Antiretroviral Therapy:

New Mechanistic and Therapeutic Insights for HIV

Single-Entity and Combination Drug Products

by

Jamie L. Austin

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Pharmaceutical Sciences)
in the University of Michigan
2015

Doctoral Committee:

Professor Gordon L. Amidon, Chair
Associate Professor A. Oveta Fuller
Jinrong Liu AbbVie Inc.
Professor David E. Smith
Professor Duxin Sun

DEDICATION

This dissertation is dedicated to those who told me, “No”, “You can’t” or “You will never be”. You were right; *I* can’t. But with God’s help: “Yes”, “I did”, and “I am”.

TABLE OF CONTENTS

List of Figures

List of Tables

CHAPTER 1

BACKGROUND AND INTRODUCTION

Introduction

The Acquired Immune Deficiency Syndrome (AIDS) has killed more than 39 million people since it was identified and clinically defined in 1981 and 1982, respectively [1] [2] [3] [4] [5]. The Human Immunodeficiency Virus (HIV) has been known as a causative agent for AIDS for over 30 years but it continues to have a major impact on global health [6]. Thirty-five million people are currently infected with HIV and each year 2.7 million new HIV infections are expected to occur [4] [7] [8].

In order to contribute to the advancement of antiretroviral therapy, this dissertation presents research pertaining to both early- and late-stage antiretroviral product development by: (1) highlighting opportunities for molecular targeting to impede HIV infection and (2) providing a biopharmaceutical rationale to accelerate the development of co-formulated antiretroviral drug products. The first aspect of this body of work presents research focused on understanding how the early events of the HIV lifecycle

influence the infection process. Single-cycle, fluorescently-tagged HIV and virus-like particles were produced and characterized in order to determine factors that may account for the observed low infectivity of the virus, quantitate HIV infection efficiency, and identify some of the clinically-relevant molecular mechanisms that underlie early infection/entry events. The results from these studies will clarify the mechanisms and efficiency of virus binding and infectivity, which may influence the development of better strategies for therapeutic intervention and/or vaccine development.

The second aspect of this work presents a provisional assessment of the biopharmaceutical characteristics of drug products in the late stages of clinical development and/or in the post-market phase for antiretroviral indications in order to emphasize how *in vitro* dissolution strategies can be scientifically valid approaches for demonstrating bioequivalence between co-formulated drug products and the reference, single-entity products administered in combination. Baseline recommendations are presented as a starting place for developing *in vivo* predictive and scientifically valid *in vitro* dissolution methodology to assess fixed dose combinations of various BCS classes. Thus, by promoting the utility of theoretically viable scientific rationale to support simpler and abbreviated development pathways, this research highlights potential opportunities to increase the availability of co-formulated antiretroviral drug products and describes mechanisms by which drug manufacturers and regulators can implement science-based approaches to address unmet medical needs and

effectively treat some of the world's deadliest diseases – especially in developing countries.

History and Overview of HIV

In 1983, Human Immunodeficiency Virus (HIV) was discovered to be the etiological agent that causes the Acquired Immune Deficiency Syndrome (AIDS) [6]. There are two known types of HIV: HIV-1 and HIV-2, which are further classified into groups of genetically similar isolates (see Figure 1 below). The M-group of HIV-1, however, comprises the majority of globally prevalent strains of HIV [1]. Therefore, the HIV-1 was chosen as the focus of the work described herein.

HIV-1 infects cells involved in the immune system, with $CD4^+$ T lymphocytes (i.e., helper T cells) and macrophages acting as the primary reservoirs of infection [9]. Following productive infection (i.e., integration of viral genome to support viral replication), the host cell is usually destroyed and/or impaired so that it can no longer function properly within the immune system. As a result of HIV infection, $CD4^+$ T lymphocytes are characteristically depleted [10]. Thus, the immune response worsens as the infection spreads, which eventually leads to an acquired, immune deficiency (AIDS) as depicted in Figure 2 .

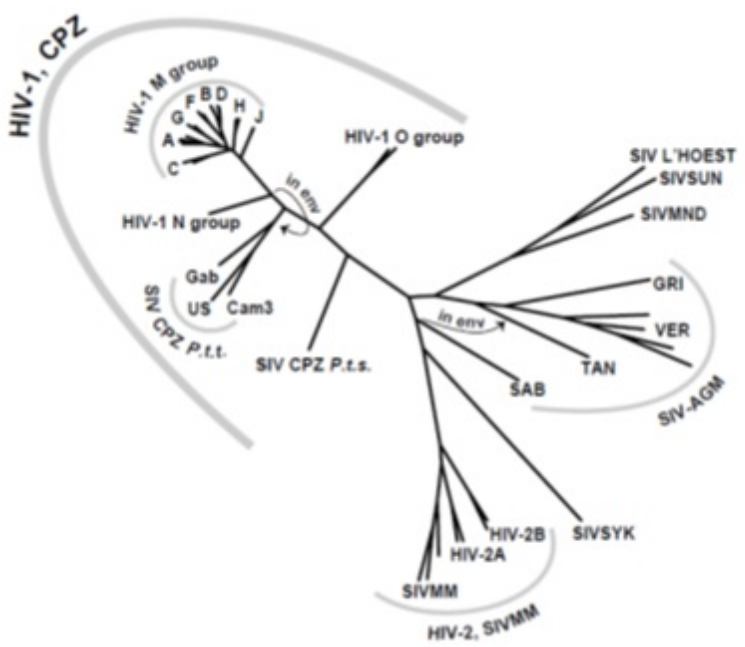


Figure 1 Primate Lentivirus Phylogenetic Relationships [11]

Highlights genetic relationship between different strains of HIV and the Simian Immunodeficiency Virus (SIV).

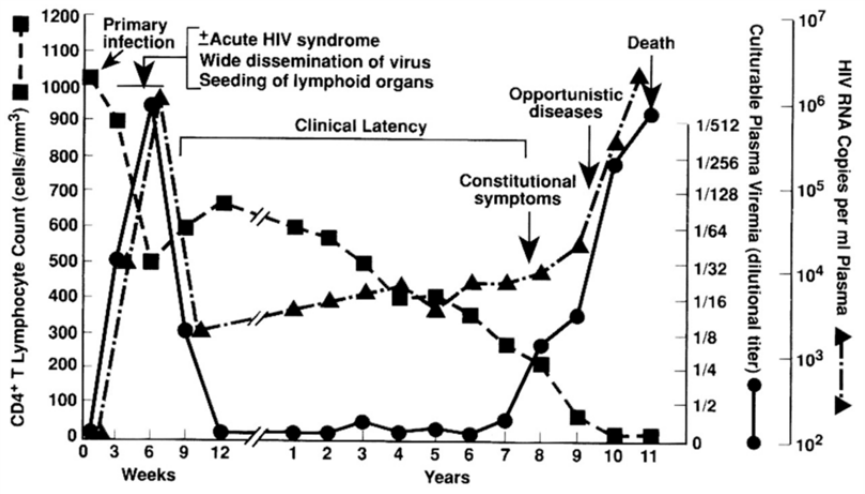


Figure 2 Typical Course of HIV Infection [12].

HIV viral load in the blood increases as the number of CD4$^+$ T cells dramatically declines. As the immune system deteriorates, it is unable to combat other infections. An HIV-infected individual is diagnosed with AIDS when their CD4$^+$ count is ≤ 200 cells/mm^3 and they have one or more opportunistic infections [13]**.**

HIV-1 is an enveloped retrovirus that possesses a single-stranded RNA genome of approximately 9kb in length (depicted in Figure 3). Each virion carries a non-covalently-associated dimer of genomic RNA. Coding regions comprise the majority of the HIV-1 genome, and these regions are flanked by characteristic Long Terminal Repeats (LTRs) at the 5' and 3' ends. The HIV-1 genome encodes for 15 mature viral proteins, as described in Table 1 and Table 2. For HIV to establish an infection, at least one copy of its genomic RNA must be reverse transcribed (i.e., produce complimentary DNA from the RNA template) into linear proviral cDNA that is subsequently integrated into the host cell's genome.

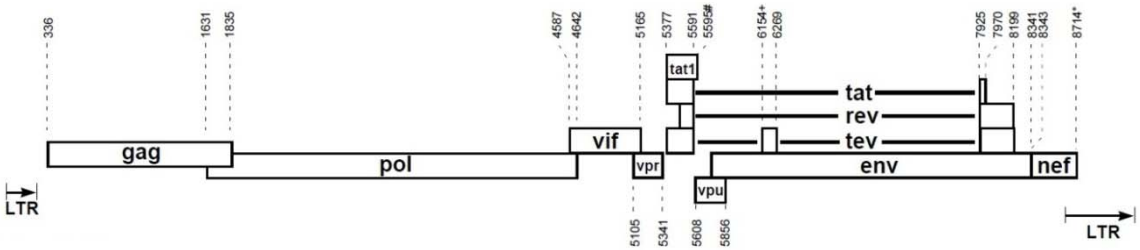


Figure 3 Genomic Arrangement of HIV-1 Landmark Proteins [14]

Table 1 HIV-1 Structural Proteins [15]

Precursor/ Region of Genome	Protein Name (Abbreviated)	Nomenclature by Protein Size	Known Functions	Primary Localization
Gag	Matrix (MA)	p17	Membrane anchoring; Env interaction; Nuclear transport of viral core (myristylated protein)	Virion
	Capsid (CA)	p24	Core capsid	Virion
	Nucleocapsid (NC)	p7	Nucleocapsid, binds RNA	Virion
	p6	p6	Binds Vpr	Virion
Pol[1]	Protease (PR)	p15	Gag-Pol cleavage; Maturation	Virion
	Reverse Transcriptase (RT)	p66	Reverse transcription; RNAse H activity	Virion
	Integrase (IN)	p31	Integration of proviral cDNA into host DNA	Virion
Env	Envelope Surface Protein (SU)	gp120	External glycoprotein that binds to CD4 and secondary receptors	Virion Envelope
	Envelope Transmembrane Protein (TM)	gp41	Transmembrane glycoprotein that mediates membrane fusion	Virion Envelope

[1] Synthesized as Gag-Pol polyprotein

Table 2 HIV-1 Regulatory and Accessory Proteins [15]

Protein Name (Abbreviated)	Nomenclature by Protein Size	Known Functions	Primary Localization
Negative Regulatory Factor (Nef)	p27	CD4 and MHC I downregulation (myristylated protein)	Host Cell Membrane and Cytoplasm
Regulator of Virion (Rev)	p19	RNA transport and stability factor	Host Cell Nucleus
Transactivator of Transcription (Tat)	p16	Viral transcriptional transactivator	Host Cell Nucleus
Viral Infectivity Factor (Vif)	p23	Virion maturation; Infectivity	Host Cell Cytoplasm; Virion
Viral Protein R (Vpr)	p10	Nuclear localization of preintegration complex; Inhibits cell division	Virion Core
Viral Protein U (Vpu)	p16	Release of viral particles; CD4 degradation	Host Cell Integral Membranes

HIV-1 infection is believed to be initiated via interactions with cell surface receptors such as CD4, which is the primary receptor recognized by the virus. The CD4 receptor is commonly expressed on the surface membranes of $CD4^+$ T lymphocytes and monocytic cells [16]. Interactions between CD4 and the viral envelope glycoprotein, gp120, have been noted as the principal means to mediate HIV binding and entry [17]. However, coexpression of chemokine co-receptors CXCR4 and/or CCR5 is necessary to permit entry into the target cells [18] [19] [20] [21].

Although the CD4, CXCR4, CCR5 and HIV-1 gp120 interactions are well documented, there are still several questions pertaining to HIV entry and infectivity. The literature consistently reports HIV infectivity (a unitless number that is calculated by normalizing the virus titer according to the physical number of virus particles) to be <0.1% [22] [23] [24]. To-date, however, there is not a clear understanding of why HIV possesses such low infectivity. Some within the field have proposed that HIV-1 and other retroviruses, are predominantly defective as the extent of rapid mutations (most notably occurring during reverse transcription) may lead to the production of noninfectious virus particles [25]. Others contest that there are host cell factors that limit HIV-1 infectivity by interfering with and/or disrupting the replication cycle [26]. Overall, however, there is a great deal of literature suggesting that one of the primary limitations in the establishment of a productive HIV infection is the target cell engagement and/or entry step. Specifically, several engineered/artificial methods for

increasing interactions between virions and target cells have been documented to result in greater infection efficiency. Such methods include:

- Spinoculation – method of inoculating cells with virions using centrifugation, which results in greater amounts of cell-associated virus and a proportional increase in virus replication [27].
- Use of polycations – inoculating virions in the presence of positively-charged molecules (e.g., polybrene, DEAE-dextran) to enhance adsorption of virus particles onto target cells and result in greater infectivity [24] [28] [29].
- Overexpression of adhesion molecules – overexpressing adhesion molecules on virion-producer cells results in virions that possess more adhesion molecules (acquired from budding out of producer cells) and enhanced ability to interact with and infect target cells [30] [31] [32].

Therefore, it is very plausible that HIV-1 infectivity is primarily limited by the ability to efficiently engage target cells. In fact, there have been some initial reports demonstrating that envelope density may affect infectivity for Simian Immunodeficiency Virus (SIV) [33] [34]. However, much of the existing HIV-related literature concerns target cell receptor density rather than the density of envelope proteins on the virus [35].

A study by Platt et al in 2010 also supports the hypothesis that the virus' inability to engage and/or enter target cells may be a main limitation in the establishment of a productive infection [36]. They observed virus particles

rapidly dissociating from target cells and proposed that the true, inherent level of infectivity is masked because of HIV's inefficient interactions with host cells [36]. While the results of the study are intriguing, the molecular mechanism behind this phenomenon remains unclear. Furthermore, the data are limited by their methods for quantitating the virus titer by counting foci of infected cells (which may underestimate the number of infections) instead of counting actual number of infected cells (which can be achieved using other assays). Most importantly, the quantitative nature of this study is also limited because it only quantitated the infectivity of virions that were pre-adsorbed onto target cells (rather than quantitating based on the entire virion population). Therefore, to-date, there has been no confirmatory study to quantitatively describe the molecular mechanisms for HIV entry and infectivity.

Evolution of Therapeutic Approaches to Treat HIV Infection

Numerous antiretroviral drugs have been approved to treat HIV/AIDS, but a complete cure has yet to be discovered. Highly active antiretroviral therapy (HAART), or combinations of antivirals of different drug classes and mechanisms of action, has become the standard of care to treat HIV infection. The current U.S. Department of Health and Human Services HIV treatment guidelines recommend starting treatment with a regimen of three HIV medicines from at least two different drug classes among non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors

(PIs), fusion inhibitors, entry inhibitors, and integrase strand transfer inhibitors (INSTIs). In general, the treatment guidelines recommend that patients naïve to antiretroviral therapy initiate therapy with a regimen that includes two NRTIs in combination with an NNRTI, a PI boosted with a pharmacokinetic enhancer, or an INSTI [37].

Approved antiretroviral drugs generally have been limited therapeutically because of drug resistance that results from the virus' ability to rapidly mutate as well as the persistence of a reservoir of latently infected cells (i.e., cells that possess an inactive, integrated copy of proviral DNA) [38]. In addition to the therapeutic limitations of current therapies, these treatment regimens require strict compliance. Unfortunately, many of these drug products have also been associated with numerous adverse effects including in some cases, but not limited to, diarrhea, nausea and vomiting, lipid abnormalities, and speculated risk for myocardial infarction [39] [40] [41] [42]. To-date, there is no prophylactic vaccine.

The success of current combination therapies for HIV-1 infection is largely due their ability to target specific viral targets (e.g., viral protease). However, with few exceptions, most of these agents are only effective following virus entry and/or infection of a host cell. The ability to block HIV entry and early infection events, on the other hand, would offer enormous advantage as a drug development strategy.

By blocking target cell entry, the extent of subsequent infections could be reduced (in a HIV-infected individual, for example), which may stabilize $CD4^+$ T counts over time and delay progression to AIDS since the virus' ability to enter and infect new cells would be diminished. Yet designing effective entry inhibitors requires sound evidence regarding the events that facilitate viral entry and lead to a productive infection. The virology field lacks clear insight about these events in the viral life cycle and the general understanding of HIV-1 infection remains highly disputed. Thus, an understanding of the molecular events of viral infectivity will be beneficial to the development of entry inhibitors.

With the intention of contributing to the discovery and early-stage development of antiretroviral therapy, the first half of this dissertation research focused on understanding how the early events of the HIV-1 lifecycle influence the infection process. Central to this research is the underlying hypothesis that HIV-1 infectivity is limited by the ability to efficiently engage target cells, which as described above, is a theory that is well-supported by various forms of evidence. In order to further investigate this theory, the first half of this body of work was intended to characterize the virus-cell interactions at the molecular level. As HIV-1 has been reported to only express an average of 14 envelope spike proteins per virion – which is an order of magnitude lower than other enveloped viruses [43] – one objective for the work presented herein was to investigate the envelope-dependent mechanisms that influence the efficiency of HIV-1 binding/infection. Thus, HIV-1 was characterized to determine factors that may

account for the observed low infectivity of the virus, quantitate HIV infection efficiency, and identify some of the clinically-relevant molecular mechanisms that underlie early infection/entry events and could potentially become drug targets to prevent infection.

Current Status of Antiretroviral Combination Drug Therapy

Given the chronic nature of HIV infection and the need for strict patient compliance in order to minimize the chances of emerging viral resistance [44], fixed dose combinations are commonly utilized for more convenient and simplified dosing regimens, which may result in greater levels of patient compliance. Several studies have demonstrated that, when compared to regimens requiring multiple tablets, single-tablet HIV regimens are associated with greater adherence; and as a result, lead to more viral suppression [45] [46] [47]. However, not all available, single-entity antiretroviral drugs have been incorporated into approved, co-formulated antiretroviral combination drug products and, although it could significantly aid the development of combination drug products globally, scientifically justified criteria for demonstrating bioequivalence to support the efficacy of fixed dose combination regimens relative to their individual drug components have yet to be established [38] [48].

In order to contribute practical scientific data to advance antiretroviral therapy in the later stages of development, the second half of this dissertation research focused on providing a biopharmaceutical rationale to accelerate the development of co-formulated antiretroviral drug products. Central to this

research is the underlying theory that, a well-designed, oral fixed-dose combination drug product, formulated with individual drugs that exhibit linear and non-interacting absorption, distribution, metabolism and excretion/elimination (ADME), in the absence of excipients that significantly affect bioavailability, should have the same *in vivo* dissolution characteristics and hence absorption profiles as the constituent, single-entity drug products in the same dosage form, and, as a result, will be bioequivalent, regardless of the biopharmaceutical classification of the active ingredients. 34 single-entity drug products and 22 fixed dose, co-formulated, combination drug products with HIV indications in late stage clinical development (Phase 2 or beyond) or post-marketing phase were assessed in order to promote the utility of this theoretically-viable scientific rationale. Thus, the second half of this body of work highlights potential opportunities to increase the availability of co-formulated antiretroviral drug products – especially in developing countries – in order to meet the significant global unmet medical need for HIV/AIDS therapy.

Thesis Overview

This work is comprised of an investigation of the molecular mechanisms that form the basis of HIV-1 infectivity and an evaluation of both investigational and approved antiretroviral [combination] drug regimens. The results from the first portion of this thesis further the current understanding of the molecular mechanisms underlying HIV entry and infectivity, which may influence the development of better strategies for therapeutic intervention. In the second half of

the project, antiviral drug products that are approved or in the late-stage pipeline were provisionally classified and evaluated in relation to product substitutability and/or interchangeability standards for both single-entity and combination drug products. The results from both aspects of this research provide a current and forward-looking view of antiretroviral therapy that will aid the development of therapeutic approaches to combat one of the world's most serious viral infections.

CHAPTER 2

OPTIMIZED INFECTIVITY OF EGFP-TAGGED, SINGLE-CYCLE HIV-1

Abstract

The infectivity of cell-free HIV-1 is consistently reported to be less than 0.1% and the mechanisms influencing this low infectivity are not yet fully understood. Some hypothesize that this observed low infectivity results from the presence of defective HIV-1 particles, formed from mutations introduced in the reverse transcription step of virus replication. Using molecularly cloned HIV-1 that is capable of only a single round of infection, we can bypass the reverse transcription step during virus production in order to investigate this hypothesis and the extent to which infectivity can be influenced by other factors during virus production. Herein we show that optimal infectivity is obtained by harvesting virions from culture media 18 hours after transfection, with complete media changes 4-6 hours post-transfection. This optimal infectivity appears to primarily result from the emergence of “noninfectious” virus particles at later time points. Our data demonstrate that HIV-1 infectivity increases in the presence of

greater amounts of viral envelope glycoproteins. Thus, the larger proportion of "noninfectious" virus particles apparent in harvested virus cultures at later time points may be attributable to the production of virus lacking sufficient amounts of envelope glycoproteins. However, although we demonstrate ways by which virus infectivity can be optimized by varying parameters to enhance culture and production conditions, the overall infectivity of HIV-1 remains low. This suggests that the observed low infectivity of the virus is principally attributable to other, potentially-related, mechanism(s).

Introduction

Infectivity, a unitless number that quantitates the proportion of virus particles that are infectious, is a critical parameter for characterizing the human immunodeficiency virus type 1 (HIV-1). The infectivity of HIV-1 is consistently reported to be less than 0.1% [22] [23] [24]. There is not yet a clear, confirmatory understanding of why HIV-1 possesses such low infectivity, but there is a great deal of literature suggesting that one of the primary limitations in the establishment of a productive HIV infection is the target cell engagement and/or entry step. In fact, several engineered/artificial methods for increasing interactions between virions and target cells have been documented to result in greater infection efficiency. Spinoculation, a method of inoculating cells with virions using centrifugation, for example, results in greater amounts of cell-associated virus and a proportional increase in virus replication [27]. Furthermore, inoculating virions in the presence of positively-charged molecules

(e.g., polybrene) has been shown to enhance adsorption of virus particles onto target cells and result in greater infectivity [24] [28] [29]. And the overexpression adhesion molecules on virion-producer cells has also been shown to result in virions that possess more adhesion molecules (acquired from budding out of producer cells) and enhanced ability to interact with and infect target cells [30] [31] [32]. Therefore, it is plausible that HIV-1 infectivity is primarily limited by the ability to efficiently engage target cells.

On the other hand, however, some have hypothesized that this observed low infectivity results from the presence of defective HIV-1 particles, formed from mutations introduced in the reverse transcription step of virus replication [25]. This theory is supported by the well-documented, naturally high error rate of HIV reverse transcriptase [49] [50] [51]. Furthermore, the identification of defense mechanisms, such as APOBEC3 cytidine deaminases, in the host cell that can influence the rate of mutation during the reverse transcription step of virus replication also strongly point to the possibility that the low infectivity of HIV-1 might be due to the presence of large numbers of defective virions resulting from mutations introduced to the proviral DNA during reverse transcription [52] [53].

Using molecularly cloned HIV-1 that is capable of only a single round of infection, we can bypass the reverse transcription step during virus production and essentially eliminate the influence of reverse transcriptase errors and APOBEC3 in order to investigate this hypothesis and determine the extent to which infectivity can be influenced by other factors during virus production.

Herein we specifically demonstrate that the culture conditions commonly used to produce cell-free HIV-1 in cultured media significantly influence the resulting infectivity of virions and can result in the production of a greater proportion of defective virus over time. These results emphasize the optimal conditions for producing cell-free virus and point to a potential molecular mechanism, related to the alternate theory of inefficient virus-cell interactions, which may more significantly impede the infectivity of HIV-1 in plasma or cultured media.

Materials and Methods

Production of single-cycle HIV-1 virions.

Virions were produced by transfecting HEK 293T/17 cells (ATCC, Manassas, VA) as previously described [54]. Briefly, 293T were cultured at 37^{o}C with 5% CO_2 in DMEM supplemented with 10% FBS (HyClone Laboratories, Logan, UT) and seeded overnight in culture media. Using the TransIT LT-1 transfection reagent (MirusBio, Madison, WI), HIV virions carrying free EGFP were generated by transfection with variable amounts of pNL4-3E- plasmid, pNL4-3E-MA-EGFP-CA plasmid and pcDNA3.1REC. In certain experiments, after a specified number of hours of incubation at 37^{o}C, the culture media (with transfection reagents) was removed and replaced with fresh culture media. The 37^{o}C incubation continued for an additional period. At the experimentally-determined time point post transfection, the culture media, containing EGFP-labeled single-cycle virions was collected and filtered through a 0.45-mm syringe filter (Millex-HV PVDF, Millipore). The filtrate was then aliquoted on ice, flash-

frozen in a dry ice/ ethanol bath and stored in a -80^{o}C freezer. Subsequent analysis using p24 ELISA (HIV-1 p24 Antigen Capture Kit, Advanced Bioscience Laboratories, Rockville, MD) was conducted to determine the number of virus particles in a specified volume, assuming 2,500 molecules of p24 per virion.

Calculating the Infectivity of free-EGFP HIV-1.

Infection assay in TZM-bl cell line. The infectivity of virions was calculated by normalizing the virus titer (i.e., number of infectious units in a certain volume) to the physical number of virus particles present in the specified volume. The titer was be determined by using an established β-galactosidase-based infection assay. This assay relies upon the TZM-bl indicator cell line - a genetically engineered HeLa-derived cell line that expresses CD4, CXCR4, and CCR5 [55] [56]. TZM-bl cells function as indicators of HIV infection because they also possess Luciferase and bacterial β-galactosidase reporter genes, which are driven by an HIV LTR promoter. This LTR promoter is induced to express the reporter enzymes following HIV infection when the viral protein Tat (Transactivator of transcription) is produced [57]. Thus, in theory, only infected cells will express the reporter genes. These infected cells can be detected by providing a chromogenic substrate for either of the reporter enzymes. For these studies, infected cells will be quantitated by using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as a substrate for β-galactosidase. β-galactosidase catalyzes the hydrolysis of X-gal and produces a blue-colored byproduct, 5,5'-dibromo-4,4'-dichloro-indigo.

The infectious titer of HIV-1 virions can therefore be determined by enumerating the number of blue TZM-bl cells following inoculation with various dilutions of the virus stock in accordance with the following equation:

$$\text{Titer} = \frac{\#\,\text{Blue TZMbl Cells}}{\text{Dilution Factor}} \times \frac{1\,\text{mL}}{\text{Inoculum Volume (mL)}} = \frac{\text{Infectious Units}}{\text{mL}}$$

TZM-bl cells (cat#8129, NIH AIDS Research and Reference Reagent Program) were cultured at 37^{o}C with 5% CO_2 in DMEM supplemented with 10% FBS (HyClone Laboratories, Logan, UT). Prior to tenth passage, TZM-bl cells were trypsinized, counted, sedimented by centrifugation at 1,000g for 5 minutes and resuspended in DMEM with 10% FBS. 8X10^4 TZM-bl cells in a 1-ml culture volume were seeded in each well of a 12-well plate one day prior to infection. EGFP-labeled HIV-1 particles (obtained from stocks stored at -80^{o}C) were added to these pre-seeded aliquots of TZM-bl cells in 100 µl of DMEM with 10% FBS and DEAE dextran (final concentration 20 mg/ml). The virion and TZM-bl cell mixture was incubated at 37^{o}C for 2hrs with gentle rocking every 30 min. At the end of two hours, 1 ml of complete media was added to each well and the incubation was continued at 37^{o}C for 48 hours with 5% CO_2. After 2 days of incubation, cells were fixed in 2% gluteraldehyde at room temperature for five minutes. Cells were then washed three times with PBS, and stained for 50 min at 37^{o}C using cell staining solution provided in the beta-galactosidase staining kit (Mirus Bio, Madison, WI). Cells were washed three times with milliQ water and

the number of blue cells in each well was counted using a 10X objective on a Nikon TS100-F inverted microscope.

p24 ELISA. Calculating infectivity also depends on quantitating the number of physical virion particles in a given volume. Expression of HIV-1 p24 structural protein has been demonstrated to correlate with viral loads *in vitro* [58]. Thus, in these studies, a commercially available p24 Enzyme-Linked Immunosorbent Assay (ELISA) was utilized to quantitate the number of virions. The ELISA enables detection and quantification of p24 in a given volume through absorbance readings that can be extrapolated to a p24 concentration according to a standard curve of reference samples with known p24 concentrations. Specifically, based on the estimation of there being approximately 2.5 million virus particles per ng of p24 [59] [60], the number of HIV-1 particles in a specified volume was calculated as follows:

$$\frac{\text{pg of p24}}{\text{mL}} = \frac{\text{Sample Absorbance Value}}{\text{Slope of Standard Curve} \times \text{Dilution Factor}}$$

$$\frac{\#\text{ of HIV particles}}{\text{mL}} = \frac{\text{pg of p24}}{\text{mL}} \times \frac{1\,\text{ng}}{1000\,\text{pg}} \times \frac{2.5 \times 10^6\ \text{HIV particles}}{\text{ng of p24}}$$

Infectivity Calculation. Using the titer and p24 values, the infectivity, i.e., the fraction of single-cycle free-EGFP HIV-1 that is infectious, was quantitated as follows:

$$\text{Infectivity} = \frac{\text{Infectious Units/mL}}{\text{Total \# of HIV Particles/mL}} \times 100$$

Results and Discussion

In order to quantitate the efficiency of HIV infection, single-cycle HIV-1 pseudovirions carrying free molecules of Enhanced Green Fluorescent Protein (i.e., free-EGFP) were utilized. To produce free-EGFP virions, the proviral HIV-1 DNA was engineered to carry an EGFP coding sequence flanked by two HIV protease cleavage sites within the *gag* domain of viral genome (see Figure 4). Upon virion maturation, the Gag polyprotein is proteolytically cleaved such that free molecules of EGFP exist inside of the virions, as depicted in Figure 5.

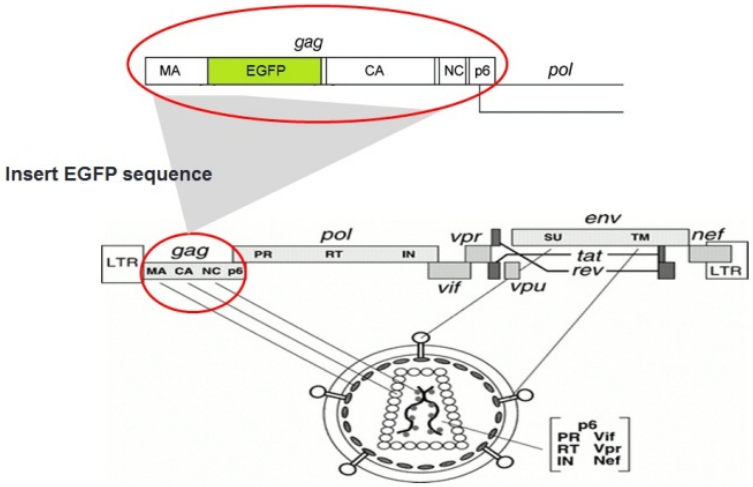


Figure 4 Depiction of EGFP Insertion in HIV-1 Genome [61]

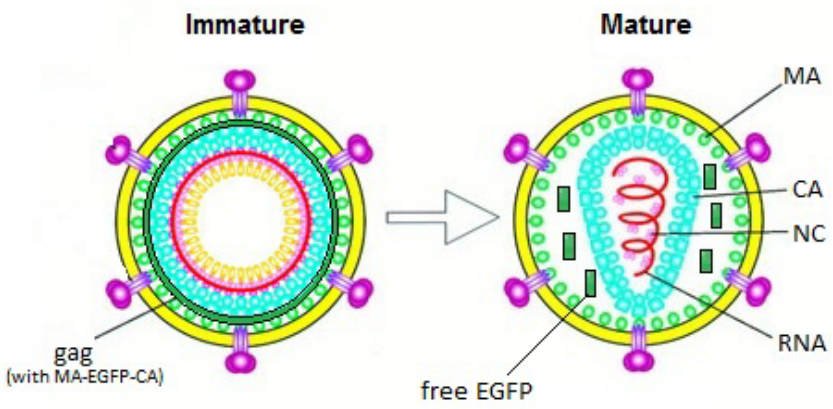


Figure 5 Viral Matruation and Production of "Free" EGFP within Virions [62]

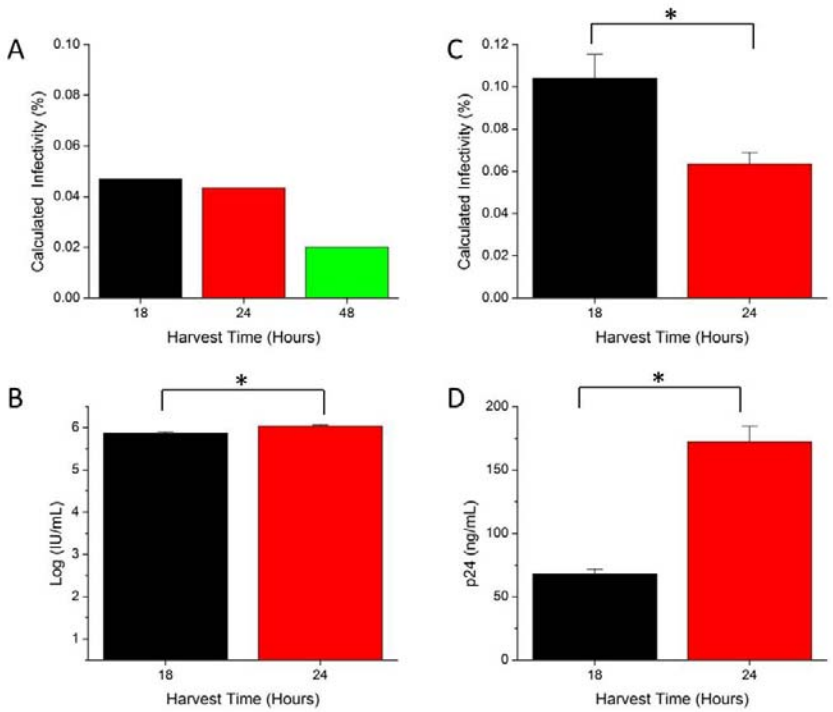
The backbone of the single-cycle, free-EGFP virions was derived from an envelope-deficient (E-) HIV-1 plasmid. Therefore, progeny virus were not capable of producing the HIV-1 envelope proteins that are necessary for subsequent rounds of infection. Instead, a single round of infection is achieved by expressing the envelope deficient proviral DNA in the presence of a separate envelope expression plasmid [63], as described in Materials and Methods.

The usual transfection procedures call for an equal weight mixture of provirus and envelope plasmids and specify that virions should be harvested 48 hours post-transfection [64]. However, there is no well-documented rationale for harvesting virus at 48 hours. To investigate this whether timing impacts infectivity during the production of cell-free single-cycle HIV-1, a range of common harvesting time points were studied. Consistent with previous reports [36], EGFP-labeled, single-cycle HIV-1 harvested from culture media after 18

hours has higher infectivity (Figure 6A) and Figure 6B). Per the modest changes in the infectious titer and corresponding significant increase in virus concentration, as shown in Figure 6C and Figure 6D, respectively, this optimal infectivity appears to primarily result from the emergence of “noninfectious” virus particles at later time points.

Consistent with the significant increase in p24 concentration (Figure 6D), this observed harvest time-dependency could be a result of the greater production of defective virions at later time points. However, it also possible that there is actual loss of virus infectivity over time. We elected to investigate our findings in greater detail to determine whether there are specific mechanisms to explain the correlation between higher infectivity and early harvesting time. For example, if the correlation is due to the actual loss of infectivity over time, it is plausible that certain “environmental factors” in the cell culture may be promoting a loss of infectivity. Virus harvested at later times would be subject to the cell culture conditions for longer periods and thus, the impact on infectivity would be more apparent and consistent with our observations. Therefore, in order to distinguish between an actual loss of infectivity and the production of defective virions, we examined the impact of various environmental factors to determine if the cell culture/virus production conditions might be promoting a loss of infectivity.

Figure 6 HIV-1 Infectivity is Dependent on Harvest Time

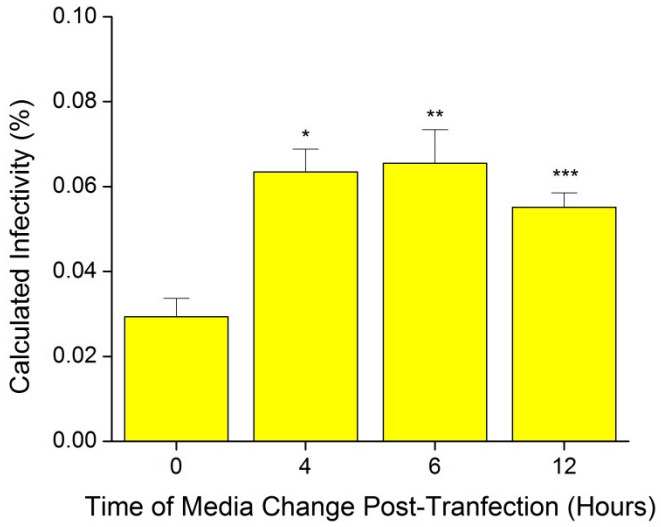


Panel (A) - Data presented are representative of the mean of 2 replicates under each experimental condition, which suggest a general trend for increased infectivity at shorter harvest times. Panels (B), (C), and (D) - Data presented are representative of the mean ± SD (for Panel (B), the SD was calculated per propagation of error in the experimental values) of 3 individual replicates (n=3), each of which was studied in duplicate for each experimental condition. Statistical significance between the experimental conditions was tested using an unpaired t test. The t-statistic was compared to the corresponding critical values for a two-tailed test and df = 4 in standard statistical tables. Asterisks denote statistical differences at a 0.05 significance level.

Per the experimental method, single-cycle HIV-1 is collected and harvested from the cell culture media. Prior to harvesting, however, virions reside in culture with the 293T producer cells and are subject to the cell culture conditions. Although media change is not required following transfection with the

TransIT LT-1 reagents (Mirus Bio, Madison, WI), the cell culture conditions may still be less than ideal. Therefore, we studied the impact of refreshing the complete media at various time points following transfection. As shown in Figure 7, infectivity increases substantially when media changes are introduced – and optimal infectivity is obtained with media changes at 4-6 hours post-transfection.

Figure 7 Media Changes Increase Infectivity



Data presented are representative of the mean ± SD (the SD was calculated per propagation of error in the experimental values) of 3 individual replicates (n=3), each of which was studied in duplicate for each experimental condition. Statistical significance between the experimental conditions was tested using an unpaired t test. The t-statistic was compared to the corresponding critical values for a two-tailed test and df = 4 in standard statistical tables. Asterisks denote statistical differences (vs. no media change, time = 0) at a 0.05 significance level.

Since changing the media post-transfection resulted in higher infectivity and pH is an important parameter for maintaining viable cell culture, we monitored the pH over time to assess whether pH changes could be promoting a loss of virus infectivity over time. As shown in Table 3, however, the pH remained relatively constant through the time of harvest. Therefore, pH changes do not explain the observed decline in HIV-1 infectivity.

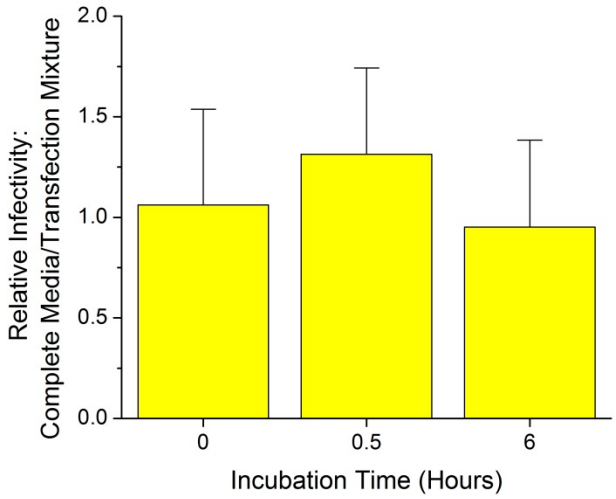
Table 3 pH Changes Do Not Promote Loss of Infectivity

Time after Transfection (Hours)	pH	
	18-hr Harvest	24-hr Harvest
0	8.1	8.1
4	8.1	8.1
6	8.1	8.1
12	8.1	8.1
18	8.1	8.1
24	N/A	8.1

Transfection reagents are well-known for cytotoxicity [65] and it is possible that HIV-1might also be sensitive to transfection reagents. To assess whether prolonged exposure to transfection reagents remaining in the cell culture might promote loss of virus infectivity, we conducted a stability assay with harvested virus. The relative infectivity, calculated as the ratio between the

infectivity of virus incubated in complete media only and the infectivity of virus subject to transfection reagent (diluted in complete media to mimic conditions of virus production) demonstrates that the infectivity was consistently comparable over time. Therefore, the presence of transfection reagents does not explain the observed decline in HIV-1 infectivity.

Figure 8 Presence of Transfection Reagents Does Not Promote Loss of Infectivity



Ratios were calculated using the means ± SD of 3 individual replicates (n=3), each of which was studied in duplicate for each experimental condition. Statistical significance between the experimental conditions was tested using an unpaired t test. The t-statistic was compared to the corresponding critical values for a two-tailed test and df = 4 in standard statistical tables at a 0.05 significance level. No statistical differences were observed (vs. control at 0 hours).

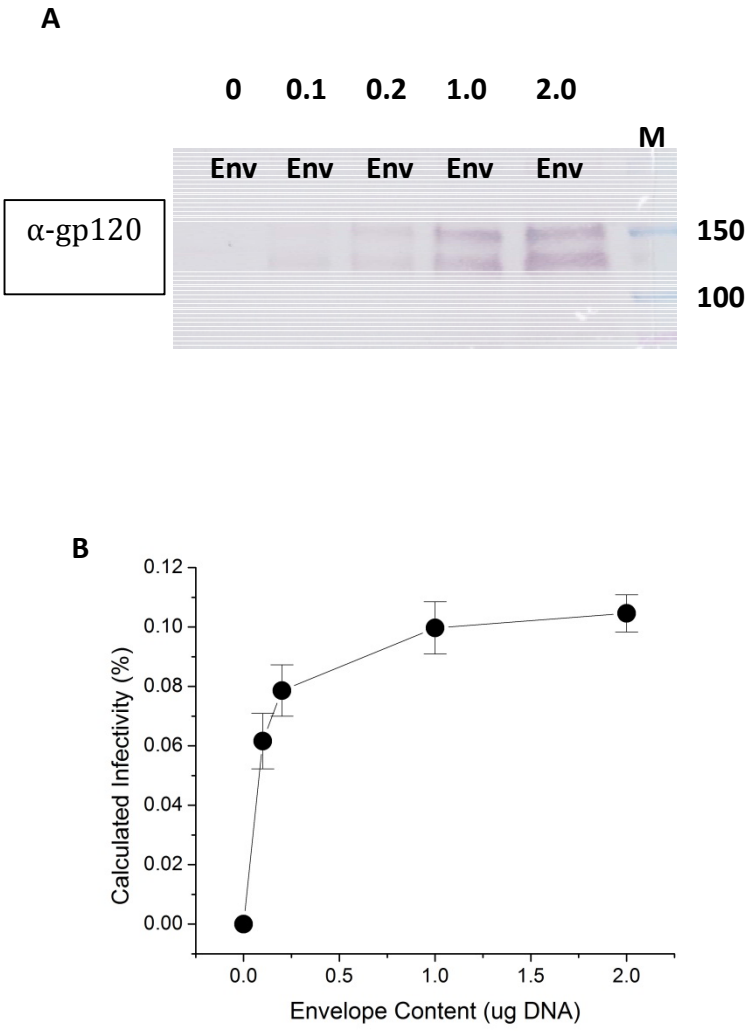
To further investigate and distinguish between low infectivity resulting from the production of greater proportions of defective virions at later time points and the loss of virus infectivity over time, we considered that the biophysical instability of virion particles is a known contributor to the infectivity decay of HIV-1 [22]. However, such reports do not yet fully explain why HIV-1 infectivity is consistently observed to be so low. Notably, even under optimal culture conditions, we continued to observe infectivity on the same low order of magnitude. We, therefore, wanted to determine whether the overall low infectivity of HIV-1 could be attributed to the presence of defective virions resulting from mechanisms other than revere transcription-associated mutations (which our experimental system bypassed) during virus production. Evidence supporting the presence of defective virions would be consistent with our observations of increased virus production without a corresponding increase in infectivity as well as the marked impact of changing the media post-transfection which, theoretically, could reduce the concentration of defective virions.

Along these lines, there are reports of how stoichiometries of viral proteins influence stability of HIV-1 and the incorporation of viral envelope glycoproteins has specifically been implicated in HIV-1 infectivity decay [66]. There are, in fact, still many questions about the distribution of viral proteins during virus assembly and it is possible that defective, less infectious, and/or unstable virions could result from progeny virus incorporating unfavorable amounts of viral proteins. Therefore, we sought to demonstrate and assess

the extent to which varying the stoichiometry of viral proteins might influence the infectivity - as this might provide a molecular explanation for the observed low infectivity of HIV-1.

Our method of producing single-cycle virus was conducive to investigating how the incorporation of variable amounts of viral proteins might influence the infectivity of HIV-1. Specifically, since viral envelope protein incorporation results from expression of HIV-1 DNA encoded on a plasmid that is separate from the remainder of the viral genome, we could vary the envelope stoichiometry by changing the amount of envelope-encoding DNA used to produce our single-cycle virus.

Figure 9 Greater Amounts of Envelope Glycoproteins Increase Infectivity



Panel (A) - Western blot for HIV-1 envelope glycoprotein 120 (gp120) confirming successful production of virus with variable amounts of envelope spike proteins. Panel (B) - The corresponding infectivity calculations, representative of the mean ± SD of 3 replicates, which suggest a general trend for increased infectivity with greater amounts of envelope incorporation.

As shown above (Figure 9A), we successfully produced virus with variable amounts of envelope spike proteins. Our data demonstrate that HIV-1

infectivity increases with greater amounts of envelope glycoprotein (Figure 9B); thus indicating that the larger proportion of “noninfectious” virus particles apparent in harvested virus cultures at later time points following transfection may be due to the production of defective virus, lacking sufficient amounts of envelope glycoproteins. Yet, because the overall infectivity still remained relatively low under these optimized conditions, this mechanism does not to fully explain the reasons underlying the consistent reports of low HIV-1 infectivity.

Conclusion

We have studied the infectivity of single-cycle HIV-1 and determined that harvesting time, media changes, and the expression of envelope proteins all impact virus infectivity and indicate that a greater proportion of defective virus is produced at later time points. Our data are consistent with prior reports regarding the role of biophysical instability and/or lack of envelope glycoproteins in diminishing virus infectivity. However, although we demonstrate ways by which virus infectivity can be optimized by varying these conditions, the overall infectivity of HIV-1 remains low – suggesting that the observed low infectivity of the virus is principally attributable to other, potentially-related, mechanism(s).

CHAPTER 3

QUANTITATION OF HIV-1 INFECTION EFFICIENCY

Abstract

There is not a clear understanding of why the infectivity of cell-free HIV-1 has been consistently reported to be several orders of magnitude lower than other enveloped viruses (i.e., HIV-1 infectivity is typically observed as less than 0.1%). In order to gain more knowledge about the factors influencing HIV-1 infectivity, we sought to quantitate the overall efficiency of infection. Specifically, by combining the well-established TZM-bl indicator assay with fluorescent techniques, we were able to quantitate the overall infection efficiency of HIV-1 as a function of virus concentration.

Introduction

Due to the apparently low infectivity in plasma or culture media, retroviruses such as HIV-1 have generally been perceived as being predominantly defective. In the case of HIV-1, for example, typically less than 0.1% of the total cell-free virus concentration will produce infections [22] [23] [24].

It was originally believed that this observed low infectivity results from the presence of defective HIV-1 particles, formed from mutations occurring during reverse transcription [25]. Yet, despite the fact that defective virus particles have been well-characterized for other RNA viruses, there is not conclusive evidence to directly associate the low infectivity of HIV-1 with the existence of large quantities of defective particles. On the contrary, there are several pieces of evidence to support the notion that the inefficiency of HIV-1 interactions with target host cells is a primary factor impacting this overall low infectivity. The use of polycations like DEAE dextran [24] [28] [29], for example, as well as the increasingly popular practice of applying centrifugal force [27] ("spinoculation") to promote virus-cell interactions, have been demonstrated to substantially increase the infectious virus titer. Furthermore, our recent studies limiting the presence of defective particles (by bypassing the reverse transcription step during virus production) also suggest that the overall observed infectivity of HIV-1 is largely influenced by host cell interactions [54]. Consistent with this finding, it has been demonstrated that, after VSV-G pseudo-typed HIV-1 enters a host cell and initiates reverse transcription, it is capable of establishing infection with approximately 13% efficiency [67]. As this infection efficiency is at least two orders of magnitude greater than the purported infectivity of the virus, it supports the notion of the primary hindrance(s) to the overall efficiency of HIV-1 infection occurring prior to the initiation of reverse transcription.

With this in mind, we sought to quantitate the overall infection efficiency of HIV-1 by determining the proportion of cells that become infected after inoculation with various concentrations of virus. By comparing the proportion of cells that become infected to the fraction of cells that originally had virions bound/internalized (as determined by flow cytometry), we also calculated the efficiency of HIV-1 infection following attachment to cells.

Materials and Methods

Production of single-cycle HIV-1 virions.

Virions were produced by transfecting HEK 293T/17 cells (ATCC, Manassas, VA) as previously described [54]. Briefly, 293T were cultured at 37^{o}C with 5% CO_2 in DMEM supplemented with 10% FBS (HyClone Laboratories, Logan, UT) and transfected at the fifth passage. 10^6 293T cells were seeded overnight in a T-75 flask with 10ml of culture media. Using the TransIT LT-1 transfection reagent (MirusBio, Madison, WI), HIV virions carrying free EGFP were generated by transfection with 4.0μg pNL4-3E- plasmid, 4.0μg pNL4-3E-MA-EGFP-CA plasmid and 0.8μg pcDNA3.1REC. After 6 hours of incubation at 37^{o}C, the culture media (with transfection reagents) was removed and replaced with 16ml of fresh culture media. The 37^{o}C incubation continued for an additional 12 hour period. At 18 hours post transfection, the culture media, containing EGFP-labeled single-cycle virions was collected and filtered through a 0.45-mm syringe filter (Millex-HV PVDF, Millipore). The filtrate was then aliquoted on ice, flash-frozen in a dry ice/ ethanol bath and stored in a -80^{o}C

freezer. Subsequent analysis using p24 ELISA (HIV-1 p24 Antigen Capture Kit, Advanced Bioscience Laboratories, Rockville, MD) was conducted to determine the number of virus particles in a specified volume, assuming 2,500 molecules of p24 per virion.

Infection assay in TZM-bl cell line.

TZM-bl cells (cat#8129, NIH AIDS Research and Reference Reagent Program) were cultured at 37^{o}C with 5% CO_2 in DMEM supplemented with 10% FBS (HyClone Laboratories, Logan, UT). Prior to tenth passage, TZM-bl cells were trypsinized, counted, sedimented by centrifugation at 1,000g for 5 minutes and resuspended in DEAE dextran (final concentration 20 mg/ml) and/or DMEM with 10% FBS. EGFP-labeled HIV-1 particles were added to aliquots of TZM-bl cells in 1.5ul Eppendorf tubes up to a total volume representative of each MOP condition. The virion and TZM-bl cell mixture was incubated in suspension on a fixed-speed nutator (Fisher Scientific) at 37^{o}C for 2hrs. Following the 2hr inoculation, samples were immediately placed on ice to halt the infection process. Samples were kept on ice/at 4^{o}C and washed (i.e., centrifugation at 1,000g for 5 minutes followed by resuspension) with DMEM containing 10% FBS three times to remove any unbound virions. However, the supernatant from the first wash was collected and flash frozen for later analysis via p24 Antigen Capture Kit (Advanced Bioscience Laboratories, Rockville, MD) in accordance with the manufacturer's instructions. Following the washing step, 2 aliquots (containing approximately 8x10^{4} cells) from the cell-virion samples were seeded in 1ml of

DMEM with 10% FBS in 24-well plates. The samples were further incubated at 37^{o}C with 5% CO_2 for 2 days. After 2 days of incubation, cells were fixed in 2% gluteraldehyde at room temperature for five minutes. Cells were then washed three times with PBS, and stained for 50 min at 37^{o}C using cell staining solution provided in the beta-galactosidase staining kit (Mirus Bio, Madison, WI). Cells were washed three times with milliQ water and the number of blue cells in each well was counted using a 10X objective on a Nikon TS100-F inverted microscope.

Flow cytometry analyses

Following inoculation in suspension and after aliquots were taken for beta-galactosidase staining, the remaining TZM-bl cells were resuspended in 4% paraformaldehyde for 10 minutes at room temperature. After fixation, the samples were washed (i.e., centrifugation at 1,000g for 5 minutes followed by resuspension) in PBS two times. The samples were kept at 4^{o}C until subsequent flow cytometry analysis (using BD FACSCanto II flow cytometer). The resulting measurements of fluorescence intensity were analyzed via original Matlab code, designed to fit data to the sum of two log-normal distributions.

Results

Herein we have utilized flow cytometry to quantitate the infection efficiency of HIV-1. Our data show that the inherent efficiency of HIV-1 infection is dependent upon the multiplicity of virus particles (MOP). Interestingly, at high MOP values, we observed a significant limitation in the infection efficiency.

MOP Dependence of HIV Infection

In order to quantitate the efficiency of HIV-1 infection, it was necessary to study both the extent of virion binding/internalization and the number of cells infected. For direct comparisons of both quantitative measures in these experiments, we utilized TZM-bl cells, which carry a β-galactosidase reporter that is expressed upon productive HIV infection [57]. Because infected cells can be stained blue following the β-galactosidase-mediated reaction with X-gal, the chromogenic substrate, this reporter allowed us to visibly determine the number of infected cells under different inoculation conditions.

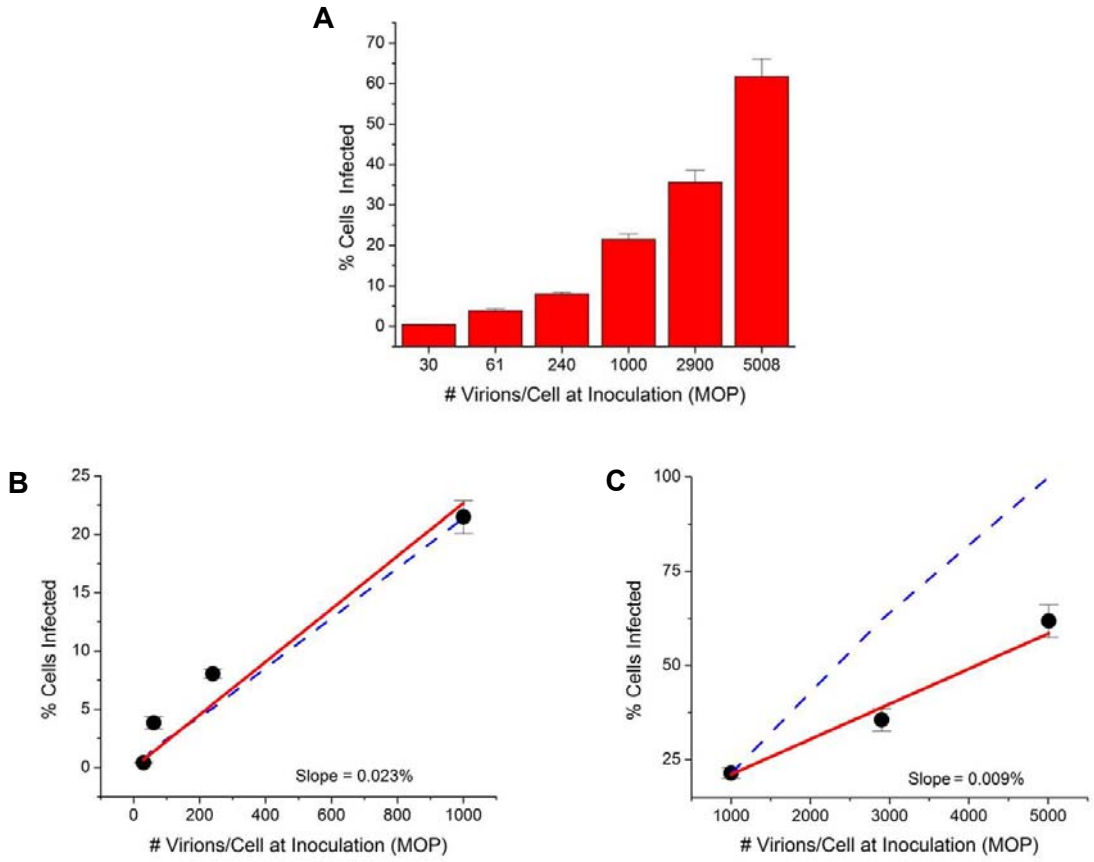
Specifically, we quantitated the fraction of blue, infected cells as a function of the multiplicity of particles (MOP). The MOP refers to the ratio between the physical number of HIV-1 particles (as determined by p24 ELISA) and the number of potential $CD4^+$ target cells. In our experiments, the MOP value is based on the total number of virus particles introduced to a defined cell population at the start of the inoculation. Clinically, it is well known that viral loads and $CD4^+$ cell counts fluctuate over the duration of infection and in accordance with disease progression in HIV-infected individuals. In fact, over a typical course of infection, the MOPs cover a wide range, potentially rising as high as 1000 virus particles/$CD4^+$ T cell or more (following the onset of AIDS) [12]. Our experimental conditions were designed to allow us to study HIV-1 infection efficiency over a range of clinically-relevant MOP values. Furthermore, because we utilized single-cycle virions (as described in Methods), we are able to obtain

an accurate measure of the overall infection efficiency of a single round of virus replication. As the fraction of infected cells is determined in accordance with the total number of virus particles in the inoculum our measure of the overall infection efficiency will also take into account any possible influences resulting from inefficient/unproductive virus binding.

As presented in Figure 10, we observed that the percentage of cells that became infected increases with higher MOP. And, per the continual increase seen through the regime of extremely high MOPs (i.e., 2900 and 5008), we projected that the infection efficiency would eventually reach a point of saturation – where basically every cell would become infected. However, because the infectivity of this single-cycle virus was estimated to be 0.02% - 0.03% (determined according to previously described protocol, see Methods), we expected to have an average 1 or more "infectious" virus particles per cell at MOP values greater than or equal to 5000. Hence, we were surprised to find that the efficiency of infection did not approach saturation within the range of MOPs tested. Although there was concordance with projected values in the low MOP regime, the actual fraction of cells that became infected was lower than anticipated at high MOP values (Figure 10B and C). In fact, as listed in Table 4, there is a large discrepancy in the projected proportion of infected cells, based on the infectivity calculated at low virus concentrations (MOP=12), and the experimentally-determined fraction of cells that became infected. It is unlikely that this unexpectedly low proportion of infected cells is attributable to limitations

in the experimental method and/or substrate availability because we observe a continual increase in the fraction of cells infected as a function of MOP overall, which we confirmed by additional, repetitive experiments. Instead, this discrepancy suggested that there were other factors influencing virion infectivity, which may become more prominent in the higher range of MOP values. Therefore, we determined the overall infection efficiency of each regime separately. A linear fit of the data (Figure 10B) revealed that the overall efficiency of HIV-1 infection is approximately 0.023% for MOP≤1000. The corresponding infection efficiency under high MOP conditions (i.e., MOP≥1000) is estimated to be 0.009% (Figure 10C). Our data demonstrate that infection was more efficient at low MOP. Additional studies were warranted to characterize this MOP dependence.

Figure 10 Fraction of Cells Infected and Overall Infection Efficiency



Panel (A) - Data demonstrates that increasing fractions of infected cells (as determined by the TZM-bl indicator assay) are observed at higher MOP. Values represent the mean of 2 replicate samples ± SD. The overall infection efficiency was determined according to a linear fit of the fraction of infected cells as a function of MOP for MOP≤1000 (Panel (B)) and MOP≥1000 (Panel(C)). Experimental values from Panel (A) are plotted as filled circles, the blue line represents the expected values (per Table 4) and the red line corresponds to the linear fit. The relative infectivity can be determined from the slope of each linear fit, as shown. The data support a general trend of greater infectivity for MOP≤1000.

Table 4 Overall Infection Efficiency: Observed Fraction of Infected Cells vs. Expected Fraction of Infected Cells

MOP	Overall Infection Efficiency (Fraction of Cells Infected)	
	Expected	Observed
30	0.6%	0.4% ± 0.04%
61	1.7%	3.8% ± 0.5%
240	5.1%	8.1% ± 0.4%
1000	21.4%	21.5% ± 1.4%
2900	62.2%	35.6% ± 3.0%
5008	100%	61.8% ± 4.3%

Comparison of the projected fraction of cells to become infected (Expected column) and the experimentally measured fraction of cells to become infected (Observed Column) reveals that values are consistent at MOP≤1000. However, there are large discrepancies at high MOP values. Expected values were projected according to the calculated infectivity (infectious titer normalized to p24) at an MOP of 12. Observed values represent the mean ± SE of 2 repeat samples.

Limited Efficiency of HIV Infection

While rapid dissociation has been previously suspected to play a role in masking the inherent infectivity of HIV-1 [36], the impact of these quick interactions is not expected to change with increasing MOPs. Therefore, we began to investigate the role of host cell binding interactions underlying our determination of the infection efficiency for HIV-1. Specifically we sought to determine the infection efficiency as: the relative ratio between the proportion of cells with virions associate (i.e., bound/internalized) immediately following inoculation and the proportion of cells that become infected. This calculation represents an alternate method for estimating the infection efficiency as a function of the extent of virion association with host cells. By comparing the infection efficiency following virion association, to the overall infection efficiency, we can elucidate the significance of limitations posed by the engaging a host cell.

To conduct this analysis, we utilized single-cycle HIV-1 virions engineered (as described in Methods) to carry free molecules of enhanced green fluorescent proteins (EGFPs). As cells with virions bound/internalized presented fluorescence values greater than those of cell-only controls (Figure 11A), we were able to quantitate the proportion of cells with virions associated by flow cytometry analysis of the measured EGFP fluorescence. Because cells may naturally express some level of autofluorescence (which we found to be well described by a log-normal distribution), we were careful to account for this background fluorescence in our analyses. With this in mind, we modeled the

fluorescence intensity profiles of inoculated cells as the sum of two log-normal distributions – where the first log normal distribution represents the population of cells without virus (with a mean corresponding to the fit for the cell-only control) and the second log-normal represents cells with virions bound/internalized, which would display a higher mean fluorescence. Representative fits for various MOPs are shown in Figure 11B and the diminishing proportion of cells without virus coinciding with the increasing MOP should be noted.

The fraction of the cells with virus associated was then determined by comparing the relative contribution from each distribution in accordance with the following expression:

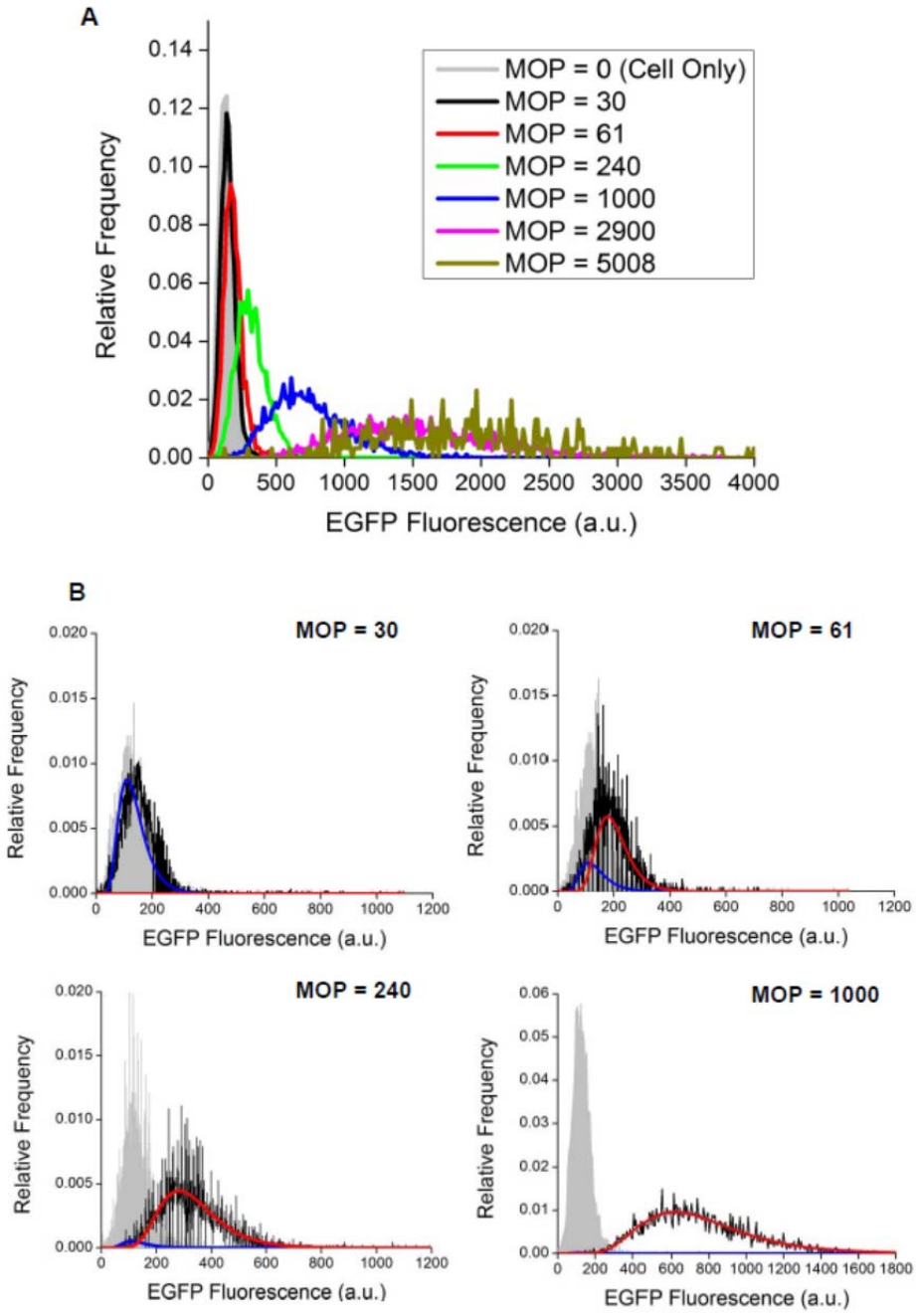
$$a_i \, LogN_1 + (1 - a_i) LogN_2$$

We determined the reliability of this deconvolution according to the confidence intervals of the a_i fit parameter. Hence, the extent of virion-cell interactions were measured over a range of MOP values (Figure 12). With increasing MOP, we found that greater fractions of cells had virions bound/internalized, confirming that, as expected, all cells had associated virions at high MOP.

Normalizing the fraction of cells that became infected, as quantitated by replicate samples that were stained for β-galactosidase activity after 2 days of incubation (Figure 10) according to the proportion of cells with associated virions immediately following inoculation (Figure 12), we determined the relative infection efficiency (shown as an overlain curve in Figure 12). Under these

conditions, we again observed that the infection efficiency continued to rise with increasing MOP. However, we did note any significant impact on the experimentally-determined infection efficiency, which still differed a great deal from the projected values at high MOP (Table 5). In fact, this observed infection efficiency continues to demonstrate that, even under conditions where there are large numbers of virions per cell, every cell will not actually become infected.

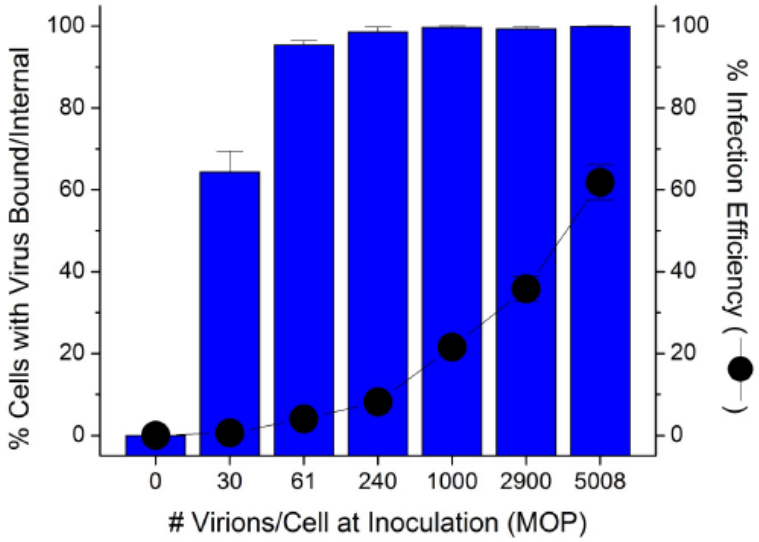
Figure 11 Representative Flow Cytometry Data



Our data demonstrate that flow cytometry can distinguish the EGFP fluorescence of cells that have associated virions. As shown in Panel (A), as the MOP increases, greater EGFP fluorescence values are observed vs. the cell only comparator (shown in gray). Panel (B) demonstrates how the

EGFP fluorescence could be modeled according to the sum of two log-normal distributions so that the relative contributions from the cell only population (blue line) and the cells with virions (red line) can be quantitated from the initial distribution of EGFP fluorescence (shown in black). The increase in EGFP fluorescence resulting from the presence of cell-associated virions can again be seen by comparing the sample distribution (in black) to a cell only comparator (shown in gray).

Figure 12 Infection Efficiency from Fraction of Cells with Associated Virions



As determined by log-normal fitting of the EGFP fluorescence measured by flow cytometry, the blue columns represent the fraction of cells that were determined to have associated virions (either bound or internalized). Increasing fractions of cells with viruses are observed with greater MOP values. The infection efficiency following virion attachment (shown in the black curve) was calculated as the ratio of the fraction of cells that became infected (Figure 10) to the fraction of cells with virions associated (blue bars). The infection efficiency increases with MOP, but does not reach 100% over this range of conditions. Displayed values were determined by log-normal fitting of the EGFP fluorescence measured by flow cytometry of at least 10^3 cells ± SD (calculated from confidence interval of log-normal fit parameter, a_i).

Table 5 Infection Efficiency Relative to Proportion of Cells with Virus Interactions

MOP	Relative Infection Efficiency (*normalized to virion-cell interactions*)	
	Expected	Observed
30	0.9%	0.6% ± 0.09%
61	1.8%	4.0% ± 0.5%
240	5.2%	8.2% ± 0.4%
1000	21.5%	21.6% ± 1.4%
2900	62.6%	35.8% ± 3.0%
5008	100%	61.9% ± 4.3%

Comparison of the projected fraction of cells to become infected (Expected column) and the experimentally measured fraction of cells to become infected relative to the proportion of cells with associated virions (Observed Column) demonstrates that MOP influences infection efficiency. Expected values were projected according to the calculated infectivity (infectious titer normalized to p24) at an MOP of 12. Observed values represent the mean ± SE of 2 repeat samples.

HIV Associates with Cells in a Proportionally-Consistent Manner

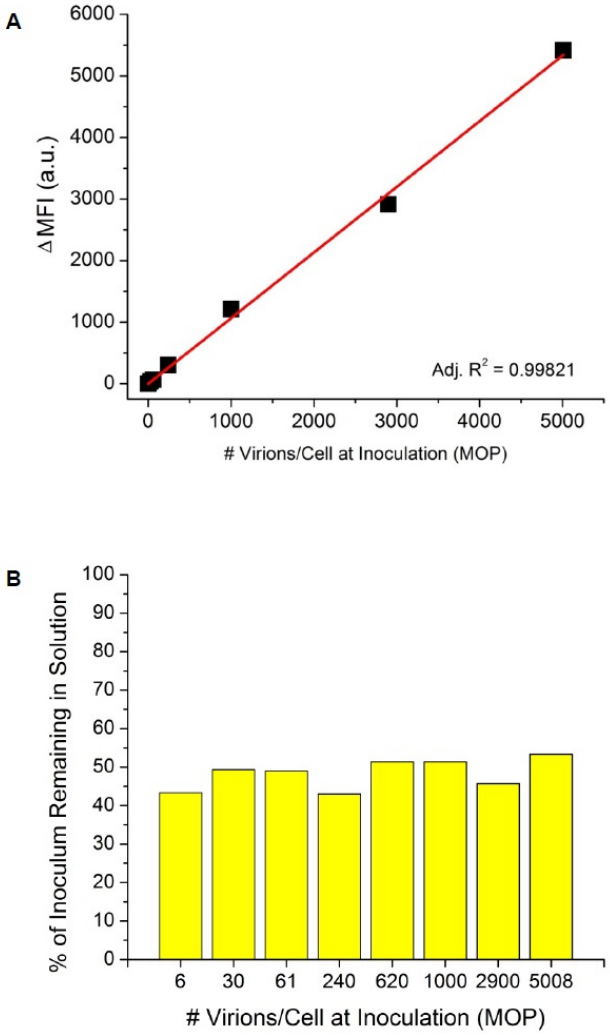
In considering possible explanations for lower infection efficiency at high MOP, we pondered the likelihood that every virus particle associates with a cell. Although our flow cytometry analysis demonstrated that, at high MOP values, all cells have virions bound, it could be that only a few virions were actually binding to each cell. So we re-examined our flow cytometry data to compare the relative increase (vs. cell-only controls) in the mean fluorescence intensities as a readout of the degree to which virions associated with cells. The linear increase in EGFP fluorescence (Figure 13A) as a function of MOP is consistent

with the presence of more virion particles at higher MOP values. This again suggests that the ability to associate with a host cell is not a significant factor in our observed limit to HIV-1 infection efficiency. We also confirmed this observation using p24 ELISA to quantitate the number of virions that remained in solution after our 2hr inoculation. Over the range of MOP values, we consistently found that nearly half (statistical mean 49.1 ± 8.9%) of the original inoculum remained in solution (Figure 13B). The accordance in this measurement of the fraction of the inoculum remaining in solution across a range of MOP conditions indicates that the MOP-dependent infection efficiency is not significantly impacted by a virion's ability to generally associate with a host cell. Hence, this consistency validates the rationality of the expected infection efficiencies (Table 4 and Table 5) because they are projected from observations under the same experimental conditions and, as such, are inherently normalized by the fraction of the inoculum associated with cells within 2 hours.

As our studies did include DEAE dextran, however, we also needed to rule out the possibility that our observations might be influenced by the presence of this polycation. A comparison of the extent of virus binding in the presence and absence of dextran (Figure 14) confirmed that the efficiency of virus and cell interactions was not significantly impacted, which is consistent with previous reports that DEAE dextran does not influence initial HIV-1 attachment onto cells [36].

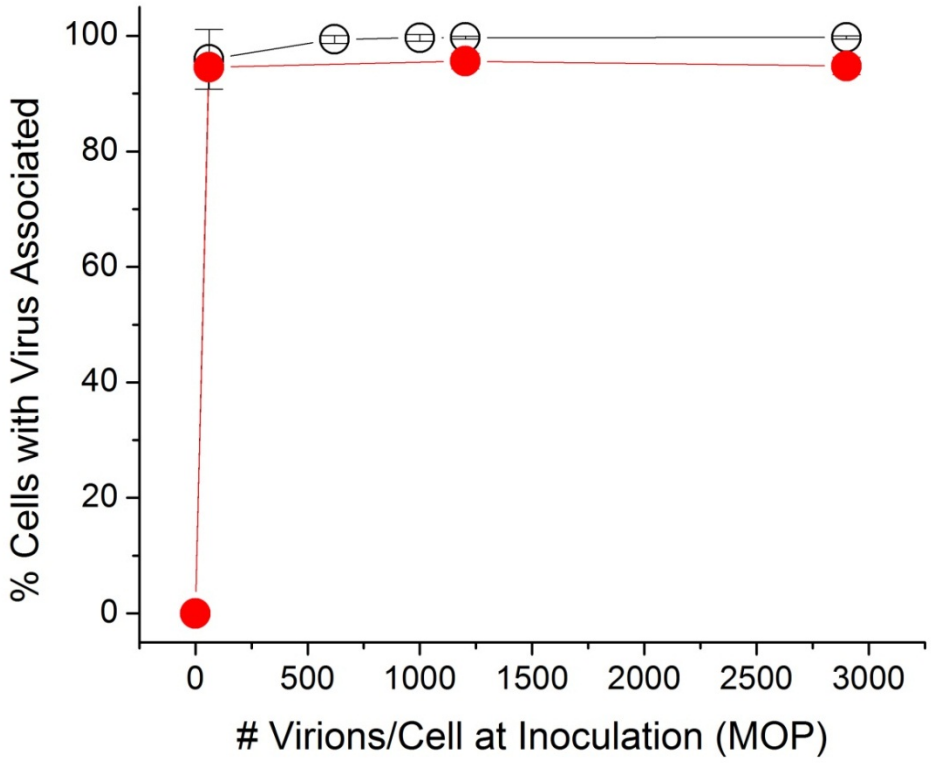
Thus, under our experimental conditions, which involve constant rocking; and therefore, could potentially minimize the distance between virus particles and target cells, HIV-1 appears to associate with host cells in a proportionally-consistent manner. Furthermore, although commonly modeled as independent events, our data suggests that HIV-1 interactions with a target cell are in fact, influenced to some extent by the presence of other virus particles.

Figure 13 Consistent Proportions of Virion Inoculum Associate with Cells



Panel (A) presents the increase in the mean fluorescence intensity (ΔMFI) of cells inoculated with EGFP-tagged virions vs. cell-only controls as a function of MOP. The linear fit (for which the Adjusted R^2 value is listed in the figure) is consistent with the presence of more virion particles at higher MOP values. Data represent analysis of at least 10^3 cells. The mean p24 ELISA data from 2 repeat samples in Panel (B) indicates that the proportion of virus remaining in solution (relative to the original inoculum) is independent of MOP. Therefore, the ability to generally associate with a host cell does not appear to be a significant factor in the observed MOP-dependent limit to HIV-1 infection because, over the range of MOPs, the proportion of the inoculum associating with cells is consistent.

Figure 14 Presence of DEAE Dextran Does Not Significantly Impact the Extent of Cells with Associated Virions



As quantitated via flow cytometry, the fraction of cells with associated HIV-1 in the presence of 20 mg/ml DEAE dextran (open, black circles) is comparable to that cells inoculated in the absence of DEAE dextran (filled, red circles). Displayed values were determined by log-normal fitting of the EGFP fluorescence measured by flow cytometry of at least 10^2 cells ± SD (calculated from confidence interval of log-normal fit parameter, a_i). The data support the hypothesis that, under our experimental conditions, DEAE-dextran is not influencing the extent of cells with associated virions.

Discussion

The infectivity of cell-free HIV-1 is consistently reported to be less than 0.1% but there is not a clear understanding of why HIV-1 possesses such low infectivity.

In order to gain more knowledge about the factors influencing HIV-1 infectivity, we first sought to quantitate the overall efficiency of infection. We were able to quantitate the overall infection efficiency by determining the fraction of cells that became infected following inoculation with a known amount of HIV-1 (Figure 10A). In fact, by varying the ratio between the number of HIV-1 particles (determined by p24 ELISA) and the number of potential $CD4^+$ target cells, we quantitated the proportion of infected cells over a range of clinically-relevant multiplicity of particle (MOP) conditions. The linear slope in the low MOP regime (Figure 10B) coincided with an overall infection efficiency of approximately, 0.023%. However, the infection efficiency at high MOP (Figure 10C), was lower - indicating that virion infectivity can be influenced by factors that may become more prominent at high MOP values.

The low infectivity of retroviruses like HIV-1 has long been purported to be associated with the presence of defective virions [25], but there is a great deal of evidence suggesting that one of the primary limitations in the establishment of a productive HIV-1 infection is engaging and/or entering a target cell. We hypothesized that, if the apparently low infectivity is a result of the inefficient manner by which virions find suitable targets, HIV-1 could actually be more infectious than reported. Thus, we began to explore the molecular mechanisms underlying the virus' low infectivity in order to elucidate the rate-limiting step in the HIV-1 infection process. HIV-1 infection is a multi-step process and because we predicted that target cell engagement to have major influence on the

efficiency of infection, we calculated the overall efficiency of HIV-1 infection (Table 4) as well as the efficiency of infection following attachment (Table 5) in order to demonstrate whether associating with a host cell poses a significant limitation. As described herein, we proceeded to quantitate virus and cell interactions through flow cytometry analyses of fluorescently-tagged HIV-1. Our results demonstrated that HIV-1 is capable of interacting with cells in an efficient manner, such that the majority of cells still have detectable amount of virions associated with them at the conclusion of the 2 hour inoculation (Figure 11). In fact, there was a linear increase in the EGFP fluorescence as a function of MOP, confirming that more fluorescently-tagged virions were interacted with cells at higher MOP values (Figure 13). A comparison of the extent of virus binding in the presence and absence of dextran (Figure 14) confirmed that the efficiency of virus and cell interactions was not significantly impacted, which ruled out the possibility that our observations were influenced by the presence of this polycation. Together, these findings suggest that the ability to attach to a potential target cell does not represent a significant limitation to HIV-1 infection.

By using TZM-bl cells, we were able to conduct a side-by-side comparison of virion binding and the resulting infection in order to determine the efficiency of HIV-1 infection. In reporting our findings, however, we realize that the observed nature of virion and cell interactions could possibly be influenced by cell-specific properties – e.g., receptor density or distribution, composition of the membrane, presence of host proteins, etc. Ultimately, to elucidate any cell-line

dependence, similar studies will need to be conducted in other cell types. With this in mind, one limitation to the interpretation of the results described herein pertains to the fact that TZM-bl cells are not natural targets of HIV-1 infection. Nevertheless, TZM-bl cells are widely used as targets to study HIV-1 infection.

Conclusion

We have quantitated the infection efficiency of HIV-1 and confirmed that, even under conditions where there are large numbers of virions per cell, such interactions will not always result in infection. On the contrary, our data show that the productivity of virion-cell interactions is significantly limited by high MOP conditions. These results suggest that the efficiency of HIV-1 infection changes according to HIV/AIDS disease progression, as viral loads increase and CD4+ T cells are depleted. Thus, this finding offers unique perspective for understanding HIV-1 infection efficiency and highlights considerations of clinical import.

CHAPTER 4

ENVELOPE-DEPENDENCE OF HIV-1 BINDING AND INFECTION

Abstract

HIV-1 particles are estimated to possess an average of only 14 envelope spike proteins [43] and by investigating the molecular mechanisms underlying virion attachment, we report evidence that the lack of binding to host cells via receptor-specific interactions poses a significant limitation in the virus' ability to establish infection. Specifically, utilizing fluorescent techniques we have gathered data showing that receptor-independent, virus-cell interactions correlate with lysosomal degradation of virions, indicating the fate of virions that are nonspecifically endocytosed.

Introduction

Productive human immunodeficiency virus type 1 (HIV-1) infection is believed to be initiated via interactions with cell surface receptors such as CD4, which is the primary receptor recognized by the virus, in conjunction with

coexpressed chemokine co-receptors, CXCR4 and/or CCR5, which are necessary to facilitate membrane fusion [17] [18] [19] [20] [21]. Although the interactions between HIV-1 envelope glycoproteins and target cell receptors have been studied extensively, there are still several questions pertaining to the early events in HIV infection and how efficiently these receptor binding events result in productive infections. Indeed, it has been reported that the virus particles rapidly dissociate from target cells in culture, which can significantly influence the observed infectivity [36]. However, the molecular mechanisms underlying these transient interactions and/or the observed low infectivity have yet to be well-documented.

Previously, we found that a high multiplicity of virus particles (MOP) limits the apparent efficiency of HIV-1 infection and demonstrated that the infectivity of HIV-1 could be enhanced with more envelope expression. As these findings are consistent with the report that HIV-1 only expresses an average of 14 envelope spike proteins [43], we have sought to closely investigate the envelope-dependence of HIV-1 binding and infection.

Materials and Methods

Production of single-cycle HIV-1 virions.

Virions were produced by transfecting HEK 293T/17 cells (ATCC, Manassas, VA) as previously described [54]. Briefly, 293T were cultured at 37^{o}C with 5% CO_2 in DMEM supplemented with 10% FBS (HyClone Laboratories, Logan, UT) and transfected at the fifth passage. 10^6 293T cells were seeded overnight in a T-75 flask with 10ml of culture media. Using the TransIT LT-1 transfection reagent (MirusBio, Madison, WI), HIV virions carrying free EGFP were generated by transfection with 4.0μg pNL4-3E- plasmid, 4.0μg pNL4-3E-MA-EGFP-CA plasmid and 0.8μg pcDNA3.1REC. After 6 hours of incubation at 37^{o}C, the culture media (with transfection reagents) was removed and replaced with 16ml of fresh culture media. The 37^{o}C incubation continued for an additional 12 hour period. At 18 hours post transfection, the culture media, containing EGFP-labeled single-cycle virions was collected and filtered through a 0.45-mm syringe filter (Millex-HV PVDF, Millipore). The filtrate was then aliquoted on ice, flash-frozen in a dry ice/ ethanol bath and stored in a -80^{o}C freezer. Subsequent analysis using p24 ELISA (HIV-1 p24 Antigen Capture Kit, Advanced Bioscience Laboratories, Rockville, MD) was conducted to determine the number of virus particles in a specified volume, assuming 2,500 molecules of p24 per virion.

Infection assay in TZM-bl cell line.

TZM-bl cells (cat#8129, NIH AIDS Research and Reference Reagent Program) were cultured at 37^{o}C with 5% CO_2 in DMEM supplemented with 10% FBS (HyClone Laboratories, Logan, UT). Prior to tenth passage, TZM-bl cells were trypsinized, counted, sedimented by centrifugation at 1,000g for 5 minutes and resuspended in DEAE dextran (final concentration 20 mg/ml) and/or DMEM with 10% FBS. EGFP-labeled HIV-1 particles were added to aliquots of TZM-bl cells in 1.5ul Eppendorf tubes up to a total volume representative of each MOP condition. The virion and TZM-bl cell mixture was incubated in suspension on a fixed-speed nutator (Fisher Scientific) at 37^{o}C for 2hrs. Following the 2hr inoculation, samples were immediately placed on ice to halt the infection process. Samples were kept on ice/at 4^{o}C and washed (i.e., centrifugation at 1,000g for 5 minutes followed by resuspension) with DMEM containing 10% FBS three times to remove any unbound virions. However, the supernatant from the first wash was collected and flash frozen for later analysis via p24 Antigen Capture Kit (Advanced Bioscience Laboratories, Rockville, MD) in accordance with the manufacturer's instructions. Following the washing step, 2 aliquots (containing approximately 8x10^{4} cells) from the cell-virion samples were seeded in 1ml of DMEM with 10% FBS in 24-well plates. The samples were further incubated at 37^{o}C with 5% CO_2 for 2 days. After 2 days of incubation, cells were fixed in 2% gluteraldehyde at room temperature for five minutes. Cells were then washed three times with PBS, and stained for 50 min at 37^{o}C using cell staining solution

provided in the beta-galactosidase staining kit (Mirus Bio, Madison, WI). Cells were washed three times with milliQ water and the number of blue cells in each well was counted using a 10X objective on a Nikon TS100-F inverted microscope.

Flow cytometry and confocal microscopy analyses

Following inoculation in suspension and after aliquots were taken for beta-galactosidase staining, the remaining TZM-bl cells were resuspended in 4% paraformaldehyde for 10 minutes at room temperature. After fixation, the samples were washed (i.e., centrifugation at 1,000g for 5 minutes followed by resuspension) in PBS two times. The samples were kept at 4^{o}C until subsequent flow cytometry analysis (using BD FACSCanto II flow cytometer). The resulting measurements of fluorescence intensity were analyzed via original Matlab code, designed to fit data to the sum of two log-normal distributions.

For confocal analyses: After PBS washing step, 100-200ul aliquots of were seeded on PLL-coated coverslips in 24 well plates and stored at 4^{o}C preceding preparation for confocal microscopy analysis. Following 4^{o}C storage for overnight (at a minimum), cell membranes were stained with 200ul of Cholera Toxin B-Alexa 555 conjugate (Item# C2284, Invitrogen) for 10 minutes at room temperature. After staining, coverslips were washed in PBS and mounted face down in 3ul of VectaShield. Confocal images were gathered using 250nm step size and 100X oil objective on Olympus FluoView 500 Laser Scanning Confocal Microscope. Confocal image stacks were analyzed via custom Matlab code to

detect cell membrane boundaries and unique EGFP virions in accordance with the intensity of the corresponding fluorescence signal.

For lysosome colocalization: Samples were prepared according to protocol for confocal analysis, except that 50nM concentration of LysoTracker Red (Item# L7528, Invitrogen) was included in the inoculum. Control experiments were conducted to confirm that the presence of LysoTracker did not influence virus titer (data not shown). Cell membrane staining was omitted but confocal images were gathered as described above. The extent of lysosomal colocalization was determined via custom Matlab code that localized EGFP-tagged virions and LysoTracker Red-stained compartments according to the intensity of the fluorescence signals. The percentage of EGFP-fluorescence that overlapped with LysoTracker Red relative to the total amount of EGFP fluorescence, M1, was determined for each z-stack, independently. Colocalization was then computed for each set of images as the average of M1 values for z-stacks where an appreciable amount of EGFP-labelled virus could be detected.

Receptor inhibition studies

Studies utilizing sCD4 (cat#7356, NIH AIDS Research and Reference Reagent Program) and AMD3100 (Item #A5602, Sigma-Aldrich) as CD4 and CXCR4 antagonists, respectively, were conducted as described above with the following steps preceding the addition of EGFP-labeled HIV-1 particles to aliquoted TZM-bl cells for inoculation: AMD3100 was added to aliquoted TZM-

bl cells to a final concentration of 1ug/ml. Treated TZM-bl cells were then incubated in suspension on a nutator at 37^{o}C for 1hr. Meanwhile, sCD4 was added to aliquots of EGFP HIV-1 particles (in 1.5ml Eppendorf tube or 15ml conical tube, if experiment required larger volumes) to a final concentration of 0.015 ug/ul. The virus-sCD4 mixture was incubated on a nutator at 37^{o}C for 1hr. Following pretreatment with AMD3100, TZM-bl cells were sedimented by centrifugation at 1,000g for 5 minutes and resuspended in DEAE dextran (final concentration 20 mg/ml) and/or DMEM with 10% FBS. Appropriate volumes of the sCD4 - HIV-1 particle mixture and/or DMEM with 10% FBS were added to aliquots of TZM-bl cells in 1.5ul Eppendorf tubes up to a total volume representative of each MOP condition.

Results

Herein we demonstrate that the majority of all virus-cell interactions are due to nonspecific, receptor- and envelope-independent binding. This nonspecific binding was found to significantly hinder the efficiency of HIV-1 infection. Furthermore, our data show that the extent of these non-specific interactions increases in accordance with the MOP – providing a mechanistic explanation for the inefficiency of HIV-1 infection at high virus concentrations.

Non-specific Binding Accounts for the Majority of Virion-Cell Interactions

Given that HIV-1 particles are estimated to possess an average of only 14 envelope spike proteins [43], the likelihood to specifically engaging the required CD4 and chemokine receptors could be limited - giving rise more non-

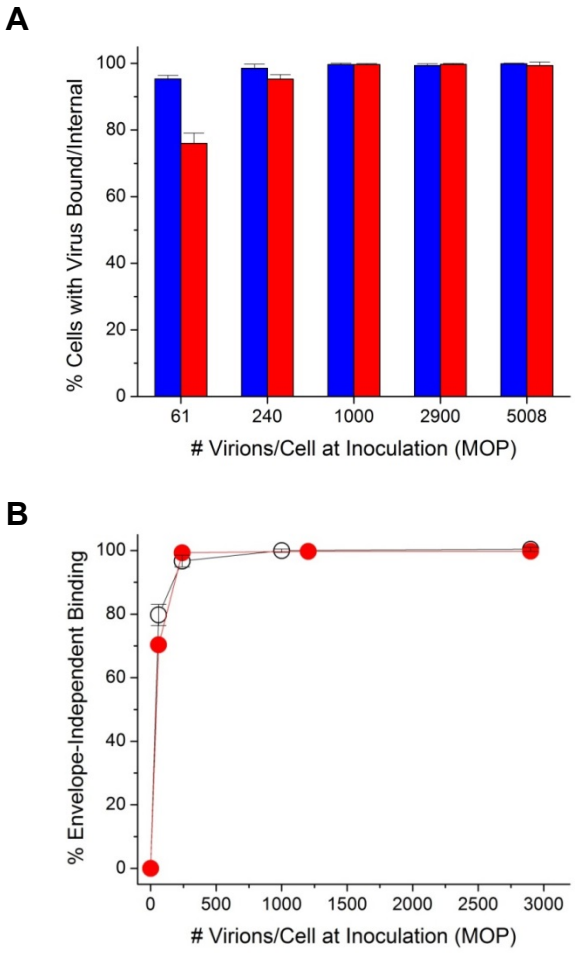
specific, receptor –independent binding/internalization. In fact, it seems very plausible that any such inefficient/non-productive virus-cell interactions could result in an “apparent” infection efficiency that is lower than expected - particularly at high MOP conditions where receptor could potential be occupied/obstructed by other virions, consistent with our previous findings. So we initiated a study to investigate the receptor-dependence of virion-cell interactions.

Indeed, we discovered that, in the presence of sCD4 and AMD3100 (to inhibit envelope-mediated interactions with both CD4 and CXCR4 receptors respectively), the extent of virion-cell interactions still continued to increase with MOP (Figure 15A), even though no infection events occurred (data not shown). In fact, at high MOP values, nearly all virion-cell interactions could be attributed to this receptor-independent, non-specific binding pathway. We confirmed this observation by conducting similar analyses with virus-like particles lacking HIV-1 envelope proteins. In this case, we observed that the extent of virion-cell interactions were comparable to the non-specific binding observed in our experiments with single-cycle HIV-1 (see Figure 15B for comparison). Thus, our data support the notion that, at least under our experimental conditions, non-specific binding accounts for the majority of all virion-cell interactions, the exact proportion of which increases with MOP.

Given that, at least in our experimental conditions, non-specific virion-cell interactions appeared to represent the majority of all virion-cell interactions, we

decided to study the fate of those virions that interact with cells in a receptor-independent manner. It has previously been shown that HeLa-derived cells can internalize NL4-3ΔEnv, virus-like-particles non-specifically via endocytosis [68]. Furthermore, the same study indicated that up to 90% of intracellular NL4-3 was internalized via endocytosis, with a large majority resulting in lysosomal degradation. Therefore, we conducted similar analyses to characterize non-specific, receptor-independent virion-cell interactions under our experimental conditions. First, to elucidate the proportion of virions that are surface-bound vs. internalized, we used confocal imaging to analyze the distribution of virions relative to the cell membrane (see representative image in Figure 16A). We utilized a custom Matlab code to detect the position of EGFP-labeled virions according to the intensity of the fluorescence signal and pinpoint the virions' location relative to the boundaries of the cell membrane. Our analyses revealed that, consistent with previous reports, an average of approximately 86% of cell-associated virions are internalized (Figure 16B, blue bars). In the presence of inhibitory concentrations of sCD4 and AMD3100, we observed that the proportion of cell-associated virions remains relatively unchanged (Figure 16B, red bars). Interestingly, these relative intracellular distributions were independent of MOP. This result, which is consistent with our flow cytometry data, reveals that the majority of virus-cell interactions are mediated in a receptor-independent fashion that results in some form of endocytosis.

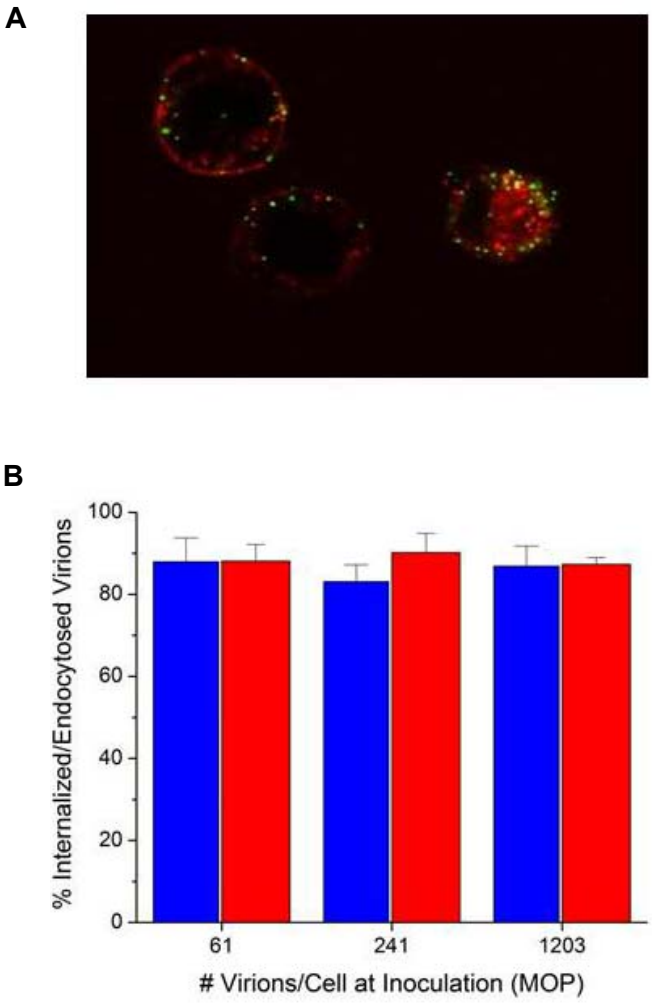
Figure 15 Non-specific Binding Accounts for the Majority of Virion-Cell Interactions



The extent of HIV receptor-independent cell interactions were quantitated via flow cytometry. Analysis of the fraction of cells with associated virions in the presence of CD4 and CXCR4 antagonists (sCD4 and AMD3100, respectively) is shown in Panel (A). The fraction of cells with associated virions in the absence (blue bars) or presence of both inhibitors (red bars) are shown for direct comparison. Under our experimental conditions, the data suggest that non-specific binding/internalization may account for a large portion (at least 86%) of all HIV-1 interactions with a target cell. Displayed values were determined by log-normal fitting (as deduced from the sum of 2 log-normal distributions) of the EGFP fluorescence measured by flow cytometric measurements of at least 10^2 cells ± SD (calculated from confidence interval of log-normal fit parameter,

a_i). As shown in Panel (B), the extent of virion-cell interactions for single-cycle HIV-1 (open, black circles) is comparable to that of virus-like particles lacking HIV-1 envelope proteins (filled, red circles). Thus, the data provide further evidence that non-specific binding accounts for the majority of all virion-cell interactions under our experimental conditions.

Figure 16 Distribution of Cell-Associated Virions



Three-dimensional, confocal analysis was utilized to study the distribution of cell-associated virions. A representative confocal image at MOP of 1203 is shown in Panel (A). EGFP-labeled virions (green particles) can be seen relative to the cell membrane, which has been stained red using cholera Toxin B-Alexa 555 conjugate. As determined by confocal image analysis, an average of approximately 86% of cell-associated virions are internalized (blue bars, Panel (B)). In the presence of inhibitory concentrations of sCD4 and AMD3100, the proportion of cell-associated virions remains relatively unchanged (red bars). The relative intracellular distributions were consistent across a wide range of MOP values. Data in

Panel (B) are representative of the mean percentage ± SE for at least 3 replicate images at each MOP.

Non-specific Binding is Associated with Virion Degradation

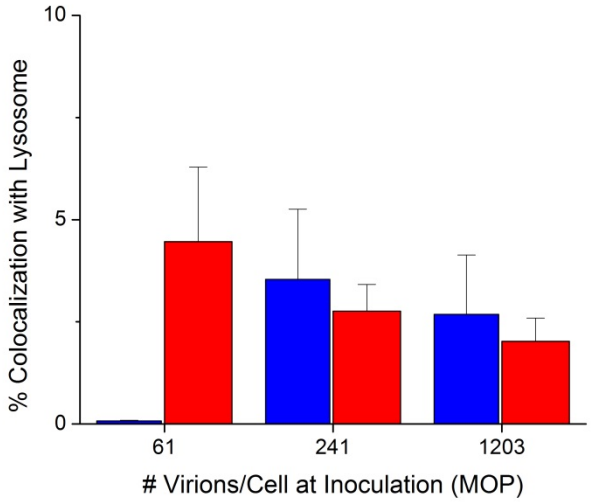
Since there were no productive infections for experiments conducted in the presence of sCD4 and AMD3100, we sought to examine the fate of internalized virions in the presence and absence of receptor antagonists to determine whether virions that were internalized via receptor-independent mechanisms would be degraded in the lysosomes. We anticipated detecting an increase in virion colocalization with lysosomal compartments as a result of sCD4 and AMD3100 pre-treatment. And so, to test this hypothesis, we labeled late endosomes/lysosomes with the membrane-penetrating stain, LysoTracker Red. As shown in Figure 17, we were only able to detect a small percentage of virions colocalizing to these acidic compartments overall. This was expected given that substantial lysosomal degradation had probably occurred prior to the end of our 2-hour inoculation period. Nevertheless, we did observe distinct behaviors between the low MOP and high MOP regimes. While we originally observed very little colocalization at an MOP of 61, the addition of CD4 and CXCR4 antagonists (Figure 17, red bars) led to an increase in the degree of colocalization. At higher MOP values, however, the addition of receptor antagonists did not result in a significant change – as there was already a detectable amount of colocalization in the absence of such inhibitors. Thus, collectively our data suggest that, for low MOP conditions, inducing non-specific, receptor-

independent endocytosis results in more virion degradation. Interestingly, at high MOP, the extent of virion degradation is greater (vs. low MOP in the absence of receptor antagonists) and remains unaffected by the inhibition of receptor-mediated interactions. This observation is consistent with our other data and suggests that there is already a good deal of virion degradation at high MOP conditions, as a result of the predominant receptor-independent interactions with the cell.

Based on these observations, we sought to quantitate the proportion of "specific", receptor-dependent binding as the relative difference between the fraction of cells with virions associated in the absence of inhibitors and the fraction of cells with virions associated in the presence of receptor inhibitors. Recalculating the infection efficiency based on this fraction of cells with virions specifically bound, could therefore, result in a quantitative measure of the true infection efficiency of HIV-1. Under our experimental conditions, however, the extent of specific binding could only be reliably quantitated for MOP values in a narrow range (i.e., 30<MOP<240). In these experiments (Figure 15, Figure 16, and Figure 17), the uncertainty in the EGFP fluorescence fit parameters suggested that the extent of binding at MOP<30, may be too low to distinguish – which is expected given that the change in mean fluorescence intensity is likely to be small. On the contrary, at high MOP values, nearly all cells have virions bound – even in the presence of receptor antagonists, making it difficult to distinguish receptor-specific binding. In fact, only at MOP of 61, was the extent of

virion-cell interactions in the presence of sCD4 and AMD3100 determined to potentially be significantly different from the extent of virion-cell interactions in the absence of these CD4 and CXCR4 antagonists. Therefore, based on our experimental observations at an MOP of 61, we determined that HIV-1 infects cells via receptor-mediated binding with an “inherent” efficiency of approximately 19% - a number that is comparable to the 13% infection efficiency previously reported for VSV-G pseudotyped HIV and 5 times greater than the observed “apparent” infection efficiency from prior studies. Thus, our results consistently demonstrate that the inability to initiate and/or sustain receptor-specific interactions with a target cell significantly hinders the efficiency of HIV-1 infection.

Figure 17 Virion Colocalization with Lysosomes



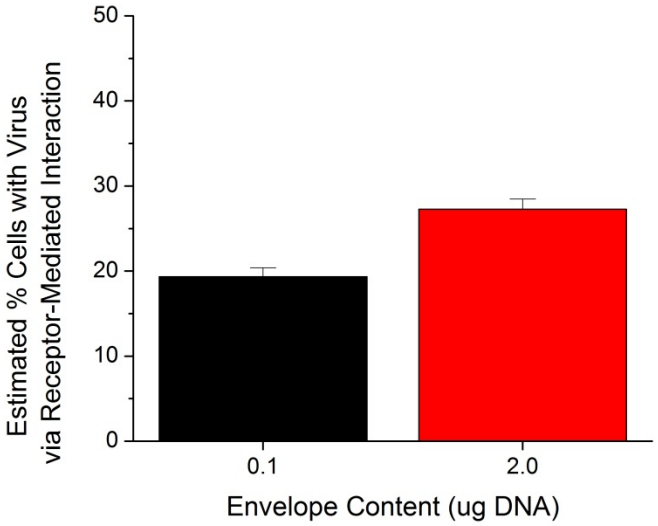
The extent of virion colocalization with late endosomes/lysosomes was determined by confocal imaging in the presence and absence of sCD4 and CXCR4 inhibitors (red bars and blue bars, respectively). Data are representative of the mean percentage ± SE for at least 3 replicate images at each MOP.

Envelope Expression Correlates with Specific Binding

Our data consistently point toward the importance of specific binding between HIV-1 envelope glycoproteins and target cell receptors and since we previously demonstrated that the infectivity of HIV-1 could be enhanced with more envelope expression, we sought to quantitate the extent of envelope-dependent, specific binding among virions engineered to express a higher density of envelope spike proteins. Specifically, using flow cytometry, we quantitated the relative difference between the extent of virion-cell interactions in the absence of sCD4 and AMD3100 and the fraction of cells interacting with virions in the presence of

these receptor antagonists. As shown in Figure 18, the results indicate that the increase in envelope expression also leads to a greater fraction of specific binding. When considered in light of our prior observations and the correlation between greater infectivity and more envelope protein expression, these data provide additional evidence of how envelope-mediated mechanisms influence HIV-1 binding, internalization, and overall infectivity.

Figure 18 Receptor-Specific Binding Correlates with Envelope Expression



Data demonstrate the extent of receptor-dependent, specific binding, quantitated as the relative difference between the fraction of cells interacting with virions in the absence of inhibitors and the fraction of cells interacting with virions in the presence of receptor inhibitors for virus with variable amount of envelope at MOP=61. The fractions of cells interacting with virus were determined by log-normal fitting of the EGFP fluorescence measured by flow cytometry of at least 10^3 cells ± SD (as calculated from confidence interval of log-normal fit parameter, a_i, for a control sample). These data suggest that increased envelope expression results in a greater proportion of specific binding, which is consistent with our previous finding that infectivity increases with envelope expression.

Discussion

Although our initial studies of the limitations in the establishment of a productive HIV-1 infection did not reveal significant influence/inefficiency due to the inability of virions to interact/associate with cells, we pursued additional experiments in order to fully investigate whether binding might influence the efficiency of HIV-1 infection in manner that would be dependent on the effective virus concentration.

Previous reports have demonstrated that HIV-1 is capable of interacting with cells in a receptor-independent manner. Non-specific interactions mediated by lectins like DC-SIGN on dendritic cells [69] or adhesion molecules such as ICAM-1 [30] have been well-described. Indeed, the presence of heparan sulfate proteoglycans on HeLa-derived cells has also been noted to play a role in virion attachment [70]. Therefore, we decided to characterize the nature of virion-cell interactions at a molecular level in order to determine the receptor-dependence under our experimental conditions. We quantitated the extent of virion-cell attachment occurring in the presence of inhibitory concentrations of CD4 and CXCR4 antagonists (Figure 15A) and in comparison to virus-like-particles lacking envelope glycoproteins (Figure 15B). Consistent with previous reports, we found that a large majority of virion interactions could be attributed to non-specific, receptor-independent attachments. Interestingly, the extent of non-specific binding appeared to increase with MOP and nearly all virion-cell interactions at high MOP values could be characterized as non-specific. Recalculating the

efficiency of HIV-1 infection according to the extent of specific interactions (which could only be determined in the low MOP regime), revealed that the efficiency of infection following specific binding is about 19%. Hence, the results of our study confirm that the lack of binding to target cells in a receptor-specific manner significantly hinders HIV-1 infection and results in an infectivity that appears much lower than the virus' inherent infectious capability.

Our results also confirm previous reports that HIV-1 can be internalized via non-specific interactions. In our experiments, we observed that approximately 89% of cell-associated HIV-1 resulting from receptor-independent uptake was detected inside of the cell. Since, under our experimental conditions, which utilize single-cycle HIV-1 virions engineered (as described in Methods) to carry free molecules of enhanced green fluorescent proteins (EGFPs), we only expect to detect punctate EGFP fluorescence from intact virions. Therefore, we can reasonably conclude that most, if not all, of these non-specifically internalized virions were taken up via some form of endocytosis as they appear as intact virions. Indeed, our data align closely with the findings of Maréchal et al, who have previously reported that approximately 90% of virions entered HeLa-derived cells via non-specific vesicular uptake and that the majority of these internalized particles would be degraded in the lysosomes [68].

Furthermore, although commonly modeled as independent events, our data suggests that HIV-1 interactions with a target cell are in fact, influenced to some extent by the presence of other virus particles. Our work shows that the

extent of non-specific interactions between HIV-1 and target cells increases in accordance with the multiplicity of virus particles. As the presence of other virions could impact the accessibility of receptors, we believe that this finding provides a reasonable explanation for the differences we observed a high MOP. In fact, at high MOP values, we observed that non-specific binding accounts for nearly all virion-cell interactions (Figure 15A) and the prevalence of this non-specific binding coincides with a significant limitation in the observed infection efficiency (prior reports) as well as an increase in the amount of lysosomal targeting (Figure 17). Therefore, the results of this study demonstrate that the inability to specifically engage the appropriate receptors on a target cell could potentially be influenced by the presence of other virus particles.

Although the observed nature of virion and cell interactions could possibly be influenced by cell-specific properties, TZM-bl cells are widely used to study HIV-1 infection. And because this HeLa-derived cell line has been genetically engineered to expresses high levels of CD4, CXCR4, and CCR5, there is still some broader relevance to our findings. According to previous reports, TZM-bl cells are estimated to express an average of ~$3x10^5$ CD4 receptors and ~$2x10^4$ CXCR4 co-receptors [71] [72], corresponding to nearly 10 times more CD4 and 3 times more CXCR4 than $CD4^+$ T cells in peripheral blood mononuclear cells (PBMC) obtained from patients [73]. Therefore, our demonstration of the lack of receptor-dependent binding, even when more receptor targets are present, is

significant for providing insight into the molecular mechanisms that may influence virus binding and ultimately, the efficiency of the infection process.

Conclusion

We have determined that nonspecific, receptor-independent binding interactions impose a significant limitation to HIV-1 infection efficiency. Although HIV-1 is capable of infecting with approximately 19% efficiency via receptor-specific binding, this infection efficiency is masked by nonspecific interactions with host cells, which become more prevalent at high MOP. Our data show that these receptor-independent interactions correlate with lysosomal degradation of virions, indicating the fate of virions that are nonspecifically endocytosed. However, HIV-1 engineered to express a greater number of envelope spike proteins display more receptor-specific binding. Thus, we report that molecular binding mechanisms, which are significantly influenced by HIV-1 envelope expression, bear substantial impact on the apparently low infectivity of HIV-1.

CHAPTER 5

BIOWAIVERS FOR COMBINATION DRUG PRODUCTS: APPLYING A PROVISIONAL BCS CLASSIFICATION TO HIV ANTIRETROVIRAL PRODUCTS

Abstract

To motivate regulatory and scientific advancement with regard to requirements for bioequivalence, we have provisionally classified and assessed the biopharmaceutical properties of and 34 single-entity and 22 fixed-dose, co-formulated, combination oral drug products in late stage clinical development (Phase 2 or beyond) or post-marketing phase for indications to treat the Human Immunodeficiency Virus (HIV). For effective treatment against this deadly disease, HIV antiretroviral therapy combines the effect of multiple mechanisms of antiviral activity through co-administration of single-entity drug products or, when available, simpler administration of fixed-dose co-formulations. As there is a significant unmet medical need for access to antiretroviral therapy globally, efforts to enable simplified and/or abridged development pathways may substantially improve the availability of these essential medicines.

Current regulatory guidelines permit waivers of *in vivo* bioequivalence studies for immediate-release orally administered products comprised of drug substances that are highly soluble, according to the Biopharmaceutical Classification System (BCS). Our provisional assessment has revealed that 38% of the single-entity drug products and 36% of the co-formulated drug combinations are comprised of high solubility compounds and are, therefore, potentially eligible for BCS-based biowaivers under the existing regulatory framework. In theory, however, *in vitro* data could be used to justify biowaivers, regardless of BCS classification. Such a theoretical opportunity is especially relevant to the development of fixed-dose combinations of single-entity products that have an established safety and efficacy profile for co-administration, which is the case for many antiretroviral drug products because the otherwise required *in vivo* studies analyzing each active moiety are inherently more complex, time consuming, and expensive. Thus, this provisional assessment is intended to highlight opportunities to utilize scientifically-valid *in vitro* dissolution strategies to demonstrate bioequivalence between co-formulated drug products and the reference, single-entity products administered in combination. More broadly, as dissolution methodologies with *in vivo* relevancy are developed, *in vitro* assessments of bioequivalence may replace the complex *in vivo* studies currently required for drug combinations; and, as a result, lead to increased availability of more effective combination drug products, especially in developing

countries where such products can help address health care disparities and significant unmet medical need.

Introduction

Unique regulatory opportunities exist to reasonably enable formulation changes and refine or amend manufacturing processes for established drug products and to expedite the development of generic drug products by demonstrating bioequivalence in comparison to a reference product. For two drug products to be considered as bioequivalent they must be pharmaceutically equivalent (i.e., containing the same active pharmaceutical ingredient(s), or API, in the same dosage form and at the same strength for the same route of administration) or alternative (i.e., same therapeutic moiety as a different molecular entity or in a different dosage form or strength) and demonstrate comparable bioavailability [74]. Standard bioequivalence studies can be conducted in healthy volunteers by administering single doses of the test and reference drug products according to a two-treatment, crossover or a four-period, replicate crossover study design [74] [75]. In order to prove bioequivalence, statistical analyses should demonstrate that the critical pharmacokinetic parameters for: a) the extent of absorption, most often determined by the area under the plasma concentration-time curve (AUC) for the last measured concentration (AUC_T) and as extrapolated to infinity (AUC_{inf}), as well as b) the rate of absorption, as measured by the maximum drug concentration (C_{max}) of

API from the test drug product are all ≥ 80.00% and ≤ 125.00% of the corresponding parameters for the API from the reference drug product.

In some cases, alternate *in vivo* or *in vitro* methods may represent more reasonable and scientifically valid bioequivalence studies. The Biopharmaceutical Classification System (BCS), a method that categorizes drugs according to their aqueous solubility and intestinal permeability, is one scientifically valid basis for justifying alternate bioequivalence approaches [76]. Specifically, for some high-solubility drugs, where the *in vivo* dissolution is rapid relative to the gastric emptying time, regulatory authorities have determined that, when certain criteria are satisfied, *in vitro* dissolution studies can be used to demonstrate bioequivalence. The European Medicines Agency (EMA)'s Committee for Medicinal Products for Human Use (CHMP) scientific guidelines, for example, state that satisfactory *in vitro* data will be accepted in place of *in vivo* bioequivalence studies for immediate release, oral drug products where the drug substance exhibits high solubility and complete absorption (BCS Class I), if both the test and reference products demonstrate *in vitro* dissolution characteristics of more than 85% dissolution within 30 minutes (similarly rapid) or 15 minutes (very rapid) and inactive ingredients potentially affecting bioavailability are qualitatively and quantitatively the same [75]. Similarly, EMA also permits BCS-based biowaivers when the drug substance exhibits high solubility and limited absorption (BCS Class III) if the *in vitro* dissolution profiles of the test and reference products show very rapid dissolution and there are no

qualitative or quantitative differences in excipients that may affect bioavailability. The United States Food and Drug Administration (FDA) also allows BCS-based waivers from *in vivo* bioequivalence studies for immediate-release solid oral dosage forms containing BCS Class I drug substances, if the excipients do not significantly affect absorption. Specifically, the FDA has indicated that biowaivers for the demonstration of *in vivo* bioavailability may be justified for a BCS Class I drug substance in an immediate release oral dosage form that exhibits rapid *in vitro* dissolution resulting in at least 85% of the drug substance dissolving within 30 minutes using the recommended test [77]. The FDA guidance on biowaivers for BCS Class I drugs has been established since 2000 and it was not until fifteen years later that the FDA issued new draft guidance extending the option for biowaivers to BCS Class III drugs [78]. The FDA and EMA note several additional factors that must be considered for determining the suitability of a BCS-based biowaiver and both agencies exclude drugs with narrow therapeutic ranges as well as drug products absorbed in the oral cavity, such as sublingual or buccal formulations.

For fixed dose combinations, when multiple active ingredients are present together in the same dosage form, demonstrating bioequivalence becomes more complex, as each API must demonstrate comparable bioavailability, and the scientific rationale for utilizing alternate and/or *in vitro* dissolution methods is especially important. Theoretically, a well-designed, oral fixed-dose combination drug product, formulated with individual drugs that exhibit

linear and non-interacting ADME, in the absence of excipients that significantly affect bioavailability, should have the same *in vivo* dissolution characteristics and hence absorption profiles as the constituent, single-entity drug products in the same dosage form, and, as a result, will be bioequivalent, regardless of BCS classification. However, to-date there is not a regulatory pathway for applying biowaivers to products containing BCS Class II or BCS Class IV drugs as the single active ingredients; thus, there is no existing basis for extending biowaivers to fixed dose combinations of BCS Class II or IV drugs that are already established as single-entity drug products that are co-administered in a comparable dosage form.

The EMA has determined that there is sufficient rationale for extending biowaivers to combinations of BCS Class I and/or Class III drugs. In Europe, BCS-based biowaivers are applicable for immediate-release, fixed-dose combination drug products if all of the active substances are BCS Class I or BCS Class III drugs, as long as the requirements regarding excipients are also satisfied [75]. The FDA recently incorporated a similar approach into their 2015 draft guidance, which documents a new pathway for applying BCS-based biowaivers to immediate-release fixed-dose combination drug products where all of the active substances are BCS Class I. If the excipients in the test product are the same as those in the reference product, the FDA's draft guidance also extends the regulatory pathway to fixed dose combinations where all of the active

constituents belong to BCS Class III or represent a combination of BCS Class I and Class III drugs [78].

The 2015 draft guidance represented major regulatory progress as, up until this time, the opportunities for BCS-based biowaivers in the U.S. were more limited than those in Europe. In fact, prior to the general guidance issued by the FDA in 2015, it was essentially unclear whether the FDA would grant biowaivers for fixed dose combinations. For example, there was no mention of biowaivers within the FDA's 2006 Guidance for Industry, *Fixed Dose Combinations, Co-Packaged Drug Products, and Single-Entity Versions of Previously Approved Antiretrovirals for the Treatment of HIV*. On the contrary, the 2006 FDA guidance merely stated that it was necessary to show that the rate and extent of absorption of each active constituent of the test, fixed dose antiretroviral combination are the same as those for each component of the reference drug combination [79]. Yet, given the chronic nature of HIV infection and the need for strict patient compliance in order to minimize the chances of emerging viral resistance [44], fixed dose combinations are commonly utilized for more convenient and simplified dosing regimens, which may result in greater levels of patient compliance. Several studies have demonstrated that, when compared to regimens requiring multiple tablets, single-tablet HIV regimens are associated with greater adherence; and as a result, lead to more viral suppression [45] [46] [47]. The current U.S. Department of Health and Human Services (HHS) HIV treatment guidelines recommend starting treatment with a regimen of three HIV

medicines from at least two different drug classes among non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors (PIs), fusion inhibitors, entry inhibitors, and integrase strand transfer inhibitors (INSTIs). In general, the treatment guidelines recommend that patients naïve to antiretroviral therapy initiate therapy with a regimen that includes two NRTIs in combination with an NNRTI, a PI boosted with a pharmacokinetic enhancer, or an INSTI [37]. Because of the clinical basis for utilizing several drugs in combination, and the wide array of established single-drug products that are co-administered [79], antiretrovirals represent a therapeutic class of drug products where an expanded regulatory framework for applying biowaivers of *in vivo* bioequivalence studies, is germane to the ability to effectively address the significant global unmet medical need.

The World Health Organization (WHO) reports that HIV is the world's leading infectious killer, with approximately 39 million people having died from AIDS-related causes so far [5]. There are currently about 35 million people living with HIV [8]. While the large majority of the people living with the virus reside in low-/middle- income countries [80] [81], only 36% of the population eligible for therapy under the WHO treatment guidelines [82] is receiving antiretroviral therapy in these regions [5]. And of the 3.2 million children infected with HIV globally, only 23% have access to treatment [83]. Thus, within the context of this disease alone, the increased availability of therapeutically equivalent drug products and co-formulated combination drug products can have

a tremendous impact on improving public health worldwide. Reducing the clinical burden, by creating a framework for the acceptability of *in* vitro data in place of *in vivo* bioequivalence data, will likely promote competition and enable additional products to enter the market from sources that may have been deterred by the costs and timelines associated with human studies [84].

As a result of recognizing the potential impact, many global health organizations and advocates have noted the importance of facilitating the development of pharmaceutically and/or therapeutically equivalent products from multiple sources in order to address global pandemics such as HIV/AIDS. The Prequalification Programme administered by the WHO, for example, was established in 2001 to help facilitate access to products designed to treat priority diseases, including HIV/AIDS. The Prequalification Programme helps address treatment gaps in countries that otherwise have limited access to quality medicines by prequalifying medicinal products that meet certain standards for safety, efficacy, and quality as acceptable for procurement by United Nations organizations, including the Joint United Nations Programme on HIV/AIDS (UNAIDS), United Nations Children's Fund (UNICEF) and United Nations Population Fund (UNFPA). Any manufacturer (innovator or generic) can apply for prequalification to supply products that are listed on the WHO's Invitation for Expression of Interest. If the WHO assessment demonstrates that the product, manufacturing facilities, and clinical sites all meet the WHO's standards, the sponsor's product will be included on the list of prequalified medicines for

procurement. The WHO assessment teams include experts from stringent regulatory authorities (such as SwissMedic, Health Canada, and national regulatory authorities within the European Union) who partner with regulators from the developing countries where the products will be utilized to complete the assessment. Both single-entity drug products and fixed dose combinations are eligible for prequalification. The WHO has been implementing BCS-based biowaivers since 2008 and their principles for assessing for fixed dose combinations are intended to align with those of the EMA and FDA. Interestingly, the WHO Prequalification Programme actually specifies the drugs that are currently eligible for BCS-based biowaivers and confirms that *in vivo* bioequivalence data are required for fixed dose combination containing any other APIs [85].

Realizing the potential for global impact and the need to motivate even more scientific advancement with regard to requirements for bioequivalence, we have conducted a provisional assessment of the biopharmaceutical properties of established and investigational antiretroviral drug products for the treatment of HIV infection. The results of our assessment can serve as the starting point for developing product-specific scientific rationale to potentially simplify and accelerate the development and availability of co-formulated antiretroviral drug products globally – especially in developing countries, where there is a significant unmet medical need.

Materials and Methods

Drug Lists

Lists of drug products approved (i.e., in the post-marketing phase in the U.S., European Union, or Canada) and/or in development (Phase 2 and beyond) for an HIV indication were obtained using clinical trial and literature review software/search engine sourced from public information. Only oral dosage forms were included. The drug list was verified and refined by reviewing clinical trial information (www.clinicaltrials.gov), published scientific literature (when available), corporate press releases, and reference labeling approved by the FDA, EMA, and/or Health Canada. The regulatory status of the drug products was determined by reviewing the FDA approvals as of December 4, 2014 (www.fda.gov).

Solubility

Solubility from Reference Literature. The aqueous drug solubility values (mg/mL) were obtained from the product labeling approved by the FDA, EMA and/or Health Canada. In cases where the aqueous solubility was described qualitatively, a conservative numerical value was assigned based on the lower limit of the range defined in the U.S. Pharmacopeia [86], in accordance with the methodology utilized by Kasim et al [87].

When unavailable from reference literature, aqueous solubility values were determined using ChemDraw software (CambridgeSoft Corp., Cambridge, MA) or sourced from ALogPS 2.1, as reported via DrugBank (www.drugbank.ca). pKa values were obtained in similar fashion.

Dose Number Calculations. Per existing FDA guidance, the solubility class of a drug substance is based on the highest dose strength. A drug is considered highly soluble when the highest dose strength is soluble in 250 ml or less of aqueous media over the pH range of 1-6.8 [78; 77]. The volume estimate of 250 ml is derived from typical BE study protocols that prescribe administration of a drug product to fasting human volunteers with a glass (about 8 ounces) of water [78; 77].

The dose number, a dimensionless solubility parameter normalized by dose, was calculated, in accordance with the method utilized by Dahan et al [88] for *in silico* BCS classification, as follows:

$$D_0 = \frac{(M_0/V_0)}{C_s}$$

where M_0 is the maximum dose strength (milligrams), C_s is either the solubility (milligrams per milliliter), and V_0 = 250 mL. Drugs where $D_o \leq 1$ were classified as high solubility and drugs with $D_0 > 1$ were categorized as low solubility.

Partition Coefficients

In silico n-Octanol/water partition coefficients of the uncharged drug were determined from several different sources. Calculations of Log P based on the chemical structure of the drug were obtained using ChemDraw software (CambridgeSoft Corp., Cambridge, MA). Log P values were also sourced from the ALogPS 2.1, as reported via DrugBank (www.drugbank.ca). Per the in silico methods utilized by Dahan et al, permeability was categorized in reference to metoprolol, as 95% of this compound is known to be absorbed from the gastrointestinal tract [89]. Accordingly, drugs with LogP values ≥ 1.632 were classified as high permeability and drugs with LogP values < 1.632 were categorized as low solubility.

Results

Mechanisms, Permeability, and Solubility of Single-Entity Antiretroviral Drug Products

Thirty-four (34) drug products with a single API were identified as either being in late stage clinical development or in post-marketing phase for an HIV

indication. As listed in Table 6, 26% of these drugs products have yet to be registered or tentatively approved. Consistent with treatment guidelines, which generally recommend nucleoside reverse transcriptase inhibitors as the backbone to an effective antiretroviral regimen in treatment-naïve patients, NRTIs represented the most common class among the single-entity drug products assessed, as the antiretroviral activity of 11 out of the 34 (i.e., 32%) can be attributed to this mechanism of action (Figure 19). Only 12% represented novel or unconventional mechanisms of antiretroviral activity: fusion inhibitors (3%), entry inhibitors (6%), or other mechanisms such as immunological boosting (3%).

Table 6 US Regulatory Status of HIV Indication for Single-Entity Drug Products

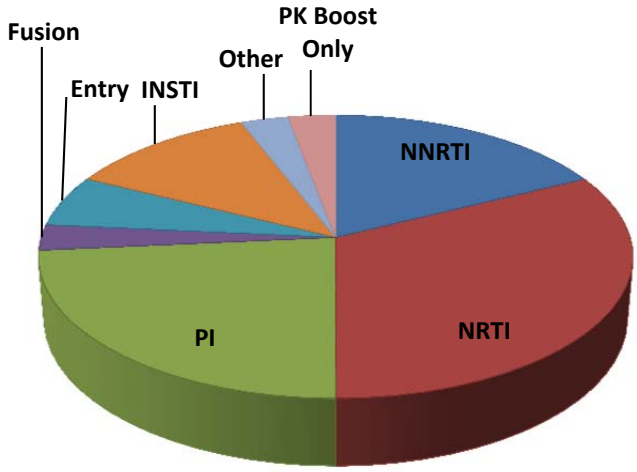
Drug Name	US Regulatory Status
apricitabine	Development
cabotegravir	Development
cenicriviroc	Development
doravirine	Development
elvucitabine	Development
festinavir	Development
fostemsavir	Development
naltrexone	Development
tenofovir alafenamide	Development
darunavir	Tentatively Approved[a]
abacavir sulfate	Approved
amprenavir	Approved
atazanavir sulfate	Approved
cobicistat	Approved
delavirdine (mesylate)	Approved
didanosine	Approved
dolutegravir	Approved
efavirenz	Approved

Table 6 Continued.

Drug Name	US Regulatory Status
elvitegravir	Approved
emtricitabine	Approved
etravirine	Approved
fosamprenavir calcium	Approved
indinavir sulfate	Approved
lamivudine	Approved
maraviroc	Approved
nelfinavir mesylate	Approved
nevirapine	Approved
raltegravir potassium	Approved
rilpivirine hydrochloride	Approved
ritonavir	Approved
saquinavir mesylate	Approved
stavudine	Approved
tenofovir disoproxil fumarate	Approved
zidovudine	Approved

[a] Tentative approval indicates that final approval of the drug product has been delayed until resolution of all patent or exclusivity issues. The product cannot be marketed or sold in the United States until final approval is granted.

Figure 19 Antiretroviral Mechanism of Action for Single-Entity Drug Products



Where:
NNRTI = non-nucleoside reverse transcriptase inhibitor
NRTI = nucleoside reverse transcriptase inhibitor
PI = protease inhibitor
INSTI = integrase strand transfer inhibitor
Fusion = fusion inhibitor
Entry = entry inhibitor

Table 7 Provisional Data for Single-Entity Drug Products

Drug Name	US Regulatory Status* (as of Dec 4 2014)	Mechanism of Action	Max Dose† (mg)	Solubility Definition (from literature)	For Do Calculation		Dose Number (Do)	Provisional BCS Class
					Solubility (mg/mL)	logP		
apricitabine	Development	NRTI	1600	-	0.65	-0.99	9.77	IV
cabotegravir	Development	INSTI	60	-	0.00008	1.04	2952.65	IV
cenicriviroc	Development	Entry	200	<0.0002	0.0002	7.5	4000	II
doravirine	Development	NNRTI	100	-	0.000007	2.23	58487.09	II
elvucitabine	Development	NRTI	10	-	13	-0.81	0.003	III
festinavir	Development	NRTI	600	-	0.08	-0.08	28.79	IV
fostemsavir	Development	Fusion	1200	-	0.00005	-0.18	101214.15	IV
naltrexone	Development	Other	3	-	100	1.36	0.0001	III
tenofovir alafenamide	Development	NRTI	25	-	0.00003	1.88	3213.66	II
darunavir	Tentatively Approved	NRTI	800	-	0.15	2.82	21.33	II
abacavir sulfate	Approved	PI	600	-	77	0.39	0.03	III
amprenavir	Approved	PI	2800	-	0.04	2.43	280	II
atazanavir sulfate	Approved	PK Boost	400	slightly soluble	1	4.54	1.6	II
cobicistat	Approved	PI	150	-	0.1	5.7	6	II
delavirdine (mesylate)	Approved	NNRTI	1200	-	2.94	1.02	1.63	IV
didanosine	Approved	NRTI	400	-	27.3	0.04	0.06	III
dolutegravir	Approved	INSTI	100	slightly soluble	1	1.1	0.4	III
efavirenz	Approved	NNRTI	600	$< 10^{-3}$	0.001	4.46	2400	II
elvitegravir	Approved	INSTI	150	$<3x10^{-4}$	0.0003	4.67	2000	II

Table 7 Continued.

Drug Name	US Regulatory Status* (as of Dec 4 2014)	Mechanism of Action	Max Dose† (mg)	Solubility Definition (from literature)	For Do Calculation		Dose Number (Do)	Provisional BCS Class
					Solubility (mg/mL)	logP		
emtricitabine	Approved	NRTI	240	-	112	-0.9	0.01	III
etravirine	Approved	NNRTI	400	practically insoluble	0.01	5.54	160	II
fosamprenavir calcium	Approved	PI	2800	-	0.31	1.98	36.13	II
indinavir sulfate	Approved	PI	2400	very soluble	1000	2.81	0.01	I
lamivudine	Approved	NRTI	300	-	70	-1.1	0.02	III
maraviroc	Approved	Entry	600	highly soluble	1000	3.63	0.002	I
nelfinavir mesylate	Approved	PI	2500	slightly soluble	1	4.72	10	II
nevirapine	Approved	NNRTI	400	practically insoluble	0.01	2.49	160	II
raltegravir potassium	Approved	INSTI	800	soluble	33	-0.39	0.097	III
rilpivirine hydrochloride	Approved	NNRTI	25	practically insoluble	0.01	5.47	10	II
ritonavir	Approved	PI	1200	practically insoluble	0.01	5.98	480	II
saquinavir mesylate	Approved	PI	2000	-	2.22	3.8	4	II
stavudine	Approved	NRTI	80	soluble	33	-0.23	0.010	III
tenofovir disoproxil fumarate	Approved	NRTI	300	-	13.4	-3.7	0.09	III
zidovudine	Approved	NRTI	600	-	20.1	-0.3	0.12	III

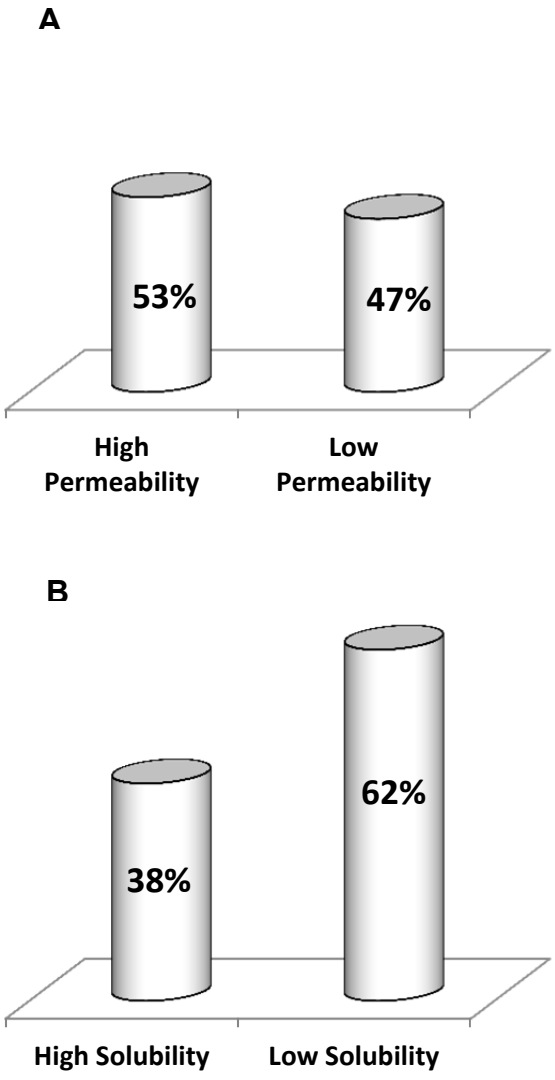
* For HIV/AIDS-related indication

† Without consideration to special populations

Each of the 34 single-entity HIV drug products was classified as either high or low permeability by comparison of the *in silico* partition coefficient value to that of the reference drug metoprolol. Drugs with LogP values greater than or equal to 1.632 were classified as high-permeability drugs. As shown in Figure 20A, 53% of the drugs in single-entity HIV drug products were categorized as high permeability.

Dose numbers were calculated using solubility values from the literature and the maximum dose strength of each oral, single-entity HIV immediate-release drug product. A total of 38% of the drugs were classified as high-solubility drugs using the maximum dose strengths (Figure 20B).

Figure 20 Permeability and Solubility of Single-Entity HIV Drug Products



Provisional BCS Classification of Single-Entity Antiretroviral Drug Products

Provisional BCS classification (Table 8) was determined using the *in silico* partition coefficients as well as the solubility data from the reference literature, as described in the

Materials and **Methods**. BCS Class II and Class IV were further subclassified as a, b, c or acid, base, neutral, respectively, according to their pKa values, per the subclassifications proposed by Tsume et al [90]. Only 6% of HIV single-entity drug products that are currently approved or in late stage development are BCS Class I and an additional 32% are BCS Class III drugs. Thus, 38% of these drug products are potentially eligible for biowaivers using dissolution data under the current regulatory framework. The establishment of suitable *in vivo* predictive, *in vitro* dissolution methods could substantially expedite the development of antiretroviral drug products.

Table 8 Provisional BCS Classification/Subclassification of Single-Entity Antiretroviral Drugs

Drug Name	Provisional BCS Class/Subclass
apricitabine	IVc
cabotegravir	IVc
cenicriviroc	IIc
doravirine	IIc
elvucitabine	III
festinavir	IVc
fostemsavir	IVc
naltrexone	III
tenofovir alafenamide	IIc
abacavir sulfate	III
amprenavir	IIc
atazanavir sulfate	IIc
cobicistat	IIb
darunavir	IIc
delavirdine (mesylate)	IVc
didanosine	III
dolutegravir	III
efavirenz	IIc

Table 8 Continued.

Drug Name	Provisional BCS Class/Subclass
elvitegravir	IIa
emtricitabine	III
etravirine	IIc
fosamprenavir calcium	IIa
indinavir sulfate	I
lamivudine	III
maraviroc	I
nelfinavir mesylate	IIb
nevirapine	IIc
raltegravir potassium	III
rilpivirine hydrochloride	IIc
ritonavir	IIc
tenofovir disoproxil fumarate	III
zidovudine	III

Assessment of Fixed Dose Combinations of Antiretroviral Drug Products

Twenty-two (22) fixed dose combinations (including single tablet regimens) of single-entity drug products were identified as being in late stage clinical development (Phase 2 or beyond) or currently marketed for an HIV indication. Of these 22 drug products, 27% have yet to be fully or tentatively approved (Table 9). Interestingly, based solely on the current general treatment guidelines [37] alone, 9 of these fixed dose combinations could potentially be developed as single tablet regimens for ART-naïve patients: efavirenz / emtricitabine / tenofovir disoproxil fumarate; lamivudine / nevirapine / stavudine; lamivudine / nevirapine / zidovudine; efavirenz / lamivudine / tenofovir disoproxil fumarate; emtricitabine / rilpivirine / tenofovir disoproxil fumarate; cobicistat/elvitegravir / emtricitabine / tenofovir disproxil fumarate; abacavir sulfate / dolutegravir / lamivudine; cobicistat / darunavir / emtricitabine / tenofovir alafenamide; and cobicistat / elvitegravir / emtricitabine / tenofovir alafenamide.

Table 9 US Regulatory Status of HIV Indication and Antiretroviral Mechanisms of Action for Fixed-Dose Combination Drug Products

Drug Combination	Mechanisms of Action	US Regulatory Status
lamivudine / raltegravir	NRTI + INSTI	Development
cobicistat / elvitegravir / emtricitabine / tenofovir alafenamide	NRTI + INSTI (+PK Boost)	Development
atazanavir sulfate / cobicistat	PI (+PK Boost)	Development
emtricitabine / tenofovir alafenamide	NRTI	Development
cobicistat / darunavir / emtricitabine / tenofovir alafenamide	NRTI + PI (+PK Boost)	Development
cobicistat / darunavir	PI (+PK Boost)	Development
lamivudine / zidovudine	NRTI	Approved

Table 9 Continued.

Drug Combination	Mechanisms of Action	US Regulatory Status
abacavir sulfate / dolutegravir / lamivudine	NRTI + INSTI	Approved
cobicistat / elvitegravir / emtricitabine / tenofovir disproxil fumarate	NRTI + INSTI (+PK Boost)	Approved
lopinavir / ritonavir	PI (+PK Boost)	Approved
abacavir sulfate / lamivudine / zidovudine	NRTI	Approved
emtricitabine / tenofovir disoproxil fumarate	NRTI	Approved
emtricitabine / rilpivirine / tenofovir disoproxil fumarate	NRTI + NNRTI	Approved

Table 9 Continued.

Drug Combination	Mechanisms of Action	US Regulatory Status
efavirenz / lamivudine / tenofovir disoproxil fumarate	NRTI + NNRTI	Tentatively Approved
lamivudine / nevirapine / zidovudine	NRTI + NNRTI	Tentatively Approved
lamivudine / nevirapine / stavudine	NRTI + NNRTI	Tentatively Approved
lamivudine / stavudine	NRTI	Tentatively Approved
efavirenz /emtricitabine / tenofovir disoproxil fumarate	NRTI + NNRTI	Approved

Table 9 Continued.

Drug Combination	Mechanisms of Action	US Regulatory Status
atazanavir sulfate / ritonavir	PI (+PK Boost)	Tentatively Approved
darunavir / ritonavir	PI (+PK Boost)	Not Approved[a]
lamivudine / tenofovir disoproxil fumarate	NRTI	Tentatively Approved
abacavir sulfate / lamivudine	NRTI	Approved

[a] Approved ex-US

Table 10 Provisional Data for Antiretroviral Fixed Dose Combinations

Combination	Components	Max FDC Dose† (mg)	Solubility Definition (from literature)	For Do Calculation		Dose Number (Do)	Provisional BCS Classification (Constituents)	Provisional BCS Classification (Combination)
				Solubility (mg/mL)	logP			
lamivudine / raltegravir	lamivudine	300	-	70	-1.1	0.02	III	III
	raltegravir	600	soluble	33	-0.39	0.07	III	
cobicistat / elvitegravir / emtricitabine / tenofovir alafenamide	cobicistat	150	-	0.1	5.7	6	II	Mixed (II, III)
	elvitegravir	150	$<3x10^{-4}$	0.0003	4.67	2000	II	
	emtricitabine	200	-	112	-0.9	0.01	III	
	tenofovir alafenamide	10	-	0.00003	1.88	1285.46	II	
atazanavir sulfate / cobicistat	atazanavir sulfate	300	slightly soluble	1	4.54	1.2	II	II
	cobicistat	150	-	0.1	5.7	6	II	
emtricitabine / tenofovir alafenamide	emtricitabine	200	-	112	-0.9	0.01	III	Mixed (II, III)
	tenofovir alafenamide	25	-	0.00003	1.88	3213.66	II	
cobicistat / darunavir / emtricitabine / tenofovir alafenamide	cobicistat	150	-	0.1	5.7	6	II	Mixed (II, III)
	darunavir	800	-	0.15	2.82	21.33	II	
	emtricitabine	200	-	112	-0.9	0.01	III	
	tenofovir alafenamide	10	-	0.00003	1.88	1285.46	II	
cobicistat / darunavir	cobicistat	150	-	0.1	5.7	6	II	II
	darunavir	800	-	0.15	2.82	21.33	II	
darunavir / ritonavir	darunavir	400	-	0.15	2.82	10.67	II	II
	ritonavir	50	practically insoluble	0.01	5.98	20	II	

Table 10 Continued.

Combination	Components	Max FDC Dose† (mg)	Solubility Definition (from literature)	For Do Calculation		Dose Number (Do)	Provisional BCS Classification (Constituents)	Provisional BCS Classification (Combination)
				Solubility (mg/mL)	logP			
efavirenz / lamivudine / tenofovir disoproxil fumarate	efavirenz	600	$< 10^{-3}$	0.001	4.46	2400	II	Mixed (II, III)
	lamivudine	300	-	70	-1.1	0.02	III	
	tenofovir disoproxil fumarate	300	-	13.4	-3.7	0.09	III	
lamivudine / nevirapine / zidovudine	lamivudine	150	-	70	-1.1	0.01	III	Mixed (II, III)
	nevirapine	200	practically insoluble	0.01	2.49	80	II	
	zidovudine	300	-	20.1	-0.3	0.06	III	
lamivudine / nevirapine / stavudine	lamivudine	150	-	70	-1.1	0.01	III	Mixed (II, III)
	nevirapine	200	practically insoluble	0.01	2.49	80	II	
	stavudine	30	soluble	33	-0.23	0.004	III	
lamivudine / stavudine	lamivudine	150	-	70	-1.1	0.01	III	III
	stavudine	30	soluble	33	-0.23	0.004	III	
lamivudine / tenofovir disoproxil fumarate	lamivudine	300	-	70	-1.1	0.02	III	III
	tenofovir disoproxil fumarate	300	-	13.4	-3.7	0.09	III	
atazanavir sulfate / ritonavir	atazanavir sulfate	300	slightly soluble	1	4.54	1.2	II	II
	ritonavir	100	practically insoluble	0.01	5.98	40	II	
lamivudine / zidovudine	lamivudine	300	-	70	-1.1	0.02	III	III
	zidovudine	600	-	20.1	-0.3	0.12	III	

Table 10 Continued.

Combination	Components	Max FDC Dose† (mg)	Solubility Definition (from literature)	For Do Calculation		Dose Number (Do)	Provisional BCS Classification (Constituents)	Provisional BCS Classification (Combination)
				Solubility (mg/mL)	logP			
abacavir sulfate / dolutegravir / lamivudine	abacavir sulfate	600	-	77	0.39	0.03	III	III
	dolutegravir	100	slightly soluble	1	1.1	0.40	III	
	lamivudine	300	-	70	-1.1	0.02	III	
cobicistat / elvitegravir / emtricitabine / tenofovir disproxil fumarate	cobicistat	150	-	0.1	5.7	6	II	Mixed(II, III)
	elvitegravir	150	$<3x10^{-4}$	0.0003	4.67	2000	II	
	emtricitabine	200	-	112	-0.9	0.01	III	
	tenofovir disoproxil fumarate	300	-	13.4	-3.7	0.09	III	
lopinavir / ritonavir	lopinavir	800	practically insoluble	0.01	4.69	320	II	II
	ritonavir	200	practically insoluble	0.01	5.98	80	II	
abacavir sulfate / lamivudine / zidovudine	abacavir sulfate	600	-	77.0	0.39	0.03	III	III
	lamivudine	300	-	70	-1.1	0.02	III	
	zidovudine	600	-	20.1	-0.3	0.12	III	
emtricitabine / tenofovir disoproxil fumarate	emtricitabine	200	-	112	-0.9	0.01	III	III
	tenofovir disoproxil fumarate	300	-	13.4	-3.7	0.09	III	
emtricitabine / rilpivirine / tenofovir disoproxil fumarate	emtricitabine	200	-	112	-0.9	0.01	III	Mixed (II, III)
	rilpivirine	25	practically insoluble	0.01	5.47	10	II	
	tenofovir disoproxil fumarate	300	-	13.4	-3.7	0.09	III	

Table 10 Continued.

Combination	Components	Max FDC Dose† (mg)	Solubility Definition (from literature)	For Do Calculation		Dose Number (Do)	Provisional BCS Classification (Constituents)	Provisional BCS Classification (Combination)
				Solubility (mg/mL)	logP			
efavirenz /emtricitabine / tenofovir disoproxil fumarate	efavirenz	600	$< 10^{-3}$	0.001	4.46	2400	II	Mixed (II, III)
	emtricitabine	200	-	112	-0.9	0.01	III	
	tenofovir disoproxil fumarate	300	-	13.4	-3.7	0.09	III	
abacavir sulfate / lamivudine	abacavir sulfate	600	-	77	0.39	0.03	III	III
	lamivudine	300	-	70	-1.1	0.02	III	

* For HIV/AIDS-related indication

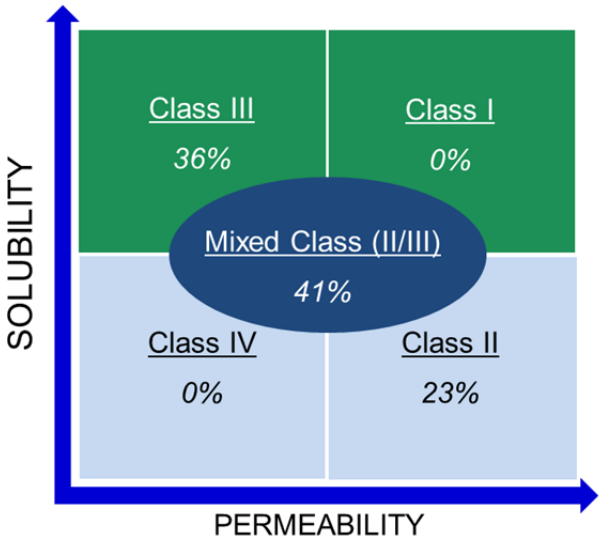
† Without consideration to special populations

^ Approved ex-US

Provisional BCS Classification of Fixed Dose Combinations of Antiretroviral Drug Products

Provisional BCS classification of each of the 22 fixed dose combinations was determined by the provisional classification of each of the constituent single-entity drugs within the combination (Figure 21). In contrast to the provisionally classified single-entity HIV drugs, none of the fixed dose antiretroviral combinations were BCS Class I. This means that none of these combination drug products would have been eligible for biowaivers in the United States prior to the implementation of FDA's 2015 draft guidance. However, 36% of the drugs were BCS Class III and now potentially eligible for biowaivers of *in vivo* bioequivalence studies from the EMA as well as the FDA. These results also point to the substantial improvement that *in vivo* predictive, *in vitro* dissolution methods could potentially offer with regard to reducing the clinical requirements, often associated with longer development timelines and higher costs [84], for fixed dose antiretroviral drug combinations of established single-entity drug products.

Figure 21 Provisional BCS Classification of Antiretroviral Fixed Dose Combinations



Provisional BCS classifications of fixed dose combinations wer determined according to the provisional BCS classifications of the constituent drugs. If all drugs in the combination were of the same BCS class, then the fixed dose combination was assigned the same provisional BCS classification. If the drugs in combination were of different BCS classes, the fixed dose combination was assigned a provisional "mixed class" that represented the BCS classification of all of the constituent drugs.

Discussion

In this work, we assessed the biopharmaceutical properties of established and investigational antiretroviral drug products for the treatment of HIV infection. Our research identified 34 single-entity drug products and 22 fixed-dose, co-formulated, combination drug products with HIV indications in late stage clinical development (Phase 2 or beyond) or post-marketing phase. Among the products assessed, no major difference was seen in the proportion of products already approved as single-entity or fixed dose combinations – 26% of single drug products and 27% of co-formulated combination drug products have yet to be registered or tentatively approved. These data suggest that, although there is increasing awareness regarding the potential advantages of fixed dose combination antiretroviral regiments, to-date there is still a comparable level of interest in developing new products with a single active ingredient for the treatment of HIV infection. However, in light of the HHS treatment guidelines and the expansion of regulatory waivers for *in vivo* bioequivalence data to fixed dose combinations comprised of BCS Class I and/or Class III drugs, there is opportunity for the development of new fixed dose combination antiretroviral regimens based on existing data from standalone, single-entity products.

For fixed dose combinations based on products with a single antiretroviral API, which have an established safety and efficacy profile for co-administration with other drug products for the treatment of HIV infection, expanding the range of BCS-based biowaiver criteria has the potential to significantly lessen the

amount of *in vivo* data needed to support literature-based marketing applications, such as those permitted in the U.S. under Section 505(b)(2) of the Federal Food, Drug and Cosmetic Act. In fact, 21% of the fixed dose combinations assessed herein were approved/tentatively approved in the United States as a 505(b)(2) application. This potential regulatory pathway has greater potential for challenges due to patent protection, but even in cases where the existence of patents and/or market exclusivity in the United States would preclude approval and market accessibility, new antiretroviral fixed dose combination products can still potentially be developed for ex-US distribution under the FDA's President's Emergency Plan for AIDS Relief (PEPFAR) initiative, which was established in 2004 to ensure that antiretroviral drugs distributed under the Presidents' Plan would be safe, effective, and manufactured to quality standards [91]. Similarly, recognizing that patent laws, which vary globally, could undermine the goal of improving access to priority medicines, the WHO Prequalification Programme does not operate with respect to product patents [92]. Of note, one-third of the fixed dose combinations included in this provisional evaluation have been tentatively approved in the U.S. under PEPFAR and 36% of the combinations are included on the WHO's 12th Invitation to Submit an Expression of Interest (EOI) for product prequalification. Thus, our provisional assessment can be utilized to build a case for how global accessibility to priority co-formulated antiretroviral drug products may be improved through the development of scientific justifications for *in vitro* methodology, in lieu of additional *in vivo* data, to

demonstrate bioequivalence between co-administered single-entity drug products and co-formulations in the same dosage form.

As exemplified by the extension of the biowaiver eligibility to BCS Class III drugs, regulatory science continues to evolve. It is generally well-recognized that regulators appear to be moving toward harmonization in their interpretations and willingness to consider alternative approaches with scientific merit. And even though the WHO currently specifies abacavir, emtricitabine, lamivudine, stavudine, zidovudine, and combinations thereof as the only antiretroviral medicines eligible for biowaivers under the Prequalification Programme, the WHO also broadly invites manufacturers to explore opportunities to demonstrate eligibility for biowaivers by providing evidence to classify the API(s) as either BCS Class I or Class III [85]. Our provisional assessment revealed that 36% of the co-formulated drug combinations (none of which were comprised of Class I drugs) were potentially eligible for BCS-based biowaivers, which emphasizes the fact that there is still significant opportunity with regard to the pharmaceutical and regulatory science surrounding fixed dose combinations. Additional scientifically sound approaches for demonstrating bioequivalence can help influence the direction in which the field progresses. Although regulatory authorities have yet to propagate guidelines to confirm the acceptability of alternate *in vitro* data in place of *in vivo* bioequivalence studies for BCS Class II and Class IV drugs, the scientific community has been making progress in establishing *in vivo* predictive and physiologically relevant dissolution methodologies [93]. If scientifically valid

in vitro approaches are developed to justify waivers for *in vivo* bioequivalence studies in support of combinations of drugs from BCS Class II and/or IV, for example, there is tremendous opportunity for simplifying the regulatory pathway and laying the ground work for advancing global access to more antiretroviral fixed-dose combination drug products.

Regardless of the drugs' BCS classifications, a well-designed oral fixed-dose combination drug product comprised of individual drugs that exhibit linear and non-interacting ADME should, in theory, demonstrate the same *in vivo* dissolution and absorption profiles as the constituent, single-entity drug products co-administered in the same dosage forms if there are no interfering excipients in the fixed dose formulation. This type of *in vitro* comparison should especially be more feasible for antiretroviral products where the *in vivo* behavior of various combinations is commonly established via clinical studies provided in marketing applications to support co-administration with other antiretroviral products in the indication and/or dosing recommendations. Therefore, appropriately-designed *in vitro* dissolution methodology comparing each API should be sufficient to demonstrate bioequivalence between co-formulated oral drug combinations and the corresponding co-administered single-entity products in the same dosage forms.

As our study revealed that the majority (41%) of the antiretroviral fixed dose drug co-formulations are mixtures of drugs of different BCS classes, combinations/hybrids of physiologically-relevant dissolution approaches

measuring each API according to the dissolution method recommended for the single-entity drug represent one scientifically valid alternative to *in vivo* bioequivalence for fixed dose combinations. Utilizing the starting recommendations for *in vivo* predictive dissolution apparatus and media proposed according to BCS subclassification by Tsume et al [90], for example, some initial dissolution parameters can be recommended as a starting place for assessing the bioavailability of fixed dose combinations with mixed BCS classification (i.e., drugs from more than one BCS Class in the combination) relative to reference single-entity products administered together(Table 11). Depending on the combination, multiple tests could be utilized or even combined so that each API is assessed according to the dissolution method recommended for the single-entity drug product.

Table 11 Starting Recommendations for Design of *in vivo* Predictive *in vitro* Dissolution Methodology for Fixed Dose Combinations Comprised of APIs with Different BCS Classifications

Mixed BCS Classifications / SubClassifications	# of Tests	Gastric Medium	Consider Gastric Compartment	Intestinal Luminal Medium	Consider Absorption Compartment
I and IIa, IVa, IIb, and/or IVb	Test 1:	250 ml PGB[a]	No	900 ml PIB[b]	No
	Test 2:	250 ml PGB	Yes	100 ml PIB	Yes
I and IIc and/or IVc	Test 1:	250 ml PGB	No	900 ml PIB	No
	Test 2:	250 ml PGB	Yes	100 ml PIB + bile acids/lipid	Yes
I and III	Test 1:	250 ml PGB	No	900 ml PIB	No
	Test 2:	250 ml PGB	No	100ml PIB	No
IIa, IIb, IVa and/or IVb	Test 1:	250 ml PGB	Yes	100 ml PIB	Yes
IIa, IIb, IVa, and/or IVb and III	Test 1:	250 ml PGB	Yes	100 ml PIB	Yes
	Test 2:	250 ml PGB	No	100ml PIB	No

[a] Physiological gastric buffer

[b] Physiological intestinal buffer

Table 11 Continued.

Mixed BCS Classifications / SubClassifications	# of Tests	Gastric Medium	Consider Gastric Compartment	Intestinal Luminal Medium	Consider Absorption Compartment
IIc and IVc	Test 1:	250 ml PGB	Yes	100 ml PIB + bile acids/lipid	Yes
IIa, IIb, IVa and/or IVb and IIc and/or IVc	Test 1:	250 ml PGB	Yes	100 ml PIB	Yes
	Test 2:	250 ml PGB	Yes	100 ml PIB + bile acids/lipid	Yes
IIc and/or IVc and III	Test 1:	250 ml PGB	Yes	100 ml PIB + bile acids/lipid	Yes
	Test 2:	250 ml PGB	No	100ml PIB	No
I and IIa, IIb, IVa, and/or IVb and IIc and/or IVc	Test 1:	250 ml PGB	No	900 ml PIB	No
	Test 2:	250 ml PGB	Yes	100 ml PIB	Yes
	Test 3:	250 ml PGB	Yes	100 ml PIB + bile acids/lipid	Yes
I and IIa, IIb, IVa, and/or IVb and III	Test 1:	250 ml PGB	No	900 ml PIB	No
	Test 2:	250 ml PGB	Yes	100 ml PIB	Yes
	Test 3:	250 ml PGB	No	100ml PIB	No

Table 11 Continued.

Mixed BCS Classifications / SubClassifications	# of Tests	Gastric Medium	Consider Gastric Compartment	Intestinal Luminal Medium	Consider Absorption Compartment
I and IIc and/or IVc and III	Test 1:	250 ml PGB	No	900 ml PIB	No
	Test 2:	250 ml PGB	Yes	100 ml PIB + bile acids/lipid	Yes
	Test 3:	250 ml PGB	No	100ml PIB	No
I and IIa, IIb, IVa, and/or IVb and IIc and/or IVc and III	Test 1:	250 ml PGB	No	900 ml PIB	No
	Test 2:	250 ml PGB	Yes	100 ml PIB	Yes
	Test 3:	250 ml PGB	Yes	100 ml PIB + bile acids/lipid	Yes
	Test 4:	250 ml PGB	No	100ml PIB	No

As a therapeutic area with an established clinical rationale [37] [82] and history of regulatory approvals for co-administered drug products, [79] we are promoting the utility of alternative approaches to justify biowaivers for fixed dose combinations of antiretrovirals – especially in cases where existing data from standalone, single-entity products can be leveraged to justify that *in vitro* bioavailability correlates with *in vivo* bioequivalence. Although antiretroviral drug products were assessed in this work, these findings and recommendations are relevant to the development of fixed-dose combination drug products in general. If scientifically-justified, *in vivo* predictive dissolution methodology can be developed, there is great potential to broadly simplify and accelerate the development of both generic formulations and new fixed dose drug combinations of established drugs. The availability of these additional drug products, developed with scientifically valid *in vitro* data to confirm the validity of extrapolating existing *in vivo* data to these new formulations, should help improve global access to priority medicines in order to address unmet medical needs.

Conclusion

This work presents a provisional assessment of the biopharmaceutical characteristics of drug products in the late stages of clinical development and/or in the post-market phase for antiretroviral indications in order to emphasize how *in vitro* dissolution strategies can be scientifically valid approaches for demonstrating bioequivalence between co-formulated drug products and the reference, single-entity products administered in combination. Baseline

recommendations have been presented herein as a starting place for developing *in vivo* predictive and scientifically valid *in vitro* dissolution methodology to assess fixed dose combinations of various BCS classes. Thus, by promoting the utility of theoretically viable scientific rationale to support simpler and abbreviated development pathways, we are highlighting potential opportunities to increase the availability of co-formulated antiretroviral drug products and describing mechanisms by which drug manufacturers and regulators can implement science-based approaches to address unmet medical needs and effectively treat some of the world's deadliest diseases – especially in developing countries.

CHAPTER 6

FINAL CONCLUSIONS

Conclusions – Part I

The first part of this body of work demonstrates the importance of molecular mechanisms mediated by HIV-1 envelope expression, which substantially impact the apparently low infectivity of HIV-1.

The initial studies to assess how defective virions might be contributing to the overall observed low infectivity of HIV-1 and demonstrated that the production of defective virions is not solely attributable to mutations introduced in the reverse transcription step of virus replication. Instead, the data clearly demonstrated that unfavorable envelope glycoprotein stoichiometry can result in "defective" HIV-1. The findings are consistent with prior reports of the role of biophysical instability and of envelope glycoprotein stoichiometry in HIV-1 infectivity decay, but demonstrate that, overall, the low infectivity of HIV-1 primarily results from other mechanism(s).

Subsequent studies help clarify these mechanisms. First by demonstrating that, although commonly interpreted as independent events, HIV-1 interactions with a target cell are in fact, influenced to some extent by the presence of other virus particles. Additional results not only show that the extent of non-specific interactions between HIV-1 and target cells increases in accordance with the multiplicity of virus particles, but that these non-specific interactions impair infection efficiency and promote the degradation of virus particles that have been endocytosed through envelope-independent mechanisms.

Hence, this work achieves its primary objective to investigate the molecular mechanisms that form the basis of HIV-1 infectivity and has proven the initial hypothesis that HIV-1 infectivity is limited by the ability to efficiently engage target cell because of the low density of envelope spike glycoproteins on virus particles. The evidence presented herein offers explanation of molecular mechanisms that underlie HIV-1 infectivity. As these results offers unique perspective for understanding HIV-1 infection efficiency and highlight considerations of clinical import, they lay the foundation for better strategies for treating HIV-1 infection.

Going forward, the results of these studies may influence the design of HIV-1 entry inhibitors and/or vaccines. In addition, in order to further understanding of the virus lifecycle and potentially discover new therapeutic

targets, future studies should explore the role of the endocytic pathways (as highlighted in this work and previous reports) in HIV-1 pathogenesis.

Conclusions – Part II

The second part of this body of work demonstrates the utility of *in vivo* predictive and scientifically valid *in vitro* dissolution methodology to assess fixed dose combinations of various BCS classes in order to support simpler and abbreviated development pathways for co-formulated, fixed-dose combination drug products.

To motivate regulatory and scientific advancement with regard to requirements for bioequivalence, the work provided herein provisionally classifies and presents an assessment of the biopharmaceutical properties of 34 single-entity and 22 fixed-dose, co-formulated, combination oral drug products in late stage clinical development (Phase 2 or beyond) or post-marketing phase for HIV indications. To be effective in treating this deadly disease, HIV antiretroviral therapy combines the effect of multiple mechanisms of antiviral activity through co-administration of single-entity drug products or, when available, simpler administration of fixed-dose co-formulations. Consistent with treatment guidelines, which generally recommend nucleoside reverse transcriptase inhibitors as the backbone to an effective antiretroviral regimen in treatment-naïve patients, NRTIs represented the most common class among the single-entity drug products assessed, as the antiretroviral activity of 11 out of the 34 (i.e., 32%) can be attributed to this mechanism of action. Based solely on the current

general treatment guidelines [37] alone, 9 of these fixed dose combinations could potentially be developed as single tablet regimens for ART-naïve patients. As there is a significant global, unmet medical need for access to antiretroviral therapy, efforts to enable simplified and/or abridged development pathways may substantially improve the availability of these essential medicines.

Current regulatory guidelines permit waivers of *in vivo* bioequivalence studies for immediate-release orally administered products comprised of drug substances that are highly soluble, according to the BCS Classification System. Our provisional assessment revealed that 38% of the single-entity drug products and 36% of the co-formulated drug combinations are comprised of high solubility compounds and are, therefore, potentially eligible for BCS-based biowaivers under the existing regulatory framework. In theory, however, *in vitro* data could be used to justify biowaivers, regardless of BCS classification. Such a theoretical opportunity is especially relevant to the development of fixed-dose combinations of single-entity products that have an established safety and efficacy profile for co-administration, which is the case for many antiretroviral drug products, because the otherwise required *in vivo* studies analyzing each active moiety are inherently more complex, time consuming, and expensive.

Thus, this provisional assessment highlights opportunities to utilize scientifically-valid *in vitro* dissolution strategies to demonstrate bioequivalence between co-formulated drug products and the reference, single-entity products administered in combination. Going forward, as dissolution methodologies with *in*

vivo relevancy are developed, this work lays the foundation for *in vitro* assessments of bioequivalence to broadly replace the complex *in vivo* studies currently required for drug combinations; and, as a result, can lead to increased availability of more effective combination drug products, especially in developing countries where such products can help address health care disparities and significant unmet medical need.

Overall Conclusions and Significance

Overall, in order to contribute to the advancement of antiretroviral therapy, this dissertation presents research pertaining to both early- and late-stage antiviral product development by: (1) emphasizing opportunities for molecular targeting to impede HIV infection and (2) providing a biopharmaceutical rationale to accelerate the development of co-formulated antiretroviral drug products.

The following research objectives were accomplished, as elaborated in this dissertation:

- Production, characterization, and optimization of the infectivity of single-cycle HIV-1,
- Quantitation of the efficiency of HIV-1 infection,
- Investigation of envelope-dependent mechanism(s) influencing HIV-1 binding/infection,
- Provisional biopharmaceutical subclassification of single-entity and fixed dose antiretroviral combination drug products, and

- Quantitative impact assessment to support scientific rationale for abbreviated bioequivalence and dissolution testing recommendations for single-entity and fixed-dose antiretroviral combination drug products.

The results from both aspects of this research provide a current and forward-looking view of antiretroviral therapy that will aid the development of therapeutic approaches to combat one of the world's most serious viral infections.

REFERENCES

1. **Joint UUNAIDS.** *Q&A II: Basic facts about the AIDS epidemic and its impact.* 2004. UNAIDS Questions & Answers.

2. **Sepkowitz.** 23, 2001, The New England Journal of Medicine, Vol. 344, pp. 1764-1772.

3. **Fauci.** 2008, Nature, Vol. 453.

4. **U.S. Department of Health & Human Services.** Global AIDS Overview. *AIDS.gov.* [Online] June 1, 2012. [Cited: 18 July, 2012.] http://aids.gov/federal-resources/around-the-world/global-aids-overview/.

5. **World Health Organization.** *HIV/AIDS Fact sheet No 360.* 2014.

6. *Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome.* **Barré-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vézinet-Brun F, Rouzioux C, Rozenbaum W, Montagnier L.** 4599, May 20, 1983, Science, Vol. 220, pp. 868-871.

7. **World Health Organization, UNAIDS, Unicef.** *GLOBAL HIV/AIDS RESPONSE Epidemic update and health sector progress towards Universal Access Progress Report 2011.* 2011.

8. **Joint United Nations Programme on HIV/AIDS (UNAIDS).** *Global Report UNAIDS Report on the Global AIDS Epidemic 2013.* 2013.

9. *Cell Biology of HIV-1 Infection of Macrophages.* **Ehrlich, Carol A. Carter and Lorna S.** 2008, Annual Review of Microbiology, Vol. 62, pp. 425–443.

10. *T cell depletion in HIV-1 infection: how CD4+ T cells go out of stock.* **Hazenberg MD, Hamann D, Schuitemaker H, Miedema F.** 4, October 2000, Nature Immunology, Vol. 1, pp. 285 - 289.

11. **Los Alamos National Lab.** Introduction (PART I. Nucleotide Alignments of HIV-1/SIVcpz Complete Genomes). *The Human Retroviruses and AIDS 1999 Compendium.* 1999.

12. *Immunopathogenic mechanisms of HIV infection.* **Fauci AS, Pantaleo G, Stanley S, Weissman D.** 7, April 1, 1996, Annals fo Internal Medicine, Vol. 124, pp. 654 - 663.

13. **National Institute of Allergy and Infectious Diseases.** Clinical Progression of HIV. *NIAID.NIH.gov.* [Online] March 28, 2011. [Cited: July 10, 2012.] http://www.niaid.nih.gov/topics/HIVAIDS/Understanding/Biology/Pages/clinicalCourse.aspx.

14. **Los Alamos National Lab.** *INTRODUCTION (The Human Retroviruses and AIDS 1996 Compendium).* 1996.

15. —. *INTRODUCTION (The Human Retroviruses and AIDS 2011 Compendium).* 2011.

16. *Quantification of CD4, CCR5, and CXCR4 levels on lymphocyte subsets, dendritic cells, and differentially conditioned monocyte-derived macrophages.* **Lee B, Sharron M, Montaner LJ, Weissman D, Doms RW.** April 1999, Proc. Natl. Acad. Sci., Vol. 96, pp. 5215 – 5220.

17. *Delineation of a region of the human immunodeficiency virus type 1 gp120 glycoprotein critical for interaction with the CD4 receptor.* **Lasky LA, Nakamura G, Smith DH, Fennie C, Shimasaki C, Patzer E, Berman P, Gregory T, Capon DJ.** 6, September 11, 1987, Cell, Vol. 50, pp. 975 - 985.

18. *CD4, CXCR-4, and CCR-5 dependencies for infections by primary patient and laboratory-adapted isolates of human immunodeficiency virus type 1.* **Kozak SL, Platt EJ, Madani N, Ferro FE Jr, Peden K, Kabat D.** 2, Feb 1997, J Virol., Vol. 71, pp. 873 - 882.

19. *CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5.* **Wu L, Gerard NP, Wyatt R, Choe H, Parolin C, Ruffing N, Borsetti A, Cardoso AA, Desjardin E, Newman W, Gerard C, Sodroski J.** 6605, November 14, 1996, Nature, Vol. 384, pp. 179 - 183.

20. *Identification of a major co-receptor for primary isolates of HIV-1.* **Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, Davis CB, Peiper SC, Schall TJ, Littman DR, Landau NR.** 6584, June 20, 1996, Nature, Vol. 381, pp. 661 - 666.

21. *HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor.* **Feng Y, Broder CC, Kennedy PE, Berger EA.** 5263, May 10, 1996, Science, Vol. 272, pp. 872 - 877.

22. *Factors underlying spontaneous inactivation and susceptibility to neutralization of human immunodeficiency virus.* **Layne SP, Merges MJ, Dembo M, Spouge JL, Conley SR, Moore JP, Raina JL, Renz H, Gelderblom HR, Nara PL.** 2, August 1992, Virology, Vol. 189, pp. 695 - 714.

23. *Quantitation of human immunodeficiency virus type 1 infection kinetics.* **Dimitrov DS, Willey RL, Sato H, Chang LJ, Blumenthal R, Martin MA.** 4, April 1993, J. Virol., Vol. 67, pp. 2182 - 2190.

24. *Quantification of infectious HIV-1 plasma viral load using a boosted in vitro infection protocol.* **Rusert P, Fischer M, Joos B, Leemann C, Kuster H, Flepp M, Bonhoeffer S, Günthard HF, Trkola A.** 1, August 15, 2004, Virology, Vol. 326, pp. 113 - 129.

25. *Relationships between infectious titer, capsid protein levels, and reverse transcriptase activities of diverse human immunodeficiency virus type 1 isolates.* **Marozsan AJ, Fraundorf E, Abraha A, Baird H, Moore D, Troyer R, Nankja I, Arts EJ.** 20, Oct 2004, J. Virol., Vol. 78, pp. 11130 - 11141.

26. *Host hindrance to HIV-1 replication in monocytes and macrophages.* **Bergamaschi A, Pancino G.** 31, April 7, 2010, Retrovirology, Vol. 7.

27. *Human immunodeficiency virus type 1 spinoculation enhances infection through virus binding.* **O'Doherty U, Swiggard WJ, Malim MH.** 21, November 2000, J. Virol., Vol. 74, pp. 10074 - 10080.

28. *Enhancement and inhibition of avian sarcoma viruses by polycations and polyanions.* **Toyoshima K, Vogt PK.** 3, July 1969, Virology, Vol. 38, pp. 414 - 426.

29. *Complexation of retrovirus with cationic and anionic polymers increases the efficiency of gene transfer.* **Le Doux JM, Landazuri N, Yarmush ML, Morgan JR.** 13, September 1, 2001, Human Gene Therapy, Vol. 12, pp. 1611 - 1621.

30. *Host-derived ICAM-1 glycoproteins incorporated on human immunodeficiency virus type 1 are biologically active and enhance viral infectivity.* **Fortin JF, Cantin R, Lamontagne G, Tremblay M.** 5, May 1997, J. Virol., Vol. 71, pp. 3588 - 3596.

31. *Level of ICAM-1 surface expression on virus producer cells influences both the amount of virion-bound host ICAM-1 and human immunodeficiency virus type 1 infectivity.* **Paquette JS, Fortin JF, Blanchard L, Tremblay MJ.** 11, November 1998, J. Virol., Vol. 72, pp. 9329 - 9336.

32. *Increased infectivity of HIV type 1 particles bound to cell surface and solid-phase ICAM-1 and VCAM-1 through acquired adhesion molecules LFA-1 and VLA-4.* **Liao Z, Roos JW, Hildreth JE.** 4, March 1, 2000, AIDS Research and Human Retroviruses, Vol. 16, pp. 355 - 366.

33. *Positive and negative modulation of virus infectivity and envelope glycoprotein incorporation into virions by amino acid substitutions at the N terminus of the simian immunodeficiency virus matrix protein.* **Manrique JM, Celma CC, Hunter E, Affranchino JL, González SA.** 20, October 2003, Journal of Viriology, Vol. 77, pp. 10881 - 10888.

34. *Modulation of Env content in virions of simian immunodeficiency virus: correlation with cell surface expression and virion infectivity.* **Yuste E, Reeves JD, Doms RW, Desrosiers RC.** 13, July 2004, Journal of Virology, Vol. 78, pp. 6775 - 6785.

35. *Cell surface CCR5 density determines the postentry efficiency of R5 HIV-1 infection.* **Lin YL, Mettling C, Portales P, Reynes J, Clot J, Corbeau P.** 24, November 26, 2002, Proceedings of the National Academy of Sciences of the United States of America, Vol. 99, pp. 15590 - 15595.

36. *Rapid dissociation of HIV-1 from cultured cells severely limits infectivity assays, causes the inactivation ascribed to entry inhibitors, and masks the inherently high level of infectivity of virions.* **Platt EJ, Kozak SL, Durnin JP, Hope TJ, Kabat D.** 6, March 2010, Journal of Virology, Vol. 84, pp. 3106 - 3110.

37. **Department of Health and Human Services.** *Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents.* 2015.

38. *HIV persistence and the prospect of long-term drug-free remissions for HIV-infected individuals.* **Trono D, Van Lint C, Rouzioux C, Verdin E, Barré-**

Sinoussi F, Chun TW, Chomont N. 5988, July 9, 2010, Science, Vol. 329, pp. 174 - 180.

39. *Risk factors for gastrointestinal adverse events in HIV treated and untreated patients.* **Hill A, Balkin A.** 1, January - March 2009, AIDS Reviews, Vol. 11, pp. 30 - 38.

40. *Dyslipidemia related to antiretroviral therapy.* **Estrada V, Portilla J.** 1, January - March 2011, AIDS Reviews, Vol. 13, pp. 49-56.

41. *Combination antiretroviral therapy and the risk of myocardial infarction.* **Friis-Møller N, Sabin CA, Weber R, d'Arminio Monforte A, El-Sadr WM, Reiss P, Thiébaut R, Morfeldt L, De Wit S, Pradier C, Calvo G, Law MG, Kirk O, Phillips AN, Lundgren JD and Group., Data Collection on Adverse Events of Anti-HIV Drugs (DAD) Study.** 21, November 20, 2003, New England Journal of Medicine, Vol. 349, pp. 1993 - 2003.

42. *Antiretroviral therapy 2010 update: current practices and controversies.* **Grant M, Samuel R, Bettiker RL, Suh B.** 7, July 2011, Archives of Pharmacal Research, Vol. 34, pp. 1045 - 1053.

43. *Few and Far Between: How HIV May Be Evading Antibody Avidity.* **Klein JS, Bjorkman PJ.** 5, May 27, 2010, Vol. 6, p. e1000908.

44. *Adherence to Protease Inhibitor Therapy and Outcomes in Patients with HIV Infection.* **Paterson, D.L., et al., et al.** 1, Jul 4, 2000, Ann Intern Med, Vol. 133, pp. 21-30.

45. *Adherence to Protease Inhibitors, HIV-1 Viral Load, and Development of Drug Resistance in an Indigent Population.* **Bangsberg, D. R., et al., et al.** 2000, AIDS, Vol. 14, pp. 357-366.

46. *Increase in single-tablet regimen use and associated improvements in adherence-related outcomes in HIV-infected women.* **Hanna, D.B., et al., et al.** 5, April 15, 2014, J Acquir Immune Defic Syndr, Vol. 65, pp. 587-96.

47. *Expanding Role of Coformulations in the Treatment of HIV Infection: Impact of Fixed-dose Combinations.* **Valenti, W.M.** 10, October 2004, AIDS Read, Vol. 14.

48. **National Institues of Health.** HIV Treatment, FDA-Approved HIV Medicines. *AIDS Info.* [Online] September 24, 2014.

49. *Frequency of direct repeat deletion in a human immunodeficiency virus type 1 vector during reverse transcription in human cells.* **An W, Telesnitsky A.** 2001, Virology, Vol. 286, pp. 475 - 482.

50. *Fidelity of HIV-1 reverse transcriptase.* **Preston BD, Poiesz BJ, Loeb LA.** 1988, Science, Vol. 242, pp. 1168 - 1171.

51. *The accuracy of reverse transcriptase from HIV-1.* **Roberts JD, Bebenek K, Kunkel TA.** 1988, Science, Vol. 242, pp. 1171 - 1173.

52. *The APOBEC3 cytidine deaminases: an innate defensive network opposing exogenous retroviruses and endogenous retro-elements.* **Chiu YL, Greene WC.** 2008, Annu Rev Immunol, Vol. 26, pp. 317 - 353.

53. *The restriction factors of human immunodeficiency virus.* **Harris RS, Hultquist JF, Evans DT.** 2012, J Biol Chem, Vol. 287, pp. 40875 - 40883.

54. *Optimized Infectivity of the Cell-Free Single-Cycle Human Immunodeficiency Viruses Type 1 (HIV-1) and Its Restriction by Host Cells.* **Kim JH, Song H, Austin JL, Cheng W.** 6, June 18, 2013, PLoS One, Vol. 8, p. e67170.

55. **Montefiori, David C.** Neutralizing Antibody Assays for HIV-1, SIV and SHIV: Recent Advances in Technology. *Curr. Protoc. Immunol.* September 16, 2006.

56. *Development and implementation of an international proficiency testing program for a neutralizing antibody assay for HIV-1 in TZM-bl cells.* **Todd CA, Greene KM, Yu X, Ozaki DA, Gao H, Huang Y, Wang M, Li G, Brown R, Wood B, D'Souza MP, Gilbert P, Montefiori DC, Sarzotti-Kelsoe M.** 1-2, January 31, 2012, Journal of Immunological Methods, Vol. 375, pp. 57 - 67.

57. *Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated beta-galactosidase gene.* **Kimpton J, Emerman M.** 4, 1992, Journal of Virology, Vol. 66, pp. 2232 -2239.

58. *Measurement of HIV-1 p24 antigen by signal-amplification-boosted ELISA of heat-denatured plasma is a simple and inexpensive alternative to tests for viral RNA.* **J, Schüpbach.** 2, April - June 2002, AIDS Reviews, Vol. 4, pp. 83-92.

59. *Three-dimensional analysis of budding sites and released virus suggests a revised model for HIV-1 morphogenesis.* **Carlson LA, Briggs JA, Glass B,**

Riches JD, Simon MN, Johnson MC, Müller B, Grünewald K, Kräusslich HG. 6, Dec 11, 2008, Cell Host Microbe, Vol. 4, pp. 592 - 599.

60. *The molecular architecture of HIV.* **Briggs JA, Kräusslich HG.** 4, Jul 22, 2011, J Mol Biol, Vol. 410, pp. 491 - 500.

61. *HIV-1: Fifteen Proteins and an RNA.* **Frankel, Young.** July 1998, Annual Review of Biochemistry, Vol. 67, pp. 1-25.

62. *Proteolytic refolding of the HIV-1 capsid protein amino-terminus facilitates viral core assembly.* **von Schwedler UK, Stemmler TL, Klishko VY, Li S, Albertine KH, Davis DR, Sundquist WI.** 6, March 16, 1998, The EMBO Journal, Vol. 17, pp. 1555-1568.

63. *Production of human immunodeficiency virus type 1 (HIV-1) pseudoviruses using linear HIV-1 envelope expression cassettes.* **Beels D, Heyndrickx L, Vereecken K, Vermoesen T, Michiels L, Vanham G, Kestens L.** 1, January 2008, Journal of Virological Methods, Vol. 147, pp. 99-107.

64. **Munk C, Landau NR.** Production and Use of HIV-1 Luciferase Reporter. *Curr Protoc Pharmacol.* 2003, 12: Unit 12.15.

65. *Breaking up the correlation between efficacy and toxicity for nonviral gene delivery.* **Breunig M, Lungwitz U, Liebl R, Goepferich A.** 36, Sep 4, 2007, Proc Natl Acad Sci U S A, Vol. 104, pp. 14454 - 9.

66. *Envelope glycoprotein incorporation, not shedding of surface envelope glycoprotein (gp120/SU), Is the primary determinant of SU content of purified human immunodeficiency virus type 1 and simian immunodeficiency virus.*

Chertova E, Bess JW Jr, Crise BJ, Sowder II RC, Schaden TM, Hilburn JM, Hoxie JA, Benveniste RE, Lifson JD, Henderson LE, Arthur LO. 11, Jun 2002, J Virol, Vol. 76, pp. 5315 - 25.

67. *Efficiency of human immunodeficiency virus type 1 postentry infection processes: evidence against disproportionate numbers of defective virions.* **Thomas JA, Ott DE, Gorelick RJ.** 8, April 2007, J Virol, Vol. 81, pp. 4367-70.

68. *Cytosolic Gag p24 as an index of productive entry of human immunodeficiency virus type 1.* **Maréchal V, Clavel F, Heard JM, Schwartz O.** 3, 1998 : s.n., March, J Virol., Vol. 72, pp. 2208-12.

69. *DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells.* **Geijtenbeek TB, Kwon DS, Torensma R, van Vliet SJ, van Duijnhoven GC, Middel J, Cornelissen IL, Nottet HS, KewalRamani VN, Littman DR, Figdor CG, van Kooyk Y.** 5, March 3, 2000, Cell, Vol. 100, pp. 587-97.

70. *Human immunodeficiency virus type 1 attachment to HeLa CD4 cells is CD4 independent and gp120 dependent and requires cell surface heparans.* **Mondor I, Ugolini S, Sattentau QJ.** 5, May 1998, J. Virol., Vol. 72, pp. 3623-34.

71. *Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1.* **Platt, E. J., K. Wehrly, S. E. Kuhmann, B. Chesebro, and D. Kabat.** 2, 1998, J Virol, Vol. 72, pp. 2855–2864.

72. *Evidence that ecotropic murine leukemia virus contamination in TZM-bl cells does not affect the outcome of neutralizing antibody assays with human immunodeficiency virus type 1.* **Platt EJ, Bilska M, Kozak SL, Kabat D, Montefiori DC.** 16, Aug 2009, J Virol, Vol. 83, pp. 8289-92.

73. *Chemokine/CD4 receptor density ratios correlate with HIV replication in lymph node and peripheral blood of HIV-infected individuals.* **Nokta MA, Li XD, Nichols J, Mallen M, Pou A, Asmuth D, Pollard RB.** 2, Jan 26, 2001, AIDS, Vol. 15, pp. 161-9.

74. **U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research/Office of Medical Products and Tobacco/Office of Generic Drugs.** *Approved Drug Products with Therapeutic Equivalence Evaluations 35th Edition.* 2015.

75. **European Medicines Agency, Committe for Medicinal Products for Human Use.** *GUIDELINE ON THE INVESTIGATION OF BIOEQUIVALENCE.* London : s.n., 2010. CPMP/EWP/QWP/1401/98 Rev. 1/ Corr.

76. *A Theoretical Basis for a Biopharmaceutic Drug Classification: The Correlation of In Vitro Drug Product Dissolution and In Vivo Bioavailability.* **Amidon G.L., Lennernas H., Shah V.P., Crison J.R.** 1995, Pharm Res, Vol. 12, pp. 413-420.

77. **U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER).** *Waiver of*

In Vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System. 2000.

78. —. *Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System Guidance for Industry, DRAFT GUIDANCE.* 2015. Revision 1.

79. —. *Guidance for Industry Fixed Dose Combinations, Co-Packaged Drug Products, and Single-Entity Versions of Previously Approved Antiretrovirals for the Treatment of HIV.* 2006.

80. **World Health Organization, UNAIDS, UNICEF.** *Global HIV/AIDS Response - Epidemic Update and Health Sector Progress towards Universal Access - Progress Report 2011.* 2011.

81. **U.S. Department of Health & Human Services.** HIV/AIDS Basics / HIV/AIDS 101: Glbal Statistics. *www.aids.gov.* [Online] November 13, 2014. [Cited: June 05, 2015.] https://www.aids.gov/hiv-aids-basics/hiv-aids-101/global-statistics/.

82. **World Health Organization.** *Consolidated Strategic Information Guidelines for HIV in the Health Sector.* 2015.

83. —. *Global Update on the Health Sector Response to HIV, 2014.* 2014.

84. *Fast Track Approaches for Drug Approval across the Globe.* **Duggal, E., et al., et al.** 1, 2014, Asian Pacific Journal of Health Sciences, Vol. 1, pp. 2-12.

85. **WHO Prequalification Team - Medicines.** *General Notes on Biopharmaceutics Classification System (BCS)-based Biowaiver Applications.* 2014.

86. *The United States Pharmacopeia, 24th ed.* 24th ed. Philadelphia : by authority of the United States Pharmacopeial Convention, Inc.; printed by National Publishing, 2000.

87. *Molecular Properties of WHO Essential Drugs and Provisional Biopharmaceutical Classification.* **Kasim, N.A., et al., et al.** 1, Jan 12, 2004, Molecular Pharmaceutics, Vol. 1, pp. 85-96.

88. *Purely in Silico BCS Classification: Science Based Quality Standards for the World's Drugs.* **Dahan, A., et al., et al.** 11, Nov 4, 2013, Molecular Pharmaceutics, Vol. 10, pp. 4378-90.

89. *Pharmacokinetic Studies on the Selective Beta1-receptor Antagonist Metoprolol in Man.* **Regårdh, C.G., et al., et al.** 4, August 1974, J Pharmacokinet Biopharm, Vol. 2, pp. 347-64.

90. *The Biopharmaceutics Classification System: Subclasses for in Vivo Predictive Dissolution (IPD) Methodology and IVIVC.* **Tsume, Y., et al., et al.** June 116, 2014, Eur J Pharm Sci., Vol. 57, pp. 152-63.

91. **U.S. Food and Drug Administration.** Approved and Tentatively Approved Antiretrovirals in Association with the President's Emergency Plan. *www.fda.gov.* [Online] May 28, 2015. [Cited: June 5, 2015.] http://www.fda.gov/internationalprograms/pepfar/ucm119231.htm.

92. **WHO Prequalification Programme.** Home - Prequalification: About. *apps.who.int.* [Online] [Cited: June 5, 2015.] http://apps.who.int/prequal/.

93. *Biorelevant in vitro performance testing of orally administered dosage forms-workshop report.* **Reppas, C., et al., et al.** 7, July 2014, Pharm Res, Vol. 31, pp. 1867-76.