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Enhancement of melphalan activity by inhibition of DNA polymerase-α and DNA polymerase-β

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Abstract Our previous studies exploring melphalan resistance in the human rhabdomyosarcoma xenograft TE-671 MR revealed elevation of DNA polymerase-α and DNA polymerase-β . The present study evaluated the alteration of melphalan activity in TE-671 (melphalan-sensitive) and TE-671 MR (melphalan-resistant) subcutaneous xenografts in nude mice after DNA polymerase-α was inhibited using aphidicolin glycinate (AG) and DNA polymerase-β was inhibited using dideoxycytidine (DDC). Administration of AG or DDC did not produce toxicity or demonstrate antineoplastic activity when given alone. AG (90 mg/m^2) enhanced the activity of melphalan against TE-671, with growth delays increasing by 8.4, 15.8, and 21.2 days over the regimen with melphalan only. AG (180 mg/m^2) only modestly increased melphalan activity against TE-671 MR, with the growth delays increasing from 9.6 and 12.1 days using melphalan alone to 12.1 and 14.5 days using melphalan plus AG. AG (180 mg/m^2) plus melphalan (the dose lethal to 10% of animals) produced greater weight loss compared with melphalan alone, whereas DDC plus melphalan produced no additional toxicity. DDC modestly enhanced the activity of melphalan plus AG against TE-671 MR. AG plus O^6-benzylguanine did not increase the activity of 1,3-bis(2-chloroethyl)-1-nitrosourea against TE-671 or TE-671 MR. AG (90 mg/m^2 and 180 mg/m^2) inhibited DNA polymerase-α to 80% and 72% of control in TE-671 and 64% and 37% in TE-671 MR, and DDC inhibited DNA polymerase-β to 59% in TE-671 and 48% in TE-671 MR. These results suggest a role for AG-mediated enhancement of melphalan activity, particularly in the treatment of newly diagnosed, melphalan-sensitive tumors.

Key words Melphalan · Drug resistance · Antineoplastic agents · DNA polymerases · Neoplasm transplantation

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Introduction

Melphalan is a bifunctional alkylating agent active against a broad spectrum of malignancies including ovarian carcinoma, rhabdomyosarcoma, medulloblastoma, and neuroblastoma [10, 14, 29, 32]. Unfortunately, sustained tumor responses are frequently limited by development of resistance to this antineoplastic agent by mechanisms including elevated levels of glutathione [13, 18, 26, 31, 33], increased activity of glutathione *S*-transferase [35], altered transport of melphalan [7, 9, 30], and enhanced repair of DNA interstrand crosslinks [3, 27, 37].

Previous studies have explored the mechanisms operating in the melphalan-resistant human rhabdomyosarcoma xenograft TE-671 MR and have demonstrated evidence for elevated glutathione levels and altered melphalan delivery [20, 31]. Additionally, DNA polymerase-α and -β are significantly increased in TE-671 MR compared with the parent xenograft TE-671, suggesting that increased repair of DNA interstrand crosslinks contributes to melphalan resistance [11].

We now report that melphalan activity in nude mice bearing subcutaneous TE-671 and TE-671 MR xenografts is enhanced by the DNA polymerase-α inhibitor, aphidicolin glycinate (AG), but it is not enhanced by the DNA polymerase-β inhibitor, dideoxycytidine (DDC).

Material and methods

Animals

Male or female athymic BALB/c mice (*nu*/*nu* genotype, 6 weeks old or older) were used for all studies and were maintained as described previously [5].

Xenograft transplantation and tumor lines

TE-671, a subline of the human rhabdomyosarcoma-derived continuous cell line RD, and TE-671 MR, a melphalan-resistant derivative of TE-671, both growing as subcutaneous xenografts, were used in all studies [22, 31, 34].

Drugs

Melphalan, kindly provided by Burroughs Wellcome Co. (Research Triangle Park, N.C.), was given to athymic mice in a single intraperitoneal injection in 17% dimethyl sulfoxide at a dose of either 71 mg/m^2 or 35.5 mg/m^2, which corresponds to 100% or 50%, respectively, of the dose lethal to 10% of injected animals (LD_{10}) [8].

1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU), provided by Bristol-Myers Squibb (Wallingford, Ct.), was given in 3% ethanol as a single intraperitoneal injection of 100 mg/m^2 alone or of 38 mg/m^2 when given in conjunction with O^6-benzylguanine. This dosing corresponds to 100% or 38%, respectively, of the LD_{10}. O^6-benzylguanine, kindly provided by Dr. Robert Moschel, was given in 40% polyethylene glycol 400 and saline as a single intraperitoneal injection of 240 mg/m^2.

Aphidicolin (NSC 234714) and AG (NSC 303812) were provided by the National Cancer Institute (Bethesda, Md.). Aphidicolin, used in initial studies, was dissolved in water to a concentration of 1 mg/ml after brief ultrasonication and given as six intraperitoneal injections of 90 mg/m^2 each. For better solubility, AG was subsequently used in the majority of studies and was dissolved in water and given by six injections at 90 mg/m^2, 180 mg/m^2, or 270 mg/m^2 each.

DDC (NSC 606170) was provided by the National Cancer Institute. It was dissolved in water to a concentration of 20 mg/ml and given in six intraperitoneal injections of 1500 mg/m^2 each (3000 mg/m^2 per day for 3 days).

[^{32}P]dCTP was obtained through Amersham Life Science (Arlington Heights, Ill.). Deoxynucleotide bases (dATP-103985, dTTP-104272, dGTP-104086) were obtained from Boehringer Mannheim (Indianapolis, Ind.). All other reagents were purchased from Sigma Chemical Company (St. Louis, Mo.).

Xenograft transplantation

Xenografts were transplanted subcutaneously into the right flanks of athymic mice as described previously, with inoculation volumes of 50 µl [8].

Tumor measurement

Tumors were measured every 3 to 4 days with vernier calipers (Scientific Products, McGraw, Ill.), and volume was calculated according to the formula [(width)^2 × (length)]/2.

Quantitation of DNA polymerase-α and DNA polymerase-β activities

Activities of DNA polymerase-α and -β in TE-671 and TE-671 MR xenografts were assayed using the method of Yamaguchi et al. [36], which was modified as previously described [2].

Tumor specimens were homogenized (Brinkman Polytron, Westbury, N.Y.) in 200–500 ml ice-cold Tris buffer (pH 7.6) containing 100 µ*M* EDTA, 200 µ*M* dithiothreitol, 500 µ*M* KCl, and 10% glycerol. The homogenates were briefly subjected to ultrasonication and were centrifuged for 30 min at 15000 *g*; the supernatant was used to measure DNA polymerase activities. Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, Calif.). A known amount of albumin was added to each cell extract to equal a total protein content of 50 µg protein per 10 µl. To determine linearity of the results, 5 and 10 µl of cell extract were used to measure polymerase-α and -β activities. The reaction mixtures for both polymerasel-α and polymerase-β assays consisted of 100 m*M* $MgCl_2$, 100 m*M* Tris HCl (pH 7.1 and 8.5 for polymerase-α and -β assays, respectively), activated calf thymus DNA, and a 500 m*M* mixture of dTTP, dATP, and dGTP. In the polymerase-α mixture, 20 m*M* dithiothreitol was added; in the polymerase-β assay, 10 m*M* *N*-ethyl maleimide was used. [^{32}P]dCTP was diluted in water to 0.1 mCi/ml, and 20 µl (~2 µCi) was added to the reaction mixture to a total volume of 100 ml. The mixture was incubated for 1 h at 37 °C and the reaction was stopped by cooling on ice for 5 min. Six replicate 10-µl aliquots of each reaction mixture were applied to DEAE-cellulose filters in a Milliport Multiscreen Assay System (Millipore Corporation, Bedford, Mass.); the filters were washed with cold 5% trichloroacetic acid with 20 m*M* sodium pyrophosphate and were counted by liquid scintillation. Polymerase activity is expressed in units per milligram of tissue. One unit of polymerase activity is the amount of polymerase needed to catalyze the incorporation of 1 nmol of [^{32}P]dCTP into newly synthesized DNA in 1 h.

Treatment regimen

Groups of eight to ten randomly assigned mice bearing TE-671 or TE-671 MR xenografts were treated with melphalan, BCNU, or vehicle when the median tumor volume exceeded 200 mm^3. In experiments evaluating inhibition of DNA polymerase-α , AG was given 4.5 and 1.5 h prior to and 1.5, 4.5, 19.5, and 22.5 h after melphalan administration. In experiments evaluating inhibition of DNA polymerase-β , DDC was given 27, 21, and 3 h prior to and 3, 21, and 27 h after melphalan administration. In experiments evaluating inhibition of O^6-alkylguanine-DNA alkyltransferase, O^6-benzylguanine was administered 1 h prior to treatment with BCNU.

Assessment of response

Response of xenografts was assessed by both growth delay and tumor regressions. Growth delay (T–C) was defined as the difference in days between the median time for tumors of treated (T) and control (C) animals to reach a volume five times greater than the volume at the time of treatment. Tumor regression was defined as a decrease in tumor volume over two successive treatments. Statistical analyses were performed using the Wilcoxon rank order test for growth delay and Fisher's exact test for tumor regressions as described previously [8].

Results

Toxicity

Melphalan given alone at 0.5 LD_{10} produced a mean weight loss of 0.83% and 0/9 deaths in TE-671 and a mean weight loss of 0.58% and 0/10 deaths in TE-671 MR. Escalation of the melphalan dosage to 1.0 LD_{10} resulted in a mean weight loss of 8.5% and 1/55 deaths in TE-671 and a mean weight loss of 9.5% and 1/32 deaths in TE-671 MR.

AG at all dosages tested produced no toxicity as determined by weight loss or death. The addition of AG (90 mg/m^2) to melphalan (1.0 LD_{10}) produced a mean weight loss of 9.3% and 2/27 deaths in TE-671 and a mean weight loss

Table 1 Treatment of TE-671 xenografts growing subcutaneously in nude mice

Experiment	Melphalan[a] (fraction of LD_{10})	Aphidicolin/AG[b] (mg/m^2)	ddC[c] (mg/m^2)	T–C[d, c] (days)	Regression
1	1.0	0	0	26.6	7/9
	0	90	0	2.6[j]	1/10[j]
	1.0	90	0	35.0[g]	10/10
2	0.5	0	0	8.3	2/10[j]
	0.5	90	0	8.4	2/10[j]
	1.0	0	0	24.95	7/8
	1.0	90	0	40.8[g]	7/9
3	1.0	0	0	31.6	10/10
	1.0	90	0	33.4	7/8
	1.0	180	0	52.8[g, h]	9/9
	1.0	270	0	Toxic	–
4	1.0	0	0	27.6	7/10
	1.0	0	3000	27.6	8/10
5	1.0	0	0	26	6/9
	1.0	180	0	34.3[g]	9/10
	1.0	0	3000	27.6[i]	7/9
	1.0	180	3000	35.2[g]	8/9
6	1.0	0	0	26.9	7/8
	1.0	180	0	39.8[g]	7/7
	1.0	180	3000	40.8[g]	7/7

[a] Given as a single intraperitoneal injection at 71 mg/m^2 (1.0 LD_{10}) or 35 mg/m^2 (0.5 LD_{10}) in 17% dimethyl sulfoxide at a volume of 90 mg/m^2

[b] Given as intraperitoneal injections 4.5 and 1.5 h prior to and 1.5, 4.5, 19.5, and 22.5 h after melphalan or vehicle administration in water at a volume of 90 ml/m^2

[c] Given as 500-mg/kg intraperitoneal injections 27, 21, and 3 h prior to and 3, 21, and 27 h after melphalan or vehicle administration in water at a volume of 90 ml/m^2

[d] Growth delay in days between median of treated (T) tumors and median of control (C) tumors to reach a volume five times larger than at the time of treatment

[e] $P < 0.001$ unless otherwise noted

[f] Tumors demonstrating reduction in size over two successive measurements

[g] $P < 0.001$ when compared with group treated with melphalan alone

[h] $P < 0.001$ when compared with group treated with melphalan and AG at 90 mg/m^2

[i] $0.001 < P < 0.005$

[j] Not significant ($P > 0.05$)

of 9.7% and 2/17 deaths in TE-671 MR. Escalation of the AG dosage to 180 mg/m^2 produced a mean weight loss of 21.0% and 3/29 deaths in TE-671 and a mean weight loss of 20.9% and 2/15 deaths in TE-671 MR. Escalation of the AG dosage to 270 mg/m^2 produced $>75\%$ mortality.

DDC Produced no toxicity alone and no additional toxicity when given in combination with melphalan or melphalan plus AG.

Effect of AG or DDC on the antitumor activity of melphalan

The response of TE-671 to chemotherapy using melphalan with and without AG is shown in Table 1. In duplicate trials, AG given at 90 mg/m^2 significantly enhanced the antitumor efficacy of the 1.0 LD_{10} of melphalan, but did not increase the efficacy of the 0.5 LD_{10}. In another trial, AG at 90 mg/m^2 had only a minimal effect on the efficacy of the LD_{10} of melphalan (33.4 days), but at 180 mg/m^2, it was highly potentiating (52.8 days). The response of the melphalan-resistant line, TE-671 MR, to melphalan at 0.5 LD_{10} or 1.0 LD_{10} was not significantly enhanced by AG at 90 mg/m^2, but was modestly potentiated by AG at 180 mg/m^2 (Table 2).

DDC was not effective either with melphalan alone or with melphalan plus AG in enhancing antitumor activity against TE-671 (Table 1). A small degree of melphalan enhancement against TE-671 MR was seen using DDC plus AG (180 mg/m^2) (Table 2).

Efficacy of BCNU plus O^6-benzylguanine plus AG

The previous observation that melphalan resistance in TE-671 MR is accompanied by development of a non-O^6-alkylguanine-DNA alkyltransferase mechanism of cross-resistance to BCNU [11] led to studies designed to evaluate whether DNA polymerase-α could be mediating this resistance. The presence of partial resistance mediated by O^6-alkylguanine-DNA alkyltransferase mandated concurrent O^6-benzylguanine to evaluate AG-mediated inhibition of DNA polymerase-α . However, no increase in response to BCNU was observed using the combination of BCNU plus O^6-benzylguanine plus AG (Table 3).

Table 2 Treatment of TE-671 MR xenografts growing subcutaneously in nude mice

Experiment	Melphalan[a] (fraction of LD_{10})	Aphidicolin/AG[b] (mg/m^2)	ddC[c] (mg/m^2)	T–C[d, f] (days)	Regression
1	0.5	0	0	0.6[k]	0/9[k]
	0.5	90	0	0.7[k]	0/9[k]
	1.0	0	0	8.2[g]	1/7[k]
	1.0	90	0	10.8	0/7[k]
2	1.0	0	0	8.9	2/10[k]
	1.0	0	3000	11.4[g]	1/9[k]
3	1.0	0	0	9.6[g]	1/8[k]
	1.0	90	0	10.1	2/10[k]
	1.0	180	0	12.1[h, i]	3/7[k]
	1.0	270	0	Toxic	
4	1.0	0	0	12.1	2/7[k]
	1.0	180	0	14.5[h]	5/8[g]
	1.0	0	3000	15.7	2/9[k]
	1.0	180	3000	18.0[h, i, j]	6/8[g]

[a] Given as a single intraperitoneal injection at 71 mg/m^2 (1.0 LD_{10}) or 35 mg/m^2 (0.5 LD_{10}) in 17% dimethyl sulfoxide at a volume of 90 mg/m^2

[b] Given as intraperitoneal injections 4.5 and 1.5 h prior to and 1.5, 4.5, 19.5, and 22.5 h after melphalan or vehicle administration in water at a volume of 90 ml/m^2

[c] Given as 500-mg/kg intraperitoneal injections 27, 21, and 3 h prior to and 3, 21, and 27 h after melphalan or vehicle administration in water at a volume of 90 ml/m^2

[d] Growth delay in days between median of treated (T) tumors and median of control (C) tumors to reach a volume five times larger than at the time of treatment

[e] $P < 0.001$ unless otherwise noted

[f] Tumors demonstrating reduction in size over two successive measurements

[g] $0.001 < P < 0.005$

[h] $P < 0.001$ when compared with group treated with melphalan alone

[i] $P < 0.001$ when compared with group treated with melphalan and AG at 90 mg/m^2

[j] $P < 0.001$ when compared with group treated with melphalan and AG at 180 mg/m^2

[k] Not significant ($P < 0.05$)

Quantitation of DNA polymerase-α and DNA polymerase-β

The activity of DNA polymerase-α and DNA polymerase-β following treatment with AG and/or DDC are shown in Table 4. AG (90 mg/m^2 and 180 mg/m^2) inhibited DNA polymerase-α to 80% and 72% of control in TE-671 and 64% and 37% in TE-671 MR. DDC inhibited DNA polymerase-β to 59% in TE-671 and 48% in TE-671 MR. Combinations of AG plus DDC did not produce appreciably different polymerase depletion compared with either drug alone.

Table 3 Treatment of TE-671 or TE-671 MR xenografts growing subcutaneously in nude mice

Experiment	Xenograft	BCNU[a] (fraction of LD_{10})	O^6-benzylguanine[b] (mg/m^2)	AG[c] (mg/m^2)	T–C[d, e] (days)
1	TE-671	0.38	0	0	–0.1[g]
		0.38	0	90	0.2[g]
2	TE-671	0.38	240	0	9.8
		0.38	0	90	0.8[g]
		0	240	90	–0.5[g]
		0.38	240	90	9.3
3	TE-671	0.38	240	0	12.5
		0.38	0	90	–0.7[g]
		0	240	90	–0.8[g]
		0.38	240	90	4.9
4	TE-671 MR	0.38	240	0	3.2[g]
		0.38	240	90	3.8[g]

[a] Given as a single intraperitoneal injection in 3% ethanol at 38 mg/m^2 (0.38 LD_{10}) in a volume of 90 ml/m^2

[b] Given as intraperitoneal injections 4.5 and 1.5 h prior to and 1.5, 4.5, 19.5, and 22.5 h after melphalan or vehicle administration in water at volume of 90 ml/m^2

[c] Given as intraperitoneal injections 27, 21, and 3 h prior to and 3, 21, and 27 h following melphalan or vehicle administration in water at a volume of 90 ml/m^2

[d] Growth delay in days between median of treated (T) tumors and median of control (C) tumors to reach a volume five times larger than at the time of treatment

[e] $0.001 < P < 0.05$ unless otherwise noted

[f] Tumors demonstrating reduction in size over two successive measurements

[g] Not significant ($P > 0.05$)

Table 4 Effect of treatment regimens on DNA polymerase-α and DNA polymerase-β content in TE-671 and TE-671 MR xenografts growing subcutaneously in athymic mice. DNA polymerase-α and -β were quantitated in TE-671 and TE-671 MR xenografts using the method of Yamaguchi et al. [36], which was modified as previously described [2] (values are means ± SD)

Regimen	DNA polymerase-α[a] (units/mg protein)		DNA polymerase-β (units/mg protein)
	TE-671	TE-671 MR	TE-671
Control	22.5 ± 5.7	26.3 ± 3.3	0.22 ± 0.04
AG (90 mg/m²)	18.2 ± 3.4	16.9 ± 3.7	0.25 ± 0.05
AG (180 mg/m²)	16.3 ± 2.7	9.7 ± 2.4	0.18 ± 0.02
ddC (1500 mg/m²)	18.8 ± 3.9	21.5 ± 4.1	0.13 ± 0.02
AG (90 mg/m²) + ddC (1500 mg/m²)	19.7 ± 6.9	10.7 ± 2.3	0.17 ± 0.02
AG (180 mg/m²) + ddC (1500 mg/m²)	19.6 ± 5.3	22.3 ± 5.5	0.23 ± 0.06

Discussion

Numerous clinical phase I, II, and III trials have demonstrated that melphalan is an active agent in a broad spectrum of human neoplasms [13, 18, 26, 31, 33]. However, both *de novo* and acquired tumor resistance to melphalan are often associated with therapy using this alkylator, which has led to several attempts to unravel the mechanisms underlying the resistance. Results to date suggest roles for a variety of mechanisms underlying melphalan resistance, including elevated levels of glutathione [10, 14, 29, 32], increased activity of glutathione *S*-transferase [35], altered drug transport [7, 9, 30], and enhanced repair of DNA interstrand crosslinks. Our recent demonstration that DNA polymerase-α and DNA polymerase-β levels are elevated in a melphalan-resistant human rhabdomyosarcoma xenograft [11] led to the current study designed to define the consequences on melphalan activity of using AG and DDC to inhibit these two DNA replication- and repair-related proteins.

AG is a tetracyclic diterpenoid derived from *Cephalosporium aphidicola* and is a specific reversible inhibitor of DNA polymerase-α and -δ [12, 15, 16, 24, 28]. Administration of AG to nude mice bearing subcutaneous xenografts at doses of 90 mg/m² and 180 mg/m² inhibited DNA polymerase-α to 80% and 72% in TE-671 and 64% and 37% in TE-671 MR, respectively, compared with controls. The activity of melphalan against TE-671 was substantially enhanced by treatment with AG, whereas a much smaller increase was seen against TE-671 MR. No activity was noted against either xenograft using AG alone. Although these results are similar to those of previous studies demonstrating enhancement of cisplatin activity in vitro [17, 19, 21] and in vivo [23], they indicate a more complex role for DNA polymerase-α in the resistance of TE-671 MR to melphalan. It is likely, however, that other mechanisms, including depletion of glutathione [31], might be required concomitantly to restore complete sensitivity to this alkylator. Indeed, glutathione depletion in human glioma cells has been shown to downregulate DNA polymerase-α, polymerase-β, and polymerase-δ activity and to inhibit DNA interstrand crosslink repair [1].

DDC is a pyrimidine analogue that is resistant to deamination by cytidine deaminase and that reversibly inhibits DNA polymerase-β [4, 6, 25]. However, despite inhibition of DNA polymerase-β to 59% in TE-671 and 48% in TE-671 MR compared with controls, no enhancement of melphalan activity against these xenografts was observed. It is likely that the approximately 50% DNA polymerase-β activity left in the tumor was sufficient to ensure effective repair of the lethal DNA interstrand crosslinks induced by melphalan.

TE-671 and TE-671 MR are both mer$^+$ lines. They contain high levels of O^6-alkylguanine-DNA alkyltransferase activity and are resistant to BCNU [11]. Whereas TE-671 could be sensitized to BCNU by using O^6-benzylguanine to deplete tumor O^6-alkylguanine-DNA alkyltransferase activity, such depletion of O^6-alkylguanine-DNA alkyltransferase activity in TE-671 MR produced only a minimal increase in the response of the tumor to BCNU. This suggests that a second, non-O^6-alkyltransferase-DNA alkyltransferase-mediated mechanism of resistance was induced when the xenograft became melphalan resistant. In this study, we examined the role of DNA polymerase-α in mediating this resistance by treatment of TE-671 MR with AG and O^6-benzylguanine simultaneously. The lack of increased sensitivity to BCNU in this approach suggests either that the additional mechanisms of resistance might not involve DNA polymerase-α or that the involvement might be more complex.

While the current studies have not clarified the potential roles of DNA polymerase-α and DNA polymerase-β in mediating melphalan resistance in TE-671 MR, the substantial enhancement of melphalan activity against TE-671 produced by AG does, however, suggest a role for this agent in combination with melphalan, particularly in the treatment of newly diagnosed melphalan-sensitive tumors. Furthermore, the use of AG in concert with other modulators of melphalan activity, such as buthionine sulfoximide [31], warrants preclinical investigation. Prior to such studies, however, it would be advantageous to examine the kinetics of DNA polymerase inhibition and possible repletion to ensure that the required window in which to achieve maximum inhibition of the repair of DNA interstrand crosslinks is established. This is particularly important since DNA crosslink repair is a relatively slow process, and a rapid recovery of polymerase activity might negate any potential gain in its initial inhibition. If this indeed is the case, a protocol of continuous (e.g. intravenous) administration of AG might be necessary to obtain significant potentiation of melphalan activity.

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