Metabolic activation of nucleoside and nucleotide reverse transcriptase inhibitors in dendritic and Langerhans cells

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Background: Langerhans cells and interstitial dendritic cells are the earliest targets for HIV infection through sexual transmission of HIV. Metabolism of nucleoside analogues markedly differs in proliferating T lymphocytes and resting monocyte/macrophages, and thus their antiviral efficacy can substantially differ between both cell types.

Methods: The metabolism of radio-labelled zidovudine (ZDV), lamivudine (3TC) and tenofovir (PMPA) to their antivirally active metabolites was studied in primary cells, representative of early *in vivo* targets of HIV [i.e. monocyte-derived dendritic cells (MO-DC), MO-derived Langerhans cells (MO-LC), PHA/IL-2-activated T-blast cells] as well as in a laboratory T-lymphocyte (CEM) cell line.

Results: Whereas lamivudine metabolism to its active triphosphate derivative (3TC-TP) did not markedly differ between T-cells and MO-derived LC and DC, zidovudine was much better converted to ZDV-TP in T-cells than in MO-LC and MO-DC. In contrast, tenofovir was markedly more abundantly converted to its antivirally active diphosphate metabolite PMPApp in MO-DC and MO-LC than zidovudine and lamivudine.

Conclusion: Our metabolic data suggest that tenofovir may be superior to zidovudine and lamivudine for inhibition of HIV replication in dendritic/Langerhans cells, the first-line cell types targeted by a primary HIV infection.

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Introduction

Both *in vitro* and *in vivo* studies suggest that intra-epithelial Langerhans cells (LC) and/or sub-epithelial dendritic cells (DC), represent critical early targets for sexually transmitted HIV [1,2]. Virus-bearing DC/LC migrate to secondary lymphoid organs to interact with CD4 + T cells, resulting in a productive HIV infection of the latter cell type [3–5]. Obviously, antiviral therapy, particularly in cases such as post-exposure prophylaxis, has to act on primary HIV-infected target cells rather than T-blast cells.

In addition to the adsorption/fusion process, reverse transcriptase (RT) may represent an ideal target enzyme for therapeutic intervention because it inhibits a pre-

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integration step in the infective cycle of HIV. Although the nucleoside RT inhibitors (NRTIs) and also the nucleotide RT inhibitor (NtRTI) tenofovir have been extensively studied on their antiviral potential and metabolic properties in T-cells, few studies were performed in HIV-infected monocyte/macrophages (M/M) [6,7]. These cells are characterized by some peculiar properties. Indeed, their resting status, and thus their limited DNA synthesis, does not require high intracellular levels of 2′-deoxynucleotide (dNTP) pools, and also, the nucleoside kinase levels that activate (phosphorylate) NRTIs are usually very low in M/M [8–10]. Therefore, in this cell type, both metabolic properties and antiviral activity of NRTIs could be strikingly different from those obtained in T-cell lines.

Given the important key role of dendritic/Langerhans cells in the first period of HIV infection and transmission, we investigated the metabolic behaviour of two NRTIs, e.g. 3′-azidothymidine (ZDV, zidovudine) and 2′,3′-dideoxy-3′-thiocytidine (3TC, lamivudine), and the recently approved NtRTI 9-(2-phosphonylmethoxypropyl)adenine [(*R*)-PMPA, tenofovir] in monocyte-derived dendritic cells (MO-DC) and Langerhans cells (MO-LC), and compared the data with those obtained in the laboratory T-cell line CEM and in PHA/IL-2-stimulated primary T-blast cells. From our studies, tenofovir had by far the highest conversion rate to its active diphosphate derivative in MO-DC and MO-LC compared with zidovudine and lamivudine, making tenofovir a prime candidate compound to be further explored for post-exposure prophylaxis of a primary HIV infection.

Materials and methods

Immuno-phenotyping of MO-DC, MO-LC, T-blast cells and CEM cells

The following antibodies were used for phenotyping of MO-DC and MO-LC: anti-CD1a-FITC, anti-E-Cadherin and Goat-anti-mouse Ig-FITC (Biosource Europe, Nivelles, Belgium), anti-CD13-PE, and anti-CD14-FITC (Becton Dickinson, Erembodegem, Belgium). We used anti-CD3-FITC and anti-CD4-PE (Becton Dickinson) for phenotyping the CEM cells and PHA /IL-2-activated T-blast cells.

Cultivation and preparation of primary cells and cell lines

Dendritic cells and Langerhans cells were generated based on previously described protocols [11–14] with minor modifications [15]. The cell sources and methods of purification are explained in detail in Van Herrewege *et al.* [16]. Briefly, 6×10^8 peripheral blood mononuclear cells (PBMC), isolated from donor buffy coats were separated into monocyte- and lymphocyte-enriched fractions by counter-flow elutration. The monocytes were further purified by sheep erythrocyte rosetting yielding $> 90\%$ CD3(−) CD4(+) MO and $< 0.5\%$ T cells, and cultured for 6–7 days in RPMI-1640 (Bio-Whittaker, Verviers, Belgium), supplemented with 10% bovine calf serum (Hyclone, Logan, Utah, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Boehringer-Mannheim, Mannheim, Germany), hereafter referred to as complete cell culture medium. In order to differentiate monocytes (MO) into interstitial-type dendritic cells (MO-DC), 20 ng/ml granulocyte monocyte-colony stimulating factor (GM-CSF) and 20 ng/ml interleukin (IL)-4 (Immunosource, Zoersel, Belgium) were added. Half of the medium, containing the same concentrations of cytokines, was replaced after 3–4 days. After 6–7 days, all cells expressed the myeloid marker CD13, over two-thirds of the cells clearly expressed the DC marker CD1a and DC-SIGN, whereas less than 10% expressed the MO marker CD14 and the LC marker E-Cadherin. For differentiation of MO into Langerhans-type cells (MO-LC), the complete cell culture medium was supplemented with 20 ng/ml GM-CSF, 20 ng/ml IL-4 and 5 ng/ml natural transforming growth factor beta1 (TGF-β1) (Immunosource). At day 7, the expression of CD1a, CD13 and CD14 was similar as in MO-DC but MO-LC also expressed E-Cadherin.

For the generation of PHA /IL-2-activated T-blast cells, buffy-coat isolated PBMC were purified by positive selection, using a CD4(+) isolation kit (Dynal, Oslo, Norway). They were $> 95\%$ CD4(+) and CD3(+), and cultured for 3 days in complete cell culture medium, supplemented with 5 ng/ml IL-2 (Immunosource) and 1 μg/ml PHA (Innogenetics, Gent, Belgium).

The CEM cell line was obtained from the American Type culture Collection in Rockville, Maryland, USA and maintained in complete RPMI-1640 cell culture medium.

Radiochemicals and drugs

[CH3-^{3}H]ZDV (radiospecificity 15 Ci/mmole), [5-^{3}H]3TC (radiospecificity 16.1 Ci/mmole) and [2,8-^{3}H]PMPA (radiospecificity 10.4 Ci/mmole) were obtained from Moravek Biochemicals (Brea, California, USA). Zidovudine, lamivudine and tenofovir were obtained from Sigma, Dr Jorg-Peter Kleim (GlaxoSmithKline, Stevenage, UK) and Dr Norbert Bischofberger (Gilead Sci., Foster City, California, USA), respectively.

Metabolism experiments

CEM T lymphocytes were suspended in complete medium at 0.5×10^6 cells/ml, whereas 1×10^6 cells/ml were used for MO-DC, MO-LC and PHA /IL-2 activated T-blast cell cultures. The cultivation and activation procedure of the MO-DC and MO-LC in these

metabolism experiments were identical to the conditions used in the antiviral (infection) experiment to enable correlation between both types of assays. Each condition was set-up in duplo (for 6 and 24 h of incubation, respectively). Five millilitres of cells were transferred in a small Petri dish and 1 μCi/ml (1 μM) of radio-labelled compound was added to each Petri dish. Cultures were incubated at 37°C and harvested after 6 and 24 h, respectively. Both time points were included in this study to cover both short-term as well as long-term drug exposure to the cells. Supernatants and cells were centrifuged (10 min, 500 *g*), supernatants were stored at −20°C and cells were washed twice with cold RPMI-1640 medium. Three hundred microlitres cold methanol was added to each pellet. High-performance liquidid chromatography (HPLC) analysis was performed on a Partisphere 10 SAX anion exchange column (Whatmann International Ltd., Maidstone, England). The cell extracts were subjected to HPLC analysis using a linear gradient of 5 mM $(NH_4)H_2PO_4$, pH 5.0 (buffer A) to 0.3 M $(NH_4)H_2PO_4$, pH 5.0 (buffer B) as follows: 5 min of 100% buffer A, 15 min of linear gradient to 100% buffer B, 20 min of 100% buffer B; 5 min of a linear gradient to 100% buffer A, and 5 min of equilibration with buffer A. The flow rate was 2 ml/min.

Results

The metabolic fate of 1 μM of the NRTI derivatives zidovudine and lamivudine and the NtRTI derivative tenofovir [(R)-PMPA] was investigated in four different cell types, including T-lymphocyte CEM cells and buffy-coat-derived dendritic, Langerhans and PHA/IL-2-activated T-blast cells. Zidovudine was detectably converted to its 5′-mono-, 5′-di- and 5′-triphosphate metabolites in all four cell types (Table 1). In all cases, ZDV-MP markedly accumulated as the predominant metabolite. Both CEM and PHA/IL-2-stimulated T-blast cells showed comparable ZDV-TP levels. In striking contrast, the dendritic and Langerhans cell cultures accumulated ZDV-TP levels that were 5- to 20-fold lower than in the CEM and PHA/IL-2-stimulated T-blast cells (Table 1). In particular the MO/LC and to some extent also the MO/DC accumulated less ZDV-MP than the T-blast and CEM cells. Given the fact that zidovudine enters the cells by passive diffusion, the lower ZDV-MP levels in MO-derived LC and DC most likely reflect lower thymidine kinase activity in MO/LC and MO/DC rather than decreased uptake of the drug by these cells.

Lamivudine was also converted in all cell types to its 5′-mono-, 5′-di- and 5′-triphosphorylated lamivudine metabolites (Table 1). In addition, a radio-labelled fraction eluting between 3TC-MP and 3TC-DP, and tentatively defined as possibly the 3TC-DP-choline/ethanolamine adduct, could be observed. An unidentified radio-labelled fraction eluted between lamivudine and 3TC-MP. However, the radio-label associated with this fraction was much lower ($<<$ 1%) than the sum of the other (phosphorylated) metabolites. The eventual 3TC-TP levels formed after 24 h did not differ very much between the four cell types. Lamivudine tended to accumulate at the 3TC-DP level in CEM and PHA/IL-2-activated T-blast cells, although the degree of accumulation of 3TC-DP versus 3TC-TP was only 2.5- to 6-fold depending the nature of the cell type. Interestingly, in the dendritic and Langerhans cells, the 3TC-MP derivative represents the predominant metabolite species, being 4- to 5-fold more abundant than the 3TC-TP levels and ∼ 2-fold more pronounced than the 3TC-DP levels. Furthermore, the dendritic and Langerhans cells tended to accumulate more of the tentatively identified 3TC-DP-choline/ethanolamine derivative than the CEM and PHA-stimulated T-blast cells (Table 1). The difference in accumulated 3TC-DP-choline/ethanolamine derivative levels between dendritic and Langerhans cells versus CEM and PHA-stimulated T-blast cells was 2- to 5-fold.

When tenofovir was exposed to the four different cell types, mono- (PMPAp) and di-(PMPApp) phosphorylated tenofovir derivatives were formed. Interestingly, whereas the PMPApp metabolite was markedly more present in the CEM and PHA/IL-2-activated T-blast cells after 6 h than after 24 h (∼ 6-fold difference) (Table 1), the PMPApp amounts were at least at equal, if not present at higher levels after 24 h in MO-derived dendritic and Langerhans cells. Furthermore, whereas PMPApp levels were quite comparable after a 6 h incubation period in all four cell types, their amounts were considerably higher (3- to 7-fold) in dendritic and Langerhans cells after 24 h than in CEM and PHA/IL-2-stimulated T-blast cell cultures. A few additional radio-labelled peaks were observed that eluted earlier than tenofovir (less polar) and one radio-labelled peak that eluted between tenofovir and monophosphorylated tenofovir (data not shown). The nature of these metabolites is currently unclear but they may well be derived from traces of contaminating [^{3}H]adenine (the free base of tenofovir) in the radio-labelled [^{3}H]PMPA samples due to radiolysis, and thus, not relevant to the current study.

Discussion

The three studied drugs belong to different pharmacological classes: Zidovudine is a thymidine analogue that needs to be activated (phosphorylated) by the cell cycle (S-phase)-dependent cytosolic thymidine kinase. Lamivudine is a 2′-deoxycytidine (dCyd) analogue that is activated by the cell cycle-independent cytosolic dCyd kinase. Tenofovir is an adenylate (AMP) analogue whose activation by cellular AMP kinase is cell cycle-indepen-

Table 1. Metabolism of 1 μM [CH_3-^{3}H] zidovudine (ZDV), [5-^{3}H] lamivudine (3TC) and [2,8-^{3}H] tenofovir (PMPA) in different cell types.

Metabolites[a] (pmoles/10^6 cells)	Time period of incubation (h)	T-lymphocyte (CEM) cells (pmoles/10^6 cells)	PHA/IL-2 T-blasts (pmoles/10^6 cells)	MO-derived dendritic cells (pmoles/10^6 cells)	MO-derived Langerhans cells (pmoles/10^6 cells)
[CH_3-^{3}H]ZDV-derived					
ZDV (fr 2-3)[b]	6	0.301 ± 0.006	0.116 ± 0.078	0.059 ± 0.010	0.037 ± 0.014
	24	0.244 ± 0.037	0.070 ± 0.018	0.070 ± 0.021	0.031 ± 0.030
ZDV-MP (fr 9-10)[b]	6	2.85 ± 0.95	2.89 ± 1.56	0.634 ± 0.34	0.186 ± 0.092
	24	4.77 ± 0.68	3.55 ± 1.62	1.30 ± 0.66	0.254 ± 0.306
ZDV-DP (fr 15-16)[b]	6	0.021 ± 0.001	0.037 ± 0.020	0.006 ± 0.002	0.007 ± 0.002
	24	0.063 ± 0.011	0.061 ± 0.038	0.008 ± 0.003	0.005 ± 0.003
ZDV-TP (fr 24-25)[b]	6	0.040 ± 0.023	0.066 ± 0.064	0.006 ± 0.002	0.008 ± 0.004
	24	0.099 ± 0.022	0.103 ± 0.065	0.007 ± 0.005	0.005 ± 0.005
[5-^{3}H]3TC-derived					
3TC (fr 2)[b]	6	0.008 ± 0.0017	0.013 ± 0.007	0.008 ± 0.005	0.008 ± 0.006
	24	0.03 ± 0.007	0.022 ± 0.01	0.017 ± 0.008	0.019 ± 0.021
(fr 4)[b]	6	0.0005 ± 0.0002	< 0.0003	0.0009 ± 0.0002	0.0006 ± 0.0001
	24	0.009 ± 0.003	0.0003 ± 0.0005	0.007 ± 0.006	0.007 ± 0.003
3TC-MP (fr 7-8)[b]	6	0.018 ± 0.0006	0.003 ± 0.001	0.033 ± 0.001	0.032 ± 0.005
	24	0.064 ± 0.016	0.014 ± 0.008	0.198 ± 0.084	0.186 ± 0.047
3TC-DP-choline/ethanolamine (fr 10-11)[b]	6	0.009 ± 0.003	0.002 ± 0.0005	0.045 ± 0.03	0.042 ± 0.014
	24	0.047 ± 0.022	0.017 ± 0.008	0.094 ± 0.021	0.070 ± 0.025
3TC-DP (fr 16-18)[b]	6	0.089 ± 0.023	0.021 ± 0.015	0.028 ± 0.023	0.049 ± 0.017
	24	0.237 ± 0.089	0.089 ± 0.108	0.109 ± 0.095	0.103 ± 0.016
3TC-TP (fr 25-26)[b]	6	0.025 ± 0.005	0.008 ± 0.007	0.011 ± 0.003	0.027 ± 0.018
	24	0.041 ± 0.009	0.031 ± 0.041	0.039 ± 0.024	0.044 ± 0.015
[2,8-^{3}H]PMPA-derived					
PMPA (fr 8-10)[b]	6	0.062 ± 0.051	0.125 ± 0.082	0.396 ± 0.538	0.457 ± 0.545
	24	0.019 ± 0.006	0.020 ± 0.029	0.054 ± 0.060	0.076 ± 0.043
PMPAp (fr 15-16)[b]	6	0.140 ± 0.098	0.140 ± 0.069	0.248 ± 0.230	0.232 ± 0.169
	24	0.034 ± 0.011	0.018 ± 0.026	0.058 ± 0.067	0.080 ± 0.008
PMPApp (fr 24-25)[b]	6	0.431 ± 0.242	0.190 ± 0.086	0.188 ± 0.0078	0.221 ± 0.045
	24	0.068 ± 0.074	0.032 ± 0.046	0.237 ± 0.022	0.228 ± 0.001

[a]Data are the mean of three independent experiments. [b]Fraction number in the high-performance liquid chromatography chromatogram at which the radio-labelled metabolite eluted. ZDV, zidovudine; 3TC, lamivudine; PMPA, tenofovir; MO, monocyte.

dent. The latter two drugs, and particularly the last one, is known to be metabolically activated in both activated (proliferating T cells) and resting (metabolically less active) cells, and strikingly differ from zidovudine which is mainly activated in proliferating cells, and poorly metabolized in resting cells. Among the three drugs evaluated, tenofovir was clearly most abundantly and efficiently converted to its diphosphorylated antivirally active derivative (equivalent of the triphosphate metabolites of the nucleoside RT inhibitors) in T-lymphocyte/T-blast cells but more importantly also in dendritic and Langerhans cells. These observations predict a favourable and durable antiviral effect in dendritic and Langerhans cells for tenofovir, and a lesser antiviral effect of lamivudine and particularly zidovudine in dendritic and Langerhans cells than in T-lymphocyte/T-blast cells. Our metabolic data are in line with our very recent findings on differences in antiviral potency found in these different cell types for zidovudine and tenofovir [16]. Tenofovir was far superior (~ 40-fold) in terms of antiviral efficacy in the dendritic and Langerhans cells than in T-lymphocyte/T-blast cells, whereas zidovudine proved (only 2-fold) more effective in T-lymphocyte CEM cells than in the monocyte-derived cell types. Tenofovir thus emerged as an interesting candidate for post-exposure prophylactic drug treatment, and this conclusion is in line with earlier findings of Tsai *et al.* [17,18] who reported the effectiveness of post-inoculation tenofovir treatment for the prevention of simian immunodeficiency virus infection in macaques.

It should be kept in mind, however, that not only the absolute intracellular drug metabolite levels are important, but also endogenous dNTP levels in the different cell types [7,10]. The competing dNTP pools have indeed been determined in macrophages versus lymphocytes, and the ZDVTP/dTTP, ddCTP/dCTP and PMEApp/dATP ratios found to be 10-, 6- and 120-fold higher in macrophages than in lymphocytes [7]. If such ratios are also relevant for MO-DC and MO-LC, they may explain the much more pronounced antiviral efficacy of tenofovir in the MO-DC and MO-LC. However, we should keep in mind that it has also been suggested that DC are not readily infected by HIV, but rather bind HIV virions on their surface through DC-SIGN. These cells then can expose the underlying draining lymph nodes to the DC-bound virus [19–21]. If this scenario is true, it is unlikely that intracellularly-activated NRTIs or NtRTIs in MO/LC and MO/DC would have an impact on HIV transmission.

Lamivudine reached triphosphate metabolite levels that

were very similar in all cell types investigated. These findings are again in agreement with the virtual independence of lamivudine metabolism on the cell cycle and thus, lamivudine metabolism is of similar efficacy in actively proliferating cells and in resting cells. As previously suggested from antiviral/metabolic studies in monocyte/macrophages and T lymphocytes, our data suggest that combination of a cell cycle-dependent drug (i.e. zidovudine) with a cell cycle-independent drug (i.e. lamivudine, tenofovir) is most advisable to afford an efficient suppression of virus replication in both (activated and resting) cell types. Tenofovir/lamivudine on the one hand, and zidovudine on the other, may be prime candidates for such a rational drug combination. In addition, our metabolic studies revealed that tenofovir should be further explored as a candidate drug for application in post-exposure prophylactic treatment. It may be advisable, however, not to use tenofovir in a monotherapeutic regimen, but to combine it with drugs that are targeted against other steps in the replication cycle but that act before proviral DNA incorporation such as adsorption/fusion inhibitors and/or integration inhibitors.

In conclusion, zidovudine, lamivudine and tenofovir have been studied for the first time in terms of their metabolic properties in MO-DC and MO-LC. Tenofovir was efficiently converted to its antivirally active metabolite in PHA/IL-2-activated T-blast cells and CEM lymphocytes, but even more in MO-DC and MO-LC. These data argue for a potential usefulness of tenofovir in post-exposure prophylactic treatment of HIV-infected individuals.

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