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Intracellular pharmacokinetics of antiretroviral drugs in HIV-infected patients and correlation with drug action

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Table of Contents

ABSTRACT 5
1 INTRODUCTION 7
2 CLINICAL PHARMACOKINETICS OF ARVS 8
2.1 ENTRY INHIBITORS 8
2.1.1 CCR5 INHIBITORS 8
2.1.2 ENFUVIRTIDE 9
2.2 NUCLEOSIDE AND NUCLEOTIDE ANALOG INHIBITORS OF REVERSE TRANSCRIPTASE 9
2.3 NON NUCLEOSIDE ANALOG INHIBITORS OF REVERSE TRANSCRIPTASE 10
2.4 INTEGRASE INHIBITORS 10
2.5 PROTEASE INHIBITORS 10
3 METHODOLOGICAL CONSIDERATIONS 11
3.1 CELL COLLECTION 11
3.2 ESTIMATION OF CELL NUMBER 12
3.3 ANALYTICAL METHODS FOR INTRACELLULAR ASSAYS 12
3.3.1 NUCLEOSIDE ANALOG INHIBITORS OF REVERSE TRANSCRIPTASE 13
3.3.2 NON NUCLEOSIDE ANALOGS AND PROTEASE INHIBITORS 15
4 MECHANISMS INFLUENCING INTRACELLULAR ACCUMULATION 16
4.1 GENERAL PRINCIPLES 16
4.2 NUCLEOSIDE AND NUCLEOTIDE ANALOG INHIBITORS OF REVERSE TRANSCRIPTASE 17
4.3 NON NUCLEOSIDE ANALOG INHIBITORS OF REVERSE TRANSCRIPTASE 18
4.4 PROTEASE INHIBITORS 18
4.5 IMPORTANCE OF GENETIC POLYMORPHISM 19
5 CLINICAL STUDIES WITH INTRACELLULAR CONCENTRATIONS 20
5.1 RELATIONSHIP BETWEEN INTRACELLULAR AND PLASMA PHARMACOKINETICS 20
5.2 DRUG-DRUG INTERACTIONS AT THE INTRACELLULAR LEVEL 21
5.3 RELATIONSHIP BETWEEN INTRACELLULAR CONCENTRATIONS AND EFFICACY 26
5.4 RELATIONSHIP BETWEEN INTRACELLULAR CONCENTRATIONS AND TOXICITY 30
6 CONCLUSION 33

Figure captions

Fig. 1. Host-cell-mediated sequential enzymatic phosphorylation steps required for activating the nucleotide- and nucleoside-analogue reverse-transcriptase inhibitors (NRTIs) to the triphosphate moiety (Reproduced from Anderson et al.[17]). ABV = abacavir; AMPD = adenosine monophosphate deaminase; AMPK = adenosine monophosphate kinase (adenylate kinase); APT = adenosine phosphotransferase; CBV = carbovir; dCK = deoxycytidine kinase; dCMPK = deoxycytidine monophosphate kinase; ddA = 2',3'-dideoxyadenosine; ddI, = didanosine; DP, = diphosphate; d4T = stavudine; FTC = emtricitabine; gK = guanylate kinase; MP monophosphate; PMPA = tenofovir (PMPA DP is a triphosphate analogue); TFV = tenofovir; TP = triphosphate; ZDV = zidovudine; 3TC = lamivudine; 5'NDPK = 5' nucleoside diphosphate kinase; 5'NT = 5' nucleotidase.

Fig. 2. Schematic representation of uptake and efflux transporters that may influence intracellular concentrations of antiretroviral drugs in peripheral blood cells. Transporters are named by gene and proteins (Adapted from Ford et al.[53] and updated[55-57]). OCT = Organic Cation Transporters, hCNT = Concentrative Nucleoside Transporter, ENT = Equilibrative Nucleoside Transporter, P-gp = P-glycoprotein, MRP = Multidrug Resistance Protein, BCRP = Breast Cancer Resistance protein.

Abstract

In patients infected by human immunodeficiency virus (HIV), the efficacy of highly active antiretroviral therapy (HAART) through the blockade of different steps of this retrovirus life-cycle is now well established. As HIV is a retrovirus which replicates within the cells of the immune system, intracellular drug concentrations are important to determine antiretroviral efficacy and toxicity. Indeed, nucleoside reverse transcriptase inhibitors (NRTI), non nucleoside reverse transcriptase inhibitors (NNRTI), newly available integrase inhibitors and protease inhibitors (PI) act on intracellular targets. NRTIs are prodrugs that require intracellular anabolic phosphorylation to be converted into their active form: the triphosphorylated drug metabolite (NRTI-TP), half-life of which being longer than plasma half-life of the parent compound for most. Activity of intracellular kinases, expression of uptake transporters which may be dependent upon cell functionality or their activation state may greatly influence intracellular concentrations of NRTI-TP. In contrast, NNRTIs as well as PIs are not prodrugs and exert their activity by inhibiting directly enzyme targets. All PIs, are substrates of CYP3A, which explains most of them display poor pharmacokinetic properties with intensive pre-systemic first pass metabolism and short elimination half-lives. There are evidences that intracellular concentration of PIs depends on P-gp and/or other efflux transporters activity, which is modulated by genetic polymorphism and co-administration of drugs with inhibiting or inducing properties. Assaying adequately the intracellular concentrations of antiviral (ARVs) drugs is still a major technical challenge, together with the isolation and the counting of peripheral blood mononuclear cells (PBMCs). Furthermore, intracellular drug could be bound to cell membranes or proteins; the amount of intracellular ARV available for antiretroviral effectiveness is never measured which is a limitation of all published studies. In this review, we summarized the results of thirty-one articles that provided results of intracellular concentrations of ARVs in HIV-infected patients. Most studies also measured plasma concentrations but few of them studied the relationship between plasma and intracellular concentrations. For NRTIs, most studies could not established significant relationship between plasma and triphosphate concentrations. Only eight published studies reported an analysis of the relationships between intracellular concentrations and virological or immunological efficacy of antiretroviral drugs in HIV patients. In prospective studies well designed and with a reasonnable

number of patients, a significant correlation between virological efficacy and intracellular concentrations of NRTIs was found with no influence of plasma concentration. For PIs, the only prospectively design trial on lopinavir found both the influence of trough plasma and intracellular concentrations. ARVs are known to produce important adverse effects through their interferences with cellular endogenous processes. The relationship between intracellular concentrations of ARVs and their related toxicity were investigated in only four articles. For zidovudine, the relative strength of the association between haemoglobin decrease and plasma zidovudine compared to intracellular zidovudine-triphosphate is still unknown. Similarly, for efavirenz and neuropsychological disorder methodological differences penalize the comparison between studies. In conclusion, intracellular concentrations of ARVs play a major role in their efficacy and toxicity and are influenced by numerous factors. However the number of published clinical studies in that area is limited; most studies were small and not always adequately designed. In addition, standardization of assays and PBMC counts are warranted. Larger and prospectively designed clinical studies are needed to further investigate the links between intracellular concentrations of ARVs and clinical endpoints.

1 Introduction

Human Immunodeficiency Virus (HIV) is a retrovirus, which replicates within the cells of the immune system. The efficacy of highly active antiretroviral therapy (HAART), is now well established, and has provided extraordinary benefits to many patients with HIV infection.[1] The morbidity and mortality related to HIV infection have dramatically decreased in countries in which HAART has been available, turning HIV infection into a chronic manageable disease.[2] Life-long antiretroviral treatment seems necessary, as viral replication and loss of CD4 cells resume when HAART is interrupted. HAART regimens have shown some limitations, the major one being the failure to eradicate HIV even after several years of therapy. One of the reasons is that despite potent antiretroviral (ARV) treatment, compartments of replication-competent virus persist, suggesting that ARVs do not reach all the infected cells: however, there are no data to support this theorical assumption. This article will focus on pharmacologic principles that govern intracellular concentrations of antiretroviral drugs and on clinical studies which aimed at assessing whether intracellular concentrations of ARVs could be a useful parameter to predict efficacy or toxicity of antiretroviral drug regimen.

The different steps of HIV replication are now well identified and understood. A number of antiretroviral drugs are now available and are grouped in five pharmacologic classes according to their mechanism of action. These drugs target essential receptors or enzymes at different steps of the life cycle of the virus and will block the production of infectious retroviral particles from the cell.[3] However virus eradication cannot be achieved with the available treatments because of the pool of latently infected CD4 cells.[4]

HAART is the standard of care to avoid selection of viral mutations. Selection of drugs for treatment naïve patients and experienced patients take into account the risk benefit ratio and the viral genotype. Current guidelines recommend in treatment-naïve patients a combination of a ritonavir boosted PI or a NNRTI plus two NRTIs and in treatment experienced patients a combination of at least two active ARV drugs from different classes based on viral genotype.[5-7]

Besides entry inhibitors which act on receptors located on cell surface, most ARV drugs inhibit viral replication inside the cell, therefore intracellular concentration should be a reliable parameter to consider when relating pharmacokinetics and

efficacy. Results from several *in vitro* studies also exist in this area. But, because of the difficulties of extrapolating the results from *in vitro* to *in vivo* studies, in this paper, we focus only on *in vivo* studies.

This article summarizes clinical trials where intracellular concentrations were measured and related to plasma concentrations, virological efficacy or toxicity. Prior to this presentation and to understand limitations of such studies, the following topics are presented and discussed: clinical pharmacokinetics of ARVs, intracellular drug assays and mechanisms influencing intracellular diffusion and accumulation.

2 Clinical pharmacokinetics of ARVs

Pharmacokinetic parameters of ARVs are summarized in table I. [6, 8-13]

2.1 Entry inhibitors

Entry inhibitors block the virus attachment on receptor of the cell surface. They have an extracellular mode of action and therefore differ from other available classes of antiretroviral agents. Two drugs of this class are available, enfuvirtide and maraviroc, considering their mechanism of action, they are outside the scope of this review. To have an exhaustive overview of ARVs, their pharmacological properties are briefly summarized below.

2.1.1 CCR5 inhibitors

CCR5 or CXCR4 chemokine co-receptor antagonists were promising entry inhibitors. Maraviroc is the first approved drug of this new class. Maraviroc inhibits CCR5 chemokine co-receptor preventing HIV binding to cell membrane. Pharmacokinetic characteristics have been summarized elsewhere.[14] In brief, maraviroc is a CYP3A substrate and dosing differs according to combined drugs (150 mg bid with ritonavir boosted PI, 600 mg bid when combined with drugs with enzyme inducing properties such as efavirenz and 300 mg bid when combined with nucleoside analogs). Maraviroc is a P-gp substrate, which limits intracellular concentrations. Concentrations in cervico-vaginal fluid and vaginal tissue are higher than in plasma.

2.1.2 Enfuvirtide

Enfuvirtide (T20) is a HIV-1 fusion inhibitor, which prevents fusion of HIV-1 and host cell membranes. It is a synthetic peptide (4492 Da), which is not bioavailable when taken orally and is administered subcutaneously twice daily (90 mg bid) which is obviously a limitation to its long-term use. Pharmacokinetic properties have been previously reported.[15]

2.2 Nucleoside and nucleotide analog inhibitors of reverse transcriptase

Zidovudine (ZDV) is the oldest antiretroviral drug; since a number of nucleoside analogs were developped (zalcitabine, didanosine (ddI), stavudine (d4T) lamivudine (3TC), emtricitabine (FTC), abacavir (ABC)). Tenofovir (TFV) is a nucleotide analog obtained after drug administration of tenofovir disoproxil (TDF), its ester prodrug. Apricitabine is a new NRTI under development.

Although absolute bioavailaility is unknown, bioavailability is supposed to be high for most nucleoside analogs but ddI, which is degraded at acid pH, and TDF. None of these drugs are highly protein bound. Elimination of parent compound occurs as unchanged drug via the kidney or non-CYP drug metabolizing enzymes, therefore potential for drug-drug interaction is low, although TDF was demonstrated to inhibit ddI metabolism.[16] Triphosphate (TP) metabolites are the active component of all nucleoside analogs.They also inhibits to varying degrees human mitochondrial polymerase γ. Phosphorylation steps occur within the cell and involved kinases, which are listed in figure 1.[17] Half-life of the active moiety is longer than plasma half-life of the parent compound for all nucleoside analogs. Long half-lifes of TP metabolites favor once daily dosing for most of nucleoside analogs except ZDV and d4T, which are administered on a twice-daily basis. TFV is a nucleotide analog for which the active form is a diphosphate (DP). All NRTIs compete with endogenous analogs and stop DNA elongation. Nucleosides such as ABC (carbovir (CBV)) and TFV are much less apt to cause mitochondrial toxicity compared with d4T.[18]

2.3 Non nucleoside analog inhibitors of reverse transcriptase

NNRTIs do not require phosphorylation to inhibit reverse transcriptase. Nevirapine (NVP) and efavirenz (EFV) are the most commonly used. Delavirdine is available in some countries, and etravirine is a new NNRTI recently approved in the European Union and in the United States. NVP and EFV have long half-life after single dose administration. They are metabolized through CYP3A and CYP2B6 and a genetic polymorphism has been described which explains at least part of interindividual variability of their total clearance. They both have enzyme inducing and autoinducing properties, which explains drug-drug interactions and their non linear pharmacokinetics.[19]

2.4 Integrase inhibitors

Integrase inhibitors represent a new class. These drugs inhibit the integration of HIV-DNA into the host genome. Raltegravir was approved in early 2008 and elvitegravir is under development. Raltegravir is rapidly absorbed and plasma concentrations decline with a terminal half-life of 7 to 12 h which supports a twice daily dosing.[20] Plasma protein binding is 83%. Biotransformation pathway involved UGT1A1 therefore drug-drug interactions are limited.[21] ATV which inhibits UGT1A1, increases raltegravir concentrations modestly.[22] Inducers such as EFV, TPV or rifampin decrease raltegravir concentrations although the clinical consequences are currently unclear.[21]

2.5 Protease inhibitors

Protease inhibitors (PI) prevent cleavage of viral precursor protein into the subunits required to form new virions. Approved PIs include amprenavir (APV), fosamprenavir, atazanavir (ATV), darunavir (DRV), indinavir (IDV), lopinavir/ritonavir (LPV/r), nelfinavir (NFV), ritonavir (RTV), saquinavir (SQV), and tipranavir (TPV).

They all are substrate and inhibitor of CYP3A, which explains part of their poor pharmacokinetic properties: pre-systemic first pass metabolism, variable plasma concentrations and short half-life in the 7 to 15 h range. RTV, which is the most potent CYP3A inhibitor, is combined to all PIs but NFV to improve their pharmacokinetic properties, increase plasma exposure and /or decrease the

administered dose.[23, 24] As basic organic chemicals they all are bound to plasma proteins, α1-glycoprotein acid and albumin. They differ for some pharmacokinetic parameters, extent of first pass metabolism, extent of protein binding (IDV 60%, LPV 98-99%) and some of them such as APV have inducing properties which make drug-drug interaction prevision very difficult.

3 Methodological considerations

All intracellular assays described to date do not discriminate between drug localised in cell membrane or in cytoplasm, either bound to intracellular proteins or truly unbound which should be the effective antiretroviral moiety. Measurement of total cell concentrations is somehow of limited value.

3.1 Cell collection

Isolation of PBMCs is the first step before analyzing the intracellular concentrations of either NRTIs, NNRTIs or/and PIs. PBMCs can be isolated either using conventional Ficoll gradient centrifugation or using cell preparation tubes (CPTs) (from Becton Dickinson). The two procedures were compared by Becher et al.[25] on phosphorylated anabolites of two NRTIs and were shown to give identical results. However using CPTs was found to be easier, less time-consuming and therefore quicker which in the case of d4T-TP was most important as the drug was shown instable in the cell ring of the Ficoll gradient (40% loss within 40 min) and lead the authors to collect the ring in less than 10 min. However before this isolation step the stability of the phosphorylated anabolites, that of NNRTIs and PIs in blood should be considered.

Regarding d4T-TP, its stability has been checked in blood before PBMCs isolation and the authors recommend to perform the isolation within 6 h after sampling.[26] Similar results were obtained with 3TC-TP and ZDV-TP.[27] It therefore seems that for the phosphorylated anabolites the storage of blood samples in CPTs before isolation could last 6 h, although it has not been thoroughly investigated for CBV-TP and TFV-DP.

Other issues during cell processing are to avoid contamination by red cells which may phosphorylate some nucleoside analogs[28] and efflux of PIs and NNRTIs out of cells. In contrast the NRTI-TPs are ion-trapped intracellularly. For NNRTIs and PIs

intracellular measurements, the collection of PBMCs has not been systematically studied. The authors mentioned that samples should be immediately taken to laboratory (within 5 min) and that all the procedures should be performed at 4℃ to inhibit enzymatic activity and to prevent active drug efflux, the time between blood sampling and the cell isolation and extraction procedure should be less than 1 h.[29, 30]

3.2 Estimation of cell number

Since the number of cells normalizes intracellular concentration, a critical step in the processus of intracellular assay is the determination of the number of cells from which the compounds were quantified.

In most studies when the information is indicated the cells were determined on a small aliquot with a Coulter Counter, or using a Malassez cell and a microscope. However this last procedure may suffer from insufficient accuracy and precision, specifically when multiple sites are involved which explains that a biochemical test was developed based on the relationship between DNA content and cells count by Malassez cell.[31] This test could be performed in the analytical laboratories where there is no Coulter Counter available.

The concentration is therefore expressed as amounts per 10^6 cells and can be converted in amount per volume on the approximation that the PBMC volume is 0.4 pL in order to compare intracellular and plasma concentrations.[32] The accuracy of this volume may be questionable as it varies according to the state of the cells (quiescent or stimulated) or to the nature of the cells (cell volume of human lymphoblast : 2.1 pL).[33] This highlights the pitfalls of the conversion. However the 0.4 pL volume is mostly used.[29, 34] This calculation step is critical for the comparison of the results from different teams, and a standardized procedure should therefore be chosen.

3.3 Analytical methods for intracellular assays

The approaches regarding the analysis of the intracellular drugs due to the difference in their concentrations (low about fmol/10^6 cells for intracellular TP anabolites and ng/3×10^6 cells i.e. about pmol/10^6 cells for PIs) are quite different.

3.3.1 Nucleoside analog inhibitors of reverse transcriptase

The major problem in measuring intracellular TP anabolites is the small amount present in cells of patients and the presence of the endogenous intracellular nucleotides able to interfere. Thus, selective and sensitive analytical methodologies should be developed.

Rodriguez et al.,[35] in 2000 reviewed the latest information regarding the intracellular *in vivo* quantification of NRTI-TP. The authors described the first methods used and pointed out all their drawbacks (lack of sensitivity, cumbersome assays, inability to differentiate NRTI-TP from the endogenous nucleotides, lack of internal standard). More recent approaches were based on the same first steps, i.e. separation of ZDV-anabolites using anion-exchange cartridges, cleavage of the phosphate group using acid phosphatase, addition of an internal standard after enzymatic digestion, desalting and quantification by HPLC-MS/MS. Moreover the calibration curve was prepared from ZDV-TP contrary to the previous procedures, which used the parent compound. The limit of detection was 4.0 fmol/10^6 cells. The authors applied the same procedure to the simultaneous determination of ZDV-TP and 3TC-TP.[36] The limit of quantification was 0.1 pmol and 4.0 pmol for ZDV-TP and 3TC-TP, respectively. Moore et al.,[37] improved this procedure describing an analytical method which allows to measure simultaneously intracellular 3TC-TP, d4T-TP and ZDV-TP with HPLC-MS/MS. The limits of detection were 5, 25, 25 pg on column for 3TC-TP, d4T-TP, ZDV-TP respectively. Similar methods were applied by King et al.,[38] to measure TFV-DP and by Robbins et al.,[39] to measure simultaneously ZDV-TP, TFV-DP and 3TC-TP in PBMC i.e. isolation by anion exchange, addition of a stable labeled isotope, dephosphorylation, desaltation and detection by LC MS/MS. The lower limit of quantification were 10 fmol/10^6 cells for TFV-DP[38] and 0.11 pmol/10^6 cells, 2 fmol/10^6cells and 3.75 fmol/10^6 cells for 3TC-TP, ZDV-TP and TFV-DP respectively for a sample size of 10^6 cells.[39] Most of these indirect methods are quite labor-intensive, involving multiple steps, which may restrict their use to specialized laboratories.

New methodologies were described based on direct HPLC-MS/MS determination on the cellular extracts without dephosphorylation. However these processes need the use of ion pairing agents to circumvent the poor retention of the nucleotides which most of them are incompatible with ionisation mass spectrometry. Pruvost et al.[26]

described the direct determination of d4T-TP as well as that of the endogenous competitor deoxythymidine triphosphate (dT-TP). Just before cell lysis, an internal standard was added. The instrument was operated in the electrospray negative ion mode under MS/MS conditions. The limit of quantification was 9.8 fmol/10^6 cells i.e. 20 pg injected for d4T-TP. In this article the authors focus on the stability of the d4T-TP at the different steps (in blood, in the cells ring, in dry cells at 4℃, after cell lysis at 4℃, in the injection solvent at room temperatur e). This procedure is very simple to perform as it does not need any extraction step. However due to the very high pH of the mobile phase (ion pairing agent: 1,5-Dimethylhexylamine) the column was changed every two weeks.[40]

With slight modifications regarding the internal standard and the chromatographic column, the same authors were able to measure simultaneously d4T-TP, 3TC-TP and ddA-TP (active anabolite of ddI) with their corresponding natural nucleotides in the same run.[25] However regarding ZDV-TP, a massive and tailing peak was observed near the retention time of ZDV-TP, which precludes the analysis of ZDV-TP simultaneously with d4T-TP, 3TC-TP and ddA-TP. To overcome this problem Becher et al.,[41] developed a specific extraction of ZDV-TP using immunoaffinity and detection of ZDV-TP using LC-MS/MS. More recently the same group improved the specificity and obtained a slightly better sensitivity for 3TC-TP, CBV-TP and TFV-TP using a positive electrospray ionization mode. [42]

Although the direct methods should be faster and more precise King et al.,[43] were unsuccessful in reproducing these methodologies. In particular they pointed out the difficulty in analyzing ZDV-TP due to the large amount of ATP and to the interference with dGTP, these latter compounds having the same precursor ion and the same product ion. This was evidenced by Compain et al.,[44] who developed an improved method to determine ZDV-TP. The authors chose a minor but specific fragment ion and had to spike their sample with a constant amount of ZDV-TP to allow the signal to emerge from background in order to increase the sensitivity.

HPLC-MS/MS is susceptible to matrix effects, i.e. co-eluting matrix components that affect the ionization of the target analyte, resulting in ion suppression, or, in some cases ion enhancement.[45] For intracellular assay the main parameter to study is the influence of the number of cells in the sample, as it cannot be fixed. The matrix effect plus recovery was tested by Becher et al.,[46] on d4T-TP and ddA-TP and the influence of the cell number was evidenced. The use of an appropriate internal

standard controlled the influence of the matrix effect between 7 and 14×10^6 cells for the simultaneous assay of d4T-TP and ddA-TP. However the use of stable isotope analog as internal standard would be the best choice to control the influence of the matrix effect.

Monitoring the very low intracellular concentrations of these active anabolites remains an analytical challenge. All the methods described have their drawbacks. However they are all based on sophisticated methods, which can be hardly reproduced, so each laboratory favors the analytical procedure in which it is familiar.

Whatever the choice regarding the procedure, indirect or direct, it appears that the quantification of the compounds using HPLC coupled to MS/MS is very specific and may circumvent all the drawbacks due to the multiple natural nucleotides that are found in the complex mixture of the intracellular medium that can interfere with the determination of intracellular phosphorylated anabolites of NRTIs.

3.3.2 Non nucleoside analogs and protease inhibitors

The measurement of intracellular concentrations of NNRTIs and PIs could be obtained using HPLC-UV detection as was it published for EFV[34] and for 10 of them (NVP, DLV, APV, IDV, metabolite of NFV (M8), RTV, LPV, EFV, SQV, NFV)[47].

However most reported data were obtained using LC-MS/MS methods either for one drug[48] or for the simultaneous measurement of several of them. The method involved automated solid-phase extraction[48], liquid-liquid extraction ((APV, LPV, SQV, EFV)[49], NVP[30], (LPV, RTV)[50]) or single-step extraction ((NVP, DLV, APV, IDV, M8, RNV, LPV, EFV, SQV, NFV)[47], (IDV, APV, SQV, RTV, NFV, LPV, ATV, EFV)[29]). Few quantitative immunoassays were published for the intracellular determination of LPV and ATV.[51, 52] These methods imply the preparation of a polyclonal antibody obtained with a synthetic antiretroviral drug derivative coupled to hemocyanin or serum albumin as the immunogen and the chemical synthesis of an enzyme tracer. Obviously these methods are not available in most laboratories, which preclude their use as useful tools to study the intracellular concentrations of NNRTIs and PIs.

4 Mechanisms influencing intracellular accumulation

4.1 General principles

As already stated, most ARVs acting on cell receptors need to enter the cell to bind to antiretroviral targets, reverse transcriptase, integrase or protease. In general, disposition from systemic circulation and capillary lumen to the extravascular compartment occurs by diffusion or involves active transporters

Simple diffusion is generally the most common mechanisms for transmembranal movement of xenobiotics in the body. The rate of diffusion is defined by Fick's law and accordingly the small, lipophilic, unionised and unbound molecules readily diffuse across the membrane. Difference in the pH gradient between plasma and lymphocytes could explain ion trapping. As reported by Ford et al.[53], the pH gradient between plasma and lymphocytes is subject to change depending on the membrane potential. Binding of drugs to plasma proteins may slow diffusion rate as only free unbound drug will cross biological membranes. However basic drugs which have higher affinity for cells or tissues proteins than for plasma proteins may leave very rapidly the blood stream and protein binding is not a limiting factor; for such drugs, volume of distribution is high, the amount of drug in plasma small compared to the amount in tissues and cells and small changes in plasma protein binding will not affect the amount in extra vascular compartments.[54] Membrane transporters (efflux, influx) are now recognized to play an important role in drug absorption and disposition and to explain, at least in part, the broad interindividual variability in intracellular concentrations of drugs. Figure 2 summarizes the different carrier proteins determining intracellular concentrations within a typical immune cell.[53, 55-57] Efflux transporters which operate at the expense of adenosine triphosphate (ATP) hydrolysis are members of ATP-binding cassette (ABC)-type transport proteins and are now well studied. P-gp was first described for its ability to reduce intracellular concentrations of anticancer compounds. Other multidrug resistance proteins (MDR) have been isolated since. They are expressed in the apical membrane of many barrier tissues such as the intestine, liver, kidney, blood-brain-barrier, placenta, testis and in immune cells. Relevance for pharmacotherapy of expression of ABC drug transporters in peripheral blood cell have been reviewed recently.[57] The Breast Cancer Resistance Protein (BCRP) was found to play a major role in nucleoside

efflux.[58, 59] Although influx transporters are not as well studied, several proteins have been identified for nucleosides transport (Solute Carrier, SLC); they differ by their mechanism of action. Some are powered by electrochemical gradient (Concentrative Nucleoside Transporter hCNT), others are Equilibrative Nucleoside Transporter (ENT), Organic Cation Transporters (OCT) or Organic Anion transporters (OAT), although this later was not found to be expressed in immune cells.[53, 60]

Several factors may influence transporters expression within cell membrane, cell subsets and functionality, activation state of cells and polymorphism of coding genes. Polymorphism in the coding region of the transporter genes has been evidenced which lead to produce functional changes in the encoded transporter protein and result in variation in drug disposition and response, however the studies are scanty.[55] Several factors which could affect intracellular concentrations of ARVs have to be taken into consideration: drug affinity and expression of the transporters according to different cells or tissues, and many of these transporters are known to be modulated by co-administrated ARVs.

4.2 Nucleoside and nucleotide analog inhibitors of reverse transcriptase

Data on nucleoside analogs (purine or pyrimidine base coupled to a sugar) cellular penetration are scarce. As they are more hydrophilic compounds, it was suggested that they could be substrates of the endogenous nucleoside transporters.[61, 62] Although studies demonstrated that cerebral penetration occurs mainly by passive diffusion and that the low concentration of nucleoside in brain is the consequence of active efflux transporters[63], expression of uptake transporters in lymphocytes could favor high intracellular concentrations. It was evidenced that ZDV-TP and 3TC-TP concentrations were effluxed by MRP4 and BCRP. TFV as a nucleotide has an ionized phosphate group, which confers acidic properties. It was demonstrated that TFV uptake in the kidney proximal tubule basolateral membrane is mediated via OAT1 and cellular efflux into the urine via MRP2 and MRP4.[64] TP concentrations differ according to cell type most likely as a consequence of influx and efflux transporters expression. In healthy volunteers, 3TC-TP concentrations were close in PBMCs and purified CD4 cells, whereas ZDV-TP concentrations were lower in CD4 cells than in PBMCs.[65] Concentrations of TFV-DP were compared in PBMC, lymph

node tissue and digestive lymphatic tissue and were higher in PBMCs than in other tissues.[66] These data strongly suggest that transporters localisation may differ according to cell functionality. Kinase activity could also influence the intracellular concentration of TP. *In vitro* experiments suggested this activity varies greatly and is lower in resting cells than in activated PBMC.[67, 68] This could have important consequences as kinases activity will govern the intracellular level of both endogenous triphosphates and NRTI-TP which compete at the level of HIV-reverse transcriptase. All NRTIs have been demonstrated to be more effective in monocyte derived macrophages which are important HIV1 reservoirs than in CD4+T lymphocytes.[69] These could also well explain the differences in NRTI-TP intracellular concentrations according to different cell types and different activation state.[28]

4.3 Non nucleoside analog inhibitors of reverse transcriptase

NNRTIs are weakly acidic and predominantly bind to albumin. Neither EFV nor NVP were thought to be substrate of P-gp.[70] In a limited number of patients, Almond et al.,[34] demonstrated a relationship between intracellular concentration of EFV and % bound EFV in plasma. Such data are in contrast with those obtained with NVP by the same team.[30] They demonstrated that intracellular concentrations of NVP are far below those measured in plasma. Intracellular concentration was negatively related to P-gp expression, but not related to plasma % unbound NVP.[30] To explain these data, the authors suggest that NVP could induce P-gp or co-regulated efflux transporter. Clearly, to understand all mechanisms, which are involved in intracellular concentrations of NNRTIs, further studies are needed.

4.4 Protease inhibitors

The intracellular pharmacology of PIs has been carefully reviewed by Ford et al.[53].

The PI physio-chemical properties are in favour of passive transfer:

- The transfer is in agreement with lipophilicity measured by the n-octanol to water partition coefficient. Accumulation of PIs in lymphocytes reflects the rank order of lipophilicity: the less lipophilic PI being IDV and the most lipophilic NFV.
- PIs are weak bases and are mostly unionized in a basic environment; intracellular sequestration is dependant upon pH gradient between plasma and cells.

- Protein binding of PIs to α1-acid glycoprotein ranged from 60% for IDV to 97-99% for RTV, LPV, SQV and NFV. However protein binding per se is not a limiting factor to intracellular diffusion as IDV, which is 60% bound, has lower intracellular concentrations than other PIs more highly bound. Within the cell PIs are bound to cell proteins and HIV proteases and therefore their relative affinity for each protein may influence their dynamic equilibrium.[53]

However active transport may play a role in the intracellular accumulation. It is now well established that PIs are substrates of P-gp and others efflux transporters such as MRP1[71] or MRP2[72]. P-gp is expressed in the gastro-intestinal tract and the liver and act with CYP3A to reduce their bioavailability. RTV combined to most PIs as a pharmacologic enhancer inhibits both CYP3A and P-gp and markedly increases the bioavailability of PIs. Such transporters are expressed on lymphocytes and may reduce cellular accumulation. Meaden et al.,[73] found a relationship between combined expression of P-gp and MRP1 on PBMCs of HIV-infected patients and intracellular accumulation of SQV and RTV. In summary, there are evidences that intracellular concentration of PIs depends on P-gp and/or other efflux transporters activity, which is modulated by genetic polymorphism and coadministration of drugs with inhibiting or inducing properties. How these transporters will control intracellular concentrations of PIs need further studies.

4.5 Importance of genetic polymorphism

At evidence, the role of transporters and their genetic polymorphism in drug disposition should be considered[74] and reviews have summarized findings from recent pharmacogenetics studies.[55, 75]

La Porte et al.,[76] studied the relationship between ABCB1 (MDR1) genetic polymorphism, P-gp expression and SQV or SQV/r pharmacokinetics in 150 healthy volunteers. No relationship was found between the C3435T, G2677T/A or C1236T polymorphisms of the ABCB1 gene and the pharmacokinetics of SQV or the expression and activity of P-gp in PBMCs. Seventy one HIV-infected children treated with a NFV backbone antiretroviral drug regimen were evaluated for MDR1 polymorphism (MDR1 C3435T), NFV plasma concentrations, CD4 cell count and HIV-RNA.[77] Children with the C/T genotypes had higher 8h post dose NFV concentrations and more rapid response to HAART. Unfortunately, intracellular

concentrations of PIs were not measured in these studies. In contrast, in 12 HIV-infected patients, Ford et al.,[78] could not evidenced higher intracellular concentrations of NFV or its M8 metabolite and lymphocyte cell surface expression of P-gp. In a cohort of 47 patients treated with PI boosted or not by RTV, Chaillou et al.,[79] demonstrated that intracellular concentration of RTV was related to undetectable plasma HIV-RNA, which was not related to MDR1 gene expression.

Interestingly the importance of MRP4 carrier was evidenced by Anderson et al.,[72], as they demonstrated that patients carrying MRP4 T4131G had elevated 3TC-TP concentrations and patients with MRP4 G3724A had a trend for elevated ZDV-TP. They also found that IDV clearance was faster in patients expressing CYP3A5 and in patients carrying the MRP2-24C/T variant[72]; whether this latter may contribute to lower intracellular concentrations remains to be established.

Recently, Kiser et al.,[80] demonstrated that intracellular concentrations of TFV-DP were higher, first with decrease in kidney glomerular filtration rate and consequently total and renal clearance of TFV (p=0.04) and second in presence of the ABCC4 3463 A>G variant (p=0.04 after adjustment for race, treatment group and glomerular filtration rate). The authors pointed out the limitation of this small sample size study for genetic association and thus results should be confirmed in larger study. Whether those exploratory data could be extrapolated to intracellular concentration of TFV-DP within renal proximal tubule cells is presently unknown.

At evidence, the control of intracellular concentrations of ARVs is complex and dependant on many factors and more work is needed in this area taking into account the differences in cell biology.

5 Clinical studies with intracellular concentrations

5.1 Relationship between intracellular and plasma pharmacokinetics

Clinical studies performed in HIV-infected patients reporting intracellular concentrations are summarized in table II.[9-13, 30, 34, 72, 78, 79, 81-101] They are displayed by antiretroviral class, then within an antiretroviral class they are listed by molecule with respect to the date of Health Authority approval. For each molecule, the more recent studies are presented first. All those studies were published after 2000, except

for ZDV for which intracellular concentrations have been studied since 1994. Most studies reported both plasma and intracellular concentrations but only few of them studied the relationship between them.

It can be seem from table II that plasma pharmacokinetic parameters are rather similar across studies but some differences are observed for intracellular parameters. For NRTIs, most studies did not established significant relationship between plasma and TP concentrations. In contrast for NNRTIs and PIs results are more conflicting, some studies evidencing correlation, while others could not. These results support the use of plasma concentration of NNRTIs or PIs but not NRTIs to monitor antiviral efficacy.

Such results are not surprising knowing first that PBMCs collection, preparation and quantification are not an easy task (see section 3) and second that many factors influence intracellular drug penetration and among them genetic polymorphism of influx and efflux carriers (see section 4). Moreover, these intracellular studies have been carried out in relatively few patients and larger studies would be needed to address consistently the relationship between plasma and intracellular concentrations of ARVs.

It is also important to note that there are potential methodological problems when studying relationship between concentrations observed at single time points, as it is done in a number of studies. Indeed, plasma and intracellular half-life are very different. It is more adequate to assess the relationship through pharmacokinetic parameters such as AUC. Surprisingly, most studies reported concentrations at some time points or PK parameters obtained by non-compartmental analysis. Population approaches were never used to analyse intracellular concentrations and their link with plasma concentrations, although this approach seems more appropriate as it allows to analyze sparse measurements.

5.2 Drug-drug interactions at the intracellular level

On a theoretical point of view, changes in the intracellular concentration of antiretroviral drugs can be secondary to modifications of (i) plasma concentration of the drug and/or the prodrug, (ii) activity of the enzymes responsible for drug anabolism/metabolism at the cellular level, (iii) activity of membrane transporters involved in cellular uptake or efflux. Since the intracellular amount of the active drug

is responsible for treatment efficacy, interactions leading to changes in intracellular concentrations are a relevant issue regarding the virologic outcome.

The clinical impact of these interactions was first evidenced by the poor efficacy of therapies combining ZDV with d4T.[102] Though the likely mechanism of this result, competitive inhibition of d4T phosphorylation by ZDV, was assessed only by *in vitro* experiments, this phenomenon highlighted the necessity to investigate the possible alteration in the intracellular concentrations of ARVs due to drug association.[103, 104]

Potential interactions involving NRTIs at the intracellular level were therefore investigated in several studies. Hawkins et al.,[82] evaluated whether the high rate of virological failure observed in patients receiving a triple NRTIs combination including TDF could be explained by modifications in the intracellular anabolism of these compounds.

So, intracellular levels of TFV-DP, CBV-TP and 3TC-TP were measured in 15 HIV-infected patients receiving a triple NRTI combination (TDF-ABC-3TC or TDF-ABC-d4T), before and after replacement of TDF or ABC by a NNRTI or a PI. No modification in the intracellular concentrations of the active anabolites of the remaining NRTIs was observed, which suggested the lack of significant interaction between the investigated drugs. Another recent study confirmed these results on 27 patients.[105] Taken together, these results suggest the clinical failure that was observed with the triple NRTI (ABC/TDF/3TC) regimen was not due to drug interactions but was more likely the consequence of lack of intrinsic power.[106] This latter study also evidenced a significant 50% increase in the intracellular concentration of TFV-DP when TFV was combined to LPV/r. However, this result could simply be the intracellular reflection of the systemic interaction between these two drugs.[107] This study found no significant difference in the intracellular concentrations of CBV-TP and 3TC-TP with respect to LPV/r use, despite a 46% decrease in ABC plasma concentration in the LPV/r group. Last, nevirapine was also found not to significantly modify the intracellular concentrations of TFV-TP, CBV-TP, and 3TC-TP.

Hoggard et al.,[108] investigated whether prior exposure to ZDV could subsequently inhibit d4T phosphorylation. The rationale for this study came from the observation that naïve patients receiving a d4T -3TC combination experienced a further one $\log_{10}$ decrease in viral DNA compared to patients previously treated by ZDV.[109] A subsequent inhibition of d4T phosphorylation due to a down regulation of thymidine

kinase induced by ZDV was one of the hypotheses raised to explain this result. However, the cellular concentration of d4T-TP measured in 7 ZDV-experienced patients was not different to the concentration measured in 20 ZDV-naïve subjects. Furthermore, the ability of PBMCs to phosphorylate d4T was not different between ZDV-experienced and ZDV-naïve subjects.[108] Similarly, no influence of prior exposure to ZDV on ZDV phosphorylation was observed during a 12 months period on 23 HIV-infected patients. [83] It is therefore likely that the decrease in efficacy observed in ZDV-experienced patients was due to the acquisition of resistance mutations rather to a modification in intracellular metabolism.

By measuring the TP moieties of ZDV and 3TC in the PBMCs of 8 patients, Fletcher et al.,[84] found a strong correlation between the intracellular concentrations of ZDV-TP and 3TC-TP. If this result suggested the existence of interplay among the cellular anabolism and/or metabolism of these drugs, its precise mechanism and possible consequences have still not been elucidated.

TDF is known to increase the plasma concentration of ddI, the most likely mechanism for this interaction being the inhibition by TVF of the enzyme responsible for the hydrolysis of guanosine and adenosine analogues, the purine nucleoside phosphorylase.[16] This interaction is clinically relevant since it is responsible for adverse effects[110-113] or treatment failure[114] which may be secondary to didanosine overexposure. Because of this, this association is currently not recommended for the initiation of HAART, but is nevertheless not countraindicated for ulterior lines of treatment.[6] Pruvost et al.,[85] investigated the possible consequences of this systemic interaction on the intracellular concentrations of the active moieties. Intracellular concentrations of ddA-TP and TFV-DP were compared between 14 patients receiving the ddI/TDF (250 mg/300 mg) combination and 16 patients receiving ddI (400 mg) without TDF or 14 patients receiving TDF (300 mg) without ddI. The measured concentrations were found to be comparable between the groups which validated the strategy consisting in decreasing ddI dose from 400 to 250 mg when it is combined with TDF.[115]

Apricitabine, a novel deoxycitidine analog currently under investigation, shares its initial phosphorylation pathway by deoxycytidine kinase with 3TC and FTC. The potential interaction between apricitabine (600 mg bid) and 3TC (300 mg qd) was evaluated in a crossover study performed on 21 healthy volunteers who received sequentially each drug separately and the combination of both. No significant

modification in the plasma pharmacokinetics of 3TC, or in the cellular pharmacokinetics of its active TP moiety was observed during the combination compared to the monotherapy period. However, if co-administration with 3TC had no influence on apricitabine plasma pharmacokinetics, cellular concentration of apricitabine TP dropped by 85% during the same period.[116] These findings strongly suggested that apricitabine should not be co-administered with deoxycitidine analogues.

Hydroxyurea is an antiproliferative drug that was shown to provide a further 0.7 $\log_{10}$ reduction in plasma HIV RNA when combined with ddI compared to patients receiving ddI alone.[117] By measuring intracellular deoxyadenosine triphosphate (dA-TP) in 69 HIV-infected sujects, it was evidenced that patients receiving the hydroxyurea-ddI combination achieved significantly lower dA-TP concentrations than patients under ddI or hydroxyurea monotherapy, whereas no modification in the plasma pharmacokinetics of the two drugs was observed.[118] If the precise mechanism of this interaction is still unknown, the likely explanation for the enhancement of ddI's efficacy is the decrease in the intracellular dA-TP/ddA-TP ratio, which would facilitate the incorporation ddA-TP in the replicating viral DNA.

Similarly to hydroxyurea, mycophenolic acid, an immunosuppressive agent, is known to decrease the intracellular concentration of an endogenous nucleotide, the deoxyguanosine triphosphate (dG-TP), which could enhance the antiviral activity of abacavir by decreasing the dG-TP/CBV-TP ratio.[119] Since this ratio could not be measured to date in patients receiving the mycophenolate mofetil–ABC combination, this hypothesis still needs to be confirmed. Nevertheless, the lack of influence of mycophenolic acid on 3TC phosphorylation was sugested by the similar intracellular concentration of 3TC-TP observed in patients receiving 3TC with or without mycophenolate mofetil.[86]

Ribavirin is a nucleoside analogue used for the treatment of hepatitis C virus (HCV) infection. Although its mechanism of action is still not fully understood, it involves at least in part an intracellular transformation into a TP moiety.[120] Thus, because ribavirin is used in HIV/HCV coinfected patients, its potential interactions with NRTIs were investigated in several studies.

Rodriguez-Torres et al.,[121] evaluated the combination of ribavirin with 3TC, d4T, or ZDV in HIV/HCV coinfected patients. Plasma concentrations of ZDV, d4T, 3TC and intracellular concentrations of ZDV-TP, d4T-TP, 3TC-TP were measured in 31

patients receiving concomitant ribavirin and compared to the concentrations obtained in 25 patients receiving a placebo instead of ribavirin. No significant difference in plasma and cellular concentrations of the measured compounds was observed, suggesting ribavirin does not modify the plasma pharmacokinetics and the intracellular phosphorylation of ZDV, d4T, 3TC. The lack of interaction between ribavirine and ZDV was confirmed in another study performed on 14 HIV-infected subjects.[122]

It is noteworthy these results are discrepant with *in vitro* data which evidenced an inhibition of the phosphorylation of ZDV[123, 124] and d4T[104] by ribavirin. However it is still unexplained whether these discrepancies are due to a poor ability of *in vitro* models to predict *in vivo* phenomenon or to some methodological drawbacks in the *ex-vivo* quantification of intracellular TP moieties.

Oppositely, ribavirin was found to potentiate *in vitro* the phosphorylation of ddI via the inhibition of inosine 5-monophosphate dehydrogenase.[125] However, despite its potential virologic interest, this interaction is also characterized by a high risk of mitochondrial toxicity[126, 127], so the ribavirin-ddI association is not recommended.

Concerning PIs, the influence of ATV on the plasma and intracellular pharmacokinetics of SQV and RTV was investigated in 9 HIV-infected patients who received the SQR/RTV (1600/100 mg qd) combination with and without ATV (200 mg qd). ATV was found to significantly increase both plasma and intracellular concentrations of SQV by a similar factor of approximately 4, but had no effect on RTV concentrations.[128] Interestingly, the cellular half-life of SQV was unaffected by ATV, which suggested the increase in the intracellular concentration was secondary to the increase in plasma concentration rather to the inhibition of a cellular transporter.

The possible modification of plasma and cellular concentrations of SQV by quercetin, a bioflavonoid displaying inhibitory properties on CYP3A4 and P-gp, was investigated on 10 healthy adults who received SQV alone (1200 mg bid) for 11 days followed by the association SQV/quercetin (1200 mg bid/500 mg bid) during the next three days.[129] If no change was observed for SQV plasma concentration, its intracellular concentration surprisingly decreased by almost 50% when combined with quercetin. However, the important intra and intersubject variability of the intracellular concentrations prevented to draw conclusions from this result.

The accumulation ratio, equal to the cellular concentration divided by the plasma concentration, of some PIs was found to be modified by low doses of RTV in HIV-infected subjects.[79] APV and IDV accumulation ratios in presence of 100 mg or 400 mg of RTV were indeed increased three-fold and five-fold respectively. However, conflicting results were obtained in another study which found that RTV did not increase the accumulation ratio of SQV and IDV.[130]

Despite these discrepencies, assessing the consequences of drug interactions at the cellular level is of great concern in order to validate new combinations. Some recent surprising results, like the possible decrease in the efficacy of HCV therapy due to ABC[131], evidenced the need for a better understanding of drug interactions.

5.3 Relationship between intracellular concentrations and efficacy

Only eight studies were published reporting an analysis of the relationships between intracellular concentrations and virological or immunological efficacy of antiretroviral drugs in HIV patients. Five studied intracellular NRTIs and three PIs. These articles are summarized in table III by molecule from the most recent to the oldest one.[8, 79, 84, 87-89, 122, 132] Of note, five were prospective study and interestingly found a significant relationship between higher intracellular concentrations and virological response.[8, 84, 87, 88, 132] The correlation with plasma concentration was not always studied but was mainly not or less significant. The three studies with non significant results were cross-sectional studies not designed for that purpose.[88, 89, 122] We start as in table II by the results on NRTIs and then on PIs, there is no such study for NNRTIs. With nucleoside analogues, it is important to notice that the relevant determinant of pharmacodynamic response is the ratio between drug triphosphate and endogenous nucleoside triphosphates rather than the absolute intracellular concentration.[133]

The study by Moore et al.,[87] was a substudy of the ACTG 862, a prospective trial where naïve patients were starting a dual NRTI therapy. A significant correlation between change in viral load between week 0 and week 28 and intracellular concentrations was found for 3TC-TP (R^2= 0.62) in the 39 patients receiving either 3TC-ZDV or 3TC-d4T, and for ZDV-TP (R^2= 0.28) in the 10 patients receiving 3TC-ZDV. No significant correlation was found for d4T-TP in the 15 patients receiving 3TC-d4T and no significant relationship was found for any drug when studying change in CD4 cells. The authors did not study the relationships between efficacy

and plasma concentration but showed a very large interpatient variability in the intracellular to plasma concentration ratio. The authors also showed that there was an important increase of intracellular concentration between first dose and week 28 only for 3TC-TP.

The study of Aweeka et al.,[122] was performed in HCV or HBV co-infected HIV patients and its primary objective was to study the influence of ribavirin on ZDV plasma and intracellular concentrations by measuring the AUC in patients before and after introduction of ribavirin. Different regimens were allowed and all patients had to receive ZDV for at least 4 weeks. In the cross-sectional analysis performed in 13 patients before receiving ribavirin, no significant relationships was found between AUC of ZDV-TP and the CD4 cell count measured the same day.

The studies by Anderson et al.,[8] and Fletcher et al.,[84] are substudies of the well known concentration-controlled randomised trial by Fletcher et al.,[134]. In this trial patients received a tritherapy with ZDV, 3TC and IDV either with a fixed dose or with a dose adapted to get trough concentrations in a define range. Intracellular concentrations of ZDV-TP and 3TC-TP were measured 2 h after dose administration at the three pharmacokinetics visits scheduled at weeks 2, 26 and 52 and at variable time post dose at the nine bimontly visits. For each patient the median intracellular concentration from all measurements was considered for the analysis of the link with efficacy. Unfortunately the authors did not analyse intrapatient variability of these concentrations.

In their first study[84], only 8 patients were studied and the efficacy criteria were the change between baseline and week 24 HIV RNA or the percent change between baseline and week 24 CD4 cell count. For ZDV-TP a significant correlation with intracellular concentrations was found both for changes in HIV RNA (R^2=0.54) and CD4 cells (R^2=0.84). For 3TC-TP a significant correlation with intracellular concentrations was found for changes in HIV RNA (R^2=0.79) but not for CD4 cells (P=0.07, R^2=0.44). There was no significant correlation between efficacy and steady-state plasma concentration of these drugs.

In the article by Anderson et al.,[8], 33 patients were studied; the analysis was very thorough with various efficacy endpoints and with several multivariate analyses. After a first simple correlation study, the authors defined thresholds for intracellular concentrations as the first quartiles, which are of 30 fmol/10^6 cells for ZDV-TP and of 7017 fmol/10^6 cells for 3TC-TP. They then studied the impact on several efficacy

endpoints of having a median intracellular concentration below or above those thresholds. The first efficacy endpoint was the time to plasma HIV RNA less than 50 cp/mL, using a survival analysis. For ZDV-TP, the median time to less than 50 cp/mL is significantly reduced in patients with ZDV-TP above the threshold than in patients below as shown.[8] A significant relationship was also found for 3TC-TP but only ZDV-TP remained in the multivariate analysis. The second efficacy endpoints were the virological status (< 50 cp/mL) at week 24 and at week 52 after starting ARVs drug regimen. For ZDV-TP, 92% of patients with concentrations above threshold were undetectable at week 24, this proportion was significantly lower (44%, P=0.009) for patients below the threshold; but this relationship was not significant at week 52. For 3TC-TP, 96% of patients with concentrations above threshold were undetectable at week 24, proportion that was significantly higher than for patients below the threshold (37.5 %, P=0.002); similarly at week 52, 91.7% of patients above threshold were undetectable which is significantly higher than for patients below the threshold (25%, P=0.0008). Another efficacy endpoint was the time to rebound (two HIV RNA greater than 50 cp/mL), this time was shorter for patients with ZDV-TP and 3TC-TP lower threshold; in the multivariate analysis only 3TC-TP remains significant (P=0.009). It is interesting to note that ZDV-TP was mostly associated with the initial viral load decrease whereas 3TC-TP with sustained response. No significant relationship was found between CD4 cell count and intracellular concentrations. However, the authors did not study the change in CD4 cell count but performed cross-sectional analyses looking for correlation at week 24 and week 52 between CD4 cell counts and corresponding intracellular concentrations. Link between plasma concentrations of ZDV and 3TC and efficacy was not studied and no significant relationships were found with IDV concentrations. The authors also found that intracellular concentrations were significantly higher in female than in male: 2.3 folds for ZDV-TP (P<0.0001) and 1.6 fold for 3TC-TP (P<0.0001), whereas no influence of gender was found on plasma concentrations. These results suggest NRTI phosphorylation differences between genders.

The first ever published article that analyzed the link between intracellular concentration and efficacy was the pharmacokinetic trial reported by Stretcher et al.,[132] where ZDV was given five times a day as a single therapy (500 mg/day) in 21 patients followed during 24 weeks. AUC of total ZDV-phosphates (ZDV-P) was evaluated at week 4 and week 24 from five samples. Efficacy was analysed mainly

through CD4 cell count, %CD4 cells and CD4/CD8 ratio. Unfortunately, in the analysis of the correlation of ZDV-P AUC and immunological efficacy, the authors pooled the observations made at week 4 and week 24, not taking into account the correlation induced by the repetition within patients. Here, only results on the analyses performed separately at week 4 and week 24 are reported. At week 4, the authors found a significant correlation of ZDV-P AUC both with %CD4 cells change from baseline (R^2=0.06, P=0.029) and with CD4/CD8 change from baseline (R^2=0.06, P=0.028) but not with the value measured at week 4. These correlations were no longer significant at week 24. No significant correlation was found with plasma AUC.

With respect to studies with PIs, the main objective of the prospective trial reported by Lamotte et al.,[89] was to investigate the concept of a once daily administration of the new galenic formulation soft gel capsule of SQV in association with RTV in PI-experienced HIV patients. The evaluation of the link between SQV intracellular concentrations and virological efficacy in 13 patients was explored as one of the secondary objectives. No significant correlation was obtained between trough SQV intracellular concentration at weeks 2, 4 or 12 and variation of plasma HIV RNA between week 0 and week 12. No significant correlation was found also for plasma SQV concentrations.

The main objective of the prospective study reported by Breilh et al.,[88] was the impact on virological success, defined as HIV less than 50 cp/mL, of intracellular and plasma trough concentrations of LPV in 38 patients receiving LPV/RTV based regimen. They found that trough intracellular concentrations of LPV at week 4 were significantly higher (12.7 μg/mL) in patients achieving virological success before week 4 than in others (4.8 μg/mL) (P<0.0002). Similarly intracellular concentrations of LPV at week 24 were significantly higher (10.5 μg/mL) in patients achieving virological success before week 24 than in others (4.6 μg/mL) (P<0.002).[88] Virological success was also significantly associated with higher plasma through concentration at week 4 ($P=10^{-5}$) and 24 (P=0.05) and with the genotype inhibitory quotient at week 4 ($P=10^{-6}$) and 24 (P=0.0004). In a multivariate analysis of virological success at week 24, the authors found the effect of baseline LPV mutations, plasma concentration at week 4 and intracellular concentration at week 24. The authors defined thresholds of 4 μg/mL and 8 μg/mL for plasma and intracellular concentration, respectively; they suggested combining plasma and

intracellular concentration of LPV for therapeutic drug monitoring. The authors derived cellular accumulation ratio but it was not used in the analysis of link with efficacy.

The cross-sectional study by Chaillou et al.,[79] included 49 patients with antiretroviral combinations containing various protease inhibitors. The first objective was to study the relationship between MDR-1 gene expression and intracellular PI concentrations and then to evaluate the correlation of PI intracellular concentrations with virological response. Efficacy was defined as undetectable HIV RNA load (<40 cp/mL) at day of study. As various PIs were analyzed, to normalize concentrations authors studied the influence of the ratio of intracellular to plasma concentrations that they defined as accumulation but which is not a measure showing the amount of drug in body. They did not found any significant correlation on the main PI. The only parameter significantly linked with efficacy was the intracellular presence of RTV (P=0.04). For the 19 patients receiving RTV as a booster, patients with undetectable HIV viral load had significantly higher RTV intracellular accumulation than patients with detectable HIV RNA (P=0.029).

In conclusion, in prospective studies well designed and with a reasonnable number of patients, all authors found a significant correlation between virological efficacy and intracellular concentrations of NRTIs with no influence of plasma concentration. For PIs, there is only one well-designed prospective trial on LPV, which found both the influence of trough plasma and intracellular concentrations at different week after treatment initiation. From these results, it is difficult to know whether the primary association is with plasma or intracellular concentrations. These findings obtained in only 38 patients should be confirmed by other studies.

5.4 Relationship between intracellular concentrations and toxicity

ARVs are known to produce important adverse effects which are the main drawback of HAART.[135] The toxicity related to ARVs is indeed an important cause of poor compliance, which is in return the main cause of treatment failure.[136] Besides, a viral rebound can be associated to the acquisition of mutation resistances by the virus, which can critically penalize the choice of the subsequent therapeutic strategy.[137-139]

Most toxicities displayed by ARVs are typical of a pharmacological class excepted for NRTIs which trend to have their own toxicities. For instance, PIs are known to induce

digestive troubles and metabolic disorders, such as hyperlipidaemia, insulin resistance, diabetes mellitus, peripheral lipodystrophy, central adiposity[140-143]. NRTIs can be responsible for lipodystrophy[144], neuropathy[145] (ddI, d4T), myopathy[146], pancreatitis[147] (ddI,d4T), anaemia and neutropenia[148],(ZDV), renal impairment and fanconi syndrome[149] (TFV), hepatic steatosis, and lactic acidosis[144]; whereas NNRTIs can provide neuropsychological disorders for EFV[150] and skin or hepatic toxicity for NVP[151-153].

To date, the mechanisms leading to these toxicities are not perfectly understood, but, the main hypotheses highly suggest interferences with some cellular endogenous processes. For example, PIs could alter adipose tissue and lipid metabolism by inhibiting the heterodimeric nuclear receptor complex composed of peroxisome proliferator activated receptor γ and the retinoid X receptor, the cellular retinoic acid-binding protein, and the synthesis of cis-9-retinoic acid.[154] PIs could also inhibit the degradation of the sterol element-binding proteins which regulate the transcription of the LDL receptor gene.[155, 156] Diabetes mellitus induced by PIs could be secondary to the direct inhibition of GLUT4, a transporter that mediates the cellular uptake of glucose stimulated by insulin.[157] Similarly, adverse effects due to NRTIs are thought to be related to michondrial damages, which are a consequence of NRTIs ability to inhibit the mitochondrial DNA polymerase.[158]

Despite these elements, the possible relationship between intracellular concentration of ARVs and their related toxicity was to date investigated for four molecules (ZDV, 3TC, TDF and EFV) only.

First, Stretcher et al.,[132] evaluated in 13 naïve patients if the intracellular concentration of ZDV-P in PBMCs was related to some markers of ZDV-induced toxicity. A negative correlation was found between intracellular ZDV-P and the decrease in haemoglobin from its baseline level. It is noteworthy that the authors did not find in this study a significant correlation between plasma ZDV and intracellular ZDV-P, and did not investigate the possible relationship between plasma ZDV and haemoglobin decrease. However, other studies evidenced an association between plasma ZDV and anaemia[159, 160], so the relative strength of the association between haemoglobin decrease and plasma ZDV compared to intracellular ZDV-P is still unknown. A different result was nevertheless found in both adults and children. Indeed, in a study performed by Anderson et al.,[8] on 33 naïve adult patients receiving a ZDV-3TC-IDV regimen, no difference in the intracellular concentrations of

ZDV-TP and 3TC-TP was observed between the 14 patients who experienced at least a grade I biological event and the 19 patients who did not. A similar result was found in 49 neonates, as the proportion of observed haematological toxicities was not related to the intracellular concentrations of ZDV-TP and 3TC-TP.[99] However, differences between the pharmacokinetic criteria and the pharmacodynamic endpoints used (see table IV[8, 90, 99, 132]) might explain the inconsistency between these studies. There are also *in vitro* data suggesting ZDV-MP is the culprit of ZDV-related anemia[161], so toxicity relationships with ZDV-TP may not be relevant.

Among NNRTIs, in a study performed on 55 patients, Rotger at al.,[90] found a significant correlation between the intracellular concentration of EFV and the risk of mood disorders. No significant correlation was found with the risk of sleep disorders and fatigue. In contrast with other studies[162, 163], no significant association was observed between the plasma concentration of EFV and the neuropsychological trouble that were investigated. Once again, methodological differences penalize the comparison between studies.

Last, Izzedine et al.,[64] found, without measuring the intracellular concentration of TFV-DP, that the risk to develop a renal proximal tubulopathy with TDF-containing treatments was significantly associated with genetic variants in the gene coding for MRP2, a transporter involved in TVF efflux from tubular cells to the urine. Since these variants are thought to be associated with a reduced activity of the transporter, this result could indicate that an accumulation in the tubular cell due to an altered MRP2-based efflux might be responsible for TDF-induced toxicity.[64] However, for obvious reasons, TFV concentration in the tubular cells could not be investigated, so this mechanistic explanation remains speculative, as are the role of mitochondria and TFV-DP in TDF toxicity.

More generally, the impossibility to investigate cellular concentration of ARVs in the tissues targeted by their toxicity is a major weakness of these studies. However, it is interesting to note the neuropsychological effect of efavirenz or the anaemia induced by ZDV are not explained by the diffusion of these compounds to the PBMCs. Significant correlations indicate that concentration in PBMCs possibly reflects the diffusion of these drugs to other tissues like central nervous system or bone morrow. Measuring the concentrations of ARVs in PBMCs could therefore be an interesting tool to predict and consequently to prevent the appearance of toxicities related to HAART in a clinical setting. Further studies are therefore warranted to validate

PBMCs as a reliable model for investigating the relationship between the intracellular concentration of ARVs and their toxicity.

6 Conclusion

In conclusion, intracellular concentrations of ARVs play a major role in their efficacy and toxicity and are influenced by numerous factors. Although measurement of intracellular concentrations needs standardisation, this review demonstrates that relationships between intracellular concentrations of ARVs and their efficacy have been evidenced. Such relationships should be interpreted with caution as intracellular concentrations reflect the total amount of drug within the cell and not the effective unbound fraction. The number of clinical studies in that area is however rather limited, most studies being small and not always adequately designed. Improved techniques measuring relevant intracellular concentrations, improved knowledge on which cells would be the best surrogate marker on antiretroviral therapy including reservoirs as well as larger and prospectively designed clinical studies are needed to further investigate the links between intracellular concentrations and clinical endpoints.

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Tables

Table I. Summary of pharmacokinetics parameters of available antiretroviral drugs adapted from [6, 8-13]

		Bioavailability (%)	t_{max} (h)	Protein Binding (%)	Elimination Pathway	Plasma $t_{1/2}$ (h)	Intracellular $t_{1/2}$ (h)
Entry Inhibitors							
Enfuvirtide	T20	70 (SC)	7	97	Peptidases -> amino acids	3 - 8	
Maraviroc		25-35%	2	76	25% renal + CYP3A	13	
Nucleosidic Reverse Trancriptase Inhibitors (NRTI)							
Zidovudine	ZDV	60	1	20	20% renal + 80% glucuronidation UGT2B7	1 - 1.5	7-11
Didanosine	ddI	40	1	< 5	50% renal	1-2	15 - 20
Stavudine	d4T	80	1	< 5	80% renal	1 - 1.5	7
Lamivudine	3TC	80	1	< 5	80% renal	2-3	22
Abacavir	ABC	75	1	49	< 5% renal + liver biotransformation	0,8 - 1,5	21
Tenofovir	TFV	40	2-3	< 10	80% renal	14	150-180
Emtricitabine	FTC	90	1	< 5	80% renal	9	39
Non Nucleosidic Reverse Trancriptase Inhibitors (NNRTI)							
Efavirenz	EFV	50	2-5	99.5	< 1% renal + CYP2B6	50	
Nevirapine	NVP	90	4	60	< 15% renal + CYP2B6+3A4	25-30	
Integrase Inhibitor							
Raltegravir		ND	3	83	< 5% renal + UGT1A1	9	
Protease Inhibitors (PI)							
Saquinavir	SQV	4-10	1-2	97	< 5% renal + CYP3A	5	
Indinavir	IDV	60	1	60	10% renal + CYP3A	1.5 - 2	2
Ritonavir	RTV	70	3	99	< 5% renal + CYP3A	3-5	
Nelfinavir	NFV	60-80	3	98	<5% renal + CYP3A	5-6	
Lopinavir/r	LPV/r	ND	5	99	< 5% renal + CYP3A	5-6	
Amprenavir	APV	30-90	2	90	< 5% renal + CYP3A	7-12	
Atazanavir	ATV	ND	2	86	< 10% renal + CYP3A	7	
Darunavir	DRV	ND	1-4	94	< 5% renal + CYP3A	10 -15	
Tipranavir	TPV	ND	3	99	< 5% renal + CYP3A	6 (single dose)	

Abbreviations: SC: subcutaneous administration; r = ritonavir low-dose; CYP = P450 cytochrome; UGT=UDP-glucuronosyltransferase

Table II. Intracellular pharmacokinetics of antiretrovirals and relationship with plasma pharmacokinetics

Drug	Dose[a]	Design			Plasma and intracellular pharmacokinetics					Correlation	Ref
		N	Plasma (n / v)	Intracellular (n / v)	Parameters[b]	Plasma	Intracellular	Ratio[c]			
NRTI											
Zidovudine (ZDV)											
	8 mg / kg / day	49	1 / 2	1 / 2	C_{trough}	0.00 – 1.29 µg/mL	16 – 385 fmol/10^6 cells	NR		Yes (R^2 = NR)	[99]
	300 mg bid	14	8 / 1	3 / 3	AUC_{0-12h}	1.8 – 1.7 h.µg/mL	1241 – 2172 h.fmol/10^6 cells	NR		No	[81]
	300 mg bid	15	2 / 2	2 / 2	C_{1h}	0.01 – 0.96 µg/mL	4 – 53 fmol/10^6 cells	9 10^{-6} – 4.2 10^{-3}		NR	[87]
					C_{4h}	0.01 – 1.08 µg/mL	5.5 – 50.7 fmol/10^6 cells	9 10^{-6} – 3.4 10^{-3}		NR	
	300 mg bid	26	-	5 / 1	C_{trough}	-	7.7 – 23.6 fmol/10^6 cells	-		-	[91]
	600 mg qd				C_{trough}	-	6.4 – 12.9 fmol/10^6 cells	-		-	
		27			C_{24h}	-	4.9 – 13.2 fmol/10^6 cells	-		-	
	200 mg tid	38	7 / 2	4 / 2	AUC_{0-8h}						[92]
					Women	0.82 – 1.34 h.µg/mL	240 – 670 h.fmol/10^6 cells	NR		NR	
					Men	0.88 – 1.35 h.µg/mL	520 – 1150 h.fmol/10^6 cells	NR		NR	
	300 mg tid	33	-	2 / 3	C_{trough} – C_{2h}	-	35 – 64 fmol/10^6 cells	-		-	[72]
	300 mg bid	23	3 / 7	3 / 7	AUC_{0-2h}			NR		Yes (R^2 = NR)	[83]
					Week 0	0.99 ± 0.71 h.µg/mL	110 ± 80 h.fmol/10^6 cells				
					Week 12	4.21 ± 1.52 h.µg/mL	130 ± 110 h.fmol/10^6 cells				
					Week 48	1.12 ± 0.41 h.µg/mL	130 ± 120 h.fmol/10^6 cells				
	200 / 300 mg bid[d]	8	1-2 / 12	1-2 / 12	C_{2h} – C_{8h}	0.18 – 0.32 µg/mL	13.8 – 96.4 fmol/10^6 cells	NR		No	[84]
					CL/F	1.16 – 2.53 L/h/kg	NR	-		-	

iv 1 / 2 mg / kg / h[e]	28[f]	>1 / 1	>1 / 1	$C_{delivery}$	0.19 – 3.66 µg/mL	11 – 127 fmol/10^6 cells	NR	No	[93]
				CL/F	0.07 – 0.89 L/h/kg	NR	-	-	
100 mg qd	10	6 / 1	6 / 1	AUC_{0-12h}	0.38 ± 0.13 h.µg/mL	420 ± 420 fmol/10^6 cells	NR	No	[94]
300 mg bid				AUC_{0-12h}	1.22 ± 0.21 h.µg/mL	610 ± 810 fmol/10^6 cells			
500 mg qd	21	6 / 2	6 / 2	AUC_{0-8h}			NR	No	[12]
				Week 4	0.71 ± 0.31 h.µg/mL	3290 ± 970 h.fmol/10^6 cells			
				Week ≥24	0.79 ± 0.41 h.µg/mL	2160 ± 1090 h.fmol/10^6 cells			
500 mg qd	6	6 / 1	6 / 1	AUC_{0-8h}	0.74 ± 0.27 h.µg/mL	4200 ± 2720 h.fmol/10^6 cells	NR	No	[13]
Didanosine (ddI)									
400 mg qd	16	-	4 / 1	$C_{trough} – C_{4h}$	-	3.8 – 13.3 fmol/10^6 cells	-	-	[85]
400 / 250 mg bid	28	1 / 1	1 / 1	$C_{2.5h} – C_{28.5h}$	0.00 – 0.16 µg/mL	0 – 23 fmol/10^6 cells	NR	No	[11]
Stavudine (d4T)									
40 mg bid	19	2 / 2	2 / 2	C_{1h}	0.04 – 1.39 µg/mL	3 – 25 fmol/10^6 cells	$5\ 10^{-6}$ – $6.5\ 10^{-5}$	NR	[87]
				C_{4h}	0.04 – 0.73 µg/mL	3.2 – 18.5 fmol/10^6 cells	$7\ 10^{-6}$ – $2.4\ 10^{-4}$	NR	
40 / 30 mg tid	28	1 / 1	1 / 1	$C_{2.5h} – C_{28.5h}$	0.04 – 0.67 µg/mL	0 – 99 fmol/10^6 cells	NR	Yes (R^2 = 0.46)	[11]
Lamivudine (3TC)									
300 mg qd	15	4 /1	4 / 1	AUC_{0-24h}	3.71 –7.14 h.µg/mL	26910 – 80810 h.fmol/10^6 cells	-		[105]
				C_{max}	1.66 – 2.59 µg/mL	-	-		
				C_{trough}	0.055 – 0.18 µg/Ml	6000 – 11460 fmol/10^6 cells	-		
4 mg / kg / day	49	1 / 2	1 / 2	C_{trough}	0.00 – 1.16 µg/mL	570 – 38900 fmol/10^6 cells	NR	Yes (R^2 = NR)	[99]
150 mg bid	14	8 / 1	3 / 3	AUC_{0-12h}	4.3 – 6.2 h.µg/mL	78.6 – 164.9 h.fmol/10^6 cells	NR	NR	[81]
150 mg bid	41	2 / 2	2 / 2	C_{1h}	0.06 – 1.42 µg/mL	42 – 4579 fmol/10^6 cells	0.01 – 1.17	NR	[87]
				C_{4h}	0.02 – 1.81 µg/mL	200 – 10730 fmol/10^6 cells	0.06 – 41.58	NR	

300 mg qd	25	-	4 / 1	C_{trough}	-	0.7 10^6 – 2.4 10^6 fmol/10^6 cells	-	-	[91]
				C_{24h}	-	1.0 10^6 – 2.5 10^6 fmol/10^6 cells	-	-	
150 mg bid	27			C_{trough}	-	0.7 10^6 – 4.0 10^6 fmol/10^6 cells	-	-	
300 mg qd	13	-	1-2 / 6	C_{trough}	-	7060 – 11600 fmol/10^6 cells	-	-	[82]
150 mg bid	32	-	2 / 3	C_{trough} – C_{2h}	-	7252 – 9313 fmol/10^6 cells	-	-	[72]
150 mg bid[e]	8	1-2 / 12	1-2 / 12	C_{2h} – C_{8h}	0.43 – 0.69 µg/mL	2352 – 13024 fmol/10^6 cells	NR	Yes (R^2 = 0.66)	[84]
				CL/F	0.30 – 0.53 µg/mL	NR	-	-	
Abacavir (ABC)									
300 mg bid	12	4 / 1	4 / 1	AUC_{0-24h}	2.25 – 7.80 h.µg/mL	656 – 2234 h.fmol/10^6 cells	-	-	[105]
				C_{max}	0.95 – 3.72 µg/mL	-	-	-	
				C_{trough}	0.03 – 0.18 µg/mL	98.3 – 472.8 fmol/10^6 cells	-	-	
600 mg qd	8	-	1-2 / 6	C_{trough}	-	88 – 200 fmol/10^6 cells	-	-	[82]
300 mg bid	9	8 / 1	4 / 1	C_{trough}	NQ	NQ	-	-	[86]
				C_{max}	1.48 – 2.35 µg/mL	-	-	-	
600 mg qd	5	-	8 / 1	C_{trough}	-	127 – 575 fmol/10^6 cells	-	-	[101]
				C_{1h}	-	107 – 670 fmol/10^6 cells	-	-	
				C_{12h}	-	188 fmol/10^6 cells	-	-	
				C_{14h} –C_{16h}	-	101 – 548 fmol/10^6 cells	-	-	
				C_{18h}	-	113 – 648 fmol/10^6 cells	-	-	
				C_{20h}	-	80 – 660 fmol/10^6 cells	-	-	
				C_{22h}	-	105 – 201 fmol/10^6 cells	-	-	
				C_{24h}	-	62 – 354 fmol/10^6 cells	-	-	
Tenofovir (TDF)									
300 mg qd	27	4 / 1	4 / 1	AUC_{0-24h}	0.45 – 1.34 h.µg/mL	476.4 – 1386 h.fmol/10^6 cells	-		[105]

				C_{max}	0.19 – 0.45 µg/mL	-	-		
				C_{trough}	0.03 – 0.119 µg/mL	116.5 – 376.5 fmol/10^6 cells	-		
300 mg qd	7	-	1-2 / 6	C_{trough}	-	85 – 110 fmol/10^6 cells	-	-	[82]
300 mg qd	8	-	4 / 1	C_{trough} – C_{4h}	-	129 – 373 fmol/10^6 cells	-	-	[85]
300 mg qd	22	7 / 1	3 / 1	AUC_{0-24h}	0.002 – 0.003 h.µg/mL	NR	NR	No	[95]
				C_{1h}	NR	71 – 130 fmol/10^6 cells	-	-	
				C_{4h}	NR	68 – 130 fmol/10^6 cells	-	-	
				C_{24h}	0.052 – 0.068 h.µg/mL	70 – 123 fmol/10^6 cells	NR	NR	
Emtricitabine (FTC)									
25 mg bid	9	8 / 1	5-2 / 2	C_{1h}	NR	0 – 1125 fmol/10^6 cells	-	-	[10,
				C_{4h}	NR	0 – 1450 fmol/10^6 cells	-	-	100]
200 mg qd	8			C_{1h}	NR	0 – 2125 fmol/10^6 cells	-	-	
				C_{4h}	NR	0 – 2250 fmol/10^6 cells	-	-	
100 mg bid	8			C_{1h}	NR	0 – 4250 fmol/10^6 cells	-	-	
				C_{4h}	NR	0 – 4625 fmol/10^6 cells	-	-	
100 mg qd	8			C_{1h}	NR	0 – 2675 fmol/10^6 cells	-	-	
				C_{4h}	NR	0 – 4000 fmol/10^6 cells	-	-	
200 mg bid	8			C_{1h}	NR	0 – 4000 fmol/10^6 cells	-	-	
				C_{4h}	NR	0 – 4100 fmol/10^6 cells	-	-	
NNRTI									
Efavirenz (EFV)									
600 mg qd	49	1 / 2	1 / 2	C_{12h}					[96]
				Week 4	1.6 – 3.1 µg/mL	2.8 – 11.5 µg/mL	2.2 – 5.4	No	
				Week 24	1.4 – 2.5 µg/mL	3.9 – 8.8 µg/mL	0.5 – 1.8	No	

600 mg qd	10	5 / 1	5 / 1	AUC_{0-24h}	36.8 – 131.9 h.µg/mL	29.8 – 176.9 h.fmol/10^6 cells	0.7 – 3.3	Yes (R^2 = 0.59)	[34]
600 mg qd	55	1 / 1	1 / 1	AUC	7.9 – 63.1 h.µg/mL	6.3 – 794.3 h.µg/mL	NR	Yes (R^2 = 0.24)	[90]
Nevirapine (NVP)									
200 mg bid	10	5 / 1	5 / 1	AUC_{0-12h}	0.05 – 0.09 h.µg/mL	0.05 – 2.9 h.µg/mL	0 – 0.05	Yes (R^2 = 0.62)	[30]
400 mg bid	10	1 / 1	1 / 1	AUC	39.8 – 398.1 h.µg/mL	0.82 – 46.0 h.µg/mL	NR	NR	[90]
PI									
Saquinavir (SQV)									
1600 mg qd (+100 mg RTV)	12	4 / 1	4 / 1	AUC_{0-24h}	5.7 – 39.3 h.µg/mL	24.7 – 114.6 h.µg/mL	1.5 – 6.7	Yes (R^2 = 0.63)	[97]
1600 mg qd (+100 mg RTV)	13	1 / 3	1 / 3	C_{24h}			1.1 – 8.7	Yes (R^2 = 0.31)	[89]
				Week 2	0.04 – 1.43 µg/mL	0.15 – 0.79 µg/mL			
				Week 4	0.04 – 1.97 µg/mL	0.11 – 0.84 µg/mL			
600 mg tid (+100 mg RTV bid)	9	2 / 1	2 / 1	C_{trough}	NR	NR	0 – 50	NR	[79]
				C_{max}	NR	NR	0 – 20	NR	
Indinavir (IDV)									
800 mg tid	10	6 / 1	6 / 1	AUC_{0-8h}	25.1 ± 4.2 h.µg/mL	7.6 ± 1.0 h.µg/mL	NR	No	[9]
				C_{trough}	10.7 ± 1.3 µg/mL	3.2 ± 0.7 µg/mL	NR		
800 mg bid (+100 mg RTV)	19	2 / 1	2 / 1	C_{trough}	NR	NR	0 – 20	NR	[79]
				C_{max}	NR	NR	1 – 5	NR	
400 mg bid (+400 mg RTV)				C_{trough}	NR	NR	2.5 – 75	NR	
				C_{max}	NR	NR	2.5 – 13	NR	
800 mg tid				C_{trough}	NR	NR	0 – 0	NR	
				C_{max}	NR	NR	0 – 7.5	NR	

Ritonavir (RTV)									
100 mg bid	11	14 / 1	4 / 1	AUC_{0-12h}	2.7 – 4.1 h.µg/mL	9.1 – 14.2 h.µg/mL	3.2 – 7.7	NR	[98]
100 mg qd	12	4 / 1	4 / 1	AUC_{0-24h}	1.5 – 14.6 h.µg/mL	3.2 – 13.7 h.µg/mL	0.8 – 4.2	No	[97]
400 / 100 mg bid	22	2 / 1	2 / 1	C_{trough}	NR	NR	0 – 3.1	NR	[79]
Nelfinavir (NVF)									
1250 mg bid	12	5 / 1	5 / 1	AUC_{0-12h}	5.6 – 50.8 h.µg/mL	5.1 – 60.8 h.µg/mL	NR	Yes (R^2 = 0.45)	[78]
				C_{trough}	NR	NR	NR	Yes	
750 mg tid	12	2 / 1	2 / 1	C_{trough}	NR	NR	0 – 37.5	NR	[79]
				C_{max}	NR	NR	0 – 11.3	NR	
Lopinavir (LPV)									
400 / 533 mg bid (+100 / 133 mg RTV)	38	1 / 2	1 / 2	C_{trough} Week 4	0.7 – 5.7 µg/mL	2.9 – 29.0 µg/mL	1.9 – 3.8	Yes (R^2 = 0.72)	[88]
400 mg bid (+100 mg RTV)	11	14 / 1	4 / 1	AUC_{0-12h}	61.8 – 82.8 h.µg/mL	63.1 – 113.8 h.µg/mL	0.7 – 2.1	NR	[98]

[a] All studies are performed at steady state

[b] Values of the pharmacokinetics parameters as published in the original article: range, IQR or mean ± SD

[c] Ratio defined by intracellular/plasma concentration

[d] Individualized regimen after the second visit

[e] Followed by a continuous infusion of 1 mg/kg/h until delivery

[f] Pregnant women

Abbreviations: AUC = area under the concentration time curve; bid = twice a day; C_{max} = maximum concentration; CL/F = clearance; C_{trough} = trough concentration; NNRTI = Non nucleoside analog inhibitors of reverse transcriptase; N = number of patients; n / v = number of samples per visit / number of visits; NRTI = Nucleoside

and nucleotide analog inhibitors of reverse transcriptase; NR = not reported in the article; NQ = not quantifiable; PI = protease inhibitors; qd = once a day; tid = three times a day.

1 **Table III**. Relationships between intracellular concentrations and efficacy of antiretroviral agents in patients
2

Study	Primary objective (yes/no) Type of trial	Intracellular moieties	Dosage regimen	Patients	Studied parameters from intracellular concentrations	Efficacy criterion	Results[a]
NRTI							
Moore et al.[87]	Yes Substudy of clinical trial	3TC-TP ZDV-TP d4T-TP	3TC 150 mg + ZDV 300 mg bid or 3TC 150 mg + d4T 40 mg bid ZDV 300 mg + 3TC 150 mg bid d4T 40 mg + 3TC 150 mg bid	39 naïve 10 naïve 15 naïve	W28: Average of 1h and 4h conc. post dose	Change between W0 and W24 in: (1) Log Plasma HIV RNA (2) CD4 cells	(1) P < 0.02 (2) NS (1) P < 0.02 (2) NS (1) NS (2) NS
Aweeka et al.[122]	No Cross-sectional analysis	ZDV -TP	Any regimen containing ZDV	13 HCV or HBV co-infected patients with stable regimen > 4 weeks	AUC (NCA) from 5 samples: pre dosing and 1,4 ,6 & 8h post dosing	CD4 cell count at time of pharmacokinetic sampling	NS
Anderson et al.[8]	Yes Substudy of clinical trial	ZDV-TP 3TC-TP	ZDV 300 mg bid + 3TC 150 mg bid + IDV 800 mg tid or Concentration-Controlled ZDV-3TC-IDV regimen	33 naïve	Median conc. above threshold (yes/no) from samples 2h post dose at W2, 28 and 56 and at 2 to 8 h post dose at 9 visits from W8 to W80 Thresholds: ZDV-TP: 30 fmol/10^6 3TC-TP: 7017 fmol/10^6	(1) Time to reach less than 50cp/mL of HIV RNA (2) Undetectable HIV RNA (< 50 cp/mL) at W24 and W52 (3) Time to loss of virological response in patients achieving undetectable (4) CD4 level at W24 and W48	(1) P = 0.01 (2) W24: P = 0.009 W52: NS (3) P = 0.02 (4) NS (1) P = 0.02 (2) W24: P = 0.002 W52: P = 0.0008 (3) P = 0.002 (4) NS

Fletcher et al.[84]	Yes Substudy of clinical trial	ZDV-TP 3TC-TP	ZDV 300 mg bid + 3TC 150 mg bid + IDV 800 mg tid or Concentration-Controlled ZDV-3TC-IDV regimen	8 naïve	Median conc. from samples 2h post dose at W2, 28 and 56 and at 2 to 8 h post dose at 9 visits from W8 to W80	Change between W0 and W24 in (1) log HIV RNA (2) CD4 cell	(1) P = 0.03 (2) P = 0.001 (1) P = 0.003 (2) NS
Stretcher et al.[132]	Yes PK trial	ZDV-P	ZDV only: 800 mg /day 100mg every 4h while awake	21 naïve of ZDV	AUC (NCA) from 6 samples: pre dosing and 1, 2, 4, 6 & 8h post dosing at W4 and >W24	Change between W0 and W4 or W0 and W24 of (1) % CD4 (2) CD4 / CD8 ratio	(1) W4: P = 0.029 W24: NS (2) W4: P = 0.028 W24: NS
PI							
Lamotte et al.[89]	No Secondary objective of clinical trial	SQV	SQV1600 mg + RTV 100 mg qd + 2 or 3 NRTI/NNRTI	13 naïve	C_{trough} (24h post dose) at W2, W4 or W12	Change in Plasma HIV RNA between W0 and W12	NS for all dates of trough measurements
Breilh et al.[88]	Yes Observational study	LPV	LPV 400 mg + RTV 100 mg bid + 2 or 3 NRTI/NNRTI	38 naïve of LPV	C_{trough} (12h post dose) at W4 and W24	Virological success: (1) HIV RNA < 50 cp/mL before W4 (2) HIV RNA < 50 cp/mL before W4 and during all follow up until W24	(1) P < 0.0002 (2) P < 0.002
Chaillou et al.[79]	No Secondary objective Cross-sectional trial	NFV, IDV, APV, SQV, RTV	NFV 750 mg tid or IDV 800 mg tid or APV 1200 mg bid or IDV 800 mg + RTV 100 mg bid or SQV 600mg + RTV 100mg bid or APV 600 mg + RTV 100 mg bid or IDV 400 mg + RTV 400 mg bid + 2 or 3 NRTI/NNRTI	49 experienced patients	Ratio of intracellular to plasma C_{trough} and C_{max} (1.5 to 3 h post dose) at day of study: (1) main PI (2) RTV	Undetectable HIV RNA (<40 cp/mL) at day of study	(1) NS for PI ratio (2) P = 0.04 for presence of intracellular RTV P = 0.029 with RTV ratio in 28 patients receiving RTV

[a] Significant results always associated better efficacy with higher intracellular concentrations

Abbreviations*:* APV = amprenavir; AUC = area under the concentration time curve; bid = twice a day; C_{max} = maximum concentration; C_{trough} = trough concentration; d4T = stavudine; d4T-TP = stavudine triphosphate; IDV = indinavir; LPV = lopinavir ; NCA= non compartmental analysis; NFV = nelfinavir; NRTI = Nucleoside and nucleotide analog inhibitors of reverse transcriptase; NNRTI = Non nucleoside analog inhibitors of reverse transcriptase; NS = non-significant ; PI = protease inhibitors; qd = once a day ; PK = pharmacokinetic; RTV = ritonavir ; SQV = saquinavir; tid = three times a day; ZDV = zidovudine; ZDV-P= total zidovudine phosphates; ZDV-TP = zidovudine triphosphate; 3TC = lamivudine ; 3TC -TP= lamivudine triphosphate; W = week.

Table IV. Relationships between intracellular concentrations and toxicity of antiretroviral agents in patients

Study	Primary objective	Intracellular moieties	Dosage regimen	Patients	Studied parameters from intracellular concentrations	Toxicity criterion	Results[a]
NRTI							
Anderson et al.[8]	Yes Substudy of clinical trial	ZDV-TP 3TC-TP	ZDV 300 mg bid + 3TC 150 mg bid + IDV 800 mg tid or Concentration-Controlled ZDV-3TC-IDV regimen	33 naïve	Median conc. above threshold (yes/no) from samples 2h post dose at W2, 28 and 56 and at 2 to 8 hr post dose at 9 visits from W8 to W80 Thresholds: ZDV-TP: 30 fmol/10^6 3TC-TP: 7017 fmol/10^6	Apparition of a grade I laboratory event (hemoglobin, absolute neutrophil count, aspartate, aminotransferase/alanine)	NS
Stretcher et al.[132]	Yes PK trial	ZDV-P	ZDV only: 800 mg / day 100mg every 4 h while awake	21 naïve	AUC (NCA) from 6 samples: pre dosing and 1, 2, 4, 6 & 8h post dosing at W4 and W24	Change between W0 and W4 or W0 and W24 of (1) neutrophils (2) red blood cells (3) hemoglobin	(1) NS (2) NS (3) P = NS
Durand-Gasselin et al.[99]	Yes PK trial	ZDV-TP 3TC-TP	ZDV (8 mg/kg/day in 4 daily doses) ± 3TC(4 mg/kg/day in 2 dayly doses)	49 neonates	Single point concentration (time of sampling not reported)	Proportions of the hematological toxicity grade between neonates with intracellular concentrations above or below the observed median	NS
NNRTI							
Rotger et al.[90]	Yes PK Trial	EFV	EFV+ZDV+3TC EFV+ABC+3TC EFV+d4T+ddI +/- PI (doses not provided)	55	Intracellular AUC obtained by Bayesian estimation (number of samples per patient and sampling times not provided)	Presence of grade I to IV of (1) sleep disorder (2) mood disorder (3) fatigue	(1) NS (2) P = 0.02 (3) NS

[a] Significant results always associated increased risk of toxicity with higher intracellular concentrations

Abbreviations: ABC = abacavir; AUC = area under the concentration time curve; bid = two times a day; tid = three times a day; ddI = didanosine; d4T = stavudine; EFV = efavirenz; IDV = indinavir; PI = protease inhibitors; PK = pharmacokinetic; NCA = non compartmental analysis; NS = non significant; ZDV = zidovudine; ZDV-P= total zidovudine phosphates; ZDV-TP = zidovudine triphosphate; 3TC = lamivudine; 3TC -TP= lamivudine triphosphate; W = week.

1

Figure 1

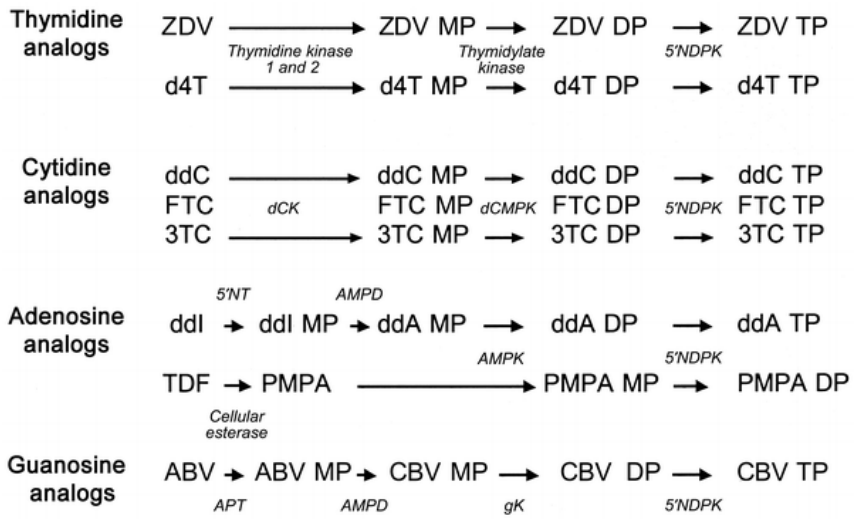
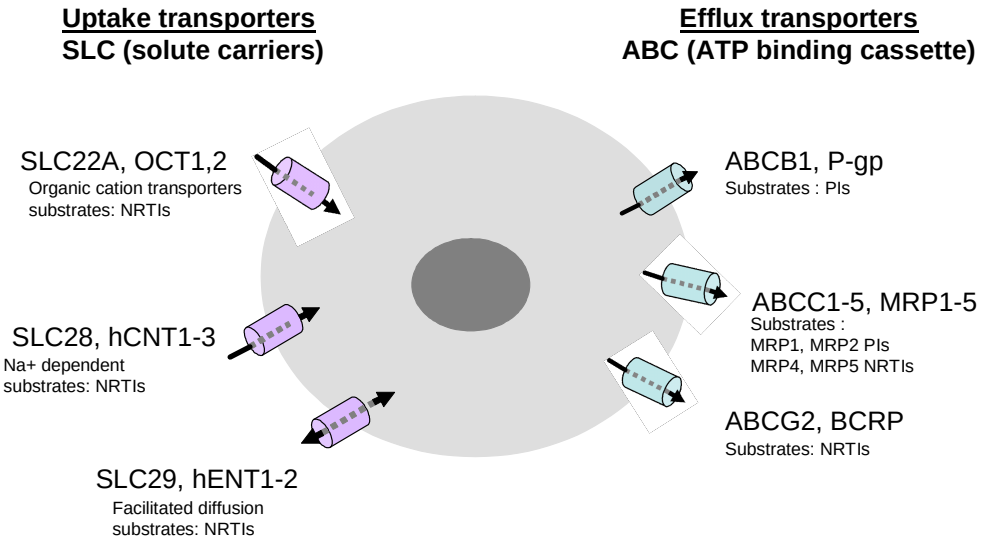

Thymidine analogs
ZDV
ZDV MP
ZDV DP
ZDV TP
Thymidine kinase 1 and 2
Thymidylate kinase
5'NDPK
d4T
d4T MP
d4T DP
d4T TP
Cytidine analogs
ddC
ddC MP
ddC DP
ddC TP
FTC
FTC MP
FTC DP
FTC TP
dCK
dCMPK
5'NDPK
3TC
3TC MP
3TC DP
3TC TP
Adenosine analogs
5'NT
AMPD
ddI
ddI MP
ddA MP
ddA DP
ddA TP
AMPK
5'NDPK
TDF
PMPA
PMPA MP
PMPA DP
Cellular esterase
Guanosine analogs
ABV
ABV MP
CBV MP
CBV DP
CBV TP
APT
AMPD
gK
5'NDPK

Figure 2


Uptake transporters
SLC (solute carriers)
Efflux transporters
ABC (ATP binding cassette)
SLC22A, OCT1,2
Organic cation transporters
substrates: NRTIs
SLC28, hCNT1-3
Na+ dependent
substrates: NRTIs
SLC29, hENT1-2
Facilitated diffusion
substrates: NRTIs
ABCB1, P-gp
Substrates : PIs
ABCC1-5, MRP1-5
Substrates :
MRP1, MRP2 PIs
MRP4, MRP5 NRTIs
ABCG2, BCRP
Substrates: NRTIs