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# Analysis of a conjugate between anti-carcinoembryonic antigen monoclonal antibody and alkaline phosphatase for specific activation of the prodrug etoposide phosphate

Hidde J. Haisma, Epie Boven, Monique van Muijen, Robert De Vries, and Herbert M. Pinedo

Department of Oncology, Free University Hospital, P. O. Box 7057, 1007 MB Amsterdam, The Netherlands

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**Summary.** The selective targeting of tumours by enzymes conjugated to monoclonal antibodies (mAb) may be an ideal approach to convert relatively nontoxic prodrugs into active agents at the tumour site. We used the anti-carcinoembryonic antigen mAb BW431/26 conjugated to alkaline phosphatase (AP) and phosphorylated etoposide (etoposide-*P*) as a prodrug to study the feasibility of this concept. Etoposide was phosphorylated with POCl<sub>3</sub>. Quantitative hydrolysis of etoposide-*P* to etoposide occurred within 10 min in the presence of AP. BW431/26 and AP were conjugated using a thioether bond. The AP conjugate retained 93% of its calculated activity. <sup>125</sup>I-labelled AP conjugate did not show a reduction of immunoreactivity as determined by a cell-binding assay. SW1398 colon cancer cells were used to analyse the cytotoxicity of etoposide and etoposide-*P*. Etoposide (IC<sub>50</sub> 22 μM) was 100 times more toxic than etoposide-*P* (20% growth inhibition at 200 μM). Pretreatment of the cells with BW431/26-AP prior to etoposide-*P* exposure resulted in a dramatic increase in cytotoxicity (IC<sub>50</sub> 70 μM). The pharmacokinetics and tumour-localizing properties of BW431/27 and the AP conjugate were assessed in nude mice bearing SW1398 tumours. BW431/26 showed excellent tumour localization (10% of the injected dose/g tissue retained from 8 h to 120 h), whereas the AP conjugate showed a reduced tumour uptake (3%–0.3% of the injected dose/g tissue at 8–120 h), a faster clearance from the circulation and a high liver uptake. Radiolabelled AP showed a similar pharmacokinetic profile to the AP conjugate. Gel filtration analysis of blood, liver, and tumour samples indicated good stability of the conjugate.

**Key words:** Monoclonal antibody – Alkaline phosphatase – Prodrug activation – Etoposide

## Introduction

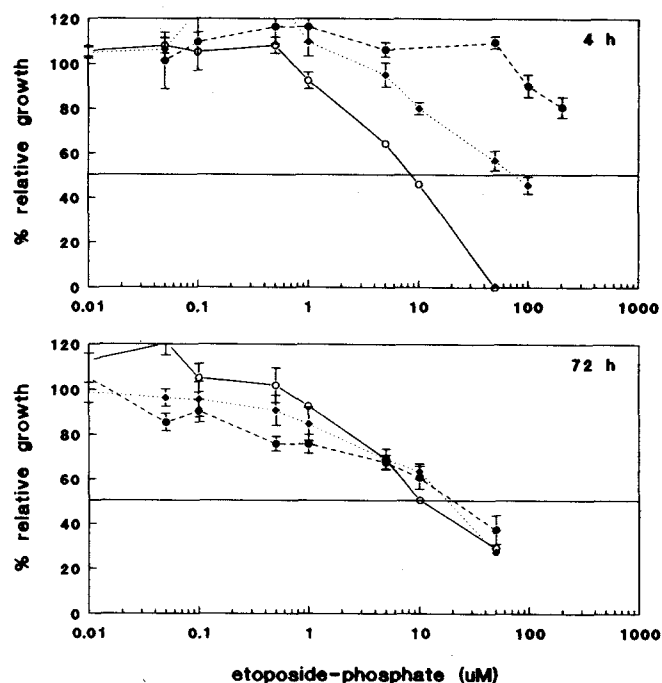
One of the major limitations of cytostatic agents for the treatment of malignancies is their lack of selectivity for cancer cells. Toxicity to normal cells limits the dose of chemotherapy required to achieve tumour cell death. Targeting of therapy is being designed to enhance the delivery of cytotoxic agents to the tumour site without increasing the side-effects. In this respect, monoclonal antibodies (mAbs) recognizing tumour-associated antigens have been successfully applied as carriers of radionuclides [2], toxins [5] or cytostatic agents [14]. The antitumour effects appear to be limited because of the relatively low concentration of the cytotoxic reagent delivered to tumours [12].

The concept of antibody-enzyme-mediated chemotherapy has been described by several investigators [1, 7, 17]. In this approach, antibodies are used to deposit enzymes at the tumour site. The enzymes convert relatively nontoxic prodrugs, to be administered after the conjugates have localized in tumours, into active cytostatic agents. Theoretically a large amount of active drug can be generated and high local concentrations will achieve better antitumour effects. Thus, an increase in the therapeutic index of the drugs is to be expected.

In our studies we have chosen the enzyme alkaline phosphatase (AP) to be conjugated to an anti-carcinoembryonic antigen mAb. The *in vivo* pharmacokinetic behaviour and biodistribution profile of the AP conjugate was determined in nude mice bearing human colon cancer xenografts. The prodrug etoposide phosphate (etoposide-*P*) was examined for its cytotoxicity before and after activation by the AP conjugate.

## Materials and methods

**Antibody and cell line.** Murine mAb BW431/26 (Behring Werke, Marburg, FRG) is specific for carcinoembryonic antigen (CEA) and does not react with granulocytes or monocytes. BW431/26 has a very high affinity



**Fig. 1.** Growth inhibition of etoposide (○), etoposide-P (●), and BW431/26-AP conjugate with etoposide-P (◆). Cells were exposed to drugs for 4 h (top) or 72 h (bottom). Cell growth was measured with a protein dye assay. Error bars are  $\pm$  SD

for cell-bound CEA:  $1 \times 10^{10}$  l/mol. Serum CEA in 20-fold molar excess cannot inhibit the binding of BW431/26 to cancer cells [3].

The human colorectal cancer cell line SW1398 [15], which expresses CEA, was grown in Dulbecco's modified Eagle's medium (Flow Laboratories, Irvine, Scotland) supplemented with 10% heat-inactivated fetal calf serum (Flow), 2 mM L-glutamine, 50 IU/ml penicillin and 50  $\mu$ g/ml streptomycin (Flow) in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. Cells were maintained as exponentially growing monolayer cultures by seeding  $1 \times 10^5$  cells/ml culture medium in a 75-cm<sup>2</sup> tissue-culture flask every 2–3 days.

**Etoposide phosphate.** Etoposide was a gift from Bristol Myers (Weesp, The Netherlands). Etoposide-P was used both as a product synthesized by ourselves or obtained from Bristol Myers (Evansville, Ind.). The synthesis of etoposide-P was carried out essentially according to Senter et al. [17]. Briefly, etoposide was phosphorylated with an equimolar amount of phosphoryl chloride in dry acetonitrile. *N,N*-Diisopropylethylamine was added at a 4-fold molar excess to neutralize the HCl produced during the reaction. The intermediate product, formed after overnight incubation, was hydrolysed with saturated NaHCO<sub>3</sub> to produce the sodium salt. The final product was purified on a C18 silica gel column (Bakerbond spe, Baker, Enschede, The Netherlands). The column was washed with H<sub>2</sub>O and the product was eluted with 20% methanol in H<sub>2</sub>O. Purity of the product was determined by reversed-phase HPLC using a C18 silica column with 50% methanol, 50% 0.1 M KH<sub>2</sub>PO<sub>4</sub>, pH 4.5, buffer at a flow rate of 1 ml/min. Etoposide-P and etoposide were monitored at 290 nm and with an electrochemical detection method [11]. Etoposide eluted after 9 min, whereas the more polar etoposide-P eluted at 5 min. Etoposide-P did not show a signal with electrochemical detection, indicating that the OH group in the phenolic ring of etoposide had been replaced. Quantitative hydrolysis of etoposide-P to etoposide occurred within 10 min in the presence of AP.

**BW431/26-AP conjugate and labelling.** AP from calf intestine (Boehringer, Almere, The Netherlands) was conjugated to BW431/26 using a thioether linkage. BW431/26 was reacted with *N*-succinimidyl 3(2-pyridyldithio)propionate (Pharmacia, Woerden, The Netherlands), which

was then reduced with dithiothreitol to introduce free thiol groups on the antibody molecule. AP was reacted with *N*-succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Pierce, Oud-Beerland, The Netherlands) to introduce a maleimide group. Both proteins were mixed and, after an overnight incubation at 4°C, conjugates consisting of one antibody molecule bound to one AP molecule were purified on a Superose 6 (Pharmacia, Woerden, The Netherlands) column. The overall protein yield after purification was approximately 20%. The AP conjugate was analysed by sodium dodecylsulphate/polyacrylamide gel electrophoresis (7.5%) according to Laemli [9] and found to be free of aggregates or unconjugated proteins. The conjugate retained 93% of the calculated AP activity as measured with *p*-nitrophenyl phosphate in 0.1 M TRIS/HCl, 1 mM MgCl<sub>2</sub>, pH 8. Co-incubation of the AP conjugate with excess cold antibody (100  $\mu$ g/ml) resulted in a complete blockade of binding of the conjugate to SW1398 cells (results not shown).

Labelling of BW431/26 with <sup>131</sup>I and of BW431/26-AP or AP with <sup>125</sup>I was performed with iodogen according to the one-vial method [6]. The specific activities of the BW431/26 and BW431/26-AP conjugate after iodination were both approximately 1 mCi/mg antibody. Precipitation with 10% trichloroacetic acid indicated that 95% of the radioactivity was bound to protein in the final preparations. The immunoreactive fractions of the antibody and the AP conjugate, determined according to the method described by Lindmo et al. [10] on SW1398 cells, were between 80% and 90%.

**In vitro cytotoxicity.** The cytotoxic effects of etoposide and etoposide-P were determined by measuring the cell growth of SW1398 cells in vitro. Cells were incubated with BW431/26-AP conjugate at 5  $\mu$ g/ml or with buffer alone for 1 h and plated in triplicate in 96-well microtiter plates. Etoposide or etoposide-P was added and the cells were exposed to the drugs for 4 h or 72 h. In the 4-h experiment, cells were washed after drug exposure and grown in tissue-culture medium up to 72 h. Cell survival in both experiments was determined at 72 h using a sulphorhodamine-B assay [18]. Briefly, the plates were centrifuged and the cells were fixed with 5% ice-cold trichloroacetic acid, washed with water and stained with 0.4% sulphorhodamine-B, dissolved in 1% (v/v) acetic acid. After rinsing with 1% acetic acid the plates were air-dried and the bound dye was solubilized with 10 mM unbuffered TRIS. The absorbance at 540 nm was read. Readings were linear with cell concentrations between 1000 cells/well and 100 000 cells/well.

**In vivo studies.** <sup>131</sup>I-labelled BW431/26 (10  $\mu$ Ci) and <sup>125</sup>I-BW431/26-AP (1  $\mu$ Ci) were diluted in 0.1 ml phosphate-buffered saline and simultaneously injected into the retro-orbital vein of nude mice bearing bilateral SW1398 xenografts with a tumour volume of 150–300 mm<sup>3</sup>. Iodinated AP was injected into normal DBA/2 mice. At time intervals ranging from 1 h to 168 h after injection, three mice were sacrificed for each determination. Blood was collected and tissues were dissected and weighed, after which the radioactivity was measured by gamma counting. The antibody, conjugate or enzyme uptake in normal tissues and tumours was calculated as the percentage of the injected dose per gram of tissue (% ID/g). Radiolabelled preparations, serum and homogenates of liver and tumour tissue were analysed by gel filtration to determine the stability of the antibody and AP conjugate.

## Results

### *In vitro cytotoxicity*

The cytotoxic effects of etoposide and etoposide-P were determined by measuring the growth of SW1398 cells with a protein dye assay. Etoposide (IC<sub>50</sub> = 22  $\mu$ M) was at least 100 times more toxic than etoposide-P (20% growth inhibition at 200  $\mu$ M) when cells were exposed to drugs for 4 h. Pretreatment of the cells with BW431/26-AP prior to etoposide-P exposure resulted in an increase in cytotoxicity (IC<sub>50</sub> = 70  $\mu$ M, Fig. 1). In contrast, the cytotoxicity of

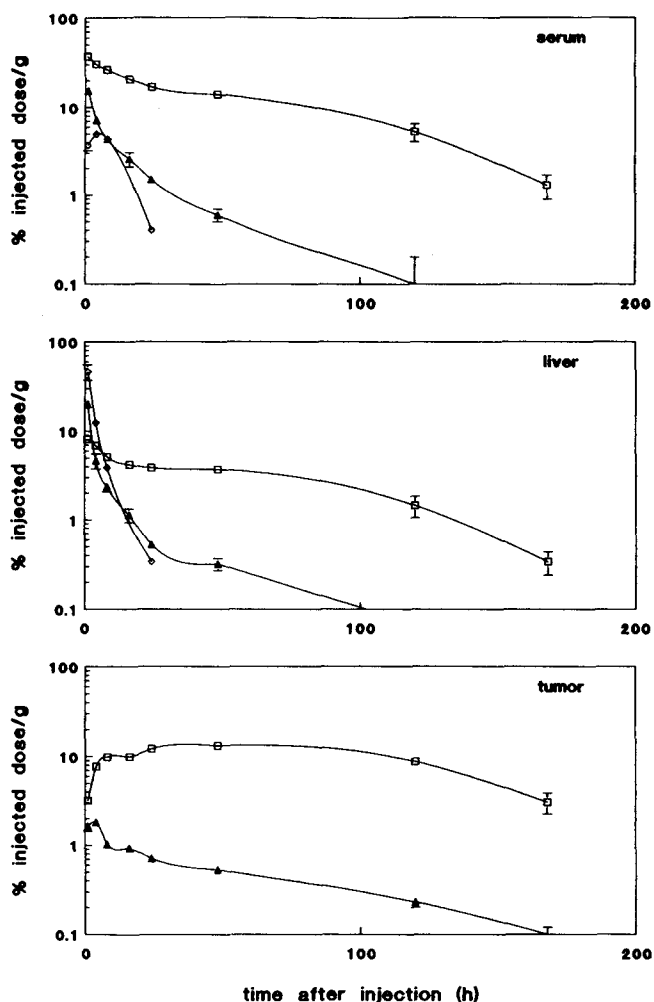


Fig. 2. Pharmacokinetic behaviour of  $^{131}\text{I}$ -BW431/26 ( $\square$ ) and  $^{125}\text{I}$ -BW431/26-AP conjugate ( $\blacktriangle$ ) in nude mice bearing SW1398 xenografts, or  $^{125}\text{I}$ -AP ( $\diamond$ ) in normal DBA/2 mice. Error bars are  $\pm$  SD

etoposide-*P* was similar to that of etoposide when pre-treated cells were exposed to the drugs for 72 h (Fig. 1). BW431/26-AP alone had no effect on cell growth.

The similar cytotoxic effects for etoposide and etoposide-*P* on cells exposed for 72 h were reason to examine the stability of etoposide-*P* in tissue-culture medium. HPLC analysis of medium at 37°C containing 1  $\mu\text{M}$  etoposide-*P* showed that 9.4%, 25.6%, and 48% etoposide was formed in 1 h, 4 h, and 24 h respectively. The fetal calf serum in the medium, which contained 28 U/l AP at pH 8.0, appeared to be responsible for the conversion of etoposide-*P* into etoposide.

#### *In vivo studies*

The pharmacokinetics and biodistribution of the mAb BW431/26, the enzyme AP, and the AP conjugate were studied in mice. Figure 2 shows the pharmacokinetic behaviour of BW431/26, AP, and BW431/26-AP in serum, liver and tumour. BW431/26 cleared from the blood with an average  $t_{1/2}$  of 38.2 h. In comparison, AP cleared faster

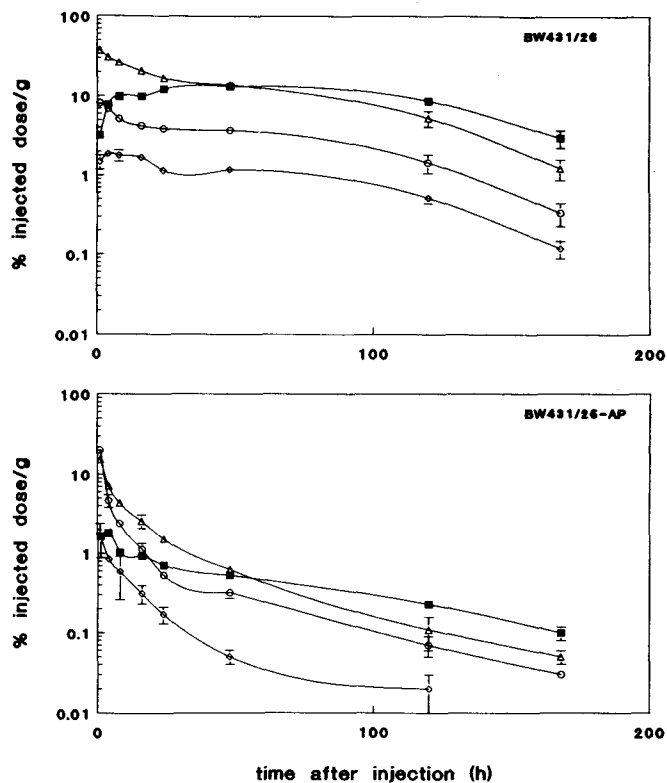


Fig. 3. Biodistribution profiles of  $^{131}\text{I}$ -BW431/26 (upper panel) and  $^{125}\text{I}$ -BW431/26-AP conjugate (lower panel) in nude mice bearing SW1398 xenografts.  $\Delta$ , Blood;  $\circ$ , liver;  $\diamond$ , colon;  $\blacksquare$ , tumour. Error bars are  $\pm$  SD

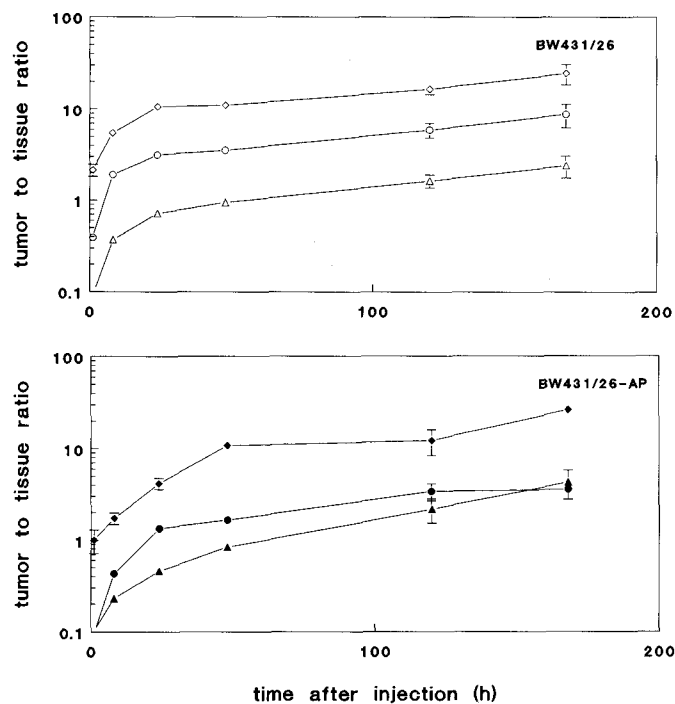


Fig. 4. Tumour-to-tissue ratios of  $^{131}\text{I}$ -BW431/26 (upper panel) and  $^{125}\text{I}$ -BW431/26-AP conjugate (lower panel) in nude mice bearing SW1398 xenografts.  $\Delta$ , Blood;  $\circ$ , liver;  $\diamond$ , colon. Error bars are  $\pm$  SD

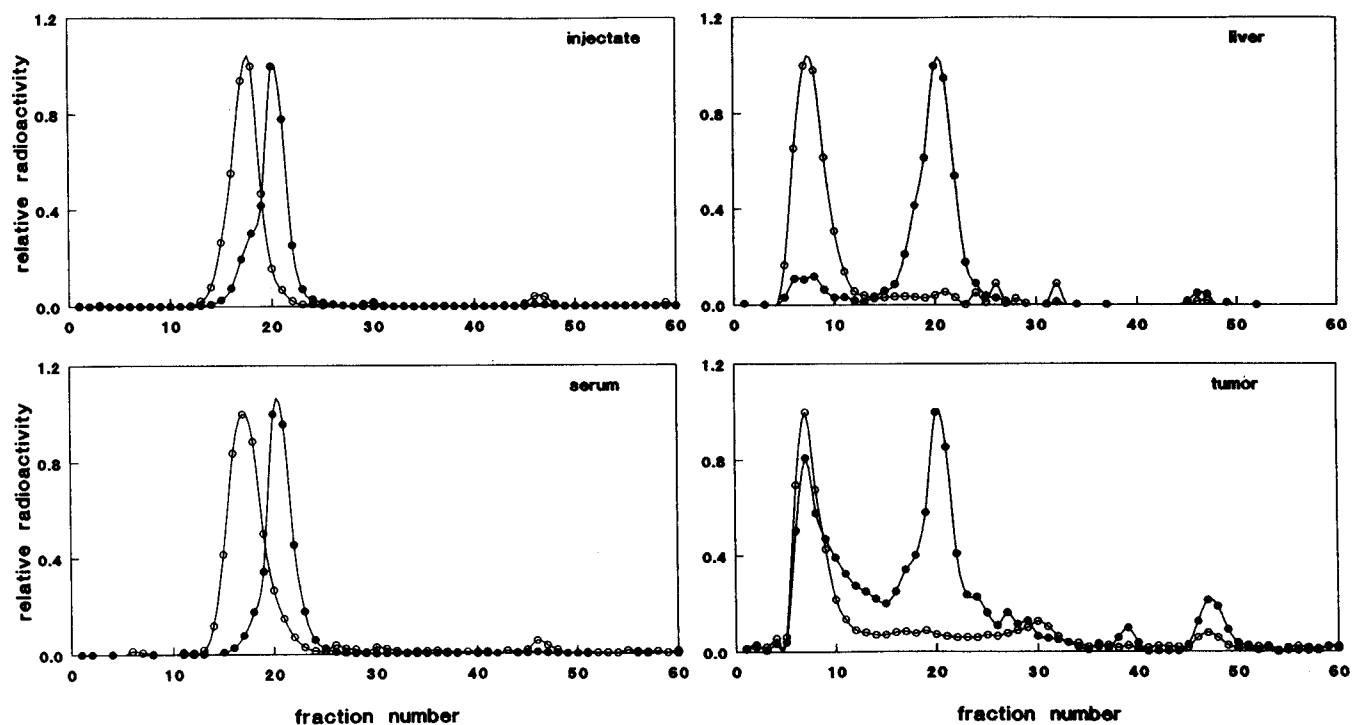


Fig. 5. Gel filtration analysis of  $^{131}\text{I}$ -BW431/26 (O) and  $^{125}\text{I}$ -BW431/26-AP conjugate (●) of the injectate, serum (8 h after injection), liver (8 h after injection), and tumour (4 h after injection) after intravenous injection

into nude mice bearing SW1398 xenografts. Molecular mass markers eluted at fractions 6 (IgM), 20 (IgG), and 24 (bovine serum albumin)

from the circulation with a  $t_{1/2}$  of 6.3 h. The conjugate showed an intermediate blood clearance resulting in a  $t_{1/2}$  of 19.5 h. High liver uptake (20.1% ID/g) was measured upon administration of the AP conjugate, which was similar to the uptake of radiolabelled AP. BW431/26 showed excellent tumour localization. Tumour tissue uptake of the antibody was 10% ID/g 8 h after injection and remained at the same level up to 120 h after injection. The uptake of the AP conjugate was less than that of the unmodified antibody, and decreased from 3% to 0.3% ID/g in the period from 8 h to 120 h after injection.

The distribution profile of both BW431/26 and BW431/26-AP in serum, liver, tumours and colon is illustrated in Fig. 3. In the first 24 h, BW431/26 antibody levels were highest in blood, intermediate in liver and lowest in other tissues. Tumour uptake increased to levels higher than liver in the first 2 h and higher than blood after 48 h. The AP conjugate showed higher levels in liver than in blood for 1 h after injection. Liver levels rapidly decreased thereafter to values lower than in blood. Tumour uptake was higher than that of liver after 24 h and higher than that of blood after 48 h. The distribution profiles of the mAb BW431/26 and the AP conjugate, shown for the colon (Fig. 3), were similar to those in other tissues, such as kidney, heart, muscle and bone. The terminal half-life of the antibody and the conjugate in normal tissues was similar: approximately 35 h. In comparison to normal tissues, clearance of BW431/26 and BW431/26-AP from tumours was slower, with terminal half-lives of 58.8 h and 50.5 h respectively.

Tumour-to-tissue ratios were calculated for blood, liver and colon. The high uptake of BW431/26-AP in the liver at

earlier time points resulted in significantly lower tumour-to-tissue ratios. At times later than 48 h, tumour-to-tissue ratios of BW431/26-AP were similar to those of the unconjugated BW431/26. Tumour-to-tissue ratios rose steadily to above 20 for colon and other tissues 7 days after injection (Fig. 4).

Blood and liver could be analysed by gel filtration up to 8 h and tumours up to 4 h to examine the *in vivo* stability of the AP conjugate. In later samples radioactivity was too low to obtain adequate results. Good stability of the bond between antibody and enzyme was demonstrated, because no shift was observed in the apparent molecular mass of the AP conjugate. In liver and tumour tissue high-molecular-mass complexes were evident for both the antibody and the AP conjugate (Fig. 5). For the mAb BW431/26, complexes accounted for 10% and 40% of the total radioactivity in liver and tumour tissue respectively. The AP conjugate was found to have formed complexes accounting for approximately 100% of the radioactivity in these tissues.

## Discussion

We have demonstrated *in vitro* that the prodrug etoposide-*P* is relatively nontoxic and can be specifically activated into an active cytostatic agent by an mAb-AP conjugate. The mAb 431/26-AP conjugate itself specifically localized in CEA-expressing human tumour xenografts. In comparison with the unconjugated antibody, uptake of the AP conjugate in the tumour was lower, liver accumulation was higher, and clearance from the circulation was more rapid.

Stability of the bond between AP and the antibody appeared to be well maintained.

Prodrugs to be activated by an enzyme, should by themselves be less toxic than the parent compound, and we could demonstrate that the antitumour activity of etoposide-*P* was at least 100 times lower than that of etoposide when cells were exposed for 4 h. However, continuous exposure to etoposide-*P* for 72 h resulted in toxicity similar to that of etoposide, which could be attributed to the AP activity present in the serum in the cell-culture media. Our results are in agreement with those of Senter et al. [17], who also showed a dramatic increase in antitumour activity of etoposide-*P* after preincubation of tumour cells with an AP conjugate.

Proper information on the retention of tumour-localizing properties of an mAb-enzyme conjugate requires *in vivo* biodistribution studies. The pharmacokinetic behaviour and the biodistribution profile give insight into the differences in clearance pattern of that conjugate as compared to those of either the mAb or the enzyme. Apparently the pharmacokinetics of the conjugate depend on the characteristics of both the antibody and the enzyme. For the enzyme, calf intestinal AP, we found a rapid clearance in mice, which has also been reported in dogs [16]. A high liver uptake of the enzyme was found, which may be explained by the fact that intestinal AP does not contain sialic acid, and therefore will bind to asialoglycoprotein receptors present in the liver [8]. The AP conjugate of BW431/26 also cleared rapidly from the circulation and showed a high liver uptake shortly after injection. The differences in pharmacological behaviour between BW431/26 and BW431/26-AP may have been the reason for a relatively low amount of AP conjugate available for uptake in tumour tissue. As with our study, Senter et al. [17] found an initial high liver uptake of an AP-mAb conjugate, and a reduced localization of the conjugate in tumour tissue when compared to that of the unconjugated antibody.

The *in vivo* stability of the conjugate was examined by gel filtration of serum, liver and tumour tissue homogenates. No evidence was found for the early release of the enzyme from the antibody. In liver and tumour tissue high-molecular-mass complexes were detectable with both the antibody and the AP conjugate. In the liver all of the conjugate appeared as a complex. Presumably, the conjugate was bound by the AP moiety to the asialoglycoprotein receptor. In the tumour, complex formation could be attributed to the binding of the BW431/26 mAb or AP conjugate to CEA. No complexes were detected in serum.

Etoposide-*P* was converted into etoposide in tissue-culture medium. After 24 h approximately 50% of the prodrug was transformed into drug. Serum and many other tissues contain AP [4] and *in vivo* it may be expected that phosphorylated prodrugs are rapidly converted into the parent compounds. In a recent report of Rose et al. [13], the antitumour activity of etoposide-*P* in animal models was described. Comparable activities for etoposide and etoposide-*P* were found, probably because of the rapid conversion of etoposide-*P* into etoposide by the AP present in many tissues. Still, Senter et al. [17] were able to show a significant therapeutic advantage with AP conjugate and

etoposide-*P* when compared to etoposide-*P* alone. A substantial amount of etoposide was apparently generated at the tumour site in their experiments. In a clinical situation, however, the amount of antibody conjugate that will localize in the tumour will be 100–1000 times less than that in animal studies. Also, the abundant presence of phosphatases in serum and other tissues will limit the amount of etoposide-*P* to be activated to etoposide in patients' tumours.

For antibody-enzyme-mediated chemotherapy phosphorylated prodrugs do not appear to be the first choice because of rapid activation in serum and other tissues. Also, the relatively unfavourable *in vivo* distribution profile of the AP-conjugated mAb may limit its therapeutic effects. However, various other enzymes are presently under investigation, which may prove of value for this elegant treatment approach.

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