NO: 22, and the VL domain may have an amino acid sequence as set out in SEQ ID NO: 23 (Figure 10). The VH and VL domains are normally joined by a peptide linker such as the 12 residue linker SEQ ID NO: 24 (Figure 10). Preferably, the scFv(L19) has the amino acid sequence as set out in SEQ ID NO: 5.

Brief Description of the Drawings

Figure 1 (A) shows sclL12-scFv(L19); **(B)** shows sclL12-SIP(L19) homodimer; **(C)** shows p40-scFv(L19)/scFv(L19)-p35 heterodimer.

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Figure 2 (A) shows SDS-PAGE analysis of the sclL12-scFv(L19) fusion protein under reduced and non reduced conditions; **(B)** shows gel filtration profile of the sclL12-scFv(L19) fusion protein under native conditions showing dimers.

15 **Figure 3** shows *in vivo* targeting of sclL12-scFv(L19) evaluated with biodistribution experiments. Accumulation is expressed in % injected dose per gram of tissue (%ID/g) after **(A)** 4 and **(B)** 24h. The tissues represented are (from left to right): tumour, spleen, liver, lung, heart, intestine, blood and kidney.

20 **Figure 4 (A)** shows SDS-PAGE analysis of the sclL12-SIP(L19) fusion protein under reduced and non reduced conditions; **(B)** shows gel filtration profile of the sclL12-SIP(L19) fusion protein under native conditions showing it to be a dimer.

25 **Figure 5** shows *in vivo* targeting of sclL12-SIP(L19) evaluated with biodistribution experiments and the accumulation expressed in % injected dose per gram of tissue (%ID/g) after **(A)** 4 and **(B)** 24h. The tissues represented are (from left to right): tumour, spleen, liver, lung, heart, intestine, blood and kidney.

30 **Figure 6 (A)** shows SDS-PAGE analysis of the p40-scFv(L19)/scFv(L19)-p35 fusion protein under reduced and non reduced conditions; **(B)** shows gel filtration profile of the p40-scFv(L19)/scFv(L19)-p35 fusion protein under native conditions showing to be a dimer.

35 **Figure 7** shows *in vivo* targeting of p40-scFv(L19)/scFv(L19)-p35 evaluated with biodistribution experiments and the accumulation expressed in % injected dose per

gram of tissue (%ID/g) after **(A)** 4 and **(B)** 24h. The tissues represented are (from left to right): tumour, spleen, liver, lung, heart, intestine, blood and kidney.

Figure 8 shows the amino acid sequence SEQ ID NO: 1. The sequence contains,

5 from the N to the C terminus:

(i) a signal peptide;

(ii) human IL12 p40 subunit (shown broken underlined - SEQ ID NO: 3);

(iii) peptide linker (SEQ ID NO: 15);

(iv) scFv(L19) (shown underlined - SEQ ID NO: 5); and

10 (v) myc;

i.e. signal peptide-human p40-linker-L19-myc. In the encoding nucleic acid sequence we used, the myc tag was followed by three stop codons.

Figure 9 shows the amino acid sequence SEQ ID NO: 2. The sequence contains,

15 from the N to the C terminus:

(i) a signal peptide;

(ii) scFv(L19) (shown underlined - SEQ ID NO: 5);

(iii) peptide linker (SEQ ID NO: 15);

(iv) human IL12 p35 subunit (shown broken underlined - SEQ ID NO: 4); and

20 (v) 6His-tag;

i.e. signal peptide-L19-linker-human p35-6xHis. In the encoding nucleic acid sequence we used, the six histidines were followed by three stop codons.

Figure 10 shows the amino acid sequence of scFv(L19) (SEQ ID NO: 5). The VH

25 and VL domains are shown separately (SEQ ID NO: 22 and SEQ ID NO: 23, respectively). The CDR1, 2 and 3 sequences in both the VH and VL domain are shown underlined. The VH and VL domains are separated by a 12 residue peptide linker sequence (SEQ ID NO: 24).

30 **Figure 11** shows the amino acid sequence of anti-tenascin-C scFv TN11 (SEQ ID NO: 21).

Aspects and embodiments of the invention will now be exemplified in the following experimental section.

Examples

Here we describe production and characterisation of IL12-L19 conjugates in three different formats, demonstrating the marked superiority of the format as claimed herein for *in vivo* targeting.

5

The three formats, as illustrated in Figure 1 A, B and C, respectively, are scIL12-scFv(L19), scIL12-SIP(L19) homodimer and p40-scFv(L19)/scFv(L19)-p35 heterodimer.

10 scIL12-scFv(L19), depicted in Figure 1 A, is a fusion protein of scIL12 and scFv(L19). This conjugate is monovalent owing to the single scFv antigen-binding site per molecule.

15 The scIL12-SIP(L19) homodimer, depicted in Figure 1 B, is a homodimer of two fusion proteins, each fusion protein containing scIL12, scFv(L19) and the CH4 domain of human IgE, respectively. The conjugate is dimerised through disulphide linkage between the two CH4 domains, forming a mini-antibody or SIP structure. This conjugate is bivalent owing to the presence of two scFv(L19) antigen-binding sites per molecule.

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The p40-scFv(L19)/scFv(L19)-p35 heterodimer, illustrated in Figure 1 C, is a heterodimer of two different fusion proteins. The first fusion protein contains the human IL12 p40 subunit fused to the N terminus of scFv(L19) through a 6 amino acid peptide linker GSADGG (SEQ ID NO: 15). The second fusion protein contains
25 scFv(L19) fused to the N terminus of human IL12 p35 subunit through a 6 amino acid peptide linker GSADGG (SEQ ID NO: 15). The two fusion proteins are heterodimerised through disulphide linkage between the p40 and p35 subunit, to form the heterodimeric p40-scFv(L19)/scFv(L19)-p35. The conjugate is bivalent, owing to the presence of two scFv(L19) antigen-binding sites per molecule.

30 scIL12-scFv(L19) without a FLAG tag

Since the original fusion protein as previously described [15] was cloned with a C-terminal FLAG tag ("scIL12-scFv(L19)-FLAG"), we should consider the possibility that the tyrosine-containing FLAG tag may contribute to artefacts in biodistribution experiments. When the tyrosines of the protein are radio labelled, the solvent-
35 exposed tyrosine of the FLAG tag may be iodinated as well. The FLAG tag may be

later proteolysed in vivo. In order to better study the tumour-targeting properties of this fusion protein, we cloned and expressed scL12-scFv(L19) fusion protein without a FLAG tag.

Cloning and expression of scL12-scFv(L19)

5 The DNA-fragment for scL12-scFv(L19) was amplified using the pCH33 vector containing the gene for scL12-scFv(L19)-FLAG [15] as a template. The PCR reaction was performed with the primer *sp40backEco* (5' ccg gaattc atg tgt cct cag aag cta acc atc 3') (SEQ ID NO: 6), which anneals to the endogenous secretion sequence of p40 and appends a restriction site for the endonuclease EcoR1 to its
10 5'end, and the primer *L19stopNotfor* (5' ttt tcc ttt t gcggccgc cta tca tca ttt gat ttc cac ctt ggt ccc 3') (SEQ ID NO: 7) appending 3 stop codons followed by a restriction site for the endonuclease Not1 to the 3'end of the scL12-L19 fusion protein, and thereby deleting the FLAG tag. The DNA fragment was cloned into the mammalian cell expression vector pcDNA3.1(+) vector (Invitrogen, Basel, Switzerland), using the
15 EcoR1 and Not1 restriction sites of the vector.

HEK 293 cells were transfected with the vector and stable transfectants selected in the presence of G418 (500µg/ml). Clones of G418-resistant cells were screened for the expression of the fusion protein by ELISA, using recombinant ED-B domain of
20 human fibronectin as an antigen. The fusion protein was purified from cell culture medium by affinity chromatography over antigen column as described previously [19, 20] and desalted by dialysis over night at 4°C. The fusion protein was frozen in aliquots at -20°C. The size of the scL12-scFv(L19) fusion protein was analyzed under reducing and non reducing conditions on SDS-PAGE and under native
25 conditions by FPLC gel filtration on a Superdex S-200 column (Amersham Pharmacia Biotech) (Figure 2). Unlike the monomeric original scL12-scFv(L19)-FLAG fusion protein, about one third of the IL12-L19 protein fraction showed to be a dimer.

Characterization of scL12-scFv(L19)

30 The in vivo targeting of scL12-scFv(L19) without a FLAG tag was evaluated with biodistribution experiments. Therefore the monomeric fraction of the fusion protein was purified by FPLC gel filtration over a Superdex S-200 column. The fraction was iodinated immediately after purification and injected in 129SvEv immunocompetent mice bearing F9 murine teratocarcinoma tumours. Mice were sacrificed 4 and 24
35 hours after injection, the organs were weighed and the radioactivity counted. The

accumulation in representative organs and the tumour was expressed in % injected dose per gram of tissue (%ID/g) (Figure 3). After 24 hours no accumulation at the tumour site could be observed. Therefore we consider the flag tag did not disturb the biodistribution results.

5 scL12-SIP(L19) homodimer

The tumour targeting properties of scFv(L19) have been shown to be improved when scFv(L19) was dimerised thanks to the CH4 domain of human IgE, building a mini-antibody structure, also termed "small immune protein" or SIP. The tumour targeting properties of SIP(L19) have been previously described [21]. Therefore, we
10 constructed a homodimer of scL12-SIP(L19) to test whether this format would also improve targeting of IL12 to tumours.

We constructed the scL12-SIP(L19) fusion protein by fusing the CH4 domain of a human IgE immunoglobulin at the c-terminus of the scL12-scFv(L19)-FLAG fusion
15 protein [22].

Cloning and expression of scL12-L19-SIP homodimer

The fusion of the CH4 domain to the c-terminus of the scL12-L19 fragment was performed by one round of PCR and inserting the PCR fragment in a vector already containing the CH4 domain.

20

The scL12-scFv(L19) fragment was amplified from the pCH33 vector containing the gene for scL12-scFv(L19)-FLAG [15]. The restriction sites were exchanged with the primer *sp40backHind* (5' ccgta aagctt atg tgt cct cag aag cta acc atc 3') (SEQ ID NO: 8) that anneals at the 5' end of the p40 secretion sequence and changes the EcoR1
25 to a HindIII restriction site, and the primer *L19forAgeI/Bsp* (5' tgt ggg accggt ttt gat ttc cac ctt ggt ccc 3') (SEQ ID NO: 9) annealing at the 3' end, deleting the FLAG tag and introducing an Age1 restriction site which provides cohesive ends with BspE1. Once ligated the Age1/BspE1 restriction site is not recleavable with BspE1 any more. This step is necessary because the scL12 sequence contains a BspE1 restriction site.
30 Using the endonucleases HindIII and BspE1 the fragment was now cloned in a mammalian cell pcDNA3.1(+) expression vector already containing a CH4 domain with a BspE1 restriction site at its 5'end [21].

The transfection, expression and purification procedure for the scL12-SIP(L19) was
35 performed as described above for the scL12-scFv(L19) fusion protein. The size of

the IL12-SIP(L19) fusion protein was as well analyzed under reducing and non reducing conditions on SDS-PAGE and under native conditions by FPLC gel filtration on a Superdex S-200 column (Figure 4).

Characterization of sclL12-SIP(L19) homodimer

- 5 The sclL12-SIP(L19) protein was purified in a second step over a gel filtration Superdex S-200 column and the dimer fraction radio iodinated immediately after collection. The labelled protein was injected in 129SvEv immunocompetent mice bearing F9 murine teratocarcinoma tumours. The mice were sacrificed 4 and 24 hours after injection, the organs were weighed and the radioactivity counted. The
10 accumulation in representative organs and the tumour was expressed in % injected dose per gram of tissue (%ID/g) (Figure 5). Although the sclL12-SIP(L19) protein displayed an improved binding to ED-B in BIAcore, an increased tumour uptake could not be observed and the tumour uptake remained poor.

p40-scFv(L19)/scFv(L19)-p35 heterodimer

- 15 The p40-scFv(L19)/scFv(L19)-p35 heterodimer consists of two subunits. One contains a scFv(L19) fragment, fused at the N-terminal extremity of the p35 subunit of IL12 (scFv(L19)-p35). In the second, the subunit p40 of IL12 is fused at the N-terminal end of scFv(L19) (p40-scFv(L19)). The fusion proteins then build the heterodimeric p40-scFv(L19)/scFv(L19)-p35 by means of the disulphide bonds
20 between p40 and p35.

This heterodimeric antibody-cytokine fusion protein conjugate is expected to have a size of 114 kD when unglycosylated. Owing to different glycosylation states, the molecular weight of the conjugate may vary according to the species in which it is
25 expressed, owing to different glycosylation patterns. Theoretical sizes for the fusion proteins, based on length of DNA sequence without post-translational glycosylation, were 65 kDa for p40-L19, 49 kDa for L19-p35 and 114 kDa for the heterodimer p40-L19/L19-p35.

Cloning and expression of p40-scFv(L19)/scFv(L19)-p35

- 30 The two subunits p40-scFv(L19) and scFv(L19)-p35 were cloned separately into two different vectors, the first containing a myc tag for detection and the second containing a his tag for detection.

The p40-scFv(L19)-myc fragment was produced by PCR assembly using as templates the pCH33 vector containing the gene encoding the scL12-scFv(L19)-FLAG protein [15] and the pLB vector containing the gene encoding the SIP(L19) fragment [21]. A first fragment A containing the p40 moiety was constructed using pCH33 as a template. The PCR reaction was performed with a first primer *HindSp40back* (5' 'ccc aagctt atg tgt cct cag aag cta acc atc 3') (SEQ ID NO: 10) annealing to the secretion sequence of the p40 subunit of IL12 introducing a restriction site for the endonuclease HindIII at its 5'end and with a second primer *Sp40HaLifor* (5' acc tcc atc agc gct tcc gga tcg gac cct gca gg 3') (SEQ ID NO: 11) annealing to the 3'end of p40, appending a part of the linker (GSADGG) to be fused to the second fragment B. This fragment B contributing the scFv(L19) moiety was constructed in 2 rounds of PCR using the template pLB with a first primer *LinkL19back* (5' gga agc gct gat gga ggt gag gtg cag ctg ttg gag tc 3') (SEQ ID NO: 12) annealing to the 5'end of the scFvL19 appending a part of the linker (GSADGG) (SEQ ID NO: 15) and a second primer *L19mycstoBamfor* (5' att cag atc ctc ttc tga gat gag ttt ttg ttc ttt gat ttc cac ctt ggt ccc ttg 3') (SEQ ID NO: 13) annealing to the 3'end of the scFv(L19) and appending a C-terminal myc tag. This fragment was finished in a second round of PCR using the primer *LinkL19back* and a third primer *mycstoBamfor* (5' cgc ggatcc cta tca tca att cag atc ctc ttc tga gat gag ttt 3') (SEQ ID NO: 14) appending 3 stop codons and a restriction site for BamH1 to the 5'end of fragment B. Using both fragments as templates a PCR assembly with the primers *HindSp40back* and *mycstoBamfor* was performed to fuse the p40 fragment to the N-terminus of the antibody fragment scFv(L19) connected by the flexible linker GSADGG producing the fragment p40-GSADGG-L19-myc. Using HindIII and BamH1 as restriction sites the fragment was then cloned into a mammalian expression vector pcDNA3.1+/Hygro providing a hygromycine resistance for selection. CHO cells were transfected with the vector and stable transfectants were selected with hygromycine (500µg/ml). Positive clones were screened with ELISA using recombinant ED-B as an antigen and anti-myc antibody for detection. The protein was expressed and purified from the cell culture medium by antigen affinity chromatography as described previously [19, 20] and desalted by dialysis over night at 4°C. The fusion protein frozen in aliquots at -20°C. An SDS PAGE size analysis showed the protein to be approximately 50% monomers in two different glycosylation states and about 50% homodimer [23].

The scFv(L19)-p35-His fragment was produced by PCR assembling as well using the same plasmids pCH33 and pLB as templates. A first fragment C corresponding to the scFv(L19) moiety was constructed from the template pLB with a first primer *EcoLonSL19back* (5' ccg gaattc gct tgt cga cca tgg gct g 3') (SEQ ID NO: 16) annealing to the secretion sequence of the scFvL19 introducing a restriction site for the endonuclease EcoR1 at its 5'end and a second primer *L19HaLifor* (5' acc tcc atc agc gct tcc ttt gat ttc cac ctt ggt ccc 3') (SEQ ID NO: 17) annealing to the 3'end of scFv(L19), introducing a part of the linker (GSADGG). A second fragment D containing the p35 moiety was constructed in two rounds of PCR using the template pCH33 with a first primer *HaLip35back* (5' gga agc gct gat gga ggt agg gtc att cca gtc tct gga 3') (SEQ ID NO: 18) annealing to the 5'end of the mature p35 subunit appending a part of the linker (GSADGG) to its 5'end and a second primer *p35hisfor* (5' gtg atg gtg atg atg atg ggc gga gct cag ata gcc 3') (SEQ ID NO: 19) annealing to the 3'end of the p35 subunit appending a his tag. In a second round of PCR 3 stop codons and a restriction site for Not1 were appended to the 3'end of fragment D using the primer *HaLip35back* and a third primer *p35hisstoNotlfor* (5' ttt tcc ttt t gcggccgc cta tca tca gtg atg gtg atg atg atg ggc 3') (SEQ ID NO: 20). The p35 cytokine subunit was now fused to the c-terminus of the scFv(L19) fragment by PCR assembly using the primers *EcoLonSL19back* and *p35hisstoNotlfor* producing L19-GSADGG-p35-his. The fragment was now cloned into a pcDNA 3.1 mammalian expression vector providing a neomycine resistance using the EcoR1 and Not1 restriction sites. HEK 293 cells were transfected and stable transfectants selected with G418 (500µg/ml). Transfected cells were screened for resistant clones expressing the fusion protein by ELISA, using human recombinant EDB as an antigen and anti-his antibody for detection. The fusion protein was purified from the cell culture medium by affinity chromatography over an antigen column as described previously [19, 20] desalted by dialyzation over night at 4°C. An SDS PAGE analysis showed the expression to be very weak and the protein was shown to consist of monomers, dimers and higher order oligomers.

30

The cotransfection of HEK 293 cells with the two vectors coding for scFv(L19)-p35 and p40-scFv(L19) was then performed simultaneously. The selection for stable transfectants that contain both fusion proteins was performed with G418 (500µg/ml) and hygromycine (500µg/ml). Cells were screened for expression of both proteins with ELISA using recombinant EDB as an antigen and anti-myc antibody as well as anti-his antibody for detection. The fusion proteins were purified in a first step by

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affinity chromatography over an ED-B antigen column as described previously [19, 20] and desalted by dialysis over night at 4°C. This first fraction was then purified in a second step over a HisTrap HP 1ml Ni²⁺ column (Amersham Biosciences) washing away the free p40-scFv(L19) fragments not bound in the heterodimer (p40-scFv(L19)/scFv(L19)-p35). For storage, 0.1% Tween 80 was added to the protein that was then frozen in aliquots at -80°C. An SDS PAGE analysis showed under non reduced conditions the protein to be a dimer in two glycosylation states and under reduced conditions the two hetero monomers of different sizes where of the p40-L19 monomer appears in its two different glycosylation states. By performing gel filtration with a Superdex S-200 column the protein showed to be a pure dimer under native conditions (Figure 6).

Characterization of p40-scFv(L19)/scFv(L19)-p35

The correct folding of the fusion protein and building of the correct disulfide bonds were determined by the IL12 activity of the purified heterodimeric protein.

15

The IL-12 activity was determined by performing a T cell proliferation assay [24]. In brief, resting human peripheral blood monocytes (PBMC) were cultured with mitogen (phytohemagglutinin (PHA) and IL-2) for 3 days and then incubated with serial dilutions of either fusion proteins or commercially available, recombinant, murine IL-12 as a standard (R&D Systems Europe Ltd, Abingdon, United Kingdom). Proliferation was subsequently measured by [³H]thymidine incorporation.

20

Biodistribution experiments were performed with the p40-scFv(L19)/scFv(L19)-p35 protein that was purified on a FPLC gel filtration Superdex S-200 column and radioiodinated. The labelled protein was injected in 129SvEv immunocompetent mice bearing F9 murine teratocarcinoma tumours. The mice were sacrificed 4 and 24 hours after injection, the organs were weighed and the radioactivity counted. The accumulation in representative organs and the tumour was expressed in % injected dose per gram of tissue (%ID/g). With the heterodimeric format we achieved a tumour uptake of almost 10% at 24 hours (Figure 7).

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Claims

1. A conjugate comprising an IL12 heterodimer having first and second subunits, wherein the first and second subunits are each conjugated to an antibody molecule lacking an Fc domain.
2. A conjugate according to claim 1, wherein the first and second subunits of the heterodimeric protein are covalently linked through a disulphide bond.
3. A conjugate according to claim 1 or claim 2, wherein the antibody molecule is a single chain Fv (scFv).
4. A conjugate according to claim 1 or claim 2, wherein the antibody molecule is a single domain antibody molecule (dAb).
5. A conjugate according to any of the preceding claims, wherein the first subunit is conjugated to the antibody molecule as a first fusion protein, and the second subunit is conjugated to the antibody molecule as a second fusion protein.
6. A conjugate according to any of the preceding claims, wherein the conjugate has a molecular weight of 250,000 or less.
7. A conjugate according to claim 6, wherein the conjugate has a molecular weight of 150,000 or less.
8. A conjugate according to claim 7, wherein the conjugate has a molecular weight of 120,000 or less.
9. A conjugate according to any of the preceding claims, wherein the two antibody molecules are identical.
10. A conjugate according to claims 1 to 9, comprising a human IL12 heterodimer conjugated to and between two antibody molecules lacking an Fc domain; wherein the IL12 heterodimer has a first and a second subunit;

the first subunit is conjugated to an antibody molecule lacking an Fc domain as a first fusion protein; and

the second subunit is conjugated to an antibody molecule lacking an Fc domain as a second fusion protein.

11. A conjugate according to claim 10, wherein the first fusion protein has an amino acid sequence as set out in SEQ ID NO: 1.
12. A conjugate according to claim 10 or claim 11, wherein the second fusion protein has an amino acid sequence as set out in SEQ ID NO: 2.
13. A conjugate according to any of the claims 1 to 10, wherein one or both antibody molecules specifically binds to an extracellular matrix component associated with neoplastic growth and/or angiogenesis.
14. A conjugate according to claim 13, wherein the component is fibronectin ED-B.
15. A conjugate according to claim 14, wherein the antibody molecule is scFv(L19) having a VH domain comprising the amino acid sequences of SEQ ID NO: 25, SEQ ID NO: 26 and SEQ ID NO: 27, and a VL domain comprising the amino acid sequences of SEQ ID NO: 28, SEQ ID NO: 29 and SEQ ID NO: 30.
16. A conjugate according to claim 15, wherein the antibody molecule is scFv(L19) having an amino acid sequence as set out in SEQ ID NO: 5.
17. A conjugate according to claim 13, wherein the component is a tenascin-C isoform.
18. A conjugate according to claim 17, wherein the antibody molecule is scFv(TN11) having an amino acid sequence as set out in SEQ ID NO: 21.
19. A method of producing a conjugate according to any of claims 5 to 18, comprising:
expressing the first and second fusion proteins; and
conjugating the first and second subunits to form the IL12 heterodimer.

20. A method according to claim 19, comprising expressing the first and second fusion proteins in a cell containing nucleic acid encoding both fusion proteins.
21. A method according to claim 19 or claim 20, further comprising formulating the conjugate into a pharmaceutical composition.
22. A composition comprising
a first nucleic acid molecule comprising a nucleotide sequence encoding a fusion protein, wherein the fusion protein comprises an antibody molecule lacking an Fc domain and an IL12 p40 subunit; and
a second nucleic acid molecule comprising a nucleotide sequence encoding a fusion protein, wherein the fusion protein comprises an antibody molecule lacking an Fc domain and an IL12 p35 subunit.
23. A composition according to claim 22, wherein the antibody molecule is an scFv.
24. A composition according to claim 22, wherein the antibody molecule is a dAb.
25. A composition according to claim 22, wherein the antibody molecule is as defined in any of claims 13 to 18.
26. A composition according to any of claims 22 to 25, wherein the first and second nucleic acid molecules are first and second vectors.
27. A host cell containing a first and a second nucleic acid molecule as defined in any of claims 22 to 26.
28. A pharmaceutical composition comprising a conjugate according to any of claims 1 to 18.
29. A conjugate according to any of claims 1 to 18 for use in treatment of the human or animal body by therapy.
30. Use of a conjugate according to any of claims 11 to 18 in the manufacture of a medicament for inhibiting angiogenesis and/or neoplastic growth in a patient.

31. Use according to claim 30, wherein the medicament is for treating a tumour, rheumatoid arthritis, diabetic retinopathy, age-related muscular degeneration or angioma:

32. A method of inhibiting neoplastic growth and/or angiogenesis in a patient, comprising administering a conjugate according to any of claims 11 to 18 to the patient.

33. A method according to claim 32, comprising treating a tumour, rheumatoid arthritis, diabetic retinopathy, age-related muscular degeneration or angioma by inhibiting angiogenesis.

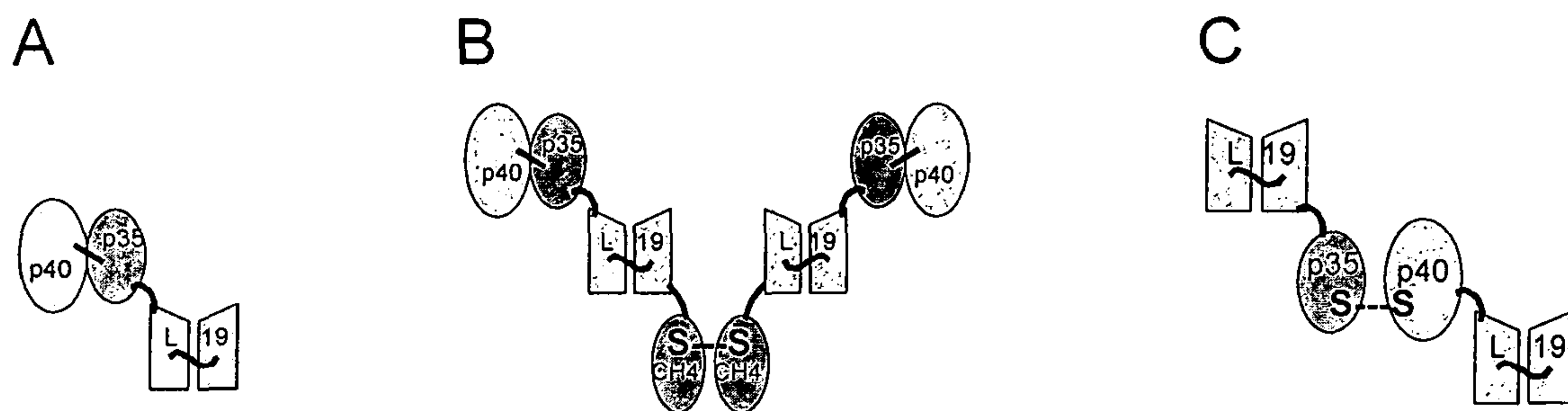


Figure 1

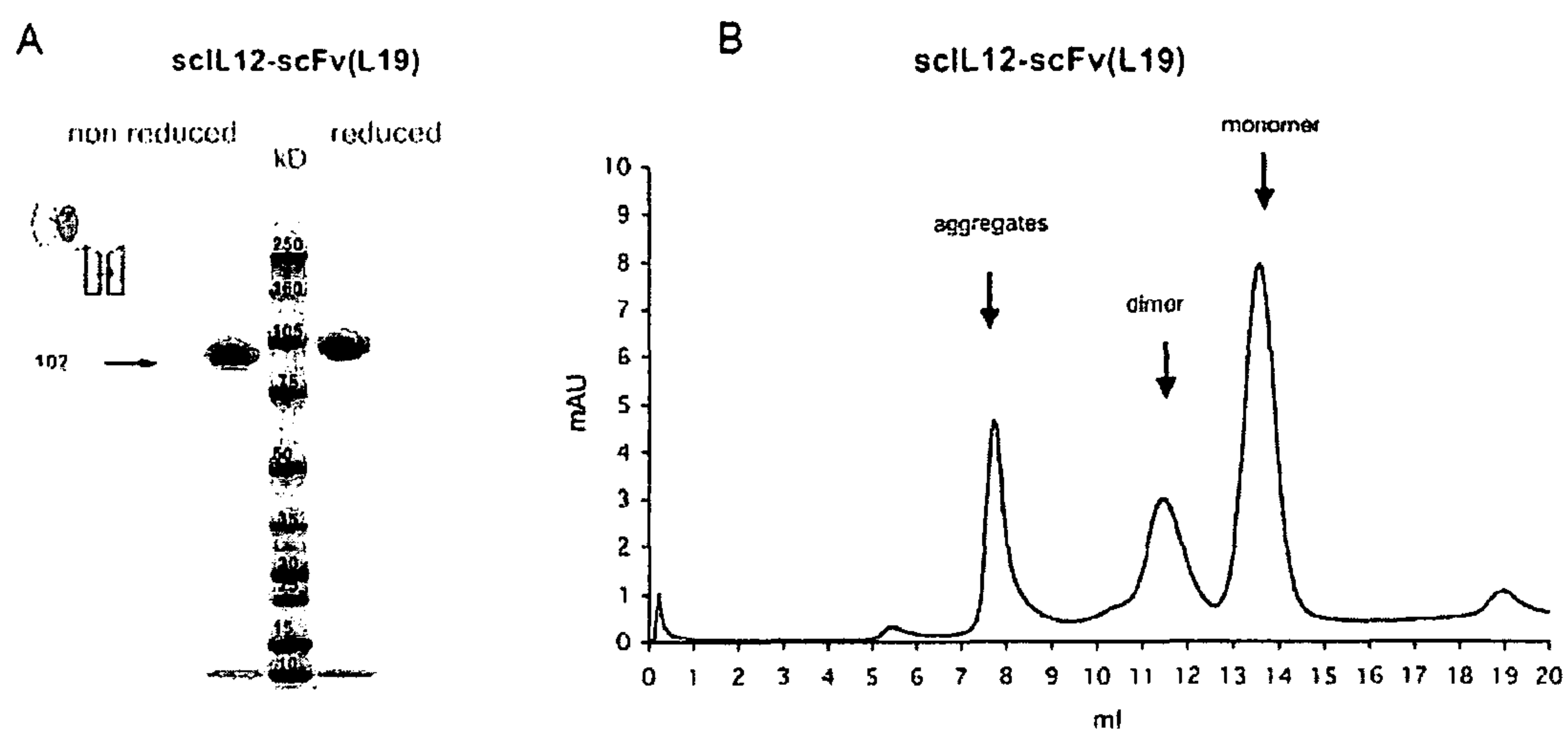


Figure 2

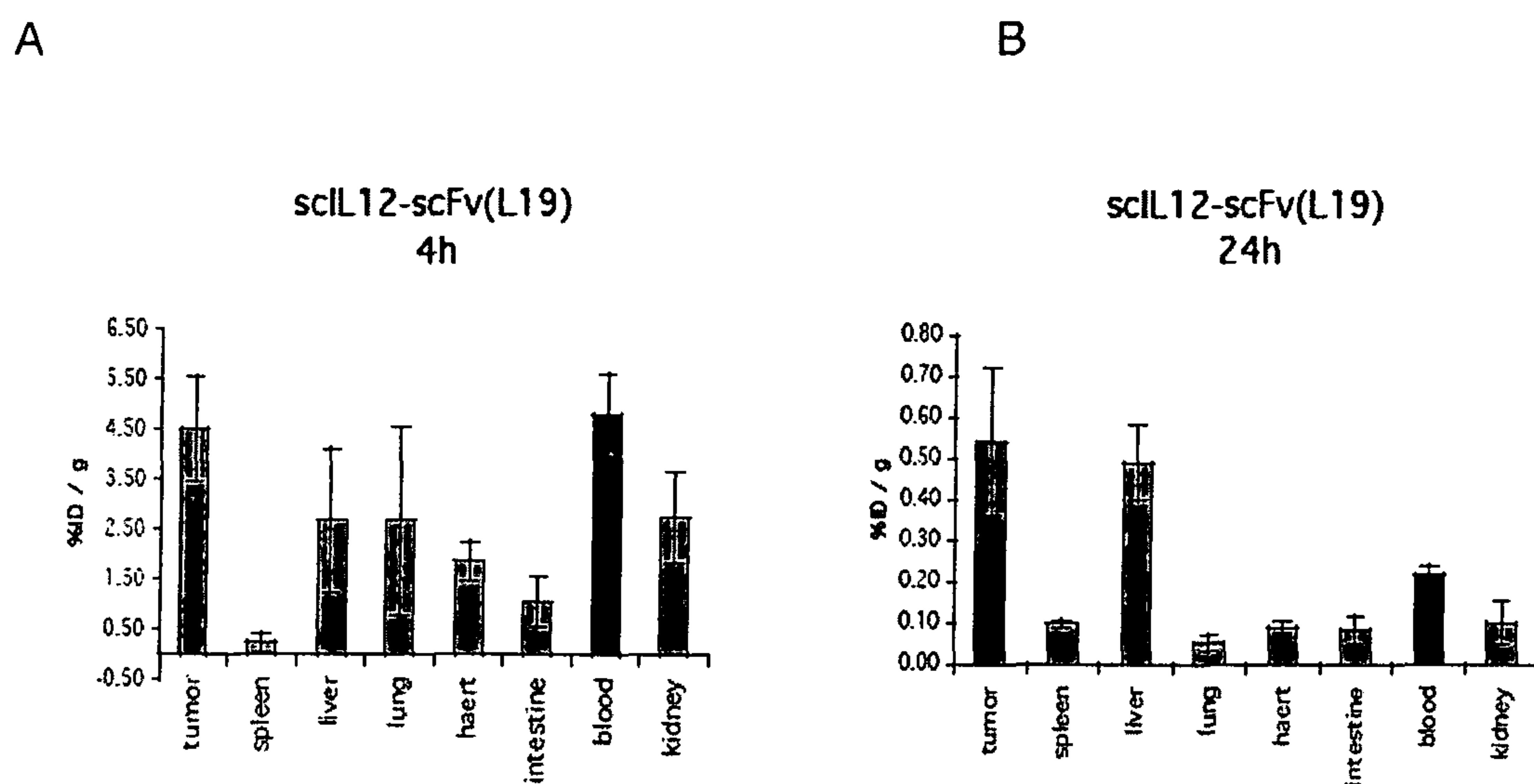


Figure 3

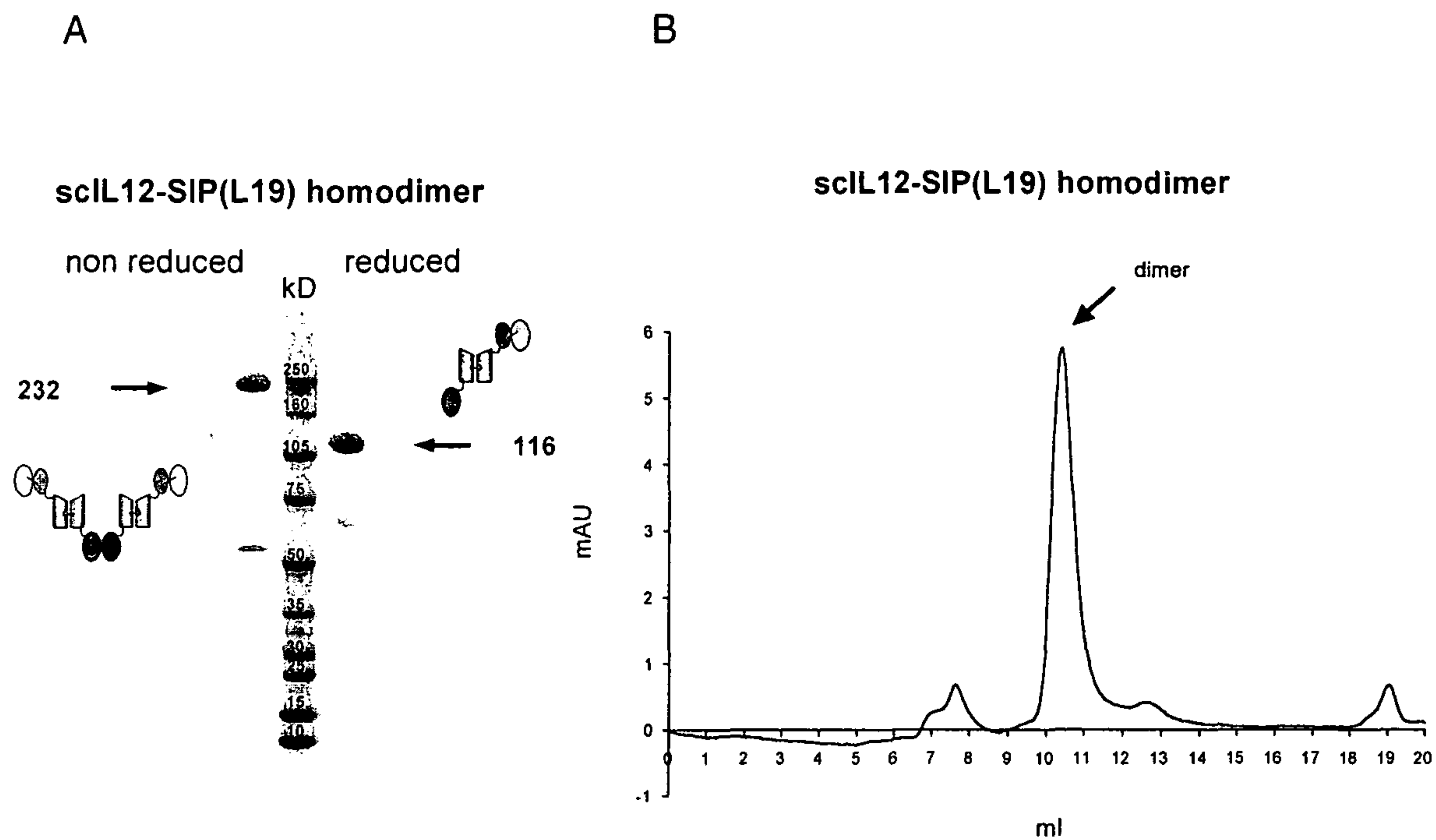


Figure 4

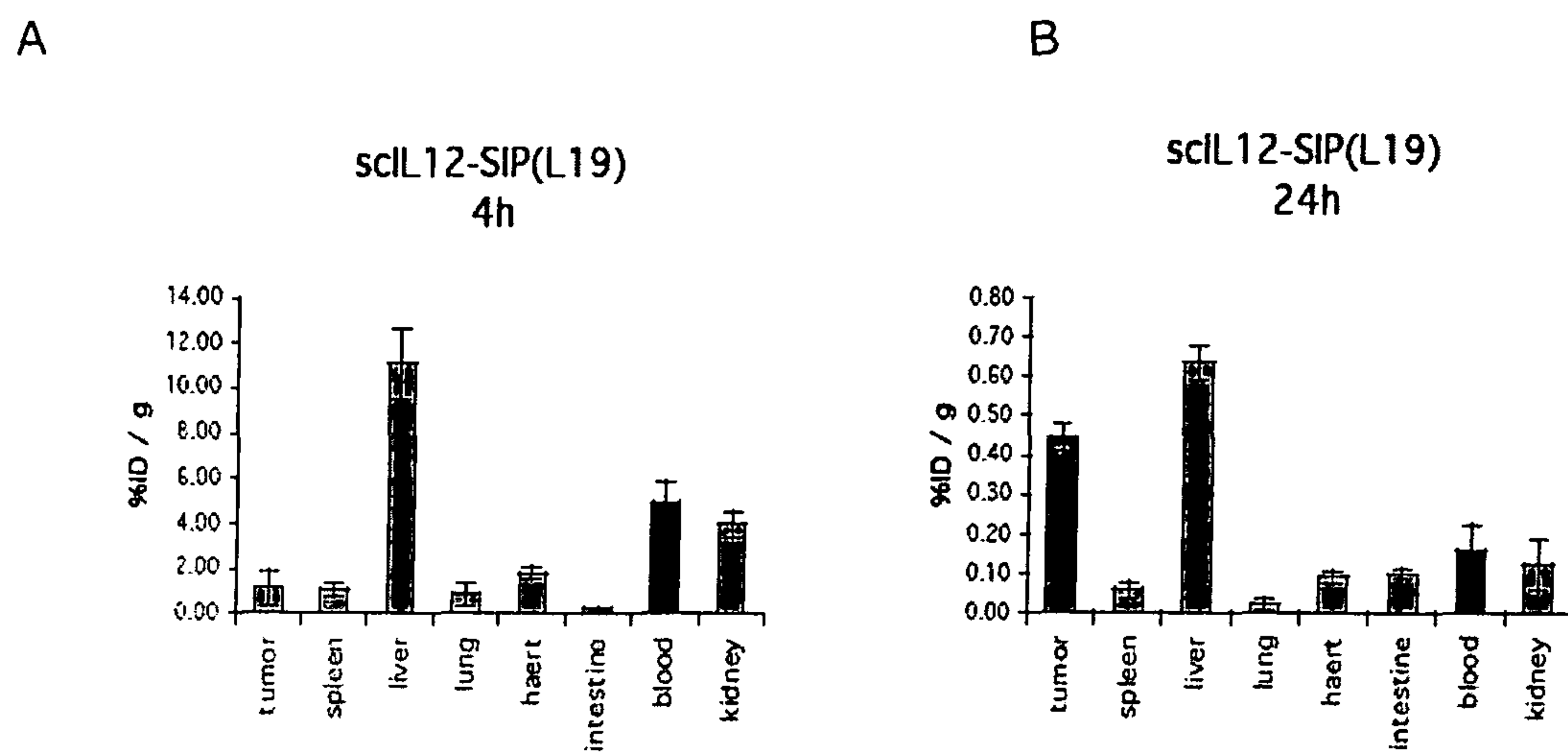
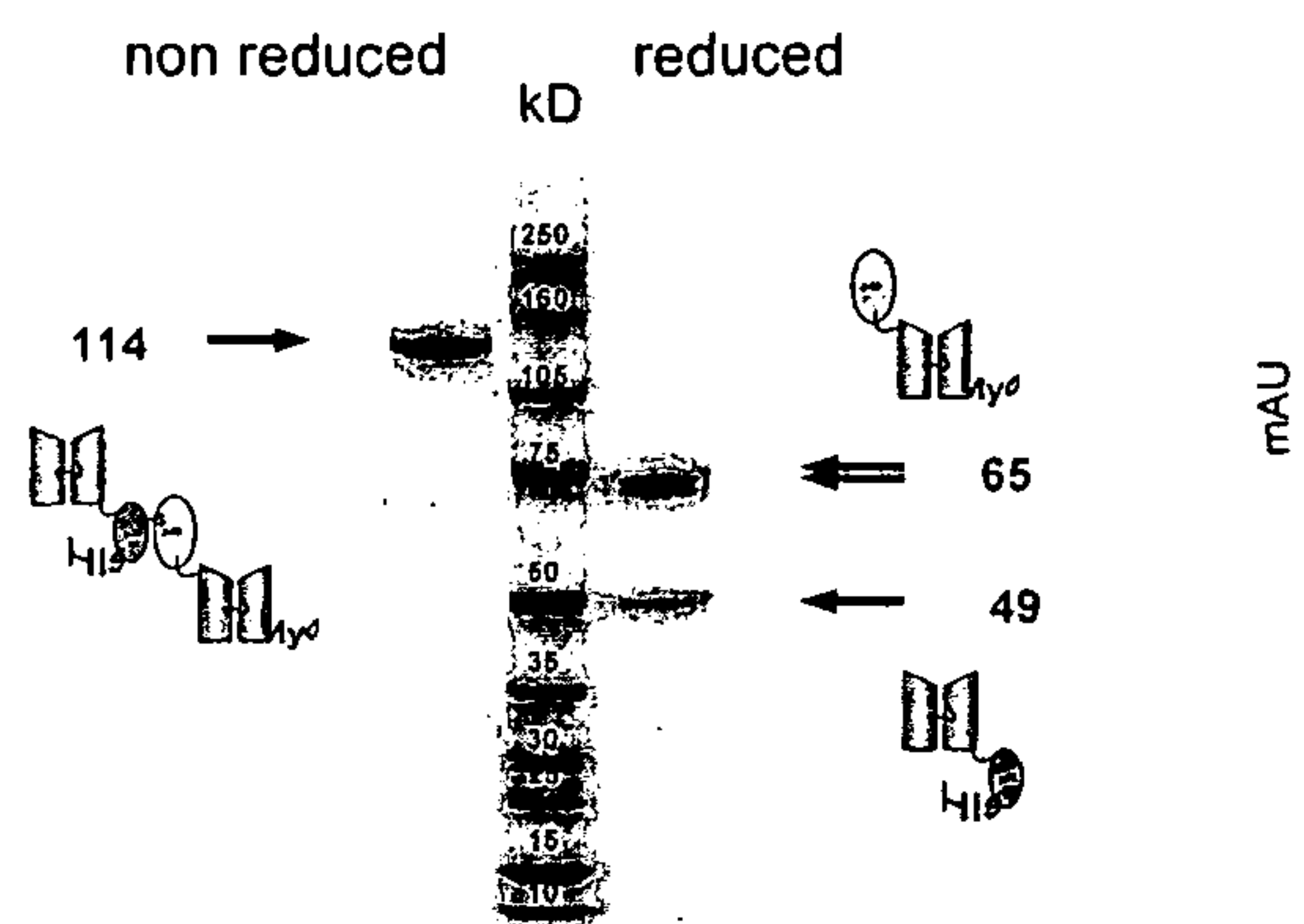


Figure 5

A

B

p40-scFv(L19)/scFv(L19)-p35 heterodimer



p40-scFv(L19)/scFv(L19)-p35 heterodimer

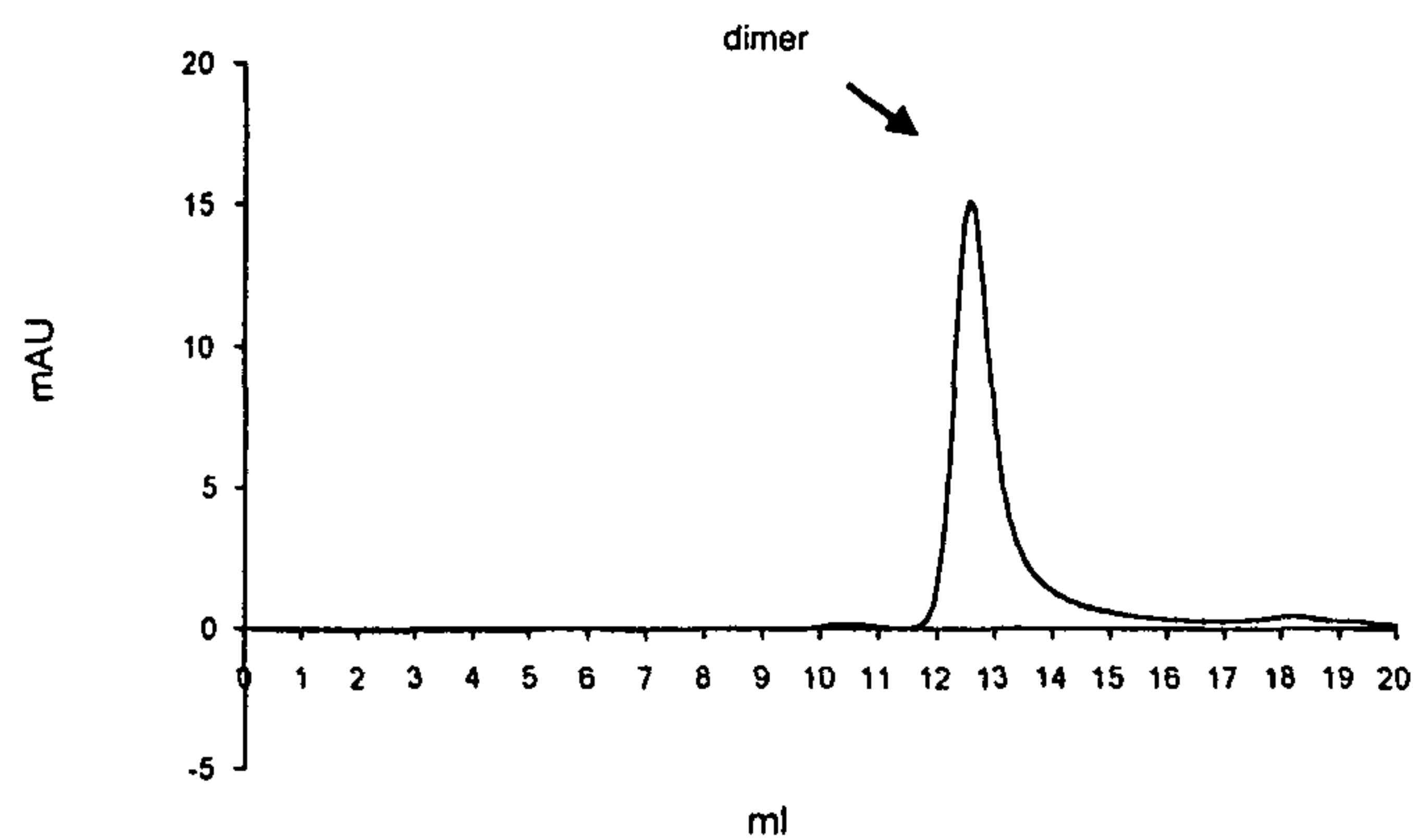


Figure 6

A

B

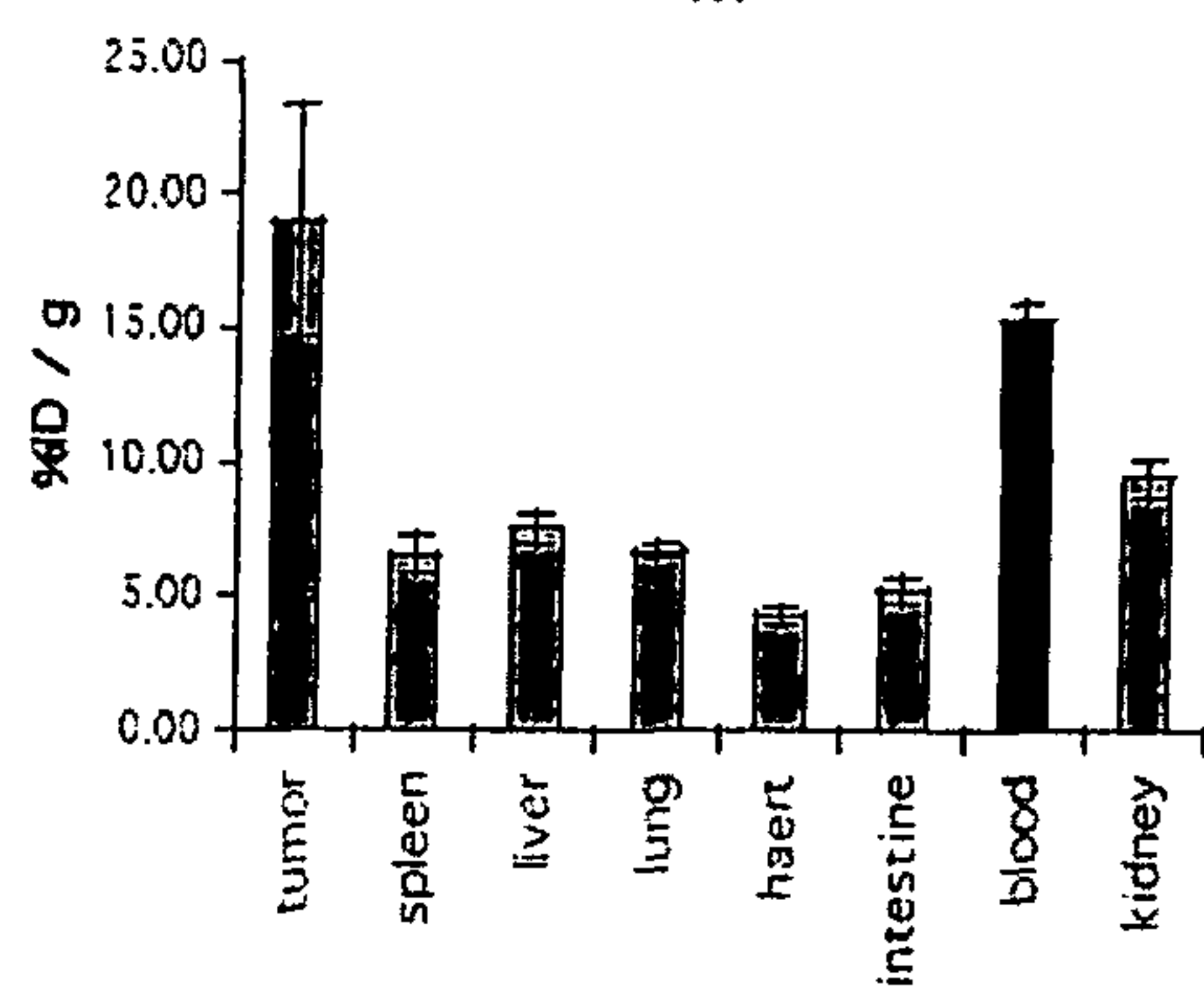
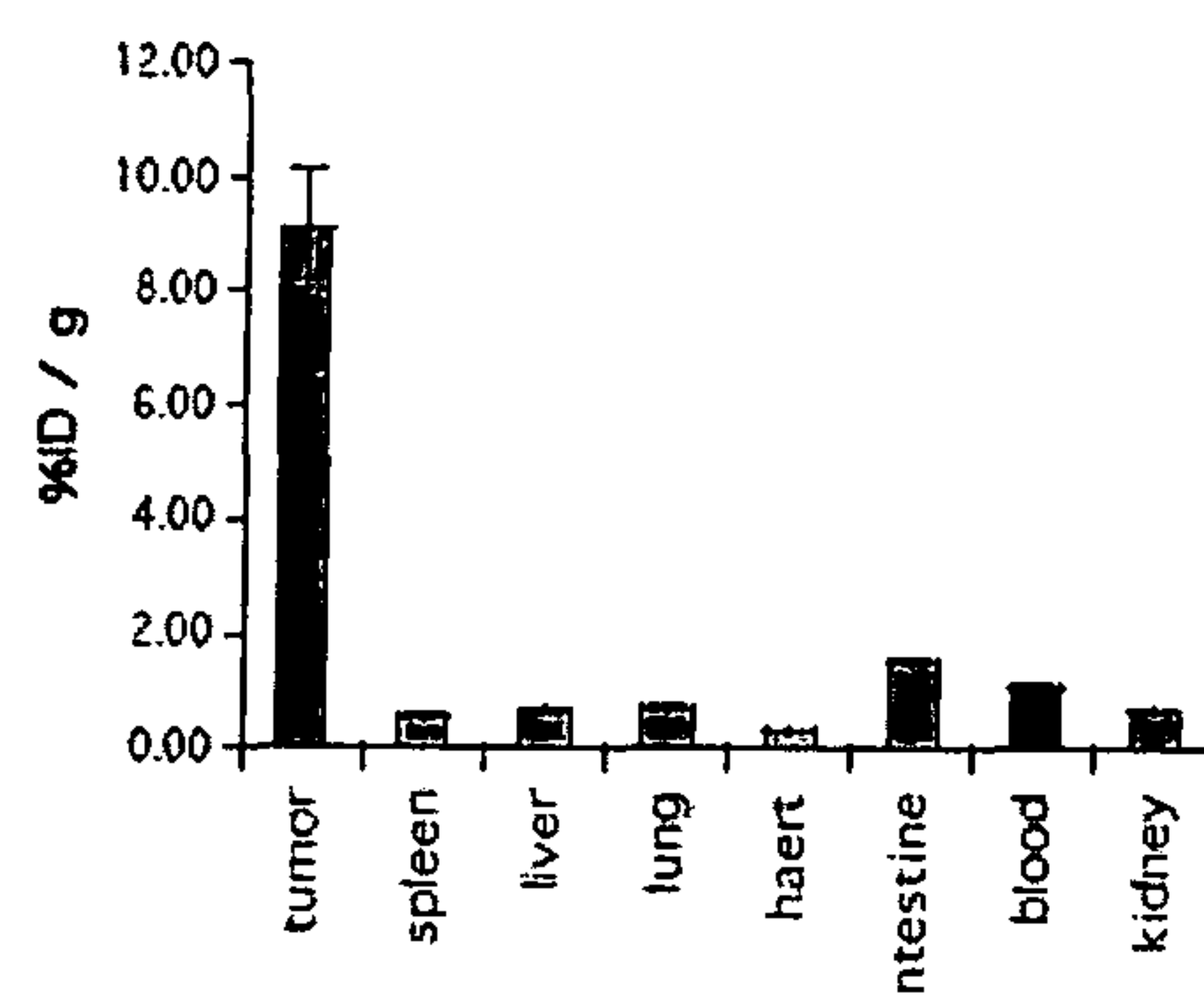
p40-scFv(L19)/scFv(L19)-p35
4hp40-scFv(L19)/scFv(L19)-p35
24h

Figure 7

Signal peptide-human p40-linker-L19-myc-3x stop

MCHQQLVISWFSLVFLASPLVAIWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDG
 ITWTLDQSSEVLGSGKTLTIQVKEFGDAGQYTCHKGGEVLSSHLLLLHKKEDGIWST
 DILKDQKEPKNKTFLRCEAKNYSGRFTCWWLTTISTDLTFSVKSSRGSSDPQGVTCG
 AATLSAERVGRDNKEYEYSVEEQEDSACPAAEESLPIEVMVDAVHKLKYENYTSFF
 IRDIIKPDPPKNLQLKPLKNSRQVEVSWEYPDTWSTPHSYFSLTFCVQVQGKSKREK
 KDRVFTDKTSATVICRKNASISVRAQDRYYSSSWSEWASVPCSGSADGGEVQLLESG
 GGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSISGSSGTTYADSVK
 GRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAKFPFYFDYWGQGT LVT VSSGDGSSG
 GSGGASEIVLTQSPGTL SLSPGERATL SCRASQSVSSSFLAWYQQKPGQAPRL LIYY
 ASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIK
 EQKLISEEDLN***

Figure 8

Signal peptide-L19-linker-human p35-6xHis-3x stop

GDNDIHFAFLSTGVHSEVQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAP
 GKGLEWVSSISGSSGTTYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAK
 PFPYFDYWGQGT LVT VSSGDGSSGSGGASEIVLTQSPGTL SLSPGERATL SCRASQ
 SVSSSFLAWYQQKPGQAPRL LIYYASSRATGIPDRFSGSGSGTDFTLTISRLEPEDF
 AVYYCQQTGRIPPTFGQGTKVEIKGSADGGRNLPVATPDPGMFPC LHHSQNLLRAVS
 NMLQKARQTLFYPCTSEEIDHEDITKDKTSTVEACLPLELTKNESCLNSRETSFIT
 NGSCLASRKTSFMMALCLSSIYEDLKMYQVEFKTMNAKLLMDPKRQIFLDQNMLAVI
 DELMQALNFNSETVPQKSSLEEPDFYKTKIKLCILLHAFRIRVTIDRVMSYLNASHH
 HHHH***

Figure 9

VH

CDR1

CDR2

EVQLLESGGGLVQPGGSLRLSCAASGFTFSSSFSMSWVRQAPGKGLEWVSSISGSSGTTYADSVKGRFT

CDR3

ISRDN SKNTLYLQMNSLRAEDTAVYYCAKPFYFDYWGQGLTVSS

LINKER

GDGSSGGSGGAS

VL

CDR1

CDR2

EIVLTQSPGTL SLSPGERATLS CRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRATGIPDRFSGS

CDR3

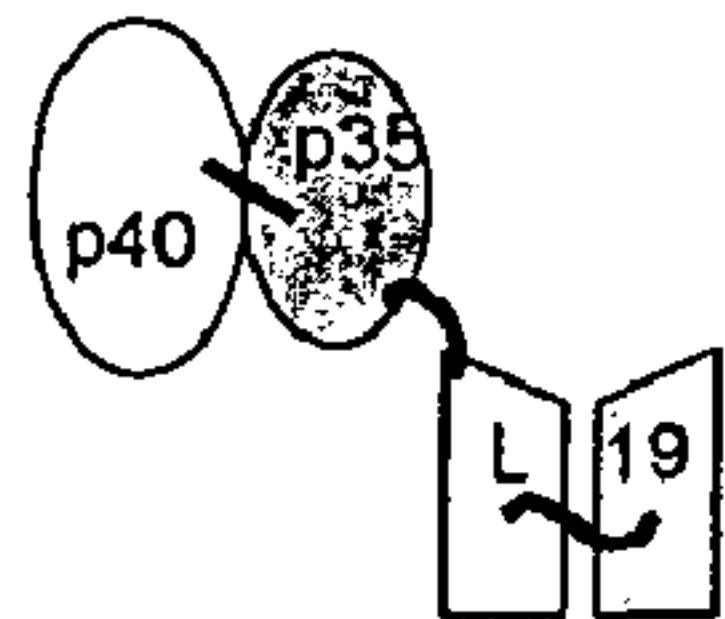
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Figure 10

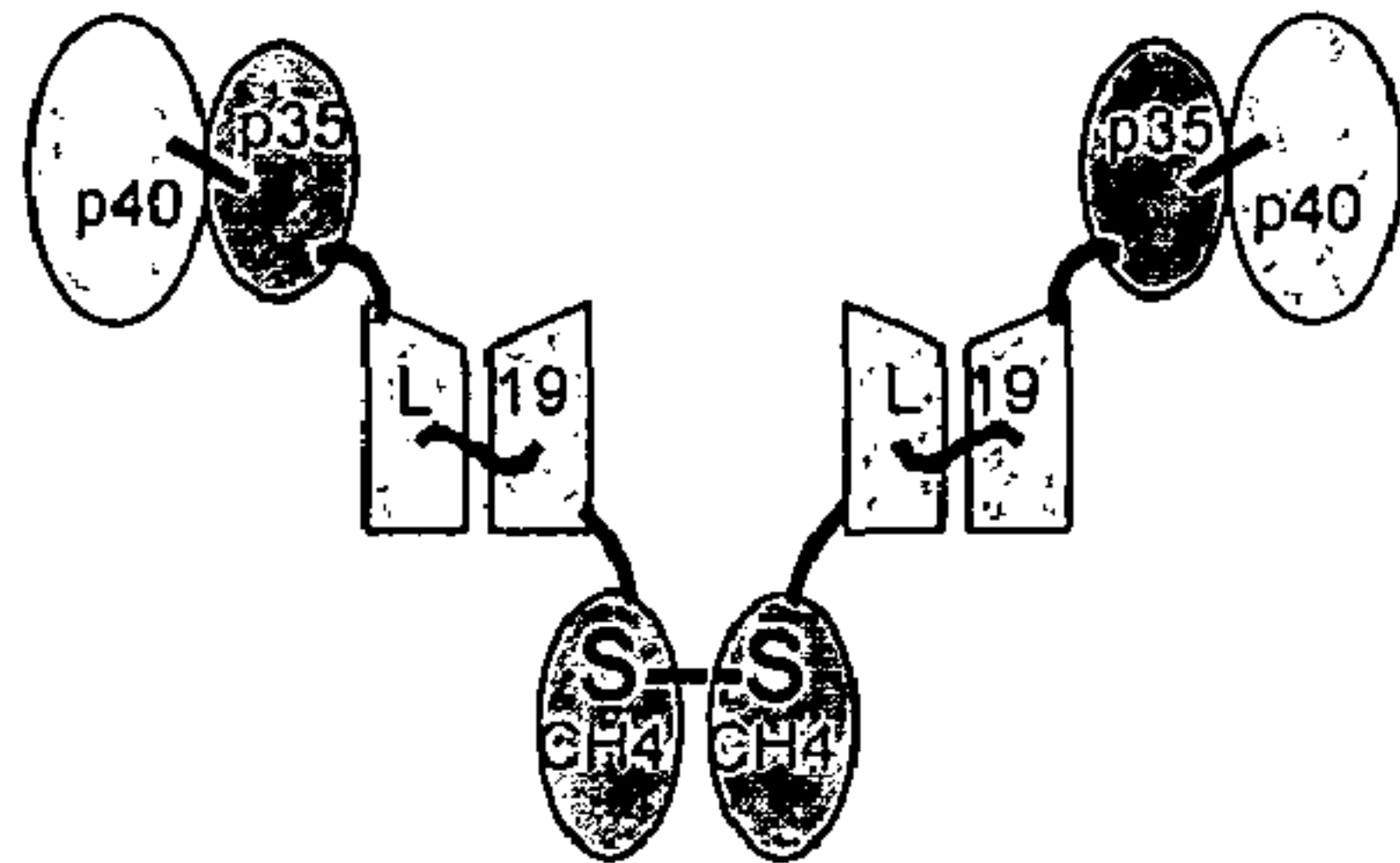
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T A D E S T S T A Y M E L S S L R S E D T A V Y Y C A R S R R I T I F
G G G A F D I W G R G T M V T V S S G G G G S G G G G S G G G S Q S
V L T Q P A S V S G S P G Q S I T I S C T G T S S D V G G Y N Y V S W
Y Q Q H P G K A P K L M I Y E G S K R P S G V S N R F S G S K S G N T
A S L T I S G L Q A E D E A D Y Y C S S Y T T R S T R V F G G G T K L
T V L G

Figure 11

A



B



C

