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Effect of O^6-benzylguanine on the response to 1,3-bis(2-chloroethyl)-1-nitrosourea in the Dunning R3327G model of prostatic cancer

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Abstract. The DNA-repair protein O^6-alkylguanine-DNA alkyltransferase is known to protect tumor cells from the antitumor effects of carmustine (BCNU). This repair protein was inactivated in Copenhagen rat prostate tumors by treatment with O^6-benzylguanine in attempts to increase the effectiveness of BCNU therapy. The alkyltransferase activity in the liver, kidney, lung, and prostate of Copenhagen rats was 66, 37, 65, and 122 fmol/mg protein, respectively. The activity in the Dunning R3327G rat prostate tumor was found to be 129 and 126 fmol/mg protein from intact and castrated animals, respectively. The level of this protein remained low in the tissues and tumors of rats for up to 24 h and slowly began to rise at 36 h following an i.p. injection of 80 mg/kg O^6-benzylguanine. Animal survival and body weight as well as tumor volumes were monitored in rats bearing prostate tumors in the flank area that had received no treatment, O^6-benzylguanine alone, BCNU alone (5.5–60 mg/kg), or 80 mg/kg O^6-benzylguanine 1 h prior to BCNU (5.5 mg/kg). When O^6-benzylguanine was combined with BCNU therapy, there was a regression in tumor growth that was not observed in animals treated with an equal dose of BCNU alone. A similar regression in tumor growth was observed in animals treated with a higher dose of BCNU alone (45 mg/kg); however, this regimen was more toxic than O^6-benzylguanine plus BCNU (5.5 mg/kg) as determined by animal weight loss. The mean weight loss observed in animals treated with BCNU alone and in those given the combination was 24% and 6%, respectively. Histopathology revealed that animals receiving either BCNU alone or the combination had a decrease in all types of bone marrow cells, a loss of intestinal crypts, and a decreased number of lymphocytes in the spleen. The enhancement of the antitumor effect on BCNU by pretreatment with O^6-benzylguanine supports a role for this therapy in the treatment of prostate cancer.

Abbreviations: AGT, O^6-alkylguanine-DNA alkyltransferase; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea

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Introduction

Efforts to utilize conventional chemotherapy in the effective treatment of human prostatic carcinoma has achieved limited success. For example, regimens that include single agents or various combinations produce objective regressions in as many as 20%–50% of patients [5, 14, 16, 18, 27]. However, even with potent combinations such as Adriamycin, cyclophosphamide, and 5-fluorouracil, the median survival is only 40 weeks [16, 18]. Treatment with nitrosoureas, particularly lomustine (CCNU), has been found to result in a response rate (partial response plus stable disease) of 33%, producing no increase in median survival over that reported for other single-agent regimens or combinations [14, 27]. The ineffectiveness of CCNU and other chloroethylnitrosoureas in destroying prostate tumor cells may be due to repair of DNA lesions prior to cell division. Chloroethylnitrosoureas exert their cytotoxic effect through the formation of interstrand DNA cross-links [20]. These cross-links are formed after initial attack at the O^6-position of guanine to form O^6-chloroethylguanine. A chemical rearrangement occurs to form, 1, O^6-ethanoguanine, and a reaction with cytosine finally takes place on the opposite strand of DNA to form the lethal cross-link [12, 19, 29]. A DNA-repair protein, O^6-alkylguanine-DNA alkyltransferase (AGT), present in most cells, is known to protect them from the toxic effect of chloroethylnitrosoureas by removing lesions at the O^6-position of guanine prior to cross-link formation. The absence or inactivation of this repair protein correlates with an increase in the number of DNA cross-links and sister chromatid exchanges and an increase in the toxic effect of the nitrosoureas [2, 22, 23, 25, 26, 28]. The level of this protein varies with cell type, tissue, and species [22, 23].

The mechanism of protection by the AGT protein involves transfer of the chloroethyl group to the active site of the protein or reaction of the repair protein with the intermediate, O^6-ethanoguanine, to produce a DNA-protein cross-link [3, 22, 23]. Reversal of resistance to nitrosoureas has been achieved in human tumor cells in culture by exposure of cells to low-molecular-weight substrates such

as O^6-benzylguanine [6]. Complete depletion of AGT activity in cells and in cell-free extracts has been observed after their exposure to micromolar concentrations of O^6-benzylguanine for minutes [6]. Evidence suggests that there is a transfer of the benzyl group to the active site of the AGT protein, which in turn inactivates the protein [8]. Antibodies directed against the protein were used to demonstrate a rapid degradation of the latter after exposure to O^6-benzylguanine [24]. Tumor cell lines including brain tumor, melanoma, and colon carcinoma lines have been sensitized to the cytotoxic effects of BCNU and other chloroethylnitrosoureas by initial exposure to O^6-benzylguanine [9]. Recently, we reported an improved antitumor response in mice bearing SF767 brain tumor xenografts after treatment with O^6-benzylguanine and BCNU as compared with an equitoxic dose of BCNU alone [21].

The objective of the present work was to compare the antitumor response of poorly differentiated Dunning R3327G rat prostatic carcinoma to treatment with the combination of O^6-benzylguanine and BCNU with the response to treatment with BCNU alone. The toxicity of the combination was compared with that of BCNU alone by histopathological evaluation of several rat tissues. The effect of O^6-benzylguanine on AGT activity was determined in the rat prostate tumor, liver, and kidney before and after treatment with O^6-benzylguanine.

Materials and methods

Animals. Adult male Copenhagen rats were obtained from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, Md.). The animals were housed in an environmentally controlled room (12 h light/12 h darkness) and were provided with food and water ad libitum. All animal procedures followed guidelines according to the *Guide for the Care and Use of Laboratory Animals* prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences. The animals were restricted to medicated water throughout the experimental period [13].

Tumor model. The Dunning R3327G tumor was originally obtained in 1985 through the generosity of Dr. Norman Block (University of Miami School of Medicine, Miami, Fla.) and has been maintained in serial passage since that time. Morphologically, the Dunning R3327G tumor is poorly differentiated, consisting of sheets of tumor cells with intervening stromal tissue [15]. Historically and in the experiments described, established Dunning R3327G tumors in intact males exhibit a tumor volume-doubling time of approximately 4 days.

Tumor-inoculation protocol. The tumors selected for inoculation into recipient animals were approximately 1.5 cm in volume. Cells for inoculation were prepared as described previously [11]. Each experimental animal was inoculated s. c. in the flank region with 9×10^6 cells.

Experimental treatment. Adult male Copenhagen rats were inoculated in the flank region with Dunning R3327G tumor cells and left undisturbed until the tumor appeared and grew to a volume of approximately 100 mm^3. At that time, the tumor-bearing animals were randomized into one of the following groups and immediately received the indicated treatment: (1) no treatment, (2) O^6-benzylguanine alone (80 mg/kg), (3) BCNU alone (5.5–60 mg/kg), and (4) O^6-benzylguanine (80 mg/kg) plus BCNU (5.5 mg/kg). All treatment groups consisted of eight animals. The O^6-benzylguanine solution was made up in 10% cremophor/saline by dissolving the drug in cremophor, then slowly adding saline [21]. The BCNU solutions were made up in ethanol, then diluted in water just before their injection into the animals such that the final solution contained 10% ethanol. Tumor volume measurements commenced at the time of treatment and continued twice weekly until the death of the animals.

Evaluation of tumor growth. Three dimensions of each tumor were serially measured using orbital calipers. From these measurements, tumor volumes were calculated using the formula $(l \times w \times h) \times 0.526$ [17]. The mean absolute tumor volume $\pm$S.D. was calculated for each experimental group weekly. The significance of differences observed among the treatment groups was determined by analysis of variance using the Student-Newman-Keuls procedure to allow for multiple comparisons.

Animal survival. The animals that survived the drug treatment were not allowed to die spontaneously but were euthanized using approved methods when the tumors had grown to such a size that the overlying skin had become ulcerated. Skin ulceration and the requisite removal of the animal was to occur when the tumors had reached a volume of between 50,000 and 70,000 mm^3, although in almost every instance the experiment ended prior to this point.

Alkyltransferase activity. Alkyltransferase activity was measured as the removal of O^6-[^{3}H]-methylguanine from a [^{3}H]-methylated DNA substrate that had been prepared by reacting [^{3}H]-methylnitrosourea with calf-thymus DNA. The extracts were incubated with [^{3}H]-methylated DNA at 37° C for 30 min. The DNA was precipitated by adding ice-cold perchloric acid at a final concentration of 0.25 N and was hydrolyzed in 0.1 *N* HCl at 70° C for 30 min [10]. The modified bases were separated by reverse-phase high-performance liquid chromatography (HPLC) [6]. Protein was quantified by the method of Bradford [1], and the results were expressed as femtomoles of O^6-methylguanine released from the DNA per milligram protein.

Results

The AGT activity was determined in the prostate, liver, lung, kidney, and tumor of Copenhagen rats bearing the Dunning G tumor (Table 1). The rat prostate tissue contained 122 fmol AGT/mg protein, which was higher than the amount found in the liver (66 fmol/mg protein), kidney (37 fmol/mg protein), or lung (65 fmol/mg protein). Rat prostate tumor obtained from both normal and castrated rats had activity similar to that found in the rat prostate tissue.

The regeneration rate of the protein in the liver, kidney, and tumor is shown in Fig. 1. Rats were injected i.p. with

Table 1. AGT activity of Copenhagen rat tissues and Dunning prostate tumor

Tissue	AGT (fmol/mg protein)
Prostate	122 ± 48
Liver	66 ± 11
Kidney	37 ± 20
Lung	65 ± 18
Tumor (intact)	129 ± 55
Tumor (castrate)	126 ± 3

Copenhagen rats carrying the Dunning rat prostatic tumor were euthanized, and tissues and tumors were immediately excised and frozen at −80° C until the alkyltransferase assay was performed. Data represent the mean values (± SD) for tissue samples run in duplicate from at least 4 separate animals

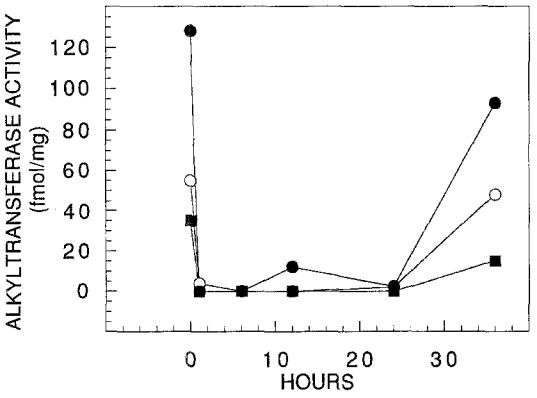

Fig. 1. Depletion of alkyltransferase activity in rat tissues and tumor by administration of O^6-benzylguanine. Copenhagen rats were treated i.p. with 80 mg/kg O^6-benzylguanine in 10% cremophor/saline. Rats were euthanized at the time shown, and the alkyltransferase activity was determined in the liver (*open circles*), tumor (*filled circles*), and kidney (*filled squares*). The mean values for duplicate determinations on 2–4 mice are shown

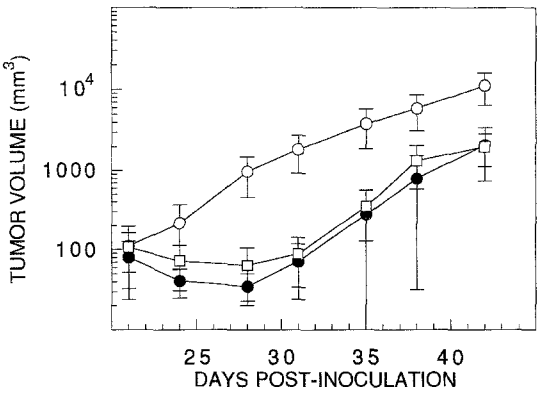

Fig. 3. Response of the Dunning rat prostate tumor carried in Copenhagen rats to O^6-benzylguanine and/or BCNU. Rats were given no treatment (*open circles*), 45 mg/kg BCNU alone (*open squares*), or the combination of 80 mg/kg O^6-benzylguanine followed 1 h later by 5.5 mg/kg BCNU (*filled circles*). The mean tumor size was determined for 8 animals/treatment group (except for BCNU alone at 45 mg/kg; $n = 4$). Error bars, ±SD

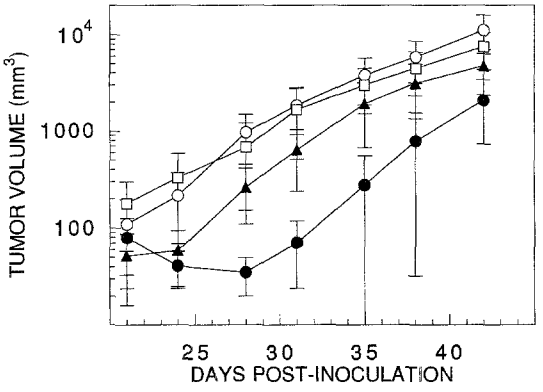

Fig. 2. Response of the Dunning rat prostate tumor carried in Copenhagen rats to O^6-benzylguanine and/or BCNU. Rats were given no treatment (*open circles*), 80 mg/kg O^6-benzylguanine alone (*filled triangles*), 5.5 mg/kg BCNU alone (*open squares*), or the combination of 80 mg/kg O^6-benzylguanine followed 1 h later by 5.5 mg/kg BCNU (*filled circles*). The mean tumor size was determined for 8 animals/treatment group. Error bars, ±SD

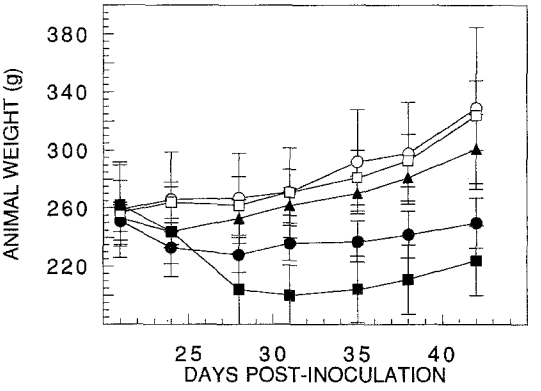

Fig. 4. Animal weight of Copenhagen rats after treatment with O^6-benzylguanine and/or BCNU. Rats were given no treatment (*open circles*), 80 mg/kg O^6-benzylguanine alone (*filled triangles*), 5.5 mg/kg BCNU alone (*open squares*), 45 mg/kg BCNU alone (*filled squares*), or the combination of 80 mg/kg O^6-benzylguanine followed 1 h later by 5.5 mg/kg BCNU (*filled circles*). The mean animal weight was determined for 8 animals/treatment group. Error bars, ±SD

80 mg/kg O^6-benzylguanine and were euthanized at various times up to 36 h. Activity in the liver, tumor, and kidney was negligible at 1 h following the injection and remained undetectable for 24 h. At 36 h, the activity in the tumor and tissues rose; however, levels remained below basal activity.

The effect of O^6-benzylguanine on the sensitivity of a Dunning G rat prostate tumor to BCNU is shown in Fig. 2. Animals were treated with BCNU alone (5.5 mg/kg) or with the combination of O^6-benzylguanine (80 mg/kg) followed 1 h later by BCNU (5.5 g/kg) on day 21 postimplantation. For the first 10 days, a regression of tumor growth was observed in animals treated with the combination of O^6-benzylguanine plus BCNU. Tumors in animals treated with the same dose of BCNU alone grew at the same rate as those in control animals. The mean tumor size in animals treated with the combination was significantly lower than that in control animals ($P = 0.01$) or in animals treated with BCNU alone ($P = 0.05$) as determined on days 28, 31, 35, and 38.

Although O^6-benzylguanine enhances the antitumor effect of BCNU at equal single-agent doses, higher doses of BCNU can be given without resulting in animal death. Toxic death occurs when animals are treated with 80 mg/kg O^6-benzylguanine plus 8 mg/kg BCNU or with 60 mg/kg BCNU alone (data not shown). Administration of BCNU alone at 45 mg/kg resulted in a tumor-growth curve similar to that obtained with the combination of O^6-benzylguanine and BCNU (5.5 mg/kg), with a temporary regression of tumor growth occurring after the administration of therapy (Fig. 3). The mean animal weights recorded for these two groups are shown in Fig. 4. At 10 days following treatment, animals receiving BCNU (45 mg/kg) alone had a weight loss of 24% as compared with the 6% weight loss in those receiving the combination of O^6-benzylguanine and BCNU (5.5 mg/kg; Fig. 4).

Table 2. Toxicity associated with BCNU alone and with O^6-benzylguanine plus BCNU

Tissue	Histopathology[a]	
	BCNU (60 mg/kg)	O^6-benzylguanine + BCNU (5.5 mg/kg)
Liver	Normal	Normal
Lung	Normal	Normal[b]
Kidney	Normal	Normal
Pancreas	Normal	Normal
Small intestine	Mild villous atrophy	Marked villous atrophy
Large intestine	Loss of crypts, increase in connective tissue	Loss of crypts, increase in connective tissue
Bone marrow	Hypocellular	Hypocellular
Spleen	Decrease in lymphocytes	Decrease in lymphocytes
Peripheral white cells[c]	$940 \pm 331/mm^3$	Not determined

[a] Animals were given either 60 mg/kg BCNU or 80 mg/kg O^6-benzylguanine 1 h prior to 5.5 mg/kg BCNU. Animals were euthanized and tissues were examined by a certified veterinary pathologist

[b] The lungs were unremarkable in 5/7 animals; however, 2 showed a mild focal accumulation of necrosis and segmented leukocytes within a bronchiole

[c] Normal value, $10{,}000/mm^3$

Table 2 lists the organ toxicity observed in Copenhagen rats in association with BCNU treatment and with the combination of O^6-benzylguanine and BCNU. It is unlikely that a significant *Streptococcus* infection was present in these animals, as lesions in the lung were not detected in either group. There was, however, extensive damage to the intestines, with a decrease in the number of periarteriolar lymphocytes being noted in the spleen and hypocellularity being observed in the bone marrow for both groups. On histopathological examination, all other organs (esophagus, stomach, pancreas, liver, brain, lung, kidney, bladder, adrenal, thryroid, pituitary, heart, and lymph nodes) were unremarkable after treatment with the combination (data not shown).

Discussion

The content of mammalian AGT is known to vary in different organs, with the liver and spleen being reported to have the highest activity and the nervous system being found to have the lowest [22]. We now report that the activity in the rat prostate is twice that in the liver. The Dunning rat prostate G tumor has equally high levels of AGT activity. To our knowledge, there is currently no report to indicate the level of this repair protein in human prostate tissue or tumor, although high levels in tumors may explain the ineffectiveness of nitrosourea therapy. This report illustrates that a reduction in the AGT activity in rat tissues and tumors can be achieved by treatment with 80 mg/kg O^6-benzylguanine. Similar to the results obtained for mouse tissues and human tumor xenografts, this AGT activity remains low for up to 24 h and begins to rise at 36 h [7]. Cross-links are thought to occur within 8–12 h of treatment and it is necessary that levels remain low prior to cross-link formation for nitrosourea enhancement [2, 12, 19, 25, 29].

The prostate tumor has extremely high levels of alkyltransferase activity relative to other tissues in the rat, particularly bone marrow [22, 23]. This is an unfavorable model, as it would require more O^6-benzylguanine to deplete the activity in the tumor than in other tissues. Our results indicate a greater extent of AGT recovery from the tumor than from the liver or kidney, two other organs with relatively high levels of AGT activity. An alternative dose schedule might include the administration of O^6-benzylguanine and a lower dose of BCNU on day 32. Cycling the combination using O^6-benzylguanine and the same dose of BCNU (5.5 mg/kg) resulted in animal death (data not shown).

There was a delay in the growth of Dunning rat prostate tumors in animals treated with O^6-benzylguanine prior to BCNU as compared with those given an equivalent dose of BCNU alone. However, increasing the BCNU dose to 45 mg/kg resulted in an equivalent inhibition of tumor growth relative to that observed in control animals. These were not quite equitoxic doses, as the toxicity measured by animal weight loss was greater for animals treated with BCNU alone (approx. 24%) as compared with those given the combination (approx. 6%). Furthermore, it is possible that deleterious effects on the host such as prolonged weight loss (>20%) can secondarily affect tumor growth.

The enhancement of BCNU toxicity when the nitrosourea was given after O^6-benzylguanine required an 8-fold reduction in the dose of BCNU. Previously, we reported that such enhancement required a 2.5- to 4-fold reduction in the BCNU dose given to mice in the same sequence of administration. [21]. The toxicity studies reported herein indicate that the organs affected by BCNU and the combination of O^6-benzylguanine plus BCNU are the intestine, spleen, and bone marrow. On histopathological examination, other tissues were unremarkable in animals treated with the combination or with BCNU alone. The difference between the BCNU dose reduction carried out in rats versus mice may reflect a difference in organ toxicity.

The dose-limiting toxicity in humans treated with BCNU alone is bone marrow suppression, with severe pulmonary toxicity being observed in some patients [4]. One approach to circumvent the toxicity associated with BCNU is through bone marrow transplantation or administration of hematopoietic growth factors. Another means of protecting bone marrow may be through the stable integration of the *ada* gene (*Escherichia coli*) into a suitable vector and expression of the bacterial alkyltransferase in bone marrow cells. We have previously shown a lack of inhibition of the Ada alkyltransferase protein by O^6-benzylguanine [8]. The achievement of high levels of the bacterial AGT in bone marrow cells would serve as a means to protect this tissue from the deleterious effects of BCNU.

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