Preclinical pharmacology of the antitumor agent *O*-6-methylguanine in CDF1 mice

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Abstract. *O*-6-methylguanine (O6-mG), a guanine analog recently shown to be a potent inhibitor of alkylguanine-DNA alkyltransferase, has been found to potentiate the antitumor activity of nitrosoureas, in particular, carmustine (BCNU), in resistant cell lines (HT-29 mer+) and is targeted for development as a modulating agent with chloroethyl nitrosoureas. A high-performance liquid chromatography (HPLC) assay of O6-mG in plasma has been developed using a μC18 reverse-phase column. O6-mG and the internal standard deoxyguanosine (dGuo) were eluted with a linear gradient of from 15% to 35% methanol in 0.5 *M* ammonium acetate (pH 6.5) at a flow rate of 1 ml/min. The assay was linear over a 4-log concentration range with a detection limit of 0.1 μg/ml. The within-run and between-run coefficients of variation (CV) were found to be 8.1% and 9.3%, respectively. The pharmacokinetics (PK) of O6-mG were investigated in healthy CDF1 mice following separate i.v. and i.p. administrations. At 20 mg/kg i.v., plasma O6-mG gave a biexponential profile with a terminal half-life ($t_{1/2}$) of 24 min and a total clearance (CLT) of 23.7 ml min^{-1} kg^{-1}. Higher doses (40 – 80 mg/kg) revealed a fluctuating third phase, probably due to enterohepatic cycling. Dose-dependent kinetics as measured by CLT and area under the plasma-concentration curve (AUC) values were also seen. Following i.p. dosing, O6-mG was completely absorbed and available to the circulation. No acute toxicity was observed in the animals, except for mild sedation, a possible side effect of the 10% ethanol used in the formulation. Studies on the cellular metabolism of highly purified [^{3}H]-O6-mG have shown that the compound is not anabolized by a human lymphoblastoid cell line (CEM). Biochemistry studies have shown that the parent molecule is inactivating the alkylguanine-DNA alkyltransferase (AGT), thus exerting its pharmacological effect.

Introduction

A major cause of tumor resistance to alkylating agents has been shown to be due to the presence of high levels of DNA repair enzymes. The repair of DNA adducts on the O^6 position of guanine [guanosine monophosphate (GMP)-DNA] formed by nitrosourea alkylation has been demonstrated in mammalian tissues and lymphoid cells [1, 2]. During its repair, the enzyme O^6-alkylguanine DNA alkyltransferase (AGT) does not release O^6-alkylguanine as a free base, and no apurinic site or break in the DNA double strand is formed [3]. On the other hand, unrepaired O6-alkylguanine adducts formed by chloroethyl nitrosoureas are converted to DNA cross-links, which appears to be the major cytotoxic action of these clinically used drugs [3 – 5]. Cells with high cellular levels of AGT are resistant, whereas those that have low levels are sensitive to nitrosoureas [6, 7]. Mammalian AGT acts as an alkyl receptor and the reaction occurs with the direct transfer of the alkyl group from the 6 position of oxygen on guanine to a cysteine residue of AGT [8, 9]. This has been defined as a "suicide" reaction of AGT because the AGT-active site becomes irreversibly alkylated and, thus, inactivated. The total copy number of AGT protein in a cell is therefore the "capacity" or "resistance factor" to alkyliotic drugs.

O-6-Methylguanine (O6-mG, NSC 37364) is a free-base analog of guanine and a biochemical modulator of

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Abbreviations: O6-mG, *O*-6-methylguanine; HPLC, high-performance liquid chromatography; PK, pharmacokinetics; CLT, total clearance; AUC, area under the plasma concentration curve; AGT, alkylguanine-DNA alkyltransferase; dGuo, deoxyguanosine

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AGT [10]. The mechanism of action of O6-mG appears to be identical to the reaction with a DNA-O^6-alkylguanine adduct [11, 12], and it enhances the cytotoxicity of compounds that induce the formation of these adducts [11 – 13].

These studies were conducted to investigate whether the concentrations of O6-mG in experimental animals, as part of the preclinical evaluation of this drug, are sufficient to induce adequate inhibition of AGT, a condition necessary to induce collateral-sensitivity regimens of O6-mG with nitrosoureas. Thus, this report presents the results of pharmacokinetics studies of O6-mG in mice, the stability and the protein-binding characteristics of the drug in animal and human plasma, as well as the cellular metabolism of O6-mG by T-lymphoblastoid cells [14].

Materials and methods

Drugs and chemicals. Nonformulated O6-mG was provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. dGuo was purchased from Sigma Co. (St. Louis, Mo.) and used without further purification. Chemicals, solvents, and reagents were of HLPC or analytical grade. [8-^{3}H]-O6-mG was purchased from Moravek Biochemicals (Brea, Calif.).

Experimental animals. CDF1 mice were supplied through the Division of Cancer Treatment, National Cancer Institute. The animals were 19 – 28 g in weight and were allowed to acclimate before randomization.

HPLC assay of O6-mG. O6-mG in plasma was quantitated by either gradient or isocratic HPLC. The gradient HPLC analysis was performed on a Waters 840 liquid chromatograph (Waters Associates, Milford, Mass.) equipped with model 510 gradient pumps, a U6K universal injector, and a 440 UV-VIS detector. The entire unit was controlled by a PC PRO 350 Controlled Equipment Digital Computer (Waters Associates, Milford, Mass.). The isocratic HPLC was performed on a chromatronix 3510 chromatograph (Spectra-Physics, Santa Clara, Calif.) coupled to a Hitachi variable UV detector set at 280 nm.

Plasma proteins were removed by a precolumn packed with Whatman Packing Gel (37 – 53 μm) and the components were then eluted onto the reversed-phase column for analysis. dGuo was used as the internal standard. Plasma samples containing increasing concentrations of O6-mG at 0, 0.2, 0.3, 0.5, 0.75, 1.0, and 2.0 μg/ml were each spiked with 20 μg of dGuo each. A 10-μl aliquot was injected directly onto a 3.9-×300-mm stainless steel column packed with 10-μm C-18 reversed-phase resin (Waters μBondapak), which was connected in series to a 3.9-×60-mm precolumn. The plasma sample was eluted over 15 min with a linear gradient solvent ranging from 15% methanol in 0.5 M ammonium acetate (pH 6.5) to 35% methanol in the same buffer at a flow rate of 1 ml/min. The column was then returned to the original solvent conditions and allowed to reequilibrate for 5 min before the next injection. Under these conditions, O6-mG and dGuo were baseline-resolved with respective retention times of 6.1 and 10.33 min on a new column. The retention times changed slightly to 5.5 and 9.0 min, respectively, after repeated use. The HPLC eluents were detected at 280 nm, the absorption maximum for O6-mG.

For the protein-binding study, since there was no interference in the ultrafiltrate, the elution was accomplished with 20% methanol in 0.5 M ammonium acetate (pH 6.5) under isocratic conditions using an Altex 10-μm C-18, 0.46- ×25-cm reversed-phase column at a flow rate of 1 ml/min..

Nucleoside and nucleotide HPLC assay. For assaying the possible nucleosides and nucleotides of O6-mG, a previously described method using an anion exchange column (SAX-10, Whatman, Inc.) was applied [14]. This method employed a 40-min linear gradient of a binary solvent system, whereby from 0 to 100% solvent B was used, followed by

maintenance at the final conditions for 50 min, after which the column was returned to the initial conditions for equilibration for 5 min. The elution buffers comprised solvent A (H_2PO_4, 0.005 M, pH 2.8) and solvent B (H_2PO_4, 0.75 M, pH 3.50), run at a combined flow rate of 2 ml/min. Under these conditions the separation of nucleosides/bases and mono-, di-, and triphosphate anabolites have been reported [15].

Stability of O6-mG in buffer and plasma. The stability of O6-mG in phosphate buffer (pH 7.4), pooled human plasma and its ultrafiltrate, and fresh human and mouse plasma was investigated. O6-mG at 20 μg/ml in pooled human plasma was incubated at 0, 5, 10, 15, 20, 30, 45, 60, 75, 90, 105, and 120 min. Following incubation, 10 μl of each sample was removed and the internal standard was added before the samples were subjected to HPLC analysis. For mouse plasma, following the incubation of O6-mG at 10 μg/ml at 37° C, a 10-μl sample was removed every 30 min for up to 5 h for HPLC analysis. Similarly, O6-mG at 2 μg/ml was incubated separately in 0.5 M sodium phosphate buffer (pH 7.4) and in pooled human plasma ultrafiltrate at the same temperature. Samples were obtained every 0.5 h for up to 6 h, and at 20 h following incubation; a 50-μl aliquot was removed and, after addition of the internal standard, analyzed by HPLC.

Protein-binding of O6-mG. Protein-binding of O6-mG in human, rat, and mouse plasma and in 5% human serum albumin (Cohn fraction V) was evaluated using ultrafiltration techniques. O6-mG was added into a known volume of the plasma to achieve final concentrations of 1 and 5 μg/ml. The plasma samples were incubated in a 37° C water bath for 1 h and then ultrafiltered at 0° C for 1 additional h on the Amicon Centrifree System. To an aliquot of the ultrafiltrate, the internal standard was added, and an aliquot of the resulting filtrate was analyzed by the isocratic HPLC procedure. Control experiments using buffer instead of plasma were run under identical conditions. These controls were analyzed at the same time as the samples. All experiments were performed in minima of triplicates and, in most cases, six replicates. O6-mG was found to bind to fresh human plasma at about 15%.

Pharmacokinetics studies in CDF1 mice. Single-dose pharmacokinetics studies of O6-mG were performed in CDF1 mice. Because of its limited aqueous solubility, O6-mG was first dissolved in a small amount of absolute ethanol and then diluted with normal saline to result in a 10% ethanol solution, which was used in all pharmacokinetics studies. CDF1 mice weighing 19 – 28 g were placed in a 3.5- ×9.5-cm plastic restraining cage (in-house custom design). The tail of each mouse was extended out through a slot in the cage to facilitate access to the caudal vein. O6-mG was formulated in normal saline containing 10% absolute ethanol at a concentration of 1.5 mg/ml.

The mice were divided into 5 groups of 90 – 100 each. Four groups received intravenous (i.v.) injections of O6-mG at doses of 20, 40, 60, and 80 mg/kg, respectively. The remaining group received an intraperitoneal (i.p.) injection of the drug at 80 mg/kg. Each animal received an injection volume ranging from 0.3 to 0.6 ml, depending on the dose delivered through the caudal vein or i.p. according to the protocol. At predetermined time points, typically 0, 2, 5, 10, 20, 30, 40, 50, 60, 100, 120, 180, 240, 300, and 360 min, blood samples of 500 – 1000 μl each were obtained by cardiac puncture following ether anesthesia and the entire process usually took less than 1 min. Six animals per time point were used. The blood samples were immediately placed in an ice bath and within 5 min were centrifuged at 5000 g at 4° C in an IEC Centra 8R centrifuge for 5 min. Immediately following the separation, the plasma was frozen in a dry-ice acetone bath and kept at −70° C until analysis. Analysis was performed within 3 days of the sample collection by the HPLC method.

Urinary excretion of O6-mG in CDF1 mice. Urinary excretion of O6-mG was studied in 2 groups of CDF1 mice containing 12 mice each. Urine samples were collected at 0 – 4, 4 – 8, and 8 – 24 h and O6-mG concentrations in these urine samples were measured by the HPLC method using dGuo as the internal standard.

Cellular metabolism studies of [^{3}H]-O6-mG in CEM cells. Human lymphocytic leukemia cells, CCRF/CEM (CEM cells), were incubated for
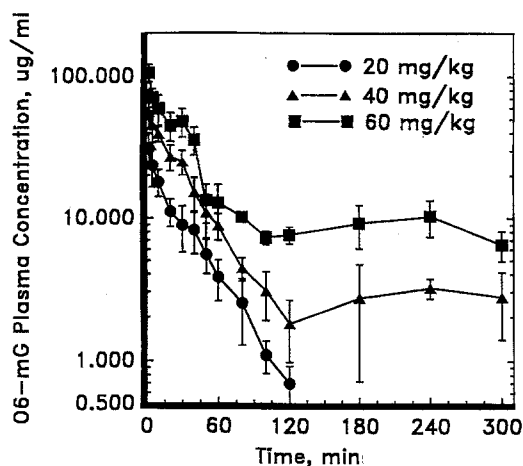
Fig. 1. Pharmacokinetic profiles of O6-mG in mice after three doses had been given i.v. Data points represent the means of $n = 3 \pm SD$ for each group

2 h with highly purified [³H]-O6-mG (0.5 µCi/µg, or 0.55×10^7 cpm/µg O6-mG) that had been premixed with cold drug to a final concentration of 0.5 mg/ml in growth media consisting of RPMI 1640 enriched with 10% heat-inactivated fetal bovine serum and 1% non-essential amino acids. After the incubation, which took place in a humidified incubator in the presence of 5% CO_2 in air at 37° C, the CEM cells were extracted with ice-cold, 0.4 N perchloric acid (PCA) twice as previously described for similar compounds [15]. This procedure extracts all the nucleoside bases, nucleosides, and nucleotides from the cells. The neutralized PCA-soluble extract was then assayed for [³H]-O6-mG and its possible cellular anabolites on an anion-exchange HPLC column as previously described. The HPLC eluates corresponding to the nucleotides, nucleosides, and mono-, di-, and triphosphate anabolites were collected every minute using a fraction collector (ISCO Retriever III). Each sample was mixed with 10 ml of liquid scintillation cocktail (BIO-SAFE II, RPI), and the radioactivity in the vials was counted by a scintillation counter.

Results

HPLC assay

With the HPLC assay developed, no interference was found in the plasma blanks in the region where O6-mG and the internal standard were eluting. Under the assay conditions, the limit of detection of O6-mG is about 0.2 µg/ml in

human plasma and 0.1 µg/ml in mouse plasma. The linearity of the assay was found to range from 1 to 1000 µg/ml; the within-run coefficient of variation of the assay was 8.1% at 0.2 µg/ml ($n = 9$) and the between-run coefficient of variation was 9.3% at 50 µg/ml ($n = 5$) in human plasma.

Stability of O6-mG in buffer and plasma

O6-mG was found to be stable in plasma ultrafiltrate and in phosphate buffer for up to 20 h as monitored. In pooled human plasma, O6-mG degraded monoexponentially with a half-life of 2.45 h. In fresh mouse and human plasma at 37° C, O6-mG concentrations appeared to remain essentially unchanged during the first 60 min, whereas at 90 min the concentration fell by approximately 50% of the original value (Table 1). The reduced concentration of O6-mG remained approximately the same for up to 180 min. Similar results were observed at 25° C and at 4° C.

Protein-binding studies of O6-mG

Protein-binding in fresh rat plasma was found to be 12% at 37° C at 5 µg/ml. At 0° C, O6-mG was found to bind to mouse plasma at 11%. In fresh human plasma the protein-binding value was determined to be 15%, and in 5% human serum albumin it was found to be about 20%. Partition of O6-mG between red blood cells and plasma was investigated in rat and mouse whole blood. The results indicated unit partition.

Pharmacokinetics studies of O6-mG in CDF1 mice

The mean plasma concentration-time profile of O6-mG in mice receiving 20 mg/kg is shown in Fig. 1. As shown, at 2 min the O6-mG plasma concentrations reached 32.5 µg/ml and then declined biexponentially, which was best fitted to a two-compartment model. The terminal half-life of elimination was found to be 24.3 min, and after 2 h the drug plasma concentrations fell below the detection limit of 0.1 µg/ml.

The mean plasma concentration-time profile of O6-mG in mice given 40 mg/kg i.v. is shown in Fig. 1. The drug

Table 1. Stability of O6-mG in human and mouse plasma

Actual initial concentration (µg/ml)	% Initial concentration remaining (time, min)									
	5	10	15	20	30	45	60	90	120	180
FHP, 4° C, 27.93	100	100	98	99	91	96	100	31	31	38
FHP, 25° C, 36.61	83	88	80	80	80	77	88	26	21	24
FHP, 37° C, 26.69	70	100	100	94	94	90	97	26	29	27
FrHP, 4° C, 21.90	100	100	100	100	100	100	100	63	49	51
FrHP, 25° C, 24.40	100	100	100	87	100	100	100	41	–	29
FrHP, 37° C, 28.49	81	86	83	75	78	84	84	21	29	29
FMP, 4° C, 35.74	62	65	66	69	19	57	59	24	27	26
FMP, 25° C, 28.06	100	100	98	100	100	100	100	38	36	39
FMP, 37° C, 24.22	100	100	100	100	100	100	100	42	46	34

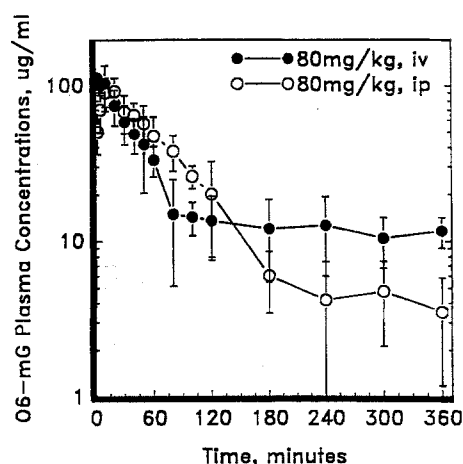FHP, Fresh human plasma; FrHP, frozen human plasma; FMP, fresh mouse plasma

Fig. 2. Comparison of the pharmacokinetic profiles of O6-mG in mouse plasma after an i.p. and an i.v. administration of the drug. Data points represent the means of $n = 6$ $\pm$ SD for each group (for more details, see Materials and methods)

level in plasma at 2 min was 47.1 µg/ml and the concentrations appeared to decline monoexponentially initially until 2 h, after which the decline became slower. At 240 and 300 min, the plasma concentrations appeared to rebound. The initial phase was regressed to a monoexponential function that gave an apparent half-time of 23.5 min.

At 60 mg/kg, the O6-mG plasma concentration at 2 min was found to be 84.7 µg/ml and the levels declined essentially at two phases (Fig. 1). However, the concentrations were found to increase at several regions. Because of this, the data were estimated by a biexponential decay for only up to 2 h, as the inclusion of data beyond this time point resulted in difficulty in nonlinear regression. The terminal slope thus estimated showed a half-time of 29 min.

After the i. v. administration of 80 mg/kg O6-mG, the plasma level at 2 min was 113 µg/ml and the drug concentration declined essentially biexponentially (Fig. 2). The initial phase gave an apparent half-time of 31 min. The latter phase showed rebound concentrations from 240 min and it was thus difficult to analyze the data by a simple exponential function. When the same dose of O6-mg was given i.p., the O6-mG plasma concentration peaked at 20 min with a value of 92.3 µg/ml and then declined monoexponentially for up to 240 min with an estimated

half-time of 40 min (Fig. 2). After 240 min, the concentrations showed an increase.

The AUC values calculated following i.v. and i.p. administration of O6-mG were computed to the last actual data point measured by the trapezoidal rule. The ratio of the AUC for i.p. dosing to that for i.v. administration produced a bioavailability of 101.7%.

The AUC values for the plasma concentration-time data of O6-mG at all doses were estimated from time zero to infinity using the actual data plus extrapolation, and the extrapolation was accomplished by approximation with the declining slope best estimated from the log-linear segments prior to the rebound region of the data. The curve-fitting from these segments of data also afforded the estimate of Co values; these Co values were also utilized in the AUC estimation. The median residence time (MRT) values were estimated similarly by a model-independent method with extrapolation, similar to the AUC calculation.

The data for AUC and Co values as a function of the dose are shown in Table 2. The mean doses for each dose-group of animals were computed by averaging the actual doses used in each animal within that group. Together with the AUC values, these data allowed the estimation of the total body clearance (CLT) of O6-mG for each dose. Additionally, the steady-state volume of distribution (Vd$_{ss}$) was computable using Vdss = CL $\times$ MRT (Table 2). The values for Co, AUC, and CLT were also plotted against the dose in milligrams per kilogram of body weight, and these are shown in Fig. 3.

The mean urinary excretion of O6-mG in 24 h was determined to be 44.8% $\pm$ 0.6% of the delivered dose, with over 60% of that being excreted in the first 4 h. Thus, only approximately 50% of the dose given was accounted for in 24-h urine samples; probably a large amount of the drug may have undergone anabolism and, perhaps, catabolism, although we have not yet obtained direct evidence of O6-mG metabolism.

Cellular anabolism studies of O6-mG in CEM cells

The HPLC profile of O6-mG with the superimposed radiogram is shown in Fig. 4. The radiogram, which has been amplified 1000-fold, clearly shows that there is only one radioactive peak associated with intact [3H]-O6-mG. No other radioactivity peaks were seen in the chromatogram.

Table 2. Pharmacokinetic parameters of O.6-mG in mice

Dose/route (mg/kg)	Amt[a] (mg)	Co (µg/ml)	"Z" (min^{-1})	$t_{1/2}$ (min)	MRT (min)	AUC (µg ml^{-1} min)	CL (ml min^{-1} kg^{-1})	Vdss (ml/kg)	Vc (ml/kg)
20/i.v.[b]	0.50	41.6	0.02826	22.5	31.6	773.4	25.9	817	481.6
40/i.v.[b]	1.02	48.0	0.02915	23.8	77.7	2140.0	18.6	1446	830.0
60/i.v.[b]	1.56	107.0	0.02322	29.7	114.2	4888.0	12.3	1401	560.8
80/i.v.[c]	1.86	164.0	0.01517	45.7	134.4	8464.0[d]	9.4	1261	484.3
80/i.p.[c]	1.68	–	0.01706	40.6	–	8054.0[e]	9.9	–	–

[a] Amount (Amt) doses used are averages with standard deviations ranging from 4.8% to 9.6%
[b] 3 mice/time point
[c] 6 mice/time point minimum
[d] 7699 µg ml^{-1} min^{-1} to 6 h
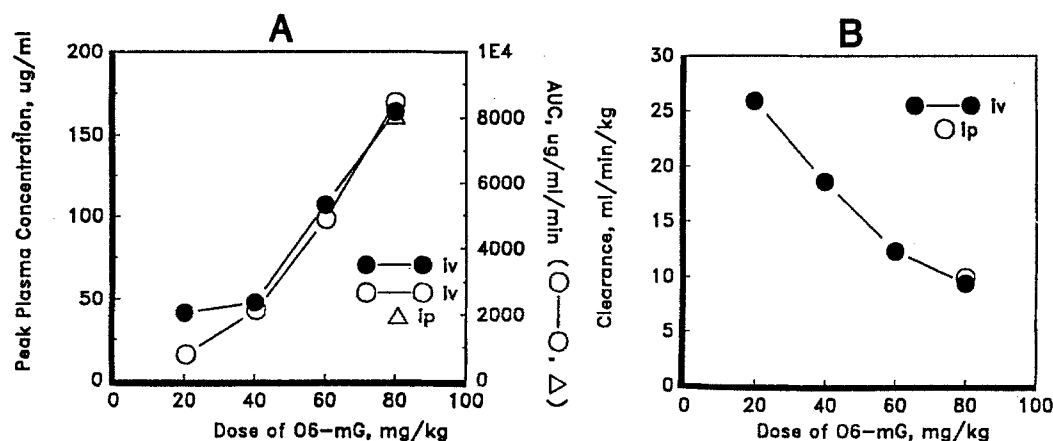[e] 7831 µg ml^{-1} min^{-1} to 6 h

Fig. 3. A Relationships between peak plasma concentrations of O6-mG, AUC, and drug dose after i.v. and i.p. administration in mice. B Relationship between the clearance and the dose of O6-mG in mice
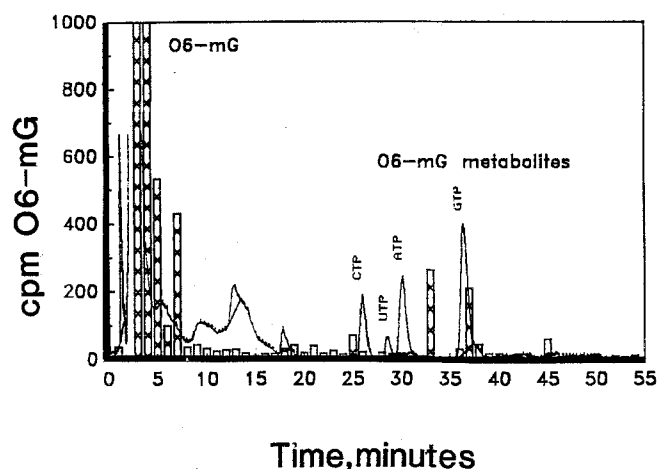

Time,minutes

Fig. 4. Cellular anabolism study of [³H]-O6-mG in human leukemic cells in vitro. Bars, Tritium radioactivity representing the parent drug and minute quantities of anabolites (for details, see Materials and methods). CTP, Cytidine triphosphate; UTP, uridine triphosphate; ATP, adenosine triphosphate; GTP, guanosine triphosphate

Less than 0.5% of the radioactive counts were associated with the triphosphate region as compared with the counts of [³H]-O6-mG. However, these data show that even if these counts do not represent a contaminant (less than 0.05% of [³H]-O6-mG radioactive purity), there is no appreciable cellular anabolism of O6-mG by this cell line.

Discussion

At higher doses of from 40 to 80 mg/kg, plasma levels rose at later time points, which hampered accurate assessment of the terminal slope by nonlinear regression using standard statistical packages. These rises were probably not due to a methodological artifact since the levels were well within the detection limits. On close examination of these profiles, especially those obtained following doses of 60 mg/kg, there appear to be distinct patterns of rise and fall within the entire profile, with the later time points being more prominent. The patterns appear to occur at approxi-

mately 30- to 60-min cycles. These values were all found to differ from each other statistically by analysis of variance. Therefore, these cyclic rises and declines may be interpreted as being due to the existence of enterohepatic cycling. The existence of this phenomenon has been reported for many drugs [15 – 20], although it has not been reported for O6-mG in the rat [21]. Cyclic fluctuation of plasma drug levels have also been attributed to the existence of circadian rhythm [22 – 23], although this possibility may be less likely, since the intervals for the cycling processes appear to be very short.

Pharmacokinetics studies of O6-mG in the rat model [21] have shown a two-compartment, open-model type of elimination kinetics after 40-mg/kg dosing, with a terminal $t_{1/2}$ of 2.3 h. In mice a similar biexponential elimination kinetics is observed, but the terminal $t_{1/2}$ is much shorter, amounting to approximately 0.3 h. A comparison of the clearance and the Vd_{ss} of O6-mG between the rat and mouse models reveals that the mice clear the drug 3 times faster and achieve a 1.5-fold higher Vd_{ss} than the rats. These enhanced parameters of O6-mG in mice explain the faster $t_{1/2}$ of elimination in this model.

Evidence of dose dependence in the pharmacokinetics of O6-mG was found. The AUC from time zero to infinity increased from 773 µg ml⁻¹ min⁻¹ at 20 mg/kg to 8464 µg ml⁻¹ min⁻¹ at 80 mg/kg i.v. The total clearance values of O6-mG were thus estimated for these doses. The values ranged from 25.9 ml min⁻¹ kg⁻¹ at the 20-mg/kg dose to 9.4 ml min⁻¹ kg⁻¹ at 80 mg/kg i.v. (Table 2). The extrapolated Co values increased from 41.6 µg/ml at 20 mg/kg to 164 µg/ml at 80 mg/kg i.v. When these values were plotted against the dose, curvilinear plots were observed at all these parameters, although the Co and AUC values for the last three doses appeared to be reasonably linearly correlated (Fig. 3). These results suggest a possible dose-dependent kinetic phenomenon. However, the dose-dependent kinetics could also be explained in part by a change in the Vdss.

We feel that the Vd_{ss} values may be less reliable because of uncertainty in MRT estimation due to the fluctuation of the later time points, presumably caused by enterohepatic cycling. Whereas it is commonly known that

AUC estimation is more sensitive to data obtained at the earlier time points but is less affected by those for the later times, the opposite effect is found for AUMC (area under the first moment curve) and, thus, MRT calculation. Since we utilized the actual data plus extrapolation for AUC calculation, on which the clearance calculation is based, these data may be more reliable. Additionally, the Vc values, which were estimated from the Co and the dose, did not show a consistent trend of increase. Thus, we favor the possibility of a dose-dependent kinetic at present.

The ratio of the AUC following i.p. injection to that following i.v. injection at the same dose gave a bioavailability value of 101.7%. This value decreased to about 95% when the areas were extrapolated to infinity using the appropriate slopes. Thus, these results indicated essentially total availability for O6-mG given by the i.p. route. Therefore, future pharmacokinetics studies in mice could readily use i.p. injection.

Following O6-mG administration at the dose ranges used, we did not detect any acute or life-threatening toxicity. However, a few animals showed transient signs of staggered motion that were probably related to the effect of alcohol, since control experiments with 10% alcohol in saline also showed similar effects in several mice. There also appeared to be some degree of sedation of the animals, which may possibly have been related to the effect of ethanol. However, we have not ruled out the possibility that this effect might have been due to O6-mG itself, since O6-mG in an ammonium acetate formulation also appears to cause sedation in nude mice (Gerson, private communication).

In a preliminary study using the synthetic O-6-deuteromethyl guanine, we could not detect any significant urinary metabolite of O6-mG in CDF1 mice. However, in the excretion study of O6-mG, only 50% of the delivered dose was accounted for as unchanged drug in 24-h urine samples. Thus, in the subsequent mouse study, tritiated O6-mG was used. O6-mG mixed with 20 μCi of [8-³H]-O6-mG at a total dose of 80 mg/kg was injected into groups of CDF1 mice. No evidence of cellular anabolism was seen in the murine tissues obtained in vivo or in the experiments in human lymphoblastic cell line (CEM). We therefore conclude that the drug is not activated either in vivo or in vitro.

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