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Thioalkyl Derivatives of Vitamin K_3 and Vitamin K_3 Oxide Inhibit Growth of Hep3B and HepG2 Cells

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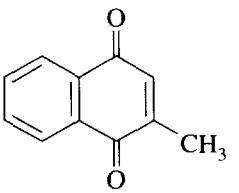
A new hypothesis regarding the effect of vitamin K_3 on hepatoma cell growth is presented. In brief, exploration of cell growth activity has been identified with the action of $p34^{cdc2}$ kinase and its associated protein tyrosine phosphatase. After exploring a series of substituted derivatives of vitamin K and vitamin K_3 oxide, we suggest a mechanism involving alkylation at the active-site cysteine for the inhibition of the protein tyrosine phosphatase which controls the activity of the $p34^{cdc2}$ kinase. © 1995 Academic Press, Inc.

INTRODUCTION

An attractive feature of vitamin K_3 **(1)** as a broad spectrum antitumor agent (*1*)



Vitamin K_3 (menadione)

1

lies with its relatively low toxicity (LD_{50}, orally in mice, 0.5 g/kg) in comparison with other quinone antitumor agents.

Juan and Wu showed that vitamin K_3 **(1)** interdicts the action of $p34^{cdc2}$ kinase (*2*) and causes cell death by an S/G2 phase delay and induction of apoptosis. Since the mechanism of action of vitamin K_3 **(1)** is not well understood in this context, (*2*) we have carried out model studies and growth inhibition experiments that may shed some light on this question.

The activity of the $p34^{cdc2}$ kinase is controlled by the phosporylation status of its Thr-14, Tyr-15, and Thr-167 residues. The $p34^{cdc2}$ kinase is transformed from the inactive to the active state by protein tyrosine phosphatase-promoted cleavage of the Tyr-15 phosphate. Therefore, if vitamin K_3 **(1)** inhibits the activity of the tyrosine phosphatase, the $p34^{cdc2}$ kinase will remain inactive, and cell growth will be inhibited.

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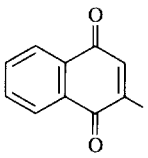
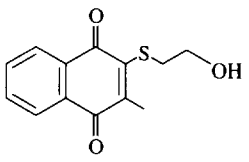
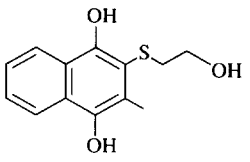
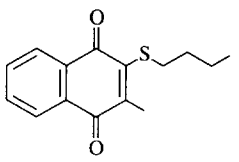
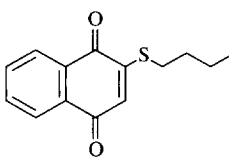
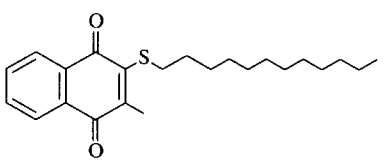
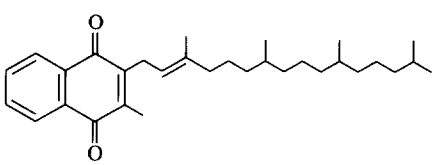
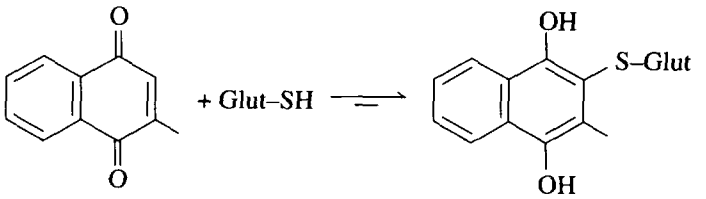
TABLE 1

Activity of Vitamin K_3 Congeners in Growth Inhibition of Hep3B Cells[a]

Compound	Activity ID_{50} (μM)
 **1**	8
 **3**	5.6[b]
 **4**	3
 **5**	15[b]
 **6**	5
 **7**	188
 **8**	>666

(*Continues*)

It has been reported that vitamin K_3 **(1)** depletes cellular pools of glutathione (*3*). This can be interpreted to mean that vitamin K_3 **(1)** forms an adduct **2** with glutathione by conjugate addition of the thiol to the quinone (Eq. [1]). This would be

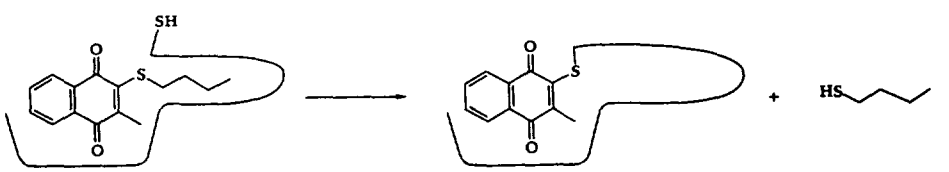


[1]

1 **2**

consistent with the propensity of quinones to undergo addition of thiols (*4*). Indeed, the glutathione adduct **2** of vitamin K has been reported in the literature (*6*). This attribute of vitamin K_3 **(1)** also provides the means to explain its capacity to inhibit the $p34^{cdc2}$ kinase. We suggest, as a working hypothesis, that vitamin K_3 **(1)** forms an adduct with the thiol group of the active-site cysteine (*5*) of the protein tyrosine phosphatase, thereby blocking hydrolysis of the Tyr-15 phosphate required for activation of the $p34^{cdc2}$ kinase.

To explore this idea, we have examined a variety of substituted naphthoquinones as growth inhibitors of HepG2 and Hep3B cell lines. The results for the Hep3B experiments are shown in Table 1. We can summarize by noting that the thionaphthoquinones with the relatively short side chains of 4–5 atoms tend to be more active than those with longer side chains, such as those that characterize **7** and vitamin K_1 **(8)**. The thioethanol derivatives **3** and **4** are noteworthy; they are somewhat more active than vitamin K_3 **(1)** and may benefit from having a hydrophilic group on the sidechain. Presumably the hydroquinone **4** is activated by air oxidation to the quinone. To accommodate these data, we suggest, as part of our hypothesis, that the protein tyrosine phosphatase may have limited space to bind the side chain, as shown in Scheme 1.

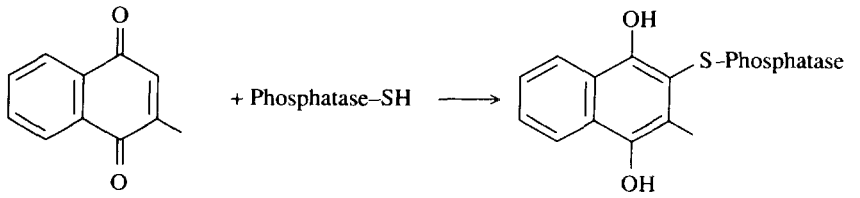


SCHEME 1

[a] The HEP 3B cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD). Hep 3B cells were cultured in MEM medium (GIBCO BRL Co., Grand Island, NY) supplemented with 10% fetal bovine serum. For measurement of cell growth, Hep 3B cells were plated in 35-mm tissue culture dishes at a concentration of 5×10^4 cells/dish. After cells attached to the dishes, fresh medium containing different concentrations of the analog to be tested was added and subsequently changed at Day 3 after attachment. Ethanol was used to dissolve the water insoluble vitamins K_1 and K_2. Ethanol was also added as a vehicle to control cells. Cells were counted at Days 3 and 6 using a hemocytometer.

[b] Prepared according to the procedure in Ref. 4c.

Following this scheme, it is likely that vitamin K_3 **(1)** acts by an addition mechanism (Eqs. [1] and [2]), while the quinone sulfides **3–7** involve an addition–elimination exchange with enzyme-bound thiol (Eq. [3]). The transformation in Eq. [2] is expected to be quite exothermic.



[2]

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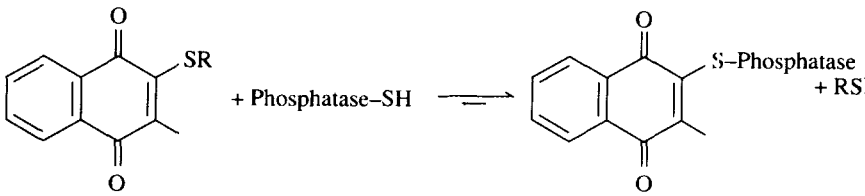
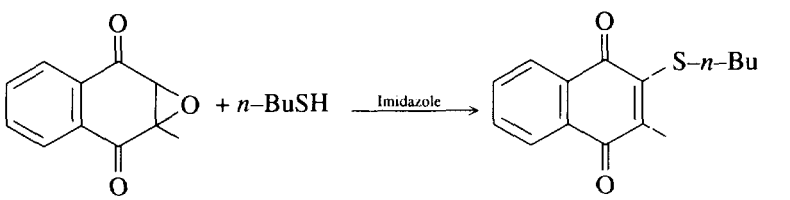


[3]

Vitamin K_3 Oxide

A likely metabolite of vitamin K_3 **(1)** or vitamin K_3H_2 is the corresponding vitamin K_3 oxide **(9).** As a consequence of the release of ring strain of the epoxide group and the subsequent elimination of water, thiol substitution at the epoxide (Eq. [5]) will have a substantial driving force. The addition of thiol to the epoxide is illustrated by the reaction between *n*-butanethiol and menadione oxide **(9),** which yields quantitative ring-opening substitution as indicated in Eq. [4].



[4]

100%

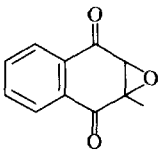
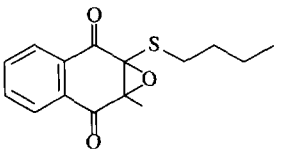
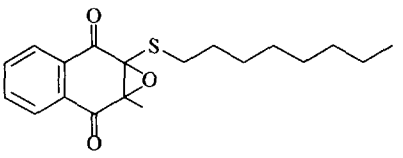
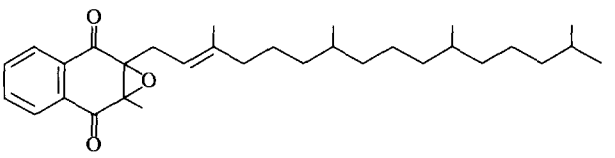
9 **5**

The consequences of using the epoxides as growth inhibitors are shown in Table 2, where vitamin K_3 oxide **(9)** is observed to be as potent as vitamin K_3 in Hep3B cell growth inhibition.

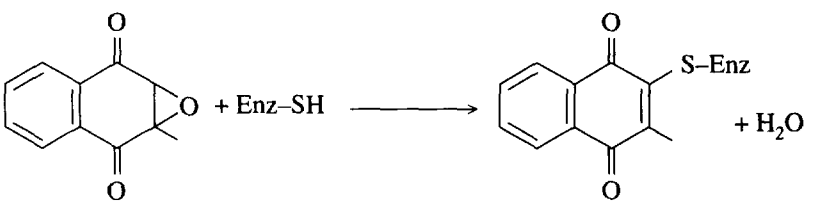
Protein tyrosine phosphatase inhibition has been considered in terms of quinone oxidation–reduction chemistry. The present work differentiates a redox path from that suggested in Eq. [2] by examining the activities of several vitamin K oxides. That vitamin K_3 oxide **(9)** is as active as vitamin K_3 supports a substitution mechanism hypothesis, the epoxide group functioning as an irreversible alkylating agent for the protein tyrosine phosphatase active site thiol (5) (Eq. [5]).

TABLE 2

Vitamin K Oxide Inhibition of Hep3B Cell Growth[a]

Compound	Activity ID_{50} (μM)
 **9** Vitamin K_3 Oxide	11
 **10**	29
 **11**	38
 **12** Vitamin K_1 Oxide	>666

[a] The same procedure was used in Table 2 as that described in the footnote to Table 1.



[5]

9

SUMMARY

This study demonstrates that a variety of thioalkyl and epoxide derivatives of vitamin K_3 will be of use in exploring the mechanism of hepatoma cell growth inhibition of vitamin K_3 **(1)**.

EXPERIMENTAL

All reactions involving moisture sensitive materials were conducted in flame-dried glassware, under argon. Ether was distilled from sodium-benzophenone ketyl, under nitrogen. All reagents from commercial sources were used without further purification, unless otherwise indicated. Melting points were recorded on a Mel-Temp apparatus and are uncorrected.

NMR spectra were recorded on Bruker AC300 or AF300 spectrometers operating at 300 MHz for proton and 75 MHz for carbon. The ^1H NMR spectra are referenced with respect to the residual proton of the solvent $CDCl_3$ at 7.27 ppm. The ^{13}C NMR spectra are referenced with respect to the middle peak of the solvent $CDCl_3$ at 77.0 ppm. Infrared spectra were recorded on IBM IR-32 Fourier transform spectrophotometer. UV spectra were obtained on a Hewlett-Packard 8451A Diode Array Spectrometer. High and low resolution mass spectra were obtained on a Varian MAT CH-5DF, VG70-SE, or VG Auto Spec.

2-(2-Mercaptoethanol)-3-methyl-1,4-naphthoquinone **(3).** 2-Mercaptoethanol (0.87 ml, 12.4 mmol) was added to a solution of vitamin K_3 oxide **(9)** (2.126 g, 11.3 mmol) and imidazole (0.841 g, 12.4 mmol) in 60 ml of ethanol at 35–40°C. After 10–15 s the colorless solution turned yellow. After 3.5 h the heat was removed and 15 ml of 3.6 M HCl was added to the solution followed by 75 ml of water. The resulting solution was extracted four times with 100 ml of ether. The combined organics were concentrated to a liquid which upon addition of 200 ml of water gave 2.370 g (85%) of a yellow solid, mp 78–79°C. ^1H NMR ($CDCl_3$) δ 8.08–0.03 (m, 2H), 7.71–7.68 (m, 2H), 3.80 (t, J = 5.7, 2H), 3.34 (t, J = 5.8, 2H), 2.50 (s, 1H), 2.38 (s, 3H). ^{13}C NMR ($CDCl_3$) 182.10, 181.42, 147.85, 145.87, 133.74, 133.41, 132.61, 131.86, 128.78, 126.55, 62.02, 37.11, 15.40. IR (KBr) cm^{-1} 3292 (m), 1657 (s), 1585 (s), 1554 (s). UV (ethanol) λ_{max} (log ε) 204 (4.21), 260 (4.22), 408 (3.33). MS (m/z): 248 (2), 230 (63), 221 (100), 197 (73). High resolution MS: Calcd for $C_{13}H_{10}O_2S$, 230.0412; found, 230.0405.

2-(2-Mercaptoethanol)-3-methyl-1,4-naphthohydroquinone **(4).** Zinc powder (0.799 g, 12.2 mmol) was added all at once to a solution of **3** (0.151 g, 0.61 mmol) and acetic acid (0.35 ml, 6.1 mmol) in ether (3 ml) at room temperature. The resulting suspension was stirred vigorously and turned colorless after 5 min. The suspension was then filtered through celite under vacuum using a cannula. Additional degassed ether was used to ensure total transfer. The filtrate was washed with three 5-ml portions of degassed water and the solvent removed under a stream of Ar to give 0.166 g of an oxygen-sensitive brown solid. This material was washed with degassed chloroform (2 × 0.25 ml) to give 0.144 g (94%) of a tan solid. ^1H

NMR (degassed $CDCl_3$) δ 8.23–8.06 (m, 2H), 7.58–7.48 (m, 3H), 4.76 (s, 1H), 3.68 (t, J = 6.0, 2H), 2.84 (t, J = 5.9, 2H), 2.61 (s, 3H) 1.8 (br s, 1H).

2-(1-Thiobutyl)-1,4-naphthoquinone (**6**). 1-Butanethiol (1.25 ml, 11.7 mmol) was added dropwise over 5 min to a degassed solution of 1,4-naphthoquinone (1.837 g, 11.6 mmol), and triethylamine (1.62 g, 11.6 mmol) in THF (30 ml) at room temperature. Stirring was maintained for 15–20 h, after which the reaction was quenched with 20 ml of a 3.6 M HCl solution. The organic and aqueous layers were separated and the aqueous layer was extracted with two 45-ml portions of ether. The combined organic layers were dried over $MgSO_4$, filtered, and the filtrate concentrated to give 3.969 g of a dark viscous liquid. Purification by flash chromatography using successive portions of 3, 5, and 10% ethyl acetate/hexanes afforded 0.676 g (24%) of a bright yellow solid, mp 99–100°C. ^1H NMR ($CDCl_3$) δ 7.98–7.92 (m, 2H), 7.67–7.57 (m, 2H), 6.49 (s, 1H), 2.74 (t, J = 7.4, 2H), 1.72–1.62 (m, 2H), 1.51–1.39 (m, 2H), 0.91 (t, J = 7.3, 2H). ^{13}C NMR ($CDCl_3$) 181.77, 181.16, 155.05, 134.09, 133.02, 131.92, 131.63, 126.77, 126.58, 126.26, 30.19, 29.18, 22.09, 13.49. IR (KBr) cm^{-1} 1668 (s), 1643 (s), 1585 (s), 1552 (s). UV (ethanol) λ_{max} (log ε) 258 (4.22), 300 (3.68), 412 (3.25). MS (*m/z*): 246 (100), 213 (50), 190 (65), 162 (65). High resolution MS: Calcd for $C_{14}H_{14}O_2S$, 246.0714; found, 246.0735.

2-Methyl-3-(1-thiododecyl)-1,4-napthoquinone (**7**). A yellow solid was obtained following the procedure above, mp 73.5–74°C. ^1H NMR ($CDCl_3$) δ 8.09–8.05 (m, 2H), 7.70–7.68 (m, 2H). 3.20 (t, J = 7.3, 2H), 2.35 (s, 3H), 1.65–1.24 (m, 23H), 0.87 (t, J = 6.1, 2H). ^{13}C ($CDCl_3$) 182.26, 181.38, 147.29, 146.55, 133.64, 133.31, 132.96, 132.14, 126.77, 126.59, 34.48, 31.97, 30.71, 29.70, 29.54, 29.41, 29.17, 28.70, 22.75, 15.30, 14.21. IR (KBr) 1657 (s), 1579 (m), 1551 (m), 1273 (s). UV (ethanol) λ_{max} (log ε) 204 (4.34), 238 (4.19), 262 (4.30), 314 (3.63), 424 (3.46). MS (*m/z*): 372 (100), 357 (7), 339 (22), 204 (90). High resolution MS: Calcd for $C_{23}H_{32}O_2S$, 372.2123; found, 372.2173.

2,3-Epoxy-2-methyl-3-(1-thiobutyl)-1,4-naphthoquinone (**10**). A solution of Na_2CO_3 (0.943 g, 8.90 mmol) and 30% H_2O_2 (1.0 ml 9.79 mmol) in water (6 ml) was added all at once to a solution of **5** (0.543 g, 2.09 mmol) in ethanol (35 ml) at room temperature. Upon addition a precipitate formed and after several minutes the solution turned pink. After 1 h the mixture was poured into 100 ml of water and extracted with two 100-ml portions of ether. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated to give 0.531 g of a clear, slightly yellow oil. Purification by flash chromatography at 0°C using 5% ethyl acetate-hexanes gave 0.436 g (76%) of a clear slightly yellow oil. ^1H NMR ($CDCl_3$) δ 7.88–7.78 (m, 2H), 7.65–7.59 (m, 2H), 2.90 (t, J = 7.4, 2H), 1.81 (s, 3H), 1.63–1.53 (m, 2H), 1.43–1.31 (m, 2H), 0.85 (t, J = 7.3, 3H). ^{13}C NMR ($CDCl_3$) 191.53, 188.47, 134.15, 131.72, 131.44, 127.46, 126.94, 73.82, 67.29, 31.86, 30.83, 21.84, 13.52, 12.62. IR (neat) cm^{-1} 1701 (s), 1595 (m). UV (ethanol) λ_{max} (log ε) 228 (4.30), 306 (3.24). MS (*m/z*) 276 (2), 243 (3), 234 (25), 178 (28), 104 (8), 84 (100). High resolution MS: Calcd for $C_{13}H_{14}O_2S$, 234.0715; found, 234.0719.

2,3-Epoxy-2-methyl-3-(1-thiooctyl)-1,4-naphthoquinone (**11**). A solution of Na_2CO_3 (0.923 g, 8.71 mmol) and 30% H_2O_2 (1 ml, 9.79 mmol) in water (6 ml) was

added all at once to a solution of 2-methyl-3-(1-thiooctyl)-1,4,naphthoquinone (0.553 g, 1.75 mmol) in ethanol (25 ml) at room temperature. Upon addition a precipitate formed and after approximately 30 s the solution turned pink. After 1 h the mixture was poured into 50 ml water and extracted with two 50-ml portions of ether. The combined organic layers were dried over $MgSO_4$, filtered, and the filtrate concentrated to give 0.451 g of a yellow oil. Purification by flash chromatography using 5% ethyl acetate-hexanes gave 0.352 g (61%) of a light yellow solid, mp 40–42°C. ^1H NMR ($CDCl_3$) δ 8.03–7.93 (m, 2H), 7.76–7.70 (m, 2H), 2.98 (t, J = 7.2, 2H), 1.92 (s, 3H), 1.73–163 (m, 2H), 1.44–1.27 (m, 10H), 0.87 (t, J = 6.9, 3H). ^{13}C NMR ($CDCl_3$) 191.87, 188.79, 134.41, 131.93, 131.66, 127.72, 127.20, 74.02, 67.51, 31.83, 31.39, 30.02, 29.15, 28.89, 22.70, 14.71, 12.81. IR (KBr) cm^{-1} 1691 (s), 1581 (m), 1271 (s). UV (ethanol) λ_{max} (log ε) 228 (4.31), 306 (3.19). MS (m/z) 332 (2.9), 316 (3.2), 290 (74), 178 (100). High resolution MS: Calcd for $C_{17}H_{22}O_2S$, 290.1341; found, 290.1317.

ACKNOWLEDGMENTS

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