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Research Article

Mutagenicity of Zidovudine, Lamivudine, and Abacavir Following In Vitro Exposure of Human Lymphoblastoid Cells or In Utero Exposure of CD-1 Mice to Single Agents or Drug Combinations

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Experiments were performed to investigate the impact of zidovudine (AZT), lamivudine (3TC), and abacavir (ABC) on cell survival and mutagenicity in two reporter genes, hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) and thymidine kinase (*TK*), using cell cloning assays for assessing the effects of individual drugs/drug combinations in (1) TK6 human lymphoblastoid cells exposed in vitro and (2) splenic lymphocytes from male CD-1 mice exposed transplacentally on days 12–18 of gestation. In TK6 cells, dose-related increases in *HPRT* and *TK* mutant frequencies were found following 3 days of exposure to AZT or 3TC alone (33, 100, or 300 μM), or to equimolar amounts of AZT-3TC. Compared with single drug exposures, AZT-3TC coexposures generally yielded enhanced elevations in *HPRT* and *TK* mutant frequencies. Mutagenicity experiments with ABC alone, or in combination with AZT-3TC, were complicated by the extreme cytotoxicity of ABC. Exposure of cells either to relatively high levels of AZT-3TC short-term (100 μM, 3 days), or to peak plasma-equivalent levels of AZT-3TC for an extended period (10 μM, 30 days), resulted in similar drug-induced mutagenic responses. Among sets of mice necropsied on days 13, 15, or 21 postpartum, *Hprt* mutant frequencies in T-cells were significantly elevated in the AZT-only (200 mg/kg bw/day) and AZT-3TC (200 mg AZT + 100 mg 3TC/kg bw/day) groups at 13 days of age. These results suggest that the mutagenicity by these nucleoside analogs is driven by cumulative dose, and raises the question of whether AZT-3TC has greater mutagenic effects than AZT alone in perinatally exposed children. Environ. Mol. Mutagen. 48:224–238, 2007.
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Key words: ***HPRT; TK;* nucleoside analog; transplacental**

INTRODUCTION

Zidovudine (3′-azido-2′,3′-dideoxythymidine; AZT), lamivudine (2′-deoxy-3′-thiacytidine; 3TC), and abacavir [(1S,4R)−4-[2-amino-6-(cyclopropylamino)−9H-purin-9-yl]−2-cyclopentene-1-methanol; ABC] are nucleoside reverse transcriptase inhibitors (NRTIs) often used as components in "highly active antiretroviral therapy" (HAART) designed to inhibit viral replication in HIV-infected patients and to reduce perinatal transmission of the virus from mother to child [USDHHS]. Most HAART regimens consist of two NRTIs and a protease inhibitor. The Centers for Disease Control recommend that HIV-infected women be given HAART regimens during the last 6 months of gestation and AZT by continuous IV infusion during labor [USDHHS]. They also recommend that AZT be administered to the infant for 6 weeks postnatally [USDHHS]. While perinatal AZT monotherapy decreased HIV transmission rates from 25 to 8%

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Abbreviations: ABC, abacavir or (1S,4R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol; AZT, zidovudine or 3′-azido-2′,3′-dideoxythymidine; 3TC, lamivudine or 2′,3′-dideoxy-3′-thiacytidine; ddI, didanosine or 2′,3′-dideoxyinosine; HAART, highly active antiretroviral therapy; *HPRT*, hypoxanthine-guanine phosphoribosyltransferase; NRTIs, nucleoside reverse transcriptase inhibitors; *TK*, thymidine kinase.

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[Connor et al., 1994; Sperling et al., 1996], administration of HAART during the prepartum period further reduced the rate of vertical transmission to $\leq$2% in infants who are not breast fed [Capparelli et al., 2005]. Although NRTI drug combinations provide enhanced anti-HIV therapy/prophylaxis, individual NRTIs have been implicated as chemical mutagens, carcinogens, and potential mitochondrial toxins [Olivero et al., 1997; Blanche et al., 1999; IARC, 2000; Wutzler and Thust, 2001; Dagan et al., 2002; Poirier et al., 2004; Walker et al., 2004; Benhammou et al., 2007; Senda et al., in press].

The mutagenic effects of NRTIs result, in part, from the mechanism by which they prevent viral replication. NRTIs are analogs of normal nucleosides that lack the 3′-OH of the deoxyribose sugar, and are therefore unable to extend the nascent DNA chain by forming a 5′ to 3′-phosphodiester bond with the proceeding nucleic acid. They are able to inhibit viral replication by taking the place of endogenous nucleotides during reverse transcription of viral RNA and, thus, cause premature termination of proviral DNA synthesis [Kakuda, 2000]. However, this mode of action also allows for the incorporation of these drugs into host cell nuclear DNA and mitochondrial DNA [Olivero et al., 1997, 1999; IARC, 2000].

AZT-3TC is the pair of NRTIs most commonly given to HIV-infected pregnant women, yet this drug combination has been associated with an increase in (i) incorporation of the NRTIs into host cell DNA, (ii) induction of mutations in reporter genes and (iii) induction and persistence of mitochondrial damage [Blanche et al., 1999; Olivero et al., 1999; O'Neill et al., 2001; Poirier et al., 2003; Bishop et al., 2004; Cooper et al., 2004; Poirier et al., 2004; Divi et al., 2004, 2007; Walker et al., 2004; Escobar et al., 2007; Meng et al., 2007]. A previous population study of newborn children exposed in utero to AZT or AZT-3TC, revealed that when AZT-3TC was given in combination, AZT incorporation into host DNA was greater than when AZT was given alone [Poirier et al., 2004; Meng et al., 2007]. Also, mutant frequencies at the hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) locus of cord blood T-cells from these AZT-3TC exposed infants were significantly greater than matched controls and equivalent to those of children aged 12–17 [O'Neil et al., 2001; Poirier et al., 2004]. Similar results were seen in *Erythrocebus patas* monkey fetuses exposed in utero to AZT-3TC, in which higher levels of AZT-DNA incorporation and more telomere damage were seen compared with that in monkeys treated with AZT alone [Olivero et al., 2002]. The findings of greater host cell AZT-DNA incorporation and mutational burden in reporter genes of fetuses exposed to AZT-3TC, compared with those exposed to AZT alone, suggest that in utero exposure to this NRTI drug pair may impose an elevated risk for cancer gene mutations and potential carcinogenesis.

The occurrence of possible synergistic mutagenic effects of AZT-3TC exposure is supported by the results of cell culture studies showing significantly greater mutagenic responses in TK6 cells exposed to AZT plus didanosine (ddI) compared with those produced by the sum of equimolar exposures to each drug alone [Meng et al., 2000b, 2002; Torres et al., 2004]. These findings suggested that the enhanced chemoprophylactic effects of the clinical use of NRTI drug pairs may be accompanied by additive or synergistic mutagenic effects compounding the long-term risk for cancer in HIV-infected patients and in uninfected children exposed in utero. The current investigation of AZT, 3TC, and ABC was undertaken to extend the current knowledge of the cytotoxic and mutagenic effects of combined NRTI exposures relative to single drug exposures in vitro and in utero, and to determine if AZT-3TC is significantly mutagenic in vitro in human cells at doses that mimic the clinical treatment of HIV-infected patients.

MATERIALS AND METHODS

Chemicals and Media Components

AZT, 3TC, and ABC were obtained from Byron Chemical Company (Long Island City, NY). Lympholyte-M was purchased from Accurate Chemical and Scientific (Westbury, NY), and β-mercaptoethanol, 6-thioguanine, cytidine, hypozanthine, aminopterin, and thymidine were purchased from Sigma Chemical (St. Louis, MO). Components for cell culture medium were purchased from the following sources: HL-1, RPMI 1640, L-glutamine, MEM nonessential amino acids, penicillin-streptomycin, and sodium pyruvate (BioWhittaker, Walkersville, MD); fetal bovine serum (FBS) (Biomeda, Foster City, CA); HEPES (Sigma Chemical); concanavalin A (Vector Laboratories, Burlingame, CA); mouse interleukin-2 (Cell Sciences, Canton, MA).

Cell Culture, Exposures, and Relative Cell Survival in TK6 Cells Exposed In Vitro

Human TK6 lymphoblastoid cells were cultured as previously described [Sussman et al., 1999]. Briefly, prior to NRTI exposure, the cells were grown in medium containing CHAT (cytidine, hypoxanthine, aminopterin, and thymidine) for 2 days followed by THC (thymidine, hypoxanthine, and aminopterin) for 2 days to reduce the frequency of background mutations. Exposures were carried out in T-flasks (n = 6–12 per group) with the cells exposed to AZT, 3TC, ABC, AZT-3TC, or AZT-3TC-ABC as described below. For exposures using a combination of NRTIs, cells were exposed to the same concentration, or equimolar amounts, of each agent. Following the exposure period (of 3 days or more, see below), the cells were washed, concentrated, resuspended, and plated to determine the acute cytotoxicity of the various NRTI treatments. The treated and unexposed cells were plated at an average of 2 viable cells per well, in the presence of 4×10^4 lethally irradiated "feeder" cells, in 96-well U-bottom microtiter dishes. Fourteen days later, the plates were scored for positive colonies, based on morphology, size, and density. Cloning efficiencies were calculated as described by Sussman et al. [1999], and then relative cell survival was computed as the ratio of the average cloning efficiency in the treated cells vs. control cells.

Cell Cloning Assay for *HPRT* and *TK* Mutant Frequencies in Cells Exposed In Vitro

The cell cloning methods for determining *HPRT* and thymidine kinase (*TK*) mutant frequencies in human lymphoblastoid cells have been previously described [Liber and Thilly, 1982; Sussman et al., 1999; Meng

et al., 2000b]. Briefly, after timed growth of TK6 cells in medium with or without NRTIs, cells were washed, concentrated, resuspended, and subcultured in nonselective medium for 7 days to allow for expression of the *HPRT* mutant phenotype. Two plates per flask were seeded as described above to determine nonselected cloning efficiencies. The remaining cells were plated in 96-well microtiter dishes at 4×10^4 cells/well in selective medium containing 6-thioguanine (1 μg/ml medium) to measure *HPRT* mutant frequencies. The microtiter dishes were scored for *HPRT* mutant colonies 14 days after plating. Observed *HPRT* mutant frequencies were calculated as the ratio of mean cloning efficiency in selective medium to that in nonselective medium [Sussman et al., 1999]. The basic technique for measuring *HPRT* mutant frequencies was used to determine *TK* mutant frequencies, except that the time allowed for phenotypic expression of *TK* mutations was 3 days, the selection agent was trifluorothymidine (3 μg/ml medium), and *TK* mutant colonies were scored at 21 days after plating.

The relationships between exposure concentration and mutagenic responses in the *HPRT* and *TK* genes were evaluated in TK6 cells exposed for 3 days to 0, 33, 100, or 300 μM AZT, 3TC, ABC, AZT-3TC, or AZT-3TC-ABC. Experiments were performed in duplicate or triplicate, and the resulting data were combined for presentation here. Observed mutant frequencies were expressed as the mean ± standard error (SE), while induced mutant frequencies were determined by subtracting the mean mutant frequencies measured in control groups from the mean mutant frequencies measured in different treatment groups.

The relationships between mutagenicity and exposure of prolonged duration at plasma equivalent levels of AZT-3TC were assessed by exposing TK6 cells to 10 μM of both drugs for up to 30 days. The molar concentration of AZT-3TC was maintained throughout the exposure period by removing excess cells and then adding appropriate amounts of the drugs based on the amount of medium needed to dilute the remaining cells in culture to a density of 400,000/ml. Aliquots of cells were removed following treatment periods of 3, 14, and 30 days to determine cloning efficiencies and *HPRT* and *TK* mutant frequencies as described above, with observed mutant frequencies expressed as the mean ± SD.

Measurement of AZT Incorporation into Nuclear DNA in Cells Exposed In Vitro

To determine whether incorporation of NRTIs into cellular DNA correlated with the accumulation of reporter gene mutations, TK6 cells exposed to 0 or 10 μM AZT-3TC for 3, 7, or 14 days were centrifuged and washed with 1× PBS, nuclear pellets were obtained, DNA was isolated using a nonorganic method (Chemicon International, Temecula, CA), and extracted DNA was treated with RNase. A competitive anti-AZT RIA was used to measure incorporation of AZT into genomic DNA, as previously described [Olivero et al., 1997; Sussman et al., 1999; Meng et al., 2000b]. The AZT-RIA assay was not used to measure levels of AZT in DNA of TK6 cells exposed in higher dose experiments because, in earlier studies, application of this method demonstrated dose-related AZT-DNA incorporation in TK6 cells exposed to ≥33 μM AZT alone, or in combination with a second NRTI [Sussman et al., 1999; Meng et al. 2000b], as well as AZT-DNA incorporation in several cell types/tissues from mice, *E. patas* monkey fetuses, and human mother-child pairs exposed perinatally to AZT only or AZT with other NRTIs [Olivero et al., 1997, 1999, 2002; Meng et al., 2007].

Animals, Husbandry, and NRTI Exposures

Date-mated female CD-1 mice were purchased from Charles River Laboratories (Portage, MI) and delivered on gestation Day 10. Pregnant mice were housed in individual cages containing heat-sterilized wood chips and nesting material, in temperature- and humidity- controlled rooms with 12-hr light/dark cycles. The animals had free access to standard chow and filter-purified tap water, and were allowed to acclimate for 2 days before treatment. Prior to treatment, pregnant mice were lightly anesthetized with isofluorane and O_2 gas, and the NRTI(s) was delivered via gavage (200 mg AZT/kg bw, 100 mg 3TC/kg bw, or the same amounts of each drug dissolved in sterile PBS) on days 12 through 18 of gestation. Control animals were anesthetized in the same manner and gavaged with vehicle alone. Doses of AZT and 3TC were set at a 2:1 ratio, as used clinically in human patients. All procedures involving the use of animals were approved by the Institutional Animal Care and Use Committee at Lovelace Respiratory Research Institute.

Isolation, Culture, and Selection of *Hprt* Mutant Splenic Lymphocytes

Groups of male pups exposed transplacentally to NRTIs or vehicle were necropsied on days 13, 15, and 21 postpartum for isolation of lymphocytes from spleen. These male offspring were available as part of another study directed at characterizing mitochondrial toxicity in their female siblings, based upon the finding that female mice were more susceptible to mitochondrial damage and cardiomyopathy than male mice following transplacental exposure to AZT [Walker et al., 2004]. At days 13 and 15 postpartum, the spleens of two male pups were pooled for overnight priming. In some cases, where the cell yield was low after overnight priming, two flasks of lymphocytes were pooled again to obtain a sufficient number of cells for plating. General procedures previously described by Skopek et al. [1992] and Sussman et al. [2001] were used for isolating lymphocytes and culturing mutant T-cell colonies. Briefly, the animals were euthanized by CO_2 anesthesia, the spleen was removed aseptically and placed in 3 ml of sterile RMPI 1640 medium in one well of a 12-well microtiter dish, and the tissue was macerated using the butt of a 5 ml syringe plunger. The cell suspension was aspirated through a 5/8″ 25-gauge needle into a sterile 1-cc tuberculin syringe and gently expelled onto a Ficoll solution (4 ml Lympholyte^M) in a 15 ml tube. The tubes were centrifuged for 15 min at 1,900*g* at room temperature using a Sorvall RT 6000D centrifuge (Sorvall, Wilmington, DE). Mononuclear cells from the interface were pipetted into a 15 ml tube containing 5 ml sterile RMPI 1640 medium. The tubes were then centrifuged for 15 min at 1,900*g* at room temperature. The medium was decanted and the cell pellet was resuspended in 5 ml of fully supplemented medium (15% FBS, 20% HL-1, 10% rat T-STIM, 25 mM HEPES buffer, 5 mM L-glutamine, 100 μM MEM nonessential amino acids, 100 units each/ml penicillin-streptomycin, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, and 20–25 units/ml IL-2). Lymphocytes were transferred to a 75-cm^2 cell-culture flask containing 15 ml of fully supplemented medium, and the mitogen concanavalin A was added at 4 μg/ml medium. The flasks were placed in an incubator at 37°C and 5% CO_2 to prime cells for plating. Following overnight priming, the cells were enumerated for plating, using a Coulter Counter (Beckman Coulter, Fullerton, CA).

Cell Cloning Assay for *Hprt* Mutant Frequencies in Splenic Lymphocytes

The cell cloning methods for determining *Hprt* mutant frequencies in splenic lymphocytes from mice exposed transplacentally have been previously described [Sussman et al., 1999]. Briefly, two 96-well U-bottom plates were seeded with 8 viable cells/well plated in the presence of 30,000 lethally irradiated "feeder" cells/well to determine the cloning efficiencies. The remaining cells were plated in 96-well U-bottom plates at 4.5×10^4 cells/well in selective medium containing 1 μg 6-thioguanine/ml. Plates were scored for mutant colony growth 14 days after plating. Mutant frequencies were calculated as described above for the in vitro cell culture studies.

Statistical Analyses

Kruskal–Wallis One Way Analysis of Variance on Ranks was used to compare differences in the mean mutant frequency values among treatment groups of cultured cells, and the Holm-Sidak or Dunn's methods were used

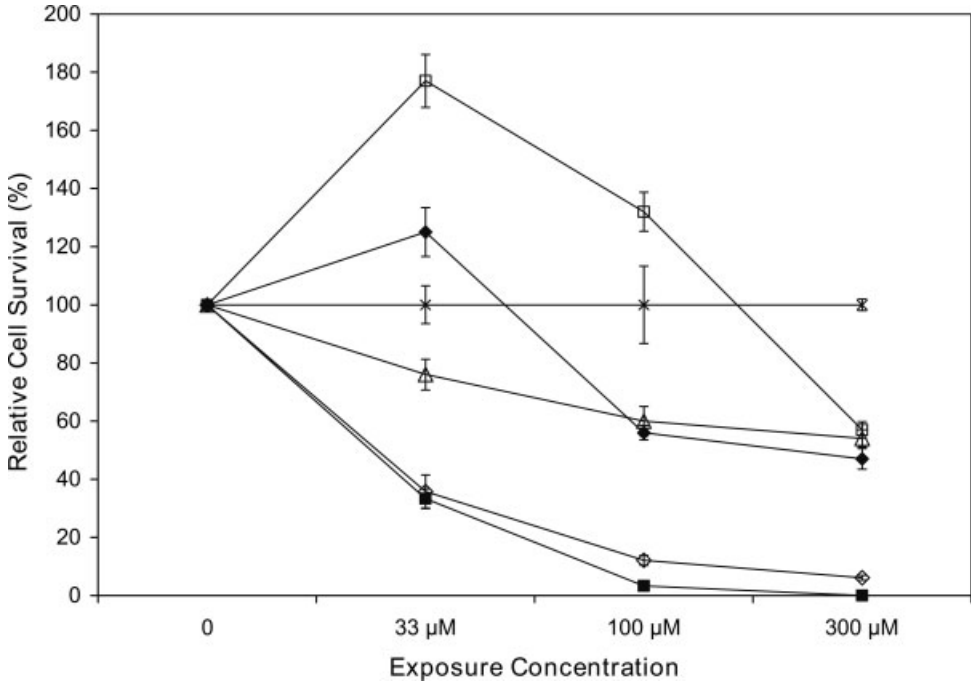

Fig. 1. Relative cell survival following 3-day exposures of TK6 cells to AZT, 3TC, ABC, AZT-3TC, or AZT-3TC-ABC. Cultures (n = 6–12/group/experiment; see Table I) were exposed to 0, 33, 100, and 300 μM of each drug alone or in equimolar combinations for 3 days. Cell survival of unexposed cells is designated as 100%, and all other groups are presented as a ratio of the measured cloning efficiencies of treated vs. control samples. Control (×); AZT (◆); 3TC (□); ABC (◇); AZT-3TC (△); AZT-3TC-ABC (■); points, averages; bars, SD.

for pair-wise multiple comparisons. The P-values used to describe the differences in *Hprt* mutant frequency values between TK6 cells exposed to vehicle or AZT-3TC for different durations of time, or between controls and mice treated transplacentally with AZT and/or 3TC at specific times postpartum, were formed by the Student's t-test or Mann-Whitney U-statistic [Sussman et al., 2001]. P-values ≤ 0.05 were considered significant.

RESULTS

Effects of 3-Day NRTI Exposures on TK6 Cell Survival

Relative survival of human lymphoblastoid TK6 cells was measured following 3 days of NRTI exposure to assess the potential cytotoxic effects of AZT, 3TC, ABC, AZT-3TC, and AZT-3TC-ABC. Figure 1 shows a graphical representation of the impact of exposure concentration on the relative cell survival. Cell survival of unexposed cells is designated as 100%, and all other groups are presented as a ratio of the cloning efficiencies of treated vs. control samples from their respective experiments (Table I). The average measured cloning efficiencies in control cells across five independent experiments was (70.4 ± 13.7)%.

Among the individual NRTIs, ABC was the most cytotoxic followed by AZT (Fig. 1, Table I). The strong cytotoxic effects of ABC on TK6 cells was evidenced by relative cell survivals of 36% and ~12% following 3-day exposures to 33 and 100 μM, respectively. The 50% inhibitory concentration (IC_{50}) at <33 μM ABC was similar to that for other types of human cells [Cihlar et al., 2002]. Three-day exposures to 100 and 300 μM AZT reduced survival of TK6 cells to 56 and 47% of control value (Table I, Fig. 1), respectively, and the observed IC_{50} was consistent with findings in previous cytotoxicity studies in AZT-exposed human lymphoid cells [Furman et al., 1986; Meng et al., 2000b]. In stark contrast, exposures to 33 and 100 μM 3TC resulted in cell survival that was significantly greater than that observed in control cells (Table I; Fig. 1), i.e., 177 and 132% of the mean control level, respectively. However, 300 μM 3TC was nearly as cytotoxic as 300 μM AZT.

In this study, the cytotoxic effects of combined NRTI drug exposures were not dramatically different from selected single drug exposures in TK6 cells (Table I, Fig. 1). For example, the relative cell survivals were not significantly different following exposures to 100 or 300 μM AZT-3TC compared with respective exposures to 100 or 300 μM AZT only. Likewise, the cytotoxicity of AZT-3TC-ABC appeared to track closely with the extreme cytotoxic effects induced by ABC alone. AZT-3TC-ABC was extremely cytotoxic to TK6 cells such that cell survival could not be measured with confidence at the 300 μM exposure level.

Effects of NRTI Exposure Concentrations on *HPRT* and *TK* Mutant Frequencies in TK6 Cells

In parallel with the cell survival studies, aliquots of the same cells exposed to AZT, 3TC, ABC, AZT-3TC, or AZT-3TC-ABC were evaluated for mutagenic responses in the *HPRT* and *TK* genes following, respectively, 7- and 3-day phenotypic expression periods post exposure (Figs. 2–5).

TABLE I. Observed Mean Cloning Efficiency and Relative Cell Survival Values following 3-Day Exposures of TK6 Cells to NRTIs[a]

	33 μM NRTI		100 μM NRTI		300 μM NRTI	
NRTI (Experiments and doses tested)	CE (%)	RCS (%)[b]	CE (%)	RCS (%)[b]	CE (%)	RCS (%)[b]
AZT (1–3 for 100 μM; & 2, 3 for 33 & 300 μM)	70.9	125	44.4	56	26.6	47
3TC (2, 3 for all doses)	100	177	74.8	132	32.3	57
AZT-3TC (2, 3 for all doses)	43.1	76	34.0	60	30.6	54
ABC (4, 5 for all doses)	29.8	36	9.9	12	5.0	6.0
AZT-3TC-ABC (4, 5 for all doses)	27.3	33	2.7	3.3	0	0

[a]TK6 human lymphoblastoid cells were exposed to vehicle or the given concentrations of NRTIs for 3 days, and then washed, diluted, and plated under nonselection conditions to measure colony outgrowth as described in the Materials and Methods. The average observed cloning efficiencies (CEs) in vehicle treated cells in order across five independent experiments were 72.9%, 60.9%, 52.5%, 86.4%, and 79.2%.

[b]Adjusted relative cell survival (RCS) values represent the ratio of observed CEs in NRTI-exposed cells versus control values for their respective experiments (shown in parentheses in the first column). There were no significant differences in the relative cell survival for a given treatment across experiments, thus, the individual relative survival values in differing experiment were averaged for presentation in Table I and Figure 1.

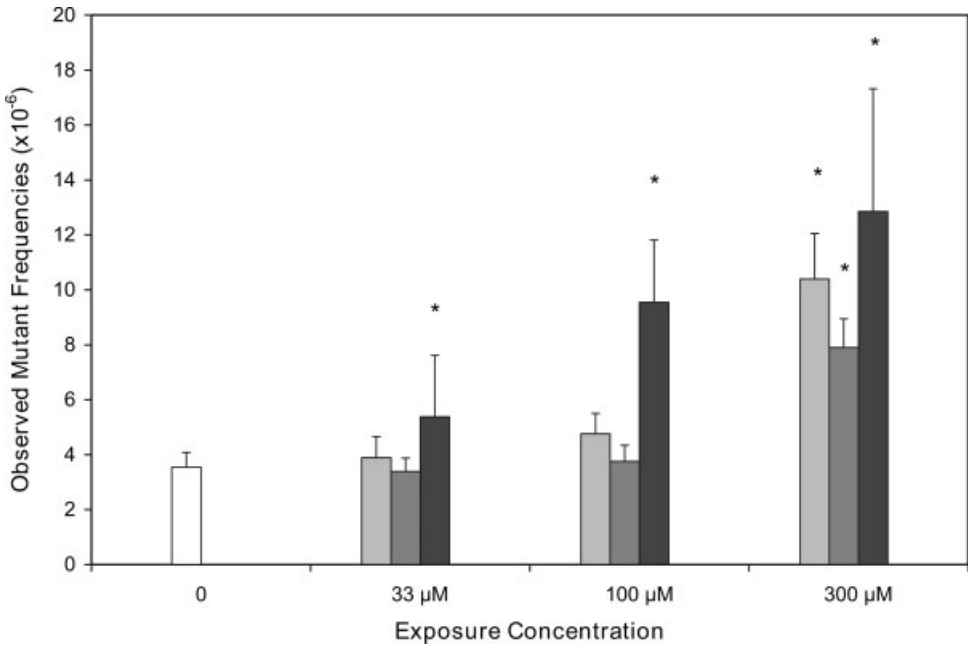

Fig. 2. Comparison of the mutagenic potencies of AZT, 3TC, and AZT-3TC at the *HPRT* locus of TK6 cells exposed in culture to 33, 100, or 300 μM AZT or 3TC alone, or in equimolar concentrations, for 3 days. Control, empty bar; AZT, light gray bar; 3TC, gray bar; AZT + 3TC, black bar; error bars represent SE. There were significant positive trends in the increases of observed mutant frequencies in cells exposed to AZT, 3TC, or AZT-3TC (P-values $\leq$0.004, Kruskal–Wallis test). Asterisks (*) indicate NRTI treatments yielding significant elevations in *HPRT* mutant frequency compared with control cells ($P < 0.05$, Dunn's or Holm–Sidak methods).

Following 3-day exposures of TK6 cells to 33–300 μM AZT or 3TC, there were significant positive trends in the increases of observed *HPRT* mutant frequencies (P-values = 0.001 and 0.004, respectively; Kruskal–Wallis test) (Fig. 2). Similar to previous results obtained following exposure of TK6 cells to AZT or ddI [Sussman et al., 1999; Meng et al., 2000b, 2002], these trends were driven largely by the significant increases in the *HPRT* mutant frequencies in cells exposed to 300 μM AZT (mean = $10.4 \pm 1.7 \times 10^{-6}$; $P < 0.05$, Dunn's method) or 3TC (mean = $7.9 \pm 1.0 \times 10^{-6}$; $P < 0.05$, Holm–Sidak method) compared with control values (mean = $3.9 \pm 0.8 \times 10^{-6}$). *HPRT* mutant frequencies were not significantly elevated above background after 3-day exposures to lower concentrations of 33 or 100 μM of AZT or 3TC. It should be noted that, in contrast to relative cell survival values in 3TC-exposed cells exceeding those for control cells immediately after 3-day treatments, nonselective cloning efficiencies in cells exposed to 33 or 100 μM 3TC were lower than those found in unexposed control cells following the phenotypic expression periods. Therefore, the elevated relative cell survivals immediately after these treatments did not impact calculation of mutant frequencies, and the interpretation of the resulting data, after plates were scored for mutant clones.

Three-day exposures of TK6 cells to AZT-3TC produced a significant dose-related trend in the increases of

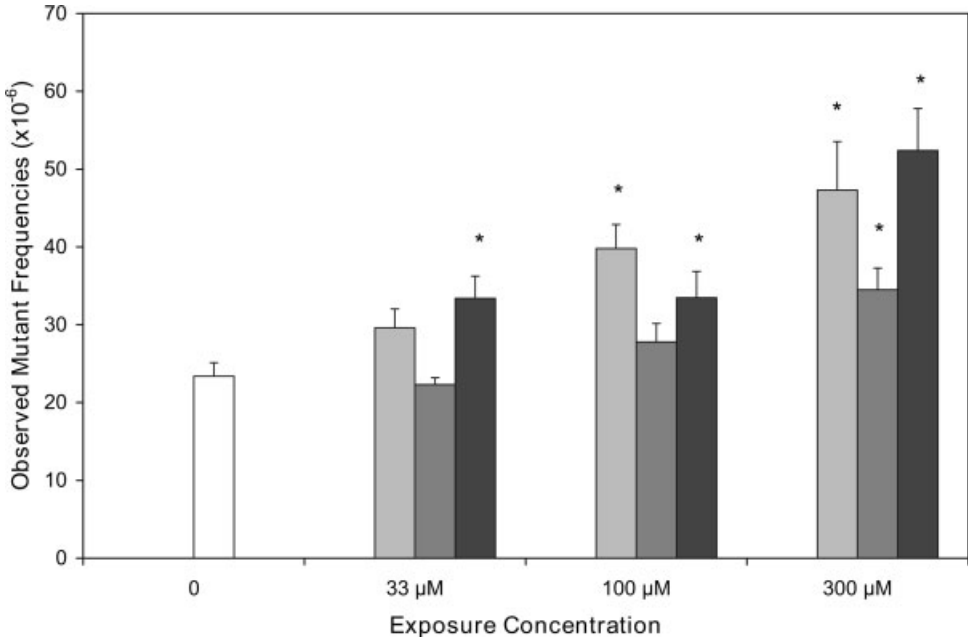

Fig. 3. Comparison of the mutagenic potencies of AZT, 3TC, and AZT-3TC at the *TK* locus of TK6 cells exposed in culture to 33, 100, or 300 μM AZT or 3TC alone, or in equimolar concentrations, for 3 days. Control, empty bar; AZT, light gray bar; 3TC, gray bar; AZT + 3TC, black bar; error bars represent SE. There were significant positive trends in the increases of observed mutant frequencies in cells exposed to AZT, 3TC, or AZT-3TC (respective *P*-values of <0.001, 0.01, and <0.001, Kruskal–Wallis test). Asterisks (*) indicate NRTI treatments yielding significant elevations in *TK* mutant frequency compared with control cells ($P < 0.05$, Dunn's or Holm–Sidak methods).

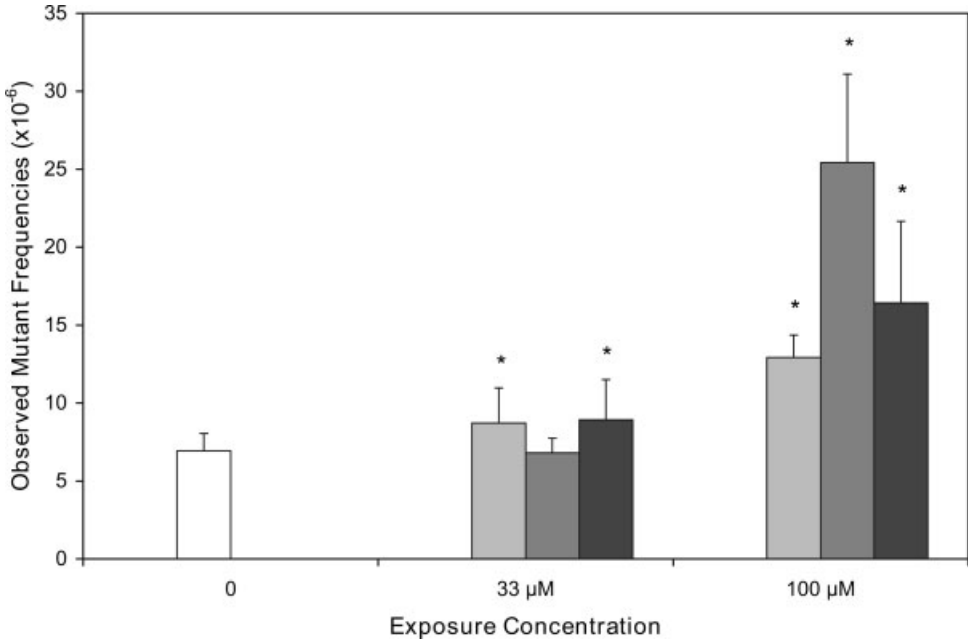

Fig. 4. Comparison of the mutagenic potencies of AZT-3TC, ABC, and AZT-3TC-ABC at the *HPRT* locus of TK6 cells exposed in culture to 33 or 100 μM ABC alone, or AZT-3TC and AZT-3TC-ABC in equimolar concentrations, for 3 days. Control, empty bar; AZT-3TC, light gray bar; ABC, gray bar; AZT-3TC-ABC, black bar; error bars represent SE. There was a significant positive trend in the increase of observed mutant frequencies in cells exposed to AZT-3TC-ABC ($P < 0.001$, Kruskal–Wallis test). Asterisks (*) indicate NRTI treatments yielding significant elevations in *HPRT* mutant frequency compared with control cells ($P < 0.05$, Dunn's or Holm–Sidak methods).

observed *HPRT* mutant frequencies ($P < 0.001$, Kruskal–Wallis test), with observed responses being significantly increased over background at 33, 100, and 300 μM [respective means = $(5.4 \pm 2.2) \times 10^{-6}$, $(9.6 \pm 1.4) \times 10^{-6}$, $(12.9 \pm 4.5) \times 10^{-6}$; $P < 0.05$, Dunn's method] (Fig. 2). Furthermore, the induced mutant frequency values in AZT-

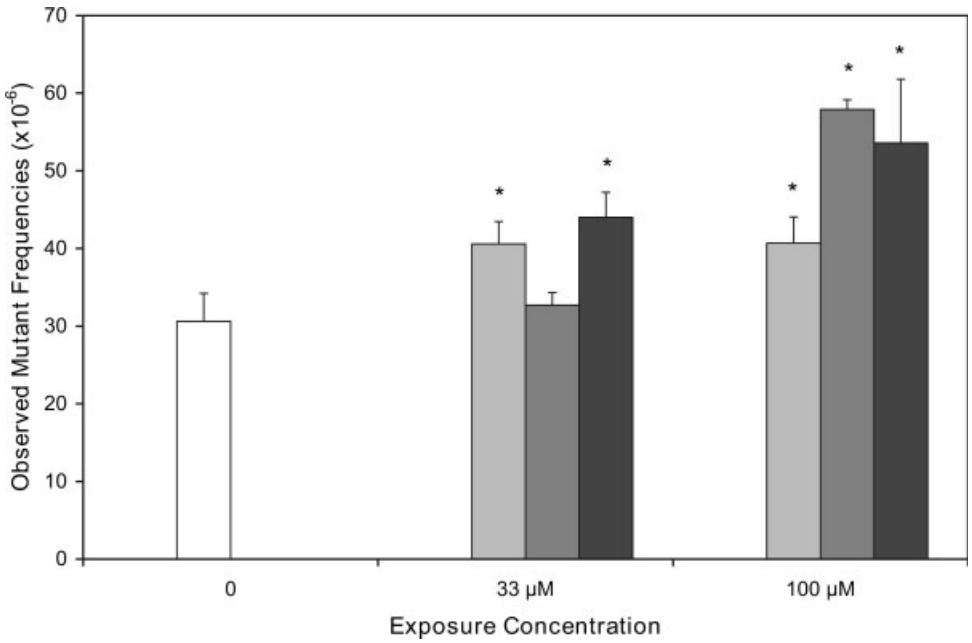

Fig. 5. Comparison of the mutagenic potencies of AZT-3TC, ABC, and AZT-3TC-ABC at the *TK* locus of TK6 cells exposed in culture to 33 or 100 μM ABC alone, or AZT-3TC and AZT-3TC-ABC in equimolar concentrations, for 3 days. Control, empty bar; AZT/3TC, light gray bar; ABC, gray bar; AZT-3TC-ABC, black bar; error bars represent SE. There was a significant positive trend in the increase of observed mutant frequencies in cells exposed to AZT-3TC-ABC ($P < 0.001$, Kruskal–Wallis test). Asterisks (*) indicate NRTI treatments yielding significant elevations in *TK* mutant frequency compared with control cells ($P < 0.05$, Dunn's or Holm–Sidak methods).

3TC exposed cells were similar to, or greater than, the additive induced mutagenic effects of single treatments with AZT or 3TC at exposures of 33, 100, or 300 μM (Fig. 2). The average induced *HPRT* mutant frequency following exposure to 33 μM AZT-3TC (mean $= 1.8 \times 10^{-6}$) was significantly greater (~6-fold) than the additive induced mutant frequency from single exposures to 33 μM AZT (mean $= 0.3 \times 10^{-6}$) and 33 μM 3TC (mean induced mutant frequency of zero) ($P < 0.05$, Holm–Sidak method). A significant synergistic mutagenic effect also was seen following coexposure to 100 μM AZT-3TC, with the induced *HPRT* mutant frequency (mean $= 6.0 \times 10^{-6}$) being fourfold greater than the additive induced effect of single exposures to 100 μM AZT (mean $= 1.2 \times 10^{-6}$) and 100 μM 3TC (mean $= 0.2 \times 10^{-6}$) ($P = 0.05$, Holm–Sidak method). On the other hand, coexposure to 300 μM AZT-3TC induced *HPRT* mutant frequencies (i.e., mean $= 9.3 \times 10^{-6}$) that were 83% of the additive induced *HPRT* mutant frequency values for single treatments with 300 μM AZT (mean $= 6.8 \times 10^{-6}$) or 3TC (mean $= 4.4 \times 10^{-6}$).

The patterns in the mutagenic responses in the *TK* gene were also defined in TK6 cells exposed to AZT, 3TC, or AZT-3TC at the same time the *HPRT* mutation studies were conducted (Fig. 3). In AZT-exposed cultures, there was a significant positive trend in the increases of *TK* mutant frequencies ($P < 0.001$, Kruskal-Wallis test), with significant elevations in observed mutant frequency values following 3-day exposures to 100 and 300 μM AZT [respective means $= (39.8 \pm 3.1) \times 10^{-6}$ and $(47.3 \pm 6.2) \times 10^{-6}$; P-values $=$ 0.05, Holm–Sidak method] compared with background [$(23.4 \pm 1.7) \times 10^{-6}$]. There was also a significant positive trend in the increases in *TK* mutant frequency values in cultures exposed to 3TC ($P = 0.01$, Kruskal–Wallis test). However, the observed mutagenic effects were significantly elevated only in cells exposed to 300 μM 3TC [mean $= (34.5 \pm 2.8) \times 10^{-6}$; $P < 0.05$, Holm–Sidak method], indicating a lower mutagenic potency for 3TC than AZT at the *TK* locus at these exposure concentrations. There was a significant positive trend for increased *TK* mutant frequencies in cells exposed to AZT-3TC ($P < 0.001$, Kruskal–Wallis test). Coexposures to AZT-3TC led to observed *TK* mutant frequencies that were significantly increased over controls at 33 μM [mean $= (33.4 \pm 2.9) \times 10^{-6}$; $P < 0.05$, Dunn's method], and the induced effect (mean $= 10.0 \times 10^{-6}$) was significantly higher than that found following exposure to 33 μM AZT alone (mean $= 6.2 \times 10^{-6}$; $P = 0.03$). Exposures to 100 and 300 μM AZT-3TC produced *TK* mutant frequencies [i.e., respective means $= (33.5 \pm 2.9) \times 10^{-6}$ and $(52.4 \pm 5.4) \times 10^{-6}$] that were significantly elevated over controls ($P < 0.05$, Holm–Sidak method). The mean induced mutant frequency at 100 μM AZT-3TC (i.e., 10.1×10^{-6}) was only 47% of the additive induced effects from single exposures to the same levels of AZT (mean $= 16.4 \times 10^{-6}$) and 3TC (mean $= 4.4 \times 10^{-6}$). The mean *TK* induced mutant frequency at 300 μM AZT-3TC (i.e., 29.1×10^{-6}) was 83% of the additive induced effects from single exposures to the same levels of AZT (mean $= 23.9 \times 10^{-6}$) and 3TC (mean $= 11.1 \times$

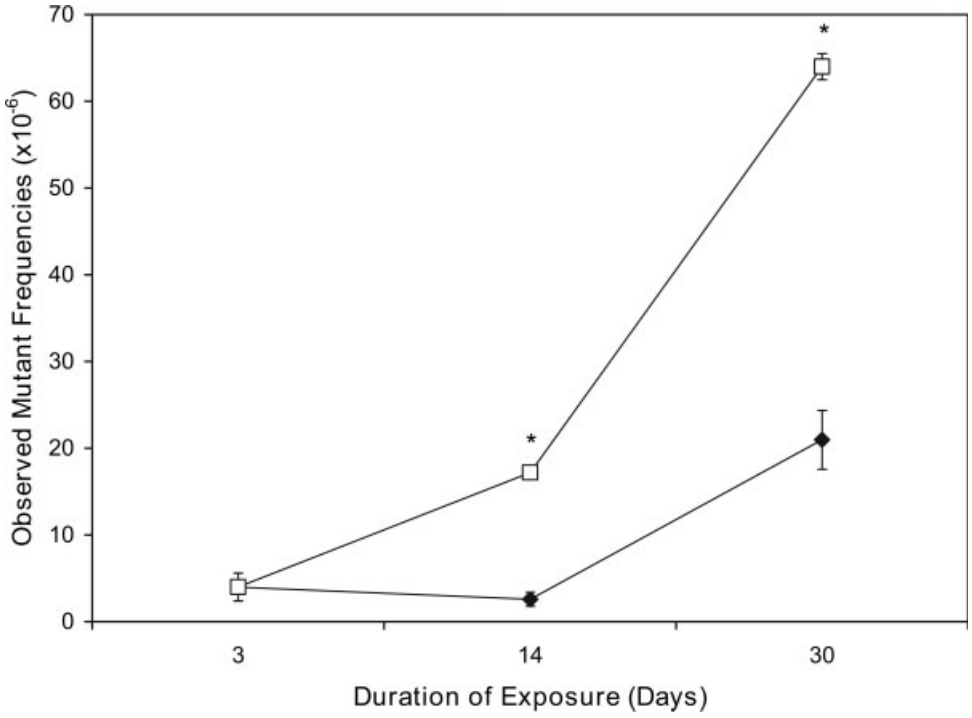

Fig. 6. Relationship between duration of exposure and observed frequency of mutations at the *TK* locus of human TK6 lymphoblastoid cells exposed to 0 (◆) or 10 μM (□) AZT-3TC for 3, 14, and 30 days. Points, average observed mutant frequency; bars, SD. Asterisks (*) indicate NRTI treatments yielding significant elevations in *HPRT* mutant frequency compared with control cells ($P < 0.05$, Student's *t*-test or Mann–Whitney U-statistic).

10^{-6}). These data suggest enhanced mutagenic responses following exposures to 33 or 300 μM AZT-3TC compared with the same exposure levels of AZT or 3TC.

Assessment of the effect of ABC exposures on *HPRT* and *TK* mutant frequencies were complicated by the high toxicity of this NRTI in TK6 cells. There were no significant mutagenic effects in either reporter gene of cells exposed to 33 μM ABC (Figs. 4 and 5). Three days of exposure to 100 μM ABC produced a substantial increase in the average observed mutant frequencies at the *HPRT* locus [$(25.4 \pm 5.7) \times 10^{-6}$; $P < 0.05$, Dunn's method] (Fig. 4) and the *TK* locus [$(57.9 \pm 1.3) \times 10^{-6}$; $P = 0.05$, Holm–Sidak method] (Fig. 5), compared with control values [$(6.9 \pm 1.1) \times 10^{-6}$ and $(30.6 \pm 3.6) \times 10^{-6}$, respectively]. These findings should be viewed with caution, however, since the relative survival in cells exposed to 100 μM ABC was only (12.1 ± 1.7)% (mean measured cloning efficiency = 9.9%). Furthermore, the 300 μM ABC exposure level was too cytotoxic to permit recovery and identification of mutant clones. Thus, it is difficult to determine whether the increase in *HPRT* mutant frequency at 100 μM ABC was a consequence of the mutagenic effect of exposure to the NRTI, or a result of artificial selection and expansion of a CHAT-treatment resistant clone that is not representative of the exposed cell population.

Data in Figures 4 and 5 permit comparisons between the mutagenic potencies of AZT-3TC-ABC vs. AZT-3TC at the *HPRT* and *TK* loci. Adding ABC to AZT-3TC treatments at concentrations of 33 μM appeared to cause little to no extra increase in *HPRT* and *TK* induced mutant frequencies compared with equimolar exposures to just AZT-3TC. Although the induced *HPRT* and *TK* mutant frequencies appeared to be greater following 3-day exposures to 100 μM AZT-3TC-ABC vs. 100 μM AZT-3TC (Figs. 4 and 5), the high degree of cytotoxicity caused by the triple drug combination at this concentration did not permit a true measure of the degree of mutagenesis caused by this exposure (i.e., 3.3% relative cell survival, or 2.7% measured cloning efficiency).

Effects of Exposure Duration at Plasma-Equivalent Concentrations of AZT-3TC on *HPRT* and *TK* Mutant Frequencies, and DNA Incorporation of AZT, in TK6 Cells

Since long-term exposure to relatively low circulating levels of NRTIs is the standard of clinical practice for treatment of HIV-infected patients, TK6 cells were exposed to equimolar, peak plasma-equivalent concentrations (10 μM) of AZT and 3TC for up to 30 days to determine if this treatment was mutagenic. *TK* mutant frequencies in exposed cells were significantly increased over those in control cells after 14 days of exposure followed by a further increase after 30 days of exposure (Fig. 6), while *HPRT* mutant frequencies were increased after 30 days of exposure

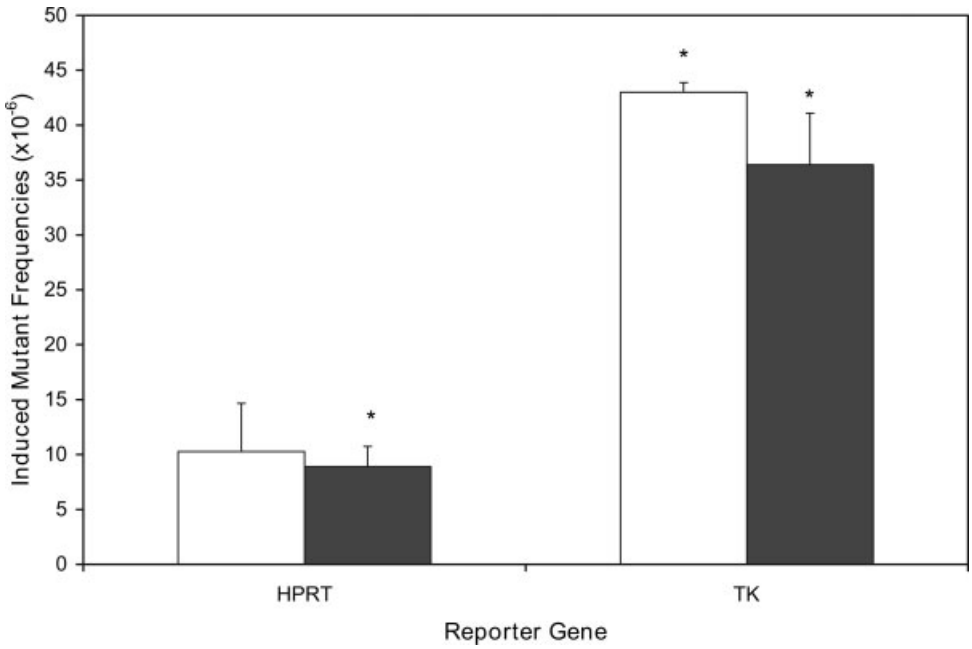

Fig. 7. Comparison of the induced mutant frequency at the *HPRT* and *TK* loci of TK6 cells exposed to 10 μM AZT-3TC for 30 days (empty bar) or 100 μM for 3 days (black bar). An asterisk (*) designates mutant frequencies that were significantly increased over control values ($P < 0.05$, Student's *t*-test or Mann–Whitney U-statistic). The induced responses at a given reporter gene were not significantly different when comparing across experiments. Error bars represent SE.

although not sufficiently so (Fig. 7). These findings demonstrated that mutant frequencies in these reporter genes increase and accumulate over time during exposure to plasma-equivalent levels of NRTIs. Furthermore, a comparison of the effects of dose rate following exposure of TK6 cells to 10 μM AZT-3TC for 30 days vs. exposure to 100 μM AZT-3TC for 3 days showed that these two very different treatment scenarios, using the same cumulative dose, caused nearly identical drug-induced mutagenic responses (Fig. 7).

To determine whether incorporation of NRTIs into cellular DNA correlated with the accumulation of reporter gene mutations induced by plasma-equivalent treatments with AZT-3TC, cellular DNA incorporation of AZT was assayed in TK6 cells exposed to 0 or 10 μM AZT-3TC for 3, 7, or 14 days (with steady state levels of AZT-DNA incorporation expected after ~6 to 7 days of treatment due to the effect of genomic equivalents [Sussman et al., 1999]). The levels of DNA incorporation of AZT were below the detection limit for the AZT-RIA method used, however, and no correlations could be tested.

Effect of Time Elapsed Since NRTI Exposure on *Hprt* Mutant Frequencies in T-Cells Isolated From Spleens of Male CD-1 Mice

To determine the relationships between transplacental exposures to NRTIs, the time elapsed since treatment, and the manifestation of *Hprt* mutant T-cells isolated from spleen, pregnant CD-1 mice were given AZT (200 mg/kg bw/day), 3TC (100 mg/kg bw/day), or AZT-3TC (200 mg AZT + 100 mg 3TC/kg bw/day) for the last 7 days of gestation. Groups of male pups were necropsied at 13, 15, and 21 days after birth (PND 13, 15, and 21) for isolation of splenic lymphocytes and performance of the T-cell cloning assay for measuring cloning efficiencies and *Hprt* mutant frequencies. The cloning efficiencies in splenic T-cells from control and NRTI-exposed CD-1 mouse pups ranged from 2.4 to 12.0%, with no apparent differences found between treatment groups at any of the three time points (Table II). On the other hand, the cloning efficiencies in PND 15 mice [mean = (6.5 ± 1.9)%] were significantly greater than those in PND 13 [mean = (3.6 ± 1.1)%] and PND 21 [mean = (3.3 ± 0.6)%] mice for unknown reasons. The mean cloning efficiencies in all groups at each time point exceeded the mean value of ~2% for splenic T-cells in control and ethylnitrosourea-treated B6C3F1 mice in the earlier transplacental study by Sussman et al. [2001]. *Hprt* mutant frequencies in PND 13 mice were significantly elevated over the background [mean = $(3.0 \pm 0.3) \times 10^{-6}$ in treatment groups exposed in utero to AZT only [mean = $(7.2 \pm 1.4) \times 10^{-6}$; $P = 0.028$, Mann–Whitney Rank Sum test] and AZT-3TC [mean = $5.3 \pm 0.6 \times 10^{-6}$; $P = 0.019$, Mann–Whitney Rank Sum test], but not to 3TC alone (Table II, Fig. 8). Although the mean induced mutant frequency in PND 13 mice exposed to AZT alone [$(4.2 \pm 1.4) \times 10^{-6}$) was greater that that observed in PND mice exposed to AZT-3TC [$(2.0 \pm 1.3) \times 10^{-6}$], the difference wasn't significant. Mutant frequencies in the AZT and AZT-3TC

TABLE II. Cloning Efficiencies and *Hprt* Mutant Frequencies in T-Cells From Spleens of 13, 15, and 21 Day-Old Male CD-1 Mice Exposed Transplacentally to AZT, 3TC, or AZT/3TC[a]

PND 13			PND 15			PND 21		
Sample No.	CE (%)	$Mf \times 10^{-6}$	Sample No.	CE (%)	$Mf \times 10^{-6}$	Mouse No.	CE (%)	$Mf \times 10^{-6}$
	Control			*Control*			*Control*	
AS 1 ($n = 4$)	4.64	2.11	AS 1 ($n = 4$)	7.58	0.38	AS 1	2.40	1.93
AS 2 ($n = 2$)	4.92	3.54	AS 2 ($n = 4$)	5.79	0.53	AS 2	3.79	1.36
AS 3 ($n = 4$)	3.23	3.09	AS 3 ($n = 2$)	4.64	2.00	AS 3	4.07	1.42
AS 4 ($n = 4$)	2.95	3.37	AS 4 ($n = 2$)	3.79	2.65	AS 4	3.51	1.10
Mean $Mf = 3.0 \pm 0.3 \times 10^{-6}$			Mean $Mf = 1.4 \pm 0.6 \times 10^{-6}$			AS 5	3.79	1.84
						AS 7	2.40	2.90
						AS 8	3.51	1.98
						AS 9	4.64	1.88
						Mean $Mf = 1.8 \pm 0.2 \times 10^{-6}$		
	AZT			*AZT*			*AZT*	
BS 1 ($n = 2$)	3.23	8.64	BS 1 ($n = 2$)	6.38	0.61	BS 1	2.67	1.73
BS 2 ($n = 2$)	2.67	3.06	BS 2 ($n = 2$)	6.68	1.60	BS 2	3.23	1.80
BS 3 ($n = 4$)	2.40	7.77	BS 3 ($n = 2$)	12.01	0.24	BS 3	3.51	1.65
BS 4 ($n = 4$)	3.51	9.37	BS 4 ($n = 2$)	7.28	1.21	BS 4	3.79	1.84
Mean $Mf = 7.2 \pm 1.4 \times 10^{-6}$ $P = 0.028$			BS 5 ($n = 2$)	4.07	0.99	BS 5	3.51	1.89
			Mean $Mf = 0.9 \pm 0.3 \times 10^{-6}$			BS 6	3.79	1.84
						Mean $Mf = 1.8 \pm 0.4 \times 10^{-6}$		
	3TC			*3TC*			*3TC*	
CS 1 ($n = 2$)	0.99	3.68	CS 1 ($n = 2$)	6.38	0.36	CS 1	4.07	1.14
CS 2 ($n = 4$)	2.67	1.86	CS 2 ($n = 2$)	6.98	0.63	CS 2	2.95	1.57
CS 3 ($n = 2$)	2.67	1.86	CS 3 ($n = 2$)	6.38	0.36	CS 3	3.23	2.70
Mean $Mf = 2.5 \pm 1.4$ (SE) $\times 10^{-6}$			Mean $Mf = 0.5 \pm 0.1 \times 10^{-6}$			Mean $Mf = 1.8 \pm 0.5 \times 10^{-6}$		
	AZT-3TC			*AZT-3TC*			*AZT-3TC*	
DS 1 ($n = 4$)	5.50	5.43	DS 1 ($n = 2$)	6.98	0.79	DS 1	3.23	2.13
DS 2 ($n = 4$)	3.79	6.70	DS 2 ($n = 4$)	5.21	2.12	DS 2	2.67	1.95
DS 3 ($n = 2$)	2.40	3.62	DS 3 ($n = 2$)	7.28	2.29	DS 3	3.79	1.36
DS 4 ($n = 2$)	5.50	5.43	Mean $Mf = 1.7 \pm 0.5 \times 10^{-6}$			DS 4	2.67	1.58
Mean $Mf = 5.3 \pm 0.6 \times 10^{-6}$ $P = 0.019$						DS 5	3.79	1.07
						DS 6	2.67	2.60
						DS 7	2.40	2.76
						Mean $Mf = 1.9 \pm 0.2 \times 10^{-6}$		

[a]Pregnant CD-1 mice were given 0, 200 mg AZT, 100 mg 3TC, or 200 mg AZT + 100 mg 3TC per kg body weight in saline by gavage daily on days 12 through 18 of gestation. Groups of male mouse offspring were necropsied on postnatal day (PND) 13, 15, or 21, and cloning efficiency (CE) and *Hprt* mutant frequency (M*f*) data were obtained using the T-cell cloning assay under nonselective and selective conditions, respectively, as described in the Materials and Methods. For PNDs 13 and 15, the number of mouse spleens pooled for measurements is shown in parenthesis after the sample number. M*f*s for individual groups and time points are expressed as means ± standard error. Only *P*-values for significant findings are listed.

groups then declined rapidly to control animal values by PND 15, and remained at background levels at PND 21 (the biological basis for this pattern is considered in the Discussion). Spontaneous *Hprt* mutant frequencies in control mice from all time points combined [$n = 16$; mean mutant frequency $= (2.0 \pm 0.9) \times 10^{-6}$] were almost identical to those previously found in similar aged mice [Sussman et al., 2001].

DISCUSSION

The current investigation of AZT, 3TC, and ABC was part of a larger effort to determine the relative degree to which different NRTIs, alone or in clinically relevant drug combinations, induce and/or potentiate mutagenic effects (i) in human TK6 lymphoblastoid cells exposed in culture at drug concentrations ranging down to peak plasma levels in humans and (ii) in mice exposed transplacentally to clinically relevant cumulative doses of AZT and/or 3TC. More than a dozen studies, including the current report, have examined the mutagenicity of AZT at the *HPRT* and *TK* loci in experimental systems, and they have consistently demonstrated that AZT causes small- and large-scale genetic alterations over a range of treatment modalities (Table III). Our research team had previously reported that the NRTI drug combination of AZT plus ddI showed significantly greater genotoxic and mutagenic responses at both the *HPRT* and *TK* loci in TK6 cells compared with those produced by the sum of equimolar exposures to each drug alone [Meng et al., 2000b, 2002]. Our initial follow-up studies were aimed primarily at investigating the in vitro or in utero mutagenicity of AZT and 3TC, so that we could correlate the responses in cultured human cells or mouse offspring to findings in recently completed population studies

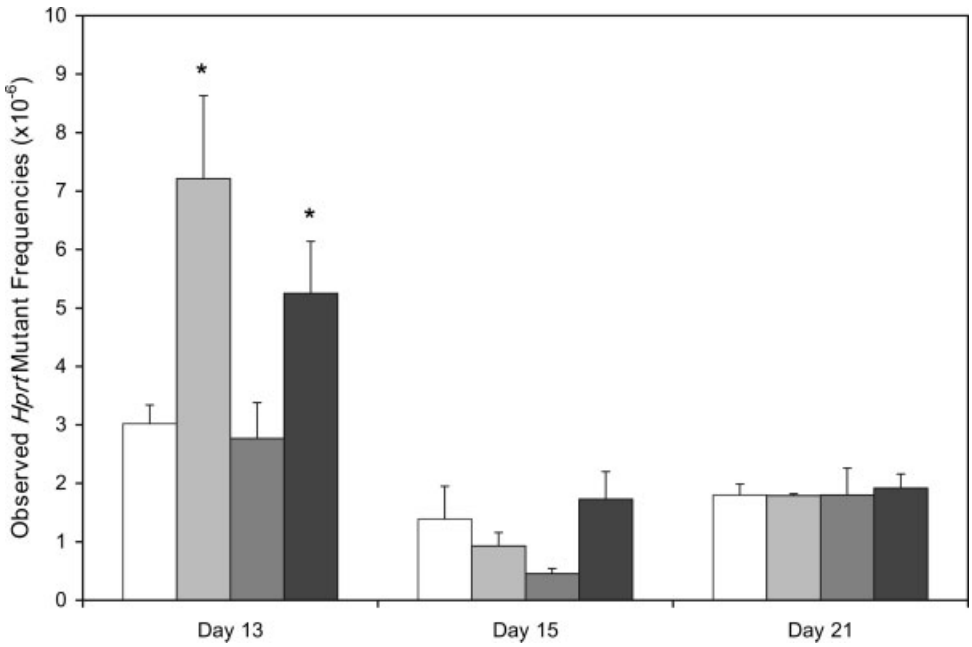

Fig. 8. Relationship between transplacental NRTI exposure and the effect of time elapsed since exposure on *Hprt* mutant frequencies in T-cells from spleens of CD-1 male mouse offspring. Pregnant CD-1 mice were dosed with sterile PBS vehicle, 200 mg AZT/kg body weight/day, 100 mg 3TC/kg body weight/day, or a combination of 200 mg AZT/kg/day + 100 mg 3TC/kg/day, on days 12 through 18 of gestation. Groups of male pups were necropsied on days 13, 15, and 21 postpartum ($n = 3$ to 14/group, see Table II). Control, empty bar; AZT, light gray bar; 3TC, gray bar; AZT/3TC, black bar; error bars represent SE. Asterisks (*) indicate NRTI treatments yielding significant elevations in *HPRT* mutant frequency compared with control cells ($P < 0.05$, Student's *t*-test or Mann–Whitney U-statistic).

of the mutagenicity of AZT or AZT-3TC in infants born to HIV+ mothers [O'Neill et al., 2001; Poirier et al., 2004; Escobar et al., 2007].

Following 3-day treatments of TK6 cells with individual NRTIs (i.e., AZT, 3TC, or ABC), the pattern in potential cytotoxic effects was atypical for cells exposed to 33 or 100 μm 3TC, with relative cell survivals being significantly greater than the mean control value. These observations suggest that 3TC may slow cell turnover and/or inhibit cellular processes involving apoptosis. The same phenomenon was found in cells exposed to 33 μm AZT-3TC but not with 33 μm AZT alone. These findings were consistent with an earlier report that treatment of asymptomatic HIV-infected patients with AZT-3TC had a stronger effect on potentially beneficial immune parameters, including more efficient IL-2 production and reduction of apoptosis in lymphocytes, compared with treatment with AZT-ddI [Piconi et al., 2000].

Cytotoxicity and mutagenicity experiments with ABC or AZT-3TC-ABC were complicated by the fact that ABC was the most cytotoxic NRTI, with the cytotoxicity of AZT-3TC-ABC tracking closely with the extreme cytotoxic effects of ABC alone (Fig. 1). This problem prevented a reliable estimation of the differences in the relative in vitro mutagenic potencies of ABC, AZT-3TC, and AZT-3TC-ABC, and precluded a reasonable prediction of the degree to which the cytotoxic effects of ABC at plasma levels may impact cell turnover and mutagenic responses in patients given AZT-3TC-ABC compared with the already significant mutagenic effects found in mother-child pairs receiving AZT-3TC [Poirier et al., 2004; Escobar et al., 2007]. Although not recommended by the Perinatal HIV Guidelines Working Group, some clinical centers in the U.S. are now administering AZT-3TC-ABC to HIV-infected pregnant women. Therefore, the mutagenic potential of ABC alone, and in combination with other NRTIs, needs to be examined using other experimental conditions (e.g., at lower concentrations of ABC for longer treatment periods) and/or model systems, and potential deleterious fetal toxicities in humans and animal models should be investigated.

Previous studies provide compelling evidence that, in the *HPRT* reporter gene, the net mutagenic effects in lymphocytes of human infants exposed prepartum to low daily doses of AZT-3TC for months of gestation are similar to those found in the current study of lymphocytes isolated from spleens of mice exposed transplacentally to high daily doses of AZT during the last 40% of an 18-day gestation [O'Neill et al., 2001; Poirier et al., 2004]. To determine if this dose rate phenomenon was reproducible in vitro, the induced *HPRT* and *TK* mutant frequencies following exposure of TK6 cells to 10 μM AZT-3TC for 30 days were compared with those after exposure to 100 μM AZT-3TC for 3 days (i.e., where the cumulative dose for each exposure scenario was the same). These exposures resulted in nearly identical drug-induced mutagenic responses (Fig. 7), indicating that mutagenesis is driven by cumulative dose and

TABLE III. In Vitro and In Vivo Mutagenicity Studies of AZT at the *HPRT* and *TK* Loci

Test system	Test modality	Reference[a]
Chinese hamster ovary cells, *Hprt* locus	AZT in vitro	Grdina et al. [1992]
Human HepG2 cells, *HPRT* locus	AZT in vitro	Grdina et al. [1992]
Mouse lymphoma L5178Y cells, *Tk* locus	AZT in vitro	Ayers et al. [1996]
Human TK6 lymphoblastoid cells, *HPRT* locus	AZT in vitro	Sussman et al. [1999]
Human TK6 lymphoblastoid cells, *TK* locus	AZT in vitro	Meng et al. [2000a]
Human TK6 lymphoblastoid cells, *HPRT* and *TK* loci	AZT and/or ddI in vitro	Meng et al. [2000b]
Mouse T-lymphocytes, *Hprt* and *Tk* loci	AZT and/or 3TC neonatally	Von Tungeln et al. [2002]
Human TK6 lymphoblastoid cells, *TK* locus	AZT and/or ddI in vitro	Meng et al. [2002]
Mouse T-lymphocytes, *Hprt* and *Tk* loci	AZT and/or ddI neonatally	Von Tunglen et al. [2004]
Mouse T-lymphocytes, *Tk* locus	AZT neonatally	Mittelstaedt et al. [2004]
Mouse T-lymphocytes, *Hprt* locus	AZT perinatally	Dobrovolsky et al. [2005]
Human TK6 lymphoblastoid cells, *HPRT* and *TK* loci	AZT and/or 3TC, ABC, or ABC-3TC-ABC in vitro	Torres et al. [current paper]
Mouse T-lymphocytes, *Hprt* locus	AZT and/or 3TC in utero	Torres et al. [current paper]
Mouse T-lymphocytes, *Hprt* and *Tk* loci	AZT and/or 3TC in utero	Von Tungeln et al. [2006]
Mouse lymphoma L5178Y cells, *Tk* locus	AZT in vitro	Wang et al. [2007]

[a]Listed in chronological order of study.

that mutations accumulate over time during exposure of human cells in vitro to plasma-equivalent levels of NRTIs. This dose rate effect helps explain the occurrence of significant increases in *HPRT* mutant frequencies in cord blood lymphocytes and glycophorin A N/N variant frequencies (i.e., frequencies of allele loss with duplication type somatic mutations) in cord blood RBCs from newborn children exposed in utero to AZT-3TC [O'Neill et al., 2001; Poirier et al., 2004; Escobar et al., 2007].

In complementary in vitro experiments to investigate the relationships between NRTI drug concentration and mutagenic effects in TK6 cells exposed for 3 days, dose-related increases in *HPRT* and *TK* mutant frequencies were observed following exposures to AZT, 3TC, or AZT-3TC. Compared with single drug exposures, the combined AZT-3TC exposures yielded additive to synergistic elevations in *HPRT* mutant frequencies following 33, 100, or 300 μM treatments, and enhanced or nearly additive increases in *TK* mutant frequencies after 33 or 300 μM treatments.

Our in vitro study results suggest the hypothesis that patients receiving HAART using combined NRTIs may be at greater risk for cancer than those given a single NRTI; support for this hypothesis comes from several recent studies that evaluated changes in non-AIDS-related cancers in HIV-infected patients in the pre-HAART vs. post-HAART eras. The potential association of HAART with cancer was assessed in two studies of causes of death in HIV-infected populations in France and Switzerland [Bonnet et al., 2004; Clifford et al., 2005]. In the pre-HAART era, deaths due to neoplasia were estimated to account for $<10\%$ of all deaths in HIV-infected patients. In the post-HAART era, this figure increased to 28%, with AIDS-related and non-AIDS-related malignancies cited as the cause of death in 15 and 13% of the cases, respectively. These studies concluded that the introduction of HAART in industrialized nations was associated with malignant disease as a major cause of death among HIV-infected patients.

Although the oldest children exposed perinatally to AZT are now 11 years of age [Connor et al, 1994], the failure to find increased incidences of any tumor type to this point in time is not indicative of the future cancer risk for this population. Quite the opposite, the overall findings in children exposed perinatally to NRTIs are troubling because (i) mutational events in developing cells were a prerequisite for transplacentally induced cancers in tissues of AZT-exposed rodents [Olivero et al., 1997; Diwan et al., 1999; NTP, 2005; Walker et al., 2007], (ii) multiple *p53* gene mutations occured in individual AZT-induced lung tumors of transplacentally exposed mice [Hong et al., 2007], and (iii) in utero AZT-3TC exposure of human infants induced *HPRT* gene transversion mutations predictive of those found in the mouse lung tumors caused by AZT [O'Neill et al., 2001].

In light of these findings, an important question is whether HAART using AZT-3TC produces greater mutagenic effects than AZT as the sole NRTI in transplacentally-exposed children. Results from studies designed to address this question have yielded some inconclusive results. Early studies showed that AZT and 3TC acted together to achieve enhanced HIV suppression in vitro and synergistic protection against HIV disease progression and vertical transmission when compared with administration of AZT alone [Hoogard et al., 2000; Snyder et al., 2000]. At the same time, greater host cell DNA incorporation of

AZT and induction of reporter gene mutations occurred in mother-child pairs exposed to AZT-3TC compared with AZT [O'Neill et al., 2001; Poirier et al., 2004; Escobar et al., 2007; Meng et al., 2007]. It is noteworthy that the elevations in *HPRT* mutant frequencies and *GPA* N/N variant frequencies in the group of infants exposed in utero to AZT-3TC was greater than those in the group exposed to AZT as the only NRTI [O'Neill et al., 2001; Escobar et al., 2007]; however, the sample size for the AZT group was too small to determine if the differences in drug-induced mutagenic responses in the two groups were significant.

This question was further addressed in this report and other recent studies that examined mutagenic responses in the *Hprt* or *Tk* genes of splenic lymphocytes from CD-1, *p53*-heterozygous, or *Tk*-heterozygous mice exposed perinatally to AZT, 3TC, or AZT-3TC. These recent mutagenicity studies used daily doses of AZT shown to be tumorigenic during four independent transplacental cancer bioassays in CD-1 mice, B6C3F1 mice, and F344 rats [Olivero et al., 1997; Diwan et al., 1999; NTP, 2005; Walker et al., 2007]. In the present study, CD-1 mice were exposed in utero to AZT and/or 3TC during the last 7 days of gestation, and the change in *Hprt* mutant frequency over time was assessed in lymphocytes isolated from spleens of mouse pups necropsied at three time points after birth to take into consideration age-dependent differences in the trafficking of T-cells. The three time points selected for measuring *Hprt* mutant frequencies in splenic T-cells were based upon the earlier findings that the maximum mutagenic response occurred at 13 days after birth in spleens of B6C3F1 mice exposed transplacentally to a single dose (on day 18 of gestation) or split doses of ethylnitrosourea (on days 12–18 of gestation) [Sussman et al., 2001]. In the current study, significant increases in *Hprt* mutant frequencies of 2.4-fold and 1.7-fold over background were found in the AZT and AZT-3TC treatment groups of 13-day-old mice, followed by a rapid decline to control animal values due to selective pressures against *Hprt* mutant cells and dilution by maturation and trafficking of new T-cells to peripheral blood. The underlying reasons for this pattern in T-cell kinetics and manifestation of *Hprt* mutants have been considered in detail elsewhere [Jones et al., 1987; Crippen and Jones; 1989; Deubel et al., 1996; Walker et al., 1999; Judice 2001].

In a preliminary report of comparable mutagenicity studies in $p53^{+/-}$ mice, *Hprt* mutant frequencies were significantly elevated above background in splenic T-cells isolated from mouse offspring immediately after the last dose of AZT (40, 80, or 160 mg/kg bw/day) or AZT-3TC (160 + 100 mg/kg bw, respectively, per day) following transplacental treatment during the last 7 days of gestation and neonatal treatment through PND 28 [Dobrovolsky et al., 2005]. In $p53^{+/+}$ mice, only the AZT-3TC combination showed a significant increase in *Hprt* mutant frequency. These combined *Hprt* mutation studies in CD-1 and $p53^{+/-}$ mice demonstrate that AZT and AZT-3TC are perinatal mutagens causing small-scale genetic alterations in mouse models.

In B6C3F1/$Tk^{+/-}$ mice, the frequencies of *Hprt* and *Tk* mutant T-cells were measured in spleens at both 3 and 5 weeks postpartum, following transplacental exposure to AZT (240 mg/kg bw/day), 3TC (120 mg/kg bw/day), or AZT-3TC (240 + 120 mg/kg bw, respectively, per day) during the last 7 days of gestation [Von Tungeln et al., 2007]. At 3 weeks after birth, mutant frequencies were significantly increased at the *Tk* locus but not the *Hprt* locus of $Tk^{+/-}$ mice exposed to AZT or AZT-3TC. Three-week-old mice, rather than younger offspring, were used in these studies to permit recovery of sufficient numbers of splenic lymphocytes for measuring both *Tk* and *Hprt* mutant cells in individual animals. While this was a commendable goal, results of earlier transplacental mutagenicity studies of ethylnitrosourea in B6C3F1 mice [Sussman et al., 2001], and the current in utero mutagenicity studies of AZT, 3TC, and AZT-3TC in CD-1 mice, provide clear evidence that measurement of mutant frequencies in splenic T-cells at PND 21 is too late for obtaining data informative about the potential mutagenicity of the selected NRTIs in utero at the *Hprt* locus. On the other hand, *Tk* mutant lymphocytes appear to persist longer than *Hprt* mutant cells following the peak mutagenic responses in the spleen, and the report of Von Tungeln et al. [2007] provided valuable and insightful evidence that AZT, 3TC, and AZT-3TC are transplacental mutagens causing large-scale genetic alterations in the mouse.

Since growing concerns about the health effects of NRTIs may pose a major threat to the lasting success of HIV treatment/prophylaxis, there is a great need for a better understanding of the potential genotoxicities of the current array of NRTI-based therapies [Wutzler and Thust, 2001; Poirier et al., 2004]. To date, however, only two experimental models, based upon the *HPRT* and *TK* reporter genes, have been used to compare the relative mutagenic potencies of individual NRTIs vs. combinations of NRTIs (Table III). Only in vitro studies in TK6 cells treated with AZT and/or ddI [Meng et al., 2000b], AZT and/or 3TC (current report), or ddI and/or 3TC [Carter et al., 2007] have shown additive to synergistic induction of small-scale mutations (as detected in the *HPRT* gene) and large-scale mutations (as detected in the *TK* gene) by combinations of NRTIs. In contrast, transplacental, neonatal, or perinatal mutagenicity studies in mice treated with AZT and/or ddI, or AZT and/or 3TC, have demonstrated the mutagenicity of individual NRTIs, or pairs of drugs, with little to no evidence of additive mutagenic effects when the agents were given in pairs [Von Tungeln et al., 2002, 2004, 2007; Dobrovolsky et al., 2005; current report]. Thus, biomarker-based clinical studies in patients receiving NRTIs, and additional studies in experimental systems, are essential in developing more definitive evidence of the relative mutagenic effects and the molecular events underlying the risks for cancer from peri-

natal prophylaxis in children, and long-term therapy in HIV-infected individuals, using one, two, or three NRTIs.

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