# Antibody Buffering: a Novel Mechanism of Drug Delivery

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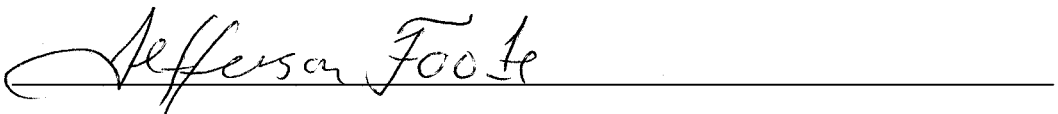

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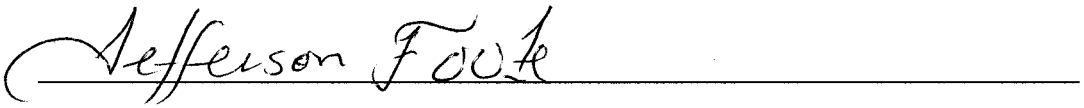

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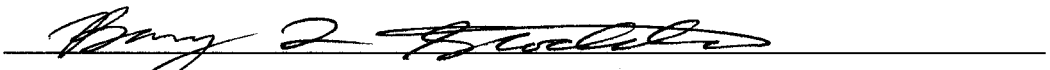

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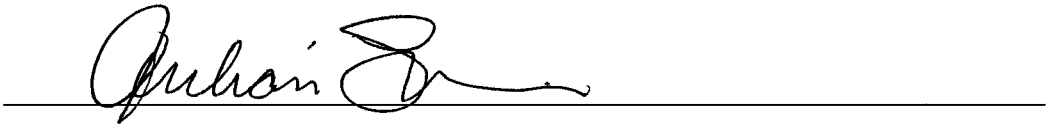

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## Abstract

Antibody Buffering: a Novel Mechanism of Drug Delivery

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Clearance results in a practical limit on drug action. Here we propose a means of slowing clearance, thereby extending drug lifetime *in vivo*, by "antibody buffering." In this process, a drug and an anti-drug antibody are co-administered. Most of the drug is bound to the antibody, preventing the drug from acting, but also preventing its elimination. A dynamic free drug pool is established by reversible dissociation from the antibody. The free drug is active and can be eliminated, but the free pool is constantly replenished by re-equilibration from the antibody-drug complex, giving a long effective lifetime. Here antibody buffering is explored experimentally using two model compounds: lysozyme, a protein, and 2-phenyl-oxazol-5-one-γ-amino butyrate (Ox), a small hapten. Lysozyme was significantly buffered in the plasma of the rat, and the amount of buffering obtained could be altered by changing the relative amounts of lysozyme and antibody administered. In addition to being able to buffer the concentration of a protein in the plasma, an antibody buffer can extend by an order of magnitude the plasma lifetime of a small molecule hapten, Ox, in rats. By employing a panel of anti-Ox antibodies, it was shown that the steady-state free Ox level depends on the molecular properties of the antibody used to buffer the Ox. Ox can also be buffered by an anti-Ox antibody within the cerebrospinal fluid compartment. In addition, the antibody can be recharged with drug *in vivo* to extend Ox lifetime without additional

antibody administration, making this technique even more suitable for possible clinical application.

## TABLE OF CONTENTS

## LIST OF ABBREVIATIONS

Ab ..... Antibody

ADCC ..... Antibody-dependent cellular cytotoxicity

AML ..... Acute myelogenous leukemia

ATBS ..... 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)

AUC ..... Area under the curve

BBB ..... Blood brain barrier

BSA ..... Bovine serum albumin

C ..... Centigrade

$^{14}C$ ..... Carbon-14

CD ..... Cluster of differentiation

CDC ..... Complement-dependent cytotoxicity

CL ..... Clearance

CLL ..... Chronic lymphocytic leukemia

CM ..... Cisterna magna

CNS ..... Central nervous system

C(0) ..... Initial concentration

cpm ..... Counts per minute

CSA ..... Chicken serum albumin

CSF ..... Cerebrospinal fluid

Da ..... Dalton

DEPC ..... Diethyl pyrocarbonate

DMF ..... N,N-dimethyl formamide

DNA ..... Deoxyribonucleic acid

dNTP ..... Deoxy nucleotide triphosphate

DTT ..... Dithiothreitol

EDC ..... 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

EDTA ..... Ethylenediaminetetraacetate

EGF ..... Epidermal growth factor

EGF-R ..... Epidermal growth factor receptor

ELISA ..... Enzyme-linked immunosorbant assay

Fab ..... Fragment, antigen-binding

FACS ..... Fluorescence activated cell sorting

Fc ..... Fragment, crystalline

FcRn ..... Neonatal Fc receptor

FDA ..... Food and drug administration

FSH ..... Follicle stimulating hormone

g ..... Gram

GABA ..... γ-aminobutyric acid

GH ..... Growth hormone

GHRF ..... Growth hormone releasing factor

$H_2O_2$ ..... Hydrogen peroxide

$H_3BO_3$ ..... Boric acid

HEPES ..... 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid

His ..... Histidine

HPLC ..... High pressure liquid chromatography

$^{125}I$ ..... Iodine-125

IGF-1 ..... Insulin-like growth factor-1

IgG ..... Immunoglobulin G

IL ..... Interleukin

$K_d$ ..... Dissociation constant

kDa ..... Kilodalton

L ..... Liter

lyso ..... Lysozyme

M ..... Molar

mAb ..... Monoclonal antibody

mCi ..... Milli Curie

MES ..... 2-(4-morpholino)-ethane sulfonic acid

μg ..... Microgram

mg ..... Milligram

$MgCl_2$ ..... Magnesium chloride

min ..... Minute

μl ..... Microliter

ml ..... Milliliter

mm ..... Millimeter

μM ..... Micromolar

mM ..... Millimolar

μmol ..... Micromole

mmol ..... Millimole

mRNA ..... Messenger ribonucleic acid

$N_2$ ..... Nitrogen

NaCl ..... Sodium chloride

$NaCNBH_3$ ..... Sodium cyanoborohydride

$NaHCO_3$ ..... Sodium bicarbonate

$NaH_2PO_4$ ..... Sodium phosphate

NaOH ..... Sodium hydroxide

ng ..... Nanogram

nm ..... Nanometer

nM ..... Nanomolar

nmol ..... Nanomole

NHL ..... Non-Hodgkin lymphoma

PAGE ..... Polyacrylamide gel electrophoresis

PBS ..... Phosphate buffered saline

PCR ..... Polymerase chain reaction

PE ..... Phycoerythrin

pg ..... Picogram

pmol ..... picomole

$OD_{600}$ ..... Optical density at 600 nanometers

Ox ..... 2-phenyl-oxazol-5-one-γ-amino butyrate

r ..... Recombinant

Req ..... Resonance units at equilibrium

RNA ..... Ribonucleic acid

RU ..... Resonance units

ScFv ..... Single chain Fv (fragment, variable)

SDS ..... Sodium dodecyl-sulfate

ss ..... Steady state

$t_{1/2}$ ..... Half-life

TAE ..... Tris-acetate-EDTA

TCA ..... Trichloroacetic acid

TNF-α ..... Tumor necrosis factor-α

Tris ..... Tris hydroxymethyl aminomethane

UV ..... Ultraviolet

Vd ..... Volume of distribution

VEGF ..... Vascular endothelial growth factor

v/v ..... Volume/volume

## LIST OF FIGURES

Figure Number

## LIST OF TABLES

Table Number

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DEDICATION

To Greg, Tyler, and Emmanuel-
I wish this research had been done sooner.

To the staff and children at Francis House Children's Hospice,
especially to Nathalie
who, in the short time I knew her,
provided me with enough love, laughter, and inspiration
to last a lifetime.

# Chapter 1:

# Introduction

## Drug concentration vs. effect

All drugs are cleared from the body. Clearance of a drug results in a major limit on its ability to act at a target site. It is nearly always a first order process, in which the amount cleared per unit time is proportional to the amount present. That is, half of the administered dose will be gone in one half-life no matter how much is initially given. Drug action, however, follows a different law from drug clearance because the biological effect of a drug is saturable. Drug action increases up to a saturating concentration, several times the receptor-drug dissociation constant ($K_d$), but beyond the saturation point, no additional positive effect can be attained no matter how much more drug is added to the system (Figure 1.1). Clinically, the desired effective concentration is achieved only transiently as drug is eliminated from the body. In order to keep drug at the concentration of desired biological effect for a longer period of time, clinical dosing typically yields an initial drug level far higher than the threshold for efficacy. Still, the drug level rapidly plummets because of first order law, and then is renewed in subsequent doses, giving a sawtooth profile of concentration vs. time (Figure 1.2). The result of the excess drug is proportionally faster drug clearance and often unwanted side-effects.

## Slow-release therapies

Chronic diseases and medical conditions often require the repeated administration of a drug. The drug concentration must remain within a therapeutic window for an extended period of time in order to have the desired effect on the illness. In the past, the

only method available to keep drug level within a therapeutic window for long periods of time was through continuous intravenous infusion. This type of therapy required constant monitoring of drug levels by health care professionals and, therefore, long hospital stays for the patient. There now exist several methods to control the rate of drug delivery without sawtooth patterned drug fluctuations, such as controlled release drug implants (Collins et al., 1997), drug-loaded liposomes (Lasic and Papahadjopoulos, 1995; Harrington et al., 2002), or slow infusion (Dash and Cudworth, 1998). Such controlled release formulations are beneficial in that they can decrease the total amount of drug necessary to treat a patient, reduce the number of doses of drug needed for therapeutic effect, and increase patient compliance with treatment (Dash and Cudworth, 1998). They can also help avoid the peaks in drug concentration that lead to toxic side-effects and the troughs in drug level that have no therapeutic benefit and can lead to drug resistance.

Controlled-release drug implants consist of various types of polymer and polymeric membrane that either enclose a compact drug core or have the drug dispersed homogenously throughout the polymer matrix (Danckwerts and Fassihi, 1991). They are surgically implanted near the desired site of action and slowly release drug over time according to the solubility and diffusion coefficients of the drug and the composition of the surrounding tissue and fluids (Dash and Cudworth, 1998). Some types of implant do not erode, thus require surgery to first place the implant and then to remove it when the drug has been released. The necessity of multiple surgeries reduces patient acceptance of this mechanism. Another problem with non-degradable implants with polymer membranes surrounding a drug core is the possibility of membrane rupture resulting in

"drug dumping" during therapy (Dash and Cudworth, 1998). Matrix formulations, in turn, have the additional problem of varied kinetic release of drug due to the volume fraction of drug in the matrix, i.e. the greater amount of dissolved drug, the greater the release from the system (Baker, 1987).

Biodegradable implants, while often preferred because the materials used to prepare the delivery system are eventually absorbed or excreted by the body, come with their own share of drawbacks as well. Polymer degradation kinetics must be taken into account and must remain at a constant rate in the face of alterations in body pH and temperature (Dash and Cudworth, 1998). The effect of decreasing surface area on drug release must also be considered, as well as the slow diffusion of drug from the polymer matrix (Graham, 1978). Also important in the use of controlled-release systems is the phenomenon of burst release, especially common in preparations with low molecular weight drugs (Huang and Brazel, 2001). For many controlled-release formulations, a large bolus of drug is released immediately upon placement in the body. This burst release may result in local or systemic toxicity, shortening of the drug release profile, and overall economic and therapeutic waste (Huang and Brazel, 2001). Despite the drawbacks, controlled-release implants are still being researched and some are being used clinically, such as the levonorgesterol sustained release birth control system (Norplant®) (Munro et al., 1996).

Liposomes are phospholipid spherules- a lipid bilayer membrane surrounding an aqueous core first described in 1965 (Bangham et al., 1965). The use of these vehicles to carry and slowly release drug has met with many difficulties. Drug entrapment

conditions must be optimized for each agent individually and their development is made more difficult by the different milieu in which the drug can localize: the aqueous core, the membrane itself, or the lipid-aqueous interface (Martin, 1997). The location of the entrapped agent within the liposome also affects the release kinetics of the drug. Thus, development of drug-loaded liposomes for clinical use is laborious and expensive (Harrington et al., 2002).

Recent advances, however, have brought the use of liposomes as slow-release vehicles for chemotherapy drugs to the forefront. It has been found that the previous problem of short circulation half-life due to opsonization of liposomes by plasma proteins can be circumvented by incorporating different glycolipids into the liposomal membrane or by pegylation (conjugating the lipids with polyethylene glycol) (Allen and Chonn, 1987; Zalipsky et al., 1999) . The extended residence time of these new liposomes in the blood allows them to extravasate into sites where the vasculature is leaky, such as within tumors (Gabizon et al., 1994). Although many of the initial clinical studies on the use of doxorubicin-loaded liposomes showed little or marginal improvement of patient response compared to free doxorubicin, there was a marked decrease in toxic side effects (cardiac, hepatic, renal, gastrointestinal) in the patients treated with the liposomal formulation (Cowens et al., 1993; Cheung et al., 1999). This is thought to be due to the reduced amount of free drug in the body compared to doxorubicin administered alone.

Despite the reduction of the usual dose-related toxicities seen in chemotherapy when liposomes were utilized, the alteration of the pharmacokinetics and biodistribution of a drug by encapsulation within liposomes caused novel toxicities to be noted. Due to

the accumulation of liposomal doxorubicin in the skin, painful swelling and erythema of the hands, feet, and sites of trauma can occur (Gordon et al., 1995; Lotem et al., 2000; Lyass et al., 2000). Mucocutaneous toxicity has often been the dose-limiting toxicity for liposomal drug use in many studies (Gordon et al., 2000). Still, the reduced extent of overall toxic side effects, occasional improved efficacy of treatment, and the ability to encapsulate different types of drugs (both water soluble and lipid soluble drugs can be encapsulated) encourage further research and use of these agents clinically.

Slow infusion can be performed either under the watchful eye of the health care professional in the hospital setting or with an implantable pump system. The implantable pump controls drug release through microtechnology and electronic systems (Dash and Cudworth, 1998). It releases drug through a pressure difference-generated gradient that yields bulk flow of a drug at a controllable rate (Ranade, 1990). Some types of implantable pumps allow for external control of the rate of drug delivery, but they are much more expensive than the kinds that require removal of the system to change the rate (Dash and Cudworth, 1998). These slow infusion delivery systems avoid the problem of burst release and steer clear of the novel toxicities associated with administration of an exogenous substance to the body, such as a pegylated liposome or degradable polymer, but they can be quite costly and, again, patients may be discouraged by the thought of multiple operations to implant, remove, or change the drug delivery rate of the device. Still, slow infusion, as well as controlled-release implants and liposomes, offers the patient a minimization of side effects, increased efficacy of treatment, and local delivery

of drugs at a constant rate compared to standard therapies with a sawtooth drug concentration profile.

**Current use of antibody therapy**

Even as far back as the early 1900s, the use of antibodies as a "magic bullet" for cancer therapy was postulated and researched (Ehrlich, 1908; Ehrlich, 1957). Little progress was made with the heterogeneous polyclonal antibodies available at the time as these preparations contained antibodies of multiple affinities and were likely reactive towards several antigens (Bigner et al., 1995). Large-scale production of a homogenous, monoclonal antibody of defined specificity was not possible until Kohler and Milstein developed the mouse hybridoma method (Kohler and Milstein, 1975). These murine antibodies still had the problems of eliciting an immune response in humans against the foreign mouse immunoglobulin, decreased ability to induce an immune response from effector cells, and short half-life in circulation (Carter, 2001). Attempts at circumventing these issues include the discovery that chimeric (Reff et al., 1994) and humanized (Jones et al., 1986; Riechmann et al., 1988) antibodies are less immunogenic than murine antibodies and interact better with human effector cells and complement. Currently, it is possible to create germline humanized (Tan et al., 2002), or even human (Griffiths et al., 1993; Lonberg et al., 1994; Fishwild et al., 1996), antibodies to further reduce immunogenic potential. These improved antibodies now enable repeated antibody administrations, offer improved capacity to recruit cytotoxic cells and complement, and have increased stability in the circulatory system. Now, phage display (Schier et al.,

1996), ribosome display (Hanes et al., 2000), and yeast display (Boder and Wittrup, 2000) methods allow for rapid selection of antibodies with desired affinities.

Currently, the standard paradigm for antibody therapy directly targets the tumor cells. The antibody is designed to react with a specific tumor antigen that is found on most tumor cells, but only rarely on normal cells. The antibodies react with their complementary target *in vivo*, causing neutralization, clearance, or destruction of the target. Accrual of IgG within tumors is facilitated by its long half-life (compared to smaller antibody fragments such as scFv and Fab), which is due both to its size and to its recycling by the neonatal Fc receptor, FcRn (Carter, 2001). One method of tumor cell destruction is via the effector functions of human IgG1 and IgG3 isotypes: antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (Figure 1.3). ADCC is triggered by the Fc region of the antibody bound to a tumor cell reacting with the Fcγ receptors on immune effector cells (neutrophils, macrophages, and natural killer cells) (Clynes et al., 2000). These cells are thus induced to act against the tumor cell, destroying it. CDC requires the complement component C1q binding to the Fc regions of IgG that are bound to tumor cells (Idusogie et al., 2000). The complement cascade is activated, resulting in the membrane attack complex inducing cell death. Tumor cell killing may also be aided by the blocking of essential survival signals from acting at a receptor site or by induction of apoptotic signals (Figure 1.3). An example is cetuximab, a recently FDA-approved antibody directed against the epidermal growth factor receptor (EGF-R) (Table 1.2). EGF-R is overexpressed in many epithelial cancers. Binding of cetuximab to the EGF-R prevents the binding of EGF to the tumor cell, and

the resulting lack of EGF-R stimulation inhibits tumor growth and leads to apoptosis (Harris, 2004).

Often a naked anticancer antibody must be used in combination with chemotherapy or external beam radiation to achieve maximum therapeutic benefit (Carter, 2001). There has been much research into other options that would avoid this need. Some of the clinical strategies to increase the antitumor activity of naked antibody therapy include enhancement of effector functions with bispecific antibodies, arming of the antibody with toxins or radionuclides (Figure 1.3), or pre-targeting for prodrugs or radionuclides (Blattman and Greenberg, 2004). The use of bispecific antibodies involves the simultaneous binding of two different epitopes, in this case a tumor antigen and a receptor on immune effector cells such as CD3. The antibody thus recruits the effector cell to target a tumor cell for destruction that it would have otherwise ignored, increasing the efficacy of the immune response in the destruction of tumor cells. A problem with the use of bispecific antibodies, however, is that widespread activation of these effector cells can lead to systemic cytokine release and resulting serious side effects (van Spriel et al., 2000). An additional difficulty with their clinical use is the cost and difficulty of producing these antibodies in sufficient quantities (Carter, 2001).

Several FDA-approved anticancer antibodies are either conjugated with a small molecule toxin or with a radionuclide (Table 1.2). For antibodies conjugated with small molecule toxins, efficient antibody internalization is necessary for effective therapy. Also, since standard chemotherapeutic agents require high doses to the tumor site, and a relatively small percent of antibodies localize to the tumor site in humans (Sedalacek,

1992), drugs with high molar toxicities are needed. Calicheamicin, which is conjugated to an anti-CD33 antibody in the chemotherapeutic agent, gemtuzumab ozogamicin, is an example of such a potent poison. CD33 is an antigen found on the surface of acute myelogenous leukemia cells, but not on stem cells or non-lymphoid tissue (Griffin et al., 1984). Binding of the antibody to the CD33 antigen on tumor cells allows for the release of calicheamicin following antibody internalization, resulting in the formation of diradicals, double strand DNA breaks, and cell death (Scheinberg et al., 1991). Thus, antibody-mediated delivery allows for the use of a drug that would have been too toxic to use systemically on its own.

The use of antibodies conjugated to radionuclides is beneficial as bystander cells are also affected by the radiation (Harris, 2004). Many tumor cells may have lost their tumor antigen that the antibody recognizes, but as long as they have neighboring cells that have retained the antigen, they will be killed as well. Radioimmunoconjugates can have severe toxicities, such as severe hematologic toxicity (Knox et al., 1996). Still, two radiolabeled antibodies have been approved by the FDA for use against Non-Hodgkin's Lymphoma: Zevalin (a $^{90}$yttrium conjugate of anti-CD20) (Witzig et al., 2002) and Bexxar (a $^{131}$I conjugate of anti-CD20) (Kaminski et al., 2001).

An additional putative use of antibodies involves pre-targeting (Carter, 2001). This strategy uses either an anti-tumor antibody conjugated to a prodrug or an antibody-streptavidin conjugate that is injected into the body. Excess antibody is washed away and another injection of an enzyme that activates the prodrug or a radionuclide-biotin complex is introduced. This would result in activation of the drug or binding of the

radionuclide specifically at the tumor site, sparing other, non-cancerous areas of the body. Use of this type of therapy has been thwarted by immunogenicity of the enzyme, often derived from a non-human source, or the potential for unwanted prodrug activation by endogenous enzymes (Sharma et al., 1992). Also, the immunogenicity of streptavidin would preclude successive treatment cycles (Chinol et al., 1998).

Antibodies are often more effective in the treatment of non-solid tumors (lymphomas and leukemias), micrometastases, and residual disease (tumor remaining post-surgery and/or chemotherapy and/or radiotherapy) than solid tumors. The large size of the IgG molecule impairs solid tumor penetration, and uptake by Fc receptors on reticulo-endothelial cells decreases half-life (Carter, 2001). Antibody binding to antigen-positive non-tumor tissue is also a problem (Tolcher et al., 1999; Ajani et al., 2000). A further drawback to the use of antibodies for treatment is their size. Antibodies are big (greater than 70 kDa), while small molecules less than 0.5 kDa, like chemotherapy drugs, circulate faster and penetrate tumor tissue more deeply (Carter, 2001). In addition, a further issue regarding the use of antibodies for cancer therapy is their cost (Harris, 2004). Despite many advances in their mass production, generation of enough of these agents for therapeutic benefit is expensive. Reducing the antibody amount necessary for patient treatment would greatly improve the ability to test these drugs in clinical trials and to use them therapeutically.

**The Concept of Antibody Buffering**

The main impetus behind explorations of antibody use for cancer therapy has been the specific targeting properties of these molecules, yet there exists another property

of antibodies that can be exploited for therapeutic use. A century of *in vitro* experimentation has shown that antibodies act as perfect exemplars of the Law of Mass Action and the Law of Chemical Equilibrium (Pecht, 1982). According to these laws, entities in a reaction are in dynamic equilibrium. The reaction proceeds according to the equilibrium constant, which is determined by the stoichiometric coefficients and concentrations of the reactants and products in solution. The reaction can proceed in either direction, with the forward reaction being favored when the equilibrium constant is greater than one.

Accordingly, antibodies release and bind antigen and antibody-antigen mixtures reliably partition between bound and free forms according to the dissociation constant of the antibody. These properties can be likened to the basic principle behind a simple pH buffer (Figure 1.4A). The theory of a pH buffer is derived most simply as a reversible binary combination, expressed by the Henderson-Hasselbalch equation:

$$pH = pK + \log_{10} [A^-]/[HA] \qquad [1]$$

As free acid is lost from the system, it is replaced through re-equilibration of the pH buffer. If acid is added, re-equilibration yields more HA form, reducing the number of free hydrogen ions in the solution. In this manner, the pH will remain within a specific range until the buffer has been used up.

Returning to the case of an antibody, a regime can be envisioned in which a ligand-specific antibody would behave like a buffering agent with respect to the ligand (Figure 1.4B). Our particular interest is in whether a drug-specific antibody can maintain a long-lived pool of uncomplexed drug within the plasma of a test subject. The equation

governing buffer-like behavior of such a system is a simple substitution of the Henderson-Hasselbalch equation:

$$p[drug] = pK_A + \log_{10}([Ab]/[Ab\text{-}drug]) \qquad [2]$$

(Here, and in subsequent equations, Ab refers to a site concentration rather than to the concentration of divalent molecules.)

When dealing with a test subject, ligand will be lost from the system. If the ligand is buffered by its specific antibody, as free ligand is lost, it will be replaced through re-equilibration of the antibody-ligand buffer. Free ligand will continue to be replaced until the antibody-ligand complex is depleted. If the antibody-ligand complex concentration is much greater than the antibody $K_d$, then most of the ligand will remain in the bound form. Thus, the complex forms a reservoir of free drug. When this concept is translated into the clinical situation where the ligand is a small molecule drug, the use of an antibody buffer could be used to deliver a stable, bioavailable concentration of a drug that is normally rapidly eliminated from the body. It is, however, important to remember that antibodies are also eliminated from the body, albeit at a much slower rate than many drugs. When choosing a drug-antibody combination that could be used therapeutically, it is necessary to select a drug that is eliminated at a much faster rate than its specific antibody.

It should also be noted that an antigen with only one combining site for an antibody is incapable of crosslinking antibodies, and thus would not be subject to immune clearance mechanisms (Mannik et al., 1971). If antibody binding does not trigger clearance, then the antibody acts as a carrier molecule for the drug. This should

be the case for low molecular weight drugs. Also, a monoclonal antibody would be unlikely to induce rapid clearance of a moderate size antigen as a monoclonal antibody can bind only one type of antigenic site. It is therefore less likely to be crosslinked by the antigen as polyclonal antibodies would be.

**Examples of Antibody Buffering**

It has long been known that serum albumin binds many therapeutic drugs, thereby reducing the amount of free drug that is able to act at the target site (Olsen et al., 2004). Albumin binding can prolong the residence time of the drug in the plasma, necessitating the need for tailoring the dose of drug administered to correlate with the known amount of protein binding of the drug *in vivo*. In fact, a key determinant of the circulating half-life of a drug is the extent to which it binds proteins in the blood (Rehlaender and Cho, 1998). In addition, while it is protein-bound, the drug molecule is usually unavailable to its site of action as well as to the excretion mechanisms of the liver and kidney (Gibaldi, 1991). While an altered drug pharmacokinetic profile due to protein binding is usually associated with albumin, the use of antibodies in this capacity has the potential for applications in pharmaceutical therapies. In fact, drug-antibody interactions leading to altered pharmacokinetics and bioactivity have been noted in various studies involving insulin, addictive drugs, peptides, hormones, and cytokines (Table 1.2).

Antibody binding has been shown to increase insulin half-life in insulin-dependent diabetics. Many patients will develop antibodies, some with high titers, to exogenous insulin beginning within 2 to 4 months of insulin therapy initiation and reaching a plateau at about 6 months as long as therapy continues (Reeves and Kelly,

1982). These insulin-binding antibodies were shown to prolong the pharmacological and biological half-lives of insulin following intravenous infusion compared to insulin administered to patients without anti-insulin antibodies (Gray et al., 1985; Van Haeften et al., 1986). It has been proposed that moderate concentrations of insulin antibodies may be beneficial for diabetics without endogenous insulin secretion because this prolonged pharmacokinetic profile of the drug may improve diabetes control (Gray et al., 1985), but several factors have contributed to their lack of use for any therapeutic benefit. Measurement of anti-insulin antibody titer in patients is complicated by the fact that some diabetics possess insulin autoantibodies that are believed to be involved in the pathogenesis of their disease (Palmer et al., 1983). Also, since their initial discovery, the development of anti-insulin antibodies in diabetic patients has decreased due to improvements in the purity of insulin preparations and because of a change in the species of the insulin used from animal-derived to human insulin. It has been shown that those who do still develop antibodies may suffer from high postprandial blood glucose levels and have an increased risk for delayed hypoglycemia if the presence of antibodies is not taken into account (Van Haeften, 1989), and this has added fuel to the debate of the benefit vs. detriment of anti-insulin antibodies. Still, insulin antibodies have never been exploited for any putative therapeutic value despite the initial thoughts that such a clinical use might be a possibility.

Antibodies have been shown to affect the biodistribution of small drugs to keep them in a specific compartment of the body. For example, nicotine is a drug that may pass freely through the blood brain barrier. This accounts for many of the physiological

and behavioral effects of the drug. It has been shown, however, that immunization of rats with a nicotine-conjugate vaccine, or passive transfer of anti-nicotine antibodies from another animal, will alter nicotine distribution within the body (Hieda et al., 1997). The anti-nicotine antibodies bind subsequently administered nicotine and sequester it within the plasma, allowing only passage of the relatively small amount of free drug into the brain. Similar results have been noted with antibodies against cocaine and heroin (Bonese et al., 1974; Killian et al., 1978; Fox et al., 1996). These not only suggest a potential role for immunization in the treatment of drug abuse (Pentel et al., 2000), but show that antibodies can sequester drug within body compartments.

Work in other laboratories has demonstrated enhanced bioactivity and in some cases prolonged lifetime of therapeutic peptides, hormones, and cytokines co-administered with their specific antibodies. Antibodies can profoundly influence the pharmacokinetics and pharmacodynamics of these types of molecules. For example, treatment with recombinant (r) hirudins, polypeptides of 65 or 66 amino acids used for their anticoagulant properties to treat venous thromboembolism, acute myocardial infarction, and unstable angina pectoris, leads to the formation of anti-hirudin antibodies in up to 74% of patients treated with r-hirudin for more than 5 days (Huhle et al., 1999). The development of anti-hirudin antibodies correlated with increased half-life and decreased total plasma clearance of r-hirudin. A decrease in total anticoagulant activity of the drug was also noted, indicating that antibody binding of r-hirudin reduces its biologic activity, which is likely because there is less free drug available to act at the target sites (Liebe et al., 2002).

Anti-hormone antibodies have been described against follicle stimulating hormone (Glencross et al., 1993; Ferasin et al., 1997), bovine growth hormone (Pell et al., 1989), human growth hormone (Holder et al., 1985), growth hormone releasing factor (Pell and James, 1995), and insulin-like growth factor-1 (IGF-1) (Hill and Pell, 1998). Complexation of the antibody with the hormone resulted in increased bioactivity of the hormone in animal models for each study. Although reduced clearance of the hormone was noted in many cases, the groups involved in the studies did not postulate a role for the antibody in acting as a reservoir of hormone, and thus potentiating hormone action until the study of Hill and Pell in 1998 of the anti-IGF-1 antibody. As yet, there has been little motion toward harnessing this anti-hormone antibody effect for any clinical use, although increased milk production in sheep has been demonstrated (Pell et al., 1989) and the development of a growth-promotion vaccine using anti-growth hormone antibodies has been proposed (Bomford and Aston, 1990).

Cytokine activity *in vivo* can be prolonged by addition of an anti-cytokine antibody. Many researchers were surprised by this finding, expecting the anti-cytokine antibody to neutralize cytokine activity (Mihara et al., 1991), but instead discovered that the cytokine bioactivity was increased by 2 to 30-fold *in vivo*. Similar results were found with interleukin (IL)-2 (Courtney et al., 1994; Sato et al., 1994), IL-3 (Jones and Ziltener, 1993), IL-4 and IL-7 (Finkelman et al., 1993), IL-6 (Mihara et al., 1991; Martens et al., 1993; May et al., 1993), and tumor necrosis factor-$\alpha$ (TNF-$\alpha$) (Rathjen et al., 1992). For example, Jones and Ziltener found that antibody complexation of IL-3 extended its $\beta$-half-life from 10 to 72.5 minutes. They also noted a seven-fold increase in biological

effect of the IL-3. Martens et al. found that mice immunized against IL-6 showed a 10-30-fold increase of IL-6 biologic activity over the IL-6 activity in non-immunized mice. Mihara et al. described a putative explanation for this phenomenon that is similar to our proposed concept of antibody buffering: (Note that a biologic activity of IL-6 is the production of a signal causing the terminal differentiation of activated B-cells into antibody-producing cells.)

> "...MH166 [the anti-IL-6 antibody] may have protected hIL-6 [human IL-6] from degradation by proteolytic enzymes in the blood stream. The complex was preserved longer in the circulating blood because mouse immunoglobulin has a long half-life... Free hIL-6 was gradually released from the preformed complex and was bound to the receptor on B cells to differentiate into immunoglobulin-producing plasma cells."

The use of cytokines for therapeutic purposes is often hindered by the short half-life of these molecules in circulation, as well as the likelihood of toxic side effects when they are administered systemically by infusion or at high single dosages (Finkelman et al., 1993). Studies on both IL-2 (Courtney et al., 1994) and TNF-α (Rathjen et al., 1992) showed that cytokine inhibition of tumor growth in mice was significantly increased in mice with anti-cytokine antibodies over mice without the antibodies. In addition, when TNF-α was used in conjunction with an anti-TNF-α antibody in a mouse tumor model, tumor regression was not only enhanced, but a reduction in toxic side effects was noted as well (Rathjen et al., 1992). Interestingly, the development of anti-cytokine antibodies occurs with high frequency in patients on cytokine therapy (Bendtzen et al., 1990). These

or exogenously prepared anti-cytokine antibodies have potential for use as a means to increase the magnitude and duration of cytokine effects *in vivo*.

**Why Antibody Buffering Over Other Treatment Regimes?**

- As previous studies have shown, antibody binding can have a profound effect of the biodistribution and pharmacokinetics of a drug.
- Controlled-release drug implants require at least one surgery for placement and often a second for removal of the implant. Antibody buffering would use a simple injection or infusion.
- Implants with polymer membranes have the risk of membrane rupture and resulting drug dumping. There is no membrane involved with antibody therapy.
- Matrix implants vary the release of drug according to the volume fraction of drug in the matrix. Antibodies release drug according to established kinetic constants.
- Preparation of a biodegradable implant must take into consideration polymer degradation kinetics, the effect of decreasing surface area on drug release, and slow diffusion of drug from the polymer matrix. All of these aspects make preparation of the system laborious and costly. Antibody buffering depends on the affinity of the antibody for the drug, which is conveniently manipulated through display technologies.
- In both controlled-release implants and slow infusions, a gradient is established between the point of entry of the unstable drug and its site of action. Antibodies equilibrate throughout and release drug uniformly within the body compartment.

- In a drug-loaded liposome, there are three different areas in which the drug can localize, each leading to different release kinetics. For an antibody buffer, the drug is either free or antibody-bound, making kinetic determinations much simpler.
- Antibodies are naturally-occurring entities within the body. As a buffer, they are being used solely to modulate the concentration of a specific drug, not targeting a self-antigen. It is less likely that they will lead to unusual, dose-limiting toxicities such as those experienced due to liposome accumulation in the skin.
- Like liposomes, antibodies can be used to deliver water-insoluble drugs since a small drug bound to an antibody will be sequestered in the binding pocket, and thus soluble with the antibody.
- Antibodies can be used to sequester a normally freely-migrating drug within a body compartment such as the cerebrospinal fluid or the plasma.
- Since antibodies both release and *rebind* drug, they have the ability to be re-charged with successive readministrations of drug without antibody. Liposomes do not have this capability.
- Current anti-cancer therapies utilizing antibodies are most often successful with non-solid tumors due to the size of the immunoglobulin. Small drugs penetrate tumor tissue more deeply, so an antibody-buffered drug may have increased efficacy in solid tumors compared to antibodies conjugated to a small molecule toxin.

- Current anticancer antibodies conjugated to toxins require antibody internalization for the toxin to be active, and thus only kill tumor cells bearing the specific tumor antigen targeted by the antibody. They do not affect neighboring tumor cells that may have lost antigen expression. Delivery of a drug via antibody buffering does not require binding and internalization so all tumor cells are affected by the toxin.
- Antibody therapy is expensive. The ability to reuse an antibody by recharging it with drug would reduce therapy cost.

**Rationale for this body of work**

Despite much chemical and *in vivo* evidence that antibodies can be used to modulate drug concentration for therapeutic benefit, there has yet to be any clinical application of the antibody buffering model to small molecule, peptide, or protein drugs. Here antibody buffering behavior is explored experimentally using drug proxies to develop quantitative tools to guide design of antibody buffers for bona fide drugs.

Table 1.1 FDA-approved anti-cancer antibodies

| Antibody | Antigen | Antibody type | Cancer | Method of Action |
|---|---|---|---|---|
| Rituximab | CD20 | Chimeric | NHL | Naked Ab |
| Trastuzumab | HER2/NEU | Humanized | Breast | Naked Ab, blocks signal transduction |
| Gemtuzumab ozogamicin | CD33 | Humanized | AML | Conjugated to toxin (calicheamicin) |
| Alemtuzumab | CD52 | Humanized | B-cell CLL | Naked Ab |
| $^{90}$Y-ibritumomab tiuexetan | CD20 | Murine | NHL | Radioimmuno-conjugate |
| $^{131}$I-tositumomab | CD20 | Murine | NHL | Radioimmuno-conjugate |
| Cetuximab | EGF-R | Chimeric | Metastatic colorectal | Naked Ab, blocks signal transduction |
| Bevacizumab | VEGF | Humanized | Metastatic colorectal | Naked Ab, prevents angiogenic signaling |

NHL= Non-Hodgkin Lymphoma, Naked Ab = not conjugated to a toxin or radionuclide, AML = acute myelogenous leukemia, CLL = chronic lymphocytic leukemia, EGF-R = epidermal growth factor receptor, VEGF = vascular endothelial growth factor.

Table 1.2 Known examples of antibody-altered pharmacokinetic drug profiles.

| Drug type | Examples | Interesting findings when antibodies present |
|---|---|---|
| Proteins/peptides | Insulin | Increased $t_{1/2}$, increased AUC, prolonged hypoglycemia |
| | r-hirudin | Increased $t_{1/2}$, decreased plasma clearance, decreased biologic activity |
| Small drugs | Nicotine<br>Heroin<br>Cocaine | Antibody-binding sequesters drugs in the plasma, affects biodistribution |
| Hormones | FSH | 3-4 fold increase in uterine weight in dwarf mice |
| | Bovine GH | Increased milk production in lactating sheep |
| | Human GH | Increased growth in dwarf mice |
| | GHRF | |
| | IGF-1 | Noted reduced ligand degradation, slower clearance |
| Cytokines | IL-2 | Enhanced tumor regression |
| | IL-3 | |
| | IL-4 | |
| | IL-6 | Bioactivity increased 10-30-fold |
| | IL-7 | |
| | TNFα | Reduction in side effects of therapy |

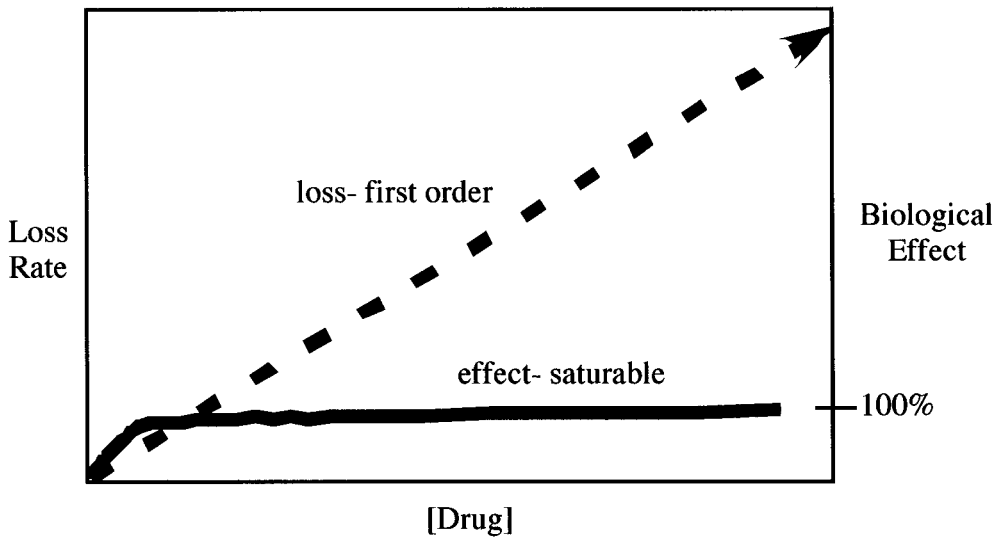


Figure 1.1 Drug loss vs. effect vs. concentration. Drug loss, or clearance, follows first order kinetics while the concentration of drug needed to achieve maximum therapeutic benefit is saturable. This concentration of maximum effect is achieved only transiently because of the continual drug loss.

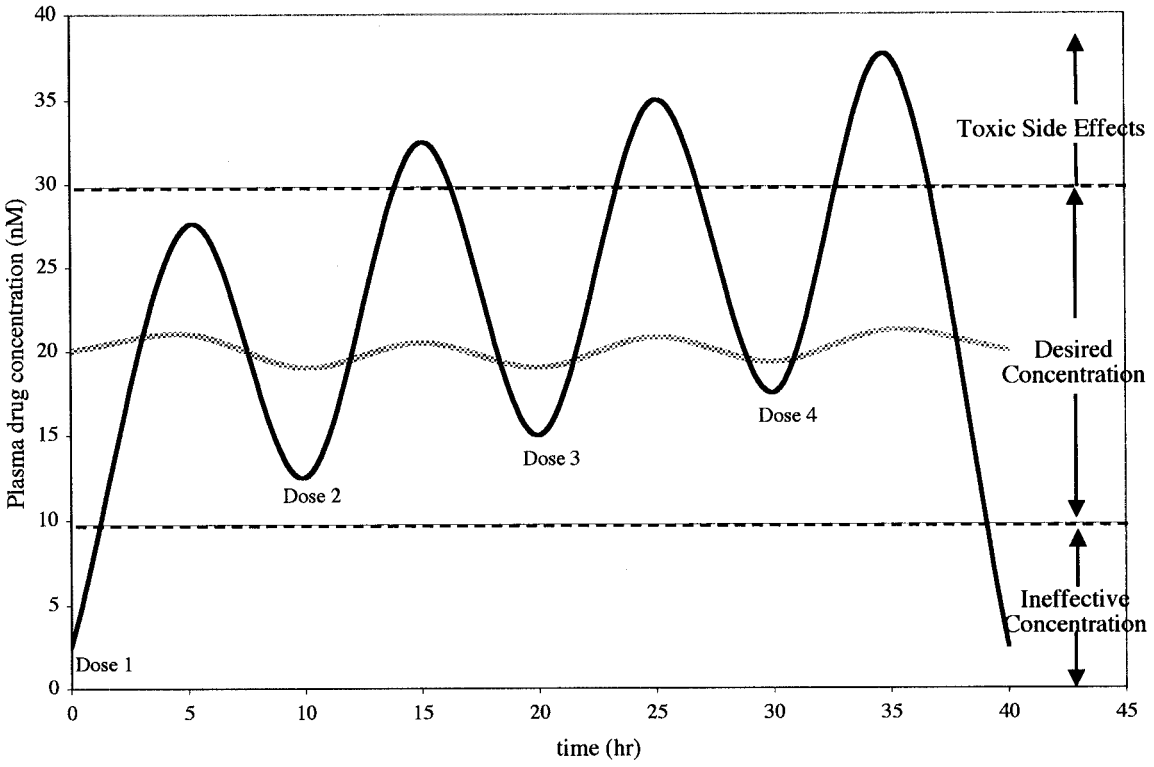


Figure 1.2 Sawtooth profile of a hypothetical drug. After each dose, the plasma drug concentration increases up to a peak. As drug is eliminated, plasma drug concentration decreases until the next dose when the concentration again rises. Since the drug was not completely eliminated from the plasma between doses, the second dose causes the concentration to rise above the desired therapeutic concentration into the range where toxic side effects are evident. Since drug is again eliminated, the concentration dips once more into the desired range before the third dose is administered. This cycle continues until drug administrations cease. The blue (solid) line shows the drug concentration. The pink (cross-hatched) line shows the plasma drug concentration that would be seen with a slow, constant release mechanism of drug administration.

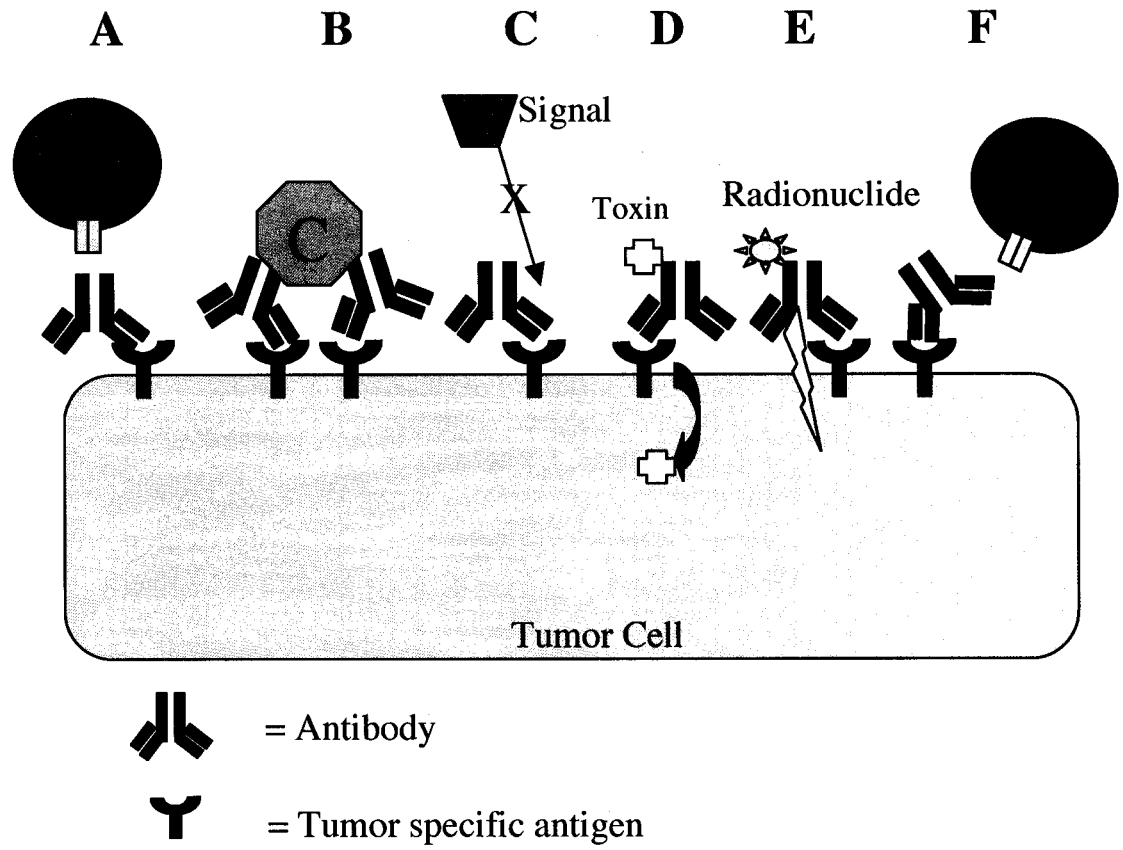


Figure 1.3 Mechanism of action of anti-cancer antibodies. There are several mechanisms currently employed to use antibodies to kill tumor cells, all involving an antibody that is specific for a tumor cell antigen. A. Antibodies bind and induce effector cell activation through Fc-Fcγ interaction. B. Antibody aggregates can also activate the complement cascade through their Fc portions. C. Antibody binding to a receptor may block survival signals from being able to bind at that site, leading to cell death. D. Antibody conjugated to a toxin is internalized and the toxin is able to kill the tumor cell. E. A radioimmunoconjugate binds to the tumor cell surface and kills the cell by DNA damage due to β particle emission. F. A bispecific antibody binds a tumor cell antigen and recruits effector cells to the location because the other arm of the antibody is specific for an effector cell receptor. E= effector cell; C= complement.

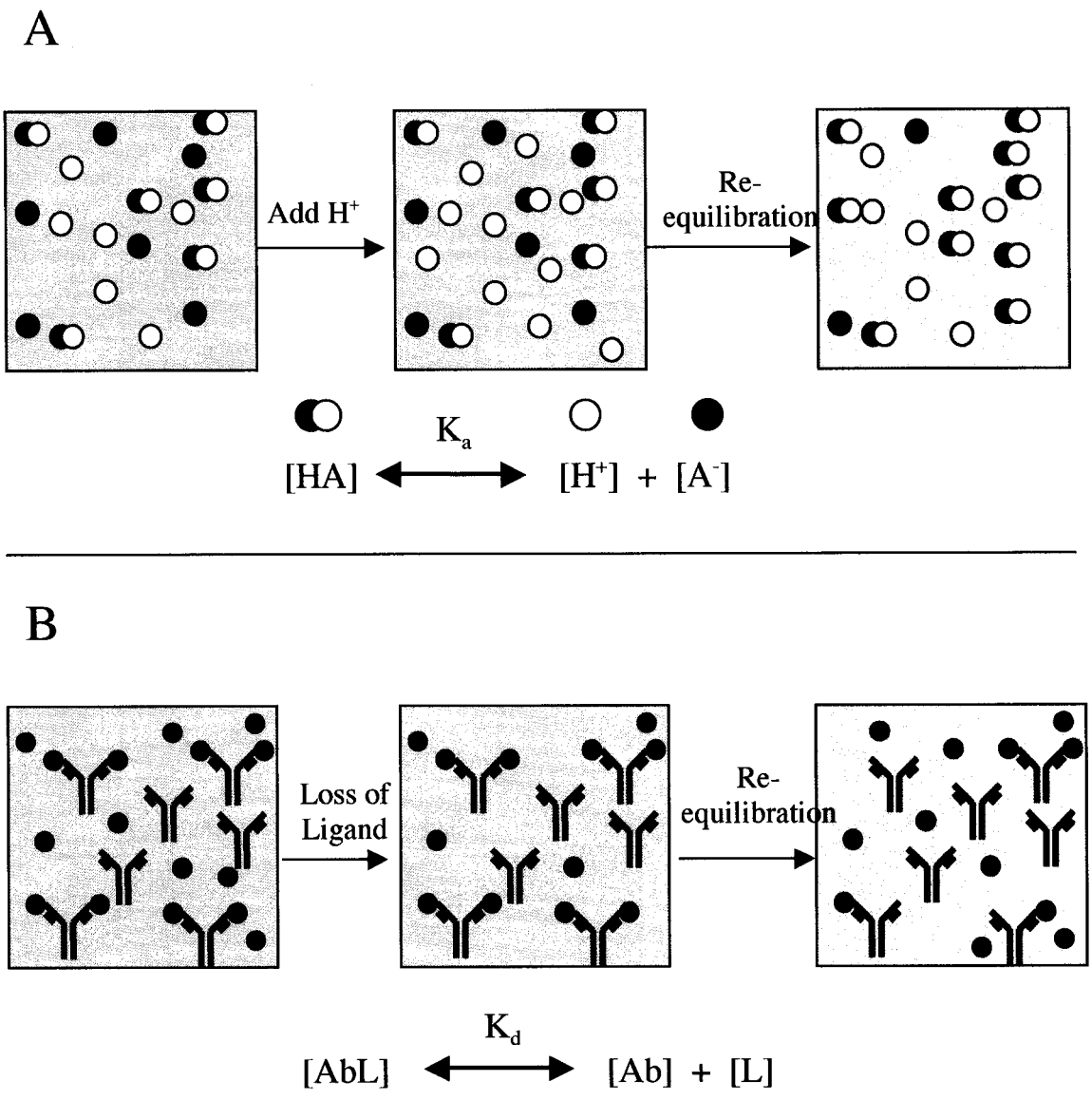


Figure 1.4 Analogy of a pH buffer to an antibody buffer. A. When acid ($H^+$) is added to a pH buffer, re-equilibration occurs with the HA buffer to bind up the excess acid. The pH, therefore, remains relatively constant until the buffer is depleted. B. An antibody buffer works in much the same fashion. In the case depicted here, when free ligand is eliminated from the system, re-equilibration occurs with the antibody buffer to release more free ligand into the system. Thus, the free ligand concentration remains relatively constant until the antibody-ligand complex is depleted of ligand.

# Chapter 2:

## Antibody Buffering of Systemically-administered Lysozyme

**Summary**

Lysozyme is an antimicrobial hydrolytic enzyme found naturally in the saliva and other bodily secretions. Although its use as an antibacterial agent for the treatment of *Listeria monocytogenes* and *Streptococcus pneumoniae* has been researched (Jado et al., 2003; Were et al., 2004), lysozyme also has an important use in the testing of controlled release systems including liposomes (Were et al., 2004), implantable systems (Kamiya and Klibanov, 2003; Singh and Singh, 2004), and a cross-linked gelatin that can be used to treat prosthetic valve endocarditis (Srinivas and Rao, 2001). Here we employ lysozyme as a model for therapeutic peptides and proteins in a different slow release therapy, antibody buffering. Lysozyme was radiolabeled with $^{14}C$ and infused with or without the murine anti-lysozyme antibody D1.3 into the jugular vein of a rat. Varying the concentrations of antibody and lysozyme was performed, as was separating the antibody and lysozyme into different infusions, to help elucidate the effect of these changes on the buffering ability of D1.3. The concentration of lysozyme in the plasma over time was followed through blood sampling at various timepoints. The results of these experiments indicate that buffering of lysozyme with an equimolar amount of D1.3 more than doubles the plasma half-life of lysozyme and addition of more antibody buffer prolongs the half-life to an even greater extent. Also, D1.3 was able to bind lysozyme that was infused several minutes after the D1.3 administration, indicating that D1.3 can

be charged with drug *in vivo*. Collectively, these results show that antibody buffering of proteins is possible and may have potential to be developed further for clinical use.

## Introduction

Lysozyme, also known as muramidase, is found naturally in mucous membranes and in secretions such as saliva, tears, and respiratory secretions. Lysozyme is known to exert wide antimicrobial activity against a number of bacterial, viral, and fungal pathogens *in vitro* (Anil and Samaranayake, 2002). It is a cationic enzyme that hydrolyzes cell wall polysaccharides, making bacteria more vulnerable to lysis due to hypo-osmotic conditions such as those found in saliva. As a human salivary protein, it is involved in the first line of defense of the oral cavity (Van Nieuw Amerongen et al., 2004). Therefore, lysozyme, alone or in combinations with other proteins, has been incorporated as a preservative in foods and pharmaceuticals as well as in oral health care products such as dentifrices, mouth-rinses, moisturizing gels, and chewing gums to restore saliva's antimicrobial capacity in patients with dry mouth (Tenovuo, 2002). New formulations containing antimicrobial peptides, such as lysozyme, have been tested for their applicability in the treatment of oral inflammatory processes such as gingivitis and periodontitis.

Administration of lysozyme in the treatment of antibiotic resistant *Streptococcus pneumoniae* has also been studied in a murine animal model with promising results (Jado et al., 2003). A specific lysozyme type, Cpl-1, greatly reduces *S. pneumoniae* titers *in vivo* and can even be used as a topical nasal treatment against colonization with *S. pneumoniae*, although it only has a serum half-life of 20.5 minutes (Loeffler et al., 2003).

However, it is known that high systemic doses of lysozyme lead to deleterious effects in systemic blood pressure, glomerular filtration, and renal blood flow (Cojocel et al., 1984; Haas et al., 2002). To extend the serum half-life and reduce the overall systemic concentration, the use of lysozyme encapsulated in liposomes to increase its activity against *Listeria monocytogenes* has been attempted. An increase in activity was seen, although it was strain dependent, and indicated that a slow-release formulation was more effective at bacterial killing than lysozyme alone (Were et al., 2004).

In addition to being used in liposomal studies, lysozyme has been adopted as a model protein for studying many implantable controlled release systems (Malzert et al., 2002; Kamiya and Klibanov, 2003; Singh and Singh, 2004). One such study reported the utilization of lysozyme in the reduction of prosthetic valve endocarditis (Srinivas and Rao, 2001). Prosthetic valve endocarditis in an infrequent but serious complication of cardiac valve replacement. It is caused by adherence of bacteria to the wall of the prosthetic valve or to the tissue at the site of implantation. As antimicrobial proteins can be used to treat this condition, the use of these proteins in a slow release system to prevent endocarditis development has been suggested (Kuijpers et al., 1998). Research into this topic has mainly involved lysozyme in a cross-linked gelatin placed into the Dacron sewing ring of the prosthetic valve with promising results in animal models (Kuijpers et al., 1998; Kuijpers et al., 2000; Srinivas and Rao, 2001).

The use of lysozyme has also been researched as a low molecular weight protein carrier for smaller compounds such as captopril and naproxen to the kidney. The lysozyme conjugate directs these compounds to the proximal tubule of the kidney where

the pro-drug is activated and the drug may have its desired action (Kok et al., 1998; Haas et al., 2002). These studies have lead to the elucidation of the mechanisms involved in lysozyme elimination in the rat. Lysozyme is rapidly cleared from the circulation with high accumulation in the kidney where the protein is endocytosed by the proximal tubular cells (Haas et al., 2002). The lysozyme molecule then migrates via endosomes to the proteolytically active lysosomes where it is degraded into small peptides and single amino acids (Hysing et al., 1990).

Here we use lysozyme in a different type of slow-release mechanism. Hen egg lysozyme was chosen as a model for antibody buffering of a systemically-administered therapeutic protein because of the availability of a murine anti-lysozyme antibody, D1.3, (Amit et al., 1986; Bhat et al., 1990) and because the pharmacokinetics of lysozyme in the rat have been elucidated (Franssen et al., 1991).

## Methods

### Preparation of $^{14}C$ lysozyme

Lysozyme was radioactively labeled with $^{14}C$ using reductive methylation, a procedure adapted from Habeeb (Habeeb, 1983). 50μCi $^{14}C$-formaldehyde was obtained from New England Nuclear (specific activity 40-60 mCi/mmol). The glass ampoule was placed on dry ice to condense the formaldehyde gas. A labeling mix consisting of 0.7 μmol lysozyme (from a 20 mg/ml solution in phosphate buffer), 3.5 μmol $NaCNBH_3$ (from a 0.1 M $NaCNBH_3$ solution, freshly prepared), and 0.1 M phosphate buffer ($NaH_2PO_4$, pH 8.0 with NaOH) to bring the total volume to 1 ml. It was added to the ampoule and the reaction was allowed to proceed for 30 minutes, mixing every 8

minutes. Then, 0.5 ml of 0.05 M borate buffer ($H_3BO_3$, pH 8.0) was added and 1 µl aliquots were withdrawn for scintillation counting for quality control purposes. The reaction mixture was dialyzed against 2 x 500 ml borate buffer, pH 7.0, followed by 5 x 500 ml of sterile PBS. Aliquots of the dialysis buffer were scintillation counted to determine when dialysis was complete. SDS-PAGE was performed to assess protein purity, and the gel was subjected to autoradiography to verify that the $^{14}C$ tag was localized only to the lysozyme band. The protein concentration was determined by UV spectroscopy. 1 µl aliquots were withdrawn for scintillation counting to determine the specific activity of the lysozyme. The antibody solution was filter-sterilized and stored at 4°C under $N_2$.

**Antibody production**

The murine anti-lysozyme hybridoma, D1.3 (Amit et al., 1986; Bhat et al., 1990), was grown in an oscillating bubble roller bottle (Pannell and Milstein, 1992). The resulting monoclonal antibody was purified from spent culture supernatant by passage over a lysozyme-Sepharose affinity column. Fractions were eluted with diethylamine (Fisher Scientific) into tubes containing 150 µl 1M Tris, pH 7.4, and dialyzed against Protein A buffer (3M NaCl, 0.1M glycine, pH 8.9). The dialysate was then passed over a Protein A-Sepharose CL-4B (Pharmacia) column. Fractions were eluted with a gradient of sodium citrate to citric acid and collected into tubes containing 100 µl 3M Tris, pH 8.8. They were then dialyzed against PBS (25 mM $NaH_2PO_4$/125 mM NaCl, pH 7.0). SDS-PAGE was performed to verify purity. Protein concentration was determined by UV spectroscopy, using an extinction coefficient calculated from sequence (Perkins,

1986). The antibody solution was filter-sterilized and stored at 4°C under $N_2$. The $K_d$ of D1.3 has been determined as 3.7 nM at 20°C (Foote and Winter, 1992).

An ELISA was performed with the D1.3 antibody to show that its ability to bind lysozyme is retained when using the $^{14}C$ conjugate. Wells in a 96-well plate were coated with 100 μg/ml lysozyme or $^{14}C$-lysozyme in a carbonate buffer (50 mM $NaHCO_3$, pH 9.6) and allowed to incubate at 37°C for 1 hour. The plate was washed with PBS, blocked with reconstituted milk, washed again with PBS, and then incubated for an hour with 20 ng/ml D1.3 in PBS with 10 mg/ml BSA added. Control wells were incubated with an antibody that does not bind lysozyme, NQ10/2.22. Following the incubation, the plate was again washed and incubated for one hour with the secondary antibody, peroxidase conjugated goat anti-mouse IgG, Fc fragment specific (Jackson ImmunoResearch). The plate was read with a kinetic microplate reader (Molecular Devices) after adding color mix (1 mM ATBS and 4 mM $H_2O_2$ in a 50 mM sodium citrate/ 50 mM citric acid buffer).

**Antibody buffering experiments**

Rats (300 g Sprague-Dawley males) implanted with a jugular vein cannula were obtained from Zivic Laboratories. The rats were freely moving throughout the experiment and allowed food and water. Samples were prepared for infusion consisting of 3 nmol $^{14}C$-lysozyme with or without 3 nmol D1.3 antibody. The volume was brought up to 1 ml using sterile normal saline and the sample was incubated at 37°C prior to use. The sample was infused through the jugular cannula over 5 minutes using a Harvard Apparatus infusion pump. Following the infusion, the cannula was flushed with 0.2 ml

of heparinized saline (250 units/ml) to reduce the risk of clotting. The end of the infusion was designated as time 0. 0.5 ml blood samples were withdrawn from the cannula at various timepoints. The blood was immediately centrifuged to separate out the plasma. 0.1 ml plasma was mixed with 5 ml scintillation fluid and counted. Another 0.1 ml was used in bound vs. free experiments described below. The cellular pellet was mixed with scintillation fluid and counted as well.

**Separation of free and bound lysozyme**

Following blood sample centrifugation to separate out the plasma, 0.1 ml of plasma was loaded onto a 1.5 ml Protein A-Sepharose mini-column. The column was washed with 3 ml of PBS followed by 3 ml of 100 mM citric acid in 0.5 ml increments. Experiments with D1.3 and lysozyme using both SDS-PAGE and UV spectroscopy indicated that these buffer amounts were sufficient to separate free and bound lysozyme. The PBS (free lysozyme) and citric acid (antibody-bound lysozyme) fractions were collected separately for each timepoint. TCA precipitation was performed on the fractions. The protein precipitate was washed with acetone and the precipitate re-dissolved in PBS (although not all of precipitate was able to be dissolved). The liquid and remaining pellet were transferred into 4 ml scintillation fluid for counting.

**Antibody Buffering with Varied Amounts of Antibody**

Sprague-Dawley rats implanted with a jugular cannula were infused with 3 nmol $^{14}$C-lysozyme and either 3 or 6 nmol of D1.3 antibody. The volume infused was brought up to 1 ml using sterile normal saline. The samples were prepared, infused, and

timepoints were taken and analyzed as described above. Free and bound separation was not performed.

**Antibody Buffering with Varied Amounts of Lysozyme**

Sprague-Dawley rats implanted with a jugular cannula were infused with 3 nmol of D1.3 antibody and either 3 nmol or 30 nmol of $^{14}$C-lysozyme. The samples were prepared, infused, and timepoints were taken and analyzed as described above.

**Double Infusion Experiment**

Two samples were prepared for infusion. The first sample, consisting of 3 nmol D1.3 antibody diluted to 0.5 ml with sterile normal saline, was infused over 4 minutes using a Harvard Apparatus infusion pump. The rat was allowed to move freely in its cage for 10 minutes to allow the antibody time to spread within the circulation. Then the second sample, consisting of 3 nmol $^{14}$C-lysozyme made up to 0.5 ml using sterile normal saline, was infused over 4 minutes. The end of the second infusion was designated as time 0. Blood samples were taken and analyzed as described above.

## Results

**$^{14}$C-lysozyme**

In the previous studies of lysozyme pharmacokinetics in the rat, the amount of lysozyme in the plasma was quantified by an enzymatic assay (Franssen et al., 1991). We were unable to employ this assay in our studies because D1.3 blocks the enzymatic activity of lysozyme (Kenett et al., 1987). Hence, lysozyme was radiolabeled with $^{14}$C so that its amount in plasma samples could be measured and followed through scintillation counting. Reductive methylation of lysozyme with $^{14}$C-formaldehyde yielded a $^{14}$C-

lysozyme conjugate with a specific activity of 7.4 x $10^7$ cpm/mg. Thus, the lysozyme was sufficiently labeled to be detected above background level in the rat plasma. The purity of the $^{14}$C-lysozyme was determined using SDS-PAGE (Figure 2.1A), and the radioactive signal that localized to the lysozyme band was shown using autoradiography (Figure 2.1B). The ability of D1.3 to bind the $^{14}$C-lysozyme conjugate effectively was determined by ELISA (Figure 2.1C). D1.3 lysozyme binding was retained with the $^{14}$C conjugate.

**Pharmacokinetics of Lysozyme in Rat Plasma**

To determine the pharmacokinetic profile of lysozyme using our radioactive method of lysozyme detection, $^{14}$C-lysozyme was infused through a jugular cannula into the bloodstream of a rat. Lysozyme elimination was followed through blood sampling at various time intervals spanning 4 hours. The plasma was collected after centrifugation, and subjected to scintillation counting. Lysozyme was found to have a plasma β-half life of 16.4 minutes (Figure 2.2). Each rat in these and in the following studies was used only once to limit the possibility of an immune response to either the lysozyme or the antibody.

**Pharmacokinetics of Antibody-buffered Lysozyme**

The murine anti-lysozyme antibody D1.3, previously kinetically-characterized (Foote and Winter, 1992), was employed as an antibody buffer for $^{14}$C-lysozyme. In order for D1.3 to provide an effective buffer, its complexation with lysozyme should block elimination of that ligand. To show that this is the case, lysozyme was administered with an equimolar amount of D1.3 through a rat's jugular cannula.

Administration with D1.3 was shown to significantly prolong the residence time of lysozyme in the rat plasma, extending the β-half life to 42.8 minutes (Figure 2.2). Although the initial concentration of lysozyme appears higher when it is administered on its own, that amount falls rapidly compared to lysozyme buffered by D1.3, indicating that D1.3 complexation is indeed decreasing the elimination rate.

Following plasma separation, the cellular pellet was counted in both this and the previous study. Little radioactivity was found in the pellet, indicating that the cellular pellet is not a significant repository for lysozyme or lysozyme-antibody complexes (data not shown).

**Pharmacokinetics of Free Lysozyme**

Following centrifugation to separate out the plasma for scintillation counting to determine the total lysozyme concentration, an aliquot of plasma was run over a Protein-A Sepharose mini-column to separate bound and free forms of lysozyme. Free lysozyme was eluted with a PBS wash while lysozyme bound to D1.3 was eluted with citric acid. The resulting large volumes of eluate precluded direct scintillation counting so TCA precipitation was performed to concentrate the proteins. The resulting protein pellet was resistant to redissolving despite attempts with tissue solubilizers and other reagents. The exact free lysozyme concentration was therefore difficult to ascertain. Nevertheless, analysis of the lysozyme alone and lysozyme-D1.3 eluates prepared identically indicates a difference in the free lysozyme levels in these samples.

For D1.3 to be a true buffer for lysozyme concentration, it must act as a reservoir of that ligand, maintaining the free lysozyme concentration at a fairly constant level. The

analysis demonstrated that a free pool of lysozyme does exist when it is administered with D1.3, and that this free pool remains at a more constant level during the course of the experiment than does free lysozyme administered alone (Figure 2.3). Therefore, the presence of D1.3 appears to provide a buffering effect.

**The Effect of Varying Lysozyme and Antibody Concentration on the Buffering Capacity of D1.3**

To determine the effect of varying the antibody concentration on the buffering of total lysozyme in the rat plasma, $^{14}$C-lysozyme was administered with either equimolar or two times the molar amount of D1.3 into the rat plasma. Blood was sampled at various timepoints over the course of four hours. Plasma was again separated through centrifugation and total lysozyme concentration was determined via scintillation counting. Administration of lysozyme with two times the molar amount of D1.3 prolonged the β-half life of lysozyme to 61.3 minutes (Figure 2.4). This was significantly different than with equimolar D1.3 and lysozyme (β-half life of 42.8 minutes). Therefore, addition of more antibody to the buffer system appears to prolong ligand half life to a greater extent.

Experiments were also performed to determine the effect of increasing the lysozyme concentration on the total lysozyme concentration over time in the rat plasma. D1.3 was infused into the jugular cannula of the rat with either equimolar or ten times the molar amount of $^{14}$C-lysozyme. Timepoints were taken and plasma analyzed for total lysozyme concentration as described above. Although the initial lysozyme concentration in the rats given ten times as much lysozyme was very high, it rapidly plummeted to a

level about twice as high as that of the rats given the lower lysozyme concentration (Figure 2.5). This indicates that while significantly increasing the amount of ligand has a large effect on the initial total lysozyme concentration, it yields only a modest increase in the ligand amount at later timepoints. The bound and free lysozyme concentrations were not determined due to difficulties with TCA precipitation, but it is very likely that the initial free ligand concentration is much higher in the rat administered ten times as much lysozyme as D1.3. This initial excess free ligand is subject to the more rapid elimination mechanisms of the kidney and rapidly leaves the rat plasma. The initial bound amounts of drug in the two groups of rats are likely fairly comparable, leading to the similar later profiles of drug elimination.

**Lysozyme administered following D1.3 equilibration within the plasma**

An additional experiment was performed where D1.3 was infused alone into the rat plasma. Ten minutes following this infusion, to allow for the spread of the antibody within the plasma, an equimolar amount of lysozyme was administered through the jugular cannula of the rat. The resulting pharmacokinetics were followed through blood sampling as described above. The initial concentration of lysozyme was high, but it quickly equilibrated to a level very similar to that attained when the D1.3 and lysozyme are administered together (Figure 2.6). This indicates that D1.3 is retained well in the circulation and its ability to bind lysozyme effectively is not altered *in vivo*.

**Discussion**

This initial study of antibody buffering using lysozyme as a model for therapeutic peptides and proteins indicates the potential for an antibody to buffer the

concentration of a protein *in vivo*, and for this type of delivery mechanism to be further developed for therapeutic use. It is shown that the anti-lysozyme antibody D1.3 is able to extend the lifetime of total lysozyme in rat plasma. Antibody complexation did not appear to potentiate removal of lysozyme from the system. This is expected since D1.3 is a monoclonal antibody directed toward one epitope on the lysozyme protein. It would be unlikely to form large complexes that would be targeted for destruction by the immune system. Also, complexation with D1.3 is likely to inhibit removal of the protein via its normal route of elimination, the kidney, because of the size of the antibody-ligand complex. This fulfills one of the criteria for a successful buffering system- that antibody binding hinders the normal elimination mechanism of the drug. That D1.3 is indeed the factor involved in lysozyme half-life prolongation is supported by the experiment where an increased amount of D1.3 was administered with $^{14}$C-lysozyme. Increasing the amount of antibody buffer increased the half life of the lysozyme, presumably because more lysozyme was antibody-bound and not subject to normal elimination mechanisms.

In addition to antibody binding slowing the elimination of total lysozyme from the plasma, it also stabilizes the free lysozyme concentration. This finding is critical to the success of an antibody buffer. There must be a free, bioavailable pool of drug able to act at the therapeutic target site. Despite problems in determining the free concentration of lysozyme in the plasma, analysis of Figure 2.3 indicates that not only does a pool of free ligand exist in the plasma, but during the timepoints where the concentration appears relatively constant (20-60 minutes), the amount of free ligand (approximately 2.0 nM) is close to the $K_d$ of D1.3 (3.7 nM). The amount of bioavailable drug appears to be dictated

by the $K_d$ of the buffering antibody. This finding is also supported by the results of the experiment where the amount of antibody administered was constant, but the lysozyme concentration was varied. The lysozyme concentration in the higher initial dosage eventually decreased to a level only moderately higher than that following the lower dosage. The free lysozyme concentrations were not determined in these studies, but it is likely that they would have shown a similar result- that the free concentration in both studies would have been similar following the initial timepoints.

Finally, like most exogenous antibodies, D1.3 has a finite lifetime in the rat plasma. Although its elimination from the plasma is slower than that of lysozyme, it is eliminated. Since antibodies are metabolized by the liver, it seemed likely that a portion of the antibody is eliminated during the first pass through that organ, and if that antibody has lysozyme bound to it, that lysozyme is eliminated as well. Allowing the antibody to first spread within the circulatory system prior to binding lysozyme may keep antibody-bound lysozyme from being eliminated during that first pass. The lysozyme administered following this equilibration period, however, may still have an initially large drop in plasma concentration due to its distribution half life as well as the faster elimination rate of unbound lysozyme, but this would soon stabilize as D1.3 binds free lysozyme in the plasma. We hoped to determine whether the effect of this putative first pass on antibody-lysozyme elimination outweighed the faster elimination of free lysozyme in the plasma when the antibody and lysozyme were administered separately. When this experiment was attempted in the rat model, the initially high lysozyme concentration quickly decreased, but it equilibrated to a level very similar to when D1.3 and lysozyme are

administered together (Figure 2.5). Thus, it appears that either the first pass effect is insignificant or that these processes cancel each other out so that it does not matter whether the infusion is done with both components together or separately. More importantly, however, this experiment shows that an antibody can bind a ligand even following its initial administration, i.e. that the antibody and ligand do not have to be administered together for buffering to occur *in vivo*. This finding leads to the proposal that an antibody buffer can actually be re-charged with fresh drug *in vivo*, that it can be re-used as a buffer as long as it remains in the circulation.

The preceding studies show the modulation of the pharmacokinetic profile of a model protein, lysozyme, by its specific antibody in the rat plasma. Lysozyme has been used as a model protein for the study of the efficacy of many different slow-release therapies (Kuijpers et al., 1998; Singh and Singh, 2004; Were et al., 2004). Here it is shown that lysozyme can also be used to test the proposed slow-release therapy of antibody buffering. An antibody can keep a free, bioavailable pool of lysozyme at a low concentration while binding, and thus inactivating, the remaining enzyme. It is likely that this low concentration is dictated by antibody $K_d$. The buffered protein is unable to act at a target site, but it is also not subject to its normal mechanism of elimination. Free lysozyme is eliminated, but is replaced through re-equilibration of the antibody-enzyme complex. This allows the pool of free lysozyme to remain in the plasma longer than if elimination was acting on the entire amount of lysozyme in the body.

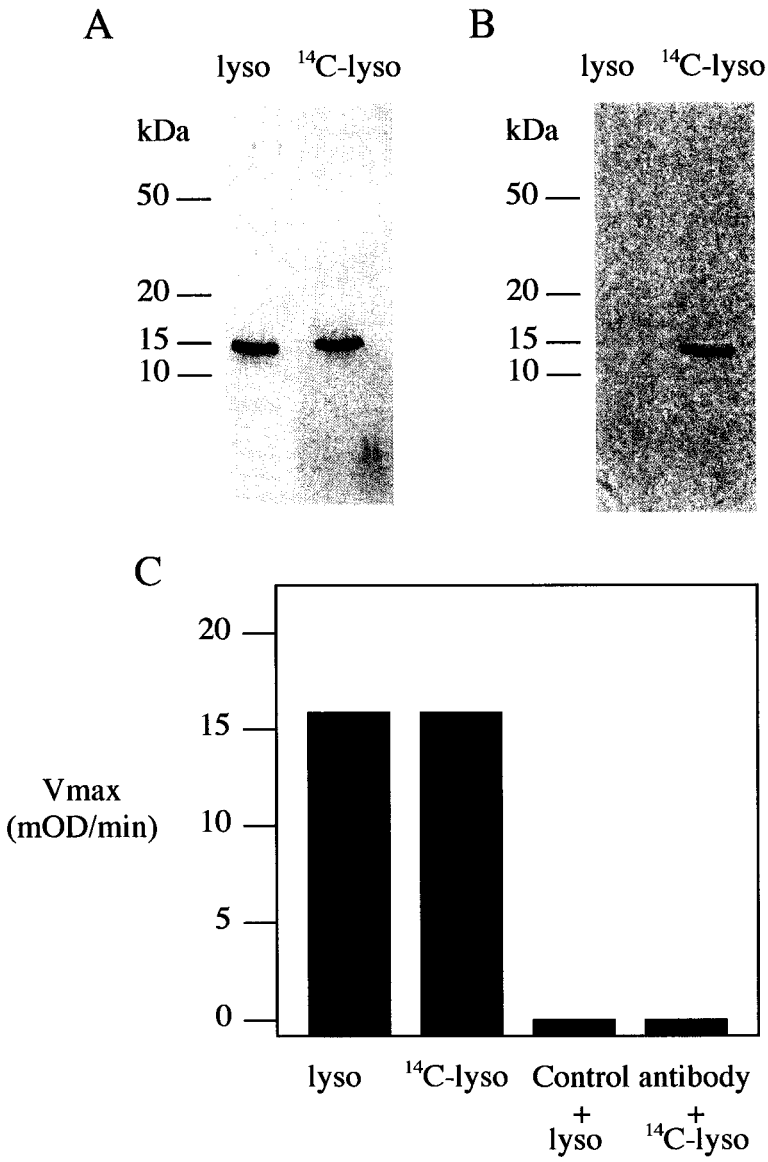


Figure 2.1 Preparation and analysis of the $^{14}$C-lysozyme conjugate. A. The purity of the $^{14}$C-lysozyme was assessed by SDS-PAGE and compared to unmodified lysozyme. B. The gel was then subjected to autoradiography to show that the radiolabel localizes to the $^{14}$C-lysozyme band only. C. An ELISA was performed to show that D1.3 retains its activity toward the $^{14}$C conjugate as compared to unconjugated lysozyme. An ELISA using a control (no activity towards lysozyme) antibody was also performed with both lysozyme and the $^{14}$C conjugate.

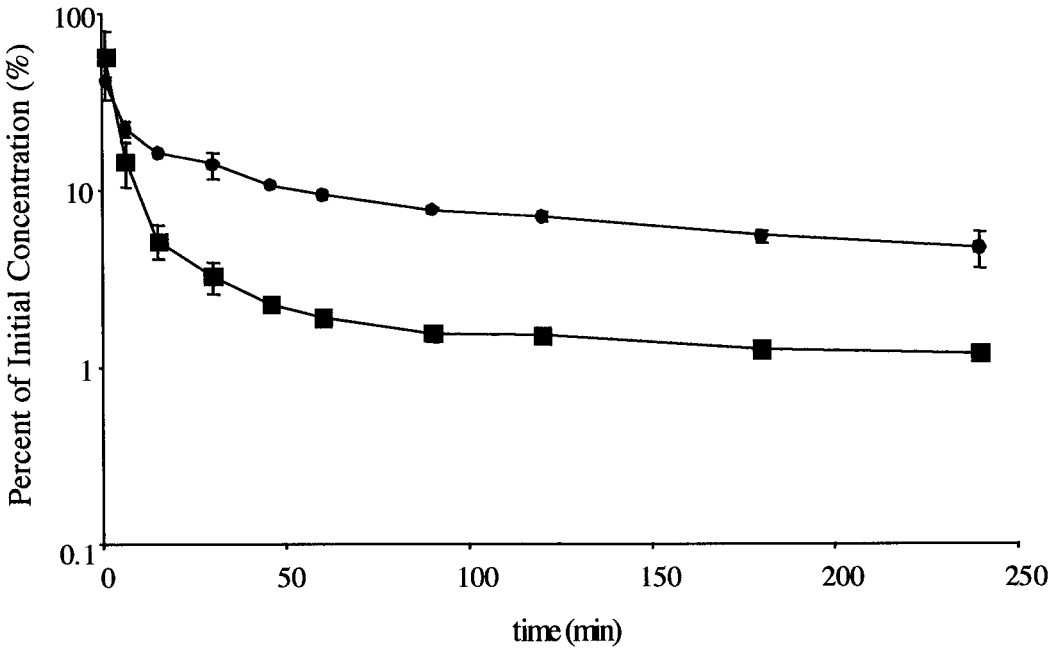


Figure 2.2 The effect of an antibody buffer on the pharmacokinetics of lysozyme. $^{14}C$-lysozyme administered alone (squares) or with an antibody buffer (circles) are compared. The concentration of lysozyme administered alone declines rapidly with an elimination half-life of 16.4 minutes. The half-life of lysozyme infused with the D1.3 antibody was extended to 42.8 minutes. Concentration of lysozyme is expressed as a percentage of initial concentration.

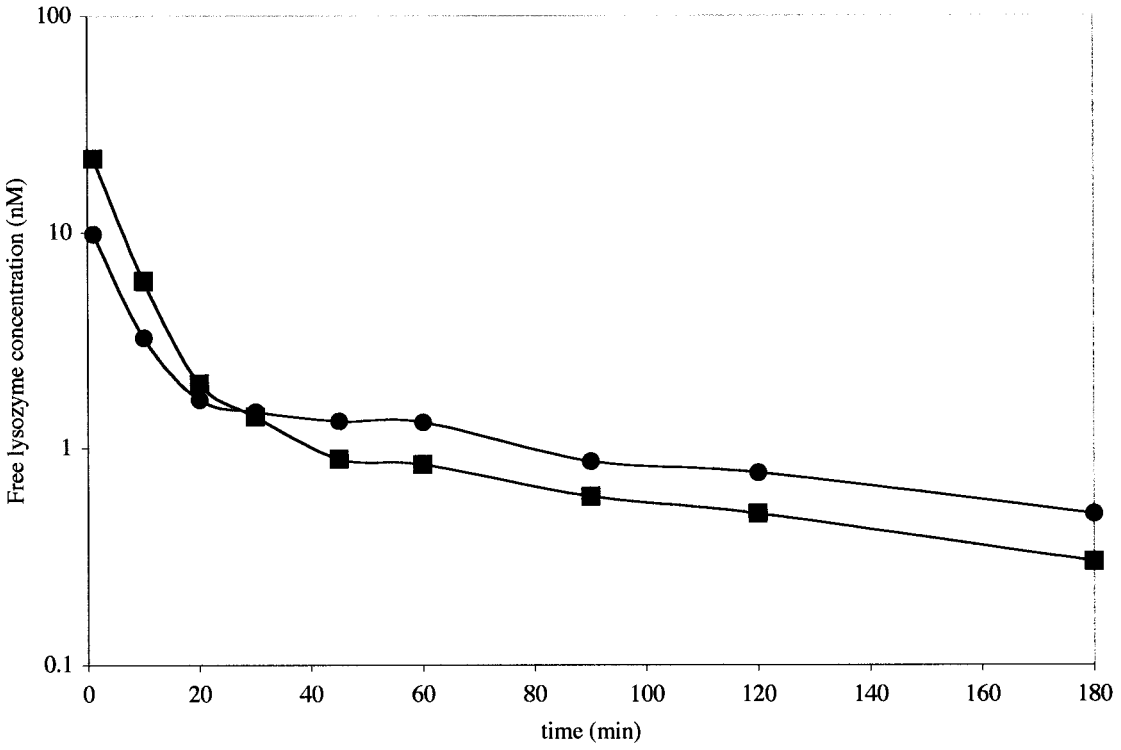


Figure 2.3 Concentration of free lysozyme administered with or without an antibody buffer. The concentration of free (not antibody-bound) lysozyme was measured when $^{14}$C-lysozyme was administered with (circles) or without (squares) an antibody buffer. The free concentration initially started high with lysozyme administered without D1.3, but rapidly declined. When lysozyme was administered with D1.3, there appears to be a period where the free concentration stays relatively constant (between 20 and 60 minutes), indicating a period of successful antibody buffering.

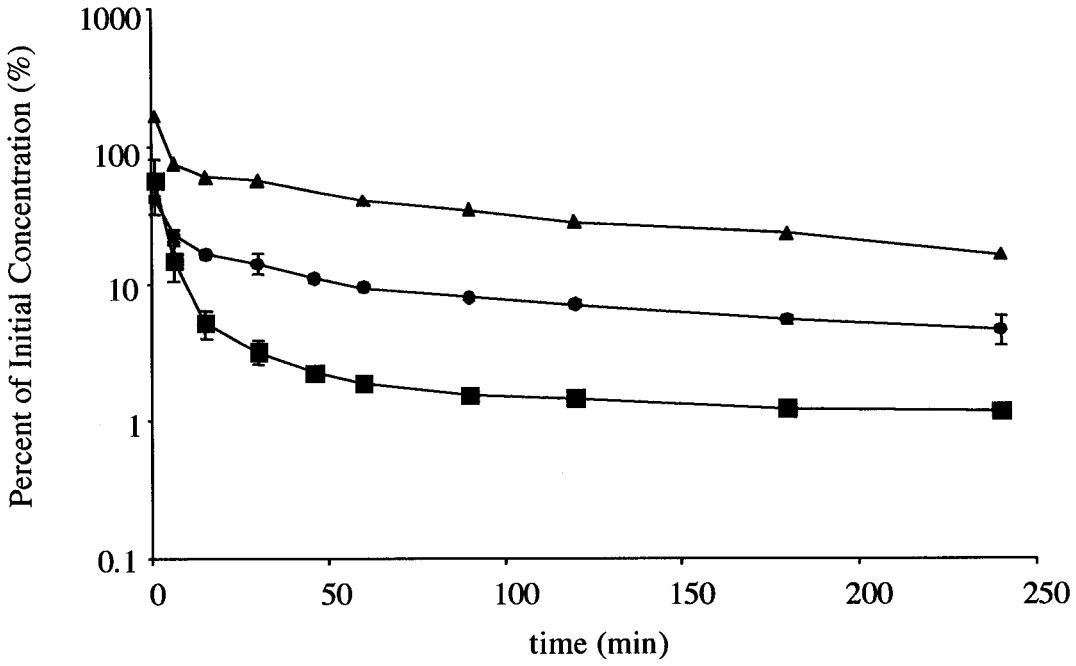


Figure 2.4 The effect of increasing antibody concentration on the half-life of systemically-administered lysozyme. $^{14}$C-lysozyme was administered with no antibody (squares), equimolar antibody (circles), or twice molar antibody concentration (triangles). Increasing the antibody concentration prolonged the elimination half-life of lysozyme from 16.4 minutes with no antibody to 42.8 minutes with equimolar antibody to 61.3 minutes with twice molar antibody.

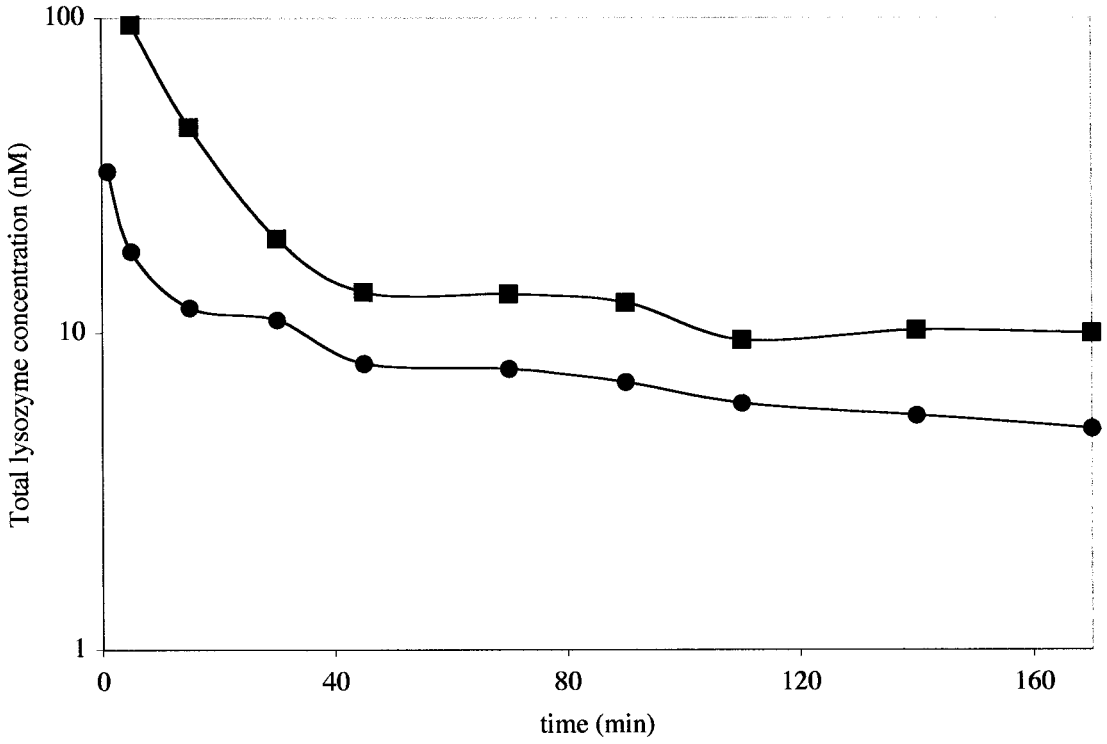


Figure 2.5 The effect of increasing lysozyme concentration on the half-life of lysozyme administered with an antibody buffer. D1.3 antibody was administered with either equimolar (circles) or ten times the molar amount of $^{14}C$-lysozyme (squares). The presence of more lysozyme greatly increased the initial concentration of lysozyme, but it was quickly eliminated to a concentration only about 2-fold higher than that of the lower lysozyme administration.

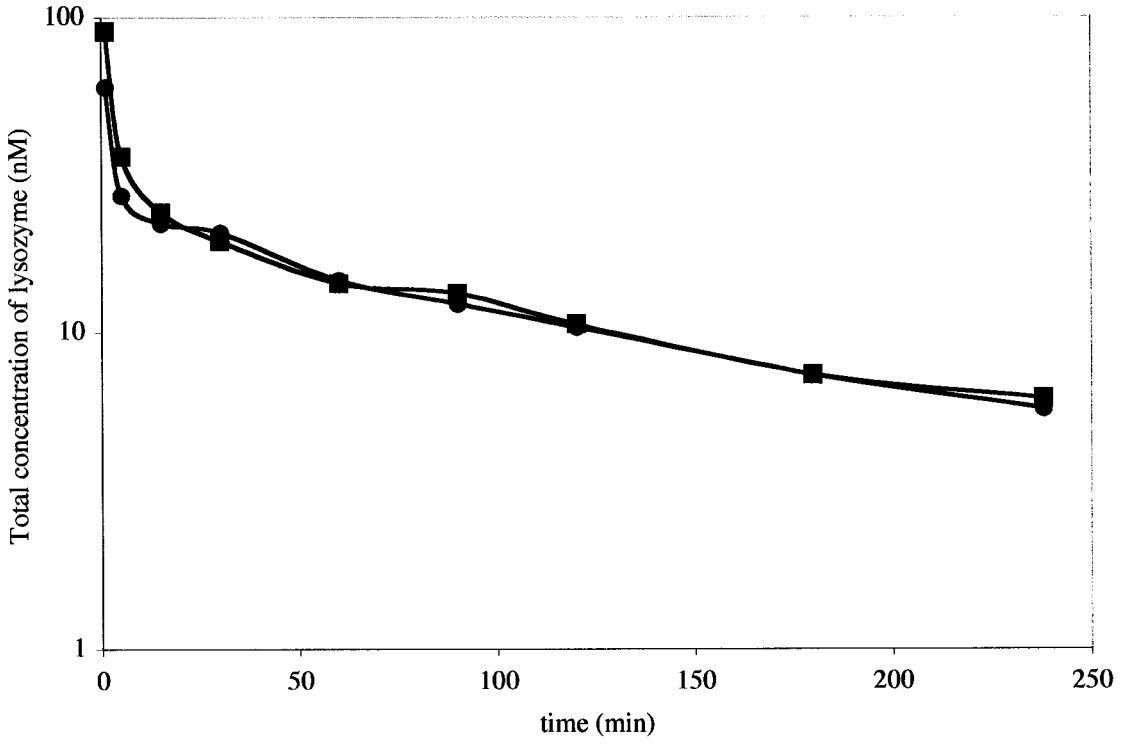


Figure 2.6 D1.3 and lysozyme infused together vs. separately. D1.3 was infused into the rat plasma with (circles) or without (squares) equimolar $^{14}$C-lysozyme. The D1.3 infused alone was followed 10 minutes later by an infusion of $^{14}$C-lysozyme. Even though the lysozyme concentration in the double infusion started higher, it rapidly approached the level of the all-in-one administration, and the two elimination patterns appear very similar after that point. This indicates that allowing the antibody to equilibrate prior to ligand infusion has little effect on ligand elimination.

# Chapter 3:

## Antibody Buffering of a Small Molecule Ligand *in vivo*


### Summary

The process of antibody buffering holds promise as a means to slow clearance, thereby extending lifetime, of a small molecule drug *in vivo*. Here antibody buffering of a small ligand is explored experimentally using a model compound, 2-phenyl-oxazol-5-one-γ-amino butyrate (Ox), as a drug proxy. Antibody buffering is shown to be able to extend by an order of magnitude the plasma lifetime of Ox in rats. In addition, through the use of a panel of three anti-Ox antibodies, the steady-state free Ox level dependence on the molecular properties of the antibody used is demonstrated. The anti-Ox antibody can be recharged with drug *in vivo* to extend Ox lifetime without additional antibody administration, making this technique even more suitable for possible clinical application.


### Introduction

A large number of therapeutic agents in clinical use are small molecule drugs. While work in other laboratories has demonstrated enhanced bioactivity, and in some cases prolonged lifetime, of hormones and cytokines co-administered with anti-hormone and anti-cytokine antibodies (Holder et al., 1985; Holder et al., 1987; Pell et al., 1989; Mihara et al., 1991; Rathjen et al., 1992; Finkelman et al., 1993; Glencross et al., 1993; May et al., 1993; Stewart et al., 1993; Pell and James, 1995; Hill et al., 1997; Pell et al., 2000), there has yet to be an antibody buffering model applied to the delivery of small molecule drugs. The utilization of an antibody to maintain a concentration of drug within a therapeutic window for an extended period of time would be of great clinical benefit,

especially as many small molecule drugs exhibit rapid clearance from the body via the kidney or liver.

Here buffering behavior is explored experimentally using Ox as a drug proxy to develop quantitative tools to guide design of antibody buffers for bona fide drugs. The γ-aminobutyric acid conjugate of 2-phenyl-oxazol-5-one was chosen as a model compound for this study because Ox has a chemical structure typical of a small molecule drug and because an existing library of anti-Ox monoclonal antibodies has been kinetically characterized (Foote and Milstein, 1991). The clearance mechanism of Ox is unknown, and it is likely that Ox is not biologically active, but these points are immaterial to the general questions of whether a specific antibody can modulate the pharmacokinetics of a small molecule ligand *in vivo* and how this buffering capability is related to the molecular properties of the antibody buffer.

## Methods

### Antibody production

A panel of three anti-Ox hybridomas (NQ11/7.12, NQ16/113.8, NQ22/16.4) have been described previously (Griffiths et al., 1984; Berek et al., 1987). A control (non-Ox binding) murine anti-lysozyme antibody, D1.3, (Amit et al., 1986) was also used. These hybridomas were grown in a oscillating bubble roller bottles (Pannell and Milstein, 1992) and the respective monoclonal antibodies were purified from spent culture supernatant by Protein A-Sepharose affinity chromatography. SDS-PAGE was performed on the antibodies to verify purity. Protein concentrations were determined by UV spectroscopy,

using extinction coefficients calculated from sequence (Perkins, 1986). Antibody solutions were filter-sterilized and stored at 4°C under $N_2$.

**Preparation of the tritiated 2-phenyl-oxazol-5-one-γ-amino butyrate conjugate (Ox)**

Crude Ox was prepared essentially as described by Berek *et al.* (Berek et al., 1987). $^3$H-γ-aminobutyric acid (GABA) (1 mCi/ml) was obtained from PerkinElmer Life Sciences, Inc., and solutions of cold GABA (0.5 mM in 1 M $NaHCO_3$) and 4-ethoxy-methylene-2-phenyl-oxazolin-5-one (0.17 M in acetone) were prepared. 5 µl of cold GABA solution was mixed with 50 µl $^3$H-GABA on ice. 10 µl 4-ethoxy-methylene-2-phenyl-oxazolin-5-one solution was added and the reaction mixture was kept on ice for one hour with occasional hand mixing. Then, another 11 µl 4-ethoxy-methylene-2-phenyl-oxazolin-5-one solution was added and the reaction mixture was left on ice for 20 minutes. 5 µl of cold GABA solution was added and the reaction was brought to room temperature for 20 hours with fast mixing. 1 µl of concentrated acetic acid was then added and the resulting precipitate was dried in a ThermoSavant SPD1010 SpeedVac System with no heating. The dried precipitate was dissolved in PBS (25mM $NaH_2PO_4$/125 mM NaCl, pH 7.0).

The resulting Ox product was purified using HPLC. 0.1 M ammonium formate, pH 4.8 (mobile phase A) and acetonitrile (mobile phase B) were vacuum-filtered through a 0.2 micron filter prior to use. Ox was applied to a Zorbax SB-C18 HPLC column with an analytical guard column from Agilent Technologies and eluted with 0.1 M ammonium formate, pH 4.8 (mobile phase A) and acetonitrile (mobile phase B). A gradient of 9.5% to 70% mobile phase B over 26 minutes was run at a flow rate of 0.5 ml/min and using a

wavelength of 348 nm. The Ox peak eluted at 20.5 minutes. Its identity was confirmed by scintillation counting of the fractions collected in that peak. Pooled fractions from the Ox peak were dried in a ThermoSavant SPD1010 SpeedVac System with no heat. After drying, the residue was resuspended in 1 ml PBS. The radiochemical purity of the Ox was confirmed by HPLC. The concentration was determined both by quantitative HPLC and by UV spectroscopy. Specific activity was determined by diluting 1 μl of Ox into 5 ml of Scintiverse II (Fisher Scientific) and counting in a Beckman LS6500 Scintillation Counter. The specific activity of Ox was 4.4 x $10^6$ cpm/nmol.

**Determination of anti-Ox antibody affinity at 37°C**

Antibody affinities were determined through fluorescence spectroscopy (Foote and Milstein, 1991) using a PerkinElmer LS 50 B luminescence spectrometer. Temperature control of the cuvette block was maintained by a circulating water bath heated to 37°C. A cuvette containing either 20 nM (NQ11/7.12) or 200 nM (NQ16/113.8 or NQ22/16.4) in PBS was placed in the spectrometer and allowed to equilibrate to 37°C. In the case of NQ11/7.12, 30 μg/ml ubiquitin was added as a carrier. An excitation wavelength of 280 nm with a bandwidth of 5 nm and an emission wavelength of 340 nm with a 10 nm bandwidth were used with an 8 second integration time. Ox-GABA solution was added in 40 nM increments and fluorescence readings were taken after allowing time for equilibration. The resulting concentration/fluorescence readings were analyzed by least squares (Foote and Winter, 1992) to determine the $K_d$ of each antibody.

**Determination of anti-Ox antibody half-life in rat plasma**

NQ11/7.12 was iodinated with $^{125}I$ using the Chloramine T method (Hunter and Greenwood, 1962; McConahey and Dixon, 1980). Iodine incorporation into the antibody was 97%, and the specific activity of the antibody solution was 1.25 x $10^6$ cpm/μg. Rats (300 g Sprague-Dawley males) implanted with a jugular vein cannula were obtained from Zivic Laboratories. They were allowed to rest at least 72 hours after delivery before experiments were started and allowed to move freely throughout the experiment. $^{125}I$-NQ11/7.12 (0.39 nmol) was mixed with cold NQ11/7.12 to a final amount of 4.54 nmol. This amount was further diluted to a final volume of 1 ml in sterile PBS. The sample was infused by hand over 1 minute through the jugular cannula and the cannula was flushed with 0.2 ml of heparinized saline (250 units/ml) to reduce the risk of clotting. The end of the infusion was designated as time 0. 0.4 ml blood samples were withdrawn from the cannula at various timepoints. The blood was immediately centrifuged to separate out the plasma. 100 μl of plasma was counted using a Packard Cobra Auto-Gamma counter to determine the concentration of NQ11/7.12 in the plasma.

**Antibody buffering experiments**

Samples were prepared for injection consisting of 2.27 nmol Ox and either 4.54 nmol anti-Ox antibody (NQ11/7.12, NQ16/113.8, or NQ22/16.4), 4.54 nmol D1.3 control antibody, or no antibody diluted to 1 ml with sterile PBS. The samples were warmed to 37°C, and then infused over 1 minute through the jugular vein cannula in Sprague-Dawley rats as described above. At various timepoints, 0.45 ml of blood was withdrawn and immediately centrifuged to separate out the plasma. 100 μl of plasma was counted

directly by adding it to 5 ml of Scintiverse II and counting in a Beckman LS6500 Scintillation Counter. 100 µl plasma was used in bound vs. free Ox experiments described below. For some rats, 0.1 ml of blood minus plasma was analyzed. These samples were first decolorized by incubating at 40°C for 15 minutes in 0.3 ml of Scintigest (Fisher Scientific):isopropanol (1:2 v/v) for one hour. Then 0.2 ml of 30% $H_2O_2$ was added dropwise and the solution was incubated at room temperature for 15 minutes. Pipetting up and down was needed to break up some clumps. The solution was incubated at 40°C before placing it into 5 ml of Scintiverse II. The samples were allowed to sit overnight before counting to reduce chemiluminescence. Control samples were prepared using known amounts of Ox to aid in analysis.

**Separation of bound and free Ox using 187.1-Sepharose affinity resin**

The rat anti-mouse kappa antibody 187.1 (obtained from ATCC) (Yelton et al., 1981) was isolated from spent culture medium and purified by passage over a Protein A-Sepharose affinity column. 10 g of CNBr-activated Sepharose 4 Fast Flow (Amersham Biosciences) were washed and coupled with the antibody according to manufacturer's protocol. 2 mg of antibody per 10 g of swelled resin were used.

The ability of immobilized 187.1 to bind anti-Ox antibodies was tested. 0.75 ml of 187.1 resin was poured into a disposable column. 1.13 nmol of NQ11/7.12 and 52.5 nmol of Ox were combined in a total volume of 0.1 ml rat plasma and loaded onto the column. 3 ml of PBS followed by 3 ml of 0.2 M glycine (pH 2.5) were run over the column in 0.5 ml increments. Every 5$^{th}$ drop eluting off the column was collected onto a 96-well ELISA plate previously incubated with Ox-CSA (Makela et al., 1978) and

blocked with reconstituted milk. The remaining 4 drops were collected into scintillation vials, each containing 5 ml of Scintiverse II. The ELISA plate was incubated for 1 hour, rinsed with PBS, and incubated again with a peroxidase-conjugated goat anti-mouse IgG, Fc fragment specific (Jackson ImmunoResearch). Color mix (1 mM ATBS and 4 mM $H_2O_2$ in a 50 mM sodium citrate/ 50 mM citric acid) was added to each well. The ELISA plate was read with a kinetic microplate reader (Molecular Devices). Higher enzyme velocities indicated more NQ11/7.12 was eluting from the column in that fraction. The Vmax values were low (less than 10 mOD/min) in the PBS (free Ox) fractions. The scintillation vials were counted to indicate the fractions in which Ox was eluting from the column.

During the antibody buffering experiments, 100 µl of plasma was loaded onto the 187.1 column immediately after centrifugation. 3 ml of PBS followed by 3 ml of 0.2 M glycine (pH 2.5) were loaded onto the column as described above. All the PBS fractions and all the glycine fractions were collected separately for each timepoint and dried down in the SpeedVac. The residue was resuspended in 150 µl PBS and then transferred to a scintillation vial with 5 ml Scintiverse II for counting.

**Long term buffering experiment**

Samples of NQ11/7.12 and Ox were prepared and infused into Sprague-Dawley rats as described previously. 0.4 ml of blood was taken at time 1, 10, 20, 50, 90, and 120 minutes. 100 µl plasma was counted directly and 100 µl was loaded onto a 187.1 column to separate bound and free Ox. 24 hours following the first administration, and again at

48 hours, 2.27 nmol Ox in 0.5 ml PBS was infused over 1 minute through the jugular cannula. Timepoints were again taken and analyzed as above.

## Results

### The effect of NQ11/7.12 on Ox lifetime in rat plasma

To determine its pharmacokinetic behavior, tritiated Ox was infused into a rat through the jugular cannula and elimination of Ox was followed through blood sampling, separation of the plasma by centrifugation, and scintillation counting of the plasma. The concentration of Ox declined rapidly with an elimination half-life of 1.2 minutes (Data not shown, but similar to Figure 3.2). This lifetime is very short; a drug candidate with a loss rate on this scale would almost certainly be disqualified from further study.

We employed a set of three kinetically-characterized monoclonal antibodies raised against Ox (Foote and Milstein, 1991). The affinities of these antibodies were redetermined at 37 $^{0}$C using fluorescence spectroscopy and found to be 1.3 nM (NQ11/7.12), 46 nM (NQ16/113.8), and 42 nM (NQ22/16.4) (Table 3.1). An isotype-matched (IgG1) anti-hen-egg lysozyme antibody, D1.3, was used as a control. Each rat in these studies was used only once to limit the possibility of a rat anti-mouse antibody response.

Antibodies are attractive therapeutic vehicles in part because of their long plasma half-life, which is far longer than the pharmacokinetic lifetime of most drugs. We confirmed this long residence time in the plasma using radioiodinated NQ11/7.12. The monoclonal antibody was infused into a rat and the elimination of the NQ11/7.12 was followed through blood sampling, separation of the plasma by centrifugation, and gamma

counting of the plasma. The concentration of NQ11/7.12 declined slowly with an elimination half-life from the plasma of 20 hours (Figure 3.1). This lifetime is so long that the anti-Ox antibody concentration is effectively constant (200 +/- 30 nM) over the course of all the pharmacokinetic studies presented here, except where noted.

For an antibody to buffer a ligand, the antibody-ligand complex must form a long-lived reservoir. In other words, antibody complexation must block ligand elimination. Ox was administered with twice the molar amount of NQ11/7.12 or the control antibody through the rat's jugular cannula (Figure 3.2). Administration of Ox with NQ11/7.12 showed a significant prolongation of Ox residence time in the plasma with an Ox elimination half-life of 20 +/- 2 minutes. Comparison of the two curves indicates that much of the Ox in the D1.3 control is cleared from the plasma before the first timepoint can be sampled. The remaining Ox administered with D1.3 showed biphasic elimination kinetics; most was eliminated from the plasma with a half-life of 1.2 +/- 0.2 minutes, and a smaller amount had a half-life of 10 +/- 2 minutes. The seventeen-fold increase in half-life indicates that NQ11/7.12 binding is opposing Ox elimination from the plasma. As a control, analysis was performed on the cellular fraction of the blood in order to determine if cells are a significant repository for the Ox. The Ox concentration in the cellular fraction ranged between 1-2% of the total Ox concentration in the plasma (data not shown). No adverse effects were noted in the animal subjects.

**The effect of NQ11/7.12 on free Ox concentration**

The principle of chemical equilibrium predicts that the Ox in the plasma should partition between free and antibody-bound forms. The buffering equation [1]

$$p[drug] = pK_A + \log_{10}([Ab]/[Ab\text{-}drug]) \qquad [1]$$

in particular predicts that a free pool should be constantly replenished, staying at a concentration near the antibody's $K_d$. To test this hypothesis, bound and free forms of Ox in plasma fractions from the previous experiment were separated on an affinity column. Samples resulting from D1.3 co-administration were processed in parallel, though no significant antibody-bound label was expected or found. An immobilized rat anti-mouse kappa antibody was used to trap antibody-Ox complexes while free Ox eluted from the column with a PBS wash (free Ox fraction). The ability of the resin to bind NQ11/7.12 and then have the antibody elute with only the glycine wash was shown (Figure 3.3A). It was also demonstrated that the wash amounts were sufficient to elute Ox from the column (Figure 3.3B).

This analysis confirmed that a free pool of Ox does indeed exist in the rat plasma whether the Ox is administered with D1.3 or with NQ11/7.12 (Figure 3.4). Initially, when Ox is administered alone or with D1.3 the amount of free Ox is high (6.8 nM), but it quickly decreases with an elimination half-life of 6.0 +/- 1 minutes. However, when Ox is administered with NQ11/7.12, the initial free concentration of Ox is low (2.2 nM). This low concentration remains fairly stable over time with an Ox elimination half-life of 41 +/- 6 minutes. Therefore, the presence of NQ11/7.12 appears to be buffering the free Ox.

**The effect of antibody $K_d$ on Ox lifetime in rat plasma**

Next, we wanted to test whether antibody $K_d$ affects the ability of an antibody to retard Ox elimination from the plasma. For this experiment, the anti-Ox antibodies

NQ16/113.8 and NQ22/16.4 were employed. Each of these antibodies has a similar $K_d$ for Ox, but this $K_d$ is significantly higher than that of NQ11/7.12. Ox was administered to rats with either NQ16/113.8 or NQ22/16.4 and the total Ox concentration was measured through blood sampling (Figure 3.5A). The concentration of Ox administered with NQ11/7.12 or with D1.3 are shown for comparison. Both NQ16/113.8 and NQ22/16.4 retard total Ox elimination from the plasma. The increase in Ox half-life, however, was unable to match the extent of the Ox half-life increase of NQ11/7.12, which has a much higher affinity for Ox. Therefore, as predicted, antibody $K_d$ is related to the elimination rate of total Ox in the plasma. The effect of the antibodies is also well-illustrated with the area under the curve (AUC) values. While the AUC of Ox administered with the D1.3 control antibody is only 253 nmol•min/L, the AUC increases to 393 and 406 nmol•min/L with NQ16/113.8 and NQ22/16.4 administration, respectively. Ox infused with NQ11/7.12 has the largest AUC at 2848 nmol•min/L.

In addition, antibody $K_d$ affects the free pool of Ox in the rat plasma (Figure 3.5B). Plasma from the previous experiment was loaded onto the rat anti-mouse kappa antibody affinity column, and free and bound Ox fractions were eluted. Initially, the free concentration of Ox administered with NQ16/113.8 or NQ22/16.4 (between 10 and 16 nM) is higher than that of Ox administered with NQ11/7.12 (2.2 nM). This free concentration declines much more rapidly than the free Ox administered with NQ11/7.12. Thus, anti-Ox antibody $K_d$ determines the buffering capacity of the antibody.

**Long-term buffering of NQ11/7.12**

Since it had previously been determined that NQ11/7.12 is retained in rat plasma for a long period of time compared to the elimination half-life of Ox, we wanted to test whether NQ11/7.12 retains its ability to buffer Ox concentration in the plasma days after the initial antibody infusion. For these experiments, NQ11/7.12 was administered only once. Ox was initially co-administered with NQ11/7.12 and additional aliquots of Ox alone were administered at 24 hours and at 48 hours. Blood sampling was done to follow total Ox elimination from the plasma. The Ox pharmacokinetic lifetime at 24 hours and 48 hours showed only small changes from the kinetics following the initial NQ11/7.12 administration (Figure 3.6A), which could be attributed to the slow clearance of circulating antibody over time. Ox co-administered with the D1.3 control antibody is shown for comparison. Free vs. bound Ox separation was also performed on these plasma samples (Figure 3.6B). Initially, the free Ox concentration at 24 hours and 48 hours was higher than when Ox was administered in conjunction with the antibody buffer. However, this free concentration quickly normalized as Ox was bound by the NQ11/7.12 already present in the plasma to follow an elimination pattern similar to Ox and NQ11/7.12 administered together. Therefore, not only can an anti-Ox antibody buffer the concentration of free Ox in the plasma, but it can do so even days after the initial antibody infusion.

**Discussion**

In this testing of a model for antibody buffering of a small molecule drug, several principles emerge. First, anti-Ox antibodies were able to extend the lifetime of total Ox

in the plasma. Antibody complexation did not facilitate removal of Ox. This result would be expected, given that haptens do not fix complement (Pressman et al., 1942) and that the major mechanism of immune complex clearance operates on aggregates of complement protein C3b and antigen-antibody complexes (Cornacoff et al., 1983). Also, antibody complexation is likely to block the two major routes of drug clearance, renal filtration and hepatic metabolism. Second, the presence of a buffering antibody prevents the spike in free ligand concentration characteristic of the unbuffered control. The concentration of uncomplexed Ox at the first timepoint in Figure 3.4 is 2.4 nM, and the remaining 97% is complexed with antibody. In a drug scenario, this property of an antibody buffer might minimize high-dose toxicities since only a fraction of the administered dose is at any one time bioavailable. Third, antibody buffering increases the pharmcokinetic lifetime of the uncomplexed ligand that would be available for receptor binding. The buffered free Ox concentration in Figure 3.4 only declines from 2.4 to 0.8 nM over the span of an hour, whereas unbuffered Ox is more than half gone before the first timepoint can be sampled. Fourth, a buffering antibody stabilizes a pool of free Ox near the antibody $K_d$. The 2.4 and 0.8 nM endpoints just cited bracket the 1.3 nM $K_d$ of NQ11/7.12. Antibodies with higher $K_d$s for Ox were weaker buffers in the ligand concentration range studied. The import of this for the use of antibody buffering in therapy is that one would determine the most advantageous *in vivo* drug level, then tune the antibody $K_d$ to an appropriate value to stabilize that level. $K_d$ can be manipulated conveniently with one of the display technologies (Lowman et al., 1991; Schier et al., 1996; Boder and Wittrup, 1997; Boder and Wittrup, 2000).

The substituted Henderson-Hasselbalch equation:

$$p[drug] = pK_A + \log_{10}([Ab]/[Ab\text{-}drug]) \qquad [2]$$

(Here, and in subsequent equations, Ab refers to a site concentration rather than to the concentration of divalent immunoglobulin molecules.)

suggests that a $K_d$ equal to the desired drug level would give the best buffer, just as a pH buffer exerts the maximum buffering power at a pH equal to its $pK_a$. A steady state model points to a similar conclusion in most cases. If we posit a steady state where dissociation of a drug from an antibody approximately balances the rates of rebinding and drug loss through clearance, then

$$k_{off}[Ab\text{-}drug] = k_{on}[Ab][drug] + k_{loss}[drug] \qquad [3]$$

If we evaluate this expression for a condition at which the antibody is half-saturated with drug, i.e. the point of maximum buffering power, and define $[Ab]_{1/2}$ as half the total antibody concentration and $[drug]_{ss}$ at the steady state free drug concentration, then

$$[drug]_{ss} = k_{off}/[k_{on} + (k_{loss}/[Ab]_{1/2})] \qquad [4]$$

The qualitative interpretation of Equation 4 is that the antibody-drug ensemble will stay close to equilibrium so long as drug clearance is slow relative to the rate of drug rebinding by the antibody.

Lastly, we have demonstrated that an antibody is able to buffer ligand concentration over multiple ligand administrations. The antibody can be "recharged" *in situ*. The buffered curves in Figure 4 represent an approach to equilibrium from opposite directions. In the initial administration, the Ox and antibody are concentrated in a small volume, and nearly all the Ox is sequestered in antibody complexes. In the re-

administrations, the antibody is already dispersed throughout the plasma and all of the Ox starts in the free state. The similarity of the buffered curves in Figure 3.6 whether the Ox was co-administered or re-administered at a later time, shows that equilibration of Ox and the antibody within the plasma is much more rapid than the elimination of free Ox.

In the preceding experiments, a model system has been used to demonstrate pharmacokinetic modulation of a ligand by an antibody. Antibody buffering has the potential to replace the sawtooth drug vs. time profile that is the current norm with a stable, long-lived drug level. The value of this steady-state level can be set to the optimum for drug action by genetic engineering of the antibody. Once the antibody has been administered, it can be re-charged *in situ* multiple times with fresh drug. These attributes of an antibody buffer offer advantages over conventional delivery vehicles that are non-genetic, non-rechargeable, and work by slow release rather than equilibrium. For example, controlled-release pellets (Collins et al., 1997) or drug-loaded liposomes (Lasic and Papahadjopoulos, 1995) can be introduced into the body, but are only useful for one drug administration. In drug delivery by slow infusion, a gradient is established between the point of entry of the drug and the site of action. Antibodies, on the other hand, are able to equilibrate within a compartment, buffering the drug concentration near the desired target. While serum albumin is known to be a circulating depot for many drugs, thereby reducing the amount of drug available for biological effect (Olsen et al., 2004), changing the structure of albumin to selectively bind a single drug with high affinity would be a formidable problem. Antibodies, on the other hand, can be more amenable to clinical use as they target a single drug at a therapeutically designated affinity. In short,

use of antibody buffering as a method of drug delivery would improve upon methods already in place by yielding a stable, bioavailable concentration of a drug that is normally rapidly eliminated from the plasma.

Table 3.1 Pharmacokinetic parameters of Ox buffered by different antibodies.

| Antibody | Affinity for Ox | t1/2 α (min) | t1/2 β (min) | C(0) (nM) | AUC nmol-min/L | Vd (ml) | CL (ml/min) |
|---|---|---|---|---|---|---|---|
| D1.3 | None | 1.2 | 9.9 | 25 | 253 | 112.5 | 9.0 |
| NQ11/7.12 | 1.34 nM | | 19.7 | 84 | 2848 | 2.26 | 0.08 |
| NQ16/113.8 | 46 nM | 1.17 | 5.3 | 71.8 | 393 | 45.0 | 5.8 |
| NQ22/16.4 | 42 nM | 1.36 | 6.1 | 58.8 | 406 | 49.1 | 5.6 |

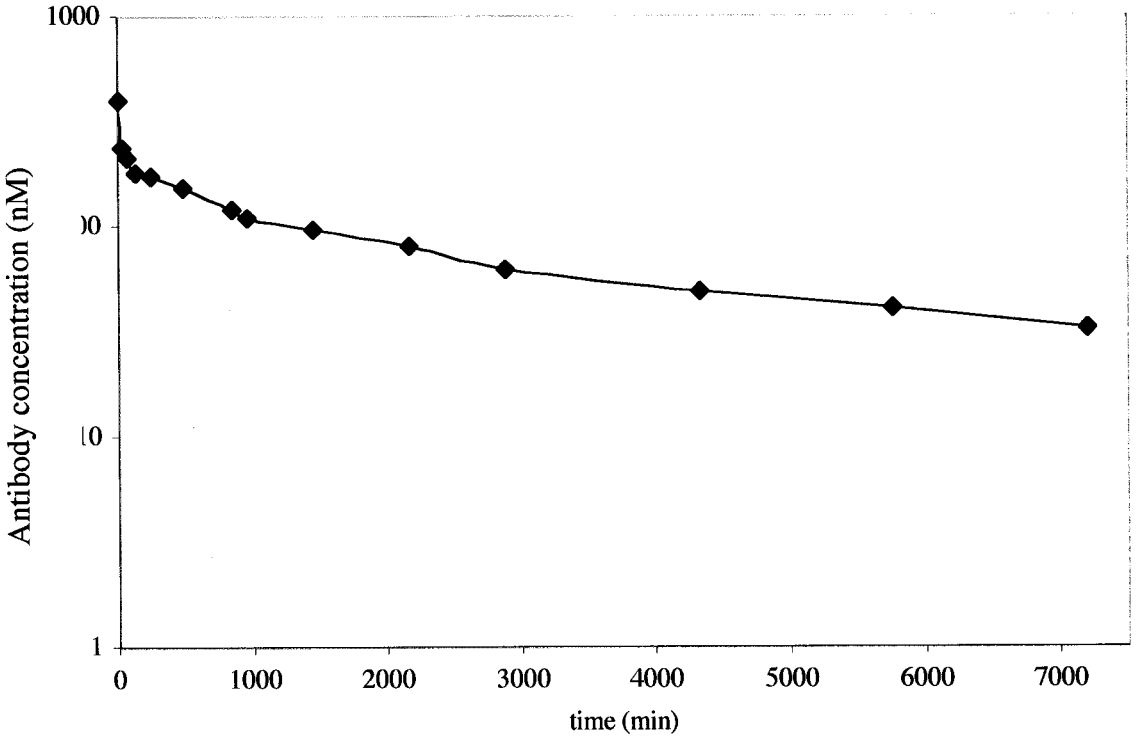


Figure 3.1 NQ11/7.12 concentration in rat plasma. NQ11/7.12 was radioiodinated and infused into rat plasma. It was eliminated with a half-life of 20 hours.

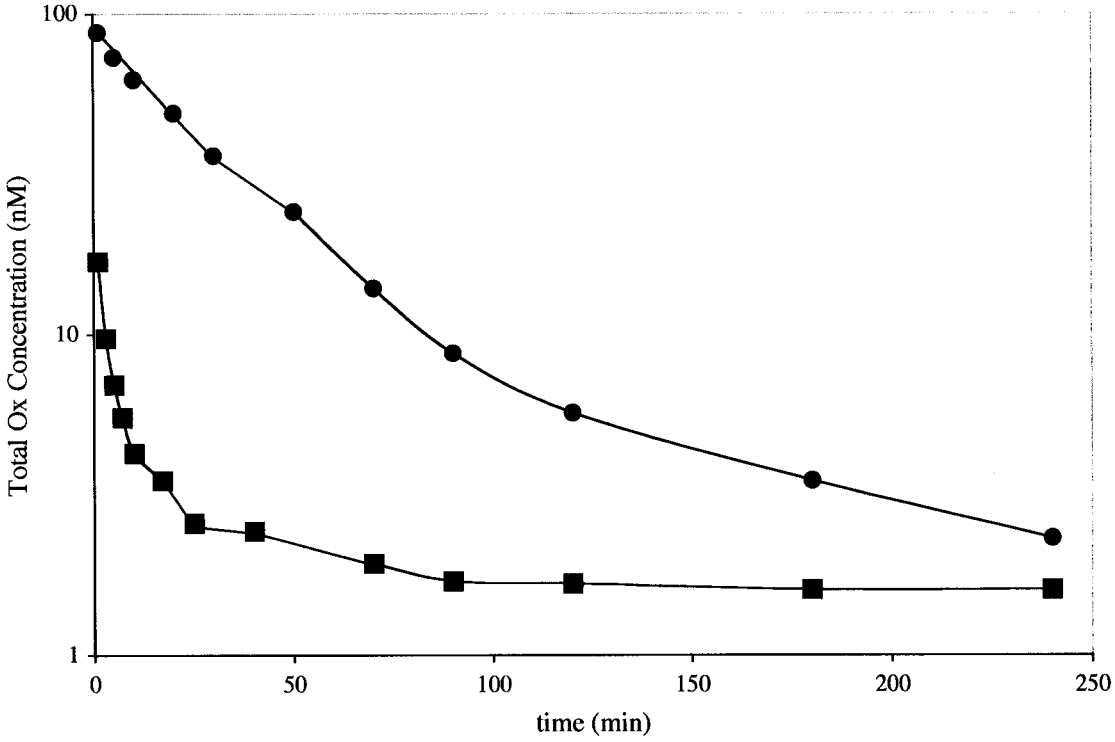


Figure 3.2 Antibody complexation opposes Ox elimination. Elimination of Ox administered with the control antibody D1.3 (squares, $t_{1/2}$= 1.2 +/- 0.2 min) occurs much more rapidly than when Ox is administered with NQ11/7.12 (circles, $t_{1/2}$= 20 +/- 2 min).

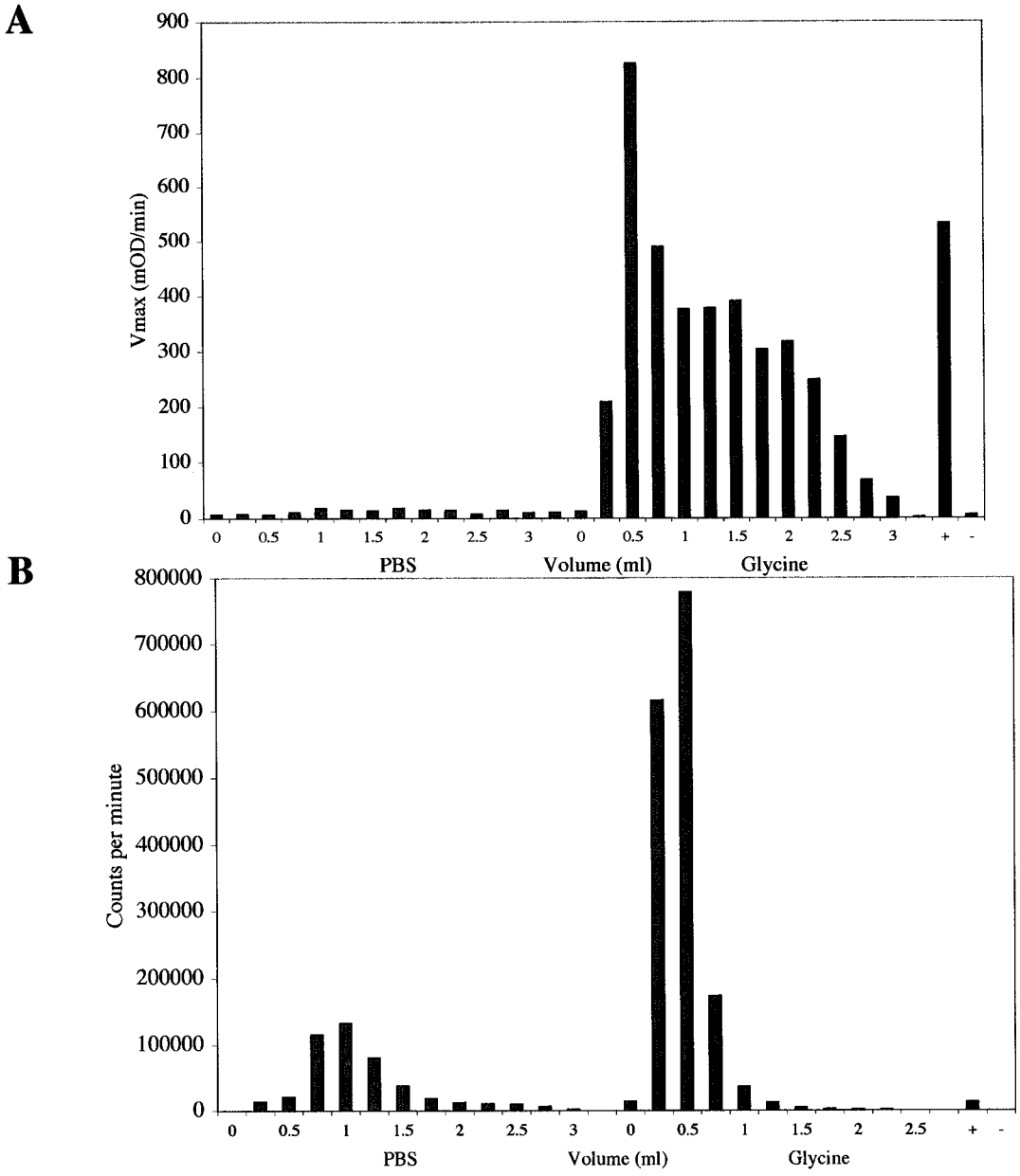


Figure 3.3 Separation of bound and free Ox using 187.1-Sepharose resin. NQ11/7.12 and Ox were combined and loaded onto a 187.1-Sepharose column followed by 3 ml of PBS and 3 ml of 0.2 M glycine (pH 2.5). A. Every 5$^{th}$ drop off the column was collected onto an ELISA plate. More NQ11/7.12 is eluting off the column at higher m/OD. This figure shows that antibody is eluting only during the glycine wash. A positive control of NQ11/7.12 only and a negative control of PBS only are shown. B. The remaining 4 drops scintillation counted to determine when Ox eluted off the column. This figure shows that approximately 1/3 Ox is eluting with the PBS wash and 2/3 with the glycine wash. A positive control of 1 μl Ox and a negative control of 4 drops of PBS are shown.

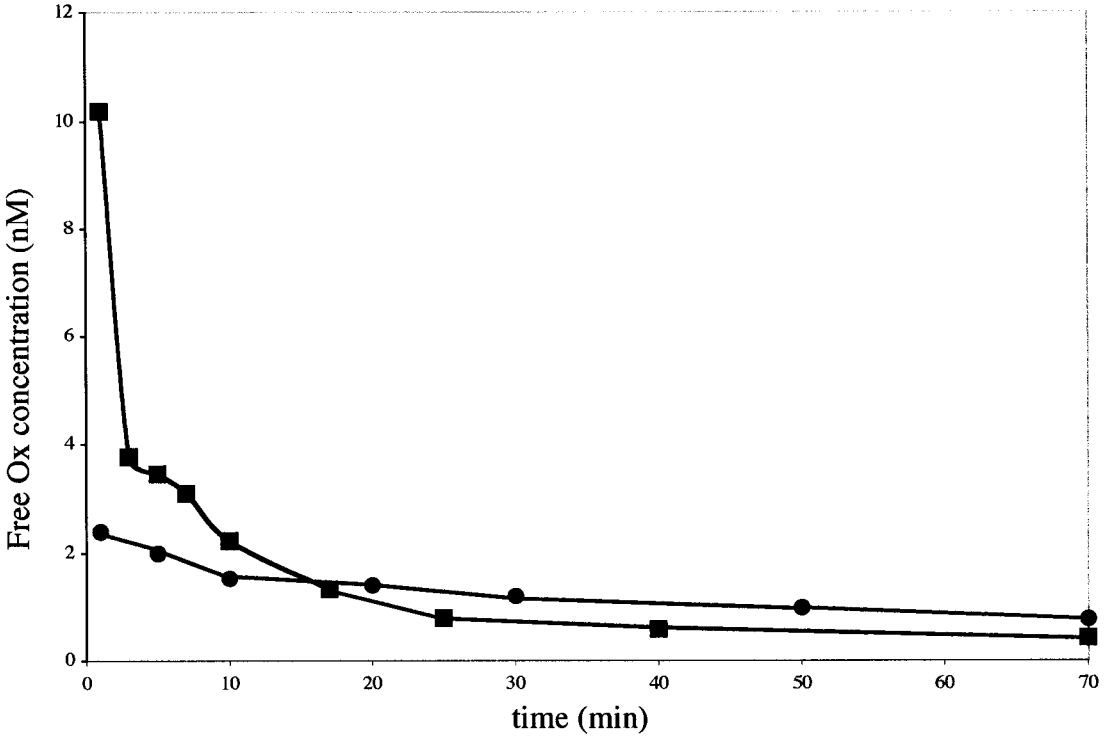


Figure 3.4 NQ11/7.12 lowers the concentration of free Ox while increasing the plasma half-life of free Ox. The free concentration of Ox is initially lower when Ox is administered with NQ11/7.12 (circles) versus D1.3 (squares). However, this free concentration with NQ11/7.12 declines more slowly than when Ox is administered without an antibody buffer.

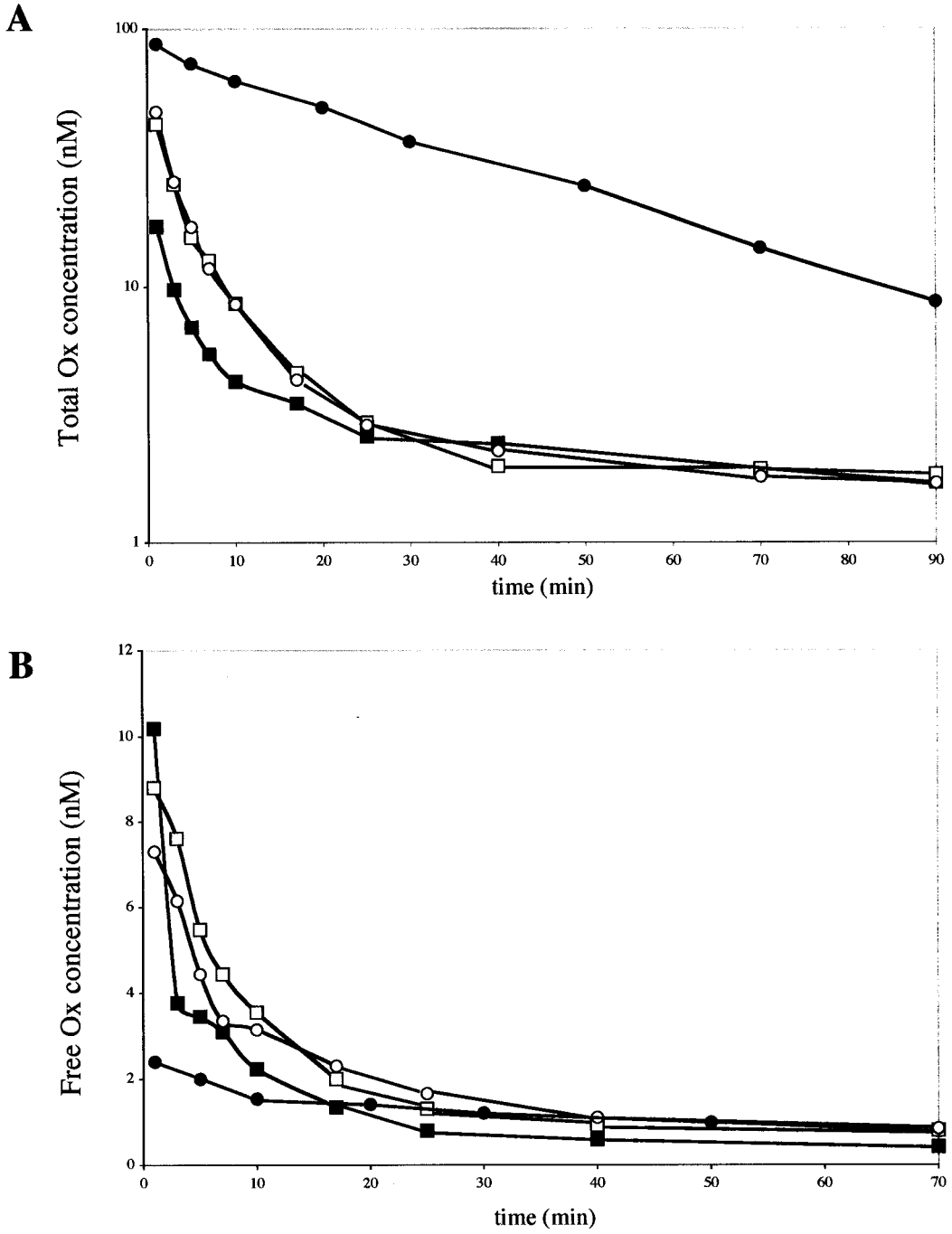


Figure 3.5 Antibody $K_d$ determines the half-life of Ox and free Ox concentration. A. Antibodies NQ16/133.8 (open circles) and NQ22/16.4 (open squares) which have higher $K_d$s than NQ11/7.12 are able to buffer Ox concentration, but to a lesser degree, than NQ11/7.12 (closed circles). D1.3 (closed squares) is shown for comparison. B. These lower affinity antibodies also reduce the free concentration of Ox in the plasma and are able to prolong the half-life of free Ox, but to a lesser extent than NQ11/7.12.

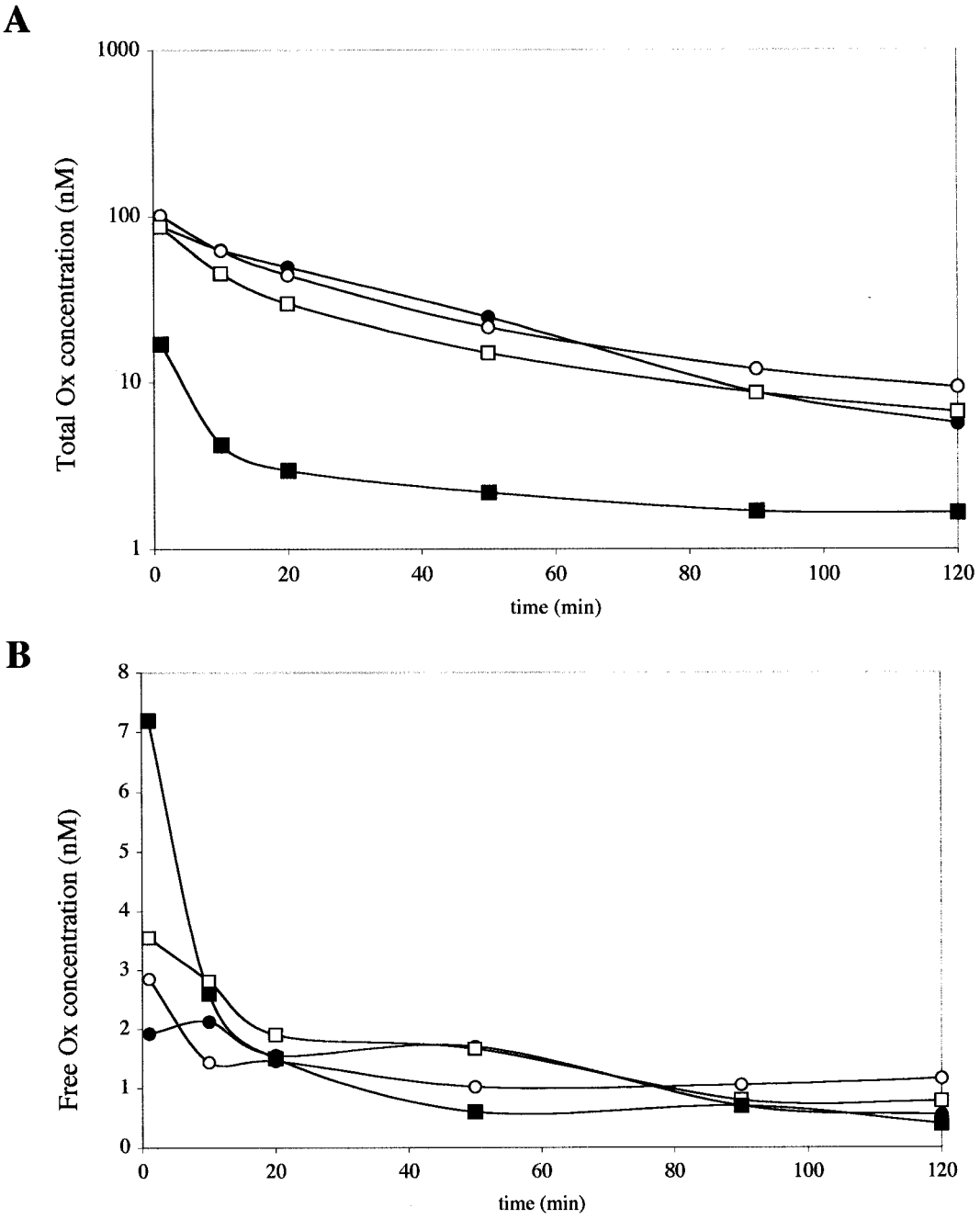


Figure 3.6 NQ11/7.12 retains the ability to buffer Ox days after initial antibody administration. Plasma half-life of total Ox (A) and free Ox (B) is prolonged whether Ox is co-administered with NQ11/7.12 (closed circles) or administered alone 24 hours after NQ11/7.12 infusion (open circles) or 48 hours after NQ11/7.12 infusion (open squares). Ox administered with a control D1.3 antibody (closed squares) is shown for comparison.

# Chapter 4:

## Antibody Buffering in the Cerebrospinal Fluid

**Summary**

The ability of an antibody to prolong ligand half-life and bioactivity in the plasma has been previously described (Mihara et al., 1991; Rathjen et al., 1992; Finkelman et al., 1993). This phenomenon has not yet been described or exploited for use in the cerebrospinal fluid (CSF) compartment. The efficacy of chemotherapy on brain tumors is often hindered by the presence of the blood brain barrier. This barrier keeps many systemically-administered substances from entering the CSF, while allowing most intrathecally-administered drugs free passage out of that compartment. Therefore, achieving therapeutic concentration of a cell cycle inhibitor in the CSF for a time period long enough to have a cytotoxic effect on slow-growing tumor cells has proven difficult. Antibodies often have a longer residence time in the CSF than do small drugs, so antibody buffering has the potential for prolonging the bioactive lifetime of a drug in the CSF. Here we describe antibody buffering of the small molecule hapten 2-phenyl-oxazol-5-one in the CSF of a rat model. Not only does the presence of an antibody buffer increase the half-life of both total and free hapten in the CSF, but the antibody can be re-charged *in situ* with fresh hapten even days after the initial antibody infusion. Antibody buffering may provide a viable option for delivering a stable concentration of a drug that is normally rapidly eliminated from the CSF.

## Introduction

Despite all the research into the treatment of brain tumors, only a subset of patients with specific types of tumors have experienced any prolonged survival (Lallana and Abrey, 2003). Since many brain-intrinsic neoplasms are characterized by relentless tumor cell infiltration of normal brain parenchyma, targeting agents require diffusive properties in order to reach invading tumor cell clusters that migrate along vascular clefts and axonal pathways (Merlo et al., 2003). Tumors of the central nervous system (CNS) represent the second most frequent malignancy in children under the age of 15 years (Heideman et al., 1997). 45% of these children will die of their disease, but not well recorded is the morbidity of the survivors- a result of the motor and intellectual deficits associated with not only the tumor but the aggressive therapies as well. Chemotherapy plays a significant role in the current treatment regime for young children with brain tumors because radiation often leads to developmental delay and neuroendocrine deficiencies in these patients (Zalutsky, 2004).

Despite their widespread application, the use of chemotherapeutic agents in treatment of solid tumors involving the brain, however, has not been very successful (Castro et al., 2003). Tumors of the CNS are usually slow-growing, so treatment with the cell cycle inhibiting drugs currently available requires these agents to remain at an effective concentration in the CSF for a lengthy period of time. Achieving this effective drug concentration at the tumor site for an extended timeframe presents a major problem (Blasberg, 1975). Additional drawbacks to the use of chemotherapy are the development of chemoresistant cells and inadequate delivery methods (Castro et al., 2003). Also, not

only do anticancer drugs kill tumor cells, but they affect hematopoietic cells, resulting in myelosuppression, often a dose-limiting toxicity.

The presence of the blood brain barrier (BBB) profoundly influences the CNS penetration of most substances (Heideman, 1997). Its purpose is to separate the brain from the blood to help regulate brain function and metabolism. The BBB is made up of tight endothelial cell junctions that limit penetration to all but small (less than 200 daltons), lipophilic, un-ionized compounds. The failure of aggressive chemotherapy in the treatment of brain tumors is in part due to the BBB (Castro et al., 2003).

CSF is the liquid that fills the ventricular system and the subarachnoid space. It is formed by the choroid plexus by processes of osmosis and active transport. Proteins usually cannot pass through the choroid plexus, which prevents the passage of immunizing substances into the CSF. The direction of CSF flow is from the lateral ventricle, through the interventricular foramen, third ventricle, cerebral aqueduct, and into the fourth ventricle. Then it passes into the subarachnoid space where it is free to flow anywhere on the surface of the brain. The arachnoid proliferates to form macroscopic patches of branched arachnoidal villi which project into spaces within the dura or into the superior saggital venous sinus. It is through these arachnoid granulations that CSF is reabsorbed into the vascular system.

IgG antibodies are large (around 150 kDa), polar proteins that do not diffuse across the tight endothelial junctions of the BBB or the choroid plexus epithelial cells that form the blood-CSF barrier (Betz, 1989; Betz et al., 1989). For example, an intact BBB prohibits significant entry of systemically-administered rituximab into the CSF

(Rubenstein et al., 2003). Antibodies can be administered directly into the CSF, however, either intrathecally or intraventricularly. Their clearance from the CSF may be influenced by the antibody class and isotype. Studies with an IgG1 antibody, an anti-human transferrin receptor monoclonal antibody conjugated to recombinant ricin A chain, exhibited an early phase half-life of 84 minutes and a late-phase half-life of 10.9 hours (Maraszko et al, 1993), while an IgG3 antibody had a half-life measured at 53.8 minutes (Bergman et al., 1998). Also, antibodies are not restricted to regions around the ventricles or the surfaces of the brain, but diffuse throughout the brain. Chauhan et al. (Chauhan et al., 2001) showed that 3 hours after injection of a horseradish peroxidase conjugated antibody into the third ventricle of the mouse brain, the antibody was localized mainly near the injection site. After 24 hours, however, the antibody had diffused throughout the brain parenchyma, while at 4 days post-injection only a few traces of antibody remained anywhere in the brain. Thus, antibodies are able to access many locations because they do not freely cross the BBB.

Although antibodies can reside in and diffuse throughout the CSF once placed there, drugs currently available for use in intrathecal chemotherapy are rapidly cleared from the CSF (Jaeckle et al., 2002). They can require repeated administrations by lumbar punctures, which are painful and inconvenient to receive. Prolonged therapeutic levels in the CSF can also be achieved by small, sequential intrathecal doses over an extended period through an implanted Ommaya reservoir (Ruggiero et al., 2001), but implantable pumps are expensive and require surgery (Chatelut et al., 1993). Liposomes have been formulated to penetrate the BBB by linkages to iron transferrin or biological toxins that

can cross the barrier (Fisher and Ho, 2002). Once inside, they can slowly release their contents. One such formulation, DepoCyte (a slow-release form of the chemotherapeutic agent cytarabine) was used in a study to treat neoplastic meningitis due to breast cancer. Although the reported results were similar to those using conventional cytotoxic agents, the results were achieved with only a fourth as many doses (Jaeckle et al., 2001). The reduction in administrations presents an important advantage to both the patient and physician.

The use of antibody buffering presents an opportunity for extending drug lifetime in the CSF. Previous studies on the intrathecal administration of antibodies showed no significant acute or delayed toxicity or neurologic effects in non-human primates (Rubenstein et al., 2003), indicating that antibody administration into the CSF is relatively innocuous. Once in the CSF, an antibody can circulate within the CSF and even penetrate into brain tissue. If the antibody was administered with its specific ligand drug, it will release the drug within the CSF, maintaining a therapeutic concentration for a given period of time.

Heretofore, prolongation of drug/hormone/cytokine activity due to antibody presence has only been described in the plasma. Here it is shown that antibody buffering can exist in a different body compartment, the CSF. Administration of an antibody with the small molecule ligand, 2-phenyl-oxazol-5-one, can extend the pharmacokinetic profile of that hapten in the rat cerebrospinal fluid.

## Methods

### Antibody Production

The anti-Ox hybridoma NQ11/7.12 has been described previously (Griffiths et al., 1984; Berek et al., 1987). A control (non-Ox binding) murine anti-lysozyme antibody, D1.3, (Amit et al., 1986) was also used. The hybridomas were grown in oscillating bubble roller bottles (Pannell and Milstein, 1992) and the respective monoclonal antibodies were purified from spent culture supernatant by Protein A-Sepharose affinity chromatography. SDS-PAGE was performed on the antibodies to verify purity. The protein concentration was determined by UV spectroscopy, using extinction coefficients calculated from sequence (Perkins, 1986). Antibody solutions were filter-sterilized and stored at $4^0$C under $N_2$.

### Preparation of the tritiated 2-phenyl-oxazol-5-one-γ-amino butyrate conjugate (Ox)

Crude Ox was prepared essentially as described by Berek *et al.* (Berek et al., 1987). $^3$H-γ-aminobutyric acid (GABA) (1 mCi/ml) was obtained from PerkinElmer Life Sciences, Inc., and solutions of cold GABA (0.5 mM in 1 M $NaHCO_3$) and 4-ethoxy-methylene-2-phenyl-oxazolin-5-one (0.17 M in acetone) were prepared. 5 μl of cold GABA solution was mixed with 50 μl $^3$H-GABA on ice. 10 μl 4-ethoxy-methylene-2-phenyl-oxazolin-5-one solution was added and the reaction mixture was kept on ice for one hour with occasional hand mixing. Then, another 11 μl 4-ethoxy-methylene-2-phenyl-oxazolin-5-one solution was added and the reaction mixture was left on ice for 20 minutes. 5 μl of cold GABA solution was added and the reaction was brought to room temperature for 20 hours with fast mixing. 1 μl of concentrated acetic acid was then

added and the resulting precipitate was dried in a ThermoSavant SPD1010 SpeedVac System with no heating. The dried precipitate was dissolved in PBS (25mM $NaH_2PO_4$/125 mM NaCl, pH 7.0).

The resulting Ox product was purified using HPLC. 0.1 M ammonium formate, pH 4.8 (mobile phase A) and acetonitrile (mobile phase B) were vacuum-filtered through a 0.2 micron filter prior to use. Ox was applied to a Zorbax SB-C18 HPLC column with an analytical guard column from Agilent Technologies and eluted with 0.1 M ammonium formate, pH 4.8 (mobile phase A) and acetonitrile (mobile phase B). A gradient of 9.5% to 70% mobile phase B over 26 minutes was run at a flow rate of 0.5 ml/min and using a wavelength of 348 nm. The Ox peak eluted at 20.5 minutes. Its identity was confirmed by scintillation counting of the fractions collected in that peak. Pooled fractions from the Ox peak were dried in a SpeedVac system with no heat. After drying, the residue was resuspended in 1 ml PBS. The radiochemical purity of the Ox was confirmed by HPLC. The concentration was determined both by quantitative HPLC and by UV spectroscopy. Specific activity was determined by diluting 1 μl of Ox into 5 ml of Scintiverse II (Fisher Scientific) and counting in a Beckman LS6500 Scintillation Counter. The specific activity of Ox was $4.2 \times 10^6$ cpm/nmol.

**Determination of anti-Ox antibody affinity at 37°C**

Antibody affinities were determined through fluorescence spectroscopy (Foote and Milstein, 1991) using a PerkinElmer LS 50 B luminescence. Temperature control of the cuvette block was maintained by a circulating water bath heated to 37°C. A cuvette containing 20 nM NQ11/7.12 in PBS was placed in the spectrometer and allowed to

equilibrate to 37°C. 30 μg/ml ubiquitin was added as a carrier. An excitation wavelength of 280 nm with a bandwidth of 5 nm and an emission wavelength of 340 nm with a 10 nm bandwidth were used with an 8 second integration time. Ox-GABA solution was added in 40 nM increments and fluorescence readings were taken after allowing time for equilibration. The resulting concentration/fluorescence readings were analyzed by least squares (Foote and Winter, 1992) to determine the $K_d$ of the antibody.

**Cannulation of the animals**

Male Sprague-Dawley rats (300 g) were obtained from Zivic Miller Laboratories. Each rat underwent surgery to place a cannula in the right lateral ventricle for injections and a second cannula in the cisterna magna for CSF sampling. Briefly, the rat was anesthetized with 2.5% isoflurane and fixed in a stereotaxic device. Normal body temperature was maintained through a heating pad. A linear midline incision exposed the frontal, parietal, and occipital bones. The lateral ventricle was located using the coordinates (1.8 mm posterior, 3.8 mm lateral, and 4 mm ventral from bregma) obtained from a stereotaxic atlas of the rat (Paxinos, 1998). A hole was drilled through the skull, a cannula was inserted, and it was fixed to the skull using VetBond (3M) and dental cement. The hole was drilled for the cisternal cannula just posterior to the occipital crest and slightly left of midline to avoid puncture of the superior saggital sinus. The cannula was inserted to a depth of 2 mm below the bottom of the skull and fixed with VetBond and dental cement. Screw-capped cannula dummy wires were inserted into the cannula guides to maintain a closed system. Rats were allowed to recover for at least 24 hours before ventricular injection.

For the experiments where injections and withdrawals of CSF were done via the same site in the cisterna magna, male Sprague-Dawley rats (250 g) were obtained from Charles River Laboratories. These rats had previously undergone surgery to implant a single cannula into the cisterna magna using a procedure similar to that described above. Rats were allowed to rest no more than three days after arrival prior to experimentation to reduce the risk of cannula blockage.

**Determination of NQ11/7.12 half-life in rat CSF**

NQ11/7.12 was iodinated with $^{125}I$ using the Chloramine T method (Hunter and Greenwood, 1962; McConahey and Dixon, 1980). Iodine incorporation into the antibody was 97%, and the specific activity of the antibody solution was 1.25 x $10^6$ cpm/μg. $^{125}I$-NQ11/7.12 (21.3 pmol) was mixed with cold NQ11/7.12 to a final amount of 104 pmol in a volume of 17 μl. The sample was infused by hand over 1 minute through the cisternal cannula of the Charles River rats and the cannula was flushed with 2 μl of normal saline. The end of the infusion was designated as time 0. 10 μl of CSF was withdrawn from the cisternal cannula at various timepoints. The entire CSF sample was counted using a Packard Cobra Auto-Gamma to determine the concentration of NQ11/7.12 in the plasma.

**Antibody buffering experiments**

Samples were prepared for injection consisting of 52 pmol Ox and either 104 pmol anti-Ox antibody, 104 pmol D1.3 control antibody, or no antibody and diluted to 10 μl with sterile PBS. The samples were warmed to 37°C and infused over 1 minute through the intraventricular or cisternal cannula in Sprague-Dawley rats. The end of the infusion was designated as time 0. At various timepoints, 10 μl of CSF was withdrawn.

For one set of rats, the 10 μl of CSF was counted directly by placing it into 5 ml of Scintiverse II and counting in a Beckman LS6500 Scintillation Counter. For another set of rats, the 10 μl CSF was diluted into 100 μl ice cold PBS and used in bound vs. free Ox experiments described below.

**Separation of bound and free Ox using 187.1-Sepharose affinity resin**

The rat anti-mouse kappa antibody 187.1 (obtained from ATCC) (Yelton et al., 1981) was isolated from spent culture medium and purified by passage over a Protein A-Sepharose affinity column. 10 g of CNBr-activated Sepharose 4 Fast Flow (Amersham Biosciences) were washed and coupled with 187.1 according to manufacturer's protocol. 2 mg 187.1 per 10 g of swelled resin were used. The ability of 187.1 resin to bind NQ11/7.12 was tested as described previously (Figure 3.2).

During the antibody buffering experiments, the CSF plus ice cold PBS sample was loaded onto the 187.1 column immediately after the dilution. 3 ml of PBS followed by 3 ml of 0.2 M glycine (pH 2.5) were loaded onto the column in 0.5 ml increments. All the PBS fractions and all the glycine fractions were collected separately for each timepoint and dried down in the SpeedVac. The residue was resuspended in 150 μl PBS and then transferred to a scintillation vial with 5 ml Scintiverse II for counting.

**Long term buffering experiment**

Samples of NQ11/7.12 and Ox were prepared and infused into the cisternal cannula of Sprague-Dawley rats from Charles River Laboratories as described previously. 10 μl of CSF was taken from the cisterna magna at time 1, 10, 20, 30, 60, 90 and 120 minutes. The 10 μl CSF was either counted directly or diluted into 100 μl ice

cold PBS and loaded onto a 187.1 column to separate bound and free Ox. At 24 hours, and again at 48 hours, following the first administration, 52 pmol Ox in 10 μl total volume (diluted with sterile PBS) was infused over 1 minute through the cisterna magna cannula. Timepoints were again taken and analyzed as above.

## Results

### The effect of NQ11/7.12 on Ox lifetime in rat cerebrospinal fluid

To determine its pharmacokinetic behavior in the CSF, tritiated Ox was infused into a rat through its intraventricular cannula, and elimination of Ox was followed through CSF sampling from the cisterna magna cannula. Sampling is done from the cisterna magna as it is the largest CSF compartment. Its location between the cerebellum and the upper brain stem and its large size make it relatively easy to access CSF and withdraw amounts adequate for sampling and scintillation counting (van den Berg et al., 2002). The concentration of Ox declined rapidly from the cisterna magna with an elimination half-life of 1.2 minutes (Figure 4.1). A compound that is cleared solely by the bulk flow of CSF has a typical half-life of 130 to 140 minutes (Bergman et al., 1998). This lifetime of Ox is so short that it indicates elimination by a mechanism much faster than the bulk flow of CSF. A drug candidate with a loss rate on this scale would have little effect on a slow-growing CNS tumor.

In order to test the effect of anti-Ox antibody addition, we employed a kinetically-characterized monoclonal antibody raised against Ox (Foote and Milstein, 1991). The affinity of this antibody, NQ11/7.12, was redetermined at 37 $^{0}$C using fluorescence

spectroscopy and found to be 1.3 nM. An isotype-matched (IgG1) anti-hen-egg lysozyme antibody, D1.3, was used as a control.

We hypothesized that an antibody in the CSF would have a significantly longer half-life than the pharmacokinetic lifetime of most intrathecally-administered drugs. We confirmed this long residence time in the CSF using radioiodinated NQ11/7.12. The monoclonal antibody was infused into a rat via the cisternal cannula, and the elimination of the NQ11/7.12 was followed through cisternal CSF sampling and gamma counting of the sample. The concentration of NQ11/7.12 declined slowly with a biphasic elimination profile. NQ11/7.12 had an early half-life from the CSF of 24 minutes, but a late-phase half-life of 12.8 hours (Figure 4.2). This rate of elimination is significantly longer than that of Ox. It is important to remember, however, that elimination of substances from a location in the CNS cannot be compared to elimination from the plasma since CSF has a unidirectional flow from the lateral ventricles through to the arachnoid granulations while plasma is circulated, propelled by the pumping action of the heart.

For an antibody to buffer a ligand, the antibody-ligand complex must form a long-lived reservoir. In other words, antibody complexation must block ligand elimination. For the first set of rats, Ox was administered with twice the molar amount of NQ11/7.12 through the intraventricular cannula (Figure 4.1). Cisternal CSF sampling showed the rise and fall of Ox concentration as it entered the cisterna magna, peaked in concentration at around 5 minutes, and then was eliminated. Ox that was not administered with an antibody buffer never attained a peak concentration in the cistern. Rather, its elimination was so rapid that much had been cleared before the Ox ever reached that location.

Due to problems with the double cannulation (difficult surgery, cannulas blocked easily), it became more cost and time-effective to continue with the buffering experiments using rats with only a cisternal cannula implanted, and to perform both the injection and CSF sampling using that one cannula. To show that the buffering effect is still able to be visualized using this method, Ox was administered with twice the molar amount of NQ11/7.12 or the control antibody through the rat's cisternal cannula (Figure 4.3). Administration of Ox with NQ11/7.12 showed a significant prolongation of Ox residence time with an Ox elimination half-life of 10 minutes. The Ox administered with D1.3 was eliminated from the CSF with a half-life of 1.0 minutes. This ten-fold increase in half-life indicates that NQ11/7.12 binding is opposing Ox elimination from the CSF even with this injection/sampling regime.

**The effect of NQ11/7.12 on free Ox concentration**

The principle of chemical equilibrium predicts that the Ox in the CSF should partition between free and antibody-bound forms, and that the free pool should be constantly replenished, staying at a concentration near the antibody's $K_d$. To test this hypothesis, the Ox in the CSF samples from another set of rats was separated into bound and free forms through passage over an affinity column. Samples resulting from D1.3 co-administered with Ox were processed in parallel, though no significant antibody-bound label was expected or found. An immobilized rat anti-mouse kappa antibody was used to trap antibody-Ox complexes (bound Ox fraction) while free Ox eluted from the column with a PBS wash (free Ox fraction).

This analysis confirmed that a free pool of Ox does indeed exist in the rat CSF whether the Ox is administered with D1.3 or with NQ11/7.12 (Figure 4.4). Initially, when Ox is administered with D1.3 the amount of free Ox is high (1.4 μM), but it quickly decreases with an elimination half-life of 0.61 minutes. However, when Ox is administered with NQ11/7.12, the initial free concentration of Ox is low (60 nM). This low concentration remains fairly stable over time with an Ox elimination half-life of 1.55 minutes. Therefore, the presence of NQ11/7.12 appears to be buffering the free Ox.

**Long-term buffering of NQ11/7.12**

Since we had previously determined that NQ11/7.12 is retained in rat CSF for a long period of time compared to the elimination half-life of Ox, we wanted to test whether NQ11/7.12 retains the ability to buffer Ox concentration in the CSF days after the initial antibody infusion, much as it is able to in the plasma (Chapter 3). For these experiments, NQ11/7.12 was administered only once into the cisterna magna. Ox was initially co-administered with NQ11/7.12 and additional aliquots of Ox alone were administered at 24 hours and at 48 hours through the cisternal cannula. Cisternal CSF sampling was done to follow total Ox elimination from the CSF. The Ox pharmacokinetic lifetime at 24 hours and 48 hours showed a kinetic profile between that following the initial NQ11/7.12 administration and that of Ox administered with D1.3 (Figure 4.5A). This is likely attributable to the slow clearance in circulating antibody over time. Free vs. bound Ox separation was also performed on another set of CSF samples (Figure 4.5B). Initially, the free Ox concentration at 24 hours and 48 hours is higher than when Ox was administered in conjunction with the antibody buffer.

However, this free concentration decreases and shows a slower elimination profile than Ox administered with D1.3. This is the result of antibody binding up the initial high free Ox concentration and then releasing it over time. Therefore, not only can an anti-Ox antibody buffer the concentration of free Ox in the CSF, but it can do so even days after the initial antibody infusion.

**Discussion**

In this first description and testing of a model for antibody buffering of a small molecule drug in the CSF compartment, several principles emerge. First, anti-Ox antibodies were able to extend the lifetime of total Ox in the CSF. Ox administered alone was rapidly eliminated from the CSF following intraventricular infusion, not even achieving a peak concentration in the cisterna magna prior to the majority of the ligand being eliminated. Antibody addition slowed Ox elimination so that the passage of Ox from the lateral ventricle to the cistern and subsequent elimination from the cistern could be visualized (Figure 4.1). Second, the buffering monoclonal antibody prevents the spike in free ligand concentration characteristic of the unbuffered control. While the concentration of free Ox at the first timepoint for the D1.3 control in Figure 4.4 is 1.4 μM, the uncomplexed Ox when administered with NQ11/7.12 is only 60 nM. In a clinical scenario, having such a large amount of a toxic drug in the CSF could cause many unwanted side effects. The presence of an antibody buffer could minimize high-dose toxicities since only a fraction of the administered dose is at any one time bioavailable. Third, antibody buffering increases the pharmacokinetic lifetime of the uncomplexed ligand that would be available for receptor binding. The buffered free Ox

concentration in Figure 4.4 ranges between 60 and 6 nM, varying little after the 10 minute timepoint. This overall 10-fold difference is miniscule compared to the 500-fold decrease in free Ox of the unbuffered control. If a drug with a similar profile to Ox without an antibody buffer were to be used, after a period where toxic side effects may occur, the drug concentration would spend very little time within the desired therapeutic range before plunging into an ineffective range. This drug would have little clinical import. With a buffer, however, it may gain therapeutic value.

Lastly, we have demonstrated that an antibody is able to buffer ligand concentration over multiple ligand administrations. The antibody can be "recharged" *in situ*. The buffered curves in Figure 4.5 represent an approach to equilibrium from opposite directions. In the initial administration, the Ox and antibody are concentrated in a small volume, and nearly all the Ox is sequestered in antibody complexes. In the re-administrations, the antibody is already dispersed throughout the CNS and all of the Ox starts in the free state. The buffered curves in Figure 4.5 show that antibody binding Ox is affecting its elimination from the CSF at both 24 and 48 hours.

The fact that CSF is found not only in the ventricles and cisterna magna, but in the subarachnoid space anywhere on the surface of the brain or intrathecal area means that injected NQ11/7.12 can be found in any of these locations. In addition, as antibodies can diffuse into the brain parenchyma (Chauhan et al., 2001), free Ox may have far to travel to find a free antibody to bind it. That Ox can still find the antibody is obvious from Figure 4.5. The elimination of both free and total Ox administered at 24 and 48 hours post NQ11/7.12 infusion is slower than Ox administered with D1.3. The buffering

result may have appeared more dramatic if the CSF compartment were more compact. Nevertheless, that buffering still occurs in the convoluted CSF space is important when trying to treat a tumor in a hard-to-access area of the brain, remote from the major areas for drug delivery such as the ventricles or intrathecal space.

NQ11/7.12 injected into the cistern has a half-life in the CSF far longer than that of Ox. It is enough of a difference to yield a ten-fold increase in total Ox half-life when Ox is administered with NQ11/7.12. It has been shown, however, than an antibody can have a more prolonged residence time in the CNS (4-6 weeks) when it is administered into a tumor cyst or a surgically-created cyst (Bigner et al., 1995). Antibody buffering of a cytotoxic chemotherapy drug placed into the cyst could be extremely effective in eradicating residual tumor cells following surgery.

Antibody buffering is not limited to small molecule drugs. Prolongation of the bioactivities of various cytokines by anti-cytokine antibody presence has been reported in the plasma (Rathjen et al., 1992; Finkelman et al., 1993; Courtney et al., 1994). Some of these cytokines, IL-2, TNF-α, and IL-4, have been investigated for the treatment of gliomas (Castro et al., 2003). Since it has been demonstrated that antibody buffering works in the CSF, it could be used to extend the bioactivity of these cytokines against tumor cells.

In the preceding experiments, a model system has been used to demonstrate pharmacokinetic modulation of a ligand by an antibody in the cerebrospinal fluid. Antibody buffering has the potential to reduce the number of intrathecal administrations needed for a drug, as well as to reduce the toxicities associated with a sawtooth drug vs.

time profile. Once the antibody has been administered, it can be re-charged *in situ* multiple times with fresh drug. In the CSF, antibodies are able to equilibrate within the entire compartment, buffering the drug concentration near the desired target. In short, use of antibody buffering as a method of drug delivery would improve upon methods already in place by yielding a stable, bioavailable concentration of a drug that is normally rapidly eliminated from the CSF.

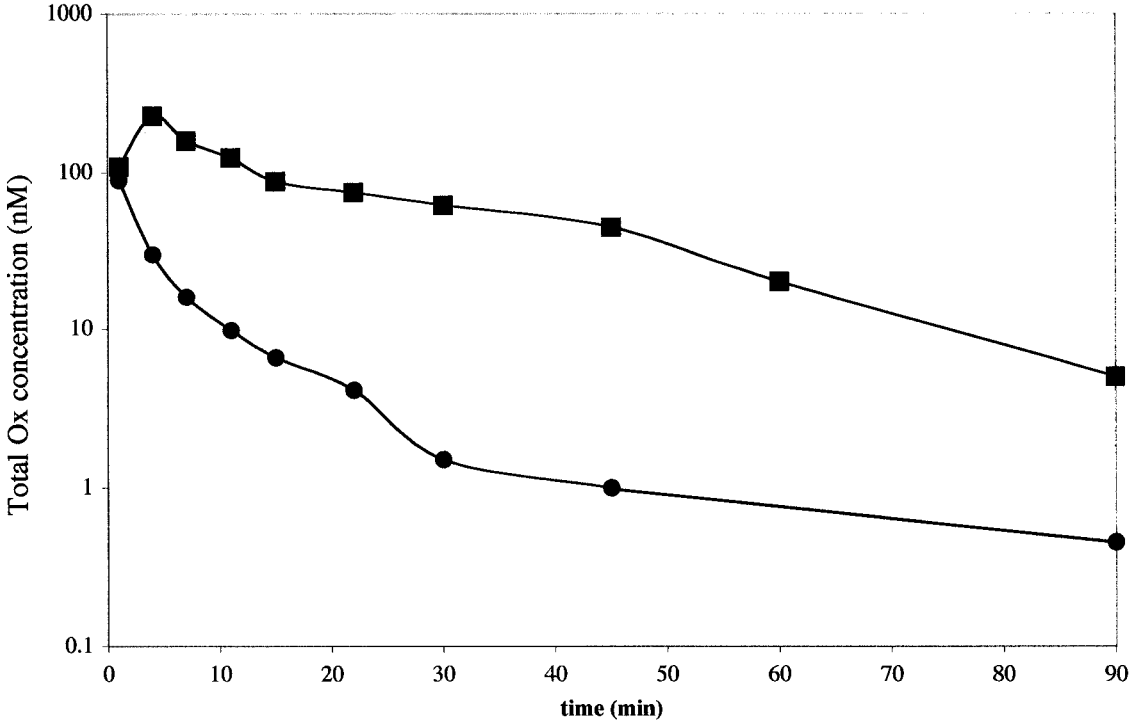


Figure 4.1 Antibody buffering of intraventricularly-administered Ox. Ox was infused, with (squares) or without (circles) an antibody buffer. CSF samples were withdrawn from the cisterna magna. When Ox was administered with an antibody, a gradual increase in Ox concentration is seen as Ox makes its way from the ventricle into the cistern, followed by a slow fall in concentration as Ox is eliminated. Ox administered alone is eliminated too quickly to exhibit a rise and fall in concentration in the cistern.

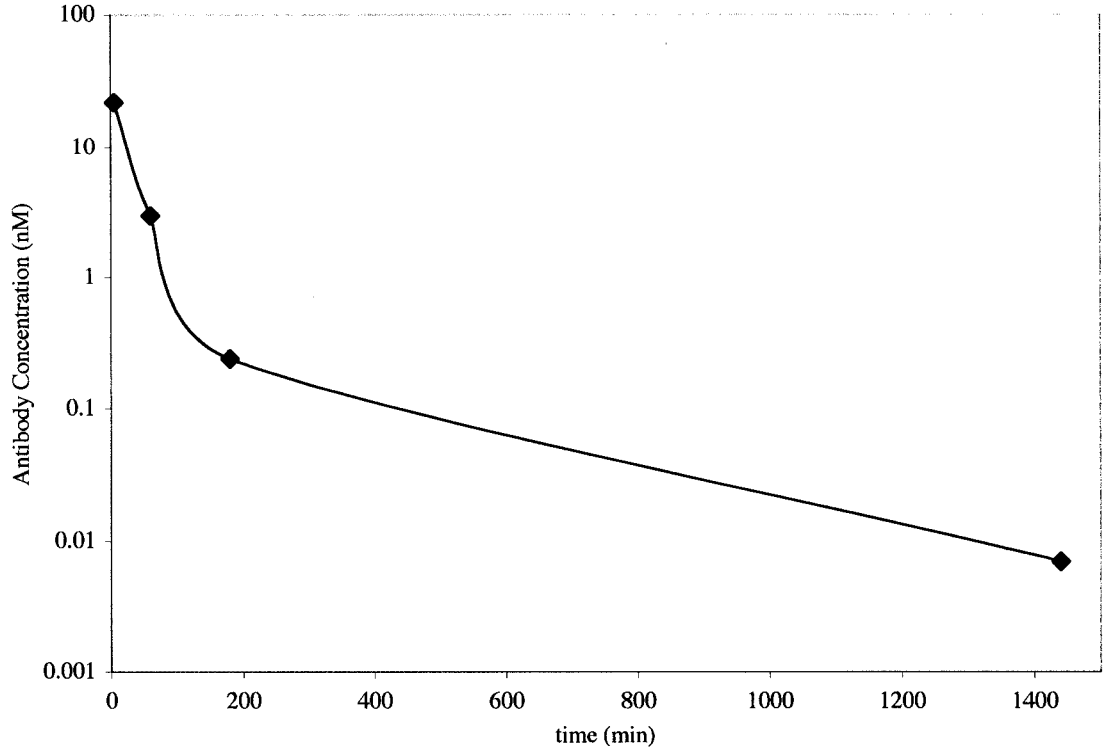


Figure 4.2 Elimination of NQ11/7.12 from the rat CSF. Radioiodinated NQ11/7.12 was infused into the cisternal cannula. CSF samples were withdrawn and gamma counted to determine antibody concentration.

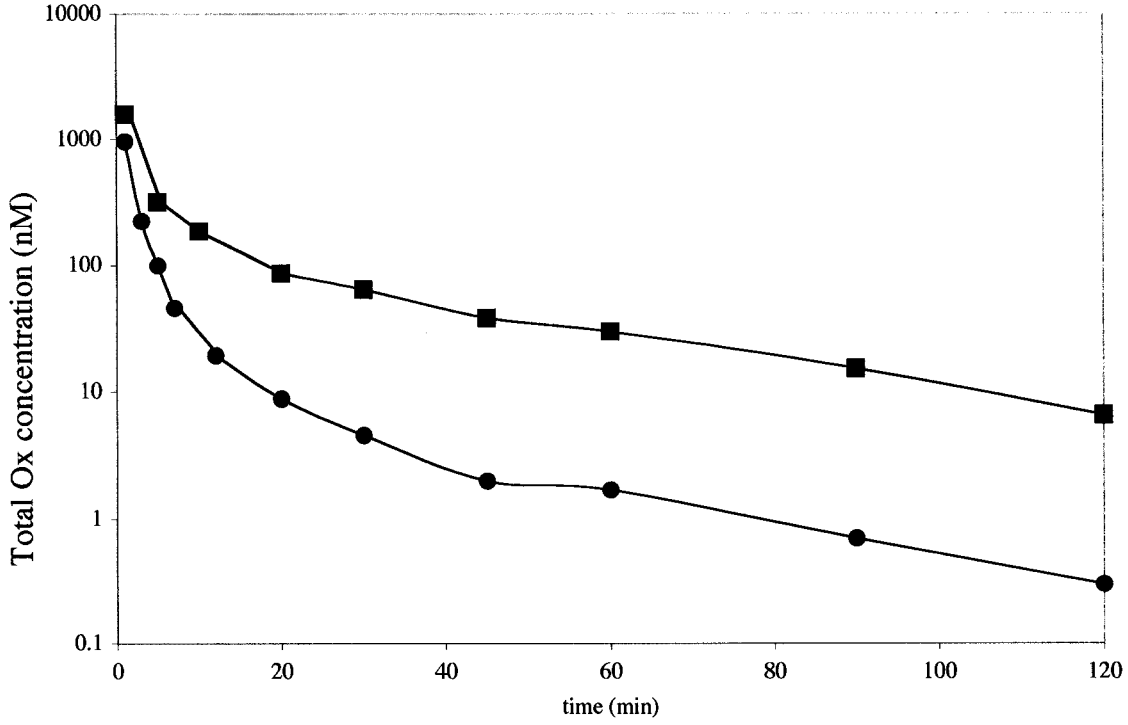


Figure 4.3 Antibody buffering of Ox in the CSF. Ox was infused with (squares) or without (circles) an antibody buffer into the cisterna magna. CSF samples were withdrawn from the cistern and scintillation counted to determine total Ox concentration. Ox administered with an antibody buffer showed a ten-fold increase in half-life as compared to Ox administered alone (10 minutes vs. 1 minute).

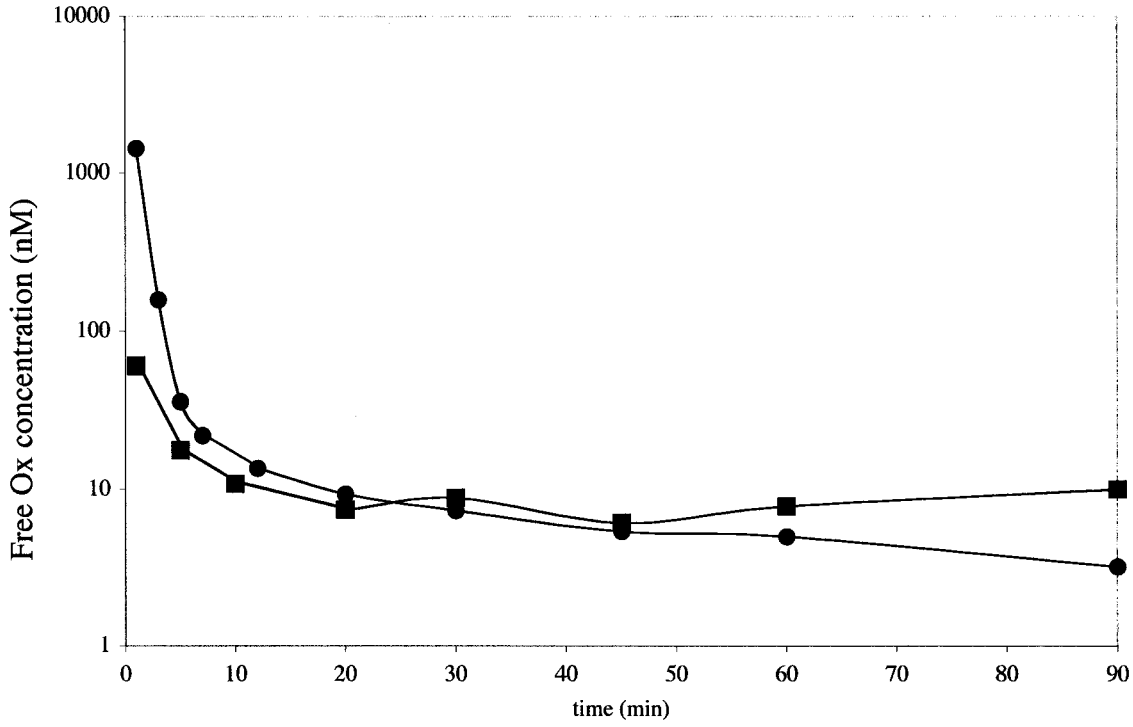


Figure 4.4 Free Ox in the CSF. Ox was administered with (squares) or without (circles) NQ11/7.12. CSF samples were withdrawn and passed over an affinity column to separate bound and free forms of Ox. In the sample with no antibody added, the free Ox concentration started high, but rapidly decreased 500-fold in just an hour. In the sample with antibody, however, the decrease was less than 10-fold in that amount of time.

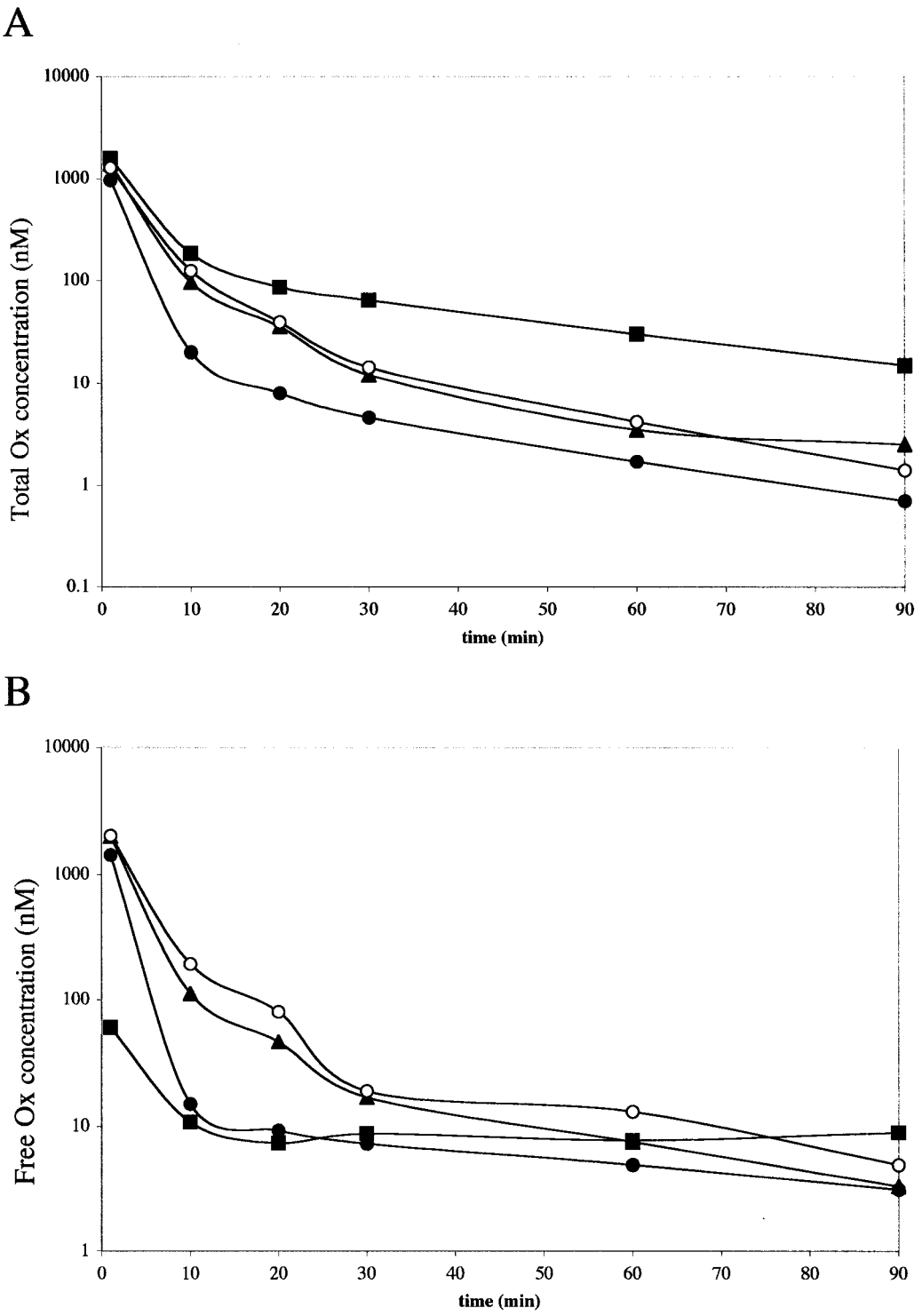


Figure 4.5 Long term buffering in the CSF. CSF half-life of total Ox (A) and free Ox (B) is prolonged whether Ox is administered with NQ11/7.12 (squares) or given alone 24 hours (triangles) or 48 hours (open circles) after the initial NQ11/7.12 infusion. Ox administered with D1.3 is shown for comparison (filled circles).

# Chapter 5:

# Conclusion

This body of work has described the concept of antibody buffering using either a protein or a small molecule hapten in the plasma or cerebrospinal fluid (CSF). This proposed method of drug delivery exploits an important property of antibody-drug interactions: that they follow the Law of Mass Action and the Law of Chemical Equilibrium (Pecht, 1982). Antibodies release and bind antigen according to the dissociation constant of the antibody. A regime is demonstrated here in which a ligand-specific antibody behaves like a buffering agent with respect to the ligand in a manner exactly analogous to a pH buffer. The antibody-ligand complex forms a reservoir of free ligand: as free ligand is lost from the system, it is replaced through re-equilibration of the antibody-ligand buffer. When this concept is translated into a clinical situation, antibody buffering could be used to deliver a stable, bioavailable concentration of a drug that is normally rapidly eliminated from the body, while reducing the occurrence of toxic side effects due to an overabundance of free drug in the body.

Although antibody prolongation of antigen half-life and bioactivity has been described in the plasma for varied substances such as insulin (Palmer et al., 1983; Gray et al., 1985), r-hirudin (Huhle et al., 1999), cytokines (Mihara et al., 1991; Rathjen et al., 1992; Finkelman et al., 1993), and hormones (Bomford and Aston, 1990; Hill and Pell, 1998); it had not been noted in other body compartments such as the CSF. Antibodies, which cannot readily penetrate the blood brain barrier following systemic administration

(Betz, 1989), have a longer residence time when placed inside the CSF compartment than most drugs that are intrathecally administered (Ruggiero et al., 2001). This allows an antibody to provide effective buffering of a small molecule drug in that compartment, as shown when NQ11/7.12 was administered with Ox in the CSF in Chapter 4.

Whether in the plasma or in the CSF, antibody buffering provides numerous potential benefits compared to slow-release therapies such as those described in Chapter 1. The kinetic determinations involved in designing an effective antibody buffer are relatively simple. One does not have to consider matrix degradation rates, volume fraction of drug in matrix, or various areas in a liposome in which drug can localize. Antibodies can be conveniently engineered to the desired dissociation constant with display technologies (Griffiths et al., 1993; Boder and Wittrup, 2000; Hanes et al., 2000). Surgery is not needed to place antibodies inside the body, as implants and pumps require. They are naturally-occurring substances in the body and are less likely to cause novel toxic side effects such as those that liposomes have been known to cause (Lotem et al., 2000; Lyass et al., 2000). Antibody buffering therapy does not require that the antibody be bound and internalized by a tumor cell. In addition, antibodies have the important property of being able to re-bind drug *in situ*. By exploiting this asset, cost of therapy can be reduced by utilizing the same antibody infusion to buffer successive drug administrations.

In many instances in these chapters, the benefits of using antibody buffering to enhance the activity of chemotherapeutic agents have been highlighted. It is important to note, however, that while the concept of antibody buffering is well suited to the delivery

of chemotherapy drugs, there are many other disease treatments that would benefit from this technique. Antibody buffering could be used to maintain digoxin concentration within its narrow therapeutic window, deliver anti-epileptic medication near a seizure focus in the brain, or decrease the incidence of dry mouth with clonidine treatment which often leads to therapy cessation. Because the free drug will be buffered at a concentration near the antibody's dissociation constant, antibody buffering will be most effective for drugs that require a nanomolar concentration for therapeutic activity. Fortunately, this is the case for several small molecule drugs. Examples of drugs that could be effectively buffered by an antibody in the body are listed in Table 5.1.

Antibody buffering is a mechanism of slowing clearance, thereby prolonging bioactivity of a drug. Here it has been shown that antibody buffering is a viable option for increasing the half-life of both a model protein, lysozyme, and a model small molecule drug, 2-phenyl-oxazol-5-one. A reservoir of free ligand exists in the antibody buffered system that is able to act on a specific target. The free pool is subject to elimination, but is replenished from the antibody-ligand buffer. Therefore, antibody buffering represents a novel mechanism of drug delivery that should be further developed for clinical use. It has the potential for many far-reaching applications in the delivery of various types of therapeutic agents.

Table 1. Examples of drugs that could be effectively buffered by an antibody.

| Name of Drug | Peak therapeutic concentration | Problems with maintaining a therapeutic concentration | Therapeutic Use |
|---|---|---|---|
| Alendronate | 5 ng/ml (oral) | | Bisphosphonate/ Paget's Disease |
| Bromocriptine | 691 pg/ml (0.92 nM) | | Neuroleptic malignant syndrome/ Parkinson's disease |
| Buspirone | 1.66 ng/ml (3.93 nM) | Undergoes extensive first pass metabolism | Anxiety/ Irritable Bowel Syndrome |
| Calcitriol | 90-460 pg/ml (0.21-1.1 nM) | | Active hormone form of Vitamin D |
| Clonidine | 0.8 ng/ml (3.0 nM) | Side effect of dry mouth | Hypertension |
| Digoxin | 0.8-2.0 ng/ml (1.0-2.6 nM) | Narrow therapeutic index | Heart failure |
| Dofetilide | 2.3 ng/ml | | Antiarrhythmic agent |
| Exemestane | 11-17 ng/ml | Extensive first pass metabolism | Breast cancer |
| Filgrastim | 4-49 ng/ml | Clearance increases with doses above 4 μg/kg | Treats chemotherapy-induced neutropenia |
| Misoprostol | 674 pg/ml | | Prevents mucosal injury caused by nonsteroidal anti-inflammatory drugs |
| Pramipexole | 1-2 ng/ml | | Parkinson's Disease |
| Raloxifene | 0.5 ng/ml (1.1 nM) | Extensive first pass metabolism | Osteoporosis |
| Selegiline | 1.1 ng/ml (5.9 nM) | Extensive first pass metabolism | Parkinson's Disease, antidepressant |

Data compiled from Goodman and Gilman's The Pharmacological Basis of Therapeutics (2001).

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# Appendix A:

# Preparation of an Anti-topotecan Antibody for Use in Antibody Buffering Therapy

## Summary

In order for antibody buffering to be used as a treatment modality, antibodies will need to be prepared against drugs used to treat diseases. One such drug that has characteristics that make it amenable to antibody buffering is the DNA topoisomerase I inhibitor topotecan. To create an anti-topotecan antibody, topotecan was conjugated to the protein lysozyme to make it immunogenic to mice. Antibodies were raised to this small molecule drug that had affinities in the micromolar range. The antibody with the best affinity, A3C1, was cloned as an scFv and incorporated into a yeast display vector. The A3C1 was subjected to random PCR mutagenesis to create a mutant library and screened for clones that had improved affinity for topotecan. Although further improvements are necessary in A3C1 affinity enhancement, an anti-topotecan antibody engineered to have an affinity near the therapeutic concentration of topotecan holds promise for clinical use in antibody buffering therapy of such cancers as central nervous system tumors.

## Introduction

The anticancer activity of camptothecin was discovered in the 1960s during a National Cancer Institute cytotoxic drug-screening program (Wall, 1966). This compound, derived from the plant *Camptotheca accuminata*, is an inhibitor of DNA topoisomerase I (Hsiang and Liu, 1988). Topoisomerases are enzymes important for the

replication, transcription, and repair of DNA. Camptothecin acts by forming a stable covalent complex with the DNA/topoisomerase I aggregate, the cleavable complex (Hsiang et al., 1985). This results in double strand DNA breaks and the subsequent disruption of the cell cycle for cells in S phase leads to apoptosis and cell death (Del Bino et al., 1991). Camptothecin underwent clinical trials for cancer treatment, but the trials were quickly halted due to high toxicity and unpredictable side effects such as hemorrhagic cystitis (Schaeppi et al., 1974). There were also additional problems with drug administration due to the insolubility of camptothecin.

Currently, several analogs of camptothecin have been synthesized and are being tested in clinical trials or being used in the treatment of different types of cancers. One such compound is the water-soluble drug, topotecan (Figure A.1). Topotecan has a saturated lactone ring that undergoes a pH-dependent reversible hydrolysis from the biologically active lactone form to a carboxylate form. At an acidic pH, the lactone form predominates (Herben et al., 1996). At physiologic pH, the carboxylate form forms a larger percentage of the equilibrium mixture. The cytotoxic activity of topotecan is determined by the concentration of the lactone form *in vivo* since it is this form that is required for both the interaction with DNA topoisomerase I and for the passive diffusion of topotecan into tumor cells (Kollmannsberger et al., 1999).

Topotecan is involved in clinical trials for many different types of cancers including small cell lung cancer, colorectal cancer, and ovarian cancer (Kollmannsberger et al., 1999). In contrast to many cytotoxic drugs, topotecan can penetrate the blood brain barrier (Blaney et al., 1995). In rat xenograft models, tumor regressions have been noted

in ependymoma, medulloblastoma, and high grade glioma following intraperitoneal administration of topotecan (Friedman et al., 1994). Topotecan is currently being used in Phase II and III trials for the treatment of various central nervous system (CNS) tumors (Baker et al., 1996).

CNS tumors are notoriously slow-growing. For a cell cycle inhibitor such as topotecan to be effective, the drug must remain at the tumor site for a long period of time. A pharmacokinetic study in primates showed that the half-life of intravenously-administered topotecan in the cerebrospinal fluid (CSF) is 80 minutes (Blaney et al., 1995). The rapid exit from the CSF could be overcome by repeated administrations of the drug intrathecally. The pharmacokinetics of the drug suggests that maintenance of a therapeutic level of topotecan in humans would require a twice-daily lumbar puncture, an impractical rate- especially for pediatric cases. Antibodies, in contrast, have been shown to have a residence half-life in the CSF of 4-6 weeks when introduced into cavities caused by a CNS tumor or tumor removal surgery (Bigner et al., 1995). Therefore, an anti-topotecan antibody buffer could extend the residence time of topotecan in the CSF, thus making it more effective against a slow-growing tumor.

In order for antibody buffering to be useful therapeutically, antibodies must be prepared against drugs that treat diseases. Creating an antibody to a small molecule drug that is not otherwise immunogenic, however, can present many problems. Here we discuss the preparation of an antibody directed against the small molecule drug topotecan. To be a potent buffer, this antibody needs to have a $K_d$ in the range of the effective concentration of topotecan in the body (less than 10 nM). This is because an

antibody will tend to buffer the free topotecan concentration in a range near the antibody's $K_d$ (Chapters 2 and 3). An antibody to topotecan in the correct affinity range has the potential for use to increase the lifetime of this drug in the cerebrospinal fluid to treat CNS tumors.

## Methods

### Preparation of the topotecan-lysozyme conjugate

For this reaction, EDC was used to crosslink the topotecan through its carboxyl group to lysine residues on the lysozyme. EDC first forms an *O*-acylisourea derivative with topotecan's carboxyl group. Then nucleophilic substitution with the amino group on lysozyme completes the crosslinking. First, 10 mg topotecan (LKT Laboratories) was dissolved in 500 μl DMF (Sigma). 1 ml 0.5 M MES, pH 8.0 was added, followed by 250 μl DMF. The solution was allowed to stir for 10 minutes before adding 0.5 ml 0.5 M MES, pH 6.0. 10 g of EDC (Pierce) was then added and the mixture was stirred for one hour. 0.5 ml of a 20 mg/ml solution of hen egg lysozyme in 10 mM MES, pH 6.5 was added and the reaction was stirred at room temperature for 24 hours. The solution was filtered through a 0.45 micron filter to remove any precipitate and dialyzed against 10 mM MES, pH 6.5 through four buffer changes. The resulting topotecan-lysozyme was filtered through a 0.2 micron filter and stored at $4^0$C. Protein concentration and amount of substitution were determined by UV spectroscopy using extinction coefficients calculated from sequence (Perkins, 1986).

**Anti-topotecan production and screening**

Two mice were injected with topotecan-lysozyme. After a booster injection one month later, the mice were sacrificed and their spleens removed. Hybridomas were prepared and grown in 96 well plates. They were screened for production of anti-topotecan antibodies by ELISA using the topotecan-lysozyme conjugate as described below. Limiting dilutions of the hybridoma cells were done to ensure monoclonality of the antibodies. The hybridomas were grown in oscillating bubble roller bottles (Pannell and Milstein, 1992) and the respective monoclonal antibodies were purified from spent culture supernatant by Protein G-Sepharose affinity chromatography. Antibody purity was assessed by SDS-PAGE and the protein concentration was determined by UV spectroscopy, using extinction coefficients calculated from sequence (Perkins, 1986). Antibody solutions were filter-sterilized and stored at $4^0$C under $N_2$.

**Characterization of anti-topotecan antibodies**

*ELISA*

A topotecan-bovine serum albumin (BSA) conjugate was prepared using a procedure similar to that described above for topotecan-lysozyme. An ELISA was performed using this conjugate in addition to the screening ELISAs using topotecan-lysozyme since the topotecan-lysozyme was the compound used to raise the anti-topotecan antibodies in the mice. Briefly, wells in a 96-well plate were coated with 100 μg/ml topotecan-BSA in a carbonate buffer (50 mM $NaHCO_3$, pH 9.6) and allowed to incubate at $37^0$C for 1 hour. The plate was washed with PBS (25mM $NaH_2PO_4$/125 mM NaCl, pH 7.0), blocked with reconstituted milk, washed again with PBS, and then

incubated for an hour with 100 ng/ml anti-topotecan antibody in PBS with 10 mg/ml BSA added. Positive control wells were coated with 100 μg/ml lysozyme and then incubated with the anti-lysozyme antibody D1.3. Negative control wells were coated with topotecan-BSA and incubated with an antibody that does not bind topotecan, NQ10/2.22. Following the incubation, the plate was again washed and incubated for one hour with the secondary antibody, a peroxidase-conjugated goat anti-mouse IgG, Fc fragment specific (Jackson ImmunoResearch). The plate was read with a kinetic microplate reader (Molecular Devices) after adding color mix (1 mM ATBS and 4 mM $H_2O_2$ in a 50 mM sodium citrate/ 50 mM citric acid buffer). The ELISA was also performed with addition of various amounts of soluble topotecan to the well with the anti-topotecan antibody to determine if competition with binding to topotecan-BSA affected the outcome. This assay also served as a preliminary estimate of anti-topotecan antibody affinity.

*Equilibrium dialysis*

100 μl of a 12.5 μM anti-topotecan antibody solution in PBS was placed into a microdialysis cell (Cambridge Repetition Engineers Ltd.) and the cell was covered with a Spectra/Por 3 (molecular weight cut-off 3500) dialysis membrane. Topotecan solutions of 50, 25, 10, 5, 2, 1, 0.5, 0.2, or 0 μM were placed into a reservoir and the microdialysis cell was added to the reservoir. The solutions were allowed to equilibrate between 24 and 48 hours. The fluorescence inside and outside each dialysis cell was quantified by fluorescence spectroscopy using a PerkinElmer LS 50 B luminescence spectrometer (excitation max of 382 nm, emission max of 522 nm).

*BIACORE*

Three of the anti-topotecan antibodies (A3C1, A3D11, A3G11) and one control, non-topotecan binding antibody (NQ22/16.4) were immobilized on a CM5 chip using the standard amine coupling kit (Biacore AB) at pH 5.0 on a Biacore 3000 machine. Immobilization levels ranged from 13,000 to 15,000 resonance units (RU). Various concentrations of topotecan were passed over the chip surface (from 800 nM to 120 μM in HBS-EP buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20)). Since the off-rate of topotecan from the antibody was so rapid, no regeneration of the chip surface was required between runs. The antibody affinities were calculated by plotting RU at equilibrium versus concentration. Experiments were also performed using topotecan-BSA as the analyte with similar results to using topotecan alone.

**Antibody affinity improvement through random mutagenesis and yeast display**

*Determination of anti-topotecan antibody sequence*

The hybridoma for the anti-topotecan antibody with the best affinity was grown in a 75 ml flask in RPMI 1640 (no HEPES) media with 1% L-glutamine, 1% 100 mM sodium pyruvate, 1% penicillin-streptomycin, 0.1% gentamicin, and 10% fetal bovine serum. To purify the RNA from the cells, the media was removed and the cells were gently washed with PBS. 5 ml of trypsin-EDTA was added and the cells were incubated for 5 minutes. 5 ml of media was added and the cells were centrifuged at 1000 rpm for 5 minutes. The media was removed and the cells were washed with 10 ml PBS. The cells were again centrifuged, the PBS removed, and the cells resuspended in 1 ml TRI reagent

(Sigma). After 5 minutes, 0.2 ml chloroform was added. After 10 minutes, the mixture was centrifuged at 14,000 rpm for 15 minutes. The aqueous phase was removed and added to 0.5 ml isopropanol. After 10 minutes, the solution was centrifuged at 14,000 rpm for 10 minutes. The resulting pellet was washed with 75% ethanol and resuspended in 150 μl of DEPC treated water. mRNA was purified from total RNA using the Oligotex mRNA MiniKit (QIAgen) following manufacturer's protocol.

Reverse transcription was performed using both the K-specific oligonucleotide GACAACGCGTCTCATTCCTGTTGAAGCTCTTGAC and an equimolar mix of three IgG-specific oligonucleotides:
GACAACGCGTCTCAATTTTTCTTGTCCACCTTGGTGC,
GACAACGCGTCTCGATTCTCTTGATCAACTCAGTCT, and
GACAACGCGTTGGAATGGGCACATGCAGATCTCT. 2 μl of each was added to 68 μl purified mRNA, 5 μl of 0.1 mM DTT, 5 μl of 20x dNTPs, and 10 μl of reverse transcriptase buffer. The mixture was incubated at 67 $^{0}$C for 5 minutes and cooled to room temperature for 15 minutes. 4 μl each of RNase inhibitor (Boehringer, 50 units/ml) and Superscript II reverse transcriptase (Invitrogen) were added. The mixture was incubated at 42$^{0}$C for one hour and heated to 100$^{0}$C for 3 minutes before using it for the PCR of antibody variable domains.

Ten individual amplifications were carried out to PCR the heavy chain gene using VHarch1-VHarch10 (Table A.1) and the equimolar mixture of oligonucleotides described above. Another ten individual amplifications were carried out to PCR the light chain gene using VKarch1-VKarch10 (Table A.1) with the single oligonucleotide described

above. 20 μl PCR reactions were prepared using 2 μl of each forward and reverse oligonucleotide, 0.5 μl Taq polymerase, 2 μl 10x PCR buffer, 0.6 μl 50 mM $MgCl_2$, 0.2 μl BSA, 1 μl 20x dNTPs, 6.7 μl $H_2O$, and 5 μl of the reverse transcription sample. PCR was performed using a PTC-100 Programmable Thermal Controller (MJ Research, Inc.). The 30 cycles consisted of: denature at 94°C for 1 minute, anneal at 60°C for 1 minute, and extend at 72°C for 2 minutes. A 5 minute denaturation at 94°C was done before the cycles began and a 5 minute extension at 72°C followed the cycles. The reactions were run out on a 1% agarose TAE gel to determine which oligonucleotide pairs should be employed for sequencing purposes. The bands were cut out and the DNA extracted using the QIAquick gel extraction kit (QIAgen) according to manufacturer's protocol. The DNA from the heavy and light chain variable regions were sequenced using the primer pair used in the PCR that generated the respective fragments.

*Preparation of a yeast display vector*

Primers were designed to link the heavy and light chain variable regions and to add BamH1 and Xho1 restriction sites to the scFv (Table A.2). The heavy chain was amplified using primers P1 A3C1 and P2 A3C1, and the light chain was amplified using primers P3 A3C1 and P4 A3C1. 100 μl PCR reactions were prepared using 4 μl DNA template, 0.5 μl of each primer, 10 μl 10 x PCR buffer, 5 μl 20x dNTPs, and 400 units Vent polymerase (NEB). The 30 cycles consisted of: denature at 94°C for 30 seconds, anneal at 50°C for 2 minutes, and extend at 72°C for 2 minutes. A 2 minute denaturation at 94°C was done before the cycles began and a 10 minute extension at 72°C followed the cycles. The PCR products were gel purified and the resulting heavy and light chain DNA

were joined into an scFv in a subsequent PCR. A 100 µl reaction was prepared consisting of 0.5 pmol heavy chain DNA, 0.5 pmol light chain DNA, 50 pmol P1 A3C1, 50 pmol P4 A3C1, 10 µl 10x PCR buffer, 5 µl 20x dNTPs, and 1 µl Vent polymerase. The cycles were run as described just above and the product was gel purified. The scFv sequence was verified by sequencing the DNA using either primer P1 A3C1 or P4 A3C1.

The antibody scFv and the PYD1 yeast display vector (Invitrogen) were both cut with restriction enzymes BamH1 and Xho1. Following digestion for 3 hours at $37^0$C , 1 µl calf intestinal alkaline phophatase (Promega) was added to the PYD1 digest only and incubation was continued for one hour. The digests were gel purified. Ligation reactions were prepared by combining 1 µl cut PYD1 DNA with 3.5 µl cut insert and 1 µl T4 DNA ligase. Ligations were carried out at $18^0$C for 12 hours. The ligation mixture was transformed into *E. coli* and plated onto 2xYT plates with ampicillin added. A colony was picked from the plate after overnight incubation and grown in 2xYT media with ampicillin. PYD1-A3C1 DNA was extracted using the QIAprep spin miniprep kit (QIAgen) according to manufacturer's protocol.

*Error prone PCR of A3C1 scFv*

Error prone PCR using the nucleotide analogs 8-oxo-dGTP and dPTP was carried out essentially as described previously by Colby et al. (Colby et al., 2004). The primers used were:

Forward- GGTGGACAGCAAATGGGTCGGGATCTGTACGACGATGACGATAAGG TACCAGGATCC

Reverse- GAGACCGAGGAGAGGGTTAGGGATAGGCTTACCTTCGAAGGGCCCT CTAGACTCGAG

The insert DNA was concentrated to 2 μg/μl using Pellet Paint (Novagen).

*Transformation of yeast*

A triple enzymatic digest of the PYD1-A3C1 yeast display vector was performed using BamH1, Xho1, and Sty1. The reaction mixture was incubated for four hours at 37°C. The QIAgen nucleotide removal kit was then used to purify the vector DNA from the enzymes. The DNA was concentrated to 2 μg/μl using Pellet Paint (Novagen).

The yeast strain EBY100 was used to prepare electrocompetent cells for transformation using the protocol from Colby et al. (Colby et al., 2004). In each of seven Eppendorf tubes, 1 μg vector, 9 μg insert, and 50 μl electrocompetent EBY100 cells were combined. The mixture was added to a 0.2 cm electroporation cuvette (Biorad) on ice. The Biorad Gene Pulser device was set to 25 μF, ∞ Ω, and 0.54 kV. The time constant should be about 30 msec with these settings. Pulsing was carried out at room temperature and 1 mL YPD medium (1% yeast extract, 2% peptone, 2% dextrose) was added immediately after pulsing. The cuvettes were incubated at 30°C for one hour with shaking at 250 rpm. The cells were spun down at 3500 rpm in a microcentrifuge, resuspended in 50 ml YNB-CAA + glucose medium (0.67% yeast nitrogen base (YNB), 0.5% casamino acids, 2% glucose), and incubated at 30°C overnight with shaking. Serial dilutions were plated on minimal dextrose + leucine plates (0.67% YNB, 2% glucose, 0.01% leucine, 1.5% agar) to determine transformation efficiency.

*Labeling of yeast cells and analysis using flow cytometry*

The transformed yeast overnight cultures were grown to an $OD_{600}$ of greater than 1. A 5 ml culture of YNB-CAA + galactose medium (0.67% YNB, 0.5% casamino acids, 2% galactose) was inoculated with the overnight culture to a final $OD_{600}$ of 1. The yeast cultures were induced at 20°C with shaking for 36 hours. 0.2 $OD_{600}$-ml yeast were collected into Eppendorf tubes and rinsed with PBS/BSA (PBS with 0.1% BSA). The cells were incubated on ice for one hour with anti-His tag antibody (1:100 dilution in PBS/BSA) (Covance). The cells were then rinsed with ice cold PBS/BSA and incubated with a mixture of 100 μM topotecan plus 20 μl of R-phycoerythrin-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) for one hour on ice. The cells were rinsed with ice cold PBS, resuspended in 0.5 ml of ice cold PBS, and transferred to a tube for flow cytometry and fluorescence activated cell sorting (FACS).

Successive rounds of FACS were performed to isolate antibodies that had improved binding to topotecan. The phycoerythrin (PE) signal was used to show that antibody was being expressed on the yeast cell surface. Since topotecan is naturally fluorescent, it was used to show antibody binding. A 410/420 filter was used to detect PE. A 510/520 filter was used on a UV laser to detect topotecan. Sort gates were drawn conservatively on initial sorts so as not to miss improved clones. Gates were narrowed in subsequent sorts. Cells were sorted directly into YNB-CAA + glucose medium containing kanamicin antibiotic to diminish the risk of bacterial contamination. They were grown overnight at 30°C with shaking before induction with YNB-CAA + galactose medium for the next round of labeling and sorting.

Following the final sort, cells were plated on minimal dextrose + leucine plates. Colonies were picked and grown in separate overnight cultures in YNB-CAA + glucose medium. The plasmid was recovered from the yeast using the Zymoprep yeast plasmid miniprep kit (Zymo Research). In order to sequence the yeast plasmid, it was first transformed into *E. coli* and re-isolated using the QIAprep spin miniprep kit. The plasmid was sequenced using the primer:

CTGTACGACGATGACGATAAGGTACCAGGATCC.

## Results and Discussion

### Isolation of anti-topotecan antibodies

In order for the small molecule drug topotecan (molecular weight 421 Da) to be immunogenic in mice, a conjugate of topotecan to the carrier protein lysozyme was prepared. UV spectroscopy revealed that the conjugate had approximately 2 topotecan molecules per lysozyme. This substitution rate is not high, but it was enough to be able to raise antibodies to this small molecule drug in mice. Mice were injected with topotecan-lysozyme, and hybridomas prepared from their spleens were analyzed for production of antibodies to topotecan. Seventeen antibodies were shown to bind to topotecan-lysozyme in preference to lysozyme alone by ELISA. The results for several of these antibodies are shown in Figure A.2. In addition, the presence of soluble topotecan in the ELISA assay was able to reduce the apparent binding to topotecan-lysozyme bound to the ELISA plate. This also indicated the preferential binding of the anti-topotecan antibody to topotecan over lysozyme.

**Characterization of the anti-topotecan antibodies**

Several methods were employed to determine the affinity of the anti-topotecan antibodies for topotecan. First, an ELISA was performed with a topotecan-BSA conjugate to further show that the anti-topotecan antibodies were indeed binding to topotecan and not to lysozyme. Then, an additional ELISA done with known amounts of soluble topotecan added. Although a solution of topotecan greater than 400 μM could not be produced due to problems with topotecan solubility, competition for the binding of anti-topotecan with topotecan-BSA was seen with as little as 4 μM soluble topotecan. A decrease in binding to topotecan-BSA was not seen with lower amounts of soluble topotecan. Therefore, it seems that many of these antibodies have affinities for topotecan in the micromolar range. Results for a representative anti-topotecan antibody, A3C1, are shown in Figure A.3.

It was difficult to elucidate actual antibody affinity more than was shown by ELISA. Equilibrium dialysis, a technique used to analyze the binding of a low molecular weight ligand to a macromolecular receptor, was performed. Here, if the antibody binds topotecan, the amount of topotecan, and hence the amount of fluorescence, will be greater inside the microdialysis cell than outside of it. In these experiments, however, fluorescence inside and outside the microdialysis cells remained fairly similar for all topotecan concentrations used (data not shown).

Biacore analysis was performed on three of the anti-topotecan antibodies: A3C1, A3D11, and A3G11. These results were only a little more conclusive than those of the previous experiments. At the higher topotecan concentrations, the RU value at

equilibrium jumped to numbers much higher than expected (Figure A.4). This could have been due to problems with topotecan solubility at the surface of the CM5 chip. Experiments using the topotecan-BSA conjugate in place of topotecan showed similar results. By ignoring the points at higher concentrations, it was possible to assign approximate affinities of 42 μM for A3C1, 47 μM for A3D11, and 126 μM for A3G11. These results merely confirm the data obtained from ELISA, that the antibodies have affinities for topotecan in the micromolar range. This seemingly low affinity, however, is not unusual for anti-hapten antibodies prepared in mice (Ternynck and Avrameas, 1986; Schier et al., 1996; Suarez et al., 1996).

**Improvement of anti-topotecan antibody affinity**

Antibody affinity for a small molecule drug must fall within certain windows for antibody buffering to be effective. Because topotecan is effective at concentrations lower than 10 nM (Akil et al., 2002) and since previous studies have shown that antibodies buffer free drug concentrations near the antibody $K_d$ (Chapter 3), an antibody with an affinity for topotecan of less than 10 nM is desired. To improve the affinity of our anti-topotecan, the antibody with the highest apparent affinity, A3C1, was chosen for use in a yeast display system. A3C1 was cloned as an scFv and ligated into the yeast display vector PYD1. The A3C1 scFv was subjected to error prone PCR according to the protocol by Colby et al. (2004) to randomly mutate residues throughout the sequence. The mutant inserts were amplified and then transformed, along with cut PYD1-A3C1 vector, into EBY100 electrocompetent yeast cells. The vector and insert underwent homologous recombination inside the yeast cell and only yeast that had an intact plasmid

were able to grow in the selective media. This method yielded an A3C1 mutant library of $2 \times 10^6$ transformants.

These transformants were screened using flow cytometry and improved clones were selected using FACS. The natural fluorescence of topotecan allowed sorting of the yeast cells for binding using the drug itself with a UV laser rather than needing to employ a topotecan conjugate and a different fluorophore to visualize binding. Five rounds of sorting were performed, although little improvement in binding was seen after the 4$^{th}$ sort. The overall affinity improvement did not appear to be large, but there was definitely a difference in binding between the original A3C1 clone and the cells taken from the 4$^{th}$ sort (Figure A.5).

The cells from the 5$^{th}$ sort were plated and 40 individual colonies were sequenced. Despite the initial promising results, however, these sequences were either exactly the same as the initial A3C1 sequence (3 clones) or had a huge deletion from the BamH1 restriction site to the His tag, a deletion that encompasses more than just the insert region of the PYD1-A3C1 vector (37 clones). The reason for the deletion is unclear. Sequencing also was attempted directly from yeast patches or from the yeast plasmid before transformation into *E. coli.* These sequencing attempts were unsuccessful. Several clones from the original mutant library were sequenced as well. About 20% of these clones had the same deletion as the majority of the 5$^{th}$ sort clones, but the preponderance had intact mutant scFv regions. Although the sorted cells appeared to have improved affinity for topotecan, no intact, mutant scFv was able to be isolated.

## Conclusions

In these experiments, an antibody to topotecan was produced. This antibody was shown to bind to topotecan rather than to the lysozyme that was used as a carrier protein in the antibody production. Both ELISA and Biacore were used to determine that the anti-topotecan antibody affinities were in the micromolar range, but exact affinity was difficult to obtain. Since an antibody affinity less than 10 nM is desired for anti-topotecan to provide an effective buffer clinically, one anti-topotecan antibody, A3C1 was cloned as an scFv and used in a yeast display system. Despite creation of a random mutation library of A3C1 and promising results from flow cytometry, an improved affinity antibody clone was unable to be isolated. This work shows that antibodies can be made to small molecule drugs, but that affinity modifications necessary for their therapeutic utility can be problematic. There are other methods that can be employed for affinity improvement such as phage display and ribosome display. These may be of use in future affinity modifications of A3C1.

Table A.1 List of oligonucleotides used in antibody cloning

| Oligo name | Sequence |
|---|---|
| VHarch1 | GACAGTGCACATGGAATGGAGCTGGGTCTTTCTCTT |
| VHarch2 | GACAGTGCACATGGAATGGAGCTGGATCTTTCTCTT |
| VHarch3 | GACAGTGCACATGGGATGGAGCTGGATCTTTCTCTT |
| VHarch4 | GACAGTGCACATGGGATGGAGCTGTATCATCCTCTT |
| VHarch5 | GACAGTGCACATGAAGTTGTGGATGAACTGGATTTT |
| VHarch6 | GACAGTGCACATGAAATGCAGCTGGGTTATCTTCTT |
| VHarch7 | GACAGTGCACATGAAGTTGTGGTTAAACTGGGTTTT |
| VHarch8 | GACAGTGCACGGGCTCAGCTTGATTTTCCTTGTCCT |
| VHarch9 | GACAGTGCACATGTTTAACATCATTATCTTCACAGT |
| VHarch10 | GACAGTGCACCTCTTCTGCCTGGTGACATTCCCAAG |
| VKarch1 | GACAGTGCACATGAAGTTGCCTTGTTAGGCTGTTGG |
| VKarch2 | GACAGTGCACATGGACATGAGGGCTCCTGCTCAG |
| VKarch3 | GACAGTGCACAGACACACTCCTGCTATGGGTGCTGC |
| VKarch4 | GACAGTGCACACTGCTCTGTGTGTCTGGTGCTCATGG |
| VKarch5 | GACAGTGCACGCTCAGTTCCTTGGTCTCCTGTTGCTC |
| VKarch6 | GACAGTGCACCTTGTGTTCTGGATCTCTGGAGCCA |
| VKarch7 | GACAGTGCACTTCCTGCTAATCATTGTCACAGTC |
| VKarch8 | GACAGTGCACATGGTATCCACACCTCAGTTCCTTG |
| VKarch9 | GACAGTGCACTGCTCTGGCTTACAGGTGCCAGATGT |
| VKarch10 | GACAGTGCACGCCCAGTTCCTGTTTCTGTTAGTGC |

Table A.2 Primers used for preparation of yeast display vector

| Primer Name | Sequence |
|---|---|
| P1 A3C1 | GTACCAGGATCCGACGTGAAGCTCGTGGAGTCT |
| P2 A3C1 | CCGCCAGAGCCACCTCCGCCTGAACCGCCTCCACCT GAGGAGACTGTGAGAGTGGT |
| P3 A3C1 | GTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGG ACATCAAGATGACCCAGTCT |
| P4 A3C1 | CCCTGTAGACTCGAGAGCCCGTTTTATTTCCAGCTT |

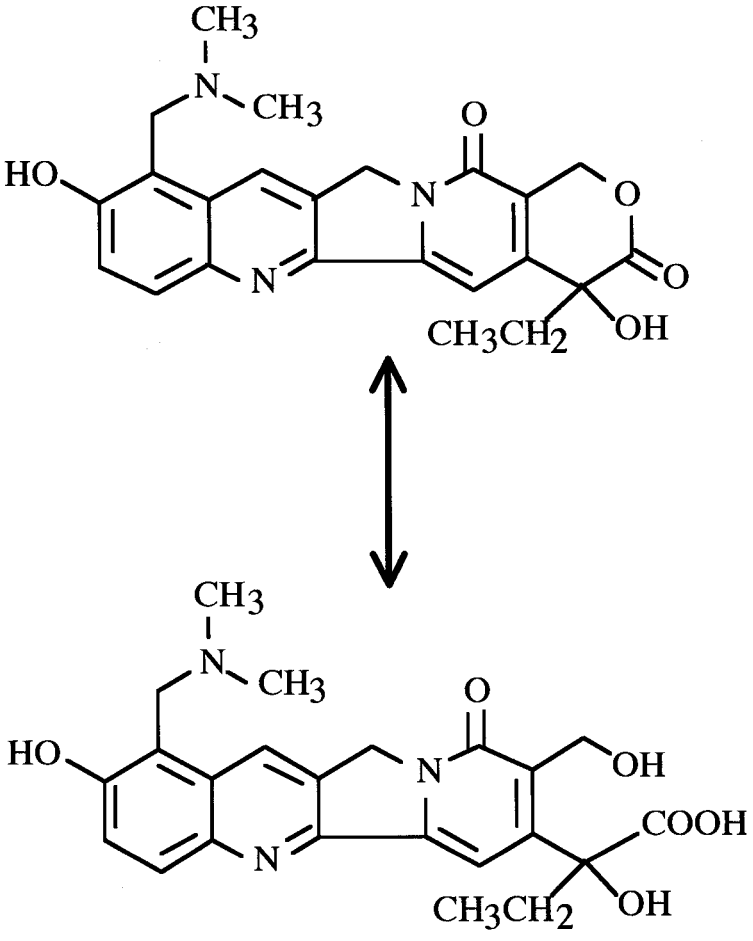



Figure A.1. The chemical structure of topotecan. Topotecan undergoes a pH-dependent, reversible conversion between the lactone form (above) and the carboxylate form (below).

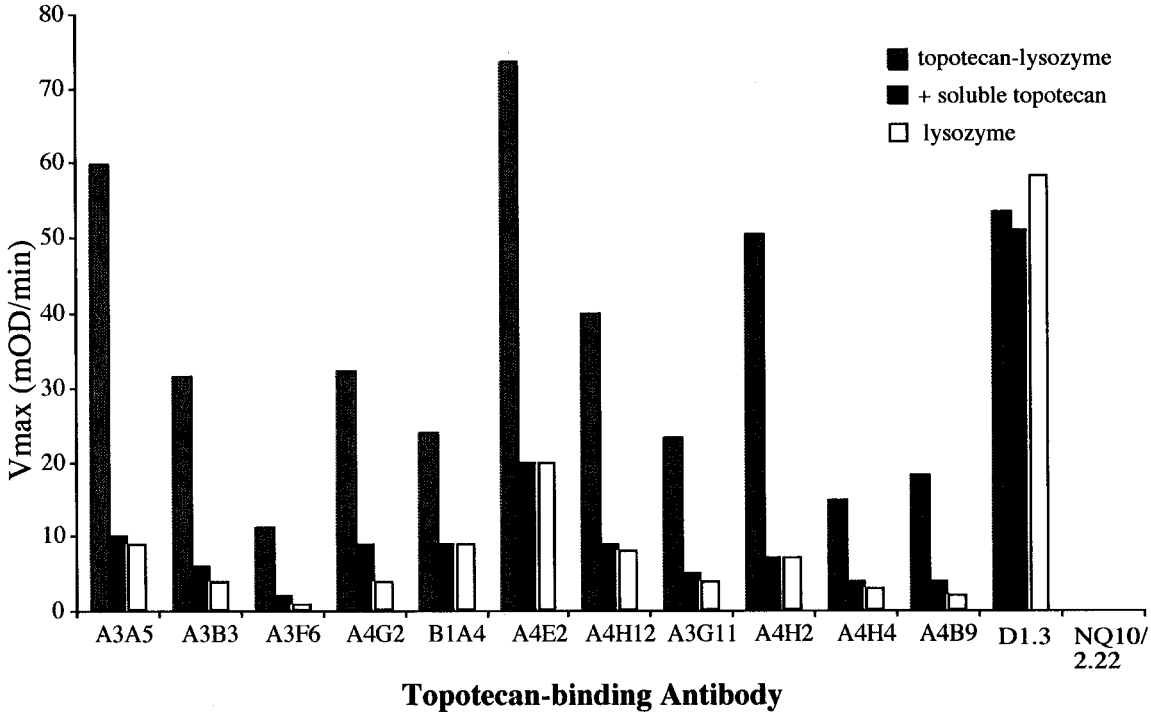



Figure A.2 Anti-topotecan antibodies selectively bind topotecan over topotecan-lysozyme. In addition, binding can be competed off by the addition of soluble topotecan. This figure shows the ELISA data obtained from some of the anti-topotecan antibodies isolated. The blue bar represents binding of the antibody to the topotecan-lysozyme conjugate. The yellow bar is binding to lysozyme alone. The red bar shows the ability of addition of soluble topotecan to compete with binding to topotecan lysozyme.

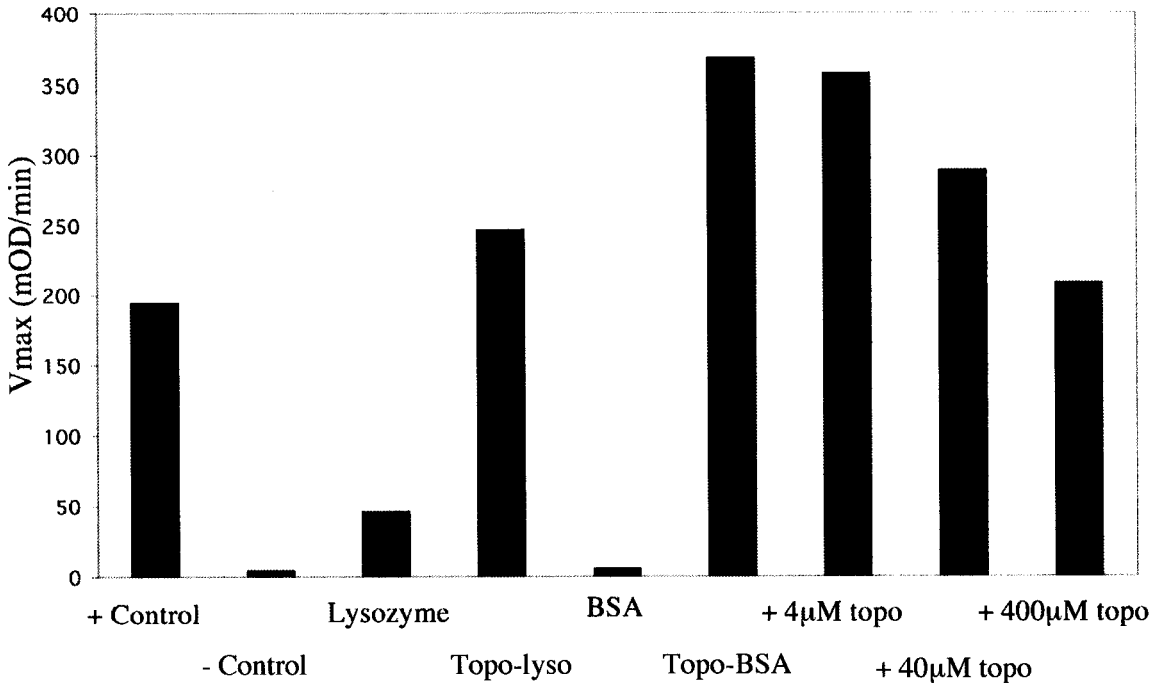



Figure A.3 Competition of soluble topotecan with topotecan-BSA conjugate. The ability of the anti-topotecan antibody A3C1 to bind to topotecan-BSA rather than to BSA is shown by ELISA. This preferential binding to topotecan-BSA is even greater than the preferential binding to topotecan-lysozyme over lysozyme. Next, various concentrations of topotecan were added to the ELISA plate well. Topotecan is able to compete off binding starting at 4 μM, but more significant results are seen at higher concentrations.

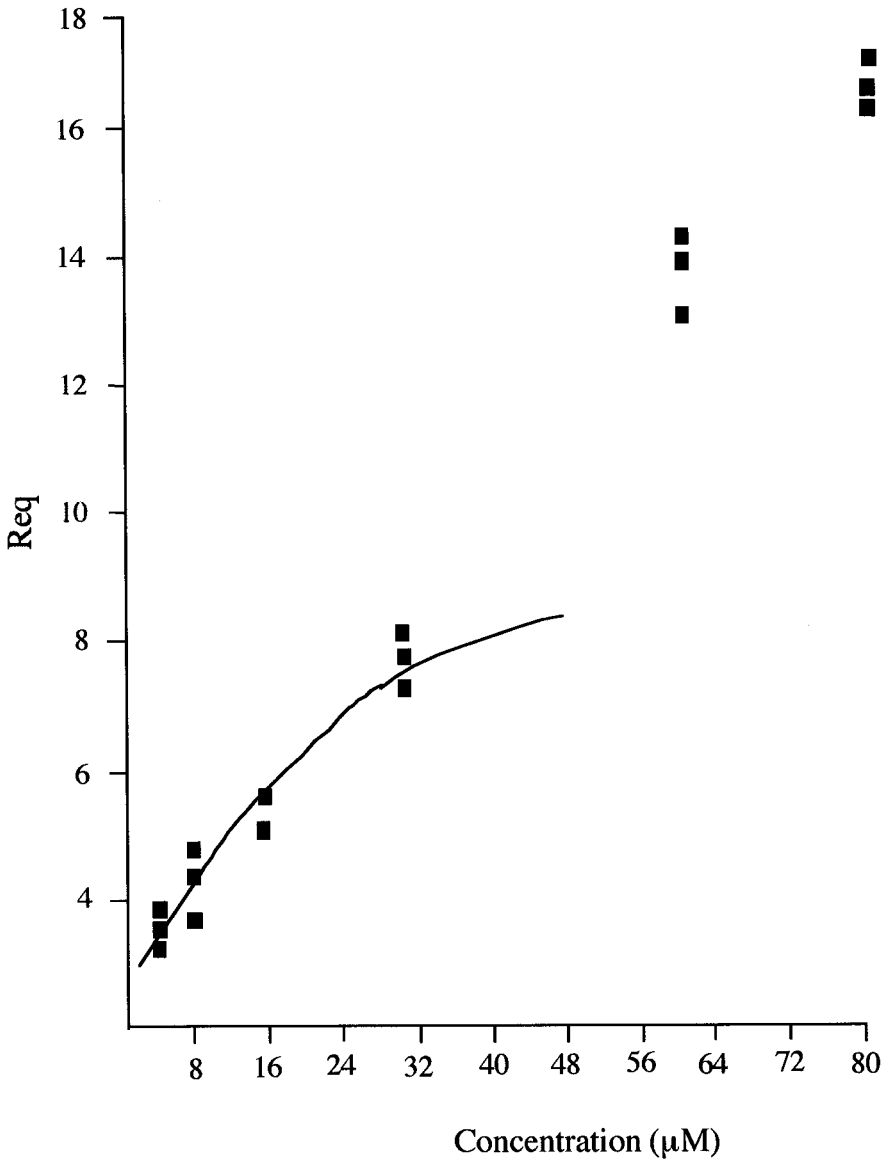
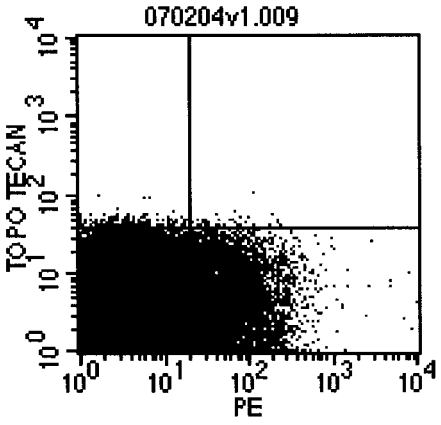



Figure A.4 Biacore data for anti-topotecan antibody A3C1. Biacore analysis was performed on three of the anti-topotecan antibodies, but possible problems with data at higher topotecan concentrations prohibited definitive affinity measurements. Here, the RU at equilibrium (Req) is plotted against topotecan concentration for the anti-topotecan antibody A3C1. By ignoring points at higher concentrations, an affinity of 42 μM is obtained.

A




B

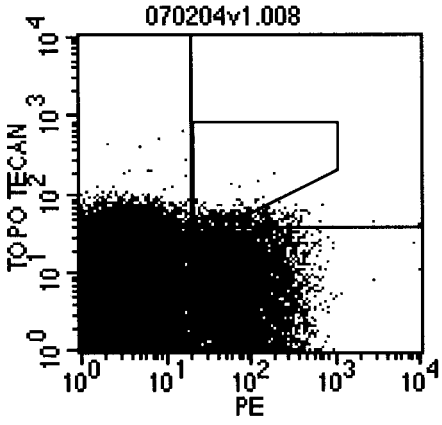



Figure A.5 Flow cytometry results for A3C1 mutant library. Expression is shown on the Y-axis. Topotecan binding is shown on the X-axis. An increase in binding is seen when the flow cytometry results for A3C1 (number in upper right quadrant=206) (A) and the $5^{th}$ sort of the mutant library (number in upper right quadrant=2168) (B) are compared. Cells in the sort gate shown in (B) were collected and plated for yeast plasmid isolation and sequencing.

# VITA

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ABSTRACTS:

O'Hear, C.E. & Foote, J. (2004) Antibody Buffering: a novel mechanism of drug delivery. J Investig Med. 52 (1). Abstract #217.

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