6.1 Introduction

Acquired immune deficiency syndrome (AIDS) is a formidable pandemic that is still wreaking havoc worldwide. There are two different species of HIV: HIV-1 and HIV-2. HIV-1 (Human Immunodeficiency Virus Type-1) is the pathogenic retrovirus (lentivirus family) and causative agent of AIDS or AIDS-related complex (ARC). HIV-2 is endemic and is mostly found in West Africa [1-3]. Currently the World Health Organization estimates that some 33 million people are infected with HIV-1, constituting a global pandemic illness with significant social and economic impact [4]. HIV infects humans, and the progression of its infection leads to AIDS. HIV can destroy entire "families" of CD4 cells. CD4 cells are a type of white blood cell that fights infection. Another name for them is T-helper cells. CD4 cells are made in the spleen, lymph nodes, and thymus gland, which are part of the lymph or infection-fighting system. For HIV negative person a normal CD4 count is in the range 460 – 1600 /μL. CD4 cells move throughout body, helping to identify and destroy germs such as bacteria and viruses [5].
HIV is spread when an infected body fluid is introduced directly into the blood stream of a non infected individual. It also transmitted through unprotected sex, shared needles, mother to child, birth, breast feeding and blood transfusions. At the final stage of this disease, the immune system of an infected person becomes severely damaged; as a result, this person becomes susceptible to a number of opportunistic infections (typically rare in people with healthy immune systems) and eventually dies [6-8]. Some opportunistic infections (CD4 Count 200-500/μL) are pneumonia (usually caused by bacteria), tuberculosis in the lungs, oral or vaginal yeast infections, shingles (viral skin infection), oral hairy leukoplaikia and Kaposi’s sarcoma [9-11].

6.1.1 Structure of HIV-1

The HIV-1 genome consists of cone-shaped nucleocapsid (core) which contains the genomic RNA molecules. There are three viral enzymes namely reverse transcriptase (RT), protease (PR), and integrase (IN) encoded by the gag and gag-pol genes of HIV play an important role in the virus replication cycle. Among them, viral reverse transcriptase (RT) catalyzes the formation of proviral DNA from viral RNA, the key stage in viral replication [12]. HIV-1 is an enveloped virus with roughly spherical virions of variable size. The envelope is studded with ten trimeric envelope (Env) proteins and various host membrane proteins. Assembly of the virus is largely driven by the viral Gag protein. Gag is a multi-domain polyprotein with the three folded domains matrix (MA), capsid (CA) and nucleocapsid (NC) and the three shorter peptides SP1, SP2 and p6 (Figure. 6.1) [13-19].
6.1.2 HIV-1 replication

When HIV infects a cell, RT copies the viral single stranded RNA genome into a double-stranded proviral DNA (Figure 6.2). The viral DNA is then integrated into the host chromosomal DNA. This insertion (integration) is catalyzed by the HIV-encoded enzyme – integrase (IN) which then allows host cellular processes, such as transcription and translation. The proviral DNA is transcribed by the cellular RNA polymerase. The mRNAs are translated by the cellular polysomes. Viral proteins and genomic RNA are transported to the cellular membrane. Immature virions are released. Polypeptide precursors are processed by the viral PR to produce mature viral particles. Viral protease (PR) initiates the essential process of virion maturation. That means transcription of the viral genome and of the viral
genes followed by translation, packaging, fusion and maturation supply the molecular components for the release of the new infectious viral particles [20-23].

**Figure 6.2** Steps in the replicative cycle of HIV (i) adsorption and cell fusion, (ii) reverse transcription, (iii) proviral DNA integration, (iv) proviral DNA transcription to viral mRNA, (v) viral mRNA translation to viral proteins and (vi) maturation and budding.

HIV-1 RT performs an integral role in virus replication and thus is a main target of current antiretroviral treatment. RT is a multifunctional enzyme that contains both polymerase and RNaseH activities to convert the single-stranded viral RNA genome into a double-stranded DNA (dsRNA) product ready for integration [24].
6.1.3 The HIV-1 RT enzyme

The HIV-1 RT is an asymmetric heterodimer, comprising of p66 subunit (560 amino acids) and a p51 subunit (440 amino acids) [25]. Both subunits are encoded by the same sequence in the viral genome. The p66 subunit is the larger of the two subunits within HIV-1 RT. This subunit contains the finger, palm, thumb, and connection sub domains as well as the RNaseH sub domain (Figure 6.3). The palm domain contains the polymerase active site with its three aspartic acids (110, 185 and 186) and the YMDD characteristic motif [26]. The p51 subunit is the smaller of the two subunits in HIV-1 RT. The p66 subunit assumes a flexible and open structure, whereas the p51 subunit is rather compact, and seems to play a structural role, devoid of catalytic activity, with the three aspartic acids buried inside. The p51 subunit is a product of the same gene as the p66 subunit, however, the RNaseH domain is absent in the p51 subunit as a result of proteolytic cleavage [27].

Figure 6.3 Ribbon representation of the active domain of RT (p66 monomer) illustrates its hand-like structure, showing fingers (blue), palm (pink) and thumb (green). DNA is elongated in the active site (red) which is in palm region, NNRTI binding pocket (yellow) [Ref.27].
RT Inhibitors block reverse transcriptase's enzymatic function and prevent completion of synthesis of the double-stranded viral DNA, thus preventing HIV from multiplying. The RT can be inhibited by two classes of drug belonging either to the nucleoside (or nucleotide) reverse transcriptase inhibitors (NRTIs and NtRTIs) or to the non-nucleoside reverse transcriptase inhibitors (NNRTIs). HIV-1 NNRTIs have become key components in the combination regimens of anti-HIV therapy [28]. Three NNRTIs are licensed for use in antiretroviral therapy: nevirapine, delavirdine, and efavirenz. Among those, efavirenz was the first NNRTI to demonstrate clinical efficacy in patients with NNRTI-resistant HIV-1 infection, and was approved by the FDA in 2008. Other NNRTIs with an improved resistance profile are currently being developed.

NRTIs and NtRTIs compete with the natural deoxynucleoside triphosphate (dNTP) for DNA incorporation by HIV-1 RT. After incorporation, they act as chain terminators. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) bind to an allosteric site termed the NNRTI-binding pocket (NNRTI-BP) approximately 10 Å from the polymerase active site and disrupt RT polymerase function [29]. This binding site is located in the palm domain of the p66 subunit of the heterodimeric protein, between the β6–β10–β9 and β12–β13–β14 sheets, and at the basis of the β4–β7–β8 sheet, at a distance of approximately 10 Å from the catalytic site of the enzyme. This pocket is hydrophobic in nature and is lined by the aromatic (Y181, Y188, F227, W229, and Y232), hydrophobic (P59, L100, V106, V179, L234, and P236), and hydrophilic (K101, K103, S105, D132, and E224) amino acids of the p66 subunit, and two amino acids of the p51 subunit (I135 and E138) [30-33]. NNRTI binding to RT induces conformational changes in the enzyme that affect
key elements of the polymerase active site and also the association between the two protein subunits [34-35].

Huang et al. reported anti-HIV activity of Dicamphanoyl khellactone (DCK), a coumarin derivative [36]. Due to the potent anti-HIV activity, many DCK derivatives were synthesized and were shown to have improved anti-HIV activity [37-38]. Rao et al. reported 2-(2, 6-Dihalophenyl)-3-(pyrimidin-2-yl)-1,3-thiazolidin-4-ones as non-nucleoside HIV-1 RT inhibitors. Structure–activity relationship studies revealed that the nature of the substituents at the 2 and 3 positions of the thiazolidinone nucleus had a significant impact on the in vitro anti-HIV activity of this class of potent antiretroviral agents [39]. A series of 2-(2,6-dibromophenyl)-3-heteroaryl-1,3-thiazolidin-4-ones were designed, synthesized and evaluated as selective human immunodeficiency virus type-1 reverse transcriptase (HIV-1 RT) enzyme inhibitors by Ravindra et al. [40]. The results of the HIV-1 RT kit and in vitro cell based assay showed that compounds effectively inhibited HIV-1 replication at 20-320 nM concentrations with minimal cytotoxicity in MT-4 as well as in CEM cells. Ma et al. performed a comparative molecular field analysis (CoMFA) like 3D-QSAR and docking studies with the objective of understanding pharmacophore properties of styrylquinoline derivatives and to design inhibitors of HIV-1 integrase [41]. Yu’ning et al. discovered novel pyridazinylthioacetamides as potent HIV-1 NNRTIs using a structure-based bioisosterism approach [42].

The treatment for HIV faces many limitations like the production cost, metabolic instability and has several side-effects. Transition metal-based drugs are better alternatives for HIV treatment. Some of the attractive features of metal based therapeutics include synthetic simplicity, solubility control, redox capability, expansion of coordination number and topography matching of the complex to the protein's active site. Building asymmetry
into the complex, which may offer better discrimination between host and rogue cell, can readily be achieved through coordination of chiral ligands to the metal centre [43-44]. The variability of the d-block metals, coupled with the availability of designer organic ligands, augers well for the future development of clinical metallo-drugs for deployment against protease-associated fatal diseases [45]. Metal complexes that interfere with the viral glycoprotein and/or cell receptors are currently based on polyanionic ligands. Metal complexes have also been found to target other sensitive parts of the viral cycle like HIV RT, IN or PR [46].

The use of metal-organic complexes is a potentially fruitful approach for the development of novel enzyme inhibitors [47]. Copper is a bio-essential element and copper complexes have been extensively utilized in metal mediated DNA cleavage for the generation of activated oxygen species [48]. Copper can be potential for anti-HIV, as copper has inhibitor activity for HIV-protease. Copper can interact with donor atoms on a biological target via the formation of coordinate bonds rather than a combination of weaker intermolecular force such as H-bonding [49]. Metal complexes hold the attractive promise of forming stronger attachments with the target by combining the co-ordination ability of metals with the unique stereoelectronic properties of the ligand [50].

This chapter deals with the HIV-1 RT inhibitory activity of heterocyclic Schiff bases and their transition metal complexes by determining their percentage inhibition of HIV-1 RT activity in HIV-1 RT kit.
6.2 Experimental

6.2.1 Methods

In vitro HIV-RT kit assay

The HIV-RT inhibition assay was performed by using an RT assay kit (Roche), and the procedure for assaying RT inhibition was performed as described in the kit protocol [51]. Briefly, the reaction mixture consists of template/primer complex, 20-deoxy-nucleotide-50-triphosphates (dNTPs) and RT enzyme in the lysis buffer with or without inhibitors. After 1 h incubation at 37°C the reaction mixture was transferred to streptavidine-coated microtitre plate (MTP). The biotin labeled dNTPs that are incorporated in the template due to activity of RT were bound to streptavidine. The unbound dNTPs were washed using buffer and antidigoxigenin- peroxidase (DIG-POD) was added in MTP. The DIG-labeled dNTPs incorporated in the template was bound to anti-DIG-POD antibody. The unbound anti-DIG-POD was washed and the peroxide substrate (ABST) was added to the MTP. A colored reaction product was produced during the cleavage of the substrate catalyzed by a peroxide enzyme.

The absorbance of the sample was determined at optical density (OD) 405 nM using ELISA reader. The resulting color intensity is directly proportional to the actual RT activity. The percentage inhibitory activity of RT inhibitors was calculated by comparing to a sample that does not contain an inhibitor. Pictorial representation of test principle was shown in Figure 6.4.

\[
\% \text{ Inhibition} = 100 \cdot \frac{\text{OD 405 nm with inhibitor}}{\text{OD 405 nm without inhibitor}} \times 100
\]
6.3 Results and discussion

The reverse transcriptase inhibitory activity of the synthesized Schiff bases and its Ni(II), Cu(II), Zn(II) complexes were studied by reverse transcriptase colorimetric assay kit (Roche). Nevirapine was used as reference drug. It has been observed from in vitro screening (Table 6.1-6.2) that, newly synthesized ligands and its Zn(II) complexes did not show any anti HIV activity. [Ni(TA)_2(H_2O)_2], [Ni(TNA)_2(H_2O)_2]-2H_2O, [Ni(PA)_2]-H_2O and
[Ni(PNA)₂]·2H₂O showed mild activity. But other two nickel complexes, [Ni(TMA)₂]·3H₂O and [Ni(PMA)₂] are inactive against reverse transcriptase. RT inhibitor activity of the tested compounds revealed that the complex formation had great positive effect on the bioactivity.

Table 6.1 Anti HIV results of Ni(II) complexes

<table>
<thead>
<tr>
<th>Compound</th>
<th>% inhibition at 10 µg/ml</th>
<th>% inhibition at 50 µg/ml</th>
<th>% inhibition at 100 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Ni(TA)₂(H₂O)₂]</td>
<td>11.4</td>
<td>20.6</td>
<td>31.2</td>
</tr>
<tr>
<td>[Ni(TNA)₂(H₂O)₂]·2H₂O</td>
<td>13.5</td>
<td>18.3</td>
<td>29.3</td>
</tr>
<tr>
<td>[Ni(TMA)₂]·3H₂O</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[Ni(PA)₂]·H₂O</td>
<td>2.8</td>
<td>7.9</td>
<td>15.9</td>
</tr>
<tr>
<td>[Ni(PNA)₂]·2H₂O</td>
<td>22.6</td>
<td>30.3</td>
<td>35.9</td>
</tr>
<tr>
<td>[Ni(PMA)₂]</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 6.2 Anti HIV results of Zn(II) complexes

<table>
<thead>
<tr>
<th>Compound</th>
<th>% inhibition at 10 µg/mL</th>
<th>% inhibition at 50 µg/mL</th>
<th>% inhibition at 100 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Zn(TA)₂]</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[Zn(TNA)₂]</td>
<td>2.0</td>
<td>0</td>
<td>7.8</td>
</tr>
<tr>
<td>[Zn(TMA)₂]</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[Zn(PA)₂]</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[Zn(PNA)₂]</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[Zn(PMA)₂]</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Cu(II) complexes possess potent RT inhibitory activity (Table 6.3). IC₅₀ was calculated by drawing a graph with concentration against % inhibition (Figure 6.5 and 6.6). Among the synthesized copper(II) complexes, [Cu(TA)₂H₂O]·H₂O showed higher RT inhibitory activity with IC₅₀ = 0.080 µM followed by [Cu(TNA)₂]·2H₂O (IC₅₀ = 0.087 µM), [Cu(PA)₂H₂O]·2H₂O (IC₅₀ = 0.092 µM) and [Cu(PNA)₂]·3H₂O (IC₅₀ = 0.11 µM). The HIV-1 RT inhibitory activity showed that all of the tested copper complexes showed...
significant potency but none of them showed higher activity than nevirapine. The square planar geometry of Cu(II) complexes allows hydrogen bonding in the active site of RT thereby increasing their inhibitory activity. Another factor responsible for activity of Cu(II) complexes against reverse transcriptase is their hydrophilicity. Anti-HIV activity was also affected by different heteroaryl moiety with or without an appropriate lipophilic substitution. Results showed that the new copper complexes effectively inhibited HIV-1 replication. Among them heterocyclic Schiff base complex with thiophene moiety showed higher anti-HIV activity than pyrole moiety. Result also indicates that the presence of electron donating group i.e., methyl group decreases anti-HIV activity. Compared to Schiff bases, metal complexes (mainly copper complexes) were found to exhibit anti HIV activity (Figure 6.7). This is probably due to their greater lipophilic nature.

Table 6.3 Anti HIV results of Cu(II) complexes

<table>
<thead>
<tr>
<th>Compound</th>
<th>% inhibition at 10 µg/mL</th>
<th>% inhibition at 50 µg/mL</th>
<th>% inhibition at 100 µg/mL</th>
<th>IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Cu(TA)2H2O]·H2O</td>
<td>1.9</td>
<td>63.3</td>
<td>85.1</td>
<td>0.080</td>
</tr>
<tr>
<td>[Cu(TNA)2]·2H2O</td>
<td>14.4</td>
<td>48.8</td>
<td>85.6</td>
<td>0.087</td>
</tr>
<tr>
<td>[Cu(TMA)2]·H2O</td>
<td>0</td>
<td>0</td>
<td>8.2</td>
<td>-</td>
</tr>
<tr>
<td>[Cu(PA)2H2O]·2H2O</td>
<td>2.1</td>
<td>46.5</td>
<td>70.2</td>
<td>0.092</td>
</tr>
<tr>
<td>[Cu(PNA)2]·3H2O</td>
<td>10.8</td>
<td>46.5</td>
<td>67.5</td>
<td>0.112</td>
</tr>
<tr>
<td>[Cu(PMA)2]·H2O</td>
<td>6.3</td>
<td>35.2</td>
<td>48.4</td>
<td>-</td>
</tr>
<tr>
<td>Nevirapine</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>
Figure 6.5 Anti HIV activity of [Cu(TA)$_2$H$_2$O]·H$_2$O and Cu(TNA)$_2$·2H$_2$O

Figure 6.6 Anti HIV activity of [Cu(PA)$_2$H$_2$O]·2H$_2$O and [Cu(PNA)$_2$]·3H$_2$O
6.4 Conclusion

HIV-1 RT kit assay was used for testing the RT inhibition of Schiff base ligands and their Ni(II), Cu(II) and Zn(II) complexes. Reference drug used was Nevirapine. Results showed that the new copper complexes effectively inhibited HIV-1 replication. Among them heterocyclic Schiff base complex with thiophene moiety showed higher anti-HIV activity than pyrole moiety. Synthesized ligands and their complexes with nickel or zinc have not shown any RT inhibition activity.
References


HIV-RT inhibitory activity studies of Schiff bases and their Ni(II), Cu(II) and Zn(II) Complexes


HIV-RT inhibitory activity studies of Schiff bases and their Ni(II), Cu(II) and Zn(II) Complexes


