

ELBOURAKADI, NAJOUA, Ph.D. A Novel Method For The Selective Elimination Of HIV Infected Cells: Dexamethasone And Procaine As A Combination Therapy Prototype. (2011)

Directed by Dr. Ethan W. Taylor. 148 pp.

It has been established that various combinations of three or more antiretroviral drugs will lead to durable inhibition of HIV replication in patients. Despite its success in controlling the infection, highly active antiretroviral therapy (HAART) does not eradicate the virus; systemic infection re-emerges upon treatment interruption in all but exceptional cases, necessitating a lifetime of expensive drug therapy with an ever-present risk of emerging drug resistance.

Using standard colorimetric and fluorometric live/dead cell labeling methods, we have developed a novel “selective cell death” assay, and identified a lead drug combination for elimination of infected cells, involving a patented combination of 2 generic FDA approved drugs or their metabolites: the glucocorticoid dexamethasone (Dex) and N,N-diethylaminoethanolamine (DEAE, a metabolite of procaine). Neither drug alone, but only the combination, is effective in cell killing, and only in HIV-infected cells ($P < 0.0001$). Furthermore, prolonged exposure of cells to this drug combination leads to a decline in viral load; two weeks of treatment results in a decrease of more than 50% relative to untreated control cells. Flow cytometric methods indicate that the mechanism of infected cell death is via apoptosis. In addition, microarray analysis has highlighted several host genes whose expression is altered by Dex/DEAE treatment, providing initial clues to understanding the pathways leading to apoptosis in infected cells.

A NOVEL METHOD FOR THE SELECTIVE ELIMINATION
OF HIV INFECTED CELLS: DEXAMETHASONE
AND PROCAINE AS A COMBINATION
THERAPY PROTOTYPE

by

Najoua Elbourakadi

A Dissertation Submitted to
the Faculty of the Graduate School at
the University of North Carolina at Greensboro
In Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

Greensboro
2011

Approved by

Committee Chair

APPROVAL PAGE

This dissertation has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair _____

Ethan Will Taylor, Ph.D.

Committee Members _____

Nadja Cech, Ph.D.

Dennis Lajeunesse, Ph.D.

Vince Henrich, Ph.D.

06/17/2011
Date of Acceptance by Committee

06/17/2011
Date of Final Oral Examination

ACKNOWLEDGMENTS

I would like to take this opportunity to thank the following people for their support patience and guidance. It is to them that I owe my deepest gratitude:

- Dr. Taylor for his unfailing support as my advisor and friend, without his guidance, wisdom and knowledge this work would not have been possible.
- The other members of my graduate research committee: Dr. Nadja Cech, Dr. Vince Henrich and Dr. Dennis LaJeunesse, for various valuable suggestions.
- The members of my lab who provided valuable help to my research especially Dr. Lijun Zhao, Dr. Jan Ruzicka and Dr. Wilson Radding.
- To all my friends & family especially my siblings Zora, Kyle and Zakaria Elbourkadi and my fiancé Yassine for the infinite love and support.

Last but not least a special thank goes to my parents Khadija and Mohamed Elbourkadi who so lovingly encouraged and believed in me in all my endeavours.

TABLE OF CONTENTS

CHAPTER	Page
I. INTRODUCTION: HIV AND AIDS EPIDEMIC	1
Basic Virology of HIV	8
Therapeutic Approaches to HIV	10
New Treatment Modalities	13
II. INNOVATION AND DRUGS USED.....	15
Innovation	15
Drugs Used and Drug Dilutions.....	17
III. HIV SELECTIVE TOXICITY (SELTOX) ASSAY	21
Materials and Methods.....	21
Results	22
IV. INHIBITION OF HIV REPLICATION	27
Materials and Methods	27
Results	28
Pro/Dex Combination to Inhibit Viral Replication in TZM-bl Cells	29
Pro/Dex Activity Against Resistant Strains	33
V. FLUORESCENCE EVIDENCE OF THE CELL VIABILITY	36
Materials and Methods	36
Results	37
Summary	43
VI. MECHANISM OF CELL DEATH	44
Annexin V FITC	45
Materials and Methods	45
Results	48
Double Stain Apoptosis Detection Assay (Hoechst 33342/PI)	53
Materials and Methods.....	53
Results	59

Discussion	63
VII. DNA MICROARRAYS AND GENES INVOLVED	64
Materials and Methods.....	65
Microarray Data Analysis	71
Results	81
VIII. SUMMARY AND FUTURE RESEARCH DIRECTIONS	85
Summary	85
Future Research Directions	87
Q-PCR/RT PCR	87
RNAi	87
REFERENCES	89
APPENDIX A: GENES AFFECTED IN INFECTED CELLS WITH DEX/DEAE.....	95
APPENDIX B: SELECTIVE TOXICITY ASSAY	113

CHAPTER I

INTRODUCTION: HIV AND AIDS EPIDEMIC

In the early 1980s, public health officials began using the term Acquired Immune Deficiency Syndrome (AIDS) to refer to a new syndrome characterized by a progressive deterioration of the immune system due to a loss of the CD4⁺ helper/inducer subset of T lymphocytes. This loss leads to a severe destruction of the immune functions, and the appearance of multiple opportunistic infections in the form of constitutional diseases, opportunistic infections, neurological complications (AIDS dementia complex), and neoplasms that rarely happen in persons with undamaged immune functions [1]. This disease state was caused by human immunodeficiency virus HIV, a lentivirus subfamily of retroviruses that produces chronic infection in the host and gradually damages the host's immune system [2]. Three major strains of immunodeficiency-associated lentiviruses have been characterized in primates: simian immunodeficiency virus (SIV) and among humans, HIV-1, the predominant type in the world, and HIV-2, mostly located in Western Africa and India[1].

Nearly thirty years after the discovery of HIV/AIDS, the number of people infected in the world has grown rapidly turning the disease into a very complex epidemic, and one of the biggest health crises facing the world today.

Estimating HIV's prevalence is notoriously complicated, frustrated by the challenge of acquiring sound statistical data from struggling clinics in developing

countries [3]. Keeping these difficulties in mind, recent estimates suggest there were 33.3 million [31.4 million–35.3 million] people living with HIV at the end of 2009 [4].

Levels of infection vary greatly across and within many regions of the globe.

Significant research has been directed at understanding geographical HIV transmission, often to aid in the design of rational prevention and treatment strategies. With an estimated 15 million HIV-positive people living in low- and middle-income countries today, well tailored means of effective transmission reduction and AIDS treatment are vital [5, 6].

Table 1: Regional HIV and AIDS statistics, 2001 and 2009[4]

		Adults and children living with HIV	Adults and children newly infected with HIV	% Adult prevalence (15–49 years)	AIDS-related deaths among adults and children
SUB-SAHARAN AFRICA	2009	22.5 million [20.9–24.2 million]	1.8 million [1.6–2.0 million]	5.0 [4.7–5.2]	1.3 million [1.1–1.5 million]
	2001	20.3 million [18.9–21.7 million]	2.2 million [1.9–2.4 million]	5.9 [5.6–6.1]	1.4 million [1.2–1.6 million]
MIDDLE EAST AND NORTH AFRICA	2009	460 000 [400 000–530 000]	75 000 [61 000–92 000]	0.2 [0.2–0.3]	24 000 [20 000–27 000]
	2001	180 000 [150 000–210 000]	36 000 [32 000–42 000]	0.1 [0.1–0.1]	8300 [6300–11 000]
SOUTH AND SOUTH-EAST ASIA	2009	4.1 million [3.7–4.6 million]	270 000 [240 000–320 000]	0.3 [0.3–0.3]	260 000 [230 000–300 000]
	2001	3.8 million [3.5–4.2 million]	380 000 [350 000–430 000]	0.4 [0.3–0.4]	230 000 [210 000–280 000]
EAST ASIA	2009	770 000 [560 000–1.0 million]	82 000 [48 000–140 000]	0.1 [0.1–0.1]	36 000 [25 000–50 000]
	2001	350 000 [250 000–480 000]	64 000 [47 000–88 000]	<0.1 [<0.1–<0.1]	15 000 [9400–28 000]
OCEANIA	2009	57 000 [50 000–64 000]	4500 [3400–6000]	0.3 [0.2–0.3]	1400 [<1000–2400]
	2001	29 000 [23 000–35 000]	4700 [3800–5600]	0.2 [0.1–0.2]	<1000 [<500–1100]
CENTRAL AND SOUTH AMERICA	2009	1.4 million [1.2–1.6 million]	92 000 [70 000–120 000]	0.5 [0.4–0.6]	58 000 [43 000–70 000]
	2001	1.1 million [1.0–1.3 million]	99 000 [85 000–120 000]	0.5 [0.4–0.5]	53 000 [44 000–65 000]

CARIBBEAN	2009	240 000 [220 000–270 000]	17 000 [13 000–21 000]	1.0 [0.9–1.1]	12 000 [8500–15 000]
	2001	240 000 [210 000–270 000]	20 000 [17 000–23 000]	1.1 [1.0–1.2]	19 000 [16 000–23 000]
EASTERN EUROPE AND CENTRAL ASIA	2009	1.4 million [1.3–1.6 million]	130 000 [110 000–160 000]	0.8 [0.7–0.9]	76 000 [60 000–95 000]
	2001	760 000 [670 000–890 000]	240 000 [210 000–300 000]	0.4 [0.4–0.5]	18 000 [14 000–23 000]
WESTERN AND CENTRAL EUROPE	2009	820 000 [720 000–910 000]	31 000 [23 000–40 000]	0.2 [0.2–0.2]	8500 [6800–19 000]
	2001	630 000 [570 000–700 000]	31 000 [27 000–35 000]	0.2 [0.2–0.2]	7300 [5700–11 000]
NORTH AMERICA	2009	1.5 million [1.2–2.0 million]	70 000 [44 000–130 000]	0.5 [0.4–0.7]	26 000 [22 000–44 000]
	2001	1.2 million [960 000–1.4 million]	66 000 [54 000–81 000]	0.4 [0.4–0.5]	30 000 [26 000–35 000]
TOTAL	2009	33.3 million [31.4–35.3 million]	2.6 million [2.3–2.8 million]	0.8 [0.7–0.8]	1.8 million [1.6–2.1 million]
	2001	28.6 million [27.1–30.3 million]	3.1 million [2.9–3.4 million]	0.8 [0.7–0.8]	1.8 million [1.6–2.0 million]

Globally, the epidemic was thought to have peaked in 1999 with 3.1 million [2.9 million–3.4 million]. Since then the number of new infections has been steadily declining and reaching an estimated 2.6 million [2.3 million–2.8 million] people annually in 2009, representing approximately one fifth (19%) fall from the peak [4].

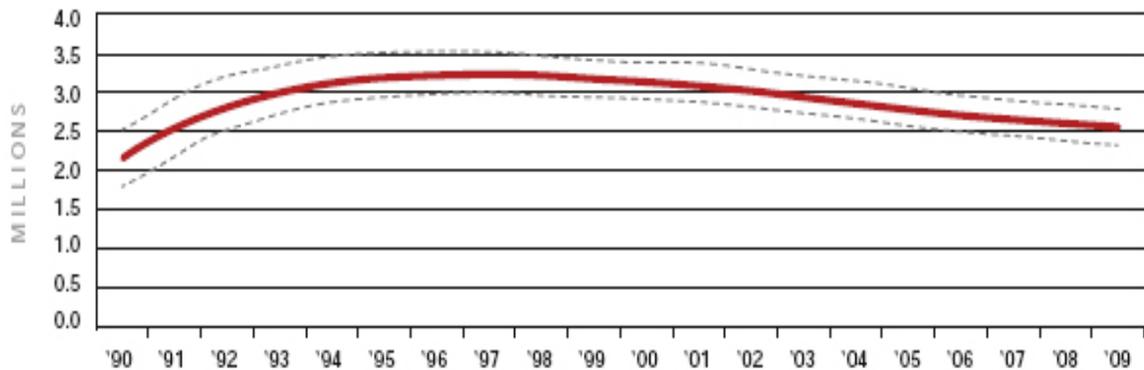


Figure 1: Number of people newly infected annually with HIV[4]

The epidemic disproportionately affects sub-Saharan Africa (SSA) a region that comprises 22 of the 33 countries most afflicted with AIDS. Despite an overall reduction in transmission rate of 25%, the number of people living with HIV keeps escalating, reaching 22.5 million [20.9 million–24.2 million] in 2009[4].

In these sub-Saharan countries, an estimated 1.8 million [1.6 million–2.0 million] people became infected in 2009; a significant improvement from 2.2 million [1.9 million–2.4 million] in 2001 [4]. These changes are the results of a combination of factors, from the impact of colossal HIV prevention efforts and the natural course of HIV epidemics.

Unfortunately not all regions and countries follow this positive overall trend. Seven countries saw an increase in HIV incidence of greater than 25% between 2001 and

2009. In other regions, such as Central Asia, North America, and Europe, the rates of new HIV infections have remained constant for the past five years [4].

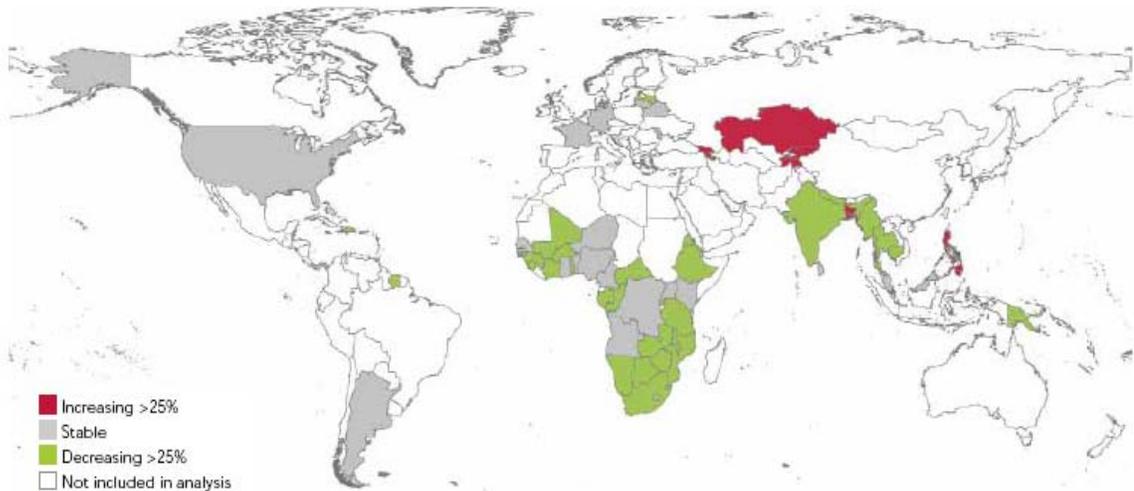


Figure 2: Changes in the incidence rate of HIV infection, 2001 to 2009, selected countries)[4]

Global HIV infection rates have declined by an impressive 19% during the last decade due to dedicated efforts to endorse and sustain HIV prevention methods that support behavioral changes among young people, such as increased condom use, delayed sexual debut, and reductions in multiple partnerships [4]. These traditional preventative methods are combined with new tools to expand and reinforce effective HIV prevention, such as:

- Prevention of mother to child transmission among women living with HIV in countries with high infection rates.
- Male circumcision: clinical trials demonstrate that male circumcision significantly decreases the probability of uninfected men catching HIV from an HIV-infected female sex partner. UNAIDS and WHO have both suggested insistent promotion

of male circumcision in areas of high HIV prevalence, where rates of circumcision have been historically low.

- Microbicides: the Centre for the AIDS Programme of Research in South Africa (CAPRISA) has announced promising results of a tenofovir-based gel for a new female-initiated prevention option that reduces HIV infection by 39% and herpes simplex virus-2 infection by 51%. The gel also passed safety tests when used once within the 12 hours before sex and once in the 12 hours after sex by women aged 18–40 years [4].

Although results of the preventative methods have been very promising, investments in HIV prevention programs are still insufficient. The trajectory of the epidemic cannot be reversed without augmented investment in additional prevention programs.

Along with advances in preventive therapy and advances in treatment, improvements in care and support services were also a considerable achievement in 2009. Among the estimated 15 million people living with HIV in low and middle income countries, 36% (about 5.2 million) had access to anti retroviral therapy. While this represents greater regional diversity than at any previous time during the epidemic period, universal access goals remain unachieved [4]. More efforts need to be done to cover the remaining two thirds of people who need HIV treatments but don't have access to them yet, and financing this expansion in access to antiretroviral therapy will require a continuous and prolonged global commitment to providing high quality HIV care for all.

Basic Virology of HIV

A thorough understanding of the biology of the virus allows a systematic development of therapeutic strategies targeted to the inhibition of virus proliferation. An understanding of the structure of HIV facilitates understanding of their high rate of mutation and the selectivity in their mode of infection and multiplication.

HIV-1 and HIV-2 are two distinct viruses with many similarities; their genomes are a mere 9.8 kilobases, coding for not more than a dozen proteins. The core and matrix of viral proteins are enclosed by a lipid envelope, that the virus particle derives from the host cell as it is detached from it. It also serves as a means of camouflage for the virus from the immune system, as it contains host cellular proteins such as HLA. The envelope also contains viral envelope glycoproteins, which are essential for infectivity and immunogenicity.

One of the main differences between the two strains (HIV-1 and HIV-2) is the fact that HIV-2 is about 55% different at the sequence levels from HIV-1 and is serologically distinct, mostly due to differences in the envelope glycoproteins [7].

Usually all retroviruses encode the genes *gag*, *pol* and *env*; in addition to those genes, lentivirus genomes encode a number of accessory and regulatory genes. HIV-1 encodes two regulatory proteins, Tat and Rev, and four accessory proteins [viral infectivity factor (Vif), viral protein R (Vpr), viral protein U (Vpu) and so-called negative factor (Nef)]. These regulatory proteins are necessary for virus replication as they control HIV gene expression in host cells. In contrast, accessory proteins are often dispensable for virus replication in vitro [8].

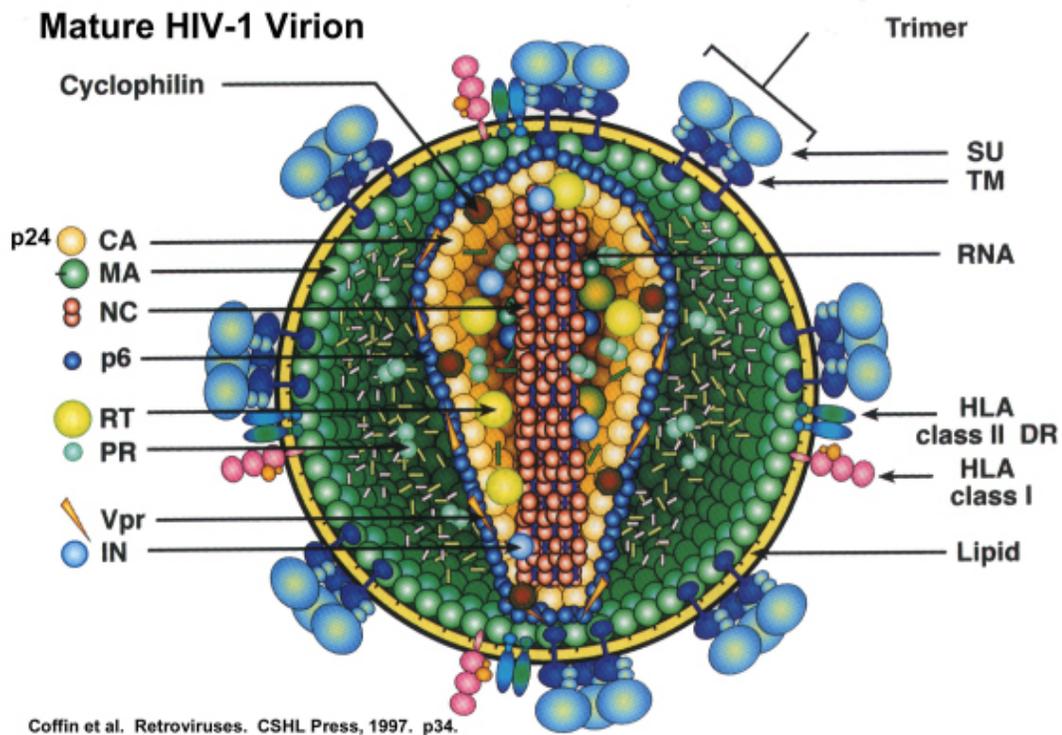


Figure 3: Virion structure: Schematic of the mature HIV-1 virion. The packaging of two copies of the viral RNA genome in each particle allows for viral recombination to occur during each replicative cycle [9].

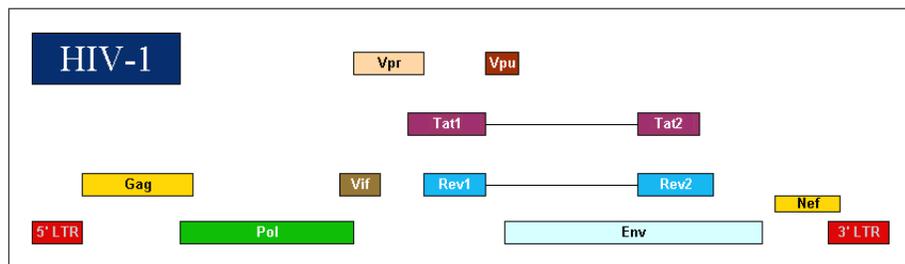


Figure 4: Genomic organization of HIV-1, shown as a map of the ~9.8 kb HIV-1 genome. A total of nine viral genes are encoded (gag, pol, vif, vpr, tat, rev, vpu, env, and nef). Gag, pol, and env are the defining features of the retroviral family, encoding capsid structural proteins, replicative enzymes, and surface glycoproteins, respectively [7].

Therapeutic Approaches to HIV

To date, more than 20 antiretroviral drugs have been approved for clinical use. These antiretrovirals fall into distinct mechanistic classes, which have been developed for their ability to reduce viral replication to clinically undetectable levels. When used in appropriate combinations, they can prevent the occurrence of AIDS-related symptoms, extending survival and improving the quality of life for HIV infected individuals. [10]

It has now been established that several different combinations of three or more antiretroviral drugs will lead to durable inhibition of HIV replication [11]. This approach for the treatment of HIV infection has become known as highly active antiretroviral therapy (HAART). The simultaneous use of multiple drugs is required due to the fact that HIV-1 can easily develop drug resistance to any single inhibitor.

Despite its success, HAART does not eradicate the virus; systemic infection re-emerges upon treatment interruption in all but exceptional cases [12]. Therefore, due to its virostatic nature, HAART can control but not cure HIV infection, necessitating a lifetime of expensive drug therapy to suppress viral replication, with an ever-present risk of emerging drug resistance.

Currently, five classes (as summarized in table 1) of antiretroviral drugs have received FDA approval: nucleoside/nucleotide analog reverse transcriptase inhibitors (NRTIs), nonnucleoside analog reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors and Integrase Inhibitors [13].

Table 2: Anti HIV FDA approved drugs

Antiretroviral drug class	First approved to treat HIV	Mechanism of action	Current Antiretrovirals Approved by the FDA
Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs, nukes)	1987	NRTIs are analogs of (RNA, DNA) that instead of the hydroxyl in the 3' position have another group that is unable to form the 5' to 3' phosphodiester linkage essential for DNA elongation. Therefore interfering with reverse transcriptase activity by competing with the natural substrates and incorporating into viral DNA to act as chain terminators in the synthesis of proviral DNA.	Zidovudine(ZDV)/ azidothymidine (AZT) Didanosine (ddI) Stavudine (d4T) Lamivudine (3TC) Abacavir (ABC) Tenofovir (TDF) Emtricitabine (FTC)
Non-Nucleoside Reverse Transcriptase Inhibitors(NNRTIs, non-nukes)	1997	NNRTIs bind to a site on the reverse transcriptase enzyme that is distinct from the substrate (dNTP) binding site and block DNA polymerase activity by causing a conformational change and disrupting the catalytic site of the enzyme.	Nevirapine (NVP) Delavirdine (DLV) Efavirenz (EFV)
Protease Inhibitors (PIs)	1995	PIs inhibit the protease enzyme responsible for the cleavage of the	Saquinavir (SQV) Indinavir (IDV)

		large viral precursor polypeptide chains into smaller, functional proteins, allowing maturation of the HIV virion.	Ritonavir (RTV) Nelfinavir (NFV) Lopinavir/r (LPV/r) Atazanavir (ATV)
		Consequently, inhibition of Protease results in the release of structurally disorganized and noninfectious viral particles.	Fosamprenavir (fAPV) Tipranavir (TPV) Darunavir (DRV)
Fusion or Entry Inhibitors	2003	Enfurvitide is a linear peptide homologous to a segment of the HR2 region of gp41. It binds to the HR1 region of gp41 and blocks the formation of the 6-helix bundle necessary for fusion.	Enfurvitide (T-20)
		Maraviroc blocks the chemokine receptor CCR5 that HIV uses as a coreceptor to bind and enter a target cell. Thus both drugs prevent HIV from binding to or entering human immune cells.	Maraviroc (Celsentri)
Integrase Inhibitors	2007	Integrase inhibitors interfere with the integrase enzyme that HIV needs to insert its genetic material within the nucleus of the T-cell.	Raltegravir

The development of drug resistance remains a central problem in the treatment of HIV, even with the use of combination chemotherapy (HAART) consisting of at least three different drugs. Because the HIV polymerase (reverse transcriptase) does not have operational proofreading mechanisms during replication of its genome, mutations at the protein level are common. Alterations in the proteins targeted by antiretrovirals can render the drugs ineffective against mutant viral progeny. Emerging resistance is often addressed therapeutically by utilizing at least three different drugs for HAART, attacking a vital viral protein at multiple sites, or targeting several proteins simultaneously.

As more people develop resistance to PIs and NRTIs/NNRTIs, the development of new treatment modalities has become critical. The anti-HIV drug discovery process, however, remains focused on screening small molecule, non-vaccine agents that are mainly targeted against viral proteins such as HIV protease and reverse transcriptase.

New Treatment Modalities

One aspect of possible HIV therapy could be the elimination of infected cells to get rid of the virus by eliminating its host cell. This is a viable strategy in the case of HIV infection, because only a small percentage of potentially infectable T cells or macrophages are actually infected at any given time. The potential drug for such use should come from a very specific cytotoxic component.

This concept is entitled “selective toxicity” that was introduced in the early 1900’s by Paul Ehrlich, and has been a cornerstone of chemotherapeutics, and has proven very successful in antibacterial treatment as well as in cancer chemotherapy [14], while it has been a neglected approach in antiviral research, mainly because for decades

virologists were taught that selective toxicity for viruses was unattainable due to their status as obligate intracellular parasites. This generalization does not exclude HIV [15]. The concept of selective toxicity was among strategies that have at least been considered, except that the research that attempted to utilize this concept vs. HIV has involved macromolecules, which are not very useful as drugs. However, the “selective toxicity” approach vs. HIV does not appear to have been significantly explored for the discovery of small drug-like molecules.

CHAPTER II

INNOVATION AND DRUGS USED

Innovation

The proposed research is innovative as it tackles the problem from a different perspective that appears to be crucial to complementing the current HIV treatment approach as it introduces a new multidrug regimen targeted toward the properties of infected cells rather than those of the virus. This class of drugs has the ability *to selectively eliminate infected cells* from the body, it is indeed sometimes spoken of almost as a “holy grail” of HIV research [12, 16] but this area still remains undeveloped, with little systematic research if any, particularly on the industrial side.

A part of the reason why this area is so unexplored is due to the fact that in traditional pharmaceutical research, the question of toxicity usually only enters into consideration as a basis for undesirable side effects, and is evaluated via a series of screenings (cellular assays, animal toxicology and Phase I trials). Thus, the existing anti-HIV drug development pipeline is inherently unlikely to identify a potential treatment that might have little or no ability to suppress HIV by inhibiting a known “drug target”, yet still be *preferentially toxic to infected cells*. Such a compound would likely fail the first anti-HIV activity screens, and never even get to the phase of toxicity testing. Furthermore, the drug discovery process focuses almost exclusively on single molecules,

whereas it is most likely that a combination of two or more small “drug-like” molecules would be required to effectively and selectively kill an infected cell.

The handful of documented attempts to utilize the concept of “selective toxicity” vs. HIV have involved high-tech engineered macromolecules, e.g., targeting HIV infected cells by conjugating a toxin to the gp120 binding region of human CD4 [17], as well as a few targeted gene-therapy based approaches (e.g. involving “cytotoxic nucleases” [18]). But what is needed for a commercially viable HIV treatment using this approach, especially for the developing world, is one involving small drug-like molecules, either alone or in combination.

Given this situation, it would not be surprising if a treatment working by this mechanism was developed serendipitously, or by some other rationale, or empirical observation of efficacy. That may apply to the case of interest here, which is a combination of the anti-inflammatory glucocorticoid dexamethasone (Dex) and the local anesthetic procaine, developed by Dr. Ruben Fabunan as a patented treatment for several types of viral infections, including HIV, dengue virus and influenza virus. The mechanism of action of this combination has been poorly understood, although it seemed probable that it could involve the procaine metabolite diethylaminoethanol (DEAE), which is formed very rapidly and quantitatively from procaine in human blood by the hydrolysis of procaine by serum esterases [19]. An early hypothesis that DEAE might have virucidal activity targeting the virion itself has been ruled out by earlier studies, as well as studies reported below because, given alone, DEAE lacks any significant activity against HIV in our assays.

The primary hypothesis of this dissertation is that Dex and DEAE achieve their apparent anti-HIV effect in humans via the mechanism of selective toxicity vs. HIV-infected cells, relative to uninfected cells. To validate this hypothesis we develop a novel drug discovery “platform technology” for identification of compounds (or mixtures of compounds) having the ability to selectively eliminate HIV-infected cells. Various studies designed to elucidate the mechanism by which this effect is achieved will also be described.

Drugs Used and Drug Dilutions

In our assays we have been using a combination of the two drugs dexamethasone sodium phosphate and procaine hydrochloride, as described by Dr. Ruben G. Fabunan [20]:

- Dexamethasone sodium phosphate is a water soluble, inorganic ester of dexamethasone. is 9-fluoro-11 β 17-dihydroxy-16 α -methyl-21-(phosphonoxy) pregna-1, 4-diene-3, 20-dione disodium salt. Its empirical formula is $C_{22}H_{28}FNa_2O_8P$ and its structural formula is:

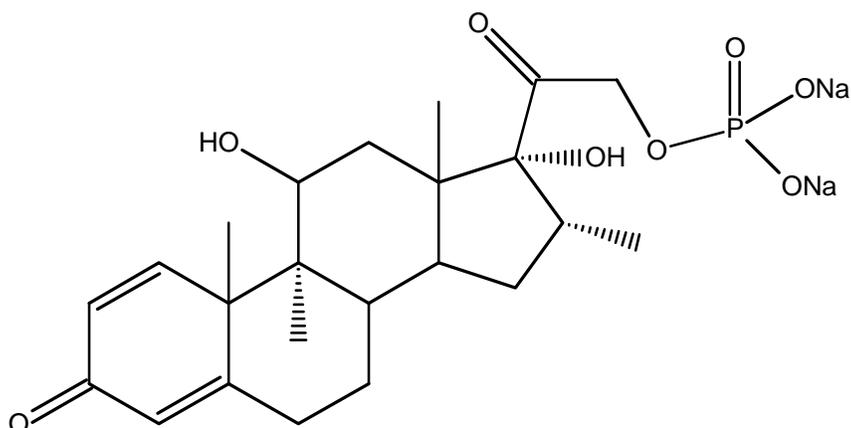


Figure 5: Dexamethasone sodium phosphate formula.

Dexamethasone is a corticosteroid hormone (glucocorticoid) that is a synthetic analog of naturally occurring glucocorticoids (hydrocortisone and cortisone). It is used to treat various conditions such as severe allergic reactions, arthritis, blood diseases, breathing problems, eye diseases, intestinal disorders, and skin diseases. It decreases the body's natural defensive response and reduces symptoms such as swelling and allergic-type reactions, to relieve inflammation. Dexamethasone is also used to treat certain types of cancer.

- Procaine is a synthetic organic compound used as an anesthetic agent indicated for production of local or regional anesthesia, particularly for oral surgery. Procaine (like cocaine) has the advantage of constricting blood vessels which reduces bleeding; chemically procaine is the PABA (p-amino-benzoic acid) ester of the amino acid alcohol DEAE (N,N diethylaminoethanol)[21].

Procaine is metabolized in the plasma at a very high rate by the enzyme pseudochoolinesterase through hydrolysis into PABA and DEAE.

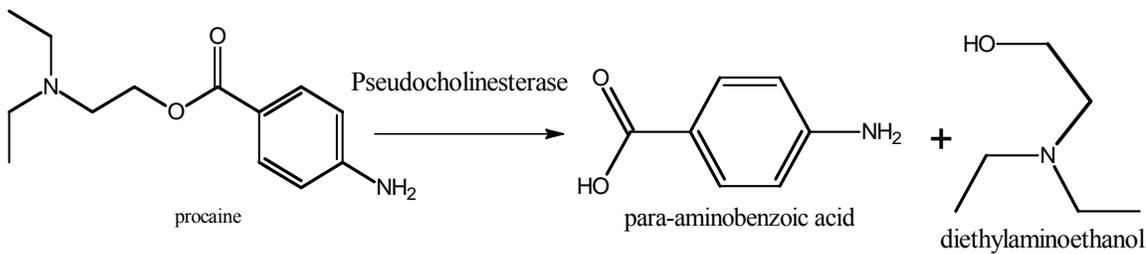


Figure 6: Hydrolysis of Procaine by Pseudochoolinesterase

In regard to the hydrolysis of procaine to generate DEAE and PABA, it must be noted that cell culture medium is not the same as human blood. Although medium is supplemented with 10% fetal bovine serum, which presumably has some ability to hydrolyze procaine, this would be significantly slower than in actual blood. Thus we have conducted many of our studies using a Dex:DEAE combination to mimic the effect of human serum[19]. Previous studies on PABA by our lab failed to detect any anti-HIV activity. We are thus currently focused on DEAE as the likely contributor to selective toxicity.

The Fabunan patent describes a preparation in which 1 volume of dexamethasone sodium phosphate (4mg/ml) is mixed with 9 volumes of 2% procaine hydrochloride for direct injection into patients [20]. This mixture gives a final molar ratio of 1:69 for Dex:Pro, reflecting the much higher potency of Dex. Based on typical blood levels of Dex that are used clinically, and an estimate of what the Fabunan injection might achieve

in human blood, we have chosen a 0.1 μ M concentration of Dex as a “standard concentration” for many of the studies described below, e.g. in the selective toxicity assays. We have also found this concentration of Dex, as well as the corresponding 6.9 μ M concentration of procaine or DEAE, to be below the threshold of toxicity in our *uninfected* cell lines.

CHAPTER III

HIV SELECTIVE TOXICITY (SELTOX) ASSAY

Materials and Methods

We have developed a simple and practical combination of cell-based assays that can demonstrate the activity of compounds or mixtures functioning via the mechanism of selective killing of HIV-infected cells. This assay is fairly simple in concept. We have used a Jurkat T-cell line (Human T cell lymphoblast-like cell line). The protocol is briefly as follows:

- Culture cells on microplates;
- Infect half of the cells with HIV-1, leave half uninfected; incubate for 4 hours.
- Expose both groups of cells to various treatments (no drug, drug A or B, etc., combination of drugs A and B).
- After 48 hours incubation, a standard cell viability assay (e.g, MTT or XTT assay) is performed [22] .

A “positive” hit is a compound or mixture that has little or no toxicity to uninfected cells, but has a significantly greater toxicity to the HIV-infected cells (fewer viable cells after treatment). To assess drug toxicity to infected cells, we compare drug-treated cells to infected cells with no drugs, because HIV infection itself can lead to a decline in cell numbers, particularly at high viral titers, that is the reason why we have avoided using

very high viral titers in the initial infections of cells, because the cytotoxic effects of HIV alone at higher MOI (“multiplicity of infection”, the ratio of infectious virions to cells) are well known, which reduces the number of viable cells that are available to detect the drug effect. So we have generally used a moderate dose of virus (MOI=0.1), and a fairly short incubation time of prior to the addition of drugs. The total time frame for the assay conducted in a microplate format is limited to three days, after which exhaustion of the media and growth capacity of the cells in a 300uL volume might confound the results. In order to give the drugs as much time as possible to act, we have reduced the incubation time with the virus to 12 hours.

Due to these factors (low MOI and short incubation time), it is very unlikely that all the cells in culture are infected after 12 hours, so *even with highly effective killing of infected cells, we do not expect to see zero viable cells in these cultures at the end of the experiment. This must be kept in mind in interpreting the results.*

Results

Figures 7-10 show various SelTox assay results, demonstrating that the synergistic action of Dex and DEAE can significantly reduce the number of viable cells in HIV-infected cell cultures. For this purpose, we have employed two measures of cell viability, the MTT assay (Figures 7 and 8), and the XTT assay (Figure 9); both yielded similar results. Figure 6 combines the data from seven completely independent experiments on the Dex-DEAE combination done over a six month period. Note the comment in the legend to Figure 8.

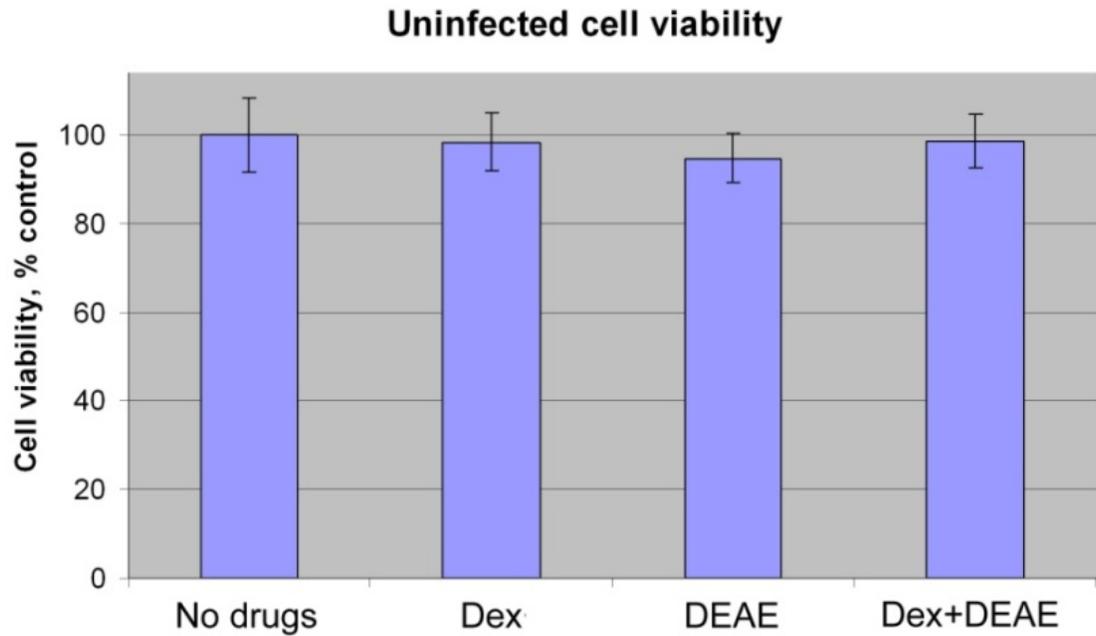


Figure 7: At the standard concentration based upon 0.1 μ M Dex, neither drug, alone or in combination, shows significant toxicity in uninfected T cells. Results are from a single experiment conducted in sextuplicate, using the MTT assay for cell viability. Drugs were incubated with the cells for 48 hrs prior to the MTT assay. Error bars in this and subsequent figures represent the S.E.M.; an asterisk is used to indicate a significant difference between means.

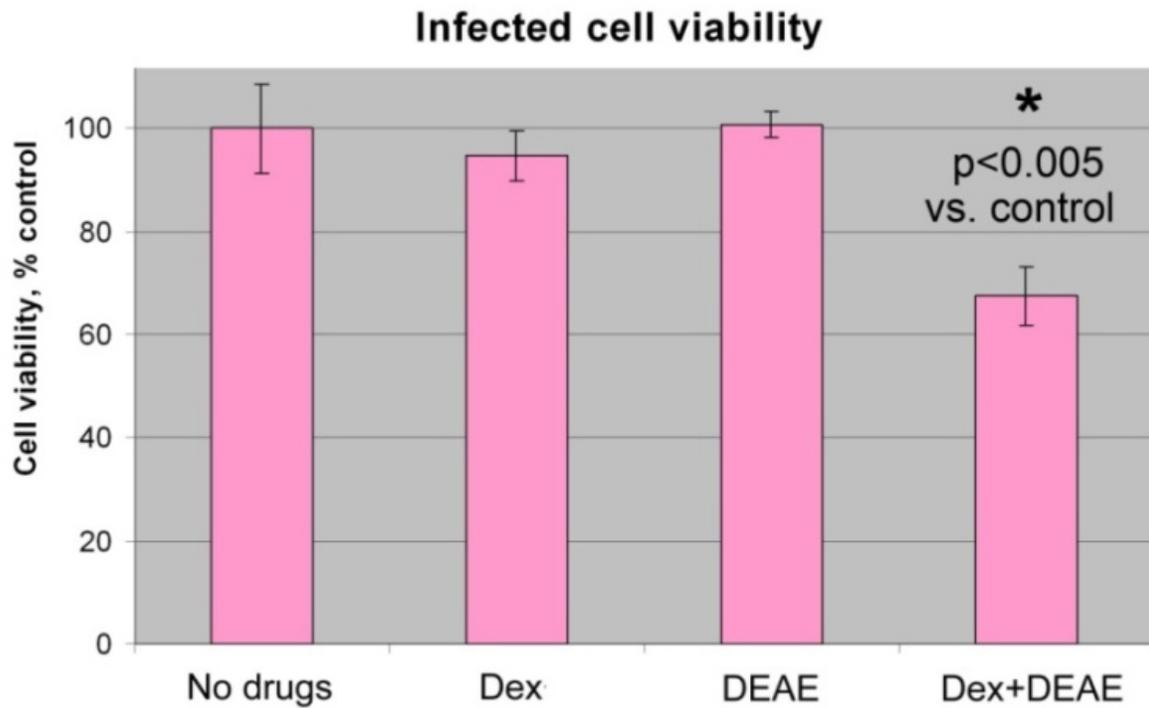


Figure 8: At the standard concentrations, neither drug alone shows significant toxicity to HIV-infected T cells. However, in combination, the drugs produce significant killing of infected cells ($P < 0.005$ compared to control). Results are from a single experiment conducted in sextuplicate, using the MTT assay for cell viability. Note: Because of the low viral titer used ($MOI = 0.1$) and the preincubation time with virus is short (12hrs), not all cells in HIV+ cultures are infected; thus, 100% cell death is not expected, since the drug combination has no effect on viability of uninfected cells (as shown in Figure 7).

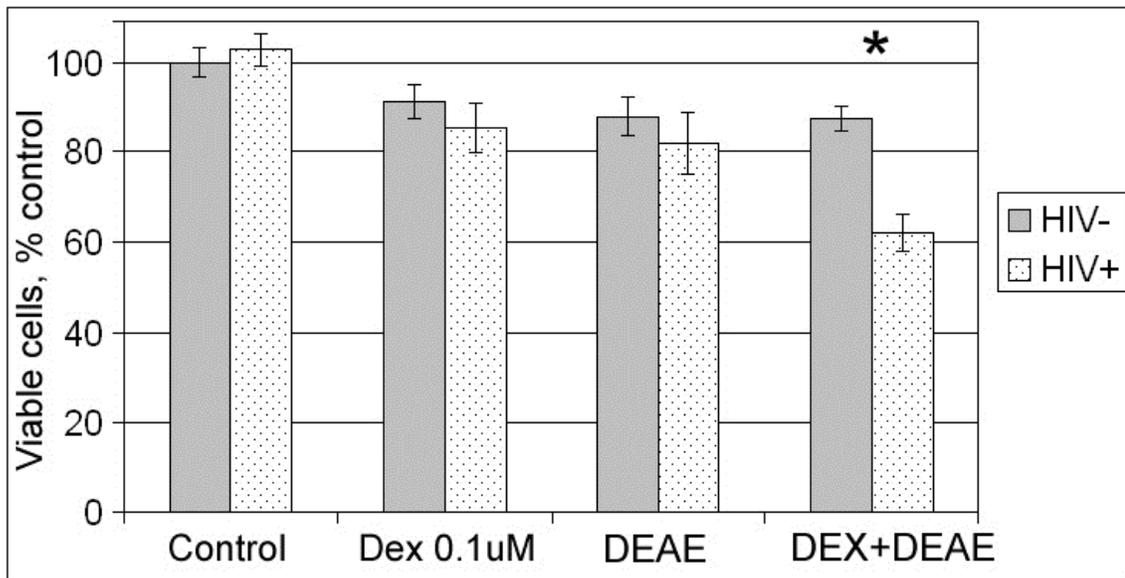


Figure 9: Essentially identical results are obtained using an alternate cell viability assay (XTT) with the SelTox assay protocol. The results shown are from a single experiment with a 12 hr. pre-incubation with virus prior to addition of drugs. The asterisk indicates that in this experiment the Dex + DEAE combo yielded significantly greater cell death in HIV+ cells than in HIV- cells (*, $P < 0.05$), and gave an overall ~40% reduction in cell viability in infected cells, compared to untreated HIV+ control cells ($P < 0.05$).

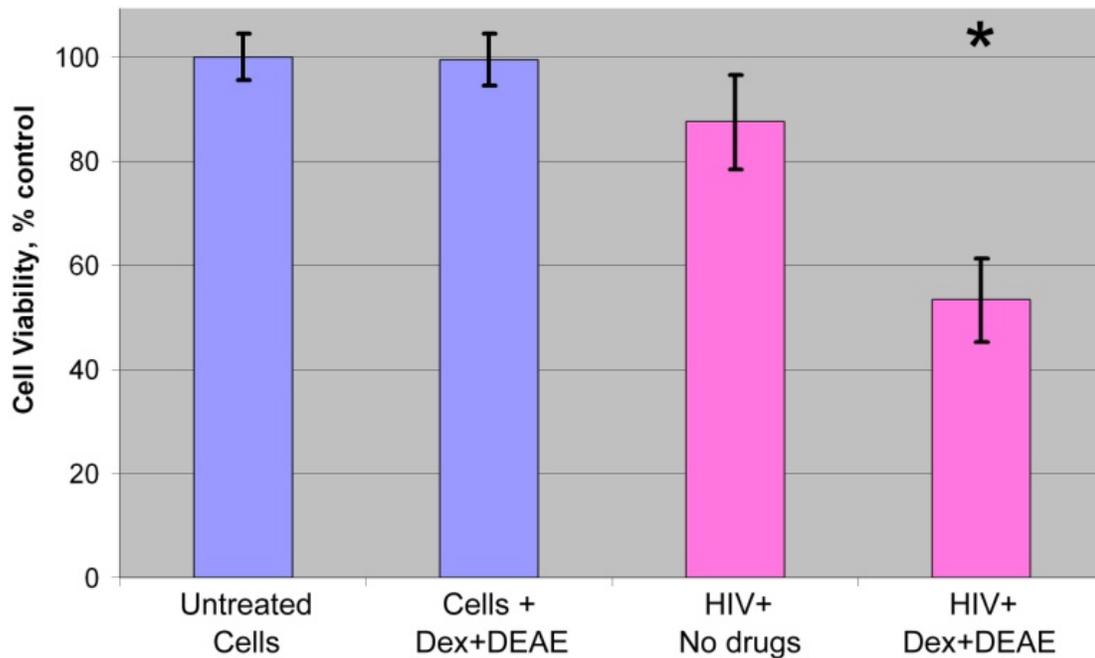


Figure 10: Summary of selective toxicity of the Dex+DEAE combination in Jurkat T cells. The results shown are the average of 7 independent experiments, 3 of which were performed using the XTT assay for cell viability, and 4 using the comparable MTT assay, from which results were not significantly different (Figures 2-4 above). After 48 hr incubation with drugs post-infection, the drug combination at the “standard concentration” based on 0.1 uM Dex did not show any toxicity to uninfected cells, but in cells preincubated with HIV (at MOI = ~0.1) for 4 to 12 hours, there was significantly more cell killing than in cells treated with drugs alone ($p < 0.0005$) or with virus alone ($p < 0.01$). A trend showing the ability of HIV alone to reduce the number of viable cells is observable, although not statistically significant at the viral titer used here.

CHAPTER IV

INHIBITION OF HIV REPLICATION

Materials and Methods

To assess the inhibition of virus replication, we used an antiviral drug assay system that has been routinely used in industry for anti-HIV drug discovery. This system uses the HeLa-CD4/CCR5 (JC53) cell line, which has been engineered to express relatively high surface levels of both the HIV receptor CD4 and co-receptor CCR5, enhancing the susceptibility of the cells to infection [23]. More specifically, we have used a variant of JC53 called TZM-bl which, when infected by HIV, are engineered to express the gene for the enzyme luciferase [24]. Luciferase serves as a reporter whose activity can be quantified using a luminometer; the activity so measured is directly proportional to the amount of virus to which the cells are exposed. Thus, this assay system can also be used for comparative measurements of viral titer, which can be regarded as proportional to viral load (see Figure 11). The potency of antiviral agents can be assessed by their ability to inhibit the production of luciferase.

Results

Active compounds showing selective toxicity to HIV-infected cells should lead to reduced virus production, due to the elimination of infected cells. We have used several protocols to verify that the level of infectious virus is actually decreasing in cell cultures in which selective killing of HIV-infected cells appears to be taking place. For this purpose we have used a “pseudo viral load” assay, which uses the TZM-bl cell line described above, one of the applications of which is to measure viral titers.

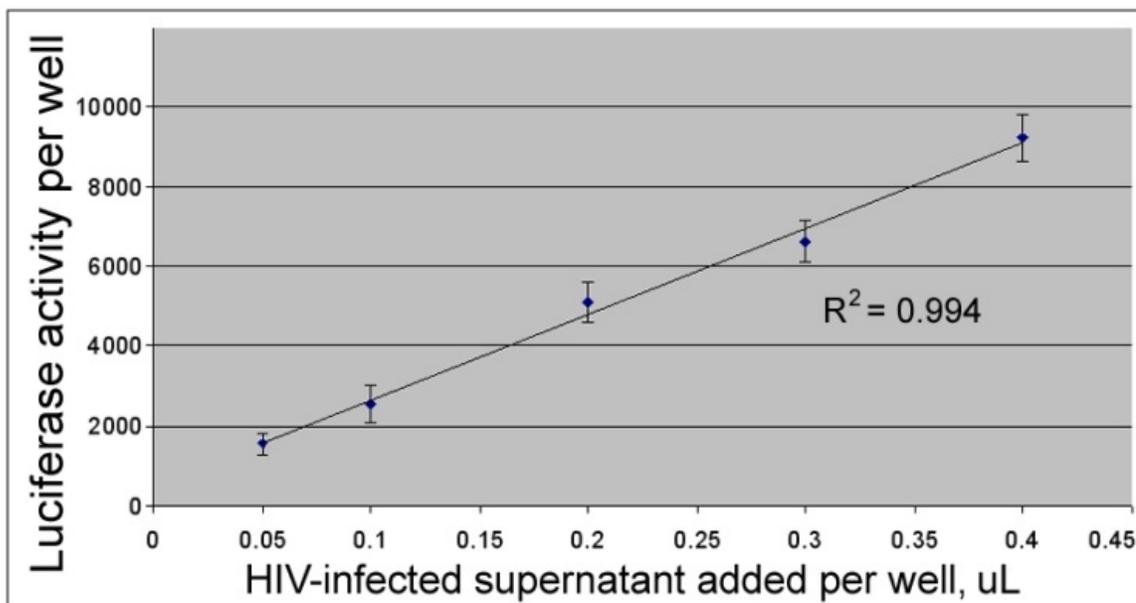


Figure 11: Standard curve for pseudo viral load assay in TZM-bl (JC53) cells. This shows that HIV Tat/LTR-driven luciferase activity in this assay responds linearly to an increasing dose of viral stock solution. This assay is used to confirm that treatments that appear to selectively kill HIV+ cells actually lead to a reduction in viral load. Inhibition of virus replication in these cells is also the basis of the “conventional” antiviral assays. Inhibitors of HIV replication lead to reduced luciferase activity.

Pro/Dex Combination to Inhibit Viral Replication in TZM-bl Cells

The procaine and dexamethasone (Pro/Dex) drug combination was found to have modest ability to inhibit HIV replication in the TZM-bl cell line, but only at low viral titers. For these experiments we used two different viral doses to infect the cultures (MOI = 0.1 and MOI = 0.01). Using the Pro/Dex drug combination at the standard concentration, we were able to achieve much greater inhibition with the lower viral titer, when expressed as a percentage of the total viral replication. Figure 8 illustrates the dependence of the antiviral activity of Pro/Dex upon the viral titer, where as the antiviral activity of AZT is independent of MOI used.

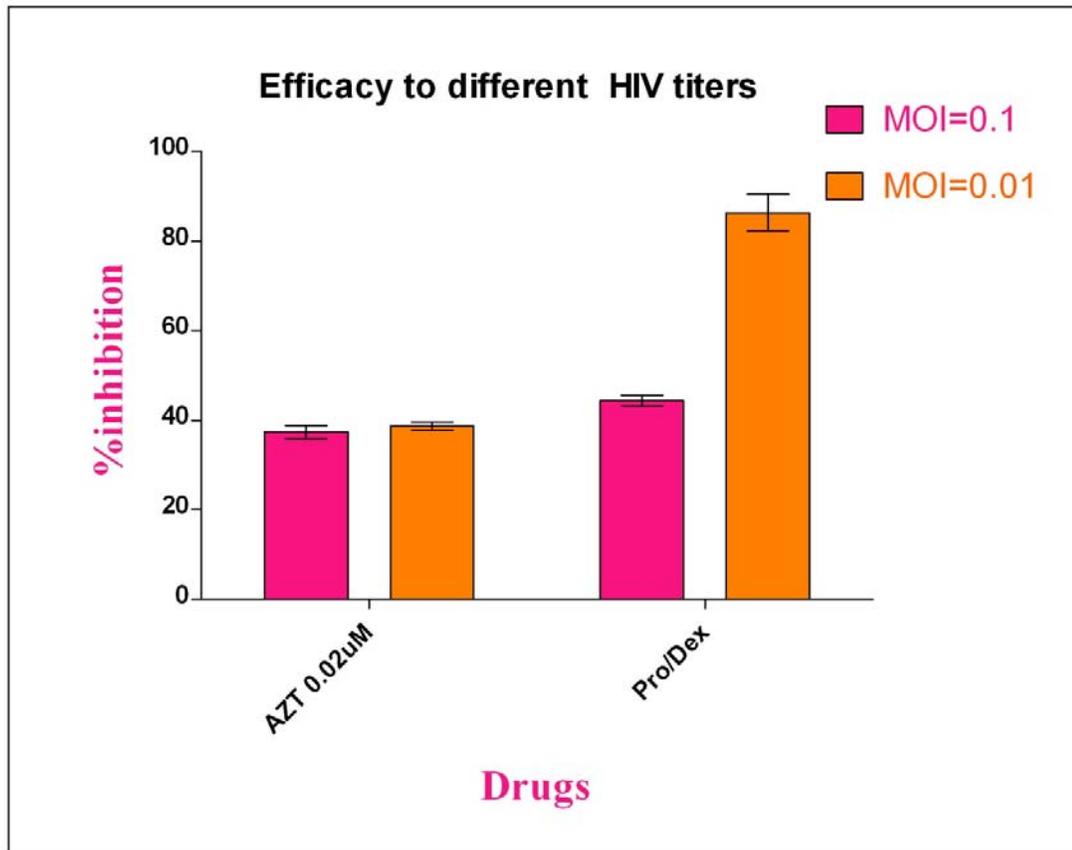


Figure 12: Inhibition of HIV replication by Pro/Dex is more effective at low viral titres. AZT at 0.02uM shows a similar inhibition, about 40%, at both high and low viral titres. At lower titer, MOI = 0.01, the Pro/Dex combination standardized to 0.1uM Dex is about twice as effective (80 % inhibition) as at high viral titer; at MOI = 0.1, the Pro/Dex combination is only able to inhibit about 40% of viral replication. Note: For Figures 8-10, data shown are the average of 2 experiments, so error bars are not shown; however, the key results are statistically significant based on the standard deviation of replicates in the individual experiments.

It is an unusual observation that when viral titer is low, the combination is able to completely inhibit HIV replication, yet, at higher viral titers; this activity appears to become saturated, so that virus replication is maximally reduced by only about 50%. This saturable inhibition of HIV replication may be associated with the previously documented ability of Dex to inhibit the transcription factor NF- κ B, the primary cellular activator of HIV [15]. Because other cellular transcription factors can independently activate HIV replication, NF- κ B inhibition alone is typically insufficient to completely suppress HIV replication. Thus, NF- κ B inhibition by Dex may at least partly explain these observations, and contribute to the HIV replication ability of the Pro/Dex mixture.

Pro/Dex and Dex/DEAE show an additive inhibitory effect with AZT:

As shown in Figure 9, both the Pro/Dex and Dex/DEAE combinations provide significant enhancement to the antiviral activity of AZT.

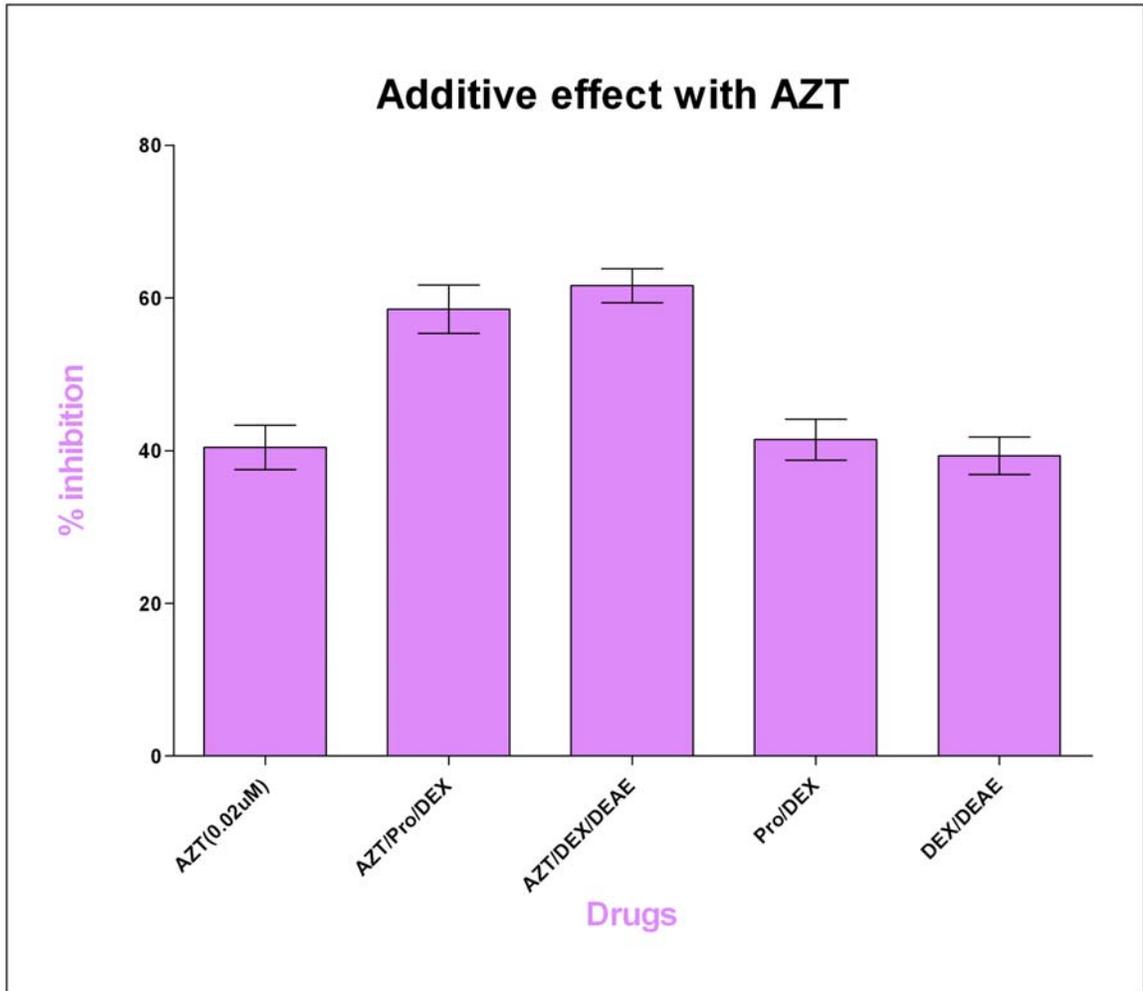


Figure 13: Enhancement of the anti-HIV activity of AZT when combined with either Pro/Dex or Dex/DEAE standardized to 0.1uM Dex. The standard antiviral assay protocol was used beginning with an inoculation of HIV at MOI = 0.1.

Pro/Dex Activity Against Resistant Strains

As shown in Figure 14, the antiviral activity of the Pro/Dex combination is the same whether or not the viral strain used is AZT resistant, whereas AZT itself is less effective against the drug-resistant virus_(4 times less effective). There is no reason to expect that the results would be any different with other common drug-resistant viral strains.

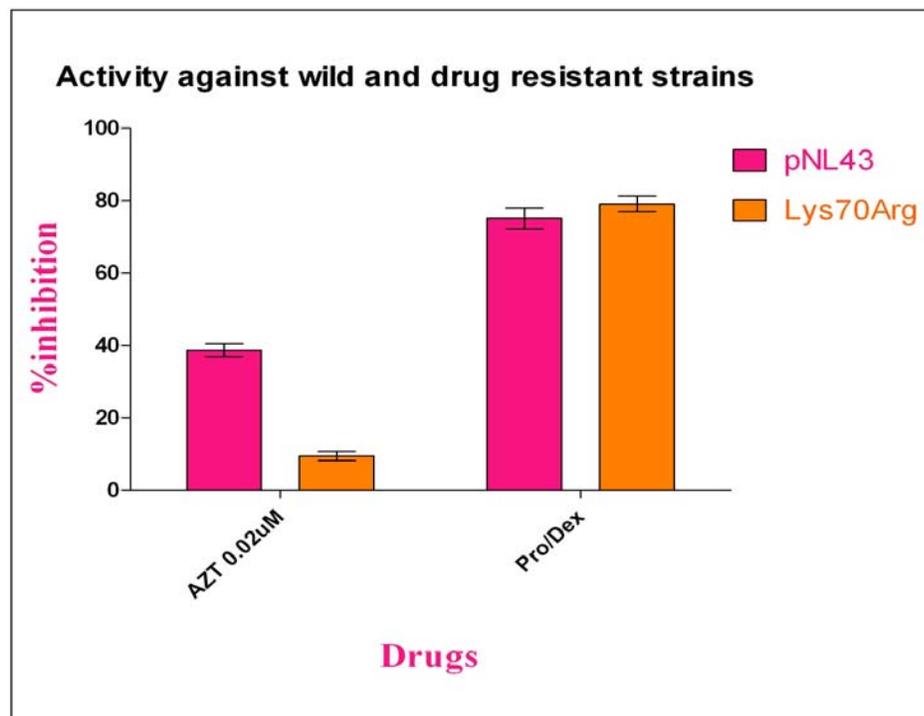


Figure 14: Anti-HIV activity of Pro/Dex versus wild type and AZT resistant strains of HIV. Pro/Dex was used at a standard concentration of 0.1uM Dex. The standard antiviral assay protocol was used beginning with an inoculation of HIV at MOI=0.01.

In order to assess whether the treatment with Dex and either DEAE or procaine could actually lead to a reduction in viral load over time, we completed two separate experiments. The first used a simple protocol. A set of wells containing HIV(+) cells

(exposed to drugs or control) from the selective toxicity protocol was maintained for up to one additional week. Fresh media was added with maintenance of the treatment conditions. This showed that Dex + DEAE gave significant reductions in “pseudo” viral load (PVL), as assessed in TZM-bl cells (using the method shown in Fig. 13). After one week of treatment, during which nothing was removed from the cultures, but only an equal volume of fresh media added after three days, supernatants of HIV+ cells treated with the Dex/DEAE combination showed a 42% reduction in relative PVL as compared to untreated HIV+ cells ($P < 0.01$).

The second experiment, with Dex and Pro instead of Dex and DEAE, used a “serial dilution” approach, in which every two days, each 2mL culture was “split”, with 1mL of the cell suspension being moved to a new well, and 1mL of fresh media added. Thus, at each “split”, half of the spent media was removed and the rest diluted with fresh media. Freshly added media always contained the same concentration of treatment, meaning either no drugs, or the standard concentration of Pro/Dex. At the end of two weeks, supernatants from each treatment were assessed in the TZM-bl PVL assay. Supernatants of HIV+ cells treated with the Pro/Dex combination showed a 59% reduction in relative PVL as compared to untreated HIV+ cells. In this case, note that procaine was used; because of the long duration of the study, it seemed more likely that substantial hydrolysis could be achieved.

However, the viral load decrease was not as much as would be expected from the Dex/DEAE result from the one week trial (two weeks with a 42% decrease each week would give over a 65% reduction at the end of 2 weeks). So again, this points to the

somewhat greater efficacy of DEAE vs. procaine, particularly in the cell culture media with only 10% serum where complete hydrolysis of procaine to DEAE may not be achieved.

Nonetheless, based on these 2 results, a 50% decrease after 2 weeks of treatment would be a conservative estimate of the attainable decrease in viral load. If that could be extrapolated in actual patients (which is speculation at this point), it would mean that if the treatment were sustained for about 4 months (8 two-week periods), viral load could potentially be reduced to less than 1% of its initial value ($0.5^8 < 0.01$).

CHAPTER V

FLUORESCENCE EVIDENCE OF THE CELL VIABILITY

Materials and Methods

The purpose of this part of the research was the determination of HIV replication in vitro and its inhibition by Dexamethasone and DEAE. For that we developed a method for monitoring HIV infection, using two different stable human T-lymphoid cell lines:

- CEM-GFP that contains an HIV-1 long terminal repeat-driven green fluorescent protein (GFP) cDNA in which GFP expression is induced by the HIV-1 Tat protein. This cell line was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: CEM-GFP from Dr. Jacques Corbeil.
- Rev-CEM which is a CEM-SS derived cell line that had been transduced with an HIV-1 Rev and Tat dependent GFP expression vector, thereby allowing GFP analysis of infected cells. These cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Rev-CEM cell line from Dr. Yuntao Wu and Dr. Jon Marsh.

Results

Figure 15 shows the production of GFP in HIV-1 infected cells. The expression of GFP by infected cells reached a maximum 4 days post infection. Note that once this protein has accumulated in a cell, it persists for some time, even in dying or dead cells. Thus in the “infected cells” panel of Figures 16 and 17, we see equivalent amounts of GFP produced in all cultures that were infected (HIV+), even though the Live/Dead cell labeling shows varying ratios of dead to live cells, as discussed below. This also reflects the fact that the cells were exposed to virus a full 3 days prior to the addition of drugs in these experiments, giving time for GFP production.

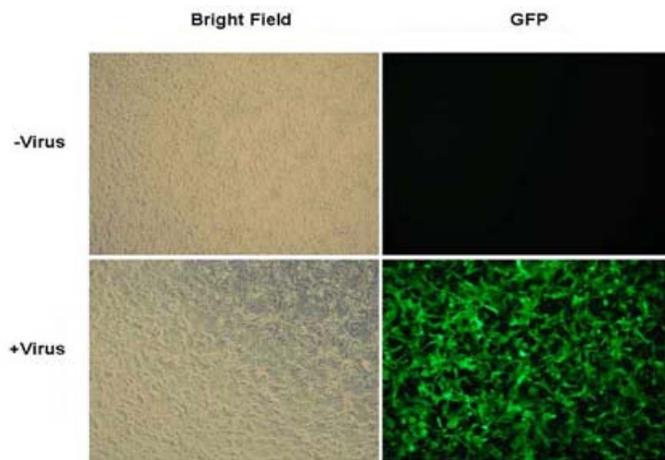


Figure 15: The expression of GFP through cultured T-cells infected with HIV-1 is observed via an engineered cell line that expresses the green fluorescent protein (GFP). Over time, the number of infected cells increases, and individual infected cells can be identified by this marker.

We combined the ability of CEM cells to fluoresce green when infected with HIV with a LIVE/DEAD® Cell Viability/Cytotoxicity Assay. This assay, as the name implies, is a fluorescence-based method of assessing cell viability. It provides means for

the simultaneous determination of live and dead cells, using two probes that measure recognized parameters of cell viability: intracellular esterase activity and outer cellular membrane integrity.

Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, which may be detected by the enzymatic conversion of the virtually nonfluorescent cell-permeant dye Calcein blue AM (excitation/emission maxima ~322/435 nm). Upon cleavage of the AM esters by intracellular esterases, this tracer becomes relatively polar and is retained by cells for several hours. In addition, its fluorescence intensity increases and the spectra shift to longer wavelengths, with excitation/emission maxima of ~360/449 nm. Dead cells are labeled by the red fluorescent dye Ethidium-1, which selectively enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thus tagging dead cells (excitation/emission~495 nm/~635 nm) [25].

The determination of cell viability depends on these physical and biochemical properties of cells. Background fluorescence levels are inherently low with this assay because the dyes are virtually non-fluorescent before interacting with cells; nonetheless the background fluorescence was subtracted from all the results shown.

We infected the CEM-GFP and Rev-CEM cells with HIV-1 virions at an input MOI of 0.01, treated them with a standard concentration of Dex and DEAE, and monitored the fluorescence via a Synergy Mx microplate reader (Biotek, Inc.). After allowing the virus to replicate in the cell culture media alone for 3 days, the different treatments (various drugs or saline as a control) were added. On the fourth day post infection, we detected a

significant increase in dead cell count among the HIV infected cells treated with “combo.” This combination of drugs showed no toxicity to the uninfected cells, and neither Dex nor DEAE alone is able to induce cell death.

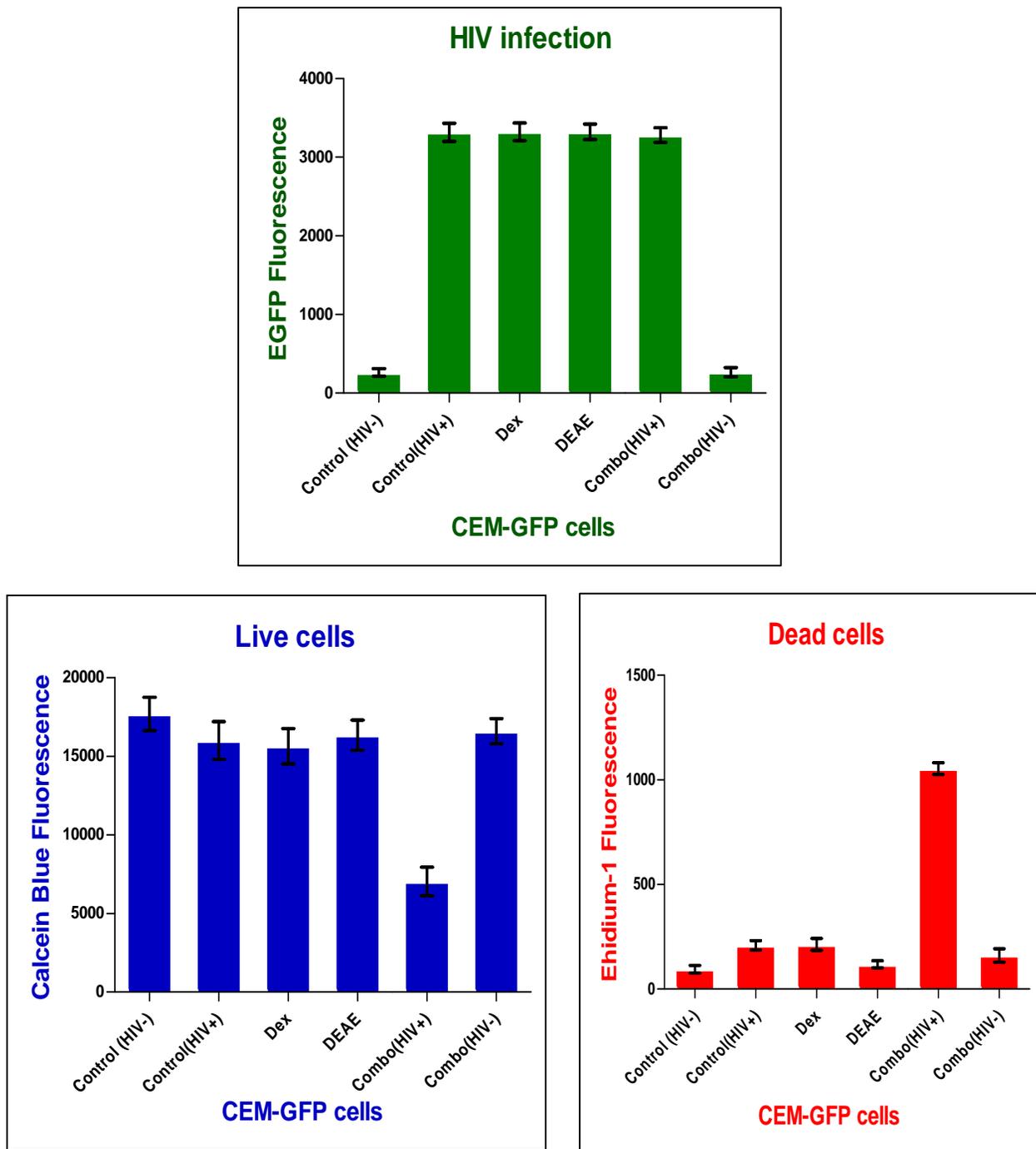


Figure 16: CEM-GFP cells read with the microplate reader for the 3 fluorophores:

- Enhanced Green Fluorescent Protein (EGFP) the level of fluorescence is relative to the level of infection with HIV-1 virus.
- Ethidium-1: its level of fluorescence corresponds to the number of dead cells.
- Calcein blue AM; its level of fluorescence increases with viability of the cells.

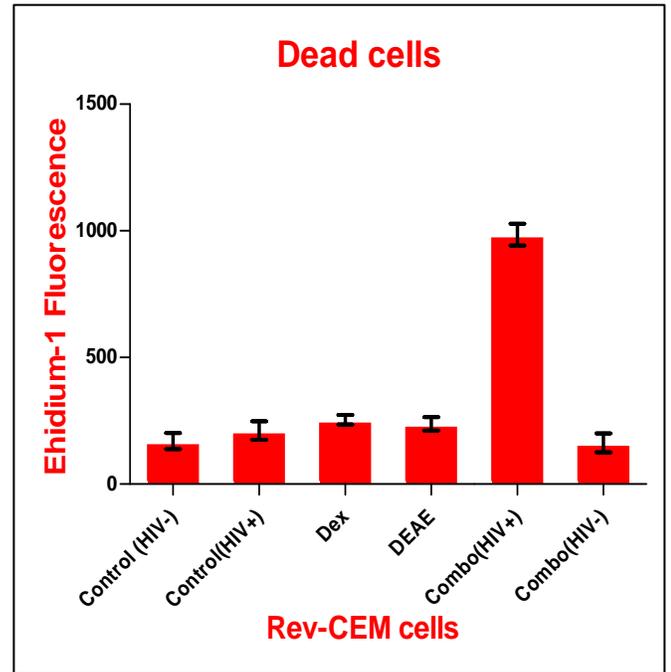
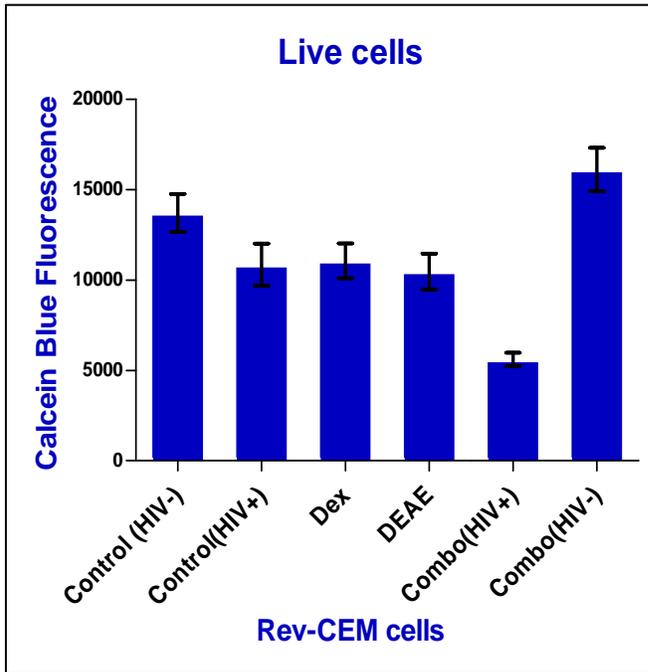
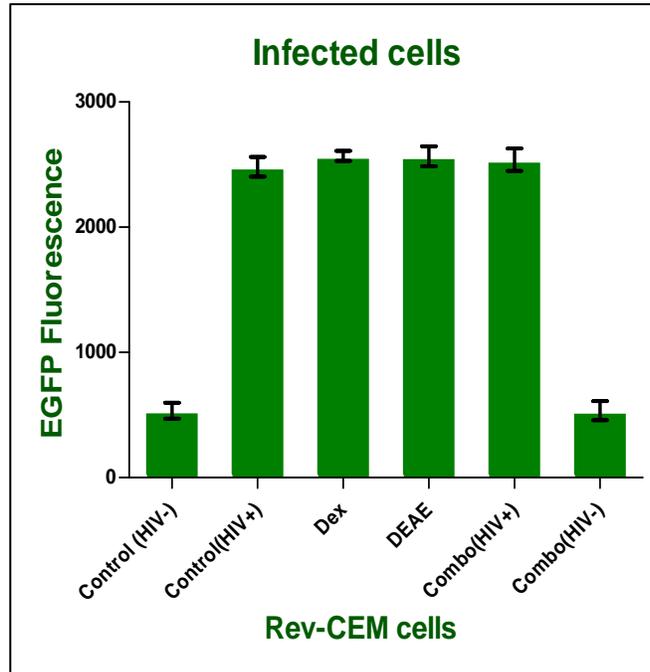


Figure 17: Rev-CEM cells read with the microplate reader

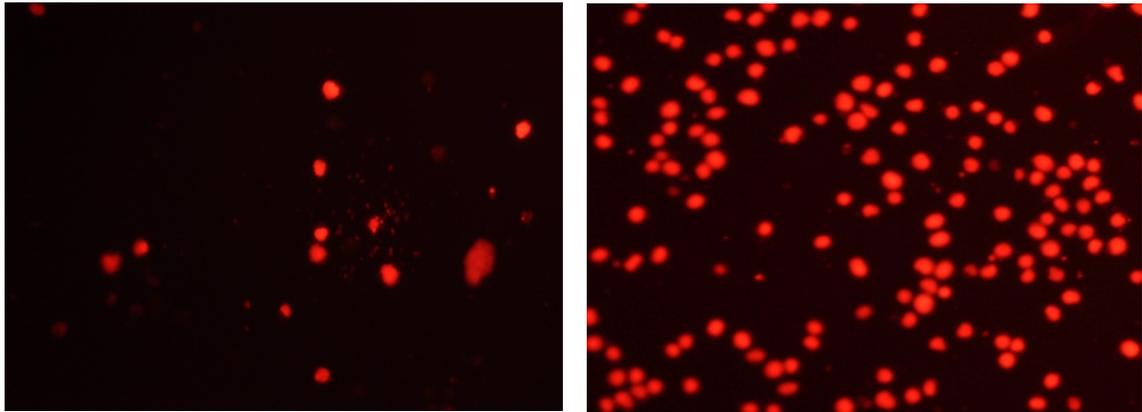


Figure 18: Ethidium-1 (DEAD dye) labeling in CEM-GFP cell line infected by HIV-1, 4 days after HIV-1 infection at an input MOI of 0.01. CEM-GFP cells were visualized by fluorescence microscopy. At the left: infected cells with no treatment. Note that even in “healthy” cell cultures, it is normal to have a small percentage of dead cells, and HIV infection alone does cause a slight increase in cell death under these cell culture conditions. At the right: infected cells with the combined Dex/DEAE treatment added after 3 days, and maintained for an additional 24 hrs. This illustrates visually the results assessed quantitatively in the “Dead cells” panels of Figs. 16 and 17, which show (by precise counting of the red fluorescent signal) that after only 24 hrs. Exposure to the drugs, there are significantly more dead cells in the Dex/DEAE treated HIV+ cell cultures as opposed to untreated HIV+ cells, or cells treated with drugs alone. This again supports our working hypothesis that the drug combination is selectively toxic to HIV-infected cells.

Summary

The results described above demonstrate that neither Dex or DEAE alone exhibits significant toxicity to either infected or uninfected cells (Figures 3-5); only the Dex/DEAE combination was active, and only in infected cells (Figures 4-6). Since the drugs alone or in combination have no effect on the number of uninfected viable cells, the most reasonable and obvious explanation of these results is that HIV-infected cells are selectively killed by the drug combination.

These results support our hypothesis that the combination of DEAE and dexamethasone has the ability to selectively kill HIV-infected cells.

CHAPTER VI

MECHANISM OF CELL DEATH

There are two major mechanisms of cell death: necrosis, which is a consequence of a passive degenerative process, and apoptosis, which is the consequence of an active process. The latter, also known as “programmed cell death”, is characterized by activation of distinct cellular pathways, e.g. involving caspases and other proteins, along with DNA fragmentation and other events. The two phenomena are morphologically different from each other; their physiological consequences are also very different.

Apoptosis is triggered by multiple stimuli, and is regulated by one of two known cell death-signalling pathways: the extrinsic and intrinsic pathways. The two pathways share similar molecules (especially caspases) and features, but differ in the initiation method. The extrinsic pathway is usually initiated by external stimuli that are sensed by cell-death receptors on the cell membrane, while in the intrinsic pathway mitochondria play the major role by releasing apoptosis triggering molecules [16] Morphological changes during apoptosis are distinguished by a loss of plasma membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA.

Apoptosis is considered a very common response of cells to drugs in the course of therapy, and its quantitative evaluation represents an issue of substantial significance. Several studies over the years have proven that flow cytometry is the preferred method

for the quantitation of apoptosis, and various established assays have been described to identify apoptotic cells by flow cytometric analysis.

For our research, since there is no unequivocal method to distinguish apoptotic from necrotic cells by flow cytometry, we chose two different flow cytometric assays where we can discriminate necrotic cells from apoptotic cells in HIV-infected cells treated with DEAE/Dex.

Annexin V FITC

Materials and Methods

We used Annexin V-FITC Apoptosis Detection kit by BD (San Jose, CA) that uses Annexin V-FITC conjugates which facilitates rapid fluorimetric detection of apoptotic cells. The principle of the assay lies in the fact that phospholipid asymmetry is disrupted early in the apoptotic process, during the phase of loss of plasma membrane, leading to the exposure of phosphatidylserine (PS) on the outer leaflet of the cytoplasmic membrane, thus exposing PS to the external cellular environment. This event is important for macrophage recognition of cells undergoing apoptosis.

Annexin V is a 35-36 kDa anticoagulant protein that preferentially binds negatively charged phospholipids in a calcium dependent manner with a high affinity for PS, and binds to cells with exposed PS. This Annexin V is conjugated to the FITC fluorochrome and retains its high affinity for PS, serving as a sensitive probe for flow cytometric analysis of cells undergoing apoptosis. This reaction is reversible, and occurs with a Kd of approximately 5×10^{-10} M.

The advantage of the FITC Annexin V method over other assays that are based on nuclear changes such as DNA fragmentation is that it can identify apoptosis at an earlier stage, because of the early externalization of PS.

FITC Annexin V staining precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. For that reason, we used FITC Annexin V in conjunction with propidium iodide (PI), which is excluded by intact membranes in viable cells, while it enters the damaged cells and has a specificity for double-stranded nucleic acids, absorbing in blue-green (493 nm) and fluorescing red. Then, the flow cytometry results will read as follows:

- Early apoptotic cells (PI negative, FITC Annexin V positive);
- Viable cells (FITC Annexin V and PI negative);
- Early apoptotic cells (FITC Annexin V positive and PI negative);
- Late apoptotic cells (FITC Annexin V and PI positive).

In order to gate the results of the flow cytometer appropriately, it is important to use adequate controls to set up compensation and quadrants:

- Unstained cells (negative control, FITC negative and PI negative; will be in lower left quadrant of histogram);
- Untreated cells stained with Annexin V-FITC alone, but not PI (to give background or nonspecific Annexin V-FITC staining; will be in lower right quadrant);

- Untreated cells stained with PI alone, but not Annexin V-FITC (to give background or nonspecific PI staining; will be in upper left quadrant);
- Apoptosis-induced cells (positive control; will be in upper right quadrant).

Apoptotic cells can be induced by multiple methods and in our research we used Camptothecin (Sigma-Aldrich); it is a cytotoxic quinoline alkaloid extracted from the bark and stem of the Chinese tree *Camptotheca acuminata*, and has been shown to induce apoptosis in a dose-dependent manner in vitro, due to its action as a potent inhibitor of the DNA enzyme topoisomerase (Topo I), a molecule required for DNA synthesis.

After performing a time course with different Camptothecin concentrations, the protocol that gave us optimal results was as follows for Camptothecin-induced apoptosis:

1. Prepare a 1 mM stock solution of Camptothecin (Sigma Cat. No. C-9911) in DMSO.
2. Add 6 μ M (final concentration) Camptothecin to cell suspension (1×10^6 cells/ml in cell culture medium).
3. Incubate for 6 hr at 37°C.
4. Proceed with assays designed to evaluate induction of apoptosis.

After apoptosis induction, generating apoptotic cells which will serve as a positive control, cells under different conditions are prepared for the staining procedure:

- Negative control (uninfected Jurkat cells);
- Positive control (apoptotic jurkat cells induced by Camptothecin);
- Uninfected Jurkat cells treated with DEX/DEAE;

- Untreated Jurkat cells infected with HIV;
- Jurkat cells infected with HIV and treated with DEX/DEAE.

The experimental staining procedure was as follows:

1. Wash Jurkat cells twice with cold phosphate-buffered saline (PBS) and then resuspend cells in 1X Binding Buffer at a concentration of $\sim 1 \times 10^6$ cells/ml.
2. Transfer 100 μ l of the solution ($\sim 1 \times 10^5$ cells) to a 5 ml culture tube.
3. Add Annexin V and Vital Dye: FITC 5 uL, PI 2 uL.
4. Gently mix the cells and incubate for 15 min at RT in the dark.
5. Add 400 μ l of 1X Binding Buffer to each tube.

Finally, the samples were analyzed using BD FACSAria III flow cytometer within 1 hour, a high performance flow cytometer analyzer and cell sorter.

Results

The mechanism by which the flowcytometer works is that when a cell passes through a laser beam in the flow cytometer, it generates light scatter. Forward scatter (FSC) provides information about the cell size, while side scatter (SSC) gives information about the cells morphological complexity, in fact cell shrinkage and increased granularity can be analysed following the FSC and SSC criteria respectively [26]. Due to the morphological changes the cell undergoes after death, there are changes in light scatter that follow and may reflect these phenomena, and these changes differ from apoptosis and necrosis as follows:

- Necrosis: both FSC and SSC increase, as a consequence of cell engorgement.
- Apoptosis: FSC and SSC decrease as a consequence of plasma membrane damage and leakage of cell constituents.

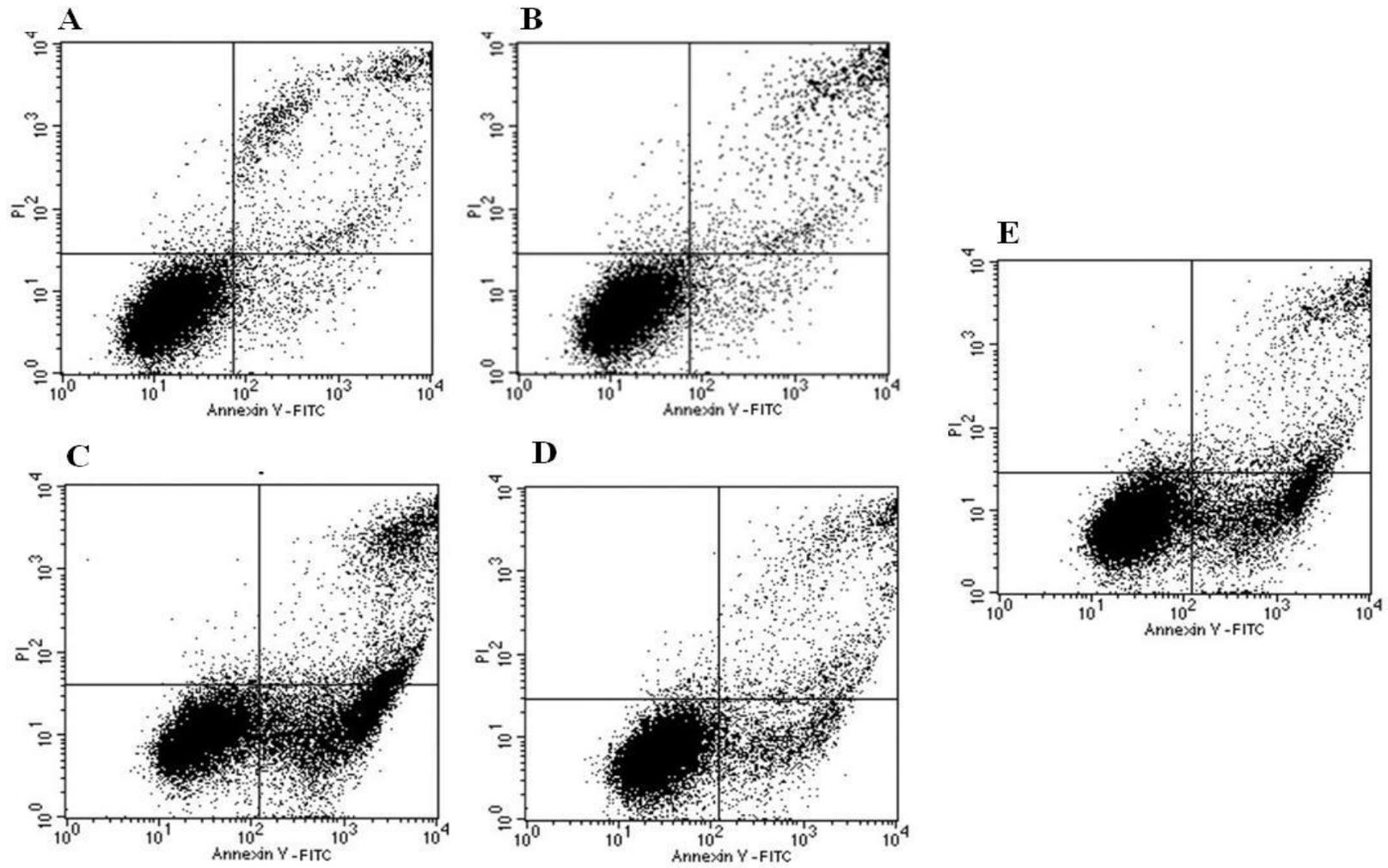


Figure 19: Flow cytometric analyses (FACS) measuring apoptosis (A, B, C, D and E).

Figure 19 shows representative scatter plots for propidium iodide (PI) (y-axis) versus Annexin V-FITC (x-axis), with different cell treatments (A-E). Lower left quadrants (absence of both markers) indicate viable cells; upper left quadrants (PI positive) indicate cellular necrosis. Lower right quadrants (PI negative, FITC positive) indicate early stage apoptosis; upper right quadrants (PI positive, FITC positive) indicate late stage apoptosis. The various treatments are: **A**: Negative control (uninfected Jurkat cells); **B**: Uninfected Jurkat cells treated with DEX/DEAE; **C**: Positive control (apoptotic Jurkat cells induced by Camptothecin); **D**: Jurkat cells infected with HIV only (no drugs); **E**: Jurkat cells infected with HIV and treated with DEX/DEAE.

The results shown above represent an average of triplicates of each sample, and we can see how the cell viability (percentage of cells in the lower left quadrant of each histogram) in the uninfected Jurkat cells after 24 hrs treatment with the combination of DEX/DEAE (**B**) is not disturbed and remains at 89% compared to the negative control (**A**) that is at 92%, while the DEAE/Dex treated HIV infected Jurkat cells (**E**) reach a viability of only 47% compared to a viability in untreated HIV-infected cells (**D**) with no treatment of 77% (showing that some cell death is induced by the virus alone). In both cases however, the cell death at the time point measurements were taken appears to be predominantly early apoptotic.

The same experiment was repeated on a different cell line CEM-SS and the results were read using a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA) equipped with a 488-nm argon laser.

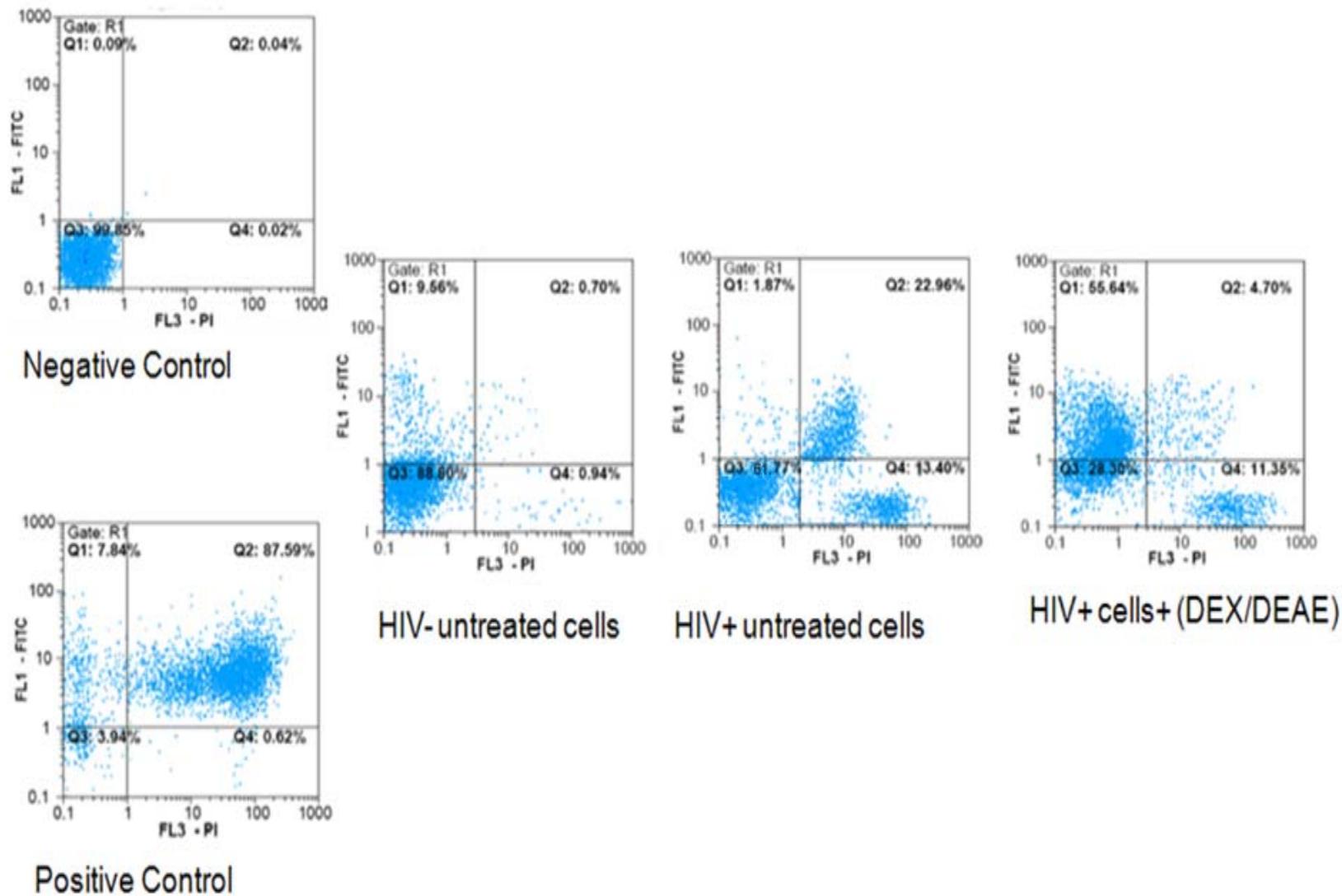


Figure 20: Flow cytometry results:

In figure 19 it is important to note that the axes have changed: PI is on x-axis, Annex V-FITC is on y-axis. Lower left quadrants, viable cells. Lower right quadrants, necrotic cells. Upper left quadrants, early apoptotic cells. Upper right quadrants, nonviable late apoptotic cells. For the positive control, apoptosis was induced using Camptothecin. Note that in the presence of Dex/DEAE, early apoptosis is considerably increased in HIV+ cells.

The results shown above are in accordance with the previous experiment, showing a total cell death of only 10.26% in the uninfected CEM-SS cells treated with Dex/DEAE, compared to 0.15% in the negative control (untreated, uninfected cells), while the HIV infected cells treated with Dex/DEAE showed a 60.34% rate of apoptosis and 11.36% necrosis, compared to 24.83% apoptosis and 13.40% necrosis in the HIV infected untreated cells.

Double Stain Apoptosis Detection Assay (Hoechst 33342/PI)

The previous Flow Cytometry experiments used populations of cells that in the case of “HIV+ cells” nonetheless contained a mixed population of infected and uninfected cells (because not all cells are infected even though the cultures were inoculated with virus). Thus, we desired to study the phenomenon of Dex/DEAE induced apoptosis in a population of sorted, uniformly infected cells.

Materials and Methods

For that purpose we used CEM-GFP cells that have the property of producing GFP post infection as a marker to differentiate between HIV+ and HIV- cells. For this, we used a BD Biosciences FACSAria III cell sorter which is considered to be BD's most

advanced cell sorter. FACS (Fluorescence-Activated Cell Sorting) is a method for separating particles from a heterogeneous suspension to one that is homogeneous based on the physical and fluorescent properties of the particles established by flow cytometric analysis of samples.

We used a cell density of 10×10^6 cells/ml filtered through a 40 μ m filter, and made sure that the cells were as healthy as possible, as the healthier they are, the better they will hold up after the sorting process. The cells were prepared in a final suspension buffer that contains 2% serum; it is important that the serum is limited to a small amount because high serum concentrations cause distortion of light scatter signals.

RPMI medium containing 10% serum was prepared and added to the collection tubes so that cells remain as healthy as possible after the sort, and were transported immediately after the sort in a sterile manner and plated into microplates.

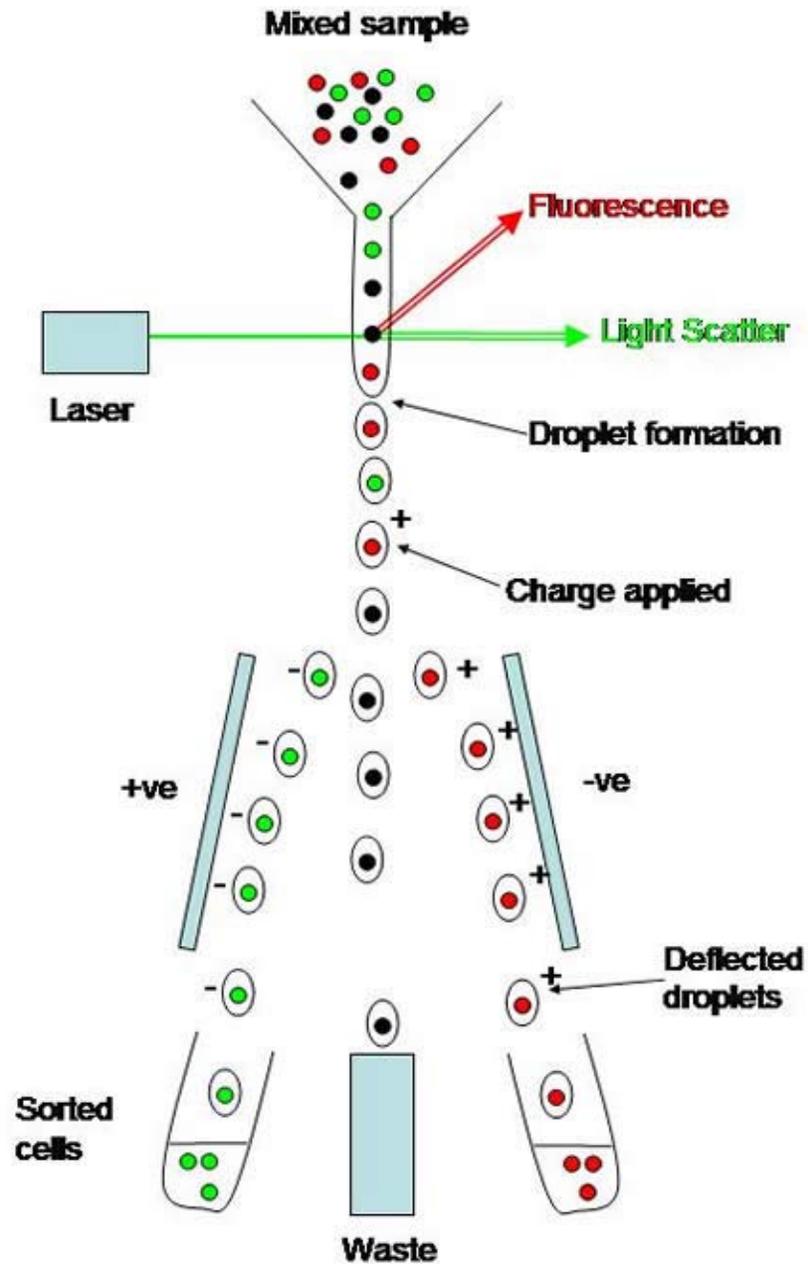


Figure 21: Schematic representing the mechanism of Flow Cytometry and Fluorescence-Activated Cell Sorting (FACS) [27]

Furthermore, different cell types sort differently, so we had to proceed with multiple sorts to optimize conditions for the CEM-GFP cells.

The optimal parameters for CEM-GFP cells were as follow:

The FACSAria has two nozzle sizes, 70 um and 100 um. To achieve good stream stability, it is suggested that the cells being sorted should be 1/4 to 1/5 the size of the nozzle; this is important because it gives the FACSAria an effective limit on sorting cells below 50 um in diameter. That is why we chose the 70um nozzle, since our CEM-GFP are about 12um in diameter. By choosing the 70 um nozzle, we had to keep in mind that the stability of the stream is related to the nozzle size. and using the 70 um nozzle, the instrument is stable at 70 PSI (high pressure sort).

At 70 psi and 90 kHz with an average threshold rate of 25,000 events per second, a two-way sort achieved a purity of >94% and a yield >80% of Poisson's expected yield as shown in figure 22. All sorts were performed at sheath pressure of 70 PSI using CEM-GFP cell line, and resulted in cells that proved to be viable and proliferated for several days post-sort.

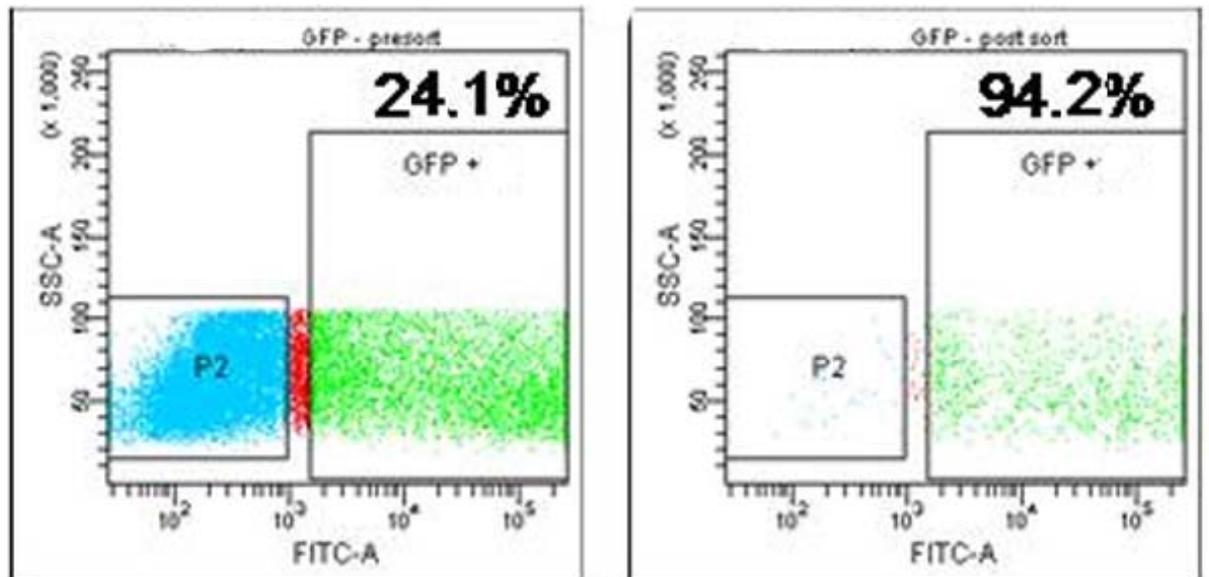


Figure 22: Purity of CEM-GFP cells pre sort (left) and post sort (right)

The Double Stain Apoptosis Detection Kit (Hoechst 33342/PI) was chosen because it provides a rapid and convenient assay for apoptosis based upon fluorescent detection for the compacted state of the chromatin in apoptotic cells, especially because it is based on two ready-to-use dyes bound to DNA:

- Hoechst 33342: a blue-fluorescence dye (excitation/ emission maxima ~350/461 nm when bound to DNA), stains the condensed chromatin in apoptotic cells more brightly than the chromatin in normal cells.
- Propidium iodide (PI): a red-fluorescence dye (excitation/emission maxima ~535/617 nm when bound to DNA), is only permeant to dead cells.

With these two dyes that have excitation/emission maxima different from the GFP range it will be easy to distinguish between the three fluorescent dyes after analysis. Furthermore, the staining pattern produced by the simultaneous use of these dyes makes

it possible to distinguish normal, apoptotic, and dead cell populations by flow cytometry and fluorescence microscopy.

Protocol:

1. Prepare a positive control: induce apoptosis in cells using Camptothecin.
1. Prepare a negative control without inducing reagent.
2. Wash the CEM-GFP cells by cold PBS and adjust the cell density to 1×10^6 cells/ml in PBS.
3. Directly collect suspension cells by centrifugation.
2. Add 10 μ l of Hoechst 33342 to each 1 ml of the cell suspension and mix thoroughly. Incubate the cells at 37°C for 5-15 minutes.
3. Centrifuge the cells at 1,000 rpm for five minutes at 4°C and discard the supernatant.
4. Resuspend cells in 1000 μ l of 1X buffer A.
5. Add 5 μ l of PI to each 1 ml of cell suspension and mix thoroughly. Incubate the cells at room temperature for 5-15 minutes and avoid exposing to light.
6. Post incubation, analyze the stained cells by flow cytometry immediately, using UV/488 nm dual excitation and measuring the fluorescence emission at ~460 nm emission of Hoechst 33342 dye and >575 nm emission of Propidium Iodide.

After this step the population can be separated into three groups: live cells will show only a low level of fluorescence; apoptotic cells will show a higher level of blue fluorescence, and dead cells will show low-blue and high-red fluorescence.

The flow cytometer results were confirmed by viewing the cells under a fluorescence microscope.

Results

The flow cytometer results showed an apparent shift in the Hoechst 33342 absorbance in the cells infected by HIV (Figure 23), proving that the cells are dying via apoptosis and the most pronounced effect was noticed in the HIV infected cells treated with Dexamethasone and DEAE, while the cells that were infected with the virus but did not receive any treatment showed a shift characterizing the cell death induced by the virus itself. The non infected cells treated with Dexamethasone and DEAE did not appear any different than the non treated cells, reinforcing our previous findings that the combination is non toxic to the non infected cells, and the apoptosis inducing effect is only witnessed in the infected cells.

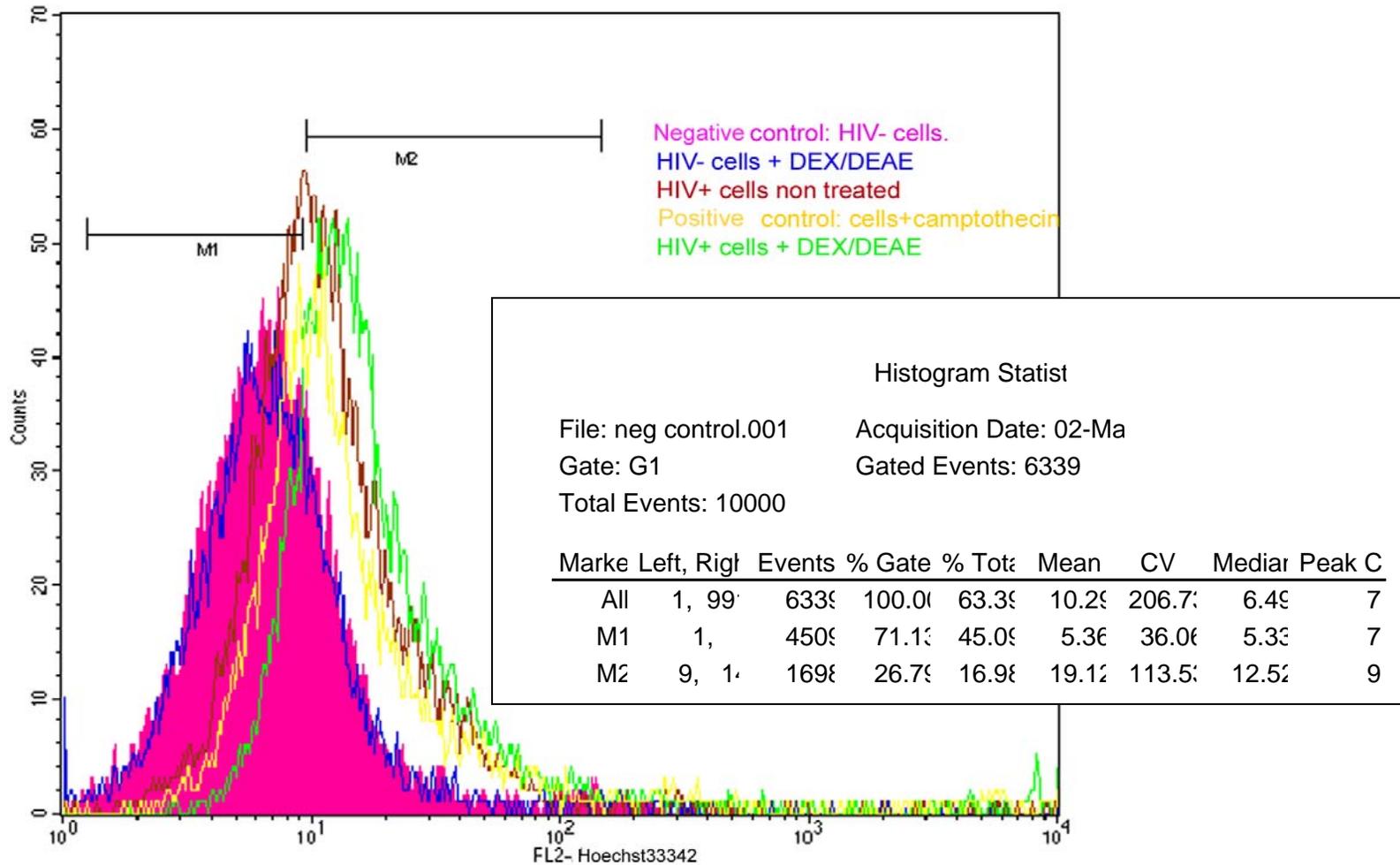


Figure 23: Histogram representation of Hoechst fluorescence representing apoptosis.

The cells were further analyzed by the FACS to look for the combined fluorescence of both Hoechst 33342 and PI. Figure 24 shows the results collected, and as shown by the statistics the HIV infected cells treated with Dex/DEAE have 47.2% of the total cell population at a late apoptosis stage and 20.19% early apoptotic, while the non infected cells treated with Dex/DEAE showed 15.35% early apoptotic and an insignificant number of cells dead via late apoptosis, and the HIV infected cells untreated had a 19.29% cell death slightly higher than the uninfected cells, which represents the effect of the virus on the cells.

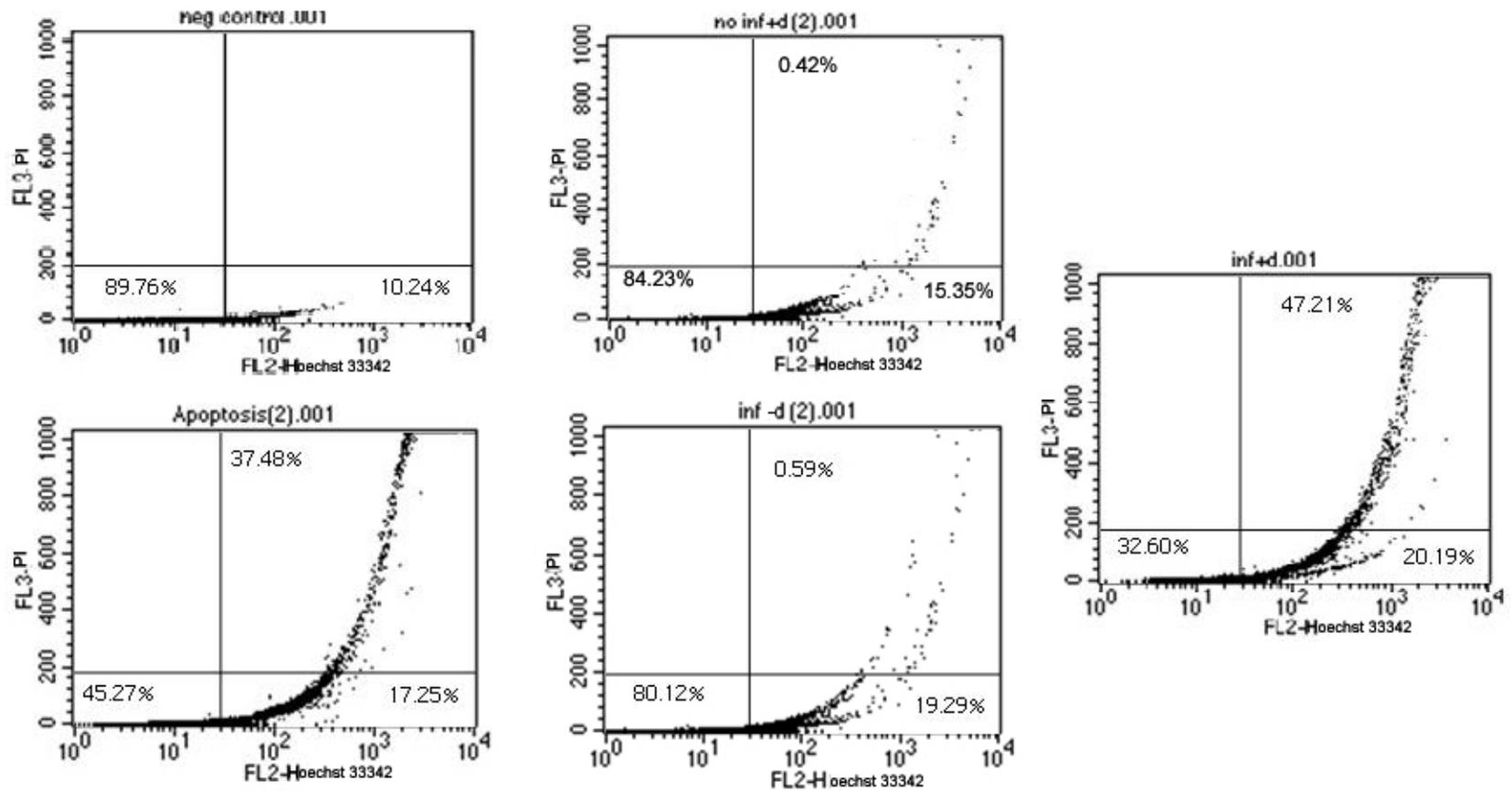


Figure 24: FACS results of Hoechst and PI combined for :
 Upper left: control non infected non treated cells.
 Upper right: non infected cells treated with DEX/DEAE.
 Lower left: apoptosis induced cells via camptothecin.
 Lower right: HIV infected cells treated with DEX/DEAE.

Discussion

The results collected by flow cytometry indicated that the combination of the two FDA approved drugs appears to be a potent inducer of programmed cell death (apoptosis) only in infected cells (killing almost 50% of the total HIV+ population) while non infected cells seem to be relatively unaffected by this combination. This suggests that Dex+DEAE triggers genes and biochemical pathways that lead to apoptosis as a response to the HIV infection in cells, and it is of great interest to identify these genes and pathways to further analyze and understand this effect. Microarray analysis seems to be the perfect method for this endeavor.

CHAPTER VII

DNA MICROARRAYS AND GENES INVOLVED

A more comprehensive picture of the mechanism can be obtained by comparing whole-genome MicroArray data. This gene expression analysis gives us a better understanding of the molecular logic underlying biological circuits involved in this mechanism of selective toxicity induced by Dex/DEAE.

Historically the use of microarray has led to the understanding and discovery of many molecular mechanisms underlying normal and dysfunctional biological processes that play important roles in cell performance of many diseases.

Microarray studies along with other genomic techniques represent an important stimulant in drug development, immunotherapeutics and gene therapy. However, microarray techniques have an advantage over other techniques that were employed for determining gene expression levels, such as Northern Blot analysis, because the later have been limited to the gene-by-gene approach, by looking for transcripts (certain RNA expression levels) one or a few at a time [28]. The invention of genome-wide transcript screening methods such as DNA microarray technology have helped surmount this restraint, and opened the door for scientists to study thousands of transcripts simultaneously [29].

Materials and Methods

Microarray is a high-throughput technology that uses an ordered array of microscopic elements on a planar substrate that allows the specific binding of analytes (molecules under analysis) [29], enabling the measurement of thousands of genes in a single RNA sample. Microarrays contain assortments of small components or spots arranged in rows and columns, and during measurements, the array is exposed to the biological sample (generally in an aqueous solution) where different analytes are present in unknown concentrations. Individual spots then capture specific analytes by affinity from the solution in the incubation phase and consequently the quantity captured is measured in real time or at the end of incubation depending on the method[29].

The microarray systems that measure mRNA concentrations are referred to as DNA microarrays or gene chips. They are ordered collections of DNA sequences (probes) deposited on and chemically bound to solid surfaces such as microscope slides (see Fig25).

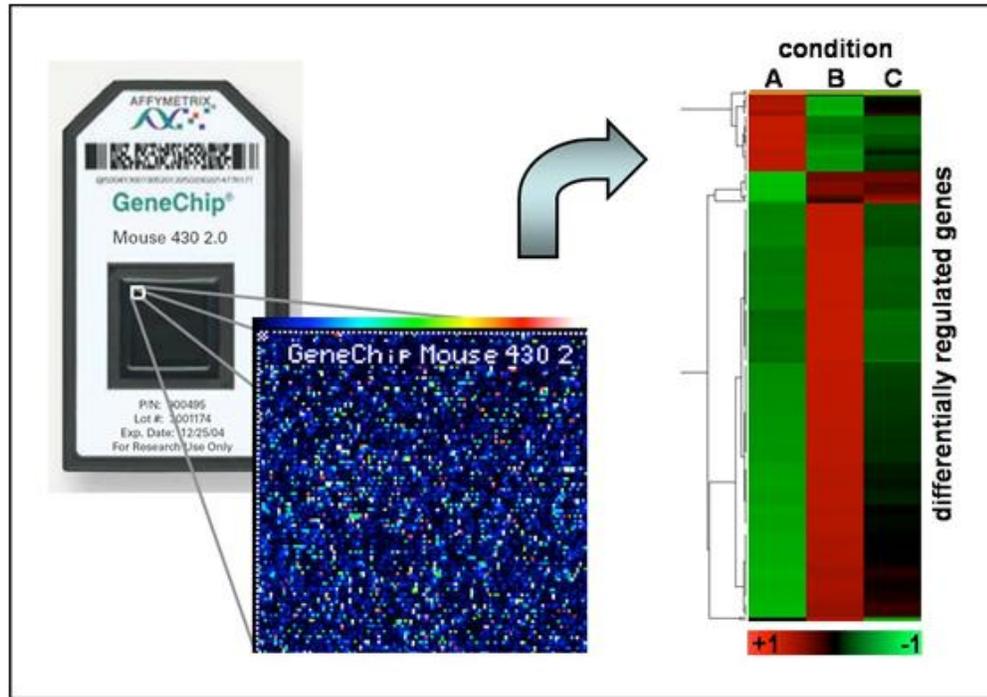


Figure 25: Affymetrix gene chip[30].

Although there are a variety of microarray platforms that have been developed and evolved significantly since their introduction in 1995[31], the most popular format is still the original gene expression microarray designed to measure the relative abundance of mRNA transcripts.

DNA microarrays are based on the principle of DNA hybridization, by which a single strand of DNA (the probe) binds to a complementary strand of DNA (the target), forming a highly stable duplex structure in a lower energy state [29].

To allow detection of this hybridization, targets are fluorescently labeled with fluorophore molecules (such as Cy3 and Cy5 dyes)[32]

A typical microarray detection procedure follows this basic methodology:

1. A slide or membrane is spotted or "arrayed" with DNA fragments or oligonucleotides that represent specific gene coding regions.
2. RNAs are initially reverse transcribed into cDNA by a simple polymerization reaction
3. RNA is purified and then fluorescently- or radioactively labeled
4. The prepared RNA is simultaneously hybridized to the slide/membrane.
5. The microarrays are thoroughly washed to eliminate any excess from the hybridization reaction then dried.
6. The raw data is obtained by laser scanning or autoradiographic imaging for detection of fluorescence on the DNA probes as represented in Figure 1).
7. The intensity in each spot is related to the expression level of the specified gene, and the data is then entered into a database and analyzed by multiple statistical methods.[33]

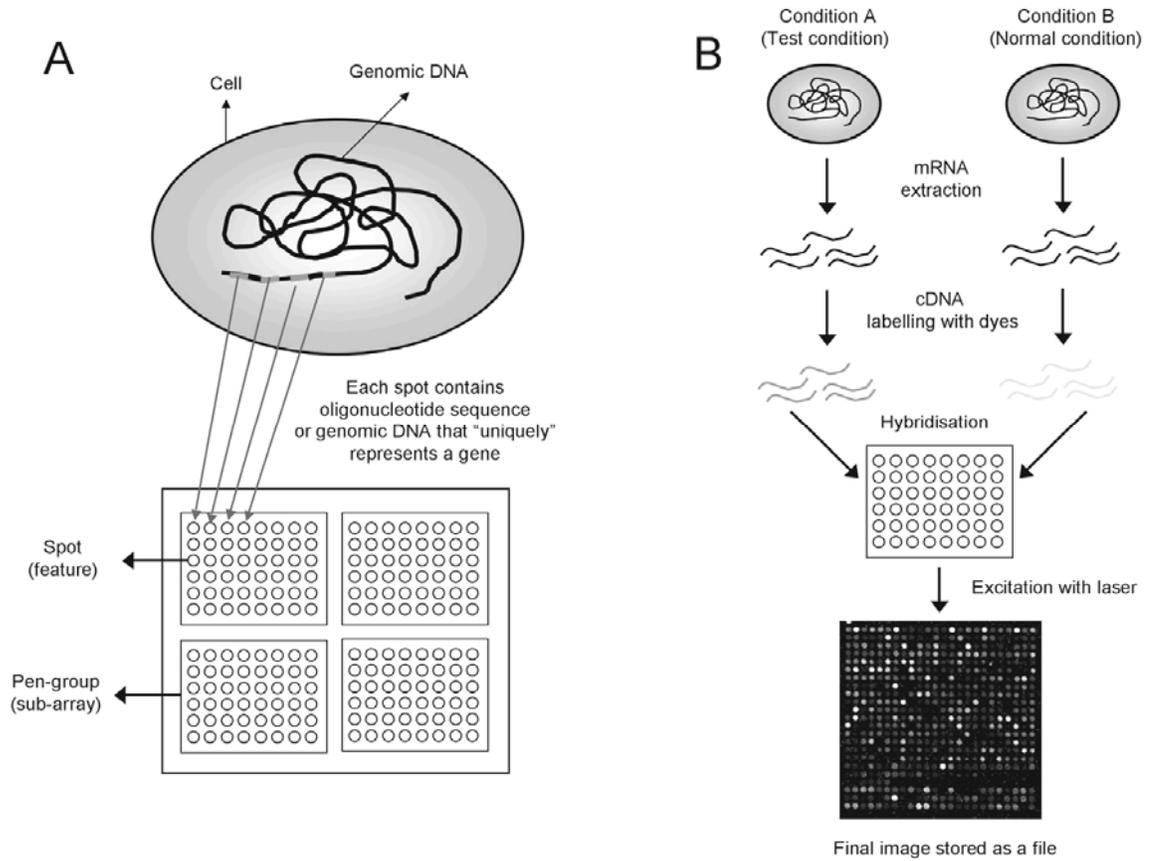


Figure 26: (A) A microarray may contain thousands of spots. Each spot contains many copies of the same DNA sequence that uniquely represents a gene from an organism. Spots are arranged in an orderly fashion into Pengroups. (B) Schematic of the experimental protocol to study differential expression of genes. The organism is grown in two different conditions (a reference condition and a test condition). RNA is extracted from the two cells, and is labelled with different dyes (red and green) during the synthesis of cDNA by reverse transcriptase [34].

Following this step, cDNA is hybridized onto the microarray slide, where each cDNA molecule representing a gene will bind to the spot containing its complementary DNA sequence. The microarray slide is then excited with a laser at suitable wavelengths to detect the red and green dyes. The final image is stored as a file for further analysis

[35]. For our experiment we prepared the samples according to the selective toxicity protocol briefly as follows:

- Culture Jurkat cells on microplates.
- Infect half of the cells with HIV-1, leave half uninfected; incubate for 12 hours.
- Expose both groups of cells to various treatments:
 - No drugs.
 - Combination of Dex and DEAE.
- After 48 hours incubation, the total cellular RNA is prepared and purified using TRIzol® Plus from Invitrogen which is considered the most trusted reagent for preparing high quality, intact RNA from cells. The TRIzol® Plus combines the property of TRIzol® Reagent to efficiently lyse tissues and cells with the silica spin-column for the isolation of total RNA using the PureLink™ RNA Mini Kit.

Total RNA extracted from the samples was checked for purity using a spectrophotometer to quantify the amount of nucleic acids via UV absorption measured at the wavelengths of 260 nm and 280. A₂₆₀ is frequently used to measure RNA concentration since RNA has its absorption maximum at 260 nm and A₂₈₀ is used to measure protein concentration. A ratio of $A_{260}/A_{280} > 1.8$ suggests little protein contamination in an RNA sample, and the concentration of nucleic acid can be determined using the Beer-Lambert law, which predicts a linear change in absorbance with concentration, and pure RNA has an A_{260}/A_{280} of 2.1 [33]. In many protocols it is indicated that a value between 1.8 and 2.0 indicates that the RNA is pure.

Our samples had A260/A280 ratio of 2, and were then packaged in 1.5 ml RNase-free microcentrifuge tubes, containing 35µg each of high quality total RNA in 100µl DEPC-treated water. The prepared total RNA was sent to The Microarray Shared Resource of the Comprehensive Cancer Center of Wake Forest University (CCCWFU) directed by Dr. Lance Miller, Associate Professor, Department of Cancer Biology. The samples were run on Affymetrix HG-U133 Plus 2.0 chips using GeneChip Scanner 3000 7G multi-color scanner.

Further quality control checks were run on all samples by the Wake Forest Core facility, and the quality and quantity of RNA was judged acceptable. The core facility provided us with Excel tables that show the prospective intensities of each one of our samples:

- Uninfected cells non treated
- Uninfected cells treated with DEX/DEAE
- Infected cells untreated
- Infected cells treated with DEX/DEAE

And from there we analyzed the microarray data which represented a huge challenge for us due to the colossal amount of data generated by this powerful technology; the analysis of the 60,000 genes was done using R software.

Microarray Data Analysis

Systematic variation between experimental conditions are unrelated to the biological differences we seek in a microarray analysis, that is why a normalization step is crucial to the analysis as it accounts for these differences and compensates for any systematic technical differences between chips, to allow us observe more clearly the systematic biological differences between our biological samples [36].

Many commercially or freely available tools exist to perform the common steps for analyzing microarray data; we chose to use BRB-ArrayTools by Richard Simon and BRB-ArrayTools Development Team at the National Cancer Institute, NIH Bethesda. BRB-ArrayTools is an integrated package for the visualization and statistical analysis of DNA microarray gene expression data. It was developed by professional statisticians experienced in the analysis of microarray data and involved in the development of improved methods for the design and analysis of microarray based experiments [37]. The array tools package utilizes an Excel layout that we are familiar with.

An important part of the microarray analysis includes the comparison of the differentially expressed genes or the change of certain expression levels in the different array corresponding to the different conditions used.

For this purpose the arrays have to be comparable. However different arrays have different intensity levels, also if the same condition is measured twice and the average intensity is the same, the distribution of intensity values may differ, [38], the difference in intensity in raw data (figure 27) cannot be taken into consideration until after normalization:

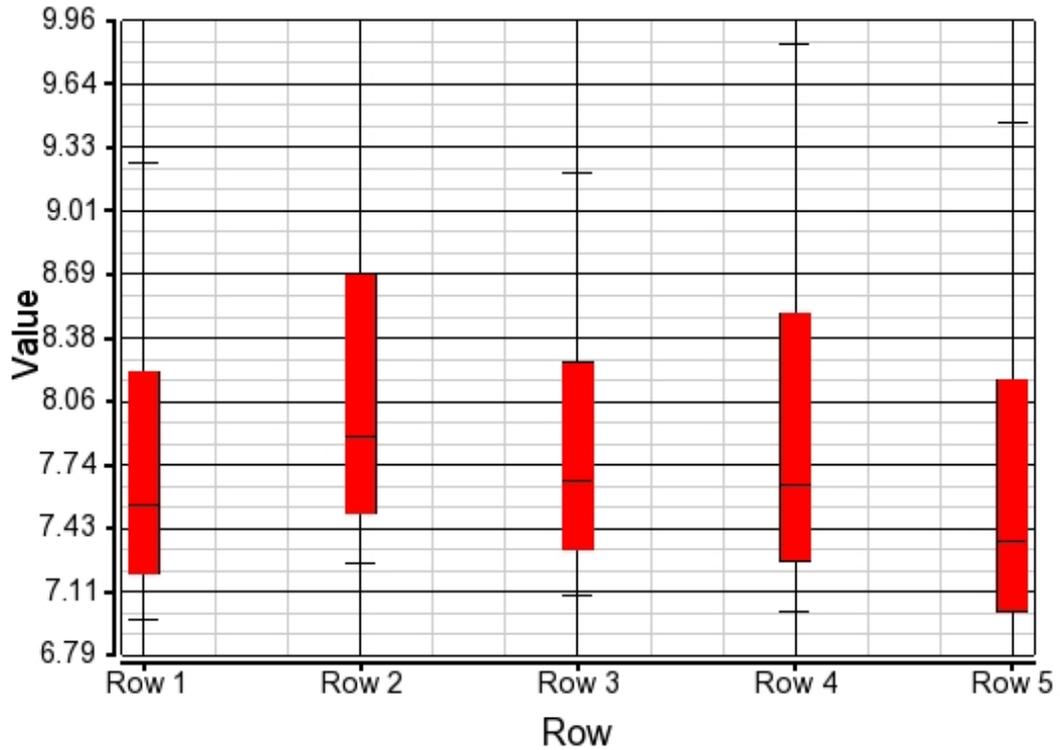


Figure 27: Box plot representation of the dispersion of raw data intensities: Rows 1, 2, 3, 4, 5 represent the different sample treatments in the experiment; Row 1: uninfected untreated Jurkat cells; Row 2: HIV infected Jurkat cells treated with Dex/DEAE; Row 3: uninfected, Jurkat cells treated with Dex/DEAE; Row 4: HIV infected untreated Jurkat cells; Row 5: HIV infected treated with Gentamicin cells (irrelevant for our experiment, belongs to another experiment but data was still used for normalization purposes).

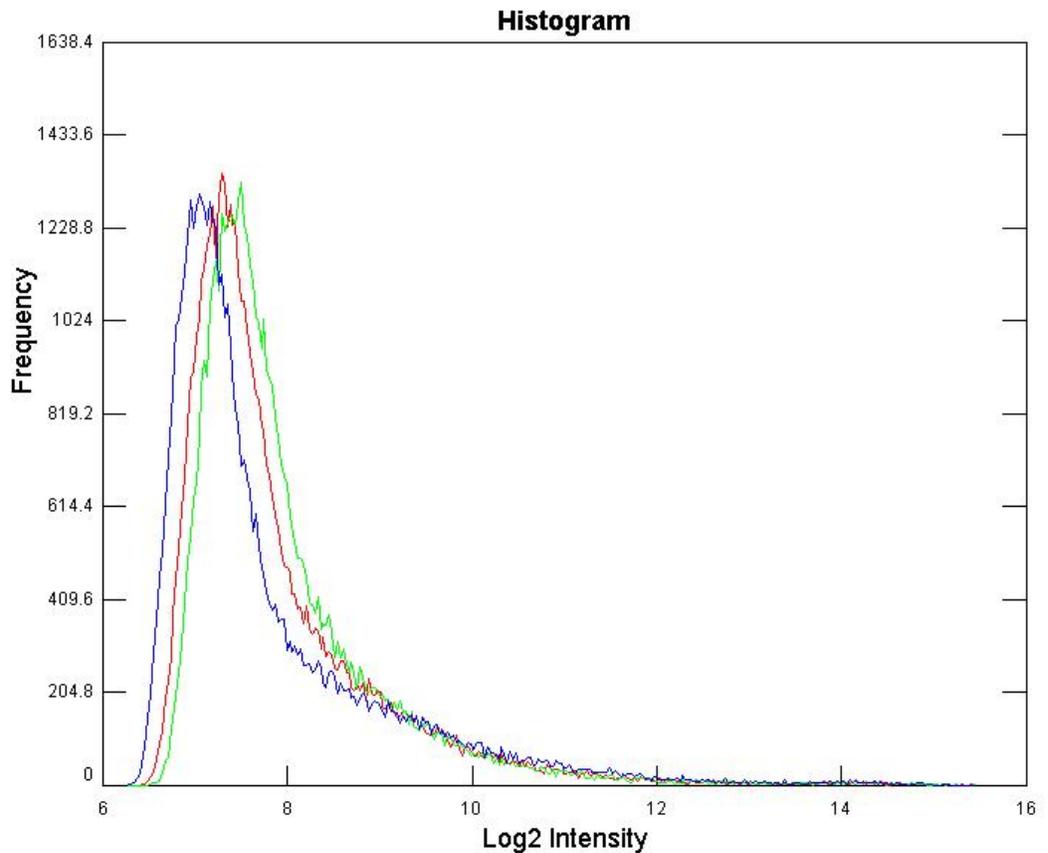


Figure 28: Histogram presentation of Log2 intensities of raw data. Only the three important conditions in the experiment were used for the Log2 normalization: Blue represents uninfected untreated Jurkat cells; red represents uninfected Jurkat cells treated with Dex/DEAE; green represents HIV infected Jurkat cells treated with Dex/DEAE.

In general, to obtain single gene expression value out of raw probe intensity measurements is called "microarray preprocessing", in order to be able to consider these results valid it is critical to proceed with pre-processing and normalization of microarray data, in order to control the effects of systematic error while retaining full biological variation [34].

Many different normalization methods or combinations of methods were proposed over the years, for our analysis we chose the three most reliable methods: Systematic Variation Normalization (SVN), Robust Multi-array Analysis (RMA) and the standard Affymetrix probe modeling algorithm MAS5:

- Systematic variation normalization (SVN) is a procedure for removing systematic variation in microarray gene expression data. This technique is based on an analysis of systematic variation as it contributes to variability in microarray data sets; it contains background subtraction determined by the distribution of pixel intensity values and log conversion, linear or non-linear regression, restoration or transformation, and multiarray normalization [39]

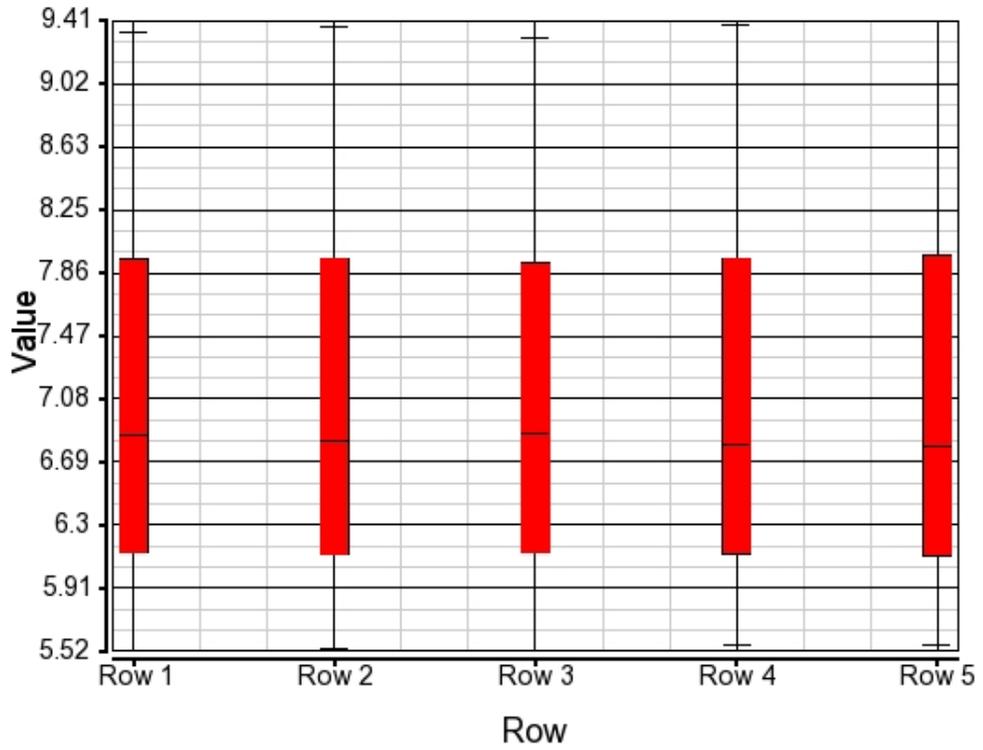


Figure 29: Box plot presentation of data intensities after SVN normalization (see Figure 25 for explanation of treatments represented by the various rows).

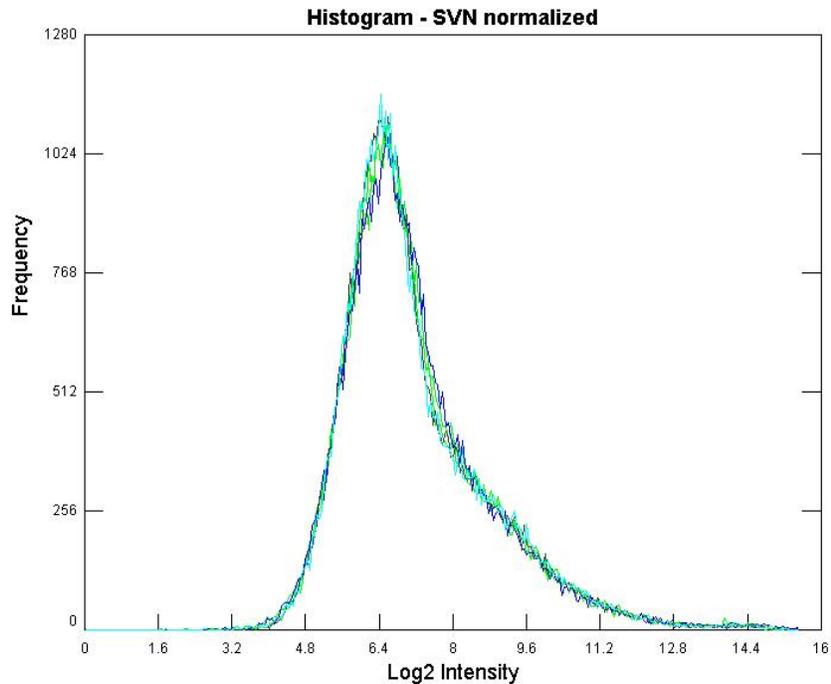


Figure 30: Histogram presentation of Log2 intensities after SVN normalization (see figure 28 for explanation of color coding).

- RMA (Robust Multi-array Analysis) is a method for normalizing and summarizing probe-level intensity measurements from Affymetrix Gene Chips®, it actually has become the de facto standard for preprocessing expression data from Affymetrix GeneChips. It uses probe-level expression measurements of all arrays to estimate expression values, starting with the probe-level data from a set of Gene Chips, the perfect-match (PM) values are background-corrected, normalized and finally summarized resulting in a set of expression measures. It comprises the three steps: Background Correction, Normalization and summarization [40].

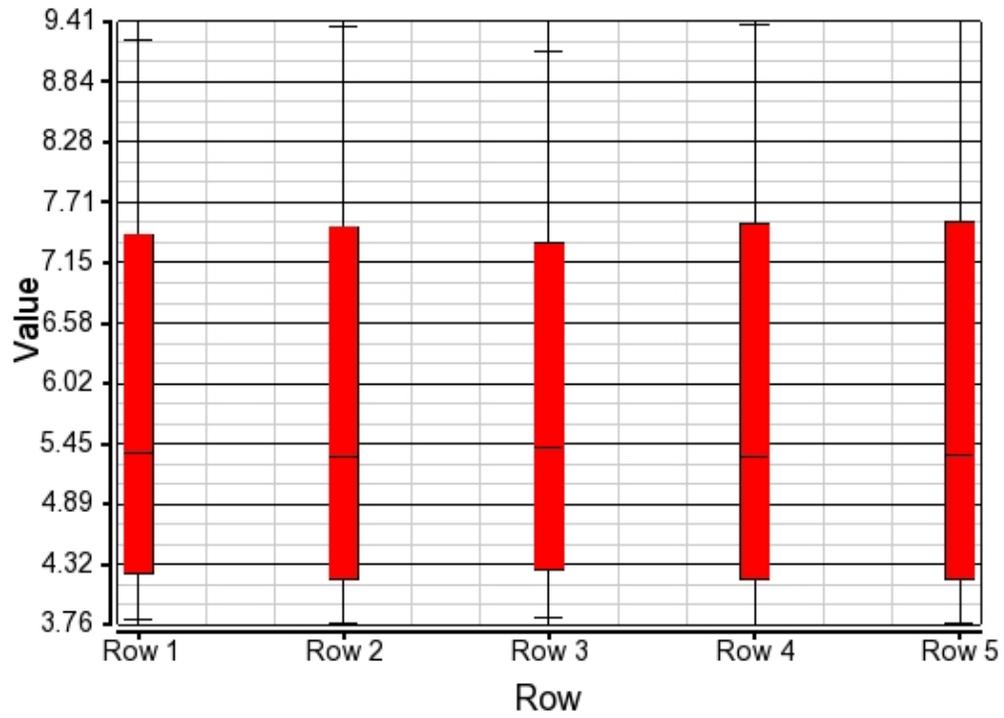


Figure 31: Box plot representation of data intensities after RMA normalization (see Figure 27 for explanation of treatments represented by the various rows).

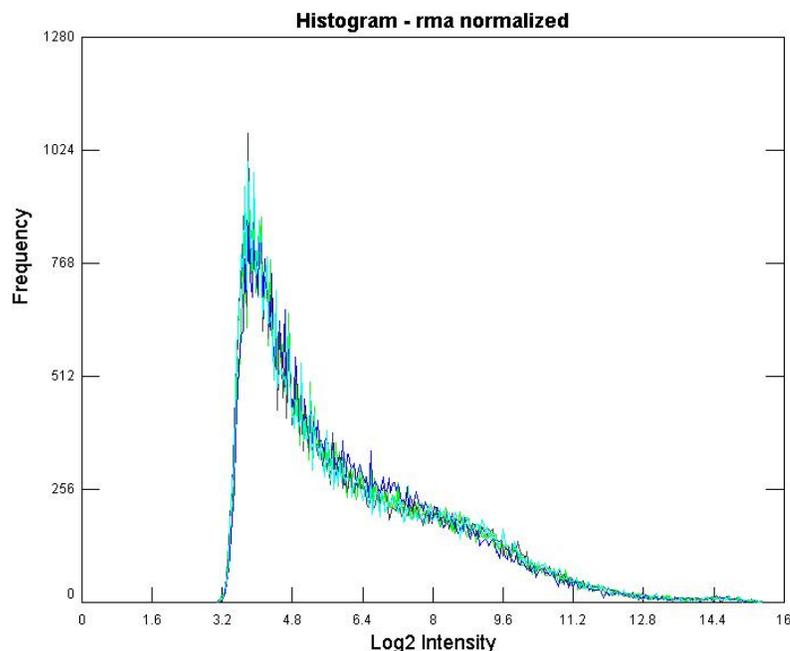


Figure 32: Histogram presentation of Log2 intensities after RMA normalization (see figure 28 for explanation of color coding).

- The standard Affymetrix probe modeling algorithm MAS5: is a basic method considered just slightly better than scaling using linear regression. This procedure is to construct a plot of each chip's probes against the corresponding probes on the baseline chip; eliminate the highest 1% of probes (and for symmetry the lowest 1%), and fit a regression line to the middle 98% of probes (ie. estimate slope and intercept: two parameters). Transform the values in each probe, by subtracting the intercept, and dividing by the slope, so that the regression line becomes the identity ($y = x$) line [36].

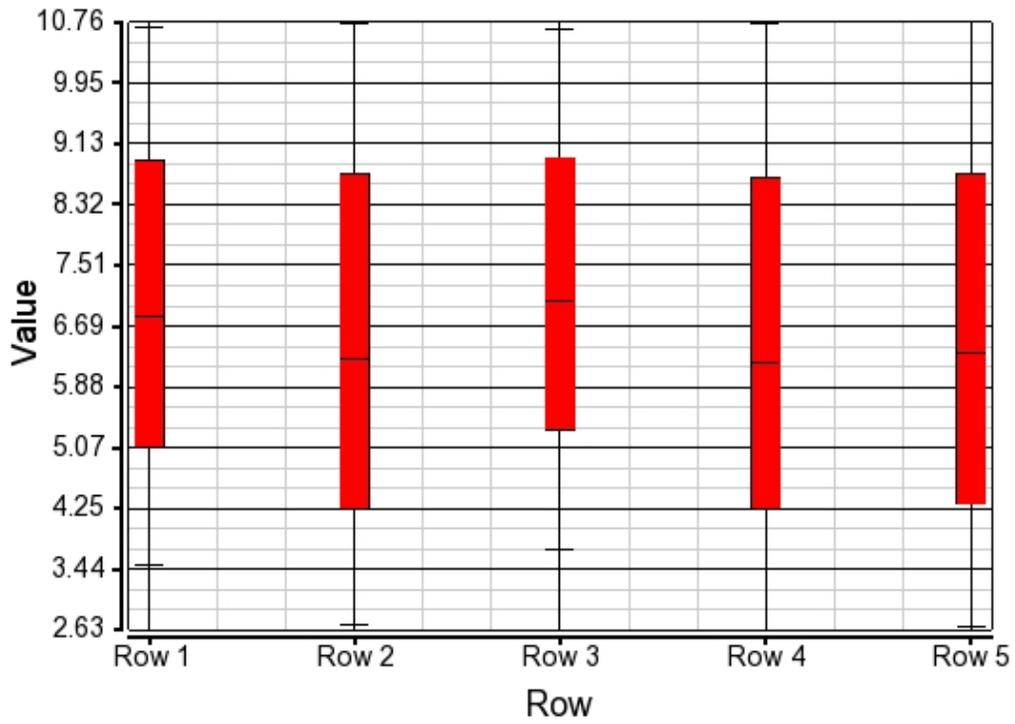


Figure 33: Box plot representation of data intensities after MAS5 normalization (see Figure 27 for explanation of treatments represented by the various rows).

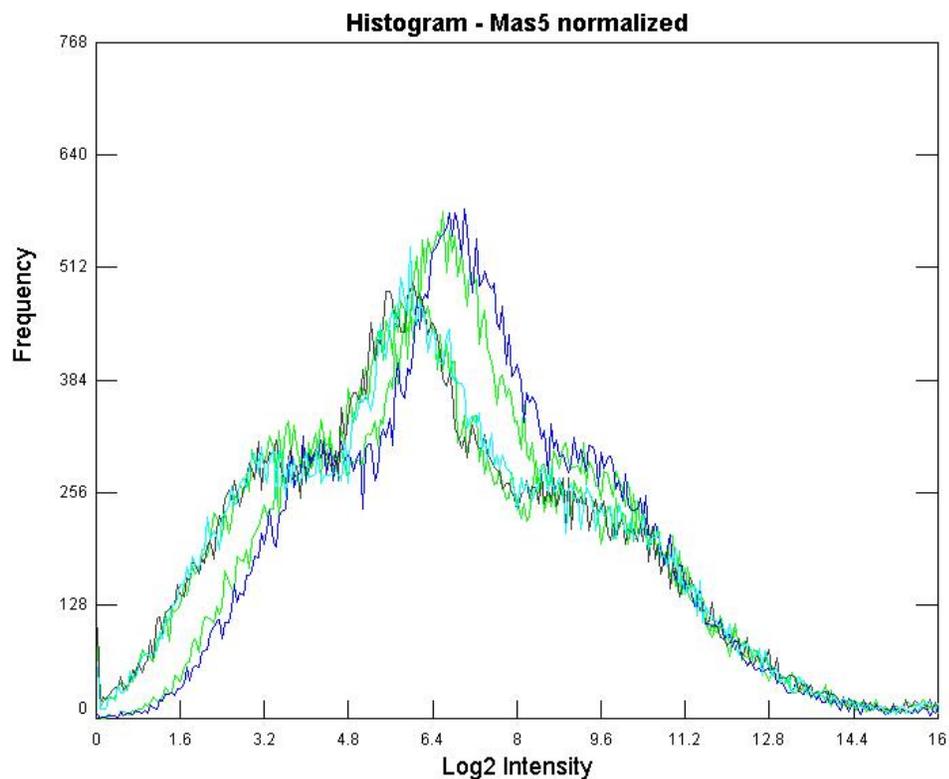


Figure 34: Histogram presentation of Log₂ intensities after MAS5 normalization (see figure 28 for explanation of color coding).

These three pre-processing methods have in common the ability to remove systematic variation in the data attributable to variability in microarray slides, assay-batches, the array process, or experimenters. Biologically meaningful comparisons of gene expression patterns between control and test channels or among multiple arrays are therefore unbiased using normalized datasets [41].

Results

After the analysis of the Microarray data compiled via the three different normalization methods, altered expression of several genes in the HIV infected cells treated with Dexamethasone and DEAE was revealed; 211 genes upregulated and 91 down regulated (figure 35), while the uninfected cells treated with Dexamethasone and DEAE showed 1 up regulated gene , and 29 down regulated (figure36). A complete list of the genes affected is listed in the appendix.

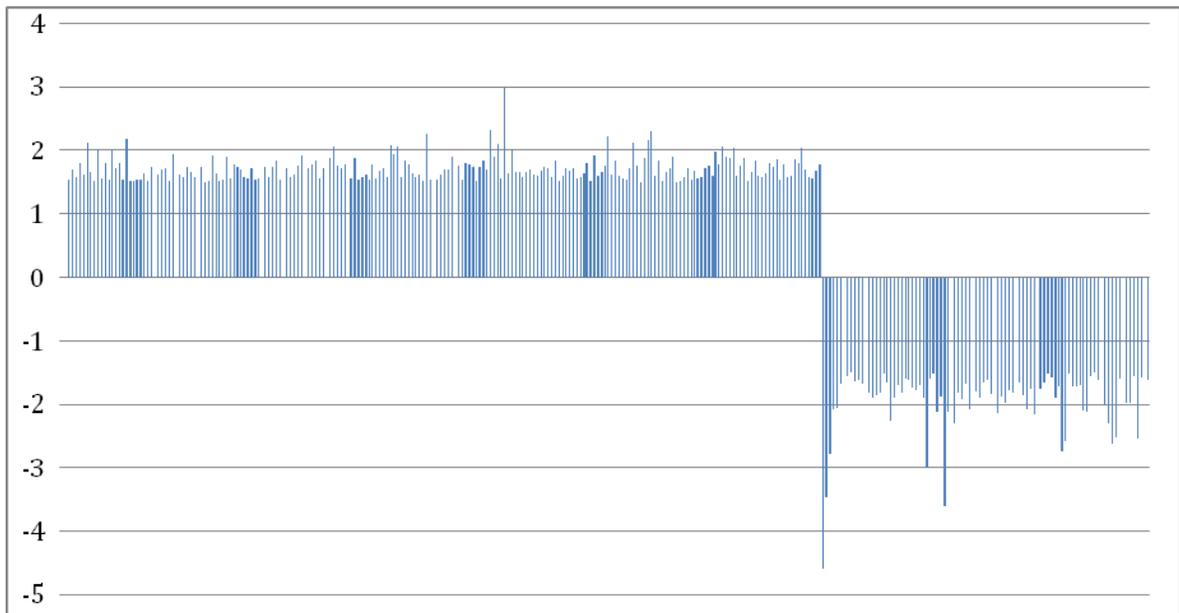


Figure 35: Genes up and down regulated in HIV+ Jurkat cells treated with Dex/DEAE. Space does not permit the labeling of individual genes, which are listed in rank order in the appendix.

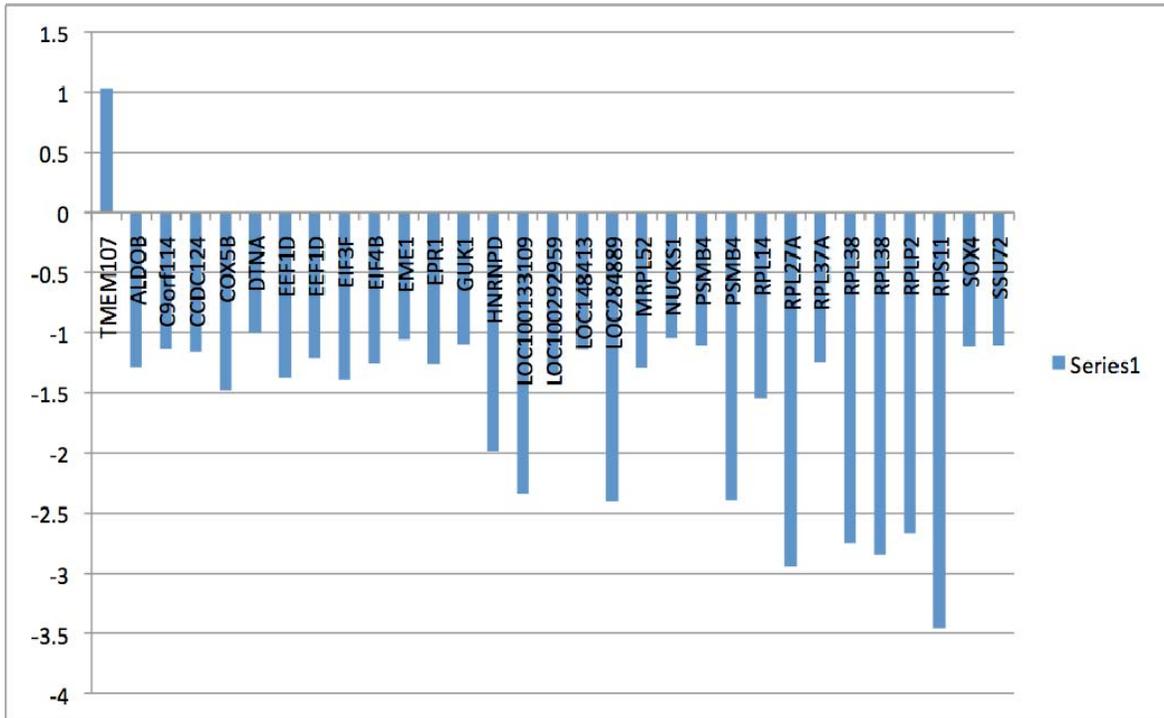


Figure 36: genes up and down regulated in the HIV- Jurkat cells treated with Dex/DEAE.

It is beyond the scope of the current project to conduct a complete analysis of this data set (which requires software for “Systems Biology” pathway analysis that we do not possess), we proceeded with a preliminary manual analysis of all these genes, to be able to single out specific genes and or clusters of genes belonging to gene networks (biochemical pathways) that are induced or down regulated only in the presence of both drugs and virus and which therefore could be central to the mechanism of selective toxicity to infected cells. (Already stated) We also looked for genes that might relate to the experimental evidence presented previously, e.g., suggesting that Dex/DEAE achieves its effects via the induction of apoptosis in HIV-infected cells (Chapter 6).

A handful of upregulated genes are specifically involved in apoptosis. These include Annexin A1 (ANXA1), which is related to the Annexin V that we used as a fluorophore in Chapter 6 to demonstrate apoptosis using flow cytometry. Another is Mudeng (“MU-2/AP1M2 domain containing, death-inducing”), a gene that encodes a protein that is known to induce cell death in cytotoxic T cells. Other important cell-death related genes that are upregulated include BCLAF1, which when overexpressed is proapoptotic via its action as a transcriptional repressor of BCL-2 family genes, and last but not least, Fas (CD95), a well known inducer of cell death [42, 43] [44].

Various other up- or downregulated genes are, if not directly involved in apoptosis, possibly indirectly involved via their roles in the cell growth cycle, e.g. hedgehog interacting protein (HHIP, upregulated); alpha thalassemia/mental retardation syndrome X-linked (ATRX, upregulated), a transcription factor involved in cell cycle progression from G1 to S phase, and cyclin-dependent kinase inhibitor 2D (CDKN2D, downregulated), a cell growth regulator that controls cell cycle G1 progression [42].

Several notable clusters of genes whose functions can also be related to apoptosis include:

1. A set of peptidyl-prolyl isomerases (PPIs, often called cyclophilins, including PPIG, PPIL1, PPWD1, all upregulated),
2. Several ubiquitin-related genes (USP15 and USP16, both upregulated), and
3. A group of genes involved in regulation of RNA splicing (splicing factors SFRS7, 11, 12, 18, all upregulated).

The peptidyl-prolyl isomerases may have functions that relate to pre-mRNA splicing (e.g. PPWD1 can be part of the spliceosome) [45, 46]. The splicing-related genes may relate to the Fas death pathway, because splicing can produce FLIPs, the short form of FLICE inhibitor [47]. Furthermore, the half life of certain protein isoforms is regulated by degradation via ubiquitination, possibly explaining cluster of ubiquitin-related genes. For example, USP15 directly regulates Caspase 3, and important mediator of apoptotic signaling [48]; furthermore, the short form of FLICE inhibitor mentioned above is degraded via ubiquitination faster than the long form, which links ubiquitination as well as RNA splicing to the regulation of apoptosis. Thus, the net effect of this cluster of genes may be to reduce the half life of an inhibitor of Fas, which combined with increased Fas expression, accelerates cell death [49].

Thus, it appears upon preliminary examination that the results of the microarray study are reasonably consistent with the experimental results reviewed previously. However, a more in depth computer assisted analysis, will be required to fully mine the microarray data set for clues as to the exact mechanism by which the intersection of HIV infection plus Dex/DEAE treatment selectively unleashes apoptosis in cells.

CHAPTER VIII

SUMMARY AND FUTURE RESEARCH DIRECTIONS

Summary

The results described in this dissertation are a proof of concept and support to the hypothesis that there are two complementary modes of action of the Dex/Procaine or Dex/DEAE drug combinations:

1. the ability to inhibit viral replication, most effectively at low viral titers (this is most likely due to the documented NF- κ B antagonism of Dex)
2. the ability to selectively kill HIV-infected cells.

This is a very unique activity and has rarely been reported, particularly for small molecule drugs. It is expected that with prolonged exposure to treatment with this drug combination, the killing of infected cells would lead to a decline in viral load. This has been confirmed in the experiments described at the end of Chapter IV. Over just a two-week period, viral load was decreased by more than 50%, compared to untreated infected cells. This reduction in viral titer by the proposed mechanism of selective killing of HIV-infected cells would be expected to enhance the effectiveness of the treatment to inhibit HIV replication, which is more effective at low viral titers (Chapter IV). Thus, these two activities could be synergistic in practice. Furthermore, the drug combination showed an additive effect when combined with an antiretroviral drug (AZT), and the antiviral activity of the Pro/Dex combination remained unchanged in an AZT-resistant strain of

HIV, suggesting that the treatment is likely to be effective against other common drug-resistant strains.

We have used 3 independent methods to confirm that the Dex/DEAE combination leads to a selective reduction in the number of viable cells in HIV-infected Jurkat cells: MTT assay, XTT assay (Chapter III) and fluorometric Live/Dead cell assays (Chapter V). Combined with evidence that, over time, the viral titer decreases in infected cells exposed to this treatment, compared to untreated infected cells (Chapter IV), these results strongly suggest that it is HIV-infected cells that are being selectively eliminated by the drug treatment.

To shed the light on the mechanism of cell death involved, several flow cytometric techniques were used (Chapter VI). These studies demonstrate that the HIV infected cells are selectively killed by Dex/DEAE by the induction of apoptosis, via several known apoptotic pathways that we have identified using microarray analysis techniques (Chapter VII). Significantly, in one study (Figure 26, Chapter VI), it was demonstrated that in a uniformly infected population of cells (almost 100% infected cells, generated by cell sorting using a FACS ARIA III), the drug treatment induced a state of early or late apoptosis in over 67% of the cells, whereas drug treated uninfected cells showed only a 5% increase in apoptosis relative to untreated uninfected cells (15% vs. 10% respectively). This definitively demonstrates that the drug treatment effectively targets HIV-infected cells for apoptosis, while having little effect on uninfected cells.

Q.E.D.

Future Research Directions

Q-PCR/RT PCR

Results obtained from MicroArray studies can be confirmed using real-time PCR or quantitative PCR to get more accurate quantitative readings of how the expression of specific genes changes in response to the various treatments. So after we identify the genes in question, we will prepare total RNA from the samples (Untreated control cells, cells treated with either virus or drugs alone, and cells treated with both virus and drugs) using Qiagen's Rneasy Kit; cDNAs will be prepared by using Qiagen's Quantitect Reverse transcription kit. Target gene expression will be determined by SYBR Green PCR. The specific primers for PCR will be picked from NIH PrimerDepot at <http://primerdepot.nci.nih.gov/>. The real time PCR products will be quantitated by generating a standard curve or quantitated relative to a control gene. From these results we shall be able to detect any abnormality to confirm the results generated with Micro array.

RNAi

The biochemical effects of the changes induced by DEAE/Dex in gene expression can be studied using RNA interference particularly short interfering RNAs (siRNA) to mimic a gene that is down regulated, so we will design and generate (siRNAs) against the specific genes that proved to be down regulated from the results collected through Microarray and real time PCR, and we will use a transfection reagent to get the siRNAs inside the cells, such as LipofectAMINE from INVITROGEN, and then since RNAi down regulates a

gene function without actually interacting with it, but via mRNA degradation, we can actually measure the degree of RNA interference achieved, by directly relating it to the level of mature mRNA and the translated proteins. The mature mRNA can then be measured by quantitative PCR using SYBR green since it is the most sensitive method and can discriminate closely related mRNAs.

By the same rationale we can mimic a gene that is upregulated by gene transfection/expression. We will transfect cells with the target gene to see if it inhibits HIV replication. Briefly, CEM cells are plated in a 24-well plate. Cells are transfected with the target gene expression vector, using an empty vector as control. 6 hours post-transfection, cells are infected with HIV-1 stock (MOI=0.1). 48 hours post-infection, luciferase activity is measured. Luciferase activity of CEM cells is used to give comparative levels of virus replication, at varied levels of the transfected gene. This experiment can establish whether the gene under study could be an intermediary between exposure to the drugs (Dex/DEAE), and increased HIV production in the infected cell. And if this approach leads to the expected result (e.g., cell death by apoptosis), we will have a plausible mechanism for how the drug treatment exerts its unique effects in HIV-infected cells.

REFERENCES

1. Hutchinson, J.F., *THE BIOLOGY AND EVOLUTION OF HIV*. Annual Review Anthropology 2001. **30**: p. 85–108.
2. Levy, J.E., *HIV pathogenesis: 25 years of progress and persistent challenges*. AIDS, 2009.
3. Boerma, et al., *Measurement of Biomarkers in Developing Countries: Opportunities and Problems*. Population and Development Review, 2001. **27**: p. 303-314.
4. UNAIDS, *UNAIDS 2010*.
5. Grassly, et al., *The Effectiveness of HIV Prevention and the Epidemiological Context*. Bulletin of the World Health Organization, 2001. **79**: p. 1121-1135.
6. Aral, S. O., and R. Roegner, *Mathematical Modeling as a Tool in STD Prevention and Control: A Decade of Progress, a Millennium of Opportunities*." Sex Transm Dis, 2000(27): p. 556-7.
7. COWLEY, S., *The biology of HIV infection*. FURTHER EDUCATION SERIES, 2001.
8. Romani, B. and S. Engelbrecht, *Human immunodeficiency virus type 1 Vpr: functions and molecular interactions*. Journal of General Virology, 2009. **90**: p. 1795–1805.

9. Coffin, o.M., S.H. Hughes, and H.E. Varmus, *Retrovirus*, ed. C.S.H. (NY). 1997: Cold Spring Harbor Laboratory Press.
10. Trono, D., *HIV Persistence and the Prospect of Long-Term Drug-Free Remissions for HIV-Infected Individuals*. SCIENCE, 2010.
11. Adolescents, P.o.A.G.f.A.a., *Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents*. 2009.
12. Geeraert, L., G. Kraus, and R.J. Pomerantz, *Hide-and-peek: the challenge of viral persistence in HIV-1 infection*. Annu Rev Med, 2008. **59**: p. 487-501.
13. Warnke, D., J. Barreto, and Z. Temesgen, *Antiretroviral Drugs*. the Journal of chemical Pharmacology, 2007.
14. Work, T.S., *Selective Toxicity, an Essential Requirement for Successful Chemotherapy*. Proceedings of the Royal Society of London, 1949. **136**(883): p. 159-166.
15. Esposito, F., L. Zinzula, and E. Tramontano, *Antiviral drug discovery: HIV-1 RNase H, the next hit target*. Communicating Current Research and Educational Topics and Trends in Applied Microbiology, 2007.
16. Williams, S.A. and W.C. Greene, *Regulation of HIV-1 latency by T-cell activation*. Cytokine, 2007. **39**(1): p. 63-74.
17. Berger, E.A., et al., *CD4-Pseudomonas exotoxin hybrid protein blocks the spread of human immunodeficiency virus infection in vitro and is active against cells expressing the envelope glycoproteins from diverse primate immunodeficiency retroviruses*. Proc Natl Acad Sci U S A, 1989. **86**(23): p. 9539-43.

18. Singwi, S. and S. Joshi, *Potential nuclease-based strategies for HIV gene therapy*. Front Biosci, 2000. **5**: p. D556-79.
19. Brodie, B.B., E.M. Papper, and L.C. Mark, *Fate of procaine in man and properties of its metabolite diethylaminoethanol*. Curr Res Anesth Analg, 1950. **29**(1): p. 29-33.
20. Fabunan, R.G., *Injection Viral treatment*. 2001.
21. Bank, D. *Procaine*. 2005 [cited; Available from: <http://www.drugbank.ca/drugs/DB00721>].
22. Denizot, F. and R. Lang, *Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability*. J Immunol Methods, 1986. **89**(2): p. 271-7.
23. Platt, E.J., et al., *Effects of CCR5 and CD4 Cell Surface Concentrations on Infections by Macrophagetropic Isolates of Human Immunodeficiency Virus Type 1*. virology, 1997.
24. Montefiori, D.C., *Measuring HIV neutralization in a luciferase reporter gene assay*. Methods Mol Biol, 2009. **485**: p. 395-405.
25. probes, M., *LIVE/DEAD ® Viability/Cytotoxicity Kit *for mammalian cells**, i.d. technology, Editor. 2005.
26. Lecoecur, H., et al., *Journal of Immunological Methods*, 1997. **209**(2): p. 111-123.
27. Francisco, U.o.S. 2007 [cited; Available from: <http://missinglink.ucsf.edu/lm/molecularmethods/flow.htm>].

28. Ausubel, F.M., et al., *Current Protocols in Molecular Biology*. 1996: Wiley, New York.
29. Hassibi, A., *INTEGRATED MICROARRAYS*, in *ELECTRICAL ENGINEERING*. 2005, STANFORD UNIVERSITY. p. 141.
30. Toled, t.u.o. *Analysis of Microarray Data – UT Genomics Core Laboratory*. 2010 [cited; Available from: http://www.utoledo.edu/med/depts/bioinfo/cores/GCL_Analysis_of_Microarray_Data.html].
31. Schena, M., et al., *Quantitative Monitoring of Gene Expression Patterns with a Complementary DNA Microarray*. *Science*, 1995. **270**(5235): p. 467-470.
32. Lakowicz, J.R., *Principles of Fluorescence Spectroscopy*. third ed, ed. P.P. 1983. 2006, New York, NY.
33. [cited; Available from: http://www.biomedicalgenomics.org/RNA_quality_control.html].
34. Zahurak, M., et al., *Pre-processing Agilent microarray data*. *BMC Bioinformatics*, 2007. **8**(142).
35. Babu, M.M., *An Introduction to Microarray Data Analysis*. 2004.
36. group, G.a.B. *Microarray Data Analysis*. 2011 [cited; Available from: <http://discover.nci.nih.gov/microarrayAnalysis/Affymetrix.Preprocessing.jsp>].
37. Simon, R. *BRB-ArrayTools*. 2011 [cited; Available from: <http://linus.nci.nih.gov/BRB-ArrayTools.html>].
38. Hochreiter, S., *Bioinformatics III*

- Structural Bioinformatics and Genome Analysis*. 2007, Institute of Bioinformatics, Johannes Kepler University Linz.
39. Health, T.n.i.o.e.h.s.-N.I.o. *Systematic Variation Normalization (SVN)*. 2005
[cited; Available from:
<http://www.niehs.nih.gov/research/resources/software/svn/index.cfm>.
40. agilent. *RMA Probe Summarization*. 2009 [cited; Available from:
http://www.chem.agilent.com/Library/technicaloverviews/Public/RMA_Probe_Summarization.pdf.
41. MATANGI, E.S., *Processing of Microarray data*, in *African Institute For Mathematical Sciences (AIMS)*. 2006, South African National Bioinformatics Institute (SANBI)University of Western Cape: Western Cape p. 49.
42. Baumann, S., et al., *Glucocorticoids inhibit activation-induced cell death (AICD) via direct DNA-dependent repression of the CD95 ligand gene by a glucocorticoid receptor dimer*. *Blood*, 2005. **106**(2): p. 617-25.
43. Lamy, L., et al., *Interactions between CD47 and Thrombospondin Reduce Inflammation*. *The Journal of Immunology*, 2007. **178**: p. 5930 -5939
44. Kogianni, G., et al., *Fas/CD95 is associated with glucocorticoid-induced osteocyte apoptosis*. *Life science*, 2004. **75**(24): p. 2879-95.
45. Krücken, J., G. Greif, and G.v. Samson-Himmelstjerna, *In silico analysis of the cyclophilin repertoire of apicomplexan parasites*. *Parasites & Vectors*, 2009.
46. Aaron, D., et al., *Structure of spliceosomal ribonucleoproteins*. *Biology reports*, 2010.

47. Maseda, D., et al., *Proteasome inhibition drastically but reversibly impairs murine lymphocyte development*. *Cell Death and Differentiation*, 2008. **10**(1038): p. 600–612.
48. Sano, Y., et al., *Photoreceptor Cell Apoptosis in the Retinal Degeneration of Uchl3-Deficient Mice*. *The American Journal of Pathology* 2006. **Volume 169**(1): p. 132-141.
49. Tripathi, P., et al., *Cutting Edge: Limiting Amounts of IL-7 Do Not Control Contraction of CD4 + T Cell Responses*. *Journal of immunology*, 2007. **178**: p. 4027-4031.
50. probes, M. *Vybrant® MTT Cell Proliferation Assay Kit (V-13154)*. 2002 [cited; Available from: <http://probes.invitrogen.com/media/pis/mp13154.pdf>].

APPENDIX A
GENES AFFECTED IN INFECTED CELLS
WITH DEX/DEAE

List of Upregulated genes arranged in decreasing intensity:

Probe Set ID	Gene Symbol	Gene Title
218718_at	PDGFC	platelet derived growth factor C
206727_at	C9	complement component 9
218886_at	PAK1IP1	PAK1 interacting protein 1
204614_at	SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2
234989_at	NEAT1	nuclear paraspeckle assembly transcript 1 (non-protein coding)
223154_at	MRPL1	mitochondrial ribosomal protein L1
223809_at	RGS18	regulator of G-protein signaling 18
231175_at	BEND6	BEN domain containing 6
1554089_s_at	SBDS /// SBDSP	Shwachman-Bodian-Diamond syndrome /// Shwachman-Bodian-Diamond syndrome pseudogene
232231_at	RUNX2	runt-related transcription factor 2
222808_at	ALG13	asparagine-linked glycosylation 13 homolog (S. cerevisiae)
232063_x_at	FARSB	phenylalanyl-tRNA synthetase, beta subunit
212718_at	PAPOLA	poly(A) polymerase alpha
208835_s_at	LUC7L3	LUC7-like 3 (S. cerevisiae)
224632_at	GPATCH4	G patch domain containing 4
203176_s_at	TFAM	transcription factor A, mitochondrial

1558111_at | MBNL1 | muscleblind-like (Drosophila)

201593_s_at | ZC3H15 | zinc finger CCCH-type containing 15

215171_s_at | TIMM17A | translocase of inner mitochondrial membrane 17 homolog A (yeast)

201012_at | ANXA1 | annexin A1

234405_s_at | PHAX | phosphorylated adaptor for RNA export

208859_s_at | ATRX | alpha thalassemia/mental retardation syndrome X-linked (RAD54 homolog, *S. cerevisiae*)

235925_at | TCF12 | Transcription factor 12

205518_s_at | CMAH | cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMP-N-acetylneuraminate monooxygenase)

235879_at | MBNL1 | Muscleblind-like (Drosophila)

1557081_at | RBM25 | RNA binding motif protein 25

1564164_at | DENND1B | DENN/MADD domain containing 1B

1559094_at | FBXO9 | F-box protein 9

212720_at | PAPOLA | poly(A) polymerase alpha

218859_s_at | ESF1 | ESF1, nucleolar pre-rRNA processing protein, homolog (*S. cerevisiae*)

201110_s_at | THBS1 | thrombospondin 1

227062_at | NEAT1 | nuclear paraspeckle assembly transcript 1 (non-protein coding)

222850_s_at | DNAJB14 | DnaJ (Hsp40) homolog, subfamily B, member 14

213649_at | SFRS7 | splicing factor, arginine/serine-rich 7, 35kDa

217147_s_at | TRAT1 | T cell receptor associated transmembrane adaptor 1

201821_s_at | TIMM17A | translocase of inner mitochondrial membrane 17 homolog A
(yeast)

222669_s_at | SBDS /// SBDSP | Shwachman-Bodian-Diamond syndrome ///
Shwachman-Bodian-Diamond syndrome pseudogene

206660_at | IGLL1 | immunoglobulin lambda-like polypeptide 1

217850_at | GNL3 | guanine nucleotide binding protein-like 3 (nucleolar)

214662_at | WDR43 | WD repeat domain 43

218386_x_at | USP16 | ubiquitin specific peptidase 16

200685_at | SFRS11 | splicing factor, arginine/serine-rich 11

218535_s_at | RIOK2 | RIO kinase 2 (yeast)

235469_at | FAM133B | family with sequence similarity 133, member B

201699_at | PSMC6 | proteasome (prosome, macropain) 26S subunit, ATPase, 6

226269_at | GDAP1 | ganglioside-induced differentiation-associated protein 1

213899_at | METAP2 | methionyl aminopeptidase 2

210793_s_at | NUP98 | nucleoporin 98kDa

203584_at | TTC35 | tetratricopeptide repeat domain 35

221970_s_at | NOL11 | nucleolar protein 11

217987_at | ASNSD1 | asparagine synthetase domain containing 1

227322_s_at | BCCIP | BRCA2 and CDKN1A interacting protein

202541_at | AIMP1 | aminoacyl tRNA synthetase complex-interacting multifunctional
protein 1

209181_s_at | RABGGTB | Rab geranylgeranyltransferase, beta subunit

201595_s_at | ZC3H15 | zinc finger CCCH-type containing 15

210681_s_at | USP15 | ubiquitin specific peptidase 15

212893_at | ZZZ3 | zinc finger, ZZ-type containing 3

230097_at | GART | Phosphoribosylglycinamide formyltransferase,
phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole

1553689_s_at | METTL6 | methyltransferase like 6

203023_at | NOP16 | NOP16 nucleolar protein homolog (yeast)

203177_x_at | TFAM | transcription factor A, mitochondrial

224870_at | KIAA0114 | KIAA0114

228050_at | UTP15 | UTP15, U3 small nucleolar ribonucleoprotein, homolog (S.
cerevisiae)

219212_at | HSPA14 | heat shock 70kDa protein 14

213092_x_at | DNAJC9 | DnaJ (Hsp40) homolog, subfamily C, member 9

1559096_x_at | FBXO9 | F-box protein 9

213262_at | SACS | spastic ataxia of Charlevoix-Saguenay (sacsin)

223267_at | RG9MTD1 | RNA (guanine-9-) methyltransferase domain containing 1

212202_s_at | TMEM87A | transmembrane protein 87A

219553_at | NME7 | non-metastatic cells 7, protein expressed in (nucleoside-diphosphate
kinase)

202930_s_at | SUCLA2 | succinate-CoA ligase, ADP-forming, beta subunit

202451_at | GTF2H1 | general transcription factor IIH, polypeptide 1, 62kDa

225200_at | DPH3 | DPH3, KTI11 homolog (S. cerevisiae)

221923_s_at | NPM1 | nucleophosmin (nucleolar phosphoprotein B23, numatrin)

226332_at | FAM133B | family with sequence similarity 133, member B

201573_s_at | ETF1 | eukaryotic translation termination factor 1

220553_s_at | PRPF39 | PRP39 pre-mRNA processing factor 39 homolog (S. cerevisiae)

202467_s_at | COPS2 | COP9 constitutive photomorphogenic homolog subunit 2
(Arabidopsis)

224523_s_at | C3orf26 | chromosome 3 open reading frame 26

229838_at | NUCB2 | nucleobindin 2

228822_s_at | USP16 | ubiquitin specific peptidase 16

225676_s_at | DCAF13 | DDB1 and CUL4 associated factor 13

1557239_at | BBX | bobby sox homolog (Drosophila)

226587_at | SNRPN | small nuclear ribonucleoprotein polypeptide N

1552480_s_at | PTPRC | protein tyrosine phosphatase, receptor type, C

201608_s_at | PWP1 | PWP1 homolog (S. cerevisiae)

1555996_s_at | EIF4A2 | eukaryotic translation initiation factor 4A, isoform 2

237466_s_at | HHIP | hedgehog interacting protein

214683_s_at | CLK1 /// PPIL3 | CDC-like kinase 1 /// peptidylprolyl isomerase
(cyclophilin)-like 3

205280_at | GLRB | glycine receptor, beta

212177_at | SFRS18 | splicing factor, arginine/serine-rich 18

233080_s_at | PRPF40A | PRP40 pre-mRNA processing factor 40 homolog A (S. cerevisiae)

226671_at | LAMP2 | lysosomal-associated membrane protein 2

1556283_s_at | FGFR1OP2 | FGFR1 oncogene partner 2

226794_at | STXBP5 | syntaxin binding protein 5 (tomosyn)

223263_s_at | FGFR1OP2 | FGFR1 oncogene partner 2

214764_at | RRP15 | ribosomal RNA processing 15 homolog (S. cerevisiae)

215719_x_at | FAS | Fas (TNF receptor superfamily, member 6)

221107_at | CHRNA9 | cholinergic receptor, nicotinic, alpha 9

208993_s_at | PPIG | peptidylprolyl isomerase G (cyclophilin G)

222837_s_at | NARG1 | NMDA receptor regulated 1

221193_s_at | ZCCHC10 | zinc finger, CCHC domain containing 10

1553179_at | ADAMTS19 | ADAM metalloproteinase with thrombospondin type 1 motif, 19

201599_at | OAT | ornithine aminotransferase (gyrate atrophy)

223087_at | ECHDC1 | enoyl Coenzyme A hydratase domain containing 1

219158_s_at | NARG1 | NMDA receptor regulated 1

221547_at | PRPF18 | PRP18 pre-mRNA processing factor 18 homolog (S. cerevisiae)

201606_s_at | PWP1 | PWP1 homolog (S. cerevisiae)

210117_at | SPAG1 | sperm associated antigen 1

218349_s_at | ZWILCH | Zwilch, kinetochore associated, homolog (Drosophila)

203202_at | KRR1 | KRR1, small subunit (SSU) processome component, homolog
(yeast)

225180_at | TTC14 | tetratricopeptide repeat domain 14

202950_at | CRYZ | crystallin, zeta (quinone reductase)

230375_at | SFRS18 | splicing factor, arginine/serine-rich 18

226459_at | PIK3AP1 | phosphoinositide-3-kinase adaptor protein 1

212591_at | RBM34 | RNA binding motif protein 34

206653_at | POLR3G | polymerase (RNA) III (DNA directed) polypeptide G (32kD)

235610_at | ALKBH8 | alkB, alkylation repair homolog 8 (E. coli)

225194_at | PLRG1 | pleiotropic regulator 1 (PRL1 homolog, Arabidopsis)

212648_at | DHX29 | DEAH (Asp-Glu-Ala-His) box polypeptide 29

220060_s_at | C12orf48 | chromosome 12 open reading frame 48

203970_s_at | PEX3 | peroxisomal biogenesis factor 3

1569136_at | MGAT4A | Mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-
acetylglucosaminyltransferase, isozyme A

209475_at | USP15 | ubiquitin specific peptidase 15

213704_at | RABGGTB | Rab geranylgeranyltransferase, beta subunit

222500_at | PPIL1 | peptidylprolyl isomerase (cyclophilin)-like 1

231784_s_at | DCAF13 | DDB1 and CUL4 associated factor 13

227277_at | MTDH | metadherin

232156_at | MUDENG | MU-2/AP1M2 domain containing, death-inducing

213188_s_at | MINA | MYC induced nuclear antigen

219015_s_at | ALG13 | asparagine-linked glycosylation 13 homolog (*S. cerevisiae*)

236620_at | RIF1 | RAP1 interacting factor homolog (yeast)

1552344_s_at | CNOT7 | CCR4-NOT transcription complex, subunit 7

203341_at | CEBPZ | CCAAT/enhancer binding protein (C/EBP), zeta

202662_s_at | ITPR2 | inositol 1,4,5-triphosphate receptor, type 2

216996_s_at | FASTKD2 | FAST kinase domains 2

204807_at | TMEM5 | transmembrane protein 5

200690_at | HSPA9 | heat shock 70kDa protein 9 (mortalin)

218512_at | WDR12 | WD repeat domain 12

201779_s_at | RNF13 | ring finger protein 13

218750_at | TAF1D | TATA box binding protein (TBP)-associated factor, RNA polymerase I, D, 41kDa

218967_s_at | PTER | phosphotriesterase related

206034_at | SERPINB8 | serpin peptidase inhibitor, clade B (ovalbumin), member 8

214943_s_at | RBM34 | RNA binding motif protein 34

201745_at | TWF1 | twinfilin, actin-binding protein, homolog 1 (*Drosophila*)

206158_s_at | CNBP | CCHC-type zinc finger, nucleic acid binding protein

213483_at | PPWD1 | peptidylprolyl isomerase domain and WD repeat containing 1

202660_at | ITPR2 | inositol 1,4,5-triphosphate receptor, type 2

205530_at | ETFDH | electron-transferring-flavoprotein dehydrogenase

201068_s_at | PSMC2 | proteasome (prosome, macropain) 26S subunit, ATPase, 2

204285_s_at | PMAIP1 | phorbol-12-myristate-13-acetate-induced protein 1

238214_at | LRRC69 | leucine rich repeat containing 69

227987_at | VPS13A | vacuolar protein sorting 13 homolog A (*S. cerevisiae*)

233251_at | STRBP | Spermatid perinuclear RNA binding protein

202939_at | ZMPSTE24 | zinc metallopeptidase (STE24 homolog, *S. cerevisiae*)

201111_at | CSE1L | CSE1 chromosome segregation 1-like (yeast)

1569484_s_at | MDN1 | MDN1, midasin homolog (yeast)

209838_at | COPS2 | COP9 constitutive photomorphogenic homolog subunit 2
(*Arabidopsis*)

215165_x_at | UMPS | uridine monophosphate synthetase

219802_at | PYROXD1 | pyridine nucleotide-disulphide oxidoreductase domain 1

204840_s_at | EEA1 | early endosome antigen 1

219002_at | FASTKD1 | FAST kinase domains 1

202542_s_at | AIMP1 | aminoacyl tRNA synthetase complex-interacting multifunctional
protein 1

212896_at | SKIV2L2 | superkiller viralicidic activity 2-like 2 (*S. cerevisiae*)

213189_at | MINA | MYC induced nuclear antigen

202146_at | IFRD1 | interferon-related developmental regulator 1

236696_at | SR140 | U2-associated SR140 protein

221782_at | DNAJC10 | DnaJ (Hsp40) homolog, subfamily C, member 10

205279_s_at | GLRB | glycine receptor, beta

214130_s_at | PDE4DIP | phosphodiesterase 4D interacting protein

235564_at | ZNF117 | Zinc finger protein 117

232630_at | APIP | APAF1 interacting protein

233819_s_at | RNF160 | ring finger protein 160

201144_s_at | EIF2S1 | eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa

213878_at | PYROXD1 | pyridine nucleotide-disulphide oxidoreductase domain 1

213424_at | KIAA0895 | KIAA0895

226776_at | ENY2 | enhancer of yellow 2 homolog (Drosophila)

204914_s_at | SOX11 | SRY (sex determining region Y)-box 11

227777_at | C10orf18 | chromosome 10 open reading frame 18

221514_at | UTP14A | UTP14, U3 small nucleolar ribonucleoprotein, homolog A (yeast)

232238_at | ASPM | asp (abnormal spindle) homolog, microcephaly associated (Drosophila)

226811_at | FAM46C | family with sequence similarity 46, member C

239835_at | KBTBD8 | kelch repeat and BTB (POZ) domain containing 8

221652_s_at | C12orf11 | chromosome 12 open reading frame 11

203095_at | MTIF2 | mitochondrial translational initiation factor 2

201083_s_at | BCLAF1 | BCL2-associated transcription factor 1

200880_at | DNAJA1 | DnaJ (Hsp40) homolog, subfamily A, member 1

221773_at | ELK3 | ELK3, ETS-domain protein (SRF accessory protein 2)

212604_at | MRPS31 | mitochondrial ribosomal protein S31

223707_at | RPL27A | ribosomal protein L27a

231863_at | ING3 | inhibitor of growth family, member 3

201873_s_at | ABCE1 | ATP-binding cassette, sub-family E (OABP), member 1

222684_s_at | NOL10 | nucleolar protein 10

209095_at | DLD | dihydrolipoamide dehydrogenase

212296_at | PSMD14 | proteasome (prosome, macropain) 26S subunit, non-ATPase, 14

1555125_at | C21orf66 | chromosome 21 open reading frame 66

59644_at | BMP2K | BMP2 inducible kinase

210346_s_at | CLK4 | CDC-like kinase 4

212721_at | SFRS12 | splicing factor, arginine/serine-rich 12

232291_at | MIR17HG | MIR17 host gene (non-protein coding)

201210_at | DDX3X | DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked

209849_s_at | RAD51C | RAD51 homolog C (*S. cerevisiae*)

212286_at | ANKRD12 | ankyrin repeat domain 12

227447_at | SKIV2L2 | superkiller viralicidic activity 2-like 2 (*S. cerevisiae*)

218462_at | BXDC5 | brix domain containing 5

218403_at | TRIAP1 | TP53 regulated inhibitor of apoptosis 1

209750_at | NR1D2 | nuclear receptor subfamily 1, group D, member 2

210116_at | SH2D1A | SH2 domain protein 1A

228395_at | GLT8D1 | Glycosyltransferase 8 domain containing 1

220330_s_at | SAMSN1 | SAM domain, SH3 domain and nuclear localization signals 1

222679_s_at | DCUN1D1 | DCN1, defective in cullin neddylation 1, domain containing 1 (*S. cerevisiae*)

List of Down regulated genes arranged by decreasing intensity:

Probe Set ID | Gene Symbol | Gene Title

202912_at | ADM | adrenomedullin

202718_at | IGFBP2 | insulin-like growth factor binding protein 2, 36kDa

202887_s_at | DDIT4 | DNA-damage-inducible transcript 4

227510_x_at | MALAT1 | metastasis associated lung adenocarcinoma transcript 1 (non-protein coding)

202022_at | ALDOC | aldolase C, fructose-bisphosphate

228770_at | GPR146 | G protein-coupled receptor 146

201170_s_at | BHLHE40 | basic helix-loop-helix family, member e40

1553185_at | RASEF | RAS and EF-hand domain containing

202856_s_at | SLC16A3 | solute carrier family 16, member 3 (monocarboxylic acid transporter 4)

1553186_x_at | RASEF | RAS and EF-hand domain containing

231721_at | JAM3 | junctional adhesion molecule 3

210512_s_at | VEGFA | vascular endothelial growth factor A

202855_s_at | SLC16A3 | solute carrier family 16, member 3 (monocarboxylic acid transporter 4)

231720_s_at | JAM3 | junctional adhesion molecule 3

242064_at | SDK2 | sidekick homolog 2 (chicken)

211454_x_at | FKSG49 | FKSG49

228499_at | PFKFB4 | 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4

223199_at | MKNK2 | MAP kinase interacting serine/threonine kinase 2

203828_s_at | IL32 | interleukin 32

227744_s_at | HNRNPD | Heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37kDa)

200872_at | S100A10 | S100 calcium binding protein A10

213350_at | RPS11 | Ribosomal protein S11

217137_x_at | LOC100291812 | hypothetical protein LOC100291812

201848_s_at | BNIP3 | BCL2/adenovirus E1B 19kDa interacting protein 3

206323_x_at | OPHN1 | oligophrenin 1

201849_at | BNIP3 | BCL2/adenovirus E1B 19kDa interacting protein 3

220232_at | SCD5 | stearyl-CoA desaturase 5

200632_s_at | NDRG1 | N-myc downstream regulated 1

205199_at | CA9 | carbonic anhydrase IX

225530_at | MOBKL2A | MOB1, Mps One Binder kinase activator-like 2A (yeast)

224321_at | TMEFF2 | transmembrane protein with EGF-like and two follistatin-like domains 2

208998_at | UCP2 | uncoupling protein 2 (mitochondrial, proton carrier)

211791_s_at | KCNAB2 | potassium voltage-gated channel, shaker-related subfamily, beta member 2

228204_at | PSMB4 | Proteasome (prosome, macropain) subunit, beta type, 4

205349_at | GNA15 | guanine nucleotide binding protein (G protein), alpha 15 (Gq class)

224288_x_at | FKSG49 | FKSG49

1556316_s_at | LOC284889 | hypothetical protein LOC284889

219599_at | EIF4B | eukaryotic translation initiation factor 4B

218205_s_at | MKNK2 | MAP kinase interacting serine/threonine kinase 2

210045_at | IDH2 | isocitrate dehydrogenase 2 (NADP+), mitochondrial

218625_at | NRN1 | neuritin 1

217294_s_at | ENO1 | enolase 1, (alpha)

211019_s_at | LSS | lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)

212813_at | JAM3 | junctional adhesion molecule 3

210132_at | EFNA3 | ephrin-A3

201313_at | ENO2 | enolase 2 (gamma, neuronal)

227799_at | MYO1G | myosin IG

208120_x_at | FKSG49 /// tcag7.1056 | FKSG49 /// hypothetical LOC402469

225762_x_at | LOC284801 | hypothetical protein LOC284801

235163_at | MOBKL2A | MOB1, Mps One Binder kinase activator-like 2A (yeast)

217202_s_at | GLUL | glutamate-ammonia ligase (glutamine synthetase)

206792_x_at | PDE4C | phosphodiesterase 4C, cAMP-specific (phosphodiesterase E1 duncce homolog, Drosophila)

220796_x_at | SLC35E1 | solute carrier family 35, member E1

203366_at | POLG | polymerase (DNA directed), gamma

203282_at | GBE1 | glucan (1,4-alpha-), branching enzyme 1

219622_at | RAB20 | RAB20, member RAS oncogene family

202028_s_at | RPL38 | ribosomal protein L38

221943_x_at | RPL38 | Ribosomal protein L38

200908_s_at | RPLP2 | ribosomal protein, large, P2

224284_x_at | FKSG49 | FKSG49

200648_s_at | GLUL | glutamate-ammonia ligase (glutamine synthetase)

213826_s_at | LOC100133109 | hypothetical LOC100133109

238701_x_at | C11orf92 | chromosome 11 open reading frame 92

234981_x_at | CMBL | carboxymethylenebutenolidase homolog (Pseudomonas)

208962_s_at | FADS1 | fatty acid desaturase 1

234562_x_at | LOC728678 | Similar to p40

234762_x_at | NLN | Neurolysin (metallopeptidase M3 family)

217635_s_at | POLG | polymerase (DNA directed), gamma

232266_x_at | CDC2L5 | Cell division cycle 2-like 5 (cholinesterase-related cell division controller)

215978_x_at | LOC152719 | hypothetical protein LOC152719

214715_x_at | ZNF160 | zinc finger protein 160

203523_at | LSP1 | lymphocyte-specific protein 1

211708_s_at | SCD | stearoyl-CoA desaturase (delta-9-desaturase)

210240_s_at | CDKN2D | cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)

211934_x_at | GANAB | glucosidase, alpha; neutral AB

218084_x_at | FXYD5 | FXYD domain containing ion transport regulator 5

212085_at | SLC25A6 | solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 6

211911_x_at | HLA-B | major histocompatibility complex, class I, B

211162_x_at | SCD | stearyl-CoA desaturase (delta-9-desaturase)

232215_x_at | PRR11 | proline rich 11

211527_x_at | VEGFA | vascular endothelial growth factor A

226549_at | SBK1 | SH3-binding domain kinase 1

1554327_a_at | CANT1 | calcium activated nucleotidase 1

208997_s_at | UCP2 | uncoupling protein 2 (mitochondrial, proton carrier)

201367_s_at | ZFP36L2 | zinc finger protein 36, C3H type-like 2

212044_s_at | RPL27A | Ribosomal protein L27a

200751_s_at | HNRNPC | heterogeneous nuclear ribonucleoprotein C (C1/C2)

201216_at | ERP29 | endoplasmic reticulum protein 29

233571_x_at | PPDPF | pancreatic progenitor cell differentiation and proliferation factor homolog (zebrafish)

226548_at | SBK1 | SH3-binding domain kinase 1

237475_x_at | CCDC152 | coiled-coil domain containing 152

217356_s_at | PGK1 | phosphoglycerate kinase 1

Genes affected in HIV infected cells treated with Dex/DEAE

List of Upregulated genes arranged in decreasing intensity:

Probe Set ID | Gene Symbol | Gene Title

217238_s_at | ALDOB | aldolase B, fructose-bisphosphate

218565_at | C9orf114 | chromosome 9 open reading frame 114

225453_x_at | CCDC124 | Coiled-coil domain containing 124

213736_at | COX5B | Cytochrome c oxidase subunit Vb

210611_s_at | DTNA | dystrobrevin, alpha

213087_s_at | EEF1D | eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein)

214395_x_at | EEF1D | eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein)

226014_at | EIF3F | Eukaryotic translation initiation factor 3, subunit F

219599_at | EIF4B | eukaryotic translation initiation factor 4B

234464_s_at | EME1 | essential meiotic endonuclease 1 homolog 1 (S. pombe)

1555826_at | EPR1 | effector cell peptidase receptor 1 (non-protein coding)

213621_s_at | GUK1 | Guanylate kinase 1

227744_s_at | HNRNPD | Heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37kDa)

213826_s_at | LOC100133109 | hypothetical LOC100133109

213789_at | LOC100292959 | similar to hCG2042049

225934_at | LOC148413 | hypothetical LOC148413
1556316_s_at | LOC284889 | hypothetical protein LOC284889
221997_s_at | MRPL52 | Mitochondrial ribosomal protein L52
222027_at | NUCKS1 | Nuclear casein kinase and cyclin-dependent kinase substrate 1
202243_s_at | PSMB4 | proteasome (prosome, macropain) subunit, beta type, 4
228204_at | PSMB4 | Proteasome (prosome, macropain) subunit, beta type, 4
219138_at | RPL14 | ribosomal protein L14
212044_s_at | RPL27A | Ribosomal protein L27a
214041_x_at | RPL37A | Ribosomal protein L37a
202028_s_at | RPL38 | ribosomal protein L38
221943_x_at | RPL38 | Ribosomal protein L38
200908_s_at | RPLP2 | ribosomal protein, large, P2
213350_at | RPS11 | Ribosomal protein S11
213665_at | SOX4 | SRY (sex determining region Y)-box 4
227244_s_at | SSU72 | SSU72 RNA polymerase II CTD phosphatase homolog (S. cerevisiae)

List of Down regulated genes arranged by decreasing intensity:

Probe Set ID | Gene Symbol | Gene Title

224496_s_at | TMEM107 | transmembrane protein 107

APPENDIX B
SELECTIVE TOXICITY ASSAY

Cell maintenance

JURKAT cells (human T-lymphocytes; ATCC) were maintained in clear RPMI-1640 without L- Glutamine and phenol red, prepared with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM Na Pyruvate. Cells were maintained at 37°C in a 5% CO₂ environment.

Seeding the cells:

100µL of media containing 300.104 cells/mL was plated in round-bottom 96-well plates (for a final concentration of 150.103 cells/well since each well is going to contain 200µL as follows: 100µL of cells in media and 100µL of drug dilutions in media with virus or PBS); leaving the outer rows to avoid the edge effect . And leave 2 rows for background reading with media only.

Infecting the cells with HIV:

A solution of media and DEAE-dextran (DD) was prepared to reach a final concentration of 40µg/mL in well. This solution was split in two; one with HIV virus added to it in order to attain 0.1 MOI in 96 well plate, while the other half had an equal volume of PBS added to it.

80µL of this respective solutions were added into two 96 well plate, adding the HIV containing solution into one plate and the HIV negative solution into the other one. The plates were incubated at 37°C in a 5% CO₂ environment for 4 hours, to give the virus enough time to infect the cells.

Treating the cells:

After 4 hours of incubation, 20 μ L of a dilution of drugs were added into the plates:

Dexamethasone (FAT080343) for a final concentration of 0.1 μ M, DEAE

Diethylaminoethanol (FAT451505) for a final concentration of 6.9 μ M, Procaine

(FAT031545) for a final concentration of 6.9 μ M, combination of DEAE/

Dexamethasone, a combination of procaine/ Dexamethasone. Each of the drugs is added in duplicate row, the final plate layout is as shown in the table below,

Then the plates were placed on a shaking table, 150rpm for 5 minutes, to thoroughly mix the samples into the media, before being incubated at 37°C in a 5% CO₂ environment for 48 hours to allow the drug to take effect.

XTT Plate for Dex DEAE SelTox expt. infected on 08.10.24

	1	2	3	4	5	6	7	8	9	10	11	12
A	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control
B	Pre-inc 2hr No HIV Dex 0.1uM	Pre-inc 2hr No HIV Dex 0.1uM	Pre-inc 2hr No HIV Dex 0.1uM	Pre-inc 2hr No HIV Dex 0.1uM	Pre-inc 2hr HIV Dex 0.1uM	Pre-inc 2hr HIV Dex 0.1uM	Pre-inc 2hr HIV Dex 0.1uM	Pre-inc 2hr HIV Dex 0.1uM	Control	Control	Control	Control
C	Pre-inc 2hr No HIV DEAE 5uM	Pre-inc 2hr No HIV DEAE 5uM	Pre-inc 2hr No HIV DEAE 5uM	Pre-inc 2hr No HIV DEAE 5uM	Pre-inc 2hr HIV DEAE 5uM	Pre-inc 2hr HIV DEAE 5uM	Pre-inc 2hr HIV DEAE 5uM	Pre-inc 2hr HIV DEAE 5uM	Control HIV+	Control HIV+	Control HIV+	Control HIV+
D	Pre-inc 2hr No HIV COMBO	Pre-inc 2hr No HIV COMBO	Pre-inc 2hr No HIV COMBO	Pre-inc 2hr No HIV COMBO	Pre-inc 2hr HIV COMBO	Pre-inc 2hr HIV COMBO	Pre-inc 2hr HIV COMBO	Pre-inc 2hr HIV COMBO	Control HIV+	Control HIV+	Control HIV+	Control HIV+
E	Control	Control	Control	Control	Control	Control	Control HIV+	Control HIV+	Control HIV+	Control HIV+	Control HIV+	Control HIV+
F	No HIV Dex 0.1uM	No HIV Dex 0.1uM	No HIV Dex 0.1uM	No HIV Dex 0.1uM	No HIV Dex 0.1uM	No HIV Dex 0.1uM	HIV+ Dex 0.1uM	HIV+ Dex 0.1uM	HIV+ Dex 0.1uM	HIV+ Dex 0.1uM	HIV+ Dex 0.1uM	HIV+ Dex 0.1uM
G	No HIV DEAE 5uM	No HIV DEAE 5uM	No HIV DEAE 5uM	No HIV DEAE 5uM	No HIV DEAE 5uM	No HIV DEAE 5uM	HIV+ DEAE 5uM	HIV+ DEAE 5uM	HIV+ DEAE 5uM	HIV+ DEAE 5uM	HIV+ DEAE 5uM	HIV+ DEAE 5uM
H	No HIV COMBO	No HIV COMBO	No HIV COMBO	No HIV COMBO	No HIV COMBO	No HIV COMBO	HIV+ COMBO	HIV+ COMBO	HIV+ COMBO	HIV+ COMBO	HIV+ COMBO	HIV+ COMBO
	1	2	3	4	5	6	7	8	9	10	11	12

Optimized protocol:

Final vol in wells is 200uL; 100uL media already added in seeding, need another 100uL total volume (drug dilutions plus media +/- virus).

DEAE-dextran (DD) stock is 100x, so for final volume of 200 uL per well, with 100 wells (20 mL), use 200 uL DD stock added to 10mL media.

44 of 96 wells have HIV; add HIV (32 uL stock) to 5.5 mL media + DD, no HIV to the other 6.5 mL

Add 80 uL of respective media mixes to HIV+ or HIV- wells as required.

Add either 20 uL of drug dilutions or 20 uL PBS to the appropriate wells

	1	2	3	4	5	6	7	8	9	10	11	12
A	Media buffer zone											
B	Media buffer zone	BLANK media only	Control HIV+	HIV+ Dex 0.1uM	HIV+ DEAE 5 uM	HIV+ COMBO	NO HIV COMBO	NO HIV DEAE 5 uM	NO HIV Dex 0.1uM	Control NO HIV	BLANK media only	Media buffer zone
C	Media buffer zone	BLANK media only	Control HIV+	HIV+ Dex 0.1uM	HIV+ DEAE 5 uM	HIV+ COMBO	NO HIV COMBO	NO HIV DEAE 5 uM	NO HIV Dex 0.1uM	Control NO HIV	BLANK media only	Media buffer zone
D	Media buffer zone	BLANK media only	Control HIV+	HIV+ Dex 0.1uM	HIV+ DEAE 5 uM	HIV+ COMBO	NO HIV COMBO	NO HIV DEAE 5 uM	NO HIV Dex 0.1uM	Control NO HIV	BLANK media only	Media buffer zone
E	Media buffer zone	BLANK media only	Control HIV+	HIV+ Dex 0.1uM	HIV+ DEAE 5 uM	HIV+ COMBO	NO HIV COMBO	NO HIV DEAE 5 uM	NO HIV Dex 0.1uM	Control NO HIV	BLANK media only	Media buffer zone
F	Media buffer zone	BLANK media only	Control HIV+	HIV+ Dex 0.1uM	HIV+ DEAE 5 uM	HIV+ COMBO	NO HIV COMBO	NO HIV DEAE 5 uM	NO HIV Dex 0.1uM	Control NO HIV	BLANK media only	Media buffer zone
G	Media buffer zone	BLANK media only	Control HIV+	HIV+ Dex 0.1uM	HIV+ DEAE 5 uM	HIV+ COMBO	NO HIV COMBO	NO HIV DEAE 5 uM	NO HIV Dex 0.1uM	Control NO HIV	BLANK media only	Media buffer zone
H	Media buffer zone											
	1	2	3	4	5	6	7	8	9	10	11	12

Live Dead assay Protocol:

Control (HIV-)	Control(HIV+)	Dex	DEAE	Combo(HIV+)	Combo(HIV-)
1mL (non infected Cells) + 1mL RPMI	1mL (infected Cells) + 1mL RPMI	1mL (infected Cells) + 0.5mL RPMI +0.5 mL Dex	1mL (infected Cells) + 0.5mL RPMI +0.5mL DEAE	1mL (infected Cells) + 0.5mL Dex +0.5mL DEAE	1mL (non infected Cells) + 0.5mL Dex +0.5mL DEAE
1mL (non infected Cells) + 1mL RPMI	1mL (infected Cells) + 1mL RPMI	1mL (infected Cells) + 0.5mL RPMI +0.5 mL Dex	1mL (infected Cells) + 0.5mL RPMI +0.5mL DEAE	1mL (infected Cells) + 0.5mL Dex +0.5mL DEAE	1mL (non infected Cells) + 0.5mL Dex +0.5mL DEAE
1mL (non infected Cells) + 1mL RPMI	1mL (infected Cells) + 1mL RPMI	1mL (infected Cells) + 0.5mL RPMI +0.5 mL Dex	1mL (infected Cells) + 0.5mL RPMI +0.5mL DEAE	1mL (infected Cells) + 0.5mL Dex +0.5mL DEAE	1mL (non infected Cells) + 0.5mL Dex +0.5mL DEAE

We need a final concentration in the wells of:

0.1 uM Dex (4mg/mL) (F.W=516.4)

6.9 uM DEAE (F.W= 117.19)

So for a final volume of 2mL per well, we need to make a solution 4* as concentrated:

0.4 uM of Dex in a total volume of 10mL RPMI

27.6 uM of DEAE in a total volume of 10mL RPMI

Determining the optimal dye concentration:

1. take 100uL per well of uninfected cells in a 96 well plates
2. kill half the number of wells by:
3. test with increasing concentration of blue Calcein:
 0.1/0.25/0.5/0.75/1/2.5/5/7.5/10
 a. EthD-1: 0.1/0.25/0.5/0.75/1/2.5/5/7.5/10

0	0.1	0.25	0.5	0.75	1	2.5	5	7.5	10
CEM-GFP+ calcein									
CEM-GFP+ EthD-1									
Rev-CEM+ calcein									
Rev-CEM+ EthD-1									

Cell Media Preparation

To make 500 mL of RPMI1640 Medium for CEM-GFP

Starting with RPMI 1640, 1X Medium
w/o L- Gln,

RPMI1640	435mL
10% FBS(fetal Bovine Serum)	50 mL
1% of 200 mM L-Glutamine	5 mL of 200 mM solution
1% Penicillin-streptomycin	5mL of Pen Strep Penicillin Streptomycin
500 ug/mL of G-418	5 mL of 50mg/mL solution

1. Remove a 50 mL aliquot of FBS from the freezer and thaw in warm water.
2. Open a 500 mL bottle of fresh RPMI under sterile conditions
3. Remove 65 mL of media.
4. Add 5 mL of 200 mM L-glutamine from the -20 freezer
5. Add 50 mL (56.8 mL) of FBS
6. Add 5mL of Pen Strep Penicillin Streptomycin
7. 5 mL of 50mg/mL of G-418solution
8. Mix well, label bottle with initials, date, contents, etc.
9. The media need not be filtered unless any of the reagents are not sterile (everything we purchase is cell culture grade)
10. Place 1 mL of newly prepared media in the incubator overnight to check sterility before storing cell, if it turns cloudy there are bacteria partying in the medium.
11. Media will last about 2 months in the refrigerator.

Procedure for Counting Cells

Supplies needed:

1 mL pipette
2 sterile bullet tubes (smaller ones (500 μ L are preferred)
Sample from cell generation (60 - 100 μ L)
Trypan Blue (TB) (40 μ L)
Hemocytometer (slide with gridlines)
Slide cover

Procedure:

1. Percuss the cells vigorously to dissociate the cells from each other.
2. Open the cell flask and remove less than 100 μ L of the media (fill the tip only).
3. Place the sample in the bullet tube (if more than one flasks of cells is being counted be sure to label the bullet tubes).
4. Place 40 μ L of TB in the other bullet tube.
5. To save plastic, reuse the pipette tip that was just used for the TB and remove 40 μ L of cells/media from the first bullet tube, being sure to mix the TB and the cells well in the bullet tube. (there is now a dilution factor of 2)
6. Take the mixture in the bullet tube to the microscope.
7. Place a cover slip over the slide and insert 20 μ L of the cell media/TB mixture in the small groove in-between the cover slip and the slide.
8. Place slide on the microscope and bring into focus the grid and cells.
9. Bring into focus the gridlines, use the 0.25 magnification which will narrow the sight to one field. Count a total of 4 different fields.

10. Counting cells:

A method is needed so that there is consistency to the count. If cells placement is on top of a gridline count only cells on right and upper gridline and ignore cells on bottom and left gridline or vice versa. Either way, be consistent.

Additionally, always move the slide in the same direction either clockwise or counter clockwise to count cells. Again, either way, be consistent.

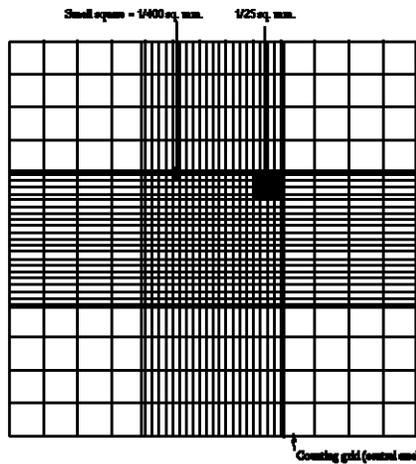
If there is difficult focusing in on the gridline start with the lowest magnification (0.10) and graduate up in magnification power. 0.25 seems to work best. At this power one can see one field (4x4 squares) fully, making counting easy.

The number between squares would be within 5% if the cells are evenly distributed.

Keep an eye out for dead cells (dark blue) if there are numerous dead cells it is time to start troubleshooting.

11. Using the counter, count number of cells in each 4x4 grid being sure to count all four of the 4x4 fields.

12. Take the total number of cells counted and calculate the total number of cells:
(# of cells counted/4) x 2 x 10⁴ c/mL



Cell senescence is characterized by changes in morphology and decreased growth rate.

Freezing Cells

1. Spilt cells into a T-75 in 15 mL of media and grow until cell count is 8×10^5 or 9×10^5 .
2. Prepare media with 10% DMSO + 90% FBS.
3. Calculate the number of cryovials (externally threaded) that will be needed to freeze cells. Each cryovial should hold 2×10^6 cells
CALCULATION: (vol. media in T-75) x cell count (cells/mL) = # cells
 $\# \text{ cells} / 2 \times 10^6 = \# \text{ vials needed}$
4. Cryovials should be sterile and endotoxin free or autoclaved with drying to destroy endotoxin.
5. Prepare the appropriate amount of STERILE freezing medium to have 1 mL per cryovial. The freezing medium is 7.5% DMSO in complete RPMI (with FBS) or the alternative mentioned above.
CALCULATION: # tubes x 1mL/tube = mL of media needed.
6. Keep media at 4° C (on ice) during procedure.
7. Fill cryocontainer with 100% isopropanol and be sure it is already cooled.
8. While medium is warming, label the cryotubes with the cell type, generation, cell number, date, your initials. Place clear tape over the labels to be sure that they don't get washed off in the liquid N₂.
9. Be sure all supplies are ready and that all labeled tubes are under hood with caps off and ready to have cells added to them before putting cells in freezing medium (this is necessary because DMSO is toxic to cells and should only be combined with cells for the minimum amount of time).
10. Spin cells in Falcon tube at 1500 rpm for 5 min. (check to be sure pellet has formed)
11. Discard supernatant from cells and add the appropriate volume of freezing medium (calculated in step 5)
12. Pipet gently up and down to resuspend cells.
13. Aliquot 1 mL of cell mixture into each cryotube, replace lids.
14. Place in -20° C for 2 hours.
15. Move the vials to -80° C freezer and allow them to cool overnight.
16. Move cells from cryostorage device to liquid nitrogen in 4 -5 hr (although cells can survive up to 5 weeks at -80° C, better results are had with a shorter time).

Thawing Cells, protocol for bringing up cells from liquid N₂

Note: Vials that have been stored in liquid N₂ can explode on warming if the vials are leaky. For this reason it is wise to wear face protection and gloves.

Be sure to test media for bacterial contamination by placing 5 mL in incubator for 24 hr.

Supplies:

1, 15 mL Falcon Tubes; 1 T-25 Flask; 10 mL media, 40 mL beaker of EtOH.

1. Turn on water bath to 37° C.
2. Prepare 1 T25 flask and 1, 15 mL falcon tube with 5 mL media each.
3. Remove cell vial from liquid N₂. Check that cap is securely fastened. Transfer *immediately* to 37° C water bath.
4. Swirl gently (bottom half only) in bath until barely thawed (like sherbert). Be sure you have recovered any information recorded on the vial.
5. Submerge vial in beaker of ETOH before placing vial under hood (water bath is a major source of contamination). Be sure the cap is on tight. Change your gloves.
6. Under sterile hood, suck cells up in 1 mL pipette and transfer to falcon tube with 5 mL media already present. Rinse pipette up and down to facilitate complete transfer.
7. *OR: Transfer cells immediately into 15 mL of media in T-25 flask place in incubator for 24 hr and then go to step 8.*
8. Spin @1500 rpm (no more than 800 rcf) for 5 min, should see pellet of cells (if media is cloudy, spin longer).
9. Remove media-discard.
10. Add ≤ 5 mL of media, gently rinse up and down in pipette to resuspend pellet. {If the cells have been frozen for a long period of time (decreased cell viability) best to suspend the cells in a smaller volume of media. You can always add more media in 24 – 48 hr}
11. Gently transfer into a T-25 falcon flask
12. Label the flask with the date, the cell type, and your initials.
13. Place in CO₂ incubator at 39.6 °C, 4.7% CO₂, 72% relative humidity.
14. Turn off water bath.
15. Check cell count every 24 – 48 hours
16. After 20 passages, cells should be discarded. Old cells should be removed from the incubator.

General Cell Culture Procedures

Helpful hints:

1. Run UV light for 30 minutes before working under the sterile hood.
2. Make sure all reagents, instruments etc are available before starting -- running back and forth between the cell culture room and a lab may introduce contamination.
3. Keep cell culture room door closed at all times.
4. Rule of thumb: Warm cells fast to wake, cool cells slow to store
5. Experiments should be done on 1st or 2nd generation cells, never the original stock
6. Incubator will need the sterile H₂O changed every 2-3 mo. Check incubator to make sure there is water present and add more if it is dry.

General Comments about Cell Health:

1. If media becomes lighter, pH has changed (more acidic) – change media
2. If cells are detached from the flask, bad sign. Check media, CO₂ concentration, humidity, temperature, etc.
3. 80 – 90% confluence is best for running an experiment, if confluence approaches 100% cells will not be able to grow.
4. Jurkats grow in clumps (T cells like to hang out together)
5. Shape of cells:

Round	low growth
Fractal shape	active growth
“giant” cells	old cells (change media) (happens with cancer cells only)

Cell Culture Lab Procedures

Helpful hints:

1. Make sure all reagents, instruments etc are available before starting, running back and forth between the cell culture room and a lab is a sure way to introduce contamination.
2. Keep cell culture room door closed at all times while working under the hood.
3. Always filter FBS to assure sterility.

Day before starting a culture, take 10 mLs of FBS and place in incubator, this will demonstrate if there is bacterial contamination in the FBS within 24 h.

If the color of the media changes, change the media!
4. when waking cells, cell growth is best in large populations, do not separate them.
5. Media will last about 2 months in the refrigerator.
6. Rule of thumb: Warm cells fast to wake, cool cells slow to store.
7. DMSO 7% (or other specification depending on cells) should be used to put cells to bed (cyrostorage).

8. Experiments should be done on 1st or 2nd generation cells, never the original stock.
9. 80-90% confluence is best for running an experiment, if confluence is at 100% cells will not be able to grow.
10. Incubator will need sterile H₂O every 2-3 mo.

Cell culture protocol for JC53

To detach the cells from the plate we use EDTA without trypsin for JC53 because trypsin removes CD4

- Remove medium from culture vessel by aspiration and wash the monolayer to remove all traces of serum.
- add 10mL of (EDTA+PBS) enough to cover all the surface (EDTA takes longer time than trypsin)
- Place culture in the 37°C incubator for 5 minutes.
- Monitor cells under microscope. Cells are beginning to detach when they appear rounded.
- As soon as cells are in suspension remove EDTA PBS, immediately add culture 5mL of medium containing serum. Wash cells very well with serum containing medium (to resuspend the cells)
- transfer to a sterile centrifuge tube

Viable cell counts. USING A HEMOCYTOMETER TO DETERMINE TOTAL CELL COUNTS AND VIABLE CELL NUMBERS

(Trypan blue is used in dye exclusion procedures for viable cell counting. This method is based on the principle that live cells do not take up certain dyes, whereas dead cells do.)

- combine 20 μ l of cells with 20 μ l of trypan blue suspension (about 20 Mix thoroughly and allow to stand for 5-15 minutes.
- With the cover slip in place, transfer a small amount of trypan blue-cell suspension (about 20 μ l) to both chambers of the hemocytometer by carefully touching the edge of the cover slip with the pipette tip and allowing each chamber to fill by capillary action. Do not overfill or underfill the chambers.
- Starting with 1 chamber of the hemocytometer, count all the cells in the 1 mm center square and four 1 mm corner square. Keep a separate count of viable and non-viable cells.
- The circle indicates the approximate area covered at 100X microscope magnification (10X ocular and 10X objective). Include cells on top and left touching middle line. Do not count cells touching middle line at bottom and right. Count 4 corner squares and middle square in both chambers and calculate the average.
- Each large square of the hemocytometer, with cover-slip in place, represents a total volume of 0.1 mm³ or 10⁻⁴ cm³. Since 1 cm³ is equivalent to approximately 1 ml, the total number of cells per ml will be determined using the following calculations: Cells/ml = average cell count per square x dilution factor x 10⁴;

Total cells = cells/ml x the original volume of fluid from which the cell sample was removed; % Cell viability = total viable cells (unstained)/total cells x 100.

- For a 96 well plate you put a low enough number of cells to grow depending on the incubation time (10-20.10³ cells per well) you make your dilution depending on how many cells you want in your medium and then you distribute them in the wells (by multipipetting).
- It take the cells 2hours to attach if they don't in 2h it means they are not in good condition (add the compounds active antiviral)
- We incubate them overnight and infect them with HIV Virus.

Infecting plated cells with HIV:

- Set absorbent pad bellow work area
- Use DEAE dextran to enhance Virus entry into cells (stock solution is 4 µg/µl , target concentration =40µg/mL in wells)
- Vial of virus stock(spray off with 70%ethanol)
 - 1µL =0.1 MOI in 96 well palte
 - 1µL = 10,000 Virus particles
 - use 20µL virus to give 200,000 per plate, which is 20,000 per well (while we have 16,000 cells per well)
- First mix the virus with 100µL/well of media per well
 - -10 mL media+9almost 10mL already on plate from seeding)
 - -20µL DEAE stock
 - -20µL virus stock
- Mix in 15mL tube , add to pipette reservoir, then pipette bleach into empty 15mL tube to kill residual virus
- Add 100µL virus/DEAE/media to each well using mltichannel pipette
- (Don't forget to not add virus to the rows that are background and uninfected controls, instead add 100µL of media alone (no virus) to these wells.
- Change pipette tips after each rows to avoid cross contamination with drug in different wells.
- Dump used pipette tips directly into beaker of bleach, rinse reservoir with bleach after use, dispose it in biohazard waste after replacing in original wrapper.

Titer the viron

You spin the infected cells with viron for 5min at 7,000 r.p.m

- you get 500µL of the supernatant that contain the virus, add 10µL of DEAE (20/40µg/mL) to it.
- add 100µL of the mixture(virus+DEAE) to the wells where you already have 100µL of the media +cells
- incubate the plate for 72hours

Luciferase assay:

Use RPMI 16-40 media with the luciferase buffer (which has lysis buffer to break down the cells, and substrate of luciferase enzyme)

We start by removing the media from the cells

In the machine (polar star Optima) we pump the media first from the hose to the pump in order to avoid the dead volume.

- you run the system washing the pumps.
- we inject 100 μL of the buffer +100 μL of the media, and measure luminescence , the plate in the computer is named NUNC96 and Layout is all of them.
- The first reading is not good so you allow 5min and then you redo the reading in order to get more accurate results.
- Then when you are done you wash the system afterwards with DI water.

Vybrant® MTT Cell Proliferation Assay Kit (V-13154) Protocol from Invitrogen [50]

Reagent Preparation:

- 1.1 Prepare a 12 mM MTT stock solution by adding 1 mL of sterile PBS to one 5 mg vial of MTT (Component A). Mix by vortexing or sonication until dissolved. Occasionally there may be some particulate material that will not dissolve; this can be removed by filtration or centrifugation. Each 5 mg vial of MTT provides sufficient reagent for 100 tests, using 10 μ L of the stock solution per well. Once prepared, the MTT solution can be stored for four weeks at 4°C protected from light.
- 1.2 Add 10 mL of 0.01 M HCl to one tube containing 1 gm of SDS (Component B). Mix the solution gently by inversion or sonication until the SDS dissolves. Once prepared, the solution should be used promptly. Each tube makes sufficient solution for 100 tests, using 100 μ L per well.

Quantitation of Jurkat cells using the Vybrant MTT Cell Proliferation Assay Kit:

Cells in the parent culture were counted in a hemacytometer and then diluted to the indicated cell numbers in 100 μ L volumes, delivered to the wells of a microplate and incubated for 4 hours to allow time for adsorption before being assayed. Absorbance measurements at 570 nm were made using a microplate reader. Each data point represents the mean value of samples in triplicate. The inset shows the data plotted for the lower cell numbers. 2 Vybrant® MTT Cell Proliferation Assay Kit data. The age of the cultures, number of passages and details of the growth medium can all be important factors. Natural variation in the requirements and growth rates of different cell lines make it difficult to provide precise guidelines for preparing your cells. In general, cells seeded at densities between 5000 to 10,000 cells per well should reach optimal population densities within 48 to 72 hours. Note that the presence of phenol red in the final assay samples can seriously affect results. We strongly recommend that the cells be cultured in medium free of phenol red, if possible. Alternatively, the final incubation with the MTT can be performed after exchanging the cells into medium free of phenol red.

Labeling Cells:

- 2.1 For adherent cells, remove the medium and replace it with 100 μ L of fresh culture medium. For non-adherent cells, centrifuge the microplate, pellet the cells, carefully remove as much medium as possible and replace it with 100 μ L of fresh medium.
- 2.2 Add 10 μ L of the 12 mM MTT stock solution (prepared in step 1.1) to each well. Include a negative control of 10 μ L of the MTT stock solution added to 100 μ L of medium alone.
- 2.3 Incubate at 37°C for 4 hours. At high cell densities (>100,000 cells per well) the incubation time can be shortened to 2 hours.

- 2.4 Add 100 μL of the SDS-HCl solution (prepared in step 1.2) to each well and mix thoroughly using the pipette.
- 2.5 Incubate the microplate at 37°C for 4–18 hours in a humidified chamber. Longer incubations will decrease the sensitivity of the assay.
- 2.6 Mix each sample again using a pipette and read absorbance at 570 nm.

Quick Protocol Option:

To shorten the time of the assay it is possible to use DMSO (not provided) as a solubilizing agent to dissolve the formazan.⁶

- 3.1 After labeling the cells with MTT, as described above, remove all but 25 μL of medium from the wells. For non-adherent cells it may be necessary to first centrifuge the plates to sediment the cells.
- 3.2 Add 50 μL of DMSO to each well and mix thoroughly with the pipette.
- 3.3 Incubate at 37°C for 10 minutes.
- 3.4 Mix each sample again and read absorbance at 540 nm not 570 nm, as above.