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ORIGINAL ARTICLE

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1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino) carbonyl]hydrazine (VNP40101M): I. Direct inhibition of O^6-alkylguanine-DNA alkyltransferase (AGT) by electrophilic species generated by decomposition

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Abstract *Purpose*: To investigate the interaction of the electrophilic species generated by the decomposition of the antineoplastic prodrug 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine (VNP40101M) on the ability of O^6-alkylguanine-DNA alkyltransferase (AGT) to repair alkylated O^6-chloroethylguanine and/or N^1,O^6-ethanoguanine DNA lesions. *Materials and methods*: The contributions of inhibitory electrophilic species generated from VNP40101M towards AGT was assessed using analogues that selectively generated either the chloroethylating or the carbamoylating components of VNP40101M. The activity of AGT was determined from the inhibition of crosslink formation from O^6-chloroethylguanine and/or N^1,O^6-ethanoguanine lesions. The half-lives of sulfonylhydrazine derivatives and isocyanates were measured using an acidification assay which gives a change in absorbance proportional to the release or consumption of small quantities of protons. *Results*: Both of the reactive components produced by VNP40101M directly inactivated cloned human AGT; the carbamoylating moiety (IC_{50} about 13 μM) was approximately seven- to eight-fold more potent than the alkylating component(s) (IC_{50} about 100 μM). These inhibitory actions were moderated by the addition of naked T5 bacteriophage DNA. Thus, AGT bound to DNA was markedly more resistant than free AGT to these electrophilic species. DNA also blocked the spontaneous loss of AGT activity which occurred upon incubation of this protein under mild conditions. *Conclusions*: The reaction of AGT with the methyl isocyanate generated from the decomposition of VNP40101M increased the net number of crosslinks generated by VNP40101M compared to a sulfonylhydrazine prodrug that formed the equivalent alkylating species in the absence of the cogeneration of methyl isocyanate. These actions may be of significance to the antineoplastic activity of VNP40101M.

Keywords VNP40101M · O^6-Alkylguanine-DNA alkyltransferase · Alkylation · Carbamoylation · Sulfonylhydrazine prodrugs

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Introduction

1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine (VNP40101M) is the first 1,2-bis(sulfonyl)-1-alkylhydrazine prodrug of a broad class of potential anticancer agents that we have synthesized [1] to enter clinical evaluation, currently being in phase I clinical trial. This agent has an exceptionally broad spectrum of antineoplastic activity, exhibiting significant activity in ten different tumor systems in vivo [1, 2]. VNP40101M decomposes with a half-life of approximately 1 h at pH 7.4 and 37°C to generate two short-lived species [3], 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine (90CE), a prodrug of hard chloroethylating intermediates ($t_{1/2}$ about 30 s, pH 7.4, 37°C) [4], and methyl isocyanate, a soft electrophilic carbamoylating agent ($t_{1/2}$ about 1.7 min, pH 7.4, 37°C) (Fig. 1) [5]. This is somewhat analogous to the decomposition of the chloroethylnitrosoureas (CNUs), which also generate both alkylating and carbamoylating

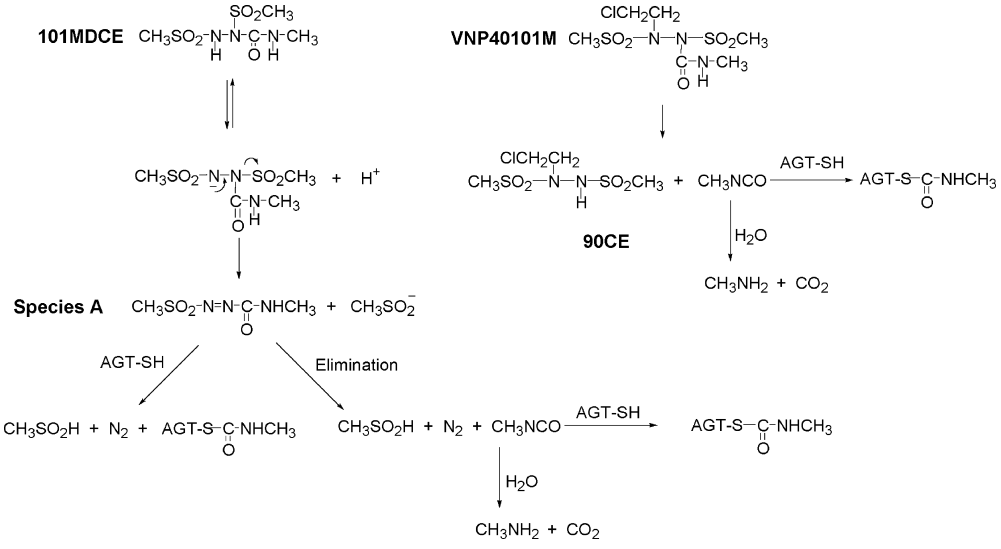


Fig. 1 Proposed schemes for the decomposition of 101MDCE and VNP40101M in neutral aqueous solution indicating the generation of species capable of AGT carbamoylation. *Species A* is a transient carbamoylating species unique to the decomposition of 101MDCE, while methyl isocyanate is generated by both agents

species [6]. The superiority of VNP40101M over the nitrosoureas in preclinical studies was clearly demonstrated by (a) its activity against tumors resistant to BCNU, as well as to cyclophosphamide and melphalan; (b) its avoidance of the mutagenic/carcinogenic activities of hydroxyethylation; (c) its oral activity; and (d) its effectiveness against tumor cells in the CNS. As an example, the therapeutic index (LD_{10}/ED_{50}) of VNP40101M against the L1210 leukemia is approximately 8 (LD_{10} of >80 mg/kg and an ED_{50} of <10 mg/kg), a value approximately twice that found for the best of 300 nitrosoureas tested, whose therapeutic indices varied from 1 to 3.9 [2, 6].

VNP40101M displays a superior therapeutic index in murine models not only to the CNUs [2] but also to other 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine prodrugs which generate 90CE and, therefore, should also produce identical DNA lesions. Moreover, VNP40101M displays similar cytotoxicity to both HT29 cells, a colon carcinoma line expressing O^6-alkylguanine-DNA alkyltransferase (AGT), and BE cells, a colon carcinoma line with little or no AGT, whereas 90CE, an agent with only the alkylating activity of VNP40101M, shows significantly less activity against the AGT-expressing HT29 cell line [2]. A major difference between these agents is in the production of a reactive isocyanate species with VNP40101M generating methyl isocyanate, a short-lived thiophilic electrophile [5], and BCNU forming 2-chloroethyl isocyanate. The findings suggest that methyl isocyanate plays a superior role in enhancing the cytotoxicity of VNP40101M, possibly by inhibiting AGT activity [3, 7].

Isocyanates can be relatively specific enzyme inhibitors [8]. Thus, in studies conducted with the 2-chloroethyl isocyanate generated during the decomposition of BCNU, a 90% inhibition of erythrocytic glutathione reductase was found after a therapeutic dose, with little or no effect on 13 other erythrocyte enzymes [9]. Furthermore, despite the presence of ten cysteine residues per subunit, only the essential cysteine residue (Cys 58) of glutathione reductase was modified to any significant extent [10]. Unlike the CNUs, the sulfonylhydrazine prodrugs are very tolerant of structural modification, allowing the ready synthesis of compounds that generate chloroethylating or carbamoylating species either individually or in combination. These agents are therefore useful tools in studying the interaction between AGT and the different electrophilic species generated from sulfonylhydrazine prodrugs (Fig. 1, Table 1).

Table 1 Structures and reactive electrophiles generated by various 1,2-bis(methylsulfonyl)hydrazine derivatives

Compound	CH_3SO_2-N(X)-N(Y)-SO_2CH_3		Chloroethylating agent	Carbamoylating agent
	X	Y		
VNP40101M	$ClCH_2CH_2$-	CH_3NHCO-	YES	YES
90CE	$ClCH_2CH_2$-	H	YES	NO
101MDCE	H	CH_3NHCO-	NO	YES
101	$ClCH_2CH_2$-	$ClCH_2CH_2NHCO$-	YES	YES
101DCE	H	$ClCH_2CH_2NHCO$-	NO	YES

It is generally accepted that events stemming from the initial chloroethylation of the O^6 position of guanine in DNA are responsible for the therapeutic efficacy of the CNUs [11]. 90CE appears to be a more selective chloroethylator of the O^6 position of guanine than the CNUs, giving twice the molar yield of DNA crosslinks. This is coupled with a much lower yield of single strand breaks, indicating fewer alkylations of the N^7 position of guanine [3]. Therefore, we have assumed that chloroethylation of the O^6 position of guanine in DNA is the primary lesion responsible for the therapeutic actions of 90CE and its prodrugs (i.e., VNP40101M).

AGT is a repair protein that catalyzes the transfer of alkyl groups from the O^6 position of guanine to cysteine 145 of the AGT molecule, restoring the O^6 position of guanine to its native state [12]. There is no known acceptor that removes the alkyl groups from cysteine 145 of AGT, so one AGT molecule is required to repair each lesion; once alkylated the AGT molecule is rapidly degraded [12]. AGT is able to remove a wide variety of alkyl groups from the O^6 position of guanine. Haloethylation, however, of the O^6 position of guanine in DNA represents a special case because a relatively rapid cyclization reaction can occur resulting in the production of N^1,O^6-ethanoguanine and halide loss [13]. N^1, O^6-Ethanoguanine is the precursor of 1-(3-cytosinyl)-2-(1-guanyl)ethane, the G-C crosslink, which is formed relatively slowly by the reaction of N^1,O^6-ethanoguanine with the N^3-position of cytosine on the opposing strand [14]. AGT can repair the lesion before or after cyclization. However, once cyclization has occurred, 'repair', while preventing the formation of a highly toxic crosslink, will result in the 26 M_r AGT molecule becoming covalently attached to the N^1-position of guanine [15, 16]. This could impair further repair of nearby lesions and/or delay any signal transduction initiated by the alkylated AGT and result in the requirement of other repair proteins to fully repair the lesion. After the G-C crosslink has been formed AGT is unable to produce a repair of any kind.

In the study reported here, we examined the inactivation of cloned human AGT by the reactive species generated by VNP40101M and related derivatives by measuring the in vitro repair of a model DNA, T5 bacteriophage DNA, which has been chemically modified to include G-C crosslink precursors. These studies demonstrated that the methyl isocyanate generated from the decomposition of sulfonylhydrazine prodrugs can effectively inactivate AGT, and may contribute to the cytotoxicity produced by the chloroethylating species [7]. In an analogous manner, the interaction of the different isocyanates generated by the various CNUs probably account for a significant proportion of the direct AGT inactivation previously reported for nitrosoureas in simple model systems [15].

Materials and methods

Chemicals

T5 DNA and all chemicals were purchased from the Sigma Chemical Company (St. Louis, Mo.), except where specified. Hoechst 33258 was obtained from Molecular Probes (Eugene, Ore.). VNP40101M was synthesized in this laboratory as previously described [1]. 101DCE and 101MDCE were synthesized using a procedure similar to that described for compounds 101 and VNP40101M, by substituting 1,2-bis(methylsulfonyl)hydrazine for 90CE [1]. Stock solutions of sulfonylhydrazine prodrugs were made in dimethylsulfoxide and diluted with ice-cold buffer, such that the final concentration of dimethylsulfoxide in the reaction mixture did not exceed 1%; these solutions were used immediately. Solutions of methyl isocyanate and 2-chloroethyl isocyanate were made by diluting the pure reagent with ice-cold buffer; these solutions were then immediately used. Cloned human AGT was purified as previously described [17].

Preparation of AGT substrate

T5 DNA containing crosslink precursors was prepared by treating the DNA (100 μg/ml) with 0.2 m*M* 90CE, a short-lived chloroethylating agent with a half-life of about 30 s, in 20 m*M* Tris-HCl and 1 m*M* EDTA buffer (pH 7.4) for 3 min at 37°C. The DNA was then stored at 0–4°C until used. After the 3 min of incubation with 90CE at 37°C the T5 DNA substrate contained crosslink precursors, without detectable crosslinks when assayed without further incubation utilizing the method described below. Furthermore, under these conditions the 90CE used to introduce the crosslink precursors was essentially completely decomposed and had no capacity to further alkylate the DNA or to inhibit AGT activity directly. The concentrations of 90CE were chosen to give a maximum of approximately 35–45% of the DNA being crosslinked (at least one crosslink in 35–45% of the molecules) after the DNA was allowed to undergo the intramolecular crosslinking reaction, which required approximately 12–15 h at 37°C or 2 h at 55°C to reach maximum levels. At lower concentrations of 90CE the eventual fluorescent signal was reduced, decreasing the signal to noise ratio, while at higher concentrations many of the molecules had multiple crosslink precursors, making small changes in their number not readily determinable.

Determination of DNA crosslinks

The crosslinking of DNA was determined utilizing an assay based upon the snap-cooling of thermally denatured T5 DNA under neutral pH conditions as previously described [18]. A single crosslink was required per DNA molecule to allow the DNA to rapidly renature under conditions of snap-cooling. Samples containing 1 μg T5 double-stranded DNA were removed at various times, diluted by mixing with 1.5 ml 5 m*M* Tris-HCl (prepared from the free base to minimize ionic strength) and 0.5 m*M* EDTA buffer (pH 8.0) containing 0.1 μg/ml of Hoechst 33258 fluorescent probe, and the fluorescence was determined before and after a heating (96°C) and chilling (0°C) cycle. The fluorescence measurements were performed using a Hoefer Scientific Instruments TKO 100 Mini-fluorometer. The percentage of DNA crosslinked was calculated using the following equation: $100(E_A/E_B - C_A/C_B)/(1 - C_A/C_B)$, where E_A is the fluorescence of the experimental sample after the heat/chill cycle, E_B is the fluorescence of the experimental sample before the heat/chill cycle, C_A is the fluorescence of the control sample after the heat/chill cycle, and C_B is the fluorescence of the control sample before the heat/chill cycle. Control T5 DNA typically lost approximately 93% of its fluorescence after the heat/chill cycle, so the percentage of DNA crosslinking is: $107.5(E_A/E_B - 0.07)$.

Determination of AGT activity

Samples to be assayed for AGT activity (10 μl) were mixed with 10 μl AGT substrate (T5 DNA containing crosslink precursors prepared as described above) and incubated for 30 min at 37°C to allow the AGT to react with the crosslink precursors. Samples were then diluted tenfold with 5 m*M* Tris-HCl and 0.5 m*M* EDTA buffer (pH 8.0) to prevent any problems caused by concentration/evaporation of the small 20-μl samples within the 1.5-ml sealed tubes during a subsequent 55°C incubation for 2 h to allow any remaining crosslink precursors to progress to DNA crosslinks. The samples were then assayed for DNA crosslinks as described above, and the values obtained were compared with those from control samples treated equivalently but lacking the addition of AGT.

AGT solutions

AGT stock solutions containing 1.0 mg/ml of AGT (1543 pmol AGT activity/mg protein) in 10 m*M* Tris-HCl, 1 m*M* EDTA, 1 m*M* dithiothreitol, 10% glycerol, and 0.02% NaN_3 (pH 7.4) were diluted tenfold with the same buffer and stored in aliquots at −70°C. The specific activity of this diluted stock was approximately 154 fmol AGT activity/μl. Unless otherwise stated this solution was diluted tenfold immediately prior to use with 20 m*M* Tris-HCl, 1 m*M* EDTA and 1 mg/ml of bovine serum albumin (BSA) buffer (pH 7.4). Approximately 31 fmol AGT (2 μl) was used per determination in the AGT inhibition studies, except in experiments in which a range of AGT levels between 0 and 62 fmol were employed.

Inhibition of AGT activity

Small samples containing approximately 31 fmol AGT in 2 μl were mixed with 8 μl 20 m*M* Tris-HCl and 1 m*M* EDTA buffer (pH 7.4) containing 1.25-fold of the desired concentration of various inhibitors or stabilizers to give 10 μl of 1× reaction mixture. This mixture was then incubated at 37°C for three times the $t_{1/2}$ value of the agent being tested (or for 5 min in the case of very short-lived species), then assayed for AGT activity by the addition of 10 μl AGT substrate T5 DNA as described above.

Determination of drug half-life

The half-lives of the various sulfonylhydrazine derivatives and isocyanates were determined using an acidification/alkalinization assay [19]. The decomposition kinetics were measured in 1 m*M* potassium phosphate buffer containing 20 μg/ml of phenol red at pH 7.4 and 37°C by following the change in absorbance of phenol red at 560 nm, which varied linearly with very small molar changes in the consumption or generation of hydrogen ions as previously described [19].

Results

In initial experiments, loss of AGT activity was noted in the absence of any drug during 5-min incubations at 37°C. While this was not a problem in measuring the interaction of very short-lived electrophiles with AGT, the spontaneous loss of AGT activity precluded studying the inactivation of this protein by much longer-lived sulfonylhydrazine prodrugs. Therefore, factors affecting the stability of AGT were investigated. The presence of various metal ions, chelating agents, and thiols had little effect on the stability of AGT. However, 1 mg/ml of BSA enhanced the stability of AGT sufficiently to permit studies involving longer-lived agents. BSA was therefore included in all experiments. The low stability of AGT and the stabilizing effects of BSA have been previously reported [15].

The incubation of AGT with T5 DNA containing crosslink precursors resulted in a titration of the crosslink precursors and a reduction in the ability to generate crosslinks upon subsequent incubation. The repair of the lesions was very rapid with the majority of lesions being repaired in 5–10 min (data not shown). Therefore, incubations of 30 min were used to allow the reaction to go to effective completion. In the 0–40% final crosslinking range, assuming a random distribution of crosslink precursors, a relatively linear relationship between the final level of crosslinking attained and the number of residual crosslink precursors should occur, because most of the DNA molecules will contain either no crosslink precursors or only a single crosslink precursor. The requirement for one AGT molecule to repair each lesion should, therefore, result in an approximately linear reduction in the final level of crosslinking in proportion to the quantity of added AGT until the supply of crosslink precursors becomes low. This theoretical expectation agrees with the experimental observations shown in Fig. 2. The inverse relationship between the eventual level of crosslinking and the quantity of added AGT allows the measurement of AGT activity in this range, and the assessment of the inhibition of AGT by reactive electrophiles.

Incubation of T5 DNA containing crosslink precursors with simple thiols such as glutathione or thioglycerol at concentrations up to 20 m*M* did not mimic the actions of AGT (data not shown). These thiol concentrations are $>10^6$ times the concentration of AGT in molar terms that abolishes DNA crosslinking, yet the thiols did not measurably titrate the crosslink precursors under the incubation conditions employed. This is contrary to earlier findings by others [20] which suggest that DNA crosslink precursors generated by BCNU can react with GSH and block crosslink formation. Such reactions, if they occurred, would give simple thiols

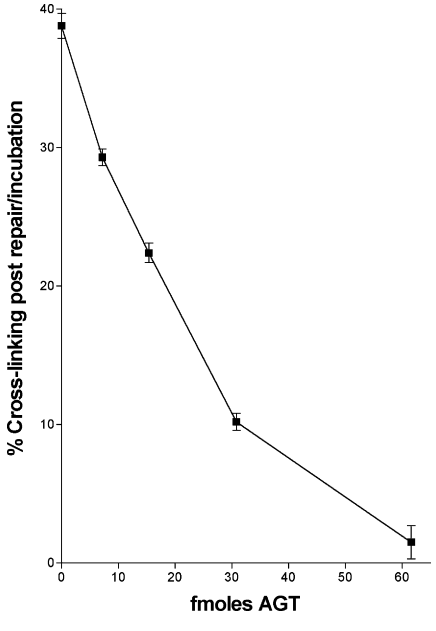


Fig. 2 Titration of DNA crosslink precursors by AGT. Titration of DNA crosslink precursors, as measured by the decrease in the eventual percentage crosslinking in the test DNA versus femtomoles of AGT added. A DNA concentration of 50 μg/ml was used in the AGT/DNA reaction mixture (volume 20 μl). Experiments were performed in 20 m*M* Tris-HCl, 1 m*M* EDTA buffer at pH 7.4 and 37°C containing 1 mg/ml of BSA. The values given are means ± SE ($n = 3$)

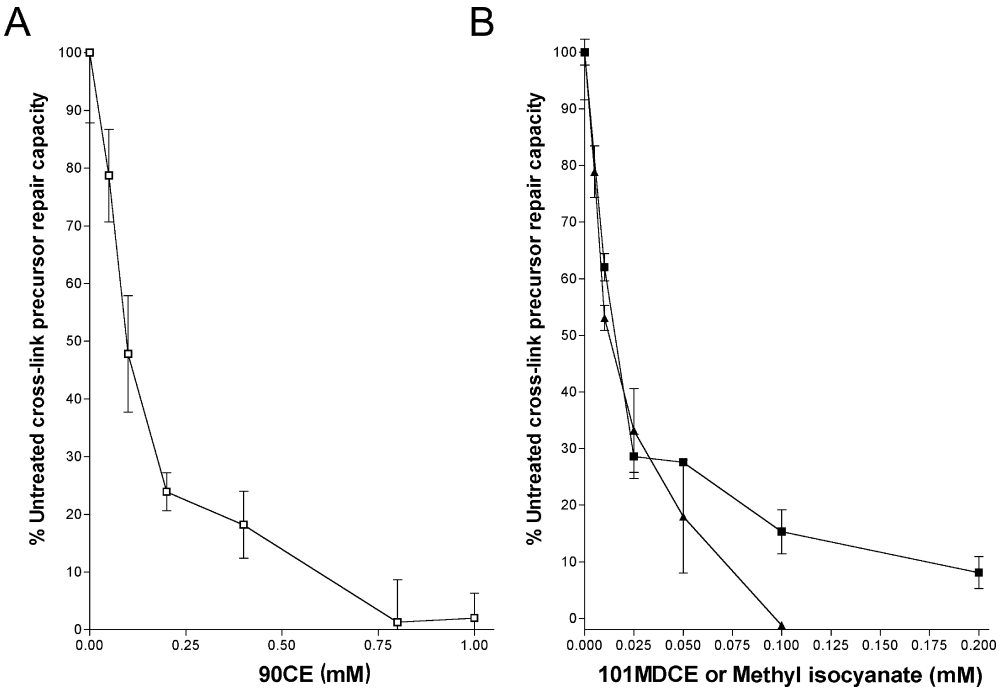
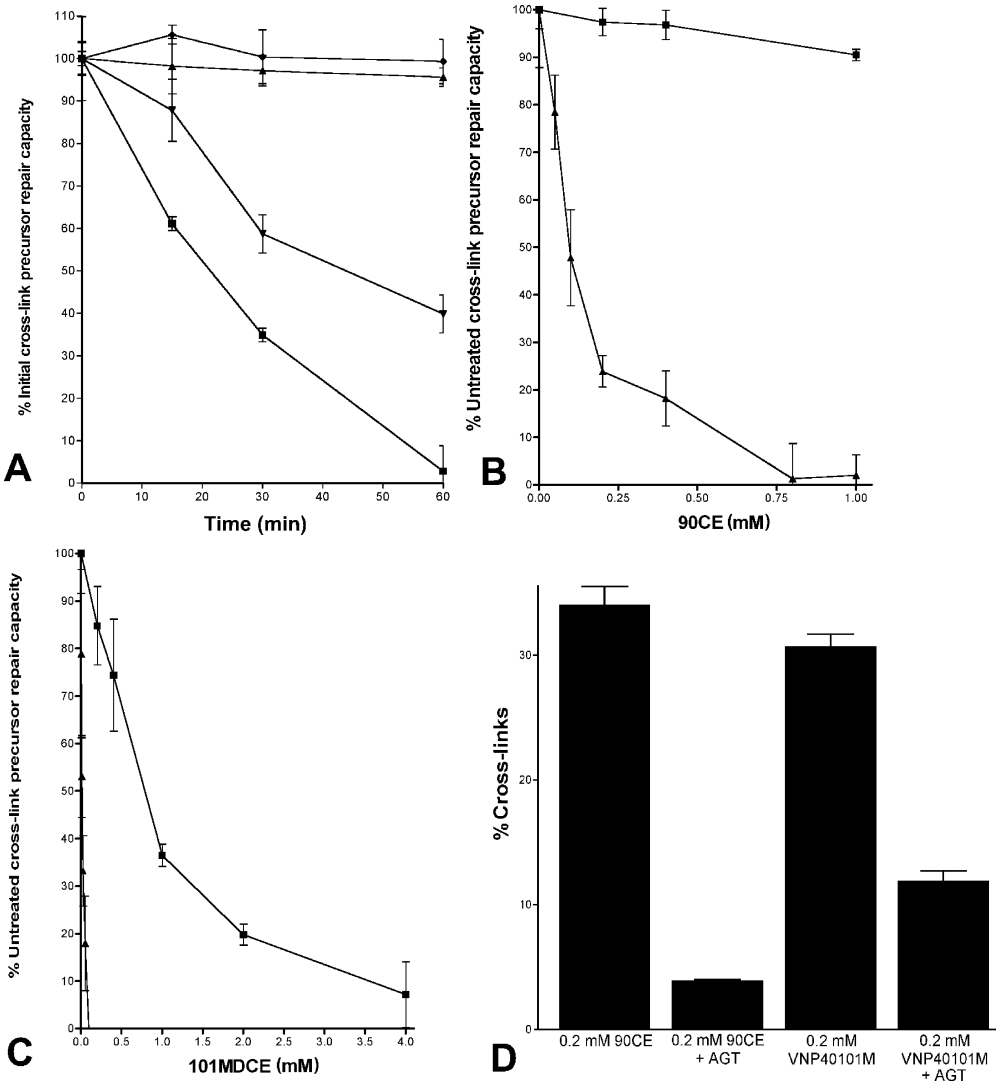


Fig. 3A, B Inactivation of AGT by 90CE, methyl isocyanate and 101MDCE. **A** The crosslink precursor repair capacity of AGT incubated for 5 min with various concentrations of 90CE, expressed as the percentage of the activity of AGT equivalently incubated in the absence of 90CE versus the concentration of 90CE. **B** ■ The crosslink precursor repair capacity of AGT incubated for 5 min with various concentrations of methyl isocyanate expressed as the percentage of the activity of AGT equivalently incubated in the absence of methyl isocyanate versus the concentration of methyl isocyanate. ▲ The crosslink precursor repair capacity of AGT incubated for 10 min with various concentrations of 101MDCE expressed as the percentage of the activity of AGT equivalently incubated in the absence of 101MDCE versus the concentration of 101MDCE. All AGT/drug reaction mixtures (10 µl) contained 31 fmol AGT. The values given are means ± SE ($n = 3$)

AGT-like ability to protect cells from agents that chloroethylate the O^6 position of DNA guanine. Since AGT appears to be the main determinant of resistance to this assault, any such effects of simple thiols in intact cells are likely to be minor.

Pretreatment of AGT with 90CE (VNP40101M is a prodrug which generates this alkylating species) resulted in attenuation of the repair capacity of AGT, with 0.1 m*M* 90CE producing an approximate 50% decrease in the repair capacity of AGT (Fig. 3A). This concentration of 90CE in our model system resulted in a molar loss of AGT activity by direct interaction with 31 fmol AGT protein in a 10-µl sample volume, comparable to what would be expected from the repair of the lesions generated by the interaction of the same concentration (0.1 m*M*) of 90CE with 100 µg/ml of T5 DNA in the 10-µl sample volume. The highest therapeutic dose of VNP40101M used in in vivo measurements of antineoplastic activity, if one assumes VNP40101M generates 90CE in high yields as observed in simple model systems, was capable of generating twice this level of 90CE. This implies that direct inactivation of AGT by the alkylating moiety could have physiological relevance, the relative importance of this effect being dependent upon the ratio of AGT to DNA.

Pretreatment of AGT for three half-life times with 101MDCE ($t_{1/2}$ about 3.5 min, pH 7.4, 37°C), an agent which only generates methyl isocyanate, the carbamoylating activity of VNP40101M, strongly impaired the repair capacity of AGT. Approximately 13 µ*M* 101MDCE resulted in a 50% reduction in the repair capacity of AGT. This experiment was repeated using pure reagent methyl isocyanate and a very similar IC_{50} value of about 15 µ*M* was obtained (Fig. 3B). While VNP40101M is a prodrug of methyl isocyanate, the decomposition of both BCNU and 101 generate 2-chloroethyl isocyanate. Therefore, the effects of 101DCE, a prodrug of 2-chloroethyl isocyanate, and reagent 2-chloroethyl isocyanate were also examined to compare the effects of 2-chloroethyl isocyanate to those of methyl isocyanate. The half-life of 2-chloroethyl isocyanate is less than one-fifth that of methyl isocyanate under the conditions employed ($t_{1/2}$ about 17 s, pH 7.4, 37°C) [21]. Both 2-chloroethyl isocyanate and 101DCE readily inactivated AGT, although an approximately twofold greater concentration than methyl isocyanate appeared to be required to achieve a 50% inactivation of AGT (data not shown). It is likely that AGT inhibitory capacity is a common feature of many isocyanate species, at least in simple model systems. None of the isocyanates or the isocyanate prodrugs tested matched the inhibitory capacity of O^6-benzylguanine, a specifically designed inhibitor of AGT [22, 23], which had an IC_{50} of approximately 1 µ*M* after a 10-min incubation at 37°C in our model system (data not shown).



Since each AGT molecule is only capable of repairing a single DNA alkylation, it would seem extremely wasteful if the AGT protein had low stability in situ, resulting in a high turnover even in the absence of performing its repair function. While the presence of BSA increased the stability of AGT, the protein still lost activity faster than one would expect under relatively mild incubation conditions in the absence of substrate. Therefore, we examined the effects of T5 DNA on the stability of AGT and found that the presence of naked T5 DNA greatly enhanced the stability of the AGT. The addition of only 5 μg/ml of T5 DNA appeared to completely block measurable AGT activity loss over a 60-min period (Fig. 4A). In a separate study, no significant AGT activity loss occurred after 3 h at 37°C in the presence of 100 μg/ml of T5 DNA (data not shown).

This potent stabilization of AGT by naked DNA, and the fact that much of the AGT in intact cells appears to be localized in the nucleus [12, 24], prompted us to examine the effects of the presence of T5 DNA on the inactivation of AGT by both the isocyanate and alkylating moieties generated by VNP40101M. In these studies, the initial incubation mixture contained AGT, 5 μg/ml of T5 DNA, and 1 mg/ml of BSA together with the agent being tested. The presence of DNA at this level was found to strongly protect the AGT from inactivation by both the isocyanate and alkylating moieties (Fig. 4B, C). While the magnitude of the protection from the carbamoylating species (about 60-fold) was easy to ascertain, the true magnitude of protection from inactivation by direct alkylation in the presence of DNA was not so readily determined. This was due to the indirect inhibition of AGT resulting from the repair of the O^6-guanine alkylations introduced into the protecting DNA, even though the concentration of the protecting DNA was kept at a low level (5% of the AGT substrate DNA concentration) to minimize this effect.

The high stability of AGT in the presence of DNA allowed a direct comparison of the crosslinking ability of the relatively long-lived VNP40101M ($t_{1/2}$ about 1 h) and 90CE in the presence and absence of AGT. In this experiment, either VNP40101M or 90CE was added

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Fig. 4A–D Effects of DNA on AGT stability and sensitivity to electrophilic species. **A** Comparison of the stability of AGT activity in the presence and absence of BSA and T5 DNA (■ the percentage of the initial AGT activity versus preincubation time in minutes, with no additional components; ▼ the percentage of the initial AGT activity versus time under identical conditions, but in the presence of 1 mg/ml of BSA; ▲ the percentage of the initial AGT activity versus time under identical conditions, but in the presence of 5 μg/ml of T5 DNA; ◆ the percentage of the initial AGT activity versus time under identical conditions, but in the presence of 1 mg/ml of BSA and 5 μg/ml of T5 DNA). **B** Protection of AGT by T5 DNA (5 μg/ml) from inactivation by 90CE. The activity of AGT incubated for 5 min with various concentrations of 90CE expressed as the percentage of the activity of AGT equivalently incubated in the absence of 90CE versus the concentration of 90CE (■) in the presence of 5 μg/ml of T5 DNA and (▲) in the absence of T5 DNA. **C** The activity of AGT incubated for 10 min with various concentrations of 101MDCE expressed as the percentage of the activity of AGT equivalently incubated in the absence of 101MDCE versus the concentration of 101MDCE (■) in the presence of 5 μg/ml of T5 DNA and (▲) in the absence of T5 DNA. All reaction mixtures described in **A–C** were 10 μl in volume and contained 31 fmol AGT. **D** Comparison of the DNA crosslinking ability of 90CE and VNP40101M in the presence and absence of AGT. The effects of the presence or absence of AGT (31 fmol) on the crosslinking of 100 μg/ml of T5 DNA by 0.2 m*M* 90CE or 0.2 m*M* VNP40101M. The reaction mixtures were incubated for 4 h at 37°C, then diluted tenfold with 5 m*M* Tris-HCl/0.5 m*M* EDTA buffer, pH 8.0, and incubated for a further 2 h at 55°C to allow the mono-adducts to progress to crosslinks. These samples were then analyzed for DNA crosslinks as previously described. The values given are means ± SE ($n = 3$)

directly to T5 DNA, which in this case was not pretreated and therefore did not initially contain any crosslink precursors, in the presence or absence of AGT. The mixture was incubated for 4 h at 37°C to allow the drugs to react with the DNA and the AGT, and the functional AGT molecules to repair the alkylations that were introduced by the two agents. The mixture was then incubated at 55°C for 2 h and assayed for DNA crosslinks. The presence of 100 μg/ml of T5 DNA represented a complex experiment because the DNA should not only stabilize the AGT and allow it to functionally persist in the mixture during the relatively protracted release of reactive components from VNP40101M, but also should impair the inactivation of AGT bound to DNA by the reactive species generated from the sulfonylhydrazine prodrugs. As shown in Fig. 4D, in the absence of AGT, over the employed time frame, VNP40101M was essentially equivalent to 90CE in its yield of DNA crosslinks. However, in the presence of AGT, VNP40101M generated a measurably greater net yield of crosslinks than 90CE presumably due to the methyl isocyanate generated from VNP40101M which interfered with the repair capacity of AGT despite the presence of a relatively high level of DNA capable of protecting DNA-bound AGT.

Discussion

VNP40101M has a half-life of 1 h at pH 7.4 and 37°C and generates two major short-lived species [1, 2], 90CE, a prodrug of hard chloroethylating intermediates [4], and methyl isocyanate, a soft electrophilic carbamoylating agent [5]. Although differences in chemical and physicochemical properties of VNP40101M may contribute to its therapeutic superiority over all other 90CE-generating prodrugs tested in our laboratory to date, we hypothesized that the cogeneration of methyl isocyanate, may be a key determinant in the observed efficacy of VNP40101M as an anticancer agent. Therefore, we examined the repair by AGT of O^6-alkylated guanine DNA lesions generated by 90CE, with and without pretreatment of AGT with the various electrophilic species generated by VNP40101M.

The O^6-(2-chloroethyl)guanine lesion appears to be short-lived and is thought to cyclize rapidly to form N^1,O^6-ethanoguanine [13, 14]. Reaction of AGT with either of these species would prevent DNA crosslink formation. However, it is likely that the lesion being repaired in these studies is N^1,O^6-ethanoguanine.

While the molar yield of crosslinks generated by 90CE and VNP40101M is greater than that seen with the CNUs, it is still extremely low (around 0.025%[1]), since the vast majority of the available alkyl groups are transferred to water. While it is tempting to speculate that direct alkylation of the essential thiol in AGT by 90CE is responsible for the observed loss of AGT activity when this protein is treated with the sulfonylhydrazine prodrug, it is possible that alkylation at other sites on the molecule are responsible for this effect, due to the low preference of the alkylating species generated from the sulfonylhydrazine prodrugs for thiol groups under the conditions used [3, 18].

AGT appeared to be far more sensitive to inactivation by the carbamoylating moieties generated by 101MDCE and 101DCE than to inactivation by the alkylating moiety generated by 90CE. Both 101MDCE and 101DCE are capable of generating unique transient carbamoylating intermediates during their decomposition that subsequently form methyl isocyanate and 2-chloroethyl isocyanate, respectively. Therefore, it could be argued that the presence of these transient intermediates is responsible for the inactivation of AGT by 101MDCE and 101DCE and that they are not true models of the carbamoylating portions of VNP40101M or 101. However, the fact that methyl isocyanate and 2-chloroethyl isocyanate behaved similarly to 101MDCE and101DCE with respect to the inactivation of AGT in our model system would argue against this possibility.

Isocyanates react readily with thiolates, thiols and amino groups and, therefore, it would be expected that the presence of species containing such groups would compete for reaction and attenuate the inactivation of AGT. Nevertheless, substantial inactivation of isocya-

[1]Ten μl of 100 μg/ml of T5 DNA = 1 μg DNA; T5 DNA M_r = 68,000,000 = 15 fmol DNA; 30–40% crosslinking = 4.5–6.0 fmol of crosslinks generated by 2 nmol 90CE (10 μl of 0.2 m*M*). Moles of crosslinks/mole alkylator = crosslink molar yield = 0.025%.

nate-sensitive enzymes in a relatively specific manner has been seen in both whole organisms and cells as a consequence of isocyanates generated by therapeutic levels of the nitrosoureas [8, 9, 25, 26, 27, 28, 29]. The reaction of isocyanates with other molecules in a reversible manner or the generation of secondary carbamoylating species have the potential to extend the inhibition of enzymes over a relatively long time scale. Such latentiation of carbamoylating activity has been described and is thought to account for the delayed and systemic toxicity observed in methyl isocyanate poisoning [5, 30].

The pronounced stabilization of AGT by naked T5 DNA (Fig. 4A) suggests that AGT interacts strongly with DNA. Such an interaction with DNA could enhance the efficiency of this protein with respect to locating its substrate. This interaction of AGT with DNA prompted us to investigate the effects of the presence of DNA on the inactivation of AGT by the alkylating and carbamoylating moieties of VNP40101M. To produce inactivation equivalent to that of free AGT over a 10-min period in the presence of 5 μg/ml of T5 DNA required an increase of about 60-fold in the concentration of 101MDCE (Fig. 4C). It was more difficult to ascertain the magnitude of protection of AGT from inactivation by direct alkylation in the presence of DNA, as alkylation of the small quantity of T5 DNA (5% of the quantity of DNA containing crosslink precursors used as AGT substrate in the AGT activity assay) present in the incubation mixture would generate some AGT-consuming lesions. This would result in an overestimate of the inhibitory action by direct alkylation of the AGT. Despite this error leading to an exaggeration of AGT inactivation by direct alkylation, very little inhibition was measured in the presence of DNA compared to its absence. Therefore, the protection of AGT from direct alkylation by DNA is substantial (Fig. 4B), and significant inactivation of DNA-associated AGT by 90CE at biologically relevant concentrations would appear to be unlikely. These findings imply that the location and the binding status of AGT in the cell could have a major effect on the sensitivity of AGT to inactivation by VNP40101M-derived electrophiles in intact cells. Since AGT appears to be predominantly located (about 70%) in the nucleus [24], possibly bound to DNA, a large portion of the cellular AGT might be expected to show reduced sensitivity to inactivation by VNP40101M. Surprisingly, the presence of 5 μg/ml of T5 DNA did not appear to substantially affect the inactivation of AGT by O^6-benzylguanine (IC_{50} about 1 μ*M*) in our assay system (data not shown).

The severity of the impact of the presence of 100 μg/ml of T5 DNA on the inactivation of AGT by the carbamoylating and alkylating moieties generated during the decomposition of VNP40101M and 90CE were estimated by comparing the resulting level of DNA crosslinking achieved by molar equivalent quantities of 90CE and VNP40101M in the presence and absence of AGT. The presence of the DNA should have two AGT-sparing effects, stabilization of the AGT, thereby allowing it to functionally persist in the mixture during the relatively protracted release of the reactive components from VNP40101M, and inhibition of the inactivation of AGT by the generated reactive species. In the absence of AGT, VNP40101M and 90CE are essentially equivalent in their yield of DNA crosslinks. However, in the presence of AGT, VNP40101M generated a measurably greater net yield of crosslinks, implying that the methyl isocyanate produced by VNP40101M compromised the repair capacity of AGT despite the presence of a relatively high level of AGT-protective DNA. The ratio of carbamoylating to alkylating moieties generated is fixed at approximately 1:1 in 1,2-bis(methylsulfonyl)-1-(alkyl)-2-[(alkylamino)carbonyl]hydrazines, and this ratio is unlikely to be optimum. The use of agents that segregate these activities would allow this ratio to be varied, which could lead to an improved response. In support of this concept, the pretreatment of parental Chinese hamster ovary (CHO/AA8) cells, devoid of AGT activity, and AGT-transfected clones thereof, with 101MDCE followed by 90CE clearly results in pronounced synergistic kill in vitro [7].

Inhibition of AGT may have net therapeutic benefit under certain circumstances. AGT-negative mice are only approximately threefold more sensitive to BCNU [31], while highly AGT-overexpressing tumor cells are much more resistant to such agents compared to normal cells [32]. Therefore, complete inhibition of AGT activity should increase the relative toxicity of such agents to resistant tumor cells relative to the host. The interaction with AGT of the various isocyanates generated during the decomposition of different nitrosoureas probably accounts for a significant proportion of the inhibition of AGT reported previously for the nitrosoureas [15], and may account at least in part for the large variation in AGT-inhibitory action between nitrosoureas which supposedly generate the same alkylating species. Simple alkylation of O^6-guanine in DNA only requires AGT for repair. However, the N^1,O^6-ethanoguanine lesion would require additional components, since interaction of this lesion with AGT results in the AGT protein being covalently linked to the N^1-position of guanine [15]. Inhibitory actions of isocyanates on other enzymes involved in DNA repair and synthesis, such as DNA polymerase, DNA ligase, and ribonucleotide reductase [27, 28, 33] could result in the enhancement of the cytotoxicity of the alkylating portion of VNP40101M by the carbamoylating moiety by AGT-independent mechanisms in intact cells.

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