Targeting antiretroviral nucleoside analogues in phosphorylated form to macrophages: *In vitro* and *in vivo* studies

(2',3'-dideoxyctydine 5'-triphosphate/human immunodeficiency virus inhibition/murine acquired immunodeficiency syndrome/erythrocytes)

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**ABSTRACT** A number of nucleoside analogues are active against the infectivity of human immunodeficiency virus (HIV); however, their use is limited by toxic side effects and by limited phosphorylation in the infected cells. In an attempt to overcome these problems, a drug delivery system has been developed. A prototype of these drugs in a form already phosphorylated (2',3'-dideoxyctydine 5'-triphosphate; ddCTP) was encapsulated into erythrocytes. Subsequently, by the addition of Zn, an arrangement of band 3 in clusters was induced (band 3 is the major transmembrane protein in erythrocytes). The immune system recognizes these clusters as nonself, promoting autologous IgG binding and phagocytosis by cells of the monocyte–macrophage lineage. In this way, ddCTP encapsulated into erythrocytes was delivered to macrophage cells, where concentrations >2 μM were found. Addition of ddCTP-loaded erythrocytes to macrophages previously infected by HIV-1 resulted in almost complete inhibition of HIV production over 3 weeks in culture. Administration of ddCTP-loaded erythrocytes to LP-BM5-infected mice at 10-day intervals over a period of 3 months resulted in reduction of lymphadenopathy, splenomegaly, and hypergammaglobulinemia. Thus, the delivery of nucleoside analogues in phosphorylated form is feasible, and selective targeting to virus reservoirs (macrophage cells) can be accomplished by the use of autologous erythrocytes.

A broad family of nucleoside analogues, particularly those with a 2',3'-dideoxyribose moiety, are able to inhibit the infectivity and replication of several human immunodeficiency virus type 1 (HIV-1) strains in vitro (1, 2). The most popular of these compounds 3'-azido-2',3'-dideoxyribosylthymine (AZT) has been found to reduce morbidity and mortality associated with severe HIV infection. However, its dose-limiting toxicity has prompted the development of other dideoxynucleosides as potential therapeutic agents. 2',3'-Dideoxyctydine was then developed and tested clinically (3, 4). Again, clinical trials have shown that dideoxyctydine can produce immunological and virologic improvement in AIDS patients, but several individuals developed a dose-limiting toxic effect. The same applies to 2',3'-dideoxyinosine (5, 6) and will probably occur for other analogues. Furthermore, drug benefit may be limited by failure, over months to years, of therapy.

To be pharmacologically useful, dideoxynucleosides must be phosphorylated to 5'-triphosphate by cellular kinases (1, 2, 4). Different cell types within the same species have different abilities to phosphorylate these compounds (7), and other drugs can potentiate or reduce the activity of these nucleoside analogues by interfering with their metabolism directly or indirectly (8, 9).

In an attempt to overcome the problem of nucleoside analogue toxicity as well as the efficiency of their phosphorylation, we have encapsulated dideoxyctydine in the active phosphorylated form in erythrocytes. Subsequently, this encapsulated drug was targeted to cells of the monocyte–macrophage (M/M) lineage, which are important hosts for HIV-1 with a key role in the propagation of virus as well as in the pathogenesis of AIDS. The results obtained show that this treatment suppresses *in vitro* HIV-1 production in infected M/M, while in a mouse model of immunodeficiency it substantially reduces signs of the disease.

**MATERIALS AND METHODS**

Cells. Mononuclear cells were prepared by Ficoll-Paque sedimentation from leukapheresed healthy HIV-1 antibody-negative donors, washed twice in sterile saline, and cultured in RPMI medium supplemented with 20% (vol/vol) heat-inactivated human serum. After 3 days in culture, nonadherent cells were removed and M/M were maintained in RPMI medium supplemented with 10% (vol/vol) fetal calf serum. These cells were found to be >95% M/M as determined by nonspecific esterase staining and Leu-M3 positivity. Cells were infected with a purified preparation of HIV-1 obtained from and propagated in CEM cells. Exposure to the virus was for 3 h at 7 ng of 300,000 × g sedimenting p24 per 10⁷ cells. Viral doses 10- to 20-fold higher provided the same viral production (3–7 ng of p24 per 10⁵ cells) 3–4 weeks after infection. Treatments of infected M/M with ddCTP-loaded erythrocytes (see below) were performed overnight just after infection. After treatment, the medium containing erythrocytes was removed, and the M/M cells were washed twice and cultured in fresh RPMI complete medium.

**Mice and Virus**. LP-BM5 viruses were prepared as cell-free supernatants from SC-1 cells as described in ref. 10. C57BL/6 mice were infected by an intraperitoneal injection of 0.1 ml of the virus stock containing 6.6 × 10⁵ cpn of reverse transcriptase.

**ddCTP Encapsulation in Erythrocytes**. Human or murine erythrocytes were washed twice in 10 mM Hepes/140 mM NaCl/5 mM glucose, pH 7.4 (buffer A), to remove leukocytes and platelets and were resuspended at 70% hematocrit in buffer A. These cells were dialyzed for 45 min using a tube with a cut-off of 12–14 kDa against 50 vol of 10 mM NaH₂PO₄/20 mM glucose/4 mM MgCl₂, pH 7.4, containing 3 mM reduced glutathione and 2 mM ATP (buffer B). The

Abbreviations: AZT, 3'-azido-2',3'-dideoxyribosylthymine; M/M, monocyte–macrophage; BS3, bis(sulfosuccinimidy)suberate; HIV-1, human immunodeficiency virus type 1.

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osmolarity of buffer B was 58 mosM. After this time, 16 μmol of ddCTP (lithium salt) was added to each ml of erythrocyte suspension that was further dialyzed against 5–10 vol of 16 mM NaH2PO4 (pH 7.4) containing 4 mM ddCTP for a further 45 min. All these procedures were performed at 4°C. Resealing of the erythrocytes was obtained by adding 0.1 vol of 5 mM adenine/100 mM inosine/2 mM ATP/100 mM glucose/100 mM sodium pyruvate/4 mM MgCl2/0.194 M NaCl/1.606 M KCl/35 mM NaH2PO4, pH 7.4, per vol of dialyzed erythrocytes and by incubating at 37°C for 20 min. Resealed cells were then washed three times in buffer A and used as they were or processed further to increase their recognition by macrophages.

**ddCTP Stability in Human Erythrocytes.** The stability of ddCTP was evaluated both in human erythrocyte lysates as well as in intact erythrocytes. Human erythrocyte lysates were prepared by adding 2 vol of 3 mM sodium potassium phosphate buffer (pH 7.4) containing 3 mM mercaptoethanol and 0.5 mM EDTA to each vol of washed and packed erythrocytes. After 30 min at 4°C, the lysates were centrifuged for 1 h at 12,000 × g to remove the cell membranes. The supernatants were then dialyzed overnight against 160 vol of 0.9% (wt/vol) NaCl containing 10 mM Tris-HCl (pH 7.5), 10 mM MgCl2, and 0.02 mM EDTA. The dialyzed lysates were then used for studies of ddCTP stability. Briefly, lysates containing 50 μM, 100 μM, 300 μM, 1.2 mM, or 1.5 mM ddCTP were incubated at 37°C. At time intervals of 0, 15, 30, and 60 min, 250 μl of the incubation mixtures was removed and extracted with 125 μl of 10% (vol/vol) HClO4. The HClO4 extract was then neutralized, processed, and analyzed by HPLC as described in ref. 11.

The stability of ddCTP (1 mM) in loaded erythrocytes was evaluated at 37°C by incubation of ddCTP-loaded erythrocytes at 6% hematocrit in 0.9% (wt/vol) NaCl containing 10 mM glucose and 5 mM sodium potassium phosphate buffer (pH 7.4). In some experiments 1HddCTP (4 Ci/mm; 1 Ci = 37 GBq; Moravek Biochemicals, Brea, CA) was used. In this case, fractions of 0.3 ml were collected at the exit of the wavelength detector of the HPLC system and assayed. Both methods (HPLC detection and liquid scintillation counting) provided similar results.

**Targeting of ddCTP-Loaded Erythrocytes to Human M/M.** Targeting of erythrocytes to M/M was obtained as follows: first, erythrocytes were subjected to the procedure of ddCTP encapsulation as specified above, and then loaded erythrocyte suspensions (10% hematocrit) in 1 mM ZnCl2 were treated with 1 mM bis(sulfosuccinimidyl)suberate (BS3) for 15 min at room temperature and washed once in buffer A containing 10 mM ethanalamine and once in buffer B containing 1% (wt/vol) bovine serum albumin. These cells were then used immediately or incubated in autologous plasma for 30–60 min at room temperature for determination of bound IgG molecules by evaluating 125I-labeled protein A (125I-protein A) binding. Briefly, loaded erythrocytes treated with ZnCl2 and BS3 and controls were incubated at room temperature in autologous plasma for 30–60 min at a hematocrit of 40% and then washed twice in 10 mM Hepes buffer (pH 7.4) containing 140 mM NaCl, 5 mM glucose, and 2% (wt/vol) bovine serum albumin (Hepes buffer). Washed erythrocytes (50 μl) were then resuspended in 100 μl of Hepes buffer containing 5 × 104 cpm of 125I-protein A (1.1 mCi per mg of protein A) and incubated at room temperature for 30 min. The erythrocytes were then extensively washed in Hepes buffer (four times) and finally assayed in a Beckman 5500 γ-counter.

**Recognition of ddCTP-Loaded Erythrocytes by M/M.** Adherent M/M cells were incubated with ddCTP-loaded erythrocytes either untreated or treated with ZnCl2 plus BS3, or they were incubated with unloaded erythrocytes overnight at 37°C at 100 erythrocytes per M/M. During the loading procedure, 125I-labeled ubiquitin (2.1 μCi/mg) was also encapsulated as an internal marker to follow phagocytosis (ubiquitin is a polypeptide of 8 kDa that is retained by the erythrocytes).

After M/M erythrocyte incubation, the dishes were extensively washed with RPMI 1640 medium followed by a 0.9% ammonium chloride washing step to remove erythrocytes adherent to M/M but not yet phagocytosed. Finally, M/M were washed in RPMI medium and assayed in a γ-counter.

**Determination of ddCTP transfered from drug-loaded erythrocytes to M/M was as described above except that 125I-ubiquitin was not encapsulated but 1HddCTP was used instead of the unlabeled drug.**

Similar experiments were also performed on mouse erythrocytes for in vivo studies. The in vivo half-life of these erythrocytes was evaluated by following the radioactivity of 125I-ubiquitin-loaded erythrocytes coencapsulated with ddCTP in untreated erythrocytes or in erythrocytes treated with ZnCl2 and BS3.

**RESULTS**

**ddCTP Encapsulation and Stability in Erythrocytes.** ddCTP was encapsulated into human and murine erythrocytes by a procedure of hypotonic dialysis, isotonic rescaling, and reannealing as described. By this procedure, human erythrocytes were routinely loaded with ddCTP to a final concentration of 1–2 mM; however, by addition of increasing amounts of ddCTP added during the dialysis step, the cellular concentrations of ddCTP can be easily varied up to 10 mM. Human erythrocytes subjected to this procedure are slightly microcytic (73 ± 1.5 fl) (normal values, 88 ± 3.5 fl) and contain 27.5 ± 2 g of hemoglobin per 100 ml of cells (normal values, 29.5 ± 2.5 g/100 ml). These cells have normal glycolytic rates (3 μmol of lactate produced per h per ml of erythrocytes) and normal ATP concentrations (1.2 mM).

Human erythrocyte lysates were found to dephosphorylate ddCTP to ddCDP with a Vmax of 3 ± 0.5 nmol per min per g of Hb and with a Km of 140 μM; then ddCDP is slowly converted to ddCMP (we were unable to determine the kinetic parameters of this reaction because of the low activity of the system), which is further converted to dioxyxycytidine in the presence of cAMP which is also present in the erythrocyte. This reaction is without effect even at 6 mM. ddCTP dephosphorylation is also very pH dependent, with a maximum dephosphorylation rate at pH 8.4 and a value of 50% of this rate at pH 7.2.

ddCTP loaded onto erythrocytes is stable at 37°C for at least 3 h and for >12 days at 4°C. Also, in intact erythrocytes

![Fig. 1](https://example.com/f1.png)

**Fig. 1.** Schematic diagram of the procedure used to induce opsonization of erythrocytes by autologous IgG. (a) Major transmembrane protein (band 3) is distributed randomly in the membrane. Addition of ZnCl2 causes band 3 clustering (b) that is made irreversible by the crosslinking agent BS3 (c). (d) Incubation of these cells in autologous serum induces IgG binding.
dephosphorylation of ddCTP started when the cellular ATP concentration decreased to 1 mM (data not shown).

**Targeting ddCTP-Loaded Erythrocytes to Human M/M.** Targeting of ddCTP-loaded erythrocytes to human M/M is obtained by promoting the clustering of band 3 (the predominant transmembrane protein that functions as an anion transport system) (12). In fact, the normal distribution of band 3 is random; however, its clustering can be induced by chemicals such as zinc (13), the peptide melittin (14, 15), and the dye acridine orange (15) that have minimal side effects or by oxidants such as phenylhydrazine (16) or diamide (17). We used 1 mM ZnCl2 as a mild agent. However, these band 3 clusters are reversible upon removal of the bivalent cation but can be made irreversible by addition of the cross-linker BS3 (18). Once these clusters are formed, they are viewed by the immune system as nonself and consequently are opsonized by autologous antibodies. The process is schematically shown in Fig. 1 and has been characterized in detail by Turrini et al. (18). In our experimental system, ddCTP-loaded erythrocytes treated as described above are opsonized by IgG, are actively phagocytosed by M/M, and can deliver ddCTP to M/M. These data are summarized in Table 1. Similar experiments performed on mouse erythrocytes provided comparable results. The mouse erythrocytes were also evaluated as a drug delivery system in vivo. Their half-life was 3.5 days against values of 10.5 days for the ddCTP-loaded erythrocytes not treated with ZnCl2 and BS3. Any leakage from the loaded cells was detected in vivo when [3H]ddCTP or the small 125I-labeled polypeptide ubiquitin was coencapsulated with ddCTP.

**In Vitro Inhibition of HIV Production by ddCTP-Loaded Erythrocytes.** Infected macrophages play a central role in the pathogenesis of AIDS (19–21), acting as reservoirs (19) and propagators of virus (21) through the immune system (22). We have added ddCTP-loaded erythrocytes treated to increase their phagocytosis to cultures of human M/M immediately after HIV-1 infection. The loaded erythrocytes were maintained in culture overnight and then the noningested erythrocytes were removed. As shown in Fig. 2, ddCTP-loaded erythrocytes dramatically reduce p24 and reverse transcriptase production by HIV-1-infected M/M. Any free drug was detected in the culture medium. Unloaded erythrocytes (i.e., erythrocytes subjected to the procedure of loading but without ddCTP addition) treated to increase their phagocytosis by M/M were found to be able to slightly reduce HIV-1 production in infected M/M. In conditions similar to those reported in Fig. 2, the peak concentration of soluble p24 was 1.8 ± 0.15 ng per 10^6 cells (mean ± SD of three experiments). This effect was also found upon phagocytosis of latex particles.

The ability of ddCTP-loaded erythrocytes to inhibit the infection of T cells cocultivated with infected M/M was also evaluated (Table 2).

**In Vivo Inhibition of LP-BM5 Infectivity by ddCTP-Loaded Erythrocytes.** C57BL/6 mice inoculated with the mixture of murine leukemia viruses known as LP-BM5 develop an immunodeficiency syndrome resembling that in humans infected with HIV-1 (23). This animal model of AIDS has already been used for preclinical studies of antiviral drugs (23–27). We have administered ddCTP-loaded erythrocytes treated with ZnCl2 and BS3 to a group of four mice infected with infected M/M.

### Table 1. Recognition of human ddCTP-loaded erythrocytes by M/M

<table>
<thead>
<tr>
<th>Erythrocytes</th>
<th>ddCTP loaded plus ZnCl2 and BS3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native IgG bound, molecules per cell</td>
<td>20-40</td>
</tr>
<tr>
<td>Phagocytosis, erythrocytes per M/M</td>
<td>0.1-0.2</td>
</tr>
<tr>
<td>[3H]ddCTP in macrophages, µM</td>
<td>ND</td>
</tr>
</tbody>
</table>

Phagocytosis assays in each experiment were conducted at the same time with the same preparation of macrophages to decrease variability. ND, not detectable.

**Table 2. Effect of ddCTP-loaded erythrocytes on p24 production in cultures of peripheral blood lymphocytes (PBL) cocultivated with infected M/M**

<table>
<thead>
<tr>
<th>p24, ng per 10^6 cells</th>
<th>M/M</th>
<th>M/M plus PBL</th>
<th>M/M treated with ddCTP-loaded erythrocytes</th>
<th>M/M plus PBL treated with ddCTP-loaded erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.1</td>
<td>58</td>
<td>0.19</td>
<td>2.1</td>
</tr>
</tbody>
</table>

PBL were added to M/M 21 days after infection. p24 values were determined 5 days after M/M plus PBL cocultivation and represent the amount of p24 in cells and in virus.
with LP-BM5. The first administration of 360 × 10^6 erythrocytes (13.5 μl) containing 5 μmol of ddCTP per ml of erythrocytes was performed 24 h after the infection, and similar administrations were repeated at 10-day intervals over a period of 3 months. Two other groups of mice served as controls and were the uninfected and infected but not treated animals. As shown in Fig. 3, the treatment dramatically reduced splenomegaly and also, as shown quantitatively in Table 3, lymphoadenopathy after the 3-month period. The amount of serum immunoglobulins is also markedly reduced (Table 4). These experiments have been repeated twice with a minimum of eight animals in each treatment group. Administration of unloaded erythrocytes to infected mice was without significant effect on all the parameters tested.

**DISCUSSION**

A broad family of nucleoside analogues, particularly those with a 2',3'-dideoxyribose moiety, are able to inhibit HIV-1 infectivity in vitro (1, 2). Several of these molecules have been reported to be active against HIV in short-term clinical trials; AZT and dideoxyinosine are the drugs currently being prescribed. All these drugs are DNA-chain terminators; thus, to be incorporated into the growing viral DNA chain (28) they must be phosphorylated in the target cells to yield the dideoxynucleoside 5'-triphosphates. Since each analogue requires a separate metabolic pathway (1, 2, 7, 28–33), the various dideoxynucleosides are not equivalent in activity. Furthermore, with different kinds of cells having different levels of specific kinases, some compounds can be more or less active even in different cells of the same organism. These considerations prompted us to investigate the possibility of using a drug delivery system to directly administer the dideoxynucleoside analogues in their active, 5'-triphosphate form. Furthermore, since recent evidence indicates a central role for infected macrophages in the pathogenesis of AIDS (19–21), we decided to target the drug to these cells. The idea of using erythrocytes as a drug delivery system has been successfully explored by several investigators (34). Erythrocytes are natural targets of the reticuloendothelial system, can be manipulated to increase their removal from circulation, can influence the biodistribution of encapsulated drugs, can decrease the drug's toxicity to nonmacrophage cells, and can prolong drug action.

<table>
<thead>
<tr>
<th>Days after infection</th>
<th>35</th>
<th>62</th>
<th>77</th>
<th>84</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>77</td>
<td>86</td>
<td>80</td>
<td>105</td>
</tr>
<tr>
<td>Infected</td>
<td>88</td>
<td>115</td>
<td>230</td>
<td>160</td>
</tr>
<tr>
<td>Infected and treated</td>
<td>80</td>
<td>88</td>
<td>88</td>
<td>120</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>70</td>
<td>105</td>
<td>105</td>
<td>115</td>
</tr>
<tr>
<td>Infected</td>
<td>84</td>
<td>480</td>
<td>240</td>
<td>175</td>
</tr>
<tr>
<td>Infected and treated</td>
<td>72</td>
<td>160</td>
<td>130</td>
<td>125</td>
</tr>
</tbody>
</table>

All values are mg per 100 ml and represent means of at least four animals in each experiment that agreed within 10% of the mean.
In our model system, we selected ddCTP as an antiretroviral drug since on a molar basis dideoxycytidine was shown to be one of the most potent dideoxynucleosides tested in susceptible cells in vitro (4). The results reported in this paper show that ddCTP can be encapsulated into erythrocytes where it is stable at least until ATP does not decrease to <1.0 mM. These ddCTP-loaded cells can be manipulated in a way that opsonization by autologous IgG and phagocytosis occurs. ddCTP remains in an active state once internalized in M/M, and the concentration of drug found in these cells (2.5 μM) is several times higher than that needed to inhibit the reverse transcriptase. That, indeed, this drug delivery system is able to inhibit HIV-1 production in infected macrophages is also evident from the data reported in Fig. 2. The importance of administering dideoxycytidine in phosphorylated form with targeting to macrophages is clearly shown by the in vivo data in the murine model of immunodeficiency. In fact, dideoxycytidine in murine cells is poorly phosphorylated (7) and of limited efficacy in in vivo studies of murine acquired immunodeficiency syndrome (26). In contrast, the administration of ddCTP-loaded erythrocytes dramatically reduces the typical signs of the disease over a period of 3 months. Although applications of this protocol remain to be done, the absence of adverse effects in vitro and in vivo, together with the positive data obtained, provide reasons for optimism that medical uses can be explored.

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