CHAPTER 4
COMPOUND-INDUCED HOST CELL RESPONSES AND EFFECTS ON WHOLE VIRUS

SUMMARY

Background: The interaction of test agents with cells in culture is an important aspect of drug discovery because it mimics the *in vivo* scenario. In addition, it avoids the costs and ethical issues involved in working with animal models especially during the early stages when drug-like properties are still being investigated. In this study, the effect of the compounds on host cells and whole virus was evaluated by monitoring cell viability, cell proliferation, viral infectivity and immunomodulatory effects on relevant cell types.

Materials and Methods: Toxicity studies were done using spectrophotometric methods (measuring absorbance) and by flow cytometry (using the annexin V and propidium iodide kit) while proliferation studies were performed with carboxyfluorescein succinimidyl ester dye and a real time cell electronic sensing device. To determine if any of the compounds could prevent whole virus from infecting host cells, luminescence measurements of luciferase reporter gene expression (indicative of HIV *Tat*-responsive gene expression by engineered TZM-bl cells) were performed. The intracellular production of cytokines, IFN-γ and TNF-α within CD4+ and CD8+ cells, was evaluated using multi-parametric flow cytometry.

Results and Discussion: The 50% cytotoxic concentrations of most of the gold complexes were in the low micromolar range (between 1 and 20 µM). Ten complexes had anti-proliferative effects on peripheral blood mononuclear cells (decreasing proliferation from the parent generation by >50%) with PFK7 being the most prominent followed by PFK190, PFK8 and EK207. Inhibition of viral infectivity was observed at non-toxic (cell viability was >80%) concentrations of complexes TTC24, EK207 and EK231 and cytostatic concentrations of PFK7 and PFK8 (seen by RT-CES analysis). CD4+ cell frequencies from PBMCs of twelve HIV infected donors were reduced by complexes EK207 and PFK7 ($p<0.05$) further confirming the cytostatic abilities of these two complexes. The cytostatic ability of PFK7 was also shown to be as a result of significant ($p = 0.003$) inhibition of RNR enzyme. The production of the pro-inflammatory cytokine (TNF-α) was elevated in the same cells (CD4+) by the complementary ligand of PFK7 (PFK5) but not by complex PFK7 suggesting that complexation with gold resulted in a drug-like property. None of the complex precursors prevented infection of host cells, illustrating the importance of metal/gold complexation in these potential drugs.

Conclusion: Complexes TTC24, EK207, EK231 inhibited viral infectivity at non-toxic concentrations (but unfortunately had poor drug-like properties, chapter 3) suggesting that

structural modifications to improve drug-likeness may be required. PFK7 and PFK8 also inhibited viral infectivity but at cytostatic concentrations. Furthermore, EK207, PFK7 and PFK8 decreased PBMC proliferation while EK207 and PFK7 suppressed the frequency of CD4+ cells without altering cytokine production. Cytostasis is an anti-HIV mechanism which is also linked to decreases in CD4+ cell numbers and to inhibition of RNR. After modification to improve drug-likeness, complex EK207 and the drug-like complexes such as PFK7 and PFK8 which inhibit viral infectivity presumably as a result of cytostatic effects stand a chance of being incorporated into virostatic combinations which will offer better resistance profiles than existing drugs.

Keywords: viability, proliferation, infectivity inhibition, immunomodulation, cytostasis

4.1 INTRODUCTION

Test agents interacting in culture with cells are highly desirable in any drug discovery paradigm because they provide a means for ready, direct access and evaluation *in vitro* (Allen *et al*., 2005). Drug-cell interactions are valuable in providing information about cytotoxicity, drug mechanism of action and allow for screening of potential therapeutic agents. With cell cultures, it is easy to manipulate the cells to mimic a disease state thereby making it possible for significant information about the effect of a test compound to be obtained (e.g. engineering cells to have surface receptors necessary for infectivity by HIV). *In vitro* analysis ensures that ethical issues related to drug testing in humans or animal models can be avoided in the initial stages of drug development research (Allen *et al*., 2005) when safety is still a concern. The result of this is that, more safe drugs get into the more costly, late drug developmental phases (Donato *et al*., 2008).

Two cell types were used in this study; primary cells and immortalized cell lines. Primary cells closely mimic the *in vivo* state and generate physiologically relevant data. These cells unfortunately tend to undergo senescence after a few divisions and cannot be maintained in culture indefinitely (Castilho *et al*., 2008). Unlike the immortalised or continuous cell lines which are homogenous (Burdall *et al*., 2003), primary cells usually contain a variety of cell types in the same mixture therefore requiring further manipulations (e.g. tagging of surface receptors with fluorescently labelled antibodies or sorting) if information on specific subsets is required. Immortalized cell lines facilitate high throughput screening since they are readily available and together with primary cells allow for extrapolation of information from *in vitro* data regarding the effect of potential drugs *in vivo*. Unfortunately, these cells could lose their genotype and/or phenotype as a result of continuous culturing (Burdall *et al*., 2003). As such, strict culture conditions such as passage number have to be adhered to, to ensure phenotypic and genotypic stability.

The study of drug-like properties of new chemicals early in drug discovery has gained significant importance over the last decade due to the high rate of late drug failures during clinical trials (Hamid *et al*., 2004) with toxicity and adverse effects being some of the reasons for the failures. While the *in silico* prediction models (covered in chapter 3) provides insights into potential toxicity and can significantly shorten the drug discovery time line (Lobell and Sivarajah, 2003), complementing these data with *in vitro* experimental studies is important because of the physiological relevance of the latter. Specific cell types are available for determining ADMET properties *in vitro.* Brain microvessel endothelial cells have been used in BBB penetration studies (Glynn and Yazdanian, 1998) and Caco-2 cells for cellular permeability (Egan and Lauri, 2002). Because cytotoxicity is one of the most critical and unpredictable of the drug-like properties and can be species and organ-specific (Ponsoda *et al*., 1995), the focus here was on the toxicity component of ADMET.

In this study, both HIV uninfected and infected cells were used. When uninfected cells were used, compound cytotoxic effect could be monitored in the absence of viral cytopathic effect. When infected cells were used, it was also important to exclude toxicity by using non-toxic viral titres in addition to including viability dyes to exclude dead cells.

Various complementary assays were used in determining compound effect on cell viability. The reason for this is because of the multiple parameters that can influence cell death making the use of multiple markers to determine viability during early drug screening a necessity (Kepp *et al*., 2011). Standard spectrophometric assays were used for determining cellular metabolism and have the advantage of being robust, inexpensive and can be easily applied in HTS (Kepp *et al*., 2011). These included the tetrazolium dyes; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 3-(4,5-dimethylthiazol-2-yl)-5-3(3-car oxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). These inexpensive 96 well format assays facilitated the determination of the 50% cytotoxic concentrations (CC_{50}s) of the compounds. This is because for determining CC_{50}, a wide range of concentrations (at least six or more) is required for producing a dose response curve. These assays are however susceptible to metabolic interference and can lead to false positive results (Kepp *et al*., 2011, Boyd, 1989, Haselsberger *et al*., 1996, Denizot and Lang 1986). For this reason, the assays were only used after thorough optimisation and only as preliminary viability screening assays. The more specific flow cytometric assay using annexin V and propidium iodide was used for validating viability dye findings. More background information on these assays will be provided in the materials and methods sections of this chapter.

Other approaches that were used to indirectly investigate viability included the use of proliferation assays such as the carboxyflourescein succinimidyl ester dye dilution assay and impedence measurement by real time cell electronic sensing. In addition to providing information regarding the viability status of the cells, these assays also provide mechanistic information e.g. cytostasis or anti-proliferative effects of test agents. Various reports have

indicated the importance of anti-proliferative properties of test agents e.g. cytostatic anti-viral effect of HU which prevents immune activation by limiting proliferation (Lori *et al*., 2005). For metal-based drugs, prevention of antigen presentation through stripping of peptide antigens from MHC class II molecules is one of the ways by which T cell activation is prevented (De Wall *et al.*, 2006).

Gold compounds with anti-proliferative or cytostatic effects which also inhibit viral replication have the potential of being incorporated into virostatic combinations (Lori *et al*., 2005). This is a treatment strategy that is encouraged as a first-line combination to reduce the emergence of resistant strains and at a point when the patients are asymptomatic so as to avoid complications that could arise in advanced disease (Lori, 1999). On the other hand stimulation of lymphocyte proliferation may mean the compounds have the potential of being antigenic and may end up having adverse effects (Best and Sadler, 1996, Verwilghen *et al*., 1992). Since in HIV infection, activation usually results in increased viral replication and progression to AIDS (Gougeon, 2005) this may mean such compounds will potentiate the chronic inflammation already present (Appay and Sauce, 2008). The side effects related to gold therapy have been linked to their ability to cause a stimulatory effect on the immune system leading to the production of pro-inflammatory cytokines (Lampa *et al*., 2002). Such adverse effects previously seen in rheumatoid arthritis treatment are not manifested in all patients and some end up being cured by the use of gold-based drugs (Sigler *et al*., 1974, Forrestier, 1935).

In HIV infection, there is immunodeficiency as a result of the loss of CD4+ cells, hyperactivity as a result of B cell activation as well as changes in cytokine production (Breen, 2002). Cytokine-based therapy has been reported to be an alternative approach to HAART since it allows for the manipulation of the immune system to attain beneficial results; exemplified for IL-2, which boosts CD4+ cell number (Alfano and Poli, 2001). Assessing the function of T lymphocytes (crucial immunological cells) is representative of the immune state and aids in the identification of correlates of protection and of disease (Heeney and Plotkin, 2006). The frequency of CD4+ and CD8+ cells as well as representative anti-inflammatory (IFN-γ) and pro-inflammatory (TNF-α) cytokine production levels was determined as a means of assessing the effect of the compounds on immune function and on the chronic inflammatory disease caused by HIV (Appay and Sauce, 2008). Gold compounds have been reported to have immunomodulatory effects (discussed in section 2.3.3.3). We envisaged similar properties for the compounds tested here and performed immunomodulatory assays as a means of ascertaining if the compounds could serve as immune therapies.

In the next sections, the effect of the compounds on cell viability, proliferation, viral infectivity and immunomodulation is described. For the inexpensive and HTS assays (such as viability using MTT and annexin V/PI, the proliferation assay using CFSE and in the infectivity assays), all the compounds were tested and only representatives from the various classes for

more expensive and non HTS assays (e.g. RT-CES and in the multi-parametric flow cytometry assay). The data from class representative compounds could be used to extrapolate or deduce similar responses for members of the same class especially since drug-likeness predictions suggested that there were similarities between groups (Table 3.8A, chapter 3).

4.2 MATERIALS AND METHODS

4.2.1 Cells

The primary cells, referred to as peripheral blood mononuclear cells were isolated from venous blood obtained from both HIV positive and negative donors. The cell lines PM1 (courtesy of Dr. Marvin Reitz, Lusso *et al*., 1995) and TZM-bl (from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc, Takeuchi *et al*., 2008, Wei *et al*., 2002, Derdeyn *et al*., 2000, Platt *et al*., 1998) were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. The PM1 cell line obtained by transforming a neoplastic T-cell line, Hut 78 (Lusso *et al.,* 1995) to display CCR5 on its surface, is susceptible to infection by CXCR4 and CCR5 isolates and therefore ideal for expansion of progeny virus. The TZM-bl cells previously designated JC53-bl (clone 13) is a HeLa cell line engineered to stably express CD4, CXCR4 and CCR5. These cells were generated from JC.53 cells by introducing separate integrated copies of the luciferase and β-galactosidase genes under the control of the HIV-1 promoter (Platt *et al*., 1998) and are highly sensitive to infection with diverse isolates of HIV-1.

4.2.1.1 Isolation of primary cells from whole blood

Ethics clearance for this research was obtained from both the Faculties of Natural and Agricultural Sciences and the Health Sciences Ethics Committees (University of Pretoria) with approval numbers EC080506-019 and 163/2008 respectively. Blood from HIV infected (HIV+) individuals was obtained from subjects attending routine check-up at clinics around Pretoria (South Africa) including the Kings Hope Development Foundation Clinic (Diepsloot), the Fountain of Hope Clinic (FOH, Pretoria Central) and the Steve Biko Academic Hospital's Division of Infectious Diseases. The University of Pretoria's clinic was the point at which uninfected (HIV-) blood samples were obtained from healthy volunteers. In sampling from HIV+ donors, blood was only obtained from volunteers who were not on antiretroviral therapy (ART) and generally had a CD4+ count of > 200 cells/µL of blood (the cut-off point used in South Africa as exclusion criteria from ART for people infected with HIV). It was important to collect blood from these treatment-naïve patients to avoid false conclusions regarding the compounds under study which might be resulting from residual effect of administered ART in the case where treatment-experienced donors were used.

Venous blood from consenting donors was collected in K_3 EDTA anti-coagulant Vacuette® tubes (Greiner Bio-one, Austria) and processed within 2 h. The separated plasma

portion (liquid portion containing clotting factors) was stored at -20 ºC and the cell-containing fraction was diluted 1:1 with RPMI-1640 medium (Sigma Aldrich, Missouri USA) supplemented with antibiotics (10 mg/mL penicillin, 10 mg/mL streptomycin sulphate, 25 µg/mL fungizone and 1% v/v gentamycin sulphate). This was followed by standard Ficoll-Histopaque®-1077 (Sigma Aldrich, Missouri USA) density centrifugation. Briefly, two parts of the diluted blood was gently overlaid on one part of ficoll (Sigma Aldrich, Missouri USA) followed by 30 min of centrifugation (1610x*g*, 25 ºc). The buffy coat (middle layer) containing the PBMCs was washed (866x*g,* 10 min, 25 ºc) with incomplete medium (RPMI containing antibiotics only) to remove platelets and further treated with 5 mL of ammonium-chloride-potassium or ACK (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2) to disrupt and lyse any residual red blood cells. Following another wash step with incomplete RPMI-1640 medium (258x*g,* 10 min), the PBMCs were resuspended in complete RPMI-1640 medium which in addition to antibiotics, also contained 10% (v/v) heat-inactivated (56 °C, 30 min) fetal calf serum. A hemocytometer count was performed using trypan blue to determine the viability and concentration of the cells. A cell viability of > 90% was considered appropriate and the cells were resuspended at a suitable concentration as needed for the particular bioassay. Phytohemagglutinin-protein (PHA-P) also known as lectin from *Phaseolus vulgaris* was used as a stimulant for enhancing *in vitro* proliferation of primary cells while phorbol myristate acetate (PMA) and ionomycin (ION) were used for enhancing cytokine production (both stimulants were obtained from Sigma Aldrich, Missouri, USA). The cells were used to determine compound effect in one or more of the following assays: viability determination, proliferation or immune effects studies.

4.2.1.2 Culturing of continuous cell lines

Two types of continuous cell lines were used; the PM1 and the TZM-bl cell line. The PM1 suspension cell line were cultured in complete RPM1-1640 medium and subcultured at a concentration of approximately $5x10^4$ cells/mL every two days. The TZM-bl cells on the other hand are adherent cells and were subcultured in T-75 tissue culture flasks (Nunc™, Roskilde, Denmark) with approximately 10^6 cells in 15 mL of complete Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine, sodium pyruvate, glucose and pyridoxine (Gibco BRL Life Technologies, Grand Island, USA) containing antibiotics (10 mg/mL penicillin G, 10 mg/mL streptomycin sulphate, 25 µg/mL fungizone and 1%-v/v gentamycin sulphate) and 10% (v/v) heat inactivated FCS. The cells were sub-cultured every two or three days when confluency (surface area of the culture flask occupied by cells) was about 90%. Generally, a concentration of $1x10^5$ cells/mL was prepared for both the PM1 and the TZM-bl cells for experimental purposes unless stated otherwise.

4.2.2 Compound Preparation

Compounds were stored desiccated at -20 ºC until needed for experiments. Sufficient quantities were dissolved in DMSO (Highveld Biologicals, Sandringham, South Africa) to a concentration of 20 mg/mL and stored as single use aliquots of 5 or 10 µL at -20 ºC and used within a week. For bioassays, each of this was made up to 1 mg/mL with either phosphate buffered saline (PBS, pH 7.4) solution or growth medium (complete RPMI-1640 or DMEM) as required. The compounds were further diluted to experimental concentrations (0.02 - 200 µM) ensuring that the final DMSO concentration was ≤0.5% (v/v). This concentration of DMSO had no discernible effect on cell viability compared to controls.

4.2.3 Cell Viability Assays

Many *in vitro* cytotoxicity assays are available for determining cell viability. Some like the MTT and MTS assays, have the advantage of being adaptable to large scale screening relevant for most cells while others e.g. flow cytometry using annexin and propidium iodide (PI) provide additional information such as the mechanism of cell death. MTT (developed by Mosmann in 1983) is widely used for the quantitative assessment of cellular viability and proliferation but has shortcomings. Some of the draw backs are poor linearity with changing cell number, sensitivity to environmental conditions and the fact that it depend on the cells' metabolism of formazan (Boyd, 1989, Haselsberger *et al*., 1996, Denizot and Lang 1986). In addition, some human cell lines metabolize these dyes very inefficiently, and in some cases such dyes are cytotoxic (Hertel *et al*., 1996). Although MTT and MTS (Buttke *et al*., 1993) are widely used, reported conditions and parameters of the assays vary widely and depend largely on cell type (Soman *et al*., 2009, Young *et al*., 2005). These limitations necessitated the inclusion of optimisation steps in the HTS assays (details for these are provided in the appendix in section 8.3) followed by the use of more specific confirmatory assays. Flow cytometry (using annexinV/PI and CFSE) as well as RT-CES analysis were used to corroborate MTT data. Although the flow cytometric method is more specific than the MTT viability dye, important criteria such as gating on the right population (inclusion of cell surface markers) and having the right forward scatter (FSC, which is related to size) and side scatter (SSC, related to granularity) scaling has to be adhered to. In the following subsections, HTS viability dye assays as well as flow cytometry methodologies are provided. An incubation time of 72 h was used because it is sufficient for monitoring early drug toxicity (Sussman *et al*., 2002) but in the real time studies, proliferation (viability) was monitored for up to 7 days. Background information on all assays precedes protocols, all of which are followed by results and discussion.

4.2.3.1 HTS dye assays for determining cell viability

Several viability dyes (MTT, MTS and annexinV/PI) and a cytotoxicity kit for measuring lactate dehydrogenase release (details in the appendix, section 8.3.1), were compared during optimisation assays and a decision was made to use MTT and MTS for viability assessment. Both MTT (Sigma Aldrich, Missouri, USA) and MTS (Promega Corporation, WI, USA) are tetrazolium salts which function on the same principle. Dehydrogenase enzymes present in the mitochondria of viable cells convert these salts to a quantitative colorimetric formazan product that can be measured spectrophotometrically. In the case of MTT, the product is insoluble and requires a solubilisation step while for MTS, an additional coupling reagent (phenazine methosulfate-PMS), confers enhanced chemical stability leading to the formation of a stable solution. MTS was found to be easily metabolised by PBMCs probably because it is a more stable dye which could withstand the heterogeneity of PBMCs while either of the two dyes were easily metabolisable by the more homogenous cells lines (PM1 and TZM-bl). The MTT assay was done according to the protocol by Mueller *et al*., (2004) with minor modifications including the removal of spent medium to exclude compound effect before dye addition. The same conditions were employed for the MTS assay.

Procedure: PBMCs ($1x10^6$ cells/mL) and PM1 cells ($1x10^5$ cells/mL) in complete RPMI-1640 medium were treated with various concentrations of the compounds (0.4-200 µM) in cell culture grade 96 well plates (Nunc™, Roskilde, Denmark) and incubated (37 ºC, 95% humidity, 5% CO_2) for 72 h. At the end of the incubation, the plates were centrifuged (258 x *g,* 10 min) and 150 µL spent medium discarded and replaced with 50 µL freshly prepared complete medium. Ten microlitres of MTS was added to the resuspended cells and reduction of the MTS tetrazolium compound to formazan was detected after colour development using a Multiskan Ascent® spectrophotometer (Labsystems, Helsinki, Finland) at 492 nm and 690 nm as reference wavelength. Readings were taken after 2 h of incubating with MTS in the case of the cell lines and 24 h later in the case of PBMCs. Viability percentages were determined relative to an untreated control of cells only.

When MTT was used, a similar protocol was implemented but with the inclusion of a solubilisation step. After incubating the cells with the compounds, 150 µL of spent medium was discarded and replaced with an equivalent amount of freshly prepared complete medium. Then 20 µL (5 mg/mL) of MTT was added to the cells and colour development analysed after 2 h (cell lines) or 24 h (PBMCs). This was followed by solubilisation of the formazan product using acidified isopropanol in a 1:9 ratio (1 part of 1 M HCl and 9 parts of isopropanol). Absorbance was measured at 550 nm and a reference wavelength of 690 nm on a Multiskan Ascent® spectrophotometer (Labsystems, Helsinki, Finland). In both cases (MTS or MTT), percentage viability was calculated using the formula:

$$\text{Viability (\%)} = \frac{\text{Absorbance of Sample – Absorbance of medium}}{\text{Absorbance of control - Absorbance of medium}} \times 100$$

CC_{50} values were then graphically obtained after generating a dose response curve using Graphpad Prism® software (California, USA). Non-toxic concentrations (>50% viability) of the compounds obtained from viability assays were subsequently used in other cell-based assays and in the direct enzyme assays.

4.2.3.2 Effect of the compounds on cell viability by flow cytometry

Flow cytometry is a technology that simultaneously measures and then analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light from a laser source. The properties measured include a particle's relative size (FSC), relative granularity or internal complexity (SSC), and relative fluorescence intensity). A schematic representation of the components of a typical flow cytometer is shown in Figure 4.1.

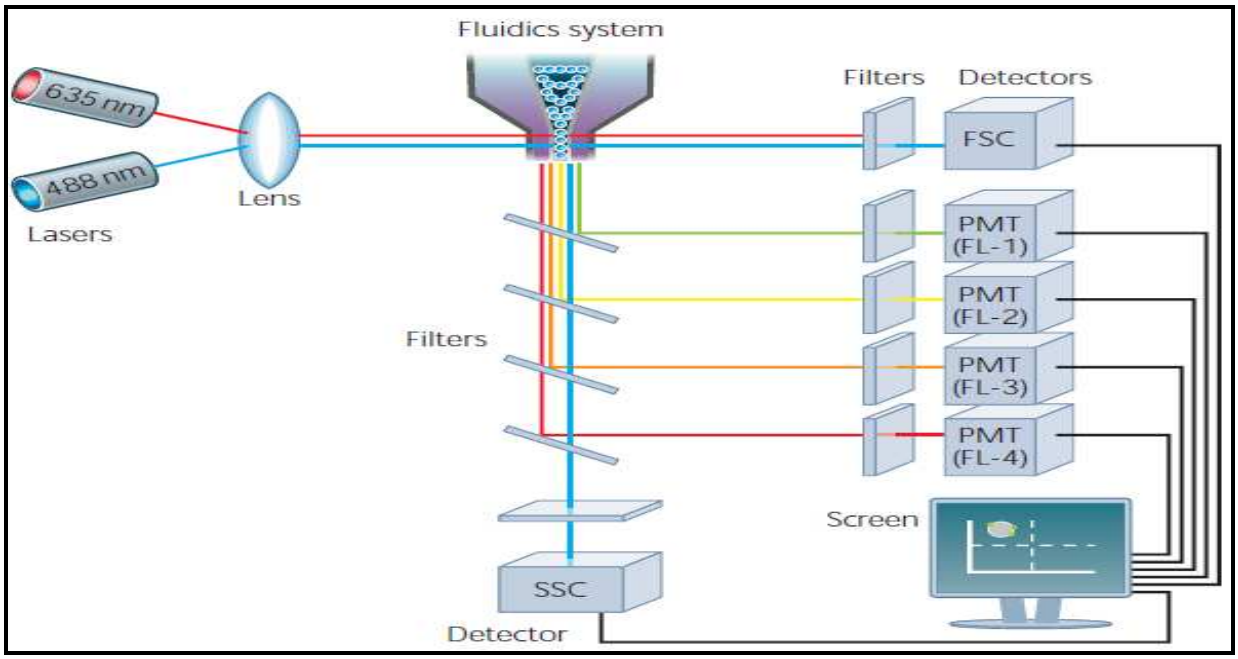

Figure 4.1: A schematic representation of the setup of a flow cytometer. Main components are lasers which generate light source, lenses which focus light onto sample, detectors for determining emitted light at specific wavelengths and this information is conveyed onto an output device represented by the screen. This figure was taken from www.ab-direct.com, accessed on the 25/04/2011

Unlike spectrophotometry (employed for the MTT and MTS assays), which measures absorption in a bulk volume, flow cytometry analyses single cells, making it more specific for determining cell viability and other cellular parameters. The major advantage of the dye assays is HTS but because of associated limitations, laborious and time consuming optimisation steps are usually required. Although the dye assays could have completely been left out, the fact that different parameters can influence cell death necessitated the use of multiple markers (Kepp *et al*., 2011) and complementary assays to determine viability. While flow cytometry is more specific, care has to be taken to ensure that the right population (especially in a situation where different cell types are involved e.g. PBMCs) is identified and gated for analysis and that the FSC (particle's relative size) and SSC (relative granularity or

internal complexity) scales are accurate. Failure to follow these requirements could result in the analysis of a completely different population than that which is desired.

In this study, the cells of interest were the lymphocytes consisting predominantly of CD4+ and CD8+ subsets of CD3+ cells. To identify these cells from PBMCs, a CD45+ marker common on lymphocytes (Stelzer *et al.*, 1993) was used to determine their position on a FSC/SSC dot plot ensuring the exclusion of monocytes and neutrophils. In the viability and proliferation studies with annexin V/PI and CFSE respectively, no further immunophenotyping of surface molecules was necessary but for the immunomodulatory assay, a CD3+ marker for T cells was used followed by CD4+ and CD8+ to differentiate T cell subsets.

The annexin-V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Becton Dickinson or BD BioSciences, California, USA) was used in determining the viability of the PBMCs using flow cytometry. The kit contains annexin V which stains cell surface phosphatidylserine indicating apoptotic cells and propidium iodide which stains the DNA of damaged cells indicating the presence of necrotic cells. Cells negative for both annexin V and PI were considered viable. In addition to being a complementary assay for the HTS viability assays, this assay provided information on the mode of cell death (necrosis or apoptosis) caused by the compounds.

Procedure: One millilitre of PBMCs ($1x10^6$ cells/mL) in complete RPMI medium was added to 1 mL of compound solutions in cell culture grade 24 well plates (Nunc^{TM}, Roskilde, Denmark). Since the CC_{50} values obtained in the HTS assays only served as indicators of non-toxic concentrations, in this study, a decision was made to use compound concentrations that resulted in >60% viability (from MTT and MTS studies) for the flow cytometric analysis and other cell-based assays. This cut-off was considered sufficient in keeping toxicity to a minimum. After 72 h of incubating the PBMCs with various concentrations of the compounds, staining of the cells was performed using the annexin V/PI detection kit according to instructions by the manufacturer (BD Biosciences, California, USA). The cells were harvested by washing (500 x *g*, 5 min) twice with ice cold PBS (pH 7.4) and transferred to plastic flow tubes (BD Biosciences, California, USA). The cell pellet was resuspended in 100 µL of binding buffer followed by the addition of a pre-titrated amount or separation titre (minimum amount of dye to achieve good separation between positive and negative cell populations, which was 2 µL each in this case) of annexin V-FITC and PI solution. After gentle mixing, the tubes were kept on ice in the dark for 15 min followed by the addition of 400 µL of ice cold binding buffer. Controls used included untreated cells, an annexin positive and a PI positive control. The annexin positive control was obtained by treating cells with 10 µM of auranofin (an anti-arthritic gold(I) compound known to have anti-tumour activity) while PI positive cells were obtained by fixing cells in ice cold methanol for 5 min. An unstained control, the annexin as well as the PI positive controls were used for setting compensation (correcting for spectral emission overlap between different dyes to eliminate false positive or negative outcomes) and quadrant

specification. The samples suspended in 500 µL of binding buffer were subjected to flow cytometric analysis with 10,000 events collected per sample within 30 min of treatment. Ten thousand events were considered sufficient for discriminating apoptosis, necrosis and viability between treatments and controls for this assay (general criterion for determining the number of events that are enough for a flow cytometry assay, Roederer, 2008). Flow cytometric profiles were determined using a FACSAria (BD Biosciences, California, USA) with the FITC and peridinin chlorophyll protein-cyanin 5.5 (PerCp.Cy5.5) detectors used for the identification of apoptotic and necrotic cells respectively. The data was analysed using the FACSDiva software (BD Biosciences, California, USA) and FlowJo version 7.6.1 (TreeStar Inc., Oregon, USA).

4.2.4 Effect of the Compounds on Cell Proliferation

The proliferation of PBMCs and TZM-bl cells was monitored by flow cytometry using the CellTrace^{Tm} CFSE kit (Molecular Probes, Oregon, USA) and by the use of a RT-CES analyser respectively. In addition to providing mechanistic information, proliferation assays also serve to confirm cell viability and can provide information on potential antigenicity of the compounds (i.e. if the compounds can cause lymphocyte proliferation, Lampa *et al*., 2002, Verwilghen *et al*., 1992).

4.2.4.1 Compound effects on the proliferation of PBMCs by use of CFSE

In its cell-free state, CFSE is non-fluorescent because of the presence of two acetate groups. This non-fluorescent form is designated CFDA-SE (carboxyflourescein diacetate succinimidyl ester). The acetate groups however result in the compound being highly membrane permeable and allow the dye to rapidly shuttle across the plasma membrane of cells (the mechanism is shown in Figure 4.2). Once inside the cells, these groups are rapidly removed by intracellular esterases to yield highly fluorescent CFSE which binds covalently with proteins and is well retained within the cells (Graziano *et al*., 1998). A fraction of the fluorescent conjugates are not stable and exit through the cell membrane while a highly stable proportion remains within the cells and is halved between daughter cells as they divide, allowing for proliferation monitoring by flow cytometry (Quah *et al*., 2007, Lyons and Parish, 1994).

Procedure: Cell proliferation was monitored using the CellTrace^{Tm} CFSE cell proliferation kit (Molecular Probes, Oregon, USA) from a “live gate” by simultaneous monitoring of viability using PI (BD BioSciences, California, USA). PI incorporation helps in the tagging and exclusion of dead cells which could potentially compromise proliferation data analysis. One microlitre of a 5 mM stock of CFSE was used in staining 1x10^{7} cells/mL of PBMCs such that the final concentration of CFSE was 5 µM. Staining was done in PBS containing 5% (v/v) FCS as staining buffer for 15 min at 37 ºC with gentle agitation every 5 min. The reaction was

quenched with 5 volumes of ice-cold complete RPMI-1640 medium with gentle mixing conditions for 5 min on ice. Unincorporated CFSE was removed by washing three times with ice cold complete RPM1-1640 medium and the stained cells were resuspended in the warm complete RPM1-1640 medium at $1x10^6$ cells/mL.

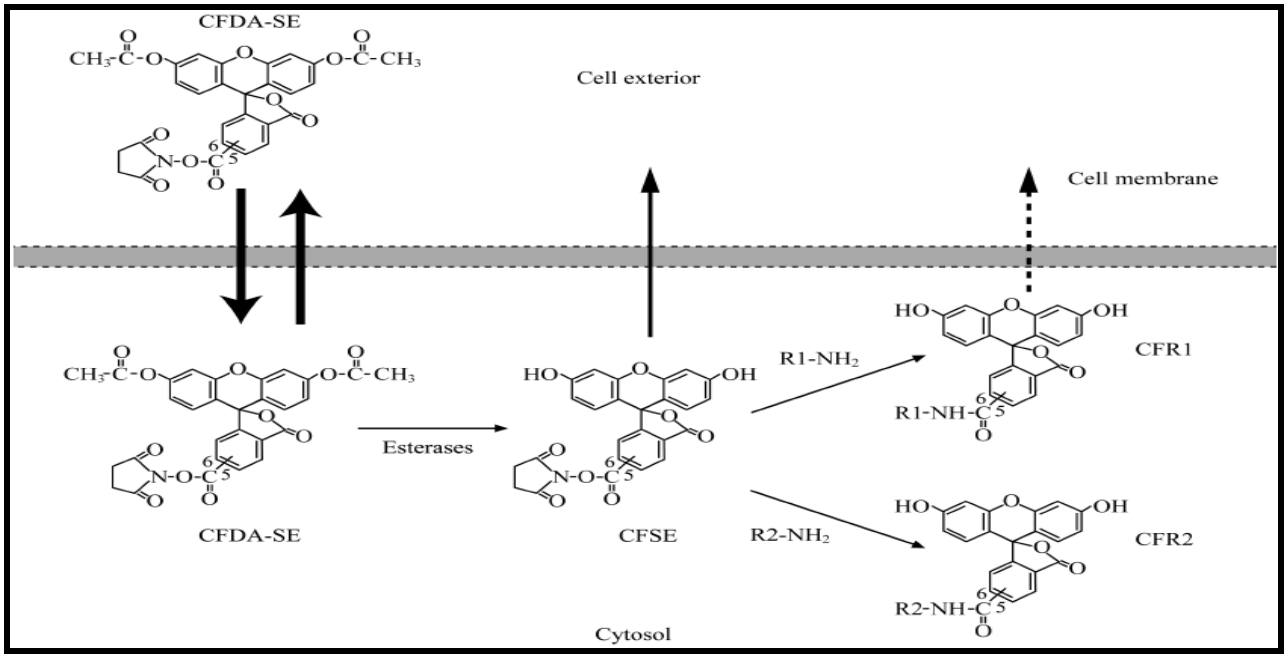

Figure 4.2: Schematic representation of the mechanism involved in fluorescent labelling of cells with CFDA-SE. The CFDA-SE readily taken up by the cell is converted to CFSE by intracellular esterases. The CFSE covalently binds to proteins (R1-NH2 or R2-NH2) and some exits the cell as CFR1 while some of it is retained as CFR2. (Figure was taken from Wang *et al.*, 2005).

To optimise the assay, various strategies were used. It was important to determine when incubations could be stopped and if compounds alone could affect cell proliferation or if stimulants were required for monitoring the effects of the compounds on cell proliferation. Details on these optimisations are provided in the appendix (section 8.3.2). Three days of incubating the compounds with cells stimulated with PHA-P (2 µg/mL) was considered optimal for subsequent tests. Three days (72 h) was used for this assay because by the $5^{th}/6^{th}/7^{th}$ day, most of the cells had lost their fluorescence through proliferation (especially for treatments where anti-proliferative effects were absent). In addition to the fact that the data could be correlated with the annexin V/PI and the MTS viability data (which also involved 72 h incubations), adequate determination of dividing cells using CFSE can only be monitored after > 48 h in culture (Quah *et al*., 2007) making 3 days ideal.

PHA-P (2 µg/mL) stimulated CFSE stained cells at a final concentration of $1x10^6$ cells/mL were treated with different compound concentrations (those that had resulted in >60% viability in the MTS assay) for 72 h in 24 well plates. At the end of the incubation period, samples were harvested and washed twice with ice cold PBS. The samples were resuspended in 100 µL of PBS and a pre-titrated amount (2 µL) of PI was added to the cell suspension. This was incubated for 15 min on ice in the dark followed by the addition of 400 µL of ice cold PBS. Instrument controls for compensation setting included an unstained

control, CFSE stained cells (detected on the FITC detector) and dead cells stained with PI (detected on the PerCp-Cy5.5 detector). The samples were placed on ice and analysed within 1 h by the acquisition of 10,000 events on a FACSAria (BD, California, USA) after compensation or exclusion of fluorescence spillover. Data analysis was done using the proliferation tool in FlowJo Version 7.6.1 (TreeStar Inc., Oregon, USA).

4.2.4.2 Compound effects on the proliferation profile of TZM-bl cells by RT-CES assessment

The xCelligence system (Roche Diagnostics, Mannheim, Germany) also referred to as a real time cell electronic sensing analyser, is a microelectronic biosensor system for monitoring cells. The system provides dynamic, real time, label-free cellular analysis for a variety of research applications in drug development, toxicology, cancer, medical microbiology and virology. Unlike standard end point assays such as MTT and flow cytometry, RT-CES allows for the capturing of more physiologically relevant data and complements these other techniques by providing additional information such as cytotoxicity start time (which will be difficult to identify in end point assays). The system uses plates known as E-plates which contain integral sensor electrode arrays that allow adherent cells (only) within each well to be monitored. The presence of cells in the wells of the E-plate affects the local environment leading to an increase in electronic impedence (resistance). The more the cells attach to the electrodes, the higher the electronic impedence which is measured as cell index (CI). Compound treated cells exhibited varying response patterns represented by CI changes which represent proliferating (increasing CI), cytotoxic (decreasing CI) or cytostatic (stable CI) behaviour of the cells when compared to untreated samples.

This assay has been described for the measurement of cytotoxicity (Boyd *et al*., 2008, Xing *et al*., 2006, Xing *et al*., 2005) and can be used to determine other cellular parameters such as cell proliferation, cytotoxicity start time, cell recovery, and cell response patterns (Xing *et al*., 2005) in real time. Figure 4.3 is a representative diagram showing the stages involved in the functioning of a RT-CES analyser. At point A where no cells are present (Figure 4.3), the CI is zero, but when cells are loaded into the well (1), the CI begins to increase gradually as the cells attach and divide (B). When a toxin or test agent is added (2), the cells can either carry on proliferating resulting in increasing CI (C) or could start dying resulting in decreasing CI (D). The effect of the test agent (stimulatory, cytostatic or toxic) could then be deduced based on the cell proliferation pattern relative to an untreated control.

Procedure: Before experiments were initiated, a cell titration experiment for the TZM-bl adherent cell line was performed in E-plates to establish ideal seeding concentrations. Based on the titration, a concentration of 10,000 cells/well was chosen for subsequent assays because it resulted in about 80% confluency after approximately 24 h (cell index was ~ 1.5) which was ideal for monitoring treatment effects. Proliferation profiles of these cells were established in the presence of selected compounds (TTL3, TTC3, EK207, MCZS2, KFK154B,

PFK5, PFK7 and PFK189) from each class and the assay was performed at least three times for each compound. Two hundred microlitres of 1 x10^5 cells/mL was seeded into the microelectronic plates and allowed to adhere for 24 h. The cells were treated with three different concentrations of the compounds and proliferation or adhesion was automatically monitored every minute (short term) for 1 h and then every 30 min (long term) for 3 to 7 days. Short term monitoring allows for the identification of immediate and transient compound effects while long term monitoring allows sufficient time for the compounds to interact with the cells and modulate their targets and also allows for distinguishing cell response patterns (Abassi *et al*., 2009).

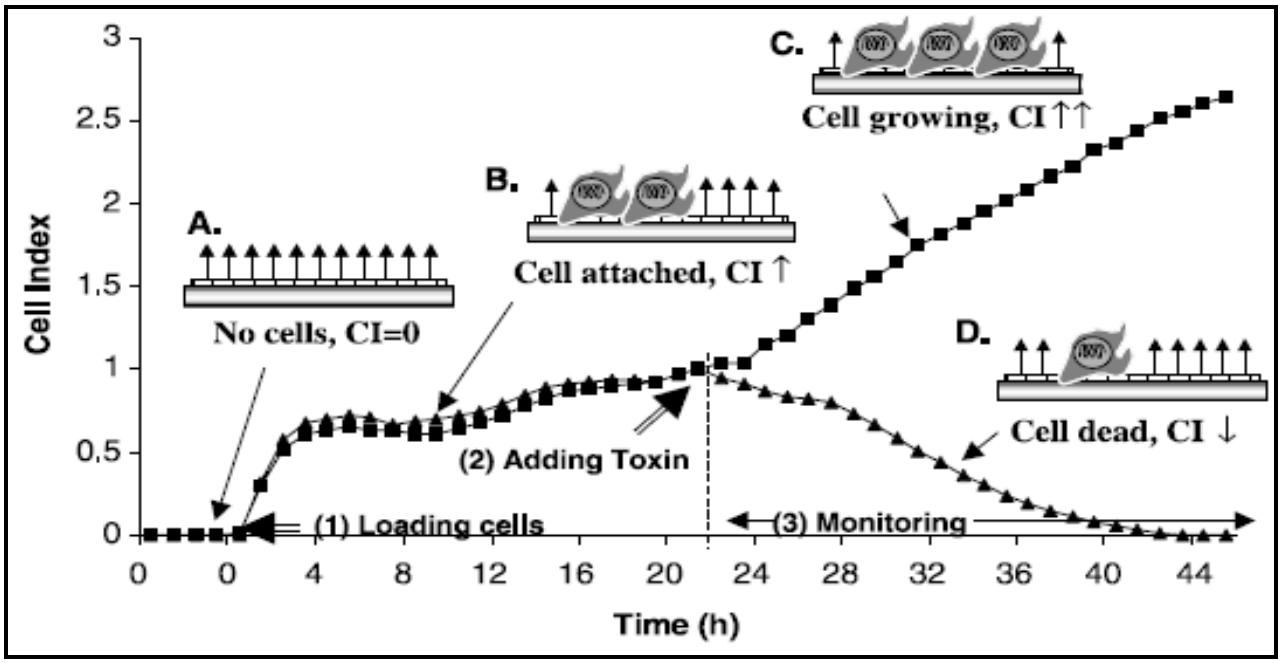

Figure 4.3: The principle of cell proliferation monitoring using the RT-CES analyser. A represents a point where no cell are present with a CI of 0, in B, cells had been added to the well and were beginning to attach and divide (the CI is steadily increasing). When a toxin is added, cells either die (D) or continue proliferating (C). This figure was taken from Xing *et al*., (2006).

4.2.5 Virus Infectivity Inhibition Ability of Compounds by Luciferase Gene Expression Assay

The previous viability and cell proliferation assays were done using uninfected cells. Here and in the next section, the effect of the compounds on cells in the presence of virus is described. Infectivity assays measure the concentration of infective virus in a sample and in this case luciferase reporter gene expression was assessed. This assay was performed at the HIV Research Laboratory of the National Institute of Communicable Diseases (NICD, South Africa) according to the protocol described by David Montefiori (2004). The assay uses molecularly cloned pseudoviruses designed to undergo a single round of infection readily detectable in genetically engineered cell lines that contain a *tat*-responsive reporter gene such as luciferase and for this, the TZM-bl cell line was used. This reporter gene permits sensitive and accurate measurements of infection and the data obtained could be used to infer whether

the compounds could be viral entry inhibitors or if other pathways of the life cycle e.g. post entry steps were inhibited.

Procedure: Du151, an isolate of two phylogenetically distinct subtype C viral strains (Coetzer *et al*., 2007), at a dilution that gave 50,000 ± 15000 relative light units (non-toxic tissue culture infectious dose - TCID) was pre-incubated with the compounds for 1 h. Following this incubation, 100 µL of TZM-bl cells at a concentration of $1x10^5$ cells/mL was added to the virus/compound mixture (final volume of 250 µL) and further incubated for 48 h. At least six concentrations of the compounds were tested in 2 fold serial dilutions with the highest final concentration being 25 µM. Controls included in the assay were a cell control (uninfected cells and growth medium), virus control (virus, growth medium and cells) and a positive inhibitor for infectivity known as BB pool (plasma with known virus neutralizing antibodies). At the end of the 48 hour incubation, 150 µL of supernatant was removed from each well and discarded. Bright Glo substrate solution (100 µL, Promega, Wisconsin, USA) was added to the wells followed by 2 min of incubation at room temperature. From each well, 150 µL of the cell lysate was transferred to equivalent wells of a 96-well flat-bottomed black plate (Nunc™, Roskilde, Denmark) and luminescence was immediately obtained on a luminometer (PerkinElmer 1420 Multilabel Counter, Victor^{3TM}, Connecticut, USA) with the luciferase activity measured in relative light units. Infectivity inhibition was determined as a percentage using the formula = 100-[(test wells-CC)/ (VC-CC) X100], where CC represents cell control and VC represents virus control. The 50% inhibitory concentrations (IC_{50}) were graphically obtained after generating dose response curves (using Graphpad Prism® software, California, USA) representing percentage control inhibition values.

In addition to pre-incubating the virus and the compounds prior to the addition of cells, an alternative incubation strategy was performed which involved pre-incubating the cells with the compounds before adding virus. By using the virus/compound pre-incubation strategy above, it was tempting to conclude that compounds that inhibited viral infectivity did so by interacting with viral surface components. Pre-incubating cells with compounds prior to the addition of virus on the other hand helps in confirming or disproving this and allows for speculation on whether the compounds might have multiple modes of inhibiting infection of the cells. In the situation where both strategies result in similar levels of inhibition, one could conclude that inhibition was at multiple targets. However, if findings lead to different levels of inhibition, then it could be speculated that the compounds affected infectivity at the level of the virus or the cells. In general, such “time of addition studies” allows for commentary on whether a viral or cellular pathway was inhibited with the important variable being the exposure time.

Concurrent cell viability studies were also performed using MTT and the same experimental procedures (incubation time, final compound concentration and cell number) to determine the viability of the TZM-bl cells. This was important so as to eliminate compound toxicity in the infectivity studies since toxicity could be misinterpreted as infectivity inhibition.

4.2.6 Effects of Compounds on Immune System Cells Using Multi-parametric Flow Cytometry

Flow cytometry is the defining tool for immune system cell studies and is currently the only technology that can analyse complex components of the immune system for clinical significance (Mahnke and Roederer, 2007, Pala *et al*., 2000). Because of the complexity of the T cell compartment, both phenotypically and functionally (Mahnke and Roederer, 2007), the frequency of immunological markers on cell surface alone are not reliable determinants of the immune state or the chronic inflammatory disease caused by HIV (Appay and Sauce, 2008). Monitoring the intracellular production of cytokines along with the phenotypic identity of a cell gives better insight about disease associations because the survival, growth, differentiation and effector function of cells and tissues are controlled by cytokines (Maino and Picker, 1998). The development of flow cytometers capable of measuring up to 20 parameters has widened the possibilities in this field (Mahnke and Roederer, 2007). Enzyme linked immunosorbent assays (ELISAs) can also be used for monitoring cytokines in culture supernatant but the method is impractical when large numbers of heterogeneous cells obtained *ex vivo* need to be analysed (Pala *et al*., 2000). Because intracellular cytokine staining (ICCS) is an end point experiment, there was the concern that the cytokines being targeted might have been secreted before incubations were stopped. As a precautionary measure, culture supernatant from cells used for ICCS was collected and used to concurrently measure secreted cytokines using ELISAs with the hope of correlating the data with the ICCS data.

A multi-parametric ICCS flow cytometric analysis was performed to determine the effect of the compounds on the production of a representative pro-inflammatory cytokine, TNF-α (increases result in disease progression in HIV, Caso *et al*., 2001) and an anti-inflammatory cytokine, IFN-γ (which has anti-viral effect, Dinarello, 2000). Simultaneous analysis of T-lymphocyte (CD3+, CD4+ and CD8+) subsets to determine cytokine levels with respect to T cell frequencies was also performed. A viability marker was included to aid in excluding dead cells. This is because false positive events resulting from antibody conjugates could bind non-specifically to dead cells resulting in misleading results (Mahnke and Roederer, 2007). Assays were performed for both HIV+ and HIV- donors so as to determine if the effects of the compounds (and therefore usefulness) on immune state was dependent or independent of infection status.

ICCS antibodies: The following monoclonal antibodies (Mabs) directed against T cell surface markers were used: CD3-pacific blue (detected on the 4',6'-diamidino-2-phenylindole or DAPI detector), CD4-phycoerythrin (PE), CD8-PerCP-Cy 5.5 as well as anti-human cytokine Mabs: anti-TNF-α-allophycocyanin (APC) and IFN-γ-FITC, all purchased from Pharmingen (BD BioScience, California, USA). The sixth fluorochrome conjugated antibody was an aqua fluorescent fixable amine reactive dye (Molecular Probes, Oregon, USA) for “live gating” to eliminate dead cells. This dye can permeate compromised membranes of necrotic cells and

react with free amines both in the interior and on the cell surface resulting in intense fluorescent staining. In contrast, only the cell surface amines of viable cells are available to react with the dye resulting in relatively dim staining. These fixable dyes help in preserving the live/dead discrimination in subsequent fixation steps in ICCS procedures during which pathogens are inactivated unlike PI which is not fixable.

ICCS Reagents: ION, PMA and PHA-P were obtained from Sigma Aldrich (Missouri, USA). Golgi stop reagent (containing monensin) as protein transport inhibitor and reagents for cell fixation and permeabilisation (Cytofix/Cytoperm and Perm/Wash, respectively) were purchased from Pharmingen/BD (California, USA).

Prior optimisation assays: Serious sensitivity issues limit the advantages of multi-parametric flow cytometry. These include cell autofluorescence in a specific region of the spectrum, the specificity or selectivity of the antibody conjugates and the presence of other antibody conjugates attached to the same cell that could result in spillover fluorescence into the same detector (Mahnke and Roederer, 2007). In order to curb these shortcomings, optimisation assays, which included Mabs titrations to determine optimal antibody concentrations were performed prior to actual experiments. Additionally, controls such as fluorescence minus one (FMO, to aid with proper gating) and compensation controls were included during data acquisition. Additional optimisation experiments were done for various conditions including the use of different stimulants (PHA-P or PMA/ION), different incubation times (6, 24, 48 and 72 h), and treatments (compounds only or compounds with stimulants). The optimisation data is shown in Figure A4.8. After the optimisations, PMA/ION stimulation in the last 6 of a 24 h treatment with the compounds was found ideal for monitoring ICC production. Stimulants were required to induce *in vitro* cytokine gene expression because unstimulated PBMCs spontaneously produce little or no cytokines (Baran *et al*., 2001) making quantification difficult. The following detailed experimental conditions were used after the optimizations.

Procedure: PBMCs isolated from both HIV+ (12) and HIV- donors (13) were prepared at a concentration of $1x10^7$ cells/mL in complete RPMI-1640 medium. The cells were incubated without or with the compounds (with at least one representative compound from each class) at concentrations ranging from 0.04 to 5 µM for 24 h in V shaped 96 well plates (Nunc™, Roskilde, Denmark) with a final volume of 200 µL and cell number of $1x10^6$ cells/well. In the last 6 h of the incubation, activators of cytokine production, PMA (10 ng/mL) and ION (1 µM) were added to the cells in the presence of 1 µL of BD GolgiStop™ (containing monensin) for preventing cytokine secretion. At the end of the 24 h incubation, the plate was centrifuged and 150 µL of culture supernatant from each well collected and stored at -20 ºC for subsequent evaluation of secreted cytokines using ELISAs. The cells were blocked with 10% (v/v) FCS in PBS for 20 min at 4 ºC to prevent nonspecific binding. Following two washes with staining buffer (PBS), the cells were stained with pre-titrated optimal concentrations of surface Mabs CD3-Pac blue (0.625 µL), CD4-PE (2.5 µL), CD8-PerCp-Cy5.5 (2.5 µL) and the aqua

fluorescent fixable amine reactive dye (0.02 µL) in 100 µL reaction volumes for 20 min. The cells were washed twice with staining buffer and then fixed and permeabilised with Cytofix/Cytoperm solution for 20 min at 4 ºC. Two more wash steps with Perm/Wash solution were performed and the pelleted cells were stained (30 min at 4 ºC) for intracellular cytokines using pre-titrated FITC and APC conjugated Mabs against IFN-γ (0.05 µL) and TNF-α (0.3 µL) respectively. After two final wash steps with Perm/Wash, the cell pellet was resuspended in 150 µL of PBS and transferred to flow tubes (BD BioSciences, California, USA). An equal amount of 6% (v/v) formaldehyde (Sigma Aldrich, Missouri USA) was added to the cells to maintain them in a fixed state. In addition to the FMOs and compensation (instrument) controls which were used for quadrant specification and for the exclusion of fluorescent spillovers, compulsory biological controls consisting of an unstained sample and a stained untreated control were included.

Flow cytometry acquisition and analysis: A six colour (multi-parametric) flow cytometry analysis was performed on a FACSAria (BD, California, USA) using FACSDiva software with a total of 30,000 events (3 times more than for the viability and proliferation assays since intracellular or rare events were being probed) collected per sample. Single cell cytokine production was evaluated after FSC and SSC gating of lymphocytes from the PBMCs population. The intracellular cytokines were determined from the CD4+ and CD8+ subpopulations of T cells (CD3+). FlowJo version 7.6.1 (TreeStar Inc., Oregon, USA) was used for data analysis and statistical evaluation was done using the stained untreated control sample as a reference for each treatment.

Statistical analysis: The frequency of CD4+, CD8+ and cytokine producing cells from untreated controls and those treated with the various compounds were expressed as a percentage. Statistical analysis was done using Graphpad Prism® (San Diego, California, USA). The Wilcoxon matched-pairs signed rank test was used in determining statistical significance between medians with a one way non-parametric statistical test used since the data did not meet normal distribution. Correlations were tested using the non-parametric Spearman correlation test. A p value of <0.05 was considered significant.

ELISA: The method and results for the ELISA performed for only 6 of the 12 HIV+ donors and 2 of the 13 HIV- donors is provided in the appendix (subsection 8.3.6.2).

4.2.7 Experimental Summary

Table 4.1 is a summary of the cell types (plus HIV status), incubation times and the concentration of compounds that were used for the various cell-based assays that have been described here. The viability of PBMCs and the PM1 cell line was monitored using MTS and the CC_{50}s of the compounds was determined from this assay. The TZM-bl adherent cell line was used for monitoring infectivity and concurrent viability using MTT (48 h) and for the RT-

CES analysis. The CC_{50}s data was subsequently used as a guide for determining non-toxic concentrations of the compounds needed in other assays.

Table 4.1: Cell-based assay summary. Cell types, cell HIV status and the various concentrations and incubations times used for the cell-based assays are shown. The asterisk (*) represents an adherent cell line unlike PM1 and PBMCs which are suspension cells. #: These were concentrations with >60% viability in MTS assay. HIV- cells were used for viability assays where only compound effect was determined. HIV+ cells were used for assays in which viral infectivity information was required and for determining compound effect on the inflammation caused by HIV.

Cell type	Assay type	HIV status	Time	[Compound] in µM
PM1	MTS	HIV-	72 h	0.2 - 200
PBMCs	MTS	HIV-	72 h	0.2 – 200
	Annexin-V/PI	HIV-	72 h	0.04, 0.2, 2.5 or 5#
	CFSE	HIV-	72 h	0.04, 0.2, 2.5 or 5#
	Immune cell effect	HIV- & HIV+	24 h	0.04, 0.2, 2.5 or 5#
TZM-bl*	RT-CES	HIV-	3-7 days	0.1, 5 and 10 µM
	MTT	HIV-	48 h	0.8 - 25
	Infectivity	HIV+	48 h	0.8 – 25

4.3 RESULTS AND DISCUSSION

For ease of reference, data presentation always appears in the order of control samples (where applicable), followed by the gold(I) phosphine chloride complexes and corresponding ligands (class I), then the BPH gold(I) chloride complexes of class II, the phosphine gold(I) thiolate complexes of class III, the Tscs-based complexes and corresponding ligands of class IV and finally the pyrazolyl gold(III) complex of class V. Where only representative compounds were tested, the order of result presentation will still be maintained in terms of classes.

4.3.1 Cell Viability Determination

A crucial step in drug discovery is screening for non-toxic and hopefully efficacious concentrations at an early stage. The effect of the compounds on the viability of relevant cells types was monitored using the MTS dye as well as the annexin V FITC apoptosis kit (BD, California, USA). The concentration of compounds which caused 50% cytotoxicity of the PBMCs and the PM1 cell line was generally in the low micromolar range (between 1 and 20 µM, Table 4.2). These are concentrations which are physiologically relevant for gold compounds (i.e. found in the serum or synovium of people on chrysotherapy, Stern *et al*., 2005, Okada *et al*., 1999, Yoshida *et al*., 1999, Mascarenhas *et al*., 1972). For this assay and all the cell-based assays, a percentage standard error of means (SEM) between experimental repeats of <20% was considered acceptable. This range is acceptable for these assays because of the inherent variabilities present in cell-based assays. For example opening and closing of incubator doors can cause slight temperatures fluctuations that can affect cell growth patterns (this was seen in the RT-CES assay and the expectation was that it was the

same for the end point assays). For the direct enzyme assays (chapter 4), a % SEM of <10% was considered acceptable because of the expected low variability in these assays.

Table 4.2: CC_{50} values indicating the effect of the compounds on the viability of PBMCs and the PM1 cell line. The cells were treated with various concentrations (0.02-200 µM) of the compounds for 72 h followed by MTS treatment. ND refers to not done. Ligands are colour coded in grey while the superscript ([a]) refers to gold complexes with >10 µM CC_{50}.

Compounds	CC_{50} (µM)		Compounds	CC_{50} (µM)	
	PBMCs	PM1		PBMCs	PM1
TTL3	52±5.6	85.7±14	MCZS1[a]	13±3.2	19.8±7.4
TTC3	4±1.3	ND	MCZS2	1.2±0.1	1.5±0.4
TTL10	65±10.1	88±7.1	MCZS3[a]	19±1.8	12.6
TTC10	4.4±0.7	<3.1	PFK174[a]	58±9.1	15±2.4
TTL17	21±6.8	35.5	PFK189[a]	103±11.8	2.5±0.1
TTC17	3.7±0.9	43±5	PFK190[a]	11±0.9	<0.4
TTL24	45.7±8.2	ND	PFK5	>200	>100
TTC24	4.6±0.4	4.8±0.1	PFK7	5.6±0.6	1.7±.03
EK207	8.5±2.3	5.3±2.2	PFK6	>200	ND
EK208	6.6±1.5	6.1±0.2	PFK8[a]	11.8±2.8	1.7±01
EK219	7.1±1.9	6.1±1.1	PFK39	<0.2	ND
EK231[a]	50±8.9	29±4.8	PFK41	0.21±0.4	ND
			PFK38	<0.07	ND
			PFK43	0.07	ND
			KFK154B[a]	27±6.3	90±7.4

Although CC_{50s} were obtained for both PBMCs and PM1 cells, values determined using PBMCs were used as a model for further studies especially because subsequent assays mostly involved the use of the latter cell type. Gold complexes EK231, MCZS1, MCZS3, PFK174, PFK189, PFK190, PFK8 and KFK154b were generally less toxic with CC_{50} of > 10 µM when PBMC viability was determined. The ligands had higher CC_{50} values (less toxic) than the corresponding complexes except for two Tscs ligands (PFK39 and PFK38) which had CC_{50} values of < 0.08. A decrease in toxicity after complexation has been reported in the literature for some Tscs ligands (Pelosi *et al.*, 2010) such that the latter findings were not surprising. The slightly higher toxicity that was generally observed for the other gold complexes compared to the ligands might be because of the fact that gold has a high affinity for sulphur and is known to undergo ligand exchange reactions with sulfhydryl groups in cysteine side chains of proteins (Shaw III, 1999, Sadler and Guo, 1998). If these interactions occur with the membrane or intracellular proteins it may be responsible for increased retention of the compound resulting in the observed increase in toxicity especially for the very lipophilic compounds (chapter 3, Table 3.8A) such as the gold(I) phosphine chloride complexes, the BPH gold(I) complexes and the bimetallic gold(I) phosphine thiolate complexes. In fact this kind of effect has been reported for the parent compound of the BPH gold(I) complexes, $Au(DPPE)_2Cl$, which demonstrated promising *in vitro* anti-cancer properties (Fricker, 1996, Berners-Price *et al.*, 1986, Mirabelli *et al.*, 1986) but led to cardiotoxicity problems in pre-

clinical studies (Hoke *et al*., 1989). This would however not be the case for complexes such as the two of the phosphine thiolate complexes (MCZS1 and 2), the Tscs-based complexes and the gold(III) pyrazolyl complex which had ideal lipophilicity values and overall good ADMET qualities with a score of 6/7 (Table 3.8A). PF174, PFK189 and PFK190 demonstrated varying CC_{50}s in the PBMCs and PM1 population (Table 4.2) and these differences may be related to the poor aqueous solubility that was observed for these complexes during wet lab dissolution procedures corroborating the ADMET predictions in chapter 3 (Table 3.8A and 3.9).

Tests with the annexin V apoptosis detection kit (Figure 4.4) confirmed that concentrations of the compounds that resulted in >60% viability of PBMCs in the MTS assay were in fact not toxic (Figure 4.3) except for two complexes (PFK189 and PFK190). These two complexes caused < 50% viability of PBMCs at 5 µM contrary to findings in the MTS assay (CC_{50}s were 103±11.8 and 11±0.9 µM respectively, Table 4.2). Again, the poor aqueous solubility predicted in the *in silico* ADMET studies (chapter 3, Table 3.8A) and in wet lab assays might be responsible for this variation. Alternatively because the apoptotic mechanism cannot be identified in the MTS assay, cells in early apoptosis may still be able to metabolise MTS giving the overall impression that the compound was not toxic. Such variations were not surprising and support the idea that more than one parameter should be investigated when determining the toxicity of potential drugs (Kepp *et al*., 2011).

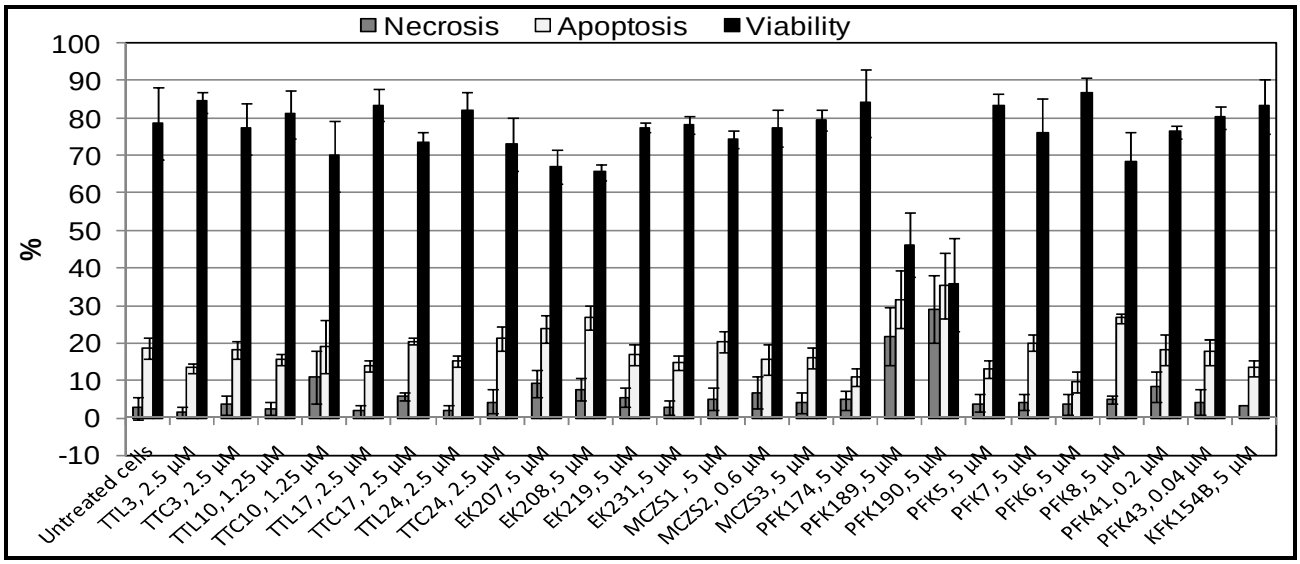

Figure 4.4: Viability profile of PBMCs treated with the compounds and analysed using flow cytometry. The cells were treated with the compounds for 72 h and stained with annexin V and PI for 15 min before flow cytometry analysis. Except for PFK189 and PFK190 which caused high apoptotic and necrotic abilities, all the other compounds did not significantly affect cell viability at the indicated concentrations (concentrations with >60% viability in MTS assay). Cells treated with ligands (e.g. TTL3, TTL10) had slightly more viable cells than those treated with corresponding complexes (e.g. TTC3, TTC10). The BPH gold complexes (EK207, and EK208) and the gold(III) thiosemicarbazonate complex PFK8 were slightly more toxic than the rest. The percentage SEMs were <20%.

With regards to mode of cell death, the BPH gold(I) complexes, EK207 and EK208, the gold(III) thiosemicarbazonate complex, PFK8, as well as complexes PFK189 and PFK190 caused the highest apoptotic cell death (>20%). Cell death by necrosis was generally below 10% for all treatments (except for PFK189 and PFK190) and untreated cells.

4.3.2 Cell Proliferation Determination

4.3.2.1 Monitoring proliferation using CFSE

The CellTrace^{Tm} CFSE kit from Molecular probes was used in determining the effect of the compounds on the proliferation of PBMCs. Representative cell proliferation histograms are shown in Figure 4.5. The various peaks in Figure 4.5 equate to different generations of daughter cells as they divide from the parent generation (orange coloured peak). The brightest peak (orange coloured) or parent generation or generation 0 consists of cells with the least proliferation while the dimmest peaks represent cells that proliferated the most (fluorescence intensity between 0 and 10^3 on the x-axis, Figure 4.5).

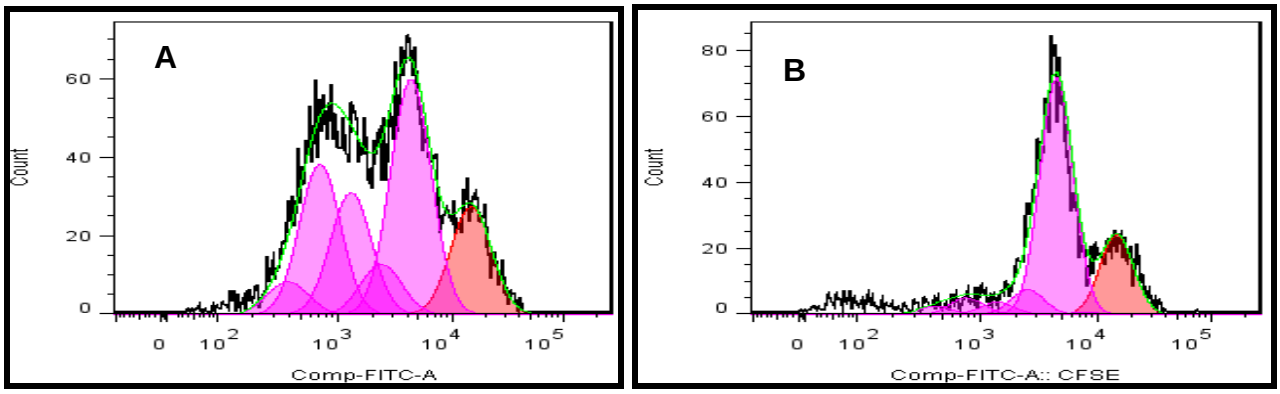

Figure 4.5: Representative proliferation histograms showing proliferation patterns of CFSE stained PBMCs. Various peaks represent generations with the brightest being cells that did not proliferate (orange colour) and the dimmest are cells that proliferated the most. In A, cells were treated with 2 µg/mL of PHA-P and in B PHA-P stimulated cells were treated with gold complex EK208. EK208 was anti-proliferative.

The software (FlowJo version 7.6.1, TreeStar Inc., Oregon, USA) that was used in analysing the proliferation of the PBMCs indicated a total of eight generations but only 6 were visible enough (Figure 4.5A and B) since events in the dimmest generations were very few especially in Figure 4.5B due to compound (EK208) effect on cell proliferation. Because of the diminished number of events observed in generations 2, 3, 4, 5, 6, and 7 for the treated cells (Figure 4.5B), the first 3 generations i.e. 0, 1 and 2 were merged to form generation 0 and the last two i.e. 6 and 7 were merged to form generation 4. Data for the resultant five generations are shown as stacked column bars for each tested compound in Figure 4.6.

The concentrations of compounds tested were those that resulted in >60% viability (PBMCs) in the MTS assay and were similar to those used in determining viability by flow cytometry (Figure 4.4). Proliferation monitoring was done from a “live gate” which was obtained by excluding PI positive events (cells) on a SSC versus PerCp-Cy5.5 dot plot.

The proportion of cells in generation 0 of the untreated sample (cells) was chosen as a reference point (indicated as a grey line spanning across compound treated cells, Figure 4.6) for determining compounds that had anti-proliferative effects on PHA-P stimulated PBMCs. The percentage anti-proliferative effect was calculated using the formula:

$$\% \text{ anti-proliferative effect} = \frac{(\text{Treatment \% in generation 0} - \text{control \% in generation 0})}{(\text{Control \% in generation 0})} \times 100$$

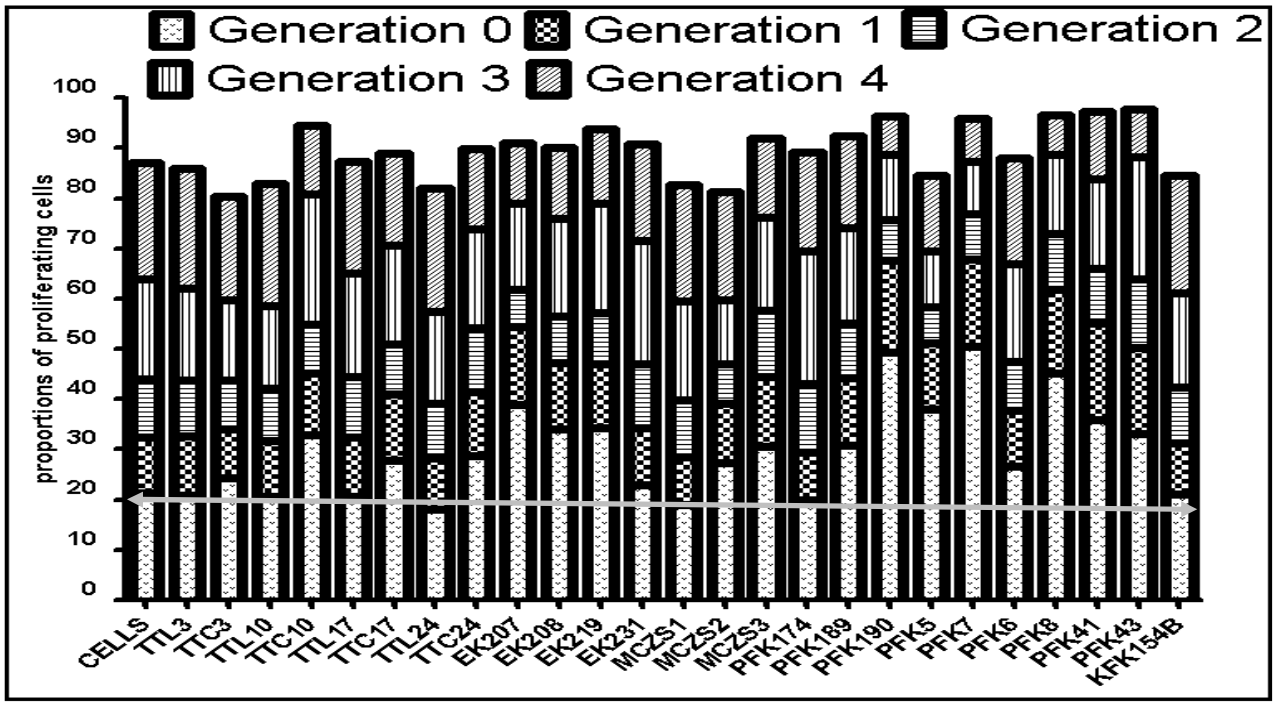

Figure 4.6: The effect of the compounds on PBMC proliferation. CFSE stained PBMCs stimulated with 2 µg/mL of PHA-P were treated with the compounds for 72 h. The cells were harvested and proliferation monitored on a FACSAria flow cytometer. The phosphine(I) complex TTC10, the BPH gold(I) complexes, EK207, EK208, EK 219 , gold(I) thiolate complex PFK190 and the gold(III) thiosemicarbazonate complexes PFK7, PFK8, PFK41 and PFK43 prevented the proliferation of >50% of the initial number of cells in generation 0 compared to the untreated control suggesting these compounds had anti-proliferative effects.

Anti-proliferative effects of >50% caused by the compounds on cells in generation 0 was considered relevant and was applicable to complexes TTC10 (55%), EK207 (83%), EK208 (60%), EK219 (61%), PFK190 (132%), PFK5 (79%), PFK7 (137%), PFK8 (112%), PFK41 (69%) and PFK43 (56%). Compounds that were the least anti-proliferative were ligands TTL3 (1.9 %), TTL10 (2.9%) and TTL17 (2.5%), and gold complexes EK231 (8.1%), PFK174 (6.7%) and KFK154b (1%), a finding which was in agreement with the observed CC_{50}s for these compounds (Table 4.2). Only two compounds; TTL24 and MCZS1 appeared to have a minor stimulatory effect with slightly less cells in generation 0 (below grey line). Generally the proportion of cells in generation 0 translated to the 1st, 2nd, 3rd and 4th generations i.e. treatments with less cells in generation 0 had more cells in either generation 1, 2, 3 or 4 and vice versa (Figure 4.6). For example, PFK7 which had the highest percentage of cells in generation 0 (137%) had far fewer cells in generation 4 compared to the untreated control. With regards to class, class IV (Tscs-based) compounds were the most anti-proliferative followed by class II (BPH-based) and finally I, III and V which were the least.

By monitoring the effect of the compounds on the proliferation of T-cells, drug mechanisms can be deduced (Brenchley and Douek, 2004). For example compounds with mitogenic (e.g. PHA-P-like compounds) tendencies which stimulate cellular proliferation or those that inhibit cell proliferation can be identified through the proliferation patterns observed.

Stimulation and therefore proliferation although beneficial in the sense that important bio-molecules and cells such as CD8+ needed by the host (especially during HIV infection)

are elevated, could also be detrimental. The increase in the proliferation of CD8+ cells is associated with the production of perforin which aids in the destruction of infected CD4+ cells, a phenomenon seen in long term non progressors (Migueles *et al*., 2002). In HIV infection, activation or stimulation of cells also leads to proliferation and is directly linked to viral pathogenesis (Douek *et al*., 2009) since it accelerates viral replication (Mcdougal *et al*., 1985, Folks *et al*., 1986, Biancotto *et al*., 2008). These reports suggest that stimulatory and anti-proliferative effects have advantages and disadvantages suggesting that an optimal state (in which there is a balance between the two) should be the ideal.

None of the compounds caused increases in PBMCs proliferation, suggesting that the compounds did not result in cell activation or stimulation, a situation which is usually associated with disease progression e.g. in rheumatoid arthritis (Lampa *et al*., 2002). This also means that the compounds (if eventually used as drugs), should not demonstrate the type of hypersensitive adverse effects (lymphocyte proliferation resulting from stimulation) usually observed as dermatitis for gold drugs (Verwilghen *et al*., 1992, Lampa *et al*., 2002).

None of the gold complexes on their own stimulated cell proliferation (like PHA-P, Figure A4.8C), but when cells were stimulated with PHA-P; it was possible to observe the compounds' anti-proliferative effects (Figure 4.6). This finding was not surprising for these complexes since gold salts have previously been reported to inhibit PHA-P stimulated proliferation of PBMCs (Sfikakis *et al*., 1993, Lipsky and Ziff, 1977).

The proliferation studies were performed on uninfected cells and so it is not clear if the anti-proliferative effects of the compounds may be related to the ability to lower viral replication. If these assumptions could be made, then the ten complexes which inhibited the proliferation of the PHA-P stimulated cells (Figure 4.6) may be capable of modulating and suppressing viral replication through the ability to prevent T cell activation. However, in the multiparametric flow cytometry assays, the effect of the compounds on the frequency of HIV+CD4+ cells could be used to deduce this.

4.3.2.2 Monitoring proliferation using a RT-CES device

Cell proliferation was also monitored using a modified HeLa cell line (TZM-bl) to determine the compounds' effects on the kinetics of cell growth using an RT-CES device. Unlike standard end point assays such as MTT, the ability to monitor cytotoxicity start time, cell recovery and cell response patterns (Xing *et al*., 2005) in real time make this technique unique. Following titration experiments, 10,000 cells were chosen as the optimum seeding density per well for the TZM-bl cells (Figure 4.7A shows a typical titration profile). This density resulted in an average cell index of 1.5 which was ideal for the assay since over confluency (100%) or under confluency (< 60%) prior to compound addition at about the 24th hour was avoided. The ideal confluency range should be between 70 and 85%.

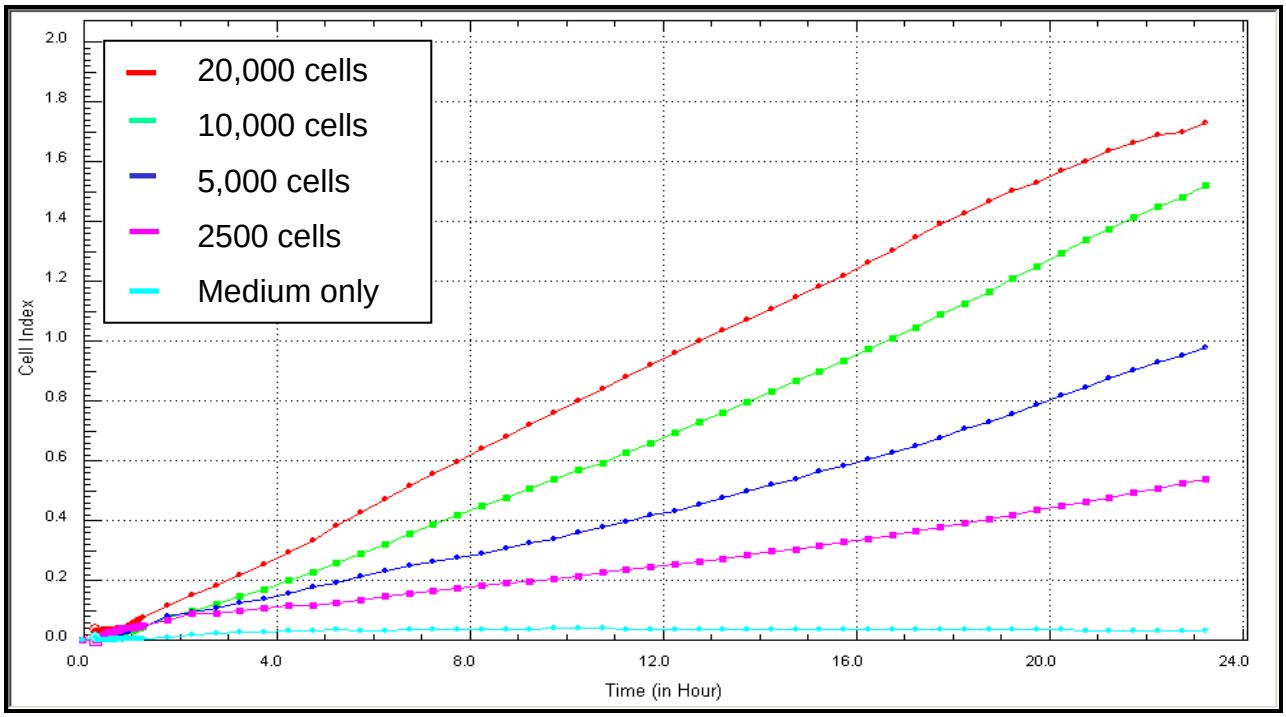

Figure 4.7A: Typical titration profile of TZM-bl cells. The proliferation of the cells was monitored for 23 h at concentrations of 2500, 5000, 10000 and 20,000 cells. A titre of 10,000 cells was chosen for experiments since it resulted in approximately 80% confluency between 22 and 24 h (time range at which test agents were added).

Selected compounds from each class were tested at 3 different concentrations. Representative profiles for all tested compounds are shown in Figure 4.7B while experimental repeats are presented in the appendix (Figure A4.11 and A4.12). The plots were constructed from normalized cell index (CI normalized against the time point when the compounds were added to the cells ~ 22-24 h post seeding) on the y-axis against time in hour on the x-axis. The normalisation is set to 1 and corrects for any small differences between cells before the addition of compounds such that only compound effects are visible. Upon the addition of compounds, the cells could either continue proliferating (increasing CI), stopped proliferating (stable CI) or started dying (decreasing CI). The assay was monitored for 7 days.

Various profiles were observed for the different compounds but there was generally a dose dependent change in CI index. As expected, the phosphine ligand, TTL3, had no adverse effect on the proliferation of these cells compared to the corresponding complex, TTC10, which at 10 µM demonstrated a cytostatic effect. The BPH gold(I) complex, EK207, initially demonstrated a significant increase in CI at 10 µM (probably as a result of compound uptake and swelling of the cells) and then after 24 h of addition (45th h on graph), a steady decrease was observed suggesting toxicity which continued until the assay was stopped at the 168th hour. A similar phenomenon was observed at 5 µM but the cytoxicity start time was at the 98th h i.e. 72 h after compound addition. The profile for the gold(I) phosphine thiolate complex, MCZS2, suggested that the complex was much more cytotoxic than the rest of the complexes with cell indices steadily dropping until they approached zero within hours of complex addition. For this compound, only the 0.1 µM concentration was non-toxic.

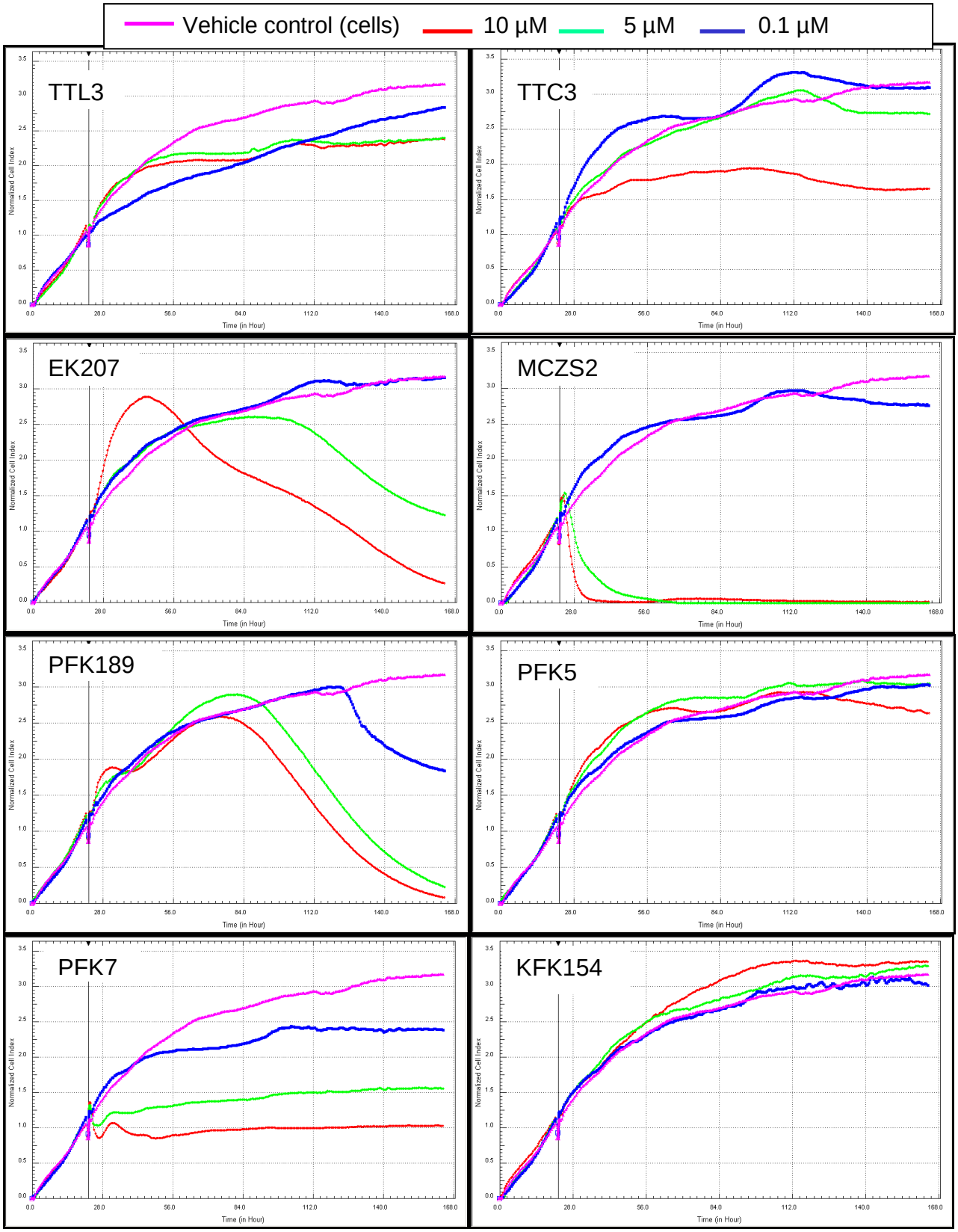

Figure 4.7B: Effect of compounds on TZM-bl cell growth pattern monitored by an RT-CES analyser. Cells were seeded into an E-plate and allowed to adhere for at least 20 h followed by treatment with compounds. Three concentrations of each compound were tested and are represented on each graph as normalized CI (y-axis) against time (h) alongside the vehicle control (cells with 0.5% DMSO, minimum DMSO concentration present in treatment, included exclude vehicle effect on cells). Compounds TTL3, PFK5 and KFK154b did not cause CI decreases. EK207 and PFK189 induced a dose dependent decrease in CI days after addition. TTC3 was cytostatic at 10 µM while PFK7 displayed a dose dependent cytostasis at all 3 concentrations which was absent for the complementary ligand, PFK5. Significant decreases in CI were observed for complex MCZS2 within hours of addition suggesting toxicity.

PFK189, on the other hand had a similar profile to that of EK207 causing significant CI decreases after 72 h (10 µM) and 94 h (5 µM). Some of the possible reasons why the cell indices for EK207 and PFK189 started dropping after an initial phase of increase could be because the cells had reached 100% confluency and were dying as a result of overcrowding. Alternatively cell death could have been triggered after the accumulation of toxic doses of the compounds. The similarity in behaviour between EK207 and PFK189 may be because both compounds are bimetallic gold complexes. These findings were not surprising considering that in the drug-likeness studies, these complexes had been predicted to have extremely higher lipophilic tendencies (outside the ideal limit, Table 3.8A). It is possible that these two complexes bind tightly and accumulate at the cell membrane and likely disrupt it over time as seen in the profiles (Figure 4.7A). TTL3, PFK5 and KFK154b, as expected (from MTS data Table 4.2), were not toxic to these cells at ≤ 10 µM.

A notable observation was the dose dependent cytostasis observed for the gold(III) thiosemicarbazonate complex, PFK7, which was absent for the complementary ligand, PFK5 (Figure 4.7B, Figure A4.11 and A4.12). The same phenomenon was noted for complex PFK8 (Figure 4.7C), also a thiosemicarbazonate complex. A similar effect was noted for the gold(I) phosphine chloride complex, TTC3 at 10 µM (Figure 4.7B) but not upon subsequent analysis (Figure A4.11 and A4.12). In an end point assay, such observations could easily have gone unnoticed and the assumption would have been that PFK7 and PFK8 were cytotoxic especially at 5 and 10 µM, which was apparently not the case as seen from these real time studies. Gold(III) complexes have been shown to have anti-cancer activity (Casini *et al*., 2008, Che *et al*., 2003) and thus cytotoxic and anti-proliferative effects (Gabbiani *et al*., 2007). The cytostatic or anti-proliferative effect noted here for PFK7 and PFK8 may mean that these complexes have potential anti-cancer activity. With regards to HIV, cytostasis has been reported as a mechanism by which some anti-viral drugs e.g. HU, trimidox and didox (Lori *et al*., 2005, Mayhew *et al*., 2005, Lori *et al*., 2007, Clouser *et al*., 2010) function. Combining optimal doses of cytostatic compounds with drugs that directly inhibit virus, e.g. didanosine, leads to an overall beneficial effect in HIV treatment (Lori, 1999, Lori *et al*, 2005, Clouser *et al*, 2010). PFK7 and to a lesser extent PFK8 were some of the complexes which significantly prevented the proliferation of PBMCs in the CFSE assay, retaining as much as 137% and 112% of the cells in generation 0 respectively at 5 µM (Figure 4.6). The RT-CES analysis for these complexes (Figure 4.7B and C) was therefore in agreement with the CFSE findings (Figure 4.6). These outstanding observations of cytostasis were compiled in a manuscript that was accepted for publication in the Journal of Inorganic Biochemistry (Fonteh *et al*., 2011) as the possible mechanism by which these compounds inhibited viral infectivity (discussed in the next section). To the best of our knowledge this is the first time a cytostatic mechanism for gold-based drugs has been demonstrated using the impedence-based technology of the RT-CES analyser. The fact that these thiosemicarbazonate gold(III) complexes also demonstrated

outstanding drug-like properties when shake flask (section 3.4.3) and *in silico* ADMET predictions (Table 3.8A) were compared makes these observations significant for HIV (and probably anti-cancer) drug discovery.

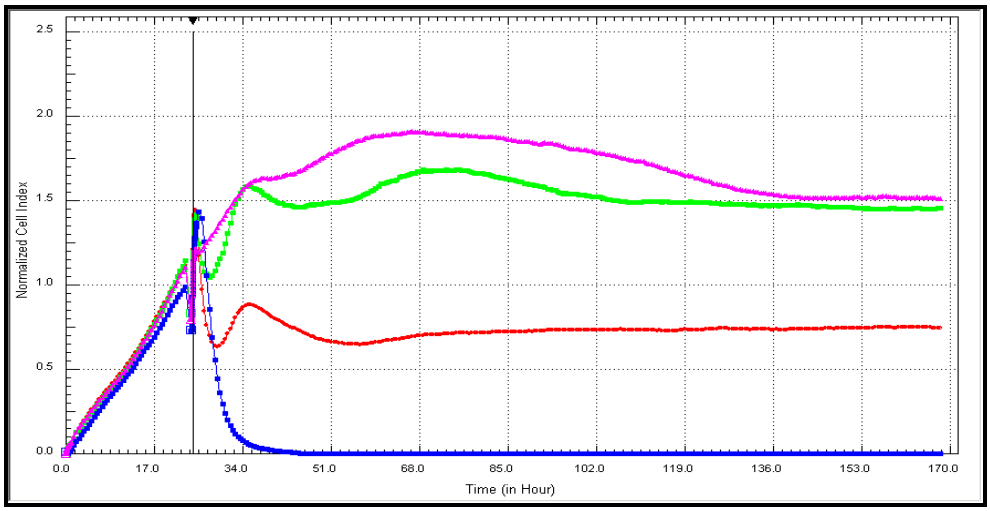

Figure 4.7C: The effect of complex PFK8 on the proliferation of TZM-bl cells monitored by RT-CES analysis. Here the vehicle control is represented by a pink line, green is 5 µM and red is 10 µM concentrations of PFK8 respectively. Auranofin (10 µM) is represented in this figure by the blue line. The cells (vehicle control) appeared to have entered the dead phase earlier (cell index was gradually decreasing after 68 h).

4.3.3 Inhibition of Viral Infectivity by Determining Luciferase Gene Expression from TZM-bl Cells

Inhibition of pseudovirus (Du 151.2) infectivity by the compounds was measured as a reduction in luciferase reporter gene expression after a single round of infection of TZM-bl cells. There was a dose dependent decrease in infection from 0.8 to 25 µM (Figure 4.8A). Viability assessment (using MTT at the same concentrations) was performed to determine whether the observed inhibition of infection was specific and not due to compound-induced cell death (Figure 4.8B).

For the analysis, a cut-off of >80% viability was considered a good point for excluding compounds which might influence infectivity through toxicity (i.e. compounds with <80% viability) since this assay is highly sensitive to toxicity. Based on this criterion, three complexes were inhibitory at non-cytotoxic concentrations. These were the gold(I) phosphine chloride complex TTC24 which only caused 10% toxicity (90% viability) at 12.5 µM with an associated inhibition of infectivity of 94% (CC_{50} =18.6±4 µM and a 50% inhibitory concentration or IC_{50} of 7±1.8 µM), two BPH gold(I) complexes EK207 with an 88% viability at 6.25 µM where viral inhibition was 84% (CC_{50} = 27±1.3 µM and IC_{50} = 3.6 ±1.1 µM) and EK231 which inhibited infectivity by 98 and 104% at 12.5 and 25 µM respectively with viability > 80% in both cases (CC_{50}>25 µM, IC_{50} = 6.8±0.8 µM). MTT viability data for the gold(III) thiosemicarbazonate complex, PFK7, suggested that the compound was toxic from 6.25 µM and higher (experimentally tested up to 25 µM). This presumed toxicity was observed as a dose dependent cytostatic effect noted especially for 0.1, 5 and 10 µM (discussed in section 4.3.2.2).

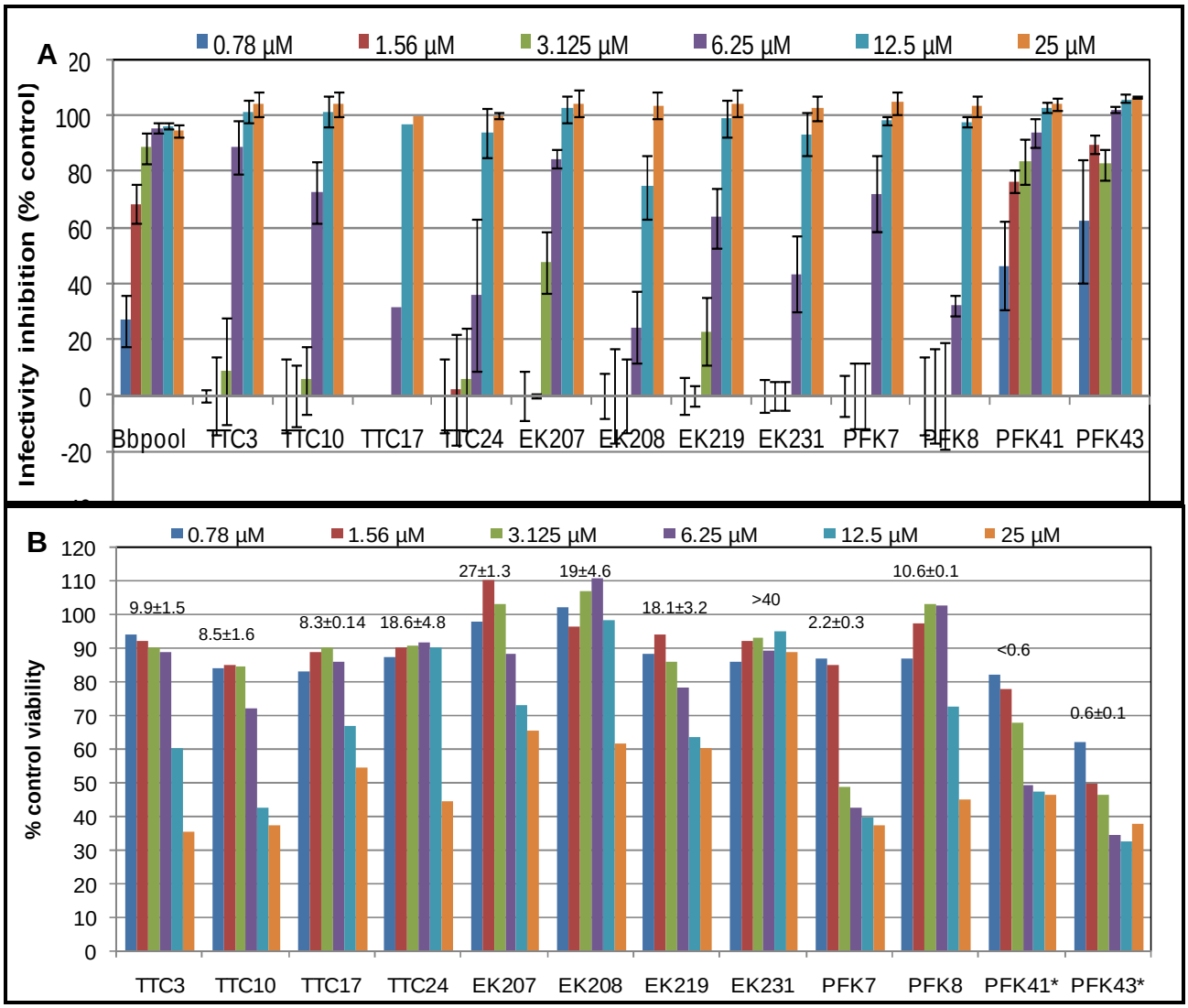

Figure 4.8: The effect of the compounds on the infectivity (A) and viability (B) of TZM-bl cells. A dual subtype C viral isolate (DU151.2) was pre-treated with the compounds and its ability to infect TZM-bl cells was monitored by determining luciferase gene expression after 48 h (A). Cell viability was monitored at the same concentrations (B). TTC24, EK207 and EK231 significantly inhibited viral replication while maintaining cell viability at >80%. Inhibition by PFK7 and PFK8 was seen at cytostatic concentrations. Bbpool was used as a positive control for inhibition of infectivity. The asterisk (*) indicates tested concentrations which were 0.04, 0.08, 0.16, 0.31, 0.625 and 1.25 µM for PFK41 and PFK43. The concentrations tested for the rest of the complexes are shown on the graph. Concentrations with negative inhibitory values were rounded up and plotted as 0% (A). In B, inserts representing the CC_{50}s of the complexes are shown.

With the RT-CES analysis data in mind, a closer look at Figure 4.8B revealed that for PFK7 (and PFK8 to a lesser extent) cytostasis played a role since viability did not significantly change for concentrations 6.25, 12.5 and 25 µM. Over this concentration range, viability was only moderately and insignificantly decreasing with percentages of 49.5, 47.5 and 46.4% respectively further confirming the cytostasis seen in Figure 4.6 for PFK7. Not only did PFK7 inhibit viral infection of the TZM-bl cell line by 72 and 98% at 6.25, and 12.5 µM respectively (Figure 4.8A) with an IC_{50} of 5.3±0.4 µM (Table A4.2), but it appeared to do so as a result of its cytostatic nature. The same principle could be applicable to PFK8 but this compound demonstrated a lower potency than PFK7. Viral infectivity inhibition by PFK8 was 98% at a non-toxic (67% viability) concentration of 12.5 µM with an IC_{50} 6.8±0.6 µM and this compound was cytostatic at 5 and 10 µM suggesting possible cytostasis at 12.5 µM. PFK7 was more

effective than PFK8 with the main difference between the two compounds being the presence of methyl groups in place of ethyl groups for latter (Table 3.4) which appear to confer unique properties to this compound. This difference led to a slightly higher lipophilicity value for PFK7 over PFK8 with the former having an AlogP prediction of 1.5 and the latter of 0.8 (Table 3.8A). The same trend was seen in the shake flask data with PFK7 having a log P value of 2.42±0.6 and PFK8 of 0.97±0.5 respectively (Section 3.4.3).

Compounds that inhibit viral infectivity through cytostasis have been extensively covered in the literature and shown to lead to better resistance profiles in clinical tests when combined with drugs that inhibit the virus directly such as didanosine (Lori *et al*., 1997, Frank, 1999, Rutschmann *et al*., 1998, Federici *et al*., 1998). The anti-viral activity of Tscs has been postulated (Easmon *et al*., 1992) and shown (Spector and Jones, 1985) to be through the lowering of nucleotide pools (needed by the virus) through inhibition of ribonucleotide reductase, an enzyme known to be inhibited by cytostatic agents such as HU (Lori *et al*., 2005, Clouser *et al*., 2010). PFK7 and PFK8 being Tscs-based compounds therefore show potential as anti-HIV agents through the observed cytostatic activity. Since cytostatic agents are known to inhibit RNR, as a confirmatory test, the effect of PFK7 and the complementary ligand were tested for this ability using the human ribonucleotide reductase M1, RRM1 ELISA kit from EIAab (USCNLIFE™, Wuhan, China). PBMCs were isolated from blood obtained from four HIV- donors and treated with the compounds for 3 days. The cells were then lysed by repeated freeze thawing and the RNR concentrations (from the lysate) determined from a standard curve after measuring absorbance at 450 nm. The results are shown in Figure 4.9. A dose dependent inhibition was observed for PFK7 with a significant p value of 0.003 at 10 µM but not for PFK5. This finding further supported the cytostatic ability of this complex. Inhibition of RNR by HU-like compounds such as PFK7 means this compound will be less susceptible to resistance since RNR is not prone to mutations like viral proteins are (Lori, 1999).

PFK41 and PFK43 (both gold(III) thiosemicarbazonate complexes as well) seemed to have cytostatic effects on the TZM-bl cell line at concentrations from 0.31 to 1.25 µM (Figure 4.8B). Unfortunately this was not confirmed with RT-CES as the initial assumption was that these two complexes were toxic. The IC_{50} and CC_{50} for all tested compounds in Figure 4.8 are shown in the appendix (Table A4.1).

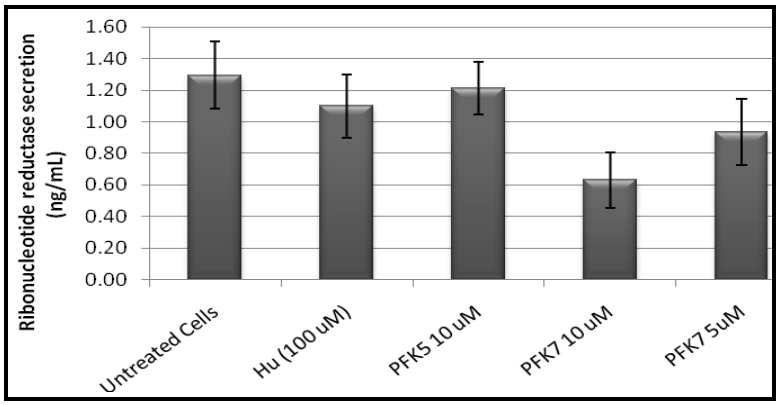

Figure 4.9: The effect of complex PFK7 on RNR production from PBMCs. PFK7 significantly (p=0.003) inhibited RNR production at 10 uM but not the complementary ligand, PFK5 at the same concentrations when compared to untreated cells. HU was used as a positive control but the concentration inhibited RNR tested only slightly.

To verify whether inhibition of infection by the compounds was through interaction with components on the surface of the virus (since the virus was pre-treated with the compounds), time of addition studies were performed for selected complexes which included; TTC24, EK231, PFK7 and PFK8. The TZM-bl cells were pre-treated with the compounds prior to addition of virus and the rest of the assay performed as previously described in section 3.2.4. The IC_{50} values obtained (5.6±0.9, 5.8±0.4, 6.2±0.5 and 6±1.3 µM respectively) were not significantly different from when virus was pre-treated with compounds (7±1.8, 6.8±0.8, 6.8±0.6 and 5.3±0.4 µM respectively). This finding suggests that the inhibition of infection was not a direct effect of the complexes on viral surface components but that it could have been by another mechanism probably at the entry or post entry steps (within the cells). Inhibition of infectivity resulting from a compound targeting multiple steps of infection has been seen in an infectivity assay for amphotericin B methyl ester (Waheed *et al*., 2006). The fact that PFK7 for example was cytostatic at inhibitory concentrations further supports the idea that the complex exhibited inhibition at the entry or post entry steps. The positive control for infectivity inhibition (Bbpool) always presented a dose related inhibition (Figure 4.8A).

4.3.4 Effects of Compounds on T Cell Frequency and on Inflammation

Using a six colour multi-parametric ICCS assay, we investigated the effect of the compounds on both phenotypic markers (cell surface markers specific for T cell subsets to determine frequencies) and cytokine production from the same cells to determine the compounds' effect on HIV inflammation. ICCS is superior over ELISAs because it allows for individual characterization of large numbers of cells and can fully display the heterogeneity of cell populations (Pala *et al*., 2000). Although ELISA assays were also performed, they were done primarily to determine any correlations with the ICCS assays since the latter measures intracellular cytokines while the former measures secreted cytokines. The ELISA assays were also performed as a precautionary measure in case there was a situation where ICCS was unable to detect cytokines because these proteins may have been released into the culture

supernatant before the addition of the protein transport inhibitor (BD GolgiStop™) to block cytokine secretion.

In Figure 4.10 (A-G), smooth dot plots displaying the hierarchical representative gating strategy that was used in determining ICC levels of IFN-γ and TNF-α within CD4+ and CD8+ cells is shown. The plots were obtained using FlowJo Software Version 7.6.1 (TreeStar Inc., Oregon, USA).

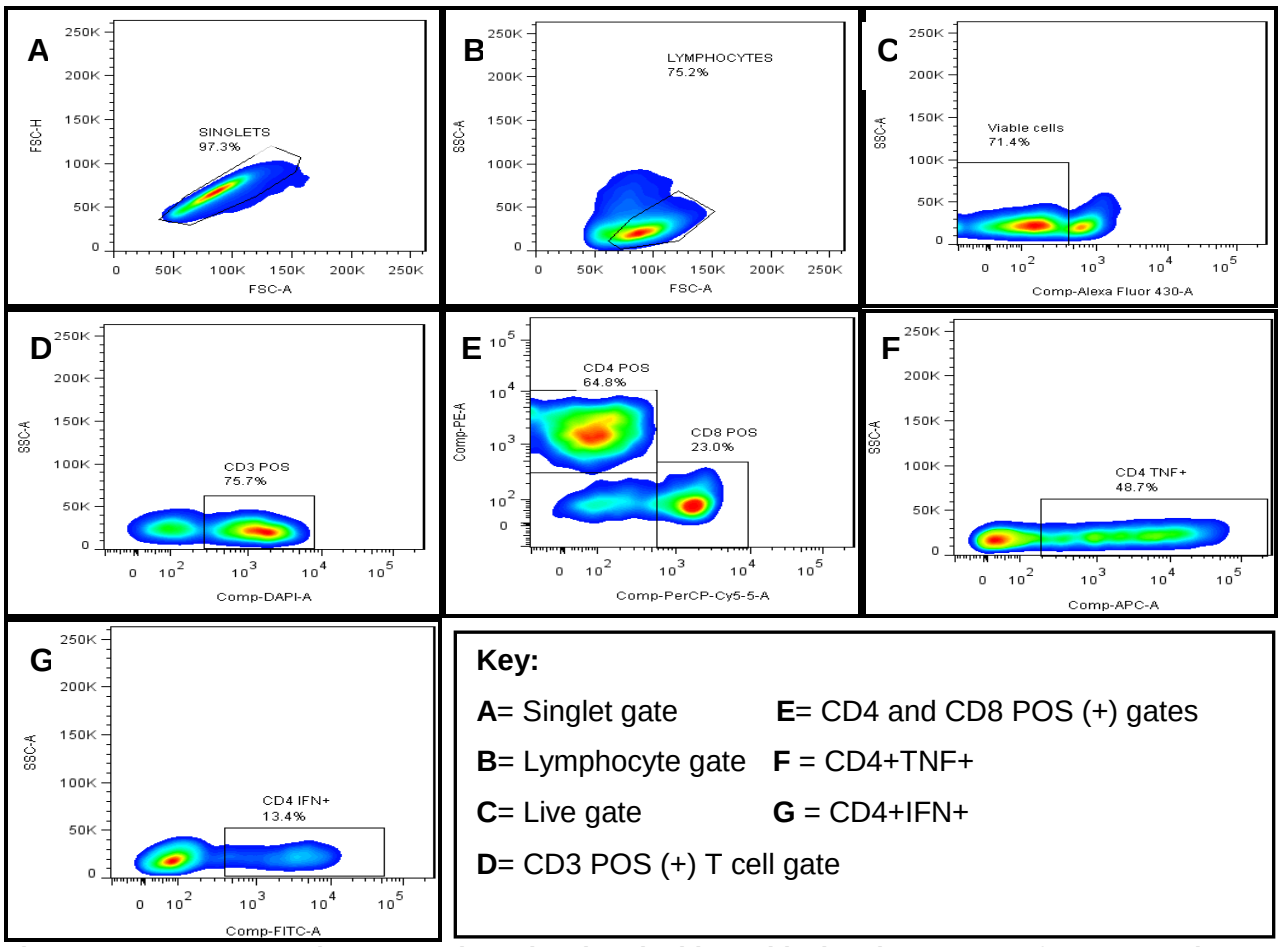

Figure 4.10: Representative FACS plots showing the hierarchical gating strategy for IFN-γ and TNF-α detection. A singlet gate (A), the lymphocyte gate (B), viable cell gate (C), T cell gate (D), T cell subset (CD4+, CD8+) gates (E), CD4+ TNF-α+ gate (F) and CD4+IFN-γ+ gate (G) are shown. Percentages of the frequencies of the cells in each gate are represented. The figures which represent smooth dot plots were obtained using FlowJo (TreeStar Inc., Oregon, USA).

In A, a singlet population consisting of single cells was obtained on an FSC height (H) and an FSC-area (A) plot followed by the gating of lymphocytes (B). This lymphocyte gate was identified using a CD45 marker so as to exclude neutrophils and monocytes. From the lymphocyte gate, a "live gate" was obtained by excluding cells positive for the aqua fluorescent fixable amine reactive dye (C) while in Figure 4.10D, T cells consisting of cells positive for the CD3+ marker were isolated. This was followed by the gating of the T subsets including CD4+ and CD8+ cells as seen in Figure 4.10E. Subsequently the percentage of TNF-α producing CD4+ cells (F) and IFN-γ producing CD4+ cells (G) were obtained. Cytokine producing CD8+ cells were also obtained in a similar manner as shown for CD4+ cells F and G. The gates were defined based on FMOs after fluorescent spillover subtraction.

The pooled data (after analysis using FlowJo) for all 12 HIV+ and 13 HIV- donors from whom samples were obtained was statistically analysed for significant differences using Graphpad Prism® (San Diego, California, USA). The effect of the compounds on the frequency of CD4+ expressing cells and on the frequency of IFN-γ and TNF-α producing CD4+ cells was analysed separately for HIV+ and HIV- donors (Figure 4.11). This was done using the Wilcoxon's matched pairs signed rank test with untreated cells as controls in each case (Figure 4.11). A similar analysis was done for the CD8+ T cell subset (Figure 4.12).

Effect of compounds on the frequency of IFN-γ and TNF-α from HIV+ and HIV- CD4+ cells: Two complexes, a BPH gold(I) complex, EK207 and the gold(III) thiosemicarbazonate complex (PFK7) significantly reduced the frequency of CD4+ cells from HIV+ donors with p-values of 0.0269 and 0.0049 respectively as seen in the box and whiskers plot (Figure 4.11A) but no such effect was observed for CD4+ cells from HIV- donors (D). A reduction of CD4+ cells has been shown to lead to AIDS in progressors since these are crucial immune system cells required by both the humoral and cell mediated arms of the immune system (Fan *et al*., 2000). On the other hand cytostatic agents such as HU (in addition to inhibiting RNR, Meyerhans *et al.,* 1994) also exert anti-HIV effect through the lowering of CD4+ count resulting in a decrease in immune activation (Frank, 1999, Rutschmann *et al*., 1998, Lori *et al.,* 1997). Trimidox and didox (Lori, 1999, Lori *et al*., 2005, Mayhew *et al*., 2005, Clouser *et al*., 2010) are also examples of cytostatic agents which like HU, at optimal non-toxic doses in combination with anti-viral agents such as ddI or indinavir (virostatics) have shown superior efficacy over regimens that did not incorporate them (Lori *et al*., 2005) in clinical trials (Lori *et al*., 1997, Frank, 1999, Rutschmann *et al*., 1998, Federici *et al*., 1998).

While lowering of CD4+ cells is not such a good quality for a drug that should potentially be administered to immunocompromised (HIV+) persons, significant benefits have been observed when cytostatic drugs (that lower CD4 cells) are combined to form virostatics. These are a decrease in the incidences of drug resistance (compared to current anti-HIV agents) and an overall increase in CD4 cell numbers (Lori *et al*., 2007). The mechanism of this action is reportedly through the inhibition of RNR thereby reducing dNTP pools required by the virus to make copies of itself (Meyerhans *et al*., 1994, Lori *et al*., 1994). When combined with a NRTI (ddI, a dNTP analogue) there is a relative increase in ddI with a resultant synergistic anti-viral effect since the natural substrate of DNA synthesis (dNTP) is lowered (Meyerhans *et al*., 1994, Lori *et al*., 1994). Accordingly, complexes EK207 and PFK7 which lowered CD4+ cell frequencies in HIV+ donors might be beneficial in virostatic combinations. In addition, this cytostatic effect (Figure 4.6, Figure 4.7 and Figure 4.9) and lowering of CD4+ cells from HIV+ donors (Figure 4.11) may play a role in the observed anti-HIV effect of these complexes (Figure 4.8). The link between inhibition of infectivity, the lowering of CD4+ cell frequencies and the cytostatic effect observed for PFK7 and it analogues forms part of a manuscript compiled from this study (Fonteh *et al*., 2011).

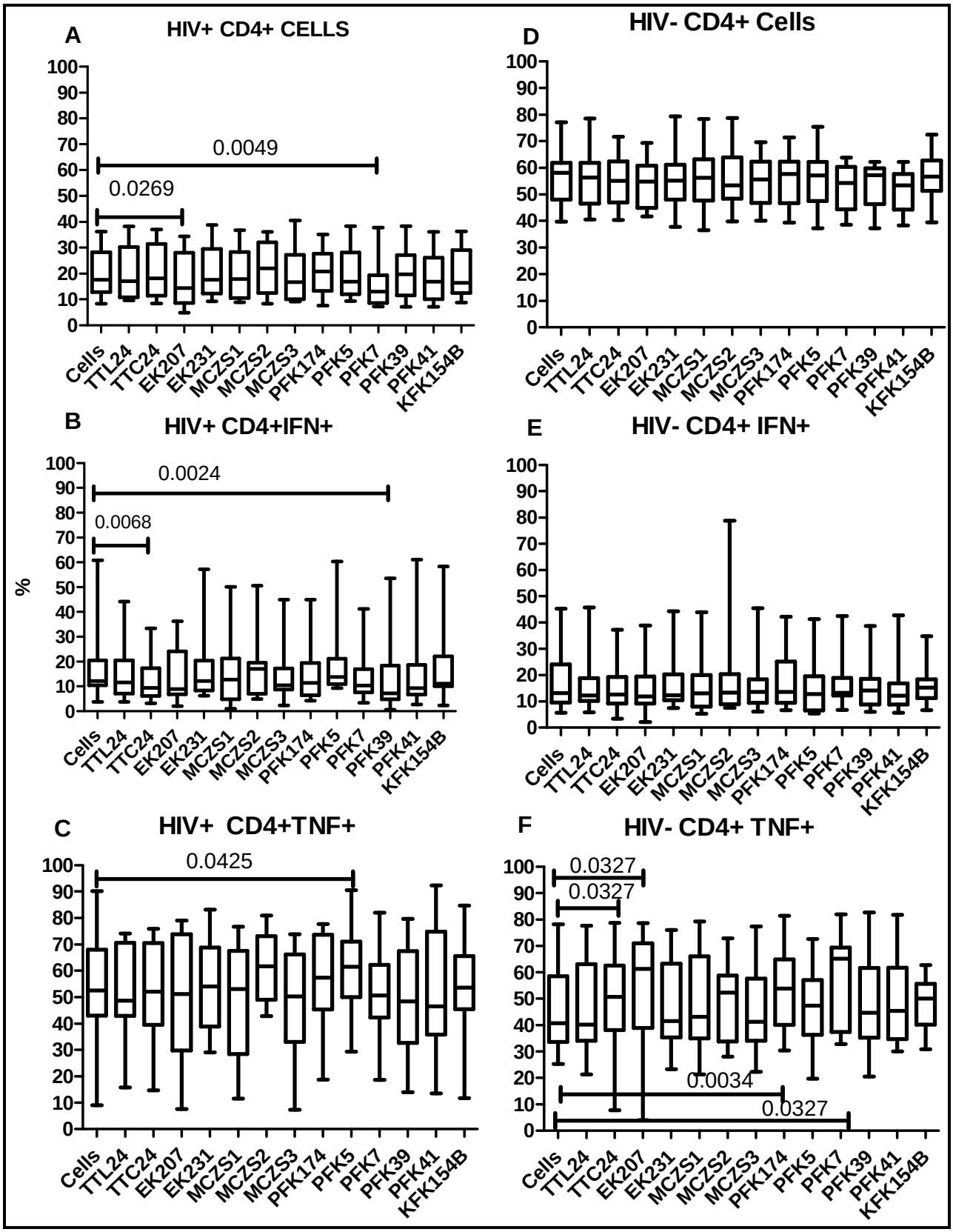

Figure 4.11: The effect of the compounds on CD4+ cells frequency and cytokine production. PBMCs from 12 HIV+ and 13 HIV- donors were treated with the compounds and the frequency of CD4+ cells as well as IFN-γ and TNF- α production from the HIV+ (A, B, C respectively) and HIV- (D, E, F respectively) donors determined. The Wilcoxon matched-pairs signed rank test was used in determining statistical significance while correlations were tested using the nonparametric Spearman correlation test. A p value of <0.05 was considered significant. Complexes EK207 and PFK7 significantly reduced the frequency of CD4+ cells from HIV+ individuals (A). TTC24 and PFK39 significantly reduce the frequency of IFN-γ producing cells while PFK5 caused an increase in the frequency of TNF- α producing cells from the HIV+ group. None of the compounds had an effect on the frequency of CD4+ cells from HIV- donors (D) or on the frequency of these cells producing IFN-γ. TTC24, EK207, PFK174 and PFK7 caused an increase in the number of CD4+ cells producing TNF- α from HIV- donors (G).

None of the compounds tested had a significant effect on frequency of CD4+ cells from HIV- individuals (Figure 4.11D) suggesting that the suppression of CD4+ cells by EK207 and PFK7 was specific for infected cells and would not occur in the absence of an infection.

The effect of the compounds on the frequency of IFN-γ and TNF-α producing cells from both HIV+ (Figure 4.11B and C respectively) and HIV- donors (Figure 4.11E and F respectively) was also investigated. TTC24 and PFK39 suppressed the production of IFN- γ from HIV+CD4+ cells (Figure 4.11B) but none of the compounds altered its production from the HIV- group (Figure 4.11E). IFN-γ is an anti-inflammatory cytokine which has been associated with a decrease in HIV disease progression and pathogenesis (Ghanekar *et al*., 2001, Francis *et al*., 1992) such that an increase in its production should be beneficial. Unfortunately, none of the compounds significantly increased the frequency of cells producing IFN-γ. Although IFN-γ was chosen as a representative anti-inflammatory cytokine in this study, this cytokine is known to have a bimodal role in HIV (both enhancement and suppression of HIV replication, Alfano and Poli, 2005) and is sometimes labelled as a pro-inflammatory cytokine because it can augment TNF-α activity and induces nitric oxide production (Dinarello, 2000). Therefore, if IFN-γ was labelled a pro-inflammatory cytokine, then the fact that TTC24 and PFK39 suppressed its production from HIV+CD4+ cells meant these compounds have potential anti-inflammatory abilities.

PFK5 which is the complementary ligand of PFK7 caused an increase in the frequency of TNF-α producing CD4+ cells (C), p=0.04. This effect was not seen for PFK7 and appeared to have been lost as a result of complexation. Four of the complexes namely: TTC24, EK207, PFK174 and PFK7 caused an increase in the frequency of HIV+CD4+ cells producing TNF-α (Figure 4.11F). Low circulating levels of the pro-inflammatory cytokine, TNF-α, has been correlated with lower viral load and slower disease progression in HIV (Than *et al*., 1997) while increases have been associated with HIV disease progression *in vivo* (Caso *et al*., 2001). The fact that TTC24, EK207, PFK174 and PFK7 caused an increase in TNF-α producing HIV-CD4+ cells is fortunately not critical since these compounds if potentially used as drugs will not be administered to uninfected people.

Effect of compounds on the frequency of IFN-γ and TNF-α from HIV+ and HIV- CD8+ cells: The effect of the compounds on the frequency of CD8+ cells, IFN-γ and TNF-α from PBMCs obtained from HIV+ and HIV- donors was also determined (Figure 4.12). EK207 also significantly (p= 0.002) reduced the frequency of CD8+ cells from PBMCs obtained from the HIV+ population (Figure 4.12A) which is not a good property considering that CTLs are required for killing activated CD4+ cells when the latter present antigen.

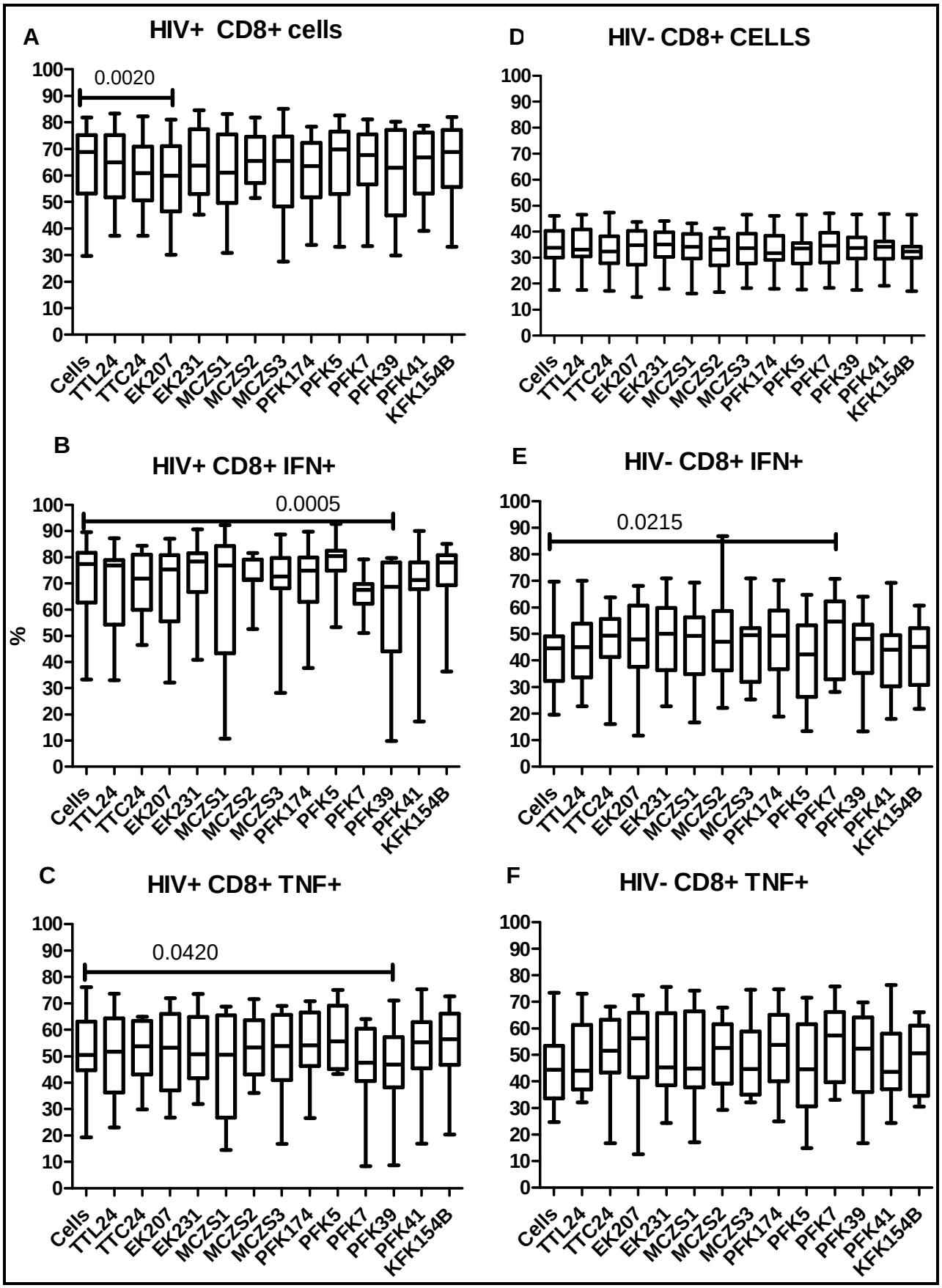

Figure 4.12: The effect of the compounds on CD8+ cell cytokine production. PBMCs from 12 HIV+ and 13 HIV- donors were treated with the compounds and the frequency of CD8+ cells as well as IFN-γ and TNF- α production from the HIV+ (A, B, C respectively) and HIV- (D, E, F respectively) donors determined. Statistical analysis was performed as for Figure 4.11. Complex EK207 significantly reduced the frequency of CD8+ cells from HIV+ individuals (A). PFK39 significantly suppressed the frequency of IFN-γ (B) and TNF- α (C) producing cells from HIV+ individuals. None of the compounds had an effect on the frequency of CD8+ cells from HIV- donors (D) or on the frequency of these cells producing TNF- α. PFK7 enhanced the production of IFN-γ from HIV-CD8+ cells with a p value of 0.022 (E).

PFK39 caused a decrease in the frequency of CD8+ cells producing both IFN-γ and TNF-α from HIV+ donors (Figure 4.12B and C respectively). A reduction in the frequency of CD8+ cells and CD8+TNF-α producing cells was not seen for the HIV- group (Figure 4.12D and F respectively). PFK7 stimulated the production of IFN-γ from the HIV- cells (Figure 4.12E), which is a good sign considering that IFN-γ is an anti-inflammatory cytokine. Compounds that suppress TNF-α production (such as PFK39, Figure 4.12C) will slow down disease progression while those that augment its production e.g. TTC24, EK207, PFK174 and PFK7 (Figure 4.11F) may be detrimental to immune function.

Interestingly though, the increase in the number of TNF-α producing cells caused by these compounds was not observed in the HIV+ group (Figure 4.11C) and appeared to have been removed for PFK7 upon complexation of PFK5 with gold. The use of anti-TNF-α drugs has had some success in limiting RA progression (Stern *et al*., 2005) and other inflammatory diseases and may play a role in lowering the inflammation caused by HIV. A summary of the effects of compounds that altered ICC production is shown in Table 4.3.

Table 4.3: A summary of the effect of the compounds on immune cell function. CD4+ and CD8+ cell frequency and that of the anti-inflammatory cytokine IFN-γ and the pro-inflammatory TNF-α frequency was assessed. Only compounds that altered the frequency of cells and cells producing cytokines are shown. ↑ represent increase while ↓ represents decreases. Complexes are representative from the different classes and there was the expectation that other members would respond the same.

Status	Cell type	Molecule	TTC24	EK207	PFK174	PFK5	PFK7	PFK39
HIV+	CD4+	CD4+		↓			↓	
		IFN-γ+	↓					↓
		TNF-α+				↑		
	CD8+	CD8+	↓					
		IFN-γ+						↓
		TNF-α+						↓
HIV-	CD4+	TNF-α+	↑	↑	↑		↑	
	CD8+	IFN-γ+					↑	

Some notable general observations were the fact that the median CD4+ count in the HIV+ group was lower (Figure 4.11A) than that of the HIV- group (Figure 4.11D) while median CD8+ cells in the HIV+ group was higher (Figure 4.12A) than for the HIV- group (Figure 4.12D). These are hallmarks of HIV infection (Forsman and Weiss, 2008, Musey *et al*., 1997, Koup *et al*., 1994) suggesting that these two groups were indeed different with regards to HIV status. The levels of the pro-inflammatory cytokine TNF-α and the anti-inflammatory cytokine IFN-γ from CD4+ cells were similar in both the HIV+ group (Figure 4.12B and C respectively) and the negative group (Figure 4.12E and F respectively). This was not the case in the CD8+ cell populations (Figure 4.12). Here IFN-γ production was generally higher in the HIV+ group (Figure 4.12B) than in the HIV- group (Figure 4.12E). The observed differences are probably because of the immune reactions mounted as a result of the infection in the HIV+ group. TNF-α levels from CD8+ cells from both groups were similar (Figure 4.12C and F). These general observations which support the fact that there are differences in systemic activation of immune

responses (Forsman and Weiss, 2008) provides further evidence that the donors in different groups were correctly assigned with respect to HIV status. This is because in HIV- individuals CD4+ cells numbers are higher than CD8+ cells and this ratio changes in HIV+ patients where CD4+ cells drop as CD8+ cells increase (Musey *et al*., 1997, Koup *et al*., 1994). Supporting data for the differences in the immunological state of the donors is shown in the appendix (Figure A4.13).

The ELISA data and detailed explanations are provided in Table A4.2 and subsection 8.3.6.2 of the appendix respectively. Although relatively fewer donors were tested, there was a pattern observed. In the absence of phenotypic identification in ELISAs, the compounds (TTC24, PFK174, PFK5 and PFK7) mostly demonstrated both anti-inflammatory and pro-inflammatory tendencies (except for PFK5 which lowered TNF-α production). In the ICCS assay, only two complexes altered cytokine production i.e. TTC24 and PFK5 (Table A4.3). TTC24 lowered IFN-γ production while PFK5 caused an increase in TNF-α suggesting poor anti-viral tendencies for both complexes with respect to the CD4+ subset that was analysed (Table A4.3). However, because phenotypic identification of the relevant subset of PBMCs (in this case T cells) was important in order that associations could be made with regards to cytokine production and cell phenotype, the ICCS data was considered more representative. The above mentioned conclusions were deduced from only six of the twelve HIV+ donors as a way of determining if cytokines had been secreted prior to the addition of the protein transport inhibitor and for checking if compound effects on cytokine production patterns were similar when ELISA and ICCS methods were compared.

4.4 CONCLUSION

Most of the gold complexes had CC_{50}s in the low micromolar range (between 1 and 20 µM) in both PBMCs and the PM1 cell line (Table 4.2). An overall summary of the effects of the compounds on the viability/proliferation of the different cell types when monitored using the different assays is provided in Table 4.3. The ligands were generally non-toxic with >20 µM CC_{50}s (Table 4.2 and 4.3) suggesting that complexation of gold to ligands resulted in increased toxicity except for ligands PFK41 and PFK43 which were more toxic that the corresponding complexes (PFK39 and PFK43). The MTS findings were confirmed for most of the compounds (except for complexes PFK189 and 190) when the annexin V and apoptosis kit was used (Figure 4.4, Table 4.3).

Table 4.4: Summary of the various effects caused by the compounds to the different cell types. In the MTS and MTT assays, compounds with CC_{50}< 1 µM are rated toxic (yes), those with CC_{50} between 1 µM and 10 µM inclusive were rated as moderately toxic while those with CC_{50} above 10 µM were rated as not toxic (no). In the annexin/PI assays, compounds either caused an apoptotic effect (% apoptotic cells> 10 % of control value), a necrotic effect (% necrotic cells > 10% control) or no effect (viability ≥ cells). In the CFSE studies, yes represents anti-proliferative and no means no effect while for the impedence measurements, no means no effect and yes means cytostatic. [#] same [ ]s like in the Ann/PI study. The ligands are coloured in grey. The asterisk (*) on TTC3 refers to the fact that cytostasis was only seen once for this complex at 10 µM. Superscript ([a]) represent cytotoxic compounds.

	PBMCs			PM1	TZM-bl	
Compound	**MTS (Toxicity)**	**Annexin/PI [ ] with >60% viability in MTS**	**CFSE Anti-proliferative[#]**	**MTS (Toxicity)**	**MTT (Toxicity)**	**RT-CES Cytostasis/ cytotoxic[a]**
Cells only	No	Viable (100%)	No	No	No	No
TTL3	No	Viable	No	No		No
TTC3	Moderate	Viable	No	ND	Moderate	No*
TTL10	No	Viable	No	No		
TTC10	Moderate	Viable	Yes	<3.1	Moderate	
TTL17	No	Viable	No	No		
TTC17	Moderate	Viable	yes	No		
TTL24	No	Viable	No	ND		
TTC24	Moderate	Viable	No	Moderate	No	
EK207	Moderate	Viable	Yes	Moderate	No	[a] over days
EK208	Moderate	Viable	Yes	Moderate	No	
EK219	Moderate	Viable	Yes	Moderate	No	
EK231	moderate	Viable	No	No	No	
MCZS1	No	Viable	No	No		
MCZS2	Moderate	Viable	No	Moderate		[a] within hours
MCZS3	No	Viable	No	No		
PFK174	No	Viable	No	No	No	
PFK189	No	Apoptotic Necrotic	No	Moderate	No	[a] over days
PFK190	No	Apoptotic Necrotic	Yes	Yes		
PFK5	No	Viable	Yes	No		No
PFK7	No	Viable	Yes	Moderate	Moderate	Yes
PFK6	No	Viable	No	ND		
PFK8	No	Viable	Yes	ND	No	Yes
PFK39	Yes	Viable		ND		
PFK41	Yes	Viable	Yes	ND	Yes	
PFK38	Yes	Viable		ND		
PFK43	Yes	Viable	Yes	No	Yes	
KFK154b	No	Viable	No	No		No

With regards to cell proliferation, ten compounds inhibited proliferation of PHA-P stimulated PBMCs by >50% with Tscs-based compounds>BPH gold(I) chloride complexes > phosphine chloride compounds> gold(I) phosphine thiolate>gold(III) pyrazolyl complex (Figure 4.6). Except for PFK5 which appeared to slow down cell proliferation, the rest of the ligands did not affect PBMC proliferation. Anti-proliferative compounds have the potential of lowering immune activation in asymptomatic patients and can reduce viral loads to undetectable levels and prevent progression to AIDS (Lori, 1999).

In the RT-CES analysis to determine the effect of the compounds on TZM-bl cell proliferation profiles, it was evident that two of the gold(III) Tscs-based complexes, PFK7 and PFK8 had cytostatic effects on this cell line (Figure 4.7B and C) and appeared to display cytostatic effects irrespective of cell type i.e. in PBMCs (Figure 4.6) and in the TZM-bl cell line (Figure 4.7B and C). Cytostasis is an important anti-HIV mechanism which when combined with an antiviral mechanism results in immune boosting capabilities and a reduction in drug resistance not seen for current HAART cocktails. The use of the impedence-based technology of a RT-CES analyser to observe the cytostatic mechanism of gold-based drugs was demonstrated here for the first time to the best of our knowledge.

The different complementary assays that were performed for determining cell viability and proliferation led to results which supported the importance of testing more than one marker in viability studies (Kepp *et al*., 2011). In the MTS and MTT assay, either toxic or non-toxic responses were obtained while in the CFSE and RT-CES assay, anti-proliferative and cytostatic conclusions were reported for some compounds initially thought to be cytotoxic when MTT and MTS assays were performed e.g. PFK7 (Figure 4.7B and 4.8A).

The gold(I) phosphine chloride complex TTC24 and two BPH gold(I) phosphine chloride complexes (EK207 and EK231) inhibited viral infectivity of the TZM-bl cell line at non-toxic concentrations (Figure 4.8A and B) while PFK7 and PFK8 (to a lesser extent) did so at cytostatic concentrations (Figure 4.7B and C and Figure 4.7A). Time of addition studies suggested that inhibition might have been as a result of the compounds inhibiting multiple targets either on the virus, on or within the cell since viral pre-treatment and cell pre-treatment resulted in similar IC_{50} values (Table A4.1). According to this finding, the differences in the exposure time appeared to have had nothing to do mechanistically. All compounds appeared to inhibit virus especially at high concentrations, this could obviously not be true and was confirmed through TZM-bl viability testing in concert with infection inhibition where cytotoxicity appeared to be the cause of the presumed infectivity inhibition responses that were observed (Figure 4.8). In the case of the cytostatic complexes (PFK7 and PFK8), where inhibition of infectivity was as a result of cytostasis (Figure 4.7B and C and Figure 4.9) the observed cytotoxicity in the MTT assay was not relevant. Although complexes TTC24, EK207 and EK231 inhibited viral infectivity at non-toxic concentrations (>80% viability), the fact that these complexes (especially EK207 and EK231) were predicted to have very poor drug-like properties (unlike PFK7 and PFK8) means to be used as drugs, structural modifications (e.g. the addition of NH groups) to increase aqueous solubility will be required. TTC24 on the other hand (with a drug score of 3/7) may stand a better chance of being developed to a drug without significant structural modifications.

Notable observations with regards to the effects of the compounds on immune cell function were the findings from ICCS assays that EK207 and PFK7 could decrease the frequency of CD4+ cells from HIV+ and not in HIV- donors (Figure 4.11). These findings

correlated with the anti-proliferative and cytostatic (PFK7 only) findings observed for these compounds (Figure 4.6 and 4.7B). The ICCS and ELISAs techniques used for determining cytokine production did not correlate (Table A4.3) since opposite trends were observed.

The most outstanding findings from experiments in this chapter are those observed for complex PFK7 and PFK8 to a lesser extent (Fonteh *et al.,* 2011). These thiosemicarbazonate complexes proved to be cytostatic when data from CFSE for PBMCs (Figure 4.6) and RT-CES for TZM-bl cells were compared (Figure 4.7B and C) and inhibited viral infectivity at these cytostatic concentrations (IC_{50}=5.3±0.4 and 6.8±0.6 µM respectively). In addition, PFK7 caused a decrease RNR levels (Figure 4.9) and in the frequency of CD4+ cells from 12 HIV+ donors (p=0.0049) and the two complexes demonstrated very favourable drug-like properties when the shake-flask and *in silico* ADMET methods were compared (Table 3.8A and Table 3.9). We suggest that these gold complexes could potentially be combined with viral inhibitory agents such as ddI to form part of the new and emerging class of anti-viral agents known as virostatics (Lori *et al*., 2005, Lori *et al*., 2007). Cytostatic drugs are not prone to the resistance issues associated with HAART and may become the combination of choice to curb the resistance associated with HAART. No evidence of cellular resistance has been revealed for HU, a cytostatic agent with more than 40 years of clinical usage (Donehower, 1992). In addition, HU has been shown to compensate for resistance that arises from the use of NRTIs when it is used in combination with ddI (Lori, 1999, Lori *et al*., 1997). The possibility that PFK7 and PFK8 could limit drug resistance is further supported by findings that the coordination of organic ligands with metals could lead to significant reduction in drug resistance (West *et al*., 1991) probably because of the stabilisation that the metal confers to the ligand. Other advantages that metal-based drugs have over organic ones are improved stability and the fact that they form covalent interactions (leading to longer lasting interactions with their active site). This may mean that PFK7 and PFK8 could be better cytostatic inhibitors than HU. A 10 µM concentration of HU inhibited cell proliferation and suppressed HIV-1 replication *in vitro* (Lori *et al*., in 2005) which is within the concentration range of viral infectivity inhibition and cytostasis that was seen for PFK7 and PFK8 (a concentration that is clinically relevant for gold compounds). The mechanism by which a cytostatic agent such as HU and potentially HU-like agents e.g. PFK7 function in a virostatic combination to curb viral replication is demonstrated in Figure 2.15.

To further investigate the potential of using these thiosemicarbazonate compounds (PFK7 and PFK8) in virostatic cocktails, the effect of PFK7 on RNR secretion was tested. PFK7 but not the complementary ligand significantly (p = 0.003) inhibited RNR secretion from PBMCs at 10 µM. However, *in vitro* combination studies with ddI must still be performed to determine if there would be a synergistic anti-viral or immunomodulatory effect.