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Effect of administration of sodium cyanate and melphalan on the lifespan of P388 tumor-bearing CD2F1 mice

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Summary. *Sodium cyanate (NaOCN) at a dose of 250 mg/kg was shown to decrease protein synthesis in P388 leukemia tumor cells to approximately 52% of control values at 2 h and 32% at 5 h after NaOCN administration, without a corresponding decrease in various normal tissues of the tumor-bearing CD2Fl mice.*

CD2Fl mice that had received P388 tumor cells IP 1 day prior to drug administration underwent various schedules of treatment with NaOCN and melphalan (MLN). NaOCN (200 mg/kg or 250 mg/kg) administered IP has no significant antitumor activity (increased mean lifespan [ILS] < 20%). The simultaneous IP administration of NaOCN (250 mg/kg) plus MLN (15 mg/kg) resulted in a significantly greater antitumor activity (approximately 265% of control, with 21 of 30 animals being long-term survivors) than MLN (15 mg/kg) alone (approximately 156% of control, with 11 of 30 animals being long-term survivors). This synergism was not observed when MLN was administered 4 h after NaOCN administration. The synergistic activity of MLN with NaOCN does not appear to be secondary to alterations in the absorption from the peritoneal cavity into the systemic circulation as determined by ^3H_2O. NaOCN does not increase the intracellular concentration of [chloroethyl-^{14}C]MLN into P388 cells. The mechanism of the synergistic antitumor activity of simultaneous IP administration of NaOCN and MLN is unknown.

Introduction

Sodium cyanate (NaOCN) is a selective inhibitor of protein synthesis in a variety of malignant tissues [1, 6]. It has been demonstrated that NaOCN administered IP at 250 mg/kg selectively inhibits amino acid incorporation in a variety of animal tumor cells without a corresponding inhibition of protein synthesis in the normal tissue of the tumor-bearing animal [6, 8]. This effect of NaOCN appears to be present in all the malignant tissues tested to date, including human tumor cell lines [1]. The mechanism by which NaOCN causes suppression of protein synthesis only in malignant tissues and not in normal tissues is unknown.

The selective suppression of protein synthesis by NaOCN in malignant tissues may be of therapeutic importance. The administration of NaOCN with known anticancer agents could potentially be synergistic if a diminution in protein synthesis augmented the anticancer activity of the particular agent. In this study, we investigated: (a) the inhibition of tumor protein synthesis by NaOCN in P388 tumor-bearing CD2Fl mice; (b) the benefits of administration of NaOCN plus L-phenylalanine mustard [melphalan (MLN)] in comparison with either agent alone in P388 tumor-bearing mice; (c) the uptake of MLN in vitro into P388 tumor cells after NaOCN was (i) administered in vivo or (ii) incubated in vitro; and (d) the uptake of ^3H_2O administered IP in P388 tumor-bearing mice, to see whether NaOCN decreases the circulation between the peritoneum and the intravascular system.

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Materials and methods

Animal studies. Male Balb/c × DBA/2Fl (hereafter called CD2Fl mice) weighing 17–25 g and maintained on Lablox laboratory chow pellets and water ad libitum, were used throughout the study.

Inhibition of protein synthesis by NaOCN in P388 tumor-bearing mice. Sodium cyanate was purchased from Sigma (St Louis, MO). It was dissolved in phosphate-buffered saline (pH 6) at a concentration of 20–25 mg/ml. CD2Fl mice received implants of 2×10^6 P388 leukemia cells IP. Four days after implantation, groups of three mice received, IP, 0.1 ml/10 g body weight of either NaOCN solution or saline. The mice then received 20 μCi L[4,5-^{3}H]leucine (sp. act. 13.2 Ci/mmol) IP at 1 and 4 h after NaOCN or saline administration. One hour after leucine administration the mice were sacrificed by cervical traction and the ascitic P388 tumor was removed from the abdomen and kept at 0° C. Red blood cells in the tumor suspension were removed by osmotic lysis. In addition, the spleen, kidney, lung, and a section of the jejunum were removed, rinsed in cold saline, and frozen at −70° C. Within 3 days of the experiment, the tissues were homogenized in 1.5 ml 5% trichloroacetic acid and washed twice with the same solution at 4° C. The final pellets were dissolved in 1 *N* NaOH at room temperature. Aliquots of each sample were utilized to determine radioactivity and micrograms of protein as previously described [10]. The results are expressed as percentages of control values (saline-treated mice).

Determination of NaOCN and MLN antitumor activity in CD2Fl mice. Groups of 10 mice were utilized for each drug dose level in each experiment. Sodium cyanate was dissolved as described above. Melphalan was dissolved in the following fashion: 20 mg was placed in a solution of 0.655 ml 90% DMSO and 10% 1 *N* HCl, plus 9.3 ml normal saline, pH 6. The MLN was then diluted in normal saline (pH 6) to the

appropriate concentration for each experimental group. Control animals received normal saline, pH 6. All compounds and saline were administered IP or SC in a volume of 0.1 ml/10 g body weight. The murine P388 leukemia system was utilized to determine antitumor activity. Treatment was administered 1 day after the IP implantation of 2×10^6 P388 leukemia cells. The percentage of increased mean lifespan (ILS) of all nonsurvivors (mice living less than 50 days) was calculated from the survival of control tumor-bearing mice that received normal saline as described. The termination day of the P388 experiments was the 50th day after tumor implantation. Melphalan, 10 mg/kg IP, is a nontoxic dose in our laboratory, while MLN 15–20 mg/kg IP is an LD_{20}–LD_{80}. Toxic deaths are defined as deaths occurring in the MLN 15–20 mg/kg groups prior to the first death in the corresponding MLN 10 mg/kg group. All experiments were carried out in duplicate or triplicate.

Absorption of ^3H_2O from the peritoneum. To determine the absorption of water from the peritoneal cavity into the systemic circulation, groups of three or four P388 tumor-bearing mice were utilized 1 day after implantation. The control group received phosphate-buffered saline IP 0.1 ml/10 g body weight, while the experimental mice received NaOCN (250 mg/kg) IP 0.1 ml/10 g body weight. At 30 min or 1 h after the saline or NaOCN injection each animal received ^3H_2O 0.1 ml/10 g body weight (100 μCi/ml) IP. Serial peripheral blood samples were obtained by retro-orbital sinus puncture. For scintillation counting, 10 μl plasma plus 100 μl 1 *N* HCl were used with 10 ml scintiverse (Fisher Scientific Corp., Rochester, NY). The radioactivity was determined in a Searle mark III liquid scintillation spectrophotometer and corrected for quenching.

Transport of MLN into P388 leukemia cells. CD2F1 mice received implants of 2×10^6 P388 leukemia cells IP. Four days after implantation, the mice received either an injection of phosphate-buffered normal saline or NaOCN at 250 mg/kg, as previously described. At 30 min or 1 h later the animals were sacrificed by cervical traction and the tumor was aspirated from their abdomens. The P388 leukemia cells were washed twice in PAG (phosphate-buffered saline pH 7.4, glucose 0.25%, and 0.1 m*M* albumin) and all subsequent incubations were done in PAG at a concentration of 2×10^6 cells/ml at 37° C. The saline control cells, the in vivo NaOCN-treated cells, and an aliquot of the saline control cells treated with NaOCN 250 μg/ml in vitro were incubated for 30 min or 1 h prior to the addition of MLN.

[Chloroethyl-^{14}C]melphalan (10.9 mCi/mmol) was kindly supplied by Dr Robert Engle, Developmental Therapeutics Branch, Divsion of Cancer Treatment, National Cancer Institute, Silver Spring, MD. Radiolabeled MLN was dissolved in absolute ethanol and stored at −20° C. Thin-layer chromatography revealed that the drug was stable at this temperature. Melphalan (5.42 μ*M*) was added to each cell suspension and aliquots of these samples were withdrawn at specified time points. At these time points, aliquots of the incubation mixture were layered on microcentrifuge tubes containing 1 ml versilube F50 silicone oil. The tubes were spun at 12,000 *g* for 1 min at room temperature. Cell pellets were solubilized in 0.2 *N* NaOH overnight at 0–4° C and then neutralized with an equivalent amount of 1 *N* HCl. To this was added 10 ml scintiverse and the samples were counted as previously described. Nonspecific absorption of radiolabeled MLN was estimated by measuring drug binding at 0–4° C following exposure of the cells to 5.4 μ*M* MLN for time periods up to 35 min. Student's *t*-test was utilized for statistical analysis of all experiments except the antitumor experiment, where an analysis of variance was utilized.

Results

Sodium cyanate suppression of protein synthesis

Table 1 demonstrates that NaOCN at 250 mg/kg (approximately LD_{10}–LD_{20}) suppresses protein synthesis in P388 leukemia cells to 52% of control at 2 h ($P < 0.025$) and 32% of control at 5 h ($P < 0.01$) after administration. In contrast, there appears to be stimulation of protein synthesis in the lungs and jejunum, while the level of protein synthesis remains stable in the kidneys.

Antitumor activity of MLN and NaOCN

Table 2 demonstrates the results of treating P388-bearing CD2F1 mice with several different combinations of saline, MLN, and/or NaOCN at different times by IP administration. Experiments were done in duplicate or triplicate. Sodium cyanate (200 or 250 mg/kg) demonstrates minimal antitumor activity with an ILS of less than 20%. Melphalan alone at its optimal antitumor dose of 15 mg/kg results in an ILS of approximately 156%, with 11 mice out of 30 being long-term survivors (greater than or equal to 50 days after tumor implantation). Melphalan 20 mg/kg was less effective than MLN 15 mg/kg, because it produced 'toxic deaths.' Simultaneous IP administration of NaOCN (250 mg/kg) and MLN (15 mg/kg) resulted in a significantly greater antitumor activity (265% ILS, with 21 of 30 animals being long-term survivors) with fewer toxic deaths than MLN (15 mg/kg IP) alone ($P < 0.01$). Administration of MLN 1 h after the administration of NaOCN resulted in a greater antitumor activity than that of either MLN (10 mg/kg) or MLN (15 mg/kg) alone, although these results were not statistically significant ($P = 0.123$ and $P = 0.053$). Also, there is a suggestion of increased toxicity with MLN (15 mg/kg) 1 h after NaOCN, as 4 of 20 animals succumbed to toxic death. When MLN was administered 4 h after NaOCN the synergistic activity was lost. Simultaneous SC administration of MLN and NaOCN was not significantly better than SC administration of MLN alone (Table 3).

Table 1. Sodium cyanate suppression of protein synthesis in tissues of P388-bearing CD2F1 mice following treatment with sodium cyanate 250 mg/kg IP[a]

Tissue	Time of ^{3}H-leucine administration after sodium cyanate treatment			
	1 h		4 h	
P388 tumor	52 ± 9[b]	(6)[c]	32 ± 6	(7)
Kidney	100 ± 16	(4)	91 ± 21	(3)
Spleen	226 ± 51	(6)	136 ± 26	(6)
Lung	187 ± 62	(7)	213 ± 57	(7)
Jejunum	166 ± 48	(7)	196 ± 74	(7)

[a] Results are expressed as percent incorporation of ^{3}H-leucine compared with control mice that received an equivalent volume of saline IP

[b] Percent of control ± SE

[c] Number of mice per group

Table 2. Antitumor activity of melphalan (MLN) and sodium cyanate (NaOCN) administered IP 1 day after IP implantation of P388 leukemia cells (2×10^6/mouse) into CD2F1 mice

Treatment		Mean lifespan of nonsurvivors (days)	% Increased lifespan (nonsurvivors)	No. of survivors[a]	Toxic deaths[b]
Mode	Drug and dose (mg/kg)				
Administration of NaOCN or MLN alone	Saline 0.9% (control)	8.4 ± 0.2	–	0/30	–
	NaOCN 250	9.6 ± 0.2	14	0/30	–
	NaOCN 200	9.9 ± 0.8	18	0/10	–
	MLN 10	25 ± 2	195	5/30 (1, 2, 2)	–
	MLN 15	22 ± 1	156	11/30 (0, 4, 7)	2/30
	MLN 20	14 ± 3	67	9/20 (–, 4, 5)	6/20
Simultaneous administration of NaOCN and MLN	NaOCN 250/MLN 10	25 ± 2	200	10/30 (4, 1, 5)	–
	NaOCN 250/MLN 15	31 ± 3	265	21/30 (6, 6, 9)	0/30
	NaOCN 250/MLN 20	19 ± 4	123	10/20 (–, 4, 6)	4/20
	NaOCN 200/MLN 10	26 ± 2	214	3/10 (3, –, –)	–
	NaOCN 200/MLN 15	25 ± 2	198	7/10 (7, –, –)	0/10
MLN administered 1 h after NaOCN	NaOCN 250/MLN 10	24 ± 2	187	7/18 (–, 3, 4)	–
	NaOCN 250/MLN 15	21 ± 4	145	12/20 (–, 5, 7)	4/20
MLN administered 4 h after NaOCN	NaOCN 250/MLN 10	22 ± 2	167	0/20	–
	NaOCN 250/MLN 15	22 ± 2	163	3/20 (1, 2, –)	2/20
	NaOCN 250/MLN 20	21 ± 2	145	9/21 (–, 3, 6)	1/20

[a] Greater than or equal to 50-day survival after tumor implantation (the number in parentheses represents the mice cured in each individual experiment)

[b] Toxic deaths are defined as deaths occurring in any MLN 15 mg/kg or 20 mg/kg groups earlier than the first death in the corresponding group receiving MLN 10 mg/kg

Table 3. Antitumor activity of melphalan (MLN) and sodium cyanate (NaOCN) injected SC into P388 tumor-bearing CD2F1 mice

Drug administered (mg/kg)	Lifespan (days)	% Increase of lifespan	Number of survivors[c]
NaOCN 250	10.7 ± 0.7[b]	27	0/20
MLN 10	16.5 ± 1	96	0/20
MLN 15	15.8 ± 0.7	88	0/20
NaOCN 250/MLN 10[a]	15.5 ± 0.8	85	0/20
NaOCN 250/MLN 15	18 ± 5	111	0/20

[a] All SC injections of NaOCN plus MLN were performed simultaneously

[b] Mean ± SE

[c] Greater than or equal to 50 days after tumor implantation

Effects of NaOCN on the absorption of ^3H_2O from the peritoneal cavity and on the transport of MLN into P388 leukemia cells

Experiments were done to determine whether IP administration of NaOCN to P388-bearing CD2F1 mice retarded the absorption of water from the peritoneal cavity into the systemic circulation as determined by means of peripheral blood samples obtained at 2–60 min after IP administration of ^3H_2O. Sodium cyanate (250 mg/kg) administered IP 30 min or 1 h prior to ^3H_2O administration did not alter ^3H_2O absorption into the system circulation compared with saline-treated control mice.

Melphalan is actively transported into cells via neutral amino acid transport systems [2, 4, 5, 11–13]. The effect of NaOCN on MLN transport was investigated in the following fashion. Sodium cyanate (50 mg/kg) was administered IP to P388-bearing CD2F1 mice 30 min or 1 h prior to sacrifice by cervical traction. Following sacrifice by cervical traction the P388 cells were collected and washed. The transport of 5.4 μ*M* [chloroethyl-^{14}C]melphalan into P388 cells in PAG solution (a solution containing no amino acids) was determined at 1–30 min. The in vivo administration of NaOCN failed to increase the in vitro transport of MLN into P388 cells over that seen in similar experiments done with mice receiving an equivalent volume of saline. Furthermore, in vitro incubation of NaOCN (250 μg/ml) at 37° C for 30 min or 1 h with P388 cells obtained from untreated P388-bearing CD2F1 mice also failed to increase the transport of 5.4 μ*M* MLN over that in appropriate control samples.

Discussion

As previously reported, NaOCN demonstrates selective inhibition of tumor protein synthesis [1, 6, 8]. In this study, NaOCN inhibited protein synthesis in the P388 leukemia cells to a greater extent at 5 h than at 2 h after IP administration, without any accompanying suppression of normal protein synthesis. This phenomenon with NaOCN is very interesting because it appears to be selective for all malignant tissues, including human tumors [1]. It is clear that the phenomenon is not simply secondary to carbamoylation of proteins, since the selective inhibiton of tumor protein synthesis requires activation of NaOCN by the cytochrome P450 S9 fraction. This activation is unnecessary for the simple carbamoylation of proteins. The correlation between NaOCN and the malignant phenotype has been further extended by the observation that sodium butyrate, which suppresses the malignant characteristics of cultured tumor cells, also prevents NaOCN suppression of protein synthesis in these cells. In addition, viral transformation of fibroblasts by Rous sarcoma virus renders these cultured tumor cells sensitive to the inhibitory effects of NaOCN [3]. It has been demonstrated that NaOCN selectively inhibits the in vivo uptake of phosphate and the nonmetabolizable amino acid, α-aminoisobutyric acid, into trans-

planted hepatomas compared with the normal liver. The decrease in protein synthesis observed with NaOCN was believed to be secondary to an amino acid membrane transport defect induced by NaOCN [9]. However, studies utilizing tritiated water (^3H_2O) have suggested that perhaps NaOCN decreases the circulation in tumors and thus results in a decrease in protein synthesis via this mechanism instead of via decreased membrane transport [7].

While NaOCN itself does not possess significant antitumor activity, its selective inhibiton of protein synthesis may be exploitable when it is combined with anticancer agents. In P388-bearing CD2Fl mice, the simultaneous IP administration of NaOCN and MLN significantly increases the antitumor activity over that of MLN alone. The synergistic activity may persist when administration of MLN is delayed until up to 1 h after administration of NaOCN, but disappears when MLN is administered 4 h after NaOCN. While part of the increased antitumor activity of simultaneously administered NaOCN and MLN may be due to a decrease in MLN toxicity, this does not fully account for it. The synergistic activity of MLN with NaOCN does not appear to be secondary to alterations in the absorption from the peritoneal cavity into the systemic circulation as determined by ^3H_2O. The transport of MLN into cells is an active carrier-mediated mechanism which utilizes the same transport as neutral amino acids [2, 4, 5, 11–13]. Pretreatment with NaOCN does not appear to increase intracellular concentrations of MLN in P388 leukemia cells.

The present study demonstrates that approximately simultaneous IP administration of NaOCN and MLN modestly augments the antitumor activity of MLN in P388-bearing CD2Fl mice. The mechanism of this synergistic activity is unknown.

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