Potentialities of *N*-succinyl-chitosan as a drug carrier for water-soluble macromolecular prodrugs

by

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DEDICATION

I wish to dedicate to my beloved families:

TO MY FATHER, for doing everything he could to insure this dream to come true.

TO MY MOTHER, for her selfless love, without her understanding and support, this work would not have been finished.

TO MY BROTHER, for his love and sharing our pleasure.

TO MY GRANDMOTHER, for her love and encouragement.

TO THE MEMORY OF MY GRANDFATHER, for his strict and encouragement, he brought me up and I could grow up to be a fine man.

= List of Publications =

The present thesis is based on the works contained in the following:

1) **Title:** Evaluation of *N*-succinyl-chitosan as a systemic long-circulating polymer
   Authors: Yoshinori Kato, Hiraku Onishi and Yoshiharu Machida
   Paper: *Biomaterials* 21, 1579-1585 (2000).

2) **Title:** A novel water-soluble *N*-succinyl-chitosan-mitomycin C conjugate prepared by direct carbodiimide coupling: Physicochemical properties, antitumor characteristics and systemic retention
   Authors: Yoshinori Kato, Hiraku Onishi and Yoshiharu Machida
   Paper: *STP Pharma Sci.* 10, 133-142 (2000).

3) **Title:** Biological fate of highly-succinylated *N*-succinyl-chitosan and antitumor characteristics of its water-soluble conjugate with mitomycin C at i.v. and i.p. administration into tumor-bearing mice
   Authors: Yoshinori Kato, Hiraku Onishi and Yoshiharu Machida
   Paper: *Biol. Pharm. Bull.* 23, 1497-1503 (2000).

4) **Title:** Biological characteristics of lactosaminated *N*-succinyl-chitosan as a liver-specific drug carrier in mice
   Authors: Yoshinori Kato, Hiraku Onishi and Yoshiharu Machida
   Paper: *J. Contr. Release* 70, 295-307 (2001).

5) **Title:** Lactosaminated and intact *N*-succinyl-chitosans as drug carriers in liver metastasis
   Authors: Yoshinori Kato, Hiraku Onishi and Yoshiharu Machida
   Paper: *Int. J. Pharm.* 226, 93-106 (2001).

6) **Title:** Efficacy of lactosaminated and intact *N*-succinyl-chitosan-mitomycin C conjugates against M5076 liver metastatic cancer

Authors: Yoshinori Kato, Hiraku Onishi and Yoshiharu Machida

Paper: *J. Pharm. Pharmacol.* in print.

Contents

Dedication i

List of Publications iii

Contents v

Abbreviations xi

General introduction 1

Chapter 1: Evaluation of *N*-Succinyl-chitosan as a Systemic Long-circulating Polymer 7

Summary 9

1-1. Introduction 10

1-2. Materials and methods 10

1-2-1. Materials 10

1-2-2. Animals 11

1-2-3. Tumors 11

1-2-4. Polymer characterization 11

1-2-5. Biodisposition studies 12

1-3. Results and discussion 14

1-3-1. Chemical and physicochemical characteristics 14

1-3-2. Recovery ratio 15

1-3-3. Biodisposition in normal mice 16

1-3-4. Biodisposition in tumor-bearing mice 17

Chapter 2: Novel Water-soluble *N*-Succinyl-chitosan-mitomycin C Conjugate Prepared by Direct Carbodiimide Coupling: Physicochemical Properties, Antitumor Characteristics and Systemic Retention 23

Summary 25

2-1. Introduction 26

2-2. Materials and methods 27

2-2-1. Materials 27

2-2-2. Animals 27

2-2-3. Tumors 27

2-2-4. Preparation of water-soluble conjugate 27

2-2-5. *In vitro* drug release 29

2-2-6. Stability at low temperature 29

2-2-7. *In vivo* antitumor effect against Sarcoma 180 30

2-2-8. Plasma residence of the conjugate 30

2-3. Results and discussion 31

2-3-1. Physicochemical characteristics of water-soluble conjugate 31

2-3-2. *In vitro* drug release 33

2-3-3. Stability in preservation at low temperature 35

2-3-4. *In vivo* antitumor effect against Sarcoma 180 37

2-3-5. Systemic residence of the conjugate 38

Chapter 3: Biological Fate of Highly-succinylated *N*-Succinyl-chitosan and Antitumor Characteristics of Its Water-soluble Conjugate with Mitomycin C at I.v. and I.p. Administration into Tumor-bearing Mice 41

Summary 43

3-1. Introduction 44

3-2. Materials and methods 45

3-2-1. Materials 45

3-2-2. Animals 45

3-2-3. Tumors 46

3-2-4. Biodisposition of Suc(II)-FTC 46

3-2-5. Antitumor effect of Suc(II)-MMC conjugate against Sarcoma 180 47

3-2-6. Disposition of Suc(II)-MMC conjugate into blood circulation and tumor 49

3-2-7. HPLC assay 49

3-2-8. Viscometric analysis 50

3-2-9. Statistical analysis 50

3-3. Results and discussion 50

3-3-1. Body distribution of Suc(II)-FTC 50

3-3-2. Urinary excretion of Suc(II)-FTC 52

3-3-3. Pharmacokinetic analysis of Suc(II)-FTC biodisposition 53

3-3-4. Antitumor effect of Suc-MMC against Sarcoma 180 55

3-3-5. Translocation of MMC and Suc-MMC to plasma and tumor 58

Chapter 4: Biological Characteristics of Lactosaminated *N*-Succinyl-chitosan as a Liver-specific Drug Carrier in Mice 61

Summary 63

4-1. Introduction 64

4-2. Materials and methods 65

4-2-1. Materials 65

4-2-2. Animals 66

4-2-3. Body distribution studies 67

4-2-4. Pharmacokinetic analysis 68

4-2-5. Urinary and fecal excretion studies 68

4-2-6. Comparison of biodistributions of Suc, Lac-Suc and galactosaminated *N*-succinyl-chitosan (Gal-Suc) 69

4-2-7. Competitive liver uptake 69

4-2-8. Statistical analysis 70

4-3. Results 71

4-3-1. Body distribution of Lac-Suc-FTC 71

4-3-2. Pharmacokinetic analysis 73

4-3-3. Urinary and fecal excretion 75

4-3-4. Comparison of biodistribution among Suc, Lac-Suc and galactosaminated *N*-succinyl-chitosan (Gal-Suc) 75

4-3-5. Competitive liver uptake 77

4-4. Discussion 79

4-5. Conclusion 83

Chapter 5: Lactosaminated and Intact *N*-Succinyl-chitosans as Drug Carriers in Liver Metastasis 85

Summary 87

5-1. Introduction 88

5-2. Materials and methods 88

5-2-1. Materials 88

5-2-2. Animals and tumors 89

5-2-3. Biodistribution studies of Lac-Suc and Suc 89

5-2-4. Pharmacokinetic analysis. 90

5-2-5. Urinary excretion 91

5-2-6. *In vivo* antitumor effects of Suc(II)-MMC and Lac-Suc-MMC conjugates against M5076 liver metastatic tumor 91

5-2-7. Distribution of free and conjugated MMCs in blood circulation and liver after i.v. injection of the conjugates 92

5-2-8. Statistical analysis 93

5-3. Results 93

5-3-1. Body distribution of Lac-Suc-FTC and Suc-FTC 93

5-3-2. Pharmacokinetics of Lac-Suc-FTC and Suc-FTC 94

5-3-3. Urinary excretion 98

5-3-4. *In vivo* antitumor effect of Suc(II)-MMC and Lac-Suc-MMC against M5076-bearing mice 98

5-3-5. Distribution of free and conjugated MMCs in systemic circulation and liver after i.v. injection of the conjugates 99

5-4. Discussion 100

Chapter 6: Efficacy of Lactosaminated and Intact *N*-Succinyl-chitosan-Mitomycin C Conjugates against M5076 Liver Metastatic Cancer 107

Summary 109

6-1. Introduction 110

6-2. Materials and methods 110

6-2-1. Materials 110

6-2-2. Animals and tumors 111

6-2-3. Accumulation of Lac-Suc-FTC into the liver at repeated administration 111

6-2-4. Pharmacokinetic data analysis 113

6-2-5. *In vitro* release characteristics of Lac-Suc-MMC 113

6-2-6. Antitumor effects of Suc(II)-MMC or Lac-Suc-MMC against M5076-bearing mice 113

6-2-7. Statistical analysis 114

6-3. Results 115

6-3-1. Accumulation of Lac-Suc-FTC in the liver 115

6-3-2. *In vitro* release characteristics of Lac-Suc-MMC 115

6-3-3. Antitumor effects of Suc(II)-MMC and Lac-Suc-MMC against M5076 116

6-4. Discussion 119

6-5. Conclusion 122

Conclusions 124

Acknowledgments 128

References 130

Abbreviations

- *AUC*: Area under the concentration-time curve
- *CL*: Clearance
- EDC: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
- EPR: Enhanced permeability and retention
- FITC: Fluorescein isothiocyanate
- -FTC: Fluorescein thiocarbamyl-
- GPC: Gel permeation chromatography
- ^{1}H-NMR: Proton nuclear magnetic resonance
- *ILS*: Increase in life span
- Lac-Suc: Lactosaminated *N*-Succinyl-chitosan
- Lac-Suc-FTC: Fluorescein isothiocyanate-labeled lactosaminated *N*-succinyl-chitosan
- Lac-Suc-MMC: Lactosaminated *N*-succinyl-chitosan-mitomycin C conjugate
- MMC: Mitomycin C
- *MRT*: Mean residence time
- MW: Molecular weight
- PBS: Phosphate buffered saline, pH 7.4
- SEC-MALS: Size exclusion chromatography-multi angle light scattering
- Suc: *N*-Succinyl-chitosan
- Suc(II): Highly-succinylated *N*-succinyl-chitosan
- Suc-FTC: Fluorescein isothiocyanate-labeled *N*-succinyl-chitosan
- Suc(II)-MMC: Highly-succinylated *N*-succinyl-chitosan-mitomycin C conjugate

General Introduction

Recently, it has attempted that drugs were selectively reached a pathological site with little distribution to undesirable site. The designed preparation system (a delivery to target site, at an appropriate dose, for required time and term) is termed as drug delivery system (DDS). DDS can be classified into (1) controlled release, (2) improvement of absorption and (3) targeting.[1,2] To target drugs into a pathological site, which reduces the side effects of drugs and enhances efficacy, is the goal of DDS.[3] The demand for DDS is increasing rapidly, especially in the United States.[4]

Targeting can be classified into active targeting and passive targeting (Figure A). The concept of a passive targeting is that drug-carrier device retains long in the body and delivers to the target site selectively by making use of EPR effect, especially tumor or inflammatory site.[5-7] Then, drug is released there gradually. It is known that the permeation from the blood stream is enhanced in tumor or inflammatory site.[8-10] These effects were named EPR effect by Maeda et al.[11] The cancer chemotherapy using anticancer drugs together with

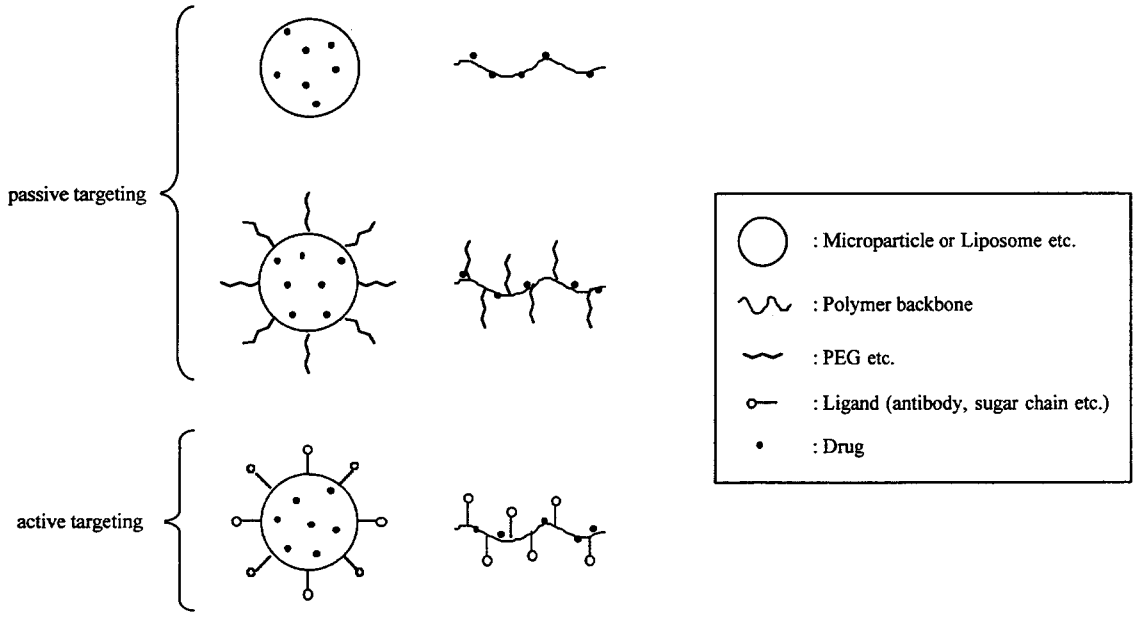


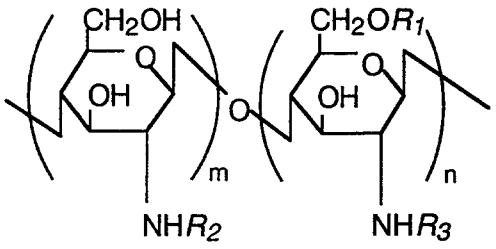
Figure A. Scheme of active and passive targeting device

angiotensin II[12,13] or angiotensin converting enzyme inhibitor[14] make effective use of EPR effect. At present, the particulates such as liposome[15-22] and microparticle,[23,24] and the macromolecular carriers such as dextran,[25-27] *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers,[28-31] albumin,[32,33] pullulan[10,34] and chitosan derivatives[35-37] are utilized. In case of the particulates, the control of the particle size can give variety to the distribution to the target tissue.[38-41] The strategy of macromolecular prodrugs is that the conjugate has the drug retained long in the body and released in the target site; although a free drug with low MW rapidly excretes by the kidney. Furthermore, it is also known that these carriers circumvent immuno-system by attaching polyethylene glycol and the retention in the systemic circulation is improved in the resultant carriers.[42-48] On the other hand, the concept of an active targeting is the system using carriers having specific affinity to target site, and the conjugate have the drug delivered to the target site positively and released there. Liposome,[49,50] microcapsule[51] and macromolecule[52-57] attached ligand such as antibody,[29,49,54,58] hormone,[59,60] growth factor,[52,53] and sugar chain[50,56,57] are used as carriers. These carriers make use of antigen-antibody reaction and biological specificity of receptor. Increasing attention has been paid in recent years to the research which has these carriers applied to gene therapy.[39,61] Based on these concepts, the following systems have been developed as commercial products: Leuprin® (poly(d,l-lactic acid-co-glycolic acid) copolymer microcapsulated leuprolide acetate),[62-64] LipoPGE$_1$® (lipid microsphere containing prostagrandin E$_1$), SMANCS/lipiodol® (conjugate of neocarzinostatin and poly(styrene-co-maleic acid/anhydride) in a lipid contrast medium)[65-67] and so on.

Among macromolecules, which may be useful as drug carriers, I have noted Suc that is chitosan derivative. Chitosan is produced by deacethylation of chitin that constitutes the exoskeleton of crustacea, insects and some fungi and that is one of the most abundant polysaccharides in nature, second only to cellulose. Biomedically, chitosan is reported to have pharmacological properties such as hypocholesterolemic action,[68] wound-healing properties,[69] antacid and antiulcer activity.[70] In addition, in the field of DDS, much interest

has been focused on chitosan and its derivatives.[71] On the other hand, Suc is obtained by introduction of succinyl groups into chitosan at the *N*-position of the glucosamine units (Figure B). Suc is water-soluble, low toxic[72,73] and less biodegradable in the body[37]; therefore, it is expected as a useful macromolecular drug carrier showing long-term retention in the body. However, further studies are needed to confer aqueous solubility to the conjugates of Suc with MMC because the conjugates were reported to be almost water-insoluble.[74]

Next, I shall confine my attention to cancer chemotherapy. Although the prevalence of cancer differs among countries, races and ethnicity,[75,76] cancer incidence and death rates



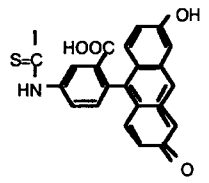
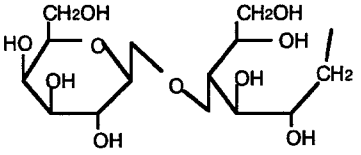
Compound	R_1	R_2	R_3
Chitin	H	$COCH_3$	$COCH_3$
Chitosan	H	H	H
N-Succinyl-chitosan	H	$CO(CH_2)_2COOH$	H
Fluorescein thiocarbamyl-*N*-succinyl-chitosan	H	$CO(CH_2)_2COOH$	
Lactosaminated *N*-succinyl-chitosan	H	$CO(CH_2)_2COOH$	

Figure B. Structures of chitin and chitosan derivatives used in this study

declined between 1990 – 1997 in USA.[75,76] On the contrary, in Japan, those rates increased and cancer has been at the head of death since 1981.[77,78] Some cancers can now often be cured due to marked medical progress if found and treated at a sufficiently early stage. However, it is still considered as one of jeopardous diseases for humankind. One of the reasons is cancer metastasis. If a tumor is not removed completely by surgical operation, the relapse or metastasis can occur, and it is then quite difficult to cure the disease. When cancer cells metastasize all over the body, it is impossible to remove them perfectly by surgical operation. Accordingly, chemotherapy with antitumor drugs is then used. However, antitumor drugs with strong cytotoxicity usually cause strong side effects because they do not kill only tumor cells but also damage some normal cells. Nausea, vomiting, loss of hair and mouth sores are frequently seen as temporary side effects of such drugs, and further lethal side effects also occur sometimes. Therefore, methods are required for the specific targeting of antitumor drugs to the tumor tissue to reduce the side effects and to make effective use of antitumor drugs.

The liver is the largest internal organ of the body. The hepatic artery supplies the liver with blood that is rich in oxygen. The portal vein carries nutrient-rich blood from the intestines to the liver. Some nutrients and vitamins absorbed by the intestines are stored in the liver until they are needed by other organs. Furthermore, the liver inactivates many drugs and toxic chemicals. If the liver is not working well, these substances can build up and interfere with many of the physiological functions. Humans cannot survive without a liver because the liver is involved in many important metabolic functions as stated above. Hepatocellular carcinoma is one of the most common malignancies, causing estimated over 100,000 deaths per year worldwide.[79] The effectiveness of chemotherapy on liver cancer is low. Chemotherapy administered via the tumor-feeding arteries suppresses the plasma concentration in comparison with systemic administration, and enhances the concentration in cancer tissue. For this purpose, macromolecular prodrugs have been developed. SMANCS/lipiodol® is now commonly utilized clinically.

In the present study, I tried to prepare a novel water-soluble macromolecular prodrug with MMC using Suc, and examined administration form (route and schedule) which reduce side effects and which show an effective antitumor activity against tumor-bearing mice.

This thesis is composed of biological characteristics of Suc as a drug carrier and antitumor properties of prodrugs of MMC with Suc(II) and lactosaminated Suc. The contents are as follows:

In Chapter 1, I examined the characteristics of Suc as a drug carrier.

The purpose of Chapter 2 was to prepare a water-soluble Suc(II)-MMC by direct carbodiimide coupling.

In Chapter 3, the biodisposition profiles of Suc(II) were compared between i.v. and i.p. administration, and the antitumor characteristics of its conjugate with MMC against subcutaneous Sarcoma 180 solid tumor were examined by both administration routes.

In Chapter 4, the introduction of lactose to Suc by reductive amination was attempted. The product, Lac-Suc was examined for pharmacokinetics and biodistribution in mice. Furthermore, the specific binding properties of Lac-Suc to the asialoglycoprotein receptor of the liver were examined.

In Chapter 5, the biodistribution of Lac-Suc and Suc was examined using mice bearing experimental liver metastasis of M5076 cells in the early and advanced stage of the disease. Further, conjugates of MMC were investigated *in vivo* for antitumor characteristics using M5076-bearing mice.

In Chapter 6, the differences in biodistribution of Lac-Suc between single and repeated administration was investigated. Further, antitumor effects of Lac-Suc-MMC against M5076 cells as a liver metastatic tumor model were examined after intravenous administration at single or repeated administration. Similarly, the antitumor effects of water-soluble Suc(II)-MMC against M5076 cells were also examined.

Chapter 1

Evaluation of *N*-Succinyl-chitosan as a Systemic Long-circulating Polymer

Summary

The water-soluble, low toxic and less biodegradable chitosan derivative Suc was investigated for body distribution and urinary excretion on a long time scale (24 – 72 h after i.v. injection) using a fluorescein-labeling technique. Suc-FTC was characterized for MW, succinylation degree and FTC content. Systemic retention and tissue distribution of Suc-FTC were examined after i.v. administration to normal and Sarcoma 180 tumor-bearing mice. Suc-FTC was sustained at a high level in the circulation over 72 h; that is, the plasma half-life in normal mice was 100.3 h and that in tumor-bearing mice was 43.0 h, which was longer than those of other long-circulating macromolecules reported to date. As to the tissues except blood circulation, Suc-FTC was distributed very little in tissues other than the tumor. Although the total amount of Suc-FTC residing in tested tissues decreased gradually, urinary excretion did not increase from 24 h after injection. These observations suggested that Suc-FTC may be eliminated by mechanisms other than in the urine or moved to tissues other than those tested. The ratio of tumor accumulation reached a plateau at 48 h after injection, and the accumulation level, ten and a few percent, was similar to those observed for other reported long-circulating macromolecules.

1-1. Introduction

Chitin and chitosan are produced as major components of the exoskeleton of Crustacea Pennant such as shrimp and crab. Chitosan is a D-glucosamine polysaccharide polymerized by β-1,4 linkage and easily obtained by deacetylation of chitin. Suc synthesized by introduction of succinyl groups into chitosan at the *N*-position of the glucosamine units is water-soluble, low toxic[72,73] and less biodegradable in the body;[37] therefore, it is expected as a useful macromolecular drug carrier showing long-term retention in the body. A macromolecular prodrug of MMC with Suc has been reported to permit MMC to remain for long periods in the body.[35,36,74,80,81] Systemic long-circulating water-soluble macromolecules are known to be concentrated into diseased parts such as tumor tissues where vascular permeability is enhanced.[5] The biodisposition characteristics of Suc with 55 % *N*-succinylation degree at 0 – 24 h were reported previously.[37] The results raised the question of how Suc could be retained for longer periods and how the further retention would influence tumor accumulation. Thus, it is important to perform more exact and detailed evaluation of the *in vivo* behavior of Suc to elucidate its biodistribution and excretion characteristics on a longer time scale. In this study, such properties were examined using Suc-FTC after its physicochemical characterization.

1-2. Materials and methods

1-2-1. Materials

Suc was obtained from Katakura Chikkarin Co., Ltd. (Tokyo, Japan). FITC was purchased from Sigma Chemical Company (St. Louis, U.S.A.). Deuterium oxide (D_2O) was obtained from Merck (Darmstadt, Germany). Twenty % deuterium chloride (DCl) and 3-(trimethylsilyl)propionic 2,2,3,3-d_4 acid sodium salt (TSP) were purchased from Aldrich Chemical Co., Inc. (Milwaukee, U.S.A). All other chemicals were of reagent grade.

Suc-FTC was synthesized according to the method by Tanaka et al.[82] and Kamiyama et al.[37] Briefly, FITC (4 mg) was reacted with Suc (100 mg) in 14 ml of 0.5 *M* carbonate buffer (pH 8.94), and then the reaction mixture was divided into 7 ml fractions. The Suc-FTC thus produced was purified by gel-filtration of each fraction with a Sephadex® G-50 column (3.5 × 30 cm) using 0.5 *M* carbonate buffer as an elution solvent and subsequent dialysis against water. Finally, Suc-FTC was obtained as a powder by lyophilization of dialyzed solution.

1-2-2. Animals

Male ddY mice (6 weeks old) weighing 24 – 28 g were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Hoshi University.

1-2-3. Tumors

Sarcoma 180 cells were maintained by weekly transplantation of 1×10^6 cells suspended in Hanks' balanced solution (0.1 ml) into the peritoneal cavity of each mouse. Sarcoma 180 cells (1×10^6) suspended in 0.1 ml of Hanks' balanced solution, which were obtained from the above tumor-bearing mice, were inoculated subcutaneously into each mouse at the axillary region.

1-2-4. Polymer characterization

Suc used in this study was examined first for degree of deacetylation by ^{1}H-NMR spectra (D_2O-DCl; pD 5-6; 90 °C; reference TSP) with a JEOL JNM-LA500 spectrometer. The *N*-succinylation degree of Suc was investigated by elemental analysis (Yanako Analytical Industrial Co., Japan) after extensive drying at 80 °C in desiccator with P_2O_5 for 3 h, and it was also checked by ^{1}H-NMR spectra (D_2O-DCl; pD 5-6; 90 °C; reference TSP). The FTC content of Suc-FTC was calculated from the absorbance at 495 nm using a DU®640 spectrophotometer (Beckman, USA) in PBS based on the absorbance of FITC at 495 nm in

the same buffer, and it was also determined from the fluorescence intensity at 520 nm with excitation at 495 nm using an FP-777 spectrofluorometer (Jasco Co., Japan) in PBS based on that of FITC in the same buffer. The MW of Suc was checked by simple GPC or SEC-MALS. The simple GPC was performed at room temperature using a Shimadzu LC-6AD equipped with a Shimadzu SPD-10AV for UV absorption, a Shimadzu RF-10AXL for fluorescence intensity and a Shimadzu RID-10A for differential refractive index. TSK-gel G3000SW (7.5 mm I.D. × 60 cm; Tosoh Co.; Tokyo, Japan) and TSK-gel G5000PW (7.5 mm I.D. × 60 cm; Tosoh Co.; Tokyo, Japan) were used as columns using 0.2 *M* NaCl-containing and non-containing 0.05 *M* phosphate buffer, pH 7.0, respectively, as the elution solvent at the flow rate of 0.8 ml/min. Blue-dextran 2000 (MW 2×10^6), catalase (MW 2.32×10^5), bovine serum albumin (MW 6.7×10^4), ribonuclease A (MW 1.3×10^4) and uracil (MW 112.1) were used as a MW calibration for protein markers, and polyethylene oxides with established MWs (PEO; MW 9.2×10^5, 2.5×10^5, 10.7×10^4 and 2.4×10^4; Tosoh, Japan) were applied for a MW calibration as linear macromolecule markers. The former and the latter markers were checked by UV absorption at 280 nm and by RID, respectively, and Suc-FTC was determined fluorometrically at 520 nm with an excitation wavelength of 495 nm. SEC-MALS was carried out using a Shodex DS-4 (Shoko Co., Ltd., Tokyo) apparatus equipped with a Shodex OH-pak SB-805HQ column. The flow rate was 1.0 ml/min, and the temperature of the column was 40 °C. A Wyatt DAWN E and Shodex RI-71 were used as a multi angle light scattering detector and a differential refractive index detector, respectively. The elution solvent was 0.05 *M* phosphate buffer, pH 7.0. Suc was dissolved in 0.05 *M* phosphate buffer at an appropriate concentration, and aliquots of 200 μl of these samples were applied on SEC-MALS. The MW of Suc was determined as 310,000.

1-2-5. Biodisposition studies

Body distribution of Suc-FTC in normal and Sarcoma 180 tumor-bearing mice was examined by i.v. administration. The recovery ratio was determined using tumor-bearing mice according to the method by Kamiyama et al.[37] Briefly, the mice underwent fast for 24

h, then blood was withdrawn. The liver, kidneys, spleen and tumor were excised, washed with PBS and wiped briefly with a filter paper. Fifty μl of Suc-FTC solution in PBS each at the concentration of 0.1, 1 and 10 mg/ml was added to the blood (1 ml) and mixed. PBS was added to the tissue by a three-fold volume of the tissue weight, 50 μl of Suc-FTC solution in PBS each at the concentration of 0.1, 1 and 10 mg/ml was added to the mixture, and then the mixture was homogenized using a glass homogenizer with a Teflon pestle and mixed. These tissue homogenates and blood containing a certain amount of Suc-FTC were centrifuged at 3000 rpm for 10 min. The fluorescence intensity of the supernatant diluted with PBS was measured at 520 nm with excitation at 495 nm. Blank samples were prepared in the same way except that 50 μl of PBS alone was added to the tissue or blood instead of Suc-FTC solution, and fluorescence was checked fluorometrically under the same conditions. The concentration of Suc-FTC in each sample was calculated from the net fluorescence intensity obtained by subtracting the fluorescence intensity of the blank from that of each sample based on the standard calibration curve. The ratio of the observed concentration to the concentration calculated from the added amount and the dilution degree (ideal concentration) was determined as the recovery ratio. As to the recovery ratio in plasma, the ratio of transfer from whole blood to plasma was treated as the recovery ratio.

Suc-FTC was administered to normal mice at the dose of 1 mg (0.2 ml) by injection into the tail vein after fasting for 24 h. The mice were sacrificed at 24, 48 and 72 h after administration, blood samples were withdrawn and several tissues (liver, kidneys and spleen) were excised. The blood volume and tissue weight were measured. The subsequent procedure was the same as for the recovery ratio except that addition of 50 μl Suc-FTC solution was not performed. Briefly, the tissue was washed with PBS and wiped with a filter paper, then PBS was added by a three-fold volume of the weight of the tissue, and then homogenized. After centrifugation of the homogenate or blood, the supernatant was diluted with PBS, and then the diluted sample was measured fluorometrically at 520 nm with excitation at 495 nm. The concentration of Suc-FTC in the sample was determined from the net fluorescence intensity obtained by subtracting the fluorescence intensity of the blank from

that of each sample based on the standard calibration curve. The distributed amount was calculated from the concentration and tissue weight. The concentration and amount were corrected by the recovery ratio. Therefore, the corrected plasma concentration means that given provided Suc-FTC is completely transferred from blood to plasma.

At 9 d after subcutaneous tumor inoculation, Suc-FTC was injected into the tail vein at a dose of 1 mg (0.2 ml) in tumor-bearing mice that had undergone fast for 24 h. The mice were sacrificed at 24, 48 and 72 h after administration, and blood samples were withdrawn and several tissues (liver, kidney, spleen and tumor) were excised. The blood volume and tissue weight were measured. The concentration and amount of Suc-FTC distributed to plasma and tissues were determined in the same way as in normal mice.

The collection of urine was performed simultaneously in the biodistribution studies as described above. Namely, the urine was collected for 24, 48 and 72 h after i.v. injection, and its volume was measured. The urine was filtered using a membrane filter (0.45 μm pore diameter), and the filtrate was appropriately diluted with PBS and measured fluorometrically at 520 nm with excitation at 495 nm. After the blank test, the concentration of Suc-FTC in urine was determined from the net fluorescence intensity using a calibration curve in the same manner as in the distribution study. The total excreted amount in urine was calculated from the concentration and urinary volume.

1-3. Results and discussion

1-3-1. Chemical and physicochemical characteristics

The results on elemental analysis and signal assignment of ^{1}H-NMR spectrum for Suc were described in Table 1-1. Methyl protons derived from acetyl groups were not observed in ^{1}H-NMR spectra. Namely, the deacetylation degree was very high and near 100 % against sugar unit. The result was consistent with the data by the supplier, which indicated that the deacetylation degree of the starting polymer, chitosan, for Suc was 96 % against sugar unit.

The too small ratio of acetyl groups may cause the detection of their protons to be impossible. Thus, Suc was considered to possess the deacetylation degree of 96 – 100 % against sugar unit. *N*-Succinylation degree of Suc (81 % against sugar unit) could be calculated from the C/N ratio in elemental analysis as 81 % against sugar unit, when the deacetylation degree was treated as 100 % against sugar unit. The result was consistent with that obtained by ^{1}H-NMR spectra, which indicated that *N*-succinylation degree was 79 % against sugar unit from the ratio of the integrated intensity of succinyl protons to that of other protons. This value was larger than that (55 %, w/w) of Suc used previously by Kamiyama et al.[37]

1-3-2. Recovery ratio

Table 1-2 shows the recovery ratio of Suc-FTC in the tissues tested (n=2). Recovery ratios were very high, that is, more than 85 % (w/w). The recovery ratios were a little higher in plasma and tumor, equivalent in kidney and a little lower in liver and spleen compared with those of Suc-FTC (55 % (w/w) *N*-succinylation) in the previous study.[37] These differences in recovery were considered to be due to the differences in chemical or physicochemical

Table 1-1.
Elemental analysis and signal assignment of ^{1}H-NMR spectrum for Suc

Elemental analysis	Found			Calcd.*		
	C	H (w/w%)	N	C	H (w/w%)	N
	41.4	6.1	5.2	42.6	5.2	5.4

^{1}H-NMR spectrum	δ (ppm)	Origin of peak
	2.3-2.6	multiplet, CH_2CH_2 of succinyl
	2.6-2.8	broad, H-2 of D-glucosamine unit
	3.4-4.0	multiplet, H-2 of *N*-succinyl-D-glucosamine unit, H-3, H-4, H-5, H-6
	4.4-4.5	broad, H-1 of D-glucosamine unit
	4.5-4.7	broad, H-1 of *N*-succinyl-D-glucosamine unit

* These values were calculated for $(C_{10}H_{14}NNa)_{0.81}(C_6H_{11}O_4N)_{0.19}$.

properties such as degree of *N*-succinylation and MW between Suc-FTC molecules used. However, the recovery ratios in this study were considered similar to those reported previously, suggesting that small differences in the polymer properties should not markedly affect the recovery ratio.

1-3-3. Biodisposition in normal mice

Figure 1-1 shows the body distributions at 24, 48 and 72 h after i.v. administration of Suc-FTC in normal mice. The concentration was expressed as the percentage of dose per ml of plasma or per g of tissue. Suc-FTC was sustained at a high level in the circulation over 72 h and was distributed very little in other tissues. It showed long systemic retention; that is, more than 25% of dose/ml was maintained even at 72 h after injection. The plasma level was significantly higher than that in each tissue for 72 h ($p<0.01$). The retention of Suc-FTC in blood was much higher than that in other tissues even at 72 h after injection. The half-life was calculated to be 100.3 h by log-linear elimination analysis.

The amount of Suc-FTC distributed in tested tissues at 24, 48 and 72 h after i.v. injection to normal mice and the total amount excreted into urine throughout 24, 48 and 72 h after i.v. injection to normal mice are shown in Figure 1-2. The amount of Suc-FTC in

Table 1-2.
Recovery ratios of Suc-chitosan-FTC in tested tissues

Tissue	Recovery ratio (%) *
Plasma	91.47 ± 0.65
Liver	89.23 ± 0.47
Kidney	87.47 ± 1.95
Spleen	93.05 ± 0.99
Tumor	91.08 ± 0.78

* The results are expressed as the mean ± (the difference between two observations)/√2(n=2).

plasma was determined based on the reported volume of mouse plasma, 48.8 ml/kg.[83] Since fluorescein itself was recognized to hardly affect the distribution or elimination of Suc-FTC,[37] these results indicated that Suc showed little distribution to the liver, kidneys and spleen and was slightly excreted into urine. The urinary excretion scarcely increased from 24 h to 72 h after injection, that is, it seemed to be almost over at 24 h after injection. The part of Suc-FTC with a relatively smaller MW, which corresponded to a dozen percent of the dose, was considered to be excreted into urine, and half of the dose was found to remain in the body even at 72 h after injection. Elimination of the total amount for the tested tissues was due to that from the circulation. Further, it can indicate the movement of Suc-FTC to other tissues or excretion in other forms so that urinary excretion was not increased.

1-3-4. Biodisposition in tumor-bearing mice

Figure 1-3 shows the body distribution at 24, 48 and 72 h after i.v. administration of Suc-FTC in tumor-bearing mice. Suc-FTC remained in the circulation at the concentration

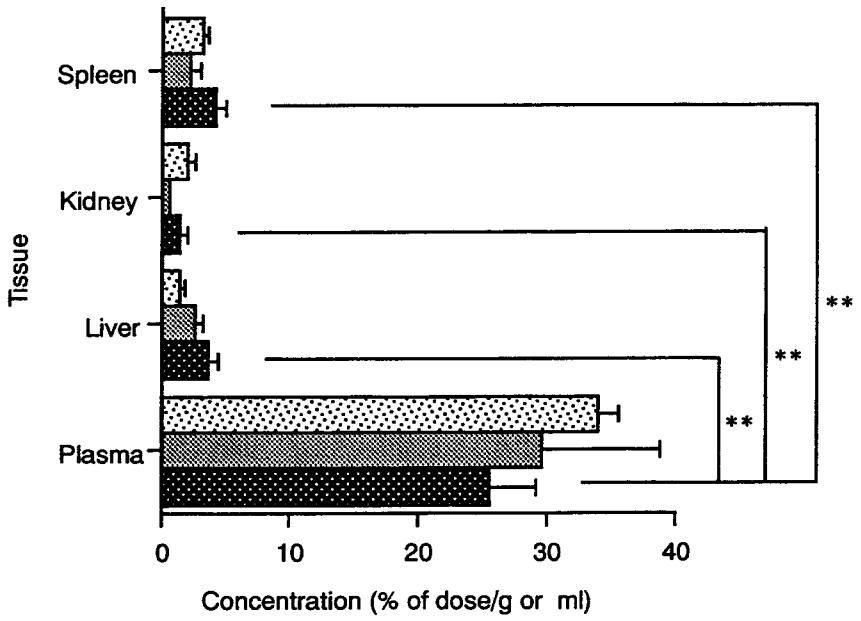


Figure 1-1. Plasma concentration and tissue distribution of Suc-FTC after i.v. injection at a dose of 1 mg (0.2 ml) per normal mouse: ▒, 24 h ; ▒, 48 h; ▒, 72 h. Each column represents the mean ± S.D. (n=5). **: $p<0.01$ for comparison at 72 h after injection.

of more than 20 % of dose/ml at 48 h after injection and at the concentration of more than 10 % of dose/ml even at 72 h after injection. The plasma concentration in tumor-bearing mice declined faster than that in normal mice. The distribution of Suc-FTC to tumor reached approximately 10 % of dose/g at 48 h after injection. This indicated that Suc-FTC should accumulate in the tumor. The accumulation to tumor seems to reach a plateau within 24 h after injection and to increase very slowly after that. The tumor level was close to the plasma level at 72 h after injection; the concentrations were not significantly different (p>0.05). The retention of Suc-FTC in blood was much higher than that in other tissues except tumor even at 72 h after injection. The plasma half-life was calculated to be 43.0 h by log-linear elimination analysis. Accumulation to the spleen, kidneys and liver was low in tumor-bearing mice.

The amount of Suc-FTC distributed in the tissues tested at 24, 48 and 72 h after i.v. injection to tumor-bearing mice and total amounts excreted into urine throughout 24, 48 and

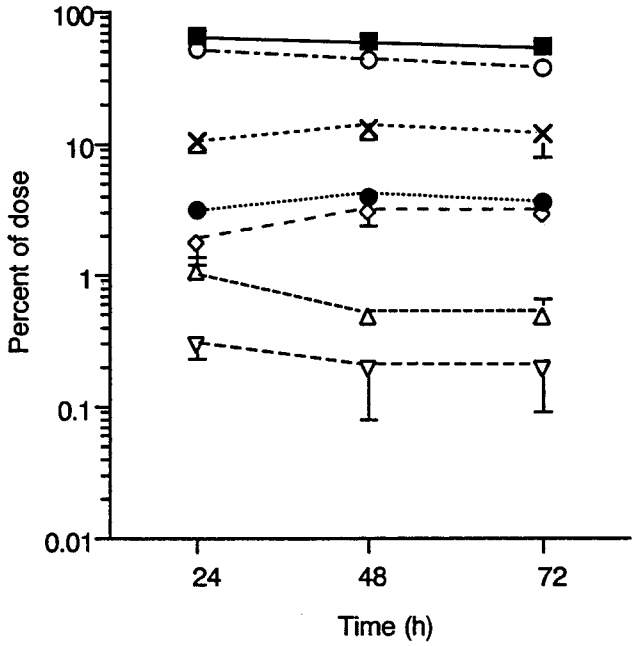


Figure 1-2. Distribution amount of Suc-FTC after i.v. injection at a dose of 1 mg (0.2 ml) per mouse: —■—, Plasma+liver+kidney+spleen (total in tested tissue not including urine); —○—, Plasma; —×—, Urine; —□—, Liver; —△—, Kidney; —●—, Liver+ kidney+spleen; —▽—, Spleen. Each point represents the mean ± S.D. (n=5).

72 h after i.v. injection to tumor-bearing mice are shown in Figure 1-4. The amount of Suc-FTC in plasma was determined in the same manner as in normal mice. Suc-FTC showed little distribution to the liver, kidneys and spleen and showed slight excretion into urine. The urinary excretion did not increase from 24 h to 72 h after injection, that is, it seemed to be almost finished at 24 h after injection. Retention in the blood circulation was longer and urinary excretion was less compared with the previous observations with Suc-FTC (55 % (w/w) *N*-succinylation).[37] The chemical and physicochemical properties of those Sucs used were different from each other. Especially, the degree of *N*-succinylation of the Suc used in the present study was higher, and thus the polymer would have a more negative charge. Generally, derivatives related to chitin and chitosan are supposed to undergo more degradation in tumor-bearing mice because the elevated lysozymic activity is known in Sarcoma 180 tumor- bearing mice.[84,85] As to the present Suc-FTC, the urinary excretion was near or less in tumor-bearing mice than that in normal mice, although neither was significantly different(p>0.05). This suggests that the excretion of Suc used in this study

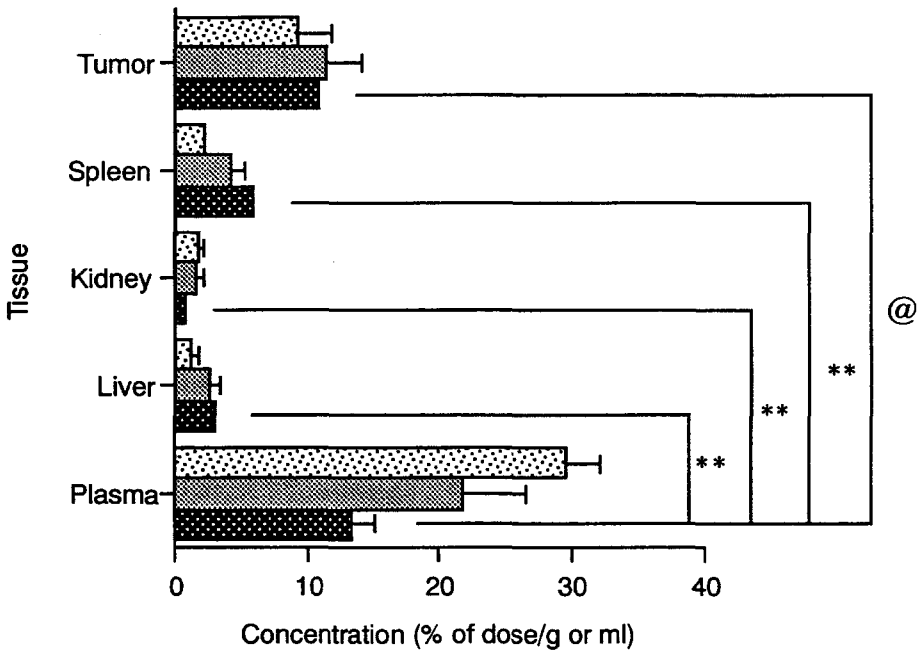


Figure 1-3. Plasma concentration and tissue distribution of Suc-FTC after i.v. injection at a dose of 1 mg (0.2 ml) per Sarcoma 180 tumor-bearing mouse: ▨, 24h; ▩, 48h; ■, 72h. Each column represents the mean ± S.D. (n=5). **: p<0.01 for comparison at 72 h after injection. @: p>0.05 for comparison at 72 h after injection.

should not be affected by the elevated enzymatic activity in tumor-bearing mice. The present Suc with higher degree of *N*-succinylation was considered to be protected against enzymatic degradation and to maintain much higher MW than the glomerular filtration limit (MW 4 - 5 × 10^4) in the body. The part of Suc-FTC with a relatively smaller MW, which was supposed to correspond to about 10 % of the dose, might be excreted into urine. The decline of the total amount of Suc-FTC for the tissues tested, which was parallel to the elimination from the blood circulation, was faster than that in normal mice. Nearly two thirds of the dose was recognized to move to tissues other than those tested, suggesting that the movement to other tissues or excretion in other forms such as the feces should be caused. Further studies are needed to clarify the reasons for this mass balance.

The systemic retention of Suc-FTC was compared with other macromolecules that have been reported to exhibit relatively long systemic circulation.[82,86-88] The systemic half-life of Suc-FTC was more than 4 d in normal mice and more than 40 h in tumor-bearing mice. On the other hand, the systemic half-lives of glutaryl-BSA (MW 70,000~80,000; tumor-bearing),

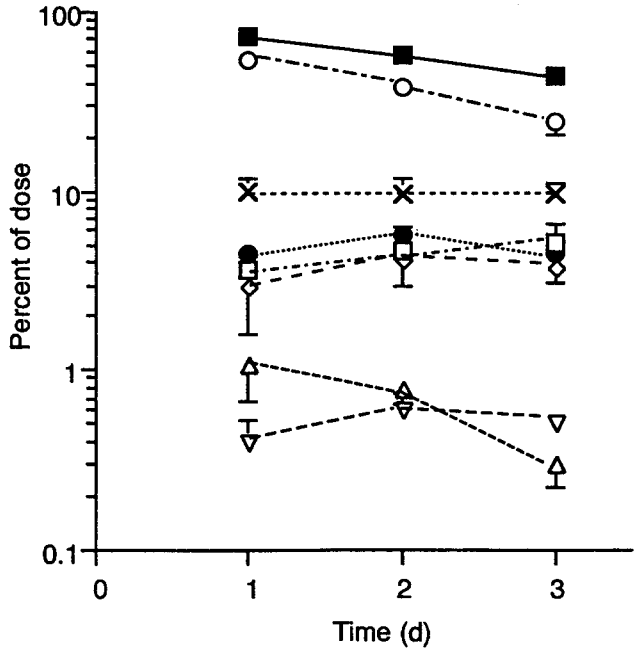


Figure 1-4. Distribution amount of Suc-FTC after i.v. injection at a dose of 1 mg (0.2 ml) per Sarcoma 180 tumor-bearing mouse: ■, Plasma+tumor+liver+kidney+spleen (total in tested tissues not including urine); ○, Plasma; ×, Urine; □, Tumor; ●, Liver+kidney+spleen; ◇, Liver; △, Kidney; ▽, Spleen. Each point represents the mean ± S.D. (n=5).

sulfoethyl-dextran (MW 197,000; normal), carboxymethyl-dextran (MW 70,000; tumor-bearing), BSA (MW 67,000; tumor-bearing) and carboxymethyl-dextran (MW 500,000; tumor-bearing) were reportedly around 2, 5, 9, 10 and 11 h, respectively. Thus, Suc exhibited much longer retention in the systemic circulation than other macromolecules having been reported before.[82,86-88] This was probably because Suc has a high MW and is hardly biodegradable and less interactive to tissues due to its high negative charge. The tumor accumulation of Suc-FTC was moderate and comparable to that of other macromolecules.[86,87] This study demonstrated that Suc is polymer with very long systemic circulation time. Since the tumor accumulation, which is known to be related to EPR in the diseased part,[5] is considered to be affected by MW as well as systemic circulation time, it may be controlled by changing the MW of Suc. More detailed analyses will allow the refinement of tumor-targeting ability of Suc as a drug carrier.

Chapter 2

Novel Water-soluble *N*-Succinyl-chitosan-mitomycin C Conjugate Prepared by Direct Carbodiimide Coupling: Physicochemical Properties, Antitumor Characteristics and Systemic Retention

Summary

Preparation of water-soluble conjugate of MMC with Suc by direct carbodiimide coupling was attempted. Smaller amounts of EDC, more advanced succinylated Suc, Suc(II), and shorter coupling reaction time were selected as reaction conditions. Almost half the obtained conjugate was water-soluble. The water-soluble conjugate, named Suc(II)-MMC, showed a high drug content of greater than 10 % (w/w). Suc(II)-MMC exhibited a pH-dependent drug release; i.e. the MMC release rate constants were 0.007, 0.018 and 0.114 (h^{-1}) at pH 6.2, 7.4 and 9.0, respectively at 37 °C. Addition of mouse plasma barely accelerated the release of MMC. Suc(II)-MMC was very stable at low temperatures (7 °C), but showed gel-formation when left to stand at that temperature for 2 weeks. The gel-formation was not observed during the usual *in vitro* and *in vivo* operations. Suc(II)-MMC showed less toxicity than MMC and a strong in vivo antitumor effect against Sarcoma 180 at a high dose in i.v. administration. MMC disappeared quickly from systemic circulation after i.v. administration, while Suc(II)-MMC was retained at a high concentration of greater than 15 % of the dose/ml in plasma even at 8 h after i.v. administration.

2-1. Introduction

Chitosan is a weakly basic polysaccharide, which is extensively used in various fields. One of its derivatives, Suc, is water-soluble, has low toxicity[72] and is retained for long periods in the blood circulation;[37] therefore, it has been proposed as a useful macromolecular drug carrier. It is known that macromolecules with carboxylic acid can easily react with MMC via carbodiimide coupling.[32,89,90] It was previously reported that the conjugate prepared by the direct coupling of MMC with Suc using EDC was obtained as a solid due to crosslinking among or within the polymer supports.[35,74,80] This insoluble property should influence the biopharmaceutical behaviors of the polymer supports as well as its physicochemical properties. Therefore, to make effective use of the physicochemical characteristics of the polymer itself for controlling the conjugate biopharmaceutically, the preparation of water-soluble conjugates is required. An activated ester of glutaric MMC (glu-MMC-OSU) was utilized in the conjugation reaction as a method to avoid the formation of the insoluble product of the conjugate.[36,91] Although the reaction between Suc and glu-MMC-OSU formed water-soluble conjugates, it was difficult to obtain the conjugates with a high load of MMC.[36] The low drug content of the conjugate caused the problem that the conjugate could not be injected at a high dose because the high concentrated conjugate solution was too viscous for safe injection intravenously.[36] Therefore, a water-soluble conjugate with high drug content has been required to increase the usefulness of the conjugate of MMC with Suc. Since Suc is known to exhibit a very long systemic retention after i.v. injection,[37,92] intravenously injectable water-soluble Suc-MMC conjugate with high drug content is considered to be valuable. Therefore, in this study, preparation of water-soluble Suc(II)-MMC conjugate with high drug content was attempted, and its basic characteristics were examined.

2-2. Materials and methods

2-2-1. Materials

MMC was obtained as a powder by solvent evaporation after extraction using methanol from Mitomycin Kyowa S (Kyowa Hakko Kogyo Co., Tokyo, Japan) which contained only NaCl in addition to MMC. Suc (*N*-succinylation degree per glucosamine unit, 0.81) was kindly supplied from Katakura Chikkarin Co., Ltd. (Tokyo, Japan). EDC was purchased from Dojindo Laboratories (Kumamoto, Japan). Deuterium oxide (D_2O) was obtained from Merck (Darmstadt, Germany). Twenty percent deuterium chloride (DCl) and 3-(trimethylsilyl)propionic 2,2,3,3-d_4 acid sodium salt (TSP) were purchased from Aldrich Chemical Co., Inc. (Milwaukee, U.S.A). All other chemicals were of reagent grade.

2-2-2. Animals

Male ddY mice (6 weeks old) weighing 24 – 28 g were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan), and used soon after being supplied. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Hoshi University.

2-2-3. Tumors

Sarcoma 180 cells were maintained by weekly transplantation of 1×10^6 cells suspended in Hanks' balanced solution (0.1 ml) into the peritoneal cavity of each mouse. In the *in vivo* tests of antitumor effect, 1×10^6 Sarcoma 180 cells suspended in Hanks' balanced solution (0.1 ml), which were obtained from the above tumor-bearing mice, were inoculated subcutaneously at the axillary region in each mouse.

2-2-4. Preparation of water-soluble conjugate

First, Suc(II) was prepared by reaction of Suc with succinic anhydride. Suc (750 mg) was dissolved in 0.1 *M* NaCl aqueous solution (100 ml) with pH adjusted to 8.0 using 0.1 *M*

NaOH aqueous solution. Succinic anhydride (1.47 g), which was approximately 15-fold to glucosamine molar ratio, was gradually added to Suc solution, maintaining pH between 7.5 to 8.5 by addition of 3 *M* NaOH aqueous solution. The solution was stirred at 15 × 1 °C for 30 min. After the reaction mixture was divided into small aliquot, the product, Suc(II), was separated by gel-filtration of each portion. Namely, Suc(II) was separated from small molecules containing free succinic anhydride by gel-filtration of the portion using a Sephadex® G-50 column (3.5 cm (inner diameter) × 27 cm (length)) with 0.1 *M* NaCl aqueous solution as an elution solvent. High MW fractions were collected and dialyzed against water to remove salts. Finally, Suc(II) was obtained as a powder by lyophilization of the dialyzed solution. The structure of Suc was characterized before,[92] and Suc(II) were investigated by ^{1}H-NMR spectra using a JEOL JNM-LA500 spectrometer (Tokyo, Japan). The findings on Suc(II) were as follows: ^{1}H-NMR (D_2O-DCl, pH 6), reference TSP, δ (ppm): 2.3 - 2.7 (multiplet, CH_2CH_2 of succinyl), 3.4 - 4.0 (multiplet, H-2, H-3, H-4, H-5, H-6), 4.5 - 4.8 (broad, H-1). The MW of Suc(II) determined by GPC using PEO standards or by SEC-MALS was about 310,000.

Water-soluble conjugate, Suc(II)-MMC, was prepared as follows: Suc(II) (50 mg) and MMC (20 mg) were dissolved in water (12.5 ml), and EDC (50 mg) was added, and then the pH of the mixture was adjusted to 6.1 by addition of 0.1 *N* HCl aqueous solution. The solution was stirred at room temperature for 15 min. Immediately after the reaction, the mixture was filtered using a fine filter (pore diameter 10 μm). Suc(II)-MMC was separated from small molecules containing free MMC and EDC using a gel-chromatograph of the filtrate on a Sephadex® G-50 column (2.6 × 14 cm) with 0.1 *M* NaCl aqueous solution as an elution solvent. High MW fractions were collected, concentrated by evaporation at 35 °C and washed with normal saline using an ultrafiltration unit USY-5 (MW cut-off limit 50,000; Toyo Roshi Kaisha, Ltd.) to give the conjugate solution in saline. The obtained conjugate solution in saline was diluted with a 100-fold volume of 1/15 *M* phosphate buffered solution, pH 7. The diluted solution was measured at 363 nm using a DU® 640 spectrophotometer (Beckman, USA), and the amount of MMC in 1 ml of the conjugate solution in saline was

calculated based on a calibration curve. In addition, after removing salts from the conjugate solution in saline (1 ml) by ultrafiltration with water, the remaining solution was freeze-dried. The weight of the obtained powder was measured as the Suc(II)-MMC amount in 1 ml of the conjugate solution in saline. Thus, the content of MMC per conjugate (w/w %) was calculated from the amounts of MMC and conjugate in 1 ml of the conjugate solution in saline. Also, the conjugate solution in saline was utilized in the following *in vitro* and *in vivo* studies.

2-2-5. *In vitro* drug release

The release of MMC from Suc(II)-MMC in 1/15 *M* phosphate buffer (pH 6.2, 7.4 and 9.0) and the mixture of 1/15 *M* phosphate buffer (pH 7.4) and mouse plasma (4 : 1, v/v), named 20 % (v/v) mouse plasma, were investigated. Suc(II)-MMC was dissolved at a concentration of 0.86 mg/ml in 3 ml of 1/15 *M* phosphate buffer or 20 % (v/v) mouse plasma, and incubated at 37 °C and 60 rpm. The amount of free MMC released in the media was determined using HPLC, which was carried out using a Shimadzu LC-6AD apparatus equipped with a SUMIPAX Nucleosil $5C_{18}$ reversed phase column (4 × 250 mm) and an SPD-10AV UV detector (Shimadzu) set at 365 nm. The mobile phase was a mixture of 0.01 *M* phosphate buffer, pH 6.0, and methanol (65 : 35, v/v). As to the samples obtained from the buffers, the sample solution was directly injected on the HPLC system. Concerning the samples obtained from 20 % (v/v) mouse plasma, they were treated following the method of Den Hartigh et al.;[93] briefly, the sample was mixed to a 10-fold volume of the mixture of chloroform and 2-propanol (1 : 1, v/v). After centrifugation of the mixture, the whole supernatant was taken and evaporated to dryness below 40 °C under nitrogen gas. The residue was dissolved in methanol, and the solution was analyzed for MMC in the HPLC system stated above.

2-2-6. Stability at low temperature

The chemical and physical stabilities of Suc(II)-MMC were checked after storage at

low temperatures (7 °C). The physical situation of Suc(II)-MMC, especially its aggregation property, was observed for 2 weeks, and the amount of released MMC at that low temperature during preservation was checked using HPLC in the same manner as mentioned above for the *in vitro* release study in the buffers.

2-2-7. *In vivo* antitumor effect against Sarcoma 180

At 9 d after subcutaneous (s.c.) inoculation, MMC and Suc(II)-MMC were administered intravenously into the tail vein to Sarcoma 180-bearing mice at a single dose of 5 mg eq. MMC/kg. In addition, MMC and Suc(II)-MMC were administered repeatedly at the dose of 5 mg eq. MMC/kg at 9, 13 and 17 d after inoculation, i.e. at 3 × 5 mg eq. MMC/kg in total. Further, Suc(II)-MMC was administered at a single dose of 10 mg eq. MMC/kg and repeatedly at the dose of 10 mg eq. MMC/kg at 9, 13 and 17 d after inoculation, i.e. at 3 × 10 mg eq. MMC/kg in total. As to the control mice, saline alone was injected in similar volumes and times. All mice were observed for tumor volume. The tumor volume immediately before administration was used as the initial tumor volume, and the tumor volume of each group was measured for 30 d after administration. The ratio of the tumor volume to the initial volume was calculated for 30 d after administration. The length (L, cm) of the tumor major axis, the longest axis, and width (W, cm) of the tumor minor axis, the vertical axis to the major one, were measured with slide calipers. The tumor volume (V, cm^3) was calculated based on the equation of Takakura et al.[86] using L and W as follows:

$$V = L \times W^2/2 \qquad (1)$$

At the same time, the change in body weight of each group was measured to evaluate the toxic side effect.

2-2-8. Plasma residence of the conjugate

MMC and Suc(II)-MMC were administered to Sarcoma 180-bearing mice at 5 and 10 mg eq. MMC/kg. At 8 h after i.v. administration of MMC, the blood was taken after sacrifice. Blood was taken and centrifuged (3,000 rpm for 10 min), and plasma was

collected. Plasma was treated following the method of Den Hartigh et al.[93] as stated above for the sample incubated in 20 % (v/v) plasma for *in vitro* release study. The sample solution obtained by this process was analyzed for MMC using HPLC in the same manner as stated above. Similarly, plasma was obtained from blood sample taken after sacrifice at 8 h after i.v. administration of Suc(II)-MMC. One part of this plasma was analyzed for the determination of free MMC in plasma as in the case of administration of MMC. In addition, a four-fold volume of 1 *M* carbonate buffer (pH 9.0) was added to the remaining part of the plasma. This plasma-buffer mixture was heated at 90 °C for 5, 10 and 15 min. The heat-treated samples were treated as in the case of administration of MMC, and analyzed for MMC using HPLC in the same manner as stated above in the *in vitro* release study. The latter measurement permits the calculation of the total MMC derived from free MMC and Suc(II)-MMC residing in plasma.

2-3. Results and discussion

2-3-1. Physicochemical characteristics of water-soluble conjugate

Preparation of water-soluble conjugate was attempted by the direct coupling of MMC with Suc using EDC. However, the preparation procedure based on a previous report[74] presented a water-insoluble conjugate because of crosslinking within or among the polymer supports. Since the crosslinking was considered formed among amino groups and carboxyl groups of Suc, increased *N*-succinylation of Suc was expected to reduce the crosslinking. Therefore, further succinylation of Suc was attempted. Moreover, for improved suppression of the condensation reaction among amino groups and carboxyl groups of Suc, reduced amounts of EDC were used. In addition, a reduction of reaction time was also applied to help suppress the crosslinking. Thus, the reaction scheme described in Figure 2-1 was proposed for preparation of water-soluble conjugate of MMC with Suc by EDC coupling. Suc and Suc(II) were found to be completely deacetylated from the ^{1}H-NMR spectra, which

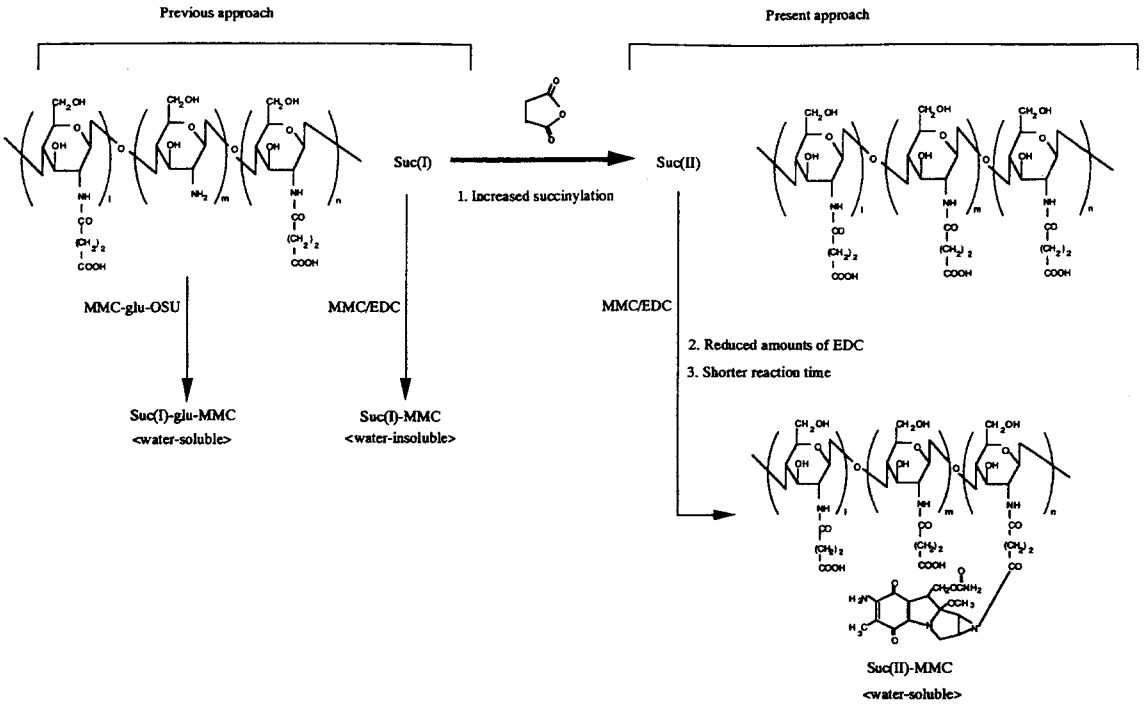


Figure 2-1. Synthetic approach for novel water-soluble conjugate of MMC with Suc

was consistent with a high degree of deacetylation (95 – 96 % against sugar unit) indicated by the supplier based on the deacetylation degree of the starting polymer, chitosan. Succinylation degree of Suc and Suc(II) was determined from ^{1}H-NMR spectra by comparison of the integrated intensity of methylene protons of the succinyl groups with that of protons of sugar moieties other than succinyl groups. Free amino groups were hardly observed in Suc(II) from its ^{1}H-NMR spectra (data not shown). Furthermore, it has been reported that *O*-deacylation could occur selectively by stirring with alkali-methanol solution overnight.[94] According to the report,[94] Suc(II) was dissolved in the mixture of 1 *N* KOH (3 ml) and methanol (3 ml), and then was stirred at room temperature overnight. The reaction mixture was evaporated, neutralized using HCl, and then underwent gel-filtration using a Sephadex® G-50 column. After dialyzed and lyophilized, the alkali-treated Suc(II) was checked by ^{1}H-NMR. These results showed that the degree of succinylation decreased by about 10 % compared with the intact Suc(II) (data not shown). Therefore, the small portion of the attached succinyl groups, approximately 10 %, is considered bound to the sugar

hydroxyl groups.

The reaction conditions, based on the previous study of EDC coupling, exclusively gave a solid product (98 % (w/w) <). However, in the novel reaction condition, more than one-third of the product obtained was water-soluble conjugate, Suc(II)-MMC. The MMC content of Suc(II)-MMC was 12 % (w/w), which was lower than Suc-MMC. It was considered due to the reduced amounts of EDC used and shorter reaction time in the preparation of Suc(II)-MMC. However, the MMC content of Suc(II)-MMC was much higher than that of the water-soluble conjugate prepared by reaction of Suc and glu-MMC-OSU; the latter conjugate showed the MMC content of 4.7 % (w/w).[36] The content of 12 % (w/w) led to a reduction in the mass of the conjugate at administration, suggesting it should be possible to inject the conjugate solution with a relatively low viscosity even at a high dose. Thus, the present preparation is considered available for preparation of water-soluble conjugate with a high content of MMC.

2-3-2. *In vitro* drug release

Drug release from Suc(II)-MMC was examined at 37 °C in 1/15 *M* phosphate buffer (pH 6.2, 7.4 and 9.0) and 20 % (v/v) mouse plasma in pH 7.4. The conjugate showed a pH-dependent release (Figure 2-2). The drug release was analyzed based on the pseudo-first order kinetics as described below:

$$\begin{array}{ccccc} & & \overset{k_2}{} & & \overset{k_1}{} \\ \text{Suc(II)-MMC} & & \rightarrow & \text{MMC} & \rightarrow \quad \text{decomposition products} \\ & \downarrow k_3 & & & \\ & \text{decomposition products} & & & \end{array}$$

The free MMC (%) appearing in the media at time t is given by the following equation:

$$[MMC] = (k_2 A_o/(k_1 - k_2 - k_3)) \times (\exp(-(k_2 + k_3)t) - \exp(-k_1 t)) + (100 - A_0) \times \exp(-k_1 t) \quad (2)$$

where $[MMC]$ means the percentage of the appearing free MMC against the total initial contained MMC (t = 0), k_1, k_2 and k_3 are first-order rate constants, and A_0 represents the

conjugated MMC (%) against the total initial MMC. The conversion rate constants (k_2, k_3) were determined by curve fitting using equation 2. At that time, the decomposition rate constant (k_1) was fixed based on the reported pH-rate constant profile of MMC in 1/15 *M* phosphate buffer at 37 °C;[81,91,95] that is, k_1 values were fixed at 0.016, 0.004 and 0.004 (h^{-1}) to pH 6.2, 7.4 and 9.0, respectively. The curve fitting was executed using the non-linear regression program MULTI.[96] The results are shown in Table 2-1. The calculated profiles were well fitted to the observed ones. Especially, the concentration of free MMC in the medium of pH 9.0 was lower at 48 h than at 24 h in pH 9.0, and this situation was simulated satisfactorily by equation 2. Fifty percent degradation time of the conjugate at 37 °C was calculated as 98, 40 and 6 h at pH 6.2, 7.4 and 9.0, respectively, from the k_2 value. The addition of plasma barely affected the release rate. The release rate of Suc(II)-MMC was different from other types of conjugates, i.e. water-soluble *N*-succinyl-chitosan-glutaryl-MMC

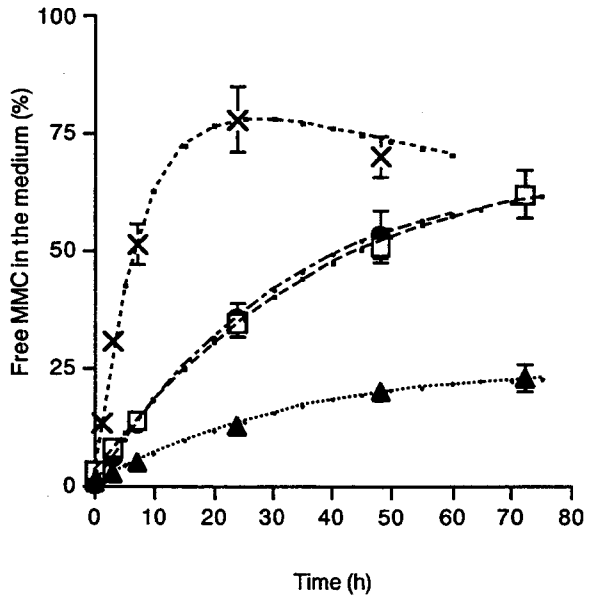


Figure 2-2. Release of MMC from Suc(II)-MMC in 1/15 *M* phosphate buffer (pH 6.2, 7.4 and 9.0) and the mixture of 1/15 *M* phosphate buffer (pH 7.4)-mouse plasma (4:1, v/v) at 37 °C: △, pH 6.2; □, pH 7.4; ×, pH 9.0; ●, plasma-buffer mixture. Each point represents the mean ± S.D. (n=3). The broken or clotted lines show the profiles calculated from equation 2 using the parameters in Table 2-1.

conjugate (Suc-glu-MMC)[91] and water-insoluble *N*-succinyl-chitosan-MMC conjugate.[74] This is possibly because the release rate should depend on the subtle change in circumstances around the bound MMC. The results suggest that Suc(II)-MMC should exhibit gradual release of MMC in the body. Suc(II)-MMC has potency to act as a slow release water-soluble macromolecular prodrug.

2-3-3. Stability in preservation at low temperature

The chemical and physical stabilities of Suc(II)-MMC in saline were evaluated under low temperature conditions (7 °C) using the final conjugate solution in saline. Suc(II)-MMC was investigated for the release of MMC in saline and the physical state of the saline solution at low temperature in the refrigerator (7 °C) during preservation. The results are described in Table 2-2. Around 10 % of MMC from Suc(II)-MMC was regenerated after 1 week preservation. Further, no gel-formation was observed after 1 week preservation. However, gel-formation was found after 2 weeks preservation. Suc(II)-MMC appears to have characteristics to form gel relatively in the concentrated solution. The MW of Suc(II)-MMC may be somewhat higher than that of the polymer support itself Suc(II) because some linking can be caused among the polymer support. Furthermore, Suc(II)-MMC, with a high content

Table 2-1.
Conversion rate constants for Suc(II)-MMC in various media

Rate constant [a] (h^{-1})	One-fifteenth *M* phosphate buffer			Twenty percent plasma in 1/15 *M* phosphate buffer, pH 7.4 [b]
	pH 6.2	pH 7.4	pH 9.0	
k_2	0.007	0.018	0.114	0.020
k_3 [c]	0	0	0.017	0.002

a) The k_1 values were fixed to 0.016, 0.004 and 0.004 at pH 6.2, 7.4, and 9.0, respectively, based on the known pH-rate constant profiles.[81,91,95]

b) The k_1 value was fixed to 0.004; degradation effect by plasma was regarded as slight.

c) The k_3 values at pH 6.2 and 7.4 were fixed to 0 because they converged to very small minus values.

Table 2-2.
Chemical and physical stabilities of Suc(II)-MMC in its saline solution during preservation at 7 °C

	Preservation time (d)				
	1	3	5	7	14
Appearing free MMC (%)	1.11	3.24	5.33	9.02	——
Gel formation	non-detected	non-detected	non-detected	non-detected	detected

of MMC, may exhibit increased interaction among the moieties of the conjugates due to the introduction of lots of MMC moieties. These chemical and physicochemical changes of the polymer may cause the characteristics of gel-formation. The remainder of the reactive carboxylic acid-carbodiimide intermediate on the conjugate may be another reason for the gel-formation, because its intermediate can react with amino groups. Since the possibility was considered that a few amino groups remained in the conjugate and that the reactive carboxylic acid-carbodiimide intermediate on the conjugate reacted intermolecularly or intramolecularly with the amino groups, suppression of the reaction between the carboxylic acid-carbodiimide intermediate and the amino group was attempted. Namely, excessive amounts of aminoethanol were added to the conjugate solution for complete reaction of the possibly remaining reactive carboxylic acid-carbodiimide intermediate. The reaction mixture was stirred, dialyzed, and then the dialyzed solution was freeze-dried. However, the resulting powder was hardly soluble in water. This suggests the conjugate may possess solubility characteristics different from those of Suc(II) itself and that the gel-formation may not be caused by the remainder of the reactive intermediate but by the solubility characteristics of the conjugate. However, the detailed mechanism of the gel-formation remains unclear. These results imply that the conjugate solution in saline should cause no problems in use if used within 1 week after preparation. Actually, through all experiments in this study, the Suc(II)-MMC conjugate solution was used within 4 days after preparation.

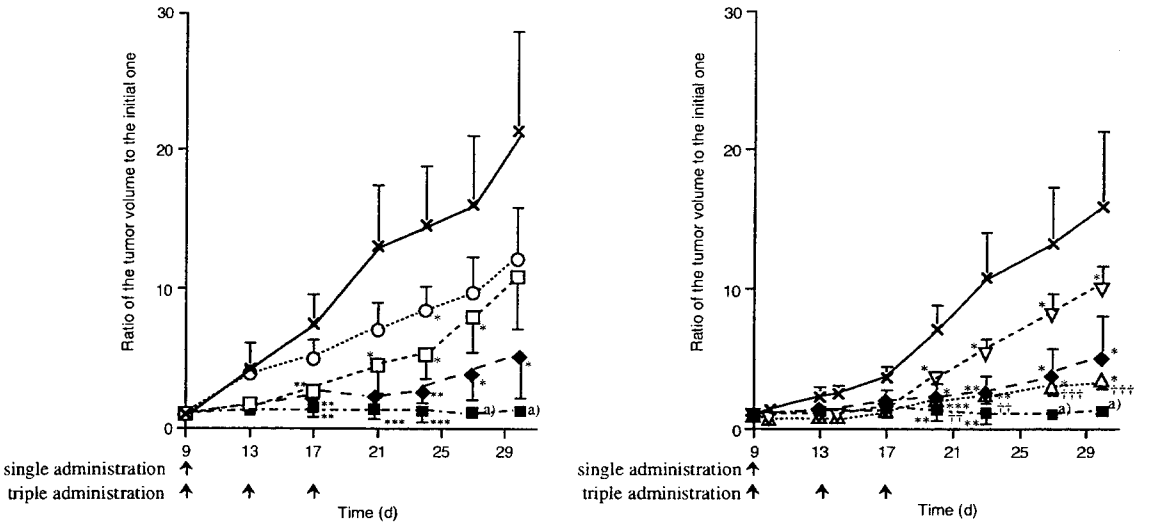


Figure 2-3. ***In vivo*** **growth inhibitory effect of Suc(II)-MMC and MMC against Sarcoma 180 solid tumor:** —×—, control; - -◆- -, MMC 5 mg/kg; - -■- -, MMC 3 × 5 mg/kg; - -□- -, Suc(II)-MMC: 5 mg/kg; ·····○·····, Suc(II)-MMC: 3 × 5 mg/kg; ·····▽·····, Suc(II)-MMC: 10 mg/kg; ·····△·····, Suc(II)-MMC: 3 × 10 mg/kg. The tumor volume immediately before administration is used as the initial one. Each point represents the mean ± S.D. (n=5). At 9 d after inoculation, a solution of the testsubstance was injected intravenously. a) Four mice died (n=1).
*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ vs control. ††: $p<0.01$, †††: $p<0.001$ vs single dose (Suc(II)-MMC).

2-3-4. *In vitro* antitumor effect against Sarcoma 180

The efficacy of Suc(II)-MMC at i.v. administration was examined using mice bearing Sarcoma 180 solid tumor subcutaneously at the axillary part. The tumor growth profiles are shown in Figure 2-3. The tumor was ellipsoidal in shape but somewhat spherical. Marked tumor growth was observed in the control mice. MMC exhibited good tumor growth suppression at 5 mg/kg, but was lethally toxic in the three times repeated administration of 5 mg/kg (3 × 5 mg/kg in total). The dose of 3 × 5 mg/kg caused a decrease in body weight (Figure 2-4), indicating overdose. On the other hand, the Suc(II)-MMC group brought about tumor growth suppression both at 5 mg eq. MMC/kg and at 3 × 5 mg eq. MMC/kg without any decrease in body weight (Figure 2-4). These results indicate Suc(II)-MMC should exhibit less acute toxicity than MMC. The previous water-soluble conjugate, Suc-glu-MMC, showed a lower drug content,[36] and its solution was too viscous at a dose greater than 2.5 mg eq. MMC/kg to be injected safely. However, Suc(II)-MMC could be safely administered intravenously at a higher dose because of its high drug content. The difference in the effect

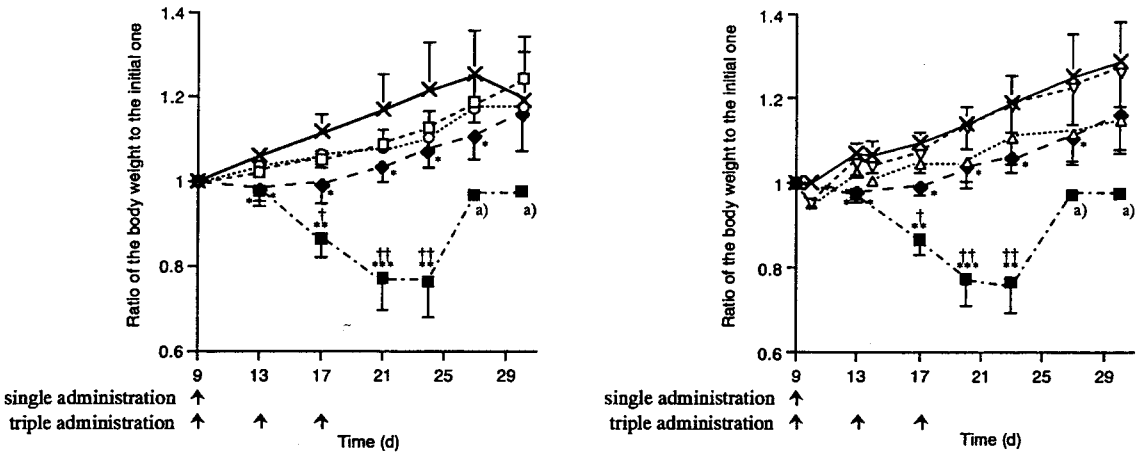


Figure 2-4. Change in the mean body weight of mice after MMC or Suc(II)-MMC injection: —×— , control; - -◆- - , MMC 5 mg/kg; - -■- - , MMC 3 × 5 mg/kg; - -□- - , Suc(II)-MMC: 5 mg/kg; ·····○····· , Suc(II)-MMC: 3 × 5 mg/kg;·····▽····· , Suc(II)-MMC: 10 mg/kg; ·····△····· , Suc(II)-MMC: 3 × 10 mg/kg. The body weight immediately before administration is used as the initial one. Each point represents the mean ± S.D. (n=5). At 9 d after inoculation, a solution of the testsubstance was injected intravenously. a) Four mice died (n=1). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ vs control. †: $p<0.01$, ††: $p<0.01$ vs single dose (MMC).

of Suc(II)-MMC was not observed between 5 mg eq. MMC/kg and 3 × 5 mg eq. MMC/kg. In higher dose tests, Suc(II)-MMC was administered at a single dose of 10 mg eq. MMC/kg and at three times repeated doses of 10 mg eq. MMC/kg (3 × 10 mg eq. MMC/kg in total). Under these conditions, a marked tumor growth suppression was observed in the 3 × 10 mg eq. MMC/kg group. A decrease in body weight was not observed even at 3 × 10 mg eq. MMC/kg in Suc(II)-MMC. Thus, Suc(II)-MMC could be administered at a high dose, and it was recognized highly effective and low toxic. Further, tests by various administration schedules will be needed to more fully understand the detailed antitumor activity of Suc(II)-MMC.

2-3-5. Systemic residence of the conjugate

Blood was withdrawn at 8 h after i.v. injection of MMC (5 mg/kg) and Suc(II)-MMC (10 mg eq. MMC/kg) to Sarcoma 180-bearing mice, and the plasma was obtained using centrifugation. The concentrations of MMC and the conjugate in the plasma were examined. The conjugated MMC was recognized as almost completely liberated as free MMC after the

Table 2-3.
Plasma concentration of bound or free MMC at 8 h after i.v. injection of MMC and Suc(II)-MMC at 5 mg/kg and 10 mg eq. MMC/kg, respectively, in mice

	Concentration in plasma (μg/ml) [a]		
	Bound MMC	Free MMC	Total
MMC	————	Non-detected	0
Suc(II)-MMC	53.3 ± 13.7** (15.2 % of dose/ml)	8.8 ± 4.2 (2.5 % of dose/ml)	62.0 ± 17.7 (17.7 % of dose/ml)

MMC lowest detection limit in HPLC: 0.04 μg/ml.
a) The results are expressed as the mean ± S.D. of four mice.
**: $p<0.01$ vs free MMC.

treatment for 5 min at 90 °C and pH 9, while as treating time was further increased, decrease in recovery of MMC was observed (data not shown). Therefore, the total amount of MMC in plasma could be determined from that of MMC existing in the sample after such heat treatment for 5 min at 90 °C and pH 9. The lowest detection limit of MMC in HPLC was 0.04 μg/ml. The results are described in Table 2-3. No MMC was detected in the administration of free MMC, indicating MMC had decreased at the level lower than 0.04 μg/ml at 8 h after injection. On the contrary, free MMC and bound MMC in the conjugate were observed at much higher concentrations than the lowest detection limit. The main part of MMC was found retained as a conjugated form. The total residing amount at 8 h was calculated to be nearly 18 % of dose/ml, which was consistent with the long residence characteristics of the polymer in the blood circulation.[37,92] The systemic retention of Suc(II) will be described in another article. Since a large part of MMC is expected to remain in a conjugated form at 8 h after administration due to its slow release under physiological conditions as based on *in vitro* release studies, Suc(II)-MMC is considered to be found at a high level at 8 h after administration. These results indicate that Suc(II)-MMC should circulate well in the blood stream and that MMC should be gradually regenerated there.

Thus, Suc(II)-MMC was recognized to be a water-soluble conjugate exhibiting long-

term circulation in the blood stream and a gradual drug release. The long residence of the conjugate in the blood circulation may realize the localization of the drug to the diseased parts, such as tumor site, based on the EPR effect.[5,97)]

Chapter 3

Biological Fate of Highly-succinylated *N*-Succinyl-chitosan and Antitumor Characteristics of Its Water-soluble Conjugate with Mitomycin C at I.v. and I.p. Administration into Tumor-bearing Mice

Summary

Suc(II) was fluorescein-labeled, and the labeled product (Suc(II)-FTC) was examined for biodisposition in Sarcoma 180-bearing mice after i.v. and i.p. administration. Suc(II)-FTC injected intravenously was sustained at a high level in the blood circulation and showed little distribution to tissues other than tumor. On the other hand, it took a few hours for Suc(II)-FTC to be transferred to the blood circulation after i.p. administration. There were no marked differences in the distribution of Suc(II)-FTC between i.v. and i.p. administration routes except in the early stage. The urinary excretion of Suc(II)-FTC following both i.v. and i.p. administration was small, but the excretion tended to be suppressed after i.p. administration. Water-soluble Suc(II)-MMC conjugate prepared using water-soluble carbodiimide exhibited marked effect at a high dose and suppressed the acute toxic side effect of MMC. Suc(II)-MMC tended to be more toxic at i.p. administration than at i.v. administration. The difference in biodisposition between the two administrations was thought to affect the toxic side effect. The plasma levels of conjugated and free MMCs at 8 h after i.v. administration were higher than those at 8 h after i.p. administration. These suggested more localization of the conjugate in peripheral tissues and less excretion at i.p. administration, which might result in greater toxicity.

3-1. Introduction

There have been a number of studies concerning modification of anticancer drugs with macromolecules.[98-101] One of the aims of drug-carrier conjugation by combining an anticancer drug with a carrier is to obtain agents that selectively attack cancers. Drug-carrier conjugates often improve the less selective toxicity of many anticancer agents.[5,102] Drug-carrier conjugates generally have characteristics such as gradual drug release because anticancer drugs display their effects when liberated from the carrier. Dextran,[103] albumin,[33] chitosan derivatives[91] and others have been used as carriers.

Suc, synthesized by introduction of succinyl groups into chitosan at the *N*-position of the glucosamine unit, is water-soluble, less toxic,[72,73] poorly biodegradable in the body[81] and long retained in the systemic circulation after i.v. administration.[37,92] Thus, Suc is expected to be a useful macromolecular drug carrier with a long retention time in the body.

Macromolecules with carboxylic acid are known to be easily combined with MMC. Previous studies showed that a water-soluble Suc(II)-MMC conjugate could be prepared by the direct coupling of MMC with highly-succinylated Suc using water-soluble carbodiimide (EDC) under specific reaction conditions, and that the water-soluble conjugate released MMC by about 13, 35 and 78 % after incubations at 37 °C in 1/15 *M* phosphate buffer for 24 h at pH 6.2, 7.4 and 9.0, respectively.[104] Further, the addition of plasma had little influence on the drug release rate, and actually the gradual *in vivo* release of MMC from the conjugate was recognized.[104] The water-soluble conjugate by the direct coupling was demonstrated to be a useful prodrug of MMC.

Hitherto, the biodisposition characteristics of Suc have been examined after i.v. administration.[37,92] The differences in the administration route can modify the efficacy and toxicity of the conjugates.[25] Such phenomena seem to be related to the biodisposition properties of the conjugates, which are highly dependent on the characteristics of the carriers.[26,105,106] Also, even molecules with a very high molecular weight can be quickly transferred from the intraperitoneal part to the systemic circulation.[107] Further, the i.p.

administration of these conjugates is often useful for high antitumor efficacy.[25,98] Therefore, how Suc with a very high MW and its drug-conjugate behave in the body following i.p. administration are viewed as very interesting. The biodisposition characteristics of Suc(II) and antitumor properties of its conjugate with MMC following i.p. administration have not been investigated. Therefore, the present study compared the biodisposition profiles of Suc(II) after i.v. and i.p. administration, and determined the antitumor characteristics of its conjugate with MMC against subcutaneous Sarcoma 180 solid tumor by both administration routs.

3-2. Materials and methods

3-2-1. Materials

MMC was used after extraction by methanol from Mitomycin Kyowa S (Kyowa Hakko Kogyo Co., Tokyo, Japan) which consisted of NaCl and MMC. Suc was a kind gift from Katakura Chikkarin Co., Ltd. (Tokyo). Its succinylation and deacetylation degrees were 0.81 mol/sugar unit and 1.0 mol/sugar unit, respectively, by proton nuclear magnetic resonance,[92] and the average molecular weight was 3.4×10^5 (the range of MW: $5 \times 10^4 - 1.5 \times 10^6$) by SEC-MALS. EDC was purchased from Dojindo Laboratories (Kumamoto, Japan). FITC was purchased from Sigma Chemical Company (St. Louis, U.S.A.). All other chemicals were obtained commercially as reagent-grade products.

3-2-2. Animals

Male ddY mice (6 weeks old) weighing 27 – 28 g were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo), and were used in all experiments. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Hoshi University.

3-2-3. Tumors

Sarcoma 180 cells were maintained in ddY mice by weekly i.p. transfer of 1×10^6 cells obtained from ascitic fluid. In the *in vivo* antitumor tests, 1×10^6 Sarcoma 180 cells suspended in 0.1 ml of Hanks' balanced salt solution, which were obtained from the above tumor-bearing male ddY mice, were inoculated subcutaneously into each male ddY mouse at the axillary region.

3-2-4. Biodisposition of Suc(II)-FTC

First, Suc(II) was prepared by reaction of gifted Suc with succinic anhydride. Briefly, the Suc (1.5 g) was dissolved in 0.1 *M* NaCl aqueous solution (200 ml) with the pH adjusted to 8.0 by 0.1 *M* NaOH aqueous solution. Succinic anhydride was gradually added at a 15-fold molar excess to the glucosamine units, while the pH was maintained at between 7.5 to 8.5 by addition of 3 *M* NaOH aqueous solution. The solution was stirred at 15 ± 1 °C for 30 min. The product was separated on a Sephadex® G-50 column (3.5 cm in inner diameter × 27 cm in length) using 0.1 *M* NaCl aqueous solution as an elution solvent. The eluted solution at the high MW fractions was collected, and dialyzed against water for 3 d to remove salts. Finally, Suc(II) was obtained by freeze-drying the dialyzed solution. The succinylation degree was elevated to approximately 1.1 mol/sugar unit, which was due to succinylation to the hydroxy groups at approximately 0.1 mol/sugar unit in addition to almost complete succinylation to the amino groups.[104)]

Next, Suc(II)-FTC was prepared according to the methods reported by Tanaka et al.[82)] and Kamiyama et al.,[37)] and its distribution in the body was investigated. Briefly, FITC (4 mg) was reacted with Suc(II) (100 mg) in 14 ml of 0.5 *M* carbonate buffer (pH 8.9), and then the reaction mixture was divided into portions of 7 ml. The produced Suc(II)-FTC was purified by gel-filtration of portions with a Sephadex® G-50 column (3.5 cm in inner diameter × 30 cm in length) using 0.5 *M* carbonate buffer as an elution solvent and subsequent dialysis against water. Finally, Suc(II)-FTC was obtained as a powder by lyophilization. The FTC content of Suc(II)-FTC was calculated from the absorbance at 495 nm using a DU® 640

spectrophotometer (Beckman, USA) in PBS by referring to the absorption coefficient of FITC at 495 nm in the same buffer. The FTC content of Suc(II)-FTC was 0.1 % (w/w).

At 9 d after inoculation, Suc(II)-FTC was administered intravenously or intraperitoneally at a dose of 1 mg per mouse. The mice were sacrificed at 1, 8 and 24 h after injection, blood samples were withdrawn and several tissues (tumor, liver, kidneys, spleen and lung) were excised. The tissues were homogenized using a glass homogenizer with a Teflon pestle. The tissue homogenate was diluted with PBS, and the supernatant was obtained by centrifugation. Plasma was obtained by centrifugation of the blood. The supernatant and plasma were diluted appropriately with PBS, and their fluorescence intensities were investigated (Ex=495 nm, Em=520 nm). The concentrations and amounts of Suc(II)-FTC distributed in the blood and tissues were calculated from these fluorescence intensities and the recovery ratios for blood and each tissue.[92] Further, the urine was collected for 8 or 24 h, and diluted appropriately with PBS. Its fluorescence intensity was measured under the same conditions as described above to determine the amount of Suc(II)-FTC excreted into urine.

3-2-5. Antitumor effect of Suc(II)-MMC conjugate against Sarcoma 180

First, Suc(II)-MMC was prepared in the same manner as reported previously.[104] Briefly, Suc(II) (100 mg) and MMC (40 mg) were dissolved in water (25 ml), EDC (100 mg) dissolved in water (5 ml) was added, and then pH of the mixture was adjusted to 6.1 by addition of 0.1 *N* HCl aqueous solution. The solution was stirred at room temperature for 15 min. The water-soluble conjugate was separated from free MMC and EDC on a Sephadex® G-50 column (2.6 cm in inner diameter × 14 cm in length) using 0.1 *M* NaCl aqueous solution as an elution solvent. The eluted solutions at the high MW fractions were mixed, concentrated by evaporation of solvent and washed with saline using an ultrafilter unit USY-5 with a MW cut-off limit of 50,000 (Toyo Roshi, Japan). The product solution (conjugate solution) in saline was used for the following experiments. It was diluted 100-fold with 1/15 *M* phosphate buffer (pH 7.0) for the determination of MMC content. The diluted solution

was measured spectrophotometrically at 363 nm, and the concentration of MMC in the conjugate solution was calculated from a standard calibration curve of MMC. The weight of the conjugate contained in 1 ml of the conjugate solution was measured after removing the salt and subsequent freeze-drying. The drug content (%, w/w) was calculated based on MMC concentration and the amount of conjugate contained in 1 ml of the conjugate solution in saline.

Antitumor effect was examined using the mice at 5 d after inoculation. Namely, at 5 d after inoculation, MMC was intravenously administered at a single dose of 5 mg/kg, and Suc(II)-MMC was intravenously administered at a single dose of 10 mg eq. MMC/kg. Further, MMC was intravenously administered repeatedly at a dose of 5 mg/kg at 5, 9 and 13 d after inoculation, i.e. at a dose of 3 × 5 mg/kg, and Suc(II)-MMC was intravenously administered repeatedly at a dose of 10 mg eq. MMC/kg at 5, 9 and 13 d after inoculation, i.e. at a dose of 3 × 10 mg eq. MMC/kg. Controls were injected with a similar volume of saline alone according to the same schedules. Tumor volume was investigated in all mice. The tumor volume immediately before administration was used as the initial volume, and that of each group was measured for 30 d after administration. The ratio of the tumor volume to the initial volume was examined after administration. The length (L, cm) of the tumor major axis (longest axis), and width (W, cm) of the minor axis (vertical to the major axis) were measured with slide calipers, and the tumor volume (V, cm^3) was calculated as follows based on the report by Takakura et al.:[86]

$$V=L \times W^2/2 \qquad (1)$$

At the same time, the changes in body weight of each group were measured to evaluate the toxic side effects.

Next, at 5 d after inoculation, MMC and Suc(II)-MMC were administered intraperitoneally to the tumor-bearing mice at the same dose and according to the same schedule as in the i.v. administration experiment described above. Antitumor and toxic side effects were examined in the same way as described for the i.v. administration experiment.

3-2-6. Disposition of Suc(II)-MMC conjugate into blood circulation and tumor

In this experiment, mice at 9 d after inoculation were used. Suc(II)-MMC (10 mg eq. MMC/kg) was injected intravenously or intraperitoneally. The animals were sacrificed at 8 h after injection, a blood sample was withdrawn, and the tumor was excised. Plasma was obtained by centrifugation of the blood. One *M* sodium carbonate buffer (pH 9.0) was added to the tumor by the three-fold volume, and the mixture was homogenized using a glass homogenizer with a Teflon pestle.

Free MMC contained in the sample solution (pH 9) was extracted according to the method of Den Hartigh et al.[93] This operation allowed the complete recovery of MMC from the sample. Briefly, one milliliter of a mixture of chloroform and isopropanol (1 : 1, v/v) was added to 0.1 ml of plasma sample or homogenate. The obtained mixture was shaken vigorously for 3 min, then centrifuged to obtain the supernatant. All the supernatant was transferred to a glass tube and evaporated to dryness at 35 °C under nitrogen gas. The residue was dissolved in 100 μl of methanol, and the resultant solution was analyzed by HPLC to determine free MMC in the sample solution.

To determine the total amounts of free and conjugated MMCs in the sample solution, the solution was heated at 90 °C for 5 min. Then, 1 ml of a mixture of chloroform and isopropanol (1 : 1, v/v) was added to aliquots of 0.1 ml of the treated sample, and the mixture was shaken vigorously for 3 min. After centrifugation, all the supernatant obtained was transferred to a glass tube and evaporated to dryness at 35 °C under nitrogen gas. The residue was dissolved in 100 μl of methanol, and the resultant solution was analyzed by HPLC to determine the total amounts of free and conjugated MMC in the sample solution. The amount of conjugated MMC was calculated by subtracting the amount of free MMC from that of total MMC.

3-2-7. HPLC assay

HPLC analysis was performed at room temperature. Twenty μl of the final solution in methanol was subjected to HPLC using a Shimadzu LC-6AD apparatus equipped with a

SUMIPAX Nucleosil $5C_{18}$ reversed phase column (4 mm in inner diameter × 250 mm in length) and an SPD-10AV UV detector (Shimadzu) set at 365 nm. The mobile phase was a mixture of 0.01 *M* phosphate buffer, pH 6.0, and methanol (65 : 35, v/v).

3-2-8. Viscometric analysis

Suc(II)-FTC and Suc(II)-MMC were examined for the viscosity of their solution using an Ubbelohde viscometer (Shibata, Japan) according to JPXIII. The viscometry was performed at 37 ± 0.1 °C. Namely, the solution of Suc(II)-FTC (0.33 mg/ml) or Suc(II)-MMC (0.33 and 0.67 mg/ml) in saline was prepared and kept at 37 ± 0.1 °C. The solution (13 ml) was poured into the viscometer fixed in a container, and then the time (t) for the surface of the solution to move between the specified lines of the viscometer was measured. The kinematic viscosity (ν) was calculated by the following equation:

$$\nu = K \times t \tag{2}$$

where K was a viscometer constant (cSt/s).

3-2-9. Statistical analysis

The significant difference was checked using the Student's t-test. The data were evaluated to be significantly different when the p-value was less than 0.05.

3-3. Results and discussion

3-3-1. Body distribution of Suc(II)-FTC

Suc(II)-FTC contained FTC at 0.1 % (w/w). This lower introduction of FTC into Suc(II) was considered because very few free amino groups existed in Suc(II). However, in spite of the low FTC content, there was no problem in determination of the tissue concentration. Figure 3-1 shows the biodistribution at 1, 8 and 24 h after i.v. administration of Suc(II)-FTC. The concentration was expressed as the percentage of dose per ml of

plasma or per g of tissue. Suc(II)-FTC was sustained at a high level in blood circulation but little distributed in tissues other than the tumor. The distribution of Suc(II)-FTC in tumor tissue reached approximately 15 % of dose/g at 24 h after injection. The tumor level of Suc(II)-FTC was close to that in the plasma at 24 h after i.v. injection; their concentrations at that time were not significantly different (p>0.05). These findings indicated that Suc(II)-FTC could accumulate in the tumor tissue similarly to other FITC-labeled Suc with succinylation degrees of 0.51 mole/sugar unit[37] and 0.81 mole/sugar unit.[92] Namely, it was demonstrated that further succinylation of *N*-succinyl-chitosan to 1.1 mol/sugar unit little influenced its biodisposition characteristics.

Figure 3-2 shows biodistribution at 1, 8 and 24 h after i.p. administration of Suc(II)-FTC. The concentration was expressed as the percentage of dose per ml of plasma or per g of tissue. At 1 h after i.p. administration, the plasma concentration was much lower than that by i.v. administration, and after 8 and 24 h, it was a little lower than that by i.v.

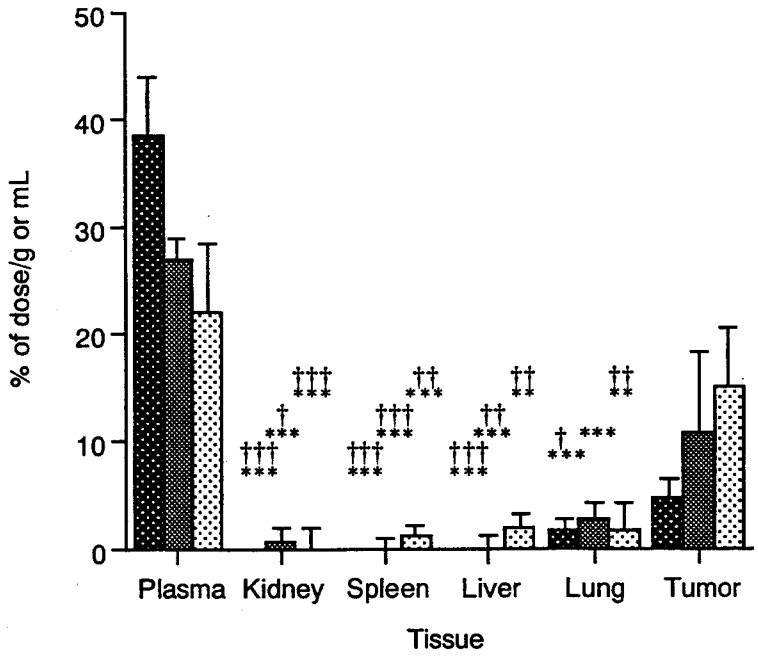


Figure 3-1. Plasma concentration and tissue distribution of Suc(II)-FTC after i.v. administration at a dose of 1 mg (0.2 ml) per Sarcoma 180-bearing mouse: ▩, 1 h; ▩, 8 h; ▩, 24 h. Each column represents the mean ± S.D. (n=5). *: p<0.05, **: p<0.01, ***: p<0.001 vs. plasma. †: p<0.05, ††: p<0.01, †††:p<0.001 vs. tumor.

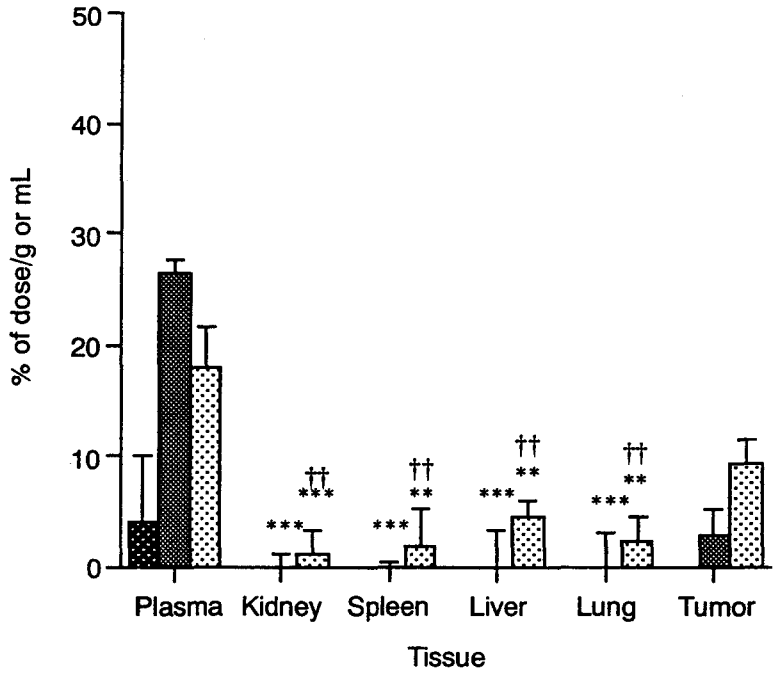


Figure 3-2. Plasma concentration and tissue distribution of Suc(II)-FTC after i.v. administration at a dose of 1 mg (0.2 ml) per Sarcoma 180-bearing mouse: ▩ , 1 h; ■ , 8 h; ⁙ , 24 h. Each column represents the mean ± S.D. (n=5). **: $p<0.01$, ***: $p<0.001$ vs. plasma. ††: $p<0.01$ vs. tumor.

administration. The amounts distributed in other tissues following i.p. administration were small, but the pattern differed slightly from that after i.v. administration: Suc(II)-FTC administered intraperitoneally tended to be distributed in a somewhat lower amount to the tumor tissue, but in a somewhat higher amount to the tissues other than plasma and the tumor tissue.

3-3-2. Urinary excretion of Suc(II)-FTC

Table 3-1 shows urinary excretion at 8 and 24 h after the i.v. and i.p. administration of Suc(II)-FTC. The amount was expressed as a percentage of dose. The urinary excretion was very low by both administration routes. The urinary excretion of Suc(II)-FTC tended to be lower at i.p. than at i.v. administration, although the difference in the excreted amount between the two administration routes was not significant at any time point examined ($p>0.05$). This suggested that transfer of Suc(II)-FTC from the intraperitoneal part into the

Table 3-1.
Urinary excretion of Suc(II)-FTC at 8 h and 24 h after i.p. administration in Sarcoma 180-bearing mice

Administration route	Urinary excretion (% of dose)[a)]	
	8 h	24 h
I.v.	5.0 ± 2.3	9.4 ± 4.1
I.p.	3.0 ± 2.1	4.9 ± 3.4

a) Each value represents the mean ± S.D. (n=5).

blood circulation might not be complete, which was supported by the plasma levels of Suc(II)-FTC after i.p. and i.v. injections (Figs. 3-1 and 3-2). These results also proposed that the localization of Suc(II)-FTC in peripheral tissues might be greater and continue for longer periods at i.p. than at i.v. administration.

As to FITC-labeled Suc with similar MW and succinylation degree of 0.81 mole/sugar unit, its excretion to the feces was hardly observed at 8 h after injection to normal mice, and a few dozen percent was thought to be localized to the parts other than lung, liver, spleen, kidney, urine and feces (data not shown). In the present study, the total recovery of Suc(II)-FTC from the tested tissues and urine was near or less than 50 % of dose at 8 and 24 h after injection. Although the excretion to the feces was not checked, the fair distribution to the tissues other than those tested was thought probably due to the similarity of the biodisposition among Sucs with succinylation degrees of 0.51 – 1.10 mole/sugar unit.

3-3-3. Pharmacokinetic analysis of Suc(II)-FTC biodisposition

Since the number of the sampling time points was small, the clearance of Suc(II)-FTC from the systemic circulation and the translocation of Suc(II)-FTC from the site of injection to the blood circulation cannot be discussed in detail. However, the plasma concentration profile at i.v. administration appeared to be approximated to the linear decline. Also, the plasma concentration profile at i.p. administration seemed to show the simple transfer from

the injected site to the blood and subsequent elimination from the systemic circulation. The transfer from the injected site to the systemic circulation was analyzed with a linear translocation model described in Figure 3-3(A) by referring to Yamaoka et al.[107] The mean values of the concentrations or amounts located in the plasma or tissues were used for analysis.

The plasma concentration (C_{iv}) of Suc(II)-FTC at the time t after i.v. injection was analyzed by the following equation of mono-exponential decline:

$$C_{iv} = (D/V_d) \times \exp(-k_e t) \quad (3)$$

where D, V_d and k_e were dose, distribution volume and elimination rate constant, respectively. Further, the plasma level (C_{ip}) of Suc(II)-FTC at the time t after i.p. injection could be obtained by the analysis using a linear translocation model as follows:

$$C_{ip} = (DFk_a/(V_d \times (k_e - k_a))) \times (\exp(-k_a t) - \exp(-k_e t)) \quad (4)$$

where F and k_a were transfer efficiency and transfer rate constant from the peritoneal cavity to the blood circulation, respectively. First, profile fitting was performed to the profile observed after i.v. administration using equation 3 to calculate V_d and k_e. Next, F and k_a were calculated by profile fitting using equation 4 with V_d and k_e being fixed. The calculation was performed using the non-linear least squares program MULTI.[96] Further, as to each tissue, average tissue clearance (CL_{tissue}) for 0 – 24 h was estimated by dividing the amount located in each tissue after 24 h (T_{tissue}(24 h)) by the area under the plasma concentration-time curve for 0 – 24 h ($AUC_{0\text{-}24\,h}$) as shown in the following equation:[86]

$$CL_{tissue} = T_{tissue}(24\text{ h}) / \int_0^{24h} C\,dt \quad (5)$$

where equations 3 and 4 were utilized for calculation of $AUC_{0\text{-}24\,h}$.

The calculated pharmacokinetic parameters are described in Tables 3-2 and 3-3, and the plasma concentration profiles were simulated based on equations 3 and 4 (Figure 3-3(B)). The calculated profiles were well fitted to those observed. The transfer efficiency (F) from the peritoneal cavity to the blood circulation was fairly large (0.871), suggesting that transfer from the site of injection to the bloodstream should be incomplete but relatively high. The elimination rate constant (0.0253 h^{-1}) was very small. The clearance from plasma, CL_p, was

much larger than average tissue clearances of each tissue tested; these clearances were large in tumor, urine and liver with tumor exhibiting the largest clearance per unit weight. Average tissue clearances were a little higher following i.p. administration as compared with i.v. administration in all the tested sites except tumor tissue and urine. Translocation via routes other than the blood circulation may occur following i.p. administration, however, the contribution of other routes seemed small. Transfer to tumor tissue and urine paralleled plasma concentration. Overall, the biodisposition of Suc(II)-FTC at i.p. administration still appeared to be dominated primarily by the plasma level.

3-3-4. Antitumor effect of Suc(II)-MMC against Sarcoma 180

First, at 5 d after inoculation, MMC and Suc(II)-MMC were injected intravenously at a single dose or triple doses at 4-d intervals. The tumor growth profiles after i.v. administration are shown in Figure 3-4. Marked tumor growth was observed in the control mice. The MMC group exhibited good tumor growth suppression, but the dose of 3 × 5 mg/kg was lethal. Suc(II)-MMC showed good growth suppression at 3 × 10 mg eq.

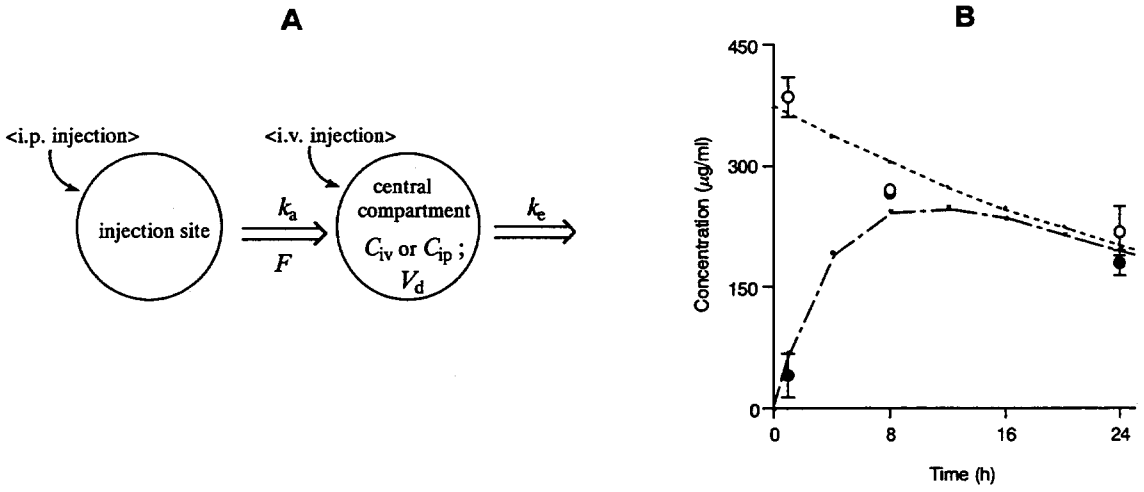


Figure 3-3. Translocation model of Suc(II)-FTC (A) and its plasma concentration profiles (B) after i.v. and i.p. administration: (A) k_a and k_e : first order rate constant, F: transfer efficiency from injection site to blood circulation, C_{iv} and C_{ip} : plasma concentration, V_d: distribution volume; (B) ○ : i.v. injection (mean ± S.E. (n=5)), ● : i.p. injection (mean ± S.E. (n=5)), - - - - : calculated i.v. profile, ·—·—·— :calculated i.p. profile.

Table 3-2.
Plasma pharmacokinetic parameters of Suc(II)-FTC after i.v. and i.p. administration

Parameter[a)]	Estimated value	Unit
Dose	1.00×10^3	μg per mouse
V_d	2.68×10^3	μl per mouse
k_e	0.0253	h^{-1}
k_a	0.245	h^{-1}
F	0.871	——
AUC_{calc}(i.v.; 0 - 24 h)	6.72	$\mu g \times h/\mu l$
AUC_{calc}(i.p.; 0 - 24 h)	5.04	$\mu g \times h/\mu l$
CL_p	67.8	μl/h per mouse

a) V_d, k_e, k_a and F were calculated based on the mean plasma concentrations of Suc(II)-FTC. CL_p is the clearance from plasma and estimated as ($k_e \times V_d$).

Table 3-3.
Average tissue clearances of Suc(II)-FTC for 0 - 24 h after i.v. and i.p. administration

Tissue	i.v.		i.p.	
	μl/h per mouse	μl/h per g tissue	μl/h per mouse	μl/h per g tissue
Kidney	0.0	0.0	1.52	2.38
Spleen	0.422	1.83	0.759	3.94
Liver	5.14	3.07	14.6	9.12
Lung	0.585	2.77	0.927	4.56
Tumor	14.0	22.3	10.4	18.5
Urine	14.0		9.77	

MMC/kg, similar to MMC at 5 mg/kg. Suc(II)-MMC showed a slow drug release as reported previously.[104] Further, the conjugate taken up by the tumor was considered to regenerate MMC there more slowly due to the weakly acidic lysosomal pH. Such a slow

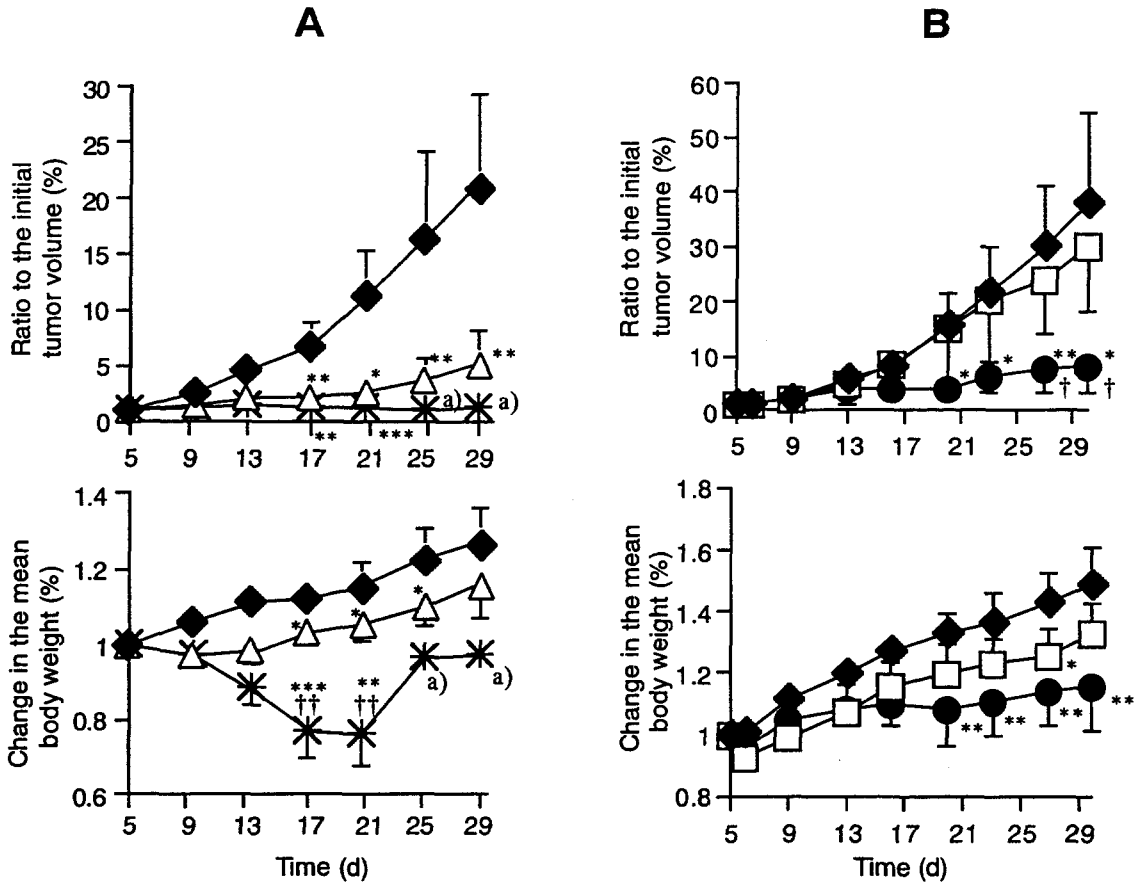


Figure 3-4. ***In vivo*** **growth inhibitory effect against Sarcoma 180 and changes in the body weight of Sarcoma 180-bearing mice after i.v. administration of MMC (A) and Suc(II)-MMC (B):** —◆—, Control; —△—, MMC: 5 mg/kg; —✳—, MMC: 3 × 5 mg/kg; —□—, Suc(II)-MMC: 10 mg/kg; —●—, Suc(II)-MMC: 3 × 10 mg/kg. Sample solution was injected at 5 d after inoculation (single dose), and at 5, 9 and 13 d after inoculation (triple dose). Each point represents the mean ± S.D. (n=5). a) Four mice died (n=1). *:$p<0.05$, **:$p<0.01$, ***:$p<0.001$ vs. control. †:$p<0.05$, ††:$p<0.01$ vs. single dose.

drug supply was considered to be the reason the higher dose of the conjugate was required to achieve good efficacy. No lethal toxicity of Suc(II)-MMC was observed even at the dose of 3× 10 mg eq. MMC/kg.

Next, MMC and Suc(II)-MMC were injected intraperitoneally at a single dose and at triple doses at 4-d intervals at 5 d after inoculation. The profiles of tumor growth and body weight change after i.p. administration are shown in Figure 3-5. The results were similar to those after i.v. administration stated above; however, at i.p. administration, lethal toxicities were observed both at the dose of 3 × 5 mg/kg for MMC and at the dose of 3 × 10 mg eq.

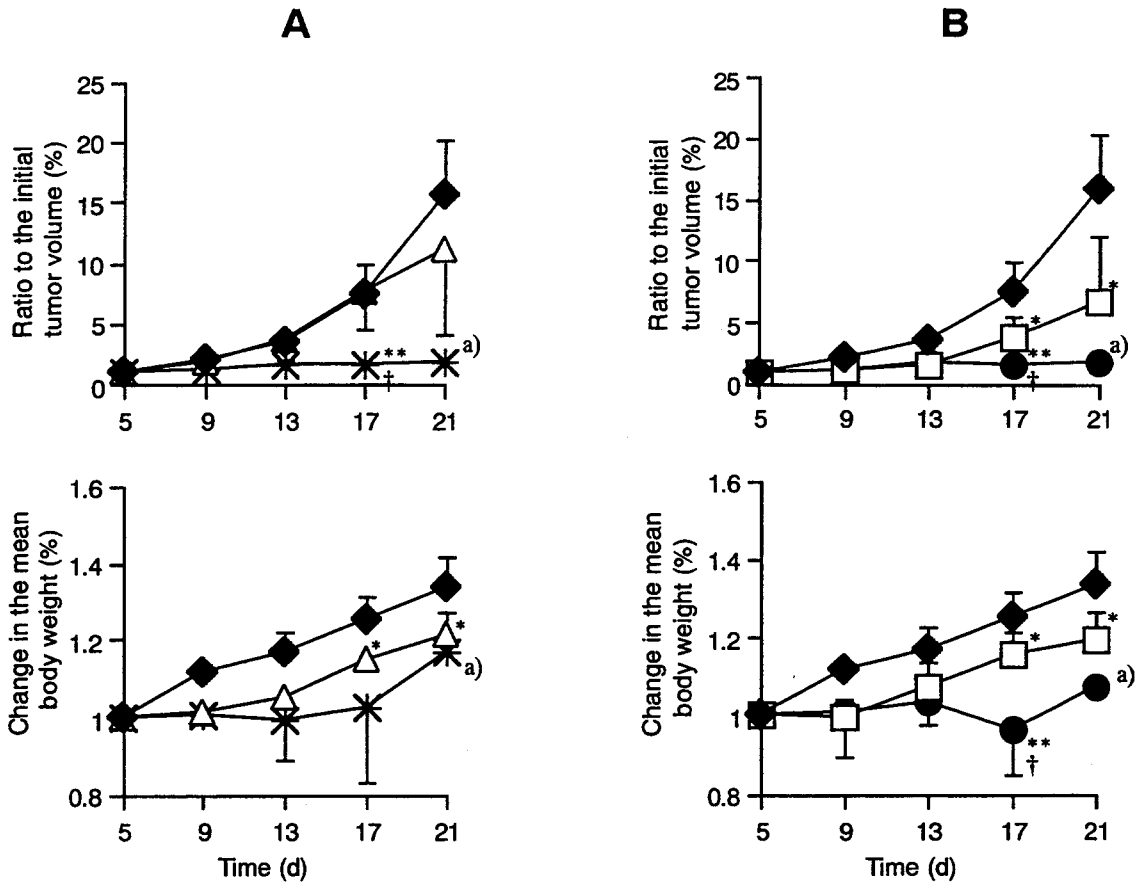


Figure 3-5. ***In vivo*** **growth inhibitory effect against Sarcoma 180 and changes in the body weight of Sarcoma 180-bearing mice after i.p. administration of MMC (A) and Suc(II)-MMC (B):** —◆—, Control; —△—, MMC: 5 mg/kg; —✳—, MMC: 3 × 5 mg/kg; —□—, Suc(II)-MMC: 10 mg/kg; —●—, Suc(II)-MMC: 3 × 10 mg/kg. Sample solution was injected at 5 d after inoculation (single dose), and at 5, 9 and 13 d after inoculation (triple dose). Each point represents the mean ± S.D. (n=5). a) Four mice died (n=1). *: $p<0.05$, **: $p<0.01$ vs. control. †: $p<0.05$ vs. single dose.

MMC/kg for Suc(II)-MMC. The lethal toxic side effect of Suc(II)-MMC following i.p. administration suggested that this might be more toxic than i.v. administration. This may be related to the greater peripheral localization or less elimination of the polymer support after i.p. administration as described above in the biodistribution studies of Suc(II)-FTC.

3-3-5. Translocation of MMC and Suc(II)-MMC to plasma and tumor

At 8 h after i.v. injection of free MMC at the dose of 5 mg/kg, the plasma level of MMC was less than the detection limit 0.04 μg/ml (data not shown). This indicated MMC should be eliminated quickly from the systemic circulation. On the other hand, at 8 h after i.v. and

i.p. administration of Suc(II)-MMC at 10 mg eq. MMC/kg, both conjugated and free MMCs were observed at levels much higher than the detection limit (Figure 3-6). It qualitatively indicated that Suc(II)-MMC was well retained in the plasma and that the translocation of the MMC and conjugate from the intraperitoneal part to the blood circulation was caused. However, the levels of conjugated and free MMCs following i.p. administration were approximately one-third of those after i.v. administration, respectively, suggesting that Suc(II)-MMC might not exhibit the same translocation rate or profile as Suc(II)-FTC (Figs. 3-1 to 3-3). Further, when the tumor level of MMC at 8 h after i.v. and i.p. administration of Suc(II)-MMC was checked in a similar manner after homogenization, free MMC and conjugated MMC were hardly detectable (data not shown). This also was not in accord with the results in Suc(II)-FTC which clearly exhibit the localization of the polymer support to the tumor. One of the reason might be the rapid clearance of MMC and Suc(II)-MMC in the tumor; this speculation will have to be confirmed by an investigation such as a stability study in the tissue. Another reason might be the difference in the experimental conditions. Namely, Suc(II)-FTC was examined at the i.v. injection of 1 mg per mouse, while Suc(II)-MMC was used at approximately 2 mg per mouse, which indicated that Suc(II)-MMC should exist in a more viscous condition in the body. This more viscous condition might affect the tissue localization including EPR effect. Considering their high localization in the plasma, Suc(II)-FTC and Suc(II)-MMC were investigated for the viscosity of their solution. As a result, the viscosity of Suc(II)-FTC solution in saline was 0.85 cSt at 0.33 mg/ml at 37 °C, while that of Suc(II)-MMC solution in saline was 0.87 and 0.97 cSt at 0.33 and 0.67 mg/ml at 37 °C, respectively. Further, Suc(II)-MMC was not necessarily equivalent to Suc(II)-FTC chemically or physicochemically, for example, in chemical structure and MW. These might make the disposition behavior of Suc(II)-MMC different from that of Suc(II)-FTC. As to the antitumor effect of Suc(II)-MMC, the slight but prolonged supply of MMC from systemic circulation to the tumor site was believed to play a role in the efficacy, and it might be importantly related to the effect because little MMC or Suc(II)-MMC was detected in the tumor as stated above.

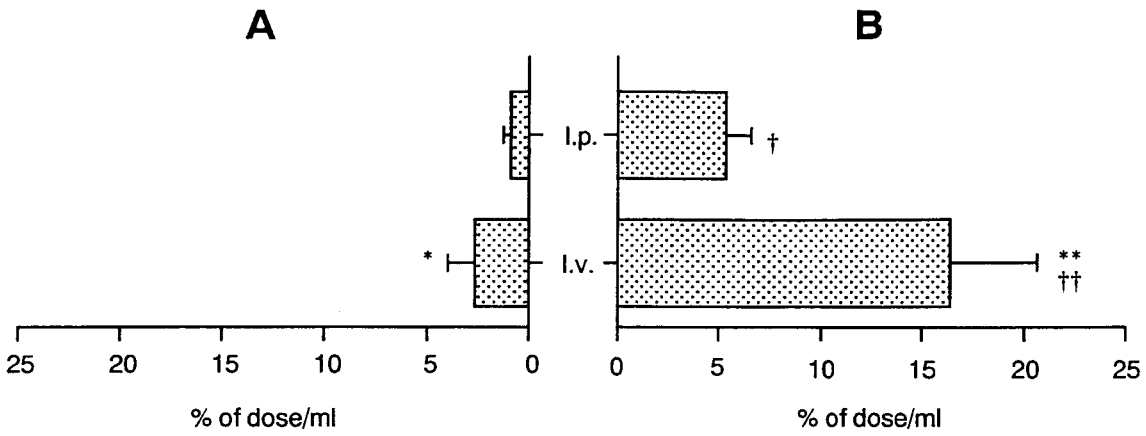


Figure 3-6. Plasma concentrations of free (A) and conjugated (B) MMCs at 8 h after i.v. and i.p. administration of Suc(II)-MMC at 10 mg eq. MMC/kg in mice: Each column represents the mean ± S.D. (n=4). *: $p<0.05$, **: $p<0.01$ vs. i.p. injection. †: $p<0.05$, ††: $p<0.01$ vs. free MMC.

The difference in the administration route influenced the biodisposition properties of Suc(II); transfer of Suc(II) into the blood circulation following i.p. administration took a few hours and seemed high but not complete. The urinary excretion of Suc(II) tended to be lower following i.p. than i.v. administration. Therefore, the efficacy of Suc(II)-MMC was considered to depend on the administration route. Suc(II)-MMC exhibited a high antitumor effect at a high dose by both i.v. and i.p. injections, but tended to be less toxic following the former. These results suggested that there might be greater invasion of Suc(II)-MMC into peripheral tissues following i.p. than following i.v. administration. Thus, it was proposed that i.v. administration would be preferable for antitumor chemotherapy by Suc(II)-MMC against subcutaneous Sarcoma 180 solid tumor. The present study provides valuable information on the usage of Suc as a drug carrier of antitumor agents.

Chapter 4

Biological Characteristics of Lactosaminated *N*-Succinyl-chitosan as a Liver-specific Drug Carrier in Mice

Summary

Lac-Suc was prepared by reductive amination of Suc and lactose using sodium cyanoborohydride. Six-day reaction using lactose (12.8-fold (w/w)) yielded Lac-Suc with lactosamination degree of 30.1 % (mol/sugar unit). Lac-Suc-FTC was prepared by labeling Lac-Suc with FITC. Lac-Suc-FTC was injected intravenously at a dose of either 1 (high dose) or 0.2 (low dose) mg/mouse. At both doses, Lac-Suc-FTC initially underwent fast hepatic clearance, showed maximum liver localization at 8 h, and the amounts localized there were maintained even at 48 h post-injection. Very slow excretion into feces and urine was observed. The ratio of liver $AUC_{0\text{-}48\,h}$ to plasma $AUC_{0\text{-}48\,h}$ at low dose was three times higher than that at high dose. On the other hand, the Suc derivative, Gal-Suc, obtained by reductive amination of Suc/galactose showed very little distribution to the liver similarly to Suc itself. Further, since the liver uptake of Lac-Suc-FTC was inhibited by asialofetuin, it was suggested that the liver distribution of Lac-Suc should be concerned with asialoglycoprotein receptor. Thus, Lac-Suc was found available as a carrier exhibiting a high affinity to and long retention in the liver.

4-1. Introduction

Recently, there have been many studies of liver-targeting systems using methods such as passive trapping of microparticles by reticuloendothelium[38,41] or active targeting based on hepatic receptor recognition.[95,108-114] The biodisposition characteristics of the microparticles after i.v. administration are influenced extensively by their sizes and surface properties,[115] and their passive targeting to the liver is also dependent on particle diameter. On the other hand, active targeting utilizing receptor recognition can be attained using molecules with receptor-specific ligands. The biochemical characteristics of the hepatic asialoglycoprotein receptor, localized on the liver parenchymal cells, have been investigated in detail,[116-120] and this receptor has been utilized as a useful site for liver targeting by many researchers.[95,108-114] Since the galactose moiety is recognized specifically by the asialoglycoprotein receptor, macromolecules used as carriers for macromolecule-drug conjugates have often had a galactose moiety added, and the products have been examined for their usefulness as liver-specific drug carriers. For example, when galactose moieties were introduced into serum albumin,[108,109] *N*-(2-hydroxypropyl)methacrylamide copolymer,[110,111] asialofetuin[95] and poly-L-glutamic acid,[112] the products functioned well as liver-specific drug carriers.

Suc has been demonstrated to be highly safe[72,73] and to be available as a macromolecular drug carrier showing very long-term retention in the systemic circulation.[37,92] This long-term retention is based on the low biodegradability as well as high MW, and thus Suc is available for passive targeting to disease regions with the highly vascular permeation.[37,92] This property is also very useful for active targeting from the systemic circulation to the target site based on receptor recognition of ligands because the long-term circulation allows prolonged supply of conjugated drugs to the target site. Furthermore, when Suc is trapped by the target site cells, it is considered to remain longer in them due to low biodegradability; the long retention in the target cells is supposed to be responsible for the good action of Suc-drug conjugates. Such long retention effect may not be achieved in the case of proteins or synthetic polypeptides that are degraded relatively

quickly. Therefore, Suc is an attractive macromolecule for receptor targeting from the systemic circulation to the target site. Suc possesses many functional groups such as carboxyl groups and amino groups, which are considered to facilitate the introduction of galactose moieties. In this study, the introduction of lactose to Suc by reductive amination was attempted. The product, Lac-Suc, was labeled with FITC and the pharmacokinetics, i.e. biodistribution, elimination and excretion, of Lac-Suc-FTC were examined in mice. Furthermore, the specific binding of Lac-Suc to the asialoglycoprotein receptor of the liver was examined by the competitive binding study with asialofetuin.

4-2. Materials and methods

4-2-1. Materials

Suc (MW 3×10^5 (averaged); degree of *N*-succinylation 0.81 mol/sugar unit; degree of deacetylation 1.0 mol/sugar unit) was kindly donated by Katakura Chikkarin Co., Ltd. (Tokyo, Japan). β-Lactose (lactose), β-galactose (galactose), FITC, asialofetuin (type I), collagenase (type Ⅶ), bovine serum albumin (fraction V), HEPES buffer (1 *M*) were purchased from Sigma Chemical Company (St. Louis, U.S.A.). Hanks' balanced salt solution was purchased from Life Technologies, Inc., (MD, U.S.A.). Sodium cyanoborohydride was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). All reagents were of the highest grade available.

Suc (100 mg) was dissolved in 3 ml of 1/15 *M* phosphate buffer (pH 6.0) with stirring, and then 3 ml of methanol was added. To the solution was added lactose (1280 mg) dissolved in 1 ml of the same buffer. After stirring, sodium cyanoborohydride (320 mg) dissolved in 1 ml of the same buffer was added to the solution, followed by stirring at 400 rpm at room temperature for 6 d. After the reaction, methanol was removed from the mixture on a rotary evaporator under reduced pressure with slight warming in a water bath, and then separated on a Sephadex® G-50 column (3.5 cm in inner diameter × 10.5 cm in

length) using 1/15 *M* phosphate buffer as an elution solvent. The eluted solution at the high MW fractions were gathered and dialyzed for 3 d against water using Seamless Cellulose Tubing (MW cut off limit = 12,000 ~ 14,000; Viskase sales Corp., U.S.A.). Finally, lactosaminated Suc (Lac-Suc) was obtained by lyophilization of the dialyzed solution. The content of lactose residues in Lac-Suc was examined by elemental analysis (Yanako Analytical Industrial Co., Japan) after extensive drying at 80 °C in desiccator with P_2O_5 for 3 h. The degree of lactose in Lac-Suc could be calculated from the C/N ratio in elemental analysis as 0.30 mol/sugar unit.

Lac-Suc-FTC was prepared to monitor Lac-Suc in the body as follows: FITC (4 mg) dissolved in 4 ml of 0.5 *M* carbonate buffer (pH 8.94) was added to Lac-Suc (100 mg) in 10 ml of the same buffer, and the mixture was stirred at room temperature in the dark for 20 h. The product, i.e. Lac-Suc-FTC was purified by gel-filtration with a Sephadex® G-50 column (3.2 cm in inner diameter × 10.5 cm in length) using 0.5 *M* carbonate buffer as the elution solvent and subsequent dialysis against water. Finally, Lac-Suc-FTC was obtained as powder after lyophilization. The FTC content of Lac-Suc-FTC was calculated from the absorbance at 495 nm using a DU®640 spectrophotometer (Beckman, USA) in PBS based on the absorbance of FITC at 495 nm in the same buffer. The profiles for fluorescence quenching of Lac-Suc-FTC in aqueous solution or biological media were checked during the incubation period. The Lac-Suc-FTC solutions of 1 and 0.2 mg dissolved in 0.2 ml of PBS were put into the glass tubes shielded from the light, which included 1 ml of PBS or of 20 % (v/v) plasma in PBS, and the mixtures were incubated at 37 °C at 60 rpm.

4-2-2. Animals

Male C57BL/6 mice (6 weeks old, specific pathogen-free) weighing around 20 g were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Hoshi University. These animals were kept on the breeding diet MF (Oriented Yeast Co., Ltd., Tokyo) with water *ad libitum* in a room maintained at 23 ± 1 °C and

a relative humidity of 60 ± 5 %. They were used soon after purchase. Three animals were used in each point on experiments except for the recovery ratios of Lac-Suc-FTC from tissue homogenate and plasma (n = 3).

4-2-3. Body distribution studies

The body distribution of Lac-Suc-FTC was investigated after administration of Lac-Suc-FTC dissolved in normal saline at a dose of 1 or 0.2 mg per mouse. The recoveries of Lac-Suc-FTC from each tissue homogenate and plasma were determined in advance as reported previously.[92] The recoveries of Lac-Suc-FTC from each tissue homogenate and plasma were obtained as the mean of two experiments as follows: 108 % (liver), 87 % (spleen), 90 % (kidney), 77 % (lung), 130 % (feces) and 105 % (plasma); the difference of the individual data was very little. Normal mice received Lac-Suc-FTC via the lateral tail vein at a dose of 1 or 0.2 mg (0.2 ml) without anesthetization. The mice were sacrificed at 5 min, 0.5, 1, 8, 24 and 48 h after injection, blood samples were withdrawn and several tissues (liver, kidney, spleen and lung) were excised. Each organ except the blood was washed with PBS, gently blotted using filter paper and weighed. A three-fold volume of PBS was added, and the mixture was homogenized using a glass homogenizer with a Teflon pestle. The supernatant was obtained by centrifugation (3,000 rpm, 10 min). Plasma was obtained by centrifugation of the blood. The supernatant and plasma were diluted appropriately with PBS, and their fluorescence intensities were investigated (Ex = 495 nm, Em = 520 nm). The blank sample was obtained by injecting normal saline alone into mice instead of Lac-Suc-FTC solution, and blood and tissues not containing Lac-Suc-FTC were taken from the mice and treated in the same way. The diluted supernatant and plasma were measured fluorometrically under the conditions described above. The concentration of Lac-Suc-FTC in the sample was determined from the net fluorescence intensity obtained by subtracting the fluorescence intensity of the blank from that of each sample based on the standard calibration curve. The concentration was corrected by the recoveries stated earlier. The distributed amount was calculated from the concentration and tissue weight.

4-2-4. Pharmacokinetic analysis

In the initial phase (0 – 1 h post injection), the elimination rate constant (k_P) for the plasma concentration was estimated from the slope of the best-fitted mono-exponential decline curve, the distribution volume (V_d) was by dividing the dose by the concentration estimated at time = 0, and the plasma clearance (CL^{plasma}) was given by $V_d \times k_P$. The area under the plasma concentration-time curve for 0 - t h ($AUC_{0\text{-}t\ h}^{plasma}$), the area under the liver concentration-time curve for 0 - t h ($AUC_{0\text{-}t\ h}^{liver}$), the mean residence time given by the plasma concentration-time curve for 0 – t h ($MRT_{0\text{-}t\ h}^{plasma}$) and the mean residence time given by the liver concentration-time curve for 0 – t h ($MRT_{0\text{-}t\ h}^{liver}$) were calculated using the trapezoidal method. Further, as to the liver, average hepatic clearance for 0 - t h ($CL_{0\text{-}t\ h}^{liver}$) was estimated by dividing the amount located in the liver at time t (X_t^{liver}) by $AUC_{0\text{-}t\ h}^{plasma}$ as shown in the following equation:

$$CL_{0\text{-}t\ h}^{liver} = X_t^{liver} / AUC_{0\text{-}t\ h}^{plasma} \quad (1)$$

The initial liver uptake rate (v_L) was determined from the slope of the initial hepatic accumulation. Concerning the hepatic clearance stated above, the outflow from the liver was assumed to be negligible at early time points judging from general hepatic uptake kinetics based on asialoglycoprotein.[118-120] The elimination rate from the liver (k_L) was analyzed by assuming a terminal decrease following a mono-exponential decline. The half-lives in the plasma were calculated as 0.693/(elimination rate constant).

4-2-5. Urinary and fecal excretion studies

Cumulative collection of urine and feces was performed simultaneously in the biodistribution studies as described above. Animals were kept separately in metabolite cages immediately after administration. Urine and feces were collected for 8, 24 and 48 h after i.v. administration, and then urinary volume and fecal weight were measured. Each urine sample was filtered using a membrane filter (0.45 μm pore diameter). A nine-fold volume of PBS was added to feces, and the mixture was ultrasonicated at 28 kHz for 40 min. The

supernatant was obtained by centrifugation (3,000 rpm, 10 min). The supernatant and urine filtrate were diluted appropriately with PBS, and their fluorescence intensities were measured fluorometrically (Ex = 495 nm, Em = 520 nm). After the blank test, the concentration of Lac-Suc-FTC was measured from the net fluorescence intensity using a calibration curve in the same manner as in the distribution study. The total amounts excreted in urine and feces were calculated from the concentration and urinary volume or fecal weight.

4-2-6. Comparison of biodistributions of Suc, Lac-Suc and galactosaminated *N*-succinyl-chitosan (Gal-Suc)

Galactosaminated Suc (Gal-Suc) was also synthesized in the same manner as Lac-Suc, i.e. reductive amination, except using galactose instead of lactose. Gal-Suc was reacted with FITC to obtain FTC moiety-carrying Gal-Suc, abbreviated as Gal-Suc-FTC. Normal mice received Suc-FTC or Gal-Suc-FTC via the lateral tail vein at a dose of 0.2 mg (0.2 ml) with no anesthetization, and were kept separately in metabolite cages immediately after administration. The mice were sacrificed at 8 h after injection, blood samples were collected and several tissues (liver, kidney, spleen and lung) were removed. Furthermore, urine and feces were collected for 8 h after administration, and then urinary volume and fecal weight were measured. The following procedures and measurements were performed in the same way as described in the body distribution studies and urinary and fecal excretion studies.

4-2-7. Competitive liver uptake

In vivo accumulation to the liver: Lac-Suc-FTC and asialofetuin[121,122] in saline were co-administrated intravenously to mice. Namely, the dose of asialofetuin was 0, 0.1, 1 and 10 mg per mouse, while Lac-Suc-FTC was injected at a constant dose of 0.1 mg per mouse. At appropriate time after injection, the mice were sacrificed, blood samples were collected and liver was enucleated. The concentration of Lac-Suc-FTC in the plasma and liver was determined in the same manner as described in Section 4-2-3.

Liver perfusion studies: Lac-Suc-FTC and asialofetuin in saline were co-administered

intravenously to mice in the same dose as described above. At 60 min after administration, mice were laparotomized, and the portal vein, vena cava and aorta were cut. The liver was perfused with PBS through vena cava and aorta for 8 min and then with 0.1 *M* HEPES buffer containing collagenase at 0.05 % (w/v) for 8 min. The perfusion rate was kept at 3 – 4 ml/min. After the perfusion was finished, the liver was removed from the mice. As to the isolation of liver cells, the method by Nilsson and Berg[122] was modified and adapted. Briefly, the cells were suspended in 20 ml of 0.1 *M* Hanks'-HEPES buffer containing 0.1 % BSA, referred to hereafter as H-H buffer, by gentle stirring at 4 °C overnight. The initial cell suspension was filtered through four layers of gauze (Nishio eizai Co., Ltd., Japan). The cell number was counted by microscopy (Olympus BX50F, Japan). The cell suspension was then centrifuged at 500 rpm for 2 min. The supernatant, containing most of the non-parenchymal cells (NPC), was first centrifuged further for 2 min at 500 rpm to remove parenchymal cells (PC). Cells remaining in the supernatant were then centrifuged at 1750 rpm for 3 min. The pellet was resuspended in H-H buffer and washed by centrifugation for 3 min at 1750 rpm. The pellet was resuspended in H-H buffer and was again centrifuged at 500 rpm for 2 min. The supernatant was sedimented at 1750 rpm for 3 min, and the pellet (NPC) was finally resuspended in 3 ml of PBS. On the other hand, the first pellet containing most of PC was washed twice by centrifugation at 500 rpm for 2 min. The pellet (PC) was finally resuspended in 3 ml of PBS. The cell number in final suspension of PC or NPC was again counted by microscopy, and the recovery ratio of cell number was calculated from those before and after isolation. Each final cell suspension was homogenized, and the concentration of Lac-Suc-FTC in the suspension was determined fluorometrically as described in Section 4-2-3. The concentration of Lac-Suc-FTC in the sample was corrected by the recovery ratio of cell number to obtain the distributed concentration in PC and NPC of the initial liver.

4-2-8. Statistical analysis

Student's *t*-test was used to determine significant difference. Differences were

considered significant when the p value was less than 0.05.

4-3. Results

4-3-1. Body distribution of Lac-Suc-FTC

The synthetic approach of Lac-Suc is illustrated in Figure 4-1. Lactose is a disaccharide that consists of galactose and glucose. Amino groups of Suc were combined with an aldehyde group of the glucose moiety of lactose, and the Schiff bases formed were reduced by cyanoborohydride into secondary amino groups,[122] which meant that the glucose moiety was changed into another structure. Therefore, as shown in Fig. 4-1, only the

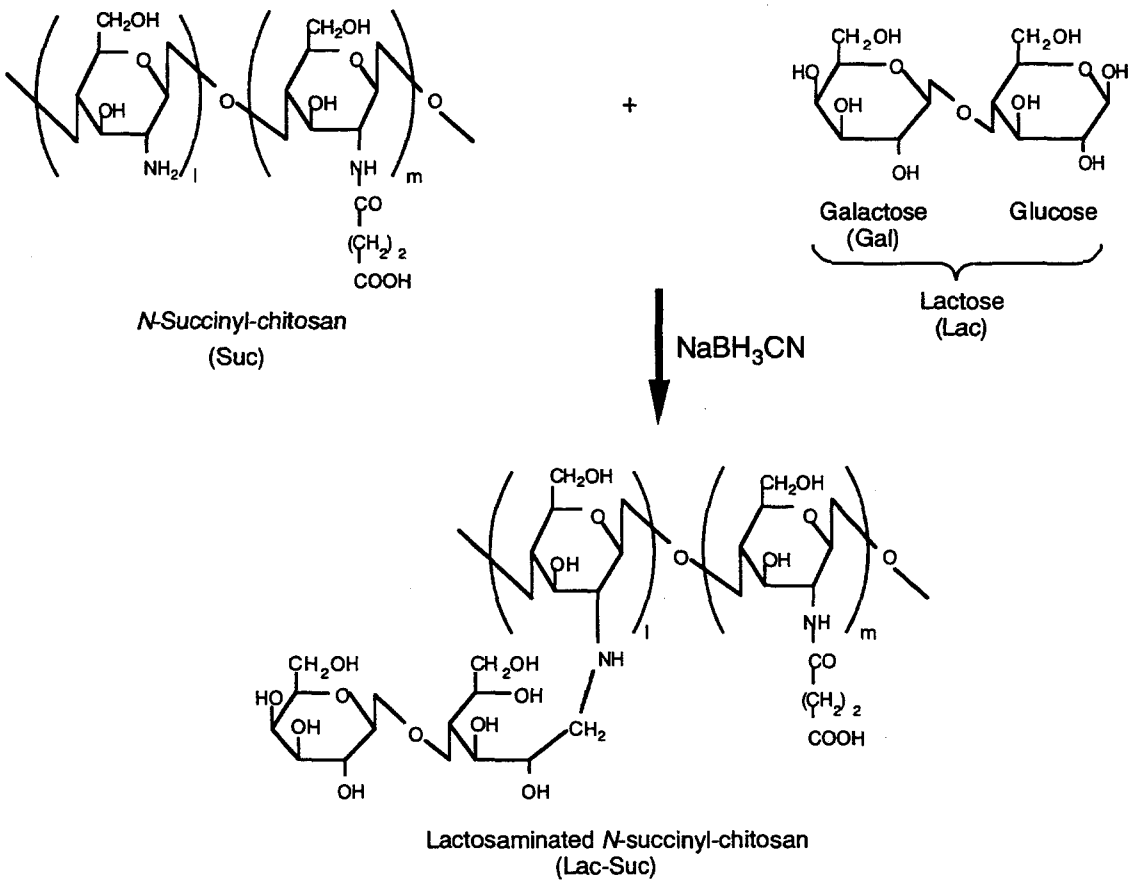


Figure 4-1. Synthetic scheme of Lac-Suc

structure of galactose remained intact.

The long exposure of fluorescent molecules in solution results in the possibility of quenching. Therefore, elucidation of this property was considered necessary for quantification based on the fluorescent moiety. The fluorescence intensity of Lac-Suc-FTC in PBS alone was quenched to around 90 % after 24 h incubation and to around 80 % after 48 h. However, in 20 % (v/v) plasma, no quenching was observed after 24 h incubation, and quenching was seen very little even after 48 h incubation (data not shown). From these results, the quenching effect was considered to be negligible for *in vivo* quantitative analysis of biodistributed Lac-Suc-FTC in this experiment. The bond between Lac-Suc and fluorescein moiety was considered to be stable in the body by referring to the previous results.[37] Namely, when Suc-FTC was administered into mice, only Suc-FTC was observed in urine and plasma but no free fluorescein was detected there; further, since free fluorescein itself was quite susceptible to urinary excretion, non-detection of free fluorescein suggested that its liberation should not be caused.[37] Therefore, liberation of free fluorescein from Suc-FTC was considered to be negligible in the body. Similarly, Lac-Suc-FTC obtained in the same labeling method was considered not to relieve fluorescein in the body.

Figure 4-2(A) shows the time course of biodistribution of Lac-Suc-FTC after i.v. administration at a dose of 1 mg per mouse. The results were expressed as the percentage of dose in plasma or each tissue. The elimination from plasma occurred much faster compared with that reported previously for Suc-FTC.[92] Namely, Lac-Suc-FTC had almost completely disappeared from blood circulation at 24 h after injection, while Suc-FTC was retained at 34 % of dose in plasma at 24 h post-injection. The distribution of Lac-Suc-FTC in the liver rapidly reached approximately 12.6 % (w/w) of the dose administered at 1 h post-injection, and reached the maximum of 16.6 % (w/w) of the dose administered at 8 h post-injection. Lac-Suc-FTC was sustained in the liver at a similar high level, and 14.0 % (w/w) of the dose administered remained even at 48 h post-injection. On the other hand, Lac-Suc-FTC was present at very low levels or not at all in other tissues throughout the experimental period.

Figure 4-2(B) shows the time course of changes in body distribution of Lac-Suc-FTC

after i.v. administration at a dose of 0.2 mg per mouse. The results are expressed as the percentage of dose in plasma or each tissue. Lac-Suc-FTC was eliminated almost completely from plasma within 8 h post-injection. The amount of Lac-Suc-FTC distributed in the liver rapidly increased and reached 22.3 % (w/w) of dose at 1 h post-injection, and reached the maximum of 23.0 % (w/w) of dose at 8 h after injection. The level of Lac-Suc-FTC in the liver was then gradually reduced to 10.4 % (w/w) of dose at 48 h post-injection. The amounts in other tissues were very low. Thus, the elimination rate from plasma was more rapid at 0.2 mg than at 1 mg per mouse, and the ratio of Lac-Suc-FTC distributed in the liver to the dose was higher at 0.2 mg than at 1 mg per mouse. However, as to elimination from the liver, Lac-Suc-FTC underwent faster reduction at 0.2 mg than at 1 mg per mouse. As to the sensitivity in this fluorometrical analysis, for example, it was possible to detect the concentration of at least 0.2 μg/ml or g for plasma and liver, indicating sensitivity of less than 0.1 % of dose/ml or g at the dose of 0.2 mg per mouse in them.

4-3-2. Pharmacokinetic analysis

Figure 4-3(A) shows the semi-logarithmic plots of plasma concentration of Lac-Suc-FTC. Approximation to first-order decrease kinetics provided elimination rate constants (k_P)

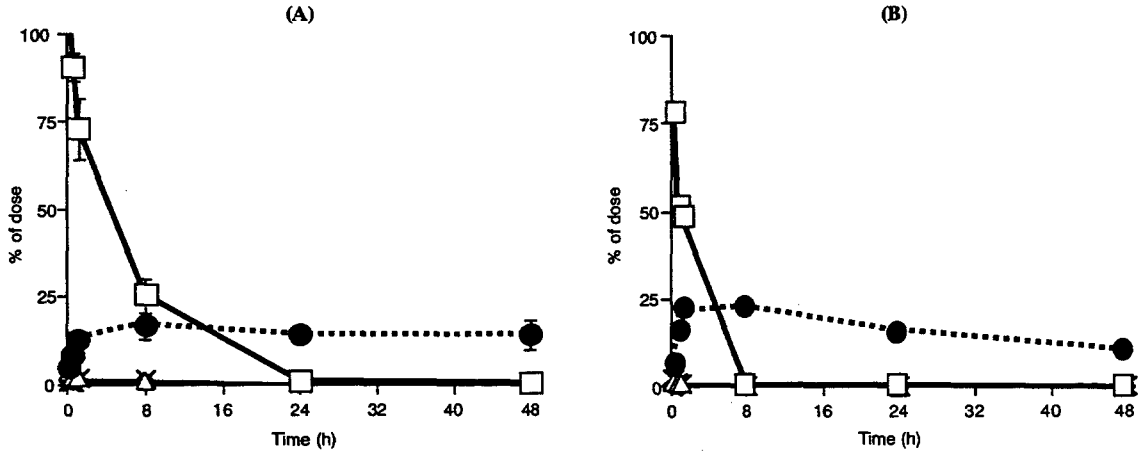


Figure 4-2. Plasma concentration and tissue distribution of Lac-Suc-FTC after i.v. administration at a dose of 1 mg (A) or 0.2 mg (B) (0.2 ml) per normal mouse (20 g): —□— ; Plasma, ····●···· ; Liver, —△— ; Kidney, —×— ; Lung, —◇— ; Spleen. Each point represents the mean ±S.D. (n=3).

of 0.43 and 0.54 h^{-1} at doses of 1 and 0.2 mg per mouse, respectively. The distribution volumes (V_d), obtained from the dose and calculated initial plasma concentration in Fig. 4-3(A), were not markedly different between the two doses. The initial plasma clearances ($CL_{0\text{-}1\ h}^{plasma}$) indicated that elimination from the plasma was non-linear. During the initial 1 h after injection, the amount accumulated in the liver increased approximately linearly with time (Fig. 4-3(B)). The initial uptake rates (v_L) by the liver were 110 and 42 μg/h per mouse, that is 1.9 and 0.70 μg/min per mouse, at the doses of 1 and 0.2 mg per mouse, respectively. Fig. 3(C) shows the relationship between the $AUC_{0-t\ h}^{plasma}$ value and the amount (X_t^{liver}) accumulated in the liver at time t. The average hepatic uptake clearances ($CL_{0\text{-}1\ h}^{liver}$) for the initial 0 – 1 h were calculated as 0.12 and 0.33 ml/h per mouse at doses of 1 and 0.2 mg per mouse, respectively, using equation (1). These $CL_{0\text{-}1\ h}^{liver}$ values were around one third and a half of the $CL_{0\text{-}1\ h}^{plasma}$ values at doses of 1 and 0.2 mg per mouse, respectively. These kinetic parameters for the plasma clearance and hepatic clearance for 0 – 1 h are summarized in Table 4-1. The moment values in a long scale (0 – 48 h) for the plasma and liver were compared between the doses of 1 and 0.2 mg per mouse (Table 4-2). The $AUC_{0\text{-}48\ h}^{plasma}$ values of Lac-Suc-FTC at doses of 1 and 0.2 mg per mouse were about 7.3×10^3 and 5.5×10^2 h·μg/ml, respectively. Also, the $MRT_{0\text{-}48\ h}^{plasma}$ of Lac-Suc-FTC at doses of 1 and 0.2 mg

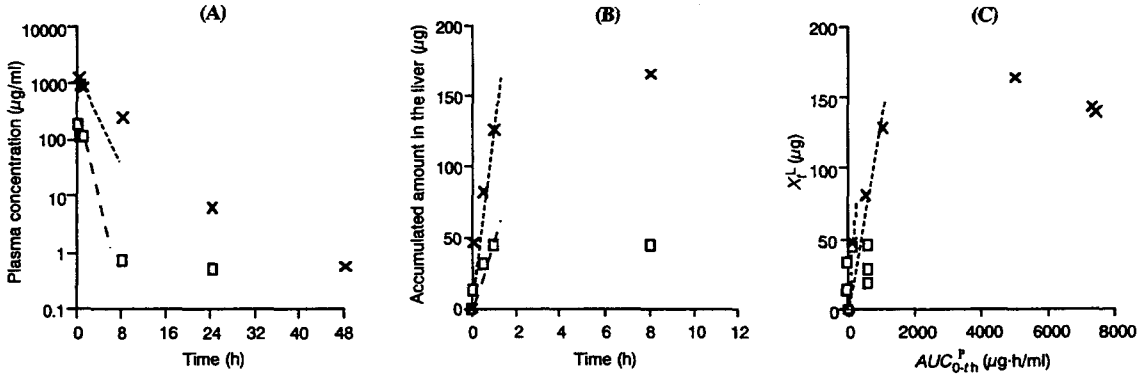


Figure 4-3. Pharmacokinetic profiles of Lac-Suc-FTC in the plasma and liver after i.v. administration at a dose of 0.2 mg or 1 mg per normal mouse (20 g): **(A):** Plasma concentration after injection, **(B)** :Accumulated amount in the liver per mouse after injection, **(C):** Accumulated amount (X_t^L) in the liver per mouse at time t after injection vs. area under the plasma concentration-time curve for 0 - t h ($AUC_{0\text{-}t\ h}^P$) per mouse: ✕ , ✕ , ····· ; 1 mg, □ , □ , - - - - ; 0.2 mg. The broken lines are the linear curves best-fitted in the initial phase (0 - 1 h).

per mouse were approximately 24 and 19 h, respectively. The $AUC_{0\text{-}48\,h}^{liver}$ and $MRT_{0\text{-}48\,h}^{liver}$ of Lac-Suc-FTC at a dose of 1 mg were calculated to be about 7.7×10^3 h·μg/ml and 4.4 h, respectively. The $AUC_{0\text{-}48\,h}^{liver}$ and $MRT_{0\text{-}48\,h}^{liver}$ of Lac-Suc-FTC at a dose of 0.2 mg were calculated to be about 1.9×10^3 h·μg/ml and 1.5 h, respectively. Thus, the $AUC_{0\text{-}48\,h}^{plasma}$ of Lac-Suc-FTC (1 mg per mouse) was about thirteen times larger than that of Lac-Suc-FTC (0.2 mg per mouse), while the $AUC_{0\text{-}48\,h}^{liver}$ of Lac-Suc-FTC (1 mg per mouse) was about four times greater than that of Lac-Suc-FTC (0.2 mg per mouse). That is, the ratios of $AUC_{0\text{-}48\,h}^{liver}$ to $AUC_{0\text{-}48\,h}^{plasma}$ were 1.1 and 3.4 at doses of 1 and 0.2 mg per mouse, respectively. The very slow elimination of Lac-Suc-FTC from the liver was observed at each dose.

4-3-3. Urinary and fecal excretion

Urinary and fecal excretions are shown in Table 4-3. The urinary volume collected until 1 h after i.v. injection was too small to determine the amount of excreted Lac-Suc-FTC. Therefore, urine collection was performed over longer time intervals of 8, 24 and 48 h after injection. The urine stored in the cage bottle and that withdrawn from the bladder were combined to obtain the total urine excreted. The level of excretion increased gradually in both urine and feces. The urinary and fecal excretion rates tended to be faster at a dose of 0.2 mg than 1 mg. However, the percentages of dose excreted did not show significant differences between urine and feces ($p>0.05$).

4-3-4. Comparison of biodistribution among Suc, Lac-Suc and galactosaminated *N*-succinyl-chitosan (Gal-Suc)

Suc-FTC and Gal-Suc-FTC were investigated for biodisposition at 8 h after i.v. injection at a dose of 0.2 mg per mouse. The results are shown in Figure 4-4. As expected, Suc-FTC exhibited a high distribution in the systemic circulation and was scarcely distributed to the liver, where the distributed amount was significantly lower than that of Lac-Suc-FTC ($p<0.01$). Most of the injected Gal-Suc-FTC was eliminated from the plasma at 8 h after injection, and it showed little distribution in the tissues tested, urine or feces. The amount of

Table 4-1.
Pharmacokinetic parameters of Lac-Suc-FTC for plasma clearance and hepatic uptake in the initial phase (0 - 1 h) in the normal mice

Lac-Suc-FTC	k_P [1/h]	$t_{1/2}^{plasma}$ [h]	Vd [ml]	CL_{0-1h}^{plasma} [ml/h]	v_L [μg/h]	CL_{0-1h}^{liver} [ml/h]
1 mg	0.43	1.6	0.78	0.34	110	0.12
0.2 mg	0.54	1.3	1.1	0.60	42	0.33

Vd, $CL_{0\text{-}1h}^{plasma}$, v_L and $CL_{0\text{-}1h}^{liver}$ are expressed as the values per normal mouse.

Table 4-2.
Pharmacokinetic parameters of Lac-Suc-FTC for long-term biodistribution and hepatic elimination in the latter phase (8 - 48 h) in normal mice

Lac-Suc-FTC	AUC_{0-48h}^{plasma} [h•μg/ml]	MRT_{0-48h}^{plasma} [h]	AUC_{0-48h}^{liver} [h•μg/ml]	MRT_{0-48h}^{liver} [h]	$\frac{AUC_{0-48h}^{liver}}{AUC_{0-48h}^{plasma}}$	CL_{0-48h}^{liver} [ml/h]	k_L [1/h]
1 mg	7300	4.4	7700	24	1.1	0.02	0.005
0.2 mg	550	1.5	1900	19	3.4	0.04	0.025

$AUC_{0\text{-}48h}^{plasma}$, $AUC_{0\text{-}48h}^{liver}$ and $CL_{0\text{-}48h}^{liver}$ are expressed as the values per normal mouse.

Table 4-3.
Urinary and fecal excretion of Lac-Suc-FTC at 8, 24 and 48 h after i.v. administration in normal mice

			Excretion [% of dose]		
			8 h	24 h	48 h
Lac-Suc-FTC	1 mg	Feces	0.13 ± 0.09	0.7 ± 0.2	2.7 ± 0.8
		Urine	1.1 ± 0.6	1.8 ± 1.0	3.3 ± 0.9
	0.2 mg	Feces	0.09 ± 0.05	1.7 ± 0.8	5.0 ± 2.1
		Urine	1.2 ± 0.7	2.8 ± 1.7	6.3 ± 3.5

Each value represents the mean ±S.D. (n=3).

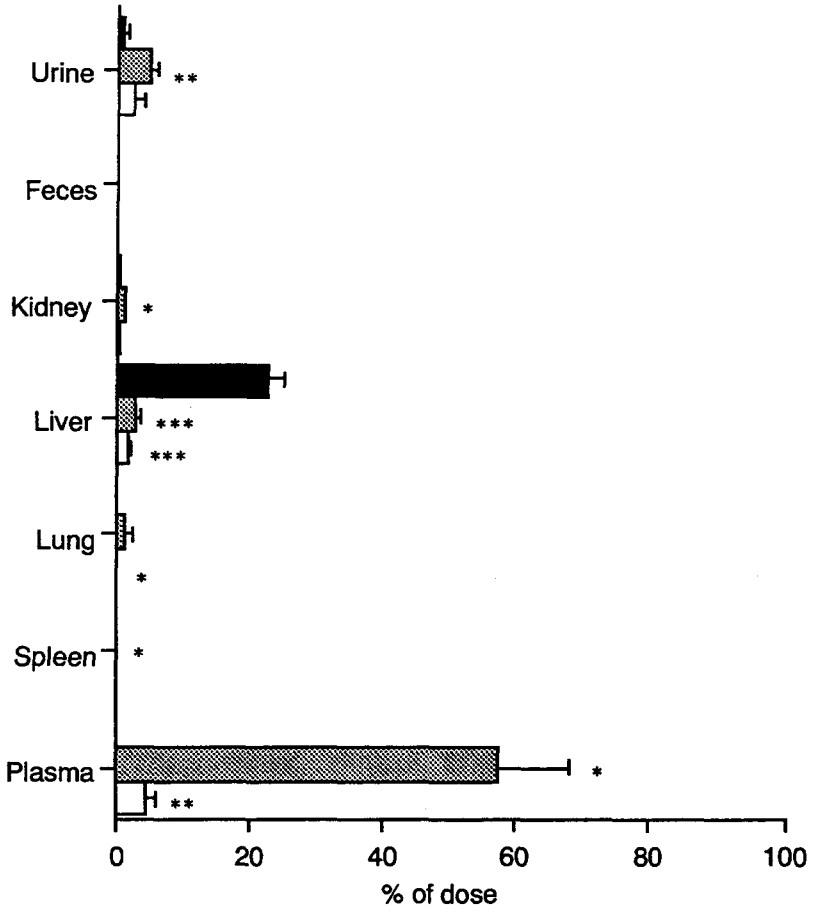


Figure 4-4. Comparison of biodispositions among Lac-Suc-FTC, Suc-FTC and Gal-Suc-FTC at 8 h after i.v. administration at a dose of 0.2 mg (0.2 ml) per normal mouse (20 g): ■ ; Lac-Suc-FTC, ▨ ; Suc-FTC, □ ; Gal-Suc-FTC.
Each column represents the mean ± S.D. (n=3). *; $p<0.05$, **; $p<0.01$, ***; $p<0.001$ vs. Lac-Suc-FTC.

Gal-Suc-FTC distributed to the liver was significantly lower than that of Lac-Suc-FTC ($p<0.001$). Thus, Gal-Suc-FTC was suggested to be distributed to tissues other than those tested in this study. Further studies are therefore required to determine the tissue distribution of Gal-Suc-FTC. These results of the present study demonstrated that Lac-Suc-FTC was superior as a drug carrier for liver targeting.

4-3-5. Competitive liver uptake

The initial cell suspension (before isolation) contained about 3.7×10^7 PC and 1.6×10^7 NPC, respectively.

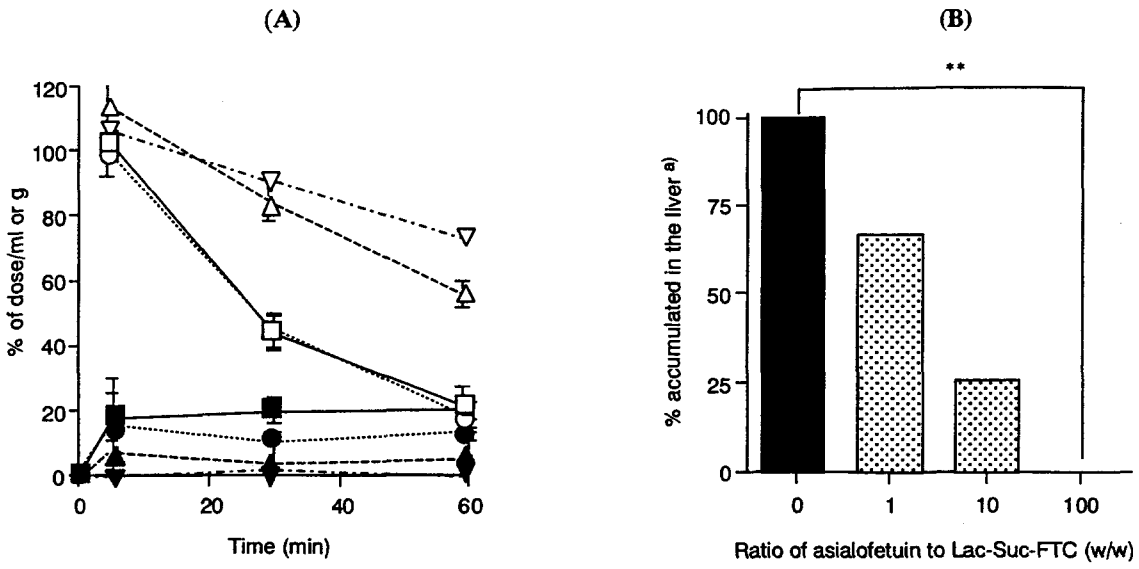


Figure 4-5. Competitive liver uptake of Lac-Suc-FTC after i.v. administration at a dose of 0.1 mg (0.2 ml) by co-administration of various amounts of asialofetuin per normal mouse (20 g): (A) Plasma concentration and liver distribution profiles of Lac-Suc-FTC. □, ■ ; Lac-Suc-FTC only. ○, ● ; Lac-Suc-FTC : asialofetuin = 1 : 1 (w/w). △, ▲ ; Lac-Suc-FTC : asialofetuin = 1 : 10 (w/w). ▽, ▼ ; Lac-Suc-FTC : asialofetuin = 1 : 100 (w/w). Open and closed symbols expressed plasma and liver concentration, respectively. Each point represents the mean ±S.D. (n=3). (B) Liver accumulation of Lac-Suc-FTC at 60 min post-injection based on (A). a) The results are expressed as the percentage to the accumulation of Lac-Suc-FTC without asialofetuin. **; $p<0.01$ vs. Lac-Suc-FTC only.

Figure 4-5(A) shows the distribution of Lac-Suc-FTC after i.v. administration at a dose of 0.1 mg/mouse by co-administration of various amounts of asialofetuin. When asialofetuin was added at a dose of 0.1 mg, the liver distribution of Lac-Suc-FTC was inhibited at 60 min post-injection to a certain extent, while the plasma profile scarcely changed. On the contrary, at a dose of 1 and 10 mg of asialofetuin, the elimination of Lac-Suc-FTC from the plasma was suppressed with the inhibition of its liver uptake. Especially, in case of co-administration of 10 mg of asialofetuin, the liver uptake of Lac-Suc-FTC was completely inhibited (Fig. 4-5(B)).

The hepatic cellular uptake of Lac-Suc-FTC is shown in Figure 4-6. The uptake into PC was inhibited with the increase of the ratio of asialofetuin to Lac-Suc-FTC. On the other hand, the uptake into each NPC was approximately 7 % of dose/liver except the ratio of asialofetuin to Lac-Suc-FTC being 100.

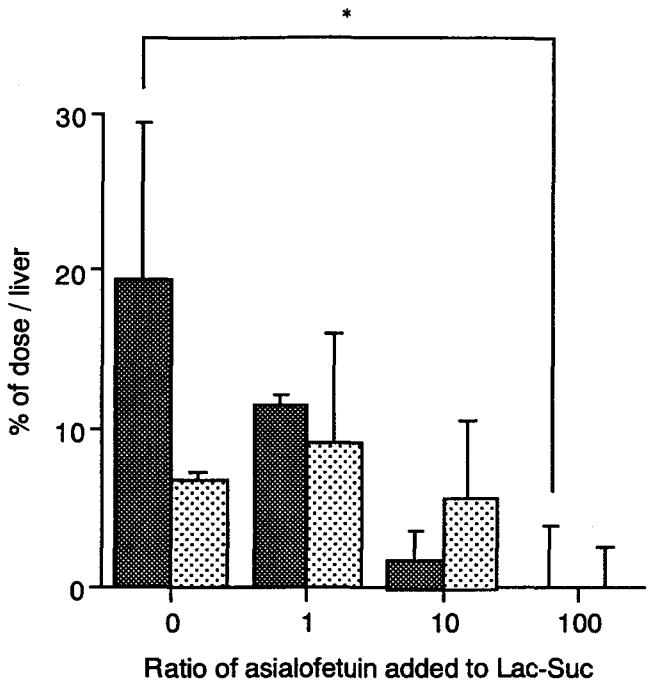


Figure 4-6. Hepatic cellular localization of Lac-Suc-FTC after i.v. administration at a dose of 0.1 mg (0.2 ml) by co-administration of various amounts of asialofetuin per normal mice (20 g): Each bar represents the mean ±S.D. of three mice. ▩ ; PC, ⊡ ; NPC. *; $p<0.05$ vs. Lac-Suc-FTC only.

4-4. Discussion

In recent years, drug carriers such as macromolecules have been combined with sugar chains to allow targeting to the liver. Since Lac-Suc used in this study has many carboxyl groups, it was thought to be able to react with drugs with amino functional groups. In fact, we developed a Lac-Suc-antitumor drug conjugate and confirmed that the conjugate exhibited antitumor activity against a liver metastatic tumor (unpublished observations), supporting the availability of Lac-Suc for liver targeting of the drug based on recognition of the galactose residue. Galactose has been introduced to dextran, poly(L-lysine) (PLL), poly(L-glutamic acid) (PLGA) etc. using 2-imino-2-methoxyethyl 1-thiogalactoside (IME-thiogalactoside).[113,114,125,126] In the present study, reductive amination using lactose was selected for the introduction of galactose to Suc; because Suc possessed amino groups by

about 20 % (mol/sugar unit), special reagents such as IME-thiogalactoside were not required and the reaction could occur under mild conditions in aqueous solution (Fig. 4-1). The FTC content of Lac-Suc-FTC was 1.53 % (w/w). This value was lower than that (1.88 % (w/w)) of Suc-FTC obtained in the same synthetic procedure,[92] which might have been based on the decrease of amino function of Suc due to lactosamination. Lac-Suc-FTC in the biological media showed very little quenching of its fluorescence, and the quenching was negligible for *in vivo* quantitative analysis of Lac-Suc-FTC in biodistribution studies.

The biodistribution studies were performed by i.v. injection at 1 and 0.2 mg of Lac-Suc-FTC per mouse. These doses were considered to be practical, e.g. the doses of loaded drugs were calculated to be 10 and 2 mg/kg at 1 and 0.2 mg per mouse (20 g) with Lac-Suc-drug conjugates containing the drug at 20 % (w/w). Therefore, the biodisposition profiles obtained at these doses were supposed to be directly available for evaluation of efficacy and side effects of Lac-Suc-drug conjugates. Lac-Suc-FTC was eliminated much faster from the systemic circulation than Suc-FTC (Figs. 4-2 and 4-4).[92] Irrespective of dose, after 30 min post-injection, Lac-Suc-FTC showed significantly higher distribution to the liver in comparison with other tissues (in Fig. 4-2: $p<0.001$ liver vs. other tissues at a dose of 0.2 mg after 30 min post-injection; $p<0.01$ or $p<0.001$ liver vs. other tissues at a dose of 1 mg after 30 min post-injection). The maximum accumulation in the liver was observed at 8 h post-injection at both doses. Distribution of Lac-Suc-FTC to the liver may have been inferior to that of other glycosylated macromolecules reported to date, but that of Lac-Suc-FTC to other tissues appeared to be suppressed.[113,114] Namely, accumulation extents of galactosylated PLL (Gal-PLL) and galactosylated PLGA (Gal-PLGA) in the liver were approximately 70 and 50 % of dose at 60 min post-injection, respectively, and those of Gal-PLL and Gal-PLGA in the kidney were both 10 % of dose at 60 min post-injection in case of a dose of 1 mg/kg, while that of Lac-Suc-FTC in the tissues other than liver was less than 1.9 % of dose at 60 min post-injection in case of a dose of 1 mg/mouse. Since the total amount of Lac-Suc-FTC found in the blood and tested tissues was much smaller than the injected amount, it is conceivable that undetected Lac-Suc-FTC was distributed to tissues that were not tested in

this study such as prostate and testes known to express galactosyl receptor.[127] Lac-Suc-FTC showed a long-term retention in the liver. At a dose of 1 mg Lac-Suc-FTC per mouse, it was calculated to be retained in liver to about 150 μg per mouse even at 48 h post-injection (n = 3). This was considered to be due to the biological stability of Suc, while proteins are cleared soon due to lysosomal degradation.[118-120] These properties are considered to result in a long half-life of the drug in the liver. Once Lac-Suc is taken up by the liver, it is eliminated very slowly, and this elimination seems to occur faster at the lower dose such as 0.2 mg per mouse (Fig. 4-2). The gradual increase of Lac-Suc-FTC in fecal excretion indicated that Lac-Suc-FTC was slowly excreted via biliary excretion from the liver, which was consistent with the slow elimination from the liver. The urinary excretion was also increased gradually. The whole urine was obtained by combining the urine collected in the cage bottle and that in the bladder, while feces were collected only in the cage bottle. Since the feces remaining in the intestine was not taken, the total fecal excretion was considered to be higher than that shown in Table 4-3. Therefore, the results in Table 4-3 do not necessarily indicate that urinary excretion was higher than fecal excretion.

To confirm whether the liver uptake of Lac-Suc is concerned with the asialoglycoprotein receptor, competitive liver uptake was investigated by the co-administration of asialofetuin, which is known to be taken up by the liver PC via the asialoglycoprotein receptor. Reportedly, asialofetuin is accumulated very rapidly in the liver and begins to be degraded from 15 min after addition.[128] Therefore, the competitive binding study was carried out in the initial phase, i.e. 0 – 60 min. It was observed that the liver distribution of Lac-Suc-FTC was inhibited with the increase of the ratio of asialofetuin to Lac-Suc-FTC (Fig. 4-5(B)). This observation suggested that the liver uptake of Lac-Suc-FTC should be concerned with the asialoglycoprotein receptor. Furthermore, the uptake of Lac-Suc-FTC by the liver cells was investigated using a fluorescent microscope. As a result, PC exhibited the fluorescence due to the uptake of Lac-Suc-FTC; on the contrary, such fluorescence was hardly observed in NPC (data not shown). However, the sample of isolated NPC included cells emitting fluorescence. Since most of the dead cells and cell

debris, as well as NPC, were transferred to supernatant and were collected by centrifugation,[129] it was conceivable that the distribution of Lac-Suc-FTC in NPC portion shown in Fig. 4-6 might be caused by the contamination of PC debris with Lac-Suc-FTC.

The pharmacokinetic parameters of Lac-Suc-FTC at doses of 1 and 0.2 mg were calculated from the results of distribution studies (Tables 4-1 and 4-2). The plasma clearance indicated that the elimination from the systemic circulation was non-linear. At both doses, the initial hepatic clearance was considerably large but was smaller than the initial plasma clearance; that is, the $CL_{0\text{-}1\ h}^{liver}$ values were around one third and a half of the $CL_{0\text{-}1\ h}^{plasma}$ values at doses of 1 and 0.2 mg per mouse, respectively. These observations suggested that although the liver should be a major clearance tissue, other tissues also play a role in clearance from the plasma. The hepatic uptake rates for the initial period of 0 – 1 h were calculated as 42 and 110 μg/h per mouse (20 g) at doses of 0.2 and 1 mg per mouse, respectively. That is, the uptake rates were calculated as 3.5 and 9.3 μg/min per 100 g body weight. The hepatic uptake rates of asialofetuin and asialoorosomucoid at the maximal eliminative capacity of the liver were reported to be 18 – 20 μg/min per 100 g body weight in rats.[129] Therefore, the uptake rate of Lac-Suc seemed to be comparable to their asialoglycoproteins although mice were used in our study. The initial hepatic clearance at a dose of 0.2 mg per mouse was about three times greater than that at a dose of 1 mg per mouse, indicating that the accumulation in the liver should be achieved more efficiently at the lower dose. After accumulation, the elimination rate constants from the liver were calculated to be very small (data not shown), suggesting that Lac-Suc could remain in the liver for long periods. This observation may show that liver behaves as a depot due to a slow drug release from carrier, because Suc(II)-MMC conjugate showed a slow drug release in acidic condition.[104]

Gal-Suc, the macromolecule synthesized by reductive amination of Suc and galactose itself, showed no accumulation to the liver (Fig. 4-4). Since the integrity of galactose is broken by reductive amination, the specific recognition by the asialoglycoprotein receptor is considered not to be caused in Gal-Suc. In addition, the residence of Gal-Suc-FTC in

plasma was much lower than that of Suc-FTC. Galactosamination affected the properties of Suc differently from lactosamination. The low recovery in the tissues tested and low excretion in urine and feces suggested that major part of Gal-Suc should be distributed to tissues other than tested ones in this study. Thus, Lac-Suc-FTC was considered to be an advantageous drug carrier for targeting to and subsequent retention in the liver.

4-5. Conclusion

Suc can be easily modified into Lac-Suc tagged with lactose by reductive amination using sodium cyanoborohydride. After i.v. injection, Lac-Suc is cleared much faster from the systemic circulation than Suc itself, and simultaneously a considerable amount of Lac-Suc is quickly taken up by the liver via the asialoglycoprotein receptor. At practical doses, the liver accumulation of Lac-Suc is dependent on the dose, and the Lac-Suc localized into the liver is retained there for a long period. These results can provide valuable information for development of carrier-drug conjugate systems using Lac-Suc as a drug carrier in future studies.

Chapter 5

Lactosaminated and Intact *N*-Succinyl-chitosans as Drug Carriers in Liver Metastasis

Summary

The biodistributions of Suc-FTC and Lac-Suc-FTC after i.v. administration to mice intravenously inoculated with M5076 cells were investigated at 3 and 12 d post-inoculation. At both time points, Lac-Suc-FTC was specifically localized to the liver. However, *AUC* in the liver decreased gradually by progress of the liver metastasis. At 3 d post-inoculation, Suc-FTC showed good retention in the systemic circulation and was little distributed to the liver. However, at 12 d post-inoculation, Suc-FTC was eliminated relatively fast from the systemic circulation and gradually accumulated in the liver. The antitumor effects of MMC, Lac-Suc-MMC and Suc(II)-MMC were examined on single i.v. administration for both metastatic stages. For administration at 3 d post-inoculation, Lac-Suc-MMC alone tended to elongate significantly the lifespan at a lower dose (0.4 mg eq. MMC/kg), and MMC, Suc(II)-MMC and Lac-Suc-MMC increased significantly the lifespan at a higher dose (10 mg eq. MMC/kg). However, at 12 d post-inoculation (late stage of metastasis), neither MMC nor the conjugates were effective even at the higher dose (10 mg eq. MMC/kg). Both carriers, Suc showing systemic long-circulation and Lac-Suc with an ability of liver-specific localization, are thought to be drug carriers with potentialities for therapeutics at early stage of metastasis.

1-1. Introduction

In cancer chemotherapy, it is important that the antitumor drugs are delivered to the tumor sites efficiently in order to reduce the severity of side effects. Many systems have been used to deliver drugs to the target sites.[67,130] Above all, we have focused on Suc as a drug carrier for active and passive targeting. Suc has excellent characteristics as a drug carrier, i.e. long-term retention in the systemic circulation,[37,92] low toxicity[72] and good drug loading.[74] In fact, when a water-soluble macromolecular prodrug of MMC with Suc(II), Suc(II)-MMC, was injected into Sarcoma 180-bearing mice, it was accumulated in Sarcoma 180 solid tumor due to the EPR effects, and good antitumor activity was found with reduced side effects.[104] Furthermore, it was demonstrated in normal mice that the introduction of lactose to Suc enabled liver targeting of the carrier.[131] However, it is known to be difficult for the macromolecules with galactose moieties to target to the liver effectively under diseased conditions.[111,132] It is also known that the angioarchitecture of hepatocellular carcinoma changes markedly as the cancer progresses.[133,134] Therefore, the biodistribution of macromolecules with galactose residues is considered to be affected by liver metastasis. In addition, it is unknown how Suc possessing no lactose behaves in mice with liver metastasis. Thus, in the present study, the biodistribution of Lac-Suc and Suc has been examined using mice bearing experimental liver metastasis of M5076 cells[135,136] in the early and advanced stage of the disease. Further, conjugates of MMC were investigated in vivo for antitumor characteristics using M5076-bearing mice.

5-2. Materials and methods

5-2-1. Materials

MMC was purchased from Kyowa Hakko Kogyo Co. (Tokyo, Japan). MMC was used after extraction by methanol. Suc (succinylation degree 0.81 mol/sugar unit, deacetylation

degree 1.0 mol/sugar unit, MW 3.4×10^5) was kindly supplied by Katakura Chikkarin Co., Ltd. (Tokyo, Japan). Lactose and FITC were purchased from Sigma Chemical Company (St. Louis, U.S.A.). Sodium cyanoborohydride was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). All other chemicals were obtained commercially as reagent-grade products.

5-2-2. Animals and tumors

Specific-pathogen-free male mice of the inbred strain C57BL/6 weighing approximately 20 g (at the age of 6 weeks) were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan) and used soon after for the experiments. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Hoshi University. Four animals were used for each time point in distribution experiments on Lac-Suc-MMC and Suc(II)-MMC (n = 4), and five animals in each group for *in vivo* antitumor tests (n = 5). For all other experiments, three animals were used (n = 3).

Murine reticulum cell sarcoma M5076 cells were used as tumor cells. These cells metastasize to several organs including the liver. M5076 cells were maintained in C57BL/6 mice by intraperitoneal transfer of 1×10^5 cells obtained from ascitic fluid every other week. In the *in vivo* biodistribution experiments and antitumor activity tests, 1×10^5 M5076 cells suspended in 0.1 ml of Hanks' balanced salt solution, which were obtained from the above mice bearing the tumor intraperitoneally, were inoculated intravenously into each mouse.

5-2-3. Biodistribution studies of Lac-Suc and Suc

Lac-Suc was synthesized by reductive amination using sodium cyanoborohydride according to a method reported previously.[131] The lactose residue content of Lac-Suc was determined to be about 0.30 mol/sugar unit by elemental analysis (Yanako Analytical Industrial Co., Japan).

Lac-Suc-FTC and Suc-FTC were used throughout the biodistribution experiments. Lac-Suc-FTC and Suc-FTC were prepared as reported previously.[92] The FTC contents of

Lac-Suc-FTC and Suc-FTC, examined according to the previous report,[92] were 1.5 and 2.3 % (w/w), respectively.

The recoveries of Suc-FTC and Lac-Suc-FTC from various tissues were determined using normal mice according to the method of Kamiyama et al.[37]

To investigate the biodistribution of Lac-Suc-FTC administered at an early stage of liver metastasis, at 3 d after i.v. tumor inoculation, M5076-bearing mice received Lac-Suc-FTC via the lateral tail vein at a dose of 0.2 mg (0.2 ml) per mouse without anesthetic. The mice were sacrificed at 5 min, 0.5 h, 1 h, 8 h, 24 h and 48 h after injection, blood samples were withdrawn and several tissues (liver, kidney, spleen and lung) were excised. The subsequent procedure was performed as reported previously.[92] The concentration was corrected by the recoveries. The distributed amount was calculated from the concentration and tissue weight. The corrected plasma concentration means the concentration given provided Lac-Suc-FTC was completely transferred from blood to plasma. The amount of Lac-Suc-FTC in plasma was calculated using the reported volume of mouse plasma, 48.8 ml/kg.[83]

To investigate the biodistribution of Lac-Suc-FTC when administered at an advanced stage of liver metastasis, at 12 d after i.v. tumor inoculation, M5076-bearing mice received Lac-Suc-FTC via the lateral tail vein at a dose of 0.2 mg (0.2 ml) per mouse with no anesthetic. The subsequent procedures were the same as those described for the early stage of liver metastasis.

The biodistribution of Suc-FTC was examined in the same way as that of Lac-Suc-FTC except that the body distribution at 5 min and 0.5 h after i.v. injection was not investigated.

5-2-4. Pharmacokinetic analysis

The areas under the plasma or liver concentration-time curves for 0 - 48 h ($AUC_{0\text{-}48\ h}$) and the mean residence time ($MRT_{0\text{-}48\ h}$) were calculated by the trapezoidal method.[96] The following equation was employed to determine the relative effectiveness of liver targeting (R_{et}):

$$R_{et} = AUC_{0\text{-}48\,h}{}^{liver} / AUC_{0\text{-}48\,h}{}^{plasma}$$

5-2-5. Urinary excretion

The cumulative collection of urine was performed simultaneously with the biodistribution studies as described above. Mice were placed separately in metabolite cages immediately after administration. Urine was collected for 8, 24 and 48 h after i.v. administration, and then urinary volume was measured. Each urine sample was filtered using a membrane filter (0.45 μm pore diameter). The filtrate was diluted appropriately with PBS, and fluorescence intensities were measured fluorometrically (Ex. 495 nm, Em. 520 nm). The total amount excreted in urine was calculated from the concentration and urinary volume.

5-2-6. *In vivo* antitumor effects of Suc(II)-MMC and Lac-Suc-MMC conjugates against M5076 liver metastatic tumor

First, in order to give a water-soluble conjugate, Suc(II) was prepared by reaction of Suc with succinic anhydride as reported previously.[104] Namely, although most of the conjugate prepared by the direct coupling of MMC with original Suc using water-soluble carbodiimide was water-insoluble due to crosslinking among or within the polymer supports, water-soluble conjugate could be obtained to a much larger extent by using Suc(II) instead of Suc.[104] The preparation of the water-soluble Suc(II)-MMC followed the previous manner.[104] Lac-Suc-MMC was also prepared by the same procedure except that Lac-Suc was used instead of Suc(II). Suc(II)-MMC and Lac-Suc-MMC showed the drug contents of 12 and 20 % (w/w), respectively, which were measured in the manner previously reported.[104] These were used throughout the study. The dose of MMC conjugates was expressed in terms of amount of parent MMC.

Antitumor effects were examined using the mice at 3 and 12 d after intravenous inoculation with M5076 cells. Namely, at 3 or 12 d post-inoculation, MMC was intravenously administered at a single dose of 0.4, 5 and 10 mg/kg, and Suc(II)-MMC or Lac-Suc-MMC were intravenously administered at a single dose of 0.4 and 10 mg eq. MMC/kg,

respectively. Controls were injected with a similar volume of saline alone. For all the mice, the survival time after inoculation was observed for 50 d post-inoculation. The antitumor effects were obtained by comparing the mean survival time of the treated mice after inoculation (*T*) with that of the control mice after inoculation (*C*), that is, from *ILS* calculated by the following equation:

$$ILS = (T/C - 1) \times 100\ (\%)$$

At the same time, the changes in body weight of each group were measured to evaluate the toxic side effects.

5-2-7. Distribution of free and conjugated MMCs in blood circulation and liver after i.v. injection of the conjugates

In this experiment, the mice were used at 3 and 12 d post-inoculation. Lac-Suc-MMC or Suc(II)-MMC (4 mg eq. MMC/kg) was intravenously injected into M5076-bearing mice. The mice were sacrificed at 8 and 24 h after injection, the liver was enucleated, and plasma was obtained. To the excised liver was added the same volume of 1 *M* sodium carbonate buffer (pH 9.0), and the mixture was homogenized with a glass homogenizer with a Teflon pestle.

Free MMC contained in the obtained sample mixture (pH 9.0) was extracted according to the method of Den Hartigh et al.[93] This operation allowed the complete recovery of MMC from the sample. The extracted MMC was finally dissolved in methanol. The amount of free MMC in the sample was determined by HPLC.

To determine the total amounts of free and conjugated MMC in the obtained sample mixture (pH 9.0), heating treatment (90 °C, 5 min) was performed;[104,137] this operation allowed the almost complete release of MMC from the conjugates. Then, MMC was extracted by the same method, and finally dissolved in methanol. The total amounts of free and conjugated MMC in the sample were determined by HPLC.

HPLC was executed at room temperature, and the absolute calibration curve method was used for analysis.[93] Samples (20 μl of the final solution in methanol) were injected into

a SUMIPAX Nucleosil $5C_{18}$ reversed phase column (4 mm in inner diameter × 250 mm in length) and eluted at a flow rate of 0.4 ml/min with a mobile phase of a mixture of 0.01 *M* phosphate buffer, pH 6.0, and methanol (65 : 35, v/v). The HPLC operation was performed using a Shimadzu LC-6AD apparatus equipped with an SPD-10AV UV detector (Shimadzu) set at 365 nm. The concentration of MMC was calculated using a standard curve.

5-2-8. Statistical analysis

Student's *t*-test was performed to determine the level of significance without survival analysis. As to survival, the Mantel-Cox log-rank test was applied to check for a significant difference. The data were considered to be significantly different when the *p*-value was less than 0.05.

5-3. Results

5-3-1. Body distribution of Lac-Suc-FTC and Suc-FTC

The concentrations of Lac-Suc-FTC distributed after i.v. administration at a dose of 0.2 mg per M5076-bearing mouse at 3 and 12 d after i.v. tumor inoculation are shown in Figures 5-1(A) and (B), respectively. Lac-Suc-FTC was quickly accumulated in the liver and showed very little accumulation in other tissues. Furthermore, only less than 2.5 % of the dose administered remained in plasma at 8 h after injection. At 1 h post-injection, the amount in liver was more than 15-fold that in any other tissue except plasma (Fig. 5-1(A-2)). Even at 12 d post-inoculation, Lac-Suc-FTC was rapidly accumulated in the liver and showed little distribution to other tissues except the kidney at 1 h (Fig. 5-1(B)); however, the liver concentration at 12 d post-inoculation was lower than that at 3 d post-inoculation. Furthermore, less than 2.3 % of the dose remained in plasma at 8 h after injection.

Figures 5-2(A) and (B) also show the concentrations of Suc-FTC distributed after i.v. administration at a dose of 0.2 mg per M5076-bearing mouse at 3 and 12 d post-inoculation,

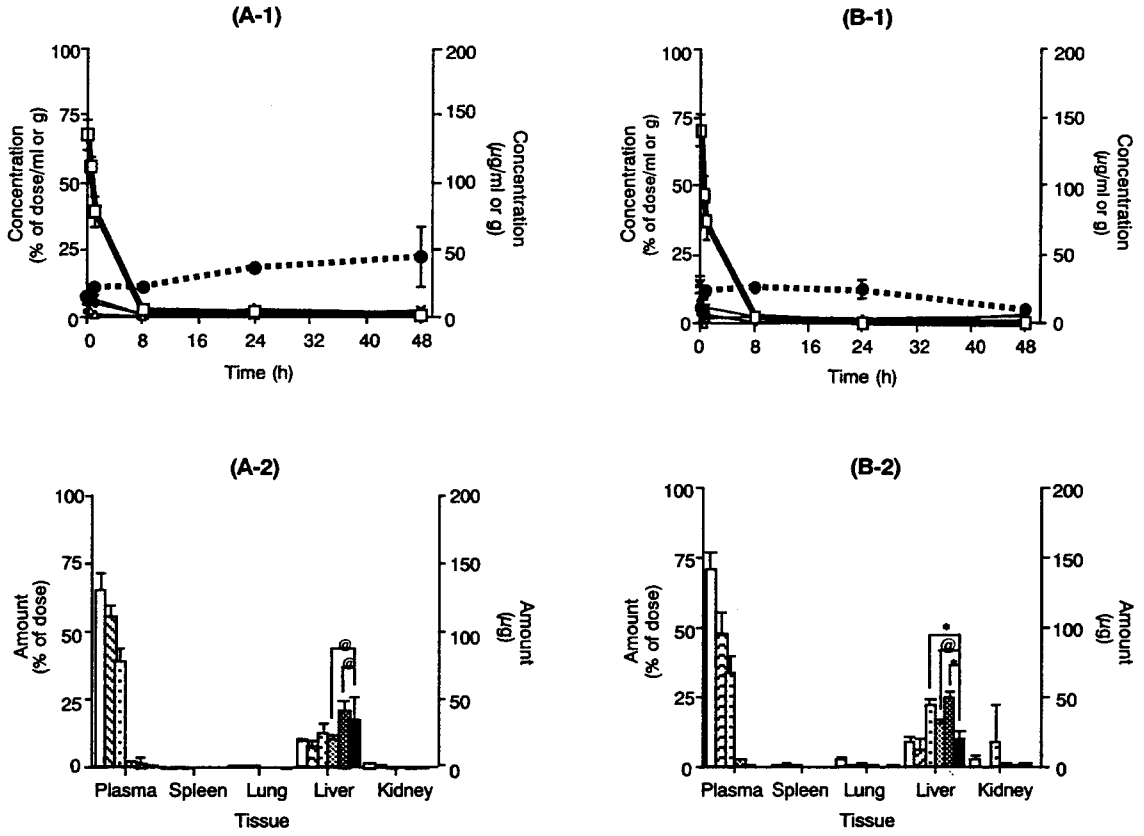


Figure 5-1. Plasma concentration and tissue distribution of Lac-Suc-FTC after i.v. administration at a dose of 0.2 mg (0.2 ml) per M5076-bearing mouse: —□— ; Plasma, ▪▪▪●▪▪▪ ; Liver, —△— ; Kidney, —×— ; Lung, —◇— ; Spleen. At 3 d ((**A-1**), (**A-2**)) or 12 d ((**B-1**), (**B-2**)) after i.v. inoculation, test substance (0.2 ml) was injected intravenously. Each point represents the mean ± S.D. (n = 3).
(**A-2**) 3 d(□ : 5 min, ▨ : 0.5 h, ⊡ : 1 h, ▩ : 8 h, ■ : 24 h, ■ : 48 h.).
(**B-2**) 12 d(□ : 5 min, ▨ : 0.5 h, ⊡ : 1 h, ▩ : 8 h, ■ : 24 h, ■ : 48 h.).

respectively. In contrast to Lac-Suc-FTC, at 3 d post-inoculation, Suc-FTC was retained in the blood circulation at a high level for at least 48 h and was slightly distributed to the liver (Fig. 5-2(A-1)). On the other hand, at 12 d post-inoculation, Suc-FTC was eliminated more rapidly from the systemic circulation (Fig. 5-2(B-1)). The amount distributed in the liver was greater at 12 d than at 3 d post- inoculation. The localization to the spleen was also increased at 12 d post-inoculation.

5-3-2. Pharmacokinetics of Lac-Suc-FTC and Suc-FTC

The pharmacokinetic parameters of Lac-Suc-FTC at a dose of 0.2 mg per mouse, estimated from the mean concentration-time curves in the plasma and liver, are shown in

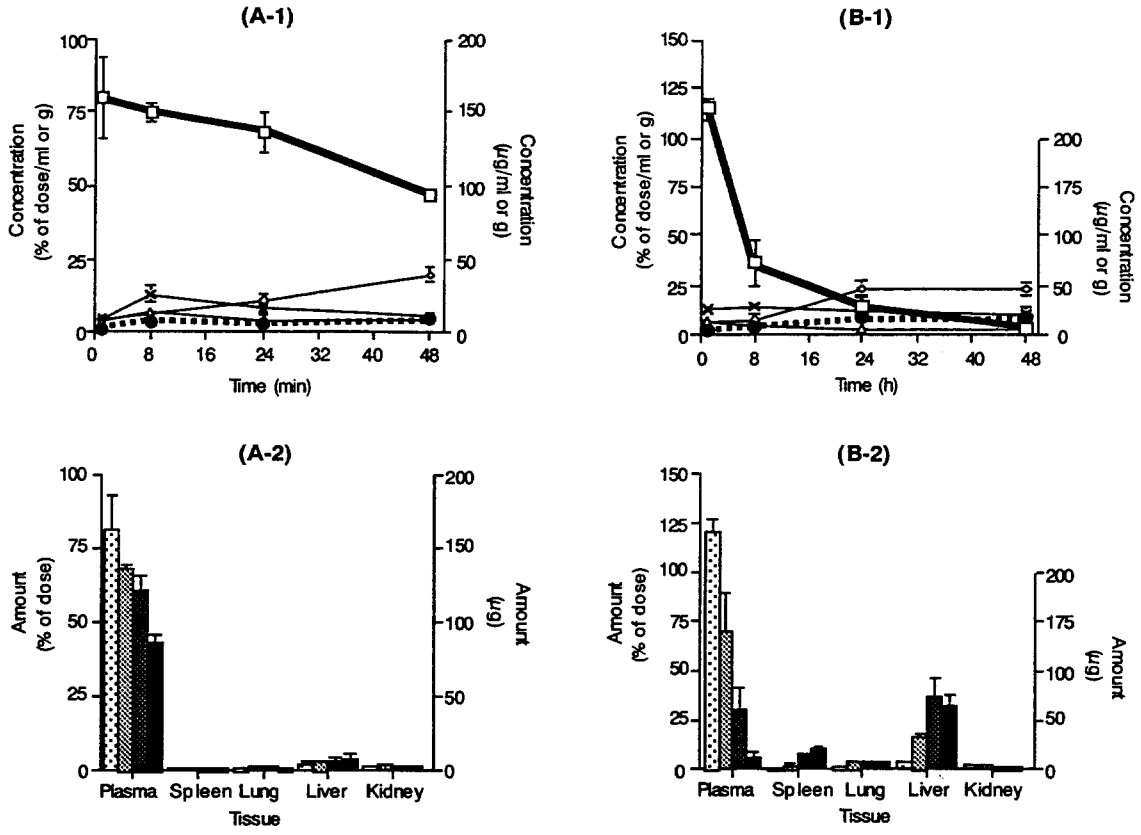


Figure 5-2. Plasma concentration and tissue distribution of Suc-FTC after i.v. administration at a dose of 0.2 mg (0.2 ml) per M5076-bearing mouse: —□— ; Plasma, ▪▪▪●▪▪▪ ; Liver, —△— ; Kidney, —×— ; Lung, —◇— ; Spleen. At 3 d ((**A-1**), (**A-2**)) or 12 d ((**B-1**), (**B-2**)) after i.v. inoculation, test substance (0.2 ml) was injected intravenously. Each point represents the mean ± S.D. (n = 3).
(**A-2**) 3 d (▩ : 1 h, ▩ : 8 h, ▩ : 24 h, ■ : 48 h.).
(**B-2**) 12 d (▩ : 1 h, ▩ : 8 h, ▩ : 24 h, ■ : 48 h.).

Table 5-1(A). The data at 0 d, corresponding to the data for normal mice, are from the previous results obtained using normal mice.[131] *AUC*, *MRT* and R_{et}, calculated based on the concentration-time curve, were abbreviated to *AUC*(C), *MRT*(C) and $R(C)_{et}$, respectively. $AUC(C)_{0\text{-}48\ h}^{liver}$ at 12 d post-inoculation was two-thirds of that at 3 d post-injection. Moreover, $AUC(C)_{0\text{-}48\ h}^{plasma}$ at 12 d post- inoculation was four-fifths of that at 3 d post-injection. After all, the $R(C)_{et}$ value at 3 d post-inoculation was greater than that at 12 d post-injection. The $MRT(C)_{0\text{-}48\ h}$ values in the plasma and liver were larger at 3 d post-inoculation. Both $AUC(C)_{0\text{-}48\ h}$ and $R(C)_{et}$ values decreased gradually with progression of liver metastasis.

The pharmacokinetic parameters of Lac-Suc-FTC at a dose of 0.2 mg per mouse, estimated from the mean amount-time curves in the plasma and liver, are shown in Table 5-1(B). *AUC*, *MRT* and R_{et}, calculated and evaluated based on the amount-time curve, were abbreviated as *AUC*(M), *MRT*(M) and $R(M)_{et}$, respectively. The $AUC(M)_{0\text{-}48\ h}^{liver}$ values slightly increased with the progression of liver metastasis. The $AUC(M)_{0\text{-}48\ h}^{plasma}$ value was a little lower at 12 d than at 3 d post-inoculation. Accordingly, the $R(M)_{et}$ value for Lac-Suc-FTC increased slightly with the progression of liver metastasis.

Therefore, when liver metastasis was progressed, the concentration data indicated small decrease in ability of Lac-Suc-FTC for liver targeting, but the amount localized to the liver was rather raised to some extent. This increase in the amount localized to the liver was found to be due to liver size, which was increased with progression of liver metastasis.

$AUC_{0\text{-}48\ h}^{liver}$, $AUC_{0\text{-}48\ h}^{plasma}$, $MRT_{0\text{-}48\ h}^{liver}$, $MRT_{0\text{-}48\ h}^{plasma}$ and R_{et} values of Suc-FTC at 3 and 12 d post-inoculation were calculated as shown in Table 5-2. In the advanced stage of liver metastasis at 12 d post-inoculation, the $AUC(C)_{0\text{-}48\ h}^{plasma}$ and $AUC(M)_{0\text{-}48\ h}^{plasma}$ were about two-fifths of those at 3 d post-injection, respectively. On the other hand, at the

Table 5-1.
Pharmacokinetic parameters of Lac-Suc-FTC after i.v. administration at a dose of 0.2 mg (0.2 ml) per M5076-bearing mouse

Injection day post-inoculation		(A) Concentration			(B) Amount		
		AUC(C)$_{0\text{-}48h}$ [h•μg/ml or g]	*MRT*(C)$_{0\text{-}48h}$ [h]	$R(C)_{et}^{a}$	*AUC*(M)$_{0\text{-}48h}$ [h•μg per mouse]	*MRT*(M)$_{0\text{-}48h}$ [h]	$R(M)_{et}^{a}$
0 d [b]	Liver	1860	19	3.4	1580	20	3.3
	Plasma	550	1.5		480	1.5	
3 d	Liver	1610	28	2.9	1620	26	3.1
	Plasma	550	7.0		530	6.8	
12 d	Liver	1020	20	2.4	1770	22	4.3
	Plasma	430	2.7		410	2.8	

[a] R_{et} means the relative effectiveness of liver targeting, and was calculated using the following equation:

$$R_{et} = \frac{AUC_{0\text{-}48h}^{liver}}{AUC_{0\text{-}48h}^{plasma}}$$

[b] The data in 0 d were from the previous results[131] using normal mice.

advanced stage of liver metastasis at 12 d post-inoculation, the $AUC(C)_{0\text{-}48\,h}^{liver}$ and $AUC(M)_{0\text{-}48\,h}^{liver}$ were increased by 2- and 4.7-fold the values at 3 d post-inoculation, respectively. As shown in Table 2, the $R(C)_{et}$ and $R(M)_{et}$ values at 12d post-inoculation were 5 and 10 times

Table 5-2.
Pharmacokinetic parameters of Suc-FTC after i.v. administration at a dose of 0.2 mg (0.2 ml) per M5076-bearing mouse

Injection day post-inoculation		(A) Concentration			(B) Amount		
		$AUC(C)_{0\text{-}48h}$ [h•μg/ml or g]	$MRT(C)_{0\text{-}48h}$ [h]	$R(C)_{et}^{a}$	$AUC(M)_{0\text{-}48h}$ [h•μg per mouse]	$MRT(M)_{0\text{-}48h}$ [h]	$R(M)_{et}^{a}$
3 d	Liver	320	28	0.05	280	26	0.05
	Plasma	6270	21		5770	21	
12 d	Liver	640	29	0.25	1340	28	0.52
	Plasma	2530	9.9		2600	9.9	

[a] R_{et} means the relative effectiveness of liver targeting, and was calculated using the following equation:

$$R_{et} = \frac{AUC_{0-48\,h}^{liver}}{AUC_{0-48\,h}^{plasma}}$$

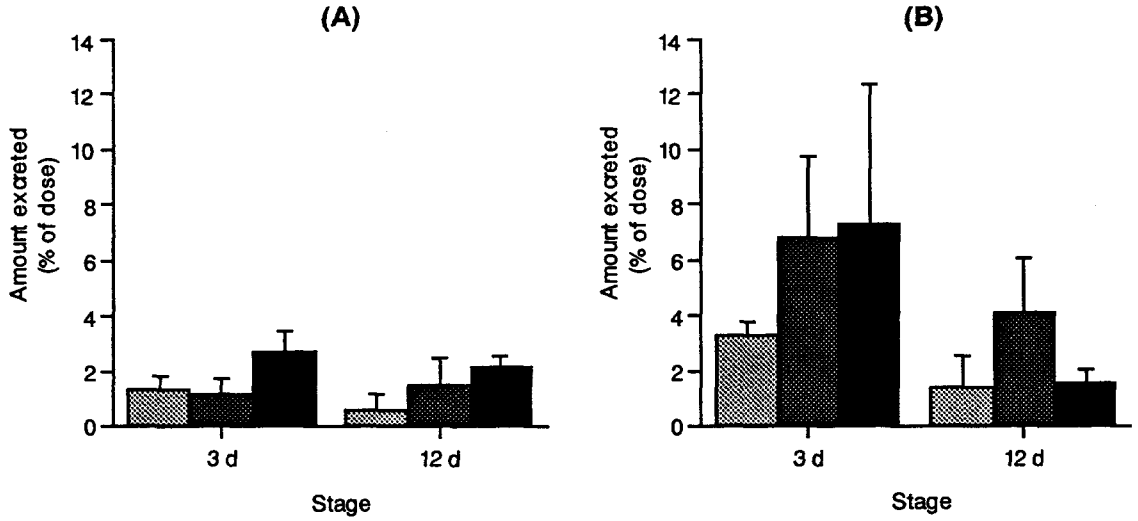


Figure 5-3. Urinary excretion of Lac-Suc-FTC and Suc-FTC after i.v. administration at a dose of 0.2 mg (0.2 ml) per M5076-bearing mouse:
(A) Lac-Suc-FTC (: 8 h, : 24 h, : 48 h.).
(B) Suc-FTC (: 8 h, : 24 h, : 48 h.).
Each column represents the mean ± S.D. (n = 3).

larger than those at 3 d post-injection, respectively. Therefore, the liver localization of Suc-FTC increased as to both concentration and amount.

5-3-3. Urinary excretion

The levels of urinary excretion of Lac-Suc-FTC and Suc-FTC are shown in Figure 5-3. The urine collected in the cage bottle and that withdrawn from the bladder were combined to obtain the total urinary excretion. As shown in Fig. 5-3(A), the amounts of Lac-Suc-FTC excreted were not different between the early and advanced stages of liver metastasis. The urinary excretion of Suc-FTC tended to be lower at 12 d post-inoculation than at 3 d post-inoculation (Fig. 5-3(B)), but it was not significantly different between the two stages ($p>0.05$).

5-3-4. *In vivo* antitumor effect of Suc(II)-MMC and Lac-Suc-MMC against M5076-bearing mice

Figure 5-4(A) illustrates the ratio of survival and the change of body weight when MMC, Suc(II)-MMC or Lac-Suc-MMC was administered at 3 d post-inoculation. Lac-Suc-MMC at a dose of 10 mg eq. MMC/kg was observed to produce the highest *ILS* value, i.e. 91.3 %. The survival on administration of MMC (5 mg/kg), Suc(II)-MMC (10 mg eq. MMC/kg) and Lac-Suc-MMC (10 mg eq. MMC/kg) was significantly different from that of control. However, a significant difference in survival was not found among MMC (5 mg/kg), Suc(II)-MMC (10 mg eq. MMC/kg) and Lac-Suc-MMC (10 mg eq. MMC/kg). A fair loss of body weight was observed in these groups (Fig. 5-4(A-2)). At 10 mg eq. MMC/kg, MMC was lethally toxic, but Suc(II)-MMC and Lac-Suc-MMC were not. The conjugates were considered to be less toxic than MMC. Figure 5-4(B) shows the therapeutic efficacy of both conjugates when administered at 12 d post-inoculation. No markedly long survival was observed in any of the groups. Since lethal toxicity was not observed (Fig. 5-4(B-2)), each drug was considered not to work well against the tumor at the doses tested.

5-3-5. Distribution of free and conjugated MMCs in systemic circulation and liver after i.v. injection of the conjugates

The distribution of free and conjugated MMCs was examined after i.v. administration of Suc(II)-MMC and Lac-Suc-MMC at the dose of 4 mg eq. MMC/kg; this dose was chosen as intermediate dose between low and high doses used in the antitumor tests. Therefore, the result did not reflect exactly the drug distribution in the antitumor tests. However, it should give the biodistribution features of free and conjugated MMCs and permit qualitative evaluation of the distribution of free and conjugated MMCs. The antitumor properties at the dose, 4 mg eq. MMC/kg, will be reported elsewhere. Table 5-3 indicates the plasma and liver concentration of free MMC and total (free and conjugated) MMC at 8 and 24 h after i.v.

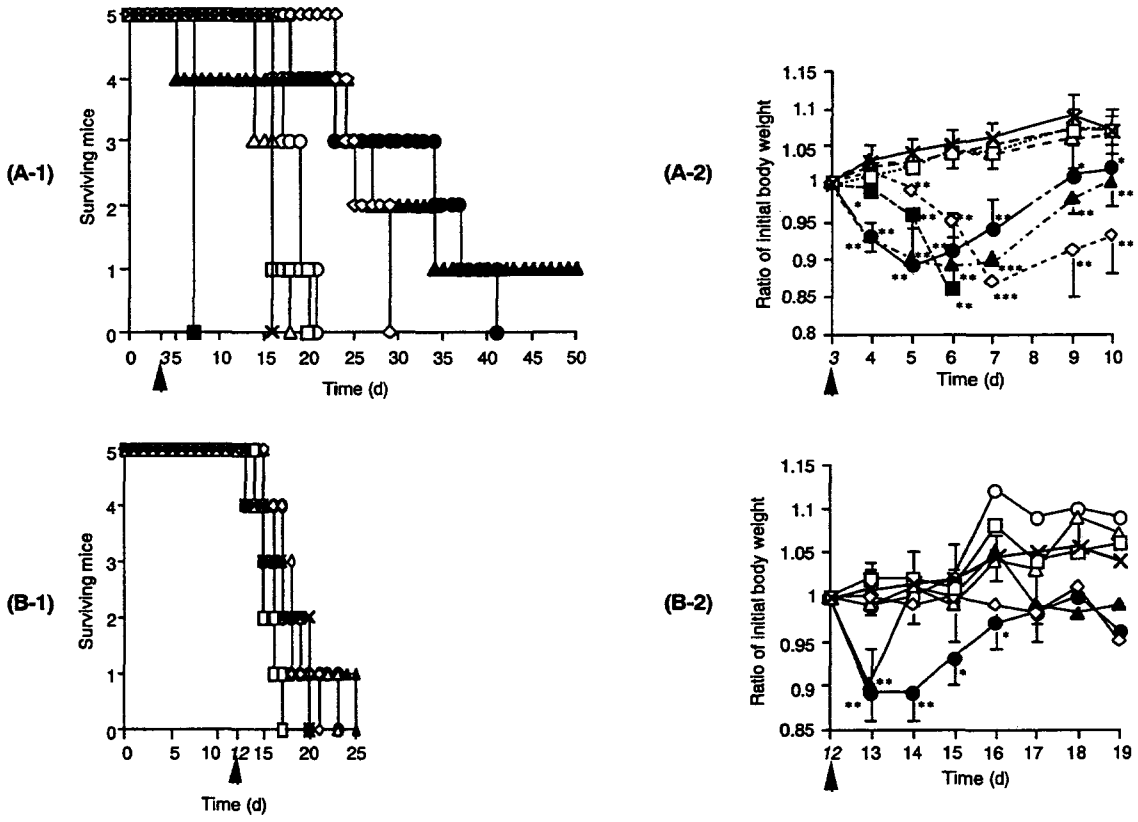


Figure 5-4. Effect of MMC, Suc(II)-MMC and Lac-Suc-MMC on the survival of M5076-bearing mice (A-1, B-1) and change in the mean body weight of mice bearing M5076 (A-2, B-2) after i.v. administration at 3 or 12 d post-inoculation:
Control: × ; MMC 0.4 mg/kg: □ , 5 mg/kg: ◇ , 10 mg/kg: ■ .
Suc(II)-MMC 0.4 mg eq. MMC/kg: △, 10 mg eq. MMC/kg: ▲.
Lac-Suc-MMC 0.4 mg eq. MMC/kg: ○ , 10 mg eq. MMC/kg: ● .
At 3 d (▲) **(A)** or 12 d (▲) **(B)** after inoculation, test substance (0.2 ml) was injected intravenously.
(A-2), (B-2): Each point represents the mean ± S.D. (n = 5). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ vs. control.

administration of Lac-Suc-MMC and Suc(II)-MMC. As to Lac-Suc-MMC, at both 3 and 12 d post-inoculation, MMC was observed in the liver mainly as a conjugated form, and free MMC was scarcely detected; though free MMC was detected at 8 h after injection at 12 d post-inoculation. On the other hand, for Suc(II)-MMC, MMC was hardly observed at all in the liver. At both 3 and 12 d post-inoculation, MMC was detected in plasma as a conjugated form at 8 h after administration. Neither free nor conjugated MMC was detected in the other time points.

5-4. Discussion

In liver metastasis induced by inoculation of M5076, amber flecks were spread out over the liver surface, and the liver became hypertrophic with progression of liver metastasis. In fact, at 12 d post-inoculation the liver was approximately 1.5 times heavier than that of normal mice (data not shown). The liver surface, which is normally glossy, became rugged

Table 5-3.
Plasma or liver concentration of free and total MMCs at 8 and 24 h after i.v. administration of Lac-Suc-MMC and Suc(II)-MMC at 4 mg eq. MMC/kg in mice

Conjugate			Concentration (μg/ml or g)[a]			
			3 d		12 d	
			8 h	24 h	8 h	24 h
Lac-Suc-MMC	Plasma	Free MMC	N.D.[b]	N.D.	N.D.	N.D.
		Total MMC	N.D.	N.D.	N.D.	0.06
	Liver	Free MMC	N.D.	N.D.	0.13 ± 0.08	N.D.
		Total MMC	0.39 ± 0.09	0.21 ± 0.03	0.14 ± 0.09	0.17 ± 0.31
Suc(II)-MMC	Plasma	Free MMC	N.D.	N.D.	N.D.	N.D.
		Total MMC	0.11 ± 0.16	N.D.	0.04 ± 0.05	N.D.
	Liver	Free MMC	N.D.	N.D.	N.D.	N.D.
		Total MMC	N.D.	N.D.	N.D.	N.D.

The detection limit for MMC in HPLC: 0.04 μg/ml.
[a] The results are expressed as the mean ± S.D. of four mice.
[b] N.D. means "non-detected".

with the progression of liver metastasis, and the liver became fragile and crumbly in the advanced stage of metastasis. In normal animals, Lac-Suc was selectively distributed to the liver, and it was shown to be able to behave as a liver targeting drug carrier.[131] However, it is possible that the biodistributions of Lac-Suc-FTC and Suc-FTC are influenced by the pathological state after M5076 inoculation. Therefore, we investigated the biodistributions of Lac-Suc-FTC and Suc-FTC in mice inoculated with M5076 as a liver metastasis tumor model. The experiments were performed at both the early and advanced stages of liver metastasis. There is a possibility that tissue weight, in particular the liver weight, may influence the evaluation of the distribution. Both the concentration and amount of Lac-Suc-FTC or Suc-FTC were checked at each stage of metastasis and the results were evaluated taking the above into account.

It is well known that FITC tagged substances have been found to be stable, non-reactive, non-toxic, little quenching and to behave similarly to the non-tagged parent substance *in vivo*.[8,138] In addition, it was also confirmed that the bond between Suc and fluorescein moiety was stable in the body[37] and that Lac-Suc-FTC was little quenched *in vitro*.[131] Therefore, the stability and the quenching effect of both fluorescent-carriers were considered to be negligible for *in vivo* quantitative analysis in this experiment. Moreover, biodistribution of Suc(II)-FTC was investigated in our previous report.[137] That result indicated that further succinylation of *N*-succinyl-chitosan to 1.1 mol/sugar unit little influenced its biodisposition characteristics. Therefore, Suc-FTC used in this study reflects nearly the biodistribution of Suc(II).

As to the biodisposition of Lac-Suc-FTC, the liver concentration at 3 d post-inoculation at 1 h post-injection, being 11.3 % of dose/g (Fig. 5-1(A-1)), was lower than that at 1 h post-injection in normal mice in which 22.8 % of dose/g.[131] The $AUC(C)_{0\text{-}48\ h}^{liver}$ value at 3 d post-inoculation was slightly lower than that of normal mice (Table 5-1). The hepatic uptake profile might be reduced somewhat by liver tumor metastasis. The total amount recovered from the tested tissues at 48 post-injection of Lac-Suc-FTC was much lower than the dose. Since urinary excretion was also very small, the localization of Lac-Suc-FTC to untested

tissues was possibly high; experiments to test this speculation are in progress. The chitin or chitosan derivatives are supposed to undergo more degradation in tumor-bearing mice because of the elevated lysozymic activity in these animals.[84,85] However, the level of urinary excretion was very low in these metastatic mice (Fig. 5-3) and similar to or less than that in normal mice,[131] which suggested that the biodegradation of Lac-Suc-FTC is hardly altered at all by metastasis.

The biodistribution of Suc-FTC at 3 d post-inoculation was similar to that in normal mice.[37,92] Suc-FTC showed high-level and long-term retention in plasma (Fig. 5-2(A)). However, a small distribution of Suc-FTC was observed in the spleen, lung, liver and kidney. The distribution in the lung was greater initially and decreased from 8 h post-injection. The distribution to the liver and spleen tended to increase with time following i.v. injection. The majority of M5076 cells are distributed to the lung immediately after i.v. inoculation, and then are gradually distributed to other tissues including the liver, kidney and spleen.[139] At the early stage of liver metastasis, i.e. 3 d post-inoculation, M5076 cells are distributed to the tissues mentioned above and are also capable of recirculation. Accordingly, it is conceivable that the phagocytosis of carriers, in this case Suc-FTC, by M5076 cells and the subsequent transfer of the cells may be partly responsible for the tissue distribution. Anyway, at 3 d post-inoculation, the targeting ability of Suc-FTC to the liver was minimal. On the other hand, at 12 d post-inoculation, Suc-FTC was gradually distributed to the liver and spleen and was eliminated from plasma relatively quickly (Fig. 2(B)). The $AUC_{0\text{-}48\ h}^{plasma}$ at 12 d post-inoculation was less than that at 3 d post-inoculation. The liver localization was increased at 12 d post-inoculation; especially, the localized amount was much raised. The increase in liver size was partly responsible, and further the EPR effects may be related to the increase in localization at the advanced stage. At 12 d after inoculation, the amount of Suc-FTC accumulated in the liver (32.4 ± 5.6 μg) was higher than that of Lac-Suc-FTC (20.3 ± 4.8 μg) at 48 h post-injection (Fig 5-2(B-2)), although this difference was not significant (p>0.05). It might be thought that Suc is available for liver targeting at the advanced stages of metastasis, i.e. in the deteriorated liver. The urinary excretion was small at 12 d as well as at

3 d post-inoculation, suggesting that the biodegradation or excretion of Suc is not promoted by metastasis.

One mouse died at 2 d post-administration when treated with Suc(II)-MMC at a dose of 10 mg eq. MMC/kg at 3 d post-inoculation (Fig. 5-4(A-1)). This rapid death was supposed to be due to the reason other than drug toxicity, because Suc(II)-MMC was not lethally toxic at 10 mg eq. MMC/kg according to the previous study on antitumor effect against Sarcoma 180-tumor bearing mice.[104] As previously reported,[36] it was difficult to inject the high viscous conjugated drug solution safely into the vein probably because of prevention of blood flow. The tested samples, i.e. Suc(II)-MMC, were also too viscous especially at a dose of 10 mg eq. MMC/kg. Further, the patterns of the loss of body weight were different between MMC and conjugates. Therefore, it was supposed that the sample viscosity should be responsible for the above rapid death of one mouse with the conjugate solution. The *ILS* values in MMC at 5 mg/kg, Suc(II)-MMC and Lac-Suc-MMC at 10 mg eq. MMC/kg are 62.5, 75.0 and 91.3 (%), respectively. The *ILS* values of Lac-Suc-MMC in M5076-bearing mice tended to be higher than those of Suc(II)-MMC at low and high doses, respectively (data not shown). However, one mouse showed a long survival, i.e. more than 50 d, when administered Suc(II)-MMC at a dose of 10 mg eq. MMC/kg. In addition, if *ILS* value in Suc(II)-MMC at 10 mg eq. MMC/kg was calculated using four mice except one that died at 5 d, the value is more than 110 %. Although MMC at a dose of 5 mg/kg exhibited the similar survival when compared with the conjugates at a dose of 10 mg eq. MMC/kg, both conjugates extremely show the elongation of the lifespan by other schedule (unpublished observation); this observation will be reported elsewhere. On the other hand, when administered at 12 d post-inoculation, no significant elongation of survival was observed with either conjugate (Fig. 5-4(B-1)). The administration of conjugates at 12 d post-inoculation appeared to be too late to cure the mice.

The distribution of MMC after i.v. injection of free MMC was not performed, because no MMC was detected at 8 h when injected to mice at 5 mg/kg.[104] The liver localization of Lac-Suc-MMC was superior to that of Suc(II)-MMC, which was consistent with the

biodistribution profiles of their carriers; however, these values in Table 5-3 were lower than those calculated from the biodistribution study using FITC-labeled polymers. One explanation for these results may be that MMC, whether large or small, was degraded in acidic conditions or by enzyme.[81,140] Table 5-3 also suggested that not only the concentration of MMC in the liver but also that in the systemic circulation is one of major factors affecting the antitumor activity because Suc(II)-MMC, not detected in the liver at both tested time points, exhibited a good antitumor effect similar to Lac-Suc-MMC. Long systemic circulation of MMC might be available for efficacy on liver metastasis; long systemic retention of Suc(II)-MMC permitted the long retention of free MMC by the gradual release in the blood stream.[137] Further, Lac-Suc-MMC was considered to be targeted predominantly to the liver parenchymal cells. For Lac-Suc-MMC to be effective against a tumor, the drug targeted to the parenchymal cells must escape inactivation and diffuse to the diseased region. Although Lac-Suc-MMC was well targeted to the liver, it did not show antitumor effect surpassing that of Suc(II)-MMC, which might be related to instable properties of MMC in acidic or biological medium.[81,140] In short, although Lac-Suc-MMC was taken up by liver cells, MMC released from Lac-Suc-MMC there might be easily degraded in cells by acidic pH and metabolic enzymes before exhibiting action at the surrounding diseased part. Such inactivation is more serious for Lac-Suc-MMC localized into liver cells in comparison with Suc(II)-MMC residing long in the blood flow.

In conclusion, this study clarified the localization characteristics of Lac-Suc and Suc in the liver at the early and advanced stages of liver metastasis. Lac-Suc was concentrated more effectively in the early metastatic stage, while Suc was localized more to the liver in the advanced stage of liver metastasis. The conjugates of MMC with Lac-Suc and Suc functioned effectively in the early metastatic stage. Namely, in early metastatic stage, it was considered that both systemic long-retention and liver localization of MMC tended to be available for effectiveness against M5076 metastasis. Thus, both carriers were found potentialities as drug carriers for therapeutics of liver metastatic tumor in the early stage. The examination of therapeutic efficacy of Lac-Suc-MMC and Suc(II)-MMC with various

schedules is taken up in the next chapter.

Chapter 6

Efficacy of Lactosaminated and Intact *N*-Succinyl-chitosan-mitomycin C Conjugates against M5076 Liver Metastatic Cancer

Summary

In this chapter, Lac-Suc was investigated for its liver targeting ability in the early liver metastatic stage, and subsequently Lac-Suc-MMC and Suc(II)-MMC were examined for efficacy against the liver metastasis. Mice to which M5076 cells were inoculated intravenously were used as liver metastatic animals. Lac-Suc-FTC was intravenously administered at a daily dose of 0.2 mg/mouse × 4 d or at a single dose of 0.8 mg/mouse × 1 d at 3 d post-inoculation. At a dose of 0.2 mg/mouse × 4 d, liver accumulation of Lac-Suc-FTC was increased after all except the fourth injection, indicating that the capacity of accumulation might be limited to around 110 μg per mouse with repeated daily administration at 0.2 mg/mouse. As to efficacy in i.v. administration at 7 d post-inoculation, Lac-Suc-MMC was less effective at 1 mg/kg × 4 d than at a single dose of 4 mg/kg × 1 d. This result was not in accordance with that expected from the biodistribution study. On the other hand, in i.v. administration at 3 d post-inoculation, Suc(II)-MMC was more effective at repeated administration, and it showed higher efficacy than Lac-Suc-MMC at both 1 mg/kg × 4 d and 4 mg/kg × 1 d. Further, in i.v. administration at 3 d post-inoculation, Suc(II)-MMC exhibited much higher survival effect at a dose of 4 mg/kg × 4 d.

6-1. Introduction

We have been studying the possible usefulness of the chitosan derivative Suc as a drug carrier.[36,37,72,81,92,104,131] Since it is retained for long periods in the systemic circulation after i.v. administration[37,92] and has low toxicity,[72,73] Suc is expected to be useful as a safe systemic long-circulating carrier. Recently, there has been a great deal of interest in liver targeting using various glycosylated macromolecules,[56,57,111,113,114,141] microparticles with suitable diameter[41,142] and liposomes[50,143] as drug carriers. Similarly, it was confirmed that connecting lactose to Suc enabled selective distribution to the liver in mice.[131]

It is important for cancer chemotherapy to determine the administration schedules that present higher efficacy and lower toxicity.[144-146] In the case of polymer-drug conjugates, biodistribution of the polymer is importantly related to the therapeutic efficacy. In the liver metastatic tumor model used in this study, i.e. M5076 cells,[135,139,147] differences in the timing of injection following inoculation influenced the biodistributions of Suc and Lac-Suc.[148] Therefore, biodistribution characteristics dependent on the administration schedule, i.e. single or repeated administration, have to be elucidated. We investigated differences in biodistribution of Lac-Suc between single and repeated administration. Further, in the present study, antitumor effects of Lac-Suc-MMC against M5076 cells as a liver metastatic tumor model were examined after intravenous administration at single or repeated administration. Similarly, the antitumor effects of water-soluble Suc(II)-MMC against M5076 cells were also examined. Finally, administration schedules of Lac-Suc-MMC and Suc(II)-MMC were evaluated based on these results from the viewpoint of efficacy.

6-2. Materials and methods

6-2-1. Materials

MMC was purchased from Kyowa Hakko Kogyo Co. (Tokyo, Japan). Suc

(succinylation degree 0.81 mol/sugar unit, deacetylation degree 1.0 mol/sugar unit) was kindly provided by Katakura Chikkarin Co., Ltd. (Tokyo, Japan). Its degrees of succinylation and deacetylation were measured using proton nuclear magnetic resonance,[92] and the average MW was determined to be 3.4×10^5 (range: $5 \times 10^4 - 1.5 \times 10^6$) by SEC-MALS. EDC was purchased from Dojindo Laboratories (Kumamoto, Japan). Lactose and FITC were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Sodium cyanoborohydride ($NaBH_3CN$) was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). All other chemicals were purchased as reagent-grade products.

6-2-2. Animals and tumors

Male C57BL/6 mice weighing approximately 20 g at the age of 6 weeks were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Hoshi University. All mice were housed in a pathogen-free environment. Five or six animals were used in each group to examine *in vivo* antitumor effects (n = 5, 6). Four or five mice were used at each time point in distribution experiments (n = 4, 5).

Murine reticulum cell sarcoma M5076 cells were used as tumor cells. M5076 cells were maintained in C57BL/6 mice by i.p. transfer of 1×10^5 cells obtained from ascitic fluid every other week. In the *in vivo* antitumor activity tests, 1×10^5 M5076 cells suspended in 0.1 ml of Hanks' balanced salt solution, which were obtained from tumor-bearing mice, were inoculated intravenously into each male C57BL/6 mouse.

6-2-3. Accumulation of Lac-Suc-FTC into the liver at repeated administration

Lac-Suc was prepared by reductive amination between Suc and lactose using $NaBH_3CN$, and Lac-Suc-FTC was prepared by reaction of Lac-Suc and FITC as described previously.[131] The lactosamine residue content of Lac-Suc was determined to be approx. 0.30 mol/sugar unit by elemental analysis (Yanako Analytical Industrial Co., Japan). Lac-Suc-FTC was used throughout the biodistribution studies. The distribution of Lac-Suc-FTC

in M5076-bearing mice was examined as follows: After i.v. inoculation with M5076 cells, 0.8 mg of Lac-Suc-FTC was administered intravenously on day 3 (single dose) or 0.2 mg of Lac-Suc-FTC was intravenously injected repeatedly on each of days 3 – 6 (repeated dose). In the case of repeated administration, the mice were sacrificed at 1, 8 or 24 h after each injection, blood samples were withdrawn and the liver was enucleated. The liver was washed with PBS gently blotted using filter paper and weighed. A three-fold volume of PBS was added, and the mixture was homogenized using a glass homogenizer with a Teflon pestle. The supernatant was obtained by centrifugation (3,000 rpm, 10 min). Plasma was obtained by centrifugation of the blood. The supernatant and plasma were diluted appropriately with PBS, and their fluorescence intensities were investigated (Ex = 495 nm, Em = 520 nm). The blank sample was obtained by injecting normal saline alone into mice instead of Lac-Suc-FTC solution. The concentration of Lac-Suc-FTC in the sample was determined from the net fluorescence intensity obtained by subtracting the fluorescence intensity of the blank from that of each sample based on the standard calibration curve. The concentration was corrected by the recoveries calculated in advance.[131] The distributed amount was calculated from the concentration and tissue weight. The amount of Lac-Suc-FTC in plasma was calculated using the reported volume of mouse plasma, 48.8 ml/kg.[83] To determine the biodistribution of Lac-Suc-FTC administered at a single dose, blood and liver samples were taken at 1, 8, 24, 48, 72 and 96 h after i.v. injection. The distributed Lac-Suc-FTC was measured and determined in the same manner as described for the repeated administration schedule.

The cumulative collection of urine was executed simultaneously in the biodistribution studies. Mice were placed separately in metabolite cages immediately after administration. Urine was collected for 24 h after each i.v. injection (repeated dose) or 24, 48, 72 and 96 h after i.v. administration (single dose), and then urine volume was measured. The subsequent procedure was performed as reported previously.[92] The total amount excreted in urine was calculated from the concentration and urine volume.

6-2-4. Pharmacokinetic data analysis

The areas under the plasma or liver concentration-time curves for t_1 - t_2 h ($AUC_{t1\text{-}t2\ h}$) and the mean residence time ($MRT_{t1\text{-}t2\ h}$) were calculated by the trapezoidal method.[96] The following equation was employed to determine relative effectiveness of liver targeting (R_{et}):

$$R_{et} = AUC_{t1-t2\ h}^{liver} / AUC_{t1-t2\ h}^{plasma}$$

6-2-5. *In vitro* release characteristics of Lac-Suc-MMC

Lac-Suc-MMC conjugate was synthesized as reported previously except that Lac-Suc was used instead of highly-succinylated Suc.[104] The release of MMC from Lac-Suc-MMC in 1/15 *M* phosphate buffer (pH 6.0, and 9.0) and in the mixture of 1/15 *M* phosphate buffer (pH 7.4) and mouse plasma (4 : 1, v/v), named 20 % (v/v) mouse plasma, were investigated according to the method described in the previous report.[104] Shortly, the amount of free MMC released was measured using high performance liquid chromatography (HPLC), which was carried out using a Shimadzu LC-6AD apparatus equipped with a SUMIPAX Nucleosil $5C_{18}$ reversed phase column (4 × 250 mm) and an SPD-10AV UV detector (Shimadzu) set at 365 nm. The mobile phase was a mixture of 0.01 *M* phosphate buffer, pH 6.0, and methanol (65 : 35, v/v). As to the samples obtained from the buffers, the sample solution was directly injected on the HPLC system. As regards the samples obtained from 20 % (v/v) mouse plasma, they were firstly mixed to a 10-fold volume of the mixture of chloroform and 2-propanol (1 : 1, v/v). After centrifuging the mixture, the whole supernatant was decanted and evaporated to dryness below 40 °C under nitrogen gas. The residue was dissolved in methanol, and the solution was analyzed for MMC in the HPLC system stated above. All determinations were performed using three samples.

6-2-6. Antitumor effects of Suc(II)-MMC or Lac-Suc-MMC against M5076-bearing mice

Suc(II))was prepared by reaction of Suc with succinic anhydride and then a water-soluble Suc(II)-MMC was also prepared.[104] MMC contents of Suc(II)-MMC and Lac-Suc-MMC, measured in the manner previously reported,[104] were about 12 and 20 % (w/w),

respectively. The doses reported for MMC conjugates refer to the quantity of parent MMC contained in the conjugate.

Antitumor effects were examined using mice intravenously inoculated with M5076 cells 7 d previously. Namely, at 7 d post-inoculation, MMC, Suc(II)-MMC and Lac-Suc-MMC were intravenously administered at a single dose of 4 mg/kg or repeatedly at a dose of 1 mg/kg on each of days 7 – 10 following inoculation, i.e. at a total dose of 4 mg/kg. Controls were injected with a similar volume of saline alone according to the same schedules. For all mice, the survival time after inoculation was observed for 50 d after inoculation. The antitumor effects were measured by comparing the mean survival time of the treated mice (T) with that of controls (C), i.e. from ILS calculated by the following equation:

$$ILS = (T/C - 1) \times 100\ (\%)$$

At the same time, the changes in body weight of each group were measured to evaluate the toxic side effects.

Antitumor effects using mice intravenously inoculated with M5076 cells 3 d previously were also investigated in the same way as stated above for treatment at 7 d post-inoculation. In the mice treated at 3 d post-inoculation, the efficacies of Suc(II)-MMC and Lac-Suc-MMC were also examined by i.v. administration at a dose of 4 mg/kg × 4 d.

6-2-7. Statistical analysis

With the exception of the survival tests, the statistical analysis was performed using Student's t-test for unpaired data. For the survival tests, Kaplan-Meier curves were constructed and the survival ratios were compared by the Mantel-Cox log-rank test. Differences were considered significant when the p-value was less than 0.05.

6-3. Results

6-3-1. Accumulation of Lac-Suc-FTC in the liver

The distribution profiles to the liver and the elimination from plasma of Lac-Suc-FTC after i.v. administration are shown in Figure 6-1. The amount of Lac-Suc-FTC in plasma was calculated using the reported volume of mouse plasma, 48.8 ml/kg.[83] The distribution of Lac-Suc-FTC to the liver at a single dose reached 66 μg per mouse at 8 h after injection, and remained at this level throughout the 96 h observation period. On the other hand, following repeated administration the plasma level of Lac-Suc-FTC declined rapidly at a similar rate after each injection, and accumulation in the liver showed a similar profile until 72 h. The distribution profile after the fourth injection suggested that the accumulation might reach a plateau approximately 110 μg per mouse.

Pharmacokinetic parameters of Lac-Suc-FTC administered at single dose or by repeated injection are summarized in Table 6-1. $AUC_{0\text{-}96h}^{liver}$ following injection four times at a dose of 0.2 mg was larger than that after a single injection at a dose of 0.8 mg. Conversely, $AUC_{0\text{-}96h}^{plasma}$ of the former was lower than that of the latter. R_{et} following repeated administration was double that following a single injection; i.e. repeated administration of Lac-Suc-FTC is considered more effective for liver targeting than a single injection.

Table 6-2 shows the cumulative urinary excretion of Lac-Suc-FTC. The urinary excretion of Lac-Suc-FTC was much lower with repeated administration.

6-3-2. *In vitro* release characteristics of Lac-Suc-MMC

Figure 6-2 shows the in vitro release properties of Lac-Suc-MMC. The release profiles from Suc(II)-MMC were described in the same figure by referring to the previous paper.[104] Lac-Suc-MMC showed pH-dependent release similarly to Suc(II)-MMC. Lac-Suc-MMC showed faster release to some extent than Suc(II)-MMC. Fifty % MMC release time of Lac-Suc-MMC was about 1 d in 20 % (v/v) mouse plasma. Under each condition, similar release profiles were observed for both the conjugates.

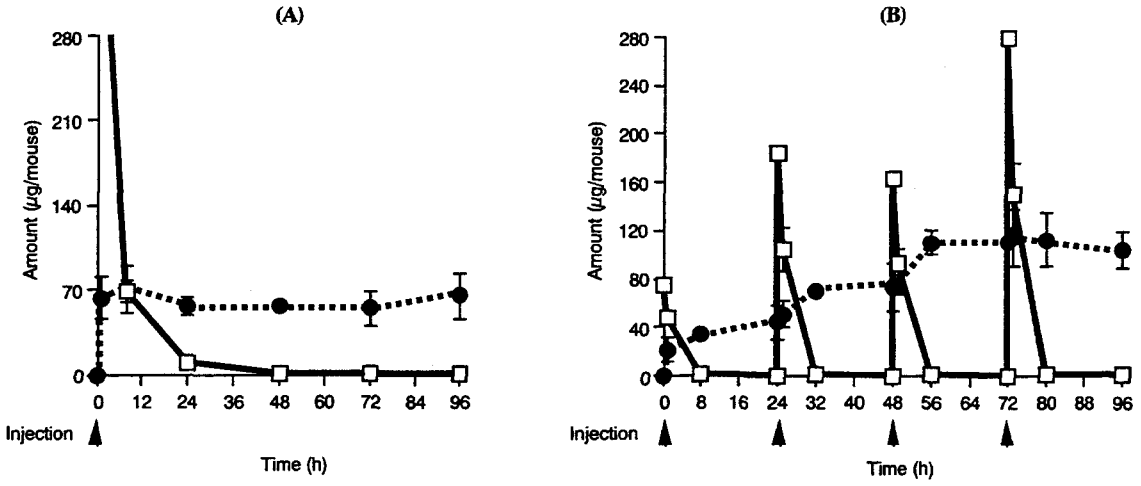


Figure 6-1. Plasma level and accumulation of Lac-Suc-FTC to the liver after i.v. administration at a dose of 0.8 mg × 1 d (A) or 0.2 mg × 4 d (B) per M5076-bearing mouse: —□— ; plasma, —●— ; liver. The arrow shows i.v. injection. At 3 d after i.v. inoculation, test substance (0.2 ml) was injected intravenously. Each point represents the mean ± SD (n = 4 - 5).

Table 6-1.
Pharmacokinetic parameters of Lac-Suc-FTC after i.v. administration at a single dose of 0.8 mg (0.2 ml) or repeated injection at 0.2 mg (0.2 ml) × 4 d per M5076-bearing mouse at 3 d post-inoculation.

Dose		$AUC_{0\text{-}96\,h}$ [h•μg/ml or g]	$MRT_{0\text{-}96\,h}$ [h]	R_{et}[a]
Single	Liver	5330	47	1.6
	Plasma	3400	5.5	
Repeated	Liver	8010	59	3.2
	Plasma	2530	5.0	

[a] R_{et} values indicate the relative effectiveness of liver targeting, and were calculated by the following equation:

$$R_{et} = \frac{AUC^{liver}_{0\text{-}96\,h}}{AUC^{plasma}_{0\text{-}96\,h}}$$

6-3-3. Antitumor effects of Suc(II)-MMC and Lac-Suc-MMC against M5076

Table 6-3 shows the therapeutic efficacy of Suc(II)-MMC and Lac-Suc-MMC at a

Table 6-2.
Urinary excretion of Lac-Suc-FTC after i.v. administration at a single dose of 0.8 mg (0.2 ml) or repeated injection at 0.2 mg (0.2 ml) × 4 d per M5076-bearing mouse at 3 d post-inoculation.

Dose	Urinary excretion (μg)			
	24 h	48 h	72 h	96 h
Single	20.1 ± 9.4	26.9 ± 11.5	32.8 ± 6.9	38.0 ± 6.9
Repeated	3.1 ± 2.4	2.6 ± 1.4	7.1 ± 5.0	9.5 ± 6.7

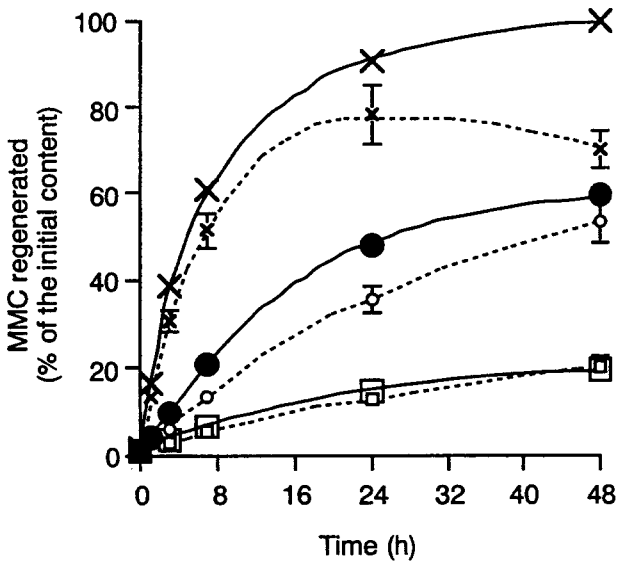


Figure 6-2. Release of MMC from Lac-Suc-MMC and Suc(II)-MMC in 1/15 *M* phosphate buffer (pH 6.0 and 9.0) and the mixture of 1/15 *M* phosphate buffer (pH 7.4)-mouse plasma (4 : 1, v/v) at 37 °C: □ , pH 6.0; × , pH 9.0; ● , ○ , plasma-buffer mixture. Each point represents the mean ± SD (n = 3). Large symbols show the release of MMC from Lac-Suc-MMC, whereas small symbols show that from Suc(II)-MMC. The data of Suc(II)-MMC are from our previous report.[104]

single or repeated dose using mice inoculated 7 d previously with M5076 cells. In this case, the best *ILS* value was observed at a dose of 4 mg/kg × 1 d in MMC; however, this dose caused the greatest loss of body weight, which did not lead to death (data not shown). Suc(II)-MMC at a dose of 4 mg/kg × 1 d showed a similar good *ILS* value, with no significant loss of body weight. Lac-Suc-MMC exhibited a lower *ILS* value than MMC and Suc(II)-

MMC at a dose of 4 mg/kg × 1 d. On the whole, the groups treated with a single injection, i.e. 4 mg/kg × 1 d, exhibited better survival than those at given repeated injection. No drug exhibited an increase in *ILS* at a dose of 1 mg/kg × 4 d.

The therapeutic efficacy of Lac-Suc-MMC and Suc(II)-MMC at a single (1 mg/kg × 4 d) or repeated dose (1 mg/kg × 4 d) using mice inoculated 3 d previously with M5076 cells is also shown in Table 6-3. The *ILS* value of the mixture of Lac-Suc and MMC at a dose of 4 mg/kg × 1 d was over 100 %, which was observed as the best *ILS*. The survival patterns given by MMC at 3 d post-inoculation were similar to those at 7 d post-inoculation; that is, a single dose showed a high degree of efficacy but repeated administration was hardly effective. On the other hand, for Lac-Suc-MMC, a moderate *ILS* was obtained following repeated injection and was similar to that following a single injection. The best *ILS* value was observed in the group treated with Suc(II)-MMC at a dose of 1 mg/kg × 4 d, and two of 5 mice in this group survived for over 50 d. Suc(II)-MMC showed better therapeutic efficacy than MMC. Thus, the pattern of efficacy of Suc(II)-MMC at 3 d post-inoculation was markedly different from that at 7 d post-inoculation. The loss of body weight was observed in the groups treated with MMC, Lac-Suc plus MMC, Suc(II) plus MMC and Suc(II)-MMC at a dose of 4 mg/kg × 1 d, but it was not lethal (data not shown). In the group treated with Suc(II)-MMC at a dose of 1 mg/kg × 4 d at which the best *ILS* value was observed, loss of body weight was slight.

The therapeutic efficacy of Lac-Suc-MMC and Suc(II)-MMC against M5076-bearing mice when test solutions were administered intravenously at a dose of 4 mg/kg on each of days 3 – 6 following inoculation, i.e. at a dose of 4 mg/kg × 4 d is also shown in Table 3 and Figure 6-3. The *ILS* values of both groups treated with both conjugates were over 100 %. Four of 6 mice in the group treated with Suc(II)-MMC survived for over 50 d. Control mice died within 17 d post-inoculation. No loss of body weight was observed in the group treated with Lac-Suc-MMC (data not shown). In the group treated with Suc(II)-MMC, an initial loss of body weight was observed a little, but the body weight recovered to the initial level at 1 week after treatment (data not shown).

Table 6-3.
***ILS* values of MMC, Suc(II)-MMC and Lac-Suc-MMC in different schedules**

Administration schedule	Substances	Single administration[a]		Repeated administration[b]	
		Dose	*ILS* (%)	Dose	*ILS* (%)
7 d post-inoculation	Control	——	——	——	——
	MMC	4 mg/kg × 1 d	59.5**	1 mg/kg × 4 d	0
	Suc(II)-MMC	4 mg/kg × 1 d	54.8**	1 mg/kg × 4 d	3.6
	Lac-Suc-MMC	4 mg/kg × 1 d	23.8	1 mg/kg × 4 d	-6.0
3 d post-inoculation	Control	——	——	——	——
	MMC	4 mg/kg × 1 d	98.6**	1 mg/kg × 4 d	18.8**
	Lac-Suc-MMC	4 mg/kg × 1 d	56.5**	1 mg/kg × 4 d	42.0*
	Lac-Suc	(16 mg/kg)[c]	8.7		
	Lac-Suc + MMC	4 mg/kg × 1 d	156.5**		
	Control	——	——	——	——
	MMC	4 mg/kg × 1 d	37.8**		
	Suc(II)-MMC	4 mg/kg × 1 d	> 97.3**	1 mg/kg × 4 d	> 185.1**
	Suc(II)	(29 mg/kg)[c]	16.2		
	Suc(II) + MMC	4 mg/kg × 1 d	> 91.9**		
	Control			——	——
	Suc(II)-MMC			4 mg/kg × 4 d	> 192.5**
	Lac-Suc-MMC			4 mg/kg × 4 d	118.8**

[a] At 3 or 7 d post-inoculation, each substance was intravenously administered at a dose of 4 mg/kg.
[b] Each substance was intravenously administered repeatedly at a dose of 1 mg/kg on each of days 3 - 6 or 7 - 10 post-inoculation.
[c] Lac-Suc or Suc(II) was administered at a dose of 16 or 29 mg polymer eq/kg, respectively.
*: $p<0.05$, **: $p<0.01$ vs. control [Mantel-Cox log-rank test].

6-4. Discussion

As shown in Figure 6-1(B), accumulation of Lac-Suc-FTC into the liver according to the repeated administration schedule implied that the capacity of accumulation might be limited to around 110 μg per mouse in the case of daily repeated administration of Lac-Suc-

FTC at 0.2 mg/mouse. Table 6-1 also suggested that repeated administration was superior to a single injection with regard to liver distribution. Lac-Suc-MMC and Suc(II)-MMC were shown to act as prodrugs, and both showed a similar release profile (Figure 6-2). Intravenous administration of MMC at 2 – 5 mg/kg was known effective *in vivo*;[104,137,145] therefore, in this study, the dose of MMC was selected at 4 mg/kg. The dose of Suc(II)-MMC and Lac-Suc-MMC was chosen at 4 mg eq. MMC/kg for comparison with free MMC (Table 6-3). As to repeated administration, this dose was divided into 1 mg eq. MMC/kg/day for the consecutive 4 d (Table 6-3). The further trial was planned based on these results (Figure 6-3). With administration at 7 d post-inoculation, the therapeutic efficacy of Lac-Suc-MMC was in contrast to the results of the biodistribution study. Further, the observations that the antitumor effect of Lac-Suc-MMC was less than that of MMC suggested that Lac-Suc-MMC is taken up by hepatic cells but not targeted to tumor cells, resulting in insufficient drug concentration in tumor sites. The antitumor effect of Suc(II)-MMC, acting as a prodrug of MMC, was similar to that of MMC. However, MMC caused a decrease in body weight, while Suc(II)-MMC did not (data not shown). Therefore, Suc(II)-MMC was considered to be a better antitumor agent due to its reduced side effects. Further, the timing of administration was considered to be an important factor because chemotherapy was hardly effective in the highly advanced metastasis stage.[148] Namely, since the disease severity was lower in the early stage, earlier treatment was supposed to result in better efficacy of the drugs. In the groups treated at 3 d post-inoculation, Lac-Suc-MMC showed similar survival-enhancing effects with both single and repeated injections (Table 6-3). In these experiments, the mixture of MMC and Lac-Suc showed a very high *ILS* value (over 100 %). This phenomenon may be explained by a synergistic effect between the immunostimulatory effect of Lac-Suc and antitumor activity of MMC because some chitin and chitosan derivatives tend to stimulate the immune system including macrophages.[149,150] Mice treated with Suc(II)-MMC showed longer survival when treated by repeated administration rather than at a single dose. Suc(II)-MMC showed a much higher *ILS* value than MMC and Lac-Suc-MMC when administered according to the repeated treatment schedule. These results suggested the

better availability of Suc(II)-MMC at a multiple dose in the early metastasis stage. To pursue further therapeutic efficacy, Lac-Suc-MMC and Suc(II)-MMC were intravenously administered repeatedly at a daily dose of 4 mg/kg on days 3 – 6 post-inoculation, i.e. at a dose of 4 mg/kg × 4 d in M5076-bearing mice. The survival of mice treated with each conjugate was markedly improved in comparison with those treated at a dose of 1 mg/kg × 4 d or 4 mg/kg × 1 d. This indicated increases in dose raised the therapeutic efficacy. Especially, Suc(II)-MMC exhibited a very strong antitumor effect. This conjugate may become a useful prodrug for metastatic cancer if the loss of body weight can be improved. A good result was also obtained with Lac-Suc-MMC, which was associated with no loss of body weight; Lac-Suc-MMC was considered to be less toxic than Suc(II)-MMC, which might be due to the suppression of the systemic flow of MMC in Lac-Suc-MMC.

On the whole, the antitumor effects of Lac-Suc-MMC against M5076-bearing mice were inferior to those of Suc(II)-MMC. Duncan et al reported that hepatic targeting via asialoglycoprotein receptors of the liver was not necessarily effective against liver metastasis, and indicated that a simple macromolecular conjugate not containing galactose might act better in animal models of liver metastasis.[151] This was considered to be because the conjugate was not targeted directly to the metastatic cells but to the parenchymal cells. For effectiveness against metastasis, the drug targeted to the parenchymal cells must escape from inactivation and diffuse to the diseased region. Therefore, the following considerations are proposed based on the results described above. Lac-Suc-MMC is rapidly eliminated from the systemic flow and distributed to the liver parenchymal cells by specific binding to the asialoglycoprotein receptors. Drug released in or on the liver parenchymal cells is related to the efficacy. Therefore, the biological stability of the drug will influence its effects on the surrounding tumor tissue. Since MMC is very unstable in the liver,[90,81] MMC released from Lac-Suc-MMC in the liver parenchymal cells is thought not to be able to reach the tumor cells sufficiently. On the other hand, intact Suc is much less interactive with body tissues and is maintained for long periods in the systemic circulation after i.v. injection,[37,92] which is considered to be due to the lack of specific ligands and its physicochemical properties such as

anionic charge. Therefore, drugs combined with Suc can be liberated on or in various cells around the sites at which the carrier is distributed. The systemic long-term circulation at higher level of Suc(II)-MMC may permit its effective and long-term access to the whole liver and consequently facilitate the supply of liberated MMC or endocytosis of the conjugate by liver metastatic M5076 cells. Thus, the antitumor characteristics of both the conjugates could be evaluated well by the physiological behaviors of their carriers.

6-5. Conclusion

Following administration at 7 d post-inoculation, the therapeutic efficacy of repeated injection of Lac-Suc-MMC (1 mg/kg × 4 d) was inferior to that of Lac-Suc-MMC at a single dose (4 mg/kg × 1 d), and Lac-Suc-MMC was much less effective than MMC. Lac-Suc-MMC showed similar antitumor effects between single and repeated injection schedules with administration at 3 d post-inoculation. These results were not in accordance with those of the biodistribution studies. In contrast, at both 7 d and 3 d post-inoculation, Suc(II)-MMC showed similar or higher therapeutic efficacy in comparison with MMC and Lac-Suc-MMC. Further, the efficacy of Suc(II)-MMC at a repeated dose was better than that at a single dose except for the administration at 7 d post-inoculation. At 3 d post-inoculation, Suc(II)-MMC exhibited very high survival effect at a dose of 4 mg/kg × 4 d. The present results demonstrated the high therapeutic efficacy could be achieved at repeated administration of Suc(II)-MMC in the early metastatic stage.

Conclusions

In this thesis, potentialities of *N*-succinyl-chitosan as a water-soluble drug carrier were demonstrated from the following findings: i) a long systemic circulation, ii) accumulation in the solid tumor, iii) ability to combine conjugates with MMC, iv) active targeting to the liver by attaching lactose and v) a high antitumor activity of its conjugates with MMC without lethal side effects against Sarcoma 180 solid tumor and M5076 liver metastasis. The findings of the present study are summarized as follows:

Chapter 1:

i) Suc-FTC was sustained at a high level in the circulaton over 72 h; that is, the plasma half-life in normal mice was 100.3 h and that in tumor-bearing mice was 43.0 h.

ii) Suc-FTC was distributed very little in tissues other than the tumor.

Chapter 2:

i) Smaller amounts of EDC, more advanced succinylated Suc (Suc(II)) and shorter coupling reaction time were selected as a conjugation reaction condition. Almost half the obtained conjugates was water-soluble.

ii) Suc(II)-MMC showed less toxicity than MMC and a strong *in vivo* antitumor effect against Sarcoma 180 at a high dose in i.v. administration.

iii) MMC disappeared quickly from systemic circulation after i.v. administration, while Suc(II)-MMC was retained at a high concentration of greater than 15 % of the dose/ml for MMC.

Chapter 3:

i) It took a few hours for Suc(II)-FTC to be transferred to the blood circulation after i.p. administration. There were no marked differences in the distribution of Suc(II)-FTC between i.v. and i.p. administration routes except in the early stage.

ii) The urinary excretion of Suc(II)-FTC following both i.v. and i.p. administration was small, but the excretion tended to be suppressed after i.p. administration.

iii) Suc(II)-MMC tended to be more toxic at i.p. administration than at i.v. administration.

iv) More localization of the conjugate in peripheral tissues and less excretion were found at i.p. administration, which might result in greater toxicity.

Chapter 4:

i) Suc could be easily modified into Lac-Suc tagged with lactose by reductive amination using sodium cyanoborohydride.

ii) After i.v. injection, Lac-Suc was cleared much faster from the systemic circulation than Suc itself, and simultaneously a considerable amount of Lac-Suc was quickly taken up by the liver via the asialoglycoprotein receptor. At practical doses, the liver accumulation of Lac-Suc was dependent on the dose, and the Lac-Suc localized into the liver was retained there for a long period.

Chapter 5:

i) Lac-Suc was concentrated more effectively in the early metastatic stage, while Suc was localized more to the liver in the advanced stage of liver metastasis.

ii) The conjugates of MMC with Lac-Suc and Suc(II) functioned effectively in the early metastatic stage. Namely, in early metastatic stage, it was considered that both systemic long-retention and liver localization of MMC tended to be available for effectiveness against M5076 metastasis. Thus, both carriers were found potentialities as drug carriers for therapeutics of liver metastatic tumor in the early stage.

Chapter 6:

i) Following administration at 7 d post-inoculation, the therapeutic efficacy of repeated injection of Lac-Suc-MMC (1 mg/kg × 4 d) was inferior to that of Lac-Suc-MMC at a single dose (4 mg/kg × 1 d), and Lac-Suc-MMC was much less effective than MMC.

ii) Lac-Suc-MMC showed similar antitumor effects between single (4 mg/kg × 1 d) and repeated injection (1 mg/kg × 4 d) schedules in administration at 3 d post-inoculation. These results were not in accordance with those of the biodistribution studies.

iii) At both 7 d and 3 d post-inoculation (4 mg/kg × 1 d; 1 mg/kg × 4 d), Suc(II)-MMC showed similar or higher therapeutic efficacy in comparison with MMC and Lac-Suc-MMC.

iv) The efficacy of Suc(II)-MMC at a repeated dose (1 mg/kg × 4 d) was better than that at a single dose (4 mg/kg × 1 d) except for the administration at 7 d post-inoculation.

v) At 3 d post-inoculation, Suc(II)-MMC exhibited very high survival effect compared with Lac-Suc-MMC at a dose of 4 mg/kg × 4 d.

vi) The high therapeutic efficacy could be achieved at repeated administration of Suc(II)-MMC in the early metastasis stage.

Chitin and chitosan derivatives are considered to play an important role in drug carrier system using macromolecule because these are one of the most abundant polysaccharides in nature. Especially, *N*-succinyl-chitosan is thought to be very useful as a drug carrier due to the very long systemic circulation in comparison with other macromolecular carriers. Further, Suc can bind to many kinds of drugs because Suc has many carboxyl groups and a few amino group. For the perspective, the present results which the high therapeutic efficacy could be achieved in the solid tumor and the liver metastasis show that Suc has the possibility for a drug carrier.

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