Azidocytidine is incorporated into RNA of 3T6 mouse fibroblasts

Lennart Åkerblom*

Department of Biochemistry I, Medical Nobel Institute Karolinska Institutet, S-104 01 Stockholm, Sweden

Received 11 September 1985

Earlier work has shown that azidocytidine inhibits the growth and DNA synthesis of 3T6 mouse fibroblasts by inactivation of the enzyme ribonucleotide reductase. RNA synthesis, as measured by incorporation of [^{3}H]cytidine was not affected. Here I show that azidocytidine is incorporated into RNA, but not into DNA. Incorporation of the analogue into RNA may under special circumstances contribute to the biological effect of the nucleoside.

2'-Azido-2'-deoxycytidine *Ribonucleotide reductase inhibitor* *Cs_2SO_4 density gradient* *Reverse-phase HPLC* *3T6 cell*

1. INTRODUCTION

Azidocytidine contains an azido (= N_3) group at the 2'-position of ribose and can be regarded as an analogue of both cytidine and deoxycytidine [1]. In 3T6 cells phosphorylation of the nucleoside by deoxycytidine kinase is a prerequisite for its biological activity [2]. The diphosphate of azidocytidine is a suicide inhibitor of ribonucleotide reductase from *E. coli* and acts by scavenging the tyrosyl free radical of the B2 subunit of this enzyme [3,4]. Also the mammalian reductase is inhibited by azidoCDP, probably via a similar mechanism [5].

Azidocytidine inhibits the growth of cells in culture. Earlier work suggested that inhibition was caused by interference with DNA strand elongation [6] as well as by inhibition of ribonucleotide reductase [2]. More recently it was discovered that commercial preparations of azidocytidine were contaminated by trace amounts of arabinosylcytosine and that this contaminant was responsible for the observed effects on strand elongation. In 3T6 cells azidocytidine purified by HPLC and free from arabinosylcytosine inhibited DNA synthesis only indirectly by inhibition of ribonucleotide reductase [7].

Early on it seemed possible that azidocytidine might also exert its effect on DNA replication by incorporation into the polynucleotide. I started experiments to investigate this point but found no incorporation into DNA. Instead, I discovered a small amount of incorporation of the analogue into RNA.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Azidocytidine and all enzymes were obtained from Boehringer, Cs_2SO_4 (ultrapure) from Merck.

The labelling of azidocytidine with tritium was carried out by the tritium labelling service at Amersham. The crude radioactive reaction mixture was then adsorbed to a column of Dowex-1. After extensive washing with water, azido[^{3}H]cytidine (spec. act. 560 cpm/pmol) was eluted with 30% methanol. A short time before use, the nucleoside was purified further by HPLC on a

* Present address: Department of Virology, Veterinary Faculty and National Veterinary Institute, Box 585, Biomedical Center, S-751 23 Uppsala, Sweden

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

semipreparative C_{18} column with 20% methanol in the water.

2.2. *Cell growth and separation of nucleotides from nucleic acids*

3T6 cells were grown on 5-cm petri dishes in Dulbecco's modification of Eagle's medium containing 10% heat-inactivated horse serum to a density of $0.7-1.0 \times 10^6$ cells/dish [8]. Parallel dishes were then incubated for different periods with azidocytidine and, after removal of medium, the cells were extracted with 60% methanol at $-20°C$ for 24 h [9]. The supernatant solution after centrifugation was used to determine the amount of azido[^{3}H]CTP formed during incubation. To this purpose portions were spotted on PEI-cellulose plates and chromatographed in a system containing 0.5 M $LiCl_2$ in M HCOOH [10].

2.3. *Purification of RNA and DNA*

The cell pellet after methanol extraction was dissolved in 0.5 ml of 0.5% SDS in 20 mM Tris-HCl, pH 7.5, containing 2 mM EDTA and proteinase K (1 mg/ml). The samples were incubated for 2 h at 37°C and then extracted with an equal volume of chloroform/isoamyl alcohol (25:1) to remove proteins. RNA and DNA were precipitated with ethanol and the precipitates were dissolved in 0.2 ml of 20 mM Tris-HCl, pH 7.5, containing 2 mM EDTA. After addition of 0.2 ml formamide the samples were kept at 80°C for 5 min and then applied at room temperature to a neutral Cs_2SO_4 density gradient (2.5 ml saturated Cs_2SO_4 + 2.1 ml Tris-HCl, pH 7.5, containing 5 mM EDTA) and centrifuged for 60 h at 35000 rpm (20°C) in an SW 50.1 rotor. Fractions (0.3 ml) were collected from the bottom of the centrifuge tubes and portions (0.15 ml) of each fraction were precipitated with 10% trichloroacetic acid to measure acid insoluble radioactivity. Fractions containing RNA or DNA were combined, equilibrated with 50 mM Tris-HCl (pH 7.5) on a Biogel P-30 column, and precipitated with ethanol. After centrifugation, the precipitates were dissolved in 0.1 ml of 20 mM Tris-HCl, pH 7.5.

2.4. *Enzymatic digestion of RNA and DNA*

Each sample containing RNA was digested in a final volume of 0.15 ml by addition of 5 μg each of RNase A and T_1, snake venom phosphodiesterase and alkaline phosphatase in 5 mM $MgCl_2$ and 5 mM $CaCl_2$. Incubation was for 2 h at 37°C. Conditions for the digestion of DNA were the same as for RNA, except that DNase 1 was used instead of RNases.

Approx. 50% of the radioactivity present in the combined RNA fractions of the Cs_2SO_4 gradient was recovered as nucleoside after the enzymatic digestion. The indentity of this material with azidocytidine was established by chromatography on silica thin-layer plates with a methanol:chloroform (1:1) mixture [10].

3. RESULTS AND DISCUSSION

In preliminary experiments I found that on incubation of 3T6 cells with azido[^{3}H]cytidine a considerable amount of radioactivity was incorporated into the RNA fraction of the cells. However, when enzymatic digests of RNA were analyzed by chromatography most of the radioactivity comigrated with cytidine and only a small part occupied the position of azidocytidine. It then became apparent that the preparation of azido[^{3}H]cytidine was contaminated by [^{3}H]-cytidine (fig.1). In spite of the fact that this contaminant only amounted to a few tenths of a percent it influenced the outcome of the experiment and had to be removed. This was done routinely by HPLC chromatography on a semipreparative C_{18} column and all the results described below were obtained with the preparation of azido[^{3}H]cytidine purified in this way.

In the experiment described in fig.2 and table 1 parallel plates of growing 3T6 cells were incubated for different times with 2 concentrations of azido[^{3}H]cytidine. At the indicated times the medium was removed from the cells and the soluble nucleotide fractions were separated from the polynucleotides by extraction with 60% methanol as described in section 2. After removal of proteins, RNA and DNA were further purified and separated by centrifugation on Cs_2SO_4 density gradients (fig.2). This figure demonstrates that with time increasing amounts of radioactivity accumulate at the position of RNA in the gradient. No similar accumulation is found at the position of DNA. Compared to the bandwidth of the ultraviolet markers the radioactivity showed a much broader distribution. This indicates that ^{3}H

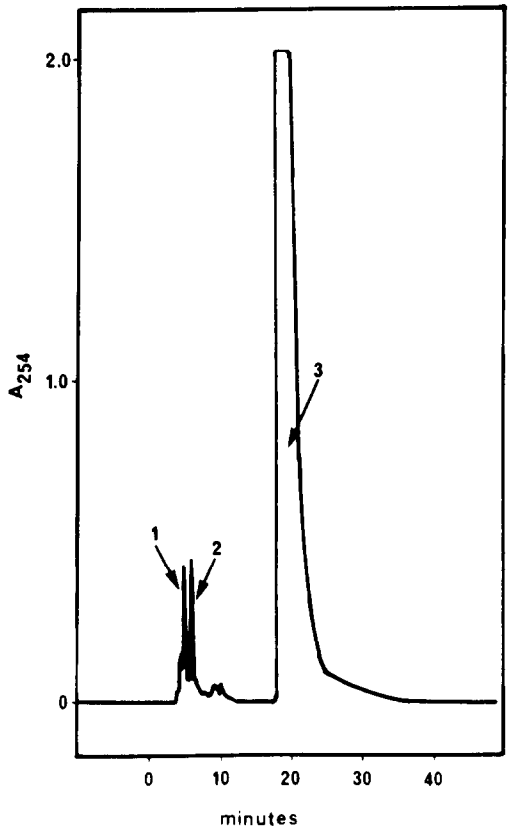

Fig.1. Purification of azido[^{3}H]cytidine by reversed-phase chromatography on a C_{18} column. ^{3}H-labelled azidocytidine purified by chromatography on Dowex-1 was chromatographed on a semipreparative C_{18} column with 20% methanol in water. The flow rate was 3 ml/min. Absorbance was recorded at 254 nm. The two main impurities are 2-amino-cytidine (1) and cytidine (2), well separated from azidocytidine (3).

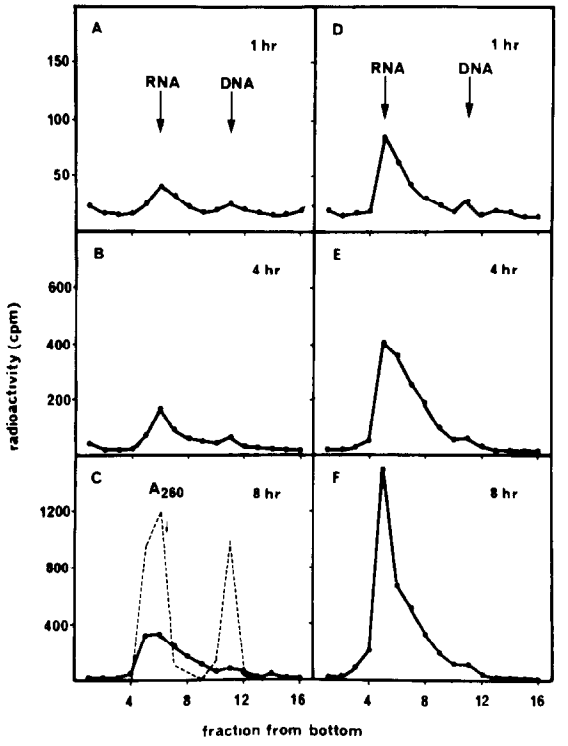

Fig.2. Separation on neutral Cs_2SO_4 density gradients of RNA and DNA labelled from azido[^{3}H]cytidine. The nucleic acids were labelled by incubation of growing 3T6 cells with 53 μM azido[^{3}H]cytidine (panels A–C) or 158 μM azido[^{3}H]cytidine (panels D–F) for the indicated times. Fractions were collected from the bottom of centrifuge tubes and analyzed for isotope. The broken line in panel C shows the analysis of the absorbance at 260 nm corresponding to carrier RNA and DNA.

was incorporated into RNA of low M_r. Since we show below that isotope was incorporated as azidocytidine into RNA a possible explanation appears to be that azidocytidine acts as a chain terminator. Such a result was previously found when azidoCTP was incorporated by primase into the RNA priming DNA synthesis in *E. coli* [11].

The amount of azidocytidine incorporated into RNA can be calculated from the total amount of radioactivity present in the RNA peak, making the reasonable assumption that the specific activity of the incorporated azidocytidine is identical to that of azidocytidine in the medium. Table 1 compares the amounts of azidocytidine incorporated into RNA at different time points and at 2 concentrations of azidocytidine with the corresponding amounts of azidoCTP present in the nucleotide fraction. It appears that incorporation into RNA is strongly dependent on the concentration of azidoCTP and always only amounts to a small fraction of the free nucleotide. At most, only 10 pmol azidocytidine are incorporated into RNA during a 4 h period into 10^6 cells. For comparison we estimate from previous experiments [7] that these cells during 4 h incorporate 20 nmol CMP. It is, however, possible that only a particular subset of RNA molecules contains azidoCMP. A second point emerging from the table is that the increase

Table 1

Distribution between azidoCTP and RNA (azidoCMP) of azido[^{3}H]cytidine incorporated by 3T6 cells

Azidocytidine in medium (μM)	Incubation time (h)	AzidoCTP (pmol/10^6 cells)	AzidoCMP in RNA (pmol/10^6 cells)
53	1	73.5	0.2
53	4	201	1.45
53	8	368	3.1
158	1	180	0.65
158	4	756	5.2
158	8	1484	14.8

The RNA values were calculated from the Cs_2SO_4 experiment described in fig.2. The azidoCTP values were obtained from PEI cellulose chromatograms of the methanolic extract

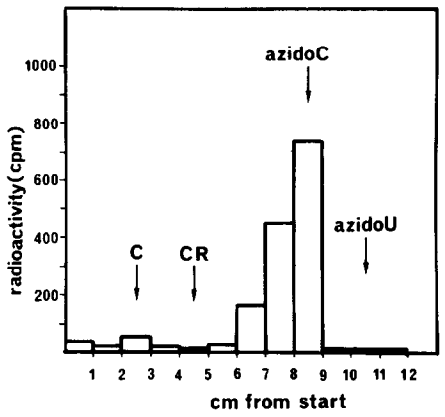

Fig.3. Chromatography on silica thin-layer plates of an RNA digest. The RNA fractions from a Cs_2SO_4 separation of the nucleic acids from cells labelled with azido[^{3}H]cytidine was digested to nucleosides and separated on a silica TLC plate. The radioactivity on each 1 cm area was determined. The arrows indicate the positions of various marker nucleosides: C (cytosine), CR (cytidine), azidoC (azidocytidine) and azidoU (azidouridine).

of the amount of azidoCTP formed is essentially linear with time. 8 h after addition of 158 μM azidocytidine 3T6 cells contain approximately equal amounts of azidoCTP and CTP.

To determine whether azidocytidine was incorporated directly into RNA, growing 3T6 cells were incubated for 20 h with 0.2 or 0.8 mM azido[^{3}H]cytidine and RNA and DNA were extracted and purified by Cs_2SO_4 density centrifugation as described above (not shown). The radioactive materials were then degraded enzymatically and chromatographed on silica thin-layer plates as described in section 2. The results from the TLC analysis of RNA are shown in fig.3. Clearly the radioactivity from the RNA digest cochromatographed with authentic azidocytidine. Too little radioactivity was present in DNA to give meaningful results.

While this work demonstrates that very small amounts of azidocytidine are incorporated into RNA it is not clear whether such an effect contributes to the inhibition of cell growth by the nucleoside analogue. Incorporation into RNA is minute at concentrations of azidocytidine that give a profound inhibition of ribonucleotide reductase. Overall RNA synthesis, as measured by incorporation of [^{3}H]cytidine, is not effected. All this might argue against the importance of the incorporation into RNA. However, it cannot be excluded that incorporation occurs into a subset of RNA molecules and may contribute to the effects of the analogue.

ACKNOWLEDGEMENTS

I wish to thank Dr F. Eckstein, Max-Planck-Institut für Experimentelle Medizin, Abteilung Chemie, Göttingen, FRG, for advice in connection with the labelling and purification of azidocytidine. This work was supported by grants from the Wallenberg foundation, the Medical Faculty of Karolinska Institutet, and the Swedish Medical Research Council to Peter Reichard.

REFERENCES

[1] Hobbs, J., Sternbach, H., Sprintzl, M. and Eckstein, F. (1973) Biochemistry 12, 5138–5145.
[2] Åkerblom, L., Pontis, E. and Reichard, P. (1982) J. Biol. Chem. 257, 6776–6782.
[3] Thelander, L., Larsson, B., Hobbs, J. and Eckstein, F. (1976) J. Biol. Chem. 251, 1398–1405.
[4] Sjöberg, B.-M., Gräslund, A. and Eckstein, F. (1983) J. Biol. Chem. 258, 8060–8067.
[5] Engström, Y., Eriksson, S., Thelander, L. and Åkerman, M. (1979) Biochemistry 18, 2941–2948.

[6] Bjursell, G., Skoog, L., Thelander, L. and Söderman, G. (1977) Proc. Natl. Acad. Sci. USA 74, 5310–5313.
[7] Åkerblom, L. and Reichard, P. (1985) J. Biol. Chem. 260, 9197–9202.
[8] Nicander, B. and Reichard, P. (1985) J. Biol. Chem. 260, 5376–5381.
[9] Skoog, L. (1970) Eur. J. Biochem. 17, 202–208.
[10] Randerath, K. and Randerath, E. (1967) Methods Enzymol. 12, 323–347.
[11] Reichard, P., Rowen, L., Eliasson, R., Hobbs, J. and Eckstein, F. (1978) J. Biol. Chem. 253, 7011–7016.