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# 8-AZIDOADENOSINE AND RIBONUCLEOTIDE REDUCTASE

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**SUMMARY:** Inhibitors of ribonucleotide reductase are potential antiproliferative agents, since they deplete cells from DNA precursors. Substrate nucleoside analogues, carrying azido groups at the base moiety, are shown to have strong cytostatic properties, as measured by the inhibition of the incorporation of thymidine into DNA. One compound, 8-azidoadenosine, inhibits CDP reduction in cytosolic extracts from cancer cells. The corresponding diphosphate behaves as a substrate for ribonucleotide reductase while the triphosphate is an allosteric effector. 

Ribonucleotide reductase plays a central role in DNA biosynthesis, catalyzing and regulating the conversion of ribonucleotides to deoxyribonucleotides (1). In mammals, viruses and some bacteria, such as *Escherichia coli*, the enzyme consists of a 1 : 1 complex of two non-identical homodimer subunits (2). Redox-active cysteines of the large subunit, named $R_1$, reduce both the purine and pyrimidine ribonucleoside diphosphate substrates at the same site. Protein $R_1$ also contains binding sites for nucleoside triphosphates which function as allosteric effectors. Protein $R_2$, the small subunit, carries a binuclear iron center and a tyrosyl radical within each of its polypeptide chains (2). The tyrosyl radical is absolutely required for ribonucleotide reduction.

Since depletion of DNA precursors halts cell proliferation, inhibitors of ribonucleotide reductase are under study as potential drugs in antineoplastic and antiviral chemotherapy (3). Hydroxyurea, an excellent scavenger of the tyrosyl

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**ABBREVIATIONS:** CDP, cytidine 5'-diphosphate; ADP, adenosine 5'-diphosphate; 8-$N_3$ADP, 8-azidoadenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; 8-$N_3$ATP, 8-azidoadenosine 5'-triphosphate; dGTP, 2'-deoxyguanosine 5'-triphosphate.

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radical, is the only specific inhibitor under clinical application. Substrate analogues, which react as mechanism-based inhibitors, have been extensively studied during the past few years (4). New 2'-modified ribonucleosides, 2'-deoxy-2'-methylenecytidine and 2'-deoxy-2',2'-difluorocytidine (5-8), have been reported to have broad spectrum inhibitory activities. On the other hand, only very few base-modified nucleosides have been studied as inhibitors of ribonucleotide reductase and we thus started recently the synthesis of series of such nucleosides to be tested as anticancer agents.

Here we report that nucleoside analogues, carrying azido groups on the base moiety (Scheme 1), have strong antiproliferative properties. Their effects with respect to ribonucleotide reductase activity are discussed, considering that 8-$N_3$ADP was shown to be a substrate for the enzyme and 8-$N_3$ATP an allosteric effector. Both nucleotides might be useful probes for studying substrate and effector sites on protein $R_1$.

## MATERIALS AND METHODS

**Materials.** 5-azidomethyluridine 4 (Scheme 1) was synthesized from uridine according to a procedure developed by Ikeda et al. to prepare 5-cyanomethyluridine (9). 2',3'-O-isopropylidene-5-hydroxymethyluridine 1, prepared as previously described (10), was reacted with hydrogen chloride in dioxane at 0-5°C. The unstable resulting chloromethyl derivative 2 was treated without purification with 5 equivalents of sodium azide in refluxing acetone and transformed to 3. Deprotection was achieved by treating 3 with aqueous trifluoroacetic acid at 0°C and afforded 4 in 16% yield from 2. The preparation of 6-azidopurine riboside 5 was achieved by treatment of 6-chloropurine riboside with 10 equivalents of sodium azide in DMF at 60°C (90% yield after crystallization from water). This reaction has been previously described to proceed with decomposition in ethanol (11,12). 5 exists predominantly in the tetrazole form .8-azidoadenosine 6, 8-azido-2'-deoxyadenosine and 8-azidoguanosine 7 were.prepared as previously described (13,14).

CDP, ADP, 8-$N_3$ADP, ATP, 8-$N_3$ATP and dGTP were obtained from Sigma. Radioactive CDP was purchased from Amersham France (specific activity: 680 GBq/mmol). *E. Coli* thioredoxin was a gift from J.P. Jacquot (Université Paris Sud, Orsay). *E. Coli* thioredoxin reductase was obtained from A. Holmgren (Karolinska Institute, Stockholm, Sweden). The murine adenocarcinoma $R_2$-overproducing TA3 cell line was provided by Pr. L. Thelander (University of Umea, Sweden) and cytosolic extracts were prepared as previously described (15) and concentrated by centrifugation into a Centricon 30 tube (Amicon). Protein $R_1$ and protein $R_2$ were prepared from overproducing strains of *E. Coli* as previously described (16,17).

**Assays for ribonucleotide reductase activity.** Ribonucleotide reductase activity in concentrated TA3 cytosols was measured by reduction of [5-$^3$H] CDP as previously described (15), in the presence of various concentrations of base-modified nucleosides. Inhibitors were preincubated for 15 min at 37°C with 80 µl of extracts in 100 mM hepes, pH 7.6, 15 mM Mg acetate, 10 mM dithioerythritol and 5 mM ATP. Then 200 µM CDP and 74 kBq of [5-$^3$H] CDP (final concentration) were added in 10 µl. The reaction was allowed to proceed for 30 min. Activity of ribonucleotide reductase from *E. coli* was assayed spectrophotometrically

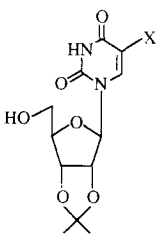
monitoring NADPH consumption at 340 nm ($\varepsilon_{340}$ = 6220 $M^{-1}.cm^{-1}$) (18). Standard conditions were thioredoxin 0,16 mg/ml, thioredoxin reductase 0.025 mg/ml, DTT 0.5 mM, $MgCl_2$ 10 mM, NADPH 0.32 mM, $R_1$ 0.1 mg/ml, $R_2$ 0.02 mg/ml in 35 mM Hepes buffer pH 7.5. Effectors were ATP, dGTP or 8-$N_3$ATP and substrates were CDP, ADP or 8-$N_3$ADP at concentrations indicated in the results section. Units are defined as nmoles of NADPH oxidized per minute.

**Determination of [$^3$H] Thymidine incorporation.** Murine adenocarcinoma EMT6 cells were plated in flat-bottomed microtiter plates (Nunclon) at $10^4$ cells per well, in 100 µl of RPMI 1640 medium (GIBCO BRL) plus 5 % heat-inactivated fetal calf serum and antibiotics. After overnight incubation at 37°C in a 5 % $CO_2$ incubator, 100 µl of fresh medium containing appropriate concentration of inhibitor were added into triplicate wells. Cultures were further incubated for 40 h and then pulsed for additional 8 h with 57 kBq of [$^3$H] thymidine (CEA Saclay, France) with a specific activity of 37 GBq/mmol. Radioactivity incorporated into DNA was counted as previously reported (15). Results are expressed as the percentage of the radioactivity incorporated in the treated samples, compared to untreated controls.

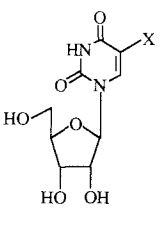
## RESULTS

**Cytostatic properties of azido nucleosides.** The azido nucleosides,5-azidomethyluridine 4, 8-azidoadenosine 6 and the corresponding deoxy compound, 8-azidoguanosine 7 (scheme 1), were tested for their ability to inhibit the growth of murine adenocarcinoma EMT6 cells. The $IC_{50}$ values for these compounds were determined from the thymidine incorporation into DNA assay and are summarized in Table 1. 8-azidoadenosine and 5-azidomethyluridine had much stronger inhibitory activity than hydroxyurea. Remarkable is the absence of activity of 8-azido-2'-deoxyadenosine and 8-azidoguanosine.

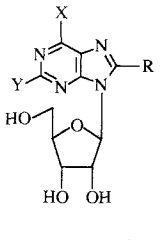
**Inhibition of ribonucleotide reductase *in vitro* by azido nucleosides.** CDP reductase activity in cytosolic extracts from TA3 cells was determined in the



1: X= $CH_2OH$
2: X= $CH_2Cl$
3: X= $CH_2N_3$



4: X= $CH_2N_3$



5: X= $N_3$, Y=R = H
6: X= $NH_2$, Y= H, R= $N_3$
7: X= OH, Y= $NH_2$, R= $N_3$

Scheme 1.

Table 1. Inhibitory effects of azido nucleosides and hydroxyurea on the growth of EMT6 cells

| Compound | $IC_{50}$ (μM) |
|---|---|
| Hydroxyurea | 80 |
| 8-$N_3$adenosine | 3 |
| 5-$CH_2N_3$uridine | 7 |
| 8-$N_3$-2'-deoxyadenosine | > 1000 |
| 8-$N_3$guanosine | > 1000 |

$IC_{50}$ was given as the concentration of drug at 50% inhibition of thymidine incorporation into DNA during cell growth, assayed as described in Materials and Methods.

presence of various concentrations of 8-azidoadenosine. The cytosols contain the phosphorylating activities required for the transformation of the nucleosides into the corresponding, mono, di and triphosphates. However, it should be noted that the balance between phosphorylating and dephosphorylating activities in the extracts is not well defined. Under the conditions described in Materials and Methods, inhibition of CDP reduction was demonstrated, with an $IC_{50}$ value of 0.6 mM. 8-azidoguanosine gave slightly less inhibition : 89 % for 2 mM compared to 98 % at 2 mM 8-azidoadenosine. On the other hand, 8-azido-2'-deoxyadenosine, 5-azidomethyluridine and 6-azidopurine riboside had no effect on the enzyme activity.

**8-$N_3$ADP is a subtrate and 8-$N_3$ATP an allosteric effector of ribonucleotide reductase.** The demonstration that 8-azidoadenosine was capable of inhibiting CDP reductase and cell growth *in vitro* led us to investigate the reactions of the phosphorylated states of the compound with ribonucleotide reductase. This study then required pure preparations of protein $R_1$ and protein $R_2$. Figure 1A shows that, in the presence of ATP, dGTP and an electron transfer chain consisting of thioredoxin and thioredoxin reductase, proteins $R_1$ and $R_2$ purified from *Escherichia coli* were able to catalyze the reduction of 8-$N_3$ADP by NADPH. The reaction can be monitored spectrophotometrically since NADPH is strongly absorbing at 340 nm and since oxidation of NADPH results in the loss of that absorption. No oxidation of NADPH could be detected when protein $R_1$ or protein $R_2$ or any component of the incubation mixture was omitted (data not shown). Moreover, no oxidation of NADPH could be observed when protein $R_2$ was replaced by met$R_2$, the tyrosyl radical-free inactive form of $R_2$. These results show that the mechanism for the reduction of natural ribonucleotides by ribonucleotide reductase is retained in the case of 8-$N_3$ADP. Figure 1A also shows that reduction of 8-$N_3$ADP was possible only if dGTP was present in the

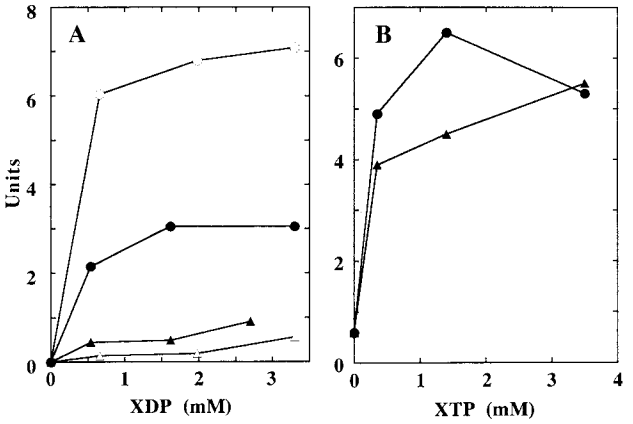



Figure 1. 8-$N_3$ADP as a substrate and 8-$N_3$ATP as an allosteric effector of ribonucleotide reductase. Assays were performed at 37°C as described in Materials and Methods. A, reduction of ADP (open symbols) or 8-$N_3$ADP (closed symbols) in the presence of 1.4 mM ATP and with (○,●) or without (△,▲) 0.6 mM dGTP. B, reduction of 0.63 mM CDP in the presence of ATP (●) or 8-$N_3$ATP (▲).

reaction mixture. dGTP is the specific allosteric effector that drives the reduction of the natural substrate ADP .

ATP is a positive allosteric effector that drives the reduction of CDP by NADPH (Figure 1B). In Figure 1B we show that ATP can be omitted and replaced by slightly larger concentrations of 8-$N_3$ATP.

## DISCUSSION

In this paper we show that two nucleoside analogues, carrying an azido group on the base moiety, 8-azidoadenosine and 5-azidomethyluridine, have strong cytostatic effects. Only in the first case this might be, at least partly, related to the apparent inhibition of CDP reduction in cell extracts. It has, indeed, to be noted that cell growth was much more sensitive to 8-azidoadenosine than CDP reduction was. These effects on both CDP reduction and cell growth absolutely require the presence of an OH group at the 2'-position since 8-azido-2'-deoxyadenosine was completely inactive.

We have also demonstrated that the presence of an azido group at the 8-position of adenosine did not strongly affect the interaction of the corresponding nucleotide (di-or tri-phosphate) with ribonucleotide reductase. Actually, 8-$N_3$ADP was recognized by protein $R_1$ substrate sites and thus efficiently reduced in the presence of dGTP, which acts as an allosteric effector. The fact that 8-azidoADP binds to the active site might explain why reduction of CDP in cell extracts is affected by the presence of increasing concentrations of 8-azidoadenosine.

However, reduction of CDP and 8-azidoADP does not require the same allosteric conditions, since in the presence of ATP as the only effector, CDP is reduced while 8-$N_3$ADP is not (Figure 1A). The figure is even more complicated if one considers our observation that 8-$N_3$ATP is able to bind to an allosteric site of protein $R_1$, probably the ATP-binding site since both triphosphates stimulate CDP reduction.

8-azidoADP and 8-azidoATP are well-known and widely utilized photaffinity labelling agents in the case of nucleotide binding proteins (19) However, they have never been used to probe the nucleotide binding sites of ribonucleotide reductase. Our results strongly suggest that they might be efficient labelling agents also in this case and thus should be used for identifying both substrate and allosteric sites of protein $R_1$. This is currently under investigation in our laboratory.

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