Vol. 166, No. 1, 1990
January 15, 1990

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 273-279

THE METABOLISM OF 3'-AZIDO-2',3'-DIDEOXYGUANOSINE IN CEM CELLS

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Received November 27, 1989

When CEM cells were incubated with [^{3}H]-labeled 3'-azido-2',3'-dideoxyguanosine (AzddGuo), the isotope was largely recovered as ribo- and deoxyribonucleotides in the acid soluble fraction of CEM cells, due to extensive catabolism of AzddGuo and recycling of the guanine formed by purine nucleoside phosphorylase. Only 10% was found as the AzddGuo nucleotides, with the diphosphate of AzddGuo as the dominating nucleotide at all time points. Thus nucleoside diphosphate kinase was rate limiting for the formation of AzddGuo triphosphate, responsible for the toxic and antiviral activity of the nucleoside. Inhibition of *de novo* deoxyribonucleotide synthesis with hydroxyurea increased the phosphorylation of AzddGuo twofold.

AzddGuo inhibits the replication of human immunodeficiency virus (HIV) *in vitro* (1,2). Its selectivity index of 140, based on the ratio between the 50% cytotoxicity dose (190 μM) and the 50% effective antiviral dose (1.4 μM), is similar to that of dideoxyadenosine and gives AzddGuo interest in the treatment of HIV infection. Similar to other dideoxynucleoside analogues, the therapeutic effect of AzddGuo requires its transformation to the triphosphate. Here, we study the ability of human CEM cells to phosphorylate the drug and also investigate whether treatment with hydroxyurea increased the phosphorylation. With the two pyrimidine analogues, 3'-azidothymidine and 3'-fluorothymidine, inhibition of the *de novo* synthesis of deoxyribonucleotides by hydroxyurea was earlier shown to increase their phosphorylation (3).

0006-291X/90 $1.50

When CEM cells were incubated with AzddGuo labeled in the guanine moiety, only 10 % of the isotope of the nucleotide fraction was recovered as AzddGuo nucleotides, the remainder being present as ribo- and deoxyribo-nucleotides. In contrast, when a CEM mutant lacking the enzyme hypoxanthine- guanine phosphoribosyl transferase (HGPRT) was used, all radioactivity was present as AzddGuo nucleotides. These results suggest that AzddGuo is readily cleaved by purine nucleoside phosphorylase (PNP) and that the released guanine is subsequently reutilized for the synthesis of ribonucleotides. Hydroxyurea increased the phosphorylation of the drug in both lines.

Materials and Methods

Compounds. Nonlabeled AzddGuo was synthesizes as described(4). The material was labeled by Amersham Int., Amersham, U.K. by exchange with ^3H_2O at 95°C (TR8) and had a final specific activity of 5000 cpm/pmol.Hydroxyurea was obtained from Sigma Chemicals,MO, U.S.A.

Cell culture procedures. CCRF-CEM cells, a human T-lymphoblastoid line, and a HGPRT- mutant derived from the same cell line, was a gift from D. Carson (Scripps Clinic and Research Foundation, La Jolla, Calif., U.S.A) (5). The cells were grown at 37° C in 7.5% CO_2 atmosphere in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum. After addition of drugs the incubation of the cells continued for the indicated times. The cells were harvested by centrifugation. Nucleotides were extracted with 0.3 M perchloric acid. After centrifugation, acid was removed with freon-octylamin (6). The acid insoluble fraction of the cells was dissolved in 0.6 M NaOH and used for RNA and DNA analyses (7).

Analyses of nucleotides. Separation was achived by HPLC on a Partisil SAX column with a 20 to 500 mM ammonium phosphate, pH 3.8 gradient containing 7% methanol. For identification, nucleotides present in the separated peak fractions were adsorbed to charcoal, eluted with 0.1 M NH_3 in 50% ethanol, evaporated to dryness and dephosphorylated with alkaline phosphatase. The identity of the obtained nucleoside was established by HPLC on a C18 column with a 20 mM ammonium acetate : 40 % methanol-water gradient.

Results

Incubation of wild type CEM cells with 5 μM [^{3}H]-AzddGuo resulted in a large time dependent incorporation of isotope into both RNA and DNA (Fig.1). Since the substitution at the 3'-carbon of the analogue excluded its extensive incorporation into nucleic acids this result suggested recycling of the free base via condensation with phosphoribosyl pyrophosphate. Furthermore, a mutant cell line lacking the appropriate transferase did not incorporate any isotope. A direct demonstration of the recycling came from the radioactivity pattern of the nucleotide fraction of the labeled CEM cells (Fig. 2A). Separation by HPLC resulted in the recovery of at least 7

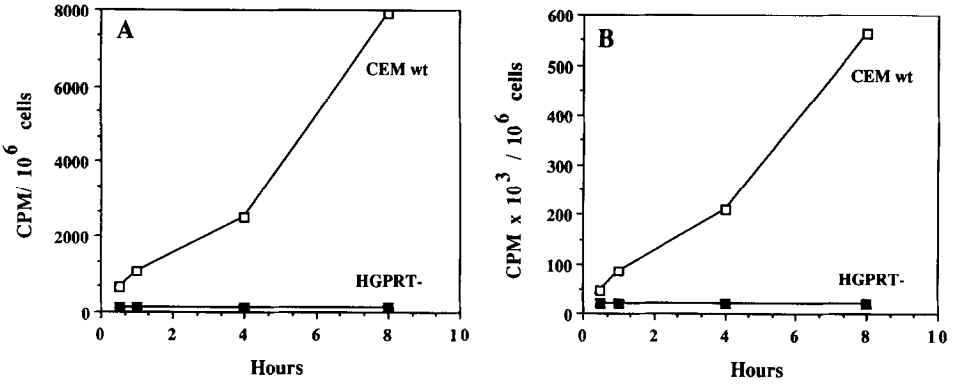

Figure 1. Incorporation into DNA and RNA. CEM wild type cells and CEM HGPRT- mutant cells were incubated with 5 μM ^{3}H-AzddGuo for different timeperiods. The incorporation of tritium into DNA (A) and RNA (B) were determined.

radioactive peaks. The nature of the nucleoside in each nucleotide peak was identified by HPLC after dephosphorylation of the nucleotide with phosphatase. These analyses indicated that between 2 and 8 hours after addition of labeled AzddGuo only 10% of the total radioactivity of the nucleotide fraction corresponded to AzddGuo

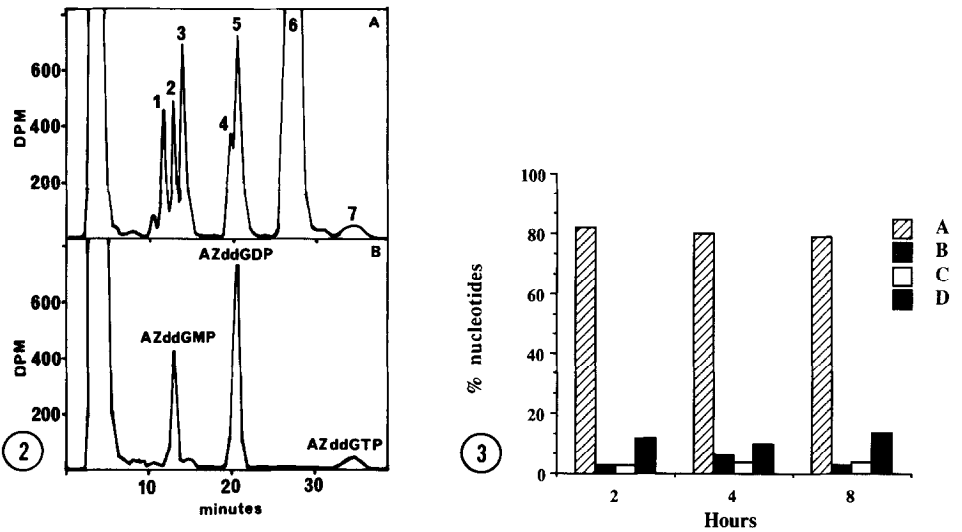

Figure 2. Separation of labeled nucleotides by HPLC. The acid soluble fraction of CEM wt cells (A) and CEM HGPRT- mutant cells (B) was analysed on a Partisil-SAX column as described in Methods. Peak number: 1= GMP, 2= AzddGMP, 3= GDP, 4= ATP, 5= AzddGDP, 6= GTP+dGTP, 7= AzddGTP.

Figure 3. AzddGuo anabolites in CEM wild type cells. CEM cells were incubated with 5 μM ^{3}H-AzddGuo for 2, 4 and 8 hours. After dephosphorylation the extracts were analysed by reversed phase HPLC as described in Methods. The amounts of (A) guanosine-, (B) deoxyguanosine-, (C) adenosine- and (D) azidodeoxyguanosine were determined.

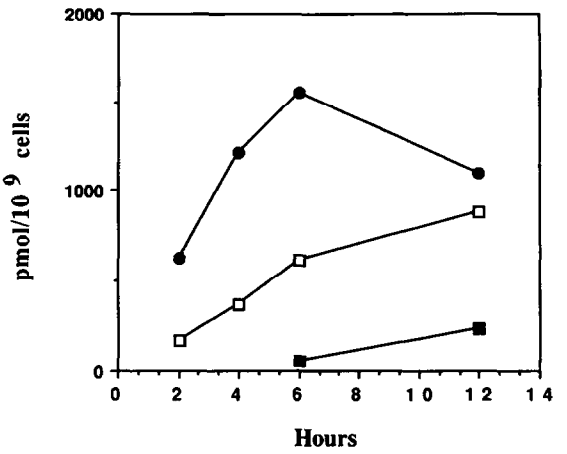

Figure 4. AzddGuo nucleotides in CEM cells. CEM cells were incubated with 5 μM ^{3}H-AzddGuo for 2, 4, 6 or 12 hours. Cell extracts were analysed by HPLC and the levels of mono-, di- and triphosphates were determined. (□); AzddGMP, (●); AzddGDP and (■); AzddGTP.

nucleotides, the rest being accounted for by guanosine-, adenosine- and deoxyguanosine nucleotides (Fig. 3). The situation changed in the HGPRT- line (Fig. 2B). Now all isotope was recovered in the three AzddGuo peaks. These results clearly indicate that guanine was released from the drug, in all probability by purine nucleoside phosphorylase, and subsequently reused for ribonucleotide synthesis by HGPRT in the CEM line.

The results of Fig. 2 show an unusual distribution of isotope between the various AzddGuo nucleotides since after 12 hours the majority of the label was

Table 1. Effects of hydroxyurea on AzddGuo nucleotide pools

	AzddGMP+DP+TP after incubation with 1 μM AZddGuo	AzddGMP+DP+TP after incubation with 5 μM AZddGuo	AzddGTP after incubation with 5 μM AZddGuo
	(pmol / 10^{6} cells)		
control	0.6	2.2	0.2
200 μM hydroxyurea	1.1	4.0	0.4

CEM cell extracts were analysed by HPLC after 12 hours of incubation with 1 or 5 μM of [^{3}H]-AzddGuo. The cell cultures were preincubated with hydroxyurea for 15 hours before addition of the nucleoside analogue.

recovered as the diphosphate. A time curve of the various labeled nucleotides is shown in Fig.4. At all time points from 2 to 12 hours after addition of the drug AzddGuo diphosphate is the dominating nucleotide. In this experiment the triphosphate could be demonstrated only after 6 hours and even after 12 hours it amounted to as little as 10% of the total AzddGuo nucleotides. At this time 0.2 pmol of the triphosphate was present in 10^6 cells.

In a final experiment we tested the effect of hydroxyurea on the phosphorylation of AzddGuo (Table 1). At two concentrations of the nucleoside analogue, the presence of hydroxyurea caused a doubling of the phosphorylation of AzddGuo. The increase occurred both in the wild type and mutant CEM lines at all levels of phosphorylation and thus suggests that it affected the first step in the chain, i.e. the phosphorylation of the nucleoside.

Discussion

Up to now, azidothymidine is the only dideoxynucleoside approved for therapy even if several other such nucleosides are of potential clinical interest (8,9). The general believe is that in all cases dideoxynucleosides must be phosphorylated to the corresponding triphosphates in order to exert their antiviral effect. Their ability to be phosphorylated by the cellular machinery is thus a critical factor to be considered. Azidothymidine is a thymidine analogue and as such a substrate for thymidine and thymidylate kinases (10). Extensive studies of its metabolism have demonstrated that the nucleoside is quite readily phosphorylated to the monophosphate, but that the next step, phosphorylation to the diphosphate is deficient, at least in human cell lines. At therapeutic concentrations of the analogue most of it is therefore recovered as the monophosphate inside human cells (3,10).

Our results now demonstrate that AzddGuo is a still more sluggish substrate for the cell's kinases. It seems possible - but is not proven - that deoxycytidine kinase is the enzyme responsible for the phosphorylation of AzddGuo. This enzyme has a low affinity for deoxyguanosine and the phosphorylation of the analogue is certainly also slow (Fig. 4). The next step, phosphorylation to the diphosphate, should be catalysed by (d)GMP kinase and apparently proceeds quite smoothly. In contrast, the final phosphorylation to the triphosphate is very slow. This was a surprising finding since

the relevant enzyme, the unspecific nucleoside diphosphate kinase, in all other to us known instances is not rate limiting. As a result, the diphosphate dominates the picture at all time points and the buildup of the triphosphate is very slow.

In comparison to azidothymidine, a 500 fold higher concentration of AzddGuo was required to give an intracellular concentration of 0.2 pmol/ 10^6 cells (5 μM AzddGuo vs 0.01 μM azidothymidine) (3). This difference reflects the abilities of the various kinases to accept the respective analogues as substrates but may also be influenced by the ability of purine nucleoside phosphorylase to catabolize AzddGuo. The two azidonucleosides also show an approximately 500 fold difference with respect to their toxicity and antiviral effects, in line with the generally held belief that the nucleoside triphosphates are the metabolically active derivatives (1,11,12).

Inhibition of *de novo* synthesis with hydroxyurea increased the phosphorylarion of AzddGuo. The twofold increase was smaller than the corresponding effect on azidothymidine. Hydroxyurea has, however, also an effect on the endogenous deoxynucleoside triphosphate pools: the drug increases the size of the dTTP pool but decreases the size of the dGTP pool (13). This favours the inhibitory effects of AzddGuo but counteracts the effects of azidothymidine, since inhibition by dideoxynucleoside triphosphates depends on their competition with the corresponding natural polymerase substrates. If AzddGuo should be used in therapy its combination with hydroxyurea might therefore be considered.

Acknowledgments

We thank F. Benseler for the synthesis of AzddG. This work was supported by grants from the Swedish Medical Research Council, the Medical Faculty of the Karolinska Institute and the Swedish Society of Medicine (A. Karlsson).

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