Biochemical Pharmacology 80 (2010) 1317–1325

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Quantitative relationship between guanine O^6-alkyl lesions produced by Onrigin™ and tumor resistance by O^6-alkylguanine-DNA alkyltransferase

Kimiko Ishiguro, Yong-Lian Zhu, Krishnamurthy Shyam, Philip G. Penketh, Raymond P. Baumann, Alan C. Sartorelli *

Department of Pharmacology and Developmental Therapeutics Program, Cancer Center, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06520, United States

ARTICLE INFO

Article history:
Received 19 May 2010
Accepted 8 July 2010

Keywords:
Onrigin™ (laromustine
cloretazine
VNP40101M
101M)
O^6-Alkylguanine-DNA alkyltransferase
(AGT)
O^6-Benzylguanine
Guanine O^6-chloroethyl and O^6-methyl
lesions
Carmustine (BCNU)
Temozolomide

ABSTRACT

O^6-Alkylguanine-DNA alkyltransferase (AGT) mediates tumor resistance to alkylating agents that generate guanine O^6-chloroethyl (Onrigin™ and carmustine) and O^6-methyl (temozolomide) lesions; however, the relative efficiency of AGT protection against these lesions and the degree of resistance to these agents that a given number of AGT molecules produces are unclear. Measured from differential cytotoxicity in AGT-ablated and AGT-intact HL-60 cells containing 17,000 AGT molecules/cell, AGT produced 12- and 24-fold resistance to chloroethylating (90CE) and methylating (KS90) analogs of Onrigin™, respectively. For 50% growth inhibition, KS90 and 90CE generated 5,600 O^6-methylguanines/cell and ~300 O^6-chloroethylguanines/cell, respectively. AGT repaired O^6-methylguanines until the AGT pool was exhausted, while its repair of O^6-chloroethylguanines was incomplete due to progression of the lesions to AGT-irreparable interstrand DNA cross-links. Thus, the smaller number of O^6-chloroethylguanine lesions needed for cytotoxicity accounted for the marked degree of resistance (12-fold) to 90CE produced by AGT. Transfection of human or murine AGT into AGT deficient transplantable tumor cells (i.e., EMT6, M109 and U251) generated transfectants expressing AGT ranging from 4,000 to 700,000 molecules/cell. *In vitro* growth inhibition assays using these transfectants treated with 90CE revealed that AGT caused a concentration dependent resistance up to a level of ~10,000 AGT molecules/cell. This finding was corroborated by *in vivo* studies where expression of 4,000 and 10,000 murine AGT molecules/cell rendered EMT6 tumors partially and completely resistant to Onrigin™, respectively. These studies imply that the antitumor activity of Onrigin™ stems from guanine O^6-chloroethylation and define the threshold concentration of AGT that negates its antineoplastic activity.

1. Introduction

Onrigin™ (laromustine; cloretazine; VNP40101M; 101M; 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino) carbonyl]hydrazine) is an active antitumor agent in humans, designed and synthesized in our laboratory as a prodrug that upon base catalyzed fragmentation releases chloroethylating (alkylating) and carbamoylating species (Fig. 1) [1]. Onrigin™ was conceived through incorporation of a methylaminocarbonyl residue into the prototype chloroethylating molecule 90CE (1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine) (Fig. 1A) [2]. The methylaminocarbonyl residue in which carbamoylating activity resides, acts as a masking group, thereby delaying the rapid fragmentation reactions observed with 90CE. The half-lives of 90CE and Onrigin™ at physiological pH and 37 °C are ~0.5 and ~60 min, respectively [3]. This modification results in a dramatic increase in antitumor activity in preclinical tumor models, presumably due to improved distribution *in vivo* [1–4].

Alkylating agents have occupied an important position in cancer chemotherapy, being among the most extensively used anticancer agents [5]. However, alkylating agents such as the nitrogen mustards whose antitumor effects are largely attributable to their reactivity with the *N-7* position of guanine in DNA, are exceedingly toxic resulting in relatively low therapeutic indices [5,6]. Onrigin™ appears to be an exception in that, in phase II clinical studies, Onrigin™ as a single agent produced 28% complete response rates in elderly high-risk myelodysplasia and acute myelogenous leukemia patients with limited extramedullary toxicity [7]. Defining the underlying mechanisms of tumor selectivity manifested by Onrigin™ in this subset of patients [6,8,9] has been a major focus of our laboratory.

Abbreviations: AGT, O^6-alkylguanine-DNA alkyltransferase; ^{3}H-BG, [benzene-^{3}H]O^6-benzylguanine; O^6-BG, O^6-benzylguanine; IC_{50}, concentration giving 50% inhibition; 90CE, 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine; 101MDCE, 1,2-bis(methylsulfonyl)-1-[(methylamino)carbonyl]hydrazine; KS90, 1,2-bis(methylsulfonyl)-1-methylhydrazine.

* Corresponding author. Tel.: +1 203 785 4533; fax: +1 203 737 2045.
E-mail address: alan.sartorelli@yale.edu (A.C. Sartorelli).

0006-2952/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2010.07.022

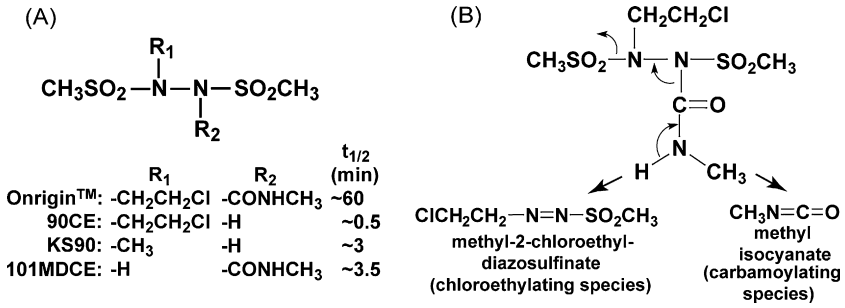

Fig. 1. The structures of Onrigin™ and its analogs used in this study (A) and the fragmentation pattern of Onrigin™ generating chloroethylating and carbamoylating species (B). 90CE and KS90 are chloroethylating and methylating agents, respectively, without the carbamoylating moiety. 101MDCE is a carbamoylating agent without the alkylating moiety. The half-lives ($t_{1/2}$) of these compounds measured in phosphate buffer, pH 7.4 at 37 °C are listed.

Using Onrigin™, 90CE and the carbamoylating-only analog 101MDCE (1,2-bis(methylsulfonyl)-1-[(methylamino)carbonyl]hydrazine) that lacks the alkylating moiety (Fig. 1A), the mode of action of the chloroethylating and carbamoylating species of Onrigin™ has been dissected [10–12]. As exemplified by the chloroethylating agent carmustine (BCNU; *N,N′*-bis(2-chloroethyl)-*N*-nitrosourea) [13], the antitumor activity of Onrigin™ derives from its ability to chloroethylate the *O-6* position of guanine, ultimately yielding interstrand DNA cross-links between complementary G-C base pairs, a proposition supported by a series of observations. First, interstrand DNA cross-link formation by 90CE *in vitro* can be essentially prevented by the presence of the repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) [3,14] which transfers guanine O^6-alkyl groups to the AGT active site cysteine and restores the *O-6* position of guanine to the native state [15,16]. In addition, interstrand DNA cross-link formation is demonstrated in cells exposed to Onrigin™ and 90CE, but not to 101MDCE, in alkaline comet assays [12]. Second, the cytotoxicity of Onrigin™ and 90CE is markedly attenuated by expression of AGT in cultured cells [11,17].

The carbamoylating species generated from Onrigin™ is methyl isocyanate with affinity for nucleophiles such as sulfhydryls and amines in amino acid side chains of cellular proteins [18]. Consistent with this reactivity, the carbamoylating-only agent 101MDCE causes inhibition of DNA, RNA and protein syntheses in a non-selective manner and is cytotoxic by itself [12]. In AGT negative cells, the chloroethylating agent 90CE is more potent than 101MDCE in producing cytotoxicity [11].

Convincing evidence has demonstrated that AGT is a major factor in the production of resistance to the clinically active antitumor alkylating agents that generate chloroethyl (Onrigin™ and carmustine) and methyl (temozolomide) adducts at the *O-6* position of guanine [19]. However, AGT is likely to repair guanine O^6-chloroethyl and O^6-methyl lesions with different degrees of effectiveness. Moreover, these guanine O^6-targeting agents are known to exert their cytotoxicities by different mechanisms, with chloroethylating agents through generation of interstrand DNA cross-links and methylating agents via an intact mismatch repair system [19,20]. For better understanding of the role of AGT in conferring resistance to Onrigin™, we compared the effectiveness of AGT repair of guanine O^6-chloroethyl and O^6-methyl lesions.

AGT is a unique DNA damage repair protein in that the transfer of an alkyl group to an AGT active site cysteine results in a stoichiometric irreversible inactivation of the protein [15,16]. Thus, pretreatment of AGT positive cells with the relatively potent pseudosubstrate inhibitor of AGT O^6-benzylguanine (O^6-BG, IC_{50} = 0.05 μM) [21,22] was used to create AGT-ablated conditions in cultured cells. Two additional approaches unique to this laboratory were employed. The first was the availability of the chloroethylating agent 90CE and the methylating agent KS90 (1,2-bis(methylsulfonyl)-1-methylhydrazine) [23], analogs of Onrigin™ differing in the alkylating structure in the absence of the carbamoylating moiety (Fig. 1A), enabling exclusive analyses on these alkylating functions. The second was the availability of an AGT assay based upon the covalent transfer of the benzyl moiety from [benzene-^{3}H]O^6-benzylguanine to AGT developed in this laboratory [24]. Unlike conventional AGT assays using O^6-methylguanine containing DNA as substrates, this assay employed the small chemical substrate O^6-BG, enabling (a) multiple assays to be conducted using a relatively small number (2×10^6 cells/assay) of intact cells, (b) direct measurement of AGT activity as the number of AGT molecules/cell, and (c) quantification of guanine O^6-alkyl adducts generated in cells by guanine O^6-targeting agents without DNA extraction and chromatographic analyses. These analyses have uncovered the distinct ways in which AGT produces resistance to chloroethylating and methylating agents.

The present report measures the relationship between tumor AGT content and AGT mediated tumor resistance to Onrigin™ both in cultured cells and in a murine tumor model, using tumor cell lines expressing AGT at various levels, AGT negative transplantable tumor cell lines and those transfected with human or murine AGT. These studies demonstrated that maximum resistance to Onrigin™ is reached by AGT at a level of ~10,000 molecules/cell both *in vitro* and *in vivo*. These findings underscore the importance of determining the tumor AGT content prior to treatment with Onrigin™ and analyzing the relationship between the tumor AGT level and effectiveness of Onrigin™ in clinical trials.

2. Materials and methods

2.1. Chemicals

Onrigin™, 90CE, 101MDCE and KS90 were synthesized in our laboratory as previously described [1–3,23]. Carmustine (C0400) and O^6-BG (B2292) were obtained from Sigma (St. Louis, MO). Temozolomide was purchased from LKT Laboratories, Inc. (St. Paul, MN). All agents were dissolved in anhydrous DMSO; Onrigin™, 90CE, 101MDCE, carmustine and temozolomide at 200 mM, KS90 at 2 M and O^6-BG at 20 mM. These stock solutions were serially diluted with DMSO and the same volume of DMSO or drug solution was added to control or treated samples to produce a constant final DMSO concentration in all assays. The half-lives of Onrigin™ (~60 min), 90CE (~0.5 min), 101MDCE (~3.5 min) and KS90 (~3 min) in phosphate buffer, pH 7.4, at 37 °C have been described elsewhere [3,25]. The half-lives of carmustine (50 min) and temozolomide (74 min) at physiological pH have also been reported [26,27].

2.2. Tumor cell lines

HL-60 human promyelocytic leukemia cells and EMT6 murine mammary carcinoma cells were obtained from Drs. Robert C. Gallo

and Sara C. Rockwell, respectively. Madison 109 (M109) murine lung carcinoma cells and U251 human glioblastoma cells were from the DCTD Tumor Repository, National Cancer Institute at Frederick (Frederick, MD). Human carcinoma cell lines, HCT 116 (colon), A549 (lung), HeLa S3 (cervix) and DU145 (prostate) were obtained from the American Type Culture Collection (Manassas, VA). Suspension and attached cell lines were maintained in RPMI 1640 and McCoy's 5A media, respectively, supplemented with 10% fetal bovine serum (FBS) in a humidified 5% CO_2 incubator.

2.3. Growth inhibition assays

Growth inhibition assays were conducted based upon cell number using 24-well plates in a volume of 1 ml/well. HL-60 cells at an initial density of 7×10^4 cells/ml, with or without pretreatment with 10 μM O^6-BG for 1 h, were incubated with various guanine O^6-targeting agents for 3 days and cell numbers were determined using a Beckman Coulter Counter (Hialeah, FL). For attached cell lines (U251, HCT 116, A549, HeLa S3, DU145, EMT6 and M109), 2×10^4 cells (except for 3×10^4 cells for U251) were seeded per well and cultured overnight prior to drug exposure. O^6-BG (20 mM) was added to cultures at 0.5 μl/well. Guanine O^6-targeting agents were added to cultures at 1 μl/well. The final concentration of DMSO in control and treated cultures was 0.15%. DMSO at this level had no effect on cell growth. The percent growth inhibition was calculated using the formula: [log(final density of the control culture) − log(final density of the treated culture)]/[(log(final density of the control culture) − log(initial density of the control culture)] × 100.

2.4. AGT assays

AGT levels were measured using [benzene-^{3}H]O^6-benzylguanine (^{3}H-BG) as a substrate as described previously [24]. Briefly, 2×10^6 intact cells were incubated with 1 μCi of ^{3}H-BG in a volume of 100 μl at 37 °C for 2 h, and radioactivity in 70% methanol precipitates was measured after extensive washing. The cellular AGT content was expressed as the number of molecules/cell [24]. In AGT inactivation assays, 2×10^6 HL-60 cells containing 17,000 AGT molecules/cell were treated with AGT inactivators (0.5 μl) in a total volume of 100 μl at 37 °C. The final DMSO concentration in control and treated samples was 0.5%. Cells were then incubated with 1 μCi of ^{3}H-BG for 1 h to measure the remaining levels of AGT.

2.5. Transfection of human and murine AGTs into AGT negative tumor cells

Human and murine AGT cDNAs (BC000824 and BC031888, respectively) in pCMV-SPORT6 were obtained from Open Biosystems (Huntsvill, AL). Each AGT coding region was amplified by PCR and inserted into pCRII-TOPO by TA cloning (Invitrogen, Carlsbad, CA). The fragments generated by PCR were verified by DNA sequencing. Human and murine AGT coding sequences were subcloned into mammalian expression vector p75/15 [28] containing the human metallothionein IIA promoter using *Bam*HI (5′) and *Xba*I (3′) sites.

The vector p75/15 contained the neo^r selection marker [28]. Non-transfected cell lines, EMT6, M109 and U251, were 100% non-viable at 0.8–1.0 mg/ml of G418. For transfection, $(5–7.5) \times 10^5$ cells were plated in 25 cm^2 flasks and cultured overnight. Cells were then washed with Opti-MEM (Invitrogen) and exposed to a mixture of 4.8 μg of plasmid DNA and 30 μl of lipofectamine (Invitrogen) in 3.6 ml of Opti-MEM for 3 h. After incubation in serum-containing medium for 1 day for expression, cells were plated in 6-well plates for selection at densities of 1×10^4/6 wells, 4×10^4/6 wells and 1.6×10^5/6 wells in the presence of 0.8–1 mg/ml of G418. After selection for 7 (EMT6 and M109) and 12 (U251) days, well-separated colonies were isolated, expanded and subjected to AGT assays.

2.6. Treatment of EMT6 tumors by Onrigin™ in vivo

Animal experiments were reviewed and approved by Yale University's Institutional Animal Care and Use Committee. EMT6/wild-type, EMT6/mAGT4 and EMT6/mAGT10 cells were implanted by s.c. inoculation of 0.1 ml of Dulbecco's phosphate buffered saline containing 10^5 tumor cells into the flank of 8–10-week-old female BALB/c mice (Charles River Laboratories, Wilmington, MA). When tumor volumes reached 100–120 mm^3 after 7 days of tumor inoculation, mice were randomized into treatment groups, each consisting of 5 mice, and treatment with Onrigin™ (10 mg/kg, i.p., *q2d x 10*) was initiated. Onrigin™ was dissolved in anhydrous DMSO at 50 mg/ml, freshly diluted 10-fold with water and administered at 20 μl/10 g of body weight using insulin syringes. A vehicle control (2 μl of DMSO in 18 μl of water/10 g of body weight, i.p., *q2d x 10*) did not produce weight loss in host animals; the LD_{50} for a single i.p. injection of DMSO is 126 μl/10 g in mice [29]. Tumor volume was estimated from caliper measurements of the length and width using the formula: $V = (1 \times w)/2$.

2.7. Statistical and mathematical analyses

AGT assays and growth inhibition assays were repeated at least three times and values of IC_{50} (mean) ± standard deviations are shown. IC_{50} values were derived from logistic 3-parameter regression analyses using KaleidaGraph software (Synergy Software, PA).

3. Results

3.1. Cytotoxicity of chloroethylating and carbamoylating moieties of Onrigin™ in AGT-intact and AGT-ablated HL-60 cells and rationale for using 90CE and KS90 to analyze AGT mediated resistance

AGT transfers a guanine O^6-alkyl group onto an AGT active site resulting in a stoichiometric (1:1) inactivation of the protein [15,16]. Since regeneration of the cellular AGT pool is a relatively slow process dependent upon new *de novo* protein synthesis [15], pretreatment of human AGT positive cells with the pseudosubstrate O^6-BG, whose IC_{50} value for AGT inhibition is 0.073 μM in our assay (Fig. 2B), at a level of 10 μM for 1 h and subsequent growth inhibition assays in the continuous presence of O^6-BG are capable of generating a complete AGT-ablated environment.

The cytotoxicities of the two electrophiles (i.e., chloroethylating and carbamoylating species) generated from Onrigin™ were evaluated using HL-60 human promyelocytic leukemia cells expressing 17,000 AGT molecules/cell [24] exposed to Onrigin™, the chloroethylating-only agent 90CE, or the carbamoylaing-only agent 101MDCE for 3 days with or without pretreatment with O^6-BG. The AGT ablating treatment sensitized HL-60 cells to Onrigin™ (IC_{50} values from 32 to 6.0 μM) and to 90CE (IC_{50} values from 83 to 6.8 μM), but not appreciably to 101MDCE (IC_{50} values from 33 to 29 μM) (Fig. 2).

Based upon the finding that Onrigin™ (IC_{50}: 32 μM) produced more cytotoxicity than 90CE (IC_{50}: 83 μM) in AGT-intact cells, we suggested earlier the possibility that the carbamoylating species derived from Onrigin™ inactivated AGT via carbamoylation of the AGT active site cysteine thiol and sensitized AGT positive cells to the chloroethylating species of Onrigin™ [10,14]. We addressed this possibility by directly measuring AGT levels in HL-60 cells exposed to 101MDCE for 1 h (Fig. 2B). 101MDCE inactivated AGT at well above 100 μM (IC_{50}: ∼1000 μM), a level much greater than

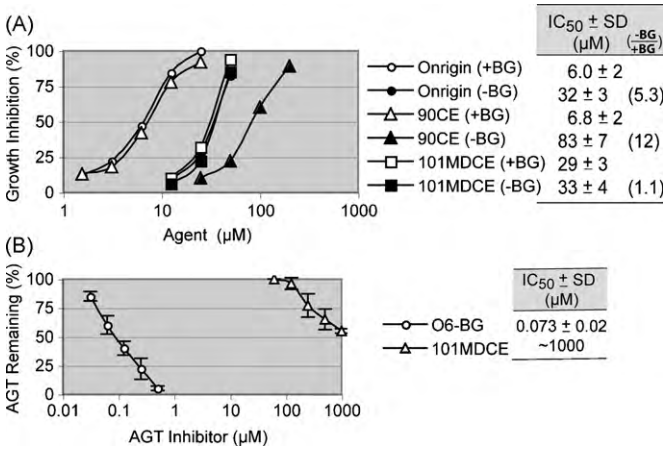

Fig. 2. Dissection of the cytotoxicity stemming from chloroethylating and carbamoylating species of Onrigin™ using its mono-functional analogs (A) and AGT inhibition caused by O^6-BG and 101MDCE (B). Panel A, HL-60 cells containing 17,000 AGT molecules/cell were pretreated with either vehicle (−BG) or 10 μM O^6-BG (+BG) for 1 h. Cells were then exposed to Onrigin™, 90CE, or 101MDCE for 3 days and cell numbers were determined. Growth inhibition (%) was calculated using the log of the cell number. Standard deviation (SD) bars at each drug concentration are omitted in the graph for clarity, as IC_{50} value ± SD for each agent is provided. Panel B, HL-60 cells were exposed to O^6-BG or 101MDCE for 1 h. Thereafter, cells were incubated with ^{3}H-BG for 1 h to measure AGT levels.

that required for the growth inhibitory effect (i.e., IC_{50}: 33 μM). Since the IC_{50} values for Onrigin™ and 101MDCE in AGT-intact conditions (−BG) were equivalent (32 and 33 μM, respectively), while the IC_{50} value for 90CE in AGT-intact conditions was much higher (83 μM), these results collectively implied that the carbamoylating species of Onrigin™ became the predominant cytotoxic entity in AGT-intact HL-60 cells, and that sensitization to the chloroethylating species of Onrigin™ through AGT inhibition by the carbamoylating species did not occur.

Because measurement of AGT mediated resistance to the alkylating function of Onrigin™ was not possible in the co-presence of the carbamoylating species that caused cytotoxicity by itself, the chloroethylating agent 90CE and the methylating agent KS90, analogs of Onrigin™ that differed in alkylating activity in the absence of the carbamoylating moiety (Fig. 1A) were employed. Since the half-lives of 90CE and KS90 in aqueous solution were less than 5 min (Fig. 1A), these agents provided a practical advantage for *in vitro* experiments, in that their alkylation reactions were completed within a short incubation time.

3.2. Quantification of guanine O^6-alkyl lesions generated by 90CE and KS90 using AGT inactivation assays

Because (a) the reaction of AGT with guanine O^6-alkyl adducts generated by guanine O^6-targeting agents leads to stoichiometric (1:1) inactivation of AGT [15,16], (b) unlike bacterial AGT, mammalian AGT does not repair O^4-methylated thymine effectively [30,31], and (c) thymine O^4-methylation (0.1%) is much less frequent than guanine O^6-methylation (7.5%) by *N*-methyl-*N*-nitrosouurea [32], the number of AGT molecules inactivated roughly reflects the number of DNA guanine O^6-alkylations generated by guanine O^6-targeting agents. HL-60 cells expressing 17,000 AGT molecules/cell were incubated with the chloroethylating agent 90CE or the methylating agent KS90 for 1 h to allow guanine O^6-alkylation and AGT repair, and then subjected to AGT assays for 1 h to measure remaining AGT levels. As shown in Fig. 3A, the methylator KS90 (IC_{50}: 53 μM) generated guanine O^6-alkylations 5.5 times more effectively than the chloroethylator 90CE (IC_{50}: 290 μM).

Using this methodology, guanine O^6-alkylations generated by the clinically active chloroethylating (Onrigin™ and carmustine) and methylating (temozolomide) agents were also estimated. The half-lives of Onrigin™, carmustine and temozolomide in aqueous solution are 60, 50 and 74 min, respectively [3,26,27]; therefore, longer incubation periods (2 h for Onrigin™ and carmustine and 4 h for temozolomide) were employed. As shown in Fig. 3C, temozolomide (IC_{50}: 120 μM) generated guanine O^6-alkylations ~3.5 times more effectively than Onrigin™ (IC_{50}: 410 μM) and carmustine (IC_{50}: 440 μM). The IC_{50} values for all of the guanine O^6-targeting agents used in AGT inactivation assays are summarized in Table 1.

3.3. Determination of IC_{50} values for guanine O^6-alkylators in AGT-intact and AGT-ablated HL-60 cells

HL-60 cells were treated with the chloroethylator 90CE or the methylator KS90 for 3 days with or without O^6-BG pretreatment

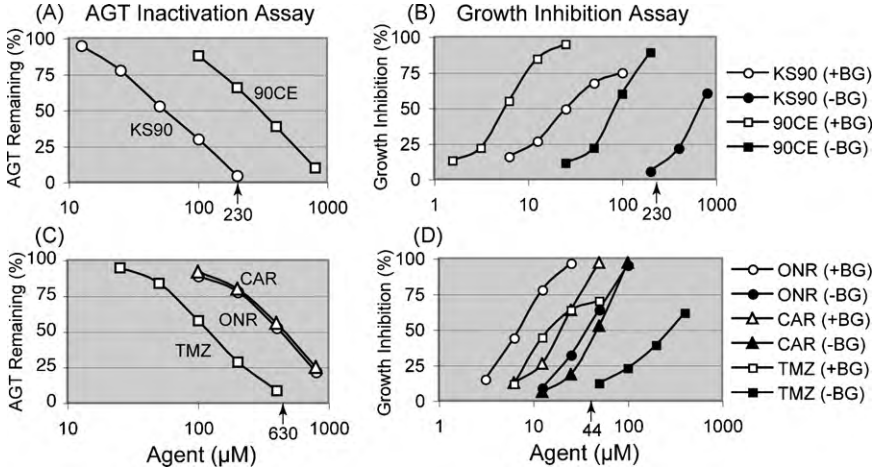

Fig. 3. AGT inactivation assays to measure guanine O^6-alkyl lesions generated by guanine O^6-targeting agents and growth inhibition assays to determine IC_{50} values for these agents in AGT-intact and AGT-ablated HL-60 cells. Panels A and B show the results with 90CE and KS90. Panels C and D show the results with Onrigin™ (ONR), carmustine (CAR) and temozolomide (TMZ). For AGT inactivation assays, HL-60 cells were exposed to 90CE or KS90 for 1 h, to ONR or CAR for 2 h, and to TMZ for 4 h. Cells were then subjected to AGT assays to measure remaining AGT levels. For growth inhibition assays, HL-60 cells, with or without pretreatment with 10 μM O^6-BG for 1 h, were treated with various agents for 3 days. The bold numbers with arrows on the *x*-axis indicate 100% AGT-depleting concentrations in AGT inactivation assays and growth inhibition initiating concentrations in AGT-intact conditions (−BG) in growth inhibition assays for KS90 and TMZ. SD bars at each drug concentration are omitted in the graph for clarity, as IC_{50} value ± SD for each agent is provided in Table 1.

Table 1
Guanine O^6-alkylation events at the IC_{50} values and repair efficiency of AGT for guanine O^6-methyl and guanine O^6-chloroethyl lesions estimated in HL-60 cells.

Agent	AGT inactivation[a] IC_{50} (μM)	Growth inhibition[a] IC_{50} (μM)			Alkyl events at IC_{50} (sites/cell)	AGT protective repair efficiency (alkyl site:AGT)
		+BG	−BG	(−BG/+BG)		
90CE	290 ± 24	6.8 ± 2	83 ± 7	(12)	~300	1:19[b]
KS90	53 ± 7	28 ± 3	670 ± 45	(24)	5,600	1:1
ONR	410 ± 42	6.0 ± 2	32 ± 3	(5.3)		
CAR	440 ± 61	19 ± 4	45 ± 4	(2.4)		
TMZ	120 ± 10	19 ± 5	280 ± 31	(15)		

Abbreviations: ONR, Onrigin™; CAR, carmustine; TMZ, temozolomide.
[a] The IC_{50} values are derived from the data shown in Fig. 2.
[b] The AGT protective repair efficiency (1:19) for 90CE was estimated from a ratio of alkyl events at IC_{50} for KS90 and 90CE (5,600/300 = 19).

and degrees of growth inhibition caused by these agents under AGT-ablated (+BG) and AGT-intact (−BG) conditions were measured (Fig. 3B). The AGT ablative treatment increased the cytotoxicity of 90CE by 12-fold (IC_{50} values from 83 to 6.8 μM) and the cytotoxicity of KS90 by 24-fold (IC_{50} values from 670 to 28 μM) (Table 1). The AGT ablative treatment also increased the sensitivity to the clinically active agents Onrigin™ by 5.3-fold (IC_{50} values from 32 to 6.0 μM), carmustine by 2.4-fold (IC_{50} values from 45 to 19 μM) and temozolomide by 15-fold (IC_{50} values from 280 to 19 μM) (Fig. 3D and Table 1).

3.4. Estimation of the numbers of guanine O^6-chloroethylations and guanine O^6-methylations necessary to produce 50% growth inhibition

Calculations were made from the % inactivation values obtained from AGT inactivation assays (Fig. 3A) and the IC_{50} values derived from growth inhibition assays (Fig. 3B) for 90CE and KS90. The methylating agent KS90 caused 50% growth inhibition at 28 μM under AGT-ablated conditions (+BG) in HL-60 cells. Since AGT assays demonstrated that KS90 caused 33% inactivation of AGT at this concentration in HL-60 cells containing 17,000 AGT molecules/cell, the combined results implied that 5,600 guanine O^6-methylations/cell were needed by KS90 to produce 50% growth inhibition. In contrast, the chloroethylating agent 90CE caused 50% growth inhibition at 8.2 μM under AGT-ablated conditions (+BG). Because 90CE (IC_{50}: 290 μM) was 5.5 times less effective in producing AGT-depleting guanine O^6-alkylations than KS90 (IC_{50}: 53 μM) in AGT inactivation assays, but 90CE (IC_{50}: 8.2 μM) was 3.4 times more growth inhibitory than KS90 (IC_{50}: 28 μM) under AGT-ablated conditions (+BG) in growth inhibition assays, the combined results suggested that only ~300 AGT-depleting guanine O^6-alkylations (5,600/5.5/3.4) were required to produce 50% growth inhibition by 90CE.

3.5. Comparative efficiencies of AGT repair of guanine O^6-methyl lesions and guanine O^6-chloroethyl lesions

KS90 in AGT-intact conditions (−BG) became growth inhibitory at 230 μM (Fig. 3B), the concentration corresponding to that producing 100% inactivation in AGT inactivation assays (Fig. 3A), indicating that AGT repaired guanine O^6-methyl lesions until the AGT pool (17,000 AGT molecules/cell in HL-60 cells) was exhausted before KS90 became growth inhibitory. Compared to the enormous efficiency of AGT repair of guanine O^6-methyl lesions, AGT mediated removal of guanine O^6-alkyl lesions generated by 90CE was inefficient, because the initial chloroethyl lesion progressed through the formation of a cyclic intermediate to an AGT-irreparable form, i.e., an interstrand DNA cross-link that does not involve the *O-6* position of guanine (Fig. 4). Thus, in contrast to AGT repairing the O^6-methylguanine damage at a ratio

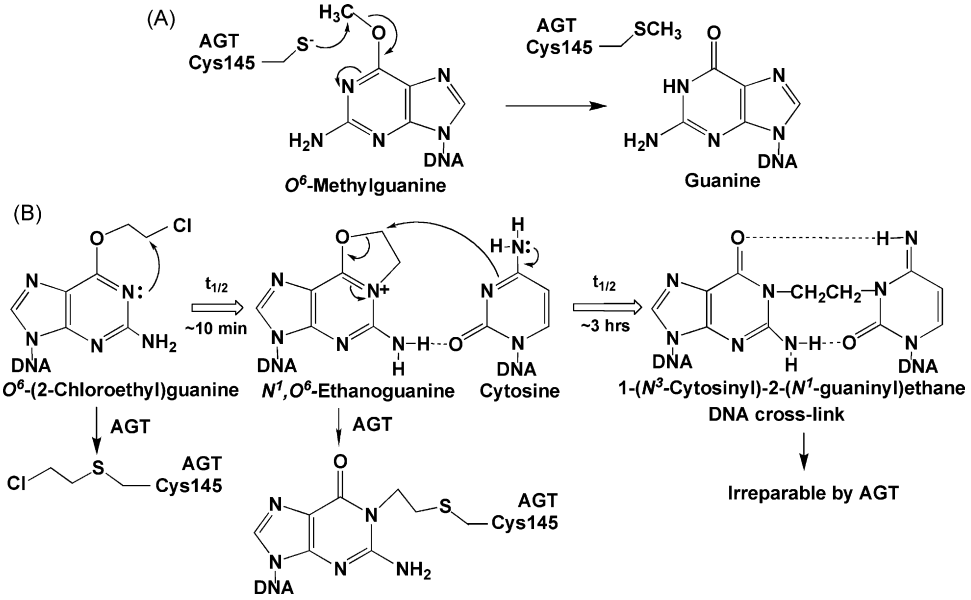

Fig. 4. AGT repair of guanine O^6-methyl and guanine O^6-chloroethyl lesions. Panel A, direct reversal of a guanine O^6-methyl lesion by AGT. Panel B, progression of a guanine O^6-chloroethyl lesion via the formation of a cyclic intermediate, N^1,O^6-ethanoguanine, then to an interstrand DNA G-C cross-link and AGT reparability of these lesions. Note that repair of the cyclic intermediate by AGT yields AGT tethered to guanine *N-1* and that AGT does not produce repair of the DNA cross-link, because the ethylene linkage between the two DNA strands does not involve guanine *O-6*.

of 1:1, the protective repair efficiency of AGT for guanine O^6-chloroethyl lesions, i.e., the number of AGT molecules needed to guard against the damage from one guanine O^6-chloroethylation was estimated to be 19 (5600/300), taking from a ratio of alkyl events at the IC_{50} values for KS90 and 90CE (Table 1).

Unlike KS90, whose 100% AGT inhibitory concentration in AGT inactivation assays coincided with its growth inhibition initiating concentration under AGT-intact conditions in growth inhibition assays, the methylating agent temozolomide exhibited its growth inhibitory activity in AGT-intact conditions at 44 μM (Fig. 3D), a concentration much below that causing 100% AGT inhibition (630 μM) in AGT inactivation assays (Fig. 3C). This result suggests that the cytotoxicity of temozolomide is derived from a mixture of events dependent upon and independent of guanine O^6-methylation.

3.6. Generation of tumor cell lines transfected with human or murine AGT and the relationship between AGT content and the sensitivity to 90CE in vitro

The growth inhibition studies with five human tumor cell lines with naturally occurring AGT levels ranging from none (U251) to 42,000 (DU145) molecules/cell clearly demonstrated an inverse relationship between the content of AGT and sensitivity to 90CE (Fig. 5A). Since this relationship was more accurately examined in an isogenic background, AGT was transfected into three transplantable AGT negative tumor cell lines, i.e., EMT6 murine mammary carcinoma, M109 murine lung carcinoma and U251 human glioblastoma. Both human and murine AGTs were transfected into EMT6 and M109 cells to examine species differences in the ability to repair guanine O^6-chloroethyl lesions. Transfectants were denoted as cell type/mAGT for murine AGT or cell type/hAGT for human AGT and the AGT content expressed as the number of molecules $\times 10^{-3}$/cell. Transfection of AGT generated clones expressing AGT at levels ranging from 4,000 (EMT6/mAGT4) to 700,000 (U251/hAGT700) molecules/cell. The rate of cell growth was not significantly altered by the expression of human or murine AGT at any level in any cell type with the doubling times of EMT6, M109, and U251 cells being ~10, ~10 and ~17 h, respectively. AGT expression in the transfectants was extremely stable upon repeated subcultures in the absence of G418 for more than 6 months.

Growth inhibition assays for 90CE using AGT transfectants from EMT6, M109 and U251 cells are shown in Fig. 5B–D. The degree of resistance to 90CE caused by the expression of AGT, based upon the lowest and highest IC_{50} values, were approximately an order of magnitude in an isogenic background, 9.2 (220/24) for EMT6, 12 (390/32) for M109 and 12 (140/12) for U251. As shown in Fig. 5B, resistance to 90CE was proportional to the AGT level up to approximately 10,000 molecules/cell and AGT levels above this concentration resulted in only a relatively minor further increase in the degree of resistance in EMT6 cells. AGT levels of M109 and U251 transfectants were all above 18,000 molecules/cell (M109/hAGT18); these transfectants demonstrated semi-saturability in AGT mediated resistance to 90CE at extremely high AGT levels (Fig. 5C and D). These findings implied that the cytotoxicity of 90CE at high concentrations was derived from alkylations at positions other than the *O-6* position of guanine in DNA. Thus, these studies defined the cytotoxic ranges of 90CE dependent upon and independent of DNA guanine O^6-alkylations in EMT6, M109 and U251 cells as depicted in Fig. 5B–D.

Resistance to 90CE caused by 21,000 murine AGT molecules/cell (M/m21) and by 18,000 human AGT molecules/cell (M/h18) was both in the semi-saturable range in M109 transfectants (Fig. 5C), suggesting human and murine AGTs repaired guanine O^6-alkyl adducts generated by 90CE with comparable efficiencies.

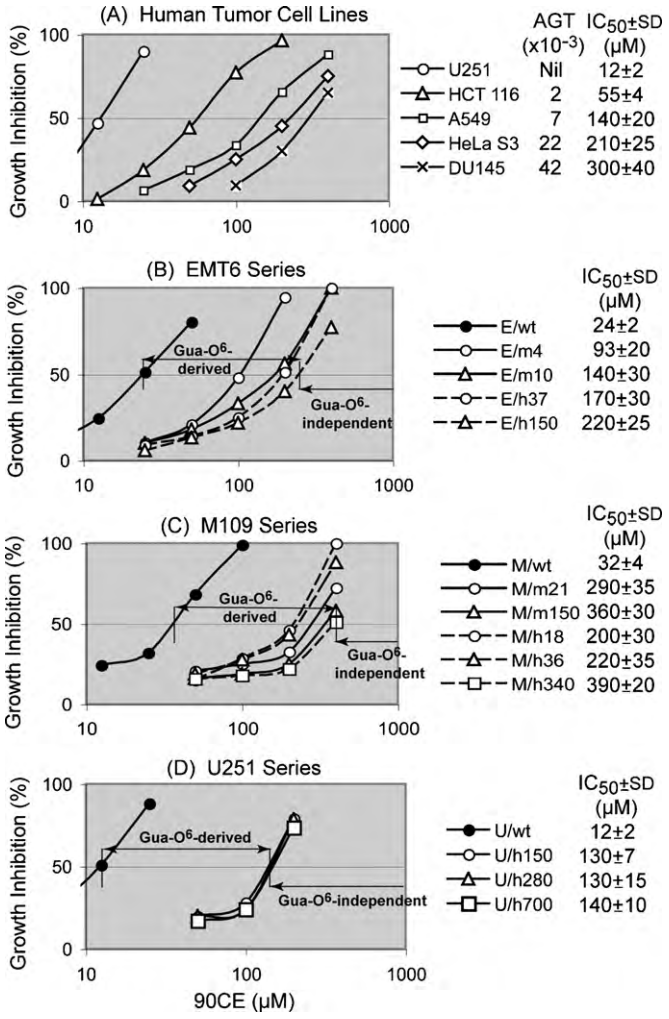

Fig. 5. Relationship between the cellular AGT content and the sensitivity to the chloroethylating agent 90CE *in vitro*. Cells were treated with 90CE for 3 days and the % growth inhibition was calculated using the log of the cell number. Transfectants were denoted as E for EMT6, M for M109 or U for U251, and m or h to represent murine or human AGT followed by the AGT level expressed as the number of molecules $\times 10^{-3}$/cell. In all three types of cells, growth inhibition curves of non-transfected (wild-type) and empty vector-transfected cells were indistinguishable. Hence, results using wild-type cells are shown.

3.7. Relationship between the tumor AGT content and resistance to Onrigin™ in vivo

To examine the relationship between the content of AGT and tumor resistance to Onrigin™ treatment *in vivo*, BALB/c mice were transplanted with wild-type EMT6, EMT6/mAGT4 and EMT6/mAGT10, expressing murine AGT at levels of 0, 4,000 and 10,000 molecules/cell, respectively. The treatment schedule of Onrigin™ employed was 10 mg/kg, i.p., *q2d x 10*. AGT caused tumor resistance to Onrigin™ in an AGT concentration dependent manner *in vivo*, with tumor growth delays produced by Onrigin™ being 11, 3 and 0 days in wild-type EMT6, EMT6/mAGT4, and EMT6/mAGT10 tumors, respectively (Fig. 6).

4. Discussion

In this report, we analyzed the quantitative relationships between tumor AGT content and the degree of tumor resistance to the prodrug Onrigin™ *in vitro* and *in vivo*. Onrigin™ is composed of both chloroethylating and carbamoylating functions. Using the mono-functional analogs of Onrigin™, i.e., the chloroethylating-

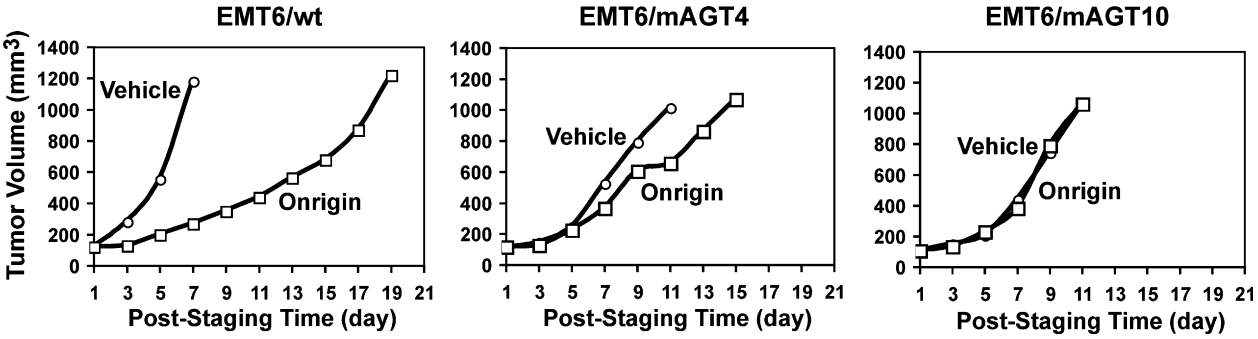

Fig. 6. Relationship between tumor AGT content and resistance to Onrigin™ *in vivo*. EMT6/wt, EMT6/mAGT4 and EMT6/mAGT10 cells were implanted into the flank of BALB/c mice. Onrigin™ treatment was initiated when average tumor volumes reached 100–120 mm^3 (Day 1). Non-transfected (wild-type) and empty vector-transfected EMT6 tumors exhibited equal sensitivity to Onrigin™ and thus only results with wild-type EMT6 tumors are shown.

only agent 90CE and the carbamoylating-only agent 101MDCE, we demonstrated that the carbamoylating species became the dominant cytotoxic entity in AGT-intact HL-60 cells containing 17,000 AGT molecules/cell. Because quantification of AGT mediated resistance to the chloroethylating function of Onrigin™ was not possible in the co-presence of the cytotoxic carbamoylating species, 90CE instead of Onrigin™ was used for *in vitro* growth inhibition studies.

Using human tumor cell lines with natural AGT levels ranging from 0 to 42,000 AGT molecules/cell and AGT negative transplantable tumor cells transfected with human or murine AGT at levels ranging from 4,000 to 700,000 AGT molecules/cell, we demonstrated that AGT caused resistance to 90CE in a concentration dependent manner up to approximately 10,000 AGT molecules/cell and that further increases in the level of AGT resulted in only minor increases in the degree of resistance. These findings were consistent with those from *in vivo* studies where the expression of 4,000 and 10,000 murine AGT molecules/cell in EMT6 tumors conferred partial and complete tumor resistance to Onrigin™, respectively. These studies provide evidence that the antitumor activity of Onrigin™ stems from its ability to chloroethylate the *O*-6 position of guanine in DNA. They also demonstrate that the antineoplastic effectiveness of Onrigin™ is compromised by the presence of even low levels of AGT, emphasizing the advantage of determining tumor AGT levels in humans prior to treatment with Onrigin™ and the need to select patients with AGT negative tumors for optimum results.

In phase II clinical studies, Onrigin™ (cloretazine) as a single agent was reported to produce a 28% complete response rate in elderly high-risk myelodysplasia and acute myelogenous leukemia patients with modest extramedullary toxicity [7]. AGT assays conducted for randomly chosen human leukemia cell lines revealed that 3 out of 9 lines (33%) completely lacked AGT activity [24]. Therefore, a retrospective study is necessary to determine (a) the range of tumor AGT levels and frequency of AGT negative tumors in these disease categories, (b) the relationship between the tumor AGT content and the clinical effectiveness of Onrigin™, (c) whether the upper threshold concentration of AGT that causes maximum resistance to Onrigin™ is 10,000 molecules/cell, corresponding to 100 fmoles/mg of protein based upon the protein content of HL-60 cells being 160 pg/cell, and (d) whether tumor selectivity manifested by Onrigin™ in responsive patients [6,8,9] is due to a differential AGT content in tumor and host tissues.

The finding that AGT levels above 10,000 AGT molecules/cell did not result in a further increase in resistance to 90CE in cultured cells implies that the cytotoxicity of 90CE at high drug concentrations is caused by lesions other than guanine O^6-alkyl adducts. Kaina et al. [33] also observed the saturability of AGT mediated resistance to both the methylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine and the chloroethylating agent *N*-hydroxyethyl-*N*-chloroethylnitrosourea using Chinese hamster ovary cells transfected with human AGT at various levels. These results collectively suggest that both guanine O^6-targeting chloroethylating and methylating agents are capable of causing cytotoxicity through the formation of DNA guanine O^6-alkyl lesions at relatively low drug concentrations in the absence of AGT and that they cause cytotoxicity through AGT-irreparable lesions such as *N*-alkylpurines and alkylphosphotriesters at high drug concentrations irrespective of the presence or absence of AGT.

Using the chloroethylating agent 90CE and the methylating agent KS90 in the absence of the carbamoylating moiety, we conducted analyses on the effectiveness of the repair of the guanine O^6-chloroethyl lesion by AGT employing the repair of the guanine O^6-methyl lesion by AGT as a reference. AGT inactivation assays combined with growth inhibition assays revealed that as many as 5,600 guanine O^6-methylations/cell and only ~300 initial guanine O^6-chloroethylations/cell were needed to produce a 50% growth inhibition. Using human AGT negative cells exposed to [methyl-^{3}H]*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine followed by DNA extraction and modified base analyses, Rasouli-Nia et al. [34] reported a value of 6,650 guanine O^6-methylations/cell for 1 lethal event (the effect producing 63% lethality). This value is roughly comparable to that derived from the present study using AGT inactivation assays (5,600 guanine O^6-methylations/cell for 50% growth inhibition of AGT-ablated HL-60 cells). For chloroethylating agents, to our knowledge, this is the first report to show the number of AGT-depleting guanine O^6-alkylations necessary for cytotoxicity (~300 initial guanine O^6-chloroethylations/cell for 50% growth inhibition of AGT-ablated HL-60 cells).

Structural studies demonstrate that AGT catalyzes a unique, single-step, direct DNA damage reversal repair by flipping an O^6-alkylguanine moiety out of the DNA base stack into the active site of AGT and transferring the alkyl group to an internal cysteine residue in an S_N2 reaction [16]. Although AGT is capable of producing substantial resistance to both the chloroethylating agent 90CE (12-fold) and the methylating agent KS90 (24-fold) in HL-60 cells, the underlying mechanisms involved are quite different. As shown in Fig. 4, the repair of guanine O^6-methyl lesions by AGT is relatively simple, since this lesion is a "dead end" target for AGT. Thus, in HL-60 cells expressing 17,000 AGT molecules/cell, AGT inactivation assays indicated that KS90 generated 17,000 guanine O^6-methyl lesions at 230 μM, while the growth inhibition assay indicated that the growth inhibitory activity of KS90 started at 230 μM in AGT-intact conditions. These results implied that AGT repaired guanine O^6-methyl lesions until the 17,000 AGT molecules existing in HL-60 cells were entirely consumed. In contrast, the repair of guanine O^6-chloroethyl lesions by AGT is complex, since the initial guanine O^6-chloroethyl lesion consecutively progresses to N^1,O^6-ethanoguanine and then to an AGT-irreparable interstrand G-C cross-link (Fig. 4). Therefore, AGT

is relatively ineffective in protecting cells from guanine O^6-chloroethylation induced damage, because the initial guanine O^6-chloroethyl lesion is a "moving" target for AGT and DNA cross-link formation competes with the repair of N^1,O^6-ethanoguanine by AGT. However, since only a relatively small number of initial guanine O^6-chloroethylations is necessary to produce cytotoxicity, AGT is capable of producing significant resistance to guanine O^6-chloroethylating agents. AGT repair of N^1,O^6-ethanoguanine results in AGT tethered at the *N-1* position of guanine [35]; although this repair does not regenerate the native form of guanine, it prevents N^1,O^6-ethanoguanine from progressing to a deadly interstrand DNA cross-link.

We estimated the protective repair efficiency of AGT for guanine O^6-chloroethyl lesions to be 19 by using the ratio of alkyl events at the IC_{50} values for KS90 and 90CE (5,600/300, Table 1). Although the number of AGT molecules needed to guard against the damage induced by one O^6-chloroethylguanine is high, the ratio of AGT molecules to guanine O^6-alkyl lesions engaged in the actual repair process resulting in inactivation of AGT protein remains 1:1. Since the number of O^6-methylguanines required to produce 50% growth inhibition (5,600/cell) is 19 times greater than that of O^6-chloroethylguanines (~300/cell), these findings explain differences in the clinical outcome of cellular AGT levels before and after treatment with methylating and chloroethylating agents in that, at therapeutic doses, the methylating agent temozolomide causes a sizable depletion of the AGT pool [36]; whereas, the chloroethylating agent Onrigin™ has been reported to not produce measurable AGT depletion in humans [37].

Onrigin™ bears a functional resemblance to carmustine in that both agents generate chloroethylating and carbamoylating species, and produce interstrand DNA cross-links as their primary mechanism of action. However, carmustine undergoes complex decomposition patterns generating chloroethylating, hydroxyethylating and vinylating species with each pathway accompanied by production of the carbamoylating species, chloroethyl isocyanate [38]. Moreover, greater than 90% of the DNA adducts generated by carmustine occur at the *N-7* position of guanine (55% N^7-hydroxyethylguanine and 38% N^7-chloroethylguanine) [39], base modifications known to result in spontaneous and enzymatic depurination [40]. In contrast, Onrigin™ was shown to have considerably greater specificity for the *O*-6 position of guanine than carmustine [3]. Furthermore, Onrigin™ consistently displayed much greater differential cytotoxicity in AGT-intact and AGT-ablated HL-60 cells than carmustine (5.3-fold for Onrigin™ versus 2.4-fold for carmustine), although the contribution of cytotoxicity from the carbamoylating species (methyl isocyanate for Onrigin™ and chloroethyl isocyanate for carmustine) to the overall cytotoxicity of each agent was not taken into consideration. In preclinical tumor models, Onrigin™ was found to be more potent, more efficacious and less toxic than carmustine [4].

Unlike KS90, whose 100% AGT inhibitory concentration coincided with its growth inhibition initiating concentration in AGT-intact HL-60 cells, temozolomide initiated growth inhibition at 44 μM in AGT-intact HL-60 cells (Fig. 3D), much below its 100% AGT inhibitory concentration (630 μM) (Fig. 3C). This result suggests that the cytotoxicity of temozolomide stems from a combination of guanine O^6-methyl lesions and lesions unrelated to O^6-methylguanine. Temozolomide generates major adducts at guanine *N-7* (70%) and at adenine *N-3* (9%), base modifications subject to base excision repair; treatment with temozolomide in the presence of an inhibitor of various components of the base excision repair system has been shown to bypass resistance caused by AGT and a defect in the mismatch repair system [41].

In this report, we also ruled out the intriguing possibility that Onrigin™ can bypass AGT mediated resistance through inhibition of the AGT protein by its carbamoylating species. The findings that (a) 101MDCE inactivated AGT in HL-60 cells at concentrations greater than 100 μM, with an IC_{50} value of ~1,000 μM and (b) Onrigin™ and 101MDCE caused growth inhibition of HL-60 cells with comparable IC_{50} values (32 and 33 μM, respectively), indicated that sensitization to the chloroethylating species of Onrigin™ through inhibition of AGT by the carbamoylating species did not occur.

It is noteworthy that even among AGT negative cell lines, considerable variability existed in the basal level of sensitivity to the chloroethylating agent 90CE. For example, the IC_{50} values for 90CE are 6.5 μM in L1210 cells [11], 6.8 μM in HL-60 cells measured under AGT-ablated conditions, 12 μM in U251 cells, 24 μM in EMT6 cells and 32 μM in M109 cells. On the same treatment schedule *in vivo*, Onrigin™ is capable of producing complete tumor regression of the "relatively sensitive" U251 tumor xenograft in nude mice [4], while the effectiveness of Onrigin™ is recognized as tumor growth delay in "relatively resistant" EMT6 and M109 [4] syngeneic mouse tumor models. Elucidation of the mechanism(s) underlying variable sensitivity/resistance to the chloroethylating agent intrinsic to each cell type in the absence of AGT is currently underway.

Conflict of interest statement

The potential anticancer agent Onrigin™, designed and synthesized in Dr. Sartorelli's laboratory, had been licensed to Vion Pharmaceuticals, Inc., no longer a viable company involved in the development of Onrigin™, by Yale University. Dr. Sartorelli in the past served as a Director and Chairman of the Scientific Advisory Board of this company, had common stock in Vion and, several years ago, his laboratory received gift monies in support of new research. In addition to Dr. Sartorelli, two of the other authors (K. Shyam and P.G. Penketh) also owned stock in Vion in the past.

Acknowledgments

This work was supported in part by USPHS grants CA-090671, CA-122112 and CA-129186 from the National Cancer Institute and a grant from the National Foundation for Cancer Research. We express our gratitude to Dr. Rick A. Finch for his helpful advice on animal experiments and to Dr. Kevin P. Rice for his help in using KaleidaGraph software to obtain IC_{50} values.

References

[1] Shyam K, Penketh PG, Loomis RH, Rose WC, Sartorelli AC. Antitumor 2-(aminocarbonyl)-1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazines. J Med Chem 1996;39:796–801.
[2] Shyam K, Penketh PG, Divo AA, Loomis RH, Patton CL, Sartorelli AC. Synthesis and evaluation of 1,2,2-tris(sulfonyl)hydrazines as antineoplastic and trypanocidal agents. J Med Chem 1990;33:2259–64.
[3] Penketh PG, Shyam K, Sartorelli AC. Comparison of DNA lesions produced by tumor-inhibitory 1,2-bis(sulfonyl)hydrazines and chloroethylnitrosoureas. Biochem Pharmacol 2000;59:283–91.
[4] Finch RA, Shyam K, Penketh PG, Sartorelli AC. 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-(methylamino)carbonylhydrazine (101M): a novel sulfonylhydrazine prodrug with broad-spectrum antineoplastic activity. Cancer Res 2001;61:3033–8.
[5] Colvin M, Chabner BA. Alkylating agents. In: Chabner BA, Collins JM, editors. Cancer chemotherapy: principles and practice. Philadelphia: J.B. Lippincott Company; 1990. p. 276–313.
[6] Giles FJ. Bendamustine and cloretazine: alkylators with sharply contrasting activity in AML. Leuk Lymphoma 2007;48:1064–6.
[7] Giles F, Rizzieri D, Karp J, Vey N, Ravandi F, Faderl S, et al. Cloretazine (VNP40101M), a novel sulfonylhydrazine alkylating agent, in patients age 60 years or older with previously untreated acute myeloid leukemia. J Clin Oncol 2007;25:25–31.
[8] Giles FJ. Laromustine: the return of alkylators to non-myeloablative therapy of AML. Leuk Res 2009;33:1022–3.
[9] Vey N, Giles F. Laromustine (cloretazine). Expert Opin Pharmacother 2010;11:657–67.

[10] Baumann RP, Seow HA, Shyam K, Penketh PG, Sartorelli AC. The antineoplastic efficacy of the prodrug Cloretazine is produced by the synergistic interaction of carbamoylating and alkylating products of its activation. Oncol Res 2005;15:313–25.
[11] Ishiguro K, Shyam K, Penketh PG, Sartorelli AC. Role of O^6-alkylguanine-DNA alkyltransferase in the cytotoxic activity of cloretazine. Mol Cancer Ther 2005;4:1755–63.
[12] Ishiguro K, Seow HA, Penketh PG, Shyam K, Sartorelli AC. Mode of action of the chloroethylating and carbamoylating moieties of the prodrug cloretazine. Mol Cancer Ther 2006;5:969–76. PMC2680221.
[13] Ludlum DB. The chloroethylnitrosoureas: sensitivity and resistance to cancer chemotherapy at the molecular level. Cancer Invest 1997;15:588–98.
[14] Penketh PG, Shyam K, Baumann RP, Remack JS, Brent TP, Sartorelli AC. 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 2004;53:279–87.
[15] Pegg AE. Repair of O^6-alkylguanine by alkyltransferases. Mutat Res 2000;462:83–100.
[16] Tubbs JL, Pegg AE, Tainer JA. DNA binding, nucleotide flipping, and the helix-turn-helix motif in base repair by O^6-alkylguanine-DNA alkyltransferase and its implications for cancer chemotherapy. DNA Repair (Amst) 2007;6:1100–15.
[17] Baumann RP, Shyam K, Penketh PG, Remack JS, Brent TP, Sartorelli AC. 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine (VNP40101M): II. Role of O^6-alkylguanine-DNA alkyltransferase in cytotoxicity. Cancer Chemother Pharmacol 2004;53:288–95.
[18] Rice KP, Penketh PG, Shyam K, Sartorelli AC. Differential inhibition of cellular glutathione reductase activity by isocyanates generated from the antitumor prodrugs Cloretazine and BCNU. Biochem Pharmacol 2005;69:1463–72.
[19] Gerson SL. Clinical relevance of MGMT in the treatment of cancer. J Clin Oncol 2002;20:2388–99.
[20] Margison GP, Santibanez-Koref MF. O^6-Alkylguanine-DNA alkyltransferase: role in carcinogenesis and chemotherapy. Bioessays 2002;24:255–66.
[21] Dolan ME, Moschel RC, Pegg AE. Depletion of mammalian O^6-alkylguanine-DNA alkyltransferase activity by O^6-benzylguanine provides a means to evaluate the role of this protein in protection against carcinogenic and therapeutic alkylating agents. Proc Natl Acad Sci USA 1990;87:5368–72.
[22] Moschel RC, McDougall MG, Dolan ME, Stine L, Pegg AE. Structural features of substituted purine derivatives compatible with depletion of human O^6-alkylguanine-DNA alkyltransferase. J Med Chem 1992;35:4486–91.
[23] Shyam K, Hrubiec RT, Furubayashi R, Cosby LA, Sartorelli AC. 1,2-Bis(sulfonyl)hydrazines. 3. Effects of structural modification on antineoplastic activity. J Med Chem 1987;30:2157–61.
[24] Ishiguro K, Shyam K, Penketh PG, Sartorelli AC. Development of an O^6-alkylguanine-DNA alkyltransferase assay based on covalent transfer of the benzyl moiety from [benzene-^{3}H]O^6-benzylguanine to the protein. Anal Biochem 2008;383:44–51. PMC2773450.
[25] Penketh PG, Shyam K, Sartorelli AC. Studies on the mechanism of decomposition and structural factors affecting the aqueous stability of 1,2-bis(sulfonyl)-1-alkylhydrazines. J Med Chem 1994;37:2912–7.
[26] Wiencke JK, Wiemels J. Genotoxicity of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). Mutat Res 1995;339:91–119.
[27] Stevens MF, Hickman JA, Langdon SP, Chubb D, Vickers L, Stone R, et al. Antitumor activity and pharmacokinetics in mice of 8-carbamoyl-3-methyl-imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(^{3}H)-one (CCRG 81045; M & B 39831), a novel drug with potential as an alternative to dacarbazine. Cancer Res 1987;47:5846–52.
[28] Koay DC, Sartorelli AC. Functional differentiation signals mediated by distinct regions of the cytoplasmic domain of the granulocyte colony-stimulating factor receptor. Blood 1999;93:3774–84.
[29] Bartsch W, Sponer G, Dietmann K, Fuchs G. Acute toxicity of various solvents in the mouse and rat. LD50 of ethanol, diethylacetamide, dimethylformamide, dimethylsulfoxide, glycerine, N-methylpyrrolidone, polyethylene glycol 400, 1,2-propanediol and Tween 20. Arzneimittelforschung 1976;26:1581–3.
[30] Brent TP, Dolan ME, Fraenkel-Conrat H, Hall J, Karran P, Laval L, et al. Repair of *O*-alkylpyrimidines in mammalian cells: a present consensus. Proc Natl Acad Sci USA 1988;85:1759–62.
[31] Sassanfar M, Dosanjh MK, Essigmann JM, Samson L. Relative efficiencies of the bacterial, yeast, and human DNA methyltransferases for the repair of O^6-methylguanine and O^4-methylthymine. Suggestive evidence for O^4-methylthymine repair by eukaryotic methyltransferases. J Biol Chem 1991;266:2767–71.
[32] Pegg AE. Methylation of the O^6 position of guanine in DNA is the most likely initiating event in carcinogenesis by methylating agents. Cancer Invest 1984;2:223–31.
[33] Kaina B, Fritz G, Mitra S, Coquerelle T. Transfection and expression of human O^6-methylguanine-DNA methyltransferase (MGMT) cDNA in Chinese hamster cells: the role of MGMT in protection against the genotoxic effects of alkylating agents. Carcinogenesis 1991;12:1857–67.
[34] Rasouli-Nia A, Sibghat U, Mirzayans R, Paterson MC, Day 3rd RS. On the quantitative relationship between O^6-methylguanine residues in genomic DNA and production of sister-chromatid exchanges, mutations and lethal events in a Mer- human tumor cell line. Mutat Res 1994;314:99–113.
[35] Brent TP, Remack JS. Formation of covalent complexes between human O^6-alkylguanine-DNA alkyltransferase and BCNU-treated defined length synthetic oligodeoxynucleotides. Nucleic Acids Res 1988;16:6779–88.
[36] Spiro TP, Liu L, Majka S, Haaga J, Willson JK, Gerson SL. Temozolomide: the effect of once- and twice-a-day dosing on tumor tissue levels of the DNA repair protein O^6-alkylguanine-DNA-alkyltransferase. Clin Cancer Res 2001;7:2309–17.
[37] Gururangan S, Turner CD, Stewart CF, O'Shaughnessy M, Kocak M, Poussaint TY, et al. Phase I trial of VNP40101M (Cloretazine) in children with recurrent brain tumors: a pediatric brain tumor consortium study. Clin Cancer Res 2008;14:1124–30.
[38] Lown JW, Chauhan SM. Mechanism of action of (2-haloethyl)nitrosoureas on DNA. Isolation and reactions of postulated 2-(alkylimino)-3-nitrosooxazolidine intermediates in the decomposition of 1,3-bis(2-chloroethyl)-, 1-(2-chloroethyl)-3-cyclohexyl-, and 1-(2-chloroethyl)-3-(4′-trans-methylcyclohexyl)-1-nitrosourea. J Med Chem 1981;24:270–9.
[39] Tong WP, Kohn KW, Ludlum DB. Modifications of DNA by different haloethylnitrosoureas. Cancer Res 1982;42:4460–4.
[40] Liu L, Yan L, Donze JR, Gerson SL. Blockage of abasic site repair enhances antitumor efficacy of 1,3-bis-(2-chloroethyl)-1-nitrosourea in colon tumor xenografts. Mol Cancer Ther 2003;2:1061–6.
[41] Tentori L, Graziani G. Recent approaches to improve the antitumor efficacy of temozolomide. Curr Med Chem 2009;16:245–57.