HIV-1 Resistance to Dolutegravir Is Affected by Cellular Histone Acetyltransferase Activity

Kaitlin Anstett,^{a,b} Bluma Brenner,^b Thibault Mesplède,^{a,b} Mark A. Wainberg^{a,b}

Department of Microbiology and Immunology, Faculty of Medicine, McGill University, Montreal, Canada^a;
McGill AIDS Centre, Lady Davis Institute of the Jewish General Hospital, Montreal, Canada^b

ABSTRACT Integrase strand transfer inhibitors (INSTIs) are the newest class of antiretrovirals to be approved for the treatment of HIV infection. Canonical resistance to these competitive inhibitors develops through substitutions in the integrase active site that disrupt drug-protein interactions. However, resistance against the newest integrase inhibitor, dolutegravir (DTG), is associated with an R263K substitution at the C terminus of integrase that causes resistance through an unknown mechanism. The integrase C-terminal domain is involved in many processes over the course of infection and is posttranslationally modified via acetylation of three lysine residues that are important for enzyme activity, integrase multimerization, and protein-protein interactions. Here we report that regulation of the acetylation of integrase is integral to the replication of HIV in the presence of DTG and that the R263K mutation specifically disrupts this regulation, likely due to enhancement of interactions with the histone deacetylase I complex, as suggested by coimmunoprecipitation assays. Although no detectable differences in the levels of cell-free acetylation of the wild-type (WT) and mutated R263K enzymes were observed, the inhibition of cellular histone acetyltransferase enzymes sensitized the NL4.3_{WT} virus to DTG, while NL4.3_{R263K} was almost completely unaffected. When levels of endogenous acetylation were manipulated in virus-producing cells, inhibitors of acetylation enhanced the replication of NL4.3_{R263K}, whereas inhibition of deacetylation greatly diminished the replication of NL4.3_{WT}. Taken together, these results point to a pivotal role of acetylation in the resistance mechanism of HIV to some second-generation integrase strand transfer inhibitors, such as DTG.

IMPORTANCE This is, to our knowledge, the first report of the influence of post-translational modifications on HIV drug resistance. Both viral replication and resistance to second-generation integrase strand transfer inhibitors of both WT and INSTI-resistant HIV strains were differentially affected by acetylation, likely as a result of altered interactions between integrase and the cellular deacetylation machinery. Many “shock and kill” strategies to eradicate HIV manipulate endogenous levels of acetylation in order to reactivate latent HIV. However, our results suggest that some drug-resistant viruses may differentially respond to such stimulation, which may complicate the attainment of this goal. Our future work will further illuminate the mechanisms involved.

KEYWORDS antiretroviral agents, antiretroviral resistance, dolutegravir, drug resistance mechanisms, histone acetylation, human immunodeficiency virus, integrase

Human immunodeficiency virus (HIV) has claimed the lives of over 35 million people worldwide since the beginning of the epidemic (1). Despite the advent of effective antiretroviral medications (ARVs), drug resistance remains a prime concern in the fight against AIDS (2, 3). The integrase strand transfer inhibitors

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Address correspondence to Thibault Mesplède, tibo_mes@hotmail.com.

This article is dedicated to the memory of Mark Wainberg, our friend and mentor, who relentlessly fought for those affected by HIV.

(INSTIs) represent the most recently approved drug class for the treatment of HIV infection. They act to inhibit the second reaction catalyzed by the HIV integrase enzyme (IN, or INB to denote subtype B), i.e., the insertion of the viral DNA (vDNA) genome into the cellular chromatin, an essential step in the viral replication cycle (4). Raltegravir (RAL) was approved by the U.S. Food and Drug Administration in 2007, followed by elvitegravir (EVG) in 2012 (5, 6). Although they are highly effective, both drugs are susceptible to the emergence of drug resistance substitutions within IN in treated patients. Moreover, most of these substitutions provide cross-resistance against both compounds (7).

In 2013, dolutegravir (DTG) was approved for use in treatment-naïve individuals, and it has a higher genetic barrier to the selection of resistance than that of RAL/EVG and retains activity against most RAL/EVG-resistant viruses (8). Indeed, instead of selecting for resistance substitutions that disrupt the active site of IN to which INSTIs (such as RAL and EVG) bind, DTG has not yet selected for resistance-associated substitutions in treatment-naïve patients (9). In treatment-experienced INSTI-naïve patients and in cell culture, DTG selected for the novel R263K substitution, which led to a low fold change in the 50% inhibitory concentration (IC_{50}) for DTG and diminished the viral replicative capacity (10, 11). When individuals who previously experienced treatment failure with RAL or EVG and the emergence of drug resistance mutations subsequently failed DTG-based regimens, further development of classical RAL/EVG-associated mutations, not the R263K mutation, was observed (12–14). Located in the C terminus of the enzyme, the R263K substitution potentially causes resistance through a mechanism different from that of other INSTI resistance substitutions. However, as the C terminus of IN is an unstructured region and the crystal structure of HIV IN remains elusive, homology modeling has thus far been unable to rule out an interaction between the R263K substitution and the catalytic core domain of the protein (15, 16).

The IN protein can be modified posttranslationally, including being phosphorylated, ubiquitinated, SUMOylated, and acetylated, by various host proteins (17). In regard to acetylation, IN is known to be acetylated both *in vitro* and *in vivo* by the cellular histone acetyltransferase (HAT) enzymes p300 and GCN5, at residues K264, K266, and K273; this acetylation enhances DNA binding, enzyme activity, and integration (18, 19). It has also been shown that a protein termed KAP1 recruits the histone deacetylase (HDAC) I complex to HIV IN, catalyzing its deacetylation and decreasing integration rates (20). Residues K264 and K266 have also been implicated in viral preintegration complex (PIC) nuclear import and the multimerization of IN (21, 22). The present study was designed to evaluate whether the R263K substitution affected the acetylation of nearby residues and/or whether the regulation of this epigenetic event might be a cause of R263K mutation-mediated DTG resistance.

We used multiple approaches to show a lack of detectable differences between levels of acetylation of wild-type INB (INB_{WT}) and INB_{R263K}. However, when cells were infected with NL4.3_{WT} and subsequently treated with HAT inhibitors (HATi), a significant decrease in the IC_{50} for DTG occurred that was not observed for NL4.3_{R263K}. Similar patterns were observed for another second-generation INSTI, cabotegravir (CTG). In contrast, HIV inhibition by RAL was unaffected by the use of HATi. Consistently, NL4.3_{WT} virus produced under conditions of HDAC inhibition showed a reduced peak of replication, while NL4.3_{R263K} was unaffected. Conversely, HATi increased the peak of replication for NL4.3_{R263K} but did not affect NL4.3_{WT}. Lastly, coimmunoprecipitation assays showed that INB_{R263K} bound to KAP1 with a higher affinity than that of INB_{WT}, while binding to p300 was unaffected. Thus, wild-type (WT) and R263K mutation-containing HIV-1 strains appear to react in opposite ways to modulation by acetylation, which may be due, at least in part, to an altered interaction with KAP1 and a subsequent increase in the deacetylation of INB_{R263K}.

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FIG 1 The R263K substitution does not diminish IN acetylation in cell-free assays. Acetylation of the HIV-1 integrase protein was measured as the counts per minute (CPM) of ³H-labeled acetyl-CoA. (a) CPM as a function of [³H]acetyl-CoA concentration. (b) CPM with 20 nmol of [³H]acetyl-CoA. Data are means $\pm$ standard deviations (SD). (c) Acetylation assay by Western blotting. Acetylation of the HIV-1 integrase protein was measured by Western blotting with an anti-acetyl-lysine primary antibody in a 12% SDS-PAGE gel.

RESULTS

In vitro acetylation of HIV-1 integrase. The R263K substitution introduces a lysine residue into a domain of IN that can be acetylated both *in vitro* and *in vivo* (18, 19). We thus investigated whether the R263K substitution can interfere with IN acetylation, since it provides an additional lysine substrate for the histone acetyltransferase p300. To test this, we measured levels of acetylation of the WT and R263K mutation-containing IN proteins in cell-free acetylation assays using p300 in the presence of ³H-labeled acetyl-coenzyme A (acetyl-CoA). The results are summarized in Fig. 1. No differences in the radiolabeling of IN were detected in experiments performed with a gradient of acetyl-CoA (Fig. 1a) or acetyl-CoA at a fixed concentration (Fig. 1b). We next investigated the cell-free acetylation of IN proteins by Western blot analysis using a primary anti-acetylation antibody and were again unable to detect differences between IN_{WT} and IN_{R263K} (Fig. 1c). It is worth noting that various IN oligomers of different sizes were visible after SDS-PAGE under denaturing conditions (Fig. 1c).

Modulation of acetylation affects INSTI resistance in cell culture. Given that (i) IN acetylation contributes to DNA binding and (ii) INSTIs are competitive IN inhibitors, we hypothesized that acetylation might influence HIV susceptibility to INSTI inhibition in cell culture. To study this, we used a variety of small chemical compounds to interfere with cellular acetylation and deacetylation and probed the impact of this on HIV-1 infectivity. We first used the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay as previously described (23) to determine the 50% cytotoxic concentration (CC₅₀) in TZM-bl cells of anacardic acid (AA), an allosteric inhibitor of p300; C646, a competitive p300 inhibitor (collectively termed HATi); and vorinostat (a pan-HDAC inhibitor; referred to as SAHA). The results are summarized in Table 1. The effects of these drugs on the ability of HIV-1 to infect TZM-bl cells were also tested (Fig. 2). Using both of these assays, CC₅₀ values that had few or no cytotoxic effects on HIV-1 infectivity in TZM-bl cells were determined for use in further experiments.

To verify that modulation of INSTI susceptibility by acetylation specifically affected integration, we measured the DTG susceptibility of NL4.3 viruses in the presence of

TABLE 1 Summary of CC₅₀ values for each inhibitor in TZM-bl cells as measured using the MTT assay

Inhibitor	CC ₅₀	95% confidence interval
Anacardic acid (AA)	47.44 mM	41.89–53.73 mM
Vorinostat (SAHA)	3.974 μM	3.015–5.238 μM
C646	35.38 μM	28.24–44.32 μM

HATi or SAHA at either 2 or 12 h postinfection. As shown in Fig. 3 and summarized in Table 2, the addition of HATi at 2 h postinfection to the NL4.3_{WT} infection resulted in a lower IC₅₀ for DTG than that with the addition of SAHA or the no-drug control. It was possible that the modulation of cellular acetylation would also affect later stages in the virus’s life cycle, such as the activity of the transactivating viral protein Tat, and that this would confound observations of decreased infectivity upon HATi treatment. However, this result was not observed when the HATi was added at 12 h postinfection. Moreover, no such decrease in IC₅₀ was seen for NL4.3_{IN(R263K)} at any time point. There was, however, a trend toward sensitization with HATi for the NL4.3_{IN(G140S/Q148H)} mutant (harboring a common combination of RAL resistance substitutions).

There was a general lack of effect on HIV-1 susceptibility to RAL (Table 3). For CTG, a novel INSTI currently in clinical development, the effects were mixed. AA had no effect on HIV-1 susceptibility to CTG, whereas C646 sensitized NL4.3_{WT} and NL4.3_{IN(G140S/Q148H)} to both drugs while having no effect on NL4.3_{IN(R263K)}.

Disruption of acetylation in producer cells differentially affects HIV replication and drug resistance. Having determined in single-cycle replication assays that HATi sensitized WT but not R263K mutation-containing HIV to DTG added early but not late during infection, we next investigated whether a disruption of acetylation in viral producer cells might also influence HIV replication. 293T cells were transfected with either NL4.3_{WT} or NL4.3_{IN(R263K)} in the presence of HATi, SAHA, or a no-drug control. When viral stocks were collected, they were filtered to remove all inhibitors, diluted to 100,000 RT units, and used to subsequently infect TZM-bl cells. While no effect was seen

FIG 2 Modulation of acetylation in TZM-bl cells does not affect luciferase production at noncytotoxic concentrations. Titrations of anacardic acid (AA) (a), C646 (b), and vorinostat (SAHA) (c) were performed in TZM-bl cells infected with NL4.3_{WT}. The infectivity of virus over various drug concentrations was measured by luciferase production at 48 h postinfection. The dotted line shows the highest concentration of each drug used in subsequent assays. Means were plotted (*n* = 6), and error bars represent SD.

FIG 3 Inhibition of acetylation sensitizes WT HIV to DTG added early during infection. NL4.3 infectivity was measured by luciferase luminescence (relative light units [RLU]) and normalized to that of the no-drug control for each virus. Anacardic acid (AA) (10 mM) was added at 2 h postinfection (hpi) (a) or 12 hpi (b). Means were plotted ($n > 6$), and error bars represent SD.

on the IC_{50} of DTG (data not shown), SAHA significantly decreased the infectivity of WT viruses but had no effect on R263K viruses. The decrease in the infectivity of WT viruses was shown by a 3.18-fold, significant increase in the 50% effective concentration (EC_{50}), which means that 3.18 times more WT viruses were needed in the presence of SAHA than in its absence to reach half-maximal infectivity levels. In contrast, C646 had the opposite effect, i.e., the infectivity of NL4.3_{IN(R263K)} was increased 2-fold, without any significant effect on the infectivity of NL4.3_{WT} (Table 4).

Single-cycle infectivity assays do not always recapitulate prolonged effects relating to viral infectivity. Therefore, we also infected PM1 cells with the various filtered virus preparations at 150,000 RT units per well and quantified RT enzyme activity as a function of virus production over the course of 14 days. A peak of replication under all conditions for both viruses was seen at day 5 postinfection. However, the viruses responded differently to HATi or SAHA treatment, with SAHA reducing the peak of replication for NL4.3_{WT} but not NL4.3_{IN(R263K)}, whereas HAT inhibition had no effect on NL4.3_{WT} but greatly enhanced peak replication for NL4.3_{IN(R263K)} (Fig. 4). These results are consistent with those of the infectivity assays with TZM-bl cells as summarized in Table 4.

The R263K substitution increases IN binding to the HDAC I complex protein KAP1. HIV integrase has been shown to interact with and be modified by a number of host cell proteins (reviewed in reference 17). Because proper acetylation is important for IN enzymatic activity and the inhibition of HATs and HDACs has such markedly different effects on WT versus R263K mutation-containing viruses, we investigated whether the INB_{R263K} protein interacted with the components of the cellular acetylation machinery differently than the INB_{WT} interaction. Figure 5 shows the results of a coimmunoprecipitation experiment using 293T cells transfected with green fluorescent protein (GFP)-tagged INB constructs. The results show that INB_{R263K} pulled down more KAP1/TRIM28 than did INB_{WT}, while no difference in the interaction with p300 was observed. Accordingly, the differential response of WT and R263K mutation-containing

TABLE 2 Relative IC_{50} s of DTG in TZM-bl cells treated with either AA, C646, vorinostat (SAHA), or no inhibitor^a

Virus genotype	Relative DTG IC_{50} (95% confidence interval) with inhibitor addition at:					
	2 h postinfection				12 h postinfection	
	AA	C646	SAHA	NA	AA	NA
WT	0.41 (0.31–0.55)*	0.34 (0.17–0.67)*	0.71 (0.50–1.01)	1.00 (0.56–1.80)	1.14 (0.86–1.51)	1.00 (0.80–1.24)
R263K mutant	0.94 (0.64–1.39)	0.79 (0.54–1.15)	1.19 (0.70–2.01)	1.00 (0.68–1.48)	1.77 (1.39–2.26)*	1.00 (0.74–1.35)
G140S/Q148H mutant	0.67 (0.46–0.98)	0.57 (0.36–0.92)	0.82 (0.52–1.28)	1.00 (0.50–1.99)	ND	ND

^a IC_{50} s were normalized to the no-inhibitor control (NA) for each virus. *, significantly different from the control by Student's *t* test ($P < 0.05$). ND, no data.

TABLE 3 Relative IC₅₀s of select antiviral drugs in TZM-bl cells treated with either AA, C646, vorinostat (SAHA), or no inhibitor^a

Virus genotype	Relative IC ₅₀ (95% confidence interval) with inhibitor addition at 2 h postinfection											
	3TC						RAL					
	CTG			AA			NA			SAHA		
WT	1.45 (1.35–1.55)*	0.76 (0.66–0.87)	1.17 (0.90–1.52)	1.00 (0.79–1.25)	1.12 (0.77–1.62)	0.19 (0.15–0.27)*	1.19 (0.58–2.38)	1.00 (0.73–1.38)	1.21 (0.936–1.57)	0.80 (0.50–1.27)	0.90 (0.61–1.32)	1.00 (0.65–1.54)
R263K mutant	1.02 (0.82–1.25)	0.56 (0.44–0.72)*	1.17 (0.98–1.38)	1.00 (0.80–1.26)	1.41 (1.06–1.84)	1.59 (1.03–2.44)	0.71 (0.46–1.68)	1.00 (0.60–1.68)	0.71 (50.12–1.02)	0.54 (0.30–0.97)	1.00 (0.66–1.50)	1.00 (0.71–1.40)
G140S/Q148H mutant	1.37 (1.08–1.72)	0.57 (0.69–1.12)	2.14 (1.55–2.94)*	1.00 (0.74–1.36)	1.06 (0.75–1.49)	0.45 (0.28–0.72)*	0.98 (0.67–1.43)	1.00 (0.71–1.41)	~1 ^b	~1 ^b	~1 ^b	~1 ^b

^aIC₅₀s were normalized to the no-inhibitor control (NA) for each virus. *, significantly different from the control by Student's *t* test (*P* < 0.05).

^bNL4.3_{NI(G140S/Q148H)} RAL IC₅₀s were all >5 μM (NL4.3_{WT} IC₅₀ = 55.75 nM).

TABLE 4 Relative EC₅₀ values for NL4.3 viruses transfected from 293T cells treated with either AA, C646, vorinostat (SAHA), or no inhibitor^a

Genotype	Condition	Relative EC ₅₀ (95% confidence interval)
NL4.3 _{WT}	+AA	1.66 (1.42–1.94)*
	+C646	0.95 (0.84–1.07)
	+SAHA	3.18 (2.74–3.70)*
	NA	1.00 (0.76–1.31)
NL4.3 _{IN(R263K)}	+AA	0.77 (0.70–0.86)
	+C646	0.40 (0.36–0.46)*
	+SAHA	1.31 (1.19–1.44)
	NA	1.00 (0.65–1.53)

^aEC₅₀s were normalized to the no-drug control (NA). *, significantly different from the control for each virus by Student's *t* test (*P* < 0.05).

HIV-1 strains to modulation of cellular acetylation may be due, at least in part, to altered interactions with the HDAC I complex.

DISCUSSION

This is only the second report of drugs targeting IN posttranslational modification in a manner that can have a significant effect on HIV drug susceptibility. Indeed, small

FIG 4 WT and R263K mutation-containing NL4.3 viruses produced in the presence of HATi/SAHA respond in opposite ways over the course of long-term infection. (a) NL4.3_{WT} + AA; (b) NL4.3_{WT} + C646; (c) NL4.3_{WT} + SAHA; (d) NL4.3_{R263K} + AA; (e) NL4.3_{R263K} + C646; (f) NL4.3_{R263K} + SAHA. Reverse transcriptase (RT) activities (in counts per minute) were plotted (*n* > 3), and error bars represent SD.

FIG 5 HIV-1 integrase with the R263K substitution has a higher affinity for KAP1. Coimmunoprecipitation of GFP-tagged integrase (GFP-INB) was performed with 293T cells. (Left) GFP-INB was immunoprecipitated from transfected 293T cells by use of anti-GFP agarose beads. Both p300 and KAP1 were copurified. (Right) Input of cellular lysates.

ubiquitin-like modifier (SUMO)-specific protease (SEN) inhibitors were previously shown to also interfere with HIV-1 integration (24). Whether such compounds may synergize with INSTIs is an intriguing possibility. Here we demonstrated that inhibiting p300 can change the level of resistance against certain INSTIs and that these variations depend on specific integrase substitutions. Modulation of acetylation by C646 sensitized NL4.3_{WT} but not NL4.3_{R263K} to DTG and CTG. It was also observed that disruption of cellular acetylation in virus-producing cells had marked effects on long-term infectivity that were specific to the viral strain tested. We have also shown that the mutant HIV IN protein containing the R263K substitution associated with DTG resistance interacts more favorably with KAP1 than does INB_{WT}.

Previous work showed that acetylation of lysine residues at the C terminus of HIV IN enhanced enzyme activity both *in vitro* and *in vivo* (18). It has been shown for many DNA binding proteins and transcription factors that acetylation of lysine residues enhances their interactions with DNA (25). Hence, we hypothesized that the R263K substitution, which lies proximal to the acetylated residues K264 and K266, might be able to interfere with the acetylation of IN because of its additional lysine or through altered interactions with cellular proteins that bind to this domain. Our results suggest that K263 is not a major substrate for acetylation in cell-free assays (Fig. 1). However, we have shown an enhanced interaction between HIV INB_{R263K} and KAP1 compared to the interaction with WT integrase. KAP1 was previously shown to bind preferentially to acetylated IN and to recruit the other components of the HDAC I complex to deacetylate the protein; however, whether the interaction we observed is due generally to increased acetylation of the C terminus or to the specific amino acid change is unknown (20). This enhanced interaction with the mutated enzyme suggests that INB_{R263K} may be deacetylated more readily than INB_{WT}, which should decrease its DNA binding activity and therefore overall enzyme proficiency, consistent with previous findings (11, 15).

Why decreased DNA binding would be selectively advantageous in the presence of DTG is unclear and is the subject of current research in our laboratory. Perhaps spatial or temporal regulation of integrase acetylation within infected cells plays a role in viral infectivity and altering this regulation creates a superior ability for the R263K mutation-containing virus to replicate in the presence of DTG. Clearly, when cellular acetylation was inhibited, we witnessed an increased sensitivity to second-generation INSTIs for WT virus; therefore, acetylation must play a role in the interaction between HIV and these inhibitors. This phenomenon seems to be specific to second-generation INSTIs, as there was no decrease in susceptibility to RAL under the same conditions. The RAL-resistant G140S/Q148H virus behaved similarly to the WT virus in these assays, in contradistinction to R263K viruses in the presence of DTG and CTG. This demonstrates that these effects were specific for IN and were not caused by broad modulation of cellular processes. In contrast, the effects of acetylation on HIV susceptibility to the reverse

transcriptase inhibitor lamivudine (3TC) seemed to be inhibitor specific. Indeed, AA and SAHA showed trends toward increased IC_{50} s that were statistically significant for the WT and G140S/Q148H viruses, respectively, whereas C646 sensitized HIV-1 to 3TC inhibition, a trend that was statistically significant only for the R263K virus (Table 3). Although not fully understood, these differences may have to do with the fact that C646 is preferentially selective for p300, whereas AA inhibits p300 and p300/CBP-associated HATs and possesses tyrosinase- and lipoxygenase-inhibitory activities. This may have also influenced results obtained with CTG, whereby C646 seemed to sensitize the WT and G140S/Q148H viruses, while AA had no effect (Table 3). However, and in contrast to the case for 3TC, for CTG and DTG, the effects of acetylation on HIV-1 susceptibility to INSTIs were specific for IN. The fact that R263 is located close to acetylated residues provides a potential explanation for this specificity. Future *in silico* docking, cocrystallization, or cryo-electron microscopy studies of inhibitors bound to IN may provide further information on this topic (16, 26, 27).

Such *in silico* docking studies have shown that different INSTIs may bind within the IN catalytic pocket with slightly different conformations. This is well documented for RAL and EVG and has been associated with the development of primary resistance mutations at position Y143 in patients experiencing treatment failure with RAL, but not when EVG was used (28–30). In addition, and despite their high structural homology, DTG and CTG have been modeled to bind IN with different conformations (31). This in turn was used to explain differences between these two drugs in regard to the selection of resistance mutants after treatment failure (31). We hypothesize that differences in INSTI-IN binding may contribute to differences in the susceptibility to acetylation of RAL, DTG, and CTG (Tables 2 and 3). In this regard, it would be important to include both EVG and bictegravir in our future studies. In addition, the effects of acetylation on the *in vitro* and *in silico* binding of INSTIs to IN should be investigated.

In addition, cell culture selection experiments may inform whether the modulation of acetylation can decrease the HIV-1 genetic barrier to resistance against DTG and other INSTIs. This is particularly important given that HDAC inhibitors are currently being clinically tested in the context of HIV infection. Another question pertains to the nature of the drug resistance mutations that may be selected in the presence of acetylation inhibitors. Indeed, rare DTG failures are often associated with the R263K substitution, whereas RAL and EVG select for classical resistance mutations, including mutations at positions G140 plus Q148 or the N155H mutation. We previously reported that the R263K mutation may in large part be incompatible with classical resistance mutations for reasons of decreased replicative fitness and low-level resistance (32–34). Whether the resistance levels of mutants combining the R263K mutation with classical resistance mutations are also susceptible to acetylation inhibition remains to be investigated.

The dysregulation of cellular acetylation in virus-producing cells also had marked effects on the long-term infectivity of HIV. HDAC inhibitors reduced the peak of replication for WT but not R263K mutation-containing HIV, while HATi reciprocally enhanced the peak of replication for NL4.3_{IN(R263K)} but had no effect on the replication of NL4.3_{WT}. HDAC inhibitors are well known as latency reversing agents and have been used widely in so-called “shock and kill” strategies to reactivate and purge the latent reservoir, as HDAC-mediated chromatin silencing perpetuates HIV long terminal repeat (LTR) downregulation (reviewed in reference 35). Here we showed that HDAC activity may also be important for production of infectious WT virions, although the mechanism through which this occurs requires further investigation. These results again suggest that a lack of acetylation is advantageous for the survival of the R263K virus and that this effect is exerted in both single-cycle and protracted infections. Another interesting observation is that AA decreased the susceptibility to DTG of the R263K virus (Table 2) when the former drug was added 12 h after infection. This result suggests that the R263K and WT viruses differ in postintegration processes, such as viral reactivation. This in turn may be due to differences in integration sites or kinetics between these two viruses.

This study did have its shortcomings. First, we were unable to definitively determine whether or not the R263K substitution was itself acetylated or whether acetylation of IN affected the inhibitor dissociation rate from the IN-DNA complex (36). Further investigation is under way to pinpoint the exact cause of our observed results through use of mass spectrometry analysis and the study of IN-DNA interactions by use of multiple C-terminal mutants. We also previously reported an effect of the R263K substitution on IN DNA binding activity in cell-free assays (11, 32). While this may also be considered a mechanism through which the substitution provides resistance, it is important that these studies were performed with recombinant enzymes purified from *Escherichia coli* that most likely lack proper posttranslational modifications (37). Thus, the effect of acetylation on IN DNA binding may not have been captured accurately in the previous reports.

Taken together, our results suggest a fundamental role for the acetylation of the integrase protein in HIV resistance against INSTIs and raise two additional questions. First, future studies should examine the feasibility of combining HAT/HDAC inhibitors with INSTI-based therapy for the treatment of HIV infection. In this regard, our study may inform the development of better therapeutic strategies aimed at inhibiting viral integration. For example, the use of histone acetyltransferase inhibitors may be beneficial to individuals treated with DTG. Second, “shock and kill” studies should carefully take into account the nature of the antiretroviral therapy (ART) regimen, as considerations of acetylation may conceivably affect the treatment outcomes.

MATERIALS AND METHODS

Experimental design. The research objectives of this study were to evaluate whether the R263K substitution affected the acetylation of the C terminus of the HIV integrase protein and whether the resulting aberrant posttranslational modifications might be a mechanism of resistance to DTG.

Cells and reagents. PM1, 293T, and TZM-bl reporter cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, from Marvin Reitz (PM1), from Andrew Rice (293T) and from John C. Kappes, Xiaoyun Wu, and Tranzyme Inc. (TZM-bl) and were cultured as previously reported (11, 38–43). Merck & Co., Inc., Viiv Healthcare Ltd., GlaxoSmithKline PLC, and Toronto Research Chemicals Inc. supplied raltegravir, dolutegravir, lamivudine, and cabotegravir, respectively. Anacardic acid, C646, and vorinostat were obtained from Sigma-Aldrich Co.

³H acetylation assay. The generation of the pET15b_{WT} and pET15b_{R263K} plasmids was described previously (15). Recombinant integrase proteins were expressed in *E. coli* BL21(DE3) bacterial cells and purified as published previously (11). Integrase reaction mixtures were assembled on ice with 10% glycerol, 50 mM HEPES, pH 8.0, 1 mM dithiothreitol (DTT), 10 mM sodium butyrate, 0.1 nmol integrase, 17 pmol p300 (Enzo Life Sciences), and between 0 and 20 nmol ³H-labeled acetyl-CoA (PerkinElmer) in a final volume of 20 μ l. Reaction mixtures were incubated at 30°C for 45 min, after which 50 μ l 25% trichloroacetic acid (TCA) was added and counts per minute were measured after an additional 30 min of incubation at 4°C, using a Millipore multiscreen filtration plate system per the manufacturer's instructions.

Coimmunoprecipitation. The pACGFP-1C_{IN(WT)} plasmid was obtained from Xiao-Jian Yao at the University of Manitoba, and pACGFP-1C_{IN(R263K)} was created as described previously (11). 293T cells were transfected with either expression construct, and cells were collected after 24 h into 1 ml ice-cold phosphate-buffered saline (PBS) and spun at 500 $\times g$ for 3 min at 4°C. Cells were washed twice with 500 μ l PBS and then lysed in 200 μ l RIPA buffer (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, protease inhibitor cocktail pellet [Sigma-Aldrich], 1 mg/ml DNase I [Invitrogen], 2.5 mM MgCl₂), placed on ice, and pipetted vigorously every 10 min for 30 min. Samples were spun at maximum speed for 20 min at 4°C, and then lysates were diluted in 300 μ l wash buffer (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA). Twenty-five microliters of Chromotek GFP-trap_A beads (Chromotek, Munich, Germany) per sample was vortexed, diluted in 500 μ l wash buffer, and then spun at 2,500 $\times g$ for 2 min at 4°C. This wash was repeated twice before cell lysates were added to the beads, and the samples were incubated with inversion for 1 h at 4°C. Samples were then spun at 2,500 $\times g$ for 2 min at 4°C, supernatants were discarded, and beads were washed three times with 500 μ l wash buffer. Proteins interacting with the beads were then separated by SDS-PAGE and used for further analysis.

Western blot analysis. Acetylation assays were performed as described above, with the exception that nonradiolabeled acetyl-CoA was used and reactions were stopped by freezing at –20°C for 30 min. Four percent SDS-PAGE loading dye (200 mM Tris-Cl, pH 6.8, 400 mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol) was added to coimmunoprecipitation eluates or acetylation reaction mixtures, and the samples were boiled at 95°C for 10 min and run in a 12% SDS-PAGE protein gel. Western blots were performed on a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Primary antibodies against acetyl-lysine (mouse; Thermo Fisher), HIV-1 integrase (rabbit; NIH), KAP1 (rabbit; Abcam), and p300 (mouse; EMD Millipore) were used. Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit secondary antibodies were obtained from Thermo Fisher.

MTT cytotoxicity assay. The cytotoxic concentrations of anacardic acid, C646, and vorinostat in TZM-bl cells were determined as described previously (23). Briefly, compounds were serially diluted 1:10 and then added to TZM-bl cells in a 96-well plate. Cells were incubated for 48 h at 37°C, 10 μ l 5% MTT reagent (Invitrogen) in PBS was added, and the cells were incubated at 37°C for 3 h prior to the addition of 110 μ l lysis buffer (10% [vol/vol] Triton X-100 in acidified isopropanol) and shaking overnight in the dark at room temperature. The absorbance at 570 nm (540 nm and 690 nm) was determined using a microplate reader.

Generation of NL4.3 HIV-1 clones. The generation of the pNL4.3_{IN(WT)} and pNL4.3_{IN(R263K)} plasmids has been reported previously (11). Similar methods were used to generate the pNL4.3_{IN(G140S/Q148H)} plasmid through site-directed mutagenesis using the following primers: G140S-sense (5'-GGGGATCAAGCAGGAATTTAGCATTCCTACAATC-3'), G140S-antisense (5'-GATTGTAGGGAATGCTAAATTCCTGCTTGATC CCC-3'), Q148H-sense (5'-CTACAATCCCCAAGTCACGGAGTAATAGAATCTATG-3'), and Q148H-antisense (5'-CATAGATTCTATTACTCCGTGACTTTGGGGATTGTAG-3'). Genetically homogenous viral stocks were produced as described previously (11). Briefly, 293T cells were transfected with the various pNL4.3 plasmids by use of Lipofectamine 2000. Four hours after transfection, the medium was changed to Opti-MEM plus 10% FBS, with or without 10 mM anacardic acid, 10 μ M C646, or 0.5 μ M vorinostat. Forty-eight hours after transfection, cell culture supernatants were collected and filtered at 0.45 μ m to remove plasmids and cell debris, and then epigenetic inhibitors were filtered out (if necessary) by use of Centrifree ultracentrifugation devices (EMD Millipore). Viruses were aliquoted and stored at -80°C. Viral stocks were quantified by measuring cell-free reverse transcriptase (RT) activity in culture fluids.

HIV susceptibility to integrase strand transfer inhibitors. HIV susceptibilities to DTG, RAL, lamivudine (3TC), and CTG were measured by infection of 30,000 TZM-bl cells with 100,000 RT units per well of each virus in the presence of 1:10 (RAL, DTG, and 3TC) or 1:4 (CTG) serial dilutions of drugs. Neither elvitegravir nor bictegravir was tested. After 48 h, cells were lysed, and luciferase production was measured using a luciferase assay system (Promega, Madison, WI). For synergy assays, anacardic acid, C646, or vorinostat was also serially diluted 1:10 at 2 h postinfection and then added to cells. To investigate the effects of epigenetic inhibitors on ARV IC₅₀s, 10 mM anacardic acid, 10 μ M C646, 0.5 μ M vorinostat, or Dulbecco's modified Eagle's medium (DMEM; control) was added at 2 or 12 h postinfection.

Long-term infectivity assay. PM1 cells were diluted to 20,000 cells/well in a 96-well plate and then infected with WT and mutant NL4.3 viruses that were produced under various conditions in triplicate per plate per assay. Samples were collected at days 3, 5, 7, 11, and 14 postinfection for RT quantification.

HIV infectivity and replication capacity. HIV-1 infectivity was measured through infection of 30,000 TZM-bl cells per well by use of serial 1:4 dilutions of the various NL4.3 viral clones. Levels of infection were measured as described above. The 50% effective concentration (EC₅₀) was calculated as the amount of virus (measured by RT activity) needed to reach half-maximal infectivity (measured by TZM-bl cell assay) by use of Prism software. EC₅₀s were normalized against the EC₅₀ of each virus in the absence of drug. Accordingly, an increase in EC₅₀ indicates a decrease in infectivity.

Statistical analysis. The data from each experiment are averages for at least two replicates performed in triplicate ($n = 6$). EC₅₀, IC₅₀, CC₅₀, and 95% confidence interval values were calculated using Prism 7.0 software, and all figures were created using the same software. Student's *t* tests were performed using the OpenEpi toolkit, accessible freely online at www.openepi.com.

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