Published in final edited form as:

J Virol Methods. 2008 March ; 148(1-2): 132–145.

Controlled Inactivation of Recombinant Viruses with Vitamin B2

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Abstract

Inactivated viruses are important tools for vaccine development and gene transfer. 8-methoxypsoralen (8-MOP) and long-wavelength ultraviolet irradiation (LWUVI) inactivates many viruses. Toxicity limits its use in animals and humans. Toxicological and photosensitizing properties of riboflavin make it suitable for virus inactivation in preparations for biological use. Viruses expressing beta-galactosidase were mixed with either 8-MOP (1.5 mM) or riboflavin (50 μ M) and exposed to LWUVI (365 nm) for 2 hours. Virus activity was determined by limiting dilution. The half-life of the adenovirus preparation treated with 8-MOP was 8.28 nanoseconds⁻¹ (ns⁻¹) and 36.5 ns⁻¹ after treatment with riboflavin. Despite the difference in half-life, both preparations were completely inactivated within 45 minutes. In contrast, the half-lives for adeno-associated virus (AAV) preparations were similar (63 ns⁻¹ 8-MOP vs. 67 ns⁻¹ riboflavin). Each AAV preparation was fully inactivated within 90 minutes. The half-life of lentivirus was 193.4 ns⁻¹ after treatment with 8-MOP and 208 ns⁻¹ after exposure to riboflavin. Virus treated with riboflavin was inactivated within 20 minutes. Virus exposed to 8-MOP was inactivated in 90 minutes. DNA and RNA viruses can be inactivated by riboflavin and LWUVI and used in physiological systems sensitive to other photochemicals.

Keywords

adenovirus; adeno-associated virus; lentivirus; inactive control; riboflavin; psoralen

Introduction

Recombinant adenoviruses are one of the most widely used vectors for gene transfer in the clinic (No Authors Listed 2007). Their use, however, is limited by strong innate and adaptive immune responses that occur soon after administration of a single dose by the systemic route (Sakurai, 2007). A single dose of virus also significantly alters the expression and function of several key cytochrome P450 (CYP) enzymes in the liver and the kidney (Callahan, 2005; Le, 2006). It is not clear if this effect is related to the innate immune response against the virus or if certain components of the viral genome are responsible for this effect. In order to investigate

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this further, a preparation of inactive, yet intact, virus capsids is needed. Exposure to 8-methoxy psoralen (8-MOP) and ultraviolet light is one of the most commonly used methods for inactivation of pathogens in blood products (Lin, 2005; Seghatchian, 2006; 2003) and has often been used to inactivate adenovirus to create biologically inert, immunologically active controls in pre-clinical gene transfer and vaccination protocols (Borgland, 2000; Cotten, 1994; Cotten, 1992; Kafri, 1998; Schnell et al., 2001; Thomas, 2000).

Psoralens are photochemically active tricyclic compounds initially used in ancient Egypt and India to treat depigmented lesions (Pathak, 1992). They continue to be used for this indication today and are an important tool in nucleic acid research. Their chemical structure allows them to easily fit between adjacent thymine base pairs so that, when exposed to UV radiation, they irreversibly link two DNA strands. In an initial pilot study, the combined treatment of the virus with 8-MOP and UV light effectively and efficiently inactivated the virus, however, administration of 8-MOP alone significantly altered the expression and activity of hepatic CYP3A2 as early as six hours after administration in the male Sprague Dawley rat (Figure 1).

The human correlate of this isoform, CYP3A4, responsible for metabolizing approximately 40% of all currently marketed medications (Plant, 2006), can convert 8-MOP to metabolites that irreversibly bind to and alter the expression and function of many CYP isoforms (Fouin-Fortunet et al., 1986; Mays, 1989; Tinel et al., 1987; Yang, 2007). This effect would skew results obtained from animals treated with virus inactivated in this manner unless excess 8-MOP was removed from the preparation by size-exclusion chromatography or dialysis. Although each of these methods would most likely remove the compound from the preparation, it is difficult to confirm since 8-MOP absorbs light within the same range as the virus itself.

Riboflavin, vitamin B₂, is a molecule capable of intercalation and oxidation of DNA and RNA in the presence of UV light (Kumar et al., 2004). Unlike psoralen, riboflavin is an elemental dietary component with a low toxicity profile (Corbin, 2002; Unna, 1942). To date, changes in the expression and function of CYP in the presence of riboflavin have not been described. Use of riboflavin in combination with UV light has effectively inactivated a wide array of pathogens such as extra- and intracellular human immunodeficiency virus (HIV), pseudorabies virus, West Nile virus, parvovirus, gram positive bacteria (*Staphylococcus epidermidis*), gram negative bacteria (*Escherichia coli*) and *Leishmania* protozoa without compromising blood components, producing toxic byproducts or inducing adverse side effects (Cardo, 2006; Corbin, 2002; Pelletier, 2006; Ruane, 2004). In this report, the effect of riboflavin and UV light on the infectivity of a first generation recombinant adenovirus, a non-enveloped virus with a double stranded DNA genome was assessed. Aggregation of virus particles was monitored throughout the inactivation process by field emission scanning electron microscopy (FESEM) and dynamic light scattering. Capsid integrity was confirmed by transmission electron microscopy (TEM).

The *in vivo* performance of the inactive virus was compared to the same dose of active virus by histochemical staining for the beta-galactosidase transgene in liver sections obtained from treated animals six hours after administration. Serum cytokines (IL-6, IL-12 and IFN- γ) and CYP activity was also assessed. The utility of this combination for inactivation of adeno-associated virus, a non-enveloped virus with a single-stranded DNA genome and lentivirus, an enveloped virus with a RNA genome is also described.

Materials and Methods

Materials

All chemicals were purchased in the highest purity available from EMD Chemicals (Gibbstown, NJ), unless specified otherwise. Phosphate-buffered saline (PBS), riboflavin, 8-

methoxypsoralen (8-MOP), and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Mediatech (Herndon, VA). 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) was purchased from Gold Biotechnology (St. Louis, MO). Tissue-tek[®] optimal cutting temperature (O.C.T.) embedding compound was purchased from Fisher Scientific (Pittsburgh, PA).

Virus Preparation

Adenovirus—First-generation adenovirus expressing *E. coli* beta-galactosidase under the control of a cytomegalovirus (CMV) promoter was amplified in 293 cells and purified from cell lysates by banding twice on cesium chloride gradients according to established methods (Croyle, 1998). Virus was desalted on Econo-Pac 10DG disposable chromatography columns (Bio-Rad, Hercules, CA) equilibrated with PBS. Virus concentration was determined by UV spectrophotometric analysis at 260 nm and by limiting dilution.

Adeno-Associated Virus—Recombinant AAV2 containing the beta-galactosidase (*lacZ*) transgene under control of a CMV promoter was produced by transfecting 293 cells with a 1:1:1.2 molar ratio of *cis* plasmid containing AAV ITRs flanking the transgene cassette (pAAVLacZ (from the AAV Helper-Free System, Stratagene, La Jolla, CA), *trans* plasmid, containing AAV2 *Rep* and *Cap* genes, (pAAV-RC, Stratagene) and helper plasmid, containing adenovirus genes VA, E2A and E4 (pHelper, Stratagene). Transfection was performed using a CalPhos Mammalian transfection kit (BD Biosciences, Palo Alto, CA). A portion of the DNA mixture (120 μ g) was added to each 150 mm plate of 293 cells. Seventy-two hours after transfection, cells were harvested, resuspended in serum-free DMEM and lysed by three freeze/thaw cycles. RNase A and DNase I (Roche Applied Science, Indianapolis, IN) were added (8 U/ml, final concentration each) and the lysate placed at 37°C for 30 minutes. Lysate was clarified by centrifugation at $3,700 \times g$ for 20 minutes. Supernatant was loaded on a heparin-agarose column (Sigma Aldrich, St. Louis, MO) equilibrated with PBS (pH, 7.4, Sigma) containing 1 mM MgCl₂ and 2.5 mM KCl (Mallinckrodt Baker, Paris, KY) according to established methods (Auricchio, 2001). The column was washed with 3 column volumes of equilibration buffer and virus eluted with equilibration buffer containing 400 mM NaCl. Excess salt was removed by dialysis against sterile PBS at 4 °C.

Lentivirus—Recombinant lentivirus expressing beta-galactosidase was produced by mixing the helper packaging construct, pCMV Δ R8.2, encoding for the HIV helper function, the transfer vector, pHxLacZWP, encoding for the beta-galactosidase transgene and the plasmid encoding the VSV-G envelope, pMD.G, in a 1:2:3 molar ratio. This mixture was used to transfect 293T cells by calcium phosphate precipitation as described previously (Croyle et al., 2004). Forty-four hours after transfection, fresh media was added to each plate and the virus harvested 16 hours later. Cell-free supernatants were concentrated by ultracentrifugation at 28,000 r.p.m for 2 hours at 4 °C in a SW28 rotor (Beckman, Fullerton, CA). Virus was resuspended in 100 mM potassium phosphate buffered saline (pH 7.4). All experiments were performed with freshly purified viruses.

Virus inactivation—Freshly purified virus preparations containing either: 50 μ M riboflavin or 1.5 mM 8-MOP in DMSO (3%) were added to appropriately sized polystyrene dishes (Fisher Scientific, Pittsburgh, PA), surrounded by ice and placed directly on top of a transilluminator emitting UV light (365 nm, 7000 μ W/cm², Ultra-Lum, Claremont, CA). A hand held device (UVP, Upland, CA) emitting UV light (365 nm, 1000 μ W/cm²) was placed directly on top of the lid of each dish. Each preparation was exposed to UV light for a period of 2 hours. The effective dose of radiation for the adenovirus preparations was 317 J/ml while that of the AAV and lentiviral preparations was 43.2 J/ml based upon the final volume of each purified preparation. Samples were taken for assessment of titer and aggregation immediately after the

addition of either riboflavin or psoralen before exposure to UV light (t=0). Additional samples were then taken at approximately 1, 5, 10, 15, 20, 30, 33, 36, 39, 42, 45 minutes, 1, 1.5 and 2 hours after exposure.

Limiting Dilution Assay—The term lac-forming unit (lfu) defines the number of infectious particles present in a preparation of beta-galactosidase (lacZ) expressing virus. In this assay, it is assumed that one lac-forming unit is equivalent to one active virion capable of producing one positive (lac⁺) cell in a cell population treated with a given preparation determined by histochemical staining. At each timepoint, 10 µl were taken from each preparation. Serial dilutions were made in DMEM supplemented with 2% fetal bovine serum (FBS, Cambrex BioScience Inc., Walkersville, MD). One hundred microliters of each dilution was added in triplicate to HeLa cells (ATCC CCL-2.2) seeded in 24 well tissue culture dishes (BD Biosciences, Bedford, MA) and incubated at 37°C for 2 hours. One milliliter of DMEM, supplemented with 10% FBS, was then added to each well. Twenty-four hours later (48 hours for AAV preparations), cells were fixed with 0.5% glutaraldehyde and beta-galactosidase activity determined by incubation of infected cells with the substrate 5-bromo-4-chloro-3-indolyl-beta-galactoside (X-gal) for 4 hours at 37 °C in the dark. Staining medium was removed and blue colored positive cells tallied from a minimum of 20 microscope fields (approximately 48,000 cells). Lac-forming units were calculated as described previously (Croyle, 1998). This assay can detect virus concentrations that fall within the range of 10 to 1×10^{12} lfu/ml.

Initial inactivation rates for each preparation were calculated from the slope of the lines generated by linear regression analysis of the first 4 data points of each concentration vs. time curve with standard data analysis software. The half-life of each preparation was calculated using the following formula for first order degradation processes:

$$t_{1/2} = 0.693/k$$

where k is the initial rate of inactivation.

Virus Aggregation—Field emission scanning electron microscopy (FESEM) and dynamic light scattering were used to assess aggregate formation during the inactivation process.

FESEM—Samples were prepared at 25°C as described previously (Obenauer-Kutner, 2002). Samples were absorbed onto 1% poly-L-lysine carbon slides, fixed with 1.5% glutaraldehyde in phosphate buffered saline (pH 7.6) and stained with 1% aqueous uranyl acetate. Particles were visualized at 5 kilovolts on a JEOL model 6335F field emission scanning electron microscope (JEOL USA, Inc., Peabody, MA). Gold particles (80 ± 5 nm, BB International, Golden Gate, UK), an internal control for sizing of virus particles, were prepared in a similar manner on separate grids. Digitized images prepared from sample grids were then rapidly processed using an automated macro program within Image-Pro Plus, IPP, v.4.1 (Media Cybernetics, Silver Spring, MD) as described previously (Obenauer-Kutner, 2005).

Dynamic Light Scattering—Particle size was assessed with a DynaPro LSR laser light scattering device and detection system (Wyatt Technology formerly Protein Solutions, Santa Barbara, CA) as described previously (Quick, 2003). Regularization histograms and assignment of hydrodynamic radii values to various subpopulations within the sample were calculated using either Dynamics or DynaLS software (Wyatt Technology). Each analysis was qualified by assessment of particle size of a standard solution of polystyrene beads with a hydrodynamic radius approximately the size of each respective virus (Duke Scientific, Palo Alto, CA). Readings within ± 2 nm of the labeled value of the bead standard were the criteria for qualification of each analysis.

Transmission Electron Microscopy (TEM)

Adenovirus Samples—Ten microliters of each sample was placed on parlodion carbon coated 300-mesh copper grids (Ted Pella, Inc., Redding, CA) for 2 minutes to facilitate adsorption of virus particles onto the substrate. Excess virus particles were wicked from the grid with filter paper. Samples were fixed with 1.5% glutaraldehyde (electron microscopy-grade, Sigma Aldrich) in phosphate buffered saline (PBS) for 5 minutes, rinsed once in PBS and twice in distilled water. Virus particles were then stained with 1% uranyl acetate (Electron Microscopy Sciences, Hatfield, PA) for 30 seconds and grids air dried at room temperature. Negative stained virus particles were examined with a Hitachi H600 (Tokyo, Japan) electron microscope.

AAV and Lentivirus Samples—Samples were prepared by absorbing 4 microliters of sample onto glow discharged 400-mesh copper grids pre-coated with Formavar/thin carbon film (Ted Pella, Inc., Redding, CA). Grids were rinsed with distilled water and stained with 3% uranyl acetate for 10 seconds. Particles were visualized at 60–90 kilovolts on a Philips EM 208 transmission (Philips, Eindhoven, The Netherlands) electron microscope.

In vitro Testosterone Hydroxylation Assay

Metabolic activity for CYP3A2 and 2C11 was determined by *in vitro* analysis of testosterone hydroxylation as previously described (Callahan, 2005). Reaction mixtures, 1 ml final volume, contained 0.1 M potassium phosphate, 250 μ M testosterone in methanol, 200 μ g hepatic microsomal protein, 5 mM NADP⁺, 100 mM glucose-6-phosphate, and 100 mM magnesium chloride. Samples were incubated with 250 μ M testosterone for 3 minutes at 37°C with agitation (100 rpm/min) following the addition of glucose-6-phosphate dehydrogenase (1 unit/ μ l). The reaction was allowed to continue for an additional 15 minutes under the same conditions. The reaction was quenched with dichloromethane (5 ml) and 11 α -hydroxyprogesterone (1.2 μ g) added as the internal standard. The organic phase was transferred to a clean tube, evaporated under a constant stream of air, dissolved in 200 μ l of methanol and stored in a sealed tube at 4°C until further analysis. Testosterone metabolites were separated and quantified by HPLC according to a previously described method (Lu, 2003). Peak areas of corresponding hydroxylation metabolites were measured and compared to peak areas of the internal standard within the same run.

Evaluation of Serum Cytokines

Blood was collected from rats 6 hours after treatment. Serum was obtained by centrifugation and stored at –80 °C until analysis. Samples were analyzed for IL-6 (Endogen, Pierce Biotechnology, Rockford, IL), IL-12 (p70, Biosource International, Camarillo, CA) and IFN- γ (Endogen, Pierce Biotechnology, Rockford) using an enzyme-linked immunosorbent assay (ELISA) according to each of the manufacturer's instructions.

Statistical Analysis

SigmaStat software (Systat Software Inc., Point Richmond, CA) was used to perform a one-way analysis of variance with a Bonferroni/Dunn post-hoc analysis to determine the differences between control and treatment groups. Differences were determined to be significant when the probability of chance explaining the results was reduced to less than 5% ($p < 0.05$).

Results

Effect of Riboflavin on the Expression and Function of Hepatic CYP3A2 and 2C11 in the Male Sprague Dawley Rat

Five hundred microliters of either riboflavin (50 μ M, effective dose 36.2 μ g/kg) and 8-MOP (psoralen, 1.5 mM, effective dose 625 μ g/kg) were administered intravenously to male Sprague Dawley rats at concentrations documented to significantly inactivate several different pathogens (Cotten, 1994; Cotten, 1992; Ruane, 2004). The activity of CYP3A2 and 2C11 was assessed by separation and quantification of isoform-specific testosterone metabolites, 6 β -hydroxytestosterone and 2 α -hydroxytestosterone respectively. Samples obtained from animals given phosphate buffered saline served as controls and could convert testosterone to 6 β -hydroxytestosterone at a rate of 846.8 ± 152.2 pmol/min/mg protein which was not statistically different from that seen in samples from animals treated with riboflavin ($1,036.6 \pm 95.7$ pmol/min/mg protein, $p = 0.07$, Figure 1A). In contrast, the ability to process testosterone to the 6 β -hydroxy- metabolite was significantly elevated in samples obtained from animals treated with psoralen ($1,737.1 \pm 247.6$ pmol/min/mg protein, $p \leq 0.05$). Treatment with either reagent did not significantly alter the function of hepatic CYP2C11 ($p = 0.26$, Figure 1B).

Effect of Riboflavin and Exposure to Ultraviolet Light on the Inactivation Kinetics of Recombinant Adenovirus

Freshly purified recombinant adenovirus expressing *E. coli* beta-galactosidase was inactivated by the addition of 1.5 mM 8-MOP and exposure to UV light according to established protocols (Cotten, 1994; Cotten, 1992). The initial rate of decline in titer of the virus after treatment with 8-MOP and UV light was approximately 5.02×10^9 lfu/ml/min and the projected half-life ($t_{1/2}$) 8.2 ns^{-1} . Infectivity was significantly compromised within the first 5 minutes of treatment as virus concentration fell from 1.14×10^{10} lfu/ml to 3.09×10^8 lfu/ml (Figure 2A). Virus titer continued to rapidly drop to 1.15×10^6 lfu/ml and 2.47×10^4 lfu/ml after 10 and 20 minutes of exposure. Titer of virus treated with 8-MOP hovered between 25–50 lfu/ml after 30 minutes of exposure and fell to undetectable levels within 45 minutes.

Riboflavin was added to the adenovirus preparation at a concentration documented to significantly inactivate several different pathogens (Ruane, 2004). The initial rate of decline in titer of virus in the presence of riboflavin and UV light was approximately 1.14×10^9 lfu/ml/min and the projected $t_{1/2}$ was 36.5 ns^{-1} . Virus titer dropped from an initial concentration of 1.55×10^{10} lfu/ml to 4.56×10^9 lfu/ml within 5 minutes (Figure 2A). The concentration of infectious virus particles fell to 2.12×10^6 lfu/ml after 20 minutes of exposure and to 8.75×10^4 lfu/ml after 30 minutes. The titer hovered around this concentration until the 40 minute timepoint when it fell to 103 lfu/ml. Infectious virus particles could not be detected after 45 minutes.

Exposure of the virus to UV light in the absence of either photosensitizer, did not significantly compromise virus infectivity ($p=0.07$). A slight drop from 6.06×10^{10} lfu/ml to 1.93×10^{10} lfu/ml between the 10 and 15 minute timepoints was the only notable change observed throughout the entire study period (Figure 2A). Mixing the virus with either 8-MOP or riboflavin and storing the preparation at room temperature also did not significantly affect the overall infectious titer within the same time interval ($p=0.08$), suggesting that exposure to UV light in the presence of each photochemical was necessary for virus inactivation (Figure 2B).

Effect of Photosensitizers on Aggregation of Adenoviral Particles

Physical and chemical factors associated with the production and processing of recombinant viruses can alter the stability of the virus capsid and facilitate aggregate formation (Konz, 2005). Administration of a preparation containing aggregates can potentiate a severe immune

response and, if the aggregates are sufficiently large, can facilitate clot formation and cause phlebitis (Brange, 1997; Braun, 1997; Thornton, 1993; Tomazic-Jezic, 2001). To confirm that virus inactivation with riboflavin and UV light did not induce aggregation of virus particles beyond that associated with standard purification techniques, characterization of each preparation was performed at the following timepoints: 0, 10, 30, and 45 minutes. Multiplet order was determined by field emission scanning electron microscopy (FESEM), an analytical method that allows for simultaneous visualization and quantification of submicron particles (Obenauer-Kutner, 2002).

Over the course of the study, neither reagent induced significant aggregate formation (Figures 3 and 4). The percentage of single particles in preparations treated with riboflavin increased slightly from 73.3% of the population at the beginning of the study ($t=0$) to 82.6% of the population at 45 minutes (Figure 3A). The presence of doublets declined from 20.4% ($t=0$) to 10.9% at 45 minutes. A slight increase in multiplet formation after treatment with riboflavin from 3.2% initially to 4.7% was also noted. Transmission electron microscopy confirmed the presence of non-aggregated virus particles and revealed that capsid integrity was maintained throughout the exposure period (Figures 3B–3D). In contrast, the percentage of singlets detected in preparations treated with psoralen declined over time falling from 82% of the population ($t=0$) to 64.3% at the end of the study (Figure 4A). A corresponding increase in doublets (15.8% at the start to 24.8%), triplets (1.31% to 7.33%) and multiplets (0.9% to 3.6%) was also detected in these preparations. Electron microscopy revealed that treatment with psoralen and UV light did not significantly compromise virus capsid integrity (Figures 4B–4D). Clusters of aggregated virus particles were noticeable at later timepoints (Figure 4D, 45 minutes of exposure).

***In vivo* Performance of Inactivated Adenovirus**

As a final test to confirm that treatment of recombinant adenovirus with riboflavin and UV light completely inactivated all virus particles, beta-galactosidase transgene expression was evaluated in tissue sections obtained from the livers of male Sprague Dawley rats after systemic administration of virus (5.7×10^{12} vp/kg) inactivated by either riboflavin or psoralen and UV light (Figure 5). Animals were sacrificed 4 days after virus administration, when transgene expression peaks. Beta-galactosidase could not be detected in sections of livers from animals treated with virus inactivated by either method (psoralen, Figure 5B, riboflavin, Figure 5C), in contrast to that seen in animals given active virus taken from the same production lot (Figure 5D). Serum levels of cytokines commonly produced in response to systemic administration of recombinant adenoviruses (IL-6, IL-12 and IFN- γ), were comparable to those reported in the literature in rodent models treated with a similar dose of virus (Sakurai, 2007; Manickan, 2006; Smith, 2004), suggesting that the inactivation process did not affect the innate immune response against virus capsid proteins (Table 1). Samples taken at this time from animals treated with virus inactivated with riboflavin were capable of converting testosterone to the CYP3A2-specific metabolite, 6 β -hydroxytestosterone, at a rate of $1,089 \pm 133.9$ pmol/min/mg which was significantly lower than that detected in samples obtained from animals given PBS ($1,731.1 \pm 174.73$ pmol/min/mg, $p \leq 0.01$) but not significantly different from that seen in samples from animals given active virus (983.3 ± 129.9 pmol/min/mg, $p = 0.06$). Conversion of testosterone to the CYP2C11-specific metabolite, 2 α -hydroxytestosterone, was also significantly compromised by the virus inactivated with riboflavin (678.6 ± 15.5 pmol/min/mg, Ad-riboflavin vs. $1,027.3 \pm 125.14$ pmol/min/mg, PBS $p \leq 0.01$). This was somewhat higher than that seen in samples from animals given the active virus (414.9 ± 66.9 pmol/min/mg, $p = 0.04$). mRNA levels for each isoform were unaffected by the administration of virus inactivated by riboflavin, suggesting that virus transcription may be necessary for changes in CYP3A2 and 2C11 gene expression.

Inactivation Kinetics of Recombinant Adeno-Associated Virus in the Presence of Riboflavin

Recombinant adeno-associated virus (AAV) expressing the beta-galactosidase transgene was mixed with 1.5 mM psoralen and exposed to UV light in the same manner as described for adenovirus. Within 5 minutes, the infectious titer of the preparation fell from 1.33×10^{10} lfu/ml to 4.92×10^9 lfu/ml (Figure 6A). Titer continued to drop to 6.19×10^7 lfu/ml after 30 minutes and 6.32×10^6 lfu/ml after 45 minutes. The last measurable concentration of this preparation (53,500 lfu/ml) was recorded after 60 minutes of exposure. The overall inactivation rate of AAV in the presence of psoralen and UV light was 6.60×10^8 lfu/ml/min and the $t_{1/2}$ 63 ns^{-1} . Recombinant AAV was also mixed with riboflavin (50 μM) in a manner similar to adenovirus. The overall inactivation rate of AAV in the presence of riboflavin and UV light was 6.13×10^8 lfu/ml/min and the $t_{1/2}$ 67 ns^{-1} . Titer consistently dropped over time from 1.33×10^{10} lfu/ml prior to exposure, to 4.35×10^8 lfu/ml after 20 minutes and then to 1.52×10^5 lfu/ml at one hour (Figure 6A). Active virus was not detected after 90 minutes of treatment with riboflavin and UV light. Exposure to UV light in the absence of a photosensitizer did reduce the titer from 1.33×10^{10} lfu/ml to 1.64×10^9 lfu/ml after 30 minutes. Titer fell by one log unit to 1.72×10^8 lfu/ml after one hour of exposure and to 4.31×10^7 lfu/ml after 2 hours. Incubation of the virus in either photosensitizer alone also significantly reduced AAV infectivity. Psoralen reduced titer from 1.33×10^{10} lfu/ml to 1.07×10^8 lfu/ml after 2 hours, while riboflavin reduced titer from 1.33×10^{10} lfu/ml to 3.83×10^8 lfu/ml at the same timepoint ($p = 0.04$, Figure 6B).

Effect of Riboflavin and Exposure to Ultraviolet Light on the Inactivation Kinetics of Recombinant Lentivirus

Despite the fact that the initial inactivation rate of recombinant lentiviral preparations treated with either psoralen or riboflavin were quite similar (riboflavin, 2.0×10^7 lfu/ml/min, psoralen 2.15×10^7 lfu/ml/min), distinct differences in the calculated half-life (riboflavin, 208 ns^{-1} , psoralen 193.4 ns^{-1}) and the time that it took to achieve complete inactivation were observed (Figure 6C). This first became evident at the 15 minute timepoint when the titer of the preparation treated with riboflavin and UV light fell from an initial concentration of 1.57×10^8 lfu/ml to 2.98×10^5 lfu/ml while that treated with psoralen only fell from 1.57×10^8 lfu/ml to 3.30×10^6 lfu/ml. The titer of the riboflavin treated preparation fell to 2,710 lfu/ml after 20 minutes of treatment. Infectious virus particles could not be detected beyond this timepoint. In contrast, the titer of the preparation treated with psoralen fell to 2.76×10^5 lfu/ml after 20 minutes and to 73,100 lfu/ml after 30 minutes. The titer of this preparation continued to drop steadily to 65,000 lfu/ml after 45 minutes and 8,900 lfu/ml at 90 minutes. Infectious virus could not be detected after 2 hours. Treatment with either UV light or each photosensitizer alone for 2 hours significantly reduced the titer of the lentivirus preparation ($p = 0.05$, Figure 6D). Exposure to UV light reduced the titer of the preparation from 1.57×10^8 lfu/ml to 1.25×10^7 lfu/ml within 2 hours. Treatment with either psoralen or riboflavin reduced the titer from 1.57×10^8 lfu/ml to 1.94×10^7 lfu/ml and 2.12×10^7 lfu/ml respectively.

Effect of Inactivation on Aggregation Patterns of Recombinant AAV

Aggregation patterns for recombinant AAV preparations were characterized by dynamic light scattering, a technique capable of identifying and quantifying different molecular species within a given preparation based upon their ability to disperse light (Bloomfield, 1981). In an effort to present results consistent with that reported for adenovirus, data for AAV are described as singlets (average hydrodynamic radius $12.2 \pm 1.5 \text{ nm}$), doublets ($26.2 \pm 2.1 \text{ nm}$), triplets ($35 \pm 3.4 \text{ nm}$) and multiplets ($> 60 \pm 5 \text{ nm}$). Each preparation initially contained a number of large aggregates, which is commonly associated with purification techniques that use elution buffers that contain high salt concentrations (unpublished observations and (Wright, 2005)). The AAV preparation exposed to psoralen and UV light was aggregated at the start of the study (17.5%

singlets, 82.5% multiplets, Figure 7B) and remained so without significant change throughout the entire treatment period. The preparation treated with riboflavin and UV light, however, started with equal amounts of singlets and aggregated species (52.2% singlets, 47.8% multiplets, Figure 7A). Electron micrographs of this preparation prior to exposure to UV light also displayed the presence of a widely disperse population of intact virus particles (Figure 7C). The distribution of virus particles within the riboflavin treated preparation changed within the first 10 minutes of exposure to 22.8% singlets and 77.2% multiplets. Electron micrographs at this time revealed large networks of intact virus particles with a few single capsids throughout (Figure 7D). There was no significant difference between preparations with respect to aggregation at 90 minutes (riboflavin: 16.8% singlets, 83.2% multiplets, psoralen: 10.1% singlets, 89.9% multiplets).

Inactivation Patterns of Recombinant Lentiviruses in the Presence of Photosensitizers and UV Light

Aggregation of lentivirus was also assessed by dynamic light scattering. The criteria for categorizing the types of particles found in lentiviral preparations differed from that of AAV preparations, based upon differences in the hydrodynamic radius of the virus particles. Singlets were particles having an average hydrodynamic radius of 51.2 ± 5.3 nm, with doublets as 106 ± 7 nm and triplets as 120 ± 9 nm. The “multiplet” descriptor was assigned to particles with a hydrodynamic radius greater than 200 nm. Species with hydrodynamic radii less than 10 nm were assigned the “subparticle” descriptor. Approximately 15% of the virus population of each preparation initially consisted of these subparticles, which was considered to be debris that accumulated after harvest and concentration (Figure 8A and 8B). Despite this, each preparation consisted primarily of single intact particles prior to inactivation (71.6%) (Figure 8C). The primary difference observed between the two treatments was the development of a marked amount of subparticles in the preparation exposed to riboflavin and UV light with 18% and 30.3% of the population falling in this category at the 60 minute and 2 hour timepoints respectively (Figure 8A). Representative electron micrographs confirmed the presence of disrupted particles (asterisk, Figure 8D), which seem to facilitate formation of larger protein aggregates and single, intact particles (arrows, Figure 8D). The most notable change in preparations treated with psoralen and UV light was a shift in the amount of multiplet species over time. Only 12.1% of the population consisted of multiplets after 10 minutes of exposure. This increased to 34.8% of the population after 30 minutes and continued to be high throughout the study (Figure 8B).

Discussion

Initial observations that a single dose of recombinant adenovirus expressing the beta-galactosidase transgene significantly compromised hepatic cytochrome P450 (CYP) enzymes 3A2 and 2C11 and renal 2E1 for a period of 14 days led to additional studies to identify components of a recombinant virus that might be responsible for these effects (Callahan, 2006; Callahan, 2005; Le, 2006). In order to determine how the immune response against virus proteins *in vivo* affects the expression and function of CYP enzymes and if simple engagement of virus receptors affects CYP expression and function in an *in vitro* system, a method to inactivate the virus without introducing any extraneous components that could mask results was required. Psoralens are used for medicinal purposes and have a profound effect on the expression and function of hepatic cytochrome P450 enzymes (Bethea, 1999; Fouin-Fortunet et al., 1986; Mays, 1989; Pathak, 1992; Tinel et al., 1987; Yang, 2007). This effect is so striking that subjects enrolled in clinical trials are restricted from eating foods containing psoralens such as grapefruit, parsley, parsnips, celery and some herbal medicines to prevent extraneous changes in drug levels (Guo, 2004; Ho, 2001). Despite the fact that these characteristics make

this compound unsuitable for studies assessing CYP activity and expression, it effectively inactivated the viruses employed in the studies outlined in this manuscript.

Few reports outlining protocols for the inactivation of microorganisms describe complete inactivation of a given pathogen as done here. This may be due in part to the sensitivity of the assay developed for each respective pathogen. In order to increase the probability of detecting very low numbers of virus particles, a wide range of serial dilutions were prepared for each sample at a given timepoint. It is also important to realize that the assay used to assess the infectivity of each virus was based upon histochemical staining of cells infected with samples taken from preparations during the inactivation process. Quantitation of active virus particles was achieved by visual inspection of cell monolayers and calculations based upon the assumption that one cell positive for expression of the beta-galactosidase transgene was equal to one active virus particle. This may essentially underestimate the number of active virus particles present in a given sample for several reasons. More than one virion could potentially infect a cell and induce the same level of transgene expression. Virus capsids that do not contain genetic material and therefore cannot induce transgene expression are a common contaminant of all recombinant virus preparations and were present to some degree in each of the virus preparations used to develop the inactivation technique outlined in this manuscript (Osten, 2007). These particles are already inactive and thus could also minimize the actual number of particles inactivated by each of the techniques described in this manuscript. Considering these two factors together, the inactivation process could be more efficient than what the data included in this manuscript conveys.

Although each compound could completely inactivate each of the viruses employed in these studies, there were distinct differences in the kinetics associated with inactivation process. While the initial inactivation rate of the riboflavin and UV light treatment for adenovirus was slightly slower than that observed after the psoralen treatment, the half-life for each preparation was similar and both methods were capable of completely inactivating the virus within 45 minutes (Figure 2). There was no significant difference between the rate of inactivation nor the half-lives of adeno-associated virus particles induced by either riboflavin or psoralen (Figure 6). Both treatments fully inactivated AAV within 90 minutes, twice the time it took to inactivate adenovirus. In contrast, lentiviral preparations were completely inactivated by exposure to riboflavin and UV light within 20 minutes (Figure 6), while it took 2 hours to completely inactivate the virus with psoralen. The disparity between the half-life for each preparation and the time it took for each compound to inactivate each respective virus may be due to differences in the ability of each compound to penetrate the capsid and interact with each viral genome and their effect on capsid structure over time.

A significant number of large aggregates were detected in the AAV preparations and to a lesser extent the lentivirus preparations used in these studies prior to treatment with either photosensitizer (Figures 7 and 8). Several groups have shown that aggregation of virus particles can hinder and even prevent inactivation of virus particles during chemical treatment and exposure to UV light (Hijnen, 2006; Madge, 2006; Templeton, 2005), suggesting that this may in part be responsible for differences in the time it took to inactivate each virus. It has also been shown that riboflavin generally has a lower binding affinity for genetic material than psoralen and that psoralen is also capable of binding to proteins as well as charged and uncharged lipids (Dardare, 2002). Thus, much of this compound may have been bound to the lipids and proteins associated with the lentivirus envelope, which delayed inactivation with respect to virus treated with riboflavin. Differences in inactivation rates may also be attributed to the difference in structure of the virus genomes. Riboflavin and psoralen primarily exert their effect by irreversibly crosslinking strands of genetic material. This process is facilitated by the proximity of strands in a double stranded genome, like that found in the adenovirus. Disruption of the single stranded genomes of the AAV and lentiviral vectors most likely occur through oxygen

radical formation and subsequent base damage and strand breaks, a process that may be more complex and take longer to complete (Kumar et al., 2004).

Differences in the structure of each virus genome may also explain differences in their sensitivity to UV light. The titer of the recombinant adenovirus preparation did not change after exposure to UV light for 2 hours (Figure 2), confirming reports that this virus is one of the most resistant to UV inactivation (Gerba, 2002;Nwachuku, 2005). This effect has largely been attributed to the fact that the virus can use host cell enzymes to repair damages to its double stranded genome caused by UV irradiation (Day, 1984;Day, 1975). Exposure of AAV and lentivirus to UV light did reduce titer by several logs but did not completely eliminate infectious virions from each preparation (Figure 6). Exposure of these viruses to each photosensitizing agent alone reduced the concentration of active virus in AAV and lentivirus preparations to a similar degree. These results not only reflect differences in susceptibility of the single stranded genomes to chemical disruption induced by each component of the inactivation process, but reflect differences in inactivation as a function of different initial concentrations of each virus preparation. The starting concentration for each preparation was based upon the yield for a given production lot and the amount of virus needed for *in vivo* applications. The effect of starting concentration on the inactivation kinetics for each of these viruses is currently under investigation.

It is also important to note that treatment with either photosensitizer did not significantly compromise the integrity of virus capsids nor induce marked virus aggregation, making the final preparations suitable for *in vivo* and *in vitro* assessment of biological processes associated with virus binding and entry in the absence of transcription (Figures 3, 4, 7 and 8). The only significant observation noted throughout the studies outlined in this report with respect to capsid integrity was that the riboflavin treatment seemed to disrupt and destroy the lentivirus vector (Figure 8). It is not clear why this occurred. This phenomenon, however, did not contribute significantly to the reported inactivation kinetics of the vector since an increase in subparticle species did not occur until after virus infectivity dropped below detectable levels.

Inactive virus preparations can serve as important controls in pre-clinical pharmacological and toxicological assessment for many vaccination and gene therapy protocols. In this report, we have illustrated the importance of assessing the biological properties of reagents used to inactivate viruses for these purposes. Psoralen, commonly used for inactivation of adenovirus, significantly altered the activity of hepatic CYP3A2, an enzyme that plays a key role in drug metabolism. Riboflavin, a reagent with similar properties but which does not alter CYP3A2 was found to successfully inactivate a variety of recombinant viruses with minimal changes in capsid structure. Most importantly, viruses inactivated with riboflavin were capable of inducing a strong immune response as indicated by significant levels of serum cytokines in animals treated with the virus (Table 1). Inactivated virus also affected CYP3A2 and 2C11 activity in a manner similar to that seen after administration of active virus despite the fact that expression of virus-mediated transcription of transgene product was not detected (Figure 5). The results included here also illustrate that an inactivation protocol for one pathogen may not effectively inactivate another within a given timeframe. The advantages to using this approach for inactivation of microorganisms is that riboflavin is inexpensive, non-toxic and poses no risk to biological systems, medical personnel and the environment. The inactivation process described here with some modifications is currently in the phase I/II level of pre-clinical testing for pathogen reduction in vital blood components such as platelets and red blood cells (Klein, 2005). The utility of this approach in the reduction of pathogen burden in other biological products for human use and consumption is currently under investigation in our laboratories.

Acknowledgements

The authors would like to thank Dr. Gary Kobinger of the National Microbiology Laboratory, Public Health Agency of Canada, in Winnipeg, Manitoba for kindly providing the plasmids necessary for producing the recombinant lentivirus used in these studies. We also thank Ms. Hong Le of The University of Texas at Austin College of Pharmacy for assistance with production of recombinant adeno-associated virus and Dr. Gary Vellekamp of SPRI for assistance in the compilation of this manuscript. This work was supported by research grant R21GM69870 from the National Institutes of Health (MAC). SMC was the recipient of a University of Texas at Austin Continuing Fellowship.

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Figure 1. Riboflavin Alone Does not Affect the Activity of Hepatic A) CYP3A2 and B) 2C11
Rats were treated with: phosphate buffered saline (Vehicle), riboflavin (50 μ M, effective dose 36.2 μ g/kg), or psoralen (1.5 mM, effective dose 625 μ g/kg) and sacrificed six hours after treatment. *In vitro* catalytic activity of hepatic CYP3A2 microsomal proteins was measured by the production of the testosterone metabolite, 6 β -hydroxytestosterone. Hepatic CYP2C11 activity was determined by measuring the production of the isoform-specific testosterone metabolite, 2 α -hydroxytestosterone. Values are presented as the mean $\pm$ standard error of 4 animals/treatment/time point. Statistical significance was determined between individual treatment groups and saline controls by one-way analysis of variance with a Bonferroni/Dunn post-hoc analysis. * $p \leq 0.05$.

Figure 2. Treatment with Riboflavin and Exposure to Long-Wavelength Ultraviolet Irradiation Can Fully Inactivate Recombinant Adenovirus Particles. A) Time Course of Virus Inactivation A first generation recombinant adenovirus expressing *E. coli* beta-galactosidase was mixed with either psoralen (1.5 mM) or riboflavin (50 μ M) and exposed to UV light. Exposure to long wavelength UV light (UV alone) did not significantly alter virus infectivity, suggesting that use of either riboflavin or psoralen in the presence of UV light is required for efficient inactivation within 45 minutes. **B) Photosensitizing Agents Alone Do Not Compromise Virus Infectivity.** Treatment of recombinant adenovirus with either riboflavin (50 μ M) or psoralen (1.5 mM), in the absence of UV light did not significantly compromise infectivity for 45 minutes. Data in each panel are presented as mean values obtained from three independent studies $\pm$ standard error.

Figure 3. Exposure of Recombinant Adenovirus to Riboflavin and UV Light Does Not Significantly Alter Capsid Integrity and Induce Aggregation. A) FESEM Species Distribution of Riboflavin/UV Inactivated Adenovirus

Data represent the percent of subspecies characterized as singlets, doublets, triplets, and multiplets of recombinant adenovirus at four representative time points: 0, 10, 30, and 45 minutes after treatment with riboflavin and UV light. Gold particles served as an internal standard. Transmission electron microscopy confirmed that preparations treated with riboflavin in the presence of UV light were predominantly single, intact particles prior to treatment (panel B), as well as after 10 minutes (panel C) and 45 minutes (panel D). Magnification in all panels: 60,000 ×

Figure 4. Exposure of Recombinant Adenovirus to Psoralen and UV Light Does Not Significantly Alter Capsid Integrity. A) FESEM Species Distribution of Psoralen/UV Inactivated Adenovirus Data represent the percent of subspecies characterized as singlets, doublets, triplets, and multiplets of recombinant adenovirus 0, 10, 30, and 45 minutes after exposure to psoralen and UV light. Gold particles served as an internal standard. Transmission electron microscopy confirmed that these preparations primarily consisted of single, intact particles prior to treatment (Panel B), as well as after 10 minutes (Panel C). Some large virus complexes were noticeable upon the completion of the study (Panel D, 45 minutes). Magnification in all panels: 60,000 ×

Figure 5. Recombinant Adenovirus Inactivated by Riboflavin Fails to Induce Transgene Expression in the Liver After Systemic Administration

Hepatic sections from adult Sprague-Dawley rats four days after treatment with A) Phosphate buffered saline (Vehicle control) or 5.7×10^{12} vp/kg of either B) recombinant adenovirus expressing beta-galactosidase (AdlacZ) inactivated by treatment with 1.5 mM psoralen and UV light, C) AdlacZ inactivated by 50 μ M riboflavin and UV light or D) AdlacZ prior to inactivation (positive control). Magnification in all panels: 400 $\times$.

Figure 6. Riboflavin Can Effectively Inactivate Both Recombinant Adeno-Associated Viruses and Lentiviruses. **A) Time Course of Inactivation for Recombinant Adeno-Associated Virus (AAV)** Recombinant AAV expressing the beta-galactosidase transgene was mixed with either psoralen (1.5 mM) or riboflavin (50 μ M) and exposed to UV light. Exposure to long wavelength UV light (UV only) did alter virus infectivity but did not completely inactivate the virus. **B) Treatment with Either Riboflavin or Psoralen Alone Can Reduce the Infectious Titer of Recombinant AAV.** AAV preparations were incubated with each reagent at room temperature for 2 hours. Titer of each preparation was assessed before and after treatment. There was no significant difference in titer of a preparation incubated in buffer in the absence of photosensitizer for the same time period (data not shown). **C) Kinetic Profile of Lentivirus Inactivation in the Presence of Photosensitizers.** Preparations treated with 50 μ M riboflavin in the presence of UV light were completely inactivated within 20 minutes while treatment with 1.5 mM psoralen and UV light inactivated the virus in approximately 2 hours. **D) Exposure of Lentivirus to UV light or Photosensitizers Alone Can Compromise Infectious Titer.** Lentivirus was incubated with each photochemical at room temperature or exposed to UV light for 2 hours. Titer of each preparation was assessed before (Pre-Tx) and after treatment. Data in each panel are presented as mean values obtained from three independent studies $\pm$ standard error. ** $p=0.04$, * $p=0.05$, one-way analysis of variance with a Bonferroni/Dunn post-hoc test.

Figure 7. Inactivation of Recombinant Adeno-Associated Viruses by Exposure to Photosensitizers and UV Light Does Not Compromise Capsid Structure Nor Induce Significant Aggregation of Virus Particles

A) Particle size distribution of recombinant AAV mixed with 50 μ M riboflavin and exposed to UV light as determined by dynamic light scattering. This preparation initially consisted of a mixture of single, intact virus particles and large aggregates in equal proportion. At later time points, the majority of the preparation consisted of large viral aggregates with an average hydrodynamic radius greater than 60 nm. B) Particle size analysis of an AAV preparation mixed with 1.5 mM psoralen and exposed to UV light. No significant change in particle size distribution could be detected in the preparation during the inactivation process. C) Representative electron micrograph illustrating the presence of single intact particles and large aggregates present in an AAV preparation prior to treatment with riboflavin and UV light. Magnification: 80,000 $\times$. D) Representative electron micrograph illustrating the presence of few intact single virus particles and many large aggregates after 90 minutes of exposure to riboflavin and UV light. Magnification: 50,000 $\times$.

Figure 8. Long-Term Treatment of Recombinant Lentivirus with Riboflavin and UV Light Compromises Virus Particle Integrity

A) Particle size analysis of a lentivirus preparation during inactivation by exposure to 50 μ M riboflavin and UV light as determined by dynamic light scattering. The preparation consisted primarily of single, intact particles throughout the study. However, a significant number of “subparticle” species were detected at later timepoints, suggesting that extended exposure of the virus to riboflavin and UV light significantly disrupts virus particles. B) Particle size distribution of lentivirus treated with 1.5 mM psoralen and UV light. This preparation consisted primarily of single, intact virus particles, however, a significant rise in large aggregates was noted toward the completion of the study. C) Representative electron micrograph of a lentivirus preparation prior to treatment with riboflavin and UV light. The preparation consisted primarily of large, intact virus particles. Magnification: 100,000 $\times$. D) Representative electron micrograph of a lentivirus preparation 2 hours after exposure to riboflavin and UV light illustrating the presence of some intact virus particles (arrows), degraded virus capsids (asterisk) and large protein aggregates (center of image). Magnification: 140,000 $\times$.

Table 1

Cytokine Secretion Profiles of Rats Six Hours After Systemic Administration of Adenovirus Inactivated by Riboflavin (Ad + Ribo) or Active Virus (AdlacZ)^a

	IFN- γ (pg/ml)	IL-6 (pg/ml)	IL-12 (p70) (pg/ml)
PBS	0	0	0
AdlacZ + Riboflavin	3.732 $\pm$ 467.1	374.22 $\pm$ 46.63	53.47 $\pm$ 5.1
AdlacZ	5,585.9 $\pm$ 331.9	525.53 $\pm$ 97.82	75.45 $\pm$ 4.96

^aValues were determined by ELISA assays of serum collected from 4 animals per treatment group and reflect the average measurement $\pm$ the standard error of the mean.