Frequency of Tk and Hprt lymphocyte mutants and bone marrow micronuclei in mice treated neonatally with zidovudine and didanosine

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The nucleoside analog zidovudine (3’-azido-3’-deoxythymidine, AZT), by itself or in combination with other anti-retroviral drugs, is used perinatally to prevent mother to child transmission of human immunodeficiency virus type 1. AZT is mutagenic in vitro and mutagenic and carcinogenic when administered to neonatal mice. A previous study indicated that the anti-retroviral agent didanosine (2’,3’-dideoxyninosine, ddI) potentiated the mutagenicity of AZT in the thymidine kinase (TK) gene of cultured human TK6 lymphoblastoid cells. We have evaluated whether or not ddI affects the in vivo genotoxicity of AZT by breeding C57Bl/6N/Tk+/+ female mice with C3H/HeNMTV male mice and treating the offspring daily on postnatal days 1–8 with 200 mg/kg ddI alone or in combination with 200 mg/kg AZT. One day after the last dose, bone marrow polychromatic erythrocytes (PCEs) were obtained to assess the induction of micronuclei; 3 weeks following treatment, the induction of mutants was determined in the hypoxanthine-guanine phosphoribosyltransferase (Hprt) and Tk genes of splenic T lymphocytes from B6C3F1/Tk+/+ mice. The mixture of AZT and ddI, but not ddI alone, caused a significant increase in micronucleated PCEs. When assessed 3 weeks after dosing, ddI did not induce mutations in the Hprt or Tk genes. The mixture of AZT and ddI also did not induce mutations in the Hprt gene, but did induce a significant increase in Tk mutants, similar to that observed previously with AZT alone. The induction of mutations in the Tk gene by the mixture of AZT and ddI was associated with loss of the wild-type Tk+ allele. These data indicate that, under the conditions of this experiment, ddI is not mutagenic in neonatal B6C3F1/Tk+/+ mice and that it does not potentiate the mutagenicity of AZT.

Materials and methods

Chemicals
AZT was obtained from Glaxo Wellcome (Research Triangle Park, NC) and ddI was acquired from Bristol-Meyers Squibb (Princeton, NJ). N-Ethyl-N-nitrosourea (ENU) was purchased from Sigma Chemical Co. (St Louis, MO).

Animals
Transgenic mice heterozygous for the Tk gene were created using an embryonic stem cell line in which one allele of the endogenous Tk gene was inactivated by targeted homologous recombination (Dobrovolsky et al., 1999a). The Tk+/- genotype has been gradually transferred to a C57Bl/6N background by breeding Tk+/- male mice to C57Bl/6N female mice. The offspring were genotyped by allele-specific PCR, as described previously (Dobrovolsky et al., 1999b), and Tk+/- males from the cross were bred with C57Bl/6N females. To generate the mice used in this study, female C57Bl/6N/Tk+/- mice, obtained transplacentally or neonatally (Olivero et al., 1997; Diwan et al., 1999), a response that has been correlated with the incorporation of AZT into DNA (Olivero et al., 1997). AZT is also incorporated into the DNA of non-human primates (Poirier et al., 1999) and humans, including DNA from the cord blood leukocytes of infants exposed to AZT in utero (Olivero et al., 1999).

Didanosine (2’,3’-dideoxynosine, ddI), when combined with AZT, delays HIV-1 disease progression and death as compared with AZT alone (HIV Trials’ Collaborative Group, 1999). As with AZT, a number of side-effects occur from the use of ddI, including neuropathy, hepatic steatosis and lactic acidemia and pancreatitis (Carr and Cooper, 2000). These toxicities appear to be a consequence of inhibiting mitochondrial DNA polymerase γ with a resultant impairment of mitochondrial enzyme synthesis and oxidative phosphorylation (Carr and Cooper, 2000). ddI also appears to potentiate the mutagenicity of AZT because when human TK6 lymphoblastoid cells were incubated with AZT, ddI or a mixture of AZT and ddI, the combination of AZT and ddI increased the incorporation of AZT into cellular DNA and the mutant frequency within the hypoxanthine-guanine phosphoribosyltransferase (HPRt) and thymidine kinase (Tk) genes (Meng et al., 2000). The increase in mutant frequency was attributed to an increase in point mutations (Meng et al., 2002).

In previous work we demonstrated that AZT, but not the nucleoside analog lamivudine [(±)2’,3’-dideoxy-3’-thiocytidine, 3TC], was genotoxic to neonatal mice, as indicated by an increased mutant frequency in the Tk gene and an increased frequency of micronucleated polychromatic erythrocytes (PCEs). Furthermore, 3TC in combination with AZT did not alter the responses observed with AZT alone (Von Tungeln et al., 2002). In a subsequent study we found that the major mechanisms of mutation induction by AZT involved deletion and recombination (Mittelstaedt et al., 2004). We have now extended these studies to investigate the induction of mutants and micronuclei in mice treated neonatally with ddI or ddI in combination with AZT.
from eight generations of backcrossing, were mated with male C3H/HeNMTV mice. The offspring of this mating are referred to as B6C3F1/Tk mice (i.e. a combination of both B6C3F1/Tk+/- and B6C3F1/Tk+/- mice). This combination was used for the micronucleus assays, while B6C3F1/Tk+/- mice were used for the mutagenesis assays.

**Treatment of B6C3F1/Tk neonatal mice**

Male and female B6C3F1/Tk mice (45–94 per group) were treated daily on postnatal days (PNDs) 1–8 with 200 mg/kg ddI or a mixture of 200 mg/kg AZT and 200 mg/kg ddI. The total doses of each drug were 4 mg. These doses are identical to those used in our previous study with AZT and 3TC (Von Tungeln et al., 2002). The compounds were dissolved in dimethylsulfoxide (DMSO) and administered in a 5 µl volume by i.p. injection. Solvent-treated control mice received daily injections of 5 µl of DMSO. A single injection of 167 µg ENU in 5 µl of 50% aqueous DMSO was administered at PND 8 to serve as a positive control for the mutagenesis assays. Two separate mutagenesis assays were conducted. Each assay included ddI or AZT and ddI, a solvent-treated negative control group and an ENU-treated positive control group.

**Analysis of micronuclei**

One day after the last dose (PND 9), bone marrow was collected from B6C3F1/Tk mice, applied to microscope slides, fixed and stained with acridine orange as described by Tinwell and Ashby (1989). Micronuclei were scored, without knowledge of the treatment, in 1000 PCEs from each mouse.

**In vivo mutagenesis**

Three weeks after the last treatment (PND 28), the mutant frequency was determined in the Hprt and Tk genes of splenic T lymphocytes from B6C3F1/Tk+/- mice. This time point corresponds to that used in our previous study with AZT and 3TC (Von Tungeln et al., 2002) and is approximately the time of maximum mutation induction in the Hprt gene of splenic T lymphocytes from preweanling male mice administered ENU (Walker et al., 1999). Assays for the frequency of Hprt mutants, as indicated by resistance to 6-thioguanine (6-TG), and Tk mutants, as indicated by resistance to 5-bromodeoxyuridine (BrdU), were conducted as described by Meng et al. (1998) and Dobrovolsky et al. (1999b), respectively, with the modifications indicated in Von Tungeln et al. (2002). Loss of heterozygosity (LOH) in BrdU-resistant (BrdU+) T lymphocyte clones was measured by allele-specific PCR (Dobrovolsky et al., 1999b) as outlined in Von Tungeln et al. (2002).

**Statistical analyses**

Statistical analyses of cloning efficiency, mutation frequency and micronucleus data were conducted by analysis of variance (ANOVA). Comparisons between individual treatments and the control were conducted by one-tailed unpaired t-tests; two-tailed unpaired t-tests were used for comparisons between treatment groups. When necessary, the data were log transformed before the analyses to maintain homogenous variances, a normal data distribution or both. If a suitable transformation could not be found, the data were analyzed by a Kruskal–Wallis ANOVA and comparisons between individual treatments were made using the Mann–Whitney test. Analyses of LOH and intragenic mutation frequencies were conducted by χ² tests. Bonferroni-type adjustments (Wright, 1992) were applied to correct for multiple comparisons.

**Results**

**Animal treatment**

Male and female B6C3F1/Tk mice were treated daily on PNDs 1–8 with ddI or a mixture of AZT and ddI. Fifty-nine percent of the solvent-treated control mice died during the 8 day dosing period. The corresponding values for mice treated with ddI and the mixture of AZT and ddI were 55 and 63%. Most of the deaths occurred during the first 3 days of dosing. A similar mortality was observed when using other solvents, including saline, water and 50% aqueous DMSO (Von Tungeln and Beland, unpublished observations), thus, the high mortality appears to be due to stress placed upon the neonatal mice rather than the use of DMSO as a solvent.

**Hprt mutant frequency**

Three weeks after the last treatment, the Hprt mutant frequency was assessed in splenic T lymphocytes from B6C3F1/Tk+/- mice by determining the number of clones that grew in the presence of 6-TG. None of the responses was sex dependent, thus the data from the male and female mice were combined. Likewise, none of the treatments had a significant effect upon the cloning efficiencies of the T lymphocytes (Table I). When compared with the solvent-treated control mice, neither treatment with ddI nor a mixture of AZT and ddI significantly increased the frequency of 6-TG-resistant (6-TG+) clones (Table I). A significant increase in 6-TG+ clones was observed with the positive control ENU.

**Tk mutant frequency**

The Tk mutant frequency was assessed in splenic T lymphocytes from B6C3F1/Tk+/- mice by determining the number of clones that grew in the presence of BrdU. Compared with the solvent-treated controls, ddI did not affect the Tk mutant frequency (Table I). Treatment with the mixture of AZT and ddI resulted in a significant increase in the frequency of BrdU-resistant (BrdU+) T lymphocyte clones that was similar in extent to that previously reported for AZT (Von Tungeln et al., 2002). A significant increase in mutant frequency was also observed with the positive control ENU (Table I).

**LOH analysis of BrdU+ lymphocyte clones**

The Tk gene of BrdU+ clones was analyzed for LOH using allele-specific PCR. The data are summarized in Table II. As previously described (Von Tungeln et al., 2002), the PCR results were divided into three classes. In instances where the intensity of the wild-type Tk+ allele was greatly diminished compared with the disrupted Tk- allele the clone was classified as having LOH. When the alleles had a similar intensity, the clone was considered to contain an intragenic mutation, such as a point mutation or frameshift. When results were ambiguous (~5% of the samples) the clone was classified as ‘other’. The distribution of LOH and intragenic mutation in BrdU+ clones from mice treated with ddI alone did not differ from the solvent-treated control mice. In contrast, BrdU+ clones from

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>Cloning efficiency (%)</th>
<th>Hprt mutant frequency (6-TG+ lymphocytes/10⁶ cells)</th>
<th>Tk mutant frequency (BrdU+ lymphocytes/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28</td>
<td>7.3 ± 0.4</td>
<td>0.3 ± 0.1</td>
<td>39 ± 5</td>
</tr>
<tr>
<td>AZT±</td>
<td>17</td>
<td>5.5 ± 0.3</td>
<td>2.7 ± 0.8</td>
<td>77 ± 17</td>
</tr>
<tr>
<td>ddI</td>
<td>19</td>
<td>8.0 ± 0.5</td>
<td>1.0 ± 0.6</td>
<td>37 ± 6</td>
</tr>
<tr>
<td>AZT + ddI</td>
<td>17</td>
<td>6.5 ± 0.7</td>
<td>2.6 ± 2.1</td>
<td>87 ± 12</td>
</tr>
<tr>
<td>ENU</td>
<td>4</td>
<td>5.4 ± 1.1</td>
<td>70 ± 13</td>
<td>73 ± 23</td>
</tr>
</tbody>
</table>

*Male and female B6C3F1/Tk+/- mice were treated daily on PNDs 1–8 with 200 mg/kg ddI or a mixture of 200 mg/kg AZT and 200 mg/kg ddI. Three weeks after the last treatment the Hprt (6-TG+) and Tk (BrdU+) mutant frequencies were assessed in splenic T lymphocytes. The data are expressed as means ± SEM.

**Wright, 1992** were applied to correct for multiple comparisons.

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mice administered the mixture of AZT and ddI had a significantly greater percentage of LOH and a concomitant reduction in intragenic mutation, with the distribution being similar to that observed previously (Von Tungeln et al., 2002) with AZT alone.

**Analysis of micronucleated PCEs**

The induction of micronuclei in PCEs from B6C3F1/Tk mice was assessed 1 day after the last dose (Figure 1). Previous experiments indicated that AZT-induced micronuclear responses were equivalent in Tk<sup>+</sup> and Tk<sup>-</sup> mice (Heflich, unpublished data), therefore, these experiments were performed without reference to the Tk genotype of the mice. The responses did not differ between sexes. Compared with the solvent-treated controls, the administration of ddI did not increase the frequency of micronucleated PCEs. The mixture of AZT and ddI significantly increased the micronucleus frequency, to an extent that did not differ significantly from that previously found (Von Tungeln et al., 2002) for AZT alone.

**Discussion**

The activation of AZT to an anti-retroviral agent involves sequential phosphorylation to AZT 5'-monophosphate, diphosphate and triphosphate, which is catalyzed by thymidine kinase, thymidylate kinase and pyrimidine nucleoside diphosphate kinase, respectively (reviewed in Peter and Gambertoglio, 1998; IARC, 2000). AZT 5'-triphosphate is thought to inhibit viral reverse transcriptase directly, with a resultant termination of proviral DNA replication. AZT 5'-triphosphate also inhibits mammalian DNA polymerases (Furman et al., 1986; Cheng et al., 1987; Huang et al., 1990; Vazquez-Padua et al., 1990; Nickel et al., 1992), in particular polymerase γ, the DNA polymerase found in mitochondria (Lewis and Dalakas, 1995). Furthermore, the incorporation of AZT into mammalian DNA has been demonstrated in vitro and in vivo (reviewed in IARC, 2000).

ddI is also activated by sequential phosphorylation. The initial step involves 5'-nucleotidase-catalyzed formation of ddI 5'-diphosphate, which is converted to 2',3'-dideoxyadenosine (ddA) 5'-phosphate by the action of adenylyl cyclase and adenylyl lyase (Kewn et al., 1999). ddA 5'-phosphate is then converted to ddA 5'-diphosphate and ddA 5'-triphosphate by adenylyl kinase and, perhaps to a lesser extent, by 5'-phosphoribosyl-1-pyrophosphate synthetase (Navé et al., 1994; Kewn et al., 1999). As with AZT 5'-triphosphate, ddA 5'-triphosphate inhibits viral reverse transcriptase and is incorporated into viral DNA (Faulds and Brodgen, 1992); it is also possible that ddA 5'-triphosphate becomes incorporated into mammalian DNA (Faulds and Brodgen, 1992).

Meng et al. (2000) have examined the genotoxic and mutagenic effects of AZT and ddI in TK6 human lymphoblastoid cells. At equimolar concentrations, ddI was considerably more cytotoxic than AZT, as measured by cell survival. Both drugs increased the HPRT mutant frequency, whereas AZT, but not ddI, induced mutants in the TK gene. Interestingly, when the cells were incubated simultaneously with AZT and ddI there was a marked reduction in cytotoxicity compared with ddI alone and a substantial increase in both the HPRT and TK mutant frequency. The increase in mutant frequency was associated with an increased incorporation of AZT into cellular DNA; ddI incorporation was not measured. The mechanism for this synergistic increase in the mutant frequency with AZT and ddI is not known. AZT increased the conversion of ddI to ddA 5'-triphosphate in some experiments (Palmer and Cox, 1994, 1995), but not others (Kewn et al., 1999); nonetheless, ddI has no effect upon the phosphorylation of AZT (Palmer and Cox, 1994; Veal et al., 1994). While the increase in ddA 5'-triphosphate could explain the synergistic effect of ddI and AZT upon HIV-1 (Dornsife et al., 1991), it cannot account for the increased incorporation of AZT into DNA induced by ddI. Co-incubation of AZT and ddI could alter the size of the deoxynucleotide triphosphate pools, with a resultant increase in AZT incorporation into DNA. Although combinations of AZT and ddI did not decrease deoxynucleotide triphosphate pools at low concentrations (1 µM) (Palmer and Cox, 1994), at higher concentrations of AZT (i.e. 100 µM), a significant reduction in the level of deoxadenosine 5'-triphosphate was observed (Kewn et al., 1999). Inasmuch as the synergism observed by Meng et al. (2000) was most evident at AZT and ddI concentrations >100 µM, this suggests that the effect could be a result of alterations in the size of deoxynucleotide triphosphate pools.
In order to determine whether AZT and ddI show synergism in vivo, as was observed in vitro, we treated neonatal B6C3F1/Tk mice with ddI or a combination of AZT and ddI and assessed the mutation frequency in the Hprt and Tk genes of splenic T lymphocytes and the frequency of micronucleated PCEs. In contrast to results observed in human TK6 cells, ddI did not increase the mutant frequency in either the Hprt or Tk gene of B6C3F1/Tk<sup>+</sup> mice, and while the combination of ddI and AZT did increase the Tk mutant frequency, the response was not greater than that observed with AZT alone (Table 1). Likewise, ddI did not increase the percentage of PCEs with micronuclei in B6C3F1/Tk mice and the frequency of micronuclei induced by the combination of AZT and ddI did not exceed that observed with AZT alone (Figure 1).

The lack of a synergistic effect between AZT and ddI in vivo when one was observed in vitro may be due to differences in the metabolic processing of AZT and ddI between these two models. In the experiments conducted by Meng et al. (2000), human TK6 cells were exposed to up to 300 μM AZT and 300 μM ddI for 3 days. Although the extent of metabolism of AZT was not measured, in other studies with human lymphoblastoid cells, intracellular levels of AZT remained relatively constant for at least 24 h (Avramis et al., 1989). Pharmacokinetic parameters for AZT and ddI have not been reported for neonatal mice, however, in adult female C57Bl/6N mice treated orally with 400 mg/kg AZT, peak serum levels of AZT (~1000 μM) occurred within 5 min and the half-life of the drug was ~30 min (Williams et al., 2003). Likewise, peak levels of AZT 5'-phosphate occurred within 15 min of dosing in both serum and spleen and, as with AZT, AZT 5'-phosphate was rapidly eliminated from both compartments (Williams et al., 2003). Similar pharmacokinetic parameters have been reported for lower doses of AZT administered to adult female B6C3F1 mice (Trang et al., 1993). The pharmacokinetics of ddI and the interaction of AZT and ddI do not appear to have been assessed in B6C3F1 mice, nonetheless, in male CD-1 mice the half-life of ddI in serum was three-fold shorter (8 min) than that of AZT (23 min) (Russell and Klunk, 1989). In rats, the half-lives of ddI and AZT in plasma were similar (20–30 min) and there were no pharmacokinetic interactions apparent between the drugs (Wienjtes and Au, 1992). While the concentrations of AZT and ddI in the neonatal mice may be comparable with those used to treat the TK6 cells, the in vivo concentrations decrease rapidly due to rapid elimination of the drugs. This rapid elimination compared with what is observed in vitro may explain the lack of synergism in the mutagenic response of the drugs in vivo.

In summary, ddI was not mutagenic when administered to neonatal B6C3F1/Tk<sup>+</sup> mice on PNDs 1–8. Furthermore, ddI did not potentiate the mutagenic response observed with AZT.

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References


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