Role of Integrase Acetylation in HIV-1 Replication Cycle and Search for Acetylation Inhibitors

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PhD Thesis in Molecular Biology

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2013

ABSTRACT

HIV-1 integrase catalyzes the integration of the viral DNA into the genome of the host cells. This irreversible event is crucial to the pathogenesis of the infection and complicates its eradication both by the immune systems and by pharmacological treatments.

The mode of action of this viral enzyme is still not completely characterized, although full understanding of some key aspects, as the mechanism of integration site selection, are relevant both for the development of new anti-integrase drugs and for potential application of HIV-derived vectors for gene therapy.

Our group has demonstrated that integrase is post-translationally acetylated by two cellular histone-acetyl transferases (HATs), chromatin-modifying enzymes whose major role is that of transcriptional co-activators. Integrase acetylation is important for the viral infectivity and interaction with HATs might be one of the determinants of HIV-1 preferential integration in actively transcribed genomic regions.

Integrase is a poorly exploited target of anti-HIV drugs, while traditional therapies based on combinations of reverse transcriptase inhibitors and protease inhibitors are facing the rapid diffusion of multi-drugs resistant viral variants. This pushes research towards new drugs and new targets, including integrase and, even better, its interactions with cellular cofactors like, for instance, HATs.

This thesis deals with the selection of novel inhibitors of integrase acetylation, to be used as lead compound for the development of new generation anti-integrase drugs.

A selective inhibitor of integrase acetylation was identifyied through *in vitro* screening of a library of synthetic compounds, designed based on the

structures of natural HAT inhibitors. Structure-Activity-Relationships (SAR) studies led to the rational design of a smaller set of compounds, whose activity was tested with *in vitro* and *in vivo* assays. Finally, one molecule was chosen for further studies with HIV-1 derived lentiviral vectors. This cinnamoil compound was able to inhibit integrase acetylation in the virus and reduced viral integration in infected cells. In a reciprocal experiment, viral vectors containing hyper-acetylated integrase were generated by trans-incorporation of fusion integrase-HAT proteins, or of isolated HAT domains. The enhanced infectivity of these virions confirmed the role of acetylation for integrase function.

Table of contents **Page**

1 INTRODUCTION

1.1 HIV-1 and AIDS: epidemiology and disease

Human immunodeficiency virus type 1 (HIV-1) is the main cause of HIV disease, which can progress with variable dynamics to its end stage, the Acquired Immunodeficiency Sindrome (AIDS). AIDS and HIV-1 infection represent global health problems and complex scientific dilemmas, which raise enormous social, ethical and economical issues, thus they are obvious targets for drug discovery.

First reported in 1981 in a small number of patients, after three decades AIDS has become a major epidemic, which account for about 33 million people infected worldwide, according to the 2010 UNAIDS Report on the global AIDS Epidemic (UNAIDS, 2010).

The clinical profile of the infection caused by HIV is specific. Upon an initial HIV-1 infection, there is a period of strong viral replication and immune activation, which results in a relatively low steady state of viraemia. Afterwards, the infection enters a chronic stage, characterized by a limited virus replication and absence of evident symptoms of disease. This phase can persist for many years, ultimately leading to an irreversible damage of the immune system characterized by a total loss of CD4+ T cells. This results in the onset of the AIDS stage, wherein repeated opportunistic infections can become lethal for the vast majority of untreated patients. In a very small proportion of infected patients, the so-called ‘long-term non-progressors’, the CD4 T cells count remains stable and normal, and no signs of disease occur. These individuals are able to control viral replication to low levels without undergoing antiretroviral treatments and represent one of the models of immune control of HIV-1 (Pantaleo, 1995). Elite controllers or suppressors (ES) represent a distinct subset of untreated patients, who appear to be able to control viral replication at undetectable levels (Thiébaut, 2011; Blankson, 2010; Hatano, 2009).

HIV-1 seems to be highly adapted for life in the host, taking advantage of cellular machinery to promote replication and transmission while possessing adequate equipment for immune evasion strategies (Douek, 2002; Kwong, 2002; Yue, 2005). In most individuals HIV-1 induces a generalised immune activation that involves not only the main target of infection (i.e. CD4+ T lymphocytes and monocyte/macrophages) but also B lymphocytes, natural killer cells, and antigen-presenting cells (Lawn, 2001).

Human Immunodeficiency Virus–1 is a member of the lentivirus genus of the *Retroviridae* family, a large group of single stranded RNA viruses endowed with the unique property of retro-transcribing their RNA into complementary cDNA, a process that is carried out by a virus-encoded enzyme called Reverse Transcriptase (RT).

According to The Universal Virus Database of the International Committee on Taxonomy of Viruses (ICTVdB) the Retroviridae family is currently classified into 7 genera.

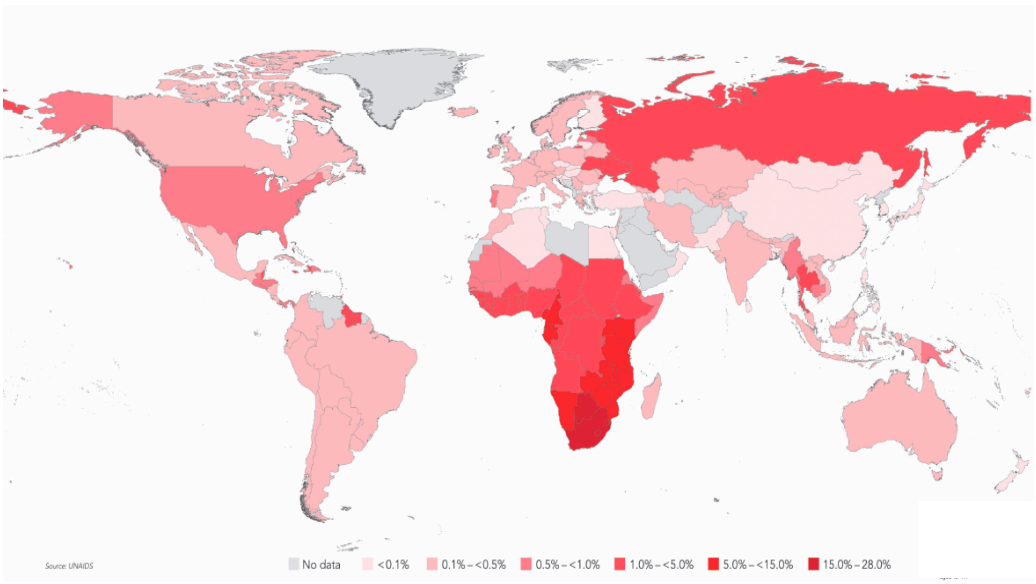


Figure 1-1. A global view of HIV-1 infection. According to the World Health Organization, 34 million people were living with HIV-1 at the end of 2010 (WHO – UNAIDS).

One of the features that distinguish HIV-1, as well as the other lentiviruses, from other members of the retroviridae family, is their ability to productively infect non-dividing, terminally differentiated cells, without a requirement for cell passage through mitosis to establish productive infections (Lewis, 1994).

1.2 HIV-1 virion structure

Like other retroviruses, HIV is an enveloped virus with a central, cone-shaped core surrounded by a lipid bilayer enriched in cholesterol and sphingomyelin, derived from the membrane of the host cell (Chan, 1998; Liao, 2001; Pierson, 2003; Krogstad, 2003). Embedded in this viral envelope are the 2 envelope glycoproteins, gp120 Surface (SU), exposed to the extra-cellular environment and the gp41 Trans-Membrane (TM) anchoring protein, as well as numerous cellular membrane proteins derived from the infected cells. A protein shell composed of many copies of the matrix (MA) protein separates the viral lipid envelope by the capsidic core. The core is composed of approximately 2,000 molecules of the 24 kD capsid (CA) protein (Gelderblom and Gottlinger, *http://www.hiv.lanl.gov;* Krogstad, 2003). The viral genome is composed of 2 copies of the positive-sense ribonucleic acid (RNA) packaged within this core. The RNA molecules are held together as a dimer, coated and protected by multiple copies of the nucleocapsid (NC) protein. The viral core also contains the viral integrase (IN) and reverse transcriptase (RT) proteins, which play essential roles in early steps of virus replication (Kaplan, 2002).

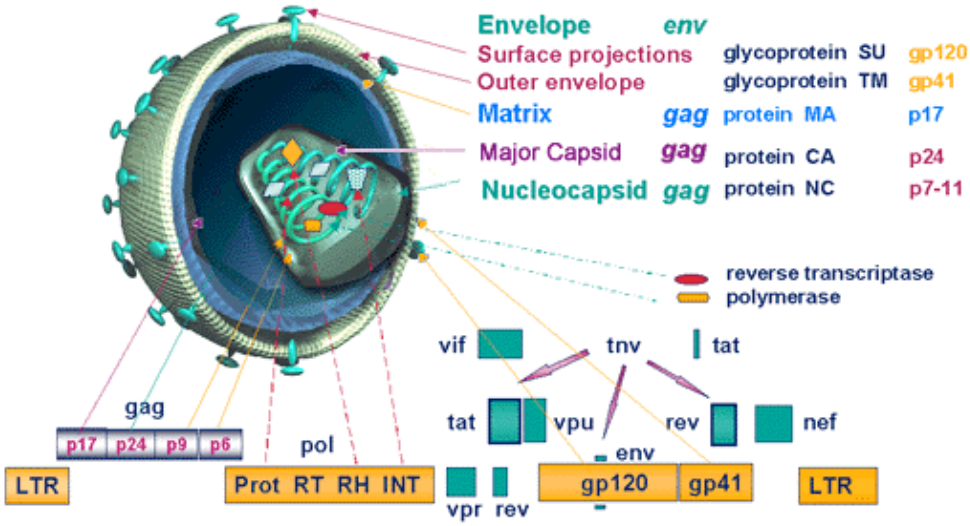


Figure 1-2. Genetic organization of HIV-1 (from the ICTV database)
The ~9.7 kb provirus comprises two identical LTRs (long terminal repeats) flanking the internal unique sequence. The 5′ LTR is a promoter for transcription; the 3′ LTR ensures polyadenylation. Genome regions encoding Gag, Pol and Env and the accessory proteins are shown.

The retroviral genome is about 9-kb of RNA, and encodes nine open reading frames. Three of these encode the Gag, Pol, and Env polyproteins, which are subsequently proteolyzed into individual proteins common to all retroviruses by the viral protease (Coffin, 1997; Zuckerman, 2004).

The four *Gag* proteins, matrix (MA or p17), capsid (CA or p24), nucleocapsid (NC or p7), and p6, and the two *Env* proteins, gp120 and gp41, are structural components that make up the core of the virion and outer membrane envelope, respectively.

The *Pol* gene encodes three enzymes that define the replicative strategy of the retrovirus: reverse transcriptase (RT) copies the viral RNA genome into DNA, and integrase (IN) mediates the insertion of that DNA into the genomic DNA of an infected cell to establish the provirus (and persistent infection). The

third enzyme, protease (PR), is necessary for maturation of virions into an infectious form.

Of the remaining six regulatory/accessory genes of HIV-1, *tat* and *rev* are crucial for virus replication, whereas *vif*, *vpr*, *vpu*, and *nef* are thought to have modulatory functions on the immune system *in vivo* (often in a species-specific manner).

1.2.1 *Gag* poliprotein

Gag is a multidomain polypeptide that constitutes the major structural constituent of all retroviruses. Indeed, Gag is capable of assembling into virus-like particles when expressed in various cell types in the absence of other viral constituents (Gheysen, 1989). HIV-1 Gag is synthesized as a precursor polyprotein, Pr55Gag, which consists of four major domains. Concomitant with or soon after virion budding, Pr55Gag is cleaved by the virally-encoded protease (Gelderblom, 1991) into its mature products p17 matrix, p24 capsid, p7 nucleocapsid, the carbossi (C)-terminal p6, and several small polypeptides including p1 and p2.

Matrix (p17, MA), situated at the amino (N)-terminal domain of the gag polyprotein (Freed, 1998), is, in mature virions, a 132-aa polypeptide (Göttlinger, 1989; Bryant, 1990), which forms a protective shell associated directly with the inner layer of the viral membrane (Gelderblom, 1991).

The matrix protein serves several functions in the viral replication cycle. MA is important for targeting Gag and Gag-Pol precursor polyproteins to the plasma membrane prior to viral assembly (Flint, 2004). In addition, this protein appears to help incorporate Env glycoproteins into viral particles (Mammano, 1995). Furthermore, MA is part of the pre-integration complexes (PICs) (Bukrinsky,

1993) and contains two nuclear localization signals (NLS) (Haffar, 2000) that may facilitate the nuclear import (Gallay, 1995). It has been recently shown that HIV-1 MA displays biological activities also outside infected cells, in particular it is able to activate the transcription factors c-Myc and CREB in human B cells, suggesting a potential mechanism of B cell lymphomagenesis during HIV-1 infection (Li, 2010).

Capsid (p24, CA) is the second component of the Gag polyprotein and forms the core shell of the HIV-1 viral particle with about 2000 molecules per virion (Scarlata, 2003). This protein is responsible for the morphogenesis of the mature, cone-shaped core and for assembly and particle production (Dorfman, 1994). Capsid is also important for infectivity, by participating in viral uncoating, and it has been reported to be the major determinant for the unique ability of HIV-1 to access the nucleus independent of the cell cycle (Yamashita, 2007). Two cellular proteins, cyclophilin A (CypA) and TRIM5α, regulate infection at the uncoating step. CypA binds capsid acting as a viral cofactor, increasing the viral infectivity (Kootstra, 2003; Saphire, 2002; Towers, 2007). Indeed CypA may participate as an uncoating factor and modulate CA disassembly (Li, 2009) or protect the viral core by binding of cellular restriction factors (Sokolskaja, 2006), leading to an increased infectivity. Interestingly, in African Green Monkey the interaction between CypA and HIV-1 CA decreases infectivity, as it facilitates restriction mediated by TRIM5α. This is due to the existence, in old world primates, but not in humans, of a TRIM5α-CypA fusion protein, which is responsible for the post-entry restriction (Sayah, 2004; Sokolskaja, 2004). In human cells, instead, TRIM5 and CypA seem to act independently one from the other (Sokolskaja, 2006; Hatziioannou, 2005).

Nucleocapsid (NC) protein is the third component of the Gag polyprotein and it is complexed to the genomic RNA inside the viral core. The NC domain

is required for genomic RNA packaging and primer placement and it has a role in viral RNA dimerization (Frankel, 1998; Adamson, 2007; Bampi, 2004). The mature NC protein, which is released in a late cleavage reaction, plays a major role in assuring the specificity and efficiency of reverse transcription and is also important for other events in the virus life-cycle including maturation of the genomic RNA dimer, integration of proviral DNA into the host genome and budding (Popova, 2010). NC's function in virus replication is correlated with its ability to act as a nucleic acid chaperone (Williams, 2001).

P6 protein comprises the C-terminal 51 amino acids of Gag and is important for incorporation of Vpr during viral assembly (Cohen, 1990). In addition, p6 is required for efficient viral particle release (Demirov, 2002; Huang, 1995; Stuchell, 2004).

1.2.2 *Env* gene

The *Env* gene encodes the mature TransMembrane gp41 (TM) and the Surface gp120 (SU) envelope glycoproteins, cleaved by cellular enzymes from the gp160 precursor (Zuckerman, 2004). The cellular enzyme responsible for the processing of the gp160 precursor is furin or a furin-like protease (Hallenberger, 1992). Cleavage of gp160 is required for Env-induced fusion activity and virus infectivity (Freed, 1989; McCune, 1988). The proteins gp120 and gp41 are located on the viral membrane surface and their function is to bind the CD4 receptor of the target cells and mediate fusion between viral and cellular membranes, respectively (Frankel, 1998).

1.2.3 *Pol* poliprotein

The *Pol* poliprotein harbors the viral enzymes protease, reverse transcriptase and integrase, which are processed by cleavage by the viral protease. These three enzymes are not active in their monomeric forms, but need to oligomerize as dimers or tetramers to be catalitically active.

Reverse Transcriptase (RT) protein catalyzes both RNA-dependent and DNA-dependent DNA polymerization reactions and contains an RNase H domain that cleaves the RNA portion of RNA-DNA hybrids generated during the reaction (Coffin, 1997). RT is characterized by a high error rate when transcribing RNA into DNA, since it lacks a proofreading function (Coffin, 1997).

Protease (PR) is activated during or shortly after budding of virions from the cell, and cleaves Gag into the virus structural proteins. Cleavage occurs sequentially and in a highly ordered manner.

The first cleavage event catalyzed by PR during or immediately after virion release from the cell serves to release PR itself from the Gag-Pol polyprotein. Following its own cleavage from the precursor, the dimeric enzyme cleaves a number of sites in both Gag and Gag-Pol. PR activity does not seem to target a consensus sequence, but it appears to cleave different targets with varying efficiencies, so that Gag cleavage takes place as an ordered, step-wise cascade. Mutations in Gag that disrupt the ordered nature of PR-mediated processing severely disrupt virus assembly or subsequent maturation (Krausslich, 1991).

1.2.3.1 Integrase

HIV-1 integrase (IN) is an essential viral enzyme that is required to catalyze the specific and efficient insertion of the viral DNA product of reverse

transcription into the host cell genomic DNA (Bushman, 1990; Goff, 1992; Vink, 1993).

Integrase participates also in other steps of the viral replication cycle, playing a role in the uncoating of the viral core (Leavitt, 1996; Nakamura, 1997; Li, 2009; Briones 2010), in nuclear import of the viral DNA (Gallay, 1997; Tsurutani, 2000; Ikeda, 2004) and in viral DNA synthesis (Masuda, 1995; Engelman, 1995; Wu, 1999).

Integrase structure

The integrase enzyme is a 288 amino acids, 32 KDa protein encoded by the 3′-end of the pol gene and approximately 50-100 copies of the integrase enzyme are packaged per virion particle (Flint, 2003).

Integrase is comprised of three structural and functional domains: an N-terminal domain (NTD), a catalytic core domain (CCD), and a C-terminal domain (CTD). The functional integrase enzyme is composed of integrase homodimers that are proposed to further associate with each other to form a multimer complex in solution (Ellison, 1995; Engelman, 1993).

The N-terminal domain (NTD) of integrase includes amino acids 1 to 50 and it contains two pairs of highly conserved hystidines (residues 12 and 16) and cysteines (residues 40 and 43) that form a zinc finger motif that has been demonstrated *in vitro* to involve the chelation of zinc ions (Burke, 1992; Zheng, 1996). The N-terminal domain is required for high-order multimerization that is stimulated by zinc (Zheng, 1996; Cai, 1997). A zinc atom is required for proper NTD folding and it is necessary for optimal enzymatic activity (Burke, 1992; Zheng, 1996).

The core domain (CCD) contains the catalytic site and consists of aminoacids 50 to 212. It is characterized by three invariant and essential acidic

residues (D64, D116 and E152), which forms the catalytic triad indispensable for the enzymatic activity. Crystal structures of several integrase catalytic core domains, obtained as dimers or trimers (Goldgur, 1999; Lubkowski, 1998; Chen, 2000; Molteni, 2001), show that it consists of five β-sheets flanked by six α-helices that are connected by flexible loops.

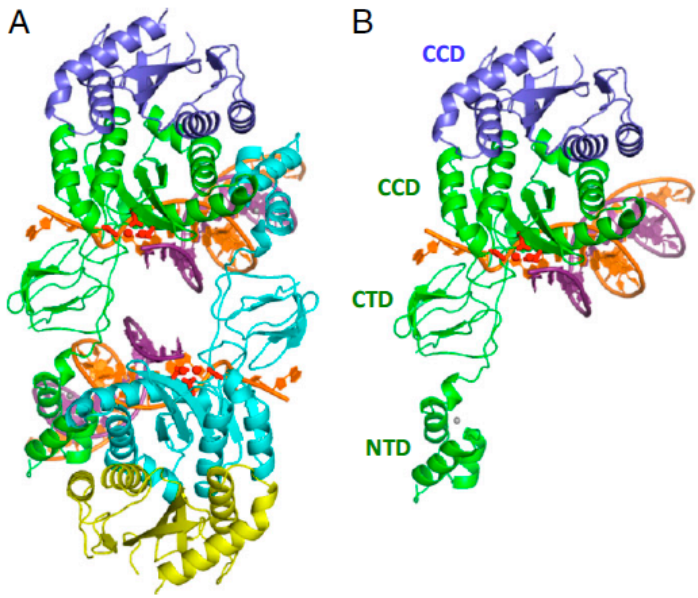


Figure 1-3. Structure of HIV-1 Integrase. (A) Green and cyan: inner residues of the IN tetramer, engaged with viral DNA; blue and yellow: outer IN CCDs domain; Magenta: cellular DNA; Orange: viral DNA. (B) Resection of the upper IN dimer from A, highlighting the position of canonical IN domains: The three domains appear to be stably folded when prepared separately. The amino-terminal domain is characterized by pairs of histidine and cysteine residues (HHCC) that are universally conserved among retroviral integrases. The core domain contains the catalytic site, which includes the so-called catalytic triad, formed by universally conserved and essential residues: an aspartate, and at some distance another aspartate and a glutamic acid, separated by 35 amino acids (DD35E) (Krishnan, 2010; Coffin, 1997).

The less conserved carboxy-terminal domain (CTD) consists of 76 aminoacids (aminoacids 212 to 288) and adopts an SH3-like fold (aminoacids 220 to 270) (Cai, 1997; Eijkelenboom, 1995). The C terminus of IN is the less conserved region of the protein and it is required both for 3'end processing and

integration activity (Coffin, 1997). Hindmarsh and colleagues showed that an HIV-1 IN fragment representing residues 235 to 288 binds nonspecifically to DNA (Hindmarsh, 1999). Interpreting the DNA binding activity of integrase CTD is not obvious, since integration involves two different DNA substrates, which have different structural requirements: the viral cDNA and the host genomic DNA (Coffin, 1997). The isolated CTD binds well to simple linear double-stranded DNA oligonucleotides (Engelman, 1994; Lutzke, 1994; Vink, 1993), suggesting that it may contribute to binding the viral cDNA ends (att sites) (Coffin, 1997). In addition, the C-terminus seems to enhance the multimerization of IN (Hindmarsh, 1999) (Asante-Appiah, 1999; Engelman, 1999). The CTD domain contains 3 lysines residues which are acetylated by both p300 and GCN5 histone acetyl-transferases (K264, K266 and K273) (Cereseto, 2005) and a fourth lysine acetylated exclusively by GCN5 (Terreni, 2010).

The crystal structure of a full-lenght retroviral integrase (from the Prototype Foamy Virus) has been recently characterized. Hare and colleagues showed that the retroviral intasome (the nucleoprotein complex needed for integration of the viral DNA into the host genome) is comprised of an integrase tetramer that tightly binds the two viral DNA extremities (Hare, 2010).

The N- and C-terminal domains of IN are essential for proper interactions with substrates and they are needed for 3’ processing and strand transfer, presumably because without them the CCD cannot correctly position the viral cDNA termini at the active site (Chiu, 2004).

Integrase enzymatic activity

Following reverse transcription, IN catalyzes a series of reactions to integrate the viral genome into a host chromosome. Initially, in a reaction termed 3'-processing, IN removes two or three nucleotides from one or both viral DNA ends to expose the 3'hydroxyl groups of the invariant CA dinucleotides. Next, after import of the viral DNA into the nucleus, IN inserts both 3'ends of the viral DNA into opposing strands of cellular genomic DNA (Coffin, 1997).

Mechanistically and structurally, IN belongs to a diverse family of polynucleotidyl transferases (Dyda, 1994), which notably includes RNaseH (Nowotny, 2005), the transposases from Tn5 (Davies, 2000) and eukaryotic mobile element Mos1 (Richardson, 2009; Jaskolski, 2009; Nowotny, 2009; Engelman, 1991). The reactions catalysed by these enzymes proceed by SN2-type nucleophilic substitution, assisted by divalent metal cofactors (Nowotny, 2005; Engelman, 1991). In retroviral integrase, a pair of divalent metal cations (Mg or Mn) is thought to be coordinated by three carboxylates of the invariant DD35E motif within the catalytic core domain (CCD). In vivo, integrase acts within a large nucleoprotein complex that contains viral DNA and several virus- and host cell-derived components called the Pre-Integration Complex (PIC). PICs include viral proteins, such as the viral matrix, vpr and nucleocapsid (Miller, 1997), and several host proteins, such as barrier to autointegration (BAF [Lin, 2003]), high mobility group proteins (HMGs [Farnet, 1997]), and LEDGF/p75 (Llano, 2004) (a detailed list of integrase cofactors is the subject of a dedicated paragraph ahead).

Integrase multimerization

Studies with purified recombinant protein and model DNA substrates indicated that integrase does not function in its monomeric form, but individual protein monomers establish complementary contacts both with DNA substrates and with the other integrase subunits, to form the functional nucleoprotein complexes (Engelman, 1993; van Gent, 1993; van den Ent, 1999; Zhao, 2008; Kessl, 2009; Hare, 2010). Although a dimeric protein is sufficient to process each 3'-end, a tetramer is needed to carry out the concerted integration of both viral ends (Faure, 2005; Guiot, 2006; Li, 2006; Hare, 2010). A dynamic interaction between integrase subunits is essential for the assembly of the fully functional nucleoprotein complex and restricting the molecular movement of individual subunits within a multimer could compromise catalytic processes.

Cellular proteins interacting with integrase

Because of retroviruses' limited genome size and content, each step in the elaborate replication cycle of HIV-1 requires the assistance of multiple host proteins. In particular the factors described hereafter have been shown, at different extents, to have a role at the integration step.

Ini1 (Integrase Interactor 1), also known as hSNF5, was first identified as a cellular cofactor of IN by two-hybrids screening (Kalpana et al., 1994). Ini-1 is the human homolog of yeast SNF5, a transcriptional activator and component of the chromatin remodeling SWI/SNF complex (Carlson, 1994) and it was similarly shown to be part of the mammalian SWI/SNF complex (Wang, 1996).

The exact role of Ini1 in HIV-1 replication is presently unclear. The first reports about it shown that recombinant Ini1 directly binds Integrase and stimulates IN catalytic activity in vitro (Kalpana, 1994).

Interestingly, evidence for a possible role for Ini1 in the post-integration steps of HIV-1 replication is stronger. Indeed, Ini1 is incorporated into the virions and it is necessary for efficient viral particle production (Yung, 2004), while a cytoplasmic fragment of Ini1 (S6), when over-expressed, was able to interact with IN in the context of the Gag-pol precursor and it was reported to inhibit viral particle production, thus suggesting a role for Ini-1 during the late stage of HIV-1 replication (Yung, 2001).

On the other hand, another report suggested a role for Ini-1 as an inhibitor of the early steps of HIV-1 replication. Maroun and co-workers, showed that siRNA mediated silencing of SWI/SNF complex expression increased the formation of 2-LTR circles and integrated forms of viral DNA, (Maroun, 2006). In fact a single amino acid change, K71R, in integrase reduced its ability to interact with Ini1, leading to an increased viral infectivity (Maroun, 2006).

Ku, a chromatin-associated protein which is part of the double-stranded DNA break recognition and repair system known as non-homologous end-joining (NHEJ), has also been identified in PICs (Li, 2001; Lin, 2003). This protein seems to enhance viral DNA circularization in infected cells after reverse transcription. In this way Ku might protect cells from apoptosis induced by linear unintegrated viral cDNA forms, allowing the remaining integrated viral DNA copies to efficiently complete the viral replication cycle (Li, 2001).

BAF-1 (Barrier to autointegration 1), is a small DNA-binding protein identified as a component of the MLV and HIV-1 PIC (Chen, 1998; Suzuki, 2002; Lin, 2003; Mansharamani, 2003). The association of BAF-1 with the PIC might be mediated by interactions with DNA, Gag or IN. BAF-1 seems to function by bridging and condensing DNA helices (Zheng, 2000; Umland, 2000) and by doing so on viral DNA it would render it inaccessible to autointegration reactions. Indeed, removing BAF-1 from the PIC by using a

high-salt wash activates the suicidal autointegration of the viral termini into internal sites on the viral DNA in *cis*. With the same bridging mechanism, BAF could promote anchoring of the PIC to the target DNA, as demonstrated by the fact that its presence promotes the integration to target DNA in *trans* (Suzuki, 2002; Lee, 1998).

LAP2α (lamina-associated polypeptide 2α) is a laminin-associated component of the nuclear envelope and it is another host component of the PICs. LAP2α binds to BAF-1 (Shumaker, 2001) and promotes productive PIC integration (Suzuki, 2004). LAP2α is required for infection by MLV, and by HIV-1 entering the cell using its own envelope protein but not by HIV-1 pseudotyped with VSV G protein (Suzuki, 2004).

Emerin, a component of the inner nuclear membrane, has been reported to be necessary for HIV-1 infection of nondividing macrophages and dividing HeLa cells (Jacque, 2006). However, Emerin role in HIV-1 infection is controversial (Shun, 2007; Mulky, 2008). The localization of emerin in the nuclear membrane is mediated by BAF-1 (Haraguchi, 2001; Lee, 2001), and is regulated by BAF-1 phosphorylation (Bengtsson, 2006; Hirano, 2005). Emerin itself is phosporilated by the ERK2/MAP Kinase (Bukong, 2010). According to Jacque and coworkers, Emerin seems to function to mediate the association of the PIC with chromatin after nuclear entry of the PIC, thereby enhancing viral DNA integration. The association of emerin with viral DNA is mediated by BAF, which binds to the PIC in the cytoplasm, and the two proteins seem to work together to promote HIV-1 integration into chromatin (Jacque, 2006).

HMGA1 and HMGA2 (high mobility group chromosomal protein A1 and A2), are non-histone DNA-binding proteins that can modulate transcriptional regulation and chromatin structure (Farnet, 1997). They have sequence-specific binding sites on chromatin and seem to function by facilitating the binding of

transcription factors to the cellular genome (Thomas, 2001). HMGA1 and HMGA2 have been identified within the PICs of MLV and HIV-1, and have the capability to favor retroviral integration (Farnet, 1997; Li, 2000). However, a recent report investigating the role of these cellular proteins during the viral replication cycle has indicated that they actually are dispensable for retroviral integration, probably due to redundancy with other factors (Beitzel, 2003).

EED (Embryonic Ectoderm Development protein) is a chromatin-remodeling protein. It belongs to the broadly conserved Polycomb family of proteins, and has recently been found to interact with integrase (Violot, 2003). Like BAF and HMGs, EED is associated to condensed chromatin. According to data acquired so far, these proteins' primary effect is on donor viral DNA, and not on the acceptor cellular genome; however, this coincidence raises the hypothesis of a major involvement of these factors at the level of the integration site in vivo. This involvement could also be indirect, with these proteins acting as bridges for the interaction with other factors (e.g. transcriptional factors) that, in turn, could favor integration, as well as transcription.

Components of the DNA damage response system, including **DNA-PK, ATM, ATR, Ku80 and XRCC4/ligase IV**, have all been suggested to be important for HIV-1 DNA integration (Daniel, 1999; Smith, 2006). Recent work indicates that these proteins are not directly involved in the integration reaction (Ariumi, 2005; Dehart, 2005), though they are probably required to induce the post-integration DNA repair systems that are responsible for filling in the single-stranded gaps and sealing the nicks that are left at the sites of viral DNA insertion by IN.

LEDGF/p75 (lens epithelium-derived growth factor), assists the integration process by tethering integrase to the host chromosomal DNA (Maertens, 2003; Cherepanov, 2003). LEDGF/p75 is a ubiquitously and

constitutively expressed nuclear transcriptional co-activator and it is the 530 amino acid product of the gene PSIP1 (Ge, 1998). A 333-aminoacids splice variant, LEDGF/p52 (p52), shares p75's N-terminal 325 residues. The two proteins differ in their C-terminal portion, derived by alternative splicing, which consists of 8 amino acids in the case of p52 and 205 amino acids for p75 (Ge, 1998). The C-terminal portion of LEDGF/p75 contains the integrase binding domain (IBD) (Cherepanov, 2004). Both LEDGF/p52 and LEDGF/p75 are chromatin-associated proteins, which have been implicated in transcriptional regulation, cell survival and autoimmunity. P52 seems to be the more active one and also has a more restricted intranuclear distribution during the different phases of the cell cycle (Nishizawa, 2001).

LEDGF/p75 is necessary for the nuclear and chromatin localization of PICs for HIV-1 and other lentiviruses. To exert this function, LEDGF/p75 acts as a receptor that tethers HIV integrase to chromatin and stabilize it by protecting it from degradation, while strongly influencing the genome-wide distribution of HIV integration. In the absence of LEDGF/p75, lentiviral IN proteins are cytoplasmic (Maertens, 2003). LEDGF/p75 enhances *in vitro* strand transfer activity of integrase from HIV and from other lentiviruses, but have no effect on integrase from other types of retroviruses (Cherepanov, 2007). LEDGF's feature common to its viral and cellular roles is its ability to act as a molecular adaptor and tether proteins to the chromatin fiber.

Transportin-SR2 (TRN-SR2), one of the alternate splicing product of the gene TNPO3, is a protein belonging to the importin-beta family of proteins (Lai, 2000; Kataoka, 1999) and it has been recently identified as the nuclear import factor of HIV in cycling cell lines as well as in macrophages (Christ, Thys, 2008). TRN-SR2 was first identified as the shuttle transporter for the splicing factors SR (Serine/Arginine) proteins (Lai, 2000; Lai, 2001), but we

now know that it can shuttle other proteins as well, as it does for instance with the RNA-binding motif protein 4 (RBM4) (Lai, 2003). In the study of Christ *et al*. it was shown that TRN-SR2 interacts with the viral integrase (Christ, 2008; Thys, 2011; Luban, 2008) and that silencing of TRN-SR2 interferes with HIV replication by inhibiting the nuclear import of viral particles (Christ, 2008; Thys, 2011). These findings are confirmed by two independent genome-wide RNAi screenings (Brass, 2008; Konig, 2008). Notably, the impact of TRN-SR2 on nuclear import is specific for lentiviruses, as it does not seem to influence MoMLV, which is known to be dependent on nuclear envelope breakdown during mitosis (Christ, 2008; Levin, 2010). Recently Ocwieja *et al*. suggested that the steps of HIV import through the nuclear pore may influence subsequent integration site preference both in non-cycling and in dividing cells (Ocwieja, 2011), showing that in transportin knockdown cells the distribution patterns of integration site was altered for HIV but not for MLV infections, in line with first results by Christ *et al*.. In particular they showed that, in the absence of TRN-SR2, HIV redirects its integration preference towards chromosomal regions with low gene density, as opposite to its usual behaviour, thus originating less productive infection events.

P300 histone acetyl transferase, a well-known transcriptional co-activator, has been discovered by our group as an integrase cofactor (Cereseto, 2005), which binds and acetylates HIV integrase, positively influencing viral DNA integration and infectivity. These findings were later confirmed by two other groups (Topper, 2007; Apolonia, 2007) and by subsequent studies by our group (Allouch, 2009; Terreni, 2010) and are discussed ahead throghout this thesis.

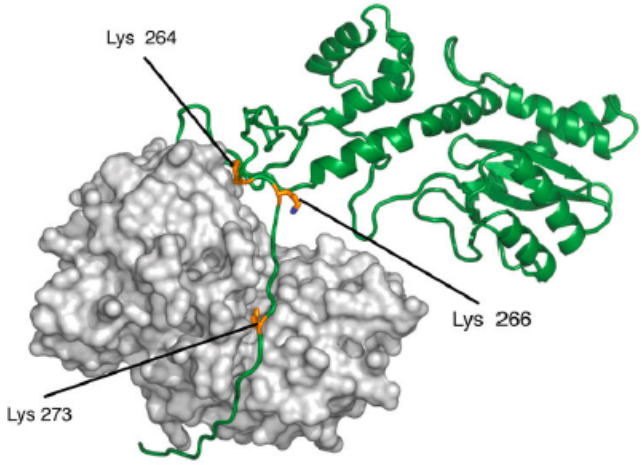


Figure 1-4. Three-dimensional models of IN complexed with p300. IN is represented in green and p300 in light grey. The three lysine residues in the C-terminal domain of IN that are acetylated by both GCN5 and p300 (Lys 264, Lys 266, and Lys 273) are shown in yellow. P300 is rendered as surface, while IN as a cartoon to highlight the C-terminal unfolded portion which inserts in the binding pockets of the HAT (Terreni, 2010).

KAP1, a protein involved in transcriptional silencing thanks to its interaction with other chromatin modifying proteins (among which the histone deacetylase complex HDAC1), has been recently identified as a novel HIV-1 restriction factor, targeting specifically acetylated integrase (Allouch, 2011). It has been demonstrated that KAP1 binds to acetylated integrase and recruits HDAC1, which in turns deacetylates integrase and reduces integration. The authors propose a model for the virus to escape KAP1 restriction, in which KAP1 is inactivated by phosphorilation operated by ATM, a protein involved in the DNA double-strand break repair system, which is activated upon HIV-1 infection.

Cofactor	Other names	Physiological role	Role in HIV-1 infection	References
Integrase Interactor 1 (Ini1)	hSNF5	Chromatin remodelling, transcriptional silencing	stimulates IN catalytic activity in vitro virion assembly	Kalpana, 1994 Yung, 2004
Ku	XRCC	double-stranded DNA break recognition and repair (non-homologous DNA end joining)	enhance the circularization of viral DNA	Li, 2001
Barrier to autointegration (BAF1)	BANF1	DNA-bridging protein. Attaching chromatin to the inner nuclear membrane?	promotes the integration to target DNA in *trans*	Suzuki, 2002 Lee, 1998 Wilson, 2002
Lamina-associated polypeptide 2 (LAP2α)	TMPO; LEMD4;	laminin-associated component of the nuclear envelope	promotes productive integration by binding to BAF1	Shumaker, 2001 Suzuki, 2004
Emerin	EMD; LEMD5;	component of the inner nuclear membrane	mediates association of PICs with chromatin	Jacque, 2006
High mobility group chromosomal protein A1 (HMGA1)	HMGI(Y)	modulation of transcriptional regulation and of chromatin structure	stimulates integration; redundant?	Farnet, 1997 Li, 2000 Beitzel, 2003
Embryonic Ectoderm Development protein (EED)	HEED; WAIT-1	chromatin-remodeling protein	interacts with Integrase and with viral DNA; integration site selections?; interaction with other cofactors?	Violot, 2003
DNA-PK, ATM, ATR, Ku80 and XRCC4/ligaseIV		components of the DNA damage response system	post-integration DNA repair	Smith, 2006 Ariumi, 2005 Dehart, 2005
Lens epithelium-derived growth factor (LEDGF/p75)	PSIP1	transcriptional regulation, cell survival and autoimmunity	tethers the IN to the host chromosomal DNA	Maertens, 2003 Cherepanov, 2003
Transportin-SR2 (TRN-SR2)	TNPO3; IPO12; MTR10A	shuttling protein through the nuclear membrane	nuclear import factor of HIV	Christ, 2008 Thys, 2011 Luban, 2008
p300	EP300; KAT3B	transcriptional coactivator	Acetylates In and is required for efficient integration;	Cereseto 2005 Allouch, 2009
GCN5	KAT2A	transcriptional coactivator	Acetylates In and is required for efficient integration	Terreni 2010
KAP1	TRIM28; Tif1β	gene silencing	Inhibits integration through deacetylation of IN	Allouch, 2011

Figure 1-5. Partial list of cellular proteins interacting with HIV-1 integrase

1.2.4 Regulatory and accessory proteins

In addition to *gag, pol* and *env*, common to all members of the family *Retroviridae,* HIV-1 also encodes six regulatory and accessory proteins.

Tat (TransActivator of Transcription) gene encodes a small protein essential for efficient transcription of viral genes and for viral replication (Cann, 1985; Kessler, 1992; Marcello, 2001), which is able to increase viral gene expression (Ratnasabapathy, 1990; Zhou, 1995).

Tat binds to a structured RNA element (TAR, transactivation-responsive region) present at the 5'-end of viral leader mRNA (Wei, 1998) and recruits a series of transcriptional complexes and P-TEFb (Positive Transcription Elongation Factor b), which stimulates RNA polymerase II phosphorylation by Cdk9, increasing the processivity of the enzyme complex (Bieniasz, 1998; Shilatifard, 2003; Wei, 1998). Moreover, due to its efficient cell membrane transduction properties, Tat is released into the microenvironment and the circulation, and then taken up by the surrounding cells (Westendorp, 1995).

Rev (Regulator of Expression of Virion) is a sequence-specific RNA binding phospho-protein that is expressed during the early stages of HIV-1 replication (Malim, 1989). Rev is required for expression of the viral structural proteins Gag, Pol and Env from the integrated proviral DNA. By binding to the Rev-Responsive Element (RRE), an RNA structure present on the unspliced RNA encoding Gag and GagPol and on singly spliced RNAs encoding Env, Rev tethers these transcripts to the cellular CRM-1-mediated nuclear-export pathway, leading to enhanced cytoplasmic levels of these RNAs and increased expression of the encoded proteins. Rev has also recently been shown to be able to enhances encapsidation of the genomic RNA into virions (Blissenbach, 2010).

Due to its roles in nuclear RNA export, in the increase in translational efficiency of viral structural proteins, and in the stimulation of encapsidation, Rev has been thought for long time to be essential for the late phase of the virus replication cycle. However, Rev plays also a role during the early phase of infection, as it can also interfere with integration of the reverse-transcribed cDNA into the host-cell genome, by promoting dissociation of the IN–LEDGF/p75 complex, with consequent blocking IN activity and preventing tethering of the pre-integration complex to the host-cell chromosome. Since Rev is presumably present in the infected cell at sufficiently high levels only after integration has already taken place, the main function of Rev during the early phase might be to impede that superinfection of the same cell by subsequent viruses leads to excessive integration and consequent genotoxicity (Levin, 2009; Grewe, 2010).

Nef (Negative regulatory Factor) is a 27 KDa protein highly conserved in all primate lentiviruses, that is abundantly produced during the early phase of viral replication cycle. Nef has different roles in HIV-1 replication and disease pathogenesis. It down-regulates CD4 (Garcia, 1991), which limits the adhesion of a Nef-expressing T cell to the antigen-presenting cell, thus promoting the movement of HIV-infected cells into circulation and spread of the virus. Nef also down-modulates MHC-I (Schwartz, 1996) cell surface expression, protecting HIV-infected cells from host CTL response. In addition, it interferes with cellular signal transduction pathways and it enhances virion infectivity and viral replication, since it induces actin remodeling and facilitates the movement of the viral core past a potentially obstructive cortical actin barrier (Campbell, 2004; Chowers, 1994).

Vpr (Viral Protein R) is a 96 aa small basic protein. Despite its small size, Vpr has been shown to have multiple activities during viral replication. Vpr appears to participate in the anchoring the PICs to the nuclear envelope and to

be involved in the nuclear translocation of the viral DNA (Heinzinger, 1994). An important function of Vpr is that of facilitating the infection of non-dividing cells, like macrophages (Connor, 1995). This viral protein is cytopathic to cells, although there has been some debate as to whether the cells dye from apoptosis (Muthumani, 2005) or necrosis (Sakai, 2006). Nevertheless, one well demonstrated attribute of Vpr expression is its ability to delay or arrest cells in the G2 phase of the cell cycle (Bartz, 1996; Di Marzio, 1995). The biological significance of Vpr-induced arrest during viral infection is not well understood. However, HIV-1 LTR seems to be more active in the G2 phase, implying that Vpr induced G2 arrest may confer a favorable cellular environment for efficient transcription of HIV-1 (Goh, 1998). Vpr concentrates at the nuclear membrane by interacting with the nuclear pore complex components (Vodicka, 1998) and even more specifically with nucleoporins. These interactions seem to indicate that Vpr is involved in docking of the PIC to the Nuclear Pore Complex (NPC) (Jacquot, 2007). Interaction of Vpr with nucleoporin hCGI also contributes to the G2-arrest mediated by Vpr (Jacquot, 2007). Next to interactions with the NPC, Vpr was shown to interact with importin α. Given that importin α also binds other components of the PIC such as integrase or matrix, it was suggested that Vpr acts like an importin β like protein (Vodicka, 1998). A second theory suggests that Vpr facilitates nuclear import by stabilizing the interactions of matrix or integrase with the nuclear import machinery (Popov, 1998).

Vpr binds to the p6 protein (Bachand, 1999; Paxton, 1993) and this property can be exploited to trans-incorporate other proteins in the nascent viral particle (Wu, 1995; Wu, 1997; Fletcher, 1997; Liu, 1997).

Vpu (Viral Protein U) is a 9 KDa membrane protein that induces the degradation of the CD4 receptor. Vpu is involved in ubiquitination of CD4 that leads to their degradation. In addition, Vpu increases progeny virus secretion from infected cells. This function is related to the ability of Vpu to self-

assemble into homooligomeric complexes that in vitro function as ion-conductive membrane pores (Bour, 2003). Vpu counteracts an inhibitory cellular factor, TASK-1, an acid-sensitive K+ channel that, in the absence of Vpu, inhibits virus release (Hsu, 2004). TASK-1 is structurally homologous to Vpu, suggesting oligomerization as a possible mechanism of inactivation of ion channel activity of these proteins (Hsu, 2004; Li, 2005). Vpu antagonizes also another cellular restriction factor, Tetherin, a membrane protein that, in the absence of Vpu, inhibits the release of viral particles, by retaining them at the cell membrane and subsequently in endocytic vescicles (Neil, 2008).

Vif (Virus Infectivity Factor) is a 192 aa protein that is expressed at high levels in the cytoplasm of infected cells. Vif was thought to be important because it is essential for the replication of HIV-1 in the peripheral blood lymphocytes, macrophages, and certain cell lines known as “nonpermissive” cells (Strebel, 1987). Indeed vif antagonizes a host cellular restriction factor, APOBEC3G (apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G), which inhibits HIV infection in nonpermissive cells (Harris, 2002; Jarmuz, 2002; Sheehy, 2003). APOBEC3G is a member of the cytidine deaminase family, which prevents viral cDNA synthesis by deaminating deoxycytidines in the minus-strand retroviral cDNA replication intermediate (Harris, 2003; Yu, 2004). As a result, it creates stop codons or G to A transitions in the newly synthesized viral cDNA, which is then subjected to elimination by host DNA repair machinery (Zhang, 2003). Vif induces the ubiquitination and thus the degradation of APOBEC3G (Li, 2005), permitting the completion of HIV replication cycle.

1.3 HIV-1 replication cycle

The early events of the retrovirus life cycle begin upon fusion of the virus with the host cell plasma membrane. After fusion of viral envelope with the membrane of the host cell, the virus starts the reverse transcription of its ssRNA genome to a double strand DNA, by forming the reverse transcription complex (RTC). The RTC comprises genomic viral RNA associated with nucleocapsid protein (NC), cellular tRNA primer, enzymes reverse transcriptase, integrase and protease, viral protein R (VPR) and matrix protein (MA, p17) (Bukrinsky, 1992, 1993; Fassati, 2001; Briggs, 2003). There is a general agreement on the notion that reverse transcription initiation occurs within the intact capsid cores. However, there are different models to describe the following steps of the journey of the RTC to the nuclear membrane. In an earlier view, capsid was believed to disassemble as soon after fusion of the virion with the cellular membrane and the start of reverse transcription (Miller, 1997; McDonald, 2002; Auewarakul, 2005), so to release free RTCs into the cytoplasm (Freed, 1998; Narayan, 2004). In this view, while completing the reverse transcription process, the RTCs would travel towards the nucleus exploiting the microtubule network, which would help them to overcome the high viscosity of the cytoplasm (Bukrinskaya, 1998; McDonald, 2002).

However, several recent findings support a different model for capsid disassembly, which also implies a role for capsid in the replication cycle (Arhel, 2010), which is far from being merely structural, as initially believed. In this view, capsid integrity would be essential for the completion of the reverse transcription, as it would allow for an appropriate concentration of reverse transcriptase to remain in the proximity of the viral RNA, while at the same time allowing for the diffusion of the necessary cellular factors (like deoxyribonucleotides) through its permeable structure. Indeed, the completion of reverse transcription is necessary for capsid disassembly (Arhel, 2007).

Interactions of some of the nuclear import components with capsid has also been reported, and may have a role to drive and coordinate a timely capsid disassembly prior to nuclear import (Arhel, 2007; Arhel, 2010). In accordance with this model, CA has been reported to play a role in PICs nuclear import (Dismuke, 2006; Yamashita, 2007; Yamashita, 2004). Moreover, premature capsid disassembly induced by some restriction factors (like TRIM5α), impairs reverse transcription (Stremlau, 2006; Perron, 2007; Black, 2010).

Conversion of the viral genomic RNA into DNA is accompanied by reduction of the size of the RTC. At the end of reverse transcription, the complex becomes integration-competent and it is termed preintegration complex (PIC). PIC comprises viral cDNA, integrase, NC, RT, MA, Vpr and some cellular proteins. The PIC protects viral DNA from degradation and facilitates its integration into the host cell chromosome (Miller, 1997; Turelli, 2001). To cross the intact nuclear membrane, the virus exploits the components of the cellular nuclear transport machinery (De Rijck, 2007). Several viral proteins possess nuclear localization signal (Vpr, integrase and matrix protein), therefore these proteins, as well as the central DNA flap (an intermediate triple-stranded cDNA product of reverse transcription) might also be involved into PIC nuclear import (Bukrinsky, 1993; von Schwedler, 1994; Heinzinger, 1994; Gallay, 1997; Nie, 1998; Zennou, 2000; Sherman, 2002; Bukrinsky, 2004; Butterfield-Gerson, 2006). Facilitated by the karyophilic property of the PIC, the lentivirus subfamily is unique in its ability to access the nucleus without requiring the breakdown of the nuclear envelope during mitosis, thus independently of the phase of the cell cycle (Lewis, 1992). The next step to establish a productive infection is the integration of the viral cDNA into the host genome. This process maintains the viral information life-long in the infected cell and it is carried out by the viral protein integrase (IN).

Transcription of the integrated proviral DNA marks the start of the late phase (Freed, 2001). In this phase of the life cycle, the viral DNA is transcribed by the host RNA polymerase II (RNAP) system, and the viral RNAs are processed and exported back to the cytoplasm by regulated trafficking mechanisms. The three viral structural protein precursors — group-specific-antigen protein (Gag), Gag-polymerase (Gag-Pol) and the envelope protein (Env) — are translated in the cytoplasm and transported to the plasma membrane by vescicular, cytoskeletal or other routes.

The process of assembly of the viral particles starts when the precursors processing has not yet been completed: the Gag precursor Pr55 plays a central role in assembly and it is sufficient for viral assembly and production of non-infectious virus particles in the absence of the other viral proteins (Gheysen, 1989; Wills, 1991). Assembly starts with Gag dimerization and multimerization, followed by binding of Gag complexes to genomic viral RNA. These Gag/RNA complexes, together with Gag/Pol, Gag p55 and Env are then transported to the site of assembly, which can be lipid rafts within plasma membrane (Freed, 1998; Gottlinger, 2001) or endosomal vacuoles (Pelchen-Mattheus, 2003; Ono, 2004), depending on the cell types.

Gag gene partially overlaps with Pol and is translated as Gag or GagPol fusion precursors at a Gag/GagPol ratio of 20:1 (Liao, 2004; Arrigo, 1995; Hill, 2001; Shehu-Xhilaga, 2001). The complexes containing Gag and GagPol are rapidly and almost completely associated with host cell membranes (Halwani, 2003). The assembly is initiated by the interaction of Gag NC with the viral RNA as a scaffold, and the complex promotes subsequent Gag–Gag association (Sandefur, 2000; Khorchid, 2002). If NC is deleted from Gag, the virus uses the RNA-binding region of MA for Gag multimerization (Burniston, 1999; Ott, 2005). In the absence of viral RNA, the cellular RNAs (possibly tRNA) are used and incorporated into the virus particle (Muriaux, 2004). The NC–RNA

complex promotes dimerization of CA domains. These observations led to a dimerization model of Gag protein assembly, where formation of the Gag Pr55 dimers leads to the assembly of higher-order products (Alfadhli, 2005).

Gag multimerization takes place at the plasma membrane but more recently has been suggested to commence at intracellular membranes, in multivesicular bodies (Nydegger, 2003; Ono, 2004). In primary macrophages, Gag p55 is found in late endosomes, and viral particles are budding from intracellular membranes into intracellular vesicles (Pelchen-Mattheus, 2003). In T cells, virus assembly utilizes specific microdomains in the plasma membrane known as lipid rafts, which contain a high concentration of cholesterol and saturated lipids. Gag and Env are bound to lipid rafts via lipid interactions of their acylated residues (Ono, 2001; Ding, 2003; Halwani, 2003; Bhattacharya, 2004). Cholesterol depletion and Gag binding to non-raft domains of the membrane

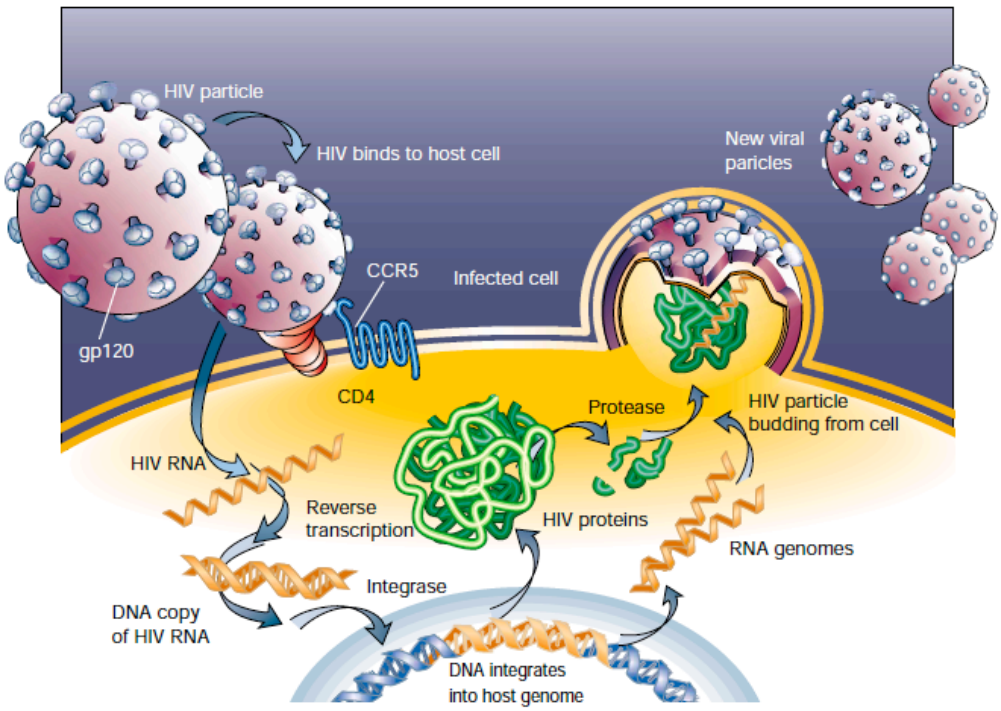


Figure 1-6. Schematic representation of HIV-1 replication cycle. (Weiss, 2001)

severely inhibit production of virus particles, and the perturbation of plasma lipids causes Gag to be redirected away from lipid rafts towards endosomal membranes (Ono, 2001, 2004; Ono, 2005). Gag dimerization and multimerization enhance membrane binding and the association with lipid rafts, and lipid rafts may also serve as concentration platforms for Gag, thereby facilitating higher-order Gag multimerization. Gag and Env form complexes with ganglioside M1, a constituent of lipid rafts, which are revealed within supramolecular structures termed virological synapse (VS) located at the cell–cell interface (Jolly, 2005). Binding to the plasma membrane of the Gag precursor precedes budding of virus particles (Ono, 1999; Paillart, 1999). A domain within Gag, called late domain, interacts with cellular proteins to efficiently release virions from the surface of the cells. The L domain is centered around a PTAP sequence in the p6 region of Gag. This sequence acts in concert with the cellular protein-sorting machine of the ESCRT complex (endosomal-sorting complex required for transport and removal of damaged or misfolded cellular membrane proteins) to promote viral release (Freed, 2002, 2003; Goff, 2003; Strack, 2003; Martin-Serrano, 2003; Martin-Serrano, 2005; Ott, 2005; Gottwein, 2005; Bieniasz, 2006). Gag interacts also with the components of adaptor protein complexes, including AP-2 and AP-3 subunits that control endocytic trafficking (Dong, 2005; Batonick, 2005). The final step of assembly involves a set of large assembly complexes comprising viral and cellular components (Lingappa, 1997; Morikawa, 2004; Alfadhli, 2005; Ono, 2000; Halwani, 2003; Ott, 2005).

Virus particles are released at budding as immature particles containing a spherical shell of structural proteins, not shaped as a central cone-like core. Virions subsequently undergo a maturation step, triggered by the viral protease, which results in a drastic reorganization of the core, with condensation of the inner core, formation of the core shell and convertion of the virus particle into

an infectious virion, ready for disassembly in a newly infected cell. However, the structural principles governing particle maturation have not yet been fully elucidated, and virus maturation is still one of the less known steps in HIV life cycle.

1.3.1 Integration

Integration of the viral genome is a key step of retroviral infection because it is responsible for the stable maintenance of viral genetic information in infected cells and it ensures at the same time the expression of viral genes, and thus production of new progeny viruses.

Integration is performed by the viral enzyme integrase in two well-characterized catalytic steps, referred to as end processing and end joining (Coffin, 1997; Hindmarsh, 1999). A third step, namely gap repair, is carried out by yet poorly known cellular enzymes (Hindmarsh, 1999, Skalka, 2005).

A blunt-ended linear viral genome cDNA is the precursor to integration. 3'End processing occurs largely or entirely before nuclear entry for most retroviruses, including lentiviruses. This step involves removal of a dinucleotide, adjacent to a highly conserved CA dinucleotide, from the 3' strand of the U3 and U5 viral DNA LTRs in a reaction involving a water molecule or other nucleophile (Engelman, 1991). This exposes a 3' hydroxyl group, whose oxygen is used as an attacking nucleophile on the target DNA during the joining reaction, in which the viral DNA is inserted into the cellular DNA. It is believed that one Mg^{++} atom coordinated in the active site of IN facilitates the deprotonation of the water to activate it as a nucleophile. This first reaction step may serve to remove extra nucleotides occasionally added by reverse transcriptase (Patel, 1994) and promote stable complex formation (Li, Mizuuchi, 2006; Ellison, 1994).

The DNA-joining or strand transfer step of integration, which involves the formation of new phosphodiester bonds joining the viral and host DNAs, proceeds without an extrinsic source of chemical energy. This suggests that the energy from the target DNA bonds that need to be broken in this step is used to form the new bonds that join the viral and target DNAs. This cleavage-ligation reaction proceed via a transesterification reaction and not via a covalent intermediate between IN and DNA (Engelman, 1991), as it happens, for example, between topoisomerases and DNA (Champoux, 1977). The joinings occur on the same face of the double helix, flanking a major groove.

Integration is accompanied by duplication of a short sequence from the target site, which flanks the integrated provirus as a direct repeat of 4-6 bp (Coffin, 1997). The 5' ends of the viral DNA and the 3' ends of the host DNA remain unjoined. In the third main step of integration, gap repair, extra nucleotides are trimmed from the 5' ends of the viral cDNA, and these are joined to host DNA 3'ends. This closing of the second joint generating the integrated provirus involves host cell DNA repair enzymes, but the full details remain to be elucidated (Hindmarsh, 1999, Skalka, 2005).

Alternatively, the viral DNA may follow three different fates, all of which do not lead to the formation of a functional provirus. The ends of viral DNA may join to form a 2-LTR ring or the viral genome may undergo homologous recombination producing a single LTR ring. Two-LTR circles are viral cDNA molecules that fail to integrate (Coffin, 1997; Engelman, 1999) and become circularized likely by the non-homologous end joining (NHEJ) cellular repair (Li, 2001). Therefore, 2-LTR circles are a surrogate marker of retrovirus nuclear import (Coffin, 1997) and are indicative of an abortive integration (Engelman, 1999).

Finally, the viral DNA may integrate into itself (autointegration) leading to the formation of a rearranged circular structure (Coffin, 1997). None of these circular forms serve as precursor to integrated provirus, and none appear to

contribute significantly to viral replication, even though they are transcriptionally active (Cara, 1996). Rather, they all appear to be dead-end by-products of aborted infections (Coffin, 1997).

The numerous survival advantages that follow from integration include acquisition by an RNA virus of the long-term stability of chromosomal DNA, the capacity to replicate through mitosis, and the ability to parasitize the elaborate cellular transcriptional apparatus. Thus, while the exceptional ability of HIV-1 to evade and slowly destroy human immunity rests on many mechanisms, the most fundamental may be integration. A stably integrated provirus can occupy a spectrum of transcriptional states, allowing it to evade immune surveillance through latency, while retaining the capacity to scale up transcription rapidly and initiate progeny production (Han, 2007; Bisgrove, 2005) Additionally, integrated proviruses render impossible the clearing of the virus.

For all these reasons integration represent a favorable target for a therapeutic strategy development.

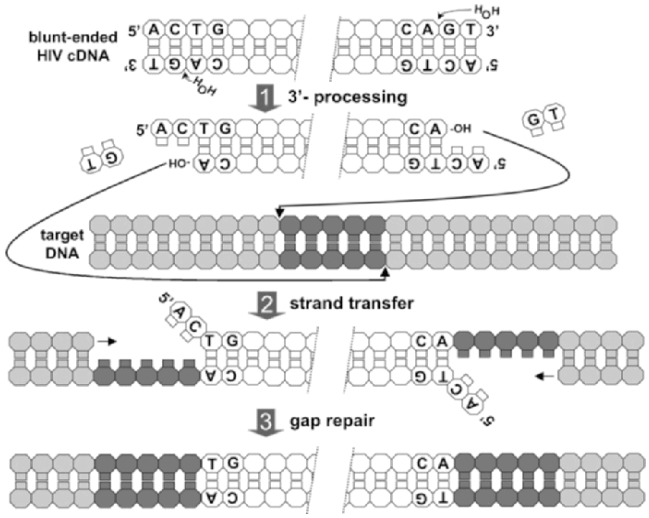


Figure 1–7 The integration reaction. The three main step of end-processing, strand transfer and gap repair are schematized. The five dark shaded bases show the duplication of host DNA flanking the provirus (Poeschla, 2008).

1.3.2 Integration site selection

Integration is not a random process. Each retrovirus genera displays a distinct and specific pattern of integration, which is regulated by viral and cellular factors as well as by local DNA conformation at the site of integration. The unique property of retroviruses to integrate their genome constitutes a major advantage for retrovirus-based gene therapy, which aims at long-term correction of genetic defects. However, the risk of insertional mutagenesis is dramatically real: indeed, in a clinical trial when a murine leukemia virus (MLV)-derived vector carrying the γ-chain cytokine receptor gene was used to treat children suffering from X-linked severe combined immunodeficiency syndrome (Cavazzana-Calvo, 2000), proviral integration near essential cellular genes led to uncontrolled cell proliferation and thus, after initial remission, leukemia-like disorders arose in some cases (Hacein-Bey-Abina, 2003a, 2003b; Cavazzana-Calvo, 2004). It is therefore extremely important to unravel the mechanism of integration site preference of retroviruses.

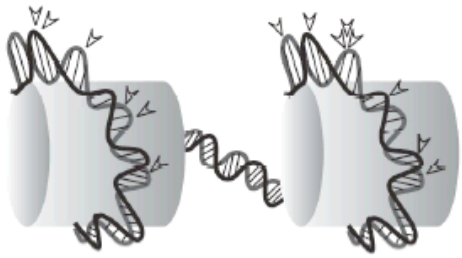


Figure 1-8. Preferred DNA integration sites into nucleosomal DNA. The arrows indicate favorable sites for retroviral integration. These are located into the major groove on the exposed face of the DNA, as it bends around nucleosomes (schematically represented by cylinders). (Cereseto and Giacca, 2004).

So far no primary sequence in the cellular genome has been identified as the preferential binding site for IN but integration does not seems to occur at random on DNA molecules.

Initial experiments with murine retroviruses revealed that DNA assembled with nucleosomes constitutes a better substrate for integration as compared to naked DNA. Analysis of integration hotspots in chromatinized DNA indicates that these are sites at which DNA is probably distorted and exposed, due to the wrapping of DNA around nucleosomes (Bushman, 2005; Bushman, 1994; Muller, 1994; Pruss, 1994; Pryciak, Sil, 1992). Thus, integration preference at this spots can be explained by the fact that the outside surface of the bend is easily accessible for integration.

Although extensive analyses of the sequences flanking the integration sites have revealed some weak biases due to different primary sequence (Carteau, 1998; Pryciak, 1992; Stevens, 1996), a real consensus DNA sequence for retrovirus integration has not been identified (Bor, 1996; Fitzgerald, 1994; Goodarzi, 1997), corroborating other evidences that the structure of the integration target site, more than the primary sequence, has the major influence on site selection during infection. In this view, the influence of certain

sequences on integration efficiency can be explained by the modifications of the local DNA structures induced by these sequences (Muller, 1994; Pruss, 1994). Indeed, several reports have correlated integration in vivo with the presence of nearby repeated sequences, including LINE-1 elements (Stevens, 1994), clusters of Alu repeats (Alu islands) (Stevens, 1996), or topoisomerase II cleavage sites (Howard, 1993). However, in all these studies the number of integration sites analyzed was relatively low. In addition, their conclusions are challenged by another report in which no strong bias could be detected in favor of, or against, integration near Alu or LINE-1 elements (Carteau, 1998). Considered together, the overall conclusion of these studies is that DNA secondary structure, and not DNA primary sequence, is a major determinant for integration site selection.

On the other hand, more recent studies exployting extensive mapping of proviral integration sites underscored the existence of a weak palindromic consensus at the site of proviral insertion for both HIV-1 (Grandgenett, 2005; Holman, 2005; Wu, 2005) and FVs (Nowrouzi, 2006). This, as well as the symmetry observed at integration sites, indicates that IN might posses the intrinsic ability to bind preferentially symmetric DNA sequences.

It has been observed that centromeres are disfavored integration targets in vivo (Carteau, 1998). Inside the cells, centromeres assume a tightly wrapped heterochromatic conformation and this chromatin environment is unfavourable for the expression of most genes. Moreover, alphoid sequences become more resistant to digestion with DNase I than most DNA in isolated nuclei. This indicates that the packing of DNA into centromeric heterochromatin renders it less accessible, and so it disfavours integration.

The improvement of high-throughput sequencing methodologies has recently allowed obtaining a global picture of the integration pattern of several retroviruses (Lewinsky, 2006; Mitchell, 2004; Narezkina, 2004; Schroder,

2002; Wu, 2003). This technology has permitted to map over 500 integration events following infection of a human T cell line with HIV-1 and HIV-1-derived vectors and revealed that integration preferentially takes place in genes highly transcribed by RNA PolII (Mitchell, 2004; Schroder, 2002). This specific profile might indicate that, although deleterious for host cell survival, efficient HIV-1 gene expression is favored to maximize virus propagation. While HIV-1 proviruses are found along the entire length of transcription units (TU), MLV integration within TU is only slightly favored. FVs integration profile is similar to that of MLV (Trobridge, 2006; Nowrouzi, 2006), while Avian sarcoma–leukosis virus (ASLV) shows the most random pattern of proviral insertions since TU and transcription start sites are only weakly or not favored (Mitchell, 2004; Narezkina, 2004). Following analysis of the genomic distribution of proviruses, retroviruses have been clustered in three groups according to their integration preferences: SIV and HIV, MLV and FVs, HTLV-I and ASLV (Derse, 2007).

Transcriptional profiling analysis has been carried out in some of the cell types studied as integration targets, allowing the influence of transcriptional activity on integration-site selection to be assessed. Some of these transcriptional profiling studies were carried out on retrovirus-infected cells (Lewinski, 2005; Mitchell, 2004; Schroder, 2002), so that the data reflected the influence of infection on cellular gene activity (Bushman, 2005; Corbeil, 2001; Mitchell, 2003; Schroder, 2002; van 't Wout, 2003). Analysis of the microarray data revealed that the median expression level of genes hosting HIV integration events was consistently higher than the median expression level of all the genes assayed on the microarray. Transcriptional profiling studies for HIV vector integration in SupT1 cells also indicates that genes that are activated by infection are favoured integration targets (Schroder, 2002). Since the majority of the HIV-1 infected cells die very shortly after infection due to cytopathic

effects or by immunoclearance, it has been hypothesized that the bias for integration into transcriptionally active regions is a strategy to maximize its expression to produce viral progeny. Conversely, a latent infection can be established by silencing the basal viral expression by integration into heterochromatic regions (Jordan, 2001). Taken together, these data indicate that the integration is favoured in the transcriptionally active genes. These regions are characterized by an open chromatin structure, which is more accessible to the integration apparatus. Since histone posttranslational modifications are involved in chromatin condensation and gene regulation (Allis, 2007), a role for acetylation and methylation of histones in directing retroviral integration has been investigated (Wang, 2007). In this study 40,569 unique sites of HIV-1 integration have been sequenced. Analysis of integration site positions in the densely annotated ENCODE (Encyclopedia of DNA elements) regions revealed that integration was favoured near transcription associated histone modifications, including H3 acetylation, H4 acetylation, and H3 K4 methylation, but was disfavored in regions rich in transcription-inhibiting modifications, which include H3 K27 trimethylation and DNA CpG methylation.

Specific interactions between PIC components, host proteins and/or chromatin architecture could critically contribute to integration target-site selection. It is well established for instance that LEDGF/p75 is a key cellular partner of HIV-1 IN and an essential player in HIV-1 integration target-site specificity (Cherepanov, 2003; Maertens, 2003; Turlure, 2004). Following depletion of LEDGF/p75, proviral insertion within TU is drastically reduced (Cherepanov, 2003; Ciuffi, 2006) and, despite integration does not become completely random, new trends appear, including integration near CpG islands. In addition, bioinformatics study of 15 HIV-1 integration site data sets in different cell types showed that frequency of integration within transcription

units is correlated with the expression levels of LEDGF/p75 (Marshall, 2007). The finding that IN proteins belonging to animal retroviruses other than lentiviruses does not display an evident affinity for LEDGF/p75 suggests that other cellular and/or viral proteins might act as cofactors during proviral integration (Cherepanov, 2007; Busschots, 2005). This hypothesis could, at least in part, explain the differences in retroviral integration patterns observed so far.

1.4 Current anti-retroviral therapies

HIV is the primary etiological agent of AIDS. Search for effective treatment of HIV remain a top priority for the international research community. Nowadays, although the use of antiretroviral drugs remains an effective treatment to control AIDS progression, new strains of HIV subtypes continue to evolve. They are demonstrating drug resistance due to their effective and continuous mutations in vivo. Similarly, failure of HIV vaccine development is due to the unique mechanisms of HIV in evading the host immune response. Thus, it is imperative to further investigate the molecular basis of HIV pathogenesis and identify new targets for therapeutic intervention.

Currently, 23 antiretroviral drugs are approved for the treatment of HIV-1 infection, the majority of which target two essential viral enzymes: reverse transcriptase and protease. Reverse transcriptase inhibitors (RTI) can be subdivided into two classes, based their distinct mechanisms of action, and include nucleoside/nucleotide reverse transcriptase inhibitors (NRTI) and nonnucleoside reverse transcriptase inhibitors (NNRTI). Several RTI have been combined into fixed-dose combination tablets, which contain either two or more NRTI, or two NRTI and the NNRTI efavirenz. The protease inhibitors (PI) comprise the third class of approved antiretroviral drugs, all of which

inhibit the essential proteolytic processing of viral proteins. Other classes of drugs act extra-cellularly to prevent the entry of the virus into the host cell (fusion or entry inhibitors). Enfuvirtide (T-20, Fuzeon™, Roche Laboratories Inc. and Trimeris, Inc. USA), a fusion inhibitor, was introduced in 2003, and acts to mimic the viral gp41 polypeptide, thereby blocking the fusion of the viral and cellular membranes. Maraviroc (UK-427857, Selzentry™, Pfizer Inc. USA) a newly approved member of the entry inhibitors class, targets coreceptor binding by HIV-1 (CCR5 antagonist).

For the last decade, combination therapy, known as highly active antiretroviral therapy (HAART), has been the gold standard of care for HIV-1 infected individuals in most developed countries. HAART has been credited with a highly significant reduction in HIV/ AIDS mortality by reducing plasma viremia, increasing CD4+ lymphocytes count, reducing immune activation, and restoring lymph node architecture (Gulick, 1997; Hammer, 1997; Ledergerber, 1999; Lederman, 1997). Current frontline HAART includes combinations of small-molecule inhibitors of PR and RT (nucleoside RT inhibitors, NRTIs; non-nucleoside RT inhibitors, NNRTIs). A peptide inhibitor of the viral TransMembrane Glycoprotein (TM) that inhibits viral entry is used in salvage therapy, and a small-molecule IN inhibitor, Raltegravir, was recently approved by the FDA, as discussed in the next section.

On the other hand, many currently available antiretroviral drugs have also been associated with long-term side effects, inability to eradicate latent reservoirs of HIV-1 (Chun, 2000; Finzi, 1997), development of drug resistance, and eventual failure of therapy (Deeks, 2003; Little, 2002). Moreover, new infections with HIV-1 strains exhibiting multiclass drug resistance, together with the continual evolution of drug-resistant virus strains (Blankson, 2002; Saksena, 2003) highlights the urgent need to develop novel antiretroviral drugs. Several potential targets for the development of new antiretroviral drugs have

been identified due to the substantial increase in the knowledge of the structural biology of HIV-1 and its interaction with host cells (Turner, 1999). Among the viral targets being evaluated for new drug development, the most exciting opportunities currently under consideration include (i) viral entry, with a major focus on blocking the interaction of the virus with its two major coreceptors, CCR5 and CXCR4, (ii) integration of viral DNA into the human genome, and (iii) maturation of the viral particle. The use of different compounds to block the interaction of the viral envelope with its major receptor (the CD4 protein) or coreceptors (CCR5 or CXCR4) is relatively advanced (Poveda, 2006; Weber, 2006). Inhibition of virion maturation, that is, the blockage of the cleavage of Gag (p55) and Gag/Pol (p160) precursor polyproteins into structural proteins and enzymes (i.e. protease, reverse transcriptase, and integrase), represents another intriguing opportunity to develop antiretroviral drugs. Although viral maturation inhibitors are less advanced in clinical development than protease, reverse transcriptase and entry inhibitors, the first member of this drug class (PA-457, bevirimat) has been shown to reduce plasma viral RNA load by > 1 log10-fold in phase IIa clinical trials (Li, 2003).

The HIV-1 integrase inhibitors act at a point in the viral lifecycle following classical antiretroviral drugs such as NRTI, NNRTI and the most recently developed entry inhibitors, but prior to the effect of PI. Viral integration is a particularly desirable target because HIV-1 integrase has no known human equivalent and offers the possibility of high drug specificity with limited cellular toxicity. Typical products of the HIV-1 integration process include linear and nonintegrated DNA, which are degraded in cells within 24 hours, plus 1- and 2-LTR circles (formed by the ligation of the long terminal repeat ends of the linear HIV-1 genome) (Barbosa, 1994; Pauza, 1994; Shin, 1994).

Finally, blocking virus-host interactions is an important objective for the future. Indeed viral proteins are specific targets with no cellular equivalents, but

are also able to evade the pharmacological blockage because of the fast evolution of resistant mutants driven by the selective pressure of the drug. Conversely, cellular proteins which interacts with the virus evolve slowly and would be an ideally stable target for new drugs, if it was possible to block specifically the interaction of the cellular protein with the virus and not its, often essential, physiological function (Al-Mawsawi, 2007). Frontline research in this field has therefore concentrated efforts in the search of **inter-face inhibitors,** i.e. compound able to disrupt the interaction of a viral protein with its cellular co-factor, without interfering with the cellular function of the latter (see paragraph 1.4.2).

1.4.1 Integrase inhibitors

Integrase inhibitors belong to a new class of antiretroviral compounds (integrase strand transfer inhibitors, InSTIs) that offer an attractive alternative to other antiretrovirals in the setting of salvage therapy and in treatment-naïve patients, firstly and most importantly, because of their different target enzyme and, as a consequence, potent activity against virus strains that carry resistance mutations against drugs from other classes (Hazuda, 2000; Hazuda, 2004). Raltegravir (RAL) was the first drug in this class to be approved by the United States Food and Drug Administration (FDA) for use in highly treatment-experienced HIV-1-infected patients in October 2007 (Grinsztejn, 2007). In January 2009, the FDA granted traditional approval for the 400mg RAL tablets (Isentress; Merck and Company, Whitehouse Station, New Jersey, USA) for HIV-1 treatment in treatment-experienced individuals in combination with other antiretrovirals. In July 2009, the FDA extended approval for Isentress for the treatment of treatment-naive patients. RAL interferes with the strand-transfer reaction of viral integrase and it markedly reduces viral load.

A second drug in this class, Elvitegravir, is in the late stages of clinical development and currently in phase III clinical trials (Klibanov, 2009).

Other InSTIs, for example, MK-2048 (Merck, NJ, USA) and GSK1349572 (GlaxoSmithKline, NC, USA) (Glaxo-SmithKline, London, UK) are in early clinical development. InSTIs interact directly with the active site of HIV-1 integrase, and this binding only occurs in complex with reverse transcribed viral DNA.

Current InSTIs seem to have a relatively low threshold for drug resistance.

Resistance against RAL is conferred through one (of at least three currently identified, N155H, Q148R and Y143R/C) key mutation, with secondary mutations balancing and increasing the replicative capacity (Malet, 2008; Malet, 2009; Ceccherini-Silberstein, 2009; Canducci, 2009). Of the two InSTIs that are currently in clinical development (MK-0248 and GSK-364735), MK-0248 may prove to be beneficial for the treatment of HIV strains that carry resistance mutations to RAL and Elvitegravir.

1.4.2 Integrase inter-face inhibitors

The concept of rational designing inhibitors of protein-protein binding by targeting the interface between the two interacting molecule is relatively recent, owing to the technical complexity of defining finely the dynamic interactions at the contact surface between two macromolecules (Hajduk, 2002; Ryan, 2005; Whitty, 2006). Indeed usually protein-protein interactions (PPIs) involve large areas without obvious features to target or classical binding pockets, into which a small molecule inhibitor could dock easily. Nevertheless, in the last decades some small molecules which functions as PPI inhibitors has been found for use as potential cancer therapeutics – for instance targeting the p53/MDM2 or the Myc/Max interactions (Arkin, 2004; Pagliaro, 2004; Vassilev, 2004; Fotouhi,

2005; Laurie, 2007; Patel, 2008) – or as modulators of nervous signaling in the central nervous system (Blazer, 2009). More recently, two PPIs inhibitors reached clinical testing in humans (Arkin, 2009).

The main step forward in the development of small molecule PPIs inhibitors came from the discovery that protein interaction surfaces contain 'hot spots', that is, small regions of the interaction interface responsible for a great part of the binding energy in the interaction. These regions are often enriched in aromatic and positively charged residues (Bogan, 1998; Delano, 2002; Darnell, 2008; Shulman-Peleg, 2008; Fletcher, 2006).

The ongoing search for potent second generation integrase inhibitors, as alternative or complementary to the InSTIs class, has only recently started to exploit the possibilities disclosed by the knowledge in the field of protein-protein interactions inhibitors. A few reports of PPI inhibitors in the field of anti-HIV research exist for other HIV enzymes: a peptide inhibitor of reverse transcriptase dimerization had been described early in 1999 (Morris, 1999), and other groups are working on small molecules inhibitors of HIV protease dimerization (Shultz, 2004; Lee and Chmielewski, 2010). However, in both cases these molecules are directed at inhibiting the interaction between the two subunits of a viral enzyme, rather than that between a viral and a cellular protein. The latter approach offers the advantage of decreasing the rapidity the virus will develop resistance, since the selective pressure from the drug will have no influence on the cellular protein. Thus, the virus should evolve some more complicated evasion strategy to circumvent the obstacle of a viral-cellular PPI inhibitor.

The first application of this principle to anti-HIV research came recently from Debyzer's group. They designed by virtual screening a series of 2-(quinolin-3-yl)acetic acid derivatives (LEDGINs) that resulted to be potent inhibitors of the LEDGF/p75-integrase interaction, at submicromolar

concentration. These molecules are able to inhibit HIV-1 replication by blocking the integration step. These inhibitors do not target the catalytic domain of the enzyme, but act on the protein-protein interaction between the viral protein and a cellular host factor, being the first HIV-1 integrase inhibitors functioning with a genuine allosteric mechanism. As a consequence, this class of molecules did not show cross-resistance with two clinical integrase inhibitors, Raltegravir and Elvitegravir (Christ, 2010).

The interaction between integrase and other known cellular co-factors can be similarly addressed. For instance Transportin SR2 (TRN-SR2) can be a promising target for the development of novel PPI inhibitors interfering with the nuclear entry step of HIV (Thys, 2009).

1.5 Integrase post-translational modifications

Post-translational modifications (PTMs) represent a versatile, rapid and generally reversible mechanism to finely modulate protein functions and properties, such as enzymatic activity, protein structure, subcellular localization, stability and interaction with other binding partners, and can have therefore a regulatory function in many biological processes. PTMs alter the chemical nature of an amino acid residue either by chemical moieties (acetylation, phosphorylation, amidation, methylation, nitrosylation, ADP-ribosylation, N- and O-linked glycosylation, and carbonylation) or by other proteins (ubiquitin, SUMO and FAT10, among others) (Deribe, 2010; Walsh, 2005). Some PTMs are specifically cross-related to others, creating a rational "code" for the regulation of protein function according to the different contexts.

Integrase is post-translationally modified during the viral replication cycle. Manganaro *et al.* (Manganaro, 2010) shown that integrase is phosphorilated at Serine 57 by the c-Jun N-terminal kinase (JNK), an enzyme expressed in

activated peripheral blood T lymphocytes (PBL) but not in resting T cells. This modification permits efficient HIV-1 infection in activated PBLs, as opposite to impaired infections observed in resting PBLs. Indeed, Manganaro *et al*. (Manganaro, 2010) demonstrated that phosphorilated integrase is modified by Pin-1, which catalyzes a conformational change. This enhances integrase stability and allows for integration to occur.

Our and other groups (Cereseto, 2005; Terreni, 2010; Topper, 2007; Apolonia, 2007) have demonstrated that integrase is post-translationally modified by acetylation on at least three lysine residues (264, 266 and 273), and that this modification finely tunes integrase function in the context of the viral infection. The importance and details of this PTM in the viral replication cycle is discussed exstensively in the following chapters.

Recently it has been shown that integrase is positively regulated also by SUMOylation, which seems to influence the interaction of integrase with other cellular co-factors required for efficient viral replication, similarly to what already reported for integrase acetylation (Allouch, 2009). Indeed SUMOylation defective IN mutants, even though retaining wt catalytic activity, displayed impaired integration (Zamborlini, 2011).

Moreover, it was found that HIV-1 integrase is also ubiquitinated and subsequently degraded by the proteasome (Mousnier, 2007; Mulder, 2000; Devroe, 2003)

1.6 Histone post-translational modifications: Acetylation

DNA is present in the nucleus in the form of chromatin, the basic unit of which is the nucleosome core particle, which consists of 147 base pairs of DNA wrapped in 1.65 left-handed superhelical turns around of an octamer of histone molecules, which is composed of two molecules each of four types of core

histones: H2A, H2B, H3 and H4 (Davey, 2002). The fact that eukaryotic DNA is packed into chromatin constitutes a physical barrier to enzymes and regulatory factors to reach the DNA molecule for replication, transcription, recombination and repair. Histones, the main protein component of chromatin, not merely play a role in packaging DNA. The tails and the globular domains of histones are subjected to reversible covalent modifications by acetylation, phosphorylation, methylation, ubiquitination, sumoylation, and less commonly by citrullination and ADP-ribosylation. These posttranslational modifications (PTMs) can alter DNA-histone interactions or the binding of proteins to chromatin constituting a chromatin remodeling mechanism that can dynamically change and regulate the accessibility of chromatinized DNA to regulatory factors. In particular, the N-terminal tails of the core histones extend beyond the nuclesomes and can have their characteristics significantly altered by PTMs (Figure 1-8). H3 has the greatest number of modifications currently identified, followed by H4, H2B, and H2A. The C-terminal tails also contain PTMs, but they are few in number, as are those for the non-tail regions.

The term “histone code” has been coined for the combinatorial diversity of post-translational histone modifications (Fischle, 2003; Strahl, 2000).

Lysine acetylation weakens electrostatic DNA–histone interations and provides a more open chromatin structure correlating with gene transcription.

Acetylation is mediated by acetyl-CoA–dependent histone acetyl-transferases (Marmorstein, 2001) and reversed by zinc-dependent histone deacetylases or NAD dependent sirtuins (Denu, 2005).

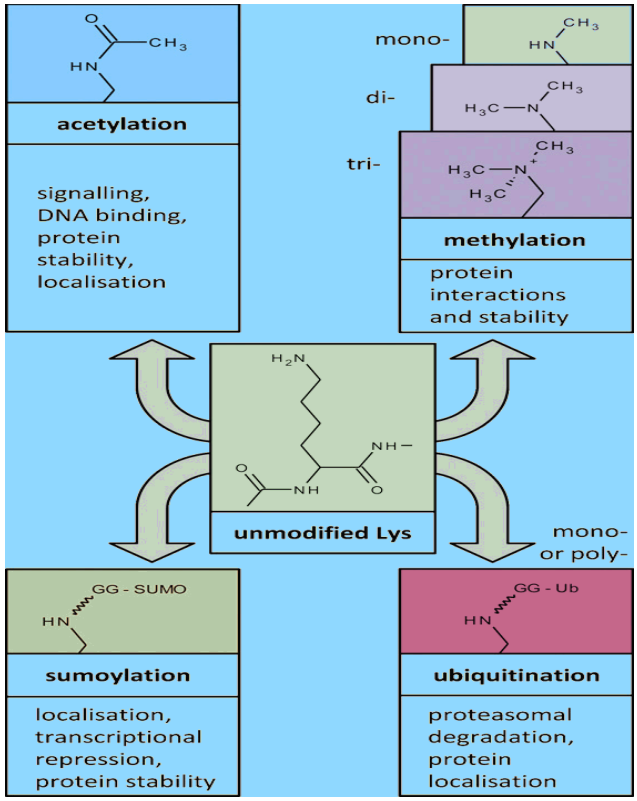


Figure 1–9. Lysine modifications. Lysine residues are subject to posttranslational modifications with varying functional consequences. Switching between modifications allows to alter protein function (Spange, 2009).

The correlation between histone acetylation and increased transcription has been known for many years (Allfrey, 1964; Vidali, 1968; Allfrey, 1996). This is due to reduced ionic interactions of the positively charged histone tails with the negatively charged DNA backbone and reduced internucleosomal interactions. Additionally, modified histones generate specific binding sites for protein interactions (Lohrum, 2007), for example with transcription factors and histone acetyltransferases (HATs). Binding of HATs to acetylated lysine moieties via bromodomains can provide a feed-forward mechanism for acetylation.

Post-translational ε-amino lysine acetylation of histone and other proteins is highly reversible. In humans, there are 18 potential deacetylase enzymes, HDAC1 to HDAC11 and SIRT1 to SIRT7, which are responsible for the removal of acetyl groups and maintenance of the equilibrium of lysine acetylation in histones. Like HATs, histone deacetylases (HDACs) also possess substrate specificity and accumulating evidence suggests that many, if not all, HDACs can deacetylate non-histone proteins at least *in vitro*.

1.7 Histone Acetyl-Transferases: p300 and GCN5

Histone Acetyl Transferases (HATs) are evolutionarily conserved from yeast to man and form multiple subunit complexes (Kimura, 2005). Unlike HDACs, HATs are more diverse in structure and function (Yang, 2004). In mammals, over 30 HATs display distinct substrate specificities for histones and non-histone proteins.

They are grouped into two general classes: A- and B-type HATs, of which A-type HATs mainly carry out transcription-related acetylation. Based on the protein homology, substrate specificity, and functional consequences, nuclear HATs can be further classified under different groups (Batta, 2007): p300/CBP family (Roth, 2001); MYST family that consists of Sas2, Sas3, Esa1, MOF, Tip60, MOZ, MORF, HBO1 (Sterner, 2000); the GNAT superfamily that includes Hat1, Gcn5, PCAF, Elp3, Hpa2 (Iyer, 2004); Nuclear receptor coactivators like SRC-1, ACTR, TIF2 (Goodman, 2000); TAFII250 family (Kundu, 2000); and TFIIIC family (Sterner, 2000).

Family	HAT	Targets	Function
GNAT	Gcn5	H2B, H4, c-Myc	Coactivation
	PCAF	H3, H4, c-Myc, p53, MYOD, E2F	Coactivation
	Elp3	H2A, H2B, H3, H4	Elongation
	ATF-2		Activation
MYST	MOZ	H3, H4	Coactivation
	Ybf2/Sas3	H3, H4	Elongation
	Sas2	H3, H4	Silencing
	Tip60	H2A, H3, c-Myc, AR	DNA-repair, apoptosis
	Esa1	H2A, H3, H4	Cell cycle progression
	MOF	H3, H4	Dosage compensation
p300/CBP	CBP	H2A, H2B, H3, H4, pRb, E2F, p53, AR, c-Myb, MYOD, FOXO	Global coactivation
	p300	H2A, H2B, H3, H4, pRb, E2F, p53, AR, c-Myb, MYOD, FOXO	Global coactivation
Nuclear receptors coactivators	SRC-1	H3, H4	Steroid receptor coactivator
	ACTR	H3, H4	Steroid receptor coactivator
$TAF_{II}250$	$TAF_{II}250$	H3, H4	Transcriptional coactivator
TFIIIC	TFIIIC220		RNA polimerase III transcription initiation
	TFIIIC110		RNA polimerase III transcription initiation
	TFIIIC90	H3	RNA polimerase III transcription initiation

Figure 1-10. A-type HAT families and function of selected members (von Wantoch Rekowski, 2010; Sterner, 2000).

Nuclear A-type HATs are found in cells as components of evolutionarily conserved and cooperatively acting high-molecular-weight complexes (Grant, 1999). The cytoplasmic B-type HATs acetylate *de novo* synthesised free histones, promoting their nuclear localization and deposition onto newly synthesised DNA (Allis, 1985; Ruiz-Carrillo, 1975). Many HATs show a distinct pattern of substrate specificity, even towards histones, depending on the subunit composition of HAT complexes and the specific recruitment to the target sites of acetylation (Waterborg, 2002). The HAT domain consists of a conserved core lying at the bottom of a deep hydrophobic cleft and is responsible for binding to the acetyl-CoA. The N- and C-terminal regions flanking the core are less conserved and bind to the substrate: it is due to their variability that HATs can discriminate between different targets (Bottomley, 2004).

HAT complexes also affect chromosome decondensation, DNA-damage repair and the acetylation of non-histone targets (Lee, 2007). The ability of HAT to acetylate non-histone substrates such as transcription factors or chromatin-related proteins, is referred to as factor acetyltransferase (FAT) activity (Sterner, 2000). The acetylation of such substrates can modify protein-DNA or protein-protein interaction. In some cases acetylation increases the DNA-binding capacities of the substrate, as it occurs with the transcriptional activators GATA-1 (Boyes, 1998), p53 (Gu, 1997; Sakaguchi, 1998; Liu, 1999), E2F (Martinez-Balbas, 2000; Marzio, 2000) and TAL1 (Huang, 2000). In other cases, acetylation inhibits the interaction of the substrate with a co-repressor, as it happens when P/CAF acetylates E1A 12S and TAL1 (Huang, 2000; Zhang, 2000). Conversely, the acetylation of the chromatin associated protein HMG-17 by P/CAF decreases its affinity for nucleosomes (Herrera, 1999). Acetylation of other proteins is also involved in other cellular functions,

such as the control of protein stability or nuclear import of proteins (Martinez-Balbas, 2000; Bannister, 2000).

HATs are themselves regulated by phosphorylation and by the interaction with other proteins (Berger, 1999).

Many HATs possess an evolutionarily conserved protein module specifically recognising acetyl-lysines, the bromodomain, which directs chromatin associated proteins to acetylated histones (Dhalluin, 1999; Lee, 2007).

Two HATs belonging to two different enzyme families have been shown to be involved in acetylation of the C-terminal tail of HIV-1 integrase, that is p300 and GCN5.

P300 (alternative name: E1A-associated protein p300; gene name: EP300) and its close homolog CBP are probably the most widely studied histone acetyltransferases. Both contain a bromodomain and are often found within the same complexes. HAT p300 preferentially acetylates histone H2B lysine 12 and 15, H3 lysine 14 and 18 and H4 lysine 5 and 8 (Schiltz, 1999) but seems to have a broad substrate acceptance for histones and non-histone proteins (Kimura, 2005; Liu, Wang, 2008).

P300 has been implicated in a number of diverse biological functions such as proliferation, cell-cycle regulation, apoptosis, differentiation, and DNA damage response (Yier, 2004; Goodman, 2000). It is a potent transcriptional coactivator, which is recruited to specific promoters through interaction with constantly expanding array of transcription factors, like E1A, c-Jun, c-Myc, c-Fos, TFIID, MyoD, nuclear hormone receptor and E2F-1, through which it may integrate several signaling pathways with transcriptional responses (Kundu, 2000; Chan, 2001; Vernarecci, 2010). Both p300 and CBP have been found to possess intrinsic HAT activity (Bannister, 1996; Ogryzko, 1996). Unlike most

of the other HATs, that have limited substrate specificity for histone and nonhistone proteins, p300 and CBP are capable of acetylating all the four core histones and also a wide variety of nonhistone proteins with functional consequences in several cases (Das, 2005). P300 is a multifunctional protein and not all of its functions are HAT activity dependent. However, several important cellular functions are regulated by p300-mediated protein (both histone and nonhistone) acetylation including DNA repair, cell cycle, differentiation, and establishment of retroviral pathogenesis (Giordano, 1999; Quivy, 2002). Lysine-specific acetylation of histone H3 and H4 by p300 in conjunction with chromatin remodeling and other covalent modifications establishes the active state of chromatin in a gene-specific manner (Barrero, 2006).

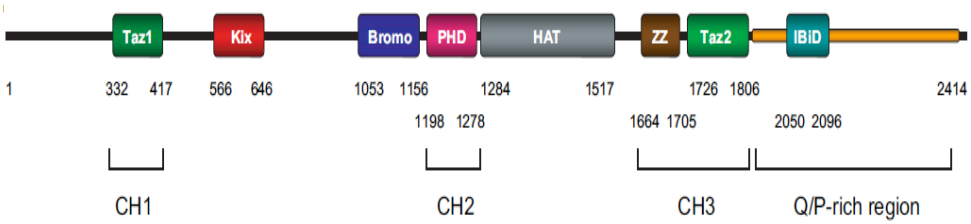


Figure 1-11. Domain structure of human p300 (Teufel, 2007).

Gcn5 (general control nonderepressible 5), together with PCAF (p300/CBP associated factor), is part of the GNAT family of acetyltransferases, which are important for transcriptional initiation.

GCN5 comprises a N-terminal domain, a catalytic core domain (the HAT domain) and a C-terminal bromo-domain.

The catalytic site and mechanism of histone acetylation by Gcn5 have been defined as a result of structural determinations and mutational analyses. The

acetylation reaction involves the formation of a ternary complex between histones, acetyl-CoA, and enzyme. The e-amino group of histone lysine residue is deprotonated by the enzyme and then carries out a nucleophilic attack to the acetyl-CoA cofactor (Rojas, 1999; Tanner 1999). Residue Glu 173 within the cleft region of Gcn5 HAT domain is conserved among the various Gcn5 homologs and it is potentially critical for function, since replacing glutamate with glutamine yielded a mutant yeast impaired in HAT activity *in vitro* (Tanner, 1999), as well as inefficient for growth and transcription *in vivo* (Trievel, 1999). Residue Glu 173 seems to act by deprotonating the lysine substrate through its carboxyl moiety (Tanner, 1999).

The N and C-terminal domains of GCN5 diverge from other structurally related HATs and seem to contribute to substrate specificity (Poux, 2002). In particular, the N-terminal domain seems to be involved in chromatin recognition, as deletion mutants of the N-terminal portion are unable to acetylate nucleosomal histones, while retaining their enzymatic activity on free histones (Xu, 1998). The C-terminal bromodomain of GCN5 was shown to participate in cooperative acetylation of nucleosomes by facilitating the acetylation of the N-terminal tail of H3 when this is part of a nucleosome where the other H3 is already acetylated (Li, Shogren-Knaak, 2009).

Gcn5 is highly conserved in evolution and shows a global role in acetylation of histone H3 and H4 lysines on the N-terminal tail (Kuo, 1996; Brownell, 1996) but also targets non histone transcription factors, like Myc, (Liu, 2008) BRCA1 (Oishi, 2006) and p53 (Gamper, 2008). Acetylation of these transcription factors often leads to a change in their stability or activity (Berger, 1999). A recent study showed that Gcn5 is also involved in telomere maintenance and that its deletion leads to embryonic lethality, chromosomal fusions and dysfunctional telomeres (Atanassov, 2009).

Both p300 and Gcn5 had already been reported to play some role in HIV infection. Indeed, they are recruited by Tat and trigger chromatin remodeling by acetylating nucleosomes at the viral LTR promoter, which in turn activates transcription of proviral genes (Benkirane, 1998; Hottiger, 1998; Marzio, 1998; Col, 2001; Lusic, 2003). The ability of Tat to recruit histone acetyltransferases explains the previous observation that Tat transactivation is accompanied by chromatin remodeling at the LTR insertion site (Verdin, 1993; Van Lint, 1996). In addition, it has been demonstrated that both p300 and GCN5 acetylate Tat itself on the same residues (lysine 50 and 51), and that this post-translational modification enhances Tat transactivation activity (Kiernan, 1999; Col, 2001; Ott, 1999). In particular, both HATs acetylate the TAR binding domain of Tat at the early phase of transcriptional elongation and this increases the rate of dissociation of Tat from the TAR region, leading to increased activation of transcription from the LTR (Kiernan, 1999; Col, 2001).

1.8 Acetylation of non-histone proteins

Post-translational acetylation on the ε-amino group of lysines prevents positive charges from forming on the amino group, and as a result, has a significant impact on the electrostatic properties of a protein.

Following the identification of nuclear histone acetylases, a number of non-histone proteins have been identified as substrates for PCAF and/or p300/CBP (Kim, 2006). Many of these substrates are involved in the regulation of transcription and include p53, E2F1, EKLF, TFIIEβ, TFIIF, TCF, GATA1, HMGI(Y) and ACTR (Gu, 1997; Imhof, 1997; Boyes, 1998; Munshi, 1998; Waltzer, 1998; Zhang, 1998; Chen, 1999; Martınez-Balbas, 2000; Marzio, 2000).

P53 is acetylated by p300/CBP at multiple lysine residues at the C-terminal DNA binding regulatory domain. Acetylation of p53 by p300/CBP activates its sequence-specific DNA binding activity and, consequently, increases activation of its target genes.

DNA binding proteins such as HMG1 and even non-nuclear proteins such as α-tubulin were already known to be modified by acetylation (Sterner, 1979; L'Hernault, 1985). Thus, substrates for acetylation now include DNA-binding proteins (histones and transcription factors), non-nuclear proteins (tubulin) and proteins that shuttle from the nucleus to the cytoplasm, such as the importin-α family of nuclear import factors (Bannister, 2000).

Acetylated targets can be specifically recognized by bromodomain-containing proteins (Mujtaba, 2007). However, the molecular mechanisms by which acetylation may control protein function and effect cellular regulation are still poorly known.

1.8.1 Acetylation and protein function

The consequences of acetylation depends on where within the protein acetylation takes place. In the case of four site-specific DNA-binding transcription factors, p53, E2F1, EKLF and GATA1, the acetylation site falls directly adjacent to the DNA-binding domain and acetylation results in stimulation of DNA binding (Gu, 1997; Boyes, 1998; Zhang, 1998; Martınez-Balbas, 2000). In contrast, the lysines acetylated within the HMGI(Y) transcription factor fall within the DNA-binding domain and result in disruption of DNA binding.

Besides affecting DNA binding, acetylation also regulates protein–protein interactions. Acetylation of histones seems to generate a recognition site for the

bromodomain, a structure conserved in many proteins, including acetylases (Dhalluin, 1999).

A third function regulated by acetylation is protein stability. Analysis of in vivo acetylated E2F1 shows that the acetylated version has a longer half-life (Martınez-Balbas, 2000). Acetylation of cyclin A regulates its degradation (Mateo, 2009). For α-tubulin also, the correlation has been made between acetylated α-tubulin and microtubule stability (Takemura, 1992).

1.8.2 Acetylation of viral proteins

Recent investigations revealed that several virally encoded proteins are substrates for acetylation by cellular HATs (Alfonso, 2007; Cereseto, 2005; Col, 2001; Kiernan, 1999; Madison, 2002; Marzio, 2000; Mu, 2002; Ott, 1999; Shimazu, 2006; Topper, 2007; Xie, 2002; Zhang, 2000).

HIV-1 Tat can be acetylated by three different HATs at three specific lysine residues: lysine 28 is targeted by PCAF, while lysines 50 and 51 are substrates for p300/CBP and GCN5 (Marzio, 1999; Pagans, 2005; Col, 2001; Kiernan, 1999; Ott, 1999; Van Duyne, 2009). Most interestingly, the acetylation of each individual lysine differently affects Tat functionality at the molecular level. Lysine 28 acetylation enhances the ability of Tat to recruit the P-TEFb complex (Kiernan, 1999), while modification of lysine 50 leads to Tat dissociation from TAR RNA (Deng, 2000). Moreover, the acetylation at lysine 50, but not that at lysine 28, creates a high-affinity binding site for the bromodomain of PCAF (Dorr, 2002; Mujtaba, 2002).

The accepted model explaining the role of Tat acetylation in the regulation of its activity is as follows (Nakatani, 2002): Tat acetylated at lysine 28 would efficiently recruit P-TEFb, through its interaction with Cyclin T1 (Peterlin, 2006), and interact with TAR to enhance the processivity of RNA Pol II

(D'Orso, 2009). Acetylation of Tat at lysine 50 would then help the recycling of Tat by inducing its release from TAR. This release seems to be promoted by the bromodomain of PCAF, which competes with TAR for the binding to acetylated lysine 50. In agreement with the proposed model, the three HATs capable of acetylating Tat all efficiently cooperate to stimulate transcription from the 5′ LTR of HIV-1 proviral DNA (Kaehlcke, 2003)

In addition, it has been recently shown that Tat acetylation at K28 enhances its effect on microtubule dynamics and thereby promotes the activity of Tat to induce apoptosis in T lymphocytes (Huo, 2010).

Adenovirus protein E1A is also acetylated by cellular HATs, resulting in a wide variety of functional effects. The oncoprotein E1A is known to play a key role in the dramatic alteration of various essential cellular activities, leading to cell transformation and tumorigenicity (Gallimore, 2001). E1A was found to be acetylated by p300/CBP and PCAF at a lysine residue (located at position 239 in E1A 12S) in the C-terminal domain (Madison, 2002; Zhang, 2000), which is involved in the interaction with the transcriptional corepressor C-terminal binding protein (CtBP) (Boyd, 1993). One study showed that acetylation at lysine 239 inhibits the interaction between E1A and CtBP, leading to the loss of CtBP-mediated transcriptional repression and the increase in the transforming potential of E1A (Zhang, 2000). Conversely, a subsequent report revealed that, rather than interfering with CtBP recruitment, acetylation at lysine 239 prevents the nuclear import of E1A by abrogating its interaction with importin α (Madison, 2002). According to this hypothesis, acetylation may act to either attenuate the nuclear functions of E1A or redirect a portion of the protein to cytoplasmic targets.

African swine fever virus (ASFV) protein pE120R, essential for virus transport from assembly sites to plasma membranes, is acetylated at the N-terminal Ala residue during infection (Alfonso, 2007).

HIV-1 integrase has been recently shown to be the target of acetylation by two different cellular HATs, p300 and GCN5 (Cereseto, 2005; Topper, 2007; Apolonia, 2007; Terreni, 2010). In particular three lysines at the carbossi-terminal domain of the viral enzyme (K264, K266, K273) are acetylated by both the aforementioned HATs, while a fourth lisyne, K258, is acetylated esclusively by GCN5 (Cereseto, 2005; Terreni, 2010). The acetylated form of integrase has been shown to have a higher affinity for DNA and enhanced strand transfer activity *in vitro*. Moreover, impairment of integrase acetylation *in vivo* lead to decreased viral infectivity. Indeed a mutant virus carrying substitutions at the lysines targeted for acetylation displayed reduced replicative efficiency. In a reciprocal experiment, infection of cells knocked down for GCN5 by a vector containing wild type integrase resulted less efficient (Cereseto, 2005; Terreni, 2010).

To get a deeper insight in the mechanisms by which acetylation influences integrase activity, a two-hybrids screening was performed on a human lymphocytes cDNA library, using as a bait acetylated integrase, in order to elucidate how acetylation would modulate the interaction of integrase with other cellular proteins (Allouch, 2009). To obtain the constitutively acetylated enzyme, a tethered catalysis system was set up, where a recombinant integrase was fused to the HAT domain of p300, separated by the latter through a TEV (Tobacco Etch Virus) proteolitic site. This screening identified 13 cellular cofactors, which bind acetylated integrase with higher affinity as compared to the unmodified enzyme. Preferential binding to acetylated integrase was further validated by binding assay. The factors identified include transcription regulatory and chromatin remodeling factors, translation regulatory and RNA binding proteins and nuclear import–export proteins and might therefore be variously implicated at different step in HIV infection, from DNA tethering to

transcription units (remarkably, one of the hits from the two-hybrid screening is LEDGF/p75) to nuclear import of the virus (Allouch, 2009).

Interestingly the screening identified also a member of the TRIM family of antiretroviral proteins, TRIM28 or KAP1, a transcriptional corepressor, which functions through the recruitment of HDAC complexes to chromatin. KAP1 has been demonstrated to bind preferentially acetylated integrase both *in vitro* and *in vivo*, and to be a novel restriction factor acting specifically at the integration step (Allouch, 2011). Indeed, in KAP1 knockdown cells, viral integration is enhanced, as demonstrated both by the activity of a reporter gene inserted in the viral vector and by quantitative PCR of integrated and unintegrated viral DNA forms. Conversely, the integration of a virus mutated at the lysines target of acetylation is unaffected by KAP1 knockdown, further corroborating the finding that KAP1 interferes with viral infection by specifically acting on acetylated integrase. KAP1 recruits HDAC1 and induces integrase deacetylation, thus interfering with its activity. Unfortunately, the virus is able in some way to evade this cellular defense mechanism. One possible mechanism would depict KAP1 as inactivated through phosphorilation, triggered by the ATM DNA damage response system, which is activated upon viral infection (Allouch, 2011).

Therefore, taken together, all these observation evidence that acetylation is important for HIV-1 replication cycle, with p300 gaining the role of integrase cofactor / tethering factor during HIV-1 infection, as shown and discussed ahead in the results and discussion sections.

1.9 Acetylase inhibitors

Because of p300 HAT involvement in the important cellular events described above, dysfunction of this enzyme may be the underlying causes of

several diseases, including a few types of cancers, cardiac hypertrophy, asthma, and diabetes (Yier, 2004; McKinsey, 2004; Barnes, 2005; Selvi, 2009). HAT activity of p300 is therefore being considered as a target for new generation therapeutics. Unlike histone deacetylase inhibitors, the number of HAT modulators (activator and inhibitors) discovered so far is less copious and it is only recently increasing.

1.9.1 Naturally occurring HAT inhibitors and their derivatives

The first specific natural modulators of HATs identified was anacardic acid from cashew nut-shell liquid, which resulted a potent inhibitor of p300 and PCAF. (Balasubramanyam, 2003; Sung, 2008). Subsequent studies on anacardic acid have revealed that this natural product is also able to inhibit the MYST HAT Tip60 in vitro and also blocks Tip60-dependent activation of ATM and DNA-PK protein kinases by DNA damage *in vivo* (Sun, 2006). In the effort to obtain more selective and cell-permeable anacardic acid derivatives, benzamide analogs of this natural compound were synthesized, leading to different results. Indeed, CTPB (N-[4-chloro-3-trifluoromethyl-phenyl]-2-ethoxy-6-pentadecyl-benzamide), remarkably activates p300 HAT activity, while the related cyano-benzamides (compounds 1, 2 and 3 in figure 1-10), with different alkyl chain lengths and different substituents, are as potent as anacardic acid itself in cell cultures experiments (Souto, 2008). Recently, another study reported that a long-chain alkylidenemalonate is a small-molecule modulator of the HATs p300 and CBP, with potency approximately equal to that of anacardic acid (Sbardella, 2008).

Other groups reported of various anacardic acid derived HAT inhibitors (Ghizzoni, 2010). Biological studies of these compounds showed an inhibition of HAT activity up to 95% in vitro, and a correlation of their inhibitory potency

and cytotoxicity toward an array of cancer cells. Moreover, the compounds were relatively nontoxic to non-malignant human cell lines and inhibited significantly p300 activity, although in a lower degree than anacardic acid. Nevertheless, these compounds suffer from low solubility in aqueous medium (Eliseeva, 2007).

Taken together, the main problem related to anacardic acid and its derivatives seems to be their poor cell-membrane-permeability potential. Moreover, a limitation for further studies on structure-activity relationships (SAR) based on anacardic acid is the absence of published structural information to enable structure-based optimization of the inhibitory potency (Dekker, Haisma, 2009).

An important development in the field was initiated when another natural small-molecule inhibitor of HATs, garcinol, a polyisoprenylated benzophenone derivative from *Garcinia Indica* fruit rind was discovered (Balasubramanyam, Altaf, 2004). Garcinol was found to be a nonspecific HAT inhibitor (HATi) but highly permeable to cultured cells and a potent inhibitor of histone acetylation *in vivo,* able to inhibit histone acetylation in cells treated with the HDAC inhibitor, TSA. The nonspecific nature of garcinol made it highly cytotoxic, indeed it was shown that it induces apoptosis and predominantly downregulates global gene expression in HeLa cells (Balasubramanyam, Altaf, 2004). Another study confirmed that garcinol and isogarcinol inhibit p300 and PCAF and exhibit high cytotoxicity (Mantelingu, 2007). A more specific garcinol derivative, LTK-14, prepared by monomethylation of the catechol functionality of garcinol, resulted able to inhibit p300 but not PCAF in the concentration range studied. This garcinol derivative is nontoxic to T-cells, inhibits histone acetylation in HIV-infected cells and it was also shown to inhibit the multiplication of HIV, although the precise mechanism of anti-viral activity was not investigated by the authors (Mantelingu, 2007). A more recent study

describes the mechanism of HAT inhibition of garcinol, isogarcinol and LTK-14 (Arif, 2009). Another group identified two garcinol derivatives, Nemorosone and Guttiferone A, able to activate and inhibit, respectively, p300 enzymatic activity in cell culture (Dal Piaz, 2010).

Unfortunately, the synthetic complexity of garcinol limits studies on structure activity relationships. This limits the potential use of garcinol to target p300 for understanding the physiological role of HATs or to develop a therapeutic molecule.

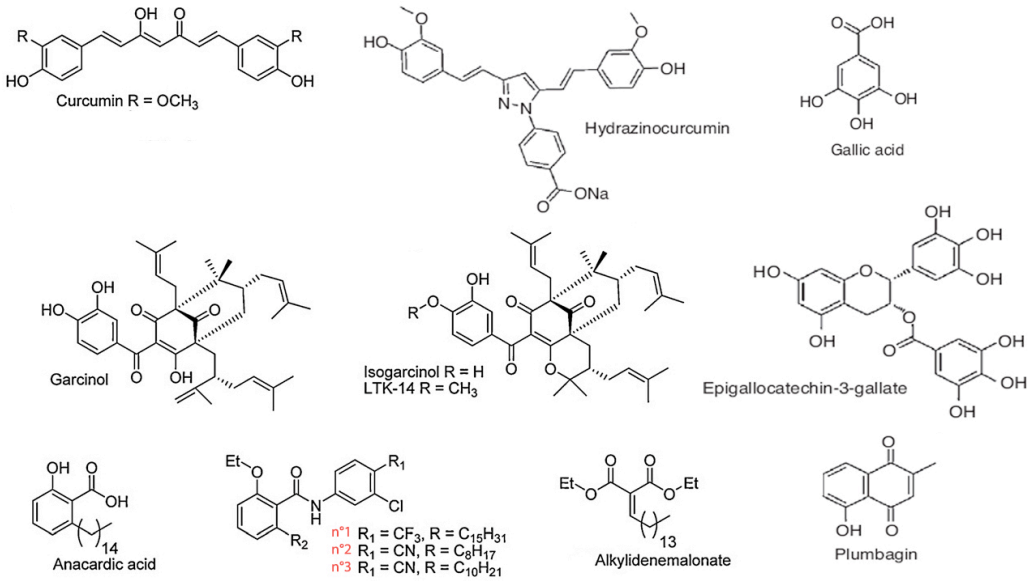


Figure 1-12. Natural products inhibitor of Histone acetyl transferases and their derivatives (adapted from Ghizzoni, 2011).

A few years ago, a screening of plant extracts from *Curcuma longa* rhizome led to the discovery of curcumin as a potent and specific inhibitor of p300 (Balasubramanyam, Varier, 2004). Curcumin has been widely used in Indian medicine and culinary traditions and possesses antiproliferative, antiangiogenetic, antioxidative, anti-inflammatory, antiinfective and antiseptic properties (Maheshwari, 2006).

In cell cultures experiments, Curcumin treatment caused hypoacetylation not only of the histones substrates (H3 and H4) of p300/CBP, but also of p53. Exposure of tumor cells to curcumin resulted in the inhibition of cell proliferation and induction of apoptosis, without cytotoxic effects on healthy cells (Kang, 2005; Tourkina 2004). Because of this promising pharmacological profile it is currently tested in phase II/III of clinical trials for cancer therapy, as well as in phase II for Alzheimer's disease and psoriasis (Marcu, 2006). Recently, it has been described that curcumin inhibition of HAT activity *in vivo* prevents heart failure in rats (Morimoto, 2008; Sun, 2010).

The kinetics of p300/CBP inhibition by curcumin suggests that curcumin does not bind to the binding sites of either histone or acetyl CoA, but to some other site of the enzyme. A synthetic derivatives of curcumin, hydrazino-curcumin, also inhibits HAT (Arif, 2010). Marcu et al. have shown that, apart from its direct effect on the acetyltransferase activity of p300, curcumin promoted proteasome-dependent degradation of p300 and the closely related CBP protein, and did so without affecting the HATs PCAF or GCN5 (Marcu, 2006).

However, curcumin is chemically not stable and has a rapid metabolism and thus, displays a relatively low *in vivo* bioavailability (Kang, 2005). On the other hand, its simple structure provides easy derivatization, thus several curcumin analogues are already described in literature, tested on different target enzymes (Anand, 2008; Mishra, 2005).

Another naturally occurring compound with HAT inhibitory properties recently found is plumbagin, which was isolated from *Plumbago rosea* root extract, (Ravindra, 2009). This molecule inhibits p300 but not PCAF mediated acetylation *in vivo*, in a non-competitive manner (Ravindra, 2009). Although plumbagin is a putative anticancer agent (Kuo, 2006; Hsu, 2006;

Gomathinayagam, 2008; Acharya, 2008), its major limitation for use as therapeutic molecule could be the cellular toxicity (Ravindra, 2009).

Recently, natural products gallic acid and epigallocatechin-3-gallate (EGCG), a constituent of green tea, where shown to be non-selective HAT inhibitors (Choi, Jung, 2009; Choi, Lee, 2009). Interestingly, EGCG showed to possess global specificity for the majority of HAT enzymes but no activity toward other epigenetic enzymes, like HDAC, SIRT1, and HMTase. Further studies revealed that EGCG generally induces hypoacetylation of p65 by directly inhibiting the activity of HAT enzymes. Moreover, EGCG suppresses viral protein-induced acetylation of p65 which leads to the inhibition of EBV induced B-cell transformation (Choi, Lee, 2009).

A drawback for these compounds is their extreme unspecificity: indeed, epigallocatechin-3-gallate also inhibits phosphorilation of several proteins such as MAP kinases (Adachi, 2009).

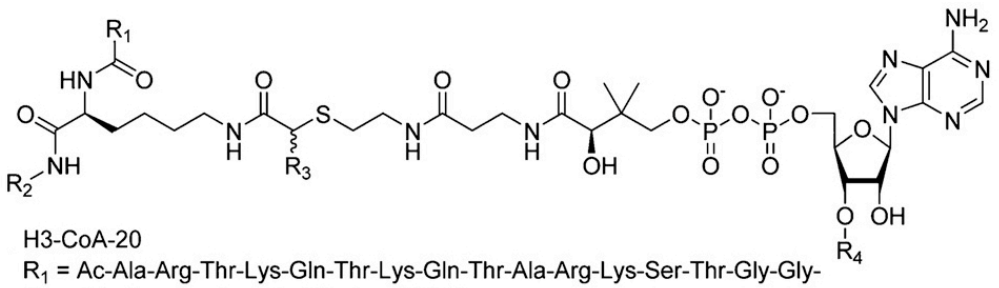
Given the structural complexity and often insufficient structural information on natural anti-p300 agents, the studies on structure-activity relationships (SAR) in this field are still limited, and only a few analogs of natural compounds have been described (Cebrat, 2003; Sagar, 2004, Dekker, Haisma, 2009). The structures of natural HAT inhibitors identified so far and of some of their derivatves is illustrated in figure 1-12.

1.9.2 Synthetic HAT inhibitors

Among the synthetic HAT modulators, the first group to be described for the HATs p300 and PCAF were the bisubstrate inhibitors peptide Co-A conjugates (Figure 1-13). The development of these inhibitors was based on the understanding that the target enzyme employs a ternary complex mechanism with substrate binding. Hence, these compounds represent structural mimics of

the simple, covalent linkage between H3 peptide substrates and acetyl CoA. Owing to their chemical nature, they form a strong and selective bond to the binding site of the enzyme and, consequently, act as potent inhibitors (Thompson, 2001; Poux, 2002).

Of these, Lys Co-A, a lysine analog of HAT substrate acetyl-CoA, was specific for p300, and H3Co-A20 was specific for PCAF (Lau, Kundu, 2000; Thompson, 2001; Poux, 2002; Sagar, 2004). The major limitation of bisubstrate inhibitors is their lack of cell-permeability. To address this problem, another study reported the generation of a bisubstrate inhibitor derivative linked to an arginine-rich peptide sequence. This construct resulted to be cell-permeable, although it loss some potency and selectivity, thus it did not originate, so far, a drug-like inhibitor (Zheng, 2005).



H3-CoA-20
R_1 = Ac-Ala-Arg-Thr-Lys-Gln-Thr-Lys-Gln-Thr-Ala-Arg-Lys-Ser-Thr-Gly-Gly-
R_2 = -Ala-Pro-Arg-Lys-Thr-Gln-Leu-COOH
R_3 = H, R4 = PO_3^{2-}

Lys-CoA
$R_1 = CH_3$, $R_2 = R_3 = H$, $R_4 = PO_3^{2-}$

Lys-CoA-Me
$R_1 = CH_3$, $R_2 = H$, $R_3 = CH_3$, $R_4 = PO_3^{2-}$

Lys-Phe-CoA
R_1 = Phe, $R_2 = H$, $R_3 = H$, $R_4 = PO_3^{2-}$

Lys-CoA-3'dephospho
$R_1 = CH_3$, $R_2 = R_3 = R_4 = H$

Figure 1-13. Bisubstrate inhibitors for the HATs p300 and PCAF (adapted from Dekker, 2009)

Latest studies in the field of bi-substrate inhibitors involved the design of constructs in which the Lys and CoA portion are separated by linker of different

lengths, in the attempt to obtain better inhibitors by providing the optimal distance for the correct orientation of the two ligand moieties (Karukurichi, 2011). Unfortunately these class of molecules, although displaying a discrete inhibitory activity, which was directly proportional to the length of the linker introduced, are inhibitors a few fold weaker than the parent compound Lys-CoA.

Furthermore, α-methylene-γ-butyrolactones were found as first-known small-molecule inhibitors of human GCN5 HAT activity (Biel, 2004). In this class, MB-3 was found by rational design and resulted a cell-permeable inhibitor, with an IC50 of 100 μM. Although this could appear to be a modest potency, it is worth to note that in the presence of acetyl-CoA the Kd value for binding Gcn5 or PCAF to the natural substrate histone H3 is approximately 100 μM, therefore comparable to the inhibitor MB-3. (Biel, 2004).

More recently, isothiazolones-based modulators have also been identified by high-throughput screening of a library of about 70 thousand molecules (Stimson, 2005). The compounds selected are able to inhibit both p300 and PCAF (Stimson, 2005). The isothiazolone core structure has been used as a starting point to generate more PCAF inhibitors (Clerici, 2003; Dekker, Ghizzoni, 2009; Gorsuch, 2009). Unfortunately, the high reactivity of isothiazolones has limited their applications in cell-based studies (Ghizzoni, 2009; Gorsuch, 2009).

Very recently, a promising HAT inhibitor was discovered using virtual ligand screening (Bowers, 2010). This compound is a potent and selective inhibitor of p300 and it can reduce histone acetylation and cancer cell growth. Another group used phenotypic screening for the identification of new HAT inhibitors (Mai, 2006; Chimenti, 2009; Mai, 2009).

Small-molecule HAT inhibitors are useful tools to unravel the functions of HATs, where classical genetic methods fail. Moreover, they can represent starting points for the design of novel epigenetic drugs. In contrast to HDACs, the different types of HATs exhibit only minor homology in sequence and structure (Vetting, 2005). Furthermore, the acetylation mechanism in the catalytic site of the enzymes showed to be chemically simple, since the amide bond is formed directly, without the generation of an acetylated enzyme intermediate (Von Wantoch Rekowski, 2010).

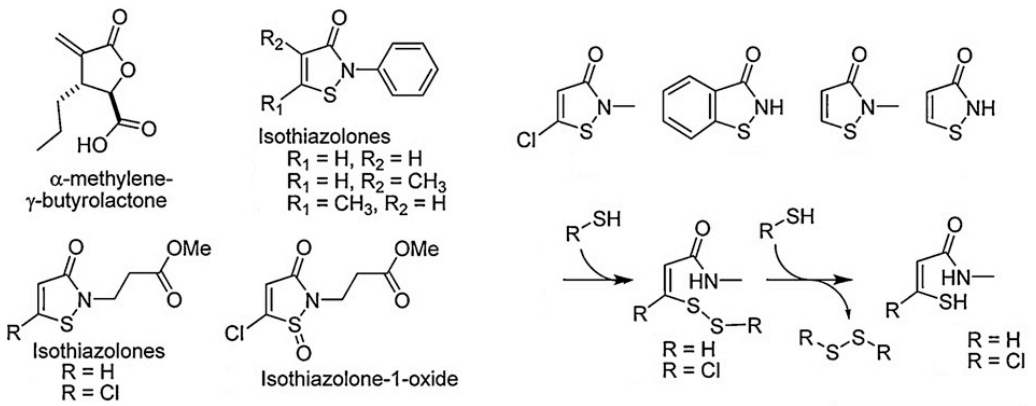


Figure 1-14. Structures of small molecules synthetic HAT inhibitors reported so far (adapted from Dekker, 2009)

For these reasons, it seems possible to design specific and effective HAT inhibitors to block the acetylation of a determined substrate or class of substrates.

However, redundancy of acetyl transferases complicates somehow the scenery in *in vivo* settings, and the currently described inhibitor classes suffer either from low potency, or lack of specificity or low cell-permeability.

2 – AIMS OF THE THESIS & EXPERIMENTAL STRATEGY

2 Aims of the thesis and experimental strategy

Integration of the viral genome into the DNA of the infected cells is a crucial and peculiar event in the life cycle of HIV.

Our group has previously shown that the viral enzyme integrase, responsible for the integration reaction, is post-translationally modified by two histone-acetyl-transferases (HAT), p300 and GCN5, and that this modification enhances its catalytic activity and is important for the viral infectivity. As both p300 and GCN5 are chromatin-modifying enzymes and transcriptional co-activators, the interaction of integrase with these enzymes has also been suggested to facilitate integration in active regions of chromatin, another characteristic of HIV-1, essential to the virus for high expression of its genome, but whose mechanism is still poorly understood.

The aim of this thesis was to select new molecules capable of selectively blocking the acetylation of integrase by p300. Such chemicals should represent lead compounds for the development of new generation anti-integrase drugs, targeting the interaction of the enzyme with a cellular cofactor, rather than the enzyme itself, thus less amenable to the development of pharmaco-resistance.

Moreover, the aim of this study was to further confirm and characterize the role of acetylation in the virus life cycle, exploring the effect of integrase acetylation in the context of an infectious viral particle. A better understanding of integrase functioning is essential not only for the developments of anti-integration therapies, but also for the research on safe gene therapy vectors derived from lentiviruses, as the ability to control integration and integration site selection would be essential to minimize the risk of insertional mutagenesis, inherently associated with this type of vectors.

In order to select an efficient acetylation inhibitor, first a library of small molecules synthetic compounds, whose chemical structures derived from that of more complex naturally occurring p300 inhibitors, was screened in an *in vitro* acetylation assay. The data obtained in this initial screening were used for Structure-Activity Relationships (SAR) studies and led to the rational design of a smaller set of molecules, which combine the chemical moieties resulted important for activity. *In vitro* testing of this set of molecules led to the selection of two most active molecules for further *in vivo* testing on cellular histone acetylation. In these preliminary *in vivo* tests, cell coltures were treated with the potential inhibitor, immunostained with a fluorescent antibody specific for acetyated histone H3, and visualized by confocal microscopy. One of the two molecules tested in cell coltures resulted active and was further tested to inhibit acetylation of the viral integrase. In order to dissect the step of the viral life cycle in which acetylation occurs and our inhibitor exerts its effect, we adopted an experimental design in which either the virus producing cells or the cells target of infection were treated with the inhibitor. In this way it was possible to observe that integrase is acetylated inside the viral particle. This led to the design of experiments aimed at generating hyper-acetylated viruses, using two different approaches. First, DNA construct coding for chimeric protein consisting of integrase fused to the HAT domain of p300, and thus constitutively acetylated, were incorporated *in trans* in the viral particle. In a second set of experiments, more conservative to the viral enzyme morphology and functioning, the HAT domain of p300 alone was trans-incorporated in the virions, in order to exert is action on integrase in the narrow space of the viral particle. The infectivity of these virions was finally studied in cell coltures, to confirm the data obtained with the acetylation inhibitor previously selected.

3 – MATERIALS AND METHODS

3.1 Plasmids

pFlag-IN codon optimized (c.o.) was kindly provided by A. Engelman. pLKO1p300, pGIPZMM09 and pGIPZGCN5 lentiviral vectors plasmids were purchased from Open Biosystems (Huntsville, AL).

The NL4.3-Luc env-deleted virus expressing the luciferase reporter gene was produced from the pNL4.3.Luc.R-E- molecular clone obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

The envelope plasmid pMDG and the packaging plasmid pCMVΔR8.91 were kindly provided by Z. Debyser.

3.2 Antibodies

Primary antibodies used for western blot or immunofluorescence were: rabbit anti-acetyl-integrase, generously supplied by M.I. Gutierrez (Terreni, 2010); mouse mAb AG3.0 anti-HIV p24CA and rabbit anti-HIV p17MA (AIDS Research and Reference Reagent Program); mouse anti-IN 8G4 obtained from the AIDS Research and Reference Reagent Program; rabbit anti-HA Clone 3F10 (Roche Diagnostics); rabbit anti-GCN5 H-75 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); rabbit anti-p300 N15 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); mouse anti-a-tubulin Clone B-5-1-2 (Sigma, Inc.).

Secondary antibodies used were: anti-rabbit or anti mouse conjugated with Alexa-594, Alexa-633 and Alexa-647 (Molecular Probes, Eugene, OR) and anti-goat conjugated with Alexa-680 (Molecular Probes, Eugene, OR), anti-mouse and anti-human conjugated with HRP (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

3.3 Recombinant proteins production and purification.

The GST-p300 HAT domain (aa 1195-1810), GST-PCAF and GST-GCN5 were purified from Escherichia Coli BL21 cells transformed with the respective plasmids. Bacterial cultures were grown at 37 °C in terrific broth supplemented with 100 mg/ml ampicillin until reaching an absorbance of 0.6 OD600. Protein expression was induced with 1 mM IPTG and the incubation was continued for 3 h at 30 °C. Bacterial cells were pelleted, resuspended in cold lysis buffer (1× PBS pH 7.4, 50mM EDTA pH 8.0, 1% Triton X-100, 2 mM DTT, 1 mM PMSF), and sonicated by 4 pulses of 15 s each. Cleared lysates were mixed with a 50% slurry of glutathione Sepharose beads, and GST fusion proteins were allowed to bind to the resin at 4 °C on a rotating wheel. After 2 h of incubation, the beads were spun down by centrifugation and washed for three times with 10 bead volumes of lysis buffer. For the elution of GST fusion proteins, 1 bead volume of elution buffer (50 mM Tris-HCl pH 8.0, 25 mM reduced glutathione) was added to the resin. After incubation of the mixture at 4 °C on a rotating wheel for 15 min, the beads were spun down by centrifugation and the supernant (containing the eluted GST fusion proteins) was collected. The elution procedure was repeated for three times. Eluted proteins were dialysed overnight at 4 °C against one liter of dialysis buffer (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 20% glycerol).

Full length p300 was purified from Sf9 eukaryotic cells via a baculovirus expression vector (Ogryzko, 1996).

N-terminal 6×His-tagged IN proteins used in the strand transfer assay were expressed in Escherichia Coli BL21 strain and purified by metal ion affinity chromatography (BD TALON Metal Affinity Resin, BD Biosciences, Palo Alto, CA) according to a previously reported protocol {Bushman, 1993 #140}, with minor modifications. Briefly, bacterial pellets were resuspended in cold

lysis buffer (20 mM Tris-HCl, pH 8.0, 1 M NaCl, 0.5% Triton X-100, 1 mM PMSF, 5mM imidazole) and sonicated by 4 pulses of 15 s each. Cleared lysates were incubated with a 50% BD TALON Resin slurry at 4 °C on a rotating wheel for 2 h. The lysate/BD Talon Resin suspension was loaded into an empty plastic column (Bio-Rad, Richmond, CA), letting the unbound proteins to pass through, and the resin was washed for three times with 10 bed volumes of wash buffer (20 mM Tris-HCl, pH 8.0, 1 M NaCl, 0.5% Triton X-100, 10 mM imidazole). 6×His-tagged proteins were eluted with 1 bed volume of elution buffer (20 mM Tris-HCl, pH 8.0, 1 M NaCl, 0.1% Triton X-100, 200 mM imidazole), repeating the procedure for four times. Eluted proteins were dyalised against one liter of dialysis buffer (20 mM Tris-HCl, pH 8.0, 1 M NaCl, 10% glycerol, 1 mM DTT) overnight at 4 °C.

The purity and integrity of purified proteins were checked by SDS-PAGE followed by Coomassie Blue staining.

3.4 *In vitro* acetylation assay (HAT assay) to test the efficacy of curcumin and its derivatives.

To test the efficacy of derivatives 1a-c, and 2a-d, the catalytic activity of p300 in the presence of these molecules has been measured by an in vitro acetylation assay. Derivative 2c was additionally tested on PCAF and GCN5.

To test the inhibitory effect of curcumin on p300 in vitro acetylation assays were performed using both p300HAT domain (aa 1195-1810) fused to GST purified from bacteria as well as full length p300 purified from Sf9 eukaryotic cells via a baculovirus expression vector.

One hundred fifty ng of recombinant histones (H1, H2a, H2b H3 and H4-Sigma) were incubated with 25 ng of p300 HAT domain fused to GST in 30 μL

final volume of HAT buffer (50 mM Tris pH 7, 10% glycerol, 0.1 mM EDTA and 1mM DTT, 5 mM sodium butyrate) containing 2 μL of 56 mCi/mmol 14C AcetylCoA (Amersham Biosciences). To each reaction curcumin derivatives, resupended in DMSO, where added to obtain final concentration of 400 μM, 200 μM, 100 μM and 25 μM or lower concentration of 12.5 μM, 3.125 μM, 0.78 μM and 0.195 μM for derivatives with higher inhibitory activity. Control reactions were performed either with DMSO alone or with Lys-CoA. Derivative 2c was additionally tested on PCAF and GCN5.

Reaction mixtures were incubated at 30˚C for 1h and then loaded onto a 15% SDS-PAGE gel. Following staining with Coomassie Blue, the gels were dried and exposed overnight on a Packard film type SR for Cyclone. The optical density of the bands corresponding to acetylated histones was measured using Cyclone software.

The optical densities corresponding to each concentration of curcumin derivative (expressed as percentage of the positive control) were plotted versus concentration of the curcumin derivative expressed in logaritmic scale. The curves were calculated using the equation:

$$a0/(1+10\text{^}((\log(a1)-\log(x))*-1))$$

where x indicates the inhibitor concentration and a the optical density data. This analysis was performed using the SlideWrite 5.0 software which calculates the IC50 value, indicated as a1 and the maximum of absorbance of the positive control indicated as a0.

3.5 *In vivo* acetylation assays to test the efficacy of the curcumin derivatives in mammalian cells.

HeLa cells stably transfected with histones H2B fused to EYFP20 were cultured in 10% FCS DMEM.

Treatments with derivative 2c were performed by plating 60.000 cells in 8-chamber slides the day before treatment. Forty hours following drug treatment the slides were processed for immunostaining to analyse histone H3 acetylation. Briefly, cells were fixed in 2% parafolmaldheyde and incubated with anti-acetylated H3 (Santa Cruz) for 1 hours followed by incubation with secondary antibodies labelled with Alexa 647. Slides were then analyzed by confocal microscopy (Leica) using 667-790 nM or 525-556 nM wavelengths for Alexa 647 staining or EYFP respectively.

3.6 Strand Transfer assay

Oligonucleotide substrates for IN reaction assays were as follows: (1) 5'GTGTGGAAAATCTCTAGCA3' and (2) 5'ACTGCTAGAGATTTTCCACAC3' (Parissi, 2001). Oligonucleotide 1 was labeled with ^{32}P using polynucleotide kinase and annealed to the complementary oligonucleotide 2. Strand transfer reaction was carried out in 20 mM Hepes, pH 7.2, 7.5 mM $MnCl_2$, 0.05% NP-40 and 10 mM DTT, in the presence of the 1/2 substrate. [^{32}P]-labeled duplex DNA (1 pmol) was incubated in a final volume of 20 µl with 50 or 200 ng of 6xHis-IN proteins at 37 °C for 1h, in the presence of different concentration of molecule 2c or RDS1983. The reaction products were separated by electrophoresis on a 15% polyacrylamide gel with 7M urea in Tris-Borate-EDTA buffer, pH 7.6, and then visualized by phosphoimaging (Cyclone).

3.7 Cell culture and transfection

HeLa, 293T and HeLa-H2B-EYFP cells (generously supplied by Jörg Langowski) were maintained in DMEM supplemented with 10% FCS. HeLa-H2B-EYFP cells were cultured in medium containing 500 μg/ml of G418 (Gibco BRL, Milan, Italy).

HEK 293T cells stably transduced with GIPZ lentiviral vectors were grown with the addition of puromycin 2 mg/ml. Transfections were performed by the standard calcium phosphate coprecipitation procedure, or by using the polyethylenimine (PEI) reagent (MW 25000, Sigma, Inc., St Louis, MO) according to a previously reported protocol (Durocher, 2002) with a few adaptations for lentiviral vectors production, as illustrated hereafter.

3.8 Lentiviral vectors production

HEK 293T cells growing in standard DMEM medium additioned of 10% calf serum and antibiotics were seeded 24 hours prior to transfection in an adequate number in order to reach 80% confluency at the moment of transfection (6.000.000 cells for a p100 plate). DNA mix for transfection were prepared in 2ml eppendoorf tube containing 1ml DMEM without serum and without antibiotics. For wt virus production DNA mix contained 20 μg of pNL4.3 Luc R-E- and 5 μg of pMDG, while for trans-incorporated virions (Liu, 1997) it contained 12μg of pD64E, 12 μg of the appropriate construct to be trans-incorporated and 2μg of pMDG. Following the addition of 45 μl of 10 μM PEI, the tube was immediately vortexed for 1 second, incubated at room temperature for 10 minutes, then vortexed again for 1 second and added to the plates, were DMEM medium had been replaced by Optimem. Medium was changed 16h post-trasnfection and replaced with fresh optimem. Viral supernatant were harvested at 48 hours post-transfection, filtered through 0,45

(m filters and stored in 1ml aliquot at -80°C or concentrated by ultracentrifugation at 100.000g for 2 hours at 4°C, resuspended in fresh optimem and stored at -80 °C.

3.9 Stable and transient knockdown of GCN5 expression

For production of stably silenced cell lines, HEK 293T cells, seeded in 24-well plates the day before transduction (5(104 cells/well), were incubated for 4 h with 1 mg p24 antigen of the appropriate GIPZ lentiviral vector (encoding GCN5 shRNAmir, p300 shRNAmir or a mismatch non silensing insert). Two days after transduction, selection with 2 mg/ml puromycin was initiated. For selection of GCN5 knockdown cell clones, the clones with the highest GFP expression levels, as determined by fluorescence-activated cell sorting analysis (FACS), were chosen.

To obtain transiently silenced cells lines 40.000 HEK 293T or HeLa cells were transduced with lentiviral vectors encoding an appropriate shRNAmir (500.000 RTcpm pGIPZ-GCN5 + 500.000 RTcpm pKLO1-p300) or a mis-match control shRNAmir insert (1.000.000 RTcpm). Alternatively, 293T cells were directly transfected with pGIPZ-GCN5 and pKLO1-p300 plasmids or with the control plasmid pGIPZ-MM09. Cells were harvested two days post-transduction or post-transfection and western blot on cell lysates was performed using (-p300 or (-GCN5 antibodies.

3.10 HIV-1 infectivity assays

For single-round viral replication assays, HeLa cells (2.5(106/well) were seeded in 6-well plates and incubated for 3 h, in a total volume of 500 ml, with normalized p24 antigen amount of NL4.3-Luc wt virions (produced in treated

or control cells) or trans-incorporated virions. Cells were collected 48 h after infection and lysed for measurement of luciferase activity (Luciferase Assay System, Promega Corp.). Luminometer readouts, expressed as relative light units (RLU), were normalized with respect to protein concentration in each sample.

Viral stocks used in infections for measurement of HIV-1 DNA species by real time quantitative PCR (RT-Q-PCR) were pre-treated for 1 h at 37 °C with 160 U/ml Turbo DNase (Ambion, Inc., Austin, TX).

3.11 RT-Q-PCR analysis

Total DNA was extracted from HEK 293T cells with the DNeasy Tissue Kit (QIAGEN GmbH, Hilden, DE) at different time points after infection. Amplification reactions were performed with the Light Cycler 480 instrument (Roche Diagnostics). Quantification of total HIV-1 DNA was performed at 24 h post infection with a pair of primers (LucFw, LucRev) and a fluorogenic hybridization probe (LucProbe) annealing to the luciferase reporter gene of NL4.3-Luc viral clone. Reaction mixtures contained 500 ng of total cellular DNA, 1× Light Cycler 480 Probe Master (Roche Diagnostics), 300 nM each forward and reverse primers and 200 nM probe in a total volume of 20 ml. After an initial denaturation step (95 °C for 10 min), the cycling profile was 40 cycles consisting of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s.

Quantification of proviral DNA at 48 h post infection in HEK 293T cells was performed by Alu-LTR nested PCR. In the first amplication step, two outward-facing primers annealing within the highly repeated chromosomal Alu element (Alu 1 and Alu 2) were used together with a HIV-1 LTR-specific primer containing a lambda phage-specific heel sequence at its 5′ end (L-

M667). Alu-LTR sequences were amplified from 500 ng of total cellular DNA in a 20 ml reaction mixture comprising 1× Light Cycler 480 Probe Master (Roche Diagnostics), 100 nM L-M667 primer, and 300 nM (each) primers Alu 1 and Alu 2. The first-round PCR cycle conditions were as follows: a denaturation step of 8 min at 95°C followed 12 cycles of amplification (95°C for 10 s, 60°C for 10 s, and 72°C for 170 s). In the second round of PCR, a lambda-specific primer (Lambda T) and a HIV-1 LTR-specific primer (AA55M) were used, so that only LTR-containing products from the first-round PCR could be amplified. Nested PCR was performed on 1/10 of the first-round PCR product in a mixture comprising 1× Light Cycler 480 Probe Master (Roche Diagnostics), 300 nM Lambda T primer, 300 nM AA55M primer, and 200nM (each) hybridization probes LTR FL and LTR LC. The nested-PCR cycling profile began with a denaturation step (95°C for 8 min), followed by 50 cycles of amplification (95°C for 10 s, 60°C for 10 s, and 72°C for 9 s).

Two-LTR circles were detected with primers spanning the LTR-LTR junction (HIV F and HIV R1). Reaction mixtures contained 1× Light Cycler 480 Probe Master (Roche Diagnostics), 300 nM (each) forward and reverse primers, and 200 nM (each) fluorogenic hybridization probes HIV FL and HIV LC in a final volume of 20 ml. After an initial denaturation step (95°C for 8 min), the cycling profile was 15 cycles consisting of 95°C for 10 s, 66°C for 10 s, and 72°C for 10 s, followed by 35 cycles at the beginning of which the annealing temperature was decreased by 0.5°C per cycle to the secondary target temperature (59°C).

As an internal standard for normalizing the amount of cellular genomic DNA, the level of human b-globin DNA was quantified. The reaction was carried out using 1× Light Cycler 480 Probe Master (Roche Diagnostics), 400 nM of forward primer BGF, 400 nM of reverse primer BGR, and 200 nM of

BGX-P fluorescent probe. The amplification conditions included a hot start at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and extension at 60 °C for 1 min.

3.12 Western blotting

Cell pellets were lysed 30 h after transfection in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycolic acid) containing 10 mM sodium butyrate (Sigma, Inc.) and protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche Diagnostics, Indianapolis, IN). The protein concentration of cell extracts was determined by Bradford assay (Bio-Rad). Lysates were run on SDS-page and analyzed with the appropriate antibody.

4 – RESULTS

4.1 A new class of small molecules is able to inhibit p300 Histone Acetyl Transferase

4.1.1 Screening for new HAT inhibitors

An *in vitro* HAT assay (Cereseto, 2005) was used to screen a number of potential inhibitors of the acetyltransferase activity of p300.

To this aim, a construct corresponding to the catalytic domain of p300, the HAT domain (aminoacids 1195 to 1810 of the full length protein), fused to GST was expressed and purified from bacteria. Full length p300 was expressed, instead, via a baculovirus expression vector in Sf9 eukaryotic cells. The purified enzymes obtained were assayed to measure their acetylation activity on recombinant histones in the presence of the molecules to be screened. Scalar amounts of each potential inhibitor were added to the reaction mixture containing the HAT domain of the enzyme, recombinant histones (H1, H2A, H2b, H3 and H4) and radioactively labeled Acetyl-CoA. After the completion of the acetylation reaction, mixtures were separated on SDS-page and gels were exposed to measure, by densitometric analysis, the amount of radioactivity incorporated in histones. Based on the signal obtained, it is possible to evaluate the inhibitory activity of each compound.

The initial screening focused on about 50 structurally related compounds carrying different substituents synthesized by the group of R. Di Santo and R. Costi (University La Sapienza, Rome).

The synthetic compounds in the starting library were polyhydroxylated aromatic derivatives whose chemical structures were based on that of known p300 inhibitors described in the introduction, that is, curcumin, garcinol and anacardic acid. Indeed, an examination of the structures of these natural

products, led to the identification of some structural features that characterize these compounds: (i) a α,γ-diketo group; (ii) a cinnamoyl moiety; (iii) a catechol ring; (iv) a salicylic acid portion. These features were used as a starting point for choosing the molecules to include in the preliminary screening.

Each of the 50 compounds was tested in a concentration range from 100μM to 400 μM. Compounds which resulted active already at a concentration of 100 μM were tested again at a lower concentration range, in order to define more precisely their IC50 (that is, the concentration at which the enzymatic activity in the presence of the inhibitor is reduced by 50%).

This initial large screening served to obtain preliminary Structure-Activity Relationship (SAR) data to be used for the design of a smaller group of molecules carrying the most promising chemical features.

From this preliminary screening, in particular two chemical features came out to be important for the inhibitory activity, that is a simmetric, bi-substituted structure, and the presence in ortho position to OH groups of lipophilic and withdrawing bromine atoms.

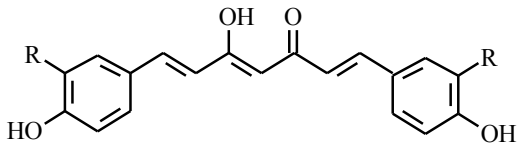
Based on these findings and on the structures of the most active molecules, Di Santo's group designed and synthesized 7 novel compounds, whose structures are shown in figure 4.1, which can be divided in two classes:

1) curcumin derivatives with different substituents on the aromatic moieties (**1a-c**);
2) 2,6-bis-arylidene cyclohexanone derivatives (**2a-d**).

Of course compounds of both classes share and variously combine the chemical features identified by the SAR analysis on the data from the in vitro screening, as well as from the analysis of the naturally occurring p300 inhibitors as described above. For instance, all compounds share a simmetric, bi-substituted

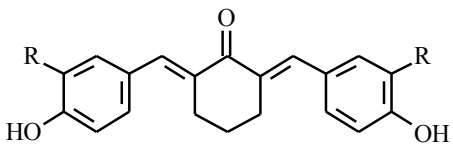
structure; compound **1a (**related to curcumin), contains an α,γ-diketo group; compounds **1a**, **2a** and **2d** (cyclohexanone derivatives) contain the cinnamoyl moiety; compounds **1a** and **2a** contain the catechol ring; finally, compounds **1c** and **2c** share the presence in ortho position to OH groups of bromine atoms

The two series of compounds **1a-c** and **2a-d** were tested for their inhibitory activities against p300 in the *in vitro* acetylation assay as described above (Figure 4.2). As shown in detail ahead, all synthesized molecules, at different extent, inhibited p300 enzymatic activity (Figure 4.2C).



1a-c

R = OH (**a**), COOH (**b**), Br (**c**)



2a-d

R = OH (**a**), COOH (**b**), Br (**c**), OCH_3 (**d**)

Figure 4.1. Structures of the cinnamoyl derivatives **1a-c** and **2a-d** tested as p300 inhibitors.

The inhibitory activity of each compound was tested with concentration ranging from 400 μM to 25 μM (data not shown). Figure 4.2A shows a comparison of the inhibitory activity of all the compounds at the highest concentration tested (400 μM). The most potent derivative, 2C, inhibited almost completely acetylation also at the lower concentration tested (25 μM).

Therefore, to determine its IC_{50} value, it was further tested up to a concentration of 0.19 μM (Figure 4.2B).

For each molecule inhibition curve were drawn, which allowed for the calculation of the IC_{50} values (figure 4.2C and table 1).

In general, compounds **1a-c** and **2a-d** showed good activities against p300, with IC_{50}s ranging from 5 to 233 μM (Table 1). Derivative **2c** was the most potent compound of these series (IC50 = 5 μM), being 6 times more potent than Lys-CoA used as a reference drug.

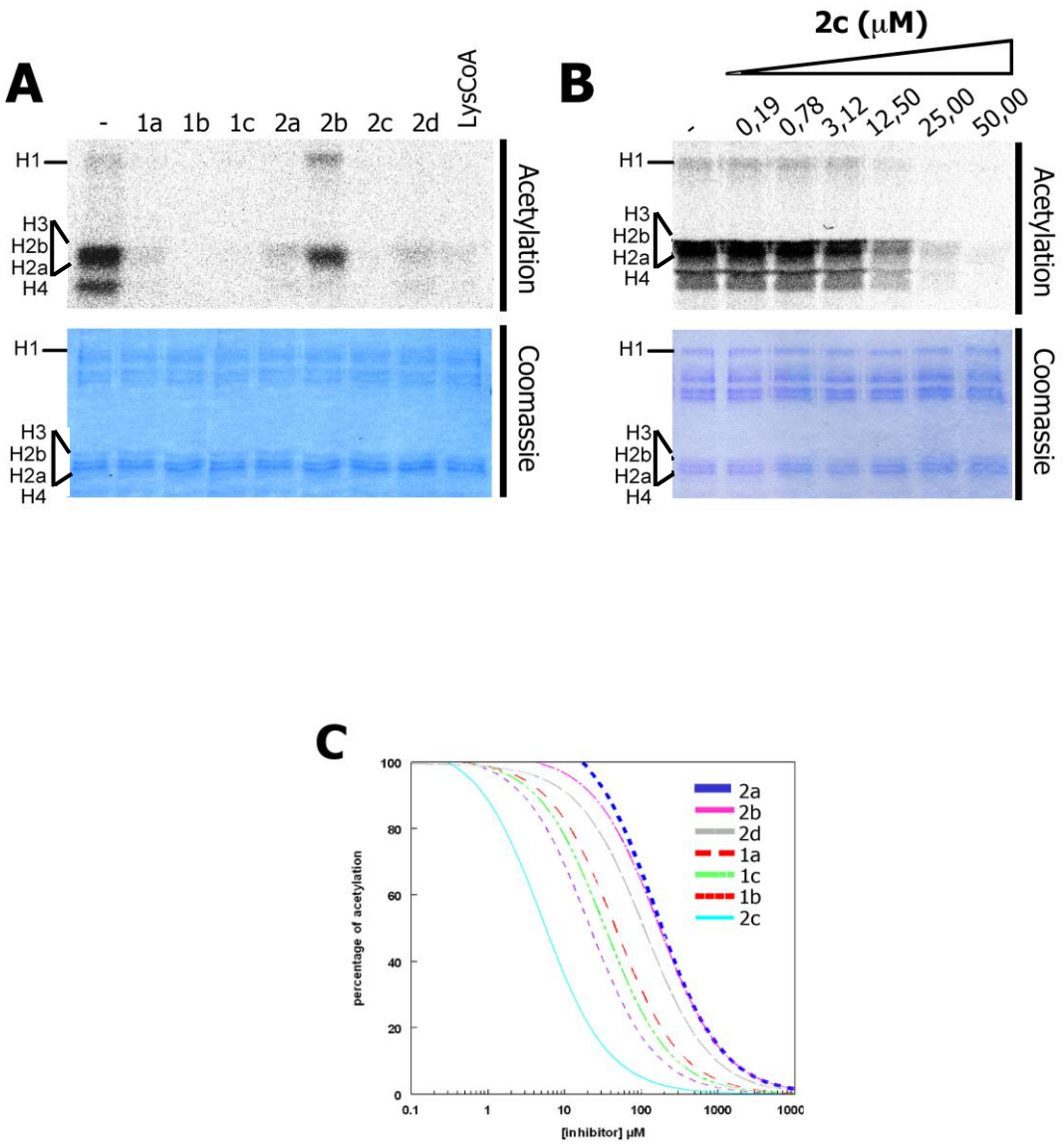


Figure 4.2. Inhibitory effects of compounds **1a-c**, **2a-d** on p300 activity. (A) Upper panel: autoradiography of a gel showing acetylated histones following incubation with p300 and ^{14}C Acetyl-CoA in the presence of 400 μM of each indicated derivative, Lys-CoA (last lane) or DMSO (first lane). Lower panel: Coomassie blue staining of the same gel showing the total amounts of histones. (B) Upper panel: autoradiography of a gel showing acetylated histones following incubation with p300 in the presence of the indicated concentration of **2c**, or DMSO alone (first lane). (C) Dose response curves obtained by densitometric analysis of the levels of histone acetylation mediated by p300 in the presence of **1a-c**, **2a-d**. The graph summarizes the results obtained from three independent experiments.

Surprisingly, in our assays curcumin was inactive at concentrations up to 400 μM. Due to this result we tested a new stock of commercial curcumin (Fluka) after a further chromatography purification and ^{1}H NMR identification and used both HAT core (aa 1195-1673) as well as the full length p300 enzyme (Ogryzko, 1996). In spite of this, the inactivity of curcumin was confirmed (Figure 4.3).

Interestingly, the curcumin derivatives **1a-c** were potent p300 inhibitors showing IC_{50} from 21 to 46 μM, thus comparable to that found for Lys-CoA used as a reference drug in the same experiment (IC_{50}= 30 μM). The most active derivative among this group of molecules was **1b**, which was characterized by salicylic groups (1.4 times more potent than Lys-CoA). Replacement of carboxylic function with a bromine or hydroxyl groups led to **1c** and **1a** that were 1.5 and 2 times less potent than parent derivative **1b**, respectively. In general, the activities in this series decreased if the electron-withdrawing groups (COOH, Br) were replaced by electron-donor ones (OH, OCH_3), and the following order depending by substituents in the 3-positions of the aromatic rings was observed: COOH > Br > OH > OCH_3.

The cyclohexanone derivatives **2a-d** were active against p300, as well. The IC_{50} values obtained in the enzyme assays ranged from 5 to 233 μM. The activities of compounds **2a-d** decreased based to the substituents in 3-positions of the aromatic rings in the following order: Br > OCH_3 > COOH > OH. In conclusion, the replacement of hydrophilic groups (COOH, OH) with the lipophilic ones (Br, OCH_3) in **2a-d** series led to increased anti-p300 activities. In particular, the highest potency was obtained with the introduction of the lipophilic and electron-withdrawing bromine atom on the cinnamoyl portion.

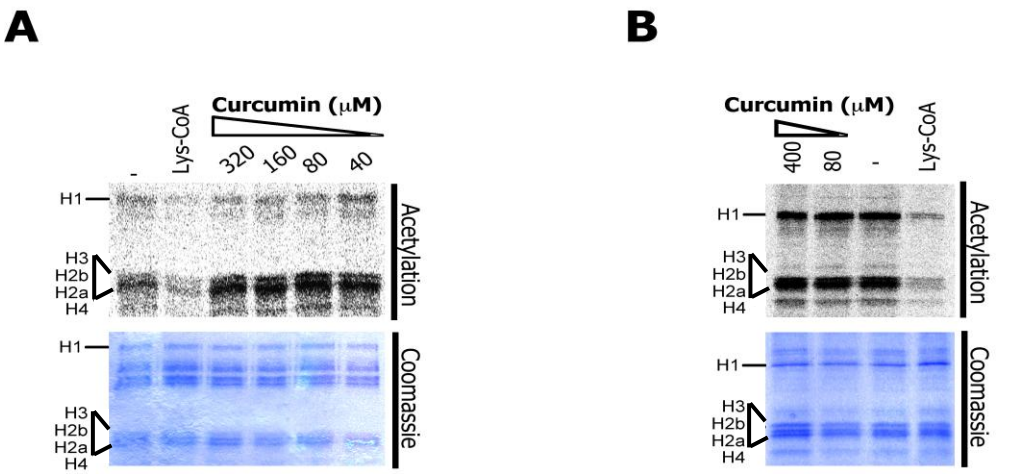
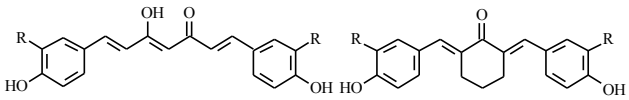


Figure 4.3. Curcumin does not inhibit p300. (A) Upper panel: autoradiography of a gel showing acetylated histones following incubation with p300HAT, purified from bacteria, in the presence of ^{14}C Acetyl-CoA and the indicated amounts of curcumin, DMSO (first lane) or Lys-CoA (second lane). Lower panel: Coomassie blue staining of the same gel showing the total amounts of histones. (B) Upper panel: autoradiography of acetylated histones following incubation with full length p300, purified from Sf9 cells, in the presence of ^{14}C Acetyl-CoA and the indicated amounts of curcumin, DMSO (third lane) or Lys-CoA (last lane).

The preliminary SAR in the series of cyclohexanone derivatives (compounds **2a-d**) were different if compared with those found in the curcumin series (compounds **1a-c** and curcumin). A direct comparison among the two series led to the following conclusions: (i) compounds **2a-d** were generally less potent than **1a-c** derivatives, showing IC_{50}s from 111 to 233 μM, with the exception of **2c** that was the most potent derivative described in this work (IC_{50} = 5 μM); (ii) introduction of bromine atoms in 3 position of benzene rings gave derivatives **1c** and **2c**, which were both endowed with good activities; (iii) introduction of OH or COOH groups in the same positions, within the curcumin series, gave compounds **1a** and **1b** that showed good anti-p300 potency; opposite results were found when the same groups were introduced in 3 position of benzene rings in the cyclohexanone series (**2a**, IC_{50} = 233 μM; **2b**, IC_{50} = 168 μM).

Table 1. Inhibitory activity of compounds **1a-c** and **2a-d** against p300 enzyme



Comp	R	IC_{50}[a]
1a	OH	46±3.9
1b	COOH	21±8.7
1c	Br	33±5.2
Curcumin	OCH_3	>400
2a	OH	233±120
2b	COOH	168 ±12
2c	Br	5±1.3
2d	OCH_3	111±45
Lys-CoA		30±1.6

[a]Inhibitory concentration 50% (μM) determined from dose-response curves. Data represent mean values of at least three separate experiments.

4.1.2 Efficacy of inhibitors 1b and 2c on different HATs and in cell culture conditions.

Derivatives **2c** and **1b**, which showed the highest inhibitory activity, were additionally tested on PCAF and GCN5, both belonging to a different class of HAT factors. The assays were performed using concentrations of **2c**

and **1b** corresponding to the IC_{50s} formerly determined against p300 (5 μM and 21 μM for **2c** and **1b**, respectively; Table 1). As expected, the activity of p300 was reduced of 50% with both compounds, while the same concentration of derivative **2c** showed no effect on PCAF (100%) and only partial inactivation of GCN5 (70%), indicating a selective inhibition of p300 activity. Conversely, **1b** is partially active on PCAF (68%) and shows on GCN5 (38%) the same efficacy as for p300 indicating that this compound is active on HATs other than p300 (Figure 4.4).

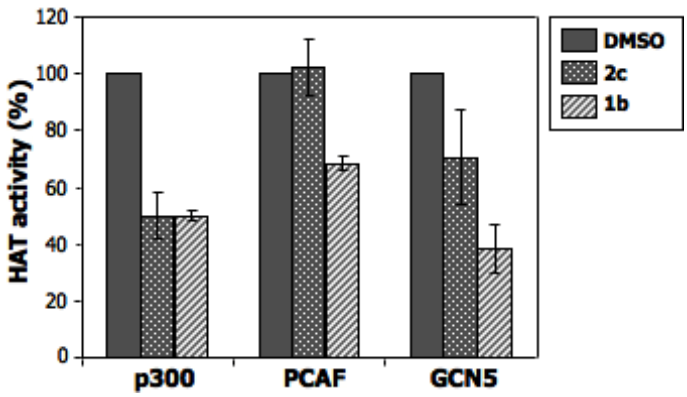


Figure 4.4. Inhibitory activities of derivatives **2c** (5 μM) and **1b** (21 μM) on different HATs were tested in *in vitro* assays using equal molar amounts of recombinant p300, PCAF and GCN5. HAT activity for each enzyme is expressed as percent variation as compared to DMSO treated sample. The graph summarizes the mean densitometric values from three independent experiments (mean ± standard error).

Several previously described HAT inhibitors such as Lys-CoA are not cell permeable, and cannot thus be used for *in vivo* studies. Therefore, we have tested for its anti-acetylase activity in a cell culture system derivatives **1b** and **2c**, which had shown, *in vitro*, the most potent inhibitory effect against p300. HeLa cells stably expressing fluorescent H2B histones (HeLa – H2B-EYFP) (Ogryzko, 1996) were treated with various concentrations of **2c** and **1b** and

subsequently analyzed by immunostaining using antibodies against acetylated H3 histones. The fluorescent H2B histones were used as internal control to monitor protein expression levels. Derivative **1b** at concentrations up to 200 μM did not alter either the H3 acetylation levels or the H2B protein expression. This experiment led us to hypothesize that **1b** is not cellular permeable (data not shown). Conversely, we found that at 20 μM - 40μM of **2c** the levels of H3 acetylation decreased, while the H2B expression remained unaltered indicating specificity of anti-acetylase treatment (Figure 4.5A).

At concentrations lower than 20 μM it could not be observed any effect on the acetylation levels, while over 40 μM cell toxicity was noticed, as indicated by decreased H2B-EYFP fluorescence (data not shown). Interestingly, the effect of **2c** is not homogeneous in cell culture. Indeed, a high percentage of cells (estimated around 24%) showed no detectable H3 acetylation even though the H2B expression remained unaltered (Figure 4.5A, cell in the upper-center in the middle panels as a representative image). Finally, we observed that **2c** determined an overall increased of H2B-EYFP fluorescence intensity. This observation is indicative of decreased histone acetylation that results in chromatin condensation. This effect was visualized by increased fluorescence of the exogenously expressed histones. In order to quantify the different levels of histone H3 acetylation the average fluorescence intensity obtained from the immunostaining with antibodies against acetylated H3 was measured and normalized with values obtained in the same cells with fluorescent histones H2B-EYFP. Results summarized in Figure 4.5B indicate that the acetylation levels were 30% reduced in cells treated with 20 μM of **2c**, and a reduction higher than 50% was observed using 40 μM as compared to DMSO control cells.

In light of these *in vitro* and *in vivo* promising results, we decided to investigate if compound 2c was able to inhibit also acetylation of integrase and eventually study the effect of reduced IN acetylation on viral infectivity.

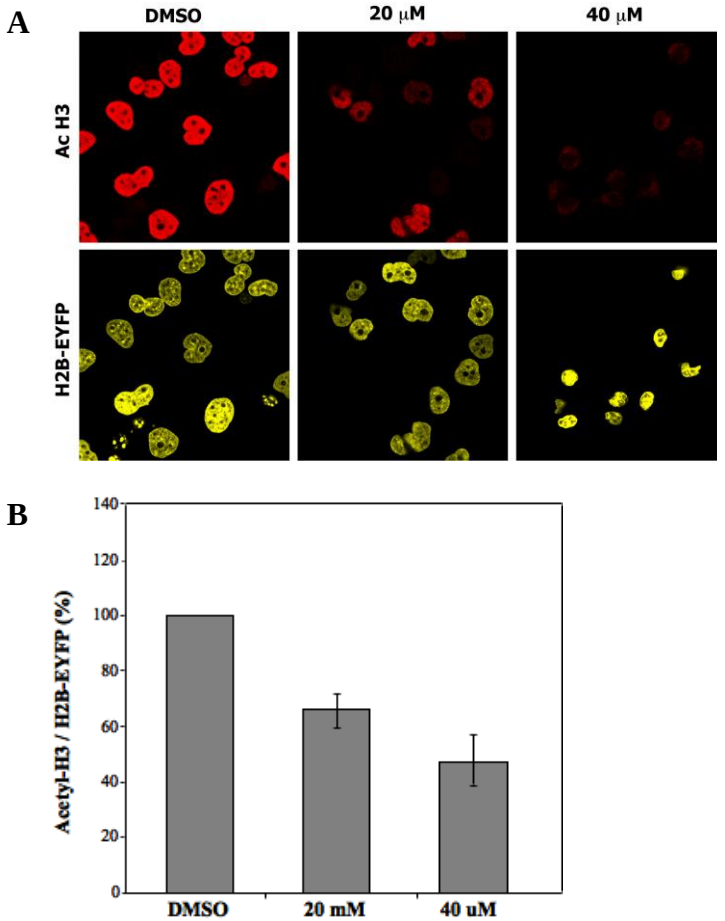


Figure 4.5. (A) HeLa–H2B-EYFP cells treated with derivative **2c** (20 μM or 40 μM) or DMSO were immunostained with antibodies anti-acetyl H3 and analyzed with appropriate wavelength to visualize acetylated H3 or H2B-EYFP total protein levels. (B) The percent inhibition of histone H3 acetylation was obtained by measuring the mean fluorescence intensity with anti-acetyl H3 antibodies relative to the mean fluorescence intensity values of H2B-EYFP from the same cells. The graph summarizes data obtained from three independent experiments. Mean and standard error were derived analyzing 150 cells per experiment.

4.2 *In vivo* inhibition of Integrase acetylation

HIV-1 Integrase is acetylated by p300 and GCN5 (Cereseto, 2005; Terreni, 2010).

Since compound 2c showed a good HAT inhibitory activity on histones, we investigated whether this molecule could inhibit also acetylation of integrase.

Initially we tested *in vitro* the activity of 2C on integrase function. To this aim, we performed a strand transfer assay (figure 4.6) using recombinant His-tagged integrase in the presence of varying amount of compound 2c or of a known integrase inhibitor (RDS1983) as a positive control. The pure solvent used to dissolve both 2c and RDS1983 was instead used as a negative control.

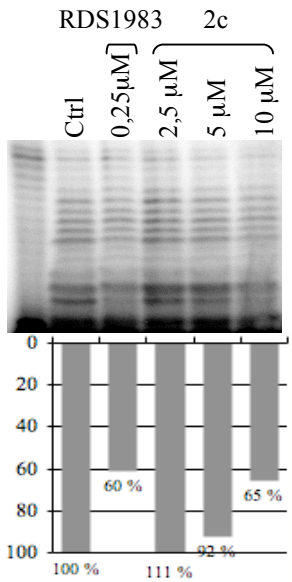


Figure 4.6. Integrase cataliytic activity is not affected by compound 2c. *In vitro* strand transfer assay was performed using recombinant integrase (His-IN) in the presence of increasing amounts of compound 2c (2.5, 5, 10 µM). The activity of integrase in the presence of compound 2c was compared with the activity obtained with a known integrase inhibitor, the dicheto-acid (DKA) RDS1983, at a concentration equal to its *in vitro* IC50 (0,25µM). Integrase activity remained unaltered at low concentration of compound 2c (2,5 and 5µM) also used for subsequent *in vivo* infectivity experiments, while at the highest concentration (10 µM) the activity was 35% reduced, similarly to the reduction obtained at the IC50 of the DKA derivative.

As shown in figure 4.6, low concentration of 2c (comparable to those used in subsequent *in vivo* experiments on viral infectivity) had no effect on integrase catalytic function (lanes 3 and 4). Conversely, a ten times lower concentration of RDS1983 decreased integrase activity by 40%, as expected since this concentration corresponds to the IC*50* value for this compound (lane 2). When 2c was used at higher concentration (10 μM), there was instead some effect on integrase function (35% reduction, lane 5).

Results from the strand transfer assay lead to the conclusion that any effect on integrase activity subsequently observed *in vivo* should come from inhibition of acetylation of the viral enzyme, since molecule 2c does not alter integrase catalytic activity *per se*.

Next, to test the efficacy of compound 2c on integrase acetylation, 293T cells were transiently transfected with an expression vector encoding for the viral integrase codon-optimized on eukaryotic cells and fused to a Flag tag (pFLAG-IN codon-optimized). At the same time a plasmid encoding for p300 (pCMV-p300) was co-transfected to achieve over-expression of the endogenous protein. Over-expression of p300 was necessary in order to maximize integrase acetylation. In fact, preliminary experiments have shown that the acetylated fraction of integrase is low (data not shown) probably due to continuous de-acetylation activity, as demonstrated for other HAT substrates (Huo, 2005; Glozak, 2005).

Twenty-four hours before transfection cells were treated with 5μM of compound 2c, while control cells where treated with an identical volume of DMSO that is the solvent in which molecule 2c was dissolved. Western Blot on cell lysates harvested 24 hours after transfection shows that integrase acetylation is efficiently inhibited in the presence of compound 2c (Figure 4.7A). As expected, due to the role of histone acetylation in transcriptional

activation, overall protein expression in treated cells was also slightly reduced. Therefore the amounts of cell lysate loaded on the gel were normalized according to IN expression level in treated and untreated cells, as verified by western blot with anti-IN antibody, prior to the analysis of the acetylation level.

In order to evaluate the acetylation levels of integrase incorporated in viral particles, HIV-1 virus stocks were produced in cells treated with compound 2c.

To this aim, cells were treated with 5μM of 2c, 24 hours before co-transfection with the plasmid mix used to produce viruses (pNL4.3 Luc R- E-; pVSVG). Viral supernatants were harvested at 48 hours from transfection, concentrated by ultracentrifugation and resuspended in loading buffer for SDS-page. Control viral supernatants were produced in cells treated with DMSO alone and concentrated with the same experimental procedures. Viral supernatants from treated and from control cells were titrated by p24 assay before western blot, in order to load on the SDS-page gel the same amount of test and control sample. The amount of virus loaded on the gel was normalized by hybridizing the membrane with antibodies against the viral capsid (p24) and resulted equal in test and control samples (figure 4.7B, lower panel); similarly, the amount of integrase incorporated in the virions was normalized using anti-IN antibodies (figure 4.7B, middle panel).

Conversely, western blot using anti-acetylated integrase antibody (Terreni, 2010) showed that integrase acetylation is undetectable in virions produced in treated cells (Figure 4.7B, upper panel).

These results show that it is possible to detect acetylated integrase directly in the natural context of the viral particle. This has been possible thanks to the use of a specific anti-acetylated integrase antibody (Terreni, 2010) and it is a new data in the field of biology of HIV-1.

Moreover, these data demonstrate that 2c is able to inhibit integrase acetilation in the viral particle.

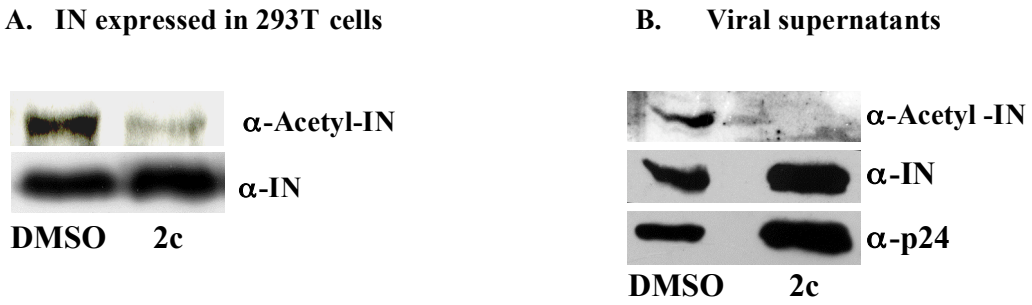


Figure 4.7. Integrase acetylation is inhibited by treatment with compound 2c. **(A)** 293T cells were co-trasfected with Integrase and p300. Acetylation of Integrase was reduced in cells treated with 5 μM of compound 2c (second lane, upper panel), as compared to control cells treated with DMSO (first lane, upper panel). To normalize the amount of transfected Integrase, the same membrane was hybridized with α-IN antibody. **(B)** Viral supernatants produced in cells treated with 5 μM of compound 2c show a marked decrease in the acetylation signal (second lane, upper panel), as compared to viruses produced in control cells treated with DMSO (first lane, upper panel). To normalize the amount of virus loaded and the amount of Integrase included in the two viruses, the same membrane was hybridized with α-p24 and α-IN antibodies, respectively.

4.3 Experimental design to evaluate the activity of compound 2c

In order to further characterize the novel inhibitor of integrase acetylation, we set up the series of experiments described hereafter. In particular, our aim was to determine the precise step in the viral life cycle in which our acetylase inhibitors acts and blocks integrase function.

To address this issue we decided to inhibit acetylation in different steps of the viral replication using the experimental scheme illustrated in figure 4.8. Thanks to the use of single-round replication virions, we could first inhibit the acetylation in virus producing cells, to obtain virions containing hypo-acetylated integrase molecules. Viral supernatants produced in these treated cells were ultracentrifuged and viral pellets were re-suspended in fresh medium, to remove 2c interference in the subsequent infection of untreated cells (Figure 4.8A). In a reciprocal experiments, virus produced in untreated cells were used to challenge treated target cells (Figure 4.8B).

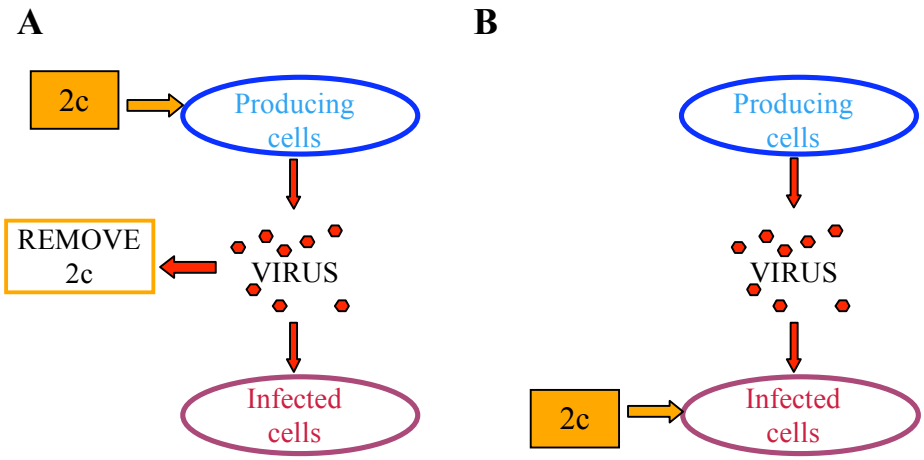


Figure 4.8. Experimental scheme

4.3.1. Infectivity of virions produced in cells treated with the HAT inhibitor 2c

Having verified the presence of acetylated integrase inside the virions and having succeeded to inhibit acetylation during virion formation (as shown in section 4.2), we decided to study the infectivity of virions produced in cells treated with the HAT inhibitor 2c (figure 4.8A).

Single round infectious HIV-1 derived vectors bearing a Luciferase reporter gene copy (NL4.3 Luc R-E-) were produced in 293T cells treated with 5 μM of compound 2c or in control cells treated with DMSO 24 hour prior to viral DNA transfection, in the same experimental conditions used to produce the virions previously analyzed by Western blot.

Viral supernatants were harvested at 48h post-transfection, concentrated by ultracentrifugation, re-suspended in fresh medium and used to infect untreated HeLa cells at MOI 0,1 (56 ng p24 for 30.000 cells). Cells were harvested at 20 hours and 48 hours post-infection to analyze the infectivity of the two viral samples.

Infectivity was first estimated by luciferase reporter activity at 48 hours post-infection. At this time point most of the non-integrated viral DNA copies should have been eliminated by cellular division, thus luciferase activity gives a good estimation of the transcriptional activity of integrated viral copies. As shown in figure 4.9A, the luciferase expression of cells infected with viruses produced in treated cells and thus hypo-acetylated is 70% reduced compared to the one of cells infected with control viruses. In order to better define which step of the viral life cycle was affected by the inhibition of p300, we analyzed the different viral DNA species by real time quantitative PCR (RT-Q-PCR). Integrated DNA copy number was 70% reduced in cells infected with hypo-acetylated virions in comparison with cells infected with control viruses, in

accordance to luciferase results (figure 4.9B). Cells were harvested also at 20 hours post-infection to measure the other viral DNA species and a slighter reduction in total HIV-1 DNA (30%) and in 2LTR circles (40%) copies was again observed (figures 4.9C and 4.9D). This might indicate that viral life cycle steps other than integration, i.e. reverse transcription and/or nuclear import, could also be partially affected by the treatment and contribute in part to the decreased infectivity. This would not be surprising, since integrase is known to interact with the reverse transcription machinery and it takes also part in the nuclear import as part of the PICs. These results show that viruses produced in cells treated with the HAT inhibitor 2c, thus harboring hypo-acetylated integrase molecules, are less infective than viruses produced in cells in which there are physiological levels of acetylase activity. These data show that this effect is due to the lower acetylation level of integrase, since the decreased infectivity seems to be due mainly to an integration defect.

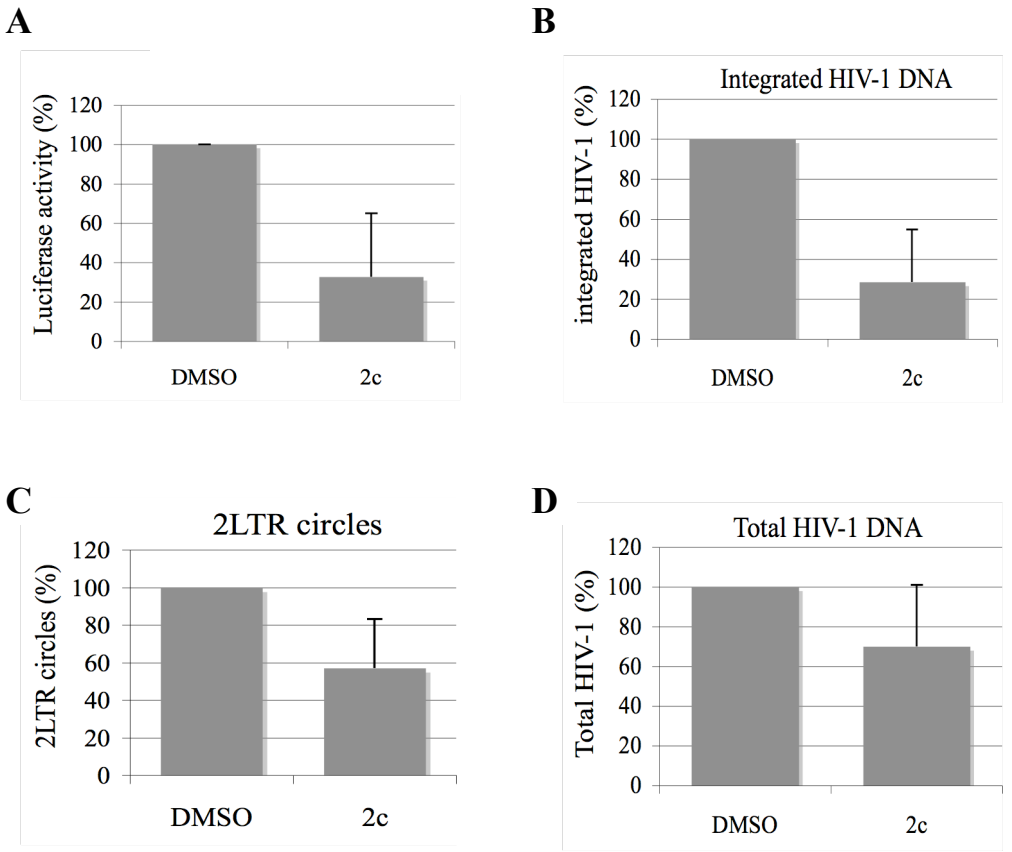


Figure 4.9. Virus produced in cells treated with compound 2c is less infectious. Luciferase reporter activity in cells infected with viruses produced in treated cells is reduced 70% compared to control infections (A). Accordingly, integrated viral DNA copy number is reduced to the same extent (B). A slighter reduction in the other viral DNA species (2LTR and total HIV-1 DNA, C and D) might indicate involvement also of reverse transcription and/or nuclear import in the effect observed. Average data and standard deviations from three independent experiments.

4.3.2. Infectivity in cells treated with the HAT inhibitor 2c.

In order to verify the effect of the HAT inhibitor 2c on cells to be infected with HIV-1 derived virions, we used viruses produced in untreated cells and infected target cells treated with the HAT inhibitor (figure 4.8B).

293T cells were treated with 5μM of compound 2c or with DMSO (control cells) for three days before the infection with single round replication HIV-1 virions produced in un-treated cells. The duration of the treatment (three days) was chosen in order to resemble the conditions of the previous experiment, in which virus-producing cells were treated for a total duration of three days before harvest of viral supernatants. Figure 4.10 shows that infectivity is reduced at about 50% in cells treated with 2c.

In order to determine which was the precise step in the viral replication cycle responsible for the reduced infectivity, quantitative Real Time PCR was performed to measure the amounts of the various viral DNA species. Results show that all the viral DNA species were reduced to some extent. However the reproducibility of this experiment was intrinsically limited by the experimental conditions. In fact, cells treated with compound 2c have a slower growth kinetic compared to normal cells, due to the fact that p300 is an important transcriptional co-activator. Consequently, it was difficult to infect and analyze the same number of treated and control cells. This problem could be circumvent in the previous experiment (Figure 4.9) since viruses produced were quantified and normalized to perform infections with the same amounts of treated and control viruses.

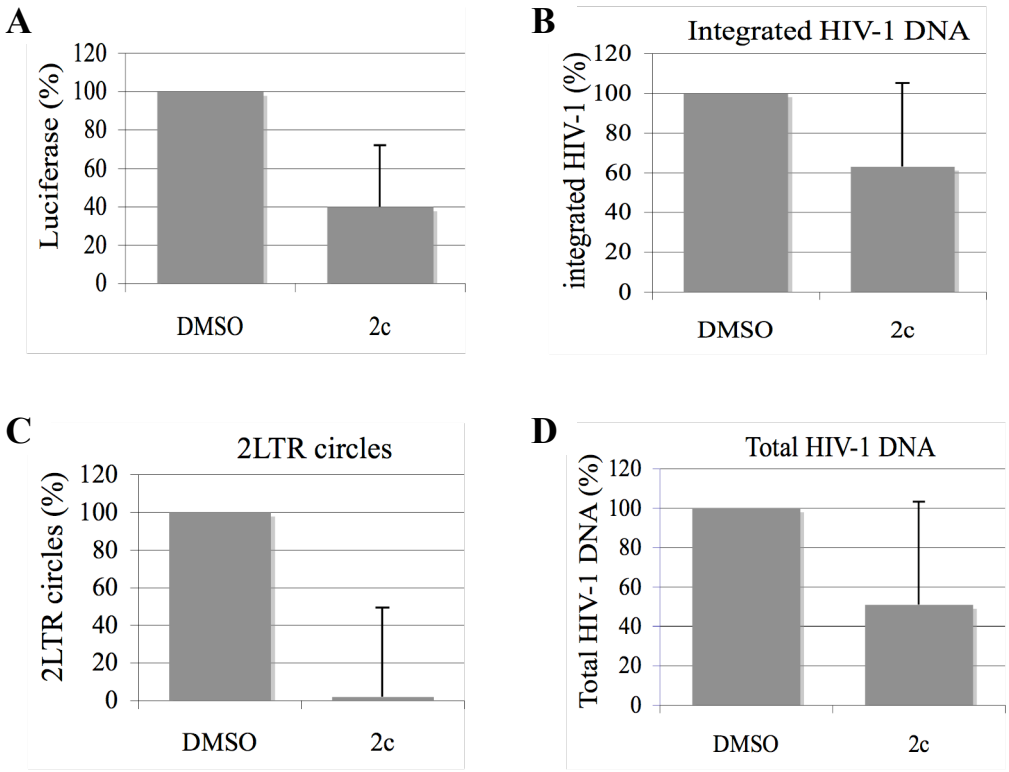


Figure 4.10. 293T cells were treated with 5μM of compound 2c for three days before the infection with single round replication HIV-1 virions. Control cells were treated with DMSO and infected with the same viral stocks. Luciferase activity measured 48h post infection is 60% lower in treated cells compared to control cells (A). Parallely, all the viral DNA species as measured by Real Time PCR are also reduced (B, C and D).

4.4 Transient and stable knock-down of p300 and GCN5

To confirm that the reduced infectivity of hypo-acetylated virions was indeed primarily due to the inhibition, obtained with compound 2c, on the acetyl-transferase activity of p300, we attempted to produce hypo-acetylated virions in cells that were knocked-down for p300. Since compound 2c is also active on GCN-5, that has been demonstrated to acetylate integrase (Terreni, 2010), we tried to obtain double knock-down cells in which both HATs are down-regulated.

HeLa or 293T cells were transduced with lentiviral vectors (GIPZ or

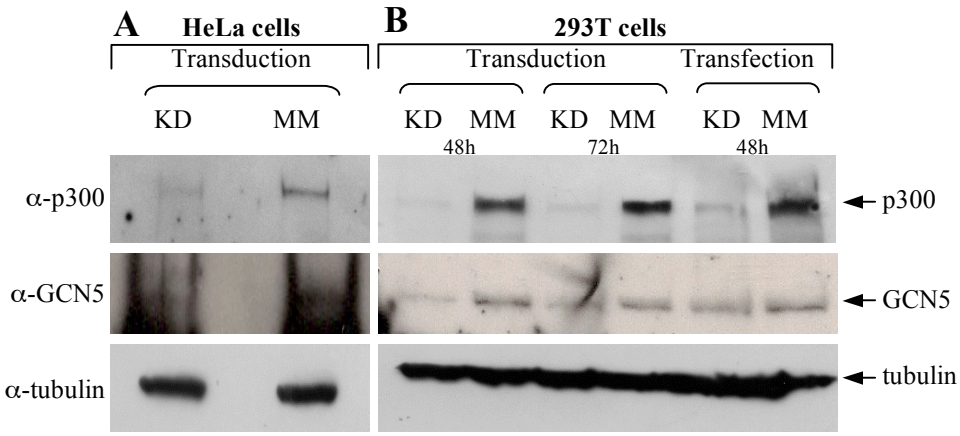


Figure 4.11 Transient knockdown of p300 and GCN5. **(A)** HeLa cells were transducted with GIPZ-GCN5 and LKO1-p300 lentiviral vectors containing short-hairpin RNA against the transcript of the two HATs. Control cells were transduced with GIP-Z MM09 lentiviral vector containing a mis-match shRNAmir. Knock-down of p300 is quite efficient (first lane, upper panel) while simultaneous knock-down of GCN5 is less efficient (first lane, middle panel). **(B)** 293T cells were transducted with GIPZ-GCN5 and LKO1-p300 lentiviral vectors or with GIP-Z MM09 mis-match control and harvested at 48h (lines 1 and 2, each panel) or at 72h (lines 3 and 4, each panel). Alternatively, 293T cells were directly transfected with pGIPz-GCN5, pLKO1-p300 or pGIPZ-MM09 plasmids (lines 5 and 6, each panel). In both cases, knock-down of p300 is very efficient (first, third and fifth lanes, upper panel) and simultaneous knock-down of GCN5 is quite efficient (first, third and fifth lanes, middle panel).

LKO1) encoding an appropriate shRNAmir to simultaneously silence GCN5 and p300 (500.000 RTcpm GIPZ-GCN5 + 500.000 RTcpm LKO1-p300 for 40.000 cells) or a mis-match control shRNAmir insert (1.000.000 RTcpm for 40.000 cells). Alternatively, 293T cells were directly transfected with pGIPZ-GCN5 and pLKO1-p300 plasmids or with the control plasmid pGIPZ-MM09. Cells were harvested two days post-transduction or post-transfection and western blot on cell lysates was performed using α-p300 or α-GCN5 antibodies.

Figure 4.11 shows that silencing of p300 was very efficient both in HeLa and in 293T cells, either through transduction or direct transfection of the shRNAmir encoding plasmids. Simultaneous silencing of GCN5 was less efficient in HeLa cells, while, in 293T cells, GCN5 silencing was more evident by using transduction conditions.

Transduced 293T cells were tested at different time points post-transduction and the silencing resulted stable for at least 72 hours (data not shown).

Silenced cells were then infected 48 hours post-silencing with NL4.3 Luc R-E- VSVG pseudotyped virions at MOI 0,04 (45ng p24 / 60.000 cells) for 2 hours. The combination of the double knockdown plus the subsequent infection resulted in severe toxicity for both type of cultured cells, regardless on whether the silencing had been obtained by transduction or by transfection. Therefore the analysis of the infectivity of HIV-1 in transiently silenced cells could not be performed.

We thus tried to produce stable clones silenced for both HATs.

As shown in Figure 4.12, it was not possible to obtain stable clones that were double knocked down for p300 and GCN5, likely due to the important

role of histone acetylation in DNA organization and functioning. Single knockdown clones for p300 were obtained, in which silencing of the sole p300 was quite efficient, but the level of GCN5 resulted unaffected. These clones were thus not useful for our scope.

We conclude that depletion of both p300 and GCN5 is not tolerated by cells and therefore this experimental strategy cannot be exploited. Thus, the use of a drug acetylase inhibitor, without the need for further cell treatments (transfection or transduction with shRNA), is the only experimental tool available to block acetylation. This further remarks the importance of selecting an efficient drug inhibitor of these enzymes.

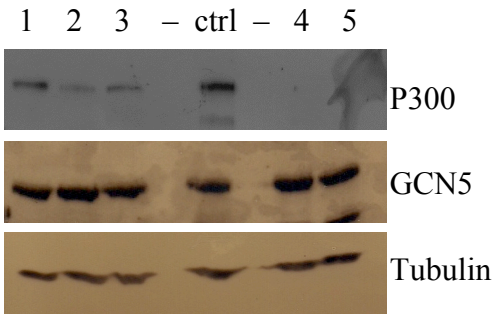


Figure 4.12. 293T clones knock-down for p300 and GCN5. Clones 1-5 display variable level of expression of p300 (upper panel), while it was not possible to simultaneously silence GCN5 (middle panel). Loading of the sample is normalized by tubulin expression (lower panel).

4.5 Infectivity of hyperacetylated virions

Having assessed that hypo-acetylated virions are less infectious than control viruses with physiological level of integrase acetylation, we sought to study the effect on viral infectivity of increased integrase acetylation. To this aim we exploit the system of vpr-mediated protein trans-incorporation (Liu, 1997) in order to insert functional HAT domain of p300 in the viral particle and enhance the acetylation of the viral enzyme.

4.5.1 Generation of virions containing hyper-acetylated integrase through IN-HAT chimeras trans-incorporation

The first attempt was to exploit a method previously set up in our lab to obtain integrase hyperacetylation by mean of a tethered catalysis system (Allouch, 2009). This system consists in fusing integrase to the HAT domain of p300, thus obtaining a chimera in which integrase is constitutively acetylated. As a control, chimeric constructs containing integrase fused to a point-mutated, catalytically inactive HAT domain, were generated (Figure 4.13).

These constructs were fused to a vpr encoding portion that serves to facilitate the incorporation of the protein expressed *in trans* during the assembly of the viral particle (Wu, 195; Wu, 1997; Fletcher, 1997). A proteolytic cleavage site recognized by the HIV-1 protease has been introduced to permit the separation of the vpr portion from the chimeric protein after the incorporation in the virion. An HA tag was attached to the C-terminal part of the HAT domain.

Figure 4.13. Integrase-HAT chimera. The chimeric construct is separated from vpr domain by a proteolytic cleavage site recognized by the HIV-1 protease, so that the free chimeric protein is released in the virion after vpr-mediated transincorporation.

The viral particles were produced through trans-incorporation in integration defective HIV-1 virions containing a catalytically inactive integrase (D64E).

To test for incorporation of integrase and for the level of acetylation, western blot analysis was performed on viral supernatants. As shown in figure 4.14, chimeric IN-HAT proteins, both wt and mut, could be efficiently trans-incorporated in the viral particles, since the HA antibody detects a band (lower panel).

Remarkably, integrase acetylation (at physiological levels) was observable also in the absence of the chimeric construct (upper panel, third lane). Moreover, the protein containing a HATwt domain was efficiently hyper-acetylated, as it becomes evident comparing the first two lanes in the upper panel of figure 4.14.

Interestingly, also the band corresponding to integrase alone (IN), likely expressed by the viral clone, resulted hyper-acetylated in virions carrying the wt IN-HAT chimera, while virions containing the mutated IN-HAT chimera displayed basal levels of integrase acetylation. Since integrase is known to oligomerize and to be active in its oligomeric form, we speculate that the IN-HAT chimera in virions is able to interact with the integrase expressed by the viral clone and to induce hyper-acetylation of integrase.

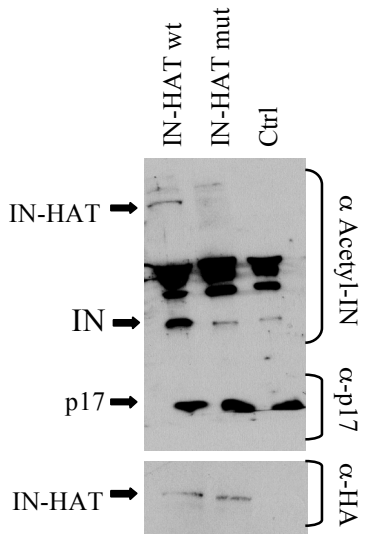


Figure 4.14. Trans-incorporated (TIC) virions with chimeric IN-HAT constructs contain hyper-acetylated integrase. Western blot on concentrated viral supernatants show the hyper-acetylated IN-HAT chimera (first lane, upper band) in TICs containing the IN-HATwt construct, while acetylation in control virion containing IN-HATmut constructs is not appreciable (second lane). Amount of virions loaded is normalized by the expression of the matrix protein p17, while the amount of trans-incorporated chimeric protein is normalized by WB to the HA tag fused to the HAT portion (lower panel, first and second lane). As an additional control, a non trans-incorporated virion was also loaded (third lane).

However, the above-described hyper-acetylated virions resulted not more infectious than control virions not carrying a trans-incorporated integrase (data not shown). This is likely due to the fact that the chimeric IN-HAT protein is much larger than the wt IN (about 4 times), arising steric hindrance problems which become even more evident in its oligomeric form and impair the enzymatic activity of the trans-incorporated enzyme.

4.5.2 Generation of virions containing hyper-acetylated integrase through HAT domains trans-incorporation

Since previous data indicated that integrase expressed from the viral clone is highly acetylated by the transincorporated IN-HAT chimera, we modified the strategy aimed at the production of virions containing acetylated integrase. Vpr-

HAT proteins (wt or mutated) were trans-incorporated in virions carrying a functional integrase (pNL4.3 Luc R-E-), in order to obtain the hyperacetyation of the integrase produced by the viral clone, as schematized in figure 4.15.

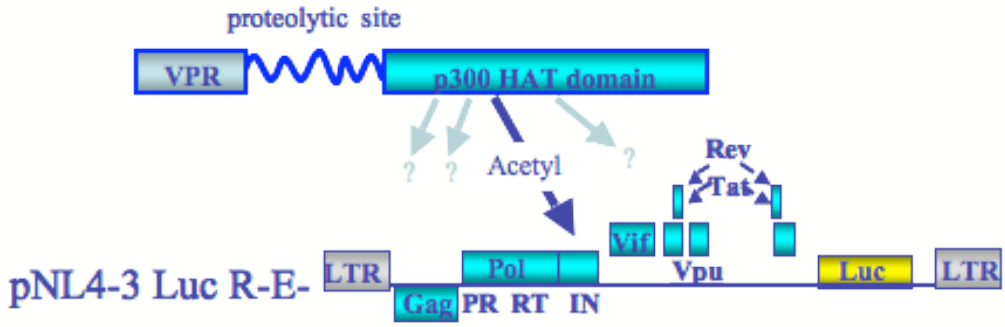


Figure 4.15. Generation of Trans-Incorporated (TIC) wt viruses

To verify for integrase acetylation western blot analysis was carried out on concentrated viral supernatants containing either the wt or the mutated form of the vpr-HAT protein. As shown in figure 4.16, hybridization with HA antibodies demonstrates that both the wt and the mutated form of the vpr-HAT proteins are efficiently trans-incorporated in the viral particle (upper panel). The amount of virus in the two lanes was normalized by p24 on the viral supernatants before loading the gel and then verified by hybridization with IN antibodies (figure 4.16, lower panel).

Hybridization with antibody recognizing acetylated integrase (middle panel in figure 4.16) shows that, similar to what obtained with the previous experimental settings (paragraph 4.5.1), it was possible also in this case to obtain virions in which integrase was acetylated above its physiological level.

HeLa cells were then infected with hyper-acetylated and control viruses and the infectivity was estimated by the luciferase reporter expression level. Additionally, quantitative measurements of the different HIV-1 DNA species

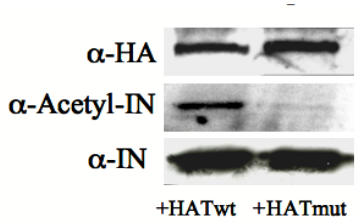


Figure 4.16. Integrase in wt virions trans-incorporated by vpr-HATwt construct is hyper-acetylated. Western blot on concentrated viral supernatants shows that integrase is acetylated above its physiological level when vpr-HATwt is trans-incorporated (first lane, middle panel), while this does not happen when catalytically inactive vpr-HATmut is used (second lane, middle panel). Level of trans-incorporated HAT protein are verified by western blot against the HA tag fused to HAT (upper panel). Unmodified integrase level are also verified (lower panel).

was performed through Real Time PCR on infected cells lysates. As illustrated in figure 4.17, infectivity of hyper-acetylated virions is enhanced, compared to control virions in which the trans-incorporated HAT domain was catalytically inactive.

While total viral DNA level in infections with both trans-incorporated virions resulted similar (figure 4.17D, first and second bars), integrated DNA copy number was higher in infections with hyperacetylated viral stocks (figure 4.17B, first and second bars). 2LTR circles level in cells infected with hyper-acetylated virions were reduced compared to infections with control virions (figure 4.17C, first and second bars), which confirmed that the effect observed is due to enhanced integration.

It is also interesting to notice that luciferase activity of infections with viral stocks containing HATwt domains resulted even higher than that of wt non trans-incorporated virions (figure 4.17A, first and third bars). Since this is not paralleled by an increase in integrated HIV-1 copy number of the virions containing IN-HATwt, compared to the wt virus (figure 4.17B, first and third bars), this luciferase increase might be due to an additional transcriptional effect caused by free HAT entering the infected cells with the virus.

As an additional control, parallel experiments were performed trans-incorporating the same vpr-HAT constructs in control virions containing integrase carrying three Lysine to Arginine point mutations at the three lysines targeted by p300 acetylation (NL4.3 Luc R- E- K 264, 266, 273 R).

As expected, both luciferase activity and integrated viral DNA measured in cells infected with these mutant viruses was drastically decreased (about 5 fold) as compared with the virions carrying wt integrase (lanes 4, 5 and 6 in figure 4.17A and 4.17B).

In addition, since these viruses carry non-acetylable integrase, integration efficiency was not affected by acetylation mediated by the trans-incorporated HATwt domain (compare lanes 4 and 5 in both figure 4.17A and B).

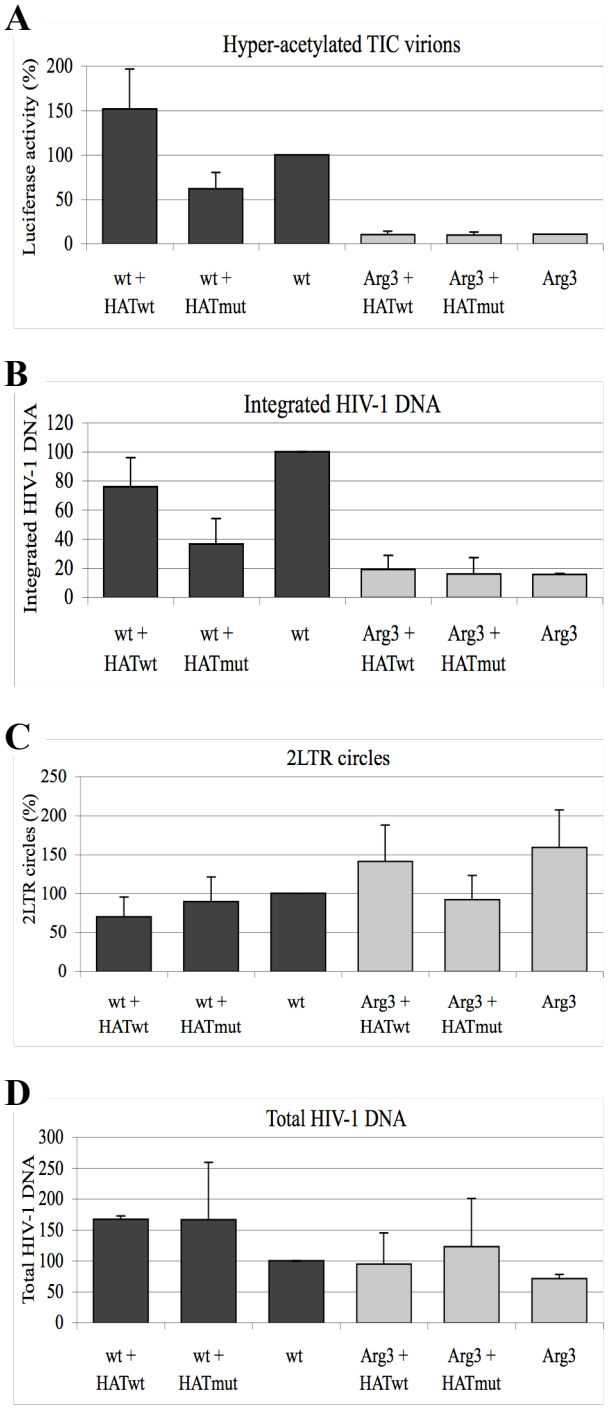


Figure 4.17. Infectivity of hyperacetylated virions is enhanced. NL4.3 Luc R-E- wt virions were trans-incorporated with vpr-HATwt or with catalitycally inactive vpr-HATmut proteins. (first and second bars respectively, in each graph). As an additional control, wt non trans-incorporated virions were used (third bar, each graph). Luciferase activity of TIC containing HATwt is higher than that of TIC containing mutated HATmut and also of that of non-TIC wt viruses (A). Integrated viral copy number of hyper-acetylated TIC is accordingly enhanced compared to control TICs, while is not higher than that measured in infections with non-TIC wt viruses (B). This discrepancy with the luciferase result might be due to an additional transcriptonal effect in TIC containing wt HAT, coming from free HAT entering the infected cells together with the virus. Total DNA level in the three infections were similar, while 2LTR circles in infections with hyperacetylated TICs resulted decreased by about 30% compared to control TICs (C), being in perfect accordance with the increase in the integrated copy number described above. Control viruses carrying a Lys to Arg mutation at the three acetylable lysines (Arg3) were not affected by HAT trans-incorporation (light gray bars, each graph).

5 DISCUSSION

5.1 A new class of small molecules is able to inhibit p300 Histone Acetyl Transferase

Among histone tails modifications, acetylation has been most widely studied in the context of gene expression. The dynamic equilibrium between acetylation and deacetylation is maintained by the activity of histone acetyltransferases (HATs) and deacetylases (HDACs) that regulate the expression of the genome. Mutations in these enzyme have been proven to be associated with certain cancers and other human disease processes (Giles, 1998). Therefore, these enzymes could be considered useful target for a novel approach in chemotherapy. During the last decade, significant progress has been made in the field of HDAC inhibitors as antineoplastic agents and some of these compounds are already in clinical trial as anticancer drugs (Richon, 2001). Conversely, specific inhibitors for p300 were not identified until recently.

The aim of the present work was the identification of novel anti-p300 agents that could be useful as potential lead molecules for anticancer as well as antiviral drug discovery. The anti-p300 agents so far identified have been described in the introduction (paragraph 1.9).

We reported herein a new class of small synthetic molecules that inhibited the p300 activities in *in vitro* assays. In particular, we described the discovery of cinnamoyl compounds **1a-c** and some **2a-d** as inhibitors of p300 enzyme. Among them, derivative **2c** was proven the most potent anti-p300 agent that was 6 times more active than Lys-CoA used as a reference drug, and with a high selectivity for p300 as demonstrated by comparative *in vitro* assays performed on different HATs belonging to another family of enzymes. Most notably, derivative **2c** was active in mammalian cells as demonstrated by the

downregulation of histone H3 acetylation. For all the above mentioned reasons derivative **2c** might be considered a lead compound for further studies in this field. Remarkably for the scope of this study, HAT inhibitor **2c** efficiently decreased HIV infectivity in cell based assays, acting mainly via inhibition of HIV integrase acetylation.

Another inhibitor of p300 has been used during HIV infectivity studies by Mantelingu and co-workers. They reported that an iso-garcinol-derived specific inhibitor of p300 is able to reduce HIV-1 multiplication in SupT1 cells as measured by reduced syncytia formation. They attributed the effect to reduced histone acetylation in the target T-cells but did not try to analyze the viral replication step affected by the presence of the inhibitor, hypothesizing but did not demonstrating the involvement of integrase (Mantelingu, 2007).

It is also interesting to notice the observation that, in immunofluorescence experiments, decreased acetylation caused by compound **2c** is accompanied by an overall increased of H2B-EYFP fluorescence intensity. This observation is in accordance to what previously reported in similar cellular systems (Kanda, 1998; Weidemann, 2003), where chromatin condensation resulting from decreased histone acetylation was visualized by increased fluorescence of the exogenously expressed histones and validates the experimental settings we employed.

The molecules object of this study have been designed using as a starting point the structure of curcumin, which had been reported to be a specific inhibitor of p300 HAT activity (Balasubramanyam, Varier, 2004). However, in our *in vitro* assay, curcumin resulted unable to inhibit histone acetylation mediated by both full lenght and HAT domain of p300. This finding is in contrast to what reported by Balasubramanyam and co-workers. We hypothesized that this discrepancy might be due to the difference in curcumin

used, being ours derived from chemical synthesis, in contrast to the one isolated from its natural source used by Balasubramanyam. The latter might thus contain other un-identified adjuvant molecules, which would contribute to the observed effect. As a strange coincidence, it is interesting to notice that curcumin analogs had been called as possible anti-integrase drugs in the far 1995 by Mazumder and co-workers, which had identified curcumin as an HIV integrase inhibitor *per se*, acting directly on the viral enzyme catalytic core, also in the absence of the acetylated domain (Mazumder, 1995).

Extensive SAR, as well as molecular modeling studies are ongoing to increase the knowledge within the here reported series of p300 inhibitors. Due to the vital role of p300 in the reversible processes of acetylation of histones and other cellular proteins, the development of these inhibitors might result in novel approaches to antitumor and antiviral chemotherapies.

5.2 Integrase acetylation inside the viral particles.

Our group has previously found that HIV-1 integrase is acetylated at lysines 264, 266 and 273 by both p300 and GCN5 (and additionally on Lysine 258 exclusively acetylated by GCN5) and that these post-translational modifications positively regulates viral integration and infectivity (Cereseto, 2005, Terreni, 2010). Other groups have also reported on integrase acetylation (Topper, 2007, Apolonia, 2007). This study expands our previous findings, showing for the first time integrase acetylation inside the virions. Although integrase acetylation had been reported *in vitro* and *in vivo (*Cereseto, 2005; Terreni, 2010; Topper, 2007; Apolonia, 2007), the possibility to observe it in the context of the viral particle was not obvious because this post-translational modification is not a stable one but it is reversible and in a dynamic equilibrium with de-acetylation: two groups of enzymes, histone acetyltransferases (HATs)

and deacetylases (HDACs), balance the acetylation levels as required for cellular function.

This temporary nature of acetylation somehow complicated the experimental settings when studying intracellular protein acetylation, with the need of introducing deacetylases inhibitors in the cell lysates or during cell culture, in order to maximize the detection of acetylated proteins. Moreover, co-transfection with a plasmid encoding for p300 was also necessary, in order to achieve over-expression of the enzyme. Here, concentrated viral supernatants were analyzed by western blot as they were, without further treatment to enhance the visualization of acetylation and, nevertheless, the acetylated fraction was well visible. This seems to indicate that integrase is preferentially incorporated in its acetylated form during the formation of the viral particle, where it is afterwards protected from the action of cellular de-acetylases.

We have shown that the HAT domain of a chimeric IN-HAT protein trans-incorporated in a virion is able to hyperacetylate also integrase produced by the viral clone. This is likely due to oligomerization between the trans-incorporated chimeric integrase and integrase produced by the virus. This observation is in accordance with previous reports showing that integrase oligomers form already during maturation of the viral particle, after proteolytic cleavage of the Gag-Pol precursor (Petit, 1999; Petit, 2000; Berthoux, 2007).

5.3 Molecular engineering of viral particles containing hyper-acetylated integrase

In order to obtain viral particles containing hyper-acetylated integrase we exploited two different strategies, described hereafter.

First, we used a method previously set-up by our group (Allouch, 2009) to produce constitutively acetylated integrase by mean of a tethered catalysis system, which consists in fusing integrase to the HAT domain of p300. To avoid disturbing the viral clone replication, we chose not to fuse the sequence of the HAT domain directly at the C-terminus of integrase in the context of the viral genome. Indeed, previous attempts to produce retroviruses that contained integrase fusion proteins were unsuccessful due to loss of virus infectivity after transfection (Bushman, 1997) or loss of fusion protein expression during viral replication owing to reversion (Katz, 1996). The difficulty in encoding the fusion protein in the viral genome is probably due to the fact that the 3'coding region of integrase overlaps with vif, thus fusion of the extra sequence interferes with splicing (Purcell, 1993). We thus decided to incorporate IN-HAT fusion protein *in trans* into HIV-1 viral clones carrying a catalytically inactive point-mutated D64E integrase. This approach exploits the vpr property to interact with p6 region of gag (Bachand, 1999; Paxton, 1993) to shuttle exogenous proteins fused to vpr inside the viral particles (Wu, 1995; Wu, 1997; Fletcher, 1997; Liu, 1997). The trans-incorporation system has been successfully used to incorporate chimeric proteins IN-LexA (Goulaouic, 1996; Holmes-Son, 2000) or IN-E2C (Tan, 2006), while Lu *et al.* showed that a viral clone carrying a catalytically inactive integrase mutant could be efficiently complemented by a trans-incorporated vpr-fused integrase (Lu, 2004). In our system, trans-incorporation of the chimeric IN-HAT protein was efficient and the tethered catalysis system succeeded to achieve constitutively acetylated integrase, as demonstrated by western blot on viral supernatants (figure 4.14). However our IN-HAT chimeric protein was much larger than the previously reported LexA or E2C fusion proteins (Holmes-Son, 2000; Tan, 2006) due to the big size of the HAT domain, and thus it hampered the catalytic function of

integrase, probably by steric interference with either integrase folding or multimerization.

The trans-incorporation system was also the basis for the second experimental approach (paragraph 4.5.2), in which the sole HAT domain was shuttled in a viral clone carrying, this time, a catalytically active wt integrase. Trans-incorporated HAT efficiently acetylated integrase produced by the viral clone due to the proximity of the two proteins in the narrow space inside the viral particle, thus the HAT catalysis was obtained without the need for fusing the two proteins. This second set of experiments left as intact as possible the replication cycle of HIV, as it exploited viral clones containing hyper-acetylated wild type integrase, not fused to any other potentially interfering sequences and free to complex in its active oligomeric form with other integrase subunits, to exert its catalytic functions. The resulted increased integration should therefore be interpreted by resulting solely from increased acetylation of the viral enzyme.

5.4 Importance of integrase acetylation during the replication cycle of HIV-1

The here presented data further support and expand previous findings by our group on the positive role of protein acetylation in viral infectivity (Cereseto, 2005, Terreni, 2010).

Here conditions of reduced acetylation were obtained without depletion of cellular HATs and using un-modified integrase, thus in situation very close to physiological, thanks to the use of a new p300 inhibitor selected in this study.

In accordance to previous reports from our group (Cereseto, 2005; Terreni, 2010), we could show that reduced integrase acetylation impairs viral infectivity.

Additionally, in a reverse experiment, we were able to enhance integrase acetylation above its physiological level and show that this improves viral performance, specifically acting on the integration step. In particular, enhanced acetylation was obtained inside the viral particle by means of trans-incorporated HAT domain in an infectious viral clone, notably in conditions of minimal interference with the viral life cycle or integrase enzymatic function. In fact nor integrase nor the viral genome needed to be mutated, and integrase was not fused to other proteins.

In our opinion, the results obtained in this study, together with those presented in previous reports, support the notion of integrase CTD acetylation by cellular HATs representing a mechanism which contributes to finely regulate the efficiency of HIV integration and consequently positively influences viral infectivity.

5.5 Inhibitors of integrase acetylation as potential lead compounds for the design of second generation integrase inhibitors

As already outlined in the introduction, integrase is still a poorly exploited target for anti-retroviral therapy.

Raltegravir (RAL) was the first drug in this class to be approved by the United States Food and Drug Administration (FDA) for use in highly treatment-experienced HIV-1-infected patients in October 2007 (Grinsztejn, 2007; Summa, 2008). Unfortunately, contrary to the expectations coming from preliminary *in vitro* results, viral strains resistant to Raltegravir rapidly emerged

during treatments. (Malet, 2008). Similarly to Raltegravir, current integrase inhibitors target the catalytic site of the enzyme, and are therefore intrinsically more prone to induce resistance.

Since integrase, like the other viral proteins, interacts with a number of cellular co-factors, an emerging strategy for the design of new anti-retroviral drugs with delayed onset of resistance is to target the interaction between the viral and the cellular proteins (Christ, 2010).

The molecule selected in this study, by inhibiting a post-translational integrase modification, while not affecting the viral enzyme's catalytic activity (see figure 4-6), has the potential to become a lead compound for the development of second generation anti-integrase drugs of this kind. On the other hand, given its inhibitory effect on a cellular enzyme, it raises obvious concerns on its potential cyto-toxicity, as acetylation/deacetylation patterns in the cell are among the major determinants of the epigenetic control of gene expression. Indeed, drugs specifically designed to block enzymes of the HAT (Ghizzoni, 2010; Kang, 2005; Tourkina 2004; Kuo, 2006; Hsu, 2006; Gomathinayagam, 2008; Acharya, 2008) or HDAC (reviewed in Bolden, 2006; Ma, 2009) families have been studied as potential anti-cancer agents due to their ability to disrupt the epigenetic changes associated to tumor development and to induce apoptosis in cancer cells by mechanisms related to the acetylation state of both histone and non-histone cellular proteins.

These issues push to direct future efforts towards the development of more selective derivatives of compound 2c, able to target specifically the modification of the viral enzyme, reducing the interference with the cellular functions of p300. Nevertheless, it is also worth to recall here that the abundance and redundancy of histone acetyl-transferases offers to the cell the possibility to handle a partial block of one enzyme of this group by simply

activating alternative pathways to achieve the needed acetylation patterns. Indeed 2c resulted almost non-toxic in cell coltures at the concentration used to observe its HAT inhibitory activity, which corresponded to its IC_{50}, thus it represents, also in respects of these toxicity issues, a good starting point for further optimization.

6 – CONCLUSIONS AND FUTURE DIRECTIONS

Our and other groups have recently investigated post-translational modifications of HIV-1 integrase as possible targets for future anti-retroviral therapy. In particular, our group has focused on the acetylation of integrase, operated by two different classes of histone acetyl-transferases, and suggested a model to explain HIV-1 integration preference for transcriptionally active genomic regions, through its interaction with chromatin modifying HAT complexes.

In this study we have reported the biological activity of a new synthetic inhibitor of p300 Histone Acetyl-Transferase, selected by an *in vitro* screening.

We showed that this molecule is capable of inhibiting also the acetylation of HIV-1 integrase. We have used this molecule as a basic research tool aimed at investigating the role of integrase acetylation and this allowed us to confirm previous findings from our group highlighting the importance of this post-translational modification for the viral performance. We have further confirmed this data in a reciprocal experimental setting, by generating a system which allowed us to obtain hyper-acetylated virions, and showing that this was beneficial to viral infectivity.

Structure-Activity Relationships studies are ongoing, aimed at optimizing this compound in order to obtain a second generation anti-integrase drug, targeting the interaction of the viral enzyme with one of its cellular co-factor, p300.

7 – REFERENCES

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Cinnamoyl Compounds as Simple Molecules that Inhibit p300 Histone Acetyltransferase

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Received August 4, 2006

Cinnamoly compounds **1a**−**c** and **2a**−**d** were designed, synthesized, and in vitro tested as p300 inhibitors. At different degrees, all tested compounds were proven to inactivate p300, particularly, derivative **2c** was the most active inhibitor, also showing high specificity for p300 as compared to other histone acetyltransferases. Most notably, **2c** showed anti-acetylase activity in mammalian cells. These compounds represent a new class of synthetic inhibitors of p300, characterized by simple chemical structures.

Introduction

DNA is a charged polymer that is highly packaged in the nucleus of eukaryotic cells. This extreme compaction is achieved through association of DNA with a set of basic histone proteins to form a structure known as chromatin. The fundamental repeat unit of chromatin is the nucleosome, in which 146 base pairs of DNA are wound around a histone octamer comprising two copies of each histones H2A, H2B, H3, and H4.[1,2] Nucleosomes are in turn folded into progressively higher-order structures. Though apparently repressive, the precise organization of chromatin is essential for replication, repair, recombination, and chromosomal segregation. Modification in the chromatin organization modulates the expression of underlying genes. The dynamic changes in the chromatin structure are brought about by post-translational modifications of the amino terminal tails of the histones and the ATP-dependent chromatin remodeling. Specific amino acids within the histone tails are the sites of a variety of modifications including phosphorylation, acetylation, methylation, ADP-ribosylation, and ubiquination.[3] Among these modifications, acetylation has been most widely studied in the context of gene expression.

The dynamic equilibrium between acetylation and deacetylation is maintained by the activity of hystone acetyltransferases (HATs[a]) and deacetylases (HDACs) that regulate the expression of the genome. Specifically, HATs function enzymatically by transferring an acetyl group from acetyl-coenzyme A to the ϵ-amino group of certain lysine side chains within a histone's basic N-terminal tail region.[4] HATs are divided into five families including the GNAT family, the MYST group, p300/CBP HATs, the general transcription factor, and the nuclear hormone-related HATs.[5] p300 is a ubiquitously expressed global transcriptional coactivator that has critical roles in a wide variety of cellular phenomena including cell cycle control, differentiation, and apoptosis.[6] Mutations in p300 enzyme have been proven to be associated with certain cancers and other human disease processes.[7] Therefore, these enzymes could be considered useful targets for a novel approach in chemotherapy. During the past decade, significant progress has been made in the field of HDAC inhibitors as antineoplastic agents, and some of these compounds are already in clinical trial as anticancer drugs.[8] Conversely, specific inhibitors for p300 were not identified until recently.

The aim of the present work was the identification of novel anti-p300 agents that could be useful as potential lead molecules for anticancer as well as antiviral drug discovery. The anti-p300 agents so far identified are (i) the natural products garcinol,[9] anacardic acid,[10] and curcumin[11] and (ii) the synthetic derivative Lys-CoA,[12] a lysine analog of HAT substrate acetyl-CoA (Figure 1).

In particular, a screening of plant extracts from *Curcuma longa* rhizome led to the discovery of curcumin as a potent and specific inhibitor of p300.[11] Interestingly, in the course of our studies aimed at the discovery of antiviral agents targeted to HIV-1 integrase, we reported a group of curcumin-like derivatives characterized by a 3,4-dihydroxycinnamoyl pharmacophore.[13] Based on this preliminary evidence and because the studies on structure−activity relationships (SARs) in the field of anti-p300 agents are still limited and only a few Lys-CoA analogs have been described,[14] we set out to identify new synthetic polyhydroxylated aromatic derivatives related to curcumin, garcinol, and anacardic acid as p300 inhibitors. The results of this study may represent a groundwork for the development of novel anti-p300 agents as potential leading molecules for anticancer as well as antiviral drug discovery.

An examination of the chemical structures of these natural products led us to identify some structural features that characterize these compounds: (i) a α,γ-diketo group; (ii) a cinnamoyl moiety; (iii) a catechol ring; and (iv) a salicylic acid portion. Therefore, we decided to test the activity against p300 of cinnamoyl compounds, such as **1a (**related to curcumin), **2a**, and **2d** (cyclohexanone derivatives), previously reported by us in the course of our studies aimed at the discovery of antiviral agents targeted to HIV-1 integrase.[13] In fact, **1a**, **2a**, and **2d** share some of the above chemical features such as (i) the α,γ-diketo group (**1a**), (ii) the cinnamoyl moiety (**1a**, **2a**, **2d**), and (iii) the catechol ring (**1a**, **2a**). Moreover, as a preliminary SAR study, we designed and synthesized salicylic derivatives **1b** and **2b** and compounds **1c** and **2c** that are characterized by the presence in the ortho position to OH groups by lipophilic and withdrawing bromine atoms. Compounds **1a**−**c** and **2a**−**d** (Figure 2) were tested in in vitro assays for their inhibitory

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[a] Abbreviations: HAT, histone acetyl transferase; HDAC, histone deacetylase; GNAT, Gcn5-related *N*-acetyltransferase; P300/CBP, p300/CREB binding protein; SAR, structure−activity relationship; H2B-EYFP, H2B-enhanced yellow fluorescent protein; FCS-DMEM, fetal calf serum-Dulbecco's modified eagle's medium.

10.1021/jm060943s CCC: $37.00
Published on Web 03/10/2007 PAGE EST: 4.7

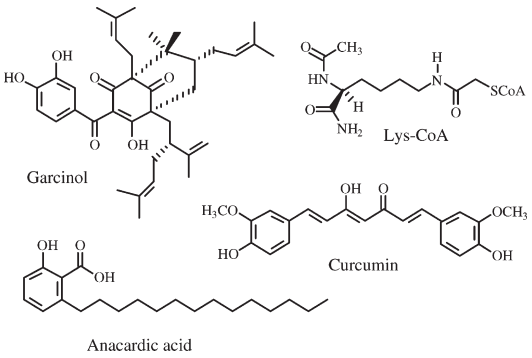
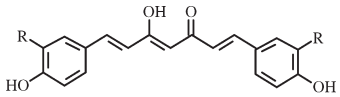
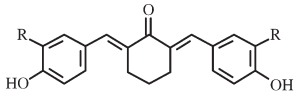


Figure 1. Structures of p300 inhibitors reported in literature.



1a-c

R = OH (**a**), COOH (**b**), Br (**c**)

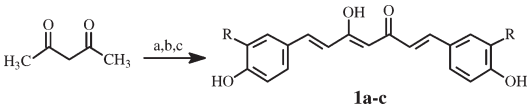


2a-d

R = OH (**a**), COOH (**b**), Br (**c**), OCH_3 (**d**)

Figure 2. Structures of the cinnamoyl derivatives **1a**−**c** and **2a**−**d** reported in the present study and tested as p300 inhibitors.

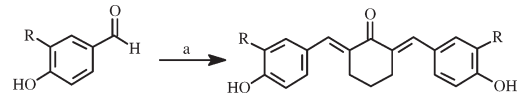
Scheme 1[a]



1a-c

[a] Reagents and conditions: (a) $B(OH)_3$ DMF, 100 °C, 5 min; (b) arylaldehydes, 1,2,3,4-tetrahydroquinoline, AcOH, DMF, 100 °C, 4 h; (c) AcOH, room temp, 1 h. Yields for the three-steps, one-pot synthesis: **1a**,[14] 10%; **1b**, 50%; and **1c**, 65%.

Scheme 2[a]



2a-d

[a] Reagents and conditions: (a) cyclohexanone, montmorillonite K-10, 5 min, 100 W, 100 °C. Yields: **2a**, 75%; **2b**, 50%; **2c**, 89%; **2d**, 75%.

activities against p300. To different extent, all synthesized molecules inhibited p300 enzymatic activity. The most active compound, **2c**, was selective for p300 as compared to other HATs and, most notably, was cell permeable, as demonstrated by decreased histones acetylation.

Results and Discussion

Chemistry. Synthesis of derivatives **1a**−**c** and **2a**−**d** is outlined in Schemes 1 and 2. The curcumin analogs **1a**−**c** were synthesized according to the Babu and Rajasekharan method (Scheme 1).[15]

The fundamental step in this reaction is the protection of the active methylene group by reacting with acetylacetone in the presence of boric acid to get acetylacetone−boric acid complex and reacting less reactive methyls with the appropriate aldehyde using 1,2,3,4-tetrahydroquinoline as a catalyst. Notably, the synthesis of derivative **1b** has already been reported in very low yields (<9%) by Subaraju[16] in three steps involving protection/deprotection procedures of carboxylic groups. However, we obtained **1b** in higher yields (50%) by the application of the Babu method to 5-formylsalicylic acid in the three steps, one-pot synthesis (Scheme 1).

Bis-arylidene derivatives **2a**−**d** were synthesized by condensation of cyclohexanone with the appropriate benzaldehyde (Scheme 2). A new procedure that did not require the preliminary protection of the OH groups was developed. In particular, a dispersion of cyclohexanone and the substituted benzaldehyde in montmorillonite K-10, was submitted to microwave-assisted heating (100 °C, 100 W) for 5 min. Interestingly, montmorillonite K-10 was used in this reaction as both an environmentally benign solid support and a heterogeneous acid catalyst. This procedure allowed (i) increased yields of these condensations if compared to those previously reported,[13] (ii) a reduction in the synthetic pathway from three steps to one step, and (iii) a minimization of the reaction time.

Evaluation of Biological Activities. The p300 inhibitory activities of the newly synthesized cinnamolyl compounds **1a**−**c** and **2a**−**d** were tested in an in vitro acetylation assay[17] using recombinant histones (H1, H2A, H2B, H3, and H4) and the HAT domain of p300 (Figure 3A).

The inhibitory activity of each compound was tested, with concentration ranging from 25 μM to 400 μM (data not shown), or starting with 0.19 μM for the derivative **2c**, to determine the IC_{50} value (Figure 3C and Table 1). Figure 3B shows the histone acetylation levels following incubation with p300 in the presence of scalar amounts of the derivative **2c**.

Compounds **1a**−**c** and **2a**−**d** could be divided into the following: (i) curcumin derivatives with different substituents on the aromatic moieties (**1a**−**c**) and (ii) 2,6-bis-arylidene cyclohexanone derivatives (**2a**−**d**). In general, compounds **1a**−**c** and **2a**−**d** showed good activities against p300, with IC_{50} values ranging from 5 to 233 μM (Table 1). Derivative **2c** was the most potent compound of these series (IC_{50} = 5 μM), being six times more potent than Lys-CoA used as a reference drug. Surprisingly, in our assays, curcumin was inactive at concentrations up to 400 μM. Due to this result, we tested a new stock of commercial curcumin (Fluka) after a further chromatography purification and ^{1}H NMR identification and tested with both HAT domain as well as the full length p300 enzyme.[18] In spite of this, the inactivity of curcumin was confirmed.

Interestingly, the curcumin derivatives **1a**−**c** were potent p300 inhibitors showing IC_{50} values from 21 to 46 μM, comparable to that found for Lys-CoA used as a reference drug in the same experiment (IC_{50} = 30 μM). The most active derivative among this group of molecules was **1b**, which was characterized by salicylic groups (1.4 times more potent than Lys-CoA). Replacement of the carboxylic function with a bromine or hydroxyl groups led to **1c** and **1a**, which were 1.5 and 2 times less potent than parent derivative **1b**, respectively. In general, the activities in this series decreased if the electron-withdrawing groups (COOH, Br) were replaced by electron-donor groups (OH, OCH_3). The following order, depending on substituents in the 3-positions of the aromatic rings, was observed: COOH > Br > OH > OCH_3.

The cyclohexanone derivatives **2a**−**d** were active against p300 as well. The IC_{50} values obtained in the enzyme assays ranged from 5 to 233 μM. The activities of compounds **2a**−**d**

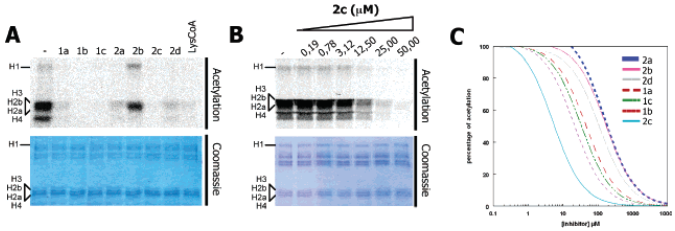
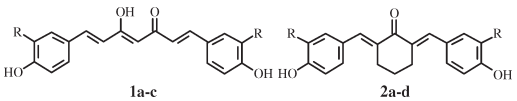


Figure 3. Inhibitory effects of compounds **1a**−**c** and **2a**−**d** on p300 activity. (A) Upper panel: autoradiography of a gel showing acetylated histones following incubation with p300 and ^{14}C acetyl-CoA in the presence of 400 μM of each indicated derivative, Lys-CoA (last lane) or DMSO (first lane). Lower panel: Coomassie blue staining of the same gel showing the total amounts of histones. (B) Upper panel: autoradiography of a gel showing acetylated histones following incubation with p300 in the presence of the indicated concentration of **2c** or DMSO alone (first lane). (C) Dose response curves obtained by densitometric analysis of the levels of histone acetylation mediated by p300 in the presence of **1a**−**c** and **2a**−**d**. The graph summarizes the results obtained from three independent experiments.

Table 1. Inhibitory Activity of Compounds **1a**−**c** and **2a**−**d** against p300 Enzyme



1a-c **2a-d**

cmpd	R	IC_{50}[a]
1a	OH	46 ± 3.9
1b	COOH	21 ± 8.7
1c	Br	33 ± 5.2
curcumin	OCH_3	>400
2a	OH	233 ± 120
2b	COOH	168 ± 12
2c	Br	5 ± 1.3
2d	OCH_3	111 ± 45
Lys-CoA		30 ± 1.6

[a] Inhibitory concentration of 50% (μM) determined from dose−response curves. Data represent the mean values of at least three separate experiments.

decreased based on the substituents in the 3-positions of the aromatic rings in the following order: Br > OCH_3 > COOH > OH. In conclusion, the replacement of hydrophilic groups (COOH, OH) with the lipophilic ones (Br, OCH_3) in the **2a**−**d** series led to increased anti-p300 activities. In particular, the highest potency was obtained with the introduction of the lipophilic and electron-withdrawing bromine atom on the cinnamoyl portion.

The preliminary SARs in the series of cyclohexanone derivatives (compounds **2a**−**d**) were different if compared with those found in the curcumin series (compounds **1a**−**c** and curcumin). A direct comparison among the two series led to the following conclusions: (i) compounds **2a**−**d** were generally less potent than **1a**−**c** derivatives, showing IC_{50} values from 111 to 233 μM, with the exception of **2c**, which was the most potent derivative described in this work (IC_{50} = 5 μM); (ii) introduction of bromine atoms in the 3 position of the benzene rings gave derivatives **1c** and **2c**, which were both endowed with good activities; and (iii) introduction of OH or COOH groups in the same positions within the curcumin series gave compounds **1a** and **1b**, which showed good anti-p300 potency; opposite results were found when the same groups were introduced in the 3 position of benzene rings in the 2,6-bis-arylidene cyclohexanone series (**2a**, IC_{50} = 233 μM; **2b**, IC_{50} = 168 μM).

Derivatives **2c** and **1b**, which showed the highest inhibitory activity, were additionally tested on PCAF and GCN5, both belonging to a different class of HAT factors. The assays were performed using concentrations of **2c** and **1b** corresponding to

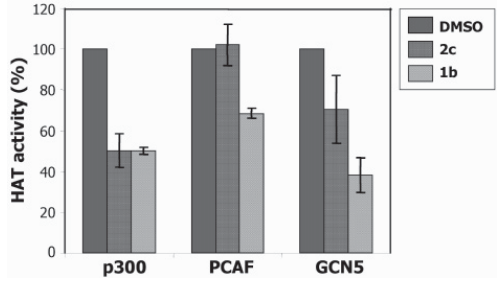


Figure 4. Inhibitory activities of derivatives **2c** (5 μM) and **1b** (21 μM) on different HATs were tested in in vitro assays using equal molar amounts of p300, PCAF, and GCN5. HAT activity for each enzyme is expressed as percent variation as compared to that of the DMSO-treated sample. The graph summarizes the mean densitometric values from three independent experiments (mean ± standard error).

the IC_{50} values formerly determined against p300 (5 μM and 21 μM for **2c** and **1b**, respectively; Table 1). As expected, the activity of p300 was reduced to 50% with both compounds, while the same concentration of derivative **2c** showed no effect on PCAF (100%) and only partial inactivation of GCN5 (70%), indicating a selective inhibition of p300 activity. Conversely, **1b** is partially active on PCAF (68%) and shows on GCN5 (38%) the same efficacy as for p300, indicating that this compound is active on HATs other than p300 (Figure 4).

Several previously described HAT inhibitors, such as Lys-CoA, are not cell permeable and cannot thus be used for in vivo studies. Therefore, we have tested for its anti-acetylase activity in cell culture system derivatives **1b** and **2c**, which showed the most potent inhibitory effect against p300. HeLa cells stably expressing fluorescent H2B histones (HeLa−H2B-EYFP)[18] were treated with various concentrations of **2c** and **1b** and subsequently immunostained with antibodies against acetylated H3 histones. The fluorescent H2B histones were used as internal control to monitor protein expression levels. Derivative **1b** at concentrations up to 200 μM did not alter either the H3 acetylation levels or the H2B protein expression. This experiment led us to hypothesize that **1b** is not cellular permeable (data not shown). Conversely, we found that at 20 μM and 40 μM of **2c** the levels of H3 acetylation decreased, while the H2B expression remained unaltered, indicating specificity of anti-acetylase treatment (Figure 5A).

Concentrations lower than 20 μM did not have any effect on the acetylation levels, while over 40 μM cell toxicity was observed as indicated by decreased H2B-EYFP fluorescence (data not shown). Interestingly, the effect of **2c** is not homogeneous in cell culture. Indeed, a high percentage of cells

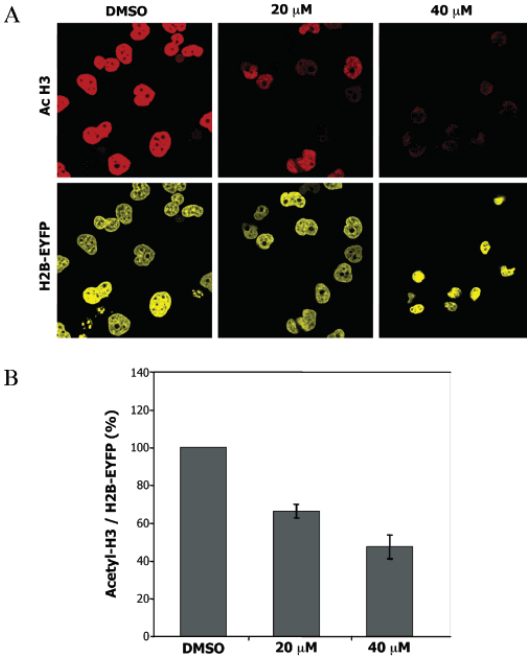


Figure 5. (A) HeLa−H2B-EYFP cells treated with derivative **2c** (20 μM or 40 μM) or DMSO were immunostained with antibodies anti-acetyl H3 and analyzed with appropriate wavelengths to visualize acetylated H3 or H2B-EYFP total protein levels. (B) The percent inhibition of histone H3 acetylation was obtained by measuring the mean fluorescence intensity with anti-acetyl H3 antibodies relative to the mean fluorescence intensity values of H2B-EYFP from the same cells. The graph summarizes data obtained from three independent experiments. Mean and standard error were derived analyzing 150 cells in each experiment.

(estimated around 24%) showed no detectable H3 acetylation even though the H2B expression remained unaltered (Figure 5A, cell in the upper-center in the middle panels as a representative image). Finally, we observed that **2c** determined an overall increase of H2B-EYFP fluorescence intensity. This observation is indicative of decreased histone acetylation that results in chromatin condensation. This effect was visualized by increased fluorescence of the exogenously expressed histones as previously reported in similar cellular systems.[19,20] To quantify the different levels of histone H3 acetylation, the average fluorescence intensity obtained from the immunostaining with antibodies against acetylated H3 was measured and normalized with values obtained in the same cells with fluorescent histones H2B-EYFP. Results summarized in Figure 5B indicate that the acetylation levels were 30% reduced in cells treated with 20 μM of **2c**, and a reduction higher than 50% was observed using 40 μM as compared to that of DMSO control cells.

Similar results were obtained in parallel where the H3 acetylation level was normalized with the level of expression of the nuclear lamina in HeLa cells not expressing H2B-EYFP (data not shown).

Conclusions

In conclusion, herein we reported a new class of small synthetic molecules that inhibited the p300 activities in in vitro assays. In particular, we described the discovery of cinnamoyl compounds **1a**−**c** and **2a**−**d** as inhibitors of the p300 enzyme. Among them, derivative **2c** was proven the most potent anti-p300 agent, which was six times more active than Lys-CoA used as a reference drug and with a high selectivity for p300, as demonstrated by comparative assays performed with different HATs belonging to another family of enzymes. Most notably, derivative **2c** was active in mammalian cells, as demonstrated by the downregulation of histone H3 acetylation. For all the above-mentioned reasons, derivative **2c** might be considered a lead compound for further studies in this field.

Extensive SARs, as well as molecular modeling studies, are ongoing to increase the knowledge within these series of p300 inhibitors. Due to the vital role of p300 in the reversible processes of acetylation of histones and other cellular proteins, the development of these inhibitors might result in novel approaches to antitumor and antiviral chemotherapies.

Experimental Section

Chemistry. General. Melting points were determined with a Büchi 530 capillary apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Spectrum-one spectrophotometer. ^{1}H NMR spectra were recorded on a Bruker AC 400 spectrometer, using tetramethylsilane (Me_4Si) as an internal standard. All compounds were routinely checked by TLC and ^{1}H NMR. TLC was performed by using aluminum-baked silica gel plates (Fluka F_{254}) and aluminum-baked aluminum oxide plates (Fluka F_{254}). Concentration of solutions after reactions and extractions involved the use of a rotatory evaporator operating at a reduced pressure of approximately 20 Torr. Organic solutions were dried over anhydrous sodium sulfate. The microwave reactions were performed in a Discover CEM, which produced controlled irradiation with a power of 0−300 W.

Syntheses. Specific examples presented below illustrate general synthetic procedures.

1,7-Bis(3-bromo-4-hydroxyphenyl)-1,6-heptadiene-3,5-dione (1c). A solution of 3-bromo-4-hydroxybenzaldehyde (1.0 g, 5.0 mmol) and acetylacetone (250 mg, 2.5 mmol) in *N*,*N*′-dimethylformamide (0.5 mL) was treated with boric acid (490 mg, 8.0 mmol), and the mixture was heated at 100 °C for 5 min. After this time, a solution of 1,2,3,4-tetrahydroquinoline (0.5 mL, 530 mg, 4.0 mmol) and acetic acid (0.15 mL) in *N*,*N*′-dimethylformamide (0.5 mL) was added. The resulting mixture was heated at 100 °C for 1.5 h, then cooled, diluted with 20% acetic acid (25 mL), and stirred at room temperature for 1 h. The precipitate that formed was extracted with ethyl acetate (3 × 50 mL), and the organic extracts were collected, washed with brine (3 × 100 mL), and dried. Evaporation of the solvent gave crude product, which was chromatographed on a silica gel column (chloroform/methanol, 20:1, as eluent) to obtain pure **1c** (760 mg, 65% yield); mp 175−176 °C (isopropanol/isopropyl ether). Anal. ($C_{19}H_{14}BrO_4$) C, H, Br. This procedure was used for the synthesis of compounds **1b** starting from 5-formylsalicylic acid. **1b**: 50%; mp >270 °C (dioxane). Anal. ($C_{21}H_{16}O_8$) C, H.

2,6-Bis(3-bromo-4-hydroxybenzylidene)cyclohexanone (2c). 3-Bromo-4-hydroxybenzaldehyde (300 mg, 1.5 mmol) was dissolved in MeOH and treated with montmorillonite K-10 (600 mg). Evaporation of the solvent gave a dispersion that was placed in a 5 mL glass tube and treated with cyclohexanone (75 mg, 0.75 mmol). The vessel was sealed with a septum and placed into the microwave cavity. Microwave irradiation of 100 W was used, the temperature being ramped from room temperature to 100 °C. Once 100 °C was reached, the reaction mixture was held at this temperature for 5 min. The reaction vessel was opened, and the mixture was diluted with methanol and filtered. Evaporation of the solvent gave crude product, which was chromatographed on a silica gel column (chloroform/methanol, 20:1, as eluent) to obtain pure **2c** (310 mg, 89% yield); mp 201−202 °C (isopropanol/water). Anal. ($C_{20}H_{16}Br_2O_3$) C, H, Br. This procedure was used for the synthesis of compounds **2a**, **2b**, and **2d** starting from 3,4-dihydroxybenzaldehyde, 5-formylsalicylic acid, and 4-hydroxy-3-methoxybenzal-

dehyde, respectively. Yield, mp, and recrystallization solvent are reported for each compound. **2a**: 75%, 244−246 °C, and methanol/water. **2b**: 50%, >270 °C, and DMF/water. Anal. ($C_{22}H_{18}O_7$) C, H. **2d**: 75%, 179−181 °C, and acetic acid.

Biological Assays. Acetylation Assay To Test the Efficacy of Curcumin Derivatives. To test the efficacy of derivatives **1a**−**c** and **2a**−**d**, the catalytic activity of p300 has been measured by an in vitro assay as previously reported.[17]

Acetylation Assays To Test the Efficacy of the Curcumin Derivatives in Mammalian Cells. HeLa cells, stably transfected with histones H2B fused to EYFP,[20] were cultured in 10% FCS DMEM. Histone H3 acetylation was analyzed as described in Supporting Information.

Acknowledgment. This project was supported by Ministero della Sanità, Istituto Superiore di Sanità, "Programma Nazionale di Ricerca sull'AIDS" (Grant No. 30F.19 and 40F.25), Italian MIUR (PRIN 2006), and Fondazione Cassa di Risparmio di Pisa (Grant No. 120/06).

Supporting Information Available: Spectroscopic data for derivatives **1b**,**c** and **2a**−**d**, elemental analyses for derivatives **1b**,**c** and **2b**,**c**, and biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM060943S

Terreni et al. Retrovirology 2010, 7:18
http://www.retrovirology.com/content/7/1/18

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GCN5-dependent acetylation of HIV-1 integrase enhances viral integration

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Abstract

Background: An essential event during the replication cycle of HIV-1 is the integration of the reverse transcribed viral DNA into the host cellular genome. Our former report revealed that HIV-1 integrase (IN), the enzyme that catalyzes the integration reaction, is positively regulated by acetylation mediated by the histone acetyltransferase (HAT) p300.

Results: In this study we demonstrate that another cellular HAT, GCN5, acetylates IN leading to enhanced 3'-end processing and strand transfer activities. GCN5 participates in the integration step of HIV-1 replication cycle as demonstrated by the reduced infectivity, due to inefficient provirus formation, in GCN5 knockdown cells. Within the C-terminal domain of IN, four lysines (K258, K264, K266, and K273) are targeted by GCN5 acetylation, three of which (K264, K266, and K273) are also modified by p300. Replication analysis of HIV-1 clones carrying substitutions at the IN lysines acetylated by both GCN5 and p300, or exclusively by GCN5, demonstrated that these residues are required for efficient viral integration. In addition, a comparative analysis of the replication efficiencies of the IN triple- and quadruple-mutant viruses revealed that even though the lysines targeted by both GCN5 and p300 are required for efficient virus integration, the residue exclusively modified by GCN5 (K258) does not affect this process.

Conclusions: The results presented here further demonstrate the relevance of IN post-translational modification by acetylation, which results from the catalytic activities of multiple HATs during the viral replication cycle. Finally, this study contributes to clarifying the recent debate raised on the role of IN acetylated lysines during HIV-1 infection.

Background

Integration of reverse transcribed HIV-1 DNA into the cellular genome is catalyzed by the viral IN protein. Even though *in vitro* integration can be solely driven by IN, cellular cofactors are required to complete the reaction *in vivo*. It was recently reported that the cellular HAT p300 interacts with IN and regulates its function through acetylation [1,2]. HATs are enzymes able to transfer acetyl groups from acetyl coenzyme A (acetyl-CoA) to specific lysine residues within the N-terminal tails of nucleosomal histones, leading to chromatin decondensation and transcriptional activation [3,4]. HATs can also acetylate non-histone substrates, such as transcription factors and other nuclear proteins, as well as cytoskeletal components, metabolic enzymes and signalling regulators in the cytoplasm [5]. Acetylation has been reported to regulate the activity of these factors by modulating DNA binding [6-8], protein-protein interactions [9-12], protein stability [13-15], and subcellular localization [16-19]. Growing evidence now indicates that acetylation significantly participates in signaling pathways ultimately regulating viral infectivity [20-26]. Among the viral factors functionally modulated by acetylation is the HIV-1 protein Tat. Tat is acetylated at lysine 28 by PCAF, while residues 50 and 51 are substrates for p300/CBP and GCN5 [27-29]. Acetylation of lysine 28 enhances the ability of Tat to recruit the P-TEFb complex [28], while modification of lysine 50 leads to Tat dissociation from TAR RNA [28,30]. Therefore, even though the final effect of acetylation is an increased transactivation activity on the viral LTR promoter, the modification of each individual lysine differently affects Tat functionality at the molecular level.

We have recently discovered that another HIV-1-encoded protein, IN, is a substrate for p300-mediated

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acetylation. Three lysine residues, located at positions 264, 266, and 273 in the C-terminal domain of IN, were identified as the target sites for modification [1,2]. Acetylation by p300 was shown to increase both IN affinity for DNA and strand transfer activity [1], thus suggesting a potential role for this post-translational modification during viral integration. The importance of IN acetylation for HIV-1 replication was further highlighted by the finding that the mutant virus, in which arginine substitutions were introduced at p300-targeted IN lysines, integrated less efficiently than the wild type [1].

Since proteins modified by acetylation are often substrates for multiple HATs, we sought to investigate whether IN might be acetylated by enzymes other than p300. It has already been reported that MOZ and PCAF (belonging to the MYST and GNAT families of HATs, respectively) are incapable of efficiently acetylating the IN C-terminal domain *in vitro* [2]. Therefore, in this study, another member of the GNAT family, GCN5, was examined. Here we demonstrate that GCN5 binds and acetylates IN both *in vitro* and *in vivo*. GCN5 expression is functionally relevant to HIV-1 infectivity and specifically affects the integration process, likely by modulating the catalytic activity of IN. Interestingly, the four lysines targeted by GCN5 partially overlap with those modified by p300 in the C-terminal domain of IN. A comparative analysis of viral clones mutated at IN lysines acetylated by GCN5 or p300 revealed the same replication defect at the step of integration, thus indicating common roles for the two HATs in regulating IN function.

Results

HIV-1 IN is acetylated by GCN5

To examine whether IN is acetylated by GCN5, *in vitro* acetylation assays were performed with recombinant IN and GCN5, both purified as GST fusion proteins. Incubation of the single GST domain with GCN5 in the presence of [^{14}C]-acetyl-CoA, and subsequent protein resolution by SDS-PAGE followed by autoradiography, revealed a unique band at the same size as GST-GCN5, corresponding to the auto-acetylation product of the enzyme (Figure 1A, lane 1). Incubation of GST-IN with GST-GCN5 resulted in two major radiolabeled bands, the higher one corresponding to auto-acetylated GST-GCN5 and the lower one to GST-IN (Figure 1A, lane 2), thus demonstrating that GCN5 specifically acetylates IN *in vitro*.

To define which region of IN is acetylated by GCN5, GST-IN fragments with progressive deletions starting from the C-terminus (as schematized in Figure 1C) were used as substrates in *in vitro* acetylation assays, and the corresponding acetylation signals in the autoradiograms were evaluated by densitometric analysis (Figure 1B, left histogram). GST-IN fragment 1-272 was acetylated to a similar extent as full-length IN (Figure 1A, compare lanes 2 and 3, and Figure 1B, left histogram). Acetylation of fragment 1-263 (Figure 1A, lane 4) was reduced by 30% (Figure 1B, left histogram), while a more significant decrease in the signal (ranging from 60% to 70%) was observed using shorter fragments (1-243, 1-234 and 1-212) (Figure 1A, lanes 5-7, and Figure 1B, left histogram). These results indicated that IN is acetylated by GCN5 within the region located between amino acids 244 and 288. As schematically represented in Figure 1C, this region contains five lysines at positions 244, 258, 264, 266, and 273 as possible targets for acetylation. Therefore, in order to exclude that the reduced acetylation of the deleted IN forms resulted from improper protein folding, each of these lysines was replaced with an arginine, an amino acid that cannot be acetylated and conserves a positively charged side chain. The resulting mutants were then tested *in vitro* as substrates for GCN5 activity. In this experiment, IN was tagged with a 6× His epitope in place of GST, in order to obtain better SDS-PAGE resolution between acetylated GCN5 and IN (Figure 1A, lane 8). As reported in the right histogram of Figure 1B, densitometric analysis of radioactivity incorporation highlighted that the mutation of the individual lysines 258, 264, 266, and 273 (Figure 1A, lanes 10-13) caused a reduction in the acetylation level of IN ranging from 40% to 50%, while no significant decrease in the signal was detected upon mutation of lysine 244 (Figure 1A, lane 9). These data suggested that GCN5 acetylates IN at residues 258, 264, 266, and 273. Notably, previous reports demonstrated that another HAT, p300, acetylates lysines 264, 266, and 273 of IN [1,2]. To confirm that GCN5 acetylates lysine 258 in addition to the above-mentioned residues, two mutant forms of IN were assayed for *in vitro* acetylation: one containing mutations at the sites acetylated by both GCN5 and p300 (IN K264,266,273R), and the other carrying these same amino acidic substitutions, with the additional mutation of lysine 258 specifically targeted by GCN5 (IN K258,264,266,273R). The decrease in the radioactive signal detected with IN K264,266,273R was similar to the one obtained with the single-mutated forms (compare lane 14 with lanes 10-13 in Figure 1A, and right histogram of Figure 1B), while the residual acetylation level of IN K258,264,266,273R dropped to 20% with respect to wild type (Figure 1A, lane 15, and Figure 1B, right histogram). These results demonstrated that GCN5 acetylates lysines 264, 266, and 273 of IN, also targeted by p300, and lysine 258 as a specific site of modification.

Next, we investigated whether IN is also acetylated by GCN5 *in vivo*. Codon-optimized Flag-IN [31] was expressed in HEK 293T cells, alone or together with HA-GCN5 wild type or mutated in the catalytic domain

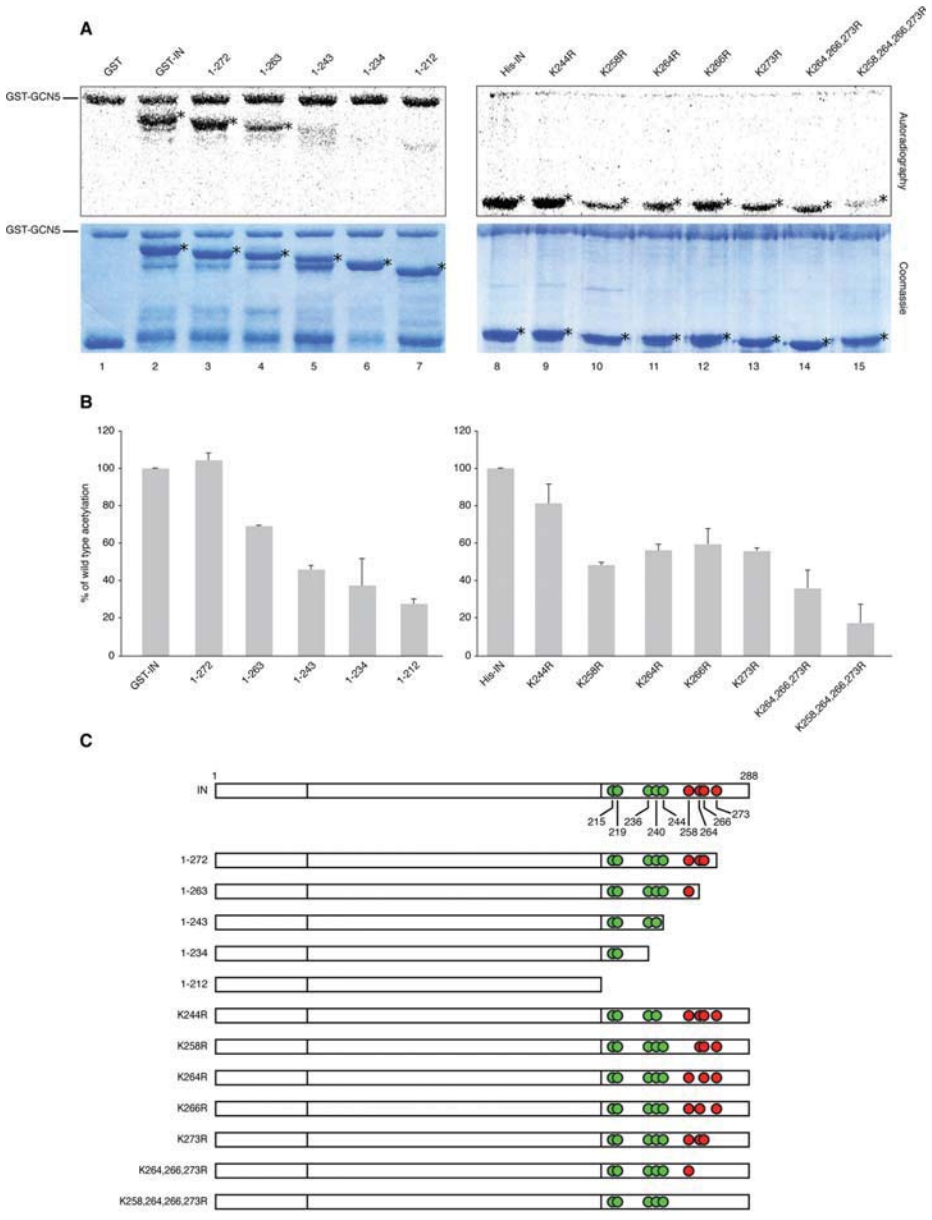

Figure 1 HIV-1 IN is acetylated by GCN5 *in vitro*. (A) Autoradiography (upper panels) and Coomassie blue staining (lower panels) of *in vitro* acetylation assay with recombinant GST-GCN5 and IN wild type or mutant proteins. Lanes 1-7: GST fusion IN proteins; lanes 8-15: 6× His-tagged IN proteins. In the Coomassie panels, IN proteins used as acetylation substrates are indicated by asterisks; in the autoradiograms, IN proteins found positive for GCN5-mediated acetylation are indicated in the same way. Presented results are representative data from triplicate *in vitro* acetylation assay experiments. (B) Results of densitometric analysis of autoradiograms derived from three independent experiments (means ± standard errors of the means [SEM]) expressed as percent wild type IN acetylation. (C) Schematic representation of IN proteins used for the acetylation assays. The positions of lysines in the C-terminal domain of IN are indicated. Lysines positive for acetylation are shown in red.

(Y260A/F261A) [32]. Immunoprecipitation of IN and subsequent detection by Western blotting with an antibody specific to acetylated lysines revealed the highest acetylation signal in the sample corresponding to IN co-expressed with wild type GCN5 (Figure 2A, upper panel, lane 3). Conversely, expression of IN alone or together with catalytically inactive GCN5 resulted in a lower acetylation signal, likely derived from the activity of endogenous HATs (Figure 2A, upper panel, lanes 2 and 4). In this experiment, the total amounts of immunoprecipitated IN and the expression levels of wild type and mutant GCN5 were verified by Western blot analysis with anti-Flag and anti-HA antibodies, respectively (Figure 2A, middle and lower panels).

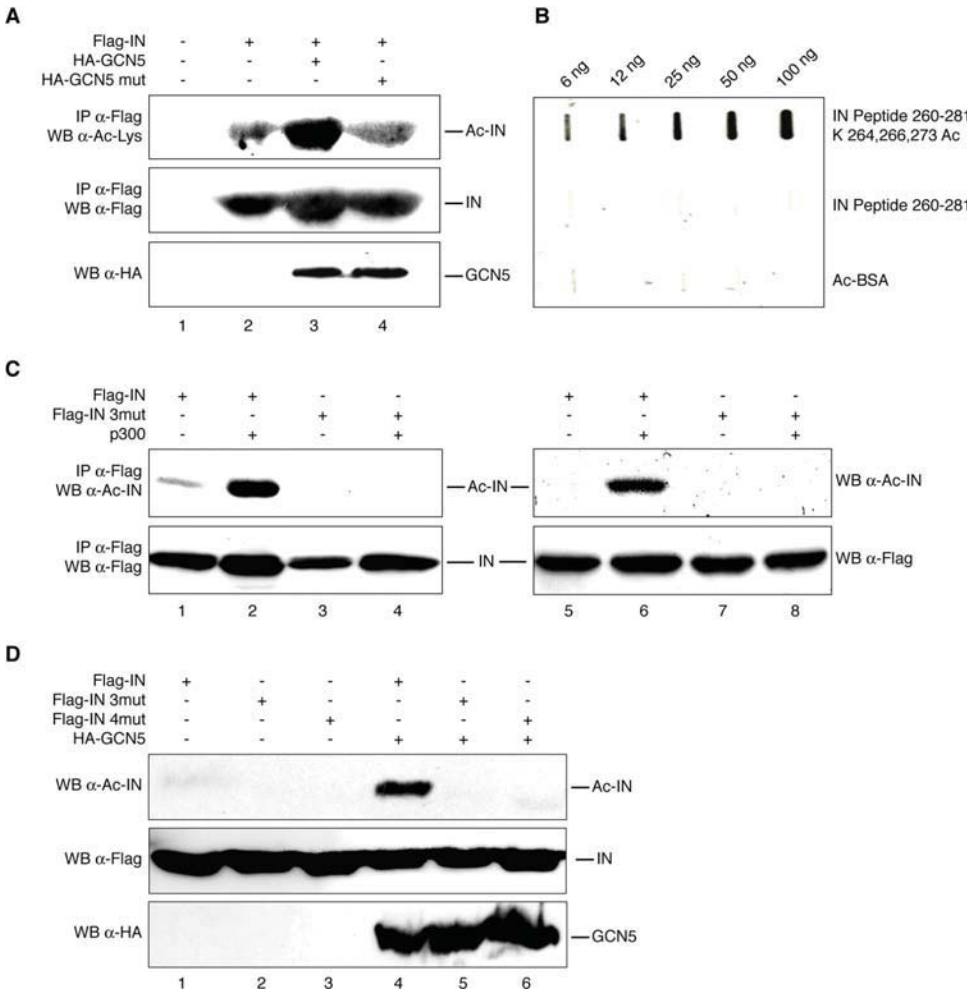

Figure 2 IN is acetylated by GCN5 *in vivo*. (A) Extracts from HEK 293T cells transfected with the indicated plasmids were immunoprecipitated using anti-Flag antibody and analyzed by Western blotting with anti-acetyl-lysine antibody (upper panel) or anti-Flag antibody (middle panel). Lower panel: cell extracts immunoblotted with anti-HA antibody. (B) Acetylated BSA and peptides corresponding to IN amino acids 260-281, either chemically acetylated at lysines 264, 266, and 273, or not acetylated, were blotted onto a nitrocellulose filter and incubated with anti-acetylated IN antibody. (C) Left panels (lanes 1-4): extracts from HEK 293T cells transfected with the indicated plasmids were immunoprecipitated using anti-Flag antibody and analyzed by Western blotting with anti-acetylated IN antibody (top panel) or anti-Flag antibody (bottom panel). Right panels (lanes 5-8): extracts from HEK 293T cells transfected with the indicated plasmids analyzed by Western blotting with anti-acetylated-IN antibody (top panel) or anti-Flag antibody (bottom panel). (D) Extracts from HEK 293T cells transfected with the indicated plasmids analyzed by Western blotting with anti-acetylated-IN antibody (upper panel), anti-Flag antibody (middle panel), or anti-HA antibody (lower panel).

Detection of *in vivo* IN acetylation by a novel anti-acetylated IN antibody

To confirm the *in vitro* observation that IN is a substrate for both GCN5 and p300, an antibody specific to acetylated IN was produced by using an IN-derived peptide for immunization. The IN-derived peptide was chemically acetylated at lysines 264, 266, and 273, which are targeted in common by the two HATs (see the Methods section). As shown in Figure 2B, the purified antibody specifically recognized the acetylated IN peptide in dot blot experiments, while no cross-reactivity was detected with the unmodified peptide or acetylated BSA. This antibody allowed detecting basal levels of IN acetylation by endogenous HATs following immunoprecipitation (Figure 2C, top-left panel, lane 1); additionally, high levels of IN acetylation were detected from cells overexpressing p300 (Figure 2C, top-left panel, lane 2). This result is consistent with our previous study showing that p300 mediates IN acetylation *in vivo* at positions 264, 266, and 273 [1]. Conversely, no signal, expressed either alone or together with p300 (Figure 2C, top-left panel, lanes 3 and 4), was detected with IN K264,266,273R, thus revealing the high specificity of the antibody. In this experiment, the amount of IN (wild type or mutated) immunoprecipitated in each sample was verified by Western blotting with an anti-Flag antibody (Figure 2C, bottom-left panel). The anti-acetylated IN antibody was also used for direct Western blot analysis of cell lysates, producing a strong acetylation signal in the sample corresponding to IN co-expressed with p300 (Figure 2C, top-right panel, lane 6). Therefore, the newly developed antibody showed higher sensitivity than the standard anti-acetyl-lysine antibodies, which require an immunoprecipitation step to reveal IN acetylation. Given the high specificity and sensitivity of the anti-acetylated IN antibody, it was used to confirm the *in vivo* acetylation of IN by GCN5, as well as the mapping of the *in vitro* targeted lysines. As shown in the upper panel of Figure 2D, extracts from cells co-expressing wild type IN and GCN5 revealed a remarkable signal corresponding to IN acetylation (lane 4); while, consistent with the data reported in Figure 2C (top right panel, lane 5), acetylation of the viral enzyme by endogenous HATs was almost undetectable (lane 1). Conversely, no signal with triple- and quadruple-mutant IN, expressed either alone (lanes 2 and 3) or together with GCN5 (lanes 5 and 6) was detected. In this experiment, Western blot analysis of the cell lysates was also performed with anti-Flag and anti-HA antibodies to control the levels of exogenously expressed proteins (Figure 2D, middle and lower panels). Taken together, these data demonstrated that IN is acetylated by GCN5 both *in vitro* and *in vivo*, and the targeted lysines are located in the C-terminal domain at positions 258, 264, 266, and 273.

IN interacts with GCN5

Since IN is acetylated by GCN5, the interaction between these two factors was investigated. To this aim, HEK 293T cells were transfected with Flag-IN together with HA-GCN5 wild type or mutated in the catalytic domain. After immunoprecipitation with an anti-Flag antibody, both wild type and mutant GCN5 were found to co-precipitate with IN, as demonstrated by Western blot analysis using an anti-HA antibody (Figure 3A, upper panel, lanes 3 and 4). Accordingly, in the reciprocal experiment, where immunoprecipitation was performed with an anti-HA antibody, IN was found to associate with GCN5 (both wild-type and mutant forms) (Figure 3B, upper panel, lanes 3 and 4). In both experiments, the total amounts of immunoprecipitated proteins and the expression levels of IN and GCN5 were verified by Western blotting (Figures 3A and 3B, middle and lower panels).

To map the region of IN mediating the interaction with GCN5, pull-down assays were carried out between GST-GCN5 immobilized on glutathione-Sepharose beads and IN deletion mutants labeled with [^{35}S]-Met by *in vitro* translation. As shown in Figure 3C, the affinities of IN fragments 1-272 and 1-263 to GST-GCN5 (13% binding efficiency) were similar to that of full-length IN (16% binding efficiency). Conversely, the GCN5/IN interaction significantly decreased using fragments containing further deletions towards the N-terminus (1-243 and 1-234). These results indicated that the C-terminal region of IN located between amino acids 244 and 288 is involved in binding to GCN5.

Acetylation by GCN5 increases IN catalytic activity *in vitro*

To explore the effect of GCN5-mediated acetylation on the catalytic activity of IN, constitutively acetylated recombinant IN was produced by exploiting the "tethered catalysis" approach [33,34]. This method allows the production of a constitutively acetylated protein by tethering the factor of interest to the catalytic domain of a specific HAT enzyme. Based on this approach, as schematized in Figure 4A, a chimeric construct was generated where 6× His-tagged IN was fused at its C-terminal end with the HAT domain of GCN5 (amino acids 6-300). To obtain a control that cannot be acetylated, the same chimera was constructed using the inactive mutant of GCN5 Y260A/F261A. In addition, a sequence coding for Tobacco Etch Virus (TEV) protease recognition site was inserted between IN and GCN5 coding sequences to allow for the separation of the two domains. The fusion proteins expressed from the two chimeric constructs were purified, digested with TEV protease, and the acetylation levels of the resulting IN proteins analyzed by Western blotting with an anti-

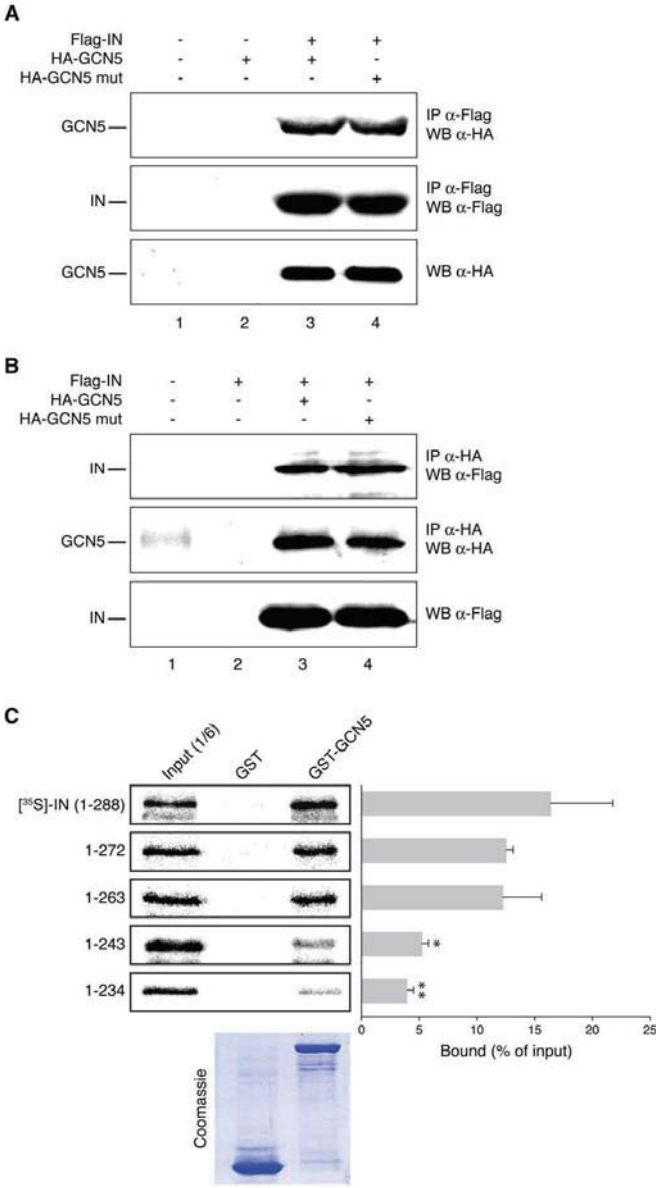

Figure 3 IN interacts with GCN5 both *in vitro* and *in vivo*. (A) Extracts from HEK 293T cells transfected with the indicated plasmids were immunoprecipitated using anti-Flag antibody and analyzed by Western blotting with anti-HA antibody (upper panel) or anti-Flag antibody (middle panel). Lower panel: extracts immunoblotted with anti-HA antibody. (B) Extracts from HEK 293T cells transfected with the indicated plasmids were immunoprecipitated using anti-HA antibody and analyzed by Western blotting with anti-Flag antibody (upper panel) or anti-HA antibody (middle panel). Lower panel: extracts immunoblotted with anti-Flag antibody. (C) Autoradiography and Coomassie Blue staining of *in vitro* binding assays with GST-GCN5 and ^{35}S-IN or the indicated ^{35}S-IN fragments. The histogram represents the results of three independent experiments (means ± SEM), where the amounts of bound proteins are expressed as percentages of the corresponding radiolabeled inputs. Statistical significance of the binding percentages was calculated by using the Student's two-sided *t* test. Asterisks directly above bars indicate differences in binding efficiency to GST-GCN5 between IN deleted forms and full-length IN. **, $P < 0,01$; *, $P < 0,05$. Conversely, where asterisks are not present, values obtained did not significantly differ ($P > 0,05$) from those obtained with control, non-silenced cells.

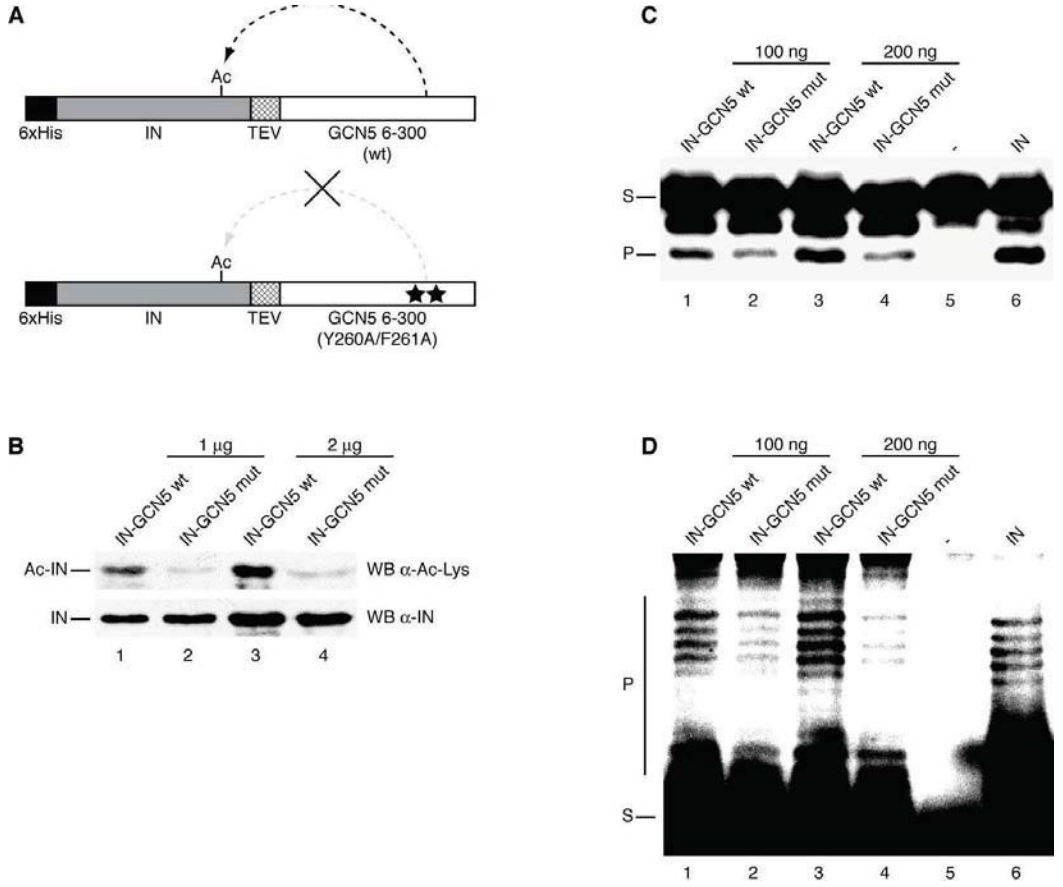

Figure 4 GCN5-mediated acetylation increases the catalytic activity of IN. (A) Schematic representation of IN-GCN5 tethered catalysis constructs. Full-length IN, tagged with a N-terminal 6× His epitope, is fused in frame with TEV proteolytic site and cloned upstream of the 6-300 amino acid region of wild type GCN5 (IN-HAT wt) or its catalytically inactive allele (IN-HAT mut). (B) 1 µg and 2 µg of IN derived from IN-HAT wt (lanes 1 and 3, respectively), or 1 µg and 2 µg of IN derived from IN-HAT mut (lanes 2 and 4, respectively) were analyzed by Western blotting with anti-acetyl-lysine antibody (top panel) or anti-IN antibody (bottom panel). (C) 3' -end processing activity of IN derived from IN-HAT wt (lane 1: 100 ng; lane 3: 200 ng) or IN-HAT mut (lane 2: 100 ng; lane 4: 200 ng). Lane 5: DNA substrate; lane 6: DNA substrate with 40 ng of 6× His-tagged IN. (D) Strand transfer activity of IN derived from IN-HAT wt (lane 1: 100 ng; lane 3: 200 ng) or IN-HAT mut (lane 2: 100 ng; lane 4: 200 ng). Lane 5: DNA substrate; lane 6: DNA substrate with 40 ng of 6× His-tagged IN. In (C) and (D), the DNA substrate (S) and the catalytic products (P) are indicated.

acetyl-lysine antibody. IN derived from the wild type GCN5 fusion scored positive for acetylation, while no significant signal was detected with IN derived from the GCN5 mutant chimera (Figure 4B, top panel, compare lanes 1 and 3 with lanes 2 and 4). In this experiment, the levels of loaded proteins were verified by incubating the same membrane with an antibody directed against IN (Figure 4B, bottom panel).

Constitutively acetylated recombinant IN and the non-acetylated control were tested *in vitro* for 3'-end processing and strand transfer activities. In the 3'-end processing reaction, recombinant IN was incubated with a [^{32}P]-labeled DNA substrate (S) and the excision of 2 nucleotides evaluated by measuring the radioactive signal of the shorter product (P). In Figure 4C the comparative analysis by densitometry of the bands corresponding to the 3'-end processed template, indicated that acetylated IN (100 ng in lane 1 and 200 ng in lanes 3) was two- to three-fold more active than non-acetylated controls (lanes 2 and 4 respectively). In the strand transfer assay, a [^{32}P]-labeled oligonucleotide was used as a substrate (S) and IN activity was evaluated by

measuring the radioactive signal derived from the ladder of higher molecular weight products (P). Constitutively acetylated IN, at two different doses (100 ng and 200 ng), was more active than non-acetylated IN (Figure 4D, compare lanes 1 and 3 with lanes 2 and 4). This was consistent with the 3'-end processing results. Finally, densitometric analysis of the autoradiograms indicated that the two amounts of acetylated IN were five- to ten-fold more active than the corresponding non-acetylated controls.

Taken together, these results demonstrated that GCN5-mediated acetylation enhances the catalytic activity of IN *in vitro*.

HIV-1 infectivity is reduced in GCN5 knockdown cells

In order to assess the physiological relevance of the IN/GCN5 interaction during HIV-1 replication cycle, viral infectivity upon GCN5 depletion in target cells was monitored. Transient knockdown of GCN5 expression was obtained in HeLa cells using a specific short interfering RNA (siRNA), while stably silenced HEK 293T cell clones were selected after transduction with a lentiviral vector (pGIPZ from Open Biosystems, Inc.) encoding a short hairpin RNA (shRNA) targeting GCN5 (GCN5 shRNAmir). As a control for the transient knockdown experiments, HeLa cells were transfected with a non-targeting siRNA (unrelated to any human genomic sequence), while stable silencing experiments were checked by using two HEK 293T polyclonal cell lines, one expressing a mismatched, non-targeting GCN5 shRNAmir (GCN5 shRNAmir mut) and the other carrying an empty pGIPZ vector. As shown in the top panels of Figure 5A, siRNA- and shRNAmir-mediated knockdown reduced GCN5 expression to a similar extent. Silenced cells were then infected with an *env*-deleted, VSV-G pseudotyped NL4.3 virus expressing the luciferase reporter gene (indicated hereafter as NL4.3-Luc), and luciferase activity was measured 48 hours after infection. As shown in Figure 5B, a two- to three-fold reduction in luciferase activity was detected in both transiently and stably silenced cells, thus indicating that knockdown of GCN5 expression in target cells reduces HIV-1 infectivity. To determine which step of viral replication was affected by GCN5 depletion, cells were collected at various time points after infection, and measurements of the different HIV-1 DNA species were performed by real time quantitative PCR (RT-Q-PCR). Total HIV-1 DNA was quantified with the use of primers annealing to the luciferase reporter gene, in order to avoid cross-reaction with the integrated pGIPZ lentiviral vectors present in stably transduced cell lines. As shown in Figure 5C, no significant alterations in total HIV-1 DNA levels were detected in cells either transiently or stably silenced, thus indicating that reverse transcription was not affected by the reduction of GCN5 expression. SiRNA-treated cells were analyzed 48 hours post-infection by Alu-LTR nested PCR to detect integrated HIV-1 DNA, while stable knockdown cell clones were processed two weeks after infection using primers specific to the luciferase gene. This was necessary in order to dilute non-integrated HIV-1 DNA and avoid cross-reaction with the integrated pGIPZ lentiviral vectors. Proviral DNA was about two-fold less in all GCN5 knockdown cells, either treated with siRNA or transduced with shRNAmir-encoding lentiviral vectors (Figure 5D). Finally, a two-fold increase in the amount of two-LTR circles was detected in both stably and transiently silenced cells (Figure 5E). Since the increase in two-LTR circles often correlates with a defect at the step of integration [35], these data are collectively consistent with reduced integration efficiency in GCN5 knockdown cells.

Mutations at IN acetylation sites cause a defect in HIV-1 replication at the integration step

Since the IN lysines acetylated by GCN5 partially overlap with those targeted by p300, a comparative analysis was performed to evaluate the role of these residues during the HIV-1 replication cycle. To this aim, single-round infections were performed, using *env*-deleted NL4.3-Luc viruses expressing either IN K264,266,273R (NL4.3-Luc-3mut), or IN K258,264,266,273R (NL4.3-Luc-4mut). Luciferase activity was measured 48 hours after infection, revealing an average five-fold reduction in infectivity for both mutant viruses as compared to wild type (Figure 6A). To determine which step of viral replication was affected by the lysine-to-arginine substitutions, DNA was extracted from cells at several time points after infection and the different HIV-1 DNA species were measured by RT-Q-PCR. Infection with NL4.3-Luc-3mut and 4mut, as well as with wild type virus, resulted in similar levels of total HIV-1 DNA at 24 hours post-infection (Figure 6B), indicating that reverse transcription was not affected by the amino acidic substitutions. Integrated HIV-1 DNA was quantified at 48 hours post-infection by Alu-LTR nested PCR, showing a five-fold reduction in the number of proviruses for both mutant clones with respect to wild type (Figure 6C). These data indicated decreased integration efficiency upon mutation of IN lysines targeted by acetylation. Consistently, a three-fold increase in the amount of two-LTR circles was detected at 24 hours post-infection with both NL4.3-Luc-3mut and 4mut (Figure 6D), confirming a specific defect at the step of integration and no alterations during viral nuclear import.

To investigate the role of IN acetylated lysines during HIV-1 replication in a T-cell line, two NL4.3-derived clones were generated, expressing either the triple- or

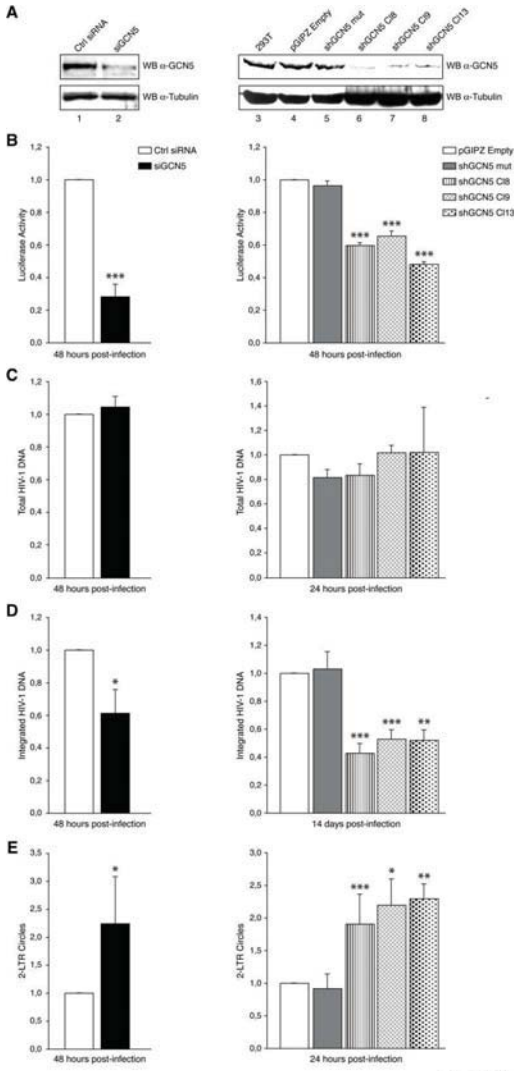

Figure 5

Figure 5 GCN5 depletion in infected cells reduces HIV-1 integration. (A) Left panels: extracts from siRNA-treated Hela cells analyzed by Western blotting with anti-GCN5 antibody (top) or anti-α-tubulin antibody (bottom). Lane 1: cells transfected with non-targeting siRNA (Ctrl siRNA); lane 2: cells transfected with GCN5-targeting siRNA (siGCN5). Right panels: extracts from stable GCN5 knockdown HEK 293T cell clones or control cells immunoblotted with anti-GCN5 antibody (top panel) or anti-α-tubulin antibody (bottom panel). Lane 3: untransduced HEK 293T cells; lane 4: HEK 293T cells carrying empty pGIPZ vector; lane 5: HEK 293T cells expressing mutant, non-targeting GCN5 shRNAmir; lanes 6-8: HEK 293T clones (Cl8, Cl9 and Cl13) expressing GCN5 shRNAmir. (B) siRNA-treated Hela cells (left histogram) or HEK 293T cells stably transduced with pGIPZ lentiviral vectors (right histogram) were infected with NL4.3-Luc and analyzed for luciferase activity 48 hours after infection. The histograms represent percentages of luciferase activity relative to control, non-silenced cells. Means ± SEM from three independent experiments are reported. (C-E) Total DNA extracted from siRNA-treated HeLa cells (left histograms) or HEK 293T cells stably transduced with pGIPZ lentiviral vectors (right histograms) was analyzed by RT-Q-PCR for total HIV-1 DNA (C), integrated HIV-1 DNA (D), and two-LTR circles (E). In (C-E), results are presented as percentages relative to control, non-silenced cells. Reported values are means ± SEM from three independent experiments. Statistical significance values shown in (B-E) were calculated by using the Student's two-sided *t* test. Asterisks directly above bars indicate differences between knockdown and control, non-silenced cells. ***, $P < 0{,}001$; **, $P < 0{,}01$; *, $P < 0{,}05$. Conversely, where asterisks are not present, values obtained did not significantly differ ($P > 0{,}05$) from those obtained with control, non-silenced cells.

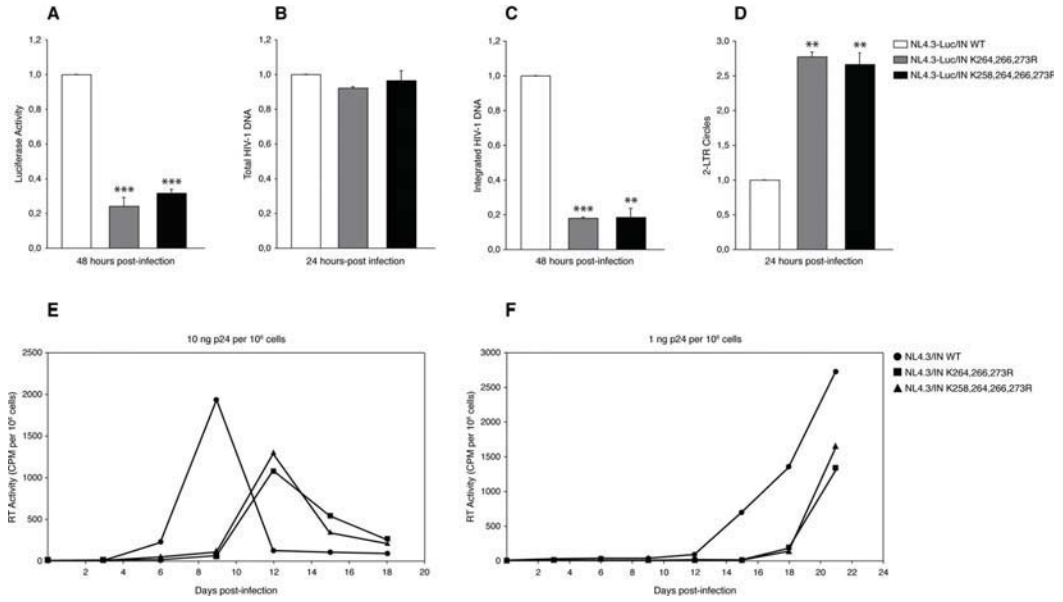

Figure 6 Mutations at IN acetylation sites cause a replication defect at the step of integration. (A) HEK 293T cells infected with NL4.3-Luc/IN WT, NL4.3-Luc/IN K264,266,273R, or NL4.3-Luc/IN K258,264,266,273R were analyzed for luciferase activity 48 hours after infection. (B-D) Total DNA extracted from HEK 293T cells infected with the same viral clones as in (A) was analyzed by RT-Q-PCR for total HIV-1 DNA at 24 hours after infection (B), integrated HIV-1 DNA at 48 hours after infection (C) and two-LTR circles at 24 hours after infection (D). In (A-D), results are presented as percentages relative to cells infected with NL4.3-Luc/IN WT virus. Reported values are means ± SEM from three independent experiments. Statistical significance values shown in (A-D) were calculated by using the Student's two-sided *t* test. Asterisks directly above bars indicate differences between cells infected with mutant viruses and cells infected with wild type virus. ***, $P < 0,001$; **, $P < 0,01$. Conversely, where asterisks are not present, values obtained did not significantly differ ($P > 0,05$) from those obtained with cells infected with wild type virus. (E) RT activity detected in the culture supernatants of CEM cells at different time points after infection with NL4.3/IN WT, NL4.3/IN K264,266,273R, or NL4.3/IN K258,264,266,273R. (F) Infections performed as in (E), using 10-fold lower viral loads.

quadruple-mutant IN (NL4.3-3mut and NL4.3-4mut, respectively). One million CEM T-cells were infected with the resulting viruses using two different amounts of p24 antigen (10 ng or 1 ng). Viral replication was followed by measuring HIV-1 reverse transcriptase (RT) activity in the culture supernatants every three days over a period of 21 days. As shown in Figure 6E, cells infected with the higher viral load (10 ng of p24) of wild type virus showed a peak of HIV-1 replication around day 9 post-infection. Conversely, infections with the same amounts of NL4.3-3mut and -4mut resulted in delayed peaks at day 12. Notably, at the infectivity peak, the RT amounts produced by both mutant HIV-1 clones were approximately half of that obtained with wild type virus. By using the lower viral load (1 ng of p24), the replication curve of wild type virus started to raise quite steeply around day 12 post infection, while for both mutant clones the curves started to appear at day 15. Detectable RT production was observed for both mutant viruses at day 18, thus with 6 days of delay compared to the kinetics of the wild type virus (Figure 6F). In conclusion, mutations introduced in the virus at IN acetylation sites targeted by both GCN5 and p300 (K264, K266, and K273), or additional mutation at lysine 258 specifically acetylated by GCN5 *in vitro*, determined similar decreases in viral integration and infectivity.

Discussion

The results presented in this study reveal that GCN5 is a novel HAT which interacts with IN. GCN5 binding to the C-terminal domain of IN leads to the acetylation of IN at lysines 258, 264, 266 and 273, located within the same region required for the two proteins to interact. We have recently demonstrated that the carboxy terminus of IN is a substrate for another cellular HAT, p300, which acetylates IN lysines at positions 264, 266, and 273 [1], a finding that was also later confirmed by Topper and coworkers [2]. Therefore, based on previous and present studies, three IN lysines (K264, K266, and K273) are acetylated by both HATs, while lysine 258 appears to be specifically targeted by GCN5. Our mapping of the HAT-interacting regions of IN based on

in vitro binding assays is consistent with a recent report which presented two models of full-length IN complexed with GCN5 and p300 [36]. Both models predict that the IN C-terminal tail located between amino acids 271 and 288, due to its high flexibility, could easily adapt to the binding pocket of GCN5, as well as to that of p300 (Figure 7). Interestingly, lysine 273 is included in this unstructured region and is therefore expected to be the residue most prone to acetylation. In fact, since lysines 264 and 266 are located in close proximity to a sandwich of two three-stranded antiparallel β-sheets, their binding and acetylation would require a more complex unfolding of this stable secondary structure. Based on this model, we may hypothesize that IN lysine 273 is the first residue contacted and acetylated by the HAT enzyme, whether GCN5 or p300. This event might in turn induce a conformational change in the C-terminal portion of IN, which could facilitate the modification of the other two lysines. This hypothesis is also compatible with the data reported by Topper and coworkers, demonstrating a hierarchy of reactivity between the three residues modified by p300, with lysine 273 as the key site targeted for acetylation [2].

A comparative analysis, aimed at establishing the roles of the two HATs during the HIV-1 replication cycle, revealed that the mutant viruses expressing either IN K264,266,273R or IN K258,264,266,273R exhibited the same replication deficiency, specifically affecting the step of integration. These results indicated that acetylation of IN C-terminal lysines 264, 266, and 273 is required for maximal HIV-1 integration efficiency, while acetylation of lysine 258, although observed *in vitro*, does not appear to play any significant role during infection.

Proteins modified by acetylation, including viral factors, are often targeted by multiple HATs in a redundant manner. For instance, HIV-1 Tat is acetylated at lysines 50 and 51 by p300/CBP and GCN5, leading in both cases to an increased transactivation activity of the modified protein on the viral LTR promoter [27-30]. The action of two different HATs on common sites of the same substrate may be ascribed to the importance of acetylation for the functionality of the target protein. However, in the case of IN, the reduced viral integration capacity detected in GCN5 knockdown cells indicated that endogenous p300 is not able to fully compensate for the lack of GCN5 so as to completely restore HIV-1 infectivity.

The role of IN acetylation at lysines 264, 266 and 273 during the HIV-1 replication cycle has been the subject of a recent debate. Our former study showed that the replication level of a HIV-1_{BRU} clone expressing a triple-mutant Flag-tagged IN (Flag-IN K264,266,273R) was severely impaired, and that the replication deficiency was specifically due to a block at the integration step [1]. In subsequent reports, the untagged triple-mutant virus showed either no replication defect [2], or a five-fold infectivity decrease in single-round infections [37]. Moreover, by using a genetic assay where integration was evaluated through the number of cell clones containing proviruses, one report [2] detailed almost half decreased integration efficiency, while the other [37] indicated a 14-fold lower residual integration rate. In

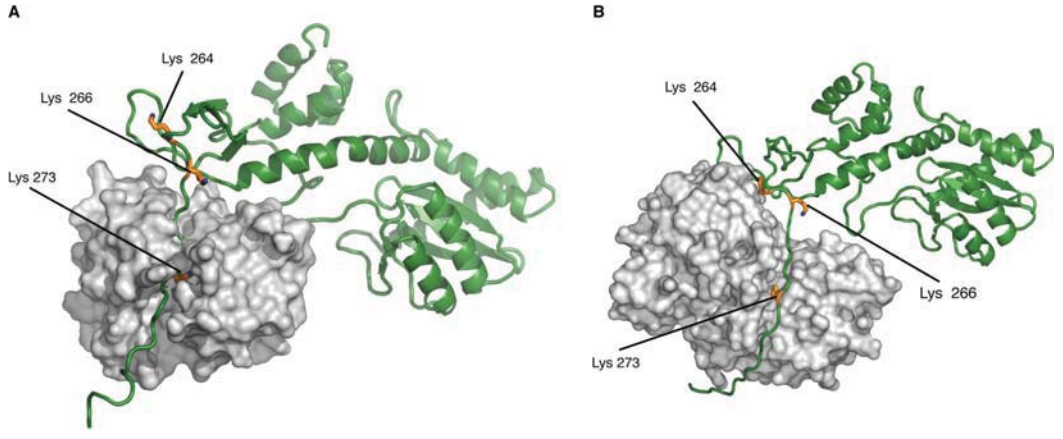

Figure 7 Three-dimensional models of IN complexes with GCN5 and p300. (A) Three-dimensional model of the IN/GCN5 complex. IN is represented in green and GCN5 in light grey. (B) Three-dimensional model of the IN/p300 complex. IN is represented in green and p300 in light grey. In (A) and (B), the three lysine residues in the C-terminal domain of IN that are acetylated by both GCN5 and p300 (Lys 264, Lys 266, and Lys 273) are shown in yellow. GCN5 and p300 are rendered as surfaces, while IN as a cartoon to highlight the C-terminal unfolded portion which inserts in the binding pockets of the two HATs.

the present study, we performed single- and multiple-round infections with HIV-1 clones encoding IN either mutated at the positions targeted by both GCN5 and p300 (IN K264,266,273R), or carrying an additional lysine-to-arginine substitution at the site specifically modified by GCN5 (IN K258,264,266,273R). In multiple-round replication experiments, both mutant clones showed reduced virus production and delays in the peaks of infectivity with respect to wild type. The discrepancy of these findings with the data reported by Topper *et al.* [2] might be due to the different time-courses of analyses: although working in the same experimental conditions (10 ng of p24 antigen per 1×10^6 CEM cells), the detection of RT activity in the culture supernatants over a period of 21 days allowed us to monitor the peak of HIV-1 replication, while Topper and coworkers terminated the replication curve before the highest point of viral infectivity was reached (at 12 days post infection).

Moreover, consistent with Apolonia *et al.* [37], we detected a five-fold infectivity decrease in single-round replication assays performed with IN triple- and quadruple-mutant viruses. The five-fold infectivity decrease paralleled a five-fold reduction in the number of proviruses, as measured by RT-Q-PCR. Taken together, the results presented in all the different reports suggest that acetylation of IN C-terminal lysines 264, 266, and 273 represents a mechanism which, by finely regulating the integration process, contributes to determine the efficiency of HIV-1 replication.

Identification of lysines 258, 264, 266, and 273 as the targets of GCN5 activity on IN does not exclude that additional residues might be acetylated, as indicated by the residual acetylation level of the quadruple-mutant IN (Figure 1A, lane 15). Finally, IN could also be subject to different post-translational modifications, such as methylation, sumoylation, or ubiquitination [38-41], which might open up new mechanisms of modulation of IN function.

Conclusions

This study demonstrates that, in addition to the formerly reported p300, another HAT, GCN5, acetylates the C-terminal domain of IN. Similar to p300, GCN5-mediated acetylation is required for efficient viral integration, thus reinforcing the role of this post-translational modification for HIV-1 replication.

Methods

Plasmids

Construction of pGEX-IN has already been described [1]. pcDNA3-HA-IN was obtained by subcloning IN sequence from pGEX-IN plasmid into pcDNA3-HA vector. pGEX-IN and pcDNA3-HA-IN deletion mutants were produced by PCR amplification of IN with primers specific to the deleted versions. pASK-IBA37-IN was constructed by subcloning IN sequence from pGEX-IN plasmid into pASK-IBA37 vector (IBA GmbH, Göttingen, DE). pFlag-IN codon optimized (c.o.) was kindly provided by A. Engelman. pASK-IBA37-IN point mutants and pFlag-IN c.o. K264,266,273R or K258,264,266,273R were obtained by PCR-based site-directed mutagenesis starting from the corresponding plasmids encoding wild type IN.

pGEX-GCN5 was a kind gift of M. Benkirane. pGEX-GCN5 deletion mutants were produced by PCR amplification of GCN5 with primers specific to the truncated forms. pcDNA3-HA-GCN5 was constructed by subcloning GCN5 sequence from pGEX-GCN5 plasmid into pcDNA3-HA vector. pcDNA3-HA-GCN5 (Y260A/F261A) [32] was obtained by PCR-based site-directed mutagenesis starting from the plasmid encoding wild type GCN5.

For production of IN-GCN5 tethered catalysis constructs, the sequence coding for the 6-300 amino acid region of GCN5 was amplified by PCR from pcDNA3-HA-GCN5 or pcDNA3-HA-GCN5 (Y260A/F261A) and cloned into a pASK-IBA37 vector in frame with c.o. IN. The sequence encoding TEV protease recognition site was inserted by PCR between IN and GCN5 cDNAs.

pGIPZ and pGIPZGCN5 lentiviral vectors were purchased from Open Biosystems (Huntsville, AL). The sequence of GCN5 shRNAmir inserted into the pGIPZGCN5 vector is as follows: 5'-CCCATTCATT CCCTGGCATTAATAGTGAAGCCACAG ATGTATT AATGCCAGGGAATGAATGGT-3'. For production of the pGIPZGCN5 mut vector, four point mutations were introduced in the shRNAmir cassette of pGIPZGCN5, obtaining the following sequence: 5'-CCCATTCA**AA GG**CTGGCA TTAATAGTGAAGCCACAGATGTATT AATGCCAG**CCTT**TGAATGGT-3', where mutated nucleotides are underlined.

The NL4.3-Luc *env*-deleted virus expressing the luciferase reporter gene was produced from the pNL4.3.Luc. R-E- molecular clone obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. IN sequence was subcloned from the molecular clone pHXB2 for construction of pNL4.3.Luc.R-E-/IN WT and pNL4.3/IN WT plasmids. The IN mutations in pNL4.3.Luc.R-E-/IN K264,266,273R, pNL4.3.Luc.R-E-/IN K258,264,266,273R and in pNL4.3/IN K264,266,273R, pNL4.3/IN K258,264,266,273R were introduced by PCR-based site-directed mutagenesis using either pNL4.3.Luc. R-E-/IN WT or pNL4.3/IN WT as template.

The envelope plasmid pMDG and the packaging plasmid pCMVΔR8.91 were kindly provided by Z. Debyser.

In vitro acetylation assay

HAT assays were performed as previously described [1], with minor modifications. Briefly, GST or 6× His-tag fusion proteins were incubated with GST-GCN5 and [^{14}C]-acetyl-CoA in HAT buffer (50 mM Tris-HCl, pH 8.0, 5% glycerol, 0.1 M EDTA, 50 mM KCl and 2 mM sodium butyrate) in a final volume of 30 μl for 45 min at 30°C. Acetylated proteins were visualized by phosphoimaging (Cyclone) after separation by SDS-PAGE.

In vitro binding assay

[^{35}S]-labeled IN proteins used for *in vitro* binding assays were produced from the corresponding pcDNA3-HA plasmids by using the TNT Reticulocyte Lysate System (Promega Corp., Madison, WI). Analysis of *in vitro* binding between GST fusion proteins and [^{35}S]-IN or [^{35}S]-IN fragments was performed as previously described [1]. Briefly, GST fusion proteins (1 μg) immobilized on agarose beads, after pre-treatment in a solution containing DNase I 0.25 U/μl and RNase H 0.25 U/μl, were incubated with 600 c.p.m. of *in vitro* translated [^{35}S]-proteins in a solution containing 0.2 mg/ml ethidium bromide. Following extensive washes, the reaction mixtures were resolved by SDS-PAGE and radiolabeled proteins visualized by phosphoimaging (Cyclone).

Recombinant proteins production and proteolytic processing

GST fusion proteins were expressed and purified from *Escherichia Coli* BL21 as already described [1].

N-terminal 6× His-tagged IN proteins were expressed in *Escherichia Coli* BL21 and purified by metal ion affinity chromatography (BD TALON Metal Affinity Resin, BD Biosciences, Palo Alto, CA) according to a previously reported protocol [42]. Proteolytic processing of IN-GCN5 chimeras was performed by incubating 20 μg of fusion protein with 30 U of TEV protease (AcTEV Protease, Invitrogen, Inc., Carlsbad, CA) in a buffer containing 50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.1 M NaCl, 1 mM DTT and 10% glycerol, overnight at 4°C. 6× His-tagged IN was then recovered from the reaction mixture by adsorption on BD TALON Resin.

Immunoprecipitation and Western blotting

For immunoprecipitation, cell pellets were lysed 36 hours after transfection in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycolic acid) containing 10 mM sodium butyrate (Sigma, Inc.) and protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche Diagnostics). Anti-Flag M2 affinity resin or rat monoclonal anti-HA antibody were incubated overnight at 4°C with the cell lysates (2 mg for coimmunoprecipitation or 4 mg for in vivo acetylation experiments). The HA-immune complexes were precipitated by incubation with Ultra-Link Immobilized Protein G (Pierce Biotechnology, Inc., Rockford, IL). The precipitated complexes were then extensively washed and analyzed by Western blotting using the appropriate antibodies.

Antibodies

The following primary antibodies were used: rabbit anti-acetylated-lysine (Cell Signaling Technology, Inc., Danvers, MA); mouse anti-Flag M2 (Sigma, Inc., St Louis, MO), either free or bound to agarose beads; rat anti-HA Clone 3F10 (Roche Diagnostics, Indianapolis, IN); mouse anti-IN 8G4, obtained from the AIDS Research and Reference Reagent Program; rabbit anti-GCN5 H-75 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and mouse anti-α-tubulin Clone B-5-1-2 (Sigma, Inc.).

For the production of a polyclonal, anti-acetylated IN antibody, three rabbits were immunized with a peptide corresponding to amino acids 261-280 of the IN sequence, chemically acetylated at lysines 264, 266 and 273, after conjugation with Maleimide Activated mcKLH (Pierce Biotechnology, Inc.). The IgG fraction was obtained from collected sera with the use of ImmunoPure (A) IgG Purification Kit (Pierce Biotechnology, Inc.). The purified samples were then passed over a column conjugated with the unmodified IN peptide to remove the antibody cross-reacting with non-acetylated IN.

Secondary horseradish peroxidase (HRP)-conjugated antibodies against mouse or rabbit Igs were purchased by Santa Cruz Biotechnology, Inc. For Western blot analysis with anti-acetylated-lysine antibody, Biotin-SP-conjugated AffiniPure F(ab')2 Fragment Donkey Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and ECL Streptavidin-HRP conjugate (Amersham Biosciences Corp., Piscataway, NJ) were employed.

Cell cultures and virus production

HeLa and HEK 293T cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. HEK 293T cells stably transduced with pGIPZ vectors were grown with the addition of puromycin 2 μg/ml. CEM cells were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

To produce *env*-deleted, VSV-G pseudotyped NL4.3-Luc viruses, 6× 10^6 HEK 293T cells were transfected with 20 μg of pNL4.3.Luc.R-E- (wild-type or mutated) and 5 μg of the envelope plasmid pMDG using the PEI reagent (Sigma, Inc.). The cell culture supernatant was collected 48 h after transfection and filtered through a 0.45 μM pore size filter.

NL4.3 replication competent viruses were prepared as described for NL4.3-Luc viral clones, using 25 μg of pNL4.3 plasmid (wild-type or mutated) for transfections.

For the generation of viral vector stocks, HEK 293T cells were transfected with 10 μg of the packaging plasmid pCMVΔR8.91, 5 μg of pMDG and 20 μg of the gene transfer plasmid (pGIPZ, pGIPZGCN5, or pGIPZGCN5 mut), following the protocol used for virus production. The cell culture supernatant was collected twice, at 48 h and 72 h after transfection, filtered through a 0.45 μM pore size filter and concentrated by ultracentrifugation at 110000 × g for 2 h at 4°C.

Both viruses and viral vectors were titered by quantification of p24 antigen in cell culture supernatants with an enzyme-linked immunoabsorbent assay (Innogenetics, Gent, Belgium).

Transient and stable knockdown of GCN5 expression

GCN5-targeting siRNA (Dharmacon Research, Boulder, CO) had the following plus-strand sequence: 5'-AACCAUGGAGCUGGUCAAUGAAA-3'. As a non-silencing control, Dharmacon ON-TARGETplus siCONTROL Non-Targeting Pool was employed.

HeLa cells, seeded in 6-well plates (1.5×10^6 cells/well), were transfected twice at a 24 h interval with 150 nM siRNA using Gene Silencer reagent as recommended by the manufacturer (Gene Therapy Systems, Inc., San Diego, CA). Cells trypsinized after 20 h were either plated for infections, or lysed for Western blot analysis.

For production of stably silenced cell lines, HEK 293T cells, seeded in 24-well plates (5×10^4 cells/well), were transduced with shRNAmir-encoding pGIPZ lentiviral vectors and grown in medium containing 2 μg/ml puromycin.

Infectivity and IN activity assays

For single-round replication assays, siRNA-treated HeLa cells (2.5×10^6/well) or HEK 293T cells (5×10^6/well) were seeded in 6-well plates and incubated for 3 h, in a total volume of 500 μl, with 50 or 100 ng p24 antigen of NL4.3-Luc virus (wild type or mutated), respectively. Cells were collected 48 h after infection for measurement of luciferase activity (Luciferase Assay System, Promega Corp.).

Viral stocks used in infections for measurement of HIV-1 DNA species by RT-Q-PCR were pre-treated for 1 h at 37°C with 160 U/ml Turbo DNase (Ambion, Inc., Austin, TX).

For multiple-round infections, 1×10^6 CEM cells were incubated with 1 ng or 10 ng p24 antigen of NL4.3 virus (wild-type or mutated) in a total volume of 500 μl for 3 h. Every 3 days, supernatants were collected and viral titers determined by a ^{32}P-based RT assay performed by standard procedures.

To evaluate IN catalytic activity *in vitro*, 3'-end processing and strand transfer reactions were performed with recombinant IN proteins as previously described [1].

Real-time quantitative PCR analysis

Total DNA was extracted from HEK 293T cells with the DNeasy Tissue Kit (QIAGEN, Valencia, CA) at different time points after infection. Amplification reactions were performed with the Light Cycler 480 instrument (Roche Diagnostics). Quantification of total HIV-1 DNA was performed with a pair of primers and a fluorogenic hybridization probe annealing to the luciferase reporter gene of NL4.3-Luc viral clone. The sequences of the primers and the probe are as follows: forward primer, LucFw, 5'-GAAGAGATACGCCCTGGTTCC-3'; reverse primer, LucRev, 5'-TGTGATTTGTATTCAGCCCATATCG-3'; and probe, LucProbe, 5'-FAM-TTCATAGCTTCTGCCAACCGAACGGACA-3' - BlackBerry Quencher. Reaction mixtures contained 500 ng of total genomic DNA, 1× Light Cycler 480 Probe Master (Roche Diagnostics), 300 nM each forward and reverse primers and 200 nM probe in a total volume of 20 μl. After an initial denaturation step (95°C for 10 min), the cycling profile was 40 cycles consisting of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Quantifications of proviral DNA at 48 h post infection (Alu-LTR nested PCR) and of two-LTR circles were performed according to previously described protocols [43]. For detection of integrated HIV-1 DNA in HEK 293T cells transduced with pGIPZ vectors, cells were maintained in culture for two weeks and proviruses were quantified using LucFw, LucRev primers and LucProbe.

As an internal standard for normalizing the amount of cellular genomic DNA, the level of human β-globin DNA was determined in each sample using primers and fluorogenic hybridization probe that were previously described [44]. The amplification conditions included a hot start at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and extension at 60°C for 1 min.

Statistical analysis

Paired comparisons were carried out using two-tailed Student's *t*-tests, assuming equal variance between samples to determine differences at the 5% level; all data points (including outliers) were included in the analysis for significance.

Acknowledgements

This work was supported by grants from the EU FP7 (THINC, Health-2008-201032), the Fondazione Cassa di Risparmio di Pisa (grant number 2006.120) and the ISS Italian AIDS Program (grant number 40G.17).

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Authors' contributions
MT designed and performed the experiments, analyzed the data, wrote the manuscript; PV performed the experiments and analyzed the data; VL designed the experiments and analyzed the data; MIG performed the experiments and analyzed the data; CDP performed the experiments and helped in the design of the study; ADF performed the computational analysis; VT performed the computational analysis; AAll performed the experiments and analyzed the data; AAlb performed the experiments and analyzed the data; MG designed the research and analyzed the data; AC designed the research, analyzed the data and wrote the manuscript

Competing interests
The authors declare that they have no competing interests.

Received: 17 July 2009 Accepted: 12 March 2010
Published: 12 March 2010

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doi:10.1186/1742-4690-7-18
Cite this article as: Terreni *et al.*: GCN5-dependent acetylation of HIV-1 integrase enhances viral integration. *Retrovirology* 2010 **7**:18.

Acknowledgements

I'm thankful to Scuola Normale Superiore of Pisa, who gave me the possibility and the support to carry out this work.

I want to thank my supervisor, Prof. Anna Cereseto, for believing in me and giving me the possibility to live this professional experience and for the open scientific discussions that always stimulated me to find the correct answers.

I'm grateful to Prof. Arturo Falaschi, whose dedication to science and passion for research was a constant inspiration for me during these years.

I thank all the integrase group members, Sara, Fabrizio, Awatef, Cristina, MariaElena, Valentina and Muthu, and all the people of the Molecular Biology lab of SNS, for being good colleagues and good friends, making the atmosphere in the lab enthusiastic and energetic even when we all were tired and it was really time to go home. A special thank goes to Vania, Antonella and Monica, for their invaluable technical support and constant helpfulness.

I want to thank my students, Antonio and Valentina, with their enthusiasm in approaching science and their dedication. I also would like to thank the many people that during these years I met at the CNR-IFC, somebody just for short time, but all of them contributing to my good memories of those days.

Ringrazio la mia famiglia, per aver sempre creduto in me ed avermi sempre incoraggiata, e per avermi trasmesso valori forti come l'onestà intellettuale ed il bisogno di fare bene il proprio lavoro per essere utili alla società, valori che sono importanti in ogni campo ma soprattutto per fare ricerca nel campo delle scienze della vita senza perdere di vista l'obiettivo finale, che è diminuire la sofferenza legata alle malattie.