Department of Chemistry, University of Jyväskylä

DERIVATIVES OF DEXTRAN
SYNTHESIS AND APPLICATIONS IN ONCOLOGY

JIN DU

Academic Dissertation
for the Degree of
Doctor of Philosophy

Jyväskylä, Finland 2001

Research Report No. 85

URN:ISBN:978-952-86-0145-6
ISBN 978-952-86-0145-6 (PDF)
ISSN 0357-346X

University of Jyväskylä, 2024

OPPONENT

Docent Kim A. Bergström, University of Kuopio

SUPERVISOR

Professor Jussi Valkonen, University of Jyväskylä
Dr. Anders R. Holmberg, Karolinska Institute, Sweden
Docent Jukka Hiltunen, University of Helsinki

REVIEWERS

Professor Stig A. Larsson, Karolinska Hospital, Sweden
Docent Aapo Ahonen, University of Turku

DEPARTMENT OF CHEMISTRY, UNIVERSITY OF JYVÄSKYLÄ
RESEARCH REPORT No. 85

Derivatives of Dextran

Synthesis and Applications in Oncology

By

JIN DU

Academic Dissertation for the Degree of
Doctor of Philosophy

To be presented, by permission of the Faculty of Mathematics and Natural Sciences of the University of Jyväskylä, for public examination in Auditorium KEM4, on June 9th, 2001, at 12 noon

Jyväskylä, Finland
ISBN 951-39-0992-1
ISSN 0357-346X

PREFACE

The work described in this thesis was carried out in MAP Medical Technologies Oy during the years 1997-2001.

First of all, I wish to express my gratitude to Professor Jussi Valkonen Ph.D., for being my formal supervisor in University of Jyväskylä.

I am deeply indebted to my second supervisor Anders R. Holmberg Ph.D., for his inexhaustible patience and guidance. His thorough knowledge of dextran chemistry and his ability to suggest solutions have been most helpful in carrying out this study.

I am deeply indebted to my co-supervisor Docent Jukka Hiltunen, Ph.D., the President of MAP Medical Technologies Oy for providing unique working and studying opportunity and sharing his experience in radiochemistry with me. His encouragement, support and especially his warm friendship made this study possible.

I wish to thank the official referees of the thesis, Professor Stig A. Larsson, Ph.D. and Docent Aapo Ahonen, M.D. Ph.D. for their valuable criticism and comments. I also wish to thank Grayce Kautzer for revising the language of this thesis.

I am very grateful to all my collaborators, particularly to Marcela Marquez Ph.D., Martin Behe, Ph.D., and Professor Sten Nilsson, M.D., Ph.D.

I want to express my thanks to my teacher, Professor emeritus Jouni Tummavuori Ph.D. for teaching me the knowledge on analytic chemistry and for his encouragement during this study.

I want to use this chance to express my grateful to Professor Xiaohai Jin in China Institute of Atomic Energy for introducing me to the field of radiopharmaceutical chemistry.

I wish to thank Aki Savonen M.Sc. for his encouragement and friendship during this study.

I sincerely thank all the personnel at MAP Medical Technologies Oy for being so helpful throughout this study. In particular, I want to express my gratitude to Jaana Kumpulainen Ph.D., Pirkko Penttilä M.Sc., Timo Nelimarkka B.Sc., Mika Nyyssönen M.Sc., Jarmo Haukka M.Sc. and Jukka Siikki M.Sc. for their encouragement, collaboration and friendship.

Finally, there is no way I can express my deepest gratitude to my wife Yumei, my son Cong and the whole family in China for their patient waiting, understanding and support during the past four years.

Financial support from MAP Medical Technologies Oy, the Cancer Society in Stockholm and the King Gustaf V Jubilee Fund, Sweden is gratefully acknowledged.

Jyväskylä, February 2001

Jin Du

ABSTRACT

Targeted therapy for cancer treatment using cytostatic drugs and radionuclides is becoming an increasingly important tool in clinical oncology. Much research is focused on the development of effective tumour targeting compounds. The present study describes the construction of dextran based tumour targeting conjugates and examples of their applications in oncology.

The behaviour of charge-modified dextran was investigated in patients with superficial urinary bladder cancer. Cationic dextran showed a strong tumour selective accumulation indicating possible to use it as a vehicle for therapeutic compounds. Epirubicin was coupled to the cationic dextran resulting in a cytotoxic formulation. The cationic epirubicin-dextran conjugate was tested *in vitro* and showed a marked inhibitory effect on the growth of three human bladder cancer cell-lines. Interestingly, the cationic moiety of the conjugate enhanced the inhibitory effect. The cytotoxicity of cationic epirubicin-dextran was comparable to free epirubicin.

To allow rhenium-188 chelation, cysteine was coupled to dextran resulting in free sulfhydryl side-groups. The cysteine-dextran was labelled with ^{188}Re using ^{188}Re-gluconate as transchelator. The labelling efficiency was 60-70%, and radiochemical purity was > 95%. In the presence of an antioxidant, the ^{188}Re-dextran had a high stability.

The ^{99m}Tc-tricabonyl method was adapted for tecnetium-99m labelling of a somatostatin-dextran conjugate. The *in vitro* stability of the labelled conjugate was evaluated by size-exclusion high performance liquid chromatography (HPLC). The labelled conjugate interacted with serum protein (~30% of radioactivity was associated with serum protein after 24 h incubation at 37°C).

The biodistribution and blood half-life of somatostatin-dextran^{70} were investigated in normal mice. The results showed enhanced plasma half-life, ~27 h after subcutaneous administration. The biodistribution showed uptake in liver, spleen and kidneys indicating the route of digestion and excretion. The conjugate showed high somatostatin receptor-binding affinity when tested *in vitro* on rat brain cortex membranes.

The different dextran conjugates described in this study have a potential of becoming tools in clinical oncology, such as the cationic dextran derivatives for superficial bladder cancer and radioactive/non-radioactive somatostatin-dextran for systemic tumour targeting. The clinical relevance of non-radioactive somatostatin-dextran^{70} in the treatment of somatostatin receptor positive tumours is currently being investigated in clinical phase I-II studies.

LIST OF ORIGINAL PAPERS

This thesis is based on the following original papers, which are referred to in the text by their Roman numerals (I – V).

I. ION EXCHANGE TUMOR TARGETING: A NEW APPROACH

Anders R. Holmberg, Meir Wilchek, Marcela Marquez, Jan-Erik Westlin, Jin Du, and Sten Nilsson.

Clinical Cancer Research, 5: 3056s-3058s, 1999 (Suppl.).

https://aacrjournals.org/clincancerres/article/5/10/3056s/288222

II. CYTOTOXIC EFFECTS OF CATIONIC DEXTRAN, IN VITRO STUDIES

Marcela Marquez, Jin Du, Sten Nilsson, Lena Lennartsson, Jukka Hiltunen, Jan-Erik Westlin, Teuvo Tammela, Mika Raitanen, Matti Laato, and Anders R. Holmberg.

Manuscript, 2000.

https://europepmc.org/article/med/12014645

III. RADIOLABELLING OF DEXTRAN WITH RHENIUM-188

Jin Du, Marcela Marquez, Jukka Hiltunen, Sten Nilsson, and Anders R. Holmberg.

Applied Radiation and Isotopes, 53: 443-448, 2000.

https://doi.org/10.1016/S0969-8043(99)00283-3

IV. TECHNETIUM-99M LABELLING OF GLYCOSYLATED SOMATOSTATIN-14

Jin Du, Jukka Hiltunen, Marcela Marquez, Sten Nilsson, and Anders R. Holmberg.

Applied Radiation and Isotopes, 55: 181-187, 2001.

https://doi.org/10.1016/S0969-8043(01)00046-X

V. BIODISTRIBUTION, BLOOD HALF-LIFE AND RECEPTOR BINDING OF A SOMATOSTATIN-DEXTRAN CONJUGATE

Martin Behe, Jin Du, Wolfgang Becker, Thomas Behr, Christa Angerstein, Marcela Marquez, Jukka Hiltunen, Sten Nilsson, and Anders R. Holmberg.

Medical Oncology, accepted, 2000.

https://doi.org/10.1385/MO:18:1:59

TABLE OF CONTENTS

ABBREVIATIONS

BFCA	bifunctional chelating agent
BSA	bovine serum albumin
CDAP	1-cyano-4-dimethylaminopyridinium
cpm	counts per minute
DTPA	diethylenetriaminepenta-acetic acid
Dx	dextran
EGF	epidermal growth factor
HPLC	high performance liquid chromatography
IC_{50}	concentration causing 50% inhibition
ITLC	instant thin-layer chromatography
kDa	kilo dalton
MAG_3	mercaptoacetyltriglycine
MBq	Mega Becquerel (10^6 Bq)
MMCD	dextran-mitomycin C
PBS	phosphate buffered saline solution
s.c.	subcutaneous
sms	somatostatin
smsr	somatostatin receptor
SPDP	N-succinimidyl-3-(2-pyridyldithio) propionate
Tris	tris (hydroxymethyl)aminoethane

1. INTRODUCTION

1.1 Purpose of the present study

Dextran has been in clinical use for several decades, and its pharmacological and biological properties are well documented. It can be modified (chemical activation) allowing coupling of compounds that can be of medical interest. An interesting and potentially useful property of such dextran conjugates is that biologically active substances are given a prolonged *in vivo* half-life. Dextran may also act as a carrier for pharmaceutical drugs, e.g. cytostatic drugs or radionuclides. An attractive strategy in the development of compounds for tumour targeting in cancer therapy is to use dextran as a construct backbone.

The present study describes the construction of dextran based tumour targeting conjugates and examples of their applications in oncology. Four amino acids, a cytostatic drug, a neuropeptide and different radionuclides were the components of the different conjugates. The purpose of the study was to construct dextran based conjugates with a potential of becoming tools in clinical oncology.

1.2 Tumour targeting

Conventional cancer treatment, i.e. surgery, chemotherapy, and external beam radiotherapy continues to play a primary role in cancer therapy. However new approaches are becoming increasingly important, e.g. specific delivery of cytotoxic substances to cancer cells causing minimal damage to healthy tissue is an attractive approach in cancer therapy ("tumour targeting"). Tumour targeting means that a specific ligand carrying cytotoxic agents is delivered selectively to the tumour tissues due to its inherent specificity for the tumour. The ligand can also target tumour tissues by physicochemical or other mechanisms. The damage to healthy tissues is limited while the tumour tissues are optimally sterilised. In general, the targeting conjugate is administrated intravenously; however, local or intratumoural administrations may also be considered (Wahl et al., 1988; Perkins et al., 1989; Hird et al., 1990; Wang et al., 1998).

For targeting to be effective, specific markers or structures (i.e. targets) that do not exist on normal cells, or are present in very low amounts, need to be expressed by the tumour cells.

Targeting molecules may be directed against these targets. Examples of such a system are monoclonal antibodies directed against tumour-associated antigens, natural peptides or glycoproteins, which bind to specific receptors on the cancer cell surface.

1.2.1 Receptors as tumour targets

Paul Ehrlich first introduced the term "receptor" to describe the interaction of the tetanus toxin with its specific protoplasmic site (Ehrlich and Heter, 1904). He also speculated that the molecules with affinities for certain organs could transport therapeutically active substances to the target organ. Traditionally, biochemists define receptors as entities that can recognise a receptor ligand with high affinity and selectivity. The receptors are usually proteins that are embedded in a double layer of lipid molecules, the cell membrane, which separates the extracellular environment from the intracellular compartment (Bretscher, 1985). Since there are only a few structural determinations of receptors, a receptor is often characterised by its biological properties, including high ligand affinity, specificity, saturability, and distribution in relation to the physiological response (Eckelman, 1994). In general, the receptor protein is present in a limited concentration (10^{-6} – 10^{-10} M per gram of tissue) (Eckelman, 1992; Langstorm and Hartvig, 1992).

In a multicellular organism, receptors fulfil important functions, i.e. they act as a "molecular antennae" in the communication between cells and specific ligands. When a ligand is attached to the specific receptor protein on the outside of the target cell, the signal is transduced through the membrane by a family of guanyl nucleotide regulatory binding proteins (Gilman, 1987). After ligand binding, these become active due to a change in conformation, resulting in activation or inhibition of a number of specific second messengers and proteins. They in turn activate or inhibit a characteristic cellular process such as contraction, secretion, metabolism or growth (Berridge, 1985).

A wide range of biological actions is mediated by specific receptors. Some specific receptors are expressed in many different tissues, i.e. normal and tumour tissue. In some cases, tumours express receptors in higher densities than normal tissues. Recent studies show that a variety of receptors with high affinity for regulatory peptides or other ligands are over-expressed on tumour cells. Such as somatostatin receptors (Reichlin, 1983), vasoactive intestinal peptide

receptors (Reubi, 1995), substance P receptors (Hennig et al., 1995), alpha-melanocyte stimulating hormone receptors (Eberle et al., 1993), and epidermal growth factor (EGF) receptors (Liberman et al., 1984). Over-expressed tumour specific receptors represent potential molecular targets for selective delivery of therapeutic compounds.

1.2.2 Targeting molecules

When using a receptor as tumour target, the ligand itself or a receptor specific monoclonal antibody can be chosen as the tumour-targeting molecule. Antibodies may have high receptor binding affinity and high specificity. They are in fact dominating as vehicles in tumour targeting (Bale and Spar, 1967; Gregoriadis, 1977; Ghose and Blair 1978; Goldenberg, 1988; Parker, 1990; Pietersz, 1990). However, monoclonal antibodies have some disadvantages, e.g. they show poor penetration into solid tumours due to their high molecular weight (Wheldon and O'Donoghue, 1990). Clinical studies with radiolabelled antibodies have often demonstrated limited accumulation in the target and relatively slow blood clearance, resulting in only modest target-to-background ratios (Vaughan et al., 1987).

Compared to antibodies, small peptides offer several advantages. Peptides are necessary elements in fundamental biological processes. In many cases the binding affinity of peptides are higher than that of antibodies and their fragments (Fischman et al., 1993). Peptides often tolerate chemical modification better than antibodies (McAfee and Neumann, 1996). Therefore they usually remain active after modification. Additionally, they are less likely to be immunogenic and they show rapid blood clearance. All these factors make peptides interesting candidates for use in tumour targeting constructs. However, there are also disadvantages, e.g. they often have short *in vivo* half-life due to enzymatic degradation. When doing direct conjugation with bifunctional chelating agent (BFCA) and radiolabelling they may loose some of their receptor binding affinity.

1.2.3 Somatostatin and its receptors

Somatostatin (sms) is a natural cyclic disulphide-containing peptide hormone having 14 or 28 amino acids. It is found in a number of organ systems, such as the central nervous system, the hypothalamus pituitary system, the gastrointestinal tract, the exocrine and endocrine pancreas

and the immune system. Somatostatin is an important regulating peptide, which exerts inhibitory effects on the secretion of growth hormones (Reichlin, 1983). The somatostatin receptors (smsr) are over-expressed on most human cancers, e.g. small cell lung cancers, breast tumours, brain tumours, lymphomas, prostate cancer etc. (Reubi et al., 1990). Until now, five different human somatostatin receptor subtypes, smsr1-smsr5, have been cloned and characterised (Reisine and Bell, 1995). Natural somatostatin has too short a biological half-life (< 3 min) to be clinically useful. Therefore many somatostatin analogues with extended half-life have been developed. The most well known and clinically established is the octreotide, a cyclic octapeptide with an improved specificity and a half-life of about 90 min (Bauer et al., 1982; Weckbecker et al., 1992). Recently it was reported that "octreotate", the carboxylic acid derivative of octreotide, offers improved pharmacological properties and could therefore be an interesting alternative to octreotide (De Jong et al., 1998).

1.3 Conjugate design

A tumour-targeting compound often consists of three basic parts, i.e. targeting molecule, carrier, and drug. The targeting molecule serves as the tumour specific moiety, the carrier as the vehicle, and the drug as the therapeutic moiety. Examples are macromolecules, such as antibodies (both targeting molecule and carrier) and its fragments, or small biomolecules like peptide constructs and other synthetic constructs. Drug in this context has a general meaning, i.e. toxin, chemotherapeutic agent, or radionuclide. The different parts of a targeting conjugate are linked together by chemical methods, e.g. a drug linked directly to the peptide, or a drug coupled to a spacer that is linked to the peptide. The linker between two parts can be a simple hydrocarbon chain, polymer, or bifunctional chelating agent (BFCA) depending on the nature of the constituents.

The choice of chemical method should be based on appropriate criteria, for example, the nature of the reactive groups on the targeting molecule and the drug, the *in vivo* stability of the conjugate etc. The possibility of polymer formation during the coupling reaction and possible adverse effects on the biological activity of conjugate need also to be considered.

The reactive groups in antibodies and peptides that can be used for modification are found in the side chains of the amino acids. They include amino, carboxyl, disulphide, hydroxyl,

imidazol, phenol, and thiol groups. The most widely used are carboxyl and amino groups. Some reactive groups may also be introduced to antibodies by the use of heterobifunctional cross-linkers (Means and Feeney, 1990). These methods have been applied most frequently in protein conjugation, e.g. the use of N-suucinimidyl-3 (2-pyridyldithio) propionate (SPDP) for the linking of toxins to antibodies (Carlsson et al., 1977).

In order to increase the number of drug molecules that can be carried by targeting molecules, inert spacers or intermediaries can be used. The drug is reacted with the intermediary and the resulting complex is then coupled resulting in the targeting conjugate. Several intermediaries have been used, e.g. modified dextran (Rowland 1977; Manabe et al., 1984), poly-amino acids (Kato et al., 1984), and human serum albumin (Pietersz et al., 1988).

When the drug is a chemotherapeutic agent with low molecular weight, its functional groups require careful consideration in order to preserve the drug activity (Pietersz, 1990). The carboxyl group has often been used because it allows mild coupling methods, e.g. the water-soluble carbodiimide, which links the carboxyl group with a free amine group of the counterpart (Hurwitz et al., 1975). Other useful reactive groups include amino, hydroxyl, and vicinal dihydroxyl groups. Incorporation of functional reactive groups to drugs may also be possible, e.g. by introduction of protected thiols using SPDP.

When the drug is a radiometal, the bifunctional chelating agent (BFCA), which strongly coordinates to metal ion, is attached to the targeting molecule. The resulting conjugate can subsequently be labelled with the appropriate radioactive metal ion, e. g. coupling of DTPA to antibodies and subsequent labelling with ^{111}In (Hnatowich et al., 1983). The selection of BFCA is largely determined by the nature and oxidation state of the radiometal.

Antibodies and peptides have been radiolabelled with reduced technetium-99m. Two labelling methods have been used, the direct method and the bifunctional chelates method. In the direct method, reduced ^{99m}Tc is bound to sulfhydryls produced by the reduction of disulphide bridges in an antibody or a peptide (Thakur et al., 1993). This method is easy to carry out. However, very little is known about the number of donor atoms and the coordination geometry around the Tc centre. There is little control over the stability of the ^{99m}Tc complex (Eckelman, 1990). This method applies only to antibodies, antibody fragments and some

peptides, since small peptides do not always have disulphide bonds or in some cases the disulphide bond may be essential for the biological activity.

Examples of bifunctional chelators are DTPA (Lanteingne and Hnatowich, 1984), N_2S_2 (Rao et al., 1990), N_3S (Fritzberg et al., 1988), BATOs (Linder et al., 1991) and HYNIC (Abrams et al., 1990). These BFCAs can be used in two ways; the prelabelling approach and the postlabelling approach. In the prelabelling approach, the BFCA is labelled with ^{99m}Tc first and then conjugated to an antibody or a peptide (Fritzberg et al., 1988). In this approach, the chemistry is better defined, and the protein or peptide is not exposed to the adverse conditions that might be used in the chelation step. However, it is too complex and time-consuming for routine clinical use. In the postlabelling approach, a BFCA is covalently attached to an antibody or a peptide to form a conjugate and then radiolabelled with ^{99m}Tc. This approach combines the ease of direct labelling with well-defined chemistry of the preformed chelate approach. Indirect labelling is thus the most practical method.

The technetium core determines the design of the BFCA framework and the choice of donor atoms. Most of ^{99m}Tc complexes are based on Tc(V)oxo or octahedral Tc(III) cores (Johannsen and Spies, 1996). The organometallic low-oxidation state ^{99m}Tc agent is less common, but the Tc(I) oxidation state has particular advantages due to the kinetic inertness inherent in its low-spin d^6 configuration (Salmain et al., 1993; Top et al., 1995). The convenient and fully aqueous synthesis of $[^{99m}Tc(OH_2)_3(CO)_3]^+$, Tc(I) core has recently been developed (Alberto et al., 1998). The three water molecules coordinated to the highly inert $^{99m}Tc(CO)_3$ core are readily substituted by a variety of functional groups such as amines, thioethers, thiols and phosphines (Resigys et al., 1998; Alberto et al., 1999; Schibli et al, 1999; Pietzsch et al, 2000). The $^{99m}Tc(CO)_3$ core has been used to label derivatized antibodies and peptides to form *in vivo* stable radioconjugates (Egli et al., 1999, Waibel et al, 1999).

Reduced technetium also forms stable complexes with carbohydrates (De Kievet, 1981).

Since the chemistry of rhenium is similar to that of technetium, it may be possible to adapt some of the labelling methods that have been developed for ^{99m}Tc. However, the synthesis of rhenium complexes is considerably more difficult because rhenium is more prone to reoxidation and transchelation (Deutsch et al., 1986).

Also other metallic radionuclides have been used to label antibodies or peptides by the BFCA approach, e.g. ^{111}In (Bakker et al., 1991), ^{90}Y (De Jong et al, 1997; Otte et al., 1997), ^{64}Cu (Anderson et al., 1995), and $^{67/68}$Ga (Smith-Jones et al., 1994).

Radioiodine can label antibodies or peptides through their existing tyrosine residues using the Chloramine-T method (Greenwood et al., 1963) and Iodogen method (Salacinski et al., 1981). If tyrosine residues are missing, radiolabelling is carried out via lysine residues by using the Bolton-Hunter reagent, which is a radio-iodinated tyrosine linked with a N-hydroxysuccinimide ester reactive with amine side groups (Bolton and Hunter, 1973).

1.4 Dextran

Dextran is a synthetic polymer of α-D-glucose, presenting 95% of α-1,6-linked linear glucose units with 5% of α-1,3-linked moieties as side chains. A part of a dextran chain is shown in Figure 1. The dextran is synthesised from sucrose by microorganisms of the lactobacillus family or by cell-free systems containing dextran sucrase. The native dextran is a mixture of polymers of different molecular weights ranging from oligo- to macropolysaccharides. Fractions of dextran with a narrow molecular weight range are produced by acid or alkaline hydrolysis of the native dextran mixture, and then by repeated fractionation and precipitation of the hydrolysate (Molteni, 1979; Schacht, 1987). The term "weight average molecular weight" is normally used to define dextran. This means that the average molecular weight of the macromolecules is determined in proportion by weight (Molteni, 1979). Dextrans with broad and narrow average molecular weights are commercially available.

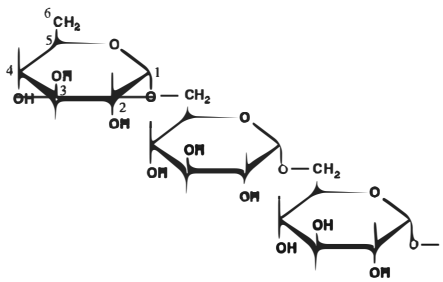


Figure 1. The structure of dextran chain

Dextrans are uncharged and freely soluble in aqueous solution. They are very stable in solution. Dextrans are inert in biological systems and do not affect cell viability. Dextran has been clinically used as a plasma expander to improve blood circulation, to prevent blood platelet aggregation and as blood substitute for several decades (De Belder, 1990).

Dextran has low immunogenicity. Clinical dextran (40 and 70 kDa) does not stimulate formation of antibodies. However, immunogenicity increases with increasing molecular weight and the degree of branching of the polysaccharide chain (Schacht, 1987). The risk of side effects when using clinical dextran is now very low. In very rare cases, anaphylactic reactions have occurred and to prevent this a dextran hapten (m.w. 1000) was given prior to the injection of clinical dextran.

The fate of dextran in the blood circulation depends on its molecular weight. The dextran 40kDa is completely excreted through the kidneys within 48 h. Dextran above the kidney threshold (~50 kDa) can remain in the blood circulation for two or three days and is slowly oxidised (Molteni, 1979; Schacht, 1987). Eventually they are internalised by the reticuloendothelial system (RES) where they are slowly degraded to glucose units by dextranases (Molteni, 1979).

Because of excellent physicochemical properties and numerous reactive hydroxyl groups available for drug fixation, dextran has been used as a macromolecular carrier of drugs, enzymes, hormones, and antibodies (Molteni, 1979). The aims of conjugation to dextran might be to prolong *in vivo* half-life, to increase the stability *in vivo*, to facilitate targeting of a drug, or to decrease antigenicity of the protein moiety.

Different methods have been developed for chemical modification, i.e. activation of the hydroxyl groups in the dextran, making them reactive towards ligands with the appropriate function group.

One method employs cyanolating agents such as cyanogen bromide (CNBr) or 1-cyano-4-dimethylamino pyridinium (CDAP) (Kagedahl and Akerstrom, 1970; Kohn and Wilchek, 1984). The dextran is activated with CDAP or CNBr to yield cyanate esters and imidocarbonates that react with primary amines of a ligand to form isourea bonds. The most

frequently used method is the periodate oxidation method (Foster, 1975; Bernstein et al., 1978; Hurwitz et al., 1978). Dextran is activated with sodium periodate to yield aldehydes that can react with amines of a ligand to form unstable Schiff bonds. The Schiff bonds are reduced with cyanoborohydride to form stable amine bonds (Borch et al., 1971). Both of these common methods have advantages and disadvantages. All cyanogen bromide based methods are more or less toxic and may be difficult to control when activating a soluble polymer like dextran resulting in unwanted cross-linking. The periodate oxidation method is mild, and the polyaldehyde-dextran is relatively stable in aqueous solution, but requires a second step, reductive amination.

1.5 Dextran conjugates

1.5.1 Conjugates with drugs

Dextran has been used in tumour targeting for therapeutic applications (Bernstein et al., 1978; Hurwitz, 1983). The approach has been both "passive" targeting and specific targeting. Passive targeting means the targeting depends on unspecific interactions (Maeda et al., 1992; Seymour, 1992), and specific targeting means dextran-drug conjugates coupled to tumour specific moieties (Noguchi et al., 1992). The drugs utilised were different chemotherapeutic agents.

Several drugs have been coupled to dextran, alone or in association with antibodies (Bernstein et al., 1978; Kojima et al., 1980; Hurwitz, 1983; Manabe et al., 1984; Sezaki and Hashida, 1984; Takakura et al., 1987a; Heindel et al., 1990; Noguchi et al., 1992). For example, daunomycin, doxorubicin, methotrexate, and mitomycin C have been coupled to dextran and tested as polymeric chemotherapeutic agents.

In the late 1970s, Bernstein showed that dextran coupled to daunomycin, produced a conjugate with greater therapeutic activity than the free drug and with decreased toxicity (Bernstein et al., 1978), subsequently, doxorubicin was coupled to dextran 70 kDa. The conjugate had higher plasma concentration, persisted longer in the circulation and an improved therapeutic efficacy compared to the free drug (Fujita et al., 1991).

Methotrexate-dextran conjugates have been tested on mice inoculated with L1210 tumour cells. The conjugates were significantly more effective. The ability of macromolecules to remain in the peritoneal cavity has been postulated as an important factor for their action (Sezaki and Hashida, 1984).

The chemical properties of dextran-drug conjugates are of importance for their ability to target tumours *in vivo*. Studies have revealed that the electric charge and molecular weight of the dextran drug conjugate affects its pharmacokinctic behaviour (Takakura et al., 1987a, b). Dextran-mitomycin C (MMCD) conjugate with a cationic charge exhibited a high affinity for cell surface *in vitro* and showed stronger cytotoxicity compared with free mitomycin C or anionic MMCD conjugate. It is more suitable for local than for intravenous administration because of rapid removal from circulation, due to interaction with the reticuloendothelial system (RES). When the conjugates were injected intravenously, the corresponding anionic MMCD conjugate had a longer circulation time due to less interactions with RES. This resulted in higher tumour accumulation. It showed a stronger anti-tumour effect on the subcutaneous S-180 tumour in mice than an equitoxic dose of free mitomycin C (Takakura et al., 1987a, b).

The anionic MMCD was also conjugated to a monoclonal antibody mAb A7 to enhance the delivery of mitomycin C to the target tumour tissues. The pharmacological activity of mitomycin C in A7-MMCD conjugate was preserved. In addition, A7-MMCD exhibited about a 14-fold greater cytotoxicity than anionic MMCD (Noguchi et al., 1992).

Other examples of dextran-linked drugs coupled to antibodies are cytosine arabinoside (Hurwitz et al., 1985), bleomycin (Manabe et al., 1983), and chlorin e_6 (Oseroff et al., 1986).

1.5.2 Conjugates with peptides

Dextran protects conjugated drugs and polypeptides from biodegradation (Sherwood et al., 1977; Melton et al., 1986). This may be an important advantage, enabling the use of peptides with short *in vivo* half-lives coupled to dextran for tumour targeting applications. For example, epidermal growth factor (EGF) and somatostatin have been coupled and tested as prototypes of tumour targeting compounds. The dextran moieties provide a spacer to attach

therapeutic radionuclides or other cytotoxic substances at a suitable distance from the antigen- or receptor-binding site.

The EGF-dextran conjugate bound specifically to EGF receptors on cultured glioma cells and showed prolonged blood retention time compared to free EGF, especially when the dextran chain was long (Andersson et al., 1991; Lindstrom and Carlsson, 1993). The EGF-dextran conjugates were radiolabelled with ^{76}Br for positron emission tomography (Zhao et al., 1999) and with ^{131}I for radionuclide therapy of EGF-receptor expressed tumours. ^{131}I radiolabelled EGF-dextran conjugate was to a large extent internalised in tumour cells, and the associated radioactivity was retained inside the cells for at least 20-24 h. Therapeutic effect improved compared with native EGF (Andersson et al., 1992). The boron compound, sulfhydryl boron hydride has also been coupled to EGF-dextran for the boron neutron capture therapy application (Gedda et al., 1996). However, with therapeutic amounts of boron atoms coupled to the EGF-dextran, there are significant problems associated with preserving EGF receptor binding activity.

The somatostatin-dextran conjugate retained specific binding to somatostatin receptors and showed a strong enhancement of the *in vivo* half-life (Holmberg et al., 1994, 1995a). The dextran protected the peptide moiety from enzymatic degradation. This conjugate has also been radiolabelled with ^{99m}Tc via the dextran spacer (Holmberg et al., 1995b).

The insulin-dextran conjugate showed increased plasma half-life compared to free insulin. The mean absorption time of the insulin-dextran conjugate was not significantly different from that of free insulin. The insulin receptor-binding constant of the conjugate compared well with that of native insulin (Baudys et al., 1998).

1.5.3 Other dextran conjugates

Dextran-DTPA-Gd^{3+} has been used as contrast agents for nuclear magnetic resonance imaging with good paramagnetic properties and long intravascular persistence (Rebizak et al., 1997, 1998). Radiolabelled dextran with ^{99m}Tc has been used for angiocardiography and lymphoscintigraphic applications (Henze et al., 1982a, b). Recently, its application has been extended to diagnosis of intestinal inflammation (Lahoti et al., 1999).

2. AIMS OF THE STUDY

1. To investigate the behaviour of charge-modified dextran after intravesical instillation in patients with superficial urinary bladder cancer.

2. To develop a cytotoxic formulation of cationic dextran using three human urinary bladder cancer cell-lines.

3. To develop a simple and reproducible method for labelling dextran derivatives with rhenium-188.

4. To develop an alternative method for labelling somatostatin-dextran conjugates with technetium-99m.

5. To investigate the *in vivo* properties of somatostatin-dextran^{70} conjugates in normal mice and evaluate its *in vitro* receptor binding properties.

3. MATERIALS AND METHODS

3.1 Chemicals and reagents

Freeze-dried dextran with a mean molecular weight of 40 kDa or 70 kDa was used as conjugate backbone (Centre for Surface Biotechnology, Uppsala, Sweden). Sodium metaperiodate (Merck AG, Darmstadt, Germany) was used for dextran activation. Sodium cyanoborohydride (Chemicon, Stockholm, Sweden) was used for reductive amination. L-lysine, L-cysteine, L-histidine, taurine (Merck-Schuchardt, Darmstadt, Germany), epirubicin (Pharmacia & Upjohn, Stockholm, Sweden) and somatostatin (Ferring, Arnzneimittel, Germany) were used for dextran conjugation. ^{125}I-Bolton-Hunter regent was obtained from Amersham International (Buckinghamshire, Great Britain). ^{99m}Tc and ^{188}Re were eluted from alumina-based ^{99}Mo/^{99m}Tc and ^{188}W/^{188}Re generator with saline solution, respectively (manufactured at MAP Medical Technologies Oy, Finland). All other reagents were purchased from commercial suppliers and were of analytical grade and used without further purification.

3.2 Conjugate preparation

3.2.1 Activation and conjugation procedures

All the conjugates described in this thesis were synthesised based on the dextran periodate oxidation method. The activation and conjugation procedures for each conjugate are described in detail in papers I-V. Briefly, 20 mg of dextran were dissolved in 1.0 ml 0.1 M sodium acetate buffer at pH 5.5. Thereafter, 6.0 mg of sodium periodate was added, the solution was then incubated under a magnetic stirring in the dark at room temperature. The reaction was allowed to process for 5-16 h. After incubation, the activated dextran was purified on a PD-10 disposable Sephadex G25 column (Pharmacia Amersham Biotech AB, Uppsala, Sweden) equilibrated with buffer solution. To 10 mg of activated dextran in 1.0 ml buffer solution, the conjugated ligand (see Table 1), 2.0 mg of sodium cyanoborohydride were added. The solution was incubated with gentle shaking in the dark at room temperature or at 5°C for 5 - 24 h. Finally, the reaction mixture was purified on a PD-10 column. The conjugates prepared in this study are summarised in Table 1. Column A lists the dextran molecular weight and

activation time. Column B lists the ligand solutions to which the activated dextran was added. In column C, the solution added to the conjugation vials after the respective incubation times given. Total conjugation time is obtained by addition of incubation times given in columns B and C (in parenthesis).

Table 1. Summary of the conjugates used in papers I-V.

Conjugate	A	B	C
Paper I			
Lysine-Dx^{40} (cationic)	Dx^{40} (24 h)	lysine[1] (24 h)	
Taurine-Dx^{40} (anionic)	Dx^{40} (24 h)	taurine[1] (24 h)	
Paper II			
Lysine-Dx^{40} (cationic)	Dx^{40} (18 h)	lysine[2] (5 h)	
Epirubicin-Dx^{40}	Dx^{40} (18 h)	epirubicin[2] (18 h)	
Epirubicin-Dx^{40}-lysine (cationic)	Dx^{40} (18 h)	epirubicin[2] (18 h)	lysine[2] (3 h)
Paper III			
Cysteine-Dx^{70}	Dx^{70} (5 h)	cysteine[1] (16 h)	
Paper IV			
Somatostatin-Dx^{40}-histidine	Dx^{40} (15 h)	somatostatin[3] (4 h)	histidine[3] (3 h)
Somatostatin-Dx^{40}	Dx^{40} (15 h)	somatostatin[3] (4 h)	
Paper V			
Somatostatin-Dx^{70}-taurine	Dx^{70} (15 h)	somatostatin[3] (4 h)	taurine[3] (3 h)

1. ligand in 0.1 M pH 8.2 $NaHCO_3$ buffer, incubated at room temperature
2. ligand in 0.1 M pH 6.5 sodium acetate buffer, incubated at room temperature
3. ligand in 0.2 M pH 6.0 sodium acetate buffer, incubated at 5°C

3.2.2 Conjugate analysis

3.2.2.1 Dextran determination

The dextran content of the conjugate was determined by using the Anthrone method (Scott and Melvin, 1953). A standard solution was prepared contained 5 –100 μg/ml dextran. To 1.0 ml of purified conjugate was added 2.0 ml of Anthrone reagent. The blue-green colour formed was compared with that of the standards.

3.2.2.2 Determination of ligand concentrations

The content of epirubicin (paper II) and somatostatin (papers IV and V) in the dextran conjugate was determined by UV spectrophotometer at 280 nm. The standard curves were prepared. The conjugate sample was measured at the same absorbency, compared with the standards, and the ligand concentration of conjugate was determined.

The sulfhydryl concentrations in cysteine-dextran[70] conjugate (paper III) was determined with Ellamn's reagent, 5,5'-dithio bis(2-nitrobenzoic acid) (DTNB), in a colorimetric assay (Ellman, 1959). The conjugate samples were incubated with an excess of DTNB and the absorbency at 412 nm was measured after 20 min. Absorbency readings were correlated with a standard curve generated from a series of cysteine standards (8 -80 μM).

3.3 Radiolabelling of conjugates

3.3.1 Radiolabelling with ^{99m}Tc

The charge-modified dextran conjugates (paper I) were labelled with ^{99m}Tc as previously described (Holmberg et al., 1995b). Briefly, to 0.5 ml of conjugate (100 μg dextran) in 0.15 M NaCl solution was added 40 μg of $SnCl_2$ in 10 μl of ethanol. After mixing, 150 MBq of $^{99m}TcO_4^-$ solution was added, and the solution was incubated for 15 min at room temperature. After incubation, the radiolabelled conjugate was purified on a PD-10 column (eluted in 0.15 M NaCl). The eluate was sterilised with 0.22 μm filter and diluted in 150 ml saline.

The somatostatin-dextran-histidine conjugate was labelled with ^{99m}Tc by using $^{99m}Tc(CO)_3$ core, based on technetium organometallic chemistry (paper IV). The organometallic precursor $^{99m}Tc(CO)_3$ was prepared according to a published procedure (Alberto et al., 1998). A 0.5 ml of somatostatin-dextran-histidine conjugate (5.0 mg/ml in 0.2 M acetate buffer pH 6.0) was mixed with 0.5 ml of an aqueous solution of $^{99m}Tc(CO)_3$. The solution was incubated for 30 min at 37°C under nitrogen atmosphere. After labelling, the radiolabelled conjugate was purified on PD-10 column. More details of the method are described in paper IV.

3.3.2 Radiolabelling with ^{188}Re

The cysteine-dextran conjugate was labelled with ^{188}Re by a transchelation method using ^{188}Re-gluconate as transchelator (paper III). Complexation of ^{188}Re was achieved with the sodium gluconate in 0.2 M sodium acetate buffer at pH 5.0. Reduced ^{188}Re in 0.5 M gluconate were transchelated to the cysteine-dextran conjugate in 0.2 M sodium acetate at pH 5.5 by incubating equivalent volumes in a sealed vial at room temperature for 90 min. This was followed by filtration through a 0.22 μm filter. Purification of the final product, ^{188}Re-cysteine-dextran, was achieved by using a PD-10 column. To the purified ^{188}Re-cysteine-dextran was added 0.5 ml ascorbic acid solution (120 mg/ml in saline solution) to protect the conjugate from radiolysis. The details of labelling methods are presented in paper III.

3.3.3 Radiolabelling with ^{125}I

The radiolabelling of somatostatin-dextran^{70}-taurine conjugate (paper V) with ^{125}I was performed according to Bolton-Hunter method (1973). Briefly, the conjugate was dissolved in a 0.2 M borate buffer pH 8.5. Five hundred μCi of ^{125}I-Bolton-Hunter reagent were added to 100 μg of the conjugate in 500 μl borate buffer and incubated at room temperature for 30 min. The reaction was terminated by adding 500 μl 0.1 M phosphate buffer pH 7.0 containing 0.05% Tween 20. The solution was purified on a PD-10 column. The eluate was sterile-filtrated with 0.22 μm filter. The radiolabelling of Tyr^3-octreotide with ^{125}I was performed by chloramine-T method as described previously (Bakker et al., 1990). The details of labelling methods are described in paper V.

3.3.4 Quality control

The techniques used for quality control of radiolabelled conjugates in the present study are instant thin-layer chromatography (ITLC, Gelman Science Inc., Ann Abbor, MI) with 85% methanol as eluant, size exclusion chromatography (PD-10 column) and high performance liquid chromatography (HPLC). A size-exclusion HPLC was carried out on a Superdex 200 HR 10/30 column (Pharmacia Biotech AB, Uppsala, Sweden) with 0.05 M sodium phosphate buffered saline solution at pH 7.4 as a mobile phase. The apparatus was Gilson system (USA)

with a Jasco UV-975 for the absorbency detection and a radioactive detector (Bioscan, USA). The details of the methods are presented in papers III, IV and V.

3.3.5 Stability of the ^{99m}Tc and ^{188}Re radiolabelled conjugates

The *in vitro* stability of ^{99m}Tc and ^{188}Re labelled conjugates were determined using cysteine challenge assay as previously reported (Hnatowich et al., 1994). Briefly, a fresh cysteine solution in phosphate buffered saline at pH 7.0 was prepared and serially diluted with the same buffer. The different amounts of radiolabelled conjugate in cysteine solution were incubated at 37°C for 1 h. After incubation, each solution was analysed by size-exclusion chromatography (PD-10 column) or ITLC (85% methanol as solvent). The details of the methods are described in papers III and IV.

The stability of ^{99m}Tc labelled somatostatin-dextran-histidine conjugate in serum was determined by size-exclusion HPLC (paper IV). Typically, 500 µl of radiolabelled conjugate was added to 500 µl fresh human serum. The solution was incubated at 37°C, and samples were taken at different time points for HPLC analysis (5 min – 24 h).

3.4 *In vitro* testing

3.4.1 Cytotoxic effect of cationic dextran (paper II)

The cytotoxic effect of cationic dextran derivatives on urinary bladder cancer cell-lines was investigated by using a fluorometric cytotoxicity assay as described by Larsson and Nygren (1989). Briefly, three human bladder cancer cell-lines J82, TCCsup and 5637 (ATTC, Manassas, USA) were used. About 10,000 cells in culture medium were seeded into the wells of flat-bottomed 96-well microtiter plates (Falcon, Becton Dickinson, Meylan, France). Dextran, cationic dextran, cationic epirubicin-dextran, epirubicin-dextran and epirubicin (at equimolar concentration) were added at two concentrations, 6 µM and 12 µM. After 72 h of incubation the microtiter plates were centrifuged (200 g for 3 min), and the medium was removed. The cells were washed once with 0.15 M phosphate buffered saline at pH 7.4. A 200µl of fluorescein diacetate (Sigma, Sweden) solution in PBS (10 µg/ml) was added to each well. The plates were then incubated for 30 min at 37°C. The emitted fluorescence of the

samples was counted with a scanning fluorometer (Fluoroscan 2, Labsystems, Finland). The data were transferred to a Macintosh SE computer and the results were calculated.

3.4.2 Receptor-binding assay of somatostatin-dextran^{70} (paper V)

The radioligand binding studies were performed as described previously (Maina et al., 1994). Briefly, the rat cortex membranes were diluted to 50 μg protein per assay tube with binding buffer (50 mM Tris-HCl, pH 7.5; 2 mM $MgCl_2$; 0.5% BSA; 1.0 μg/ml aprotinin). Binding assays consisted of 50 μl of ^{125}I-Tyr^3-octreotide (100 000 cpm) and 50 μl binding buffer or increasing concentration of somatostatin-dextran^{70} and 200 μl membrane suspension. The tubes were incubated for 60 min at room temperature. The incubation was stopped by rapid filtration over glass fibre filters (Whatman GF/C, pre-soaked in 1% BSA) and subsequently washed with 40 ml of 154 mM NaCl/10 mM Tris buffer (pH 7.5, 4°C). Specific radioligand binding was defined as total binding minus non-specific binding. The experiments were carried out in triplicate. The data were analysed by competition curve analysis.

3.5 *In vivo* testing

3.5.1 *In vivo* behaviour of ^{99m}Tc labelled charge modified dextran (paper I)

Ten patients with diagnosed (biopsy proven) superficial bladder cancer were included. They were all males and had a mean age of 64 years. Four of them were instilled with cationic dextran, three with anionic dextran, and three with neutral dextran. Approximately 60 MBq of ^{99m}Tc labelled charged-modified dextran (100 μg) diluted in 150 ml of saline, was instilled intravesically through an urethral catheter and then kept in the bladder for 30 min. During the 30 min incubation, the patient was encouraged to move about in bed to improve the incubation conditions. After incubation, the bladder was washed carefully with 200 ml of saline. The patient was then moved to the operation theatre. Biopsy samples were taken from normal and tumour tissue. The samples were weighed and measured in a gamma counter. The radioactivity per gram of tissue and the quotient of radioactive uptake in tumour tissue to uptake in normal tissue were calculated.

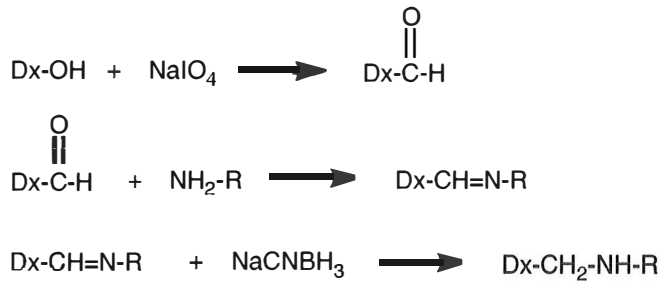
3.5.2 Biodistribution of ^{125}I labelled somatostatin-dextran^{70} (paper V)

The ^{125}I labelled somatostatin-dextran^{70} conjugate was diluted to 1.85 MBq/ml with saline. Normal mice were injected subcutaneously (s.c.) with 0.1 ml of the conjugate solution. The mice (three at each time point) were sacrificed by cervical dislocation after 1 h, 4 h, 6 h, 24 h and 48 h post injection. Samples of blood and organs were collected, i.e. liver, spleen, stomach, intestine, kidney, adrenals and heart. The organs were carefully rinsed with saline solution, weighed and the radioactivity was measured in an automatic NaI (Tl) gamma counter. The percentage of the injected dose per gram of tissue (%ID/g) was calculated.

4. RESULTS

4.1 Preparation of dextran conjugates

The general activation and coupling reactions is illustrated in scheme 1. Dextran is activated with sodium periodate yielding aldehydes that react with amines forming unstable Schiff bonds. The Schiff bonds are reduced with cyanoborohydride forming stable amine bonds.



Scheme 1. Dx, dextran; R, ligand.

The coupling yield was different for each conjugate, depending on varying activation and coupling conditions. In papers I and II, the ambition was to obtain charge-modified dextran with a maximum coupling yield. The molar ratio between sodium periodate and dextran glucose units was 0.5–1. With this activation degree, 10 lysine residues per dextran and 2 epirubicin per dextran were coupled. The lysine coupling to dextran resulted in cationic dextran derivatives.

In paper III, activated dextran was incubated with cysteine in molar excess, yielding a coupling efficiency of 10%, i.e. 10-12 cysteine residue were coupled to one dextran chain.

In the papers IV and V, the degree of dextran activation was kept low to minimise changes in the original dextran structure (cross-linking, partial polymerisation). The molar ratio between sodium periodate and the dextran glucose units were 0.25. With this activation degree, on average, three to four somatostatin molecules were coupled per dextran chain. This yield was highly reproducible and HPLC analysis revealed no polymerisation. The coupling time was optimised to prevent lysine residues located in the receptor-binding site of somatostatin to be

involved in the coupling reaction. Coupling for more than 4 h resulted in increasing involvement of the lysine residues (Figure 2).

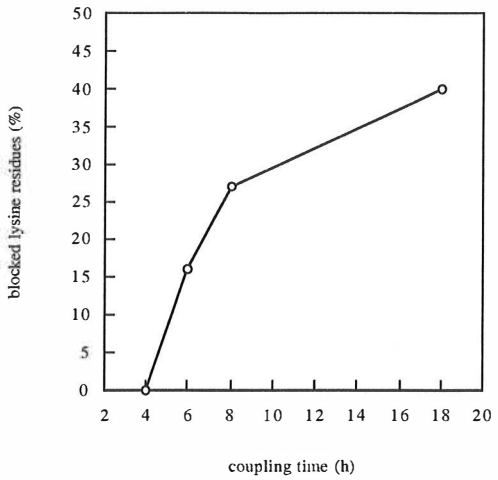


Figure 2. Dextran coupling of somatostatin at pH 6. Relationship between coupling time and lysine involvement

4.2 Radiolabelling of the conjugates

Two different ^{99m}Tc labelling techniques were used for dextran conjugate labelling. In paper I, the dextran moiety of the conjugate was labelled with reduced technetium. The mechanism responsible for labelling of dextran is similar to that in the labelling of glucoheptonate (De Kievet, 1981). The labelling yield was 70-90% determined by ITLC and gel filtration. The *in vitro* and *in vivo* stability of ^{99m}Tc-dextran complex is reported to be very good (Henze et al., 1982a, b).

In paper IV, the histidine-tagged somatostatin-dextran conjugate was labelled with ^{99m}Tc using $^{99m}Tc(CO)_3$ core. The organometallic precursor $^{99m}Tc(CO)_3$ was produced in 90-95% yield. Only small traces of $^{99m}TcO_4^-$ or ^{99m}Tc-tartrate could be detected. The $^{99m}Tc(CO)_3$ was stable at neutral pH and can be used without purification. The conjugate labelling efficiency was 65-80% and the radiochemical purity >95% determined by HPLC. Non-specific labelling of dextran and somatostatin-dextran were < 2%. The radiolabelled conjugate showed similar HPLC profile as the unlabelled conjugate. The *in vitro* stability of radiolabelled conjugate in saline and PBS at pH 5.0, 7.5 and 9.0 was excellent (24 h incubation at room temperature). In

the cysteine challenge assay, 25% of radiolabel was released from conjugate after 1 h incubation at 37°C (at 1000:1 cysteine to conjugate molar ratio). In serum, 30% of radioactivity was associated with serum proteins after 24 h incubation at 37°C, but radiolabelled conjugate revealed no significant reoxidation to $^{99m}TcO_4^-$.

In paper III, the free sulfhydryl of cysteine-dextran conjugate was labelled with ^{188}Re using ^{188}Re-gluconate as the transchelator. In optimised conditions, the labelling efficiency was 60-70% and the radiochemical purity > 95%. ^{188}Re-dextran was stable in 1.0 mM cysteine. More than 95% of the radioactivity remained associated with the conjugate after 1 h at 37°C. The presence of an antioxidant (ascorbic acid) prevented radiolysis of the ^{188}Re-dextran (Figure 3), and good stability was achieved (> 80% intact after storage at 25°C for 24 h).

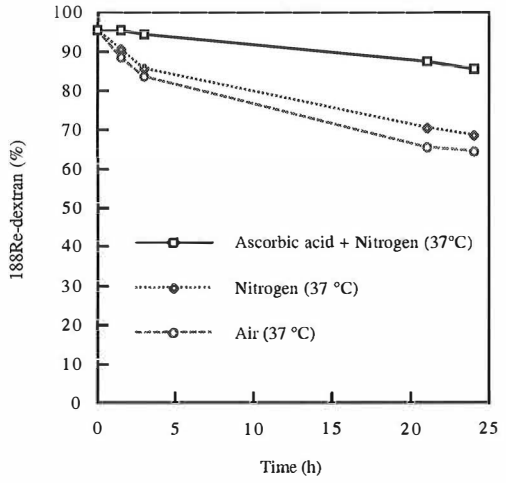


Figure 3. Stability of ^{188}Re-dextran under different storage conditions

In paper V the radiolabel was ^{125}I (chloramine T and Bolton-Hunter methods).

4.3 *In vitro* testing

In the fluorometric cytotoxicity assay, the reference epirubicin showed 90-100% growth inhibition in all three human bladder cancer cell-lines at 6 and 12 μM concentration. Dextran alone had no effect on the cell growth. Epirubicin-dextran without cationic moiety showed 35-65% growth inhibition (concentration dependent in all three cell-lines). Cationic

epirubicin-dextran showed 40-95% growth inhibition (concentration dependent). Cationic dextran (dextran-lysine) showed 5-90% growth inhibition, with a strong effect on the 5637 cell-line (concentration dependent only in 5637). The results are shown in Figure 4.

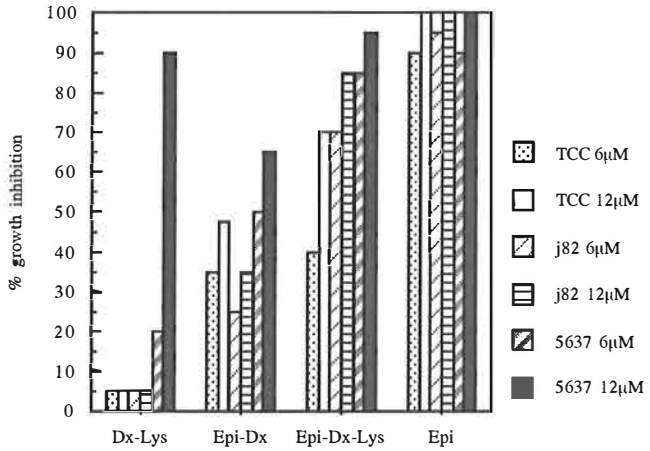


Figure 4. Growth inhibition of three bladder cancer cell-lines by dextran-lysine (Dx-Lys), epirubicin-dextran (Epi-Dx), epirubicin-dextran-lysine (Epi-Dx-Lys) and epirubicin (Epi). All at two concentrations, 6 and 12μM

In Paper V, the binding experiments were performed with rat brain cortex membranes using ^{125}I-Tyr^3-octreotide, a specific ligand for somatostatin receptors. The somatostatin-dextran^{70} showed high somatostatin receptor binding affinity in the low nanomolar range (IC_{50} ~2.5 nM). The affinity was in the same range as for the octreotide (Figure 5).

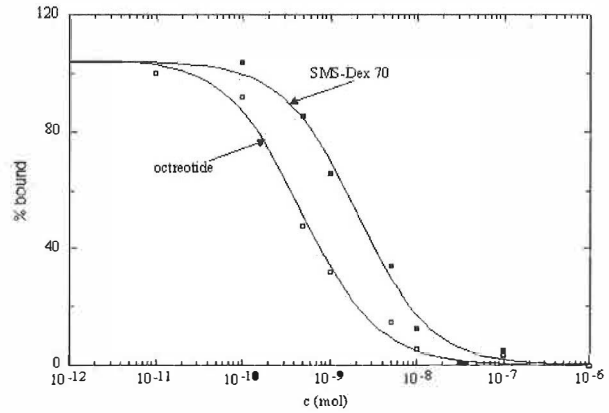


Figure 5. Displacement of ^{125}I-tyr^3-octreotide with increasing concentration of sms-dxtran^{70} and octreotide (IC_{50} octreotide 0.45 ± 0.12, slope 1.1; IC_{50} sms-dextran^{70} 1.9 ± 0.6, slope 1.0)

4.4 *In vivo* testing

In paper I, the behaviour of ^{99m}Tc labelled charge modified dextran after intravesical instillation in patients with superficial bladder cancer was investigated. Table 2 shows the quotient and the mean quotient of radioactive uptake in tumour tissue divided with radioactive uptake in normal bladder tissue. Instillation of cationic dextran yielded very high quotients. Normal tissue had no uptake (i.e. normal background activity, < 50 cpm/g tissue). Instillation of anionic dextran yielded low quotients with increased uptake in normal bladder tissue (~6000 cpm/g tissue). Neutral dextran yielded intermediate quotients with normal background activity. No radioactivity could be detected in blood. The bladder tumours in this investigation apparently had cation-exchanging properties; their anionic charge interacted with the cationic charge of the dextran.

Table 2. Quotient and mean quotient of radioactive uptake

Quotient					
Dextran	Patient 1	Patient 2	Patient 3	Patient 4	Mean / SD
Cationic	550	1510	1750	3000	1702 / 1008
Anionic	1.5	1.8	2.5	---	1.93 / 0.51
Neutral	75	90	110	---	91.7 / 17.56

In paper V, the ^{125}I-somatostatin-dextran^{70} conjugate was injected s.c. in normal mice. The half-life in blood and the biodistribution in organs were studied. The blood concentration of ^{125}I-sms-dextran^{70} after the s.c. administration gradually increased, reaching a maximum concentration after 24 h post injection (4.90 % ID/g). After this time point, the blood half-life was ~27 h (Figure 6).

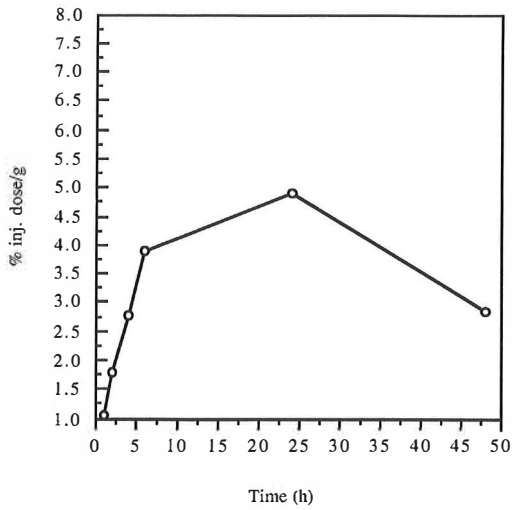


Figure 6. The radioactivity in blood at different time points after s.c. injection of ^{125}I-sms-dextran^{70}

In the biodistribution study, the relatively high uptake in the adrenals was seen at all time points. An uptake in stomach was seen indicating certain de-iodination of the radiolabelled conjugate. The activity in spleen, liver and kidneys increased until 24 h post injection and then decreased (Figure 7).

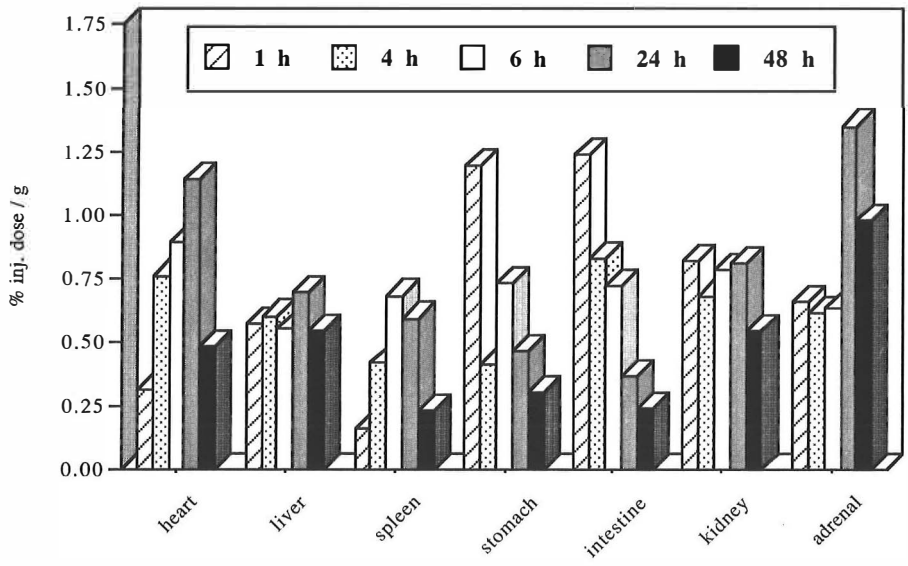


Figure 7. Organ distribution of ^{125}I-sms-dextran^{70} at different time points after s.c. injection

5. DISCUSSION

This study demonstrates the versatility of dextran as a backbone in tumour targeting compounds. Five compounds have been constructed, cationic dextran, cationic epirubicin-dextran, rhenium-188 labelled cysteine-dextran, somatostatin-dextran[70], and technetium-99m labelled somatostatin-dextran[40]. Each conjugate has interesting applications in oncology, the cationic dextran derivatives in the treatment of superficial bladder cancer, radioactive derivatives of somatostatin-dextran for receptor targeting, and somatostatin-dextran as a non-radioactive pharmaceutical.

Cationic dextran. The finding that superficial bladder tumours could be effectively targeted by using electrostatic force may have important general implications (paper I). Other tumours growing in body cavities, e.g. metastatic ovarian cancer (Chen and Berek, 1998; Hofstra et al., 2000), certain brain tumours (Erlanson et al., 1992; Galanis and Buckner, 2000) may show the same electrostatic properties as shown in the bladder tumours. This would expand the relevance of the "ion exchange tumour targeting" concept that was demonstrated in paper I. There is reason to believe that the charge phenomenon is also present in most other solid tumours. The "tumour physiognomy" seems to be general, i.e. the blood vessels, the interstitium, sialic acid on cell membranes, pH etc. (Tannock and Rotin, 1989; Jain, 1990; Konukoglu et al., 1992). Focusing on superficial bladder cancer, a therapeutic derivative of cationic dextran was developed in paper II. On all three bladder cancer cell-lines cationic epirubicin-dextran inhibited cell growth. The cationic moiety of the conjugate enhanced the growth inhibitory effect. Interestingly, cationic dextran alone without the epirubicin moiety had a strong growth inhibitory effect on one cell-line. Regarding the mechanism: why did the cationic moiety increase the growth inhibition? Probably because the positive charge could interact with the negative charge of the cell membrane thus facilitating the internalisation. Why did the cationic moiety alone inhibit growth and in only one cell-line? This could be related to the growth pattern of this cell-line and a certain cytotoxicity of the lysine side group as demonstrated earlier by Szende and co-workers (Szende, 1993; Szokan et al., 1997). The reference substance in these experiments, free epirubicin, showed stronger growth inhibition than the cationic derivative. However this was an *in vitro* study, and in the *in vivo* situation the cationic dextran derivative may have an advantage due to its capacity to accumulate selectively in the tumour tissue.

Rhenium-188 labelled dextran. In nuclear oncology, rhenium-188 is a useful radionuclide because of its nuclear properties. It has beta radiation suitable for therapy but also gamma radiation making it possible to do imaging simultaneously (Knapp et al., 1997).

In paper III, dextran was labelled with ^{188}Re in a similar way as with the antibody direct labelling method employed by Rhodes et al. (1996). Cysteine residues were coupled to the dextran resulting in free sulfhydryls available for rhenium chelation. ^{188}Re-gluconate was used as transchelator. There are advantages when using this method, e.g. simple procedures and reproducible results. The labelling yield was good, 60-70%. In the presence of an antioxidant like ascorbic acid, the ^{188}Re-dextran was very stable. The relative drawback of this method is that an inert atmosphere during all the conjugate synthesis and the labelling procedure is necessary. The presence of oxygen results in oxidation of the sulfhydryls resulting in poor radiolabelling yield. It would be desirable and would improve the versatility of the method if the labelling efficiency could be improved (i.e. >95%). This would exclude the last purification step. To improve the efficiency of the dextran labelling, a new ^{188}Re transchelator may be employed during the ^{188}Re reduction. Another possibility could be to couple a suitable ^{188}Re chelator to dextran, such as the MAG_3 facilitating the ^{188}Re complex (Guhlke et al., 1998).

It would be relatively easy to make the rhenium-188 labelled dextran cationic by coupling an additional side group, for example lysine. That would yield a ^{188}Re labelled cationic dextran with several interesting oncology applications as previously discussed. It may not be suitable for bladder cancer application, however for treatment of metastatic ovarian cancer and possibly certain brain tumours it could become useful. For intratumoural treatment of inoperable solid tumours (intratumoural injection) the ^{188}Re labelled dextran derivative could also be an alternative.

Somatostatin dextran. Papers IV and V deal with somatostatin-dextran[70]. This construct, a conjugate between natural somatostatin and dextran, was first described by Holmberg et al. (1994, 1995a). The construct described in this thesis is of high molecular weight (~75 kDa) intended to have long *in vivo* half-life. In paper IV the conjugate served as a template for radiolabelling with technetium-99m. The ^{99m}Tc-tricarbonyl method was adapted for technetium-99m labelling of the somatostatin-dextran. Histidine residues were coupled to the

dextran backbone resulting in free N-heterocycles for $^{99m}Tc(CO)_3$ chelation. One major advantage of this method is that it employs "non-reducing" conditions thus not affecting sensitive S-S bonds essential for the biological activity of the peptide (Marmion et al., 1999; Knight et al., 2000). Another advantage is that since Tc(I) complexes possess a d^6 low-spin electronic configuration they are kinetically very stable. This methodology could be possibly used for labelling with $^{186/188}Re$ for therapeutic applications. This is due to the similar physical and chemical properties of technetium-99m and rhenium-188 (Deutsch et al., 1986). Preliminary experiments indicate that this is indeed possible.

The general ambition is to develop a "kit formulation" for the radiolabelling of somatostatin-dextran allowing technetium labelling for diagnosis and rhenium labelling for therapy. However one important drawback of this method was discovered, radiolabelled conjugate was not stable in serum environment, i.e. the radiolabel interacted with serum proteins (30% after 24 h incubation at 37°C). This could be explained by the coordination properties at the Tc centre. The histidine residues in the somatostatin-dextran-histidine conjugate were similar to that of C-terminal histidine within a peptide chain and provided a bidentate coordinating ligand system for radiolabelling. The radiolabelled complex offers one free coordination site for free ligands. This free coordination site resulted in the exchange of the substitution of the labile water molecules by functional groups of serum proteins. A possible improvement could be the use of a tridentate coordinating ligand system, since then there is no free coordination site for incoming ligands. Further development is necessary to obtain an ideal formulation.

In paper V the goal was to determine the blood half-life, the biodistribution and obtain an indication of the relative receptor affinity of the somatostatin-dextran conjugate. Since this formulation is intended as a "cold" pharmaceutical with long *in vivo* half-life, a large clinical dextran was used as backbone (on average 70 kDa). The resulting blood half-life was ~27 h after s.c. administration and degradation followed the normal route of a dextran above the kidney threshold (Arturson and Wallenius, 1964). The binding affinity to the somatostatin receptor was in the nM range, approximately the same as the reference ligand (i.e. octreotide). Rat brain cortex membranes were used as the receptor source (expressing receptor subtype 2). The long blood half-life and high receptor affinity are important findings in this study. Long half-life makes it practical to use in the clinic and increases the probability to reach therapeutic concentrations. High affinity to the target receptor is of importance and since

somatostatin receptors have five subtypes, a ligand having affinity to all five subtypes would be desirable. In a recent study, the somatostatin-dextran^{70} showed indeed high affinity to all five subtypes (Wulbrand et al., 2000). These properties of somatostatin-dextran^{70}, long *in vivo* half-life and "pan-affinity" are unique for this compound. Additionally, the pan-affinity has important implications also for the radioactive derivative indicating new possibilities for somatostatin receptor scintigraphy/therapy. The non-radioactive somatostatin-dextran^{70} is currently being evaluated in a clinical phase I-II study.

Conclusion. This thesis demonstrates the versatility of dextran in the context of tumour targeting. A new approach to targeting has been established together with new dextran derivatives for therapy (I, II). Radiochemistry for rhenium-188 and technetium-99m has been developed for the dextran derivatives (III, IV). Important properties of the somatostatin-dextran have been determined (V). The clinical relevance of these properties is currently being evaluated in phase I-II studies. The development of radioactive derivative of somatostatin-dextran is in progress.

Each part of the study indicates and exemplifies new possibilities and applications for treatment and diagnosis of cancer.

6. REFERENCES

Abrams M.J., Juweid M., tenKate C.I., et al. (1990) Technetium-99m-human polyclonal IgG radiolabeled via the hydrazino nicotinamide derivative for imaging focal sites of infection in rats. J. Nucl. Med. **31**, 2022-2028

Alberto R., Schibli R., Egli A., et al. (1998) A novel organometallic aqua complex of technetium for the labeling of biomolecules: synthesis of $[^{99m}Tc(OH_2)_3(CO)_3]^+$ from $[^{99m}TcO_4]^-$ in aqueous solution and its reaction with a bifunctional ligand. J. Am. Chem. Soc. **120**: 7987-7988.

Alberto R., Schibli R., Schubiger P.A., et al. (1999) First application of fac-$[^{99m}Tc(OH_2)_3(CO)_3]^+$ in bioorganometallic chemistry: design, structure and *in vitro* affinity of a 5-HT1A receptor ligand labeled with ^{99m}Tc. J. Am. Chem. Soc. **121**, 6076-6077.

Anderson C.J., Pajeau T.S., Edwards W.B., et al. (1995) *In vitro* and *in vivo* evaluation of copper-64-octreotide conjugates. J. Nucl. Med. **36**, 2315-2325.

Andersson A., Capala J. and Carlsson J. (1992) Effects of EGF-dextran-tyrosine-^{131}I conjugate on the clonogenic survival of cultured glioma cells. J. Neuro-Oncology. **14**, 213-223.

Andersson A., Holmberg A., Carlsson J., et al. (1991) Binding of epidermal growth factor-dextran conjugate to cultured glioma cells. Int. J. Cancer. **47**, 439-444.

Arturson G. and Wallenius G. (1964) The renal clearance of dextran of different molecular sizes in normal humans. Scandinav. J. Clin. Lab. Investig. **1**, 81-86.

Bakker W.H., Albert R., Bruns C., et al. (1991) [^{111}In-DTPA-D-Phe^1]-Octreotide, a potential radiopharmaceutical for imaging of somatostatin receptor-positive tumors, synthesis, radiolabeling and *in vitro* validation. Life Sci. **49**, 1583-1591.

Bakker W.H., Krenning E.P., Breeman W.A.P., et al. (1990) Receptor scintigraphy with a radioiodinated somatostatin analogue: radiolabelling, purification, biological activity, and *in vivo* application in animals. J. Nucl. Med. **31**, 1501-1509.

Bale W.F. and Spar I.L. (1967) Towards the use of antibodies as carriers of radioactivity for therapy. Monogr. In Adv. In Biol. Med. and Phys. **5**, 286-356.

Baudys M., Letourneur D., Liu F., et al. (1998) Extending insulin action *in vivo* by conjugation to carboxymethyl dextran. Bioconjugate Chem. **9**, 176-183.

Bauer W., Briner U., Doepfner W., et al. (1982) SMS 201-995: A very potent and selective octapeptide analogue of somatostatin with prolonged action. Life Sci. **31**, 1133-1140.

Bernstein A., Hurwitz E., Maron R., et al. (1978) Higher antitumour efficacy of daunomycin when linked to dextran: *in vivo* and *in vitro* studies. J. Natl. Cancer Inst. **60**, 379-384.

Berridge M.J. (1985) The molecular basis of communication within the cell. Sci. Am. **253**, (10), 124-134.

Bolton A.E. and Hunter W.M. (1973) The labeling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. Biochem. J. **133**, 529-539.

Borch R.F., Bernstein M.D. and Durst H.D. (1971) The cyanohydridoborate anion as a selective reducing agent. J. Am. Chem. Soc. **93**, 2897-2904.

Bretscher M.S. (1985) The molecules of the cell membrane. Sci. Am. **253**, (10), 86-90.

Carlsson J., Drevin H. and Axen R. (1978) Protein thiolation and reversible protein-protein conjugation. N-Succinimidyl 3-(2-pyridyldithio)propionate, a new heterobifunctional reagent. Biochem. J. **173**, 723-737.

Chen L.M. and Berek J.S. (1998) Progress in ovarian cancer. Curr. Opin. Obstet. Gynecol. **10**, 51-55.

De Jong M., Bakker W.H., Krenning E.P., et al. (1997) Yttrium-90 and indium-111 labelling, receptor binding and biodistribution of [DOTA^0,D-Phe^1,Tyr^3]octreotide, a promising somatostatin analogue for radionuclide therapy. Eur. J. Nucl. Med. **24**, 368-371.

De Jong M., Breeman W.A., Bakker W.H., et al. (1998) Comparison of ^{111}In-labeled somatostatin analogues for tumor scintigraphy and radionuclide therapy. Cancer Res. **58**, 437-441.

De Belder A.N. (1990) Dextran, 2nd Ed. Pharmacia Print, Uppsala, Sweden.

De Kievet W. (1981) Technetium radiopharmaceutical: chemical characterisation and tissue distribution of Tc-glucoheptonate using Tc-99m and carrier Tc-99. J. Nucl. Med. **22**, 703-709.

Deutsch E., Lisbon K., Vanderheyden J.L., et al. (1986) The chemistry of rhenium and technetium as related to the use of isotopes of these elements in therapeutic and diagnostic nuclear medicine. Nucl. Med. Biol. **13**, 465-477.

Eberle A.N., Siegrist W., Bagutti C., et al. (1993) Receptors for melanocyt-stimulating hormone on melanoma cells. Ann. N.Y. Acad. Sci. **680**, 320-341.

Eckelman W.C. (1990) Development of radiochemically pure antibodies. Cancer Res. **50** (3), 780s-782s.

Eckelman W.C. (1992) In Radiopharmaceuticals. Chemistry and Pharmacology; Nunn, A.D., Ed.; Dekker: New York. 167-219.

Eckelman W.C. (1994) The application of receptor theory to receptor-binding and enzyme-binding oncologic radiopharmaceuticals. Nucl. Med. Biol. **21**, 759-769.

Egli A., Alberto R., Tannahill L., et al. (1999) Organometallic ^{99m}Tc-aquaion labels peptide to an unprecedented high specific activity. J. Nucl. Med. **40**, 1913-1917.

Ehrlich P. and Heter C.A.Z. (1904) Physiol. Chem. **61**, 379.

Ellman G.L. (1959) Tissue sulfhydryl group. Arch. Biochem. Biophys. **82,** 70-77.

Erlanson M., Daniel-Szolgay E. and Carlsson J. (1992) Heterogeneity in penetration and accumulation of cytostatics in human glioma and human colon carcinoma spheroids. Cancer Chemotherapy and Pharmacology, **29**, 343-353.

Fischman A.J., Babich J.W. and Strauss H.W. (1993) A ticket to ride: peptide radiopharmaceuticals. J. Nucl. Med. **34**, 2253-2263.

Foster R.L. (1975) Preparation and properties of a soluble trypsin-dextran conjugate. Experentia. **31**, 772-773.

Fritzberg A.R., Abrams P.G., Beaumier P.L., et al. (1988) Specific and stable labelling of antibodies with technetium-99m with a diamide dithiolate chelating agent. Proc. Natl. Acad. Sci. USA **85**, 4025-4029.

Fujita H., Okamoto M. and Takao A. (1991) Pharmacokinetics of adiramycin-oxidized dextran conjugate (ADM-OXD) in experimental animals (in Japanese). Drug Delivery Syst. **6**, 133-138.

Galanis E. and Buckner J. (2000) Chemotherapy for high-grade gliomas. Br. J. Cancer. **82**, 1371-1380.

Gedda L., Olsson P., Ponten J., et al. (1996) Development and *in vitro* studies of epidermal growth factor-dextran conjugates for boron neutron capture therapy. Bioconjugate Chem. **7**, 584-591.

Ghose T. and Blair A.H. (1978) Antibody linked cytotoxic agents in the treatment of cancers: Current status and future prospects. J. Natl. Cancer Inst. **61**, 657-676.

Gilman A.G. (1987) G proteins: transducers of receptor-generated signals. Ann. Rev. Biochem. **56**, 615-649.

Goldenberg D.M. (1988) Targeting of cancer with radiolabelled antibodies. Prospects for imaging and therapy. Arch. Path. Lab. Med. **112**, 580-587.

Greenwood W.C., Hunter W.M. and Glover J.S. (1963) The preparation of ^{131}I labelled human growth hormone of high specific radioactivity. Biochem. J. **89**, 114-123.

Gregoriadis G. (1977) Targeting of drugs. Nature. **265**, 407-411.

Guhlke S., Schaffland A., Zamora P.O., et al. (1998) ^{188}Re and ^{99m}Tc-MAG_3 as prosthetic groups for labeling amines and peptides: approaches with pre- and postconjugation labeling. Nucl. Med. Biol. **25**, 621-631.

Heindel N.D., Zhao H., Leiby J., et al. (1990) Hydrazide pharmaceuticals as conjugates to polyaldehyde dextran: syntheses, characterization and stability. Bioconjugate Chem. **1**, 77-82.

Hennig I.M., Laissue J.A., Horisberger U., et al. (1995) Substance P receptors in human primary neoplasms. Tumoural and vascular localisation. Int. J. Cancer. **61**, 786-792.

Henze E., Robinson G.D., Kuhl D.E., et al. (1982a) Tc-99m Dextran: a new blood-pool-labeling agent for radionuclide angiocardiography. J. Nucl. Med. **23**, 348-353.

Henze E., Schelbert H.R., Collins J.D., et al. (1982b) Lymphoscintigraphy with Tc-99m-labeled dextran. J. Nucl. Med. **23**, 923-929.

Hird V., Steward J.S.W., et al. (1990) Intraperitoneally administrated ^{90}Y-labelled monoclonal antibodies as a third line of treatment in ovarian cancer. A phase 1-2 trail: problems encountered and possible solutions. Br. J. Cancer. **62** Suppl. 10, 48-51.

Hnatowich D.J., Layne W.W., Childs R.L., et al. (1983) Radioactive labeling of antibody: A simple and efficient method. Science. **220**, 613-615.

Hnatowich D.J., Virzi F., Fogarasi M., et al. (1994) Can a cysteine challenge assay predict the *in vivo* behavior of ^{99m}Tc labeled to antibodies? Nucl. Med. Biol. **21**, 1035-1044.

Hofstra L.S., de Vires E.G., Mulder N.H. et al. (2000) Intraperitoneal chemotherapy in ovarian cancer. Cancer Treat. Rev. **26**, 133-143.

Holmberg A., Marquez M., Westlin J-E., et al. (1994) Preparation, *in vitro* binding, and technetium-99m labelling of somatostatin-dextran conjugates. Antibody Immunoconj, and Radiopharmaceut. **7**, 253-259.

Holmberg A., Ljungstedt-Pahlman I., Marquez M., et al. (1995a) *In vivo* properties of ^{125}I-somatostatin-dextran conjugates. Antibody Immunoconj. and Radiopharmaceut. **8**, 171-177.

Holmberg A., Marquez M., Westlin J-E., et al. (1995b) Labelling of polypeptides with technetium-99m by using a dextran spacer. Cancer Res. (Suppl) **55**, 5710-5713.

Hurwitz E. (1983) Specific and nonspecific macromolecule-drug conjugates for the improvement of cancer chemotherapy. Biopolm. **22**, 557-567.

Hurwitz E., Kashi R., Arnon R., et al. (1985) The covalent linking of two nucleotide analogues to antibodies. J. Med. Chem. **28**, 137-140.

Hurwitz E., Levy R., Maron R., et al. (1975) The covalent binding of daunomycin and adriamycin to antibodies, with retention of both drug and antibody activities. Cancer Res. **35**, 1175-1181.

Hurwitz E., Maron R., Arnon R., et al. (1978) Daunomycin-immunoglobulin conjugates, uptake and activity *in vitro*. Eur. J. Cancer. **14**, 1213-1220.

Jain R.K. (1990) Vascular and interstitial barriers to delivery of therapeutic agents in tumours. Cancer Metastasis Rev. **9**, 253-266.

Johannsen B. and Spies H. (1996) Technetium (V) chemistry as relevant to nuclear medicine. In Topics in current chemistry, Yoshihara K., and Omori T. Ed., Springer-Verlag Berlin, Heidelerg. **176**, 77-121.

Kagedal L. and Akerstrom S. (1970) A method for coupling biologically important molecules to polysaccharides. Acta Chem. Scand. **24**, 1601-1608.

Kato Y., Umemoto N., Kayama Y., et al. (1984) A novel method of conjugation of daunomycin with antibody with a poly-L-glutamic acid derivative as intermediate drug carrier. An anti-α-fetoprotein antibody-daunomycin conjugate. J. Med. Chem. **27**, 1602-1607.

Knapp Jr. F.F., Beets A.L., Guhlke S., et al. (1997) Availability of rhenium-188 from the alumina-based tungsten-188/rhenium-188 generator for preparation of rhenium-188-labeled radiopharmaceutical for cancer treatment. Anticancer Res. **17**, 1783-1796.

Knight L.C., Baldoo K.E., Marmino M.E., et al. (2000) Tc-99m tricarbonyl labeling of DTPA-bitistatin for imaging thrombi and emboli. Nucl. Med. Commun. **21**, 589.

Kohn J. and Wilchek M. (1984) The use of cyanogen bromide and other novel cyanolating agents for the activation of polysaccharide resins. Appl. Biochem. Biotech. **9**, 285-305.

Kojima T., Hashida M., Muranishi S., et al. (1980) Mitomycin C-dextran conjugate: a novel high molecular weight pro-drug of mitomycin C. J. Pharm. Pharmacol. **32**, 30-34.

Konukoglu D., Sonmez H., Uslu E., et al. (1992) Sialic acid in various types of cancer. Cancer Biochem. Biophys. **13**, 57-64.

Lahoti D., Bhatnagar A., Singh A.K., et al. (1999) Tc-99m dextran: a new and sensitive general purpose scintigraphic agent for diagnosing intestinal inflammation. Clin. Nucl. Med. **24**, 424-427.

Langstorm B. and Hartvig P. (1992) In Radiopharmaceuticals. Chemistry and Pharmacology; Nunn, A.D., Ed.; Dekker: New York, 21-66.

Lanteingne D. and Hnatowich D.J. (1984) The labeling of DTPA-coupled proteins with ^{99m}Tc. Int. J. Appl. Radiat. Isot. **35**, 617-621.

Larsson R. and Nygren P. (1989) A rapid fluorometric method for semiautomated determination of cytotoxicity and cellular proliferation of human tumor cell lines in microculture. Anticancer Res. **9**, 1111-1119.

Libermann T.A., Razon N., Bartal A.D., et al. (1984) Expression of epidermal growth factor receptors in human brain tumors. Cancer Res. **44**, 753-760.

Linder K.E., Wen M.D., Nowotnik D.P., et al. (1991) Technetium labeling of monoclonal antibodies with functionalized BATOs: 2. $TcCl(DMG)_3CPITC$ labeling of B72.3 and NP-4 whole antibodies and NP-4 $F(ab')_2$. Bioconjugate Chem. **2**, 407-414.

Lindstrom A. and Carlsson J. (1993) Penetration and binding of epidermal growth factor-dextran conjugates in spheroids of human glioma origin. Cancer Biother. **8**, 145-158.

Maeda H., Seymour L.W. and Miyamoto Y. (1992) Conjugates of anticancer agents and polymers: Advantages of macromolecular therapeutics *in vivo*. Bioconjugate Chem. **3**, 351-362.

Maina T., Stolz B., Albert R., et al. (1994) Synthesis, radiochemistry and biological evaluation of a new somatostatin analogue (SDZ 219-387) labelled with technetium-99m. Eur. J. Nucl. Med. **21**, 437-444.

Manabe Y., Tsubata T., Haruta Y., et al. (1983) Production of a monoclonal antibody-bleomycin conjugate utilizing dextran T-40 and the antigen-targeting cytotoxicity of the conjugate. Biochem. Biophys. Res. Commun. **115**, 1009-1014.

Manabe Y., Tsubota T., Haruta Y., et al. (1984) Production of a monoclonal antibody-methotrexate conjugate utilizing dextran T-40 and its biological activity. J. Lab. Clin. Med. **23**, 445-454.

Marmion M., Alberto R., Bugaj J., et al. (1999) Preparation and biodistribution of $[^{99m}Tc(CO)_3His^0,Tyr^3]$Octreotate. J. Labelled Cpd. Radiopharmo. **42**, Suppl. 1, 231-233.

McAfee J.G. and Neumann R.D. (1996) Radiolabeled peptide and other ligands for receptors overexpressed in tumor cells for imaging neoplasm. Nucl. Med. Biol. **23**, 673-676.

Means G.E. and Feeney R.E. (1990) Chemical modifications of proteins: History and applications. Bioconjugate Chem. **1**, 2-12.

Melton R.G., Wiblin C.N. Foster R.L., et al. (1987) Covalent linkage of carboxypeptidase G2 to soluble dextrans. Properties of conjugates and effects on plasma persistence in mice. Biochem. Pharm. **36**, 105-112.

Molteni L. (1979) Dextrans as drug carriers. In " Drug carriers in biology and medicine" Editor Gregory Gregoriadis, Academic Press: 107-125.

Noguchi A., Takahashi T., Yamaguchi T., et al. (1992) Preparation and properties of the immunoconjugate composed of anti-human colon cancer monoclonal antibody and mitomycin C-dextran conjugate. Bioconjugate Chem. **3**, 132-137.

Oseroff A.R., Ohuoha D., Hasan T., et al. (1986) Antibody-targeted photolysis: selective photodestruction of human T-cell leukaemia cells using monoclonal antibody-chlorin e_6 conjugates. Pro. Natl. Acad. Sci. U.S.A. **83**, 8744-8748.

Otte A., Jermann E., Behe M., et al. (1997) DOTATOC: a powerful new tool for receptor-mediated radionuclide therapy. Eur. J. Nucl. Med. **24**, 792-795.

Parker D. (1990) Tumour targeting. Chem. Br. **26**, 942-945.

Perkins A.C., Pimm M.V. Fells H.F., et al. (1989) Intrapertoneal ^{131}I and ^{111}In-791T/36 monoclonal antibody in recurrent ovarian cancer: imaging and biodistribution. Nucl. Med. Commun. **10**, 577-584.

Pietersz G.A. (1990) The linkage of cytotoxic drugs to monoclonal antibodies for treatment of cancer. Bioconjugate Chem. **1**, 89-95.

Pietersz G.A., Cunningham Z. and McKenzie I.F.C. (1988) Specific *in vitro* anti-tumour activity of methotrexate monoclonal antibody conjugates prepared using human serum albumin as an intermediary. Immunol. Cell Biol. **66**, 43-49.

Pietzsch H.J., Gupta A., Reisgys M., et al. (2000) Chemical and biological characterization of technetium(I) and rhenium (I) tricarbonyl complexes with dithioether ligands serving as linkers for coupling the $Tc(CO)_3$ and $Re(CO)_3$ moieties to biologically active molecules. Bioconjugate Chem. **11**, 414-424.

Rao T.N., Adhikesavalu D., Camerman A., et al. (1990) Technetium (V) and rhenium (V) complexes of 2,3-bis(mercaptoacetamido) propanoate. Chelate ring stereochemistry and influence on chemical and biological properties. J. Am. Chem. Soc. **112**, 5798-5804.

Rebizak R., Schaefer M. and Dellacherire E. (1997) Polymer conjugate of Gd^{3+} - Diethylenetriaminepentaacetic acid and dextran. 1. Synthesis, characterization and paramagnetic properties. Bioconjugate Chem. **8**, 605-610.

Rebizak R., Schaefer M. and Dellacherire E. (1998) Polymer conjugate of Gd^{3+} - Diethylenetriaminepentaacetic acid and dextran. 2. Influence of spacer arm length and conjugate molecular mass on the paramagnetic properties and some biological parameters. Bioconjugate Chem. **9**, 94-99.

Reichlin S. (1983) Somatostatin (part 1). N. Engl. J. Med. **309**, 1495-1501.

Reichlin S. (1983) Somatostatin (part 2). N. Engl. J. Med. **309**, 1556-1563.

Reisine T. and Bell G.I. (1995) Molecular biology of somatostatin receptors. Endocr. Rev. **16**, 427-442.

Resigys M., Wust F., Alberto R., et al. (1997) Synthesis of rhenium (I) and technetium (I) carbonyl dithioether ligand complex bearing 3, 17β-estradiol. Bioorg. Med. Chem. Lett. **17**, 2243-2246.

Reubi J.C. (1995) *In vitro* identification of vasoactive intestinal peptide receptors in human tumors: implications for tumor targeting. J. Nucl. Med. **36**, 1846-1853.

Reubi J.C., Kvols L., Krenning E., et al. (1990) Distribution of somatostatin receptors in normal and tumour tissue. Metabolism. (Suppl 2) **39**, 78-81.

Rhodes B.A., Lambert C.R., Marek M.J., et al. (1996) Re-188 labelled antibodies. Appl. Radiat. Isot. **47**, 7-14.

Rowland G.F. (1977) Effective antitumour conjugates of alkylating drug and antibody using dextran as the intermediate carrier. Eur. J. Cancer. **13**, 593-596.

Salacinski P.R.P., Mclean C., Sykes J.E.C., et al. (1981) Iodination of proteins, glycoproteins, and peptides using a solid-phase oxidizing agent, 1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril (Iodogen). Anal. Biochem. **117**, 136-146.

Salmain M., Gunn M., Gorfi A., et al. (1993) Labeling of proteins by organometallic complexes of rhenium (I), synthesis and biological activity of the conjugates. Bioconjugate Chem. **4**, 425-433.

Schacht E. (1987) Polysaccharides macromolecules as drug carriers. In "Polymers in controlled drug delivery" Editors Illum L. and Davis S.S. Wright Bristol: 131-151.

Schibli R., Katti K. V., Higginbotham C., et al. (1999) *In vitro* and *in vivo* evaluation of bidentate, water-soluble phosphine ligands as anchor groups for the organometallic fac-$[^{99m}Tc(CO)_3]^+$-core. Nucl. Med. Biol. **26**, 711-716.

Scott T.A. and Melvin E.H. (1953) Determination of dextran with anthrone. Anal. Chem. **25**, 1656-1661.

Seymour L. (1992) Passive tumour-targeting of soluble macromolecules and drug conjugates. Crit. Rev. Ther. Drug Carrier Syst. **9**, 135-187.

Sezaki H. and Hashida H. (1984) Macromolecule-drug conjugates in targeted cancer chemotherapy. Biodegradable polymers in controlled drug delivery. Crit. Rev. Ther. Drug Carrier Syst. **1**, 1-38.

Sherwood R.F., Barid J.K., Atkinson T., et al. (1977) Enhanced plasma persistence of therapeutic enzymes by coupling to soluble dextran. Biochem. J. **164**, 461-464.

Smith-Jones P.M., Stolz B., Bruns C., et al. (1994) Gallium-67/Gallium-68-[DFO]-octreotide: a potential radiopharmaceutical for PET imaging of somatostatin receptor-positive tumors: synthesis and radiolabeling *in vitro* and preliminary *in vivo* studies. J. Nucl. Med. **35**, 317-325.

Szende B. (1993) The effect of amino acids and amino acid derivates on cell proliferation. Acta Biomedica de L'Ateneo Parmense. **64**, 139-145.

Szokan G., Almas M., Krizsan K., et al. (1997) Structure determination and synthesis of lysine isopeptides influencing on cell proliferation. Biopolymers. **42**, 305-318.

Takakura Y., Kitajima M., Matsumoto S., et al. (1987a) Development of a novel polymeric prodrug of mitomycin C, mitomycin C-dextran conjugate with anionic charge, physicochemical characteristics and *in vivo* and *in vitro* antitumor activities. Int. J. Pharmaceutics. **37**, 135-143.

Takakura Y., Takagi A., Hashida M., et al., (1987b) Disposition and tumour localization of mitomycin C-dextran conjugates in mice. Pharm. Res. **4**, 293-300.

Tannock I.F. and Rotin D. (1989) Acid pH in tumours and its potential for therapeutic exploration. Cancer Res. **49**, 4373-4384.

Thakur M.L., Eshbach J., Wilder S., et al. (1993) Tc-99m labeled Sandostatin: preparation and preliminary evaluation. J. Labeled. Cmpd. Radiopharm. **32**, 365-367.

Top S., Hafa E.H., Vessieres A., et al. (1995) Rhenium carbonyl complexes of β-estradiol derivatives with high affinity for the estradiol receptor: An approach to selective organometallic radiopharmaceuticals. J. Am. Chem. Soc. **117**, 8372-8380.

Vaughan A.T.M., Andersson P., Dykes P.W., et al. (1987) Limitations to killing of tumours using radiolabelled antibodies. Br. J. Radiology. **60**, 567-578.

Wahl R.L., Barret J., et al. (1988) The intraperitoneal delivery of radiolabeled monoclonal antibodies: studies on the regional delivery advantage. Cancer Immunol. Immunother. **26**, 187-201.

Waibel R., Alberto R., Willuda J., et al. (1999) Stable one-step technetium-99m labeling of His-tagged recombinant proteins with a novel Tc(I)-carbonyl complex. Nature Biotechnol. **17**, 897-901.

Wang S.J., Lin W.Y., Chen M.N., et al. (1998) Intratumoural injection of rhenium-188 microspheres into an animal model of hepatoma. J. Nucl. Med. **39**, 1752-1757.

Weckbecker G., Liu R., Tolcsvai L., et al. (1992) Antiproliferative effects of the somatostatin analogue octreotide (SMS 201-995) on ZR-75-1 human breast cancer cells *in vivo* and *in vitro*. Cancer Res. **52**, 4973-4978.

Wheldon T.E. and O'Donoghue J.A. (1990) The radiobiology of targeted radiotherapy. Int. J. Radiat. Biol. **58**, 1-21.

Wulbrand U., Feldman M., Pfestroff A., et al. (2000) High affinity binding to somatostatin receptors, subtypes 1-5. Abstract, The eighth conference on radioimmunodetection and radioimmunotherapy of cancer, 2000, October 12-14, Princeton, USA.

Zhao Q., Tolmachev V., Carlsson J., et al. (1999) Effects of dextranation on the pharmacokinetics of short peptide. A PET study on mEGF. Bioconjugate Chem. **10**, 938-946.

PAPER I

Ion Exchange Tumor Targeting: A New Approach

by

Anders R. Holmberg, Meir Wilchek, Marcela Marquez, Jan-Erik Westlin,
Jin Du, and Sten Nilsson

Clinical Cancer Research 5: 3056s-3058s, 1999 (Suppl.)

https://aacrjournals.org/clincancerres/article/5/10/3056s/288222

PAPER II

Cytotoxic Effects of Cationic Dextran, In Vitro Studies

by

Marcela Marquez, Jin Du, Sten Nilsson, Lena Lennartsson, Jukka Hiltunen, Jan-Erik Westlin, Teuvo Tammela, Mika Raitanen, Matti Laato, and Anders R. Holmberg

Manuscript, 2000

https://europepmc.org/article/med/12014645

PAPER III

Radiolabelling of Dextran with Rhenium-188

by

Jin Du, Marcela Marquez, Jukka Hiltunen, Sten Nilsson, Anders R. Holmberg

Applied Radiation and Isotopes 53: 443-448, 2000

https://doi.org/10.1016/S0969-8043(99)00283-3

PAPER IV

Technetium-99m Labelling of Glycosylated Somatostatin-14

by

Jin Du, Jukka Hiltunen, Marcela Marquez, Sten Nilsson, Anders R. Holmberg

Applied Radiation and Isotopes 55: 181-187, 2001

https://doi.org/10.1016/S0969-8043(01)00046-X

PAPER V

Biodistribution, Blood Half-life and Receptor Binding of a Somatostatin-Dextran Conjugate

by

Martin Behe, Jin Du, Wolfgang Becker, Thomas Behr, Christa Angerstein, Marcela Marquez, Jukka Hiltunen, Sten Nilsson and Anders R. Holmberg

Medical Oncology, accepted, 2000

https://doi.org/10.1385/MO:18:1:59