CHAPTER 6
CONCLUDING DISCUSSION & FUTURE WORK

Three decades following the discovery of the link between HIV and AIDS, the best option for long-lasting viral suppression which eventually leads to a reduction in morbidity and mortality is the use of HAART (Simon *et al*., 2006). Unfortunately, latent reservoirs of the virus, which persist within the host's genome, re-emerge and start replicating once treatment is stopped (Finzi *et al*., 1997). So far, there is no viable cure for HIV/AIDS (the stem cell transplantation report of Hutter *et al*., in 2009 came close) and advances in vaccine development still require significant research effort to improve safety and efficacy.

HAART continues to play a vital role in sustaining the lives of people infected with HIV but unfortunately, the virus develops resistance to these drug cocktails (Simon *et al*., 2006, Svarovskaia *et al*., 2003). In addition, toxicity to the host is also a major problem together with uncomfortable side effects of the drugs (Yeni, 2006, Montessori *et al*., 2004, Montaner *et al*., 2003). These limitations greatly affect treatment options, which are further complicated by the fact that therapy has to be life-long. The need to increase the repertoire of drugs available for treatment therefore remains a priority. These new drugs should inhibit both wild type and resistant viral strains or should be capable of targeting different points of the life cycle or points of host cell interactions which had hitherto not been explored. Recent findings by the HIV Prevention Trials Network that early initiation of ARV therapy can curb transmission of HIV to partners of men and women infected by the virus by 96% (www.hptn.org, accessed 5/6/2011) is a finding that further supports the importance for identifying new drugs.

Twenty seven compounds (nineteen gold complexes and eight free ligands, synthesized by chemists from the Project AuTEK consortium) were screened for potential inhibition of HIV. *In silico* and *in vitro* drug-likeness (ADMET) studies of the compounds were performed, interactions with host cells and whole virus as well as the compounds' effects on viral enzymes were also evaluated.

Eight (Table 3.9) of the nineteen gold complexes demonstrated drug-like properties that were similar to those of auranofin (an anti-arthritic gold drug in clinical use) and in some cases better than that of the currently available anti-viral agent, nevirapine (Table 3.8B). The gold(III) thiosemicarbazonate complexes, PFK7 and PFK8, had very good drug-like qualities and presented as cytostatic complexes through RT-CES and flow cytometry evidence. According to bioassay studies, none of the compounds (some recently synthesised and others older) had any usable RT or PR inhibitory ability; a finding that was supported by *in silico* docking studies. No inhibition of IN was observed when both dual (3'P and ST) and ST specific assays were performed. However, *in silico* predictions studies, favourable size-shape

complementarity predictions were obtained for the binding of PFK7 to the LEDGF binding pocket of IN. The data so far suggests that PFK7 and PFK8 which had favourable drug-like properties (Table 3.8A), inhibited viral infectivity (Figure 4.8), demonstrated cytostatic effects (Figure 4.7B and C) and lowered the frequency of CD4+ cells in HIV+ donors (PFK7 only, Figure 4.10), are possible lead compounds. PFK7's cytostatic effect was supported by RNR inhibitory effects (Figure 4.9). TTC24 had a drug score of 3/7 and inhibited viral infectivity at non-toxic concentrations. This compound could be a potential lead compound after structural modification to improve drug-likeness. With regards to class, the Tscs class of complexes (class IV) was superior in drug-likeness and in the inhibition of HIV (infectivity and enzyme inhibition) followed by the gold(I) phosphine chloride containing class (class I) with TTC24 being the most favoured. Although the gold(I) phosphine thiolate class (class III, except the bimetallic complexes) and the gold(III) pyrazolyl complex of class V had very favourable ADMET properties, no significant inhibition of HIV was observed. The BPH gold(I) phosphine chloride class (II) of complexes were the least drug-like.

In the following sections, a summary of the major findings for each of the main topics will be provided followed by answers to the research questions that were posed as well as recommendations for future directions. A highlight of the novel contribution of the project and an overall conclusion section will then follow.

6.1 COMPOUNDS: STRUCTURE AND DRUG-LIKE PROPERTIES

Drug-likeness predictions were done using *in silico* computer simulations and by *in vitro* viability studies. The ^{1}H and ^{31}P NMR chemical shifts of six complexes (from each of the classes) on day zero, 24 h and 7 days after dissolution and storage at -20 and at 37 ºC in DMSO suggested that the backbone structure of all the complexes tested were intact (summarised in section 3.4.1.5, Table 3.7). The main difference was the presence of water peaks (in the ^{1}H NMR at 3.33 ppm, Gottlieb *et al*., 1997) in the day zero spectrum of four of the complexes (i.e. TTC3, MCZS3, PFK174 and PFK7, Table 3.7), suggesting hygroscopic tendencies. In three of these complexes (except MCZS3 which was only analysed on day zero), the water peak became more prominent after 24 h and at 7 days but was absent in the ^{1}H spectrum complex KFK154b over time. The increase in the water peak area and the new water peak in the spectrum of complex EK231 after 24 h and later were supposedly as a result of DMSO's hygroscopic nature.

Although compounds dissolved in DMSO can degrade when water is present (Ellson *et al*., 2005), the main problem usually encountered is the precipitation of compounds out of solution (Ellson *et al*., 2005) which could result in concentration discrepancies in bioassays. To minimise such problems in this study, DMSO stocks were aliquoted and together with the dissolved compounds, stored in single use vials. In addition, the compounds were stored desiccated at -20 ºC and samples were prepared fresh and used within a week. In this form,

compounds from classes I, II, III and V which were analysed for stability approximately 4 years after synthesis, maintained relevant chemical shifts (but not inhibitory activity, see RT studies in Table A5.1) with the only noticeable impurity being a water peak on day zero (Table 3.7). Gold complexes EK231 and KFK154b appeared very stable on day zero but subsequently (24 h and 7 days) both spectra had a water peak (EK231) and a D_2O peak (in KFK154b) as impurities. The ^{31}P chemical shifts of complexes TTC3 and EK231 remained intact which was in agreement with the idea that covalent interactions with S, P or C containing ligands lead to stabilising interactions with gold (Parish and Cottrill, 1987). While the backbone structure of all the complexes were represented, the presence of water in the ^{1}H spectra of complexes TTC3, MCZS3, PFK174 and PFK7 (on day zero) is suggestive of inherent hygroscopic abilities. This means that bioassay activity (as seen in the RT studies) could potentially be affected through compound precipitation in DMSO solutions with the end result being concentration differences. Alternatively, H-bond donors and acceptors present in the compounds with inherent hygroscopic abilities may form interactions with water molecules and hence not be available for interacting with enzyme active sites (even when freshly prepared). Although degradation products were not detectable in the NMR spectra, there is the possibility that this could have occurred. This is because NMR can be limited in sensitivity and there is the possibility of spectral overlap where chemical shifts of degradation products are masked by those of backbone compounds. This may explain why some of these compounds inhibited RT when freshly prepared and analysed after synthesis but not after three years even though NMR analysis presented presumably stable structures (chapter 3 in section 3.4.1).

In the ADMET prediction studies, eight of the nineteen gold complexes had drug-like properties which were comparable to known drugs. These predictions were confirmed for two complexes when the traditional shake flask method was used (section 3.4.3).

6.2 EFFECTS OF COMPOUNDS ON HOST CELLS AND WHOLE VIRUS

A variety of assays were performed to determine the interaction of the compounds with host cells ranging from viability, proliferation, infectivity and the immunomodulatory assays (specifically on T cell frequency and inflammation). The *in vitro* ADMET studies showed physiologically relevant CC_{50}s in the range of 1 and 20 µM for most of the complexes (except for complexes PFK41 and PFK43 whose CC_{50}s values were < 1 µM, Table 4.2). The ligands were less toxic than the gold complexes suggesting that complexation increased toxicity, a finding likely more significant in cancer studies where gold is considered for this property (Che *et al*., 2003, Marcon *et al*., 2002, Messori *et al*., 2000).

Ten complexes inhibited the proliferation of PBMCs by >50% in the CFSE proliferation assay with PFK7 being the most prominent (Figure 4.6). The data correlated with the RT-CES analysis where significant cytostasis was observed for PFK7 and PFK8 (Figure 4.7B and C). None of the compounds stimulated T cell proliferation suggesting that the compounds will not

be potentially antigenic which is the case for some clinically available gold complexes (a situation that is also linked to the side effects that gold complexes have, Lampa *et al*., 2002, Verwilghen *et al*., 1992).

Inhibition of viral infectivity was observed at non-toxic concentrations (>80% viability) of complexes TTC24, EK207 and EK231 (Figure 4.8) and cytostatic concentrations of PFK7 (seen by RT-CES analysis, Figure 4.8 and 4.7B respectively)**.** Unfortunately, the very poor drug-likeness predictions for complexes EK207 and EK231 (Table 3.8A) limits their potential as infectivity inhibitory agents. Time of addition studies suggested that inhibition of infectivity was either due to interactions of the compounds with entry or post entry steps as seen from similarities of the IC_{50} values (Table A4.1) implying that differences in exposure time did not affect mechanism of action.

In the immunomodulatory assays (summarised in Table 4.3), the most significant findings were the observed decreases in the frequency of CD4+ cells from 12 HIV+ treatment-naive patients caused by complexes EK207 and PFK7 ($p=0.03$ and 0.005 respectively). TNF-α production was elevated from the same cells by PFK5 and this effect appeared to be removed upon complexation since it was not observed in the complementary complex (PFK7). Cytokine detection by ELISAs from culture supernatant indicated that most of the compounds had stimulatory effects (causing increases in both anti-inflammatory and pro-inflammatory cytokines). However, because these were integrated cytokines from all PBMCs, the ICCS assay findings were considered over the latter because of phenotypic relatedness to T cell type.

None of the ligands demonstrated anti-viral activity supporting the importance of gold complexation in these potential drugs. PFK7 was noted as a lead compound which inhibited viral infectivity at cytostatic concentrations and lowered the frequency of CD4+ cells (and hence activation) without altering cytokine production. These finding suggests that this compound (which also had a good ADMET score, Table 3.9) could be incorporated into the emerging class of anti-HIV agents known as virostatics, a combination which has been found to lead to long term anti-viral efficacy (Lori *et al*., 2002). Other compounds with potential were PFK8 (an analogue of PFK7) and TTC24 which also inhibited viral infectivity at non-toxic concentrations and had ADMET scores of 6/7 and 3/7 (close to 50%) respectively (Table 3.8A). Although the ADMET score of TTC24 was slightly below average, it will be easier to enhance the drug-likeness of this compound compared to its analogues for which overall drug scores of 0 or 1/7 were noted (Table 3.9). The solubility of this complex could be improved by adding H-bond donors and acceptors such as OH and NH_2 groups, and by reducing the lipophilicity (Kerns and Di, 2008).

6.3 EFFECTS OF COMPOUNDS ON VIRAL ENZYMES

None of the eleven new compounds tested for inhibited RT in the direct enzyme assays (Table 5.2). Eight compounds which previously inhibited RT (Fonteh and Meyer, 2009, Fonteh *et al*., 2009) were re-tested as controls. It was found that these complexes had lost their RT inhibitory abilities (Table A5.1). This finding was attributed to a number of possible factors such as poor aqueous solubility which is known to affect the reproducibility of bioassay data (Di and Kerns, 2006), degradation, aged compounds and to the possibility that solvents used in the synthetic process (see section 8.4.1 of the appendix for more details) may have contributed to compound effect. The poor size shape complementarity observed for the compounds which interacted favourably with the RNase H site was also thought to be a contributing factor since any conformation change by the receptor to accommodate the ligand could have led to the latter being dislodged. These compounds also lack metal-chelating groups (e.g. carbonyl groups) and therefore could not interact with the crucial active site Mn^{2+} ions such that repulsive forces possibly prevailed.

Except for PFK7 which inhibited PR at a cytotoxic concentration, PR and IN inhibitory activities were absent. The gold(III) thiosemicarbazonate complexes (particularly PFK7) interacted favourably with the LEDGF-IN site but these findings must be confirmed using *in vitro* assays.

In silico predictions suggested that the binding of the ligands to RT was at the RNase site while for IN, the ligands interacted more with the LEDGF binding site. Although the enthalpic contributions for both sites were overall not very favourable (negative binding energies are considered favourable), the size-shape complementarity that was observed for PFK7 with this site may play a role in the infectivity inhibition that was observed for this compound but this must still be confirmed experimentally.

The representative gold starting material, $HAuCl_4H_2O$ that was tested in this study showed no outstanding inhibition. This was not surprising since it has generally been reported that it is the gold complex and not the ligand or gold starting material that is involved in the biological activity noted for gold complexes (Sun *et al*., 2004, Traber *et al*., 1999).

6.4 ANSWERS TO RESEARCH QUESTIONS

In this study, it was hypothesized that “gold-containing compounds could inhibit HIV replication directly through action on viral enzymes and indirectly through action on host cells (e.g. immune modulation) and could serve as drug leads for further analysis and development”. In order to verify this hypothesis, three main research questions were posed. In the following subsections, quick responses will be provided for these questions and other secondary questions that arose.

6.4.1 Were the Compounds Drug-like?

Eight of the nineteen complexes had drug-like properties which were similar to those of auranofin, a gold compound in clinical use for rheumatoid arthritis treatment. Some complexes appeared to have inherent hygroscopic abilities as seen from ^{1}H NMR spectra but overall the backbone structure of all the analysed compounds were intact for both ^{1}H and ^{31}P NMR spectra (for compounds dissolved in DMSO and analysed immediately and at 24 h and 7 days later following storage at -20 and 37 ºC respectively).

6.4.2 What Were the Effects of the Compounds on Host cells and Whole Virus?

Except for two of the complexes which had CC_{50}s below 1 µM, most of the complexes had CC_{50}s between 1 and 20 µM which were within the physiologically relevant concentration for gold compounds. At these same concentrations, ten of the complexes inhibited T cell proliferation (a mechanism by which gold compounds are thought to exhibit their anti-inflammatory effect (Matsubara and Ziff, 1987). Inhibition of viral infectivity at non-toxic concentrations was observed for complexes TTC24, EK207 and EK231. The gold(III) thiosemicarbazonate complexes, PFK7 and PFK8, inhibited viral infectivity at cytostatic concentrations and also lowered the frequency of HIV+CD4+ cells (shown for PFK7 only, p= 0.005) suggesting potential for incorporation into virostatic cocktails.

6.4.3 Were the Compounds Capable of Inhibiting Viral Enzymes and How?

None of the compounds inhibited RT while one inhibited PR but at a toxic concentration. Compounds with prior anti-RT activity were predicted to bind with the enzyme by interacting with the RNase H site. These interactions unfortunately resulted in poor size-shape fit and poor binding free energies.

None of the compounds inhibited INs' 3'P or ST activities but predictions for the interaction of the gold(III) thiosemicarbazonate comlplexes (particularly PFK7) with LEDGF hotspots on IN were observed.

6.4.4 Other Questions

Answers to some of the secondary questions are provided in the next subsections.

6.4.4.1 Did complexation enhance anti-viral activity?

The advantages of complexation (detailed in Chapter 2), were observed across the chapters. None of the free ligands tested in this study inhibited virus both in the infectivity inhibition assays and in the direct enzyme assays. In the CFSE assay, anti-proliferative effects were noticed more for the complexes than for the ligands e.g. PFK7 retained 137% cells in generation 0 while the complementary ligand retained only 79% (Figure 4.6). Cytostasis was observed for PFK7 and not for PFK5 (Figure 7B) which also lowered the frequency of CD4+

cell presence in HIV+ PBMCs. In the *in silico* docking studies, ligand binding energies were usually higher (suggesting poor binding affinity) than those of complementary complexes. Some examples are the binding free energies obtained for TTL10 and TTL24 (76.6 and 78.6 kcal/mol respectively) and TTC10 and TTC24 (10.9 and 12.3 kcal/mol respectively) in the RNase H studies (Table 5.4). In all, complexation enhanced biological activity and improved binding mode interactions of the gold complexes with host cells in cell-based assays and in *in silico* predictions. Since toxicity checks were implemented in all bioassays, the observed differences in activity were considered not toxicity related.

6.4.4.2 Was activity class and oxidation state related?

The five different classes of compounds (I-V, Table 3.6) that were assayed showed class dependent similarities possibly because of the interclass similarities in precursor structures. With regards to drug-likeness, the gold(I) thiolate complexes of class III, the gold(III) thiosemicarbazonate complexes and the gold(III) pyrazolyl complexes were the most drug-like with ADMET scores of 6/7 each. The gold(I) phosphine chloride complexes and corresponding ligands and the BPH gold(I) chloride complexes were the least drug-like and the most lipophilic. With regards to oxidation state, the gold(III) complexes were the most favoured in terms of drug-likeness with all four complexes in class IV and one in class V having drug scores of 6 out of 7 compared to the gold(I) complexes. Cytostasis was observed for the gold(III) thiosemicarzanate complexes and not for any of the other class representatives in the RT-CES studies.

6.4.4.3 What was the effect of complexation on drug-likeness?

With regards to *in silico* ADMET predictions, there were no differences in the ADMET scores of ligands and complementary complexes e.g. TTL24 and TTC24 had similar drug scores (Table 3.9). The same applied to PFK5 and PFK7 and the other ligand-complex pairs.

With regards to *in vitro* ADMET studies (particularly in the cytotoxicity studies), toxicity was observed to increase upon complexation. CC_{50} values for the complexes were generally lower than for the free ligands (Table 4.2). The stability that comes with complexation, although advantageous in improving binding affinity to active sites, is known to be detrimental in the sense that the drug accumulates more in the cells and ends up affecting cell viability. This is the more reason why potential drugs need to be fine tuned to obtain ideal lipophilicity levels and overall drug-like properties.

In the absence of the *in vitro* cytotoxicity studies, the differences in toxicity between ligands and complexes would otherwise not have been obvious from the *in silico* ADMET prediction studies further suggesting the need for complementing both approaches.

6.5 RECOMMENDATIONS

6.5.1 Bioassays should be Complemented with *In Silico* Molecular Modelling Studies

In this study, traditional drug design methods (based on literature and structure) followed by biological screenings were performed. *In silico* predictions studies (although introduced a little latter) helped in the optimisation and filtering of potential drug-like candidates. Their importance as complementary methods to the experimental assays was observed. *In silico* data provided additional explanations for the RT bioassay inconsistencies (the predicted binding site studies suggested poor binding affinity to the RNase H active site). In addition, the observed differences in cytotoxicity patterns between ligands and complexes would not have been noticed. It is therefore recommended that where possible, *in silico* ADMET predictions always be performed alongside high throughput experimental assays or used for eliminating compounds with very poor aqueous solubility properties prior to biological screening. This should increase hit rate and potential drug candidates can be prioritized (Hou and Xu, 2003, Pirard, 2004) which should hopefully lead to a reduction in late-stage drug failures (O'Brien and Groot, 2005). This approach supports the "fail fast" "fail cheap" concept that has been adopted over the past decade by pharmaceutical companies (Egan *et al*., 2000).

6.5.2 Incorporate Real Time Techniques in Drug Discovery Studies

The use of real time assays such as the RT-CES analysis (which is non invasive) in this study was very valuable in the identification of the mechanism of action of the thiosemicarbazonate complexes, PFK7 and PFK8. Although the CFSE studies (endpoint) suggested that PFK7 and other complexes had anti-proliferative effects on PBMCs, it was the RT-CES studies with the TZM-bl cell line, which provided convincing evidence on the cytostatic effect (for PFK7, Figure 4.7B). With the MTT study, also endpoint, the only deduction that could be made was that the compounds were toxic at concentrations which were also inhibiting infectivity. The absence of an additive in the RT-CES analysis eliminates the shortcomings usually associated with MTT making the former data more reliable.

6.5.3 The Need for Therapies to Inhibit Immune Activation

In HIV infected people, activated CD4+ cells presenting virus are primed for killing by CTLs leading to a decrease in CD4+ cells. Concerns regarding cytostatic compounds like HU and the HU-like compound, PFK7, which suppresses CD4+ cell frequency and limit HIV infectivity through a cytostatic mechanism have been raised since these cells are needed by immunocompromised individuals. Several clinical trials (Lori *et al*., 1997, Frank, 1999, Rutschmann *et al*., 1998, Federici *et al*., 1998) have however shown that the use of an optimal cytostatic dose of HU in combination with anti-viral agents such as ddI results in superior efficacy over clinical trial arms that did not incorporate it (Lori *et al*., 2005). The explanation for

this is that, when a compound suppresses the activity of CD4+ cells, it is likely that activated cells (antigen presenting) and bystander cells (which are mostly affected by HIV apoptosis, Veazey *et al*., 2000) are reduced in numbers. The result is that these cells are not primed for killing by HIV leading to an overall steady state that is beneficial to the host. Therapies aimed at targeting immune activation have thus been recommended as a remedy for the severe chronic immune activation noted throughout the course of infection (Forsman and Weiss, 2008).

6.5.4 Test the Prodrug in Bioassays

Although auranofin has been reported to demonstrate anti-HIV activity *in vivo* (Lewis *et al.,* 2011, Shapiro and Masci, 1996), it did not exhibit this property in the inhibitory assays that were performed in this study. Most drugs and particularly metal-based compounds such as gold drugs are known to be prodrugs (Tiekink, 2003, Shaw III, 1999, Parish and Cottrill, 1987), and it is therefore possible that the active component of auranofin in the *in vivo* findings was not the one administered and that instead, its metabolites were. In fact metabolites of gold compounds such as diacyanogold(I), $Au(CN)_{2,}$ have demonstrated anti-RT activity *in vitro* (Tepperman *et al.,* 1994). Some authors have therefore suggested that for *in vitro* studies, the drug metabolites should be tested (Parish and Cottrill, 1987). While this suggestion may be valid theoretically, in practice, it is not easy to comply with since the exact metabolic format of each drug may not be easy to determine without *in vivo* analysis which unfortunately have ethical limitations.

6.5.5 Management of DMSO Compound Stocks

Compounds for HTS are usually dissolved in DMSO and stored frozen. The hygroscopic nature of DMSO could however affect bioassay performance significantly because water in DMSO can accelerate degradation and in many cases, cause compound precipitation thereby affecting concentration (Ellson *et al*., 2005). Additionally, in the absence of water, compounds dissolved in DMSO could precipitate out of solution within three weeks (Waybright *et al*., 2009). These limitations can result in underestimated activity, variable data, inaccurate SAR, discrepancies in enzyme and cell-based assays and inaccurate *in vitro* ADMET data (Di and Kerns, 2006). To avoid this problem, DMSO and compounds dissolved in DMSO should be stored in single use aliquots. In addition, short term working stocks should be prepared and DMSO dissolved compounds should not be used for assays after > one week while undissolved compounds should be stored desiccated at -20 ºC. The significance of this is to limit or avoid the uptake of water (except in the case where the compounds themselves were hygroscopic e.g. MCZS3) before dissolution for use in assays (Janzen and Popa-Burke, 2009). Alternatively where possible, the compounds should be dissolved and used fresh since compounds prepared fresh maintain activity better (Kerns and Di, 2008).

6.5.6 Structural Modification of the Gold(I) Phosphine Chloride Complexes

Compounds with poor ADMET predictions require structural modification to enhance drug-likeness. The most important of the drug-like properties is lipophilicity since it influences the rest of the parameters such as tissue distribution, receptor binding, cellular uptake, metabolism and bioavailability (Ghose *et al*., 1998). Compounds in class I, II and the bimetallic complexes of class III (Table 3.6) are those requiring such modifications since the overall drug scores were below 4 (Table 3.9). These compounds had very high lipophilicity values and it would therefore be important to include groups that would result in the reduction of the log P or AlogP98 to the ideal range (0≥3, Di and Kerns, 2006). In other words the hydrophilicity or aqueous solubility of the compounds needs to be enhanced. Some suggestions for improving aqueous solubility include the addition of ionisable groups such as a basic amine and carboxylic acid moieties (which will be charged in pH buffers with a resultant increase in solubility), a reduction in log P, introduction of H-bond donors and acceptors, adding polar groups (e.g. ester group and carboxylic acid group) and reducing molecular weight (Kerns and Di., 2008). By improving aqueous solubility, compound toxicity can be reduced while the addition of metal chelating groups to TTC24 for example could enhance interactions with the RNase H site of RT both *in silico* and in direct enzyme assays.

6.5.7 Solvent Effect on Enzyme Activity should be considered During Synthesis

One of the reasons suggested for the absence of RT activity after 3 years for compounds previously shown to inhibit the enzyme when freshly prepared after synthesis was the possibility that solvents used during the synthetic process may have contributed to the inhibition. Solvents such as DMSO, methanol and ethanol have been reported to inhibit RT (Tan *et al*., 1991). Tan *et al*., (1991) showed that ethanol inhibited RT activity more than methanol and DMSO, with the latter being the least inhibitory when concentrations from 2-10% (v/v) were tested. In the study, 6 % (v/v) ethanol inhibited RT by up to 50%. For our assays, DMSO concentrations were always kept to the minimum (≤ 0.5 % in cell-based assays) and in the RT assays concentrations of 1.5% had no effect on RT activity (Fonteh and Meyer, 2008). In the synthetic processes, chemists used various solvents either in the complexation process or in the synthesis of gold starting material e.g. dichloromethane and ethanol (Kriel *et al*., 2007). Synthetic products sometimes resulted in different colours (e.g. TTC3 was cream white at one point and white at another and TTC24 was purple at some point and yellow at another) which the chemists indicated had no effect on analytical data but was mostly linked to the solvent used. While this is true (since backbone NMR chemical shifts were maintained), the fact that these compounds inhibited RT when freshly made up after synthesis and not subsequently may suggest solvent effect and not compound effect may have been at play. We postulate that the loss of activity over time may have resulted from the fact that

inhibitory solvents had evaporated and that the absence or minimal concentrations left had no inhibitory effect on RT.

6.6 NOVEL CONTRIBUTIONS

In this study, the anti-HIV activity of nineteen gold complexes and eight ligands were evaluated. Assays performed were focused towards determining the effect of the compounds on viral infectivity of host cells and on viral targets (RT, PR and IN). Inhibition of viral infectivity was observed for three of the compounds at non-toxic concentrations and for two compounds at cytostatic concentrations. No direct anti-viral activity was noted in direct enzymes assays but favourable predictions were observed for the RNase H site of RT and the LEDGF binding site of IN when computer aided simulations were performed.

Intensive literature review revealed that cytostasis was an anti-viral mechanism in which compounds inhibited viral replication by inhibiting the enzyme, ribonucleotide reductase, thereby reducing dNTP pools required by the virus for replication. Based on the knowledge that gold(III) compounds have anti-cancer activity through cytostatic or anti-proliferative effects (Casini *et al*., 2008, Che *et al*., 2003), we postulated and showed for the first time (to the best of our knowledge) that inhibition of HIV-infectivity by the novel group of gold(III) thiosemicarbazonate compounds was related to cytostasis. This cystostatic mechanism was determined in both an adherent cell line using impedence-based technology (RT-CES) and in primary cells using CFSE dye dilution technology. Further confirmation of the cytostatic effect for PFK7 was observed where the frequency of CD4+ cells from twelve treatment-naive HIV+ donors was significantly reduced ($p = 0.0049$) as well as in the inhibition of RNR ($p = 0.003$). These findings were in accordance with reports documented for HU which is also a cytostatic anti-HIV agent with both *in vitro* and *in vivo* activity (Lori *et al*., 1997, Frank, 1999, Rutschmann *et al*., 1998, Federici *et al.*, 1998). Cytostatic agents also prevent viral replication by reducing cell activation caused by HIV thereby lowering CD4+ cell frequency. This inhibition of immune activation by cytostatic agents such as HU is known to reduce viral replication and we postulate this to be the possible mechanism by which PFK7 (and PFK8) inhibited DU 151.2s' infectivity of the TZM-bl cell line. While this is not the ideal scenario for anti-HIV agents since an increase in CD4+ cell count is usually recommended (Lori *et al*., 2005), clinical trials have shown that the combination of cytostatic agents such as HU with drugs that directly target the virus such as ddI in virostatics combinations result in longer term efficacy (Lori *et al*. 2002). Some of the benefits are a decrease in immune activation and a reduction in immunodeficiency with an overall increase in CD4+ cell numbers. Additionally the incidence of drug resistance in such combinations is limited compared to current HAART combinations because cytostatic agents target a cellular protein and not a viral one. Since dNTP pools are reduced when cytostatic agents are administered, it means there is a relative

increase in the dNTP analogue (ddI) and an overall longer sustainable efficacy in such combinations (Lori *et al*. 2002).

These postulations were further supported by literature confirming that the anti-viral effects of thiosemicarbazones are as a result RNR inhibition (Easmon *et al*., 1992, Spector and Jones, 1985). Complexes PFK7 and PFK8 are both thiosemicarbazone-based complexes. The thiosemicarbazone ligands tested here did not inhibit viral infectivity but upon complexation with gold, an overall synergistic anti-viral effect was observed.

Other novel contributions were the findings that the modification of $Au(DPPE)_2Cl$ through the use of nitrogen heteroatoms to increase hydrophilicity in the ethane bridge was not sufficient to render the compounds drug-like as seen from the *in silico* lipophilicity predictions (AlogP98 was >5, Table 3.8A). This finding supports reports by Kriel *et al*., (2007) that the addition of the hydrazine bridge in synthesizing analogues of $Au(DPPE)_2Cl$ did not result in increased specificity for these compounds as anti-cancer agents.

6.7 FUTURE WORK

6.7.1 Structural Modification To Improve Solubility and Activity

The gold(I) phosphine chloride complex (TTC24) which had a drug-likeness score of 3 out of 7 and inhibited viral infectivity at non-toxic concentrations could be structurally modified to enhance its drug score by increasing aqueous solubility. This same complex and its analogues TTC3, TTC10 and TTC17 also interacted favourably with the RNase H site of RT but will require the addition of metal chelating moieties to increase the affinity of the compounds for this site which contains two Mn^{2+}.

The phosphine complexes, the BPH complexes and the bimetallic gold(I) phosphine thiolate ligands were not sufficiently soluble in biological media (precipitating), also seen in the aqueous solubility predictions. This means these compounds will not readily enter cells but may instead assemble at the cell membrane (high lipophilicity) and cause cell death due to irreversible damage to the membrane. Moderating the lipophilicity of these complexes which had AlogP98 predictions of >5 might improve the bioassay activity especially in cell culture. This could potentially solve the problem of inconsistencies in bioassay data (if aqueous solubility was the cause) that was obtained for these complexes especially in the RT assays.

Solubility also appeared to be influence by geographical location. Compounds with previous anti-RT activity were tested soon after synthesis at the University of Johannesburg, Auckland Park Campus in Johannesburg (1753 m above sea level). The re-tests were done at the University of Pretoria, Pretoria (1271 m above sea level). Differences in geographical location are known to affect air pressure and thus solubility. These differences might have been the cause of the discrepancies in the RT data. To manage this, CO_2/O_2 levels in the dissolved compounds could be altered to improve solubility.

6.7.3 Determine the Oxidation State of Gold Within Cells

The gold complexes that were tested here were either gold(I) or gold(III) complexes. Uptake studies have previously been shown for some of these compounds using ICP-AES (Fonteh and Meyer, 2009). It will be of interest to determine what the oxidation states of the complexes are after uptake using mössbaur spectroscopy. Gold(III) compounds are prone to reduction by thiols in biological media (Fricker, 1996). Gold(I) complexes are more stable than gold(III) complexes when soft ligands such as the phosphine gold and triethylphosphine thiol ligands are used for coordinating the gold nucleus. For gold(III), the use of hard donor ligands containing N and O enhances stability in the biological environment, which was the case in this study. By determining the oxidation states, information on the stability of the complexes and the active form (prodrug form) can be deduced.

6.7.4 RT-CES Analysis

The proliferation profiles of each compound especially the remaining thiosemicarbazonate complexes (PFK41 and PFK43), should be determined using RT-CES. These compounds showed considerable toxicity in the MTT assay and very high inhibitory effects on viral infectivity (Figure 4.8). It will be interesting to verify if these complexes perhaps had a cytostatic effect on these cells since the analogues, PFK7 and PFK8 did.

6.7.5 Combination Studies of PFK7 and PFK8 with dNTP Analogues.

The anti-viral activity of the cytostatic agent, HU, was potentiated when combined with dNTP analogues such as ddI and indinavir both *in vitro* (Lori *et al*., 2005) and *in vivo* (Lori *et al*., 1997, Frank, 1999, Rutschmann *et al*., 1998, Federici *et al.*, 1998). Complex PFK7 inhibited viral infectivity of TZM-bl cells at cytostatic concentrations (Figure 4.8 and 4.7 respectively), and like HU lowered the frequency of CD4+ cells from HIV+ donors (Figure 4.11). Combinations studies of PFK7 with ddI or indinavir should hopefully result in synergistic effects both in anti-viral activity and in improving immune responses.

6.7.6 Determine if Compounds with Anti-proliferative Effects can Prevent T Cell Activation

Not all the compounds which had anti-proliferative effects on PBMCs were tested in the immunomodulatory assays where compound effects on virus was assessed with regards to T cell frequency and cytokine production. These were complexes EK208, EK219, PFK190, PFK8 and PFK43. It would be interesting to know if these complexes (particularly PFK8 and PFK43 which are drug-like and structurally similar the gold(III) compound, $KAu^{III}Cl_4$, known to prevent T cell activation, De Wall *et al*., 2006) can affect T cell frequency and alter the chronic inflammatory effect caused by HIV. Immune activation by HIV leads to clonal expansion and proliferation of T cells. Compounds with anti-proliferative effects may be capable of preventing

this activation and as a result alter T cell frequency and possibly cytokine production. The incorporation of activation markers such as CD69 to monitor these effects will thus be useful.

6.7.7 Determine Viral Core Protein (p24) Secretion as Measure for Viral Infectivity

Viral core protein (p24) was not directly analysed in this study but the expectation was that inhibition of infectivity by the cytostatic complexes such as PFK7 should lead to a reduction of p24 antigen secretion. It would be important to verify this assumption by assessing the level of p24 antigen secretion from infected cells treated with the promising compounds.

6.7.8 Cell Cycle Analysis to Determine the Phase Affected by Cytostatic Compounds

Cells treated with the cytsotatic agent, HU, are arrested between the G_1 and S phases or enter and accumulate in the S phase (Maurer-Schultze *et al*., 1988). It will be useful to determine if the HU-like compounds (PFK7 and 8) inhibit cell proliferation by arresting growth in the same phase as HU does. This will further confirm whether the compounds block dNTP production thereby impairing DNA synthesis (Lori, 1999).

6.7.9 Preselect T Cells Prior to Treatment

The heterogeneous nature of PBMCs together with interperson differences means using these cells for viral quantification can result in data variation (Trkola *et al*., 1999). For the immunomodulatory studies, cells were tagged with Mabs and cell frequencies and cytokine production levels monitored. The heterogeneous nature of the cell population means compound action could be limited for the cells of interest due to interactions with cells from the different subpopulations prior to analysis. To alleviate this and increase specificity for the immunomodulatory assays, the cells should be pre-sorted using the sorting function on a flow cytometer such as the FACSAria (Becton Dickinson or BD BioSciences, California, USA) or using other cell separating tools such as magnetic beads prior to treatment.

6.7.10 Docking Considerations

Since metals form covalent bonds with ligands and gold complexes are known to undergo ligand exchange reactions, it is possible that a putative inhibitor in a docking study might bind to the protein covalently (Höltje *et al*., 2003). This could be possible especially for the gold(I) phosphine chloride complexes which have a chloride ion since this ion is a good leaving group (Allaudeen *et al*., 1985) that would potentially leave the gold atom ionised. Determining the binding affinity of the complexes in the ionised form might result in different outcomes from those that were obtained and might prove more promising for the gold complexes considering that gold could easily form covalent bonds with sulhydryl groups of cysteine residues in receptor active sites. Unfortunately *in silico* docking strategies for such

situations are still being developed and as mentioned in Chapter 5, most *in silico* packages have not yet incorporated metals into their atom base. This is because of the enormous diverse structure of coordination compounds available making it difficult for the development of reference values for such packages (Comba and Hambley, 1995, Hay, 1993).

The modification of the subtype B crystal structures through site directed mutagenesis to include amino acid mutations found in the subtype C strain prior to docking should be considered. While direct enzyme assays for RT inhibition for subtype C and B viral strains have been reported to result in similar susceptibilities for commonly used NRTIs and NNRTIs (Xu *et al*., 2010), such modifications might lead to different outcomes for the gold-based compounds both in direct enzyme and *in silico* studies.

In the docking studies, metal-based drugs which have previously inhibited viral enzymes should be used as controls.

6.8 CONCLUSION

While finding new medication for HIV remains a major concern for researchers and the pharmaceutical industry, identifying an ideal drug is never easy (Joshi, 2007). A list of criteria has to be met for a lead candidate to successfully navigate through the drug discovery time line phases (Figure 2.18) which can be up to 10 years or more. This has been clearly demonstrated in this study where in an attempt to answer some major research questions, more questions were raised.

A total of 27 compounds were analysed from several angles to determine toxicity, effect on cell proliferation and antiviral abilities. Three promising candidates were singled out; TTC24 which inhibited viral infectivity at non-toxic concentrations with a fairly reasonable drug score and PFK7 and PFK8 which inhibited viral infectivity at cytostatic concentrations and had drug scores similar to clinically available drugs. The latter two (both gold(III) Tscs-based compounds) can be incorporated into virostatic combinations but assays to show favourable responses in immune parameters (e.g. CD4+ cell increases) upon combination with direct viral inhibitory agents must first be done to determine usefulness in such cocktails.