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O^6-Methylguanine as a modulator of antitumor activity of *N*-alkyl-*N*-nitrosoureas *in vivo*

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The effect of O^6-methylguanine (O^6-MeG) on the therapeutic efficiency of 1-methyl-1-nitrosourea (MNU) and 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) against the murine L1210 leukemia and B16 melanoma was studied *in vivo*. Although the level of O^6-alkylguanine-DNA alkyltransferase (AGT) in L1210/BCNU leukemia cells was three times higher than in L1210 leukemia cells, no enhancement of the antitumor activity of MNU and BCNU in L1210 leukemia cells with acquired resistance to the *N*-nitrosoureas was observed. Pretreatment of mice bearing L1210 leukemia or B16 melanoma with O^6-MeG or MNU potentiated markedly the cytotoxic effects of MNU and BCNU. Administration of O^6-MeG to mice bearing L1210 leukemia led to delayed inhibition of DNA synthesis and appearance of long-term single-strand breaks in DNA of tumor cells.

Key words: *N*-Alkyl-*N*-nitrosourea, resistance, O^6-methylguanine, DNA synthesis.

Introduction

N-Alkyl-*N*-nitrosoureas exhibit a broad spectrum activity against a number of experimental tumors and some human neoplasms, such as lung small-cell carcinomas, disseminated melanomas, gliomas and lymphoproliferative diseases.[1,2] Their use in clinical practice, however, is limited by the rapid development of tumor cell resistance. Under physiological conditions, the nitrosoureas decompose spontaneously to yield an alkyldiazohydroxide precursor of the chloroethyl(methyl) carbonium ion, alkylating the nucleophilic centers of biomacromolecules and isocyanates, carbamoylating proteins.[3,4] Among the various DNA adducts produced by *N*-nitrosoureas, O^6-methylguanine (O^6-MeG) is thought to have a cytotoxic effect.[5] O^6-MeG is repaired by the action of a DNA repair protein—O^6-alkylguanine-DNA alkyltransferase (AGT)—which is present in mammalian normal and tumor cells in varying amounts.[6–9] Much evidence supports the idea that cells with a high level of AGT are resistant to cytotoxic effects of nitrosoureas.[10] The AGT may be permanently inactivated by the exogenous O^6-MeG.[11,12] The possibility of the use of O^6-MeG for enhancement of the therapeutic efficiency of methyl- and chloroethylnitrosourea *in vivo* has not been proved.

In the present study, we examined the effects of O^6-MeG pretreatment on the therapeutic efficiency of 1-methyl-1-nitrosourea (MNU) and 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) against murine L1210 leukemia cells resistant to MNU and BCNU or against B16 melanoma cells *in vivo*. We also investigated the response of L1210 leukemia to O^6-MeG, MNU, BCNU or their combinations and the effects of O^6-MeG and MNU on DNA synthesis and DNA secondary structure in leukemia L1210 cells *in vivo*.

Materials and methods

2-[^{14}C]Thymidine (54 Ci/mmol) was purchased from Izotop (USSR), [^{3}H]MNU (17 Ci/mmol) was obtained from Amersham. MNU was synthesized at the institute of Chemical Physics, BCNU was provided by Dr L Radina. O^6-MeG was synthesized by us as described in Ref. 13. All other biochemicals were obtained from Sigma. MNU was dissolved in 0.9 (w/v) saline; BCNU was dissolved in 10%

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Table 1. Response of L1210/MNU and L1210/BCNU leukemia to O^6-MeG, MNU, BCNU and their combinations

Resistant leukemia	Drug, dosage (mg/kg)	Mean survival time (days)[a]	Increased life span (%)
L1210/MNU	O^6-MeG, 75	13.1	29.6
	MNU, 80	10.5	0.0
	O^6-MeG, 75 + MNU, 80	12.7	25.5
L1210/BCNU	O^6-MeG, 75	14.6	46.6
	BCNU, 20	10.3	0.0
	O^6-MeG, 75 + BCNU, 20	12.6	26.9

[a] Mean survival times of control mice bearing L1210/MNU and L1210/BCNU implants were both 10.0 days.

ethanol. O^6-MeG was suspended in 0.1% agarose immediately before use.

For evaluation of the sensitivity of transplantable murine tumors to O^6-MeG, MNU, BCNU or their combinations, C57B1 × DBA2 mice were implanted with 3×10^5 L1210 leukemia cells i.p. and C57B1 × CBA2 mice were inoculated with 0.5 ml of 1:10 B16 melanoma brei i.p. Tumor-bearing mice received all drugs as a single i.p. injection 48 h or 144 h after transplantation of leukemia L1210 or melanoma B16 cells, respectively. Both O^6-MeG and MNU were administered to tumor-bearing mice 3 h before *N*-nitrosoureas when combinations of two compounds were used. Antitumor activity of drugs was assessed on the basis of increased life span (%), mean tumor volumes and tumor-free survivors. DNA synthesis was measured by 2-[^{14}C]thymidine incorporation into DNA.[14] Damage of parental DNA structure was determined by the nucleoid sedimentation assay.[15,16] Extracts of L1210 leukemia cells for determination of AGT activity were prepared as described previously.[17] The substrate DNA containing [^{14}H]O^6-MeG was prepared by incubation of calf thymus DNA with [^{14}H]MNU, followed by heating the alkylated DNA at 80°C for 1 h. The AGT level was determined by ^{3}H-activity, which was transferred from the substrate DNA to an acid-insoluble protein fraction in cell extracts.[18] L1210/MNU and L1210/BCNU leukemia strains were developed by *in vivo* treatment.[14]

Table 2. O^6-Alkylguanine-DNA alkyltransferase activity in L1210 and L1210/BCNU leukemia cells

Sensitive and resistant leukemia	AGT activity (pmol/mg protein)
L1210	0.21 + 0.02
L1210/BCNU	0.56 + 0.03

Results

Pretreatment of L1210/MNU- and L1210/BCNU-bearing mice with O^6-MeG did not increase the cytotoxicity of MNU and BCNU (Table 1), although a significantly increased level of AGT activity was found in L1210/BCNU cell extracts as compared to L1210 cells (Table 2).

The effect of pretreatment with O^6-MeG or MNU *in vivo* on the response of L1210 leukemia and B16 melanoma tumors to *N*-nitrosoureas (MNU or BCNU) was investigated. Both O^6-MeG and MNU administered as single i.p. injections (75 and 40 mg/kg, respectively) were about equally effective against L1210 leukemia (Table 3) and effective to a lesser degree against melanoma B16 (Figure 1). O^6-MeG itself was active against parent L1210 leukemia and L1210 leukemia cells with an acquired resistance to MNU or BCNU (Tables 1 and 3). Good antitumor activity of combinations of O^6-MeG or MNU with BCNU against leukemia L1210 was observed (Table 3, Figure 1).

The effects of O^6-MeG and *N*-nitrosoureas on DNA synthesis and production of single-strand

Table 3. Response of L1210 leukemia to O^6-MeG, MNU, BCNU and their combinations

Drug, dosage (mg/kg)	Mean survival time (days)[a]	Increase life span (%)	60-Day tumor-free survivors
MNU, 40	11.5	29.7	0/10
BCNU, 5	12.7	42.7	0/10
BCNU, 10	12.4	38.8	0/10
O^6-MeG, 75	12.3	38.1	0/10
MNU, 40 + BCNU, 5	15.0	68.5	0/10
MNU, 40 + BCNU, 10	18.0	102.3	2/10
O^6-MeG, 75 + BCNU, 10	19.6	120.2	2/10

[a] Mean survival time of control tumor-bearing mice was 7.9 days.

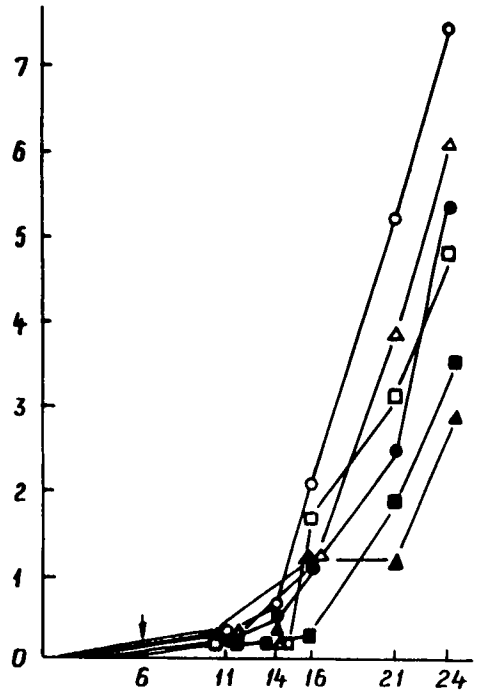

Figure 1. Response of the melanoma B16 to O^6-MeG, MNU, BCNU and their combinations *in vivo*. 1—control (○); 2—O^6-MeG, 75 mg/kg (●); 3—MNU, 40 mg/kg (△); 4—BCNU, 10 mg/kg (□); 5—O^6-MeG, 75 mg/kg + MNU, 40 mg/kg (▲); 6—O^6-MeG, 75 mg/kg + BCNU, 10 mg/kg (■). Ordinate—mean tumor volume (cm^3); abscissa—days post-implant.

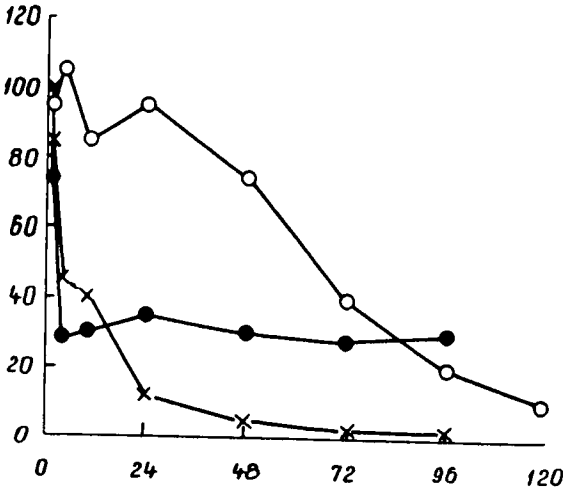

Figure 2. Effect of the single i.p. doses of O^6-MeG (75 mg/kg) (○); MNU (80 mg/kg) (●); and BCNU (20 mg/kg) (×) on the incorporation of 2-[^{14}C]thymidine into DNA of melanoma B16; Ordinate—percentage of control; abscissa—time after injection of the drugs (h). Control data (time 0): 27 205 cpm/μg of DNA

breaks in leukemia L1210 cells *in vivo* were studied. O^6-MeG is characterized with more delayed inhibitory action on DNA synthesis than MNU and BCNU (Figure 2). Appearance of single-strand breaks in DNA of L1210 leukemia cells produced by MNU was observed when mice were sacrificed 1 h after MNU administration (Figure 3). The sedimentation pattern of L1210 cell lysates 24 h after treatment *in vivo* with MNU was similar to that of untreated mice. O^6-MeG induced production of single-strand breaks in DNA of cells only 24 h after treatment. The damage DNA structure during the subsequent 72 h after the administration of drug was increased significantly (Figure 3).

Discussion

Some modulators for overcoming resistance to *N*-nitrosoureas have been proposed. *N*-Nitroso-compounds such as streptozotocin, MNU and *N*-methyl-*N*-nitro-*N'*-nitrosoguanidine form O^6-MeG adducts which can saturate the AGT repair system, rendering tumor cells more sensitive to chloronitrosoureas. There is considerable evidence of the possibility to increase cytotoxicity of *N*-nitrosoureas by exposure of human tumor cells to O^6-MeG or methylating *N*-nitrosocompounds *in vitro*.[11,12,19] Very recently, Gerson[20] and Dolan *et al.*[21] have reported on the dose-dependent decrease in

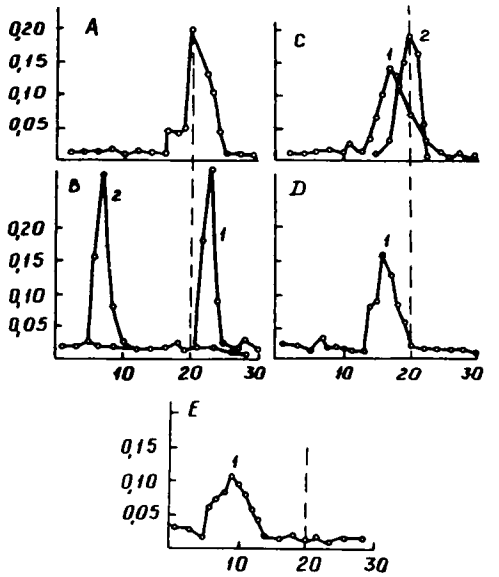

Figure 3. Sedimentation of nucleoids on neutral sucrose gradients of pre-existing DNA of L1210 leukemia cells at various intervals after treatment *in vivo* with O^6-MeG (1) and MNU (2). (A) Time 0 without treatment; (B, C, D, E) 1, 24, 48 and 72 h after treatment with O^6-MeG (1) or MNU (2) leukemia L1210 cells *in vivo*. Ordinate—percentage of total radioactivity; abscissa—fraction number.

AGT activity in human lymphocytes and human tumor xenografts in nude mice after treatment with streptozotocin and O^6-MeG *in vivo*. However, therapeutic efficiency of 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (MeCCNU) against HT29 tumors resistant to the drug after pretreatment with O^6-MeG *in vivo* has not been shown.[21]

In our experiments, pretreatment of mice bearing leukemia L1210 cells resistant to MNU or BCNU with O^6-MeG also did not increase cytotoxicity of MNU and BCNU. Dolan *et al.* believe that the absence of enhancement of cytotoxicity of MeCCNU by pretreatment with O^6-MeG is due to a very high level of AGT in the HT29 tumor. Levels of AGT in both L1210 and L1210/BCNU leukemia cells have also been high. However the enhancement of therapeutic efficiency of BCNU by pretreatment with MNU or O^6-MeG has been demonstrated only in experiments with mice bearing parent L1210 cells.

Thus the higher level of AGT in tumor cells resistant to *N*-nitrosoureas declines by pretreatment with O^6-MeG *in vivo* and is insufficient for overcoming resistance to the drugs. Factors other than the level of AGT in tumor cells may be responsible for cytotoxicity of *N*-nitrosoureas *in vivo*.[22]

Effective combination of O^6-MeG and *N*-nitrosoureas in experiments with L1210 leukemia and B16 melanoma-bearing mice may be due to the differences in mechanisms of action of the antimetabolite and the *N*-nitrosocompound. It is possible that O^6-MeG after its transformation to O^6-methyldeoxyguanosine triphosphate incorporates into the newly replicated DNA of tumor cells.[23,24] Sufficiently long persistence of the modification correlates with delayed appearance of single-strand breaks in DNA of L1210 leukemia cells. However, we could not exclude that other mechanisms may underlie the additive antitumor effect, including more effective inhibition of DNA repair by O^6-MeG in parent L1210 cells with decreased AGT activity.

Conclusion

There is much evidence that it is possible to increase the cytotoxicity of *N*-alkyl-*N*-nitrosoureas by exposure of human tumor cells resistant to these drugs to O^6-MeG *in vitro*. Our results, and those of others, indicate that higher levels of AGT in tumor cells resistant to *N*-nitrosoureas decrease by pretreatment with O^6-MeG *in vivo* and do not lead to overcoming of resistance to the drugs. O^6-MeG itself reveals the cytotoxic activity *in vivo* both against parent leukemia L1210 cells and L1210 cells with acquired resistance to MNU or BCNU, as first was shown by us. Effective combination of MeG and MNU or BCNU has been demonstrated in experiments with L1210 leukemia and B16 melanoma-bearing mice. This, more than the additive therapeutic effect, may be due to the differences in biochemical mechanisms of action for the antimetabolite and *N*-nitrosoureas.

References

1. *Nitrosoureas in Cancer Treatment*. Serrou B, Schein PS, Imbach JL, eds. Amsterdam: Elsevier North-Holland Biomedical Press 1981.
2. Emanuel NM, Vermel EM, Ostrovskaya LA, Korman NP Experimental and clinical studies of the antitumor activity of 1-methyl-1-nitrosourea (NSC-23909). *Cancer Chemother Rep* 1974; **48**: 135–48.
3. Montgomery JA, James R, McCaleb GS, Johnston TP. Decomposition of *N*-(2-chloroethyl)-*N*-nitrosoureas in aqueous media. *J Med Chem* 1975; **18**: 568–71.
4. Colvin M, Brundrett RB, Cowens W, Jardine I, Ludlum DB. A chemical basis for the antitumor activity of chloroethylnitrosoureas. *Biochem Pharmacol* 1976; **25**: 695–9.
5. Singer B. Alkylation of the O^6-guanine is only one of many chemical events that may initiate carcinogenesis. *Cancer Invest* 1984; **2**: 233–8.
6. Brent TP. Suppression of cross-link formation in chloroethylnitrosourea-treated DNA by an activity in extracts of human leukemic lymphoblasts. *Cancer Res* 1984; **44**: 1887–92.
7. Bodell WJ, Aida T, Rosenblum ML. Increased repair of O^6-alkylguanine DNA adducts in glioma-derived human cells resistant to the cytotoxic and cytogenetic effects of 1,3-bis-(2-chloroethyl)-1-nitrosourea. *Carcinogenesis* 1986; **7**: 879–83.
8. Domoradzki J, Pegg AE, Dolan ME, Maher VM, McCormick JJ. Correlation between O^6-methylguanine-DNA methyltransferase activity and resistance of human cells to the cytotoxic and mutagenic effect of *N*-methyl-*N*-nitro-*N'*-nitrosoguanidine. *Carcinogenesis* 1984; **5**: 1641–7.
9. Erickson LC, Bradley MO, Ducore JM, Ewig RA, Kohn KW. DNA cross-linking and cytotoxicity in normal and transformed human cells treated with antitumor nitrosoureas. *Proc Natl Acad Sci USA* 1980; **74**: 467–71.
10. Lindhal DB. Regulation and deficiencies in DNA repair. *Br J Cancer* 1987; **56**: 91–5.
11. Dolan ME, Corsico CD, Pegg AE. Exposure of HeLa cells to O^6-alkylguanine increases sensitivity to the cytotoxic effects of alkylating agents. *Biochem Biophys Res Commun* 1985; **132**: 178–85.
12. Yarosh DB, Hurst-Calderone S, Babich MA, Day RS III. Inactivation of O^6-methylguanine-DNA methyltransferase and sensitization of human tumor cells to killing by chloroethylnitrosourea by O^6-methylguanine as a free base. *Cancer Res* 1986; **46**: 1663–8.

13. Nasutavicus WA, Love J. The preparation of 2-amino-6-chloropurine, a safe improved procedure. *J Heterocyc Chem* 1974; **11**: 77–78.
14. Kukushkina GV, Peretolchina NM, Verovsky VN, Sofina ZP, Gorbacheva LB. *In vivo* damage of DNA structure and DNA synthesis in murine leukemia L1210 cells sensitive and resistant to 1-methyl-1-nitrosourea and 1,3-bis-(2-chloroethyl)-1-nitrosourea. *Biokhimia* (in Russian) 1984; **49**: 1189–98.
15. Hartwig M. Organization of mammalian chromosomal DNA: supercoiled and folded circular DNA subunit from interphase cell nuclei. *Acta Biol Med Germ* 1978; **37**: 421–32.
16. Gorbacheva LB, Kukushkina GV, Durdeva AD, Ponomarenko NA. *In vivo* DNA damage and resistance to 1-methyl-1-nitrosourea and 1,3-bis-(2-chloroethyl)-1-nitrosourea in L1210 leukemia cells. *Neoplasma* 1988; **35**: 3–14.
17. Catapano CV, Broggini M, Erba E, Ponti M, Citti L, D'Incalci M. *In vitro* and *in vivo* methazolastone-induced DNA damage and repair in L1210 leukemia sensitive and resistant to chloroethylnitrosoureas. *Cancer Res* 1987; **47**: 4884–9.
18. Myrnes B, Norstrand K, Giercksky K, Sjunneskog C, Krokan H. A simplified assay for O^6-methylguanine-DNA methyltransferase activity and its application to human neoplastic and non-neoplastic tissues. *Carcinogenesis* 1984; **5**: 1061–4.
19. Zlotogorski C, Erickson LC. Pretreatment of human colon tumor cells with DNA methylating agents inhibits their ability to repair chloroethyl monoadducts. *Carcinogenesis* 1984; **5**: 83–7.
20. Gerson SL. Regeneration of O^6-alkylguanine-DNA alkyltransferase in human lymphocytes after nitrosourea exposure. *Cancer Res* 1988; **48**: 5368–73.
21. Dolan ME, Larkin GL, English HF, Pegg AE. Depletion of O^6-alkylguanine-DNA alkyltransferase activity in mammalian tissues and human tumor xenografts in nude mice by treatment with O^6-methylguanine. *Cancer Chemother Pharmacol* 1989; **25**: 103–8.
22. Hall J, Kataoka H, Stephenson C, Karran P. The contribution of O^6-methylguanine and methylphosphate triesters to the cytotoxicity of alkylating agents in mammalian cells. *Carcinogenesis* 1988; **9**: 1587–93.
23. Hall JA, Saffhill R. The incorporation of O^6-methyldeoxyguanosine and O^4-methyldeoxythymidine monophosphates into DNA by DNA polymerases I and α. *Nucleic Acids Res* 1983; **11**: 4185–93.
24. Duan Dah-Shun, Sadee W. Guanine for DNA synthesis. *Biochem J* 1988; **255**: 1045–8.

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