CHAPTER 5
SUMMARY AND CONCLUSION

Soon after the discovery of HIV-1 as causative agent of AIDS, Zidovudine was the first drug made available for the treatment of AIDS patients (Hirsch & Kaplan, 1987a; Hirsch & Kaplan, 1987b). Understanding the requirement and necessity of targeting specific stages in HIV life cycle several molecules that target various HIV-1 enzymatic proteins like Reverse Transcriptase, integrase, and protease were developed in the last twenty-five years (MaltezDoroana et al., 2011a). Later, focus was shifted to target HIV-1 at very early stage of lifecycle i.e., during the process of receptor binding and entry, which led to development of Maraviroc, Enfuvirtide (T20) and several Mabs (GranichCrowley et al., 2010).

Entry inhibitors have been also evaluated for development of effective microbicide. Failure of surfactants and polyanionic inhibitors as microbicide candidates in clinical trials induced the development of Reverse Transcriptase inhibitors as microbicide candidates. This approach seems to be successful as use of Tenofovir as a microbicide formulation in healthy women significantly reduced the risk of infection. Currently available HAART therapy utilizes a combination of antiretroviral drugs that can inhibit reverse transcription, protease activity and fusion. A large number of approaches have been designed by researchers all around the globe to inhibit HIV-1 replication. Small molecule inhibitors, antisense RNAs, siRNAs, shRNAs, peptides and proteins have been identified as anti-HIV-1 agents.

Many natural products and natural product derived compounds are under development as anti-HIV-1 agents and are also being evaluated as HIV-1 microbicide candidates (Kelly & Shattock, 2011a; Olsen Easterhoff et al., 2011). Buchapine, a known natural quinoline alkaloid has been reported to hamper HIV-1 Reverse Transcriptase activity. Our group has earlier reported anti-HIV-1 activity of Buchapine and its analogues.
7 dimericphloroglucinol derivatives were assessed for anti-HIV-1 activity in TZM-bl cells infected with HIV-1NL4-3. M3 and M4 had shown moderate anti-HIV-1 activity while M1, M5, M6 and M7 derivatives inhibited HIV-1 infection by more than 90%. M1 having cyclohexanecarbaldehyde substitution and M7 having pyridine-2-yl substitution were most potent with IC50 values 2.41 µM and 1.49 µM. M1 and M7 exhibited better safety index compared to other analogues.

In order to further explore spectrum of activity of M1 and M7, we used X4 tropic, R5 tropic and X4R5 dual tropic viruses. M1 and M7 exhibited inhibition of replication of all these viruses in hPBMCs. M7 and M1 showed more or less similar anti-HIV activity against all the virus strains used independent of their coreceptor use.

In order to see the effects of M1 and M7 on replication of clinical isolates, we used subtype C clinical isolates VB51 and VB52. Subtype C HIV isolates are more prevalent in Asia, Africa (Lakhashe Thakar et al., 2008; Lihana Ssemwanga et al., 2012). Both the compounds exhibited significant inhibition of replication of these isolates in hPBMCs culture.

We have also evaluated 11 buchapine (quinoline 2,4-diol) analogues for anti-HIV-1 activity. The prenyl substituted quinolinediol analog (B1) was found to be more potent than Buchapine even in TZM-bl assay. Compound B4 showed higher safety index. Anti-HIV-1 activity of compound B1 can be attributed to its 4-carbon side chain and prenyl group at C3 position. Compound B4 which is mono O alkyl derivative has shown potent anti-HIV activity with better safety index. In an effort to define and prioritize a lead microbicide candidate for further development, Buchapine and its two analogues were further evaluated in a series of in vitro antiviral evaluation using TZM-bl and hPBMC cell system.

All three compounds showed significant efficacy against laboratory and clinical virus isolates but compound B1 seems to be better as it exhibits significant activity in the range of 5-8 µM. We have further characterized these two novel analogues of
Buchapine for identification of their mechanism of action and evaluation of their use as potential microbicide candidates.

The two analogues of buchapine have side chain modifications. We wanted to see if this modification has altered the mechanism of inhibition as Buchapine is known to be a RT inhibitor. In an in vitro Reverse Transcriptase assay, compound B4 showed an EC50 value four fold higher than Buchapine while for B1, the EC50 value was found to be 200 µM. This result indicates that though B1 exhibits anti-HIV-1 activity at much lower concentration in cell based assay, it does not inhibit reverse transcription at that concentration in in vitro enzymatic assay.

We then performed time of addition experiment in TZM-bl cells. Our data indicates that all three compounds are active at early phase of life cycle of HIV-1, however, B7 exhibited better activity than B4 and Buchapine when added during infection or immediately after infection. Most of the compounds which act at early stages of infection generally interact with viral or cellular proteins that facilitate entry of the virus into the cell (Hertje Zhou et al., 2010; Tilton & Doms 2010). However, when compounds were incubated with cells prior to addition of virus to see effect on CD4 binding, no change in infectivity of the virus was observed.

This data indicated that compounds have no effect on CD4 receptor binding and thus may target viral proteins. Thus these results indicate that B7 probably inhibits HIV-1 infection by interacting with viral surface proteins. We then used these compounds in a cell-cell fusion assay, where 70% reduction in virus-cell fusion by B7 was observed at similar concentration that was used for anti-HIV assay. Buchapine and B4 did not affect gp120-CD4 binding. Thus our results suggest that B7 inhibits gp120-CD4 interaction probably by interacting with gp120. Side chain modification plays an important role in the activity of compounds. Several Betulinic acid derivatives and trifluoromethyl pyridinone (PF-46396) were reported to possess diverse mechanism of action.

Some derivatives of Betulinic acid inhibit entry while some derivatives act during budding and release of virus from infected cells (Aiiken & Chen 2005a; Blair, Cao et
This kind of behavior of B7 may be attributed to side chain modifications.

In time of addition experiment, (cyclohexylmethylene) bis-diacylphloroglucinol (M1) exhibits anti-HIV activity in TZM-bl tissue culture only if pre-incubated with virus and added after infection of target cells. We have shown that M1 interferes with HIV-1 integrase activities in vitro.

HIV infection in vivo can be caused by cell-free and cell-associated viruses. Both forms of HIV are carried by semen. Studies have shown that semen helps in enhancing attachment of virions to target cells. The surface of the cervicovaginal mucosa provides a large portal of entry for HIV.

It has been demonstrated that virus can pass through thingaps between squamous epithelial cells (Hladik and Hope, 2009) and also by endocytosis and this penetration exposes the initial target cells such as intraepithelial Langerhans cells (LCs) and CD4+ T lymphocytes to the virus (Permanyer-Ballana et al., 2010; Pritschet, Donhauser et al., 2012).

Microbicides are a class of compounds/formulations that can inhibit transmission of HIV and thereby prevent infection. We explored the preventive capacity of Buchapine and its analogues in vitro conditions to mimic those under which the virus can readily infect the target cells. It has been shown that an epithelial synapse is formed when HIV-1 infected PBMCs come in contact with uninfected epithelial cells. The cell membranes of both cells undergo modification and signal transduction occur from the infected cell to the epithelial cell. The signal transduction facilitates efficient HIV-1 endocytosis and transcytosis (Bobardt-Chatterji et al., 2007; Hocini & Bomse, 1999). B1 has shown better activity than Buchapine by inhibiting transmission of virus from infected hPBMCs to ME180 cells.

We also confirmed the microbicidal activity of Buchapine and its analogues against cell-free virus in a dual chamber trans-well system. In an in vitro dual chamber model for evaluating microbicidal activity, M7, the dimeric phloroglucinol derivative exhibited moderate microbicidal activity. From our data, it
is clear that M7 moderately affected transmission of HIV-1 and infection of hPBMCs.

NNRTIs like UC-781 and several diaryl triazine (DATA) and diaryl pyrimidine (DAPY) compounds block transmission of HIV to epithelial cells from T-cells infected with virus and prevent infection of T-cells. Polyanaionic inhibitors block transmission and infection of cell free virus more efficiently. This difference in the activity of both types of inhibitor is due to their solubility. The polyanionic inhibitors are hydrophilic and the above mentioned NNRTIs are lipophilic in nature. The lipophilic nature of NNRTIs allows them to easily penetrate the membrane of epithelial cells or hPBMCs or T-cells (Herrera, Cranage et al., 2009; Pirrone Wigdahl et al., 2011; Roth Monsour et al., 2007).

This supports the activity of B7 in both cellfree and cell associated virus transmission assay. Nonoxynol 9 was originally considered a potent microbicide candidate; however, its toxicity to mucosal tissue caused the inflammation of vaginal epithelium and also facilitated virus transmission. Hence before going for ex vivo evaluation of any microbicide candidate in animal models, it is suggested to assess its effect on cytokine secretion by vaginal epithelial cells (Poynten, Millwood et al., 2009). We analyzed a panel of pro-inflammatory and inflammatory cytokines from culturesupernatant of ME 180 cells.

We did not see stimulatory effect on secretion of cytokines rather in presence of B7, significant reduction in levels of pro-inflammatory cytokines like TNFα and RANTES were observed. Neither M7 nor tenofovir were found to activate cytokine secretion by ME180 cells.

Our results indicated that B7 and M7 do not have toxic effect on epithelial cells.

In conclusion, we evaluated broadspectrum anti-HIV activity of Buchapine and its analogues and dimeric phloroglucinol derivatives. The spectrum of activity is independent of cell type and virus tropism. Further we identified B7 as dual inhibitor of HIV entry and Reverse Transcriptase. B7 efficiently prevented transmission and infection of epithelial cells by cell associated and cell free virus. We have also identified M1 as HIV-1 Integrase Inhibitor.