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Radioimmunodetection of Human Small Cell Lung Carcinoma Xenografts in the Nude Rat Using ^{111}In-labelled Monoclonal Antibody MOC-31

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The applicability of mouse monoclonal antibody MOC-31 for *in vivo* radioimmunodetection of human small cell lung cancer (SCLC) was investigated in a nude rat xenograft model. MOC-31 is reactive with a 38 kD pan-carcinoma membrane antigen. [^{111}In]DTPA-MOC-31 showed good *in vivo* immunolocalisation to xenografted SCLC cells, whereas antigen-related uptake was low in normal rat tissues and in a control antigen-negative, human-derived tumour. Non-antigen-related uptake in the liver could be blocked by pretreatment with irrelevant antibody. It is concluded that MOC-31 can be used for radioimmunodetection of SCLC *in vivo* and may be suitable as a targeting device in patients.
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INTRODUCTION

MONOCLONAL ANTIBODIES (Mab) provide good opportunities for diagnostic and therapeutic applications in patients [1–3]. Most patients examined so far in radioimmunodetection trials suffered from melanoma, lymphoma, colon, breast or ovarian carcinoma. Although lung cancer is also an important target for radioimmunodetection or radioimmunotherapy, few studies have been published on this type of cancer [4, 5]. Based on histological and clinical findings, lung cancer can be divided into small cell lung carcinoma (SCLC) and non-SCLC [6]. SCLC is characterised by a high metastatic capacity and a high initial sensitivity to chemo- or radiotherapy. Although good initial responses to chemo- or radiotherapy are obtained in most SCLC patients, almost all patients show therapy-resistant recurrences within 0.5–1 year and as a result survival is very low [7, 8]. To overcome the bad prognosis for SCLC patients, additional treatment modalities are needed. Mabs might be helpful for such a requirement in the future. A number of Mabs directed against SCLC-associated antigens has been developed [7–14]. One of these, MOC-31, which has been clustered as an SCLC-cluster 2 antibody during

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the first international workshop on small cell lung cancer antigens [15], might be applicable for *in vivo* use. MOC-31 is reactive with a membrane antigen (38 kD), abundantly present on a variety of normal epithelial cells and almost all carcinoma cells [9, 15]. The present paper assesses the *in vivo* diagnostic potential of this antibody in a SCLC xenograft nude rat model.

MATERIALS AND METHODS

Rats

Wag/Rij/rnu rats (age 8–10 weeks; weight 230 ± 43 g) were used for all experiments.

Tumour cell lines

Cell line GLC-28 was established from a patient (male; 59 years old), suffering from histologically confirmed SCLC. After five courses of combination chemotherapy (cyclophosphamide, doxorubicin and etoposide), resulting in a partial remission, a biopsy sample was taken from the remaining primary tumour site, put into tissue culture medium, and the tumour cells were adapted to growth *in vitro* according to described procedures [16], resulting in the establishment of cell line GLC-28. GLC-28 is a 'classic' SCLC-derived cell line and grows loosely attached to the bottom of the culture flask. GLC-28 is MOC-31 antigen-positive (MOC-31$^+$). The human-derived melanoma cell line IGR37, a kind gift of Dr Aubert, Marseille, France, is MOC-31 antigen-negative (MOC-31$^-$) and was used as a (negative) control cell line.

Tumour xenografts

In vitro grown SCLC-derived GLC-28 cells or melanoma-derived IGR37 cells were collected by tapping the flask or by trypsinisation, respectively, followed by centrifugation. Subsequently, the cells were suspended in RPMI-1640 medium, supplemented with 10% fetal calf serum and antibiotics. Tumour cells were xenografted by injection of 1×10^7 viable cells subcutaneously (s.c.) in the flank, hind, or neck of nude rats, resulting in a tumour take of approximately 80% ($n = 29$). In some experiments, GLC-28 and IGR37 cells were injected separately in the same animal at the right and left-lateral areas, respectively. After about 6 weeks, s.c. growing tumours with a diameter of approximately 2 cm (i.e. 1–2 g) had developed. Tumour masses could increase to 10 g after about 9 weeks, but these contained considerable central necrosis.

Purification of Mab

MOC-31, isotype IgG1, is reactive with SCLC but not with melanoma cells. Mab 2g12, isotype IgG1, is directed against a 240 kD membrane-associated antigen present on human-derived melanoma (C. Berends, M.W.A. de Jonge, L. de Leij, unpublished results), and is not reactive with SCLC. As an irrelevant Mab, HIS30 was used, which is unreactive with both SCLC and melanoma. Mab HIS30 (IgG2a) is directed against the LAMA tumour (pre B-cell leukaemia) in the rat [17]. Mab MOC-31 IgG was concentrated from tissue culture supernatant by ammonium sulphate precipitation (30–55% fraction), whereas isolation of HIS30 and 2g12 was started from ascites fluid. Isolation was done by affinity chromatography on protein A-Sepharose (Pharmacia, Uppsala, Sweden) according to standard procedures [18]. Purified Mabs were dialysed against 0.01 mol/l phosphate-buffered saline (PBS), pH 7.2, and stored at −70°C until use.

Conjugation and radiolabelling of Mab

The bicyclic anhydride of diethylenetriaminepentaacetic acid (DTPA) (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) was conjugated to IgG according to Hnatowich *et al.* [19] at a molar ratio of DTPA to Mab of 5 : 1. The conjugate was isolated by gel filtration (Sephadex G-50, PD-10, Pharmacia, Uppsala, Sweden) and used for radiolabelling immediately after preparation.

One hundred microlitres of $^{111}InCl_3$ (37 MBq/0.02 μg in HCl, Amersham, U.K.) were added to 100 μl of 1.0 mol/l sodium acetate, pH 9.2. Subsequently, 300 μl of DTPA-conjugated Mab (approximately 1 mg/ml) were added and incubated at room temperature (RT) for 30 min. Radiolabelled Mab was purified from free ^{111}In by gel filtration (Sephadex G-50, PD-10). Specific activity of [^{111}In]DTPA-MOC-31 preparations varied from 74 to 185 kBq/μg IgG. Specific activity of [^{111}In]DTPA-2g12 was 63 kBq/μg IgG. [^{111}In]DTPA-Mab were subsequently filter-sterilised (0.22 μm filter, Millex GV) and immediately used for further experiments.

Determination of the immunoreactivity of radiolabelled Mab in vitro

The immunoreactivity of [^{111}In]DTPA-MOC-31 was determined in various cell binding assays. GLC-28 cells (antigen-positive) and IGR37 cells (antigen-negative) were used as target cells in a concentration of 1×10^7 cells/ml in fresh RPMI-1640 medium, supplemented with 10% fetal calf serum and antibiotics. Increasing concentrations of radiolabelled Mabs in PBS ranging from 0.01 μg to 1.5 μg of IgG were added to a fixed amount of 1×10^6 cells/sample of 100 μl, mixed and incubated at 20°C for 1 h. Incubations were performed in duplicate. Subsequently, cells were washed three times with PBS and both cell-bound radioactivity (final cell pellet) and non-bound radioactivity (total of wash supernatants) were measured in a 3-channel gamma-counter (SRL4, Searle, U.S.A.; or Konntron MR 1032 automated gamma counter, W + W Electronic Inc., Switzerland). The immunoreactive fraction of radiolabelled antibody preparations was determined as described by Lindmo *et al.* [20].

Biodistribution of radiolabelled MOC-31 in vivo

Nude rats, bearing s.c. growing tumour xenografts (GLC-28 tumour and/or IGR37 tumour), were given intravenous (i.v.) injections of 3.7 MBq of [^{111}In]DTPA-MOC-31, at a specific activity of 74 to 185 kBq/μg of IgG. Animals were killed at several time points postinjection. The tumour(s) and a number of normal tissues or organs like liver, kidney, spleen, lung, heart, stomach, colon, adrenal gland, skin, bone, striated muscle, brain tissue and testicle were extirpated and weighed. The uptake of radioactivity in these specimens was measured in a gamma-counter (see above). Likewise, the radioactivity of blood samples was counted. In blocking experiments, a similar procedure was followed except that GLC-28 tumour-bearing animals were pretreated by i.v. injection of 300 μg of (non-radioactive) In-DTPA-HIS30. After 24 h, [^{111}In]DTPA-MOC-31 was i.v. injected.

Radioimmunodetection of GLC-28 tumour xenografts in vivo

Nude rats bearing s.c. growing GLC-28 tumour xenografts were given i.v. injections of 3.7 MBq of [^{111}In]DTPA-MOC-31, at a specific activity of 74–185 kBq/μg of IgG. At several time points postinjection (1, 24, 48, 72 or 144 h), the animals were anaesthetised by intraperitoneal (i.p.) injection of chloralhydrate

(60 mg/ml in 0.9% NaCl) at a dose of approximately 360 mg/kg of body weight. Scintigraphic imaging was performed using a gamma-camera (LFOV Scintiview, Siemens, U.S.A.) equipped with a median energy parallel collimator. The 172 keV and 247 keV photon energy peaks of ^{111}In were recorded, during 10–30 min (depending on the radioactivity present at time of recording). After the last image was made, the animals were killed, and various organs and tissues were extirpated to assess the biodistribution at this time point as described above for the biodistribution experiments.

RESULTS

Immunoreactivity of radiolabelled [^{111}In]DTPA-MOC-31 in vitro

A linear relation was found between added and cell-bound radioactivity after addition of increasing concentrations of [^{111}In]DTPA-MOC-31 to a fixed amount of GLC-28 cells. Apparent saturation of available binding sites for MOC-31 was obtained at an added concentration of 1 μg of IgG/10^6 cells. Binding of [^{111}In]DTPA-MOC-31 to GLC-28 cells appeared to be specific since no such binding was found when control Mab [^{111}In]DTPA-2g12 was used. In addition, when IGR37 cells (MOC-31$^-$, 2g12$^+$) were used as irrelevant target cells, no binding of [^{111}In]DTPA-MOC-31 could be detected, while addition of control Mab [^{111}In]DTPA-2g12, showed positive binding to its antigen.

To assess the immunoreactivity of [^{111}In]DTPA-MOC-31 in more detail, a competition assay was performed, in which a fixed amount of GLC-28 cells was incubated with various ratios of [^{111}In]DTPA-MOC-31 and unlabelled MOC-31 IgG. A linear correlation was found between radiolabelled MOC-31/unlabelled MOC-31 versus cell-bound radioactivity, indicating that [^{111}In]DTPA-MOC-31 had retained its immunoreactivity after radiolabelling. Using Scatchard analysis, approximately 3×10^6 antibody-binding sites were estimated to be present per GLC-28 tumour cell *in vitro*, whereas the apparent association constant of antibody binding (K_a) was calculated to be 3.5×10^8/mol/l. The immunoreactive fraction of the radiolabelled antibody preparations was shown to be 0.65 to 0.85 [see also the experiment given in Fig. 1, where the immunoreactive fraction (r) was determined to be 0.79].

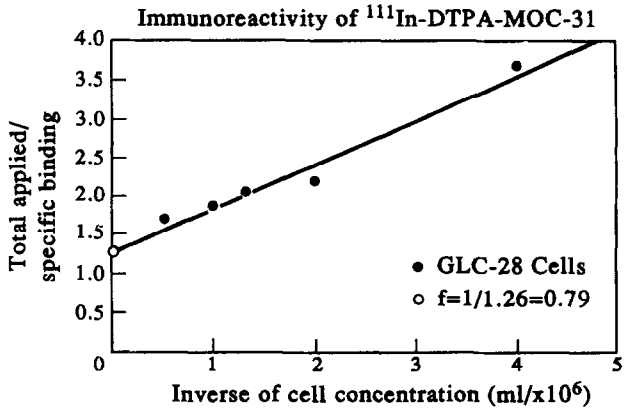

Fig. 1. Determination of the immunoreactive fraction of [^{111}In]DTPA-MOC-31 towards GLC-28 tumour cells according to Lindmo *et al.* [20], (experiment performed in duplicate).

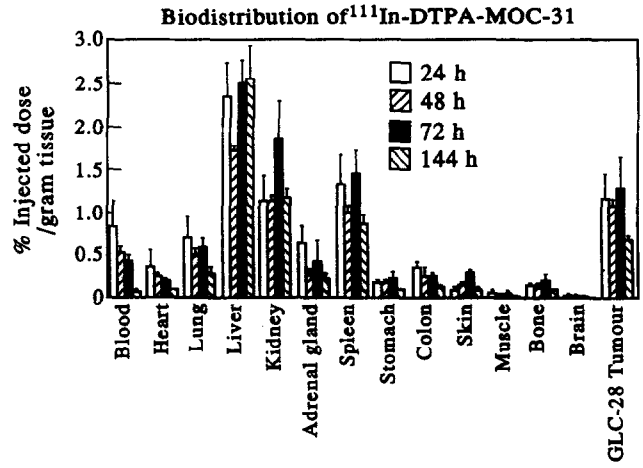

Fig. 2. Biodistribution of [^{111}In]DTPA-MOC-31 to several tissues and organs in GLC-28 tumour-bearing nude rats at several time points after injection, presented as percentage of injected dose per gram tissue. Injected dose expressed as mean value (± S.E.): 1769 kBq ± 39 kBq/17.8 μg ± 1.0 μg; specific activity: 100 kBq/μg. Values represent mean values (± S.E.) of three animals each.

Biodistribution of radiolabelled MOC-31 in vivo

At 24–72 h after injection of [^{111}In]DTPA-MOC-31 an amount of radioactivity equivalent to 1.2–1.4% of the injected dose had been localised into the GLC-28 tumour per gram tumour (see Fig. 2). A sharp decrease of radioactivity in the circulation occurred with time, with increasing radioactivity until day 3 in liver, kidney, spleen and tumour. This resulted in an increase in tumour to blood ratio varying from 1.5 after 24 h, to a ratio of approximately 3.0 after 72 h, and up to a ratio of more than 10.0 at 6 days after injection. The spleen, the kidney and the liver showed relatively high and persistent uptakes of radioactivity (Figs 2 and 3).

To further assess the immunospecificity of [^{111}In]DTPA-MOC-31 *in vivo*, rats (n = 2) bearing both GLC-28 (SCLC) and IGR37 (melanoma) tumours at different locations were used. Biodistribution of [^{111}In]DTPA-MOC-31 in these two rats 3

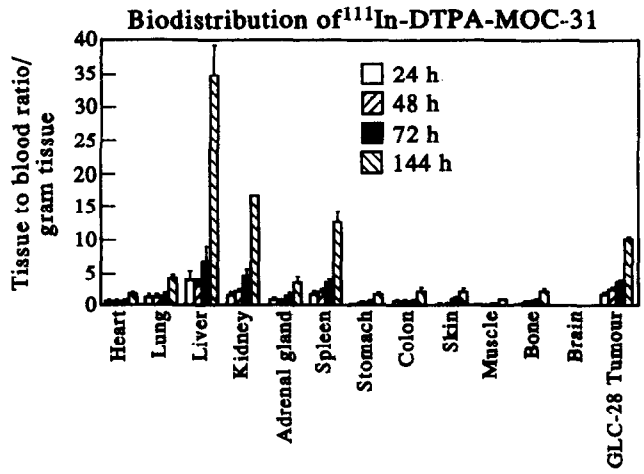

Fig. 3. Biodistribution of [^{111}In]DTPA-MOC-31 to several tissues and organs in GLC-28 tumour-bearing nude rats at several time points after injection, presented as tissue to blood ratio per gram tissue. Injected dose expressed as mean value (± S.E.): 1769 kBq ± 39 kBq/17.8 μg ± 1.0 μg; specific activity: 100 kBq/μg. Values represent mean values (± S.E.) of three animals each.

days post injection, showed a GLC-28 tumour to blood ratio of 3.0 and 6.0, respectively, whereas the IGR37 tumour to blood ratio was found to be 0.2 and 0.5, respectively. This indicated localisation of [^{111}In]DTPA-MOC-31 to the tumour expressing the specific antigen (i.e. GLC-28) and not to the antigen-negative tumour (IGR37).

Radioimmunodetection of GLC-28 tumour xenografts in vivo

Results of *in vivo* radioimmunoscintigraphy of a GLC-28 tumour-bearing nude rat at 24, 48, 72, and 144 h postinjection of [^{111}In]DTPA-MOC-31 are shown in Fig. 4. An increase of accumulation of [^{111}In]DTPA-MOC-31 into the GLC-28 tumour and in the liver region can be seen with time. In contrast, the amount of radioactivity in the heart + lung region, representing to a certain extent free circulating [^{111}In]DTPA-MOC-31, decreased with time. Most favourable images were obtained around day 3.

The imaging results as quantified by determination of counts per pixel, correlated well with results obtained by direct counting of the tissues after animals were killed. The result of *in vivo* radioimmunoscintigraphy (Fig. 4), can be superimposed on the results obtained with biodistribution (see Fig. 3). This can be illustrated from the image obtained in Fig. 4 at 144 h after injection. The absolute number of counts measured in the circulation amounts to only 12 000 cpm (per 5.3 g of blood), and 15 500 cpm in the both lungs (1.47 g) and a very low value of 2500 cpm in the heart (0.78 g) (see Fig. 3 showing tissue to blood ratios per gram tissue). Visualisation of these organs in the scintigraphic image was low. High values were found in the tumour (142 000 cpm per 6.2 g) and in the liver (605 000 cpm per 8.0 g) with corresponding high visualisation in the image.

Mab [^{111}In]DTPA-MOC-31 showed good immunoselective properties *in vivo* too, since it localised to a GLC-28 tumour (antigen-positive) and not to a IGR37 tumour (antigen-negative) in a nude rat bearing separately both types of tumour (data not shown).

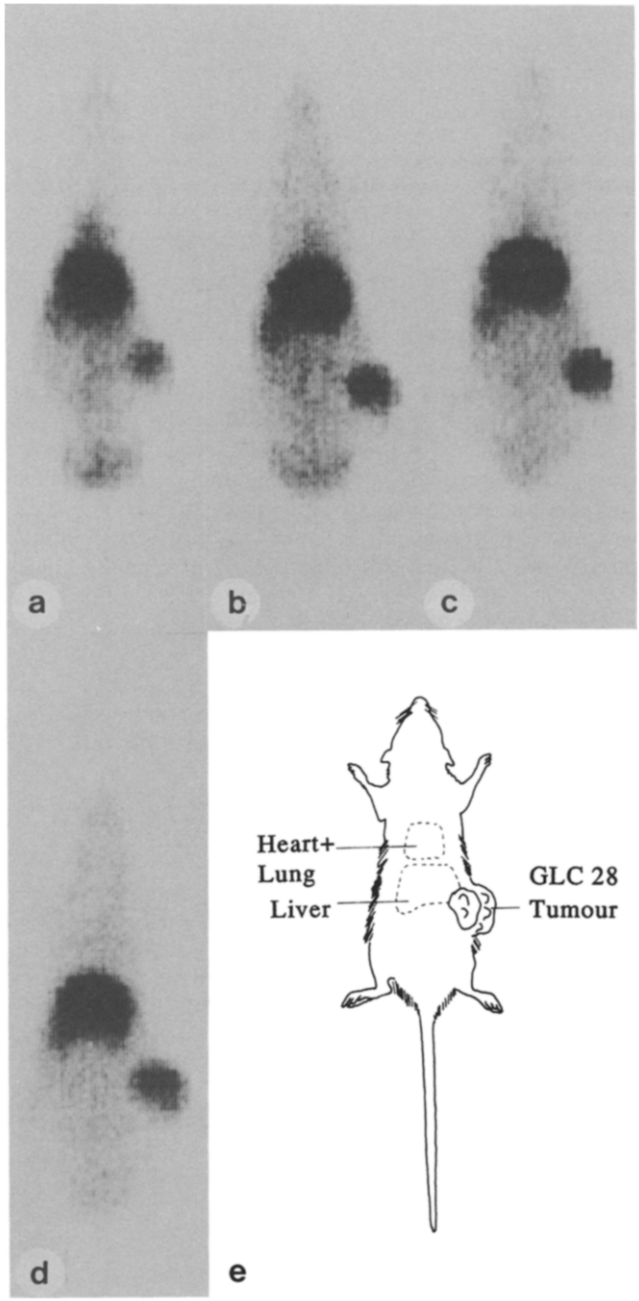

Fig. 4. Radioimmunodetection of [^{111}In]DTPA-MOC-31 of GLC-28 tumour-bearing nude rats at 24 h (a), 48 h (b), 72 h (c), and 144 h (d) after injection of 1791 kBq/17.9 μg; specific activity: 100 kBq/μg. The schematic drawing (e) represents the location of the GLC-28 tumour, heart + lung and liver.

Biodistribution of radiolabelled MOC-31 in vivo *after antibody pretreatment*

To assess the possibility of reducing the uptake of [^{111}In]DTPA-MOC-31 into the liver, GLC-28 xenografted animals were pretreated with a 15-fold excess (300 μg IgG) of irrelevant, non-radioactive antibody In-DTPA-HIS30. Figure 5 shows that this resulted in a strong decrease in the uptake of radioactivity in the liver, a small decrease of uptake in the spleen and an increase in the kidney. Localisation of [^{111}In]DTPA-MOC-31 to the tumour was almost unaffected.

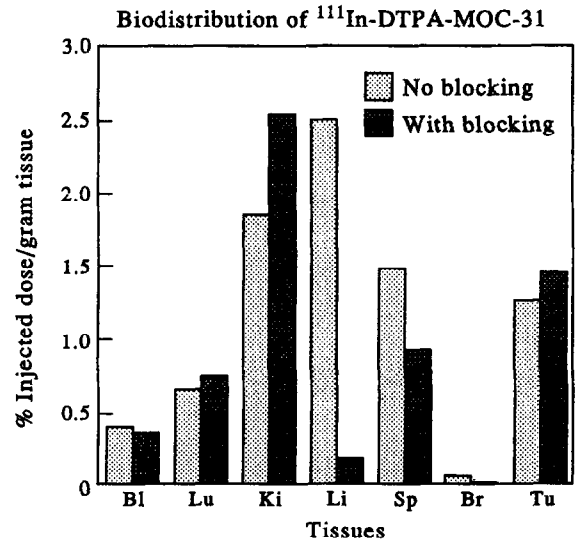

Fig. 5. Biodistribution of [^{111}In]DTPA-MOC-31 to several tissues and organs in GLC-28 tumour-bearing nude rats at several time points after injection, after pretreatment with 300 μg non-radioactive labelled irrelevant IgG (In-DTPA-HIS30). Results are presented as injected dose per gram tissue. Injected dose: 6549 kBq/20.0 μg; specific activity: 326 kBq/μg. Abbreviations: Bl = blood; Lu = lung; Ki = kidney; Li = liver; Sp = spleen; Br = brain; Tu = GLC-28 tumour.

DISCUSSION

This paper describes radiolabelling and quality assessment of Mab MOC-31 *in vitro* and *in vivo* in a nude rat experimental model.

Mab MOC-31 was selected because of a number of favourable characteristics. Firstly, a high number of potential antibody binding sites appears to be present in SCLC (circa 3×10^6/cell). Secondly, in SCLC, but also in other carcinomas, virtually all tumour cells in the specimens examined so far show strong reactivity with MOC-31 [11]. Thirdly, the antigen detected by MOC-31 is firmly membrane-bound and cannot be detected in serum from patients suffering from carcinoma. The antigen detected by MOC-31 is probably similar to the one detected by AUA-1 [21] and GA-733 [22].

Human tumours xenografted in nude rats may offer several advantages over the more generally used nude mice. Firstly, most Mab used for the detection of human tumours are mouse-derived. In the nude rat model, mouse Mabs can be visualised after they have attached *in vivo* to the xenografted tumour by means of anti-mouse IgG immunoperoxidase staining.

Secondly, a surplus of free Mab Ig can be removed from the circulation in the rat model by applying an anti-mouse Ig preparation. Thirdly, although even the size of the nude rat is rather small for assignment of discrete regions of interest (ROI) in the gamma-camera images, relative activities could be calculated (data not shown).

In the present article, we have shown that [^{111}In]DTPA-MOC-31 specifically localised to xenografted, antigen-positive tumours. However, considerable uptake of radioactivity was also observed in liver, spleen and kidney. This may partly be explained by transchelation of a fraction of ^{111}In to transferrin in the blood, and subsequently to ferritin in a.o. liver and spleen. Additionally, to a certain extent, Fc-receptor-mediated binding may contribute to this high uptake of radiolabelled Mab in liver and spleen into the cells of the reticuloendothelial system (RES) as suggested by others [e.g. 23–25]. Most likely, ^{111}In in the liver does not exist as the intact [^{111}In]DTPA-MOC-31 immunoconjugate, but will be rapidly degraded after uptake in the liver [26]. We have found that after injection of unlabelled MOC-31 no mouse antibody could be visualised in tissue sections of the liver using immunoperoxidase staining aimed at the detection of the whole antibody molecule, by incubation with horseperoxidase (HRPO)-conjugated rabbit-anti-mouse immunoglobulins (results not shown), which is in favour of such an explanation.

Pretreatment with non-radioactive irrelevant antibody can block Fc-receptor-mediated binding of [^{111}In]DTPA-MOC-31 as is shown in the experiment in which animals were pretreated with a 15-fold excess of irrelevant antibody (In-DTPA-HIS30) (Fig. 5). A similar reduction of the uptake of ^{111}In in the liver after pretreatment with specific antibody has been reported for anti-melanoma antibody 96.5 in humans [27].

This article also illustrates the differences in the amount of uptake of radioactivity by tumour cells *in vitro* as compared to the accretion into the xenograft *in vivo*. Using a number of assumptions, it might be estimated that about 1000-fold less radiolabelled Mab molecules had been bound per cell *in vivo* than *in vitro*. This indicates that methods for selective immunolocalisation can still be improved for better delivery of radiolabelled antibodies. For example, a better saturation of the tumour might be accomplished by injection of higher amounts of antibody [28].

A number of factors may influence this lack of complete immunolocalisation or may cause heterogenous distribution of antibody into tumour tissue [29]. Intrinsic factors like the presence of certain physiological barriers inhibiting diffusion of antibodies into tumour tissue, i.e. inadequate blood supply, elevated interstitial pressure, and large transport distances in the interstitium will diminish antibody penetration [29]. Typically, necrotic areas of GLC-28 tumour showed a significantly lower uptake of radioactivity (up to 50%) compared with viable tumour tissue. For compensation, it has been shown by quantitative analysis of viable and necrotic regions of tumours, that antibody directed to tumour surface antigen preferably localised to the peripheric rim of the tumour and less to the necrotic centre [30]. Immunolocalisation of antibodies is feasible in necrotic regions, as is shown for antibodies directed against intracellular (nuclear) antigens, because of availability and exposure of relevant antigens in permeable, dying cells [31]. Apparently, binding of antibody occurs if the (intact) antigen is still present.

In summary, it is concluded that ^{111}In-radiolabelled MOC-31 is a suitable antibody for radioimmunodetection of experimental xenografted antigen-positive tumours. Radiolabelled [^{111}In]-DTPA-MOC-31 retains its immunoreactivity and specificity, and has good localisation properties, with high affinity to target antigens *in vivo*. Therefore, MOC-31 seems to be suitable for application as a targeting-device in patients and will be studied further.

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Evaluation of the Time-schedule Dependency for the Cytotoxic Activity of the New Vinca Alkaloid Derivative, S 12363 (Vinfosiltine)

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S 12363 is a new vinca alkaloid derivative obtained by appending an optically active α-aminophosphonate at the C23 position of 04-deacetyl vinblastine. The present study concerns four different human tumour cell lines, which represent the spectrum of vinca alkaloid clinical activity. The influence of time exposure on S 12363 growth inhibition was studied *in vitro*. Cells were exposed to the drug during the following exposure times : 5, 15, 30 min and 1, 3, 6, 12, 24, 48, 72, 144 h. The concentrations of S 12363 applied were between 1×10^{-2} and 1×10^{3} nmol/l. The cytotoxic effects were assessed by using the methyltetrazolium (MTT) semi-automated test. Considering the IC_{50} values in terms of concentration (C) × time (T), I $(C \times T)_{50}$, it was shown that for an equal growth inhibitory effect (50% of cell death) the increased exposure times required higher cumulative drug exposures. More precisely, only very long exposure (greater than 24 h) resulted in very high I $(C \times T)_{50}$. The drug exposure ratios which correspond to I $(C \times T)_{50}$ values for 144 h divided by the I $(C \times T)_{50}$ values for 0.25 h ranged between 2.8 and 18.3. If T and C had symmetrical effects on the final growth inhibition, the I $(C \times T)_{50}$ ratios should have been equal to one. For all cell lines investigated there were similar dose–response curves following two types of S12363 exposure: a single day exposure or three successive daily exposures, the total C × T values being the same in both experimental situations. The basic pharmacological information provided by the present study may encourage further clinical trials of this potentially interesting new vinca alkaloid.
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INTRODUCTION

S 12363 (VINFOSILTINE) IS A new vinca alkaloid derivative obtained by appending an optically active α-aminophosphonate at the C23 position of 04-deacetyl vinblastine. This compound is very promising, since it is 72 and 36 times more cytotoxic than vincristine and vinblastine, respectively, when tested on a panel of murine and human tumour cell lines using the methyltetrazolium (MTT) assay [1]. It is the objective of preclinical investigations for a new anticancer drug to supply rational guidelines which may be useful in providing the basis for future clinical trials [2]. More precisely, these guidelines could outline suggestions for an optimal administration schedule based on