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(54) Title: ENZYMATIC CLEAVABLE REAGENTS FOR SPECIFIC DELIVERY TO DISEASE SITES

(57) **Abrégé/Abstract:**

A therapeutic reagent to control one or more reactions of the immune system in a host, or to deliver anti-tumour agents, or disease treatment agents to the host. The therapeutic reagent comprises a regulatory moiety and a carrier protein that inactivates or substantially reduces the activity of the regulatory moiety. Also, the therapeutic reagent includes a cleavage site at which cleavage of the therapeutic reagent can occur to free the regulatory moiety from the carrier protein so that the therapeutic reagent can act at a diseased site in the host.

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(54) Title: ENZYMATIC CLEAVABLE REAGENTS FOR SPECIFIC DELIVERY TO DISEASE SITES

(57) Abstract: A therapeutic reagent to control one or more reactions of the immune system in a host, or to deliver anti-tumour agents, or disease treatment agents to the host. The therapeutic reagent comprises a regulatory moiety and a carrier protein that inactivates or substantially reduces the activity of the regulatory moiety. Also, the therapeutic reagent includes a cleavage site at which cleavage of the therapeutic reagent can occur to free the regulatory moiety from the carrier protein so that the therapeutic reagent can act at a diseased site in the host.



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Cleavable reagents for specific delivery to disease sites

Field of the Invention

This invention relates to therapeutic reagents that can be manipulated to have a reduced effect or activity when circulating in the body of a host, but which are designed to be released in an active form at specific sites and times when needed. In particular, the invention relates to regulators of immune functions such as anti-complement reagents, which may be used to regulate the negative roles of immune molecules or cells in the host. Further, the invention relates to the use of said therapeutic reagents, pharmaceutical compositions including same and methods of medical treatment.

Background of the invention

The complement (C) system is an important component of the immune system of hosts, including humans and animals. C is known to consist of a number of proteins that act in a proteolytic cascade to target antigens or cells. During this sequence, one protein activated through binding antigen cleaves the next reacting protein to generate a new activated proteolytic enzyme, which cleaves and thereby activates the next protein in the sequence. Proteolytic fragments and protein complexes generated during activation have phlogistic activity, and cause inflammatory tissue changes such as increased vascular permeability and attraction of polymorphonuclear leukocytes.

Examples of such proteins include C5a, C3a and MAC (cytolytic macromolecular membrane attack complex). Targeting of a cell by C results in cell damage or death directly through formation of MAC, or indirectly through initiation of inflammation due to production of the inflammatory mediators (C5a, C3a and MAC) and phagocytosis of C targeted cells.

To protect themselves from this potentially harmful defence mechanism, cells express, on their surface, complement regulatory proteins (CRegs) which rapidly and efficiently inactivate 'accidental' foci of C activation (Morgan, B & Harris C., Complement Regulatory Proteins (1999)).

CRegs function either by inactivating enzymes, such as the C3 and C5 convertases, which are formed during C activation and which are responsible for cleavage of C3 and C5, or by interfering with MAC formation. In humans, the C regulators membrane cofactor protein (MCP;CD46), decay accelerating factor (DAF;CD55) and complement receptor 1 (CR1;CD35) inhibit C by accelerating the decay of or (with factor I) irreversibly inactivating the C3 and C5 convertase enzymes. A fourth regulator, CD59, inhibits MAC formation by binding C8 and/or C9, and inhibiting C9 polymerisation during MAC formation.

In normal circumstances, these control mechanisms are sufficient to protect cells from damage by homologous C. However, evidence of C activation is abundant in diverse inflammatory diseases including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), glomerulonephritis, adult respiratory distress syndrome (ARDS), ischemia-reperfusion injury, demyelination, myasthenia gravis, Arthus reaction, rejection in transplantation, lupus nephritis and multiple sclerosis. For example, in RA, soluble products of C activation are abundant in the synovial fluid of affected joints. Complement deposits are evident in synovial tissue, together with leukocytes (neutrophils and T cells) attracted to the site by a gradient of C5a and other chemo-attractants.

Whilst C itself is not always the primary cause of disease, it acts to sustain the pro-inflammatory cycle, can exacerbate the disease and perpetuate and extend tissue damage due to non-targeted activity.

Description of the Prior Art

Research has been carried out to find therapeutic reagents capable of inhibiting the C cascade and preventing the formation of pro-inflammatory mediators. Some groups have concentrated on high-throughput screening to identify small chemical compounds that might inhibit C. Others have exploited the naturally occurring cell-associated CRegs by generation of soluble, recombinant forms of these molecules that inhibit C through their native activities. Yet others have developed antibodies that block activation of specific components in the C system.

Anti-C reagents are known, most of which inhibit production of C5a and MAC, the key active by-products of C activation. The best-described examples are, first, a scFv that binds C5 and prevents its enzymatic cleavage, and second, a soluble, recombinant form of CR1 (sCR1) that inhibits the amplification enzymes in the activation pathways of C. Both of these reagents have been used in acute conditions, such as adult respiratory distress syndrome (ARDS), or ischaemia-reperfusion injury, which occurs in many clinical contexts, including myocardial infarction following cardiopulmonary bypass.

Known reagents, with the exception of sCR1, have low molecular weights, for example, from 12 kDa for CD59 to 40-50 kDa for DAF, MCP and Crry, therefore, these reagents are rapidly cleared from the body *via* the kidneys.

In addition to development of sCR1 for anti-C therapy, soluble recombinant forms of other human and rodent C regulators have been generated and tested in C-mediated inflammatory conditions, such as the Arthus reaction and rejection in xenotransplantation. In most combinations, human C regulators also inhibit rodent C and rodent C regulators control human C. For example, it has been shown that human and rodent DAFs are not species-specific in their complement inhibiting activities (Harris *et al* Immunology 100 462-470 (2000)). However, administration of a foreign C regulator results in a prompt immune response in the recipient, limiting its function to just a few days. This has restricted the ability to test human C regulators, such as sCR1, in chronic disease models in rodents.

Various modified forms of the C regulators have been produced and tested. Attempts have been made to combine two regulatory activities into one reagent, but these attempts have resulted in linear, inflexible molecules where the CRegs are fused end-to-end, making them unsuitable for targeted action (Higgins *et al* in J Immunol 158 2872 (1997)). A chimeric molecule, in which mouse Crry has been fused to mouse IgG1 domains has been produced (Quigg *et al* J Immunol 4553 (1998)). This has been used in the therapy of murine glomerulonephritis.

Known reagents, including sCR1, have short half-lives *in vivo* (minutes to hours), requiring frequent systemic administration and limiting their roles to the therapy of acute situations. They are not suitable for long-term use in the treatment of chronic disease.

5 In the aforementioned paper by Harris *et al* (2000), human DAF-Ig has been produced, in which DAF is tethered to the Fc fragment of an immunoglobulin molecule. Tests *in vitro* and *in vivo* have demonstrated that Ig fusion proteins, such as DAF-Igs, have anti-C activity. Subsequent *in vivo* tests have demonstrated the ability of fusion proteins to remain in the circulation and to inhibit plasma C activity over longer periods of time.

10

A confounding problem with current CReg-based anti-C reagents is that C activity is inhibited systemically. Although this may be of little consequence in acute situations, long-term reduction in systemic C activity is not desirable. This is because long-term inhibition of C may predispose individuals to infection, and also severely compromise the C-dependent process of immune complex solubilization and clearance.

15

We set out to overcome the problems of known reagents, specifically short half-life *in vivo* and systemic inhibition of C, by engineering a therapeutic reagent that is long-lived in the circulation and has little or no C inhibitory activity while in the circulation, but which can be activated at sites of inflammation and/or C activation. Such an anti-C “pro-drug” would offer advantages over current reagents, which treat acute situations of C activation, and allow for the first time use of an anti-C therapeutic reagent in the treatment of chronic illnesses in which C activation is implicated.

20

25 We have taken advantage of our observation that, in some CReg-Ig fusion proteins, the C regulator has much reduced or absent C regulatory function. Release of the CReg from the Ig moiety restores C regulatory capacity. Inclusion of one or more specific enzyme cleavage sites between the CReg and the hinge region of the Ig moiety can provide a therapeutic reagent that can possess the desirable properties of a long half-life *in vivo*, minimal systemic disturbance and efficient C regulation at the site of inflammation or disease.

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Summary of the Invention

Accordingly, the present invention seeks to provide therapeutic reagents having a long plasma half-life, minimal systemic effects and an efficient function at the site of pathology. Delivery to the appropriate site may be enhanced by the inclusion of targeting elements. These reagents include anti-C agents. These therapeutic agents differ from previously-described reagents, in that the intact therapeutic reagent or 'prodrug' is designed to express little or no systemic activity. Instead, sites are engineered between the active agent, which is a regulatory moiety, and a carrier moiety, such as an Ig, whereby the agent is released in an active form at the site of pathology to mediate its therapeutic effect. For example, inhibition of the C cascade at sites of inflammation can be achieved using a prodrug comprising a CReg attached *via* a cleavable sequence to a Ig Fc domain. The Ig moiety is chosen both to minimise C regulatory function of the attached CReg in prodrug form and to maximise the half-life of the CReg-Ig prodrug in the circulation. The therapeutic reagents may therefore be viewed as prodrugs when circulating in the body and active drugs following release of the CReg or other active agent at the target site.

In addition, the therapeutic reagent preferably further comprises a specific targeting sequence that can enhance delivery to the site of disease.

According to the present invention there is provided a therapeutic reagent to control one or more reactions of the immune system in a host, said therapeutic reagent comprising:

- i. at least one regulatory moiety that is an immunoregulatory protein (IRP) or a functional fragment thereof;
- ii. a carrier protein which renders said IRP inactive or substantially inactive; and
- iii. positioned therebetween at least one cleavage site;

whereby

when the regulatory moiety is at, or adjacent, target organ or tissue in the host, said cleavage site is cleaved, freeing the regulatory moiety from the carrier protein and restoring its regulatory activity.

5 According to a yet further aspect of the invention there is provided a therapeutic reagent that is inactive systemically comprising:

- i. at least one regulatory moiety that has a therapeutic activity;
- ii. a carrier protein which renders said regulatory moiety inactive or substantially inactive; and
- 10 iii. positioned therebetween at least one cleavage site characterised in that said cleavage site is a substrate for a matrix metalloproteinase (MMP) or an aggrecanase; whereby
at sites where MMP's or aggrecanase are active in the host said cleavage site is cleaved and said regulatory moiety is freed from the carrier protein and so
15 able to perform its therapeutic function.

According to a yet further aspect of the invention there is provided a therapeutic reagent to control one or more reactions of the immune system in a host, said therapeutic reagent comprising:

- 20 i. at least one regulatory moiety that is an immunoregulatory protein (IRP) or a functional fragment thereof;
- ii. a carrier protein which renders said IRP inactive or substantially inactive; and

- iii. positioned therebetween at least one cleavage site characterised in that said cleavage site comprises a substrate for at least one enzyme of the Complement system; whereby

when said therapeutic reagent is at or adjacent a site in the host where Complement is active said cleavage site is cleaved so freeing the immunoregulatory moiety from the carrier protein and enabling it to perform its immunoregulatory activity.

The term "regulatory moiety" is used to mean the part of the therapeutic reagent that acts or causes an effect in the host.

Preferably, the cleavage site is between the regulatory moiety and the carrier protein. More preferably, the cleavage site is positioned between the regulatory moiety/CReg and a hinge region of the carrier protein/Ig.

It is preferred that the cleavage site comprises an extrinsic moiety at which the therapeutic reagent can be cleaved under cleavage conditions. More preferably, it comprises an extrinsic amino acid or protein sequence, which is inserted between the regulatory moiety and the carrier protein. By extrinsic, is meant that the amino acid or protein sequence is not naturally a component of the regulatory moiety or the carrier protein and must therefore be inserted in the therapeutic reagent together with the regulatory moiety and carrier protein. Ideally, the cleavage site is susceptible to cleavage by enzymes of the matrix mettaloproteinase (MMP) and aggrecanase families. Secondly, it is susceptible to enzymes of the complement system. It therefore follows that a therapeutic reagent employing these latter cleavage sites will become active at locations where complement is active because complement enzymes will cleave the cleavage site and so release the regulatory moiety which can then function in a regulatory manner to achieve its therapeutic effect.

Nevertheless, the cleavage site may comprise intrinsic amino acids or proteins that are already present, for example as part of the carrier protein or regulatory moiety. Again,

the amino acids or proteins may be acted on by enzymes at a particular site so that the regulatory moiety and the carrier protein become cleaved, thereby enabling the therapeutic agent to act at a site in the host's body. However, in either case, it must be ensured that no cleavage site is present in the therapeutic reagent that would result in significant cleavage thereof to release the regulatory moiety in active form other than at or adjacent the target organ or tissue; which, in the instance where the cleavage site is susceptible to cleavage by complement enzymes would be at sites where complement was active.

Preferably the regulatory moiety is an immunoregulatory protein, such as a C regulatory protein (CReg). Alternatively, the regulatory moiety may be a regulatory protein involved in other types of immune response. Ideally, the CReg is an immunoregulatory protein that acts either as a decay accelerating factor or a cofactor for the plasma protease factor 1 or to inhibit formation of membrane attack complex. In one embodiment of the invention the regulatory moiety may also be a combination of CReg and other regulatory proteins. It is envisaged that the CReg may be any of those described (Morgan & Harris in Complement Regulatory Proteins (1999)), including those mentioned above, especially DAF, CD59, Crry, active fragments of CR1 and MCP, and may also include active fragments of factor H (FH) or other C regulators. Further, the regulatory moiety may comprise more than one active agent, such as more than one CReg. Alternatively, and more preferably, a therapeutic reagent comprising a single active agent may be co-administered with another therapeutic reagent containing a single, but different, regulatory moiety.

It is preferred that the carrier protein is an immunoglobulin (Ig) Fc fragment. Suitable Igs include IgG1, IgG2, IgG3 or IgG4, with IgG4 being a preferred Ig and IgG2 especially preferred. Modifications of the Ig to minimise activity of the prodrug-bound CReg, to extend plasma half-life or to minimise effector functions of the Fc are included. In one embodiment of the invention the Fab arms of the Ig may be replaced by two CReg moieties. Ideally, the immunoglobulin is human immunoglobulin.

The therapeutic reagent may comprise an immunoglobulin in which one arm comprises a CReg, while the other may comprises a targeting moiety such as a Fab specific for a certain cell or tissue or an adhesion molecule specific for a certain cell or tissue.

5 Targeted reagents may also include a Fab on one arm of the Ig while the other arm comprises a different regulatory moiety, such as a protein that modulates other types of immune response, *eg* an anti-cytokine agent or a cytokine receptor blocker.

It is preferred that the cleavage site of the therapeutic reagent is enzyme based.

10 The cleavage site may comprise a polypeptide/amino acid sequence in the prodrug susceptible to a specific enzymatic cleavage. The enzyme is that present at sites of inflammation or immuno pathology, for examples matrix metalloproteinases (MMPs) and/or aggrecanases. The cleavage site(s) in the therapeutic reagent can be cleaved by specific enzymes such as MMPs and aggrecanases at the target site to release the CReg,
15 or other active agent acting as an immunoregulatory moiety, from the carrier protein, such as an Ig Fc domain, in order to restore function of the active agent.

In a preferred aspect, the therapeutic reagent comprises a cleavage site that itself comprises a polypeptide/amino acid sequence incorporated between the regulatory
20 moiety/CReg and the hinge region of an Ig, the cleavage site being susceptible to cleavage by one or more enzymes, selected from: MMP3, MMP8 and other members of the MMP family, and those of the aggrecanase family. Examples of such are mentioned by Mercuri *et al* in J Biol Chem 274 32387 (1999). Several publications describe the preparation of recombinant aggrecanase, such as Tortorella *et al* in Science 284 1664
25 (1999) [aggrecanase-1] and Horber *et al* in Matrix Biol 19 533 (2000) Other enzymes expressed specifically or in increased abundance at the target site may also be utilised, with appropriate modification of the cleavage site.

In the Examples hereinbelow, a preferred cleavage sequence is a part of the inter-
30 globular-domain (IGD) of aggrecan, which comprises approximately 120 amino acids. Ideally said cleavage sequence comprises the minimum number of amino acids needed for cleavage to occur. It is preferred that the cleavage sequence for aggrecanase or

MMPs comprises in the range of from 17-75 amino acids. More particularly, the cleavage sequence may comprise the aggrecanase IGD cleavage site itself.

Preferred MMP or aggrecan cleavage amino acid sequences comprise:

IGD1 having the amino acid sequence RNITEGEARSVILTVK;

IGD2 having the amino acid sequence TTFKEEEGLGSVELSGL; and

IGD75 having the amino acid sequence: GYTGEDFVDIPENFFGVGG-

EEDITVQTVTWPDMEPLPRNITEGEARGSVILTVKPIFEVSPSPLEPEEPFTFAP.

It is preferred that the therapeutic reagent is functionally substantially inactive prior to cleavage. By 'substantially inactive' is meant that the therapeutic reagent has no, or at least a much reduced ability to act on the host, compared to when the regulatory moiety is in its free and/or solubilised state. In this state, the therapeutic reagent can therefore be described as a prodrug. In the case of the therapeutic reagent comprising a CReg, the reagent has a significantly reduced ability to act on the host's C system compared to when the CReg is not bound to carrier. For example, at least an order of magnitude reduction in activity can be observed, such as in the range of from a 10- to 60-fold reduction. When comparing molecules on a 'moles of CReg' basis (*ie* taking into account the mass of the Ig domains), not by mass of reagent, then:

- (1) Rat DAF-IgG1 has a 10-fold decrease in ability to regulate the classical pathway of complement, compared to soluble DAF (comprising four short consensus repeats [SCRs]);
- (2) Rat CD59-IgG1 has a 35-fold decrease in ability to regulate the terminal pathway of complement, compared to free soluble CD59;
- (3) Human DAF-IgG2 or DAF-IgG4 has a 10-fold decrease in activity (classical pathway), compared to soluble DAF (four SCRs); and
- (4) Human DAF (3 amino terminal SCR)-IgG2 has a 60-fold decrease in ability to regulate classical pathway, compared to soluble DAF (three SCRs).

When cleavage occurs, the therapeutic reagent is activated, by release of the immunoregulatory moiety/CReg from the carrier protein, so that, in the case of the

CReg, for example, it can participate in the control of C. The principle applies equally to other immune regulators delivered as Ig fusion proteins. By 'activated', we therefore mean that the activity of the CReg or other active agent is more or less equivalent to that of the agent in its non-prodrug-bound/soluble form.

5

For human DAF, in particular, it has been noted that the choice of antibody isotype greatly influences flexibility at the hinge region of the DAF-Ig fusion protein, which in turn influences the activity of the DAF *in vitro* and *in vivo*. Altering the activity of the regulatory moiety can be used to control its effect on the host when circulating in the body. Fusion to either IgG2 or IgG4 Fc domains has the most restrictive effect on function of DAF. This is likely to be due to steric hindrance around the hinge region. Similar principles will apply to the design of other prodrugs to provide reagents with markedly restricted function but that are activated upon removal of the Fc by cleavage.

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It is envisaged that the cleavage site is positioned between the regulatory moiety/CReg and a joining or hinge region of the carrier protein/antibody.

Preferably, the therapeutic reagent includes the minimal portion of the CReg or other agent necessary for function upon release.

20

A further embodiment of the invention relates to a targetable therapeutic reagent comprising a regulatory moiety-carrier protein prodrug as described above, in which one of the Fab arms of the Ig is replaced by a CReg or other immune regulatory molecule and the other by a targeting moiety comprising either a Fab or another protein that confers specific binding in the target tissue.

25

Accordingly, the present invention further provides a therapeutic reagent to control one or more pathologies in a host, said reagent comprising an immunoregulatory moiety and a carrier protein, characterised in that there is a cleavage site between the immunoregulatory moiety and the carrier protein, whereby, when the immunoregulatory moiety is at or adjacent a target in the host, cleavage of the therapeutic reagent occurs at

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the cleavage site, freeing the regulatory moiety from the carrier protein, wherein the therapeutic reagent is combined with a tissue or cell-specific targeting moiety.

Preferably, the targeting moiety is one or more membrane targeting molecule(s). These enable the therapeutic reagent to be localised to cell membranes. For example, a targeting moiety may comprise an addressin (described below) which is incorporated into the therapeutic reagent between the regulatory moiety and the cleavage site such that, following cleavage, the regulatory moiety and the addressin are released in a bound form, and the addressin is thus able to direct, or target, the regulatory moiety to a cell membrane.

For example, in the case of CReg-Ig fusion proteins, incorporation of a membrane-targeting molecule can yield therapeutic reagents that have minimal systemic anti-C activity, and that can bind to membranes but only become active when released at sites of expression of the relevant enzymes. Membrane targets might include adhesion molecules, or C fragments deposited in and around inflamed tissue.

As mentioned, membrane targeting may involve the engineering of a myristate group together with a stretch of negatively charged amino acids into the protein, termed an 'addressin' (Smith & Smith in Mol Immunol 38 249-55 (2001)). Together, these modifications confer upon the protein the propensity to associate with lipid membranes through insertion of the myristate and charge interactions of the amino acids with negatively charged phospholipid headgroups. Just one example of a CReg modified with an addressin is APT070, which comprises the three amino-terminal SCR of CR1 attached to an addressin at the carboxy terminus. Anti-C prodrugs modified in this way will bind lipid membranes and subsequent enzymatic cleavage will release active C regulator at tissue site, or visa versa. Additional targeting strategies may include the sLe^x carbohydrate moiety, a ligand for E- and P-selectins on activated endothelia.

Yet a further embodiment of the invention relates to DNA coding for a therapeutic reagent as described above. In particular, the invention provides a method for preparing such a therapeutic reagent, which method comprises ligating DNA molecules each

encoding the regulatory moiety, the carrier protein and the cleavage site comprising the therapeutic reagent and giving expression to the DNA sequence thereby encoding the therapeutic reagent.

5 Preferably, the therapeutic reagent protein is expressed in a eukaryotic system using a high expression vector. A preferred expression vector is pDR2ΔEF1α (as described by Charreau in Transplantation 58 1222 (1994)), although other vectors may also be used.

10 A further embodiment of the invention relates to a culture system comprising the cDNA encoding the therapeutic reagent protein as described above inserted in a high expression vector and transfected in CHO cells or other appropriate eukaryotic expression systems, including DNA encoding a regulatory moiety and a carrier protein, separated by DNA encoding a cleavage site.

15 According to a further aspect of the invention there is provided the use of a therapeutic reagent, as aforescribed, in the preparation of a medicament for the treatment of disease.

20 A further aspect of the invention includes a method of treating disease in a host, comprising administering a therapeutically effective amount of a therapeutic reagent according to this invention to the host.

It is preferred that the therapeutic reagent is suitable for treating humans and therefore a preferred host is man.

25 Diseases which may be treated include all those in which complement plays a role in pathology. Such diseases include inflammatory diseases, such as rheumatoid arthritis; immunological disorders *eg.* Arthus reaction; ischaemic disorders or cancer. Further conditions that may be treated include adult respiratory distress syndrome (ARDS),
30 systemic lupus erythematosus, multiple sclerosis and other demyelinating disorders, glomerulonephritis, ischemia-reperfusion injuries, such as stroke and myocardial

infarction, myaesthesia gravis, allergic reactions such as asthma and dermatological disorders, and rejection in transplantation.

5 It is envisaged that the therapeutic reagent may be administered systemically, such as *via* the intravenous, intramuscular or subcutaneous routes. Intravenous administration is particularly applicable where multiple sites in the body are involved, as, for example, in autoimmune disease. In some circumstances, the agent may be injected directly to a site of inflammation, such as intra-articularly in an inflamed joint in arthritis.

10 According to a further aspect of the invention there is provided a pharmaceutical composition including the therapeutic reagent, as aforescribed, which is combined with a pharmaceutically acceptable carrier, which carrier comprises those conventionally known in the art.

15 Although the invention has been described with particular reference to certain CRegs it is envisaged that the invention may apply to other regulators of the immune response, where the regulatory moiety is, for example, a molecule that can have immunoregulatory effects on the body. The reagents could be used for a range of diseases for both human and veterinary applications.

20

The invention will now be illustrated by the following Examples, in which reference is made to the accompanying Figures.

Brief Description of the Figures

25

Figure 1 shows the results of new studies of in vivo half-life of DAF-Ig and soluble DAF (sDAF). Radiolabelled DAF-Ig (●) or sDAF (○) was administered to rats, blood was removed at certain timepoints and protein bound radioactivity was determined. Results are expressed as percent of levels at 3 minutes and represent the means of five
30 animals $\pm$ SD.

Figure 2 illustrates the therapeutic effect of DAF-Ig on antigen induced arthritis, a rat model of rheumatoid arthritis. Methylated BSA was introduced into the right knee of immune rats. DAF-Ig (○) or saline control (●) was administered to the joint at the same time. Swelling of the joint was measured daily and compared to that of the left knee.

Results represent the mean of five animals $\pm$ SD. These results show a reduction in swelling and disease severity in treated compared with control animals from day 2 onwards. * $p < 0.01$, * * $p < 0.001$

Figure 3 shows new studies concerning in vitro complement regulatory function of DAF-Ig and the effect of cleavage by the enzyme, papain, that cleaves the DAF-Ig and releases active DAF. a) Antibody sensitised erythrocytes were incubated in GVB with rat serum and different concentrations of sCR1 (□), DAF-Ig (Δ), sDAF (∇) or a non-regulatory Ig fusion protein (O). b) shows results after treatment with papain.

Haemolysis was assessed by release of haemoglobin to the supernatant and percent lysis was determined. Results represent the mean value $\pm$ SD of three determinations.

Figure 4 a) shows the results of new studies concerning the in vitro complement regulatory function of CD59-Ig and the effect of using a spacer in CD59 fusion proteins. Guinea pig erythrocytes bearing C5b-7 sites were incubated in PBS/EDTA with rat serum and different concentrations of test protein. Results represent the mean value $\pm$ SD of three determinations showing the functional comparison of CD59-Ig (□), CD59-spacer-Ig (◇), a non-regulatory Ig fusion protein (O) and sCD59 (●). b) shows results after treatment with papain. As seen, cleaved CD59 activity is comparable with sCD59 activity.

Figure 5 shows an example of the results of a haemolytic assay showing the ability of different human DAF-Ig fusion proteins (DAF-G2, DAF-G4, S3-G4, S3-G2) to inhibit complement, compared with inhibition achieved by sCR1 and soluble DAF with no Fc attached. DAF-G2, DAF-G4, four SCR of DAF attached to Fc of IgG2 and IgG4 respectively; S3-G2, S3-G4, three SCR of DAF attached to Fc of IgG2 and IgG4

respectively; SCRs1-3, SCRs1-4, soluble DAF with three and four SCR domains respectively and no Fc.

5 **Figure 6** shows a schematic representation of a therapeutic reagent according to the invention having CReg and IgFc moieties, together with a cleavage site.

10 **Figure 7** shows the portions of IGD of aggrecan (from 17-75 amino acids) incorporated into DAF-Ig of the invention, between the antibody hinge and DAF. Single underlined: major MMP cleavage site (including MMP3 and MMP8). Double underlined: major aggrecanase cleavage site, also cleaved by MMP8. Dotted underlined: alternative aggrecanase cleavage site.

15 **Figure 8** DNA sequences encoding different lengths of the IGD of aggrecan were cloned into the expression vector between human DAF and human IgG4 hinge. Lane (1) no IGD, (2) IGD 1 (3) IGD 2 (4) a control 'scrambled' polypeptide sequence (5) 75 amino acids of IGD. Supernatant from expressing cells were subject to SDS-PAGE and Western blot. Blots were probed with (a) anti-human Fc or (b) anti-human DAF.

20 **Figure 9** Human DAF-Ig containing 75 amino acids of IGD was purified by protein A affinity chromatography and subjected to SDS-PAGE. The lower band represents DAF-Ig which is not disulphide bonded at the hinge, this is characteristic of human IgG4. Gel filtration studies indicate that the 'half-forms' are linked through non-covalent bonds under non-denaturing conditions.

25 **Figure 10** The prodrug shown in figure 9 (1.5 μ g) was digested with MMP3 or MMP8 and subjected to non-reducing SDS PAGE and Western blot. Blots were probed with polyclonal anti-human DAF. Lane 1: no enzyme; Lanes 2-4: 414, 138 and 46ng cdMMP3, respectively; Lanes 5-7; 300, 100 and 37ng MMP8 respectively.

Figure 11 shows a schematic of the cleavage sites on a therapeutic reagent following cleavage of the prodrug described in the invention, detected by anti-neoepitope antibodies. BC3, new N-terminus at site 1; BC4, new C-terminus at site 3; BC14, new N-terminus at site 3.

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Figure 12 shows a prodrug as shown in Figure 6, digested with MMP8 or aggrecanase and subjected to reducing SDS PAGE and Western blot. Anti-neoepitope antibodies were used to detect the new N-termini following cleavage as described above.

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Figure 13 shows detection of the new C-terminus of a prodrug as shown in Figure 6, following cleavage at the major MMP site.

Figure 14 shows cleavage of DAF (4 SCR)-IGD75-IgG4 with MMP3 and MMP8 using silver stained SDS PAGE gels.

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Figure 15 shows cleavage of DAF (4 SCR)-IGD1-IgG4 with MMP8 using silver stained SDS PAGE gels.

Figure 16 schematically shows human S3-DAF-Ig2 (three SCR of DAF attached to IgG2 Fc) incorporating a DIPEN cleavage site.

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Figure 17 graphically shows the results of functional tests using the prodrug of Figure 16.

Figures 18 to 22 show results of tests on the prodrug of Figure 16 as follows.

25

Figure 18: gel filtration to purify.

Figure 19: resistance of parent molecule to MMP3 cleavage at 37°C and stability of prodrug (no degradation) at 37°C.

Figure 20: cleavage of prodrug by incubation with MMP3 at various doses at 37°C for up to 24 hours.

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Figure 21: detection of neoepitopes following cleavage by MMP3.

Figure 22: detection with anti-DAF mAb of release of DAF from prodrug.

Figure 23 graphically shows restoration of function and inhibitory activity of the prodrug versus controls. Controls: comprised the prodrug without MMP3. Inset: gel to illustrate cleavage of prodrug by MMP3 at 3, 6 and 24 hours.

5

Figure 24 comprises three sets of results (A B and C) on the prodrug comprising 4 SCR of DAF, 75 amino acids of IgD and IgG4 Fc, to determine whether enzyme released from chondrocytes treated with inflammatory cytokines, rather than purified enzyme, could cleave the prodrug. As can be seen, in each instance, the enzymes are effective and the prodrug is activated.

10

EXAMPLES

Preparation of recombinant proteins

Method Example 1: - Rat DAF-Ig and CD59-Ig

DNA encoding the four SCRs of rat DAF was cloned into the expression vector SigpIg (R&D Systems) and that encoding the signal peptide and entire extracellular domain of CD59, omitting the GPI anchor signal sequence, was cloned into the vector pIgPlus (R&D Systems). In both cases DNA encoding the regulator was cloned upstream of and in frame with DNA encoding the hinge and Fc Domains of human IgG1. In order to achieve high levels of expression, DNA encoding the signal peptide, regulator and Ig domains was then sub-cloned using PCR into the high expression vector pDR2 Δ EF1 α . CHO cells were transfected using lipofectamine (Life Technologies) according to the manufacturer's instructions and stable lines were established by selection with 400 μ g/ml Hygromycin B (Life Technologies). Supernatant was collected and passed over a Prosep A column (Bioprocessing Ltd, Consett, UK) to purify the fusion protein. The column was washed with PBS and with 0.1M citrate buffer pH5.0 to remove contaminating bovine Ig and the fusion protein was eluted with 0.1M Glycine/HCl pH2.5. Eluted protein was neutralised with Tris, concentrated by ultrafiltration and dialysed into PBS.

Method Example 2:- Soluble Rat DAF and soluble CD59

DNA encoding the signal peptide and four SCRs of rat DAF (C-terminal residue Lys254) was cloned directly into the expression vector pDR2 Δ EF1 α . CHO cells were transfected as described above. sDAF was prepared by affinity chromatography on a monoclonal anti-DAF (RDII-24) column. Protein was eluted using 50mM diethylamine pH11 and immediately lyophilised. The dried protein was solubilised in phosphate buffer with 1M NaCl and was applied to a Superose 12 gel filtration column (Amersham-Pharmacia Biotech AB, Uppsala, Sweden). Proteins were eluted with PBS and fractions containing DAF were identified. The pure DAF was concentrated by ultrafiltration. Soluble CD59 containing the entire extracellular portion (omitting the

GPI anchor) was also produced in transfected CHO cells and was obtained from idENTIGEN^{cyf} (Cardiff, UK).

Method Example 3:- Control SCR Fusion Protein

5 A control SCR-containing fusion protein was also prepared in an identical manner to that of Example 1. This protein had no C-regulatory function.

Method Example 4: CD59-Spacer-Ig according to the Invention

10 A CD59-containing fusion protein was also prepared in which the amino acids (Ser-Gly-Gly-Gly-Gly)₂-Ser were inserted between CD59 and the antibody hinge using two stage PCR. Briefly, DNA encoding CD59 and the Ig domains was reamplified in two separate reactions using new primers that incorporated the sequence of the spacer domain at the 3' end of CD59 and at the 5' end of the Ig hinge. The two PCR products were mixed together and allowed to anneal at complementary DNA sequences encoding the spacer domain. Following PCR using outside primers, the product was ligated into the expression vector pDR2 Δ EF1 α . Cells were transfected and the second CD59-Ig protein was purified as described above. Protein concentrations were determined using Pierce Comassie assay (Perbio Science UK Ltd, Tattenhall, UK) using bovine serum albumin as a standard.

20

CD59-Ig has a mass of 77Kda, CD59-spacer-Ig has a mass of 78.5Kda and DAF-Ig has a mass of 122Kda, these masses being confirmed by mass spectrometry.

Method Example 5:- Human DAF-IgG2 and Human DAF-IgG4 according to the invention

25

Human DAF-IgG1 was generated as described by Harris CL et al. (2000), Immunology, 100, 462. Fusion proteins consisting of human DAF and either IgG2 or IgG4 hinge were generated as follows:

30

DNA encoding the hinge and Fc of human IgG4 or IgG2 were amplified by RT-PCR from human peripheral blood lymphocyte RNA. The amino terminal sequences of the antibody hinges are as follows:

IgG4 short hinge: KYGPPC ...
 IgG4 long hinge: VDKRVES ...
 IgG2: ERKCCV ...

5 Primers incorporated restriction sites to enable later ligation into a high expression vector pDR2 Δ EF1 α (BamH1 at the 5' end and EcoRV at the 3' end). DNA encoding the signal peptide and either the first three or four SCR domains of hDAF was amplified by PCR using plasmid containing DAF sequences as a template. The carboxy-terminal sequences of the DAF domains are as follows:

10

3SCR form: ... PECREIY
 4SCR form (with IgG4 long hinge): ... KSLTSK
 4SCR form (with IgG4 short hinge and IgG2): ... PPPECRG

15 Amplified DNA was separated from the template by electrophoresis on an agarose gel. The insert was extracted from the gel, cut with suitable restriction enzymes at sites encoded on the PCR primers (BamH1 at the 3' end and XbaI at the 5' end) and ligated into pDR2 Δ EF1 α upstream of and in frame with DNA encoding the hinge and Fc domains of the human immunoglobulin. DNA proof-reading polymerase was used in
 20 the PCR reactions and sequencing confirmed that no errors had been introduced by PCR. CHO cells were transfected using lipofectamine (Life Technologies) according to the manufacturer's instructions and stable lines were established by selection with 400 μ g/ml Hygromycin B (Life Technologies). Supernatant was collected and passed over a Prosep A column (Bioprocessing Ltd, Consett, UK) to purify the fusion protein.
 25 The column was washed with PBS and with 0.1M citrate buffer pH5.0 to remove contaminating bovine Ig and the fusion protein was eluted with 0.1M Glycine/HCl pH2.5. Eluted protein was neutralised with Tris, concentrated by ultrafiltration and dialysed into PBS. The purified proteins were analysed by SDS PAGE.

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Functional Assay Protocols

Protocol Example 1: - Functional Analysis of DAF-Ig and sDAF

5 In order to assess function of rat DAF, antibody coated sheep erythrocytes (E; 2% (v:v)) were prepared by incubating cells in PBS for 30 minutes with 1/500 dilution of rabbit anti-sheep E (Amboceptor, Behring Diagnostics GmbH). Sensitised E were washed three times in GVB (Gelatin Veronal Buffer comprising CFD [C-fixation Diluent with added 0.1% (w/v) gelatin (Immunol 100 463 (2000))]) and re-suspended to 2%. In order
10 to determine a concentration of rat serum giving partial lysis (50-80%), antibody coated sheep E (EA) were incubated for 30 minutes at 37°C with different dilutions of serum. Following pelleting of cells by centrifugation, amount of lysis was quantitated by adding an aliquot of supernatant (50 µl) to water (100 µl) and measuring absorbance at 415nm. Control samples were prepared by adding buffer only (0% control) or 0.1% Triton X100
15 (100% control) to the E instead of serum. % lysis was calculated as follows: % lysis = $100 \times (A_{415} \text{ sample} - A_{415} 0\% \text{ control}) / (A_{415} 100\% \text{ control} - A_{415} 0\% \text{ control})$. To test function of the recombinant inhibitors, EA were incubated with the predetermined dilution of rat serum giving 50-80% lysis and different dilutions of the test protein. Following incubation at 37°C, % lysis was determined as described above.

Protocol Example 2: - Functional Analysis of CD59

20 Guinea pig E(GPE) were washed and re-suspended in GVB at 2% (v:v). These were incubated for 30 minutes at 37°C with an equal volume of 25% (v:v) normal human serum from which C8 had been depleted by passage over a monoclonal anti-C8 affinity
25 column. The resulting cells (GPE-C5b7) were washed and re-suspended at 2% in PBS.10mM EDTA. The amount of rat serum giving 50-80% lysis was determined by incubating GPE-C5b7 for 30 minutes at 37°C with dilutions of rat serum in PBS/EDTA. In order to assess function of soluble CD59, GPE-C5b7 were incubated in PBS, EDTA with dilutions of the test reagent and the predetermined concentration of rat serum. 0%
30 and 100% controls were included and %lysis was determined as described above.

Illustrative Results of Known Studies

5 Illustrative Example 1: - In vivo clearance of DAF-Ig is slowed compared to sDAF

In order to study the effect of the Fc domain (immunoglobulin crystallisable fragment) on clearance of soluble DAF (sDAF), DAF-Ig and sDAF were radiolabelled with ¹²⁵I. Animals were administered with a single dose of either reagent and samples of blood were removed at certain timepoints. Protein was precipitated using TCA and protein
10 bound counts were measured in a gamma counter. At 1 hour following administration, sDAF levels were down to 20% of that seen at 3 minutes, DAF-Ig levels were still 80% of those at 3 minutes, demonstrating the enhancement of half life as a consequence of fusion to Ig domains (**Figure 1**).

15 Illustrative Example 2: - DAF-Ig delays onset and inhibits progression in antigen induced arthritis (AIA)

AIA was induced in Wistar rats (Goodfellow et al, Clinical and Experimental Immunology (1997), 110, 45). Briefly, methylated BSA (mBSA) was introduced into the right knee of five rats pre-immunised with mBSA; 0.45mg DAF-Ig or the same
20 volume of saline (control animals) was included with the antigen. Disease progression was monitored by comparing swelling of the right knee to that of the left, over the course of a week. Rat DAF-Ig caused a significant reduction in swelling and disease severity compared to control animals from day 2 onwards (**Figure 2**). These results indicate the long-term effects of DAF-Ig fusion proteins in vitro.

25

Results of New Studies Relating to the Invention

Example 1:- *In vitro* functional analyses of DAF-Ig, sDAF, CD59-Ig and CD59 – Cleavage by Papain

30 The ability of DAF-Ig and sDAF to inhibit the classical pathway of C was analysed using a haemolysis assay and was compared to inhibition of lysis achieved with sCR1. Both sDAF and sCR1 were powerful inhibitors of lysis, while DAF-Ig showed a reduced

ability to inhibit lysis (**Figure 3**). Tests using papain to cleave Ig from DAF indicated that the functional activity of DAF *in vitro* could be restored by removal from the Ig. This was shown by the fact that DAF released from Ig domains by digestion with papain, had identical activity to sDAF secreted from CHO cells.

The ability of CD59-Ig and CD59-spacer-Ig to inhibit C was also tested using haemolysis assays specific for the terminal pathway. Again, the fusion protein showed a much lowered ability to inhibit MAC formation when compared to CD59 released from CD59-Ig using papain. This, like the DAF analysis, indicated that cleavage of the Ig from the CD59 increased activity (**Figure 4**). Further, the presence of the spacer domain indicated its ability to modify the activity of the regulatory moiety, and implicated steric factors in loss of regulatory function in the prodrug. The presence of a spacer domain enhanced regulatory function of CD59-Ig although activity was still low compared to sCD59.

Figures 3 and 4 show that both DAF-Ig and CD59-Ig had less complement regulatory capacity than the soluble forms lacking the Fc. It is likely that this is due to steric constraints in which the active site of the regulatory proteins cannot access and bind the large multimolecular substrate, be it the C3/C5 convertase or MAC. This is supported by the observation that enzymatic removal of the Fc domains restores full function to the released regulatory protein.

In addition to modification of activity of a therapeutic reagent using a spacer domain as described above and shown on **Figure 4**, the type of antibody used as the carrier protein can influence the effect of a therapeutic reagent. This is presumably due to the steric influence of variations in the hinge region of an antibody. Studies as shown in **Figure 5**, indicate that Ig with less flexible hinge regions, for example IgG2, cause more restriction on the functional activity of a linked therapeutic reagent *in vitro*. Deletion of the fourth SCR of human DAF further restricted functional activity of the CReg (**Figure 5**).

Example 2: Preparation of New Fusion Proteins incorporating IGD1, IGD2 and IGD75 Cleavage Sites

Figure 6 shows a diagrammatic representation of a prodrug form of a therapeutic reagent according to the invention, showing the position of targeted enzyme cleavage sites between a regulatory moiety and the hinge region of a carrier protein.

5

In this Example, studies were made using cleavage sites appropriate to enzymes involved in inflammatory disease such as arthritis. MMPs and aggrecanases are involved in inflammation of the joints and they destroy cartilage by proteolysis of the major constituent proteoglycan, aggrecan. Polypeptides containing three different cleavage sites for some of these enzymes (MMP3, MMP8 and aggrecanase (ADAM-TS4)) were incorporated into CReg-Ig fusion proteins between the CReg and the hinge.

10

The length of the polypeptide was restricted to 17 amino acids each (termed here IGD1 and IGD2, each incorporating a different cleavage site) or 75 amino acids (termed here IGD75, incorporating two cleavage sites: the site from IGD1 and another site). Scrambled IGD is a control sequence containing no cleavage sites. **(Sequences shown in Figure 7).**

15

In the case of IGD1, the amino acid sequence is RNITEGEARGSVILTVK;
IGD2 has the amino acid sequence TTFKEEEGLGSVELSGL; and
IGD75 has the amino acid sequence: GYTGEDFVDIPENFFGVGGEE-
DITVQTVTWPDMEPLPRNITEGEARGSVILTVKPIFEVSPSPLEPEEPFTFAP.

20

To incorporate the enzyme sites, complementary DNA oligomers encoding the short IGD sites with suitable restriction sites at both ends were used (BamH1). These were annealed together, restricted with BamH1 and ligated into the expression vector between DNA encoding DAF and the antibody hinge. The longer stretch of DNA encoding 75 amino acids of IGD was amplified using PCR from a plasmid template and similarly ligated into the vector at the BamH1 site (primers incorporated the restriction site). CHO cells were transfected as described above and the culture supernatant was collected. **Figure 8** shows Western Blot analysis using anti-human Ig (goat anti-human Fc – HRPO conjugated; 1 : 1000 dilution, available from Sigma) to demonstrate the

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presence of DAF-Ig in culture supernatant which contained target enzyme cleavage sites. In the case of aggrecan, several portions of the IGD can be included. **Figure 9** shows a SDS-PAGE analysis of the purified DAF-Ig prodrug. **Figure 10** shows digestion of the DAF-Ig prodrug with MMP3 and MMP8 enzymes and detection of released DAF in a western blot.

A schematic showing various cleavage sites of a therapeutic reagent according to the invention and their detectability by anti-neo-epitope antibodies is shown in (**Figure 11**).

Secreted fusion proteins were purified by Protein A affinity chromatography. The fusion protein containing IGD 75 was further purified by gel filtration on a Superose 12 gel filtration column (Amersham-Pharacia Biotech AB), equilibrated with 50mM Tris pH 7.5, 100mM NaCl, 10mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (**Figure 9**). The eluted protein was digested with MMP3, MMP8 and aggrecanase (**Figure 10**).

The therapeutic reagent (3.5 μg) was incubated at 37°C for 24 hours with 0.3 μg neutrophil MMP8 (Calbiochem) or with recombinant aggrecanase. The sample was lyophilised, re-dissolved in reducing SDS PAGE loading buffer and subjected to SDS PAGE and Western blot (1 μg protein/lane). The blots were probed with anti-neoepitope antibodies that recognise the 'cut-ends' of aggrecan. Primary antibodies were detected with HRPO-linked secondary antibodies and bands were visualised using enhanced chemiluminescence (ECL). The antibody BC3 (Hughes *et al* Biochem J 305 700 (1995)) recognises the new N-terminus formed following cleavage at the major aggrecanase cleavage site (site 1 in **Figure 11**); the antibody BC14 (Caterson *et al* in Acta Orthop Scand Suppl 266 121 (1995)) recognises the new N-terminus formed following cleavage at the major MMP site (FFG - site 3 in **Figure 11**). BC13 recognises the new C-terminus following cleavage at the major aggrecanase site (EGE). BC4 recognises the new C-terminus created following cleavage at the major MMP site (PEN – site 3 in Figure 7). The antibodies were used to probe the blots at 1 : 100 (tissue culture supernatant). Cleavage of the prodrug by MMP8 at both enzymes sites was detected (**Figure 12**, lanes 1 and 2) and also cleavage by aggrecanase at the aggrecanase site (**Figure 12**, lane 3).

The therapeutic reagent in prodrug form was digested with MMP8 and analysed by Western blot as described above. The blot was probed with an anti-neo-epitope antibody that recognises the new C-terminus formed following cleavage at the major MMP site, BC4 recognises the new C-terminus formed following cleavage at the major MMP site (PEN – site 3); this protein fragment comprises hDAF and a small stretch of aggrecan IGD (**Figure 13**).

The results shown in **Figures 11 to 13** demonstrate that short enzyme sites can be incorporated into Ig-fusion proteins and that the active agent can be released following cleavage by the target enzyme. **Figure 14** shows a silver-stained SDS-PAGE analysis of the cleavage by MMP8 and MMP3 of the prodrug comprising DAF attached to the IgG4 Fc via the IGD75 linker (**Figure 7**). **Figure 15** shows a silver-stained SDS-PAGE analysis of the cleavage by MMP8 of the prodrug comprising DAF attached to the IgG4 Fc via the IgD1 linker (**Figure 7**).

Example 3: Generation of S3-DAF-IgG2 prodrug containing 'DIPEN' enzyme site

DNA encoding the hinge (starting amino acids ERKCCV...), CH2 and CH3 domains of human IgG2 was amplified by RT-PCR from peripheral blood mononuclear cell total RNA and ligated into the high expression vector PDR2 Δ EF1 α (Charreau *et al* in Transplantation 58 1222 (1994)). DNA encoding the signal peptide and first three SCR of human DAF (finishing amino acids ...CREIY) was amplified by PCR from plasmid template and ligated into the vector upstream of and in frame with DNA encoding the antibody hinge, as described in Method Example 5. CHO cells were transfected and fusion protein (S3 DAF-IgG2; also termed 'parent' molecule, not having cleavage site) was purified as described in Method Example 1. A prodrug form (termed the 'DIPEN prodrug') of the reagent was prepared as described in Example 2 using BamH1 restriction sites by inserting DNA (purchased oligomers) encoding the enzyme site here termed 'DIPEN' between DNA encoding DAF and the Ig. The sequence of the inserted

enzyme site is GEDFVDIPENFFGVGGEED; this is illustrated in **Figure 16** where the cleavage site is indicated (immediately upstream of the DIPEN sequence). This site is cleaved by most MMPs.

5 Functional activity of S3 DAF-IgG2 and corresponding DIPEN prodrug

S3 DAF-IgG2 (parent) and the DIPEN prodrug were purified by protein A affinity chromatography and gel filtration on a Superose 12 column as described in Example 2. Functional activity was assessed by inhibition of lysis of antibody coated sheep
10 erythrocytes (EA) essentially as described in Protocol Example 1, ensuring that the buffer composition (GVB, PBS or Tris/Ca²⁺) was equivalent in each incubation (**Figure 17**). Control incubations included a non-regulatory SCR-containing fusion protein (negative control) and a three SCR form of DAF produced in yeast. Calculation of IH₅₀ and adjustment for molarity (equivalent moles of DAF) indicated that the DIPEN
15 prodrug was approximately 20 fold less active than the three SCR form of DAF. Incorporation of the enzyme site acted as a 'spacer' domain and restored some activity to S3 DAF-IgG2 (compare 'parent' to DIPEN prodrug).

20 Stability of S3 DAF-IgG2 and DIPEN prodrug

Both reagents were gel filtered into Tris/NaCl/Ca²⁺ as described in Example 2 (**Figure 18**, filtration of prodrug), and incubated for 24 hours at 37°C. S3 DAF-IgG2 was incubated in the presence of MMP3 (Calbiochem, recombinant catalytic domain) to
25 assess non-specific cleavage by the target enzyme. Aliquots were removed from the incubations at the specified time-points and stored frozen in reducing SDS PAGE loading buffer until the end of the experiment. Samples were run on a 10% gel and silver stained according to the method of Morrissey in Anal Biochem 117 307-10 (1980) (**Figure 19**). Both molecules were stable stored at 37°C, the parent molecule was also
30 stable in the presence of MMP3.

Cleavage of DIPEN prodrug with MMP3

The prodrug was incubated for 1, 2.5, 5, 7.5 and 24 hours with MMP3 at the concentrations specified in the following table.

5

Prodrug (µg/ml)	MMP3 (µg/ml)	Ratio Prodrug : MMP3
100	10	10 : 1
100	2	50 : 1
200	2	100 : 1

Aliquots of each incubation were analysed by silver staining as described above. The prodrug was cleaved by MMP3 even at ratios of 100:1 (w:w) (**Figure 20**). The upper cleavage band ($\approx 35\text{kDa}$) represents the released Fc domains (confirmed by Western blot); the lower cleavage product ($\approx 30\text{kDa}$) is the released DAF (three SCRs). The DIPEN prodrug was also cleaved by MMP8 (not shown) and could therefore form a target for a multitude of metalloproteases.

10

Western blot detection of released DAF and neo-epitope formation

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In order to confirm that the prodrug had been cleaved at the metalloprotease site, portions of the incubations described above (**Figure 20**) were run on an 11% reducing SDS PAGE gel, Western blotted and probed with BC14 to detect the neo-epitope (antibody 'side') formed following cleavage (as described in Example 2 and **Figure 11**). BC14-reactive neo-epitope was detected following incubation of DIPEN prodrug with MMP3, but not when the prodrug was incubated alone (prodrug control) or when the parent molecule was incubated with MMP3 (**Figure 21**). Samples from a similar incubation (DIPEN prodrug at $200\mu\text{g/ml}$, MMP3 at $5\mu\text{g/ml}$) were analysed by non-reducing SDS PAGE and Western blot using a monoclonal anti-DAF antibody. The prodrug was incubated in the absence of MMP3 as a control. Released DAF was detected when the prodrug was incubated with enzyme (**Figure 22**).

20

25

Restoration of function following cleavage

5 In order to demonstrate that incubation of the DIPEN prodrug with MMP3 restored complement-regulatory activity, the following incubations were set up:

- (1) Prodrug (200µg/ml)
- (2) Prodrug (200µg/ml); MMP3 (5µg/ml)
- (3) BSA (200µg/ml); MMP3 (5µg/ml)

10

Proteins were incubated for up to 24 hours and analysed by SDS PAGE and silver stain (inset to **Figure 23**). 3 hour and 6 hour incubations of prodrug (with and without MMP3) and 6 hour incubation of BSA (with MMP3) were analysed by haemolysis assay as described in Protocol Example for complement-regulatory activity. Per cent lysis and inhibition (compared to negative control: non-regulatory fusion protein) were calculated (15 **Figure 23**). MMP3 in the BSA incubation had no effect on complement-mediated lysis of EA. As can be seen in **Figure 23**, incubation of the DIPEN prodrug with MMP3 for 3 hours restored almost all DAF activity.

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Cleavage of anti-complement prodrug using native enzyme release from activated chondrocytes

25 In order to determine whether enzyme released from chondrocytes treated with pro-inflammatory cytokines, rather than purified enzyme, could cleave the prodrug, experiments were carried out using the therapeutic reagent described in **Example 2** (comprising 4 SCRs of human DAF fused to human IgG4 and 'IGD75' as the inserted enzyme site (**Figure 7**)).

30

By a method analogous to that described by Hughes *et al* in J Biol Chem 272 20269 (1997)), pig chondrocytes were embedded in agarose and cultured in the presence of

various cytokines (retinoic acid, IL-1, TNF- α) and prodrug. After 4 days, media samples were dialysed against water, lyophilised to dryness and reconstituted with reducing SDS- PAGE loading buffer containing 10% (v/v) mercaptoethanol. Samples were separated on 10% SDS-PAGE gels, transferred to nitrocellulose membranes and Western blot analysis was performed with anti-neo-epitope antibodies. BC3 detects cleavage at the aggrecanase site, whereas BC4 (Hughes *et al* Biochem J 305 700 (1995)) and BC14 detect cleavage at the MMP site (Figure 11).

Figures 24A illustrate cleavage of the prodrug by aggrecanase released from cultures stimulated with either retinoic acid, IL-1 α or TNF using 3.4 μ g of prodrug and anti-ARG (BC3) monoclonal (1:100). Cleavage of the prodrug at the aggrecanase site was only evident in the presence of stimulatory cytokines.

In addition, **Figure 24B** illustrates an IL-1 dose-dependent cleavage by aggrecanase of prodrug, detected using BC3. **Figure 24C** illustrates cleavage at the MMP site using BC14; cleavage was evident in the presence of stimulating cytokines.

Conclusion

These data illustrates the preparation of a new prodrug, ideally based on an IgG2 backbone, and containing a short cleavage site for metalloproteases. Whilst insertion of the enzyme site into the 'parent' molecule restored some function, the prodrug showed a marked reduction in activity compared to released DAF. This enzyme site could be further truncated to retain as much inhibition of function as possible in the prodrug; the parent molecule (having no enzyme site) showed almost two logs reduction in function. The DIPEN prodrug was susceptible to cleavage by several metalloproteases tested; release of DAF and formation of neo-epitopes following digestion was demonstrated by silver stain and Western blot. The cleavage reaction was almost complete as assessed by silver stain. Incubation of the prodrug with MMP3 restored almost complete complement-regulatory function to the reagent. Importantly, analysis of the cleavage of these reagents using native enzyme released from target cells, chondrocytes is given.

Using a culture system, cleavage of DAF-IGD75-IgG4 at both the metalloprotease and aggrecanase site has been demonstrated. Cleavage was triggered using various pro-inflammatory cytokines.

CLAIMS

1. A therapeutic reagent to control one or more reactions of the immune system in a host, said therapeutic reagent comprising:

- 5 i. at least one regulatory moiety that is an immunoregulatory protein (IRP) or a functional fragment thereof;
- ii. a carrier protein which is an antibody, or a fragment thereof, and which renders said IRP inactive or substantially inactive; and
- iii. positioned therebetween at least one cleavage site;

10 whereby

when the regulatory moiety is at, or adjacent, a target organ or tissue in the host, said cleavage site is cleaved, freeing the regulatory moiety from the carrier protein and restoring its regulatory activity.

15 2. A therapeutic reagent that is inactive systemically comprising:

- i. at least one regulatory moiety that has a therapeutic activity as an immunoregulatory agent;
- ii. a carrier protein which renders said regulatory moiety inactive or substantially inactive; and
- 20 iii. positioned therebetween at least one cleavage site characterised in that said cleavage site is a substrate for a matrix metalloproteinase (MMP) or an aggrecanase; whereby

at sites where MMP's or aggrecanase are active in the host said cleavage site is cleaved and said regulatory moiety is freed from the carrier protein and so able to perform its therapeutic function.

5 3. A therapeutic reagent to control one or more reactions of the immune system in a host, said therapeutic reagent comprising:

i. at least one regulatory moiety that is an immunoregulatory protein (IRP) or a functional fragment thereof:

ii. a carrier protein which renders said IRP inactive or substantially inactive; and

10 iii. positioned therebetween at least one cleavage site characterised in that said cleavage site comprises a substrate for at least one enzyme of the Complement system; whereby

when said therapeutic reagent is at or adjacent a site in the host where Complement is active said cleavage site is cleaved so freeing the
15 immunoregulatory moiety from the carrier protein and enabling it to perform its immunoregulatory activity.

4. A therapeutic reagent according to Claim 1 or Claim 3, wherein the immunoregulatory protein (IRP) is a complement regulatory protein (CReg) or a functional fragment
20 thereof.

5. A therapeutic reagent according to Claim 4, wherein the (CReg) acts either as: a decay accelerating factor, or a cofactor for the plasma protease factor I, or to inhibit formation of membrane attack complex, or a combination thereof.

6. A therapeutic reagent according to any preceding Claim, wherein said reagent has more than one regulatory moiety, two of which have different activities.

5 7. A therapeutic reagent according to any preceding Claim, wherein said cleavage site is an enzymatically cleavable site.

8. A therapeutic reagent according to any preceding Claim, wherein said cleavage site is a substrate for an enzyme of the Complement system.

10

9. A therapeutic reagent according to any preceding Claim, wherein said cleavage site comprises a plurality of cleavage sites.

10. A therapeutic reagent according to any preceding Claim, wherein at least one of said
15 cleavage sites comprises a substrate for a matrix metalloproteinase (MMP) or an aggrecanase.

11. A therapeutic reagent according to Claim 10, wherein said cleavage site comprises a substrate for MMP3 or MMP8.

20

12. A therapeutic reagent according to any preceding Claim, wherein at least one of said cleavage sites comprises a part of the inter-globular-domain (IGD) of aggrecan.

13. A therapeutic reagent according to Claim 12, wherein said cleavage site comprises

17-75 amino acids of said IGD.

14. A therapeutic reagent according to Claim 12, wherein the cleavage site comprises the minimal aggrecanase cleavage site in said aggrecan IGD (inter globular domain).

5

15. A therapeutic reagent according to Claim 10, wherein the cleavage site includes the amino acid sequence DIPEN.

10

16. A therapeutic reagent according Claim 10 or Claim 15, wherein the cleavage site includes the amino acid sequence GEDFVDIPENFFGVGGEED.

17. A therapeutic reagent according to Claim 10, wherein the cleavage site includes the amino acid sequence RNITEGEARGSVILTVK.

15

18. A therapeutic reagent according Claim 10, wherein the cleavage site includes the amino acid sequence TTFKEEGLGSVELSGL.

19. A therapeutic reagent according to Claim 10, wherein the cleavage site includes the amino acid sequence

20

GYTGEDFVDIPENFFGVGGEEDITVQTVTWPDMEPLPRNITEGEARGSVILTVK
PIFEVSPSPLEPEEPFTFAP.

20. A therapeutic reagent according to Claims 2-19, wherein the carrier protein is an antibody, or a fragment thereof.

21. A therapeutic reagent according to Claim 20, wherein said antibody is human immunoglobulin IgG4, IgG2 or IgG1.

5 22. A therapeutic reagent according to Claim 20 wherein one or more Fab arms of said immunoglobulin contains, or is replaced by, a further regulatory moiety.

23. A therapeutic reagent according to Claim 20, 21 or 22, wherein one or more arms of the Fab of the immunoglobulin contains or is replaced by a targeting moiety.

10

24. A therapeutic reagent according to Claim 23, wherein the targeting moiety comprises one or more membrane targeting molecules.

15

25. A therapeutic reagent according to Claim 24, wherein the targeting moiety comprises one or more addressins that is incorporated between the regulatory moiety and the cleavage site so that, following cleavage, said addressin directs the regulatory moiety to its target site.

20

26. A therapeutic reagent according to Claim 25, wherein the addressin is APT542.

27. A therapeutic reagent according to any preceding claim, wherein the cleavage site is positioned between the regulatory moiety and a hinge region of the carrier protein.

28. A method of making a therapeutic reagent according to any preceding Claim, comprising expressing protein from cells transformed or transfected with at least one nucleic acid molecule encoding said therapeutic reagent.

5 29. Use of a therapeutic reagent according to Claims 1 - 27, in the preparation of a medicament for the treatment of disease.

10 30. Use according to Claim 29 wherein the disease to be treated includes one or more of: inflammatory, immunological, traumatic, ischaemic or disorders in which Complement contributes to pathology such as rheumatoid arthritis, systemic lupus erythematosus, glomerulonephritis, multiple sclerosis, adult respiratory distress syndrome (ARDS), ischemia-reperfusion injury, demyelination, myasthenia gravis, Arthus reaction or rejection in transplantation

15 31. A pharmaceutical composition including a therapeutic reagent according to Claims 1-27, which is combined with a pharmaceutically acceptable carrier.

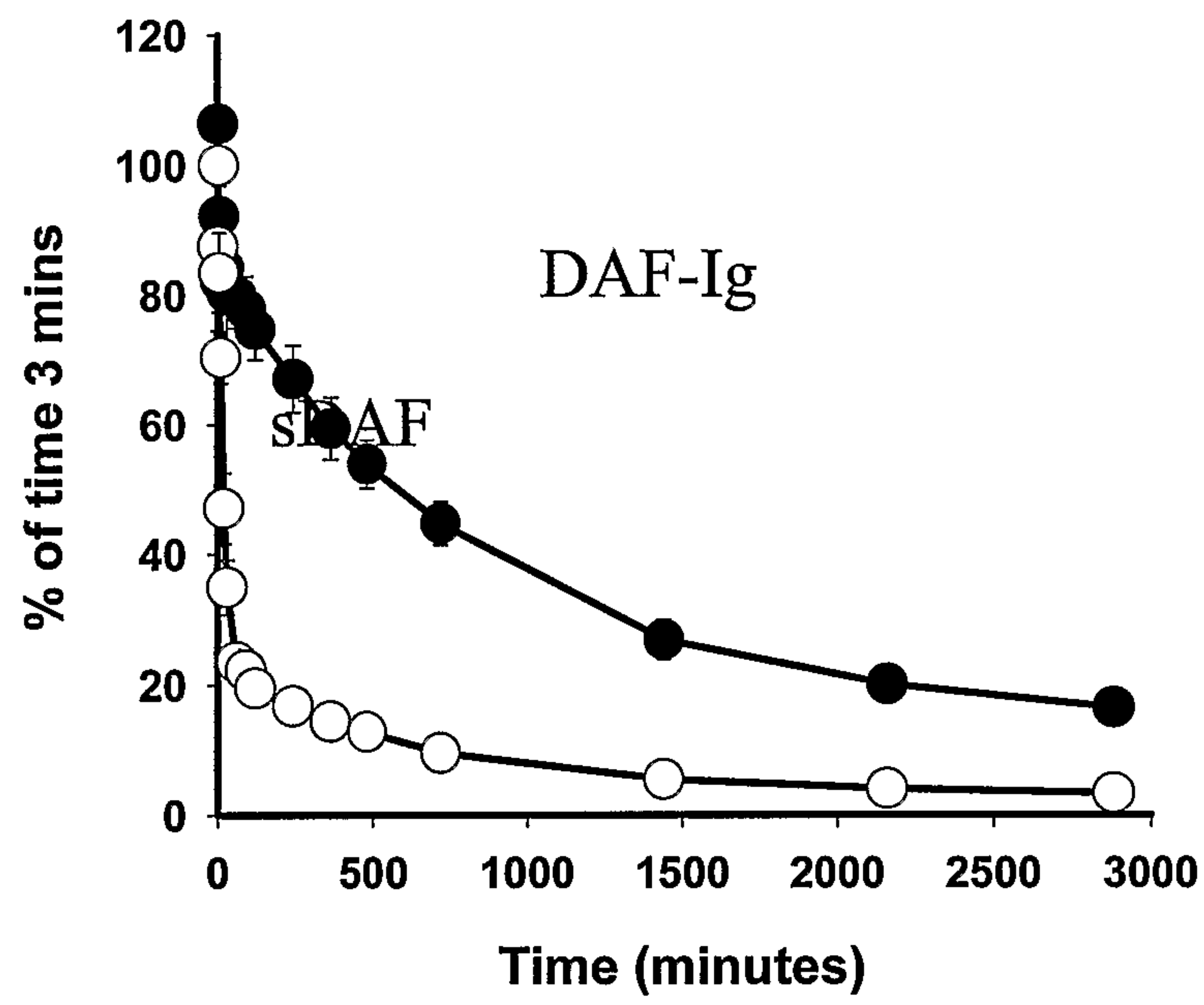
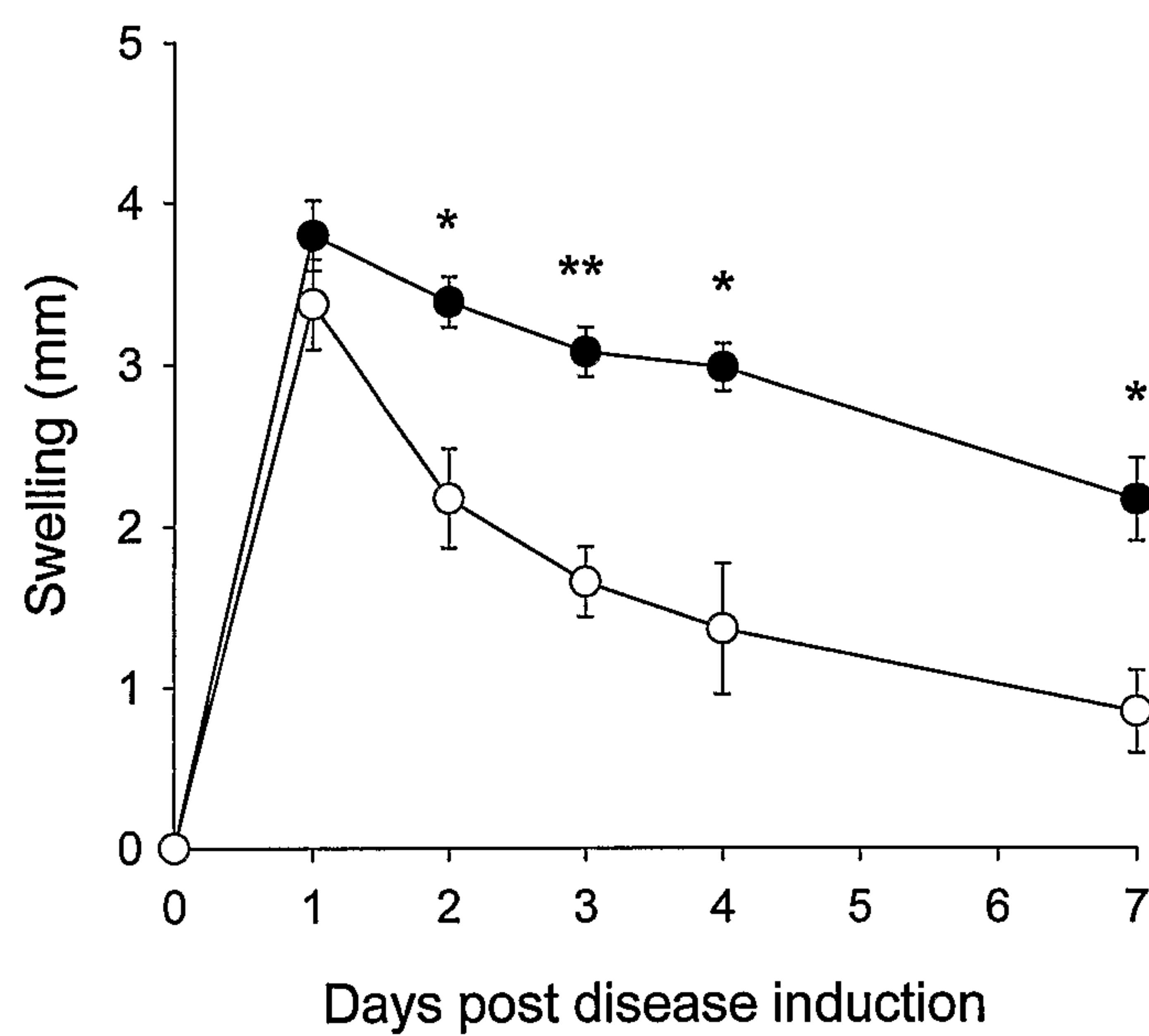
20 32. A method of treating an individual comprising administering to said individual a therapeutic reagent according to Claims 1-27 or a pharmaceutical composition according to Claim 30.

33. A therapeutic reagent, pharmaceutical composition, Use and Methods as substantially described herein and as illustrated by, and with reference to, the accompanying figures.

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Figure 1

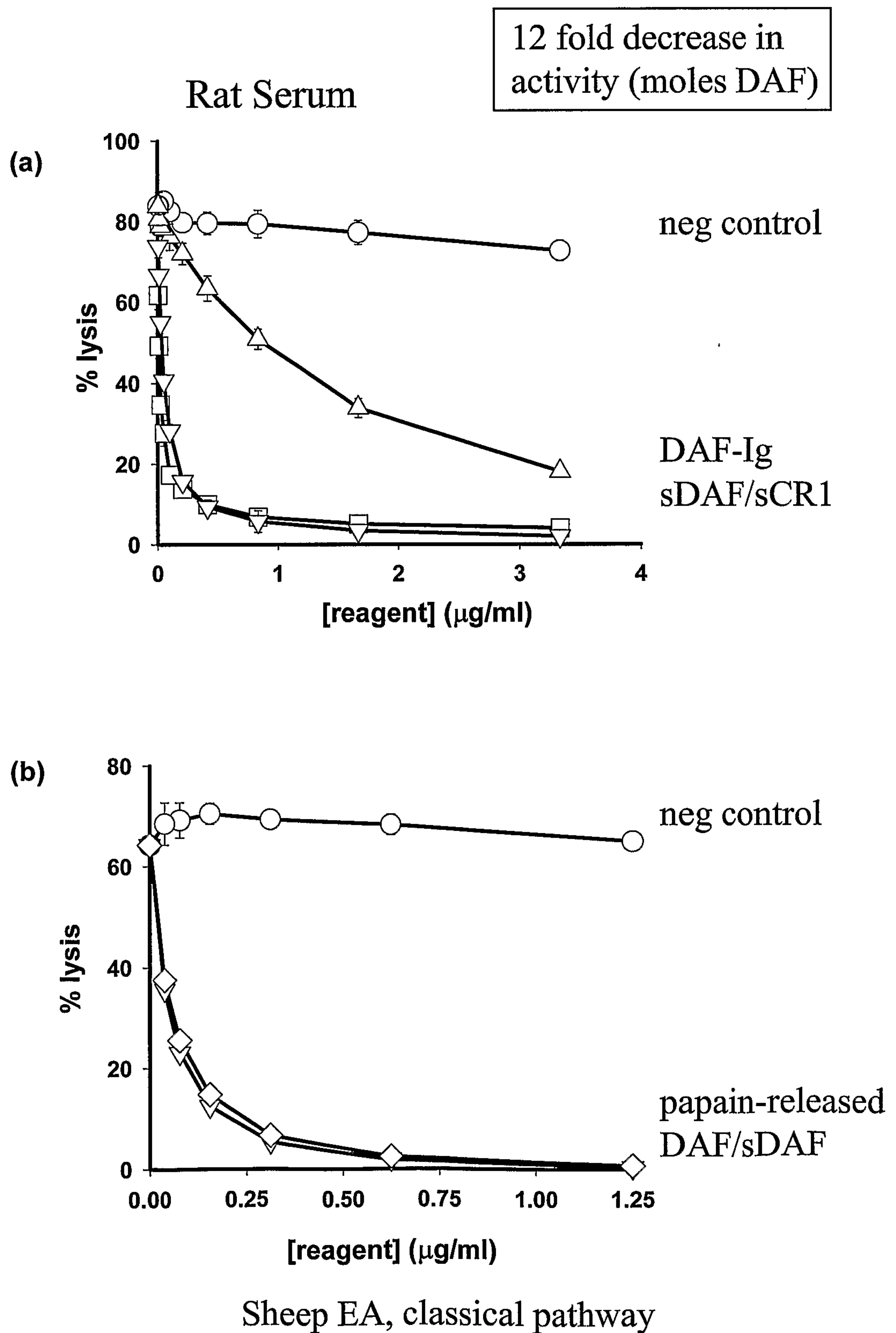
DAF-Ig demonstrates extended half-life in vivo

**Figure 2**

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Figure 3

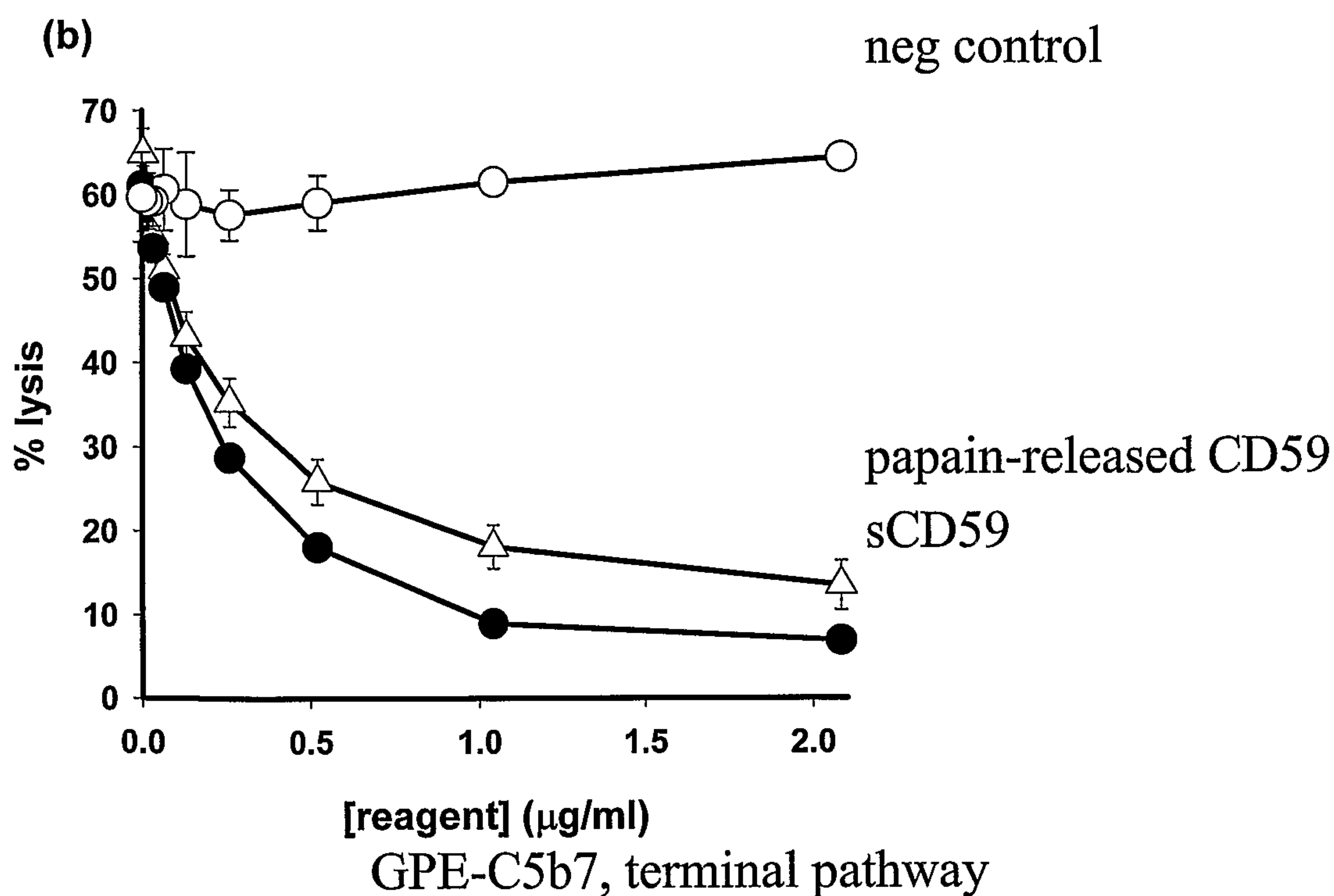
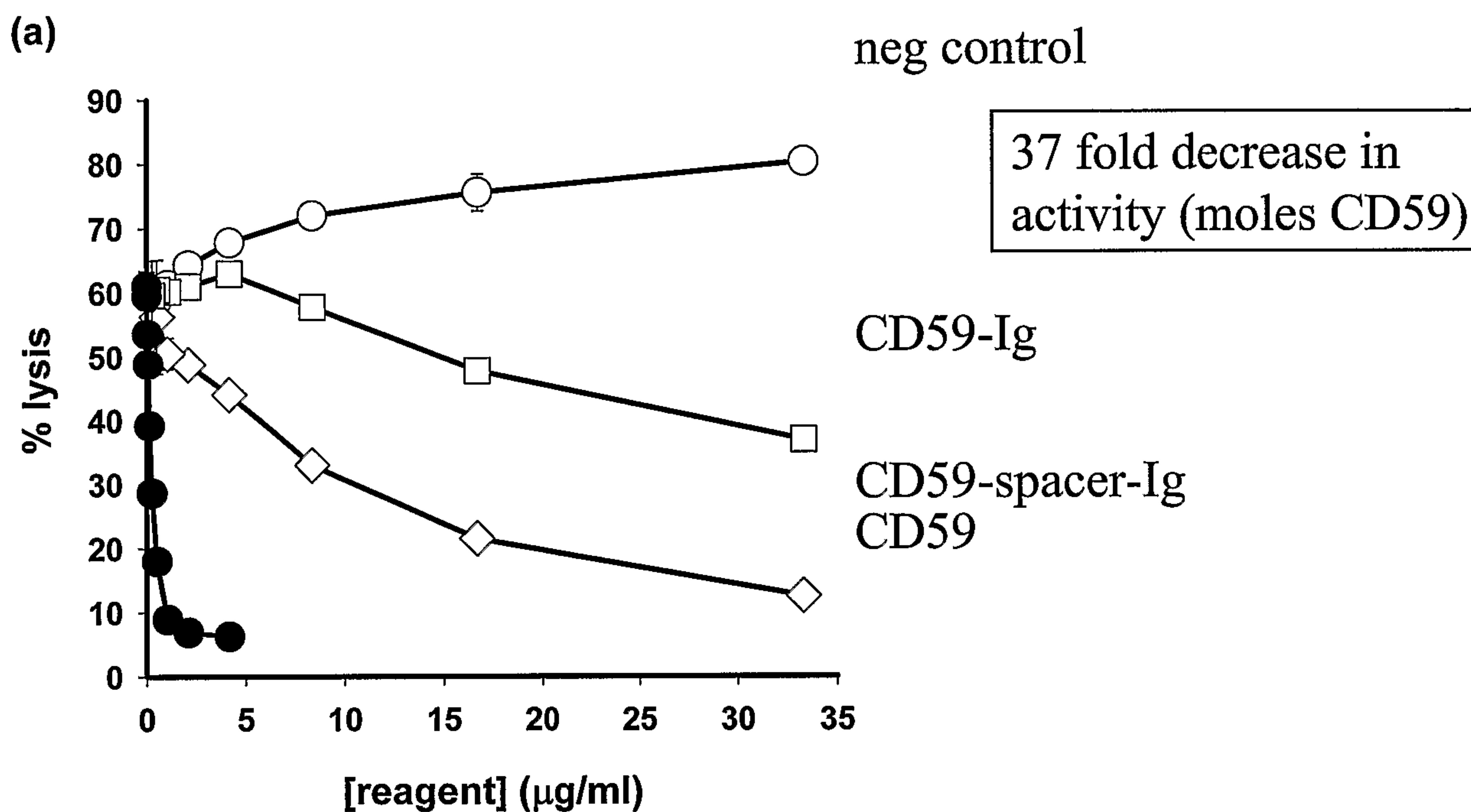
- Fusion of rat DAF to Ig domains can restrict function
- Release of DAF using papain restores function



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Figure 4

- Fusion of rat CD59 to Ig domains can restrict function
- Release of CD59 using papain restores function



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Figure 5

Effect of Ig domains and SCR4 deletion on regulatory function

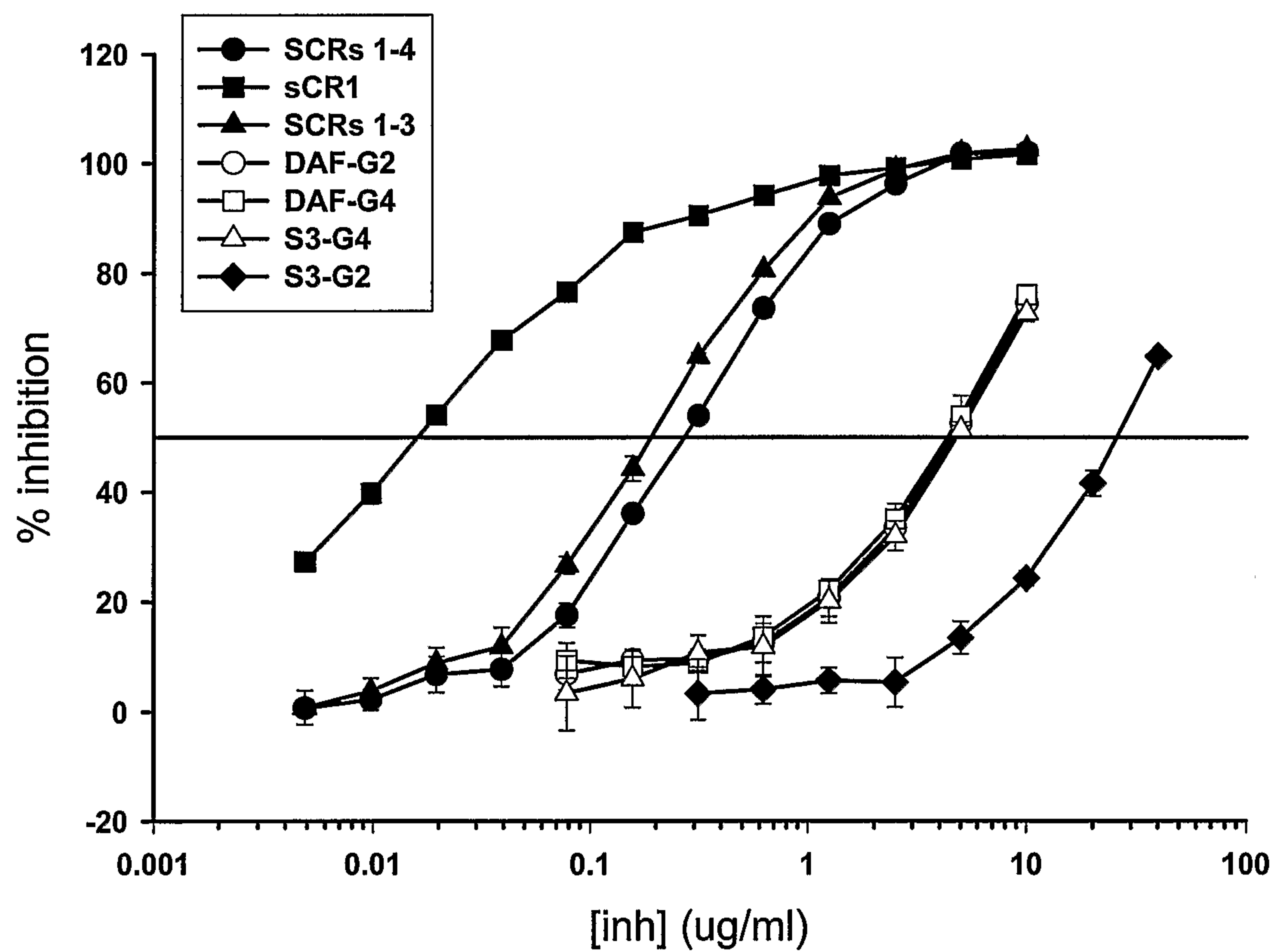
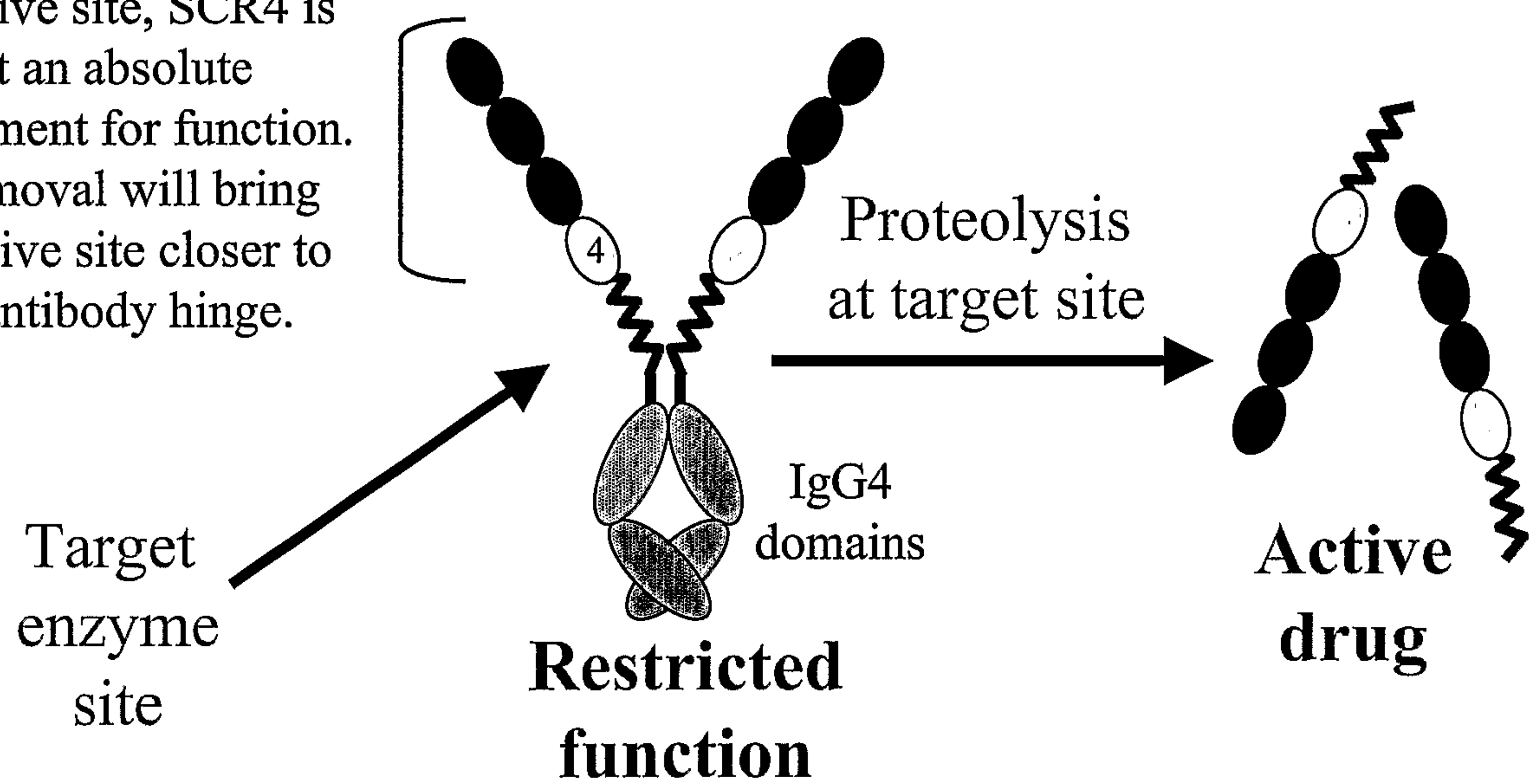


Figure 6

Cleavage of prodrug by target enzymes releases active CRP

SCRs 2 and 3 contain the active site, SCR4 is not an absolute requirement for function. Its removal will bring the active site closer to the antibody hinge.



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Figure 7

site 1
'IGD 1': RNITEGEEARGSVILTVK

site 2
'IGD 2': TTFKEEEGLGSVELSGL

'IGD75':
site 3 site 1
GYTGEDFVDIPENFFGVGGEEDITVQTVTWPDMEPLPRNITEGEEARGSVILTVKPIFEVSPSPL
EPEEPFTFAP

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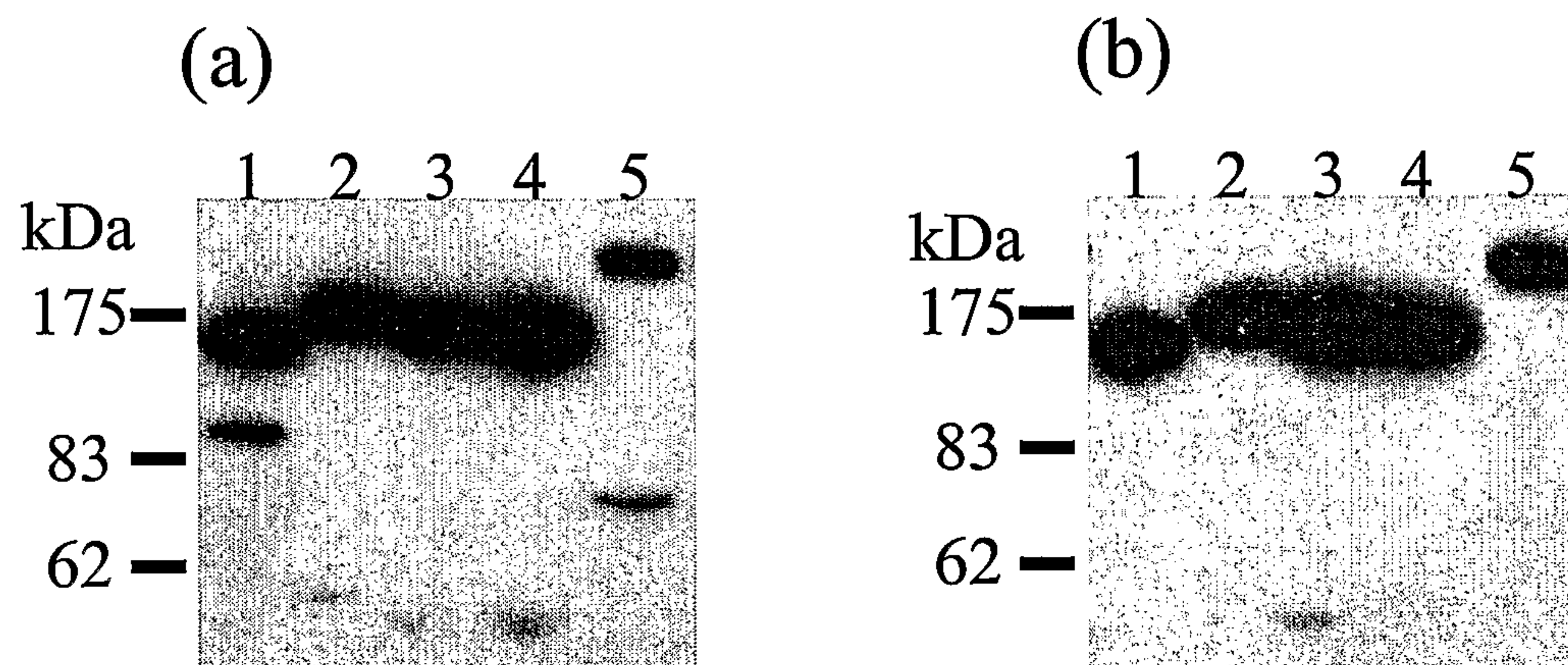
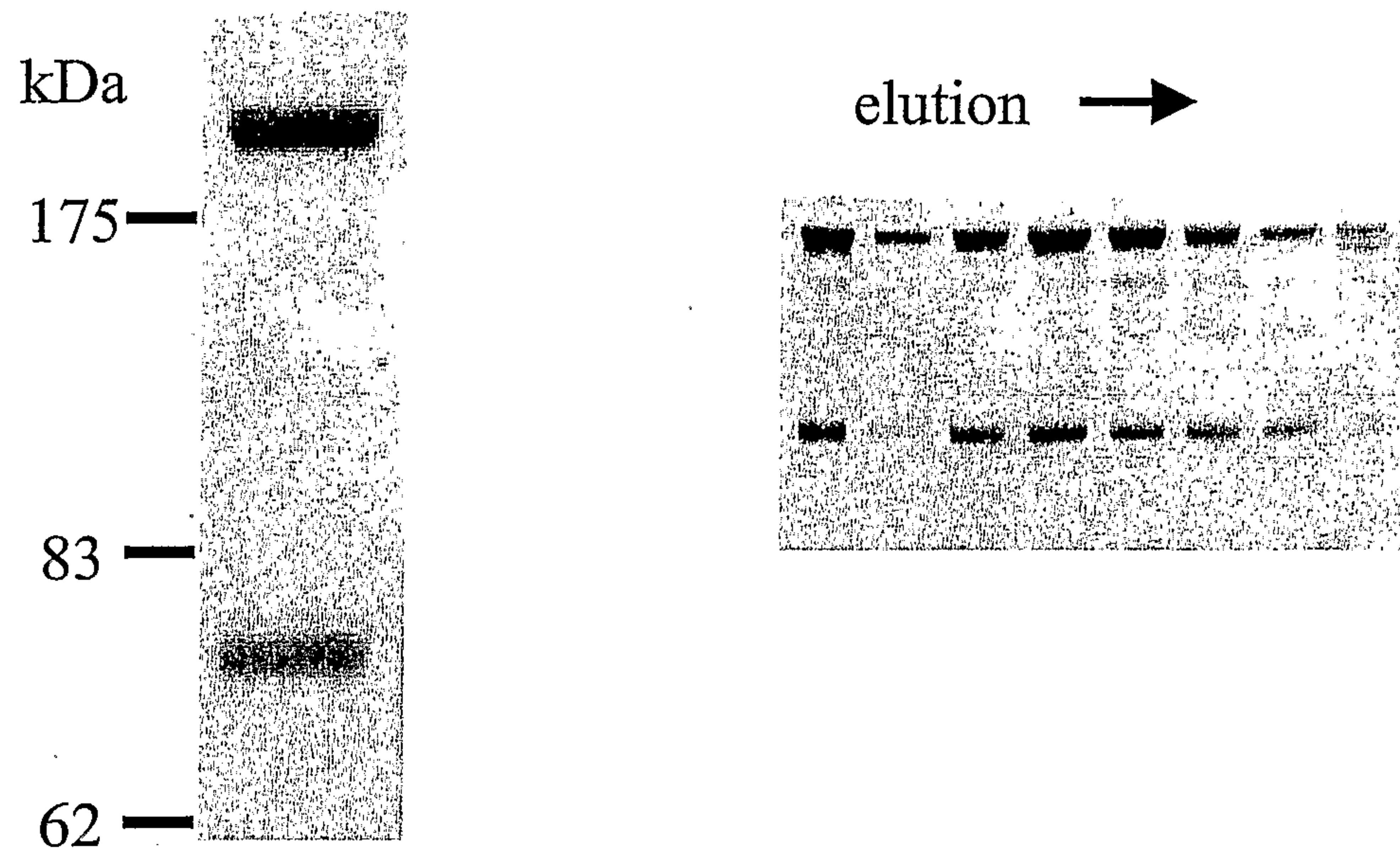
Figure 8

Figure 9

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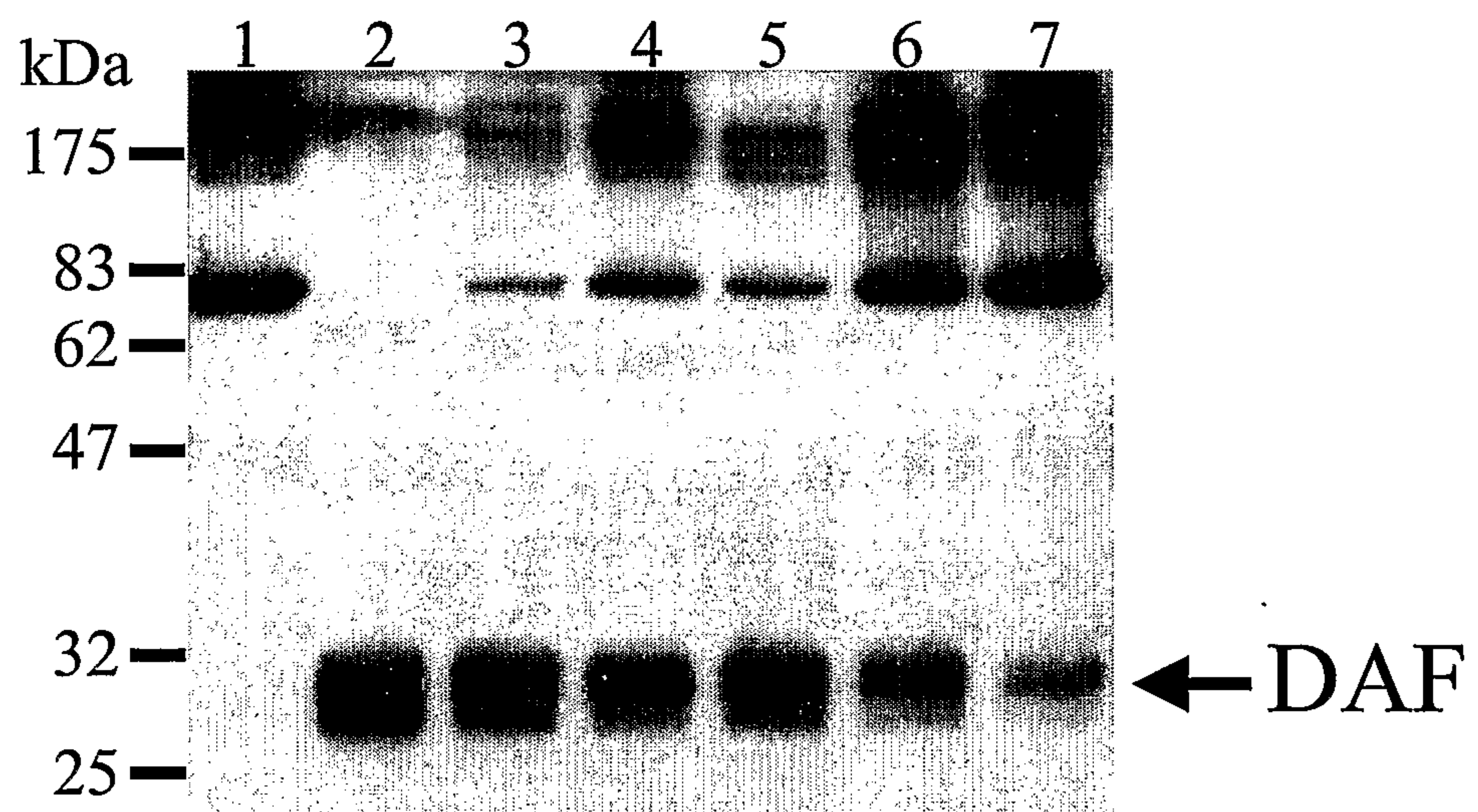
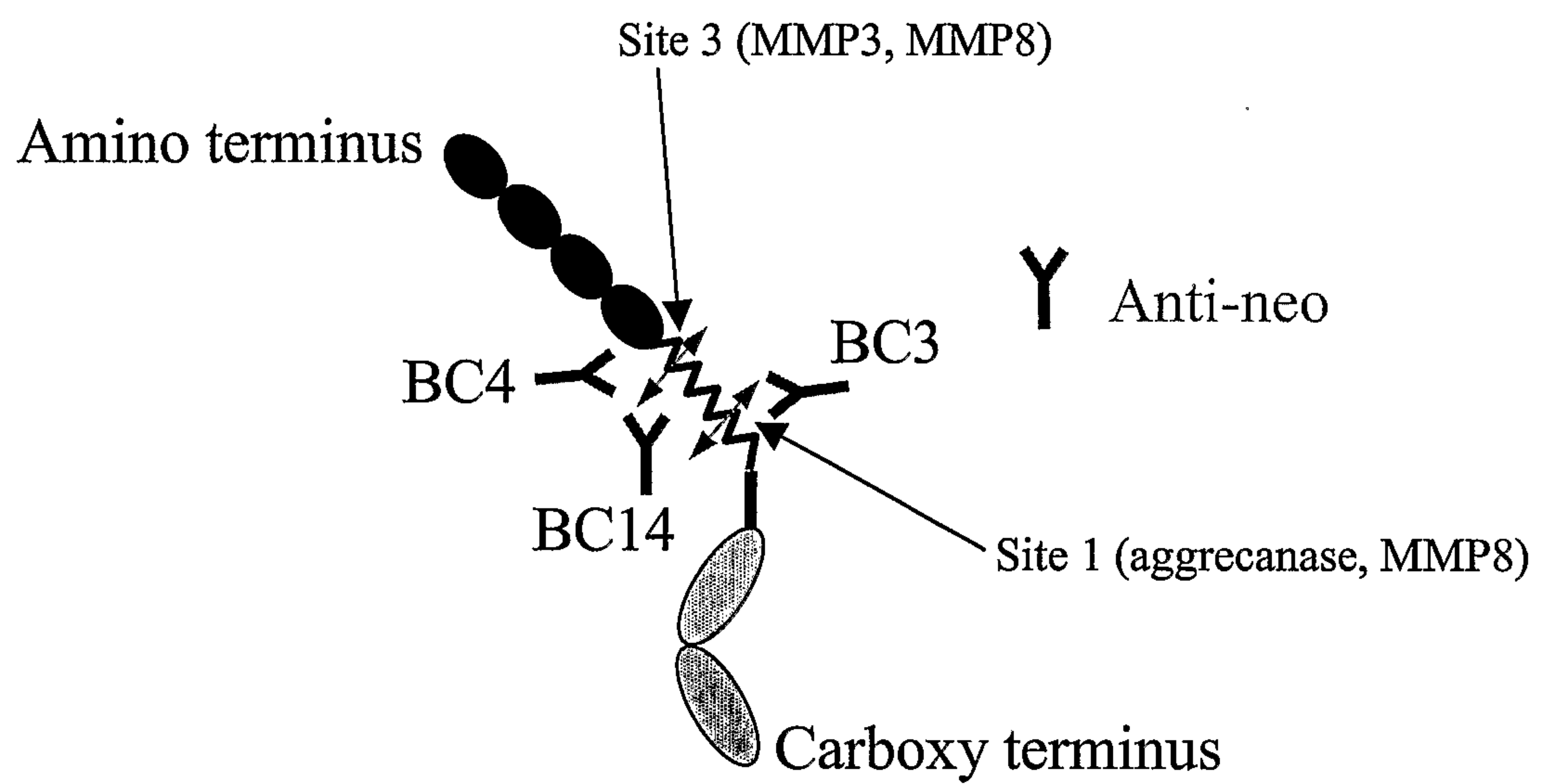
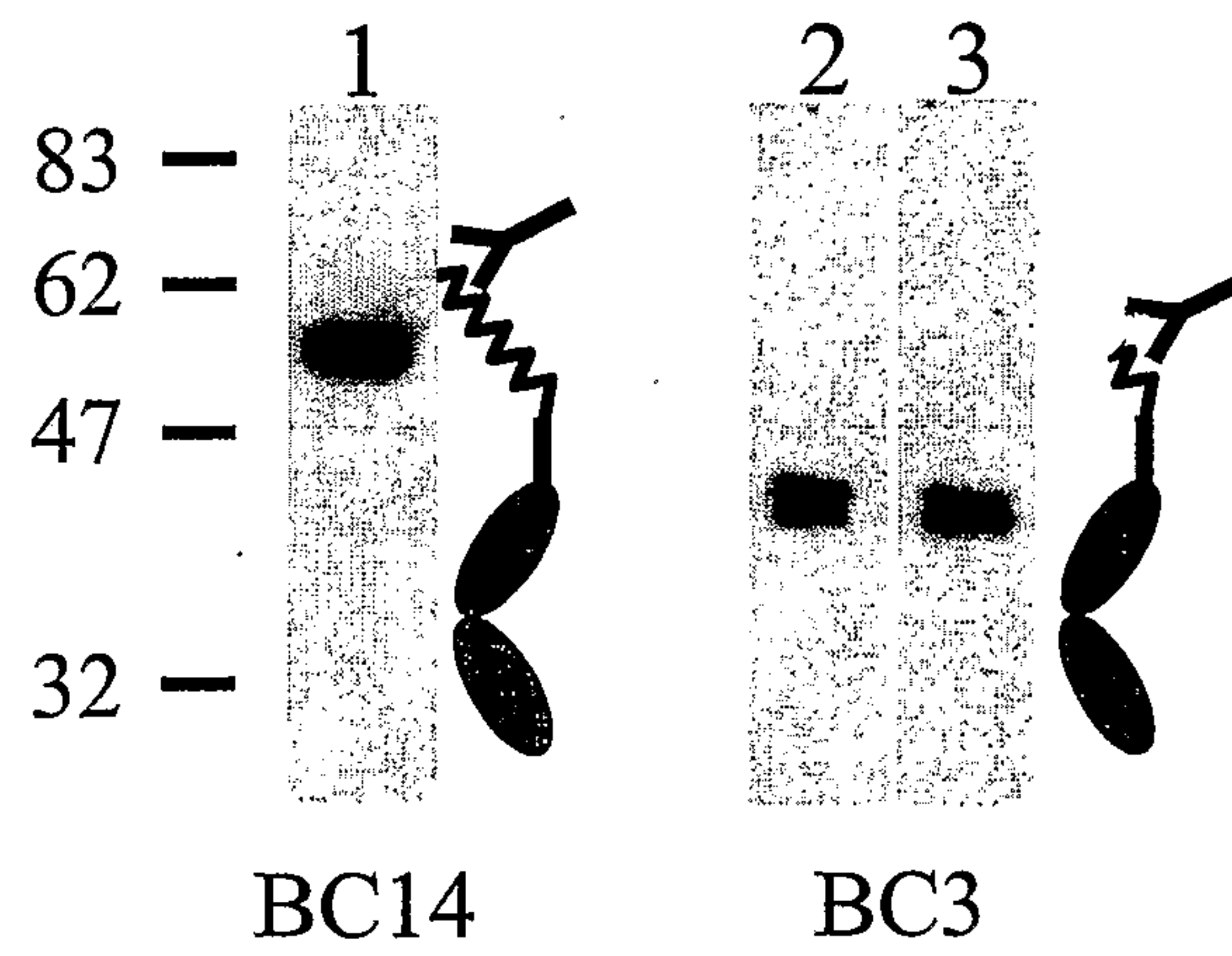
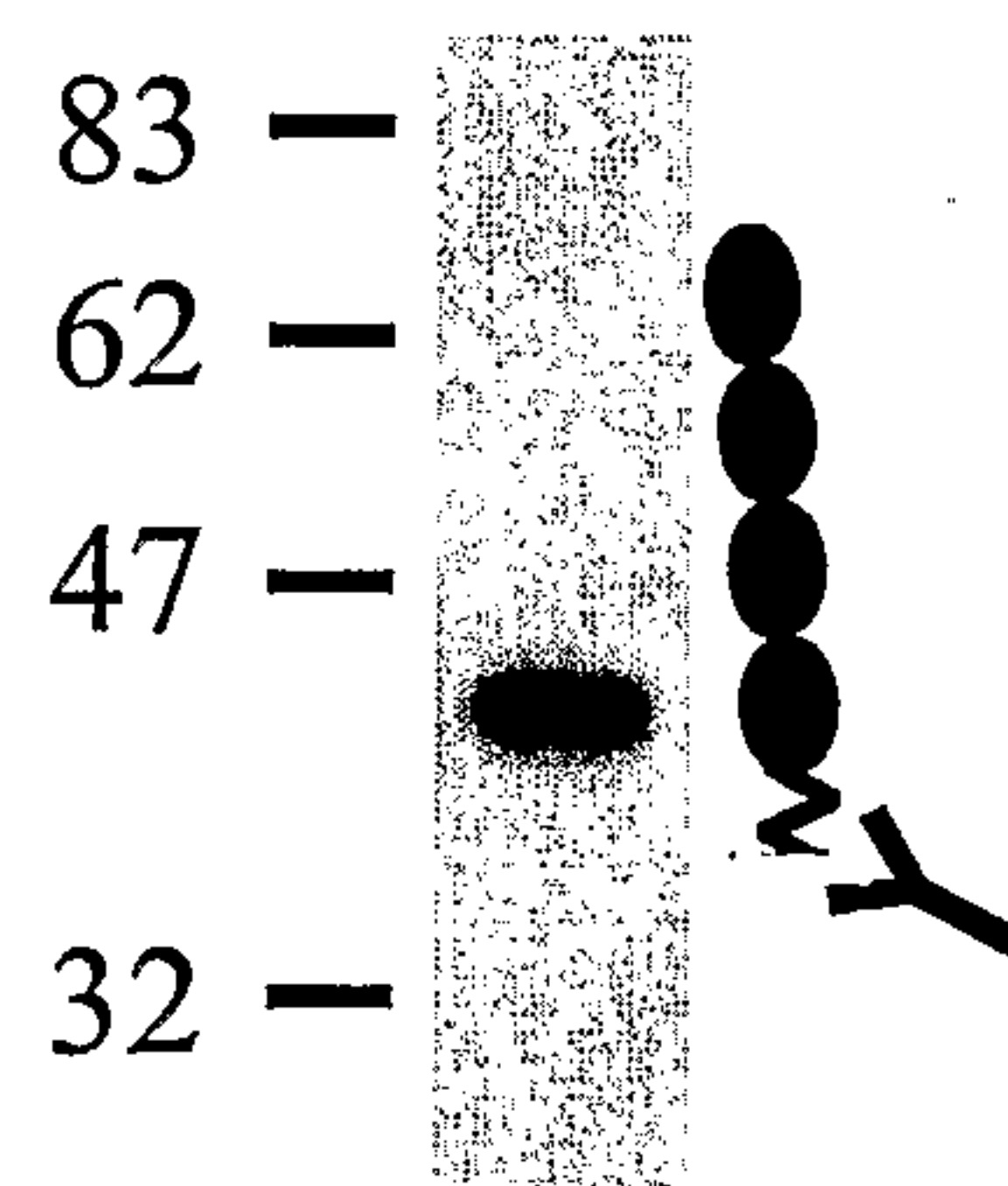
Figure 10

Figure 11

Cleavage sites on prodrug detected by anti-neoepitope antibodies.

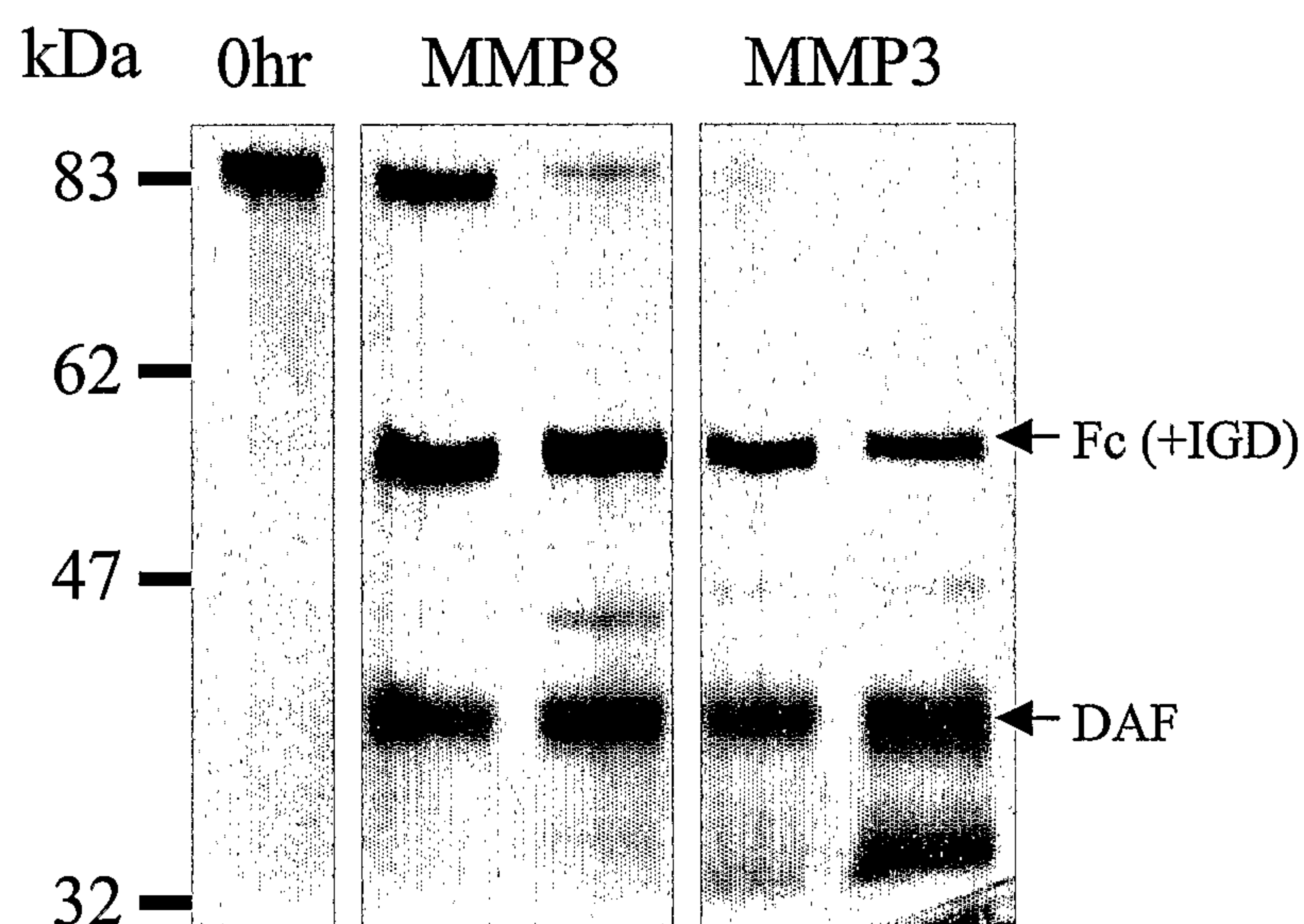
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Figure 12**Figure 13**

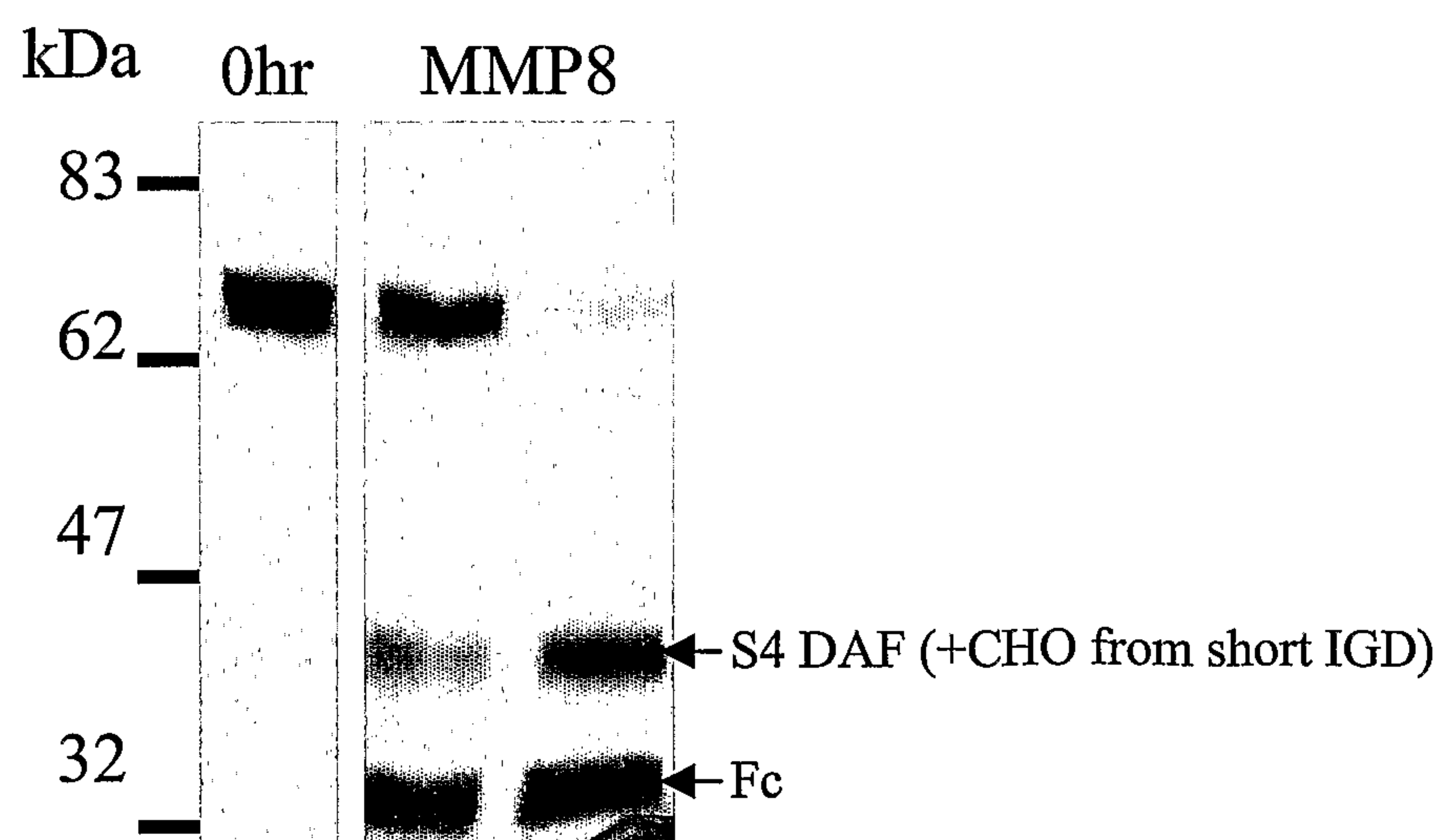
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Figure 14: Silver Stained SDS PAGE gel

Cleavage of DAF(4 SCRs)-IGD75-IgG4 with
MMP3 and MMP8

**Figure 15: Silver Stained SDS PAGE gel**

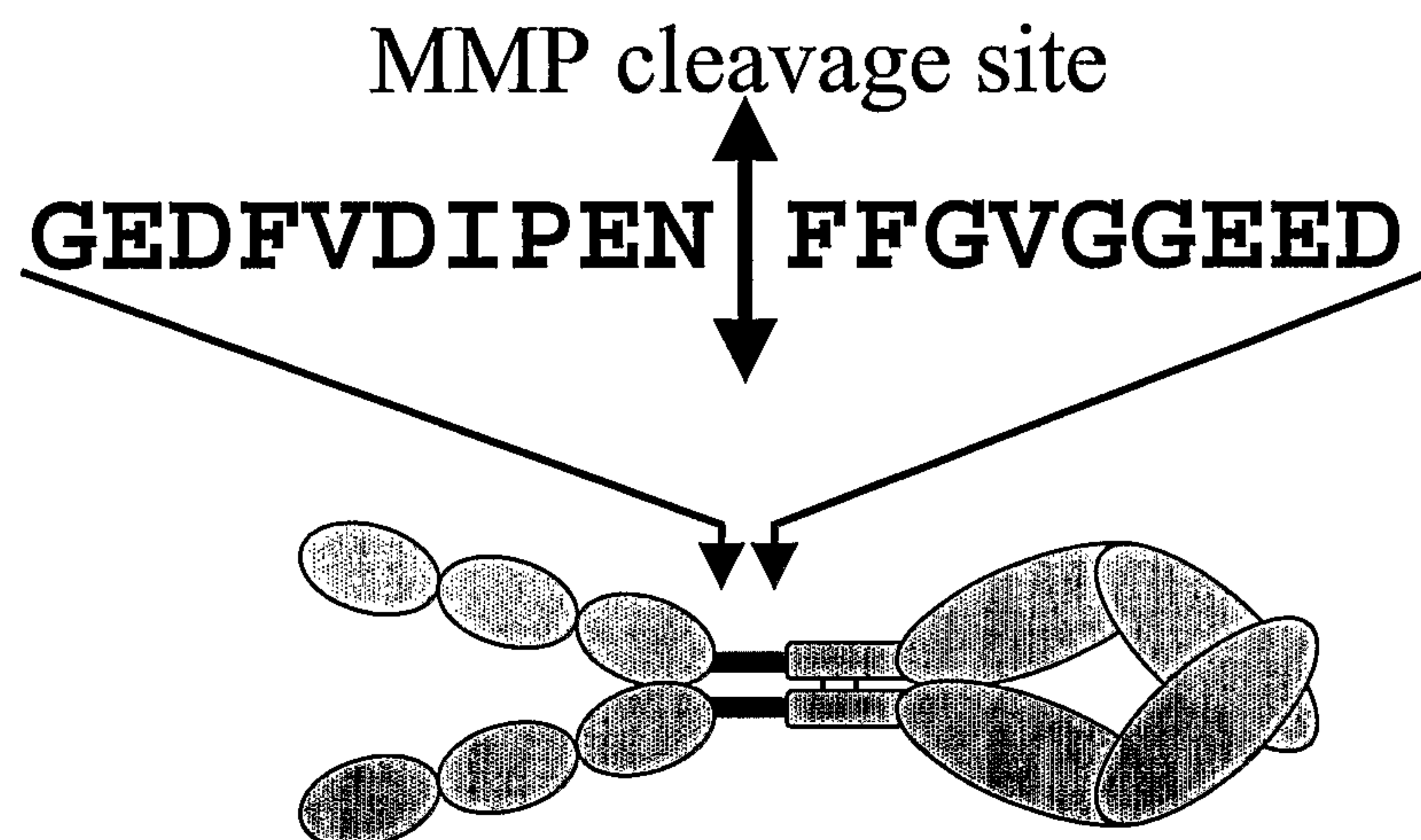
Cleavage of DAF(4 SCRs)-IGD1-IgG4
with MMP8



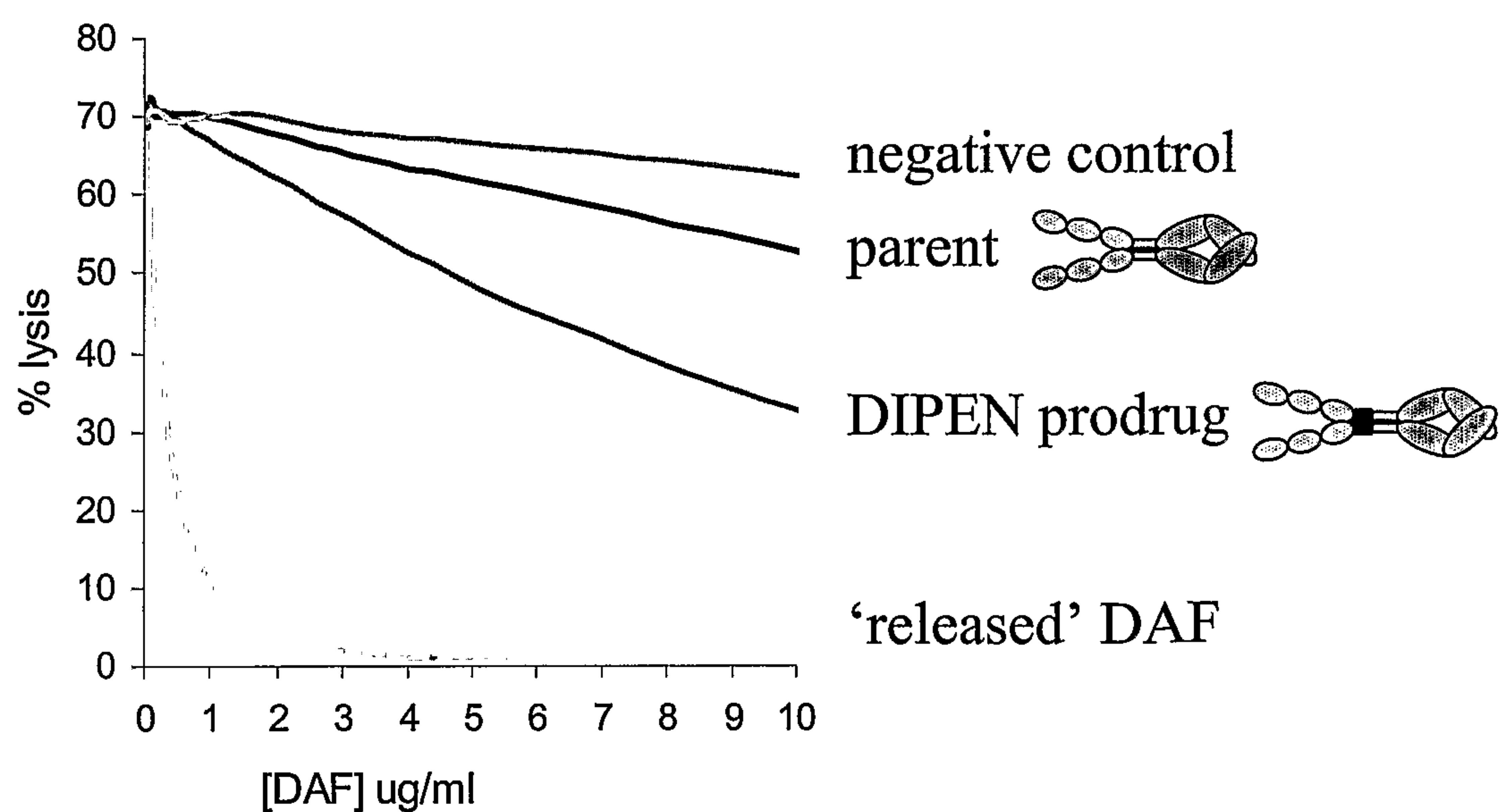
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Figure 16

Prodrug design:

**Figure 17**

Functional activity:



Prodrug is 20x less active than released DAF (on a molar basis)

Figure 18

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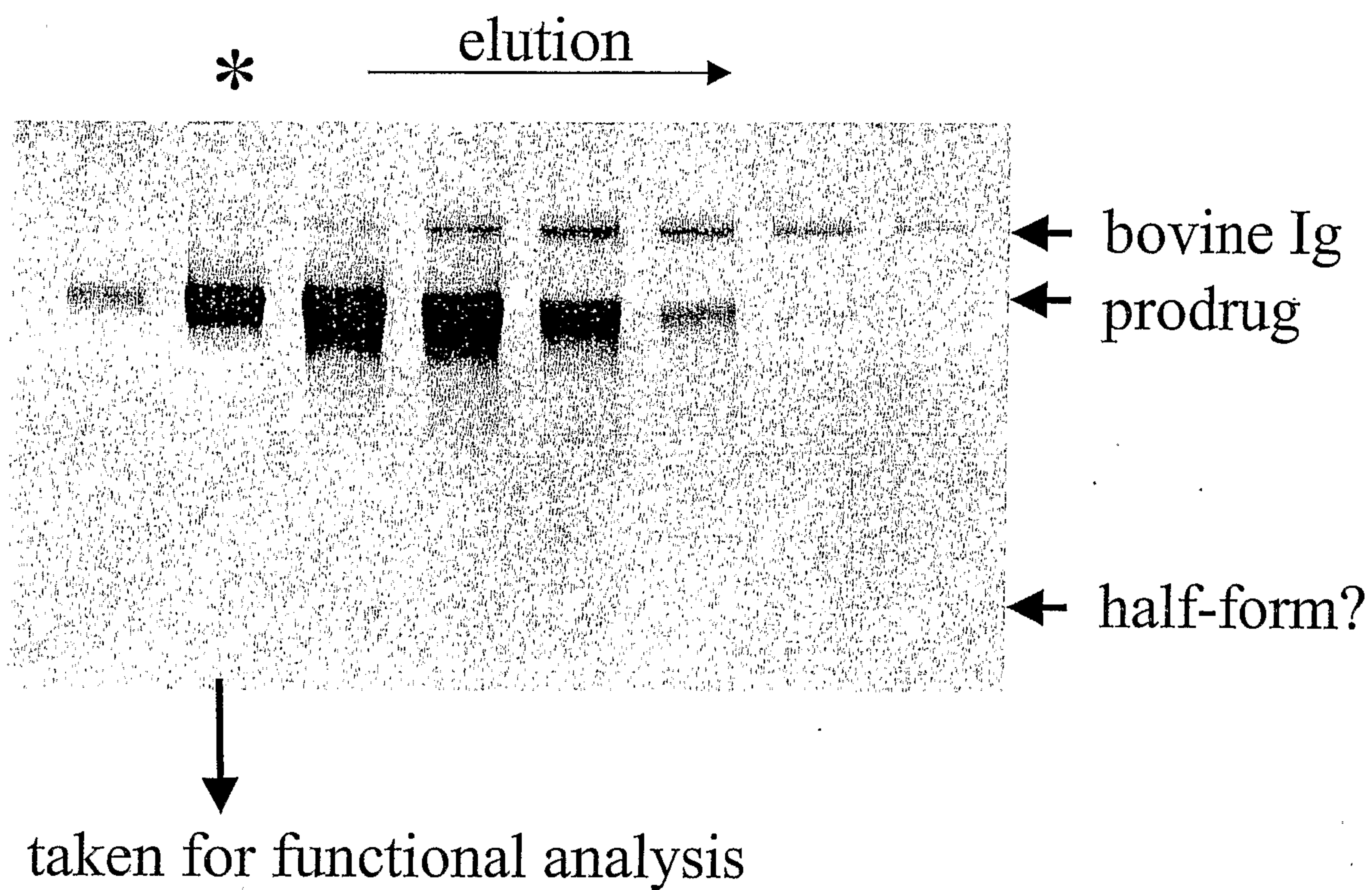
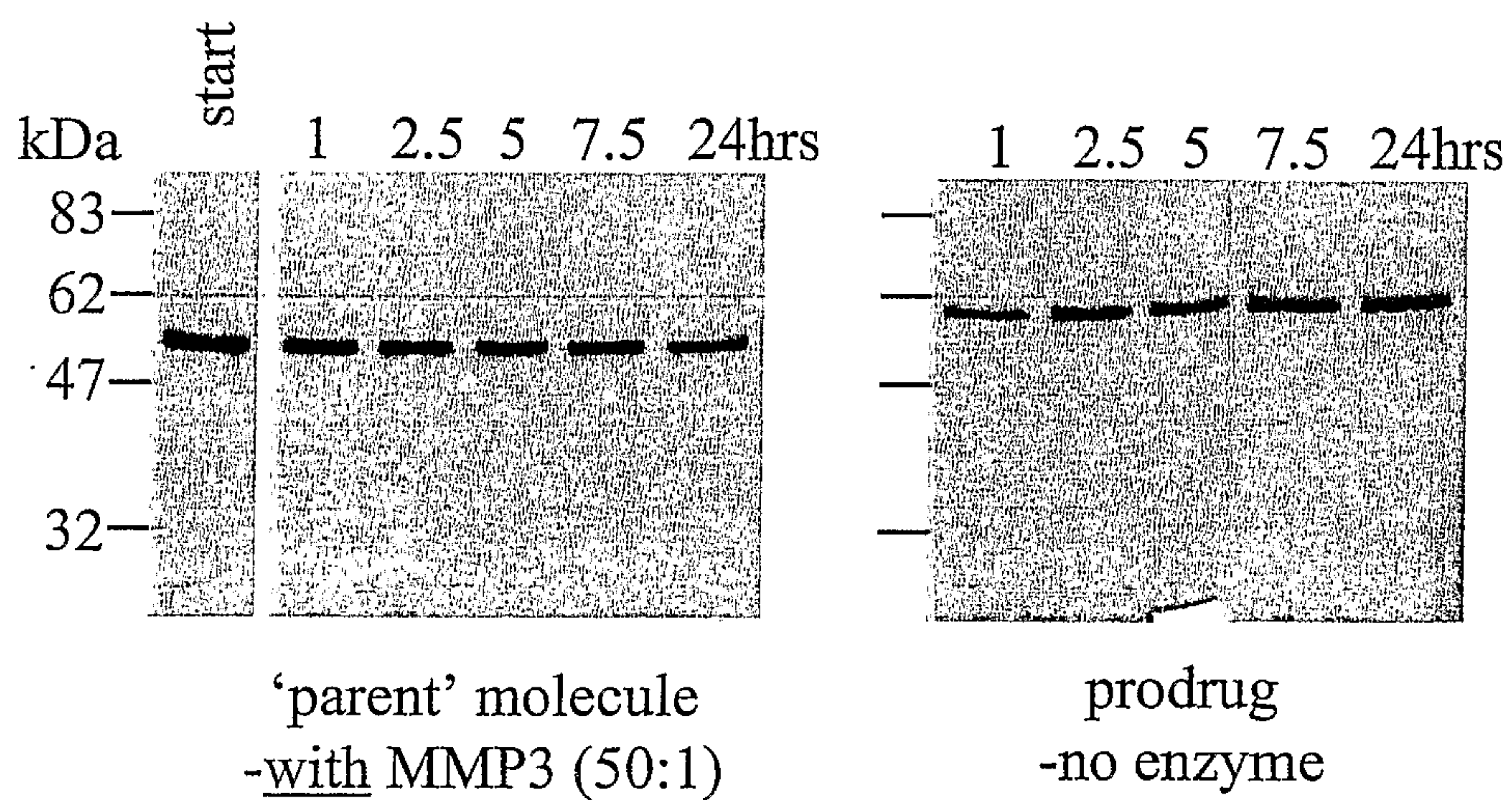
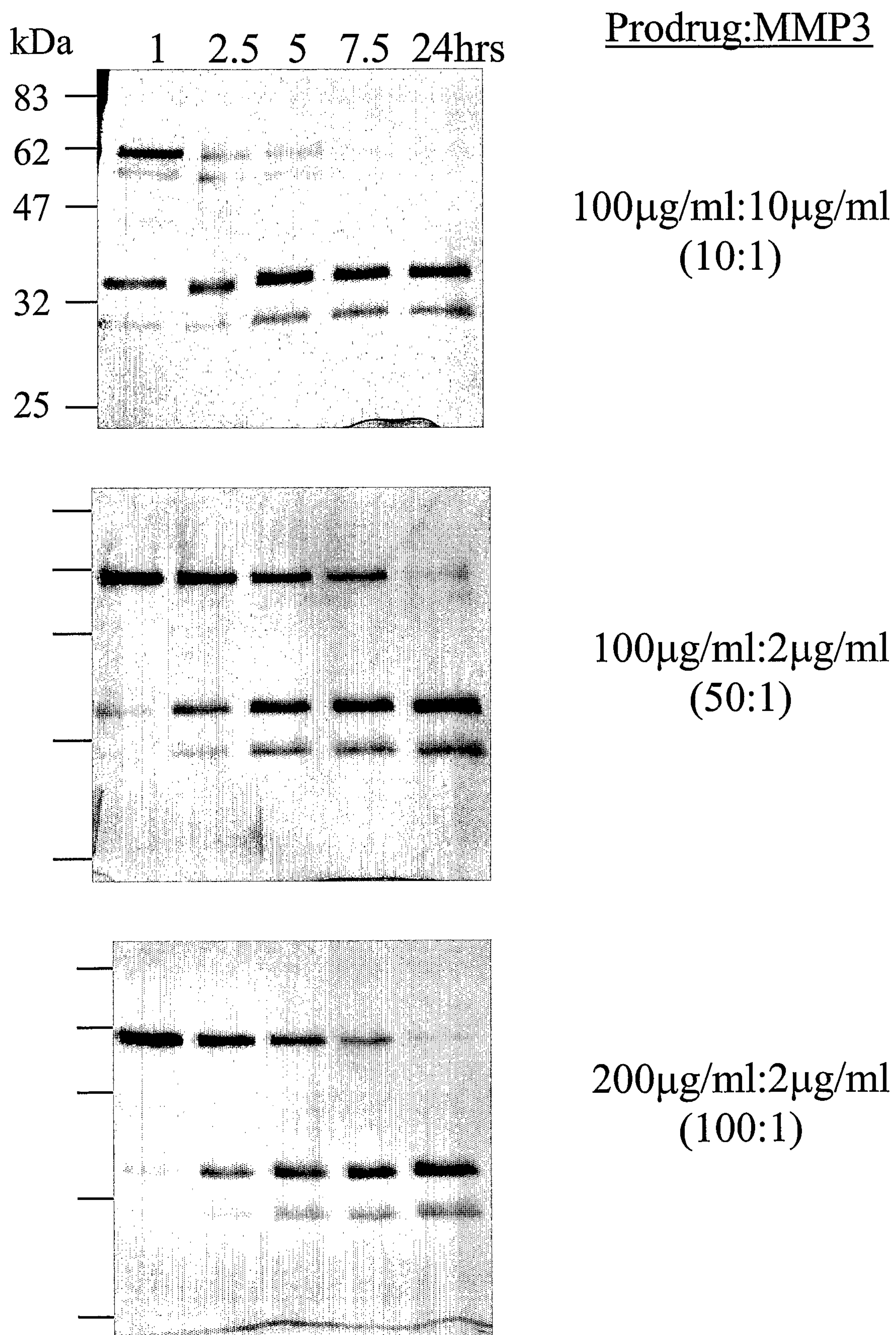
Gel filtration prodrug:**Figure 19****Stability:**

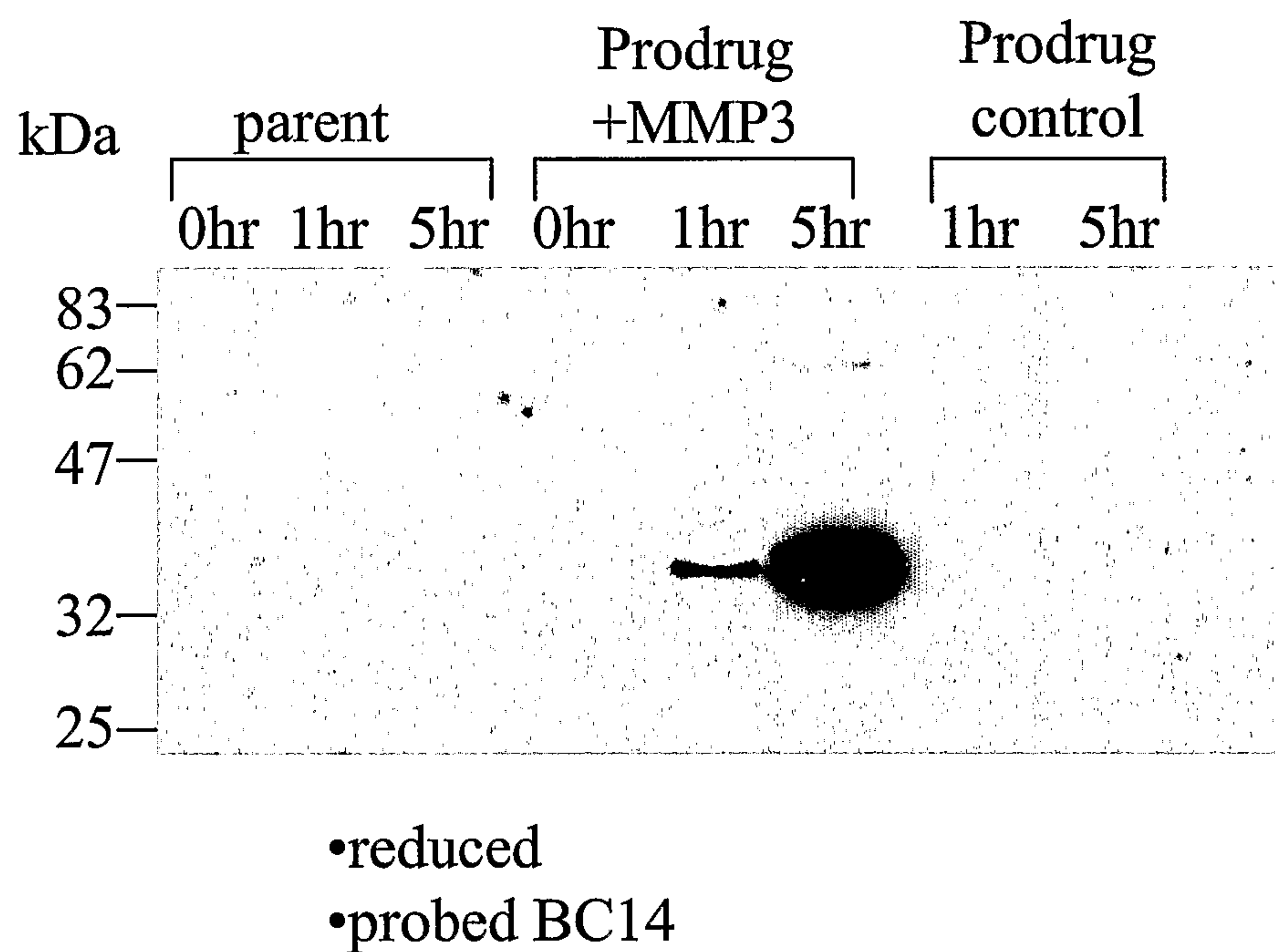
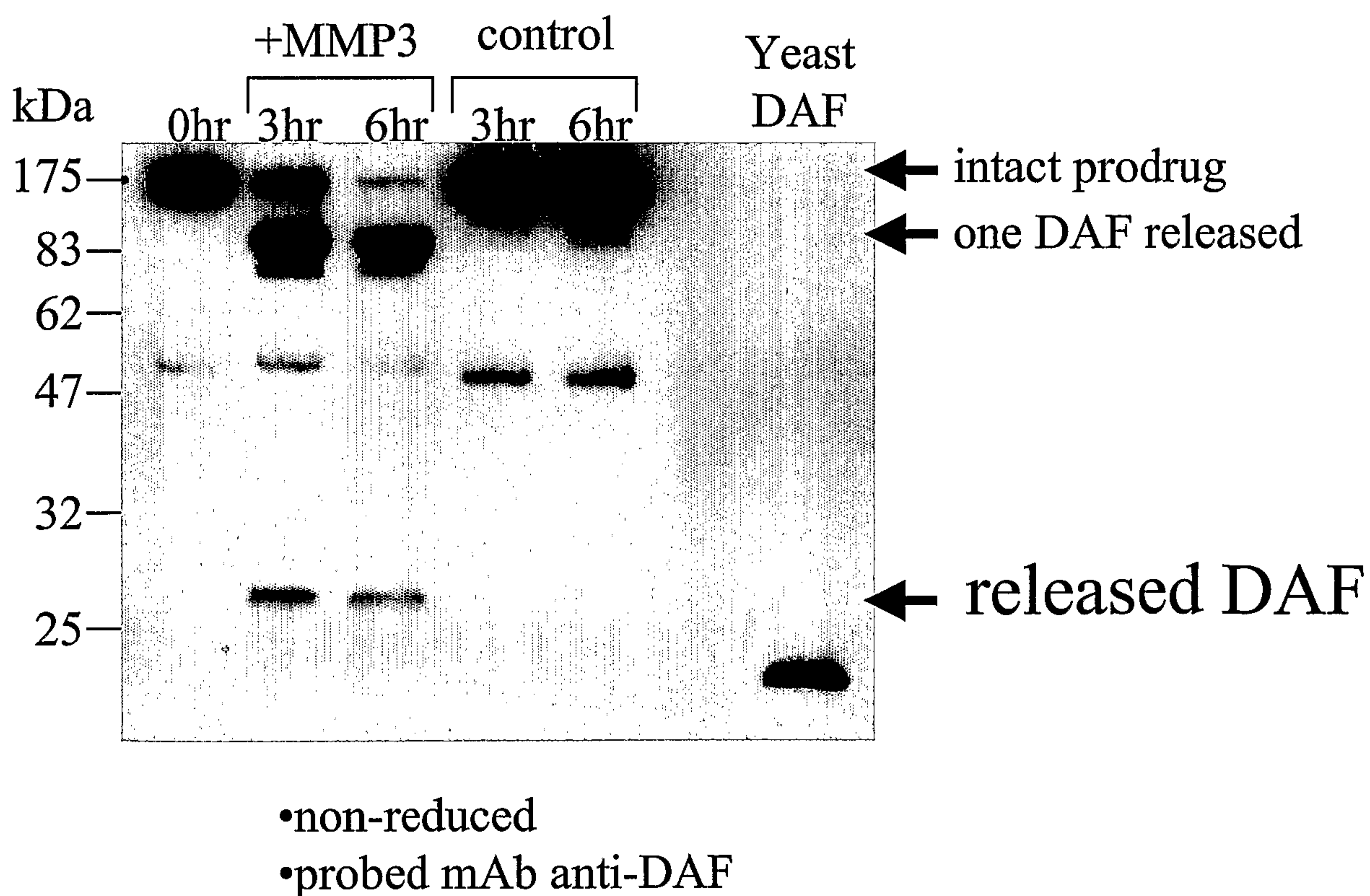
Figure 20

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Cleavage of prodrug with MMP3:



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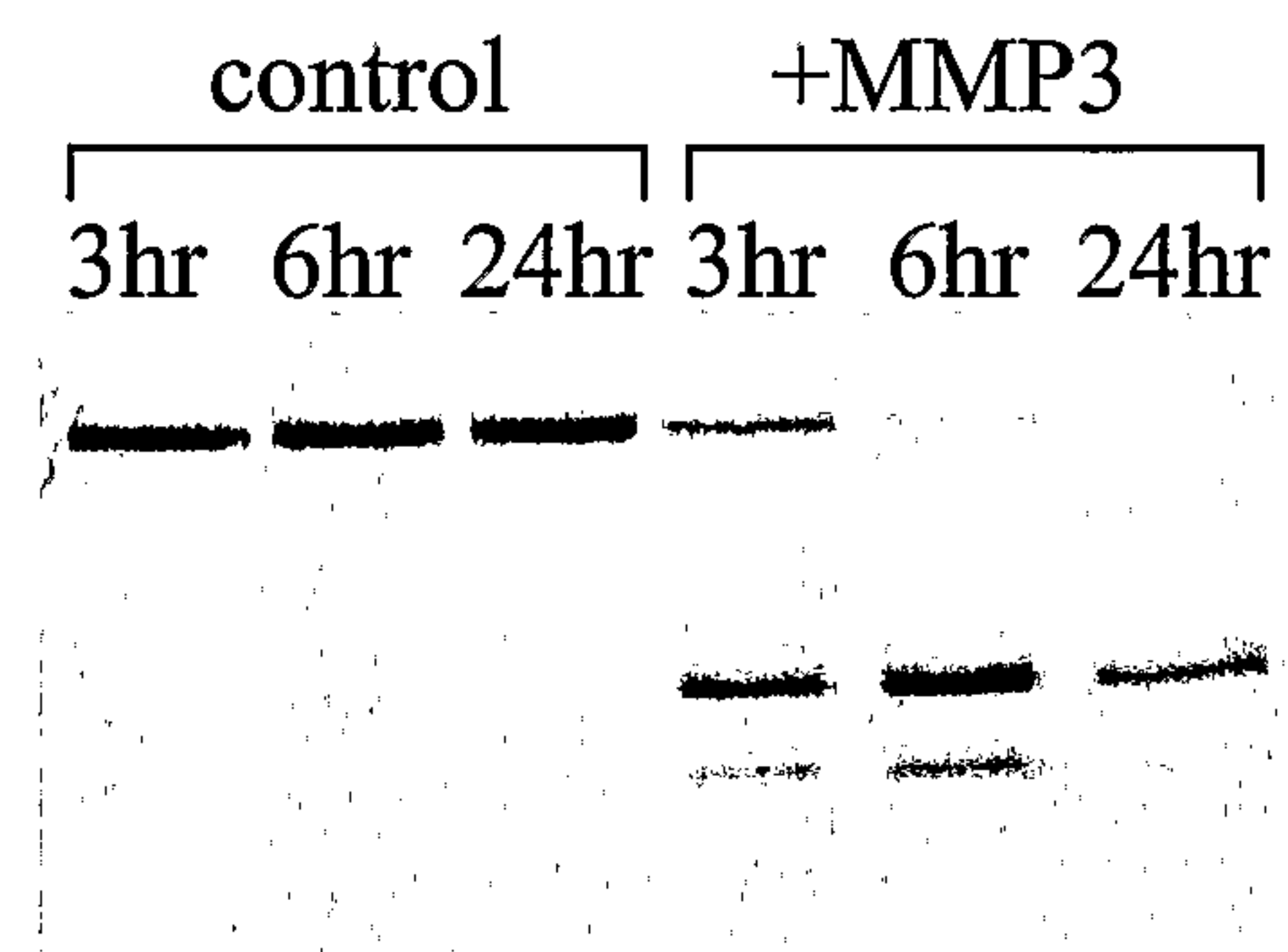
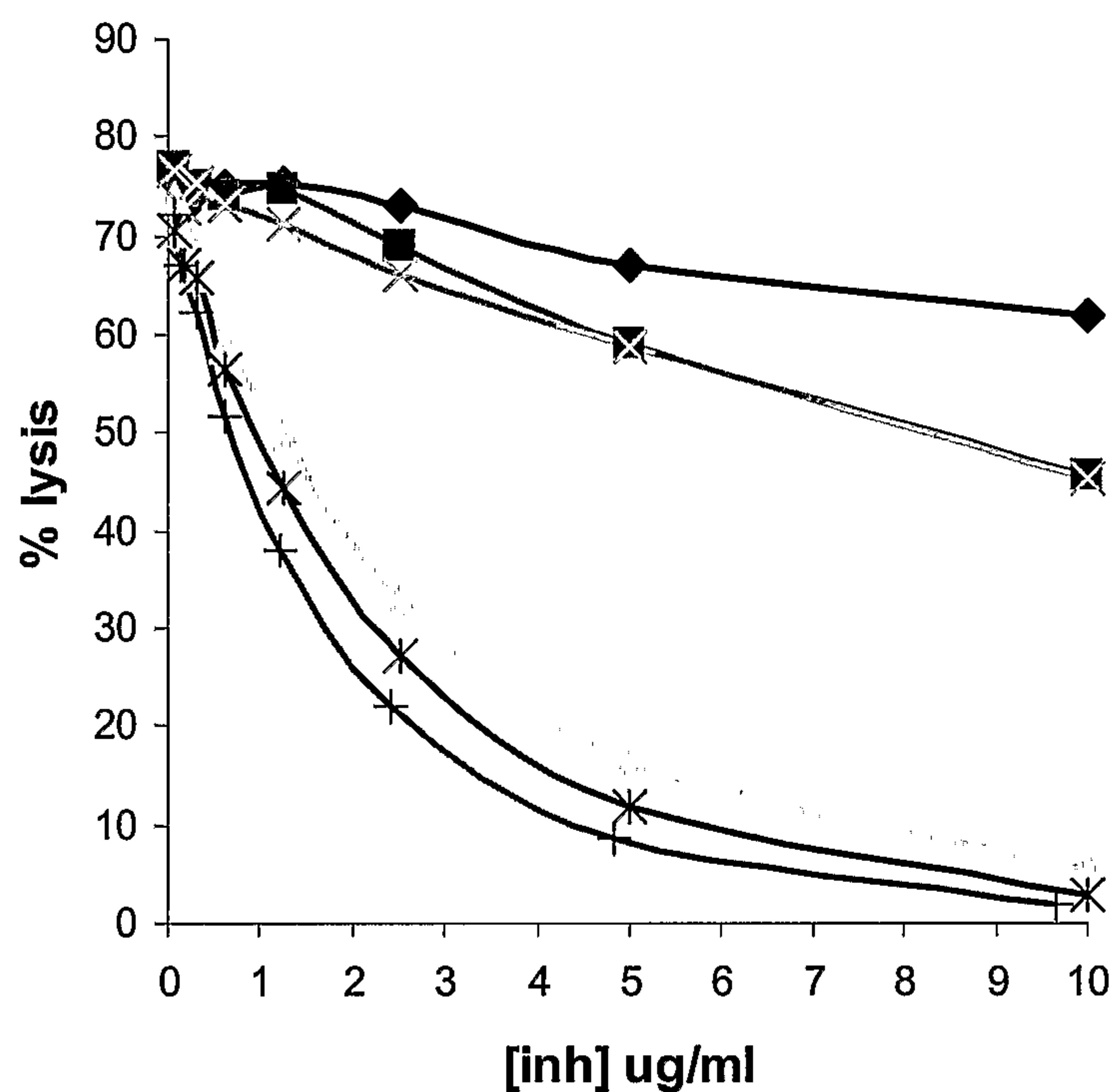
Figure 21**Detection of released DAF and neoepitopes:****Figure 22**

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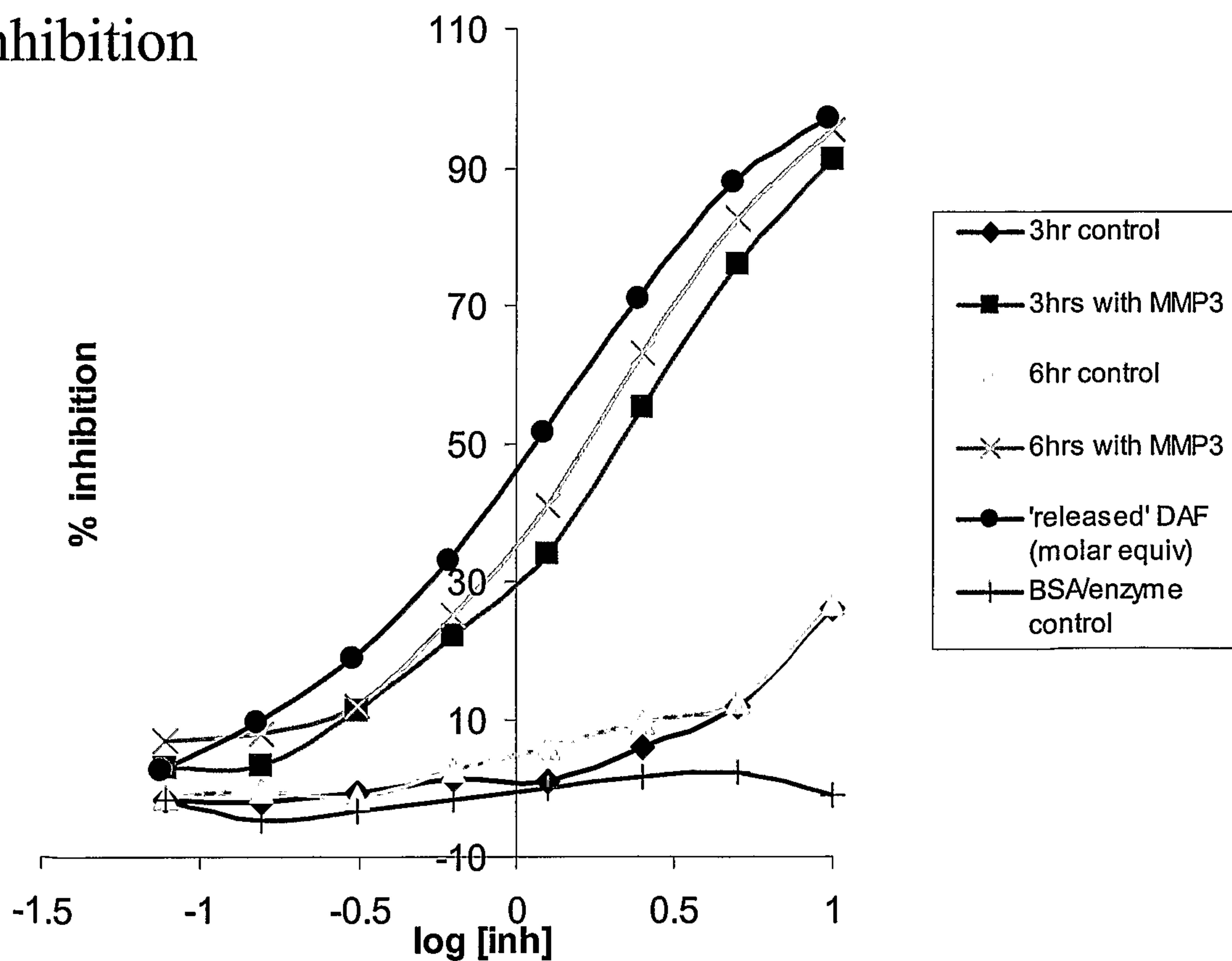
Figure 23

Restoration of function:

Lysis



Inhibition



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Figure 24**Chondrocytes stimulated with cytokines release enzymes which activate the prodrug**