Cancer Immunol Immunother (2004) 53: 411–421
DOI 10.1007/s00262-003-0457-9

ORIGINAL ARTICLE

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Cytotoxic properties of immunoconjugates containing melittin-like peptide 101 against prostate cancer: in vitro and in vivo studies

Received: 7 July 2003 / Accepted: 9 September 2003 / Published online: 13 January 2004
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Abstract *Background*: Monoclonal antibodies (MAbs) can target therapy to tumours while minimising normal tissue exposure. Efficacy of immunoconjugates containing peptide 101, designed around the first 22 amino acids of bee venom, melittin, to maintain the amphipathic helix, to enhance water solubility, and to increase hemolytic activity, was assessed in nude mice bearing subcutaneous human prostate cancer xenografts.
Methods: Mouse MAbs, J591 and BLCA-38, which recognise human prostate cancer cells, were cross-linked to peptide 101 using SPDP. Tumour-bearing mice were used to compare biodistributions of radiolabeled immunoconjugates and MAb, or received multiple sequential injections of immunoconjugates. Therapeutic efficacy was assessed by delay in tumour growth and increased mouse survival.
Results: Radiolabeled immunoconjugates and antibodies showed similar xenograft tropism. Systemic or intratumoural injection of immunoconjugates inhibited tumour growth in mice relative to carrier alone, unconjugated antibody and nonspecific antibody-peptide conjugates and improved survival for treated mice.
Conclusions: Immunoconjugates deliver beneficial effects; further peptide modifications may increase cytotoxicity.

Keywords Melittin peptides · Monoclonal antibody targeted therapy · Prostate cancer · DU-145 cells · LNCaP-LN3 cells

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Introduction

Prostate cancer (CaP), the second highest cause of cancer mortality in men in Western society, is heterogeneous. Early disease is treatable, but once the cancer has spread, hormonal ablation therapy is limited by the development of androgen-independent CaP. New treatment strategies are needed. Monoclonal antibodies (MAbs) against prostate tissue or tumours may be used in early detection, imaging and—by conjugation with toxins, drugs or radionuclides—treatment [4]. Selective concentration of MAbs in tumour tissue allows immunoconjugates to deliver killing agents to cancers, while minimising normal tissue exposure. These therapies may be limited if cellular internalisation is required for cell killing. In this study, we have determined the efficacy of anti-CaP immunoconjugates containing a synthetic analogue of Melittin, peptide 101, that does not require internalisation for activity.

Melittins, a family of peptides from honey bee (*Apis mellifera*) venom, have been studied as potential toxins, and progress has been made in defining structural features essential for cytolytic and cytotoxic activity [10, 19, 25]. The major melittin species, a 26 amino acid peptide, forms an amphipathic helix with a highly charged carboxyl terminus [10]. We have previously shown that the

carboxyl terminal residues are not essential for activity, provided that the molecule remains positively charged [25]. While native melittin contains a highly cationic C-terminus that is immunogenic and potentially allergenic, modified peptide 101 has a truncated C-terminus devoid of this region [19]. Melittin and its analogues have properties advantageous for their use as toxins in immunoconjugates. These are (1) low toxicity of the peptides, limiting undesirable side effects; (2) their mode of action via interaction of the peptides with the plasma membrane that is independent of internalisation [8, 23, 25]; (3) the fact that they are cytolytic, not cytostatic, and their half-lives are limited in vivo by proteolysis, limiting toxicity of peptide released from its carrier. Tumour-specific antibody-melittin conjugates have been shown to selectively kill tumour cells in vitro [6].

We have previously shown that two MAbs against CaP, BLCA-38 and J591, have excellent properties for in vivo targeting, localising to DU-145 and LNCaP-LN3 human CaP xenografts, respectively, with no unusual localisation in nontumour tissues (Carter et al., submitted). BLCA-38 is an IgG_1 murine MAb [24] raised against the human bladder cancer cell line, UCRU-BL-17CL, that detects a cell surface glycoprotein. A single intraperitoneal (i.p.) dose samarium 153–labeled BLCA-38 provided sustained growth delay of well-established subcutaneous (s.c.) bladder cancer xenografts [14]. BLCA-38 also binds to most human CaP cell lines and CaP biopsy samples (Russell et al., submitted). J591, an IgG_1 MAb raised against LNCaP cells, binds to two extracellular epitopes of prostate-specific membrane antigen (PSMA), now known as folate hydrolase 1 (FOLH1) [11], that was identified as a membrane antigen on LNCaP CaP cells [9]. Its expression is higher on CaP cells [22] than on normal prostate [2] and is upregulated during androgen deprivation therapy [27]. J591 is internalised after binding [15]. Labeled-MAbs to FOLH1 (internal domain) (ProstaScint; Cytogen, Princeton, NJ) can image occult CaP in men [7], and recent data suggest that MAbs to extracellular epitopes, like J591, are clinically more useful in vivo than those against internal domains [20].

J591 and BLCA38 were conjugated with peptide 101, using N-succinimidyl 3-(2-pyridyldithio) propionate, SPDP, which cross-linked activated amino-functionalities on the antibody to carboxyl terminal cysteine on the peptide. The peptide sequence was designed to maintain the amphipathic helix with enhanced water solubility and increased haemolytic activity. In vitro, these immunoconjugates showed nonspecific binding to target and nontarget cells, possibly due to the affinity of the amphipathic helices of the peptides for cell membranes. Cytotoxicity studies showed that the extent of killing of cultured cell lines was equivalent to the molar peptide content of the conjugates. Killing was nonspecific, affecting antigen-positive and antigen-negative cells similarly, when differential sensitivities of the cell lines to free peptide were considered. This may reflect the geometry of in vitro testing (monolayer cultures), where the peptide can access cells regardless of the antibody moiety. We wished to ascertain the therapeutic efficacy of immunoconjugates of J591 and BLCA-38 with peptide 101 in vivo. J591 binds to LNCaP cells and shows increased binding to the LNCaP-LN3 derivative (abbreviated LN3) [18] that was used in our experiments. BLCA-38 binds to PC-3 and DU-145 cells (Russell et al., submitted), but as DU-145 cells form more vascular xenografts, they were selected as the target in vivo.

Materials and methods

Cell lines

LNCaP-LN3 (abbreviated LN3) cells were from C. Pettaway (MD Anderson Cancer Center, Houston, TX) and DU-145 cells from American Type Culture Collection (ATCC, Rockville, MD). C3 cells, a clonal subline of UCRU-BL-17/2, a human bladder cancer cell line [3], do not express the BLCA-38 antigen (unpublished data). SKMEL (human melanoma) and L929 (mouse leukaemia cells) provided negative controls for J591 MAb. Cell lines were maintained in RPMI 1640 medium (Gibco BRL Life Technologies, Rockville, MD), with 10% fetal bovine serum (FBS; Trace Biosciences), penicillin (50 U/ml), streptomycin (0.05 mg/ml) and 0.2-mM L-glutamine (Gibco BRL Life Technologies, Rockville, MD) at 37°C in 5% CO_2 and passaged with trypsin / ethylenediamine tetra-acetic acid (EDTA) (Gibco).

Monoclonal antibody production and purification

Three IgG_1 MAbs were used. J591 binds to FOLH1-expressing cells [9], BLCA-38 to human CaP cell lines and biopsy specimens (Russell et al., submitted), and DS-1 was used as a negative control. J591 hybridoma was provided by BZL, through N Bander, New York Presbyterian Hospital / Weill Medical College of Cornell University, NY, USA; BLCA-38 hybridoma and DS-1 were in-house. J591 and DS-1 hybridomas were grown serum-free with 0.01% glutamax II, penicillin (51 U/ml), and streptomycin (5 μg/ml) at 10 ml/l (each Gibco BRL Life Technologies, Rockville, MD) and glucose 2 g/l (Sigma St. Louis, MO) in 0.05% CO_2 at 37°C, but BLCA 38 hybridoma required 2% ultralow IgG FBS (Gibco BRL). Hybridoma cells (4×10^7) grown in roller bottles for 9 days yielded MAbs at 0.1–0.2 mg/ml. MAbs were isolated by affinity chromatography using a protein A (Prosep A, Novachem, Australia) column, pH 7.4. Bound IgG was eluted with 0.2-M glycine buffer, pH 3.0, neutralised, concentrated to ~5 mg/ml by ultrafiltration using an Amicon 10-kDa cutoff membrane and dialysed into PBS, pH 7.4. Purity was monitored by SDS-PAGE and size exclusion chromatography on a Superose 12 HR10.30 column, pH 7.4, at a flow rate of 0.5 ml/min. MAb binding was assessed by flow cytometry. $F(ab')_2$s were prepared as previously described (Carter et al., in press).

Peptide 101 synthesis

Peptide 101, H-GIRRVLRVLRRGLPALIRWIC-NH_2, was synthesized with an AIB synthesizer with Fmoc_RINK resin or with a PerSeptive Pioneer peptide synthesizer with Fmoc-PAL-PEG-PS resin according to manufacturer's instructions. Arginine was protected with Pmc (2,2,5,7,8-pentamethyl chroman-6-sulfonyl-); cysteine sulfhydryl was protected with Trt (trityl); solvent was NMP (*N*-methyl pyrrolidone). At each cycle the carboxyl group of the Fmoc amino acid was activated with HBTU/HOBt/DIEA reagent (HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium

hexafluorophosphate; HOBt, *N*-hydroxy-benzotriazole; DIEA, di-isopropylethylamine) and after coupling, the Fmoc group was removed with 20% piperidine ready for the next cycle. After synthesis the peptide was cleaved from the resin and deprotected for 5 h with trifluoroacetic acid containing phenol (0.5 g/10 ml), triisopropylsilane (0.2 ml/10 ml) and water (0.5 ml/10 ml) as scavengers. The product was precipitated and washed with ether, then dried in vacuo to a white powder. Its purity was assessed by HPLC analysis, mass spectroscopy and amino acid composition. Peptide 101 was also custom synthesised by AUSPEP.

Immunoconjugation

Peptides were conjugated using SPDP (*N*-Succinimidyl 3-(2-pyridyldithio) propionate; Amersham Pharmacia Biotech), a heterobifunctional reagent that linked the carboxyl terminal cysteine to amino functionalities on the antibody. Activation and conjugation were performed and monitored as previously described [16]. Protein was activated by reaction with SPDP at a 1:20 to 1:30 molar ratio. Unreacted SPDP was removed on a 10-ml desalting column (cross-linked dextran Presto Desalting Column, prod. no. 43233; Pierce Chemical Company), then peptide was added in a 1.5:1 (peptide to SPDP) molar ratio, to give conjugates with a ~10:1 ratio of peptide to antibody. The characteristics of selected batches are shown in Table 1. SDS-PAGE (nreduced gels) was used to show that the conjugates had a higher molecular mass than parent MAbs or $F(ab')_2$ fragments. The molar ratio of conjugated peptide to antibody was determined by amino acid analysis of the purified product. The degree of conjugation was dependent on the thiolation ratio achieved during the reaction of SPDP with the antibody, but conjugation levels were consistently lower than the molar thiolation ratio.

Flow cytometric analysis of antibody and immunoconjugate binding

Assays for antibody and conjugate binding were carried out by flow cytometry using target (antigen positive) and nontarget (antigen negative) cells. Cells at 5×10^5, harvested in 0.02% EDTA/PBS (ICN, Costa Mesa, CA), were stained for analysis by standard methodology using purified BLCA-38 or J591 MAbs (optimal concentrations 1 μg/ml) or immunoconjugates followed by fluorescein-conjugated sheep antimouse immunoglobin (FITC-SaMIg; Amrad, Melbourne, Victoria, Australia). Surface immunofluorescence of cells fixed with 1% paraformaldehyde (Sigma, St. Louis, MO) was assessed in a Fluorescence Activated Cell Scanner (FACScan; Becton Dickinson, Mountain View, CA) or using a Coulter EPICS Elite flow cytometer (Beckman-Coulter, Miami, FL).

Flow cytometric analysis of cell killing by immunoconjugates

Cytotoxicity was measured by flow cytometry on a Coulter EPICS Elite flow cytometer and analysed using WinMDI (Scripps Research Institute, La Jolla, CA). Live/dead cell discrimination was carried out using light scatter and propidium iodide uptake. Events with low light scatter were assumed to be debris and were excluded from the analysis by gating. Live and dead cells remaining after treatment were estimated from the number of propidium iodide negative and positive events, respectively, within a light scatter gate set from the distribution shown by live cells in an untreated sample [6].

Preparation of labeled immunoconjugates

^{14}C-labeling of peptide 101 (done by Dr L. Sparrow, CSIRO) was by acetylation on the protected peptide attached to the synthesis resin peptide using 0.5-mCi 1-^{14}C-labeled acetic anhydride in DMF and triethylamine. This directed the acetyl group exclusively to the N-terminus of the peptide. The acetylated peptide was cleaved with deprotection, purified by RP-HPLC (Vydac C-18 semi-prep column) and lyophylised giving 9.0-mg peptide at 6×10^6 counts/min (cpm). Immunoconjugates of BLCA-38, J591 or control IgG_1 MAb containing ^{14}C-labeled peptide were labeled with iodine 125 (^{125}I) (2 mCi ml^{-1}; Amersham, Bucks, UK) at 0.2 mCi/100 μg using the Iodogen method, as before [14]. Labeling efficiencies of BLCA-38, J591 and DS-1 MAb–peptide 101 conjugates, determined by protein estimation after a trichloracetic acid assay [14] were 84%, 85% and 83%, respectively. Counts per minute of 1 μl standards of ^{14}C-peptide–^{125}I-MAb conjugates were used to correct for decay.

Animal Studies

Athymic BALB/c nu/nu (nude) male mice aged 6–8 weeks from Biological Resources Centre, University of New South Wales (UNSW) at Little Bay, NSW, Australia, were maintained according to the code of practice of the National Health and Medical Research Council, Australia. Ethics approval was obtained from the Animal Care and Ethics Committee, UNSW. Mice were kept

Table 1 Conjugation of peptide to antibodies. Data of batches used in in vivo studies. Summary of DS-1 IgG conjugates. Characteristics of selected pooled batches of peptide antibody conjugates are shown. The coupling parameters were estimated by amino acid analysis

Conjugate batch	Activation ratio	Thiolation ratio	Coupling ratio	Coupling efficiency (%)
152	30:1	6.01	5.7	94.8
153	30:1	9.53	6.8	71.3
155 (pool 152 & 153)			8.4	
168	30:1	8.7	11.1	127.6
171[a] (pool 155, 161 & 168)		-	10.7	
Summary of BLCA-38 conjugates				
147	25:1	6.8	6.7	98.5
148	30:1	7.9	6.8	86.1
150	30:1	10.6	9.7	91.5
151	25:1	7.9	6.9	87.3
154 (pool 147, 148, 150 & 151)		-	9.4	
162	30:1	11.6	12.5	107.7
167	30:1	10.7	15.4	143.9
170[a] (pool 154, 162, 167)		11.4		
Summary of J591 conjugates				
130	25:1	10.5	10.4	99
132	20:1	9.6	13.7	143
137	20:1	7.5	7.3	97
140	25:1	11.6	14.4	124

[a]Pools 170 and 171were used for the prophylactic studies using BLCA-38 immunoconjugates

sterilely, housed in cages with filter tops and fed sterile mouse chow and water ad libitum. Drinking water was supplemented with potassium iodide (0.05%; BDH Chemicals, Port Fairy, Vic, Australia) to block thyroid uptake of radioiodine prior to injection of labeled MAbs. After injection, mice were kept behind lead shielding and cage litter was changed regularly to minimise label ingestion.

Xenografts

LN3 or DU-145 human CaP cell xenografts were established in male nude mice by s.c. injection into the right flank of $1.5x10^6$ to $2x10^6$ tumour cells in serum free media (100 µl), mixed with an equal volume of Matrigel (Integrated Sciences, Sydney, Australia) on ice.

Biodistributions

Biodistributions were done using DU-145 or LN3 xenograft-bearing nude mice (8 mice/group) 8 or 7 days (the time taken for maximal MAb localisation; see Carter et al., in press) after i.p. injection of 150-µg MAbs or 2 days after injection of 50-µg $F(ab')_2$s to assess uptake of ^{125}I-labeled MAbs, $F(ab')_2$s and conjugates or ^{14}C-labeled peptide—^{125}I-test MAb, or ^{14}C-labeled peptide–^{125}I-control MAb. The conjugation ratio was 9–11 in those used for biodistribution studies. Tissue to blood (T/B) ratios and percentage injected dose corrected for decay/gram (%ID/g) were calculated (Carter et al., in press). At sacrifice, tissues were removed, divided, and placed into preweighed gamma tubes or scintillation vials for gamma or beta counting (1470 Wizard gamma counter; Wallac Oy, Finland), that were re-weighed to determine tissue mass. For ^{14}C counting, tissues were dissolved in 1-ml tissue-dissolving solution (TDS, Soluene-350 or Soluene-350/isopropanol 1:2) incubated overnight at 40°C, then H_2O_2 (300 µl) was added and incubated for 30 min, followed by 10-ml Hionic-Fluor scintillant.

Therapeutic studies

Every batch made was tested for its activity in vitro. Below a coupling ratio of 5:1, binding and cytotoxicity by the immunoconjugates was affected. Hence, only conjugate batches with a coupling ratio of 10–12 M of peptide to antibody and in vitro activity comparable on a molar basis to that expected by free peptide, and a MAb binding and efficacy that was not significantly decreased by peptide conjugation, were used for in vivo studies. Two treatment strategies were pursued:

1. Systemic efficacy: The therapeutic efficacy of BLCA-38 or J591-MAb-peptide 101 conjugates was compared with that of non-specific control (DS-1 MAb) conjugates in nude mice carrying s.c. DU-145 or LN3 xenografts, respectively. Treatment was started when the tumours reached ~5×5 mm (taken as day 0), a few days after tumour cell inoculation. DU-145 tumour-bearing mice (21/group) were injected i.p. with 1.5 mg of either BLCA-38 MAb- or DS-1 MAb-peptide 101 conjugate or 10-mM sodium acetate buffer, pH 5.3 (700 µl) on days 0, 2, 6, 10, 17, 24 and 31 (total 10.5 mg/mouse). Groups of 15 mice with LN3 tumours ~5×5 mm received five i.p. injections, each 1.5 mg (total 7.5 mg) of J591MAb- or DS-1-MAb-peptide conjugate i.p. on days 0, 2, 5, 7 and 9; 3 LN3-bearing controls were injected i.p. with 1 ml 10-mM sodium acetate buffer, pH 5.0, over the same period.
2. Intratumoural efficacy: Using antibody conjugates with a conjugation ratio of 11.5 peptides/mole of antibody, we calculated that the dose/mouse i.p. was 1.5-mg conjugatex5 (or 7) injections (mainly MAb on a weight basis), or 10 nM (based on 150,000-g MAb = 1 mole)x5 (or 7), i.e. 50 nM (or 70 nM, respectively) of MAb, and 575- or 805-nM peptide. Based on data from biodistributions of ^{125}I-labeled MAb alone or immunoconjugates (4% ID/g of tumour) (Carter et al., in press), total peptide delivered to the xenograft after 5 or 7 i.p. injections was 23 or 32 nM, respectively. We injected 500 µg immunoconjugate (equivalent to 3.33-nM MAb and 38.3-nM peptide) intratumurally over 5 doses, a dose roughly equivalent to that given i.p. LN3-tumour (s.c.) bearing mice (16/group) received J591-MAb–peptide 101 conjugate, J591 MAb alone or sodium acetate buffer in 5 intratumoural injections (each 100 µg) on days 0, 3, 5, 7 and 14 (total 500 µg). Treatment began when tumours were ~5×5 mm, on three separate occasions, as the tumour growth lag period differed in different mice. The start of treatment (tumour = 5×5 mm) was considered to be day 0. On days 21 and 35, three mice/group were sacrificed for histology; all were sacrificed on day 72.

Tumour volume and growth delay

Mice were weighed and tumour measurements taken twice/week using vernier calipers; tumour volume, V $(mm^3) = \pi/6(d_1.d_2)^{3/2}$, where d_1 and d_2 are diameters (mm) at right angles [21]. Under guidance from the Animal Care and Ethics Committee, UNSW, mice were euthanatised if they lost 20% of body weight or if tumours reached 15×15 mm. At sacrifice, tumours were fixed (DU-145) or frozen in OCT (LN3) and stained for MAb uptake. Tissues were H&E stained and assessed by Dr W Delprado, pathologist, Douglass Hanly Moir, Sydney, Australia. A Mann-Whitney *U*-test determined statistical significance of differences in tumour volumes between groups.

Tumour growth delay was assessed from percentage reduction in tumour volume between treatment groups, calculated by determining the number of days taken to reach 50% maximum mean tumour volume, assessed from a line of best fit constructed to fit each set of data. For each experiment, the maximum tumour volume was the volume reached by the largest control tumours when the mice were euthanatised. The maximum tumour volume differed in the two xenograft models due to differences in the biology of the tumours and their differing growth rates. In each case, the percentage tumour reduction = difference between time taken for control and treatment groups to reach 50% maximum volume divided by time taken for controls to reach 50% maximum volume x 100. The rate of tumour growth was assessed for clinical significance (partial response, measured by reduction in tumour size by more than 50%; stable or progressive disease).

Results

Peptide 101 is a C-terminal truncated melittin analogue [19, 26] designed with extensive arginine substitution on the hydrophilic face of the helix to increase its water solubility. Haemolytic assays indicated that peptide 101 was ~4× as active as Melittin on a w/w basis (~6× activity, mole/mole, data not shown). The peptide was synthesised with a carboxyl terminal cysteine residue for use in conjugation. Efficiency of conjugation to MAbs, BLCA38, J591 and DS-1, was determined on the final product by amino acid analysis. The conjugation levels were dependent on, but consistently lower than, the molar thiolation ratio achieved during the reaction of SPDP with MAb (Table 1).

In vitro cell binding

Peptide 101 immunoconjugates were shown to have increased binding to test cell lines, compared with MAbs alone when analysed by flow cytometry (Coulter EPICS

Elite machine). Thus geometric mean fluorescence of binding to LN3 cells by J591 was 31.2, compared with J591 peptide 101 conjugate of 63.2. Similarly that of the DS-1 (nonspecific) peptide 101 conjugate was 17.3, compared with 3.2 by MAb alone. The increased binding was thought to be due to the excessive stickiness of the peptide. Conjugate binding was reduced by including FBS in the binding buffer. In 100% serum or whole blood, the binding of J591 and BLCA-38 conjugates approached that of unconjugated MAb, but the binding of J591 conjugates in 100% serum decreased markedly with increasing dilution of conjugate, while unconjugated antibody showed saturated binding at all protein concentrations tested (data not shown).

In vitro cell killing

Because no cell lines were available with identical genetic backgrounds except for the presence or absence of the target antigens, the antigen positive and negative cell lines used were necessarily diverse. These target and control cell lines had differential inherent sensitivities to the peptide component of the immunotoxins (Fig. 1) that had to be taken into account when comparing conjugate activity. J591 MAb–peptide 101 conjugates were tested against LN3 (antigen positive) and SKMEL (antigen negative, high sensitivity to peptide) or L929 (antigen negative mouse origin, low sensitivity to peptide) cells (Fig. 2B). The melittin peptide molar equivalents in the added conjugates are plotted against cytotoxicity measured by propidium iodide uptake. At lower peptide levels, LN3 cells were more sensitive to both free and conjugated peptide than were L929 cells, while the difference disappeared at higher peptide concentrations. Conjugates were less cytotoxic than equivalent free peptide at low concentrations, but slightly more effective at higher concentrations. There was no selectivity of killing of target (LN3) versus nontarget (L929) cells. BLCA38 conjugates were tested against DU145 cells (antigen +ve) and C3 cells (antigen −ve). As for J591 conjugates, free peptide showed slightly enhanced killing potential at low concentrations compared with conjugate containing equivalent quantities of peptide (Fig. 2A). Target (DU145) and nontarget (C3) cells were similarly sensitive to peptide, but killing of DU145 and C3 cells by BLCA-38 conjugates did not differ significantly (Fig. 2A).

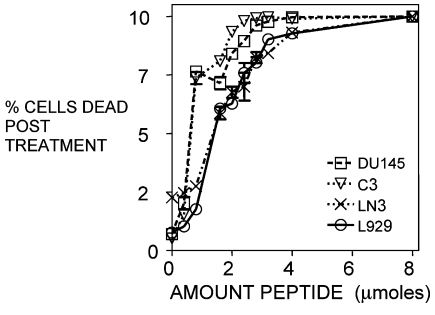


Fig. 1 Cytotoxicity of peptide 101 on DU145, C3, LN3 and L929 cells was measured from propidium iodide uptake as described in "Materials and methods". The results are shown from triplicate samples

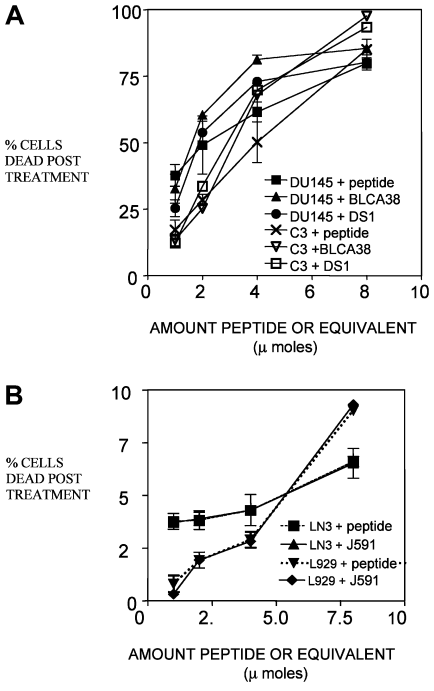


Fig. 2 **A** Cell killing by free peptide, DS1 control antibody–peptide101 conjugate and BLCA38-peptide conjugate performed on DU145 (test) and C3 (control) cells. The conjugates contained between 8 and 10 moles/mole of peptide and the titration curves for the antibody conjugates have been adjusted to peptide equivalents. The results are shown from duplicate samples. **B** Cell killing by free peptide and J591 MAb–peptide 101 conjugate on LN3 (test) and L929 (control) cells. The conjugates contained between 8 and 10 moles/mole of peptide and the titration curves for the antibody conjugates have been adjusted to peptide equivalents. The results are shown from duplicate samples

The inclusion of FBS or whole blood in the cytotoxicity assay reduced the toxicity of J591 conjugate (Fig. 3), but did not enhance its killing of LN3 cells compared with that by DS-1 control antibody conjugates containing equivalent quantities of peptide.

Antigen expression by xenografted tissue

Before injection, each cell culture batch was monitored for surface expression of target antigens by flow cytometry (FACScan, Becton Dickensen). In each case, DU-145 cells expressed BLCA-38 antigen with a mean fluorescence intensity (MFI) of 150–170 and LN3 cells had high FOLH1 expression (MFI 660–804). Both antigens maintained strong expression by immunohistochemistry when the cells were xenografted [7].

Biodistributions were performed to ensure that the coupling of peptide 101 to MAbs or $F(ab')_2$s did not inhibit their ability to localise specifically to xenografts

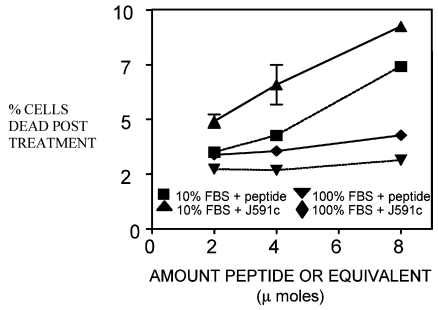
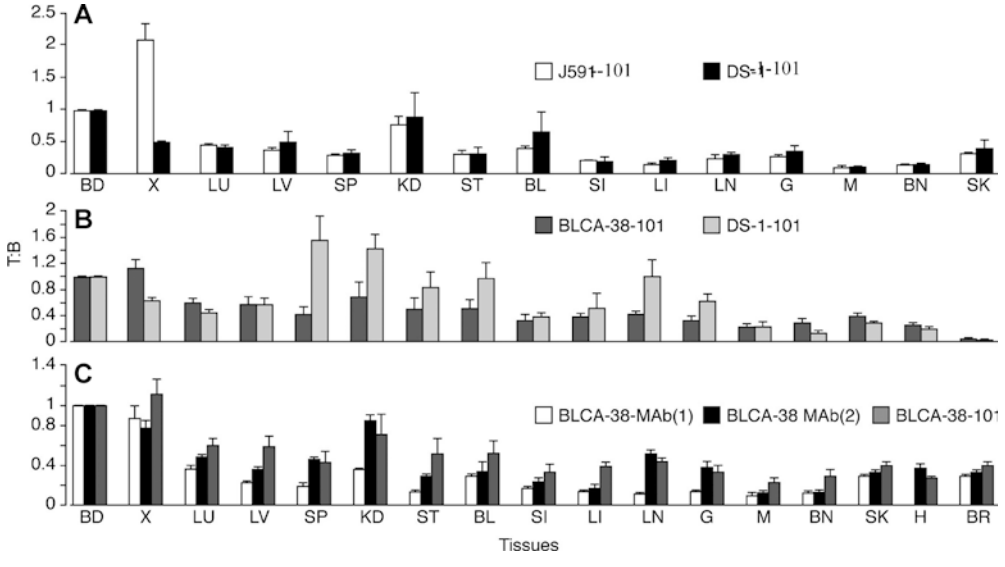


Fig. 3 The effect of serum on cell killing by peptide conjugates. Cytotoxicity assays were performed using free peptide and J591 peptide conjugate on LN3 cells as described in "Materials and methods", in the presence of 10% or 100% fetal bovine serum. The conjugates contained between 8 and 10 moles/mole of peptide and the titration curves for the antibody conjugates have been adjusted to peptide equivalents. The results are shown from duplicate samples

in vivo. We labeled the peptide with ^{14}C and the antibody moiety with ^{125}I to show by beta and gamma counting of tissues that the conjugates remained intact. Previous studies had indicated that optimal localisation of these MAbs or $F(ab')_2$s alone following i.p. injection of s.c. tumor-bearing mice (Carter et al., in press) was obtained 8 or 7 days after BLCA-38 MAb or J591 MAb administration, respectively, or 2 days after administration of $F(ab')_2$s. Gamma cpm values of all tissues indicated preferential localisation of BLCA-38 MAb-peptide 101 immunoconjugates (IC) compared with DS-1-MAb-peptide immunoconjugates at day 8 and of BLCA-38 $F(ab')_2$ peptide-101 immunoconjugates compared with DS-1-$F(ab')_2$-peptide immunoconjugates at day 2 in DU-145 xenografts (BLCA-38 MAb IC: % injected dose / gram (%ID/g) = 0.40 + 0.08 (MAbs), 0.69 + 0.10 $F(ab')_2$; BLCA-38 MAb IC: xenograft to blood (X/B) = 1.11 + 0.59 (MAb), versus DS-1 MAb IC: 0.43 + 0.02; BLCA-38 $F(ab')_2$s IC: X/B = 1.31 + 0.20, versus DS-1 $F(ab')_2$s IC: 0.79 + 0.11). Similarly, J591-peptide-101 immunoconjugates showed preferential localisation compared with DS-1-peptide-101 immunoconjugates in LNCaP-LN3 xenografts on day 7 (MAbs) and day 2 ($F(ab')_2$s), respectively (J591 IC: %ID/g = 2.19 + 0.28 (MAbs), 0.74 + 0.15 ($F(ab')_2$s); J591-MAb IC: X/B = 2.08 + 0.25, versus DS-1 MAb IC: 0.48 + 0.03; J591 ($F(ab')_2$s IC: X/B = 1.96 + 0.18, versus DS-1 ($F(ab')_2$s IC: 0.88 + 0.08). Thus, in each case, the specific MAbs showed a X/B ratio around 2–4 times that of the DS-1 immunoconjugates that tended to remain in the circulation, or bound preferentially in lung, liver, spleen, kidney and bladder and gonads (Fig. 4). The presence of blood in the tissues quenched the beta counts, so that no T/B ratios could be assessed for the ^{14}C component. We were unable to overcome this problem by addition of hydrogen peroxide or tissue-dissolving reagents. We therefore compared the distribution using MAb alone with that obtained using MAb-conjugates using the gamma counts (Fig. 4C). The biodistribution of the ^{125}I-labeled BLCA-38 MAb was performed on two occasions; both showed similar tropism, although the %ID/g differed, possibly reflecting the age of the iodine when it was used (Fig. 4C). It was clear that both BLCA-38 and J591 MAb conjugates showed similar tissue tropism as had been previously reported (Carter et al., in press) for the MAbs alone (Fig. 4C), indicating that the presence of the peptide did

Fig. 4A–C Tissue to blood ratios of gamma cpm values following i.p. injection of **A** 150 μg ^{14}C-peptide 101–^{125}I-J591 and ^{14}C-peptide 101–^{125}I-DS-1 MAbs, 7 days postadministration into nude mice bearing human prostate cancer LNCaP-LN3 xenografts; **B** 150 μg ^{14}C-peptide 101–^{125}I-BLCA-38 and ^{14}C-peptide 101–^{125}I-DS-1 MAb–peptide 101 conjugates, 8 days postadministration into nude mice bearing human prostate cancer DU-145 xenografts; **C** 150 μg ^{14}C-peptide 101–^{125}I-BLCA-38 (BLCA-38-101), BLCA-38 MAb (experiment 1) and 200 μg BLCA-38 MAb (experiment 2), 8 days postadministration into nude mice bearing human prostate cancer DU-145 xenografts. *Vertical bars* represent standard error of the mean (8 mice/group); *BD* blood, *X* xenograft, *LU* lung, *LV* liver, *SP* spleen, *KD* kidney, *ST* stomach, *BL* bladder, *SI* small intestine, *LI* large intestine, *LN* lymph nodes, *G* gonads, *M* muscle, *SK* skin, *H* heart and *BR* brain



not interfere with the tissue binding of the MAbs. While it is feasible that, if the SPDP bonds were labile in vivo, the MAb immunoconjugates may have become dissociated by days 7 or 8 when the biodistributions were performed, we feel that this is unlikely to be the case for the $F(ab')_2$-immunoconjugates that were tested for biodistribution after only 2 days when it would be anticipated that they would remain intact.

Therapeutic studies

Antibody immunoconjugates have potential for eliminating a low tumour burden, such as micrometastases, or after the tumour volume has been decreased by other therapy. Because we lacked suitable experimental models, our first approach was "prophylactic". As we found previously that the amount of radioactivity delivered by MAbs compared with $F(ab')_2$s was considerably higher (Carter et al., in press), we decided to use MAb-immunoconjugates for our therapeutic studies. MAb-peptide 101 immunoconjugates were injected 4 days after inoculating tumour cells, to try to prevent further tumour growth. For ease of measurement, s.c. tumours were used; treatment was systemic (i.p.). Preliminary experiments showed that mice could tolerate repeated i.p. injections every 2 days for 8 days of 1-ml DS-1 MAb–peptide 101 conjugate (total 4.32 mg) without weight loss. We detected no abnormalities in heart, liver or kidney taken 18 days after starting treatment.

Prophylactic effects of MAb–peptide 101 immunoconjugates given systemically (i.p.)

BLCA-38 MAb-peptide 101 conjugates versus DU-145 xenografts

The therapeutic efficacy of BLCA-38 MAb–peptide 101 conjugate (pool 170; Table 1; total 10.5 mg/mouse), started 4 days after mice were injected with DU-145 cells, was compared to that of unconjugated BLCA-38 MAb, DS-1 MAb–peptide 101 conjugate (pool 171; Table 1) and sodium acetate buffer in 21 mice/group over 72 days. The percentage reduction in tumour volume was calculated by determining the time to reach 50% of the maximum mean tumour volume. The maximum tumour volume in this case was selected as 1,527 mm^3, the volume on day 52, since after this time, some mice were sacrificed due to large tumor sizes. Figure 5 shows that the mean tumour volume for the BLCA-38 MAb–peptide 101 conjugate group was lower than for the other groups up to day 52. By the Mann-Whitney *U*-test, there were statistically significant differences in tumour volumes between mice treated with BLCA-38 MAb–peptide 101 or buffer on days 14 ($p < 0.02$),17 ($p < 0.02$), 21 ($p < 0.01$) and 24 ($p < 0.02$). No significant differences were observed between other groups. Tumours of mice injected with the BLCA-38 MAb–peptide 101 conjugate reached 50% maximum volume on day 44, compared with those receiving BLCA-38 MAb, DS-1 Mab conjugates and buffer on days 31, 37 and 31, respectively. This was a 30% reduction in tumour growth in mice treated with test conjugates compared with either BLCA 38 MAb or sodium acetate buffer, whereas mice treated with DS-1 MAb–peptide 101 conjugate showed a 16% reduction in tumour growth.

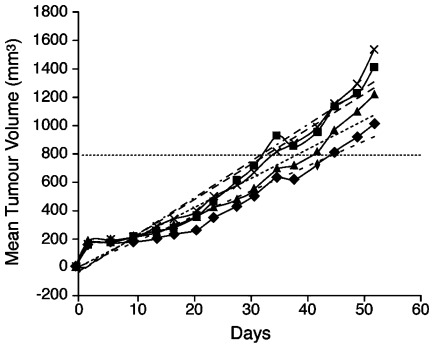


Fig. 5 Mean tumour volumes of DU-145 xenograft–bearing mice which were injected i.p. with 10.5 mg of either BLCA-38 MAb–peptide 101 conjugate *(solid diamond)*, BLCA-38 MAb *(solid square)*, DS-1 MAb–peptide 101 conjugate *(solid triangle)* or sodium acetate buffer *(x)*. A line of best fit was obtained for each group: BLCA-38 MAb–peptide 101 conjugate *(short-dash line)*; DS-1 MAb–peptide 101 conjugate *(dotted line)*; BLCA-38 MAb *(medium-dash line)*; and sodium acetate buffer *(long-dash line)*. The percentage reduction in tumour volume, calculated by determining the time to reach 50% of the maximum mean tumour volume, was based on a maximum volume on day 52 (794 mm^3), shown as a *horizontal line*

By 3 weeks, there was a partial response or stable disease in 38% of mice treated with BLCA-38 MAb–peptide 101 conjugate compared with 19% in mice treated with DS-1 MAb–peptide 101 conjugate, and only stable disease in those receiving BLCA-38 MAb (14%) or buffer alone (19%) (Table 2). However, by 7 weeks, nearly all mice had progressive disease. Thus the treatment was effective initially, but failed when treatment stopped (day 31). There was no difference in host survival of the four groups (data not shown). Macroscopic post mortem examination indicated liver and splenic swelling at days 21 and 35 of mice injected with either BLCA-38 or DS-1 MAb–peptide 101 conjugates, histologically identified as minor cytoplasmic oedema; but livers, spleens, kidneys and lungs from mice killed on day 72 were normal. Tumours of mice treated with BLCA-38 MAb–peptide 101 conjugates showed increased necrosis and haemorrhage compared with those from mice given other treatments.

Effects of J591 MAb–peptide 101 immunoconjugates on LN3 xenografts

Preliminary data indicated that J591 MAb alone did not affect tumour growth of LN3-bearing mice. Ten LN3-

Table 2 Effects of prophylactic treatment with BLCA-38 MAb–peptide conjugates on tumour growth in DU-145 tumour-bearing nude mice. *PR* partial response (reduction in tumour volume of greater than 50%); *SD* stable disease; *PD* progressive disease; *N* number of tumours remaining. DU-145 tumour-bearing mice were injected i.p. with 1.5 mg of either BLCA-38 MAb–peptide 101 conjugate, DS-1 MAb–peptide 101 conjugate, BLCA-38 MAb or sodium acetate buffer, pH 5.3, on days 0 (4 days post s.c. tumour cell injection), 2, 6, 10, 17, 24 and 31 (total 10.5 mg/mouse). Tumour volumes were assessed twice weekly thereafter. Mice were euthanatised if they lost 20% of their body weight, or if the tumours reached 15×15 mm

	BLCA-38 MAb–peptide 101 conjugate			BLCA-38 MAb			DS-1 MAb–peptide 101 conjugate			Sodium acetate buffer		
	3 weeks	4 weeks	7 weeks	3 weeks	4 weeks	7 weeks	3 weeks	4 weeks	7 weeks	3 weeks	4 weeks	7 weeks
PR	2/21 (9%)	1/21 (5%)					1/21 (5%)					
SD	6/21 (29%)	4/21 (19%)		3/21 (14%)	1/18 (6%)		3/21 (14%)	2/17 (12%)	1/16 (6%)	4/21 (19%)		
PD	13/21 (62%)	16/21 (76%)	15/15 (100)	18/21 (86%)	17/18 (94%)	16/16 (100)	17/21 (81%)	15/17 (88%)	15/16 (94%)	17/21 (81%)	17/17 (100)	16/16 (100)
N	21	21	15	21	18	16	21	17	16	21	17	16

tumour-bearing mice / group were treated with five sequential injections (days 0, 2, 4, 6 and 8) totalling 7.5 mg of J591 MAb, or with buffer (5 mice) or saline (5 mice). No statistically significant differences in tumour volume were found at any time point between mice treated with J591 MAb and controls treated with sodium acetate buffer or saline i.p. (data not shown). Subsequently, the therapeutic efficacy of J591 and DS-1 MAb–peptide 101 conjugates was compared in LN3-tumour-bearing mice followed for 30 days. Mice injected with either J591 MAb or saline reached 50% of the maximum tumour volume (1,147 mm^3) 12.5 days after the first injection, the DS-1 MAb–peptide 101 conjugate group by 18 days and the J591 MAb–peptide 101 conjugate group by 24 days. Compared with J591 MAb, the percentage reduction in mean tumour volume of the DS-1 MAb–peptide 101 conjugate group was 31%, and that of the J591 MAb–peptide 101 conjugate was 48%. The difference in tumour volumes between mice receiving J591 MAb–peptide 101 conjugate and DS-1 MAb–peptide 101 conjugate was statistically significant (Mann-Whitney *U*-test) on days 12 ($p = 0.02$) and 15 ($p = 0.03$). The differences between J591 MAb and J591 MAb–peptide 101 conjugate were statistically different from day 7 onward, whereas there was a stastistically significant difference between the J591 MAb and the DS-1 conjugate groups only on day 9. Figure 6 shows the number of mice in each group with tumour volumes above or below the 50% maximum volume at times after treatment began. PSA values, assessed in only three mice/group were decreased after treatment with J591 MAb immunoconjugates compared with DS-1 MAb conjugates or buffer (data not shown). Histology on day 15 indicated that tumours from treated mice contained more necrosis and haemorrhage than those from control mice that received DS-1-1-1 conjugates. This experiment was repeated, starting 3 days after inoculation of LN3 cells (data not shown).

Intratumoural injection of J591 conjugates into LN3 xenografts

While systemic injection using immunoconjugates inhibited prostate tumour growth, the levels of inhibition needed improvement. This may have reflected limitations of the amount of immunoconjugate that could be injected, accessibility of the tumour, or a need for a longer exposure of the tumour to the conjugates, as may be achieved using a pump. As the human prostate can be injected directly by procedures like brachytherapy, we tested whether J591 MAb–peptide immunoconjugates could inhibit tumour growth after direct injection into the tumours of mice carrying s.c. LN3 tumours. We calculated that 500 μg/mouse given intratumourally was about equivalent to the dose administered by systemic therapy. The therapeutic efficacy of J591 MAb–peptide 101 conjugate was compared with that of J591 MAb and sodium acetate buffer after five sequential

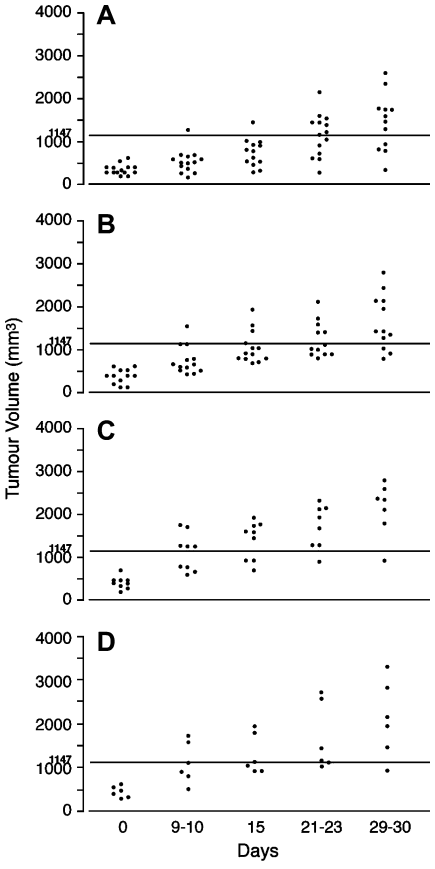


Fig. 6A–D Comparison of tumour volumes in LN3-tumour-bearing nude mice at various times after start of treatment (day 0), initiated when tumours were 5×5 mm. Mice were treated with five i.p. injections of **A** J591 MAb–peptide 101 conjugate, **B** DS-1 MAb–peptide 101 conjugate, **C** J591 MAb, or **D** sodium acetate buffer/saline. Fifty percent maximum tumour volume was 1,147 mm^3

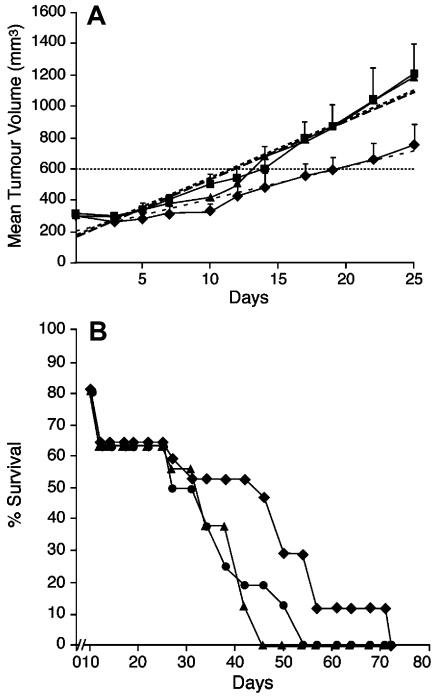


Fig. 7 A Mean tumour volumes of mice bearing LNCaP-LN3 xenografts which were injected intratumorally with 500 μg of either J591 MAb conjugate *(solid diamond)* or J591 MAb *(solid square)* and 250-μl sodium acetate buffer *(solid triangle)*. *Vertical bars* represent standard error of the mean where 16 mice were used in each group. A line of best fit was obtained for each group: J591 MAb conjugate *(thickened dash line)*, J591 MAb *(longer separated dashes)*, and sodium acetate buffer *(fine-dash line)*. **B** Survival curve for LN3 xenograft-bearing mice injected intratumourally with either J591 MAb–peptide 101 Conjugate *(solid diamond)*, J591 MAb alone *(solid square)* or sodium acetate buffer *(solid triangle)*

intratumoural injections (total 500 μg) into LN3 xenografts over 71 days. J591 MAb–peptide 101 conjugate showed consistent effects on tumour growth compared with J591 MAb and sodium acetate buffer (Fig. 7A), with an increase in partial response or stable disease compared with the other groups (Table 3). There was a 40% reduction in tumour growth on day 25, when the 50% of maximum tumour volumes had been reached. There was a statistical significance between the groups on days 10, 19, 22 and 31. The treated mice survived longer than those treated with unconjugated J591 MAb or sodium acetate buffer (Fig. 7B). Despite these promising effects, the inhibitory effects of the J591 MAb–peptide 101 conjugate were not prolonged. Histology of tissues of three mice/group killed on days 6 and 10 and on all mice at the end of the experiment, confirmed a localised effect but it was not sufficient to stop tumour growth, as the tumours continued to grow around areas of dead cells (data not shown). However histology also showed that tumour volume in some cases represented dead cells, indicating an effect on tumour growth.

Discussion

New strategies are needed to overcome CaP that has spread beyond the prostate. The use of immunoconjugates that incorporate tumour-specific or prostate tissue–specific MAbs or ligands for therapy of CaP has been explored [1, 5, 17]. Because they selectively concentrate in tumour tissue, immunoconjugates can deliver substantial doses of a radionuclide, drug or toxin to the cancer, while minimising exposure of normal tissue. In this study, we have determined the therapeutic efficacy of two anti-CaP immunoconjugates containing peptide 101, a Melittin analogue. Such immunoconjugates should overcome disadvantages associated with MAb-targeted therapy. Melittin peptides show relatively low toxicity and their half-lives are limited in the body by proteolysis, limiting undesirable side effects. Moreover, these peptides cause cytolytic effects on plasma membranes independent of internalisation [8, 19, 23].

Both MAbs studied have excellent targeting abilities against CaP xenografts in vivo (Carter et al., in press; Fig. 4) and work therapeutically in in vivo models when labeled with samarium 153 [14] or bismuth 213 [13]. We

Table 3 Effects of intratumoral injection of J591 MAb–peptide 101 conjugates on tumour growth in LN3-tumour-bearing nude mice. *PR* partial response (reduction in tumour volume of greater than 50%); *SD* stable disease; *PD* progressive disease. LN3-tumour-bearing mice were injected intratumorally with 100-μg MAb–peptide 101 conjugate or J591 MAb alone on five occasions (total 500 μg) or an equivalent volume of sodium acetate buffer, pH 5.3. Tumour volumes were assessed twice weekly thereafter. Mice were euthanatised if they lost 20% of their body weight, or if the tumours reached 15×15 mm

	J591 MAb conjugate		J591 MAb		Sodium acetate buffer	
	Day 7	Day 22	Day 7	Day 22	Day 7	Day 22
PR	7/17 (41%)	4/11 (36%)	2/14 (14%)	0/10	1/16 (6%)	0/10
SD	1/17 (6%)	1/11 (9%)	1/14 (7%)	1/10	0/16	0/10
PD	9/17 (53%)	6/11 (54%)	11/14 (79%)	10/10 (100%)	15/16 (94%)	10/10 (100%)

did not attempt to compare these immunoconjugates; rather we were attempting to determine whether they could target and kill tumour cells. As prostate cancers are very heterogeneous, it is not expected that all cancers would react with the same antibody. We have already described differences between the antibodies used. J591 is internalised after binding, whereas BLCA-38 is not. In the case of melittin peptides, this theoretically should not make a difference, as the peptides are cytolytic to plasma membranes [8, 19, 23]. In both cases, we used models that show good vascularisation (LNCaP-LN3 for J591-related studies and DU-145 for BLCA-38 studies). We could also have used PC-3 tumours that are highly reactive with BLCA-38, but these tumours show poor vascularisation, and it was felt that DU-145 cells provided a more appropriate model for study.

We can produce chemical conjugates containing a tumour-specific antibody and up to 12 moles/mole of a truncated melittin analogue. The conjugates retain substantial antigen-binding capacity, although results with one (J591), indicated some reduction in binding capacity, as evidenced by a more rapid decrease of binding on dilution compared with the unconjugated antibody. A high proportion of the cytolytic activity of the attached peptide was retained on a molar basis. Interestingly, cell killing by the conjugates had a sigmoid relationship to conjugate concentration (Fig. 2), while free peptides did not, over the concentration range used. It was thought that in vivo conditions might be more conducive to the induction of specific killing. We have shown beneficial effects against xenografts when CaP-targeting MAbs–peptide 101 conjugates were injected i.p. or directly into the tumour.

The conclusions from in vitro experiments indicate that attachment of peptide to antibody molecules induces a high degree of nonspecific binding, and therefore killing, presumably through the high affinity of the peptide moieties for lipid bilayer membranes. The conjugates showed similar cytotoxicity regardless of antigen status. Some specificity of antibody binding could be restored by including serum in the assays, probably through binding of hydrophobic serum proteins to the peptides, but this reduced the cytotoxic activity of the conjugates, and selective killing was not seen. Cell killing by melittin–antibody conjugates produced by expression from recombinant DNA did not create this problem [6]. The relatively high level of substitution we used in our conjugates may have exacerbated the problem. This was considered necessary to deliver sufficient toxin to the target tumour in vivo.

Ideally, antibody immunoconjugates should be used to target tumours of low burden (e.g. micrometastases, cancer cells shed during surgery) or after the tumour volume has been decreased by radiotherapy, brachytherapy or hormonal treatment. Our first approach was "prophylactic", to prevent further growth of small tumours targeted by i.p. injection 4 days after inoculation of cells into nude mice. In both tumour models, specific antibody–peptide 101 conjugates significantly inhibited tumour growth compared with buffer, unconjugated antibody or peptide alone; partial responses or development of stable disease occurred in some mice 3 weeks after initiation of treatment. Control antibody–peptide 101 conjugates showed more limited efficacy. Test-MAb–peptide 101 conjugates induced necrosis and haemorrhage in tumours, but sometimes with new growth around the dead areas, suggesting that the effects were limited and did not affect all tumour cells or that the effects were temporary. We did not see toxicity in organs examined histologically (heart, kidney, liver), and while the peptide 101 has haemolytic properties in vitro, selective targeting through the use of MAbs prevented any long-term toxic effects, except in the tumours. Injection of peptide 101 alone caused necrosis/haemorrhage around the site of injection (not shown), but tumour volume was not affected; it would be anticipated that peptide 101 would be rapidly cleared as it is small. Intratumoral delivery of J591 MAb–peptide 101 immunoconjugates also provided tantalising benefits and prolonged survival (Fig. 7).

The limited response may have reflected dosage. The dose was restricted by conjugate solubility; refinement of peptide 101 design to improve its solubility may allow higher doses to be administered in vivo with increased efficacy. The conjugates were soluble at the dose administered. The in vivo data to some extent reflected the nonspecific cell killing seen in vitro, thought to reflect the stickiness of peptide 101 and steric effects that allowed attachment of the complexes to cells grown in two dimensions. We reasoned that the three-dimensional growth in vivo would allow higher

specificity to be achieved. Indeed, immunoconjugates of peptide 101 with prostate tissue–specific MAbs were more efficient than those containing control MAb, but these did have some effect. We maximised the number of peptide 101 moities / MAb to increase delivery in vivo, possibly exacerbating their nonspecificity. Peptides with considerably greater toxicity than melittin and peptide 101 can be designed [12]; such peptides may have greater potential as immunoconjugate partners.

In conclusion, our data indicate that sequential treatment with prostate-directed MAbs conjugated with melittin-based peptide 101 is cytotoxic against human CaP xenografts in nude mice, but these immunoconjugates require refinement to maximise their potential.

Acknowledgements This work was supported by a grant from AusIndustry, and by CSL Limited. We wish to thank Dr Lindsay Sparrow for preparation of ^{14}C-labeled peptide 101, Dr Neil Bander for making available the J591 hybridoma, Drs Simon Green and Robyn McLachlan from CSL Limited for helpful discussions and expert advice, and Ms Mila Sajinovic, Oncology Research Centre, Prince of Wales Hospital, for excellent animal husbandry. Dr Warick Delprado from Douglass Hanly Moir Pathology kindly assessed our slides for pathologic effects.

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