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Azidothymidine Homodinucleotide-Loaded Erythrocytes as Bioreactors for Slow Delivery of the Antiretroviral Drug Azidothymidine

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A new Azidothymidine derivative, di-(thymidine-3′-azido-2′,3′-dideoxy-D-riboside)-5′-5′-p^1-p^2-pyrophosphate ($AZT_{p2}AZT$), was encapsulated in human erythrocytes according to a conservative procedure of hypotonic shock-isotonic resealing and reannealing. Like in erythrocyte lysates supplemented with 1 mM ATP, intact red cells too were found to convert $AZT_{p2}AZT$ to 3′-Azido-3′-deoxythymidine which was then released linearly in plasma. The major metabolic pathway involved in this conversion was the symmetrical hydrolysis of $AZT_{p2}AZT$ to yield two 3′-Azido-3′-deoxythymidine-5′-phosphate molecules which were then dephosphorylated to 3′-Azido-3′-deoxythymidine. At late times of incubation, also a limited asymmetrical hydrolysis of $AZT_{p2}AZT$ became apparent in the intact erythrocytes, yielding 3′-Azido-3′-deoxythymidine-5′-diphosphate that was then converted to the triphosphorylated derivative. Therefore, erythrocytes loaded with $AZT_{p2}AZT$ act "in vitro" as bioreactors ensuring sustained and potentially useful release of 3′-Azido-3′-deoxythymidine.

Properly engineered erythrocytes can behave as slow delivery systems for cytotoxic drugs, improving their pharmacokinetic patterns and therapeutic performances (1–8). There are two major ways of achieving erythrocyte-based release of drugs. One is direct encapsulation of membrane-diffusable drug molecules. The second way is internalization of impermeant pro-drugs susceptible to be metabolically converted by endogenous erythrocyte enzymes to membrane-releasable active drugs. The latter approach is more versatile since the choice of the pro-drugs to be synthesized will depend on the properties of the erythrocyte enzymes responsible for their bio-activation. Furthermore, the pro-drugs can be structurally tailored in order to meet pharmacokinetic requirements, e.g. by designing a multi-step bioconversion pathway instead of direct activation of a pro-drug by a single enzyme (9).

In both cases of engineered erythrocytes, either passive carriers or bioreactors, the release of drugs can be obtained in the circulatory system or at specific sites. While targeting of loaded erythrocytes to selected organs may meet problems related to species-specific differences (10), the natural property of erythrophagocytosis of monocytes/macrophages makes these cells ideal compartments for erythrocyte-based delivery of drugs. A successful example of this strategy is the targeting of ddCTP, the pharmacologically active form of the antiretroviral nucleoside analogue 2′,3′-dideoxycytidine, to macrophage cells (11).

We have recently synthesized a new molecule, a homodinucleotide of 3′-Azido-3′-deoxythymidine derivative ($AZT_{p2}AZT$) which, following encapsulation in macrophage-targeted erythrocytes, conferred protection to human, feline and murine macrophages from infection by

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Abbreviations: $AZT_{p2}AZT$, di-(thymidine-3′-azido-2′,3′-dideoxy-D-riboside)-5′-5′-p^1-p^2-pyrophosphate; AZT, 3′-azido-3′-deoxythymidine; AZT-MP, 3′-azido-3′-deoxythymidine-5′-phosphate; AZT-DP, 3′-azido-3′-deoxythymidine-5′-diphosphate; AZT-TP, 3′-azido-3′-deoxythymidine-5′-triphosphate; ddCTP, 2′,3′-dideoxycytidine-5′-triphosphate; HIV, human immunodeficiency virus; FIV, feline immunodeficiency virus; FCS, fetal calf serum; PBS, phosphate-buffered saline; 5-FdUR, 5-Fluoro-2′-deoxyuridine.

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HIV, FIV and LP-BM5, respectively (12). Another study has shown that AZT-MP, when internalized in human erythrocytes, is converted to AZT that then diffuses outside the red cells (13). The present investigation addressed the question of possible metabolic degradation of $AZT_{p2}AZT$ inside human erythrocytes, which would allow them to behave as bioreactors for the delivery of AZT in the circulatory system, in addition to their demonstrated role of targeted carriers of $AZT_{p2}AZT$ to the macrophage compartment. The results obtained demonstrate that $AZT_{p2}AZT$-loaded human erythrocytes can be used for both purposes, thereby affording a potential multi-target cell protection against HIV infection.

MATERIALS AND METHODS

Synthesis of $AZT_{p2}AZT$. $AZT_{p2}AZT$ was synthesized and characterized as previously described (12).

$AZT_{p2}AZT$ metabolism in intact erythrocytes. Human erythrocytes were loaded with $AZT_{p2}AZT$ at a final concentration of 0.15±0.005 μmoles/ml erythrocytes as reported in ref. 12. Drug-loaded erythrocytes, prepared under sterile conditions, were resuspended at 0.5% hematocrit in RPMI 1640 medium containing 2 mM glutamine and 10% (v/v) fetal calf serum (FCS) and incubated at 37°C for up to 140 hrs. At time intervals as indicated in Fig. 2, samples (3 ml) were collected, centrifuged at 1,500 × g and the cell pellets and the medium separately extracted with perchloric acid as described (12). Neutralized perchloric acid extracts were submitted to solid-phase extraction using isolute TM C18 columns (International Sorbent Technology, Mid-Glamorgan, UK) according to the instructions of the manufacturer. $AZT_{p2}AZT$ and its metabolites were determined by HPLC chromatography essentially as described (12).

Synthesis and characterization of AZT-MP and AZT-DP. AZT-MP was synthesized as previously reported (13).

AZT-DP was obtained according to Michelson (14). Briefly, 0.25 mmoles of AZT-5′-p^1,p^2-diphenylpyrophosphate [obtained as described in (12)] were dissolved in 1.8 ml of anhydrous pyridine and added to 0.5 mmoles of tri-*n*-octylammonium orthophosphate together with 0.32 ml of hexamethylphosphotriamide. The mixture was dried under reduced pressure and the synthesis was carried out as in the $AZT_{p2}AZT$ preparation (12). AZT-DP was then purified using a HRLC MA7Q Anion Exchange Column (Biorad, Richmond, Virginia, USA, 100 × 19 mm).

Elution was carried out stepwise as follows: 20% CH_3OH in water (sol. A) for 10 min, then a linear gradient starting from sol. A up to 30% sol. B (0.5 M LiCl in sol. A) for 30 min, at a constant flow of 7 ml/min. The eluted compounds were detected by an HP 1040 A diode array spectrophotometric detector set at 260 nm. The fractions containing AZT-DP were collected and vacuum dried and the residue was washed 6 times with 5 ml of a solution of ethanol-acetone (1:1). Finally AZT-DP was resuspended in 1 ml water, lyophilized and submitted to mass spectrometry analyses.

Mass spectra were acquired in the negative ion mode using a single quadrupole HP Engine 5989-A equipped with an electrospray ion source. The mass spectrum of the purified product showed a molecular ion at m/z 426.7, consistent with the $[M-H]^-$ ion of the expected AZT-DP molecule. AZT-DP stability and metabolism in the human erythrocyte lysate and in intact erythrocytes were evaluated as reported for $AZT_{p2}AZT$.

Upgrading of erythrocyte loading. Encapsulation of $AZT_{p2}AZT$ in human erythrocytes at a final concentration of 3.9±0.1 mmoles/ml erythrocytes was obtained as reported in ref. 9.

RESULTS

Metabolism of $AZT_{p2}AZT$ in erythrocyte lysates. Preliminary experiments showed that $AZT_{p2}AZT$ has a considerable stability in erythrocyte lysates, particularly in the presence of 1 mM ATP. Thus, after two hours at 37°C, 90% of $AZT_{p2}AZT$ was still present. Without addition of ATP, $AZT_{p2}AZT$ decayed down to 62% at two hours. As shown in Fig. 1, the decrease of $AZT_{p2}AZT$ was paralleled by progressive increase of AZT-MP and by a remarkably lower appearance of AZT. No apparent formation of AZT-DP was observed in the lysates either in the presence or absence of ATP, this indicating symmetrical rather than asymmetrical hydrolysis of $AZT_{p2}AZT$ under these conditions.

Metabolism of $AZT_{p2}AZT$ in intact erythrocytes. Encapsulation of $AZT_{p2}AZT$ did not result in any appreciable alteration of erythrocyte morphology and metabolism (not shown). In order to evaluate the intraerythrocytic metabolism of $AZT_{p2}AZT$ over long times of incubation and under sterile conditions, sub-optimal concentrations of internalized $AZT_{p2}AZT$ and hematocrit values as low as 0.5% in FCS were used. As shown in Fig. 2, a slow and linear decrease of intraerythrocytric $AZT_{p2}AZT$ was observed in these conditions, down to 12% of the starting levels at 140 hrs of incubation. The production of AZT-MP within red cells started almost immediately, peaking at 50 hrs. The output of AZT from red cells began at 24 hrs and progressed linearly over the incubation

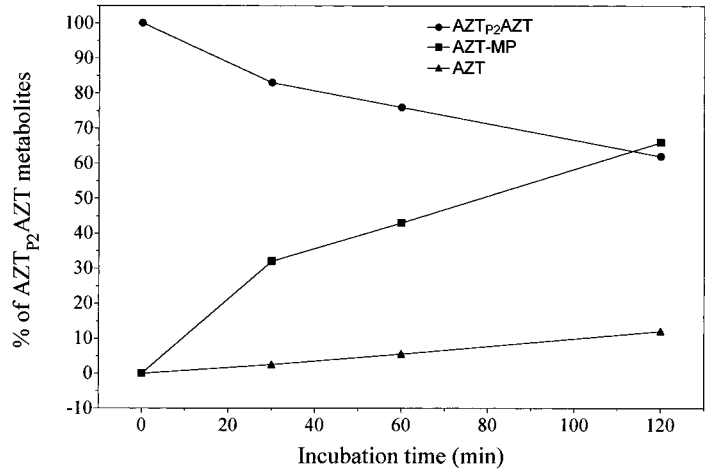

FIG. 1. $AZT_{p2}AZT$ metabolism in erythrocyte lysates. Human red blood cells were washed twice in PBS, and lysed by addition of two volumes of distilled water containing 3 mM 2-mercaptoethanol and 0.5 mM EDTA. After 30 min on ice the cell lysate was centrifuged at 4°C and 12,000 × g for 60 min to remove the cell membranes. The membrane-free hemolysate was then dialyzed overnight against 200 volumes of 0.9% (w/v) NaCl, containing 10 mM Tris-HCl, 10 mM $MgCl_2$, 0.02 mM EDTA, pH 7.5, and incubated at a concentration of 100 mg of hemoglobin per ml at 37°C in the presence of 0.2 mM $AZT_{p2}AZT$. At times 0, 30, 60 and 120 min, aliquots were removed and extracted with perchloric acid as described (13). Neutralized extracts were then used for HPLC determination of $AZT_{p2}AZT$ and its metabolites (12). Results of a representative experiment are shown. In two other experiments variability never exceeded 10%.

time. At the late time intervals (since 70 hrs onwards), a small yet appreciable formation of both AZT-DP and AZT-TP was consistently detected.

Since human erythrocytes are unable to phosphorylate AZT-MP to AZT-DP (13), the appearance of the latter metabolite should derive from an asymmetrical hydrolysis of $AZT_{p2}AZT$ that becomes evident upon prolonged incubation, yielding low amounts of AZT and AZT-DP. In order to

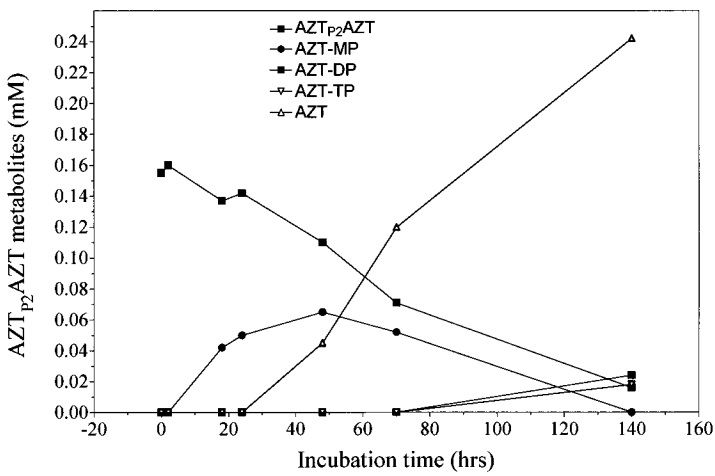

FIG. 2. $AZT_{p2}AZT$ metabolism in intact erythrocytes. Human red blood cells were loaded with $AZT_{p2}AZT$ by a procedure of hypotonic dialysis and isotonic resealing to a final concentration of 0.15 mM (12). At the time intervals indicated, $AZT_{p2}AZT$ and its metabolites were determined as described under Materials and Methods. AZT and low amounts of AZT-MP (5%–10% of the total) were found in the incubation medium, while all other metabolites were present in the erythrocyte extracts. For sake of clarity, results of a representative experiment are shown, out of three different ones in which variability never exceeded 12%.

evaluate the pathway of AZT-TP formation, as observed at these long times, erythrocyte lysates were incubated with AZT-DP in the presence and in the absence of 1 mM ATP. As shown in Fig. 3, the progressive decay of AZT-DP was accompanied by AZT-TP formation in the presence of ATP only. Evaluation of some kinetic properties for this reaction provided Km values for AZT-DP of 1.5±0.1 mM and a Vmax of 0.15±0.02 nmoles min^{-1} mg $hemoglobin^{-1}$. Intact erythrocytes loaded with 0.4 μmoles AZT-DP/ml erythrocytes and incubated at 37°C up to 4 hrs, provided results similar to those shown in Fig. 3 (not shown).

AZT_{p2}AZT-loaded erythrocytes as AZT-releasing bioreactors. The potential of the AZT_{p2}AZT-loaded red cells to behave as bioreactors suited to the delivery of AZT in circulation was explored by upgrading the starting intraerythrocytic AZT_{p2}AZT levels as compared to Fig. 2 and following the release of AZT in the surrounding plasma (Table I). Indeed, the loaded erythrocytes showed a high efficiency of AZT formation and export, but a significant release of AZT-MP was also observed, as previously reported for erythrocytes (13) loaded with high AZT-MP concentrations.

DISCUSSION

The present data demonstrate that human erythrocytes can metabolize the newly designed and synthesized AZT dinucleotide to yield AZT that is eventually released by simple diffusion (15). The major pathway of such bioconversion is symmetrical degradation to two AZT-MP molecules that are then dephosphorylated by a 5′-nucleotidase (13). Hydrolysis of the pyrophosphate bond of AZT_{p2}AZT is most probably catalyzed by a dinucleotide pyrophosphatase previously identified and characterized in human erythrocytes (16), as suggested by inhibition of AZT_{p2}AZT degradation by ATP. A slower and delayed asymmetrical hydrolysis of AZT_{p2}AZT can produce AZT and AZT-DP (Fig. 2), the latter compound being then phosphorylated to AZT-TP by a kinase activity (Fig. 3). AZT-DP and AZT-TP might represent storage metabolites for delayed formation and sustained delivery of AZT, as previously demonstrated for intraerythrocytic 5-FdUR metabolites (9). Permeation of AZT-MP across the erythrocyte membrane has already been observed in AZT-MP-loaded human red cells (13), and human plasma is able to dephosphorylate AZT-MP, this contributing to the total AZT pool present in plasma.

The efficiency of AZT_{p2}AZT in releasing AZT can be modulated by encapsulating different amounts of the dinucleotide. Thus, sub-millimolar concentrations of internalized AZT_{p2}AZT seem

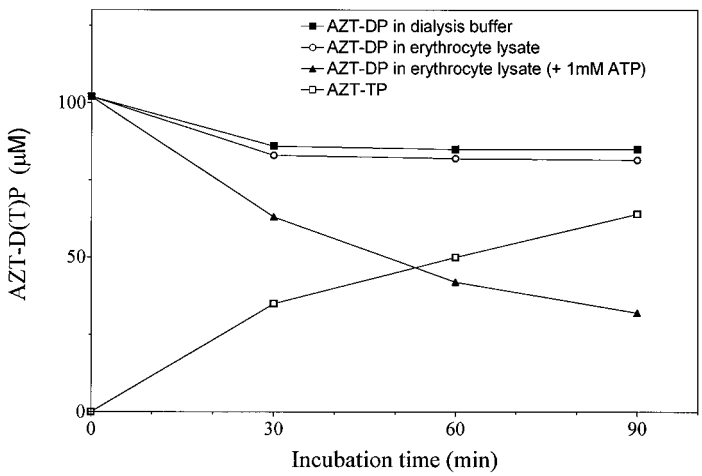

FIG. 3. AZT-DP metabolism in human erythrocyte lysates. AZT-DP (0.1 mM) was incubated at 37°C in the dialysis buffer or in human erythrocyte lysate (see legend to Fig. 1) in the absence or in the presence of 1 mM ATP. Under the latter conditions the disappearance of AZT-DP was paralleled by the formation of AZT-TP. Results of a representative experiment are shown, out of three different ones in which variability never exceeded 12%.

TABLE 1
Patterns of AZT Release from $AZT_{p2}AZT$-Overloaded Human Erythrocytes[a]

Time (hrs)	$AZT_{p2}AZT$[b]	AZT-MP[b]	AZT[b]
0	4.5	21.9	4.6
2	3.7	28.2	14.4
6	5.3	24.7	62.4
24	5.3	67.1	355.8

[a] The methodology of encapsulation (12) was upgraded to result in starting concentrations of intraerythrocytic $AZT_{p2}AZT$ as high as 3.9 μmoles/ml packed red cells, as described in Materials and Methods. Incubation was at 37°C in autologous plasma at a 10% hematocrit. The stably low levels of $AZT_{p2}AZT$ in plasma are apparently due to microhemolysis of the loaded red cells, while the concentrations of AZT-MP (13) and of AZT reflect output from the cells. The release of AZT during time shows upconcave patterns. For sake of clarity, results of a representative experiment are shown, out of four different ones in which variability never exceeded 11%.

[b] Values are expressed in nanomoles/ml plasma.

to be ideal to achieve the slow release patterns shown in Fig.2. Millimolar $AZT_{p2}AZT$ would conversely result in a more rapid AZT delivery (Table I). Probably, the inhibitory effects of ATP on the rate of $AZT_{p2}AZT$ degradation can in part explain these different patterns.

In conclusion, the $AZT_{p2}AZT$-loaded erythrocytes seem to have a two-fold potential in the therapy of HIV infection. Their membrane perturbation to elicit protein clustering and subsequent phagocytosis is a means to achieve targeted delivery to macrophages, with significant protection against retroviral infection (12). On the contrary, unmodified, $AZT_{p2}AZT$-loaded red cells are efficient bioreactors for the untargeted production and output of AZT. Sustained release of AZT in circulation is advocated to avoid some toxic effects, mostly myelosuppression, which are induced by conventional (oral or intravenous) administration of this molecule. Therefore, a single batch of $AZT_{p2}AZT$-loaded erythrocytes might be subdivided and processed to induce macrophage targeting on one hand (12) or used as an AZT-releasing bioreactor on the other. In the latter case, the efficiency of the bioreactor (see Table I) is such that less than two ml of $AZT_{p2}AZT$-overloaded erythrocytes would be sufficient to produce and release within 24 hrs the same total amount of plasma AZT detected after 1 h in subjects receiving a bolus of 100 mg of AZT (17). Although proper allowance should be made for renal clearance of AZT "in vivo", the slow delivery patterns observed in this study hold promise for therapeutic use of limited volumes of autologous $AZT_{p2}AZT$-overloaded erythrocytes.

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