Biochemical Pharmacology, Vol. 40, No. 4, pp. 867–870, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00
Pergamon Press plc

EFFECT OF DIPYRIDAMOLE ON TRANSPORT AND PHOSPHORYLATION OF THYMIDINE AND 3′-AZIDO-3′-DEOXYTHYMIDINE IN HUMAN MONOCYTE/MACROPHAGES

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(*Received* 30 *October* 1989; *accepted* 19 *January* 1990)

Abstract—Dipyridamole (DPM), a commonly used coronary vasodilator and antithrombotic drug, was shown recently to potentiate the antiviral effect of 3′-azido-3′-deoxythymidine (AZT) in HIV-1 infected human monocyte-derived macrophages (M/M) *in vitro*. We report in the present study that in uninfected M/M, DPM markedly inhibited cellular uptake of [^{3}H]thymidine (dThd) and its incorporation into the nucleotide pools, particularly the dThd-triphosphate pool. In contrast, DPM did not affect cellular uptake and phosphorylation of [^{3}H]AZT. Since dThd counteracts the phosphorylation and antiviral action of AZT, these findings support the hypothesis that the potentiation of the anti-HIV effect of AZT is due, at least in part, to differential inhibition of nucleoside salvage.

Dipyridamole (DPM§) is a widely used coronary vasodilator and antithrombotic drug [1]. For the past few years considerable attention has also focused on its ability to potentiate the cytotoxic effects of a range of antitumor drugs [2]. Recently, a further possible use of DPM emerged in the treatment of human immunodeficiency virus type-1 (HIV-1) infections. We reported that DPM potentiates the antiviral effects of 3′-azido-3′-deoxythymidine (AZT) and 2′,3′-dideoxycytidine in HIV-infected human monocyte-macrophages (MM) *in vitro* [3]. The mechanism of this effect, however, remained unclear.

It has been established that AZT and dThd share the same phosphorylating enzymes in the cell (i.e. thymidine and thymidylate kinases) and that dTTP antagonizes the incorporation of AZT-TP into viral DNA by reverse transcriptase (RT). For these reasons dThd counteracts the phosphorylation and antiretroviral action of AZT [4–9]. It has also been shown that DPM inhibits carrier-mediated uptake of dThd in several cell types [1, 2, 10–13], and that AZT penetrates the membranes of red blood cells and lymphocytes through non-carrier mediated, passive diffusion [13, 14]. On these grounds, we suggested that the potentiation of the anti-HIV effect of AZT by DPM in M/M might be due to a differential inhibition of dThd uptake, with subsequent suppression of its antagonistic influence on the action of AZT [3]. To test this hypothesis, we measured in the present study the effect of DPM on uptake and phosphorylation of dThd and AZT by uninfected M/M, under conditions similar to those in the viral studies.

MATERIALS AND METHODS

Materials. DPM {2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido[5,4-*d*]pyrimidine} was obtained from the Sigma Chemical Co. (St. Louis, MO). It was dissolved in ethanol, and a 0.01 M stock solution was diluted to the test concentration (ethanol = 0.02%). AZT was prepared by Ash Stevens (lot HLR 0221) and obtained through the Developmental Therapeutics Branch, AIDS Program (National Institutes of Allergy and Infectious Diseases). [*Methyl*-^{3}H]dThd (108.8 Ci/mmol, radiochemical purity 97.8 to 99%) and [*methyl*-^{3}H]AZT (3 Ci/mmol, radiochemical purity ≥ 99%) were obtained from Moravek Biochemicals (Brea, CA).

Preparation of adherent monocyte-macrophage cultures. Peripheral blood monocytes were obtained by counterflow centrifugal elutriation as previously described [15, 16], except that pyrogen-free phosphate-buffered saline (PBS) (B & P/Scott, Carson, CA) was used in the elutriation procedure. Purified monocytes were plated in 6-well Costar plates (5×10^6 cells/well) in serum-free Dulbecco's Modified Eagle's medium (D-MEM) containing 2 mM glutamine and antibiotics. After overnight incubation, the medium was changed and supplemented with 10% fetal calf serum (FCS).

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§ Abbreviations: AZT, 3′-azido-3′-deoxythymidine; AZT-MP, AZT-monophosphate; AZT-DP, AZT-diphosphate; AZT-TP, AZT-triphosphate; D-MEM, Dulbecco's modified Eagle's medium; DPM, dipyridamole; FCS, fetal calf serum; HIV-1, human immunodeficiency virus type-1; M/M, monocyte-derived macrophages; PBS, phosphate-buffered saline; RT, reverse transcriptase; TCA, trichloroacetic acid; dThd, thymidine; dTMP, thymidine-monophosphate; dTDP, thymidine-diphosphate; and dTTP, thymidine-triphosphate.

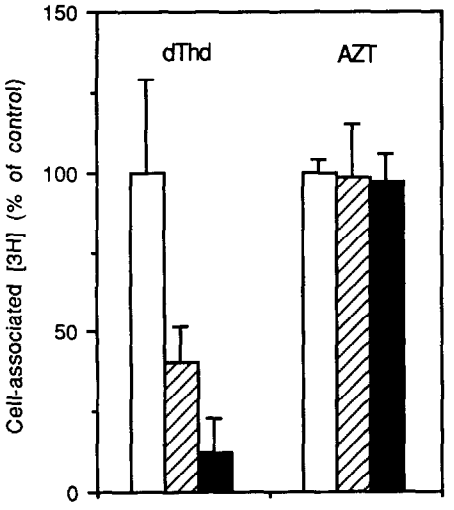

Fig. 1. Effects of dipyridamole (DPM) on cellular uptake of [^{3}H]dThd and [^{3}H]AZT by adherent monocyte-macrophages. Cells were exposed to the isotopes in the presence (cross-hatched) or absence (empty bars) of 2 μM DPM, as described in Materials and Methods. After 1 min of incubation, the cells were washed and analyzed for accumulated [^{3}H] activity. Nucleoside uptake is expressed as percent of control. Black bars represent cells preincubated with DPM for 10 min prior to adding the nucleosides. Means ± SD are shown for 2–3 independent experiments (with duplicate samples in most experiments). Control values (i.e. nucleoside uptake in the absence of dipyridamole taken as 100%) in a representative experiment with [^{3}H]dThd and [^{3}H]AZT were 15 and 29 fmol/10^6 cells/min respectively. The control error bars represent the mean coefficient of variation.

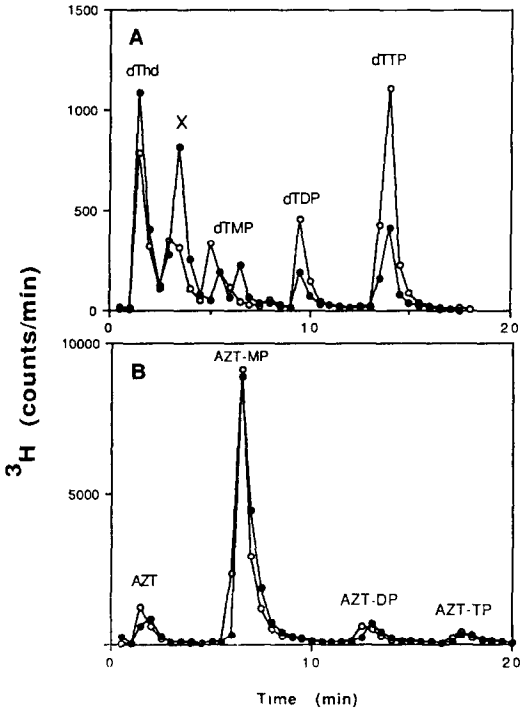

Fig. 2. Effects of 2 μM DPM on the incorporation of [^{3}H]dThd (A) and [^{3}H]AZT (B) into the nucleotide pools of adherent monocyte-macrophages. Typical HPLC chromatograms were recorded after a 3-hr incubation. Closed and open circles show DPM-treated and control cells, respectively. "X" in Panel A is an unidentified metabolite of [^{3}H]dThd. As thymine co-eluted with dThd, it could be ruled out as peak X. dThd and AZT nucleotide peaks were identified with authentic standards. Details of HPLC analysis are described in Materials and Methods. Splitting of the monophosphate peak, as shown for the treated cells in panel A, was also observed in other experiments, irrespective of treatment with DPM. For the purpose of this study, however, we did not differentiate a further nucleotide species and pooled these counts as monophosphate.

Uptake studies. After 1-day cultivation, nonadherent cells were removed by washing with medium, and the adherent cells were exposed to ≈0.8 μM [^{3}H]dThd (25 μCi/well, 0.25 μM as carrier dThd plus 0.5 μM dThd in 10% FCS [2]) or to 1 μM [^{3}H]AZT (3 μCi/well) in 1 mL medium with or without 2 μM DPM. The latter was either preincubated with the cells at 37° for 10 min or added together with the nucleosides. After a 1-min incubation at room temperature, cells were rapidly washed four times with ice-cold D-MEM containing 20 μM DPM (to inhibit efflux of the nucleoside during washing). Cells were detached from the plate with 2% sodium dodecyl sulfate, and aliquots were counted in a liquid scintillation counter. The choice of a 1-min incubation was dictated by the fact that nucleoside transport across cell membranes equilibrates in minutes. Longer incubations reflect cellular accumulation of nucleoside phosphates [2, 12].

HPLC analysis of [^{3}H]dThd and [^{3}H]AZT phosphorylation in monocyte/macrophages. In experiments similar to those described above, cells were incubated with [^{3}H]dThd or [^{3}H]AZT with or without 2 μM DPM at 37° in a humidified incubator (95% air/5% CO_2). At intervals the cells were washed as described above, and after exposure for 10 min to 400 μL of iced 5% trichloroacetic acid (TCA), they were scraped off the plate using a Costar scraper. This process was repeated with 200 μL TCA, the extracts were pooled, and the precipitates formed were pelleted by centrifugation. The supernatant fractions were neutralized by thorough mixing with 700 μL of ice-cold 20% tri-*n*-octylamine (Sigma) in freon. After phase separation, the upper, aqueous phase was collected and kept frozen until it was analyzed by HPLC with an HP 1090 chromatograph, using a Vydac 303NT405 nucleotide column. The column was eluted at a flow rate of 1 mL/min for 13 min, with a linear gradient of ammonium formate (pH 4.65) from 10 mM to 0 and of sodium phosphate (pH 2.8) from 0 to 0.5 M. Fractions of 0.5 mL were collected, and the radioactivity was measured in a liquid scintillation counter.

RESULTS AND DISCUSSION

The effects of DPM on uptake of [^{3}H]dThd and [^{3}H]AZT by M/M are shown in Fig. 1. At 1-min incubation, DPM inhibited [^{3}H]dThd uptake by ≈60% if it was added together with [^{3}H]dThd and

Table 1. Effect of 2 μM dipyridamole on cellular accumulation and incorporation of [^{3}H]dThd and [^{3}H]AZT into different nucleotide pools in adherent monocyte-macrophages

HPLC peak	[^{3}H]dThd		[^{3}H]AZT	
	Peak ratio (DPM/no-DPM)	Peak integral (% of total)	Peak ratio (DPM/no-DPM)	Peak integral (% of total)
3-hr Incubation (N = 3–4)				
Nucleoside	1.16 ± 0.11	25 ± 4	1.05 ± 0.08	22 ± 12
Peak "X"	1.62 ± 0.12*	10 ± 5		0
Monophosphate	0.73 ± 0.09†	15 ± 2	0.91 ± 0.06	72 ± 5
Diphosphate	0.77 ± 0.11	12 ± 3	0.95 ± 0.08	7 ± 1
Triphosphate	0.37 ± 0.07‡	37 ± 4	0.92 ± 0.06	3 ± 1
6-hr Incubation (N = 5–6)				
Nucleoside	0.80 ± 0.06*	42 ± 12	0.92 ± 0.03	13 ± 2
Peak "X"	>2.32 ± 0.34*§	20 ± 6		0
Monophosphate	0.59 ± 0.15*	16 ± 7	0.91 ± 0.05	74 ± 2
Diphosphate	0.53 ± 0.10‡	8 ± 2	0.91 ± 0.05	9 ± 1
Triphosphate	0.42 ± 0.12‡	17 ± 5	0.91 ± 0.05	4 ± 0

"Peak ratio" represents the ratio of the individual HPLC peak integral in DPM-treated samples to that in the non-treated (control) samples. Peak integrals were obtained by summing [^{3}H] counts under the individual peaks, identified with authentic standards (shown in Fig. 2). "Peak integral" gives the relative size, expressed as percent of recovered counts for the different HPLC peaks in the control, non-treated samples. "0" indicates values below the limit of detection. Means ± SE of N samples were obtained in two to four independent experiments. Other experimental details are described in Materials and Methods.

* Significantly different from unity at $P < 0.05$ (unpaired, two-tail Student's *t*-test).

† $P = 0.06$

‡ Significantly different from unity at $P < 0.01$.

§ Peak ratio was infinite in one experiment (peak "X" increased from 0 to 17%), which was not included in the calculation.

by ≈90% if the cells had been exposed to the drug for 10 min prior to adding the nucleoside. In contrast, [^{3}H]AZT uptake was not changed by DPM, with or without pretreatment. These observations indicate that in M/M. as in several other cell types [1, 2, 10–13], DPM inhibits dThd transport. They also suggest that AZT bypasses the nucleoside carrier in these cells, as it has been reported to do in erythrocytes and lymphocytes [13, 14]. The effect of preincubation argues against competitive, reversible inhibition of nucleoside transport [2], but it is consistent with the hypothesis that DPM partitions into the membrane and interacts with hydrophobic domains of the transporter protein [2, 10].

Next, we asked how DPM influences nucleoside salvage, that is, incorporation of [^{3}H]dThd and [^{3}H]AZT into nucleotide pools. As shown in Fig. 2A and Table 1, cell-associated [^{3}H]dThd levels were not lower in DPM-treated cells than in control cells at 3 hr, and they were decreased only slightly at 6 hr. The [^{3}H]dTMP and [^{3}H]dTDP peaks appeared smaller by 3 hr, and they were significantly smaller than the corresponding peaks in control cells at 6 hr. The [^{3}H]dTTP peaks were decreased significantly (by ≈60%) both at 3 and 6 hr in the presence of DPM. In contrast, neither the cellular levels nor the incorporation of [^{3}H]AZT into nucleotides was altered significantly by DPM at these times. Figure 2A and Table 1 show a further DPM effect: enhancement of the incorporation of [^{3}H]dThd into an unidentified peak ("X"), which eluted from the nucleotide column between [^{3}H]dThd and [^{3}H]dTMP. This peak was present and was increased by DPM in three out of four experiments, but nothing resembling peak "X" was seen in cells treated with [^{3}H]AZT. Its identity and possible role in the antiviral effect of DPM are currently under study.

These observations, taken together with the key role that dThd-phosphates play in antagonizing the antiviral effect of AZT [4–9], lend support to the hypothesis that differential effects on nucleoside transport and/or phosphorylation could play a role in the potentiating influence of DPM on the antiviral action of AZT [3]. However, the relationship between transport and phosphorylation is not clear. In particular, it is not known whether inhibition of transport can account, by itself, for inhibition of the formation of dThd nucleotides or whether other effects of DPM are also involved in this phenomenon. As mentioned earlier, carrier-mediated transmembrane flux of nucleosides equilibrates in minutes, and it is not known to be rate-limiting to the subsequent phosphorylation process that occurs on a time scale of hours. In fact, we observed little or no decrease of cell-associated [^{3}H]nucleoside level in DPM-treated cells at times when the [^{3}H]dThd-phosphates were decreased substantially. This observation is compatible, among other possible explanations, with a direct influence of DPM on dThd phosphorylation.

In summary, short-term incubations provided evidence that DPM inhibits the uptake of [^{3}H]dThd in cultured M/M, and longer-term incubations suggest that DPM also perturbs nucleoside metabolism in these cells. These effects led to decreased formation of [^{3}H]dThd-nucleotides from exogenous [^{3}H]dThd,

i.e. decreased dThd salvage. At the same time, the uptake and phosphorylation of [^{3}H]AZT were not affected. The effect of decreased nucleoside salvage on dThd-nucleotide pool sizes and the extent by which dThd suppresses the antiviral action of AZT in M/M are not known. However, the findings of this study support the hypothesis that the potentiation of the anti-HIV effect of AZT by DPM [3] is due, at least in part, to a suppression of the antagonistic influence of dThd-phosphates (and possibly other physiological nucleoside phosphates) on the antiviral activity of AZT.

Acknowledgements—We are grateful to Drs. R. J. Parker for help in HPLC analysis, P. S. Torrence and Terez Kovacs for providing phosphorylated AZT standards, and S. M. Wahl for helpful discussion. S.S.P. was supported by a fellowship from the Howard Hughes Medical Institute. This work was supported in part by a grant from the National Institutes of Health Intramural AIDS Targeted Antiviral Program.

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