Estimation of efficacy of HIV nucleoside-analogue reverse transcriptase inhibitor (AZT) via stochastic modeling

Samira Khalili, James M. Monaco, and Antonios Armaou†, *Senior Member IEEE & Member AIChE, SIAM*

***Abstract*— In this work, the mechanisms by which nucleoside-analogue reverse transcriptase inhibitors (NRTIs), the most common class of drugs used in the treatment of HIV-1, exert their antiviral effects are analyzed and methods in which those known mechanisms could be employed to generate mathematical models for drug efficacy in terms of measurable physical values are identified. Drug concentration is considered as a time variant parameter which depends on the drug administration time and dosage.**

I. Introduction

In order to describe the dynamics of the HIV infection, causative agent of AIDS, numerous mathematical models of varying detail have been proposed in the open literature to capture different aspects of disease progression. Currently, physically informed mathematical models have been developed for many aspects of the HIV reproductive cycle and the majority include drug efficacy as time-invariant constants [1], [2], [3], [4]. Such mathematical models have been employed to control disease progression and optimize medication schedules [5], [3]. In [6], a model predictive control based method for determining optimal treatment interruption of HAART was developed to schedule HIV therapy. In [7] and [8], optimal medication strategies were scheduled for the primary stage of infection. The role of mathematical modeling on the optimal control of HIV-1 pathogenesis was reviewed in [9].

Studies such as [10], [11] use broad empirical models, as summarized by Greco, et. al. in [12]. These models estimate the correlation between drug concentration and efficacy. However, no models have attempted to reflect the class-specific mechanisms by which the major types of antiretroviral drugs exert their inhibitory effect. Nucleoside analogue RT inhibitors (NRTIs) are prodrugs and they must undergo three enzyme-catalyzed phosphorylations before reaching their active anabolite form. In the next step, the drugs must compete with natural deoxynucleotides to exert their effects. This prevents easy correlation of plasma concentrations with instantaneous efficacy. Still, there are techniques for quickly and directly measuring the intracellular concentration of NRTI triphosphates and natural dNTPs [13], [14], [15], [16].

S. Khalili and A. Armaou are with the Department of Chemical Engineering, Pennsylvania State University, University Park, PA-16802

†Corresponding author, email: armaou@psu.edu. The author gratefully acknowledges the financial support of the National Science Foundation Career Award # CBET 06-44519 and American Chemical Society, Petroleum Research Fund, grant PRF # 44300-G9.

In this study, a mechanistically informed model for the intracellular interaction of HIV-1 and NRTIs was developed which provides necessary tools to more accurately simulate the progression of the HIV infection and its response to treatment. The detailed explanation of the model and more results can be find in [17]. The development of this type of mechanistic model can help guide future experimental investigations by highlighting the key parameters that ultimately determine the drug's efficacy. The efficacy was linked with time-varying RTI triphosphate concentration from physiologic data on intracellular triphosphate concentration maximums and half lives, such as that collected in [15].

II. Reverse transcription process

The reverse transcription of viral RNA to DNA initiates after attachment of virus to host and fusion of viral components into the host cytoplasm. The process of reverse transcription is not linear; up to three strands, a negative sense strand and two halves of the positive sense strand, may be transcribed at once. The process, as described in detail in [18] and shown in figure 1, includes these steps: (1) Host-provided tRNA binds to the primer binding site (PBS) and (-) strand transcription begins. (2) Upon reaching the 5' end of the RNA template, the (-) strand is transferred to the 3' end. (3) Initiation of the U(+) and D(+) strands begins as the (-) strand reaches the two purine-rich primer sites. (4) After the U(+) and the (-) strand reach the the 5' end of their respective templates, another strand transfer occurs.This allows each to use the other as a template and continue transcription. (5) The ring structure is opened as the (-) strand continues to the 5' end of the U(+) strand and the D(+) strand continues to the 5' end of the (-) strand. (6) Transcription is complete once the U(-) strand displaces 100 bases of the D(+) strand to reach the central termination site (CTS).

After approximately 20000 reverse-transcriptase mediated nucleotide polymerization events, the complete double-stranded copy of viral DNA which is called a provirus is ready for being integrated into the host genome. During those 20000 nucleotide additions, NRTIs can exert their desired effects.

NRTIs compete with natural nucleotides for addition into the HIV reverse transcription complex. They inhibit proviral production through chain termination. However, addition of an NRTI molecule to the growing HIV genome does not guarantee permanent chain termination in the intracellular environment. HIV reverse transcriptase enzyme can also

ISBN 978-963-311-370-7

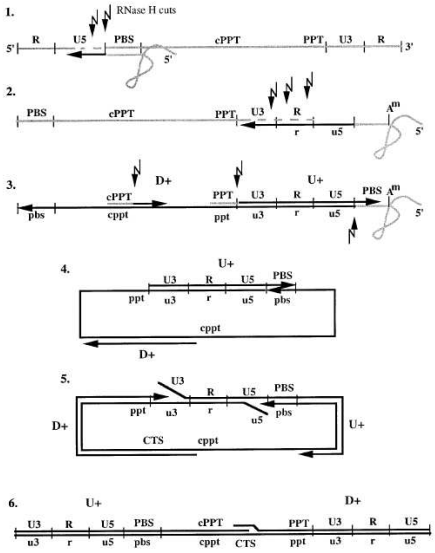

Fig. 1. An outline of critical reverse transcription events. Figure reproduced from (Gotte and Li, 1999).

catalyze the reverse reaction which results in the removal of the terminal nucleotide.

A. *Efficacy: measurement of drug effectiveness*

There are several measures used to determine the efficacy of therapy: changes in T-cell counts, log-reductions in viral titers, and IC_{50} [11], [12], [13]. Here, the concept of efficacy outlined by Perelson et al. [1] is used. In this definition, the efficacy of an NRTI is the percentage by which it reduces the apparent rate of conversion of healthy T-cells to infected T-cells over the untreated case. This definition correlates most closely with drug IC_{50}, which is defined as the plasma concentration of drug that results in a 50% reduction in the appearance of infected cells over a set incubation period in an *in vitro* culture.

Addition of NRTI triphosphate occurs by the same mechanism as addition of deoxynucleotides. Hence, there are two possible "reactions" in the system: natural nucleotide addition and NRTI addition. A good first step in determining efficacy would be to quantify the average probability that an NRTI triphosphate is added at each "vulnerable" nucleotide in the genome. It is expected that this probability to be dependent upon three factors: the intracellular concentration of NRTI triphosphate, the concentration of the deoxynucleotide with which it competes, and the relative selectivity (α) of the RT-Genome complex for the NRTI. The next step then is describing these quantities for the intracellular environment.

There are numerous experiments which directly measure the in vivo concentration of the triphosphate products for a variety of RTIs [13], [14], [15], [19], [20], [21]. The same techniques that are used to determine intracellular NRTI TP concentrations are also used to determine natural deoxynucleotide concentrations [16], [22], [21].

The binding of nucleotide to the complex is a reversible process. Since the conformational change is significantly rate limiting, the concentration of nucleotide-RT complex can be expressed in terms of its dissociation constant, as well as the concentrations of free nucleotide and RT transcription complex. As such, the overall rates of nucleotide and NRTI addition could be expressed as:

$$rate(+dNTP) = k_{rl}(\frac{1}{K_D}[dNTP][RT.DNA]) \tag{1}$$

$$rate(+RTP) = k_{irl}(\frac{1}{K_i}[RTP][RT.DNA]) \tag{2}$$

In which K_D and K_i are the dissociation constants for the natural nucleotide and the drug, and k_{rl} and k_{irl} are the respective rate constants for the rate limiting step. Equations 1 and 2 can be used to calculate the probability of NRTI triphosphate addition:

$$p = \frac{rate(+RTP)}{rate(+RTP) + rate(+dNTP)} = \tag{3}$$

$$\frac{\frac{k_{irl}}{K_i}[RTP]}{\frac{k_{irl}}{K_i}[RTP] + \frac{k_{rl}}{K_D}[dNTP]} \tag{4}$$

which is equivalent to:

$$p = \frac{\frac{K_D k_{irl}}{K_i k_{rl}}[RTP]}{\frac{K_D k_{irl}}{K_i k_{rl}}[RTP] + [dNTP]}, \tag{5}$$

Since the intracellular concentration is a function of time, p is time dependent as well. α, the relative affinity is then:

$$\alpha = \frac{K_D k_{irl}}{K_i k_{rl}} \tag{6}$$

It can be thus concluded that the probability of NRTI addition can be expressed in terms of measurable physical constants. Consequently, if the concentration vs. time curves for these two species may be predicted, then trends in probability of NRTIs addition over time should be likewise predictable. The effect of this probability on overall efficacy depends upon many additional factors, most importantly the stability of the NRTI once included in the HIV genome.

B. *Stability of NRTI as a chain terminator*

As mentioned earlier, RT can catalyze the reaction to remove chain-terminating NRTIs. The stability of the Ternary Dead End Complex that forms varies enormously between drugs. As reported by Isel et al. [23], HIV RT complexes terminated by different NRTIs demonstrated varied results when incubated in the presence of the next incoming nucleotide. Isel et al. kindly provided the data of +1 rescue (see [23], figure 3) for AZT which was studied in this work. The raw data was used to evaluate the parameters to equation $A(1-\exp(-k_{ext}t))$, where A is the amplitude of the reaction and k_{ext} is the apparent repair rate constant.

Presence of NRTIs results in the reduction in the number of new cells that HIV can infect in a given period of time. This is achieved through two mechanisms:

Mechanism 1: Peripheral Blood Mononuclear Cells have a set lifespan in vivo, and express certain natural defense factors that may potentially degrade the HIV RT complex. If an NRTI's TDEC is stable, then the reduction in infection rate might be the result of a certain fraction of HIV fusions being effectively arrested during reverse transcription. The apparent rate of infections decreases by the percentage reduction in completed reverse transcriptions.

Mechanism 2: If TDECs are not stable, each addition of an NRTI to the HIV genome would still represent a period of holdup in the reverse transcription phase, extending the time between viral fusion and active infection. When multiple NRTI additions occur, the holdup time may exceed the time it would take for the host cell to die or clear the RT complex. This, also will reduce the number of cells that become infected in a given period of time. We can consider two types of time delay: time delay which exceeds the life span of RNA in cytoplasm, which we define as mechanism 2.I, and time delay which does not exceed the life span of the RNA in cytoplasm, which we define as mechanism 2.II.

In other words, the source of NRTI efficacy is likely due to a combination of both mechanisms (1) and (2): NRTI induces a moderate delay in reverse transcription, increasing the chance of a viral fusion failing to produce an actively infected T-cell while causing those that do succeed to take longer in doing so. Such a process defies reduction to a simple mathematical description, but is approachable via *in sillico* stochastic modeling of the individual events that occur during reverse transcription.

C. Estimation of NRTI efficacy

In [11], plasma time-course of drug concentration is described:

$$C(t) = \begin{cases} C(T) + \frac{t}{t_p}(C_{max} - C(T)) & 0 \le t \le t_p \\ C_{max}\exp(-\omega(t - t_p)) & t_p < t < T \end{cases} \quad (7)$$

where t_p and T represent dosing interval (administration time) and time to peak, respectively; and $\omega = \frac{\log(2)}{T_{1/2}}$ where $T_{1/2}$ is plasma half life of the drug. It was assumed that the drug concentration peaks instantaneously when the dose is taken, i.e., $t_p = 0$.

In this work, we have attempted to develop a more accurate model which considers not only changes in drug concentration, but also specific drug properties like the probability of forming dead end complex. Each time the idealized NRTI is added to the growing HIV provirus, it is either removed by pyrophosphorolysis or successfully forms a stable dead-end complex. Under cellular conditions, both phosphorolysis and TDEC formation can be considered first-order reactions that compete for NRTI-terminated RT complexes. Thus, the formation of a stable complex can again be calculated as a propensity function in terms of these rates, yielding some probability p_{TDEC} that a dead end complex will form. The overall probability of permanent elimination of the RT complex, which is equivalent to efficacy in this case, can be expressed as:

$$1 - \epsilon_{DC} = (1 - p \cdot p_{TDEC})^B \quad (8)$$

In which ϵ_{DC} is the efficacy due to dead end complex formation, p is the probability of NRTI addition each time a vulnerable base is transcribed (expressed in equations 5 and 6) and B is the number of vulnerable bases in the complete genome. It is important to note that only formation of indefinitely stable TDECs (mechanism 1) contributes to the calculation of efficacy in the above equation. The effect of time delay (mechanisms 2.I and 2.II) on efficacy needs detailed simulation.

There are a number of shortcomings to expression 8. As discussed earlier, the delay of reverse transcription (mechanism 2), might be as important as permanent termination of the RT complex (mechanism 1) which can not be included in our proposed equation in a straightforward manner. Fortunately, stochastic simulation provides us with the ability of calculating the time delay associated with a certain concentration as well as the permanent termination. Furthermore, since addition of NRTI is a discrete event, and $p(t)$ depends on the concentration of NRTI which changes with time, stochastic simulation can provide a more accurate estimation of the probability of NRTI addition over the transcription process. The results of stochastic simulation will be discussed in detail in section IV.

III. Stochastic model of reverse transcription process

An investigation of the problem might persuade one to wonder if the overall delay in reverse transcription resulting from NRTI could be calculated simply by taking the expected number of NRTI additions per genome and multiplying by the expected time that each chain termination will last. Unfortunately, As discussed in section II, the reverse transcription is a nonlinear process. The inhibition of one strand will not necessarily prevent the others from being elongated. As such, the location and time when NRTI addition occurs affects just how long it delays the entire process. The proposed stochastic model accurately simulates the entire process of reverse transcription on an event-by-event basis, "building" the genome one base at a time with probabilistic addition of NRTIs followed by stochastic analysis of the ultimate path to chain-terminator removal. Figure 2 shows the algorithm of the simulation. Specifically this algorithm is based on Gillespie's next reaction algorithm to the RT process. The "nonlinear guideline" in figure 2 eludes to choosing which strand of the DNA the simulation is currently transcribing. It is a sequence of events which is described in section II and graphically presented in figure 1. A random sequence was chosen from the NIH HIV sequence database for simulation. For each drug one million simulation runs were used to compute the time distributions as well as percentage of TDEC formation.

IV. Results and discussions

In this section, the simulations' results are discussed. The reverse transcription (without considering the effect of inhibitors) is a stochastic process. As a result, the reverse transcription process acquires a time distribution rather than

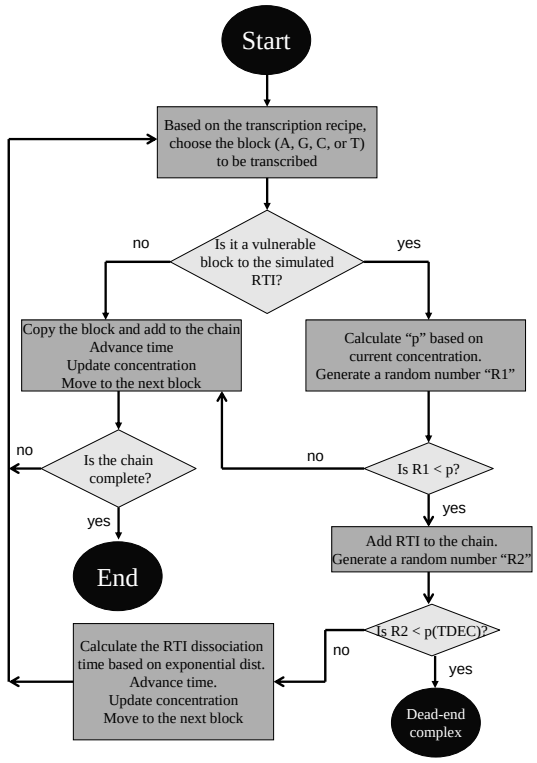

Fig. 2. Algorithm of the stochastic simulation. p and p_{TDEC} show the probability of NRTI addition to the chain and the probability of forming a dead end complex, respectively. "Nonlinear guideline" refers to which strand of the DNA is currently being considered as described in [18] and figure 1.

a precise time: $t_{RT} = 221.14$ min with variance of 5.48 min. The distribution is shown in figure 3.

A. Effect of reverse transcription inhibitors on reverse transcription time

In this study AZT which is T-analogue is simulated. The parameters used for simulation are: $\alpha = 10$, $k_{ext} = 1.76 \times 10^{-3}[1/s]$, and $p_{TDEC} = 8.1\%$.

Although the value of affinity is significant, one should note that the concentrations of drug and the natural nucleotide play an important role in determining the probability of inclusion of NRTI in the chain. Once included in the chain, it is more beneficial if 1) the extension time is long (small k_{ext}) and 2) the probability of forming TDEC, p_{TDEC} is high.

Half-life and the initial drug concentration are two very important inputs for the simulations. Because of the variety in reported half-life values in literature, various values were simulated: $h = 1, 2, 4[hr]$. The IC_{50} values in literature for AZT are quite similar and the value of $IC_{50} = 10[nM]$ was used in the simulations. It is important to emphasize here that C_{max}, which is the maximum drug concentration at the time of administration, is the input for the simulation, not the IC_{50}. However, C_{max} should be chosen considering the IC_{50}. Six different ratios of C_{max} to IC_{50} were simulated: (0.1, 0.25, 0.5, 1, 2, 4).

In Table I summary of the simulations results are presented. Investigating the one million runs for each case, four different outcomes can be identified: NRTI additions which lead to dead end complex formation (mechanism 1), NRTI additions which delay the process so that the overall time exceeds the life span of RNA (mechanism 2.I), NRTI additions which delay the overall process but not enough to exceed the life span of RNA (mechanism 2.II), and finally no NRTI addition. Although mechanism 2.II results in delaying the reverse transcription process, viral DNA will be produced and eventually integrated and transcribed to generate new virus particles. In other words, mechanism 2.II and no NRTI addition can be considered as "unsuccessful inhibition" cases and mechanism 1 and 2.II as "successful inhibition" cases. Consequently, we defined the successful inhibition or the overall efficacy of stochastic simulation, $\epsilon_{si}\%$, as the sum of mechanism 1 ($\epsilon_{DC}\%$) and mechanism 2.I. $\epsilon_{si}\%$ is shown in the last column in Table I.

TABLE I

EFFECT OF CONCENTRATION AND HALF LIFE ON AZT INHIBITORY EFFECT FOR WILD TYPE VIRUS. THE OVERALL EFFICACY $\epsilon_{si}\%$ IN THE LAST COLUMN IS THE SUM OF $\epsilon_{DC}\%$ AND THE PERCENT OF 2.I. NO NRTI ADDITION PERCENTAGE AND 2.II PERCENTAGE REPRESENT THE FAILURE OF THE NRTI TO INHIBIT REVERSE TRANSCRIPTION.

$\frac{C_{max}}{IC_{50}}$	h	no NRTI addition %	dead-end formation ($\epsilon_{DC}\%$)	time delay		$\epsilon_{si}\%$
				2.I %	2.II %	
0.1	2	69.02	3.78	1.0E-4	27.20	3.78
	4	61.98	5.05	4.0E-4	32.97	5.05
0.5	2	15.75	17.12	7.2E-3	67.12	17.13
	4	9.11	22.35	0.03	68.51	22.38
1	2	2.50	30.20	0.08	67.22	30.28
	4	0.82	38.89	0.31	59.98	39.20
2	2	0.06	48.66	0.83	50.45	49.49
	4	7.8e-3	60.81	2.90	36.28	63.71

It was assumed that the administration time is every eight hours, and two different half lives: 2 and 4 hours were investigated. As discussed before in section II-C, a longer half life means higher p and consequently higher efficacy. Efficacy also increases with drug concentration as expected.

In order to show the effect of drug administration time as well, a_t, we chose a value for maximum concentration (here $\frac{C_{max}}{IC_{50}} = 2$) and simulated six cases: adminstration time of 4 and 8 hours; and half lives of 1, 2, and 4 hours (the results are not shown here). It is interesting to note that when the half life is very short (comparing to a_t), ϵ_{si} is slightly larger than ϵ_{DC}; however, when the half life is larger, the difference between ϵ_{si} and ϵ_{DC} becomes significant.

It is also interesting to investigate the time distribution of reverse transcription process under treatment and compare it with no treatment time distribution. It is important to

note that the TDEC forming cases were not considered in time distribution graphs. No NRTI addition cases also were not included in order to see the effect of time delays more vividly. One may need to consider the TDEC and no NRTI addition percentages with the graphs to gain an overall insight of each drug. Data with $\frac{C_{max}}{IC_{50}} = 0.5$ were chosen to create figure 3. Comparing the time distributions of no treatment and AZT treatment in figure 3, it can be observed that the AZT distribution is shifted to the right towards longer times. This is the effect of (single or multiple) NRTI inclusions into the chain and the time needed for their dissociation.

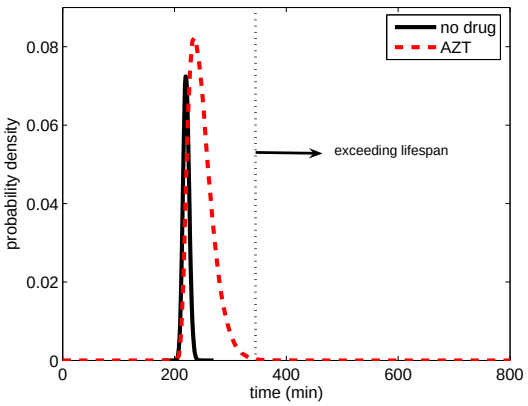

Fig. 3. Distribution of time to complete reverse transcription process with no drug (solid black line) and under treatment with AZT (dashed red line). Only mechanisms 2.I and 2.II are considered. The dotted vertical line represents the life span of RNA in cytoplasm.

V. Conclusion

In conclusion, we showed that stochastic simulation can be employed to investigate the mechanism of inhibition of the RT process. It helped us investigate the formation of TDEC as well as time delay associated with NRTI inclusion. The most important challenge in widely employing this model is the lack of various necessary quantitative data for different drugs. In vitro experiments could be employed to decipher what will take place in vivo. Due to the fact that mutation rate of HIV is quite high and resistance to treatments decreases the effect of treatment, it is also important to quantify the necessary parameters of resistant strains. Currently, quantitative data which can be used in mathematical simulations are scarce and limited to a few types of currently used drugs. Once accurate data are available, such predictions for combinations of NRTIs will be feasible; we will then become able to ascertain the effectiveness of HAART through simulation. For future studies, we seek to include this model in an intracellular model of HIV infection which initiates by attachment of a virus to the host to production and budding of new virions. Such an exclusive model provides the opportunity to investigate the combined effects of different classes of HIV drugs such as Protease Inhibitors, Fusion Inhibitors, and Integrase Inhibitors.

VI. Acknowledgment

Financial support from NSF, CAREER Award #CBET 06-44519, is gratefully acknowledged. The authors would like to thank Dr. Isel for very helpful discussions and also providing the experimental data necessary for this study.

References

[1] A. S. Perelson and P. W. Nelson, "Mathematical analysis of HIV-1 dynamics in vivo," *SIAM review*, vol. 41, no. 1, pp. 3–44, 1999.

[2] A. A. Ding and H. Wu, "Relationships between antiviral treatment effects and biphasic viral decay rates in modeling HIV dynamics," *Math. Biosci.*, vol. 160, no. 1, pp. 63–82, 1999.

[3] S. J. Snedecor, "Comparison of three kinetic models of HIV-1 infection: Implications for optimization of treatment," *J. Theor. Biol.*, vol. 221, pp. 519–541, 2003.

[4] M. A. Nowak, S. Bonhoeffer, and et al, "Anti-viral drug treatment: Dynamics of resistance in free virus and infected cell populations," *J. Theor. Biol.*, vol. 184, no. 2, pp. 205–216, 1997.

[5] D. Kirschner, S. Lenhart, and S. Serbin, "Optimal control of the chemotherapy of HIV," *J. Math. Biol.*, vol. 35, pp. 775–792, 1997.

[6] R. Zurakowski and A. R. Teel, "A model predictive control based scheduling method for HIV therapy," *J. Theor. Biol.*, vol. 238, pp. 368–382, 2006.

[7] S. Khalili and A. Armaou, "Sensitivity analysis of HIV infection response to treatment via stochastic modeling," *Chem. Eng. Sci.*, vol. 63, pp. 1330–1341, 2008.

[8] S. Khalili and A. Armaou, "An extracellular stochastic model of early HIV infection and the formulation of optimal treatment policy," *Chem. Eng. Sci.*, vol. 63, pp. 4361–4372, 2008.

[9] M. Joly and J. M. Pinto, "Role of mathematical modeling on the optimal control of HIV-1 pathogenesis.," *AICHE J.*, vol. 52, pp. 856–884, 2006.

[10] Y. X. Huang and S. L. Rosenkranz, "Modeling HIV-1 dynamics and antiviral response with consideration of time-varying drug exposure, adherence, and phenotypic sesitivity," *Math. BioSci.*, vol. 184, pp. 165–186, 2003.

[11] L. M. Wahl and M. A. Nowak, "Adherence and drug resistance: predictions for therapy outcome," *Proceedings of the Royal Society of London Series B-Biological Sciences*, vol. 267, pp. 835–843, 2003.

[12] W. R. Greco and G. Bravo, "The search for synergy - a critical review from a response-surface perspective," *Pharmacolo. Reviews*, vol. 47, pp. 331–385, 1995.

[13] J. Balzarini and P. Herdewijn, "Differential patterns of intracellular metabolism of $2^{'},3^{'}$-didehydro-$2^{'},3^{'}$-dideoxythymidine and $3^{'}$-azido-$2^{'},3^{'}$-dideoxythymidine, 2 potent anti-human immunodeficiency virus compounds," *J. Bio. Chem.*, vol. 264, pp. 6127–6133, 1989.

[14] F. Becher and A. Pruvost, "A strategy for liquid chromatography/tandem mass spectrometric assays of intracellular drugs: Application to the validation of the triphosphorylated anabolite of antiretrovirals in peripheral blood mononuclear cells," *J. Mass Spect.*, vol. 38, pp. 879–890, 2003.

[15] F. Becher and R. Landman, "Monitoring of didanosine and stavudine intracellular trisphosphorylated anabolite concentrations in HIV-infected patients," *AIDS*, vol. 18, pp. 181–187, 2004.

[16] A. Meyerhans and J. P. Vartanian, "Restriction and enhancement of human-immunodeficiency-virus type-1 replication by modulation of intracellular deoxynucleoside triphosphate pools," *J. Virol.*, vol. 68, pp. 535–540, 1994.

[17] S. Khalili, J. Monaco, and A. Armaou, "Development of a stochastic model for the efficacy of nrtis using known mechanisms of action," *Journal of Theoretical Biology*, vol. in press, 2010.

[18] M. Gotte and X. G. Li, "HIV-1 reverse transcription: A brief overview focused on structure-function relationships among molecules involved in initiation of the reaction," *Arch. Biochem. & Biophys.*, vol. 365, pp. 199–210, 1999.

[19] C. Y. Solas and F. Li, "Intracellular nucleotides of 2',3'-deoxy-3'-thiacytidine in peripheral blood mononuclear cells of a patient infected with human immunodeficiency virus," *Antimicrob. Agents & Chemo.*, vol. 42, pp. 2989–2995, 1998.

[20] C. V. Fletcher and E. P. Acosta, "Concentration-controlled zidovudine therapy," *Clinical Pharmaco. & Therapeutics*, vol. 64, pp. 331–338, 1998.

[21] W. Y. Gao and T. Shirasaka, "Differential phosphorylation of azidothymidine, dideoxycytidine, and dideoxyinosine in resting and activated peripheral blood mononuclear cells," *J. of Clinical Investigation*, vol. 91, pp. 2326–2333, 1993.

[22] J. D. Chi and A. Jayewardene, "A direct determination of thymidine triphosphate concentrations without dephosphorylation in peripheral blood mononuclear cells by lc/ms/ms," *AIDS*, vol. 26, pp. 829–836, 2001.

[23] C. Isel and C. Ehresmann, "The emergence of different resistance mechanisms toward nucleoside inhibitors is explained by the properties of the wild type HIV-1 reverse transcriptase," *J. Biol. Chem.*, vol. 276, pp. 48725–48732, 2001.