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Intrathecal 6-Mercaptopurine: Preclinical Pharmacology, Phase I/II Trial, and Pharmacokinetic Study

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ABSTRACT

For over 30 years, oral 6-mercaptopurine (6-MP) has been a mainstay of systemic maintenance therapy for acute lymphoblastic leukemia. Despite its efficacy as an antileukemic agent, 6-MP has not been previously administered by the intrathecal (IT) route. In anticipation of a clinical trial of IT 6-MP, preclinical cytotoxicity and pharmacology studies were performed to define a safe, effective dose. The optimal concentration (>1 μM) and duration of exposure (>12 h) to 6-MP required for cytotoxicity were determined *in vitro* using human leukemia cell lines. The dose required to achieve the desired cerebrospinal fluid concentrations in humans was derived from pharmacokinetic parameters determined in rhesus monkeys. A phase I/II study was then performed in pediatric patients with refractory meningeal leukemia. Nine patients (aged 3.5 to 16 years) with chronic meningeal leukemia (2 to 6 central nervous system relapses) were entered onto the study. All had previously failed, at a minimum, IT methotrexate, IT cytarabine, and cranial (± spinal) radiation. A 10-mg IT dose of 6-MP (calculated to produce cytotoxic cerebrospinal fluid levels for 12 h) was administered twice weekly for 4 weeks. There were four complete responses and three partial responses. The duration of complete responses ranged from 7 to 22 weeks. Observed toxicities were not dose limiting and included mild headache (three patients) and minimal nausea (two patients). Pharmacokinetic studies performed in patients confirmed that cerebrospinal fluid concentrations of 6-MP were >1 μM for 12 h. These results indicate that the IT administration of 6-MP is feasible, is not associated with significant toxicity, and has definite activity in patients with refractory meningeal leukemia.

INTRODUCTION

Despite the success of central nervous system preventive therapy in children with acute lymphoblastic leukemia in reducing the incidence of meningeal recurrence, meningeal relapse remains an important cause of treatment failure (up to 10% of patients) (1). Although overt meningeal leukemia is responsive to standard intrathecal therapy with drugs such as methotrexate, long-term control is generally ineffective, and the majority of patients succumb to their disease (2).

Whereas systemic chemotherapy for acute lymphoblastic leukemia usually uses combination regimens of six or more antileukemic agents, only one to three drugs (methotrexate, cytarabine, and hydrocortisone) are currently administered intrathecally for the treatment or prevention of meningeal leukemia. The development of new, active intrathecal agents may decrease the incidence of meningeal relapse as well as improve the treatment of overt meningeal disease.

6-Mercaptopurine has been in clinical use for over 30 years and is currently a mainstay of maintenance chemotherapy for children with acute lymphoblastic leukemia. When administered p.o. in conventional doses of 75 mg/m^2, the peak drug concentration in the cerebrospinal fluid is extremely low (<0.1 μM) or undetectable (3–5) and thus is unlikely to play a significant role in the treatment or prevention of meningeal leukemia. Despite its proven antileukemic activity, consideration has not been given to administering 6-MP[4] by the intrathecal route. This report describes preclinical studies and a clinical trial of IT 6-MP. A target cytotoxic concentration of the drug was identified *in vitro* using human leukemia and lymphoma cell lines, the pharmacokinetics was evaluated in the nonhuman primate, and a safe and potentially effective dose was defined and subsequently evaluated in a pediatric phase I/II trial.

MATERIALS AND METHODS

Cell Lines

All cell lines were of human origin and passaged twice weekly in RPMI 1640 plus 10% dialyzed fetal calf serum to maintain cells in logarithmic growth phase. CCRF-CEM and MOLT-4, two acute lymphoblastic leukemia lines, were obtained from the American Type Culture Collection (Rockville, MD). Wilson, a Burkitt's lymphoma cell line, was kindly provided by Dr. Ian Magrath (Pediatric Branch, National Cancer Institute, Bethesda, MD).

In Vitro Cytotoxicity Assay

All chemicals were obtained from Sigma Chemical Company (St. Louis, MO). 6-MP for *in vitro* experiments was dissolved in 0.02 N NaOH, and the pH was adjusted to 8.5 using concentrated HCl.

A modified MTT assay (6, 7) was used to determine the sensitivity of the cell lines to 6-MP. The assay is based on the ability of viable cells to reduce MTT to formazan. One hundred thirty-five μl of 4 × 10^4 cells/ml of each cell line were plated into 96-well microtiter plates. Twenty-four h later, 6-MP, at specified concentrations, was added to each well, in replicates of six. Cells were exposed to 6-MP for 48 h, at which time the number of surviving cells was quantitated with MTT. Briefly, 15 μl of MTT (5 mg/ml) was added to each well, and the plates, protected from light, were agitated for 10 min. Following 4 h of incubation at 37°C, plates were centrifuged at 400 × *g* for 10 min, the medium was aspirated to waste, and 150 μl of dimethyl sulfoxide were added to each well to solubilize the formazan. Plates were shaken for 10 min, and the absorbance was measured at 540 and 690 nm using a microplate spectrophotometer (Bio-Tek EL 312; Bio-Tek Instruments, Winooski, VT). Cell survival was calculated by subtracting the background absorbance of media alone and then dividing the absorbance of test wells by the absorbance of the control (untreated) wells. The concentration producing 50% inhibition of growth was determined by regression analysis using points on the steep portion of the dose-response curve.

To determine the schedule dependence of 6-MP cytotoxicity, more detailed studies were performed with the MOLT-4 cell line. Prior to drug exposure, 4.5 ml of 4 × 10^4 MOLT-4 cells/ml were placed in 15-

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[4] The abbreviations used are: 6-MP, 6-mercaptopurine; CSF, cerebrospinal fluid; IT, intrathecal; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; AUC, area under the concentration time curve; HPLC, high-pressure liquid chromatography; CR, complete response; PR, partial response.

ml conical tubes and incubated in a humidified 5.5% CO_2 atmosphere at a temperature of 37°C. Twenty-four h later, 6-MP, at specified concentrations, was added to each tube. Cells were exposed to the drug for intervals of time ranging from 4 to 24 h, then washed twice in ice-cold phosphate-buffered saline, and resuspended in RPMI 1640 plus 10% dialyzed fetal calf serum. One hundred fifty-μl aliquots were transferred to 96-well microtiter plates and incubated until analysis. Seventy-two h from the start of the experiment, the number of surviving cells in the microtiter plates was quantitated with the MTT assay as described above. To determine whether cytotoxicity was schedule dependent, survival was plotted against the *in vitro* AUC, calculated by multiplying the drug concentration by the duration of exposure. All results presented are the mean of two separate experiments.

To determine whether the metabolism of 6-MP caused significant decreases in drug concentration over time, 6-MP concentration in media was determined by HPLC (described below) 2, 4, 6, 8, and 24 h after addition of the drug. Mean steady-state *in vitro* drug concentrations were calculated by dividing the area under the concentration time curve by the time interval.

Pharmacokinetic Studies in Rhesus Monkeys

Adult male rhesus monkeys (*Macaca mulatta*) weighing 7.1 to 9.1 kg were obtained from the NIH Primate Center. Each animal was housed individually and fed Purina monkey chow and water *ad libitum*. A silicone Pudenz catheter was surgically placed into the fourth ventricle and attached to a s.c. implanted Ommaya reservoir as previously described (8). 6-MP as the sodium salt was provided by Burroughs Wellcome in sterile vials containing 500 mg/vial. The drug was reconstituted in Elliott's B solution to a final concentration of 10 mg/12 cm^3. Animals received a 3.2-mg dose of 6-MP administered via the Ommaya reservoir. This dose, equivalent to approximately one-half of a standard dose p.o. of 6-MP, was chosen to achieve CSF and plasma 6-MP concentrations that could be accurately quantified for pharmacokinetic calculations. Ventricular CSF was sampled prior to, and 0.5, 1, 2, 5, 6, 8, and 12 h after dosing. Plasma samples were obtained prior to and 2, 5, 6, 8, and 12 h following drug administration. Samples were stored at −20°C until assayed. Animals were observed for a minimum of 4 weeks for clinical signs of toxicity.

Pharmacokinetic parameters were determined by model-independent methods. Area under the CSF concentration time curve from time 0 to 12 h was derived by the trapezoidal method and extrapolated to infinity ($AUC_{0-\infty}$) (9). Clearance of 6-MP from the CSF was calculated by dividing the dose by the $AUC_{0-\infty}$. The terminal half-life was determined by regression analysis.

6-MP Sample Analysis

The concentration of 6-MP in cell culture media, CSF, and plasma samples was measured by a previously described reverse-phase HPLC method (5, 10). To extract 6-MP from plasma, 1-ml samples were loaded onto Waters C_{18} Sep-pak cartridges (Milford, MA) after adding 10 μl of 1.0 M dithiothreitol and 20 μl of 20 μg/ml 6-thioguanine (internal standard). Cartridges were rinsed with 1 ml of 35 mM acetate buffer, and samples were eluted with a 2-ml methanol wash. Samples were evaporated to dryness under a gentle stream of nitrogen, reconstituted with 125 μl of mobile phase, and injected onto the HPLC system. The HPLC system included a Waters model 510 pump, a Waters WISP 712 automated sample injector, and a C_{18} Beckman steel column (5-μm particle size). The mobile phase consisted of 0.2% acetate buffer and acetonitrile (97:3) at a flow rate of 1.4 ml/min. Eluant was monitored with a Waters 490 multiwavelength detector at wavelengths of 331 and 340 nm. Retention times under these conditions were approximately 5.2 min for 6-MP and 8.6 min for 6-thioguanine.

Phase I/II Trial

Patient Eligibility. Patients between the ages of 3 and 25 years with meningeal spread of leukemia, lymphoma, or other malignancies refractory to conventional therapy (radiation therapy and intrathecal chemotherapy) were eligible for the study. The presence of meningeal disease was confirmed by examination of a cytocentrifuge preparation of CSF obtained from both the lumbar and, in patients with Ommaya reservoirs, ventricular CSF spaces. All patients had adequate renal and hepatic function as defined by a serum creatinine <1.5 mg/dl, a serum bilirubin <2.0 mg/dl, and serum transaminases <3 times normal for their age. Patients must have recovered from the toxic effects of prior therapy before receiving intrathecal 6-MP. Patients receiving other therapy designed specifically to treat their central nervous system disease (intrathecal or systemic therapy) were not eligible.

Prior to entry on study, informed consent was obtained from the patient or his/her parent in accordance with individual institutional policies.

Study Design. The primary objectives of this phase I/II trial were to determine the safety and toxicity of IT 6-MP and to define its therapeutic efficacy when administered as a bolus dose to patients with meningeal malignancies refractory to conventional therapy.

Patients received 6-MP intrathecally twice weekly for 4 weeks. In those patients who achieved a complete response, additional therapy consisted of four weekly followed by monthly intrathecal doses. Patients achieving a partial response after the first 4 weeks of therapy received 2 additional weeks of twice-weekly therapy. If the patient did not achieve a CR, he/she was removed from the study. Patients who progressed or relapsed at any time or experienced unacceptable toxicity were removed from the study.

A CR was defined as a total clearing of all malignant cells on cytocentrifuge preparation of lumbar CSF. A PR was defined as a 50% reduction in the total CSF blast count in lumbar CSF.

Patients were monitored with complete blood counts, electrolytes, calcium, phosphate, uric acid, serum transaminases, and urinalysis weekly and were closely monitored for clinical signs of toxicity. CSF for total cell count and differential, protein, and glucose was obtained prior to each intrathecal dose.

Drug Formulation and Administration. 6-MP as the sodium salt was reconstituted in Elliott's B solution to a final concentration of 10 mg/12 cm^3. Drug administration was isovolumetric; *i.e.*, an amount of CSF equivalent to the volume administered was removed prior to drug injection.

Following lumbar administration of the drug, patients were instructed to lie prone for 1 h. For patients with an Ommaya reservoir, the drug was administered via a 25-gauge needle into the reservoir and flushed with 2 cm^3 of Elliott's B or CSF, and the reservoir was then pumped 4–6 times.

Three patients were treated with 5-mg bolus doses of 6-MP. This dose was equivalent to approximately one-sixth of the dose that proved safe in the rhesus monkey experiments. All subsequent patients entered onto the protocol were treated with 10-mg doses of 6-MP, a dose calculated to achieve optimally cytotoxic 6-MP concentrations in the CSF.

Pharmacokinetic Studies

Pharmacokinetic studies of IT 6-MP were performed in selected patients with an Ommaya reservoir. One ml of ventricular CSF was drawn from the reservoir (after being pumped 4–6 times) prior to the first dose of 6-MP, and 0.5, 1, 3, 5, 8, and 12 h following intraventricular administration of the drug. Plasma samples were obtained simultaneously. Pharmacokinetic calculations were performed as described above.

RESULTS

In Vitro Cytotoxicity

The 50% inhibitory concentrations for the MOLT-4, CCRF-CEM, and Wilson cell lines exposed to 6-MP for 48 h were 1.5, 2.0, and 2.4 μM, respectively.

The dose-response curves for MOLT-4 cells exposed to 6-MP for 4, 6, 8, 12, and 24 h are shown in Fig. 1. No cytotoxicity was detected when the 6-MP concentration in the media was

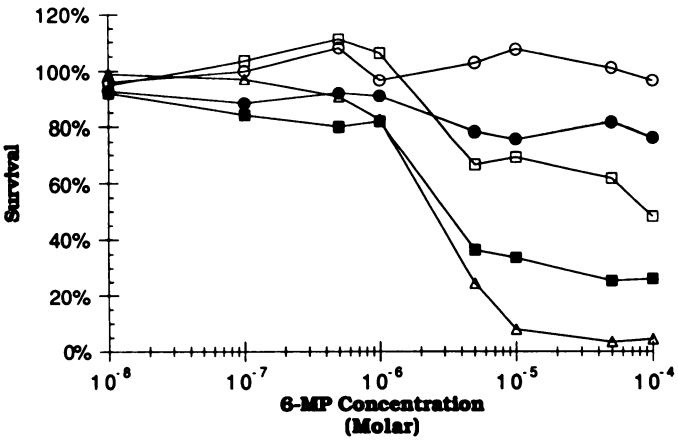

Fig. 1. Dose-response curves for 6-MP in the MOLT-4 cell line. Cells were exposed to the drug *in vitro* for 4 to 24 h. *Ordinate*, percentage survival as compared to untreated control cells. Durations of exposure: ○, 4 h; ●, 6 h; □, 8 h; ■, 12 h; △, 24 h.

below 1 μM. At concentrations >1 μM, cytotoxicity increased with increasing durations of exposure. A significant schedule dependency was found when survival was plotted against the *in vitro* AUC (Fig. 2). A cell kill >50% was obtained only in cells exposed to 6-MP concentrations between 1 and 5 μM for a minimum of 12 h.

Metabolism of 6-MP *in vitro* did not deplete 6-MP in the media with starting concentrations ≥10 μM. At starting concentrations ≤1 μM, metabolic depletion of 6-MP was detected. The mean steady-state 6-MP concentration during exposures of 2, 4, 6, 8, and 24 h was 0.82, 0.71, 0.62, 0.54, and 0.31 μM for 1 μM starting solutions, and 0.075, 0.068, 0.058, and 0.054 μM for 0.1 μM starting solutions (the 24 h concentration was below the limit of detectability). The 1 μM threshold of cytotoxicity for 6-MP defined here may thus be a slight overestimate.

Pharmacokinetic Studies in Rhesus Monkeys

No animal developed any signs of neurotoxicity following IT administration of 6-MP. The geometric mean CSF and plasma concentration time curves for the three animals studied are shown in Fig. 3, and the pharmacokinetic parameters derived from these concentrations are listed in Table 1. 6-MP was cleared from the ventricular CSF at a rate exceeding the CSF bulk flow rate. The volume of CSF and the rate of CSF production in monkeys are approximately 10-fold less than in humans (11). Based on this and the pharmacokinetics of 6-MP determined in rhesus monkeys, it was estimated that, in humans, an IT dose of 10 mg of 6-MP would be required to maintain CSF concentrations above 1 μM for approximately 12 h. This dose was chosen as the target dose for the phase I/II trial.

Phase I/II Trial

Patient Characteristics. The characteristics of the twelve patients treated are listed in Table 2. These patients had experienced a median of 4 relapses each (range, 2 to 6) prior to entry on study. All had, at a minimum, failed IT methotrexate, IT cytarabine, and cranial ± spinal radiation.

Toxicity. The only toxicities observed were mild headache ($n = 3$) and mild nausea ($n = 2$) in patients treated with the 10-mg dose. No patient developed chemical meningitis or other neurotoxicity, and no systemic effects of the 6-MP were detected.

Responses. Of the three patients treated at the 5-mg IT 6-MP dose level, there was one PR. Of the nine patients treated at the 10-mg IT 6-MP dose level, there were four CRs and three PRs, for an overall response rate of 78%. The duration of the CRs ranged from 7 to 22 weeks.

Pharmacokinetics. Three patients treated with 5-mg doses and four patients treated with 10-mg doses had pharmacokinetic studies performed. The geometric mean CSF concentration-time curves of 6-MP for these two dose groups are shown in Fig. 4, and the pharmacokinetic parameters derived from the CSF concentrations are listed in Table 1.

The CSF concentration of 6-MP remained >1 μM (the *in vitro* cytotoxic threshold concentration) for >8 h following a 5-mg bolus dose and for >12 h following a 10-mg bolus dose. The clearance of 6-MP from the CSF was approximately 9-fold greater in humans than that found in monkeys. The 3.2-mg IT dose of 6-MP administered to monkeys is thus equivalent to a dose of approximately 29 mg in humans, and the resultant pharmacokinetic parameters can be compared on this basis.

The plasma pharmacokinetics of 6-MP following a 10-mg IT dose were studied in two patients (Fig. 4). The geometric mean peak plasma concentration was 0.32 μM, and the area under the plasma concentration time curve was 2.68 μM·h.

DISCUSSION

Evaluating the safety and effectiveness of chemotherapeutic agents administered intrathecally presents unique challenges

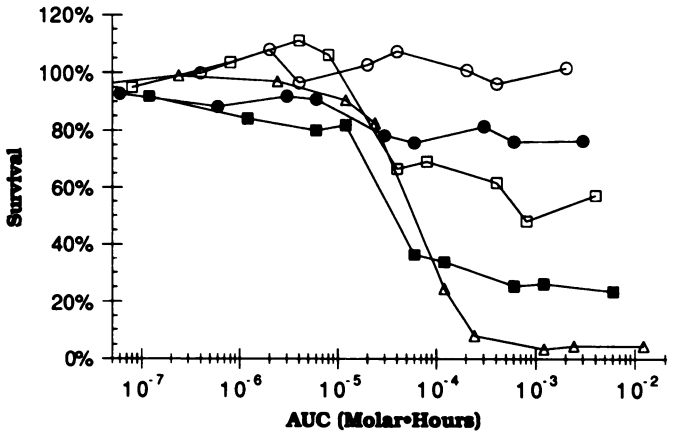

Fig. 2. Survival *versus in vitro* AUC of 6-MP in the MOLT-4 cell line. Exposure times are the same as in Fig. 1.

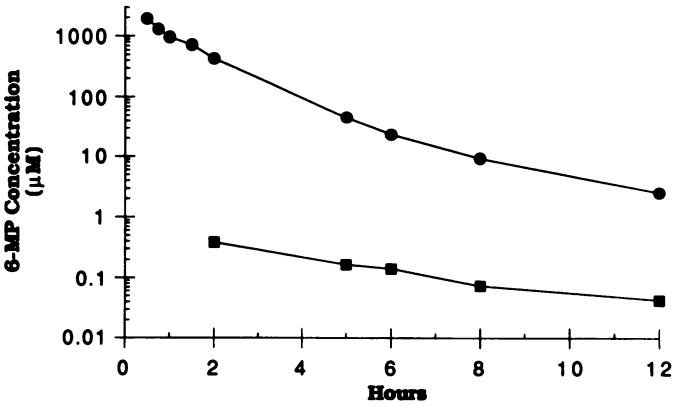

Fig. 3. CSF (●) and plasma (■) concentration-time curves for 6-MP in monkeys treated with a 3.2-mg IT dose ($n = 3$).

Table 1 *Pharmacokinetics of intrathecal 6-mercaptopurine*

	CSF $AUC_{CSF_{0\rightarrow12}}$[a] ($\mu M \cdot h$)	Plasma $AUC_{CSF_{0\rightarrow12}}$[a] ($\mu M \cdot h$)	Peak CSF concentration[b] (μM)	Peak plasma concentration[b] (μM)	CSF clearance[a] (ml/min)	Half-life[c] (h)
Monkey						
3.2-mg dose ($n = 3$)	3548 ± 377	1.49 ± 0.68	1882	0.37 (0.25–0.46)	0.071 ± 0.013	1.8 (1.5–2.1)
Patients						
5-mg dose ($n = 3$)	1507 ± 552	ND[d]	613 (275–1355)	ND	0.63 ± 0.17	1.4 (1.1–3.3)
10-mg dose ($n = 4$)	1941 ± 1637	2.68 ± 1.4	762 (177–2553)	0.32 (0.15–0.66)		

[a] Mean ± SD.
[b] Geometric mean (range).
[c] Harmonic mean (range).
[d] ND, not determined.

Table 2 *Patient characteristics*

Age	
Median (range)	12 years (3.5–16 years)
Sex	
M/F	8/4
Diagnosis	
Acute lymphoblastic leukemia	11
Non-Hodgkins lymphoma	1
No. of prior relapses	
Median (range)	3 (2–6)
Prior therapy	
Intrathecal chemotherapy	
Methotrexate + ara C	12
Hydrocortisone	7
Diaziquone	3
Thiotepa	4
Cranial ± spinal x-ray therapy	
>2400 cGy	4
≤2400 cGy	8

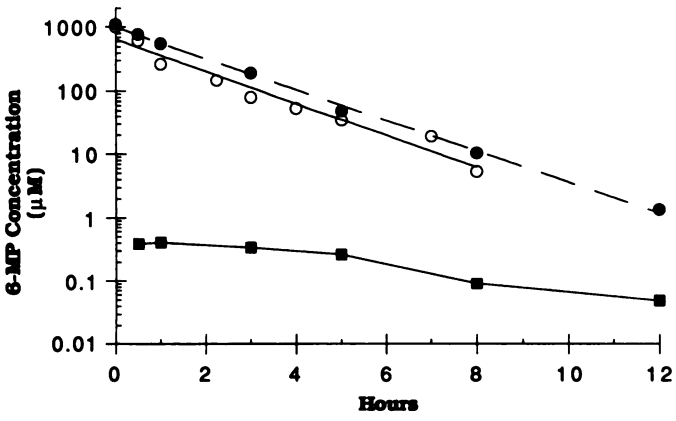

Fig. 4. CSF concentration-time curves for 6-MP in patients treated with a 5-mg (○, $n = 3$) or 10-mg dose (●, $n = 4$). Plasma concentrations following an IT 10-mg dose are also shown (■, $n = 2$)

not encountered in evaluating systemically administered drugs. Following an extensive preclinical evaluation, a potential new systemic anticancer drug undergoes a phase I clinical trial to determine its toxicity spectrum and to estimate its maximum tolerated dose. The dose is escalated until dose-limiting toxicities are consistently observed in a cohort of patients. Clinicians are willing to tolerate these toxicities, since they usually are readily reversible.

For phase I development of a new IT agent, the classic drug development approach is not acceptable. The wide experience with IT methotrexate provides evidence for exercising extreme caution in the escalation of intrathecal doses of drug. In studies of patients treated with doses of IT methotrexate based on body surface area, neurotoxicity was found to correlate with elevated CSF drug concentration (12, 13). The manifestations of neurotoxicity were often irreversible or fatal and included patients with severe meningismus, sustained grand mal seizures, irreversible cerebellar dysfunction, quadriplegia, and fatal myelopathy. In addition, in the evaluation of other antimetabolites for IT administration performed in our laboratory, we have observed irreversible and in some instances fatal neurological sequelae in experimental animals during dose escalation. Given the type of toxicity likely to be encountered, performance of a classic phase I trial for an IT drug, in which cohorts of patients receive progressively escalated doses until toxicity is consistently observed, would be unethical.

For this reason, the approach taken in the present study appears to be the most appropriate one. Initially, the optimum cytotoxic concentration of 6-MP was determined *in vitro*. The safety of administering 6-MP intrathecally was then demonstrated in rhesus monkeys. The pharmacokinetic parameters derived from the animal experiments formed the basis for calculating the IT dose that would be required to achieve the desired 6-MP CSF concentrations in humans.

The rhesus monkey model accurately predicted the pharmacokinetics of IT 6-MP when administered to humans, considering the known differences in CSF volume and bulk flow rate. Drugs that are administered intrathecally are usually cleared from the ventricles by bulk flow, which in humans is approximately 0.4 ml/min (14, 15). The rate of clearance of 6-MP from the ventricles (0.63 ml/min) exceeded bulk flow, suggesting an additional mechanism of elimination. The peak plasma concentration (0.32 μM) and the plasma AUC (2.68 $\mu M \cdot h$) following a 10-mg IT dose were similar to the mean peak plasma concentration (0.89 μM) and AUC (2.32 $\mu M \cdot h$) found following administration of a standard 75-mg/m^2 dose p.o. of 6-MP (16). It thus appears reasonable to withhold the dose p.o. of 6-MP on days that patients receive an IT dose.

6-MP *in vitro* behaved similarly to other antimetabolites, demonstrating both schedule and dose dependency (17). In the three human cell lines studied, the 50% inhibitory concentrations for 6-MP following 48 h of drug exposure were between 1 and 3 μM, findings which are consistent with previous studies (18, 19). No significant cytotoxicity occurred at 6-MP concentrations <1 μM, regardless of duration of drug exposure, suggesting a concentration threshold for 6-MP cytotoxicity. The steep portion of the dose-response curve, which was independent of the duration of drug exposure, occurred between concentrations of 1 and 10 μM. The level of the plateau in cytotoxicity (maximum cytotoxicity), which occurred at concentrations >10 μM, was proportional to the duration of drug exposure, with a

cell kill >50% occurring only with durations of exposure ≥12 h.

A 10-mg IT dose of 6-MP in patients sustained CSF 6-MP concentrations >1 μM for 12 h and thus exceeded the cytotoxicity threshold of 6-MP for this interval. This dose is recommended in view of (*a*) the *in vitro* data demonstrating that no increase in cytotoxicity is observed at 6-MP concentrations exceeding 10 μM, (*b*) the risk of irreversible or fatal neurotoxicity with dose escalation, and (*c*) the demonstrated clinical activity of 6-MP administered at this dose. Based on the *in vitro* data demonstrating the importance of prolonging the exposure to concentrations of 6-MP achieved with a 10-mg dose, we are currently performing an additional study that administers IT 6-MP doses on a more frequent schedule.

Intrathecal 6-MP demonstrated impressive activity in this cohort of heavily pretreated patients, with four of nine patients achieving a CR. Importantly, no patient experienced any significant toxicity. It thus appears that 6-MP can be safely and effectively administered to patients with meningeal malignancy. 6-MP may soon be added to the list of IT agents that are effective in the treatment of meningeal leukemia.

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