

HBV transgenic mice for evaluation of therapeutics

Description. The transgenic mouse model, developed by Dr. Frank Chisari and associates (The Scripps Research Institute) (3, 8), has been established in our laboratory for over 7 years, in which many potential therapeutics have been evaluated (4-6, 11-13). The transgenic mice express infectious hepatitis B virus (HBV) from a transgene. Unlike the natural infection, HBV produced from the transgene in mice cannot infect mouse cells for successive rounds of viral replication. In the natural infection, HBV RNA and proteins are derived from successive rounds of replication to produce increasing levels of cccDNA, and consequently, increasing levels of HBV RNA and proteins. In this regard the use of the HBV transgenic mice is analogous to HBV stably transfected cells such as HepG 2.2.15 cells (7). Since the transgenic mice are immunotolerant to HBV, the mice do not get any disease signs that normally would be caused by immunopathological responses.

Uses. The uses are the evaluation of therapeutics that act directly on the virus replication, such as polymerase inhibitors, or evaluation of cytokine or innate immune responses for non-cytopathic inhibition of virus replication. Theoretically, therapeutic vaccines could be evaluated in these mice with the intent of breaking immunotolerance.

Parameters. Liver HBV DNA is the primary assay for efficacy. Other viral parameters can be measured, i.e., liver core antigen, liver HBV RNA, serum HBe and HBs. Serum HBV DNA is not measured in these mice because of low levels and contamination with transgenic DNA from degraded cells in the serum. No disease occurs in the mice, so no disease parameters are measured.

Hydrodynamic injection mouse model for evaluation of therapeutics for HBV

Description. The hydrodynamic injection technique allows for the delivery and expression of naked DNA into an animal in the absence a vector (1, 2, 9, 10, 14). A large volume of DNA solution, typically 1.6 mL/20 g mouse, is injected within 4-6 sec into a tail vein (15), which probably causes pores in cellular membranes. The DNA is mainly taken up by liver cells. Infectious clones of HBV have been used with this procedure to achieve virus production in the liver of immunocompetent mice (14). A range of 5% - 20% of the hepatocytes can be infected depending on variations of the procedure. If an immunocompromised mouse (NOD/SCID) is used, the virus is persistent for >1 month. Eventual elimination of the virus from immunocompetent mice is consistent with the appearance of antibodies, liver infiltrates and HBV-specific cytotoxic T lymphocytes (CTLs) beginning at 5, 7 and 7 days post-injection, respectively. This has provided the opportunity to measure the efficacy of therapeutics (1, 10) in the context of viral and immunological parameters. This may also provide the opportunity to express different clones of HBV, such as drug-resistant HBV.

Uses. The uses are the evaluation of therapeutics that act directly on the virus replication, such as polymerase inhibitors, or evaluation of cytokine or innate immune responses for non-cytopathic inhibition of virus replication. Immunological studies are limited because of the use of immunosuppressed mice.

Parameters. Liver HBV DNA is the primary assay for efficacy. Other viral parameters can be measured, i.e., liver core antigen, liver HBV RNA, serum HBe and HBs.

Methods

Liver HBV DNA using Southern blot hybridization. Liver tissue was homogenized in lysis buffer immediately upon necropsy. The tissue (approximately 0.1 g) was ground with a well-fitted pestle in a microcentrifuge tube containing lysis buffer (1 mM EDTA, 10 mM Tris, 10 mM NaCl, 0.5% SDS, proteinase K). After incubation for 5 - 10 min at room temperature, the tubes were snap-frozen in liquid nitrogen for storage. For extraction of the DNA, the samples were incubated at 55°C for 2 to 4 hours. Samples were poured into Phaselock™ Gel tubes containing 150 µL water, 350 to 500 µL phenol. After shaking and centrifuging at 12,000 rpm for 15 m, contents were poured into a second tube containing 350 to 500 µL chloroform. After centrifuging, the DNA was precipitated with 50 µL 5 M NaCl and 650 µL isopropanol and washed with 70% ethanol. The dried pellets were suspended in 500 µL TE buffer containing 1 µL RNase and incubated overnight at 55°C with occasional shaking. A specified volume of DNA solution typically containing 40 µg was digested with Hind III enzyme (New England Biolabs, Beverly, MA) for an incubation period of 3 h at 37°C. Hind III has been shown not to cut within the HBV transgene sequence. Digested DNA was separated by electrophoresis utilizing a 1% TAE-buffered agarose gel at 80 V for 3-4 h. DNA was then transferred to BioDyne™ B positive-charged nylon membrane by alkaline transfer method with the following modifications: 1) the gel was soaked in 0.4 M NaOH for 15-30 min, 2) the nylon membrane was soaked in water followed by 0.4 M NaOH for 5 min. The sponge used for transfer was also saturated in 0.4 M NaOH. The treated gel was placed (well-opening-side down) upon absorbent paper on the sponge. Transfer occurred over a 3 h period, after which the gel was discarded and the nylon membrane was washed in a solution of 0.2 M Tris (pH 7.6), 2x SSC, and 0.1% SDS. The membrane was baked for >30 min at 80°C and UV- fixed using UV Stratalinker™ 1800 (Stratagene, La Jolla, CA). Prior to hybridization, the filter was rinsed twice for 30 min in a neutralizing solution of 0.1x SSC and 0.1% SDS. Hybridization using a [³²P] CTP-labeled, HBV genomic probe (digested with Hae III) cloned into the pBluescript plasmid (gift of Dr. Luca Guidotti, The Scripps Institute, LaJolla, CA) occurred overnight at 60°C in a solution of 10% PEG-8000, 0.05 M NaPO₄, 0.21 mg/ml salmon sperm DNA, and 7% SDS.

The radioactive signals were measured using a Phosphor Imaging method (Optiquant). An image of the radioactive filter was exposed overnight to a Cyclone™ Storage Phosphor Screen (Perkin Elmer Multisensitive Medium, Type MS PPN 7001723). The exposed screen was transferred to the Cyclone™ drum and read using the 600 dpi setting. The ratio of the viral DNA bands to the transgene band was used to determine the concentration of viral DNA per host DNA. This calculation was based upon the knowledge that there were 1.3 copies of the transgene present per host cell with this line of transgenic mice (personal communication, F. Chisari). The transgene was used as an internal indicator to calculate the pg of HBV DNA per µg of homozygous cellular host DNA.

Liver HBV DNA using qPCR.

Liver HBV RNA using qRT-PCR. Real-time RT-PCR was used to assay HBV-specific RNA in liver biopsies. Total RNA from tissues was extracted using Trizol™ reagent. Primer-pairs (HBV3 forward ATAAAACGCCGCAGACACATC, HBV3 reverse AACCTCCAATCACTACCAACC) and HBV3 Taq-man probe [6~FAM]-AGCGATAACCAGGACAAGTTGGAGGACA-[BHQ1a~6FAM]. A second primer-pair (HBV4 forward GGACAAACGGGCAACATACT, HBV4 reverse TCTTCCTTTCATCCTGCCTGCT) and HBV4 Taqman probe [6~FAM]TCCAGAAAGAACCAACAAGAAGATGAGGCA[BHQ1a~6FAM] were used, without a noticeable difference between the two sets, so the HBV3 probe/primer set was used. A duplex reaction was done with the internal control, mouse GAPDH primers/probe. The primers and probe were the forward GCATCTTGGGCTACACTGAGG, reverse GAAGGTGGAAGAGTGGGAGTTG, and probe [5~HEX]-ACCAGGTTGTCTCCTGCGACTTCAACAG-[BHQ1a~5HEX]. The one-step FullVelocity™ QRT-PCR Master Mix (Stratagene, La Jolla, CA) was used for RT and amplification of HBV RNA and mouse GAPDH with primers and probe at a final concentration of 0.1 µM. Two microliter of total cellular RNA, extracted from infected or control tissues was used. Samples were run on a DNA Engine Opticon 2 (MJ Research Inc, Waltham, MA). A 25 µL reaction consists of 12.5 µL FullVelocity™ QPCR Master Mix, 0.375 µL dilute reference dye (1:500), 0.25 µL Stratascript™ RT/RNase Block Enzyme Mixture, and 0.5 µL FullVelocity™ Enzyme. The reaction contained 0.25 µL of both HBV-primers, 0.25 µL of both GAPDH-primers and 0.25 µL of both probes, all having a stock concentration of 10 µM. Reverse transcription of cellular RNA were performed for 30 min at 50°C followed by PCR, which consisted of 1 cycle of 2 minutes at 95°C, then 40 cycles of 10 sec at 95°C and 30 sec at 60°C. The assay was run with a series of 10-fold dilutions of pooled liver RNA from HBV transgenic mice to obtain a standard curve. The y axis was the log dilutions of the standard and the x axis was the C(t) values. R² values were used to measure the quality of the curve, which was always above 0.098. Mean C(t) values were obtained for duplicates of each sample. The mean C(t) values of each sample were used to obtain the log relative RNA value using a formula of the fit line of the standard curve.

Liver HB core immunohistochemistry. For detection of hepatitis B core antigen (HBcAg), liver biopsies were first paraffin-embedded. The paraffin was then removed from the sections by using two 5 min treatments with xylene. Tissues were fixed with two 3 min treatments with 95% ethanol. Sections were treated with deionized water for 3 min, exposed to 3% hydrogen peroxide for 5 min, and Biotin-block (#X0590, Dako Corporation) for 5 min. The primary antibody, rabbit anti-HBcAg (1:100 dilution) (#B0586, Dako Corporation), goat anti-rabbit secondary antibody (#k684 Dako, LSAB Peroxidase Kit), streptavidin peroxidase (#K684 Dako, LSAB Peroxidase Kit), and substrate-chromogen solution (3-amino-9-ethylcarbazole, AEC) were added for durations of 30, 30, 10 and 10 min, respectively. Sections were counterstained with Mayer's hematoxylin before being mounted.

Three different parameters were obtained from each tissue section. The first two measurements are based on the observation that cells surrounding the central veins of the liver are more strongly stained than in other areas of the liver, and that drug administered intraperitoneally should have ready access to the luminal cells of the veins. The first two parameters were obtained from counting cells surrounding central veins as follows. The total number of cells, the number of cells with stained nuclei, and the number of cells with stained cytoplasm were counted around central veins. The stained nuclei counts or the stained cytoplasm counts were divided by the total cells. Three central vein areas were counted with each slide sample. For the third parameter, a field, not in a central vein area, was counted for the total number of stained nuclei. One quarter of the field was counted. Three such fields were counted per liver section. The identity of the samples were blinded to the person reading the slides.

Serum HBeAg. Whole blood samples were obtained during necropsy by cardiac puncture. The whole blood was then transferred to heparin containing vials and centrifuged for the collection of the serum component. Ten microliters of serum was then diluted into 90 μ L of negative control serum, resulting in a 1:10 dilution of each sample. These samples along with a serial dilution of a positive control and a calibrator were run on an HBeAg-specific ELISA (International Immuno Diagnostics, Foster City CA) per manufacturer's instructions. Using the known PEI units for the Calibrator, PEI units were formulated for the serial dilutions of the positive serum. A graph was generated and extrapolation was used to assign a PEI unit value for each sample with a high degree of confidence (R^2 value of 0.9816). The ELISA manufactures cut-off was utilized.

Serum HBsAg. Sample collection is the same for HBsAg ELISA. Fifteen microliters of serum was diluted in the same manner and ran on an HBsAg specific ELISA (International Immuno Diagnostics, Foster City CA) per manufacturer's instructions. Interpretations were then made from both the manufacture's cut-off chart (with blank correction) and the manufacturer's cut-off formulae (without blank correction).

Cytokine array. Liver punches (approximately 35 mg each) were collected during necropsy and quickly homogenized in sterile PBS containing 0.1% NP-40. These homogenized sample were then snap frozen in liquid nitrogen until the assay was performed. Just prior to performing the assay, the sample were rapidly thawed and centrifuged at 3000 rpm's for 20 minutes to remove any solid matter. The supernatant was then diluted 1:5 into sample dilution buffer and run on a Q-Plex™ Mouse Cytokine Array (Quansys Biosciences, Logan, UT) per manufacturer's instructions. The fully developed cytokine plate was then captured as a .tif image on a Fuji LAS-3000 Luminescent Image Analyzer (Fuji Life Sciences, Stamford, CT) and analyzed with Quansys Array Software™, version 1.3.

Chemistry panel. A VetScan® Chemistry, Electrolyte and T4 Analyzer, specifically designed for veterinary medicine, was used in our BL-2 laboratory to process samples for the “comprehensive diagnostic profile”, which consists of ALT, BUN, creatinine, total bilirubin, albumin, alkaline phosphatase, globulin, glucose, Na⁺, K⁺, phosphorous, total protein. Protocols with the instrument were used.

Liver biopsies. Liver biopsies are done to screen the liver HBV DNA for entry of mice into experiments. The fur of Avertin-anesthetized mice was clipped from the abdomen and scrubbed for surgery. Within a sterile field and using sterile instruments, the ventral skin was cut longitudinally, followed by the peritoneum. While holding the liver with forceps, a portion as much as half the lobe was cut with Teflon-coated scissors that had been heated in a bead-sterilizer at 250°C. The scissors immediately cauterized the liver. As quickly as possible a second person slid ambient-temperature forceps along both sides of the scissors to easily separate the liver and biopsy from the hot scissors. The biopsy was processed as usual. The peritoneum was closed with one suture (3.0 Vicryl) and the skin was joined with wound clips. The wound was saturated with Betadyne™.

Diet restriction. Pups up to 3 weeks of age are restricted from solid food provided to the dam by placing the food in a solid tray at the top of the cage that could only be accessed by the dam. The small amount of food dropped by the dam into the bottom of the cage is not controlled. Pups raised in this way have higher liver HBV DNA titers in their livers when they become adults. This allows us to use higher numbers of the transgenic mice that we raise.

Statistical analysis. Kruskal-Wallis, Mann-Whitney, rank sum non-parametric test or Student's t-test are done with the statistical programs in JMP™ Software version 6.0 (The Statistical Discovery Software, SAS Institute, Inc.) or Prism 4 (GraphPad Software, Inc.). Power analysis is done to ensure sufficient numbers of animals.

Example experiments.

A. Toxicity range-finding studies.

1. Approach

In the event that a suitable dosage is not known from the literature or from the supplier of the compound, a toxicity range-finding study will be performed in order to identify a suitable dose of the drug. In these studies, a toxic dose should be identified to better determine potential therapeutic doses for animals in efficacy studies and to calculate a therapeutic index (TI). The TI will then be used to compare values between drugs. In our experience and per the RFP, we expect that one toxicity study will be performed per year.

Sub-acute toxicity studies will be performed using 5 animals per treatment group with $1/2 \log_{10}$ dilutions of the drug. Intraperitoneal (IP) or oral gavage administration is typically done unless otherwise specified. Intravenous or intramuscular routes can also be used if deemed necessary for specific drugs. The drug toxicity in these preliminary studies will be monitored by mortality, morbidity and degree of weight gain or loss. In addition, a blood chemistry profile (VetScan Chemistry Analyzer, Abaxis, Inc., Union City, CA) will be done. The parameters measured on the panel by the autoanalyzer (VetScan Chemistry Analyzer, Abaxis, Inc., Union City, CA) are albumin for liver function, alkaline phosphatase for cellular function, alanine aminotransferase (ALT) to measure enzyme leakage, amylase for pancreas, BUN for kidney function, Ca^{+2} for electrolyte status, creatinine for kidney filtration, globulin immune function, glucose for metabolism, K^{+} for electrolyte and kidney function, Na^{+} for electrolyte status, phosphorous for electrolyte status and kidney function, total bilirubin for hepatocellular function, and total protein for liver, kidney and nutritional status. Gross pathology and histopathological evaluations will be done by Dr. Thomas Baldwin, collaborator and Director of the Veterinary Diagnostics Laboratory. Five $1/2 \log_{10}$ dilutions of the drug starting at a high dosage of 100 mg/kg/day will be used. A placebo-treatment group will be used in the experiment. The maximum tolerated dosage (MTD) will be identified as the highest $1/2 \log_{10}$ dosage not causing toxicity. The Project Officer or Supplier, together with the PI and a toxicologist, Dr. Jeff Hall (collaborator), can modify this protocol to suit the specific requirements of the material being evaluated.

2. Method – toxicity protocol

To determine the MTD, the toxicity range-finding study will be set up using five C57BL/6 non-transgenic mice per group. The following dosages and treatment regimens will be used unless otherwise specified:

- *Group 1:* Highest designated dosage of test drug or 100 mg/kg/d using recommended treatment route and schedule or intraperitoneal injections qd X 21 days.
- *Group 2:* $1/2 \log_{10}$ dilution of the highest dosage (e.g., 32 mg/kg/d), same treatment schedule as #1.
- *Group 3:* $1/2 \log_{10}$ dilution of that used in Group #2 (e.g., 10 mg/kg/d), same treatment schedule as #1.

- *Group 4:* 1/2 log₁₀ dilution of that used in Group #3 (e.g., 3.2 mg/kg/d), same treatment schedule as #1.
- *Group 5:* 1/2 log₁₀ dilution of that used in Group #4 (e.g., 1 mg/kg/d), same treatment schedule as #1.
- *Group 6:* Vehicle only of drug, same treatment schedule as #1.

Mice will be monitored daily for morbidity and mortality, and weight will be obtained before and after treatment for each individual mouse. Morbidity will include signs such as ruffled fur, hunching, tremors, lethargy, hyperactivity, and diarrhea, in addition to reduction in weight gain. In addition, the VetScan™ serum chemistry profile will be done. If merited, gross and histopathological evaluations will be done. Whole blood will be processed for complete blood cell count (CBC) at the USU Veterinary Diagnostic Laboratory. This assay includes RBC, WBC, hematocrit, and hemoglobin. The highest dosage showing no toxicity will be identified as the maximum tolerated dosage (MTD). This dosage will serve to determine the dosages for the Pharmacokinetic Studies below.

B. Pharmacokinetic studies.

1. Approach

The purposes of the pharmacokinetic (PK) studies are to determine parameters such as the maximum concentration in plasma or tissues (C_{max}), time required to see the maximum concentration (T_{max}), the half-life ($t_{1/2}$), rate of absorption (k_a), rate of distribution (k_d), rate of elimination (k_e), or other parameters requested by the Project Officer such as the area under the curve for the plasma concentration-versus-time curve (AUC) [102, 103]. This information will be used to best identify a suitable dosage, route of administration, and treatment schedules for initial efficacy studies. Mortality, morbidity and rate of weight gain, as described in the previous section, will be recorded.

Pharmacokinetic studies will use the C57BL/6 non-transgenic mice, which have the same genetic background as the HBV transgenic mice. C57BL/6 mice will be used for pharmacokinetic studies and for toxicity control mice in antiviral studies for several reasons; namely, a) they are genetically identical to the HBV transgenic mice except for the presence of the transgene, b) there is not sufficient remaining serum in the HBV transgenic mouse after HBV antigen and DNA assays to evaluate pharmacological/toxicological parameters, and c) samples from non-transgenic mice are safer and easier to send to the location where drug-tissue assays are to be done.

A pharmacokinetic study will involve the administration of a single dosage of one-half the maximum tolerated dose (MTD) and then following the concentration of the drug in plasma, or if methodology for detection of the drug exists, in the liver or other tissues. Plasma and tissues will be collected at times early and later after the single drug injection [103]. Intravenous injections will be used as a necessary route to determine true rates of elimination. We have considerable skill with tail vein injection of black C57BL/6 mice. Other routes of administration, such as oral, IP, or intramuscular, can be tested if requested by the Project Officer or drug Supplier. As indicated in the RFP, we will anticipate that 1 to 2 PK studies will be done each year.

2. Method – PK protocol

The following dosages and treatment regimens will be used unless otherwise specified:

- *Group 1:* Single i.v. dosage of $1/2 \log_{10}$ MTD, 50 C57BL/6 mice.
- *Group 2:* Vehicle only of drug (placebo), single i.v. dosage, 50 C57BL/6 mice.

Plasma from 5 mice per group will be collected at 3 min, 6 min, 12 min, 24 min, 40 min, 1 h, 3 h, 6 h, 12 h, and 24 h (47). If the methodology exists for the particular drug being evaluated, liver or other tissues will be collected. The samples will be rapidly frozen and stored until shipment to other locations for drug determinations per the Project Officer's or Supplier's instructions. Based on the drug concentration in tissues, the C_{max} , T_{max} , $t_{1/2}$, k_a , k_d , k_e , and AUC will be calculated [102, 103] by Dr. Jeffery Hall (toxicologist, collaborator) and used for determination of effective treatment regimen. We anticipate that two PK studies will be done each year as indicated in the RFP.

C. Efficacy studies.

1. Approach

Per instructions from the Project Officer or using data obtained from the pharmacokinetic study, a treatment regimen will be selected for the efficacy determination. For example, if the $t_{1/2}$ is found to be 18 hours, once daily administration of the drug would be appropriate. Basic design will be the same as the HBV therapeutic studies done by us in the Proof-of-Concept section. Examples of treatments based upon our previous studies [4] might be oral treatment once daily for 21 days or intraperitoneal injections every 2 days as used with interferon [3]. A positive control treatment group, utilizing an effective dose of Hepsera® (drug supplied with their approval from Gilead Pharmaceutical), and a placebo-treated control group will be included in each experiment. The safety of these compounds will be monitored by tracking survival each day and by weighing individual mice before and after treatments. Toxicity controls using non-transgenic mice are not necessary because HBV in the transgenic mice is non-pathogenic. If more in-depth safety/toxicity data is requested, a full Toxicity Study measuring other parameters will be done using non-transgenic C57BL/6 mice.

Based on our experience, liver HBV DNA and liver HBV RNA will be the initial parameters measured because they are markers for viral production and viral transcription, respectively. If the test agent is efficacious in reducing either of these two parameters, the other parameters will be determined. If a test agent is not efficacious in reducing the initial two parameters, then no other parameters will be measured.

2. Method – transgenic mouse efficacy protocol

The following experiment is based on the protocol we have been using for the previous Contract [11]. Ten mice will be used per group. At least two weeks before initiating the experiment, liver biopsies will be obtained and assayed for HBV DNA. Low-expressing mice will not be used. Mice will be block-randomized across treatment groups.

- *Group 1:* Test drug with appropriate dosage and treatment for 14 to 21 days depending on the drug.
(Another dilutions of the drug could be used.)
- *Group 2.* Positive control drug, Hepsera® (adefovir dipivoxil), p.o., qd, 10 mg/kg/day [11].
- *Group 3.* Vehicle only, same treatment schedule as #1.

Animals will be uniquely identified with ear tags and each mouse will be weighed before and after the experiment. Three to four hours after the last treatment, weights will be obtained and necropsies will be performed to obtain serum, liver punch using 4-mm skin punch homogenized in proteinase K/SDS solution and snap-frozen for DNA extraction, 4-mm liver punch homogenized in Trizol™ and snap-frozen for RNA extraction, three 4-mm snap-frozen liver punches as backup tissues, and the remaining of the liver fixed in formaldehyde for immunocytochemistry. Liver HBV DNA and RNA will be assayed on all tissues because these parameters represent the production of virions and transcription of the virus from the transgene, respectively. If the drug was NOT efficacious in reducing either of these two viral parameters, no further parameters will be assayed unless directed otherwise by the Project Officer. All samples will be collected so that all assays can be done if needed. The criteria to determine efficacy will include the following in order of priority:

Two most prominent viral parameters:

- Significant reduction of liver HBV DNA.
- Significant reduction of liver HBV RNA.

Toxicity:

- Mortality and reduced weight change.

Secondary viral parameters if needed prominent parameters are positive:

- Significant reduction of liver HBcAg.
- Significant reduction of Serum HBeAg and HBsAg.
- Significant reduction of serum HBV DNA.

3. Method - hydrodynamic injection protocol

Much of the design and strategy for an Efficacy Study is the same as for the HBV transgenic mouse study above, except that non-transgenic C57BL/6 mice will not require pre-experiment liver biopsy surgery. To select for mice in which hydrodynamic injection was successful, 5 µg of an unrelated expression vector, such as pTHAAT (secreted human alpha-1-antitrypsin), will be co-injected with 12 µg of HBV clone. Extra animals will be entered into each group so that mice that express low levels of alpha-1-antitrypsin will be removed from analysis [8]. As needed, clones of drug-resistant HBV [28] (gift of Dr. S. Xiong, Gilead Pharmaceuticals) expressed from transthyretin liver-specific promoter [24] (gift of Teri Van Dyke, University of North Carolina at Chapel Hill) will be used in the place of the wild-type HBV. Construction of these vectors is currently underway. Sample collection and analysis will be the same as for the transgenic mice.

D. Optimized dosage studies.

1. Approach

The purpose of these studies is to identify the best treatment schedules, routes or dosages of a certain drug. We have found that the best way to compare drugs or treatment regimens is to identify the minimal effective dose (MED) of a drug, which is similar to many viral cell culture assays where low-effective dosages (e.g., 50% effective concentration) are identified and compared [84]. The dynamic range of the liver HBV DNA is not large in transgenic mice, so differences in drug activities is not best identified by using fold-reduction of liver HBV DNA titers. Finding the end-point of activity is the best way to compare compounds. For example, we have determined that the MED for entecavir is 0.1 mg/kg compared with 1.0 mg/kg for Hepsera® using the same treatment schedule (see Proof-of-Concept section).

Using the same treatment regimen as utilized in the initial Efficacy Study, a range of doses will be evaluated in the HBV transgenic mice. As a general guideline, five $1/2 \log_{10}$ dilutions will be used. Liver HBV DNA or liver HBV RNA, which ever is reduced by the therapeutic compound, will be used to determine the minimal effective dose. The optimized dosage is defined as the most active treatment regimen using $1/2 \log_{10}$ of the maximum tolerated dose (MTD) identified in the Toxicity and Efficacy Studies above.

2. Method – optimization protocol

At least two weeks before initiating the experiment, liver biopsies will be obtained and assayed for HBV DNA or RNA. Low-expressing mice will not be used. Mice will be block-randomized across treatment groups. Using the same treatment regimen as utilized in the Efficacy Study, treatment groups with 5-10 mice each will be as follows. As in the Efficacy Study, the biopsies of all mice will have been assayed for liver HBV DNA to eliminate low-expressors.

Transgenic mice (30-60 total):

- *Group 1:* Test drug at $1/2 \log_{10}$ MTD.
- *Group 2:* Test drug at $1/10 \log_{10}$ MTD.
- *Group 3:* Test drug at $1/2 \log_{10}$ of Group #2 dose.
- *Group 4:* Test drug at $1/10 \log_{10}$ of Group #2 dose.
- *Group 5:* Test drug at $1/2 \log_{10}$ of Group #4 dose.
- *Group 6:* Drug vehicle

Animals will be uniquely identified with ear tags and each mouse will be weighed before and after the experiment. Since these animals do not get disease, the test animals themselves can be used as toxicity controls. All

samples will be prepared so that all assays can be done if needed. Liver HBV DNA or RNA will be the primary parameters.

E. Evaluation of drug combinations

1. Approach

It is presumed that each drug used in a Combination experiment will have already been evaluated when used alone, so the range of doses (including minimum effective dose) and treatment schedule which render a significant HBV-inhibitory effect will be known. A 3 X 3 matrix design, or larger as may be need, will be used with two dosages per compound flanking the MED's and their respective placebo groups.

The data will be analyzed to determine if the combination therapies resulted in lower mean viral parameters as compared to monotherapies. Until the actual data are obtained, it is difficult to know which statistical methods described under "Concepts in Combination Therapy" section will be used. We anticipate the most appropriate tests will be the Berenbaum FIC Index procedure [81] and the Prichard/Shipman 3-D assay [82]. Use of multiple dosages will provide results for a 3-D portrayal of data, although an additional dose in each group would be better for this purpose, but less likely because of the large number of animals that would be required.

2. Method – combination study protocol

At least two weeks before initiating the experiment, liver biopsies will be obtained and assayed for HBV DNA. Low-expressing mice will not be used. Mice will be block-randomized across treatment groups. The following groups will be used in a 3 X 3 matrix design. Five to ten mice, selected by HBV DNA liver biopsy assays, will be used per group.

Transgenic mice (45 – 90 total):

- *Group 1:* Drug A, using MED and the appropriate treatment schedule.
- *Group 2:* Drug A using the $1/2 \log_{10}$ MID and the same treatment schedule as #1.
- *Group 3:* Drug B, using MED and the appropriate treatment schedule.
- *Group 4:* Drug B, using $1/2 \log_{10}$ MID and the same treatment schedule as #3.
- *Group 5:* Drug A using MED and treatment schedule of Group #1 + Drug B using MED and treatment schedule of Group #3.
- *Group 6:* Drug A using MED and treatment schedule of Group #1 + Drug B using $1/2 \log_{10}$ MED and treatment schedule of Group of #3.
- *Group 7:* Drug A using $1/2 \log_{10}$ MED and treatment schedule of Group #1 + Drug B using MED and treatment schedule of Group #3.
- *Group 8:* Drug A using $1/2 \log_{10}$ MED and treatment schedule of Group #1 + Drug B using $1/2 \log_{10}$ MED and treatment schedule of Group #3.
- *Group 9:* Vehicle only of Drug A using treatment schedule of Group #1 + vehicle only of Drug B using treatment schedule of Group #3.

Non-transgenic mice (45 each): The same treatments can also be done with non-transgenic mice to determine any synergistic toxicity. This is optional depending on the nature of the drug combinations and the direction of the Project

Officer and the Supplier of the drugs. The same parameters described in the toxicity range-finding study protocol (Toxicity Study) would be used.

All samples will be prepared so that all assays can be done if needed. Liver HBV DNA or RNA will be the primary parameters. Analysis is described under the previous section and in the Background section.

F. Therapeutic vaccine study.

1. Approach

Therapeutic vaccine studies will be based on the previous approach used by us [19, 16] (see Proof-of-Concept section) and others [90, 91, 93-95]. Based on the knowledge of the supplier of the vaccine, a vaccine schedule will be selected for vaccination of the HBV transgenic mice. It may involve, as was the case with Stressgen (see results below), a single injection or multiple long-term injections. At various days post-vaccine injection, the liver and serum will be collected for viral parameters and the splenocytes will be removed. The splenocytes of individual mice will be cultured and the supernatant removed for cytokine profile determinations. Particular attention will be given to Th1 or Th2 responses. If tolerance appears to be broken, then H&E histological examination, an automated chemistry panel, liver HB core or surface antigen, liver HBV DNA and anti-HBe and –HBs antigen assays will be done to correlate the tolerogenicity with liver pathology, and viral parameters.

Since few, if any, studies have been able to sufficiently break tolerance in HBV transgenic mice to affect viral liver pathology parameters, an alternate approach may be used. The approach is to vaccinate naïve C57BL/6 non-transgenic mice and adoptively transfer splenocytes to the HBV transgenic mice where the pathological and viral parameters will be evaluated in the HBV transgenic mice and cytokine profiles will be evaluated in the vaccinated naïve non-tg mice. This study, however, will not determine the ability of the vaccine to break tolerance, but it will allow an evaluation of the potency of the vaccine.

2. Method – therapeutic vaccine protocol

HBV transgenic mice will be entered into the experiment and block-randomized between groups with the use of pre-experiment liver biopsies. The vaccine will be administered s.c. or by the prescribed route on day 0 or with subsequent boosters over a long duration of time. Seven days after the last injection, all tissues will be collected. Additionally, spleens will be obtained and cultured for detection of immunological responses.

- *Group 1:* vaccine administered to HBV transgenic mice
- *Group 2:* vaccine administered to C57BL/6 mice
- *Group 3:* placebo administered to HBV transgenic mice
- *Group 4:* placebo administered to C57BL/6 mice

The primary set of parameters will be splenocyte cytokine profile responses, with particular attention to interferon-gamma and Th1 response, as compared with placebo-treated mice, as an indication of viral-specific T cell responses such as with CTL's. If tolerance appears to be broken, then H&E histological examination, an automated chemistry panel, liver HB core or surface antigen,

liver HBV DNA and anti-HBe and –HBs antigen assays will be done to correlate the tolerogenicity with liver pathology, and viral parameters.

References

1. **Bordier, B. B., J. Ohkanda, P. Liu, S. Y. Lee, F. H. Salazar, P. L. Marion, K. Ohashi, L. Meuse, M. A. Kay, J. L. Casey, S. M. Sebti, A. D. Hamilton, and J. S. Glenn.** 2003. In vivo antiviral efficacy of prenylation inhibitors against hepatitis delta virus. *J Clin Invest* **112**:407-14.
2. **Chang, J., L. J. Sigal, A. Lerro, and J. Taylor.** 2001. Replication of the human hepatitis delta virus genome is initiated in mouse hepatocytes following intravenous injection of naked DNA or RNA sequences. *J Virol* **75**:3469-73.
3. **Guidotti, L. G., B. Matzke, H. Schaller, and F. V. Chisari.** 1995. High-level hepatitis B virus replication in transgenic mice. *Journal of Virology* **69**:6158-6169.
4. **Iyer, R. P., A. Roland, Y. Jin, S. Mounir, B. Korba, J. G. Julander, and J. D. Morrey.** 2004. Anti-hepatitis B virus activity of ORI-9020, a novel phosphorothioate dinucleotide, in a transgenic mouse model. *Antimicrobial Agents and Chemotherapy* **48**:2318-2320.
5. **Julander, J. G., R. J. Colonno, R. W. Sidwell, and J. D. Morrey.** 2003. Characterization of antiviral activity of entecavir in transgenic mice expressing hepatitis B virus. *Antiviral Research* **59**:155-161.
6. **Julander, J. G., R. W. Sidwell, and J. D. Morrey.** 2002. Characterizing antiviral activity of adefovir dipivoxil in transgenic mice expressing hepatitis B virus. *Antiviral Research* **55**:27-40.
7. **Korba, B. E.** 1995. In vitro evaluation of combination therapies against hepatitis B virus replication. *Antiviral Research* **29**:49-51.
8. **Korba, B. E., and J. L. Gerin.** 1992. Use of a standardized cell culture assay to assess activities of nucleoside analogs against hepatitis B virus replication. *Antiviral Research* **19**:55-70.
9. **Liu, F., Y. Song, and D. Liu.** 1999. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther* **6**:1258-66.
10. **McCaffrey, A. P., H. Nakai, K. Pandey, Z. Huang, F. H. Salazar, H. Xu, S. F. Wieland, P. L. Marion, and M. A. Kay.** 2003. Inhibition of hepatitis B virus in mice by RNA interference. *Nat Biotechnol* **21**:639-44.
11. **Morrissey, D. V., P. A. Lee, D. A. Johnson, S. L. Overly, J. A. McSwiggen, L. Beigelman, V. R. Mokler, L. Maloney, C. Vargeese, K. Bowman, J. T. O'Brien, C. S. Shaffer, A. Conrad, P. Schmid, J. D. Morrey, D. G. Macejak, P. A. Pavco, and L. M. Blatt.** 2002. Characterization of nuclease-resistant ribozymes directed against hepatitis B virus RNA. *J Viral Hepat* **9**:411-8.
12. **Pan, W.-H., P. Xin, J. D. Morrey, and G. A. Clawson.** 2004. A self-processing ribozyme cassette: utility against human papillomavirus 11 E6/E7 mRNA and hepatitis B virus. *Molecular Therapy* **9**:596-606.

13. **Radhakrishnan, P. I., S. P., G. Z., J. D. Morrey, and B. E. Korba.** 2005. Nucleotide analogs as novel anti-hepatitis B virus agents. *Current Opinions in Pharmacology* **5**:520-528.
14. **Yang, P. L., A. Althage, J. Chung, and F. V. Chisari.** 2002. Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection. *Proc Natl Acad Sci U S A* **99**:13825-30.
15. **Zhang, G., X. Gao, Y. K. Song, R. Vollmer, D. B. Stolz, J. Z. Gasiorowski, D. A. Dean, and D. Liu.** 2004. Hydroporation as the mechanism of hydrodynamic delivery. *Gene Ther* **11**:675-82.