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TOXICITY AND METABOLISM IN MICE OF 2,6-DITHIOPURINE, A POTENTIAL CHEMOPREVENTIVE AGENT

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ABSTRACT:

2,6-Dithiopurine (DTP) has been proposed as a possible chemopreventive agent because of its facile reaction with the electrophilic ultimate carcinogen, benzo[*a*]pyrene diol epoxide, and other reactive electrophiles. Previous studies in mouse skin indicated almost complete inhibition of benzo[*a*]pyrene diol epoxide-induced tumorigenesis by DTP, suggesting the possible utility of this compound as a chemopreventive agent. However, little is known of the metabolism of DTP or of its possible long-term toxicity. Mice were fed diets containing up to 4% DTP in AIN-76A for a period of 7 weeks, and possible toxicity was monitored by weight gain and histopathological examination of all major tissues. No toxicity was observed at any dose of DTP. DTP was found to be a good substrate *in vitro* for two enzymes known to metabolize 6-mercaptopurine: xanthine oxidase and thiopurine methyltransferase. The *in vitro* metabolites were 2,6-dithiouric acid and an apparent monomethylated derivative, respectively. *In vivo*, the major urinary metabolite was 2,6-dithiouric acid, which attained levels as high as 34 mM in the urine of mice receiving the 4% DTP diet. DTP was also excreted unchanged in the feces and urine. DTP, 2,6-dithiouric acid, and an unidentified, relatively nonpolar metabolite were also detected in the serum of experimental animals. Although large interindividual variation in the serum DTP concentration was found, there was a dose-dependent increase in serum DTP as the dietary level of DTP was increased. These results suggest that neither toxicity nor metabolism will severely limit the utility of DTP as a chemopreventive agent.

Most chemicals that are known to be carcinogenic are metabolized in humans and other mammals to highly reactive electrophiles that covalently modify cellular macromolecules (1, 2). In particular, damage to the nuclear DNA has been implicated in chemical carcinogenesis, because unrepaired lesions can lead to mutations in critical cellular genes (3). Thus, a suitable target for chemoprevention of cancer that is common to many chemical carcinogens is the production and disposition of the electrophilic intermediates that are responsible for DNA damage. Three strategies for blocking DNA damage have been outlined (4): *1*) block the production of the electrophilic "ultimate" carcinogen by phase I enzymes; *2*) increase the rate of detoxification of the ultimate carcinogen by phase II enzymes; and *3*) provide an exogenous, nucleophilic target for the ultimate carcinogen to scavenge the DNA-damaging activity. Chemopreventive agents targeted for each of these strategies have been described (4–6).

We have previously reported a new class of potential chemopreventive agent, the thiopurines, that act as nucleophilic scavengers for electrophilic diol epoxides that are the ultimate carcinogenic forms of several polycyclic aromatic hydrocarbon carcinogens (7–10). The most active of these, DTP[1] (fig. 1), has been shown to react facilely with BPDE, forming a thioether adduct, and to block the reaction of BPDE with DNA *in vitro*, in cultured cells and *in vivo*. Because the major detoxification pathway for BPDE is through conjugation with GSH (11), the nucleophilic scavenging strategy is likely to be most useful in tissues such as epidermis and mammary gland that lack high levels of GSH and GSH *S*-transferase (12). Initial tumorigenesis studies in the mouse skin two-stage carcinogenesis model indicated a high level of chemopreventive activity (8). Topical application of 10 μmol DTP produced >90% inhibition of BPDE-induced skin tumors.

We are interested in the possible chemopreventive activity of this compound when administered in the diet. However, several factors, including metabolic disposition and toxicity, could limit the utility of dietary DTP. In particular, the extent to which the compound is absorbed and metabolized must be determined. It is known that thiopurines in general are substrates for the cellular purine transport mechanism (13). Many purines, including 6-MP, are oxidized by XO [EC 1.2.3.2 (14–17)]. Furthermore, Weinshilboum and coworkers (18–20) have described an enzyme that can be purified from several human tissues, including kidney [TPMT (EO 2.1.1.67)], that metabolizes 6-MP to form a methyl-conjugated metabolite.

Also of importance is the possible toxicity of DTP, because the related compound, 6-MP, is a cytotoxic chemotherapeutic agent. Initial studies of DTP given as a bolus dose by intraperitoneal injec-

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[1] Abbreviations used are: DTP, 2,6-dithiopurine; BPDE, 7*r*,8*t*-dihydroxy-9*t*,10*t*-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; GSH, glutathione; 6-MP, 6-mercaptopurine; XO, xanthine oxidase; TMPT, thiopurine methyltransferase; XAN, xanthine; TP, thiopurinol; PMA, phenylmercuric acetate; DUA, 2,6-dithiouric acid; UA, uric acid; MTD, maximum-tolerated dose.

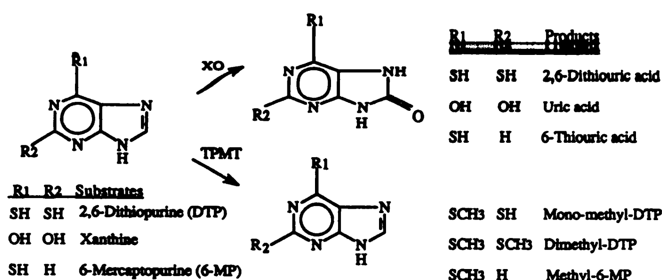

FIG. 1. *Metabolic pathways of thiopurines and XAN.*

tion in mice indicated much lower toxicity than that of 6-MP (21). Preliminary, short-term studies of the toxicity of DTP as a dietary or drinking water additive were similarly negative.[2] This encouraged us to go on with longer term studies of the toxicity and metabolic fate of DTP. In the current studies, we have evaluated the toxicity of DTP in mice receiving this thiopurine as a major component (up to 4% by weight) of their diets for a 7-week period. In addition, two pathways of metabolism of DTP have been established *in vitro*, and levels of DTP and a major metabolite in serum, urine, and feces of the experimental mice have been measured.

Methods and Materials

Chemicals. DTP was synthesized by an established method from XAN and phosphorus pentasulfide in tetramethylene-sulfone as solvent (22). DTP was analyzed qualitatively by IR, UV, and NMR spectroscopy, and was determined by HPLC to be at least 95% pure. Pellets of AIN-76A semipurified diet containing different concentrations of DTP were formulated by Dyets, Inc. (Bethlehem, PA) and stored at 4°C before use. Weighed portions of each diet were extracted with 0.1 N NaOH at the beginning of the experiment, and the recovery of DTP was determined by absorbance spectroscopy. Mean DTP concentrations were within 10% of the values expected by formulation. HPLC analysis of the extracts (see data herein) indicated a single component with retention time identical to pure DTP. XAN, XO (from buttermilk), 6-MP, TP, PMA, thiocresol, and toluene were from Aldrich (Milwaukee, WI) or Sigma Chemical Co. (St. Louis, MO). 1-Heptanesulfonic acid sodium salt was obtained from Eastman Kodak Co. (Rochester, NY).

Animal Treatment. Female SENCAR mice (4 weeks of age) were obtained from the NCI-Frederick Cancer Research Facility. AIN-76A semipurified diets containing 0, 0.5, 1, 2, and 4% by weight of DTP were given *ad libitum* to five groups of mice (10 in each group). HPLC and spectrophotometric analyses of alkaline extracts of portions of the 0.5% and 2% DTP diets after 4 days exposure to the animals at room temperature indicated no breakdown of DTP under these conditions. Body weight and food intake were recorded twice weekly for 7 weeks; animals were examined daily for any symptoms of toxicity. All animals were kept in metabolic cages for 24 hr before killing by CO_2 euthanasia to collect urine and feces. Blood samples were obtained by heart puncture, and lung, liver, kidney, spleen, heart, stomach, and a segment of intestine were removed and fixed in 10% buffered formalin for routine histopathological examination.

Determination of DTP in Serum, Urine, and Feces. To 0.5 ml of serum from treated mice were added 30 μl of 50 μM TP as an internal standard and 80 μl of 5 mM PMA. PMA reversibly binds thiopurines such as DTP and TP, allowing efficient transfer of the complexes to the organic phase in a subsequent extraction (23–25). One ml of methanol was then added to denature serum proteins, and the complexes of PMA with thiopurines were extracted with 2 ml toluene. After mixing and phase separation, the toluene phase was recovered and mixed with 0.1 ml 10% thiocresol/ether solution and 0.5 ml water. The excess of thiocresol effectively replaces the thiopurines in the PMA complexes, releasing free thiopurines, DTP and TP, into the water. After a second-phase separation, a 200 μl aliquot of the water phase was analyzed by HPLC (mobile phase: 0.2% acetic acid, 0.1% heptanesulfonic acid sodium salt; column: Aquapore RP 300, 220 × 4.6 mm, 7 μm) (24).

[2] K. L. Powell and M. C. MacLeod, unpublished data.

For urine samples, a similar method was used initially. However, direct analysis of urine after dilution (2,500- to 12,500-fold) gave identical results. To alleviate possible contamination of the urine with fecal metabolites or food particles, urine collected directly from the animals at the time of euthanasia was used for the HPLC analyses. For quantitation, 6-thioguanine was added as an internal standard. To identify the metabolites of DTP in urine, HPLC peaks were collected, dried, and redissolved in alkaline methanol or acidic methanol. UV absorption spectra were measured in a Hewlett-Packard 8450 spectrophotometer and compared with standard UV spectra (26).

Weighed portions of feces were extracted exhaustively with either 0.1 N NaOH or 0.1 N HCl, and the extracts were analyzed by UV spectrophotometry and HPLC as indicated.

Metabolism of DTP by XO *In Vitro*. XO is known to metabolize many purines to 8-oxo-derivatives, and the possibility existed that DTP is also a substrate for this enzyme. To analyze for possible metabolites, reaction mixtures containing 50 μM DTP in 50 mM phosphate buffer (pH 7.4) were incubated at 37°C for 0.5 or 1 hr with 0.05 unit XO. Using the same procedures as for urine samples, HPLC peaks were collected, dried *in vacuo*, and the residue dissolved in 1 ml alkaline methanol or acidic methanol for measurement of absorption spectra.

To determine Michaelis constants (K_M) of XO for DTP and XAN, different concentrations of substrate in 50 mM phosphate buffer (pH 7.4) were incubated with 0.05 units of enzyme. Absorption spectra were recorded after various incubation periods at room temperature. At each time point the extent of DUA or UA formation was calculated from the following equations:

$$[\mathrm{DUA}] = \frac{\varepsilon_{\mathrm{DTP},348} \times A_{358} - \varepsilon_{\mathrm{DTP},358} \times A_{348}}{\varepsilon_{\mathrm{DUA},358} \times \varepsilon_{\mathrm{DTP},348} - \varepsilon_{\mathrm{DUA},348} \times \varepsilon_{\mathrm{DTP},358}} \quad (1)$$

$$[\mathrm{UA}] = \frac{\varepsilon_{\mathrm{XAN},271} \times A_{290} - \varepsilon_{\mathrm{XAN},290} \times A_{271}}{\varepsilon_{\mathrm{UA},290} \times \varepsilon_{\mathrm{XAN},271} - \varepsilon_{\mathrm{UA},271} \times \varepsilon_{\mathrm{XAN},290}}. \quad (2)$$

The following extinction coefficients were obtained from the literature: $\varepsilon_{\mathrm{DTP},348}$ = 12,700 (10); $\varepsilon_{\mathrm{DUA},358}$ = 19,000 (41); $\varepsilon_{\mathrm{XAN},271}$ = 8,870 (33); and $\varepsilon_{\mathrm{UA},290}$ = 11,800 (32). The remaining extinction coefficients were measured in this study, based on the previous cited-values: $\varepsilon_{\mathrm{DTP},358}$ = 9,810; $\varepsilon_{\mathrm{DUA},348}$ = 12,700; $\varepsilon_{\mathrm{XAN},290}$ = 2,514; and $\varepsilon_{\mathrm{UA},271}$ = 4,500. Note that DUA and DTP exhibit an isobestic point at 348 nm. K_M's were calculated by double-reciprocal analysis of the initial velocity data.

Metabolism of DTP by TPMT *In Vitro*. The ability of DTP to act as a substrate for TPMT was measured using a modification of the method of Weinshilboum *et al.* (18). This assay is based on the conversion of substrate to radioactively labeled methylated product, with [^{14}C]*S*-adenosyl-L-methionine (Ado-Met) as the methyl donor. The [^{14}C]methyl-Ado-Met (60 μCi/μmol) was obtained from New England Nuclear-Dupont (Boston, MA). Blank samples contained no methyl acceptor substrate. The major modification of the assay described previously was termination of the enzyme reaction with 1 N HCl, rather than with 0.5 M borate buffer (pH 10). Enzymic methylation of one thiol moiety of DTP leaves one ionizable thiol on the molecule. At pH 10, this thiol is expected to be ionized (10) and, therefore, the metabolite would be poorly organic solvent-soluble in the subsequent extraction step. Radioactive reaction product was isolated by organic solvent extraction performed with 20% isoamyl alcohol in toluene. The partition coefficient of the methylated reaction product was determined as described previously (18). One unit of enzyme activity represented the formation of 1 nmol of methylated product/hr of incubation at 37°C. Protein concentrations were measured by the dye-binding method of Bradford (27), with bovine serum albumin as a standard. The enzyme source was recombinant human TPMT transiently expressed in COS-1 cells as described in detail elsewhere (20). Preliminary experiments were performed with DTP as a substrate by testing a series of concentrations that differed by several orders of magnitude. Experiments were then performed in which the effect of a series of concentrations of DTP on TPMT activity was determined. The apparent K_M value was estimated by the method of Wilkinson (28), with a computer program written by Cleland (29). Data used to calculate the V_{max} value were corrected for the partition coefficient of the methylated reaction product into the organic solvent phase.

[^{14}C]Methyl-labeled metabolites of DTP were separated by HPLC using the HPLC method described herein. Fractions were collected, and radioactivity

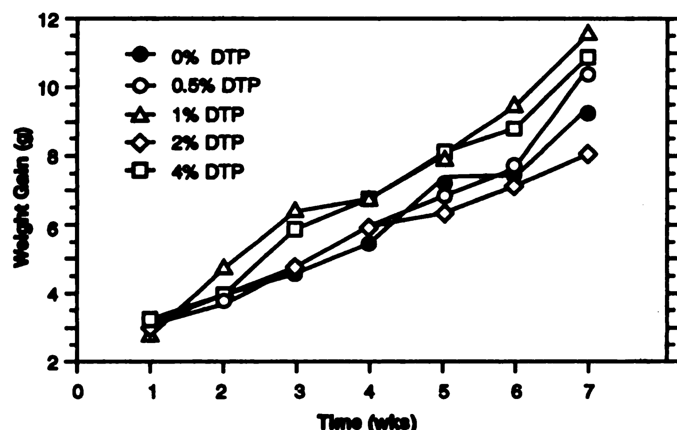

FIG. 2. *Weight gain of mice consuming DTP-containing diets.*

Groups of 10 female SENCAR mice were fed AIN-76A diet containing the indicated weight fractions of DTP *ad libitum*. The average cumulative weight gain is plotted for each week.

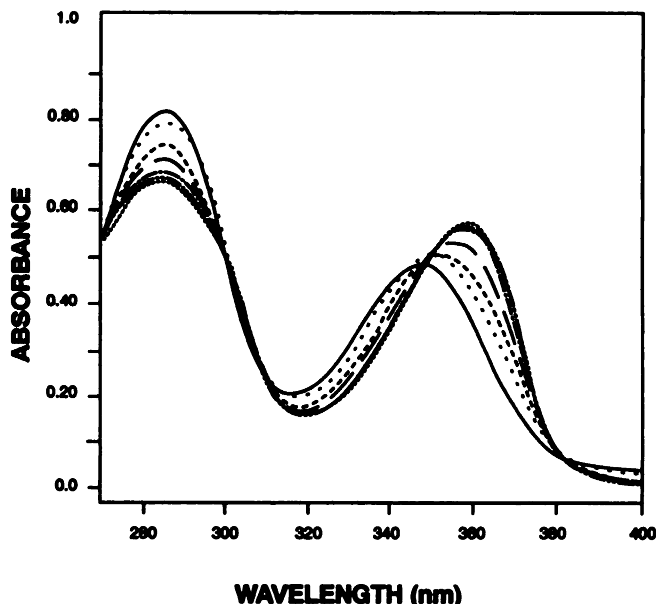

FIG. 3. *Metabolism of DTP by XO.*

A reaction mixture containing purified XO and DTP in phosphate-buffered saline was incubated at room temperature in a quartz cuvette, and absorption spectra were recorded at intervals from 4 to 200 sec. The long wavelength absorption of DTP at 348 nm visible at 0 time shifts to ~358 nm with incubation time.

was measured to determine the retention times of the metabolites. As a reference compound for the HPLC procedure, dimethyl DTP was prepared by reaction of DTP with dimethyl sulfate[3] and was detected by absorbance.

Results

Lack of Overt Toxicity. We attempted to determine the MTD of DTP (30, 31) by feeding groups of 10 mice AIN-76A diet containing different concentrations of DTP (0, 0.5, 1, 2, and 4%). Food intake was the same for all groups. Mean weight gain data for the five groups are plotted in fig. 2. At the end of the experiment, there were no significant differences in weight gain between groups (ANOVA, Bonferroni-Dunn test). When the entire time course was analyzed by two-way ANOVA, only the 1% DTP group was significantly different than the control group. Because the mean weight gain in the 1% DTP group was actually higher than the control, we conclude that these data provide no evidence for toxicity because of consumption of DTP.

We also measured the wet weights of spleen, liver, heart, lung, and kidney at the conclusion of the experiment (data not shown). In agreement with the weight gain data, there were no significant differences between groups in the major organ weights when tested by ANOVA. Samples of these organs, plus stomach and intestine from three mice/group, were examined histopathologically after hematoxylin-eosin staining. There was no evidence of histopathological changes caused by treatment. Because no evidence for toxicity was obtained, we conclude that the MTD of DTP for mice is in excess of 4% of the diet.

***In Vitro* Metabolism.** To prepare for analysis of the *in vivo* metabolites of DTP obtained in the feeding study, we first studied the *in vitro* metabolism of DTP by two enzymes suspected to be important *in vivo:* XO and TPMT. To determine whether XO utilizes DTP as a substrate, reaction mixtures containing DTP and purified XO were prepared, and UV spectra were recorded after different times of incubation. As shown in fig. 3, a distinct time-dependent red shift of the DTP absorbance spectrum was observed. Similar spectral changes have been reported for the oxidation of XAN, and these changes can be used to obtain the initial velocities of the enzymic reaction (32, 33). Using a double wavelength method, initial velocities of product formation were determined for a range of concentrations of both DTP and XAN. The apparent K_M values of XO for DTP and XAN were determined by plotting $1/V$ *vs.* $1/[S]$ (fig. 4). Because the reaction with XAN is known to exhibit substrate inhibition at high substrate concentrations (34, 35), data obtained at XAN concentrations >20 μM were excluded from the analysis. The apparent K_M for DTP (33.8 μM) was only 2.5-fold higher than that of XAN (13.0 μM), suggesting that XO has relatively high affinity for DTP. At concentrations up to 50 μM, DTP did not exhibit any evidence of substrate inhibition.

The products of this reaction were analyzed by HPLC with absorbance detection. Figure 5 (*trace A*) shows the HPLC profile of pure DTP, exhibiting a single peak with a retention time of ~7 min. In reaction mixtures containing XO and DTP in phosphate buffer (pH 7.4) and incubated at 37°C for 0.5 hr (fig. 5, *trace B*), we can see that no DTP remained unchanged, and a single peak was obtained with a retention time of ~3 min. By comparison to the known metabolism of XAN and 6-MP by XO (fig. 1), this compound is expected to be DUA. Absorbance spectra of the isolated peak confirm this tentative assignment (see data herein).

The possibility that TPMT might also metabolize DTP (36) was studied using purified, recombinant human TPMT and an assay based on the transfer of radioactivity from [^{14}C]methyl-Ado-Met to DTP. Initial velocities were measured using this assay for a range of concentrations of DTP, and the apparent K_M was determined from the double-reciprocal plot (fig. 6). The kinetics were consistent with a single methyl transfer and gave an apparent K_M of 34 μM and V_{max} of 276 units/mg protein. This compares quite favorably with the reported apparent K_M of the recombinant enzyme for 6-MP [710 μM (20)]. The products of this reaction were also analyzed by HPLC, with ^{14}C radioactivity detected by collecting fractions and liquid scintillation counting. A single peak was seen with a retention time of 11 min (fig. 7, *thin line*). The radiolabeled component was distinct from authentic DTP (retention time of ~7 min; fig. 5*A*) and from the dimethyl derivative (retention time of ~50 min; data not shown). No radioactive peaks were detected in extracts of control reactions that contained no DTP (fig. 7, *thick line*).

[3] W.-G. Qing *et al.*, manuscript in preparation.

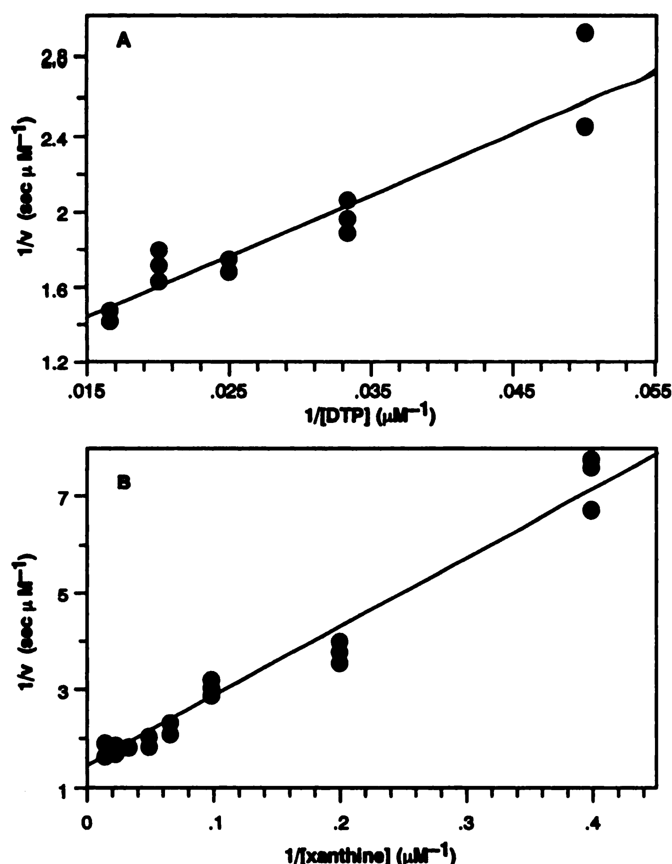

FIG. 4. *Kinetics of XO metabolism of DTP.*

Using a double wavelength algorithm, initial velocities of metabolism of (*A*) DTP or (*B*) XAN by purified XO were determined over a range of substrate concentrations. Data were plotted in double-reciprocal form to determine the apparent K_M and V_{max} values.

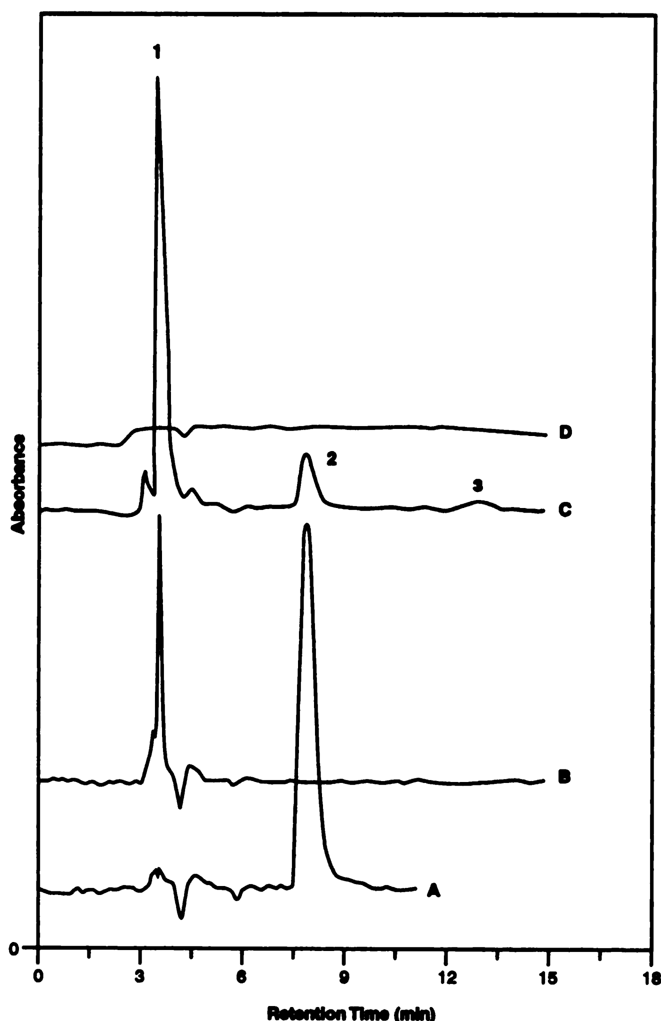

FIG. 5. *HPLC analysis of DTP and metabolites.*

Using the HPLC system described in *Materials and Methods*, (*A*) pure DTP or (*B*) a DTP-XO reaction mixture were analyzed, with detection by absorbance at 348 nm. In (*C*), urine from mice consuming the 4% DTP diet was analyzed; for comparison, control urine (*D*) was analyzed under the same conditions.

***In Vivo* Metabolism.** To detect DTP excretion, we extracted feces with 0.1 N NaOH and then analyzed the extracts by absorption spectroscopy and HPLC. The HPLC analysis of the alkaline extract showed only one peak with a retention time similar to that of pure DTP (data not shown). The UV absorption spectrum of this peak was identical to that of pure DTP, suggesting that unmetabolized DTP was the major component detectable in alkaline extracts of feces. The amount of DTP excreted in feces in a 24-hr period at the end of the 7 weeks exposure (table 1) increased in a markedly nonlinear manner with the dietary dose of DTP. The concentration of DTP rose from <2% of the wet weight of feces of mice consuming the 0.5% DTP diet, to almost 40% of the wet weight in the group consuming 4% DTP. This dose-response suggests that, at dietary doses above 1% DTP, the efficiency of uptake of DTP decreased, such that most of the DTP in the 4% diet passed through the gastrointestinal system unchanged.

Urine samples were also collected from animals after 7 weeks on DTP-containing diets. Figure 5 (*trace C*) shows a typical HPLC profile of a urine sample obtained from mice receiving the 4% DTP diet. We found two peaks with retention times similar to those of DTP (peak 2) and the major *in vitro* XO metabolite, presumed to be DUA (peak 1), and a third minor peak with a retention time of ~13 min (peak 3). None of these components were present in samples of urine from control mice (fig. 5, *trace D*). The nature of the third peak is currently unknown. However, based on the relative sizes of the two absorbance peaks, the unidentified metabolite was present at less than one-tenth the level of unmetabolized DTP. To confirm that the polar metabolite was DUA, we collected peaks 1 and 2 from urine samples (fig. 5, *trace C*), the *in vitro* XO metabolite peak (fig. 5, *trace B*), and a sample of pure DTP (fig. 5, *trace A*); dried them; and redissolved them in alkaline methanol or acidic methanol. The spectrum in alkaline methanol of peak 1 from urine was very similar to that of the *in vitro* XO metabolite of DTP; these spectra were both similar to the published spectrum of DUA in alkaline methanol (26). A similar spectral correspondence was also obtained in acidic methanol. The spectrum of urinary peak 2 was identical to that of pure DTP in both alkaline and acidic methanol (data not shown). This suggests that DTP can be metabolized by XO both *in vivo* and *in vitro*, forming DUA.

HPLC data were collected using 6-thioguanine as an internal standard to determine urinary concentrations of DTP and DUA; these are given in table 1. Extremely high levels of both compounds were attained in the urine of these mice; DTP concentrations of 5–6 mM are close to the solubility limit of the compound. Although the urinary concentration of DUA seems to have reached a plateau at ~30 mM, the total amount excreted did not. This is because the volume of urine

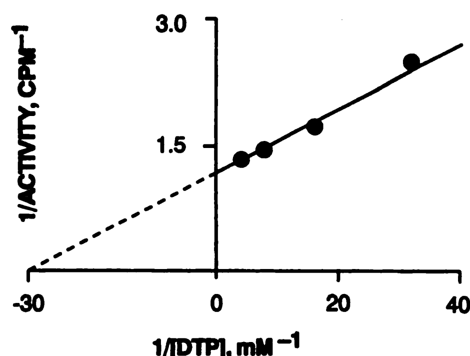

FIG. 6. *Kinetics of TPMT metabolism of DTP.*

Recombinant human TPMT was incubated with a range of concentrations of DTP in the presence of [^{14}C]methyl-Ado-Met, and the initial velocity of reaction was determined as described in *Materials and Methods*. Data were plotted in double-reciprocal form to determine apparent K_M and V_{max} values.

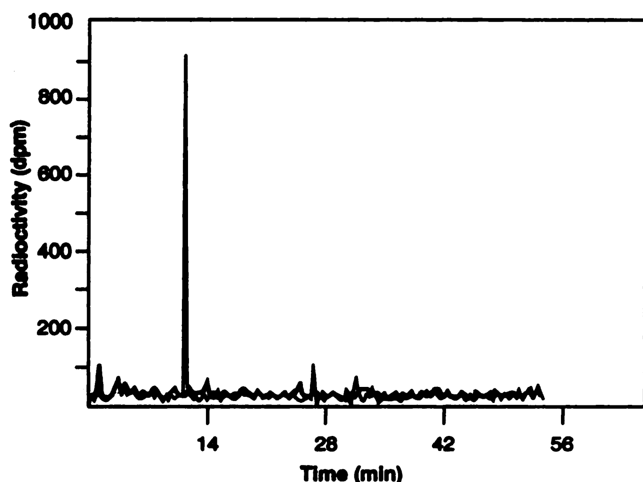

FIG. 7. *HPLC analysis of TPMT metabolites.*

The metabolites produced by the action of TPMT on DTP (*thin line*) or a no-DTP blank reaction (*thick line*) were extracted into isoamyl alcohol:toluene, the organic solvent was evaporated, and the residue was analyzed by HPLC. Fractions were collected, and radioactivity derived from [^{14}C]methyl-Ado-Met was determined by liquid scintillation counting.

recovered in a 24-hr collection in a metabolic cage was higher in the mice receiving the 4% DTP diet than in control mice.

The presence of DUA as a major urinary metabolite prompted us to reexamine fecal excretion. When fecal samples were extracted with 0.1 N HCl, small amounts of DTP were recovered, but larger amounts of a polar metabolite were obtained. We tentatively identify this metabolite as DUA, based on identity of HPLC retention times and UV absorbance spectra (data not shown). The levels of fecal DUA did not vary more than 2-fold, with changes in the dietary concentration of DTP (table 1). Data in table 1 indicate that overall excretion of DUA (urinary + fecal) accounted for ~44% of the total excretion at dietary DTP levels of 0.5 and 1%. However, at the higher levels, the fraction metabolized decreased to only ~10% in mice receiving 4% DTP.

We also analyzed extracts of serum samples from mice that had consumed control and experimental diets; fig. 8 shows typical HPLC data from several experimental groups. There are three peaks in the HPLC profile (fig. 8, *top two traces*) that are DTP-specific, with retention times of ~3, 7, and 13 min. The peak at ~9 min is TP, which was added to the serum as an internal standard for quantitation. The retention time of peak 2 is the same as that of pure DTP (fig. 5, *trace A*). The other two peaks (labeled peaks 1 and 3, retention times ~3 and 13) are metabolites of DTP, because they are absent from the extracts of control mice (fig. 8, *bottom trace*). The more polar component (peak 1) is likely to be DUA, because its retention time matches that of DUA generated *in vitro*. Metabolite peak 3 has a retention time similar, but not identical, to that of the methylated metabolite obtained *in vitro* with TPMT, and identical to the minor urinary metabolite.

Using a standard curve prepared by addition of known amounts of DTP to serum samples from control animals, the concentration of DTP in the serum of experimental animals could be measured. Determinations in five individual animals in each dose group gave the following serum concentrations of DTP: 0.5% DTP, 1.08 ± 0.28 μM; 1% DTP, 2.11 ± 1.19 μM; 2% DTP, 2.71 ± 0.64 μM; and 4%, 5.65 ± 4.87. The concentration of DTP in serum seemed to increase with dose, but exhibited great variability between individuals. Linear regression analysis gave an R value of 0.593 ($p = 0.0058$), suggesting that there was a dose-dependent increase in serum DTP levels.

Discussion

Three major factors affecting the utility of a potential chemopreventive agent are its potency, toxicity, and metabolic fate. We have addressed the latter two factors in the present study. Thiopurines were originally developed for their chemotherapeutic efficacy in treating cancer patients, and 6-MP is still used for treatment of leukemia (37). Because of this, toxicity of other thiopurines is an important issue. Numerous 6-MP analogs were tested for chemotherapeutic potential in the 1950s, and our initial interest in DTP was because of its lack of toxicity in these assays (17, 21). The current results extend this work by indicating a lack of apparent toxicity when DTP is fed to mice at high levels for 7 weeks. Future studies will be needed to address the question of possible toxicity in a lifetime exposure.

Based on previous studies of the metabolism of 6-MP (15–18), we expected that DTP would be a substrate for XO and TPMT. The

TABLE 1

Excretion of DTP and DUA[a]

Diet	Urinary Excretion (μmol/mouse/day)		Fecal Excretion (μmol/mouse/day)		Total DUA[b]
	DTP	DUA	DTP	DUA	Total Excreted
0.5%	1.2 ± 0.1	7.9 ± 1.4	28.6 ± 6.3	15.3 ± 16.9	0.44
1.0%	3.2 ± 0.1	15.1 ± 1.0	38.8 ± 1.3	17.0 ± 1.4	0.43
2.0%	4.2 ± 0.3	20.3 ± 2.3	192.7 ± 12.5	31.2 ± 4.6	0.21
4.0%	5.7 ± 0.3	50.1 ± 3.8	673.6 ± 65.2	21.7 ± 1.8	0.10

[a] Groups of five mice were kept in a metabolic cage for 24 hr at the end of a 7-week feeding period, and the weight of feces and volume of urine recovered was measured. Urinary and fecal levels of DTP and DUA were measured as described in the text.

[b] The daily excretion of DUA in urine + feces has been normalized to the total excretion of DTP + DUA.

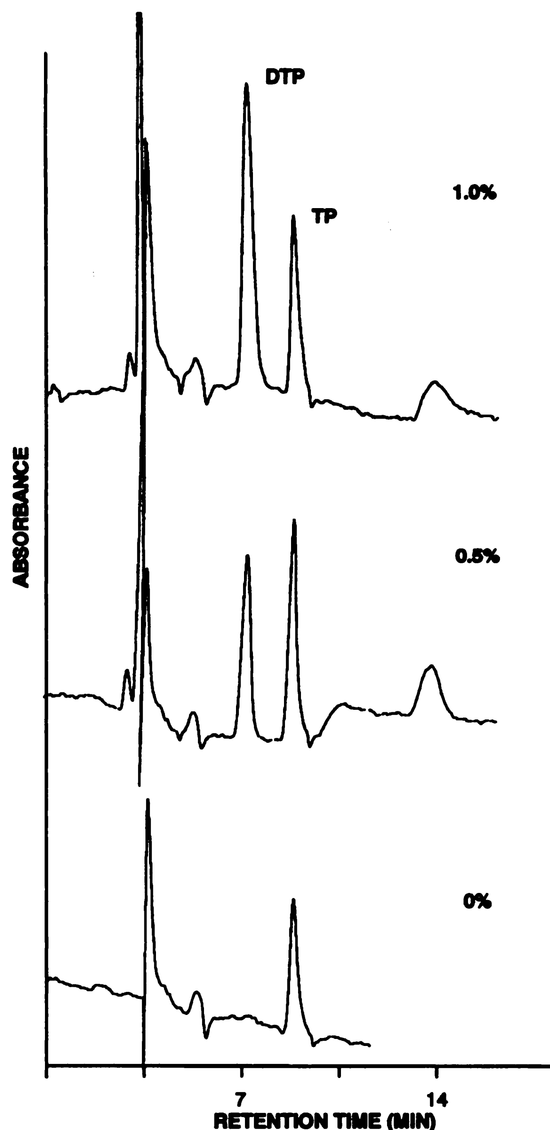

FIG. 8. *HPLC analysis of serum metabolites.*

DTP and metabolites were extracted from serum samples as described in *Materials and Methods* and analyzed by HPLC. TP was added as an internal standard before extraction.

results of the *in vitro* experiments with purified enzymes confirmed this, indicating apparent K_M's for both enzymes in the range of 30–35 μM. The products of these reactions were DUA and a monomethylated metabolite of DTP, respectively. DUA was also found as a major *in vivo* metabolite in serum and urine. Indeed, urinary levels of DUA reached above 30 mM. Oxidation at the C8 position by XO is also known to be the major metabolic pathway for 6-MP in mice and rats (15–17). Because SENCAR mice exhibit significant levels of XO (38), both in tissues and in serum, we assume that this is the enzyme activity responsible for the majority of the urinary excretion of DUA in mice ingesting DTP. However, hepatic aldehyde oxidase activity (39) may also contribute to the overall metabolic products. Interestingly, preliminary studies of the ability of DUA to act as a nucleophilic scavenger for the electrophilic carcinogen, BPDE, indicate that it reacts almost as well as DTP.[4] Thus, the possibility exists that the primary metabolic conversion of DTP may have little effect on the chemopreventive potency of the compound.

A second metabolite with a relatively long retention time in our HPLC analyses was detected in both serum and urine. However, the retention did not match that of either the monomethyl derivative found *in vitro* with TPMT or a dimethylated derivative prepared chemically. Metabolism of 6-MP in rodents has been shown to be fairly complex (14–16), with the possibility of desulfuration in addition to oxidation or methylation. Multiple modifications have also been observed (*e.g.* production of 6-methylthio-8-hydroxypurine). Furthermore, the *in vitro* TPMT metabolite was prepared using recombinant human enzyme. Differences have been noted between the human and mouse enzymes in 6-MP metabolism (19, 40), and mouse hepatic and renal extracts produce at least two methylated 6-MP metabolites under some conditions (40). It is possible, therefore, that the unidentified product of DTP in serum and urine is derived from a combination of metabolic steps that may involve the enzyme TPMT, or from methylation at a different position than that favored by the human enzyme. Furthermore, it should be noted that the metabolites recovered so far do not account for the total amount of DTP ingested, especially at the lower doses. Sulfinyl and glucuronyl derivatives of 6-MP have been reported, and similar products derived from DTP may have escaped our preparative methods.

The current preliminary toxicity and metabolism studies of DTP in mice suggest that neither of these factors severely limit the utility of the compound. Toxicity is low, and the major metabolite may have efficacy similar to the parent compound. More rigorous lifetime studies of toxicity and a thorough investigation of metabolic pathways will be needed in the future. In addition, a better idea of the chemopreventive potency of the compound in several tumor models is needed to judge the need for further mechanistic studies.

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[4] W.-G. Qing and M. C. MacLeod, unpublished results.

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