The Metabolism of 9-Butyl-6-thioguanine in Normal and Neoplastic Tissues*

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SUMMARY

The metabolism of 9-butyl thioguanine (9-BTG)1 was studied in normal tissues and the Ehrlich ascites tumor with and without pretreatment with azaserine. The study of the distribution and metabolism of the drug was facilitated by the synthesis of 9-butyl-6-thioguanine-8-C14.

The results showed that 9-BTG was rapidly cleared from the blood, rapidly catabolized, and that the metabolites were rapidly excreted in the urine. Pretreatment with azaserine resulted in a longer retention of radioactivity in the acid-soluble fraction of the tissues.

Radioactivity was also found in the lipide fraction of the tissues. It was confirmed that 9-BTG was neither dealkylated nor incorporated into nucleic acids.

Treatment with toxic doses produced bone marrow depression with a terminal agranulocytosis.

It has become evident that a broad spectrum of drugs with differing mechanisms of action will be required for the chemotherapy of cancer (6, 11) because of the diversity in responses to drugs and because of the development of drug resistance. Drugs of closely related structure may differ in metabolic effects. 6-Thioguanine apparently acts as a "feedback inhibitor" of purine synthesis and is incorporated into nucleic acids (5, 7, 10). Both of these effects require the nucleotide form of the drug. Purine analogs blocked from ribosidation by alkyl constituents in the 9-position must have a mechanism of action differing from that indicated for 6-thioguanine (5, 7, 10), since it has been shown that dealkylation does not occur (7). The utility of such alkylated derivatives in dealing with derived drug resistance has recently been illustrated (2).

The alkylated purinethiol, 9-butyl-6-thioguanine, was found to give a synergistic response with azaserine in combination therapy of the Ehrlich ascites tumor in Swiss mice (7, 8). The survival time of tumor-bearing mice increased linearly with the number of treatments given at 12-hour intervals, in contrast to the response to thioguanine, which was maximal with three to four such treatments. This might result from rapid excretion or rapid metabolism of the drug. A study of the distribution and rate of metabolism of the drug was therefore undertaken. This was facilitated by the synthesis of labeled 9-butyl-6-thioguanine, and a synthesis procedure is briefly described.

MATERIALS AND METHODS

Synthesis of 9-butyl-6-thioguanine-8-C14.—A typical preparation was carried out as follows: Five hundred mg. of 2-amino-4-butylamino-5-formylamino-6-hydroxypyrimidine2 was dissolved in 7.5 ml. of 10 per cent methanolic HCl and heated under reflux for 20 minutes. The reaction mixture

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was immediately cooled in an ice-bath. The crystals of the product, 2,5-diamino-4-butylamino-6-hydroxy pyrimidine dihydrochloride, were filtered on a sintered glass funnel and washed with 2 ml. of ice-cold anhydrous methanol. This product was dried in vacuo over NaOH. The yield was 350 mg. of white crystals, which gave purple pigments on brief exposure to air or base. Two hundred and fifty-four mg. of the 2,5-diamino-4-butylamino-6-hydroxy pyrimidine dihydrochloride (0.94 mmole) was reacted with 81 mg. (1.17 mmole) of sodium formate-C\textsuperscript{14}, 50 \mu l. of formic acid (1.21 mmole), and 2 ml. of water for 20 minutes under reflux.\textsuperscript{3}

The reaction mixture was allowed to cool and was evaporated in vacuo over concentrated H\textsubscript{2}SO\textsubscript{4} and NaOH. The crude product, 2-amino-4-butylamino-5-formylamino-C\textsubscript{14}-6-hydroxy pyrimidine, was used without purification for the ring closure to 9-butyl guanine-8-C\textsubscript{14}. The ring closure was accomplished by refluxing in 2.5 ml. of formamide for 2 hours. The hot reaction mixture was then poured into 4 ml. of ice-cold water contained in a centrifuge tube, the contents centrifuged, the supernatant discarded, and the crude 9-butyl guanine-8-C\textsubscript{14} washed twice with water. The precipitate was dissolved in 2 ml. of boiling 4 \textit{N} hydrochloric acid, treated with approximately 50 mg. of Norit A, and centrifuged. The supernatant was pipetted into another centrifuge tube, made alkaline with concentrated ammonium hydroxide, and the resulting precipitate was washed twice with water. The product was then dissolved in 3 ml. of boiling 10 per cent potassium hydroxide solution, treated with approximately 50 mg. of Norit A, and centrifuged; the supernatant was pipetted off into a centrifuge tube and the supernatant made acidic with glacial acetic acid. The resulting precipitate was washed twice with water and dried at 100\textdegree C. Ninety-one mg. of 9-butyl guanine-8-C\textsubscript{14} was obtained. Thiation of the 91 mg. of 9-butyl guanine-8-C\textsubscript{14} was accomplished by refluxing in 2.5 ml. of formamide for 2 hours. The hot reaction mixture was then poured into 4 ml. of ice-cold water contained in a centrifuge tube, the contents centrifuged, the supernatant discarded, and the crude 9-butyl guanine-8-C\textsubscript{14} washed twice with water. The precipitate was dissolved in 3 ml. of boiling 4 \textit{N} hydrochloric acid, treated with approximately 50 mg. of Norit A, and centrifuged. The supernatant was pipetted into another centrifuge tube, made alkaline with concentrated ammonium hydroxide, and the resulting precipitate was washed twice with water. The product was then dissolved in 3 ml. of boiling 10 per cent potassium hydroxide solution, treated with approximately 50 mg. of Norit A, and centrifuged; the supernatant was pipetted off into a centrifuge tube and the supernatant made acidic with glacial acetic acid. The resulting precipitate was washed twice with water and dried at 100\textdegree C. Ninety-one mg. of 9-butyl guanine-8-C\textsubscript{14} was obtained. Thiation of the 91 mg. of 9-butyl guanine-8-C\textsubscript{14} was accomplished with 400 mg. of phosphorus pentasulfide in 4 ml. of pyridine heated under reflux for 2 hours. Two ml. of water was slowly added to the reaction mixture, which was then pipetted onto a watch glass and evaporated at 100\textdegree C. The dry material was taken up with 7 ml. of boiling pyridine. The pyridine solution was centrifuged, the insoluble matter discarded, and the supernatant evaporated at 100\textdegree C. The final purification of the 9-butyl-6-thioguanine-8-C\textsubscript{14} was accomplished by recrystallization from the solvent pair dimethylformamide-water. The final yield of pure 9-butyl-6-thioguanine-8-C\textsubscript{14} was 57 mg., with a specific activity of 0.5 \mu C/\mu mole.\textsuperscript{4}

The 9-butyl-6-thioguanine-8-C\textsubscript{14} (9-BTG-8-C\textsubscript{14}) was dissolved in 2 equivalents of NaOH and made up to volume with isotonic saline as needed for injection.

\textit{Preparation of tissues for distribution studies.---} Female Swiss mice,\textsuperscript{5} 22–30 gm., were each given an inoculation of 1 \texttimes{} 10\textsuperscript{6} Ehrlich ascites cells and used 5–6 days later for tissue distribution studies. At designated times after single injections of radioactive drug at 2 mg/kg to 4 mg/kg, the mice were sacrificed by cervical dislocation. In each case the chest was opened and the vena cava cut. Blood was pipetted from the thoracic cavity into a centrifuge tube, and an aliquot of whole blood was plated immediately for the radioactivity measurement. Care was taken not to cut the diaphragm to prevent contamination of the blood with ascitic fluid. The Ehrlich ascites cells and ascitic fluid were removed with Pasteur pipettes after laparotomy, centrifuged, and the ascitic fluid was separated from the cells. The cells were extracted with 7 volumes of cold 5 per cent trichloroacetic acid (TCA) and washed once with cold TCA. The various solid tissues were removed as rapidly as possible, placed on cracked ice, weighed, and homogenized in 7 volumes of cold 5 per cent TCA in loose Potter-Elvehjem homogenizers and extracted again with cold TCA. Bone marrow was obtained from the femurs and tibiae of each mouse by flushing with isotonic saline into graduated centrifuge tubes, and was extracted with cold 5 per cent TCA. The weight of bone marrow was estimated from the tissue left after TCA extraction. Aliquots of tissue extracts were plated for radioactivity measurements. Urine was collected on Whatman No. 3 MM filter papers placed on the bottom of circular cages. The papers were dried and were eluted with 20–25 ml. of water for radioactivity measurements. The eluates were dried in vacuo and subjected to various identification procedures which will be described later.

\textsuperscript{3} Sodium formate-C\textsuperscript{14} was obtained from Hazelton Nuclear Science Corporation.

\textsuperscript{4} The 9-butyl-6-thioguanine-8-C\textsubscript{14} was spectrochemically pure, and 99.6 per cent radiochemically pure as determined by paper chromatography.

\textsuperscript{5} Swiss mice were obtained from Simonsen Laboratories, Gilroy, California.
Tests for possible dealkylation with subsequent incorporation into nucleic acids.—Tissues from distribution studies were extracted 3 times with 7 volumes of cold 5 per cent TCA to remove the acid-soluble fraction. The tissues were then washed twice with 10 volumes of cold ethanol to remove acid. Lipides were extracted by two successive treatments with 7 volumes of ethanol-ether (3:1) at 45° C. The tissues were partially dried in vacuo and the nucleic acids extracted by heating in 10 per cent NaCl (5). Subsequently, these tissues were heated in 4 volumes of 10 per cent TCA to extract any 9-butyl-6-thioguanine-8-C\textsuperscript{14} (9-BTG-8-C\textsuperscript{14}) which might be bound to protein.

RESULTS AND DISCUSSION

BLOOD LEVELS

Female Swiss mice with 5-day Ehrlich ascites carcinoma implants were given 75-\textmu g., 50-\textmu g., or 25-\textmu g. doses of 9-BTG-8-C\textsuperscript{14} intravenously through the tail veins, and the radioactivity of the whole blood was determined at 1-, 15-, and 30-minute intervals after injection.

These results are shown in Chart 1 in which per cent of dose (9-BTG-8-C\textsuperscript{14} or radioactive equivalents) per ml. of blood is plotted against time. Each point represents the average of analyses on two mice. Within 1 minute after injection, 94-98 per cent of the drug had been cleared from the blood, assuming a total blood volume of 2 ml. The 25- and 50-\textmu g. doses apparently were cleared at about the same rate. The 25-, 50-, and 75-\textmu g. doses correspond to approximately 1.5, 3, and 4.5 times the single drug dose used in multiple-dose therapy (7). By the end of 30 minutes, at all three drug doses, the blood level had decreased to about 0.5 per cent of the initial dose.

The drug absorption from the peritoneal cavity is shown in Chart 2. In addition, this chart gives a comparison of drug levels in the blood of mice with and without pretreatment with azaserine. Each point represents the average of separate analyses on the blood of mice bearing 5-day implants of Ehrlich ascites tumor after each had received an intraperitoneal dose of 100 \textmu g. 9-BTG-8-C\textsuperscript{14}. Pretreatment with azaserine appeared to produce a delay in the absorption of 9-BTG-8-C\textsuperscript{14} from the ascitic fluid into the blood. After 15 minutes the blood levels of both series were comparable to blood levels of mice which had received intravenous drug injections (see Chart 1), whereas at 30 minutes the blood levels of mice receiving intraperitoneal injections were somewhat higher than those receiving intravenous injections—but not significantly so.

It was of interest to determine whether the 9-BTG-8-C\textsuperscript{14} was present in the blood cells or only in the plasma. Chart 3 shows the distribution of the drug in whole blood and in plasma after the intravenous injection of 75 \textmu g. of 9-BTG-8-C\textsuperscript{14}. In this case, the whole blood was plated immediately after withdrawal. The plasma was separated by centrifugation and plated immediately for analysis of radioactivity. The curves are essentially superimposable, indicating that the drug is freely diffusible in the fluids of the blood cells and plasma.
The *in vitro* recovery of 9-BTG-8-C\textsuperscript{14} in whole blood and plasma is shown in Table 1. Ninety-four per cent of the drug precipitates in TCA with the whole blood proteins after a 2-minute incubation. This may indicate that 9-BTG is transported in the blood plasma in a form bound to proteins.

**ABSORPTION AND EXCRETION**

Chart 4 shows the rate of absorption of 9-BTG-8-C\textsuperscript{14} from the peritoneal cavity and the rate of urinary excretion (9-BTG-8-C\textsuperscript{14} equivalents of radioactivity) of a 100-µg. and a 50-µg. intraperitoneal dose, respectively. The radioactivity of the peritoneal fluid had decreased to about 2 per cent of the dose at 30 minutes from about 30 per cent at 1 minute. Approximately 50 per cent of the radioactivity was found in the urine at 1 hour after injection. Studies continued for 6 hours showed that from 60 to 95 per cent of the injected radioactivity could be recovered in the urine. Thus, the drug is rapidly absorbed from the peritoneal cavity, and its metabolites are rapidly excreted in the urine.

Paper chromatography was used for preliminary identifications of the radioactive compounds excreted in the urine. A typical experiment was as follows. Urine was collected for 6 hours from a Swiss mouse which had received 125 µg. of 9-BTG-8-C\textsuperscript{14}. Radioactivity equivalent to 75 µg. of 9-BTG-8-C\textsuperscript{14} was recovered from the paper by elution with 50 ml. of water. This water eluate was taken to dryness in *vacuo*, the residue taken up in 1.0 ml. of water and applied across the origin of Whatman No. 8 MM paper, and the chromatogram was developed by the descending technic in an n-propanol-1 m ammonium hydroxide (PA) (3:1) solvent. A half-inch strip was cut from the side, and radioactivity measurements showed that there were four radioactive bands with R\textsubscript{F}'s of 0.10, 0.45, 0.50, and 0.75. (The R\textsubscript{F} of the standard 9-BTG-8-C\textsuperscript{14} in this solvent was 0.72.) The band with R\textsubscript{F} 0.75 was removed from the paper by elution with 1 m HCl, the eluate taken to dryness in *vacuo*, the residue taken up in 0.3 ml. of water, placed on Whatman No. 3 MM paper, and developed in water-saturated n-butanol (BW) by descending technic.

The radioactive band rechromatographed in BW with R\textsubscript{F} 0.75 in the PA solvent had an R\textsubscript{F} of 0.59 in the BW solvent. (9-BTG-8-C\textsuperscript{14} has an R\textsubscript{F} of 0.72. The table below shows the results of the experiments:

**Table 1**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>9-Minute incubation (per cent recovery)</th>
<th>50-Minute incubation (per cent recovery)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood</td>
<td>75</td>
<td>68</td>
</tr>
<tr>
<td>Plasma</td>
<td>69</td>
<td>72</td>
</tr>
<tr>
<td>5% TCA extract of whole blood</td>
<td>6</td>
<td>14</td>
</tr>
</tbody>
</table>
of 0.68 in this solvent.) In addition, the spot showed no ultraviolet absorption. Thus, there were at least four metabolic products of the 9-BTG-8-C$^{14}$ found in the urine, little or none of which was the original compound. Further work will be required for the identification of these metabolites.

**Tissue Distribution**

*Acid-soluble fraction.*—Charts 5 and 6 compare the drug concentrations in the acid-soluble fractions of several tissues after the intraperitoneal and intravenous injection of 9-BTG-8-C$^{14}$, respectively, with and without pre-treatment with azaserine. Each point represents the average of analyses on two tumor-bearing mice. After intraperitoneal injection, bone marrow and tumor cells show the highest concentration of the drug at 1 minute. After 15 and 30 minutes liver, kidney, and bone marrow showed the highest concentration of acid-soluble radioactivity. Also, at 30 minutes the

**Chart 5.**—Concentration of radioactivity in mouse tissues after the intraperitoneal injection of 2 mg/kg or 4 mg/kg of 9-BTG-8-C$^{14}$.

Radioactivity was measured on 5 per cent TCA extracts of tissues. Values are averages of analyses on two mice.

**Chart 6.**—Concentration of radioactivity in mouse tissues after the intravenous injection of 4 mg/kg of 9-BTG-8-C$^{14}$.

Values are averages of determinations on two mice.
Drug concentration had increased in the liver, kidneys, small intestine, and spleen. In general, after intravenous drug injection, the drug concentrations in the tissues decreased. This may merely mean that, after intraperitoneal drug injection, availability of the drug to tissues is limited by absorption from the peritoneal cavity into the bloodstream. This is given credence by the generally reversed order of drug concentration in the tissues, and particularly the kidney, after intravenous injection. The higher concentrations in the kidney may be correlated with the rapid urinary excretion of 9-BTG-8-C14 metabolites.

### Table 2
**Survival Time of Mice Given 9-Butyl Thioguanine Intravenously**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. mice per group</th>
<th>Survival time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline control</td>
<td>10</td>
<td>15.8 ± 2.0</td>
</tr>
<tr>
<td>Azaserine</td>
<td>10</td>
<td>24.3 ± 5.1 (1)</td>
</tr>
<tr>
<td>Azaserine+9-BTG</td>
<td>10</td>
<td>39.3 ± 10.7 (5)</td>
</tr>
</tbody>
</table>

Female Swiss mice were each given intraperitoneal transplants of 1 X 10⁶ Ehrlich ascites cells. Treatment, which was started 1 day later, was twice daily (8 hours apart) for twelve consecutive treatments.

Figures given are average survivals and average mean deviations. The figures in parentheses indicate the number of mice surviving 50 days. Fifty-day survivors are computed as surviving only 50 days.

Dosages were: azaserine, 0.2 mg/kg, intraperitoneally; 9-butyl thioguanine (9-BTG), 0.65 mg/kg, intravenously.

The most striking feature to be noted in Charts 5 and 6 is the greater retention of the drug in most of the tissues of mice pretreated with azaserine. The notable exceptions are the lung at 1 minute after intravenous injection and the liver at 15 minutes after intraperitoneal injection.

The radioactivity of the acid-soluble fraction was negligible in mouse muscle, heart, and brain at the time tested, with or without pretreatment with azaserine. Also, the drug concentration in the ascites cells after intravenous injection appeared to be negligible. The question then arose whether the drug would be effective against the Ehrlich ascites tumor when given intravenously. Table 2 shows the results of intravenous therapy on mice bearing the Ehrlich ascites tumor. It is evident that the intravenous injections produced a response comparable to that obtained with intraperitoneal treatments (7, 8), in spite of the relatively low concentrations of drug reaching the tumor cells.

Chart 7 illustrates the paper chromatography of acid-soluble extracts of Ehrlich ascites cells of mice given a single large dose of 9-BTG-8-C14 (4 mg/kg), with (I) and without (II) azaserine pretreatment. A chromatogram of the extracts from two livers (2.67 gm.) taken 15 minutes after 8-mg/kg doses from two mice is also included. Chromatography on Dowex 50 columns, 10 X 30 mm., was used to purify the acid extracts (4) before paper chromatography. The two peaks at 1 and 14 cm. of the open trace of section I absorbed in the ultraviolet at 350 mμ after elution with 0.1 N HCl. The major peak at 14 cm. corresponds to the peak at 13 cm. of the standard 9-BTG-8-C14 (closed trace) of section I, as does the single peak of sec-
tion II at 14 cm. Comparison of sections I and II would indicate that 9-BTG-8-C\textsuperscript{14} is metabolized differently in the Ehrlich ascites cells of mice pretreated with azaserine as regards the minor component at 1 cm. Neither of the two main peaks in section III (liver extract) has the same R\textsubscript{f} as the standard 9-BTG-8-C\textsuperscript{14}. This is not unexpected, since it may be recalled that at least four metabolites were found in the urine, little or none of which was 9-BTG-8-C\textsuperscript{14}.

Alcohol-ether soluble fraction.—Radioactivity was found in the lipide fraction. Chart 8 shows the amount of drug found in the alcohol-ether-soluble fractions and acid-soluble fractions of the small intestine and Ehrlich ascites cells at various times after an intraperitoneal dose of 93 \mu g. The radioactivity in the alcohol-ether-soluble fraction in the ascites cells at 1 minute was about 30 per cent of that in the acid-soluble fraction for mice pretreated with azaserine. The concentration of the drug in the alcohol-ether-soluble fraction of the ascites cells was approximately the same at 15 and 30 minutes. Measurements on the lipide fractions of the small intestine showed that the amount of radioactivity was essentially constant throughout the time studied.

It is not surprising that this drug is found in the alcohol-ether-soluble fraction and is presumptively dissolved among the cellular lipides, since the butyl moiety of the molecule would tend to make it more lipide-soluble.

Nucleic acid fraction.—As a further test of a possible dealkylation of the drug, two Swiss mice bearing 5-day implants of Ehrlich ascites cells were each given an intraperitoneal dose of 116 \mu g. of 9-BTG-8-C\textsuperscript{14}. After 40 minutes, the mice were sacrificed, 1.5 ml. of cells harvested, and the cells fractionated as described earlier. No radioactivity was found in the nucleic acid fraction. Also, the nucleic acids extracted from the small intestine of mice from the time study described immediately above showed no radioactivity present. This corroborates the previous findings in this laboratory that 9-BTG is not incorporated into nucleic acids (7).

TOXICITY

9-Butyl-6-thioguanine, like thioguanine (1, 9), appears to exert a toxic effect primarily on the bone marrow. Female Swiss mice given 8 mg/kg twice daily for 7 days developed a marked granulocytopenia. The marrow spaces of the long bones and the sternum were found to be hemorrhagic at autopsy. Bone marrow smears revealed that little of the blood-forming elements remained. Just prior to death the principal white blood cell component present in the peripheral blood was the lymphocyte.

Evidence is available to indicate that bone marrow cells are dependent on preformed purines (3) from the liver. The toxicity of this drug to the bone marrow suggests an interference with preformed purine utilization.

REFERENCES


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