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In vivo Effects in Mice Produced by a 3′-Branched Homologue of α-2′-Deoxythioguanosine

G. A. LePage, P. A. Banks, M. J. Noujaim, and G. R. Buzzell

The University of Alberta Cancer Research Unit, McEachern Laboratory and the Department of Anatomy, University of Alberta, Edmonton, Canada T6G 2H7

Summary. *Studies with a 3′-branched chain homolog (α-3′-BCTGdR) of 2′-deoxythioguanosine (α-TGdR) showed that it did not prolong the survival of mice bearing the Mecca lymphosarcoma. Host toxicity was quite profound and resembled that seen with 6-thioguanine (6-TG). Evidence was obtained that this nucleoside derivative was not appreciably converted to 6-TG in the mouse. Mice treated with toxic doses of 6-TG or α-3′-BCTGdR were found to have very similar pathological changes. The granulocytes were eliminated from the peripheral blood, bone marrow was acellular, and some more limited damage was seen in the intestinal crypts. Experiments with radiosulfur-labeled drugs demonstrated that α-3′-BCTGdR was incorporated into the DNA of mouse bone marrow, predominantly in the chain-terminating position, with the result that shorter chains of DNA accumulated. The new homolog, unlike α-TGdR, was phosphorylated in bone marrow as well as in tumor, and incorporated well into the DNA both of bone marrow and of the neoplastic cells. In devising other homologs attention must be given to the specificity of the kinases, i.e., to whether phosphorylation is superior in tumor cells or in the growing normal cells.*

Introduction

The purine analog 6-thioguanine (6-TG) has been widely used as an antileukemic agent, and nucleosides of it, viz. α,β-deoxythioguanosine (α,β-TGdR) have been examined in clinical trials [6, 7]. α-TGdR has been found to be without appreciable host toxicity [6] and to affect some experimental tumors by being incorporated in the terminal position on the DNA chain [9]. Recently, 3′-branched chain derivatives of α,β-TGdR have been described [1]. Fig. 1 shows the structure of α-BCTGdR. These derivatives (α,β-3′-BCTGdR) were approximately twice as effective as α-TGdR in inhibiting the growth of the human lymphoblastoid cell line W1-L2. They were phosphorylated and incorporated into DNA in the murine lymphosarcoma, Mecca, about twice as well as was α-TGdR, and like α-TGdR, were shown to be DNA-chain terminators. When larger amounts of α-3′-BCTGdR became available, toxicity studies in mice and therapy tests against the Mecca lymphosarcoma were conducted. Unlike α-TGdR, the α-3′-BCTGdR was toxic to the mice, with profound body weight loss and death in a pattern indentical with that observed for 6-TG. Since 6-TG was found to be toxic mainly to the bone marrow, and mice given lethal doses of 6-TG could be saved with infusions of syngeneic bone marrow [8], studies of α-3′-BCTGdR toxicity to bone marrow were carried out. It appeared that, without prior conversion to 6-TG, α-3′-BCTGdR was converted to nucleotide and

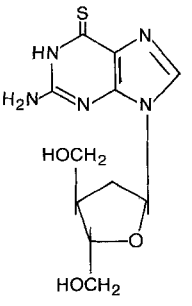

Fig. 1. Structure of 6-thioguanine-α-2,3 dideoxy-3-(hydroxymethyl)-D-erythro-pentofuranose

Reprint requests should be addressed to: G. A. LePage

Abbreviations used are: 6-thioguanine, 6-TG; α,β-2′-deoxythioguanosine, α,β-TGdR; 6-thioguanine-α-2,3-dideoxy-3-(hydroxymethyl)-D-erythro-pentofuranose, α-3′-BCTGdR; tritium-labeled (methyl) thymidine, ^{3}H-TdR

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incorporated in terminal positions on the DNA chain, and produced lethal effects in mouse bone marrow. These findings will be valuable in guiding the development of other thioguanine nucleoside analogs.

Materials and Methods

Supplies of α-3′-BCTGdR were generously provided by Dr. E.M. Acton, S.R.I. International, Menlo Park, Calif. Radiosulfur was purchased from New England Nuclear Corp., Dorval, Quebec. This radiosulfur was used to label α-3′-BCTGdR and α,β-TGdR, as described earlier [4]. The radioactive nucleoside was purified by chromatography on Dowex-1-formate (8% cross-linked, 200–400 mesh) obtained from the Sigma Chemical Co., St. Louis. Elution was with 0.4 *M* formic acid. The fraction containing α-3′-BCTGdR-^{35}S or α,β-TGdR-^{35}S was evaporated to dryness in vacuo.

Indications were obtained in incubation of α-3′-BCTGdR with W1-L2 cells (human lymphoblasts) and Mecca lymphosarcoma cells (murine) that the nucleoside was not cleaved to 6-TG. Evidence as to whether this was also true for α-3′-BCTGdR in vivo in the mouse was sought by administering α-3′-BCTGdR-^{35}S and α,β-TGdR-^{35}S IP to AKD2F1 (DBA/2 × AKR) female mice purchased from the Roscoe B. Jackson Laboratory, Bar Harbor, Maine, USA. Urines were collected from groups of such mice sacrificed at intervals. Aliquots of the urines were chromatographed on sheets of Whatman 3 MM paper developed with 95% ethanol – 1 *M* ammonium acetate (5 : 2; v : v). Unlabeled 6-TG, α,β-TGdR, α-3′-BCTGdR, thioxanthine, and thiouric acid were used as markers.

Dithioerythritol and mercaptoethanol were used in incubations and chromatographic procedures to prevent oxidation of the thioguanine compounds. These were obtained from the Sigma Chemical Co., St. Louis, USA, as were DNAase and snake venom phosphodiesterase used to degrade DNA samples. The latter was freed of phosphomonoesterase by chromatography on powdered cellulose, as described earlier [5]. Degradation of DNA samples was as described earlier [1].

Toxicity tests were conducted by IP administration of solutions of α-3′-BCTGdR, 6-TG, or α-TGdR in alkaline saline (pH 9) to groups of five AKD2F1 female mice twice daily for 4 days. Body weights and fatalities were recorded. Groups of mice given vehicle only (alkaline saline), 6-TG or α-3′-BCTGdR at 100 μmol/kg on this schedule were sacrificed when considerable weight loss had occurred but the mice were not yet moribund. Blood and marrow smears were stained with May-Grundwald-Giemsa [2]. Tissues were fixed in Bouin's fluid and 5-μm sections were stained with hematoxylin and eosin.

Tests for antitumor activity were conducted with groups of six AKD2F1 female mice 10–12 weeks old. Each mouse received Mecca lymphosarcoma ascites cells by IV injection and treatment was given twice daily, beginning 24 h after the tumor transplantation. Controls received vehicle only.

Studies were conducted on DNA from the bone marrows of control and treated AKD2F1 female mice. Bone marrow from the long bones of the hind legs was flushed out by injecting isotonic saline at one end of each. Suspensions of 5×10^5 cells/ml were made. These were incubated with the test compound (100 μ*M*) for 5 min at 37° C, then ^{3}H-TdR (50 Ci/mmol, obtained from Moravek Biochemicals, City of Industry, CA, USA, as a sterile water solution) was added and incubation continued for 3 min. The reaction was stopped and alkaline sucrose gradient centrifugation carried out by the method of Walters and Hildebrand [10]. The gradients were fractionated from the bottom by means of paraffin oil, and fractionated and counted as described by Tamaoki and LePage [9].

Results

Tests for possible therapeutic effects with α-3′-BCTGdR were conducted with mice bearing the Mecca lymphosarcoma, an experimental tumor unresponsive to 6-TG and responsive to α-TGdR. In the range of doses tested, as seen in Table 1, no response was obtained with α-3′-BCTGdR or 6-TG, though α-TGdR did increase survival time. The regimen used is not optimum for α-TGdR, but could not be continued beyond 4 days in the higher dosage of α-3′-BCTGdR because of host toxicity. Toxicity tests conducted with AKD2F1 female mice not bearing tumors are shown in Table 2. Because of the negative results of therapy and the limited supply of α-3′-BCTGdR, more extensive toxicity tests were not done. The observed toxicity, high weight loss and death at 6–9 days after treatment, was very similar to the toxicity from 6-TG [3]. It therefore became necessary to determine whether any major part of the α-3′-BCTGdR was cleaved to 6-TG in the host. AKD2F1 female mice given α-3′-BCTGdR-^{35}S were monitored for urinary metabolites in comparison with β-TGdR-^{35}S, which is known to be cleaved to 6-TG. The results are shown in Table 3. This method is not very sensitive, because (a) the radiosulfur-labeled preparations of the nucleosides were contaminated with a small amount of 6-TG formed chemically as a result of the labeling procedure*; and (b) small amounts of 6-TG formed could be retained by tissues as nucleotides. However, 6-TG was a major metabolite in the urine of mice given β-TGdR-^{35}S and was a very minor component in the urines of mice given α-3′-BCTGdR-^{35}S or α-TGdR-^{35}S.

α-TGdR has been shown to be a DNA-chain terminator in susceptible tumors where it was phosphorylated [5], and is apparently not toxic to bone marrow. Since α-3′-BCTGdR was apparently a better DNA-chain terminator than α-TGdR in one experimental tumor [1], we determined what effects α-3′-BCTGdR had on DNA in mouse bone marrow. AKD2F1 female mice received α-3′-BCTGdR-^{35}S at 100 μmol/kg by IP injection and the dosage was repeated 2 h later; the mice were sacrificed after a further 2 h and bone marrow was obtained for analysis. This bone marrow contained the equivalent of 3.8 μg 6-TG per mg DNA present as acid-soluble

* No 6-thioguanine was found present in the nucleoside preparations until they were subjected to the radiosulfur-labeling procedure

Table 1. Tests for therapeutic effects of α-3′-BCTGdR on mice bearing Mecca lymphosarcoma

No. of mice	Treatment[a]	Max. weight loss (%)	Average survival (days)[b]
16	Control-alkaline saline i.p.	− 4	11.5 ± 0.9
10	α-3′-BCTGdR, 25 μmol/kg, 2 × daily, 4 days	7	11.4 ± 0.8
10	α-3′-BCTGdR, 15 μmol/kg, 2 × daily, 4 days	2	11.4 ± 0.9
10	α-3′-BCTGdR, 9 μmol/kg, 2 × daily, 4 days	1	11.7 ± 1.0
6	α-3′-BCTGdR, 100 μmol/kg, 2 × daily, 4 days	30	8.3 ± 0.3
6	α-TGdR, 100 μmol/kg, 2 × daily, 4 days	8	14.9 ± 0.9
6	6-TG, 100 μmol/kg, 2 × daily, 4 days	30	8.1 ± 0.2

[a] Groups of AKD2F1 female mice each weighing 24–25 g were each injected IP with 7.5×10^6 Mecca lymphosarcoma cells. Treatment was begun 24 h later

[b] Results are expressed as average survival ± SD

Table 2. Toxicity tests on mice with α-3′-BCTGdR

No. of mice	Treatment[a]	Body weight loss (%)	No. of survivors
5	α-3′-BCTGdR, 25 μmol/kg, 2 × daily, 4 days	7	5
5	α-3′-BCTGdR, 50 μmol/kg, 2 × daily, 4 days	20	4
5	α-3′-BCTGdR, 50 μmol/kg, 2 × daily, 6 days	25	0
5	α-3′-BCTGdR, 100 μmol/kg, 2 × daily, 4 days	30	0
5	6-TG, 100 μmol/kg, 2 × daily, 3 days	25	1

[a] Drugs were given IP in alkaline (pH 9–10) saline to AKD2F1 female mice, 12–14 weeks old

Table 3. Urinary excretions of mice treated with thioguanine nucleosides

Product measured (nmol)	Nucleoside injected[a]								
	β-TGdR-^{35}S			α-TGdR-^{35}S			α-3′-BCTGdR-^{35}S		
	15 min	30 min	60 min	15 min	30 min	60 min	15 min	30 min	60 min
Thiouric acid	73.5	182	286	1.1	2.1	4.6	1.4	2.7	5.2
Thioxanthine	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
Thioguanine	412	878	925	2.8	5.3	9.8	5.3	8.1	10
Nucleoside	140	167	163	420	703	1,235	556	989	1,460

[a] β-TGdR-^{35}S, α-TGdR-^{35}S, and α-3′-BCTGdR contained 7.0, 7.5, and 16.3 mCi/mmol, respectively, and total counts appearing in the urine in 60 min amounted to 55%, 50%, and 59% of the administered doses, respectively. Groups of 3 AKD2F1 female mice were injected with 100 μmol drug/kg body weight in each case. Figures cited are averages from the analyses of 3 mice. Rf values for TU, TX, TG, and nucleosides were 0.22, 0.38, 0.43, and 0.50, respectively. Paper chromatograms were run off the ends of the sheets to provide sufficient separations. The 3 nucleosides contained small amounts of 6-TG-^{35}S. Other radioimpurities appeared to run near the solvent front or remain at the origin

nucleotide. The DNA contained the equivalent of 1.22 μg 6-TG per mg DNA and 87% of this was present in the terminal position. Thus, as in the Mecca lymphosarcoma, α-3′-BCTGdR was phosphorylated well in mouse bone marrow and appeared to be a DNA-chain terminator. α-TGdR-^{35}S in a similar experiment labeled DNA to a negligible extent (< 0.02 μg 6-TG equivalent/mg DNA), presumably because it is not phosphorylated in bone marrow.

Mouse bone marrow cells were incubated in vitro and used to conduct alkaline sucrose gradient centrifugation experiments. Typical gradients obtained are illustrated in Figs. 2 and 3. The gradient for cells treated with 100 μ*M* α-TGdR did not differ significantly from a control gradient. The gradient for cells incubated with 100 μ*M* α-3′-BCTGdR showed a change of longer-chain DNAs to an intermediate length and a decrease in the peak of short-chain DNAs.

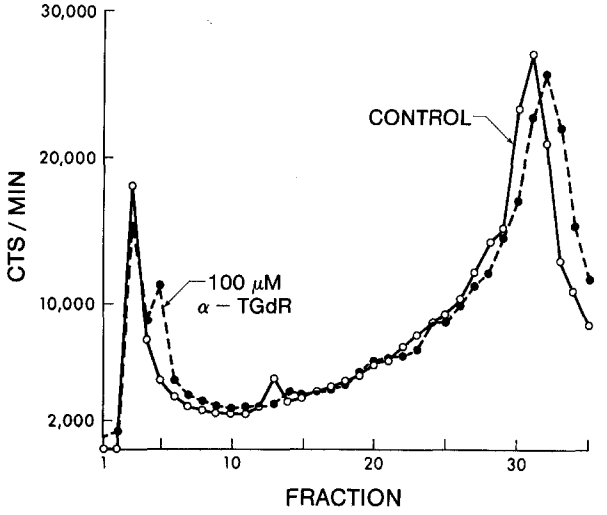

Fig. 2. Alkaline sucrose gradient centrifugation profile of DNA from murine bone marrow cells with and without treatment in 100 μ*M* α-TGdR and labeling with ^{3}H-TdR

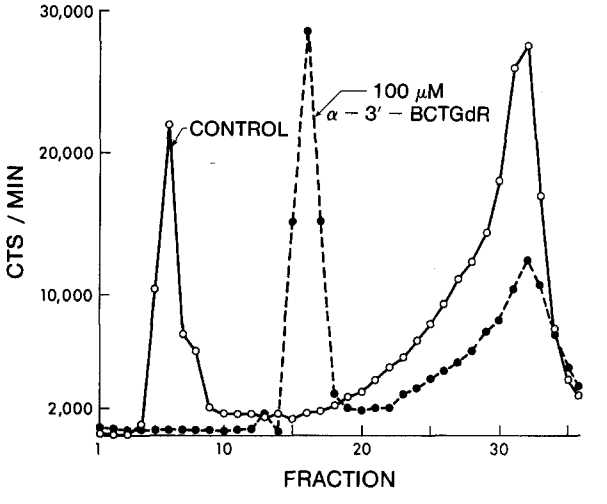

Fig. 3. Alkaline sucrose gradient centrifugation profile of DNA from murine bone marrow cells with and without treatment in 100 μ*M* α-3′-BCTGdR and labeling with ^{3}H-TdR

Control AKD2F1 female mice and groups treated with α-3′-BCTGdR or 6-TG to the point where severe toxicity was evident were sacrificed (treated twice daily at 100 μmol/kg for 3 days, sacrificed on day 6) and smears or sections of stained tissues were examined.

Blood. Control mice had normal WBC levels with all types evident. Mice treated to toxicity with either 6-TG or α-3′-BCTGdR showed almost complete deletion of the granulocytes.

Bone Marrow. Controls appeared normal, with all types of hematopoietic cells recognizable. Sections from mice treated with 6-TG or α-3′-BCTGdR were indistinguishable; both had more erythrocytes than normal. No recognizable hematopoietic cells were present.

Intestinal Mucosa. In sections from controls, mitotic figures were common. Cells were quite small and closely grouped. Again, sections from mice treated with 6-TG and those treated with α-3′-BCTGdR were very similar. Mitoses were not abundant. Some epithelial cells were enlarged and some dead cells were present. The connective tissue elements appeared normal.

Discussion

The nucleoside studied (α-3′-BCTGdR), unlike α-TGdR, does not appear to have any specificity toward neoplastic cells. Rather it is toxic to growing cells that have the kinases necessary to phosphorylate it. This kinase activity is apparently abundant in both bone marrow and Mecca lymphosarcoma cells. The lack of specificity was evident earlier [1], when Mecca lymphosarcoma cells phosphorylated both α- and β-anomers equally. It appears that the nucleoside analog has the desirable characteristic that it is not appreciably cleaved to 6-TG in the mouse or in the cultured human cells examined [1]. In selecting other such analogs for study, early study of their phosphorylation in various growing tissues appears important.

This analog, α-3′-BCTGdR, appears to produce cell kill in murine bone marrow elements by terminating DNA chains rather than by incorporation into the chain in place of β-2′-deoxyguanosine, as appeared to be the case for 6-TG [3].

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