The Utilization of α-2′-Deoxythioguanosine by Murine Tumor Cells

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SUMMARY

The α- and β-anomers of 2′-deoxythioguanosine were prepared with a radiocarbon label in the sugar moiety and a radiosulfur label in the base. *In vivo* experiments were conducted with Mecca Lymphosarcoma ascites cells in mice. Determination of the distribution of radioactivity in base and sugar of material incorporated into deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), relative to the distribution in the precursors, indicated that both anomers can be incorporated into the DNA without the separation of sugar and base. Degradation of DNA containing label from these nucleotide precursors, with deoxyribonuclease and phosphodiesterase, showed that the β-anomer was almost all present in the nucleotide chain. The incorporation of α-anomer into DNA gave a much higher proportion of terminal labeling, but also indicated that some α-nucleoside had entered the nucleotide chain. The data also indicated that entry of α-deoxynucleoside into the RNA can occur without exchange of the sugar.

INTRODUCTION

The nucleoside, β-2′-deoxythioguanosine was reported to be carcinostatic to several mouse tumors that were resistant to 6-thioguanine (1). This nucleoside was phosphorylated by a kinase and incorporated into deoxyribonucleic acid in cells lacking guanosine monophosphate pyrophosphorylase, or deficient in ability to reduce thioguanine ribosides to deoxyribosides. Its efficacy was limited in certain cell lines (e.g., L1210R), where a high level of nucleoside phosphorylase led to extensive conversion to thioguanine. The toxicity to marrow elements appeared to be equal, on a molar basis, to that of thioguanine. The chemical synthesis of this nucleoside also made α-2′-deoxythioguanosine available. This α-anomer was also converted to nucleotide in certain mouse tumor cell lines and produced carcinostatic effects. It was at least an order lower in toxicity to the host mice, apparently because it was not appreciably converted to nucleotide by the kinases of mouse marrow cells (1). The carcinostatic effects of the α-anomer were correlated with incorporations of radioactivity, from base-labeled nucleoside, into DNA.[1] This latter finding appeared significant because it was obtained in a tumor cell line, Mecca Lymphosarcoma, that had very limited ability to incorporate thioguanine into DNA. Mecca Lymphosarcoma formed relatively large amounts of thioguanine riboside from thioguanine, but little or no thioguanine deoxyriboside. More direct evidence was needed to determine whether the α-anomer actually entered the polynucleotide chain of DNA without transglycosidation or reutilization through the free base. In order to obtain such evidence, we decided to synthesize the deoxythioguanosine anomers with radioactive labels in both base and sugar. Labeling with tritium appeared to be the most

[1] Abbreviations used are: deoxyribonucleic acid, DNA; ribonucleic acid, RNA; 6-thioguanine, TG; α,β-2′-deoxythioguanosine, α,β-TGdR; tris-(hydroxymethyl)aminomethane, Tris; thioguanosine, TGR; thioguanosine monophosphate, TGMP; 2′-deoxythioguanosine monophosphate, TGdMP.

economical course, and this was attempted first. The attempt failed because of the instability of label sites during the synthesis, such that the tritium was lost by a proton exchange with the solvent (2). Since 2-deoxyribose-1-^{14}C had become available (Nuclear Chicago Corporation), a second and successful synthesis was carried out, in which the sugar component was labeled with radiocarbon and the base with radiosulfur. In order to assure complete separation of the two anomers, a system of thin-layer chromatography on silica gel was used for analysis.

Experiments carried out in Mecca Lymphosarcoma cells with these doubly labeled nucleosides support the concept that α-2′-deoxythioguanosine does enter DNA intact, possibly as the terminal and/or near terminal nucleoside of the newly synthesized DNA.

MATERIALS AND METHODS

Synthesis and purification of doubly labeled nucleosides. The procedures used were essentially those of Iwamoto, Acton, and Goodman (3), suitably scaled down: 1.0 mC of 2-deoxyribose-1-^{14}C (Nuclear Chicago Corporation) was diluted with unlabeled 2-deoxyribose to a total of 7.83 mmoles. This was converted to the 2-deoxy-3,5-di-*O*-*p*-toluoyl-D-ribofuranosyl chloride in 48% yield. This chlorosugar, 3.76 mmoles, was allowed to react in benzene with 3.76 millimoles of 2-acetamido-6-chloropurine mercury salt. The procedures described earlier (3) were followed, and the mixed α,β-anomers of the nucleoside were obtained in 24% overall yield. The crystallization from chloroform yielded 268 mg of β-anomer. Thin-layer chromatography on silica gel[2] established that this material was free of α-anomer and appeared to have no other significant impurities. The residual material, approximately 300 mg, was found to contain mainly α-anomer. This α-anomer, however, contained approximately 14% β-anomer and two smaller components that moved much faster in the chromatographic system.

For our biological experiments with doubly labeled nucleosides, it was important to have each anomer essentially free of the other. Since we had no facile means for analysis or separation of the anomers in the form of the deblocked 2′-deoxythioguanosine, the analyses and purifications were conducted with the blocked nucleosides. However, a mixture of the blocked nucleoside anomers, that by analysis on silica gel was found to be 14% β-anomer and 86% α-anomer, was carried through the thiation and deblocking procedure in 75% yield. The material was used to conduct toxicity tests in female C3H mice. Groups of 3 mice receiving different levels of this preparation were compared with groups receiving various levels of β-TGdR. Toxicity was evaluated by weight losses and fatalities. These tests indicated that, assuming all the toxicity in the α-TGdR to be due to residual β-TGdR, the preparation could not contain more than 2% β-TGdR. That is, a preparation containing 14% β-anomer was converted in the deblocking, thiation, and isolation to 2% β-anomer or less. Contamination with β-anomer was probably less than 2%, since we have found that the addition of α-anomer

[2] We are indebted to Mr. John P. Marsh, Jr., Bioorganic Chemistry Department, Stanford Research Institute, for perfecting this chromatographic system and for applying it on a preparative scale to the purification of the radioactive-tracer-labeled α-anomer. The analytical method consisted of spotting 50–200 μg quantities, in methyl ethyl ketone solution, on thin-layer strips of activated silica gel 8 inches long. These were set in a jar with the lower edge immersed in a solvent made up of 40 volumes of ethyl acetate and 60 volumes of cyclohexane. The solvent was allowed to ascend to the top of the plate; the plate was removed and air-dried. This process was repeated 3–4 times as needed to give adequate separations. The α-anomer moved the more rapidly and clearly separated from the β-anomer. The spots were visualized with an ultraviolet light source; each was separately scratched off the plate, eluted into chloroform and measurements made in a Beckman DU spectrophotometer at 290 mμ. It was established that minor quantities of one anomer could be detected and quantitatively recovered in the presence of dominant quantities of the other anomer.

to a dosage of β-anomer increased the toxicity.

The preparation containing the ^{14}C-labeled α-anomer was subjected to chromatography on silica gel.[3] When we felt that further procedures would lead to unacceptable losses, we had obtained 205 mg of blocked nucleoside, containing 97.3% α-anomer and 2.7% of β-anomer. This material was used for the deblocking and thiation procedure, as was the 268 mg of β-anomer. The method used was that described by Iwamoto *et al.* (3), except that $H_2{}^{35}S$ was used in the reaction mixture, the methanol was saturated with H_2S, but no further supply of H_2S was passed in during the reaction. Conditions were arranged so as to have $H_2{}^{35}S$ at approximately 1 mC/mmole. Applying these methods to the β-anomer yielded 110 mg of β-2′-deoxythioguanosine-$^{14}C^{35}S$, a 78% yield calculated as the monohydrate. Chromatography on paper with 5% Na_2HPO_4 gave one spot at $R_f = 0.52$ and indicated only a minor radioimpurity of 1–1.5% at $R_f = 0.95$. The thiation and deblocking of the α-anomer initially yielded 127 mg. But a major impurity appeared at $R_f = 0.95$ on the paper chromatographic system. It has since been indicated that this impurity was probably the 6-methoxy derivative.[4] The preparation was dissolved in a small volume of dilute (1 M) ammonium hydroxide, and an insoluble residue was discarded. Then the α-TGdR was precipitated by the addition of acetic acid to pH 5. Two such reprecipitations finally provided 77 mg of α-2′-deoxythioguanosine-$^{14}C^{35}S$, a 75% yield as the monohydrate. Chromatography on paper, with 5% Na_2HPO_4, now showed only one spot at $R_f = 0.52$ and radioactive impurity at $R_f = 0.95$ amounting to only 1% of the total.

The two nucleoside preparations were characterized as to their radioactive-tracer content by chromatography on paper (5% Na_2HPO_4), with and without acid-hydrolysis and addition of carrier thioguanine. Evaluation was made on the basis of extinction in ammonium hydroxide at 325 mμ and scintillation counting in a polar solvent system giving 50% counting efficiency. Direct counting of paper spots in a toluene scintillation system also gave 50% efficiency. Initially, the α-anomer was found to give 540,000 cpm/μmole in the base and 51,000 cpm/μmole in the sugar; the β-anomer showed 429,000 cpm/μmole in the base and 51,000 cpm/μmole in the sugar. Because of the limited half-life of the base-labeling, these were redetermined in each experiment.

RESULTS

In Vivo Metabolism of α,β-2′-Deoxythioguanosine-$^{14}C^{35}S$

Ascites cell growths of Mecca Lymphosarcoma appeared to be the best material for these experiments. Mecca Lymphosarcoma cells have been shown to incorporate the α,β-TGdR into DNA and RNA (1). Conversion to thioguanine by nucleoside phosphorylase does occur in these cells and would lead to reutilization of the thioguanine for RNA synthesis, but the extent of reutilization for DNA synthesis would be very small in this tumor. Several

[3] The 300 mg of impure α-anomer was applied in a thin (1–2 mm) line on silica gel (Brinkman Silica Gel HF) plates 200 × 200 × 1.25 mm, approximately 100 mg per plate. This plating was made possible by the use of an automatic sample-streaking device obtained from the Rodder Instrument Co., 775 Sunshine Drive, Los Altos, California. The plates were run with the same solvent system as that used in the analytical method. The band of α-anomer from each plate was removed from the plates and eluted in chloroform; the chloroform was evaporated off *in vacuo*. The material was then free of the fast-moving components, but a reduced amount of β-anomer was still present because of the wide bands obtained. The chromatography was repeated so that remaining β-anomer could be removed.

[4] Personal communication with Mr. Fred Keller, Senior Chemist, Riker Laboratories, Northridge, California, yielded the information that in scaling up preparation of α-TGdR, he found that a somewhat limited supply of H_2S resulted in substitution of a methoxyl group at the 6-position instead of a thiol group.

experiments were conducted with varied conditions. Data from one that gave results typical of the several experiments is described. AKD2F1[5] female mice weighing 28–30 g were each injected intraperitoneally with 2×10^7 Mecca Lymphosarcoma cells. On day 4, groups of 4 mice were selected. Each mouse received 2 injections of 2.0 μmoles of labeled substrate intraperitoneally in 0.40 ml of isotonic saline, with an 8 hr interval. On day 5 (16 hr later) each received a third injection and was sacrificed 2 hr later. The cells were removed and pooled from 2 mice for each sample. Approximately 0.70 ml of packed cells (1470 *g*, 7 min) were obtained for each sample. The samples were extracted twice in the cold with 0.4 M perchloric acid. The two extracts from each sample were put in a 20-ml beaker, neutralized to pH 7 with 2 M KOH, and carrier compounds (TG, 2′-TGdR, 2′TGdMP)[6] were added. The samples were evaporated to dryness *in vacuo* at 2°. Then each was taken up in 1.0 ml of 0.1 M NH_4OH. A 0.25-ml aliquot of each was spotted on paper and developed with 5% Na_2HPO_4. The base, nucleoside, and nucleotide spots from each (R_f's 0.28, 0.52, 0.78) were cut from the papers and counted directly in a toluene scintillation fluid (50% efficiency) as a measure of the amount of each acid-soluble component. The amount of radioactivity present as free base or nucleoside was negligible. The amounts present in nucleotides from cells given α-TGdR, β-TGdR, or TG are shown in Table 1. Mono-, di-, and triphosphate forms of the nucleotide would not be separated in this system. The extracted tissue residues were washed several times with cold ethanol, partially dried *in vacuo*, and extracted at 100° with 10% NaCl to obtain the nucleic acids. The nucleic acid preparations were separated into DNA and RNA fractions as described earlier (4). The DNA and RNA solutions were assayed for radioactivity by scintillation counting. Then aliquots were subjected to acid hydrolysis in the presence of carrier TG and chromatographed on paper with Na_2HPO_4 to determine the fraction of the radioactivity present in the base (TG). The balance was found to be due to the radioactivity of the sugar and moved rapidly in this solvent ($R_f = 0.95$). The distribution of radioactivity in the

TABLE 1

Distribution of radioactive label in Mecca Lymphosarcoma cells after treatment with labeled nucleosides

In the first experiment, mice bearing Mecca Lymphosarcoma ascites cell growths were each injected intraperitoneally with 2.0 μmoles of precursor at 26, 18, and 2 hr before sacrifice. Cells from 2 mice, approximately 0.35 ml/mouse, were pooled for each sample. Each result is an average of analyses from duplicate samples in a typical experiment. In the second experiment, 3 mice bearing Mecca Lymphosarcoma ascites cell growths were each injected intraperitoneally with 5.5 μmoles of labeled α-TGdR per mouse and sacrificed after 2 hr. The tumor cells from the 3 mice were pooled (1.12 ml) and used for isolation of the cell components.

Precursor used	Radioactivity found (cpm/min/g wet wt.)						Percentage of the radioactivity in the base			Acid-soluble nucleotides formed
	Precursor[a]		DNA		RNA					
	Total	Base	Total	Base	Total	Base	Precursor	DNA	RNA	(μmoles/g)
I α-TGdR	247,000	196,000	4,860	3,520	2,200	1,020	79.3	72.5	46.3	0.15
β-TGdR	183,500	132,500	12,700	4,300	12,400	7,200	72.3	72.6	58.1	0.22
TG	1,200,000	1,200,000	8,080	8,150	33,300	33,000	100	100	100	0.14
II α-TGdR	176,000	120,500	6,040	4,250	5,000	3,530	71.0	70.5	70.5	0.20

[a] Counts per minute per micromole.

[5] AKR × DBA_2J F1, referred to as AKD2F1.

[6] Samples of thioguanine ribonucleotide and deoxyribonucleotide were supplied by the Cancer Chemotherapy National Service Center.

precursors and in the isolated nucleic acids is indicated in Table 1. The results support the conclusion that incorporation of α-TGdR and β-TGdR into DNA occurred with intact nucleosides, since ratios of the base:sugar labeling changed relatively little. In RNA, the ratios had changed from that in the nucleoside precursors, indicating some cleavage and reutilization. Since the ratios decreased, some loss of base and reutilization of the sugar must have resulted. This is credible, since the sugar would be in the phosphorylated form.

In the type of experiment described, a relatively long incubation was involved and three separate doses were administered to a cell population in rapid growth. This experimental design was used in order to involve a relatively large part of the cell population in a relatively high level of precursor utilization. The levels of incorporation obtained were relatively high, but the time scale (26 hr) was such as to allow a considerable chance for cleavage and reutilization of the nucleoside components. A short incubation time with a single dose would have the advantage of providing data under conditions where a minimum of cleavage and reutilization would occur, though a smaller amount of utilization would be expected. Table 1 also contains some data from a typical experiment conducted with a short incubation time. No significant change in isotope distribution occurred with incorporation of α-TGdR into either DNA or RNA, indicating that the incorporation into both occurred with the intact α-nucleoside. The acid-soluble extract from Mecca Lymphosarcoma cells used in the latter experiment was chromatographed on a Dowex 1 formate column according to conditions described earlier (4), except that a refrigerated fraction collector (Gilson Medical Electronics, Madison, Wisconsin) was used and the procedures carried out at 2°C. Only one peak of radioactivity could be detected, and this contained 87% of the radioactivity present in the extract. This peak was eluted just slightly ahead of a marker peak of thioguanosine triphosphate, as detected by the extinction at 345 mμ. This indicated that the nucleoside was present, under these conditions, largely or exclusively as the triphosphate. The quantity, instability, and salt content of this fraction made it impractical to determine the distribution of the radioactivity between base and sugar.

Distribution of the Labeled Nucleosides in DNA and RNA

It was difficult to visualize how an α-nucleoside could be accommodated in the helical structure of the nucleic acids. Utilization of the α-TGdR in chain growth at a terminal position might lead to cessation of chain growth. In this event, α-TGdR would appear only in the terminal nucleoside. An experiment was carried out to test this possible explanation. Three groups of 12 female AKD2F1 mice, each weighing 28–30 g were used. Each mouse was given an intraperitoneal injection of 1.5×10^7 Mecca Lymphosarcoma cells. On day 4, each mouse received injections at approximately 8:00 AM, 11:00 AM, and 2:00 PM and was sacrificed 2 hr after the last injection. Group I received 3 injections with α-TGdR-^{35}S; Group II received β-TGdR-^{35}S, and Group III received injections with TG-^{14}C. Each injection consisted of 2.6 μmoles of labeled material in saline solution. Cells from the 12 mice of each group were pooled, except for those of 1 mouse in the group receiving α-TGdR, which were discarded because of contamination with blood.

The samples were centrifuged, and the fluid was discarded. The packed cells were in each case extracted twice with 0.4 *M* perchloric acid and washed 3 times with cold ethanol. The precipitated material was allowed to air-dry 66 hours at 2° and used to isolate DNA and RNA as described earlier (4). Dithioerythritol (5 μmoles) was added to each sample before incubation in NaOH to minimize oxidation of the thiols.

The DNA samples were each treated with Tris buffer to a final pH of 7.4 and 0.05 M, and 5 μmoles of dithioerythritol was added to each. The solution, in a final volume of 4.0 ml, in each case, also contained $MgCl_2$, 0.002 M, and 2 mg of

deoxyribonuclease.[7] These samples were incubated 60 min at 38°. Then each was treated with more Tris to adjust the pH to 9.0. Purified snake venom phosphodiesterase (2 mg protein) was added to each and monophosphates from polynucleotide units other than those terminal in the chain, which would become nucleosides. In the alkaline degradation of RNA, one should obtain nucleoside 2′,3′-monophosphates,

TABLE 2

Measurements of terminal and central labeling in DNA and RNA of Mecca Lymphosarcoma cells

Precursor used	Packed cell volume (ml)	DNA		RNA	
		Cpm	μgTG/ml cells	Cpm	μgTG/ml cells
α-TGdR-^{35}S	2.45	7,790	1.14	7,550	1.10
β-TGdR-^{35}S	3.20	45,400	3.30	43,400	3.15
TG-^{14}C	3.05	3,880	0.29	40,500	3.09

Distribution of label in degraded nucleic acids

Precursor used	DNA			RNA		
	TGdR (cpm)	TGdMP (cpm)	Recovery (%)	TGR (cpm)	TGMP (cpm)	Recovery (%)
α-TGdR-^{35}S	1,915	3,935	75	3,390	2,500	78
β-TGdR-^{35}S	2,315	36,280	85	15,800	20,450	83
TG-^{14}C	190	2,520	70	2,990	30,200	82

Groups of 12 female AKD2F1 mice bearing ascites cell implants of Mecca Lymphosarcoma were each injected intraperitoneally at approximately 8:00 AM, 11:00 AM, and 2:00 PM with 2.6 μmoles of labeled precursor and sacrificed at approximately 4:00 PM. Cells from each group were pooled and centrifuged (7 min at 1470 *g*); the fluid was discarded. The samples were used to isolate DNA and RNA, and these were degraded to mononucleotides. Samples of the degraded nucleic acids were treated with carrier nucleosides and nucleotides and chromatographed on paper with 5% KH_2PO_4. The nucleoside and nucleotide spots were counted in a scintillation system to ±3% at 50% efficiency.

incubation at 38° continued for 2 hr. The pH had declined to a minimum of approximately 8.6 in each case. The volumes were reduced *in vacuo* to 3.00 ml. This degradative procedure should yield nucleoside with terminal nucleosides appearing as free nucleosides. TG, TGdR, and TGdMP were added to the solutions of degraded DNA as carriers. TG, TGR, and TGMP were added to the solutions of degraded RNA as carriers. Separate aliquots (0.30 ml) of each solution were counted in a polar scintillation counting fluid (50% efficiency) and spotted on Whatman No. 1 papers. The papers were developed with 5% KH_2PO_4.[8] The spots of base, nucleoside, and nucleotide were visualized with an ultraviolet light source, cut from the papers

[7] Deoxyribonuclease I, 150,000 units/mg was obtained from Sigma Chemical Company, St. Louis, Missouri, and was used as such. Phosphodiesterase from *Crotalus adamanteus* was obtained from the same source. Since it contained some monoesterase activity, it was chromatographed on a Whatman powdered cellulose column 10 × 160 mm. When this was eluted with water, a protein peak, detected by extinction of 280 mμ, was obtained. Elution with 0.1% NaCl gave no peak. Elution with 0.9% NaCl gave a second peak. These 2 peaks were tested with adenosine 5′-monophosphate as substrate and had no detectable monoesterase activity. They were pooled and used for the degradation of DNA.

[8] This acid system was found to decrease the oxidation of the thiols to a negligible level during chromatography. Alkaline systems had the disadvantage of leading to some oxidation. R_f's in this system were: TG, 0.25; TGR, TGdR, 0.40; TGMP, TGdMP, 0.60.

and counted directly in a toluene scintillation fluid (50% efficiency). Counts in TG were negligible in all cases. Counts in nucleotide fractions are reported in Table 2. The overall recoveries of radioactivity obtained in the nucleoside and nucleotide carriers are reported. These range from 70 to 85% for DNA, from 78 to 83% for RNA. With β-TGdR or TG as precursor, 94 and 93%, respectively, of the counts from DNA appeared in the nucleotide carrier spots. With α-TGdR as the precursor, only 67.3% of the recovered counts were in nucleotide, 32.7% in nucleoside. The distribution for RNA is probably compromised by nucleoside cleavage and reutilization of the base, a process which could have little influence on incorporation into the DNA of these tumor cells.

DISCUSSION

It appears that the labeling of terminal nucleoside in DNA was markedly increased, with α-TGdR as precursor, relative to that obtained with β-TGdR as precursor. But two-thirds of the labeling from α-anomer was still in the mononucleotide fraction. This supports the concept that terminal labeling was involved when α-TGdR was incorporated, but it seems inescapable that the nucleoside did also enter the chain. Perhaps this occurred near the terminus, and allowed some growth beyond, with this additional growth lying free from the template, i.e., "dangling" away from the template.

The data presented do not establish without question that α-TGdR entered the nucleotide chain of DNA. To establish this would require that the labeled nucleotide from DNA be isolated and its anomeric configuration determined. The minute quantities available and the stability are such that this would be impractical. A model experiment with a mixture of blocked nucleosides known to be 86% α-anomer and 14% β-anomer yielded a final product containing less than 2% β-anomer. The tracer-labeled α-nucleoside preparation with 2.7% β-anomer present was deblocked and sulfonated on the same scale and in the same volumes. The final preparation of α-anomer might conceivably contain 0.4% β-anomer. Since it has been demonstrated that the incorporation into DNA is proportional to dose (1), a contaminant of this magnitude would not yield the levels of incorporation that were obtained with the labeled α-TGdR. Combinations of α- and β-anomers increased incorporation only slightly (1). It thus seems unlikely that contamination of the α-TGdR with β-TGdR can explain the findings.

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