

Pharmacological Mechanisms Leading to Early Virologic Failure of Two Antiretroviral Regimens: Didanosine, Lamivudine, and Tenofovir, and Abacavir, Lamivudine, and Tenofovir

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Background

Two triple nucleoside analog (NA) regimens; one using didanosine (ddI), lamivudine (3TC), and tenofovir (TFV) and the other using abacavir (ABC), 3TC, and TFV resulted in a significant number of early virologic failures. This study comprehensively evaluated the pharmacologic factors that may lead to virologic failures, including intracellular (IC) drug-drug interaction, changes in endogenous nucleotide levels, and efflux transporter expression.

Methods

U937 and CEM cell lines were used to determine whether an intracellular (IC) drug-drug interaction existed in either combinations: ddI, 3TC, TFV, and TFV, ABC, and 3TC. Nucleoside-resistant variants were developed using serial passage with increasing nucleoside exposure. U937 and CEM cells were treated alone, in dual, or in triple combination at 2 and 20 μ M. The IC NA triphosphate analogs (ddNTP) and their respective endogenous triphosphate levels were also determined using indirect LC-MS-MS. Western analysis for MRP2 and MRP4 expression was evaluated after nucleoside treatment.

Results

Treatment	Ratio of ddNTP in Combination Treatment as Compared to Control					
	3TC-3TP		CBV-3TP		TFV-DP	
	Mean \pm Std	Median	Mean \pm Std	Median	Mean \pm Std	Median
ABC/TFV	0.961 \pm 0.255	1.046	1.105 \pm 0.407	1.034	0.773 \pm 0.147P	0.800
ABC/3TC	0.792 \pm 0.204	0.725	0.769 \pm 0.194P	0.805	0.945 \pm 0.165	0.900
TFV/3TC	0.796 \pm 0.217	0.866	1.028 \pm 0.193	1.042	0.886 \pm 0.174	0.827

Table 1 Changes in intracellular ddNTP: U937 cells were treated with the respective NRTI combination at 5 μ M for 24 hours and the level of ddNTP ratios were determined using a validated LC-MS/MS method. The ratio is the ddNTP level when compared to ddNTP achieved in cells using the respective NRTIs alone. Statistical analysis was performed using a Wilcoxon signed rank test ($P < 0.05$).

Treatment	Ratio of dNTP/dNTP in Combination Treatment as Compared to Control					
	3TC-3TP		CBV-3TP		TFV-DP/dATP	
	Mean \pm Std	Median	Mean \pm Std	Median	Mean \pm Std	Median
ABC/TFV	0.865 \pm 0.169	0.856	0.769 \pm 0.194P	0.805	0.947 \pm 0.074	0.926
ABC/3TC	0.959 \pm 0.204	1.013	0.826 \pm 0.159P	0.792	0.846 \pm 0.14P	0.877
TFV/3TC	0.969 \pm 0.204	1.013	0.826 \pm 0.159P	0.792	0.846 \pm 0.14P	0.877

Table 2 Changes in intracellular ddNTP/dNTP Ratios: U937 cells were treated with the respective NRTI combination at 5 μ M for 24 hours and the level of dNTP/dNTP ratios were determined using a validated LC-MS/MS method. The ratio for the ddNTP/dNTP level was calculated using the ddNTP achieved in cells when treated with the NRTIs alone. Statistical analysis used a Wilcoxon signed rank test ($P < 0.05$).

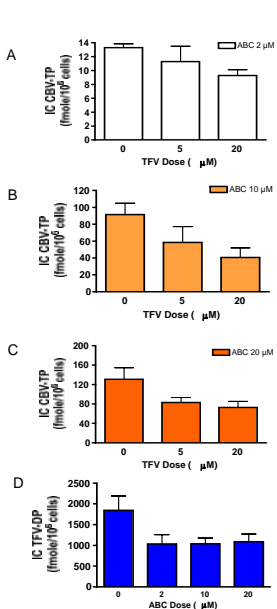


Figure 1 Intracellular Interaction between TFV and ABC: ABC concentrations at 2, 10, 10, and 20 μ M were combined with either 5 or 20 μ M of TFV in CEMss and incubated for 24 hours and the intracellular concentration of carbonyl-triphosphate (CBV-TP) and tenofovir-diphosphate (TFV-DP) were evaluated (Panel A to C). When ABC was treated with either 0, 5 or 20 μ M of TFV, the IC level of CBV-TP decreased in a concentration dependent manner. The IC of TFV-DP was evaluated using 20 μ M of TFV and an escalating level of ABC (Panel D) where a decline IC of TFV-DP was detected at 2 μ M of ABC, where the drop in TFV-DP was approximately 50% when compared to no ABC was added (Panel D).

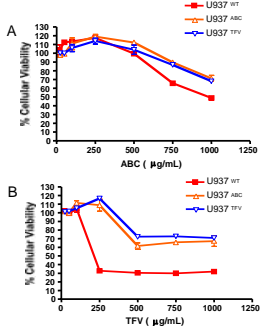


Figure 2 Cellular Viability of ABC and TFV Resistant Variants: Cellular viability of U937^{WT} and its resistant variants, U937^{ABC} and U937^{TFV}, were determined using increasing concentrations of ABC and TFV (Panel A and B). All three cell variants were treated for 24 hours with either ABC or TFV, respectively, and cellular viability was assessed using both MTT and Alamar Blue assays. The IC₅₀ for U937^{WT} cell were 150 and 1000 μ g/mL after 24 hour treatment with TFV and ABC, respectively. However, the IC₅₀ for U937^{ABC} and U937^{TFV} cells after 24 hour treatment with TFV or ABC were not achieved at 1000 μ g/mL, which was the highest concentration used in this study due to drug solubility.

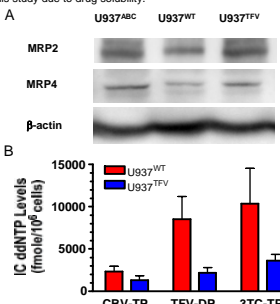


Figure 3 Cellular Properties of Resistant Variants: To investigate the molecular mechanism(s) leading to resistance towards TFV and ABC, immunoblot analysis was performed to assess the expression of efflux transporters in these cells. In U937^{TFV} and U937^{ABC} cells, expression of both MRP2 and MRP4 were enhanced as compared to that of U937^{WT} (Panel A). The ability to accumulate nucleosides to form the triphosphate moiety was assessed by treating U937^{TFV} with 20 μ M of ABC, 3TC and TFV alone for 24 hours, and the IC CBV-TP, 3TC-TP and TFV-DP was determined (Panel B). When, the IC of CBV-TP, 3TC-TP and TFV-DP levels in U937^{TFV} were compared to U937^{WT}, a reduction of 44%, 65% and 78% in the formation of CBV-TP, 3TC-TP and TFV-DP in U937^{TFV} were noted, respectively.

Treatment	Ratio of Triphosphate Nucleoside as Compared to Controls					
	3TC-3TP		TFV-DP		ddATP	
	Mean \pm Std	Median	Mean \pm Std	Median	Mean \pm Std	Median
TFV + 3TC	0.872 \pm 0.101	0.90	0.867 \pm 0.025	0.857	0.833 \pm 0.15	0.87
ddI + 3TC	1.04 \pm 0.11	1.09	1.00 \pm 0.20	0.92	1.04 \pm 0.02	1.03
ddI + TFV	0.87 \pm 0.19	0.84	0.82 \pm 0.07	0.86	0.82 \pm 0.03	0.81

Table 3: CEMss or U937 were incubated for 24 hours with the designated nucleosides and IC TP were determined using a multiplex LC-MS/MS assay (N=5 for each cell line). The ratio of triphosphate is compared to controls, which is the cells treated with the designated nucleoside at 5 μ M for 24 hours.

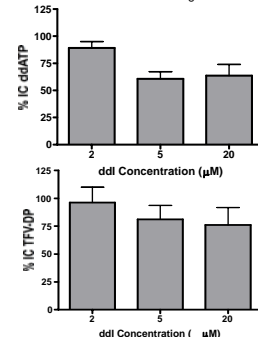


Figure 4 Interaction between ddI and TFV: CEMss cells were incubated with 20 μ M of TFV in combination with increasing concentrations of ddI. The intracellular concentration of ddATP and TFV-DP was compared to cells treated with either ddI or TFV alone (n=5). Increasing concentrations reduced both ddATP and TFV-DP production in a concentration dependent manner. A reduction of ddATP was observed despite a reported increase in ddI in the presence of TFV.

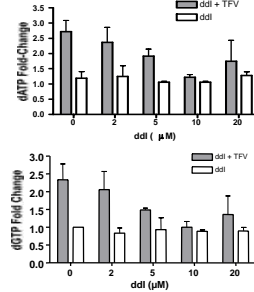


Figure 5 Impact of TFV and ddI on the production of endogenous nucleotide triphosphates: CEMss cells were incubated with increasing concentrations of ddI alone or in combination with 20 μ M TFV. Treatment with ddI does not affect endogenous nucleotide pools (dGTP [A] and dATP [B]) when compared to no treatment. No change in either dGTP or dATP levels was seen with increasing concentrations of ddI [white bars]. When CEMss cells were treated with 20 μ M TFV alone [gray bars at 0] there was a 2.4- and 2.7-fold increase in dGTP and dATP, respectively. When CEMss cells were treated with both TFV and ddI, the addition of ddI ameliorated the TFV-mediated increases in dGTP and dATP in a concentration dependent manner.

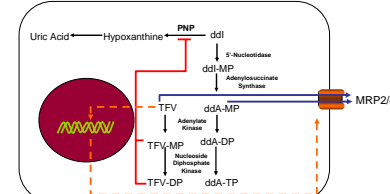


Figure 6 Proposed Mechanism of NRTI Interaction: The addition of TFV will lead to an increase in the production of TFV-MP and TFV-DP, which can inhibit PNP activity. Blockade of PNP may reduce degradation of ddI, which is a purine. When ddI and TFV were combined at 5 μ M, no changes in ddATP or TFV-DP were noted. However, increased concentrations of TFV in combination with ddI revealed significant reduction in intracellular levels of dATP. This reduction may be due to competition for cellular enzymes necessary for conversion into NTPs for each NRTI. However, the ability of NRTIs to induce MRP2 and MRP4 expression may reduce the levels of precursors and thus reduce the level of the active triphosphate product.

Conclusion

These findings suggest there is competitive inhibition between TFV and ABC and also between TFV and ddI. Moreover, long-term exposure with either TFV or ABC can increase expression of both MRP2 and MRP4 which corresponds with reduced IC levels of ddATP. Additionally, TFV at supraphysiologic concentrations was shown to increase endogenous dGTP and dATP levels. These findings taken together may provide significant insights into the pharmacologic mechanisms leading to clinical virologic failures.

Figure 5 Expression of Transporters in the presence of TFV: CEMss cells were treated with either 10 or 50 μ M TFV for 24 hours, and the expression of MRP2 and MRP4 was compared to untreated controls. The level of expression for both MRP2 and MRP4 was enhanced when exposed to TFV for CEMss cells. The induction of MRP2 and MRP4 expression appears to be dependent on the concentration of TFV employed.