IN VITRO CYTOTOXICITY FOLLOWING SPECIFIC ACTIVATION OF AMYGDALIN BY β-GLUCOSIDASE CONJUGATED TO A BLADDER CANCER-ASSOCIATED MONOCLONAL ANTIBODY

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We describe a novel version of antibody-directed enzyme prodrug therapy (ADEPT), with the use of amygdalin as prodrug. Amygdalin is a naturally occurring cyanogenic glycoside, which can be cleaved by sweet almond β-glucosidase to yield free cyanide. If amygdalin could be activated specifically at the tumour site, then malignant cells would be killed without the systemic toxicity usually associated with chemotherapy. To this end, we have conjugated β-glucosidase to a tumour-associated monoclonal antibody (MAb) (HMFG1) and the conjugate has been tested *in vitro* for specificity and cytotoxicity subsequent to activation of amygdalin. Amygdalin was cytotoxic to HT1376 bladder cancer cells only at high concentrations, whereas the combination of amygdalin with HMFG1-β-glucosidase enhanced the cytotoxic effect of amygdalin by 36-fold. When 2 concentrations of HMFG1-β-glucosidase were compared, the toxic effect was dose dependent. The cytotoxicity of amygdalin was also enhanced by the MAb–enzyme conjugate even when the unbound conjugate was removed from the medium prior to exposure to amygdalin and the cells were washed. In addition to the cytotoxic effect, we also demonstrated specificity, using a MAb–enzyme conjugate that does not recognise the HT1376 bladder cancer cells. Finally, we studied the cytotoxic effect of the conjugate in co-culture of HMFG1-positive and -negative cell lines (HT1376 and U87MG cells). We demonstrated that the rate of surviving cells corresponds well to the percentage of U87MG (HMFG1-negative) cells in the flask. Our findings indicate that ADEPT is more effective than non-directed enzyme activation of a prodrug and can result in a non-toxic cancer therapy. *Int. J. Cancer* **78:712–719, 1998.**
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Bladder cancer is a common human malignancy, being the 5th most common cancer in men in Europe and North America, accounting for 2% of all human malignancies. The disease originates from the transitional epithelium of the urinary tract and is commonly papillary and multicentric. The tumour is usually controlled by transurethral resection, although recurrences occur in 80% of cases and 10% progress to a higher grade, invasive carcinoma with a poor prognosis (Newling, 1990). Intravesical administration of several chemotherapeutic and biological agents (thiotepa, epirubicin, mitomycin C, doxorubicin, bacillus Calmette-Guérin vaccine and interferon) has been used in addition to endoscopic resection as a means of reducing recurrence rate (Tolley *et al.*, 1996). There are some disadvantages to these agents, as absorption of the drugs from the bladder that may lead to systemic toxicity. Such local agents are not usually curative, indicating the need for novel therapeutic strategies.

The development of hybridoma technology and the resulting tumour-associated monoclonal antibodies (MAbs) has led to the possibility of treating cancers by exploiting the antigenic differences between malignant and normal cells. One concept currently under investigation is the use of antibodies to carry enzymes to tumour sites, where they activate a prodrug (Philpott *et al.*, 1973*a*, 1973*b*) and is known as antibody-directed enzyme prodrug therapy (ADEPT). In this process, tumour-associated antibodies, directed against tumour antigens, are linked to enzymes and administered systemically. A prodrug is then administered and converted by the enzyme into an active cytotoxic agent at the tumour site. The interval between the 2 administrations is optimised to achieve minimal systemic toxicity by accomplishing satisfactory accumulation of the conjugate in the tumour with clearance from blood and normal tissues. In addition, as the active drug diffuses throughout the tumour, it provides a bystander effect, killing antigen-negative cells.

This approach has the advantage of reduced toxicity, because the systemic effect is minimised by optimising the interval between the 2 steps. Theoretically, it can also overcome the problem of low absolute uptake of antibody by the tumour, because a single molecule of enzyme can activate numerous prodrug molecules. It is of particular interest that the chemotherapeutic agents generated at the surface of tumour cells are more tumouricidal than equivalent concentrations of circulating free drug (Haenseler *et al.*, 1992).

Several prodrug systems have been developed over the past few years. These systems exploit the properties of enzymes such as carboxypeptidase G2, which cleaves a glutamate moiety from benzoic acid mustard (Springer *et al.*, 1991); alkaline phosphatase, which activates phosphorylated derivatives of mitomycin, etoposide and doxorubicin (Senter *et al.*, 1988); and the penicillin-V amidase, which can be used to hydrolyse the phenoxyacetamide group of doxorubicin- and melphalan-amide derivatives to release doxorubicin and melphalan, respectively (Kerr *et al.*, 1990).

However, all the above prodrug systems use established chemotherapeutic agents and drug resistance may be an issue (Baldini, 1997). Therefore, the development of a prodrug system that activates an agent with a different mode of action and without any known resistance would be highly desirable. Ideally, such an enzyme system should not have a human homologue with similar activity, in order to minimise prodrug activation outside the tumour site and reduce systemic toxicity.

Recently, a new prodrug system, known as antibody-guided enzyme nitrile therapy (AGENT), has been developed. This system exploits the activation of amygdalin, a naturally occurring cyanogenic glucoside, instead of using a modified chemotherapeutic agent. The hydrolysis of amygdalin by the enzyme β-glucosidase results in the release of the powerful metabolic poison cyanide (Rowlinson-Busza *et al.*, 1993).

In the present study, we have investigated the efficacy of this prodrug activation system *in vitro*, examining the activity, the cytotoxic effect and the specificity of this enzymatic system under physiological conditions in a bladder cancer cell line.

MATERIAL AND METHODS

Material

Amygdalin, [(6-*O*-β-D-glucopyranosyl-β- D-glucopyranosyl) oxy]benzene acetonitrile, a 457-Da naturally occurring cyanogenic glycoside, was purchased from Sigma (Poole, UK). The enzyme β-glucosidase (m.w. = 65.000) catalyses the removal of the 2

glucose residues of amygdalin to yield benzaldehyde and cyanide. It was purified from sweet almonds (EC 3.2.1.21) and purchased from Sigma.

Antibodies

All antibodies used throughout this study were supplied by the Imperial Cancer Research Fund (London, UK).

*HMFG1.*This IgG$_1$ mouse MAb was raised against a high m.w. (>400,000) glycoprotein, polymorphic epithelial mucin (PEM) (Taylor-Papadimitriou *et al.*, 1981), which is overexpressed and aberrantly glycosylated in tumours compared with normal tissues. HMFG1 binds to this aberrantly glycosylated mucin and reacts strongly with a wide range of human carcinomas (Arklie *et al.*, 1981).

*H17E2.*This is an IgG$_1$ antibody raised in mice immunised with human term placental plasma membranes and recognises human placental alkaline phosphatase (PLAP) as well as the leucine-inhibitable form of the enzyme found at low levels in the healthy testis (Travers and Bodner, 1984). H17E2 reacts strongly with normal testis as well as with testicular, ovarian and endometrial carcinomas (Epenetos *et al.*, 1987).

Tumour cell lines

*HT1376.*The HT1376 cell line was obtained from a transurethral resection of invasive, moderately pleomorphic (Grade III) transitional cell carcinoma of a female Caucasian patient. The patient had not received chemotherapy or radiotherapy prior to the removal of the tumour. The patient had also not been exposed to environmental or occupational carcinogens (Rasheed *et al.*, 1977). The HT1376 cells were shown by immunostaining and enzyme-linked immunosorbent assay (ELISA) to bind the anti-PEM HMFG1 antibody.

*U87MG.*The U87MG cell line was derived from a human glioblastoma tumour (Fogh *et al.*, 1977). As this cell line does not express the PEM, it was used as a negative control of the HMFG1-β-glucosidase conjugate.

*KB.*The KB cell line was derived from an epidermoid carcinoma involving the floor of the mouth of a male Caucasian patient (Eagle, 1955). These cells have been shown to secrete 19.9 U/ml of PLAP into the tissue culture medium in which they grow. They also express the antigen on the cell surface. The KB cells were shown by immunostaining and ELISA to bind the H17E2 antibody and were used to test the H17E2-β-glucosidase conjugate.

Conjugation of the MAb and the enzyme

Derivatisation of β*-glucosidase.*A method similar to that described by Searle *et al.* (1986) was used. β-Glucosidase was dissolved at 5 mg/ml in 0.05 M sodium phosphate, containing 1 mM ethylenediamine tetraacetic acid (EDTA). To block any free thiol groups, *N*-ethylmaleimide (Sigma) was added at 0.2 mg/ml for 1 hr, at room temperature under constant rotation. The heterobifunctional cross-linker m-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (Sulfo-MBS; Pierce, Rockford, IL) was dissolved at 10 mg/ml in the same buffer and added to β-glucosidase at a molar ratio of 40:1 MBS:enzyme and reacted for 1 hr. At this molar ratio, we have previously determined that 1–2 MBS molecules are incorporated per Ab molecule (Bamias, 1993). The incubation was carried out at room temperature, under constant rotation. Free *N*-ethylmaleimide and sulfo-MBS were separated by size exclusion chromatography, using a 50-ml Sephadex G25 column (Pharmacia, Uppsala, Sweden). Collection of enzyme-containing fractions was performed by monitoring absorbance at 280 nm. The protein-containing peak was collected. The derivatised enzyme was kept at 40°C under nitrogen, until used.

*Thiolation of monoclonal antibodies.*A modification of the method described by Jue *et al.* (1978) was used. 2-Iminothiolane (2IT; Sigma) was used as the free thiol group donor. The reaction conditions consisted of 2IT:antibody molar ratios of 50:1 (Bamias, 1993), and incubation for 1 hr, in 0.1 M triethanolamine buffer, pH 8.0, containing 1 mM of EDTA, at room temperature under constant rotation. The buffer was thoroughly de-gassed with helium to avoid oxidation at the thiol groups and cross-linking of antibodies. Free 2IT were separated from thiolated antibodies by size exclusion chromatography, using a 50-ml Sephadex G25 column. The thiolation resulted in 1.5–2 thiol groups/antibody molecule.

*Conjugate production and purification.*Thiolated antibody, after being separated from free 2IT using a 50-ml G25 Sephadex column, was eluted with conjugation buffer and was run directly into the β-glucosidase-MBS solution, to avoid oxidation. The amount of antibody used in the reaction was half the amount of enzyme used on a weight basis. This results in a 4:1 enzyme: antibody molar ratio. The reaction mixture was concentrated under nitrogen until the protein concentration was 5 mg/ml. The 2 proteins were reacted for 2 hr at room temperature under rotation.

The conjugate was separated from unreacted proteins by size exclusion chromatography, using a S-300 Sephacryl column (Pharmacia, Milton Keynes, UK). Phosphate-buffered saline (PBS) was used as the elution buffer and selection of conjugate-containing fractions was performed by estimating enzyme and antibody activity in each fraction.

Testing of the fractions for enzyme and antibody activity

Colorimetric assays were used in order to estimate enzyme activity in the S-300 fractions collected. 2-Nitrophenyl-glucopyranoside (ONPG, Sigma) was used as the chromogenic substrate. In the presence of β-glucosidase, ONPG is hydrolysed to 2-nitrophenol (ONP), which has a yellow colour in solution. ONPG was diluted in 0.05-M acetate buffer, pH 6.0, to 40 mM. Fifty microlitres of a sample of each S-300 fraction were reacted with 50 μl of the substrate for 10 min at room temperature in 96-well plates. The reaction was stopped by adding 0.2-M sodium bicarbonate, pH 12. The absorbance of the product was measured at 405 nm in a Titertek Multiscan MCC/340 plate reader (Flow, Helsinki, Finland). Unconjugated β-glucosidase was used as a standard. The activity of the native enzyme ranged from 22 to 35 U/mg, depending on the batch used in each experiment and was determined by the manufacturer. The activity of the sample was found by interpolating the absorbance at 405 nm on the standard curve and was expressed as units of β-glucosidase activity/ml.

Antibody activity was estimated using either antigen-coated plates, or cell-coated plates. For testing of HMFG1-β-glucosidase, plates were coated with a peptide containing the sequence Pro-Asp-Thr-Arg-Pro (PDTRP), which is the epitope on PEM recognised by the antibody HMFG1 (Nishimori *et al.*, 1994), diluted in 1% bovine serum albumin (BSA) in PBS. For testing of the H17E2-β-glucosidase, plates were coated with KB cells. Each fraction was tested in duplicate. Incubation lasted for 1 hr at room temperature. The rest of the assay was performed as in ELISA (see below).

Although the above methods demonstrated that some S-300 fractions have both antibody and β-glucosidase activity, this finding does not prove in any way that antibody-β-glucosidase conjugate is present in these fractions, because a mixture of free antibody and enzyme in these fractions could yield the same results. Therefore, a method was developed to test both activities simultaneously: Antigen-coated plates were incubated with 100 ml from each S-300 fraction for 1 hr at room temperature. Only the fractions with antibody activity would react with the antigen and be immobilised on the solid phase. The plate was washed with PBS/Tween and then ONPG solution, in 0.05-M acetate buffer, pH 6.0, was added as chromogenic substrate. From the molecules bound to the solid phase (conjugate and free antibody), only those with dual activity would produce the yellow colour. The reaction was stopped 10 min later, by adding 0.2-M sodium bicarbonate, pH 12. The absorbance of the product was measured at 405 nm.

β-Glucosidase assay

A colorimetric assay was used to estimate enzyme activity of the MAb-β-glucosidase conjugate, using the method outlined above.

ELISA

The immunoreactivity of the conjugate was compared with that of the native antibody by ELISA using, as antigen, HT1376 cells fixed with glutaraldehyde to 96-well plates.

Tissue culture

All tumour cell lines were cultured in RPMI 1640 medium containing 100 U/ml penicillin and 100 mg/ml streptomycin and supplemented with 10% foetal calf serum (FCS) (GIBCO, Paisley, UK). Cells were grown at 37°C, in a humidified atmosphere of 5% CO_2 in air. Cells were subdivided 1:4 twice weekly until there were sufficient numbers for experiment. Harvesting of the cells was achieved by incubating them with 5 ml of a mixture of 0.02% EDTA:trypsin (3:1) for 5 min at 37°C, until most of the cells were detached from the culture flasks. At the end of the incubation, 5 ml of culture medium were added to stop the reaction and the cells were centrifuged at 180 *g* in a Centaur 2 bench centrifuge (MSE, Loughborough, UK) for 5 min. A single-cell suspension was obtained by repeated pipetting on resuspension in 10 ml of medium and cells were counted using a haemocytometer.

Culture medium, as well as 0.02% EDTA and trypsin were supplied by the Media production Unit, Imperial Cancer Research Fund, Clare Hall (London, UK).

Cytotoxicity studies

Simultaneous incubation of enzyme and prodrug. HT1376 cells growing in culture were harvested and seeded at 5×10^5 cells/flask into 25 cm² Falcon flasks and were allowed to grow for 24 hr. The cells were then incubated in quadruplicate, for 24 hr, with one of the following agents, at various concentrations:

    (i) Potassium cyanide alone;
   (ii) Amygdalin alone;

  (iii) Amygdalin plus unconjugated β-glucosidase;
  (iv) Amygdalin plus HMFG1-β-glucosidase conjugate.

When enzyme or MAb–enzyme conjugate was used, the enzyme activity was the same in both preparations. After 24-hr exposure, the medium containing the drug was discarded and the flasks were washed 3 times with culture medium and were allowed to grow for a further 24 hr with fresh medium. Cells were then harvested and counted using a haemocytometer. Trypan blue exclusion was used as the criterion of cell integrity. Cells incubated without any agent were used as controls (4 flasks). Results were expressed as treated/control cell survival ratios. The IC_{50} was defined as the concentration of the cytotoxic agent required for the reduction of viable cells to 50% of that of the controls.

Consecutive exposure to the enzyme and the prodrug. Cells growing in culture were seeded in 25 cm² flasks (5×10^5 cells/flask) and were allowed to grow for 24 hr. They were then incubated for 2 hr with one of the following agents:

    (i) HMFG1 alone;
   (ii) β-Glucosidase alone;
  (iii) HMFG1-β-glucosidase alone;
  (iv) H17E2-β-glucosidase alone, as non-specific conjugate;
   (v) Unconjugated HMFG1 and β-glucosidase.

After incubation with these agents, cells were washed 3 times with culture medium and then amygdalin diluted in culture medium was added at the highest concentration that, according to the previous experiment, had proved to be non-toxic (10 mM). Cells were allowed to grow for a further 24 hr, then were washed 3 times with culture medium and were allowed to grow for a further 24 hr. Cells
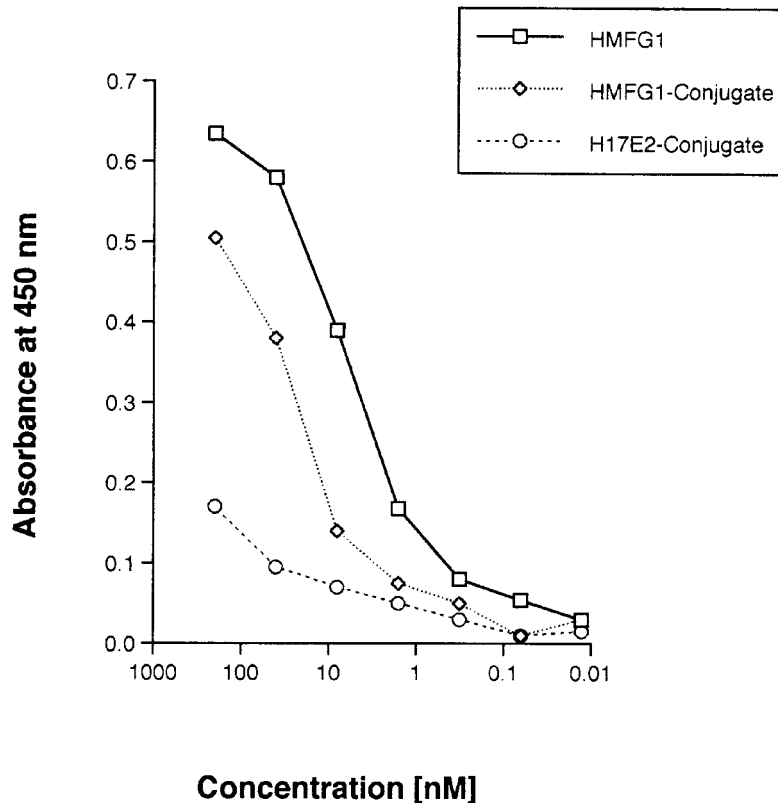

FIGURE 1 – Enzyme-linked immunosorbent assay (ELISA) showing the immunoreactivity of the native HMFG1, the HMFG1–β-glucosidase conjugate and the H17E2–β-glucosidase conjugate against immobilised HT1376 bladder cancer cells.
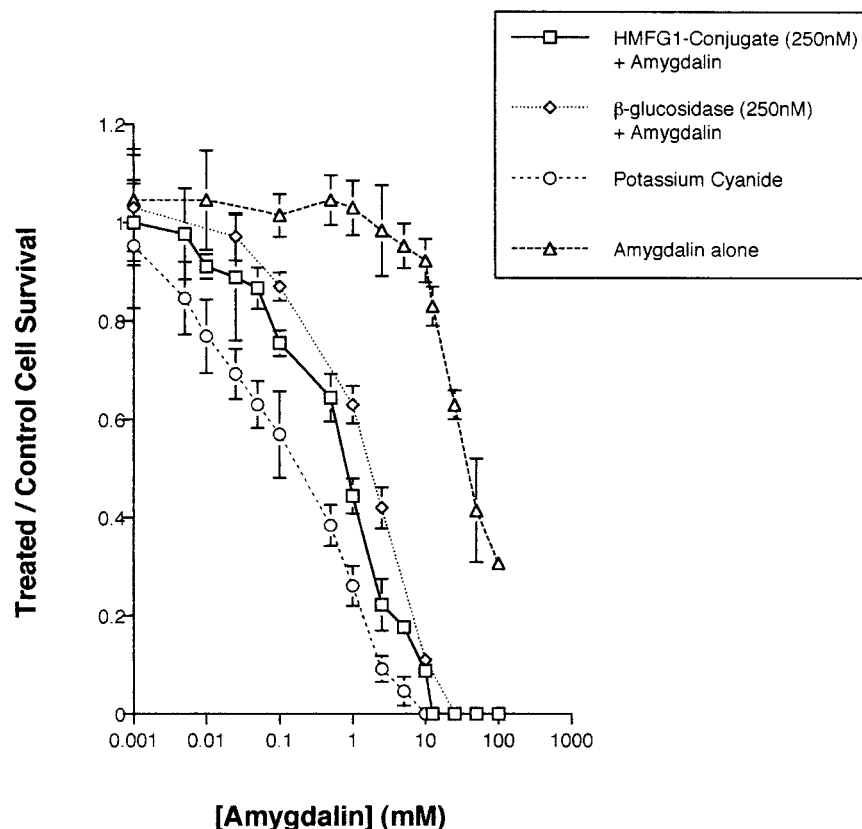
FIGURE 2 – Cytotoxicity expressed as treated/control cell survival of HT1376 cells exposed to potassium cyanide alone, amygdalin alone, amygdalin plus unconjugated β-glucosidase (250 nM) and amygdalin plus HMFG1–β-glucosidase conjugate (250 nM).

were harvested, counted and analysed as already described. Each concentration was tested in quadruplicate.

*Cytotoxicity studies on co-cultured cells consecutively exposed to the enzyme and the prodrug.*Two cell lines were grown in the same culture flask, as an *in vitro* model of the bladder environment, where malignant cells co-exist with the normal urothelium. These cell lines were the HT1376 bladder cancer cell line (which is HMFG1 positive) and the HMFG1-negative glioma cell line U87MG. These cell lines were chosen because they have similar growth rates, as shown in a previous experiment. In the 25-ml flask, the 2 cell lines were mixed: HT1376/U87MG at a ratio of 100%/0%, 90%/10%, 70%/30%, 50%/50%, 30%/70%, 10%/90% and 0%/100%. Cells were allowed to grow for 3 days to form distinct, non-overlapping colonies. Cells were then incubated with HMFG1-β-glucosidase for 2 hr. Cells were then washed 3 times with culture medium and amygdalin diluted in culture medium was added at the highest concentration that, according to the previous experiment, had proved to be non-toxic (10 mM). Cells were allowed to grow for a further 24 hr, were washed 3 times with culture medium and were allowed to grow for a further 24 hr. Cells were harvested, counted and the mean values were analysed statistically. The experiment was carried out in quadruplicate. Cells incubated with no agent were used as controls (4 flasks).

The aim of the above experiment was to test the selectivity of the HMFG1-β-glucosidase conjugate.

Statistical analysis

Differences between means were analysed using the one-way analysis of variance (ANOVA), $p < 0.05$ being considered significant.

RESULTS

The conjugate was successfully constructed and tested for dual biological activities (antibody and enzyme activity). The fractions of S-300 that were collected contained conjugate with one enzyme molecule per antibody molecule. The m.w. of the proteins in the preparation was 215 kDa, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Native HMFG1 and HMFG1-β-glucosidase immunoreactivity on the cell line HT1376 was tested by the use of ELISA plates. We found that the affinity of the conjugate was similar to that of native HMFG1, although there was slight loss of immunoreactivity ($K_d = $ 17.6 nM *vs.* 4.2 nM). The H17E2-β-glucosidase conjugate, on the contrary, showed a very poor affinity for the HT1376 cells and therefore could be used as negative control (Fig. 1).

An HMFG1-negative cell line was used to examine the specificity of the cytotoxic effect of the HMFG1-β-glucosidase conjugate. The U87MG cell line was checked for the expression of PEM, by an ELISA. It was shown that U87MG glioma cell line is HMFG1 and HMFG1-β-glucosidase negative and therefore could be used as a control cell line.

Cytotoxicity studies with simultaneous exposure of the cells to the enzyme and the prodrug

Figure 2 shows the results of the cytotoxicity against HT1376 cells, when exposed to potassium cyanide, amygdalin alone, amygdalin plus β-glucosidase or amygdalin plus HMFG1-β-glucosidase conjugate. The cells were found to be sensitive to potassium cyanide in concentrations as low as 0.01 mM, with an IC_{50} of 0.2 mM. Amygdalin was cytotoxic to HT1376 cells only at high concentrations, with an IC_{50} of 40.4 mM. When β-glucosidase was present at a concentration of 250 nM, the cytotoxicity of
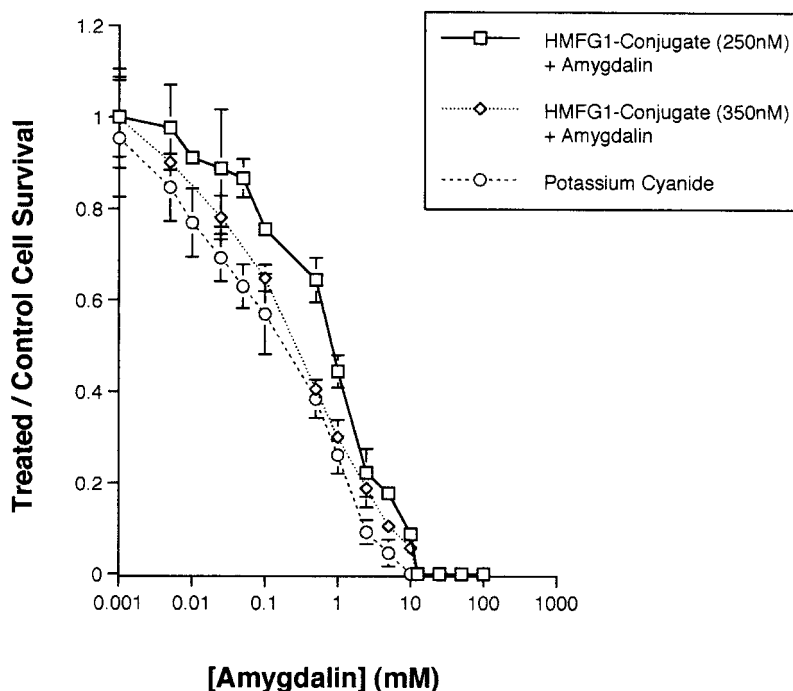
FIGURE 3 – Cytotoxicity expressed as treated/control cell survival of HT1376 cells exposed to potassium cyanide alone, amygdalin alone, amygdalin plus HMFG1–β-glucosidase (250 nM) and amygdalin plus HMFG1–β-glucosidase conjugate (350 nM). The IC_{50} values were 1.1 mM and 0.9 mM, respectively.

amygdalin was increased by 25-fold and the IC_{50} was found to be 1.6 mM. The combination of amygdalin with 250 nM HMFG1-β-glucosidase enhanced the cytotoxic effect of amygdalin by 36-fold (IC_{50} 1.1 mM), ($p < 0.001$, compared with amygdalin alone), suggesting that conjugated β-glucosidase retains its capacity of hydrolysing amygdalin, resulting in the production of cyanide, under physiological conditions of temperature and pH. This combination was more effective than the combination of amygdalin with unconjugated enzyme (IC_{50} 1.1 mM *vs.* 1.6 mM), indicating the specific character of this agent (HMFG1-β-glucosidase) for HT1376 cells.

When 2 concentrations of HMFG1-β-glucosidase were compared (250 nM and 350 nM), the toxic effect was greater at the higher concentration, indicating a dose-dependent effect (Fig. 3). In all cases, potassium cyanide was more effective, although the IC_{50} of potassium cyanide and of HMFG1-β-glucosidase at a dose of 350 nM were similar ($IC_{50} = 0.2$ mM and 0.35 mM, respectively) (Fig. 3). The molarity of potassium cyanide corresponds well to the molarity of amygdalin because the hydrolysis of 1 molecule of amygdalin results in the production of 1 molecule of cyanide (Schmidt *et al.*, 1978).

Cytotoxicity studies with consecutive exposure of the cells to the enzyme and the prodrug

Figure 4 demonstrates the cytotoxicity of HT1376 cells after incubation with HMFG1, β-glucosidase, HMFG1-β-glucosidase, H17E2-β-glucosidase (as non-specific conjugate) and unconjugated HMFG1 plus β-glucosidase. Cells were washed before addition of 10 mM amygdalin (Fig. 2).

When the enzyme or unbound conjugates were removed from the medium prior to exposure to amygdalin, and the cells were washed with culture medium, HMFG1- β-glucosidase enhanced the cytotoxicity of amygdalin, whereas amygdalin following incubation with β-glucosidase, HMFG1 alone and the combination

of unconjugated HMFG1 and β-glucosidase did not have any effect. Pre-incubation of cells with H17E2-β-glucosidase a high concentrations also had some effect on the toxicity of amygdalin, although the difference was not statistically significant (Fig. 4).

Specificity of the MAb-β-glucosidase-conjugate

Figure 5 shows the cytotoxic effect of the HMFG1-β-glucosidase conjugate in co-culture of the HT1376 and U87MG cells. The cytotoxicity of amygdalin on the HT1376/U87MG co-culture exposed to the HMFG1-β-glucosidase conjugate decreased as the proportion of HT1376 cells in the incubation mixture was reduced. The figure also demonstrates that the rate of surviving cells corresponds well to the percentage of U87MG cells (HMFG1-negative) in the flask. This finding provides evidence that when the flask was washed with medium, there was enough HMFG1-β-glucosidase conjugate bound to the HT1376 cells to exert a cytotoxic effect when amygdalin was added. Enzyme activity and the cyanide release were restricted mainly in the area of the HT1376 cells, leaving most of the U87MG cells unaffected.

When U87MG cells were grown alone in the flasks, there was a small cytotoxic effect of amygdalin that was not statistically significant. This finding could be attributed to non-specific binding of the HMFG1-β-glucosidase conjugate to the U87MG cells. The ratio of surviving cells of both cell lines in each flask to the cells alive in the flask with U87MG alone is shown in Figure 6. This figure demonstrates the linear correlation between the percentage of cells alive and the percentage of U87MG cells originally placed in each flask.

DISCUSSION

We have constructed a stable MAb–β-glucosidase conjugate, which retains dual biological activities, and we investigated its
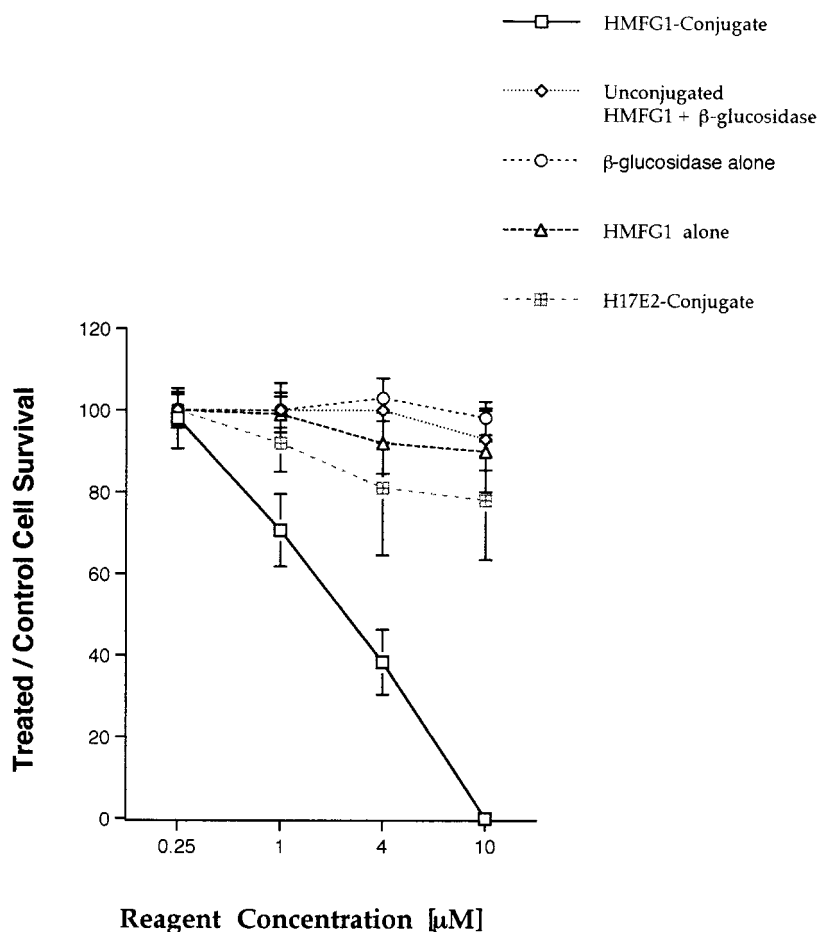
FIGURE 4 – Cytotoxicity expressed as treated/control cell survival of HT1376 cells exposed to HMFG1–β-glucosidase, unconjugated HMFG1 and β-glucosidase, β-glucosidase, HMFG1 and H17E2–β-glucosidase for 2 hr. Cells were then washed to remove unbound enzyme activity and incubated with 10 mM amygdalin.

therapeutic potential as part of a prodrug activation system. The conjugate retained dual biological activity, was stable *in vitro* and capable of delivering β-glucosidase to the surface of tumour cells growing in culture.

The results of the cytotoxicity studies demonstrate that amygdalin alone is virtually non-toxic, having some cell killing effect only at high concentrations (>10 mM). The enzyme β-glucosidase is also non-toxic, when amygdalin is not present. When amygdalin is exposed to the enzyme β-glucosidase, either in its free form or conjugated to an antibody, then its cytotoxicity is considerably increased, by 25- to 40-fold. It has also been shown that conjugated, as well as unconjugated, β-glucosidase can catalyse the hydrolysis of amygdalin under physiological conditions of temperature and pH. The above findings suggest that the amygdalin-β-glucosidase system is suitable for *in vivo* prodrug activation strategies.

The MAb–enzyme conjugate was non-toxic in the absence of amygdalin, suggesting that sufficient amounts of the conjugate could be administered *in vivo*, to achieve the desired concentration at the tumour sites, without the risk of significant toxicity. Our findings are of particular interest because, if confirmed by further studies, they indicate a dose-dependent effect of the β-glucosidase immunoconjugate. This observation is also in accordance with other prodrug activation systems (Kerr *et al.*, 1990).

In our current study, amygdalin was found to be significantly more toxic *in vitro* in combination with the specific antibody–enzyme conjugate than with the unconjugated enzyme at the same concentration. This finding could be explained by the fact that, as the MAb–enzyme conjugate is bound to the bladder cancer cells, most of the cyanide is released in the vicinity of the tumour cell and is therefore readily available to exert its lethal action. This observation suggests that one molecule of enzyme, once bound at the tumour site, can catalyse more than one molecule of amygdalin, and this action results in an amplification of effect. This observation also suggests that the combination of amygdalin and the enzyme immunoconjugate could overcome the problems caused by antigen heterogeneity, by a bystander effect of the cyanide released. The above finding is of particular interest and has potential clinical value, because it has been shown that the drug generated at the surface of tumour cells is more effective than equivalent concentrations of free drug (Haenseler *et al.*, 1992). The need for internalisation is a factor that has hampered the efficiency of the antibody–drug immunoconjugates in the past. However, we have shown that the MAb–β-glucosidase conjugate can achieve its cytotoxic effect, in the presence of amygdalin, because the cyanide generated at the cell surface diffuses through the cell membrane, causing cell death.

The selectivity of the HMFG1–β-glucosidase conjugate/amygdalin combination was investigated by a co-culture experiment, in which HT1376 cells were mixed in various ratios with U87MG cells, which are HMFG1 negative. This system is an *in vitro* tumour model, in which clusters of antigen-positive tumour cells exist within antigen-negative environment of normal cells. Because the morphology of the HT1376 and U87MG cells after
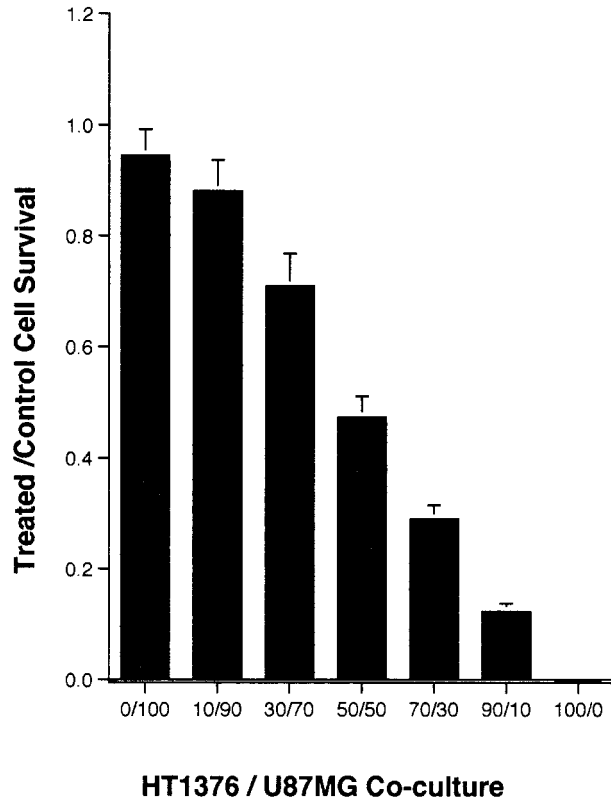
FIGURE 6 – Cytotoxicity expressed as combined/control cell survival of HT1376 and U87 cells. Flasks containing U87 cell alone were used as controls. All flasks were exposed to HMFG1–β-glucosidase for 2 hr. Cells were then washed to remove unbound enzyme activity and incubated with 10 mM amygdalin.

HT1376 / U87MG Co-culture

FIGURE 5 – Cytotoxicity expressed as treated/control cell survival of combined HT1376 and U87 cells exposed to HMFG1–β-glucosidase for 2 hr. Cells were then washed to remove unbound enzyme activity and incubated with 10 mM amygdalin.

trypsinisation is identical, the specificity of the killing can be assessed only indirectly, by estimating the percentage of cells alive. According to our results, the cytotoxicity of the conjugate was proportional to the percentage of HMFG1-positive cells in the co-culture. These observations support the specificity of ADEPT. The above data suggest that there was no significant killing of HMFG1-negative U87MG cell colonies, as a result of generation of cyanide from conjugate bound to the HT1376 cell colonies. However, the 2 cell types in this experiment grew as separate clusters, as would occur in bladder cancer, in which the malignant cells grow as discrete tumour masses within the normal urothelium. The cyanide is generated at high concentration in the colonies of antigen-positive cells, resulting in cell killing within these clusters. On the contrary, the cyanide concentration in the antigen-negative colonies would be low, because none is generated there, but only diffuses from the sites where the conjugated enzyme is bound. Figure 2 shows very steep concentration dependence for cyanide toxicity. This finding explains the survival of the antigen-negative clusters (specificity effect), whereas antigen-negative single HT1376
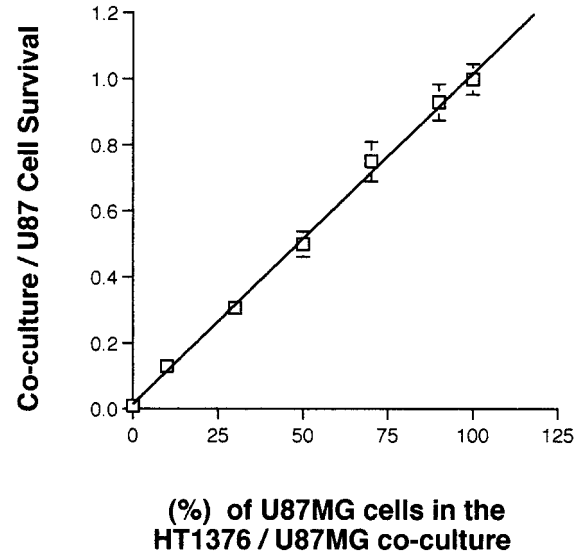
cells within the bladder tumour colonies would be killed by the high local cyanide concentration (bystander effect).

Non-specific immunoconjugate produced some cytotoxic effect, which was sufficient only at the highest concentration (10 μM). This finding could be attributed to non-specific binding of the H17E2–β-glucosidase conjugate on the surface of the HT1376 bladder cancer cells. The cytotoxic effect against the HMFG1-negative cell line U87MG by the HMFG1–β-glucosidase conjugate was not statistically significant. This finding could also be explained by the non-specific binding of the HMFG1–β-glucosidase conjugate on the surface of the U87MG cells. *In vivo* toxicity should therefore be minimal and could be eliminated by modifying the time between the administrations of the MAb–enzyme conjugate and the prodrug.

In conclusion, our studies suggest that, under physiological conditions of temperature and pH, the amygdalin-β-glucosidase system is suitable for *in vivo* prodrug testing. The combination of amygdalin with a specific MAb–β-glucosidase conjugate increased the activity of amygdalin up to 115-fold in a dose-dependent fashion. The amygdalin–MAb–β-glucosidase conjugate system has the additional advantage of specificity, as compared with conventional chemotherapy. Our results form the basis for animal and preclinical studies to evaluate this system. They also suggest that intravesical administration of the MAb–β-glucosidase conjugate, followed by washing of the bladder and subsequent instillation of amygdalin, should be able to achieve the concentrations of prodrug and enzyme required for tumour therapy.

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