Kinetics of the Reaction of a Potential Chemopreventive Agent, 2,6-Dithiopurine, and Its Major Metabolite, 2,6-Dithiouric Acid, with Multiple Classes of Electrophilic Toxicants

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Purinethiols are a class of potential cancer chemopreventive agents that exhibit nucleophilic scavenging activity against the carcinogenic electrophile benzo[*a*]pyrene diol epoxide (BPDE). Of the purinethiols tested previously, 2,6-dithiopurine (DTP), exhibited the highest scavenging activity for BPDE when tested either *in vitro* or *in vivo*. Sulfur-based nucleophiles are typically classified as "soft" nucleophiles, showing selectivity in nucleophilic substitution reactions for "soft", easily polarizable electrophiles. It was of interest to determine whether electrophilic toxicants other than BPDE react facilely with DTP, and whether 2,6-dithiouric acid (DUA), the major *in vivo* metabolite of DTP, also has scavenging activity. Four diverse toxicants tested in the present work, acrolein, melphalan, dimethyl sulfate, and cisplatin, all react facilely with DTP *in vitro* near neutral pH. These toxicants are expected to react as "soft" electrophiles. Furthermore, each of these compounds, as well as BPDE, reacts with DUA with rate constants comparable to the analogous rate constants for reaction with DTP. In contrast, several toxicants classified as "hard" electrophiles (ethyl methanesulfonate, methylnitrosourea, ethylnitrosourea, 1-methyl-3-nitro-1-nitrosoguanidine) show no appreciable reaction with DTP. These results suggest that both DTP and its major metabolite act as "soft" nucleophiles in nucleophilic substitution reactions and may be effective in scavenging a wide range of toxicants that react as "soft" electrophiles.

Introduction

It is well-known that the mechanism of action of many different classes of chemical carcinogens involves electrophilic intermediates that react with nucleophilic sites in DNA (*1, 2*). These electrophiles are often detoxified by Phase II enzymes such as the glutathione *S*-transferases (GST)[1] which catalyze the nucleophilic addition of the thiol moiety of glutathione (GSH) to a variety of toxic electrophiles (*3*). However, some tissues, including mammary and epidermal epithelia (*3*), lack high concentrations of GST and GSH and are, therefore, deficient in this detoxification pathway. Two promising strategies of chemoprevention seek to increase the rate of nucleophilic scavenging in these situations, either by increasing GST levels (*4, 5*) or by supplying an exogenous nucleophile that can react directly with the electrophilic toxicants (*6*−*9*).

Previous studies from this laboratory (*8, 10*) demonstrated that several nucleophilic purine derivatives that contain thiol moieties linked to the 2- and/or 6-carbon positions react facilely with the electrophilic, ultimate carcinogen 7*r*,8*t*-dihydroxy-9,10*t*-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE). These reactions were characterized as S_N2 substitutions, with addition of a thiol to the 10-carbon of BPDE. One of the most active of these nucleophiles was 2,6-dithiopurine (DTP). Presumably due to this scavenging reaction, several purinethiols, including 6-mercaptopurine and DTP, and the related compound thiopurinol blocked the binding of BPDE to DNA *in vitro*, in Chinese hamster ovary cells, and in mouse skin (*8*−*11*) in a dose-dependent manner. The finding that DNA binding in cells can be virtually abolished by purinethiols and the identification of purinethiol−BPDE adducts in treated cells and tissue (*9, 11*) indicate that purinethiols can be an effective complement to endogenous nucleophiles in reducing DNA damage from electrophilic toxicants. These results, along with a demonstrated ability to block BPDE-initiated tumorigenesis in mouse skin (*9*) and a lack of toxicity after oral administration (*12*), suggested that DTP has promise as a nucleophilic scavenger in chemoprevention. We therefore felt it was of interest to determine whether other electrophiles are also targets for the nucleophilic scavenging activity of DTP.

Recent studies of the metabolism of dietary DTP in SENCAR mice (*12*) indicated that the major metabolite was 2,6-dithiouric acid (DUA), presumably a product of oxidation by the enzyme xanthine oxidase/dehydrogenase (*13*). In mice fed a diet containing 1% DTP, both DTP and DUA were easily detectable in the serum, and the urinary concentration of DUA reached 37.7 mM, 7-fold higher than the urinary DTP concentration. Since DUA retains both of the nucleophilic thiol moieties of DTP, it

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[1] Abbreviations: GST, glutathione *S*-transferase; GSH, glutathione; BPDE, 7*r*,8*t*-dihydroxy-9,10*t*-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; DTP, 2,6-dithiopurine; DUA, 2,6-dithiouric acid; DMS, dimethyl sulfate; EMS, ethyl methanesulfonate; MNNG, 1-methyl-3-nitro-1-nitrosoguanidine; MNU, *N*-methylnitrosourea; ENU, *N*-ethylnitrosourea; PB, 50 mM sodium phosphate buffer, pH 7.5.

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is conceivable that this major metabolite may also exhibit nucleophilic scavenging activity. It was, therefore, also important to determine whether DUA reacts with BPDE and other electrophiles.

The selectivity of reactions between nucleophiles and electrophiles has been characterized classically in terms of a conceptual scale of "hardness" versus "softness" (*14, 15*). Chemical hardness or softness are functions of the charge density and the polarizability of the electrophilic/nucleophilic center. Hard nucleophiles, such as the oxygen atoms of the phosphate group of DNA, have highly polarized negative charge density, whereas soft nucleophiles have more diffuse but readily polarizable negative charge density, e.g., the polarizable "lone pair" of electrons of the sulfur atoms of thiol groups. Similarly, hard electrophiles, such as alkyl carbonium ions, have highly polarized positive charge density, and soft electrophiles have readily polarized positive charge density, e.g., the polarized double bond in acrolein (*14*).

A group of direct-acting, electrophilic toxicants representing several different chemical classes and including both hard and soft electrophiles was selected for this study. Short chain alkylating agents included dimethyl sulfate (DMS), a representative soft electrophile, and a series of alkylating agents of increasing hardness (*16, 17*; ethyl methanesulfonate (EMS), 1-methyl-3-nitro-1-nitrosoguanidine (MNNG), *N*-methylnitrosourea (MNU), and *N*-ethylnitrosourea (ENU)). Acrolein, a toxic metabolite of the chemotherapeutic agent cyclophosphamide, and melphalan, another antineoplastic alkylating agent used in the clinical treatment of multiple myeloma, ovarian cancer, lymphoma, and other cancer (*18, 19*), are slightly larger and presumably softer electrophiles. Both of these compounds are known to react primarily with nitrogens in DNA (*19, 20*), and acrolein has been implicated as a possible intermediate in endogenous DNA damage (*21*), presumably as a product of lipid peroxidation. Cisplatin, another antitumor agent used to treat many kinds of human cancers (*22*), produces both intra- and interstrand cross-links in DNA (*23*). Finally, BPDE was included as a bulky electrophile that reacts via an S_N2 mechanism (*8*).

Materials and Methods

Chemicals. DMS, acrolein, melphalan, cisplatin, 2-mercapto-4-hydroxy-5,6-diaminopyrimidine, MNNG, MNU, ENU, and EMS were obtained from Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO). DTP was synthesized by an established method from xanthine and phosphorus pentasulfide in tetramethylene sulfone as solvent (*24*). DUA was synthesized from 2-mercapto-4-hydroxy-5,6-diaminopyrimidine and phosphorus pentasulfide as described (*25*). AFFI-501 gel was obtained from Bio-Rad Laboratories (Richmond, CA). All HPLC analyses were conducted using a 0.47 × 25 cm RP-300 column (Brownlee, Santa Clara, CA) maintained at 35 °C. BPDE was obtained from ChemSyn Laboratories (Lenexa, KS) and was handled as previously described (*26*).

Caution: *The electrophiles described in this study are strong mutagens and in many cases carcinogens, and must be handled with appropriate precautions. All solutions and materials containing electrophiles should be disposed as biohazardous waste or chemically detoxified.*

Overall Strategy of Analyses. The test electrophiles were incubated with DTP *in vitro*, and the reaction mixtures were tested for the formation of adducts by several techniques, including UV spectral changes, the appearance of new peaks in an HPLC chromatogram, or simply the formation of a precipitate. To quantitatively analyze the binding reaction of DTP with electrophiles, rate constants were determined. All of the tested compounds may have two pathways of reaction *in vitro*: hydrolysis (rate constant: k_0) or binding to DTP (rate constant: k_1) The overall velocity of reaction (V) is given by:

$$V = V_{\text{hydrolysis}} + V_{\text{binding}} = -\text{d[DTP]}/\text{d}t = k_0[\text{E}] + k_1[\text{E}][\text{DTP}] \quad (1)$$

where E = electrophile and t = time. If [E] ≫ [DTP], and the hydrolysis is sufficiently slow, at early times of reaction the change of [E] may be neglected. In that case, the binding reaction rate just depends on [DTP]:

$$-\text{d[DTP]}/\text{d}t = k_1E_0[\text{DTP}] \quad (1a)$$

where E_0 is the initial concentration of electrophile. It can easily be shown that for an appropriate spectral parameter, A, that changes as the reaction progresses:

$$\ln A(t) = C - k_1E_0t \quad (2)$$

where C is an arbitrary constant. The pseudo-first-order rate constant k_1 may be determined by measuring changes in the UV absorption spectrum of the reaction mixture due to loss of DTP if other reaction components do not interfere.

For some compounds, the change of [E] cannot be neglected or the adducts or electrophiles affect the UV measurement of DTP. In these cases, the rate constants k_1 can be determined in an alternative way. When [DTP] ≫ [E], the fraction of product formation (f_c) at the completion of the reaction is given by:

$$f_c = [\text{A}]/([\text{A}] + [\text{H}]) = k_1[\text{DTP}]/(k_0 + k_1[\text{DTP}]) \quad (3)$$

where [A] = adduct concentration and [H] = hydrolyzed product concentration. [A] and [H] can often be measured independently by HPLC or fluorescence methods to get f_c (*8, 10*), and k_0 can be measured in the absence of DTP. The ratio k_0/k_1 can then be obtained by plotting $1/f_c$ versus 1/[DTP].

Since we have generally used assays that do not distinguish between reactions at the S^2 and S^6 thiols, the results of the kinetic analyses are presented as the apparent rate constant, k_{app}, which is the sum of the k_1's for the independent reactions at each of the two positions. The expected effects of this simplification will be described further in the Discussion.

Reaction of DTP with DMS. DTP (1000 μM) in 3 mL PB was added to 5 μL of DMS (final concentration 10.6 mM) and incubated at 37 °C. At intervals, aliquots were removed and diluted 20-fold with 5% acetic acid, effectively quenching further reaction as monitored by the absence of further absorbance changes. HPLC analysis (data not shown) indicated the presence in the quenched reaction mixtures of dimethyl-DTP (see below), which was the only reaction product after reaction times longer than 30 min, and a major intermediate, which we tentatively identified as a monomethyl derivative. Absorption spectra of the isolated HPLC peaks indicated that the mono- and dimethyl derivatives had approximately equal extinction coefficients at 292 nm. Since DTP exhibited much greater absorbance at 292 nm, the initial methylation reaction could be analyzed conveniently at that wavelength using eq 2 to determine the rate constant (k_{app}). Residual absorbance of the products of reaction at 292 nm, determined upon completion of reaction, was subtracted from the absorbance measurements.

When the reaction was carried out at higher DTP concentration (4 mM), a precipitate formed after 4 h of reaction. The reaction products were recovered by centrifugation. The pellet was recrystallized with hot water and analyzed qualitatively by nuclear magnetic resonance spectroscopy, HPLC (mobile phase: 7 min 100% 1.7 mM trifluoroacetic acid, 20 min 80% 1.7 mM trifluoroacetic acid/20% acetonitrile, detector wavelength 344 nm), and UV spectroscopy.

Reaction of DTP with Acrolein. Acrolein was diluted 50-fold into PB and then immediately diluted further with a

solution of DTP in PB at 37 °C to give a final acrolein concentration of 13.5 mM and a final DTP concentration of 47.5 μM. Absorbance spectra were recorded every 5 s beginning 15 s after the initial mixing, and k_{app} was determined using eq 2.

Reaction of DTP with Cisplatin. Different concentrations of cisplatin were incubated with a single concentration of DTP (50 μM) at 37 °C in PB overnight, and precipitate formation was observed. To measure the binding rate constant, 10 μM DTP was mixed with 100 μM cisplatin at 37 °C, and spectral changes were recorded at different times. These conditions did not promote precipitate formation.

Reaction of DTP with Melphalan. For measurement of DTP binding to melphalan, different concentrations of DTP in 1.0 mL of PB were reacted with a known concentration of melphalan at 37 °C for 4 h. To detect the adduct qualitatively, the precipitate that formed was recovered by centrifugation, redissolved in 0.1 N NaOH, diluted with PB, and analyzed by UV and HPLC (mobile phase: 88% acetic acid/12% acetonitrile for 15 min; linear gradient 12−80% acetonitrile for 30 min, detector wavelength 260 nm) (*27*). To detect hydrolyzed melphalan qualitatively, 40 μL of 20 mM melphalan/methanol solution was added to 1 mL of PB and incubated at 37 °C for 4 h. The solution was diluted 1000-fold with 30% methanol and analyzed by HPLC (mobile phase: 30% methanol). Melphalan is known to hydrolyze in aqueous solution, and the hydrolysis products are fluorescent (*28*). For measurement of the rate constant for hydrolysis of melphalan, fluorescence was recorded at different times after 60 μL of 500 μM melphalan was added to 3 mL of PB (λ_{ex} = 260 nm, λ_{em} = 360 nm). The fluorescence intensity (F) was fit to pseudo-first-order kinetics according to the equation $F(t) = F_{max}(1 - e^{-k_0 t})$.

Analysis of the starting material and the precipitate formed at high reactant concentrations indicated that neither melphalan itself nor the melphalan−DTP adduct were fluorescent under the conditions used to detect hydrolysis products. However, the concentrations of fluorescent hydrolysis products could not be monitored directly in reaction mixtures because the UV absorption of DTP interferes with fluorescent quantitation of the hydrolysis products (the "inner filter" effect). To overcome this problem, 1 mL aliquots of the above reaction mixtures were adsorbed to AFFI-501 gel to remove free DTP; AFFI-501 gel contains a mercurial reagent that binds thiol groups but does not adsorb melphalan hydrolysis products. The concentration of hydrolyzed melphalan in the supernatant was determined by fluorescence spectroscopy with reference to a standard curve. The standard curve was constructed by allowing melphalan to hydrolyze completely, then adding DTP and analyzing the mixture by AFFI-501 gel adsorption and spectroscopy. The adduct concentrations in DTP−melphalan reactions were obtained from the known starting concentrations of melphalan minus the hydrolyzed melphalan concentrations. This allowed us to determine the product ratio, f_c, and the binding rate constants were obtained from double reciprocal plots according to eq 3 (*8, 10*).

Reaction of DTP with Nitrosamides and EMS. Different concentrations of MNU, ENU, MNNG, or EMS (0−1600 μM) were reacted with 1000 μM DTP at 37 °C in PB for 4 h or longer. Reaction mixtures were diluted, and UV spectra were determined.

Reaction of DUA and DTP with BPDE. Solutions of 0−300 μM DTP or DUA in 10 mM Tris (pH 8.0) were adjusted to 1 mM β-mercaptoethanol, and BPDE (1600 μM in tetrahydrofuran) was added to a final concentration of 40 μM. After 4 h at 25 °C, iodoacetamide was added to a final concentration of 1 mM to block remaining thiol groups. Samples were prepared for and analyzed by HPLC as previously described (*8*).

Results

Reaction of DTP with DMS. When DMS was added to DTP in PB, a gradual but distinct UV spectral shift was observed during the following 960 s (Supporting Information, Figure S1). Since DMS does not absorb appreciably in this wavelength range, the shift of the long wavelength maximum of DTP (348 nm) to shorter wavelength indicated a new product was formed. When the reaction was repeated at a higher concentration of DTP (4 mM), a visible precipitate formed. After recrystallizing this precipitate from water, we obtained a product that exhibited the same UV absorption maxima in acidic, neutral, and alkaline solutions as those of 2,6-dimethyl-DTP (*29*). HPLC analysis of this product showed a single peak with much lower polarity than DTP (retention time was 14.7 min, compared to 5.3 min for DTP; Supporting Information, Figure S2). Proton NMR spectra (500 MHz) were obtained in deuterated methanol at 29 °C. In addition to the C8 proton peak (8.12 ppm), two well-defined methyl peaks (integrating for three protons each) were observed at 2.68 and 2.60 ppm, confirming the identification of this adduct as 2,6-dimethyl-DTP.

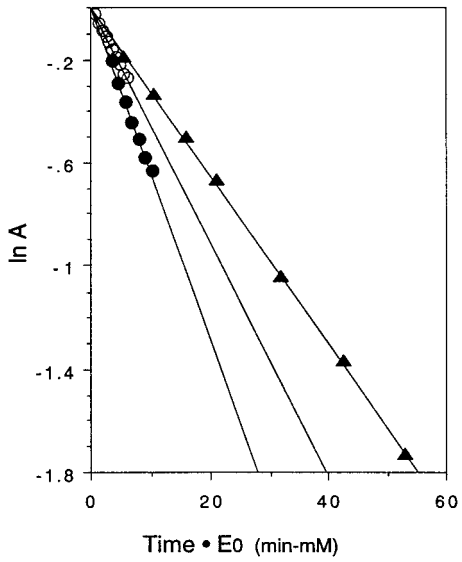

Figure 1. Kinetics of the reactions of DTP with DMS, acrolein, and cisplatin. The reactions of DMS (closed triangles), acrolein (closed circles), and cisplatin (open circles) with DTP were followed by absorbance spectroscopy as described in Materials and Methods. The natural logarithm of an appropriate spectral parameter for each reaction was plotted against the product of reaction time and the initial concentration of electrophile. The arbitrary constant in eq 2, *C*, was adjusted to give ln $A = 0$ at time zero; this does not affect the value obtained for the slope. These procedures allow the data for all three compounds to be included in one graph. Linear least squares analysis was used to obtain the apparent rate constants from the slopes of the lines; data from representative experiments are shown.

The apparent rate constant for the reaction of DMS with DTP was measured as described in Materials and Methods. Changes in [DMS] were neglected based on the published rate constant for DMS hydrolysis (expected hydrolysis in 5 min <10%; *30*). The reaction was pseudo first order, and k_{app} could be obtained by linear least squares analysis (Figure 1, closed triangles); we found $k_{app} = 0.58 \pm 0.03$ M^{-1} s^{-1} ($n = 3$).

Reaction of DTP with Acrolein. Initial tests indicated that at room temperature the addition of acrolein to a DTP solution led to time-dependent changes in the absorption spectrum (Supporting Information, Figure S3). Several isosbestic points were observed, and the decrease in the DTP absorbance at 285 nm appeared to be a useful reaction parameter. When the reaction was carried out at 37 °C, the logarithm of the corrected A_{285} decreased linearly for about 45 s (Figure 1, closed circles),

allowing determination of the rate constant: $k_{app} = 1.10 \pm 0.11$ M^{-1} s^{-1} ($n = 5$).

Reaction of DTP with Cisplatin. When cisplatin (0−80 μM) was added to DTP (50 μM) at 37 °C and incubated overnight, precipitates were observed at cisplatin concentrations of 40 μM or higher. Although there were no qualitative changes in the absorption spectra of the supernatants (Supporting Information, Figure S4), which were identical to that of unreacted DTP, there was a dose-dependent, quantitative reduction in absorbance, indicating that adducts must have formed (UV absorption of cisplatin was negligible). Unfortunately, we were unable to dissolve the precipitates obtained at high cisplatin concentrations in any of numerous solvents tested, and it was impossible to further analyze the properties of this product. When we plotted the difference between the initial and final [DTP] in the supernatant (determined by absorbance measurements) against the initial concentration of cisplatin, a straight line with a slope close to 1 was obtained (slope = 0.91, $r^2 = 0.989$), suggesting that this is a stoichiometric reaction: one molecule of cisplatin can be trapped by one molecule of DTP. At lower concentrations of DTP (10 μM), the k_{app} could again be obtained by plotting $\ln[A_{348}]$ vs time as described above for DMS and measuring the slope of the line (Figure 1, open circles). This analysis gave a value of 0.70 ± 0.05 M^{-1} s^{-1} for k_{app} ($n = 3$).

Reaction of DTP with Melphalan. When 1.6 mM melphalan reacted with 1 mM DTP for 4 h at 37 °C in PB, a yellow precipitate was obtained. Formation of a precipitate was absolutely dependent upon the presence of DTP. The redissolved precipitate had relatively more absorbance at 305 nm compared to the A_{260} than did melphalan itself, and the spectrum was also dissimilar to that of the hydrolysis products of melphalan (Supporting Information, Figure S5), suggesting that adducts had formed. HPLC analysis indicated that the reaction products consisted of three components with much less polarity than either melphalan, hydrolyzed melphalan, or DTP (Figure 2).

Since melphalan is known to hydrolyze appreciably in aqueous solution, the relatively simple analysis used to obtain the rate constants for DMS and cisplatin reaction had to be modified to take into account the alternate reaction pathway. The rate constant of hydrolysis, k_0, was first measured in order to quantitatively analyze the binding reaction later. The hydrolysis products of melphalan are fluorescent, whereas neither melphalan, DTP, nor the products of DTP−melphalan reaction (viz., the redissolved precipitate described above) exhibited appreciable fluorescence under these conditions. The time-dependent increase in fluorescence intensity after addition of melphalan to aqueous solution in the absence of DTP was analyzed to obtain k_0: $5.85 \times 10^{-4} \pm 0.43 \times 10^{-4}$ s^{-1} (Supporting Information, Figure S6).

We also used the measurement of fluorescence of hydrolyzed melphalan to determine an apparent rate constant for the binding reaction, k_{app}, as described in Materials and Methods. In Figure 3 (closed triangles), double reciprocal analysis following eq 3 was used to determine k_{app}: 2.31 ± 0.08 M^{-1} s^{-1} ($n = 3$).

Reaction of DTP with Nitrosamides and EMS. Varying concentrations of three nitrosamides, MNU, ENU, and MNNG, and a fourth alkylating agent, EMS, were incubated with DTP (1000 μM) for 4 h or longer, at 37 °C at pH 7. No significant UV spectral changes were seen, suggesting that DTP did not react appreciably with any of these compounds (data not shown). Alteration of the pH between 5 and 8 did not appear to accelerate these reactions. Based on our experience with the methylation of DTP by DMS, product formation amounting to ~5% of the initial DTP would have been easily detectable. We conclude that if these alkylation reactions take place under these conditions, the rate constants must be lower than 0.03 M^{-1} s^{-1}.

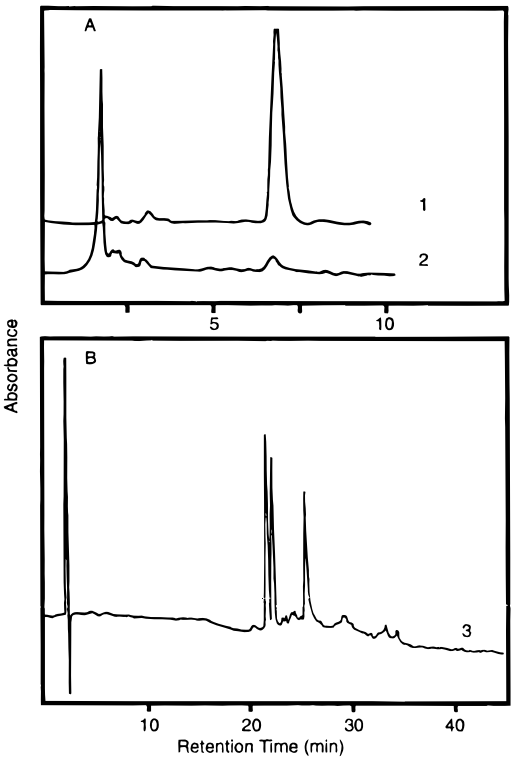

Figure 2. HPLC analysis of melphalan reaction products. Panel A: In trace 1, melphalan was diluted with 30% methanol and immediately analyzed by HPLC, using 30% methanol as the mobile phase; the major peak had a retention time of 6.9 min. Alternatively, melphalan was allowed to hydrolyze in PB for 4 h at 37 °C before analysis (trace 2). The hydrolysis product(s) exhibited a major peak with a retention time of 1.9 min. Panel B: A reaction mixture containing 1.6 mM melphalan and 2.0 mM DTP was incubated at 37 °C for 4 h, and the precipitate was collected as described in Materials and Methods. In preliminary analyses under the conditions of panel A, no absorbance peaks above background were seen. Use of an acetic acid/acetonitrile gradient (see Materials and Methods) resolved three major products with retention times between 20 and 30 min (trace 3).

Reaction of DTP and DUA with BPDE. When DTP was fed to mice in short term toxicity experiments (*12*), the major metabolite detectable in serum and urine was DUA. We compared the rates of reaction of DTP and DUA with the ultimate carcinogen, BPDE. In reactions without purinethiols, two major BPDE-derived tetrols were observed with retention times of 4.0 and 4.9 min (Figure 4, trace A). As shown previously under different reaction conditions, addition of DTP to the reaction mixture gave rise in a dose-dependent manner to an adduct peak (retention time = 8.9 min) and to a diminution in the tetrol peaks (Figure 4, trace B). In similar reactions with DUA, a major adduct peak was seen at a retention time of 7.5 min (Figure 4, trace C); again, the appearance of this peak was dose-dependent and associated with a decrease in the height of the tetrol peaks. The relative amounts of the adducts and tetrols were estimated by integrating the peak areas on chromatograms, and f_c was calculated as adduct/(tetrol + adduct).

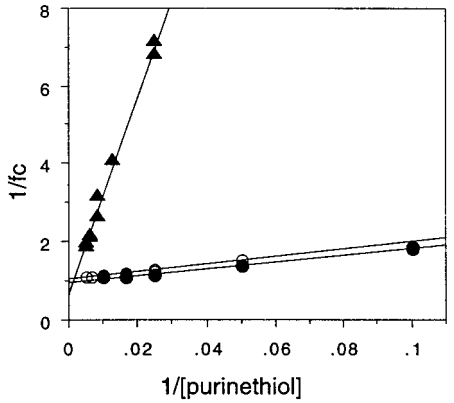

Figure 3. Kinetics of melphalan−DTP and BPDE−purinethiol reactions. A series of reactions containing 10 μM melphalan and from 40 to 200 μM DTP were incubated 5 h at 37 °C, and precipitated products were removed by centrifugation. The fraction of the original melphalan that had reacted with DTP (f_c) was calculated as described in Materials and Methods. The rate constant could then be determined from the slope of a double reciprocal plot of $1/f_c$ vs 1/[DTP] (closed triangles). To determine the rate constants for BPDE−purinethiol reactions, chromatograms such as those shown in Figure 4 were integrated and f_c was calculated. The slopes of the double reciprocal plots were used to obtain the apparent rate constants for reaction with DTP (open circles) or DUA (closed circles), which are given in Table 1.

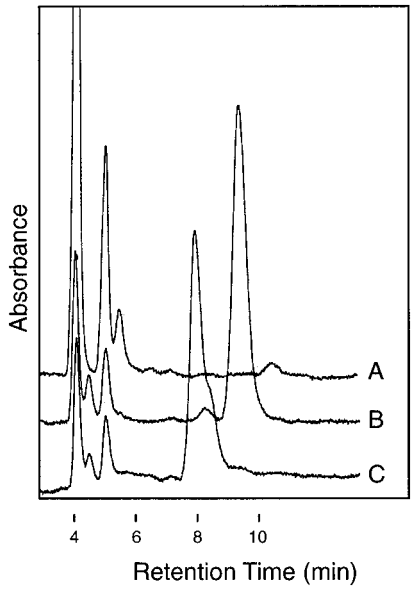

Figure 4. HPLC analysis of BPDE−purinethiol reactions. HPLC analysis was carried out as described in Materials and Methods on products from reactions containing 40 μM BPDE and (**A**) no added purinethiol (**B**) 20 μM DTP; and (**C**) 20 μM DUA.

Double reciprocal analysis (Figure 3) allowed calculation of the respective rate constants: k_{app}(DUA) = 18.5 ± 0.5 M^{-1} s^{-1}; k_{app}(DTP) = 16.1 ± 0.9 M^{-1} s^{-1}. Clearly, DUA reacts facilely with BPDE, with a rate constant similar to that found for DTP.

This encouraged us to probe the reaction of DUA with other classes of electrophiles. Using methods analogous to those described above, rate constants for the reaction of DUA with DMS, acrolein, and cisplatin were determined (Table 1). In all cases, DUA was found to have rate constants for reaction with the electrophiles similar (i.e., within an order of magnitude) to the rate constants for DTP. Reaction of DMS and cisplatin with DUA was actually faster than with DTP, whereas the reaction with acrolein was slightly slower.

Table 1. Rate Constants for Reaction of Electrophiles with DTP, DUA, or H_2O

electrophile	k_{app} (M^{-1} s^{-1}) DTP	k_{app} (M^{-1} s^{-1}) DUA	$10^4 k_0$ (s^{-1})
MNU	nd[a]	nt[b]	4.61[c]
ENU	nd	nt	4.74[c]
MNNG	nd	nt	0.39[c]
EMS	nd	nt	0.10[c]
DMS	0.58 ± 0.03	2.29 ± 0.06	3.45[c]
cisplatin	0.70 ± 0.05	1.03 ± 0.11	0.25[c]
acrolein	1.10 ± 0.11	0.89 ± 0.03	nt[d]
melphalan	2.30 ± 0.08	nt	5.87 ± 0.43
BPDE	16.1 ± 0.9[e]	18.5 ± 0.5[e]	1.56[e]

[a] nd, no detectable reaction with DTP. We estimate that a rate constant greater than 0.03 M^{-1} s^{-1} would have been detected in these experiments. [b] nt, not tested. [c] Rate constants from literature (*10, 30−34*). [d] Measurements of k_{app} using acrolein that had been preincubated in PB for 60 min were about 10% lower than with freshly diluted acrolein, indicating that the hydrolysis rate is slow compared to reaction with purinethiols. [e] Measurements of the reaction of BPDE with purinethiols were conducted in 10 mM Tris, pH 8.0 at 25 °C, to minimize the rate of hydrolysis, which is accelerated both by low pH and by phosphate buffer (*35*).

Discussion

The primary purpose of this study was to determine whether the nucleophilic scavenging capacity of DTP was unique for BPDE, or extended to other potent electrophiles. We have found that DTP reacts facilely with several electrophilic toxicants, including DMS, acrolein, melphalan, cisplatin, and BPDE. In related studies, diol epoxides derived from two other polycyclic aromatic hydrocarbons, benz[*a*]anthracene and 7,12-dimethylbenz-[*a*]anthracene, were also shown to react with DTP (*36*). This suggests that a wide range of electrophiles can potentially be scavenged by DTP. However, a series of small alkylating agents that have classically been thought to react primarily via alkylcarbonium ions (MNU, ENU, EMS, MNNG) do not appear to react with DTP at near neutral pH. These compounds react by an S_N1 reaction scheme and are generally classified as "hard" (*15*) or high "oxyphilic" (*37*) electrophiles, alkylating DNA both at oxygen residues (e.g., O^6-dG) and at ring nitrogens (e.g., N7-dG).

The toxicants shown here to react well with DTP (DMS, acrolein, melphalan, cisplatin, and BPDE) appear to be "softer" electrophiles than the small alkylating agents. Many of them are larger molecules, allowing more delocalization of developing charge density. DMS is known to react with DNA through a predominantly S_N2 mechanism (*16*), and we previously found the reaction between DTP and BPDE to be S_N2 (*8*). Pt^{2+} compounds are known to be soft electrophiles, reacting faster with sulfur-based, soft nucleophiles than with harder, nitrogen-based nucleophiles (*14*). All five of these electrophiles tend to react with DNA at the "softer" nucleophilic positions (ring and exocyclic amino moieties) rather than at oxygen residues (*16, 20, 23, 38*). Classically, nucleophiles such as the thiolate anion in which sulfur is the electron donor are classified as "soft" due to their high polarizibility (*14, 15*). These findings support the classification of DTP as a "soft" nucleophile and suggest that other "soft" or "low oxyphilic" electrophiles may also be targets for scavenging by DTP. Further detailed studies of the reaction products and mechanisms will be needed to validate this hypothesis. Since many PAH and other procarcinogens such as aflotoxin B_1 cause

DNA damage and mutation through epoxides (*38, 39*), our results suggest that DTP will prove to be a potent broad spectrum scavenger of biologically important electrophiles.

Although the data are more limited, all of the "soft" electrophiles tested to date also react well with DUA. Since DTP is an excellent substrate for xanthine oxidase/dehydrogenase and DUA is the major serum metabolite of DTP after oral administration in mice (*12*), this is a striking finding. It indicates that at least the first step along the major catabolic pathway for DTP does not necessarily impair its chemopreventive potential. Whether the *in vitro* ability of DUA to react with electrophiles will have practical importance *in vivo* remains to be determined. Since many electrophilic toxicants are converted to their active forms by cellular metabolism, the ability of a potential scavenger to enter cells is a major determinant of efficacy. DTP is known to be a good substrate for the endogenous purine transporter (*40*), facilitating its transport into cells, but whether DUA is also a substrate for this transport system is unknown.

The data in Table 1 are presented as apparent rate constants because they actually represent the sum of the rate constants for reaction at each of the two thiol groups. Based on previous studies of the reaction of BPDE with DTP (*8*), the 2-thiol is expected to be ionized at neutral pH and will therefore be the major reactant. HPLC analyses of DTP/DMS reaction mixtures at early times indicate that two intermediates are formed, presumably 2-methyl- and 6-methyl-DTP, at about a 16:1 ratio.[2] Our previous studies of the major BPDE−DTP adduct (*8*) suggested the presence of a minor adduct, presumably from reaction of the 6-thiol, at about 5% the level of the major adduct. Thus we expect that the apparent rate constants in Table 1 reflect primarily the k_1 for reaction of the 2-thiol group of DTP or DUA with the electrophiles, with a contribution at the level of 5−6% from reaction at the 6-position.

Our previous data indicated that purinethiols form adducts with BPDE *in vitro*, in CHO cell culture, and after topical application to mouse epidermis (*9*−*11*). This was correlated with decreases in DNA binding and with decreases in the biological activity of BPDE: cytotoxicity and mutagenicity in CHO cells (*11*), and initiation of tumorigenesis in mouse skin (*9*). Although biological systems contain endogenous nucleophilic scavenging moieties (e.g., GSH, protein thiols, other nucleophilic amino acid side chains in proteins), the fact that BPDE can form high levels of DNA adducts in cells (cf. ref 41) indicates that these natural defenses are generally not completely efficient. Purinethiols are an effective addition to endogenous defenses and can completely inhibit BPDE−DNA adduct formation in CHO cells and mouse epidermis (*9, 11*). The studies reported here indicate that the chemical potential for DTP to scavenge a variety of electrophiles *in vitro* is excellent and suggest that DTP may also be active *in vivo* against a wide range of chemicals that produce toxic and carcinogenic effects through "soft" electrophilic intermediates. Indeed, we have recently found that DTP, administered either in the diet or by injection, dramatically inhibits bladder toxicity in mice receiving cyclophosphamide by ip injection.[3] The range of toxicants that are subject to *in vivo* scavenging by purinethiols such as DTP remains to be determined.

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

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Supporting Information Available: Six figures, as described in the text (4 pages). Ordering information is given on any current masthead page.

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