Carcinogenesis vol.21 no.5 pp.1059–1062, 2000

SHORT COMMUNICATION

Oxidative DNA damage in fetal tissues after transplacental exposure to 3′-azido-3′-deoxythymidine (AZT)

Aneta Bialkowska[1], Karol Bialkowski[2,3], Mariana Gerschenson[4], Bhalchandra A.Diwan[1], Ann Butler Jones[2], Ofelia A.Olivero[4], Miriam C.Poirier[4], Lucy M.Anderson[2,6], Kazimierz S.Kasprzak[2] and Marek A.Sipowicz[2,5]

[1]Intramural Research Support Program, SAIC-Frederick Inc., Frederick Cancer Research and Development Center (FCRDC), [2]Laboratory of Comparative Carcinogenesis, National Cancer Institute, FCRDC, Frederick, MD 21702, USA, [3]Department of Clinical Biochemistry, University School of Medical Sciences, Bydgoszcz, Poland and [4]Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, Bethesda, MD 20892, USA

[5]Present address: Servier Poland, International Center for Therapeutic Research, ul. Sienna 75, 00-833 Warszawa, Poland

[6]To whom correspondence should be addressed
Email: andersol@mail.ncifcrf.gov

The nucleoside analogue 3′-azido-3′-deoxythymidine (AZT) has been used successfully to reduce the incidence of transplacental and perinatal transmission of the HIV virus. However, prolonged treatment with high doses of AZT is utilized in this therapy, and AZT has been found to be a perinatal carcinogen in mice. Any possible perinatal carcinogenic side effects in the human can best be managed if the mechanism is understood. AZT targets mitochondria and might cause increased intracellular production of reactive oxygen species (ROS). We tested whether transplacental AZT may cause oxidative damage in nuclear DNA of fetal tissues. CD-1 Swiss pregnant mice were treated with the transplacental carcinogenesis regimen (25 mg/day AZT, for gestation days 12–18) and tissues collected on the day of birth. Significant increases in 8-oxo-2′-deoxyguanosine (8-oxo-dG) were found in the livers, a target tissue for transplacental carcinogenesis, and in the kidneys. A non-significant increase occurred in brain, with no change in lung. Tissues were also obtained from fetal patas monkeys (*Erythrocebus patas*), whose mothers had received 10 mg AZT/day during the last half of gestation. Although limited numbers of samples were available, possible increases in 8-oxo-dG were noted, relative to controls, for placenta and for fetal lung and brain ($P = 0.055$ for treatment-related increases in these tissues). These results suggest that an increase in reactive oxygen species could contribute to the mechanism of transplacental carcinogenesis by AZT in mice, and that this may also occur in primates.

The number of women infected with human immunodeficiency virus (HIV), with potential prenatal transfer of this virus to fetuses, is still increasing worldwide (1,2). The nucleoside analogue 3′-azido-3′-deoxythymidine (AZT) was found to be an efficient drug in acquired immunodeficiency syndrome (AIDS) therapy, as an inhibitor of HIV replication. Mechanisms implicated in the therapeutic action of this nucleoside analogue include the incorporation of the AZT triphosphate into newly synthesized viral DNA template, resulting in chain termination of DNA synthesis, and inhibition of viral reverse transcriptase (3,4). Use of AZT during human pregnancy has considerably reduced the risk of perinatal HIV transmission of the virus to fetuses (5–7).

However, AZT also has been demonstrated to have genotoxic and carcinogenic effects *in vivo* and *in vitro* (8–10), including transplacental effects. Incorporation of AZT into mitochondrial and nuclear DNA was observed in multiple organs from fetal mice and fetal patas monkeys (*Erythrocebus patas*) (11), and in leukocyte DNA from cord blood of HIV-1-positive mothers given AZT therapy (12). Offspring of pregnant mice exposed transplacentally to AZT were shown to have increased incidences of liver, lung, skin, histiocytic and reproductive system tumors (11,13). Thus the possibility exists of long-term toxic side effects in children prenatally exposed to AZT treatment.

Understanding of the mechanism of the transplacental carcinogenicity of AZT in mice could assist in predicting and containing any human risk. Skin tumors initiated in mouse fetuses by AZT were found to have a significant incidence of G→A and G→T mutations in codons 12 and 13 of the H-*ras* oncogene (14). This finding was not consistent with the predicted base changes due to misincorporation of AZT in place of thymidine. On the other hand, these observed mutations commonly result from oxidative damage to guanine; this is a mechanism that has been implicated in oxidative damage to the *ras* oncogene (15,16).

Oxidative DNA damage is plausible, since mitochondria have been found to be cellular targets for AZT, resulting in ultrastructural, biochemical and molecular abnormalities (17). Mitochondrial diseases can cause an increase in production of reactive oxygen species (ROS) (18), and increases in formation of 8-oxo-2′-deoxyguanosine (8-oxo-dG) were found in liver mitochondrial DNA after treatment of mice with AZT (19,20). Elevated urinary excretion of 8-oxo-dG was found also in the urine of HIV infected patients and mice treated with AZT (21). Transplacental oxidative DNA damage has previously been demonstrated in mouse fetuses exposed to the tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (22).

We tested whether transplacental AZT exposure would cause an increase in 8-oxo-dG in fetuses of mice and monkeys. This adduct is mutagenic (15) and, in addition, is a good biomarker of overall oxidative damage to DNA (23).

8-Oxo-dG was synthesized by Dr Victor Nelson, SAIC-Frederick (Frederick, MD). Sodium acetate was obtained from

Abbreviations: 8-oxo-dG, 8-oxo-2′-deoxyguanosine; AZT, 3′-azido-3′-deoxythymidine; HIV, human immunodeficiency virus; ROS, reactive oxygen species.

EM Science (Cincinnati, OH). AZT and other chemicals and reagents were purchased from Sigma (St Louis, MO).

CD-1 mice 6–8 weeks old were obtained from Charles River Inc., Raleigh, NC. Animal care was provided in accordance with recommendations of the National Institutes of Health (24) in a facility approved by the American Association for the Accreditation of Laboratory Animal Care. Mice were fed standard NIH-31 diet (Zeigler Brothers, Gardner, PA) and were given water *ad libitum*. They were mated (day of vaginal plug = day 1) and the pregnant females were randomly divided into two groups, consisting of five animals each. One group was given 25 mg AZT/day orally in distilled water from gestation day 12 to 18, following our reported transplacental carcinogenic regimen (11,13). The control group of mice received no treatment. After birth on day 19, livers, lungs, kidneys and brains were removed and frozen in liquid nitrogen.

Patas monkeys were maintained at BioQual, Inc., Shady Grove, MD, a facility accredited by the American Association for Accreditation of Laboratory Animal Care. Pregnant patas females were treated with 10 mg AZT mixed with mashed banana daily, 5 days/week, for the last 9–11 weeks of the 23 week gestation period. This was comparable to ~20% of the daily human dose; the total dose of ~0.08 g AZT/kg body wt received by the monkeys was considerably lower than that given to pregnant women, which may total 1.4 g/kg. Controls received no treatment. Fetuses were removed by Caesarian section on gestation day 149–151. The antibiotic, cefazolin, and the analgesic, buprenorphine, were given just before surgery; anesthetics included telezol, atropine, ketamine and isoflurane and were used to effect. Tissues were frozen in liquid nitrogen and stored at –80°C until DNA extraction. Availability of pregnant patas monkeys was limited. The tissues utilized were those remaining from studies of AZT incorporation into fetal tissue DNA (11).

The DNA was extracted from tissues and the 8-oxo-dG levels were determined using the enzymatic hydrolysis procedure, according to methods described by Adachi *et al.* (25). DNA hydrolysates were separated by an HPLC system consisting of a Waters 2690 Separations Module, a Waters 490E Programmable Multiwavelength Detector, an ESA Culochem II 5200A electrochemical detector (guard cell model 5020: 700 mV, analytical cell model 5010: working electrode E1 at 300 mV), and a reversed-phase Supelcosil LC-18-S (250×4.6 mm, 5 μm grains) column equipped with a 2 cm guard column. Aliquots of 20 μl DNA hydrolysates were assayed at 1 ml/min flow rate, using 100 mM sodium acetate/orthophosphoric acid pH 5.2/methanol (92:8) as eluent. The electrochemical detector was used to measure 8-oxo-dG, while the UV detector (254 nm) measured 2′-deoxyguanosine (dG). The measured parameters were quantified by reference to separate dilutions of external dG and 8-oxo-dG standards; calibration curves were completed on each day of analysis. Chromatograms were acquired and integrated by Millenium 32 software. For the monkey fetal material, duplicate samples were assayed for some of the tissue and the mean values utilized for further analyses.

Statistical analyses were carried out using software from Instat Inc. and the Statistical Package for the Social Sciences Inc., as indicated below and in Tables I and II.

As shown in Table I, transplacental exposure of mouse fetuses to 25 mg AZT/day in the last half of pregnancy caused significant increases in 8-oxo-dG levels in DNA from newborn livers and kidneys. In the newborn livers AZT treatment resulted in a significant 68% increase above the control value. A significant 38% increase in 8-oxo-dG was also found in the newborn kidneys. The amount of 8-oxo-dG in the newborn brains was slightly higher by ~15% in the AZT-treated group but this difference did not approach statistical significance. While fetal and newborn livers and lungs were targets for perinatal carcinogenicity of AZT in mice, kidneys were not. Mouse kidney is a relatively unresponsive target tissue for perinatal carcinogens. No increase in 8-oxo-dG was observed in newborn lungs after AZT treatment, though this was a target tissue for tumorigenicity (11,13). In view of the large number of types of cells present in lung, more detailed studies would be required to rule out increased 8-oxo-dG as a factor in perinatal initiation of lung tumors by AZT. The other target tissues for perinatal carcinogenicity of AZT, i.e. epidermis, mammary gland and histiocytes (11,13), were available in quantities too small for effective utilization of present techniques for 8-oxo-dG analysis.

More limited material was available from the monkey fetuses (see above). Although none of the differences between individual treated and control tissues was of statistical significance, there was a possible trend to increase in 8-oxo-dG for all AZT-exposed tissues except fetal liver, ranging from 21% (fetal kidney and placenta) to 84% (fetal lung) (Table II). There were sufficient numbers of measurements for further analysis of placenta, fetal lung and brain cerebrum. Extensive differences in the physiology of these organs may justify treating them as independent units for purposes of

Table I. Levels of 8-oxo-dG[a] in mice offspring tissues[b]

	Lung	Liver	Kidney	Brain
Control	1.41 ± 0.25	1.34 ± 0.10	0.97 ± 0.09	1.07 ± 0.09
AZT	1.28 ± 0.47	2.25 ± 0.31[c]	1.34 ± 0.11[d]	1.24 ± 0.20

[a]Expressed as 8-oxo-dG per 10^5dG, mean ± SE.
[b]Each sample consisted of the pooled tissues from one litter (n = 5).
[c]Significantly higher than control (P = 0.02, Student's t-test).
[d]Significantly higher than control (P = 0.03, Student's t-test).

Table II. Levels of 8-oxo-dG[a] in monkey fetal tissues and placenta

	Lung	Liver	Kidney	Brain	Placenta
Control	1.59 ± 0.28 (2)	2.47 (1)	1.80 (1)	1.81 ± 0.10 (3)	3.66 ± 0.39 (6)
AZT	2.93 ± 0.87[b] (3)	2.49 (1)	2.18 (1)	2.43 ± 0.36[c] (4)	4.43 ± 0.55[d] (5)

[a]Expressed as 8-oxo-dG per 10^5dG, mean ± SE; number of samples is in parentheses.
[b]P = 0.32; Student's t-test.
[c]P = 0.20; Student's t-test.
[d]P = 0.27; Student's t-test.

statistical analysis. Also, there were no correlations of statistical significance between adduct levels in different tissues in the same feto-placental unit (*P*-values for correlations: fetal lung and brain, 0.20; placenta and fetal lung, 0.65; placenta and fetal kidney, 0.84). Use of two-way ANOVA indicated a significant difference among tissues ($P = 0.001$), confirming the significance of the higher levels in placenta. By this test there was a treatment-related increase in 8-oxo-dG, in placenta and fetal lung and brain, of borderline statistical significance ($P = 0.055$).

Our study provides the first evidence that prenatal exposure of mammalian fetuses to AZT may cause oxidative damage to nuclear DNA in several tissues. AZT incorporation into fetal tissue DNA has been demonstrated for mice (11) and for the same fetal monkey tissues as those used here (11, and unpublished data). It is possible that resulting disruption in DNA synthesis increases the vulnerability of the DNA to oxidative damage, due to reduced complex formation with protective proteins such as polymerases. A possible source of the reactive oxygen leading to this is mitochondria. This would be consistent with mitochondria being quantitatively an important intracellular source of ROS (26,27), and the accumulating evidence that mitochondria are a main cellular target for toxic effects of AZT (17), with an increase in ROS resulting (19–21). Rodent liver and kidney are among the tissues in which these effects have been seen (19,20,28,29), consistent with the increases in 8-oxo-dG in the newborn mouse livers and kidneys in our study.

In summary, the data together are consistent with increased ROS as a component of the mechanism of perinatal carcinogenesis by AZT in mice, with potential for contributing to perinatal toxicity in the human context.

Acknowledgements

The authors thank Drs Thomas Moskal, Marisa St. Claire, Boris Skopets and Jeffery and Steven Harbaugh for monkey treatment and surgery, Dr Douglas Powell for consultation in statistics, and Drs Gregory S.Buzard and Larry K.Keefer for critical comments on this manuscript. This project has been funded in part by the National Cancer Institute, under contract NO1-CO-56000.

References

1. Ickovics,J.R. and Yoshikawa,H. (1998) Preventive interventions to reduce heterosexual HIV risk for women: current perspectives, future directions. *AIDS*, **12**, S197–S208.
2. Colognesi,C., Tresoldi,E., Salvatori,F. and Scarlatti,G. (1997) Mother-to-child transmission of HIV-1: risk factors and prevention. *J. Biol. Regul. Homeost. Agents*, **11**, 7–10.
3. Furman,P.A., Fyfe,J.A., St. Clair,M.H., Weinhold,K., Rideout,J.L., Freeman,G.A., Lehrman,S.N., Bolognesi,D.P., Broder,S., Mitsuya,H. and Barry,D.W. (1986) Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc. Natl Acad. Sci. USA*, **83**, 8333–8337.
4. Cheng,Y.C., Dutschman,G.E., Bastow,K.F., Sarngadharan,M.G. and Ting,R.Y. (1987) Human immunodeficiency virus reverse transcriptase. General properties and its interactions with nucleoside triphosphate analogs. *J. Biol. Chem.*, **262**, 2187–2189.
5. Connor,E.M., Sperling,R.S., Gelber,R., Kiselev,P., Scott,G., O'Sullivan, M.J., Van Dyke,R., Bey,M., Shearer,W., Jacobson,R.L., Jimenez,E., O'Neill,E., Bazin,B., Delfraissy,J., Culnane,M., Coombs,R., Elkins,R., Moye,J., Stratton,P. and Balsley,J. (1994) Reduction of maternal–infant transmission of human immunodeficiency virus type 1 with zidovudine treatment. Pediatric AIDS Clinical Trials Group Protocol 076 Study Group. *N. Engl. J. Med.*, **331**, 1173–1180.
6. Sperling,R.S., Shapiro,D.E., Coombs,R.W., Todd,J.A., Herman,S.A., McSherry,G.D., O'Sullivan,M.J., Van Dyke,R.B., Jimenez,E., Rouzioux,C., Flynn,P.M. and Sullivan,J.L. (1996) Maternal viral load, zidovudine treatment, and the risk of transmission of human immunodeficiency virus type 1 from mother to infant. Pediatric AIDS Clinical Trials Group Protocol 076 Study Group. *N. Engl. J. Med.*, **335**, 1621–1629.
7. Simonds,R.J., Steketee,R., Nesheim,S., Matheson,P., Palumbo,P., Alger,L., Abrams,E.J., Orloff,S., Lindsay,M., Bardeguez,A.D., Vink,P., Byers,R. and Rogers,M. (1998) Impact of zidovudine use on risk factors for perinatal transmission of HIV. *AIDS*, **12**, 301–308.
8. Cid,M.G. and Larripa,I. (1994) Genotoxic activity of azidothymidine (AZT) in *in vitro* systems. *Mutat. Res.*, **321**, 113–118.
9. Ayers,K.M., Clive,D., Tucker,W.E.Jr, Hajian,G. and Miranda,P. (1996) Nonclinical toxicology studies with zidovudine: genetic toxicity tests and carcinogenicity bioassays in mice and rats. *Fundam. Appl. Toxicol.*, **32**, 148–158.
10. Olivero,O.A., Beland,F.A., Fullerton,N.F. and Poirier,M.C. (1994) Vaginal epithelial DNA damage and expression of preneoplastic markers in mice during chronic dosing with tumorigenic levels of 3′-azido-2′,3′-dideoxythymidine. *Cancer Res.*, **54**, 6235–6242.
11. Olivero,O.A., Anderson,L.M., Diwan,B.A., Haines,D.C., Harbaugh,S.W., Moskal,T.J., Jones,A.B., Rice,J.M., Riggs,C.W., Logsdon,D., Yuspa,S.H. and Poirier,M.C. (1997) 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.
12. Olivero,O.A., Shearer,G.M., Chougnet,C.A., Kovacs,A.A.S., Landay,A.L., Baker,R., Stek,A.M., Khoury,M.M., Proia,L.A., Kessler,H.A., Sha,B.E., Tarone,R.E. and Poirier,M.C. (1999) Incorporation of zidovudine into leukocyte DNA from HIV-1-positive adults and pregnant women, and cord blood from infants exposed *in utero*. *AIDS*, **13**, 919–925.
13. Diwan,B.A., Riggs,C.W., Logsdon,D., Haines,D.C., Olivero,O.A., Rice,J.M., Yuspa,S.H., Poirier,M.C. and Anderson,L.M. (1999) Multiorgan transplacental and neonatal carcinogenicity of 3′-azido-3′-deoxythymidine in mice. *Toxicol. Appl. Pharmacol.*, **161**, 82–99.
14. Zhang,Z., Diwan,B.A., Anderson,L.M., Logsdon,D., Olivero,O.A., Haines,D.C., Rice,J.M., Yuspa,S.H. and Poirier,M.C. (1998) Skin tumorigenesis and Ki-*ras* and Ha-*ras* mutations in tumors from adult mice exposed *in utero* to 3′-azido-2′,3′-dideoxythymidine. *Mol. Carcinog.*, **23**, 45–51.
15. Cheng,K.C., Cahill,D.S., Kasai,H., Nishimura,S. and Loeb,L.A. (1992) 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G→T and A→C substitutions. *J. Biol. Chem.*, **267**, 166–172.
16. Kamiya,H., Murata-Kamiya,N., Koizume,S., Inoue,H., Nishimura,S. and Ohtsuka,E. (1995) 8-Hydroxyguanine (7,8-dihydro-8-oxoguanine) in hot spots of the c-Ha-*ras* gene: effects of sequence contexts on mutation spectra. *Carcinogenesis*, **16**, 883–889.
17. Barile,M., Valenti,D., Quagliariello,E. and Passarella,S. (1998) Mitochondria as cell targets of AZT (zidovudine). *Gen. Pharm.*, **31**, 531–538.
18. Esposito,L.A., Melov,S., Panov,A., Cottrell,B.A. and Wallace,D.C. (1999) Mitochondrial disease in mouse results in increased oxidative stress. *Proc. Natl Acad. Sci. USA*, **96**, 4820–4825.
19. Hayakawa,M., Ogawa,T., Sugiyama,S., Tanaka,M. and Ozawa,T. (1991) Massive conversion of guanosine to 8-hydroxyguanosine in mouse liver mitochondrial DNA by administration of azidothymidine. *Biochem. Biophys. Res. Commun.*, **176**, 87–93.
20. García de la Asunción,J., Del Olmo,M.L., Sastre,J., Pallardó,F.V. and Viña,J. (1999) Zidovudine (AZT) causes an oxidation of mitochondrial DNA in mouse liver. *Hepatology*, **29**, 985–987.
21. García de la Asunción,J., Del Olmo,M.L., Sastre,J., Millán,A., Pellín,A., Pallardó,F.V. and Viña,J. (1998) AZT treatment induces molecular and ultrastructural oxidative damage to muscle mitochondria. Prevention by antioxidant vitamins. *J. Clin. Invest.*, **102**, 4–9.
22. Sipowicz,M.A., Amin,S., Desai,D., Kasprzak,K.S. and Anderson,L.M. (1997) Oxidative DNA damage in tissues of pregnant female mice and fetuses caused by the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). *Cancer Lett.*, **117**, 87–91.
23. Shigenaga,M.K. and Ames,B.N. (1991) Assays for 8-hydroxy-2′-deoxyguanosine: a biomarker of *in vivo* oxidative DNA damage. *Free Radic. Biol. Med.*, **10**, 211–216.
24. NIH (1985) *Guide for the Care and Use of Laboratory Animals*. National Institutes of Health Publication No. 86-23, Bethesda, MD.
25. Adachi,S., Zeisig,M. and Möller,L. (1995) Improvements in the analytical

method for 8-hydroxydeoxyguanosine in nuclear DNA. *Carcinogenesis*, **16**, 253–258.
26. Pitkänen,S. and Robinson,B.H. (1996) Mitochondrial complex I deficiency leads to increased production of superoxide radicals and induction of superoxide dismutase. *J. Clin. Invest.*, **98**, 345–351.
27. Kowaltowski,A.J. and Vercesi,A.E. (1999) Mitochondrial damage induced by conditions of oxidative stress. *Free Radic. Biol. Med.*, **26**, 463–471.
28. Modica-Napolitano,J.S. (1993) AZT causes tissue-specific inhibition of mitochondrial bioenergetic function. *Biochem. Biophys. Res. Commun.*, **194**, 170–177.
29. Pereira,L.F., Oliveira,M.B. and Carnieri,E.G. (1998) Mitochondrial sensitivity to AZT. *Cell Biochem. Funct.*, **16**, 173–181.

Received October 12, 1999; revised January 12, 2000;
accepted January 21, 2000