Radioimmunotherapy in Experimental Head and Neck Squamous Cell Carcinoma

Tumour-targeting in vitro and in vivo

JUNPING CHENG
Abstract


Radioimmunotherapy (RIT) has been shown to be a practicable way to treat head and neck squamous cell carcinoma. A specific antibody recognizes the charasteristic structure of tumour cells when loaded with cytotoxic agents (toxins, drugs, radionuclides, etc). But RIT kills not only tumour cells with attached radionuclides but also adjacent tumour cells due to the “cross fire effect”. To be efficacious, RIT depends closely on suitable monoclonal antibody, on the properties of the chosen radionuclides, and on a suitable labelling method for attaching radionuclide to antibody.

In this study we initially used radionuclide-labelled cMAB U36, via linker DABI in order to improve the retention of radio-conjugates in the tumour cells. Improved retention is important because the longer the radionuclide remains in tumour cells, the more effective will the tumour cells be eradicated. In the investigation, both normal mice and HNSCC-bearing nude mice were used to compare our form of treatment against other radio-iodination methods. In the biodistribution study, normal mice showed that radioactive uptake in organs diminished with time, irrespectively of whether the conjugate was directly or indirectly labelled. But in thyroid, there was a tenfold greater accumulation of direct-labelled than of indirectly labelled conjugate.

In tumour-bearing nude mice, by contrast, the results showed promising uptake of radioactivity, but little uptake in direct-labelled conjugate in thyroid. Significant differences were observed on comparing tumour: organ ratios between 131I-cMAB U36 vs. 125I-DABI-cMAB U36.

In the present study, cMAB U36 labelled with 211Atstatine was initially used to treat HNSCC in nude mice. The biodistribution of 211At-cMAB U36 did not reveal any significant difference between an antibody-blocked group and a non-blocked group. But it did highlight the characteristics of a successful targeting conjugate in HNSCC-bearing nude mice.

In the subcutaneous therapy experiment, most of the treated tumours (n=18) had disappeared by the 26th day, in both U36-blocked and non-blocked groups. Treatment in the intravenous therapy experiment had also proved effective. In the antibody non-blocked group, the smallest tumour volume was 25 mm3 (average 111 mm3) vis-à-vis 65 mm3 (average 145 mm3) in the blocked group. None of tumours grew again following treatment.

Keywords: squamous cell carcinoma, radioimmunotherapy, cell line, tumour targeting, radionuclide, nude mice

Junping Cheng. Department of Surgical Sciences, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden

© Junping Cheng 2005

ISSN 1651-6206
ISBN 91-554-6277-4
urn:nbn:se:uu:diva-5834 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-5834)
To my family
Original papers

This thesis is based on the following papers, which will be referred to in the text by their roman numbers I-IV:


Offprints have been included with kind permission from the publishers, the American Chemical Society (I) and Taylor & Francis (II)
Contents

Abbreviations 6

Introduction 7

Head and Neck Squamous Cell Carcinoma 7
Antibody for HNSCC 7
Radionuclides for targeting HNSCC 8
Labelling linker 9

Purpose of this study 10

Materials and Methods 11

Materials 11
Chemical products (Paper I, II, III, IV) 11
cMAb U36 (Paper I, II, III, IV) 11
\(^{211}\)Astatine (Paper IV) 11
SCC25 cell line (Paper II) 11
UT-SCC7 (Papers III, IV) 12
Animals (Papers I, II, III, IV) 12

Methods 12
Direct labelling with \(^{125}\)I (Paper I) 12
Indirect labelling with PIB (Papers I, II) 12
Indirect labelling with DABI (Papers I, II, III) 13
Direct labelling with \(^{131}\)I (Paper III) 13
\(^{211}\)Astatine labelling (Paper IV) 14
Biodistribution (Papers I, II, III, IV) 14
Tumour models 15

Results 17

Antibody biodistribution 17
Radionuclide labelling 24
Tumour models 24
\(^{211}\)Astatine therapy study 25

Discussion 27

Biodistribution in normal mice (Paper I) 27
Biodistribution in HNSCC-bearing mice (Papers II, III) 28
Therapeutic experiments in HNSCC-bearing mice (Paper IV) 29

General summary 31

Acknowledgements 33

References 36
Abbreviations

%ID/g       Unit referring to activity expressed as a percentage of injected dose per gram of tissue
AJCC       American Joint Committee on Cancer
ATCC       American type culture collection
CAT      Chloramine-T
cMAb U36   chimeric monoclonal antibody U36
CT       Computerized tomography
DABI     (4 isothiocyanatebenzyl-amino)-ido-decahydro-closo-dodecaborate (I-DABI)
DT       Doubling time
18F-FDG   18F-Fluorodeoxyglucose
HNSCC    Head and neck squamous cell carcinoma
i.p.     Intraperitoneal
i.v.     Intravenous
LET      Linear Energy Transfer
MAb      Monoclonal antibody
MRI      Magnetic Resonance Imaging
MTD      Maximum Tolerant Dose
PBS      Phosphate-Buffered Saline
PET      Positron Emission Tomography
PIB      Para-iodobenzoate, N'-succinimidyl 4-(trimethylstannyl) benzoate
p.i      Post-injection
RBE      Relative Biological Effect
RIT      Radio-immunotherapy
s.c.     Subcutaneous
SD       Standard Deviation
SPECT   Single Photon Emission Computerized Tomography
TNM      Tumour, node, metastasis
UT-SCC7   University Turku-Squamous Cell Carcinoma
Introduction

Monoclonal antibodies (MAbs) with radioactive particles attached are referred to as radio-immunoconjugates. Ideally, the objective of radio-immunotherapy (RIT) - or any other therapeutic modality - is to irreversibly impair the DNA of cancer cells, leading to their death or sterilization, while sparing normal cells or tissues.

Since the introduction of hybridoma technology by Kohler and Milstein [1], tremendous effort has been invested in effectuating Ehrlich's concept of the “magic bullet”, dating from the early 1990s [2-8]. Currently, immunotherapy is regarded as the fourth way to treat cancer, besides the three common forms: surgery, radiation, and chemotherapy.

An attractive features of RIT is that, in contrast to other immunotherapeutic approaches, there is considerable variability in the characteristics of cytotoxins that can be applied, by virtue of the availability of hundreds of radionuclides with widely differing decay properties [9]. To achieve clinical RIT, one needs to select not only a suitable radionuclide but also an appropriate antibody to obtain an acceptable clinical treatment effect by applying practicable radionuclide labelling methods.

In this thesis, cMAb U36 labelled with $^{125}$I via a linker DABI was investigated by comparison with other radio-iodination methods. cMAb U36 labelled with $^{211}$At was used for HNSCC therapy in the nude mice model.

Antibody for HNSCC

Since 1993, when Schrijvers et al. [10] initially reported a novel MAbU36 targeting HNSCCs, numerous trials have been performed both in vitro and in vivo [7, 8, 11-13]. The murine MAb U36 (Centocor B.V., Leiden, The Netherlands) was generated by immunizing BALB/c viable mouse cells of the HNSCC cell line UM-SCC-22B [10]. In order to reduce the immunogenicity of MAb U36, a chimeric form was devised aimed at the human $\gamma$-heavy chain and human $\gamma$-light chain constant regions, using recombinant DNA technology.

cMAb U36 recognizes the CD44 splice variant – CD44v6, a 200-kDa glycoprotein located on the outer cell surface. Immunohistochemistry has revealed that cMAb U36 is reactive with 99% of primary and metastatic HNSCC and has a homogeneous reactivity pattern with 96% these tumours, making it an ideal candidate antibody for targeting nearly all HNSCC.
Radionuclides for targeting HNSCC

Currently three types of radionuclide can be considered for therapeutic purposes in RIT: alpha-emitters ($^{211}$At, $^{212}$Bi, $^{213}$Bi), beta-emitters ($^{90}$Y, $^{186}$Re, $^{131}$I, $^{177}$Lu) and Auger electron-emitters ($^{111}$In, $^{125}$I). The optimal radionuclide type for RIT is dependent on tumour volume, its histological type, the targeting agent used, the characteristics of the target, etc.

Auger-emitters emit low-energy electrons with a short path length range of 0.06-17 µm, which is too short to reach the DNA of a tumour cell unless the radionuclide is internalized into the cells [14]. $^{125}$I is used mainly in a lab work [15] while $^{111}$In is commonly applied for scintigraphic imaging for SPECT [16].

Beta-emitters with a radioactive range of 0.5-0.9 cm are suitable for targeting large tumour clusters [17]. To some extent, they might be superior to alpha-emitters, due to the ‘cross-fire’ effect, i.e. the targeting toxic agent labelled with radionuclide that can kill adjacent cells. Cross-fire effect could be helpful to overcome the problem of the variability of targeting agents. However, $^{131}$I is marred by the disadvantages of abundant gamma emission and poor tumour cell retention of conventional radio-iodine label [18].

Wessels and Rogus examined the physical characteristics of several beta-emitting radionuclides in conjunction with known pharmacokinetics of antibodies and concluded that $^{186}$Re could be an effective radionuclide for RIT of non-haematological tumours [19]. No ideal half-life or energy of radionuclides has been defined, but until greater penetration of antibody into macroscopic tumours can be achieved, the physical characteristics of the radionuclide may be of secondary importance [20].

Alpha-particle emitting RIT is becoming a possible option in the clinic, offering significant therapeutic benefits [9]. Alpha-emitters seem to be the most attractive nuclide when single cells or small tumour clusters need to be treated, as their range is quite short, only a few cell diameters in path length (usually 50-100 µm). These particles do not require internalization, unlike Auger electrons (having a range of less than 0.1 µm). Of the alpha-emitters, $^{211}$At is one of the most promising so far studied for cancer therapy [21-23].

As the tumour size decreases, the potential advantage of alpha-particles over beta-particles is assumed to increase. The main disadvantages regarding the use of alpha-emitters are their poor cost effectivity and short physical half-lives [24-25].
Labelling linker

Radiohalogens such as $^{125}$I and $^{131}$I are rapidly excreted from tumour cells when conventional radio-iodination methods are used. The reason for this poor cellular retention of radioactivity is the leakage of radio-catabolites following degradation of the radio-immunoconjugate in lysosomes.

Attaching nuclides to an antibody via an appropriate linker molecule will help to optimize in vivo properties of the targeting agent, giving a higher tumour uptake and label retention and a rapid whole-body clearance of labelled catabolites. In this respect, indirect labelling methods via linker molecules that use hydrophilic anchor molecules may be better than direct labelling with tyrosine-labelled compounds.

In order to avoid the ‘halogen leakage’ phenomenon, in which radio-catabolites can leak from tumour cells after radio-immunoconjugate degradation in lysosomes, resulting in poor intracellular retention, we used DABI (closo-dodecaborate-containing) as a linker that carries a negative charge at lysosomal pH, in order to compare it with the conventional labelling method. As charged substances are not soluble in lipids, it is plausible that their use as a linker could improve the intracellular retention of radio-halogens.
Purposes of this study

The concept of this thesis was to devise a link between preclinical research and clinical application of radio-immunotherapy (RIT) in HNSCC. To accomplish this we used the cMAb U36 which can detect a 200 kDa surface antigen of HNSCC and which, in normal tissue, is present only in stratified and transitional epithelia.

To analyse the labelling methods we used $^{125}\text{I}$ and $^{131}\text{I}$, in order to compare the biodistribution characteristics of the radio-nuclide-labelled cMAb U36 in normal mice. For the therapeutic experiment we used $^{211}\text{At}$.

The investigations were designed to answer the following questions:

1. does the radio-iodinated cMAb U36 retain its capacity to target HNSCC xenografts \textit{in vivo} when DABI is used as a linker molecule?

2. does use of DABI as a linker provides better tumour retention of cMAb U36 radioactivity, compared with PIB.

3. is UT–SCC7 a suitable xenograft model for studies on targeting of HNSCC?

4. does DABI provide better HNSCC-targeting than conventional direct radio-iodination, when the dual labelling method is used?

5. does cMAb U36 retain its capacity for tumour targeting and attain therapeutic efficacy, when labelled with $^{211}\text{At}$?
Materials and Methods

Materials

Chemical products (Papers I, II, III & IV)
Sodium metabisulphite and organic solvents were purchased from Merck (Darmstadt, Germany). Chloramine-T and sodium metabisulphite were obtained from Sigma Chemical Company (St Louis, Mo., USA). All chemicals were of analytical grade or better.

cMAb U36 (Papers I, II, III & IV)
The selection and production of cMAb U36 has been described previously. It has been shown that the cMAb U36 recognizes the CD44 splice variant CD44v6 [10, 26]. The antibody was first stored in citrate buffer, and then separated by size-exclusion chromatography on a NAP-5 column pre-equilibrated with purified (ELGA) water. It was then freeze-dried overnight and stored at -20°C.

$^{211}$Astatine (Paper IV)
$^{211}$At was produced at the PET and Cyclotron Unit, Copenhagen University Hospital, Denmark, using $^{209}$Bi ($\alpha$, 2n) $^{211}$At nuclear reaction. The target was mounted at a water cooled probe for internal irradiations and irradiated with beam currents of 18 µA for 4 h, using the Scanditronix MC32 cyclotron. After delivery to Uppsala University, the $^{211}$At was separated from target material by dry distillation, as previously described by Sjöström et al. [27], and recovered as solution in 200 ml methylene chloride, with a typical yield of about 50% (not decay corrected).

SCC-25 Cell line (Paper II)
The HNSCC cell line SCC-25 (obtained from American Type Culture Collection) was derived from a 70-year-old male with squamous cell carcinoma of the tongue. SCC-25 were cultured in a 1:1 mixture of Ham’s F12 and Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 10% fetal calf serum, 2 ml L-glutamine and antibiotics (100 IU penicillin
and 100 mg/ml streptomycin). Cells were grown at 37 °C in an atmosphere consisting of humidified air with 5% CO₂.

**UT–SCC7 Cell line (Papers III & IV)**

UT–SCC7 line cells, derived from squamous cell carcinoma of the tongue, were cultured in DMEM, supplemented with 10% fetal calf serum, 2 ml L-glutamine and antibiotics (100 IU penicillin and 100 mg/ml streptomycin) and non-essential amino acids (10 ml). Cells were grown at 37°C in an atmosphere consisting of humidified air with 5% CO₂.

**Animals (Papers I, II, III & IV)**

Normal mice were adult NMRI females (BK Universal AB, Stockholm) (Paper I). The nude mice were 5-weeks-old females (NMRI nu/nu) (BK University AB, Stockholm) (Paper II). Nude mice bearing subcutaneously implanted human xenografts of the cell line UT–SCC7 were prepared. Adult females (balb/c nu/nu, around 25 g; Harlan CPB, Zeist, The Netherlands) were 10-weeks-old at the time of the experiments (Papers III - IV). All studies were performed with appropriate licences from the local Ethics Committee for Animal Research (Project No. C157/2).

**Methods**

**Direct labelling with ¹²⁵I (Paper I)**

Freshly prepared solution of cMAb U36 in PBS (60 µl, 5 mg/ml) was mixed with ¹²⁵I iodine solution (5 MBq). Reaction was initiated by adding CAT in PBS (10 µl, 2 mg/ml), and quenched after rigorous vortexing for 5 min by adding sodium metabisulphite (20 ml, 2 mg/ml). Labelled antibody (¹²⁵I-cMAb U36) was separated from non-reacted ¹²⁵I and low-molecular-weight reaction components by size-exclusion chromatography on a NAP-5 column pre-equilibrated with PBS (Fig. 1a).

**Indirect labelling with PIB (Papers I & II)**

Stock solution of ¹²⁵I (15 µl) was mixed with 10 µl of 0.1% acetic acid. A solution of N-succinimidyI 4-(trimethylstannyl)benzoate in 5% acetic acid in methanol (5 µl, 1 mg/ml) prepared as described in [28] was then added. Labelling was started by adding CAT in water.
(10 µl, 4 mg/ml) and quenched after 5 min of vortexing by adding sodium metabisulphite (10 µl, 2 mg/ml water). Freshly prepared solution of cMAb U36 in borate buffer, pH 9.1 (30 µl, 10 mg/ml) was added. The solution was carefully stirred, and the coupling reaction proceeded for 30 min at 37°C. Separation of labelled cMAb U36 (125I-PIB-cMAb U36) was performed by size-exclusion chromatography on a NAP-5 column pre-equilibrated with PBS (Fig. 1b).

**Indirect labelling with DABI (Papers I, II & III)**

A solution of potassium salt of DABI in water (3 µl, 1 mg/ml), prepared as described in [29] was mixed with stock solution of 125I (20 µl). Labelling was started by adding CAT in water (10 µl, 2 mg/ml) and quenched after 5 min of vortexing by adding sodium metabisulphite (10 µl, 4 mg/ml water). Freshly prepared solution of cMAb U36 in borate buffer, pH 9.1 (30 µl, 10 mg/ml) was then added. The solution was carefully stirred, and the coupling reaction proceeded for 30 min at 37°C. Separation of labelled cMAb U36 (125I-DABI-cMAb U36) was performed by size-exclusion chromatography on a NAP-5 column pre-equilibrated with PBS (Fig. 1c).

**Direct labelling with 131I (Paper III)**

Freshly prepared solution of cMAb U36 in PBS (60µl, 5 mg/ml) was mixed with 131I iodine solution (5 MBq). Reaction was initiated by adding CAT in PBS (10 µl, 2 mg/ml), and quenched after rigorous vortexing for 5 min by adding sodium metabisulphite (20 ml, 2 mg/ml). Labelled antibody (131I-cMAb U36) was separated from non-reacted 131I and low-molecular-weight reaction components by size-exclusion chromatography on a NAP-5 column pre-equilibrated with PBS (Fig. 1 d).
Fig. 1. Structures of the three different $^{125}$I-antibody conjugates used in the first paper. In the directly (CAT) labelled antibody (a), the $^{125}$I is attached directly to the tyrosine residues of the antibody. In the indirectly PIB-labelled conjugate (b), the $^{125}$I is attached to the antibody via the PIB linker molecule to the lysine residues of the antibody. In the case of the indirectly DABI-labelled conjugate (c), the $^{125}$I is attached via the DABI linker molecule to the lysine residues of the antibody (d), and the $^{131}$I is attached via the DABI linker molecule to the lysine residues of the antibody.

$^{211}$Astatine labelling of cMAb U36 (Paper IV)

Between 50 and 100 MBq $^{211}$At in methylene chloride was evaporated using argon gas, and re-solubilized in 10 µl of 0.1% acetic acid. A solution of $N$-succinimidyl 4-(trimethylstannyl) benzoate, prepared as previously described [28], in 5% acetic acid in methanol (5 µl, 1 mg/ml) was then added. The labelling reaction was started by adding chloramine T (CAT) in water (10 µl, 3 mg/ml) and terminated after 5 min by adding sodium metabisulphite (80 µg, 6 mg/ml water). A freshly prepared solution of cMAb U36 in borate buffer, pH 9.1 (30 µl, 10 mg/ml) was added to the reaction mixture, and the coupling reaction proceeded for 30 min at room temperature during continuous shaking. Purification of labelled cMAb U36 was performed by size-exclusion chromatography on a NAP-5 column pre-equilibrated with PBS. Radiochemical purity was checked with ITLC, using 70% acetone in water as a solvent.

Biodistribution (Papers I, II, III & IV)

Paper I. Groups of 4 mice per time point were injected via tail vein with 15 µg of 100-120 kBq $^{125}$I labelled cMAb U36 diluted in PBS. Animals were sacrificed at 1.5, 6, 24 and 96 hours after injection. 16 mice for each method (direct labelling method and two indirectly labelling methods). Altogether 48 normal mice were used in this study.

Paper II. Groups of 3 mice per time point were injected with cMAb U36 labelled with $^{125}$I. $^{125}$I, using either DABI or PIB linker molecules, was diluted in PBS and injected into mice via the tail vein, 118-154 kBq and 100-120 kBq respectively. Biodistribution was measured at 24, 96, and 192 h after injection. Altogether 18 nude mice (study for two indirectly labelling methods) were used in this study (as only 19 of 35 mice had growing tumours).
**Paper III.** Each mouse was injected with 100kBq radioactivity via the tail vein with a mixture, diluted in PBS, of 60 kBq $^{125}$I-labelled with cMAb U36 via DABI linker, 40 kBq $^{131}$I-labelled cMAb U36 respectively. Biodistribution was measured at 3, 7, 24, 96, 192 h after injection (6 mice for each time point). Comparison with directly radio-iodinated cMAb U36 was performed using dual-label technique. Altogether 30 nude mice were used in this study.

**Paper IV.** Before injection, animals were randomized for each time point. cMAb U36 labelled with $^{211}$At was diluted in PBS and injected into the mice via tail vein at a radioactivity of 70 kBq. Biodistribution was measured at 3, 7, and 21 h after injection. Furthermore, 4 mice were given 100-fold higher cMAb U36 i.p. 3 hours before $^{211}$At-cMAb U36 was injected via the tail vein. Altogether 16 nude mice were used in this biodistribution study.

**Sampling of material.** At indicated time points, mice were anaesthetized with a lethal dose of sodium pentobarbital, sacrificed by heart puncture, and dissected. Urine (Paper I) and blood were collected, as well as the major organs and samples of brain, muscle, skeleton, skin (Papers I-IV) and tumours (Papers II-IV). Samples were then weighed and the activity measured. To obtain a quantitative measure of the injected radioactivity, the radioactivity of each syringe was measured before and after injection, and the radioactivity in the tails was measured after dissection. The accumulation of radioactivity expressed as a percentage of the injected dose per gram of tissue (% ID/g) was then calculated (Papers I-IV).

**Tumour models (Papers II, III & IV)**

**Paper II.** The animals used in this study were 5-week-old female NMRI nu/nu nude mice (BK Universal AB, Stockholm). Subcutaneous transplantation was performed by injecting $10^7$ cells of SCC-25 into the left flank (near thigh) of 35 nude mice.

**Papers III-IV.** Adult female mice (balb/c nu/nu, around 25 g; Harlan CPB, Zeist, The Netherlands) were 10 weeks old at the time of the experiments. Nude mice bearing s.c.
human xenografts of the cell line UT–SCC7 were prepared. Altogether 30 nude mice were prepared for comparison biodistribution by using dual-label technique and 6 mice for each time point (Paper III). 16 mice were prepared for $^{211}$At biodistribution study and 4 mice for each time point (Paper IV). 32 mice for s.c. therapy and 26 mice i.v. therapy studies with $^{211}$At were performed (Paper IV).

Papers II-IV. Animals were weighed and tumours were measured 3-4 times a week in three dimensions with calipers. The tumour volume ($V$) was calculated by using the ellipsoid formula: $V = \frac{\pi}{6} \times \text{length} \times \text{width} \times \text{height}$. Doubling time was determined after fitting an exponential equation $V = k_1 e^{k_2 t}$ ($k$ is constant and $t$ stands for time) to the dynamic tumour volume data. From the fitted curve, the doubling time (DT) = $\ln \left(\frac{2}{\ln \frac{V_2}{V_1}}\right)$ was calculated, where $V_2$ and $V_1$ are volumes at $t_2$ and $t_1$, respectively.
Results

Antibody biodistribution (Papers I, II, III & IV)

In normal mice the biodistribution of three radio-conjugates in various organs was illustrated in Fig 2. (Paper I). Generally speaking, the radioactivity in blood and organs decreased with time, whereas there was an accumulation in the thyroid gland and in the skin. The indirectly labelled conjugates showed minor differences in tissue distribution, except in the liver and spleen. The $^{125}$I-DABI-cMAb U36 conjugate also showed greater radioactivity in blood than did the other conjugates at the 96 h time point (Figs. 2-3).

Fig. 2. Comparison of the biodistribution of $^{125}$I-cMAb U36 (--X--), $^{125}$I-DABI-cMAb U36 (□) and $^{125}$I-PIB-cMAb U36 (○) in various organs. Animals were sacrificed at 1.5, 6, 24 and 96 hours after injection. The unit %ID/g denotes the radioactivity, expressed as a percentage of the injected dose per gram of tissue. In these organs, no significant difference ($P > 0.05$) was observed between the conjugates. No accumulation could be seen in any organs except skin, where some accumulation was observed from the 24 h time point. Error bars represent standard deviations. $N = 4$ at each time point.
Tumour-to-blood ratio and tumour-to-organ ratios (Paper II) are illustrated in Figs. 4-5. Influence of tumour xenograft size on uptake of radio-conjugate is shown in Fig 6. Biodistribution was measured at 24, 96 and 192 h after injection. Average values of radionuclide uptake were used to construct biodistribution graphs in Figs. 7-8.

![Graphs showing biodistribution of radiolabeled conjugates in various organs](image)

---

**Fig. 3.** Comparison of the biodistribution of $^{125}$I-cMAb U36 (---X---), $^{125}$I-DABI-cMAb U36 (☐) and $^{125}$I-PIB-cMAb U36 (☐) in blood, liver, spleen and thyroid. Animals were sacrificed at 1.5, 6, 24 and 96 h after injection. The unit %ID/g denotes the activity expressed as a percentage of the injected dose per gram of tissue. Significant differences ($P < 0.05$) were observed at the 1.5 and 96 h time points in liver and spleen and at 96 h in blood, where the DABI conjugate displayed higher values. In thyroid, the directly labelled conjugate displayed significantly higher values than the indirectly labelled conjugates at the 24 and 96 h time points. Error bars show standard deviations. $N = 4$ at each time point.

---

**Fig. 4.** Tumour-to-blood ratios for $^{125}$I-DABI-cMAb U36 (left panel) and $^{125}$I-PIB-cMAb U36 (right panel) at different time points after i.v. injection.

---

18
Fig. 5. Tumour-to-organ ratios for $^{125}$I-DABI-cMAb U36 at different time points. Data are presented as average ± standard deviation.

Fig. 6. Influence of tumour xenograft size on uptake of radiolabelled antibodies. Tumour-to-blood ratios are plotted against tumour weight. Trend lines (………………) were drawn using an integral function in Microsoft Excel.

There was a definite tendency to less accumulation in larger tumours, for both $^{125}$I-DABI-cMAb U36 and $^{125}$I-PIB-cMAb U36 (Fig. 6). In principle, such an effect is well documented both in experimental animal models [30] and in patients [8]. However, it was clear that in vivo targeting was successful for both conjugates. For example, at later times,
namely 96 and 192 h post-injection (p.i.), there was more radioactivity in tumours (all but one) than in blood (Fig. 4).

The tumour-to-organ ratio of $^{125}$I-DABI cMAb-U36 was more than at least 4 at later time points for kidneys, spleen, liver, heart, lung, thyroid, submandibular glands and skin (Fig. 5).

The comparison of biodistribution of $^{125}$I-DABI- cMAb U36 vs. $^{125}$I-PIB- cMAb U36 in tumour-bearing mice was illustrated in Fig. 7. The radioactivity uptake in almost all organs was similar at all times (any difference within error range), for both radiiodinated conjugates. It was noticeable, however, that the thyroid uptake of $^{125}$I-DABI-cMAb U36 was lower at all time points (Fig. 8).

Biodistribution for radioactivity average uptake (Paper III) was measured at 3h, 7h, 24h, 96h and 192h. Generally speaking, the radioactivity decreased with time in the different organs. The lowest uptake was registered in brain for both compounds. A significant biodistribution difference ($P<0.05$) between the both conjugates was seen in Figs 9-10 in most of the organs and tissues in at least one time point, except in lung, stomach, colon and bladder.

---

**Fig. 7.** Comparative distribution of $^{125}$I-DABI-cMAb U36 vs. $^{125}$I-PIB-cMAb U36 at 24 h (upper panel), 96 h (middle panel) and 192 h after i.v. injection. Data are presented as average ± standard deviation.
Fig. 8. Comparison of thyroid uptake of $^{125}$I-DABI-cMAb U36 vs. $^{125}$I-PIB-cMAb U36 at different time points after i.v. injection. Data are presented as average ± standard deviation.

Fig. 9-10. Biodistribution for $^{131}$I-cMAb U36 (Fig.9) vs. $^{125}$I-DABI-cMAb U36 (Fig.10) in various organs at different time points. Mice were sacrificed 3, 7, 24, 96 and 192 h after injection. The unit %ID/g denotes to the activity, expressed in percentage of injected dose per gram of tissue. Data are presented as average standard deviation.

The tumour-organ ratio using $^{131}$I-cMAb U36 and $^{125}$I-DABI-cMAb U36 labelling is shown in Figs. 11-12, respectively.

In Fig. 11, the tumour:organs ratio of $^{125}$I-DABI-cMAb U36 and $^{131}$I-cMAb U36 shows significant differences ($P < 0.05$) in the blood, brain, submandibular gland, stomach, bone, liver and spleen, at least at one time point. In Fig. 12, tumour:organs ratio of two conjugates shows non-significant differences ($P \geq 0.05$) at all time points, in the heart, lung, colon, kidney, muscle and bladder (Paper III).
Fig. 11. Comparison of the tumour-to-blood ratio for $^{131}$I-cMAb U36 vs. $^{125}$I-DABl-cMAbU36 in various organs at different time points. Mice were sacrificed 3, 7, 24, 96 and 192 h after injection. The unit %ID/g denotes the activity, expressed as a percentage of injected dose per gram of tissue.

Fig. 12. Comparison of the tumour-to-blood ratio for $^{131}$I-cMAb U36 vs. $^{125}$I-DABl-cMAbU36 in various organs at different time points. Mice were sacrificed 3, 7, 24, 96 and 96 h after injection. Error bars represent standard deviations.
Fig. 13 shows the ratio of DABI to CAT concerning the tumour-organs-ratio.

There is a tendency that, after time points 96h and 192h, the ratio of DABI to CAT exceeds 1 in most organs, except in the liver and spleen.

![DABI / CAT (Tumour / organ ratio)](image)

Fig. 13. Localization of tumour-to-organs ratio for $^{125}$I-DABI-cMAb U36 vs. $^{125}$I-PIB cMAb U36 at different time points after i.v. injection.

![Fig. 14](image)

Fig. 14. Biodistribution of $^{211}$At-labelled cMAb U36 at set time points. The unit %ID/g denotes the activity, expressed as a percentage of injected dose per gram of tissue. Error bars represent standard deviations.

![Fig. 15](image)

Fig. 15. Specificity of $^{211}$At-labelled cMAb U36 in the biodistribution experiment, 21h post-injection. The unit %ID/g denotes the activity, expressed as a percentage of injected dose per gram of tissue. Error bars represent standard deviations.

The biodistribution of $^{211}$At-labelled -cMAb U36 in various organs (Paper IV) is shown in Fig. 14. Generally speaking, radioactivity in blood and other organs decreased
with time, with the exception of the thyroid. The lowest uptake was recorded in brain and muscles. Fig. 15 compared the specificity of $^{211}$At-labelled cMAb U36 uptake in the organs, between cMAb U36 blocking group and non-blocking group in which no significant biodistribution difference ($P$<0.05) could be seen between these two. By contrast, the specificity shows the property of tumour targeting concerning $^{211}$At-labelled-cMAb U36.

**Radionuclide labelling**

PIB labelling was performed with a labelling efficiency of 60%, and with a substitution ratio of 1.8 – 2.5 PIB/cMAb U36. DABI labelling was performed with an efficiency of 68% and a substitution ratio of 4.1 DABI/cMAb U36 (*Paper I-II*). Labelling with $^{125}$I and $^{131}$I in this setting gave overall yields of 94% for $^{131}$I-cMAb U36, and 49% for $^{125}$I-DABI-cMAb U36, resulting in a DABI:cMAb U36 molar ratio of 2.6:1. For DABI labelling totally 300 µg of cMAb U36 was used and 100 µg for CAT labelling. A radiochemical purity of > 95% for both conjugates is achieved, with preservation of cMAb U36 integrity and immunoreactivity (*Paper III*).

$^{211}$At labelling of cMAb U36 was performed with an efficiency of around 31–33%. Typically, 100-250 MBq $^{211}$At for labelling of 300 µg cMAb U36 resulted in a conjugate with a specific activity of $7\times10^4$ – $2\times10^5$ Bq / µg antibody. This resulted in an attachment of 1-2.1 prosthetic group per cMAb molecule. Radiochemical purity was 97-98%. Using labelling yield data, biodistribution labelling is 31%. For therapeutic experiment no.I, the labelling was 35%; for experiment no.II, labelling was 32% (*Paper IV*).

**Tumour models (Papers II, III & IV)**

Tumour growth (with SCC25) was obtained in about half of the animals (*Paper II*). Calculated from the fitted curve, the tumour doubling time was approximately 32 days. This xenograft model was evidently uneven and tumour growth was very slow. The doel was thus unsuitable for biodistribution studies. Consequently, in the following studies (*Papers III-IV*), the UT-SCC7 cell line replaced the SCC7 cell line.
Tumour growth (with UT-SCC7) was obtained in almost 100%. Calculated from the fitted curve, the tumour doubling time was approximately 4 days. A feature of this xenograft model was the even and rapid growth of the tumours. Subcutaneous tumours became palpable after 3-5 days, but were permitted to grow until day 8 after inoculation, by which time, when the mice were injected with $^{211}$At-cMAb U36 into tail veins, the estimated xenograft volume had attained 191±56 mm$^3$ (mean ± SD, $n$ =18, Two mice were excluded from the data analysis as the tumours were still growing.

$^{211}$At therapy study (Paper IV)

Alpha emitter therapeutic efficacy had been obtained in both experiment I and experiment II, when radio-immunoconjugates had been administered s.c. and i.v.

Fig.16. Efficacy of labelled cMAb U36-$^{211}$At therapeutic experiment I. Groups were injected with tumour cells (X), mixture of tumour cells and “naked” cMAb U36 (γ), mixture of tumour cells and $^{211}$At labelled cMAb U36 (●), or mixture of tumour cells, “naked” cMAb U36 and $^{211}$At labelled cMAb U36 (γ). Tumour size is expressed as average tumour volume (±SD). Error bars represent standard deviations.

Fig.17. Efficacy of labelled cMAb U36-$^{211}$At therapeutic experiment II. $^{211}$At treatment was given day 8. $^{211}$At labelled cMAb U36 was given i.v. and “naked” cMAb U36 for blocking i.p. The control group was not given treatment (●), therapy groups received $^{211}$At-labelled cMAb U36 (●), or $^{211}$At-labelled cMAb U36 combined with blocking (γ). Tumour size is expressed as an average tumour volume (±SD). Error bars represent standard deviations. Data from control group are not shown after volume reaches 1000 mm$^3$.

respectively. There was a significant difference between therapy groups and control groups in both experiments. The results were illustrated in Figs. 16 and 17.
The obvious treatment effect was achieved thanks to the property of $^{211}$At, with high linear energy transfer (LET) and high relative biological effectiveness (RBE) in killing tumour cells. This suggests that a range alpha emitter such as $^{211}$At could be an alternative for RIT of HNSCC.
Discussion

In our study, we investigated a novel radionuclide labelling method by using DABI compared with other radio-iodination methods in order to improve radio-conjugate retention in tumour cells. Radio-immunoconjugates (using cMAb U36) successfully targeted the HNSCC-bearing nude mice, thus providing a good xenograft model for antibody-based study of radio-immunoscntigraphy and radio-immunotherapy.

Biodistribution in normal mice (Paper I)

Three differently radio-iodinated cMAb U36 conjugates showed similar affinities but different biodistribution patterns in normal mice. Generally speaking, the radioactivity in blood and organs decreased with time. The direct-labelled conjugate (using CAT) showed a marked and high accumulation in thyroid, in contrast to the indirectly labelled conjugates. These results are consistent with those described in [31-34]. Since the thyroid is known to accumulate free radio-iodine, this could indicate a different catabolic pathway for the direct-labelled conjugate.

It has been hypothesized that the lesser uptake of radiolabel in thyroid for the indirectly labelled compounds via benzoic derivatives is due to rapid urinary excretion of the intracellular catabolic products. Such excretion may prevent further in vivo transformation of the catabolites and the release of free halide, resulting in a lower thyroid uptake [33, 35].

In conclusion, among three differently radio-iodinated cMAb U36 conjugates, all three conjugates showed high specific binding and similar affinities but displayed different biodistribution patterns. This study also indicates that radio-iodinated DABI conjugated to an appropriate tumour-seeking molecule can provide a stable residualizing iodine label which, if attached to an appropriate targeting agent, will be useful for imaging and therapy. Apart from the specific results obtained, these findings also demonstrate how the appropriate linker molecule will offer additional opportunities to further improve the properties of an antibody-radionuclide conjugate.
Biodistribution in HNSCC-bearing mice (Papers II - III)

A feature of the SCC25 cell line xenograft model was the uneven and very slow growth (DT=32 days) of the tumours, which made it unsuitable for the biodistribution study (Paper II). The UT-SCC7 cell line, with even and rapid tumour growth (DT=4 days) replaced the SCC7 model in Papers III and IV.

In tumour-bearing studies, the ability of $^{125}$I-DABI-cMAb U36 to target HNSCC \textit{in vivo} was demonstrated. Furthermore, DABI-based labelling conjugates showed the ability of a consistently low thyroid uptake [15, 36], indicating high stability of the radiolabels and different radiocatabolites from the directly labelled conjugate.

The ratio of tumour to organs (tumour localization) exceeded 1 in most of the organs at 96 and 192 h, in comparison with direct-labelled conjugate - except liver and spleen - which makes DABI more promising for further radio-immunodetection and antibody-based RIT (Paper III). However, a rapid blood clearance and an elevated uptake in liver and spleen were characteristic of DABI-labelled cMAb U36 (Papers I-III). The reason for this phenomenon is not clear which leaves more scope for further study.

Analysis of literature data shows that residual label accumulates in liver more than its non-residual counterparts [37]. This can be explained by the fact that liver is a major site of normal catabolism of antibodies [38]. Again in the literature, it has been noticed that excess coupling of MAG3 chelators to some antibodies including U36, can cause increased uptake of $^{186}$Re-MAbs in the reticuloendothelial system (viz. liver and spleen) immediately after injection of the conjugates [12, 39].

As kidney might be a dose-limiting organ during radionuclide therapy in the case of high uptake of residual radiocatabolites, it has attracted our attention. Renal excretion is a most important pathway for disposal of low molecular weight hydrophilic radiocatabolites. Fortunately this does not happen with DABI. In fact, kidney uptake was even lower at 24 h p.i. for $^{125}$I-DABI-cMAb U36.

In conclusion, DABI may well be a promising molecule for further radio-immunodetection and antibody-based RIT.
Therapeutic experiments in HNSCC-bearing mice (Paper IV)

In clinical phase I studies, stable disease and anti-tumour effects have been seen in incurable patients with HNSCC and bulky disease [40], when using the beta emitter $^{186}$Re. Combination with an alpha emitter such as $^{211}$At might prove even more effective.

Therapeutic experiment no. I was performed with $^{211}$At in 32 HNSCC-bearing nude mice, randomized into four groups for injection with: 1. labelling conjugate mixed with tumour cells ($n=10$), 2. labelling conjugate and blocker mixed with tumour cells ($n=10$), 3. blocker mixed with tumour cells ($n=6$), 4. tumour cells were injected ($n=6$).

When tumour-bearing mice were treated with $^{211}$At injected s.c., 18 of the 20 tumours in the therapy groups (cMAb U36 blocked or non-blocked disappeared between the 12th and 26th day after inoculation. The remaining two had volumes of 0.52 and 1.77 mm$^3$ respectively on the 26th day. Tumours continued to grow in non-treated groups.

The obvious treatment effect achieved was due to two properties of $^{211}$At - high linear energy transfer (LET) and high relative biological effectiveness (RBE) useful for killing tumour cells [9]. $^{211}$At is a short-range emitter having only a few cell diameters path length (usually 50-100 µm) which could be a drawback when dealing with larger tumours with varying haemodynamics and antigen concentrations [41-42].

Most investigations using RIT for solid tumours have been made with the radioisotope injected at the site of the tumour [43] (as our design in this experiment) or injected with the tumour growing intraperitoneally [44]. In order to ascertain whether or not the tumour cells were killed before the blocking antibodies found their antigens, we designed therapeutic experiment II which investigated the therapeutic efficacy of $^{211}$At intravenously administered in tumour-bearing nude mice.

In therapeutic experiment no. II, the animals were randomly divided into three groups. $^{211}$At was given to the two therapy groups. In therapy group 1, an excess of “naked” cMAb U36 was given i.p. 3 hours before $^{211}$At injection in an attempt to block CD44v6 in the tumour cells. $^{211}$At - cMAb U36 was then injected i.v. in therapy groups 1 ($n=10$) and 2 ($n=10$). 6 tumour-bearing mice, left without therapy, were regarded as a control group.

Effective treatment was achieved in the therapeutic groups in experiment no. II as well. The smallest tumour volumes were: 25 mm$^3$ (mean 111 mm$^3$) in the group without blocking, and 65 mm$^3$ (mean 145 mm$^3$) in the blocked group.
Successful RIT in animal models can be found in the literature, but the data show that tumours started to grow again around the 12th-17th day p.i. [45-46]. In our experiment, none of the tumours regrew after treatment. They even showed a tendency to continue diminishing, which indicates an efficient targeting therapy.

Most treated tumours had ulcers at the endpoint, possibly the result of a combination of a direct effect of radioactivity on the tumour cells and indirect vascular effect. Tumour blood vessels are often tortuous, have poor wall structure and fenestrations that increase vascular permeability [47]. Antibody conjugates injected i.v. reach the tumour within minutes, but antibody accumulation in solid tumours is slow, in contrast to saturation of endothelial binding sites [48-49]. Before the accumulation of labelled conjugates peaks in the tumour, they pass continuously through the vascular endothelial cells, destroying them in the process.
General summary

The objective of this thesis was to develop a novel DABI-based radionuclide labelling method by using cMAb U36 intended for tumour targeting, as a therapeutic application of an alpha emitter in HNSCC-bearing nude mice.

Anti-CD44v6 cMAb U36 is over-expressed on the cell surface of HNSCC. cMAb U36-based radio-immunoconjugates have a potential application for RIS and RIT which are adjuvant means of imaging and therapy for HNSCC patients.

Radio-halogens, such as $^{125}$I and $^{131}$I are characterized by diffusing rapidly out of cells, once the targeting agent is internalized and degraded. This leads to a decrease in tumour-associated radioactivity. One way to counteract this excretion of radio-halogens is that, when cMAb U36 is labelled with a radio-nuclide linker such as DABI, the radio-conjugates are retained inside lysosomes and in this way increase exposure time.

In paper I, the biodistribution of radio-iodinated cMAb U36 was analysed by using the DABI linker, compared with the other radio-iodination methods in normal mice. In papers II & III, DABI-based radio-labelling methods were evaluated regarding their ability to target HNSCC-bearing nude mice. In paper IV, therapeutic experiments with alpha emitter $^{211}$At were performed in HNSCC-bearing nude mice.

Indirect radio-immunoconjugates, such as the DABI linker, had only one-tenth of the thyroid uptake of a direct labelling method at time point 96h, p.i., indicating different catabolites for these conjugates. Radio-immunoconjugates retained the ability to target tumours in HNSCC-bearing nude mice. DABI-related radio-immunoconjugates improved the ratio of tumour to blood and organs - except liver and spleen.

Alpha-emitter treatment effect had been achieved in HNSCC-bearing nude mice by administering by both s.c. and i.v. routes. With subcutaneous therapy, 18 of 20 tumours in the treated groups had disappeared between the 12th and 26th day after inoculation. With intravenous therapy, a significant difference was evident between the control group and the treatment groups.
It is concluded that the use of UT-SCC7 cell line constitutes a suitable animal model giving an almost 100% tumour growth-rate and formation of uniform tumours in the HNSCC targeting study. DABI appears to be a promising linker molecule for radio-immunoconjugates in order to improve tumour cell retention. A short-range alpha-emitter, such as $^{211}$At, could be an alternative for RIT of HNSCC.
Acknowledgements

The underlying work for this thesis was completed on the basis of a collaboration between the Department of Oto-Rhino-Laryngology and Head & Neck Surgery and the Biomedical Radiation Science (BMC) Division at University of Uppsala, Sweden. Financial support was received partly from the Medical Faculty, Uppsala University and the Swedish Medical Research Council (project number 17X-7305) and partly by the grants from Cancerfonden Sweden (project nos 4462-B01-02PAA and 3980-B00-04XBB).

I wish to express my sincere appreciation and thanks to the following:

Professor Matti Anniko, my Chief Supervisor and the Head of the ENT Department, Uppsala University Hospital in Sweden: for unfailing academic – and financial - support, for your encouraging attitude and for believing in me and my ability to conclude the work in some way – especially initially in view of my poor grasp of the English language. I was in the midst of this project, facing a collapsing experiment and ultimately the prospect of completing my dissertation. Thank you for daring to accept me as a Ph.D. student!

Professor Baoqi Yang, my previous Supervisor and Head of ENT Department, Tianjin First Central Hospital, Tianjin, P.R. China; your Department always had a wonderful, academically international ambience which made my project seems feasible, thanks to the collaboration between Tianjin First Central Hospital and the ENT Department at University Hospital, Uppsala. Your steadfast support helped me greatly in completing this project in Sweden.

Associate Professor Mats Engström – your support kept my project “on track” right to the finish; thank you for your patience in all circumstances, I really do appreciate that.

Associate Professor Vladimir Tolmachev – this work could not have been completed without your not inconsiderable assistance and contribution.
My colleague, Dr. Tomas Ekberg - thanks for your help with the Astatine experiment. I must mention here your son’s contribution – he followed us to the end of this work when I was in a “handicapped” state!

My co-workers Marika Nestor and Mikael Persson – thanks for your practical assistance in the animal experiments and with cell culturing.

Professor Jörgen Carlsson – thanks for valuable ideas regarding the astatine therapeutic experiment and for offering me such an excellent working environment.

Associate Professor Kalevi Kairemo – thanks for initial assistance in getting the project on the move.

Professor Guus van Dongen – thanks for your valuable advice regarding paper III.

Assistant Professor Birgitta Linder – thanks for always helping me to obtain and prepare all the necessary materials for the experiments animals for training work, medicine and all other agents and all the instruments.

Assistant Professor Leif Nordang - thank for your help and for trying to anticipate all the things I needed!

Ms Anneli Persson – thanks for your not inconsiderable administrative and secretarial assistance.

Åsa, Ylva, Ann-Charlotte, Veronika and the staff at Rudbeck laboratory, Uppsala, for always offering help when I needed it.

I would like here to offer my thanks to all present colleagues at the ENT Department, Akademiska Hospital, Uppsala, for supporting me through to the conclusion of this work,
and to my former colleagues at the Tianjin First Central Hospital Hospital, for sharing my pleasure when I returned to China for a break.

**Dr. Rafael Acosta and his family** — I am so grateful for your company on many holidays in Sweden; I have lots of happy memories of our time together.

**Juan Ramon Lopez-Egido** - thanks for your help and for trying to solve any problem when I asked; I really appreciate that.

**Qiwen Cheng, Yuying Yan**, my parents - and other relatives, who always gave me such unwavering support.

**Chunping Zhao and Sicong Zhao**, my husband and daughter, who have always been so supportive to me, encouraging me to complete this project in Sweden. Your contributions have been invisible, yet so indispensable. There would be NO Ph.D DEGREE WITHOUT YOU!
References


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title "Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine").

Distribution: publications.uu.se
urn:nbn:se:uu:diva-5834