The Hong Kong Polytechnic University

Department of Optometry & Radiography

Study of an Interleukin-2 Immunoconjugate as a Novel Tumor Vasoactive Agent in Animal Models

By

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS OF THE DEGREE OF MASTER IN PHILOSOPHY

October, 2003

Declaration

CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written nor material which has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

Signed _____

Cheung Wai Kwan

Acknowledgement

My completion of this thesis would become impossible without the support of a number of people. First and foremost I would like to express my deepest gratitude to my supervisor Dr. Cheuk-Sang Kwok, Associate Professor, Department of Optometry and Radiography, for his insightful comments and guidance throughout my research study.

A special acknowledgement is forwarded to Dr. Wai-Hon Lau, COS of Clinical Oncology, Queen Elizabeth Hospital, for his kindness and permission in using the Radiobiology laboratory. I am immensely grateful to Dr. Timothy Yip, Florence So and Victor Ma of the Radiobiology Lab, Queen Elizabeth Hospital, for their technical advice. Valuable contributions from Monica are highly appreciated.

My sincere thanks also go to my mentor, Ms Maria Y.Y. Law, Assistant Professor, Department of Optometry and Radiography, who has nurtured my interest in research and continuously inspired me to greater efforts. I would like to extend my hearty appreciation to the Department of Clinical Oncology of the Pamela Youde Nethersole Eastern Hospital for providing me an excellent work environment and supporting my postgraduate studies.

Especially, I wish to record my acknowledgement to my family for their unconditional love and support. Besides all, I thank the Almighty God who granted me the strength and perseverance to finish this work.

Abstract of thesis entitled "Study of an Interleukin-2 Immunoconjugate as a Novel Tumor Vasoactive Agent in Animal Models" submitted by Cheung Wai Kwan for the degree of Master of Philosophy at The Hong Kong Polytechnic University in October 2003.

Abstract

The elevated levels of the HER2/*neu* protein in 60% of patients with ductal carcinoma in situ and in 30% of patients with invasive breast cancer make this oncogene product an attractive target for tumor-specific therapeutic agents. Despite some successes to date, vascular inaccessibility and tumor heterogeneity are two main factors that hindered the efficacy of anti-HER-2 antibody therapy. There is ample evidence suggesting that recombinant interleukin-2 (rIL-2) has profound antitumor properties, but was limited by its severe systemic toxicity known as vascular leakage syndrome (VLS). A fusion protein H520C9sFv-rhIL-2 has been constructed by combining the humanized V_H and V_L portions of a murine monoclonal antibody 520C9 specific for human HER2 with human rIL-2 to deliver effective concentration of IL-2 to HER2 positive tumors whilst avoiding toxic effects on normal tissues.

In this study in vitro characteristics of the H520C9sFv-rhIL-2 were investigated. Western blot of partially purified conditioned medium

from 293 cells transfected with the cDNA of the H520C9sFv-rhIL-2 revealed one major protein band with a molecular weight of 45 kDa, indicating that the fusion protein was expressed in the transfected 293 cells. The H520C9sFv-rhIL-2 was shown to preserve both immuno-stimulatory activities of IL-2 as measured by an IL-2 dependent CTLL2 cell proliferation assay and antigen binding specificity against HER2 positive SKOV3 and B16/*neu* cells. These in vitro results have stimulated interest in the in vivo application of the H520C9sFv-rhIL-2.

Present results demonstrated that a single intravenous dose of H520C9sFv-rhIL-2 to mice bearing HER2 positive tumors could preferentially increase the permeability of blood vessels in the tumors in a time- and dose-dependent manner. Using C57/BL mice bearing B16/*neu* s.c. tumors as a model, 18 μg of H520C9sFv-rhIL-2 and 24 h post injection of a vascular permeability tracer resulted in maximal tumor: nontumor uptake ratios of the tracer. Compared to a saline injection control group, the tumor: nontumor uptake ratios in bone, blood, kidney, lung, muscle and spleen ranged from 2.1 to 12.2. Nine μg of H520C9sFv-rhIL-2 only resulted in 4.5-fold and 2.3-fold increase respectively for tumor: bone and tumor: muscle uptake ratios when compared to the saline group 24 h post tracer injection. At 12-h and 72-h post tracer injection, no significant difference in tumor:nontumor uptake ratios was observed between the 9 μg

H520C9sFv-rhIL-2 group and the saline group. Some fatality was observed at 18 μg dose of H520C9sFv-rhIL-2.

Using nude mice carrying SKOV3 s.c. tumors as another model, a single intravenous dose of 9 μg H520C9sFv-rhIL-2 could not preferentially increase the permeability of blood vessels in the tumors 24 h post injection of a vascular permeability tracer, although a single intra-tumor dose of 9 μg of H520C9sFv-rhIL-2 could significantly increase tumor:nontumor uptake ratios for bone, kidney and spleen relative to a saline injection group. Although the mechanisms underlying IL-2 mediated VLS have not been investigated in detail in this study, present results suggest that at least one of the mechanisms depends on T cells. Another mechanism does not depend on T cells because vascular leak could be induced in the athymic nude mice lacking functional T cells by intra-tumoral injection of the fusion protein.

Taken together, these encouraging in vitro and in vivo results of the H520C9sFv-rhIL-2 suggest that this fusion protein might provide a good approach to selectively increasing the permeability of tumor vasculature. It is hoped that this will translate into further investigation into its clinical application.

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List of Abbreviations

ADCC	:	Antibody-Dependent Cellular Cytotoxicity
BCG	:	Bacillus Calmette-Guerin
BSA	:	Bovine Serum Albumin
C- termini	:	Carboxyl Termini
CD	:	Cluster of Differentiation
CDC	:	Complement-Dependent Cytotoxicity
CMF	:	Cyclophosphamide, Methotrexate and 5 Fluoro-uracil
CTLs	:	Cytotoxic T Lymphocytes
ECs	:	Endothelial Cells
EGFR	:	Epidermal Growth Factor Receptor
ELISA	:	Enzyme Linked ImmunoSorbent Assay
Fc	:	Crystallizable Fragment
FDA	:	Food and Drug Administration
H520C9sFv -rhIL-2	:	Humanized 520C9sFv / Human Interleukin-2 Fusion Protein
HAMA	:	Human AntiMouse Antibody
HRP	:	Horse-Radish Peroxidase
IFN-γ	:	Interferon-Gamma
IgG	:	Immunoglobulin G
IL-2	:	Interleukin-2
IL-2R	:	Interleukin-2 Receptor
i.p.		Intraperitoneal
i.t.	:	Intratumorly
i.v.	:	Intravenously
kDa	:	kiloDalton
KI	:	Potassium Iodine
LAK cells	:	Lymphokine-activated Killer Cells
MAbs	:	Monoclonal Antibodies
M.W.s		Molecular Weights
N- termini	:	Amino Termini

NaCl:	: Sodium Chloride
NK cells	: Natural Killer Cells
OD	: Optical Density
PBS	: Phosphate Buffered Saline
PBST	: Phosphate Buffered Saline containing 0.05%Tween-20
rhIL-2	: Recombinant Human Interleukin-2
s.c.	: Subcutaneously
SCA	: Single Chain Antibody
SDS	: Sodium Dodecyl Sulfate
SDS-PAGE	: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
sFv	: Single-chain Fv
T_H cells	: T-helper Cells
TAA	: Tumor Associated Antigens
TBS	: Tris Buffered Saline
TBST	: Tris Buffered Saline containing 0.05%Tween-20
TIL cells	: Tumor Infiltrating Lymphocytes Cells
TLC	: Thin Layer Chromatography
TNF-	: Tumor Necrosis Factor- alpha
Tukey's HSD test	Tukey's Honestly Significant Difference Test
V_H	: Variable Region of Heavy Chain
V_L	: Variable Region of Light Chain
VLS	: Vascular Leak Syndrome

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CHAPTER 1

Introduction

Cancer has become the top killer in Hong Kong since 1991 (Zhang 2001). According to the Census and Statistics Department, the proportionate mortality by neoplasms gradually increased from 30.9% in 1991 to 32.5% in 1997. In order to improve the prognosis, multidisciplinary approach has been realized in choosing the best-combined treatment modality. The mainstays of traditional treatment for malignant tumors are surgery, radiotherapy and chemotherapy. These approaches have met with significant success but also have demonstrated several shortcomings.

1.1 Role of Surgery in Cancer Treatment

Surgery is the oldest form of cancer therapy facilitating total clearance of the primary lesion and determination of staging and diagnosis. As a

local treatment, surgical dissection may involve simple excision of a tumor and removal of the invaded organs and regional tissues at risk of metastasis (Neal 1997). This approach obviously requires a precise knowledge of the location and extent of the cancer, because only limited amounts of tissue can be excised (Paty 2002). In the past, there have been advocates of extensive local surgery in the hope of improving overall survival (Taat 1995). However, such approach is not justified since many early tumors have already been associated with distant micrometastases (Badellino 1997). For this reason, regional macroscopic clearance, which can achieve optimal locoregional control with the minimal psychological trauma should be performed (Mirsky 1997).

1.2 Role of Radiotherapy in Cancer Treatment

Ionizing radiation has been used in cancer therapy since 1896 and currently remains one of the potentially curative treatments for many cancer patients (Mokbel 2001). Radiotherapy is useful where an

important organ must be cleared of cancer cells, but subsequently can function without extensive cell division. For example, radiotherapy for laryngeal carcinoma leaves the patient with intact, functioning vocal cords, whereas the surgical approach usually does not (Neal 1997). Preoperative radiotherapy may succeed in rendering inoperable nodes operable. For patients with multiple lymph node metastasis and extensive invasion beyond the primary tumor site, postoperative radiotherapy following surgical dissection is often administered.

1.3 Role of Chemotherapy in Cancer Treatment

Because of the systemic nature of metastatic neoplasms, chemotherapy has emerged as the major systemic weapon in preventing cancer cells from multiplying, invading adjacent tissue and developing metastasis (Mokbel 2001). Since the 1940s, there has been a widespread use of chemotherapy for the treatment of both micrometastatic and metastatic diseases. Chemotherapeutic drugs are most frequently given in combinations to maximize the therapeutic

response by addressing the diversity of cellular response. Combination chemotherapy is designed to incorporate drugs from different classes thereby maximizing tumor killing with both phase- and cycle-specific agents (Neal 1997). Additional benefits include minimization of toxicity to normal cells and slowing of the development of new resistant lines (Hall 1998). Several studies have demonstrated improved antitumor efficacy in treating breast cancer, lymphoma, testicular cancer, bladder cancer and acute leukemia with such synergistic drug combinations (Hall 1998, Maezawa 2002 and Teske 2002).

1.4 Limitations of the Standard Cancer Therapies

Even with aggressive approaches, the majority of advanced cancers fail to respond to all standard therapies. Major causes are (a) the occurrence of metastases that are inoperable, (b) the intrinsic insensitivity of the malignant clone to both radiation and most chemotherapeutic agents, (c) the rapid induction of resistance

mechanisms in tumor cells during treatment and (d) the poor ability of chemotherapeutic agents to differentiate between malignant and normal cells (Goldenberg 1993 and Kosmas 1993).

Resistance to both radiation therapy and chemotherapy can be an intrinsic property of a malignant cell but can also be acquired after exposure to these treatments. Tumors that demonstrate intrinsic resistance fail to respond from the onset. This may be due to insufficient dosing, tumor hypoxia and tumor heterogeneity (Hall 1998). Bulky tumors are frequently associated with necrosis and hypoxia, making drug access problematic (Gruber 1996). As a result, both pH and pO2 decreases lead to minimized efficacy of chemo- and radio-therapy (Mokbel 2001). Besides, tumors are generally made up of a number of subpopulations of cells with different genetic characteristics and thereby the radio- and chemo-sensitivities vary within the tumor.

Acquired resistance can develop after exposure of cells to treatments.

It is not uncommon to find that a tumor responds to a particular drug for a period of time and then ceases to do so. Both chemotherapy and radiotherapy are known to induce mutants that are not only resistant to chemotherapy but also lack certain surface markers (Mokbel 2001). Possible mechanisms include (1) decreased drug activation, (2) improved DNA repair, (3) increased competing biochemical pathways and (4) defects in drug transport (Neal 1997).

Lack of specificity causing systemic toxicity is one of the fundamental problems of chemotherapy. Since the chemotherapeutic agents cannot be delivered to tumor cells preferentially over normal cells, dose escalation is usually prohibited to prevent unacceptable side effects. Therefore, the need for new, modified anticancer therapies with greater tumor specificity, effectiveness and tolerability to patients is profound.

1.5 Role of Immunologic Therapy in Cancer Treatment

The intensive developments in immunology and rapid advances of genetic engineering have greatly stimulated studies on the possibility of cancer immunotherapy. The primary goal of immunologic therapy is to unleash the latent powers of the host's natural defense mechanism, the immune system, to combat the invading malignancy (Foss 2002 and Jaffee 1999). It has focused on augmenting the immune response to cancer either by active immunotherapy through vaccination with tumor cells or administration of cytokines, or by passive immunotherapy with monoclonal antibodies targeted directly to tumor cells or adoptive transfer of activated effector T cells (Indar 2002).

1.5.1 Active Immunotherapy

Active immunotherapy consists of the administration of substances

designed to provoke an antitumor response by the tumor-bearing host's own immune cells (Hsueh 2002 and Jaffee 1999). It is mediated by antibody and activated helper and cytotoxic T lymphocytes. The major advantage of acquired immunity is that resistance is long-term while its main disadvantage is its slow onset, especially the primary response (Riethmuller 1993). Generally it is necessary for two successive doses of the vaccine to be given. In some cases it is necessary to provide continued immunity by giving 'booster' doses of the vaccine at regular intervals.

Non-specific vaccination aims at general immune activation. This approach has been very successful in the treatment of bladder cancer with Bacillus Calmette-Guerin (BCG) (Bassi 2002). In recent years, vaccines have evolved from non-specific immune stimulants to much more specific and potent strategies. Numerous approaches require stimulation of potent antigen-specific T-cell responses. Encouraging data have been seen in the treatment of melanoma with vaccination of the GM2 ganglioside (Lode 2000 and Naramura 1993). Dendritic

cells pulsed with tumor associated antigens (TAAs) have also attracted wide interest because of their unique capacity to elicit primary and secondary antitumor responses (Bachleitner-Hofmann 2002 and Insug 2002). Administration of dendritic cells loaded with prostate cancer-specific tumor antigens was assessed in clinical trials (Tjoa 2000). Understanding the mechanisms of antitumor immunity and identifying relevant tumor-specific antigens will likely improve these vaccine strategies and provide them with a niche in the future of cancer therapy.

These therapies can be modified using cytokines and other immune modulating agents. The most widely adopted are interferon-α (IFN-α) and interleukin-2 (IL-2). Large-scale clinical trials utilizing IL-2 have been initiated since 1984 (Herberman 1984 and Pizza 1984). The use of IL-2 as an antitumor agent has been demonstrated to be effective in the management of renal cell carcinoma and melanoma (Albertini 1996, Hall 1998 and Xu 2000). However, the clinical utility of high-dose IL-2 has been limited by significant adverse effects,

including vascular leak syndrome (Ravaud 1991). Targeting of IL-2 into the tumor microenvironment using antibody-IL-2 fusion proteins may, therefore, offer an innovative approach in cancer immunotherapy for achieving effective immune stimulation at the site of tumor while minimizing systemic toxicity.

1.5.2 Passive Immunotherapy

Immunity created by the transfer of exogenous reagents to a tumor-bearing host is termed passive immunity. It confers a temporary, but immediate resistance to infection, but are gradually catabolized by the susceptible host (Hay 2002 and Riethmuller 1993). One hundred years ago, Paul Ehrlich put forward his "magic bullet" concept of using antibodies to selectively target cancer cells (Levinson 1998). Several clinical trials indicate that this approach has potential value in the treatment of B-cell lymphoma and metastatic breast cancer (Fagnoni 2001, Hall 1998 and Morse 1999). Some promising clinical data have also been obtained by using monoclonal antibodies in

patients with colon carcinoma and neuroblastoma (Lode 2000).

Passive immunotherapy with monoclonal antibodies is promising when the problem of poor penetration into a tumor mass can be circumvented (Hay 2002). Low antibody uptake in the tumor is a significant problem in antibody-directed therapy. Antibody uptake in tumor is governed by the vascular permeability of tumor endothelium and the tumor blood flow rate (LeBerthon 1991). Several approaches like hyperthermia, administration of bradykinin, histamine and such biologic response modifiers as IL-2, tumor necrosis factor-α (TNF-α) and IFNs have been studied extensively in the hope of altering the vascular permeability to enhance the antibody uptake (Ghose 2002).

1.5.3 Recombinant Antibody-interleukin 2 Fusion Proteins

Recombinant antibody-interleukin 2 fusion proteins are designed for the purpose of targeting sufficient concentration of recombinant human IL-2 (rhIL-2) in the tumor microenvironment, where it can

elicit an immune response, inflammatory reaction, or vascular changes that can destroy tumor cells or inhibit their proliferation. With the aim of concentrating IL-2 activities in HER-2 positive tumors, a recombinant humanized single-chain Fv (sFv) antibody/ IL-2 fusion protein has been constructed. It consists of the humanized variable heavy (V_H) and light (V_L) domains of a murine MAb 520C9 directed against the human HER-2/*neu* proto-oncogene product p185 and human IL-2 (Li 1999). Advantages of this immunoconjugate include improved tissue penetration and lower immunogeneicity (Li 2000). Therapeutic potential of this H520C9sFv-rhIL-2 fusion protein had been evaluated previously by means of a syngeneic mouse tumor model and in immuno-suppressed mice carrying subcutaneous and metastatic human HER-2 positive tumors (Kwok 2001). The effective inhibition of SKOV3 lung metastases in SCID mice treated with H520C9sFv-rhIL-2 and human PBM cells was evaluated by immuno-histological staining of lung sections removed from the mice for the HER2/*neu* protein as shown Appendix 4. The staining results indicated the absence of HER2 positive cells within the lung sections

of mice treated with a mixture of 6 μg H520C9sFv-rhIL-2 and 0.2 x 10^6 human PBM cells (Kwok 2003). On the contrary, tumor nodules in non-treated mice showed intense expression of the HER2 protein. Like the antibody-interleukin 2 fusion protein produced by Gillies et al. (1992), this antibody-IL-2 fusion protein is expected to not only target IL-2 to tumor sites and thereby activate immune effectors, but also can alter tumor vascular physiology to improve the delivery of therapeutic agents. This study was designed to seek evidence for this possibility.

Results of this thesis indicate that this H520C9sFv-rhIL-2 fusion protein was stably expressed in 293 cells transfected with the cDNA of the fusion protein and retained the immunostimulatory effects of IL-2 as shown by cell proliferation assay. In addition to IL-2 activity, the fusion protein possessed full binding activity to the erbB-2 proto-oncogene product as shown by SKOV3 cells mediated enzyme linked immunosorbent assay (ELISA). Vascular changes induced by the fusion protein at the tumor site and various normal tissues,

characterized by leakage of i.v. injected ^{125}I-labeled albumin into the interstitial space, were investigated in both black and nude mice carrying p185 positive tumors. The effect of fusion protein pretreatment on increased tumor uptake of this blood vessel permeability tracer was maximal 24 h post injection of the tracer. These results provide proof to the hypothesis that this fusion protein can pretarget p185-positive tumors and induce a localized vasopermeability effect. Therefore, this fusion protein might be applied as a precursor to enhance antibody uptake in tumors.

1.6 Statement of Purpose

The purpose of this study was to investigate the in vivo properties of H520C9sFv-rhIL-2 fusion protein as a specific tumor vasoactive agent in animal models. The objectives of the study were to:

1. To prepare purified fusion proteins by
   (a) culturing cell lines which allow stable expression of the fusion protein in conditioned medium, and

(b) collecting and concentrating the conditioned medium, and

2. To examine the in vitro properties of the fusion proteins, in term of

   (a) the specificity of antigen-binding activity

   (b) the detection of IL-2 moiety

   (c) the IL-2 bioactivity assay

3. To examine the in vivo effectiveness of the fusion proteins as a vasoactive agent, in term of

   (a) the time dependence , and

   (b) the dose dependence of the fusion protein in tumor bearing mice

CHAPTER 2

Literature Review

2.1 Overview of the Immune System

The immune system is a complex, dynamic system made up of a number of different physical barriers, cell types, and blood-borne proteins that protect us from pathogenic microorganisms, such as viruses, bacteria, fungi and parasites (Hay 2002). There are two types of immunity: innate and adaptive.

Innate immune responses include anatomic, physiologic, endocytic and phagocytic, and inflammatory barriers that help prevent the entrance and establishment of infectious agents (Playfair 2001). When an individual's mechanical barriers are breached, an early defense mechanism known as acute inflammatory response follows. The three

major events in acute inflammation are by 1) vasodilation causes increased blood flow, 2) increase in vascular permeability facilitates the escape of plasma proteins and leukocytes from the circulation, and 3) leukocytes emigration from the capillaries and accumulation at the site of infection (Hay 2002). These responses are manifested clinically as the 5 cardinal signs of inflammation: heat, redness, swelling, pain and loss of function.

The aim of the inflammatory response is to recruit cells and other factors from the bloodstream to the infection site to aid in the removal of pathogens. Phagocytic cells, such as macrophages, monocytes and polymorphonuclear neutrophils use the primitive non-specific recognition systems to mediate the innate immune response (Playfair 2001). A host of toxic chemical reactions may occur in phagocytes including biomolecular breakdown of pathogens by digestive enzymes and chemical modification by highly reactive oxygen and nitrogen intermediates. One consequence of phagocytosis is that macrophages and dendritic cells present antigens on their cell surfaces

corresponding to the chemical breakdown products of the organism. These antigenic fragments are able to deliver a co-stimulatory signal that is necessary for T-helper (T_H) cells activation. This in turn leads to acquired immunity as defined by the antigen-specific activities of the immune lymphocytes and their effects on other cells (Wood 2001).

The key features of the acquired immune response are its diversity, specificity, memory and self/nonself recognition. The immune system produces both humoral and cell mediated responses. The humoral immune system is dominated by circulating antibodies which are produced primarily by B lymphocytes. In contrast, the effector cells of the cell mediated immunity are activated T_H cells which secrete various cytokines, and cytotoxic T lymphocytes (CTLs) which can destroy altered self-cells (Gallucci 2001).

2.2 Monoclonal Antibody

The discovery of antibodies by Emial von Behring in 1890 was followed by Paul Ehrlich's proposal in 1906 to apply them as "magic

bullets" and "poisoned arrows" to specifically direct toxic substances to pathogenic targets. However, it was nearly a century before antibody-based therapies became established. This development was aided considerably by the advent of the hybridoma technology described by Kohler and Milstein for the production of monoclonal antibodies (MAbs) in 1975 (DiJulio 2001).

By fusing a normal activated, antibody-producing B cell with a myeloma cell, they were able to generate a hybrid cell, called a hybridoma, that possessed the immortal-growth properties of the myeloma cell and secreted the antibody produced by the B cell (Roitt 1998). The resultant hybridoma cells could be single-cell cloned and then expanded as individual clones, which secrete only one antibody type. All of these antibodies are identical, with specific property of antigen recognition and are known as MAbs (Gruber 1996).

2.2.1 Immunoglobulin Structure

Antibodies are immunoglobulins that react specifically with the antigen which stimulate their production. Immunoglobulins are glycoproteins made up of light and heavy polypeptide chains. The terms "light" and "heavy" refer to molecular weight. Immunoglobulin G (IgG) which is the most common class of immunoglobulin, consists of two identical light chains of molecular weight 23,000 Daltons and two identical heavy chains of molecular weight 53,000 Daltons (Roitt 1998). The four polypeptide chains are held together by covalent disulfide bridges and noncovalent bonds. This molecule is represented schematically in the form of a Y, with the amino (N-) termini of the chains at the top of the Y and the carboxyl (C-) termini of the two heavy chains at the bottom of the Y-shape (Levinson 1998).

In both heavy and light chains, at the N-terminal portion, the sequences vary greatly from polypeptide to polypeptide. The

heterogeneity in the amino acid sequences accounts for the great diversity of antigen-specificities among antibody molecules. In contrast, the C-terminal portion of both heavy and light chains which carry out the effector functions are common to all antibodies of a given class (Roitt 1998). Hence, these two segments of the molecules are designated variable and constant regions. The variable regions are responsible for antigen-binding, whereas the constant regions are responsible for complement activation, resulting in membrane damage and cell lysis (Wood 2001).

2.2.2 Single Chain Antibody

Monoclonal antibody-based therapies are particularly appealing because of their specific antigenic binding properties. However, the whole immunoglobulin molecules are very immunogenic and their crystallizable fragment (Fc) contributes to poor pharmacokinetics and nonspecific uptake by Fc receptors. The intact immunoglobulin molecules have high molecular weight and large size, which often

impede their diffusion into bulky tumors. Antibody fragments are therefore preferable. The use of small single chain antibody (SCA) for cancer therapy has several advantages. With the advent of genetic engineering techniques, the V_H and V_L chains can be linked together by a flexible peptide spacer (Hudson 1999). By removing the Fc portions from the antibody molecules, SCAs have found to exhibit low immunogenicity while specific antigenic binding properties are retained (Savage 1993). Because of their small size, they should have better tumor penetration together with a shorter half-life. Rapid clearance from the blood pool can thus reduce the toxicity to normal tissues (Gillies 1992). These recombinant antibody fragments directed against HER-2 have been shown to induce remission in patients with HER2-overexpressing metastatic breast cancer (Baselga 2000).

2.2.3 Chimeric Antibody

Generally, most MAbs are generated from murine sources and do not function well with human effector cells. After repeated exposure to whole murine antibodies, human antimouse antibody (HAMA) response could be induced which inactivates subsequent antibody doses. In the case of HAMA, reactions are manifested by anaphylaxis, serum sickness, fever, and hypotension, usually occur 2 to 3 weeks after the first injection (Goldenberg 1993). The immunogenicity of mouse monoclonal antibodies, where undesirable, can be overcome by the production of more human-like antibodies, such as recombinant mouse-human chimeric antibodies (Kosmas 1993). These humanized antibodies can be made by replacing the constant region of the mouse MAb with a human constant region by genetic engineering (Losman 1999). Both preclinical and clinical studies have demonstrated that chimeric antibodies could retain their specificity for their targets and may be more active than their murine counterparts (Krauss 2003).

2.2.4 Clinical Trials

Over the past ten years, MAbs have yielded promising results in both unconjugated and conjugated forms for the treatment of cancer. Monoclonal antibodies directed at tumor-associated antigens can activate both complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) specific for the tumor (Esteva 1998 and Linardou 1996). Rituxan antibody is the first monoclonal antibody to obtain the approval of the Food and Drug Administration (FDA) for the treatment of relapsed or refractory low-grade or follicular, cluster of differentiation (CD) 20-positive, B cell non-Hodgkin's lymphoma (Sacchi 2001). Monoclonal antibodies selectively produced for breast cancer-associated growth factor receptors have also been shown to induce cytotoxicity in animal models (DiJulio 2001and Mottolese 1994).

Alternatively, MAbs have been used as carriers of potent agents, such as chemotherapy drugs, radioisotopes, or toxins for selective tumor

treatment. By using MAbs that bind selectively to tumor cells, the cytotoxic activity should be focused onto tumors, thereby sparing healthy tissues (Multani 1998). Radioimmunotherapy can also overcome the problem of heterogeneous antigen expression. The antigen-negative tumor variants will also be killed if they are in the proximity of antibody-binding tumor cells. Radiolabelled anti-B cell antibodies have been associated with complete remission of lymphomas (Davis 1998).

The ongoing effort for the identification of pertinent tumor-associated antigens and further developments in recombinant DNA technology, will enable the more effective application of monoclonal antibodies for cancer therapy.

2.3 HER-2/*neu* Oncogene

The HER-2/*neu* oncogene, also known as c-erbB-2 encodes a 185kDa transmembrane tyrosine kinase receptor p185 that has partial homology with other members of the epidermal growth factor receptor

(EGFR) family (Cefai 1999). Mutational or ligand-induced activation of these receptors is required for malignant transformation of the cell. HER-2/*neu*, although having no known soluble ligand, is transactivated by heterodimerization with other members of the EGFR family. This results in transphophorylation and initiation of signals that ultimately lead to mitogenesis and altered gene expression, which are of clear important for both carcinogenesis and tumor progression (Hung 1999 and Sethi 2000).

2.3.1 Prognostic Factor

Overexpression of HER-2, which occurs in 60% of patients with ductal carcinoma in situ and in 30% of patients with invasive breast cancer, has been shown to be associated with adverse prognosis (Scott 1997 and Sahin 2000). Amplification of HER2/*neu* gene is prognostically and therapeutically significant for patients with breast cancer (Cirisano 1996 and Sethi 2000). The data regarding the potential interaction of HER-2 expression and response to chemotherapy are inconclusive. Although previous studies suggested

that patients with HER-2 overexpressing breast cancers are less responsive to Cyclophosphamide, methotrexate and 5 Fluoro-uracil (CMF), contradictory data from Moliterni (2003) have supported the hypothesis of benefit from CMF in HER-2 positive breast cancer (Thor 1998).

2.3.2 Therapeutic Target

HER-2 not only helps to predict the cancer prognosis and chemoresponsiveness, but also increasingly serves as a potential target for antibody-directed therapy. Novel treatment approaches have been developed that can take advantage of HER-2 overexpression. Herceptin (Trastuzumab), a humanized antibody against HER-2, is the first clinically available humanized MAb for treatment of solid tumors (Leyland-Jones 2002). Clinical trials have demonstrated that it is effective either applied as a single strategy or in combination with chemotherapeutic agent when administered to patients with HER2-overexpressing recurrent breast cancer (Slamon 2001). Addition of Herceptin to weekly paclitaxel resulted in a 25%

improvement in overall survival compared with chemotherapy alone (Fornier 2000, Leyland-Jones 2002 and Thomssen 2001). Herceptin was well tolerated with low incidence of severe adverse events. Cardiotoxicity could occur in patients exposed to anthracyclines but it was generally manageable (Smith 2001). Despite the achievement of tumor-specific cell killing, several problems must be resolved in order to optimize the therapeutic efficacy of antibodies.

2.4 Limitations in Antibody-directed Therapy

One of the most critical problems in antibody based therapeutic regimens is the low antibody uptake in tumor (Kemshead 1993). Only 0.01-0.1% of the injected antibody dose per gram of tumor actually binds and accumulates at the site of tumor (Penichet 1997). Factors contributing to poor tumor uptake of antibodies include antibody-antigen complexes formation, antigenic modulation, antigenic heterogeneity, heterogeneous vascular permeability within tumors and macromolecular size of antibodies (Murray 1992).

2.4.1 Antigen-antibody Complex Formation

After injection into the blood pool of an antibody, the initial problem is whether it binds with tumor antigen present in certain normal organs or with circulating antigen. Such antigen-antibody complexes could result in non-specific toxicity against normal tissues and reduction of available antibody (Goldenberg 1993).

2.4.2 Antigenic Modulation

Tumor antigen modulation is a process by which tumor cells "hide" surface antigen by internalization, sometimes spontaneously and sometimes in response to bound antibody. Therefore, tumor can escape the immune surveillance as they no longer present a target for immune attack. Furthermore, antigen expression can be temporally modulated, i.e., antigens may be expressed at one time but not be expressed at others (Grossman 1988 and Linardou 1996).

2.4.3 Antigenic Heterogeneity within Tumor

Human breast tumors are known to exhibit marked heterogeneity of TAAs among different cells within the same tumor . Still, studies have shown that immunoconjugate treatment could eradicate animal tumors completely which show only 30% antigen-positive cells Heterogeneous antigen expression should not, therefore, preclude the antibody-directed therapy from consideration (Esteva 1998). Extensive efforts are currently under way to demonstrate preclinical efficacy against heterogeneous tumors.

2.4.4 Heterogeneous Vascular Permeability

An injected antibody has to pass through a number of vascular and extravascular compartments before it can target to the tumor antigenic site. Its movement is governed by the blood flow rate and the vascular morphology (Goldenberg 1993). The blood flow in tumor vessels is

usually inadequate and intermittent. The tortuous tumor blood vessels together with the avascular necrotic central region especially in bulky tumors often make antibody access problematic (Kosmas 1993). Despite the increased overall vascular permeability of tumors compared with that of normal tissue, not all tumor blood vessels are leaky. A monoclonal antibody at the tumor periphery has to overcome the high intratumoral interstitial fluid pressure which would oppose its influx (Linardou 1996). As tumor mass increases, the ability of transvascular exchange will reduce (Penichet 1997).

2.5 Interleukin-2

2.5.1 Overview

In order to enhance tumor targeting by MAbs, vasoactive agents like IL-2 could potentially be used to increase the tumor vascular permeability (Smith 1990).

IL-2 is a cytokine produced by T helper cells with the ability to stimulate the proliferation and activation of T cells, natural killer (NK) cells and cytotoxic macrophages (Sharp 2001). The M.W. of the recombinant form of IL-2 is 15 kDa. IL-2 also induces LAK cells development, tumor infiltrating lymphocytes (TIL) cells proliferation, triggers off differentiation of B cells and acts as a stimulus for antibody synthesis (Reichert 2000 and Schmidt-Wolf 1995). The activation of different leukocytes at the tumor site results in secretion of various cytokines and expression of adhesion receptors. Once stimulated by IL-2, NK cells produce IFN-γ, TNFα and granulocyte-macrophage colony-stimulating factor (GM-CSF) which are powerful activators of macrophages (Mehrotra 1998). The continued recruitment and stimulation of macrophages result in a rise in the concentration of IL-1, IL-8 and TNFα. Both IL-8 and TNFα have chemotactic effects on monocytes and neutrophils (Epling-Burnette 1993). This provides an amplification loop to maintain macrophage and NK activation. Chan (2002) reported that the SKOV3 tumors have elevated levels of IL-6 in the presence of

IL-2, which appear to contribute greatly to the overexpression of inflammatory cytokines and adhesion molecules. These properties suggest that IL-2 could be effective in enhancing the host immune response for cancer-fighting activities.

2.5.2 The Human IL-2 Receptor System

The biological effects of IL-2 is mediated by binding to the IL-2 receptor (IL-2R), which has three component proteins alpha (α), beta (β) and gamma (γ) (Sharp 2001). Historially, the first receptor subunit identified was the α subunit, a 55 kilodalton (kDa), 251 amino acid (aa) residue glycoprotein that contains a very short 13 aa cytoplasmic tail. Although this receptor is specific for IL-2, its affinity is low (Kd = 10^{-8} M) and it apparently has no signal transducing capability. The second subunit to be isolated was the β subunit, a 70 kDa, 525 aa residue glycoprotein that possesses an extensive 286 aa cytoplasmic region. The β subunit binds IL-2 (Kd = 10^{-9} M) and is involved in the signal transduction mechanisms of both IL-2 and the newly

discovered IL-15. The last subunit to be discovered is known as the γ chain. This is a 64 kDa, 347 aa residue glycoprotein that contain an 86 aa cytoplasmic region (Johnson 1994 and Sugamura 1992).

The existence of three distinct subunits allows for multiple subunit combinations. The α-β-γ heterotrimer is generally considered to be the high affinity (Kd $=10^{-11}$ M), signal transducing receptor for IL-2. Cells known to express this combination of subunits include activated T cells and monocytes. Sugamura (1994) has demonstrated that IL-2Rβ and IL-2Rγ expressing cells, such as most large granular lymphocytes, display intermediate affinity IL-2 binding (Kd = 10^{-9} M) and are effective in IL-2 mediated signal transduction.

2.5.3 Clinical Trials

Because of the central role of the IL-2 system in mediation of the immune response, it is obvious that monitoring and manipulation of this system has important diagnostic and therapeutic implications. At

present, the FDA has given approval for the use of IL-2 in the treatment of metastatic renal cancer (Lee 1998 and Parmiani 2000). Ongoing trials with colorectal cancers and ovarian cancers are in progress (Margolin 2000 and Sharp 2001). IL-2 has shown considerable promise as an anti-cancer drug by virtue of its ability to stimulate tumor-attacking LAK and TIL cells proliferation (Parmiani 1992 and Rosenberg 1984). IL-2 also acts synergistically with other cytokines including TNF-α and IFN-α as well as the adoptive transfer of antitumor monoclonal antibodies, LAK or TILs (Papamichail 1992 and Whittington 1993). Maas (1993) demonstrated that administration of IL-2 along with TIL was more effective than IL-2 with LAK because TILs include CD8+ T cells which could become specific cytotoxic T lymphocytes. Nevertheless, extension of IL-2 therapy to other more common cancers has been limited by severe vascular leakage in normal tissues (Atzpodien 1996).

2.5.4 Vascular Leak Syndrome

Because of its extremely short half-life in vivo, high dose of IL-2 is required. However, the in vivo efficacy is limited by the severe systemic toxicity associated with high dose IL-2 therapy. It results in a systemic vascular leak syndrome (VLS) which manifests itself as peripheral edema, weight gain, hypotension, oliguria and respiratory failure (Siegel 1991 and Vial 1992). VLS has also been observed in cancer patients treated with other cytokines such as IL-3, IL-4, IFN-α and immunotoxins (Baluna 1997 and Kuan 1995). With regard to IL-2 therapy, VLS occurs when IL-2 is administered either alone or in combination with LAKs, TILs or cyclophosphamide (Sharp 2001). The symptoms usually start 3-4 days after the initiation of therapy (Rosenstein 1986).

2.5.4.1 IL-2 Mediated VLS Mechanisms

The mechanisms underlying IL-2 mediated VLS are only partially understood. Early studies suggested that IL-2 had a direct effect on the morphology of endothelial cells (ECs) causing gaps to appear between

them (Vial 1992). IL-2 could bind to the ECs and induce a toxic side effect. Direct evidence for the involvement of LAKs, particularly NK cells, has been reported. Some studies showed that anti-NK-1.1 antibodies had a protective effect against the vascular damage (Baluna 1997). Arguing against this, Ohkubo (1991) and Edwards (1992) reported that morphological changes in ECs were observed within 2 hours of injection of the IL-2, whereas proliferation of LAK cells required 2-3 days of IL-2 stimulation. It has, therefore, been suggested that such early changes were unlikely to be mediated by LAK cells. To date, there has been a general consensus that the IL-2 induced VLS was not attributable to a direct effect of the rIL-2 itself because no directly toxic activity was seen when IL-2 was applied to cells in vitro (DeJoy 1995 and Vial 1992).

Interleukin-2 induced VLS may be associated with several types of lymphocytes. Both NK cells and CTLs can mediate endothelial damage by granule exocytosis (Damle 1989). Low doses of IL-2 effectively stimulate T cell activation and proliferation, whereas NK

cells are more effectively activated at higher doses (London 1986). Although no unique mechanism for the induction of VLS by IL-2 has been described, activation of neutrophils and release of TNF-α appear to be crucial events in IL-2 induced VLS (Carey 1997 and Edwards 1992). Interleukin-2 can induce adherence of neutrophils and platelets to the ECs. The adhered neutrophils could then release hypochlorous acid and proteases to local concentrations sufficient to cause endothelial damage (Baars 1992, Baluna 1997 and Carey 1997). Finnegan (2002) proved that taurine could prevent IL-2-induced endothelial injury by attenuating neutrophil to endothelial adhesion and migration. A strong correlation between serum levels of TNF-α and VLS has also been reported by Dubinett and his colleagues (1994).

2.6 Antibody-cytokine Fusion Proteins

In an effort to concentrate and prolong action of IL-2 at the site of tumor while minimizing systemic toxicity, it is an attractive idea to

target it there via an antibody delivery system (Savage 1993and Xu 2000). Recombinant antibody-cytokine fusion proteins are immunocytokines that achieve sufficient concentrations of specific cytokines in the tumor microenvironment to effectively stimulate a cellular immune response against tumors. They combine the unique targeting ability of antibodies with the multifunctional activity of cytokines (Penichet 2001).

2.6.1 Antibody-interleukin-2 Fusion Proteins

One of the best studied cytokines in this regard is rhIL-2, which by itself is capable of activating and expanding a variety of immune effectors, including T lymphocytes, NK cells, and possibly granulocytes (Budagian 2002). In most instances, fusion proteins with an IL-2 component have been studied mainly for the purpose of augmenting antitumor responses presumably by enhancing IL-2

deposition at tumor sites (Becker 1996, Penichet 2001 and Xiang 1999). The efficacy of these antibody IL-2 fusion proteins in inhibiting tumor growth in mice with established melanoma, colorectal carcinoma, ovarian carcinomas, neuroblastoma, B cell lymphoma, and carcinoembryonic antigen-expressing tumors have been demonstrated (Lode 2000 and Xu 2000). Although it is widely accepted that these fusion proteins had therapeutic potential in the treatment of cancer, little literature has been published concerning their properties as specific tumor vasoactive agents. To our knowledge, only three of the reported fusion proteins could be used to increase preferentially the vascular permeability of solid tumors and therefore tumor uptake of a subsequent dose of therapeutic agent (Hornick 1999, Hu 1996 and LeBerthon 1991).

Advantages of an immunoconjugate consisting of recombinant antibody fragments include improved tissue penetration, rapid renal clearance of non-localized protein and potentially lower immunogenicity (Savage 1993). Taking advantage of the targeting

specificity of MAb directed to TAAs, a fusion protein consisting of a rhIL-2 linked to a humanized V_H and a V_L domain of murine monoclonal antibody 520C9 against the human HER-2 proto-oncogene product p185 (H520C9sFv-rhIL-2) has been constructed (Li 2000). The immunostimulatory effects of IL-2 together with the binding activity to the p185 of this fusion protein were examined.

Recognizing that blood flow and vascular permeability are key parameters controlling the egress of therapeutic molecules into tumors, this thesis further explored the functions of this fusion protein in terms of its potential for targeting and causing vascular leak in p185 positive tumor-bearing mice. Pretreatment with this H520C9sFv-rhIL-2 fusion protein may potentially improve the delivery of therapeutic agents to tumor sites. If this is the case, one of the most important limitations of using MAbs, namely low tumor uptake, could be solved.

CHAPTER 3

Materials and Methodology

3.1 Materials

3.1.1 Antibodies and Interleukin-2

The murine anti-human HER-2 529C9 antibody was a gift from Chiron Corp., Emeryville, CA, US. Other antibodies such as polyclonal rabbit anti-human IL-2 antibody and horse-radish peroxidase (HRP) -conjugated goat anti-rabbit IgG antibody were supplied by DAKO (Copenhagen, Denmark). Human recombinant IL-2 used in these experiments was purchased from PeproTech Corporation (London, UK). It was reconstituted with phosphate buffered saline (PBS) at a concentration of 10,000 IU per μl as a stock solution and stored at 4°C until use.

3.1.2 Cell Lines

The mouse melanoma B16/*neu* cells, human ovarian carcinoma SKOV3 cells and cervical carcinoma HeLa cells (gifts from Department of Pathology, McMaster University, Hamilton, Ontario, Canada) were maintained in RPMI 1640 culture medium (Gibco BRL) supplemented with 10 % fetal calf serum (Gibco BRL) at 37°C in a 5% CO_2 incubator. Once these cells were grown to 90% confluency, they were detached with Trypsin-EDTA (Gibco) and washed twice with PBS. Viable cells were determined by trypan blue exclusion staining and suspended in physiological saline for injection. Both B16/*neu* and SKOV3 cells are p185-positive while the HeLa cells are p185-negative.

The CTLL-2 cells, which bear the high affinity IL-2 receptor, were purchased from the American Type Culture Collection. The cells were maintained in RPMI media supplemented with 20 units/ml rhIL-2,

10% fetal calf serum (Gibco), 1% penicillin streptomycin (Gibco) and 0.05 mM 2-mercaptoethanol (Sigma Chemical, USA).

3.1.3 Nude and C57/BL Mice

Both C57/BL mice and nude mice were generously provided by the Radiobiology Unit of the Queen Elizabeth Hospital, at 6-7 weeks of age, and housed under specific pathogen-free conditions in a laminar flow rack. All mice were given free access to sterilized mouse diet and water.

3.2 Methodology

3.2.1 Preparation of Fusion Proteins H520C9sFv-rhIL-2 and H520C9sFv-mrhIL-2

3.2.1.1 Establishment of Stable 293 Cell Transfected with Fusion Protein cDNAs

Human embryonic kidney 293 cells used for stable expression of the cDNAs encoding for H520C9sFv-rhIL-2 and H520C9sFv-mrhIL-2 were grown in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10 % fetal calf serum (Gibco) and 8 μg/ ml G418 (Gibco). H520C9sFv-rhIL-2 was a fusion protein consisting of a rhIL-2 molecule, a humanized V_H and a V_L domain of murine MAb 520C9 directed against the human HER-2/*neu* (c-erbB2) proto-oncogene product p185. Both the IL-2 activity and p185 binding affinity were retained. Another fusion protein, H520C9sFv-mrhIL-2, comprising the H520C9sFv and a mutant-human IL-2 that has lost the biological function of the IL-2 moiety as a disulphide bond in the mrhIL-2 was eliminated was also constructed (Li 1999).

3.2.1.2 Collection and Purification of Fusion Proteins

When the transfected 293 cells became 70% confluent in a culture flask, its medium was removed and fresh 293 SFM II (Gibco) supplemented with 2% GlutaMax (Gibco) and 8 μg/ ml G418 was added. After 3 days, the spent cultured medium was harvested. The collected conditioned medium was concentrated up to 200-fold with the use of a centrifuge type concentrator with 30,000-dalton exclusion limit (Millipore Corp., Bedford, MA). The concentrated conditioned media were dialysed with a seamless cellulose dialysis tubing (Sigma) in a changing buffer of 0.9% sodium chloride (NaCl) to remove G418. The G418 concentration in the concentrated conditioned media was decreased by 8000 folds after three dialyses against 20-fold volume excess of the buffer. The concentrate was filtered through a 0.22-μm Millipore filter (Millipore) and then refrigerated at 4°C until use. Fusion protein in the conditioned medium was analyzed by Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

3.2.2 Examination of In Vitro Properties of Fusion Proteins

3.2.2.1 SDS-PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

Samples of H520C9sFv-rhIL-2, H520C9sFv-mrhIL-2 and standard rhIL-2 were solubilized at 95°C for 5 minutes in sample buffer containing 0.5M Tris HCl at pH 6.8 and 10% sodium dodecyl sulfate (SDS) (w/v) and then loaded onto a 1 mm-thick 12% SDS separating gel topped with a 4% stacking gel. The molecular weight determination on SDS-PAGE was resolved by simultaneously running rainbow colored protein molecular weight markers (New England Biolabs, Beverly, MA). Electrophoresis was performed in the polyacrylamide non-reducing gel for 1.5 hours under 180 V, with a Tris-glycine buffer system in an electrophoresis unit. Proteins on the gel were visualized by coomassie blue staining according to the manufacturer's instructions (Amity; Amersham Life Science). The gel was then scanned using the Personal Densitometer SI (Amity; Amersham Life Science) to determine protein quantities in specific bands within the gel.

3.2.2.2 Western Blotting Analysis

In addition to using conventional staining techniques to detect protein components in gels, immunoblotting was used to detect the presence of a particular protein on the basis of both its interaction with a specific antibody and its relative molecular weight. After SDS-PAGE electrophoresis, the gel was washed two times with Tris-buffered saline (TBS) and its embedded proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, Calif.) using standard techniques. The membrane was blocked overnight with Blotto (5% (w/v) solution of non-fat milk powder in TBS/0.05% (v/v) Tween 20). To immunoprobe the IL-2 component of the fusion protein, a polyclonal rabbit anti-human IL-2 antibody, diluted to 2.5 μg/ml in Blotto, was added and incubated for 2 hr at 37°C. Following three washes in TBS/ 0.05% (v/v) Tween 20 (TBST), the membrane was then incubated with a goat anti-rabbit IgG antibody coupled to HRP for 1 hr, which had been diluted to 2μg/ml in Blotto. After three

further washes in TBST, the membrane was tested with chemiluminescence detection solutions (Santa Cruz, Carpinteria, Calif) and exposed to a Hyperfilm (Amity; Amersham Life Science) for 30 seconds. The film was then removed and developed immediately.

3.2.2.3 Determination of the Antigen-binding Specificity of Fusion Proteins

The specific antigen-binding activity of fusion proteins H520C9sFv-rhIL-2 and H520C9sFv-mrhIL-2 were measured by cell mediated ELISA using cultured SKOV3, B16/*neu* and HeLa cells. Human ovarian carcinoma SKOV3 cells and mouse melanoma B16/*neu* cells were shown to express high levels of p185 while HeLa cells were p185 negative.

A C8 Maxisorp Nunc Immunomodule Plate (Nunc, Roskilde, Denmark) was firstly precoated with poly-D-lysine hydrobromide (Sigma) to promote cell attachment. Each well was incubated with 50

μl of poly-D-lysine (0.1 mg/ml) at 37°C for 15 min. The solution was then aspirated and the plate was allowed to dry for two days. Thereafter, 1 x 10^4 live SKOV3, B16/*neu* or HeLa cells in 100 μl culture medium were loaded into each well of the coated plate overnight at 37°C. The cells were fixed in 4% (w/v) paraformaldehyde/phosphate-buffered saline for 10 min at 37°C and then washed three times with phosphate-buffered saline containing 0.05% (v/v) Tween-20 (PBST). Afterwards, 100 μl of serially diluted samples of the H520C9sFv-rhIL-2 or H520C9sFv-mrhIL-2 were added to each well of the plate and incubated at 37°C for 2 h. After washing three times with PBST, each well was blocked with 100μl of Blotto at 37°C for 1 hr. After additional three washes with PBST, 100 μl of a rabbit anti-human IL-2 antibody (1μg/ml) was added to the cells in each well and incubated for 2 h. Following three further washes with PBST, 100 μl of a HRP-conjugated goat anti-rabbit IgG polyclonal antibody (DAKO) (0.5 μg/ml) was added to the cells in each well. After 2 h incubation at 37°C, the cells were washed three times with PBST. Color was developed with the addition

wells with PBST, color development was performed with peroxidase substrate. The resulting OD was then determined at 450nm in a microplate reader.

3.2.2.5 Determination of the IL-2 Bioactivity in Fusion Proteins

Biological activity of the fusion proteins H520C9sFv-rhIL-2 and H520C9sFv-mrhIL-2 was determined by a standard IL-2 dependent T-cell proliferation assay using the murine T cell line CTLL-2. CellTiter 96® AQueous Assay (Promega, Madison, WI) comprising solutions of a MTS tetrazolium compound and an electron coupling reagent PMS was adopted for measurement of cell viability according to the manufacturer's instructions. The assay was conducted in flat-bottomed 96-well plates and results were directly quantified using a standard ELISA plate reader without prior washing or cell harvesting. The conversion of MTS into aqueous, soluble formazan was accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of

living cells in culture. As an indirect measure of viable cell number, the overall metabolic activity in a cell population was evaluated.

Since the nonadherent CTLL-2 cells showed poor viability after being spun down and resuspended in new medium, they were not removed from the spent medium before ELISA was carried out. The cells were deprived of IL-2 for 3 days before seeding into a flat-bottomed 96-well plate at 5 X 10^3 cells per well. Serially diluted samples of the H520C9sFv-rhIL-2, H520C9sFv-mrhIL-2 and standard samples of rhIL-2 were incubated with the cells in each well for 2 days at 37°C. Twenty μl of chromogen solution (MTS/PMS) was added to each of the wells and incubated for 2 h before determining absorbance of the wells at 490 nm using an ELISA plate reader. The activities of the fusion proteins were calculated from the rhIL-2 standard curve.

3.2.3 Examination of In Vivo Properties of Fusion Proteins

3.2.3.1 Subcutaneous Tumor Model in Nude and C57/BL Mice

The ability of fusion proteins H520C9sFv-rhIL-2 and H520C9sFv-mrhIL-2 pretreatment to enhance the uptake of radiolabeled albumin in p185 positive tumors was evaluated in both B16/*neu* tumor-bearing C57/BL mice and SKOV3 ovarian tumor-bearing nude mice. Each C57/BL mouse received a subcutaneous (s.c.) injection of a 0.3 ml inoculum containing 5 X 10^6 B16/*neu* cells at the back. SKOV3 tumors were induced at the back of the athymic nude mice by s.c. injection to each animal 0.3 ml of physiological saline consisting of 6 X 10^6 SKOV3 cells. The tumors were grown for about 2 weeks when they reached 7 mm in diameter.

3.2.3.2 Preparation of ^{125}I-labeled Mouse Albumin

Mouse albumins (Sigma) were radioiodinated with $^{125}I^-$ to 2 μCi/μg using the IODO-GEN method. Iodogen solution (0.025 mg/ml) was prepared by dissolving 0.1 mg of Iodogen (Pierce Chemical Co., Rockford, IL) in 4 ml Chloroform (Sigma). Thereafter, 200 μl of the iodogen solution was added to each 1.5 ml eppendorf tube. The tubes were then dried under a gentle controlled stream of nitrogen for 1 hr. Five hundred μg of mouse albumin was premixed with 55 μl of 0.1 M Hepes, pH 7.4, in a clean uncoated eppendorf tube. The reaction mixture together with 50 μl of carrier-free ^{125} I (1 mCi; Amersham Biosciences China Ltd, HK) were then allowed to react in an iodogen coated tube for 10 min in a 37°C water bath. The reaction was quenched by transferring the reaction mixture to a clean uncoated eppendorf tube. The efficiency of the iodination was examined by running 1 μl of radioiodinated albumin solution on a strip of thin layer chromatography (TLC) paper (Whatman) using 10 ml of 5% (w/v)

potassium iodine (KI) as the mobile phase for 30 min. The paper strip was then cut into 2 pieces (1/3 total length for the origin and the remaining 2/3 for the elution front) and the radioactivity embedded in each piece was measured by a gamma counter. The labeled albumins were finally purified by elution over a 10-cm Sephadex G-25 column with PBS, if the efficiency of iodination was less than 80%.

3.2.3.3 Vascular Permeability Studies of Fusion Proteins

To investigate if pretreatment of a tumor bearing mouse with H520C9sFv-rhIL-2 leads to increased vascular permeability at the tumor site and hence increased uptake of ^{125}I-labeled albumin in the tumor, the following experiments were undertaken. All the experiments were repeated at least twice with three mice per group in each experiment.

3.2.3.3.1 Preliminary Studies Using ^{125}I-labeled BSA

The ability of the fusion protein H520C9sFv-rhIL-2 to increase vasopermeability at the tumor site was assessed in C57/BL mice using ^{125}I-labeled bovine serum albumin (BSA) (Perkin Elmer, London, UK). Fourteen days post implantation of B16/*neu* tumor cells, 7-week-old tumor-bearing C57/BL mice were randomly assigned into 2 groups of 6 as shown:

Group	Tumor model	Treatment agent	Administration route
A	C57/BL mice	9 μg of H520C9sFv-rhIL-2 in 0.3 ml volume	Intravenously
B		0.3 ml of SFM without G418	

Two and a half hours post injection of each of the treatment agents, each animal was anesthetized with intraperitoneal (i.p.) injection of 0.3 ml avertin consisting of 20 g of avertin / liter in PBS followed by an intravenous (i.v.) administration of 0.1-ml inoculum containing 30

μCi ^{125}I-labeled BSA (specific activity = 1 mCi in 1 mg of BSA). Three animals from each group of mice were then sacrificed by cervical dislocation 2.5 and 5 h post injection of the ^{125}I-labeled albumin. The tumors, kidneys, livers, lungs, spleens, long bones and muscle were removed and weighed. The uptake of ^{125}I-labeled albumin in the samples was counted in an automatic gamma counter and expressed as % injected dose per gram of tissue. Sixty μl of blood from each animal was also collected by cardiac puncture and transferred to a heparinized glass tube. All blood samples were individually chromatographed on a TLC paper (Whatman) using 10 ml of 5% (w/v) KI as the mobile phase for 30 min. The paper was analyzed as in section 3.2.3.2. Tumor:nontumor uptake ratios were determined by dividing the % injected dose per gram of tumor by the corresponding values for normal tissues. From these data, the means and standard error were calculated for each group.

3.2.3.3.2 Time-dependence Study Using ^{125}I- mouse Albumin

In the second experiment, the effect of H520C9sFv-rhIL-2 on tumor uptake of ^{125}I-labeled mouse albumin was evaluated in the C57/BL syngeneic mouse tumor model. To determine an optimal administration route and the time course of tumor uptake, 0.3 ml each of 2 different treatment agents was administered either intravenously (i.v.) or intratumorly (i.t.) for various times before the animals were sacrificed for biodistribution analysis of ^{125}I-labeled mouse albumin. The study was carried out in 3 groups of 9 C57/BL mice bearing the B16/*neu* s.c. tumors as follow:

Group	Tumor model	Treatment agent	Administration route
A	C57/BL mice	0.3 ml of SFM without G418	Intravenously
B		9 μg of H520C9sFv-rhIL-2 in 0.3 ml volume	
C			Intratumorly

Two and a half hours post injection of each of the treatment agents, each mouse received an i.p. injection of 0.3 ml avertin followed by an

i.v. injection of 30 μCi ^{125}I-labeled mouse albumin (specific activity = 1 mCi in 1 mg of mouse albumin). Three animals of each group of mice were sacrificed for biodistribution analysis 12 h, 24 h and 72 h post injection of ^{125}I-labeled mouse albumin. The time course of vascular permeability change in tumors and normal organs post injection of the fusion protein could therefore be investigated.

3.2.3.3.3 Dose-dependence Study

To establish an optimal dose of H520C9sFv-rhIL-2 in causing vascular leak in tumor, the following set of experiments was performed. The study was carried out in 3 groups A to C of 3 C57/BL mice bearing B16/*neu* s.c. tumors.

Group	Tumor model	Treatment agent	Administration route
A	C57/BL mice	0.3 ml of SFM without G418	Intravenously
B		9 μg of H520C9sFv-rhIL-2 in 0.3 ml volume	
C		18 μg of H520C9sFv-rhIL-2 in 0.3 ml volume	

Each mouse received an i.v. injection of 30 μCi of ^{125}I-labeled albumin 2.5 h after the administration of a treatment agent and the biodistribution at the time t_{max} for causing maximum vaso-permeability change was measured as in section 3.2.3.3.1.

3.2.3.3.4 Nude Mice Model

After knowing t_{max} due to 9 μg of fusion protein, the study was extended to evaluate whether this vascular permeability change also takes place in other experimental tumor models overexpressing p185. Vascular permeability studies were also performed in athymic nude mice bearing SKOV3 s.c. tumors. The mice were grouped into A to C as follow:

Group	Tumor model	Treatment agent	Administration route
A	Athymic nude mice	0.3 ml of SFM without G418	Intravenously
B		9 μg of H520C9sFv- rhIL-2 in 0.3 ml volume	
C			Intratumorly

Two and a half hours after administrating each of the treatment agents, each of the animals was injected with 0.3 ml avertin and 30 μCi ^{125}I-labeled mouse albumin. The mice were sacrificed at t_{max} for biodistribution analysis of the labeled albumin.

3.2.3.3.5 Study of H520C9sFv-mrhIL-2

To examine whether pretreatment with H520C9sFv-mrhIL-2 could also lead to increased vascular permeability at the tumor site, 3 groups of 3 C57/BL mice bearing B16/*neu* s.c. tumors were administered various doses of H520C9sFv-mrhIL-2 2.5 h before i.v. injections of ^{125}I-labeled mouse albumin as follow:

Group	Tumor model	Treatment agent	Administration route
A	C57/BL mice	0.3 ml of SFM without G418	Intravenously
B		9 μg of H520C9sFv-mrhIL-2 in 0.3 ml volume	
C		18 μg of H520C9sFv-mrhIL-2 in 0.3 ml volume	

All groups of mice were subjected to ^{125}I-labeled mouse albumin biodistribution analysis at t_{max}.

3.2.3.3.6 Study of rhIL-2

To determine if the H520C9sFv-rhIL-2 but not free IL-2 causes preferential increase in the permeability of tumor blood vessels, 2 groups of 3 C57/BL mice bearing B16/*neu* s.c. tumors were injected with 10 μl of standard rhIL-2 (specific activity = 1 X 10^4 IU/μl) or SFM (without G418) 2.5 h before the administration of ^{125}I-labeled mouse albumin as follow:

Group	Tumor model	Treatment agent	Administration route
A	C57/BL mice	10 μl of standard rhIL-2 in 0.3 ml volume	Intravenously
B		0.3 ml of SFM without G418	

Because of the rapid clearance of rhIL-2 from the animals, all mice were sacrified 2.5 h postinjection of 30 μCi of ^{125}I-labeled mouse albumin for biodistribution study. All data are presented as the mean ± standard error.

3.2.3.3.7 Statistical Analysis

In section 3.2.3.3.1, the means of tumor: nontumor ratios between two groups of mice were compared using the Independent Samples T-Test. For other experiments, one-way ANOVA test was conducted to determine if the means of tumor: nontumor ratios differed significantly among various groups of mice. If the overall ANOVA was significant, a post-hoc Tukey's honestly significant difference (Tukey's HSD) test was then followed to compare pairs of group means. The statistical findings were regarded as significant if p values were <0.05.

CHAPTER 4

Results

4.1 Examination of In Vitro Properties of Fusion Proteins

4.1.1 SDS-PAGE and Western Blotting Analysis of Fusion Proteins

Approximately 1000 ml of spent culture medium was collected and concentrated 200 folds from each type of 293 cells transfected with the cDNA of either H520C9sFv-rhIL-2 or H520C9sFv-mrhIL-2. Samples of the fusion proteins in each of the concentrated conditioned media were analyzed by a 12% SDS-PAGE under non-reducing condition and Western blotting. Result of the SDS-PAGE showed that the conditioned media from 293 cells expressing either H520C9sFv-rhIL-2 (Lane A) or H520C9sFv-mrhIL-2 (Lane B) produced a major band at 45 kDa, corresponding to the molecular weights (M.W.s) of the fusion proteins (Fig. 1). Further densitometric analysis of lanes A and B revealed that more than 90% of all protein corresponded to the major band at 45 kDa, suggesting that the concentrated fusion proteins were quite pure.

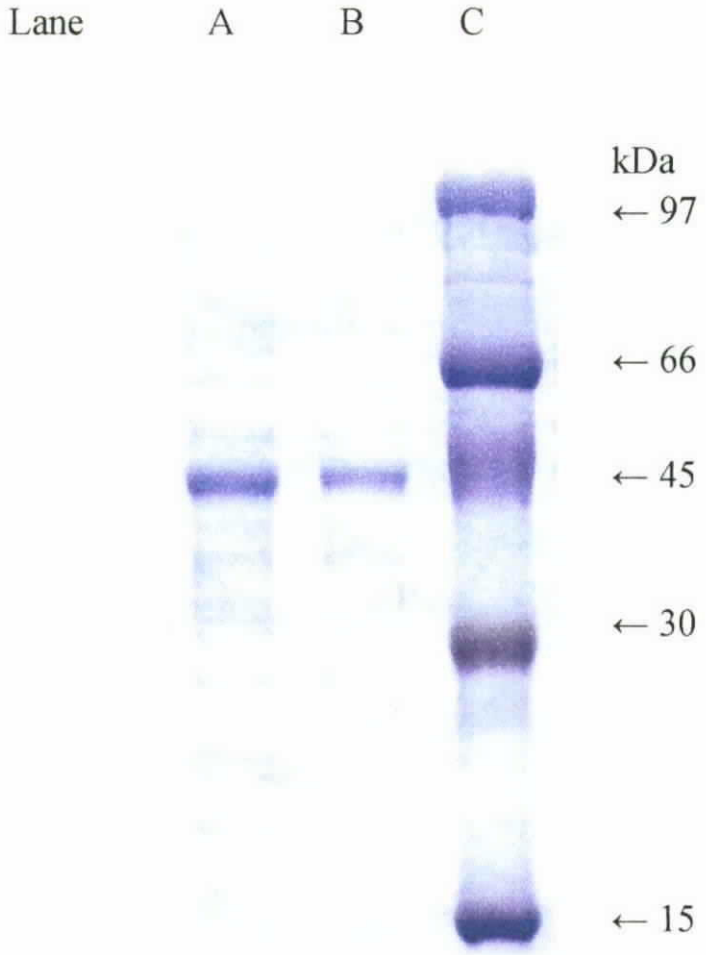

Figure 1: SDS-PAGE gel analysis of fusion proteins. Proteins were boiled without reduction and analyzed on a 12% SDS gel with coomassie blue staining. Lane A, 10 μl of concentrated cultured medium from 293 cells transfected with H520C9sFv-rhIL-2; Lane B, 10 μl of concentrated cultured medium from 293 cells transfected with H520C9sFv-mrhIL-2; Lane C, Rainbow marker

As shown in Figure 2, the rabbit anti-hIL-2 antibody stained positively the 45 kDa bands in the concentrated conditioned media from 293 cells transfected with the cDNA of either H520C9sFv-rhIL-2 (Lane A) or

H520C9sFv-mrhIL-2 (Lane B), indicating the presence of rhIL-2 or mrhIL-2 in these bands. Standard rhIL-2 showed two well-defined bands at 15 kDa and 30 kDa (Lane C), which closely agreed with the predicted molecular weights of rhIL-2 and rhIL-2 dimer. These findings confirmed that the fusion proteins were stably expressed in the transfected 293 cells and remained intact after concentration.

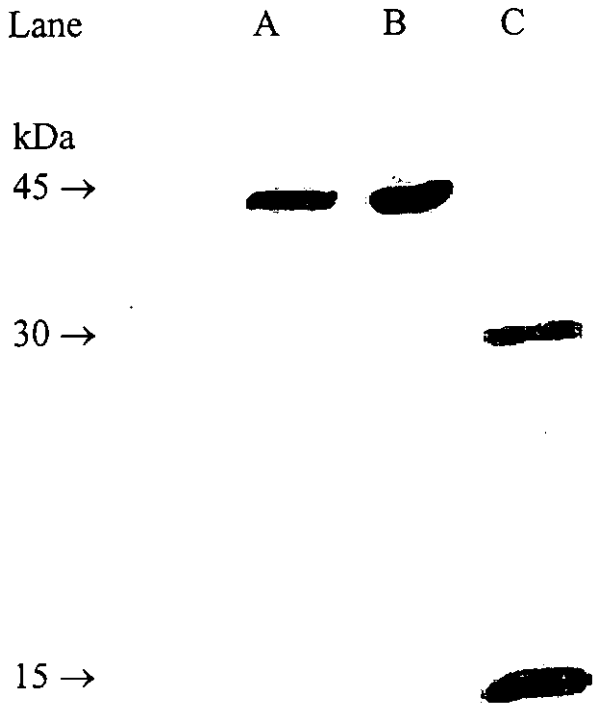

Figure 2: Western blotting analysis of fusion proteins. A polyclonal rabbit anti-hIL-2 antibody and a goat anti-rabbit IgG alkaline phosphate conjugated antibody were used to detect the IL-2 moiety. Lane A, 10 μl of concentrated cultured medium from 293 cells transfected with H520C9sFv-rhIL-2; Lane B, 10 μl of concentrated cultured medium from 293 cells transfected with H520C9sFv-mrhIL-2; Lane C, 5 μg of rhIL-2

4.1.2 Determination of the Antigen-binding Specificity of Fusion Proteins

To demonstrate the specificity of the H520C9sFv-rhIL-2 and H520C9sFv-mrhIL-2 for p185 binding, cellular ELISA was performed using cultured SKOV3, B16/*neu* and HeLa cells. As shown in Figure 3, the "parent" antibody of 520C9sFv, 520C9, displayed binding activity for p185 on the cell surface of SKOV3 and B16/*neu* cells but less binding on the p185 negative HeLa cells. Both H520C9sFv-rhIL-2 and H520C9sFv-mrhIL-2-containing supernatants were able to bind specifically for p185-expressing cells in a dose-dependent manner as did the original 520C9 antibody. However, the binding reactivity for the B16/*neu* cells was relatively lower than that of the SKOV3 cells (Figure 4).

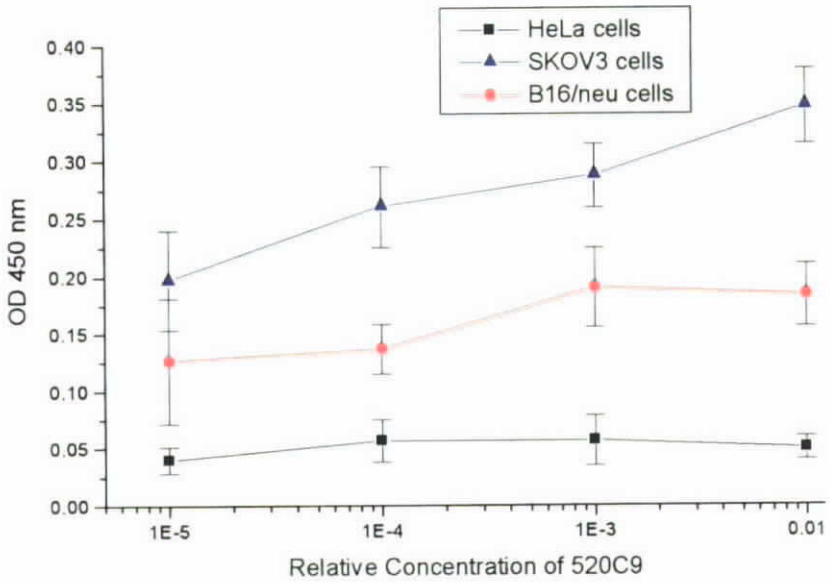

Figure 3. Determination of the p185 antigen binding activity of parental monoclonal antibody 520C9. The 520C9 with serial 10-fold dilutions from the original concentration showed specific antigen binding to p185 positive SKOV3 and B16/*neu* cells, but not to p185 negative HeLa cells. Each data point is the mean of triplicate wells. The error bars show ± one standard error.

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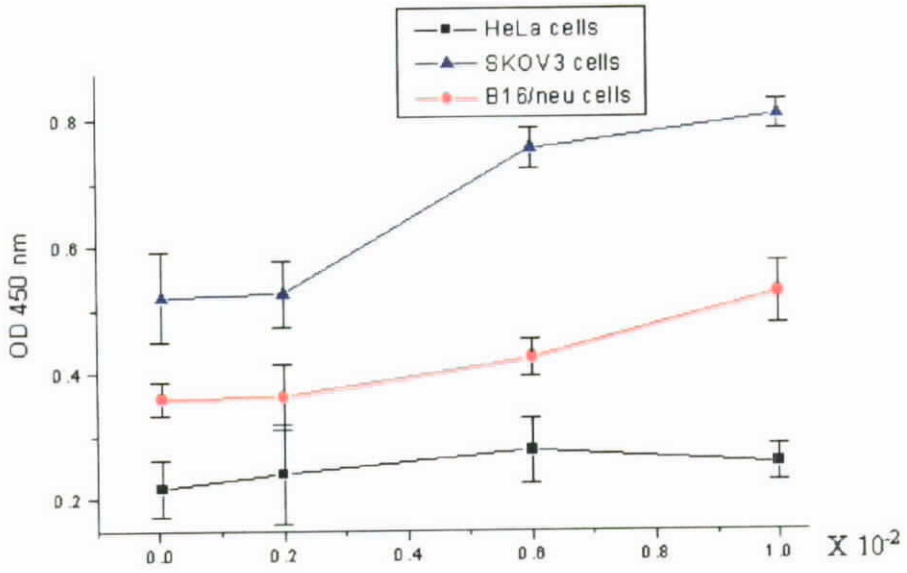
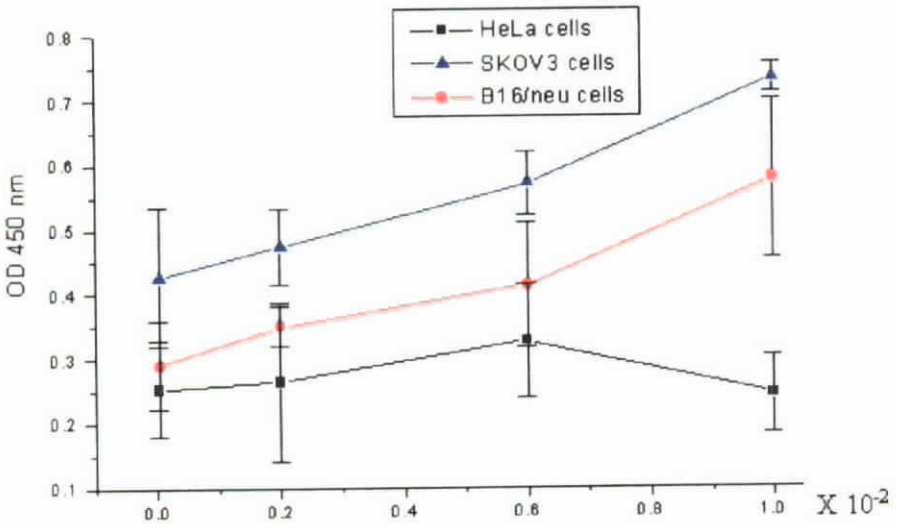


Concentration relative to the concentrated H520C9sFv-rhIL-2

II



Concentration relative to the concentrated H520C9sFv-mrhIL-2

Figure 4. Determination of antigen binding activity of (I) H520C9sFv-rhIL-2 and (II) H520C9sFv-mrhIL-2 for the p185 by indirect cellular ELISA using cultured SKOV3, B16/*neu* and HeLa cells. The H520C9sFv-rhIL-2 and H520C9sFv-mrhIL-2 with various dilutions as indicated from the concentrated supernatants were shown to bind to p185 positive B16/*neu* and SKOV3 cells, but not p185 negative HeLa cells. Each data point is the mean of triplicate wells. The error bars show ± one standard error.

4.1.3 Determination of the IL-2 Moiety in Fusion Proteins

As shown in Figure 5, the dose-response curves of both H520C9sFv-rhIL-2 and H520C9sFv-mrhIL-2-containing supernatants were superimposable with that obtained for the standard rhIL-2 as measured by the IL-2 ELISA for IL-2 concentrations < 1 μg/ml. However, the measured OD readings for the concentrated H520C9sFv-rhIL-2 and H520C9sFv-mrhIL-2 leveled off when their relative concentrations were over 0.1. As the IL-2 component of the H520C9sFv-rhIL-2 and H520C9sFv-mrhIL-2 accounts for only 1/3 of their molecular mass, the estimated concentration of the concentrated fusion proteins was 30 μg ml^{-1}.

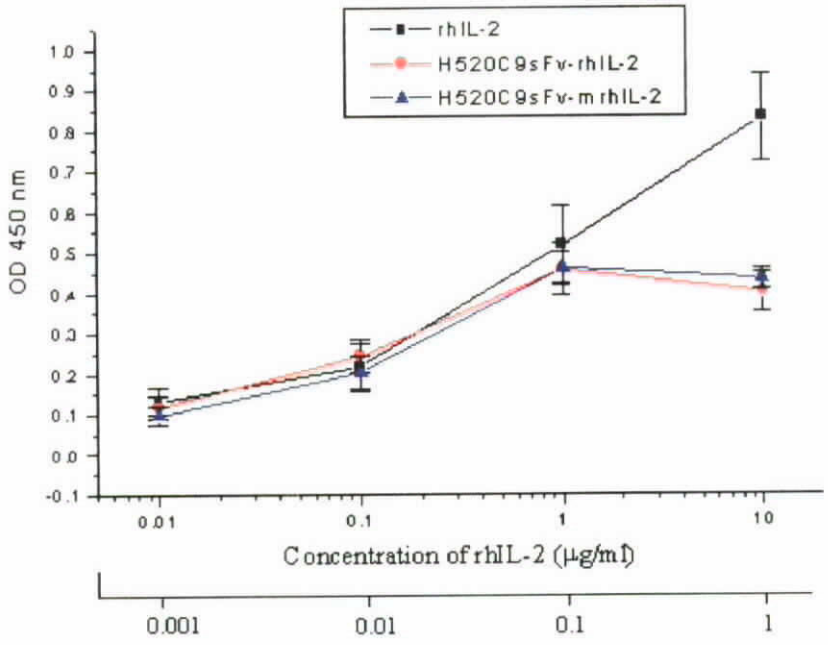


Figure 5 The IL-2 activity of standard rhIL-2 and concentrated H520C9sFv-rhIL-2 or H520C9sFv-mrhIL-2-containing supernatants with serial dilution of 10-folds. Each data point is the mean of triplicate wells. The error bars show ± one standard error.

4.1.4 Determination of the IL-2 Bioactivity in Fusion Proteins

The biological activity of the IL-2 moiety in H520C9sFv-rhIL-2 and H520C9sFv-mrhIL-2 was determined by a standard IL-2-dependent T-cell proliferation assay. Purified rhIL-2 and concentrated conditioned medium from 293 cells secreting H520C9sFv-rhIL-2 gave slightly different dose response curves (Figure 6). Half-maximal stimulation of the CTLL-2 cells occurred at a concentration of approximately 0.1 μg ml^{-1} rhIL-2. The H520C9sFv-rhIL-2 sample achieved the half-maximal stimulation at 0.2 μg ml^{-1}. When compared to rhIL-2 on a molar basis, H520C9sFv-rhIL-2 had an average of 50 % of the bioactivity of rhIL-2. Maximum proliferation was achieved at concentrations equal to or higher than 0.5 μg ml^{-1}. In contrast and as expected, the concentrated conditioned medium from 293 cells secreting H520C9sFv-mrhIL-2 did not have an effect on cell proliferation, indicating that its biological function of mrhIL-2 was lost.

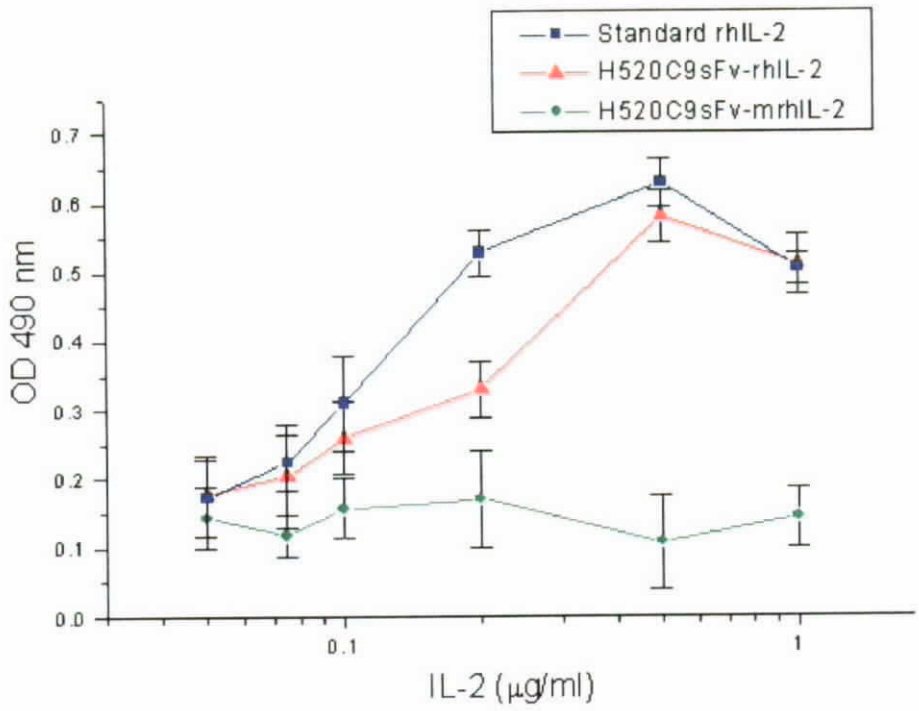


Figure 6 IL-2-dependent CTLL-2 cell proliferation assay of fusion proteins. Concentrated conditioned media from 293 cells expressing either H520C9sFv-rhIL-2 or H520C9sFv-mrhIL-2 were assayed along with rhIL-2 standard.

4.2 Determination of In Vivo Properties of Fusion Proteins

4.2.1 Preliminary Vascular Permeability Studies of the H520C9sFv-rhIL-2

The first series of studies was undertaken to determine whether commercially-available ^{125}I-labeled BSA could be used as a tracer for vascular leak measurement. Two experiments were performed under this series. Results of individual experiments were summarized in Table 1. Essential results of one of the experiments are presented here. Though pretreatment with i.v. injection of H520C9sFv-rhIL-2 generally improved the tracer localization in the tumor at 2.5 h after the injection of the tracer, there was no statistically significant difference in tumor: nontumor ratio for all organs between the H520C9sFv-rhIL-2 (i.v.) treated group and control group ($p>0.05$). As indicated in Figure 7, the tumor: blood ratios at 2.5 h were 4.1:1 and 2.68:1 for the treated and control groups, respectively. Unlike the 2.5 h biodistribution, the H520C9sFv-rhIL-2 (i.v.) treated group at 5 h exhibited marked decreases in tumor: nontumor ratios in most organs. Again, no statistically significant difference in tumor: nontumor ratios was

observed for all organs between the H520C9sFv-rhIL-2 (i.v.) treated group and control group ($p>0.05$). The result of thin layer chromatography as shown in appendix 4 illustrated that 80% of the ^{125}I-labeled BSA in the blood samples was decomposed 2.5 h postinjection of tracer.

I

	Tumor: nontumor ratio[a]			
	Saline		9 μg of H520C9sFv-rhIL-2 (i.v.)	
Organ	Individual Experiments	Overall	Individual Experiments	Overall
Bone	1.96±0.20(3),1.47±0.48(2)	1.76±0.22(5)	2.10±0.43(3),1.61±0.41(3)	1.86±0.29(6)
Blood	2.68±0.87(3),0.59±0.28(2)	1.84±0.71(5)	4.10±0.21(3),6.54±6.08(3)	5.32±2.77(6)
Kidney	1.52±0.71(3),0.51±0.08(2)	1.12±0.46(5)	3.53±0.59(3),2.53±2.05(3)	3.03±0.98(6)
Liver	1.56±0.74(3),1.32±0.39(2)	1.46±0.43(5)	1.73±0.89(3),1.55±0.67(3)	1.64±0.50(6)
Lung	0.91±0.23(3),0.78±0.15(2)	0.85±0.14(5)	1.15±0.35(3),0.55±0.09(3)	0.85±0.21(6)
Muscle	2.59±0.72(3),2.03±0.85(2)	2.37±0.50(5)	3.96±0.84(3),4.27±1.32(3)	4.12±0.70(6)
Spleen	2.36±0.63(3),1.05±0.17(2)	1.83±0.48(5)	3.29±0.32(3),4.28±3.48(3)	3.78±1.58(6)

[a] Mean ± SE (no. of mice)

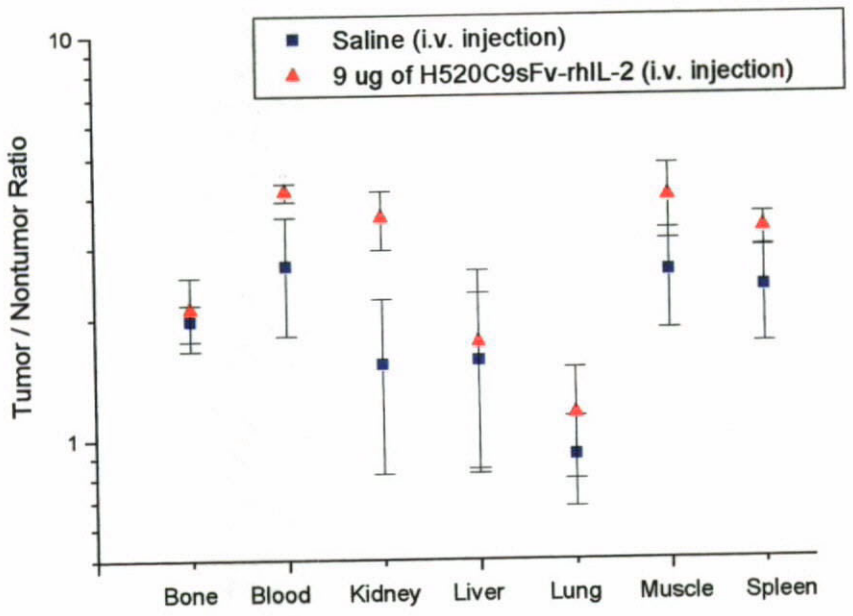
II

	Tumor: nontumor ratio[a]			
	Saline		9 μg of H520C9sFv-rhIL-2 (i.v.)	
Organ	Individual Experiments	Overall	Individual Experiments	Overall
Bone	1.61±0.56(3),1.75±0.00(1)	1.65±0.40(4)	1.95±0.70(2),0.97±0.18(3)	1.36±0.34(5)
Blood	1.68±0.60(3),0.46±0.00(1)	1.38±0.52(4)	3.17±0.48(2),3.49±2.13(3)	3.36±1.18(5)
Kidney	1.36±0.11(3),0.79±0.00(1)	1.22±0.16(4)	1.72±0.26(2),1.65±0.67(3)	1.68±0.37(5)
Liver	1.36±0.38(3),1.73±0.00(1)	1.45±0.28(4)	1.16±0.31(2),0.93±0.17(3)	1.02±0.14(5)
Lung	0.83±0.29(3),1.07±0.00(1)	0.89±0.21(4)	0.82±0.46(2),0.63±0.16(3)	0.71±0.17(5)
Muscle	2.42±0.25(3),2.50±0.00(1)	2.44±0.17(4)	2.45±0.80(2),0.99±0.19(3)	1.57±0.45(5)
Spleen	1.71±0.39(3),1.76±0.00(1)	1.73±0.27(4)	0.96±0.71(2),2.18±1.01(3)	1.69±0.67(5)

[a] Mean ± SE (no. of mice)

Table 1 Summary of uptake ratios of ^{125}I-labeled BSA in B16/*neu* s.c. tumor bearing C57/BL mice pretreated with saline or 9μg of H520C9sFv-rhIL-2 (i.v.) (I) Two and a half hours after the administration of ^{125}I-labeled BSA (II) Five hours after the administration of ^{125}I-labeled BSA

I



II

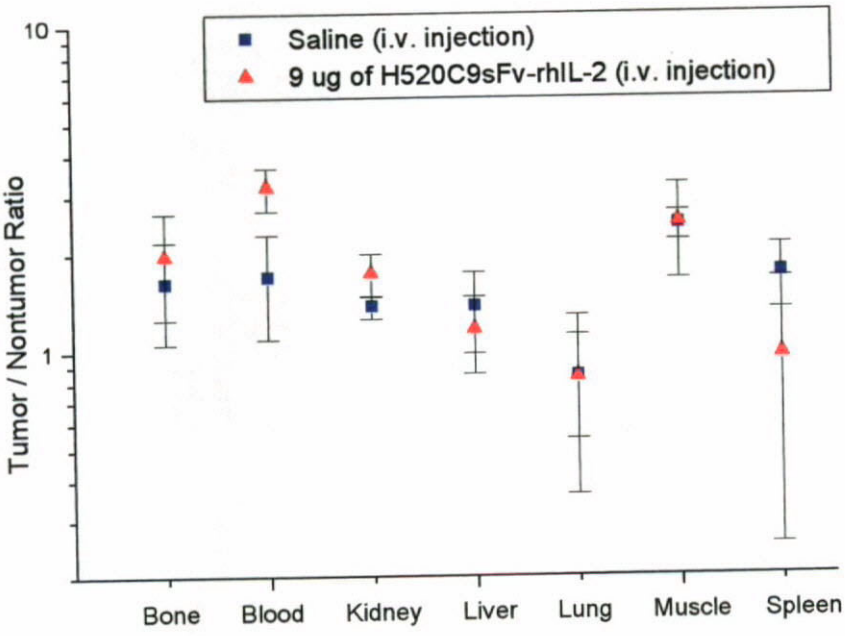


Figure 7 Vascular permeability changes in B16/*neu* s.c. tumor bearing C57/BL mice treated with saline (■) or 9 μg of H520C9sFv-rhIL-2 (i.v.) (▲) (I) Two and a half hours after injection of ^{125}I-labeled BSA. Three mice/group; $p>0.05$ Control group versus Treatment group (II) Five hours after injection of ^{125}I-labeled BSA. Three mice/group but 1 out of 3 mice in treatment group died prior to the vascular leak assay; $p>0.05$ Control group versus Treatment group. Values are expressed as means of tumor: nontumor ratio ± standard error.

4.2.2 Radioiodination of Mouse Albumin

To develop a more metabolically stable tracer for measuring vascular leak that may be induced by H520C9sFv-rhIL-2, mouse albumin was radiolabelled with ^{125}I. After radioiodination, the labeled albumin was separated from free ^{125}I by molecular sieve chromatography over a Sephadex G-25 column. The purity of the radioiodinated albumin was then analyzed using instant thin-layer chromatography and labeling efficiency greater than 85% was achieved. In the following studies, ^{125}I-labeled mouse albumin was adopted as tracer because its clearance rate was expected to be slower than that of the ^{125}I- labeled BSA.

4.2.3 Vascular Permeability Studies of H520C9sFv-rhIL-2 in C57/BL Mice Bearing Subcutaneous Tumor

4.2.3.1 Time-dependence Study

4.2.3.1.1 Twelve Hour Biodistribution Study

Three experiments were performed under this series. The results of individual experiments were summarized in Table 2 and the results of one of the experiments are presented here in figure format. As indicated in Figure 8, the H520C9sFv-rhIL-2 (i.t.) group had pronounced vascular leakage at the tumor, thereby eliciting the highest tumor: nontumor ratio for all organs among the 3 groups of mice. Analysis using one-way ANOVA (Appendix 5) demonstrated significant differences in tumor: nontumor ratio for bone, kidney, muscle and spleen among the three groups ($p<0.05$). Post-hoc multiple comparison using Tukey's HSD test suggested that significantly higher tumor: nontumor ratios were obtained in these organs for the H520C9sFv-rhIL-2 (i.t.) group when compared to the control group. The H520C9sFv-rhIL-2 (i.v.) group did not have significantly higher tumor: nontumor ratio than the control group in the organs sampled.

	Tumor: nontumor ratio[a]	
	Saline	
Organ	Individual Experiments	Overall
Bone	1.74±0.41(3),2.17±0.39(3),2.09±0.30(3)	2.00±0.19(9)
Blood	0.19±0.08(3),0.15±0.05(3),0.29±0.08(3)	0.21±0.04(9)
Kidney	0.40±0.09(3),0.49±0.08(3),0.46±0.12(2)	0.45±0.05(9)
Liver	1.54±0.30(3),2.30±0.50(3),1.95±0.44(3)	1.93±0.24(9)
Lung	0.31±0.03(3),0.34±0.16(3),0.65±0.18(3)	0.44±0.09(9)
Muscle	2.59±1.06(3),3.28±0.53(3),2.38±0.22(3)	2.75±0.37(9)
Spleen	0.79±0.16(3),0.92±0.18(3),0.92±0.17(3)	0.88±0.09(9)

	Tumor: nontumor ratio[a]	
	9 μg of H520C9sFv-rhIL-2 (i.v.)	
Organ	Individual Experiments	Overall
Bone	3.52±0.24(3),4.33±0.44(3),3.20±0.03(3)	3.68±0.22(9)
Blood	0.68±0.34(3),0.88±0.33(3),0.82±0.40(3)	0.79±0.18(9)
Kidney	0.93±0.20(3),0.80±0.22(3),1.20±0.41(3)	0.98±0.16(9)
Liver	3.97±0.31(3),3.98±0.14(3),3.08±0.25(3)	3.67±0.19(9)
Lung	1.82±0.57(3),1.92±0.38(3),1.32±0.21(3)	1.69±0.23(9)
Muscle	5.77±0.61(3),6.37±1.05(3),3.35±0.27(3)	5.16±0.59(9)
Spleen	1.85±0.06(3),1.96±0.09(3),2.70±0.29(3)	2.17±0.16(9)

	Tumor: nontumor ratio[a]	
	9 μg of H520C9sFv-rhIL-2 (i.t.)	
Organ	Individual Experiments	Overall
Bone	4.94±1.05(2),3.57±0.38(3),3.66±0.62(2)	3.99±0.39(7) *
Blood	1.96±1.49(2),2.50±1.09(3),1.59±1.17(2)	2.09±0.60(7)
Kidney	1.32±0.03(2),1.73±0.38(3),2.07±0.25(2)	1.71±0.19(7) *
Liver	3.95±1.40(2),3.74±0.54(3),3.32±0.51(2)	3.68±0.40(7)
Lung	2.68±1.67(2),2.67±0.87(3),1.54±0.28(2)	2.35±0.54(7)
Muscle	7.47±0.18(2),6.82±0.40(3),4.68±0.47(2)	6.39±0.49(7) *
Spleen	4.70±1.88(2),5.08±1.31(3),4.49±1.24(2)	4.80±0.70(7) *

[a] Mean ± SE (no. of mice)

Table 2 Summary of 12 hours biodistribution of ^{125}I-labeled mouse albumin in B16/*neu* s.c. tumor bearing C57/BL mice pretreated with saline, 9 g of H520C9sFv-rhIL-2 (i.v.) or 9 g of H520C9sFv-rhIL-2 (i.t.) 2.5 h before the administration of ^{125}I-mouse albumin. *$p<0.05$ i.t. group versus saline group.

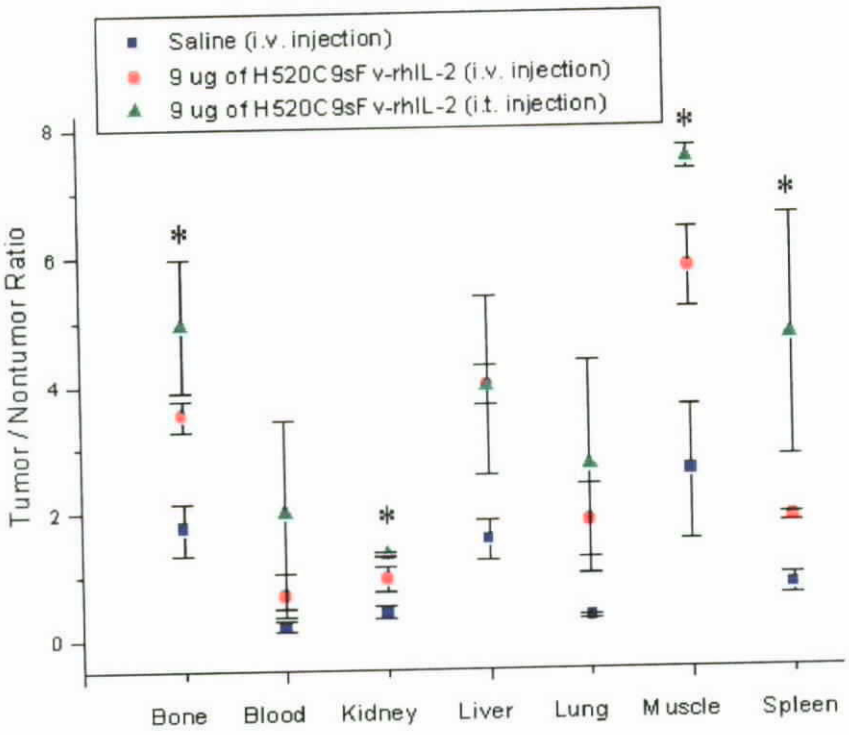


Figure 8 Twelve hours biodistribution of ^{125}I-labeled mouse albumin in B16/*neu* s.c. tumor bearing C57/BL mice pretreated with saline (▪), 9 μg of H520C9sFv-rhIL-2 (i.v.) (•) or 9 μg of H520C9sFv-rhIL-2 (i.t.) (▲) 2.5 h before the administration of ^{125}I-mouse albumin. Three mice/group but 1 out of 3 mice in H520C9sFv-rhIL-2 (i.t.) group died prior to the vascular leak assay; *$p<0.05$ i.t. group versus saline group. Values are expressed as means of tumor: nontumor ratio ± standard error

4.2.3.1.2 Twenty-four Hour Biodistribution Study

Three experiments were performed under this series. The results of individual experiments were summarized in Table 3 and the results of one of the experiments are presented here in figure format. The biodistribution profile at 24 h after ^{125}I-labeled mouse albumin injection was represented in Figure 9. Pretreatment with H520C9sFv-rhIL-2-containing supernatant either i.v. or i.t. provoked a marked increase in localization of the radiolabeled albumin to the tumor and, therefore, tumor: nontumor ratios were fairly high, ranging from 2:1 to 8:1 for most organs. Tumor: blood ratios, normally the lowest of all organs, were 0.28:1, 1.79:1 and 5.19:1 for the control, H520C9sFv-rhIL-2 (i.v.) and H520C9sFv-rhIL-2 (i.t.) groups, respectively. One-way ANOVA analysis (Appendix 5) showed that there was significant difference in tumor: nontumor ratios for bone, blood, kidney, lung, muscle and spleen among the three groups ($p<0.05$). Further analysis using the Tukey's HSD test illustrated that the H520C9sFv-rhIL-2 (i.t.) group had statistically higher

tumor:nontumor ratios in these organs when compared to the saline group but only higher tumor:blood ratio when compared to the H520C9sFv-rhIL-2 (i.v.) group. The H520C9sFv-rhIL-2 (i.v.) group had significantly higher tumor: nontumor ratios than the control group in bone and muscle only.

	Tumor: nontumor ratio[a]	
	Saline	
Organ	Individual Experiments	Overall
Bone	1.33±0.12(3),1.32±0.06(3),1.75±0.18(3)	1.46±0.09(9)
Blood	0.28±0.06(3),0.26±0.05(3),0.32±0.07(3)	0.29±0.03(9)
Kidney	0.58±0.10(3),0.64±0.12(3),0.83±0.15(3)	0.68±0.07(9)
Liver	2.61±1.52(3),2.94±0.88(3),2.53±0.20(3)	2.69±0.51(9)
Lung	0.41±0.16(3),0.44±0.06(3),0.29±0.09(3)	0.38±0.06(9)
Muscle	2.86±0.51(3),2.93±0.13(3),2.90±0.45(3)	2.90±0.20(9)
Spleen	1.21±0.10(3),1.71±0.11(3),1.99±0.09(3)	1.63±0.13(9)

	Tumor: nontumor ratio[a]	
	9 μg of H520C9sFv-rhIL-2 (i.v.)	
Organ	Individual Experiments	Overall
Bone	5.97±0.99(3),6.28±0.54(3),5.38±0.96(3)	5.88±0.45(9) @
Blood	1.79±0.85(3),2.04±0.90(3),1.67±0.84(3)	1.83±0.44(9)
Kidney	2.06±0.32(3),0.98±0.15(3),1.34±0.05(3)	1.46±0.19(9)
Liver	3.89±0.77(3),4.05±0.43(3),2.76±0.32(3)	3.57±0.34(9)
Lung	2.64±0.92(3),2.19±0.70(3),2.94±0.99(3)	2.59±0.45(9)
Muscle	6.60±0.49(3),6.40±0.64(3),5.40±0.75(3)	6.13±0.37(9) @
Spleen	4.44±1.19(3),2.71±0.56(3),3.26±0.52(3)	3.47±0.48(9)

	Tumor: nontumor ratio[a]	
	9 μg of H520C9sFv-rhIL-2 (i.t.)	
Organ	Individual Experiments	Overall
Bone	6.86±1.06(3),6.99±0.58(3),6.84±0.96(3)	6.90±0.45(9) *
Blood	5.19±0.91(3)5.74±0.82(3),5.44±0.93(3)	5.46±0.45(9) *#
Kidney	3.90±0.73(3),2.40±0.64(3),2.32±0.25(3)	2.88±0.39(9) *
Liver	6.50±0.70(3),5.12±0.35(3),3.32±0.17(3)	4.96±0.52(9)
Lung	4.43±0.64(3),3.76±0.32(3),4.89±0.52(3)	4.36±0.30(9) *
Muscle	8.10±1.07(3),7.95±0.94(3),6.31±0.23(3)	7.45±0.50(9) *
Spleen	6.09±1.08(3),4.68±0.70(3),4.87±0.36(3)	5.21±0.45(9) *

[a] Mean ± SE (no. of mice)

Table 3 Summary of 24 hours biodistribution of ^{125}I-labeled mouse albumin in B16/*neu* s.c. tumor bearing C57/BL mice pretreated with saline, 9 g of H520C9sFv-rhIL-2(i.v.) or 9 g of H520C9sFv-rhIL-2 (i.t.) 2.5 h before the administration of ^{125}I-mouse albumin. *@p<0.05* i.v. group versus saline group; **p<0.05* i.t. group versus saline group; *#p<0.05* i.t. group versus i.v. group

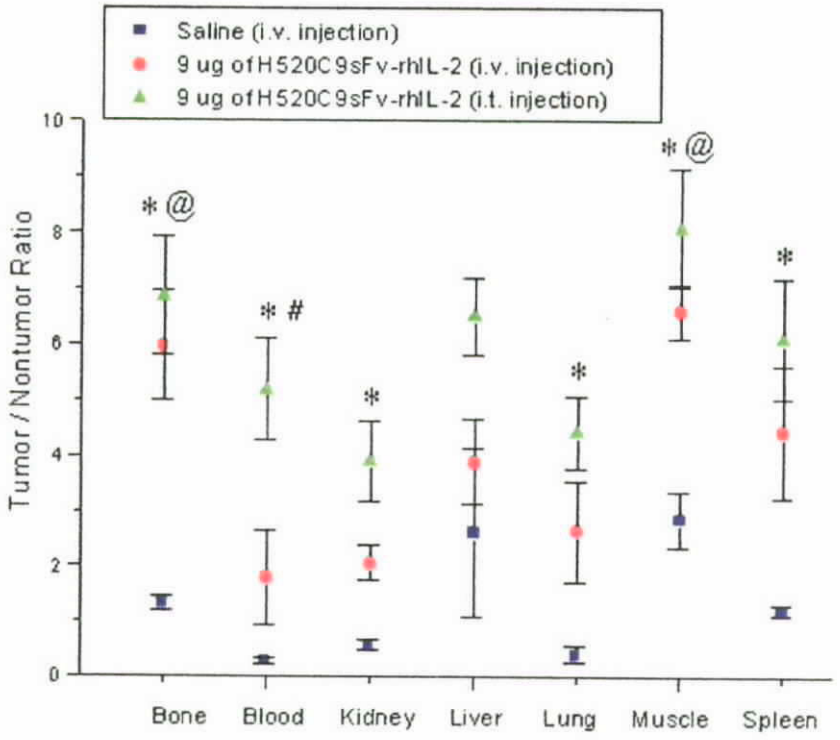


Figure 9 Twenty four hours biodistribution of ^{125}I-mouse albumin in B16/*neu* s.c. tumor bearing C57/BL mice pretreated with saline (■), 9 μg of H520C9sFv-rhIL-2 (i.v.) (●) or 9 μg of H520C9sFv-rhIL-2 (i.t.) (▲) 2.5 h before the administration of ^{125}I-mouse albumin. Three mice/group; @$p<0.05$ i.v. group versus saline group; *$p<0.05$ i.t. group versus saline group; #$p<0.05$ i.t. group versus i.v. group; Values are expressed as means of tumor: nontumor ratio ± standard error

4.2.3.1.3 Seventy-two Hour Biodistribution Study

Three experiments were performed under this series. The results of individual experiments were summarized in Table 4 and the results of one of the experiments are presented here in figure format. In Figure 10, the enhancement of tumor: nontumor ratios in the 72-h biodistribution profile was less remarkable than that obtained at 24 h. Statistical analysis from one-way ANOVA (Appendix 5) suggested that the tumor: nontumor ratios for bone, lung and muscle were significantly different among the three groups ($p<0.05$). Post-hoc Tukey's HSD test indicated significantly higher tumor:nontumor ratios for the H520C9sFv-rhIL-2 (i.t.) group in these organs when compared to the control group. However, there was no statistically significant difference between the H520C9sFv-rhIL-2 (i.v.) group and the control group.

	Tumor: nontumor ratio[a]	
	Saline	
Organ	Individual Experiments	Overall
Bone	1.74±0.30(3),1.91±0.06(3),1.84±0.12(3)	1.83±0.10(9)
Blood	0.39±0.08(3),0.42±0.12(3),0.49±0.07(3)	0.43±0.05(9)
Kidney	0.68±0.03(3),0.76±0.08(3),0.76±0.05(3)	0.73±0.03(9)
Liver	1.51±0.21(3),1.57±0.13(3),1.61±0.05(3)	1.56±0.07(9)
Lung	0.58±0.06(3),0.82±0.06(3),0.74±0.04(3)	0.71±0.05(9)
Muscle	2.95±0.19(3),3.03±0.16(3),2.90±0.21(3)	2.96±0.10(9)
Spleen	1.18±0.50(3),1.16±0.50(3),1.02±0.35(3)	1.12±0.23(9)

	Tumor: nontumor ratio[a]	
	9 μg of H520C9sFv-rhIL-2 (i.v.)	
Organ	Individual Experiments	Overall
Bone	2.78±0.13(3),2.55±0.21(2),2.06±0.18(3)	2.42±0.14(8)
Blood	0.49±0.13(3),0.44±0.08(2),0.50±0.09(3)	0.47±0.05(8)
Kidney	0.94±0.05(3),0.83±0.06(2),0.83±0.06(3)	0.86±0.03(8)
Liver	1.89±0.28(3),1.86±0.09(2),1.89±0.12(3)	1.88±0.07(8)
Lung	0.79±0.02(3),0.90±0.10(2),0.93±0.07(3)	0.88±0.04(8)
Muscle	3.36±0.03(3),3.54±0.08(2),3.70±0.14(3)	3.56±0.07(8)
Spleen	2.43±0.09(3),2.16±0.10(2),1.90±0.08(3)	2.13±0.09(8)

	Tumor: nontumor ratio[a]	
	9 μg of H520C9sFv-rhIL-2 (i.t.)	
Organ	Individual Experiments	Overall
Bone	3.53±0.41(3),2.91±0.15(3),2.54±0.10(3)	2.99±0.19(9) *
Blood	0.57±0.06(3),0.43±0.04(3),0.58±0.06(3)	0.53±0.04(9)
Kidney	1.04±0.12(3),1.03±0.06(3),0.96±0.04(3)	1.01±0.04(9)
Liver	2.22±0.17(3),1.96±0.07(3),1.84±0.08(3)	2.01±0.08(9)
Lung	0.88±0.07(3),1.18±0.02(3),1.16±0.06(3)	1.07±0.05(9) *
Muscle	4.54±0.38(3),4.48±0.37(3),4.18±0.33(3)	4.40±0.19(9) *
Spleen	2.78±0.29(3),2.41±0.17(3),1.84±0.14(3)	2.34±0.17(9)

[a] Mean ± SE (no. of mice)

Table 4 Summary of 72 hours biodistribution of ^{125}I-labeled mouse albumin in B16/*neu* s.c. tumor bearing C57/BL mice pretreated with saline, 9 g of H520C9sFv-rhIL-2(i.v.) or 9 g of H520C9sFv-rhIL-2 (i.t.) 2.5 h before the administration of ^{125}I-mouse albumin. *$p<0.05$ i.t. group versus saline group.

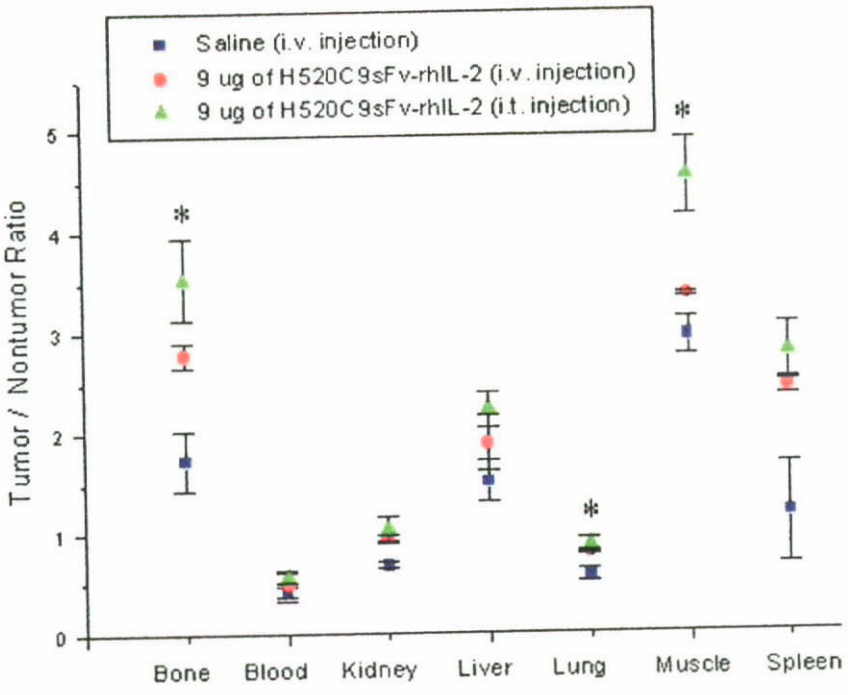


Figure 10 Seventy two hours biodistribution of ^{125}I-labeled mouse albumin in B16/*neu* s.c. tumor bearing C57/BL mice pretreated with saline (■), 9 μg of H520C9sFv-rhIL-2 (i.v.) (●) or 9 μg of H520C9sFv-rhIL-2 (i.t.) (▲) 2.5 h before the administration of ^{125}I-mouse albumin. Three mice/group; *$p<0.05$ i.t. group versus saline group. Values are expressed as means of tumor: nontumor ratio ± standard error.

4.2.3.2 Dose-dependence Study

Two experiments were performed under this series. The results of individual experiments were summarized in Table 5 and the results of one of the experiments are presented here in figure format. Figure 11 showed the relationship between the dose of H520C9sFv-rhIL-2 administrated and tumor: nontumor ratios 24 h post injection of the ^{125}I-labeled mouse albumin. One-way ANOVA analysis illustrated statistically significant difference in tumor:nontumor ratios for all organs except liver among the three groups ($p<0.05$). Post-hoc Tukey's HSD test showed that the 18 μg of H520C9sFv-hIL-2 (i.v.) group had statistically higher tumor:nontumor ratios in all these organs when compared to the saline group but only higher tumor:blood ratio and tumor:kidney ratio when compared to the 9 μg of H520C9sFv-hIL-2 (i.v.) group. The saline group had significantly lower tumor:bone ratio and tumor:muscle ratio when compared to the 9 μg of H520C9sFv-rhIL-2 (i.v.) group.

	Tumor: nontumor ratio[a]	
	Saline	
Organ	Individual Experiments	Overall
Bone	1.60±0.14(3),1.31±0.06(3)	1.45±0.09(6)
Blood	0.37±0.03(3),0.48±0.13(3)	0.43±0.06(6)
Kidney	0.64±0.04(3),0.58±0.07(3)	0.61±0.04(6)
Liver	2.70±0.04(3),2.44±0.56(3)	2.57±0.26(6)
Lung	0.38±0.02(3),0.38±0.08(3)	0.38±0.03(6)
Muscle	3.42±0.28(3),3.56±0.64(3)	3.49±0.32(6)
Spleen	1.51±0.17(3),1.58±0.15(3)	1.55±0.10(6)

	Tumor: nontumor ratio[a]	
	9 μg of H520C9sFv-rhIL-2 (i.v.)	
Organ	Individual Experiments	Overall
Bone	4.49±0.62(3),5.24±0.60(3)	4.87±0.42(6) @
Blood	1.24±0.21(3),1.47±0.28(3)	1.36±0.17(6)
Kidney	1.31±0.28(3),1.16±0.11(3)	1.23±0.14(6)
Liver	3.48±0.67(3),4.65±0.76(3)	4.07±0.52(6)
Lung	2.02±0.46(3),1.23±0.21(3)	1.63±0.29(6)
Muscle	7.07±0.12(3),7.07±0.46(3)	7.07±0.21(6) @
Spleen	3.45±0.62(3),3.10±0.54(3)	3.28±0.38(6)

	Tumor: nontumor ratio[a]	
	18 μg of H520C9sFv-rhIL-2 (i.v.)	
Organ	Individual Experiments	Overall
Bone	6.75±0.51(2),6.73±0.46(2)	6.74±0.28(4) *
Blood	2.74±0.39(2),4.94±0.26(2)	3.84±0.66(4) *#
Kidney	3.41±0.24(2),3.82±0.70(2)	3.61±0.32(4) *#
Liver	3.73±0.59(2),5.44±0.44(2)	4.58±0.58(4)
Lung	4.60±1.26(2),2.34±0.81(2)	3.47±0.89(4) *
Muscle	7.09±0.16(2),8.79±0.47(2)	7.94±0.53(4) *
Spleen	4.71±0.55(2),4.88±0.26(2)	4.80±0.25(4) *

[a] Mean ± SE (no. of mice)

Table 5 Summary of 24 hours biodistribution of ^{125}I-labeled mouse albumin in B16/*neu* s.c. tumor bearing C57/BL mice pretreated with saline, 9 g of H520C9sFv-rhIL-2 (i.v.) or 18 g of H520C9sFv-rhIL-2 (i.v.) 2.5 h before the administration of 125I-mouse albumin. *@p<0.05* 9 μg group versus saline group; **p<0.05* 18 μg group versus saline group; *#p<0.05* 18 μg group versus 9 μg group

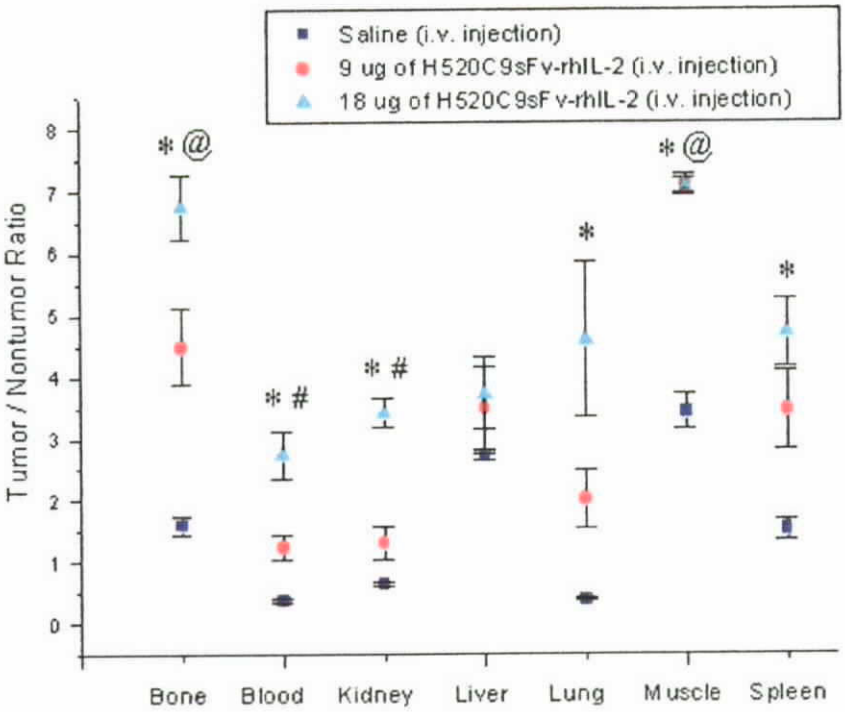


Figure 11 Twenty four hours biodistribution of ^{125}I-labeled mouse albumin in B16/*neu* s.c. tumor bearing C57/BL mice pretreated with saline (■), 9 μg of H520C9sFv-rhIL-2 (i.v.) (●) or 18 μg of H520C9sFv-rhIL-2 (i.v.) (▲) 2.5 h before the administration of ^{125}I-mouse albumin. Three mice/group but 1 out of 3 mice in 18 μg of H520C9sFv-rhIL-2 (i.v.) group died prior to the vascular leak assay; *@p<0.05* 9 μg group versus saline group; **p<0.05* 18 μg group versus saline group; *#p<0.05* 18 μg group versus 9 μg group; Values are expressed as means of tumor: nontumor ratio ± standard error

4.2.4 Vascular Permeability Studies of the H520C9sFv-rhIL-2 in Nude Mice Bearing Subcutaneous Tumor

Three experiments were performed under this series. The results of individual experiments were summarized in Table 6 and the results of one of the experiments are presented here in figure format. As shown in Figure 12, administration of H520C9sFv-rhIL-2 intratumorly in nude mice bearing the SKOV3 s.c. tumors showed improved tumor uptake of ^{125}I-labeled mouse albumin 24 h post its injection compared to the saline control group. Statistically significant differences in tumor: nontumor ratios were observed for bone, kidney and spleen among the three groups using the one-way ANOVA analysis ($p<0.05$) (Appendix 7). Post-hoc test illustrated that the H520C9sFv-rhIL-2 (i.t.) group had statistically higher tumor:nontumor ratios in these 3 organs when compared to the saline group but only higher tumor:bone ratio when compared to the H520C9sFv-rhIL-2 (i.v.) group. Tumor:nontumor ratios were not significantly different between the H520C9sFv-rhIL-2 (i.v.) and saline groups.

	Tumor: nontumor ratio[a]	
	Saline	
Organ	Individual Experiments	Overall
Bone	1.34±0.05(3),1.36±0.12(3),1.34±0.05(3)	1.35±0.04(9)
Blood	0.67±0.14(3),0.89±0.15(3),0.95±0.15(3)	0.83±0.08(9)
Kidney	0.67±0.06(3),0.67±0.05(3),0.72±0.06(3)	0.69±0.03(9)
Liver	1.34±0.12(3),1.48±0.10(3),1.54±0.08(3)	1.45±0.06(9)
Lung	0.70±0.03(3),0.93±0.15(3),0.99±0.18(3)	0.87±0.08(9)
Muscle	2.35±0.43(3),3.27±0.21(3),3.63±0.12(3)	3.08±0.24(9)
Spleen	0.73±0.13(3),0.85±0.09(3),0.99±0.18(3)	0.86±0.08(9)

	Tumor: nontumor ratio[a]	
	9 μg of H520C9sFv-rhIL-2 (i.v.)	
Organ	Individual Experiments	Overall
Bone	1.28±0.02(3),1.47±0.06(3),1.69±0.09(3)	1.48±0.07(9)
Blood	0.83±0.03(3),1.03±0.32(3),1.11±0.22(3)	0.99±0.12(9)
Kidney	0.70±0.11(3),0.91±0.21(3),1.00±0.12(3)	0.87±0.09(9)
Liver	1.28±0.22(3),1.37±0.23(3),1.32±0.20(3)	1.32±0.11(9)
Lung	1.35±0.02(3),1.27±0.02(3),1.43±0.11(3)	1.35±0.04(9)
Muscle	2.96±0.71(3),3.61±0.56(3),3.63±0.54(3)	3.40±0.32(9)
Spleen	1.43±0.19(3),1.33±0.05(3),1.35±0.09(3)	1.37±0.06(9)

	Tumor: nontumor ratio[a]	
	9 μg of H520C9sFv-rhIL-2 (i.t.)	
Organ	Individual Experiments	Overall
Bone	2.07±0.20(3),3.12±0.29(2),3.30±0.34(3)	2.92±0.24(9) *#
Blood	1.10±0.02(3),1.02±0.25(2),1.14±0.16(3)	1.08±0.10(9)
Kidney	1.10±0.02(3),1.26±0.03(2),1.36±0.08(3)	1.26±0.05(9) *
Liver	1.37±0.10(3),1.65±0.16(2),1.73±0.16(3)	1.61±0.09(9)
Lung	1.88±0.77(3),1.28±0.02(2),1.43±0.05(3)	1.48±0.17(9)
Muscle	5.01±1.32(3),4.97±0.53(2),4.76±0.53(3)	4.90±0.35(9)
Spleen	1.98±0.33(3),1.94±0.36(2),1.95±0.19(3)	1.96±0.15(9) *

[a] Mean ± SE (no. of mice)

Table 6 Summary of 24 hours biodistribution of ^{125}I-labeled mouse albumin in SKOV3 s.c. tumor bearing nude mice pretreated with saline, 9 g of H520C9sFv-rhIL-2 (i.v.) or 9 g of H520C9sFv-rhIL-2 (i.t.) 2.5 h before the administration of ^{125}I-mouse albumin. *$p<0.05$ i.t. group versus saline group; #$p<0.05$ i.t. group versus i.v. group

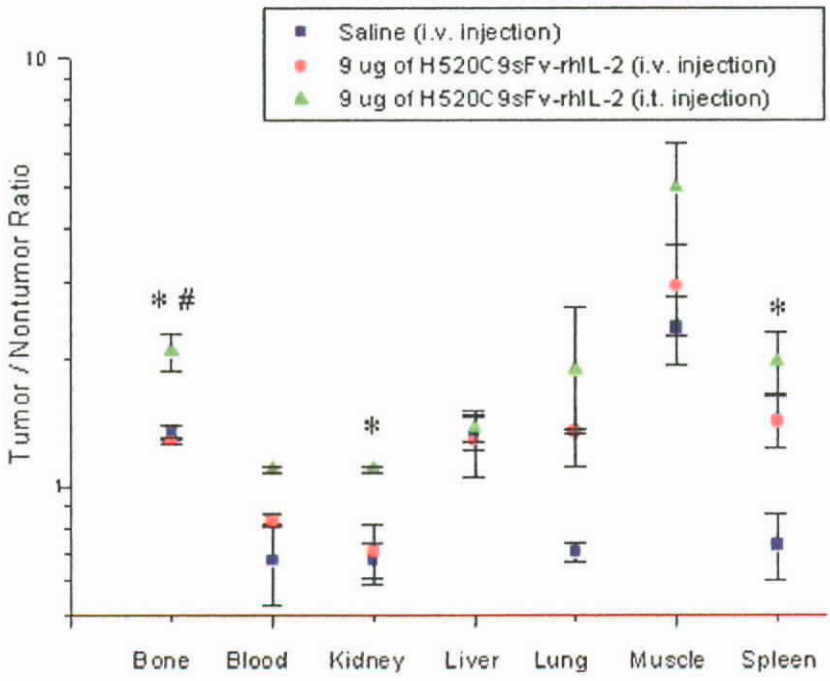


Figure 12 Twenty four hours biodistribution of ^{125}I-labeled mouse albumin in SKOV3 s.c. tumor-bearing nude mice pretreated with saline (■), 9 μg of H520C9sFv-rhIL-2 (i.v.) (●) or 9 μg of H520C9sFv-rhIL-2 (i.t.) (▲) 2.5 h before the administration of ^{125}I-mouse albumin. Three mice/group; *$p<0.05$ i.t. group versus saline group; #$p<0.05$ i.t. group versus i.v. group; Values are expressed as means of tumor: nontumor ratio ± standard error.

4.2.5 Vascular Permeability Studies of H520C9sFv-mrhIL-2 in C57/BL Mice Bearing Subcutaneous Tumor

Three experiments were performed under this series. The results of individual experiments were summarized in Table 7 and the results of one of the experiments are presented here in figure format. Figure 13 demonstrated that the tumor: nontumor uptake ratio of ^{125}I-labeled mouse albumin 24 h post its injection was greatest in the muscle, followed by bone and spleen. In comparison, blood, kidney and lung had lower tumor: organ ratios. As shown in Appendix 9, intravenous injection of up to 18 μg H520C9sFv-mhIL-2 per animal failed to show any significant increase in tumor: nontumor ratio for any of the organs sampled compared with the saline control group ($p>0.05$).

	Tumor: nontumor ratio[a]	
	Saline	
Organ	Individual Experiments	Overall
Bone	1.89±0.30(3),1.90±0.23(3),1.92±0.16(3)	1.90±0.12(9)
Blood	0.33±0.10(3),0.34±0.15(3),0.39±0.17(3)	0.35±0.07(9)
Kidney	0.51±0.10(3),0.51±0.10(3),0.53±0.05(3)	0.52±0.05(9)
Liver	1.25±0.43(3),1.07±0.17(3),1.18±0.12(3)	1.16±0.14(9)
Lung	0.56±0.05(3),0.45±0.07(3),0.58±0.12(3)	0.53±0.05(9)
Muscle	4.55±0.67(3),2.51±0.53(3),2.43±0.36(3)	3.16±0.44(9)
Spleen	1.14±0.10(3),1.27±0.17(3),1.18±0.12(3)	1.20±0.07(9)

	Tumor: nontumor ratio[a]	
	9 μg of H520C9sFv-rhIL-2 (i.v.)	
Organ	Individual Experiments	Overall
Bone	1.81±0.27(3),1.83±0.37(3),1.82±0.24(3)	1.82±0.15(9)
Blood	0.34±0.05(3),0.59±0.09(3),0.65±0.07(3)	0.53±0.06(9)
Kidney	0.55±0.06(3),0.55±0.06(3),0.43±0.08(3)	0.51±0.04(9)
Liver	0.10±0.12(3),1.27±0.49(3),0.89±0.19(3)	1.05±0.17(9)
Lung	0.40±0.04(3),0.51±0.09(3),0.54±0.17(3)	0.48±0.06(9)
Muscle	4.06±0.42(3),2.71±0.35(3),2.86±0.15(3)	3.21±0.27(9)
Spleen	1.17±0.11(3),1.00±0.09(3),1.05±0.15(3)	1.08±0.06(9)

	Tumor: nontumor ratio[a]	
	18 μg of H520C9sFv-rhIL-2 (i.v.)	
Organ	Individual Experiments	Overall
Bone	1.99±0.23(3),2.00±0.70(3),2.33±0.35(3)	2.11±0.24(9)
Blood	0.33±0.07(3),0.78±0.08(3),0.73±0.03(3)	0.61±0.08(9)
Kidney	0.60±0.08(3),0.54±0.06(3),0.62±0.13(3)	0.59±0.05(9)
Liver	1.06±0.15(3),1.42±0.34(3),1.28±0.23(3)	1.25±0.14(9)
Lung	0.48±0.05(3),0.49±0.19(3),0.57±0.18(3)	0.51±0.08(9)
Muscle	4.27±0.40(3),3.35±0.34(3),2.95±0.14(3)	3.52±0.25(9)
Spleen	1.54±0.14(3),1.54±0.16(3),1.43±0.09(3)	1.50±0.07(9)

[a] Mean ± SE (no. of mice)

Table 7 Summary of 24 hours biodistribution of ^{125}I-labeled mouse albumin in B16/*neu* s.c. tumor bearing C57/BL mice pretreated with saline, 9 g of H520C9sFv-mrhIL-2 (i.v.) or 18 g of H520C9sFv-mrhIL-2 (i.v.) 2.5 h before the administration of ^{125}I-mouse albumin.

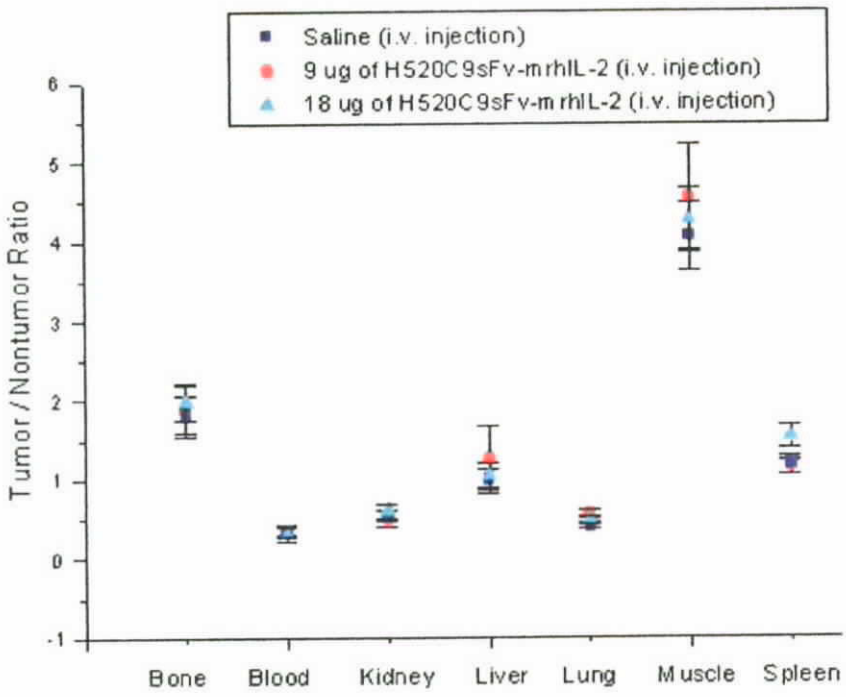


Figure 13 Twenty four hours biodistribution of ^{125}I-labeled mouse albumin in B16/*neu* s.c. tumor bearing C57/BL mice pretreated with saline (■), 9 μg of H520C9sFv-mrhIL-2 (i.v.) (•) or 18 μg of H520C9sFv-mrhIL-2 (i.v.) (▲) 2.5 h before the administration of ^{125}I-mouse albumin. Three mice/group; Values are expressed as means of tumor: nontumor ratio ± standard error

4.2.6 Vascular Permeability Studies of rhIL-2 in C57/BL Mice Bearing Established Subcutaneous Tumor

Two experiments were performed under this series. The results of individual experiments were summarized in Table 8 and the results of one of the experiments are presented here in figure format. Injection of each mouse with 10 μg of rhIL-2 (= 1 X 10^5 IU) induced significantly higher percentage uptakes of the injected ^{125}I-labeled albumin per gram of tissue for all organs than those of the control group ($p<0.05$), as can be seen in Figure 14(I). For instance, the tumor and blood uptakes in the IL-2 group were 9.47 % and 37.18 % injected dose/ gram, while in the saline group they were 2.92% and 9.38 %, respectively. However, result of T-test showed that insignificant difference in tumor: nontumor ratio was found for each of these organs between the saline and rhIL-2 treated groups (Figure 14 II), indicating that there was no preferential increase in tumor uptake for the rhIL-2 treatment.

I

	Percentage of injected dose / gram[a]			
	Saline		10 g of rhIL-2	
Organ	Individual Experiments	Overall	Individual Experiments	Overall
Bone	2.40±0.44(3),1.49±0.02(3)	1.95±0.28(6)	6.80±0.29(3),6.62±0.86(3)	6.71±0.41(6)*
Blood	9.38±1.62(3),9.28±1.15(3)	9.33±0.89(6)	37.18±2.47(3),36.29±3.60(3)	36.74±1.96(6)*
Kidney	5.85±1.04(3),4.70±0.70(3)	5.28±0.62(6)	17.11±1.72(3),16.54±0.99(3)	16.82±0.90(6)*
Liver	3.45±0.45(3),2.25±0.24(3)	2.85±0.35(6)	10.05±0.70(3),9.31±0.81(3)	9.68±0.50(6)*
Lung	4.28±0.98(3),4.34±0.73(3)	4.31±0.55(6)	21.27±0.91(3),19.90±1.25(3)	20.59±0.76(6)*
Muscle	0.47±0.16(3),0.55±0.10(3)	0.51±0.09(6)	3.32±0.37(3),2.19±0.63(3)	2.76±0.41(6)*
Spleen	2.34±0.57(3),2.37±0.40(3)	2.35±0.31(6)	8.54±1.00(3),7.85±0.76(3)	8.20±0.58(6)*
Tumor	2.92±0.57(3),2.37±0.42(3)	2.64±0.34(6)	9.47±0.59(3),8.98±0.69(3)	9.23±0.42(6)*

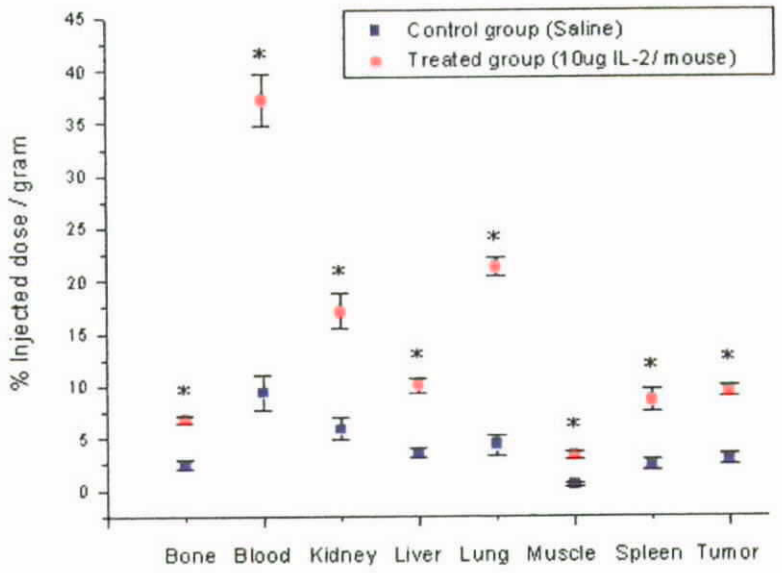
II

	Tumor: nontumor ratio[a]			
	Saline		10 g of rhIL-2	
Organ	Individual Experiments	Overall	Individual Experiments	Overall
Bone	1.37±0.44(3),1.60±0.31(3)	1.49±0.24(6)	1.39±0.07(3),1.38±0.14(3)	1.39±0.07(6)
Blood	0.32±0.07(3),0.25±0.02(3)	0.29±0.04(6)	0.26±0.02(3),0.25±0.03(3)	0.25±0.02(6)
Kidney	0.56±0.18(3),0.51±0.09(3)	0.54±0.09(6)	0.56±0.04(3),0.54±0.03(3)	0.55±0.02(6)
Liver	0.87±0.20(3),1.08±0.22(3)	0.98±0.14(6)	0.94±0.04(3),0.97±0.09(3)	0.96±0.04(6)
Lung	0.70±0.12(3),0.54±0.01(3)	0.62±0.06(6)	0.45±0.05(3),0.45±0.02(3)	0.45±0.02(6)
Muscle	7.13±1.75(3),4.65±1.22(3)	5.89±1.10(6)	2.89±0.17(3),4.56±0.81(3)	3.73±0.53(6)
Spleen	1.30±0.15(3),1.00±0.06(3)	1.15±0.10(6)	1.12±0.06(3),1.15±0.07(3)	1.14±0.04(6)

[a] Mean ± SE (no. of mice)

Table 8 Summary of 2.5 hours biodistribution of ^{125}I-labeled mouse albumin in B16/*neu* s.c. tumor bearing C57/BL mice pretreated with saline or 10 g of rhIL-2 (i.v.) (I) Means of percentage injected dose per gram of tissue. *$p<0.05$ rhIL-2 group versus saline group (II) Means of tumor to nontumor ratio

I



II

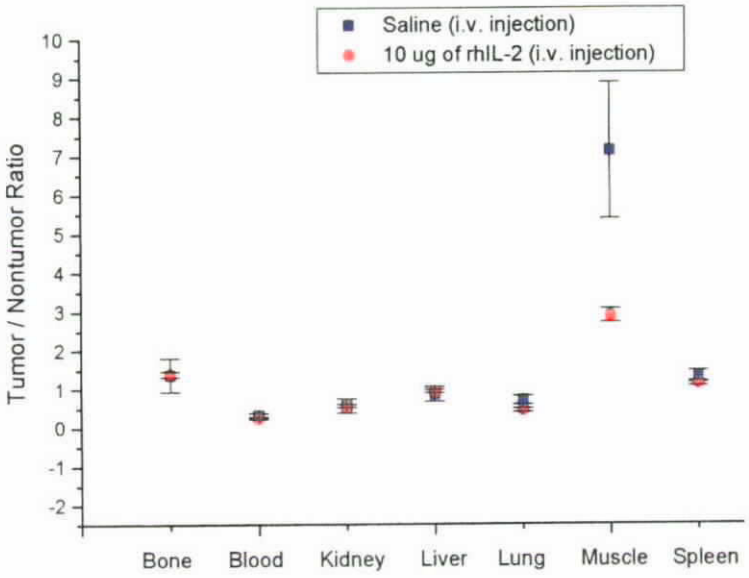


Figure 14 Effect of IL-2 on tumor uptake of ^{125}I-labeled mouse albumin. B16/*neu* s.c. tumor-bearing C57/BL mice were injected intravenously with saline (■) or 10 μg of IL-2 (•) 2.5 h before i.v. administration of 30 μCi ^{125}I-labeled mouse albumin. The mice were sacrificed 2.5 h afterward for biodistribution analysis. (I) Means of percentage of injected dose per gram of tissue. Three mice/group; *$p<0.05$ rhIL-2 group versus saline group (II) Means of tumor to nontumor ratio. Three mice/group; $p>0.05$ rhIL-2 group versus saline group. Values are expressed as means of tumor: nontumor ratio ± standard error.

CHAPTER 5

Discussion

5.1 In Vitro Characterization of the H520C9sFv-rhIL-2

This study reported some in vitro functional characteristics of a fusion protein, H520C9sFv-rhIL-2, capable of retaining both the bioactivity of IL-2 and binding specificity of the anti-p185 single chain antibody H520C9sFv. Such results were consistent with those in an earlier study by Li et al. (1999). Analysis of partially purified H520C9sFv-rhIL-2 by SDS-PAGE and Western blot revealed one major band with expected molecular weight of 45 kDa (Figs. 1 and 2). The densitometric analysis found that more than 90% of all proteins in the concentrated culture media corresponded to the H520C9sFv-rhIL-2. The fusion protein was illustrated in Figure 4 to bind specifically for p185-expressing SKOV3 and B16/*neu* cells in a dose-dependent manner, confirming that its antigen reactivity was

maintained. The presence of the IL-2 moiety in the H520C9sFv-rhIL-2 was assessed in an IL-2 binding ELISA, demonstrating a comparable dose-response effect with the standard rhIL-2 over the range of dilutions from 0.2 to 20 (Figure 5). The estimated concentration of the concentrated H520C9sFv-rhIL-2 was 30 $\mu g\ ml^{-1}$. The ability of the H520C9sFv-rhIL-2 to support the proliferative activity of the IL-2-dependent CTLL-2 cells was confirmed. As shown in Figure 6, a direct comparison with rhIL-2 suggested that the H520C9sFv-rhIL-2 on a molar basis was 50% less active than rhIL-2. The somewhat reduced biological activity of the fusion protein was likely due to steric hinderance or inaccurate determination of the concentration of the fusion protein.

5.2 Preliminary Vascular Permeability Studies of the H520C9sFv-rhIL-2

A thorough understanding of its in vivo pharmacological properties of H520C9sFv-rhIL-2 may lead to ways of increasing its therapeutic potential. In several preliminary experiments, the effect of

H520C9sFv-rhIL-2 administration on tumor uptake of the ^{125}I-labeled BSA was assessed in p185-positive tumor-bearing C57/BL mice. All mice were sacrificed 2.5 h post injection of the ^{125}I- labeled BSA as it was expected to be metabolized in the mice rapidly. Previous studies by Ettinghausen et al. (1988) have used similar methodology to quantify the vascular leak induced by IL-2 and LAK cells. Their results found that the systemic transfer of LAK cells and IL-2 produced a significantly greater extravasation of the ^{125}I- labeled BSA in the lungs, liver and kidneys than saline control administration. Although not statistically different, our data suggested that pretreatment with H520C9sFv-rhIL-2 may increase the tumor: nontumor uptake ratios of the tracer for all organs when compared with the saline treated group, implying that the H520C9sFv-rhIL-2 could increase preferentially the vascular permeability of B16/*neu* xenografts in C57/BL mice 2.5h after injection of the fusion protein (Fig. 7 I). In comparison, no preferential increase in tumor: nontumor ratio was observed if tracer uptake was measured 5 h after injection of the labeled albumin (Fig. 7 II). As the blood samples collected at 2.5 h

or 5 h postinjection of the tracer showed about 80% of the radioactivity associated with small radiolabeled fragments (Appendix 4), the rapid breakdown of such xenogenic protein in the mice prevents its use as an effective marker of vascular permeability. Since mouse albumin was successfully radioiodinated in the later stage of the project, ^{125}I- labeled mouse albumin was used for the later biodistribution analysis experiments. As shown in appendices 4 and 6, the percentage of decomposition in blood samples dramatically decreased from 80% to nearly 10% when ^{125}I- labeled mouse albumin instead of ^{125}I- labeled BSA was adopted as a blood vessel permeability tracer.

5.3 Vascular Permeability Studies of H520C9sFv-rhIL-2 in C57/BL Mice Bearing Subcutaneous Tumor

5.3.1 Time-dependence Study

To explore the optimal injection route and the time necessary to express the vascular permeability effect fully, 9 μg of

H520C9sFv-rhIL-2 was administered intravenously or intratumorly to each of a group of C57/BL mice bearing B16/*neu* tumors at various times before sacrificing the animals. At all sacrifice time points investigated, pretreatment with H520C9sFv-rhIL-2 resulted in high accumulation of ^{125}I-labeled mouse albumin in the p185-positive tumor as shown by the considerably higher tumor:nontumor ratios in some organs than those of the saline injection group, the effect being greater after i.t. injection. Compared to the saline group, statistically higher tumor:bone and tumor:muscle ratios were obtained for the H520C9sFv-rhIL-2 (i.t.) group at all time points and for the H520C9sFv-rhIL-2 (i.v.) group at the 24-h time point only. The effect of the fusion protein administration on increased tumor uptake of the permeability tracer was clearly time-dependent. Administration of the H520C9sFv-rhIL-2 resulted in the highest tumor to nontumor uptake ratios of the tracer at 24 h post injection of the tracer. At this time point, as seen in Figure 9, the mean tumor: bone ratio for control was 1.33:1 (range 1.15:1 – 1.55:1), whereas that for intravenously administered fusion protein group increased significantly to 5.97:1

(range, 4.51:1 – 7.85:1), representing approximately a 4.5-fold increase in tumor uptake relative to bone. Hu and his colleagues (1996) found that pretreatment with chLym-1/IL-2 induced a 2.5-fold increase in radiolabeled mouse albumin uptake in tumor at 24-h time point. LeBerthon (1991) and Hornick (1999) have also studied the potency of antibody-IL-2 conjugates as specific tumor vasoactive agents. The greatest increase in tumor uptake of radiolabeled antibody was seen at the 72-h time point when either Lym-1/IL-2 or chTNT-3/IL-2 was given 2.5 h before the tracer. By contrast, in our study, the increases in tumor:nontumor ratios of radiolabeled mouse albumin for the H520C9sFv-rhIL-2 pretreatment, either i.t. or i.v., for all organs at 72 h were marginal. The difference was most likely due to the variations in the tumor vasculature, antigen distribution, fusion protein dissociation rate and tracer adopted (Jackson 1999). For this reason, a 24-h interval between radiolabeled albumin injection and the sacrifice of the animals for biodistribution analysis was used for later experiments.

The serum half-life of free rhIL-2 in mice was found to be about 1.6 min while the corresponding value for H520C9sFv-rhIL-2 after i.v. administration reported by Newberry et al. (2000) was 1 h indicating that fusion of rhIL-2 to H520C9sFv significantly decreases the clearance rate (Donohue 1983, Nakagawa 1997, and Sands 1989). Despite the prolonged half-life of the H520C9sFv-rhIL-2 in serum, it was cleared rapidly by blood via kidney excretion. The percentage uptakes of the injected tracer per gram of kidney tissue after pretreatment with H520C9sFv-rhIL-2 either i.v. or i.t. at 12 h were fairly high (range, 63% - 82%). Jackson and his colleagues (1999) pointed out that the molecular weight and dissociation rate of a fusion protein were important factors in influencing its systemic clearance rate.

5.3.2 Route-dependence Study

In fact, it is still controversial regarding the optimal route of administration. Several researchers reported that regional

administration of fusion protein had proven efficacious for cancer therapy with acceptable toxicities (Goldenberg 1993, Kosmas 1993, Krauss 2003 and Maas 1993). Nevertheless, Goldenberg (1993) pointed out that the intravenous route has been the most popular means adopted to treat disseminated metastases. To determine whether the route of application influenced the effect of fusion proteins, B16/*neu* tumor-bearing C57/BL mice were treated by either i.t. or i.v. injection. Results of this work were in agreement with the observation of Christ et al. (2001), demonstrating that the effect of regional application of fusion protein regularly exceeded that of the systemic application. Twenty-four-h biodistribution analysis (Fig. 9) showed that the H520C9sFv-rhIL-2 (i.t.) group produced a nearly 3–fold increase in tumor: blood ratio when compared to the H520C9sFv-rhIL-2 (i.v.) group. The superior efficacy of i.t. compared with i.v. application can be explained by the profoundly efficient targeting of tumor-specific antigens resulting in prolonged retention of the fusion protein by the tumor cells (Christ 2001). Though intratumor administration of the H520C9sFv-rhIL-2 markedly increased tumor

uptake of the ^{125}I-labeled mouse albumin tracer, it remains unclear whether the tumor vasculature could be damaged by the direct needle injection. An alternative explanation for the inferior efficacy of i.v. application was the insufficient amount of the H520C9sFv-rhIL-2 reaching the tumor site.

5.3.3 Dose-dependence Study

To investigate this dependence, the relationship between the dose of H520C9sFv-rhIL-2 and tumor uptake of tracer was then examined. As shown in Figure 11, the tumor: blood and tumor: kidney ratios after pretreatment with 9 μg of H520C9sFv-rhIL-2 were 1.24:1 and 1.31:1 , while those for 18 μg of H520C9sFv-rhIL-2 were significantly increased (2.74:1 and 3.41:1, respectively; $p<0.05$). Pretreatment with 18 μg of H520C9sFv-rhIL-2 intravenously resulted in statistically higher tumor: nontumor ratios in bone, blood, kidney, lung, muscle and spleen, ranging from 2.1 to 12.2-fold increase, when compared to the saline control group. However, the dose level of 18 μg of H520C9sFv-rhIL-2 was not well tolerated as more than 30% of mice

passed away before the biodistribution analysis at 24 h. Christ et al. (2001) and Hornick et al. (1999) revealed that the use of high-dose fusion proteins could be limited because of the induction of toxicities to normal tissues. Previous studies by LeBerthon (1991) and Hornick (1999) demonstrated that pretreatment of antibody-IL-2 fusion proteins could specifically enhance the tumor vascular permeability in a dose-dependent manner. The tumor uptake of radiolabeled antibody was maximal at a dosage of 30 μg of Lym-1/IL-2 and 15 μg of chTNT-3/IL-2, respectively. Factors like the use of different tumor models and natures of fusion proteins may account for the difference in the dosage dependency (Dela Cruz 2000 and Xu 2000). The M.W.s of Lym-1/IL-2 and chTNT-3/IL-2 are about 166 kDa, almost 4 times as large as the 45kDa M.W. of the H520C9sFv-rhIL-2 used in this project.

5.4 Vascular Permeability Studies of H520C9sFv-rhIL-2 in Nude Mice Bearing Subcutaneous Tumor

In another series of experiment, the ability of H520C9sFv-rhIL-2 to

increase the tumor blood vessel permeability was evaluated in nude mice bearing SKOV3 s.c. tumors. Specific increase in tumor vasopermeability after H520C9sFv-rhIL-2 pretreatment was achieved in the nude mice model, although the effect was less remarkable than that seen in C57/BL mice (Figs. 9 and 12). Statistical analysis illustrated that in the nude mice the H520C9sFv-rhIL-2 (i.t.) group led to statistically higher tumor:nontumor uptake ratios of ^{125}I-labeled mouse albumin in bone, kidney and spleen when compared to the saline group and in bone when compared to the H520C9sFv-hIL-2 (i.v.) group.

There are many interacting and competing variables that affect the magnitude of permeability enhancement in the two tumor models. Several lines of evidence supported the view that the effective microvascular permeability of malignant tissues to macromolecules was associated with the rate of tumor growth and the rate of tumor angiogenesis (Graff 2001 and Eberhard 2000). The growth behaviour of different human malignancies growing subcutaneously in nude

mice has been characterized. The highest growth rate was observed in melanoma while that in the carcinoma of ovaries was intermediate (Fiebig and Burger 2002). Angiogenesis was essential for tumor growth and metastasis with characteristic and significant differences between tumor types (Polverini 2001). Vascular endothelial growth factor (VEGF) was the most potent angiogenic and microvascualr permeability factor identified so far (Senger 2001). Glioblastomas, renal cell carcinomas, and colon carcinomas were identified as the most angiogenic types of tumors (Eberhard 2000). Tumors with a high intensity of angiogenesis may benefit most from the immunoconjugate therapies because increased tumor blood flow could enhance the accumulation of immunoconjugates in the tumor, resulting in efficient targeting of tumor-specific antigens. Although the use of the B16 murine melanoma and SKOV3 human ovarian cancer cell lines as tumor models could be traced to 1970 and 1990 respectively, the degree of angiogenesis in SKOV3 and B16/*neu* tumors has not been fully elucidated. (Alvarez 2001)

One possible reason for the difference in vaso-permeability change induced by the fusion protein is the different composition of effector cells in the two animal models. The nude mice employed in this study were deficient in T cells while the C57/BL mice were immunocompetent. Greater effect observed in the C57/BL mice than in nude mice suggested that the IL-2 mediated VLS might be T-cell-dependent. The effectiveness of fusion protein in causing vascular leak was also dependent on the tumor vasculature and vessel permeability (Jackson 1999 and Jain 2001). Epstein (1995) emphasized that different tumor models varied greatly in the degree of vascularization, despite their common site of implantation. Other tumor model-dependent factors, like the circulating p185 level, are critical in determining the effect of fusion protein (Zalutsky 1999).

As the vascular leakage mechanisms have not yet been fully elucidated, it remains unclear how to prospectively identify the best-suited animal models. Numerous studies have explored the anti-tumor effect of fusion proteins using different murine models.

Penichet and colleagues (1998) demonstrated that IgG3-IL-2 fusion protein had antitumor activity for the treatment of B cell lymphoma in both syngeneic and nude mice models. In the study of Sabzevari and his colleagues (1994), the efficacy of ch14.18-IL-2 in suppressing the hepatic metastases of human neuroblastoma in human LAK-reconstituted SCID mice was reported. Though immuno-modulation by infusion of missing cell types to mice may provide an opportunity to better understand the vascular leak mechanisms, Penichet (1997) speculated that such xenografted immune system might not be able to differentiate cancerous and normal cells in the same way as the natural immune system. Based on present findings, the immunocompetent mouse model bearing p185-overexpressing tumor demonstrated more readily the vasopermeability effect of H520C9sFv-rhIL-2.

5.5 Vascular Permeability Studies of H520C9sFv-mrhIL-2 in C57/BL Mice Bearing Subcutaneous Tumor

To evaluate the importance of the bioactivity of IL-2 in inducing

vascular permeability change in tumor, C57/BL mice bearing B16/*neu* s.c. tumors were treated with H520C9sFv-mrhIL-2 as a testing fusion protein. For the H520C9sFv-mrhIL-2, its biological function of IL-2 was lost as a disulphide bond in the mrhIL-2 was eliminated. Binding assay with p185 positive cells indicated that there was no alternation in the H520C9sFv portion of H520C9sFv-mrhIL-2 as its antigen-binding property was preserved. Figure 5 detected the presence of the mutant IL-2 moiety of H520C9sFv-mrhIL-2 by ELISA using rabbit anti-human IL-2 antibodies. In contrast to H520C9sFv-rhIL-2, H520C9sFv-mrhIL-2 showed neither the ability to support the proliferation of CTLL2 cells nor the ability to increase specifically tumor vasopermeability in biodistribution study using C57/BL mice bearing p185 positive subcutaneous tumors. As depicted in figure 13, no statistical difference was observed for all organs between the saline control and H520C9sFv-mrhIL-2 (i.v.) groups, regardless of the dosage. The failure of the H520C9sFv-mrhIL-2 in modulating the tumor vasopermeability leads one to conclude that the increased tumor vasopermeability observed in the H520C9sFv-rhIL-2

experiments was related to the bioactivity of IL-2 delivered to the tumor by means of the antibody component of the fusion protein.

5.6 Limitations and Recommendations of the Study

There were several shortcomings in this study. First of all, antigenic heterogeneity of tumor has been found to be an important limitation for antibody-directed toxin therapy. It is important to assess if antibody/IL-2 fusion protein could induce cytotoxic effect not only on antigen positive target cells but also on antigen negative bystanders. Further studies involving flow cytometry will be necessary to evaluate the extent of cell surface and cytoplasmic heterogeneity of HER-2/*neu* p185 expression by SKOV3 and B16/*neu* tumor cells. Immunohistochemical staining for HER-2/*neu* p185 protein of tumor sections removed from both C57/BL mice bearing B16/*neu* s.c. tumors and nude mice bearing SKOV3 s.c. tumors should be performed if the tissue blocks are available

Besides, although our study demonstrated that pretreatment with H520C9sFv-hIL-2 enhanced accumulation of vaso-permeability tracer in the tumor, it is still possible that the effect was attributable to the increased tumor blood flow but not the enhanced tumor vasopermeability. To rule out this possibility, tumor blood flow studies should be performed to examine the characteristics of each tumor model.

Several recent reviews have surveyed and compared available in vivo and in vitro angiogenesis assays. In vitro assessments including endothelial cell proliferation and migration assays were rapid and consistently reproducible. On the other hand, in vivo chick embryo chorioallantoic membrane assays were difficult to carry out but permit the study of the complex physiological interactions that occur in vivo (Auerbach and Auerbach 2001). Further studies on the correlation of tumor angiogenesis and microvascular permeability should be carried out.

Furthermore, partially purified fusion protein was used in this study which was dialyzed against PBS with 0.9% NaCl. More positive results might be obtained if more purified fusion protein, such as that purified by affinity chromatography is used.

In addition, the dosage effect of H520C9sFv-rhIL-2 has not yet been fully analyzed due to the limitation of time. Further refinement of the dose dependence study will be necessary to determine the optimal dosage in achieving greater efficacy with acceptable toxic effects

Lastly, further researches are warranted in identifying the cells that play important roles in the IL-2 mediated VLS. Vascular permeability studies using tumor bearing SCID-Beige mice engrafted with different human cells may be used to elucidate the IL-2 mediated mechanisms.

CHAPTER 6

Conclusion

In conclusion, results of this thesis demonstrated that H520C9sFv-rhIL-2 was stably expressed in 293 cells transfected with the cDNA of the fusion protein and retained both the antigen binding specificity against p185-overexpressing cells and the IL-2 activity. Pretreatment with 9 μg of H520C9sFv-rhIL-2 i.t. per animal could significantly increase tumor:nontumor uptake ratios for bone, kidney and spleen relative to the saline injection group in both C57/BL and nude mice carrying p185 positive tumors. The effect of the H520C9sFv-rhIL-2 pretreatment on increased tumor uptake of a blood vessel permeability tracer was clearly time- and dose-dependent. The tumor: nontumor ratios of the tracer were maximal after 24 h post injection of the tracer and at a dosage of 18 μg of H520C9sFv-rhIL-2. This was the first report of an IL-2 immuno-protein that could preferentially increase the permeability of blood vessels in

p185-overexpressing tumors because the tumor: nontumor ratios in bone, blood, kidney, lung, muscle and spleen were statistically increased after the intravenous administration of 18 μg of H520C9sFv-rhIL-2. Using C57/BL mice bearing B16/*neu* s.c. tumors as a model, a single intravenous dose of 9 μg H520C9sFv-rhIL-2 could significantly increase tumor:nontumor uptake ratios for bone and muscle relative to saline injection group after 24 h post injection of the tracer. Present findings suggest that the H520C9sFv-rhIL-2 may offer a new approach to enhance the delivery of antibodies and other macromolecular therapeutic agents in p185-overexpressing tumors if more positive results are found.

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Appendices

Appendix 1

Kwok, C.S., Yip, T.C., Cheung, W.K., Leung, K.L., So, F.F., Ma, V.M. and Lau, W.H. "Recent advances of a novel vasoactive human interleukin-2/single chain antibody fusion protein for HER-2 positive tumors" *Proceedings of the 93rd American Association of Cancer Research*, San Francisco, LA, 6-10 April, 2002, p.978 (2002)

This study is a continuing effort to develop and characterize a novel vasoactive immuno-conjugate for treating HER-2 positive tumors refractory to conventional therapies. We had previously reported the construction of a fusion protein consisting of a recombinant human IL-2 and a recombinant humanized single-chain Fv (sFv) antibody which combined the V_H and V_L portions of a murine mAb (520C9) specific for human HER-2 (Li, J. et al., "Preparation and characterization of a recombinant humanized single-chain Fv antibody/human interleukin-2 fusion protein directed against the HER-2/neu (c-erbB2) proto-oncogene product, p185". *Tumor Targeting*, Vol. 4, pp.105-114 (1999). Preliminary experiments to characterize the anti-tumor effect of the fusion protein in a syngeneic mouse tumor model and in

immuno-suppressed mice carrying subcutaneous human HER-2 positive tumors were also reported (Kwok, C.S. et al., "A novel vasoactive human interleukin-2/single chain antibody fusion protein for HER-2 positive tumors". *Proceedings of the 92nd American Association of Cancer Research*, New Orleans, LA, 24-28 March, 2001, pp. 290-291 (2001). We recently extended our investigation by developing a metastatic tumor model in SCID mice by injecting intravenously into them with human SKOV3 cells that over-express HER-2. Tumor nodules in the lungs and in more severe cases the liver developed at various times after the injection of the tumor cells. The latent period of tumor development depended on the number of cells injected. The growth inhibitory effect and the vasoactive property of the fusion protein on this metastatic tumor model will be reported. Specific vascular changes at the tumor site, characterized by leakage of intravenously injected ^{125}I-labeled albumin into interstitial space of the tumor and immuno-histological staining of endothelial cells by means of a specific anti-VE-cadherin antibody, will be presented. Such studies will help gain further insight into clinical applications of the fusion protein.

Appendix 2

Yip, T.C., Kwok, C.S., So, F.F., Lau, W.H., Leung, K.L., Cheung, W.K., Ma, V.M. and Ngan, K.C. "An aminoglycoside antibiotic, Geneticin, can inhibit the growth of a HER-2 positive ovarian cancer in SCID mice model". *Proceedings of the 93rd American Association of Cancer Research*, San Francisco, LA, 6-10 April, 2002, p.923 (2002)

Tetracycline is a safe and inexpensive (less than US$1 per capsule) antibiotics that has been used for decades. Recent finding in prevent the spread of distant metatasis of breast and prostate cancers in mice probably through the inhibition of matrix metalloproteinase activity (Duivenvoorden, et al., Invasion Metatasis, Vol. 17(6), pp.312-322, 1997) has rekindled the interesst in this antibiotic for cancer treatment. Tetracycline can inhibit bacterial protein synthesis by binding to 30S ribosomal RNA. Using an aminoglycoside antibiotics, Geneticin (G418), which has the same mechanism of action, we reported in this paper the in vivo growth inhibition effect of this antibiotic in a HER-2 positive ovarian cancer, SKOV-3 in SCID mice model. In the first series of experiments, saline or Geneticin at a concentration of 30, 125 and 500mg/ml were concurrently injected intramuscularly onto the trunk of the SCID mice together with $2x10^6$ SKOV3 tumor cells. The tumor

was completely inhibited from growth by 125 and 500mg/ml Geneticin at day 45 after injection. There was a 83% reduction of tumor mass (or a growth delay of ~32 days) at 30mg/ml when compared with saline control. In comparison, injection of Tetracycline only at a concentration of 500mg/ml but not at 30 or 125mg/ml resulted in significant tumor mass reduction. To further investigate the cytotoxic effect of Geneticin, SKOV-3 tumor was grown to an average size of $76mm^3$ (SD 42mm) before Geneticin was injected. Reduction of tumor mass of 30%, 42% and 67% in a concentration dependent manner were respectively found at 30, 125 and 500mg/ml when compared with the saline control. Further findings in the extent of apoptosis and the inhibition on distant spread of tumor in lung will be presented. This tumor growth inhibition effect by a commonly used antibiotic opens up the possible usage of a much cheaper adjunct for preventing distant spread of breast and prostate cancer in conjunction with conventional primary treatment modality.

Appendix 3

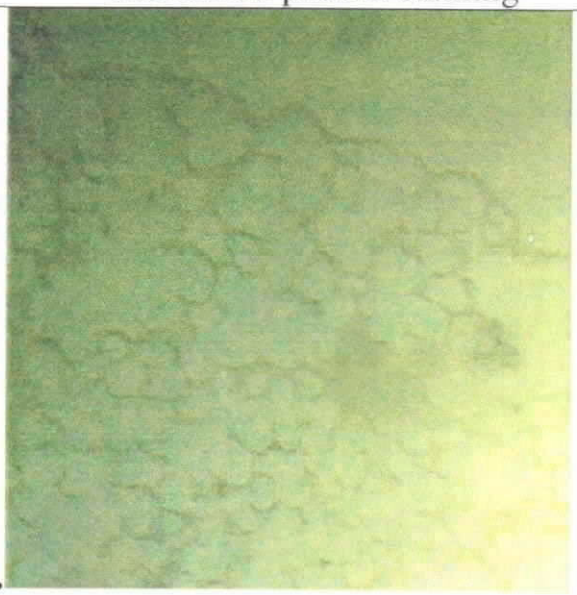
Kwok, C.S., Yip, T.C., Cheung, W.K., Leung, K.L., So, F.F., Ma, V.M. and Lau, W.H. "Preclinical study of a novel vasoactive human interleukin-2/single chain antibody fusion protein for HER-2 positive tumors" *Proceedings of the 94th American Association of Cancer Research*, Washington, DC, 11-14 July, p.1289 (2003)

This study is a continuing pre-clinical effort to characterize a novel vasoactive fusion protein (FP) for treating HER-2 positive tumors. The FP consists of a recombinant human IL-2 and a recombinant humanized single-chain Fv (sFv) antibody which combines the V_H and V_L portions of a murine MoAb (520C9) specific for human HER-2 (Li J. et al, Preparation and characterization of a recombinant humanized single-chain Fv antibody/human interleukin-2 fusion protein directed against the HER-2/neu (c-erb B2) proto-oncogene product, p185. Tumor Targeting 4: 105-114, 1999). We had previously reported preliminary experiments to characterize the anti-tumor effect of the fusion protein in an immuno-competent syngeneic mouse tumor model and in immuno-suppressed mice carrying subcutaneous human HER-2 positive tumors (Kwok C.S. et al, AACR Proceedings 42: 290-291, Abstract 1564, 2001). The investigation was later extended to a metastatic tumor model in SCID mice by injecting intravenously into them human SKOV3 cells that

over-express HER-2. Inhibitory effect of the FP on the growth of tumor nodules in the lungs was reported (Kwok C.S. et al, AACR Proceedings 43: 978, Abstract 4842, 2002). Three consecutive daily intravenous injections of 6 μg FP and 2 x 10^5 human peripheral blood mononulear cells (hPBMCs) into each of four SCID mice that had received 8 x 10^6 SKOV3 cells 3 days ago completely inhibited the development of lung metastasis four weeks later in all the animals. Treatment with 6 μg FP or 2 x 10^5 hPBMCs alone could only partially inhibit the growth of lung metastases. Vascular permeability changes in tumors and normal tissues that may be induced by FP, characterized by leakage of intravenously injected ^{125}I-labeled albumin into interstitial space of the tumors and tissues respectively at various times post intravenous injection of the FP, were also investigated in the syngeneic mouse tumor model and in nude mice carrying subcutaneous SKOV3 tumors. Specific increase in vascular permeability in the tumor compared with those in normal tissues was observed in the immuno-competent mice but not in the nude mice. Reasons for the difference in vascular permeability change caused by the FP in the two animal models are being explored. Such studies will help gain further insight into clinical applications of the FP.

Appendix 4

HER2/*neu* protein staining for evaluation of SKOV3 metastatic tumor growth in SCID mice after treatment with the H520C9sFv-rhIL-2 and human PBM cells

Treatment agents	HER2/*neu* protein staining
6μg H520C9sFv-rhIL-2 + 0.2 x 10^6 PBM cells	**A.** 
6μg H520C9sFv-rhIL-2 only	**B.** 

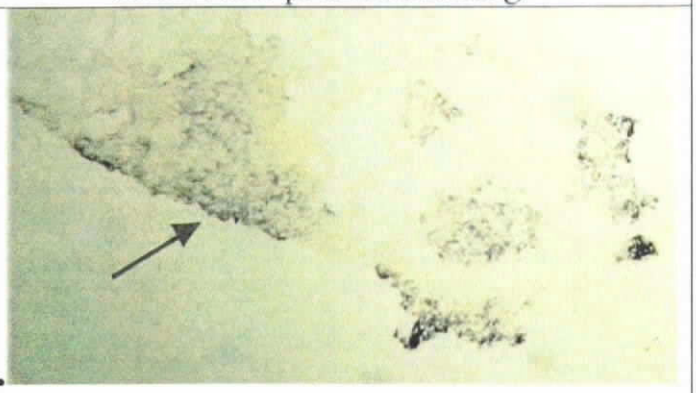
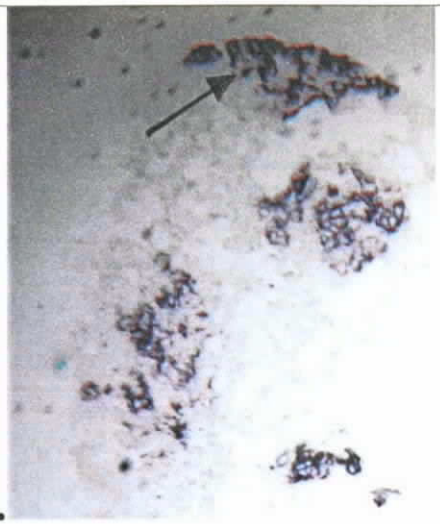
Treatment agents	HER2/*neu* protein staining
0.2 x 10^6 PBM only	 C.
saline only	 D.

Fig 15. HER2/*neu* protein staining of lung sections in evaluation of metastactic tumor growth in SCID mice after treatment with H520C9sFv-rhIL-2 and human PBM cells. SCID mice injected intravenously with 8 x 10^6 SKOV3 tumor cells each were allowed to grow for 4 days before 3 daily treatments with 6μg H520C9sFv-rhIL-2 and 0.2 x 10^6 PBM cells (panel A), 6μg H520C9sFv-rhIL-2 only (panel B), 0.2 x 10^6 PBM only (panel C) or saline only (panel D) were given. The mice were sacrificed on day 28. Arrows indicated positive staining of metastatic tumor cells in HER2/*neu* protein staining. Representative areas were photographed at a magnification of x 100.

Appendix 5

Independent Samples T-test results for preliminary vascular permeability studies of the H520C9sFv-rhIL-2

Experiment 1

(I) Two and a half hours after injection of ^{125}I-labeled BSA

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Diff	Std. Error Diff	Lower	Upper
BONE	Equal variances assumed	2.555	.185	-.278	4	.795	-.1333	.4790	-1.4634	1.1967
	Equal variances not assumed			-.278	2.847	.800	-.1333	.4790	-1.7051	1.4385
BLOOD	Equal variances assumed	2.876	.165	-1.584	4	.188	-1.420	.8965	-3.9090	1.0690
	Equal variances not assumed			-1.584	2.226	.242	-1.420	.8965	-4.9258	2.0858
KIDNEY	Equal variances assumed	.026	.880	-2.180	4	.095	-2.007	.9205	-4.5623	.5490
	Equal variances not assumed			-2.180	3.877	.097	-2.007	.9205	-4.5945	.5812
LIVER	Equal variances assumed	.182	.692	-.144	4	.892	-.1667	1.16	-3.3807	3.0474
	Equal variances not assumed			-.144	3.875	.893	-.1667	1.16	-3.4219	3.0886
LUNG	Equal variances assumed	1.257	.325	-.569	4	.600	-.2400	.4215	-1.4104	.9304
	Equal variances not assumed			-.569	3.440	.604	-.2400	.4215	-1.4894	1.0094
MUSCLE	Equal variances assumed	.110	.757	-1.232	4	.285	-1.365	1.11	-4.4410	1.7104
	Equal variances not assumed			-1.232	3.911	.287	-1.365	1.11	-4.4690	1.7383
SPLEEN	Equal variances assumed	1.952	.235	-1.310	4	.260	-.9300	.7097	-2.9005	1.0405
	Equal variances not assumed			-1.310	2.948	.283	-.9300	.7097	-3.2114	1.3514

(II) Five hours after injection of ^{125}I-labeled BSA

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Diff	Std. Error Diff	Lower	Upper
BONE	Equal variances assumed	.005	.948	-.382	3	.728	-.3400	.8905	-3.1739	2.4939
	Equal variances not assumed			-.380	2.229	.737	-.3400	.8958	-3.8389	3.1589
BLOOD	Equal variances assumed	.266	.641	-1.758	3	.177	-1.49	.8474	-4.1867	1.2067
	Equal variances not assumed			-1.949	2.951	.148	-1.49	.7645	-3.9461	.9661
KIDNEY	Equal variances assumed	5.7	.096	-1.498	3	.231	-.3600	.2403	-1.1246	.4046
	Equal variances not assumed			-1.275	1.368	.377	-.3600	.2823	-2.3080	1.5880
LIVER	Equal variances assumed	1.5	.305	.379	3	.730	.2050	.5407	-1.5157	1.9257
	Equal variances not assumed			.421	2.955	.703	.2050	.4873	-1.3593	1.7693
LUNG	Equal variances assumed	.308	.617	.013	3	.990	.0067	.5082	-1.6107	1.6241
	Equal variances not assumed			.012	1.813	.991	.0067	.5441	-2.5817	2.5950
MUSCLE	Equal variances assumed	12	.039	-.049	3	.964	-.033	.6750	-2.1816	2.1150
	Equal variances not assumed			-.040	1.191	.974	-.033	.8367	-7.3696	7.3029
SPLEEN	Equal variances assumed	.816	.433	1.045	3	.373	.7583	.7255	-1.5507	3.0674
	Equal variances not assumed			.943	1.622	.465	.7583	.8045	-3.6050	5.1217

Experiment 2

(I) Two and a half hours after injection of ^{125}I-labeled BSA

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Diff	Std. Error Diff	Lower	Upper
BONE	Equal variances assumed	.041	.852	-.233	3	.831	-.1483	.6377	-2.1779	1.8812
	Equal variances not assumed			-.236	2.388	.832	-.1483	.6280	-2.4706	2.174(
BLOOD	Equal variances assumed	8.952	.058	-.759	3	.503	-5.9583	7.8502	-30.9411	19.0244
	Equal variances not assumed			-.979	2.008	.430	-5.9583	6.0849	-32.0374	20.1208
KIDNEY	Equal variances assumed	8.892	.059	-.764	3	.500	-2.0200	2.6433	-10.4321	6.3921
	Equal variances not assumed			-.986	2.006	.428	-2.0200	2.0485	-10.8084	6.7684
LIVER	Equal variances assumed	2.320	.225	-.259	3	.812	-.2350	.9059	-3.1181	2.6481
	Equal variances not assumed			-.306	2.911	.780	-.2350	.7689	-2.7252	2.2552
LUNG	Equal variances assumed	.553	.511	1.476	3	.236	.2283	.1547	-.2639	.7205
	Equal variances not assumed			1.356	1.716	.326	.2283	.1684	-.6244	1.081(
MUSCLE	Equal variances assumed	.696	.465	-1.231	3	.306	-2.2433	1.8225	-8.0434	3.5568
	Equal variances not assumed			-1.426	2.977	.250	-2.2433	1.5731	-7.2715	2.7849
SPLEEN	Equal variances assumed	8.794	.059	-.718	3	.525	-3.2300	4.4977	-17.5437	11.0837
	Equal variances not assumed			-.926	2.010	.452	-3.2300	3.4867	-18.1641	11.7041

(II) Five hours after injection of ^{125}I-labeled BSA

Independent Samples Test

		t-test for Equality of Means						
							95% Confidence Interval of the Difference	
		t	df	Sig. (2-tailed)	Mean Diff	Std. Error Diff	Lower	Upper
BONE	Equal variances assumed	2.162	2	.163	.7800	.3607	-.7721	2.3321
	Equal variances not assumed	.	.	.	.7800	.	.	.
BLOOD	Equal variances assumed	-.711	2	.551	-3.03	4.2540	-21.330	15.2768
	Equal variances not assumed	.	.	.	-3.03	.	.	.
KIDNEY	Equal variances assumed	-.646	2	.584	-.8600	1.3305	-6.5845	4.8645
	Equal variances not assumed	.	.	.	-.8600	.	.	.
LIVER	Equal variances assumed	2.402	2	.138	.8000	.3331	-.6331	2.2331
	Equal variances not assumed	.	.	.	.8000	.	.	.
LUNG	Equal variances assumed	1.403	2	.296	.4367	.3112	-.9023	1.7756
	Equal variances not assumed	.	.	.	.4367	.	.	.
MUSCLE	Equal variances assumed	3.887	2	.060	1.5100	.3885	-.1616	3.1816
	Equal variances not assumed	.	.	.	1.5100	.	.	.
SPLEEN	Equal variances assumed	-.206	2	.856	-.4167	2.0243	-9.1264	8.2930
	Equal variances not assumed	.	.	.	-.4167	.	.	.

Appendix 6

Chromatography results of blood samples collected in C57/BL mice bearing subcutaneous tumor for preliminary vascular permeability studies of the H520C9sFv-rhIL-2

Experiment 1

(I) Two and a half hours after injection of ^{125}I-labeled BSA

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front)*100 (=Percentage decompose)
Saline	1692	6723	79.89
9 μg of H520C9sFv-rhIL-2 (i.v.)	1552	6432	80.56

(II) Five hours after injection of ^{125}I-labeled BSA

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front) *100 (=Percentage decompose)
Saline	1946	7964	80.36
9 μg of H520C9sFv-rhIL-2 (i.v.)	1864	8235	81.54

Experiment 2

(I) Two and a half hours after injection of ^{125}I-labeled BSA

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front) *100 (=Percentage decompose)
Saline	1876	7654	80.31
9 μg of H520C9sFv-rhIL-2 (i.v.)	1534	6684	81.33

(II) Five hours after injection of ^{125}I-labeled BSA

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front) *100 (=Percentage decompose)
Saline	1975	8765	81.61
9 μg of H520C9sFv-rhIL-2 (i.v.)	1845	8457	82.09

Appendix 7

One-way ANOVA test results for vascular permeability studies of H520C9sFv-rhIL-2 in C57/BL mice bearing subcutaneous tumor

(A) Time-dependence study

(I) Twelve hours biodistribution study

Experiment 1

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
BONE	Between Groups	12.716	2	6.358	9.071	.022
	Within Groups	3.504	5	.701		
	Total	16.220	7			
BLOOD	Between Groups	3.875	2	1.937	1.877	.247
	Within Groups	5.161	5	1.032		
	Total	9.035	7			
KIDNEY	Between Groups	1.057	2	.528	9.047	.022
	Within Groups	.292	5	5.840E-02		
	Total	1.349	7			
LIVER	Between Groups	11.043	2	5.521	5.508	.054
	Within Groups	5.012	5	1.002		
	Total	16.055	7			
LUNG	Between Groups	7.290	2	3.645	2.427	.183
	Within Groups	7.510	5	1.502		
	Total	14.799	7			
MUSCLE	Between Groups	31.341	2	15.670	8.670	.024
	Within Groups	9.037	5	1.807		
	Total	40.377	7			
SPLEEN	Between Groups	18.792	2	9.396	6.486	.041
	Within Groups	7.243	5	1.449		
	Total	26.035	7			

Post-hoc tests

Multiple Comparisons

Dependent Variable		(I) 24 hr- Route	(J) 24 hr- Route	Mean Diff(I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
BONE	Tukey HSD	Control	FP i.v.	-1.7803	.6836	.103	-4.0046	.4439
			FP i.t.	-3.1940*	.7642	.020	-5.6808	-.7072
		FP i.v.	Control	1.7803	.6836	.103	-.4439	4.0046
			FP i.t.	-1.4137	.7642	.247	-3.9004	1.0731
		FP i.t.	Control	3.1940*	.7642	.020	.7072	5.6808
			FP i.v.	1.4137	.7642	.247	-1.0731	3.9004
BLOOD	Tukey HSD	Control	FP i.v.	-.4890	.8295	.832	-3.1882	2.2102
			FP i.t.	-1.7755	.9274	.229	-4.7933	1.2423
		FP i.v.	Control	.4890	.8295	.832	-2.2102	3.1882
			FP i.t.	-1.2865	.9274	.414	-4.3043	1.7313
		FP i.t.	Control	1.7755	.9274	.229	-1.2423	4.7933
			FP i.v.	1.2865	.9274	.414	-1.7313	4.3043
KIDNEY	Tukey HSD	Control	FP i.v.	-.5293	.1973	.094	-1.1714	.1127
			FP i.t.	-.9160*	.2206	.020	-1.6338	-.1982
		FP i.v.	Control	.5293	.1973	.094	-.1127	1.1714
			FP i.t.	-.3867	.2206	.276	-1.1045	.3312
		FP i.t.	Control	.9160*	.2206	.020	.1982	1.6338
			FP i.v.	.3867	.2206	.276	-.3312	1.1045
LIVER	Tukey HSD	Control	FP i.v.	-2.4317	.8175	.068	-5.0917	.2284
			FP i.t.	-2.4195	.9140	.098	-5.3935	.5545
		FP i.v.	Control	2.4317	.8175	.068	-.2284	5.0917
			FP i.t.	1.2E-02	.9140	1.000	-2.9618	2.9862
		FP i.t.	Control	2.4195	.9140	.098	-.5545	5.3935
			FP i.v.	-1.E-02	.9140	1.000	-2.9862	2.9618
LUNG	Tukey HSD	Control	FP i.v.	-1.5003	1.0006	.367	-4.7563	1.7557
			FP i.t.	-2.3653	1.1188	.181	-6.0056	1.2750
		FP i.v.	Control	1.5003	1.0006	.367	-1.7557	4.7563
			FP i.t.	-.8650	1.1188	.734	-4.5053	2.7753
		FP i.t.	Control	2.3653	1.1188	.181	-1.2750	6.0056
			FP i.v.	.8650	1.1188	.734	-2.7753	4.5053
MUSCLE	Tukey HSD	Control	FP i.v.	-3.1750	1.0977	.074	-6.7467	.3967
			FP i.t.	-4.8758*	1.2272	.024	-8.8692	-.8825
		FP i.v.	Control	3.1750	1.0977	.074	-.3967	6.7467
			FP i.t.	-1.7008	1.2272	.415	-5.6942	2.2925
		FP i.t.	Control	4.8758*	1.2272	.024	.8825	8.8692
			FP i.v.	1.7008	1.2272	.415	-2.2925	5.6942
SPLEEN	Tukey HSD	Control	FP i.v.	-1.0573	.9827	.567	-4.2551	2.1404
			FP i.t.	-3.9065*	1.0987	.036	-7.4817	-.3313
		FP i.v.	Control	1.0573	.9827	.567	-2.1404	4.2551
			FP i.t.	-2.8492	1.0987	.104	-6.4243	.7260
		FP i.t.	Control	3.9065*	1.0987	.036	.3313	7.4817
			FP i.v.	2.8492	1.0987	.104	-.7260	6.4243

*. The mean difference is significant at the .05 level.

Experiment 2

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
BONE	Between Groups	4.463	2	2.231	6.755	.029
	Within Groups	1.982	6	.330		
	Total	6.445	8			
BLOOD	Between Groups	8.721	2	4.360	3.369	.104
	Within Groups	7.765	6	1.294		
	Total	16.486	8			
KIDNEY	Between Groups	2.484	2	1.242	6.258	.034
	Within Groups	1.191	6	.198		
	Total	3.675	8			
LIVER	Between Groups	4.912	2	2.456	4.363	.068
	Within Groups	3.378	6	.563		
	Total	8.290	8			
LUNG	Between Groups	8.493	2	4.247	4.584	.062
	Within Groups	5.558	6	.926		
	Total	14.051	8			
MUSCLE	Between Groups	22.353	2	11.177	7.246	.025
	Within Groups	9.255	6	1.542		
	Total	31.608	8			
SPLEEN	Between Groups	28.052	2	14.026	7.949	.021
	Within Groups	10.587	6	1.764		
	Total	38.639	8			

Post-hoc tests

Multiple Comparisons

Dependent Variable		(I) 24 hr-Route	(J) 24 hr-Route	Mean Diff	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
BONE	Tukey HSD	Control	FP i.v.	-1.0093	.4693	.159	-2.4493	.4306
			FP i.t.	-1.7160*	.4693	.025	-3.1559	-.2761
		FP i.v.	Control	1.0093	.4693	.159	-.4306	2.4493
			FP i.t.	-.7067	.4693	.353	-2.1466	.7333
		FP i.t.	Control	1.7160*	.4693	.025	.2761	3.1559
			FP i.v.	.7067	.4693	.353	-.7333	2.1466
BLOOD	Tukey HSD	Control	FP i.v.	-.7367	.9289	.721	-3.5867	2.1134
			FP i.t.	-2.3567	.9289	.097	-5.2067	.4934
		FP i.v.	Control	.7367	.9289	.721	-2.1134	3.5867
			FP i.t.	-1.6200	.9289	.265	-4.4700	1.2300
		FP i.t.	Control	2.3567	.9289	.097	-.4934	5.2067
			FP i.v.	1.6200	.9289	.265	-1.2300	4.4700
KIDNEY	Tukey HSD	Control	FP i.v.	-.3100	.3638	.687	-1.4261	.8061
			FP i.t.	-1.2367*	.3638	.033	-2.3528	-.1206
		FP i.v.	Control	.3100	.3638	.687	-.8061	1.4261
			FP i.t.	-.9267	.3638	.096	-2.0428	.1894
		FP i.t.	Control	1.2367*	.3638	.033	.1206	2.3528
			FP i.v.	.9267	.3638	.096	-.1894	2.0428
LIVER	Tukey HSD	Control	FP i.v.	-1.6733	.6126	.076	-3.5530	.2064
			FP i.t.	-1.4333	.6126	.125	-3.3130	.4464
		FP i.v.	Control	1.6733	.6126	.076	-.2064	3.5530
			FP i.t.	.2400	.6126	.920	-1.6397	2.1197
		FP i.t.	Control	1.4333	.6126	.125	-.4464	3.3130
			FP i.v.	-.2400	.6126	.920	-2.1197	1.6397
LUNG	Tukey HSD	Control	FP i.v.	-1.5807	.7858	.190	-3.9919	.8305
			FP i.t.	-2.3307	.7858	.057	-4.7419	8.1E-02
		FP i.v.	Control	1.5807	.7858	.190	-.8305	3.9919
			FP i.t.	-.7500	.7858	.629	-3.1612	1.6612
		FP i.t.	Control	2.3307	.7858	.057	-.0805	4.7419
			FP i.v.	.7500	.7858	.629	-1.6612	3.1612
MUSCLE	Tukey HSD	Control	FP i.v.	-3.0933	1.0141	.051	-6.2047	1.8E-02
			FP i.t.	-3.5467*	1.0141	.030	-6.6581	-.4353
		FP i.v.	Control	3.0933	1.0141	.051	-.0181	6.2047
			FP i.t.	-.4533	1.0141	.897	-3.5647	2.6581
		FP i.t.	Control	3.5467*	1.0141	.030	.4353	6.6581
			FP i.v.	.4533	1.0141	.897	-2.6581	3.5647
SPLEEN	Tukey HSD	Control	FP i.v.	-1.0333	1.0846	.630	-4.3612	2.2945
			FP i.t.	-4.1533*	1.0846	.020	-7.4812	-.8255
		FP i.v.	Control	1.0333	1.0846	.630	-2.2945	4.3612
			FP i.t.	-3.1200	1.0846	.063	-6.4478	.2078
		FP i.t.	Control	4.1533*	1.0846	.020	.8255	7.4812
			FP i.v.	3.1200	1.0846	.063	-.2078	6.4478

*. The mean difference is significant at the .05 level.

Experiment 3

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
BONE	Between Groups	3.394	2	1.697	6.453	.041
	Within Groups	1.315	5	.263		
	Total	4.709	7			
BLOOD	Between Groups	2.039	2	1.019	1.369	.336
	Within Groups	3.722	5	.744		
	Total	5.761	7			
KIDNEY	Between Groups	3.114	2	1.557	6.355	.042
	Within Groups	1.225	5	.245		
	Total	4.339	7			
LIVER	Between Groups	2.853	2	1.426	3.464	.114
	Within Groups	2.059	5	.412		
	Total	4.912	7			
LUNG	Between Groups	1.149	2	.574	4.561	.075
	Within Groups	.630	5	.126		
	Total	1.779	7			
MUSCLE	Between Groups	6.344	2	3.172	13.764	.009
	Within Groups	1.152	5	.230		
	Total	7.497	7			
SPLEEN	Between Groups	15.517	2	7.759	10.349	.017
	Within Groups	3.749	5	.750		
	Total	19.266	7			

Post-hoc tests

Multiple Comparisons

Dependent Variable		(I) 24 hr-Route	(J) 24 hr-Route	Mean Diff(I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
BONE	Tukey HSD	Control	FP i.v.	-1.1110	.4187	.097	-2.4735	.2515
			FP i.t.	-1.5697*	.4682	.045	-3.0930	-5.E-02
		FP i.v.	Control	1.1110	.4187	.097	-.2515	2.4735
			FP i.t.	-.4587	.4682	.619	-1.9820	1.0647
		FP i.t.	Control	1.5697*	.4682	.045	4.6E-02	3.0930
			FP i.v.	.4587	.4682	.619	-1.0647	1.9820
BLOOD	Tukey HSD	Control	FP i.v.	-.5367	.7045	.740	-2.8289	1.7556
			FP i.t.	-1.3033	.7876	.308	-3.8661	1.2595
		FP i.v.	Control	.5367	.7045	.740	-1.7556	2.8289
			FP i.t.	-.7667	.7876	.623	-3.3295	1.7961
		FP i.t.	Control	1.3033	.7876	.308	-1.2595	3.8661
			FP i.v.	.7667	.7876	.623	-1.7961	3.3295
KIDNEY	Tukey HSD	Control	FP i.v.	-.7367	.4042	.255	-2.0518	.5784
			FP i.t.	-1.6067*	.4519	.036	-3.0770	-.1364
		FP i.v.	Control	.7367	.4042	.255	-.5784	2.0518
			FP i.t.	-.8700	.4519	.226	-2.3403	.6003
		FP i.t.	Control	1.6067*	.4519	.036	.1364	3.0770
			FP i.v.	.8700	.4519	.226	-.6003	2.3403
LIVER	Tukey HSD	Control	FP i.v.	-1.1233	.5240	.175	-2.8283	.5817
			FP i.t.	-1.3617	.5858	.142	-3.2679	.5446
		FP i.v.	Control	1.1233	.5240	.175	-.5817	2.8283
			FP i.t.	-.2383	.5858	.914	-2.1446	1.6679
		FP i.t.	Control	1.3617	.5858	.142	-.5446	3.2679
			FP i.v.	.2383	.5858	.914	-1.6679	2.1446
LUNG	Tukey HSD	Control	FP i.v.	-.6767	.2898	.140	-1.6195	.2662
			FP i.t.	-.8933	.3240	.086	-1.9475	.1608
		FP i.v.	Control	.6767	.2898	.140	-.2662	1.6195
			FP i.t.	-.2167	.3240	.791	-1.2708	.8375
		FP i.t.	Control	.8933	.3240	.086	-.1608	1.9475
			FP i.v.	.2167	.3240	.791	-.8375	1.2708
MUSCLE	Tukey HSD	Control	FP i.v.	-.9733	.3920	.118	-2.2488	.3021
			FP i.t.	-2.2983*	.4382	.008	-3.7243	-.8723
		FP i.v.	Control	.9733	.3920	.118	-.3021	2.2488
			FP i.t.	-1.3250	.4382	.064	-2.7510	.1010
		FP i.t.	Control	2.2983*	.4382	.008	.8723	3.7243
			FP i.v.	1.3250	.4382	.064	-.1010	2.7510
SPLEEN	Tukey HSD	Control	FP i.v.	-1.7833	.7070	.113	-4.0837	.5171
			FP i.t.	-3.5683*	.7904	.014	-6.1403	-.9964
		FP i.v.	Control	1.7833	.7070	.113	-.5171	4.0837
			FP i.t.	-1.7850	.7904	.153	-4.3569	.7869
		FP i.t.	Control	3.5683*	.7904	.014	.9964	6.1403
			FP i.v.	1.7850	.7904	.153	-.7869	4.3569

*. The mean difference is significant at the .05 level.

(II) Twenty-four hour hours biodistribution study

Experiment 1

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
BONE	Between Groups	52.994	2	26.497	12.503	.007
	Within Groups	12.716	6	2.119		
	Total	65.710	8			
BLOOD	Between Groups	38.036	2	19.018	12.217	.008
	Within Groups	9.340	6	1.557		
	Total	47.376	8			
KIDNEY	Between Groups	16.635	2	8.317	12.855	.007
	Within Groups	3.882	6	.647		
	Total	20.517	8			
LIVER	Between Groups	23.635	2	11.818	3.489	.099
	Within Groups	20.325	6	3.387		
	Total	43.960	8			
LUNG	Between Groups	24.347	2	12.173	9.505	.014
	Within Groups	7.684	6	1.281		
	Total	32.031	8			
MUSCLE	Between Groups	43.644	2	21.822	13.370	.006
	Within Groups	9.793	6	1.632		
	Total	53.438	8			
SPLEEN	Between Groups	37.057	2	18.529	7.131	.026
	Within Groups	15.590	6	2.598		
	Total	52.647	8			

Post-hoc tests

Multiple Comparisons

Dependent Variable		(I) 24 hr-Route	(J) 24 hr-Route	Mean Diff(I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
BONE	Tukey HSD	Control	FP i.v.	-4.6397*	1.1886	.019	-8.2868	-.9926
			FP i.t.	-5.5373*	1.1886	.008	-9.1844	-1.89
		FP i.v.	Control	4.6397*	1.1886	.019	.9926	8.2868
			FP i.t.	-.8977	1.1886	.742	-4.5448	2.7494
		FP i.t.	Control	5.5373*	1.1886	.008	1.8902	9.1844
			FP i.v.	.8977	1.1886	.742	-2.7494	4.5448
BLOOD	Tukey HSD	Control	FP i.v.	-1.5087	1.0187	.363	-4.6344	1.6170
			FP i.t.	-4.9150*	1.0187	.007	-8.0407	-1.79
		FP i.v.	Control	1.5087	1.0187	.363	-1.6170	4.6344
			FP i.t.	-3.4063*	1.0187	.036	-6.5320	-.2806
		FP i.t.	Control	4.9150*	1.0187	.007	1.7893	8.0407
			FP i.v.	3.4063*	1.0187	.036	.2806	6.5320
KIDNEY	Tukey HSD	Control	FP i.v.	-1.4823	.6568	.139	-3.4975	.5328
			FP i.t.	-3.3237*	.6568	.006	-5.3388	-1.31
		FP i.v.	Control	1.4823	.6568	.139	-.5328	3.4975
			FP i.t.	-1.8413	.6568	.070	-3.8565	.1738
		FP i.t.	Control	3.3237*	.6568	.006	1.3085	5.3388
			FP i.v.	1.8413	.6568	.070	-.1738	3.8565
LIVER	Tukey HSD	Control	FP i.v.	-1.2813	1.5028	.687	-5.8922	3.3296
			FP i.t.	-3.8943	1.5028	.091	-8.5052	.7166
		FP i.v.	Control	1.2813	1.5028	.687	-3.3296	5.8922
			FP i.t.	-2.6130	1.5028	.267	-7.2239	1.9979
		FP i.t.	Control	3.8943	1.5028	.091	-.7166	8.5052
			FP i.v.	2.6130	1.5028	.267	-1.9979	7.2239
LUNG	Tukey HSD	Control	FP i.v.	-2.2317	.9240	.114	-5.0668	.6035
			FP i.t.	-4.0207*	.9240	.011	-6.8558	-1.19
		FP i.v.	Control	2.2317	.9240	.114	-.6035	5.0668
			FP i.t.	-1.7890	.9240	.209	-4.6241	1.0461
		FP i.t.	Control	4.0207*	.9240	.011	1.1855	6.8558
			FP i.v.	1.7890	.9240	.209	-1.0461	4.6241
MUSCLE	Tukey HSD	Control	FP i.v.	-3.7377*	1.0431	.027	-6.9383	-.5370
			FP i.t.	-5.2370*	1.0431	.006	-8.4376	-2.04
		FP i.v.	Control	3.7377*	1.0431	.027	.5370	6.9383
			FP i.t.	-1.4993	1.0431	.382	-4.7000	1.7013
		FP i.t.	Control	5.2370*	1.0431	.006	2.0364	8.4376
			FP i.v.	1.4993	1.0431	.382	-1.7013	4.7000
SPLEEN	Tukey HSD	Control	FP i.v.	-3.2287	1.3161	.108	-7.2670	.8096
			FP i.t.	-4.8870*	1.3161	.023	-8.9253	-.8487
		FP i.v.	Control	3.2287	1.3161	.108	-.8096	7.2670
			FP i.t.	-1.6583	1.3161	.465	-5.6966	2.3800
		FP i.t.	Control	4.8870*	1.3161	.023	.8487	8.9253
			FP i.v.	1.6583	1.3161	.465	-2.3800	5.6966

*. The mean difference is significant at the .05 level.

Experiment 2

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
BONE	Between Groups	57.291	2	28.646	45.017	.000
	Within Groups	3.818	6	.636		
	Total	61.109	8			
BLOOD	Between Groups	46.853	2	23.427	15.684	.004
	Within Groups	8.962	6	1.494		
	Total	55.815	8			
KIDNEY	Between Groups	5.271	2	2.635	5.837	.039
	Within Groups	2.709	6	.451		
	Total	7.980	8			
LIVER	Between Groups	7.130	2	3.565	3.278	.109
	Within Groups	6.526	6	1.088		
	Total	13.655	8			
LUNG	Between Groups	16.582	2	8.291	13.979	.006
	Within Groups	3.559	6	.593		
	Total	20.141	8			
MUSCLE	Between Groups	39.700	2	19.850	15.182	.004
	Within Groups	7.845	6	1.307		
	Total	47.545	8			
SPLEEN	Between Groups	13.761	2	6.881	8.499	.018
	Within Groups	4.858	6	.810		
	Total	18.619	8			

Post-hoc tests

Multiple Comparisons

Dependent Variable		(I) 24 hr-Route	(J) 24 hr-Route	Mean Diff (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
BONE	Tukey HSD	Control	FP i.v.	-4.9633*	.6513	.001	-6.9618	-2.9649
			FP i.t.	-5.6707*	.6513	.000	-7.6691	-3.6722
		FP i.v.	Control	4.9633*	.6513	.001	2.9649	6.9618
			FP i.t.	-.7073	.6513	.556	-2.7058	1.2911
		FP i.t.	Control	5.6707*	.6513	.000	3.6722	7.6691
			FP i.v.	.7073	.6513	.556	-1.2911	2.7058
BLOOD	Tukey HSD	Control	FP i.v.	-1.7733	.9979	.255	-4.8351	1.2884
			FP i.t.	-5.4767*	.9979	.004	-8.5384	-2.4149
		FP i.v.	Control	1.7733	.9979	.255	-1.2884	4.8351
			FP i.t.	-3.7033*	.9979	.023	-6.7651	-.6416
		FP i.t.	Control	5.4767*	.9979	.004	2.4149	8.5384
			FP i.v.	3.7033*	.9979	.023	.6416	6.7651
KIDNEY	Tukey HSD	Control	FP i.v.	-.3407	.5486	.814	-2.0240	1.3427
			FP i.t.	-1.7667*	.5486	.042	-3.4500	-8.3E-02
		FP i.v.	Control	.3407	.5486	.814	-1.3427	2.0240
			FP i.t.	-1.4260	.5486	.090	-3.1093	.2573
		FP i.t.	Control	1.7667*	.5486	.042	8.E-02	3.4500
			FP i.v.	1.4260	.5486	.090	-.2573	3.1093
LIVER	Tukey HSD	Control	FP i.v.	-1.1133	.8515	.442	-3.7260	1.4993
			FP i.t.	-2.1800	.8515	.095	-4.7927	.4327
		FP i.v.	Control	1.1133	.8515	.442	-1.4993	3.7260
			FP i.t.	-1.0667	.8515	.469	-3.6793	1.5460
		FP i.t.	Control	2.1800	.8515	.095	-.4327	4.7927
			FP i.v.	1.0667	.8515	.469	-1.5460	3.6793
LUNG	Tukey HSD	Control	FP i.v.	-1.7500	.6288	.071	-3.6794	.1794
			FP i.t.	-3.3233*	.6288	.004	-5.2527	-1.3939
		FP i.v.	Control	1.7500	.6288	.071	-.1794	3.6794
			FP i.t.	-1.5733	.6288	.102	-3.5027	.3561
		FP i.t.	Control	3.3233*	.6288	.004	1.3939	5.2527
			FP i.v.	1.5733	.6288	.102	-.3561	3.5027
MUSCLE	Tukey HSD	Control	FP i.v.	-3.4733*	.9336	.023	-6.3380	-.6087
			FP i.t.	-5.0233*	.9336	.004	-7.8879	-2.1586
		FP i.v.	Control	3.4733*	.9336	.023	.6087	6.3380
			FP i.t.	-1.5499	.9336	.294	-4.4146	1.3147
		FP i.t.	Control	5.0233*	.9336	.004	2.1586	7.8879
			FP i.v.	1.5499	.9336	.294	-1.3147	4.4146
SPLEEN	Tukey HSD	Control	FP i.v.	-1.0033	.7347	.414	-3.2575	1.2509
			FP i.t.	-2.9767*	.7347	.016	-5.2309	-.7225
		FP i.v.	Control	1.0033	.7347	.414	-1.2509	3.2575
			FP i.t.	-1.9733	.7347	.081	-4.2275	.2809
		FP i.t.	Control	2.9767*	.7347	.016	.7225	5.2309
			FP i.v.	1.9733	.7347	.081	-.2809	4.2275

*. The mean difference is significant at the .05 level.

Experiment 3

One-way ANOVA

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
BONE	Between Groups	41.333	2	20.666	11.027	.010
	Within Groups	11.245	6	1.874		
	Total	52.578	8			
BLOOD	Between Groups	42.309	2	21.155	13.507	.006
	Within Groups	9.397	6	1.566		
	Total	51.706	8			
KIDNEY	Between Groups	3.470	2	1.735	19.587	.002
	Within Groups	.532	6	8.859E-02		
	Total	4.002	8			
LIVER	Between Groups	.803	2	.402	2.353	.176
	Within Groups	1.024	6	.171		
	Total	1.828	8			
LUNG	Between Groups	32.033	2	16.017	12.731	.007
	Within Groups	7.549	6	1.258		
	Total	39.582	8			
MUSCLE	Between Groups	18.667	2	9.333	11.284	.009
	Within Groups	4.963	6	.827		
	Total	23.630	8			
SPLEEN	Between Groups	12.441	2	6.220	15.009	.005
	Within Groups	2.487	6	.414		
	Total	14.928	8			

Post-hoc tests

Multiple Comparisons

Dependent Variable		(I) 24 hr-Route	(J) 24 hr-Route	Mean Diff (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
BONE	Tukey HSD	Control	FP i.v.	-3.6367*	1.1178	.040	-7.0664	-.2069
			FP i.t.	-5.0967*	1.1178	.009	-8.5264	-1.6669
		FP i.v.	Control	3.6367*	1.1178	.040	.2069	7.0664
			FP i.t.	-1.4600	1.1178	.442	-4.8897	1.9697
		FP i.t.	Control	5.0967*	1.1178	.009	1.6669	8.5264
			FP i.v.	1.4600	1.1178	.442	-1.9697	4.8897
BLOOD	Tukey HSD	Control	FP i.v.	-1.3500	1.0218	.435	-4.4853	1.7853
			FP i.t.	-5.1233*	1.0218	.006	-8.2586	-1.9880
		FP i.v.	Control	1.3500	1.0218	.435	-1.7853	4.4853
			FP i.t.	-3.7733*	1.0218	.024	-6.9086	-.6380
		FP i.t.	Control	5.1233*	1.0218	.006	1.9880	8.2586
			FP i.v.	3.7733*	1.0218	.024	.6380	6.9086
KIDNEY	Tukey HSD	Control	FP i.v.	-.5133	.2430	.167	-1.2590	.2323
			FP i.t.	-1.4967*	.2430	.002	-2.2423	-.7510
		FP i.v.	Control	.5133	.2430	.167	-.2323	1.2590
			FP i.t.	-.9833*	.2430	.016	-1.7290	-.2377
		FP i.t.	Control	1.4967*	.2430	.002	.7510	2.2423
			FP i.v.	.9833*	.2430	.016	.2377	1.7290
LIVER	Tukey HSD	Control	FP i.v.	-.2300	.3374	.782	-1.2651	.8051
			FP i.t.	-.7167	.3374	.165	-1.7518	.3185
		FP i.v.	Control	.2300	.3374	.782	-.8051	1.2651
			FP i.t.	-.4867	.3374	.380	-1.5218	.5485
		FP i.t.	Control	.7167	.3374	.165	-.3185	1.7518
			FP i.v.	.4867	.3374	.380	-.5485	1.5218
LUNG	Tukey HSD	Control	FP i.v.	-2.6533	.9158	.062	-5.4634	.1567
			FP i.t.	-4.6033*	.9158	.006	-7.4134	-1.7933
		FP i.v.	Control	2.6533	.9158	.062	-.1567	5.4634
			FP i.t.	-1.9500	.9158	.164	-4.7600	.8600
		FP i.t.	Control	4.6033*	.9158	.006	1.7933	7.4134
			FP i.v.	1.9500	.9158	.164	-.8600	4.7600
MUSCLE	Tukey HSD	Control	FP i.v.	-2.4967*	.7426	.035	-4.7751	-.2182
			FP i.t.	-3.4067*	.7426	.009	-5.6851	-1.1282
		FP i.v.	Control	2.4967*	.7426	.035	.2182	4.7751
			FP i.t.	-.9100	.7426	.482	-3.1885	1.3685
		FP i.t.	Control	3.4067*	.7426	.009	1.1282	5.6851
			FP i.v.	.9100	.7426	.482	-1.3685	3.1885
SPLEEN	Tukey HSD	Control	FP i.v.	-1.2680	.5256	.114	-2.8808	.3448
			FP i.t.	-2.8733*	.5256	.004	-4.4861	-1.2605
		FP i.v.	Control	1.2680	.5256	.114	-.3448	2.8808
			FP i.t.	-1.6053	.5256	.051	-3.2181	7.E-03
		FP i.t.	Control	2.8733*	.5256	.004	1.2605	4.4861
			FP i.v.	1.6053	.5256	.051	-7.E-03	3.2181

*. The mean difference is significant at the .05 level.

(III) Seventy two hours biodistribution study

Experiment 1

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
BONE	Between Groups	4.841	2	2.421	7.564	.031
	Within Groups	1.600	5	.320		
	Total	6.442	7			
BLOOD	Between Groups	4.504E-02	2	2.252E-02	1.229	.368
	Within Groups	9.163E-02	5	1.833E-02		
	Total	.137	7			
KIDNEY	Between Groups	.207	2	.103	5.700	.051
	Within Groups	9.072E-02	5	1.814E-02		
	Total	.298	7			
LIVER	Between Groups	.770	2	.385	3.307	.122
	Within Groups	.582	5	.116		
	Total	1.351	7			
LUNG	Between Groups	.143	2	7.142E-02	7.041	.035
	Within Groups	5.072E-02	5	1.014E-02		
	Total	.194	7			
MUSCLE	Between Groups	4.016	2	2.008	9.386	.020
	Within Groups	1.070	5	.214		
	Total	5.086	7			
SPLEEN	Between Groups	4.135	2	2.068	5.121	.062
	Within Groups	2.019	5	.404		
	Total	6.155	7			

Post-hoc tests

Multiple Comparisons

Dependent Variable		(I) 72 hr-Route	(J) 72 hr-Route	Mean Diff (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
BONE	Tukey HSD	Control	FP i.v.	-1.0391	.5164	.204	-2.7195	.6412
			FP i.t.	-1.7908*	.4619	.026	-3.2938	-.2878
		FP i.v.	Control	1.0391	.5164	.204	-.6412	2.7195
			FP i.t.	-.7517	.5164	.385	-2.4320	.9287
		FP i.t.	Control	1.7908*	.4619	.026	.2878	3.2938
			FP i.v.	.7517	.5164	.385	-.9287	2.4320
BLOOD	Tukey HSD	Control	FP i.v.	-9.633E-02	.1236	.731	-.4984	.3058
			FP i.t.	-.1730	.1105	.341	-.5327	.1867
		FP i.v.	Control	9.633E-02	.1236	.731	-.3058	.4984
			FP i.t.	-7.667E-02	.1236	.816	-.4788	.3254
		FP i.t.	Control	.1730	.1105	.341	-.1867	.5327
			FP i.v.	7.667E-02	.1236	.816	-.3254	.4788
KIDNEY	Tukey HSD	Control	FP i.v.	-.2583	.1230	.184	-.6584	.1418
			FP i.t.	-.3633*	.1100	.047	-.7212	-5.E-03
		FP i.v.	Control	.2583	.1230	.184	-.1418	.6584
			FP i.t.	-.1050	.1230	.689	-.5051	.2951
		FP i.t.	Control	.3633*	.1100	.047	5.5E-03	.7212
			FP i.v.	.1050	.1230	.689	-.2951	.5051
LIVER	Tukey HSD	Control	FP i.v.	-.3777	.3114	.496	-1.3909	.6355
			FP i.t.	-.7160	.2785	.107	-1.6222	.1902
		FP i.v.	Control	.3777	.3114	.496	-.6355	1.3909
			FP i.t.	-.3383	.3114	.562	-1.3515	.6749
		FP i.t.	Control	.7160	.2785	.107	-.1902	1.6222
			FP i.v.	.3383	.3114	.562	-.6749	1.3515
LUNG	Tukey HSD	Control	FP i.v.	-.2083	9.E-02	.152	-.5075	9.1E-02
			FP i.t.	-.3033*	8.E-02	.032	-.5709	-4.E-02
		FP i.v.	Control	.2083	9.E-02	.152	-9.E-02	.5075
			FP i.t.	-9.500E-02	9.E-02	.590	-.3942	.2042
		FP i.t.	Control	.3033*	8.E-02	.032	3.6E-02	.5709
			FP i.v.	9.500E-02	9.E-02	.590	-.2042	.3942
MUSCLE	Tukey HSD	Control	FP i.v.	-.4083	.4223	.626	-1.7823	.9656
			FP i.t.	-1.5900*	.3777	.019	-2.8189	-.3611
		FP i.v.	Control	.4083	.4223	.626	-.9656	1.7823
			FP i.t.	-1.1817	.4223	.082	-2.5556	.1923
		FP i.t.	Control	1.5900*	.3777	.019	.3611	2.8189
			FP i.v.	1.1817	.4223	.082	-.1923	2.5556
SPLEEN	Tukey HSD	Control	FP i.v.	-1.2510	.5801	.173	-3.1386	.6366
			FP i.t.	-1.5977	.5188	.060	-3.2859	9.1E-02
		FP i.v.	Control	1.2510	.5801	.173	-.6366	3.1386
			FP i.t.	-.3467	.5801	.827	-2.2342	1.5409
		FP i.t.	Control	1.5977	.5188	.060	-9.E-02	3.2859
			FP i.v.	.3467	.5801	.827	-1.5409	2.2342

*. The mean difference is significant at the .05 level.

Experiment 2

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
BONE	Between Groups	1.546	2	.773	11.064	.010
	Within Groups	.419	6	6.989E-02		
	Total	1.966	8			
BLOOD	Between Groups	6.889E-04	2	3.444E-04	.016	.984
	Within Groups	.129	6	2.157E-02		
	Total	.130	8			
KIDNEY	Between Groups	.121	2	6.048E-02	4.155	.074
	Within Groups	8.733E-02	6	1.456E-02		
	Total	.208	8			
LIVER	Between Groups	.242	2	.121	4.009	.078
	Within Groups	.181	6	3.014E-02		
	Total	.423	8			
LUNG	Between Groups	.209	2	.104	7.959	.021
	Within Groups	7.873E-02	6	1.312E-02		
	Total	.288	8			
MUSCLE	Between Groups	3.217	2	1.609	9.504	.014
	Within Groups	1.016	6	.169		
	Total	4.233	8			
SPLEEN	Between Groups	2.625	2	1.313	4.581	.062
	Within Groups	1.719	6	.287		
	Total	4.344	8			

Post-hoc tests

Multiple Comparisons

Dependent Variable		(I) 72 hr-Route	(J) 72 hr-Route	Mean Diff (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
BONE	Tukey HSD	Control	FP i.v.	-.6367	.2159	.058	-1.2990	3.E-02
			FP i.t.	-1.0033*	.2159	.008	-1.6656	-.3410
		FP i.v.	Control	.6367	.2159	.058	-.0256	1.2990
			FP i.t.	-.3667	.2159	.281	-1.0290	.2956
		FP i.t.	Control	1.0033*	.2159	.008	.3410	1.6656
			FP i.v.	.3667	.2159	.281	-.2956	1.0290
BLOOD	Tukey HSD	Control	FP i.v.	-2.0000E-02	.1199	.985	-.3879	.3479
			FP i.t.	-1.6667E-02	.1199	.989	-.3846	.3512
		FP i.v.	Control	2.000E-02	.1199	.985	-.3479	.3879
			FP i.t.	3.333E-03	.1199	1.000	-.3646	.3712
		FP i.t.	Control	1.667E-02	.1199	.989	-.3512	.3846
			FP i.v.	-3.3333E-03	.1199	1.000	-.3712	.3646
KIDNEY	Tukey HSD	Control	FP i.v.	-7.0000E-02	9.851E-02	.767	-.3722	.2322
			FP i.t.	-.2733	9.851E-02	.072	-.5756	3.E-02
		FP i.v.	Control	7.000E-02	9.851E-02	.767	-.2322	.3722
			FP i.t.	-.2033	9.851E-02	.178	-.5056	1.E-01
		FP i.t.	Control	.2733	9.851E-02	.072	-.0289	.5756
			FP i.v.	.2033	9.851E-02	.178	-.0989	.5056
LIVER	Tukey HSD	Control	FP i.v.	-.2867	.1418	.188	-.7216	.1483
			FP i.t.	-.3867	.1418	.076	-.8216	5.E-02
		FP i.v.	Control	.2867	.1418	.188	-.1483	.7216
			FP i.t.	-1.0000E-01	.1418	.769	-.5350	.3350
		FP i.t.	Control	.3867	.1418	.076	-.0483	.8216
			FP i.v.	1.000E-01	.1418	.769	-.3350	.5350
LUNG	Tukey HSD	Control	FP i.v.	-8.3333E-02	9.353E-02	.665	-.3703	.2036
			FP i.t.	-.3567*	9.353E-02	.021	-.6436	-.0697
		FP i.v.	Control	8.333E-02	9.353E-02	.665	-.2036	.3703
			FP i.t.	-.2733	9.353E-02	.060	-.5603	1.E-02
		FP i.t.	Control	.3567*	9.353E-02	.021	7.E-02	.6436
			FP i.v.	.2733	9.353E-02	.060	-.0136	.5603
MUSCLE	Tukey HSD	Control	FP i.v.	-.5067	.3359	.352	-1.5373	.5240
			FP i.t.	-1.4433*	.3359	.012	-2.4740	-.4127
		FP i.v.	Control	.5067	.3359	.352	-.5240	1.5373
			FP i.t.	-.9367	.3359	.071	-1.9673	9.E-02
		FP i.t.	Control	1.4433*	.3359	.012	.4127	2.4740
			FP i.v.	.9367	.3359	.071	-.0940	1.9673
SPLEEN	Tukey HSD	Control	FP i.v.	-1.0000	.4370	.134	-2.3409	.3409
			FP i.t.	-1.2500	.4370	.065	-2.5909	9.E-02
		FP i.v.	Control	1.0000	.4370	.134	-.3409	2.3409
			FP i.t.	-.2500	.4370	.839	-1.5909	1.0909
		FP i.t.	Control	1.2500	.4370	.065	-.0909	2.5909
			FP i.v.	.2500	.4370	.839	-1.0909	1.5909

*. The mean difference is significant at the .05 level.

Experiment 3

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
BONE	Between Groups	.777	2	.388	7.038	.027
	Within Groups	.331	6	5.518E-02		
	Total	1.108	8			
BLOOD	Between Groups	1.396E-02	2	6.978E-03	.424	.672
	Within Groups	9.867E-02	6	1.644E-02		
	Total	.113	8			
KIDNEY	Between Groups	5.749E-02	2	2.874E-02	4.312	.069
	Within Groups	4.000E-02	6	6.667E-03		
	Total	9.749E-02	8			
LIVER	Between Groups	.133	2	6.643E-02	2.877	.133
	Within Groups	.139	6	2.309E-02		
	Total	.271	8			
LUNG	Between Groups	.262	2	.131	13.644	.006
	Within Groups	5.753E-02	6	9.589E-03		
	Total	.319	8			
MUSCLE	Between Groups	2.523	2	1.261	7.294	.025
	Within Groups	1.038	6	.173		
	Total	3.560	8			
SPLEEN	Between Groups	1.468	2	.734	4.885	.055
	Within Groups	.902	6	.150		
	Total	2.370	8			

Post-hoc tests

Multiple Comparisons

Dependent Variable		(I) 72 hr- Route	(J) 72 hr-Route	Mean Diff (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
BONE	Tukey HSD	Control	FP i.v.	-.2200	.1918	.523	-.8085	.3685
			FP i.t.	-.7033*	.1918	.024	-1.2918	-.1149
		FP i.v.	Control	.2200	.1918	.523	-.3685	.8085
			FP i.t.	-.4833	.1918	.100	-1.0718	.1051
		FP i.t.	Control	.7033*	.1918	.024	.1149	1.2918
			FP i.v.	.4833	.1918	.100	-.1051	1.0718
BLOOD	Tukey HSD	Control	FP i.v.	-6.6667E-03	.1047	.998	-.3279	.3146
			FP i.t.	-8.6667E-02	.1047	.701	-.4079	.2346
		FP i.v.	Control	6.667E-03	.1047	.998	-.3146	.3279
			FP i.t.	-8.0000E-02	.1047	.737	-.4013	.2413
		FP i.t.	Control	8.667E-02	.1047	.701	-.2346	.4079
			FP i.v.	8.000E-02	.1047	.737	-.2413	.4013
KIDNEY	Tukey HSD	Control	FP i.v.	-7.0000E-02	6.67E-02	.576	-.2746	.1346
			FP i.t.	-.1933	6.67E-02	.062	-.3979	1.1E-02
		FP i.v.	Control	7.000E-02	6.67E-02	.576	-.1346	.2746
			FP i.t.	-.1233	6.67E-02	.233	-.3279	8.1E-02
		FP i.t.	Control	.1933	6.67E-02	.062	-1.E-02	.3979
			FP i.v.	.1233	6.67E-02	.233	-8.E-02	.3279
LIVER	Tukey HSD	Control	FP i.v.	-.2767	.1241	.144	-.6573	.1040
			FP i.t.	-.2333	.1241	.224	-.6140	.1473
		FP i.v.	Control	.2767	.1241	.144	-.1040	.6573
			FP i.t.	4.333E-02	.1241	.936	-.3373	.4240
		FP i.t.	Control	.2333	.1241	.224	-.1473	.6140
			FP i.v.	-4.3333E-02	.1241	.936	-.4240	.3373
LUNG	Tukey HSD	Control	FP i.v.	-.1833	8.00E-02	.133	-.4287	6.2E-02
			FP i.t.	-.4167*	8.00E-02	.005	-.6620	-.1713
		FP i.v.	Control	.1833	8.00E-02	.133	-6.E-02	.4287
			FP i.t.	-.2333	8.00E-02	.060	-.4787	1.2E-02
		FP i.t.	Control	.4167*	8.00E-02	.005	.1713	.6620
			FP i.v.	.2333	8.00E-02	.060	-1.E-02	.4787
MUSCLE	Tukey HSD	Control	FP i.v.	-.8033	.3395	.121	-1.8451	.2384
			FP i.t.	-1.2833*	.3395	.021	-2.3251	-.2416
		FP i.v.	Control	.8033	.3395	.121	-.2384	1.8451
			FP i.t.	-.4800	.3395	.392	-1.5218	.5618
		FP i.t.	Control	1.2833*	.3395	.021	.2416	2.3251
			FP i.v.	.4800	.3395	.392	-.5618	1.5218
SPLEEN	Tukey HSD	Control	FP i.v.	-.8867	.3165	.070	-1.8578	8.4E-02
			FP i.t.	-.8233	.3165	.090	-1.7944	.1478
		FP i.v.	Control	.8867	.3165	.070	-8.E-02	1.8578
			FP i.t.	6.333E-02	.3165	.978	-.9078	1.0344
		FP i.t.	Control	.8233	.3165	.090	-.1478	1.7944
			FP i.v.	-6.3333E-02	.3165	.978	-1.0344	.9078

*. The mean difference is significant at the .05 level.

(B) Dosage-dependence study

Experiment 1

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
BONE	Between Groups	33.177	2	16.588	27.936	.002
	Within Groups	2.969	5	.594		
	Total	36.145	7			
BLOOD	Between Groups	6.723	2	3.361	29.935	.002
	Within Groups	.561	5	.112		
	Total	7.284	7			
KIDNEY	Between Groups	9.555	2	4.778	41.487	.001
	Within Groups	.576	5	.115		
	Total	10.131	7			
LIVER	Between Groups	1.543	2	.771	1.143	.390
	Within Groups	3.375	5	.675		
	Total	4.918	7			
LUNG	Between Groups	21.357	2	10.679	12.007	.012
	Within Groups	4.447	5	.889		
	Total	25.804	7			
MUSCLE	Between Groups	25.117	2	12.559	101.497	.000
	Within Groups	.619	5	.124		
	Total	25.736	7			
SPLEEN	Between Groups	13.113	2	6.557	10.511	.016
	Within Groups	3.119	5	.624		
	Total	16.232	7			

Post-hoc tests

Multiple Comparisons

Dependent Variable		(I) 24 hr-Dosage	(J) 24 hr-Dosage	Mean Diff (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
BONE	Tukey HSD	Control	9ug FunFP	-2.8967*	.6292	.013	-4.9439	-.8494
			18 ug FunFP	-5.1533*	.7034	.002	-7.4422	-2.86
		9ug FunFP	Control	2.8967*	.6292	.013	.8494	4.9439
			18 ug FunFP	-2.2567	.7034	.053	-4.5456	.0322
		18 ug FunFP	Control	5.1533*	.7034	.002	2.8644	7.4422
			9ug FunFP	2.2567	.7034	.053	-3.E-02	4.5456
BLOOD	Tukey HSD	Control	9ug FunFP	-.8700	.2736	.054	-1.7603	.0203
			18 ug FunFP	-2.3650*	.3059	.001	-3.3604	-1.37
		9ug FunFP	Control	.8700	.2736	.054	-2.E-02	1.7603
			18 ug FunFP	-1.4950*	.3059	.010	-2.4904	-.4996
		18 ug FunFP	Control	2.3650*	.3059	.001	1.3696	3.3604
			9ug FunFP	1.4950*	.3059	.010	.4996	2.4904
KIDNEY	Tukey HSD	Control	9ug FunFP	-.6700	.2771	.127	-1.5716	.2316
			18 ug FunFP	-2.7683*	.3098	.001	-3.7763	-1.76
		9ug FunFP	Control	.6700	.2771	.127	-.2316	1.5716
			18 ug FunFP	-2.0983*	.3098	.002	-3.1063	-1.09
		18 ug FunFP	Control	2.7683*	.3098	.001	1.7603	3.7763
			9ug FunFP	2.0983*	.3098	.002	1.0903	3.1063
LIVER	Tukey HSD	Control	9ug FunFP	-.7867	.6708	.517	-2.9695	1.3962
			18 ug FunFP	-1.0333	.7500	.418	-3.4738	1.4071
		9ug FunFP	Control	.7867	.6708	.517	-1.3962	2.9695
			18 ug FunFP	-.2467	.7500	.943	-2.6871	2.1938
		18 ug FunFP	Control	1.0333	.7500	.418	-1.4071	3.4738
			9ug FunFP	.2467	.7500	.943	-2.1938	2.6871
LUNG	Tukey HSD	Control	9ug FunFP	-1.6400	.7700	.178	-4.1455	.8655
			18 ug FunFP	-4.2183*	.8609	.010	-7.0196	-1.42
		9ug FunFP	Control	1.6400	.7700	.178	-.8655	4.1455
			18 ug FunFP	-2.5783	.8609	.066	-5.3796	.2229
		18 ug FunFP	Control	4.2183*	.8609	.010	1.4171	7.0196
			9ug FunFP	2.5783	.8609	.066	-.2229	5.3796
MUSCLE	Tukey HSD	Control	9ug FunFP	-3.6533*	.2872	.000	-4.5879	-2.72
			18 ug FunFP	-3.6700*	.3211	.000	-4.7149	-2.63
		9ug FunFP	Control	3.6533*	.2872	.000	2.7188	4.5879
			18 ug FunFP	-1.67E-02	.3211	.999	-1.0615	1.0282
		18 ug FunFP	Control	3.6700*	.3211	.000	2.6251	4.7149
			9ug FunFP	1.667E-02	.3211	.999	-1.0282	1.0615
SPLEEN	Tukey HSD	Control	9ug FunFP	-1.9433	.6449	.065	-4.0417	.1550
			18 ug FunFP	-3.2000*	.7210	.015	-5.5460	-.8540
		9ug FunFP	Control	1.9433	.6449	.065	-.1550	4.0417
			18 ug FunFP	-1.2567	.7210	.279	-3.6027	1.0893
		18 ug FunFP	Control	3.2000*	.7210	.015	.8540	5.5460
			9ug FunFP	1.2567	.7210	.279	-1.0893	3.6027

*. The mean difference is significant at the .05 level.

Experiment 2

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
BONE	Between Groups	41.012	2	20.506	39.378	.001
	Within Groups	2.604	5	.521		
	Total	43.616	7			
BLOOD	Between Groups	24.993	2	12.496	87.527	.000
	Within Groups	.714	5	.143		
	Total	25.706	7			
KIDNEY	Between Groups	13.547	2	6.773	31.285	.001
	Within Groups	1.083	5	.217		
	Total	14.629	7			
LIVER	Between Groups	12.719	2	6.360	5.570	.053
	Within Groups	5.708	5	1.142		
	Total	18.427	7			
LUNG	Between Groups	4.612	2	2.306	7.214	.034
	Within Groups	1.598	5	.320		
	Total	6.211	7			
MUSCLE	Between Groups	36.524	2	18.262	21.916	.003
	Within Groups	4.166	5	.833		
	Total	40.690	7			
SPLEEN	Between Groups	13.143	2	6.571	16.308	.006
	Within Groups	2.015	5	.403		
	Total	15.158	7			

Post-hoc tests

Multiple Comparisons

Dependent Variable		(I) 24 hr-Dosage	(J) 24 hr-Dosage	Mean Diff (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
BONE	Tukey HSD	Control	9 ug FunFP	-3.9287*	.5892	.003	-5.8459	-2.0114
			18 ug FunFP	-5.4150*	.6588	.001	-7.5585	-3.2715
		9 ug FunFP	Control	3.9287*	.5892	.003	2.0114	5.8459
			18 ug FunFP	-1.4863	.6588	.154	-3.6299	.6572
		18 ug FunFP	Control	5.4150*	.6588	.001	3.2715	7.5585
			9 ug FunFP	1.4863	.6588	.154	-.6572	3.6299
BLOOD	Tukey HSD	Control	9 ug FunFP	-.9900	.3085	.052	-1.9939	1.E-02
			18 ug FunFP	-4.4550*	.3449	.000	-5.5774	-3.3326
		9 ug FunFP	Control	.9900	.3085	.052	-1.E-02	1.9939
			18 ug FunFP	-3.4650*	.3449	.000	-4.5874	-2.3426
		18 ug FunFP	Control	4.4550*	.3449	.000	3.3326	5.5774
			9 ug FunFP	3.4650*	.3449	.000	2.3426	4.5874
KIDNEY	Tukey HSD	Control	9 ug FunFP	-.5733	.3799	.363	-1.8096	.6629
			18 ug FunFP	-3.2367*	.4248	.001	-4.6188	-1.8545
		9 ug FunFP	Control	.5733	.3799	.363	-.6629	1.8096
			18 ug FunFP	-2.6633*	.4248	.004	-4.0455	-1.2812
		18 ug FunFP	Control	3.2367*	.4248	.001	1.8545	4.6188
			9 ug FunFP	2.6633*	.4248	.004	1.2812	4.0455
LIVER	Tukey HSD	Control	9 ug FunFP	-2.2117	.8724	.111	-5.0504	.6271
			18 ug FunFP	-3.0000	.9754	.061	-6.1738	.1738
		9 ug FunFP	Control	2.2117	.8724	.111	-.6271	5.0504
			18 ug FunFP	-.7883	.9754	.715	-3.9621	2.3855
		18 ug FunFP	Control	3.0000	.9754	.061	-.1738	6.1738
			9 ug FunFP	.7883	.9754	.715	-2.3855	3.9621
LUNG	Tukey HSD	Control	9 ug FunFP	-.8567	.4616	.245	-2.3588	.6455
			18 ug FunFP	-1.9583*	.5161	.029	-3.6378	-.2789
		9 ug FunFP	Control	.8567	.4616	.245	-.6455	2.3588
			18 ug FunFP	-1.1017	.5161	.177	-2.7811	.5778
		18 ug FunFP	Control	1.9583*	.5161	.029	.2789	3.6378
			9 ug FunFP	1.1017	.5161	.177	-.5778	2.7811
MUSCLE	Tukey HSD	Control	9 ug FunFP	-3.5067*	.7453	.012	-5.9319	-1.0814
			18 ug FunFP	-5.2250*	.8333	.004	-7.9365	-2.5135
		9 ug FunFP	Control	3.5067*	.7453	.012	1.0814	5.9319
			18 ug FunFP	-1.7183	.8333	.193	-4.4298	.9931
		18 ug FunFP	Control	5.2250*	.8333	.004	2.5135	7.9365
			9 ug FunFP	1.7183	.8333	.193	-.9931	4.4298
SPLEEN	Tukey HSD	Control	9 ug FunFP	-1.5200	.5183	.071	-3.2065	.1665
			18 ug FunFP	-3.3000*	.5795	.005	-5.1856	-1.4144
		9 ug FunFP	Control	1.5200	.5183	.071	-.1665	3.2065
			18 ug FunFP	-1.7800	.5795	.061	-3.6656	.1056
		18 ug FunFP	Control	3.3000*	.5795	.005	1.4144	5.1856
			9 ug FunFP	1.7800	.5795	.061	-.1056	3.6656

*. The mean difference is significant at the .05 level.

Appendix 8

Chromatography results of blood samples collected in C57/BL mice bearing subcutaneous tumor for vascular permeability studies of the H520C9sFv-rhIL-2

(A) Time-dependence study

Experiment 1

1. Twelve hours biodistribution study

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front)*100 (=Percentage decompose)
Saline	1824	216	10.59
9 μg of H520C9sFv-rhIL-2 (i.v.)	1524	172	10.14
9 μg of H520C9sFv-rhIL-2 (i.t.)	1674	204	10.86

2. Twenty four hours biodistribution study

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front) *100 (=Percentage decompose)
Saline	1840	231	11.15
9 μg of H520C9sFv-rhIL-2 (i.v.)	1916	214	10.05
9 μg of H520C9sFv-rhIL-2 (i.t.)	2531	318	11.16

3. Seventy two hours biodistribution study

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front) *100 (=Percentage decompose)
Saline	2226	302	11.95
9 μg of H520C9sFv-rhIL-2 (i.v.)	2642	312	10.56
9 μg of H520C9sFv-rhIL-2 (i.t.)	2461	314	11.32

Experiment 2

1. Twelve hours biodistribution study

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front) *100 (=Percentage decompose)
Saline	2342	318	11.95
9 μg of H520C9sFv-rhIL-2 (i.v.)	1762	229	11.50
9 μg of H520C9sFv-rhIL-2 (i.t.)	1325	211	13.74

2. Twenty four hours biodistribution study

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front) *100 (=Percentage decompose)
Saline	1623	247	13.21
9 μg of H520C9sFv-rhIL-2 (i.v.)	1425	218	13.27
9 μg of H520C9sFv-rhIL-2 (i.t.)	1325	221	14.29

3. Seventy two hours biodistribution study

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front) *100 (=Percentage decompose)
Saline	2472	365	12.87
9 μg of H520C9sFv-rhIL-2 (i.v.)	2234	342	13.28
9 μg of H520C9sFv-rhIL-2 (i.t.)	1258	231	15.51

Experiment 3

1. Twelve hours biodistribution study

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front) *100 (=Percentage decompose)
Saline	1576	223	12.40
9 μg of H520C9sFv-rhIL-2 (i.v.)	1628	263	13.91
9 μg of H520C9sFv-rhIL-2 (i.t.)	2482	345	12.20

2. Twenty four hours biodistribution study

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front) *100 (=Percentage decompose)
Saline	2143	321	13.03
9 μg of H520C9sFv-rhIL-2 (i.v.)	2248	312	12.19
9 μg of H520C9sFv-rhIL-2 (i.t.)	2843	384	11.90

3. Seventy two hours biodistribution study

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front) *100 (=Percentage decompose)
Saline	2741	432	13.61
9 μg of H520C9sFv-rhIL-2 (i.v.)	1823	236	11.46
9 μg of H520C9sFv-rhIL-2 (i.t.)	1527	234	13.29

(B) Dosage-dependence study

Experiment 1

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front) *100 (=Percentage decompose)
Saline	2143	269	11.15
9 μg of H520C9sFv-rhIL-2 (i.v.)	1823	241	11.68
18 μg of H520C9sFv-rhIL-2 (i.v.)	1562	223	12.49

Experiment 2

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front) *100 (=Percentage decompose)
Saline	1734	227	11.58
9 μg of H520C9sFv-rhIL-2 (i.v.)	1594	236	12.90
18 μg of H520C9sFv-rhIL-2 (i.v.)	1368	185	11.91

Appendix 9

One-way ANOVA test results for vascular permeability studies of H520C9sFv-rhIL-2 in nude mice bearing subcutaneous tumor

Experiment 1

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
BONE	Between Groups	.869	2	.434	22.234	.003
	Within Groups	9.767E-02	5	1.953E-02		
	Total	.966	7			
BLOOD	Between Groups	.217	2	.108	4.340	.081
	Within Groups	.125	5	2.498E-02		
	Total	.342	7			
KIDNEY	Between Groups	.249	2	.125	6.187	.044
	Within Groups	.101	5	2.016E-02		
	Total	.350	7			
LIVER	Between Groups	1.052E-02	2	5.260E-03	.067	.936
	Within Groups	.394	5	7.889E-02		
	Total	.405	7			
LUNG	Between Groups	1.718	2	.859	3.640	.106
	Within Groups	1.179	5	.236		
	Total	2.897	7			
MUSCLE	Between Groups	8.865	2	4.433	2.906	.145
	Within Groups	7.628	5	1.526		
	Total	16.493	7			
SPLEEN	Between Groups	1.934	2	.967	9.042	.022
	Within Groups	.535	5	.107		
	Total	2.469	7			

Post-hoc tests

Multiple Comparisons

Dependent Variable		(I) 24 hr- Route	(J) 24 hr- Route	Mean Diff (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
BONE	Tukey HSD	Control	FP i.v.	6.333E-02	.1141	.849	-.3080	.4347
			FP i.t.	-.7267*	.1276	.005	-1.14	-.3115
		FP i.v.	Control	-6.3333E-02	.1141	.849	-.4347	.3080
			FP i.t.	-.7900*	.1276	.004	-1.21	-.3749
		FP i.t.	Control	.7267*	.1276	.005	.3115	1.1418
			FP i.v.	.7900*	.1276	.004	.3749	1.2051
BLOOD	Tukey HSD	Control	FP i.v.	-.1633	.1291	.470	-.5833	.2566
			FP i.t.	-.4250	.1443	.070	-.8945	4.E-02
		FP i.v.	Control	.1633	.1291	.470	-.2566	.5833
			FP i.t.	-.2617	.1443	.257	-.7312	.2078
		FP i.t.	Control	.4250	.1443	.070	-.045	.8945
			FP i.v.	.2617	.1443	.257	-.2078	.7312
KIDNEY	Tukey HSD	Control	FP i.v.	-3.0000E-02	.1159	.964	-.4072	.3472
			FP i.t.	-.4217	.1296	.050	-.8434	5.E-05
		FP i.v.	Control	3.000E-02	.1159	.964	-.3472	.4072
			FP i.t.	-.3917	.1296	.064	-.8134	3.E-02
		FP i.t.	Control	.4217	.1296	.050	.000	.8434
			FP i.v.	.3917	.1296	.064	-.030	.8134
LIVER	Tukey HSD	Control	FP i.v.	5.667E-02	.2293	.967	-.6896	.8029
			FP i.t.	-3.3333E-02	.2564	.991	-.8677	.8010
		FP i.v.	Control	-5.6667E-02	.2293	.967	-.8029	.6896
			FP i.t.	-9.0000E-02	.2564	.935	-.9243	.7443
		FP i.t.	Control	3.333E-02	.2564	.991	-.8010	.8677
			FP i.v.	9.000E-02	.2564	.935	-.7443	.9243
LUNG	Tukey HSD	Control	FP i.v.	-.6500	.3966	.314	-1.94	.6404
			FP i.t.	-1.1750	.4434	.098	-2.62	.2677
		FP i.v.	Control	.6500	.3966	.314	-.6404	1.9404
			FP i.t.	-.5250	.4434	.511	-1.97	.9177
		FP i.t.	Control	1.1750	.4434	.098	-.2677	2.6177
			FP i.v.	.5250	.4434	.511	-.9177	1.9677
MUSCLE	Tukey HSD	Control	FP i.v.	-.6033	1.01	.827	-3.88	2.6782
			FP i.t.	-2.6567	1.13	.137	-6.33	1.0122
		FP i.v.	Control	.6033	1.01	.827	-2.68	3.8848
			FP i.t.	-2.0533	1.13	.255	-5.72	1.6155
		FP i.t.	Control	2.6567	1.13	.137	-1.01	6.3255
			FP i.v.	2.0533	1.13	.255	-1.62	5.7222
SPLEEN	Tukey HSD	Control	FP i.v.	-.6967	.2670	.102	-1.57	.1722
			FP i.t.	-1.2450*	.2985	.020	-2.22	-.2736
		FP i.v.	Control	.6967	.2670	.102	-.1722	1.5655
			FP i.t.	-.5483	.2985	.250	-1.52	.4231
		FP i.t.	Control	1.2450*	.2985	.020	.2736	2.2164
			FP i.v.	.5483	.2985	.250	-.4231	1.5197

*. The mean difference is significant at the .05 level.

Experiment 2

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
BONE	Between Groups	5.827	2	2.913	29.191	.001
	Within Groups	.599	6	9.981E-02		
	Total	6.426	8			
BLOOD	Between Groups	3.762E-02	2	1.881E-02	.103	.904
	Within Groups	1.099	6	.183		
	Total	1.137	8			
KIDNEY	Between Groups	.523	2	.262	5.372	.046
	Within Groups	.292	6	4.872E-02		
	Total	.816	8			
LIVER	Between Groups	.116	2	5.803E-02	.674	.544
	Within Groups	.516	6	8.606E-02		
	Total	.632	8			
LUNG	Between Groups	.234	2	.117	4.105	.075
	Within Groups	.171	6	2.846E-02		
	Total	.404	8			
MUSCLE	Between Groups	4.880	2	2.440	3.779	.087
	Within Groups	3.874	6	.646		
	Total	8.754	8			
SPLEEN	Between Groups	1.792	2	.896	6.385	.033
	Within Groups	.842	6	.140		
	Total	2.635	8			

Post-hoc tests

Multiple Comparisons

Dependent Variable		(I) 24 hr-Route	(J) 24 hr-Route	Mean Diff (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
BONE	Tukey HSD	Control	FP i.v.	-.1147	.2579	.899	-.9061	.6768
			FP i.t.	-1.7613*	.2579	.001	-2.553	-.9699
		FP i.v.	Control	.1147	.2579	.899	-.6768	.9061
			FP i.t.	-1.6467*	.2579	.002	-2.438	-.8552
		FP i.t.	Control	1.7613*	.2579	.001	.9699	2.5528
			FP i.v.	1.6467*	.2579	.002	.8552	2.4381
BLOOD	Tukey HSD	Control	FP i.v.	-.1433	.3495	.913	-1.216	.9290
			FP i.t.	-.1300	.3495	.927	-1.202	.9424
		FP i.v.	Control	.1433	.3495	.913	-.9290	1.2157
			FP i.t.	1.333E-02	.3495	.999	-1.059	1.0857
		FP i.t.	Control	.1300	.3495	.927	-.9424	1.2024
			FP i.v.	-1.3333E-02	.3495	.999	-1.086	1.0590
KIDNEY	Tukey HSD	Control	FP i.v.	-.2333	.1802	.448	-.7863	.3197
			FP i.t.	-.5867*	.1802	.040	-1.140	-.0337
		FP i.v.	Control	.2333	.1802	.448	-.3197	.7863
			FP i.t.	-.3533	.1802	.203	-.9063	.1997
		FP i.t.	Control	.5867*	.1802	.040	3.E-02	1.1397
			FP i.v.	.3533	.1802	.203	-.1997	.9063
LIVER	Tukey HSD	Control	FP i.v.	.1133	.2395	.886	-.6216	.8483
			FP i.t.	-.1633	.2395	.782	-.8983	.5716
		FP i.v.	Control	-.1133	.2395	.886	-.8483	.6216
			FP i.t.	-.2767	.2395	.519	-1.012	.4583
		FP i.t.	Control	.1633	.2395	.782	-.5716	.8983
			FP i.v.	.2767	.2395	.519	-.4583	1.0116
LUNG	Tukey HSD	Control	FP i.v.	-.3367	.1377	.110	-.7593	9.E-02
			FP i.t.	-.3467	.1377	.100	-.7693	8.E-02
		FP i.v.	Control	.3367	.1377	.110	-.0859	.7593
			FP i.t.	-1.0000E-02	.1377	.997	-.4326	.4126
		FP i.t.	Control	.3467	.1377	.100	-.0759	.7693
			FP i.v.	1.000E-02	.1377	.997	-.4126	.4326
MUSCLE	Tukey HSD	Control	FP i.v.	-.3460	.6561	.861	-2.359	1.6670
			FP i.t.	-1.7060	.6561	.090	-3.719	.3070
		FP i.v.	Control	.3460	.6561	.861	-1.667	2.3590
			FP i.t.	-1.3600	.6561	.176	-3.373	.6530
		FP i.t.	Control	1.7060	.6561	.090	-.3070	3.7190
			FP i.v.	1.3600	.6561	.176	-.6530	3.3730
SPLEEN	Tukey HSD	Control	FP i.v.	-.4733	.3059	.336	-1.412	.4653
			FP i.t.	-1.0900*	.3059	.028	-2.029	-.1514
		FP i.v.	Control	.4733	.3059	.336	-.4653	1.4119
			FP i.t.	-.6167	.3059	.189	-1.555	.3219
		FP i.t.	Control	1.0900*	.3059	.028	.1514	2.0286
			FP i.v.	.6167	.3059	.189	-.3219	1.5553

*. The mean difference is significant at the .05 level.

Experiment 3

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
BONE	Between Groups	6.539	2	3.270	25.413	.001
	Within Groups	.772	6	.129		
	Total	7.311	8			
BLOOD	Between Groups	6.358E-02	2	3.179E-02	.332	.730
	Within Groups	.574	6	9.565E-02		
	Total	.637	8			
KIDNEY	Between Groups	.618	2	.309	11.969	.008
	Within Groups	.155	6	2.582E-02		
	Total	.773	8			
LIVER	Between Groups	.252	2	.126	1.796	.245
	Within Groups	.422	6	7.029E-02		
	Total	.674	8			
LUNG	Between Groups	.376	2	.188	4.130	.074
	Within Groups	.273	6	4.547E-02		
	Total	.648	8			
MUSCLE	Between Groups	2.561	2	1.281	2.179	.194
	Within Groups	3.526	6	.588		
	Total	6.088	8			
SPLEEN	Between Groups	1.430	2	.715	9.693	.013
	Within Groups	.443	6	7.379E-02		
	Total	1.873	8			

Post-hoc tests

Multiple Comparisons

Dependent Variable		(I) 24 hr-Route	(J) 24 hr-Route	Mean Diff (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
BONE	Tukey HSD	Control	FP i.v.	-.3567	.2929	.486	-1.2553	.5420
			FP i.t.	-1.9600*	.2929	.001	-2.8586	-1.061
		FP i.v.	Control	.3567	.2929	.486	-.5420	1.2553
			FP i.t.	-1.6033*	.2929	.004	-2.5020	-.7047
		FP i.t.	Control	1.9600*	.2929	.001	1.0614	2.8586
			FP i.v.	1.6033*	.2929	.004	.7047	2.5020
BLOOD	Tukey HSD	Control	FP i.v.	-.1667	.2525	.794	-.9415	.6081
			FP i.t.	-.1880	.2525	.748	-.9628	.5868
		FP i.v.	Control	.1667	.2525	.794	-.6081	.9415
			FP i.t.	-2.1333E-02	.2525	.996	-.7961	.7535
		FP i.t.	Control	.1880	.2525	.748	-.5868	.9628
			FP i.v.	2.133E-02	.2525	.996	-.7535	.7961
KIDNEY	Tukey HSD	Control	FP i.v.	-.2767	.1312	.168	-.6792	.1259
			FP i.t.	-.6400*	.1312	.007	-1.0426	-.2374
		FP i.v.	Control	.2767	.1312	.168	-.1259	.6792
			FP i.t.	-.3633	.1312	.073	-.7659	4.E-02
		FP i.t.	Control	.6400*	.1312	.007	.2374	1.0426
			FP i.v.	.3633	.1312	.073	-.0392	.7659
LIVER	Tukey HSD	Control	FP i.v.	.2173	.2165	.601	-.4469	.8815
			FP i.t.	-.1927	.2165	.666	-.8569	.4715
		FP i.v.	Control	-.2173	.2165	.601	-.8815	.4469
			FP i.t.	-.4100	.2165	.220	-1.0742	.2542
		FP i.t.	Control	.1927	.2165	.666	-.4715	.8569
			FP i.v.	.4100	.2165	.220	-.2542	1.0742
LUNG	Tukey HSD	Control	FP i.v.	-.4333	.1741	.104	-.9675	.1009
			FP i.t.	-.4333	.1741	.104	-.9675	.1009
		FP i.v.	Control	.4333	.1741	.104	-.1009	.9675
			FP i.t.	2.220E-16	.1741	1.000	-.5342	.5342
		FP i.t.	Control	.4333	.1741	.104	-.1009	.9675
			FP i.v.	-2.2204E-16	.1741	1.000	-.5342	.5342
MUSCLE	Tukey HSD	Control	FP i.v.	.0000	.6260	1.000	-1.9206	1.9206
			FP i.t.	-1.1317	.6260	.245	-3.0523	.7890
		FP i.v.	Control	.0000	.6260	1.000	-1.9206	1.9206
			FP i.t.	-1.1317	.6260	.245	-3.0523	.7890
		FP i.t.	Control	1.1317	.6260	.245	-.7890	3.0523
			FP i.v.	1.1317	.6260	.245	-.7890	3.0523
SPLEEN	Tukey HSD	Control	FP i.v.	-.3633	.2218	.302	-1.0439	.3172
			FP i.t.	-.9667*	.2218	.011	-1.6472	-.2861
		FP i.v.	Control	.3633	.2218	.302	-.3172	1.0439
			FP i.t.	-.6033	.2218	.077	-1.2839	8.E-02
		FP i.t.	Control	.9667*	.2218	.011	.2861	1.6472
			FP i.v.	.6033	.2218	.077	-.0772	1.2839

*. The mean difference is significant at the .05 level.

Appendix 10

Chromatography results of blood samples collected in nude mice bearing subcutaneous tumor for vascular permeability studies of H520C9sFv-rhIL-2

Experiment 1

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front)*100 (=Percentage decompose)
Saline	2834	374	11.66
9 μg of H520C9sFv-rhIL-2 (i.v.)	2369	309	11.54
9 μg of H520C9sFv-rhIL-2 (i.t.)	1985	263	11.70

Experiment 2

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front)*100 (=Percentage decompose)
Saline	2647	334	11.20
9 μg of H520C9sFv-rhIL-2 (i.v.)	1648	235	12.48
9 μg of H520C9sFv-rhIL-2 (i.t.)	1725	274	13.71

Experiment 3

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front)*100 (=Percentage decompose)
Saline	2234	341	13.24
9 μg of H520C9sFv-rhIL-2 (i.v.)	2178	315	12.64
9 μg of H520C9sFv-rhIL-2 (i.t.)	2019	302	13.01

Appendix 11

One-way ANOVA test results for vascular permeability studies of H520C9sFv-mrhIL-2 in C57/BL mice bearing subcutaneous tumor

Experiment 1

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
BONE	Between Groups	5.035E-02	2	2.517E-02	.116	.893
	Within Groups	1.305	6	.218		
	Total	1.356	8			
BLOOD	Between Groups	4.667E-04	2	2.333E-04	.013	.987
	Within Groups	.109	6	1.819E-02		
	Total	.110	8			
KIDNEY	Between Groups	1.103E-02	2	5.515E-03	.254	.784
	Within Groups	.130	6	2.172E-02		
	Total	.141	8			
LIVER	Between Groups	.105	2	5.258E-02	.240	.794
	Within Groups	1.315	6	.219		
	Total	1.420	8			
LUNG	Between Groups	3.529E-02	2	1.764E-02	2.633	.151
	Within Groups	4.020E-02	6	6.700E-03		
	Total	7.549E-02	8			
MUSCLE	Between Groups	.367	2	.184	.233	.799
	Within Groups	4.722	6	.787		
	Total	5.090	8			
SPLEEN	Between Groups	.288	2	.144	3.337	.106
	Within Groups	.259	6	4.310E-02		
	Total	.546	8			

Post-hoc tests

Multiple Comparisons

Dependent Variable		(I) 24 hr-Route	(J) 24 hr-Route	Mean Diff (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
BONE	Tukey HSD	Control	9 ug MutFP	7.567E-02	.3808	.979	-1.09	1.2442
			18 ug MutFP	-.1067	.3808	.958	-1.28	1.0619
		9 ug MutFP	Control	-7.5667E-02	.3808	.979	-1.24	1.0929
			18 ug MutFP	-.1823	.3808	.884	-1.35	.9862
		18 ug MutFP	Control	.1067	.3808	.958	-1.06	1.2752
			9 ug MutFP	.1823	.3808	.884	-.9862	1.3509
BLOOD	Tukey HSD	Control	9 ug MutFP	-1.3333E-02	.1101	.992	-.3512	.3245
			18 ug MutFP	3.333E-03	.1101	.999	-.3345	.3412
		9 ug MutFP	Control	1.333E-02	.1101	.992	-.3245	.3512
			18 ug MutFP	1.667E-02	.1101	.987	-.3212	.3545
		18 ug MutFP	Control	-3.3333E-03	.1101	.999	-.3412	.3345
			9 ug MutFP	-1.6667E-02	.1101	.987	-.3545	.3212
KIDNEY	Tukey HSD	Control	9 ug MutFP	-3.5333E-02	.1203	.954	-.4046	.3339
			18 ug MutFP	-8.5333E-02	.1203	.767	-.4546	.2839
		9 ug MutFP	Control	3.533E-02	.1203	.954	-.3339	.4046
			18 ug MutFP	-5.0000E-02	.1203	.911	-.4192	.3192
		18 ug MutFP	Control	8.533E-02	.1203	.767	-.2839	.4546
			9 ug MutFP	5.000E-02	.1203	.911	-.3192	.4192
LIVER	Tukey HSD	Control	9 ug MutFP	.2533	.3822	.792	-.9194	1.4261
			18 ug MutFP	.1933	.3822	.871	-.9794	1.3661
		9 ug MutFP	Control	-.2533	.3822	.792	-1.43	.9194
			18 ug MutFP	-6.0000E-02	.3822	.987	-1.23	1.1127
		18 ug MutFP	Control	-.1933	.3822	.871	-1.37	.9794
			9 ug MutFP	6.000E-02	.3822	.987	-1.11	1.2327
LUNG	Tukey HSD	Control	9 ug MutFP	.1533	7.E-02	.133	-.052	.3584
			18 ug MutFP	8.000E-02	7.E-02	.497	-.1251	.2851
		9 ug MutFP	Control	-.1533	7.E-02	.133	-.3584	.0517
			18 ug MutFP	-7.3333E-02	7.E-02	.550	-.2784	.1317
		18 ug MutFP	Control	-8.0000E-02	7.E-02	.497	-.2851	.1251
			9 ug MutFP	7.333E-02	7.E-02	.550	-.1317	.2784
MUSCLE	Tukey HSD	Control	9 ug MutFP	.4933	.7244	.783	-1.73	2.7159
			18 ug MutFP	.2800	.7244	.922	-1.94	2.5026
		9 ug MutFP	Control	-.4933	.7244	.783	-2.72	1.7293
			18 ug MutFP	-.2133	.7244	.954	-2.44	2.0093
		18 ug MutFP	Control	-.2800	.7244	.922	-2.50	1.9426
			9 ug MutFP	.2133	.7244	.954	-2.01	2.4359
SPLEEN	Tukey HSD	Control	9 ug MutFP	-3.0000E-02	.1695	.983	-.5501	.4901
			18 ug MutFP	-.3933	.1695	.128	-.9134	.1268
		9 ug MutFP	Control	3.000E-02	.1695	.983	-.4901	.5501
			18 ug MutFP	-.3633	.1695	.161	-.8834	.1568
		18 ug MutFP	Control	.3933	.1695	.128	-.1268	.9134
			9 ug MutFP	.3633	.1695	.161	-.1568	.8834

Experiment 2

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
BONE	Between Groups	4.348E-02	2	2.174E-02	.032	.969
	Within Groups	4.101	6	.683		
	Total	4.144	8			
BLOOD	Between Groups	.296	2	.148	4.289	.070
	Within Groups	.207	6	3.456E-02		
	Total	.504	8			
KIDNEY	Between Groups	2.115E-03	2	1.057E-03	.058	.945
	Within Groups	.110	6	1.838E-02		
	Total	.112	8			
LIVER	Between Groups	.192	2	9.621E-02	.247	.788
	Within Groups	2.334	6	.389		
	Total	2.526	8			
LUNG	Between Groups	4.622E-03	2	2.311E-03	.048	.953
	Within Groups	.289	6	4.813E-02		
	Total	.293	8			
MUSCLE	Between Groups	1.153	2	.577	1.103	.391
	Within Groups	3.137	6	.523		
	Total	4.290	8			
SPLEEN	Between Groups	.437	2	.219	3.490	.099
	Within Groups	.376	6	6.267E-02		
	Total	.813	8			

Post-hoc tests

Multiple Comparisons

Dependent Variable		(I) 24 hr-Route	(J) 24 hr-Route	Mean Diff (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
BONE	Tukey HSD	Control	9 ug MutFP	6.933E-02	.6750	.994	-2.0018	2.1405
			18 ug MutFP	-.1000	.6750	.988	-2.1712	1.9712
		9 ug MutFP	Control	-6.9333E-02	.6750	.994	-2.1405	2.0018
			18 ug MutFP	-.1693	.6750	.966	-2.2405	1.9018
		18 ug MutFP	Control	.1000	.6750	.988	-1.9712	2.1712
			9 ug MutFP	.1693	.6750	.966	-1.9018	2.2405
BLOOD	Tukey HSD	Control	9 ug MutFP	-.2500	.1518	.299	-.7157	.2157
			18 ug MutFP	-.4433	.1518	.060	-.9090	.0224
		9 ug MutFP	Control	.2500	.1518	.299	-.2157	.7157
			18 ug MutFP	-.1933	.1518	.458	-.6590	.2724
		18 ug MutFP	Control	.4433	.1518	.060	-.0224	.9090
			9 ug MutFP	.1933	.1518	.458	-.2724	.6590
KIDNEY	Tukey HSD	Control	9 ug MutFP	-3.5333E-02	.1107	.946	-.3750	.3043
			18 ug MutFP	-2.8667E-02	.1107	.964	-.3683	.3110
		9 ug MutFP	Control	3.533E-02	.1107	.946	-.3043	.3750
			18 ug MutFP	6.667E-03	.1107	.998	-.3330	.3463
		18 ug MutFP	Control	2.867E-02	.1107	.964	-.3110	.3683
			9 ug MutFP	-6.6667E-03	.1107	.998	-.3463	.3330
LIVER	Tukey HSD	Control	9 ug MutFP	-.2067	.5092	.914	-1.7692	1.3559
			18 ug MutFP	-.3567	.5092	.772	-1.9192	1.2059
		9 ug MutFP	Control	.2067	.5092	.914	-1.3559	1.7692
			18 ug MutFP	-.1500	.5092	.954	-1.7125	1.4125
		18 ug MutFP	Control	.3567	.5092	.772	-1.2059	1.9192
			9 ug MutFP	.1500	.5092	.954	-1.4125	1.7125
LUNG	Tukey HSD	Control	9 ug MutFP	-5.3333E-02	.1791	.953	-.6030	.4963
			18 ug MutFP	-4.0000E-02	.1791	.973	-.5896	.5096
		9 ug MutFP	Control	5.333E-02	.1791	.953	-.4963	.6030
			18 ug MutFP	1.333E-02	.1791	.997	-.5363	.5630
		18 ug MutFP	Control	4.000E-02	.1791	.973	-.5096	.5896
			9 ug MutFP	-1.3333E-02	.1791	.997	-.5630	.5363
MUSCLE	Tukey HSD	Control	9 ug MutFP	-.2033	.5903	.937	-2.0147	1.6080
			18 ug MutFP	-.8403	.5903	.388	-2.6517	.9710
		9 ug MutFP	Control	.2033	.5903	.937	-1.6080	2.0147
			18 ug MutFP	-.6370	.5903	.560	-2.4483	1.1743
		18 ug MutFP	Control	.8403	.5903	.388	-.9710	2.6517
			9 ug MutFP	.6370	.5903	.560	-1.1743	2.4483
SPLEEN	Tukey HSD	Control	9 ug MutFP	.2700	.2044	.435	-.3571	.8971
			18 ug MutFP	-.2700	.2044	.435	-.8971	.3571
		9 ug MutFP	Control	-.2700	.2044	.435	-.8971	.3571
			18 ug MutFP	-.5400	.2044	.085	-1.1671	.0871
		18 ug MutFP	Control	.2700	.2044	.435	-.3571	.8971
			9 ug MutFP	.5400	.2044	.085	-.0871	1.1671

Experiment 3

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
BONE	Between Groups	.432	2	.216	1.051	.406
	Within Groups	1.234	6	.206		
	Total	1.666	8			
BLOOD	Between Groups	.187	2	9.341E-02	2.650	.150
	Within Groups	.212	6	3.526E-02		
	Total	.398	8			
KIDNEY	Between Groups	5.607E-02	2	2.803E-02	1.098	.392
	Within Groups	.153	6	2.552E-02		
	Total	.209	8			
LIVER	Between Groups	.242	2	.121	1.118	.387
	Within Groups	.648	6	.108		
	Total	.890	8			
LUNG	Between Groups	2.422E-03	2	1.211E-03	.017	.983
	Within Groups	.433	6	7.220E-02		
	Total	.436	8			
MUSCLE	Between Groups	.457	2	.229	1.361	.325
	Within Groups	1.007	6	.168		
	Total	1.464	8			
SPLEEN	Between Groups	.220	2	.110	2.465	.165
	Within Groups	.267	6	4.456E-02		
	Total	.487	8			

Post-hoc tests

Multiple Comparisons

Dependent Variable		(I) 24 hr-Route	(J) 24 hr-Route	Mean Diff (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
BONE	Tukey HSD	Control	9 ug MutFP	.1000	.3703	.961	-1.0361	1.2361
			18 ug MutFP	-.4067	.3703	.549	-1.5428	.7294
		9 ug MutFP	Control	-.1000	.3703	.961	-1.2361	1.0361
			18 ug MutFP	-.5067	.3703	.413	-1.6428	.6294
		18 ug MutFP	Control	.4067	.3703	.549	-.7294	1.5428
			9 ug MutFP	.5067	.3703	.413	-.6294	1.6428
BLOOD	Tukey HSD	Control	9 ug MutFP	-.2600	.1533	.281	-.7304	.2104
			18 ug MutFP	-.3367	.1533	.150	-.8071	.1337
		9 ug MutFP	Control	.2600	.1533	.281	-.2104	.7304
			18 ug MutFP	-7.6667E-02	.1533	.874	-.5471	.3937
		18 ug MutFP	Control	.3367	.1533	.150	-.1337	.8071
			9 ug MutFP	7.667E-02	.1533	.874	-.3937	.5471
KIDNEY	Tukey HSD	Control	9 ug MutFP	9.667E-02	.1304	.750	-.3036	.4969
			18 ug MutFP	-9.6667E-02	.1304	.750	-.4969	.3036
		9 ug MutFP	Control	-9.6667E-02	.1304	.750	-.4969	.3036
			18 ug MutFP	-.1933	.1304	.363	-.5936	.2069
		18 ug MutFP	Control	9.667E-02	.1304	.750	-.3036	.4969
			9 ug MutFP	.1933	.1304	.363	-.2069	.5936
LIVER	Tukey HSD	Control	9 ug MutFP	.2867	.2684	.566	-.5368	1.1101
			18 ug MutFP	-.1000	.2684	.927	-.9235	.7235
		9 ug MutFP	Control	-.2867	.2684	.566	-1.1101	.5368
			18 ug MutFP	-.3867	.2684	.381	-1.2101	.4368
		18 ug MutFP	Control	.1000	.2684	.927	-.7235	.9235
			9 ug MutFP	.3867	.2684	.381	-.4368	1.2101
LUNG	Tukey HSD	Control	9 ug MutFP	4.000E-02	.2194	.982	-.6332	.7132
			18 ug MutFP	1.667E-02	.2194	.997	-.6565	.6898
		9 ug MutFP	Control	-4.0000E-02	.2194	.982	-.7132	.6332
			18 ug MutFP	-2.3333E-02	.2194	.994	-.6965	.6498
		18 ug MutFP	Control	-1.6667E-02	.2194	.997	-.6898	.6565
			9 ug MutFP	2.333E-02	.2194	.994	-.6498	.6965
MUSCLE	Tukey HSD	Control	9 ug MutFP	-.4267	.3346	.457	-1.4532	.5998
			18 ug MutFP	-.5167	.3346	.337	-1.5432	.5098
		9 ug MutFP	Control	.4267	.3346	.457	-.5998	1.4532
			18 ug MutFP	-9.0000E-02	.3346	.961	-1.1165	.9365
		18 ug MutFP	Control	.5167	.3346	.337	-.5098	1.5432
			9 ug MutFP	9.000E-02	.3346	.961	-.9365	1.1165
SPLEEN	Tukey HSD	Control	9 ug MutFP	.1300	.1723	.742	-.3988	.6588
			18 ug MutFP	-.2467	.1723	.385	-.7755	.2821
		9 ug MutFP	Control	-.1300	.1723	.742	-.6588	.3988
			18 ug MutFP	-.3767	.1723	.152	-.9055	.1521
		18 ug MutFP	Control	.2467	.1723	.385	-.2821	.7755
			9 ug MutFP	.3767	.1723	.152	-.1521	.9055

Appendix 12

Chromatography results of blood samples collected in C57/BL mice bearing subcutaneous tumor for vascular permeability studies of the H520C9sFv-mrhIL-2

Experiment 1

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front)*100 (=Percentage decompose)
Saline	2146	245	10.25
9 μg of H520C9sFv-mrhIL-2 (i.v.)	1832	231	11.20
18 μg of H520C9sFv-mrhIL-2 (i.v.)	1675	245	12.76

Experiment 2

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front)*100 (=Percentage decompose)
Saline	2345	273	10.43
9 μg of H520C9sFv-mrhIL-2 (i.v.)	1632	241	12.87
18 μg of H520C9sFv-mrhIL-2 (i.v.)	1271	243	16.05

Experiment 3

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front)*100 (=Percentage decompose)
Saline	2864	335	10.47
9 μg of H520C9sFv-mrhIL-2 (i.v.)	2432	352	12.64
18 μg of H520C9sFv-mrhIL-2 (i.v.)	1428	178	11.08

Appendix 13

Independent Samples T-test results for vascular permeability studies of rhIL-2 in C57/BL mice bearing subcutaneous tumor

Experiment 1

(a) Mean % injected dose/gram

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Diff	Std. Error Diff	Lower	Upper
BONE	Equal variances assumed	.963	.382	-8.352	4	.001	-4.3933	.5260	-5.8538	-2.9329
	Equal variances not assumed			-8.352	3.428	.002	-4.3933	.5260	-5.9552	-2.8315
BLOOD	Equal variances assumed	.672	.458	-9.394	4	.001	-27.80	2.959	-36.01	-19.58
	Equal variances not assumed			-9.394	3.455	.001	-27.80	2.959	-36.55	-19.04
KIDNEY	Equal variances assumed	1.41	.301	-5.601	4	.005	-11.25	2.009	-16.83	-5.6751
	Equal variances not assumed			-5.601	3.293	.009	-11.25	2.009	-17.34	-5.1693
LIVER	Equal variances assumed	1.07	.360	-7.958	4	.001	-6.6000	.8293	-8.9025	-4.2975
	Equal variances not assumed			-7.958	3.426	.003	-6.6000	.8293	-9.0631	-4.1369
LUNG	Equal variances assumed	.046	.841	-12.7	4	.000	-16.99	1.338	-20.70	-13.27
	Equal variances not assumed			-12.7	3.983	.000	-16.99	1.338	-20.71	-13.26
MUSCLE	Equal variances assumed	2.37	.199	-7.163	4	.002	-2.8543	.3985	-3.9607	-1.7480
	Equal variances not assumed			-7.163	2.738	.008	-2.8543	.3985	-4.1940	-1.5146
SPLEEN	Equal variances assumed	.850	.409	-5.377	4	.006	-6.2033	1.154	-9.4067	-2.9999
	Equal variances not assumed			-5.377	3.185	.011	-6.2033	1.154	-9.7571	-2.6495
TUMOR	Equal variances assumed	.016	.905	-8.070	4	.001	-6.5533	.8121	-8.8081	-4.2986
	Equal variances not assumed			-8.070	3.993	.001	-6.5533	.8121	-8.8096	-4.2970

(b) Mean tumor:nontumor ratio

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Diff	Std. Error Diff	Lower	Upper
BONE	Equal variances assumed	4.366	.105	-.045	4	.966	-2.0000E-02	.4439	-1.2524	1.2124
	Equal variances not assumed			-.045	2.115	.968	-2.0000E-02	.4439	-1.8337	1.7937
BLOOD	Equal variances assumed	3.177	.149	.877	4	.430	6.333E-02	.0722	-.1371	.2638
	Equal variances not assumed			.877	2.491	.457	6.333E-02	.0722	-.1954	.3220
KIDNEY	Equal variances assumed	3.194	.148	.018	4	.987	3.333E-03	.1872	-.5164	.5231
	Equal variances not assumed			.018	2.195	.987	3.333E-03	.1872	-.7373	.7439
LIVER	Equal variances assumed	2.233	.209	-.365	4	.734	-7.3333E-02	.2009	-.6310	.4843
	Equal variances not assumed			-.365	2.188	.747	-7.3333E-02	.2009	-.8703	.7236
LUNG	Equal variances assumed	5.128	.086	1.996	4	.117	.2533	.1269	-1.E-01	.6057
	Equal variances not assumed			1.996	2.586	.154	.2533	.1269	-.1897	.6964
MUSCLE	Equal variances assumed	3.583	.131	2.416	4	.073	4.2433	1.7561	-.6324	9.1191
	Equal variances not assumed			2.416	2.039	.135	4.2433	1.7561	-3.1771	11.66
SPLEEN	Equal variances assumed	4.197	.110	1.084	4	.339	.1767	.1629	-.2757	.6290
	Equal variances not assumed			1.084	2.640	.367	.1767	.1629	-.3843	.7376

Experiment 2

(a) Mean % injected dose/gram

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Diff	Std. Error Diff	Lower	Upper
BONE	Equal variances assumed	7.425	.053	-5.995	4	.004	-5.1283	.8555	-7.5035	-2.7531
	Equal variances not assumed			-5.995	2.003	.027	-5.1283	.8555	-8.8039	-1.4527
BLOOD	Equal variances assumed	6.520	.063	-7.137	4	.002	-27.010	3.78	-37.52	-16.50
	Equal variances not assumed			-7.137	2.406	.011	-27.010	3.78	-40.92	-13.09
KIDNEY	Equal variances assumed	.268	.632	-9.736	4	.001	-11.837	1.22	-15.21	-8.4613
	Equal variances not assumed			-9.736	3.591	.001	-11.837	1.22	-15.37	-8.3044
LIVER	Equal variances assumed	2.922	.163	-8.384	4	.001	-7.0583	.8419	-9.3959	-4.7208
	Equal variances not assumed			-8.384	2.364	.008	-7.0583	.8419	-10.20	-3.9215
LUNG	Equal variances assumed	1.435	.297	-10.7	4	.000	-15.565	1.45	-19.59	-11.54
	Equal variances not assumed			-10.7	3.229	.001	-15.565	1.45	-20.00	-11.13
MUSCLE	Equal variances assumed	11.1	.029	-2.555	4	.063	-1.6392	.6416	-3.4205	.1421
	Equal variances not assumed			-2.555	2.096	.119	-1.6392	.6416	-4.2823	1.0039
SPLEEN	Equal variances assumed	2.092	.222	-6.387	4	.003	-5.4867	.8590	-7.8717	-3.1017
	Equal variances not assumed			-6.387	3.053	.007	-5.4867	.8590	-8.1936	-2.7797
TUMOR	Equal variances assumed	.637	.469	-8.166	4	.001	-6.6127	.8098	-8.8610	-4.3644
	Equal variances not assumed			-8.166	3.302	.003	-6.6127	.8098	-9.0614	-4.1639

(b) Mean tumor:nontumor ratio

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Diff	Std. Error Diff	Lower	Upper
BONE	Equal variances assumed	1.042	.365	.655	4	.548	.2200	.3361	-.7131	1.1531
	Equal variances not assumed			.655	2.784	.563	.2200	.3361	-.8982	1.3382
BLOOD	Equal variances assumed	.015	.910	.090	4	.933	3.333E-03	3.712E-02	-.0997	.1064
	Equal variances not assumed			.090	3.832	.933	3.333E-03	3.712E-02	-.1015	.1082
KIDNEY	Equal variances assumed	1.701	.262	-.323	4	.763	-3.0E-02	9.286E-02	-.2878	.2278
	Equal variances not assumed			-.323	2.560	.771	-3.0E-02	9.286E-02	-.3564	.2964
LIVER	Equal variances assumed	3.248	.146	.458	4	.671	.1100	.2401	-.5567	.7767
	Equal variances not assumed			.458	2.629	.682	.1100	.2401	-.7189	.9389
LUNG	Equal variances assumed	1.180	.338	3.429	4	.027	9.000E-02	2.625E-02	2.E-02	.1629
	Equal variances not assumed			3.429	2.991	.042	9.000E-02	2.625E-02	6.E-03	.1737
MUSCLE	Equal variances assumed	.294	.616	.057	4	.957	8.333E-02	1.4624	-3.9768	4.1435
	Equal variances not assumed			.057	3.479	.958	8.333E-02	1.4624	-4.2284	4.3951
SPLEEN	Equal variances assumed	11.520	.027	-2.3	4	.085	-.1500	6.583E-02	-.3328	.0328
	Equal variances not assumed			-2.3	2.031	.148	-.1500	6.583E-02	-.4291	.1291

Appendix 14

Chromatography results of blood samples collected in C57/BL mice bearing subcutaneous tumor for vascular permeability studies of the rhIL-2

Experiment 1

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front)*100 (=Percentage decompose)
Saline	1623	436	21.18
10 μg of IL-2 (i.v.)	1365	314	18.70

Experiment 2

Blood sample	Mean net count per minute		
	Origin	Front	Front/ (Origin+Front)*100 (=Percentage decompose)
Saline	1754	267	13.21
10 μg of IL-2 (i.v.)	2148	296	12.11