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ORIGINAL ARTICLE

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1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl] hydrazine (VNP40101M): II. Role of O^6-alkylguanine-DNA alkyltransferase in cytotoxicity

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Abstract *Purpose*: VNP40101M (1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine) is a sulfonylhydrazine prodrug that possesses broad spectrum antitumor efficacy in murine models. VNP40101M activation generates chloroethylating species that alkylate DNA at the O^6-position of guanine, and a carbamoylating agent, methyl isocyanate, which inhibits O^6-alkylguanine-DNA alkyltransferase (AGT) in model systems. We determined whether expression of AGT in Chinese hamster ovary (CHO) cells decreased sensitivity to VNP40101M and explored the mechanism of VNP40101M cytotoxicity by employing analogs of VNP40101M that generate reactive intermediates with either carbamoylating or chloroethylating activity. *Methods*: AGT was overexpressed in CHO cells by transfection with an expression vector containing the human AGT gene. Cell lines expressing AGT were employed in clonogenic assays to determine the cytotoxicity of VNP40101M and its analogs. *Results*: VNP40101M was more active against AGT-expressing CHO cells than 90CE (1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine), a chloroethylating generator devoid of carbamoylating activity. Furthermore, the greater the degree of AGT expression the more resistance to VNP40101M cytotoxicity. Combination chemotherapy experiments support the conclusions that methyl isocyanate and the chloroethylating species generated from the activation of VNP40101M function synergistically to kill cells. *Conclusions*: The findings support the concept that alkylation of the O^6-position of guanine residues in DNA is the predominant lesion created by VNP40101M, and that methyl isocyanate resulting from the base-catalyzed activation of VNP40101M inhibits AGT and presumably other enzymes involved in DNA repair, thereby enhancing the yield of the DNA G-C interstrand crosslinks responsible for the antitumor activity of this agent.

Keywords AGT · VNP40101M · Sulfonylhydrazine prodrugs · Cancer therapeutics

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Introduction

Alkylating agents, which damage DNA directly, are essential components of many therapeutic drug regimens designed to combat cancer [1]. Our laboratory has synthesized a variety of sulfonylhydrazine prodrugs designed to be activated by a variety of mechanisms aimed at the preferential targeting of tumor tissue [2, 3, 4, 5]. These agents decompose to generate hard electrophilic species which alkylate DNA essentially exclusively at the O^6-position of guanine residues. The first of these agents selected for development, VNP40101M, is active against a broad spectrum of tumors in murine models, readily crosses the blood-brain barrier, and can produce cures of tumor-bearing mice over an extended dose range with minimal bone marrow toxicity in the curative range [6]. These properties have led to the clinical evaluation of VNP40101M, which is currently in phase I clinical trial.

From studies conducted with the nitrosoureas, the chloroethyl adduct at the O^6-position of guanine in DNA is recognized as one of the most cytotoxic lesions identified to date. This adduct forms a cyclic intermediate which if not repaired eventually rearranges to form an N1 to N3 G-C interstrand crosslink [7]. Once this lesion has progressed to the crosslink it is difficult to repair and fewer than ten such crosslinks appear to be

sufficient to kill a cell [8]. Cancer chemotherapeutic agents which exploit the lethality of the O^6-guanine DNA lesion for therapeutic benefit include: the chloroethylating agents, carmustine (BCNU) and lomustine (CCNU), and the methylating agents, procarbazine, temozolomide, streptozotocin and dacarbazine.

VNP40101M was designed to include the therapeutically important O^6-DNA guanine chloroethylating activity of BCNU, and to eliminate the formation of nitrosourea decomposition products with no known therapeutic benefit, such as hydroxyethylating and vinylating species, which can only contribute to host toxicity [9, 10, 11, 12, 13, 14]. Although BCNU has therapeutic utility against lymphomas and brain tumors, its use is limited by its substantial toxicity [15]. The surprising efficacy of VNP40101M and superiority over BCNU against tumors grown in mice [6] led us to investigate the mechanism by which VNP40101M exerts its cytotoxicity using isogenic Chinese hamster ovary cell lines engineered to express the repair protein AGT.

VNP40101M can be viewed as a bifunctional prodrug because of its ability to decompose under aqueous conditions to form two classes of reactive intermediates with distinct complementary properties. These consist of chloroethylating species capable of forming adducts at the O^6-position of guanine in DNA, and methyl isocyanate, a carbamoylating agent which we have shown can interfere with the repair of O^6-alkyguanine DNA lesions in vitro by directly inactivating the repair protein AGT [2, 4, 16, 17]). Isocyanates are known to react with the hydroxyl, amino and thiol groups of proteins and, in addition to AGT, have been shown to inhibit DNA polymerase, DNA ligase, and caspase activity [18, 19]. BCNU, which produces chloroethylating species, also generates 2-chloroethyl isocyanate, which is known to inhibit thioredoxin reductase, ribonucleotide reductase, and glutathione reductase, via interaction with thiol functionalities in these proteins [20].

The possibility that the extraordinary antitumor activity of VNP40101M relative to other alkylating agents of the sulfonylhydrazine prodrug class that we have synthesized is due to the co-release of methyl isocyanate, which directly inhibits the actions of AGT, the only repair protein known to protect cells from the O^6-alkylation of guanine in DNA, is intriguing and may have implications for its use against tumors that are resistant to methylating agents because of the elevated expression of AGT [21]. Because of this possibility, in the study reported here we evaluated the contribution of methyl isocyanate to the cytotoxicity of the chloroethylating species generated by the activation of VNP40101M.

Materials and methods

Cell culture

CHO/AA8 cells and cell lines derived therefrom by transfection with AGT were maintained under an atmosphere containing 5% CO_2 in alpha-MEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). All tissue culture reagents were purchased from Invitrogen (Carlsbad, Calif.).

Chemical agents

All sulfonylhydrazines were synthesized by published procedures [2, 3, 4, 5]. All other drugs and chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Transfections

Cells were transfected using lipofectamine (Invitrogen) and the appropriate plasmid construct under conditions recommended by the manufacturer. Briefly, 25 μl lipofectamine was mixed with 5 μg plasmid DNA in 0.6 ml serum-free medium for 30 min at room temperature. This cocktail was added to subconfluent monolayers of CHO/AA8 cells in 25-cm^2 plastic flasks with 2 ml culture medium for 5 h at 37°C. The medium was replaced and 48 h later cells were detached by trypsinization and seeded into 75-cm^2 flasks in the presence of G418 (1 mg/ml). After colony outgrowth under selection, single cell suspensions were sorted using a flow cytometer. The human AGT cDNA was generously provided by Dr. Sankar Mitra [22]. The cDNA was freed by digestion with HinDIII and XbaI, then inserted into the same restriction sites in the selectable vector RC/CMV (Invitrogen). A cell line containing empty vector, CHO/VEC8, was also established as an experimental control.

Assay of AGT activity

Cells ($1–2\times10^7$) were grown in 75-cm^2 flasks, detached by trypsinization, collected by centrifugation, resuspended in a minimum volume of phosphate-buffered saline and the pellet snap-frozen with liquid nitrogen, then stored at −70°C until used. AGT activity was assessed basically as previously described [23, 24]. This assay for AGT activity relies on PvuII cleavage of an oligonucleotide once an O^6-methylguanine residue within its recognition sequence has been repaired by exposure to AGT. Briefly, 0.2 pmol of double-stranded 18-mer oligonucleotide containing O^6-methylguanine within the PvuII site and labeled with [α-^{32}P]-thymidine in one strand was incubated with cell extract at 37°C for 2 h. The reaction was stopped by three phenol extractions followed by precipitation and washing with ethanol. After redissolving, the oligonucleotide was incubated with ten units of PvuII (Invitrogen) at 37°C for 1 h. The reaction was stopped with a denaturing dye (USB, Cleveland, Ohio) at 90°C for 4 min and reaction products were analyzed by 20% denaturing urea-PAGE. The radiolabeled 18-mer substrate and 8-mer digestion product bands were visualized and quantified by exposure of the Saran-wrapped wet gel using a Phosphorimager (Amersham, Piscataway, N.J.). AGT activity in picomoles was calculated as follows: (pixels of 8-mer)/(pixels of 18-mer + pixels of 8-mer)×0.2 pmol, and is expressed per milligram of extract protein in the reaction.

Drug toxicity studies

Clonogenic survival assays were performed as described previously [25]. Cells were seeded into plastic 25-cm^2 Corning tissue culture flasks at 2×10^5 cells per flask. Cells were pulsed 3 days after plating for 1 or 2 h at 37°C with drug dissolved in DMSO in a total volume of 2 ml of medium. After treatment, monolayers were rinsed, cells detached by trypsinization, suspended in culture medium and counted. Sequential cell dilutions were plated in triplicate into 60-mm dishes and 8–10 days later colonies were fixed, stained with crystal violet in 80% methanol and quantified. All analyses were corrected for plating efficiency in the presence of vehicle (DMSO) at concentrations equivalent to those used for the treatment with

drug. For studies testing drug synergism, cells were pulsed for 15 min with 101MDCE, washed and treated for 2 h with 90CE, then processed for clonogenic assays as described above. Combination index values were calculated using the program Calcusyn (Biosoft, Ferguson, Mo.).

Immunoblotting

Immunoblot analyses were performed using ECL (Amersham) as previously described [25]. Equal numbers of cells (approximately 1×10^5) were lysed by sonication and resuspended in protein sample buffer (3% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol, and 60 m*M* Tris-HCl, pH 6.8). Equal volumes were subjected to SDS/PAGE using a 10% gel and samples were transferred to nitrocellulose membranes. Blots were incubated with a 1:500 dilution of anti-AGT antibody (Neomarkers, Freemont, Calif.) in 10% non-fat dried milk for 2 h. After washing, the filters were incubated in a 1:1000 dilution of goat anti-mouse antibody conjugated to horseradish peroxidase in 10% non-fat dried milk. Bands were visualized with enhanced chemiluminescence reagent using Kodak XAR film.

Results

Development of AGT-expressing cell lines

Cell lines expressing various levels of AGT were established by transfecting CHO/AA8 cells with the RC/CMV expression vector containing the human AGT gene under the control of the CMV promoter. Cells were selected for neomycin resistance and individual cell clones were isolated by flow cytometry and expanded for further analysis. Clones were initially screened by immunoblot analysis to verify the expression of AGT and to roughly determine AGT expression levels (Fig. 1) and then AGT repair assays were performed using the oligo repair restriction enzyme inhibition method [23, 24] to precisely determine the AGT activity in individual cell lines (Table 1). CHO/AA8 cells do not express detectable levels of endogenous AGT [26] as shown in Table 1 for empty vector-transfected cells (CHO/VEC8) and, therefore, were ideal target cells for the experiments conducted in this study.

Cytotoxicity studies with chloroethylating agents

We have synthesized and evaluated a variety of sulfonylhydrazine prodrugs as potential anticancer agents [2, 3, 4, 5]. Several of these that pertain to the present studies are listed in Table 2. Among all of the compounds of this class, VNP40101M has proven to be the most efficacious against transplanted cancers. To determine if the antineoplastic activity of VNP40101M was attributable to its ability to alkylate the O^6-position of guanine residues in DNA, eventually forming a G-C interstrand covalent crosslink of DNA [17], isogenic cell lines were established which expressed various levels of AGT from a cell line that lacked endogenous AGT expression, CHO/AA8. As seen in Fig. 2, the degree of activity of AGT was directly related to the degree of sensitivity to VNP40101M, with the CHO/AGT 10-1 cell line demonstrating moderate sensitivity, and the CHO/AGT 38-4 cell line the lowest level of sensitivity to VNP40101M. In contrast, the vector control cell line CHO/VEC8 which does not have detectable AGT activity was highly sensitive to VNP40101M. Numerous additional cell lines were screened and all cell lines which expressed AGT demonstrated some decrease in sensitivity to VNP40101M when compared to vector control or parental cell lines (data not shown). AGT expressing cells

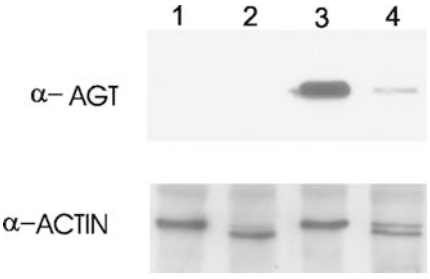

Fig. 1 Immunoblot analyses of clonal CHO/AA8 cell lines derived from cells transfected with AGT. Samples were separated by polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. The equivalent of 1×10^5 cells were loaded per lane. The filter was reacted with anti-AGT antibody (1:500 dilution) and bands were visualized using the enhanced chemiluminescent method described in Materials and methods (*lane 1* CHO/AA8, *lane 2* CHO/VEC8, *lane 3* CHO/AGT 38-4, *lane 4* CHO/AGT 10-1)

Table 1 AGT activity expressed by CHO/VEC8, CHO/AGT 10-1 and CHO/AGT 38-4 cell lines

Cell line[a]	Activity (pmol/mg AGT)
CHO/VEC8	0
CHO/AGT 10-1	0.31
CHO/AGT 38-4	3.58

[a]All cell lines were derivatives of CHO/AA8

Table 2 Comparison of the half-lives and the major reactive species generated by 1,2-bis(sulfonyl)hydrazine prodrugs

$CH_3SO_2N(X)N(Y)SO_2CH_3$					
Compound	X	Y	Chloroethylating agent	Carbamoylating agent	$T_{1/2}$ (h)
VNP40101M	$ClCH_2CH_2$–	CH_3NHCO–	Yes	Yes	0.98
90CE	$ClCH_2CH_2$–	H–	Yes	No	0.01
101MDCE	H–	CH_3NHCO–	No	Yes	0.06
101	$ClCH_2CH_2$–	$ClCH_2CH_2NHCO$–	Yes	Yes	0.25
101DCE	H–	$ClCH_2CH_2NHCO$–	No	Yes	0.06

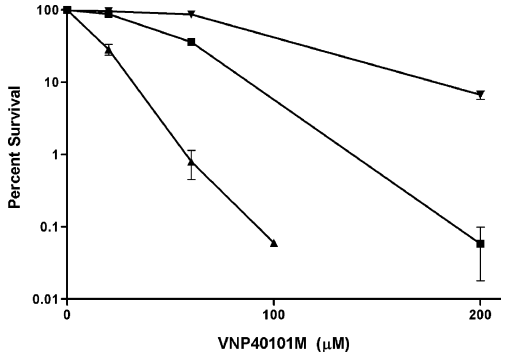

Fig. 2 Survival curves for CHO/VEC8 (▲), CHO/AGT 10-1 (■), and CHO/AGT 38-4 (▼) cells treated with various concentrations of VNP40101M. Survival of CHO/AA8 clones expressing different levels of AGT was calculated using the plating efficiencies of aerobic vehicle-treated controls. Values are geometric means of at least two determinations; SEM are shown when three or more values were obtained

were tested against additional compounds capable of alkylating O^6-guanine residues in DNA to determine if this decrease in sensitivity was unique to VNP40101M. As seen for 90CE and 101, AGT expression also decreased sensitivity to these O^6-guanine DNA alkylators (Fig. 3).

Cytotoxicity studies with isocyanate-generating agents

The sulfonylhydrazines VNP40101M and 101 are predicted to decompose into two reactive intermediates. Both generate identical O^6-guanine DNA chloroethylating species. VNP40101M also yields the carbamoylating agent methyl isocyanate while 101 generates 2-chloroethyl isocyanate, which is the carbamoylating agent produced by BCNU. The expression of AGT should confer decreased sensitivity to the O^6-guanine DNA alkylating component of VNP40101M and 101 but not to the isocyanate moiety. In agreement with this expectation, clonogenic assays demonstrated that the forced expression of AGT did not lessen sensitivity to 101MDCE, a methyl isocyanate generator, or to 101DCE, a 2-chloroethyl isocyanate generator (Fig. 4). These findings support the conclusion that the most important toxic component of VNP40101M and 101 is the activation product capable of alkylating DNA at the O^6-position of guanine. Thus, the vector control cell line demonstrated a degree of sensitivity to the isocyanate-generating agents that was similar to that of the AGT-expressing cell lines, as would be predicted, with the major toxicity being linked to the alkylation event.

Comparison of the cytotoxicity of VNP40101M and 90CE

We have previously proposed that the methyl isocyanate generated by the decomposition of VNP40101M has the potential to augment the toxicity of alkylating agents that target the O^6-guanine residues of DNA by inhibiting/inactivating AGT activity present in

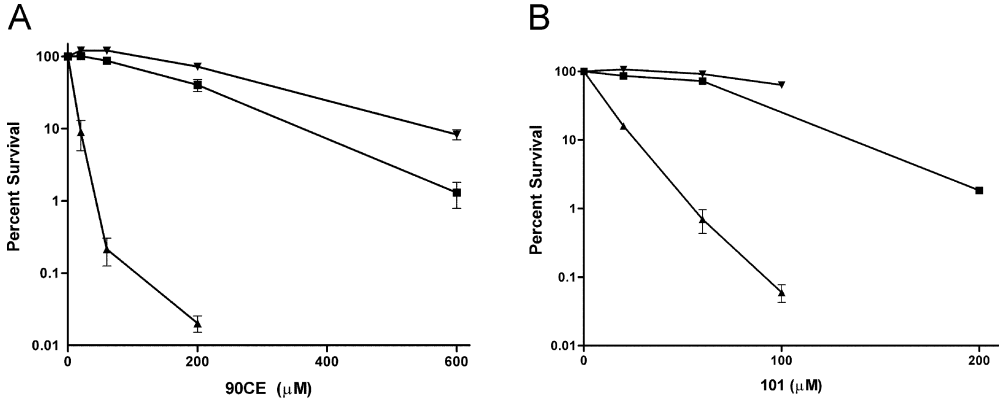

Fig. 3A, B Survival curves for CHO/VEC8 (▲), CHO/AGT 10-1 (■), and CHO/AGT 38-4 (▼) cells treated with various concentrations of 90CE (**A**) or 101(**B**). Survival of CHO/AA8 clones expressing different levels of AGT was calculated using the plating efficiencies of aerobic vehicle-treated controls. Values are geometric means of at least two determinations; SEM are shown when three or more values were obtained

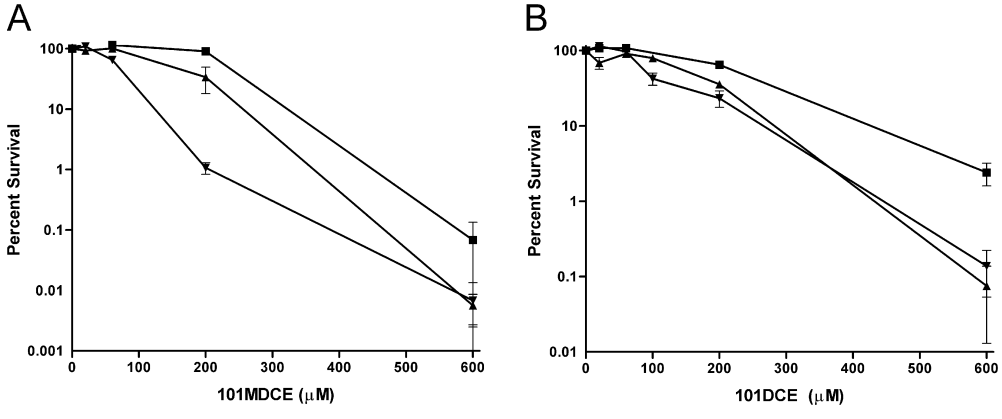

Fig. 4A, B Survival curves for CHO/VEC8 (▲), CHO/AGT 10-1 (■), and CHO/AGT 38-4 (▼) cells treated with various concentrations of 101MDCE (**A**) or 101DCE (**B**). Survival of CHO/AA8 clones expressing different levels of AGT was calculated using the plating efficiencies of aerobic vehicle-treated controls. Values are geometric means of at least two determinations; SEM are shown when three or more values were obtained

VNP40101M-treated cells [17]. Since 90CE is the initial alkylating species formed by the activation of VNP40101M but lacks the methyl isocyanate-generating portion of the molecule, one would expect that VNP40101M would be more cytotoxic than 90CE at molar equivalent levels to cells expressing AGT, and that these compounds would be essentially equitoxic to cells lacking AGT, all other things being equal. Comparison of the toxicity profiles of the AGT-overexpressing cell lines support this assumption. Thus, under similar conditions, VNP40101M was considerably more cytotoxic to AGT-expressing CHO/AGT 10-1 cells than 90CE (Fig. 5A). CHO/AGT 38-4, cells with a substantially greater level of AGT, exhibited some sensitivity to 90CE at relatively high concentrations of drug but much less sensitivity to VNP40101M than was observed with CHO/AGT 10-1 cells (Fig. 5B), presumably because sufficient residual AGT activity remained to repair the O^6-guanine lesions in DNA generated by VNP40101M. Furthermore, as expected, VNP40101M and 90CE were essentially equitoxic to CHO/VEC8 cells that lack AGT activity (Fig. 5C).

Studies with drug combinations

To ascertain whether both the chloroethylating and carbamoylating species generated by the activation of VNP40101M contribute to the observed cytotoxic activity, compounds have been designed and synthesized which produce through activation only one of the two reactive species of VNP40101M, i.e., either the methyl isocyanate or the chloroethylating species. 101MDCE generated only methyl isocyanate, while 90CE generated only the O^6-guanine DNA chloroethylating species upon activation. Therefore, if 101MDCE, which upon activation generates methyl isocyanate, inhibits AGT activity in intact cells as it does in cell-free biochemical experiments [17], then it should enhance the efficacy of the alkylating components of VNP40101M, which are also generated by 90CE. To test this possibility, experiments were performed in which CHO/AGT 10-1, CHO/AGT 38-4, and CHO/VEC8 cells were pulsed for 15 min with 101MDCE, washed and then exposed for 2 h to 90CE; cells were then plated and colony formation was quantified.

As seen in Fig. 6, a marked enhancement in cell kill occurred between these two compounds, which each generated separately the active components produced by the decomposition of VNP40101M, in all three of the cell lines. Calculation of combination index values from the data in Fig. 6, where a value of one is additive, a value greater than one is antagonistic and a value less than one is synergistic, indicated that marked synergism occurred at all concentrations of drugs employed (Table 3). In addition, synergism was also seen when the 2-chloroethyl isocyanate-generator 101DCE was used with 90CE, although the degree of synergy was not as pronounced as that produced by the combination of 101MDCE and

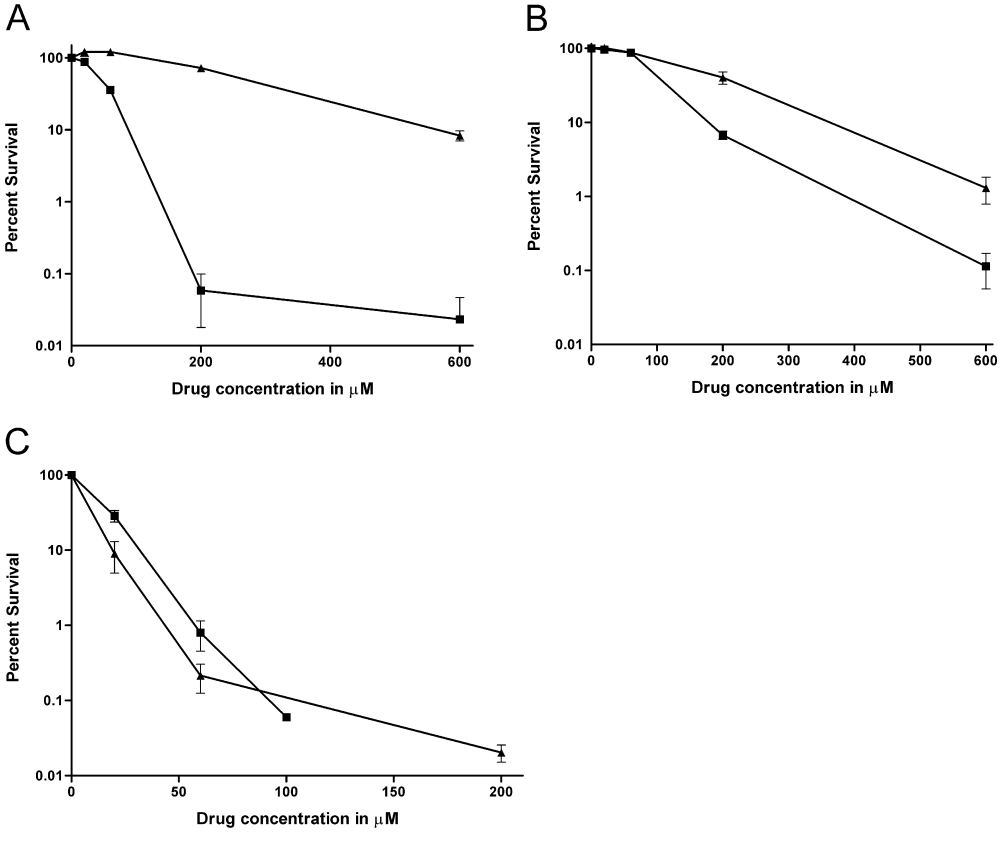

Fig. 5A–C Survival curves for CHO/AGT 10-1 (**A**), CHO/AGT 38-4 (**B**), and CHO/VEC8 (**C**) cells treated with various concentrations of 90CE (▲) or VNP40101M (■). Survival of CHO/AA8 clones expressing different levels of AGT was calculated using the plating efficiencies of aerobic vehicle-treated controls. Values are geometric means of at least two determinations; SEM are shown when three or more values were obtained

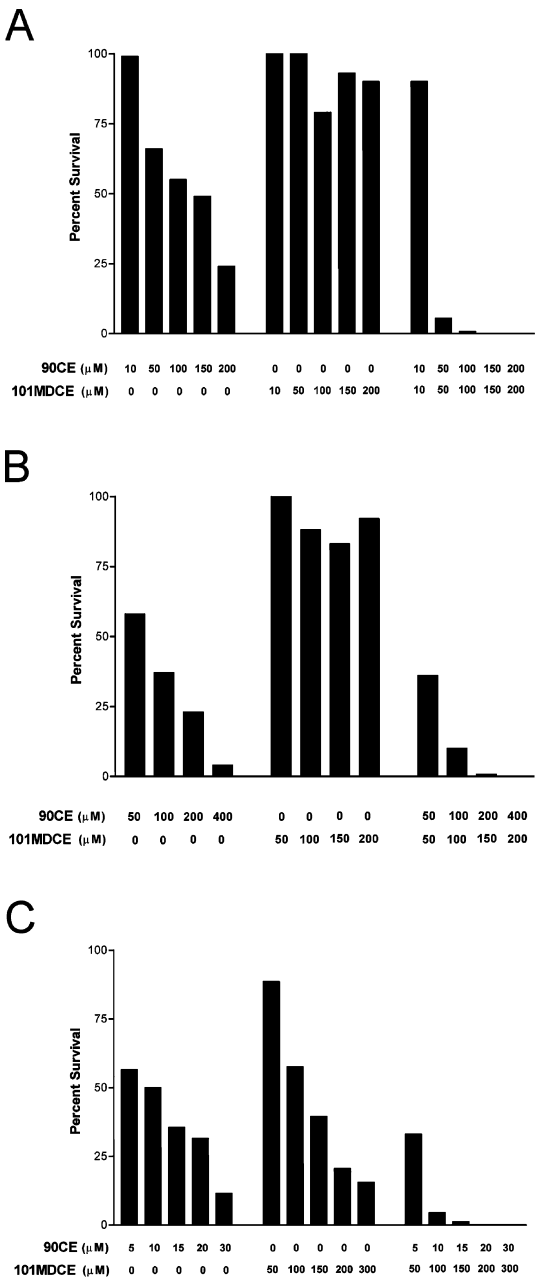

Fig. 6A–C Clonogenic assays measuring the ability of CHO/AGT 10-1 (**A**), CHO/AGT 38-4 (**B**), and CHO/VEC8 (**C**) cells to form colonies after pulsed exposure to 90CE and 101MDCE. Survival was calculated using the plating efficiencies of aerobic vehicle-treated controls. Values are geometric means of at least two determinations

90CE (data not shown) indicating that the methyl isocyanate generated by the VNP40101M was superior to the 2-chloroethyl isocyanate formed from BCNU. 101MDCE and 90CE also exhibited a synergistic kill against the vector control cell line, CHO/VEC8 (Fig. 6C and Table 3), even though CHO/VEC8 cells are devoid of AGT activity, indicating that AGT expression and inhibition thereof by methyl isocyanate is not the only factor involved in the enhanced kill of cells produced by the combination of these agents. Note that the greater sensitivity of CHO/VEC8 cells to 90CE necessitated the use of decreased concentrations of this agent with this cell line.

Table 3 Combination index values for CHO/VEC8, CHO/AGT 10-1 and CHO/AGT 38-4 cells treated with various concentrations of 101MDCE and 90CE

Cell line[a]	90CE/101MDCE drug concentrations (μM)	Combination index[b]
CHO/VEC8	5/50	0.4
	10/100	0.07
	15/150	0.03
	20/200	0.001
CHO/AGT 10-1	10/10	0.4
	50/50	0.1
	100/100	0.1
	150/150	0.06
	200/200	0.05
CHO/AGT 38-4	50/50	0.6
	100/100	0.4
	200/150	0.15
	400/200	0.07

[a]All cell lines were derivatives of CHO/AA8
[b]As determined using the Calcusyn program from data graphed in Fig. 6. CI values equal to one indicate additive toxicity while CI values less than one indicate synergistic toxicity

Discussion

VNP40101M is the initial member of a novel class of sulfonylhydrazine prodrugs to enter phase I clinical trials; this agent exhibited marked effectiveness in preclinical studies against a spectrum of transplanted tumors, readily crosses the blood-brain barrier, is curative over a range of drug concentrations and exhibits minimal toxicity at curative doses [6]. The high level of efficacy of this drug in experimental systems led us to study its mechanism of action in greater detail. VNP40101M is known to decompose in aqueous solution to yield two reactive species, the carbamoylating agent methyl isocyanate and chloroethylating species which cause alkylations at the O^6-position of guanine in DNA that eventually result in DNA crosslink formation. The protein AGT is known to mediate repair of the O^6-alkylguanine adducts by a stoichiometric noncatalytic mechanism [7, 8, 27, 28]. For these reasons, we evaluated the ability of AGT to protect cells from VNP40101M and other structurally related sulfonylhydrazine prodrugs that also alkylate the O^6-position of guanine in DNA by the forced expression of human AGT cDNA in CHO/AA8 cells which lack detectable AGT activity (Table 1).

These studies demonstrated that sensitivity to VNP40101M was directly related to the degree of AGT expression, with the lowest sensitivity being seen in cell lines with the highest levels of AGT activity. Thus, the expression of AGT almost completely blocked the cytotoxicity of VNP40101M at concentrations of drug up to 100 μM in the high AGT-expressing CHO/AGT

38-4 cell line. The expression of AGT also conferred decreased sensitivity to other sulfonylhydrazine prodrugs which exerted their cytotoxicity by chloroethylation of O^6-guanine residues in DNA including 90CE, which is generated by the activation of both VNP40101M and 101, but did not decrease sensitivity to methyl isocyanate- or 2-chloroethyl isocyanate-generating compounds, which do not act through alkylation of DNA at the O^6-position of guanine. These findings support the concept that the antineoplastic effects of VNP40101M are due to its capacity to react with the O^6-position of guanine in DNA and therefrom generate crosslinked DNA.

The results also provide evidence that methyl isocyanate, which is produced by the activation of VNP40101M, contributes to the efficacy of this agent. Thus, in both cell lines expressing AGT, VNP40101M was more toxic than 90CE, which also produces identical products that alkylate the O^6-position of guanine in DNA, but does not cogenerate methyl isocyanate. While VNP40101M and 90CE were essentially equitoxic to CHO/AA8 parental (data not shown) and CHO/VEC8 cells, both of which lack AGT activity, a differential cytotoxicity was seen with CHO/AGT 10-1 cells, which expressed a relatively low level of AGT, an observation that would be predicted if the methyl isocyanate component of VNP40101M were capable of inhibiting AGT activity or other enzymatic components involved in DNA repair.

Studies with drug combinations demonstrated that 101MDCE, which generated only methyl isocyanate, synergized with 90CE, which produced the same chloroethylating species formed by activation of VNP40101M, in all of the cell lines. Although the synergism exhibited by the combination of 101MDCE and 90CE in CHO/AGT 10-1 and CHO/AGT 38-4 cells may be the result of the alkylation of the O^6-position of guanine in DNA by products derived from 90CE, coupled with the inhibition by methyl isocyanate, produced from 101MDCE, of the repair of this lesion by AGT, this mechanism cannot explain the synergism produced in CHO/VEC8 cells, which are devoid of AGT. It is likely, therefore, that methyl isocyanate causes other effects in these vector-transfected cells, as well as in AGT-containing cells; this assumption is not unreasonable since isocyanates have been shown to react with thiol and amine functionalities on proteins. Thus, various studies have documented the ability of isocyanates to inhibit enzymes such as DNA polymerase, DNA ligase, caspase, and thiol-containing enzymes such as glutathione reductase, ribonucleotide reductase, and thioredoxin reductase [18, 19, 20]. In support of these interpretations are our observations that benzylguanine, a known inhibitor of AGT, is capable of sensitizing AGT-expressing CHO cells to drugs that alkylate the O^6-position of guanine in DNA, but does not sensitize CHO/VEC8 cells which do not express AGT (data not shown).

Recently, we have demonstrated in cell-free systems using purified AGT that methyl isocyanate is a potent inhibitor of AGT at micromolar concentrations [17]. The addition of naked T5 DNA to the AGT-containing system markedly inhibited the spontaneous loss of AGT activity during incubation, which occurred due to instability of this protein. In addition, the binding of AGT to DNA strongly impaired the inactivation of AGT by 101MDCE, suggesting that in intact cells the interaction of AGT with DNA may minimize the effectiveness of methyl isocyanate-generating prodrugs as inactivators of AGT.

In summary, while the precise mechanism(s) by which methyl isocyanate enhances the cytotoxicity of 90CE is unclear, it is important to stress that the activation of VNP40101M through decomposition simultaneously produces two synergistically interacting components that are involved in the antineoplastic action of this agent.

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References

1. Teicher BA (1997) Antitumor alkylating agents. In: DeVita VT Jr, Hellman S, Rosenberg SA (eds) Cancer: principles and practice of oncology, 5th edn, Lippincott-Raven, Philadelphia, p 405
2. Shyam K, Penketh PG, Divo AA, Loomis RH, Rose WC, Sartorelli AC (1990) Synthesis and evaluation of 1,2,2-tris(sulfonyl)hydrazines as antineoplastic and trypanocidal agents. J Med Chem 33:2259
3. Shyam K, Penketh PG, Divo AA, Loomis RH, Rose WC, Sartorelli AC (1993) Synthesis and evaluation of 1-acyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazines as antineoplastic agents. J Med Chem 36:3496
4. Shyam K, Penketh PG, Loomis RH, Rose WC, Sartorelli AC (1996) Antitumor 2-(aminocarbonyl)-1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazines. J Med Chem 39:796
5. Shyam K, Penketh PG, Shapiro M, Belcourt MF, Loomis RH, Rockwell S, Sartorelli AC (1999) Hypoxia-selective nitrobenzyloxycarbonyl derivatives of 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazines. J Med Chem 42:941
6. Finch RA, Shyam K, Penketh PG, Sartorelli AC (2001) 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine (101M): a novel sulfonylhydrazine prodrug with broad-spectrum antineoplastic activity. Cancer Res 61:3033
7. Pegg AE, Dolan ME, Moschel RC (1955) Structure, function, and inhibition of O^6-alkylguanine-DNA alkyltransferase. In: Cohen WE, Moldave K (eds) Progress in nucleic acid research and molecular biology, vol. 51. Academic Press, New York, p 167
8. Gerson S (2002) Clinical relevance of MGMT in the treatment of cancer. J Clin Oncol 20:2388
9. Pelfrene A, Mirvish SS, Gold B (1976) Induction of malignant bone tumors in rats by 1-(2-hydroxyethyl)-1-nitrosourea. J Natl Cancer Inst 56:445
10. Swenson DH, Frei JV, Lawley PD (1979) Synthesis of 1-(2-hydroxyethyl)-1-nitrosourea and comparison of its toxicity to that of 1-ethyl-1-nitrosourea. J Natl Cancer Inst 63:1469
11. Colvin M, Brundrett RB, Cowens JW, Jardine I, Ludlum DB (1976) A chemical basis for the antitumor activity of the chloroethylnitrosoureas. Biochem Pharmacol 25:695
12. Wheeler GP, Bowdon BJ, Grimsley J, Lloyd HH (1974) Interrelationships of some chemical, physicochemical and biological activities of several 1-(2-haloethyl)-1-nitrosoureas. Cancer Res 34:194

13. Panasci LC, Green D, Nagourney R, Fox P, Schein PS (1977) A structure-activity analysis of chemical and biological parameters of chloroethylnitrosoureas in mice. Cancer Res 37:2615
14. Gibson NW, Hickman JA (1982) The role of isocyanates in the toxicity of antitumor haloalkylnitrosoureas. Biochem Pharmacol 31:2795
15. Colvin OM (1997) Alkylating agents and platinum antitumor compounds. In: Holland JF, Bast RC Jr, Morton DL, Frei E III, Kufe DW, Weichselbaum RR (eds) Cancer medicine. Williams and Wilkins, Baltimore, p 983
16. Penketh PG, Shyam K, Sartorelli AC (2000) Comparison of DNA lesions produced by tumor inhibitory 1,2-bis(sulfonyl)hydrazines and chloroethylnitrosoureas. Biochem Pharmacol 59:283
17. Penketh PG, Shyam K, Baumann RP, Brent TP, Remack J, Sartorelli AC (2004) 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. Cancer Chemother Pharmacol 53, DOI 10.1007/s00280-003-0740-7
18. Baril BB, Baril EF, Lazlo J, Wheeler GP (1975) Inhibition of rat liver DNA polymerase by nitrosoureas and isocyanates. Cancer Res 35:1
19. Petak I, Mihalik R, Bauer PI, Suli-Vargha H, Sebestyen A, Kopper L (1998) BCNU is a caspase-mediated inhibitor of drug-induced apoptosis. Cancer Res 58:614
20. Schallreuter KU, Gleason FK, Wood JM (1990) The mechanism of action of the nitrosourea antitumor drugs on thioredoxin reductase, glutathione reductase and ribonucleotide reductase. Biochim Biophys Acta 1054:14
21. Kann HH, Kohn KW, Lyles JM (1974) Inhibition of DNA repair by 1,3-bis(2-chloroethyl)-1-nitrosourea breakdown product, 2-chloroethyl isocyanate. Cancer Res 34:398
22. Tano K, Shiota S, Collier J, Foote RS, Mitra S (1990) Isolation and structural characterization of a cDNA clone encoding the human DNA repair protein for O^6-alkylguanine. Proc Natl Acad Sci U S A 87:686
23. Wu RS, Hurst-Calderone S, Kohn KW (1987) Measurement of O^6-alkylguanine-DNA alkyltransferase activity in human cells and tumor tissues by restriction endonuclease inhibition. Cancer Res 47:6227
24. Marathi UK, Harris LC, Venable CC, Brent TP (1997) Retroviral transfer of a bacterial alkyltransferase gene (ada) into human bone marrow cells protects against O^6-benzylguanine plus 1,3-bis(2-chloroethyl)-1-nitrosourea cytotoxicity. Clin Cancer Res 3:301
25. Baumann RP, Hodnick WF, Seow HA, Belcourt MF, Rockwell S, Sherman DH, Sartorelli AC (2001) Reversal of mitomycin C resistance by overexpression of bioreductive enzymes in Chinese hamster ovary cells. Cancer Res 61:7770
26. Cai Y, Wu MH, Ludeman SM, Grdina DJ, Dolan ME (1999) Role of O^6-alkylguanine-DNA alkyltransferase in protecting against cyclophosphamide-induced toxicity and mutagenicity. Cancer Res 59:3059
27. Mitra S, Kaina B (1993) Regulation of repair of alkylation damage in mammalian genomes. Prog Nucleic Acids Res Mol Biol 44:109
28. Pegg AE (1990) Mammalian O^6-alkylguanine-DNA alkyltransferase: regulation and importance in response to alkylating carcinogenic and therapeutic agents. Cancer Res 50:6119