(54) Title: BIOLOGICALLY ACTIVE COMPLEX

(57) Abstract: A biologically active complex comprising alpha-lactalbumin or a variant of alpha-lactalbumin which is in the apo folding state, or a fragment of either of any of these, and a cofactor which stabilizes the complex in a biologically active form, provided that any fragment of alpha-lactalbumin or a variant thereof comprises a region corresponding to the region of α-lactalbumin which forms the interface between the alpha and beta domains, and further provided that when the complex comprises native alpha-lactalbumin, the cofactor is other than C18:1:9 cis fatty acid. These complexes have therapeutic applications for example in the treatment of cancer and as antibacterial agents.

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Biologically Active Complex

The present invention relates to biologically active complexes,
in particular complexes derived from alpha-lactalbumin (α-
5   lactalbumin), to pharmaceutical compositions containing these as
well as to their use in therapy, in particular as anti-cancer or
antibacterial agents.

Biologically active complexes obtained from milk and particularly
10   human milk, together with their use as antibacterial agents is
described for example in EP-0776214.

HAMLET (formerly known as MAL) is a molecular complex that
induces *in vitro* apoptosis selectively in tumour cells, but not
15   in healthy differentiated cells. The apoptotic activity of this
variant fold was discovered by serendipity, in a fraction of
human milk casein obtained by precipitation at low pH, and was
purified by ion exchange chromatography, eluting as a single peak
after 1M NaCl. The elute was shown by spectroscopy to contain
20   partially unfolded α-lactalbumin in an apo-like conformation (M.
Svensson, et al, (1999) *J Biol Chem*, **274**, 6388-96), with native-
like secondary structure, but lacking specific tertiary packing
of the side chains. The link between apoptosis induction and the
folding change was proven by deliberate conversion of native α-
25   lactalbumin to the apoptosis inducing form (M. Svensson, et al.,
(2000) *Proc Natl Acad Sci* USA, **97**, 4221-6). HAMLET was shown to
bind to the surface of tumour cells, to translocate into the
cytoplasm and to accumulate in cell nuclei, where it causes DNA
fragmentation (M. Svensson, et al., (2000) *Proc Natl Acad Sci*
30   USA, **97**, 4221-6).

It has also been found that other reagents and specifically lipid
such as oleic acid, are useful in the conversion of human α-
lactalbumin to HAMLET (human $\underline{\alpha}$-lactalbumin $\underline{m}$ade $\underline{l}$ethal to $\underline{t}$umour
35   cells). In particular, it has been reported previously that

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oleic acid (C18:1:9cis) is required for HAMLET production (M.
Svensson, et al., (2000) *Proc Natl Acad Sci* USA, **97**, 4221-6).

Five of the seven oxygens that ligate the Ca^{2+} are contributed by
5 side chain carboxylates of Asp residues at positions 82, 87 and
88 and by carbonyl oxygens of Lys 79 and Asp 84, and two water
molecules supply the remaining ligands (Acharya et al., 1991).
The bound Ca^{2+} brings the α-helical region and the β-sheet in
close proximity, and two disulfide bonds flanking the Ca^{2+} binding
10 site, make this part of the molecule fairly inflexible.

α-Lactalbumin is the dominant protein in human milk, where it is
present at a concentration of 2 mg/ml (140 µM). The mature
protein consists of 123 amino acid residues (14.2 kDa). Its
15 three dimensional structure has been determined to 1.7 Å
resolution and it is a globular protein with four α-helices
(residues 1-34, 86-123) and a triple stranded anti-parallel β-
sheet (residues 38-82), linked by four disulphide bonds (61-77;
73-91; 28-111 and 6-120) (K. R. Acharya, et al., (1991) *J Mol
20 Biol*, **221**, 571-81). Binding of Ca^{2+} to a single very high
affinity Ca^{2+} binding site is required for the protein to maintain
a native conformation. Five of the seven oxygens that ligate the
Ca^{2+} are contributed by side chain carboxylates of Asp residues at
positions 82, 87 and 88 and by carbonyl oxygens of Lys 79 and Asp
25 84, and two water molecules supply the remaining ligands. The
bound Ca^{2+} brings the α-helical region and the β-sheet in close
proximity, and two disulfide bonds flanking the Ca^{2+} binding site,
make this part of the molecule fairly inflexible.

30 The protein adopts the so called apo state found in HAMLET when
exposed to low pH, or in the presence of chelators, that release
the strongly bound Ca^{2+} ion (D. A. Dolgikh, et al., (1981) *FEBS
Lett*, **136**, 311-5; K. Kuwajima, (1996) *Faseb J*, **10**, 102-09).

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The applicants have found that the conversion of α-lactalbumin to HAMLET with apoptotic activity, requires both a conformational or folding change and the presence of a lipid cofactor and this may preferably be achieved using a variant of alpha-lactalbumin. The

5   conformational or folding change is conveniently effected by removal of calcium ions, or by using a variant without calcium ions. However, once the change has been effected, the presence of calcium or a functional calcium binding site does not result in any loss of activity.

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Furthermore, they have found that the optimal cofactors for the conversion of alpha-lactalbumin to HAMLET are C18:1 fatty acids with a double bond in the cis conformation at position 9 or 11. Saturated C18 fatty acid or unsaturated fatty acids in the trans

15   conformation, or fatty acids with shorter carbon chains did not form HAMLET, suggesting that highly specific inter-molecular interactions are required for lipids to act as folding partners in this system.

20   According to the present invention there is provided a biologically active complex comprising alpha-lactalbumin or a variant of alpha-lactalbumin which is in the apo folding state, or a fragment of either of any of these, and a cofactor which stabilises the complex in a biologically active form, provided

25   that any fragment of alpha-lactalbumin or a variant thereof comprises a region corresponding to the region of alpha-lactalbumin which forms the interface between the alpha and beta domains, and further provided that when the complex comprises native alpha-lactalbumin, the cofactor is other than C18:1:9 cis

30   fatty acid.

In particular the cofactors are selected from a cis C18:1:9 or C18:1:11 fatty acid or a different fatty acid with a similar configuration.

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The complex is suitably prepared by forming the apo conformation
of the protein, using conventional or molecular biological
methods, and in particular by removing calcium ions from alpha-
lactalbumin or variants, or by using variants from which calcium
5   ions have been released, or which do not have a functional
calcium binding site.

However, the applicants have found that, once formed, the
presence of a functional calcium binding site, and/or the
10   presence of calcium, does not affect stability or the biological
activity of the complex. Biologically active complexes have been
found to retain affinity for calcium, without loss of activity.

Therefore complex of the invention may further comprise calcium
15   ions but the elimination of calcium from the complex is not
essential, but provides a convenient means for the preparation of
the complex.

Thus, in a particular embodiment, there is provided a
20   biologically active complex which is obtainable by combining
(i) a cis C18:1:9 or C18:1:11 fatty acid or a different fatty
acid with a similar configuration; and
(ii) alpha-lactalbumin from which calcium ions have been removed,
or a variant of alpha-lactalbumin from which calcium ions have
25   been released or which does not have a functional calcium binding
site; or a fragment of either of any of these, provided that any
fragment comprises a region corresponding to the region of α-
lactalbumin which forms the interface between the alpha and beta
domains, and further provided that when (ii) is alpha-
30   lactalbumin, (i) is other than C18:1:9 cis fatty acid.

In particular, the complex will comprise elements (i) and (ii).

The expression "biological active" of "biological activity" as
35   used herein means that the complex has similar biological
activity to that reported for HAMLET. In other words, it will be

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effective in producing apopotosis in cancer cells and/or have
antibacterial properties.

As used herein the expression "variant" refers to polypeptides or
proteins which are homologous to the basic protein, which is
suitably human or bovine α-lactalbumin, but which differ from the
base sequence from which they are derived in that one or more
amino acids within the sequence are substituted for other amino
acids. Amino acid substitutions may be regarded as
"conservative" where an amino acid is replaced with a different
amino acid with broadly similar properties. Non-conservative
substitutions are where amino acids are replaced with amino acids
of a different type. Broadly speaking, fewer non-conservative
substitutions will be possible without altering the biological
activity of the polypeptide. Suitably variants will be at least
60% identical, preferably at least 70%, even more preferably 80%
or 85% and, especially preferred are 90%, 95% or 98% or more
identity.

When comparing amino acid sequences for the purposes of
determining the degree of identity, programs such as BESTFIT and
GAP (both from Wisconsin Genetics Computer Group (GCG) software
package). BESTFIT, for example, compares two sequences and
produces an optimal alignment of the most similar segments. GAP
enables sequences to be aligned along their whole length and
finds the optimal alignment by inserting spaces in either
sequence as appropriate. Suitably, in the context of the present
invention when discussing identity of sequences, the comparison
is made by alignment of the sequences along their whole length.

The term "fragment thereof" refers to any portion of the given
amino acid sequence which will form a complex with the similar
activity to complexes including the complete α-lactalbumin amino
acid sequence. Fragment may comprise more than one portion from
within the full-length protein, joined together. Portions will

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suitably comprise at least 5 and preferably at least 10
consecutive amino acids from the basic sequence.

Suitable fragments will be deletion mutants suitably comprise at
5   least 20 amino acids, and more preferably at least 100 amino
acids in length. They include small regions from the protein or
combinations of these.

In a particularly preferred embodiment, the variant used in the
10   method of the invention is one in which the calcium binding site
has been modified so that the affinity for calcium is reduced, or
it is no longer functional. It has been found that in bovine α-
lactalbumin, the calcium binding site is coordinated by the
residues K79, D82, D84, D87 and D88. Thus modification of this
15   site, for example by removing one of more of the acidic residues,
can reduce the affinity of the site for calcium, or eliminate the
function completely and mutants of this type are a preferred
aspect of the invention.

20   The Ca^{2+}-binding site of bovine α-lactalbumin consists of a 3_{10}
helix and an α-helix with a short turn region separating the two
helices (Acharya K. R., et al., (1991) *J Mol Biol* **221**, 571-581).
It is flanked by two disulfide bridges making this part of the
molecule fairly inflexible. Five of the seven oxygen groups that
25   co-ordinate the Ca^{2+} are contributed by the side chain
carboxylates of Asp82, 87 and 88 or carbonyl oxygen's of Lys79
and Asp84. Two water molecules supply the remaining two oxygen's
(Acharya K. R., et al., (1991) *J Mol Biol* **221**, 571-581).

30   Site directed mutagenesis of the aspartic acid at position 87 to
alanine (D87A) has previously been shown to inactivate the strong
calcium-binding site (Anderson P. J., et al., (1997) *Biochemistry*
36, 11648-11654) (Fig. 1a) and the mutant proteins adopted to the
apo- conformation.

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Therefore in a particular embodiment, the aspartic acid residue
at amino acid position 87 within the protein sequence is mutated
to a non-acidic residue, and in particular a non-polar or
uncharged polar side chain.

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Non-polar side chains include alanine, glycine, valine, leucine,
isoleucine, proline, phenylalanine, methionine, tryptophan or
cysteine. A particularly preferred example is alanine.

10   Uncharged polar side chains include asparagine, glutamine,
serine, threonine or tyrosine.

In order to minimize the structural distortion in the mutant
protein, D87 has also been replaced by an asparagine (N)
15   (Permyakov S. E., et al., (2001) *Proteins Eng* **14**, 785-789), which
lacks the non-compensated negative charge of a carboxylate group,
but has the same side chain volume and geometry (Fig. 7a). The
mutant protein (D87N) was shown to bind calcium with low affinity
$(K_{Ca}2 \times 10^5 M^{-1})$ (Permyakov S. E., et al., (2001) *Proteins Eng* **14**,
20   785-789).

Such a mutant forms a further preferred embodiment of the
invention.

25   Thus particularly preferred variants for use in the complexes of
the invention are D87A and D87N variants of α-lactalbumin, or
fragments which include this mutation.

The region which forms the interface between the alpha and beta
30   domains is, in human α-lactalbumin, defined by amino acids 34-38
and 82-86 in the structure. Thus suitable fragments will include
these regions, and preferably the entire region from amino acid
34-86 of the native protein.

35   This region of the molecule differs between the bovine and the
human proteins, in that one of the three basic amino acids (R70)

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is changed to S70 in bovine α-lactalbumin thus eliminating one
co-ordinating side chain. It may be preferable therefore, that
where the bovine α-lactalbumin is used in the complex of the
invention, an S70R mutant is used.

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It appears that three molecular events are required to form
HAMLET from α-lactalbumin. First, the tightly bound Ca^{2+}-ion is
released. The apo-protein is then allowed to bind the lipid
cofactor, for example, on an ion exchange matrix. Third, the
10   active complex is eluted at high salt and dialysed. The elutes
were characterised after repeating this procedure with 14 closely
related fatty acids as shown hereinafter. Only the C18:1:9cis
and C18:1:11cis complexes were found to cause apoptosis, and they
alone gave distinct novel signals by NMR, indicating that they
15   formed a novel molecular complex. Several other fatty acids were
capable of retaining the protein on ion exchange matrices and to
stabilize the protein in a partially unfolded conformation, but
they did not form biologically active complexes and gave sharper
NMR signals as expected from a mixture of protein and fatty acid.
20   It appears that the unsaturated C18:1cis fatty acids have unique
structural features, allowing them to form HAMLET from apo-α-
lactalbumin, and suggest that they differ from the other fatty
acids in that they offer the correct stereo-specific match. The
lack of significant HAMLET formation with a number of closely
25   related fatty acids suggests a highly selective and specific
process. Consequently, any other fatty acids used in the complex
of the invention should have essentially similar
stereospecificity to these unsaturated C18:1cis fatty acids.

30   The Ca^{2+} binding site is 100% conserved in α-lactalbumin from
different species (Acharya K. R., et al., (1991) *J Mol Biol* **221**,
571-581), illustrating the importance of this function for the
protein. It is co-ordinated by five different amino acids and
two water molecules. The side chain carboxylate of D87 together
35   with D88 initially dock the calcium ion into the cation-binding
region, and form internal hydrogen bonds that stabilise the

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structure (Anderson P. J., et al., (1997) *Biochemistry* **36**, 11648-11654). A loss of either D87 or D88 has been shown to impair Ca2+ binding, and to render the molecule stable in the partially unfolded state (Anderson P. J., et al., (1997) *Biochemistry* **36**,

5   11648-11654).

Further, as reported hereinafter, mutant proteins with two different point mutations in the calcium-binding site of bovine α-lactalbumin were used. Substitution of the aspartic acid at

10   position 87 by an alanine (D87A) totally abolished calcium binding and disrupted the tertiary structure. After substitution of the aspartic acid by asparagine, the protein (D87N) still bound calcium but with lower affinity and showed a loss of tertiary structure, although not as pronounced as for the D87A

15   mutant (Permyakov S. E., et al., (2001) *Proteins Eng* **14**, 785-789). The mutant protein showed a minimal change in packing volume as both amino acids have the same average volume of 125Å^3, and the carboxylate side chain of asparagines allow the protein to co-ordinate calcium, but less efficiently (Permyakov S. E., et

20   al., (2001) *Proteins Eng* **14**, 785-789). Both mutant proteins were stable in the apo-conformation at physiologic temperatures but despite this conformational change they were biologically inactive in the apoptosis assay. The results demonstrate that a conformational change to the apo-conformation alone is not

25   sufficient to induce apoptosis.

The structure of α-lactalbumin is known in the art, and the precise amino acid numbering of the residues referred to herein can be identified by reference to the structures shown for

30   example in Anderson et al. supra. and Permyakov et al supra.

Native bovine α-lactalbumin and the Ca^{2+} mutants could be converted to the HAMLET like complex named BAMLET, showing that the same fatty acid stabilised bovine α-lactalbumin in the BAMLET

35   conformation. The conversion yield was lower, however, suggesting that lipid binding to the bovine protein was less

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efficient.   The structural basis for this effect is not clear.
Bovine and human α-lactalbumin show 76% amino acid sequence
identity and have similar native conformations (Wijesinha-Bettoni
R., et al., (2001) *J Mol Biol* **312**, 261-273).   The lower

5   conversion yield for BAMLET suggested that the sequence
differences influenced the fit between the fatty acid and the
bovine protein.   The divergent sequences are mainly located in
the α-helical region (A-helix 57%, B-helix 50%, C-helix 23% and
3_{10}-helix 25% difference) but this region is unlikely to be

10   involved in fatty acid binding (see Example 3 below).

The applicants believe that the lipid binding site in human α-
lactalbumin may be located in the groove between the α-helical
and β-sheet domains, which becomes exposed in the apo-protein.

15   This region of the molecule differs between the bovine and the
human proteins, in that one of the three basic amino acids (R70)
is changed to S70 in bovine α-lactalbumin, thus eliminating one
potential coordinating side chain.

20   The results illustrated herein demonstrate that the change in
biologic function requires not just a conformational change of
the protein, but also the lipid cofactor.   The dual requirements
for a change in protein conformation and a lipid cofactor may be
important to achieve tissue specificity.   The active complex

25   should only be formed in local environments that favour the
altered protein fold, and where lipid cofactors are available.
In the case of HAMLET, such conditions are present in the stomach
of the breast-fed child.   The low pH precipitates casein with α-
lactalbumin in the apo-conformation, and activates pH sensitive

30   lipases that release oleic acid from the milk phospholipids.   It
is interesting to note that α-lactalbumin and oleic acid
respectively, are the most abundant proteins and fatty acid in
human milk.   The lipids thus appear to function as "post-
secretion chaperones", involved in the adaptation of proteins to

35   shifting external environment.   The need for both a folding

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change and a tissue specific lipid makes sense in order to
protect tissues from the occasional protein folding variant on
the loose, and to target the site where the novel function is
needed.

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As part of the present study, the applicants tested the calcium
affinity of the complexes. The obtained calcium affinity of α-
lactalbumin was higher than previously reported (about 2 mM as
compared to 10 mM and up), but the value found (0.15 M NaCl)

10   falls in the range reported by others at 0.1 M NaCl (Table 1
hereinafter). The difference may be explained by the lower ionic
strength as we observed that the Ca^{2+} affinity of α-lactalbumin
decreased by a factor of 200 between low and physiological ionic
strength. This is expected from salt screening of electrostatic

15   interactions, and similar decreases have been obtained with other
negatively charged proteins.

The decrease in K_a of α-lactalbumin at physiological salt may also
be due to competition between Na^+ and Ca^{2+} for the same site, as

20   Na^+ has been reported to bind to and stabilize α-lactalbumin in
vitro ($K_a = 100$ M^{-1} at 20°C,). The lack of near-UV CD signals for
D87A in the presence of NaCl was a striking contrast to wild-type
α-lactalbumin, suggesting that Na^+ binds to the Ca^{2+} site of the
wild-type protein, and that the D87 side-chain is important also

25   for coordination of this monovalent ion.

Complexes of the invention, as well as HAMLET, retained a high
affinity for Ca^{2+} at both low and physiological salt
concentrations, showing that they can bind Ca^{2+} without loss of

30   activity. This may appear surprising, as partially unfolded
conformations of α-lactalbumin usually are associated with the
Ca^{2+}-free state. Two possible explanations may be offered. In
the first and most likely scenario, α-lactalbumin converts to
HAMLET by unfolding and binding of oleic acid with little

35   disturbance of the α-helical domain. The Ca^{2+}-binding site may

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then retain a similar affinity as in the absence of oleic acid.
A second possibility is that the Ca^{2+} site is disrupted and that
the observed Ca^{2+} binding is explained by the generation of a new
Ca^{2+} site in HAMLET. The head group of oleic acid might

5   potentially coordinate calcium together with amino acid residues.
In this case, the classical Ca^{2+}-binding site in α-lactalbumin
would not be needed for Ca^{2+}- binding to HAMLET. We find this a
less likely explanation, as D87A-BAMLET has the oleic acid bound
to the unfolded protein, and thus should form the new Ca^{2+} site,

10   but does not bind Ca^{2+}. It appears therefore that the Ca^{2+} -
binding site is not involved in the conversion of α-lactalbumin
to an apoptosis-associated conformation, and that the structural
changes associated with Ca^{2+} binding to HAMLET do not hinder the
biological function.

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In HAMLET, α-lactalbumin retains a partially unfolded
conformation as well as a high affinity Ca^{2+} binding site. This
apparent paradox sheds new light on the molecular characteristics
of α-lactalbumin in the complex. The X-ray structure of the

20   native like apo form shows that the alpha and beta regions are
largely intact, while the cleft between them is widened (Chrysina
et al., J. Biol. Chem, (2000) 275, 37021-9). As discussed above,
the applicants believe that the cofactor such as oleic acid binds
in the interface between the alpha and the beta domains, and that

25   the bound cofactor acid locks this region of the molecule, while
allowing the α-domain to maintain a native-like conformation.
This is supported by the finding illustrated hereinafter that
complexes of this type such as HAMLET binds Ca^{2+} while retaining
activity against tumor cells. It would appear therefore that

30   HAMLET is therefore in a different molecular state than either
the low salt apo α-lactalbumin or the native-like apo form in
physiological salt.

Complexes of the invention are useful in a variety of therapeutic
35   applications, including anti-cancer and antibacterial treatments,

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in particular for treatment of infections of the respiratory
tract. For these purposes, the complex is suitably formulated as
a pharmaceutical composition and these form a further aspect of
the invention.

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The complex can be administered in the form of an oral mucosal
dosage unit, an injectable composition, or a topical composition.
In any case the protein is normally administered together with
the commonly known carriers, fillers and/or expedients, which are

10   pharmaceutically acceptable.

In case the protein is administered in the form of a solution or
cream for topical use the solution contains an emulsifying agent
for the protein complex together with a diluent or cream base.

15   Such formulations can be applied directly to the tumour, or can
be inhaled in the form of a mist into the upper respiratory
airways.

In oral use the protein is normally administered together with a

20   carrier, which may be a solid, semi-solid or liquid diluent or a
capsule. Usually the amount of active compound is between 0.1 to
99% by weight of the preparation, preferably between 0.5 to 20%
by weight in preparations for injection and between 2 and 50% by
weight in preparations for oral administration.

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In pharmaceutical preparations containing complex in the form of
dosage units for oral administration the compound may be mixed
with a solid, pulverulent carrier, as e.g. with lactose,
saccharose, sorbitol, mannitol, starch, such as potato starch,

30   corn starch, amylopectin, cellulose derivatives or gelatine, as
well as with an antifriction agent, such as magnesium stearate,
calcium stearate, polyethylene glycol waxes or the like, and be
pressed into tablets. Multiple-unit-dosage granules can be
prepared as well. Tablets and granules of the above cores can be

35   coated with concentrated solutions of sugar, etc. The cores can
also be coated with polymers which change the dissolution rate in

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the gastrointestinal tract, such as anionic polymers having a pk_a of above 5.5. Such polymers are hydroxypropylmethyl cellulose phthalate, cellulose acetate phthalate, and polymers sold under the trade mark Eudragit S100 and L100.

In preparation of gelatine capsules these can be soft or hard. In the former case the active compound is mixed with oil, and the latter case the multiple-unit-dosage granules are filled therein.

Liquid preparations for oral administration can be present in the form of syrups or suspensions, e.g., solutions containing from about 0.2% by weight to about 20% by weight of the active compound disclosed, and glycerol and propylene glycol. If desired, such preparations can contain colouring agents, flavouring agents, saccharine, and carboxymethyl cellulose as a thickening agent.

The daily dose of the active compound varies and is dependant on the type of administrative route, but as a general rule it is 1 to 100 mg/dose of active compound at personal administration, and 2 to 200 mg/dose in topical administration. The number of applications per 24 hours depend of the administration route, but may vary, e.g. in the case of a topical application in the nose from 3 to 8 times per 24 hours, i.e., depending on the flow of phlegm produced by the body treated in therapeutic use.

The invention further provides a method for treating cancer which comprises administering to cancer cells a complex or a composition as described above.

The invention further provides a method for treating bacterial infections which comprises administering to a patient in need thereof, a complex or a composition as described above.

In the description, the following abbreviations have been used.
GC/MS: Gas chromatography/Mass spectrometry,

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EDTA: ethylenediamintetra acetic acid, Tris

tris(hydroxymethyl)aminomethane, ANS: 8-Anilinonaphtalene-1-

sulfonic acid,

CD: circular dichroism,

5 UV: ultra violet,

NaCl: sodium chloride,

NMR: nuclear magnetic resonance, ppm: parts per million

FITC: fluorescein isothiocyanate,

TLC: thin layer chromatography,

10 DEAE: diethylaminoethyl,

HCl: hydrochloric acid,

EGTA: ethylene-bis(oxyethyleneitriol)tetraacetic acid.

FPLC; fast protein liquid chromatography;

PBS, phosphate-buffered saline.

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The invention will now be particularly described by way of

example with reference to the accompanying diagrammatic drawings

in which:

20 Figure 1 shows simplified fatty acid structures and in particular

line drawings of the unsaturated fatty acids, which were

investigated for their ability to produce a HAMLET like molecular

complex. C16:1:9cis = Palmitoleic acid, C18:1:6cis =

Petroselinic acid, C18:1:9cis = Oleic acid, C18:1:11cis = vaccine

25 acid, C20:1:11cis = Eicosenic acid, C18:1:9trans = Elaidic acid,

C18:1:11trans = Trans vaccenic acid, C20:4,5,8,11,15cis =

Arachidonic acid, C18:3:6,9,12cis = Gamma linolenic acid,

C18:3:9,12,15cis = Linolenic acid, C18:2:9,12cis = Linolenic

acid.

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Figure 2 is a series of graphs showing the retention of apo- α-

lactalbumin on ion exchange matrices conditioned with individual

fatty acids.

35 Figure 3 illustrates tumour cell apoptosis induced by the lipid-

protein complexes.

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Figure 4 shows the results of CD spectroscopy to determine the tertiary structure of the fatty acid-protein complexes.

Figure 5 shows the results of probing of the fatty acid-protein complexes by ANS spectroscopy as an indicator of hydrophobicity.

Figure 6 shows the results of NMR analysis of complexes.

Figure 7 illustrates the characterisation of the D87A and D87N mutants of α-lactalbumin, in which panel A shows the structure of the calcium-binding site.

Figure 8 illustrates biological tests carried out using mutated proteins alone, and shows that they do not induce apoptosis.

Figure 9 illustrates the conversion of bovine α-lactalbumin to BAMLET, where panel A shows elution peaks obtained during the preparation, panels B and C relate to the biological testing of BAMLET, panel D shows the results of near UV CD spectroscopy, panel E shows the results of intrinsic fluorescence spectrometry, and panel F shows the ANS spectra of HAMLET and BAMLET.

Figure 10 illustrates the production and test results for D87A and D87N to D87A-and D87N-BAMLET, where panel A shows elution peaks obtained during the preparation, panels B and C relate to the biological testing of these complexes, panel D shows the results of near UV CD spectroscopy, panel E shows the results of intrinsic fluorescence spectrometry, and panel F shows the ANS spectra.

Figure 11 shows the results of Ca^{2+} titrations in the presence of quin 2. The absorbance is shown as a function of total Ca^{2+} concentration for quin 2 mixed with human α-lactalbumin, HAMLET, or D87A-BAMLET, and quin 2 alone. The solid lines are the fitted curves. The absorbance is normalized using the fitted values for completely Ca^{2+}-free and Ca^{2+}-loaded system, respectively.

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Example 1

Structural variants of oleic acid, and other fatty acids
differing in the degree of saturation, carbon chain length and
cis/trans conformation (Fig. 1) were compared for their ability
5   to form HAMLET-like complexes from apo-α-lactalbumin.

Apo α-lactalbumin was applied to column matrices that had been
pre-conditioned with each indicated fatty acid indicated in
Figure 1 using the method described by M. Svensson, et al.,
10   (2000) *Proc Natl Acad Sci* USA, **97**, 4221-6, and the eluate after
1M NaCl was collected. Columns conditioned with C18:1:9cis were
used as a positive control (a). All unsaturated fatty acids in
the cis conformation retained apo-α-lactalbumin on the column,
but with varying efficiency. Unsaturated fatty acids in the
15   trans conformation (C18:1, 9trans, C16:1, 9trans) or saturated
fatty acids (C6:0 and C18:0) failed to retain apo-α-lactalbumin
on the column.

Results of ion exchange chromatography on fatty acid conditioned
20   matrices are shown in Fig. 2. Unsaturated C18 fatty acids in the
cis conformation formed complexes with apo-α-lactalbumin. The
C18:1:9cis fatty acid converted > 90% of the added apo-α-
lactalbumin. The C18:1:11cis fatty acid was somewhat less
efficient with a yield of about 70%, and other unsaturated C18
25   cis fatty acids (C18:1:6cis, C18:2:9,12cis, C18:3:9,12,15cis and
gamma C18:3:6,9,12cis gave considerably lower yields.

Trans conformers of C18:1 and saturated fatty acids were
practically inactive, however. Only small amounts of protein
30   eluted with high salt from columns conditioned with C18:1:9trans,
C18:1:11trans, or the saturated C18:0 fatty acid and they were
inactive.

Unsaturated cis fatty acids with shorter (C16:1:9cis) or longer
35   (C20:1:11cis and C20:4:5,8,11,15 cis) carbon chains formed

18

complexes that eluted after 1M NaCl, with yields comparable to
C18:1:11cis, but lower than C18:1:9cis. The columns conditioned
with the saturated fatty acids C6:0, C14:0 or C16:0 retained no
apo-α-lactalbumin.

5

The results demonstrate that apo-α-lactalbumin interacts in a
stereo-specific manner with C18:1 fatty acids, and that C18 fatty
acids must be unsaturated and with the double bond in the cis
conformation. Unsaturated fatty acids in the trans conformation
10   were inactive, as were the saturated fatty acids. Furthermore
the results confirmed that α-lactalbumin in its Ca^{2+}-bound form
bound only to a very low degree to the C18:1 fatty acid
conditioned column. The binding site for the unsaturated cis
fatty acid that defines HAMLET is available only in the apo-
15   conformation.

Example 2
Biological activity
Apoptosis induction was tested using the L1210 leukaemia cell
20   line. Apoptosis induction in L1210 leukaemia cells exposed to
the different protein-lipid complexes including the HAMLET
control (M. Svensson, et al, (1999) *J Biol Chem,* **274,** 6388-96).
HAMLET and the C18:1:11cis complex had killed 99-100% of the
cells after six hours (Fig. 3 Table), but the other complexes had
25   little or no effect on the cell viability. Both C18:1cis fatty
acid protein complexes induced DNA fragmentation (b), but the
C18:1trans fatty acid complexes were inactive. The C16 and C20
unsaturated fatty acid complexes caused an intermediate degree of
DNA fragmentation, but no loss of cell viability.

30

L1210 cells were exposed to lipid extracts derived from HAMLET or
from each of the other complexes. No effect on L1210 cell
viability (Fig. 3 Table) or DNA fragmentation (c) was detected
after six hours exposure to lipid concentrations corresponding to
35   the amount present in 1.0 mg of protein, even though 0.3 mg of
HAMLET was sufficient to kill the cells by apoptosis. At very

19

high lipid concentrations, the cells died of necrosis but at no
time were there evidence of apoptosis in response to lipids.

Thus the complexes formed with C18:1:9cis and C18:1:11cis fatty
5   acids induced apoptosis more efficiently than the other protein
lipid complexes (Table in Fig. 3). Cell viability was reduced
from 99% to 0% in six hours at a concentration of 0.3 mg/ml, and
DNA fragmentation was observed. Interestingly, other C18:1:cis
protein-fatty acid complexes had killed < 50% of the cells at
10 this time (Table in Fig. 3). The C18:1:trans fatty acid
complexes were inactive in the cellular assay, as were the C20
fatty acid complexes, and the C16 fatty acids complexes showed
very low effects on cell viability.

15 The C18:1:cis fatty acid complexes had induced DNA fragmentation
after six hours, suggesting that the cells were dying by
apoptosis (Fig. 3a). We were surprised to find evidence of DNA
fragmentation also in some cells exposed to the other fatty acid
complexes, even though these cells remained viable at six hours.
20
These results demonstrate that the lipids do not trigger
apoptosis, and that HAMLET is defined by both the protein and the
lipid. They further demonstrate that only C18:1:9cis and
C18:1:11cis, fulfil the criteria for a cofactor in the formation
25 of HAMLET, even though some of the other fatty acids appeared to
interact with apo-α-lactalbumin on the ion-exchange matrix.

Example 3
Structural correlates of the biologic activity
30 The ability to stabilise the protein in an apo-like conformation
was determined by CD and ANS spectroscopy, and the structural
integration was examined by NMR spectroscopy.

Conformation assessed by CD spectroscopy
35 The complexes eluting after 1M NaCl were examined by near UV CD
spectroscopy (M. Svensson, et al., (2000) Proc Natl Acad Sci USA,

20

97, 4221-6), using native or apo-α-lactalbumin and HAMLET as controls. The native α-lactalbumin control showed the characteristics of a well folded protein, with a minimum at 270 nm arising from tyrosine residues and a maximum at 294 nm arising

5   from tryptophan residues. The apo-α-lactalbumin control had lost most of the characteristic signals, indicating less restrained tyrosines and tryptophans. The C18:3:9,12,15cis, C18:3:6,9,12cis, C20:4:5,8,11,15cis and C18:1:6cis complexes resembled HAMLET with a loss of signal in the tyrosine and

10   tryptophan regions, while remaining complexes were similar to the apo-α-lactalbumin control.

       HAMLET was shown to resemble apo- α-lactalbumin, but seems to retain even less of the tertiary structure (Fig. 4a). The other

15   eluted fatty acid-protein complexes showed two main spectral patterns. The C18:1:6cis, C18:3:9,12,15cis, C18:3,6,9,12cis and C20:4:5,8,11,15cis fatty acid complexes resembled HAMLET, while the C18:1:9trans, C18:1:11:cis or trans, C18:2:9,12cis and C16:1:9cis or trans complexes were identical to apo control (Fig.

20   4b-h). Unconverted apo-α-lactalbumin that eluted in the void was shown to revert to the native state in the presence of Ca^{2+}.

       These results indicate that all fatty acids, which retain apo-α-lactalbumin on the column, stabilise the protein in a partially

25   unfolded conformation.

       Exposure of hydrophobic surfaces, as probed by ANS spectroscopy
       Apo-α-lactalbumin is known to expose hydrophobic side chains, due to the mobility of the β-sheet. The complexes eluting after 1M

30   NaCl were examined by ANS spectroscopy (M. Svensson, et al., (2000) *Proc Natl Acad Sci* USA, 97, 4221-6) using native or apo-α-lactalbumin and HAMLET as controls. Native α-lactalbumin did not bind ANS as shown by the flat curve and the low signal at 490 nm, but apo-α-lactalbumin showed significant ANS binding with

35   enhanced intensity and a maximum at 470 nm as expected from the

21

increased hydrophobicity of this fold.   All of the α-lactalbumin-
fatty acid complexes except C20:1:11cis bound ANS.   The
C18:3:9,12,15cis, gammaC18:3:6,9,12cis and C18:1:6cis complexes
resembled HAMLET, while the other fatty acid complexes showed
5   even higher ANS binding.

The apo control showed the expected ANS binding with enhanced
intensity and a maximum at 470 nm, while the native protein
failed to bind ANS as shown by the flat curve and the low signal
10   at 490 nm.   HAMLET bound ANS with a blue shift of the curve, but
the peak was lower than for apo-α-lactalbumin, (Fig. 5a).

The C18:1:6cis, C18:3:9,12,15cis, and C18:3:6,9,12cis fatty acid
complexes, bound ANS with similar spectral intensity as HAMLET.
15   Some other complexes (C18:1,9trans, C18:1,11cis and trans,
C18:2,9,12cis, C16:1,9 cis and trans, C20:4,5,8,11,15cis) showed
more intense ANS fluorescence than the apo-a-lactalbumin
control.   Finally, the C20:1:11cis complex did not bind any ANS
(Fig. 5b-h).
20

To exclude the direct binding of ANS to the fatty acids in the
protein-lipid complexes, mixtures of ANS to the fatty acid were
subjected to spectroscopy.   No ANS – fatty acid interaction was
observed (data not shown).
25

These results demonstrated that the C18:1:9cis fatty acid complex
retains the ability to interact with ANS, but the lower intensity
suggested that the fatty acid in HAMLET might modify ANS binding.
The negative results for C20:1:11cis complex suggested that the
30   longer fatty acid hindered the interaction of hydrophobic
surfaces in the protein with ANS.

^{1}H-NMR spectroscopy

^{1}H-NMR was used to resolve the structural basis for the difference
35   in activity between the C18:1cis and the inactive protein-fatty
acid complexes (Fig. 6).

22

Native α-lactalbumin showed the characteristics of a folded and
well-ordered protein with narrow lines and significant shift
dispersion, a large number of sharp signals in the aromatic
region (around 7 ppm) and several out shifted methyl signals

5   (between 0.7 and -0.6 ppm). The apo protein displayed narrow
lines and significant shift dispersion with significant
variations relative to the native state in the chemical shifts of
a large number of resonances. The aromatic and methylated
regions are shown in the left and right panels, respectively (M.

10   Svensson, et al., (2000) *Proc Natl Acad Sci* USA, 97, 4221-6).
HAMLET showed broad lines and lack of out shifted methyl signals
suggestive of a partially unfolded state, and significantly
different from the native protein. The spectrum obtained with
the C18:1,11cis protein complex was virtually identical to HAMLET

15   but the spectra of the trans fatty acid complexes showed more
narrow lines and out shifted signals suggesting that the
conformation of the C18:1:9cis or C18:1:11cis complexes are
unique and that although the trans fatty acids bind to apo-α-
lactalbumin, they do not alter the conformation so that HAMLET is

20   formed.

The spectrum of both the C18:1,9cis and C18:1,11cis complexes
showed broad lines and little shift dispersion. The lines in the
aromatic region were clustered and there were no out shifted

25   methyl signals below 0.7 ppm. The broad signals of the fatty
acid suggested that they formed an integral part of the complex.

The trans isomer complexes (C18:1:9trans and C18:1:11trans)
differed markedly from the C18:1:9cis or C18:1:11cis complexes.

30   Signals from bound fatty acid were detected, but they were
smaller than for the cis complexes. The protein lines were
narrow, and out shifted both in the methyl and the aromatic
regions. These data suggested that the trans fatty acids bind to
apo-α-lactalbumin, but do not alter the conformation so that

35   HAMLET is formed.

These results suggest that specific molecular interactions
stabilise apo-α-lactalbumin in the HAMLET conformation, and only
the unsaturated C18:1:9cis or C18:1:11cis fatty acids have the
stereo specific properties required to achieve this
5   conformational switch.

Apo-α-lactalbumin differs from other known lipid-binding proteins
in that it contains both α-helical and β-sheet domains. The
intracellular lipid-binding protein family have an all β-barrel
10 structure, forming a cavity which binds in a range of fatty acids
varying in chain length and saturation (J. Thompson, et al.,
(1997) *J Biol Chem*, **272**, 7140-7150). Typically, the carboxylate
head group of the fatty acids interacts with two to four
positively charged amino acids, usually arginines, and the carbon
15 chain is co-ordinated by six to ten hydrophobic amino acids. The
crystal structure of human serum albumin has revealed six
asymmetrically distributed, fatty acid binding sites within the
repeating α-helical domain structure of the protein (S. Curry, et
al., (1998) *Nature Struc Biol*, **5**, 827-835). Each hydrophobic
20 pocket is capped at one end by basic or polar side chains, co-
ordinating the fatty acid head group. While the binding of fatty
acids to human serum albumin causes conformational changes with
rotations of the three domains of the protein, and adjustments of
side chains to make way for incoming fatty acid (S. Curry, et
25 al., (1998) *Nature Struc Biol*, **5**, 827-835), the molecule does not
unfold or change function. We may therefore conclude that the
lipid cofactor function in the conversion of α-lactalbumin HAMLET
differs both structurally and functionally from these previously
known protein lipid interactions.
30
Tentative fatty acid binding sites were identified based on the
three-dimensional structures of native apo-α-lactalbumin. The
native α-lactalbumin molecule is a hydrophilic, acidic protein,
exposing mainly charged and polar amino acids. Two hydrophobic
35 regions are located in the interior of the globular structure.

24

One is formed by residues from the C and D helices and the β-
sheet domain in the interface between the two domains. The
second is formed by residues in the A, B and 30_{10-} helices of the
α-domain (Fig. 9) (L. C. Wu, et al., (1998) *J Mol Biol,* **280**, 175-
82; M. Saito, (1999) *Protein engineering* **12**, 1097-1104). The
crystal and NMR structures of bovine apo α-lactalbumin have
revealed a significant structural change in the cleft between the
two domains (E. Chrysina, et al., (2000) *J Biol Chem,* **275**, 37021-
37029; R. Bettoni-Wijesinha, et al., (2001) *J Mol Biol,* **307**, 885-
898) upon Ca^{2+} release. The expansion of the Ca^{2+} binding loop
tilts the 3_{10} helix towards the C helix, resulting in a disruption
of the aromatic cluster in the interface between the two domains
(Trp 60 and 104, Phe 53 and Tyr 103) (E. Chrysina, et al., (2000)
J Biol Chem, **275**, 37021-37029). The α-domain, in contrast,
remains structured in both the native and the apo-conformations,
with near native side chain packing. It seems likely therefore
that the C18:1 fatty acid binds in the interface between the α
and β domains, and thus stabilises a molten-globule like
conformation.

The shape of the hydrophobic pocket suggested that it should
favour interactions with bent molecules (Fig. 1). This may
indeed explain the inability of the C18:1 trans conformers to
form HAMLET. While fatty acids in this cis conformation are u-
shaped around the double bond, with both carbon chains projecting
in one direction, trans fatty acids are rod shaped around the
double bond due to the carbon chains on opposite sides of the
double bond. The saturated fatty acids are most flexible with no
structural constraints due to the lack of double bonds. The
results thus indicate that only the cis conformation allows fatty
acids a close stereo-specific fit, and that the additional
critical feature of the fatty acid is the carbon chain length.
In addition, the pocket is capped by basic residues, which may
co-ordinate the polar head groups of the fatty acids, thus
orienting the lipid. This interaction is, however not sufficient
for activation as the trans and saturated fatty acids, which

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possess the same charged head group failed to form the active
complex. It is highly likely that the stereo specific fit
involves both hydrophobic interactions with the lipid tail and
electrostatic interactions of the negatively charged head group
5   with basic side chains. Based on the analogy with other fatty
acid binding proteins, the fatty acid may bind to HAMLET by
electrostatic interactions between its negatively charged head
group and basic side-chains in the protein, as well as by van der
Waal's contacts and hydrophobic effects with the tail that are
10 optimized with the preferred stereo specific match (C18:1:9cis).

Example 4

Analysis of Variants of α-lactalbumin

The apo-conformation of α-lactalbumin is unstable and the protein
15 reverts to the native state at neutral pH and at the Ca^{2+}
concentrations present in the apoptosis assay. In the HAMLET
complex, the protein maintains an apo-like conformation, however.
As the lipid alone does not trigger apoptosis, it might act
simply by stabilising the apo- conformation. A conformational
20 change of the protein might then be sufficient to induce
apoptosis, but the unstable nature of the apo conformation has
precluded experiments testing the activity of the protein *per se*.

This question was addressed by site directed mutagenesis of the
25 Ca^{2+} binding site. The bound Ca^{2+} ion is co-ordinated by a
constellation of seven oxygen groups that form a distorted
pentagonal bipyramid, but a mutation of the Aspartic acid residue
at position 87 is sufficient to fully or partially inactivate the
Ca^{2+} binding site, generating mutant proteins locked in the apo-
30 conformation (Anderson P. J., et al., (1997) *Biochemistry* 36,
11648-11654; Veprintsev D. B., et al., (1999) *Proteins* 37, 65-72;
Permyakov S. E., et al., (2001) *Proteins Eng* 14, 785-789). This
study examined if apoptosis can be triggered solely by a
conformational change from the native to the apo-state.
35 Furthermore, the importance of the Ca^{2+} binding site for the
conversion to HAMLET was investigated.

26

Native human α-lactalbumin was purified from human milk by
ammonium sulphate precipitation and phenyl sepharose
chromatography as described (Svensson M., et al., (2000) *Proc
Natl Acad Sci USA* **97**, 4221-4226). Apo α-lactalbumin was
5   generated from 25 mg of native α-lactalbumin dissolved at 1.8 mM
in Tris[1] (10M Tris/HCI pH 8.5) by addition of 5 mM EDTA to remove
bound Ca^{2+}. The conformational change was confirmed by near UV CD
and ANS spectroscopy. Bovine α-lactalbumin was purchased from
Sigma, St. Louis, MO, USA and used without further purification.
10

In addition, a mutated bovine protein (D87A and D87N) was
expressed in *E. coli*, purified, folded and lyophilised as
described (Anderson P. J., et al., (1997) *Biochemistry* **36**, 11648-
11654; Permyakov S. E., et al., (2001) *Proteins Eng* **14**, 785-789).
15

A column (14 cm x 1.6 cm) packed with DEAE-Trisacryl M (BioSepra,
France) was attached to a Bio-Logic chromatography system (Bio-
Rad Laboratories, Hercules, CA), and eluted with a NaCl gradient
(buffer A: 10 mM Tris/HCI pH 8.5; buffer B: buffer A containing 1
20 M NaCl). The matrix was conditioned with oleic acid (Larodan
biochemicals, Malmo, Sweden). Ten milligrams was dissolved in
500 μl 99.5% ethanol by sonication (3 minutes using a Branson
2200 bath sonicator, Branson, Danbury, USA). After addition of
10 ml of 10 mM Tris/HC1, pH 8.5, the lipid solution was applied
25 to a newly packed DEAE-Trisacryl M matrix and dispersed through
out the matrix using a NaCl gradient.

Ten mg of each of human and recombinant and native bovine α-
lactalbumin was dissolved in 10 ml of 10 mM Tris/HC1 pH 8.5 and
30 added to the column. The protein fraction eluting after high
salt was desalted by dialysis (Spectra/Pore, Spectrum Medical
Industries, Laguna Hills, CA, membrane cut off 3.5 kDa) against
distilled water with at least four changes of water in 100-fold
volume excess, and then lyophilised.

35

27

The products were then subjected to spectroscopic analysis. The
proteins or protein fractions were dialyzed against doubly
distilled water and lyophilised. Stock solutions were prepared
by dissolving the lyophilised material in 10 mM potassium

5   phosphate buffer at pH 7.5, and concentrations determined as the
absorbance at 280 nm (A/1=C (mg/ml) where 1 mg/ml of α-
lactalbumin is 70μM). The spectra were recorded at the
appropriate dilution.

10   Circular Dichroism (CD) spectra were obtained on a JASCO J-720
spectro-polarimeter with a JASCO PTC-343 Peltier type
thermostated cell holder. Quartz cuvettes were used with 1 cm
path length and spectra were recorded at 25 °C between 240 and 320
nm. The wavelength step was 1 nm, the response time δ s and the

15   scan rate was 10 nm per minute. Six scans were recorded and
averaged for each spectrum. Baseline spectra were recorded with
pure buffer in the cuvette and substracted from the protein
spectra.

20   The mean residue ellipticity q_m (mdeg x cm^2 x dmol^{-1}) was
calculated from the recorded ellipticity, q, as

$$q_m = q \; / \; (c \cdot n \cdot 1)$$

25   where c is the protein concentration in M, n the number of
residues in the protein (123 in this case), 1 the path length in
nm and q is the ellipticy in degrees.

Fluorescence spectra were recorded at 25°C on a Perkin Elmer LS-
30   50B spectrometer using a quartz cuvette with 1 cm excitation path
length. Intrinsic (tryptophan) fluorescence emission spectra
were recorded between 305 and 530 nm (step 1 nm) with excitation
at 295 nm. The excitation bandwidth was 3 nm and the emission
was 5 nm. ANS fluorescence emission spectra were recorded at
35   25°C on a Perkin Elmer LS-50B spectrometer using a quartz cuvette
with 1 cm excitation path length, between 400 and 600 nm (step 1

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nm) with excitation at 385 nm. Both the excitation and emission
bandpass were set to 5 nm. ANS ammonium salt (Fluka, Buchs,
Switzerland) was added stepwise and the spectra at 1.5 molar
equivalents are shown.

5

The results are illustrated in Figure 7. Panel A shows ribbon
diagrams of the calcium-binding loop with the co-ordinating side
chains shown as darkly shaded lines. In the wild-type protein
calcium is co-ordinated by K79, D82,84,87 (arrow) and D88. If D87

10   is changed to A (arrow), the protein looses its ability to bind
calcium. If D87 is changed to N (arrow) the protein can still
bind calcium but with low affinity.

The results of the investigation into the tertiary structure of

15   the two mutants is shown in panel B. Spectra were recorded in
sodium phosphate buffer without EDTA. Native bovine α-
lactalbumin had a minimum at 270nm arising from tyrosine residues
and a maximum at 294 nm arising from tryptophan residues. Apo α-
lactalbumin showed the characteristic loss of signal, indicating

20   less restrained tyrosines and tryptophans. The D87A mutant
showed an almost complete loss of ellipticity consistent with a
partially unfolded conformation. The spectrum of the D87N mutant
showed decreased ellipticity in the tyrosine and tryptophan
regions, although not to the same extent as the D87A mutant.

25

The result of intrinsic fluorescence spectroscopy is shown in
panel C. Native bovine α-lactalbumin showed an intensity maximum
at 335 nm and a shoulder at 320 nm, indicative of tryptophan
residues in a folded hydrophobic core, but shifted to 350 in apo

30   α-lactalbumin indicating that the tryptophans are more accessible
to the solvent. Both the D87A and the D87N mutant showed
intensity maxima at 350 nm resembling apo-α-lactalbumin.

Fluorescence spectra at 1.5 equivalents of ANS are shown in

35   Figure 7 panel D. Native bovine α-lactalbumin did not bind ANS

29

but resembled ANS added to pure buffer. Apo α-lactalbumin bound
ANS with a maximum at 475 nm and significantly enhanced
intensity. The D87N and D87A mutants bound ANS with intensity
maxima at 475 nm, strongly resembling the spectrum of the apo
5   control.

Thus in summary, the near UV CD spectroscopy, a nearly complete
loss of ellipticity was observed demonstrating loss of tertiary
structure (Fig.7b) and the intensity maximum in the intrinsic
10   tryptophan spectrum was shifted towards higher wavelengths (Fig.
7c). The D87A mutant bound ANS as shown by the height of the
curve and the shift of the intensity maximum to shorter
wavelengths (Fig. 7d). No spectral changes were observed
following the addition of excess calcium (1mM) showing that the
15   D87A mutant did not bind calcium under these conditions (Anderson
P. J., et al., (1997) *Biochemistry* **36**, 11648-11654).

The near UV CD spectrum was intermediate between the native and
the D87A spectra, but had a significantly reduced ellipticity
20   (Fig 7b) as compared to native a-lactalbunim, and the intrinsic
fluorescence spectrum was red shifted compared to native protein
suggesting exposed tryptophans and a loss of tertiary structure
(Fig. 7c). The D87N mutant bound ANS demonstrating exposed
hydrophobic surfaces (Fig 7d). Addition of EDTA (1mM) had only a
25   marginal effect on the near UV CD ellipticity (Permyakov S. E.,
et al., (2001) *Proteins Eng* **14**, 785-789), showing that the D87N
mutant is in a molten globule like state also in the presence of
calcium.

30   It appears that the D87N protein is in an apo-like conformation,
but with better defined tertiary structure than D87A.

These results conformed that mutations in the calcium-binding
site lock the protein in a molten globule like conformation,
35   which is insensitive to the calcium conditions.

30

Example 5

Bioassays of apoptosis

The L1210 (ATCC, CCL 219) cell line was cultured in suspension, as described (Svensson M., et al., (1999) *J Biol Chem* **274**, 6388-
5   6396). The cells were harvested by centrifugation (200 g for 10 min), re-suspended in cell culture medium (RPMI 1640 supplemented with 10% fetal calf serum, non essential amino acids, sodium pyruvate and 50 µg gentamicin/ml, Life Technologies, Gibco BRL, Paisly, United Kingdom) and seeded into 24 well plates (Falcon,
10 Becton Dickinson, New Jersey, USA) at a density of 2 x 10^6/well. The different agonists were dissolved in cell culture medium, without fetal calf serum, and added to the cells (final volume 1 ml per well). Plates were incubated at 37°C in 5% CO_2 atmosphere and 100 µl of fetal calf serum was added to each well after 30
15 minutes. Cell culture medium served as a control.

Cell viability was determined by Trypan blue exclusion after six hours of incubation. For analysis, 30 µl of the cell suspension was mixed with 30 µl of a 0.2% trypan blue solution and the
20 number of stained cells (dead cells) per 100 cells was determined by interference contrast microscopy (Ortolux II, Leitz Wetzlar, Germany).

DNA fragmentation
25 Oligonucleosome length DNA fragments were detected by agarose gel electrophoresis. The cell suspension remaining after trypan blue (970 µl, 2x10^6/ml) was lysed in 5 mM Tris, 20 mM EDTA, 0.5% Triton X-100 pH 8.0 at 4°C for 1 hour and centrifuged at 13,000 x g for 15 minutes. DNA was ethanol precipitated over night in -20°C,
30 treated with proteinase K and RNAse, loaded on 1.8% agarose gels and electrophoresed with constant voltage set at 50V over night. DNA fragments were visualised with ethidium bromide using a 305nm UV-light source and photographed using Polaroid type 55 positive-negative film.
35

31

Mutant Proteins

The ability of the mutant proteins to induce apoptosis was tested
using the L1210 cell line. The proteins were suspended in cell
culture medium at 2 mg / ml and the cell viability was determined
5   after six hours of incubation as was the DNA fragmentation. The
HAMLET control induced apoptosis at 0.3 mg / ml but the mutant
proteins had no effect (Fig. 8).

Figure 8, Panel A Table 1 shows the viability of L1210 cells
10   after 6 hours' exposure to the mutant proteins. The mutants were
unable to kill the cells even at a concentration of 1.0 mg/ml
(c.f the results for BAMLET (see Fig. 9) where the viability
reduced from 98% to 4%.

15   The mutant proteins did not induce DNA fragmentation, but BAMLET
stimulated the formation of the characteristic DNA ladder as
shown in panel B.

These results demonstrate that the protein without associated
20   oleic acid is not sufficient to induce apoptosis in tumour cells.

Example 6
Preparation of BAMLET (Bovine a-lactalbumin made lethal to tumour
cells)
25   In view of the structural homology between the human and bovine
proteins it should be possible to convert bovine a-lactalbumin to
HAMLET like molecule, with apoptosis inducing properties. Hence,
bovine a-lactalbumin was subjected to the conversion conditions
previously used for human a-lactalbumin. Bovine α-lactalbumin
30   was treated with EDTA to remove Ca^{2+}, subjected to ion exchange
chromatography on a C18:1 conditioned column and eluted with a
NaCl gradient. A large portion of the applied material eluted in
the void but about 40% formed a sharp peak after 1M NaCl (arrow
Figure 9a). The eluate after high salt was saved for analysis.
35   Human α-lactalbumin was converted with higher efficiency.

32

A large proportion of the applied material eluted in the void
(about 60%), but a small sharp peak eluted after 1M NaCl (Fig.
9a).

5   The apoptosis-inducing activity of the high salt peak, named
     BAMLET, was investigated using the L1210 mouse leukemia cell line
     as described above. Loss of cell viability and DNA fragmentation
     were used as end points. The L1210 cells died rapidly when
     exposed to HAMLET (0.3 mg/ml) and DNA fragmentation was induced.
10   The L1210 cells were equally sensitive to BAMLET.

     BAMLET reduced cell viability from 99% to 12% at 0.3 mg / ml,
     after six hours incubation and induces DNA fragmentation
     (Fig.9b). There was no apparent difference in efficiency of
15   apoptosis induction between HAMLET and the bovine equivalent
     (Fig.9b).

     The tertiary structure of BAMLET was assessed using near UV CD
     spectroscopy. Native bovine a-lactalbumin shared the
20   characteristic spectrum of a well-folded protein with tyrosine
     dip and tryptophan peak, and native bovine a-lactalbumin did not
     bind ANS. The bovine apo protein had a reduced signal in both
     the tyrosine and tryptophan regions, indicative of a partially
     folded protein with flexible side chains, and significant ANS
25   binding with the maximum at 470nm and enhanced intensity. The
     bovine complex strongly resembled both HAMLET and the apo-control
     (Fig.9c). The native and apo controls were as in Fig. 7. HAMLET
     showed decreased ellipticity in the tyrosine and tryptophan
     regions characteristics of a partially unfolded protein. BAMLET
30   had spectra similar to the apo control and to .HAMLET, indicating
     flexible aromates.

     The intrinsic fluorescence spectrum native bovine a-lactalbumin
     had an intensity maximum at 320 nm, as expected from tyrptophan
35   residues in the folded protein. The apo- protein had an
     intensity-maximum at 345 nm and a shoulder at 360 nm, indicating

that the tyrptophans are more exposed (Fig. 9e). HAMLET showed
an intrinsic fluorescence intensity maximum at 345 nm and a
shoulder at 360 nm indicating solvent exposed tryptophans. The
spectrum of BAMLET was similar to that of HAMLET but without the
5   shoulder. The results indicate that tryptophan residues are
shielded from solvent in the native protein, but are more solvent
exposed in the apo control, HAMLET and BAMLET.

The ANS spectrum of HAMLET was blue-shifted with the intensity
10   maximum at 475 nm and increased quantum yield, indicative of ANS
binding. The spectrum of BAMLET was virtually identical to that
of HAMLET. The results indicate exposed hydrophobic surfaces in
the apo control, HAMLET and BAMLET but not in the native protein.
The bovine complex and HAMLET had virtually identical spectra and
15   bound ANS (Fig. 9f) resembling the apo control.

It can be concluded that bovine apo-lactalbumin can be converted
in the presence of C18:1 to a molecular complex that induces
apoptosis, and named this complex BAMLET (Bovine a-lactalbumin
20   made lethal to tumour cells).

Example 7
Conversion of the D87A and D87N mutants to BAMLET
The D87A mutant described above was applied to a C18:1
25   conditioned ion exchange column without EDTA and the column, and
most of the applied protein eluted as a sharp peak after high
salt (arrow) (Figure 10a). The D87N mutant was first treated
with EDTA to remove residual calcium and applied to the column.
A small portion of applied protein eluted in the void volume, but
30   the majority eluted as a sharp peak after high salt (arrow). The
eluted protein-lipid complexes amounting to >90% of applied D87A
and >95% of the D87N protein were named D87A- and D87N-BAMLET.

These complexes were testing in the apoptosis and DNA
35   fragmentation assays described above. Table III (Figure 10b)
shows the loss of viability after 6 hours' exposure of L1210

34

cells to d87A- and D87N-BAMLET. At 0.5 mg/ml D87A- and D87N-
BAMLET reduced the viability from 98% and 13% and 17%
respectively. The D87A-and D87N-BAMLET induced DNA fragmentation
similar to the BAMLET control (Figure 10c). The LD_{50}ies of the
5   mutant complexes (0.4 mg / ml) were slightly higher than for
BAMLET and HAMLET (0.2 mg / ml).

Spectroscopic characterisation of the D87A- and D87N-BAMLET
The conformations of D87A and D87N-BAMLET were compared to native
10   and apo-bovine a-lactalbumin and to BAMLET. Near UV CD
spectroscopy was carried out on the complexes, with the native,
apo and BAMLET controls as in Figs. 7 and 9. The D87A-BAMLET
spectrum was very similar to the unconverted D87A protein with
virtually no ellipiticy showing that D87A-BAMLET is in the apo
15   configuration. The spectrum of D87N-BAMLET was virtually
identical to that of BAMLET with reduced ellipiticy in both the
tyrosine and tryptophan region (Fig.10d).

Intrinsic tryptophan fluorescence spectroscopy was conducted with
20   the native, apo-α-lactalbumin and BAMLET controls as in figures 1
and 3. The results (Fig. 10e) with D87A- and D87N-BAMLET showed
intensity maxima at 345 nm with shoulder at 355 nm strongly
resembling BAMLET and the human apo-α-lactalbumin control,
suggesting that tryptophans are accessible to solvent.

25
ANS spectroscopy was conducted, with the native, apo- and BAMLET
controls as in Figures 7 and 9. Both D87A- and D87N-BAMLET bound
ANS, with spectra resembling BAMLET and the apo-α-lactalbumin
control. D87A-D87N-BAMLET bound ANS with the intensity maximum
30   shifted to 470 nm and an increased quantum yield compared to the
native control, indicating exposed hydrophobic surfaces in all
proteins (Fig. 10f).

These results demonstrated that D87A- and D87N-BAMLET maintain
35   the partially folded state with structural and functional
properties resembling HAMLET and BAMLET. Calcium removal prior

35

to oleic acid treatment was not required for the D87A mutant because the protein is most likely free from bound calcium and largely rests in the apo form.

5   As the complexes maintained their biologic activity, it appears that a functional calcium-binding site is not required for the apoptotic function of this complex.

Example 8

10   Calcium binding to alpha-lactalbumin and the HAMLET complex

The chromophoric chelator quin 2 was obtained from Fluka Chemie AG, Buchs, Switzerland. Other chemicals were of highest obtainable laboratory quality. To produce Ca^{2+}-free buffers, membrane tubing (M.w. cutoff: 3500, Spectrum Medical Industries

15   Inc., LA, Ca, USA, boiled four times in doubly distilled water before use) was filled with 10 ml Chelex 100 (Biorad, Richmond, Ca, USA), sealed, and stored in the solutions to absorb Ca^{2+}.

Native human α-lactalbumin was purified from human milk by

20   ammonium sulphate precipitation and phenyl sepharose chromatography as described (Svensson et al., PNAS. 2000, 97, 4221-6). Bovine α-lactalbumin was both purchased from Sigma, St. Louis, MO, USA and purified from bovine milk using ammonium sulphate precipitation and phenyl speharose chromatography

25   (Svensson et al., supra.). The mutated proteins (D87A and D87N) were expressed in *E. coli*, purified, folded and lyophilised as described (Anderson et al., 1997), (Permyakov et al., Protein Eng. (2001, 14:785-9). The purity of the protein was assayed by SDS-PAGE and agarose gel electrophoresis, and by NMR

30   spectroscopy.

Apo human or bovine α-lactalbumin for Ca^{2+}-binding studies was generated by dissolving α-lactalbumin in doubly distilled water containing a 10-fold molar excess of EGTA at pH 8.0. The sample

35   was applied to a G-25 gel filtration column after an aliquot of

36

saturated NaCl (calcium-depleted) and eluted by doubly distilled water. The sample was passed through the saturated NaCl to reduce binding of EGTA to the protein, and protein free from both Ca^{2+} and EGTA eluted in the water. The residual calcium content

5   was below 0.1 equivalents as estimated from the titration in the presence of quin2, as described below.

Calcium- and EDTA-free HAMLET was generated as described before, with the following adaptions. All buffers were stored with

10 chelex (preparation described above) on a tipping board for a minimum of five days before use. Only plastic vials were used. The FPLC system (Biorad Biologic, Richmond, Ca, USA), including the 20 ml ion exchange column and all tubings, was washed with 2 volumes of 100 mM EDTA, pH 8.5, and then rinsed with at least 10

15 volumes of millipore water. This was followed by 2 volumes of buffer before the application of oleic acid. EGTA-free apo alpha-lactalbumin (20 mg) in 80 ml calcium-free buffer was applied to the column in 4 consecutive runs (20 ml in each run). The collected fractions were pooled, dialysed, lyophilized and

20 checked for activity on tumor cells as described above. The calcium content was assayed using atomic absorption spectroscopy or titration in the presence of quin 2 (described below). The product was also characterized by agarose electrophoresis and ^{1}H-NMR.

25

To study the Ca^{2+} binding equilibrium, the proteins listed in table 1 including the D87A BAMLET obtained as described in Example 7, and HAMLET, was titrated with calcium in the presence of a chromophoric chelator, quin 2, for which the absorbance at

30 263 nm decreases (approx. 85%) upon Ca^{2+}-binding. The method relies on competition for calcium between the protein and chelator, and can be used to quantitate high affinity sites (ca. 10^7-$5 \cdot 10^9$ M^{-1} at low salt, and ca. $5 \cdot 10^5$-$1 \cdot 10^8$ M^{-1} at 0.15 NaCl) when quin 2 is the chelator. The exact concentration of the

35 chelator solution (in the range of 25-30 µM) was calculated from the absorbance at 239.5 nm in the presence of excess calcium

(using $\varepsilon_{239.5}$ = 4.2 × 10^4 L mol^{-1} cm^{-1} for quin 2). Lyophilised
protein was dissolved in calcium free (< 1 μM Ca^{2+}) chelator
solution at a concentration of 25-30 μM. The absorbance at 263
nm (A_{263}) was recorded for the protein/chelator solution using a

5   UV/Vis 920 spectrophotometer (GBC Scientific Equipment Pty Ltd,
Victoria, Australia). Calcium solution (3 mM CaCl₂ in 2 mM
Tris/HCl, pH 7.5, with or without 0.15 M NaCl) was added in
portions of 4 μl. A_{263} was recorded after each calcium addition.
The titration was continued until no absorbance change was seen

10 for the last five additions. A more concentrated Ca^{2+} stock (10
mM) was used at the end of the titrations of the proteins in 0.15
M NaCl. The data was analysed by least squares fitting directly
to the measured quantity, absorbance versus total calcium
concentration, using the CaLigator software (Andre´ and Linse,

15 2002).

The Ca^{2+} affinity of α-lactalbumin has been extensively studied,
mainly for the bovine protein. The reported values (Table 1)
vary between 2.5x10^6 and 5.7x10^8 M^{-1}, probably reflecting the

20 experimental conditions in terms of temperature, buffer, ionic
strength, salt, and protein preparation.

The titration curve was very sensitive to the differences in
affinity between chelator and protein, so this ratio can be

25 obtained from the data with high precision. Normalized data
(absorbance versus total Ca^{2+} concentration) at low salt are shown
in Figure 11, and the resulting Ca^{2+}-binding constants for the
proteins at physiological (0.15 M NaCl) and low (no added) salt
are listed in Table 1. We found a K_a of 1.8·10^9 M^{-1} at low salt (K_D

30 = 0.56·10^{-9} M). The Ca^{2+} ion bound more weakly at physiological
salt concentrations (0.15 M NaCl): K_a = 8.3·10^6 M^{-1} (K_D = 1.2·10^{-7}
M).

HAMLET and BAMLET were shown to bind Ca^{2+} (Fig. 11). The calcium-

35 binding constant for HAMLET was K_a = 5.9·10^8 M^{-1} (K_D = 1.7·10^{-9} M)
at low salt and K_a = 5.3·10^6 M^{-1} (K_D = 1.9·10^{-7} M) at physiological

38

salt concentrations. Ca^{2+}-titration data for D87A-BAMLET showed
no difference compared to the titration of quin 2 alone (Fig.
11), confirming that D87A-BAMLET has lost the functional Ca^{2+}
binding site.

It appears that HAMLET maintains a high calcium affinity in both
a low and physiological salt environment. Hence, the calcium
affinity is only 3 times lower for HAMLET than for α-lactalbumin
when no salt is added and 1.6 times lower at physiological salt
concentration.

Table 1

Calcium association constants (K$_a$) for α-lactalbumin or HAMLET at
different conditions. First five rows: this work, mean of two
experiments.

Protein	K$_a$ (M^{-1})	Buffer	Salt	pH	T (K)	Method
α-lac human	$1.8 \cdot 10^9$	2 mM Tris/HCl	–			chelator
α-lac human	$8.3 \cdot 10^6$	2 mM Tris/HCl	150 mM NaCl			chelator
HAMLET	$5.9 \cdot 10^8$	2 mM Tris/HCl	–			chelator
HAMLET	$5.3 \cdot 10^6$	2 mM Tris/HCl	150 mM NaCl			chelator
D87A-BAMLET	n.d.*	2 mM Tris/HCl	–			chelator
α-lac bovine	$4.3 \cdot 10^8$	10 mM Tris/HCl	–	7.5	298	ITC
αlac goat	$5.7 \cdot 10^8$	10 mM Tris/HCl	–	7.5	298	ITC
α-lac bovine	$2.85 \cdot 10^8$	10 mM Tris/HCl	–	8	–	DSC

39

Protein	K_a (M^{-1})	Buffer	Salt	pH	T(K)	Method
α-lac bovine	$4.8 \cdot 10^7$	10 mM Ammoinium Bicarbonate		7.8		EGTA, Fluoroscence
α-lac bovine	$2.0 \cdot 10^7$	H$_2$O	100 mM KCl	7	294	EDTA, ^{43}Ca-NMR
α-lac human	$2.0 \cdot 10^7$	H$_2$O	100 mM KCl	7	294	EDTA, ^{43}Ca-NMR
α-lac bovine	$2.7 \cdot 10^6$	20 mM Tris	–	7.5	298	1) Fluoescence 2) Hummel & Dryer method
α-lac 1) bovine 2) human, 3) goat	1) $2.5 \cdot 10^8$ 2) $3 \cdot 10^8$ 3) $2.8 \cdot 10^8$	5 mM Tris, 0.1 mM EDTA	–	7.2	298	CD 270 nm
bovine	$2.0 \cdot 10^7$	H$_2$O	100 mM KCl	8	294–298	EGTA ^{13}C- NMR
bovine	1) $2.0 \cdot 10^7$ 2) $4.0 \cdot 10^8$	50 mM Hepes	–	8	1) 310 2) 293	fluorescence
bovine	$2.5 \cdot 10^6$	20 mM Tris	–	7.5	298	microcalorimetry

40

Claims

1.   A biologically active complex comprising alpha-lactalbumin
or a variant of alpha-lactalbumin (α-lactalbumin) which is in the
5   apo folding state, or a fragment of either of any of these, and a
cofactor which stabilises the complex in a biologically active
form, provided that any fragment of α-lactalbumin or a variant
thereof comprises a region corresponding to the region of α-
lactalbumin which forms the interface between the alpha and beta
10   domains, and further provided that when the complex comprises
native α-lactalbumin, the cofactor is other than C18:1:9 cis
fatty acid.

2.   A complex according to claim 1 wherein the cofactor is a cis
15   C18:1:9 or C18:1:11 fatty acid or a different fatty acid with a
similar configuration.

3.   A biologically active complex according to claim 1 which is
obtainable by combining
20   (i) a cis C18:1:9 or C18:1:11 fatty acid or a different fatty
acid with a similar configuration; and
(ii) α-lactalbumin from which calcium ions have been removed, or
a variant of α-lactalbumin from which calcium ions have been
removed or which does not have a functional calcium binding site;
25   or a fragment of either of any of these, provided that any
fragment comprises a region corresponding to the region of α-
lactalbumin which forms the interface between the alpha and beta
domains, and further provided that when (ii) is alpha-
lactalbumin, (i) is other than C18:1:9 cis fatty acid.

30
4.   A complex according any one of claims 1 to 3 which includes
a variant of α-lactalbumin in which the calcium binding site has
been modified so that the affinity for calcium is reduced, or it
is no longer functional.

35

41

5.   A complex according to claim 4 wherein the variant as a mutation at one of the K79, D82, D84, D87 and D88.

6.   A complex according to claim 4 wherein the modification is
5   at D87 which includes a variant of α-lactalbumin having a D87A or D87N variants.

7.   A complex according to any one of the preceding claims which comprises a fragment of α-lactalbumin or a variant thereof, and
10   where the fragment includes the entire region from amino acid 34-86 of the native protein.

8.   A complex according to any one of the preceding claims wherein the α-lactalbumin is human or bovine α-lactalbumin or a
15   variant of either of these.

9.   A complex according to claim 8 wherein the α-lactalbumin is human α-lactalbumin.

20   10.   A complex according to claim 8 wherein the α-lactalbumin is mutant bovine α-lactalbumin which includes an S70R mutation.

11.   A complex according to any one of the preceding claims which further comprises calcium ions.
25

12.   A pharmaceutical composition comprising a complex according to any one of the preceding claims in combination with a pharmaceutically acceptable carrier.

30   13.   A method for treating cancer which comprises administering to cancer cells a complex according to any one of claims 1 to 11 or a composition according to claim 12.

42

14.   A method for treating bacterial infections which comprises administering to a patient in need thereof, a complex or a composition as described above.
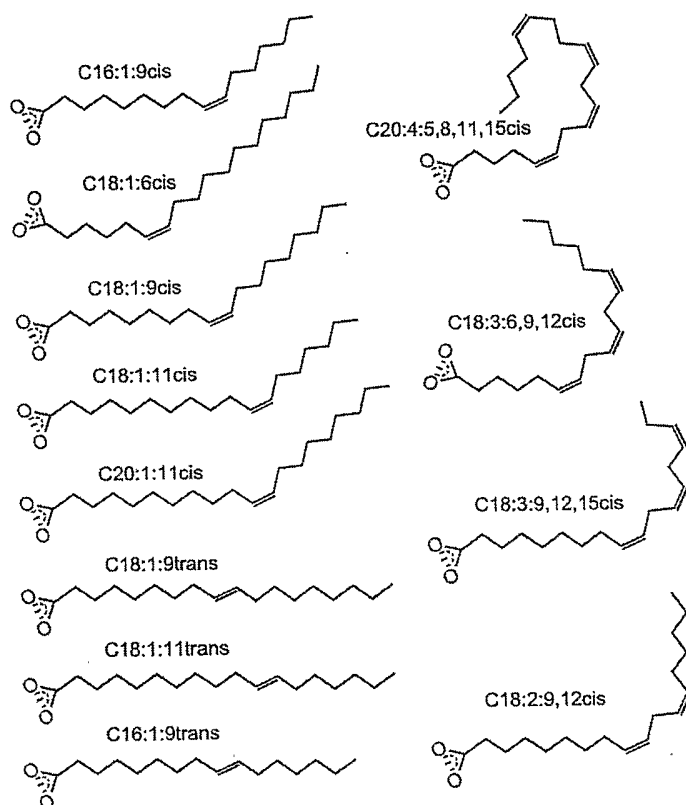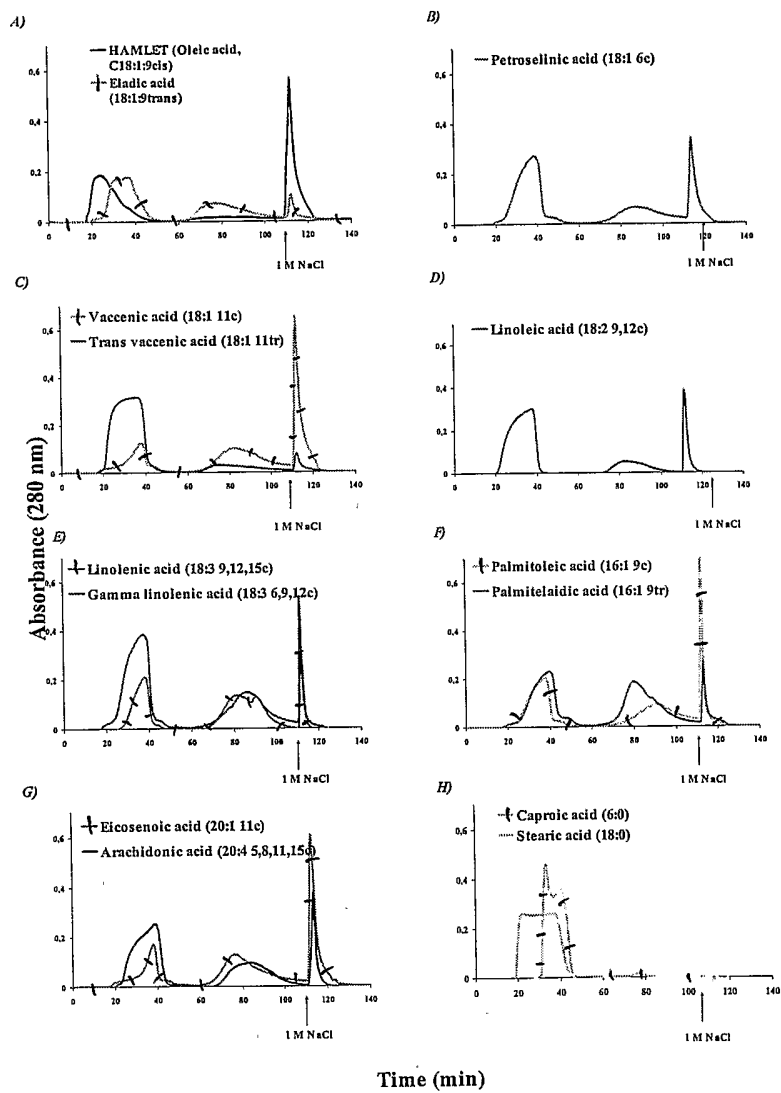
Figure 1

Figure 2

a)

The viability of L1210 cells after exposure to converted material and free fatty acids

a) Protein-lipid complexes

	Viability (%)	MW (bp)
Medium control	99	
Fatty acid/protein complexes:		
18:1: 9c (HAMLET)	0	1033 —
18:1: 9tr	98	
18:1:11c	1	
18:1:6c	60	
18:3 c	32	
γ18:3 c	43	
18:1 :11tr	98	
18:2 c	89	
		394 —
16:1:9c	73	394 —
16:1 :9tr	98	

b) Free fatty acids

Fatty acids:	Viability (%)	MW (bp)
20:1:11c	96	
20:4 c	96	
18:1:9c	99	
18:1: 9tr	98	
18:1:6c	97	1033 —
18:1: 11tr	96	
18:2 c	98	
18:3 c	99	
γ18:3 c	99	
18:3 c	99	394 —
16:1:9c	96	
16:1:9tr	98	
20:1:11c	99	
20:4 c	98	

a) Protein-lipid complexes (gel lanes):
] CT
] HAMLET
] C 18:1 9tr
] C 18:1 11c
] C 18:1 11tr
] C 18:2 c
] C 16:1 9c
] C 16:1 9tr
] C 18:1 6c
] C 18:3 c
] γC 18:3 c
] C 20:1 11c
] C 20:4 c

b) Free fatty acids (gel lanes):
] CT
] HAMLET
] C 18:1 9tr
] C 18:1 11c
] C 18:1 11tr
] C 18:2 c
] C 16:1 9c
] C 16:1 9tr
] C 18:1 6c
] C 18:3 c
] γC 18:3 c
] C 20:1 11c
] C 20:4 c
] Lip from HAMLET
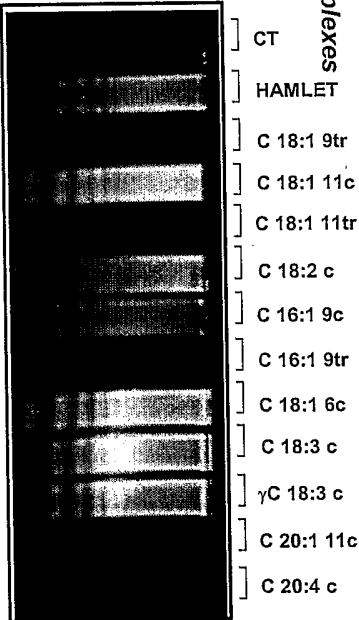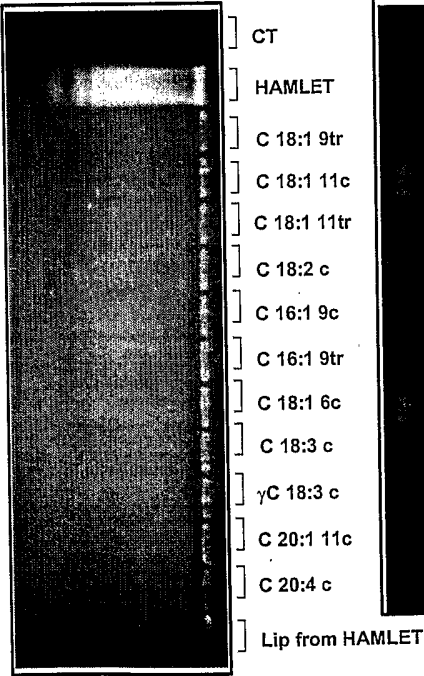
Figure 3

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Figure 4

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Wavelength (nm)
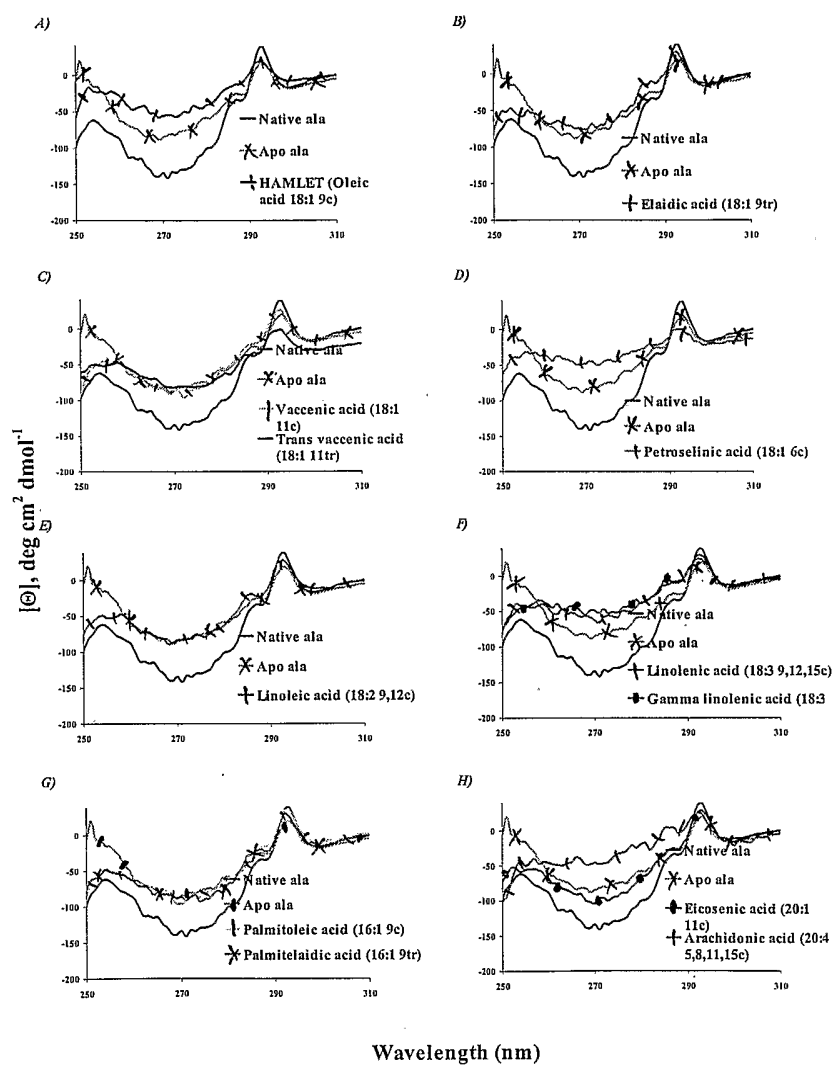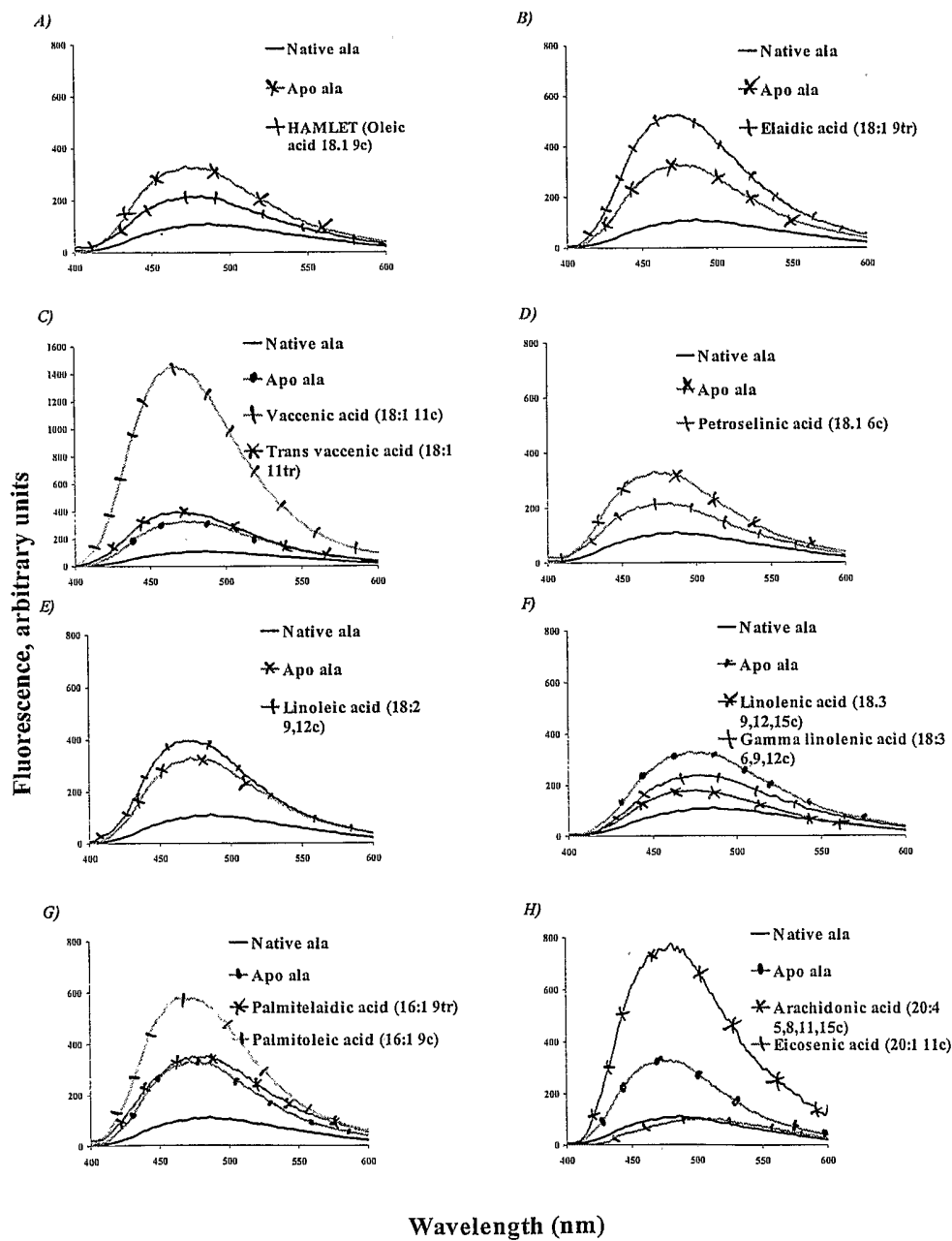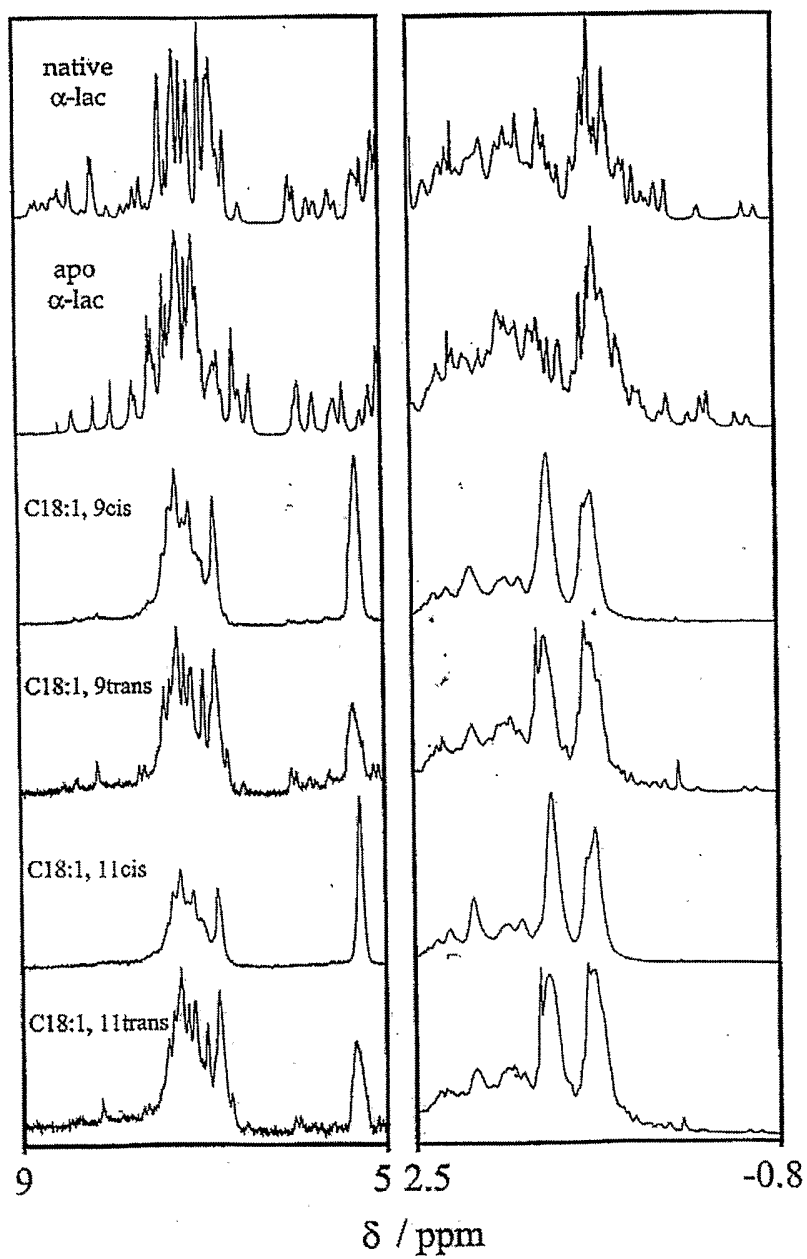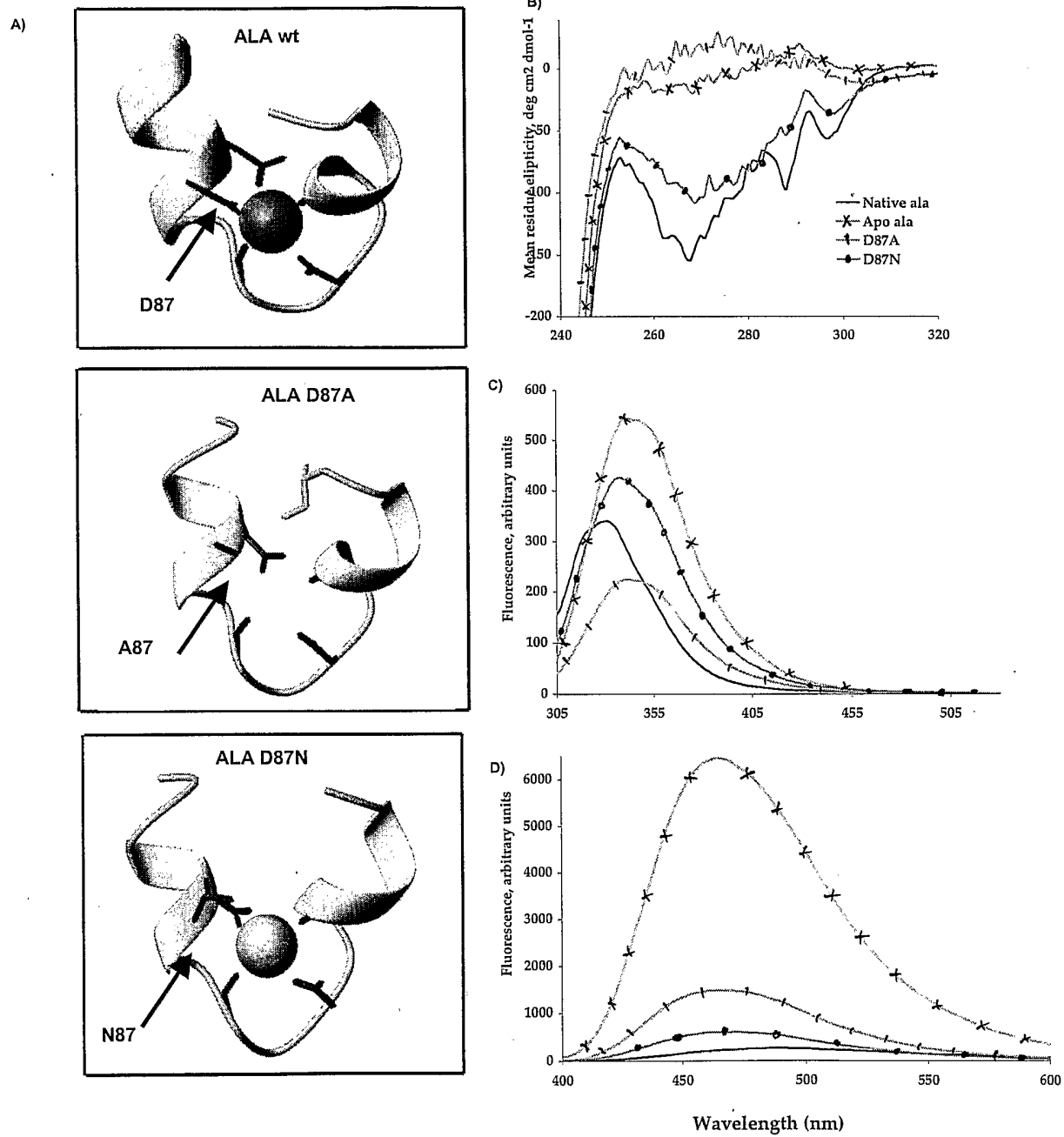
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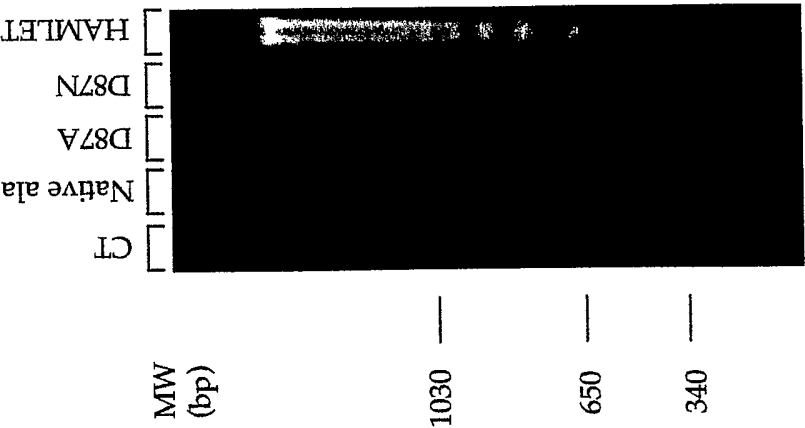
Figure 6

Figure 7

Table I. The viability of L1210 cells after treatment with various forms of α-lactalbumin.

	Cell viability (%)
Medium control	98
α-lactalbumin*:	
native	99
D87A	99
D87N	97
HAMLET†	4

* the concentration was 1.0 mg/ml
† the concentration was 0.3 mg/ml

A)

B)

MW (bp)

1030

650

340

Figure 8

A)
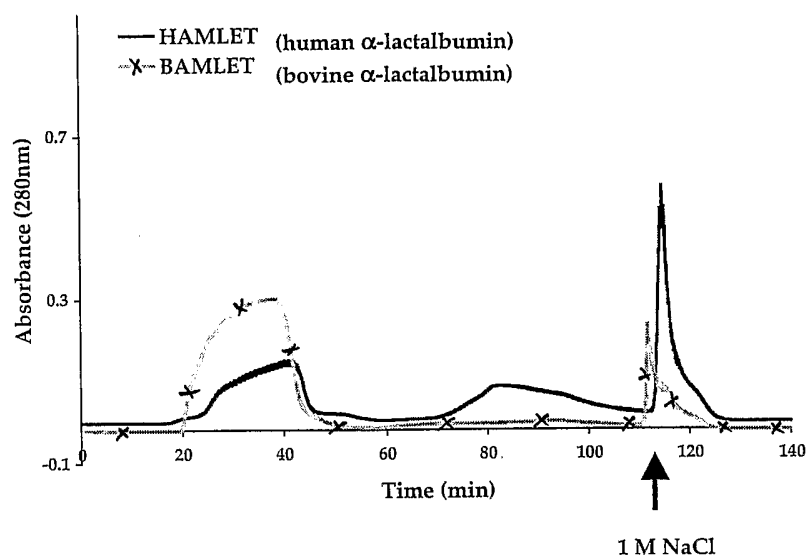
B)

Table II. Viability of L1210 cells after treatment with BAMLET and HAMLET.

	Cell viability (%)
Medium control	98
HAMLET	
0.2 mg/ml	67
0.3 mg/ml	9
BAMLET	
0.2 mg/ml	76
0.3 mg/ml	8

C)

Figure 9

D)

E)

F)

Figure 9 con't

A)
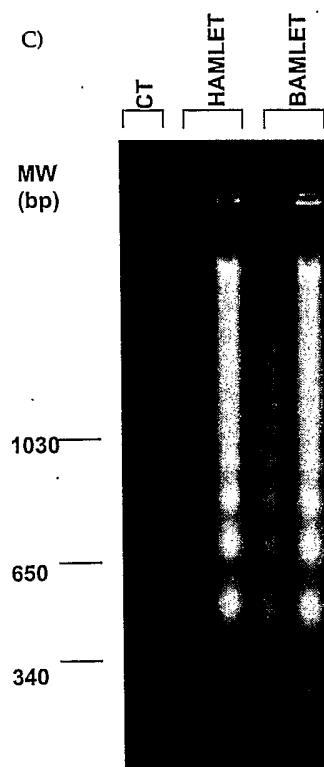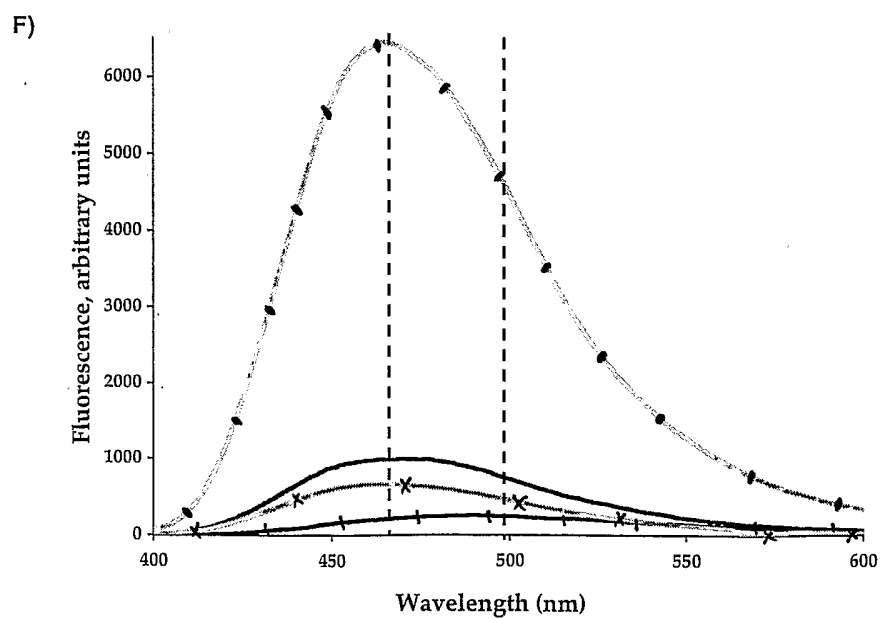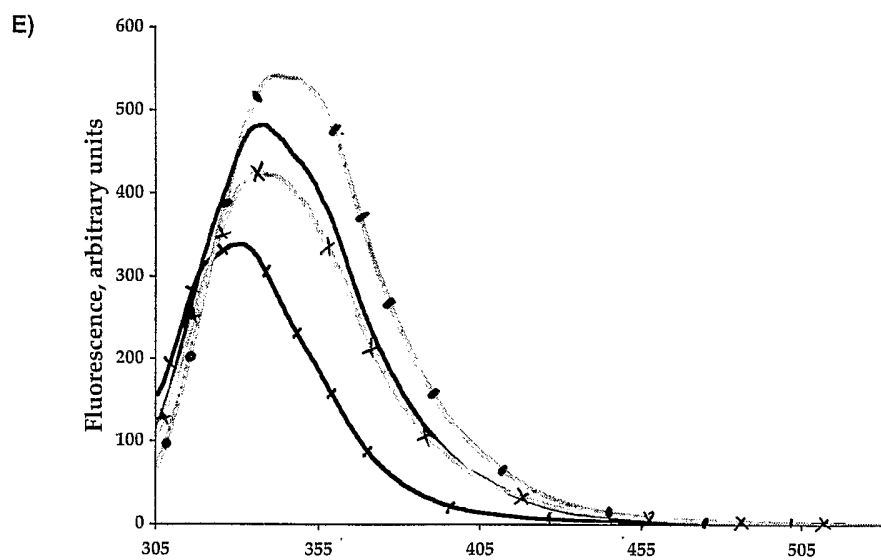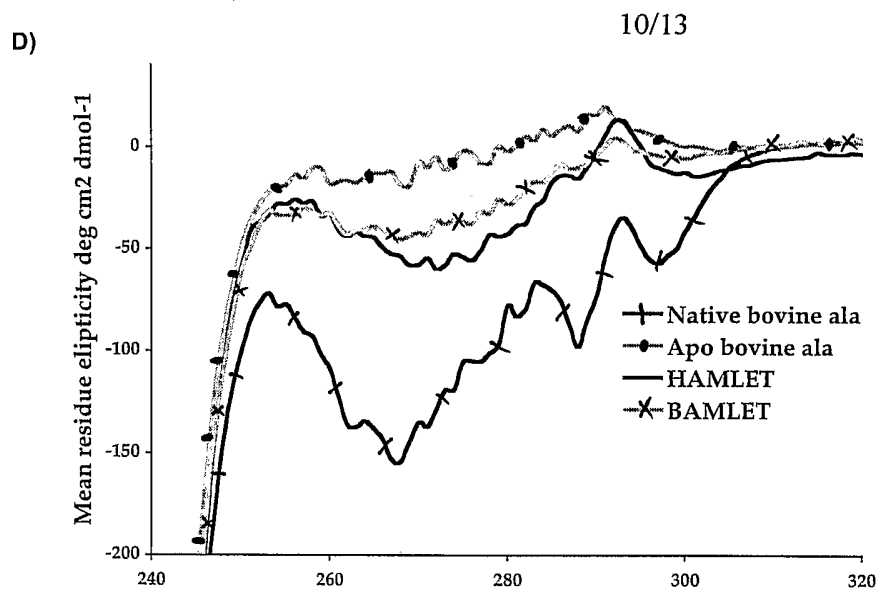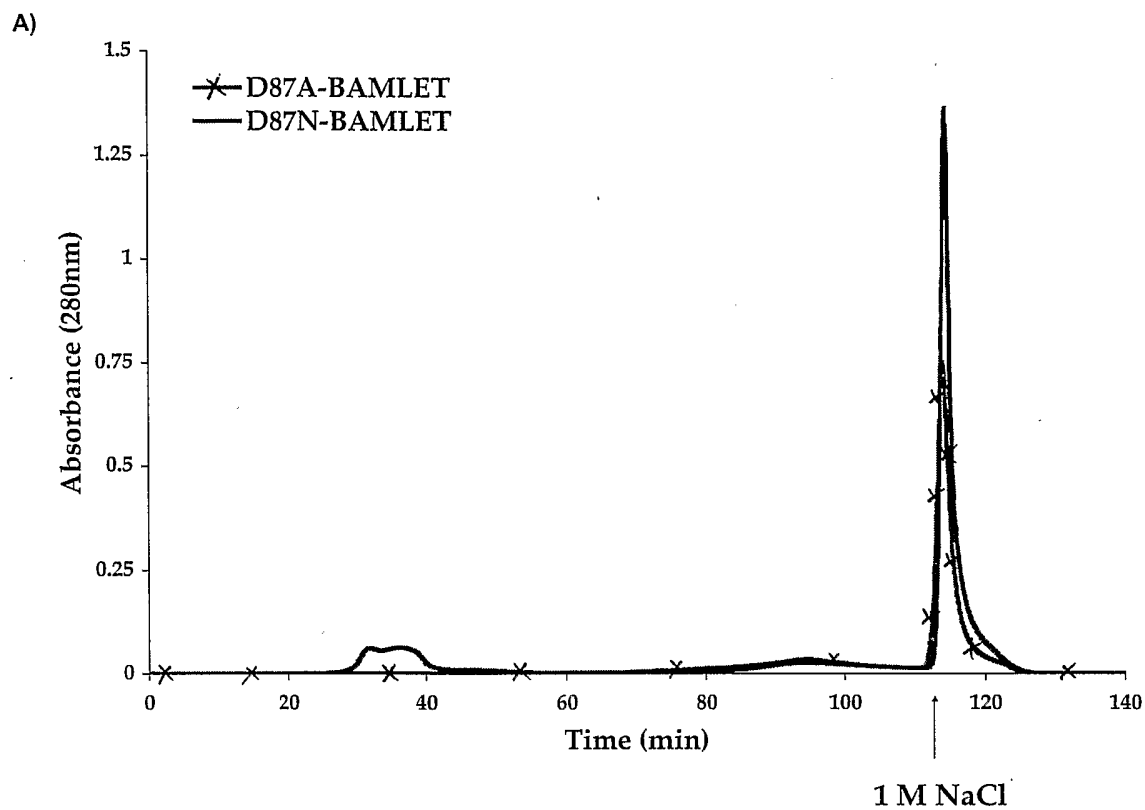
B)

Table III. The viability of L1210 cells after treatment with D87A- and D87N-BAMLET.

	Cell viability (%)
Medium control	98
α-lactalbumin*	
native	97
D87A-BAMLET	13
D87N-BAMLET	17
BAMLET†	0

*the concentration was 0.5 mg/ml
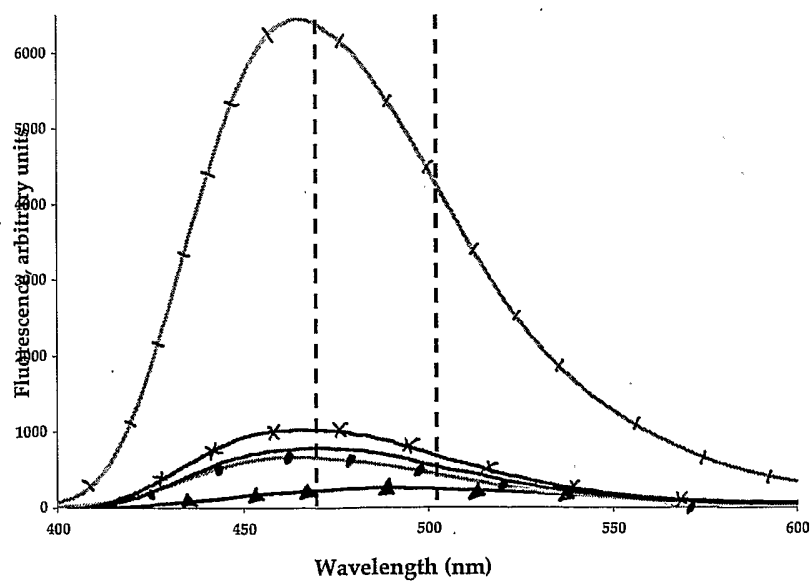†the concentration was 0.3 mg/ml

C)
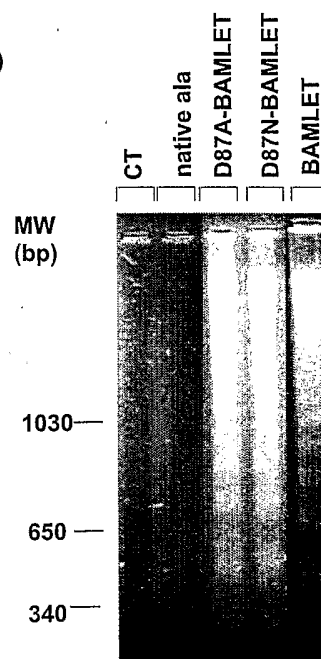
Figure 10

D)
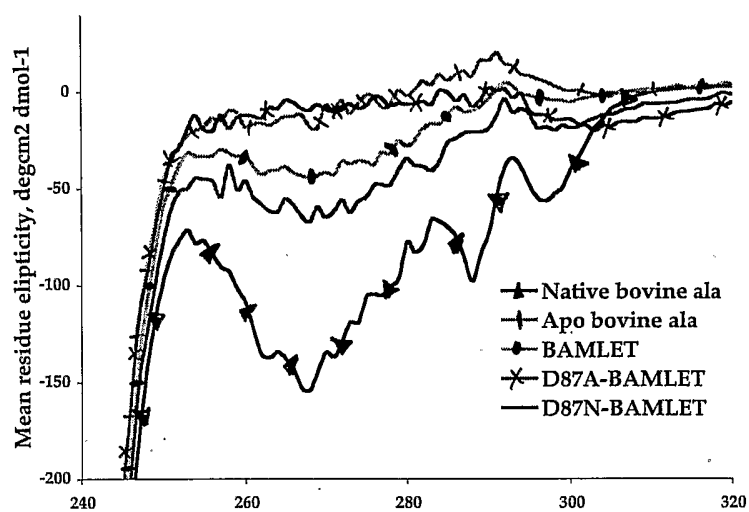
E)
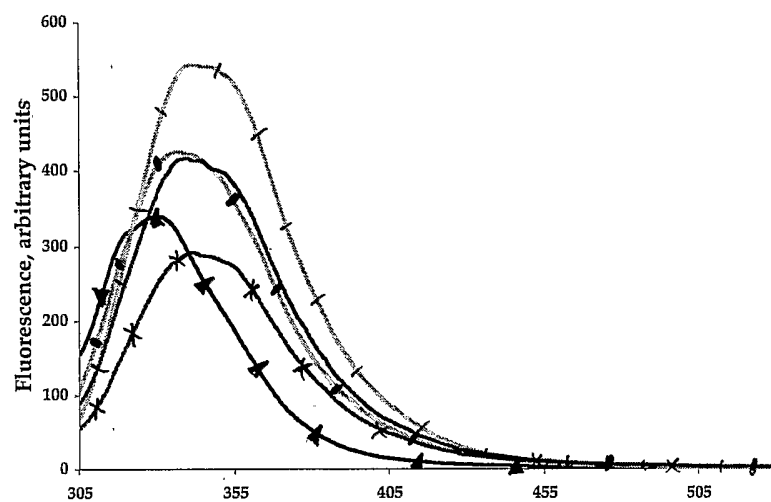
F)

Figure 10 con't

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Figure 11