

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
26 July 2007 (26.07.2007)

PCT

(10) International Publication Number
WO 2007/083100 A2

(51) International Patent Classification:
C12N 9/64 (2006.01) *C12N 9/82* (2006.01)

(21) International Application Number:
PCT/GB2007/000124

(22) International Filing Date: 17 January 2007 (17.01.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0600862.7 17 January 2006 (17.01.2006) GB
0618149.9 15 September 2006 (15.09.2006) GB

(71) Applicant (for all designated States except US): **CANCER RESEARCH TECHNOLOGY LIMITED** [GB/GB]; Sardinia House, Sardinia Street, London WC2A 3NL (GB).

(72) Inventor; and

(75) Inventor/Applicant (for US only): **SAHA, Vaskar** [GB/GB]; Teeage Cancer Trust Young Oncology Unit, Christie Hospital NHS Trust, Wilmslow Road, Withington, Manchester M20 4BX (GB).

(74) Agent: **MILES, John**; Eric Potter Clarkson LLP, Park View House, 58 The Ropewalk, Nottingham NG1 5DD (GB).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

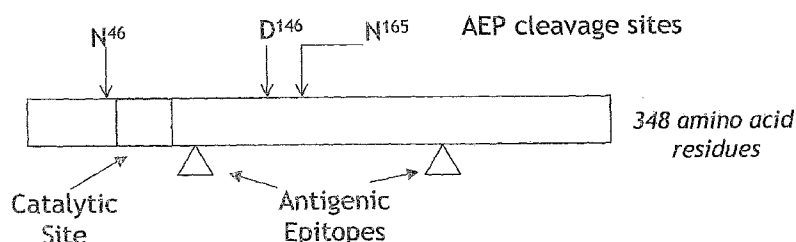
Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ENZYME

E. Coli L-Asparaginase Type II (Medac)



(57) Abstract: A modified *E. coli* L-asparaginase enzyme that is resistant to cleavage by human asparaginyl endopeptidase (also known as legumain). The amino acid residues N46, D 146 and/or N 165 have been replaced with another amino acid residue, and preferably with glycine residues.



WO 2007/083100 A2

ENZYME

This invention relates to an enzyme that is suitable for therapeutic use.

- 5 The listing or discussion of a prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge

Although the cure rate of children with acute lymphoblastic leukaemia (ALL) is
10 approximately 80%, many are not cured with today's treatments. Vincristine, L-asparaginase and daunorubicin, alone or in combination, are the most commonly administered therapies. Drug resistance is an important cause of treatment failure, but the responsible mechanisms are largely unknown.

- 15 Most mammalian cells use an asparagine synthetase enzyme to make their own asparagine. The enzyme takes aspartate and adds an amine thus forming asparagine. However, some blood cells such as leukaemic blast cells instead rely on the blood for their supply of asparagine. Asparaginase therapy, in which recombinant purified L-asparaginase is administered to patients suffering from
20 malignant haematological disease, takes advantage of this fact. When a large dose of this enzyme is introduced into the blood, it circulates and breaks-down asparagine, ultimately starving any cells that rely on the blood-borne supply (Capizzi *et al*, 1971).

- 25 The pharmaceutical industry uses two main sources to produce therapeutic L-asparaginase, *Escherichia coli* (*E. coli*) and *Erwinia chrysanthemi* (also known as *Erwinia carotovora*). The amino acid sequences of the *E. coli* L-asparaginase and the *Erwinia* L-asparaginase are only 46% identical. *E. coli* L-asparaginase has greater potency and a longer half-life than L-asparaginase from *Erwinia*
30 *chrysanthemi* and hence is a better therapy (Albertsen *et al*, 2001). However, L-asparaginases from both species are used therapeutically because the *Erwinia* L-asparaginase lacks cross-reactivity with *E. coli* enzyme. Thus patients that become resistant to *E. coli* L-asparaginase due to acquired immunity may still be

treatable with *Erwinia chrysanthemi* L-asparaginase. *Erwinia* L-asparaginase is commercially available as Erwinase[®] from OPi Ltd, UK. Native *E. coli* L-asparaginase is commercially available from Medac GmbH and from Merck & Co, USA.

5

US Patent No 6,251,388 describes an L-asparaginase enzyme from *Wolinella succinogenes* that is stated to be suitable for treating diseases that respond to L-asparaginase depletion. The *Wolinella* L-asparaginase was predicted to lack cross-reactivity with *E. coli* L-asparaginase.

10

Some patients with ALL develop resistance to *E. coli* L-asparaginase. Historically, two mechanisms of resistance to L-asparaginase have been recognised:

15

- The development of antibodies able to neutralise the enzyme.
- An increase in asparagine synthase activity.

In order to overcome resistance due to acquired immunity, polyethylene glycol (PEG) conjugation to L-asparaginase was developed to provide a form of the drug which is less likely to cause the development of neutralizing antibodies.

20

Pegylated *E. coli* L-asparaginase is commercially available as Pegaspargase (Graham, 2003). Pegaspargase has been used to treat both paediatric and adult ALL, lymphoma, non-Hodgkin's lymphoma, AIDS related lymphoma, and advanced solid tumours (see, Section 5 "Clinical Trials" in Graham (2003), and references cited therein). Pegaspargase is commercially available from Enzon

25

Pharmaceuticals as Oncaspar[™].

There is no report to date on methods to combat increases in asparagine synthase activity. However, data does suggest that its relevance is dependent on the genetic background of the patient (Stams *et al*, 2005)

30

Two recent publications from scientists at St Jude Children's Research Hospital, Memphis, Tennessee, USA, have detailed genes they believe to be involved in the development of resistance to the anti-leukaemic drugs prednisolone, vincristine,

L-asparaginase and daunorubicin in patients with ALL. Holleman *et al* (2004) used micro-array technology to identify 124 genes that are differentially expressed in ALL cells that display resistance to these four drugs (including L-asparaginase). Lugthart *et al* (2005) also used micro-array technology to determine 45 genes associated with cross-resistance to the four anti-leukaemic drugs (including L-asparaginase) and 139 genes related to a phenotype of sensitive to vincristine but resistant to L-asparaginase. Nevertheless, these studies have not contributed to an improvement in the treatment of patients with ALL using prednisolone, vincristine, L-asparaginase and/or daunorubicin, or indeed any other therapeutic agent.

Chromosome 21 is a region of general genomic instability associated with acute leukaemias (Harewood *et al*, 2003), and some patients who are hyperdiploid for chromosome 21 demonstrate resistance to L-asparaginase therapy. The inventor has identified a subset of patients who are hyperdiploid in only part of chromosome 21, and whose gene expression profile resembles that described for patients who demonstrate resistance to L-asparaginase therapy but appear resistant to vincristine (Lugthart *et al*, 2005). Genomic data from these patients was used to identify genes that may be involved in the development of, or propensity for, resistance to L-asparaginase. Analysis of these patients determined that the gene encoding human legumain (*LGMN*), an asparaginyl endopeptidase (AEP), is involved in L-asparaginase resistance. *LGMN* is not one of the genes highlighted by either Holleman *et al* (2004) or Lugthart *et al* (2005) and, to the best of the inventor's knowledge, has not previously been associated with drug resistance.

Asparaginyl endopeptidase (AEP) (EC 3.4.22.34), also known as legumain, is a lysosomal cysteine protease that cleaves protein substrates on the C-terminal side of asparagine. AEP has a strict specificity for cleavage of asparaginyl bonds at pH 5.8 (Chen *et al*, 1997) although it has also been shown to cleave aspartyl bonds slowly, especially under acidic conditions (Halfon *et al* (1998). This strict specificity is unusual among lysosomal enzymes. AEP is also known to be up-regulated in a wide variety of solid tumours and its over-expression correlates with increased invasiveness (Liu *et al*, 2003). AEP was thus proposed for use as an

enzymatic target for pro-drug therapy of solid tumours (Liu *et al*, 2003). AEP has also been shown to be involved in processing antigens for presentation within the class II MHC pathway (Manoury *et al*, 1998).

5 The inventor has now shown that AEP expression is up-regulated and the protein is active in both leukaemic cell lines and in patient samples (see, Figures 3, 4 and 5). The inventor has shown that AEP cleaves native *E. coli* L-asparaginase, thus contributing to resistance to this drug (see Figures 8 and 9 in Example 2). The inventor has also demonstrated that it is possible to modify the sequence of *E. coli*
10 L-asparaginase so that it is not susceptible to cleavage by AEP (Figure 10 in Example 3). The inventor proposes that L-asparaginase, which has been modified so that it is less susceptible, or not-susceptible, to cleavage by AEP, would have two principal benefits. Without wishing to be bound by theory, the inventor considers that a modified *E. coli* L-asparaginase which is not susceptible to AEP
15 cleavage will have a longer half-life *in vivo* than the native enzyme. In addition, a modified *E. coli* L-asparaginase which is not susceptible to AEP cleavage will also be less allergenic than the native enzyme, since the AEP will no longer be able to cleave it and present it to T-cells.

20 The inventor has identified at least three potential AEP cleavage sites in *E. coli* L-asparaginase (see Figures 6 and 7), none of which are present in the *Erwinia* L-asparaginase. This is why the *Erwinia* L-asparaginase is not susceptible to AEP cleavage, and may explain why the *Erwinia* L-asparaginase can be substituted in patients with *E. coli* asparaginase hypersensitivity.

25 The inventor has now demonstrated that altering the amino acid residues at these AEP cleavage sites in *E. coli* L-asparaginase prevents cleavage of the enzyme by AEP. Furthermore, since these AEP cleavage sites are not present at the active site of the asparaginase, the inventor considers that the modified enzyme would
30 maintain L-asparaginase activity. More particularly, replacing the amino acid residues at the AEP cleavage sites with those residues present at the equivalent sites in the *Erwinia* L-asparaginase enzyme, is expected to prevent cleavage by AEP whilst maintaining L-asparaginase activity.

A first aspect of the invention thus provides a modified *E. coli* L-asparaginase II enzyme that is resistant to cleavage by human AEP.

- 5 Human AEP (legumain) was first described by Chen *et al* (1997). The predicted 433 residue amino acid sequence of human prolegumain is listed in Figure 1 of Chen *et al* (1997), and in Genbank Accession Nos NP_005597 and CAA70989, and is shown in Figure 11 (SEQ ID No: 3).
- 10 The inactive prolegumain is processed to the active form of the AEP/legumain enzyme by autocatalytic activation (Chen *et al*, 2000) and/or by normal cellular processing (Li *et al*, 2003). Methods for determining cleavage of a given polypeptide substrate by AEP/legumain are well known in the art and are provided, for example by Chen *et al* (1997). Example 2 provides an example of a
- 15 protocol for measuring cleavage of L-asparaginase by human AEP, and is suitable for determining the resistance of a modified *E. coli* L-asparaginase to cleavage by human AEP.

- E. coli* contains two L-asparaginase isoenzymes: L-asparaginase I, a low-affinity
- 20 enzyme located in the cytoplasm, and L-asparaginase II, a high-affinity secreted enzyme. In the context of this invention, *E. coli* L-asparaginase means the periplasmic form of the enzyme (type II), and not the cytoplasmic form of the enzyme (type I). The periplasmic and cytoplasmic *E. coli* asparaginases only have about 30% amino acid identity. Thus by “*E. coli* L-asparaginase enzyme” and by
- 25 “native” *E. coli* L-asparaginase enzyme we mean the *E. coli* L-asparaginase II enzyme which has the amino acid sequence shown in Figure 7 (SEQ ID No: 1), and listed in Genbank Accession No P00805.

- By a “modified” *E. coli* L-asparaginase enzyme that is resistant to cleavage by
- 30 human AEP, we mean that the AEP is unable to cleave the enzyme at (the C-terminal side of) any one or more of the asparagine (Asn, N) or aspartate (Asp, D) residues within the L-asparaginase sequence (shown in Figures 6 and 7) that is normally cleaved by AEP. Typically, the *E. coli* L-asparaginase enzyme is

modified to be resistant to AEP cleavage by changing one or more of the Asn or Asp residues that is normally cleaved by AEP to another amino acid residue which is not susceptible to AEP cleavage. Thus by a modified *E. coli* L-asparaginase enzyme that is resistant to cleavage by human AEP, we mean that the AEP is
5 unable to cleave the L-asparaginase at (the C-terminal side of) a replacement residue which is present at any one or more of the positions in the native enzyme which is normally occupied by an Asn or Asp residue that is susceptible to AEP cleavage.

10 Preferably, each replacement residue may, independently, be any of the naturally occurring amino acids which are encoded by DNA other than Asn or Asp, and is selected from alanine (Ala, A), arginine (Arg, R), cysteine (Cys, C), glutamine (Gln, Q), glutamate (Glu, E), glycine (Gly, G), histidine (His, H), isoleucine (Ile, I), leucine (Leu, L), lysine (Lys, K), methionine (Met, M), phenylalanine (Phe, F),
15 proline (Pro, P), serine (Ser, S), threonine (Thr, T), tryptophan (Trp, W), tyrosine (Tyr, Y) and valine (Val, V).

Figures 6 and 7 illustrate three potential AEP cleavage sites within *E. coli* L-asparaginase: Asn at position 46 (N46); Asn at position 165 (N165) and Asp at
20 position 146 (D146). Accordingly, a preferred modified *E. coli* L-asparaginase enzyme that is resistant to cleavage by human AEP, is one that the AEP is unable to cleave at the C-terminal side of any one or more of positions 46, 146 and 165 as shown in Figure 7 (SEQ ID No: 1). Preferably, the AEP is unable to cleave the asparaginase at the C-terminal side of residue 46, or at the C-terminal side of
25 residue 165 or more preferably at the C-terminal side of both residues 46 and 165. Still more preferably, the AEP is unable to cleave the asparaginase at the C-terminal side of any of residues 46, 146 and 165.

In a most preferred embodiment, the human AEP does not cleave the modified *E. coli* L-asparaginase enzyme at all, or does so at an undetectable level.
30

Typically, the Asn residue at position 46 of SEQ ID No: 1 (N46) of the modified *E. coli* L-asparaginase enzyme has been replaced with another amino acid residue.

It is preferred if N46 has been replaced with a residue other than Asp, and preferably by a naturally occurring amino acid which is encoded by DNA. Suitable naturally occurring amino acids include Ala, Arg, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val. It is more preferred
5 if N46 has been replaced with a Gly residue. This is because Gly is present at the equivalent position in the *Erwinia* L-asparaginase.

Additionally or alternatively, the Asn residue at position 165 of SEQ ID No: 1 (N165) of the modified *E. coli* L-asparaginase enzyme has been replaced with
10 another amino acid residue. It is preferred if N165 has been replaced with a residue other than Asp, and preferably by a naturally occurring amino acid which is encoded by DNA. Suitable naturally occurring amino acids include Ala, Arg, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val. It is more preferred if N165 has been replaced with a Gly residue. This is because
15 Gly is present at the equivalent position in the *Erwinia* L-asparaginase.

Preferably, both N46 and N165 have been replaced with other naturally occurring amino acid residues which are encoded by DNA. It is preferred if both N46 and N165 have been replaced with residues other than Asp. It is more preferred if both
20 N46 and N165 have been replaced with Gly residues.

In an embodiment, the Asp residue at position 146 of SEQ ID No: 1 (D146) is also replaced with another amino acid residue other than Asn, and preferably by a naturally occurring amino acid which is encoded by DNA. Suitable naturally
25 occurring amino acids include Ala, Arg, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val. Preferably, D146 is replaced with a Gly residue since Gly is present at the equivalent position in the *Erwinia* L-asparaginase. However, it is appreciated that once N46 and N165 have been replaced, D146 may not need to be replaced in order to prevent cleavage of the
30 enzyme by AEP because evidence suggests that Asn residues must be cleaved before the Asp residues can be (Li *et al*, 2003).

Modified L-asparaginase enzymes according to an embodiment of the invention thus include enzymes which comprise the mutations N46G, D146G and N165G; N46G and D146G; D146G and N165G; and N46G and N165G.

- 5 It is, of course, appreciated that by a “modified” L-asparaginase enzyme we do not necessarily mean that a “native” enzyme has been physically modified. Typically, the modified enzyme is made in its modified form.

- 10 It is well known that certain polypeptides are polymorphic, and it will be appreciated that some natural variation of the *E. coli* L-asparaginase polypeptide sequence may occur. Thus, in an embodiment, the modified *E. coli* L-asparaginase is not limited to the amino acid sequence listed in Figure 7 (SEQ ID No: 1), modified at any of the Asp or Asn residues (especially at positions 46, 146 and 165) as described above, but includes naturally occurring variants of *E. coli* L-
- 15 asparaginase II, that retain asparaginase activity, in which one or more of the Asp or Asn residues have been replaced with another amino acid. Furthermore, it is possible to vary the amino acid residues at other positions within SEQ ID No: 1 without eliminating asparaginase activity of the enzyme, and without affecting its resistance to AEP cleavage. Thus the invention includes a modified enzyme that
- 20 has at least 80% sequence identity, more preferably at least 85% or at least 90% sequence identity, and still more preferably at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99% sequence identity with the amino acid sequence of native *E. coli* L-asparaginase (SEQ ID No: 1), and which is resistant to cleavage by AEP.

25

- However, preferably, residues T34, Y47, S80, T111, D112 and K184 as numbered in SEQ ID No: 1, which define the active site of the enzyme, remain unchanged. These six residues are equivalent to residues T12, Y25, S58, T89, D90 and K162 numbered according to Kozak *et al* (2000) who studied a shorter variant of *E. coli*
- 30 L-asparaginase II.

In an embodiment, any one, two or all three of the residues R217, K218 and H219 as numbered in SEQ ID No: 1, which define an antigenic region of the enzyme,

are replaced with less antigenic residues such as alanine. These three residues are equivalent to residues R195, K196 and H197 numbered according to Jianhua *et al* (2006) who studied the same shorter variant of *E. coli* L-asparaginase II as did Kozak *et al* (2000) *supra*.

5

In an embodiment, the modified *E. coli* L-asparaginase may be a modified fragment of the full-length native *E. coli* L-asparaginase II polypeptide, which fragment retains asparaginase activity, in which one or more of the Asp or Asn residues have been replaced with another amino acid. As shown in Figure 7, full-length native *E. coli* L-asparaginase II is 348 amino acid residues in length. A 326 residue fragment of full-length native *E. coli* L-asparaginase II polypeptide that has asparaginase activity is described by Kozak *et al* (2000). A suitable modified fragment is typically a contiguous portion of the native enzyme of at least 200 residues in length, more preferably at least 250 residues in length, and still more preferably at least 300, or at least 310, or at least 320, or at least 330, or at least 340 residues in length, or a sequence variant thereof. Although any one or more of the Asp or Asn residues in the modified fragment may be replaced with another amino acid, it is preferred if one or more of the residues corresponding to N46, D146 and N165 of SEQ ID No: 1 have been replaced with another amino acid as described above. The modified fragment preferably has at least 90% sequence identity with the corresponding portion of the native enzyme, and still more preferably at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99% sequence identity with the amino acid sequence of the corresponding portion of the native *E. coli* L-asparaginase. However, it is preferred if the modified fragment contains amino acid residues equivalent to T34, Y47, S80, T111, D112 and K184 as numbered in SEQ ID No: 1, which define the active site of the enzyme.

The modified *E. coli* L-asparaginase enzyme that is resistant to cleavage by human AEP has the ability to hydrolyse L-asparagine to L-aspartate, i.e. it has asparaginase activity. This can be tested for and measured using methods well known in the art. Preferably, an immunoassay such as the Medac Asparaginase Activity Test (MAAT), commercially available from Medac, is used.

Typically, the modified enzyme has at least 30% of the asparaginase activity of the native *E. coli* L-asparaginase enzyme. It is more preferred if the modified enzyme has at least 50%, preferably at least 70%, more preferably at least 90%,
5 and yet more preferably at least 99% of the asparaginase activity of the native enzyme. Most preferably, the modified enzyme has 100% or more of the asparaginase activity of the native *E. coli* L-asparaginase II enzyme.

In an alternative and less preferred embodiment, the modified *E. coli*
10 L-asparaginase may be glycosylated in order to prevent cleavage by human AEP. This embodiment is less preferred because it is more complicated and expensive. Preferably, the modified enzyme is glycosylated at, or adjacent to, any one, any two, or all three of residues N46, D146 and N165. Methods for site-specific glycosylation are known in the art and are described, for example, by Zhang *et al*
15 (2004).

In an embodiment, the modified *E. coli* L-asparaginase of the first aspect of the invention has been pegylated. Methods for pegylating an enzyme are well known in the art. Indeed, native *E. coli* L-asparaginase which has been pegylated is
20 commercially available as Pegaspargase (Graham, 2003).

A second aspect of the invention provides a polynucleotide that encodes a modified *E. coli* L-asparaginase enzyme as defined above in the first aspect of the invention.

25 The invention also includes a vector comprising a polynucleotide that encodes a modified *E. coli* L-asparaginase enzyme as defined in the first aspect of the invention. The vector is typically a plasmid vector or a viral vector. The invention further includes a cell containing the polynucleotide or the vector. The
30 cell is typically a bacterial a yeast cell, or a cell from an insect or mammalian cell line. A large number of suitable vectors and cell lines for cloning and expressing the modified *E. coli* L-asparaginase enzyme are very well known in the art. Similarly, a large number of gene therapy vectors suitable for administration to a

patient for targeted expression of the modified *E. coli* L-asparaginase in the patient, are well known in the art.

A third aspect of the invention provides a method of making a modified *E. coli* L-asparaginase enzyme as defined in the first aspect of the invention using recombinant DNA technology. Typically, the method comprises providing a plurality of cells that contain a polynucleotide that encodes the modified *E. coli* L-asparaginase enzyme under conditions suitable for expression of the enzyme from the polynucleotide encoding it, and obtaining the enzyme thus produced. Typically, the method further comprises isolating and/or purifying the enzyme thus obtained. Suitable techniques for cloning, manipulation, modification and expression of nucleic acids, and production, isolation and purification of expressed proteins, are well known in the art and are described for example in Sambrook *et al* (2001) "*Molecular Cloning, a Laboratory Manual*", 3rd edition, Sambrook *et al* (eds), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA. Indeed, recombinant native *E. coli* L-asparaginase and recombinant *Erwinia* L-asparaginase, have both been commercially available for many years.

A fourth aspect of the invention provides a modified *E. coli* L-asparaginase enzyme as defined above in the first aspect of the invention for use in medicine.

A fifth aspect of the invention provides a pharmaceutical composition comprising a modified *E. coli* L-asparaginase as defined above in the first aspect of the invention, and a pharmaceutically acceptable excipient, solvent, diluent or carrier (including combinations thereof). The carrier, diluent, solvent or excipient must be "acceptable" in the sense of being compatible with the active agents of the invention and not deleterious to the recipients thereof. Typically, the carriers will be water or saline which will be sterile and pyrogen free. Suitable excipients include mannitol and dextrose. Acceptable carriers, solvents, diluents and excipients for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro ed. 1985). The choice of pharmaceutical carrier, solvent, excipient or diluent can be selected with regard to the intended route of

administration and standard pharmaceutical practice. The pharmaceutical compositions may comprise as, or in addition to, the carrier, excipient, solvent or diluent any suitable binder, lubricant, suspending agent, coating agent, or solubilising agent. Preservatives, stabilisers, dyes and even flavoring agents may
5 be provided in the pharmaceutical composition.

Typically, the pharmaceutical composition is formulated for intravenous or intramuscular injection, or intravenous infusion. Alternatively, the modified *E. coli* L-asparaginase may be in the form of a sterile lyophilised plug or powder that,
10 once reconstituted with sterile water or saline solution, is suitable for intravenous or intramuscular injection.

A preferred formulation of the pharmaceutical composition is an isotonic sterile solution in phosphate buffered saline at pH 7-7.5.

15

A sixth aspect of the invention provides a kit of parts comprising a modified *E. coli* L-asparaginase enzyme as defined above in the first aspect of the invention, and at least one further therapeutic anticancer agent.

20 Suitable therapeutic anticancer agents include: alkylating agents including nitrogen mustards such as mechlorethamine (HN₂), cyclophosphamide, ifosfamide, melphalan (L-sarcosine) and chlorambucil; ethylenimines and methylmelamines such as hexamethylmelamine, thiotepa; alkyl sulphonates such as busulphan; nitrosoureas such as carmustine (BCNU), lomustine (CCNU), semustine (methyl-CCNU) and
25 streptozocin (streptozotocin); and triazenes such as decarbazine (DTIC; dimethyltriazenoimidazole-carboxamide); antimetabolites including folic acid analogues such as methotrexate (amethopterin); pyrimidine analogues such as fluorouracil (5-fluorouracil; 5-FU), floxuridine (fluorodeoxyuridine; FUdR) and cytarabine (cytosine arabinoside); and purine analogues and related inhibitors such as
30 mercaptopurine (6-mercaptopurine; 6-MP), thioguanine (6-thioguanine; TG) and pentostatin (2'-deoxycoformycin); natural products including vinca alkaloids such as vinblastine (VLB) and vincristine; epipodophyllotoxins such as etoposide and teniposide; antibiotics such as dactinomycin (actinomycin D), daunorubicin

(daunomycin; rubidomycin), doxorubicin, bleomycin, plicamycin (mithramycin) and mitomycin (mitomycin C); and biological response modifiers such as interferon alphenomes; and other agents including platinum coordination complexes such as cisplatin (*cis*-DDP) and carboplatin; anthracenedione such as mitoxantrone and anthracycline; substituted urea such as hydroxyurea; methyl hydrazine derivative such as procarbazine (N-methylhydrazine, MIH); and adrenocortical suppressant such as mitotane (*o,p'*-DDD) and aminoglutethimide; taxol and analogues/derivatives; and hormone agonists/antagonists such as flutamide and tamoxifen.

10

Preferred therapeutic anticancer agents, for use against leukaemia, include daunorubicin, steroids, vincristines, anthracyclines, nucleoside analogues, methotrexate, thiopurines and alkylating agents such as cyclophosphamide.

15 A seventh aspect of the invention provides a method of combating a disease or condition where asparagine depletion or deprivation would be therapeutically useful, the method comprising administering a modified *E. coli* L-asparaginase enzyme as defined above in the first aspect of the invention to an individual who has said disease or condition.

20

This aspect of the invention includes the use of a modified *E. coli* L-asparaginase enzyme as defined in the first aspect of the invention in the manufacture of a medicament for combating a disease or condition where asparagine depletion or deprivation would be therapeutically useful.

25

Typically the disease or condition is one where asparagine depletion or deprivation in the blood would be therapeutically useful.

By "combating" a particular disease or condition we include the meaning of treating, preventing or ameliorating the symptoms of that particular disease or condition.

30

In other words, the invention includes treating a disease or condition that responds to asparagine depletion or deprivation. A disease or condition which responds to asparagine depletion refers to a disorder wherein the cells responsible for the disease state either lack or have a reduced ability to synthesise asparagine.

5 Depletion or deprivation of asparagine to such cells can be partial or substantially complete, so long as the desired therapeutic benefit is achieved. In certain embodiments, more than about 50% of asparagine in the serum is depleted, preferably greater than about 75%, with depletion of more than 95% being most preferably achieved. Examples of diseases which respond to asparagine depletion

10 or deprivation include certain malignant diseases, particularly malignant hematologic diseases, including lymphomas, leukaemias and myelomas. Particular examples of leukaemias treatable according to the invention include acute lymphoblastic leukaemia (ALL), acute non-lymphocytic leukaemias, B-cell and T-cell leukaemias, chronic leukaemias, and acute undifferentiated leukaemia.

15 Non-malignant hematologic diseases which respond to asparagine depletion include immune system-mediated blood diseases, e.g., infectious diseases such as those caused by HIV infection (i.e., AIDS). Non-haematological diseases associated with asparagine dependence include autoimmune diseases, for example rheumatoid arthritis, SLE, autoimmune, collagen vascular diseases and AIDS

20 (according to US 6,251,388). Other autoimmune diseases include osteoarthritis, Issac's syndrome, psoriasis, insulin dependent diabetes mellitus, multiple sclerosis, sclerosing panencephalitis, systemic lupus erythematosus, rheumatic fever, inflammatory bowel disease (e.g., ulcerative colitis and Crohn's disease), primary billiary cirrhosis, chronic active hepatitis, glomerulonephritis, myasthenia gravis,

25 pemphigus vulgaris, and Graves' disease. Indeed, any disease the cells responsible for which cease proliferating, become senescent, undergo apoptosis or die in response to asparagine depletion, may be treated using the modified asparaginase enzyme. As will be appreciated, cells suspected of causing disease can be tested for asparagine dependence in any suitable *in vitro* or *in vivo* assay, e.g., an *in vitro*

30 assay wherein the growth medium lacks asparagine.

The invention thus includes the use of a modified *E. coli* L-asparaginase enzyme as defined in the first aspect of the invention in the manufacture of a medicament for combating a neoplastic condition in an individual.

- 5 More specifically, the neoplastic condition to be combated may be paediatric ALL, adult ALL, lymphoma, non-Hodgkin's lymphoma, AIDS related lymphoma, and advanced solid tumours.

10 The modified L-asparaginase of the invention is likely to be included in the induction, intensification and reinduction blocks for the treatment of childhood ALL and non-Hodgkin's lymphoma, and occasionally for intensification in acute myeloid leukaemia (AML).

15 In an embodiment, when the condition to be treated is a neoplastic condition, the method may further comprise administering at least one additional anticancer therapeutic agent to the individual.

20 The invention includes the use of a modified *E. coli* L-asparaginase enzyme as defined in the first aspect of the invention in the manufacture of a medicament for combating a neoplastic condition in an individual, wherein the individual is administered at least one additional anticancer therapeutic agent.

25 The invention includes the use of a modified *E. coli* L-asparaginase enzyme as defined in the first aspect of the invention and at least one additional anticancer therapeutic agent in the manufacture of a medicament for combating a neoplastic condition in an individual.

30 The invention also includes the use of at least one anticancer therapeutic agent (other than asparaginase) in the manufacture of a medicament for combating a neoplastic condition in an individual, wherein the individual is administered a modified *E. coli* L-asparaginase enzyme as defined in the first aspect of the invention.

Suitable therapeutic anticancer agents include those described above with respect to the kit of parts of the sixth aspect of the invention. Preferred therapeutic anticancer agents include daunorubicin, steroids, vincristines, anthracyclines, nucleoside analogues, methotrexate, thiopurines and alkylating agents such as cyclophosphamide.

Suitable doses of the modified *E. coli* L-asparaginase to be administered typically range from 1,000 Units to 20,000 Units. (One unit of asparaginase splits one micromole of ammonia from L-asparagine in one minute under standard conditions.) In certain embodiments, the dose of the modified *E. coli* L-asparaginase to be administered is sufficient to deplete at least 50% of the L-asparagine in the patient's serum, preferably at least 75%, and more preferably at least 90% or at least 95% of the L-asparagine in the patient's serum within 2 hours. The frequency of administration will typically range from every day, to once every three weeks. In any event, the most appropriate treatment regimen for any particular patient will be determined by the physician.

An eighth aspect of the invention provides a method of identifying an L-asparaginase enzyme that may have advantageous properties for therapeutic use, the method comprising providing a test L-asparaginase, and determining whether and/or to what extent the L-asparaginase is resistant to cleavage by human AEP, wherein an L-asparaginase that is not cleaved by human AEP may have advantageous properties for therapeutic use.

The advantageous properties for therapeutic use include a longer half-life *in vivo*, and a reduced allergenicity, compared to native *E. coli* L-asparaginase.

The step of determining whether and/or to what extent the L-asparaginase is resistant to cleavage by human AEP, is typically performed *in vitro*.

The test L-asparaginase is preferably a modified *E. coli* L-asparaginase as described above with respect to the first aspect of the invention. However, the test

L-asparaginase may be from other species, and may be a modified *Erwinia* or *Wolinella* L-asparaginase.

Thus a preferred embodiment of this aspect of the invention includes a method of
5 identifying a modified *E. coli* L-asparaginase that has a longer half-life *in vivo*
and/or a reduced allergenicity compared to native *E. coli* L-asparaginase, the
method comprising providing a modified *E. coli* L-asparaginase, and determining
whether and/or to what extent the modified L-asparaginase is resistant to cleavage
by human AEP, wherein a modified *E. coli* L-asparaginase that is not cleaved by
10 human AEP may have a longer half-life *in vivo* and/or a reduced allergenicity
compared to native *E. coli* L-asparaginase.

Not only has the inventor shown that AEP cleaves native *E. coli* L-asparaginase,
thus contributing to resistance to this drug in some patients, the inventor has
15 realised that patients with a high level of AEP are particularly susceptible to
cleavage of native *E. coli* L-asparaginase, while patients with a low level of AEP
are much less susceptible. Following from this, it is possible to use a patient's
AEP level to determine which L-asparaginase should or should not be
administered to that patient.

20

A ninth aspect of the invention thus provides a kit of parts for an assay for
determining the level of AEP in a suitable sample.

It is appreciated that AEP protein levels may be determined using any of the well-
25 known immunoassay techniques, such as an enzyme linked immunosorbent assay
(ELISA). Alternatively, Luminex or other bead-based assays for analysis of
multiple biomarkers simultaneously in one sample may be used to detect AEP in
the sample. Western blotting may also be used but this is less-preferred.

30 In one embodiment, the kit comprises anti-AEP "capture" antibodies attached to a
solid substrate, and anti-AEP "detecting" antibodies. Such a kit can be used, for
example, in a sandwich ELISA to determine the level of AEP protein in a suitable

sample. The kit is preferably for determining the level of human AEP in the sample, and the anti-AEP antibodies are preferably anti-human AEP antibodies.

Suitable anti-AEP antibodies can readily be obtained. For example, goat anti-human AEP polyclonal IgG antibodies and mouse anti-human AEP monoclonal IgG2A antibodies (R&D Systems, Catalogue Numbers AF2199 and MAB2199, respectively) are commercially available. Also, suitable anti-AEP antibodies can be made using methods well known in the art, for example by immunising an animal with an appropriate AEP peptide and deriving antibodies therefrom, or by phage display technology.

Typically, the kit will also contain detectably-labelled secondary antibodies that specifically bind to the anti-AEP detecting antibodies. The secondary antibody is specific for the mammalian species being tested, for example an anti-human antibody, as is well known in the art. Many such antibodies are commercially available. The detectable-label in the conjugate is typically an enzyme, for example horseradish peroxidase. The kit may further comprise a substrate for the enzyme. As an alternative to horseradish peroxidase, detectable labels suitable for use on the secondary antibody include FITC, biotin and alkaline phosphatase, and RPE for luminex applications. Optionally, the kit may also comprise suitable wash buffers, instructions for carrying out the assay, and/or appropriate positive and negative controls.

For the sandwich ELISA, the anti-AEP "capture" antibodies are typically coated on microtitre plates overnight at 4°C. Unbound antibody is washed off with a wash buffer such as phosphate buffered saline or Tris buffered saline. Serum or other samples are incubated on the plate, typically at 20°C for between 1 and several hours, to allow AEP in the sample to bind to the antibodies. Unbound material is washed off, and the plates are incubated with the anti-AEP "detecting" antibodies, again typically at 20°C for between 1 and several hours. Unbound material is washed off, and plates are incubated with species specific enzyme-labelled (eg horseradish peroxidase) antibody, typically anti-IgG or IgM for serum samples, for 1 to several hours at 20°C. Unbound antibody is washed off and

plates are incubated with a substrate such as TMB for about 5 minutes, and the optical density measured in a photometer.

Once the anti-human antibodies have been replaced with an appropriate antibodies
5 for the species being tested, this ELISA is suitable for detecting AEP protein in a sample from other mammalian species.

A large number of suitable conjugated and unconjugated secondary antibodies are readily available. For example, a variety of bovine, cat, dog, goat, guinea pig,
10 hamster, horse, human, monkey, mouse, pig, rabbit, rat, and sheep secondary antibodies and conjugates are commercially available from Sigma-Aldrich and from Jackson ImmunoResearch Laboratories, Inc.

In another embodiment, the kit comprises AEP, or an antigenic fragment thereof,
15 attached to a solid substrate, and anti-AEP "primary" antibodies. Such a kit can be used, for example, in a competitive ELISA to determine the level of AEP protein in a suitable sample.

The kit is preferably for determining the level of human AEP in the sample, and
20 the AEP, or antigenic fragment thereof, attached to the solid substrate is preferably human AEP and the anti-AEP antibodies are preferably anti-human AEP antibodies, as described above.

As discussed above, the amino acid sequence of human AEP well known, and is
25 shown in Figure 11 (SEQ ID No: 3). If antigenic fragments of AEP are used, it is preferred if multiple overlapping fragments that span most, if not all, of the full length AEP polypeptide are used. As is immediately evident to the skilled person, by an "antigenic fragment" of AEP, we mean that the fragment can be bound by anti-AEP antibodies, and this can readily be determined by the skilled person
30 using only standard techniques.

Preferably, the AEP, or antigenic fragments thereof are made by expression of a suitable DNA construct encoding the protein using recombinant DNA technology.

Suitable techniques for cloning, manipulation, modification and expression of nucleic acids, and purification of expressed proteins, are well known in the art and are described for example in Sambrook *et al* (2001), incorporated herein by reference.

5

Typically, the kit will also contain detectably-labelled secondary antibodies that specifically bind to the anti-AEP primary antibodies.

10

This aspect of the invention thus includes both a solid substrate with human AEP attached thereto, and a solid substrate with anti-human AEP antibodies attached thereto.

In this aspect of the invention, the solid substrate is typically a microtitre plate.

15

A further embodiment of this aspect of the invention provides a kit of parts for an assay for determining the level of AEP polynucleotide in a suitable sample.

20

The kit may comprise a pair of amplification primers that can be used to amplify a given section of the AEP polynucleotide and, optionally, a probe that specifically binds to the AEP polynucleotide within the amplified section.

25

In an embodiment, one or both of the amplification primers are fluorescently labeled. For example, DNA binding dyes such as SYBR Green I and BEBO, which become strongly fluorescent when bound to double stranded DNA, can be used. When such dyes are present in an amplification reaction, the level of fluorescence increases in proportion to the amount of double stranded products formed, which is dependent upon initial AEP polynucleotide levels.

30

In a preferred embodiment, the assay is a TaqMan[®] assay and the probe is fluorescently labeled with a reporter dye and a quenching dye.

The Taqman[®] method is now well known in the art and has been described, for example, by Van der Velden *et al* (2003), Gabert *et al* (2005) and Branford *et al* (2006).

- 5 Briefly, this method is based on the 5'-3' exonuclease activity of Taq DNA polymerase, which results in cleavage of fluorescent dye-labelled probes during PCR; the intensity of fluorescence is then measured by a detection system. The probe is located between the two PCR primers and usually has a melting temperature around 10°C higher than that of the primers. The probe has two
10 fluorescent tags attached to it. One is a reporter dye, such as 6-carboxyfluorescein (FAM), which has its emission spectra quenched due to the spatial proximity of a second fluorescent dye, 6-carboxy-tetramethyl-rhodamine (TAMRA). Degradation of the probe, by the Taq DNA polymerase, frees the reporter dye from the quenching activity of TAMRA and thus the fluorescent activity increases
15 with an increase in cleavage of the probe, which is proportional to the amount of PCR product formed. The ABI Prism 7700 is a laser-coupled spectrophotometer which is suitable for monitoring the fluorescence output of TaqMan[®] performed in a microtitre plate format in real-time.
- 20 The intersection between the amplification plot and the threshold, where the threshold is defined as 10 times the standard deviation of the background fluorescence intensity and which is typically measured between cycle 3 and 15, is known as the cycle threshold, or Ct, value. The Ct value is directly related to the amount of PCR product and therefore related to the initial amount of target DNA
25 present in the PCR reaction.

- Quantitative real-time TaqMan[®] PCR has several advantages over the classical quantitative PCR system. The use of fluorescent dye-labelled probes increases the sensitivity of the system by at several orders of magnitude and gives rise to a
30 linear relationship between copy number and Ct values. In addition, the liquid hybridisation step adds further specificity to the system. The elimination of post-amplification steps increases reliability and reproducibility of the assay. A major factor responsible for the accuracy of this method is the determination of Ct value

within the logarithmic phase of the amplification reaction, instead of the endpoint determination used by conventional quantitative PCR system systems.

Alternatively, other methods can be used such as relative quantitative (RQ) RT-PCR using locked nucleic acid (LNA) technology with or without the Taqman approach (Dreier *et al* (2006); Kivinemi *et al* (2005)).

Suitable techniques for determining levels of a given polynucleotide in a sample by quantitative PCR are discussed by Stahlberg *et al* (2005) and Goff *et al* (2004), which are both incorporated herein by reference.

It is preferred that the kit also comprises amplification primers for a control polynucleotide and, when required for the particular technique used, a probe specific for the control polynucleotide.

In an embodiment, the control polynucleotide can be beta-2-microglobulin (B2M). Other suitable controls include RPLPO, 18S, HMBS, UBC, PPIA, PGK1, IPO8, GUSB, POLR2A, TBP, ACTB, HPRT, TFRC, GAPDH and YWHAZ (Applied Biosystems, 2005) and ABL. B2M and GAPDH are preferred.

In general, a patient with a high level of AEP should not be treated with an L-asparaginase that is susceptible to cleavage by AEP, such as native *E. coli* L-asparaginase. Such a patient should be treated with an L-asparaginase that is resistant to cleavage by AEP such as *Erwinia* L-asparaginase or a modified *E. coli* L-asparaginase as defined in the first aspect of the invention. Conversely a patient with a low level of AEP can be treated with any L-asparaginase, whether or not it is cleaved by AEP. In practice, it is useful for a physician to be provided with a reference level of AEP above which is considered to be a high level of AEP.

A tenth aspect of the invention thus provides a method of assessing the optimal L-asparaginase for administration to a patient in need thereof, the method comprising:

obtaining a suitable sample from a patient;

determining the level of AEP in the sample; and
comparing the level of AEP in the sample with a reference level of AEP.

Typically, the patient is a human patient.

5

A level of AEP in the sample higher than the reference level indicates that the patient should not be treated with an L-asparaginase that is cleaved by AEP. Thus, in this embodiment, when the level of AEP in the sample is above the reference value, the patient is not treated with native *E. coli* L-asparaginase.

10

A level of AEP in the sample higher than the reference level indicates that the patient should be treated with an L-asparaginase that is resistant to cleavage by AEP. Thus, in this embodiment, when the level of AEP in the sample is above the reference value, the patient is preferably treated with an *Erwinia* L-asparaginase or a modified *E. coli* L-asparaginase as defined in the first aspect of the invention.

15

It is appreciated that the level of AEP in the sample may be measured using a kit of parts, solid substrate, or assay as described above with reference to the ninth aspect of the invention. Thus in an embodiment, determining the level of AEP in the sample may comprise determining the level of AEP protein in the sample, for example using an immunoassay. Additionally or alternatively, determining the level of AEP in the sample may comprise determining the level of AEP polynucleotide in the sample. It is preferred if the AEP polynucleotide is a single stranded mRNA or a cDNA derived therefrom.

20

25

It is appreciated that the reference level of AEP may be an absolute value of AEP protein or polynucleotide in the sample. Alternatively, the reference value may be a relative value, i.e. a level of AEP protein or AEP polynucleotide relative to a control protein or polynucleotide.

30

One of the quantitative PCR methods known in the art may be used to measure levels of AEP polynucleotide and a control polynucleotide in a sample. The $\Delta\Delta C_t$

method of comparison may be used to determine the relative level of AEP with respect to the control (see, Stalberg *et al* (2005) and Goff *et al* (2004)).

5 In an embodiment, a level of AEP polynucleotide relative to beta-2 microglobulin (B2M) of 0.1 can be used. More preferably, a relative value of 0.15, and more preferably 0.2, or 0.3, or 0.4, or 0.5 or more in bone marrow cells can be used. This can be measured, for example, in mRNA extracted from bone marrow cells, using RT-PCR and the TaqMan methods which are well known in the art.

10 Other suitable controls are known in the art and include those listed above.

It is appreciated that the reference level of AEP may depend upon the methodology used to detect AEP levels and the source of the sample. Conveniently, the sample is a fluid sample, and suitable samples include whole
15 blood, serum and plasma. It is particularly convenient if the sample is a plasma sample which may be prepared from a blood sample in a standard way (for example by collection in EDTA tubes followed by centrifugation). AEP levels can also be measured in samples of, or containing, bone marrow cells, peripheral blood cells, bone marrow plasma and peripheral plasma.

20

This aspect thus includes the use of an assay for AEP levels to assess the optimal L-asparaginase for administration to a patient in need thereof. The assay may comprise an immunoassay to measure AEP protein levels or may comprise a PCR-based assay to measure AEP polynucleotide levels. Many suitable assays are
25 known in the art and discussed above.

This aspect of the invention includes categorizing a patient in need of treatment with L-asparaginase based on his or her AEP status.

30 By a patient in need of treatment with L-asparaginase we mean a patient who has a disease or condition where asparagine depletion or deprivation would be therapeutically useful. Such diseases and conditions are described above with

respect to the seventh aspect of the invention, and includes neoplastic conditions such as both paediatric and adult ALL.

An eleventh aspect of the invention provides a method of treating a human patient who has a disease or condition where asparagine depletion or deprivation would be therapeutically useful, the method comprising:

selecting the patient on the basis that the patient has a level of AEP above a reference level; and

administering to the patient an L-asparaginase that is resistant to cleavage by AEP.

Diseases and conditions where asparagine depletion or deprivation would be therapeutically useful are described above with respect to the seventh aspect of the invention, and include neoplastic conditions such as both paediatric and adult ALL.

When the disease or condition is a neoplastic condition the method may further comprise administering at least one additional therapeutic anticancer agent to the patient, such as those described above in the sixth aspect of the invention.

When the disease or condition is a neoplastic condition, the patient may be one who is administered at least one additional therapeutic anticancer agent, such as those described above in the sixth aspect of the invention.

This aspect of the invention also provides the use of an L-asparaginase that is resistant to cleavage by AEP in the preparation of a medicament for treating a human patient with ALL, wherein the patient has been selected on the basis that the patient has a level of AEP above a reference level.

Accordingly, when the disease or condition is a neoplastic condition, the medicament may further comprise at least one additional therapeutic anticancer agent, such as those described above in the sixth aspect of the invention.

The L-asparaginase that is resistant to cleavage by AEP is preferably *Erwinia* L-asparaginase or a modified *E. coli* L-asparaginase as defined in the first aspect of the invention.

- 5 In this aspect, preferences regarding methods for obtaining information on a patient's level of AEP, reference levels of AEP, additional therapeutic anticancer agents, doses and routes of administration of the L-asparaginase, and so on, are as described above.
- 10 All of the documents referred to herein are incorporated herein, in their entirety, by reference.

The invention will now be described in more detail by reference to the following Examples, Figures and Tables.

15

Figure 1: Genomic analysis of DNA and cell suspension from patient 5989.

- (A) BAC aCGH results: chromosome 21 is positioned horizontally, with the centromeric to telomeric positions running from left to right respectively. Dye swap experiments are shown by the ▲ (for the dup(21q)) and ● (for normal controls) marked lines respectively. Double deviation of both these experiments from a normal value of 1.00 demonstrates loss or gain of DNA material. Deviation of the ▲ and ● lines greater than 1.00 shows loss or gain of copy number respectively. (B) Examples of the FISH confirmation of aCGH data: each numbered FISH probe is at the numbered position in (A).
- 20

25

Figure 2: Schematic diagram of SNP calls on chromosome 21 in patients with dup(21q).

- Lines adjacent to retained calls represent SNP calls retained in the germ-line (remission bone marrow) and the leukemia. In the shaded boxes, the LOH row shows SNPs that have become homozygous and the loss of call row shows SNPs that are unable to be called in the leukemic sample. This occurs where there are two or more copies of the amplified SNP compared against one copy of the alternate allele, so the signal given is neither heterozygous nor homozygous.
- 30

Figure 3: Box plot diagrams illustrating (A) *LGMN* expression and (B) expression of those genes within the CRA, compared to other ALL subtypes.

On the x-axis are shown seven ALL subtypes, *BCR-ABL*, *E2A-PBX1*, T-ALL, high hyperdiploid (HD), *ETV6-RUNX1*, *dup(21q)* and others. The y-axis represents the relative gene expression level of either *LGMN* (Panel A) or all those genes within the CRA (Panel B). Each box plot shows the distribution of expression levels from 25th to the 75th percentile. The median is shown as a line across the box, where the + is the calculated mean expression level for the particular subtype. The dotted line indicates the inner fence, and a far out value outside the outer fence is shown as *.

Figure 4: Comparative expression of *LGMN* in childhood ALL (real-time quantitative PCR).

On the x-axis are shown *LGMN* RNA levels normalised to B2M (house-keeping gene) in patients with ALL; normal karyotype (2 patients), *t(12;21)* (3 patients), Hyperdiploid (HD, 3 patients) and *dup(21q)* (5 patients). Expression in one normal (patient without leukaemia) and in one HRC57 sample (B-cell cell line, mature, non-malignant) are provided as controls.

Figure 5: Western blot illustrating active AEP/Legumain over-expression in *dup21(q)* patient sample.

50 µg/mL total lysates, lysed with RIPA buffer (Biosource). Samples 1-5 are patient samples (1 = hyperdiploidy, 2 = normal karyotype, 3 = normal karyotype, 4 = *dup 21(q)*, 5 = *ETV6-Runx1*). Samples 6, 7 and 8 are from leukaemic cell lines (6 = HRC57, 7 = Olga 1, 8 = REH). HRC57 and Olga-1 are B-cell cell lines, mature, non-malignant. REH is a *t(12;21)* cell line. The primary anti-legumain antibody used was mouse monoclonal antibody 6EH, used at a dilution of 1: 1000. The secondary antibody was used at a dilution of 1: 2000.

Figure 6: Schematic illustration of *E. coli* L-asparaginase II.

Figure 7: Amino acid and nucleic acid sequences of *E. coli* L-asparaginase II.

Sequences provided are the amino acid sequence (SEQ ID No: 1) and nucleic acid sequence (SEQ ID No: 2) of *E. coli* L-asparaginase II. The three potential AEP/legumain cleavage sites are indicated with an arrow.

5 Figure 8: Cleavage of *E. coli* L-asparaginase by AEP.

E. coli L-asparaginase and the tetanus toxin C fragment (TTCF) were each incubated with and without recombinant human AEP. The digestion products were separated on an SDS-PAGE gel and stained with Coomassie blue. Lane 1: protein weight markers; lane 2: L-asparaginase with AEP; lane 3: L-asparaginase alone; lane 4: TTCF with AEP; lane 5: TTCF alone; and lane 6: AEP alone. Lane 2 marked "Asp+AEP" shows cleavage products of the *E. coli* L-asparaginase by the AEP.

Figure 9: Cleavage of *E. coli* L-asparaginase by AEP.

15 Commercially available preparations of *E. coli* L-asparaginase (Medac and Kidrolase), *Erwinia* L-asparaginase (Erwinase®) and pegylated *E. coli* L-asparaginase (Pegaspargase) were each incubated with and without recombinant human AEP. The digestion products were separated on a 10% SDS-PAGE gel and stained with Coomassie blue. Lane 1: protein weight markers; lanes 2-3: Medac L-asparaginase with and without AEP; lanes 4-5 Kidrolase L-asparaginase with and without AEP; lanes 6-7: *Erwinase* L-asparaginase with and without AEP; lanes 8-9: pegylated *E. coli* L-asparaginase with and without AEP; and lane 10: Medac L-asparaginase alone. The three preparations of *E. coli* L-asparaginase (Medac, Kidrolase and Pegaspargase) are each cleaved by AEP. The *Erwinase* L-asparaginase is not cleaved by AEP.

Figure 10: Modified *E. coli* L-asparaginase is not cleaved by AEP.

A laboratory preparation of wild-type *E. coli* L-asparaginase; *E. coli* L-asparaginase with N46G and D146G mutations; *E. coli* L-asparaginase with N46G, D146G and N165G mutations; and a wild-type *E. coli* L-asparaginase preparation from Medac were each incubated with and without AEP. The digestion products were separated on a 10% SDS-PAGE gel and stained with Coomassie blue. Lanes 1-2: wild-type *E. coli* L-asparaginase (ansBpRSETB)

without (-) and with (+) AEP; lanes 3-4: *E. coli* L-asparaginase with N46G and D146G mutations (ansBpRSETBN46GD146G, $\delta 1$) without (-) and with (+) AEP; lanes 5-6: *E. coli* L-asparaginase with N46G, D146G and N165G mutations (ansBpRSETBN46GD146GN165G, $\delta 2$) without (-) and with (+) AEP; lanes 7-8: Medac *E. coli* L-asparaginase with (+) and without (-) AEP; and lane 9: Medac *E. coli* L-asparaginase without AEP. The two wild-type preparations of *E. coli* L-asparaginase are each cleaved by AEP. Neither of the mutated *E. coli* L-asparaginases are cleaved by AEP.

10 **Figure 11: Sequence of Human AEP (SEQ ID No: 3).**

Sequence provided is of human AEP (legumain) taken from Genbank Accession No. NP_005597.

15 **Table 1: BAC Array CGH and FISH results for 13 ALL patients with duplicated chromosome 21**

Gains (diagonally lined boxes) and losses (stippled boxes) of 21q material detected by aCGH. Shaded regions correspond to those area exhibiting fluorescent ratios within standard deviation limits (SDL). Ratio values were unavailable on several samples due to a lack of material, and on certain DNA clones due to poor ratio measurements (NR). Where FISH was carried out, results are shown numerically as deviations from a normal copy number of 2. The asterisks indicate cases studied for gene expression by oligonucleotide array. The # indicates those cases used for further genomic profiling with Oligo aCGH array analysis. Case 6899 and 6009 are ALL patients with an apparently normal and high hyperdiploidy (tetrasomy 21) karyotype respectively. Certain DNA clones were unavailable for FISH, so additional complementary probes were used

30 **Table 2: Significant differentially expressed genes in patients with dup(21q) (n=8)**

Example 1: Identification of Consistent Patterns of Genomic Amplification, Expression, and Chromosomal Instability in Acute Lymphoblastic Leukaemia with Duplication of 21q.

5 **Summary**

Harewood *et al* (2003) have recently identified a unique subtype of acute lymphoblastic leukaemia (ALL) characterised by duplication of 21q and amplification of RUNX1 (dup(21q)). Array-based comparative genomic hybridisation (aCGH) detected a common region of amplification (CRA) between
10 33.192 and 39.796Mb and a common region of deletion (CRD) between 43.7 and 47Mb in 100% and 70% of patients, respectively. High resolution genotypic analysis showed allelic imbalance in the CRA. These data demonstrate instability of 21q and implicates an alternative detection method for these high-risk patients. Supervised gene expression analysis showed a distinct signature for eight patients
15 with dup(21q), with 10% of over-expressed genes within the CRA. The mean expression of the genes within the CRA was significantly higher in those with dup(21q), when compared to other ALL samples (n = 41). While genomic copy number correlated with overall gene expression levels within areas of loss or gain, there was considerable individual variation. A unique subset of differentially
20 expressed genes outside the CRA and CRD was identified when gene expression signatures of dup(21q) were compared with ALL samples with ETV6-RUNX1 fusion (n=21) or high hyperdiploidy with additional chromosome 21 (n=23). From this analysis, *LGMN* was shown to be over-expressed in patients with dup(21q) (p = 0.0012). In this Example, genomic and expression data has further
25 characterised the new ALL subtype, demonstrated high levels of 21q instability in these patients leading to proposals for novel mechanisms underlying his clinical phenotype.

Introduction

30 Harewood *et al* (2003) recently defined a new recurrent chromosomal abnormality in B-lineage acute lymphoblastic leukaemia (ALL), involving duplication of the long arm of chromosome 21, dup(21q). It was identified on routine screening of ALL samples for the ETV6-RUNX1 fusion by fluorescence in situ hybridisation

(FISH). Leukaemic cells showed multiple RUNX1 signals, seen as clusters in interphase and in tandem duplication on the long arm of an abnormal chromosome 21 in metaphase, but no ETV6-RUNX1 fusion. These patients have a median age of 9 years, a low presenting white blood cell count and a poor prognosis (Robinson *et al*, 2003). Thus, on the current protocol, ALL 2003, these children are classified as high-risk and receive more intensive treatment.

Dup(21q) has also been reported in patients with acute myeloid leukaemia (AML) (Baldus *et al*, 2004). Using BAC array-based comparative genomic hybridisation (BAC aCGH), two common regions of amplification on 21q were identified in 12 AML patients. These were at 25-30Mb and 38.7-39.1Mb. Oligonucleotide expression analysis revealed that all significantly over-expressed genes were located within these amplicons, implying that the changes in gene expression were entirely related to alterations in copy number (Baldus *et al*, 2004). Similar gene expression analyses from children with high hyperdiploid ALL (Yeoh *et al*, 2002) and Down syndrome (Mao *et al*, 2003) also suggested that additional copies of chromosome 21 led to gene over-expression. In the following study, a variety of classical and innovative molecular techniques have been used to characterise the dup(21q) in patients with ALL and, in so doing, provide a plausible alternative therapeutic approach.

Methods

Patients

Patients with dup(21q) and amplification of RUNX1, in accordance with previously published cytogenetic and FISH criteria (Harewood *et al*, 2003), were identified among those registered to the national ALL trials: ALL97/99, MRD PILOT or ALL2003 for children aged 1-18 years, or UKALLXII for adults aged 15-55 years. Each center obtained informed consent from patients or their parents.

Cytogenetic Analysis

Diagnostic bone marrow and/or peripheral blood samples were analysed by standard cytogenetic methods in the UK regional cytogenetics laboratories. Where possible karyotypes were reviewed by cytogeneticists of the Leukaemia Research

Cytogenetics Group (Harrison *et al*, 2001) and described using ISCN (Mitelman, 1995). RUNX1 copy number was determined using the LSI TEL/AML1 ES Dual Color Translocation FISH probe (Abbott Diagnostics, Maidenhead, UK).

5 BAC aCGH and FISH confirmation

Genomic copy number variation was assessed using a commercially available BAC aCGH system (Spectral Genomics, Genosystems, France). The arrays comprise 2621 genomic clones positioned at approximately 1Mb intervals throughout the genome. Of these, 26 are located along 21q from position 15.1Mb (centromeric) to 46.9Mb (telomeric). The position of genes and BAC clones were
10 determined using the National Center for Biotechnology Information (NCBI) MapViewer for Homo Sapiens, Build 35, version 1 (www.ncbi.nlm.nih.gov/mapview).

15 Pooled DNA extracted from 10 healthy donors (sex matched to each ALL sample) was used as a reference (Promega, Madison, WI, USA) and samples were processed according to manufacturers' instructions. On the basis of control experiments, a normal range of 0.8-1.2 was used for the analysis of the patients with dup(21q), a range broader than one calculated on the basis of 2xSD for each
20 clone calculated, in the normal-versus-normal hybridisations. In an attempt to improve sensitivity, fluorescence ratio outside the limit of 2xSD (standard deviation limits, SDL), but within standard cut-off values of between 0.8 and 1.2, were also recorded for comparisons with FISH confirmatory data.

25 To validate DNA copy number changes detected by aCGH, the same BAC clones as spotted on the array were used as FISH probes (Genosystems, France) (see, Table 1). Where possible, 200 interphase nuclei per probe were analysed by two independent analysts and images recorded using MacProbe software (Applied Imaging, Newcastle, UK)

30

Genomic Oligonucleotide Arrays

Five patients (all with BAC aCGH of the same sample) were analysed with high-density oligonucleotide-based CGH (Oligo aCGH) arrays (NimbleGen,

Wisconsin, USA), designed to tile through chromosome 21. Sequences (NCBI Build 35.1) were repeat-masked and oligonucleotides selected at a minimal spacing distance of 60 bp, from both the forward and reverse strands, resulting in approximately 45,000 features along the length of the chromosome. The arrays
5 were synthesised as described previously (Singh-Gasson *et al*, 1999), and standard labeling, hybridisation and image capture was performed. Data were extracted from scanned images using NimbleScan extraction software (NimbleGen, Wisconsin, USA), which allows automated grid alignment, extraction and generation of data files. Segmentation analysis of data sets indicated deletion and
10 amplification breakpoints. Corrections for optical noise, background adjustments and normalisation were performed using Bioconductor as previously described (Gentleman *et al*, 2004). After a loss correction for probe GC content, the log₂ ratios were averaged in windows ranging from 500-5000 bp to produce the final segmentations using SignalMap software (NimbleGen, Wisconsin, USA; Olshen
15 *et al*, 2004).

GeneChip® Human Mapping 10K Array

The GeneChip mapping assay protocol (Affymetrix Inc., Santa Clara) was used to produce the 10,000 single nucleotide polymorphism (SNP) array results and is
20 described elsewhere (Matsuzaki *et al*, 2004). The protocol was adapted such that the purification of polymerase chain reaction (PCR) product was performed using the Ultrafree-MC filtration column (Millipore, Billerica, MA). Signal intensity data was analysed by the GeneChip DNA analysis software (GDAS), which uses a model algorithm to generate SNP calls (Kennedy *et al*, 2003). Signal values are
25 normalised across each array to the median value, and copy number ratios and changes in SNP calls between leukaemia and germ line remission bone marrow were annotated using a program written in visual basic. Noise was reduced by zeroing negative signal values, and using mean signal values in a running window of five SNPs.

30

RNA Extraction and probe preparation

Global expression profiling (GEP) was carried out on bone marrow aspirates from eight patients (seven with aCGH results). RNA was extracted with TRIzol

(Invitrogen, Paisley, UK) followed by a second ethanol precipitation, prior to quality assessment using the Agilent 2100 Bioanalyser (Agilent Technologies, Waldbronn, Germany). Fluorescently labeled cRNA probes were synthesised and hybridised to Affymetrix (Santa Clara, CA, USA) HG-U133A oligonucleotide arrays according to the manufacturers' instructions. The arrays were scanned on a GeneArray scanner (Agilent Technologies, Waldbronn, Germany), and the intensities of the fluorescent signals were captured and analysed with Affymetrix MAS 5.0 software. No scaling was applied.

10 Gene Expression Analysis

GeneSpring 6.0 (Silicon Genetics, Redwood City, CA, USA) was used for raw data normalisation. Firstly, the data were normalised to the median per sample, using all genes not marked absent. Each gene was then divided by the median of its measurements in all samples (i.e. across all arrays). If the median of the raw values was below 10, then each measurement for that gene was divided by 10. Signal intensities were log transformed for statistical analysis. Genes called absent in all samples were removed in order to exclude those with minimal variation over the experiments. Probe sets passing the filter were used to find statistically significant differentially expressed genes between the subgroups studied. Significance Analysis of Microarrays (SAM) was applied to the normalised and log transformed data. Default settings were used, and significant genes were selected based on the d-score with a maximum false discovery rate (FDR) of 5.3%. These were compared with a previously published dataset of 85 children with ALL, of whom 21 had an ETV6-RUNX1 fusion, 23 had high hyperdiploidy comprising at least one additional copy of chromosome 21 (HD+21) and 41 had no abnormality of chromosome 21 (Olshen *et al*, 2004).

Both unsupervised and supervised analyses were used and the results visualised in a 2-way hierarchical cluster (HC). Normalised gene expression values were used to obtain the mean and median expression values of genes within the defined amplicon. Significance in the differences of expression between the different subgroups was tested using t-test.

Quantitative RT-PCR

Real-Time quantitative RT-PCR (RT-RQPCR) was carried out to assess the expression of genes situated within the amplicon, using the Taqman® Gene Expression Assays (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. Appropriate positive and negative control RNA samples were tested in parallel. The comparative Ct method was used for quantitation of relative gene expression. The average Ct value of the endogenous control gene, GAPDH, was subtracted from the average experimental gene Ct value to give the Δ Ct value. Differences between control and test were carried out using $\Delta\Delta$ Ct. Concordance between the RT-RQPCR and global expression profile was demonstrated after calculation of the correlation coefficients between the level of expression as quantified by both RT-RQPCR and Affymetrix expression array.

15 Results

Patients with dup(21q) exhibit genomic amplification of 21q

Genome-wide BAC aCGH showed genomic imbalances in all 10 patients with dup(21q) analysed in this study. Patterns of imbalance corresponding to over- and under-representation of specific regions of chromosome 21 were unique to each patient (Table 1). All BAC clones on chromosome 21 showed gain in at least one patient. These gains frequently involved clones between genomic positions 22.1 and 27.8Mb (clones RP11-64I12 to RP11-90A12). The size of the amplified region varied considerably between patients, from 3-8.6 to 24.0-24.1Mb for patients 6783 and 6788 respectively. A representative aCGH result confirmed by FISH is shown in Figures 1A & B.

A common region of amplification (CRA) of approximately 8.6Mb, between clones RP11-191I6 and RP5-206A10 (genomic position 31.5 and 40.1Mb respectively), was identified in all 10 patients. Deletions were observed in seven patients. With the exception of one deletion within the centromeric region (from 15.1-20.3Mb in patient 5898), all deletions included a common deleted region (CRD) of approximately 4Mb close to the telomeric region of chromosome 21. To prove the validity of the technique, aCGH verified the presence of an entire

additional copy of chromosome 21 in seven patients with HD+21 (patient 6009) (Table 1). Furthermore, no change in copy number was observed among 50 patients with apparently normal copies of chromosome 21 (patient 6899) (Table 1). No recurrent imbalances involving chromosomes other than 21 were identified. In three patients this was the sole genomic change at 1Mb resolution.

Using tiling-path Oligo aCGH, the extent of the CRA was further refined in five patients with dup(21q) to a region of 6.527-6.604Mb in size (between genomic positions 33.192 and 39.796Mb). Using oligo aCGH, the size of the CRD was refined to a 3.541Mb region.

FISH analysis confirmed the variation in copy number along 21q in the cases analysed by BAC aCGH (Table 1 and Figure 1B). The same CRA and CRD were identified. The high concordance between the two procedures indicated the accuracy of BAC aCGH in the determination of copy number changes, while FISH analysis provided precise quantification. Between three and eight additional copies of the clones within the CRA were demonstrated by FISH, indicating a 2.5-5 fold gain. FISH data on copy number changes in a further three patients with dup(21q), provided further confirmation of the BAC aCGH results (data not included).

High resolution genotype array analysis was performed on three patients for whom both diagnostic and remission samples were available, permitting comparison of germ-line and tumor genotypes. As shown in Figure 2, the SNP analyses also identified the areas of genomic gain and loss. Within the CRA and CRD, there was loss of heterozygosity (LOH). This suggested that the amplifications were derived from a single chromosome. LOH may also imply loss of material from the complementary parental chromosome.

Global expression profiling reveals a distinct expression signature

Global gene expression profiling (GEP), using the Affymetrix U133A oligonucleotide array containing 22,283 probes sets, was performed on eight patients identified by aCGH. Their expression profiles were compared to a cohort

of 85 pediatric ALL patients in an unsupervised analysis, as previously reported (van Delft *et al*, 2005). The patients with dup(21q) did not cluster together (data not shown). Since gene expression levels correlate with loss or gain of chromosomal material, it was likely that the expression profile would be influenced by ALL samples with rearrangements and gains of chromosome 21. Thus, a supervised approach was taken. The gene expression profiles of the eight patients were compared: to the full cohort of 85; to a subgroup of 21 patients with the ETV6-RUNX1 fusion; to a subgroup of 23 patients with HD+21. When the gene list was compiled in this manner, patients with dup(21q) exhibited a distinctive expression pattern.

Using SAM, with a cut-off level for FDR of 10%, the three comparisons yielded 4174, 4768, and 5147 probe sets, respectively. The top 150 probe sets (FDR=5.3%) were used for comparison against other ALL samples, and the top 100 for comparisons against patients with the ETV6-RUNX1 fusion (FDR=.54%) and HD+21 (FDR=.81%).

When compared to all children with ALL, there were 12 genes, excluding RUNX1, located within the CRA, which were significantly over-expressed in those with dup(21q). However, when compared to those with HD+21, there were only five over-expressed genes, including RUNX1, within the CRA. Comparison of all three lists, showed that 11 genes were uniquely over-expressed, of which only two, C21orf66 and ATP50 were within the CRA. One of the over-expressed genes was *LGMN*. As shown in Figure 3, *LGMN* expression was significantly elevated in those with a dup(21q) when compared to other subtypes of ALL (t statistics = 4.38; df = 7; p = 0.0012). Using a similar approach, 12 genes outside the CRD, were shown to be expressed at significantly lower levels (Table 2).

Expression of genes contained within the common region of amplification or deletion

The CRA was represented on the Affymetrix U133A GeneChip by 96 probe sets in total, including 40 well-characterised genes and six open reading frames. From a total of 768 probe sets within the CRA analysed for eight patients, 321 (42%)

were present or marginal and up regulated. Of the 46 sequences, 13 were up regulated in at least 75% of patients. The CRD was represented on the GeneChip by 83 probe sets, containing 33 genes, three open reading frames and 3 EST's. From a total of 664 probe sets, 462 (70%) were absent. An absent flag was carried in 22 of the 39 gene sequences in at least 75% of patients. When compared to all children with ALL, 14 (10%) of the top 150 genes significantly over-expressed in those with dup(21q) were located within the CRA. In our previously reported analysis (van Delft *et al*, 2005), when comparing ALL to AML, only 1% of the top 150 genes were located within the amplicon in those with ALL. As shown in table 1, 51 (53%) of the 96 probe sets within the amplicon had a 1.5 fold increase in expression. This suggested that over-expression of the genes within the amplicon was a result of the gain of genomic material. However, 47% of the probe sets were not over-expressed. To examine the effect of the gain of chromosomal material more closely, mean and median expression of the genes within the CRA in the dup(21q) patients and 6 other subtypes of ALL (Figure 3B) were calculated. The mean expression level of the genes contained within the CRA is higher in both patients with dup(21q) (t-test, $p = 0.00903$) and HD (t-test, $p = 2.02e-7$) compared with other subtypes. Thus in ALL, while the loss or gain of chromosomal material was accompanied by a commensurate overall change of expression, there was considerable inter-genic variation.

To confirm the expression level for some of the genes contained within the amplicon, Taqman[®] Gene Expression Assays were used. The following genes were examined in cases with available cDNA: SOD1, OLIG2, IFNAR2, IL10RB, ITSN1, CRYZL1, RUNX1, TTC3, ERG and ETS2. Affymetrix called OLIG2, IFNAR2, ITSN1 and RUNX1 absent and, in confirmation, no gene expression levels were detected by the Taqman[®] assays. For all genes a strong correlation between the Affymetrix and Taqman[®] data was identified.

Discussion

The chromosomal abnormality involving dup(21q) and amplification of RUNX1 was discovered by chance, whilst screening by FISH in childhood ALL for the ETV6-RUNX1 fusion (Harewood *et al*, 2003; Soulier *et al*, 2003). Although

negative for the fusion, cells showed multiple copies of the RUNX1 gene on a duplicated chromosome 21. This abnormality cannot be defined by conventional cytogenetic analysis, since the abnormal chromosome 21 adopts a range of different morphological forms. FISH with probes directed to the RUNX1 gene is currently the only detection method, which explains its description as “amplification of RUNX1”. However, there are several reasons why FISH detection, based solely on RUNX1 copy number, may be inappropriate. Firstly, interpretation may be misleading, particularly in the presence of a high hyperdiploid cell population with multiple copies of chromosome 21. Secondly, since the observed increase in RUNX1 copy number was serendipitous, it may not be a causative mechanism. It is therefore important to characterise this abnormality to provide accurate diagnosis, particularly for ALL patients without any other high-risk clinical features.

In this study, the existence of the new chromosomal abnormality in childhood ALL has been validated, and the rearrangement characterised using whole genome analyses. Different aCGH and FISH approaches highlighted regions of variable gain along 21q in these patients. A CRA was identified, extending to 6Mb in size, from 33.2-39.8Mb, containing the RUNX1 gene. The CRA was telomeric to the first of the two amplified regions described for AML (25-30Mb) but overlapped with the second region (38.7-39.1Mb) (Baldus *et al*, 2004). The majority of patients showed a CRD, 43.7-47Mb, telomeric to the CRA.

These findings suggest that the region on 21q, from 22–47Mb, is highly unstable in acute leukaemia.

Since this CRA spans a large genomic region and reflects considerable chromosomal instability, it is difficult to determine the causative event. This supports previous reports demonstrating that large-scale genomic alteration result in changes in expression of genes within these regions (Pollack *et al*, 2002; Hyman *et al*, 2002). Like the AML study (Baldus *et al*, 2004), the inventor has now shown differential gene expression beyond those genes located in the CRA. Unlike the AML report, in which a control cohort of normal karyotype AML

patients was used, the inventor's present work has benefited from comparisons with patient groups who had either gained an entire chromosome 21, as part of a high hyperdiploid karyotype or in association with an ETV6-RUNX1 fusion. By using these subgroups for comparison, it was possible to identify a unique subset of over- or under-expressed genes in patients with dup(21q) relative to those without other chromosome 21 aberrations. This demonstrated that comparative information on the loss or gain of chromosomal material is essential when interpreting expression data. Curiously, RUNX1 expression in ETV6-RUNX1 positive and dup(21q) patients was comparable, which may be due to the inability of the GEP platform used in this study to distinguish between wild-type RUNX1 and ETV6-RUNX1 fusion transcripts. Since additional copies of the ETV6-RUNX1 fusion and RUNX1 are common findings in patients with t(12;21) (Harrison *et al*, 2005), this may have contributed to the elevated RUNX1 expression levels in ETV6-RUNX1 patients. These observations suggest that there are common processes leading to duplication and translocation, further strengthening the hypothesis that genomic instability of a region on 21q creates a cascade of events leading to or sustaining leukaemogenesis.

In previous studies, it has been demonstrated that allelic imbalances correlate with changes in gene expression (Hyman *et al*, 2002). Consequently, this may result in expression variation of many genes unassociated with, but flanking genes important in cancer pathogenesis (Masayeva *et al*, 2004). However, using genomic and genotypic profiling, we were unable to detect any consistent allelic imbalances outside the CRA and CRD. Thus, we could not correlate all gene expression changes to alteration at the genomic copy level. Not all genes within the amplicon were over-expressed and there was no linear correlation between the degree of amplification and expression. This may result from heterogeneity in amplification within the region or other regulatory mechanisms influencing gene expression, such as epigenetics and bio-feedback regulation. Our data suggest that genomic amplification within the CRA resulted from allelic imbalance. Partial uniparental disomy (UPD) has recently been reported in patients with AML (Raghavan *et al*, 2005), while others have reported disomy of chromosome 21 in

those with Down syndrome and ALL (Rogan *et al*, 1995). Thus it is plausible that this mechanism may contribute to variations in expression.

While the processes that lead to ALL appear to affect a common genomic region
5 of chromosome 21, there is disparity in the outcome to treatment. HD+21 and ETV6-RUNX1 have an excellent survival rate on current chemotherapy protocols, while dup(21q) patients have a poor outcome. Recently, two papers have correlated gene expression patterns with in vitro chemosensitivity of blast cells and both demonstrated that these patterns were predictive of outcome in childhood
10 ALL (Lugthart *et al*, 2005; Holleman *et al*, 2004). Though the expression patterns in our patients did not accurately reflect those associated with poor clinical outcome and chemo-resistance (including L-asparaginase), there were a number of similarities including over-expression (IGHM, CD44, IGFBP7, RPS9 and MAFF) and under-expression (TCF4, F8A and TAF5) of a number of genes. Of these,
15 MAFF over-expression is known to correlate with steroid resistance and F8A down regulation with insensitivity to L-asparaginase.

Resistance to asparaginase can be initiated in the presence of neutralizing antibodies, although these cellular studies clearly suggested alternative
20 mechanisms (Panosyan *et al*, 2004). We have shown that the gene *LGMN* is over-expressed in ALL samples with dup(21q) (Figures 4 and 5). *LGMN* encodes legumain (AEP), which is a lysosomal cysteine protease which specifically cleaves after the asparagine residue and participates in antigen processing (Manoury *et al*, 1998), correlating with adverse outcome in colorectal cancer
25 (Murthy *et al*, 2005). Therefore, we propose that AEP may cleave asparaginase during lysosomal processing as well as promoting antibody formation, which may contribute to the poor prognosis in patients with dup(21q). Asparagine depletion by asparaginase contributes to the current treatment protocol for these patients. Over-expression of AEP may thus be a hitherto undiscovered mechanism of drug
30 resistance, leading to therapeutic failure. AEP has previously been proposed as a candidate for targeted therapy (Liu *et al*, 2003; Ekici *et al*, 2004) and potentially such an approach offers new treatment alternatives for this high-risk group.

However, this is completely different therapeutic approach to the modified *E. coli* asparaginase of the present invention.

We conclude that the CRA represents the only detectable recurrent finding in patients with dup(21q). Gene expression within the CRA was increased significantly, suggesting that genes within this region are important in leukaemogenesis, whilst adding evidence that gain at the genomic level often leads to increased expression. Outside the CRA, over-expression of *LGMN* was demonstrated and we suggest that this gene may contribute to the poor clinical outcome and treatment response observed in these patients. Expression profiling did not show significant over-expression of *RUNX1* in our patients, suggesting that it is unlikely to be the causative gene. The study of patients with other chromosome 21 abnormalities may reveal copy number changes similar to those demonstrated here, but without *RUNX1* amplification, which may confer a poor prognosis. In addition to the 6.5-6.6Mb CRA, there was considerable associated genomic instability in patients with dup(21q), in particular deletions affecting the sub-telomeric region (CRD). The expansion of this innovative study could uncover other molecular and cellular mechanisms underlying this clinical phenotype and may demonstrate a pivotal role of chromosome 21 instability in the initiation of acute leukaemia.

Example 2A: Cleavage of *E. coli* L-asparaginase by AEP

4µg of *E. coli* L-asparaginase and 4µg of the tetanus toxin C fragment (TTCF) protein were each incubated with and without 0.4µg of recombinant human AEP in 50mM sodium citrate buffer pH 4.5 containing 5mM DTT for 3 hours at 37°C. The digestion products (20µl) were separated on a 10% SDS-PAGE gel and stained with Coomassie blue. TTCF was included as a positive control for cleavage as it is known to be cleaved by AEP (Manoury *et al*, 1998). AEP alone was run as a negative control. The results are shown in Figure 8. Lane 2 marked “Asp+AEP” shows cleavage products of the *E. coli* L-asparaginase corresponding to cleavage at the sites indicated in Figures 6 and 7 (N46, D146 and N165).

Example 2B: Cleavage of commercially available L-asparaginases by AEP

8.33 U of commercially available preparations of *E. coli* L-asparaginase (Medac from Medac UK and Kidrolase from OPi Pharmaceuticals), *Erwinia* L-asparaginase (Erwinase[®]) and pegylated *E. coli* L-asparaginase (Pegaspargase from Medac UK) were each incubated with and without 0.4µg of recombinant human AEP in 50mM sodium citrate buffer pH 4.5 containing 5mM DTT for 3 hours at 37°C. The digestion products (20µl) were separated on a 10% SDS-PAGE gel and stained with Coomassie blue. The results are shown in Figure 9.

The three preparations of *E. coli* L-asparaginase (Medac, Kidrolase and Pegaspargase) are each cleaved by AEP. The Erwinase[®] L-asparaginase is not cleaved by AEP.

Example 3: Modified *E. coli* L-asparaginase is Resistant to Cleavage by AEP

DNA from recombinant *E. coli* pKK233 containing *ansB* of *E. coli* (that encodes L-asparaginase II) was subcloned into the bacterial expression vector pRSETB (Invitrogen) using the *Nco*I and *Hind*III restriction enzyme sites. The obtained plasmid was verified by DNA sequencing and named ansBpRSETB. DNA from ansBpRSETB was then used as a template to generate two mutated versions of *ansB* using the PCR method of QuickChange Multi Site-Directed Mutagenesis (Stratagene). The following mutagenic primers were used:

5'-GACTCCGCAACCAAATCTGGCTACACAGTGGGTAAAG-3'

(SEQ ID No: 4)

5'-ACGTCTATGAGCGCAGGCGGTCCATTCAACCTGT-3'

(SEQ ID No: 5)

5'-TAAAGCCTCCGCCGGTCGTGGCGTGCTG-3'

(SEQ ID No: 6)

The mutated form of *ansB* named ansBpRSETBN46GD146GN165G contained N46G, D146G and N165G amino acid mutations. The other mutated form named ansBpRSETBN46GD146G had N46G and D146G amino acid replacements.

DNA from all three forms of ansB were transformed into BL21(DE3)pLysS *E. coli* cells and transformants were selected for ampicillin and chloramphenicol resistance. A single colony from each ansB form was grown overnight in SOB media containing 50 µg/ml ampicillin and 35 µg/ml chloramphenicol at 37°C, and
5 this was used the next day to inoculate 170 ml of fresh SOB media containing 50 µg/ml ampicillin. The culture was grown at 37°C to an OD₆₀₀ of 0.4 – 0.6 and then expression of the recombinant protein was induced by addition of 1mM IPTG. After 3.5 hours, bacterial cells were harvested by centrifugation and the recombinant protein from each ansB form was purified using Qiagen Ni-NTA spin
10 columns and concentrated using a Millipore Microcon YM-10 filter unit.

20µl of the purified recombinant protein (approximately 4µg) from ansBpRSETB, ansBpRSETBN46GD146GN165G, ansBpRSETBN46GD146G and a commercially available *E. coli* L-asparaginase preparation from Medac were each
15 incubated with and without 0.4µg of recombinant human AEP in 50mM sodium citrate buffer pH 4.5 containing 5mM DTT for 3 hours at 37°C. The digestion products (20µl) were separated on a 10% SDS-PAGE gel and stained with Coomassie blue. The results are shown in Figure 10.

20 The native wild-type preparations of *E. coli* L-asparaginase (ansBpRSETB and Medac) are each cleaved by AEP. Neither of the mutated *E. coli* L-asparaginases are cleaved by AEP.

25 **Example 4: Determination of AEP levels in Normal Individuals and Patients with ALL**

RT-PCR and the TaqMan[®] methodology were used on mRNA extracted from bone marrow cells to determine cut-off levels for normal/low, intermediate and high levels of AEP. The Taqman ready assay for AEP (probe 797894) Hs
30 00271599 – M1LGMN was used to test approximately 100 ALL patients along with 5 normal and 5 AML patients. The $\Delta\Delta C_t$ method of comparison was used with beta-2 microglobulin (B2M) as a control. AEP / B2M levels of 0 - 0.1 were

considered to be normal/low, levels between 0.1 and 0.2 intermediate, and AEP levels above 0.2 were considered to be high.

Example 5: Treatment of a Patient with ALL Using a Modified *E. coli* L-asparaginase that is Resistant to Cleavage by AEP

A nine year old child presents to a physician with symptoms of paediatric ALL, which is subsequently diagnosed by the physician. The patient is administered a modified *E. coli* L-asparaginase which has a polypeptide sequence identical to SEQ ID No: 1, but which has Gly residues at positions N46 and N165. The patient is administered the asparaginase parenterally in place of native *E. coli* L-asparaginase in a standard treatment regime, and during subsequent reinduction blocks to maintain asparagine depletion during these blocks. Once remission is obtained, maintenance therapy is instituted without asparaginase.

Example 6: Treatment of a Patient with ALL Using a Modified *E. coli* L-asparaginase that is Resistant to Cleavage by AEP

A nine year old child presents to a physician with symptoms of paediatric ALL, which is subsequently diagnosed by the physician. The patient is administered a modified *E. coli* L-asparaginase which has a polypeptide sequence identical to SEQ ID No: 1, but which has Gly residues at positions N46 and D146. The patient is administered the asparaginase parenterally in place of native *E. coli* L-asparaginase in a standard treatment regime, and during subsequent reinduction blocks to maintain asparagine depletion during these blocks. Once remission is obtained, maintenance therapy is instituted without asparaginase.

Example 7: Treatment of a Patient with ALL Using a Modified *E. coli* L-asparaginase that is Resistant to Cleavage by AEP

A nine year old child presents to a physician with symptoms of paediatric ALL, which is subsequently diagnosed by the physician. The patient is administered a modified *E. coli* L-asparaginase which has a polypeptide sequence identical to

SEQ ID No: 1, but which has Gly residues at positions N46, D146 and N165. The patient is administered the asparaginase parenterally in place of native *E. coli* L-asparaginase in a standard treatment regime, and during subsequent reinduction blocks to maintain asparagine depletion during these blocks. Once remission is
5 obtained, maintenance therapy is instituted without asparaginase.

References

- Albertsen *et al* (2001) *Br J Haematol.* **115**(4), 983-990.
- Applied Biosystems (2005) "Taqman[®] low density array: performance parameters across arrays and endogenous control panel application". (Document 127PR15-01, <http://docs.appliedbiosystems.com/pebi docs/00115036.pdf>)
10
- Baldus *et al* (2004) *Proc Natl Acad Sci USA* **101**, 3915-20.
- Branford *et al* (2006) *Methods Mol Med* **125**: 69-92.
- Capizzi *et al* (1971) *Ann Intern Med.* **74**(6): 893-901.
- 15 Chen *et al* (1997) *J. Biol. Chem.* **272**, 8090-8096.
- Dreier *et al* (2006) *Journal of Clinical Microbiology* **44**: 2714-20.
- Ekici *et al* (2004) *J Med Chem* **47**, 1889-92.
- Gabert J *et al* (2005) *Leukemia* **17**: 2318-57.
- Gentleman *et al* (2004) *Genome Biol* **5**, R80.
- 20 Goff *et al* (2004) "Evaluation of sense-strand mRNA amplification by comparative quantitative PCR" *BMC Genomics* **5**: 76.
- Graham (2003) *Adv. Drug Del. Rev.* **55**, 1293-1302.
- Halfon *et al* (1998) *FEBS Lett.* **438**, 114-118.
- Harewood *et al* (2003) *Leukaemia* **17**, 547-53.
- 25 Harrison *et al* (2001) *Br J Haematol* **113**, 3-10.
- Harrison *et al* (2005) *Br J Haematol* **129**, 520-30.
- Holleman *et al* (2004) *N Engl J Med* **351**, 533-42.
- Hyman *et al* (2002) *Cancer Res* **62**, 6240-5.
- ISCN (1995), ed. Mitelman, F. (S.Karger, Basel).
- 30 Jianhua *et al* (2006) "Probing the antigenicity of *E. coli* L-asparaginase by mutational analysis" *Mol Biotechnol.* **33**(1): 57-65.
- Kennedy *et al* (2003) *Nat Biotechnol* **21**, 1233-7.
- Kivinemi *et al* (2005) *Journal of Clinical Biochemistry* **38**: 1015 -22.
- Kozak *et al* (2000) *Acta Biochimica Polonica* **47**(3), 807-14.
- 35 Li *et al* (2003) *J Biol Chem.* **278**(40): 38980-90.

- Liu *et al* (2003) *Cancer Res* **63**, 2957-64.
- Lugthart *et al* (2005) *Cancer Cell* **7**, 375-86.
- Manoury *et al* (1998) *Nature* **396**, 695-9.
- Mao *et al* (2003) *Genomics* **81**, 457-67.
- 5 Masayeva *et al* (2004) *Proc Natl Acad Sci USA* **101**, 8715-20.
- Matsuzaki *et al* (2004) *Genome Res* **14**, 414-25.
- Murthy *et al* (2005) *Clin Cancer Res* **11**, 2293-9.
- Olshen *et al* (2004) *Biostatistics* **5**, 557-72.
- Panosyan *et al* (2004) *J Pediatr Hematol Oncol* **26**, 217-26.
- 10 Pollack *et al* (2002) *Proc Natl Acad Sci USA* **99**, 12963-8.
- Raghavan *et al* (2005) *Cancer Res* **65**, 375-8.
- Robinson *et al* (2003) *Leukaemia* **17**, 2249-50.
- Rogan *et al* (1995) *Am J Med Genet* **59**, 174-81.
- Singh-Gasson *et al* (1999) *Nat Biotechnol* **17**, 974-8.
- 15 Soulier *et al* (2003) *Leukaemia* **17**, 1679-82.
- Stahlberg *et al* (2005) "Quantitative real-time PCR for cancer detection: the lymphoma case" *Expert Rev. Mol. Diagn.* **5**(2): 1-10.
- Stams *et al* (2005) *Blood* **105**(11), 4223-5.
- van Delft *et al* (2005) *British J Haematology* **130**(1), 26-35.
- 20 Van der Velden *et al* (2003), *Leukemia* **17**: 1013-34.
- Yeoh *et al* (2002) *Cancer cell* **1**, 133-43.
- Zhang *et al* (2004) *Science* **303**: 371-3.

Table 1

Gene	BAC Clone	Clone Position (in Mkb)	3698*	4134*	4279	4405*	4414#	5898*#	6092*	6111#	6783#	6788*#	6957*	7219*	7255*
STCH	RP5-153I22	15.1		1	0	0		1	0		2	0	0	2	2
USP25	RP11-89M24	15.7													
PRSS7	RP11-15E10	18.7		3	0	1		-1	0		0	0	0	3	2
	RP11-375O2	19.3		3	1	1		-1	0		0	0	0	3	2
	RP11-49B5	20.3		3	0	1		-1	0		0	1		1	2
	RP11-49J9	21.1													
NCAM2	RP11-64I12	22.1		3	1	3		1	1		0	1	1	0	2
	RP11-97F14	22.9		3	2	3		1	4		0	1	1	1	2
	RP11-80N20	24		5	1	6		1	4		1	3	3	5	2
	RP11-13J15	24.1		5	1	5		1	4		1	3	3	4	2
GABPA	RP11-88D18	25.5		3	2	6		1	4		0	3	3	4	2
APP	RP11-15H6	26.7		4	2	7		1	4		1	3	3	5	2
	RP11-90A17	27.8		4	1	6			2		3	3	3	4	2
N6AMT1	RP11-79G23	29.4		3	1	6		3	3		0	3	3	4	2
GRIK1	RP11-30N6	29.8		3	1	7		3	2		0	3	3	5	2
TIAM1	RP11-19I16	31.5		3	4	7		3	5		1	3	3	5	2
HUNK-SOD1	RP11-147H1	32.3		6	5	8		3	4		3	3	3	5	2
OLIG2;IFNAR2;IL10RB	RP11-79D9	33.3		4	3	8		3	4		3	3	3	5	2
KCNE2;CLIC6;CRYZL1	RP11-79A12	34.7		7	3	8		3	4		3	3	3	5	2

6009							
6899		0	0	0	0		0

7255*							
7219*							
6957*							
6788*#							
6783#							
6111#							
6092*		4	0	0	0		0
5898*#							
4414#							
4405*		5	0	0	0		0
4279							
4134*		1	-1	3	1		-1
3698*							
Gene	BAC Clone	Clone Position (in Mb)					
RUNX1;ERG;TTC3;ETS2	RP11-17O20	35.3					
PCP4	RP5-206A10	40.1					
DSCAM	RP11-114H1	41.2					
MX2;MX1	RP11-120C17	41.7					
HSF2BP	RP11-88N2	43.7					
PCNT2;DIP2	P1-2H14	46.7					
HRMT1L1	RP1-63H24	46.9					

Table 2: Significant differentially expressed genes in patients with dup(21q) (n=8)

Gene	Probe identifier	Gene name	Chromosomal Location	UniGene cluster
Upregulated				
<i>LGDN</i>	201212_at	Legumain	14q32.1	Hs.18069
<i>C1orf54 (FLJ23221)</i>	219506_at	chromosome 1 open reading frame 54	1q21.2	Hs.91283
<i>STK17B</i>	205214_at	serine/threonine kinase 17b (apoptosis-inducing)	2q32.3	Hs.88297
<i>BHLHB2</i>	201170_s_at	basic helix-loop-helix domain containing, class B, 2	3p26	Hs.171825
<i>ARPC5L</i>	220966_x_at	actin related protein 2/3 complex, subunit 5-like	9q33.3	Hs.132499
<i>TBCD</i>	211052_s_at	tubulin-specific chaperone d	17q25.3	Hs.464391
<i>LSM7</i>	204559_s_at	LSM7 homolog, U6 small nuclear RNA associated (<i>S. cerevisiae</i>)	19p13.3	Hs.512610
<i>GADD45B</i>	209304_x_at	growth arrest and DNA-damage-inducible, beta	19p13.3	Hs.110571
<i>C20orf111</i>	209020_at	chromosome 20 open reading frame 111	20q13.11	Hs.75798
<i>C21orf66</i>	221158_at	chromosome 21 open reading frame 66	21q21.3	Hs.473635
<i>ATP5O</i>	200818_at	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, O subunit (oligomycin sensitivity conferring protein)	21q22.1-q22	Hs.409140
Down Regulated				
<i>NIPBL</i>	207108_s_at	Nipped-B homolog (<i>Drosophila</i>)	5p13.2	Hs.481927
<i>BAT2</i>	208132_x_at	HLA-B associated transcript 2	6p21.3	Hs.436093
<i>CDYL</i>	203100_s_at	chromodomain protein, Y-like	6p25.1	Hs.269092
<i>GFOD1</i>	219821_s_at	glucose-fructose oxidoreductase domain containing 1	6pter-p22.1	Hs.484686
<i>KIAA0265</i>	209256_s_at	KIAA0265 protein	7q32.2	Hs.520710
<i>CAMSAP1</i>	212710_at	calmodulin regulated spectrin-associated protein 1	9q34.3	Hs.522493
<i>PELL2</i>	219132_at	pellino homolog 2 (<i>Drosophila</i>)	14q21	Hs.105103
<i>KIAA0100</i>	201729_s_at	KIAA0100 gene product	17q11.2	Hs.151761
<i>SSI8</i>	216684_s_at	synovial sarcoma translocation, chromosome 18	18q11.2	Hs.404263
<i>FEM1B</i>	212367_at	Fem-1 homolog b (<i>C. elegans</i>)	15q22	Hs.362733
<i>RNF146</i>	221430_s_at	Ring finger protein 146	6q22.1-q22.3	Hs.267120
<i>MBD1</i>	208595_s_at	methyl-CpG binding domain protein 1	18q21	Hs.405610

CLAIMS

1. A modified *E. coli* L-asparaginase II enzyme that is resistant to cleavage by human AEP (legumain).
- 5 2. An enzyme according to Claim 1 in which at least one asparagine residue (Asn, N) has been replaced with another amino acid residue.
3. An enzyme according to Claim 1 or 2 in which at least one aspartate (Asp, A) residue has been replaced with another amino acid residue.
- 10 4. An enzyme according to Claim 2 or 3 in which the at least one Asn or the at least one Asp residue has been replaced with an amino acid residue selected from alanine (Ala, A), arginine (Arg, R), cysteine (Cys, C), glutamine (Gln, Q), glutamic acid (Glu, E), glycine (Gly, G), histidine (His, H), isoleucine (Ile, I), leucine (Leu, L), lysine (Lys, K), methionine (Met, M), phenylalanine (Phe, F), proline Pro, P), serine (Ser, S), threonine (thr, T), tryptophan (Trp, W), tyrosine (Tyr, Y) and valine (Val, V).
- 15 5. An enzyme according to any of Claims 1-4 in which the Asn residue at position 46 of SEQ ID No: 1 (N46) has been replaced with another amino acid residue.
6. An enzyme according to Claim 5 in which N46 has been replaced with a residue other than Asp.
- 25 7. An enzyme according to Claim 5 in which N46 has been replaced with a residue selected from Ala, Arg, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val.
- 30 8. An enzyme according to any of Claims 5-7 in which N46 has been replaced with a Gly residue.

9. An enzyme according to any of Claims 1-8 in which the Asn residue at position 165 of SEQ ID No: 1 (N165) has been replaced with another amino acid residue.
- 5 10. An enzyme according to Claim 9 in which N165 has been replaced with a residue other than Asp.
11. An enzyme according to Claim 9 in which N165 has been replaced with a residue selected from Ala, Arg, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe,
10 Pro, Ser, Thr, Trp, Tyr and Val.
12. An enzyme according to any of Claims 9-11 in which N165 has been replaced with a Gly residue.
- 15 13. An enzyme according to any of Claims 1-12 in which both N46 and N165 have been replaced with a Gly residue.
14. An enzyme according to any of Claims 1-13 in which the Asp residue at position 146 of SEQ ID No: 1 (D146) has been replaced with an amino acid
20 residue other than Asn.
15. An enzyme according to Claim 14 in which D146 has been replaced with a residue selected from Ala, Arg, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val.
25
16. An enzyme according to Claim 14 or 15 in which D146 has been replaced with a Gly residue.
17. An enzyme according to any of Claims 1-16 that has at least 90% sequence
30 identity with the amino acid sequence of native *E. coli* L-asparaginase II (SEQ ID No: 1).
18. An enzyme according to any of Claims 1-17 that has been glycosylated.

19. An enzyme according to Claim 18 that has been glycosylated at, or adjacent to, any one, any two, or all three of the residues at positions 46, 146 and 165.

5

20. An enzyme according to any of Claims 1-19 that has at least 90% of the ability of native *E. coli* L-asparaginase II (SEQ ID No: 1) to hydrolyse L-asparagine to L-aspartate.

10 21. An enzyme according to Claim 20 that has at least 99% of the ability of native *E. coli* L-asparaginase II (SEQ ID No: 1) to hydrolyse L-asparagine to L-aspartate.

22. An enzyme according to any of Claims 1-21 that has been pegylated.

15

23. A pharmaceutical composition comprising an enzyme according to any of Claims 1-22, and a pharmaceutically acceptable excipient, solvent, diluent or carrier.

20 24. A pharmaceutical composition according to Claim 23 that has been formulated for intravenous or intramuscular injection.

25. A pharmaceutical composition according to Claim 23 or 24 that has been formulated as an isotonic sterile solution in phosphate buffered saline at pH 7-7.5.

25

26. A polynucleotide that encodes a modified *E. coli* L-asparaginase II enzyme as defined in any of Claims 1-17.

27. A vector comprising a polynucleotide according to Claim 26.

30

28. A cell comprising a polynucleotide according to Claim 26 or a vector according to Claim 27.

29. A method of making a modified *E. coli* L-asparaginase enzyme as defined in any of Claims 1-17, the method comprising:
providing a plurality of cells according to Claim 28 under conditions suitable for expression of the enzyme from the polynucleotide that encodes said enzyme, and obtaining the enzyme thus produced.
30. A method according to Claim 29 further comprising isolating and purifying the enzyme.
31. A modified *E. coli* L-asparaginase according to any of Claims 1-22 or a polynucleotide according to Claim 26 for use in medicine.
32. A kit of parts comprising a modified *E. coli* L-asparaginase as defined in any of Claims 1-22 and at least one additional therapeutic anticancer agent.
33. A kit of parts according to Claim 32 wherein the at least one additional therapeutic anticancer agent is selected from daunorubicin, steroids, vincristines, anthracyclines, nucleoside analogues, methotrexate, thiopurines and alkylating agents such as cyclophosphamide.
34. A method of combating a condition where asparagine depletion or deprivation would be therapeutically useful, the method comprising administering a modified *E. coli* L-asparaginase enzyme as defined any of Claims 1-22 to an individual having said condition.
35. A method according to Claim 34 wherein the condition is one where asparagine depletion or deprivation in the blood would be therapeutically useful.
36. A method according to Claim 34 or 35 wherein the condition is a neoplastic condition.
37. A method according to Claim 36 wherein the neoplastic condition is selected from paediatric acute lymphoblastic leukaemia (ALL), adult ALL,

lymphoma, non-Hodgkin's lymphoma, AIDS related lymphoma, advanced solid tumours, and acute myeloid leukaemia (AML).

38. A method according to Claim 36 or 37 further comprising administering at
5 least one additional anticancer therapeutic agent to the individual.

39. A method according to Claim 38 wherein the at least one additional
anticancer therapeutic agent is selected from daunorubicin, steroids, vincristines,
anthracyclines, nucleoside analogues, methotrexate, thiopurines and alkylating
10 agents such as cyclophosphamide.

40. A method according to Claim 34 wherein the condition is an autoimmune
conditions selected from systemic lupus erythramatosus (SLE), rheumatoid
arthritis (RA) and AIDS.
15

41. Use of a modified *E. coli* L-asparaginase enzyme as defined in any of
Claims 1-22 in the manufacture of a medicament for combating a condition where
asparagine depletion or deprivation would be therapeutically useful.

20 42. Use according to Claim 41 wherein the condition is a neoplastic condition.

43. Use of a modified *E. coli* L-asparaginase enzyme as defined in any of
Claims 1-22 and at least one additional anticancer therapeutic agent in the
manufacture of a medicament for combating a neoplastic condition in an
25 individual.

44. Use of a modified *E. coli* L-asparaginase enzyme as defined in any of
Claims 1-22 in the manufacture of a medicament for combating a neoplastic
condition in an individual, wherein the individual is administered at least one
30 additional anticancer therapeutic agent.

45. Use of at least one anticancer therapeutic agent other than L-asparaginase
in the manufacture of a medicament for combating a neoplastic condition in an

individual who is administered a modified *E. coli* L-asparaginase enzyme as defined in any of Claims 1-22.

46. Use according to any of Claims 42-45 wherein the neoplastic condition is selected from paediatric ALL, adult ALL, lymphoma, non-Hodgkin's lymphoma, AIDS related lymphoma, advanced solid tumours and AML.

47. Use according to any of Claims 43-45 wherein the neoplastic condition is ALL, and the at least one anticancer therapeutic agent is selected from daunorubicin, steroids, vincristines, anthracyclines, nucleoside analogues, methotrexate, thiopurines and alkylating agents such as cyclophosphamide.

48. A method of identifying an L-asparaginase enzyme that may have an advantageous property for therapeutic use, the method comprising:
providing a test L-asparaginase, and
determining whether and/or to what extent the test L-asparaginase is resistant to cleavage by human AEP,
wherein an L-asparaginase that is not cleaved by human AEP may have the advantageous property for therapeutic use.

49. A method according to Claim 48 wherein the advantageous property for therapeutic use is a longer half-life *in vivo* and/or a reduced allergenicity, compared to native *E. coli* L-asparaginase.

50. A method according to Claim 48 or 49 wherein the step of determining whether and/or to what extent the L-asparaginase is resistant to cleavage by human AEP is performed *in vitro*.

51. A method according to any of Claims 48-50 wherein the test L-asparaginase is a modified *E. coli* L-asparaginase, a modified *Erwinia* L-asparaginase or a modified *Wolinella* L-asparaginase.

52. A method of identifying a modified *E. coli* L-asparaginase that has a longer half-life *in vivo* and/or a reduced allergenicity compared to native *E. coli* L-asparaginase, the method comprising:

providing a modified *E. coli* L-asparaginase, and

5 determining whether and/or to what extent the modified L-asparaginase is resistant to cleavage by human AEP,
wherein a modified *E. coli* L-asparaginase that is not cleaved by human AEP may have a longer half-life *in vivo* and/or a reduced allergenicity compared to native *E. coli* L-asparaginase.

10

53. A kit of parts for a sandwich ELISA to determine the level of AEP protein in a suitable sample, the kit comprising anti-AEP capture antibodies attached to a solid substrate and anti-AEP detecting antibodies.

15 54. A kit according to Claim 53 further comprising detectably-labelled secondary antibodies that specifically bind to the anti-AEP detecting antibodies.

55. A kit of parts for a competitive ELISA to determine the level of AEP protein in a suitable sample, the kit comprising AEP protein attached to a solid
20 substrate and anti-AEP primary antibodies.

56. A kit according to Claim 53 further comprising detectably labelled secondary antibodies that specifically bind to the anti-AEP primary antibodies.

25 57. A kit of parts according to Claim 54 or 56 wherein the detectable label is an enzyme, and the kit further comprises a substrate for the enzyme.

58. A kit of parts according to any of Claims 52–57 wherein the AEP protein is human AEP protein.

30

59. A kit according to any of claims 52–58 wherein the solid substrate is a microtitre plate.

60. A kit of parts for an assay to determine the level of AEP polynucleotide in a suitable sample, the kit comprising a pair of amplification primers that can be used to amplify a section of the AEP polynucleotide, and an AEP probe that specifically binds to the AEP polynucleotide within the amplified section.

5

61. A kit according to Claim 60 wherein the assay is a TaqMan[®] assay and the AEP probe is fluorescently labeled with a reporter dye and a quenching dye.

62. A kit according to Claim 60 or 61 further comprising amplification primers and, optionally, a probe specific for a control polynucleotide.

10

63. A kit according to Claim 62 wherein the control polynucleotide is beta-2-microglobulin (B2M).

64. A method of assessing the optimal L-asparaginase for administration to a patient in need thereof, the method comprising:

15

obtaining a suitable sample from a patient;

determining the level of AEP in the sample; and

comparing the level of AEP in the sample with a reference level of AEP.

20

65. A method according to Claim 64 wherein a level of AEP in the sample that is higher than the reference level indicates that the patient should not be treated with an L-asparaginase that is cleaved by AEP.

66. A method according to Claim 65 wherein the patient should not be treated with native *E. coli* L-asparaginase.

25

67. A method according to Claim 64 wherein a level of AEP in the sample that is higher than the reference level indicates that the patient should be treated with an L-asparaginase that is resistant to cleavage by AEP.

30

68. A method according to Claim 67 wherein the L-asparaginase that is resistant to cleavage by AEP is an *Erwinia* L-asparaginase or a modified *E. coli* L-asparaginase as defined in any of Claims 1-22.

5 69. A method according to any of Claim 64-68 wherein determining the level of AEP in the sample comprises determining the level of AEP protein in the sample.

10 70. A method according to Claim 69 wherein determining the level of AEP in the sample comprises the use of a kit according to any of Claims 53-59.

71. A method according to any of Claim 64-68 wherein determining the level of AEP in the sample comprises determining the level of AEP polynucleotide in the sample.

15 72. A method according to Claim 71 wherein determining the level of AEP in the sample comprises the use of a kit according to any of Claims 60-63.

20 73. A method according to Claim 71 or 72 wherein the reference value is an AEP / B2M value of 0.2.

74. A method according to any of Claims 64-73 wherein the suitable sample comprises blood or plasma.

25 75. A method according to any of Claims 64-73 wherein the suitable sample comprises bone marrow cells.

76. Use of an assay for measuring AEP levels to assess the optimal L-asparaginase for administration to a patient in need thereof.

30 77. Use according to Claim 76 wherein the assay comprises an immunoassay.

78. Use according to Claim 76 wherein the assay comprises a PCR-based assay.

79. A method or a use according to any of Claims 64-78 wherein the patient
5 has a condition where asparagine depletion or deprivation would be therapeutically useful.

80. A method or a use according to Claim 79 wherein the patient has a condition as defined in any of Claims 35-37 or 40.

10

81. A method for categorizing a patient in need of treatment with L-asparaginase based on his or her AEP status, the method comprising determining the level of AEP in a suitable sample obtained from the patient.

15 82. A method according to Claim 81 further comprising comparing the level of AEP in the sample with a reference level of AEP.

83. A method of treating a human patient who has a condition where asparagine depletion or deprivation would be therapeutically useful, the method
20 comprising:

selecting the patient on the basis that the patient has a level of AEP above a reference level; and

administering to the patient an L-asparaginase that is resistant to cleavage by AEP.

25

84. Use of an L-asparaginase that is resistant to cleavage by AEP in the preparation of a medicament for treating a human patient with a condition where asparagine depletion or deprivation would be therapeutically useful, wherein the patient has been selected on the basis that the patient has a level of AEP above a
30 reference level.

85. A method or a use according to Claim 83 or 84 wherein the L-asparaginase that is resistant to cleavage by AEP is *Erwinia* L-asparaginase or a modified *E. coli* L-asparaginase as defined in any of Claims 1-22.

86. A method or a use according to any of Claims 82-85 wherein the reference level is an AEP / B2M value of 0.2.

87. A method or a use according to any of Claims 81-86 wherein the patient
5 has a condition as defined in any of Claims 35-37 or 40.

Figure 1 (A)

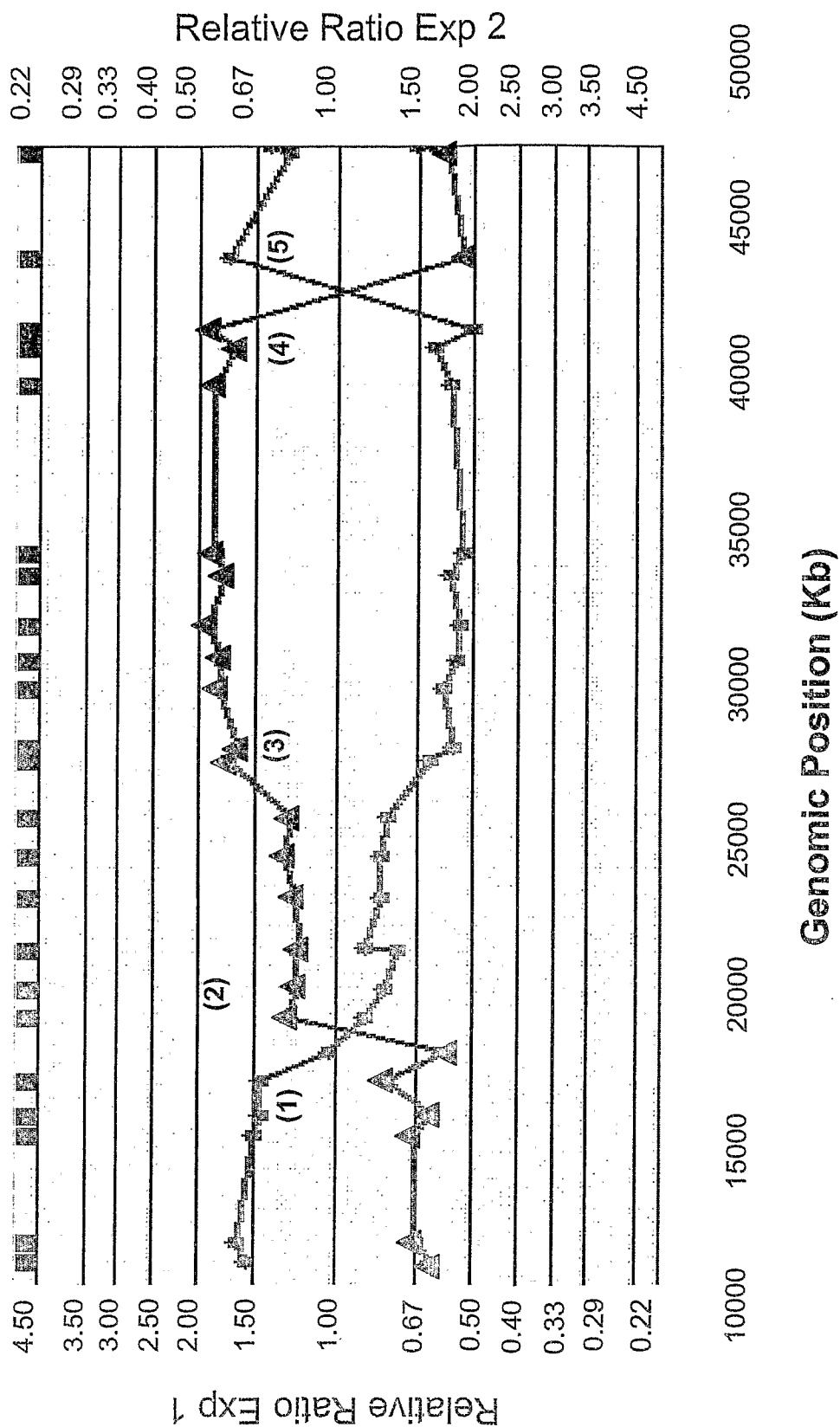


Figure 1 (B)

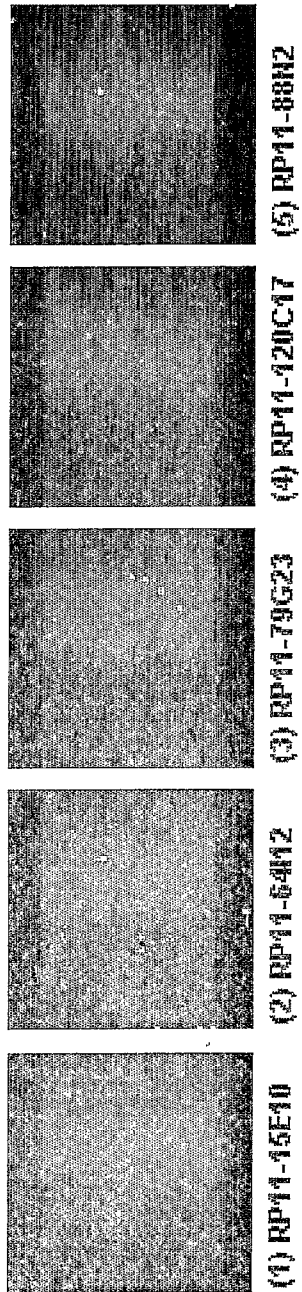


Figure 2

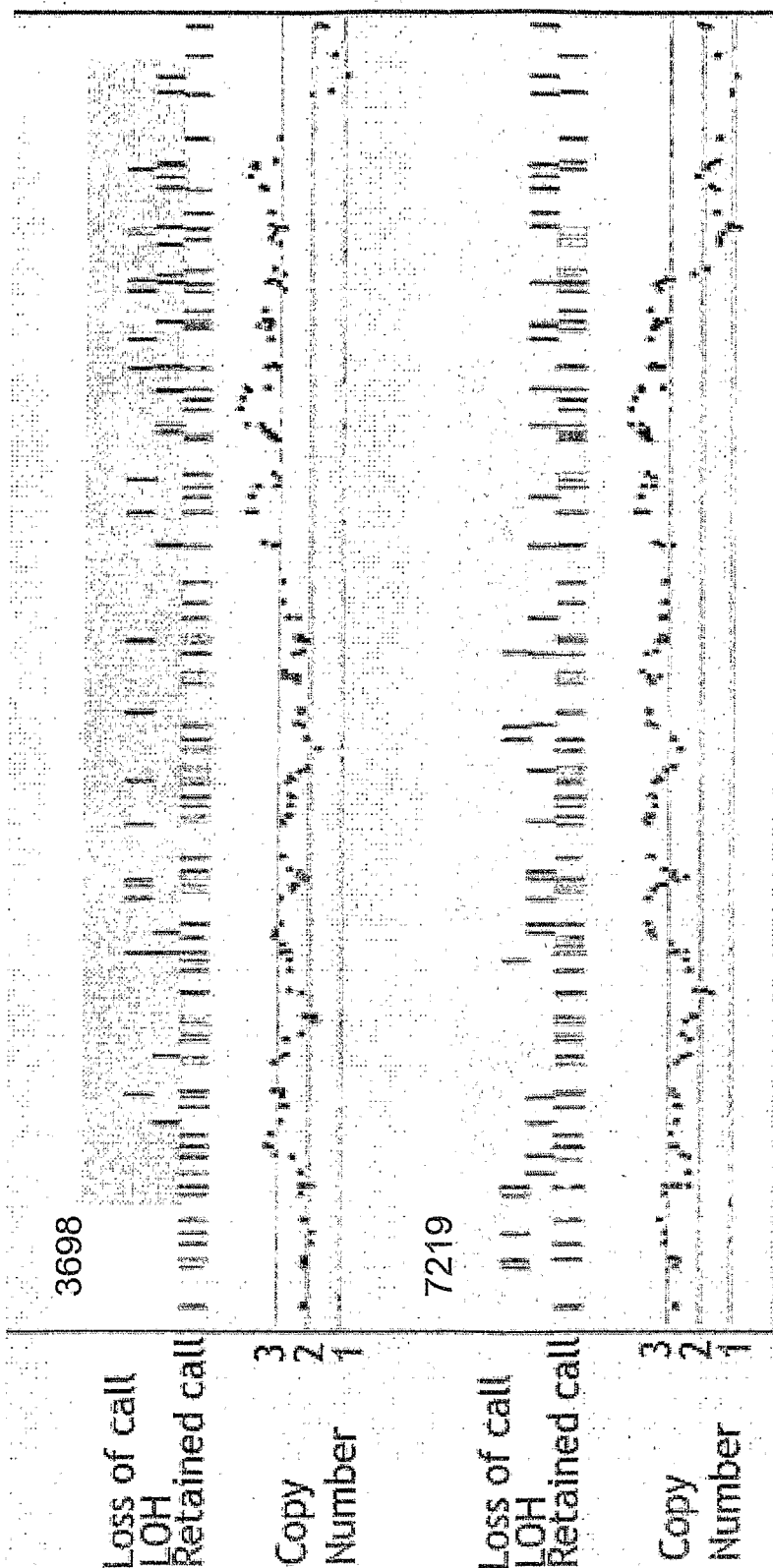


Figure 3 A

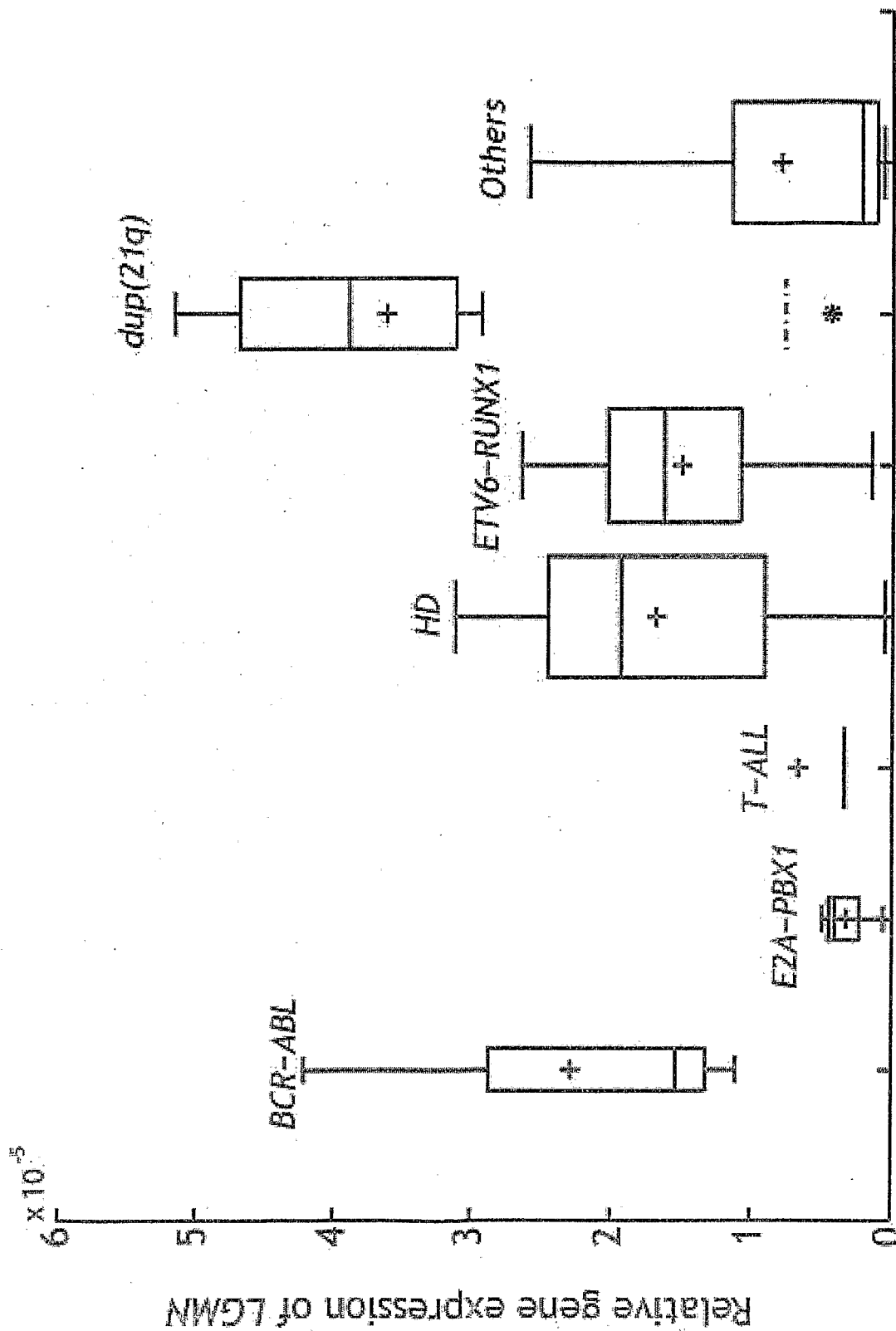


Figure 3 B

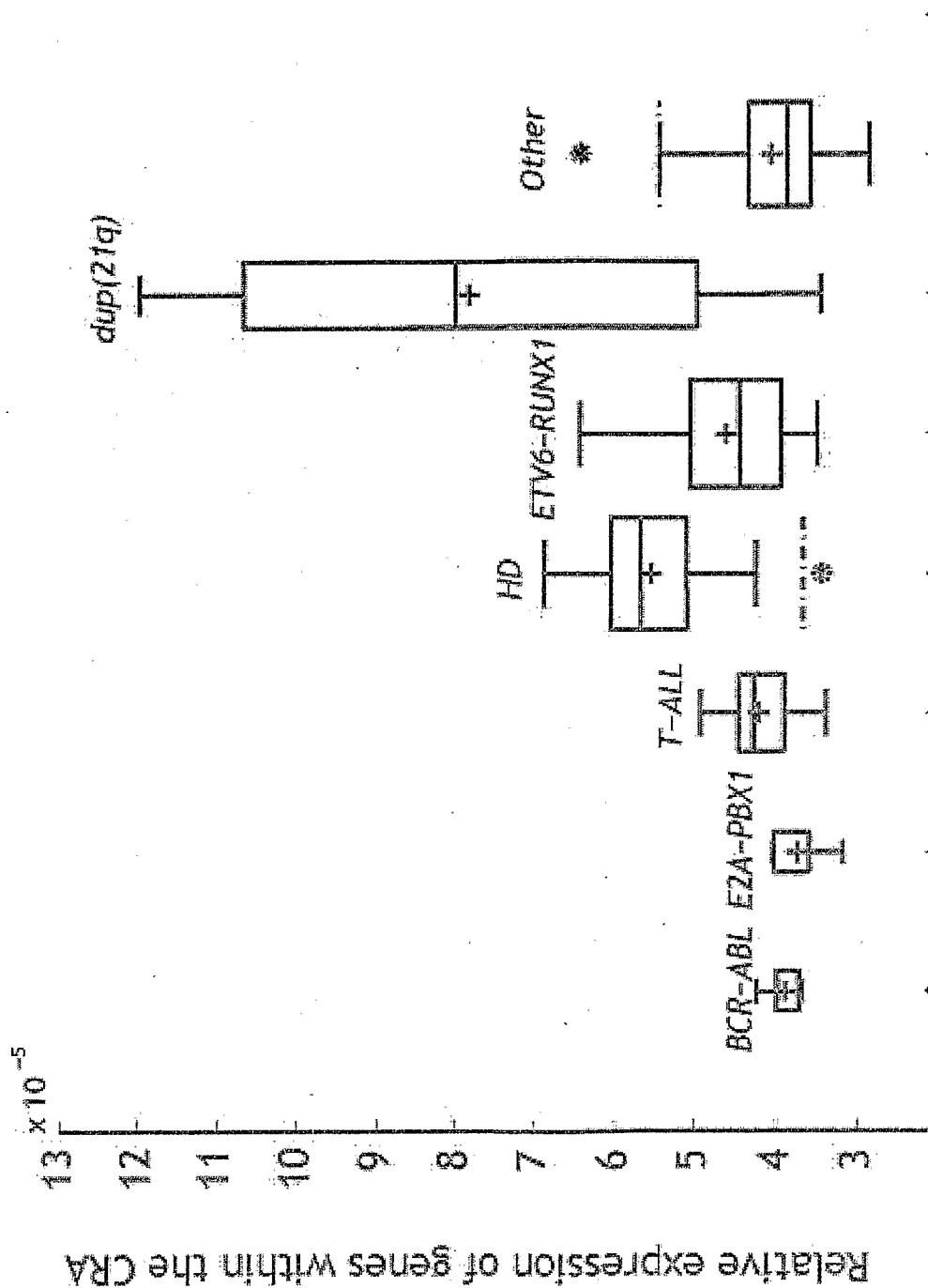


Figure 4

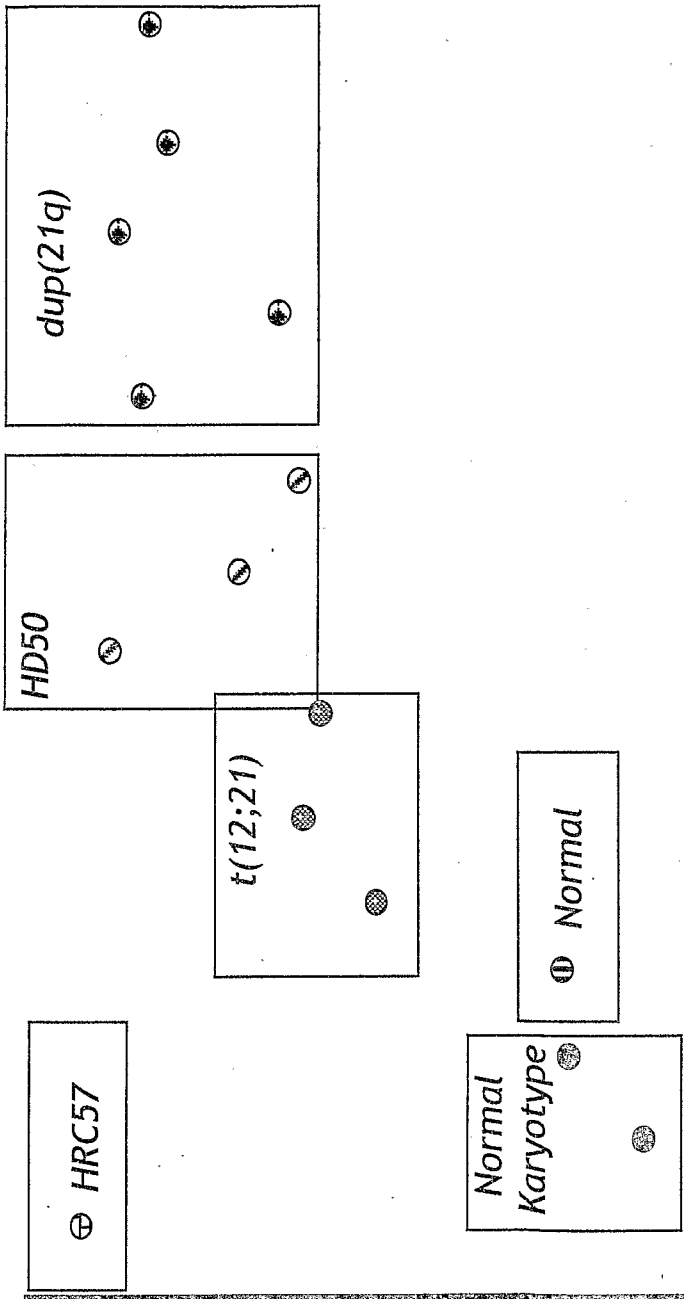


Figure 5

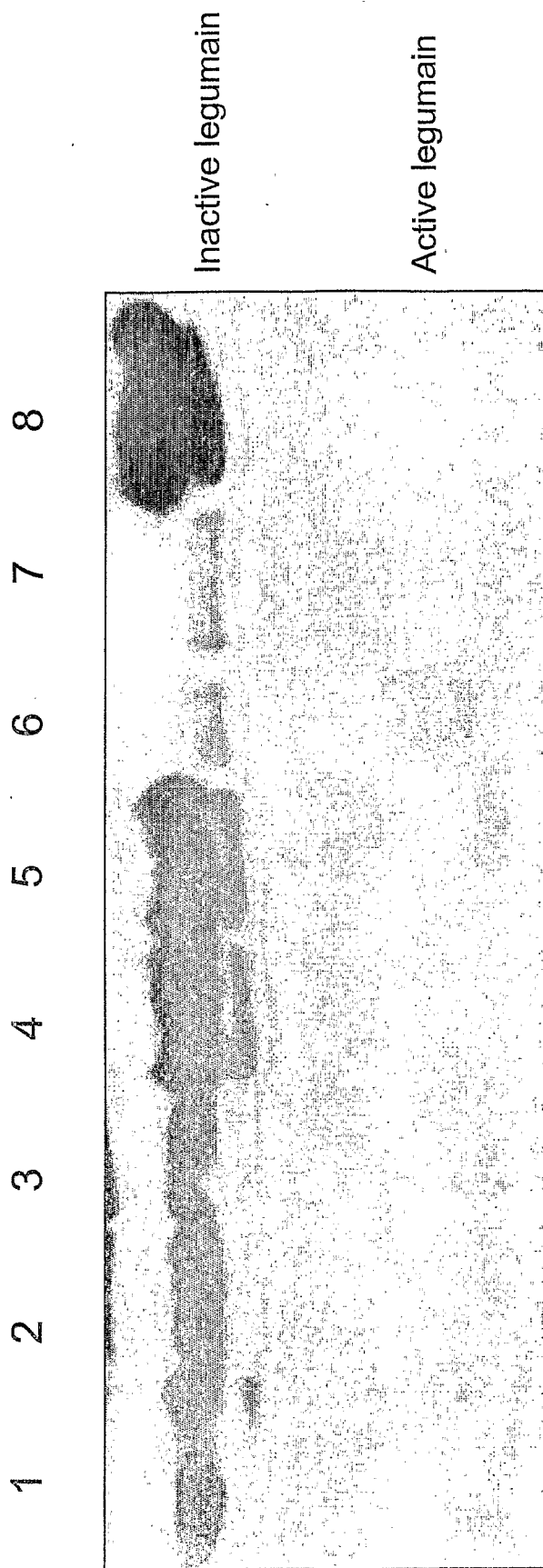


Figure 6
E. Coli L-Asparaginase Type II (Medac)

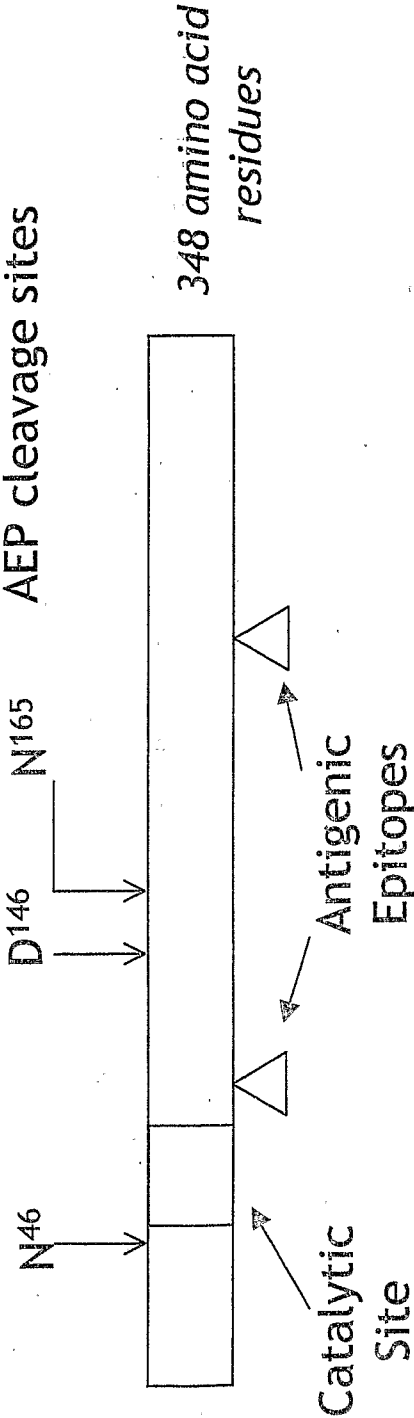


Figure 7

E.coli Asparaginase II

1 atggagtttttcaaaaagacggcacttgccgcactgggttatgggttttagtggtgcagca 60
 1 M E F F K K T A L A A L V M G F S G A A 20
 61 ttggcattaccaatataccatttttagcaaccggcgaggaccattgccggtggtggtgac 120
 21 L A L P N I T I L A T G G T I A G G G D 40
 121 tccgcaaccaaatctaaactacacagtgggttaaagttggcgtagaaaaatctggttaatgcg 180
 41 S A T K S N [▲]Y T V G K V G V E N L V N A 60
 181 gtgccgcaactaaaagacattgcgaacgttaaaggcgagcaggtagtgaatatcggtcc 240
 61 V P Q L K D I A N V K G E Q V V N I G S 80
 241 caggacatgaacgataatgtctggctgacactggcgaaaaaattaacaccgactgcgat 300
 81 Q D M N D N V W L T L A K K I N T D C D 100
 301 aagaccgacggcttcgtcattaccacgggtaccgacacgatggaagaaactgcttacttc 360
 101 K T D G F V I T H G T D T M E E T A Y F 120
 361 ctcgacctgacgggtgaatgcgacaaaccgggtggtgatggctcggcgcaatgcgtccgtcc 420
 121 L D L T V K C D K P V V M V G A M R P S 140
 421 acgtctatgagcgcagacgggtccattcaacctgtataacgcggtagtgaccgcagctgat 480
 141 T S M S A D [▲]G P F N L Y N A V V T A A D 160
 481 aaagcctccgccaaccgtggcggtgctggttagtgatgaatgacaccgtgcttgatggccgt 540
 161 K A S A N [▲]R G V L V V M N D T V L D G R 180
 541 gacgtcaccaaaaaccaacaccaccgacgtagcgaccttcaagtctgttaactacggctcct 600
 181 D V T K T N T T D V A T F K S V N Y G P 200
 601 ctgggttacattcacaacggtaagattgactaccagcgtaccccggcacgtaagcatacc 660
 201 L G Y I H N G K I D Y Q R T P A R K H T 220
 661 agcgacacgccattcgatgtctctaaactgaatgaactgccgaagtcggcattgtttat 720
 221 S D T P F D V S K L N E L P K V G I V Y 240
 721 aactacgctaacgcacatccgatcttccggctaaagcactggttagatgcgggctatgatggc 780
 241 N Y A N A S D L P A K A L V D A G Y D G 260
 781 atcgttagcgtggtgtgggtaacggcaacctgtataaatctgtgttcgacacgctggcg 840
 261 I V S A G V G N G N L Y K S V F D T L A 280
 841 accgccgcgaaaaccgggtactgcagtcgtgcgttcttcccgctaccgacgggcgctacc 900
 281 T A A K T G T A V V R S S R V P T G A T 300
 901 actcaggatgccgaagtggatgatgcgaataacggcttcgtcgcctctggcacgctgaac 960
 301 T Q D A E V D D A K Y G F V A S G T L N 320
 961 ccgcaaaaagcgcgcgttctgctgcaactggctctgacgcaaaccaagatccgcagcag 1020
 321 P Q K A R V L L Q L A L T Q T K D P Q Q 340

1021 atccagcagatcttcaatcagtactaa
341 I Q Q I F N Q Y *

1047
348

↑ Proteolytic cleavage by LGMN 36.85 kDa - 19.6; 10.85; 4.49; 1.9

Potential antigenic sites are underlined

Figure 8

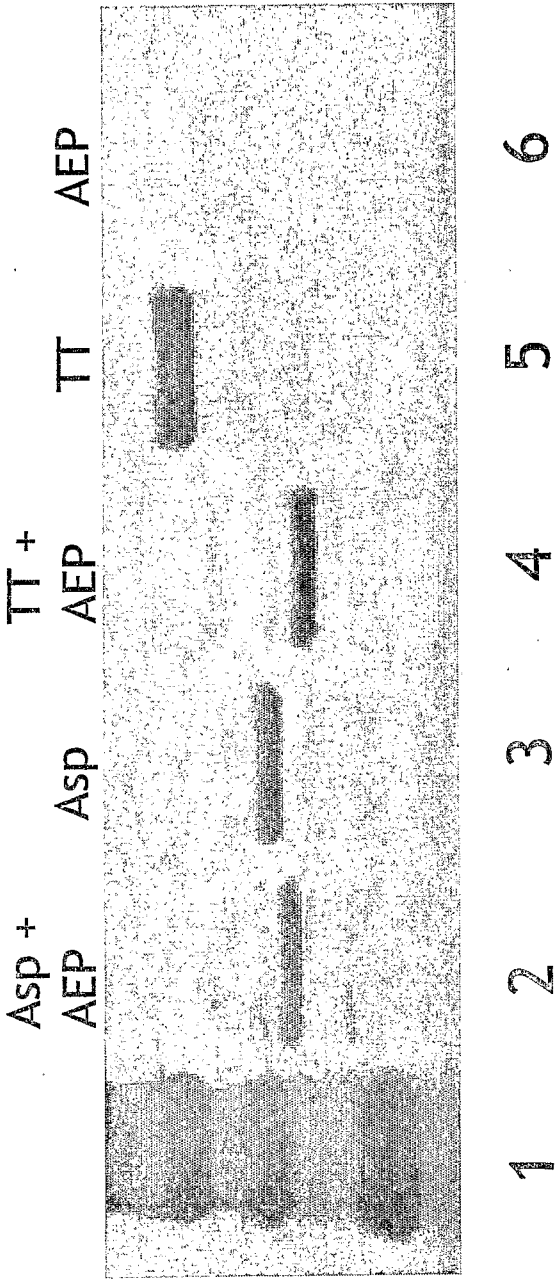


Figure 9

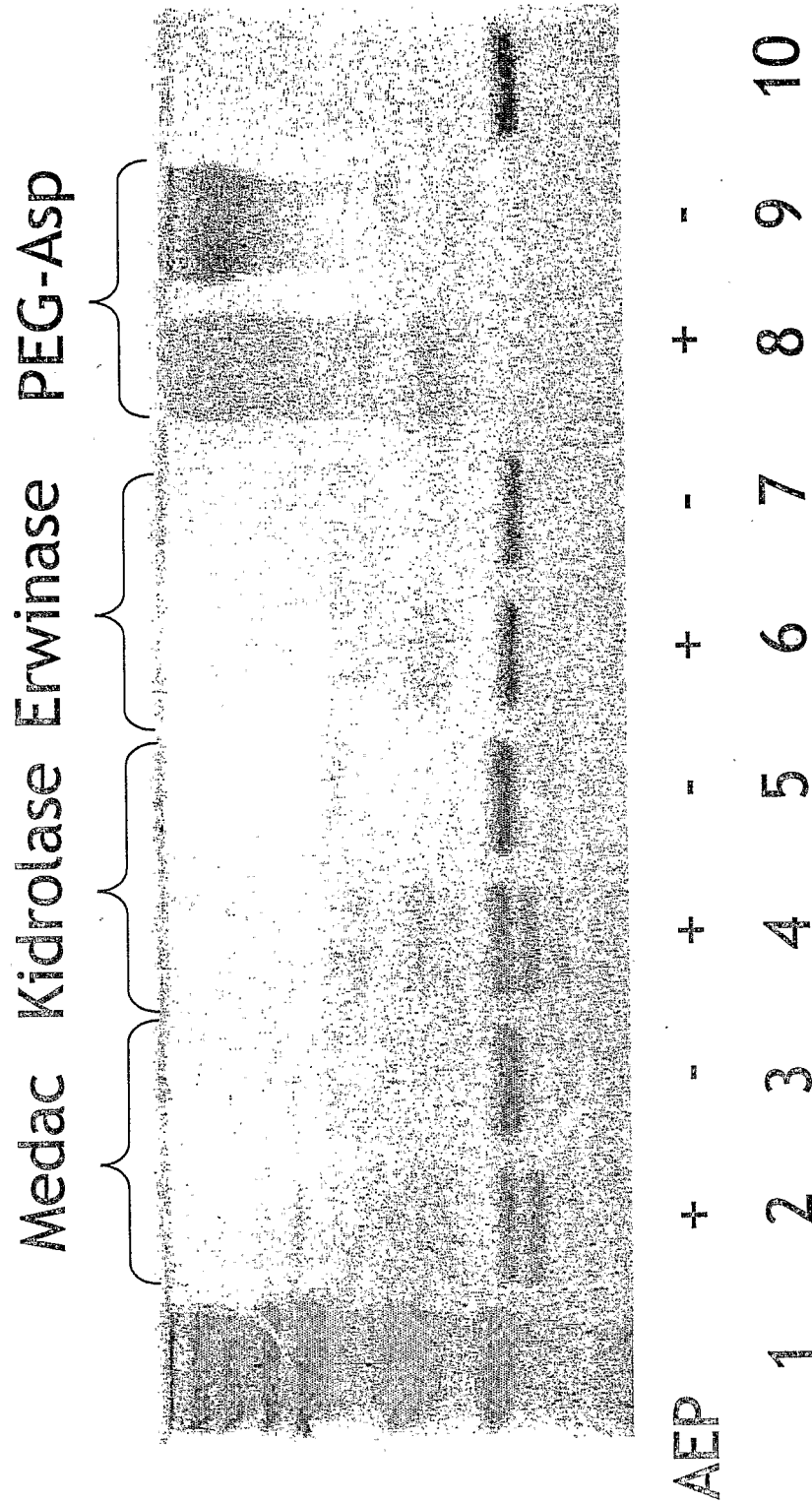


Figure 10

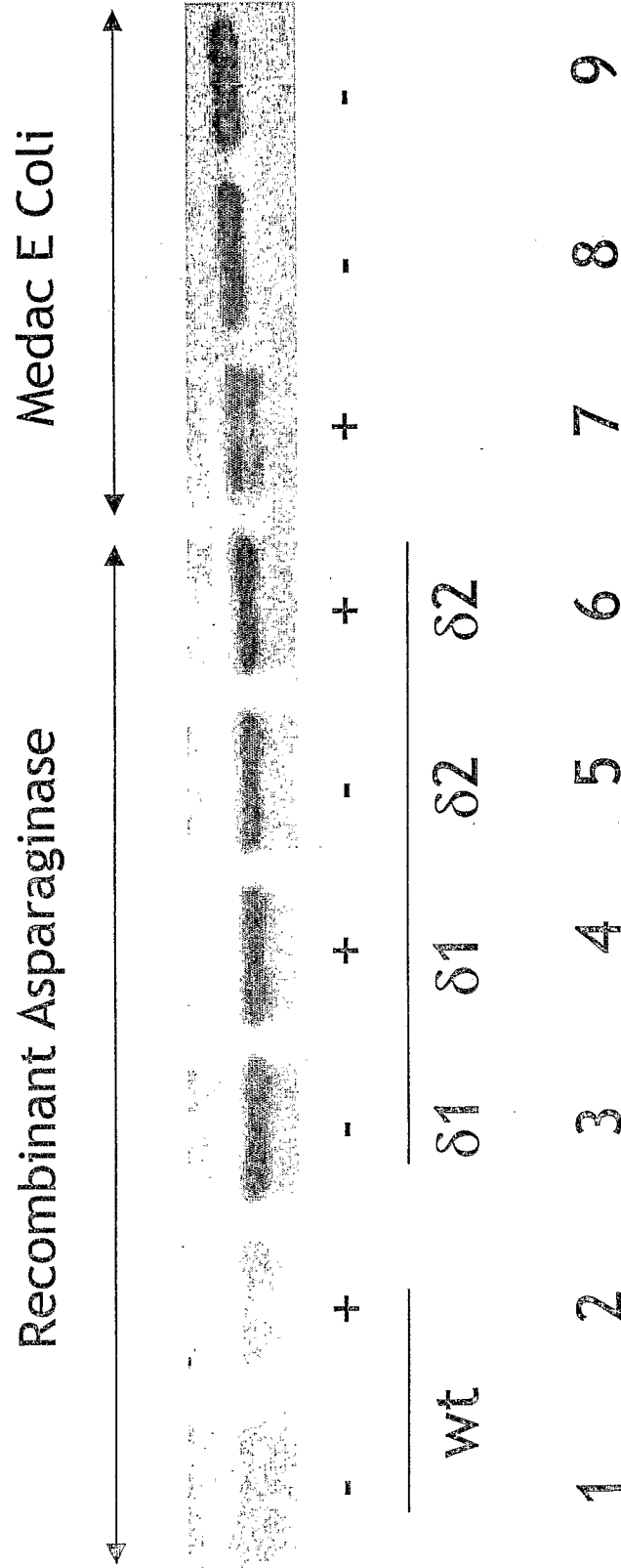


Figure 11

1 MVWKVAVFLS VALGIGAVPI DDPEDGGKHW VVIVAGSNGW YNYRHQADAC
51 HAYQIIHRNG IPDEQIVVMM YDDIAYSEDN PTPGIVINRP NGTDVYQGVP
101 KDYTGEDVTP QNFLAVLRGD AEAVKGIGSG KVLKSGPQDH VFIYFTDHGS
151 TGILVFPNED LHVKDLNETI HYMYKHKMYR KMFVYIEACE SGSMMNHLPD
201 NINVIYATTAA NPRESSYACY YDEKRSTYLG DWYSVNW MED SDVEDLTKET
251 LHKQYHLVKS HTNTSHVMQY GNKTISTMKV MQFQGMKRKA SSPVPLPPVT
301 HLDLTPSPDV PLTIMKRKLM NTNDLEESRQ LTEEIQRHLD ARHLIEKSVR
351 KIVSLAASE AEVEQLLSE APLTGHSCYP EALLHFRTHC FNWHSPTYEY
401 ALRHLYVLVN LCEKPYPLHR IKLSMDHVCL GHY