The Pharmacokinetics Of A Novel Anti-Renalase Antibody Used For The Treatment Of Melanoma In A Congenic Mouse Species

Oriyomi Alimi

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The pharmacokinetics of a novel anti-renalase antibody used for the treatment of melanoma in a congeneric mouse species

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

By
Oriyomi Alimi
YSM 2020
The pharmacokinetics of a humanized anti-RNLS monoclonal antibody used for the
treatment of melanoma in a congenic mouse species

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Cancer cells can overcome signaling that restrains their growth and promotes senescence and cell death. Renalase (RNLS) is a secreted flavoprotein that functions as a survival factor after ischemic and toxic injury, signaling through the plasma calcium channel PMCA4b to activate the PI3K/AKT and MAPK pathways. In addition, recent studies, indicate that dysregulated RNLS signaling promotes survival of melanoma cells due to its capacity to augment expression of growth-related genes and to promote macrophage polarization toward a tumor promoting phenotype. Preliminary data show single agent efficacy of the inhibitory rabbit monoclonal m28-RNLS in PD1 resistant tumors. We humanized m28-RNLS and selected a variant (K5), formatted to human IgG1 containing an Fc portion that promotes complement binding. The purpose of these experiments is to determine the pharmacokinetic (PK) profile in C57BL6 mouse of K5, a therapeutic candidate for cancer therapy. K5 was produced by transfecting human embryonic 293 kidney cells using standard methods. To determine the pharmacokinetics of K5, mice (n=28) were dosed with 6.4 mg/kg K5 either as a single intravenous injection (i.v) via tail vein or subcutaneous (s.c) injection. Blood was collected at 14 time points and stored at -20°C until assayed for K5 concentrations. K5 concentration was determined using a direct-sandwich ELISA and uses His-tag purified recombinant human renalase as capture antigen and goat anti-human Fc IgG as detection antibodies. Standards were made in duplicate using known concentrations of antibody diluted into mouse serum. Standard curves were interpolated using GraphPad Prism. Concentrations were determined from the standard curves. Concentration
versus time data were analyzed using a non-compartmental analysis in R Studio. PK data indicate that K5 may behave in vivo like other commercially available IgG1 molecules. It exhibits high bioavailability, with a rapid distribution phase. Rapid elimination of the humanized antibody may reflect formation of anti-tumor antibodies. Pharmacokinetic data in mice can be helpful in predicting the pharmacokinetics of K5 in humans using allometric scaling but may not be useful in C57BL6 mice. Pharmacokinetic testing of K5 in transgenic mice with human neonatal receptors (e.g. Tg32 homozygous mice) or other species such as non-human primates is needed before first in human doses.
ACKNOWLEDGEMENTS

I thank Dr. Gary Desir for his superb mentorship and all members of the Desir laboratory for their advice and assistance, in particular Xiaojia Guo, Heino Velasquez, Bob Safirstein, Tian-min Chen. Additionally, I thank the Howard Hughes Medical Institute Fellows program for funding and career mentorship as well as the Office of Student Research at Yale for affording me this opportunity.
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INTRODUCTION

The incidence of melanoma is rising faster than that of all malignancies as evidenced by a rise in incidence from an estimated 54,200 cases in 2003 to 91,270 in 2018 [1]. Advanced melanoma is the deadliest of skin cancers with an anticipated death rate of 9,320 in 2018 [1]. Despite approvals of systemic therapies for unresectable melanoma that demonstrate prolonged survival, incidence is rising.

Melanoma is resistant to chemotherapy and response rates (RR) for classic chemotherapy are <20% with no prolongation of survival [2],[3]. Survival is further prolonged with the addition of MEK inhibitors [3, 4] Immune checkpoint inhibitors (ICPi) are effective, regardless of BRAF mutation, and while responses are more durable, they are less frequent. Responses to CTLA-4 or PD-1 inhibitors are seen in up to 19% and 40% of melanoma patients [5-10]. The combination of CTLA-4 and PD-1 inhibitors results in a higher RR of 57.6%, with improved progression free survival (PFS) [6]

ICPi, BRAF, and MEK inhibitors are important breakthroughs for therapy but not all patients respond to immune checkpoint inhibitors or have BRAF^{V600E} mutations. Moreover, given the toxicities of the combined checkpoint inhibitors, alternative approaches might be less toxic than dual checkpoint inhibitors and equally (or more) effective in some patients, resulting in an improved therapeutic window.

Previous work from the Desir lab has shown Renalase (RNLS), a secreted flavoprotein with oxidoreductase activity [11-16], signals through the plasma membrane calcium ATPase PMCA4b and engages in the MAPK and PI3K pathways to promote cell survival in the setting of ischemia or toxic injury [16, 17]. Figure 7 shows increased RNLS expression in advanced melanoma, with
worse patient survival[18]. Survival for patients with tumors containing less infiltrating CD163+ tumor associated macrophages (TAM) was higher at 5 years (p<0.04), suggesting that high RNLS and CD163+TAM content is associated with a more aggressive phenotype.

In vitro and in vivo studies of knock-down of RNLS by siRNA inhibit melanoma cell survival. Notably, RNLS knockout mice remain fully viable suggesting targeting RNLS is a feasible strategy for treating melanoma. Allograft and metastatic murine models (B16F10 and YUMM1.7 mouse melanoma cell lines) have shown dramatic decrease in tumor burden with the administration of an inhibitory rabbit RNLS monoclonal antibody (m28-RNLS) raised against a human renalase peptide. Preliminary studies have also shown synergism between m28-RNLS and anti-PD1 in tumors resistant to anti-PD1 (Figure 8)

The mechanism by which m28 kills tumor cells has been investigated[18, 19]. It causes cell cycle arrest by markedly increasing p21 in tumors, and decreases levels of pAkt and pERK. TUNEL staining reveals a significant increase in apoptotic cells in treated tumors, temporally related to p38 MAPK phosphorylation and Bax activation[18]. Lastly, inhibition of RNLS signaling results in an increased ratio of CD86+ to CD163+ TAM, i.e. anti-RNLS results in switching of the TAM phenotype to a tumor-inhibitory rather than tumor-promoting phenotype. m28 has a favorable toxicity profile as determined by a 3-week study in WT mice, with no cardiac, liver kidney, hematologic and neuronal toxicity (blood pressure, and cardiac, liver and renal function, and pathological examination).

The data indicate monoclonal antibodies that inhibit RNLS signaling have the potential to be first in-class cancer therapeutics. m28-RNLS was selected as the lead development candidate among a panel of 36 rabbit mAbs (generated against 4 RNLS peptides and recombinant RNLS) based on
binding affinity (KD ~ 2nM), and in vitro and in vivo anti-tumor efficacy data. Therefore, the Desir lab has developed a panel of humanized m28-RNLS antibodies as therapeutic candidates. To that end, m28 was cloned and sequenced. Its complementary determining regions (CDRs) were determined. The CDRs of m28-RNLS were grafted onto the 4D5 (Herceptin) framework in the Fab antibody fragment format within a phagemid using standard methods. Oligonucleotide-directed mutagenesis was used to construct affinity maturation libraries for m28-RNLS variants. 23 variants, selected for cross reactivity to both human and mouse RNLS, were rank ordered using in-solution competitive ELISAs. Five variants, m28-K2, m28-K5, m28-13, m28-14, and m28-16, were chosen for reformattting to a- mouse IgG1 (chimera with human Fv; non-complement fixing) and to human IgG1(fully humanized; complement fixing) and human IgG4 (fully humanized, non-complement fixing) for cell-based testing, surface plasmon resonance. The mouse human chimera and fully humanized m28 variants have improved binding affinity and in vitro activity (Figure 9). M28-K5 (K5), a therapeutic candidate for cancer therapy, is the subject of this MD Thesis for in vivo pharmacokinetic (PK) testing in mouse.

Early assessment of new therapies in animal models for the prediction of PK in humans helps streamline the drug discovery process by selecting candidates with properties that have the most chance at clinical success while saving valuable time and research capital. Pharmacokinetic interspecies scaling has been used for the design of dosage regimens in untested animal models and ultimately has proven useful in identifying a human mg/kg dose [26]. Two approaches have been used; The physiologically based PK (PBPK) model and allometric scaling. The PBPK model describes drug disposition in a physiological flow model with multiple compartments.
It is useful for predicting drug concentrations in difficult to reach tissues and tumors [31]. However, the PBPK model is not readily established, requires complex computer software with simultaneous model fitting, and difficult to validate in humans [31]. The allometric scaling method views the animal as a whole entity and has been validated in humans [30,31]. The method fundamentally assumes biochemical, anatomical, and physiological similarities across animal species humans.

In monoclonal therapeutics, preclinical PK/PD studies in non-human primates are a necessary part of the pathway to first in man studies but are expensive [27]. Use of murine models with human FcRn have shown their predictive ability for the human PK properties of the tested antibody. Athymic mice and humanized murine models reduce the risk for an unknown immune response affecting experimental data when evaluating humanized mAbs in preclinical models. C57BL6 mice are wild type mice with intact immune systems and PK data is less predictive compared to humanized or athymic mice [26]. Due to my animal work experience and the early stage of the lead optimizing process for K5 it was more feasible to initially gather experimental PK data for K5 using C57BL6 wild type mice.
STATEMENT OF PURPOSE & HYPOTHESIS

Independent of its enzymatic properties (intracellular NADH oxidase), secreted renalase (RNLS) functions as a survival factor that interacts with its receptor (PMCA4b) to activate protein kinase B, and JAK/STAT, and the mitogen-activated protein kinase pathways. These signaling properties are critical to its cytoprotective effects. In addition, recent studies indicate that dysregulated RNLS signaling promotes survival of melanoma cells due to its capacity to augment expression of growth-related genes and to promote macrophage polarization toward a tumor promoting phenotype. Preliminary data show single agent efficacy of the inhibitory rabbit monoclonal m28-RNLS in PD1 resistant tumors. The projection of human pharmacokinetic (PK) profiles to help estimate dose and dosing regimens is important during clinical development, especially prior to first-in-man studies, as drug efficacy and toxicity are usually linked to drug exposure. We hypothesize that the pharmacokinetic profile of humanized variant of m28-RNLS (K5) will include a rapid distribution phase and a slow elimination phase with saturable kinetics, limited off target toxicity, high potency and a long serum half-life.

Aim 1: Investigate the pharmacokinetics of humanized m28-RNLS variants in wild type mice (C57B6) mice.

Given that anti-RNLS is a humanized monoclonal IgG1 antibody, we expect it to demonstrate a biphasic PK profile with saturable kinetics and a long serum half-life. To that end, we will administer various doses of mAb to WT mice and measure plasma levels of by ELISA.
MATERIALS AND METHODS

K5 is a novel antibody engineered by the Desir Laboratory. Its development using phage display technology was completed by Pei Li Wang and Bryce Nelson. K5 was then manufactured using recombinant DNA technology and was expressed in HEK293 cells.

E. Coli Transformation and Plasmid Purification. Plasmid DNA both light chain and heavy chain for K5 was obtained from Pei Li Wang. 5x KCM buffer (1M KCL, 1M CaCl2, 1M MgCl2, ddH20) was used to make E. Coli competent to take up the foreign plasmid DNA). For both light chain (LC) and heavy chain (HC) components of K5 an aliquot of 10µl of TOP 10 Competent Cells (ThermoScientific®) was added to a new Eppendorf tube. 5x KCM buffer (5µL) was added to each tube and at least 20ng of plasmid DNA diluted in ddH20 (qs 25µl). Cell-buffer-DNA mixture was then incubated on ice for 25 minutes. 150µl of SOC media (Invitrogen™) was then added to the mixture and incubate at 37ºC for 60 minutes. E. Coli were then plated on antibiotic Agar plates (Zeocin for HC, Blasticidin for LC) and incubated overnight at 37 ºC. Individual colonies were sampled and placed in 5mL of 2YT media (Tryptone, Yeast Extract, NaCl) with the appropriate antibiotic and then placed in a test tube on a shaker for 6-8 hours. The grown E. Coli were grown up in Erlenmeyer flasks (250mL, 500mL, 1L, 2L) overnight at 37 ºC on a shaker). Plasmid DNA was then purified using the NucleoBond® Xtra Midi/Maxi Kit (Macherey-Nagel). Plasmid DNA concentrations were determined using a BioDrop spectrophotometer.

HEK293 Transfection

Materials:

OptiPro SFM transfection media; 1L Invitrogen #12309019 (other sizes available)
K5 light chain and heavy chain were introduced into mammalian 293 cells (HEK293). HEK293 cells (Expi293F™) were grown in Expi293 Expression Medium (Gibco, Cat#A14351-01) to a density of 1.8-2.0 x 10^6 cells/mL on the day before transfection. On the day of transfection cells should be at least 2.5 x 10^6 cells/mL; preferably over 3 x 10^6 cells/mL. Cell culture sizes ranged from 15mL in 125mL flasks to 200mL in 2L flasks. Equal amounts of heavy and light chain DNA was diluted and gently mixed into OptiPro SFM in a conical tube under sterile conditions. After a 5 minute incubation at room temperature, PEI was added at a ratio of (PEI:DNA 6:1) and incubated for 10-30 minutes at room temperature. The DNA-PEI complex was then slowly added into the flask containing the cells while slowly swirling the flask. Transfected cells were then placed in an incubator at 37°C, 5% CO2, and shaken at 125rpm. Day 1 after transfection (~16-18 hours post transfection) BalanCD CHO Feed 3 + Glutamax (stock: 100mL gmax added to 1L BalanCD CHO Feed 3) and valproic acid (VPA) solution was added. Day 2 until the day cells were harvested, cell density, viability and glucose levels were monitored. IgG protein from the cell culture was harvested when viability dropped below 80%.
**Antibody Purification from Transfected Cells.** Transfected Expi293 cells were centrifuged at 7,000 rpm for 15 minutes. The supernatant was collected and passed through a 0.22µm low-protein binding filter unit (Corning Cat#430769). An aliquot of 1mL rProtein A Sepharose (GE Healthcare, Cat#17-1279-03) was washed with 49ml of PBS two times by spinning at 1,500 rpm for 5min. One milliliter of sepharose was mixed with 150L of supernatant in conical tubes for at least 2 hours. The supernatant-sepharose mix was then passed through an Econo-Pac disposable chromatography column (BioRad Cat#732-1010) by gravity. The column was washed with 20ml of PBS by gravity. IgG elution buffer (pH 2.8, 50mM NaH₂PO₄, 100mM H₃PO₄, 140mM NaCl) was added to the column and elute was collected with a clean polypropylene tube (1.3mL Elute). 1M Tris-HCl (pH 11.) was immediately added to neutralize the elute (144µL). The eluted antibody was then dialyzed in 10kDa MWCO Dialysis cassette (Thermo Scientific Cat#66810) against 2L of 0.9% NaCl + 10mM Tris, pH7.5, over an 18 hour period.

**Antibody Confirmation via SDS-Page.** 7.5% polyacrylamide gels (Bio-Rad Mini-PROTEAN® TGX™ Gels Cat#456-1024) were loaded with antibody samples purified from HEK293 cells described above. Samples were run and stained with Coomassie Blue as described by Laemmli [20]. Antibody samples were diluted to a final protein concentration of 0.2 mg/mL in both reducing and non-reducing sample buffers (containing 5% SDS, and usually containing 25% glycerol to provide the needed density for loading). Pre-stained molecular weight standards were run on each gel (10µl). K5 concentration was read by OD₂₈₀ using the IgG program in the Nanodrop. The IgG protein was available as a clear to slightly opalescent, sterile liquid at a concentration of 1mg/ml.
**Antibody Binding Affinity.** Affinities of K5 were determined with ForteBio-based bior-layer interferometry. K5 was biotinylated and loaded onto streptavidin biosensors. After washing, sensors were dipped into buffer containing renalase antigen and then dissociated in a sample dilution buffer. Data analysis was carried out on ForteBio software.

**Animal Husbandry.** Female immunocompetent mice (C57BL6 Jackson Laboratories, 19-25g) were housed as a group (5 mice per crate). Access to food and water was provided *ad libitum* to all mice. All studies were approved by the VA Connecticut Healthcare System Institutional Animal Care and Use Committee.

**Pharmacokinetics of K5.** To determine key pharmacokinetic parameters for K5, mice were dosed 6.4mg/kg K5 subcutaneously (s.c) and intravenously (i.v). Intravenous injections were administered via a tail vain, and s.c. injections were administered in the flank. Serum was harvested on day 1 pre-dose and at 30 min 1 hour (hr), 2hrs, 4hrs, 8hrs, 24hrs, 36hrs, 48hrs, 72hrs, 96hrs, 120hrs, 144hrs, 168hrs, 192hrs. At each sampling time two mice were sacrificed.

Blood samples were collected via cardiac puncture and placed in 1mL Eppendorf tubes and centrifuged immediately at 10,000rpm. The serum was taken off-the clot and placed in a separate Eppendorf tube and stored at -20 degrees Celsius until assayed for K5 concentrations.

Serum concentrations were determined by ELISA (Figure 10). Immunoreactive concentration versus time data from mice were generated using GraphPad Prism and analyzed using a non-compartment model for both i.v and s.c. dosing. Non-compartmental analysis was performed in R-Studio for the following PK parameters in mouse: total drug exposure defined as area under the serum concentration-time curve (AUC), total clearance (CLtot), terminal half-life (t1/2) and
observed maximum serum concentration (Cmax). Combined data from all animals in each dose group were used to provide one estimate of each parameter for each dose group (naïve pooled approach).

**Allometric Scaling.** Human clearance was predicted using single species with the allometry exponent fixed at 0.60, 0.80, 0.85, or 0.90. The following equation was used to calculate human clearance:

\[
\text{Clearance}_{\text{human}} = \text{Clearance}_{\text{animal}} \times (BW_{\text{human}}/BW_{\text{animal}})^b
\]  
*(Equation 1)*

Where BW is the body weight and B is the allometry exponent. Mouse weight of 0.023kg and Human body weight of 65kg was used for calculation.

**K5 ELISA.** Recombinant HIS-tagged human renalase protein that was used as the capture antigen for the ELISA assay was produced by Tian-Min Chen. The K5 antibody and subsequent analysis used in the pharmacokinetic experiments was produced by the author of this MD thesis.

**Buffers**
- Blocking Buffer, PBS/0.5% BSA
- Wash Buffer (PT) 1x PBS, 0.05% Tween 20

**Reagents**
- Anti-Human IgG (1:2000 dilution)
- Anti-Rabbit IgG (1:5000 dilution)
- Coating Solution (Recombinant Renalase Protein)
- BSA 10x
- Substrate Solution (TMB Peroxidase)
- Stop Solution (1M H₃PO₄)
**Plate Preparation**

1) Dilute the Capture Antigen (recombinant renalase) to working concentration in PBS. Immediately coat a 384well microplate with 30uL respectively of diluted Capture Antigen. Seal plate and incubate overnight at 4°

2) Block plates by adding 50uL of Blocking Buffer to each well. Incubate at room temperature (on shaker) for minimum of 1 hour

3) Aspirate each well and wash with Wash Buffer, repeating the process 3 times for a total of 4 washes. Wash by filling each well with Wash Buffer (400ul) using a multi-channel pipette, squirt bottle, manifold dispenser, or auto washer. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against a clean paper towel. The plates are now ready for sample addition.

**Assay Procedure**

1. Add 30uL/well of sample diluted in PBS or standards. Cover with an adhesive strip and incubate (on shaker) for 1 hour at room temperature.

2. Repeat the aspiration/wash as in step 3 of Plate Preparation

3. Add 30uL/well of the Detection Antibody, diluted in PBS, to each well. Cover with adhesive strip and incubate for 30 minutes.

4. Repeat the aspiration/wash as in step 3 of Plate Preparation, this time wash for a total of 5 times.

5. Wash with 1x PBS a one time.
6. Add 30uL of substrate solution to each well. Incubate for 10-20 minutes at room temperature. (avoid placing the plate in direct light).

7. Add 30uL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

8. Determine the optical density of each well immediately, using a microplate reader set to 450nm.
RESULTS

K5 Antibody Confirmation by SDS Page

K5 was purified from HEK293 cells after transfecting them with plasmid DNA purified from E. coli. K5 antibody was confirmed via SDS page and ELISA. Reduced samples of IgG class antibodies gives rise to two glycosylated-heavy chains of about 50kDa and two light chains of approximately 25kDa on SDS-PAGE (Figure 1). When disulfide bonds are not reduced, samples of IgG class antibodies give rise to a single band on SDS-PAGE representing intact antibody consisting of 2 heavy and 2 light chains weighing approximately 150kDa. This is the SDS gel after electrophoresis and commassie blue staining.

The reduced sample has two bands at 50kDa and 25kDa indicating presence of both heavy and light chain of K5. The band at 150kDa in the non-reduced samples reflects the full molecular weight of the K5 IgG1 protein in complex with both heavy and light chain. ELISA using recombinant human renalase as the capture antigen confirmed the antibody indeed did bind renalase (Fig. 2).
Pharmacokinetics of K5

Results from biolayer interferometry (BLI) measures biomolecular interactions without labeling by analyzing the interference pattern of white light reflected from two surfaces: one layer with an immobilized protein on the biosensor tip, and an internal reference layer. The number of molecules bound on the biosensor tip creates a shift in the interference pattern that can be measured in real time. Experimental data from BLI was provided by Pei Li Wang of the Desir Laboratory. Rates of association and dissociation of K5 with immobilized renalase were $1.63 \times 10^4$ (K$_{on}$) and $<9.56 \times 10^{-5}$ (K$_{off}$). The calculated K$_d$ was $5.85 \times 10^{-9}$. Rates of association and dissociation of m28 with immobilized mouse renalase were $1.35 \times 10^4$ (K$_{on}$) and $7.68 \times 10^{-5}$ (K$_{off}$). The calculated K$_d$ was $5.67 \times 10^{-9}$ (Fig. 3).
Pharmacokinetic parameters of K5 after 6.4mg/kg s.c/i.v administration in mice are provided in Table 1a and Table 1b. Data in mice are from a pooled analysis of 28 animals. All doses were well tolerated.

\[ CL = \frac{Dose}{AUC_{(0-\infty)}} \quad (Equation \ 2) \]

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<td>T1/2 (h)</td>
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Table 1(b)

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Table 2b

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Figure 4 – [K5] vs Time after s.c injection

Figure 5 – [K5] vs Time after i.v injection
The single species allometric relationships described by Ling et al. and Wang et al. were used to predict the i.v. clearance K5 in humans assuming a human body weight of 65kg and mouse body weight of 0.021kg.

There was a rapid elimination phase after a single s.c dose that occurred between day 6 and 7. Intravenous injection of K5 saw a steadier decline of K5 concentrations but the terminal elimination phase was still rapid occurring between day 7 and day 8.

![Figure 6](image.png)

**Figure 6** – [K5] 48 hours after single and two doses

When K5 concentrations 48 hours after the second dose were compared with K5 concentrations 48 hours after one dose in K5 naïve mice, the K5 concentrations were significantly lower after the second injection of K5. (Fig. 6).
DISCUSSION

Monoclonal antibodies (mAbs) represent a promising and growing class of targeted therapeutics in a variety of disease states including oncology. Renalase is a secreted protein and binding of free renalase and anti-RNLS antibody effector functions are important for the therapeutic effect in melanoma seen with m28-RNLS [18]. Different methods exist for the selection and intentional engineering of high-affinity antibodies and reformatting of m28-RNLS with a human Fc IgG1 is a step towards advancing a novel anti-renalase compound towards clinical use for treatment in melanoma. K5 was chosen from a panel of humanized variants to use for these experiments because it has the highest binding affinity for recombinant mouse and human renalase of the panel of antibodies developed (Fig 2).

Prediction of human pharmacokinetic profiles is important during clinical development to help estimate dose and dosing regimens [21]. Drug exposure is linked to drug efficacy, therefore overall drug exposure following administration, clearance, elimination half-life ($t_{1/2}$), $T_{max}$, and $C_{max}$ are the primary parameters of concern when it comes to antibody pharmacokinetics. For this reason, non-compartmental PK analysis is the preferred methodology for pharmacokinetic analysis of antibody pharmacology because it requires fewer assumptions than model based approaches [22]. Antibody pharmacokinetics is dependent on the biology of the target antigen and its pharmacodynamics effects [23]. Antibody PK is also influenced by binding to the neonatal receptor (FcRn) and the subsequent recycling contributes to a long half-life. The primary route of elimination of IgG1 is via proteolytic degradation into amino acids and monoclonal antibody clearance can be classified as specific and non-specific. Specific clearance is mediated by the interaction of the mAb with its target antigen and non-specific clearance
includes non-specific cellular uptake with subsequent catabolism[23]. Increased circulating levels of renalase has been shown in patients with melanoma. It is possible the clearance of K5 in humans may be a composite of free K5 and K5-renalase complexes. The clearance of other humanized monoclonal antibodies has been reported to depend on the concentration of the associated ligand [24].

K5 is an IgG1 isotype monoclonal antibody and was expected to behave like other IgG1 molecules, demonstrating a biphasic PK profile with saturable kinetics and a long serum half-life [26]. Monoclonal antibodies often have dose-dependent pharmacokinetics which can become significant at a lower dose (e.g bevacizumab) [25] . A high, single dose (6.4mg/kg) was used to achieve saturable levels and constant values for PK parameters. For subcutaneous injections of K5, there was a rapid distribution phase that represents K5 moving from the subcutaneous space through the lymphatics into the vascular system. Peak concentrations occurred at 24 hours and remained steady for 6 days (Fig. 4). The rapid elimination phase seen after s.c dosing raises the suspicion for the presence of anti-therapeutic antibodies (ATAs). Since K5 is a humanized IgG1 being given to immune competent mice, I suspected the C57BL6 mice were forming ATAs to the K5. If ATAs were being formed towards K5, it would mean upon injection of a second dose of K5, serum concentrations may not reach levels seen with only one dose in a K5-naïve mouse.

To test this hypothesis, I injected two K5-naïve mice with 120mcg of K5 subcutaneously. A second dose was given 1 week later.

Observation of non-linear PK with the formation of ATAs, suggests that administration of humanized m28-RNLS most likely not only elicits an immune response in these C57BL6 mice,
but this immune response causes rapid clearance of K5 and can significantly affect the pharmacokinetic and pharmacodynamics properties of the drug in C57BL6 mice. Although, immunogenicity in animals is not predictive in humans, these data obtained using C57BL6 mice as a model for pharmacokinetics may not be predictive in humans when taken alone.

PK parameters for multiple species (mouse, rat, dog, or monkey) have been used for the allometric scaling approach to predict human PK parameters with non-human primates (NHP) being the gold standard [26]. Use of allometric scaling to predict the pharmacokinetics of antibodies in humans and has been reported by several groups. [27-31] Mordenti et al showed with good accuracy in predicting human clearance and volume of distribution using interspecies scaling for five protein drugs (MW 6 to 98 kDa). Mahmood expanded the data set to 15 therapeutic proteins and reported a low prediction error of human clearance [28, 29]. He also suggested the use of at least three animal species for interspecies scaling.

Allometric scaling using multiple species pharmacokinetic data uses an empirical power function of the species body weight:

$$Y = aW^b$$

(Equation 3)

Where $Y$ is the pharmacokinetic parameter of interest (e.g., clearance), $W$ is the body weight of the species, $a$ is the allometric coefficient, and $b$ is the allometric exponent. The allometric coefficient assumes the value of the pharmacokinetic parameter when the body weight is unity. Although interspecies allometric scaling was first suggested, acceptable prediction of human clearance using single animal species for macro-molecule drugs has also been reported later by
Ling and Wang [30, 31]. Ling et al. suggested using a fixed exponent of ‘0.85’ or ‘0.90’ for human CL prediction of monoclonal antibody drugs. Use of ‘0.80’ as a fixed exponent has also been recommended for monoclonal antibodies as well as other protein drugs [31]. Although PK parameters derived from non-human primates are considered the gold standard, studies using NHP are challenging due to cost and ethical considerations particularly early in drug discovery. Avery et al. has shown transgenic mice expressing human neonatal Fc receptor (hFcRn) can be useful as a preclinical PK model to project human PK of monoclonal antibodies. The transgenic hFcRn (Tg32 homozygous) mouse predicted human CL for 100% of mAbs tested in that study relative to 93%, 93% and 82% for NHP, Tg32 hemizygous mice, and WT mice, respectively [26]. Future studies using NHPs or transgenic Tg32 mice should be used for preclinical assessment of therapeutic anti-RNLS mAbs.
REFERENCES

FIGURE REFERENCES AND LEGENDS

Figure 7

Left RNLS expression is higher in metastatic than primary specimens using a method of quantitative immunofluorescence (QIF) developed at Yale. Stars denote statistically significant differences by ANOVA. Right – higher RNLS expression in 200 primary melanomas is associated with worse survival (log rank P=0.0008). The Y axis denotes RNLS levels by QIF, X axis denotes survival in months.

Figure 8

Anti PD1 resistant mouse melanoma cell line (YUMM1.7) allograft into immunocompetent C57B6 mice

Treatment (indicated by arrows: PBS, or anti-PD1, or m28 or combination anti-PD1 and m28; anti PD1 dose held constant at 120 µg/treatment, m28 dose as indicated)

Treatment begun when tumor reaches volume ~100 mm^3

m28 dose dependent decrease in tumor volume at day 14 with combination therapy, confirming potential synergism between anti PD1 and anti renalase therapy.
Humanized variants of m28-RNLS (K2-K16) have improved binding affinity (Left panel); and K13, 14 and 16 have better in vitro efficacy than parental m28-RNLS (right panel).

**Figure 10** – K5 indirect ELISA Scheme