NOVEL ANTIVIRAL STRATEGIES TARGETING HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) VIRAL PROTEIN R AND ITS CELLULAR PARTNER, THE GLUCOCORTICOID RECEPTOR

by

Elizabeth Ann Schafer

B.S., Biology, Grove City College, 2000

B.A., English, Grove City College 2000

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This thesis was presented

by

Elizabeth Ann Schafer

It was defended on

13 July 2005

and approved by

Velpandi Ayyavoo, Ph.D
Thesis Advisor
Assistant Professor
Infectious Diseases and Microbiology
Graduate School of Public Health
University of Pittsburgh

Phalguni Gupta, Ph.D
Committee Member
Assistant Chairman and Professor
Infectious Diseases and Microbiology
Graduate School of Public Health
University of Pittsburgh

Meryl H. Karol, Ph.D
Committee Member
Professor and GSPH Associate Dean
Environmental and Occupational Health
Graduate School of Public Health
University of Pittsburgh

NOVEL ANTIVIRAL STRATEGIES TARGETING HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) VIRAL PROTEIN R AND ITS CELLULAR PARTNER, THE GLUCOCORTICOID RECEPTOR

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Most highly active anti-retroviral treatment (HAART) regimens eventually fail to provide complete and long-term suppression of virus replication due to the inability to fully clear virus from cellular reservoirs. The HIV-1 viral protein R, Vpr, increases virus replication in T cells and is necessary for the optimal infection of primary monocytes/macrophages and other non-dividing cells. In this essay, it is demonstrated that Vpr interacts with the cellular Glucocorticoid Receptor (GR) and transactivates the HIV-1 LTR through GRE and that this event can be blocked by the GR antagonist, mifepristone. Based on these observations, it is shown that targeting Vpr-mediated virus transcription with the glucocorticoid antagonist, mifepristone, can demonstrate a potent anti-retroviral therapy.

Results demonstrated that Vpr-induced transactivation of both autologous and heterologous promoters was inhibited by mifepristone in a dose-dependent manner by >90% at a 1 μM concentration. Infectivity assays using T-tropic, dual-tropic, and macrophage-tropic viruses demonstrated antiviral effects on a dose-dependent regimen of mifepristone. The effects of

mifepristone were also tested in HIV-1 latent cells that could be activated with extra-cellular viral protein and results exhibited a greater than 90% inhibition of re-activation in the presence of this antagonist. Cytotoxic effects of mifepristone demonstrated a CT_{50} from 10 to 100 μM in normal human primary cells, HeLa, HEK293, and CV-1 cells.

Statement of Public Health Relevance: By utilizing the interaction between Vpr and the glucocorticoid receptor, glucocorticoid antagonists such as mifepristone hold promise for anti-retroviral therapy by both preventing viral transactivation in currently-infected cell populations as well as preventing the reactivation of latent virus.

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excluded, so openly and whole-heartedly throughout whatever duration they may stay. I hope that I have been able to support, guide, and influence all of you in a positive manner, as your optimism and encouragement have been more than evident every day that I have stepped into the lab. So in the order in which I have known you, here are my thanks:

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Finally, I would like to thank God for the blessings with which I have been given, and to whom all credit is owed. His guidance led me to this department and has enabled me to overcome many trials and frustrations, of which I am truly thankful.

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1.1. AIDS: The Epidemic

There are currently an estimated 42 million people infected world-wide with HIV/AIDS (Human Immunodeficiency Syndrome/Acquired Immune Deficiency Syndrome), with approximately 14,000 new infections occurring every day (1) (Table 1). While current therapies including highly active anti-retroviral drug therapy (HAART) have without doubt prolonged the lives of many fighting HIV and have prevented innumerable new infections, the drug regimen is costly and adherence is generally low due to both toxicity and drug failure (2,3). Studies have shown that a regimen adherence of 95-98% is necessary to control infection and that missing even a single dose in a 28-day period can predict treatment failure (4,5). The evolution of drug-resistant viral strains has been shown to occur due to the non-adherence of therapy in a "bell-shaped manner": medium regimen adherence increases drug resistance in comparison to low or high adherence (6,7).

Four major classes of antiviral therapy are currently prescribed, which include the new fusion inhibitors, protease inhibitors, and reverse transcriptase inhibitors. Generally, HAART is administered as "triple therapy" consisting of a protease inhibitor (PI) or a non-nucleoside reverse transcriptase inhibitor (NNRTI) as well as two nucleoside reverse transcriptase inhibitors (NRTI) (8). Unfortunately, these therapies often lead to toxic side effects including mitochondrial toxicity, lipodystropy, lactic acidosis, hepatatoxicity, and cardiomyopathy (9-12). Currently, other antiviral targets are being explored including integrase inhibitors, CXCR4 and

CCR5 inhibitors, and cellular proteins including APOBEC3G and TRIM5α that demonstrate antiviral behavior (13-18).

Viral reservoirs such as macrophages and resting T cells prevent total viral clearance even during an extensive HAART regimen (19-22). Though there is some speculation that patients treated with HAART <6 months post-seroconversion are able to decrease latent viral loads to less than detectable levels, it is hypothesized that patients would have to undergo 60 years of HAART to clear all viral reservoirs (23,24). While there is great pursuit to find new therapies or vaccines, there is a substantial need to find additional novel compounds which will target areas of virus replication not yet investigated for antiviral intervention.

World Region	Adults and Children Living with HIV	Adults and Children Newly Infected with HIV	Adult Prevalence Rate (%)	Adult and Child Deaths due to AIDS
Sub-Saharan Africa	25 Million	3 Million	7.5	2.2 Million
North Africa and Middle East	480000	75,000	0.2	24,000
South and South-East Asia	6.5 Million	850,000	0.6	460,000
Latin America	1.6 Million	200,000	0.6	84,000
Caribbean	430,000	52,000	2.3	35,000
Eastern Europe and Central Asia	1.3 Million	360,000	0.6	49,000
Western Europe	580,000	20,000	0.3	6,000
North America	1 Million	44,000	0.6	15,000
Oceania	32,000	5,000	0.2	700
East Asia	900,000	200,000	0.1	44,000
Total	37.8 Million	4.8 Million	1.1%	2.9 Million

Table 1. ***Regional HIV and AIDS statistics (2003).*** **There are currently an estimated 42 million people infected with HIV/AIDS around the world. UNAIDS and WHO statistics show the largest number of HIV/AIDS cases occurring in Sub-Saharan Africa and South or South-East Asia.**

2. CHAPTER TWO: BACKGROUND

2.1. HIV-1: STRUCTURE, REPLICATION, PATHOGENESIS

The human immunodeficiency virus type 1 (HIV-1), the etiologic agent of AIDS, is classified as a member of the *Retroviridae* family; more specifically, as a member of the Lentiviral genus. The 9.8 kilobase single-stranded HIV-1 genome is composed of at least nine genes coding for structural/enzymatic (*gag, env, pol*), regulatory (*tat, rev*) and accessory (*vif, vpr, nef, vpu*) proteins (Figure 1, Table 2).

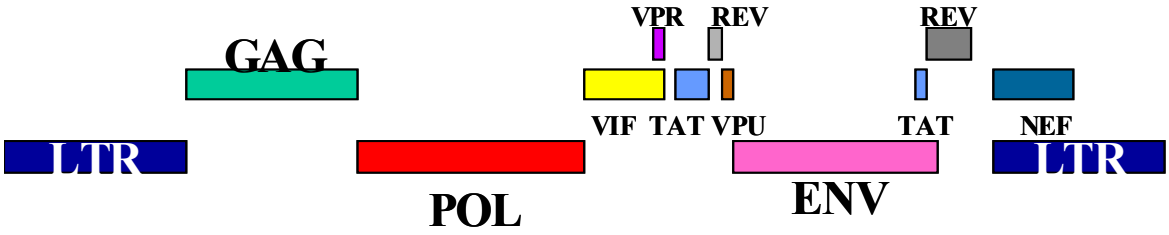


Figure 1. ***The HIV-1 Genome.*** **The schematic representation of the HIV-1 genome includes both structural and accessory genes including *gag, pol, vif, tat, vpr, rev, vpu, env,* and *nef.* These genes are flanked on both ends by the HIV-1 LTR (long terminal repeat) promoter region.**

Table 2. ***Functions of HIV-1 Proteins.*** **The nine proteins composing HIV-1 are listed along with their respective functions.**

HIV-1 Protein	Function
Vif	Viral Infectivity Factor; RT complex stability, degrades APOBEC3G
Nef	Negative Factor; Downregulates CD4 and MHCI expression, Role in viral pathogenesis
Gag	Matrix(MA), Capsid (CA), Nucleocapsid (NC) structural proteins; p24; p6 (interacts with Vpr in the capsid)
Pol	Precursor to Protease (PR), Integrase (IN), and Reverse Transcriptase (RT)
Rev	Regulator of Expression; Promotes nuclear export of RNA
Tat	Transcriptional Activator; Enhances gene transcription
Vpu	Down-modulation of CD4; Enhances virion release
Env	gp41 and gp120; comprises the viral envelope; attachment
Vpr	Viral Protein R; transport PIC, apoptosis, transactivation, G2 cell cycle arrest

The long terminal repeat (LTR) flanks both ends of the viral genome, and serves as the major promoter region for viral transcription (Figure 2). The HIV-1 LTR contains multiple binding sites for transcription factors including the glucocorticoid receptor, SP-1, NF-AT, and NF-κB.

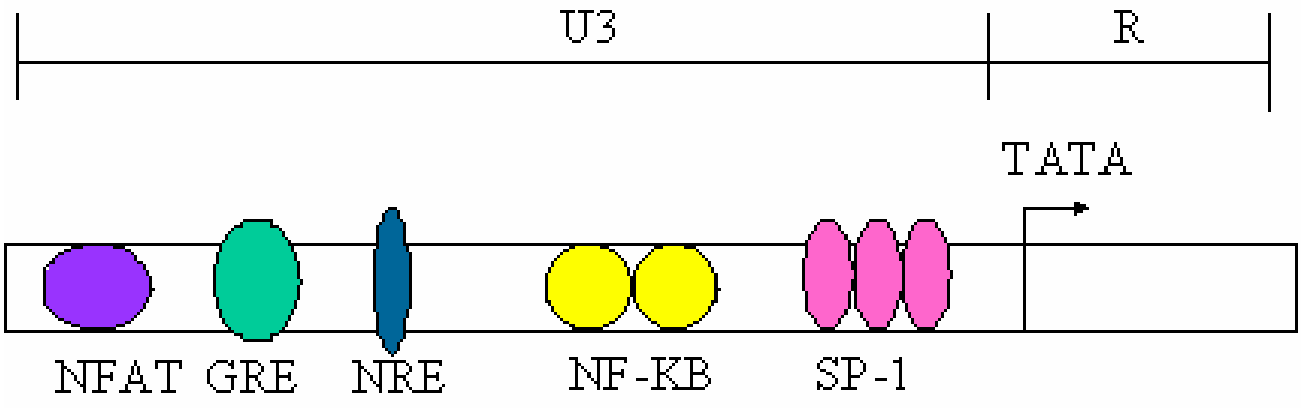


Figure 2. ***HIV-1 Long Terminal Repeat (LTR).*** **The LTR is the main promoter region for HIV-1 viral genes. The main transcription factor binding sites are found in the U3 region of the LTR and are schematically represented.**

Briefly, upon entry into host cells, HIV-1 RNA is reverse transcribed into DNA in the cytoplasm of the cell, resulting in the entry of viral DNA into the nucleus of the cell and leading to subsequent integration with host DNA (Figure 3). Target cells of HIV-1 infection include both the dividing $CD4^+$ T cells and non-dividing macrophages (25-27). Post-infection, latent reservoirs are established in various cell types including both T cells and macrophages and serve as the cause for both initial asymptomatic infection as well as the inability to fully expel virus from infected patients (28).

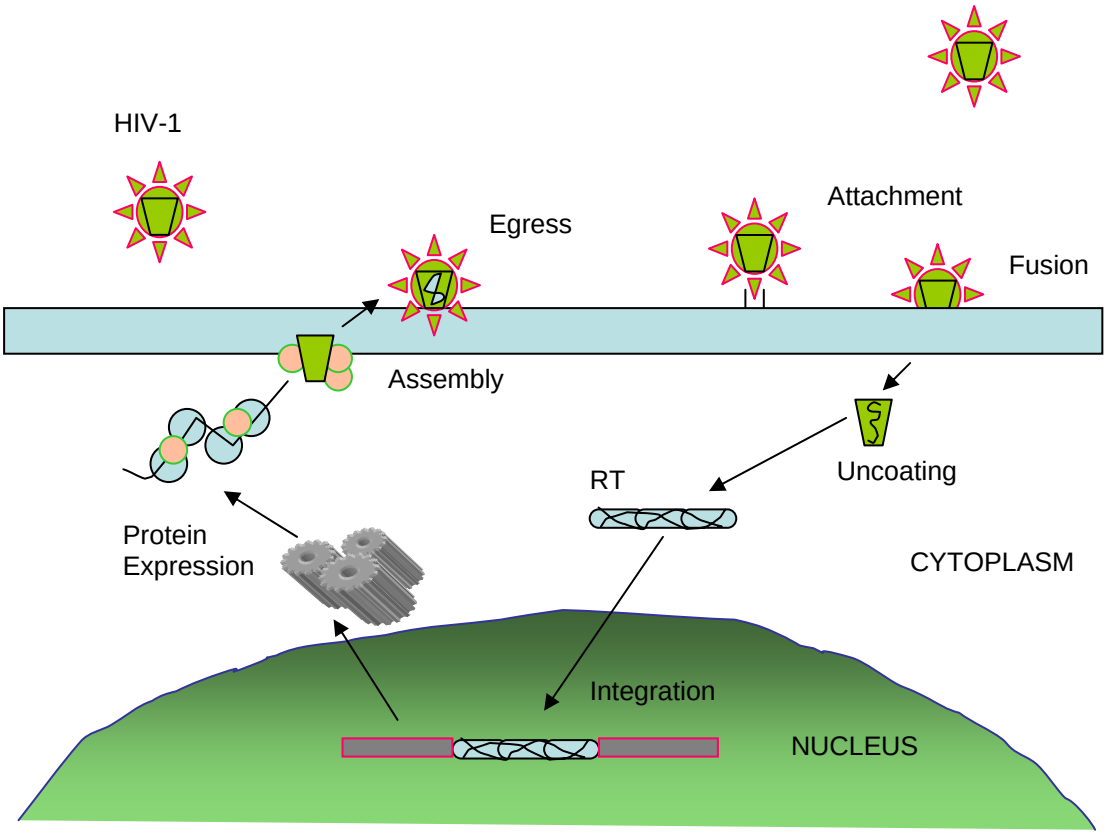


Figure 3. ***Replication of HIV-1 Genome.*** **HIV-1 attaches and fuses through the cellular membrane. The RNA uncoats in the cytoplasm and reverse transcription (RT) occurs. Viral DNA integrates with cellular DNA, leading to the production of viral proteins which in conjunction with viral RNA, assemble at the cellular membrane where the virus fully assembles, matures, and egresses into the extra-cellular environment.**

2.2. THE ACCESSORY PROTEIN, VPR

As aforementioned, HIV-1 is composed of at least four accessory proteins. One of these accessory proteins and the one in which the focus of this thesis concerns, viral protein R (Vpr), is 96-amino acids in length with a molecular weight of approximately 14 kDa. Vpr can be defined as a pleiotropic protein, as it has been shown to serve in an array of varying functions including cell cycle arrest at the G2/M phase, immune regulation and evasion, apoptosis, involvement with the pre-integration complex (PIC), and viral transactivation (29-39). It is this final function, viral transactivation, which will be further investigated throughout this essay.

While defined as an accessory protein, Vpr is evolutionarily conserved in HIV-1, HIV-2, and SIV, demonstrating its importance as a viral protein (40). Vpr is also packaged within the virion, possibly transactivating viral genes prior to the production of Tat (41). Studies have shown that Vpr, while important during infection of dividing cells such as T cells, is vital to the infection of non-dividing cells such as macrophages, possibly due to its involvement with PIC (42-44). Important to note is that macrophages are the primary initial targets of HIV-1, specifically located within the mucosa of the skin (45). The importance of Vpr in the initial stages of infection, therefore, can be demonstrated by the necessity of Vpr for optimal macrophage infection during initial virus/host cell contact.

Vpr is composed of three helices (Helix I, II, and III) connected by loop regions (Figure 4) (46). Each helix contains the motif LxxLL, which has been shown to interact with cellular proteins (47,48). These LxxLL motifs are involved in the interaction of Vpr with the cellular protein, the

glucocorticoid receptor (GR) (49). Thus the specific interaction between Vpr and GR, which increases transactivation of viral proteins, is a probable target for antiviral intervention.

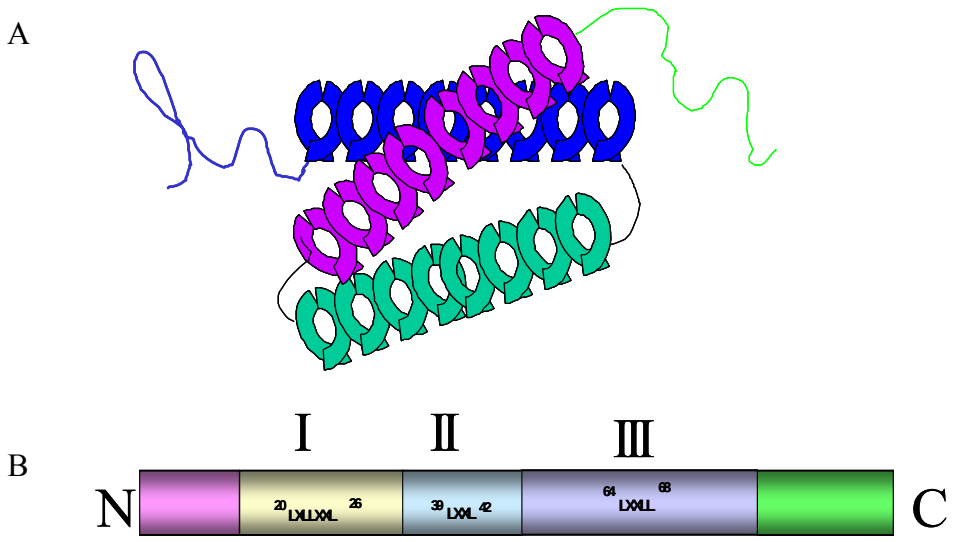


Figure 4. ***(A) Schematic Representation of Vpr. (B) Schematic Representation of Helices I, II, and III.***

2.3. THE GLUCOCORTICOID RECEPTOR AND ITS ROLE IN VPR-MEDIATED VIRAL TRANSACTIVATION

The Glucocorticoid Receptor (GR) is a host cellular ligand-dependent transcription factor and a member of the nuclear hormone receptor family. Under normal conditions, GR serves mainly as a negative regulator of NF-κB- and AP-1-induced gene transcription (cytokine production) (50,51).

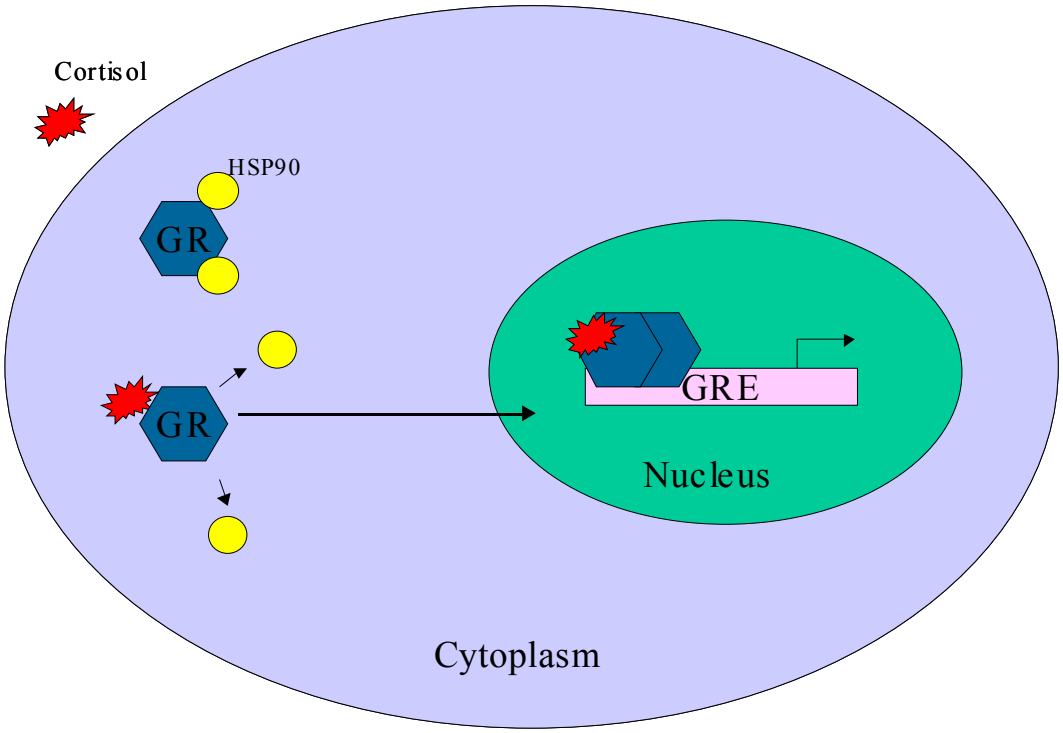


Figure 5. ***The GR Pathway.*** **The Glucocorticoid Receptor is held in the cytoplasm by heat shock proteins and other chaperones. Cortisol binds the GR within the Ligand-Binding Domain and activates GR. A conformational change after ligand binding dislodges the chaperones and presents nuclear localization signals. GR then translocates into the nucleus where it bind the Glucocorticoid Response Element (GRE) promoter region within the cellular DNA, leading to the transactivation of downstream genes. GR is also able to bind to negative GRE elements, regulating NF-κB-driven gene expression.**

GR is held in the cytoplasm of the cell by a heteromeric complex composed of several proteins including HSP90, HSP70, P23, and other chaperone proteins (52-55). Once ligand binds the GR within its ligand-binding domain region (LBD), GR is released from this complex and translocates into the nucleus of the cell where it binds specific promoter response elements termed Glucocorticoid Response Elements, or GREs (56,57) (Figure 5). One of the ways in which GR is able to negatively regulate the effects of the pro-inflammatory response is by binding to negative GREs or nGREs, which decreases gene transcription (58). GR also interacts with other transcription factors through direct protein-protein interaction, inhibiting gene

transactivation, as well as enhancing transactivation of anti-inflammatory cytokines, thereby combating the effects of cytokine-enhancing transcription factors such as NF-κB (59,60)

The Glucocorticoid Receptor contains three specific domains: AF-1 domain, DNA-binding domain, and the Ligand-binding domain (Figure 6) (61). The N-terminus AF-1 domain, consisting of amino acids 1-421, is a transcriptional activation function domain (62). The GR DNA-binding domain (DBD) is comprised of the next 65 amino acids and contains a zinc-finger DNA-binding motif which interacts with GREs (63). The C-terminus Ligand-binding domain (LBD) is made of 250 amino acids and both interacts with ligand as well as is involved in protein-protein interaction with chaperones and co-regulators (64). GR consists of at least two nuclear localization signals found in both the DBD and LBD regions (65).

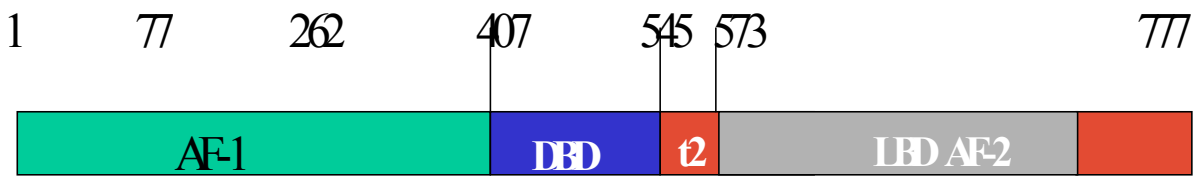
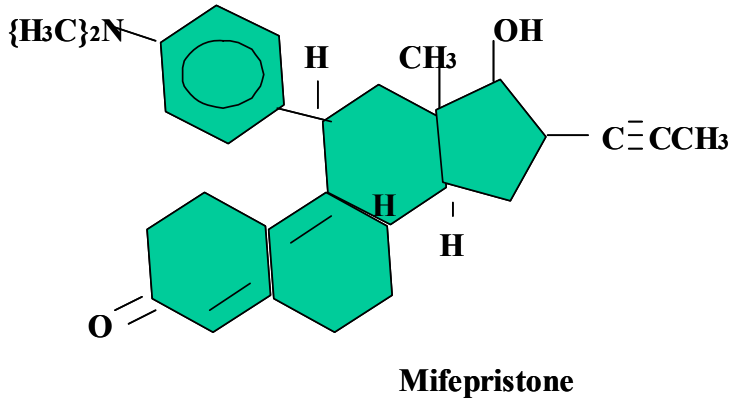


Figure 6. ***Schematic Representation of the Glucocorticoid Receptor.*** **The Glucocorticoid Receptor is made of the N-terminus AF-1 domain, the DNA binding domain, and the C-terminus Ligand binding domain.**

Vpr is able to directly interact with GR and act as ligand, increasing the transactivation of GRE-driven downstream genes. While it is currently unknown where Vpr binds GR, we have shown that the Vpr and GR interaction is mediated through the LxxLL motifs present in Vpr helices II and III (49).

2.4. THE GR ANTAGONIST, MIFEPRISTONE

Mifepristone, (11β-[*p*-(Dimethylamino)phenyl]-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one), empirical formula $C_{29}H_{35}NO_2$, and a derivative of norethindrone, is a yellow powder with a molecular weight of 429.6 and a melting point of 191-196°C (Figure 7). It is soluble in methanol, chloroform, and acetone (66).



Mifepristone

Figure 7. ***Structure of mifepristone.***

Mifepristone was originally synthesized by scientists at Roussel-Uclaf in Romainville, France while searching for steroidal compounds with anti-hormone properties (67-69). Defined as a class II GR antagonist, mifepristone demonstrates strong antagonistic effects on GR and the Progesterone Receptor (PR) with some sited instances of agonist activity towards GR, dependent upon receptor concentration (70,71). Mifepristone, which binds at the GR ligand-binding domain in the same pocket as dexamethasone, is able to exert antagonistic effects on GR-mediated transactivation by both tightening the bind between GR and GRE, thereby decreasing

the rate of speed in which gene transcription occurs (72,73), and by recruiting co-repressors rather than co-activators to the transcription complex, thus decreasing transactivation (74). It is this interaction between mifepristone and GR that produces a possible target for antiviral therapy.

2.5. THE PROPOSED RELATIONSHIP BETWEEN VPR, GR, AND MIFEPRISTONE

We have shown that Vpr and GR specifically interact, and that Vpr is able to act as ligand in a similar manner to that of dexamethasone, increasing GR-mediated transactivation (49). Looking more specifically at viral transcription, found within the HIV-1 LTR promoter region are GRE sequences (75). Therefore, during infection, GR-mediated transactivation of GRE-promoted downstream gene does not only occur in the context of cellular DNA, but can also occur with the integrated HIV-1 proviral DNA, thereby increasing viral transcription (viral production). We therefore propose that Vpr, either nascently transcribed within the host cell, or Vpr which has transduced through the cellular membrane (free Vpr), is able to bind GR similar to ligand, leading to the binding of GR to the GRE promoter region found within the HIV-1 LTR, thereby promoting the transactivation of viral genes. And that the GR antagonist, mifepristone, is able to therefore decrease or inhibit Vpr/GR-mediated viral gene transactivation in infected cells (Figure 8). The decrease or prevention of Vpr-mediated transactivation of viral genes also allows for the opportunity to inhibit or decrease reactivation from viral reservoirs, as the integrated proviral DNA will be less likely to transactivate (reactivate) in the presence of the GR antagonist, mifepristone. This is especially important in the context of macrophages in which Vpr is necessary to infect, and in which viral reservoirs are often present.

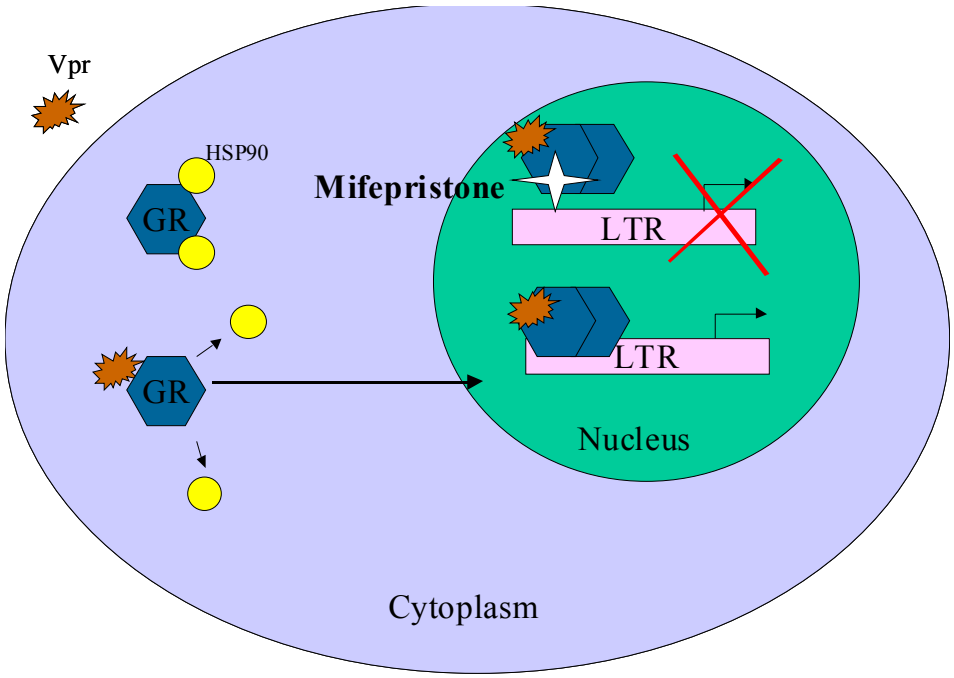


Figure 8. ***Schematic Representation of Vpr-mediated LTR transactivation.*** **Vpr interacts with GR as ligand, similar to cortisol, leading to the transactivation of GRE-driven genes, including those found in the HIV-1 LTR. Mifepristone is able to decrease this transactivation via several different mechanisms.**

2.6. CURRENT ANTI-RETROVIRAL THERAPY

Currently, there are four classes of anti-viral therapy targeting different steps in HIV-1 replication within the host cell (Table 3, Figure 9). Briefly, these therapies target either reverse transcription (AZT, efavirenz, 3TC), protease maturity (indinavir, ritonavir), or fusion (Fuzeon). Other classes of drugs are being investigated and offer hope for HIV-1 infected patients, as drug-resistant viral strains are becoming more and more frequent within infected populations, and as the adverse effects to the drugs themselves often cause patients to fail therapy.

Table 3. ***Four Classes of Retroviral Therapy and Associated Drugs.*** **The current HIV-1 antiviral drugs which are FDA-approved are listed as follows along with their common names and abbreviations.**

Common Name	Abbreviation	Drug
Nucleoside and Nucleotide RT Inhibitors (NRTIs)Trade name		
Combivir	CBV	AZT+3TC
Emtriva	FTC	Emtricitabine
Epivir	3TC	3TC, lamivudine
Hivid	ddC	ddC, zalcitabine
Kivexa		3TC+ABC
Retrovir	AZT	AZT, zidovudine
Trizivir	TZV	AZT+3TC+ABC
Truvada		FTC+TDF
Videx	ddI	ddI, didanosine
Viread	TDF	Tenofovir
Zerit	d4T	d4T, stavudine
Ziagen	ABC	Abacavir
Non-Nucleoside RT Inhibitors (NNRTIs)		
Rescriptor	DLV	Delavirdine
Sustiva	EFV	Efavirenz
Viramune	NVP	Nevirapine
Protease Inhibitors (PIs)		
Agenerase	APV	Amprenavir
Crixivan	IDV	Indinavir
Fortovase	SQV-FTV	Saquinavir soft gel
Invirase	SQV-INV	Saquinavir hard gel
Kaletra	LPV	Lopinavir/ritonavir
Lexiva/Telzir	FPV	Fosamprenavir
Norvir	RTV	Ritonavir
Reyataz	ATV	Atazanavir
Viracept	NFV	Nelfinavir
Fusion inhibitors		
Fuzeon	T-20	Enfuvirtide

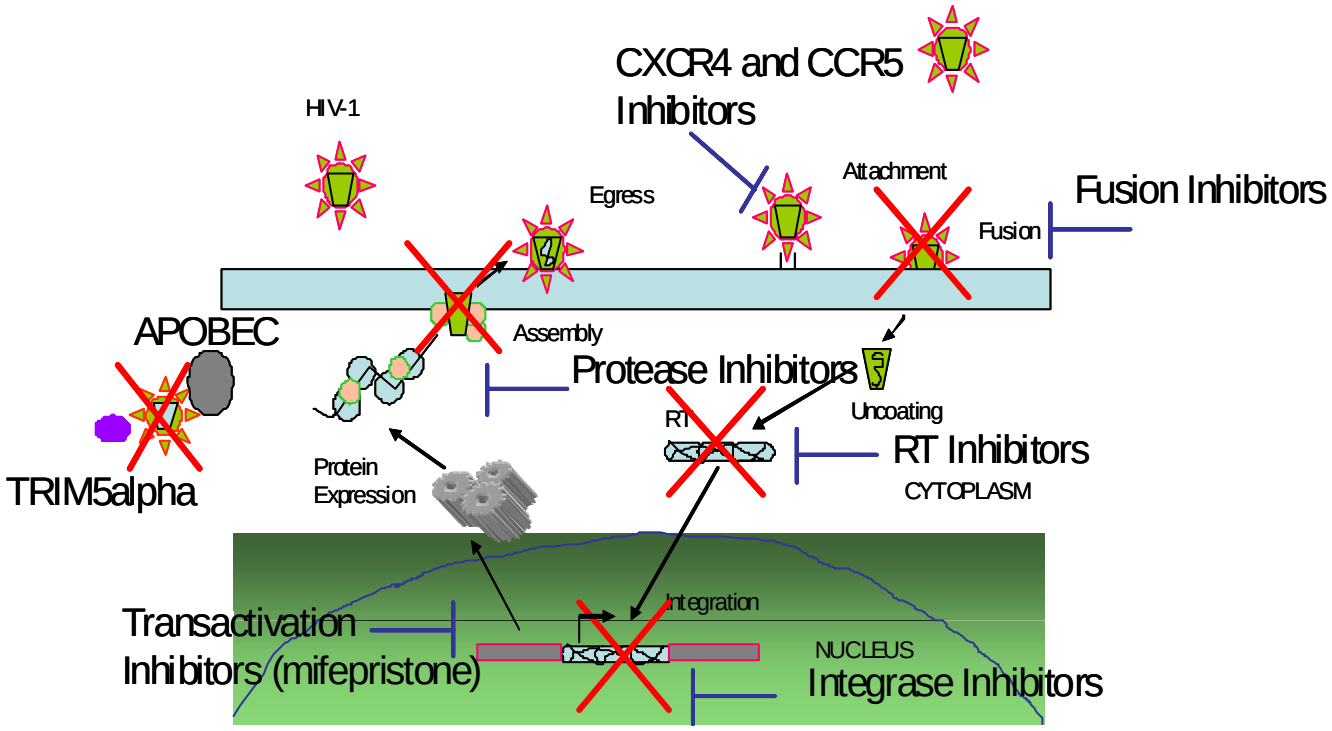


Figure 9. ***Targets for Antiviral Therapy within the HIV-1 Life Cycle.*** **Current antiviral therapy (Fusion Inhibitors, RT Inhibitors, and Protease Inhibitors) in conjunction with possible antivirals (Integrase Inhibitors, CXCR4 and CCR5 Inhibitors, Cellular Inhibitors, and Transactivation Inhibitors) are schematically represented.**

2.7. PUBLIC HEALTH SIGNIFICANCE

With an estimated 42 million people infected with HIV/AIDS around the world, there is great need for new therapies targeting HIV-1 infection that are both inexpensive as well as easily accessible. While current HAART therapy has increased the lifespan of thousands of people and prevented many new infections by both vertical and horizontal transmission, the inability of many infected patients to correctly follow the drug regimen along with the development of viral reservoirs and drug-resistant strains, results in the need for the presentation of new antivirals,

targeting different stages of the viral lifecycle. Recently, accessory proteins such as Vif and Nef have been investigated to determine if these proteins hold any answers for finding new antiviral drugs. Along that line, the interruption of Vpr-mediated transactivation via mifepristone could give rise to a new class of antiviral drugs, one which could target both active replication as well as viral reservoirs. Conceding that this therapy would not be the end-all-be-all of therapies, we propose that it could, in fact, be incorporated into current HAART therapy as a combative antiviral.

3. CHAPTER THREE: THESIS AIMS

With the development of HAART, HIV-1-infected patients have been able to prolong their lives as well as help prevent the spread of virus to others. However, HAART therapy almost always eventually fails leading ultimately to the death of the patient due to complications of opportunistic infection. Viral reservoirs are often established within the patients and viral-resistant species develop within those infected, leading to a down-hill battle concerning overcoming the fight with HIV-1.

The necessity of developing new antiviral compounds to combat HIV-1 infection has led to the investigation of specific proteins encoded by the virus, including the accessory protein Vpr. Vpr has been implicated in a variety of functions including G2M cell cycle arrest, apoptosis, immune regulation, viral escape, and finally, an increase in viral transactivation. It has been determined that one of the mechanisms underlying Vpr-mediated transactivation is through the utilization of the pathway established by the Glucocorticoid Receptor (GR). Vpr is able to activate GR in a ligand-dependent manner, similar to that of cortisol, leading to the transactivation of GRE-driven genes. The HIV-1 LTR, which contains GRE sequences, promotes the transcription of viral genes in the presence of Vpr, mediated by the interaction between Vpr and GR. The GR antagonist, mifepristone, is able to inhibit Vpr-mediated transactivation by interfering on several levels with the transcription of GRE-promoted genes.

The three major aims of this thesis included first defining the relationship between Vpr and GR and the subsequent increase in viral transactivation. Secondly, we wanted to assess whether or

not the GR antagonist, mifepristone, was able to inhibit viral gene transactivation and viral replication in both transactivation models and infectivity models. Third, we wanted to view the effects of mifepristone in a clinical manner, to determine if mifepristone worked differently in a pre- versus post-treatment regimen and to investigate the effects of mifepristone on reactivation from latency. Mifepristone analogs were also investigated to assess their ability to inhibit HIV-1 LTR-mediated transactivation as well as viral replication.

AIM #1: To determine the relationship between Vpr and GR and their role in virus replication

A) To test the effects of Vpr on LTR-mediated transactivation
B) Determine how Vpr increases LTR-mediated transactivation
C) Determine the specific interaction between Vpr and GR resulting in downstream transactivation

AIM #2: To assess whether the GR antagonist, mifepristone, is able to inhibit viral transactivation and virus replication

A) Determine the cytotoxicity of mifepristone
B) Study effects of mifepristone on transactivation models
C) Assess effects of mifepristone in T-tropic, dual-tropic, and M-tropic infection models
D) Determine effects of mifepristone patient isolate infection models

AIM #3: Clinical Applications of Mifepristone

A) Determine if mifepristone works differently in a Pre vs. Post Antiviral Infection Study
B) Determine if mifepristone is able to prevent reactivation of virus from latency
C) Study the effects of mifepristone analogs on viral transactivation and infection

4. CHAPTER FOUR: MATERIALS AND METHODS

4.1. Cell Lines

4.1.1. Established Cell Lines

HeLa, HEK293, HEK293T, and TZM cell lines were grown in DMEM (Gibco, Carlsbad, California) supplemented with 10% FBS (HyClone, Logan, Utah), 1% Penicillin-Streptomycin, and 1% L-Glutamine. Both HeLa and TZM cells were grown in 5% CO_2 at 37˚ C; HEK293 and HEK293T cell lines were grown in 10% CO_2 at 37˚ C. CV-1 cells were sustained in DMEM supplemented with 10% charcoal/dextran-stripped FBS (HyClone, Logan, Utah) at 5% CO_2 at 37˚ C. HeLa cells were obtained from the NIH AIDS Research and Reference Reagent program from Dr. Richard Axel (76). CV-1 cells were obtained from Dr. Frank Jenkins at the University of Pittsburgh and TZM-bl cells were received from the AIDS Research and Reference Reagent program from Drs. John C Knappes, Xiaoyun Wu, and Tranzyme, Inc. HEK293T cells were a generous gift from Dr. Michelle Calos, Stanford University, CA. HEK293 cells were from NIH AIDS Research and Reference Reagent Program. U1/HIV-1 and CEM cell lines were grown in RPMI 1640 (Gibco, Carlsbad, California) and supplemented with 10% FBS, 1% L-Glutamine, and 1% Penicillin-Streptomycin as. 174xCEM cells were provided by Dr. Peter Cresswell (77) and were received from the AIDS Research and Reference Reagent Program. The U1/HIV-1 cell line was acquired through the AIDS Research and Reference Reagent Program as contributed by Dr. Thomas Folks (78).

4.1.2. Primary Cells

PBMCs: Peripheral Blood Mononuclear Cells (PBMCs) were isolated using blood from healthy male HIV-1-negative donors. Male donors were used to avoid the irregulation of hormone cycling in women. Blood was isolated using Lymphocyte Separation Media (Mediatech, Herndon, Virginia) gradient centrifugation. Post-isolation, PBMCs were resuspended in RPMI 1640 supplemented with 10% FBS and stimulated with phytohemoagglutinin (PHA-P) (Sigma, St. Louis, Missouri) (5µg/mL) for three days. After stimulation, cells were cultured in RPMI supplemented with 10% FBS in the presence of IL-2 (Chiron, Emoryville, California) (5U/mL).

Human Monocyte-Derived Macrophages: Monocyte-derived macrophages were isolated from PBMCs by adhesion. PBMCs were resuspended in DMEM supplemented with 1% Penicillin-Streptomycin and 1% L-Glutamine without the presence of serum and were plated in either 100mM plates (20×10^6), 6-well plates (3×10^6/well), 12-well plates (1.5×10^6/well), or 96-well plates (1.5×10^5/well) for two hours at 37˚ C, to allow the monocytes to settle and adhere to the plates. After the two-hour incubation, non-adherent cells (primary blood lymphocytes) were excluded by removing the cells and the adherent cells were washed two times in phosphate buffered saline (PBS) to remove all particulates including fat, debris, and other cells attached to the monocytes. DMEM supplemented with 10% FBS, 10% either horse or human sera, 1% Penicillin-Streptomycin, 1% L-Glutamine, 500 U/mL GM-CSF (granulocyte-macrophage colony stimulating factor) (Berlex Labs, Richmond, California), and 15ng/mL M-CSF (macrophage colony stimulating factor) (Amgen, Thousand Oaks, California) were added to the cells to begin the differentiation of the monocytes into macrophages. After five days, the monocyte media was

removed and spun down to remove any floating cells (not of monocyte lineage) and other debris. Half of this media was placed back on the cells and an equal quantity of fresh monocyte media was placed on the cells to ensure the proper cytokines and nutrients were administered to the cells. After eight to ten days, macrophages were differentiated as determined by visual assessment (Figure 10) and CD14 FACS analysis.

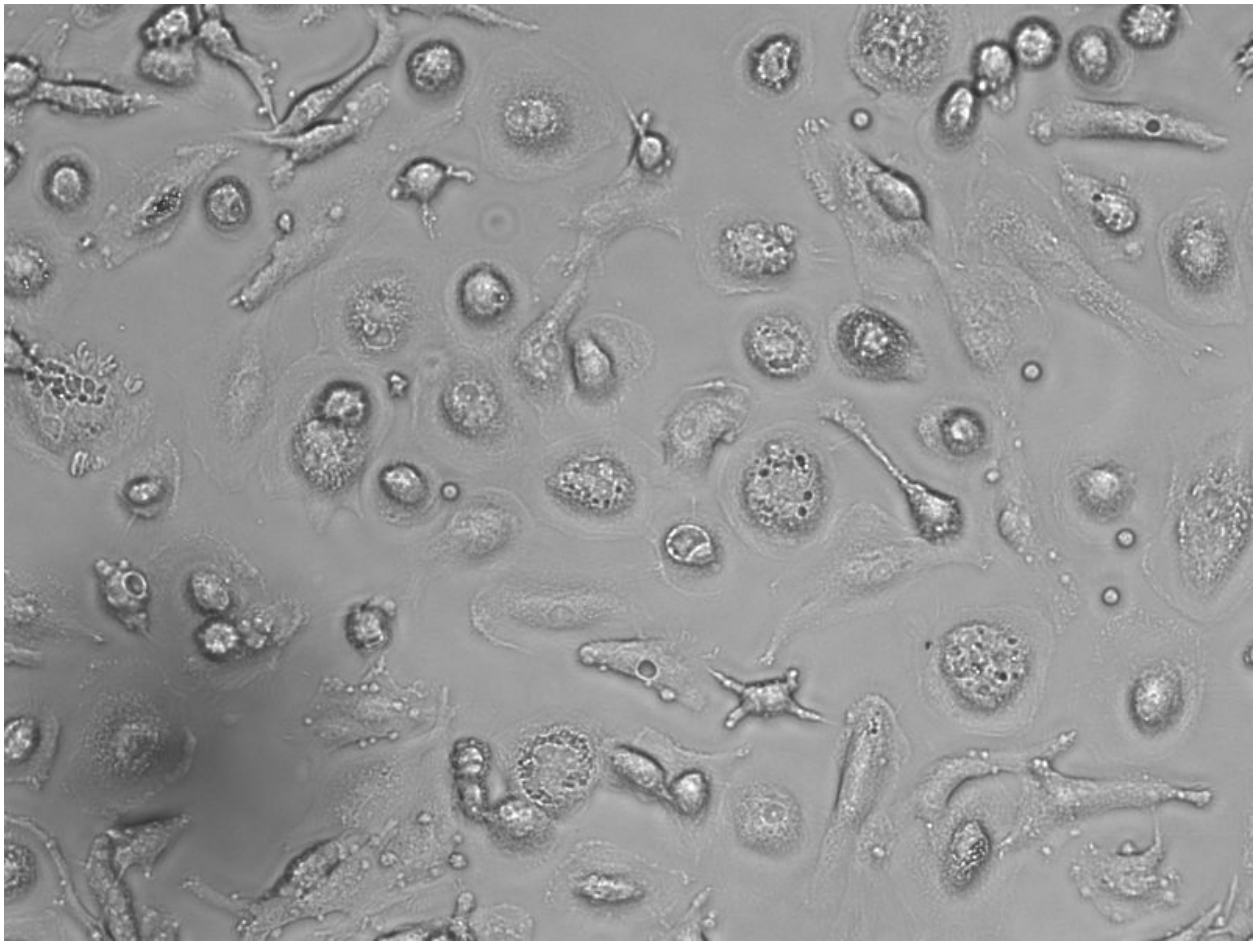


Figure 10. ***Fully Differentiated Macrophages.*** **Human monocytes were isolated as described and differentiated into macrophages in the presence of GM-CSF and M-CSF for approximately 8-10 days. When macrophages were fully differentiated, which was determined by both CD14 and visual assessment ("pancake" morphology), they were able to be infected with virus.**

4.2. Plasmids

For transactivation studies, the HIV-1 LTR reporter construct was obtained from NIH AIDS Research and Reference Reagent Program. The GRE (5X)-Luciferase reporter plasmid was constructed by PCR amplification of the 5X GRE consensus sequence from pGRE5/EBV vector (USB, CA) using specific forward (5' ATACGCGGATCCTCTAGA AGATCCGCT3') and reverse (5' ATCATACTCGAGGGCCCTCGCAGACA3') primers. Amplified PCR product (290bp) was cloned upstream of a firefly luciferase gene reporter construct. The human Glucocorticoid Receptor-alpha gene (hGRα) was PCR amplified using specific forward (5' ATCGGGGATCCGATGGACTCCAAAGAATCA3') and reverse (5' GTGGTCCTCGAGCTTGATGAAACAGAAG 3') primers and cloned into the pcDNA 3.1 (Invitrogen) vector for futher expression studies. Mutants of HIV-1 Vpr were generated using overlap PCR and/or by Quick-change mutagenesis (79) and were cloned under the control of the CMV promoter in pCDNA3.1 (Invitrogen, California) with a flag epitope. All plasmids were generated by propagation in DH5α bacterial cells and DNA was extracted by using either DNA-binding columns (Qiagen, Valencia, California) The integrity of the plasmid DNA was tested by both digest and electrophoresis followed by DNA sequence analysis (ABI 7700, CA).

4.3. Transfection Methods

4.3.1. Calcium Phosphate

The calcium phosphate method was used on HEK293 and HEK293T cell lines. Cells were plated to reach an approximate confluency of 75%. Three to four hours prior to cell transfection, old media was removed and 9mL fresh DMEM supplemented with 10% FBS was added to a 100mM plate; plates were moved from 10% CO_2 to 5% CO_2. The transfection was conducted adding DNA (450µL) to water (450µL) to 2.5M $CaCl_2$ (50µL). To the DNA-water-$CaCl_2$ mix, 50 mM BES (BES, 250mM NaCl, 0.5mM Na_2HPO_4) (500µL) was added and the mixture was incubated at room temperature for 30 minutes. After incubation, the mixture was added drop-wise to the surface of the cell media. Within 12-16 hours, the media on the cells was removed, the cells were washed two times with PBS to remove all precipitate and excess DNA, and fresh DMEM supplemented with 10% FBS, 1% Penicillin-Streptomycin, and 1% L-Glutamine was added. For transfections conducted in a smaller plate, all volumes were proportioned so that volume ratios were equivalent.

4.3.2. Lipid-Mediated Transfection

Lipid-mediated transfection was used on HeLa and CV-1 cells. Cells were plated and grown in penicillin-streptomycin-free media overnight to 85% confluency. DNA was mixed with Lipofectamine (3µL per approximately 3-5µg DNA) (Invitrogen, Carlsbad, California) and either OptiMEM (GIBCO, Carlsbad, California) or DMEM was mixed to a total volume of 200µL. The DNA-Lipofectamine complex was incubated at room temperature for 20 minutes. Post-

incubation, cell media was removed and cells were washed two times to remove any serum. The DNA-Lipofectamine complex was added drop-wise to the cell surface and OptiMEM or DMEM without penicillin-streptomycin was added to a volume just covering the cell monolayer. The cells were incubated at 37˚C for 3-4 hours. After incubation, the DNA-Lipofectamine-containing media was removed, cells were washed to remove any excess DNA or Lipofectamine, and fresh media was added.

4.4. Virus

HIV-1 89.6 was received from the NIH AIDS Research and Reference Reagent Program from contributor Dr. Ronald Collman (80) and propagated in PBMCs for two weeks with the addition of feeder PBMCs after seven days of infection. 89.6 is a dual-tropic virus specific for R5- and X4-expressing cells. HIV-1 Ba-L was received from the NIH AIDS Research and Reference Program, contributed by Drs. Suzanne Gartner, Mikulas Popovic, and Robert Gallo (81), and was propagated in PBMCs for two weeks, with the addition of feeder PBMCs after seven days. Ba-L utilizes the R5 receptor. The patient isolate, H112, was from the NIH AIDS Research and Reference Reagent Program. pNL4-3^{wt} was contributed by NIH AIDS Research and Reference Reagent Program. pNL4-3-EGFP was a kind gift from Dr. David Levy (University of Alabama) and was constructed as described (82). For all viruses, viral titer was determined through p24 ELISA and number of infectious particles were determined by TZM assay.

4.5. Mifepristone and Analogs

Mifepristone was purchased from Sigma, St. Louis, Missouri. Mifepristone analogs were a generous gift from the National Cancer Institute from Dr. Robert Schultz. Analogs were dissolved in DMSO. The compound mifepristone will not dissolve in water, and is dissolved in methanol. Initial studies involved a comparison between dissolving mifepristone with both methanol and ethanol in both transactivation studies as well as antiviral studies. Results demonstrated no significant difference in the effects of mifepristone on either transactivation or antiviral assays, therefore ethanol was used to dissolve mifepristone for all subsequent assays. In order to assess the effects of ethanol on transactivation and antiviral studies, ethanol controls were used. With the exception of U1 latent reservoir studies, ethanol demonstrated no significant effect in any assay conducted.

4.6. TZM Assay

The TZM cell line is an R5 and X4 indicator cell line that enables the analysis of HIV-1-infected cells containing both the luciferase and β-gal reporter genes. Receptor-mediated viral entry results in the transactivation of the luciferase and β-gal reporter genes which are downstream of the LTR promoter region. TZM cells were plated in a 96-well plate (15,000 cells/well) overnight to allow adherence. After adherence, virus was added to the cells in triplicate in serial dilutions. Cells and virus were incubated for eight hours in 37˚C to duplicate the normal amount of time cells were infected in subsequent assays. After incubation, the virus-containing media was removed and cells are washed two times in PBS to remove all excess virus and fresh media were

then added to the cells. In order to determine the actual number of infectious particles prior to virus replication, twenty-four hours post-viral addition, cells were fixed in 0.5% Gluteraldehyde for 15 minutes and washed two times in PBS after fixation. In order to determine the number of infectious particles as determined by β-gal, an X-gal reaction mix (40mg/mL $MgCl_2$, 0.5M X-gal, K Ferrous Cyanide, K Ferric Cyanide) as added to the cells for thirty minutes at 37˚ C. After color had developed, cells were washed once in PBS and PBS was then added to the cells to allow for counting. Cells were counted per well and the number of infectious particles/mL were calculated.

4.7. Cytotoxicity Assay

To determine the percent of cytotoxicity *in vitro* of cells in the presence of mifepristone and analogs, the MTT Tetrazolium Assay, developed by T. Mosmann (83) was performed as follows:

Adherent cells: HeLa, HEK293, or CV-1 cells were plated (15,000/well) in a 96-well plate overnight for adherence. Mifepristone and analogs were then administered in triplicate in serial dilutions of differing concentrations for three days in a total volume of 200µL. After the three-day incubation, MTT (3-(4,5,dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye solution (5mg/mL in phosphate buffer pH 7.4) (Sigma, St. Louis, Missouri) was added (20µL/well) for 4-5 hours at 37˚C in 5% CO_2. After incubation with the MTT dye solution, cell media was removed and 200µL of DMSO was added by flushing so that the MTT salt crystals would fully dissolve. The cells were placed back in 37˚C for five minutes and read on a colormetric plate reader at 490nm.

Macrophages: After isolation, PBMCs were plated on a 96-well plate (150,000/well) for two hours while monocytes adhered to the plate. After two hours, non-adherent cells (PBLs) were removed and macrophage media (containing GM-CSF and M-CSF) was added to the cells. After five days, half the media was removed and fresh macrophage media was added. Once full differentiation of monocytes to macrophages had occurred (approximately 8-10 days), mifepristone and analogs were added in triplicate in serial dilutions in varying concentrations for three days. After the three-day incubation with the compounds, 20µL MTT dye solution was added to the media. The solution was incubated with the cells at 37˚C in 5% CO_2 for 4-5 hours. Cell media was then removed and DMSO was flushed to dissolve MTT crystals. Cells were incubated an additional 5 minutes at 37˚C to fully dissolve all crystals and results were determined by reading optical density on a colormetric plate reader at 490nm. For the seven-day timepoint, mifepristone was administered at day one and at day four . After seven days, the MTT assay was performed as described and percent viability was calculated based on O.D.

PBMCs: After PHA-P stimulation, PBMCs were resuspended in IL-2-containing media and plated (15,000 cells/well) in a 96-well plate. Mifepristone and analogs were added in triplicate in serial dilutions to the cells in varying concentrations for three days. After three days, MTT dye solution was added (20µL) and incubated at 37˚C for 4-5 hours. Cells were then spun down at 1000 rpm for 5 minutes and media was removed. DMSO (200µL) was added to the cells by flushing to dissolve all MTT crystals. Cells were incubated an additional 5 minutes at 37˚C to further dissolve salt crystals and results were determined by reading on a colormetric plate reader at 490nm. In order to determine percent viability at a seven-day timepoint, infected cells were counted by trypan blue exclusion and results presented as percent viability.

Calculations: To determine percent cytotoxicity, the O.D. results from the samples were divided by the O.D. from the positive control (cells without the presence of compound) and multiplied by 100.

4.8. Luciferase Reporter Assay

Dose Parameters: HeLa, CV-1, and HEK293 cells were co-transfected with the HIV-1 LTR-luciferase reporter construct (2.5μg) or the GRE-luciferase reporter construct (2.5μg) and varying doses of pVpr (0.1-5.0μg) in order to determine the optimal transactivation response. For the CV-1 cell line, phGRα was co-transfected (2.0μg) with reporter and Vpr or vector constructs. Forty-eight hours post-transfection, cells were washed and lysed using 500μL of 1X Reporter Lysis Buffer (Promega, Madison, Wisconsin). Cell lysates were spun down to remove cell debris and supernatant was collected for further use. Luciferase substrate was added to 20μL cell lysate. (Promega, Madison, Wisconsin). Luciferase was analyzed for expression by a Veritas luminometer and results were based on Relative Light Units (RLU). Protein estimation of the cell lysate was determined by the Bradford Protein Assay (Bio-Rad, Richmond, CA) and results were quantitated accordingly. Transfection efficiency was normalized by transfecting with pEGFP and analyzed by fluorescence expression.

Effects of Mifepristone on HIV-1 LTR or GRE-mediated Transactivation: HeLa, CV-1, or HEK293 cells were co-transfected with the HIV-1 LTR-luc (2.5μg) or GRE-luc (2.5μg) reporter constructs in the presence of pVpr. For CV-1 cell transfection, phGRα was also added (2.0μg).

Mifepristone, mifepristone analogs, and/or dexamethasone were added twenty-fours hours prior to cell lysis in a dose-dependent manner. Since mifepristone and analogs were solubilized in ethanol, all concentrations were in an equal volume to ensure that the presence of the solvent was not a variable to any results. Forty-eight hours post-transfection, cells were washed in PBS and lysed in 500µL 1X Reporter Lysis Buffer. Luciferase activity was measured by RLU and quantitated by protein estimation.

4.9. PBMC Antiviral Assay

PBMC Infection: To determine the effects of mifepristone on wild-type infection, PBMCs were isolated as described and stimulated with PHA-P (5µg/mL) for three days. After stimulation, cells were washed two times in PBS and resuspended in IL-2-containing media. For viral infection, total cells were infected with an MOI of 0.1 of either the 89.6^{wt}, pNL4-3, or H112 viruses for eight hours at 37˚C. After infection, virus-containing media was removed and cells were washed two times in PBS and re-suspended in fresh IL-2-containing media. Cells were plated in 6- or 12-well plates at 5 x 10^6 cells/well in a total volume of 3mL. Mifepristone or its analogs or the EtOH control were added in a dose-dependent manner to each well directly after infection. Supernatant samples (500µL) were removed every two to three days at which time mifepristone, compounds, or EtOH were re-administered due to the half-life (48-72 hours) of the compounds. To determine the concentration of viral p24, an ELISA (NIH, AIDS Vaccine Program, Frederick, MD) was performed as per the manufacturer's directions.

Pre-Cellular and Pre-Viral Treatment with Mifepristone: In order to determine if mifepristone decreased transactivation of viral genes more efficiently when either the cells or the virus were pre-treated with mifepristone, prior to infection cells were washed in PBS and re-suspended in IL-2-containing media. For cellular pre-treatment, PBMCs (5×10^6/well) were plated in 6- or 12-well plates in a total volume of 1mL and mifepristone was added in a dose-dependent manner to the cell media for two hours at 37˚C prior to infection. Cells were infected with an MOI of 0.1 of either 89.6^{wt}, pNL4-3, or H112 viruses for eight hours at 37˚C. Post-infection, virus-containing media was removed and cells were washed two times in PBS. Cells were re-suspended in 3mL of IL-2 containing media and mifepristone was re-administered to the cells in a dose-dependent manner similar to initial treatment with the compound. Every two to three days, supernatant samples were collected for analysis by p24 and mifepristone was re-administered in a dose-dependent manner. For viral pre-treatment, virus-containing media was incubated at 37˚C in the presence of mifepristone in a dose-dependent manner for two hours. Post-incubation, virus-containing media in the presence of mifepristone was added to the cells at an MOI of 0.1 and incubated for eight hours at 37˚C. After infection, cells were washed two times in PBS and re-suspended in IL-2-containing media. Mifepristone was administered in a dose-dependent manner similar to initial treatment with the compound. Samples were taken every two or three days for analysis by p24 ELISA and mifepristone was re-administered at that time.

4.10. Macrophage Antiviral Assay

Macrophage Infection: Monocyte-derived human macrophages were isolated from PBMCs by adhesion as described and plated in either 6- or 12-well plates. After 7-10 days differentiation by

GM-CSF and M-CSF, macrophages were infected with an MOI of 0.1 of either Ba-L, 89.6^{wt}, or H112 viruses for eight hours at 37˚C. After infection, virus-containing supernatant was removed and cells were washed two times to remove excess virus. 1.5mL fresh macrophage media (DMEM supplemented with 10% FBS, 10% Human or horse serum, 1% penicillin-streptomycin, 1% L-Glutamine) and 1.5mL media collected from macrophages prior to infection were added to the cells for a total volume of 3mL. Mifepristone, compounds, or the EtOH control were added in a dose-dependent manner post-infection. Supernatant samples were collected every two to three days for analysis by p24 ELISA and mifepristone, compounds, or the EtOH control were re-administered during that time.

Pre-Cellular or Pre-Viral Treatment: Prior to infection, cells were washed in PBS and 1mL macrophage media was added to the cells. Mifepristone, mifepristone analogs, or the EtOH control were administered to the cell media in a dose-dependent manner for two hours at 37˚C pre-infection. After the two-hour incubation, virus was added at an MOI of 0.1 of either Ba-L, 89.6^{wt}, or H112, and cells were incubated at 37˚C for eight hours. Post-infection, virus-containing media was removed and cells were washed two times in PBS. 1.5mL fresh macrophage media and 1.5mL macrophage media taken from cells prior to infection were added to the cells for a total volume of 3mL. Mifepristone or the EtOH control were added in a dose-dependent manner similar to original administration. Supernatant samples (500µL) were taken every two to three days for p24 analysis and mifepristone or the EtOH control were re-administered at that time.

4.11. CEM Antiviral Assay and FACS analysis

To demonstrate the effects of mifepristone specifically within a T-cell population, 174xCEM T-cells were infected with an MOI of 0.5 of the pNL4-3-EGFP wild-type virus for eight hours. Post-infection, cells were spun down and washed two times with PBS to remove all excess virus. Cells were plated in a 12-well plate ($2.5x10^5$/well) in a 2mL total volume and mifepristone was added in a dose-dependent manner to the cells. Mifepristone was re-administered to the cells after two days and at three days post-infection, cells were collected, washed two times in FACS buffer (PBS, 1% FBS, 1% Sodium Azide) and fixed in 2% paraformaldehyde for 30 minutes at 4˚C. After fixation, cells were washed to remove paraformaldehyde, resuspended in 500µL FACS buffer, and analyzed by FACS analysis.

5. CHAPTER FIVE: RESULTS

5.1. AIM#1: To Determine the Relationship between Vpr and GR and Their Resulting Role in Virus Replication

5.1.1. Effects of Vpr on Heterologous Transactivation

It is well-established by our group and others that Vpr increases the transactivation of viral genes. To demonstrate the effects of Vpr on viral gene transactivation, a luciferase reporter assay was utilized to measure the amount of transactivation occurring downstream from the heterologous promoter region, HIV-1 LTR. The HIV-1 LTR promoter region was constructed upstream of a firefly luciferase gene therefore upon promoter activation, downstream genes are transactivated, resulting in the production of the luciferase enzymatic protein. When substrate is added to this luciferase protein, light wavelengths are emitted and are quantitated in the form of Relative Light Units (RLU). To determine the effects of Vpr on promoter-driven transactivation, HEK293 or HeLa cells were co-transfected with the HIV-1 LTR-luciferase reporter construct (2.5μg) in the presence of varying doses of the Vpr expression plasmid (pVpr) (Figure 11). Figure 11 demonstrates that Vpr was able to increase the transactivation of heterologous promoter-controlled genes approximately 3.5-fold as represented by the downstream production

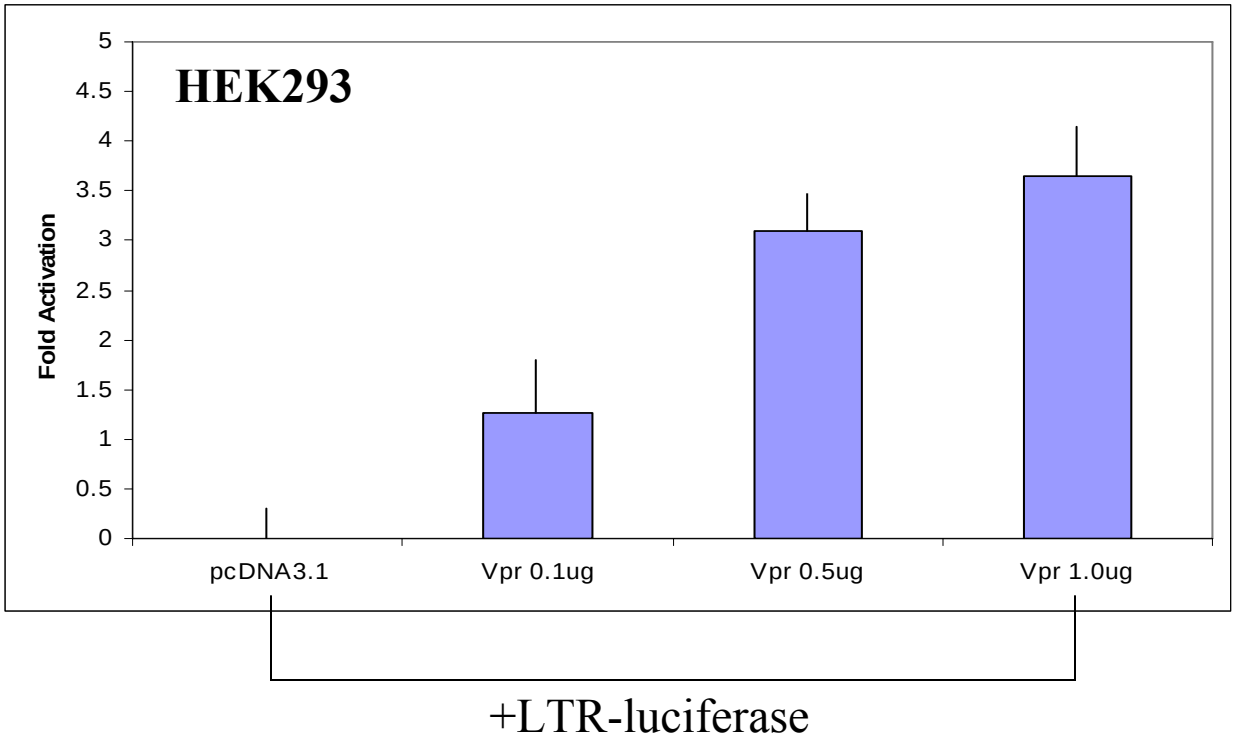


Figure 11. ***Effects of Vpr on HIV-1 LTR-mediated transactivation*** **The reporter construct, HIV-1 LTR-luc, was transfected in HEK293 cells in the presence of different concentrations of Vpr (0.1, 0.5, and 1.0µg) to determine the effects of Vpr on transactivation. Forty-eight hours post-transfection, cell lysates were analyzed for luciferase activity. pcDNA3.1 represents vector control; Vpr represents the Vpr expression plasmid.**

of luciferase driven by the HIV-1 LTR promoter. These results exemplify that the presence of Vpr is able to increase the transactivation of the LTR promoter driven reporter in a dose-dependent manner. Since Vpr is present in the virion prior to the production of Tat, this initial transactivation may in part play a role in the initial production of viral proteins.

5.1.2. Parameters for Transactivation Reporter Assays

Initial transactivation studies prompted the analysis of the optimal concentrations of DNA to administer to the reporter-based transactivation models. In order to ensure that the appropriate concentrations of plasmid DNA were transfected, and therefore achieve optimal transactivation,

parameters were determined to standardize all luciferase reporter assays specifically in the presence of pVpr, as this protein demonstrates toxic behavior at higher concentrations. To investigate the appropriate concentrations of plasmid DNA, dose-dependent transactivation assays were conducted in which one specific parameter was varied in each set of assays in order to test for optimal reporter activity in the various cells lines.

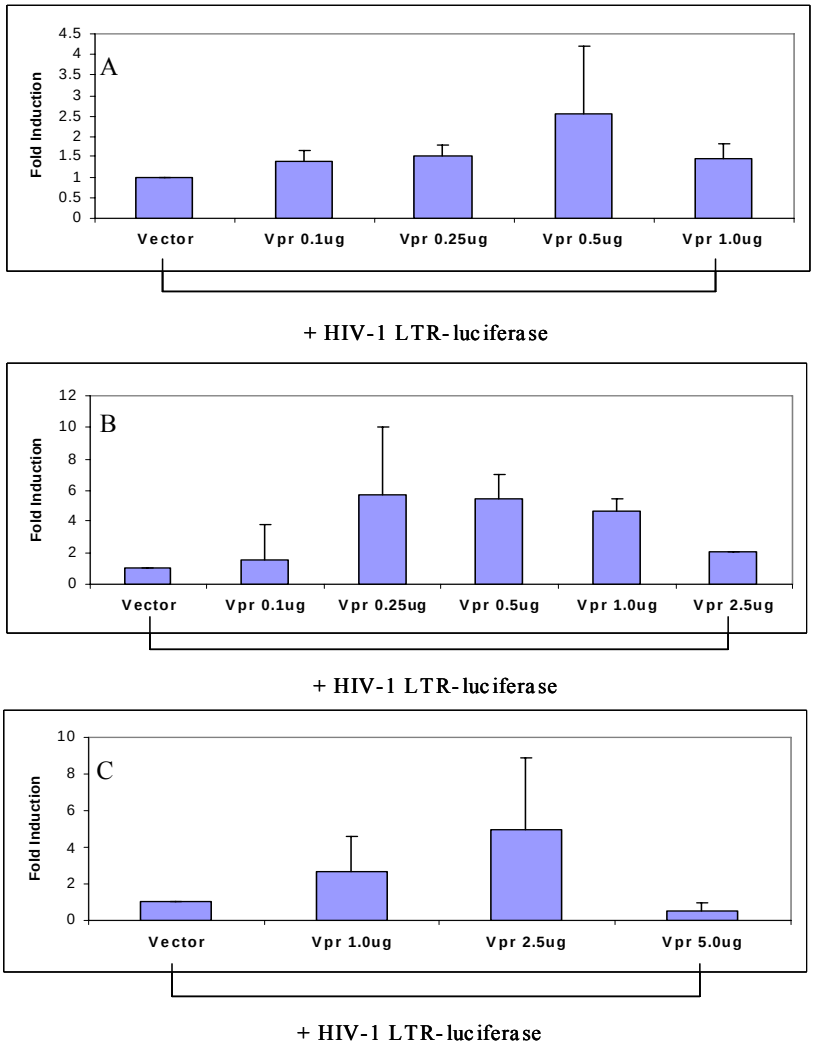


Figure 12. ***Optimal Concentrations of pVpr for Reporter Transactivation Assays in Different Cell Types.*** **(A) HeLa, (B) CV-1, or (C) HEK293 cells were transfected with the heterologous promoter HIV-1 LTR in the presence of varying doses of pVpr. Forty-eight hours post-transfection, cells were lysed and luciferase activity was determined as RLU. Fold induction is represented. All assays were conducted at least three times.**

Figure 12 demonstrates cell-dependent effects of varying doses of pVpr on HIV-1 LTR promoter-driven transactivation. In HeLa cells, the optimal concentration of pVpr was 0.5µg, leading to an approximate 3.5-fold Vpr-mediated increase in transactivation. CV-1 and HEK293 cells demonstrated optimal concentrations at 0.25µg and 2.5µg leading to a 7-fold and 7.5-fold increase, respectively. Similar results were noted when both the GRE-luciferase promoter construct and HIV-1 LTR-luc promoters were transfected. All three cell types, HeLa, CV-1, and HEK293, demonstrated a loss of transactivation activity when the pVpr concentrations reached a higher level. This could be due to the toxicity of Vpr at high concentrations. Interestingly, this was also cell-dependent; HeLa cells lost transactivation activity at 1.0µg, CV-1 cells at 0.5µg, and HEK293 cells at 5µg concentrations.

5.1.3. Vpr-Mediated Transactivation of the Autologous GRE Promoter

Since the presence of Vpr increased transactivation in a dose-dependent manner, establishing the importance of Vpr to promoter-driven transactivation, it was important to next determine a mechanism by which Vpr was able to drive transactivation of promoter-driven genes. The structure of Vpr includes three helices, each containing LxxLL motifs, which have been established as protein-interacting motifs, including in the context of transcription factors. We had shown previously that Vpr interacted directly with GR, and that this interaction led to the transactivation of the cellular Glucocorticoid Response Element (GRE) promoter-driven genes (49,75). To further exemplify that Vpr interacted with GR, and that this relationship led to promoter transactivation, reporter assays were conducted using the endogenously GR-negative cell line, CV-1 (Figure 13). The heterologous promoter, GRE, was constructed as described

containing the downstream luciferase reporter gene (GRE-luc). CV-1 cells were co-transfected with pGRE-luc in the presence or absence of pGR. When pGRE-luc was transfected without pGR, there was little to no promoter activation. When pGR was added in the presence of pGRE-luc alone, there was minimal transactivation. However when pVpr was co-transfected with both pGRE-luc and pGR, transactivation increased in a dose-dependent manner up to 3-fold, demonstrating that Vpr-mediated transactivation occurred in the presence of GR.

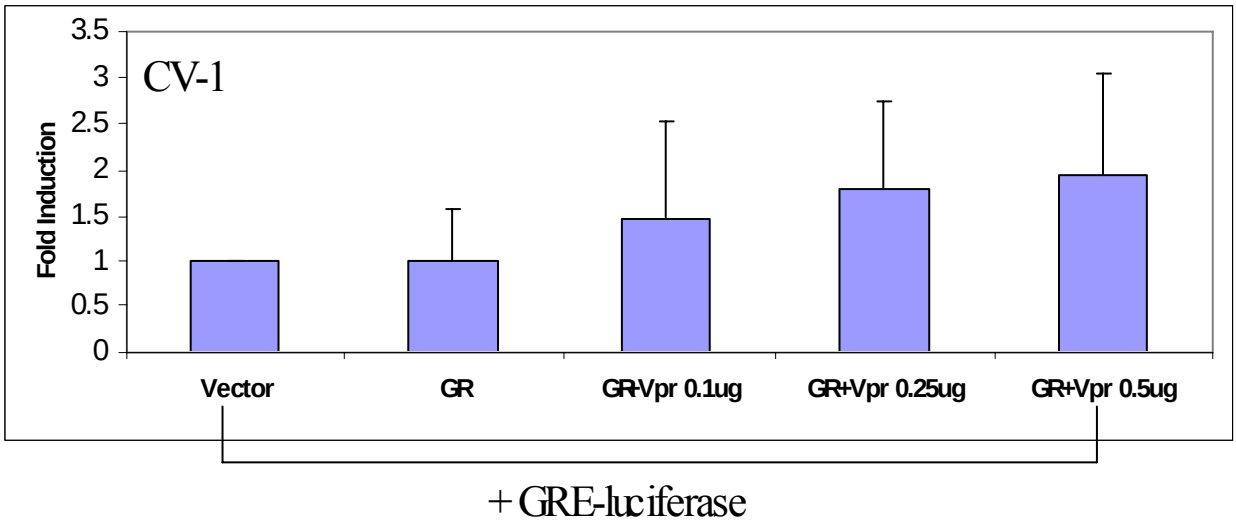


Figure 13. ***Effects of Vpr on the heterologous promoter, GRE*****. CV-1 cells were transfected with the reporter construct GRE-luciferase in the presence of GR alone as well as in the presence of Vpr. Forty-eight hours post-transfection, cells were lysed and analyzed for luciferase activity as determined by RLUs. Results are shown as fold induction with the vector alone control as one. Results represent three separate experiments.**

5.1.4. Interaction between GR and Vpr Occurs at the LxxLL Motifs of Vpr

To investigate the Vpr domains involved in the interaction between Vpr and GR, Vpr leucine mutants were constructed by site-directed mutagenesis as described (Figure 14).

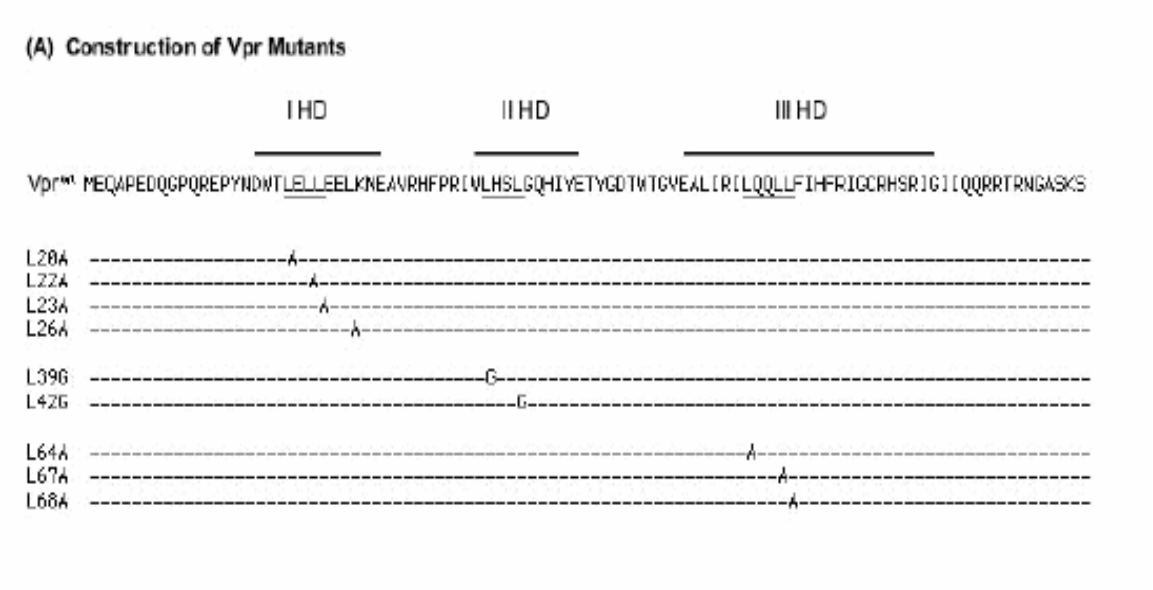


Figure 14. ***Representation of Leucine Mutants.*** **Diagram of leucine mutants as constructed by site-directed mutagenesis. Leucines within the LxxLL motifs on each of the three helices of Vpr were point mutated to either alanine (A) or glycine (G).**

As was previously stated, each of the three Vpr helices contains the LxxLL motif, which have been shown to be involved in protein-protein interaction. It is also known that several transcription factors are able to bind directly with this motif. By mutating each of the leucines within the Vpr protein, if in fact GR did interact with these motifs, we hypothesized that this interaction could be disrupted when the LxxLL motifs were altered. Using reporter transactivation assays, Vpr^{wt} and Vpr mutants were co-transfected with either the GRE-luciferase reporter construct or the HIV-1 LTR-luciferase reporter construct in both HeLa and CV-1 cells (Figure 15). As demonstrated in Figure 15A and 15B, Vpr-mediated transactivation of GRE-driven genes was altered in the presence of different Vpr mutants in both HeLa and CV-1 cell

lines. For example, the leucine mutant L22A increased transactivation approximately 11-fold. In contrast, the leucine mutants L42A and L67A decreased transactivation by 50% in comparison to wild-type Vpr. Similar results were seen in Figure 15C and 15D in the context of Vpr-mediated HIV-1 LTR promoter transactivation in both HeLa and CV-1 cells.

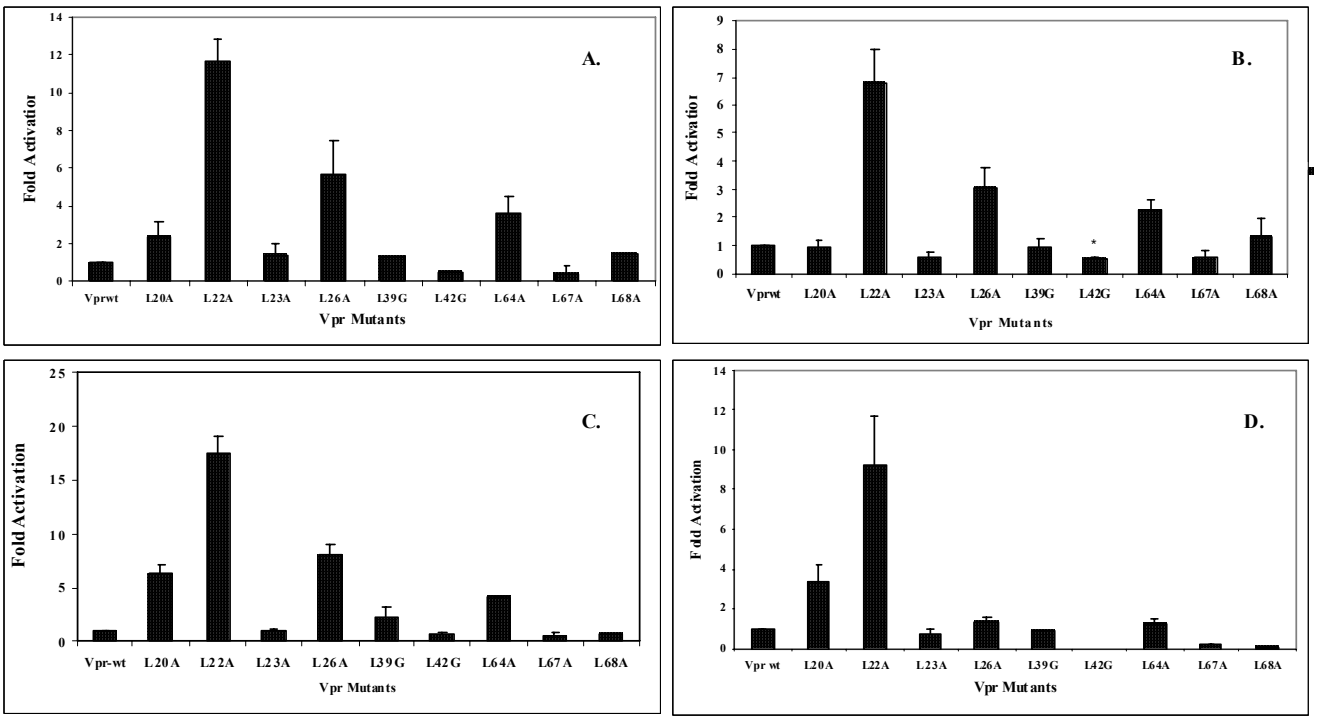


Figure 15. ***Effect of HIV-1 Vpr leucine mutants on GRE and HIV-1 LTR-mediated Transactivation.*** **(A) HeLa cells were transfected with the GRE-luciferase reporter construct in the presence of Vpr and Vpr leucine mutants. (B) CV-1 cells were transfected with GRE-luciferase reporter construct in the presence of GR and Vpr mutants. (C) HeLa cells were transfected with the reporter construct HIV-1 LTR-luciferase in the presence of Vpr mutants. (D) CV-1 cells were transfected with the HIV-1 LTR-luciferase reporter, GR, and Vpr mutants.**

5.1.5. Summary to Aim#1

The focus of AIM#1 was to determine the role of Vpr in HIV-1 LTR-mediated transactivation. By utilizing the luciferase reporter assay, the presence of Vpr demonstrated an increase in HIV-1 LTR-mediated transactivation in a dose-dependent manner. Optimal transactivation parameters were determined, specifically in the context of pVpr, in order to accurately conduct all following reporter assays. Furthermore, Vpr increased transactivation through the GRE region located within the HIV-1 LTR. More specifically, Vpr was able to interact with the cellular Glucocorticoid Receptor, leading the transactivation of GRE-driven gene expression. The LxxLL motifs found within the three helices of Vpr were implicated in the interaction with GR as determined by utilization of Vpr leucine mutants, leading to variances in transactivation levels of both HIV-1 LTR and GRE promoter-driven genes.

5.2. Aim#2: To assess whether the GR antagonist, mifepristone, is able to inhibit Vpr-mediated transactivation and virus replication

5.2.1. The Effects of Mifepristone on Vpr-mediated Transactivation

The ability of Vpr to transactivate viral genes by hijacking the cellular transfection pathway utilized by GR, possibly initiating viral protein production by transactivating viral genes prior to the production of Tat, allows for the infiltration by an antiviral prohibiting or decreasing this transactivation. The GR antagonist, mifepristone, is able to decrease GR-mediated transactivation of GRE-dependent downstream genes via several mechanisms (73). In order to determine if mifepristone was able to inhibit the transactivation of viral genes driven by the HIV-1 LTR promoter, a series of reporter assays were conducted to determine the effects of mifepristone on Vpr-mediated autologous promoter-activated gene transcription (Figure 16). The HIV-1 LTR-luciferase construct was transfected in the presence of Vpr in both HeLa (Figure 16A) and HEK293 (Figure 16B) cell lines. Results demonstrated that mifepristone was able to inhibit the transactivation of HIV-1 LTR-driven genes in a dose-dependent manner by greater than 95% at concentrations of 1, 5, and 10μM, thereby demonstrating the effectiveness of this compound in decreasing the Vpr-mediated transactivation of viral promoters as represented by HIV-1 LTR.

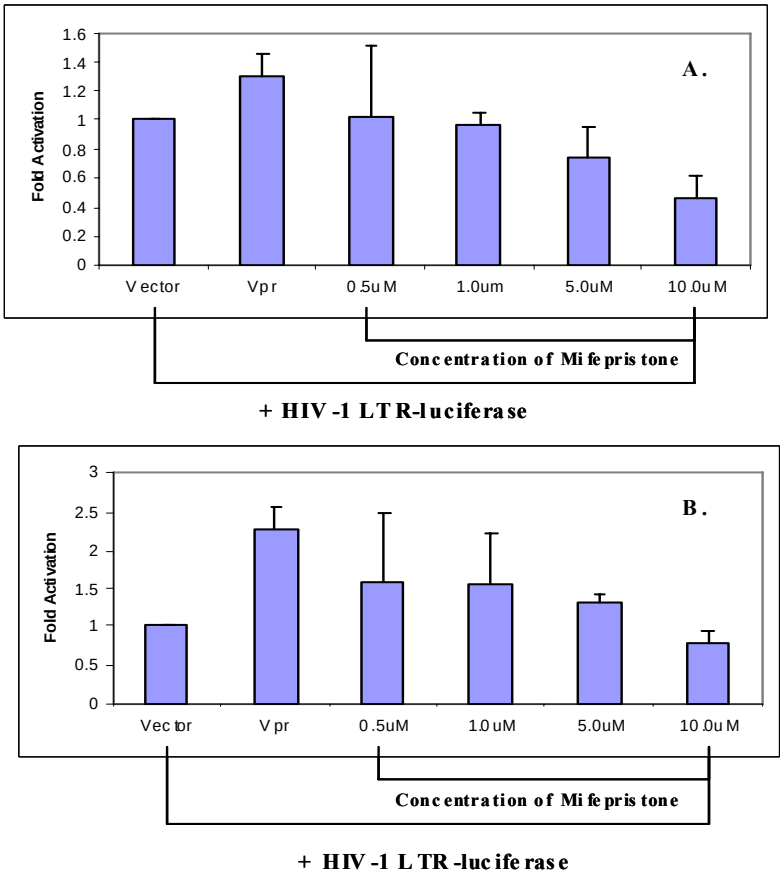


Figure 16. ***Effects of Mifepristone on HIV-1 LTR promoter activity.*** **(A) HeLa cells were transfected with HIV-1 LTR and Vpr in the presence of different concentrations of mifepristone. (B) HEK293 cells were transfected with HIV-1 LTR-luc in the presence of Vpr and varying concentrations of mifepristone. Results represent the results of at least three separate experiments.**

5.2.2. Effects of Dexamethasone on Vpr-mediated Transactivation

Under normal conditions, the cellular GR is held in the cytoplasm by chaperones. Upon binding of ligand, heat shock proteins and other chaperones are dislodged from GR allowing the transcription factor to translocate into the nucleus and bind its associated promoter response element, GRE. GR has also been shown to translocate into the nucleus without the presence of ligand and impact gene transactivation in a similar manner to that of ligand-bound GR. In order

to determine the role of ligand in Vpr-mediated transactivation, it was important to first establish what role the hormones and steroids in the cell media played in Vpr-mediated transactivation. A series of reporter assays were thus conducted, in both HEK293 and CV-1 (no endogenous GR present) cell lines, in the presence of either DMEM supplemented with 10% fetal bovine serum (FBS) or in DMEM supplemented with charcoal-dextran-treated stripped serum (stripped), in which the majority of hormones and steroids were removed (Figure 17).

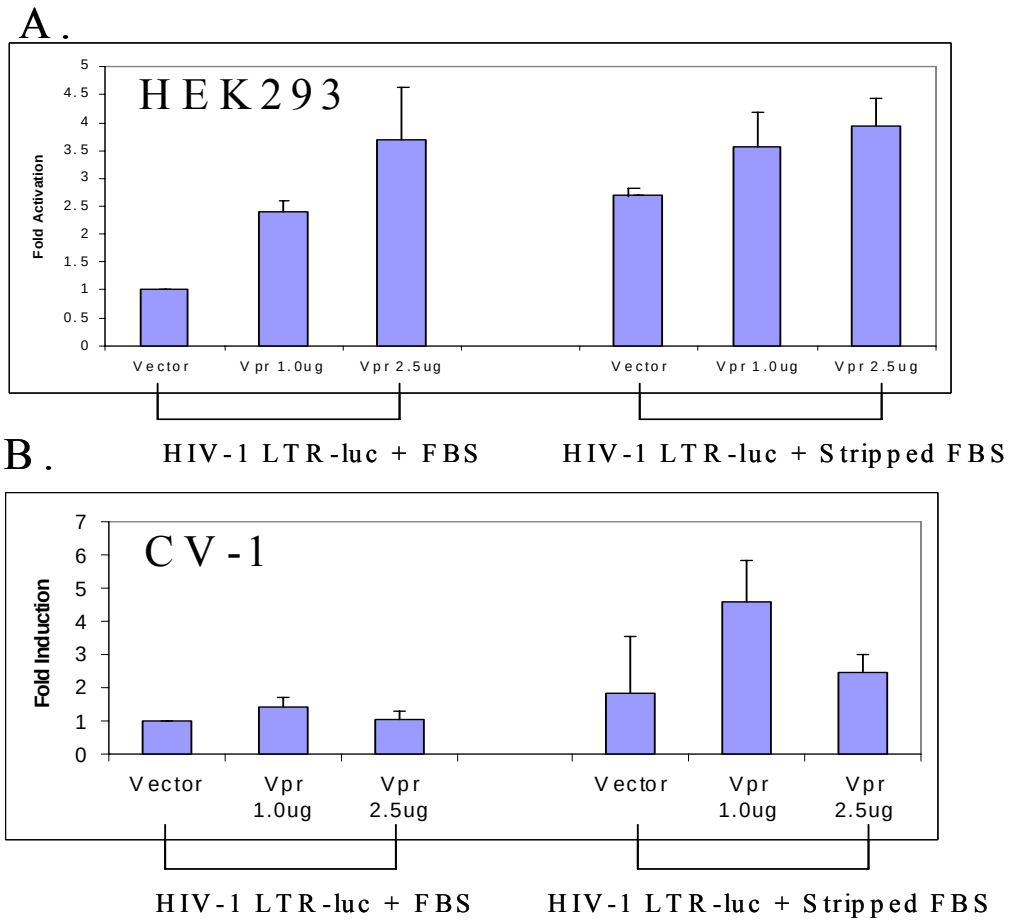


Figure 17. ***Effects of Media on Vpr-mediated Transactivation*****. (A) HEK293 cells were transfected with HIV-1 LTR-luciferase in the presence of Vpr in both DMEM supplemented with 10% FBS (FBS) and DMEM supplemented with 10% dextran charcoal-stripped serum (stripped). (B) CV-1 cells were transfected with HIV-1 LTR-luciferase in the presence of Vpr in both DMEM supplemented with 10% FBS (FBS) or DMEM supplemented with 10% dextran charcoal-stripped serum (stripped). Results reflect at least three separate experiments in each cell line.**

Results demonstrated that in the presence of either FBS-supplemented DMEM or dextran stripped-supplemented DMEM, HEK293 cell transactivation only varied slightly, with greater dose-dependent Vpr-mediated transactivation demonstrated in FBS-containing media (Figure 17A). In the presence of stripped serum, there was slight elevation of transactivation levels, noted also in the vector control (HIV-1 LTR-luciferase alone). In the case of CV-1 transfection (Figure 17B), there was a significant increase in the amount of transactivation demonstrated when cells were transfected in stripped serum; transactivation increased an average of 3-4-fold. Because the stripped serum lacked steroid and hormone ligand for GR to bind thereby enforcing the transactivation of GRE-controlled genes, it is unknown as to why there was such an increase in transactivation of the reporter gene. Quite possibly, other factors which limit GR-controlled transactivation, such as NF-κB, are also prevented from entering the nucleus thereby preventing the control of GR-mediated transactivation of NF-κB-regulated gene expression.

To futher determine the extent to which ligand influences Vpr-mediated transactivation, the steroid dexamethasone was used, a well-known and well-proven agonist of GR-mediated transactivation. Our group had shown previously that Vpr and dexamethasone bind GR and mediate the GR interaction with GRE in a similar manner (49). In order to establish if Vpr and dexamethasone transactivate HIV-1 promoter-driven genes in a similar manner, HEK293 cells were transfected with the reporter HIV-1 LTR-luc in the presence of either Vpr or dexamethasone or both Vpr and dexamethasone (Figure 18).

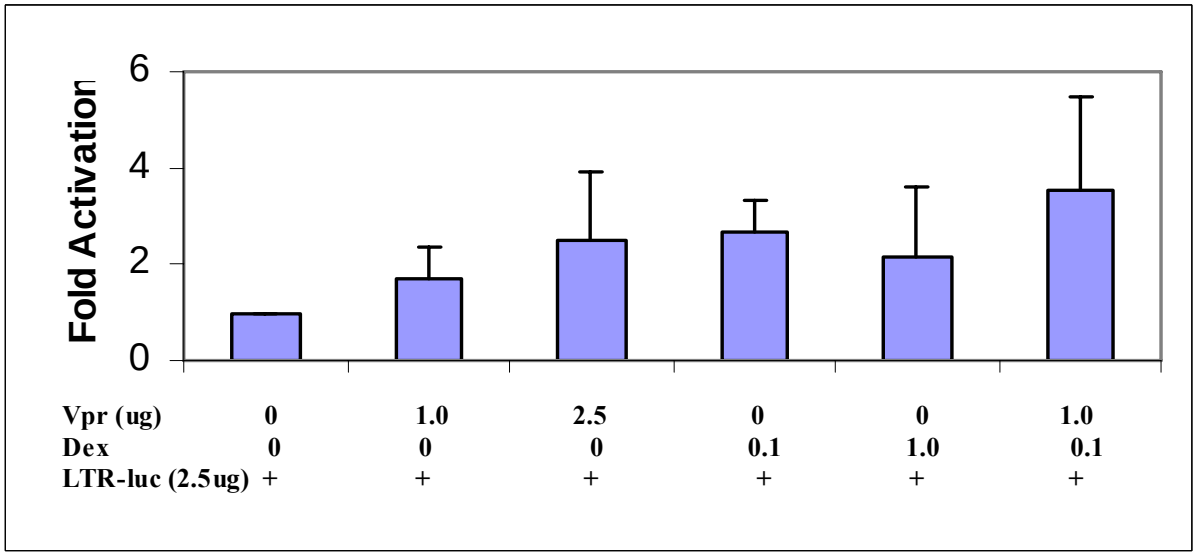


Figure 18. ***Comparison between the Effects of Vpr and Dexamethasone on HIV-1 LTR-driven transcription.*** **HEK293 cells were transfected with HIV-1 LTR-luciferase in the presence of either Vpr, dexamethasone, or both. Forty-eight hours post-transfection, cells were lysed and assayed for luciferase expression. Results are representative of three separate trials.**

Results indicated that Vpr was able to transactivate GRE- or HIV-1 LTR-driven transactivation in a dose-dependent manner similar to that of dexamethasone, correlating with results previously demonstrated by EMSA (49). Since it was not known exactly where Vpr binds GR, it was unknown as to if the mediating of transactivation would occur in a competitive manner, or in an additive manner. To investigate this further, HEK293 cells were transfected with the reporter HIV-1 LTR-luc in the presence of Vpr, and Vpr and dexamethasone added together (Figure 19).

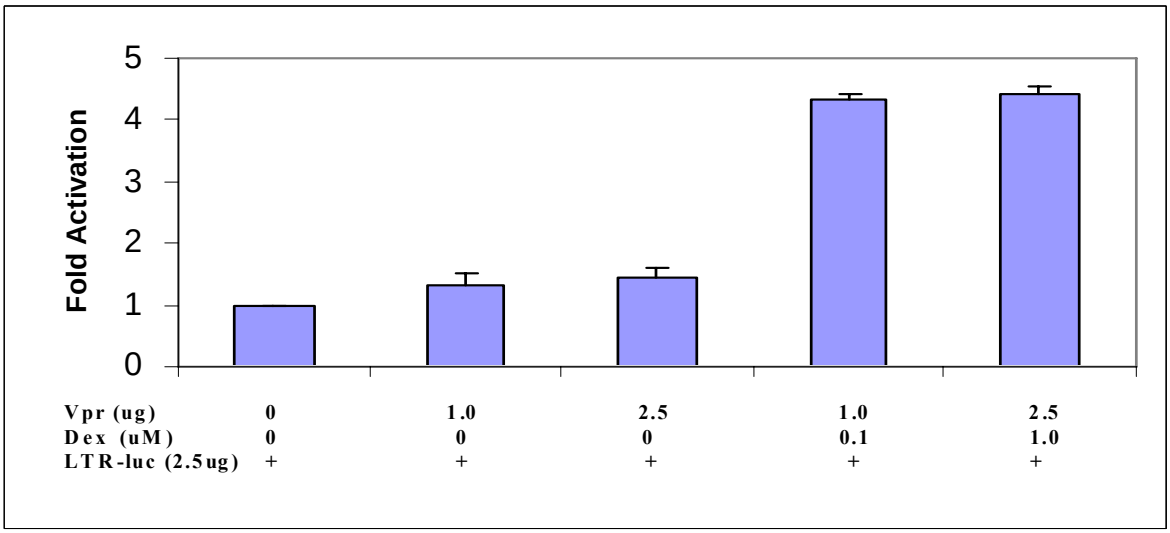


Figure 19. ***Vpr and Dexamethasone Transactivate HIV-1 LTR-promoted transcription in an additive manner.*** **HEK293 cells were transfected with HIV-1 LTR-luc in the presence of Vpr or Vpr and dexamethasone. Results reflect the analyses of at least three separate experiments.**

Results indicated that dexamethasone and Vpr were able to increase transactivation of HIV-1 LTR-driven genes in an additive manner. Interestingly, when both Vpr and dexamethasone were added together, there was a saturation in promoter activity. In other words, when both Vpr and dexamethasone were added with increasing concentrations, there was no further increase in transactivation. Taken together, it is possible that Vpr and dexamethasone do not bind in the same ligand-binding domain pocket and are therefore are able to instigate HIV-1 LTR-driven transactivation in an additive manner.

Dexamethasone and mifepristone share a binding pocket in the ligand binding domain (LBD) of the glucocorticoid receptor. To determine if mifepristone would be able to inhibit the transactivation of HIV-1 LTR-driven genes in the presence of both Vpr and dexamethasone, HIV-1 LTR-luc was transfected in the presence of pVpr in HeLa and HEK293 cells (Figure 20).

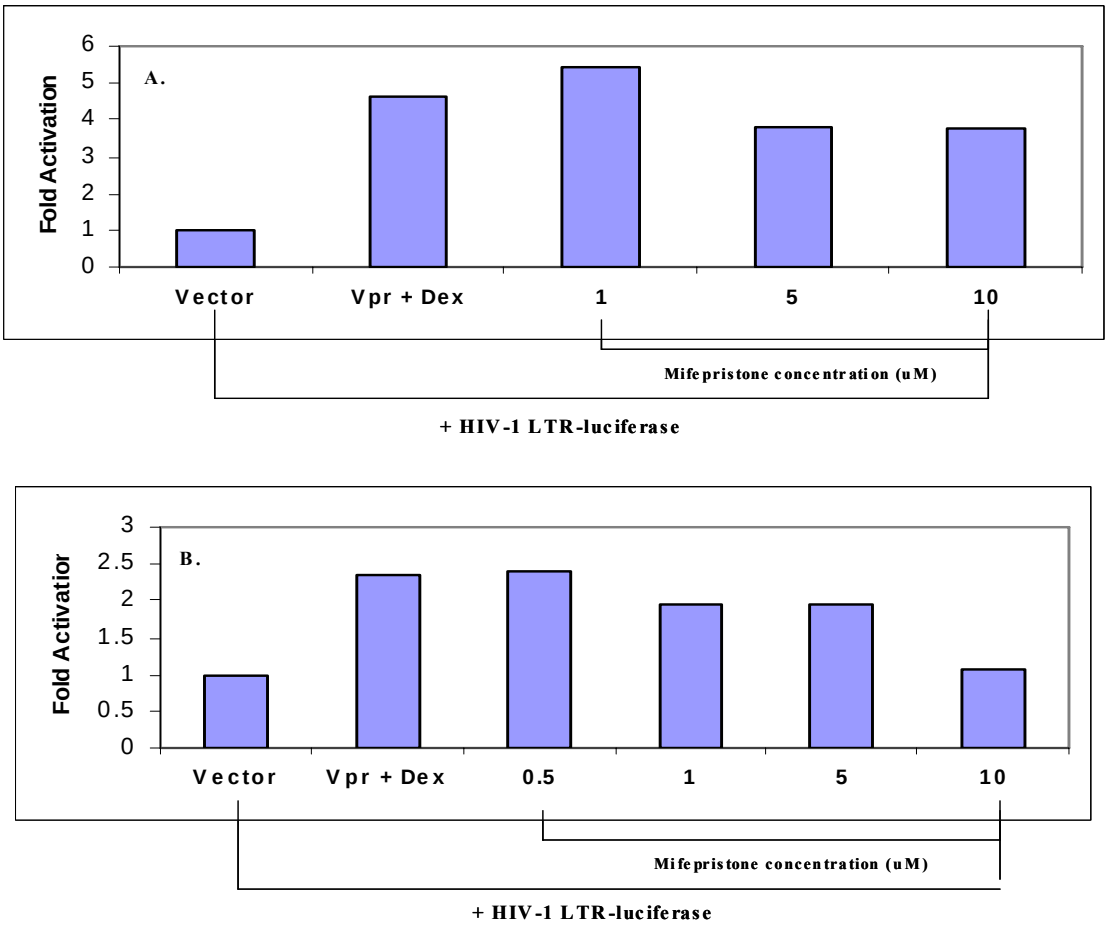


Figure 20. ***Effects of Mifepristone on Vpr + Dexamethasone-Mediated Transactivation.*** **(A) HeLa or (B) HEK293 cells were transfected with the HIV-1 LTR-luc promoter construct in the presence of both Vpr and Dexamethasone (1µM). Mifepristone was added in a dose-dependent manner. Forty-eight hours post-transfection, cells were lysed and analyzed for luciferase activity.**

Even when both Vpr and dexamethasone, two complimenting agonists of GR, were present, mifepristone was able to inhibit transactivation of HIV-1 LTR-driven genes in a dose-dependent manner. While there was slight inhibition of transactivation demonstrated when both Vpr and dexamethasone were present in HeLa cells (Figure 20A), mifepristone was still able to inhibit transactivation by >90% in HEK293 cells (Figure 20B). These results demonstrated the effects of mifepristone even when both Vpr and dexamethasone instigated HIV-1 LTR promoter-driven transactivation.

5.2.3. Cytotoxicity of Mifepristone

To assess the toxicity of mifepristone *in vitro*, trypan blue exclusion or MTT assays were conducted in HeLa, HEK293, CV-1, PBMCs, and monocyte-derived macrophages for three days as described (Table 4). For both PBMCs and macrophages, cytotoxicity was measured for one week to determine if mifepristone was toxic when more than one dose was administered (mifepristone was added every three days as described) (Figure 21). For the seven-day PBMC trypan blue exclusion assay, cells were infected with either 89.6^{wt} virus or the patient isolate H112 virus in the presence of mifepristone. Results indicated that even after seven days in the presence of mifepristone, both PBMCs and macrophages demonstrated little cell death, with the exception of the 10μM dose of mifepristone administered to PBMCs, which after one week resulted in 80% toxicity.

	CT_{25} (μM)	CT_{50} (μM)	CT_{95} (μM)
HeLa	25	35	50
HEK293	12	50	95
CV-1	20	48	80
Macrophage	75	100	200
PBMC	5	25	45

Table 4. *In Vitro Cytotoxicity of Mifepristone*. Three-day MTT assay was conducted as described in HeLa, HEK293, CV-1, monoctye-derived macrophages, and PBMCs. Results reflect concentration dose to incur 25% cytotoxicity (CT_{25}), 50% cytotoxicity (CT_{50}), and 95% cytotoxicity (CT_{95}).

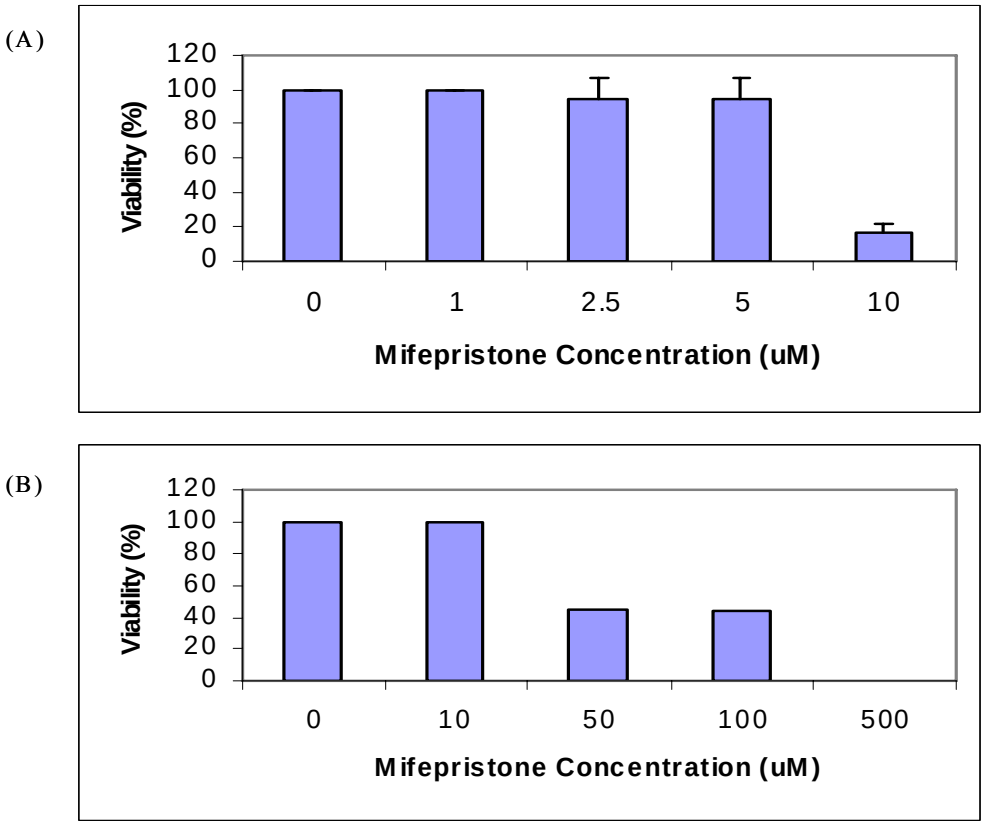


Figure 21. ***Percent Viability of Mifepristone after Seven Days.*** **(A) PBMCs. Percent Viability of PBMCs was determined through trypan blue exclusion while infected using two separate viruses. (B) Macrophages. Percent Viability was determined by MTT assay as described.**

5.2.4. Effects of Mifepristone on Virus Infection

Vpr-mediated transactivation occurs through the interaction of Vpr with the cellular Glucocorticoid Receptor. By use of the GR antagonist mifepristone, the transactivation of HIV-1 LTR promoter-driven genes is able to be repressed by greater than 90%. In assessing mifepristone for its efficacy as compound with possible antiviral properties, a series of infection assays were conducted in CEMx174 (established T cell line), PBMCs, and monocyte-derived macrophages to determine if, in fact, mifepristone was able to inhibit replication-competent viruses of different tropism.

5.2.4.1. Antiviral Properties of Mifepristone in the established T-cell line, CEMx174

In order to determine if mifepristone could inhibit viral replication in T cells, the T-cell line CEMx174 was infected with the $NL4.3^{wt}$ virus which also expressed the fluorescent protein EGFP (NL4.3-EGFP) at an MOI of 0.5. Eight hours post-infection, cells were washed to remove unbound virus and mifepristone was added to the cells in a dose-dependent manner. At day three, cells were analyzed for the expression of EGFP as a measure of virus replication. Figure 22 demonstrates a dose-dependent decrease in cell infectivity in the presence of mifepristone as determined by FACS analysis. Mifepristone, when administered at a 10μM concentration, was able to inhibit viral replication by as much as 88% compared to the untreated culture or vehicle-treated culture. Even at the 0.5μM concentration, mifepristone was able to inhibit viral replication by 51%. FACS analysis also demonstrated little to no cell death in the presence of mifepristone at the 3-day timepoint, exemplifying that there were no toxic effects that played a role in the inhibition of viral replication. To further confirm these results, p24 samples were taken at the time of FACS analysis (Figure 23). Results demonstrated by p24 that mifepristone was able to inhibit the production of p24 in a dose-dependent manner.

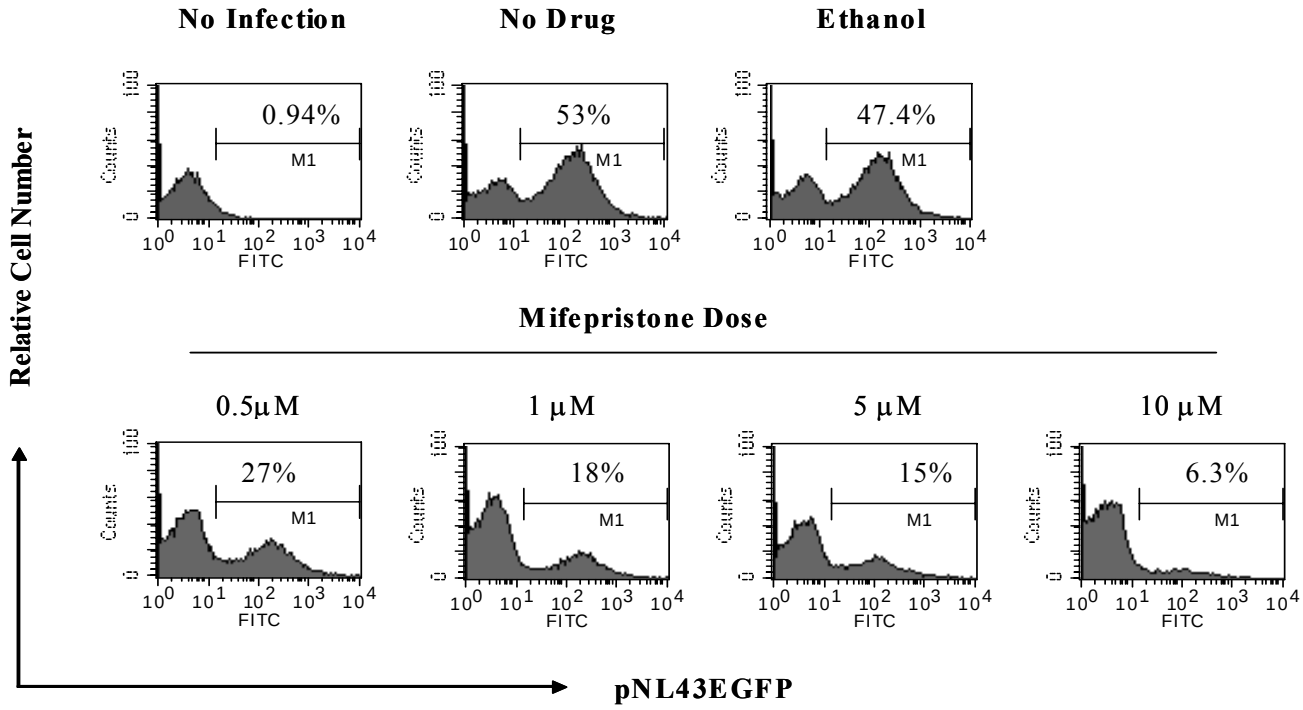


Figure 22. ***Effects of Mifepristone on NL4-3-EGFP infection as determined by FACS analysis.*** **CEM cells were infected with an MOI of 0.5 of the EGFP-expressing wild-type NL4-3 virus. Mifepristone was added post-infection in a dose-dependent manner. Samples were taken three days post-infection and analyzed by FACS analysis. Percentages reflect the number of cells infected. Results are representative of two separate experiments.**

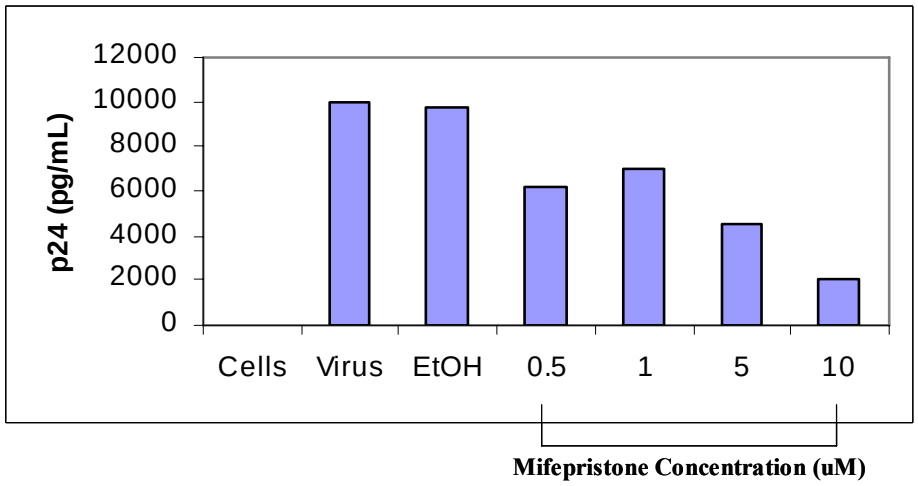


Figure 23. ***Effects of Mifepristone on CEMx174 infected with NL4-3-EGFP by p24 analysis.*** **CEM were infected with the NL4-3 virus at an MOI of 0.5 and treated with mifepristone in a dose-dependent manner. Three days post-infection, samples were taken and analyzed by p24 ELISA.**

5.2.4.2. Antiviral Effects of Mifepristone in PBMCs Infected with Replication Competent Dual-tropic 89.6 Virus

To further assess the properties of mifepristone as an antiviral in the context of infection in primary cells, PBMCs from normal healthy donors were isolated by the lymphocyte gradient isolation method. The virus isolate, 89.6, is a commonly used strain to demonstrate wild-type infection as it is dual-tropic in nature and therefore able to infect both X4 and R5 receptor-expressing cells, including both T-cells and macrophages. PBMCs were stimulated with PHA-P (5ug/mL) for three days. Post-stimulation, cells were infected with the 89.6^{wt} virus at an MOI of 0.1 for eight hours. Cells were then washed two times in PBS to remove excess virus. PBMCs were cultured at a concentration of 5×10^6 and mifepristone was added in a dose-dependent manner. Supernatant samples were taken every two to three days for p24 analysis and mifepristone was re-administered at that time. Figure 24 shows that mifepristone was able to inhibit viral replication in a dose-dependent manner in three separate donors. The highest concentrations of mifepristone, 10 and 5μM inhibited viral replication by greater than 90%, similar to results seen in transactivation assays.

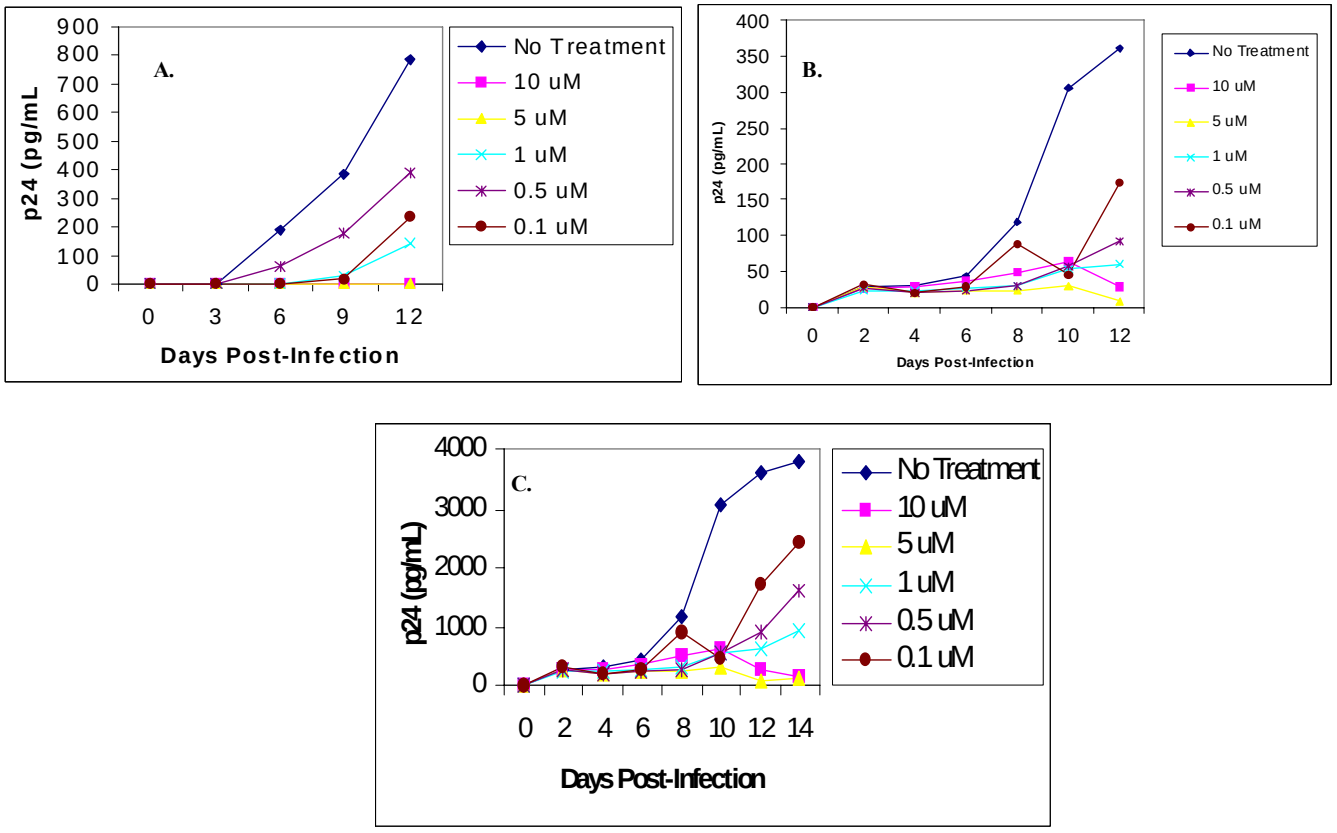


Figure 24. ***Antiviral Effects of Mifepristone on 89.6wt-infected PBMCs in three separate donors.*** **PBMCs were infected with an MOI of 0.1 of the dual-tropic 89.6. Post-infection, mifepristone was added in a dose-dependent manner and supernatant samples were collected every two or three days and analyzed for p24. Panels A-C represent the antiviral effect of mifepristone in PBMCs from different donors.**

5.2.4.3. Antiviral Effects of Mifepristone on PBMCs infected with the Patient Isolate, H112

To further determine if mifepristone would be able to inhibit viral replication of primary isolates, PBMCs were infected with the patient isolate H112. Briefly, PBMCs were infected with the H112 isolate at an MOI of 0.1 for eight hours. Mifepristone was administered in a dose-dependent manner to the cells and samples were removed every three days for analysis by p24 ELISA (Figure 25). Results demonstrate that mifepristone is able to inhibit viral replication of this patient isolate in a dose-dependent manner. While viral replication is not totally inhibited by

mifepristone at the 10μM concentration as was demonstrated in previous experiments using 89.6^{wt} , this concentration was able to prevent viral replication by 85% while the 5μM concentration was able to prevent replication by approximately 45%.

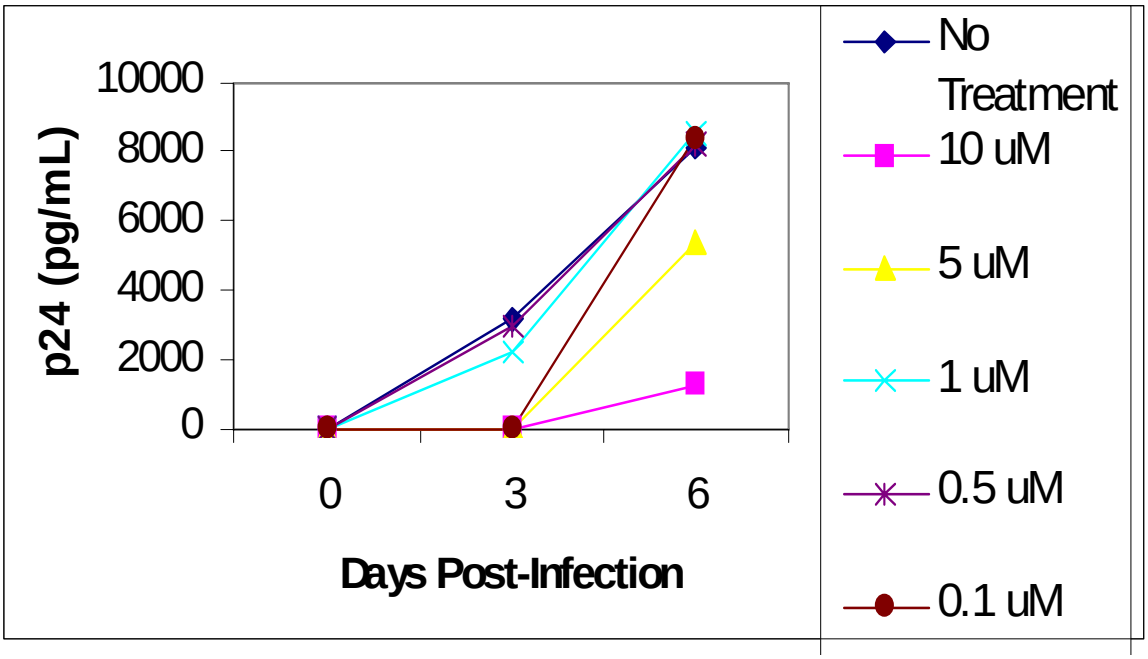


Figure 25. ***Antiviral Effects of Mifepristone on PBMCs infected with the Patient Isolate H112.*** **PBMCs were infected with an MOI of 0.1 of the patient isolate H112 virus. Mifepristone was added in a dose-dependent manner and samples were collected every three days and assessed for the presence of p24. Results representative of two separate experiments.**

5.2.4.4. Antiviral Effects of Mifepristone in Human Macrophages Infected with the Macrophage-Tropic Viral Isolate, Ba-L

As was previously stated, macrophages are initial targets of HIV-1, specifically in mucosal tissues (45). Vpr has been shown to be necessary for the infection of macrophages (42-44). Therefore, the finding of an antiviral specifically targeting Vpr-mediated viral replication could

possibly prevent and/or decrease the infection of these target cells. To assess the antiviral properties of mifepristone in macrophages, infection using the R5-tropic virus Ba-L was conducted to determine if similar results of dose-dependent mifepristone inhibition, as seen in both T-cells and PBMCs, could also occur in the infection of macrophages. Macrophages were isolated by adherence, as described in methods. Briefly, normal human PBMCs were isolated using the lymphocyte gradient isolation method. Directly after isolation, PBMCs were plated in either 6- or 12-well plates (3.5×10^6 or 1.5×10^6 PBMCs per well, respectively) for two hours, during which time the monocytes adhered to the plates, consisting of approximately 10% of the total PBMC population. Non-adherent cells were removed. Monocytes were differentiated into macrophages for approximately eight to ten days by the addition of GM-CSF and M-CSF, macrophage-stimulating cytokines. After monocytes were fully matured into macrophages, which was determined by both CD14 staining and cell morphology, macrophages were infected with an MOI of 0.1 of the Ba-L^{wt} virus for eight hours. Post-infection, cells were washed to remove all virus and mifepristone was added in a dose-dependent manner. Supernatant samples were taken every two to three days, and mifepristone was re-administered to the cells at the time of sample collection. Figure 26 demonstrates the effects of mifepristone on macrophage infection with Ba-L virus.

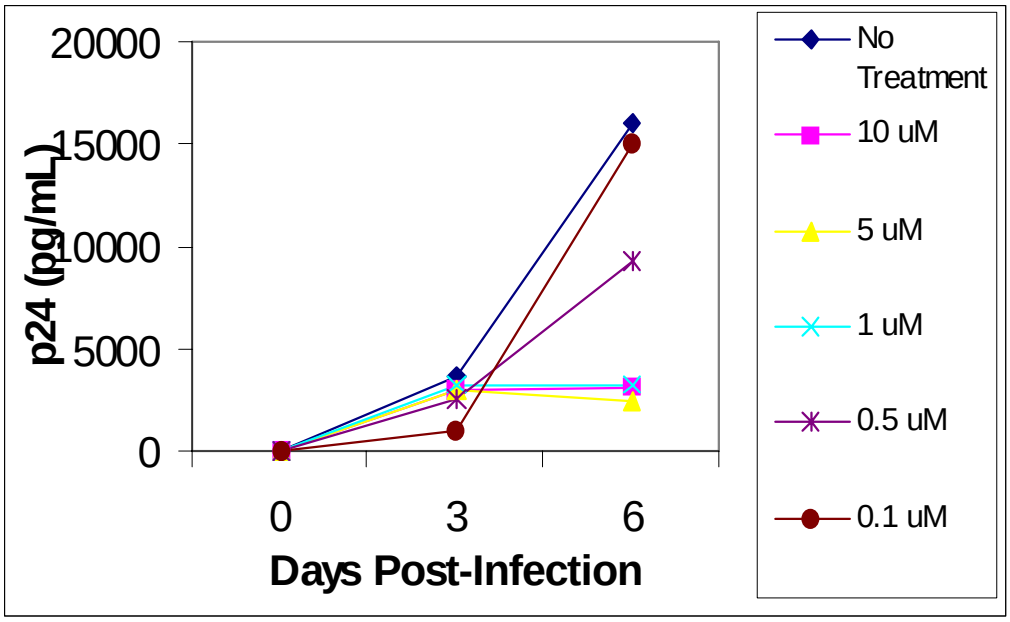


Figure 26. ***The Effects of Mifepristone on Macrophage Infection with the Ba-L Virus Isolate.*** **Monoctye-derived macrophages were infected with an MOI of 0.1 Ba-L^{wt}. Post-infection, mifepristone was added to the cells in a dose-dependent manner. Supernatant samples were collected every three days and assessed for the presence of p24. Results are representative of three separate experiments.**

Results from Figure 26 indicate that similar to T-cell and PBMC infection with wild-type virus, mifepristone inhibits the replication of macrophage-tropic Ba-L virus in the context of macrophage infection. At a 5μM concentration of mifepristone, infection was inhibited by greater than 70% compared to the untreated control. These results further support the antiviral effect of mifepristone in macrophages, in a manner similar to established CD4+ T cells and primary PBMCs.

Summary for AIM#2

The GR antagonist, mifepristone, was tested as a possible antiviral compound interrupting the Vpr-mediated transactivation of genes promoted by the HIV-1 LTR promoter specifically through the GRE promoter region. It was established both in reporter transactivation models, and in antiviral models, that mifepristone disrupted HIV-1 LTR-promoted transactivation and thereby inhibited the replication of HIV-1. The ability of mifepristone to disrupt Vpr-mediated transactivation in the context of GR demonstrates a possible mechanism for antiviral treatment. Combined with low toxicity levels established in primary cells, mifepristone has the potential of becoming a new antiviral, adding a new class of therapeutics to the currently used therapies. The next chapter with futher discuss the potential of mifepristone in clinical application by analyzing the effects of this compound on viral re-activation from latency as well as investigate the possibility of utilizing mifepristone analogs as therapeutics.

5.3. AIM#3: Clinical Applications of Mifepristone

5.3.1. Pre- versus Post-Treatment with Mifepristone

The hope for mifepristone originated with the idea that this compound could be used not only to treat infection, but to prevent infection as well. Initial studies included adding mifepristone post-infection, and not prior to or during infection. While most antivirals are used to treat patients post-infection, few are used in emergency prophylaxis, such as AZT, and none are currently utilized as a preventative drug. Since mifepristone is able to inhibit Vpr-mediated transactivation, this compound holds the possibility of being used to prevent infection by not allowing the initial transactivation of viral-promoted genes, especially in the context of macrophages where the presence of Vpr is imperative for infection. To determine if mifepristone was able to inhibit the infection of cells when the compound was present either prior to or during infection, assays were constructed to compare the amount of inhibition of viral infectivity in the presence of mifepristone. Furthermore, virus was also pre-treated with mifepristone to determine the effects of viral replication in the context of compound-treated virus.

5.3.1.1. Pre-Treatment of PBMCs and Macrophages with Mifepristone Prior to Infection

To assess the difference between pre-treatment of PBMCs prior to and during infection and post-treatment of PBMCs after infection, cells at a concentration of 5×10^6 were treated with

mifepristone in a dose-dependent manner two hours prior to infection with 89.6^{wt} virus. Similarly, monoctye-derived macrophages were pre-treated with mifepristone for two hours prior to infection with the macrophage-tropic Bal virus. During the eight-hour infection, mifepristone was present in the media to ensure the presence of the compound throughout the process of infection. Post-infection, cells were washed and mifepristone was re-administered in a dose-dependent manner. As controls, a second set of PBMCs or macrophages were infected simultaneously with the pre-treatment PBMCs without the presence of mifepristone. Post-infection, cells were washed and mifepristone was added in a dose-dependent manner. Similar to previous antiviral experiments, supernatant samples were collected every two to three days and assessed for the presence of p24 by ELISA (Figure 27).

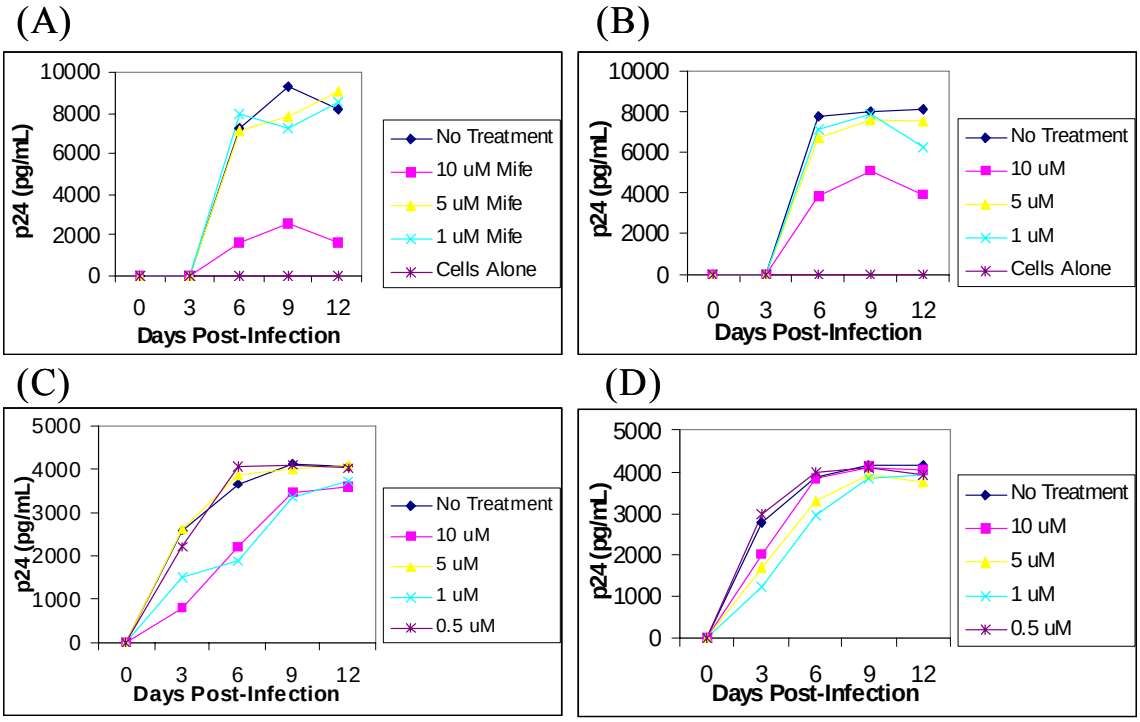


Figure 27. ***Pre-Treatment versus Post-Treatment of PBMCs and Macrophages with Mifepristone.*** **(A) PBMCs were pre-treated with mifepristone prior to and during infection and compared to (B) PBMCs treated with mifepristone post-infection. (C) Macrophages pre-treated with mifepristone prior to and during infection compared to (D) Macrophages treated with mifepristone post-infection. Supernatant samples from both sets of PBMCs and macrophages were taken every three days and analyzed for the presence of p24. Results are representative of at least two experiments for both PBMCs and macrophages.**

Results presented in Figure 27 demonstrated no significant difference between pre- and post-treatment of cells with mifepristone either in PBMCs (panels A and B) or monocyte-derived macrophages (panels C and D). Further studies need to be conducted to determine if, in fact, mifepristone is able to inhibit viral replication to a greater extent when cells are pre-treated with this compound prior to infection.

5.3.1.2. Effects of Pre-Treatment of the Virus in Comparison to Cell Pre-Treatment in PBMCs

Mifepristone has been demonstrated to alter the effects of transcription in several ways, including recruiting different co-factors to the transcriptional machinery and altering the conformation of GR around GRE, slowing down the transcriptional process. In order to determine if mifepristone could work through a different mechanism directly affecting the virus, PBMCs were pre-treated with a dose-dependent regimen of mifepristone two hours prior to PBMC infection with the dual-tropic 89.6wt virus. Cells were infected for eight hours, washed to remove excess virus, and mifepristone was added post-infection. To compare the effects of viral pre-treatment to cellular pre-treatment, a second set of PBMCs were similarly pre-treated with a dose-dependent regimen of mifepristone for two hours, infected in the presence of mifepristone for eight hours, washed to remove excess virus, and mifepristone was re-administered in a dose-dependent manner. Supernatant samples were removed from both sets at three-day intervals and analyzed by p24 ELISA; mifepristone was re-administered in a dose-dependent manner at that time (Figure 28).

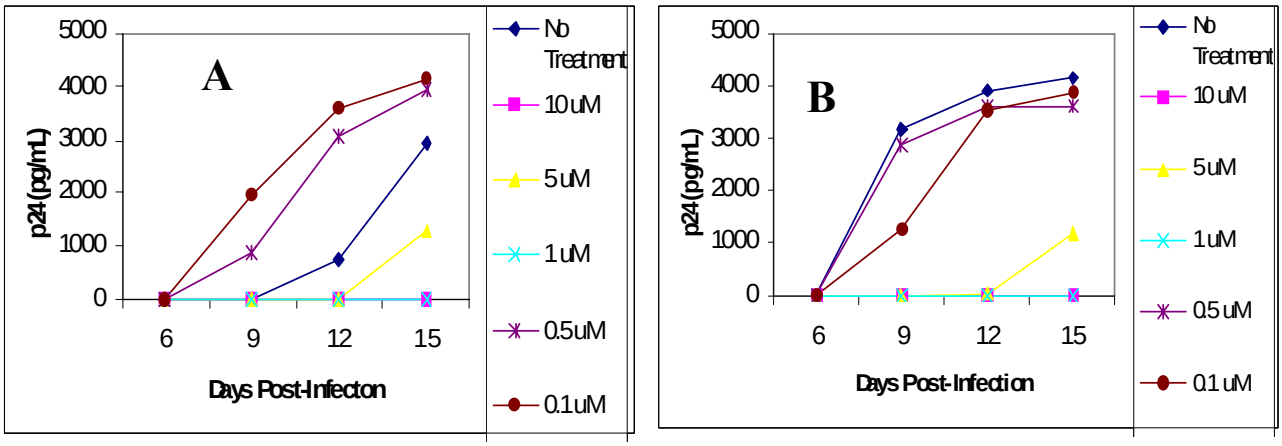


Figure 28. ***Effects of Mifepristone on Viral Pre-Treatment in Comparison to Cellular Pre-Treatment in PBMCs.*** **(A) PBMCs were infected with an MOI of 0.1 of the wild-type 89.6 which had been pre-treated in a dose-dependent manner with mifepristone. (B) PBMCs were pre-treated with mifepristone in a dose-dependent manner and subsequently infected with an MOI of 0.1 of 89.6^{wt}. Supernatant samples were removed every three days and assessed for the presence of p24.**

Results from this experiment demonstrated that 89.6^{wt} virus pre-treatment in comparison to PBMC pre-treatment with mifepristone yielded no significant difference in viral replication post-infection. Therefore, there is no conclusive evidence demonstrating the viral pre-treatment with mifepristone is able to increase the inhibition of viral replication in the context of explicative virus.

5.3.2. Effects of Mifepristone on Latent Viral Reservoirs

One of the mechanisms in which HIV-1 remains elusive to total viral clearance during therapy is due to its ability to establish viral reservoirs (19-22). In order to determine if mifepristone was able to prevent reactivation from latency, the promonocytic cell line U1/HIV-1 was used. This cell line contains two copies of the proviral HIV-1 DNA which upon stimulation by viral proteins or other factors such as TNF-α, leads to the production of virus, which can be

quantitated by p24 ELISA (86,87). In this setting, U1/HIV-1 cells are able to be defined as latent HIV-1 reservoirs. To establish if mifepristone was able to prevent the re-activation of the proviral DNA contained within the U1/HIV-1 cells, cells were activated by both TNF-α and by AT-2-treated inactivated NL4-3 virus. Therefore, any activation by the AT-2 inactivated virus is due to the presence of viral proteins in the particle and not due to infection. Mifepristone was added in a dose-dependent manner during activation by both TNF and AT-2 –treated virus (Figure 29). Results demonstrated that both U1/HIV-1 cells activated by TNF-α and AT-2 -treated NL4-3 virus led to the production of p24, which was inhibited in a dose-dependent manner by mifepristone. Interesting to note, the addition of EtOH (used as a vehicle control) increased viral production by the U1/HIV-1 cells, demonstrating further the effectiveness of mifepristone.

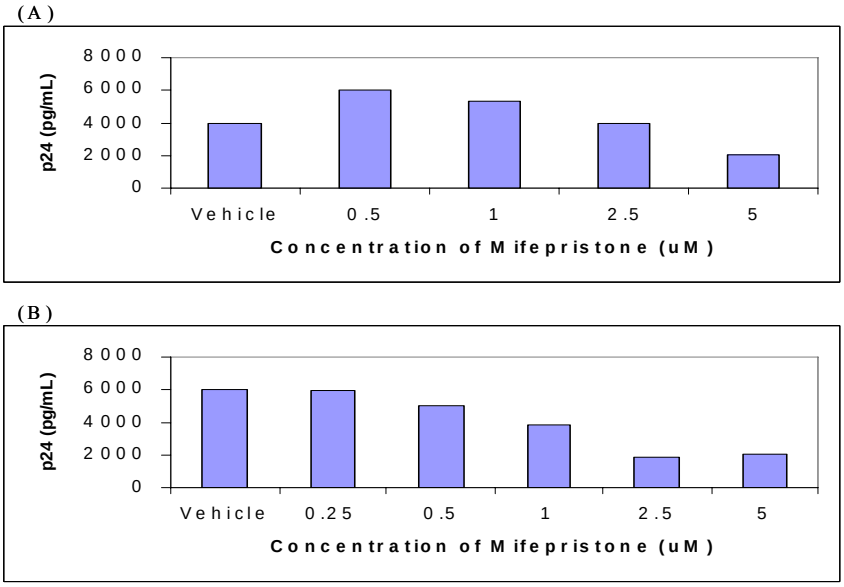


Figure 29. ***Effects of mifepristone on re-activation from latency as determined by U1/HIV-1 viral production.*** **(A) TNF-alpha stimulated U1/HIV-1 cells or (B) AT-2 treated U1/HIV-1 cells. Cells were treated with mifepristone in a dose-dependent manner. Virus replication was assayed on day three following activation and samples were assessed for the presence of p24.**

5.3.3. The Effects of Mifepristone Analogs on Promoter Transactivation and Antiviral Activity

Mifepristone is able to inhibit the transactivation of GRE-driven downstream genes, and consequently is able to inhibit the transactivation of LTR-driven downstream genes, thereby preventing viral gene expression. Mifepristone binds to the glucocorticoid receptor in a manner similar to that of dexamethasone, binding in the same pocket within the ligand binding domain of GR in a competitive manner. To identify a more potent and less toxic compound, we collaborated with the NCI drug discovery program. Based on the structure of mifepristone, analogs were composed with slight differences in structure (Figure 30). To determine if mifepristone analogs, similar to mifepristone with few molecular changes, would work in a manner similar to that of mifepristone, possibly with better efficacy in preventing viral transcription, several mifepristone analogs were assessed for their ability to inhibit HIV-1 LTR-transactivation by Vpr.

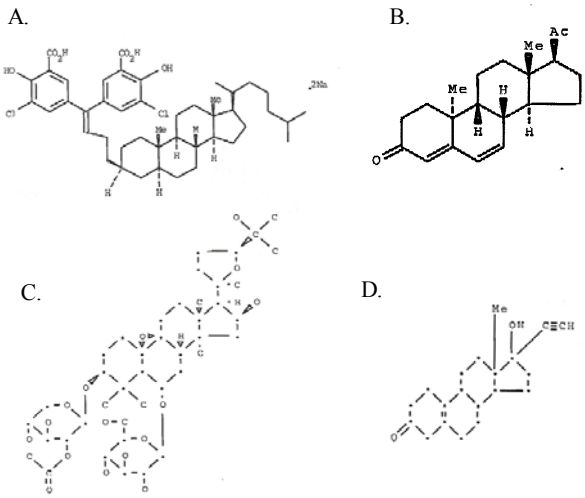


Figure 30. ***Structure of mifepristone analogs.*** **(A) Analog 666510, (B) Analog 92336, (C) Analog 641295, and (D) Analog 15432.**

5.3.3.1. Cytotoxicity of Mifepristone Analogs

As these mifepristone analogs were previously unexplored compounds, MTT cytotoxicity assays were performed as described to determine if these analogs would prove to be toxic to HeLa, PBMCs, or macrophages (Figure 31). The CT_{50} of analogs in PBMCs and HeLa cells is approximately 45µM, in macrophages approximately 60µM. In comparison, the CT_{50} in the presence of mifepristone is approximately 35µM in HeLa, 25µM in PBMCs, and 100µM in macrophages. Therefore, in PBMCs and HeLa these analogs are less toxic when compared to mifepristone however in macrophages there is an increase in toxicity demonstrated in the presence of analogs.

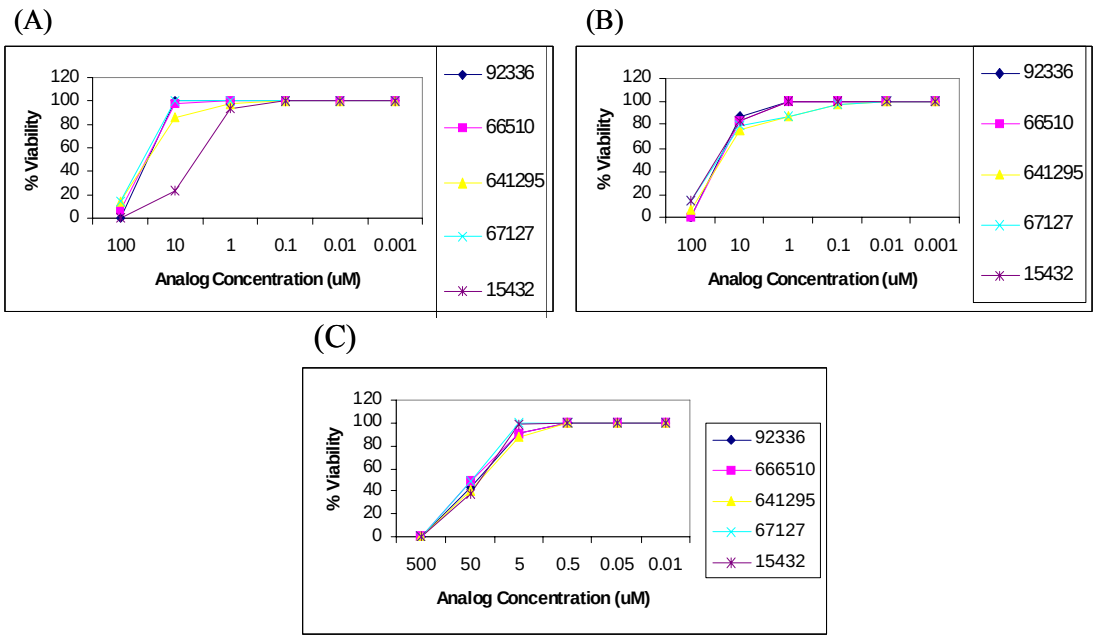


Figure 31. ***Cytotoxic effect of mifepristone analogs in PBMC, HeLa, and Macrophages.*** **MTT assays were performed as described in methods in (A) PBMC, (B) HeLa, and (C) Macrophages. Percent viability (%) was calculated based on considering untreated cells as 100%. Results reflect average of at least three separate experiments.**

5.3.3.2. Effects of Mifepristone Analogs on Promoter-Driven Transactivation

In order to assess whether or not mifepristone analogs inhibited the transactivation of either autologous or heterologous promoters, a series of reporter assays were established in HeLa, CV-1, and HEK293 cell lines. Briefly, the reporter constructs GRE-luciferase or HIV-1 LTR-luciferase were transfected with pVpr (and pGR in the context of CV-1) and a dose-dependent concentration of mifepristone analogs in these cell lines. Figure 32 demonstrates the effects of mifepristone analogs on HIV-1 LTR promoter transactivation in HeLa cells. Initial experiments were conducted on each of the compounds to determine their effects on transactivation. All compounds demonstrated a dose-dependent decrease in transactivation similar to mifepristone, though not all compounds inhibited transactivation to the same degree.

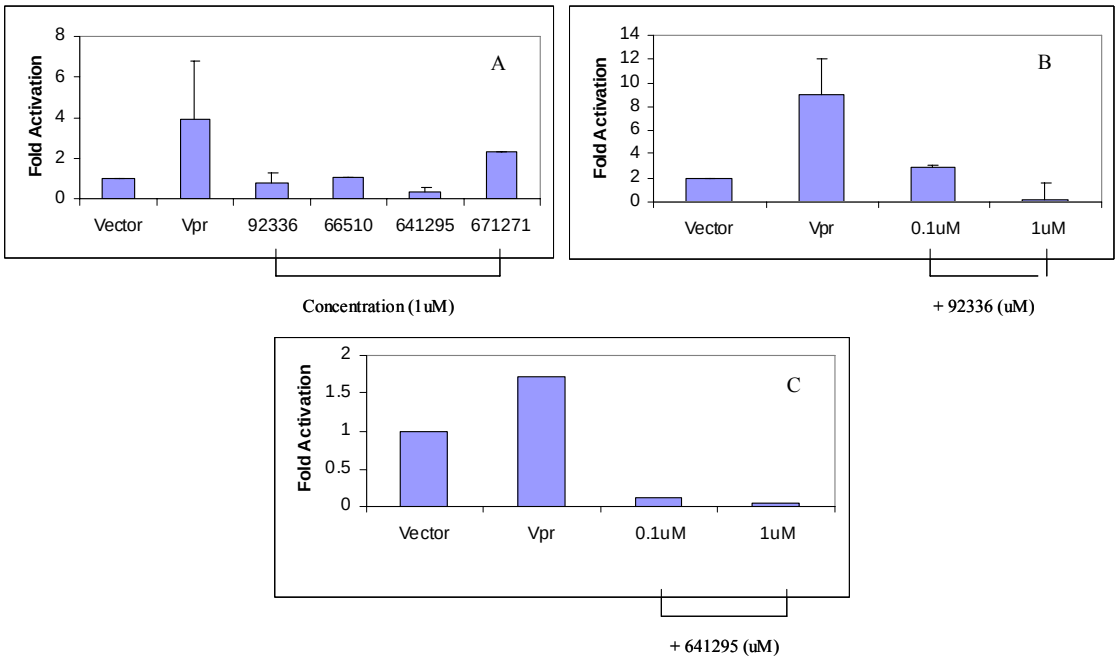


Figure 32. ***Effects of Mifepristone Analogs on HIV-1 LTR Transactivation in HeLa Cells.*** **(A) Comparison of the effects of analogs 92336, 66510, 641295, and 671271 on HIV-1 LTR-driven transactivation. (B) Dose-dependent effect of analog 92336 on HIV-1 LTR-driven transactivation. (C) Dose-dependent effect of analog 641295 on HIV-1 LTR-driven transactivation. Fold activation is calculated considering the vector control as one.**

Similar results were demonstrated in CV-1 cells. Cells were transfected with HIV-1 LTR and Vpr in the presence of 10μM of mifepristone and analogs. Post-transfection, cell lysates were analyzed for the presence of luciferase. Percent inhibition was calculated for compounds mifepristone, 641295, 666510, and 92336. Results demonstrated that mifepristone was able to inhibit viral transactivation at a greater extent in CV-1 cells than the mifepristone analogs (Figure 33).

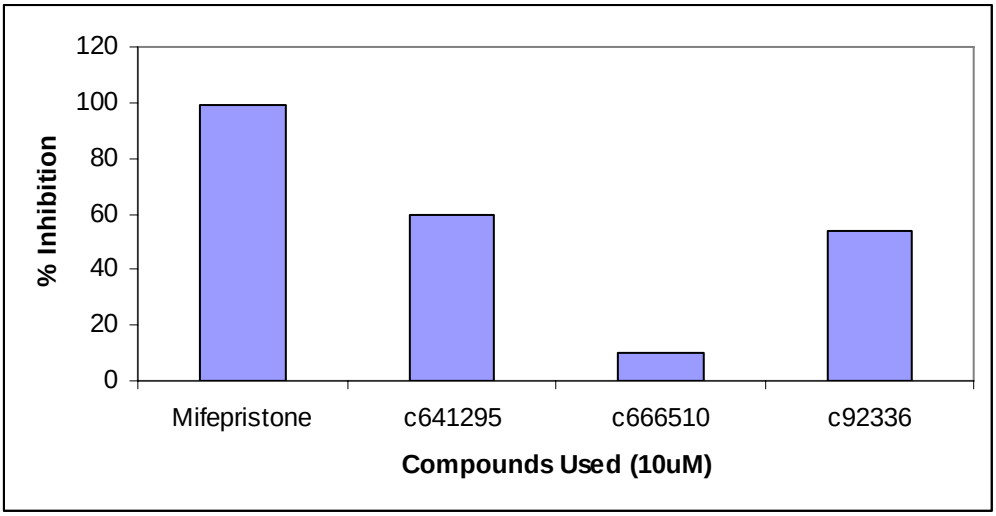


Figure 33. ***Effects of Mifepristone and Analogs on HIV-1 LTR-mediated transactivation in CV-1 cells.*** **CV-1 cells were transfected with the HIV-1 LTR luciferase reporter construct in the presence of pVpr and pGR. Cell lysates were analyzed for the presence of luciferase.**

While mifepristone analogs do inhibit transactivation, a comparison to the inhibition induced by mifepristone on viral transactivation demonstrates that mifepristone decreases transactivation more effectively (greater than 90% inhibition by mifepristone, and only 15-60% inhibition by analogs 641295, 666510, and 92336) in CV-1 cells.

Final transactivation studies to determine the effectiveness of mifepristone analogs on HIV-1 LTR-driven transcription included the addition of dexamethasone. Dexamethasone, which, as stated before, acts as ligand similar to cortisol, increases transactivation by binding to the glucocorticoid receptor and subsequently transactivating genes downstream of the GRE promoter region. In order to determine if mifepristone analogs would be able to interfere with HIV-1 LTR promoter transactivation, CV-1 or HEK293 cells were transfected with the HIV-1 LTR-luciferase reporter construct in the presence of pVpr, pGR in CV-1 cells, and the addition of dexamethasone 24 hours prior to cell lysis (Figure 34). Results indicated that mifepristone analogs, while able to inhibit the transactivation of HIV-1 LTR-driven genes, were not able to inhibit transactivation to the extent that was seen when mifepristone was added.

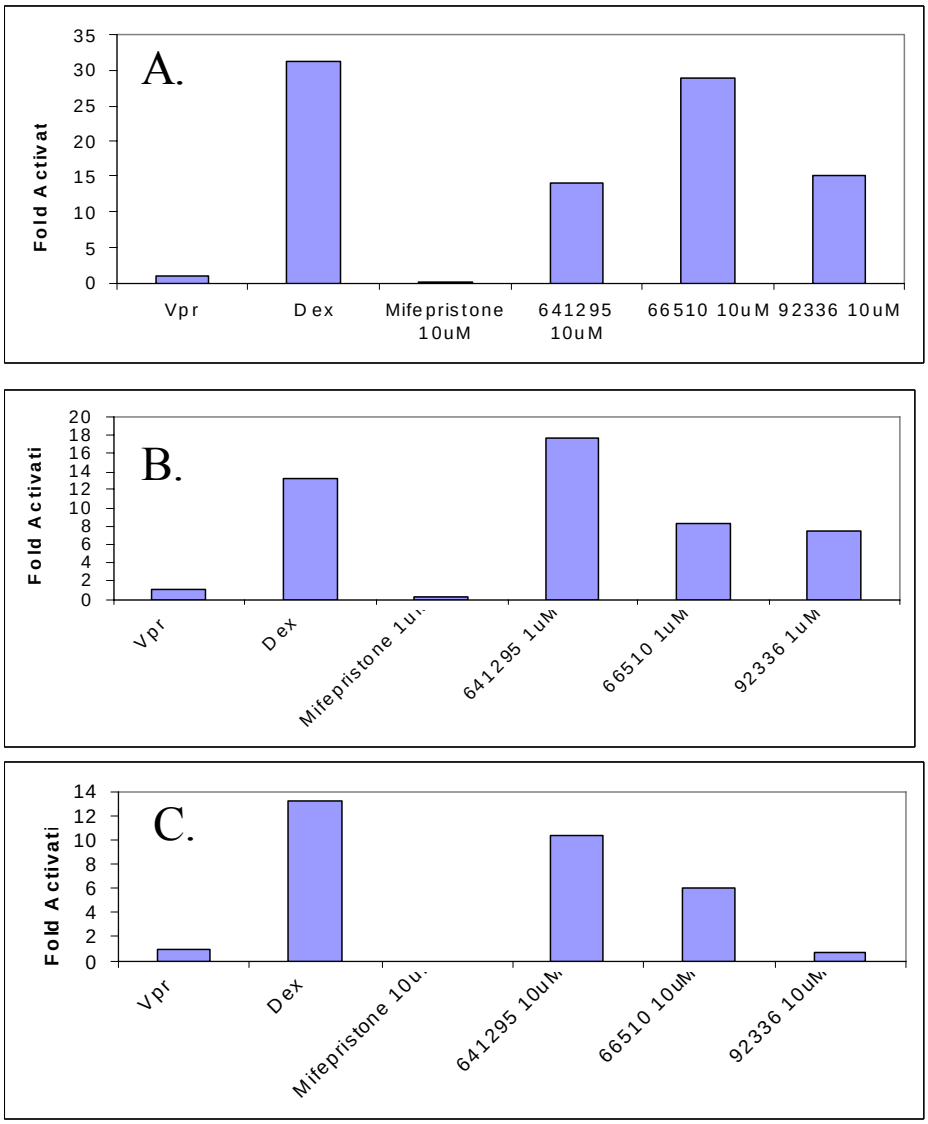


Figure 34. ***Effects of Mifepristone Analogs on HIV-1 LTR-mediated transactivation in CV-1 and HEK293 cells.*** **(A) CV-1 cells were co-transfected with the HIV-1 LTR luciferase construct in the presence of pGR and pVpr. Mifepristone and analogs were added at a concentration of 10μM in the presence of dexamethasone. (B) HEK293 cells were transfected with HIV-1 LTR luciferase in the presence of pVpr. Mifepristone and analogs were added at a 1μM concentration and dexamethasone was added 24 hours prior to cell lysis. (C) HEK293 cells were transfected with the HIV-1 LTR luciferase in the presence of pVpr. Mifepristone and analogs were added at a 10μM concentration and dexamethasone was added 24 hours prior to cell lysis. Forty-eight hours post-transfection, cells were lysed and luciferase concentration was determined. Final results are presented as fold induction, the HIV-1 LTR control as having a fold of one.**

5.3.3.3. Effects of Mifepristone Analogs on Virus Infection in PBMCs

After determining that mifepristone analogs were able to inhibit the transactivation of the HIV-1 LTR promoter-driven genes, antiviral assays were performed using $NL43^{wt}$-infected PBMCs. Cells were infected with virus for eight hours and washed to remove all residual virus. Post-infection, cells were treated with similar doses of mifepristone and analogs. Samples were taken after three days and assessed for inhibition of viral replication (Figure 35).

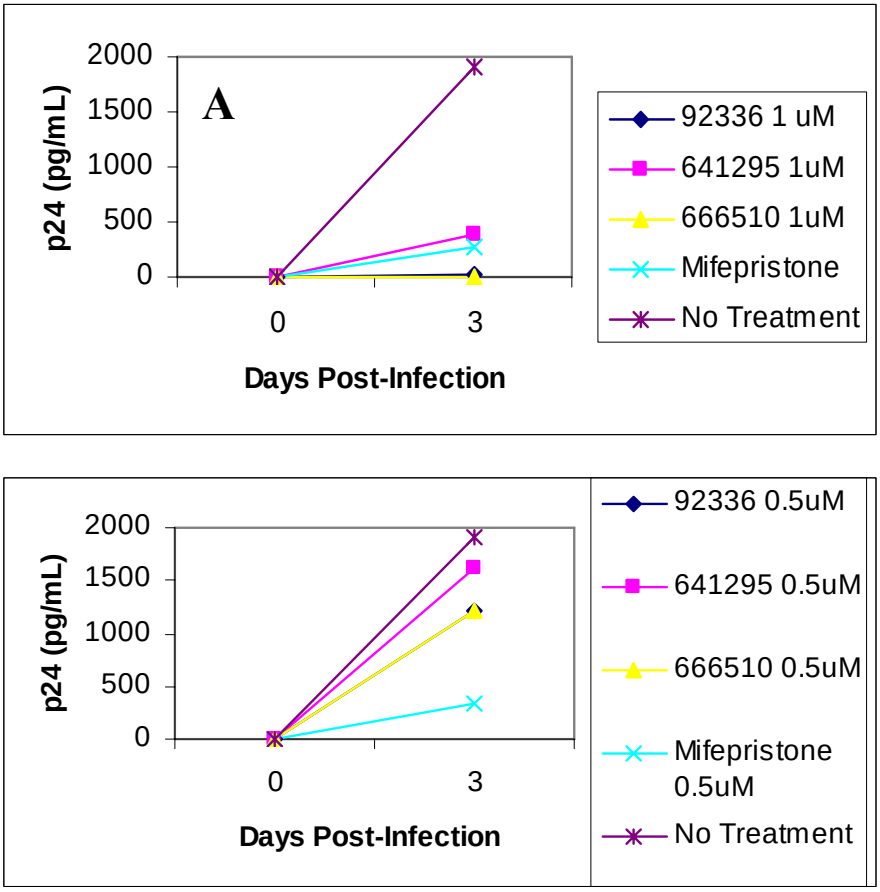


Figure 35. ***Three-day Effects of Mifepristone Analogs on NL4.3 wild-type infection of PBMCs.*** **Cells were infected with an MOI of 0.1. Post-infection, mifepristone and analogs were administered in (A)1.0uM and (B) 0.5uM concentrations. Supernatant samples were taken at day 3 and assessed for the presence of p24.**

Results demonstrated that analogs were able to inhibit wild-type virus in PBMCs. At a 1µM concentration (panel A), analogs 92336 and 66510 were able to inhibit virus greater than 90%. At a 0.5µM concentration, however (panel B), only mifepristone was able to inhibit viral replication greater than 90%.

To further determine the effects of mifepristone analogs in virus infection, PBMCs were infected with 89.6^{wt} for eight hours and post-infection mifepristone analogs were added at a concentration of 1µM (Figure 36). The analog 671271 which is included in this study is AZT and was used as a blind-folded control.

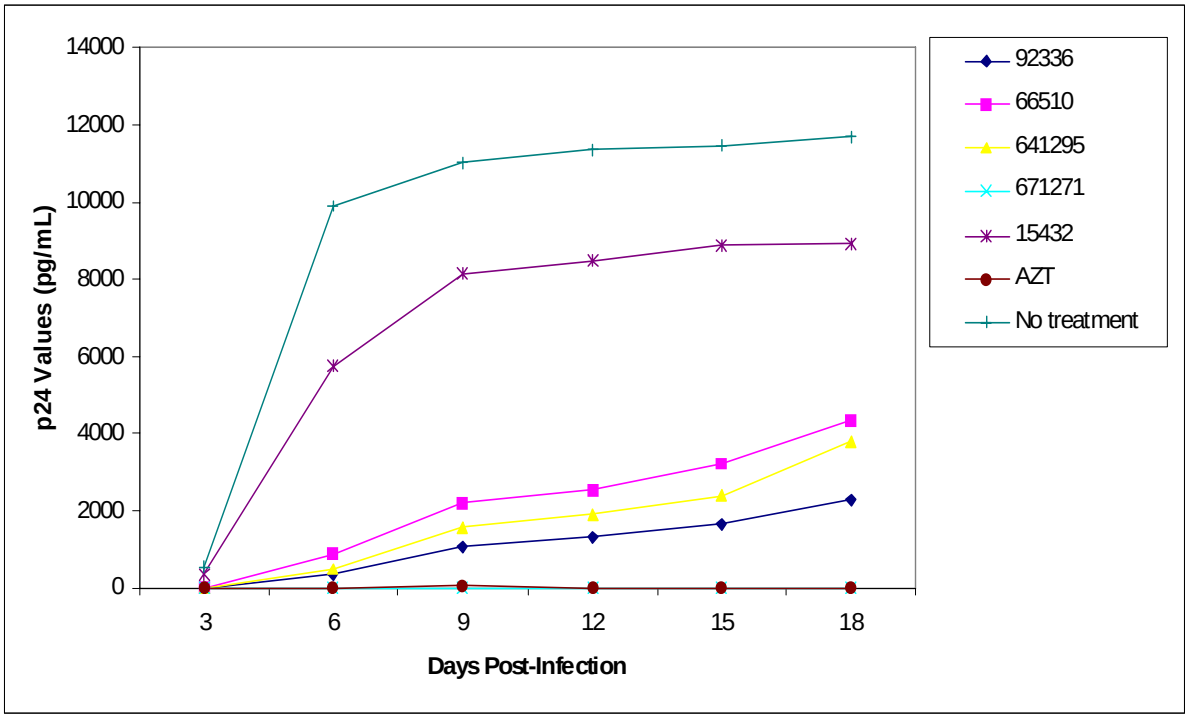


Figure 36. ***Effects of Mifepristone Analogs on Viral Replication in PBMCs infected with 89.6.*** **PBMCs were infected with an MOI of 0.1 of the dual-tropic 89.6 wild-type virus. Post-infection, compounds were added at a 1uM concentration. Every three days, supernatant samples were collected and assessed for the presence of p24.**

Results demonstrated that the mifepristone analog 92336 was able to inhibit viral replication by 80%. Analogs 641295 and 66510 were able to inhibit virus by 70 and 60%, respectively. Therefore, it is possible to conclude that mifepristone analogs are able to inhibit viral replication in a manner similar to that of mifepristone, however mifepristone inhibits replication at a higher efficiency.

5.3.3.4. Effect of Mifepristone Analogs on Ba-L Macrophage Infection

In order to fully determine the effects of mifepristone analogs on viral infection, macrophages were infected with the Ba-L virus and the inhibitory effects of mifepristone analogs were analyzed. Monoctye-derived macrophages were infected with an MOI of 0.1 for eight hours. Post-infection, cells were washed to remove residual virus and mifepristone analogs were added in a dose-dependent manner. Every three days supernatant samples were collected, analogs were re-administered, and p24 analysis was conducted to determine the presence of viral peptides released in the media. Figure 37 demonstrates the effects of each compound individually on macrophage infection based on a dose-dependent regimen.

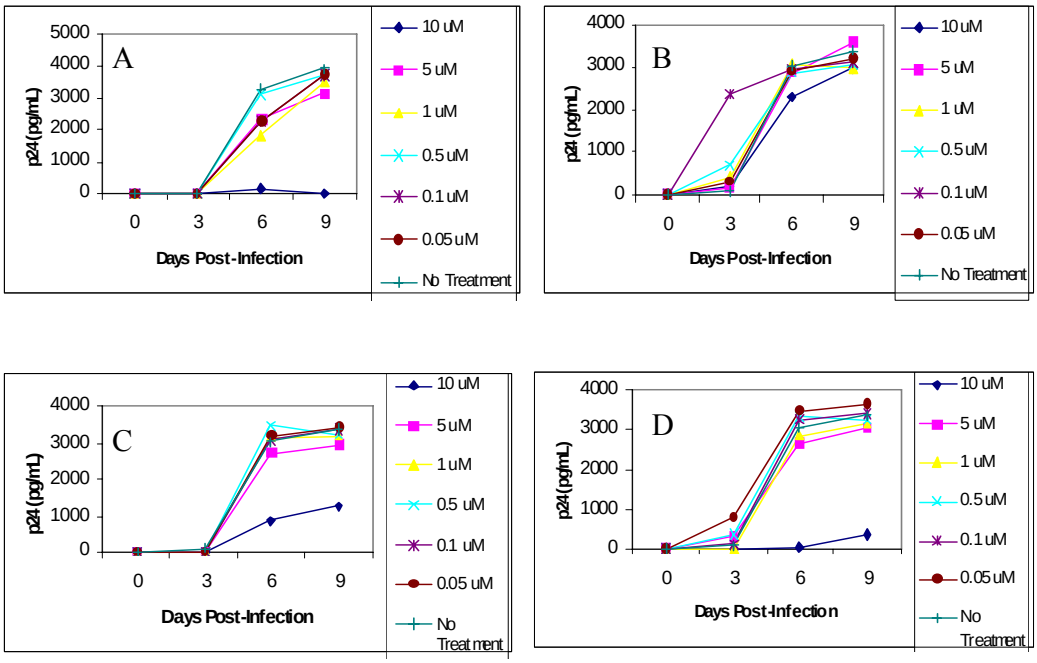


Figure 37. ***Effects of Mifepristone Analogs on Macrophages infected with Ba-L.*** **Macrophages were infected with an MOI of 0.1 of the wild-type Ba-L virus. Post-infection, mifepristone analogs were added in a dose-dependent manner. Supernatant samples were collected every three days and analyzed for p24 by ELISA.**

Results demonstrated the dose-dependent inhibition of virus replication by individual analogs. With the exception of 10μM, other concentrations, while still maintaining dose-dependent inhibition, are not able to completely inhibit the virus. This assay was conducted using two separate donors with similar results.

5.3.4. Summary to AIM#3

Because viral reservoirs are often established within macrophages, mifepristone was analyzed for the ability to prevent the reactivation of virus from latency using the promonocytic U1/HIV-1 cell line. Results demonstrated in a dose-dependent manner that mifepristone was able to inhibit reactivation from latency. To further pursue the development of antivirals targeting viral transcription, mifepristone analogs were analyzed to determine cytotoxicity, inhibition of transactivation, and inhibition of viral replication. Results exemplified that while mifepristone analogs were able to inhibit both transactivation and replication, none of the chosen analogs were able to work in a more effective manner than mifepristone. Taken together, mifepristone demonstrates characteristics complementary to the development of this compound as a new antiviral, in conjunction with the current HAART regimen.

6. CHAPTER SIX: DISCUSSION

There exists an obvious and urgent need to find the elusive "cure" for HIV/AIDS, whether it be by vaccine, antiviral, or microbicide. Frustrations have been mounting in the scientific and political communities over the lack of such a cure, even after now over twenty years of HIV research and funding. The phenomenon of HAART, prolonging the lives of possible millions and preventing the spread of this virus, was and is an amazing and currently evolving achievement. The problem arises, however, with the fact that HAART is not the "cure" that so many are searching for; instead it is a way to prolong the life of those infected. Unfortunately, the inability to follow the toxic and strict drug regimen that is HAART, combined with the evolution of HIV leading to drug-resistant strains as well as viral reservoirs, leads to an often similar outcome: the eventual succumbing of the patient to the viral parasite that ultimately leads to death.

The relatively small virus that is HIV is composed of only nine proteins, functioning in structural, enzymatic, and accessory roles. HAART focuses on only a few of these proteins: the envelope, reverse transcriptase and protease, along with targeting nucleotide/nucleoside addition involved with basic replication. While vaccine studies currently involve few genes found within the viral genome, as of yet there have been no vaccines that have fully protected human subjects, though studies in non-human primates have demonstrated otherwise (86-90).

The 96-amino acid HIV-1 accessory protein Viral Protein R (Vpr) is evolutionarily conserved within the various species of virus, including HIV-1, HIV-2 and SIV (40). Further studies into

the relevance of this protein, however, have demonstrated that Vpr is active in several different pathways and serves a variety of functions including locating the pre-integration complex, G2/M cell cycle arrest, apoptosis, evasion strategies, regulation of cellular immune function, and increasing transactivation (29-39).

The focus of this thesis is two-fold: First, to identify the role of Vpr in viral transactivation and secondly, to determine a way of inhibiting this transactivation. Vpr is present in the virion prior to the production or presence of Tat, therefore Vpr may help transactivate initial viral genes. Vpr has also been shown as necessary for the optimal infection of non-dividing cells such as macrophages. Macrophages are initial targets of the virus, specifically in the mucosa in both vertical and horizontal transmission (25). Taken together, the presence of Vpr in the virion as well as the necessity of Vpr to infect initial targets such as macrophages, enables one to both investigate the mechanism(s) underlying Vpr-mediated transactivation as well as view this protein as a possible target for antiviral therapy.

Initial studies were performed in order to demonstrate an increased transactivation of the autologous promoter HIV-1 LTR in the presence of Vpr. By exampling an increase in transactivation directly correlating with an increase in the concentration of Vpr, or put more simply, a dose-dependent increase in transactivation in the presence of Vpr, this strong example was demonstrative of the role of Vpr in HIV-1 LTR-driven transactivation (Figure 11). The question subsequently and obviously became then how exactly was Vpr able to increase transactivation?

Previous work from our group determined that the GRE sequences present within the HIV-1 LTR is the region specifically influenced by the presence of Vpr (49,75). To further this investigation, a series of reporter assays were constructed utilizing the reporter construct GRE-luciferase in the presence of Vpr in the CV-1 cell line, chosen for its lack of endogenous GR (Figure 13). Results demonstrated that Vpr was specifically influencing the promoter response element GRE by increasing transactivation in a dose-dependent manner, further confirming our published reports (49).

Vpr, comprised of three helices each containing the motif LxxLL, is known to interact with a variety of proteins, supporting studies that implicate the LxxLL motif in protein-protein interactions (47,48). Transcription factors are also known to interact with this LxxLL motif. The knowledge that Vpr was able to increase the transactivation of GRE-driven genes as determined by reporter assay, combined with studies on transcription factors implementing their interaction with LxxLL motifs, supported the theory that the transcription factor interacting with the GRE promoter region, the Glucocorticoid Receptor (GR), could be the key factor to the increased transactivation. Protein-interaction studies where therefore completed, showing a direct interaction between Vpr and GR (49). Further studies also demonstrated that Vpr increased the binding of GR to its response element, GRE, in a similar manner to that of dexamethasone (49).

It was of the utmost importance to determine the appropriate concentrations of DNA to add to luciferase reporter assay transfections. As seen in Figure 12, each cell line necessitated a different concentration of Vpr to achieve optimal transactivation. Results showed that the

highest amount of Vpr-mediated transactivation occurred when using 0.5µg pVpr in HeLa and 0.25µg in CV-1 cells, whereas 2.5µg of pVpr was needed to observe optimal transactivation in HEK293 cells. These studies were conducted using both autologous and heterologous promoters in HeLa, CV-1, and HEK293 cells, with similar results independent of promoter used. Interestingly, in comparing both GRE- and HIV-1 LTR-promoted transactivation, the HIV-1 LTR promoter activation gave a much higher luciferase reading than that of GRE. This could be due to the fact that GRE promoter activation is dependent mainly on the presence of GR whereas the HIV-1 LTR promoter contains various binding areas for a number of different transcription factors. Therefore, the HIV-1 promoter will ultimately have a much higher transactivation of downstream genes than that of GRE alone, as there are many more factors that are able to activate the promoter region in an additive manner.

After establishing that Vpr increased transactivation through an interaction with the cellular GR, leading to the increased transcription of GRE- ,or subsequently HIV-1 LTR-, driven genes, the next aim of this thesis became finding out how to disrupt this increase in transcription. Because Vpr-mediated viral transactivation occurred in the presence of GR, the GR antagonist mifepristone was investigated, to determine if this compound could intervene with the Vpr-GR interaction, leading to a decrease in viral transcription. Mifepristone has been well-studied, at least in the context of short-term use, and is currently being researched for a variety of reasons including having anti-cancer properties, for neuroprotection, and for the treatment of Alzheimer's disease (91-93). When applied to reporter transactivation models, mifepristone was able to decrease Vpr-mediated transactivation by greater than 90% in different cell lines (HeLa, and HEK293; Figure 16). As the mifepristone-induced inhibition of transactivation was dose-

dependent, it was therefore concluded that the effects of this GR antagonist were able to decrease, almost completely, the effects of Vpr-mediated transactivation.

Dexamethasone is the researcher's cortisol for *in vitro* studies, as it acts in a similar manner to cortisol and other steroids in the presence of hormone receptors such as GR. While initial results complemented by theory demonstrated that Vpr was able to work in a manner similar to that of dexamethasone, it was important to determine how effective both ligands were in mediating transactivation. As was concluded, Vpr did work in a manner of effectiveness similar to dexamethasone. And most interesting was the fact that when both Vpr and dexamethasone were added together, rather than acting as ligand competitors, Vpr and dexamethasone worked in an additive manner, leading to an eventual saturation, if you will, of transactivation (Figures 18 and 19). The inhibitory effects of mifepristone on promoter-driven transactivation were further exemplified when even in the presence of both dexamethasone and Vpr, a dose-dependent inhibition of transactivation was still demonstrated in the presence of the GR antagonist.

Initial results from the transactivation models gave rise to the further investigation into mifepristone as a possible antiviral compound. The fact that mifepristone demonstrated relatively low toxicity in both MTT and trypan blue exclusion assays allowed the continuation of the conducting of antiviral assays. To investigate the ability of mifepristone to inhibit HIV-1 infection, wild-type infectivity assays were developed In order to assess the efficacy of mifepristone in the context of wild-type infection, the T-cell line, CEMx174, was infected with an MOI of 0.5 of the EGFP-expressing virus, NL4-3-EGFP^{wt}. This NL4-3 wild-type virus expresses the EGFP protein, in which infected cells can be analyzed by fluorescent microscopy

as well as FACS analysis. Mifepristone was added post-infection to the CEM cells and samples were taken 24 hours after a second dose of mifepristone on day three. FACS analysis and p24 analysis revealed that mifepristone was able to inhibit viral replication in a dose-dependent manner, even at low concentrations of the compound (0.5μM) (Figures 22 and 23).

To further determine the effects of mifepristone on the inhibition of infection, it was determined to next investigate the effects of this compound on virus-infected PBMCs. The use of PBMCs demonstrates a relevant system in which to test mifepristone, as these cells include varying populations of initial viral targets, including both CD4-expressing T cells as well as monocytes. To conduct these assays, initial trials were conducted to determine the appropriate MOI in which to infect the donor PBMCs. After comparing p24 to MOI, it was determined that the only accurate way to infect cells was to determine the number of infectious particles per mL of each virus rather than p24, as comparative studies between p24 and MOI demonstrated that the number of infectious particles could change dramatically within different stocks of virus, even if the p24 was equivalent. In several different donors, cells were infected with various MOIs of virus, to see which would be most appropriate for this compound. If the viral titer was too high, infection overcame the cells immediately, with no chance of mifepristone, AZT, or analogs working appropriately. If the MOI was too low, there was little to no infection, which would obviously not prove either way if mifepristone was or was not able to inhibit virus replication. Each donor was different, however; some would infect at a much higher rate than others, and others would not infect at all even after the correct MOI was determined and used. Such are the trials of research.

After the appropriate MOI was established, PBMCs were infected with the dual-tropic 89.6 virus, as this virus is able to infect both R5 and X4 receptor-expressing cells including both initial target populations, T-cells and monocytes. Mifepristone was added post-infection in a dose-dependent manner to the PBMCs and supernatant samples were analyzed for the presence of p24, indicative of viral replication. Results demonstrated that mifepristone was able to inhibit the viral replication within these cells in a dose-dependent manner, exemplifying the efficacy of this compound.

To take this PBMC infection antiviral one step further, the patient isolate H112 was used to infect PBMCs at an MOI of 0.1. Post-infection, mifepristone was administered in a dose-dependent manner and supernatant samples were taken every three days and analyzed for p24. Results demonstrated that mifepristone was able to inhibit viral replication in a dose-dependent manner. Though the treatment with mifepristone did not inhibit replication when the lower concentrations were used, this compound was still effective at inhibiting viral replication when the higher concentrations were administered. This demonstrates that mifepristone was still able to combat patient isolates in a similar manner to the often-used lab isolate 89.6.

Vpr has an integral role in the infection of macrophages. In order to assess the effects of mifepristone on macrophage infection, macrophages were infected with the macrophage-tropic viral strain, Ba-L at an MOI of 0.1. Post-infection, mifepristone was added in a dose-dependent manner and similar to the PBMC antivirals, supernatant was collected every two or three days and p24 concentration was determined by ELISA. Again, mifepristone was able to inhibit viral replication in a dose-dependent manner as seen in several different donors. These experiments

were useful in determining the efficacy of mifepristone when used specifically for not only an initial cellular target of HIV-1, but one in which Vpr plays a major role in allowing viral infection.

Mifepristone inhibits the transactivation of viral genes, based on its interference with the mechanism of promoter activation. In order to determine if there was a difference in the efficacy of mifepristone dependent on when it was administered in comparison to when supernatant samples were taken, several trials were conducted by altering not only the time mifepristone was added (every two days versus every three days), but also by altering the time samples were taken (24 hours post-compound addition versus 48 hours post-compound addition). Results concluded that there was no significant difference when any of the parameters were altered as to the amount of inhibition that was demonstrated in the presence of mifepristone.

The possibility of mifepristone as an antiviral in its ability to decrease transactivation was demonstrated in these experiments. However, in order to determine if structural analogs of mifepristone exhibited less toxicity or greater efficacy at inhibiting virus, mifepristone analogs were assessed to determine the effects of compounds on both viral promoter transactivation as well as viral replication. Several compounds were acquired from NCI: mifepristone analogs 92336, 641295, and 666510. These compounds were analyzed in the context of both inhibition of viral transactivation as well as inhibition of viral replication. Toxicity results of these compounds were very similar to those of mifepristone, though they were slightly more toxic to macrophages. Though transactivation models demonstrated a dose-dependent decrease in Vpr-mediated

transactivation and antiviral models demonstrated similar inhibition, mifepristone was able to work in a more effective manner than any of these compounds. Therefore, it was concluded that these mifepristone analogs, while inhibiting the transactivation of viral genes, were not as effective as mifepristone.

Since mifepristone specifically targets Vpr-mediated transactivation in the presence of GR, initial aspirations on the purpose of mifepristone were as a microbicide. In order to determine the effects of mifepristone on a pre- versus post-treatment regimen, cells or virus were pre-treated with the compound prior to infection and were compared to cells or virus treated post-infection. Results demonstrated that there was no significant difference in the effects of mifepristone on PBMCs or macrophages.

One of the major dilemmas in combating HIV-1 infection is the presence of viral reservoirs. As Vpr is present in the virion prior to the production of Tat, initial transactivation of HIV-1 LTR-driven genes may in fact not only jumpstart viral transcription, but may re-activate latent virus hidden in these reservoirs. Vpr has already been shown to re-activate latent virus (84, 85). To assess the efficacy of mifepristone in preventing the reactivation of viral production from reservoirs, U1/HIV-1 cells were activated by TNF-α as well as by AT-2 –inactivated NL4-3 in the presence of a dose-dependent regimen of mifepristone. TNF-alpha was used as it is one of the major cytokines which regulates viral transcription mediated by the HIV-1 LTR through the NF-κB transcription factor. AT-2 -inactivated virus particle-triggered reactivation is due solely to the proteins present within the virion, one of which is Vpr. Tat is not present as the virus is non-functional and Tat is a late expressing protein. Therefore, we are able to implicate Vpr as a

transactivating factor leading to the re-activation of latent virus. After four days, samples were taken for p24 analysis. Results demonstrated that mifepristone was able to prevent the re-activation of viral production from latency in a dose-dependent manner, not only in the presence of cytokine-induced re-activation, but also by viral protein-induced reactivation. These initial studies demonstrated the effectiveness of mifepristone in combating viral reservoirs, and possibly allowing the prevention of reactivation from latency.

Taken together, Vpr was able to mediate transactivation by acting as ligand and utilizing the Glucocorticoid Receptor pathway. The GR antagonist, mifepristone, inhibited Vpr-mediated transactivation of both autologous (GRE) and heterologous (LTR) promoter-driven genes in a dose-dependent manner. Mifepristone was also able to inhibit wild-type and viral isolate infection in primary cells in a dose-dependent manner. Finally, this compound effectively decreased the re-activation of latent viral reservoirs in a dose-dependent manner. Therefore, we conclude that mifepristone could possibly compose a new class of antivirals, targeting the accessory protein Vpr and preventing transcription of viral genes, both in active infection as well as viral reservoirs. While mifepristone alone would be unable to fully combat infection, this compound could be used in conjunction with current HAART regimens to not only prevent viral transcription, but also combat viral reservoirs, preventing the reactivation from latency.

7. FUTURE DIRECTIONS

Initial studies on the antiviral effects of mifepristone include the mechanism behind Vpr-induced transactivation and the effects of mifepristone on the transactivation of autologous and heterologous promoters, the inhibition of viral replication in wild-type infectivity assays, and the inhibition of viral reactivation from latency. To further these studies:

1) A more detailed determination of the interaction between Vpr and GR should be conducted based on GR mutant studies including reporter transactivation assays, protein-protein interaction studies, and immunofluorescence to determine co-localization of GR and Vpr within the cell. In this way, the location of the interaction between Vpr and GR within the GR protein could be established, and possible other antivirals preventing this interaction could be investigated.

2) An animal model such as rhesus macaques could be investigated in order to determine the effects of mifepristone using SHIV. Mifepristone could be given in pre- versus post-treatment both as a microbicide as well as orally in order to determine if this compound can prevent viral replication.

3) The effects of mifepristone on latent viral reservoirs could be more fully established. For example, rather than using U1/HIV-1 cell lines, macrophages could be infected and brought to latency. Re-activation could be induced in the presence of mifepristone and the effects of this

compound could be determined. Also, resting T-cell reservoirs could be investigated as well as macrophage reservoirs.

4) The effects of mifepristone on pregnancy. Because mifepristone has abortive properties, pregnant women should not take this compound as an antiviral. Further studies need to be conducted to determine the effects of mifepristone on late stages of pregnancy.

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