Toxicity and Pharmacokinetics of a New Antifolate, 2,4-Diamino-5-adamantyl-6-methylpyrimidine, in Dogs

Sigmund F. Zakrzewski, Zlato Pavelic, William R. Greco, Gary Bullard, Patrick J. Creaven, and Enrico Mihich


ABSTRACT

The toxicology of a potentially useful antitumor agent, 2,4-diamino-5-adamantyl-6-methylpyrimidine (DAMP), and its ethanesulfonate salt has been studied in beagle dogs after 1 to 20 doses. Two types of toxicity could be discerned: the acute central nervous system toxicity manifested by vomiting, convulsions, and minor hypothermia; and the antiproliferative toxicity. Characteristically, the animals could evidence of a cumulative effect of the drug with respect to antiproliferative toxicity. Characteristically, the animals could be protected against the antiproliferative toxicity by simultaneous administration of folic acid.

The pharmacokinetics of the ethanesulfonate salt of DAMP was studied after i.v. administration of sublethal doses (5 mg/kg) of tritium-labeled drug. Sixty-three percent of the administered dose was excreted in the urine and 10% was excreted in the feces within 48 hr after drug administration. Thus, about 27% of the drug was not recovered, and it is possible that it persists in the tissues for a period of several days. Analysis of the plasma and urine revealed that DAMP was metabolized rapidly. At least 2 metabolites were found in plasma and urine, one lipophilic and one hydrophilic, the latter being the predominant form. Pharmacokinetic data were successfully fitted to a model consisting of central and peripheral DAMP compartments and a DAMP metabolite compartment. DAMP was very rapidly sequestered in the peripheral compartment with a rapid phase half-life of 23 sec. The slower phase of DAMP plasma disappearance had a half-life of 3 hr. The short plasma half-life and rapid metabolism distinguished this drug from other lipophilic antifolates.

INTRODUCTION

Diaminopyrimidines substituted in position 5 with a lipophilic group are potent inhibitors of dihydrofolate reductase (1), and some of them have found clinical application. The best known are the antimalarial drug pyrimethamine (4), the antibacterial agent trimethoprim (9), and DDMP (13), which has received extensive clinical evaluation as an antineoplastic agent (15). Compounds of this class resemble methotrexate in having the 2,4-diaminopyrimidine moiety but differ from it by the absence of the strongly polar and negatively charged acidic groups. The presence of the hydrophobic 5-substituent renders these compounds lipid soluble (11). These structural features make them, like methotrexate, strong inhibitors of dihydrofolate reductase (11, 12), but they also enhance their ability, unlike methotrexate, to penetrate cell membranes (7, 8). Their facile uptake by the cell and their lipid solubility suggest their possible use against solid tumors and meningeal leukemia. In fact, limited success has been reported in clinical trials with pyrimethamine against meningeal leukemia (5). The ability to penetrate the blood-brain barrier, although sometimes desirable, may in some cases be associated with CNS toxicity. In the case of DDMP and several other pyrimidines, inhibition of brain histamine methyltransferase, an enzyme responsible for inactivation of histamine, has been observed (3). DAMP is one of the newer diaminopyrimidine antifolates and shares the general properties of this class of compounds, namely, strong inhibition of dihydrofolate reductase (10), lipid solubility (11), and rapid uptake by the cell (7). DAMP has been found to have antitumor activity against a methotrexate-resistant rat tumor, Walker carcinosarcoma 256 (18). At higher doses in rats, it produced CNS toxicity manifested by convulsions in which some cases were followed by death. DAMP was metabolized and excreted more rapidly than was DDMP; thus, these compounds may be expected to differ in their overall pharmacological behavior. The antitumor activity and toxicity (18) as well as pharmacokinetics, tissue distribution, and disposition of DAMP in rats (7) have been reported previously. In this report, the toxicity, pharmacokinetics, and disposition of DAMP in dogs will be described. A preliminary report on this topic has been published (16).

MATERIALS AND METHODS

Chemicals. DAMP was custom prepared by Starks Associates, Inc., Buffalo, N. Y., according to a published procedure (12) and was converted to DAMP-ES as described elsewhere (13). [3H]DAMP was prepared by the Radiochemical Centre, Amersham, Buckinghamshire, England, and was purified in this laboratory by recycling chromatography on columns of Sephadex G-25 (19). The radio purity of the final product was determined by TLC. All radioactivity was located in a single spot, and the purity could be estimated at 93 to 99% depending on the batch. The specific radioactivity of the purified [3H]DAMP-ES was about 30 mCi/μmol.

Sephadex G-25 fine grade was obtained from Pharmacia Fine Chemicals, Piscataway, N. J. The precoated TLC plates of silica gel on aluminum sheets (20 x 20 cm) containing fluorescent indicator were obtained from E. Merck Laboratories, Inc., Darmstadt, Germany, and those precoated with cellulose with fluorescent indicator on plastic sheets were obtained from Eastman Kodak Co., Rochester, N. Y.

In order to establish whether or not tritium is released from [3H]-DAMP during metabolism, [3H]DAMP was incubated with rat liver homogenates, NADPH, and a NADPH-regenerating system as described elsewhere (17). An aliquot of the supernatant of this incubation...
mixture was used for radioactivity. Subsequently, the supernatant was lyophilized, the residue was reconstituted to the original volume with water, and an aliquot was counted again. The supernatant was now chromatographed on Sephadex G-25 (17). Two peaks of radioactivity were obtained, one representing unchanged DAMP and the other representing its metabolite or metabolites. The total radioactivity eluted from the column was the same as that of the supernatant before and after lyophilization. Thus, it appears that there was no significant loss of tritium during the biotransformation of DAMP.

Animals. For toxicology studies, adult beagles of both sexes were used only if free of obvious signs of illness and after the animals had been isolated and observed for at least 3 weeks. At the beginning of the isolation period, the dogs were examined for ecto- and endoparasites. The dogs were fed Purina dog chow and were given water ad libitum.

For pharmacokinetic studies, only male beagles weighing between 8 and 12 kg were used. They were fed Wayne dog food and were given water ad libitum.

Toxicological Studies. Both DAMP and DAMP-ES were studied. DAMP was administered p.o. in gelatin capsules (Eli Lilly and Co., Indianapolis, Ind.). Either DAMP-ES was given p.o. as described for DAMP, or it was dissolved in sterile distilled water (DAMP-ES is readily soluble in water but not in 0.9% saline) at concentrations ranging from 5.0 to 10.0 mg/ml and injected i.v. in volumes ranging from 0.08 to 1.25 ml/kg. The drug was always given prior to the daily feeding.

Food intake and body weight were measured daily.

Hematological and biochemical parameters were measured at different times before, during, and after treatment according to standard procedures. They were hematocrit, hemoglobin, RBC, WBC, reticulocytes, platelets, differential count, prothrombin time, and the serum levels of alkaline phosphatase, bilirubin, calcium, chloride, cholesterol, creatinine, glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, LDH, Mg++, K+, Na+, phosphate and blood glucose, and urea nitrogen.

Tissues for microscopic examination were obtained only in dogs which were sacrificed. Specimens were fixed in Zenker-formol (10% formalin). All sections were stained with hematoxylin-eosin unless otherwise specified. The tissues studied were heart, aorta, lips, tongue, duodenum, jejunum, colon, liver, gall bladder, pancreas, adrenal, para-thyroid, pituitary, thyroid, bone marrow, spleen, liver, mammary gland, thymus, bronchial mesentery and mandibular lymph nodes, dia-phragm, cerebrum, cerebellum, medulla, spinal cord, peripheral nerve, gonads, prostate, uterus, trachea, lung, kidney, ureter, and bladder.

Pharmacokinetic Study. Five dogs were studied. They received i.v. injection into a forepaw vein of an aqueous solution of [1H]DAMP-ES, 5 mg/ml, (5 mg/kg = 13.5 μmol/kg) over a period of 20 to 30 sec. The specific radioactivity of the drug varied between 2.2 and 2.7 μCi/μmol, except for the first experiment in which a specific radioactivity of 0.62 μCi/μmol was used. Blood (1 to 2 ml for radioactivity determination or 3 to 9 ml for chromatography) was drawn at intervals, either from the opposite forepaw or from the jugular vein, and placed immediately in the tubes containing heparin as anticoagulant. After centrifugation, the plasma was removed and refrigerated. Urine was collected at 6, 24, 30, and 48 hr, and feces were collected at 24 and 48 hr.

Extraction of DAMP from Plasma. Aliquots of plasma (500 μl) were transferred into glass-stoppered tubes and diluted with 500 μl of 0.1 M NaOH. One ml of dichloroethane was added to each tube, and the tubes were rotated for 15 min in a tube rotator (Scientific Equipment Products). The tubes were then placed vertically to allow the layers to separate. For counting of radioactivity, 500 μl of each layer was used. (There was usually a foamy interphase which contained finely dispersed precipitated protein. This interphase was discarded.) Samples for chromatography were prepared in the same way except that the procedure was scaled up. In each case, 3 ml of plasma were mixed with 3 ml 0.1 M NaOH and extracted with 6 ml dichloroethane. For chromatography, 4 ml of each aqueous phase were removed, acidified with 250 μl 1.0 M HCl, and clarified by centrifugation. Also, the organic phase was carefully removed with Pasteur pipets and evaporated to dryness in a stream of air, and the residues were redisssolved in about 4 ml of 1.0 M HCl. The chromatography was carried out as described below. About 50 three ml fractions were collected. Aliquots (500 μl) of fractions were counted with the exception of the organic phases from the 3- and 6-hr plasma samples where 1- and 2-ml aliquots were used, respectively.

Extraction of DAMP and its Metabolites from Urine. Urine samples (6 ml) were adjusted to pH 10 and extracted with dichloroethane (6 ml) in a rotary shaker for 15 min. The aqueous layer was removed with a Pasteur pipet and adjusted to pH 3 with 5 μl HCl, 500 mg of charcoal (Darco G-60) were added, and the mixture was stirred for 10 min. After filtration and washing with water, the charcoal was suspended in 6 ml of ethanol:ammonium hydroxide:water (100:25:75), stirred for 15 min, and filtered off. The eluate was concentrated in a vacuum at 50° to about 1 ml.

The dichloroethane extract was used directly for TLC chromatography. For chromatography on Sephadex G-25, it was evaporated to dryness, and the residue was resuspended in 0.1 M HCl (1 ml).

Chromatographic Procedures. It has been established earlier (17) that DAMP is retained on a Sephadex G-25 column beyond the total column elution volume, and thus it can be separated from its metabolites which have less affinity for the column than does DAMP. Thus, for chromatography, Sephadex G-25 columns (1.6 x 37 cm) were used. To the solution to be chromatographed, nonradioactive DAMP-ES (0.5 to 1.0 mg) was added as a marker. The eluting solvent was 0.1 M HCl daily (17). The UV absorbance of the effluent was continuously monitored at 254 nm with a Gilson UV monitor connected to a recorder. Fractions were collected in a Gilson Mini Escargot fraction collector, and aliquots of the fractions were counted for radioactivity.

Urine samples were chromatographed either on Sephadex columns as described above or on TLC silica gel with ethoxyethanol:ethyl acetate:4% formic acid (2:3:1); this mixture was separated into 2 phases, and the upper phase was used) or on TLC cellulose with 50% ethanol. The strips were first viewed under UV and then cut into 0.5-cm segments. Each segment was counted for radioactivity using 0.5 ml of water and 10 ml of scintillation solvent.

Determination of Radioactivity in Erythrocytes. To determine radioactivity in the blood cells, the cell pellet was reconstituted to the original volume of the blood sample with water. This caused hemolysis of the cells but, because of the release of hemoglobin, the direct counting of this liquid was impractical. Therefore, 250-μl aliquots of this suspension were burned in a Packard Tri-Carb Model 306 sample oxidizer.

Determination of Radioactivity in Feces. The whole feces of each collection was homogenized in a Waring blender with 350 ml of water. The homogenate was then lyophilized, and the weight of the dry powder was determined. For determination of radioactivity, samples of 100 mg were burned in the sample oxidizer.

Counting of Radioactivity. All counting was performed in a Packard Tri-Carb Model 2450 liquid scintillation spectrometer. The liquid samples like urine, plasma, their extracts, and fractions from the chromatographic columns were counted directly using Hydromix (Yorktown Research, Hackensack, N. J.) as scintillation solvent. Counting efficiency was calculated using a prerecorded program on the WANG 600 desk top computer.

Statistical Analysis of Data. Pharmacokinetic data were fit to the appropriate equations with nonlinear regression curve fitting using the NONLIN software package (14) on a Unicomp 90/80 mainframe computer.

RESULTS

Toxicity of DAMP. The lethal effects observed after p.o. administration of the compound are summarized in Table 1. A suggestion of cumulative effects is evident insofar as 25 mg/
kg were not lethal to a dog when given as a single dose whereas 30 mg/kg were lethal to 3 dogs when given divided into 5 doses over 5 consecutive days. It is apparent that the total dose of 15 mg/kg was not lethal whether given over a period of 4 or 10 days.

The course of intoxication in the dog given a single dose of DAMP consisted of vomiting, convulsions, and a transient minor drop in body temperature. All of these effects occurred within 0.5 hr after administration and lasted for a few hours, with complete recovery 24 hr after dosing. The rapidity of onset of the convulsions is consistent with absorption of the compound by the p.o. route, sufficient to reach systemic drug levels causing an acute effect. Because of the convulsions, in all subsequent cases, the daily dose of DAMP was given divided into 2 half-doses.

The course of intoxication in the 6 dogs given 5-day courses of lethal doses, namely, 25, 12.5, and 6.3 mg/kg/day, were generally comparable and indicated a similarity of lethal syndromes. Vomiting and diarrhea were noted in each dog; anorexia and loss of body weight were noted in most of these dogs. Hematological changes consisted of leukopenia. In 2 cases, lymphocytopenia and reticulocytopenia were noted; in the other 4 dogs, neutropenia was also seen. In most cases, these hematological changes started to become apparent as early as Day 2.

Relatively slight blood chemistry changes were noted terminally in 3 of these dogs, namely, in the dog treated with 25 mg/kg/day and in one dog each treated with 12.5 and 6.3 mg/kg/day. They consisted of increases in serum glutamic-pyruvic transaminase LDH, urea nitrogen, magnesium, and phosphate and a decrease in chlorides and blood glucose, not all of these changes occurring in the same or in all of these dogs. Because of their terminal occurrence, the specificity of these small changes is doubtful in comparison with the hematological and gastrointestinal toxicities seen.

The toxicological syndrome seen in the dog treated with 6.3 mg/kg/day for 8 days was quite comparable to that noted in the 6 dogs described above.

In the 6 dogs treated for 5 or 10 consecutive days with nonlethal doses (3.1 or 1.6 mg/kg/day), vomiting, diarrhea, anorexia, and loss of body weight were usually less marked and transient. Hematological changes similar to those described above were noted in the 2 dogs treated with 3.1 mg/kg/day but were completely reversible after the end of treatment; no significant change was noted in the other dogs. No significant blood chemistry change was observed in these animals.

Toxicity of DAMP-ES. The lethal effects observed after p.o. or i.v. administration of the compound are summarized in Table 2. By the p.o. route, DAMP-ES appeared to be somewhat less toxic than DAMP as reflected by the fact that only one of 2 dogs treated with 12.5 mg/kg died, whereas 5 of 5 dogs treated with this or one-half of this dose of DAMP, died (Table 1). By the i.v. route, a suggestion of cumulative effect was indicated by the fact that 30 mg/kg total dose appeared to be lethal to about one-half of the dogs regardless of whether treatment was over a period of 5, 10, or 20 days. The total dose of 15 mg/kg was lethal to only one of 9 dogs treated over these different periods of time. It is of interest that the lethal doses of DAMP-ES by the i.v. route were in the same range as the lethal doses of DAMP by the p.o. route.

The course of intoxication consisted of diarrhea in 3 of the 4 dogs treated p.o., with bloody diarrhea consistently seen from Day 4 on in the dog which died and occasionally in the other 2 dogs. Vomiting and anorexia were also seen in these 3 dogs. Lymphocytopenia was noted in the 2 dogs treated with 12.5 mg/kg, and it was reversible in the dog which recovered. No other significant sign of intoxication was noted.

The course of intoxication observed in the 30 dogs treated i.v. was as follows. In the 3 dogs treated with 12.5 mg/kg/day, convulsions were seen within minutes; in the dog which survived for 6 days, this effect occurred daily. Vomiting and diarrhea were also noted, and in the dog which survived for 6 days diarrhea became bloody from Day 4 onwards. Among the 8 dogs treated with 6.3 mg/kg/day for 5 days, convulsions or tremors were seen in 5; vomiting, diarrhea, and anorexia in 7; hypothermia in 7; and loss of body weight in 7. In the surviving animals, all of these symptoms were reversible. Hematological changes in these 8 dogs consisted of leukopenia which involved both neutrophils and lymphocytes and was reversible in the 2 dogs sacrificed on Days 14 and 17. Blood chemistry changes varied in extent and significance and included increases of serum creatinine, LDH, glutamic-oxaloacetic transaminase pyruvic transaminase LDH, urea nitrogen, magnesium, and phosphate and a decrease in chlorides and blood glucose, not all of these changes occurring in the same or in all of these dogs. Because of their terminal occurrence, the specificity of these small changes is doubtful in comparison with the hematological and gastrointestinal toxicities seen.
aminase chlorides, and phosphate and decreases of blood glucose; these changes were in most cases either erratic or terminal, and no pattern of organ-specific dysfunctions appeared to emerge.

The course of intoxication in the 5 dogs which died after treatment with 10 doses of DAMP-ES was essentially similar to that outlined above except that convulsions were not seen. Leukopenia was noted and was progressively more severe until death. Blood chemistry changes were minor, erratic, or terminal and did not appear to provide evidence for specific effects. In the dog treated with 20 doses of DAMP-ES which was sacrificed when moribund on Day 23, the course of intoxication was very similar to that described above except that it was slower to develop and was protracted in time.

Except for the 2 dogs treated with DAMP-ES (0.8 mg/kg/day) for 20 days, all the other animals which survived treatment and were sacrificed 1 week after the end of treatment or later showed signs of intoxication qualitatively similar to those described for the dogs which died; they had recovered or were recovering at the time they were sacrificed. The 2 dogs treated with DAMP-ES (6.3 mg/kg/day) for 5 days had early signs of intoxication comparable to those of the 2 dogs treated with the same dose for 10 days. However, whereas the former recovered, the latter developed a progressive syndrome and died. This further indicates the intrinsic reversibility of the action of the compound.

Folinic Rescue in Dogs. Because of the proven antifolate activity of DAMP-ES, it was of interest to see whether folinic acid could reverse the toxicities of the compound. This would provide added safety to the Phase I trial. Four dogs were treated with DAMP-ES i.v. and, 1 hr after each injection, with folinic acid at 5 mg/kg/day (Table 3).

The acute toxicity of DAMP-ES was not affected by folinic acid because the dog treated with 12.5 mg/kg died on Day 0 after convulsions which did not appear to be reduced by the rescue agent. In contrast, the antiproliferative toxicities of DAMP-ES were essentially completely prevented by folinic acid; it should be stressed that 10 daily courses of DAMP-ES alone (6.3 mg/kg) were lethal to 2 of 2 dogs and that significant antiproliferative toxicity was also evident in all the 4 dogs treated with 3.1-mg/kg/day doses of the compound alone.

It is tentatively concluded, therefore, that the antiproliferative toxicity of DAMP-ES is a major cause of death (except for the acute convulsive syndrome) and that it can be prevented by the administration of folinic acid.

Pathological Findings. The pathological effects of DAMP and DAMP-ES were studied in 49 dogs treated either p.o. or i.v. with 0.8 to 25 mg/kg/day.

In the 7 dogs treated with lethal doses of DAMP, pulmonary edema and signs of gastrointestinal toxicity were the most frequent autopsy findings; atrophy of lymphoid tissues was seen in 2 dogs, pancreatic necrosis was seen in 2 dogs, and thrombi were seen in the right heart of 2 dogs. Microscopic findings included pancreatic necrosis or atrophy in 5 dogs, severe enterocolitis in 6 dogs, lymphoid atrophy or necrosis in 5 dogs, bone marrow depression or atrophy in 5 dogs, necrosis of the ovaries in the dog treated with 25 mg/kg/day, and degenerative changes in the bladder of 3 dogs. Except for the pancreatic effects seen, the other signs were consistent with the well-known antiproliferative toxicity of antifolates.

In the 6 dogs which were treated with nonlethal doses, only minor pathological changes were noted.

As a whole, pathological findings in dogs treated with DAMP-ES are similar in all the lethally intoxicated animals regardless of route or schedule of drug administration.

Among the dogs treated p.o., lung edema, enterocolitis, lymphoid atrophy, occasional epithelial damage, and bone marrow necrosis or atrophy were seen only after treatment with 12.5 mg/kg/day; bone marrow damage was particularly severe in the dog which died. No significant changes were observed in the 2 dogs which had been treated with a lower dose.

After i.v. treatment, 5 dogs died on Day 6 (12.5 or 6.3 mg/kg/day for 5 days), and 2 were sacrificed for pathology on Day 5, when they were showing a well-established toxicological syndrome similar in its characteristics to that of the dying dogs. In general, all of these dogs showed similar changes which included enterocolitis with typical changes in the intestinal mucosa, pancreatic alterations up to necrosis, lymphoid atrophy, various epithelial changes (e.g., in bladder) and, consistently, bone marrow hypoplasia or atrophy. In most males, testicular atrophy or hypoplasia was also noted. After 10 daily courses, the same changes were noted in the dogs which died on Days 7 to 12 but were usually more severe (in some of these dogs, postmortem changes prevented the pathologist from reaching meaningful conclusions). It is of interest that the same types of changes were noted in the dog treated with 20 daily courses which was sacrificed when moribund on Day 23.

Pathological changes were minor or absent in the dogs which survived the effects of treatment.

Pharmacokinetics of DAMP-ES in Plasma. The level of radioactivity in plasma following rapid i.v. injection of \(^{3}H\)-DAMP-ES increased gradually with time, reaching a peak at about 3 hr after drug administration (an example in Dog 1 is given in Table 4). At the same time, the content of radioactivity

<table>
<thead>
<tr>
<th>Table 3</th>
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<tbody>
<tr>
<td><strong>Folinic rescue from DAMP-ES toxicity in dogs</strong></td>
</tr>
<tr>
<td><strong>Folinic acid calcium salt was given to each dog in the same number of doses as for DAMP-ES and fractionated the same as for DAMP-ES at a dose of 5 mg/kg/day. Daily dose given fractionated into 2 daily injections.</strong></td>
</tr>
<tr>
<td><strong>Treatment (mg/kg/day) i.v.</strong></td>
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<tr>
<td>---</td>
</tr>
<tr>
<td>12.5</td>
</tr>
<tr>
<td>6.3</td>
</tr>
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<td>6.3</td>
</tr>
</tbody>
</table>

\(a\) Given daily for 5 days followed by 2 days of rest for the number of doses indicated or until death.

\(b\) Counted from day of first dose.

\(c\) Sacrificed for pathology. Numbers in parentheses, day of sacrifice of survivors.

<table>
<thead>
<tr>
<th>Table 4</th>
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<tbody>
<tr>
<td><strong>Distribution of the radioactivity between plasma and RBC after i.v. administration of (^{3}H)DAMP-ES, 5 mg/kg, with a specific activity of 2.2 (\mu)Ci/(\mu)mol in dog 1</strong></td>
</tr>
<tr>
<td><strong>Time after injection (hr)</strong></td>
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<tr>
<td>---</td>
</tr>
<tr>
<td>Plasma</td>
</tr>
<tr>
<td>0.3</td>
</tr>
<tr>
<td>0.4</td>
</tr>
<tr>
<td>3.0</td>
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<tr>
<td>6.0</td>
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<tr>
<td>24.0</td>
</tr>
</tbody>
</table>

\(a\) Sum of DAMP and its metabolites.
in RBC (Table 4, Column 3) decreased steadily with time. Table 4, Column 4, shows that the 1:1 initial distribution of radioactivity between cells and plasma changed to 1:2.

A control experiment with \(^{[3H]}\)DAMP-ES revealed that more than 95% of DAMP could be removed from an alkaline aqueous solution by a single extraction with an equal volume of dichloroethane. In the presence of plasma, the efficiency of extraction was determined in 7 experiments to be 92 ± 3.5 (S.D.)%. Deproteinization of plasma before extraction was not practical because considerable and variable amounts of radioactivity (30 to 80%) were trapped in the precipitated protein. Therefore, it was decided to use direct extraction of plasma with dichloroethane as a method of separation of DAMP from its metabolites.

Chart 1 shows a typical series of chromatographic profiles of the aqueous and organic phases of plasma at different times after administration of \(^{[3H]}\)DAMP-ES. No DAMP is present in the aqueous phase in plasma. In the organic phase, in addition to DAMP another peak of radioactivity appears, and its proportion increases with time. Judging by its elution volume, this compound is different from that present in the aqueous phase.

To evaluate further the unusual observation that total radioactivity in plasma after i.v. \(^{[3H]}\)DAMP-ES increased with time from 6 min to 4 hr postinjection, a detailed pharmacokinetic analysis was carried out on the plasma data from 3 dogs (Dogs 3, 4, and 5). The results were similar for the 3 dogs; therefore the parameter estimates and time courses are reported for only one dog (Dog 5). The aliquots of plasma samples, obtained at frequent intervals, were first counted for total radioactivity. The plasma samples were then extracted with dichloroethane, and the content of DAMP in each sample was determined from the chromatographic analysis as described above.

The data for DAMP and its metabolite were then simultaneously fit to the set of Equations A to D using 1/\(C_0^2\) as a weighing factor.

\[
C_0 = \frac{C_0(a - k_21)e^{-at}}{a - b} + \frac{C_0(k_21 - b)e^{-bt}}{(a - b)} \tag{A}
\]

\[
C_M = \frac{C_M(k_21 - a)e^{-at}}{(km - a)(b - a)} + \frac{C_M(k_21 - b)e^{-bt}}{(km - b)(a - b)} + \frac{C_M(k_21 - km)e^{-km}}{(a - km)(b - km)} \tag{B}
\]

where

\[a + b = k_{12} + k_{10} + k_1 \tag{C}\]

\[ab = k_{21}(k_{10} + k_l) \tag{D}\]

\(C_0\) is the plasma concentration of DAMP; \(C_M\) is the plasma metabolite concentration; \(C_0\) is the DAMP concentration at Time 0; \(k_{12}, k_{21}, k_{10}, k_1\), and \(k_m\) are rate constants for the model in Chart 3, defined in the legend to Chart 2. These equations were derived by the method of Laplace transforms from the model in Chart 2. The fitted curves are displayed in Chart 3. The estimated parameters are: \(C_0\), 12.7 µM; \(k_{10}\), 0.815 hr\(^{-1}\); \(k_{12}\), 98.8 hr\(^{-1}\); \(k_{21}\), 5.56 hr\(^{-1}\); \(k_m\), 3.76 hr\(^{-1}\); and \(k_m\), 0.446 hr\(^{-1}\). The corresponding half-time for the transfer of DAMP from the central to the peripheral compartment was 25 sec. The empirical rate constant for the rapid phase of drug disappearance was 109 hr\(^{-1}\), the empirical rate constant for the second phase was 0.234 hr\(^{-2}\), the rapid phase half-life was 23 sec, the second half-life was 2.96 hr, and the volume of distribution was 10.6 liters.

Excretion of DAMP and Its Metabolites. Table 5 shows the excretion of the radioactivity in urine and feces after a dose of \(^{[3H]}\)DAMP-ES. DAMP and its metabolites were removed from urine by extraction of urine samples with dichloroethane, and
removal of the nonextractable radioactivity on charcoal was as described in "Materials and Methods."

Chart 4 represents radioactive profiles of TLC chromatograms of the dichloroethane extracts of 6- and 25-hr urine samples of one dog (Dog 1). The relative quantities of each compound could be estimated by integrating the areas under the peaks. The charcoal eluate of the aqueous phase when chromatographed in the same system produced a single peak at the origin of the strip. When chromatographed on TLC cellulose with 50% ethanol, it migrated as a broad peak at Rf 0.5. The quantitative distribution of radioactivity in 25-hr urine of 2 dogs is presented in Table 6. To compare DAMP metabolites from urine to those found in plasma, the dichloroethane extract and the eluate from charcoal were chromatographed in sequence on the same Sephadex G-25 column. The radioactive profiles of these chromatograms are presented in Chart 5.

Table 5
Excretion of radioactivity in urine and feces after i.v. administration of [3H]DAMP-ES, 5 mg/kg, to 3 dogs

<table>
<thead>
<tr>
<th>Time after injection (hr)</th>
<th>% of administered dose</th>
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<tbody>
<tr>
<td></td>
<td>Dog 1</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>6</td>
<td>21.4</td>
</tr>
<tr>
<td>24</td>
<td>50.3</td>
</tr>
<tr>
<td>30</td>
<td>61.0</td>
</tr>
<tr>
<td>48</td>
<td>63.3</td>
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</tbody>
</table>

% of administered dose

Feces

The possibility of some contamination of feces with urine could not be excluded.

Table 6
Distribution of radioactivity in urine 25 hr after administration of [3H]DAMP-ES, 5 mg/kg, with specific radioactivities of 0.62 and 2.56 μCi/μmol, respectively, in 2 dogs

<table>
<thead>
<tr>
<th>% of total radioactivity recovered</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog 1</td>
<td>Dog 2</td>
</tr>
<tr>
<td>2.4</td>
<td>5.3</td>
</tr>
<tr>
<td>11.8</td>
<td>16.7</td>
</tr>
<tr>
<td>73.6</td>
<td>66.3</td>
</tr>
<tr>
<td>1.9</td>
<td>4.5</td>
</tr>
<tr>
<td>10.3</td>
<td>7.0</td>
</tr>
</tbody>
</table>


Chart 5. Chromatographic profiles of the organic phase (top) and aqueous phase (bottom) of a urine sample of Dog 1 collected 25 hr after drug administration. Column: Sephadex G-25 eluted with 0.1 M HCl, 4-ml fractions; 50-μl aliquots were used for counting.

Judging by the elution volume, it is evident that the same compounds are present in plasma as in urine.

DISCUSSION

As is evident from the results obtained, DAMP and DAMP-ES appear to be essentially identical in terms of the qualitative aspects of the toxicological syndrome elicited. When comparing the 2 preparations after p.o. administration and when comparing the effects of DAMP p.o. and those of DAMP-ES i.v., it is apparent that the syndromes elicited are quite similar. In general, DAMP-ES is somewhat more acutely toxic after i.v. administration than is DAMP after p.o. administration. If one considers the fact that the molecular weight of DAMP is 258.4 and that of DAMP-ES is 368.5, the equal doses contain about 30% less of the active moiety in DAMP-ES than in DAMP. This may be the reason why DAMP-ES appears somewhat less toxic than DAMP p.o. The antiproliferative toxicities of the 2 preparations appear quite similar at equitoxic doses. Thus, it is reasonable to conclude that, from a qualitative point of view, the 2 preparations are similar.

The convulsions caused by DAMP and DAMP-ES are indicative of CNS toxicity. This is consistent with results obtained in rats where high levels of radioactivity were found in brains after i.v. or i.p. administration of 14C-labeled drug (17). Although the CNS toxicity was not affected by folinic acid, the antiproliferative toxicities of DAMP-ES were completely prevented by folinic acid.

The model of DAMP pharmacokinetics in Chart 2 adequately described the combined disappearance data for DAMP and its metabolites. According to the model, immediately following injection, the drug is rapidly taken up by tissue, leading to a marked fall in plasma level in the first circulation, too early to be seen in our samples. As the drug is metabolized, the metabolites distribute chiefly into the plasma and are eventually excreted, as is the parent DAMP.

The fitted curve in Chart 3 and the associated parameters are not unique. Since there are 6 parameters and only 16 data points, our reported parameter estimates are poorly defined, i.e., there are many sets of values for the 6 parameters which will adequately fit the data. However, the order of magnitude of
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the parameter values contributes to a good understanding of DAMP pharmacokinetics. The short half-time, 23 sec, for the transfer of DAMP from the central to the peripheral compartment explains the finding that the total plasma radioactivity appears to increase between 6 min and 4 hr as the peripheral biotransformed drug redistributes into plasma. Unfortunately, the first time point could not be measured early enough to give reliable information about this initial very fast phase. An additional complication is that the half-time for the distribution of DAMP into the peripheral compartment is in the same range as the time for the i.v. injection, so that the usual simplifying assumption that the injection is instantaneous is not valid. The i.v. injection was administered over a time period of 20 to 30 sec, and the first plasma sample was taken at 6 min. The estimated volume of distribution of DAMP was 10.6 liters. The problems in measuring the initial very fast phase of DAMP make an accurate estimate of the volume of distribution difficult.

After i.v. injection, [3H]DAMP is extensively localized in the tissue compartment. This has been also observed in rats by direct measurement of radioactivity in different tissues and in plasma (17). The rapid metabolism of DAMP seen in the present study in the dog was also observed in the rat. However, the pattern of plasma decay differs in the 2 species; in the rat after i.v. or i.p. administration of radioactive drug, the level of radioactivity in the plasma shows a biphasic decay while in the dog it increases to a peak at 3 to 4 hr after drug administration and then declines.

At 48 hr, 75% of the administered radioactivity is recovered in urine and feces (Table 5). Since biliary excretion of the radioactivity is small, the fact that urinary excretion is incomplete suggests the possibility of a “deep” compartment acting as a depot of the drug and metabolites.

The incomplete recovery of DAMP in urine and feces observed here is in contrast to the studies with rats where quantitative excretion of the radioactivity was observed within 27 hr after the drug administration (17).

At least 2 metabolites of DAMP are found in dog urine and plasma. One of them is hydrophobic since, like DAMP, it can be extracted from aqueous solution with dichloroethane; it occurs in a relatively small quantity and most probably represents the first step in biotransformation of DAMP. The other one, which as early as 1 hr after drug administration predominates in the plasma and at 6 and 24 hr is the main radioactive compound in urine, is nonextractable with dichloroethane. These hydrophobic and hydrophilic metabolites have been tentatively identified by mass spectroscopy as DAMP hydroxylated in the adamantyl moiety and its sulfate conjugate, respectively (6).

The biological activity of the biotransformation products of DAMP has not been established. However, it is known that the metabolite formed by rat liver microsomes in the presence of NADPH has only about 4% of the activity of DAMP against dihydrofolate reductase (17). Since DAMP biological activity is related to the inhibition of dihydrofolate reductase, it appears that the metabolism of DAMP is a detoxification process.

The data presented here indicate that DAMP is rapidly metabolized and that these metabolites constitute by far the largest proportion of the radioactivity excreted in urine. In clinical use it may, therefore, show a different spectrum of toxicity than the closely related DDMP, which has undergone clinical evaluation and produces marked and often prolonged thrombocytopenia associated with its half-life of many days. The pharmacokinetic aspects of DAMP in both the rat and the dog make it, therefore, an attractive candidate for clinical trial. Since both DAMP and its water-soluble derivative, DAMP-ES, show essentially the same toxicity and because the water-soluble substances are in general easier to handle, the latter was chosen for the Phase I clinical trial which is currently ongoing. In the dog, which is the most sensitive species of those tested (the others being monkey and rat), the dose of DAMP-ES i.v. lethal to 50% of the dogs given is 6.6 mg/kg/day or 125 mg/sq m/day. Ten mg/sq m/day or one-twelfth of the above dose lethal to 50% of the dogs was thus selected as the initial dose in human patients. In order to decrease the danger of CNS toxicity, this dose and all subsequently administered escalated doses are given by slow i.v. infusion rather than by bolus injection.

REFERENCES

Toxicity and Pharmacokinetics of a New Antifolate, 2,4-Diamino-5-adamantyl-6-methylpyrimidine, in Dogs


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