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Drug localisation and growth inhibition studies of vindesine-monoclonal anti-CEA conjugates in a human tumour xenograft

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Summary. The distribution of tritiated vindesine (^{3}H-VDS) was studied in the tissues and tumours of athymic mice bearing a human colorectal tumour xenograft. Selective tumour localisation was obtained when ^{3}H-VDS was injected as a conjugate with a monoclonal anti-CEA antibody (11.285.14) but not as a conjugate with a non-binding monoclonal IgG1 (Ag8) or as free succinoyl-VDS. The amounts of VDS that localised in the tumour following injections of ^{3}H-VDS-11.285.14 increased in proportion to the amount injected, over a wide dose range. Conjugates prepared using the Fab fragments of 11.285.14 showed no evidence of selective tumour uptake in comparison with normal tissues.

Various dose levels of VDS-11.285.14 conjugate and free VDS were studied for effects on the growth of the tumour xenograft. A growth inhibition of 50% was obtained at 1.5 mg/kg with free VDS and at 2.5 mg/kg with conjugated VDS. The conjugate was, however, considerably less toxic.

Introduction

Monoclonal antibodies recognising human tumour-associated antigens are receiving increasing attention in the fields of diagnosis and therapy of cancer. The demonstration that such antibodies, when labelled with radioactive isotopes, can localise selectively in tumours of experimental animals [2, 8, 11] and in cancer patients [1, 6] suggests that selective drug delivery could be obtained using monoclonal antibody-drug conjugates. In previous studies [15, 16] it has been shown that conjugates of the anti-cancer alkaloid vindesine (VDS) with a range of anti-tumour monoclonal antibodies produced selective tumour cytotoxic and cytostatic effects in vitro and in vivo. Although it was shown that the antibody component of these conjugates would localise in tumour xenografts [16], no direct evidence has so far been provided to show selective targeting of the drug component. In the present study, tritiated VDS (^{3}H-VDS) was conjugated to the monoclonal anti-carcinoembryonic antigen (CEA) antibody, 11.285.14, and to a non-binding monoclonal immunoglobulin, Ag8, in order to investigate drug localisation in nude mice bearing a human colorectal tumour xenograft. Localisation experiments were also performed using tritiated 4-succinoyl-VDS and VDS conjugated to the Fab fragments of the 11.285.14 or Ag8. From these studies it has been possible to determine the quantities of VDS that can be delivered to a tumour xenograft using an antibody and to relate these to the inhibition of tumour growth at different dose levels of free or conjugated VDS.

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Materials and methods

IgG and Fab fragments. The monoclonal anti-CEA antibody used in these studies was an IgG1 designated 11.285.14. This monoclonal has been shown to have strong anti-CEA binding activity, low reactivity with non-specific cross reactive antigen and a high degree of gastrointestinal tumour selectivity in comparison with a wide range of normal tissues [4, 7]. The protein secreted by the myeloma P3/X63-Ag8, an IgG1 designated Ag8, was used as a non-antibody immunoglobulin carrier. The 11.285.14 and Ag8 were isolated from ascites fluids using a Protein A affinity column. Ascites fluids were diluted with 1 volume of 0.1 M phosphate buffer pH 8.0, filtered, and absorbed onto Protein A-Agarose (Sigma Chemical Comp., Poole, England) equilibrated with the pH 8.0 buffer and maintained at 4 °C. Unabsorbed material was removed by washing with the pH 8.0 buffer, and bound immunoglobulin then eluted using 0.1 M citrate/phosphate buffer pH 3.5. The eluates were neutralised, concentrated to ca. 25 mg/ml by ultrafiltration, and dialysed against either 0.34 M borate buffer pH 8.6 for conjugation, or 0.5 M phosphate buffer pH 8.0 for the preparation of Fab fragments. For this step, a 15 mg/ml solution of the IgG in 0.5 M phosphate buffer pH 8.0 and containing 0.01 M L-cysteine and 0.002 M EDTA was treated with papain (Sigma, 1:100 w/w). The mixture was purged with nitrogen and incubated at 37 °C for 4 h. The reaction was stopped by purging with oxygen followed by gel filtration on a column of Biogel P-6 (Bio-Rad) equilibrated with 0.005 M phosphate buffer pH 8.0. The excluded material was separated into Fab and Fc fragments by anion exchange chromatography on DEAE-cellulose (Whatman, Maidstone, England, DE-52) equilibrated with 0.005 M phosphate buffer, pH 8.0. The Fab fragment was unabsorbed under these conditions, and was concentrated by dialysis against water, lyophilisation and reconstitution in 0.34 M borate buffer at ca. 25 mg/ml.

Conjugates. Conjugates were prepared by a procedure in which VDS was coupled to IgG and Fab fragments via a

succinate spacer. For distribution studies, 4-succinoyl-VDS incorporating tracer quantities of 4-succinoyl-^{3}H-VDS was activated in situ as the N-hydroxysuccinimide ester which was used without isolation. The 4-succinoyl-^{3}H-VDS was custom synthesised by Amersham International p.l.c. by tritiation of VDS followed by succinoylation using succinic anhydride in pyridine. The product isolated by preparative thin layer chromatography was 92% radiochemically pure with a specific activity of 4.8 Ci/mole and was supplied as a solution in dry DMF containing ca. 1 mCi/ml. The 4-succinoyl-VDS (20 mg) was dissolved in 0.8 ml of the 4-succinoyl-^{3}H-VDS solution and a mixture of 3.4 mg of N-hydroxysuccinimide (Sigma) and 9.9 mg of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulphonate (Sigma) in 0.2 ml of dry DMF added. After 48 h at room temperature, 0.175 ml and 0.25 ml aliquots were added respectively to stirred 1 ml samples of 20 mg/ml solutions of IgG and Fab fragment in 0.34 M borate buffer, pH 8.6. The conjugation reactions were allowed to proceed at room temperature for 4 h, after which time they were adjusted to pH 7.2, clarified by centrifugation if necessary, and chromatographed on 1.5 × 110 cm columns of Sephadex G-200 (Pharmacia Milton Keynes, England) (IgG conjugates) or Sephadex G-100 (Pharmacia) (Fab conjugates) equlibrated with 0.01 M phosphate, 0.15 M sodium chloride, pH 7.2 (PBS). Fractions corresponding to monomeric IgG or Fab conjugates were combined and characterised radiochemically. Drug and protein contents of conjugates were determined by spectrophotometry at 270 and 280 nm and relating the absorbances obtained to predetermined extinction coefficients for free drug and unconjugated IgG and Fab fragments at the two wavelengths.

Several batches of conjugates with 11.285.14 and Ag8 were prepared for tumour inhibition studies using 4-succinoyl-VDS N-hydroxysuccinimide ester kindly provided by Mr. G. Cullinan, Lilly Research Laboratories, Indianapolis. Typically, 0.4 ml of a 19.0 mg/ml solution of the ester in dry DMF was added with stirring to 5 ml of a 20 mg/ml solution of the IgG in 0.34 M borate buffer pH 8.6. The mixture was stirred at room temperature for 4 h, then 2.5 ml of 5-times concentrated PBS added and the pH adjusted to 7.2 using 1 M HCl. The solution was clarified by centrifugation and the supernatant chromatographed on a 2.2 × 26 cm column of Biogel P-6 (Bio-Rad Labs Watford, England) equilibrated with PBS. Conjugates were collected in the excluded fraction and drug and IgG content determined spectrophotometrically as described above.

Animals and xenografts. Outbred *nu/nu* athymic mice were bred and maintained in isolators (Isotec Ltd., Bicester). Mice of either sex were inoculated at two s.c. sites with the MAWI human colorectal carcinoma as previously described [16]. Mice were used in localisation studies from 4 to 6 weeks later when tumours weighed 1.5 g on average.

Localisation studies. Tumour-bearing mice were injected with ^{3}H-labelled preparations dissolved in PBS by either the i.v. or i.p. route. At various times after injection, mice were killed and blood and tissue samples taken. Weighed samples (20–400 mg) of plasma or chopped tissues were transferred to scintillation vials and decolourised by treatment with 400 µl of hydrogen peroxide (100 vols) at 55 °C for 3 h. Tissues were dissolved by incubating in 1.5 ml Soluene (Packard Inst. Co., Reading, England) at 55 °C for 18 h followed by the addition of 10 ml of Dimilume-30 scintillant (Packard Inst. Co.) and counted in a β-counter (1217 Rackbeta, LKB-Wallac). An external standard channels ratio correction was applied, based on a set of quenched tissue samples containing known amounts of a tritium standard.

Tumour inhibition studies. Mice inoculated with tumour were treated twice weekly for 5 weeks with various doses of VDS-11.285.14 or VDS sulphate in PBS by i.p. injection, the first injection being given 24 h after tumour inoculation. Mice were killed after 6 weeks, tumours excised and weighed. The percentage inhibition for each experiment was based on a mean value for the weights of tumours excised from a control group of mice treated with PBS alone.

Results

Plasma levels of radioactivity

The levels of ^{3}H-VDS found in the plasma of tumour-bearing nude mice after a single injection of free or conjugated drug are shown in Fig. 1. Free 4-succinoyl-VDS was cleared rapidly with only 0.01% of the injected dose present per ml of plasma by 3 days. By contrast, VDS-11.285.14 conjugate, injected i.p. or i.v., was present at levels above 10% of the injected dose per ml at day 3 and above 4% at day 9. Levels of VDS conjugated to the Fab fragment of 11.285.14 are also shown, falling from 0.36% on day 1 to 0.06% on day 6.

Tissues to plasma ratios

Figure 2 shows the tumour : plasma and tissue : plasma ratios of radioactivity at various times after a single injection of ^{3}H-VDS-11.285.14 conjugate. Ratios higher than 1.0 were only observed in the tumour, rising to 3.6 on day 9. The increasing ratio was a reflection of falling plasma radioactivity and selective tumour uptake maintained at a

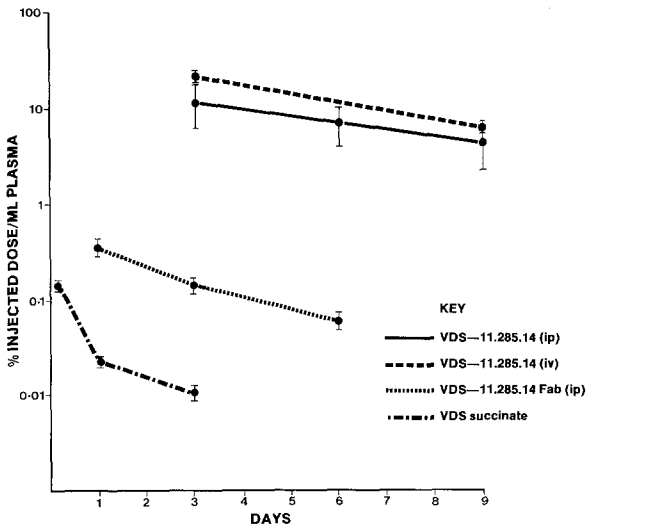

Fig. 1. Plasma clearance of ^{3}H-VDS administered in conjugated or free form to tumour-bearing nude mice. VDS-11.285.14 was injected at 0.24 mg/kg (VDS) i.p. (●———●) and 0.16 mg/kg i.v. (●– – –●). VDS-Fab 11.285.14 was injected at 0.56 mg/kg (VDS) i.p. (●·····●) and free 4-succinoyl-VDS at 2.0 mg/kg i.p. (●– · – · –●). The results are the means (±SEM) from three mice at each point

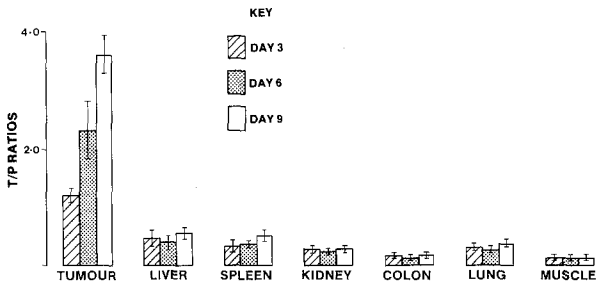

Fig. 2. Tissue:plasma ratios of ^{3}H at various times after a single i.p. injection of ^{3}H-VDS-11.285.14 into tumour-bearing nude mice at a dose of 0.24 mg/kg (VDS). Results are the means (±SEM) from three mice at each time

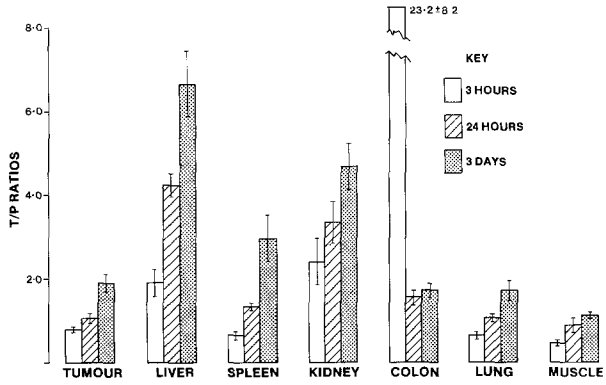

Fig. 3. Tissue:plasma ratios after injection of 4-succinoyl-^{3}H-VDS i.p. at 2.0 mg/kg. Results are the means (±SEM) from three mice at each time

steady level over the 9 days. In other tissues, radioactivity fell at approximately the same rate as in plasma.

Figure 3 shows the results of an experiment in which tumour-bearing mice were given a single i.p. injection of 4-succinoyl-VDS. In contrast with the previous experiment, no selective tumour uptake was observed. A high level of radioactivity was obtained in the colons of the three mice killed at 3 h, presumably reflecting gastrointestinal tract absorption of labelled drug from the peritoneal cavity injection site.

Antigen specificity

The selective uptake of conjugated VDS by tumour tissues was demonstrated to be due to recognition of antigen by the anti-CEA monoclonal antibody in an experiment comparing localisation of VDS-11.285.14 with VDS-Ag8, a conjugate using a mouse IgG1 with no anti-CEA reactivity. Figure 4 shows the results of tissue:plasma ratios at day 9 after a single i.v. injection. As in the study using the i.p. route, selective uptake was observed in the tumour following injection of VDS-11.285.14. No evidence of selective tumour localisation was observed when using VDS-Ag8.

Drug uptake by tumour at different conjugate dose levels

In experiments designed to demonstrate selectivity of uptake by tumours, trace amounts of radioactive antibody or conjugate are commonly used. In order, however, to determine how much drug can be delivered to a target site by

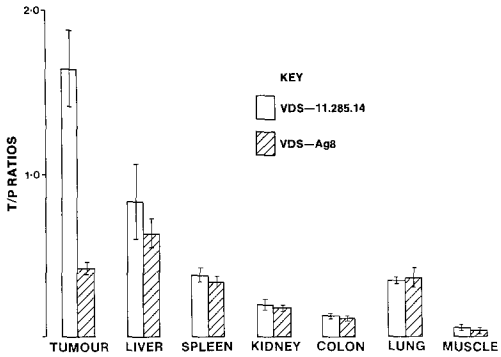

Fig. 4. Tissue:plasma ratios on day 9 after i.v. injections of ^{3}H-VDS-11.285.14 (0.52 mg/kg) or ^{3}H-VDS-Ag8 (0.61 mg/kg) into tumour-bearing nude mice. Results are the means (±SEM) from five mice for each preparation

Table 1. Concentration of ^{3}H-VDS in the MAWI xenograft on day 6 after injections of VDS-11.285.14 conjugates at a range of dose levels

VDS dose level administered (mg/kg)	Percentage injected dose detected/ g of tumour	VDS conc in tumour (ng/g)
0.13	17.4 ±4.4	566 ±143
0.33	17.5 ±2.6	1420 ±210
0.83	13.6 ±2.2	2837 ±460
2.08	16.1 ±0.9	8363 ±459

Results are the means ± SE of three mice per group injected i.p. with various volumes of a preparation containing 493 μg Ig/ml and 13 μg VDS/ml

Table 2. Tissue to plasma ratio of ^{3}H-VDS on day 6 after injection of VDS-11.285.14 at 2.08 mg/kg i.p.

Tissue	Tissue: plasma ratio
Tumour	1.26 ±0.10
Liver	0.72 ±0.12
Spleen	0.40 ±0.08
Kidney	0.19 ±0.02
Colon	0.11 ±0.01
Lung	0.31 ±0.02
Muscle	0.06 ±0.01

Results are the mean ± SE of three mice per group

Table 3. Concentration of ^{3}H-VDS in the MAWI tumour at various times after injection of free 4-succinoyl-VDS at 2.0 mg/kg i.p.

Time after injection (h)	VDS conc in tumour (ng/g)
3	54.8 ±5.2
24	12.5 ±1.8
72	9.3 ±0.3

Results are the means ± SE of three mice at each time

antibody, dose ranging experiments must be performed. Table 1 shows the result of such an experiment using ^{3}H-VDS-11.285.14. The percentage of injected dose on day 6 remained relatively constant over a 16-fold dose range, giving a calculated value for VDS of greater than 8000 ng/g of tumour following an injection at a dose of

2.08 mg/kg. Table 2 shows that the tumour selectivity was retained at this high dose level. Appreciable levels of drug were found however in the liver and spleen at this dose. Table 3 shows the tumour level of ^{3}H-VDS at various times after injecting unconjugated 4-succinoyl-VDS at 2 mg/kg. Only low levels of drug were present at 3 h compared with the levels at day 6 observed in the conjugate-treated mice (Table 1). After 3 days, free drug was virtually undetectable in the tumour.

Relationship between injected dose and inhibition of tumour growth

The results shown in Table 1 indicate increasing uptake of drug by the tumour with increasing doses of injected antibody conjugate up to 2 mg/kg in terms of VDS content. It has been shown previously that repeated injections at 5 mg/kg produce marked inhibition of tumour growth [15, 16]. In other therapeutic experiments, different dose levels have been used and compared with doses of free VDS. Figure 5 shows the combined normalised data from five separate experiments using free and conjugated VDS to treat MAWI-bearing nude mice. Both treatments produced inhibition of tumour growth in a dose dependent manner,

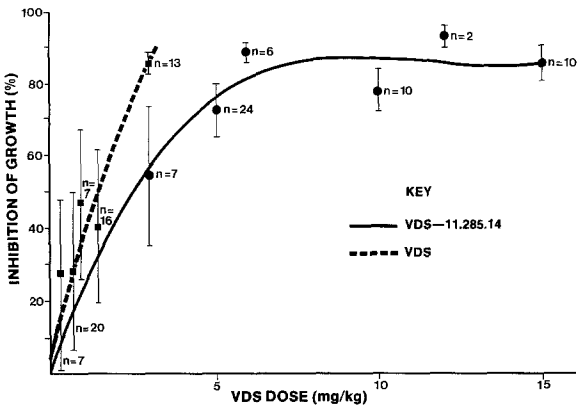

Fig. 5. Inhibition of MAWI tumour growth in nude mice following 10 bi-weekly injections i.p. of VDS-11.285.14 (●———●) or free VDS sulphate (■------■) at various dose levels. Weights of tumours excised from mice between day 42 and 63 were expressed as a percentage of a mean weight from control mice given PBS alone. The values are the means (±SEM, "*n*" as shown) and five separate experiments have been combined. Curves were fitted by polynomial regression with correlation coefficients of 0.984 (VDS-11.285.14) and 0.925 (free VDS)

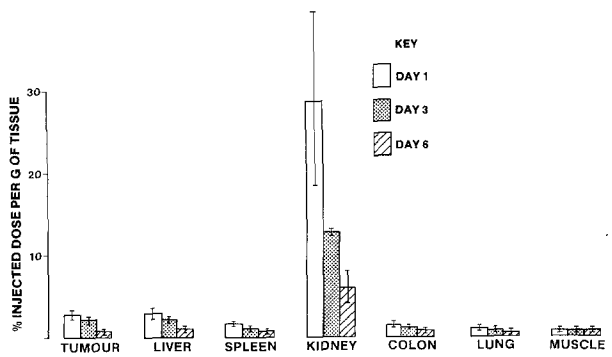

Fig. 6. Percentage of the injected dose of ^{3}H-VDS-Fab 11.285.14/g. of tumour or normal tissues at various times. Results are the means (±SEM) of three mice at each time

50% inhibition being obtained at approximately 1.5 mg/kg with free VDS and 2.5 mg/kg with VDS-11.285.14. The major difference between the two forms of treatment lay in the ability to administer higher dose levels of conjugate. Although toxicity may be observed at various conjugate dose levels, it has been shown that a level of 15 mg conjugated VDS/kg twice weekly can be well tolerated [14]. By contrast, no mice survived following repeated doses of free VDS at 6 mg/kg. Although higher levels of VDS can be given as an antibody conjugate, inhibition of tumour growth did not increase beyond a plateau of 88% in this system.

Localisation of Fab conjugates

In studies using radioiodinated monoclonal antibodies and their fragments for tumour imaging, it has been found that Fab fragments can show higher levels of tumour selectivity [2, 3]. To see if this could lead to an improved drug targeting system, conjugates of ^{3}H-VDS were prepared using Fab fragments of 11.285.14 and Ag8. A maximum tumour:plasma ratio of 16:1 was achieved with the anti-CEA conjugate on day 3, considerably higher than that obtained with the Ag8-Fab conjugate. The amount of drug localised, however, was low and as shown in Fig. 6 was no higher in the tumour than in normal tissues. A high level of radioactivity was observed in the kidneys on days 1 and 3.

Discussion

The experiments described in this study clearly demonstrate selective uptake of radiolabel by a human colorectal tumour xenograft when ^{3}H-VDS was administered as an anti-CEA monoclonal antibody conjugate. Evidence that this selectivity was due to antigen recognition by the antibody came from experiments in which VDS-11.285.14 was compared with VDS-Ag8, a monoclonal conjugate with no anti-CEA reactivity.

The in vivo distribution of ^{125}I-labelled 11.285.14 alone has previously been examined in human tumour xenografts using autoradiography [10] and measurements of tissue radioactivity [13]. These studies showed that 11.285.14 was taken up selectively by CEA positive tumours but not by tumours lacking the antigen. In these experiments, designed to demonstrate tumour imaging, microgram quantities of antibody were injected. In the present study it has been shown that selective tumour uptake of the drug component of an anti-CEA conjugate can be obtained when milligram quantities of conjugate were injected into nude mice and that the quantity of drug delivered was proportionally higher. A feature of this selective uptake was the long retention time in tumour tissue, the levels at 9 days being little lower than at 3 days. This distribution pattern contrasts markedly with the low amounts of radioactivity that were found in tumour tissue following injection of 4-succinoyl-^{3}H-VDS.

The present studies show that approximately 900 times as much VDS can be localised in MAWI tumours in the form of an anti-CEA conjugate than as free 4-succinoyl-VDS. Detailed comparative pharmacokinetics of VDS and 4-succinoyl-VDS have not been performed. However the plasma half-life of 4-succinoyl-^{3}H-VDS in the present study of ca. 20 h was similar to that of 18 h found for ^{3}H-VDS in C57BL/6 mice bearing the B-16 mouse melanoma

(H. W. Culp, unpublished observations). Although the tissue half-lives of 4-succinoyl-^{3}H-VDS are somewhat longer than those of ^{3}H-VDS in the respective mouse strains, the distribution patterns are similar, and hence the high localisation index referred to is also likely to be reflective of conjugated and free VDS. However, studies on tumour growth showed the inhibitory doses of VDS-anti-CEA and VDS sulphate to be similar (Fig. 5), suggesting that conjugates are intrinsically less potent than free drug, a conclusion supported by some previous in vitro studies [5]. On the basis of in vitro potency alone it may be tempting to discount the utility of many drug-antibody conjugates. This would, however, fail to take into account the high drug levels that can be achieved in tumour for long periods of time after treatment with such preparations (Fig. 2).

The use of fragments of monoclonal antibodies in targeting studies has been advocated by some workers as a means of improving the tumour : plasma ratios for imaging [2] and as a means of reducing the immunogenicity associated with the Fc portion of immunoglobulin [3]. In the present study ^{3}H-VDS-Fab conjugates were prepared with little loss of antigen-binding capacity. Localisation studies confirmed that tumour : plasma ratios could be obtained that were higher than with intact antibody conjugates. The absolute amounts of conjugate taken up by the tumour, however, were lower with Fab fragments and no tumour selectivity was observed with respect to other tissues. High levels of radioactivity in the kidneys on days 1 and 3 probably reflected the renal excretion of Fab conjugates. Similar results have been observed with iodinated Fab preparations of unconjugated monoclonal antibodies [9, 12]. The present studies with ^{3}H-VDS-Fab conjugates indicated that this type of preparation is likely to be of less use in therapeutic drug targeting. Conjugates of VDS and whole monoclonal anti-CEA antibody, however, appear to offer advantages of tumour selectivity and reduced systemic toxicity that could be of clinical benefit in adjuvant cancer chemotherapy of CEA-bearing tumours.

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