Plasma Drug Levels Compared with DNA Incorporation of 3′-azido-3′-Deoxythymidine (AZT) in Adult Cynomolgus (*Macaca fascicularis*) Monkeys

OFELIA A. OLIVERO,[1] MADHAVI K. REDDY, SARA M. PIETRAS, AND MIRIAM C. POIRIER

Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892-4255

Zidovudine (3′-azido-3′-deoxythymidine, AZT), widely used for the therapy of the Human Immunodeficiency Virus-1 (HIV-1), is a nucleoside analog of thymidine that becomes phosphorylated and incorporated into nuclear and mitochondrial DNA. Levels of AZT incorporation into DNA of humans, monkeys, and mice are highly variable and suggest interindividual variability in phosphorylation pathways. In addition, studies in rhesus monkeys (1) have shown a lack of correlation between levels of unbound AZT in plasma and tissue AZT-DNA. However, the correlation between plasma AZT and tissue AZT-DNA has not been previously examined in the same primate. Here we examine the relationship between AZT-DNA incorporation in leukocytes and multiple organs, and levels of the drug circulating in plasma of adult female cynomolgus (*Macaca fascicularis*) monkeys. Three monkeys were dosed with 40.0 mg of AZT/day for 30 days by naso-gastric intubation. The average daily dose of 9.9 mg of AZT/kg/body wt was similar to the ~8.6 mg of AZT/kg/body wt (600 mg/day) given to adult HIV-1-infected patients. In all three monkeys, at the time of sampling, values for AZT concentrations in plasma were similar and values for AZT incorporation into leukocyte DNA (86.1, 100.0, and 114.1 molecules of AZT/10^6 nucleotides) were also similar. AZT-DNA incorporation was detected in liver, uterus, spleen, and kidney from the three AZT-exposed animals, with values for positive samples ranging from 5.8 to 97.4 molecules of AZT/10^6 nucleotides. In brain cortex and lung DNA from AZT-exposed animals, AZT incorporation was undetectable. The data suggest that organ-specific differences in AZT uptake and/or metabolism may contribute to AZT phosphorylation and subsequent drug incorporation into DNA. In addition, AZT-DNA levels in monkey organs were similar to or lower than values observed in peripheral leukocytes of adult AIDS patients.

[Exp Biol Med Vol. 226(5):446–449, 2001]

This work was funded as part of the intramural research program of the Division of Basic Sciences, National Cancer Institute, National Institutes of Health.

[1] To whom requests for reprints should be addressed at National Cancer Institute, 37 Convent Drive, Building 37, Room 2A01, Bethesda MD 20892–4255. E-mail: oliveroo@exchange.nih.gov

Received September 19, 2000.
Accepted January 4, 2001.

0037-9727/01/2265-0446$15.00

Key words: nucleoside analogs; zidovudine; cynomolgus; genotoxicity

The nucleoside analog drug Zidovudine (3′-azido-3′-deoxythymidine, AZT), which has been used since 1987 for the treatment of HIV-1 and AIDS, has been shown *in vitro* to block the nucleoside-binding site of the viral reverse transcriptase and to inhibit DNA replication by chain termination (2). In humans, rodents, and primates AZT incorporates into both nuclear and mitochondrial DNA. Experiments performed in cultured cells demonstrated preferential AZT incorporation into the telomeric DNA of cells containing an active telomerase (3, 4). AZT has been shown to be a transplacental carcinogen in a mouse model, where offspring exposed *in utero* for the last third of gestation exhibited increased incidences of tumors in liver, lung, and reproductive organs at 1 and 2 years of age (5, 6). The same study demonstrated AZT incorporation into organ DNA of mouse offspring exposed *in utero* to tumorigenic AZT doses (5). When the drug was given to HIV-1-positive pregnant women for variable amounts of time during pregnancy (7), incorporation of AZT into DNA was demonstrated in peripheral blood mononuclear cells (PBMC) from mothers and cord blood leukocytes from infants. Additionally, AZT-DNA incorporation has been documented in leukocytes of adults exposed to therapeutic AZT doses (7). A high individual variability in the amount of AZT incorporated into DNA has been reported in the different models studied (1, 5, 7), however, none of the available models has compared AZT-DNA values in nucleated blood cells with free AZT levels in plasma in the same subject. Prior to becoming incorporated into DNA, the drug is phosphorylated and substantial interindividual variability in phosphorylation has been previously documented (8, 9), which may underlay the AZT-DNA variability. In order to explore these questions further we dosed cynomolgus (*Macaca fascicularis*) monkeys with a human equivalent dose of AZT for 30 days. Levels of free AZT were determined in plasma

and levels of AZT incorporated into DNA were determined in multiple organs and blood.

Materials and Methods

Monkey Maintenance and Dosing. Animal care for this experiment was provided at Hazleton Laboratories (Vienna, VA) in accordance with the standards established by the Association for Assessment and Accreditation for Laboratory Animal Care. The experimental protocols were approved by the Hazleton Animal Care and Use Committee. The animals were given a diet consisting of high-protein Purina monkey chow (5045 Standard), with a vitamin supplement. Three 19-year-old female cynomolgus monkeys were dosed with AZT (donation from the National Cancer Institute Drug Repository) by naso-gastric intubation. Monkeys were administered 40 mg of AZT/day, given twice daily in 20-mg doses for a period of 30 days. The euthanasia was performed 4 to 12 hr after the last dosing. One unexposed monkey was used as control. Blood was obtained immediately prior to euthanasia, which was performed with ketamine hydrochloride (15 mg/kg, intramuscular), followed by sodium thiamylal (40 mg/kg intravenous). Blood leukocytes were separated from plasma and stored frozen at −70°C. Tissues were obtained at autopsy, which was performed immediately after euthanasia, and were stored frozen at −70°C.

DNA Extraction. DNA was extracted from multiple organs (liver, lungs, kidney, spleen, brain cortex, and uterus) and peripheral leukocytes with an inorganic kit from Intergen Company (Purchase, NY). DNA was quantitated by spectrophotometry at A_{260}, and purity was assessed by $A_{260}/_{280}$ ratios. DNA samples were diluted to a final volume of 30 μg of DNA/ml and were assayed by radioimmunoassay (RIA).

AZT RIA. DNA samples were sonicated for 45 sec, boiled for 10 min, and 3 μg of DNA was assayed by anti-AZT RIA as previously described (3). Briefly, a rabbit anti-AZT antibody (Sigma, St. Louis, MO, diluted 1:5000) was incubated with either standard AZT plus 3 μg of carrier unexposed monkey DNA, or 3 μg of AZT-exposed monkey DNA, for 90 min at 37°C. Tritiated AZT tracer (11.7 Ci/mmol, Moravek Biochemicals, Mountain View, CA) and goat anti-rabbit IgG (Sigma) were added for 25 min at 4°C. After centrifugation, the supernatant was decanted and the pellets were dissolved in 100 mM NaOH and then were counted in a liquid scintillation counter. The standard curve 50% inhibition was 0.29 ± 0.10 pmol of AZT/tube (means ± SD, $n = 3$) for the plasma experiments and 1.00 ± 0.59 pmol of AZT/tube (means ± SD, $n = 9$) for the AZT-DNA incorporation experiments that contained 3 μg of carrier DNA. The lower limit of detection was ~0.075 pmol of AZT/tube for the plasma experiments and ~0.1 pmol of AZT/tube for the AZT-DNA experiments. Each sample was assayed in duplicate in three to four separate RIAs from the same tissue pool and was then compared with DNA from the unexposed monkey. The plasma samples were diluted 1:400 and 1:800 with 1mM Tris buffer, pH 8.0. Both sets of diluted plasma samples were assayed by AZT-RIA against an AZT standard curve without DNA.

Results

The four female monkeys chosen for this experiment were similar in age and were all in good health. At the time of euthanasia, the AZT-exposed monkeys (numbers 293, 297, and 304) weighed 4.6, 5.0, and 3.1 kg, respectively, while the unexposed monkey weighed 3.0 kg. Values for content of plasma protein (used to normalize plasma assays) ranged from 0.5 to 1.3 mg protein/ml of plasma for the four animals (data not shown).

The concentration of AZT circulating in the plasma of the three monkeys is shown in Table I. The values are presented as nanograms of AZT per milliliter of plasma from plasma samples diluted 1:400, and are also shown corrected for plasma protein. When normalized for protein, similar values of free circulating AZT, 121.9, 83.6, and 119.2 ng AZT/mg plasma protein, were detected in monkeys numbered 293, 297, and 304, respectively. Table I compares AZT in plasma (nanograms of AZT per milligram of plasma protein) with AZT incorporated into peripheral leukocyte DNA (molecules of AZT per 10^6 nucleotides). The AZT-DNA values were 114.1, 100.0, and 86.1 molecules of AZT/10^6 nucleotides for monkeys 293, 297, and 304 respectively. Therefore, for three primates given the same AZT dose, levels of AZT in plasma were consistent with levels of AZT incorporated into peripheral leukocyte DNA.

Incorporation of AZT into DNA of the monkey organs is shown in Table II. A high degree of interanimal variability was found for AZT-DNA incorporation in organs of the three exposed monkeys. AZT-DNA was detected in uterus from three monkeys, liver from two monkeys, spleen from one monkey, and kidney from one monkey. No AZT was detectable in brain cortex or lung DNA from any AZT-exposed animal. In two monkeys (293 and 297), the liver had the highest AZT-DNA levels, 44.3 and 97.4 molecules of AZT/10^6 nucleotides, but AZT-DNA was not detected in the liver of 304. This type of high-level interanimal variability is similar to that observed previously in other primates and may be due to differences in capacity to phos-

Table I. Levels of AZT in Plasma and AZT-DNA Incorporated into Leukocyte DNA

Monkey	Nanograms of AZT per milliliter of plasma	Nanograms of AZT per milligrams	Molecules of AZT per 10^6 nucleotides[a]
293	85.3 ± 28.5	121.9 ± 40.7	114.1 ± 46.1
297	108.7 ± 31.3	83.6 ± 24.1	100.0 ± 23.6
304	59.6 ± 25.2	119.2 ± 50.4	86.1 ± 36.9

[a] Values represent the mean ± SEM of three assays.

Table II. AZT-DNA Incorporation in Tissues of Cynomolgus Monkeys

Organ	Monkey[a,b]		
	293	297	304
Liver	97.4 ± 60.9	44.3 ± 31.9	ND[c]
Uterus	10.0 ± 2.5	10.4 ± 3.6	13.6 ± 3.4
Spleen	ND	5.8 ± 0.8	ND
Kidney	ND	ND	20.4 ± 6.8[d]

[a] Values are molecules of AZT/10^6 nucleotides.
[b] Values are expressed as the means ± SEM of three or four assays.
[c] Not detectable.
[d] One assay in three gave a non-detectable value. For statistical purposes, a value half-way between 0 and the lowest level of detection was given to the undetectable values.

phorylate the drug that occur between tissues and between animals (10–12).

Discussion

Three cynomolgus monkeys were dosed daily for 30 days with a human-equivalent dose of AZT, and the free drug was detected in plasma, while the drug was detected incorporated into the DNA of leukocytes, liver, uterus, kidney, and spleen.

The purpose of these experiments was 2-fold. First, levels of free AZT persistent in plasma several hours after AZT dosing were compared with levels of AZT incorporated into peripheral leukocyte DNA. Previous studies in humans, monkeys, and mice have not made this comparison. Second, values for AZT-DNA were determined in multiple organs, and the interorgan and interanimal variability observed confirmed previous studies in mice, cynomolgus monkeys, Patas monkeys, and humans. This study of adult cynomolgus monkeys demonstrates that at least in three primates of the same species given the same AZT dose with chronic (twice daily) dosing, there is little interanimal variability for either the plasma AZT levels or the peripheral leukocyte AZT-DNA values.

In contrast, there is more interanimal and interorgan variability in the AZT-DNA values found in particular tissues, suggesting that there may be differences in phosphorylation capacity within and between individual primates. The results are limited by the small number of animals and should be confirmed in a larger study.

AZT, a thymidine nucleoside analog becomes mono-, di- and triphosphorylated to AZT-monophosphate, AZT-diphosphate, and AZT-triphosphate by the enzymes thymidine kinase, thymidylate kinase, and pyrimidine nucleoside diphosphate kinase, respectively (2). However this pathway constitutes a fraction of the overall metabolism of the drug. The predominant metabolic pathway is glucuronidation, forming AZT-glucuronide (AZTG), which is renally excreted. A further minor metabolite, derived by reduction of the azido moiety, is AMT (3′-amino-3′-deoxythymidine). Only in the triphosphorylated form can AZT become incorporated into the nascent DNA strand and exert its antiviral action by chain termination (2, 13). The rate of AZT-triphosphate formation is cell specific, and is limited by thymidylate kinase and other biologic processes (14). For example, mitogenic-stimulated human peripheral blood mononuclear cells had elevated (150-fold) levels of AZT-triphosphate compared with resting PBMCs (15). Interaction of the drug with prokaryotic polymerases appears to influence incorporation of AZT into nuclear and mitochondrial DNA of many species, as demonstrated by experiments in cell culture and animals (3, 5, 16). The incorporation of AZT takes place ~2000-fold slower than incorporation of the natural nucleotide thymidine (17). Consequent to AZT-DNA incorporation, genotoxicity of AZT has been documented as micronuclei (18, 19), sister chromatid exchanges (20), and chromosomal aberrations (21–23) and is consistent with the tumorigenesis observed in animal models (24, 25).

Considerable interindividual variability has been observed in amounts of AZT incorporation into DNA in mice, monkeys, and humans (1, 5, 7). The origin of such variability may be attributed to individual differences in metabolism, as well as different per organ phosphorylation capacities (9). Studies showed that plasma concentration of AZT does not correlate with levels of intracellular phosphorylated AZT or clinical effect in humans (8), but rather, there is a correlation between phosphorylation ability and drug effectivity, as assessed by markers associated with drug efficiency (26).

In the present study, similar levels of free circulating AZT in plasma in the three monkeys do not correlate with the differences of incorporation in the organs analyzed, although there is an apparent correlation with the AZT-DNA incorporation in nucleated blood cells (Table I). Interestingly, levels of AZT-DNA found in cynomolgus leukocytes are in the same range as those found in humans exposed to therapeutic AZT doses of 600 mg/day for periods between 1 to 12 months. Additionally, monkey plasma levels are also comparable to the human (Table III).

In conclusion, in this set of three cynomolgus monkeys, AZT bioavailability did correlate with AZT-DNA incorporation in peripheral leukocytes, but did not appear to play a direct role in AZT-DNA incorporation in different organs. Most probably, differences in the metabolic capacities of different organs to phosphorylate AZT to AZT-triphosphate strongly influences the amount of AZT incorporated into

Table III. AZT-DNA Incorporation into Leukocyte DNA of Cynomolgus Monkeys and Human AIDS Patients

	Monkeys	Humans
Treatment (months)	1	>1
AZT-plasma (ng/ml)	~60–110	~50–206[a]
AZT-DNA (mol/10^6 nucleotides)	~85–115 (n = 3)	45–150[b] (n = 6)

[a] Pereira et al. (27).
[b] Olivero et al. (7).

DNA. These experiments also suggest that incorporation of AZT into DNA is likely to occur in multiple organs of HIV-1-infected patients receiving AZT drug therapy.

1. Poirier MC, Patterson TA, Slikker W, Jr., Olivero OA. Incorporation of 3′-azido-3′-deoxythymidine (AZT) into fetal DNA and fetal tissue distribution of drug after infusion of pregnant late-term rhesus macaques with a human-equivalent AZT dose. J Acquir Immune Defic Syndr **22:**477–483, 1999.
2. Furman PA, Fyfe JA, St. Clair MH, Weinhold K, Rideout JL, Freeman GA, Lehrman SN, Bolognesi DP, Broder S, Mitsuya H, Barry DA. Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. Proc Natl Acad Sci U S A **83:**8333–8337, 1986.
3. Olivero OA, Beland FA, Poirier MC. Immunofluorescent localization and quantitation of 3′-azido-2′, 3′-dideoxythymidine (AZT) incorporated into chromosomal DNA of human, hamster and mouse cell lines. Int J Oncol **4:**49–54, 1994.
4. Gomez DE, Kassim A, Olivero OA. Preferential incorporation of 3′-azido-2′,3′-dideoxythymidine (AZT) in telomeric sequences of CHO cells. Int J Oncol **7:**1057–1060, 1995.
5. Olivero OA, Anderson LM, Diwan BA, Haines DC, Harbaugh SW, Moskal TJ, Jones AB, Rice JM, Riggs CW, Logsdon D, Yuspa SH, Poirier MC. Transplacental effects of 3′-azido-2′,3′-dideoxythymidine (AZT): Tumorigenicity in mice and genotoxicity in mice and monkeys. J Natl Cancer Inst **89:**1602–1608, 1997.
6. Diwan BA, Riggs CW, Logsdon D, Haines DC, Olivero OA, Rice JM, Yuspa SH, Poirier MC, Anderson LM. Multiorgan transplacental and neonatal carcinogenicity of 3′-azido-3′-deoxythymidine in mice. Toxicol Appl Pharmacol **15:**82–99, 1999.
7. Olivero OA, Shearer GM, Chougnet CA, Kovacs AA, Landay AL, Baker R, Stek AM, Khoury MM, Proia LA, Kessler HA, Sha BE, Tarone RE, Poirier MC. Incorporation of zidovudine into leukoyte DNA from HIV-1-positive adults and pregnant women, and cord blood from infants exposed *in utero*. AIDS **13:**919–925, 1999.
8. Acosta EP, Page LM, Fletcher CV. Clinical pharmacokinetics of zidovudine: An update. Clin Pharmacokinet **30:**251–262, 1996.
9. Rodman JH, Flynn PM, Robbins B, Jimenez E, Bardeguez AD, Rodriguez JF, Blanchard S, Fridland A. Systemic pharmacokinetics and cellular pharmacology of zidovudine in human immunodeficiency virus type 1-infected women and newborn infants. J Infect Dis **180:** 1844–1850, 1999.
10. Hartwell LH, Kastan MB. Cell cycle control and cancer. Science **266:** 1821–1828, 1994.
11. White TJ. PCR Protocols: A Guide to Methods and Applications. New York: Academic Press, 1997.
12. Manam S, Storer RD, Prahalada S, Leander KR, Kraynak AR, Ledwith BJ, van Zwieten MJ, Bradley MO, Nichols WW. Activation of the Ha-, Ki-, and N-*ras* genes in chemically induced liver tumors from CD-1 mice. Cancer Res **52:**3347–3352, 1992.
13. St.Clair MH, Richards CA, Spector T, Weinhold KJ, Miller WH, Langlois AJ, Furman PA. 3′-Azido-3′-deoxythymidine triphosphate as an inhibitor and substrate of purified human immunodeficiency virus reverse transcriptase. Antimicrob Agents Chemother **31:**1972–1977, 1987.
14. Balzarini J, Pauwels R, Baba M, Herdewijn P, de Clercq E, Broder S, Johns DG. The *in vitro* and *in vivo* anti-retrovirus activity, and intracellular metabolism of 3′-azido-2′,3′-dideoxythymidine and 2′,3′-dideoxycytidine are highly dependent on the cell species. Biochem Pharmacol **37:**897–903, 1988.
15. Gao WY, Shirasaka T, Johns DG, Broder S, Mitsuya H. Differential phosphorylation of azidothymidine, dideoxycytidine, and dideoxyinosine in resting and activated peripheral blood mononuclear cells. J Clin Invest **91:**2326–2333, 1993.
16. Sommadossi JP, Carlisle R, Zhou Z. Cellular pharmacology of 3′-azido-3′-deoxythymidine with evidence of incorporation into DNA of human bone marrow cells. Mol Pharmacol **36:**9–14, 1989.
17. Nickel W, Austermann S, Bialek G, Grosse F. Interactions of azidothymidine triphosphate with the cellular DNA polymerases alpha, delta, and epsilon and with DNA primase. J Biol Chem **267:**848–854, 1992.
18. Dertinger SD, Torous DK, Tometsko KR. Induction of micronuclei by low doses of azidothymidine (AZT). Mutat Res **368:**301–307, 1996.
19. Stern M, Cid MG, Larripa I, Slavutsky I. AZT-induction of micronuclei in human lymphocyte subpopulations. Toxicol Lett **70:**235–242, 1994.
20. Gonzalez CM, Larripa I. Genotoxicity of azidothymidine (AZT) in *in vitro* systems. Mutat Res **321:**113–118, 1994.
21. Olivero OA, Beland FA, Fullerton NF, Poirier MC. Vaginal epithelial DNA damage and expression of preneoplastic markers in mice during chronic dosing with tumorigenic levels of 3′-azido-2′,3′-dideoxythymidine (AZT). Cancer Res **54:**6235–6242, 1994.
22. Shafik HM, Nokta MA, Pollard RB. Recombinant human interferon beta ser protects against zidovudine-induced genetic damage in AIDS patients. Antiviral Res **16:**205–212, 1991.
23. Agarwal RP, Olivero OA. Genotoxicity and mitochondrial damage in human lymphocytic cells chronically exposed to 3′-azido-2′,3′-dideoxythymidine (AZT). Mutat Res **390:**223–231, 1997.
24. Miller JA, Miller EC. Perspectives on the Metabolism of Chemical Carcinogens. Amsterdam: Elsevier/North-Holland Biomedical Press, pp25–50, 1979.
25. Poirier MC. DNA adducts as exposure biomarkers and indicators of cancer risk. Environ Health Perspect **105**(Suppl 4):907–912, 1997.
26. Stretcher BN, Pesce AJ, Frame PT, Greenberg KA, Stein DS. Correlates of zidovudine phosphorylation with markers of HIV disease progression and drug toxicity. Aids **8:**763–769, 1994.
27. Pereira AS, Kashuba AD, Fiscus SA, Hall JE, Tidwell RR, Troiani L, Dinn JA, Eron JJ, Cohen MS. Nucleoside analogues achieve high concentrations in seminal plasma: relationship between drug concentration and virus burden. J Infect Dis **180:**2039–2043, 1999.