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Research paper

A pyrophosphatase activity associated with purified HIV-1 particles

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This paper is dedicated to the memory of Valérie Goldschmidt, who was tragically taken away from all her loved ones and is sadly missed.

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ABSTRACT

Treatment of HIV-1 with nucleoside reverse transcription inhibitors leads to the emergence of resistance mutations in the reverse transcriptase (RT) gene. Resistance to 3′-azido-3′-deoxythymidine (AZT) and to a lesser extent to 2′-3′-didehydro-2′-3′-dideoxythymidine is mediated by phosphorolytic excision of the chain terminator. Wild-type RT excises AZT by pyrophosphorolysis, while thymidine-associated resistance mutations in RT (TAMs) favour ATP as the donor substrate. However, *in vitro*, resistant RT still uses pyrophosphate more efficiently than ATP. We performed *in vitro* (−) strong-stop DNA synthesis experiments, with wild-type and AZT-resistant HIV-1 RTs, in the presence of physiologically relevant pyrophosphate and/or ATP concentrations and found that in the presence of pyrophosphate, ATP and AZTTP, TAMs do not enhance *in vitro* (−) strong-stop DNA synthesis. We hypothesized that utilisation of ATP *in vivo* is driven by intrinsic low pyrophosphate concentrations within the reverse transcription complex, which could be explained by the packaging of a cellular pyrophosphatase. We showed that overexpressed flagged-pyrophosphatase was associated with HIV-1 viral-like particles. In addition, we demonstrated that when HIV-1 particles were purified in order to avoid cellular microvesicle contamination, a pyrophosphatase activity was specifically associated to them. The presence of a pyrophosphatase activity in close proximity to the reverse transcription complex is most likely advantageous to the virus, even in the absence of any drug pressure.

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) belongs to the *retroviridae* family characterised by an obligatory reverse transcription step that converts the single stranded RNA genome into double-stranded DNA capable of integrating into the host cell chromosomes. Reverse transcription is catalysed by the virally encoded reverse transcriptase (RT) bearing RNA- and DNA-dependent DNA polymerase, as well as RNase H activities [1].

The reverse transcription step was the first to be targeted by the anti-retroviral compound AZT (zidovudine) [2], approved by the Food and Drug Administration for the treatment of AIDS patients in 1987 [3]. After three decades of research and development of antivirals against HIV-1, RT still remains a major drug target. The 12 clinically approved anti-RT compounds are key backbone components of the current Highly Active Anti-Retroviral Therapies (HAART) that are administered to patients [4]. They are divided into the two broad classes of Non-Nucleoside (NNRTIs) and Nucleoside/Nucleotide (NRTIs) RT Inhibitors. After intracellular phosphorylation, all approved NRTIs are competitive analogues of the natural dNTP substrates of RT and act as chain terminators once incorporated into the elongating DNA chain (for reviews, see Refs. [5,6]).

The efficacy of anti-HIV-1 drug-therapy is limited by the unavoidable emergence of mutations that confer resistance to any of the currently available drugs. In the case of NRTIs, resistance mechanisms fall into two categories (for reviews, see Refs. [5–8]): i) *enhanced discrimination* of the NRTI as compared to its natural counterpart, due to decreased incorporation efficiency of the nucleoside analogue. This can either be attributed to decreased binding of the NRTI and/or to a diminished rate of incorporation; ii)

Abbreviations: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; AZT, 3′-azido-3′-deoxythymidine, zidovudine; d4T, 2′-3′-didehydro-2′-3′-dideoxythymidine; TAM, thymidine-associated resistance mutations; (−) ssDNA, (−) strong-stop DNA; NNRTI, non-nucleoside reverse transcriptase inhibitors; NRTI, nucleoside/nucleotide reverse transcriptase inhibitors; WT, wild-type; PPi-lysis, pyrophosphorolysis; ^{AZTR}RT, AZT-resistant reverse transcriptase; PPase, pyrophosphatase.

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improved removal efficiency of the nucleoside analogue that is positioned at the 3′ end of the elongating DNA chain. Phosphorolytic excision of the chain terminator is the reverse reaction to polymerisation performed by HIV-1 RT (Fig. 1). It primarily concerns AZT and to a lesser extent d4T and other NRTIs [9]. The ability of HIV-1 RT to perform excision is mainly linked to a set of up to 6 mutations (M41L, D67N, K70R, L210W, T215F/Y and K219E/Q) within HIV-1 RT, the so-called TAMs. Insertions at position 69, in the fingers subdomain, together or not with the TAMs and in combination with a T69S mutation, also increase the unblocking activity of RT [9–14].

The first clues to understand the TAM-associated resistance mechanism came from *in vitro* biochemical experiments showing that, despite its lack of exonuclease proof-reading activity, wild-type (WT) HIV-1 RT was capable of pyrophosphorolysis (PPi-lysis) [15,16]. Even though this data remains somewhat contradictory [17,18], increased excision with PPi as a donor substrate was reported, *in vitro* [19–21], for AZT-resistant RT (^{AZTR}RT). A general consensus has been reached now on the fact that ^{AZTR}RT is capable of enhanced excision of AZT from the growing DNA chain [18,21–24]. Although all nucleoside triphosphates can potentially be used as donor substrates *in vivo* [24], ATP, for which the concentration is greater than all other potential substrates in cells, is most likely the biologically relevant one [24,25]. The reaction, similar to PPi-lysis and thus named ATP-lysis (Fig. 1), generates a dinucleoside tetraphosphate, Ap_4AZT [23]. Noticeably, the products generated by both the PPi- and the ATP-dependent excision reactions, AZTTP [17,20,26,27] and Ap_4AZT [28] can be re-incorporated *in vitro* by WT [29] or ^{AZTR}RTs [27,28] and act as efficient chain terminators.

The influence of the physiological environment, which changes with the activation state of the cells for example, is crucial. Indeed, even though all NRTIs are potentially excised by ^{AZTR}RT, high dNTP concentrations and in particular high concentrations of the next nucleotide to be incorporated, almost completely abolish, *in vitro*, excision of all analogues except AZT and Abacavir [18,22,30,31]. This phenomenon has been explained by the formation of a so-called “dead-end” primer/template/RT/NRTI/dNTP complex that is less stable when AZT is the terminating analogue [18,22,30,32]. Recently, the comparison of the crystal structures of WT and AZT-resistant RTs, in complex with various primer/template substrates and in the presence or not of Ap_4AZT [33] (i) confirmed that AZT-resistance mutations have no effect on AZT binding or incorporation, and (ii) revealed that ATP binds differently to WT and AZT-resistant RTs, the resistance mutations, in particular K70R and T215Y, creating a new binding site for ATP.

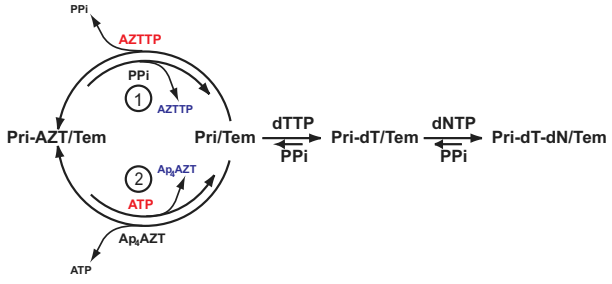

Fig. 1. AZTTP incorporation and phosphorolytic excision. Template and primer are abbreviated by Tem and Pri respectively. After addition of AZTTP to the 3′-end of a primer (Pri-AZT/Tem), unblocking can occur in two ways: pyrophosphorolysis using PPi as a donor substrate (Reaction 1) or ATP-lysis using ATP as a donor substrate (Reaction 2), respectively generating AZTTP and Ap_4AZT as well as an unblocked primer-terminus (Pri/Tem) that can resume DNA synthesis. Note that Ap_4AZT is also a substrate for HIV-1 RT. Molecules that are involved in inhibition of DNA synthesis (AZTTP) and in the release of this inhibition *in vivo* (ATP) are in red. The products that are generated by the reversal reaction to polymerisation, PPi-lysis or ATP-lysis, AZTTP and Ap_4AZT, respectively, are in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Although the evidence listed above is compelling, mechanistic [34,35] and biochemical [36] and modelling [37] data accumulated over the years continue to challenge a model that infers higher affinity of the ^{AZTR}RT for ATP compared to WT RT. Indeed, pre-steady state kinetics performed by the group of K. Anderson [35] and by us (Rigourd & Marquet, unpublished data) show that PPi-lysis always remains, *in vitro*, more efficient than ATP-lysis, for both WT RT and ^{AZTR}RT. This was further confirmed by intracellular measurements of the levels of pyrophosphorolysis donors from cells in different metabolic states, revealing that PPi levels are higher in extracts from activated T cells as compared to those in quiescent cells [24]. *In vitro* experiments at PPi and ATP concentrations matching those found in activated T cells show that PPi-lysis predominates and that AZT-resistant and WT HIV-1 RTs have overall similar excision activities [24]. In addition, no difference in ATP binding in the presence or absence of AZT resistance mutations has been reported [35,38,39], although these results are still controversial [26,40] and challenged by the fact that Ap_4AZT, the excision product of ATP-dependent AZT removal, has higher affinity for ^{AZTR}RT than AZT [28]. These findings raise the question as to why ATP-lysis, rather than PPi-lysis, is the resistance mechanism selected by ^{AZTR}RTs. The explanation which we favour and has also been put forward by others [24], is that low PPi concentration within the reverse transcription complex (RTC) would drive for the use of another abundant donor, *i.e.* ATP, for the excision reaction to occur. Such low PPi concentration could be the consequence of the presence, within the contained RTC, of a cellular pyrophosphatase (PPase) that would be packaged into the viral particles.

2. Results

2.1. AZT-resistance mutations do not improve (−) ssDNA synthesis in the presence of AZTTP and under physiological PPi and ATP conditions

In order to test the potential benefits of the AZT-resistance mutations for DNA synthesis in the presence of AZTTP, we compared the efficacy of *in vitro* (−) ssDNA synthesis by either WT RT or ^{AZTR}RT, in the absence and presence of AZTTP and in the absence and presence of physiological concentrations of PPi (150 μM) [41] and/or ATP (3.5 mM) [42,43]. A viral RNA corresponding to the first 311 nucleotides of the HIV-1 MAL isolate was used as a template and a labelled DNA oligonucleotide complementary to the primer binding site (PBS) sequence, was used as a primer. Utilisation of a labelled primer allowed direct quantification of the extended products as well as identification of AZTTP incorporation and AZTMP excision sites. Importantly, AZTTP, ATP and dNTP solutions were treated, prior to their usage, with purified commercially available PPase in order to avoid any artefacts that could potentially arise from PPi contamination.

In addition to the intermediate products and the full-length (−) ssDNA, longer products which are due to self-priming and are detected only with RNAse H(+) RT [44], were observed. Comparison of panels a of Figs. 2 and 3 as well as quantification of the final amount of (−) ssDNA and self-priming products that are synthesized (Table 1) revealed that both WT and AZT-resistant enzymes, bearing RNase H activity, behave very similarly, in the absence of AZTTP, during the first step of retroviral cDNA synthesis. Addition of 3 μM of AZTTP to the reverse transcription assay inhibited DNA synthesis by over 90% (Table 1), for both WT and ^{AZTR}RT, due to the incorporation of AZTTP at different positions during (−) ssDNA synthesis (Figs. 2 and 3, panels b). The AZTTP incorporation sites and inhibition patterns were also very similar for both enzymes