Human Placenta Does Not Reduce AZT (Zidovudine) to 3′-Amino-3′-Deoxythymidine (44134)

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Abstract. **Human placental tissue and human trophoblast cells (JAr) were examined after exposure to the anti-HIV nucleoside analog AZT (Zidovudine) for the presence of 3′-amino-3′-deoxythymidine (AMT), a toxic catabolite. Placental cells were exposed to 7.6 m*M* AZT for 48 hr, and placental lobular tissue was perfused with 3.8 m*M* AZT for 14 hr. Cell homogenates were prepared, and supernatants were subjected to HPLC analysis. Despite large cellular concentrations of AZT, AMT was not detected in any of the samples analyzed. Exposure of JAr cells to this concentration of AZT produces a 72% inhibition of cell proliferation when compared with unexposed controls. Based upon the results of the current study, AMT was not formed by placental cells exposed to AZT and, thus, not a mechanism for toxicity after *in vitro* exposure to AZT.**

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The Centers for Disease Control reported that transmission of HIV can be reduced by treating HIV-positive pregnant women with AZT (Zidovudine) during pregnancy. AZT reduced the transmission rate from 25.5% without AZT treatment to 8.3% with AZT treatment (1). In treating HIV-infected women during pregnancy, one of the concerns has been the potential toxicity of AZT to the conceptus, which includes the embryo/fetus and the placenta. Such concern may be warranted since studies with AZT in pregnant rodents have indicated increased embryonic resorptions and arrest of embryonic development (2–6). Other studies exposing human trophoblast cells of the placenta to AZT have indicated inhibited cell proliferation and inhibited DNA synthesis (7, 8). Using the human placental perfusion paradigm, AZT exposure for 14 hr resulted in reduced placental production of human chorionic gonadotropin and lactate (9). In the liver and in the gut microflora, the azido moiety of AZT is reduced to an amino group to form 3′-amino-3′-deoxythymidine (AMT), which produces myelosuppression in bone marrow cells *in vitro* (10). Formation of AMT *in vivo* (11) may be responsible for, or may contribute to, the hemotoxic side effects of AZT, which produces macrocytic anemia and neutropenia (12–14). Cretton and co-workers (10) had expressed concern that AMT production by the placenta would be important due to the potential toxicity in the developing organism.

Metabolism of parent compounds by the placenta can produce toxicity in the fetus and/or fetal malformations. All-*trans* retinoic acid, a natural derivative of Vitamin A, for example, can be oxidized by the placenta to form the teratogenic metabolite, 4-oxo-all-*trans* retinoic acid (15). Placidi and co-workers (16) determined that formation of AMT from AZT occurred utilizing specific liver microsomal enzymes, and was not simply related to the presence of reducing equivalents. AMT formation did not correlate with NADPH-dependent cytochrome P450 reductase activity, nor did AMT formation correlate with cytochrome b content. However, formation of AMT was highly correlated with total cytochrome P450 content. Thus, in the liver AZT is reduced to AMT specifically *via* the cytochrome P450 enzymes: but not to the ones typically associated with reduction reactions, NADPH-dependent cytochrome P450 reductase, or cytochrome b. To date, there have been at least

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two forms of xenobiotic metabolizing cytochrome P450 enzymes present in the human placenta, an inducible form and a constitutive form (17). The inducible form appears to be CYP1A1; however, characteristics of this enzyme may be site and substrate specific since some substrates for liver CYP1A1 are not substrates for placental CYP1A1 (18, 19). The specific cytochrome P450 enzymes which convert AZT to AMT in the liver may also be present in the placenta. As such, formation of AMT by the placenta could, therefore, account for the observed toxicity of AZT in pregnant rodents and in human trophoblast cells. The purpose of the current study was to examine whether the *in vitro* dually perfused placenta, or placental cells in culture, produced AMT when exposed to AZT. Because of AMT's cytotoxic effect upon bone marrow cells *in vitro,* AMT formation in placental cells could account for AZT's toxicity in human placental cells in culture and altered placental function in the human placental perfusion paradigm.

Materials and Methods

Chemicals. AMT, 3′-amino-3′-deoxythymidine, was obtained from Sigma Chemical Co. (St. Louis, MO). AZT was kindly provided by NIAID. Acetonitrile, ammonium acetate, and water used in dilutions were all HPLC grade reagents.

Experimental Design—Cell Culture. JAr choriocarcinoma cells were derived from human cell lines provided by Patillo and Gey (20). Small plastic culture flasks (Corning #25100, Corning, NY) were seeded with 2.5×10^5 cells/flask. Cell cultures were grown in an incubator (NAPCO Co. #6300, Chicago, IL) maintained at 37°C with a 95% air and 5% CO_2 composition. Cells were grown in culture media consisting of RPMI-1640 media (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum, 1% L-glutamine (Gibco), 1% penicillin G/streptomycin (Gibco), and 5% sodium bicarbonate. Media were changed daily. In all experiments, cultures were allowed to grow for 3 days after seeding (before drug exposure) to allow cells to acclimate after cell transfer. Cells were exposed for 48 hr to AZT, 7.6 m*M,* which represents a concentration that is 200–300 times therapeutic levels (21, 22). Such a high concentration of AZT, at which cellular toxicity was observed, was selected so that toxicity could be associated with the formation of AMT. The cellular toxicity resulted in 72% inhibition of cellular proliferation when compared with unexposed controls, yet other cellular functions such as attachment to substrate and hormone production of human chorionic gonadotropin, estradiol, and progesterone were maintained (8). An additional consideration for using a high exposure concentration of AZT was that 10% of the AZT administered to patients is converted to AMT (11). If AMT was produced in placental cells, then intracellular levels high enough to exceed the lowest limit of detectability for the HPLC system must be present for the detection of AMT. After exposure to AZT, the media was removed from the culture flask and the cell surface rinsed with two 1-ml rinses of Mg^{2+}- and Ca^{2+}-free Hanks' balanced salt solution (HBSS). Cells were separated from plastic culture flask by adding 2 ml of 1 m*M* EDTA in HBSS, and were incubated for 10 min. An additional 8 ml of HBSS was added to the cell mixture. Cell homogenates were prepared by a series of three freeze-thaw cycles followed by vortex mixing after each freeze thawing. The cell preparation was then centrifuged at 1,000*g* for 10 min. The supernatant was then subjected to HPLC analysis. One series of cell extracts (1 ml) was spiked with 2500 ng AMT/400 ng AZT to confirm that the cell extract did not affect extraction and or detection of AMT.

Experimental Design—Placental Perfusion Studies. Placental perfusions were performed according to previously described methods (23, 24). After a 14-hr total exposure to AZT, 3.8 m*M, via* two transfusions in which perfusates were replaced with new perfusates with AZT every 4 hr (9), placental tissue was obtained from the perfused region. After the placental tissue was minced into small pieces, cell homogenates were prepared using a tissue homogenizer (<200 mg samples from perfused tissues weighing 15–33 g). The homogenate was then centrifuged at 1,000*g* for 10 min. The supernatant was decanted and subjected to HPLC analysis for detection of AMT and AZT.

HPLC Analysis for AMT and AZT. One-milliliter samples of cell homogenate supernatant were subjected to analysis by HPLC (Varian 5060, Varian Corp., Palo Alto, CA). Samples were transferred to a C-18 extraction column cartridge (Varian 2325KT) which had been pretreated with 100% acetonitrile followed by 50 m*M* ammonium acetate, pH 4.5. After addition of the sample, the sample cartridge was then washed with 0.5 ml of 50 m*M* ammonium acetate, pH 4.5, with the AMT and/or AZT bound to the column cartridge. In order to maximize extraction of AMT in JAr extracts, one column was loaded with 3 ml of cell extract and then subjected to further analysis. Use of these pre-HPLC cartridges removes most of the unwanted compounds and only the compounds of interest remain. As such, using these cartridges both concentrates the sample and removes unwanted compounds, which may interfere with the analysis. The sample cartridge was then placed on the Advanced Automatic Sample Processor (AASP, Varian), which allows for on-line multiple samples to be automatically injected onto the HPLC. Separation of components in the sample by the HPLC was accomplished using a Zorbax C-18 column (250 mm in length × 4.6 mm i.d.). Detection of components is by ultraviolet absorbance at 254 nm.

During the chromatographic run, the mobile phase changes from 10% acetonitrile/5 m*M* ammonium acetate, pH 7.0, at the start time to 40% acetonitrile/5 m*M* ammonium acetate, pH 7.0, over 10 min at a flow rate of 1.2 ml/min. The retention times using these methods were approximately 4.4 min for AMT and 8.8–8.9 minutes for AZT. Thus, using these chromatographic conditions, there is an approximate 4.5-min separation between AMT and AZT.

In order to determine the lowest limit of detectability of

AMT and to construct a standard curve for AMT, 1-ml samples were prepared in HPLC grade water in the following dilutions of AMT: 50, 100, 200, 300, 500, 5,000, 25,000, 75,000, and 150,000 ng. These samples were then processed for analysis of AMT as described above. The lowest limit of detectability for AMT was 100 ng, since AMT was not detected at 50 ng. With the loading of the 3-ml sample onto the extraction column, the lowest limit of detectability for AMT was 33.3 ng. The standard curve from 100 to 150,000 ng produced a linear relationship when subjected to regression analysis ($r^2 = 0.985$, $P < 0.001$). A similar standard curve for AZT consisting of 10, 50, 100, 200, 400, 800, 1600, 3000, and 10,000 ng produced a linear relationship ($r^2 = 1.0$, $P < 0.001$) and was used for quantifying AZT in cellular extracts.

Results

Figure 1A illustrates a typical chromatogram from a JAr cell extract from one experiment after 48 hr of exposure to AZT, 7.6 m*M*. As shown, AZT is the large peak at 8.8 min. There are also large peaks at 1.9–2.4 min, which may be phosphorylated derivatives of AZT. Note, however, that there was no detectable peak at 4.4 min, which is the retention time at which AMT would be expected. Two further determinations were made to confirm that AMT was not formed in JAr cells. Three milliliters of cell extract were loaded onto the C-18 extraction column (1 ml was loaded previously) in order to maximize the amount of AMT binding if any was present at very low concentrations. The results indicated a large peak for AZT (note that this AZT peak is larger than the AZT peak in Fig. 1A) and no peak for AMT (Fig. 1B). Finally, to confirm that the cell extract had not degraded or interacted with AMT, one cell extract (1 ml) was spiked with 2500 ng AMT/400 ng AZT. These results are illustrated in the next chromatogram and indicate that the peak for AMT was present, as expected, at 4.4 min (Fig. 1C). In addition, the area for the AZT peak in the spiked cell extracts is larger than the AZT peak in Figure 1A, confirming that this peak in the cell extracts is AZT. Tissue extracts from placental perfusion studies did not contain any AMT; but did exhibit a large amount of AZT (Table I). In all instances, whether JAr cell or human placental perfusion extracts were examined, AMT was not detected despite large amounts of cellular AZT. Thus, one can conclude from these results that AMT was not formed by placental cells after exposure to AZT, and is not a mechanism for the cytotoxicity observed in JAr cells.

Discussion

The results of this study demonstrate that human placental cells, either as trophoblast cells cultured for 48 hr with AZT, or as human placental tissue directly perfused with AZT for 8 hr, do not reduce AZT to AMT. Detection of the specific cytochrome P450 enzyme(s) for reducing AZT to AMT would have suggested that AMT formation could occur. Since these cytochrome P450 enzymes have yet to be identified in those tissues which produce AMT from AZT, detection of AMT after AZT exposure would be the only indicator of AMT formation. The analytical methods used in the current study are sensitive in detecting the presence of AMT if it is formed by placental cells after AZT exposure. However, in two placental models, the JAr choriocarcinoma cell line and the dually perfused placental lobule, no AMT was detected despite high cellular concentrations of AZT. Thus, AMT formation could be directly related to a class of cytochrome P450 enzymes which the placenta or trophoblast cells simply do not possess.

The lack of AMT detected in placental cells after AZT

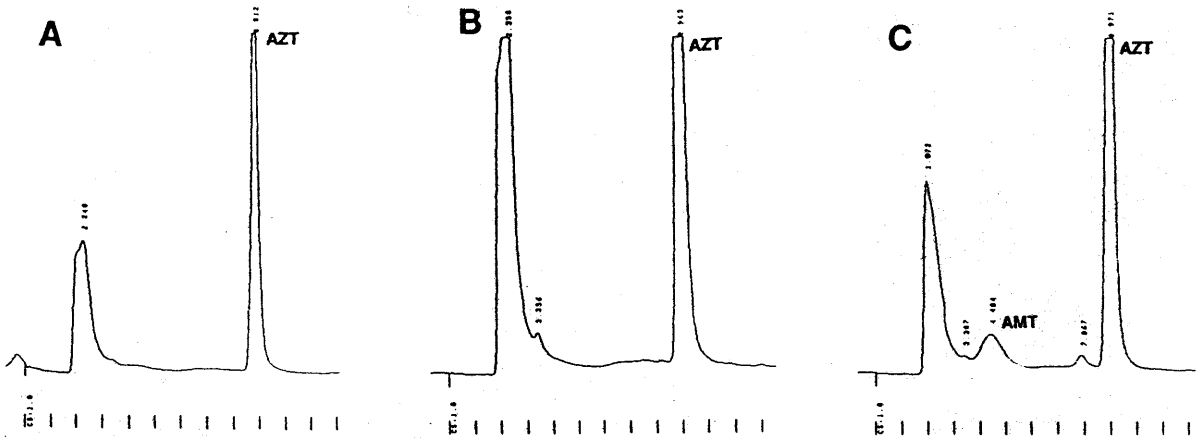

Figure 1. (A) Typical HPLC chromatogram of 1 ml of JAr cell extract sample (see text for description) illustrating peak for AZT at 8.812 min and no peak for AMT. Cell extract was obtained from JAr cells that had been exposed to 7.6 m*M* AZT for 48 hr. (B) Typical HPLC chromatogram of 3 ml of JAr cell extract sample (see text for description) illustrating peak for AZT at 8.963 min. Cell extract was obtained from JAr cells which had been exposed to 7.6 m*M* AZT for 48 hr (from same experiment as in Panel A). Note that, despite the larger AZT peak than in Panel A, there is no peak for AMT. (C) Typical HPLC chromatogram of 1 ml of JAr cell extract sample that had been spiked with 2500 ng AMT and 400 ng AZT prior to extraction and injection onto the HPLC (see text for description). Cell extract was obtained from JAr cells that had been exposed to 7.6 m*M* AZT for 48 hr (from the same experiment as illustrated in Panels A and B). AMT is now detected at 4.404 min, as expected. Tick marks indicate minutes after injection of sample onto the HPLC.

Table I. AZT/AMT Quantification/Detection in Human Placenta Using HPLC

Groups	Amount of AZT present (mean ± SD); value range (ng/ml)	Amount of AZT present (mean ± SD); value range ($\mu g/10^6$ cells for JAr Cells or µg/g for placental tissue)	Number of chromatograms with AZT present/number analyzed	Number of chromatograms with AMT present/ number analyzed
JAr extracts				
AZT exposed	4144 ± 2721; 1945–8910	26.9 ± 14.5; 12.4–50.8	12/12	0/12
Control			0/6	0/6
Placental extracts				
AZT exposed	2131 ± 285; 1611–2546	654 ± 76; 526–781	9/9	0/9
Control			0/9	0/9

Note. Data were obtained from duplicate analysis of JAr cell extracts obtained after a 48-hr exposure to 7.6 m*M* AZT or from triplicate analysis of placental tissue after 14 hr of perfusion with 3.8 m*M* AZT (See text for description). Data for JAr cells were obtained from six separate AZT exposure experiments and three separate control experiments. Data for placental extracts were obtained from three placentae from perfused tissue and from nonperfused control (unexposed) tissue.

exposure could be due to the disappearance of AMT. However, there are several characteristics of this study that would favor the formation and stability of AMT, and would allow for its detection. One characteristic is the large peak for AZT. If the specific cytochrome P450 reducing enzymes were present in placental cells, AMT formation could be expected to occur at the same rate of formation as it occurs in liver cells. If AMT formation had occurred, approximately 10% of the peak area of AZT should be in the form of AMT (11). In the examples illustrated, there should be peaks appearing for AMT that are at least two orders of magnitude higher than the lowest limit of detectability for AMT. Even a concentrated sample (Fig. 1B) does not demonstrate the presence of AMT. In this instance, the lowest limit of detectability would be 33.3 ng, since 3 ml of cell extract was passed through the pre-HPLC extraction column and 100 ng is the lowest limit of detectability. Based upon the peak area for AZT in this example, and based upon the results from Stagg *et al.* (11), 10% of this area should have been in the form of AMT. This would correspond to an area typical for approximately 7000 ng AMT, and thus would be readily detected, if present. Based on previous results using this exposure profile for AZT, cell numbers were reduced 72% when compared with controls, indicating that cytotoxicity occurs with this AZT exposure profile (8). Even if JAr cells were converting AZT to AMT, the rate of conversion would have to be less than 0.19% of the amount of AZT present. Another possibility for the disappearance of AMT is that AMT could be processed to AMT-monophosphate (MP). AMT-MP is formed utilizing cellular thymidine kinase, which also converts AZT to AZT-MP, as well as cellular thymidine to thymidine-MP. If AMT were present, it would have to compete with cellular thymidine and with a large amount of AZT in order to be converted to AMT-MP. In light of the amount of cellular AZT present, this loss of AMT to AMT-MP is unlikely.

The results of the current study indicate that placental trophoblast cells do not reduce AZT to AMT, a toxic catabolite responsible for producing cytotoxicity in bone marrow cells. Based upon results by others using liver microsomes, the reduction of AZT to AMT appears to be a specific cytochrome P450 reaction, but not related to other reducing equivalents. While the current studies were conducted *in vitro,* the possibility remains that *in vivo,* the liver may reduce AZT to AMT and AMT may circulate to the placenta and exert a toxic effect. However, *in vitro* the formation of AMT by placental cells does not occur and cannot explain the cellular toxicity observed in placental cells after exposure to AZT.

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