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BIOCHEMISTRY

Intracellular Studies of the Nucleoside Reverse Transcriptase Inhibitor Active Metabolites: a Review

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Nucleoside reverse transcriptase inhibitors (NRTIs) plasma concentrations do not correlate with clinical efficacy or toxicity. These agents need to be phosphorylated to become active against HIV-infection. Thus, the characterization of the NRTIs intracellular metabolite pharmacological parameters will provide a better understanding that could lead to the development of more rational dose regimens in the HIV-infected population. Furthermore, intracellular measurements of NRTIs may provide a better marker with respect to clinical efficacy and toxicity than plasma concentrations. Thus, in this article we review the latest information regarding the intracellular pharmacological parameters of zidovudine (ZDV) and lamivudine (3TC) active metabolites in HIV-infected patients including the results from our recent clinical studies. We will start the discussion with ZDV and 3TC clinical efficacy, followed by systemic pharmacokinetics studies. We will then discuss the *in vitro* and *in vivo* intracellular studies with particular emphasis in the method development to measure these metabolites and we will conclude with the most current data from our clinical trials. *Key words: Nucleoside, HIV, Intracellular, Zidovudine, Lamivudine.*

Nucleoside reverse transcriptase inhibitors (NRTIs) is one of the three groups of drugs used for the treatment of HIV disease, being protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) the other two groups. The mode of action of NRTIs is to inhibit HIV reverse transcriptase enzyme through their incorporation into the viral DNA, acting as a chain terminator (1). At present, there are six NRTIs approved by the US Food and Drug Administration (zidovudine (ZDV), lamivudine (3TC), didanosine (ddI), stavudine (d4T), zalcitabine (ddC), and abacavir (ABC), although *in vivo* intracellular information had been obtain mainly for ZDV and 3TC. Thus, we will limit our discussion to these two antiretroviral agents.

Zidovudine was the first drug to show clinical efficacy in the treatment of adults and children with AIDS or AIDS-related complex (2-7). In controlled trials, ZDV has shown clinical improvements resulting in a decrease of opportunistic infections and progress in neuropsychological outcomes in the adult and pediatric populations (4, 8-10). However, toxicity events such as anemia and neutropenia, were associated with the use of the medication (11). Despite the fact that ZDV toxicity to hematopietic progenitor cells has been reported to be dose related (5, 12), there was no indication that the toxicity was directly related to plasma concentrations of ZDV (13). Another report linked the occurrence of opportunistic infections in adults with ZDV exposure determined as the area under the concentration-time curve (AUC) (14), but subsequent studies showed no evidence on the relationship (15, 16).

Lamivudine (3TC) monotherapy showed improvements of surrogate markers in antiretroviral drug-naïve and -experienced patients with asymptomatic or symptomatic HIV infection (17-19). However, these improvements were transient (only observed during the first four weeks of treatment), due to rapid emergence of high level resistance of HIV to 3TC (18). In addition, there was no evidence of a correlation between surrogate marker responses and dosage of 3TC (18). The combination regimens between

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3TC and ZDV have shown to increase and sustain mean CD4+ cell counts and reduce HIV RNA levels to below 400 copies/mL (20-23). The combination of 3TC+ZDV has also resulted in reductions of mortality and disease progression in drug-naïve and -experienced patients (24, 25). Toxicity events with 3TC are less frequent than for other NRTIs, since it has little activity against mammalian DNA polymerase γ and is not incorporated into mammalian mitochondrial DNA (26, 27). Nevertheless, it is advisable that children who are antiretroviral drug-experienced and with risk factors for pancreatitis must be monitored closely (19).

Systemic Pharmacokinetics Studies

Zidovudine is the only NRTI that undergoes a significant first pass effect via hepatic glucurodination (28-30). Its oral bioavailability was measured to be approximately 60% (31). ZDV pharmacokinetics studies have shown that the drug is rapidly eliminated with an apparent terminal half-life ($t_{1/2}$) of approximately 1.0 to 1.5 hr (31-35). Total clearance was determined to be 1.3 L/h/kg, by both renal excretion and hepatic metabolism of ZDV to 5'-O-glucuronyl zidovudine. ZDV crosses efficiently the blood brain barrier as well as the human placental tissue (36, 37).

Lamivudine is well adsorbed and has good systemic bioavailability in adults (82%) and children (68%) (19, 38). Its apparent systemic $t_{1/2}$ in adults has been reported to range from 2.5 hr in patients receiving single oral doses between 0.25 and 8.0 mg/kg (38), 8.5 hr after a single 100 mg dose (39), and 11.5 hr after a single 300 mg dose (40). In children, $t_{1/2}$ was shorter than in adults, ranging from 1.6 to 1.8 hr after single oral doses between 0.5 and 10 mg/kg (19). The mean volume of distribution is 1.3 L/kg, indicates that the drug is well distributed into total body fluid (38). 3TC penetrates the CSF, although its CSF:serum ratio is lower than for other NRTIs (18).

Although a wealth of information is available regarding systemic PK parameters for ZDV and 3TC, the effectiveness or toxicity of these agents cannot be predicted with these parameters. ZDV and 3TC, as well as the other NRTIs, need to be activated to their triphosphate active anabolites (ZDV-TP and 3TC-TP) before incorporation into the target DNA (1, 41). Thus, the activity of ZDV and 3TC does not depend only on the systemic PK parameters, but also on the rate and extent of intracellular phosphorylation to form the triphosphate moieties (42, 43). Considering the possibility of inter-patient variability in the enzymatic activation of ZDV and 3TC, it is likely that plasma drug concentrations will have little value in relation to antiviral activity. Thus, the detailed evaluation

of the intracellular anabolites of ZDV and 3TC might provide a better understanding of the pharmacological properties of these drugs.

In vitro intracellular studies

In vitro studies have shed light on the intracellular biochemical pharmacology of these agents. ZDV has been studied extensively, since it was the first drug to be used against HIV infection. Intracellular levels of ZDV have been measured using [^{3}H]ZDV with appropriate cell lines for pre-determined periods of time (11, 44-46). Results from these studies indicate that the phosphorylation is highly dependent on the cell line (44), with values of total phosphorylated ZDV concentrations ranging from 0.10 μM in CEM cells (46) up to 5,700 μM in H9 cells (45). It was also established that ZDV is first phosphorylated by thymidine kinase (TK) to ZDV-monophosphate (ZDV-MP) with the same efficiency as thymidine. However, the next step of conversion, from ZDV-MP to ZDV-diphosphate (ZDV-DP), is very inefficient in human lymphocytic cells (41, 44). It is evident that the enzyme thymidylate kinase (TDK) phosphorylates ZDV-MP to a very small extent, and as a consequence there is a high accumulation of this species. It has been suggested that high concentrations of ZDV-MP inhibit 3' to 5' exonuclease activities increasing the steady-state levels of ZDV in the host DNA (47), providing an additional toxicity mechanism. The small amounts of ZDV-DP are then converted to ZDV-TP by nucleoside diphosphate kinases (NDPK), accounting ZDV-TP for approximately 5% of the total phosphorylated species. ZDV-TP half-life has been reported to be between 2.5 hr in stimulated peripheral blood mononuclear cells (PBMCs) (48), 4 hr in human lymphoid cells (49), and 10 hours in CEM$_{ss}$ cells (46). The formation of ZDV-TP is dependent on the state of the cells, producing 10 to 17-fold higher concentrations in activated cells than in resting cells (50, 51).

Lamivudine is a cytidine analogue phosphorylated by deoxycytidine kinase (dCK) to form 3TC-MP. Cytidine monophosphate kinase (CMK) and NDPK catalyze the sequential phosphorylation to 3TC-DP and 3TC-TP, respectively. The amount of 3TC-TP in activated PBMCs accounts for approximately 40% of total phosphorylated species (52, 53). The rate-limiting step is the formation of 3TC-TP from 3TC-DP. 3TC-TP has an estimated in vitro half-life of 10.5 to 15.5 hr in HIV-infected PBMCs (52, 53). The phosphorylation of 3TC is efficient in both quiescent and activated cells (51).

Although in vitro studies have provided an insightful view of the pharmacological processes of ZDV, d4T, and

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3TC, extrapolation to *in vivo* systems does not necessarily correlate. Thus, *in vivo* studies would provide a better understanding of the pharmacological parameters of intracellular anabolites (ZDV-TP and 3TC-TP). Recent studies have shown that the intracellular concentrations of the triphosphate anabolites of the nucleoside reverse transcriptase inhibitors (NRTIs) correlate better with virologic responses than plasma levels of the parent compound (54, 55).

In vivo Intracellular Studies

Method development. In recent years, the in vivo quantitation of ZDV-TP, 3TC-TP, and other NRTI-triphosphates has been a high priority in clinical pharmacology. However, one of the major problems in measuring intracellular triphosphate metabolites is due to the small amounts present in patients (low $fmol/10^6$ cells). Thus, selective and sensitive analytical methodologies need to be developed in order to measure these small quantities without drawing large amounts of blood from patients. Furthermore, the method should be one that can measure several NRTI-triphosphates simultaneously because of the present clinical strategy of using triple combination therapy (two NRTIs and one PI).

Several studies have been described in the literature for the in vivo quantitation of ZDV-TP without the use of radiolabeled drugs. The first of these studies employed a high performance liquid chromatograph (HPLC) system programmed with a column switching technique (56). They used 15 mL of blood sample for their determinations, and reported limits of detection of 3.3 pmol/injection. The ZDV-TP concentrations observed in the two HIV-infected patients they studied ranged from 5,600 to 13,000 fmol/10^6 cells. Although this study was successful in the *in vivo* determination of ZDV-TP, the use of multidimensional HPLC made the approach unattractive for routine determinations. In addition, the amount of ZDV-TP reported for HIV-infected patients was 1 to 2 orders of magnitude higher than any of the other methods reported recently. Thus, no further studies have been reported using this methodology in clinical trials.

ZDV-TP has also been measured by the combination of HPLC and radioimmunoassay (RIA), in which limits of detection were reported as low as 50 fmol/10^6 cells (57-59). However, 20 to 50 mL of blood were drawn from patients to reach this high sensitivity. The major drawback of this methodology is that the assay is cumbersome, since it requires collection and purification of ZDV anabolites from cell extracts through HPLC, treatment of extracts with alkaline phosphatase, and quantitation by RIA. Furthermore, the assay must be calibrated daily with

radiolabeled ZDV anabolites to verify the retention time for each of the species of interest, since inter-day variation in retention times could be lead to incorrect collection of anabolites. In addition, HPLC separations used in this method are very time consuming (> 50 min per sample), and cannot be implemented in routine analyses for large clinical trials. Furthermore, calibration curves prepared for the quantitation of ZDV-TP were made with standard solutions of ZDV and not standard solutions of ZDV-TP.

An alternative enzymatic assay has been reported, in which ZDV-TP was measured from PBMCs using the inhibition of HIV type 1 reverse transcriptase activity and poly (rA)-oligo(dT)$_{12-18}$ as primer template (60). The limit of quantitation (LOQ) for this method using 10 to 20 mL of blood sample was 20 to 50 fmol/10^6 cells. In this method, contrary to the previous two, standard solutions of ZDV-TP were used to prepare a calibration curve. Despite the excellent LOQ, this bioassay is limited by the inability to differentiate ZDV-TP from endogenous nucleotides, and the need to obtain patient ZDV-free PBMC extracts to produce the standard curve. Another drawback of the method is that no other phosphorylated anabolite (ZDV-MP, ZDV-DP) can be determined.

Recently, a new approach was developed in which Anion-Exchange-Solid Phase Extraction (AX-SPE) separated ZDV anabolites (ZDV-MP, ZDV-DP, ZDV-TP), followed by enzyme digestion, and RIA as the method of detection (61, 62). The change in the separation step increased the throughput, since several samples could be processed simultaneously. This method accomplished an excellent LOQ of 80 fmol/10^6 cells. A similar approach was employed by the same group to determine intracellular levels of 3TC-TP (63). The limit of quantitation (LOQ) for intracellular 3TC-TP was 212 fmol/10^6 cells, and only 4 million cells were used for the analysis. The combination of both methods was used to measure ZDV-TP and 3TC-TP in HIV-infected subjects. A total of 24 mL of blood was collected to perform both assays. The amount of blood required for this assay limits its implementation in the pediatric population. Another limitation of the method is that an internal standard in the quantitation process is not used, assuming complete recovery (61, 63). In addition, they need to split the sample to perform separate analyses for ZDV-TP and 3TC-TP.

Another approach has been proposed to measure intracellular 3TC metabolites using a combination of AX-SPE and HPLC with ultraviolet detection (HPLC-UV) (64). The use of UV detection is possible with 3TC metabolites (3TC-MP, 3TC-DP, 3TC-TP) because of the large amounts (pmol/10^6 cells instead of fmol/10^6 cells) formed *in vivo*. However, as well as the aforementioned methods, no internal standard was used with this

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methodology. In addition, this method can only be used for 3TC, since no other NRTI produces the large amounts of intracellular metabolites made by 3TC.

During this year, our group described a methodology combining an AX-SPE procedure with HPLC-MS/MS to measure intracellular ZDV-MP and ZDV-TP in PBMC using azidodeoxyuridine (AZdU) as the internal standard (65). The LOQ for this methodology was 20.0 fmol/10^6 cells with a linear concentration range of four orders of magnitude (4.0 to 10000 fmol/10^6 cells). Figure 1 shows the ion chromatograms for 3TC (from 3TC-TP), ZDV (from ZDV-TP) and AZdU (internal standard) obtained from seronegative volunteer's PBMCs incubated with 5 µM 3TC + 5 µM ZDV. A strong signal is obtained from both compounds without background interference from endogenous nucleotides. PBMCs without any drug were analyzed as negative controls and no signal was obtained in the HPLC-MS/MS system. An excellent chromatographic resolution was obtained between ZDV and 3TC with a run time of *only 6 minutes*. This short chromatographic run increases the throughput of the sample analysis.

Figures 2 and 3 show the calibration curves for ZDV-TP and 3TC-TP respectively with outstanding correlation coefficients. Contrary to other methodologies where the calibration curves were made from the parent compounds (ZDV or 3TC), ZDV-TP and 3TC-TP standard solutions processed as the samples were used to produce the calibration curves. Furthermore, we have shown that the methodology could be used not only for *in vitro* studies but also for the determination of ZDV-TP in HIV-infected patients. Table 1 shows the results from six patients in our first clinical trial. Blood samples were drawn at different time points depending on the availability of the patients in the clinic. The values shown in the Table 1 are similar to those reported previously using other methodologies. In addition, the inter-patient variability in our population is similar to other populations. The highest ZDV-TP concentration was observed for patient 1 (193 fmol/10^6 cells), who was sampled 2 hr after drug administration. This ZDV-TP concentration is similar to that reported by Fletcher et al. for patients that had an increase in the percent change of CD4+ cells from baseline (66). The only other patient that was sampled before 6 hr had also ZDV-TP concentrations above 100 fmol/10^6 cells (Patient 2 = 137 fmol/10^6 cells). All other patients were sampled after 10 hr of drug administration and had measurable ZDV-TP intracellular concentrations.

Clinical trials. Limited clinical studies have measured intracellular metabolites of NRTIs over a time course (13, 62, 67, 68). These studies have demonstrated that plasma NRTIs concentrations do not correlate with intracellular
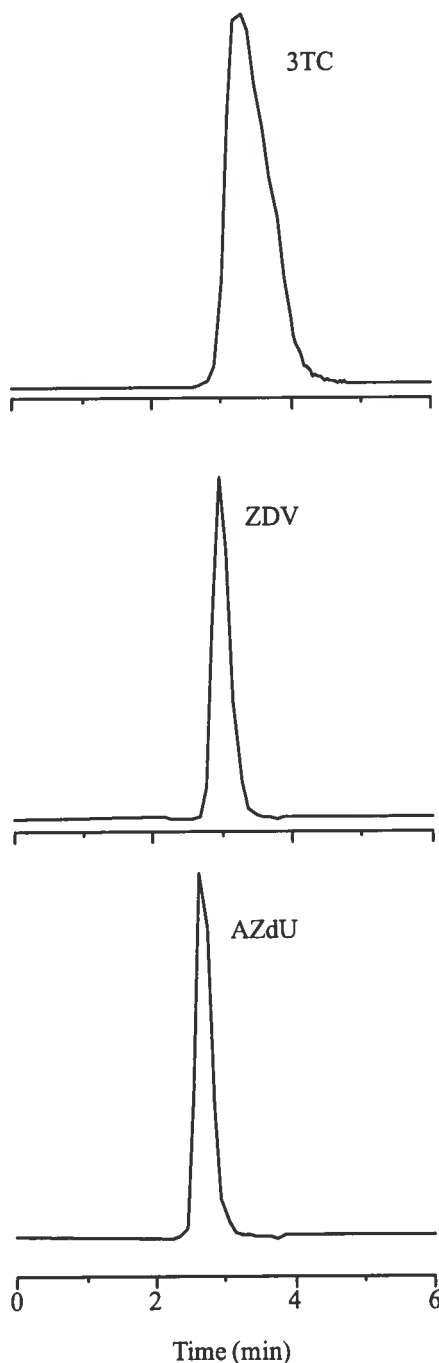

Figure 1. Chromatograms for 10^7 PBMCs incubated with 5 µM 3TC + µM ZDV for 24 hr. The top chromatogram corresponds to the signal for the amount of 3TC-TP formed during the incubation process. The middle chromatogram corresponds to the signal for the amount of intracellular ZDV-TP formed during the incubation process. The bottom chromatogram corresponds the internal standard (AZdU) added during the sample processing. Retention times for 3TC, ZDV, and AZdU were 3.3, 3.0, and 2.2 min., respectively.
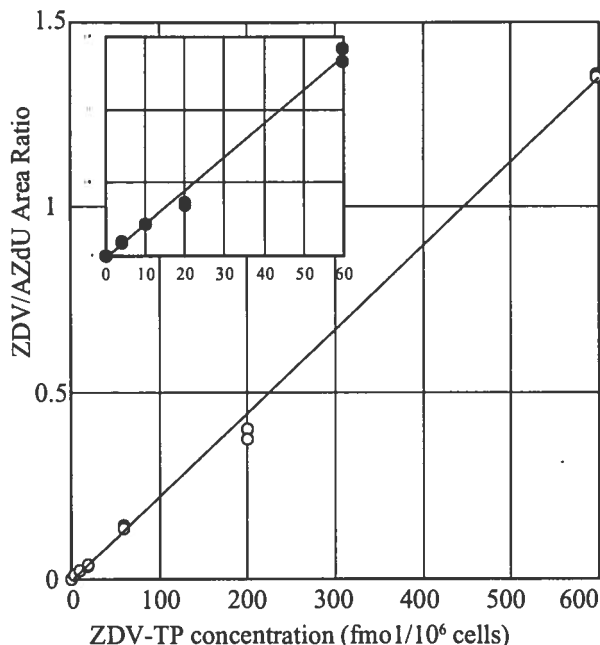
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Figure 2. Calibration standard curve for ZDV-TP using standard solutions (2, 5, 10, 50, 100 and 500 ng/mL) processed as the samples throughout the complete procedure. Each point is the average of two determininations.

nucleotide concentrations. These findings emphasize the importance of determining intracellular NRTIs pharmacokinetic parameters at the cellular level. Fridland et al. measured ZDV-TP in five patients receiving 100
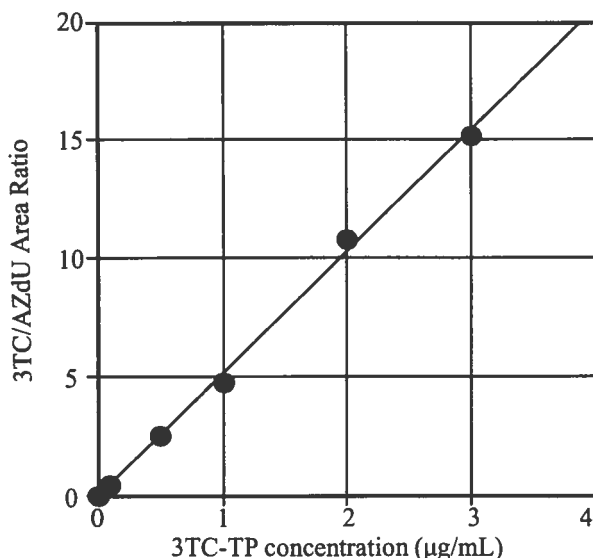

Figure 3. Calibration standard curve for 3TC-TP form standard solutions (0.05, 0.10, 0.5, 1.0 and 3.0 μg/mL) processed as the samples throughout the complete procedure. Each point is the average of two determinations.

Table 1. ZDV-TP intracellular concentration from HIV-infected patients*

Patient #	Age (yrs)	Gender	Time elapsed between drug administration and blood sampling (hr)	ZDV-TP intracellular concentration (fmol/10^6 cells)
1	35	M	2	193–4
2	52	F	4	137–1
3	39	F	11	42–1
4	38	M	11	83–16
5	43	M	12	124–4
6	66	F	15	63–4

* Patients were receiving the standard 300 mg ZDV dose b.i.d.

mg of ZDV and six patients with a dose of 500 mg ZDV (60). They found that the median intracellular ZDV-TP levels ranged from 5 to 57 fmol/10^6 cells and 42 to 92 fmol/10^6 cells for patients receiving 100 and 500 mg ZDV, respectively. They determined that intracellular ZDV-TP levels increased to a plateau by 2 hr and remained constant up to 6 hr. In a follow up study, Rodman et al. proposed a pharmacokinetic model for the systemic and cellular disposition of ZDV in HIV-infected patients (62). They described first order models that were sufficient to model ZDV-MP kinetics in PBMCs. Modeling of ZDV-TP was not feasible because sample concentrations were almost at the limit of detection of the method and were not reliable. The only conclusion that could be reached regarding ZDV-TP was that the levels were maintained constant during the time of sampling (from 1 to 6 hr).

Fletcher et al. used the enzymatic method to measure ZDV-TP concentrations in a clinical trial where ZDV therapy was concentration-controlled (66). They showed that ZDV-TP concentrations were higher (160 fmol/10^6 cells) with concentration-controlled than with standard therapy (92 fmol/10^6 cells). Furthermore, the concentration-controlled regimen increased CD4+ cell counts by 22%, compared to a 7% decrease in patients with standard therapy. This was the first evidence showing a direct correlation between intracellular ZDV-TP concentrations and drug efficacy. More recently, Rodman in collaboration with our group through the AIDS Clinical Trials Group (ACTG) studied the sysmatic pharmacokinetics and cellular pharmacology in HIV-1 infected women and newborn infants (69). We found that the substantial amount of ZDV-TP in cord-blood explained the clinical success of ZDV in reducing vertical transmission.

Barry et al. studied the intracellular metabolism of ZDV-MP, ZDV-DP, and ZDV-TP in seronegative volunteers

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and HIV-infected patients (13). They found that the total intracellular ZDV phosphorylation was higher in HIV-infected patients than in healthy volunteers. The main difference between the groups was that HIV-infected patients produced more ZDV-MP than the volunteers. ZDV-TP concentrations were similar for both groups. They also found a substantial increase of ZDV-MP in patients with CD4+ counts less than 200 cells/mm³. It was suggested that this might explain the increased toxicity found in this patient population, since ZDV-MP is associated with toxicity (47). The same group studied the effect of ZDV dose on the intracellular metabolite formation (67). They demonstrated that with a lower dose (100 mg three times daily), ZDV-MP concentrations were significantly lower than with a higher dose (300 mg twice daily). ZDV-TP remained constant at similar concentrations in both regimens. Thus, they proposed the use of ZDV 100 mg (3 times a day) instead of 300 mg (twice daily).

Peter et al. investigated the effect of long term administration of ZDV on its intracellular phosphorylation in HIV-infected patients (68). They divided their patients in two groups one that received long-term therapy (>18 months) and the other with short-term therapy (<2 months). They concluded that there were no differences between both groups in the phosphorylation process of ZDV. None of the above clinical studies found a direct relationship between plasma ZDV concentrations and ZDV-TP intracellular levels.

There are only two clinical studies that have measured 3TC-TP over a time course (70, 71). In the first study, Moore et al. found that intracellular pharmacokinetics of 3TC-TP was independent of the dose (150 vs. 300 mg twice daily), although the higher dosage tended to produce higher concentrations of 3TC-TP (70). They reported a $t_{1/2}$ of approximately 15 to 16 hr for 3TC-TP. Zhou et al. reported the intracellular pharmacokinetics of 3TC-MP and 3TC-TP in 21 HIV-infected patients taking 150 mg of 3TC twice a day (71). They found that 3TC-TP AUC_{0-10} was approximately twice as high as that of 3TC-MP. In these two studies, no direct correlation could be established between plasma 3TC concentrations and intracellular levels of its phosphates.

Our group just finished an intensive pharmacokinetic study to determine the concurrent intracellular pharmacokinetic parameters of ZDV-TP and 3TC-TP in HIV-infected patients. We collected blood samples prior to (0) and 1, 2, 4, 8, 12, 16, and 24 hr after last dose. Figures 4 and 5 show for the first time the concurrent PK profile for ZDV-TP and 3TC-TP from 5 HIV-infected patients. It is evident that the amount of 3TC-TP produced in these patients is substantially higher than ZDV-TP.
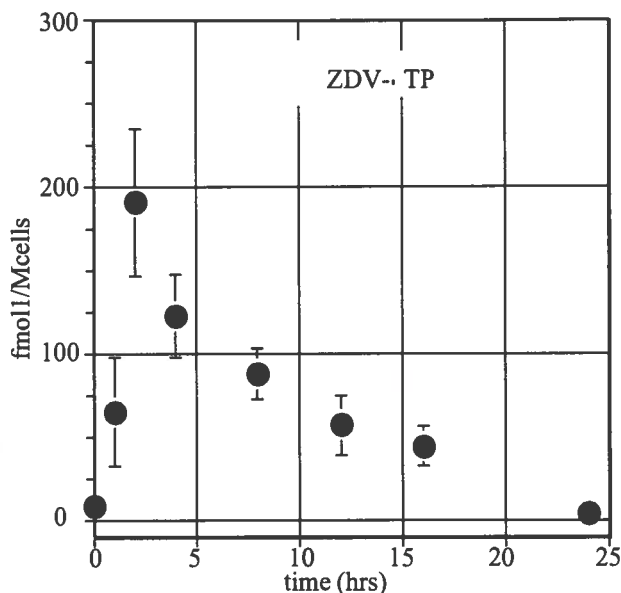

Figure 4. Intracellular pharmacokinetic profile for ZDV-TP. Values represent the mean of 5 HIV-infected patients and error bars the standard errors. Patients stayed overnight in the UPR-CRC facilities and samples were collected at 0, 1, 2, 4, 8, 12, 16 and 24 hr postdose.

These results are in accordance with previous studies. We calculate the apparent elimination constants for ZDV-TP and 3TC-TP using a one-compartment model. From this analysis, we determined that the median half-life for 3TC-
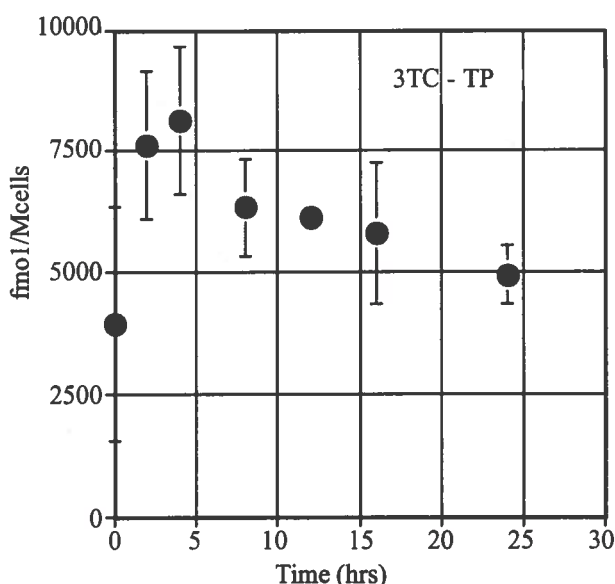

Figure 5. Intracellular pharmacokinetic profile for 3TC-TP. Values represent the mean of 5 HIV-infected patients and error bars the standard errors. Patients stayed overnight in the UPR-CRC facilities and samples were collected at 0, 1, 2, 4, 8, 12, 16 and 24 hr postdose.

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TP is longer than ZDV-TP for these HIV-infected patients by a factor of three (25 hr vs. 7 hr, respectively). The long intracellular half-life and high concentrations for 3TC-TP suggest that this anabolite could be evaluated as a biological marker for compliance. We are currently performing such clinical study. Furthermore, we are performing studies to correlate intracellular NRTI-triphosphate concentrations with clinical efficacy.

Resumen

Las concentraciones plasmáticas de los análogos de nucleosidos que son inhibidorees de la transcirptasa al reverso (NRTIs, por sus siglas en inglés) no correlacionan con la eficacia clínica ni con la toxicidad de estas drogas. Estos agentes necesitan fosforilarse para ser activos en contra de la infección del virus de immunoeficiencia adquirida (VIH). Por lo tanto, la caracterización de los parámetros farmacológicos de los metabolitos intracelulares de estas drogas ayudará al desarrollo de regímenes más racionales para la población infectada con VIH. Además, las medidas intracelulares de los NRTIs pueden ser un mejor marcador de eficacia y toxicidad que las concentraciones sistémicas. En este artículo se resume la información más reciente relacionada a los parámetros farmacológicos intracelulares de los metabolitos activos de zidovudina (ZDV) y lamivudina (3TC) en pacientes infectados con VIH, incluyendo los resultados más recientes de nuestros estudios clínicos. Se discute la eficacia clínica de ZDV y 3TC, seguido por los estudios farmacocinéticos sistémicos y luego los estudios intracelulares *in vitro* e *in vivo,* con un énfasis particular en el desarrollo de la metodología para medir los metabolitos. Finalmente presentamos los resultados de los estudios clínicos mas recientes.

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