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Modulation of thiol pools by vitamin K_3 and its effect on survival of sensitive and resistant murine tumor cells

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Cytotoxic effects of vitamin K_3 were evaluated utilizing the P388/S, L1210, EAT, S-180 and a multidrug-resistant variant of the P388 leukemia cells (P388/ADR). Antitumorigenic potential of vitamin K_3 was assessed by MTT and DNA and RNA biosynthesis inhibition assay. A dose-dependent inhibition of P388/S and P388/ADR cell survival and [^{3}H]thymidine and [^{3}H]uridine incorporation (as a function of DNA and RNA biosynthesis) was observed in tumor cell types exposed to vitamin K_3 concentrations ranging from 1 to 100 μM. One hundred mg/kg vitamin K_3 caused a 32 and 52% increase in life span of the sensitive and resistant P388 leukemia tumor-bearing mice. Induction of DNA strand breaks at 100 μM vitamin K_3 was greater in P388/S than in P388/ADR cells. *In vitro* treatment with vitamin K_3 (100 μM) reduced the intracellular levels of GSH by 40, 47, 6, 15 and 14% in P388/S, P388/ADR, EAT, S-180 and L1210 tumor cells, respectively. *In vivo* treatment with 100 mg/kg vitamin K_3 reduced the GSH content by 18 and 38% and increased the activity of the enzyme GSH-*S*-transferase and γ-glutamyl transpeptidase. Effects of free radical scavengers and of compounds that modulate the GSH metabolism on the cytotoxicity of vitamin K_3 were also investigated. Results indicate that vitamin K_3 interacts with the tumor cell thiol pools while eliciting its antitumor effects and suggest the utility of vitamin K_3 in dealing with the growing problem of multidrug resistance.

Key words: Multidrug resistance, thiol pools, vitamin K_3 cytotoxicity.

Introduction

Glutathione (GSH)—the tripeptide—has multiple cellular functions including protection of essential thiol groups on macromolecules from oxidation, detoxification of metabolites of physiological and xenobiotic origin via formation of mercapturic acids and scavenging of cytotoxic free radicals.[1] GSH through nucleophillic thioether formation or oxidation–reduction reactions participates in the detoxification and repair of cellular injuries of a variety of anti-cancer agents including alkylating drugs, quinone antibiotics and platinating agents.[1]

Overproduction of GSH in tumor cells appears to be a mechanism of resistance to various antitumor agents which are known to interact with DNA.[2] Development of resistance to one drug is often accompanied by an expression of simultaneous resistance to a variety of structurally and functionally dissimilar agents. This phenomenon is termed multidrug resistance (MDR).[3,4] An MDR cell avoids anti-cancer drug cytotoxicity by maintaining a low level of the intracellular drug concentration. This process is mediated by the overproduced, high molecular weight, P-glycoprotein (P-gp), which functions as an active efflux pump.[3,4] In spite of being a major event in the tumor cells acquiring MDR, the levels of P-gp expression have not been correlated with the degree of resistance.[5–8] This observation suggests the presence of other mechanisms functioning to aid in maintaining the MDR character. Reports have been published describing the overexpression of the GSH-based detoxification mechanism in MDR tumor.[9,10]

Several approaches have been identified to control the growing population of MDR tumor cells. These include the utility of certain chemosensitizing agents[3,4] or anti-cancer drugs which are effective in the treatment of MDR tumors. The latter class includes alkylating drugs and antimetabolites. Furthermore, synthesis of newer drugs with antitumorigenic potential has also realized a greater and better therapeutic index: toxicity ratio in the clinics.

In this study, we present our observations on menadione (vitamin K_3), a naphthoquinone, as an

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antitumor agent. The cytotoxic action of vitamin K_3 was evaluated utilizing four histologically distinct drug-sensitive tumor models (P388, L1210, EAT and S-180) and a MDR variant of the parental P388 leukemia cells (P388/ADR) resistant to adriamycin (ADR).

Materials and methods

Menadione bisulfite (vitamin K_3) and MTT (3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) were purchased from the Sigma Chemical Co. (USA). Minimum essential medium (MEM) and fetal calf serum (FCS) were procured from Centron Laboratories (Bombay) and Difco Laboratories (MI, USA), respectively. [^{3}H]Thymidine (18.5 Ci/mmol) and [^{3}H]uridine (14 Ci/mmol) were obtained from Bhabha Atomic Research Center (Bombay, India). All other reagents used were of the highest purity and grade commercially available.

Tumor system

The parental P388 murine leukemia cell line was originally obtained from the National Cancer Institute, NIH, Bethesda, USA, and was maintained *in vivo* in DBA/2 mice by weekly intraperitoneal (ip) transplantation of 10^6 cells in accordance with standard NIH protocol.[11] The subline of P388 leukemia resistant to ADR was developed as described previously.[12] The presence of MDR mediating P-gp was confirmed by utilizing JSB-1 monoclonal antibody and visualized by the immunoperoxidase staining reaction.[3] Tumor resistance was checked periodically with ADR administration, *in vivo* at a dose of 1 mg/kg through a 9 day schedule. L1210, EAT and S-180 cell lines were originally obtained from the Developmental Therapeutics Program, National Cancer Institute. L1210 tumor cells were maintained by weekly serial passage in BDF_1 mice 6–8 weeks old, weighing 18–22 g with an ip inoculum of 10^5 cells per animal. The S-180 and EAT cell lines were maintained by weekly serial passage in 8- to 10-week-old Swiss albino mice with an ip inoculum of 2×10^7 cells per animal. The ascitic fluid was collected on day 7 or 8 post-transplantation.

The contaminating erythrocytes from ascitic fluid containing tumor cells were lysed with Tris–ammonium chloride (nine parts 0.83% ammonium chloride + one part 0.17 M Tris–HCl, pH 7.3). Cell pellets after centrifugation at 300*g* were washed twice with fresh MEM and resuspended in MEM supplemented with 10% FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin. P388/S, P388/ADR and L1210 cells were suspended at a final density of 2×10^6 cells/ml. S-180 and EAT cells were adjusted to a density of 1×10^6 cells/ml. Vitamin K_3 solutions were prepared fresh in normal saline to 100 times higher than the required concentration. A stock solution of 50 μCi/ml [^{3}H]thymidine and [^{3}H]uridine was prepared to give a final concentration of 0.5 μCi/ml.

The entire procedure was carried out under sterile conditions and an aliquot of ascite cells was tested routinely for bacterial and mycoplasma contamination using thioglycolate medium. If contamination was observed the MTT assay was immediately terminated.

MTT assay

The MTT assay was performed essentially according to the method of Mosmann[13] with slight modification. Briefly, P388/S and P388/ADR cells were suspended at 1×10^6 cells in supplemented MEM and 80 μl aliquots of this suspension were dispensed into 96-well round-bottomed microtiter plates (Nunclon) which already contained 20 μl of the drug dilutions. Wells containing no drugs were used for control cell viability and wells containing no cells and no drugs for blanking the spectrophotometer. After the cells were incubated at 37°C in 5% CO_2 for 42 h, an aliquot of 10 μl of the MTT solution (5 mg/ml) was added to each well and incubated further for a period of 6 h after thoroughly mixing the contents of each well. Formazan crystals were dissolved with 100 μl of 0.04 N HCl isopropyl alcohol. The optical density (OD) of the wells was measured with a microplate spectrophotometer (Titretek Multiskan MCC) at 540 nm against a reference wavelength of 690 nm. Cell survival (CS) for a well was calculated and expressed as percentage of the control wells:

$$CS = \left[\frac{\text{OD treated wells}}{\text{Mean OD Control wells}}\right] \times 100$$

DNA and RNA biosynthesis in the presence of vitamin K_3

Ten ml cell suspensions (2×10^6 cells/ml) were added to the Erlenmeyer flasks together with

appropriate drugs. Radiolabeled precursors ([^{3}H]-thymidine for DNA and [^{3}H]uridine for RNA) at a final concentration of 0.5 μCi/ml were added to the cell suspensions and the flasks incubated at 37°C in 5% CO_2. Aliquots (750 μl) of cell suspension were removed in triplicate at regular time intervals and processed in a Millipore sampling manifold on Whatman GF/C fiberglass filter paper.[14] The radioactivity was counted in a liquid scintillation counter (LKB Rack Beta 1215 model II) equipped with automatic quench compensation.

Evaluation of antitumor activity

10^6 cells (P388/S and P388/ADR) were transplanted ip on day zero into BDF_1 mice of either sex, weighing between 18 and 22 g. Vitamin K_3 was dissolved in 0.9% NaCl solution and administered daily for 9 days, starting from the day after tumor inoculation. Vitamin K_3 was used at doses from 10 to 100 mg/kg body weight. The results are expressed as percentage increase in life span (%ILS) calculated as $[(T - C)/C] \times 100$, where T is the median survival time of treated animals and C is the median survival time of untreated animals.

Induction of DNA strand breaks

Alkaline sucrose density gradient centrifugation was carried out to evaluate the DNA strand breaks inducing ability of vitamin K_3.[15] 10^5 cells/ml of P388/S and P388/ADR tumors were cultured in supplemented MEM for a 24-h period with [^{3}H]thymidine (0.5 μCi/ml). The cells were then pelleted by centrifugation and given two washes of cold MEM. They were then resuspended in fresh supplemented MEM with 10 and 100 μM vitamin K_3. The cells were treated for 1 h, then centrifuged and washed with cold MEM. An aliquot of cell suspension containing 10^5 cells was then loaded on top of 0.2 ml of lysing medium (0.5 N NaOH, 0.1 M EDTA) which had been prelayered over 4.0 ml of a 5–20% continuous sucrose density gradient made in 0.1 M NaOH, 0.9 M NaCl and 0.01 M EDTA. The gradients were allowed to stand for 30 min in the dark to allow complete lysis of the tumor cells and further centrifuged in a Kontron ultra-centrifuge TST 60 rotor at 28 000 rev/min for 2 h at 0°C with brakes off.

Fractions were collected from the bottom of the tubes on GF/C fiberglass filter paper discs (Whatman Ltd, UK) and washed with 5% TCA for 20 min. The filter discs were then dried by washing twice with 95% ethanol.

Radioactivity in the acid-insoluble fraction was assayed as described previously.[14]

The difference in sedimentation profiles of DNA from the treated and untreated tumor cells gives a direct estimate of DNA strand breaks induced by vitamin K_3.[15]

Determination of GSH, GSH-*S*-transferase and γ-glutamyl transpeptidase (GGT) in P388/S and P388/ADR tumor cells treated *in vivo*

BDF_1 mice were inoculated with 10^6 cells (P388/S and P388/ADR) on day zero. Vitamin K_3 at a dose of 50 and 100 mg/kg was administered ip from day 1 to day 7. Tumor cells were collected from the treated mice and the contaminating erythrocytes were lysed as described earlier. Levels of GSH were determined after precipitating with 25% TCA. For evaluation of GSH-*S*-transferase and GGT, tumor cells were homogenized with a Teflon homogenizer after being suspended in KCl–Tris buffer (Tris–HCl 50 mM, KCl 150 mM; pH 7.4). The entire procedure was conducted at 4°C. The organelle-free cytosol fraction was obtained after centrifuging the homogenate at 45 000 rev/min for 60 min in a Kontron ultracentrifuge. The cytosol fraction was assayed for GSH-*S*-transferase and GGT activity as described below.

Determination of GSH

The TCA-precipitated supernatant of P388/S and P388/ADR cells was obtained by low speed centrifugation. The GSH content was estimated by the method of Moron *et al.*[16] using DTNB (5,5′-dithiobis(2-nitrobenzoic acid)) as substrate.

Estimation of GSH-*S*-transferase activity

GSH-*S*-transferase activity was measured according to the method of Habig *et al.*[17] Enzyme activity with CDNB (1-chloro-2,4-dinitrobenzene) as substrate was determined by monitoring the change in absorbance at 340 nm for at least 3 min in a Shimadzu double-beam spectrophotometer. Complete assay mixture without the enzyme source served as a control. The concentration of GSH and CDNB used was 1 mM. Values have been

expressed as nmol of CDNB conjugated/min/mg protein.

Determination of GGT activity

The assay of GGT was performed according to the method of Szasa *et al.*[18] in a totally automated, random-access Hitachi 705 analyser at 37°C and 660 nm.

Statistics

The amount of radiolabeled precursor incorporated was expressed as a precentage of counts in control sample at the end of the time course of study. Through linear regression analyses the best fit line was computed. Pearson's correlation coefficient r and its significance were also determined. The percentage inhibition was calculated as

$$\%\text{Inhibition} = 100(1 - St/Sc),$$

where St is the slope of the treated sample and Sc is the slope of the control sample. Student's t-test was utilized to find p values of differences in the levels of various cellular macromolecules due to treatment with vitamin K_3.

The significance (p values) in treated vs control median survival time was determined by the Mann–Whitney test.

Results

Dose–response activity of vitamin K_3 on the *in vitro* cell survival of P388/S and P388/ADR tumor was evaluated by the MTT assay (Figure 1). Vitamin K_3 concentration ranging from 0.1 to 1 μM affected the P388/ADR cell survival only marginally, whereas a 50% inhibition of P388/S cell survival was observed at 1 μM vitamin K_3. However, with a further increase in concentration of the vitamin a dose-dependent decrease in cell survival of P388/S and P388/ADR tumor was evidenced, with 10 μm vitamin K_3 causing a 75% inhibition of P388/S and P388/ADR cell survival (Figure 1).

Figures 2 and 3 illustate a time- and concentration-dependent effect of vitamin K_3 on the DNA biosynthesis of P388/S and P388/ADR leukemia cells, respectively. A 1 μM vitamin K_3 dose was ineffective in inhibiting the DNA biosynthesis of the sensitive and resistant P388 tumor cells. A further increase in vitamin K_3 dose revealed a concentration- and time-dependent increase in the inhibition of [^{3}H]thymidine incorporation. At maximum concentration of vitamin K_3 employed (20 μM) in this study, 88 and 96% DNA biosynthesis inhibition was observed in

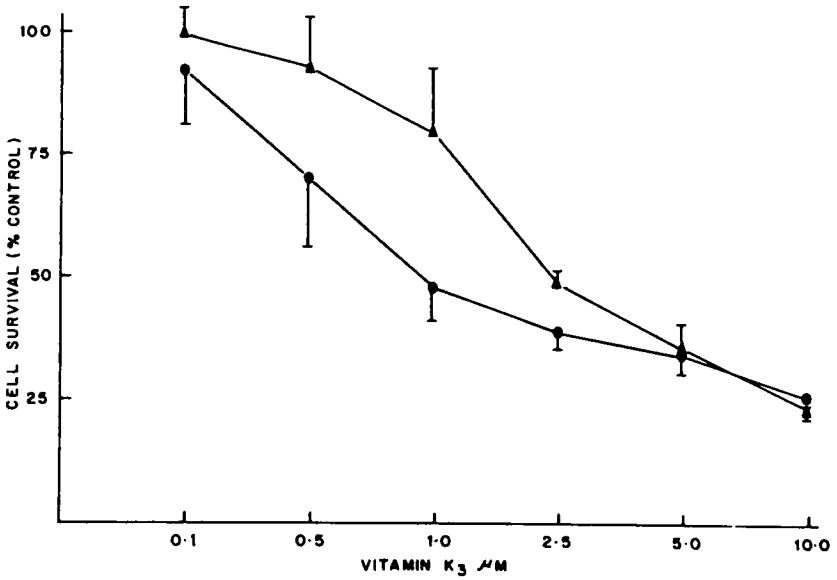

Figure 1. Dose–response activity in vitamin K_3 in P388/S (●) and P388/ADR (▲) leukemia cells. Cell survival was assessed using the MTT assay and expressed as percentage of control cell survival.

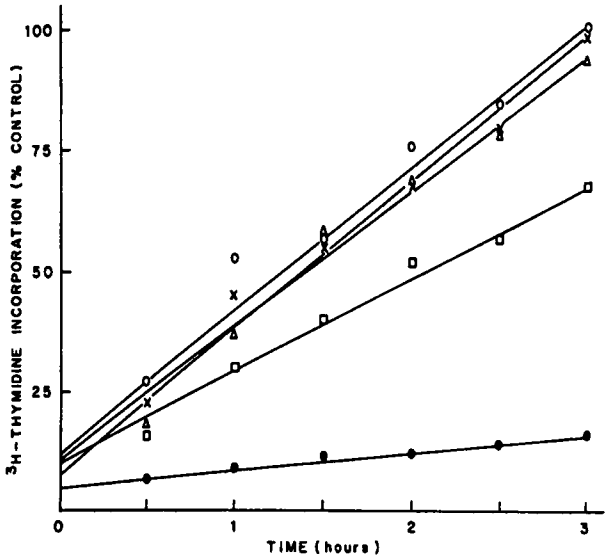

Figure 2. DNA biosynthesis in P388/S after 2 h pretreatment with different concentrations of vitamin K_3 at 37°C (total 5 h). ○, Control (26.84(X) + 20.68, $r = 0.981$, $p < 0.001$); ×, vitamin K_3 10^{-6} M (28.68(X) + 12.64, $r = 0.988$, $p < 0.001$); △, vitamin K_3 5 × 10^{-6} M (27.01(X) + 13.78, $r = 0.982$, $p < 0.001$); □, vitamin K_3 10^{-5} M (19.62 (X) + 9.17, $r = 0.992$, $p < 0.001$); ●, vitamin K_3 2 × 10^{-5} M (3.3(X) + 5.54, $r = 0.987$, $p < 0.001$).

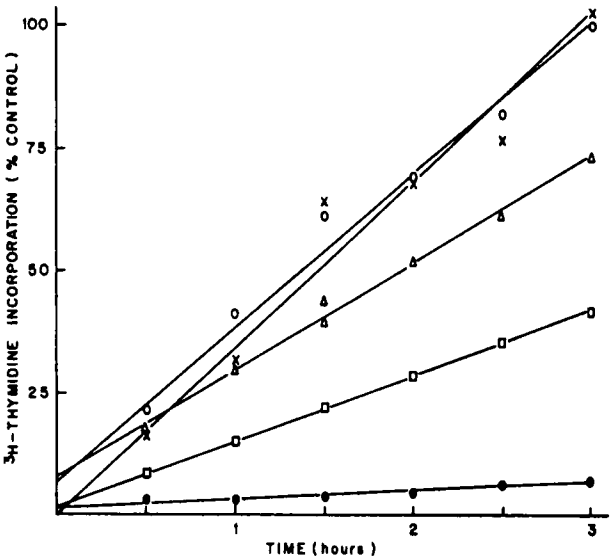

Figure 3. DNA biosynthesis in P388/ADR cells after 2 h pretreatment with different concentrations of vitamin K_3 at 37°C (total 5 h). ○, Control (29.85(X) + 10.69, $r = 0.993$, $p < 0.001$); ×, vitamin K_3 10^{-6} M (34.25(X) + 0.33, $r = 0.988$, $p < 0.001$); △, vitamin K_3 5 × 10^{-6} M (21.71(X) + 8.53, $r = 0.996$, $p < 0.001$); □, vitamin K_3 10^{-5} M (13.03 (X + 2.55, $r = 0.999$, $p < 0.001$); ●, vitamin K_3 2 × 10^{-5p} M (1.33(X) + 2.39, $r = 0.809$, $p < 0.005$).

P388/S (Figure 2) and P388/ADR (Figure 3) leukemia cells.

Vitamin K_3 concentrations ranging from 5 to 100 μM were also analysed for their effect on RNA biosynthesis. Vitamin K_3 at 5 and 10 μM concentration did not elicit RNA biosynthesis inhibition in P388/S cells, while 17 and 23% inhibition, respectively, was observed in P388/ADR cells (Figure 4). At 100 μM vitamin K_3, 91 and 88% inhibition of [^{3}H]uridine incorporation was ob-

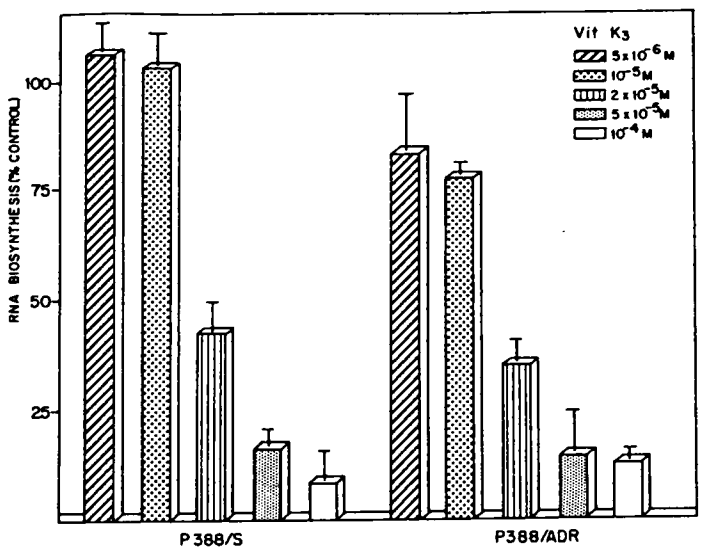

Figure 4. Dose-response activity of vitamin K_3 on the RNA biosynthesis in P388/S and P388/ADR leukemia cells pretreated with vitamin K_3 for 2 h prior to incubation with [^{3}H]uridine (0.5 μCi/ml) for 3 h at 37°C in 5% CO_2.

Table 1. Effect of vitamin K_3 on the survival of mice transplanted with P388/S and P388/ADR leukemia cells. Groups of 10 BDF_1 mice were given ip implants of 10^6 cells of P388/S and P388/ADR leukemia on day 0 and MBS was administered ip daily from day 1 to 9

Tumor model	Vitamin K_3 (mg/kg)	Median survival time (days)	Increase in life span (%)
P388/S	0	8.5	100
	10	9.0	106
	20	9.5	108
	50	10.0	118
	75	10.5	120
	100	13.0	132[a]
P388/ADR	0	11.0	100
	10	10.5	–
	20	11.5	105
	50	12.0	109
	75	14.5	129[a]
	100	17.0	152[a]

[a] $p < 0.05$ as compared with life span of untreated animals.

served in P388/S and P388/ADR cells, respectively (Figure 4).

The *in vivo* antitumor response of vitamin K_3 obtained is represented in Table 1. Vitamin K_3 dose ranging from 10 to 75 mg/kg elicited a therapeutically insignificant increase in life span of P388/S and P388/ADR tumor cell-bearing animals. In contrast, 100 mg/kg vitamin K_3 caused a 32 and 52% increased life span of the sensitive and resistant P388 leukemia-bearing mice, respectively.

In vitro effects of vitamin K_3 on DNA biosynthesis in EAT, L1210 and S-180 cells

Figure 5 is a graphical illustration of the cytotoxic effects of varying concentrations of vitamin K_3 on [^{3}H]thymidine incorporation (as a function of DNA biosynthesis) in EAT, L1210 and S-180 tumor cells. Vitamin K_3 (5 μM) effected a marginal decrease in [^{3}H]thymidine incorporation, while 100 μM vitamin K_3 demonstrated 82, 85 and 96% DNA biosynthesis inhibition in EAT, L1210 and S-180 cells, respectively. The order of sensitivity of the tumor cells to the cytotoxicity of vitamin K_3 at all concentrations analysed was L1210 > S-180 > EAT, ie L1210 lymphocytic leukemia cells were highly sensitive to vitamin K_3 and EAT cells were least sensitive.

Induction of DNA single-strand breaks by vitamin K_3 in P388/S and P388/ADR tumor cells

An increase in the induction of DNA strand breaks was observed in P388/S cells treated with 10 and 100 μM vitamin K_3. An enhanced fragmentation of the intact DNA strands was observed in the sensitive cells exposed to 100 μM vitamin K_3. In contrast, an insignificant effect of vitamin K_3 (10 and 100 μM) treatment in formation of DNA lesions was seen in P388/ADR tumor cells. The results suggest that the observed cytotoxic effects

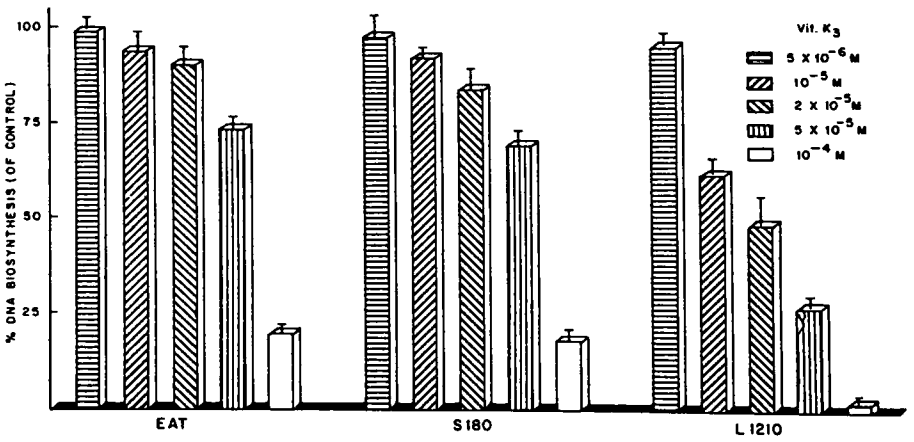

Figure 5. [^{3}H]Thymidine incorporation as a function of DNA biosynthesis in the presence of varying concentrations of vitamin K_3 in Ehrlich ascites carcinoma, S-180 and L1210 lymphocytic leukemia. Tumor cells were pretreated with vitamin K_3 for 2 h and [^{3}H]thymidine (0.5 μCi/ml) was added and cells incubated at 37°C for a further 3 h.

Table 2. Effect of vitamin K_3 treatment on the glutathione levels in murine tumor models

Treatment with vitamin K_3	Glutathione levels ($\mu g/10^6$ cells)				
	P388/S	P388/ADR	EAT	S-180	L1210
Control	0.088	0.066	0.169	0.200	0.213
SD	0.02	0.02	0.02	0.05	0.04
5 μM	0.083	0.060	0.178	0.234	0.231
SD	0.02	0.02	0.02	0.01	0.03
10 μM	0.093	0.063	0.191	0.234	0.228
SD	0.03	0.02	0.03	0.01	0.03
50 μM	0.084	0.057	0.173	0.234	0.235
SD	0.02	0.007	0.01	0.01	0.01
100 μM	0.053[a]	0.035[a]	0.159	0.170	0.183
SD	0.005	0.02	0.03	0.06	0.05

[a] $p < 0.05$ as compared with control.

of vitamin K_3 in P388/ADR cells are not mediated solely by direct interaction with double-stranded DNA.

Effect of *in vitro* vitamin K_3 treatment on the intracellular levels of GSH

Table 2 elucidates the effect of various concentrations of vitamin K_3 on the GSH levels of the histologically distinct tumor models under investigation. The untreated P388 leukemia resistant to ADR demonstrated a 25% reduced level of GSH as compared with the untreated P388/S cells. Further, it was evident that the GSH levels in the control P388/ADR cells (0.066 $\mu g/10^6$ cells) were less than the levels observed in control EAT, L1210 and S-180 tumor cells.

No significant alterations in the intracellular levels of GSH were mediated by treatment of the tumor cells with vitamin K_3 concentration ranging from 5 to 50 μM. Exposure for 3 h to 100 μM vitamin K_3 dose reduced the GSH content of P388/S, P388/ADR, EAT, L1210 and S-180 cells, by 40, 6, 14 and 15%, respectively.

Effect of *in vivo* treatment with vitamin K_3 on the levels of GSH, GSH-*S*-transferase and GGT in P388/S and P388/ADR leukemia cells

The control untreated P388/S and P388/ADR cells indicated a GSH content of 70 and 76 nmol/mg protein, respectively. Treatment with 50 mg/kg vitamin K_3 did not alter the GSH content significantly (Table 3). On administration of 100 mg/kg vitamin K_3, the GSH content of P388/S and P388/ADR leukemia cells was reduced by 18 and 38% ($p < 0.05$), respectively, as compared with levels of GSH observed in control cells (Table 3).

A 20% reduced activity of GSH-*S*-transferase was evident in untreated P388/ADR cells compared with their sensitive parental counterpart. The enzyme activity was increased by 18% in P388/S cells ($p < 0.05$) after treatment with 100 mg/kg vitamin K_3, whereas a marginal 8% increase was observed in the P388/ADR cells treated with 100 mg/kg vitamin K_3 (Table 3).

The GGT activity was enhanced by 96% in P388/ADR tumor cells after treatment with 100 mg/kg vitamin K_3 ($p < 0.05$) as compared with the basal value of 165.15 units/mg protein in the untreated resistant cells. In contrast, a highly significant ($p < 0.001$) increase of 476% in the activity of the enzyme was observed in the vitamin K_3 (100 mg/kg) treated P388/S cells compared

Table 3. Levels of glutathione, GSH-*S*-transferase and γ-glutamyl transpeptidase in the *in vivo* vitamin K_3-treated P388/S and P388/ADR leukemia cells

Tumor model	Vitamin K_3 (mg/kg)	
		GSH content (nmol/mg protein)
P388/S	0	76.19 ± 3.4
	50	70.36 ± 9.7
	100	46.90[a] ± 6.3
P388/ADR	0	70.37 ± 11.4
	50	68.74 ± 3.3
	100	57.76[a] ± 5.3
		GSH-*S*-transferase (nmol of CDNB conjugated/min/mg protein)
P388/S	0	1.039 ± 0.007
	50	0.896 ± 0.091
	100	1.229[a] ± 0.036
P388/ADR	0	0.827 ± 0.031
	50	0.912 ± 0.067
	100	0.889 ± 0.041
		γ-Glutamyl transpeptidase units/mg protein $\times 10^{-5}$
P388/S	0	157.14 ± 4.9
	50	146.19 ± 9.3
	100	909.09[b] ± 33.6
P388/ADR	0	165.15 ± 9.7
	50	177.88 ± 11.6
	100	323.14[a] ± 17.4

[a] $p < 0.05$ as compared with control.
[b] $p < 0.001$ as compared with control.

with an inherent activity of 154.74 units/mg protein present in the untreated sensitive cells (Table 3).

Effect of free radical scavenging enzymes and of compounds that modulate GSH metabolism on the cytotoxicity of vitamin K_3 in P388/S and P388/ADR leukemia cells

Vitamin K_3 at 10 μM demonstrated a 19 and 43% inhibition of DNA biosynthesis in P388/S and P388/ADR cells, respectively. The modulating agents by themselves, at the concentration utilized in this study, were found to be non-toxic (unpublished observation). Ascorbate and cysteamine at 1 mM concentration potentiated the vitamin K_3 (10 μM) induced cytotoxicity in P388/S and P388/ADR cells (Table 4). In combination, ascorbate enhanced the vitamin K_3-induced inhibition of [^{3}H]thymidine incorporation in the sensitive and resistant P388 leukemia cells by 35 and 52%, respectively. Cysteamine elicited a 145 and 186% increase in the vitamin K_3-induced DNA biosynthesis inhibition in P388/S and P388/ADR cells (Table 4).

In contrast, NAC (*N*-acetyl cysteine) (1 mM) and GSH, TU (thiourea) catalase and SOD (superoxide dismutase) when employed at 5o μg/ml antagonized the vitamin K_3 antitumor effects. NAC, GSH and TU reversed the DNA biosynthesis inhibitory capacity of vitamin K_3 (10 μM) by 50, 24 and 76%, respectively, in P388/S cells and by 14, 12 and 18%, respectively, in P388/ADR cells. When utilized in combination with catalase and SOD, the vitamin K_3-induced inhibition of [^{3}H]thymidine incorporation was decreased by 64 and 46%, respectively, in P388/S tumor cells and by 65 and 45%, respectively, in P388/ADR cells.

Discussion

Vitamin K_3 (menadione; 2-methyl-1,4-naphthoquinone) has found clinical use in combination with chemotherapeutic agents,[19] and radiation[19,20] to treat human malignancies. However, the potential of vitamin K_3 as a single-agent antineoplastic drug remains largely unexplored. In the present study, we investigated the antitumor activity of vitamin K_3 in four histologically distinct murine tumor models. Further, the importance of alteration in the cellular thiol pools due to vitamin K_3 treatment in its cytotoxicity was also evaluated.

A dose-dependent inhibition of P388/S and P388/ADR leukemia cell survival (as assessed by the MTT assay, Figure 1) was observed. Treatment of sensitive and resistant P388 leukemia cells with increasing concentration of vitamin K_3 indicated a concurrent increase in the inhibition of DNA and RNA biosynthesis (Figures 2–4). Similar findings were observed in EAT, L1210 and S-180 tumor cells exposed to increasing concentration of vitamin K_3 (Figure 5). *In vitro* studies were combined with *in vivo* investigations. A therapeutically significant ($p < 0.05$) increase in life span of P388/S and P388/ADR tumor cell-bearing mice treated with 75 and 100 mg/kg (qd 1–9) was elicited (Table 1).

Vitamin K_3, in spite of sharing structural and functional resemblance to ADR, effectively inhibited the growth of the ADR-resistant P388 leukemia cells. The P388/ADR tumor cells, which express the MDR phenotype as ascertained by the presence of P-gp, avoid the cytotoxic effects of

Table 4. Effects of various agents on the vitamin K_3-induced DNA biosynthesis inhibition after 3 h exposure at 37°C. The tumor cells were then pulse-labeled for 1 h with 0.5 μCi/ml [^{3}H]thymidine

Drugs, agents	Concentration(s)	Inhibition (%)	
		P388/S	P388/ADR
Vitamin K_3	10 μM	18.8	42.6
Vitamin K_3 + ascorbate	10 μM + 1 mM	25.3	64.6[a]
Vitamin K_3 + cysteamine	10 μm + 1 mM	46.1[a]	79.4[a]
Vitamin K_3 + *N*-acetylcysteine	10 μm + 1 mM	9.3[a]	37.0
Vitamin K_3 + glutathione	10 μM + 50 μg/ml	14.3[a]	39.0
Vitamin K_3 + thiourea	10 μM + 50 μg/ml	4.5[a]	35.1
Vitamin K_3 + catalase	10 μm + 50 μg/ml	6.7[a]	15.1[a]
Vitamin K_3 + SOD	10 μM + 50 μg/ml	9.8[a]	23.2[a]

The SD was less than 6.7% in all cases.
[a] $p < 0.001$ (effect of drug vs drug + agent).

anti-cancer drugs by keeping their intracellular concentration low. This process is mediated by the active efflux mechanism of P-gp,[3,4] a phenomenon which has been substantiated by work from our laboratory (unpublished observation), and by other investigators.[3,4] Considerable evidence has accumulated suggesting a role for 'non-P-gp'-mediated mechanism of resistance in MDR cells.[5–8] Overproduction of GSH appears to be an alternative mechanism of resistance to DNA-interactive drugs.[9,10] Mimnaugh *et al.*[21] and Sinha *et al.*[22] observed a concurrent development of resistance and tolerance to superoxide and hydroxyl radicals with an elevation in enzyme GSH peroxidase activity in the ADR-resistant MCF-7 human breast cancer cell line.

The mechanism responsible for tumor growth inhibition by vitamin K_3 involving redox cycling[23,24] and depletion of cellular thiol groups has already been reported from work with freshly isolated hepatocytes and with murine L1210 leukemia cells.[25,26] Vitamin K_3, at 10^{-5}M concentration *in vitro* did not elicit any change in levels of GSH in the tumor models studied. At 100 μM dose, vitamin K_3 reduced the GSH pools by 40 and 47% in P388/S and P388/ADR cells, respectively, while a marginal reduction was observed in EAT, L1210 and S-180 tumor cells. Although a minor difference was observed in the basal levels of GSH in P388/S and P388/ADR cells, 18 and 38% reduced levels were effected in the sensitive and resistant P388 leukemia cells, respectively, after *in vivo* treatment with 100 mg/kg vitamin K_3. GSH depletion might have been effected by adduct formation and by oxidative stress caused by oxygen radicals formed during redox cycling of vitamin K_3.[27,28]

GSH is enzymatically conjugated to a variety of electrophilic compounds,[29] a reaction catalysed by GSH-*S*-transferase. The conjugated product is further processed by GGT enzyme which is known to regulate GSH levels in the cell.[30]

Results obtained in this study indicate a 20% reduced activity of GSH-*S*-transferase in P388/ADR cells as compared to their sensitive, parental cells. On administration of 100 mg/kg vitamin K_3, an 18 and 8% increase in the GSH-*S*-transferase activity and a 476 and 96% increase in the activity of GGT were effected in P388/S and P388/ADR cells, respectively. In effect, the partial increase in the levels of GSH-*S*-transferase will affect the rate and extent of drug metabolism detoxification in a negligible manner, whereas the increased GGT activity will increase the transport of cellular GSH, leading to a depletion of the GSH pool. This assumption fits reasonably well with the observed reduction in the GSH levels after treatment with vitamin K_3.

Thiol compounds protect DNA by (a) destroying the damage-inducing reactive free radical species, (b) chemically repairing DNA, and (c) depleting oxygen stores which would otherwise form superoxide and hydroxyl radicals during the redox cycling process.[31,32]

Reduction of the GSH pool as a result of vitamin K_3 treatment results in an intracellular environment devoid of DNA-protecting components such as the thiol compounds. Thereafter, the free radicals generated by vitamin K_3 will induce a greater DNA fragmentation as observed in the present investigation, irrespective of the nature of tumor cell type employed (either sensitive or resistant).

The importance of cellular GSH level and its relation to the cytotoxic effects exhibited by vitamin K_3 have been illustrated by Nicotera *et al.*[33] and Akman *et al.*[26] Observations made in this study are consistent with those reported previously.[26,33] Co-incubation of the sensitive and resistant P388 leukemia cells with vitamin K_3 and *N*-acetylcysteine (NAC), GSH, TU, catalase and superoxide dismutase caused a reversal in the DNA biosynthesis inhibitory ability of vitamin K_3. NAC, GSH and TU are known scavengers of reactive oxygen species. Moreover, the enzymes catalase and SOD cannot traverse the membrane matrix, because of their molecular size, and therefore it is possible that the growth inhibitory effects of vitamin K_3 are mediated by metabolism at or near the cell surface independent of the depletion of cellular GSH. If so, then the lack of effect of vitamin K_3 at 20 μM concentration on GSH levels, while indicating a greater than 50% inhibition of tumor cell survival at the same concentration could have only resulted from the generation of free radicals during the redox cycling processes.

Conclusion

Vitamin K_3 via generation of free radicals induces oxidative stress, depletes cellular GSH content and causes fragmentation of intact DNA in sensitive as well as resistant tumor cells. The inhibition of cell survival due to vitamin K_3 treatment observed *in vivo* reflects the results obtained *in vitro*. An equally important clinical consideration is the collateral sensitivity of P388/ADR cells to the cytotoxic effects of vitamin K_3, suggesting a need to initiate

in-depth investigations into the possible interactions of the vitamin in MDR tumor cells which are responsible for inhibition of cell growth.

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