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ANTIBODY-GUIDED ENZYME THERAPY OF CANCER PRODUCING CYANIDE RESULTS IN NECROSIS OF TARGETED CELLS

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A number of enzyme/prodrug activation approaches for the treatment of cancer have been reported to date with varying success. We describe progress in the development of a system based on a β-glucosidase enzyme in combination with a naturally occurring "prodrug," the sugar linamarin, which releases the cytotoxin cyanide. A recombinant fusion protein, composed of an scFv (MFE-23) reactive against carcinoembryonic antigen (CEA) and a plant-derived β-glucosidase (linamarase), was produced and its cytotoxic potential was investigated. The fusion protein was expressed in a supersecretory mutant strain of *Saccharomyces cerevisiae* and purified by affinity chromatography. Extensive functional *in vitro* characterisation of the fusion protein showed that it retained antigen binding activity but that its catalytic activity was impaired, a problem not related to its fusion with the scFv. Nevertheless, we demonstrated complete tumour cell killing at doses of prodrug that are completely nontoxic to nontargeted cells. Preliminary *in vivo* characterisation showed that extensive glycosylation of the fusion protein caused its rapid clearance through the hepatic route. Aggregational properties also led to poor pharmacokinetics. Furthermore, we present some data analysing the mode of cell death resulting from exposure to this system. Enzymic catalysis of the substrate generates cyanide, a metabolic poison that asphyxiates cells and leads them to a necrotic-like cell death. This system has been called antibody-guided enzyme nitrile therapy (AGENT).
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Key words: *AGENT; ADEPT; scFv; linamarase; cyanide*

Antibodies have been widely developed as biopharmaceuticals for cancer therapy because they can effectively target tumour-related antigens. They have been used against malignant cells in their unmodified form or as delivery vehicles for cytotoxic therapy (reviewed in ref. 1). They have been shown to eliminate haematologic and solid tumours and also to seek out quiescent metastatic spreads in systemically accessible parts of the body. Recently, antibody-related drugs have been clinically approved for cancer chemotherapy and there is much optimism that many more will follow.

Combining specific antigen targeting with a cytotoxic function is an attractive concept with the potential for novel cell killing mechanisms and the promise of reduced damage to normal tissues. Genetic engineering has facilitated the creation of novel immunologically based molecules (reviewed in ref. 2). These retain the specificity and affinity characteristics of their parent molecules and have proved potentially useful for clinical applications.[3]

Antibody/enzyme-mediated prodrug activation strategies (introduced by Bagshawe[4]) are powerful approaches that exploit the selectivity of the antibody/antigen reaction to generate cytotoxic molecules specifically in tumours (reviewed in ref. 5). The antibody/enzyme fusion protein is administered and allowed to bind to the tumour. When unbound fusion protein has cleared from the circulation and normal tissues, an inert "prodrug" is administered that can be converted to a toxic drug by the enzyme part of the protein. The fact that the prodrug remains nontoxic until activation reduces the effects of nonspecific toxicity and results in minimal damage in unrelated tissues. Since the cytotoxic drug is generated locally and extracellularly, it should diffuse and kill antigen-negative tumour cells as well as cells not accessed by the fusion protein within the tumour mass. This is known as the *bystander effect*.[6] A particular advantage of this system is that it may allow the use of extremely potent agents such as nitrogen mustards and palytoxin, which are too toxic to be readily used in conventional chemotherapy. Examples of the successful implementation of this system *in vivo* and in the clinic have been described.[7,8]

The primary consideration for the choice of enzyme is that it should convert prodrug to drug with high efficiency and that the enzyme should have no accessible homologue performing a similar reaction. Also, the activity should be high under physiologic conditions, with low immunogenicity. The use of human enzymes would be a matter of great interest for the potential lack of immunogenicity, but a problem posed by such an approach is the potential prodrug activation by endogenous enzymes. A further potential drawback of this system is the phenomenon of drug resistance in tumour cells. Current prodrug activation strategies rely on the regeneration of conventional chemotherapeutic drugs such as methotrexate and doxorubicin.[9] Tumour cells that have already built up resistance to such drugs are unlikely to be susceptible to the same drugs, even if generated at higher concentrations *in situ* by targetable systems.

We describe the expression and purification of a fusion protein composed of an anti-carcinoembryonic antigen (CEA) single-chain antibody, MFE-23,[3] and the β-glucosidase enzyme linamarase.[10] The targeting of cancer cells with an antiplacental alkaline phosphatase antibody chemically conjugated to a broad substrate, sweet almond β-glucosidase, has been reported with promising results *in vivo*.[11] Recombinant fusion proteins are worth developing because they generally have advantages over chemically produced molecules in terms of product quality and yield. This is important when considering preclinical or clinical testing.

The cyanogenic β-glucosidase (linamarase) of cassava (*Manihot esculenta* Crantz) used in the present study is responsible for the first step in the sequential breakdown of 2 naturally occurring cyanoglucosides, lotaustralin and linamarin. Hydrolysis of these cyanoglucosides occurs after mechanical damage to tissues and leads to the production of hydrocyanic acid. Linamarase is an attractive candidate for enzyme-directed cancer therapy. By targeting, it could restrict the activation of the prodrug (the sugar linamarin) to the tumour site only, thus reducing any nonspecific cytotoxicity. An antibody/linamarase complex would not require internalization and the released "drug" should exemplify a bystander effect (Fig. 1). This system, called antibody-guided en-

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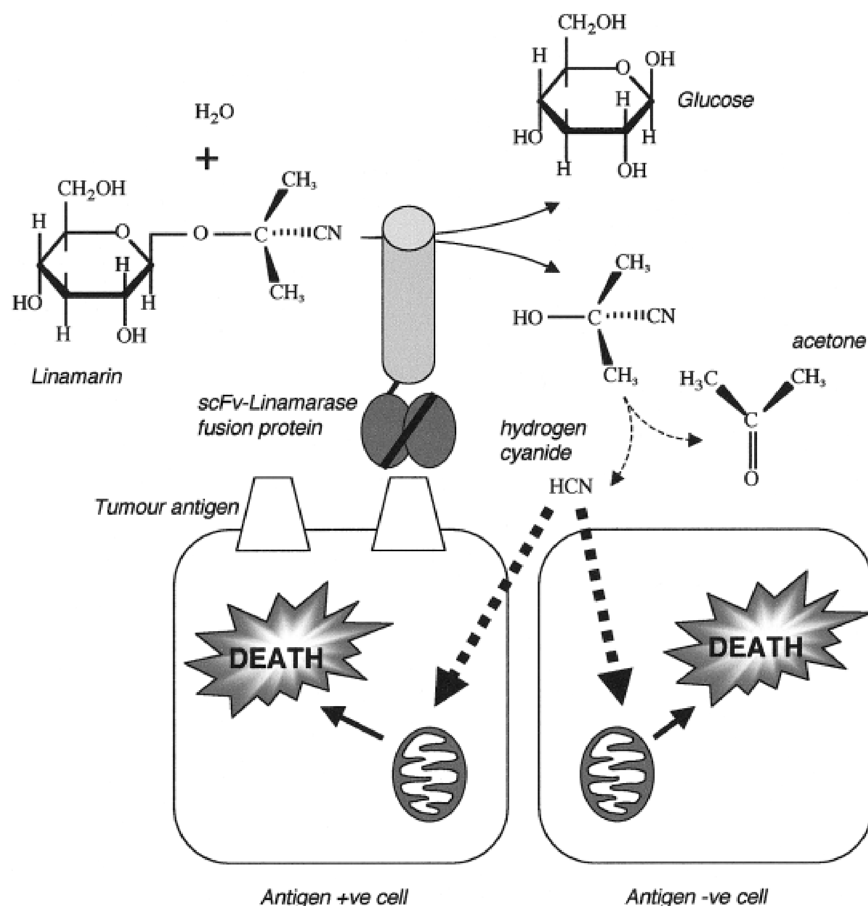

FIGURE 1 – Schematic representation of the AGENT system, which results in the conversion of the "prodrug" sugar linamarin into the "drug" hydrogen cyanide on the surface of cancer cells.

zyme nitrile therapy (AGENT), could have advantages over similar gene therapy approaches generating cyanide,[12] as the sugar does not need to be internalized and the cyanide released has a better chance of eliciting a bystander effect.

Cyanide is a rapidly acting lethal metabolic poison.[13] Intoxication occurs mainly through inhibition of cytochrome oxidase, the terminal mitochondrial enzyme of the respiratory chain. Exposure to cyanide can also result in the loss of capacity to regulate ion transport systems that control cytosolic pH, with potentially lethal effects. In mammals, most cyanide detoxification is catalysed by the enzyme rhodanase, which is normally found in the liver and the resulting nontoxic metabolites are excreted through the kidneys. Being a small molecule, cyanide can penetrate cells easily and does not require receptor-mediated internalization to exert its action. Its diffusive ability is restricted to a close cell vicinity from the tumour cells, thus limiting the damage caused to surrounding healthy tissues.

Our study tests some of the above attributes in the development of a targetable cyanogenesis system.

MATERIAL AND METHODS

Recombinant DNA technology

Recombinant constructions (Fig. 2) were carried out in *Escherichia coli* strain INVαF′ (Invitrogen, San Diego, CA) as described by Sambrook *et al.*[14] Oligonucleotides were synthesised by Sigma-Genosys (St. Louis, MO). The original vector pYX243 (R&D Systems, Minneapolis, MN) and clone pYXCAS5 were provided by Prof. M. Hughes.[15] Plasmid pYXCAS5-HA-HIS was designed to facilitate purification by immobilised metal affinity chromatography. This was achieved by inserting a cassette made from 2 oligonucleotides: HISTAGFOR 5′-TATTGTATAGTCGACCTGCCGCTTCAGGTTATCCGTATGATGTGCCTGACTACGCAGGAGGCAGTGGCGGT-3′ HISTAGREV 5′-ATAACAATACTCGA GTCAGTGATGGTGATGATGATGACCGCC ACTGCCTCCTGCGTA-3′.

Clone pYXMFE-HIS-HA was constructed by replacing the linamarase *Nco*I/*Sal*I fragment in pYXCAS5 with the MFE-23 scFv gene and HIS_6 tag-amplified pUC119-MFE (Dr. K. Chester, Royal Free Hospital, London, UK) using the following oligonucleotides: NCOIMFEFOR 5′-TATTGTTATCCATGGGGCAGGTGAAACTGCAGCAG-3′ MFEREVTWO 5′-TGTATATGTAGTCGACAAGCGGCCCCATGGTGATGATGGTGATGTGCGGCCGCCCCGTTTCAG-3′. Clone pYXMFE-LIN-HA-HIS was made similarly using the following oligonucleotides: NCOIMFEFOR 5′-TATTGTTATCCATGGGGCAGGTGAAACTGCAGCAG-3′ REVMFENCOI 5′-TATTGTTATCCATGGAACCTCCAGAACCTCCCTGTTGCAGCTC CAGCTT-3′. The clone was then inserted as an *Nco*I/*Nco*I fragment upstream of the linamarase gene in clone pYXCAS5-HA-HIS.

Saccharomyces cerevisiae *expression of recombinant proteins*

Expression studies were carried out in strains CGY 2998 (MATα, *ura*3-52, *leu*2-3) CGY1463 of *Saccharomyces cerevisiae* under the direction of a GAL promoter. This strain carries a double mutation in the secretion pathway at loci *ssc-1* and *ssc-2*.[16] This leads to bypass of a part of the glycosylation pathway, which has been demonstrated to aid the expression of heterologous proteins. Plasmids were transformed into competent yeast cells and grown on yeast selective media (YSM) agar plates, supplemented with amino acids (excluding leucine) and 2% raffinose. Re-streaked colonies were used to inoculate a 100 ml culture of YSM/-leu/2% raffinose, which was then used as a starting inoculum in 5 L shaker cultures or a 1 L culture that was later used in fermentor grow-ups.

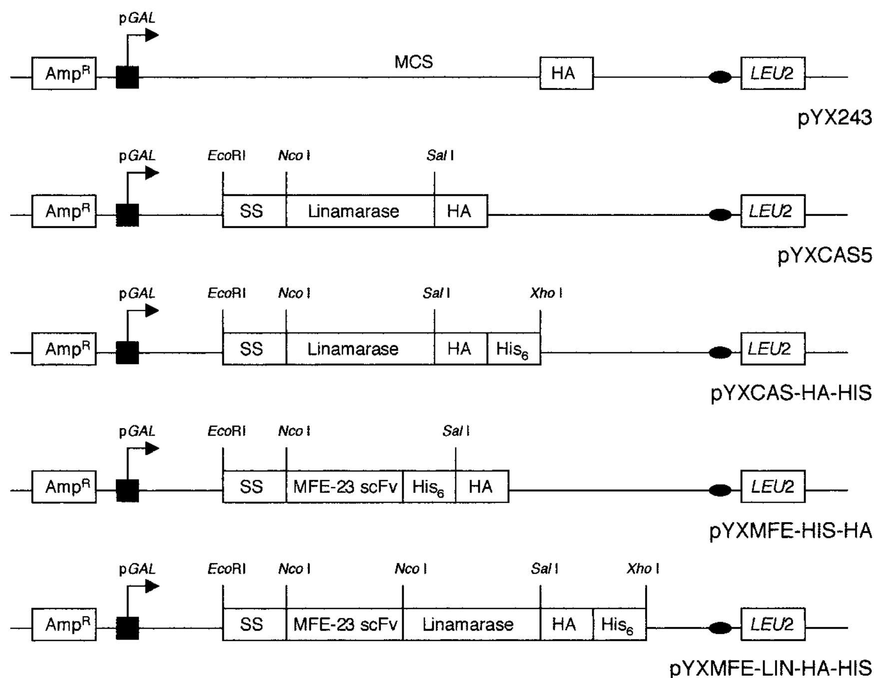

FIGURE 2 – Schematic illustration of the genetic constructs used in *Saccharomyces cerevisiae* expression experiments. Manipulations were carried out as described in the text. AMP^R, β-lactamase gene; p*GAL*, galactose promoter; HA, haemagglutinnin tag; *LEU2*, leucine marker; SS, invertase secretion signal; HIS_6, hexahistidine tag; MCS, multiple cloning site; ●, stop codon.

Shaker cultures were kept at 250 rpm aerated in baffled flasks with foam bungs. Expression of the scFv-linamarase fusion was scaled up to 15L using a fermentor for large-scale production of the protein. Fermentation conditions included shaking at 400 rpm (increased to up to 800 rpm at the end of induction), an air flow rate of 1 L/min, a ratio of O_2/CO_2 of 32:5 (CO_2 turned off after cells started respiring), pH kept at 5.0 and oxygen saturation always kept above 10%. Polypropylene glycol antifoam was added to the fermentor cultures to 0.01%. Growth was carried out for 72 hr post inoculation, after which expression was induced by the addition of a 2% final galactose solution. Induction was continued for a further 72–96 hr and was monitored at several time points for protein expression by performing a small-scale linamarase assay on cell lysates.

Isolation and purification of recombinant proteins

Proteins were extracted from the cell pellet by resuspending in 60 mM Na_2HPO_4/45 mM $Na_2H_2PO_4$ 10 mM KCl/2 mM $MgSO_4$ and French pressing in the presence of a "cocktail" of protease inhibitors consisting of 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 10 μg/ml leupeptin and 10 μg/ml aprotinin. Proteins, which included the MFE scFv, were affinity-purified by directly binding the clarified cell lysate to a CEA-coupled affinity column (Dr. K. Chester) and eluted in 50 mM diethylamine, pH 12. The eluted proteins were neutralised with 0.2 vol of 1 M phosphate buffer, pH 7.4 and dialysed exhaustively against 150 mM citrate phosphate buffer, pH 5 or 6, or against PBS. Linamarase expressed from clone pYXCas5-HA-HIS was purified on an immobilised copper-(II)-chelating sepharose column by immobilised metal affinity chromatography using a method similar to that described in Linardou *et al.*[17]

Antigen-binding activity of the fusion protein

The ability of the parental scFv and the scFv/linamarase fusion protein to bind CEA was demonstrated by ELISA on antigen-positive LS174T and antigen-negative KB cells fixed with 4% paraformaldehyde. Detection was with 1 μg/ml anti-haemagglutinin (Boehringer Mannheim, Indianapolis, IN) or anti-penta-HIS antibody (Qiagen, Chatsworth, CA) followed by anti-rat horseradish peroxidase-conjugated IgG or anti-mouse horseradish peroxidase-conjugated IgG, respectively, as described in Linardou *et al.*[17]

Binding of tested proteins on live cells was demonstrated by fluorescence-activated cell sorting (FACS) analyses. For each analysis, 10^6 cells were washed and blocked with PBS/2% FCS. Each sample of 10^6 cells was resuspended in a small volume of a 10 μg/ml solution of the test protein and left on ice for 30 min. Cells were then washed before the detection antibodies were applied. Incubations were for 1 hr on ice. Samples were FACS-analysed using a Coulter (Hialeah, FL) EPICS flow cytometer.

Kinetic analyses

Enzyme assays were carried out in 100 μl of 10 mM *p*-nitrophenyl β-D-glucopyranoside (PNP-Gluc) in 150 mM citrate-phosphate buffer, pH 6.0, at 37°C and stopped by the addition of 0.2 M sodium carbonate buffer, pH 12.0. The amount of released *p*-nitrophenolate was measured spectrophotometrically at 405 nm. When the natural substrate linamarin was used to assay for linamarase activity, the amount of liberated HCN was determined by the pyrazolon-bispyrazolon method of Mao *et al.*[18] Up to 100 μl of buffer or medium containing the linamarase enzyme to be assayed were mixed with 450 μl of substrate solution (various concentrations of linamarin in 150 mM citrate-phosphate buffer, pH 5.0) and incubated for various time intervals at 37°C. The reaction was stopped by the addition of 500 μl of a 0.2 M NaOH solution and the enzyme was stored on ice to reduce HCN evaporation.

To determine how much HCN had been released, 1 ml of 0.15 M NaH_2PO_4 and 0.5 ml of 0.14% chloramine-T were added to each sample, mixed thoroughly and incubated on ice for 5 min. Eighty-five milligrams of a 5:1 ratio of pyrazolone/bispyrazolone powder was dissolved in 25 ml of pyridine and 1 ml of this solution (freshly made) was added to each assay tube. These tubes were then incubated at 37°C for 30 min to allow the colour to develop. The colour was measured spectrophotometrically at 620 nm. The reaction was scaled down 10-fold when performed in a 96-well ELISA plate. K_m and k_{cat} values were measured, using a range of nonsaturating substrate concentrations and fit to the Michaelis-Menten equation for pseudo-first order reaction by linear regression.[19] Analysis was performed using the Sigma Plot 4.01 software package.

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Cytotoxicity assays

Purified proteins were dialyzed against PBS and tested for their cell-killing activity on the antigen-positive cell line LS174T and on the antigen-negative line KB. In each assay, 2×10^3 tumour cells/well were seeded into a 96-well microtitre plate in complete medium (DMEM containing 10% FCS and appropriate antibiotics) and grown overnight at 37°C and 5% CO_2. Next, the cells were incubated with the test protein solutions for 3 hr, followed by 3 washes in PBS. Finally, the cells were incubated in various dilutions of linamarin, in complete media. Cell death was determined by measuring the levels of lactate dehydrogenase (LDH), which is released upon cell lysis, using the CytoTox 96 assay (Promega, Madison, WI) and the manufacturer's instructions.

Apoptotic/necrotic analyses

Caspase-3 is activated during apoptotic signaling events.[20] Caspase-3 substrate I (Ac-Asp-Glu-Val-Asp-*p*NA; Calbiochem, San Diego, CA) was used to measure induction of enzyme activity after exposure to cyanide/AGENT, 5-fluorouracil (5-FU) and tissue culture media (control) according to the supplier's recommendations.

Additionally, DNA from cyanide/AGENT-treated, 5-FU-treated and control cells was extracted and analysed using the Apoptotic DNA Ladder Kit (Boehringer Mannheim), according to the manufacturer's recommendations.

Apoptotic/necrotic nuclei of treated and fixed cells were stained for 30 min with Hoechst 33342 immunofluorescent reagent (Sigma) at a concentration of 0.5 μg/ml in PBS. The stained cells were observed under a Zeiss fluorescence microscope.

In vivo *studies*

For blood clearance studies, female nude mice were injected i.v. (via lateral tail vein) with 20 μg of ^{125}I-radiolabelled protein (using the Iodogen method) in 200 μl of sterile PBS. Blood samples were taken over 2 days, weighed and counted on a γ-counter. For activity-biodistribution studies, female nude mice bearing xenografted LS174T tumours (1 cm diameter) were used. Nonlabelled samples (20 μg) were injected i.v. in 200 μl of sterile PBS. The mice were sacrificed at various time points and the tumour, blood, liver and other organs were removed and weighed. An equal volume of 150 mM citrate-phosphate buffer (pH 5.0) was added to each tissue sample and homogenised in the presence of a cocktail of protease inhibitors. The homogenate was clarified and the supernatant was used in cyanogenic linamarase assays as described above. Radiolabelled samples were used for protein biodistribution assays. In this case, tissue samples were solublized in 7 M KOH and counted as above.

RESULTS

Construction and expression of recombinant proteins

The yeast expression system based on the pYX plasmids[15] was used as a starting point for the production of chimeric molecules based on linamarase. In addition to the supersecretory mutant *S. cerevisiae* strain CGY 2998 (*ssc1*-1), other mutant strains (CGY 1468 [*ssc2*-1] and CGY 1463 [*ssc1*-1, *ssc2*-1]) were tested, to find the optimal strain. The linamarase-expression clone (pYXCAS-HA-HIS; Fig. 2) was transformed into all 3 strains and expression was analysed by Western blotting of whole cells (Fig. 3*a*). The double mutant strain produced significantly more enzyme than either of the single mutant strains and was chosen as the host for further work. The potentially reduced glycosylation of heterologous proteins from this strain may also perform better *in vivo*. It has been seen that an antibody/enzyme fusion protein produced in *Pichia pastoris*, although possessing low-level glycosylation, has very favourable *in vivo* pharmacokinetics. It accumulates in tumours but clears from the circulation rapidly enough to allow early administration of the prodrug.

Protein purification resulted in the isolation of approximately 150 μg/l of yeast culture of linamarase and scFv/linamarase fusion proteins, whereas production of scFv alone resulted in approximately 3 mg pure protein/l of yeast culture. Proteins were pure as judged by SDS-PAGE and Coomassie staining (Fig. 3*b–d*) and had the predicted subunit molecular weight. When analysed by gel filtration, the enzyme and enzyme-containing fusion proteins were shown to exist as a mixture containing oligomeric forms, similar to the wild-type enzyme.[15] Small amounts of dimeric enzyme could be isolated for further studies, but this was found to oligomerize upon storage, a feature that was not reversible by the addition of mild detergents or other agents (results not shown). The scFv/linamarase fusion protein was proteolytically stable, whereas the reverse fusion of linamarase/scFv was sensitive to degradation during production and characterization (data not shown).

Antigen binding and enzyme catalysis

ELISAs were used to evaluate the ability of the scFv/linamarase fusion protein to bind to the surface of fixed antigen-positive (LS174T) and -negative (KB) cells. The binding curves on cellular CEA for scFv alone, the enzyme alone and the scFv-enzyme chimera are shown in Figure 4*a* and the binding on KB cells is shown in Figure 4*b*.

The scFv MFE-23 and the scFv/linamarase fusion protein demonstrated their ability to bind the CEA antigen on fixed antigen-positive cells, whereas no binding was detected on a non-CEA-expressing cell line. The enzyme alone did not shown any binding affinity for either the positive or the negative cell line. The reduction in the fusion protein's affinity for CEA when compared with scFv alone was estimated to be 6-fold, probably due to the aggregation of the enzyme (see Discussion). The relative affinities

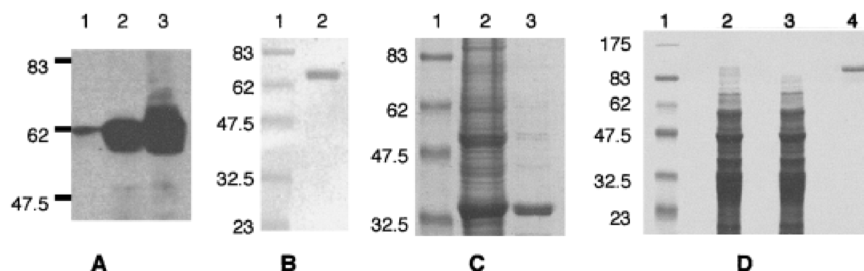

Figure 3 – Recombinant expression and purification of scFv, linamarase and scFv/linamarase fusion proteins. (*a*) Expression analysed by Western blotting of linamarase in *S. cerevisiae* strains CGY 2998 (lane 1), CGY 1468 (lane 2) and CGY 1463 (lane 3). (*b*) Coomassie-stained purified linamarase (lane 2) and molelcular weight markers (lane 1). (*c*) Coomassie-stained gel of the purification of *S. cerevisiae*-expressed scFv, with molecular weight markers (lane 1), cell lysate (lane 2) and pure scFv from a CEA column (lane 3). (*d*) Coomassie-stained gel of the purification of the scFv/linamarase fusion protein. Molecular weight markers (lane 1), cell lysate (lane 2), unbound fraction from CEA column (lane 3) and pure scFv-fusion protein from CEA column (lane 4).

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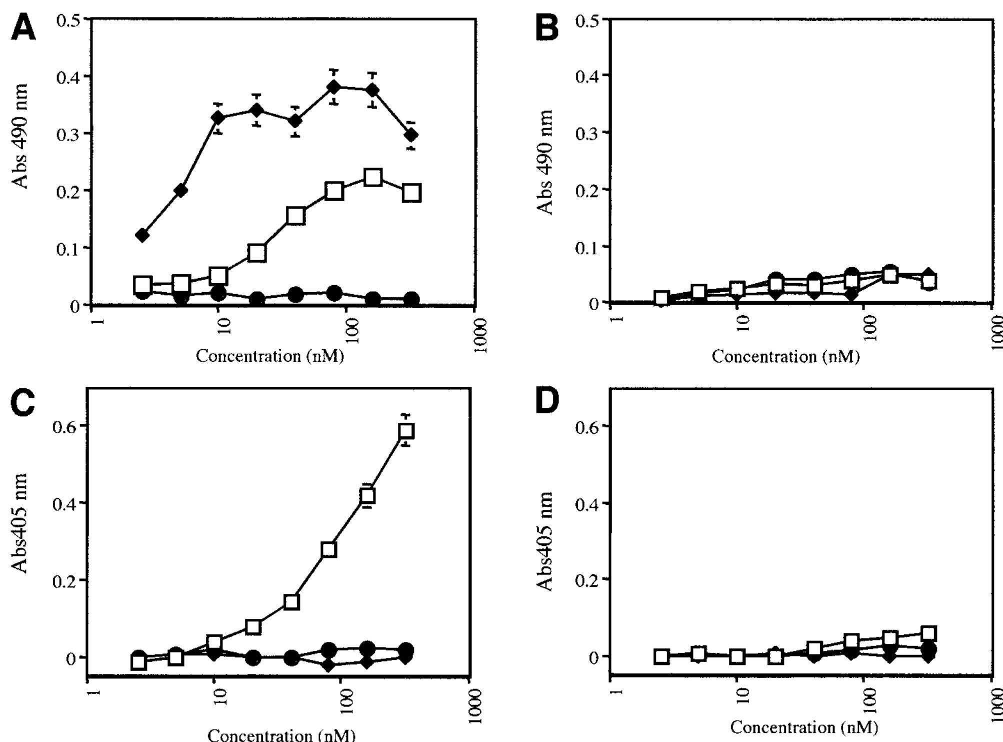

FIGURE 4 – Antigen-binding ELISA of the recombinant proteins on fixed cells. (*a*) ELISA analysis illustrating binding of the scFv/Linamarase fusion protein, the enzyme alone and the parental scFv MFE-23 alone on antigen-expressing LS174T cells. (*b*) ELISA analysis illustrating binding of the scFv/linamarase fusion protein, the enzyme alone and the parental scFv MFE-23 alone on antigen-negative KB cells. Catalytic ability of the scFv/linamarase fusion protein on fixed cells is also shown. (*c*) Analysis of the catalytic ability of the scFv/linamarase chimeric protein, the enzyme alone and the parental scFv alone after they have been incubated on fixed antigen-expressing LS174T cells. (*d*) Analysis of the catalytic ability of the scFv/linamarase chimeric protein, the enzyme alone and the parental scFv alone after they have been incubated on fixed antigen-negative KB cells. Squares, scFv/linamarase fusion protein; diamonds, scFv; circles, linamarase.

observed were more comparable when purified antigen was used (2.5 nM for scFv and 10 nM for scFv/linamarase; data not shown).

A modified ELISA system was used to evaluate whether the protein that bound to the cells possessed enzymic activity, demonstrating bifunctionality. Fixed LS174T and KB cells were incubated with the purified linamarase, scFv and the scFv/linamarase fusion proteins. Activity of bound molecules was demonstrated by incubating the plate with 10 mM PNP-glucose, a β-glucoside substrate analogue. Figure 4*c* illustrates the profile of enzymic activity on the CEA-expressing cell line. Only the fusion protein demonstrated binding to fixed cells and substrate hydrolysis, thereby indicating bifunctionality. No catalytic activity was observed when enzyme or antibody alone were incubated with LS174T cells, or on the negative control cells (Fig. 4*d*)

Binding on live antigen-expressing cells was analysed by FACS analyses, which showed significant mean fluorescence shifts upon incubation with the various antibody-containing molecules (Fig. 5). Due to the likely oligomerization of the enzyme and fusion proteins, it was decided to confirm that there was no or little background binding to cells, as can happen with aggregated molecules. The results show very little background binding of the scFv and scFv-linamarase proteins.

Kinetic parameters

Kinetic parameters for the hydrolysis of linamarin were determined using the plant-derived, wild-type enzyme (provided by Prof. M. Hughes, University of Newcastle, Newcastle, UK), the recombinant enzyme and the antibody/enzyme and enzyme/antibody fusions. Studies were carried out using several different substrate concentrations in the range of 0.1–15 × the estimated K_m, in duplicate. The results of the kinetic investigations are summarized in Table I. Our kinetic data are comparable to those already published, but what is clear is that the construction of an scFv/fusion protein with linamarase does not significantly impair its activity, as the K_m and k_{cat} are only slightly altered. The activity compared with the plant purified material is reduced by about 40%, suggesting that the yeast expression system is not reproducing the enzyme completely. These reactions were carried out at the pH optimum for the enzyme (6.0).

In vitro *cell cytotoxicity of the scFv/linamarase fusion protein*

A number of different conditions for cytotoxicity were tried, using LS174T and KB cells. The best conditions were found to be short (3–4 hr) incubation times with the fusion protein (100 μg/ml) and prolonged incubation with linamarin (24–48 hr). The number of cells in the assay were also a factor: too many resulted in the culture media becoming exhausted rapidly, even though a lot of cyanide was being generated. It is thought that the longer exposure to the linamarin substrate was needed due to the lower activity of the enzyme in culture media compared with the pH 6.0 assay conditions. Cytotoxicity of the "active drug" (potassium cyanide) was measured and was assumed to be equivalent to the potential cytotoxicity observed from complete linamarin hydrolysis, since the hydrolysis of 1 molecule of linamarin results in the production of 1 molecule of cyanide.

Cells were harvested and the media from the samples were used in a CytoTox 96 assay and results were expressed as cell survival ratios (Fig. 6). The IC_{50} is defined as the concentration of the cytotoxic agent required for the reduction of viable cells to 50% of that of the controls. LS174T cells were found to be sensitive to potassium cyanide in concentrations as low as 0.06 mM, with an IC_{50} of 0.4 mM. Linamarin was not cytotoxic to LS174T cells

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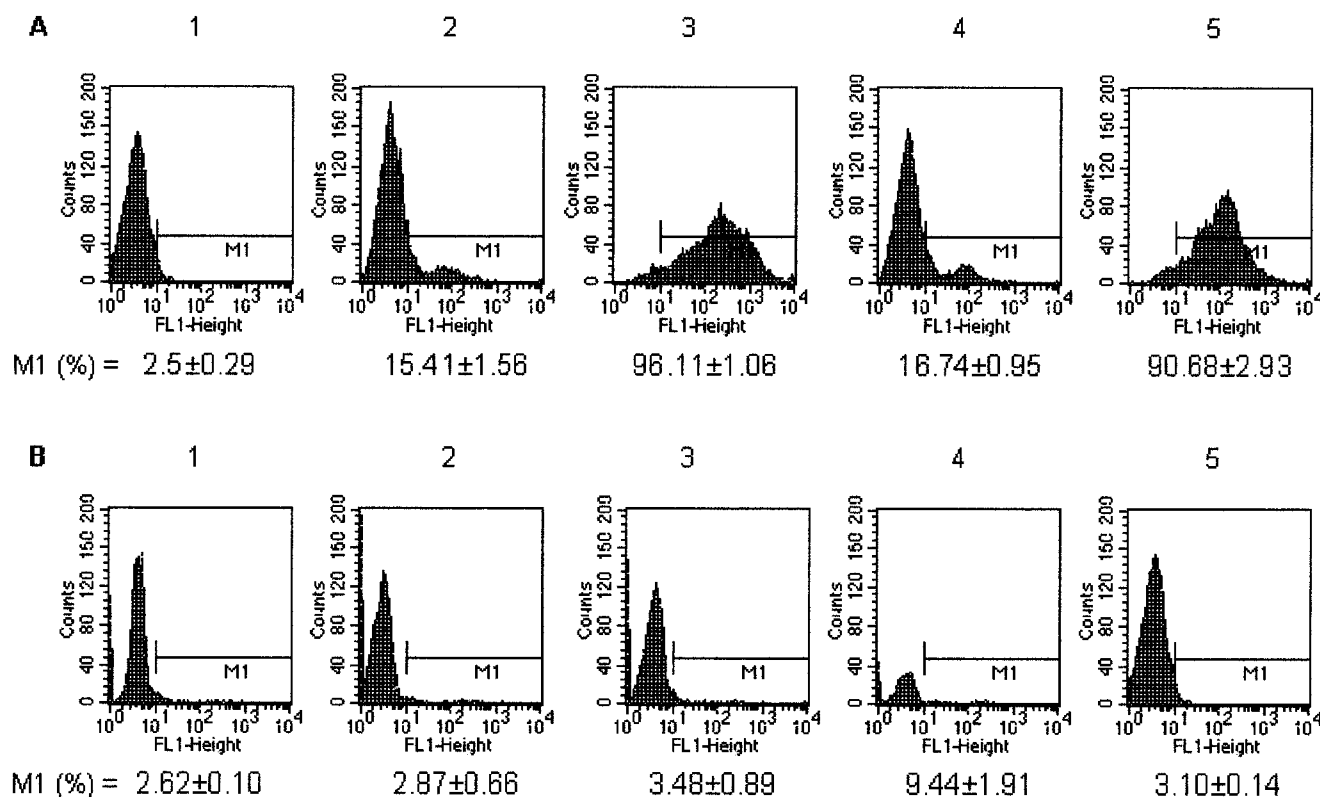

FIGURE 5 – FACS analyses on LS174T and KB cells. (*a*) FACS analyses on LS174T, CEA-expressing cells. Region M1 represents the gated area. 1, untreated cells (no detection antibodies); 2, anti-penta-HIS and anti-mouse FITC detection antibodies control; 3, scFv MFE, followed by anti-penta HIS and anti-mouse FITC detection antibodies; 4, linamarase, followed by anti-penta HIS and anti-mouse fluorescein isothiocyanate (FITC) detection antibodies; 5, MFE/linamarase fusion protein, followed by anti-penta HIS and anti-mouse FITC detection antibodies. (*b*) FACS analyses on KB, CEA-negative cells. Region M1 represents the gated area. 1, untreated cells (no detection antibodies); 2, anti-penta-HIS and anti-mouse FITC detection antibodies control; 3, scFv MFE, followed by anti-penta HIS and anti-mouse FITC detection antibodies; 4, linamarase, followed by anti-penta HIS and anti-mouse FITC detection antibodies; 5, scFv/linamarase fusion protein, followed by anti-penta HIS and anti-mouse FITC detection antibodies.

TABLE I – SUMMARY OF THE KINETIC ANALYSES OF THE VARIOUS PROTEINS USING THE NATURAL SUBSTRATE LINAMARIN[1]

Protein	k_{cat} (sec^{-1})	K^4_m (mM)
Native, wild-type linamarase[1]	$6 \pm 0.3 \times 10^{-3}$	1.2 ± 0.1
E. coli-expressed linamarase[1]	$0.03 \pm 0.001 \times 10^{-3}$	0.88
Pichia-expressed linamarase[1]	$25.9 \pm 1.7 \times 10^{-3}$ [3]	1.05
Recombinant linamarase[4]	$3.5 \pm 0.25 \times 10^{-3}$	1.02 ± 0.25
ScFv-linamarase[4]	$3.2 \pm 0.24 \times 10^{-3}$	2.16 ± 0.37

[1]Keresztessy et al., 1996 Ref. 24.–[2]Keresztessy et al., 2001. Ref. 15.–[3]Note that these values differ from the values obtained when assays were reproduced in our hands.–[4]This work.

even at very high concentrations (IC_{50} above 100 mM). The control (KB) cells were intoxicated by cyanide with an IC_{50} of about 1 mM. The AGENT approach resulted in the destruction of antigen-positive cells with an IC_{50} of about 6 mM. It is clear from these results that intoxicating cyanide is produced from nontoxic linamarin. However, the toxicity profile of the AGENT treatment is still about 10-fold higher than that of pure cyanide, indicating that not enough cyanide is being produced or not rapidly enough. As 10 mM of linamarin is not toxic, this finding suggests that complete cell killing could be achieved *in vivo*, despite the less than optimal activity of the enzyme under these conditions.

Determination of mode of cell death

As proteases are involved in the early stages of apoptosis,[20] their activation can be used to differentiate between necrosis and apoptosis. A colourimetric caspase-3 substrate was used to measure induction of the enzyme's activity after exposure to a known inducer of apoptosis, 5-FU,[21] the AGENT/cyanide system and tissue culture media (control). Results indicated the appearance of caspase-3 activity after incubation with 5-FU, as expected. When cyanide was generated, cell death was confirmed using LDH release, but it was not accompanied by caspase-3 activation, as indicated in Figure 7. This result indicated necrotic cell death.

Further evidence of necrotic cell death induced by cyanide was obtained by chromosomal DNA analysis. When apoptosis has occurred, a characteristic DNA ladder is observed,[20] whereas with necrosis, a DNA smear is observed. After lysis of cultured cells that have been exposed to media or to the death-inducing agent, DNA was prepared and analysed. Results shown in Figure 8 indicate that a DNA degradation smear is produced after treatment of cells with AGENT/cyanide, rather than the typical apoptotic DNA ladder, as seen with 5-FU treatment.

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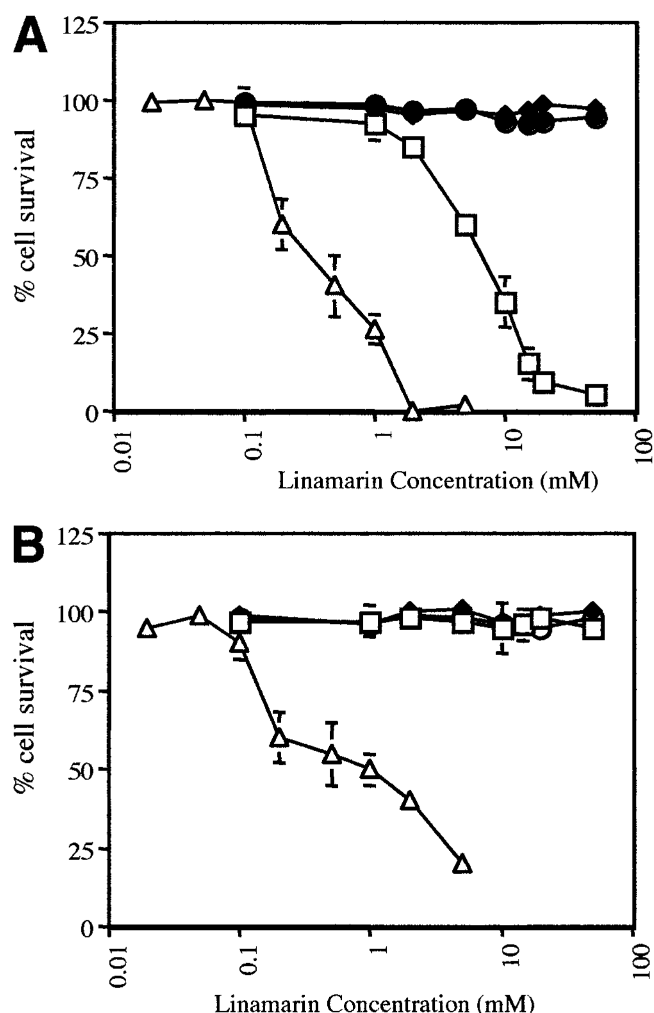

FIGURE 6 – Cytotoxicity expressed as cell survival of (*a*) LS174T cells and (*b*) KB cells exposed to scFv/linamarase, scFv alone and linamarase alone. The 3 proteins were used at a concentration of 100 μg/ml. Reactions were performed in quadruplicate. Squares, scFv/linamarase fusion protein; diamonds, scFv; circles, linamarase; triangles, KCN.

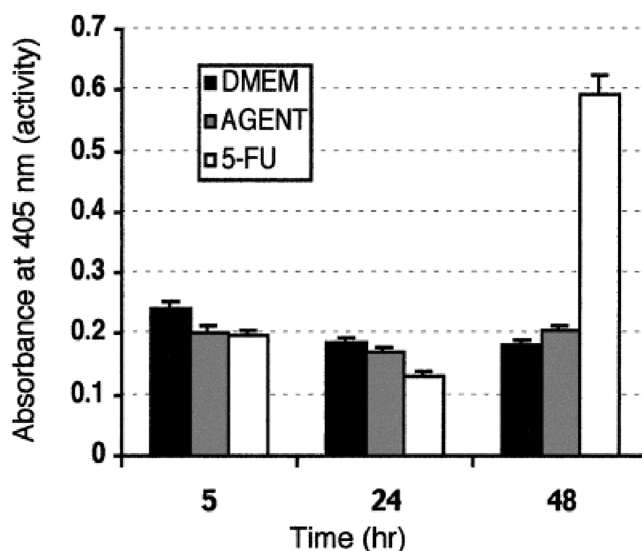

FIGURE 7 – Profile of caspase-3 activity in cells treated with media only (control), AGENT/cyanide and 5-fluorouracil (5-FU) after 5, 24 and 48 hr. The apoptosis-related caspase activity was only induced in cells treated with 5-fluorouracil.

Cells treated with AGENT/cyanide did not show the characteristic nuclear condensation when stained with Hoechst 33342 immunofluorescent reagent, as was observed for 5-FU-treated cells, further supporting the notion of necrotic death by cyanide intoxication (Fig. 9).

In vivo *tumour uptake studies*

Glycosylation and aggregation effects can be seen more clearly *in vivo*; therefore some preliminary experiments were carried out to see whether the degree of glycosylation of the scFv/linamarase fusion protein would result in impractical blood clearance rates. Radiolabelled scFv/linamarase fusion protein demonstrated more rapid clearance than expected (Fig. 10*a*), with insignificant amounts remaining in the blood by 10–20 hr. This was presumably due to the glycosylation in the *Saccharomyces* strains, even though they carried the secretory pathway mutations.

Tumour xenograft-containing nude mice were used to see whether any tumour targeting was evident, despite the rapid clearance. Significant liver accumulation was seen with approximately 5% of the injected dose of radiolabelled fusion protein present per gram of liver tissue (% i.d./g), even after 48 hr (Fig. 10*b*). Similar amounts were seen in the spleen (Fig. 10*c*). This was presumably due to the carbohydrate-mediated hepatic clearance and aggregation-induced splenocyte uptake. This resulted in significant liver and spleen to blood ratios (over 100:1; Fig. 10*f,g*). The amount of fusion protein accumulating in the tumour was poor (Fig. 10*d*), which seemed to mirror the blood clearance rate. By following radiolabelled fusion protein, the tumour/blood ratio did not climb much higher than 1 over the 72 hr study period. These observations could, to some extent, be predicted from the *in vitro* behaviour of the fusion protein.

However, a different result was noticed when linamarase activity was measured *in vivo*. Significantly more enzyme activity was found in the tumour than in blood, over the 5 hr tested. A linamarase tumour/blood activity ratio of approximately 8 was seen by 5 hr (Fig. 10*h*), suggesting that some targeting was achieved *in vivo*. This was against a background of significant liver and spleen accumulation as seen by the high linamarase liver/blood activity ratio (Fig. 10*i*). This high amount of liver activity may be an overestimation, recent research has described a human cytosolic cyanogenic β-glucosidase.[22] It is not clear whether this human enzyme will turn over linamarin.

It is possible that the observed blood levels, as measured by radioactivity, may be inaccurate, higher than the actual amount of active enzyme, possibly because some catabolised fusion protein, still containing radiolabel, may be present in the blood after passing through the liver or spleen.

DISCUSSION

We have developed an antitumour fusion protein with the ability to deliver cytotoxic cyanide specifically to malignant cells. This molecule is a recombinant scFv/linamarase fusion protein that displays antigen-binding ability and enzymic activity and kills cells by inducing necrosis.

The system is potentially an attractive candidate for targeted therapy, since cyanide is a small, soluble molecule that does not require receptor-mediated internalization to exert its action. Additionally, the released "drug" could exemplify a bystander killing effect due to its diffusive ability. It is envisaged that the targeted cells cannot develop drug resistance (a common problem in normal and directed chemotherapy). The lack of an accessible mammalian enzyme homologous to linamarase should ensure little or no non-

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specific toxicity, an issue often associated with conventional chemotherapeutic treatment modalities.

The targeting of cancer cells with an antibody chemically conjugated to a β-glucosidase has been reported[11] and was shown to result in growth delay of tumours *in vivo*. Ideally, a recombinant system needs to be developed, as this can overcome the problem of heterogeneous species that results from chemical conjugation.

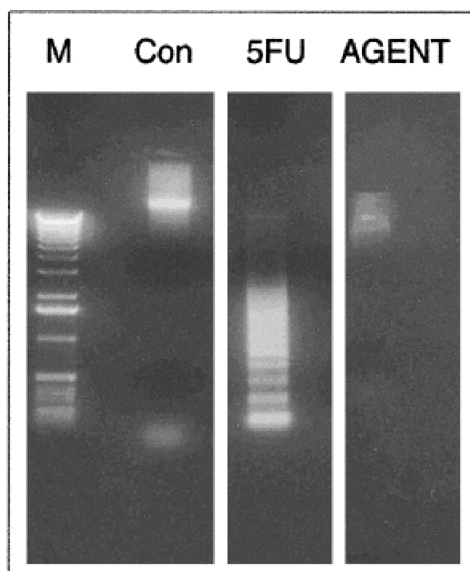

FIGURE 8 – Apoptotic DNA ladder, indicating that ordered DNA fragmentation is only observed after apoptosis induction with 5-fluorouracil (5-FU). In cells treated with AGENT/cyanide, random DNA fragmentation occurs. Con, control; M, 1 kb ladder DNA markers.

Additionally, with recombinant expression, the antibody and enzyme moieties are not in danger of being damaged during coupling and they retain binding and catalytic activities. It is generally accepted that recombinant production of such biomolecules is favourable, as this can be scaled up for clinical production. The linamarase/linamarin system described here is a favourable enzyme/substrate pair, with lower K_m and higher k_{cat} value than the β-glucosidase/amygdalin system. Therefore maximal rate values of the reaction could be reached at lower substrate concentrations and less prodrug would need to be administered *in vivo* for effective activation. The work described here represents the first steps to recombinant production, although some expression issues remain to be resolved.

Previous work has shown that functional β-glucosidases have been expressed in bacteria,[23] and linamarase was expected to give similar results. The native enzyme has already been characterised from plants.[10] Although such expression was problematic, the enzyme was finally expressed at low levels as a nonglycosylated glutathione S-transferase (GST) fusion in *E. coli*.[24] It was shown to be aggregated and to have reduced catalytic activity compared with the native material. Much of the active enzyme was found to be associated with the bacterial chaperone GroEL, a factor often associated with the protection of un/mis-folded polypeptides. Further development of an expression system led to the *Saccharomyces* system described by Keresztessy *et al.*[15]

In our hands, expression of the enzyme as a fusion in a variety of *E. coli* expression systems did not result in the production of any functional product (results not shown). Expression studies were then diverted toward the use of eukaryotic organisms as expression hosts. No functional expression was observed in *Pichia pastoris*, a methylotrophic yeast that has been successfully used in the production of several foreign proteins including plant proteins and β-glucosidases.

S. cerevisiae[16] has been well characterised as an expression host. This organism again offers the advantages of posttranslational modification, but with more elaborate carbohydrate chain

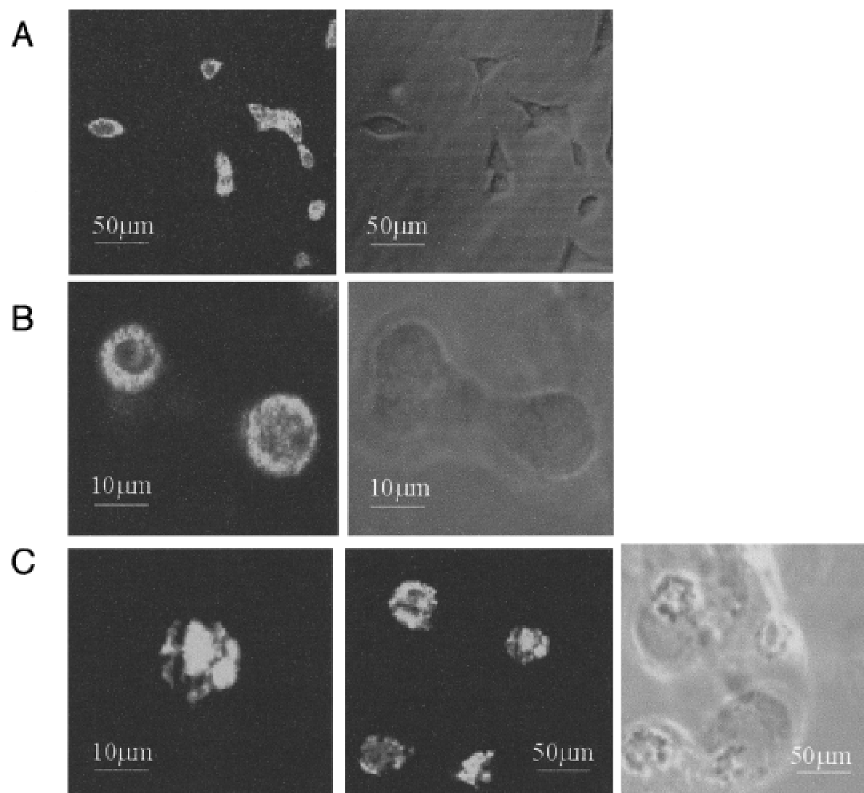

FIGURE 9 – LS174T tumour cells treated with media only (*a*), AGENT/cyanide (*b*) and 5-fluorouracil (*c*) and stained with Hoechst immunofluorescent reagent. Apopotic bodies are only visible in cells treated with 5-fluorouracil.

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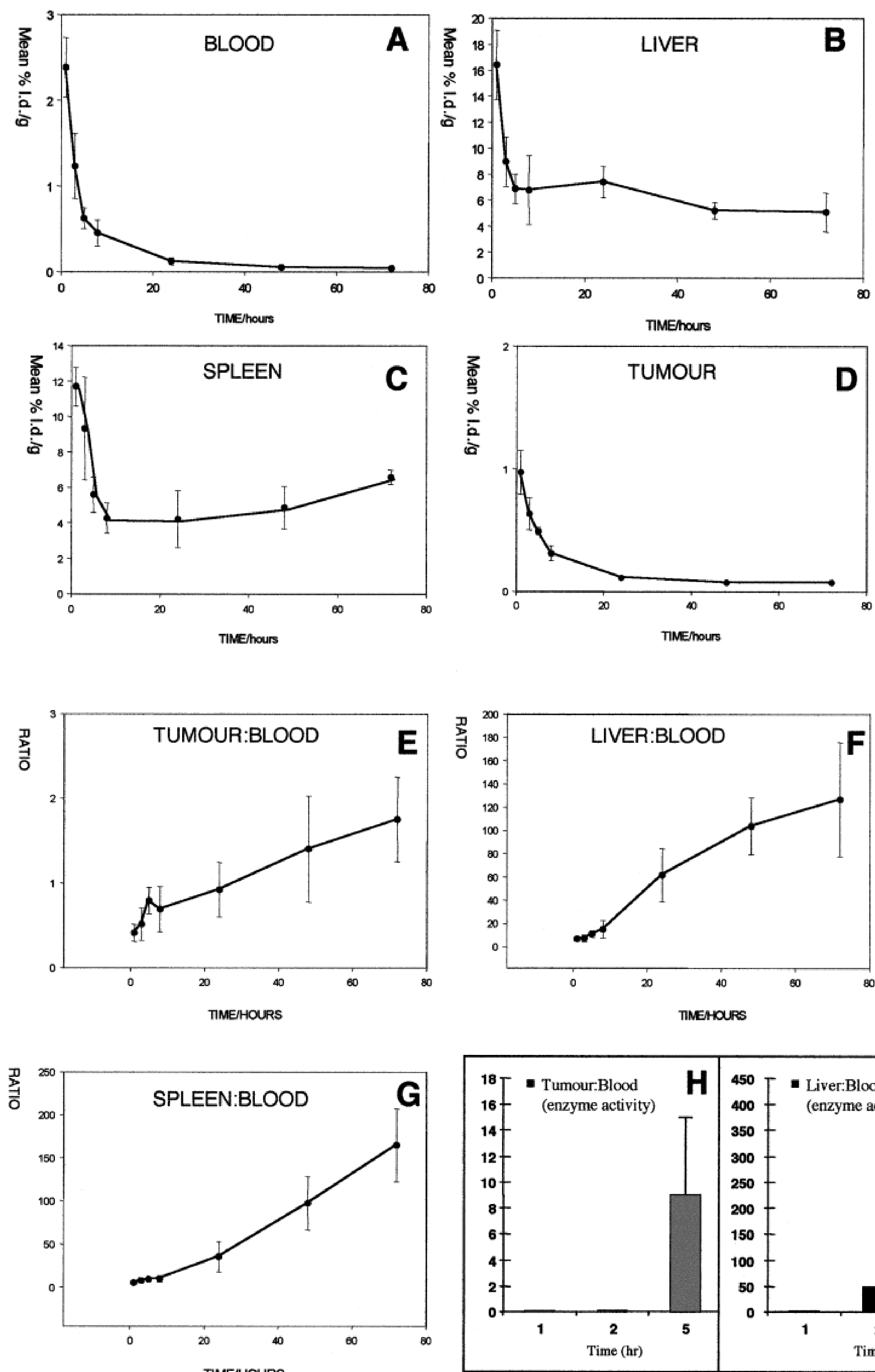

Figure 10 – (*a*) Blood clearance of ^{125}I-labelled scFv/linamarase fusion protein in non-tumour-bearing mice. (*b–d*) Tissue and tumour localisation of ^{125}I-labelled scFv/linamarase fusion protein in mice bearing tumour xenografts. (*e–g*) Tumour and tissue to blood ratios as determined by radioactive accumulation. (*h,i*) Tumour and liver to blood ratios as measured by enzyme cyanogenic activity.

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additions, a factor that was thought to be critical in expressing the scFv/Linamarase fusion protein. Expression was successful, with yields that were sufficient for characterisation. However, the molecule suffered from the same aggregational and activity problems as seen before.[15] The compromised catalytic potential of the enzyme was not due to its fusion with the antibody. Aggregation was suspected to be the result of improper folding during the production of the protein in yeast, resulting in the apparent multimerisation of the subunits. Posttranslational modifications, more specifically glycosylation differences between the yeast cells and cassava, were aspects that were thought to be responsible for the aggregation of the molecule (M.A. Hughes, unpublished results). With its catalytic ability markedly reduced compared with the wild-type linamarase, the fusion protein encountered obstacles when it was analysed in cell-killing experiments, but it still achieved significant and targeted cell killing by cyanide intoxication. This finding suggests that if the expression problems can be addressed and a more active enzyme produced, complete cell killing at lower doses of linamarin could be achieved. These experiments are currently in progress.

It has been seen by others that there is a balance between how fast a therapeutic molecule clears from the circulation *in vivo* (and thus how much accumulates in the tumour) and when the optimum time arises for drug administration (best tumour/normal organ ratios). *In vivo* targeting experiments have highlighted the severe limitations of the present molecule—the *Saccharomyces*-mediated glycosylation and aggregation. This resulted in its rapid clearance and high amounts of fusion protein in the liver and spleen. There was no accumulation in other tissues (data not shown). Against this background of high liver and spleen accumulation, there seemed to be some tumour targeting as measured by enzyme activity in the tumour compared with blood at 5 hr. This ratio (8), could be therapeutically useful and could lead to tumour destruction. However, with such a significant amount of cyanogenic activity in the liver and spleen, this would not be practical; hence there would not be an appropriate time to administer the prodrug (linamarin).

These limitations mean that the molecule described in its present state is not suitable for *in vivo* tumour ablation experiments. Alternative strategies such as "reshaping" the surface of the enzyme to have reduced aggregation properties and lower glycosylation or the use of alternative enzymes could result in a more favourable molecule.

The targeting moiety used in our system was a single-chain antibody fragment, termed MFE-23. It is a murine antibody selected from a phage display library[25] with high affinity for CEA and is found in tumours of colorectal, breast and lung origin.[26] Although the MFE-23 antibody is a suitable partner for vectoring a prodrug activating enzyme in AGENT or other prodrug activating systems, enzyme delivery can be achieved by a range of different specificity antibodies. One location that is thought to be sensitive to such a mode of treatment is the vasculature of tumours. Cyanide, the lethal drug resulting from linamarin hydrolysis, exerts its main effect on the respiratory pathway of cells, asphyxiating them to their death. Hence, well-aerated respiring cells lining tumour vascular tissue form ideal sites for targeted cyanogenesis as opposed to the hypoxic cells that carry our reduced aerobic respiration. In this instance, cytotoxicity results in the destruction of the blood supply and the starvation of the tumour. Therefore, this AGENT system, if fully developed, may work well with targeted angiogenesis.

Apoptosis and necrosis have long been considered as 2 distinct mechanisms of cell death, each with different biochemical, morphologic and functional characteristics.[27,28] The molecular changes that have been observed in the present study after cyanide intoxication suggest that cell death occurs via a necrotic pathway. Necrosis is characterised by extensive cell lysis occurring in the course of acute injury, in this case cell asphyxiation and inhibition of mitochondrial action and oxidative phosphorylation. It is accepted that high energy (ATP) levels are required for the execution of the apoptotic programme, whereas they are dissipated during necrosis.[29,30] ATP levels are suspected to drop dramatically after exposure to cyanide due to the inhibition of oxidative phosphorylation, below the threshold level essential for apoptosis; therefore necrotic cell death results. Another metabolic poison that affects the mitochondrial respiratory chain is antimycin A. Low doses of this drug result in apoptosis, higher doses lead to necrosis and intermediate doses result in a demise that shares features of apoptosis and necrosis.[31]

If cyanide intoxication had similar effects, then the relatively low amounts of the drug produced in the present study (0.1–0.2 mM at maximum doses of fusion protein and substrate; data not shown) should direct the cell into an apoptotic cascade. However, cell death analysis, as evaluated by LDH release, DNA degradation, nuclei staining and caspase-3 activity, indicated a truly necrotic death. Analysing the biologic method of cell death will allow us to develop strategies that may synergise with or complement our AGENT approach.

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