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CHROMATOGRAPHY OF FUNCTIONALIZED LIPOSOMES AND THEIR COMPONENTS

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SUMMARY

The antitumour drug 1-β-D-arabinofuranosylcytosine (ara C) was acylated by means of oleic acid anhydride, resulting in the prodrug N^4-oleoyl-ara C. Together with a lipophilic biotin derivative, this lipophilic prodrug was incorporated into the bilayer membrane of unilamellar liposomes prepared by means of the detergent dialysis method. On addition of these biotinylated prodrug–liposomes to an excess of avidin, biotin residues were complexed with avidin. The unreacted avidin was removed by chromatography on an Ultrogel AcA-22 column. The prodrug–liposome–avidin complex was coupled to biotinylated monoclonal antibodies through the free binding sites of the immobilized avidin. Unreacted antibodies were removed by chromatography on an Ultrogel AcA-22 column. *In vitro*, the liposome–antibody complexes selectively bound to cells which were recognized by the monoclonal antibodies linked to the liposomes. For this reason, a promising strategy towards a specific chemotherapy of cancer is expected.

INTRODUCTION

For the chemotherapy of cancer various attempts have been made to target drugs more efficiently to the tumour cells by means of antibodies, according to the following strategy. Antibodies which are able selectively to recognize tumour cells are linked to liposomes loaded with antitumour drugs or their prodrugs[1–3]. By means of the coupled antibodies, the drug–liposomes should select the tumour cell as a target in order to effect their action exclusively upon it.

An essential presumption for the realization of this concept is the binding of antibodies to drug–liposomes with conservation of their immunological activity. We have found that biotinylated antibodies could be coupled, without losing their immunological activity, to biotinylated drug–liposomes via avidin molecules. The linkage which is due to secondary valency is based on the fact that one avidin can complex up to four biotin residues[4].

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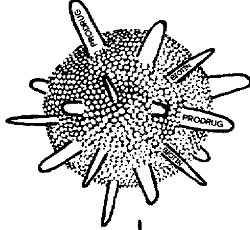
Mixture: PRODRUG + STEARYLAMINE + PE - BIOTIN
+ CHOLESTEROL + EGG PHOSPHATIDYLCHOLINE

(DETERGENS DIALYSIS METHOD)

↓

BIOTINYLATED PRODRUG-LIPOSOME

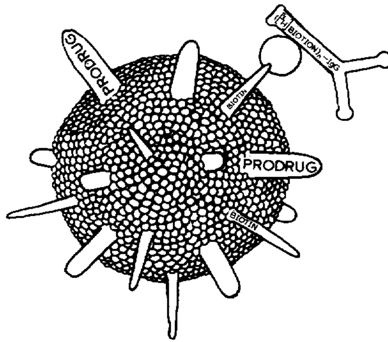


(1.) + AVIDIN ↓ (2.) ULTROGEL AcA 22-COLUMN

PRODRUG-LIPOSOME-BIOTIN···AVIDIN

(1.) + ([^{3}H]BIOTIN)$_n$-IgG ↓ (2.) ULTROGEL AcA 22-COLUMN

PRODRUG-LIPOSOME-BIOTIN···AVIDIN···([^{3}H]BIOTIN)$_n$-IgG



Scheme 1.

The coupling procedure is carried out in a very careful way in three steps, summarized in Scheme 1. In separate reactions, antibodies are biotinylated so as to effect their linkage to avidin. On the other hand, biotinylated liposomes, carrying antitumour drugs, are prepared. In the next step, biotin residues of the drug–liposomes are complexed with avidin. Finally biotinylated antibodies are linked to the remaining free binding sites of the immobilized avidin. This method is now elucidated and the functionalization and chromatographic purification of the prodrug–liposome–antibody complex and the liposomes and antibodies are described.

EXPERIMENTAL

Materials

The hydroxylapatite column (Bio-Gel HPHT) was obtained from Bio-Rad (München, F.R.G.), Sephadex G-50 from Pharmacia (Uppsala, Sweden), Ultrogel AcA-22 from IBF Biotechnics (Villeneuve La Garenne, France), hen egg avidin from Sigma (Taufkirchen, F.R.G.), d-8,9-[^{3}H]biotin succinimidyl ester, 3.7–18.5 GBq/mmol, and [^{3}H]cholesterol, 185–550 GBq/mmol, from Amersham (Braunschweig, F.R.G.), sodium cholate from Merck (Darmstadt, F.R.G.), egg phosphatidylcholine from Lipid Products (South Nutfield, U.K.), PM 30-membrane from Amicon (Witten, F.R.G.) and Aqualuma plus from Baker (Gross-Gerau, F.R.G.). The antibody B8-24-3 (anti-H-2K^b) and the mouse cell line EL 4 (H-2^b) were routinely produced in our laboratory (H.H., R.S.). The human cell line U 937 was a gift from A. Ziegler (Tübingen).

Methods

Biotinylation of B8-24-3. The biotinylation was performed on the basis of a published procedure[5]. ^{3}H-labelled biotinyl-N-hydroxysuccinimide ester (BNHS) (167 nmol, $1 \cdot 10^8$ cpm) dissolved in 19 μl dimethylformamide was added to 970 μl 0.15 *M* sodium chloride, 0.01 *M* sodium dihydrogenphosphate (PBS) (pH 7.3), containing 16.7 nmol B8-24-3 antibody. After shaking for 4 h at room temperature the mixture was chromatographed on a Sephadex G-50 column (20 cm $\times$ 1.5 cm I.D.), which was pretreated with 0.2% bovine serum albumin. The column was eluted with PBS (pH 7.3) at a flow-rate of 0.5 ml/min (see Fig. 1). Fractions of peak I were pooled, an aliquot was added to 4 ml Aqualuma plus and counted in a β-counter for 1 min. The pool was concentrated on an Amicon PM 30 membrane to a volume of 1.65 ml and stored at 4°C.

Preparation of biotinylated prodrug–liposomes. Liposomes containing the condensation product of biotin and 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylethanolamine (PE-biotin) together with the cytostatic prodrug N^4-oleoyl-1-β-D-arabinofuranosylcytosine (N^4-oleoyl-ara C) were prepared essentially according to a published procedure[6]. The mixture of egg phosphatidylcholine (EPC)–cholesterol–PE-biotin–N^4-oleoyl-ara C–α-tocopherol (molar ratio 10:2:2:2:0.01) plus sodium cholate at a molar ratio of total lipids to detergent of 0.6, all dissolved in methanol–chloroform (1:1), was evaporated to dryness with a rotatory evaporator. The liposomes were labelled on addition of [^{3}H]cholesterol. The dry lipid film was dissolved in PBS (pH 7.3) to obtain a micelle solution of 30 mg EPC/ml. The detergent was removed by dialysis during 15–20 h. The liposomes were filtered through 0.45-μm sterile filters and kept at 4°C.

Preparation of the complex prodrug–liposome–biotin–avidin. A suspension of the ^{3}H-labelled prodrug–liposome–biotin complex (48 700 cpm, 13 A_{280} units) in 0.5 ml PBS containing 688 nmol PE-biotin was added to avidin (25 mg, 370 nmol) dissolved in 1 ml PBS. After shaking for 2 h at 4°C the mixture was chromatographed on an Ultrogel AcA-22 column (30 cm $\times$ 2.5 cm I.D.) using PBS (pH 7.3) as eluent at a flow-rate of 2 ml/min (see Fig. 2). By ultrafiltration on an Amicon PM 30 membrane, the fractions of peak I (12.2 A_{280} units) containing the desired complex were concentrated to a volume of 2.44 ml. By counting of an aliquot in 4 ml Aqualuma plus

in a β-counter, the total radioactivity of peak I was calculated to be 45 320 cpm. Fractions of peak II, containing unreacted avidin (18.4 A_{280} units), were desalted and subsequently lyophilized.

Preparation of the complex prodrug–liposome–biotin–avidin–[^{3}H]biotin-B8-24-3. A mixture of the biotinylated antibody (1.47 nmol [^{3}H]biotin–B8-24-3 and 5.88 nmol B8-24-3) dissolved in 1 ml PBS (pH 7.3) was added to the complex prodrug–liposome–biotin–avidin (containing 34 nmol avidin) suspended in 2 ml PBS (pH 7.3). The reaction mixture was shaken for 2 h at 4°C and subsequently chromatographed (see Fig. 3) on an Ultrogel AcA-22 column (30 cm × 2.5 cm I.D.) using PBS (pH 7.3) as eluent at a flow-rate of 2 ml/min. Fractions of peak I, containing the desired complex (112 300 cpm, 8.96 A_{280} units) in 90% yield, were pooled and concentrated to 2 ml on an Amicon PM 30 membrane. In the fractions of peak II, 89% of the unreacted antibody (774 000 cpm, 1.29 A_{280} units) were recovered, concentrated and stored at 4°C.

Cellular radioimmunoassay. The cell lines EL 4 and U 937 were suspended in PBS (pH 7.3) and centrifuged at 160 *g* for 10 min. This washing procedure was repeated three times. Finally, the cells were resuspended in a small volume of PBS. From the cell suspension thus obtained 10^7 cells were transferred to each reaction vessel, and centrifuged for 1 min at 160 *g*. The pellet was incubated with the complex prodrug–liposome–biotin–avidin–[^{3}H]biotin–B8-24-3 (44 000 cpm) or with a mixture of biotinylated antibody ([^{3}H]biotin-B8-24-3 + B8-24-3, 1:5, 49 600 cpm) suspended in 1 ml PBS. The reaction mixtures were incubated at 4°C for 45 min and shaken occasionally. To remove the unbound components, cells were washed six times as described above until the radioactivity of the supernatant could be neglected. Finally the cell pellets were suspended in 150 μl PBS, mixed with 150 μl 1% sodium dodecyl sulphate and, after addition of 4 ml Aqualuma plus, counted in a β-counter for 1 min. The results are summarized in Table I.

TABLE I

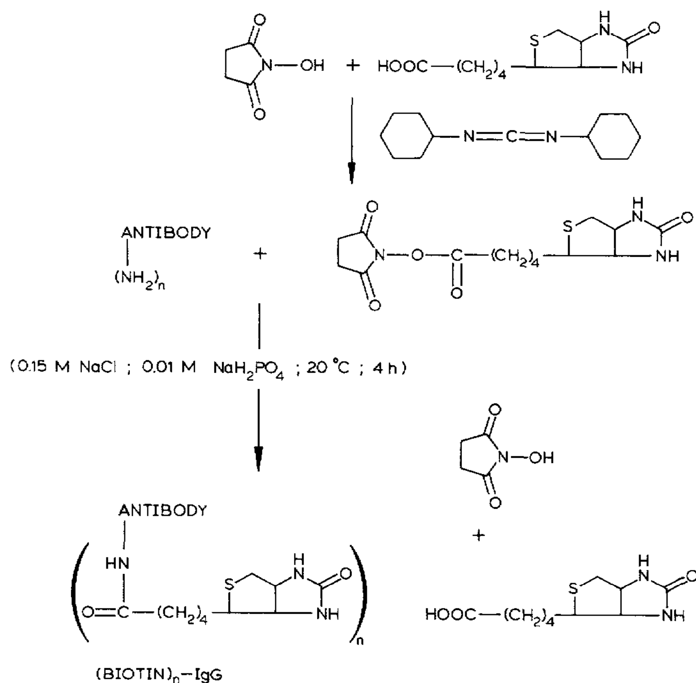
CONDITIONS AND RESULTS OF THE INCUBATION OF MOUSE-TARGET CELLS (EL 4) OR HUMAN CELLS (U 937) WITH THE 1:5 MIXTURE OF BIOTINYLATED ANTIBODY ([^{3}H]BIOTIN-B8-24-3 + B8-24-3) OR WITH THE COMPLEX PRODRUG–LIPOSOME–BIOTIN–AVIDIN–[^{3}H]BIOTIN–B8-24-3

Cell line	*Reagents*	*Incubated amounts (cpm)*	*Bound amounts [cpm (%)]*	*Calculated molecules per cell*
EL 4	[^{3}H]biotin–B8-24-3 + B8-24-3	49 600	300 (0.6)	142 000
U 937	[^{3}H]biotin–B8-24-3 + B8-24-3	49 600	50 (0.1)	24 000
EL 4	Liposome-biotin-avidin-B8-24-3-[^{3}H]biotin$_n$	44 000	1743 (4.0)	116 000
	N^4-Oleoyl-ara C	–	–	$1400 \cdot 10^6$
U 937	Liposome-biotin-avidin-B8-24-3-[^{3}H]biotin$_n$	44 000	303 (0.7)	20 000
	N^4-Oleoyl-ara C	–	–	$240 \cdot 10^6$

RESULTS AND DISCUSSION

For immobilization, monoclonal antibody B8-24-3 (anti-H-$2K^b$) was used. The antibody, classified as $IgG_{1/K}$ and specific to the mouse lymphoma cell line EL 4 (H-2^b), was precipitated from mouse ascites with ammonium sulphate. The precipitate was dissolved, dialyzed and purified by HPLC on a hydroxylapatite column according to known method[7].

The reaction of biotin residues with the antibody was performed as shown in Scheme 2. First, biotin was activated by means of N-hydroxysuccinimide in the presence of N,N-dicyclohexylcarbodiimide (DCC)[5]. The active biotin ester reacted preferably with amino functions of the antibody. For the biotinylation of B8-24-3, commercially available ^{3}H-labelled BNHS was used in a 10-fold molar excess with respect to IgG. The biotinylated antibody was purified by gel chromatography on Sephadex G-50. To avoid non-specific binding of antibodies, the column was pretreated with a 0.2% solution of bovine serum albumin. The elution of the reaction mixture was detected automatically at 280 nm. The radioactivity was determined by measuring aliquots of pooled fractions in a β-counter. The elution profile obtained (see Fig. 1) shows that the radioactively labelled biotinylated B8-24-3 contained in peak I was eluted within the exclusion volume. Low-molecular-weight reaction products were eluted in peak II. 1.6% of the radioactivity applied (10^8 cpm) were eluted in peak I, 68% in peak II. Because of this satisfactory recovery (70%) it is expected that larger amounts of reaction mixtures could be purified on Sephadex G-50. The yield of biotinylation was calculated from the radioactivity and A_{280} units of the pooled



Scheme 2.

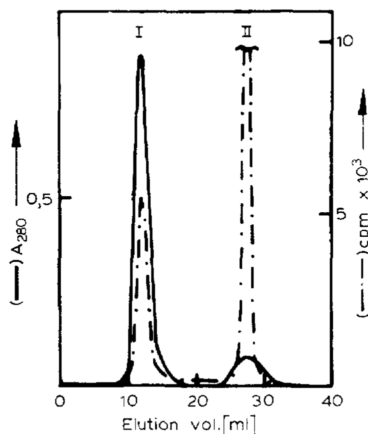
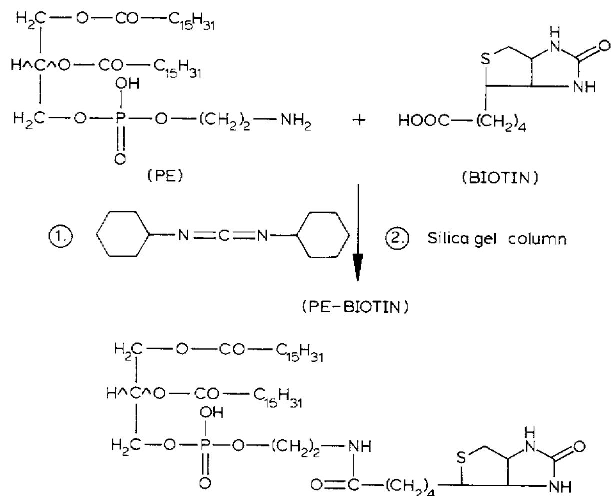


Fig. 1. Gel chromatography of the reaction mixture resulting from the biotinylation of B8-24-3 antibody. The radioactively labelled biotinylated B8-24-3 antibody ($[^3H]biotin)_n$–IgG was eluted from the Sephadex G-50 column (20 cm × 1.5 cm I.D.) in peak I. Peak II contains low-molecular-weight reaction products. The column was eluted with 0.15 *M* sodium chloride, 0.01 *M* sodium dihydrogenphosphate (pH 7.3) at a flow-rate of 0.5 ml/min.

fractions of peak I. The result demonstrated that one biotin residue was incorporated per five molecules of B8-24-3. Gel chromatography could not separate the biotinylated antibodies from the unsubstituted antibodies. Such a separation was not necessary, because only the biotinylated antibodies participated in the following reaction steps. The fractions of peak I were pooled, concentrated by ultrafiltration and stored at 4°C until further use. An aliquot of the concentrate was subjected to an immunofluorescence assay with the result that after biotinylation the biological activity as well as the specificity of the antibody were completely retained without any change after several weeks of storage at 4°C.

For the synthesis of the biotinylated prodrug–liposomes, biotin was converted into a lipophilic derivative so that it could be nearly quantitatively incorporated into the bilayer membrane of the liposomes. For this purpose, 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylethanolamine (PE) was synthesized in analogy to 1,2-dimyristoyl-*sn*-glycero-3-phosphorylethanolamine[8]. The amino group of PE was substituted with biotin in the presence of DCC resulting in the lipophilic biotin derivative (PE-biotin). The reaction summarized in Scheme 3 was realized according to a method described[9] which was modified and adapted to the gram scale. The condensation mixture was purified on a silica gel column by means of a chloroform–methanol gradient, yielding 65% of analytically pure PE-biotin.

N^4-oleoyl-ara C was used as a lipophilic prodrug which was obtained by acylation of 1-β-D-arabinofuranosylcytosine (ara C)[10]. Ara C is one of the most widely used drugs in chemotherapy. To synthesize larger amounts of N^4-oleoyl ara C (about 30 g) we have modified the method described before and purified the reaction mixture on a silica gel column using a chloroform gradient as the eluent. The chemical derivatization of ara C and its quantitative incorporation into the membrane of stable homogeneous unilamellar liposomes significantly improve the antitumour effect of the ara C[11].



Scheme 3.

The biotinylated prodrug–liposomes were prepared by means of the detergent dialysis method[12,13] starting from a mixture of N^4-oleoyl-ara C, PE-biotin, egg phosphatidylcholine, stearylamine and cholesterol/[^{3}H]cholesterol. These radioactively labelled homogeneous liposomes were obtained in sizes ranging from 70 to 90 nm. The liposomes could be stored at 4°C without noticeable degradation during about 300 days. Since 8.5 mg egg phosphatidylcholine were used for the preparation of 0.5 ml liposome suspension, it followed from the calculation of Huang and Mason[14] that $1.63 \cdot 10^{14}$ unilamellar liposomes were contained herein. Assuming that PE-biotin and N^4-oleoyl ara C were each incorporated into the liposomes at 90%, it can be concluded that one liposome contained about 8000 molecules of N^4-oleoyl-ara C and up to 2500 biotin residues.

The addition of an excess of biotinylated prodrug–liposome suspension to avidin resulted in a complex of prodrug–liposome–biotin–avidin (see Scheme I). Surplus avidin was removed by gel chromatography on an Ultrogel AcA-22 column (see Fig. 2, peak II). The fractions of peak II were desalted by ultrafiltration and lyophilized. The avidin thus regained could be used again in other reactions. The radioactivity measured in peak I was equivalent to 92% of the amount applied. Because of this high rate of recovery the Ultrogel AcA-22 column could also be applicable to the purification of larger amounts. Gel chromatography on Sephadex G-100, for example, resulted in up to 60% of the liposomes being irreversibly adsorbed.

The complexing of avidin was evidenced by the change in the absorbance ratio of 280 to 260 nm. From the absorbance ratio of the fractions of peak I, 0.7, it could be estimated that about 41 nmol avidin corresponding to 11% of the amount employed were complexed by 0.5 ml of the liposome suspension. This calculation is based on the absorbance ratios determined for avidin and the biotinylated prodrug–liposomes, 1.60 and 0.53 respectively. Furthermore, it was calculated from the amount of avidin involved in complexation that about 165 avidin molecules were bound by the approximately 2500 biotin residues of one liposome.

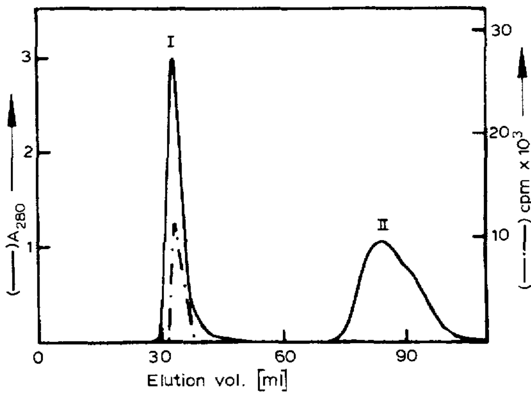


Fig. 2. Gel chromatography of the reaction mixture resulting from the preparation of the complex prodrug–liposome–biotin–avidin on an Ultrogel AcA-22 column (30 cm × 2.5 cm I.D.). The column was eluted as in Fig. 1 but at a flow-rate of 2 ml/min. Fractions of peak I, containing the desired radioactively labelled complex, were pooled and concentrated on an Amicon PM 30 membrane. The fractions of peak II, containing unreacted avidin, were desalted and subsequently lyophilized.

To immobilize the biotinylated antibody B8-24-3, the complex of prodrug–liposome–biotin–avidin suspended in PBS was added to a 1:5 mixture of [^{3}H]biotin B8-24-3 and B8-24-3 in PBS. The reaction mixture was stirred for 2 h at 4°C. Then the complex of prodrug–liposome–biotin–avidin–[^{3}H]biotin–IgG (peak I, Fig. 3) was separated from non-immobilized antibodies (peak II, Fig. 3) by gel chromatography on an Ultrogel AcA-22 column. The pooled fractions of peak I and those of peak II were concentrated by ultrafiltration. The biotinylated B8-24-3 antibody thus regained remained immunologically active and could be used again. From the radioactivity measured in peak I, 90% of the components applied to the column were eluted. Only

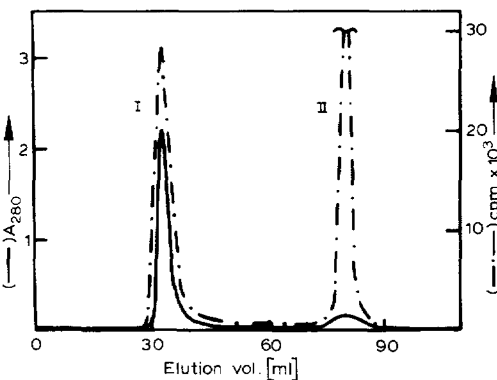


Fig. 3. Gel chromatography of the reaction mixture resulting from the preparation of the complex prodrug–liposome–biotin–avidin–[^{3}H]biotin–B8-24-3 on an Ultrogel AcA-22 column (30 cm × 2.5 cm I.D.). The column was eluted as in Fig. 2. The fractions of peak I, containing the desired complex, and the fractions of peak II, containing unreacted [^{3}H]biotin B8-24-3 and B8-24-3, were pooled and concentrated on an Amicon PM 30 membrane.

8% of the biotinylated antibody added in excess were bound by the avidin complexed with the liposomes. On the basis of this value it was calculated that on average 0.6 antibody molecules were complexed by each liposome.

Finally a cellular radioimmunoassay was performed to test the immunological activity of the immobilized B8-24-3 antibody. The results summarized in Table I showed that the antibody neither lost activity nor specificity after linkage to the liposomes. A proof of the specificity was that six times more B8-24-3 antibody as well as six times more prodrug–liposome–biotin–avidin–[^{3}H]biotin–B8-24-3 were bound by the target cells (EL 4) than by the cells of a human cell line (U 937) which served as a negative control. The activity of the coupled antibody was confirmed by the result that the EL 4 cells bound 26 times more prodrug–liposomes if these were derivatized with the B8-24-3 antibody compared to prodrug–liposomes which were not functionalized with an antibody.

CONCLUSIONS

By direct incorporation of lipophilic prodrugs into lipid membranes of liposomes, it is possible to establish a slow-release drug-carrier system with the following advantages: the efficiency of the incorporation of the prodrug into the lipid bilayer matrix is quantitative due to its lipophilic property; leakage into the aqueous environment does not occur and protection of the drug against metabolic degradation is improved and longer-lasting therapeutic levels can be achieved. As a consequence, the amount and sequence of application of the administered drug can be reduced. Drug-targeting by coupling antibodies specific for tumour cells to those prodrug–liposomes should result in a preferential binding to the tumour cell *in vivo* as well. By this very promising method, the cytostatic effect can be improved furthermore and simultaneously the systemic toxicity of the cytostatics can be reduced.

For immobilization of the antibodies to prodrug–liposomes our coupling method involving the biotin–avidin system offers considerable advantages over the chemical methods used so far. The compounds needed for the linkage can be prepared, tested and stored separately. The coupling reaction proceeds spontaneously with the addition of the reaction partners and does not require any coupling agents, contrary to the chemical condensation reactions. Thus unknown side reactions are excluded from the beginning so that complicated and time-consuming purification steps as well as loss of activity are avoided. By varying the amounts of the components needed for forming the prodrug–liposome–antibody complex, the composition of the complex can be deliberately changed within broad limits. Purification of the components on a preparative scale is possible as well as their recycling without change of activity.

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