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## A Review on Biologically Active N-Myristoylated HIV-1 Nef Protein

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### ABSTRACT

Human immuno-deficiency virus (HIV) disease continues to be a serious health issue for parts of the world. Worldwide, there were about 2.5 million new cases of HIV reported in 2011. The HIV continues to be a major global public health issue with approximately 34 million people living with HIV in 2011. However, the development of anti-viral has blunted the AIDS epidemic in the western world but globally the epidemic has not been curtailed. Negative Regulatory Factor (Nef) is one of the accessory genes found only within HIV and SIV genomes and thought to play a key role in the progression to AIDS. Nef is considered as a potential anti viral target for preventing or at least delaying HIV pathogenesis. It suggesting that, the Nef protein that was detected not identically synthesis through post translation modification though it was expressed in the cytoplasm of HEK 293 cells. The ability of not expressing the targeted myristoylated 27 kDa Nef protein was to various unpreventable factors due to time limitation and lacking of skills in the filed cloning and cell culture.

**Key words:** HIV-1, Nef protein, pathogenesis, diagnosis, HEK 293 cell

### INTRODUCTION

About 34.2 million people of the world are living with HIV. In 2010, there were about 1.8 million deaths due to Acquired Immuno Deficiency Syndrome (AIDS) and nearly 30 million people have died worldwide since the AIDS epidemic began (CDC., 2011). The major public health threat throughout the world is HIV/AIDS even though it was first recognized 28 years ago (Cohen *et al.*, 2008). HIV continues to be a major global public health issue having claimed more than 25 million lives over the past three decades there were approximately 34 million people living with HIV in 2011 (Fig. 1). Sub-Saharan Africa is the most affected region with nearly 1 in every 20 adults lives with HIV. South Africa had an estimated number of 5,600,000 in the year 2011 compared to 4400,000 in 2001 and 5,200,000 in 2006. It shows a gradual increase in new number of HIV infection cases.

According to the United Nations programme on HIV/AIDS (UNAIDS., 2012) approximately 50% know their HIV status, 2.5 million new HIV infections, number of 1.7 million AIDS-related deaths. However latest data show that a 50% reduction in the rate of new HIV infections (HIV incidence) has been achieved in 25 low and middle income countries between 2001 and 2011. In the last 10 years the landscape of national HIV epidemics has changed dramatically for the better

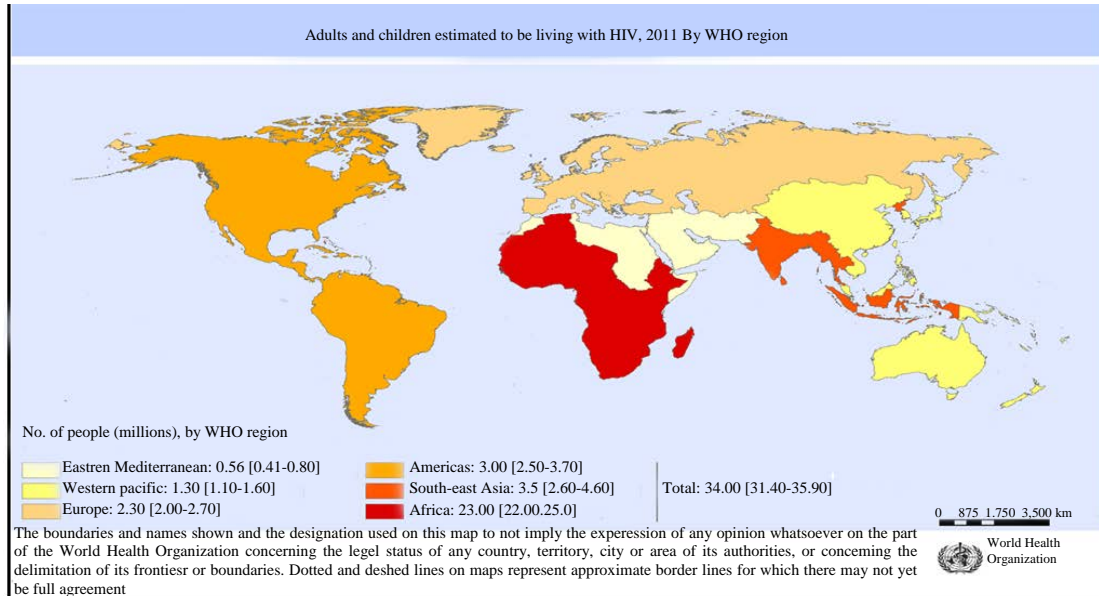


Fig. 1: Adult and children estimated to be living with HIV, 2011 (Adapted WHO., 2013) (WHO/UNAIDS/UNICEF)

in most countries, especially in sub-Saharan Africa. Countries are making historic gains towards ending the AIDS epidemic, 700,000 fewer new HIV infections across the world in 2011 showed that more than half of these countries are in sub Saharan Africa where the majority of the new HIV infections occur. In a further 9 countries the rate of new HIV infections fell steeply, by at least one third between 2001 and 2011 (UNAIDS., 2012).

This global HIV/AIDS infections also on the rise in Malaysia whereas of December, 2010, an estimated 80,922 people are currently living with HIV. By the end of 2010, Malaysia had a cumulative figure of 91,362 of HIV cases, 16,352 AIDS cases and 14,298 deaths, thus giving reported people living with HIV of 77,064. The annual number of reported new HIV cases by the Ministry of Health has been on a steady decline from a peak of 6,978 in 2002. In 2010, there were 3,652 new cases reported to the Ministry of Health, approximately halve of what was reported in 2002.

The notification rate of HIV also continues to experience a decrease from 27 in 2003 to 23.4 in 2005 and to 12.8 cases per 100,000 population in 2010. Currently, there are 10 new reported cases of HIV each day with a ratio of 2 females for every 8 males reported. In 2010, an average of 5 persons acquired HIV through injecting drug while 5 others were infected sexually. Table 1 shows an overview of the Malaysian HIV epidemic as of December, 2010 (MoH., 2011).

### **PATHOGENESIS HIV INFECTION AND Nef**

HIV infection is usually diagnosed through blood tests detecting the presence or absence of HIV antibodies. Since the initial description of the HIV-1 in 1983 (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1983) and HIV-2 in 1989 (Laraque, 1989), these two viruses have been identified as the primary cause of AIDS (WHO., 2013). HVI-1 Nef was reported to have negative effect on viral replication hence the name 'negative factor' or Nef (Terwilliger *et al.*, 1989; Ahmad and Venkatesan, 1988; Cheng-Mayer *et al.*, 1989). Although HIV Nef was originally named "Negative factor", it has been shown to have positive role in viral than in 2001. It was

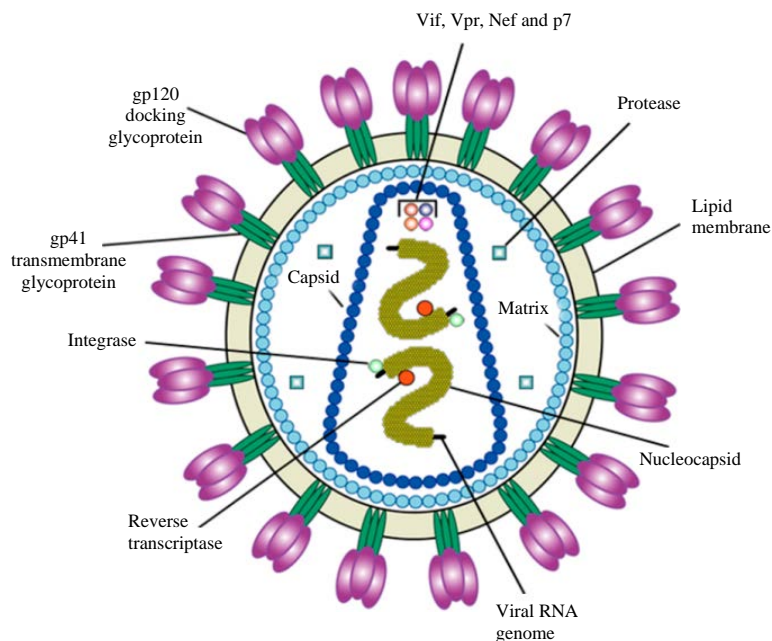


Fig. 2: Diagram of the HIV virus (Adapted from, US National Institute of Health) Source: redrawn by en: User: Carl Henderson

Table 1: Overview of the Malaysian HIV epidemic as of December, 2010 (Adapted from MoH., 2011)

Cumulative number of reported HIV infections since 1986	91,362
Cumulative number of reported AIDS related deaths since 1986	14,298
Estimated number of people living with HIV (EPP and Spectrum model 2010)	80,922
Reported number of people living with HIV	77,064
Cumulative number of women reported with HIV	8,759
Cumulative number of children under 13 with HIV	909
New HIV infections detected in 2010	3,652
AIDS related deaths in 2010	904
No. of PLHIV currently on ART in 2010	13,981
Estimated PLHIV needing ART (CD4<350) (EPP and Spectrum model, 2010)	30,600
Estimated adult (aged 15-49) years HIV prevalence (EPP and Spectrum model, 2010)	0.42%
Notification rate of HIV (per 1000,000)	12.6

Sources: Ministry of health, 2011

replication and pathogenesis. Most of the pathogenicity of HIV-1 can be attributed to the presence of six accessory genes coded within the viral genome, Nef is an accessory gene that is only present within HIV and SIV genome and thought to play a key role in the progression to AIDS (Trono, 1995). Figure 2 shows diagram of HIV virus.

Nef is 27 kDa N-terminally myristoylated accessory protein involved in post integration infection. Nef is found in the viral particles is one of protein that is expressed in abundance during the early phase of HIV infection together with Tat and Rev and Nef also induces high virus load titers both in cell culture and *in vivo* (Das and Jameel, 2005). Its mRNA is estimated to represent three quarters of the early viral load of the cell (Guy *et al.*, 1990; Klotman *et al.*, 1991). Using the Hela-CD4-LTR-b-galactosidase indicator cell line, Nef+HIV-1 was found to productively infect 5-20 fold more cells than equal amounts of Nef-defective HIV-1 (Kimpton and Emerman, 1992).

Nef showed in Fig. 3 has been indicated to induce down regulation of CD4 and HLA class I molecules (Collins *et al.*, 1998) from the surface of HIV-1 infected cells, which may represent an

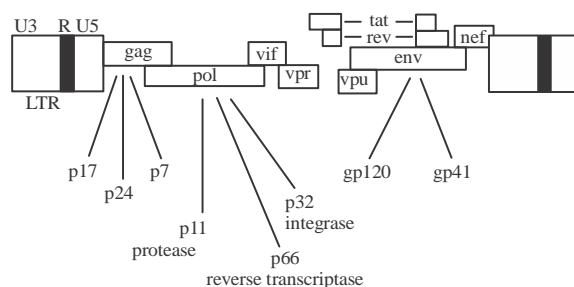


Fig. 3: HIV and its genes (Adapted from Klotman *et al.*, 1991)

important escape mechanism for the virus to evade an attack mediated by cytotoxic CD8 T cells and to avoid recognition by CD4 T cells Nef may also interfere with T cell activation by binding to various proteins that are involved in intracellular signal transduction pathways (Kwong and Wilson, 2009). A study demonstrated that Nef as accessory protein has the ability efficiently down regulate chemokine receptor CXCR4 from the surface of HIV infected primary CD4 T cells and highlight this function of Nef is important to maximize interference with super infection by additional virion already at the entry step (Freel *et al.*, 2006).

Another study suggests that the extracellular Nef protein could contribute to the decline of CD4 counts prior to and during the onset of AIDS in patients with human immunodeficiency virus type 1 infections. They showed Nef protein, in the absence of antibody was found to be apoptotic to these and other cell types through the CXCR4 receptor (James *et al.*, 2004).

Generally, few *in vivo* studies also show significant importance of Nef protein in the progression of AIDS. One of the study indicates that, intact of Nef gene was a critical for high viral loads and development of an Acquired Immunodeficiency Syndrome (AIDS) like illness in animals infected with SIV which demonstrated by the studies in Rhesus monkeys (Klotman *et al.*, 1991). In SIV-infected rhesus macaques also, an intact Nef gene was essential for a high rate of virus production and the progression of disease. HIV-1 with deletions in Nef was identified in a cohort of Australian long-term non-progressors (Kirchhoff *et al.*, 1995). However, more recent reports indicate that some of these patients are now developing signs of disease progression including a decline of CD4 T cells. Thus, although deletions of the Nef gene may slow viral replication, they cannot always prevent the eventual development of AIDS.

### Nef PROTEIN/MAMMALIAN SYSTEM

Nef is 27 kDa myristoylated HIV-1 protein and Myristoylation is an irreversible, co-translational protein modification. In this protein modification, a myristoyl group (derived from myristic acid) is covalently attached via an amide bond to the alpha-amino group of an N-terminal amino acid of a nascent polypeptide. The modification is catalyzed by the enzyme N-Myristoyl Transferase (NMT). Myristoylation of the 27 kDa Nef was demonstrated by the contranlational modification of Nef in an *in vitro* reticulocyte translation system. Myristoylation also occurs post-translationally (Kaminchik *et al.*, 1994).

*Escherichia coli* expressed Nef protein was found to bind during *in vitro* reactions with the cytoskeletal matrix to an extent of 30-50%. Cytoskeletal association of Nef was significantly enhanced by myristoylation (Niederman *et al.*, 1993). It is therefore desirable to express Nef protein in mammalian system to produce high quality protein. Important features of the mammalian cells include their ability to perform post-translational modification and to secrete glycoprotein that is correctly folded. However, the amount of stability of long term expression from these

stable cell lines may be affected by characteristics of the transfected DNA itself, mRNA structure and also chromosomal locus which it is integrated.

### **HEK 293 CELL LINE**

HEK 293 cells were generated by transformation of Human Embryonic Kidney cell cultures (HEK) with sheared adenovirus 5 DNA and were first described in 1977 (Graham *et al.*, 1977). The HEK cell line has been extensively used as an expression tool for recombinant proteins since it was generated over 25 years ago. Although of epithelial origin, its biochemical machinery is capable of carrying out most of the post-translational folding and processing required to generate functional and mature protein from a wide spectrum of both mammalian and non-mammalian nucleic acids. Though popular as a transient expression system, this cell type has also seen wide use in stably transfected forms (i.e. transformed cells) to study a variety of cell biological (Thomas and Smart, 2005). It also showed that, the human cell line HEK 293 has been used extensively for transient gene expression as a result of its good transfectability and ability to grow in suspension to high cell density in serum free media (Durocher *et al.*, 2002; Meissner *et al.*, 2001; Schlaeger and Christensen, 1999).

A study by Baldi *et al.* (2005) confirms that HEK 293 cells has the potential of adaptation in serum free suspension for the large scale production of high quality research grade material. They also evaluated that HEK 293 cells did not indicates any adverse effect on cells “Aging” on transfectability by the Ca-Pi method. Even when using cells from seed cultures that had been cultivated continuously for more than 6 months, the transfectability was excellent and the cell viability was 95%.

Apart from that, introduction of plasmid vectors such as those under the control of the CMV promoter very effectively hijack the HEK cell’s synthetic protein machinery and force the translation of gene products artificially incorporated into the plasmid (Thomas and Smart, 2005). However, HEK 293 cells do not normally produce large numbers of exosomes. In contrast to that, when the HIV-Nef gene is expressed in these cells, they produce large number of exosomes (Campbell *et al.*, 2008). Moreover, apoptosis was observed using soluble extracellular Nef expressed from HEK 293 cells transfected with HIV-1, HIV-2 and SIV Nef gene (James *et al.*, 2004). Figure 4 shows the cell morphology of HEK 293 cells.

### **DIAGNOSIS**

The first enzyme immunoassay to detect HIV antibodies was introduced in 1985. These first-generation immunoassays detected IgG antibodies to HIV-1 using viral lysate as the antigen and were unable to detect antibody response to different HIV-1 clades. They became positive approximately 6-8 weeks following infection. First-generation assays lack the sensitivity and specificity of current widely-used tests. The second-generation assays increased specificity by using recombinant proteins or peptides to produce viral antigens. They were able to detect infection approximately 1 week earlier than the first-generation assays (Kimpton and Emerman, 1992).

The development of the third-generation assays represented significant advancement since they could not only detect both HIV-1/2 IgM and IgG but also detect them as soon as 3 weeks after infection. They are also considered to be more sensitive than previous generation assays and by 2007, third-generation assays had supplanted earlier assays. Fourth-generation assays became available in the United States in 2010, although similar combination assays have been utilized in other countries since a decade earlier.

They simultaneously detect p24 antigen as well as HIV-1/2 IgG and IgM antibodies. With their ability to detect p24 approximately 5-7 days after the appearance of nucleic acid, fourth-generation

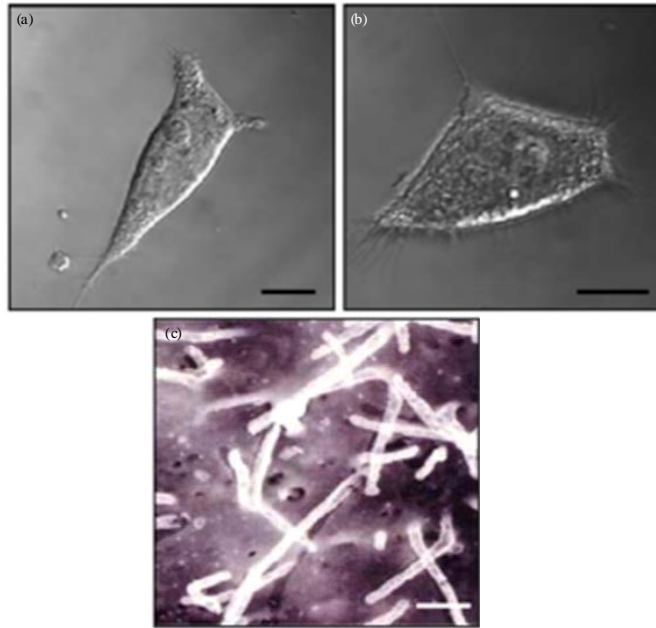


Fig. 4(a-c): (a) HEK 293 cell morphology. Transmitted light images of single, non-transfected HEK cells demonstrating their classical pyramidal or rhombic morphology and associated processes (b) Smaller hair-like filopodia around the membrane periphery and (c) which are more obvious under scanning EM visualization

assays have succeeded in significantly shortening time to diagnosis to as early as 2 weeks after infection . Published data has confirmed that fourth-generation assays can establish HIV infection in more than 80% of individuals who tested Nucleic Acid Amplification Test (NAAT) positive but either non reactive or indeterminate by other assay (Kim *et al.*, 2010).

The confirmatory test is immunoassays. When screening immunoassay tests are repeatedly reactive, Western Blot (WB) or indirect immunofluorescence assay (IFA) traditionally have higher specificity. The Western blot assay tests for antibodies that bind to fixed HIV viral proteins, which when exposed to a substrate create a pattern which can be read as positive, negative or indeterminate. The IFA, a less frequently used alternative, mixes serum or plasma samples with T-cells expressing HIV antigens to check for presence of antibodies. Bound antibodies are then identified using an anti-human antibody conjugate molecule.

The EIA/WB combination has demonstrated high sensitivity (99.3-99.7%) and specificity (99.7%) once seroconversion occurs. However, due to their ability to detect only IgG antibodies, confirmatory tests can lag behind a reactive third or fourth generation assay by as much as 3 weeks, leading to false-negative results if the test is conducted before seroconversion. False-negative results are more likely in a high prevalence population. Quantitative viral load tests are not approved in the United States for diagnosis of HIV. In 2006, the Gen-Probe an optima RNA qualitative assay was approved for diagnosis of HIV-1 infection as well as confirmation of a reactive EIA (Cornett and Kirn, 2013).

Molecular RNA assays are highly sensitive in acute HIV; however, they can be negative in 3-5% of samples with established infection. In addition, the currently available Nucleic Acid Amplification Tests (NAATs) have several limitations, including skill, expense and time necessary

to perform these tests, as well as the necessity of blood draws. Technological research is now focused on simplifying NAATs and thereby making them more suitable for point-of-care testing as well as decreasing the analytical time of real-time polymerase chain reaction assays (Cornett and Kirn, 2013).

Rapid HIV antibody tests have been FDA-approved since 2002 and typically consist of lateral-flow devices or flow-through cassettes which use porous membranes to detect anti-HIV IgG and IgM in samples of oral fluid, whole blood, plasma or serum. They offer the advantage of a turnaround time of 30 min or less, particularly important for populations with poor follow-up and women presenting in labor. Most detect both HIV-1 and HIV-2. Lateral flow tests do not require a laboratory and therefore can be performed in a variety of settings. Manufacturers have reported their sensitivities in the range of 99.3-100% and specificities as 99.7-99.9% (Cornett and Kirn, 2013).

However, various studies have reported lower sensitivities and specificities. Several studies have established performance characteristics comparable with first-generation and second-generation EIAs with a more recent study of the performance of six rapid HIV tests finding comparability to third-generation assays (Cornett and Kirn, 2013).

## **TREATMENT**

In 2012, more than 9.7 million people living with HIV were Receiving Antiretroviral Therapy (ART) in low and middle income countries. Standard antiretroviral therapy (ART) consists of the combination of at least three antiretroviral (ARV) drugs to maximally suppress the HIV virus and stop the progression of HIV disease. Huge reductions have been seen in rates of death and suffering when use is made of a potent ARV regimen, particularly in early stages of the disease (WHO., 2013)

Furthermore, expanded access to ART can also reduce the HIV transmission at a population level, impact orphan hood and preserve families (WHO., 2013). In 2011, an estimated 34 million people were living with HIV. WHO and UNAIDS estimate that at least 15 million people were in need of antiretroviral therapy in 2011. As of the end of 2011, over 8 million people had access to ART in low and middle-income countries. Figure 5 shows number of people receiving antiretroviral therapy in low and middle-income countries by region, December, 2011.

The last 10 years the landscape of national HIV epidemics has changed dramatically, for the better in most countries, especially in sub-Saharan Africa. Countries are making historic gains towards ending the AIDS epidemic: 700,000 fewer new HIV infections across the world in 2011 than in 2001. Latest data show that a 50% reduction in the rate of new HIV infections (HIV incidence) has been achieved in 25 low and middle-income countries between 2001 and 2011. More than half of these countries are in sub Saharan Africa where the majority of the new HIV infections occur. In a further 9 countries the rate of new HIV infections fell steeply by at least one third between 2001 and 2011. In particular, countries with a concurrent scale up of HIV prevention and treatment programmes are seeing a drop in new HIV infections to record lows (Foster and Garcia, 2008).

Prevention leads to behaviour change and treatment reduces a person's viral load. Both reduce the potential for the virus to be transmitted. The historic slow down indicates HIV prevention and treatment programmes are successfully reaching the people in need. However, the development of anti viral has blunted the AIDS epidemic in the Western world but globally the epidemic has not been curtailed (Foster and Garcia, 2008).



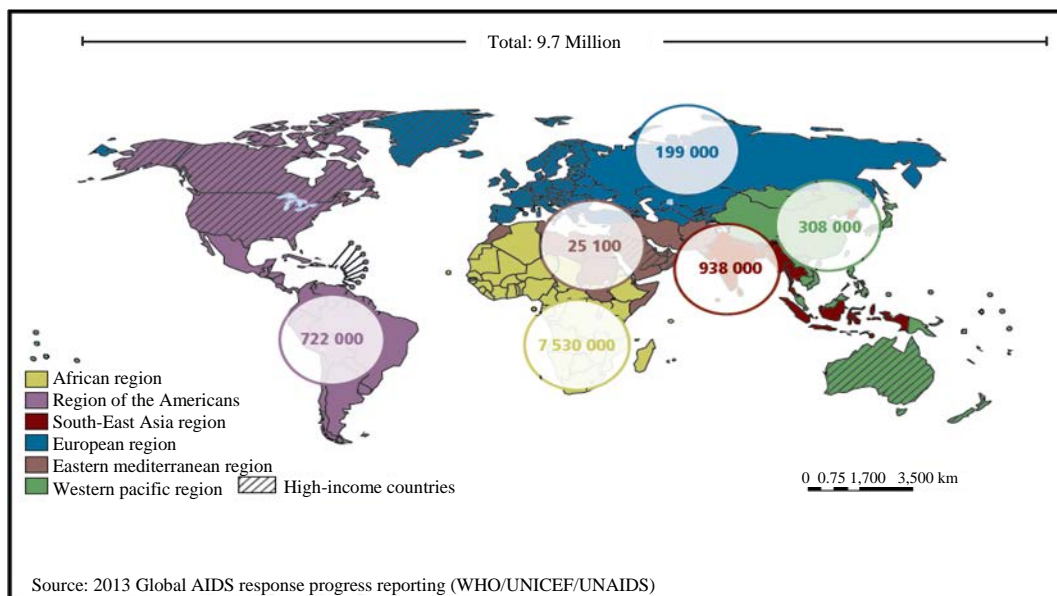


Fig. 5: Antiretroviral therapy in low and middle-income countries by region, December 2013 (Adapted from WHO, 2013)

Although highly active antiviral retroviral treatment is highly effective, its high cost and limited availability in under developed areas severely limit the success of current anti-HIV therapy. Moreover, the rapid emergence of drug resistance mutants and the increased worldwide spread of such treatment resistant HIV variants pose increasing problems to effective treatment of HIV-patients. Therefore, all possible targets for countering HIV-1 need to be considered. Given its central role in HIV pathogenesis, Nef considered as a potential anti viral target for preventing or at least delaying pathogenesis but lacks enzymatic activity is not targeted by current antiviral measures (Foster and Garcia, 2008). Recently Breuer *et al.* (2011) have inhibit Nef function by simultaneously blocking several highly conserved protein interaction surfaces. This strategy, referred to as “Wrapping Nef” is based on structure and function analyses that led to the identification of four target sites.

## CONCLUSION

Despite considerable advances in HIV science in the past 20 years, the reason why HIV-1 infection is pathogenic is still debated and the goal of eradicating HIV-1 infection remains elusive. A deeper understanding of the interplay between HIV-1 and its host will give a reasonable answer to this question. After studying the major role nef protein pathogenesis of HIV-1 and we understood the importance of production of anti viral therapy emphasizing the Nef protein to delaying pathogenesis HIV infection. This cloning and expression of HIV-1 Nef in HEK 293 cell believes to contribute a small portion to develop the anti-viral therapy against the nef protein.

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