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Biodistribution of O^6-benzylguanine and its effectiveness against human brain tumor xenografts when given in polyethylene glycol or cremophor-EL

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Abstract O^6-Benzylguanine effectively inactivates the DNA-repair protein O^6-alkylguanine-DNA alkyltransferase in tumor cells and has been shown to increase the cytotoxicity of chloroethylnitrosoureas. This study was undertaken to ascertain the optimal vehicle for further toxicological evaluation and eventual clinical trials of O^6-benzylguanine. The solubility, metabolism, bioavailability and effectiveness of O^6-benzylguanine as an adjuvant therapy with BCNU were compared using two vehicles, cremophor-EL and PEG 400. Nude mice bearing s.c. D456 MG glioblastoma xenografts were injected i.p. with 10–30 mg/kg O^6-benzylguanine dissolved in either 40% PEG 400/saline or 10% cremophor-EL/saline. The number of tumor regressions noted after treatment with 10 mg/kg O^6-benzylguanine followed by 12.7 mg/kg BCNU were 8/9 for the drug dissolved in PEG and 1/10 for the drug given in cremophor-EL. Using the same treatment regimen but increasing the dose of O^6-benzylguanine to 30 mg/kg led to a growth delay of 45.2 and 11.5 days for the drug dissolved in PEG 400 and cremophor-EL, respectively, although the number of regressions observed were the same for both treatments. 8-[^{3}H]-O^6-Benzylguanine was more rapidly distributed to the tumor when it was delivered in PEG vehicle than when it was given in cremophor-EL. In contrast, there was a 3-fold greater amount of O^6-benzylguanine in the small intestine of mice at 1 h after i.p. injection of the drug in cremophor-EL as compared with PEG 400. The rate and extent of metabolism in the liver was the same, whether the parent drug was given in PEG 400 or in cremophor-EL. These studies demonstrate that O^6-benzylguanine is a more effective enhancer of the antitumor activity of BCNU when it is given in PEG 400 than when it is delivered in cremophor-EL, which may be due to a more rapid distribution of the drug to the tumor.

Key words O^6-Benzylguanine · Human brain-tumor xenografts · Polyethylene glycol · Cremophor EL

Abbreviations BCNU 1,3-bis(2-chloroethyl)-1-nitrosourea · *CCNU* 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea · *meCCNU* 1-(2-chloroethyl)-3-methylcyclohexyl-1-nitrosourea · *PEG* polyethylene glycol · *AGT* O^6-alkylguanine-DNA alkyltransferase

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Introduction

Chemotherapeutic alkylnitrosoureas, including BCNU, meCCNU, CCNU, and streptozotocin, have limited usefulness against neoplasms such as brain tumors, pancreatic tumors, melanomas and lymphomas [25]. The therapeutic effectiveness of alkylnitrosoureas is dependent on the extent to which alkylation remains on key DNA sites prior to cell division. One of the most critical DNA sites is the O^6 position of guanine. Alkylation damage at this site is repaired by the AGT protein, which thereby limits the clinical usefulness of the alkylnitrosoureas [27, 28]. Since the vast majority of primary human tumors tested exhibit

AGT activity [6, 27, 28], the inactivation of this repair protein in efforts to increase the therapeutic index of nitrosoureas has received much attention.

We have demonstrated that O^6-benzylguanine is an extremely effective agent for inactivating the AGT protein in cells and cell-free extracts [7]. Treatment of human glioma, melanoma, and colon tumor cells in culture with micromolar concentrations of O^6-benzylguanine results in a complete loss of AGT activity and a dramatic increase in the cytotoxicity produced by the chemotherapeutic chloroethylating agents BCNU, CCNU, chlorozotocin or clomesone and the methylating agents streptozotocin or MTIC [7, 9]. Furthermore, treatment of nude mice carrying human brain- or colon-tumor xenografts with O^6-benzylguanine prior to BCNU has led to significant inhibition of tumor growth as compared with that seen in animals treated with BCNU alone [8, 10, 11, 15, 17, 19, 26].

In the preparation of O^6-benzylguanine for human clinical trials, a vehicle must be chosen that does not produce deleterious biological effects on its own. Cremophor-EL has been used in almost all our previous animal studies and is used as a vehicle in commercial formulations of taxol and teniposide. Unfortunately, significant vehicle-associated hypersensitivity reactions were reported during early clinical trials with cremophor-EL formulations of taxol [21, 31]. These cremophor-EL reactions were also seen in animal models and have been well documented [18, 20, 24]. We felt that such vehicle-related reactions should be avoided during animal toxicological evaluation and eventual clinical trials. No such problem has thus far been reported for PEG 400, which has been studied extensively in animals and has been shown to be practically non-toxic [3]. It has been given to humans orally [5] and is present in commercial drug formulations such as lorazepam for injection. To evaluate PEG 400 as a vehicle for O^6-benzylguanine, we compared the metabolism, biodistribution, and efficacy of O^6-benzylguanine in combination with BCNU when O^6-benzylguanine was dissolved in PEG 400 or cremophor-EL.

Materials and methods

Drugs

O^6-Benzylguanine was synthesized as described elsewhere [7] and was given as a single i.p. injection in 10% cremophor-EL or 40% PEG 400 in normal saline. 8-[^{3}H]-O^6-Benzylguanine was prepared by Amersham (Arlington Heights, Ill) by catalytic exchange through tritium exchange using the TR8 procedure (specific activity, 0.34 Ci/mmol). Cremophor-EL was obtained from Sigma Chemical Co. and PEG 400 was obtained from Eastman Kodak (Rochester, N.Y). BCNU, provided by Bristol-Myers Squibb (Wallingford, Conn.), was given as a single i.p. injection in 3% ethanol at a dose of 12.7 mg/kg, which corresponds to 0.38 of the dose lethal to 10% of treated animals (LD_{10}).

Animals

Male or female athymic BALB/c mice (nu/nu genotype, 6 weeks or older) were used for all studies and were maintained as described previously [4].

Evaluation of tumor

D456 MG, a xenograft derived from a frontal-lobe glioblastoma multiforme in an 8-year-old girl, was used in all studies. Subcutaneous xenograft transplantation into the right hind limb was performed as previously described with inoculation volumes of 30 µl [16]. Tumors were measured every 3–4 days with vernier calipers (Scientific Products, McGraw, Ill). The tumor volume was calculated according to the following formula: length × width^2 × 0.52.

Treatment regimens

For assessment of toxicity, animals were treated with 40% PEG 400 in normal saline containing O^6-benzylguanine (final dose: 0, 10, 20, 30, 40, 60, and 80 mg/kg) via i.p. injection in a volume of either 15 or 30 ml/kg and then treated with BCNU (12.7 mg/kg) or vehicle (3% ethanol) 1 h later. For assessment of tumor response, animals were injected with D456 MG tumor homogenates, and when the median tumor volume exceeded 200 mm^3, groups of 8–10 randomly selected mice were treated with one of the following regimens: BCNU, BCNU plus O^6-benzylguanine (10 or 30 mg/kg) in either 10% cremophor-EL/90% normal saline or 40% PEG 400/60% normal saline, or vehicle alone. For combination studies, BCNU (12.7 mg/kg) was given 1 h after O^6-benzylguanine. Animals were monitored for morbidity and mortality. For assessment of O^6-benzylguanine metabolism and biodistribution, animals were injected i.p. with 8-[^{3}H]-O^6-benzylguanine (10 mg/kg; 3.4 µCi/20-g mouse) in either 10% cremophor-EL/90% normal saline or 40% PEG 400/60% normal saline. Urine was collected at 16, 27, 39, 49, 60, 84, 108 and 120 h from animals housed in metabolic cages and the specimens were immediately frozen. A separate set of animals was euthanized at 1, 6, 12, and 18 h following O^6-benzylguanine treatment. Tissues were excised and immediately frozen in liquid nitrogen.

Assessment of response

The response of xenografts was assessed by delay in tumor growth and by tumor regression. Growth delay, expressed as T-C, is defined as the difference in days between the median time required for the tumors of treated (T) versus control (C) animals to reach a volume 5 times that measured at the time of original treatment. Tumor regression is defined as a decrease in tumor volume over two successive measurements. Statistical analysis was performed using the Wilcoxon rank-order test for growth delay and Fisher's exact test for tumor regressions as described previously [16].

Biodistribution studies

The tumor, liver, spleen, kidney, lung, brain, small intestine, and esophagus were removed from the animals at 1, 6, 12, and 18 h. Blood was obtained at simultaneous time points via cardiac puncture and was anticoagulated with heparin. Plasma was separated by centrifugation and frozen immediately. Tissue extracts were prepared by adding 2 vols. 50 m*M* TRIS (pH 7.5), 0.1 m*M* ethylenediaminetetraacetic acid (EDTA), and 5 m*M* dithiothreitol (DTT) buffer/g tissue. Tissues were homogenized for 30 s, sonicated for 1 min, and centrifuged at 15,000 *g* for 20 min and the radioactivity was measured on a scintillation counter by adding 100 µl extract to 10 ml Ultima Gold scintillant (Radiomatic). Liver homogenates were further processed for high-performance liquid chromatographic (HPLC) analysis by the addition of acetonitrile (2 times the volume of homogenate) to ice-cold samples prior to vortexing and centrifugation at 31,000 *g* for 30 min at 4° C. The supernatant was removed, evaporated, and reconstituted in mobile phase for HPLC analysis.

HPLC analysis

Methanol was added to urine to a final concentration of 35%. Samples were kept ice-cold for 1 h prior to filtering and were injected directly onto an Ultrasphere C_{18} (4.6 mm × 25 cm) reverse phase column (Beckman Instruments, Inc., San Ramon, Calif.). The mobile phase consisted of 35% methanol/0.05 *M* ammonium formate (pH 4.5) with a flow rate of 1 ml/min at room temperature. Radioactivity was monitored by scintillation counting of fractions or by use of a radioactivity flow detector (Radiomatic Instruments, Tampa, Fla.) with Flo-Scint II (Packard, Downers Grove, Ill.). Absorbance was monitored at 280 nm and a full UV spectrum was monitored at peaks of interest using a diode-array detector (Hitachi Instruments, San Jose, Calif.).

Results

O^6-Benzylguanine is only sparingly soluble in water (solubility limit at 0.1 mg/ml), but its solubility can be substantially improved using a mixed solvent. We have determined that a concentration of O^6-benzylguanine in the range of 1–5 mg/ml is necessary for both preclinical and anticipated clinical studies. Thus, its solubility in a number of vehicles suitable for human use was examined (Table 1). The two vehicles chosen for the clinical dosing formulation of O^6-benzylguanine included a mixed solvent containing either PEG 400 or cremophor-EL. Much of the previous preclinical analysis had been done with 10% cremophor-EL/saline [8, 10, 11, 15, 17, 19, 26]. Two groups of animals were injected with O^6-benzylguanine (30 mg/kg) in either cremophor or PEG 400 and were euthanized at 15 min following drug injection. There was no evidence in either cohort of animals of precipitation of O^6-benzylguanine in the peritoneal cavity.

Treatment of animals i.p. with 40% PEG 400/60% normal saline alone at a volume of 30 ml/kg was toxic but not lethal, with 3 of 4 mice displaying marked somnolence for 4–6 h. Treatment with O^6-benzylguanine (60 or 80 mg/kg) in this vehicle was lethal in 3 of 4 and 4 of 4 treated mice, respectively. Treatment at a reduced volume of 15 ml/kg resulted in no toxicity in animals receiving 40% PEG 400/saline with or without O^6-benzylguanine (doses, 10–80 mg/kg). However, the addition of BCNU was lethal in 4 of 5 animals receiving O^6-benzylguanine at 40 mg/kg and in all animals receiving 60 or 80 mg/kg (Table 2). Our previous studies with O^6-benzylguanine dissolved in 10% cremophor-EL/saline showed that animals tolerated O^6-benzylguanine doses of 80 mg/kg (plus BCNU) without developing any toxicity [17, 26].

Table 1. Solubility of O^6-benzylguanine

Vehicle	Solubility (mg/ml)
0.9% Saline	0.11
5% Cremophor-EL/5% EtOH/saline	1.1
10% Cremophor-EL/saline	2.3
40% Propylene glycol/10% EtOH/water	3.4
40% PEG 400/saline	5.0
40% *t*-Butanol/water	5.5
50% Cremophor-EL/50% EtOH	30.1

Treatment of animals bearing s.c. xenografts with i.p. injections of BCNU alone, BCNU plus O^6-benzylguanine (10 or 30 mg/kg) in either 10% cremophor-EL/saline or 40% PEG 400/saline, or vehicle revealed substantial differences in tumor-growth delays and tumor regressions (Table 3). Treatment with BCNU plus O^6-benzylguanine at a dose of 10 mg/kg was substantially more active when O^6-benzylguanine was given in 40% PEG 400 than when it was given in 10% cremophor-EL, with growth delays of 14.7 versus 4.6 days and tumor regressions in 8 of 9 versus 1 of 10 animals being obtained, respectively. Increasing the dose to 30 mg/kg produced tumor-growth delays of 45.2 versus 11.5 days and tumor regressions in 7 of 8 versus 7 of 9 animals for O^6-benzylguanine in 40% PEG 400 as compared with 10% cremophor-EL, respectively. BCNU given without O^6-benzylguanine produced growth delays of only 0.7 and 1.5 days, respectively, and no tumor regression (Table 3).

Table 2. Murine mortality of PEG 400 ± O^6-benzylguanine ± BCNU

PEG volume[a] (ml/kg)	O^6-benzylguanine alone (mg/kg)	–BCNU[b]	+BCNU[b, c] (12.7 mg/kg)
30	80	4/4	3/4
30	60	3/4	4/4
30	0	0/4[d]	4/4
15	80	0/5	5/5
15	60	0/5	5/5
15	40	0/5	4/5
15	30	0/5	0/5
15	20	0/5	0/5
15	10	0/5	0/5

[a] Given via single i.p. injection in saline
[b] Numbers of animals dying/numbers of animals treated
[c] BCNU was given at a dose of 12.7 mg/kg in a volume of 30 ml/kg at 1 h after administration of PEG 400 with or without O^6-benzylguanine
[d] All animals were somnolent for 4–6 h but recovered

Table 3. Treatment of s.c. D456 MG xenografts in athymic mice with BCNU ± O^6-benzylguanine in 40% PEG 400 or 10% cremophor-EL

Vehicle	Treatment regimen[a]		T-C (days)[b]	Regressions[c, d]
	O^6-benzylguanine (mg/kg)	BCNU (mg/kg)		
None	0	12.7	0.7	0/10
Cremophor-EL	10	12.7	4.6*	1/10
PEG 400	10	12.7	14.7*,**	8/9
None	0	12.7	1.5	0/8
Cremophor-EL	30	12.7	11.5*	7/8
PEG 400	30	12.7	45.2*,**	7/9

* $P<0.01$ versus animals receiving vehicle alone, ** $P<0.01$ versus animals treated with BCNU+O^6-benzylguanine in 10% cremophor-EL
[a] BCNU was given in 3% ethanol via a single i.p. injection at a dose of 12.7 mg/kg in a volume of 30 ml/kg. O^6-Benzylguanine was given in either 10% cremophor-EL or 40% PEG 400 in 0.9% saline in a volume of 15 ml/kg. In combination studies, BCNU was given 1 h after O^6-benzylguanine
[b] Difference in days between the median time required for the tumors of treated (T) versus control (C) animals to reach a volume 5 times that measured at the time of original treatment
[c] Decrease in tumor volume over two successive measurements
[d] Number of regressions/number of mice treated

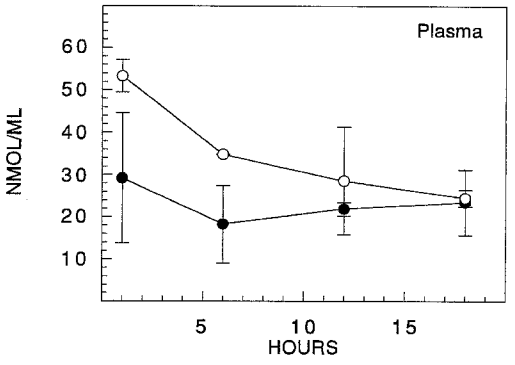
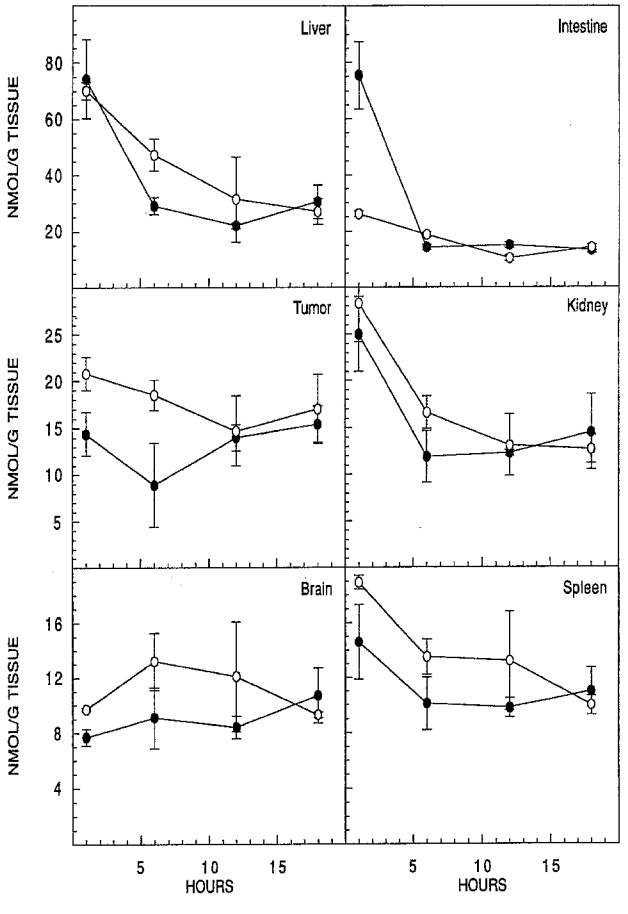


Fig. 1 Total radioactivity detected in mouse plasma after treatment with 8-[^{3}H]-O^6-benzylguanine in PEG 400 or cremophor-EL. Nude mice were injected i.p. with 10 mg/kg O^6-benzylguanine (3.4 μCi/20-g mouse) in either 40% PEG 400/saline (*open circles*) or 10% cremophor-EL/saline (*filled circles*) and were euthanized at 1, 6, 12, and 18 h. An aliquot of plasma was counted directly

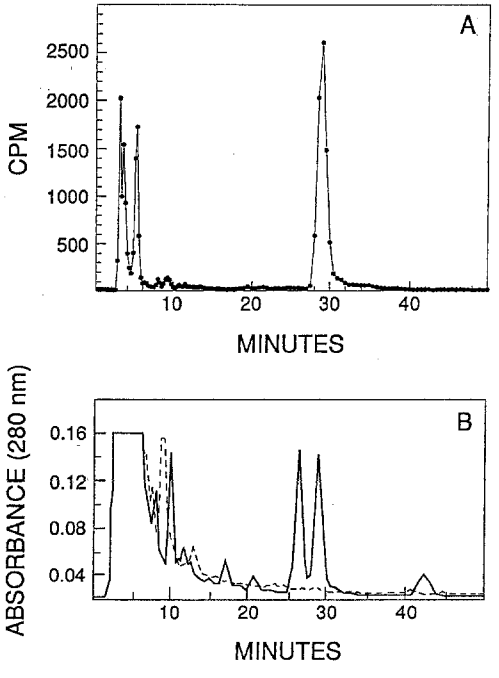
Fig. 2 Total radioactivity detected in mouse tissues after treatment with 8-[^{3}H]-O^6-benzylguanine in PEG 400 or cremophor-EL. Nude mice were injected i.p. with 10 mg/kg O^6-benzylguanine (3.4 μCi/20-g mouse) in either 40% PEG 400/saline (*open circles*) or 10% cremophor-EL/saline (*filled circles*) and were euthanized at 1, 6, 12, and 18 h. Tissue extracts from the liver, intestine, D456 MG tumor, kidney, brain, and spleen were prepared. Aliquots were counted and results were expressed as nmol parent drug and/or metabolite/g tissue



Treatment of tumor-bearing animals with 8-[^{3}H]-O^6-benzylguanine (10 mg/kg) in 10% cremophor-EL or 40% PEG 400 produced marked differences in plasma and tissue levels of radioactivity (Figs. 1, 2). At 1 h, there was 1.8 times more radioactivity in the plasma after injection of drug in 40% PEG 400 as compared with 10% cremophor-EL. Figure 2 illustrates the total radioactivity measured in the liver, intestine, tumor, kidney, brain, and spleen. There was significantly more radioactivity in the liver at 6 h and in the tumor at 1 and 6 h when animals were treated with drug in 40% PEG 400. In contrast, the intestine contained about 3 times more radioactivity at 1 h when cremophor-EL was used as the vehicle. There was no difference in the level of radioactivity detected in the kidneys or lungs of animals treated with drug in cremophor-EL or PEG 400 (data not shown). Overall, animals treated with drug dissolved in either vehicle exhibited a higher level of radioactivity in the liver than in other tissues.

The urine of mice treated with 8-[^{3}H]-O^6-benzylguanine was collected at various time points to determine the extent of metabolism of benzylated guanine. Aliquots of urine were chromatographed on a reverse-phase column and eluted with 35% methanol/0.05 *M* ammonium formate buffer, (pH 4.5). Radioactive and UV chromatograms of the 24-h mouse-urine collection are shown in Figs. 3A and 3B, respectively. The parent drug (retention time, 28.6 min) was identified by comparing the UV spectrum and retention time of samples with that of authentic O^6-benzylguanine. A

Fig. 3 A, B Reverse-phase HPLC profile of urinary metabolites detected after i.p. injection of mice with 10 mg/kg O^6-benzylguanine in PEG 400. Urine collected at 24 h was prepared by the addition of methanol followed by filtration. An aliquot was eluted with 35% methanol/0.05 *M* ammonium formate (pH 4.5) from a C18 reverse-phase column. **A** Radioactivity as determined by scintillation counting of fractions. **B** UV absorbance at 280 nm (*Solid line* mouse treated with 8-[^{3}H]-O^6-benzylguanine, *dotted line* untreated mouse)



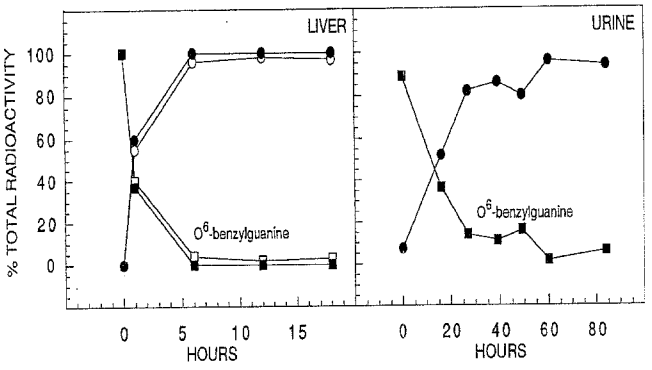


Fig. 4 Radioactivity recovered in the form of O^6-benzylguanine or metabolites from the liver and urine of mice. The percentage of total radioactivity found in the liver and urine in the form of early-eluting radioactive peaks (*circles*) and O^6-benzylguanine (*squares*) was determined after treatment of mice with the parent drug in PEG 400 (*filled symbols*) and cremophor-EL (*open symbols*)

nonradioactive metabolite eluted 2.4 min prior to the parent drug (retention time, 26.2 min). Three radioactive peaks were found early (at 3, 4, and 5 min, respectively). Following the 39-h collection, all radioactivity eluted at the earliest retention times.

Figure 4 illustrates the percentage of total radioactivity in the form of the early radioactive peak(s) and O^6-benzylguanine detected in the liver after injection of the parent drug in 10% cremophor-EL or 40% PEG 400. There appeared to be no difference in the rate or extent of conversion of O^6-benzylguanine to metabolites in mouse liver, regardless of the vehicle used. Within 1 h, most of the parent drug was converted to metabolites in the liver. Figure 4 also illustrates the percentage of total radioactivity in the form of the early radioactive metabolite(s) and O^6-benzylguanine detected in the urine after injection of the parent drug in 40% PEG 400.

Discussion

Cremophor-EL is a polyoxyethylated castor oil that has been the vehicle used in almost all previous animal studies with O^6-benzylguanine. Unfortunately, this vehicle has been shown to cause elevated cholesterol and triglyceride levels in dogs when given alone. Similar elevations have been observed in patients receiving high doses of miconazole dissolved in this solvent [1, 14, 22]. The most disturbing side effect of cremophor-EL is a hypersensitivity reaction that varies from skin flushing and itching to hypotension, bronchospasm, edema and life-threatening anaphylactoid reactions [23, 29]. Weiss and Bruno [30] reported a 1%–2% incidence rate for hypersensitivity reactions following administration of the anticancer agent teniposide dissolved in a mixed solvent vehicle containing cremophor-EL. Clinical trials with taxol also indicated that patients experienced hypersensitivity reactions that were related to the cremophor-EL vehicle [2, 21, 31]. The potential for severe anaphylactoid reactions limits the application of cremophor-EL for i.v. drug delivery in humans.

PEGs are polymers that are used in the formulation of several barbiturates, enema solutions, and antibiotics and, less frequently, anticancer drugs. Etoposide is an example of an anticancer agent that has been given in a mixed-solvent vehicle that includes PEG 300. At conventional doses of etoposide (125 mg/m^2), PEG doses are about 110 mg/kg [13].

Our studies were designed to evaluate PEG 400 as an alternative vehicle for O^6-benzylguanine therapy by evaluating the purine's solubility, bioavailability, and metabolism as well as the antitumor response when this agent was combined with BCNU. Administration of 10 mg/kg O^6-benzylguanine in PEG 400 resulted in a greater enhancement of the antitumor effect of BCNU, although equivalent antitumor activity may be observed if a higher dose of O^6-benzylguanine is given in cremophor-EL. In consistence with this observation, we found a more rapid distribution of radioactive O^6-benzylguanine to the plasma, tumor, and several other tissues when the drug was given in PEG 400 as compared with cremophor-EL at a dose of 10 mg/kg. One of the advantages of using PEG as a vehicle is that lower doses of O^6-benzylguanine can be given due to the enhanced distribution and efficacy of delivery of O^6-benzylguanine in this vehicle. However, initial clinical trials with the combination of O^6-benzylguanine and BCNU should utilize cautious doses of BCNU.

The rate of metabolism of O^6-benzylguanine was similar for either vehicle, with rapid conversion of the parent drug to a nonradioactive peak and early-eluting radioactive metabolites being detected in mouse liver and urine. Thus, although the biodistribution differs according to whether O^6-benzylguanine is given in PEG or cremophor, the metabolism is the same. After i.p. administration of O^6-benzylguanine, a dominant non-radioactive peak was observed in mouse urine that eluted 2.4 min prior to the parent drug on a reverse-phase column. Since this peak was not coincident with any radioactivity or UV peak of an untreated animal, an alteration in the parent drug must have occurred at the C-8 position of guanine. This metabolite may be an 8-hydroxylated derivative of O^6-benzylguanine. The same metabolite has been observed in rat urine and liver [12]. The early-eluting peaks may be debenzylated products such as guanine or the 8-hydroxylated derivative of guanine. Further studies to confirm the identity and activity of these compounds are under way. In terms of toxicological evaluation and possible future clinical trials, PEG 400 is a better vehicle than cremophor-EL for O^6-benzylguanine.

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