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ORIGINAL ARTICLE

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Preparation and functional evaluation of new doxorubicin immunoconjugates containing an acid-sensitive linker on small-cell lung cancer cells

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Abstract The anthracycline doxorubicin (DOX) is one of the most effective drugs for the treatment of small-cell lung cancer (SCLC), but its clinical application is limited by unspecific side-effects like cardiotoxicity. In the present study doxorubicin was conjugated to the monoclonal antibodies (mAb) SEN7, MOC31, and SWA11 via a novel acid-sensitive hydrazone linker. These mAb recognize SCLC-associated antigens of cluster 1 (NCAM), cluster 2 (EGP-2/GA733-2), and cluster 4 (CD24) respectively. To assess their potential therapeutic use against SCLC, the antigen-binding activities, the rates of internalization and the cytotoxic effects of the immunoconjugates were examined on tumour cell lines. The preparation procedure preserved the antigen-binding activities of the mAb and yielded immunoconjugates with average drug:mAb ratios of 7:1. The hydrazone linker was found to be stable at neutral pH but to release doxorubicin under acidic conditions. In contrast to SEN7-DOX, MOC31-DOX and SWA11-DOX were rapidly internalized into SCLC target cells upon binding to their specific cell-surface antigens. Accordingly, both immunoconjugates proved to be highly cytotoxic agents, inhibiting thymidine incorporation by 50% at concentrations between 0.5 μM and 1 μM and were 100-fold more selective than free doxorubicin. The results suggest that binding to selective cell-surface antigens, rapid internalization and efficient release of doxorubicin from the mAb by acid hydrolysis are required for the selective and potent function of the immunoconjugates. In particular, the use of MOC31-DOX for targeted cytotoxic therapy might be promising because of the limited cross-reactivity of the mAb with normal human tissues and its recently demonstrated tumour localization potential in SCLC patients.

Key words Small-cell lung cancer · Monoclonal antibodies · Doxorubicin · Immunoconjugates

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Introduction

Small-cell lung cancer (SCLC) accounts for more than 20% of all lung cancers [2]. At the time of diagnosis most patients already present an advanced and disseminated disease that is not amenable to surgery or irradiation. Chemotherapy is the standard treatment but, despite high initial response rates, most patients relapse within 1 year with tumours in a form refractory to conventional cytotoxic treatments [13, 28]. The long-term prognosis of SCLC is therefore poor, with a 2-year survival of less than 5% [28]. This circumstance calls for the development of novel targeted therapies with the potential to increase the concentration of cytotoxic drugs at the tumour site and to provide a high degree of selectivity.

A number of well-characterized SCLC-associated antigens have been evaluated as targets for the selective delivery to SCLC cells of cytotoxic agents attached to monoclonal antibodies (mAb). In particular, mAb recognizing the neural cell-adhesion molecule (NCAM), the EGP-2/GA733-2 antigen and CD24 proved to be efficient carriers for radioisotopes [25] and protein toxins [6, 36, 37] to SCLC. Despite their selective and potent antitumour effects, the use of radioimmunoconjugates and immunotoxins in patients is associated with less well-characterized and predictable side-effects that limit their therapeutic benefit [9]. In view of this clinical problem, immunoconjugates that employ the cytotoxic potential of well-known conventional drugs like anthracyclines, will be of therapeutic advantage.

Immunoconjugates employing the anthracycline doxorubicin have been prepared by different conjugation techniques [7, 10, 11, 22, 35]. It has been consistently found that the drug must be released from the antibody to be cytotoxically active. Stable linkage of doxorubicin to proteins through the amino sugar or the C-13 keto group frequently resulted in functional inactivation [1, 3, 14]. Therefore, cleavable linkers, like those incorporating an acid-sensitive hydrazone bond, have been developed. These provide a high degree of stability under neutral conditions

but ensure the release of active drug under acidic conditions found in the extracellular environment of solid tumours and in lysosomes [17, 18, 30].

The aim of the present study was to assess the potential use of the mAb MOC31, SWA11 and SEN7 for the development of highly selective and potent doxorubicin immunoconjugates for targeted chemotherapy of SCLC. For this purpose, doxorubicin was linked to the mAb through a novel hydrazone-thioether linker, which was synthesized by modification of a commercially available crosslinking reagent, and the immunoconjugates were tested on SCLC cell lines by detailed in vitro examinations.

Materials and methods

Cell lines and cell culture

The following SCLC cell lines were used: NCI-H69 (H69) and H249 (kindly provided by D. N. Carney, Mater Misericordiae Hospital, Dublin, Ireland). The erythroleukaemia cell line U937 was used as an antigen-negative control. Tumour cells were maintained in exponential growth in RPMI-1640 medium (Gibco, Life Technologies, UK) supplemented with 4 mM 1-glutamine and 10% fetal calf serum (cell culture medium) at 37 °C in a humidified atmosphere containing 5% CO_2.

Monoclonal antibodies (mAb)

All mAb were produced by standard hybridoma technology following immunization of mice with SCLC cells. The IgG1 MOC31 was kindly provided by Dr. L. de Leij, Academic Hospital Groningen, The Netherlands. It recognizes the epithelial glycoprotein EGP-2 (lung cancer antigen cluster 2), which is expressed on a variety of carcinomas [19]. The IgG1 SEN7 recognizing an epitope on the neural cell-adhesion molecule (NCAM, lung cancer antigen cluster 1) [33] and the IgG2a SWA11 recognizing the B-cell differentiation antigen CD24 (lung cancer antigen cluster 4) [15] were produced in our laboratory. The mAb were isolated from hybridoma supernatants by sequential affinity and size-exclusion chromatography on protein A and Superose 12 columns (Pharmacia Biotech, Uppsala, Sweden). The number of binding sites of the mAb on SCLC cells was routinely calculated by Scatchard analysis as described [24]. On H69 cells, average numbers of 4.5×10^5, 3.5×10^5 and 8×10^5 binding sites/cell were determined for MOC31, SEN7 and SWA11 respectively (data not shown).

Preparation of immunoconjugates

The doxorubicin immunoconjugates were prepared by attaching the 13-acylhydrazone derivative of the drug to sulphydryl-containing mAb essentially as described by Willner et al. [35] with slight modifications. Instead of 6-maleimidocaproic acid we used the commercially available crosslinking reagent succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC, Pierce Chemical Company, Rockford, Ill., USA) as starting reagent. Doxorubicin was kindly provided by Farmitalia Carlo Erba, Milan, Italy.

Synthesis of the hydrazone derivative of doxorubicin

The first step in the preparation of the hydrazone derivative of doxorubicin (DOX-HZN) consisted in the reaction between SMCC and *tert*-butyl carbazate, a protected form of hydrazine, to give its hydrazide derivative. A 200-mg sample of SMCC was dissolved in 3 ml dimethylsulphoxide (Sigma, St. Louis, USA) and stirred under nitrogen at room temperature. A solution containing 78 mg *tert*-butyl carbazate in 3 ml dimethylsulphoxide containing 60 µl *N*-methylmorpholine as catalyst was added dropwise. After 2 h the product was isolated by preparative high-performance liquid chromatography (HPLC) using a MN 250/21 Nucleosid C_8 300/5 column and a linear gradient of 1% trifluoroacetic acid in water to 10% (1% trifluoroacetic acid in water/90% acetonitrile) over 60 min. The flow rate was adjusted to 8.0 ml/min and the product was detected by spectrophotometry at 214 nm. To deblock the reactive amino group, the solvent was evaporated and the product was redissolved in trifluoroacetic acid. After 20 min the acid was removed under high vacuum and 97 mg hydrazide derivative of SMCC was obtained by precipitation with acetonitrile. The identity of the product was verified by mass spectroscopy (Electrospray in 50% methanol/50% water containing 1% acetic acid: M+1 = 252) and thin-layer chromatography analysis (methylene chloride/methanol = 9 : 1).

In a second step doxorubicin hydrochloride (58 mg, 940 µmol) and the hydrazide derivative of SMCC (68.1 mg, 1880 µmol) were dissolved in 11.8 ml anhydrous methanol. Trifluoroacetic acid (3.3 µl) was added and the solution was stirred under nitrogen for 24 h at room temperature. The product was isolated by crystallization in acetonitrile to give 51 mg (66 µmol). The solid hydrazone was identified and characterized by mass spectroscopy (Electrospray in 50% methanol/50% water containing 1% acetic acid: M+1 = 778) and TLC (methylene chloride: methanol = 9 : 1). The final product contained less than 5% unreacted doxorubicin, as analysed by a Waters HPLC system consisting of an ET 250/8/4 Nucleosil C_{18} 300/5 column and a mobile phase consisting in a linear gradient of 85% (3% ammonium acetate in water/15% isopropanol) to 55% (3% ammonium acetate in water/45% isopropanol) over 30 min.

Conjugation of mAb with DOX-HZN

To generate reactive sulphydryl groups, the mAb were reduced with a 10/1 molar ratio of dithiothreitol/mAb at room temperature under nitrogen. The dithiothreitol was separated 3 h later by gel chromatography on Sephadex G25 columns (NAP25, Pharmacia Biotech, Uppsala, Sweden) and the number of sulphydryl groups was quantified using Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid, Pierce, Chemical Company) as described [21]. There were six to eight sulphydryl groups, depending on the mAb. The molecular integrity of the reduced mAb was determined by polyacrylamide gel electrophoresis (PAGE) analysis under native and denaturing conditions using a PhastGel Electrophoresis System (Pharmacia). The reduced mAb were placed on ice and equimolar amounts of DOX-HZN, dissolved in anhydrous methanol, were added under nitrogen and stirring for 1 h, before the reaction was stopped by the addition of 0.5 mM cysteine. After ultracentrifugation at 45 000 rpm at 4 °C for 10 min, the mAb-DOX conjugates were purified by gel chromatography on Sephadex G-25 columns (NAP25, Pharmacia). The concentrations of doxorubicin and mAb were determined by spectrophotometry at 495 nm and 280 nm respectively and the molecular integrity of the final product was analysed by sodium dodecyl sulphate-PAGE analysis under non-reducing conditions. Contamination with unreacted doxorubicin or DOX-HZN was less than 5% as determined by HPLC analysis on a Waters system using a 250/8/4 Kromasil C_8 100/7 column (Nobel AB, Surte, Sweden) and elution at a flow rate of 0.6 ml/min with a mixture of 25% isopropanol in an aqueous solution of 3% ammonium acetate. The yields of intact immunoconjugates ranged between 70% and 90% depending on the mAb. The immunoconjugates were snap-frozen in liquid nitrogen and stored at –70 °C.

Release of doxorubicin from immunoconjugates

The release of doxorubicin from the immunoconjugates under acidic conditions was monitored by HPLC analysis. Samples were incubated at concentrations of 2 µM in 50 mM sodium acetate buffer pH 5.0 or in 50 mM phosphate buffer pH 7.4 at 37 °C. At various assay times 100-µl aliquots were analysed using a Waters HPLC system (model 600 E pump and a U6K injector) equipped with a 250/8/4 Kromasil C_8 100/7 column (Nobel AB). Doxorubicin was detected by spectrophotometry at 280 nm. The mobile phase consisted of a mixture of

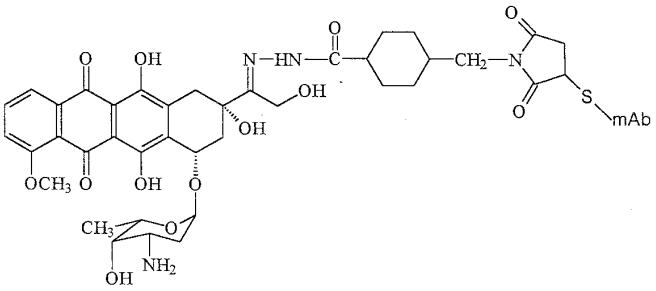

Fig. 1 Chemical structure of the doxorubicin immunoconjugates prepared in the present study

25/75 isopropanol/3% ammonium acetate and the flow rate was adjusted to 0.6 ml/min. Data acquisition and analysis were performed using a SIC Chromatocorder 12 (Sic System Instruments, Dover, Mass., USA).

Cell binding analysis

The cell-binding affinities of the mAb and the immunoconjugates were determined on H69 SCLC cells by indirect immunofluorescence analysis using fluorescence-activated cell sorting (FACScan; Becton Dickinson, Mountain View, Calif., USA) equipped with a four-log-decade full-scale amplifier gain and an analogue-to-digital converter with 1024 channels. Cells (10^6) were incubated on ice in serial dilutions of mAb or doxorubicin immunoconjugates in 1% bovine serum albumin/0.04% sodium azide in phosphate-buffered saline (PBS) in a final volume of 0.1 ml. After 1 h of incubation, cells were washed and incubated with 0.1 ml 0.2 μM solution of fluorescein-isothiocyanate(FITC)-conjugated goat $F(ab)'_2$ anti-(mouse IgG) (Jackson ImmunoResearch Lab., West Grove, Pa., USA) for 45 min. Cells were washed in PBS and the remaining fluorescence on the cell surface was quantified. The mean fluorescence intensity values were plotted against the molar concentrations and the affinity constants (given as the dissociation constant, K_d, the concentration at which 50% of the ligands were bound) were calculated from extrapolated curves.

Internalization studies

The rates and kinetics of internalization of mAb into SCLC cells were determined using ^{125}I-labelled mAb and indirect immunofluorescence analysis. The mAb SWA11, SEN7 and MOC31 were radiolabelled with ^{125}I using the Bolton and Hunter reagent (Amersham International plc, Buckinghamshire, UK) according to the manufacturer's recommendation. The specific activities of the iodinated mAb ranged between 1.5 mCi/mg and 2 mCi/mg. A sample of 10^6 H69 SCLC cells in a volume of 0.1 ml was incubated with the radiolabelled mAb at final concentrations of 10 nM for 1 h on ice. Cells were washed three times with ice-cold PBS/1%BSA to eliminate unbound mAb and further incubated at 37 °C to allow internalization of surface-bound ligands. At different assay times, cells were washed again with ice-cold PBS/1%BSA and the surface-bound radioactivity was removed by treatment of cells on ice with either 5 units pronase (Boehringer Mannheim GmbH, Germany) for 2 h (MOC31), 50 mM glycine HCl/100 mM NaCl pH 2.8 for 15 min (SEN7) or 5 units phosphatidylinositol-specific phospholipase C (Boehringer Mannheim) for 2 h (SWA11). Treatment of cells with PBS/1%BSA was used as control to determine the total cell-associated radioactivity. All cells were washed with ice-cold PBS/1%BSA and their radioactivity was measured using a gamma counter (1272 CliniGamma, Pharmacia-LKB, Uppsala, Sweden).

For indirect internalization analysis, 10^6 H69 SCLC cells in a volume of 0.1 ml were incubated with mAb, immunoconjugates or an isotype-matched control mAb (MOPC-21, Sigma, St. Louis, Mo., USA) at final concentrations of 0.1 μM for 1 h on ice. Cells were washed to remove unbound ligands and resuspended in tissue-culture medium for further incubation at 37 °C to allow internalization of surface-bound ligands. At different assay times cells were placed on ice, washed with PBS/1%BSA containing 0.05% sodium azide and stained for residual surface-bound mAb or immunoconjugates by incubation with 0.1 ml 0.2 μM solution of FITC-labelled goat $F(ab)'_2$ anti-(mouse IgG) (Jackson ImmunoResearch Lab.) for 45 min. Finally, cells were washed in PBS, fixed with 3.6% formaldehyde for 10 min and the surface-bound fluorescence was quantified by FACScan analysis.

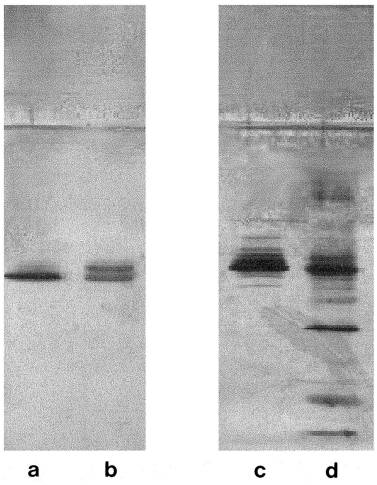

Fig. 2 Sodium dodecyl sulphate (SDS)/polyacrylamide gel electrophoresis analysis of MOC31 as a representative example after reduction of the interchain disulphide bonds with dithiothreitol. Samples were electrophoresed on a polyacrylamide gel and silver-stained. **a** MOC31 non-reduced, **b** MOC31 non-reduced treated with SDS at 95 °C; **c** MOC31 reduced with dithiothreitol, **d** MOC31 reduced with dithiothreitol and treated with SDS at 95 °C

Cytotoxicity assays

The cytotoxicity of doxorubicin and its immunoconjugates on SCLC and antigen-negative control cells was determined in [^{3}H]thymidine-incorporation assays and in limiting-dilution clonogenic assays essentially as described [37]. For [^{3}H]thymidine incorporation 0.5-ml aliquots of single-cell suspensions at a density of 2×10^5/ml in cell-culture medium were incubated in the presence of 0.5 ml serially diluted doxorubicin immunoconjugates, free doxorubicin or medium (control) for 2 h at 37 °C under cell-culture conditions. Cells were washed with PBS, resuspended in 1 ml cell culture medium and 0.2-ml aliquots were distributed into the wells of 96-well tissue-culture plates. After 42 h of incubation at 37 °C, cells were pulsed with 1 μCi/well [^{3}H]thymidine (Amersham) for 6 h and harvested onto glass-fiber discs. The concentration at which [^{3}H]thymidine incorporation was inhibited by 50% compared to controls (IC_{50}) was determined in quadruplicate by β-scintillation counting (RackBeta, LKB-Pharmacia).

For limiting-dilution clonogenic assays, 1-ml aliquots of single-cell suspensions at a density of 1×10^6/ml in cell-culture medium were placed into 6-well tissue-culture plates and an equal volume of serially diluted doxorubicin immunoconjugates, free doxorubicin or medium (control) was added to each well for 24 h. Cells were transferred into tubes, washed, resuspended in 2 ml tissue-culture medium and serially diluted tenfold into 6 wells of a 96-well tissue-culture plate for incubation for 14–20 days under cell-culture conditions.

Clonogenic tumour cells were defined as growing in colonies of at least 50 cells. The number of clonogenic units per well was calculated with the Spearman estimator [16] using diluent-treated cell cultures as standards.

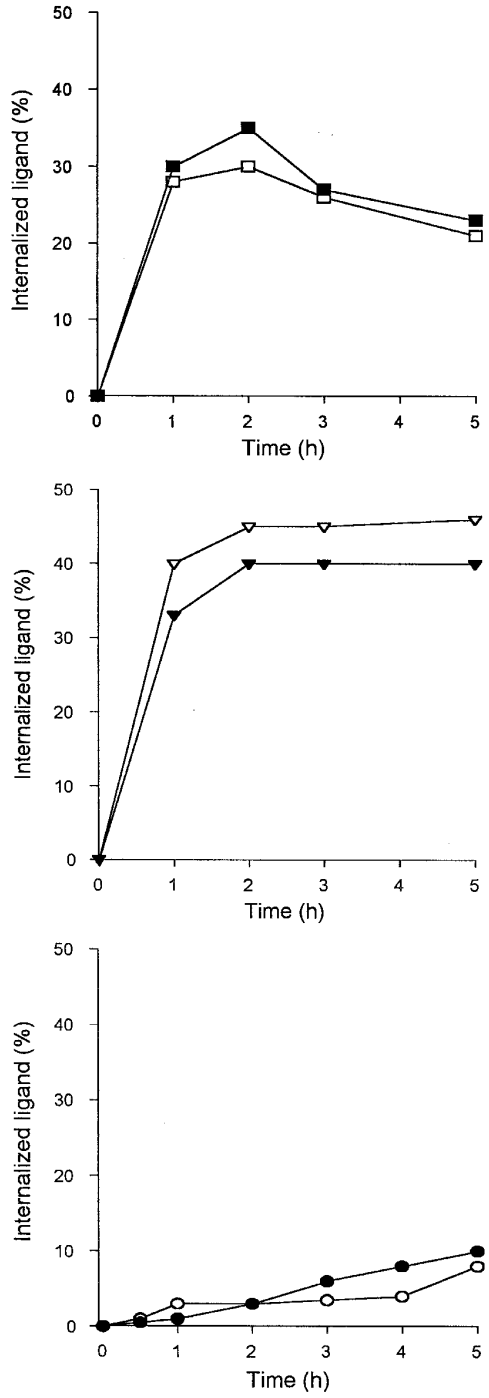

Fig. 3 Internalization of mAb and doxorubicin (DOX) immunoconjugates into H69 small-cell lung cancer (SCLC) cells. Cells were incubated on ice for 60 min with saturating amounts of either MOC31 (■), SWA11 (▼), SEN7 (●), MOC31-DOX (□), SWA11-DOX (▽), or SEN7-DOX (○). Cells were washed and incubated at 37 °C to allow internalization of the ligands. At different assay times, cells were placed on ice and stained for surface-bound ligands using fluorescein-isothiocyanate labelled anti-(mouse IgG) antibodies. Cell-surface fluorescence was determined by fluorescence-activated cell sorting (FACScan) analysis

Table 1 Cell-binding activities of mAb and doxorubicin(*DOX*) immunoconjugates on H69 SCLC cells determined by indirect immunofluorescence analysis. K_d is the concentration at which 50% of the ligands were bound on the cell surface

mAb or immunoconjugate	Affinity constant, K_d (M)
MOC31	2×10^{-9}
MOC31-DOX	2.5×10^{-9}
SWA11	1×10^{-8}
SWA11-DOX	1×10^{-8}
SEN7	1×10^{-9}
SEN7-DOX	1×10^{-9}

Table 2 Kinetics of internalization of radiolabelled mAb into H69 SCLC cells. Internalization rates were calculated by dividing the radioactivity associated with control-treated cells by the radioactivity associated with cells following treatment with pronase (MOC31), phospholipase C (SWA11) or acid (SEN7)

	Internalized mAb (%)		
Incubation time (h)	MOC31	SWA11	SEN7
0.5	12	17	0
1	25	32	
2	31	39	1
5	25	41	8

Results

Preparation and structure of immunoconjugates

In the present study doxorubicin immunoconjugates were prepared by linking a novel hydrazone derivative of the drug to the sulphydryl groups of the mAb (Fig. 1). This hydrazone derivative was prepared in a two-step reaction. First, the commercially available cross-linking reagent SMCC was reacted with a blocked form of hydrazine (*tert*-butylcarbazate). In the second step the protected reactive amino group of the SMCC derivative was subjected to acid hydrolysis and reacted with the 13-keto group of the anthracycline. The final product could be isolated by crystallization in acetonitrile with a yield of 70% and contained less than 5% free doxorubicin (not shown).

From the molecular structures of mAb of the IgG1 and IgG2a subclasses, it can be expected that splitting of the interchain disulphide bonds by mild reduction with dithiothreitol can generate seven or eight sulphydryl groups per mAb molecule. A representative SDS-PAGE analysis of the mAb MOC31 is given in Fig. 2. As can be seen, reduction of the mAb with dithiothreitol at molar ratios of 10/1 preserved their molecular integrity and only trace amounts of free heavy- and light-chain fragments could be detected. In contrast, treatment of the reduced mAb with SDS at 95 °C resulted in their disintegration. As further shown by FACScan analysis, the reduced mAb fully retained their cell-binding activities (data not shown). The reaction between the sulphydryl groups of the reduced mAb and the maleimide residue of DOX-HZN was rapid. It resulted in

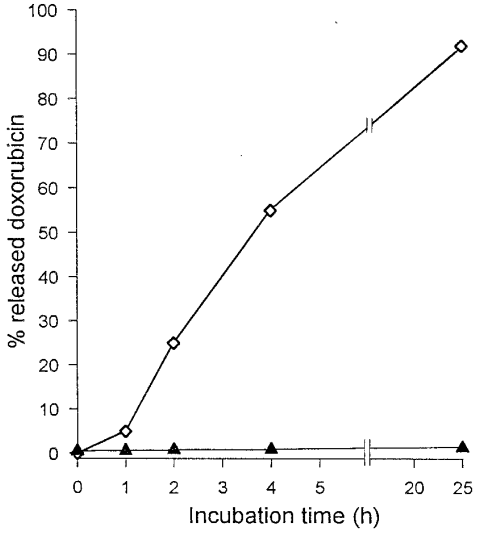

Fig. 4 pH-dependent release of doxorubicin from a representative doxorubicin immunoconjugate. SEN7-DOX was exposed to pH 5.0 (◇) or pH 7.4 (▲) at 37 °C. At different assay times, samples were analysed by HPLC to determine the amount of doxorubicin released from the antibody

high yields (80%–90%) of intact immunoconjugates and, in agreement with the number of available interchain sulphydryl groups, an average of seven to eight doxorubicin molecules per mAb. HPLC and SDS-PAGE analysis of the final products revealed immunoconjugate preparations containing only trace amounts of heavy and light chains and less than 5% of unbound doxorubicin (data not shown).

Cell-binding affinities of mAb and immunoconjugates

The cell-binding affinities of the mAb MOC31, SWA11 and SEN7 and the respective immunoconjugates were examined on H69 SCLC cells by indirect immunofluorescence staining and FACScan analysis. As shown in Table 1, the immunoconjugates fully retained the cell-binding activities of their respective unconjugated mAb.

Internalization of mAb and doxorubicin immunoconjugates

The rates of internalization of mAb and immunoconjugates into H69 SCLC cells were determined using radiolabelled mAb and indirect immunofluorescence staining of surface-

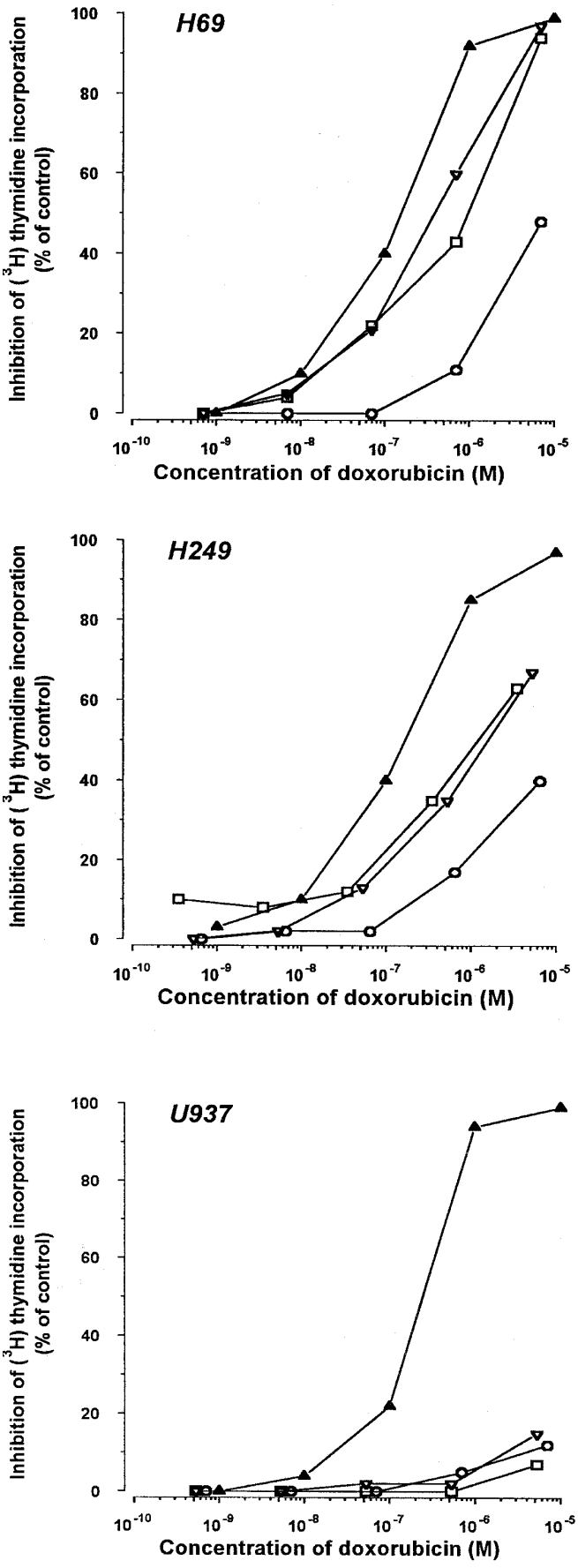

Fig. 5 Cytotoxicity of doxorubicin and doxorubicin immunoconjugates on SCLC cells in cell-proliferation assays. The antigen-positive SCLC cell lines H69 and H249 and the antigen-negative U937 control cell line were incubated with serial dilutions of doxorubicin (▲), MOC31-DOX (□), SWA11-DOX (▽) and SEN7-DOX (○) for 2 h under cell-culture conditions. Cells were washed and cultured for additional 46 h. Data are presented as the percentage inhibition of [^{3}H]thymidine incorporation relative to diluent-treated cells. Concentrations represent doxorubicin molar equivalents

bound ligands at different assay times following incubation at 37 °C. As shown in Table 2, after 2 h of incubation radiolabelled MOC31 and SWA11 were rapidly internalized (31% and 39%, respectively). Within the following 3 h, the internalized fraction of MOC31 slightly decreased to 25% whereas that of SWA11 remained stable. In contrast, the amount of internalized SEN7 was low (8%) even after prolonged incubation, and the mAb remained relatively fixed on the cell surface.

The rates of internalization of the doxorubicin immunoconjugates were determined by indirect immunofluorescence and FACScan analysis and compared with those of the unconjugated mAb. As shown in Fig. 3, the immunoconjugates MOC31-DOX, SWA11-DOX and SEN7-DOX were internalized at rates comparable to those of the respective unconjugated mAb.

Release of doxorubicin from immunoconjugates under acidic conditions

To assess the behaviour and the stability of the acid-labile hydrazone linker, the immunoconjugate SEN7-DOX was incubated either at pH 5.0 or 7.4 at 37 °C and the amount of doxorubicin released was determined by HPLC analysis at different assay times. As shown in Fig. 4, the release of doxorubicin from the antibody was strictly pH-dependent. At pH 5.0, 50% of the drug was released after 3.5 h of incubation. In contrast, the linker was very stable at pH 7.4 releasing less than 5% of the doxorubicin even after 24 h of incubation. As determined in cytotoxicity assays, the drug released from the antibody retained its full functional activity (data not shown).

Cytotoxicity of doxorubicin immunoconjugates

The cytotoxicity of the immunoconjugates was examined on antigen-positive H69 and H249 SCLC cells and on antigen-negative U937 control cells. As demonstrated in [^{3}H]thymidine incorporation assays, following a 2-h exposure the IC_{50} values of MOC31-DOX and SWA11-DOX on antigen-positive cells ranged between 0.5 μM and 1 μM (Fig. 5). In comparison, the cytotoxicity of SEN7-DOX was more than 10-fold lower (IC_{50} >10 μM). Although the cytotoxicity of free doxorubicin was approximately 5-fold higher than that of the immunoconjugates, the latter were 100-fold more selective as determined on U937 control cells (Fig. 5).

As demonstrated in clonogenic assays (Fig. 6), the killing efficiencies of MOC31-DOX and SWA11-DOX but not of SEN7-DOX could closely match that of free doxorubicin. At concentrations of 0.5 μM, on average they reduced the H69 cells in the culture by a factor of 100. The high selectivity of the antitumour effects mediated by the immunoconjugates was demonstrated on U937 control cells where 0.5 μM of either immunoconjugate reduced the cell number by less than a factor of 5. In comparison, an equivalent concentration of doxorubicin reduced the surviving fraction by more than a factor of 10^4. Significant unspecific cell killing of the immunoconjugates was only observed when the concentrations exceeded 5 μM.

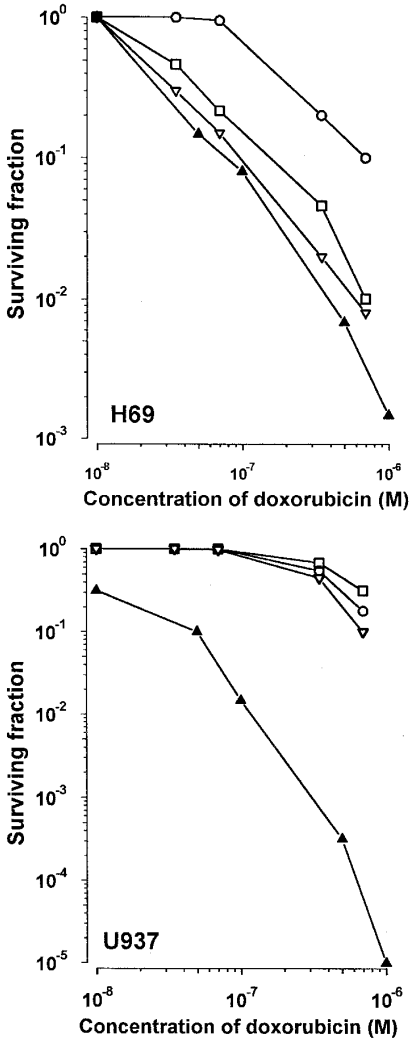

Fig. 6 Cell killing efficiencies of doxorubicin and doxorubicin immunoconjugates in limiting-dilution clonogenic assays. Antigen-positive H69 SCLC and antigen-negative U937 control cells were incubated in serial dilutions of doxorubicin (▲), MOC31-DOX (□), SWA11-DOX (▽) or SEN7-DOX (○) for 24 h under cell-culture conditions. Cells were washed and distributed into 96-well tissue-culture plates. The surviving fractions were determined as described in Materials and methods, following incubation for an additional 2–3 weeks

Discussion

In the present study we prepared acid-sensitive doxorubicin immunoconjugates and assessed their potential for therapeutic use against SCLC by detailed in vitro examinations. The immunoconjugates were composed of mAb directed against highly selective cell-surface antigens on SCLC cells and the anthracycline doxorubicin, a cytostatic drug used

for the treatment of SCLC. For synthesis, we used commercially available reagents to generate a linker containing a stable thioether and an acid-sensitive hydrazone bond. This type of linkage was designed to be stable under neutral conditions but to release active drug under acidic conditions [35].

The antigens recognized by the mAb MOC31, SWA11, and SEN7, are well-characterized cell-surface molecules, EGP-2/KSA, CD24 and NCAM respectively. They are homogeneously and abundantly expressed on SCLC cell lines and tumour tissues and show limited distribution on normal human tissues [26, 27]. The mAb MOC31, SWA11 and SEN7 have been intensively investigated in preclinical studies for targeted delivery to SCLC cells of radioisotopes and protein toxins and could demonstrate preferential tumour localization in xenograft models of SCLC [5, 24, 33]. Moreover, like other EGP-2-specific mAb, MOC31 has recently also demonstrated preferential tumour localization in SCLC patients [32][1] and can thus be considered a promising candidate for use in targeted therapies of SCLC.

Despite its ability to exert minor cytotoxic effects also on the cell surface [31], doxorubicin must be internalized into cells and gain access to its primary target in the nucleus to be potently cytotoxic. Free doxorubicin is hydrophobic enough to permeate rapidly through the cell membrane. The activity of doxorubicin, however, depends on their rates of internalization into lysosomes by receptor-mediated endocytosis, the subsequent release of the drug from the antibody and its sequestration into the nucleus [3, 9]. Therefore, the function of the linker coupling the targeting and cytotoxic moieties is an important determinant for the therapeutic efficacy of the immunoconjugates. Among the various linkers that have been employed to conjugate doxorubicin to mAb [1, 11, 23] acid-sensitive linkers especially proved to be successful in preserving the cytotoxic activity of the drug [7, 12, 30]. Acid-sensitive linkers between mAb and anthracyclines can be produced by attaching a *cis*-aconityl linker to the amino sugar of the drug [7]. However, this procedure has been shown to yield a less homogeneous product that is often contaminated with relatively large amounts of free drug [23]. A different approach is to use the 13-keto group of the anthracycline as an attachment site to produce an acid-labile hydrazone bond [12]. The chemical reactions involved in the synthesis of doxorubicin-hydrazone immunoconjugates are highly reproducible and the final product contains only trace amounts of free drug [35]. In the present study we used this technique and reacted a hydrazide derivative of the crosslinking reagent SMCC with the C_{13} carbonyl group of doxorubicin. Compared with the linker described by Greenfield et al. [12], this protocol takes advantage of a commercially available starting reagent and involves a reduced number of reaction steps.

The mAb MOC31, SWA11 and SEN7 were linked to the hydrazone derivative of doxorubicin via a nucleophilic reaction between the sulphydryl groups of the mAb and the double bond of the maleimide residue of SMCC. The sulphydryl groups were generated by mild reduction of the interchain disulphide bonds of the mAb using dithiothreitol. In agreement with the findings of Willner et al. [35] this procedure proved to be highly reproducible for all three mAb and generated seven or eight reactive groups per molecule. Despite loss of their interchain disulphide bonds, the reduced mAb fully retained their molecular integrities and their antigen-binding activities. Further treatment of these preparations with SDS and heating, however, resulted in their complete disintegration. This finding clearly confirms that the molecular integrity of the mAb did not depend on the presence of interchain disulphide bonds and was sufficiently maintained by other non-covalent forces. In contrast to the reduction of the interchain disulphide bonds we have repeatedly found that generation of sulphydryl groups using 2-iminothiolane was less reproducible and resulted in a significant loss of mAb due to aggregation and precipitation (data not shown).

The cell-binding activities and internalization potentials of the mAb were fully preserved after conjugation with doxorubicin. The rapid initial loss of surface-bound mAb and immunoconjugates detected by indirect immunofluorescence analysis was not due to shedding of mAb-antigen complexes into the culture medium. This was clearly demonstrated in a radioimmunoassay that allowed discrimination between surface-bound and intracellular ligands. Internalization of MOC31 and SWA11 occurred rapidly, reaching 25% and 32% respectively after 1 h. In contrast, internalization of SEN7 was slow and the mAb remained relatively fixed on the cell surface. Whereas uptake of SWA11 reached a plateau of 40% within 2 h, a small fraction of intracellular MOC31 reappeared on the cell surface during the last 3 h of incubation. This might be intact mAb or mAb fragments [4, 20]. In addition to the proper functioning of the targeting moiety, the necessary attributes of the hydrazone linker, namely to remain stable under neutral conditions and to release the anthracycline under acidic conditions mimicking those found in various tumours and in lysosomes, were also demonstrated. Doxorubicin was rapidly liberated from the antibody by acid hydrolysis at pH 5.0 with a half-life of 3.5 h.

As expected from their rates of internalization, MOC31-DOX and SWA11-DOX but not SEN7-DOX could prove potent small drug immunoconjugates in cytotoxicity assays. Although a lower number of binding sites was available for MOC31 and the mAb was recirculated to the cell surface, the cytotoxic potency of MOC31-DOX could closely match that of SWA11-DOX. This suggests that sequestration into lysosomes and subsequent release of doxorubicin from the mAb was at least as efficient for MOC31-DOX than for SWA11-DOX. The 10-fold difference in the cytotoxic potencies of MOC31-DOX and SEN7-DOX, for which comparable numbers of antigenic sites were available, correlated well to their different rates of internalization.

[1] While our manuscript was in press this paper was published: Kosterink JGK, et al. (1995) Pharmacokinetics and scintigraphy of indium-111-DTPA-MOC31 in small-cell lung carcinoma. J Nucl Med 36: 2356

As a consequence of antigen-specific binding to tumour cells and internalization by receptor-mediated endocytosis the immunoconjugates were 100-fold more selective than the free drug. Unspecific cytotoxicity was observed only at concentrations (above 6 μM) exceeding those normally found in the serum of patients after systemic application. This observation might be due to either binding of doxorubicin immunoconjugates to cells by non-specific hydrophobic interactions [29] or to the premature release of drug from the mAb [31].

Using an optimized and reproducible preparation procedure, we have demonstrated that conjugation of doxorubicin to the mAb MOC31 and SWA11 via an acid-sensitive hydrazone linker resulted in highly selective and potent immunoconjugates. In particular the limited crossreactivity of MOC31 with normal human tissues and its efficient localization to SCLC in xenograft models and in patients [5][1] support the potential application of MOC31-DOX in targeted chemotherapy of SCLC.

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[1] Footnote see page 61

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