

Vitamin K2-derived Compounds Induce Growth Inhibition in Radioresistant Cancer Cells

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A strategy to overcome radioresistance in cancer treatment has been expected. To evaluate the strategy, appropriate experimental models are needed. Radioresistant tumour models were originally established from human colon cancer cells, and we evaluated their molecular basis. Next, the growth inhibitory effects of newly synthesized vitamin K2 (VK2)-related compounds were tested. Here, we showed that these novel compounds have growth inhibitory effects not only on cancer cells of various origins, but also on radioresistant cells, through the generation of reactive oxygen species (ROS).

Human colon, lung, and breast cancer cell lines were used for testing the growth inhibitory activities of several chemical compounds. Radioresistant tumour models were established by fractionated radiation exposure. Irradiated cells were selected by a single cell cloning method, and their sensitivity to ionizing radiation was evaluated by a colony-forming assay. The VK2 derivatives (named MQ-1, MQ-2, and MQ-3) were chemically synthesized. To evaluate the generation of ROS, flow cytometer analyses were performed.

A radioresistant tumour model was established from the HCT116 human colon cancer cell line. The radioresistant cells from HCT116 also showed resistance to cisplatin. In the radioresistant cells, NF- κ B was highly activated. MQ-1, MQ-2, and MQ-3 showed greater growth inhibitory activities than VK2 not only in various cancer cells but also in radioresistant cells through the generation of ROS.

In conclusion, a radioresistant tumour model was originally established from colon cancer cell lines through NF- κ B activation, and it could be a useful tool for evaluating anti-tumour agents. Newly synthesized VK2 derivatives (MQ-1, MQ-2 and MQ-3) seemed to be potential anti-tumour agents in various cancers and radioresistant cancers. The efficacy of those compounds was related to the generation of ROS. These findings together might pave the way for the treatment of radioresistant or recurrent cancers.

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Vitamin K is a generic term for certain derivatives of 2-methyl-1,4 naphthoquinone, which was discovered in 1929 by Henrik Dam as a fat-soluble anti-hemorrhagic agent (5). There are two naturally occurring forms of vitamin K. Phylloquinone, also known as vitamin K1 (VK1), is found in higher plants. The menaquinone series are collectively referred to as vitamin K2 (VK2) and are synthesized by bacteria. Menadione (vitamin K3: VK3) is a synthetic derivative of beta-naphthalene (4-5, 12-13, 19, 28). Compounds with a quinone structure are reported to play a prominent role in cancer chemotherapy (5). VK2 is known to have a marginally active growth inhibitory activity, whereas VK3 is a distinct inhibitor of cell growth, both in vitro and in vivo (9, 12, 14, 24, 38-41, 44-45).

Although several reports suggested that vitamin Ks might induce cell cycle arrest or cell death, their mechanisms of growth inhibition remain largely undetermined (5, 12, 19). There are several reports concerning vitamin K and its analogues showing that the growth inhibitory activity decreased with increasing side-chain length (28). VK3 has the shortest side-chain and is reported to show a distinct cytotoxicity that is equal or superior to a number of standard antitumor agents. However, VK3 induced severe adverse effects in vivo, including haemolytic anaemia; consequently, VK3 was left out of mainstay cancer treatment regimens (5-6, 22, 25-26). VK2 is less toxic and has been reported to induce apoptosis or autophagy in certain cancer cell lines (9, 12, 14, 24, 38-41, 44-45). Although VK3 was believed to combat oxidative stress via redox cycling of the quinone to produce reactive oxygen species (12, 19, 38), it has not been clarified that the cytotoxic action of VK2 was due to the same mechanism as that of VK3.

In our previous report, we demonstrated that VK3 showed excellent cytotoxicity through the generation of reactive oxygen species (ROS) by affecting mitochondria (36). In the study, VK2 showed weaker but apparent cytotoxicity in certain cell lines, and Shibayama also reported similar results in different cancer cell lines (39). We further hypothesized that compounds that shortened the side chain of VK2 might confer both advantages of the growth inhibitory effect of VK3 against cancerous cells and a lesser toxicity of VK2 to normal cells and that those VK-2 related compounds might function as anti-tumour agents. Compounds were originally generated from VK2, and we tested those growth inhibitory activities. Here, we showed that those novel compounds have growth inhibitory effects not only on cancer cells of various origins, but also on radioresistant clones, through the generation of ROS.

MATERIALS AND METHODS

1. Cell lines and chemicals

HCT116 human colon carcinoma cell lines with wild-type p53 (HCT116 p53+/+) and their isogenic derivatives that lack p53 (HCT116 p53-/-) were a kind gift from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore). The cells were maintained in McCoy's 5A medium containing 10% foetal bovine serum (FBS) or in McCoy's 5A-based enriched medium with 10% FBS, 2 mM sodium pyruvate, 50 µg/ml uridine added to the normal medium at 37°C with 5% CO₂. H1299 and MCF-7 were obtained from ATCC and cultured in RPMI-1640 and DMEM medium. VK1, VK2, VK3, doxorubicine, gemcitabine, taxol, TNF- α and N-acetylcysteine (NAC) were purchased from Sigma (St. Louis, MO, USA). Dihydroethidine (HE) and 5-carboxy-2',7'-dichlorodihydrofluorescein diacetate (c-DCF) were obtained from Molecular Probes (Eugene, OR, USA). Aminophenyl fluorescein (APF) was purchased from Sekisui Medical (Tokyo, Japan) (37). All other reagents were purchased from Nakalai Tesque Ltd. (Kyoto, Japan)

2. Preparation of vitamin K2 derivatives (MQ-1, MQ-2, MQ-3)

The VK2 derivatives (named MQ-1, MQ-2, and MQ-3) were chemically synthesized according to the procedure reported by Mayer and Isler with minor modifications (23). The structures of these compounds were confirmed by comparison of the spectral data with those reported. All reactions were monitored by thin liquid chromatography (TLC), which was carried out on Silica Gel 60 F₂₅₄ plates (Merck, Germany). Flash chromatography separations were performed on PSQ 100B (Fuji Silysia Co., Ltd., Japan). ¹H and ¹³C NMR spectra were recorded on a JEOL 270 MHz spectrometer (EX-270W), using CDCl₃ (with TMS for ¹H NMR and chloroform-d for ¹³C NMR as the internal reference) solution unless otherwise noted. Chemical shifts were expressed in δ (ppm) relative to Me₄Si or residual solvent resonance, and coupling constants (J) were expressed in Hz. Melting points were determined with a Yanaco MP-3S and were uncorrected. Infrared (IR) spectra were recorded on a Jasco FT/IR-410 spectrometer using NaCl (neat) or KBr pellets (solid) and were reported as wavenumbers (cm⁻¹). Mass spectra (MS) were obtained on an Applied Biosystems mass spectrometer (APIQSTAR pulsar i) under high-resolution conditions, using polyethylene glycol as an internal standard.

3. Analyses of cellular superoxide, hydrogen peroxide, and the hydroxyl radical

Cellular superoxide and hydrogen peroxide were measured by flow cytometer analyses using HE and c-DCF (33, 36). HE was dissolved in DMSO (100 mg/ml stock) and further diluted with PBS at 1:10,000. The diluted dye was added to the cell culture at a final concentration of 50 ng/ml and incubated at 37°C during the last 60 min. c-DCF was also dissolved in DMSO (20 mM stock) and used for staining at 50 μM at 37°C for 60 min. Cellular hydroxyl radical were also measured by flow cytometer according to the manufacturer's instructions (37).

4. Electrophoretic mobility shift assay (EMSA) for NF-κB

Nuclear extracts were prepared, and EMSAs were performed essentially as described previously (2). Binding reaction mixtures (20 μl) containing 5 μg protein of nuclear extract, 2 μg poly(dI-dC) (Pharmacia, Sweden), ³²P-labeled NF-κB p65 probe (Santa Cruz, CA, USA), 50 mM NaCl, 2 mM MgCl₂, 0.2 mM Na₂EDTA, 1 mM dithiothreitol (DTT), 10% (v/v) glycerol and 4 mM Tris-HCl (pH 7.9) were incubated for 30 min at room temperature. Proteins were separated by electrophoresis in a native 6% polyacrylamide gel using a Tris-borate-EDTA running buffer (12.5 mM Tris-borate containing 0.25 mM Na₂EDTA, pH 8.0) followed by autoradiography.

5. Western blot analysis

Total cell lysates were prepared and separated by electrophoresis on 8-12% SDS-PAGE, essentially as described previously (1). Antibodies for NF-κB p65 (Santa Cruz, CA, USA) and β-actin (Sigma) were used. The primary antibodies were diluted at 1:1,000-1:5,000 and detected using appropriate horseradish peroxidase-conjugated secondary antibodies, followed by detection with a SuperSignal enhanced chemiluminescence kit (Pierce, Rockford, IL, USA). Immune complexes were detected by chemiluminescence and then by fluorescence using a LAS3000 mini lumino image-analyzer (FUJI FILM, Tokyo, Japan). The density of each band was quantitatively evaluated using Multi Gauge Version 3.0 (FUJI FILM).

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6. Cytotoxicity (MTT) assay and long-term survival (colony-forming) assay

Cytotoxicity was determined by a 3-(4,5-dimethyl thiazol-2)-2,5-diphenyltetrazolium bromide (MTT) assay (72 h), as described previously (36). For a longer-term survival analysis, a colony-forming assay was performed. Cells were incubated for 10-14 days. Fixation and staining were according to previously described methods (8, 34-35).

RESULTS

1. Establishment of radioresistant cells from human colon cancer cell lines.

The first objective of our experimental protocol was to establish an in vitro model that would mimic our clinical observation in terms of the variable response to ionizing radiation. We used two kinds of HCT116 cells: one that is p53 wild type (HCT116 p53+/+) and the other that is p53-deficient (HCT116 p53-/-). After exposure to fractionated irradiation, surviving cells were tested as to whether they could grow by themselves and form a colony (single cell cloning method). Several colonies were isolated as candidates for radioresistant clones. As a result, irradiated clones HCT116 p53+/+ were more resistant than the parental cells, whereas irradiated clones from HCT116 p53-/- showed similar sensitivities to those of their parental cells (Figure 1). Next, the sensitivities of those radioresistant clones to anti-tumour agents were tested (Table I). As for cisplatin, resistant clones of HCT116 p53+/+ showed more than 10-fold greater resistance than their parental cells and resistant clones of HCT116 p53-/- showed 3-fold greater resistance, whereas those clones had similar sensitivities to doxorubicin, gemcitabine, and taxol as their parental cells.

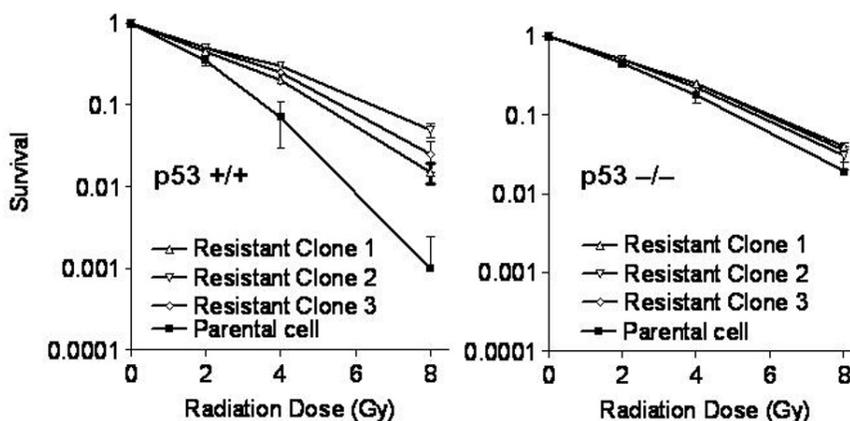


Figure 1. Establishment of radioresistant clones from HCT116 human colon cancer cells. Error bars from 3 independent experiments.

Table I. Comparison of drug sensitivities between HCT116 and radioresistant cells.

	IC ₅₀ (μM)			
	HCT116 p53+/+		HCT116 p53-/-	
	WT	Resistant	KO	Resistant
Doxorubicine	0.05	0.15	0.12	0.15
Cisplatin	1.5	18	3.5	10.5
Gemcitabine	0.008	0.012	0.015	0.015
Taxol	0.005	0.015	0.007	0.008

Footnotes: WT=wild type, KO=knockout

2. NF- κ B activation in radioresistant cells

Characteristics of HCT116 parental cells and their radioresistant clones were investigated, especially in terms of the relationship to NF- κ B activation. First, the binding activity of parental cells of HCT116 p53^{+/+} and of HCT116 p53^{-/-} and their resistant clones were tested. Resistant clones of HCT116 p53^{+/+} showed higher binding activity compared to the parental cells (Figure 2A). In contrast, the parental HCT116 p53^{-/-} and their resistant clones had slightly higher binding than the HCT116 p53^{+/+} cells but similar to HCT116 p53^{+/+} resistant clones. Next, responses to ionizing radiation in HCT116 p53^{+/+} parental and resistant clones were tested by western blotting (Figure 2B). The amount of NF- κ B increased in response to the irradiation in the parental cells. The amounts of activated NF- κ B in HCT116 p53^{+/+} resistant clones were higher than those in the parental cells (Figure 2B).

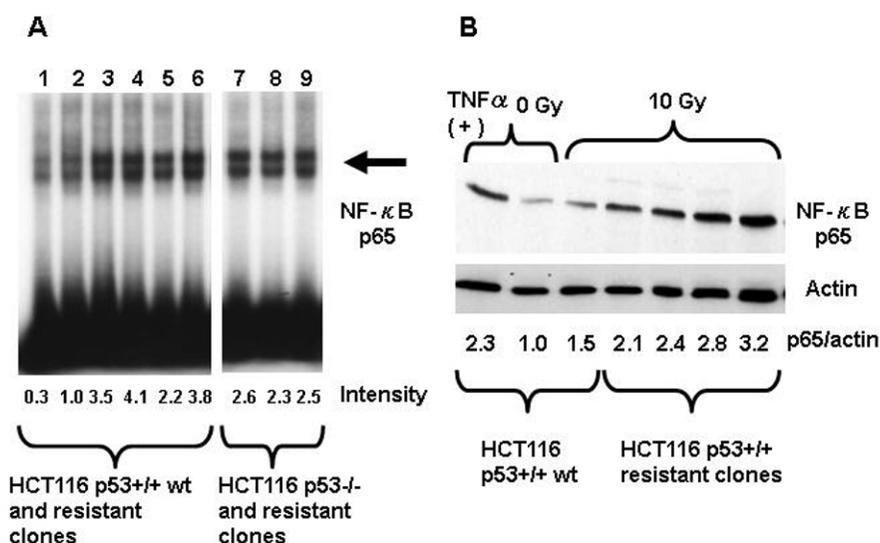


Figure 2. Radioresistance acquired through NF- κ B activation. A. EMSA assay for NF- κ B (Lane 1: cold probe, L2: HCT116 p53^{+/+}, L3-L6: HCT116 p53^{+/+} resistant clones, L7: HCT116 p53^{-/-}, L8-L9: HCT116 p53^{-/-} resistant clones, B. Western blotting analysis for NF- κ B with or without 10 Gy of irradiation (TNF α for positive control). Both EMSA and western blotting assays were performed at least twice and a representative datum was shown.

3. Generation of vitamin K2 derivatives

Three VK2 derivatives were chemically synthesized according to the procedure shown in the Methods and Materials. Three derivatives were established, named MQ-1, MQ-2, and MQ-3, which all shared a methylated naphthoquinone ring structure and were varied in the isoprenoid side chains. The molecular weights of MQ-1, MQ-2, and MQ-3 were 240.3, 308.4, and 376.5, respectively (Figure 3).

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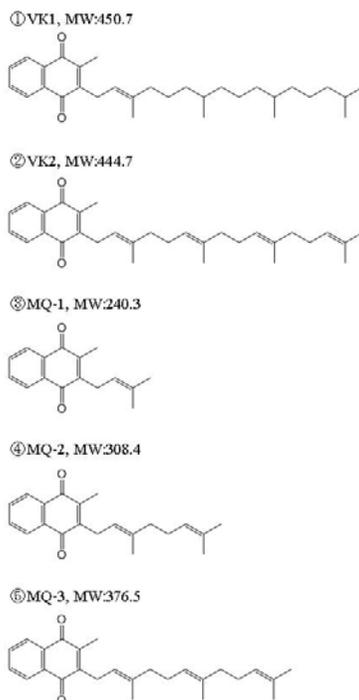


Figure 3. Structures and molecular weights of vitamin K1, K2, and VK2 derivatives (MQ-1, MQ-2, and MQ-3).

4. Growth inhibitory effects of MQ-1, MQ-2, and MQ-3, in various cancer cells

Growth inhibitory activities of MQ-1, MQ-2, and MQ-3 were assessed in various human cancer cells in comparison with that of VK2. All of those derivatives obtained greater growth inhibitory activities than VK2 in HCT116 human colon cancer cells, H1299 human lung cancer cells, and MCF-7 human breast cancer cells (Figure 4); therefore, the length of isoprenoid residue of VK2 must be important for the activity. Surprisingly, radioresistant clones of HCT116 showed similar growth inhibitory effects to those derivatives as HCT116 wt did (Table II). This result suggested that those derivatives could be promising compounds against radioresistant tumours. In addition, those growth inhibitory activities of MQ-1, MQ-2, and MQ-3 to HCT116, H1299, and MCF-7 cells were much weaker than that of VK3 (data not shown). Therefore, those derivatives showed intermediate growth inhibitory activities between those of VK2 and VK3.

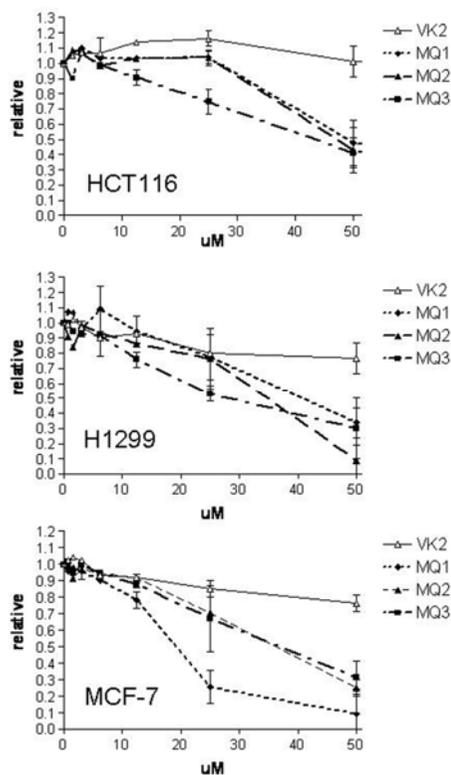


Figure 4. Comparison of growth activities between VK2 and VK2 derivatives in various cancer cells. Error bars from 3 independent experiments.

Table II. Comparison of the growth inhibitory activities of vitamin K1, K2, and VK2 derivatives.

Cell Type	Tissue origin	IC50 (μM)				
		VK1	VK2	MQ1	MQ2	MQ3
HCT116 wt	Colon	>100	>100	50	46	42
HCT116 Resistant clone	Colon	>100	>100	52	35	40
H1299	Lung	>100	>100	40	33	29
MCF-7	Breast	>100	>100	20	38	40

5. Reactive oxygen species (ROS) generation by vitamin K2 derivatives in HCT116 cells

Because the mechanisms of cytotoxicity of VK3 were determined to occur through reactive oxygen species generation in the previous study (36), the actions of MQ-1, MQ-2, and MQ-3 for free radical generation were investigated. These compounds induced greater amounts of superoxide, hydrogen peroxide, and hydroxyl radicals compared to VK2 in HCT116 cells (Figure 5). Next, free radical generation was tested in radioresistant clones of HCT116. The induction of superoxide, hydrogen peroxide, and hydroxyl radicals by

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superoxide, hydrogen peroxide, and hydroxyl radical in the radioresistant clones was almost similar to that in HCT116 cells (Figure 5). To clarify whether the free radical generation affected the cytotoxicities of MQ-1, MQ-2, and MQ-3, a ROS scavenger, N-acetylcysteine (NAC), was tested. With 5 mM of NAC administration, the IC_{50} of those VK2 derivatives in HCT116 cells were decreased (50 μ M by MQ-1 only versus 100 μ M by MQ-1 and NAC; 46 μ M by MQ-2 only versus 85 μ M by MQ-2 and NAC; 42 μ M by MQ-3 only versus 56 μ M by MQ-3 and NAC). These data indicated that the cytotoxicities of VK2 derivatives proceeded through the generation of ROS.

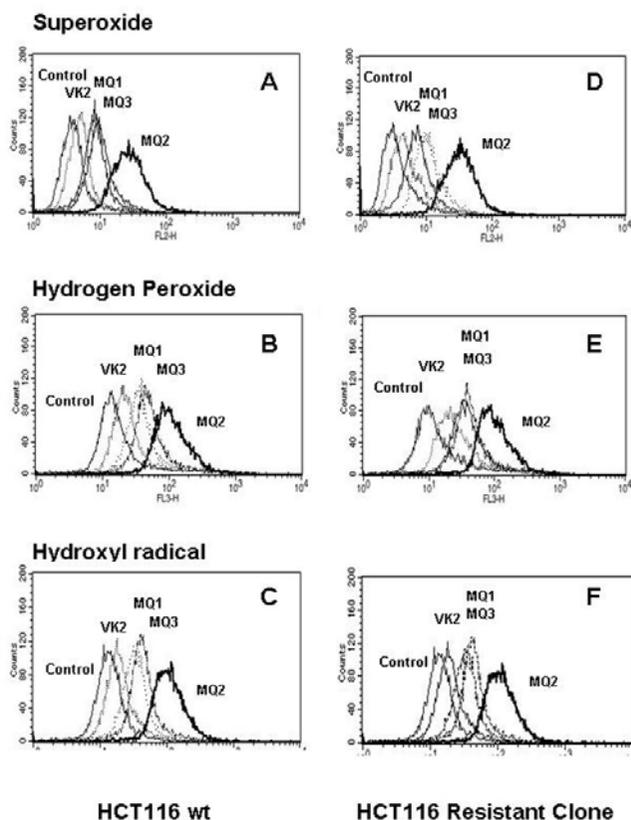


Figure 5. Generation of reactive oxygen species induced by VK2 and VK2 derivatives in HCT116 and radioresistant cells.

DISCUSSION

Although many factors have been reported to be candidates for determining cellular radioresistance, how cancer cells obtain radioresistance is largely unknown (29). The p53 protein has been intensively assessed in this regard. It has been reported that p53 mutations in several cancer cells were associated with increased radioresistance because of the failure to induce apoptosis following radiation (3, 7, 21, 35). Our data using the human colon cancer cell HCT116 and the counterpart of p53-deficient HCT116 cells strongly supported the notion that p53 was a major determinant of radioresistance. However, in the process of establishing radioresistant models, only wild-type HCT116 became resistant to ionizing radiation, and normal p53 function was retained. Therefore, we tested an alternative major

determinant of radioresistance, NF- κ B. NF- κ B activity has been widely reported to be linked to chemoresistance in multiple tumours (31), as well as with radioresistance in experimental models (17-18, 20, 27, 42-43). We first reported in the clinical setting that NF- κ B was the strongest factor predicting radioresistance in early stage laryngeal squamous cell carcinomas and that more than 90% of recurrent laryngeal cancer after curative radiotherapy showed highly activated NF- κ B (46). In this study of colon adenocarcinoma, NF- κ B was increased in the process of obtaining radioresistance in wild-type HCT116 cells and was already activated in p53-deficient cells. Although both p53 and NF- κ B are important transcriptional factors for various genes (10, 18, 27, 29), NF- κ B might affect the acquisition of radioresistance in the situation where p53 is normal.

Several investigators reported that vitamin K analogues have growth inhibitory effects, especially on hepatoma cells and hematopoietic or myeloma cells (12, 24, 28, 41, 44). Carr and co-workers investigated the growth inhibitory effects of their originally synthesized vitamin K-related compounds (28). Although they synthesized several vitamin K analogues of side chains with a sulphur (thioether) or oxygen atom (*O*-ether) at the site of attachment of the side chain to the ring, the potency to induce apoptosis in hepatoma cells was correlated with the decreasing length of the side chain. In contrast, Shibayama and co-workers reported that VK2 induced apoptosis through the generation of ROS in ovarian cancer cells (39). Ozaki and co-workers reported that vitamin K2 inhibited hepatoma cell proliferation by regulating cyclin D1 expression through the inhibition of NF- κ B activation (31). In this study, we originally synthesized a VK analogue from VK2, and those compounds had cytotoxic effects not only in various cancer cells but also in radioresistant cells. These seemed to be novel findings, and these observations might pave the way in the novel strategy against recurrent cancer after radiotherapy.

Although acquired tumour radioresistance during radiotherapy is believed to be due to tumour repopulation (15), the exact molecular mechanisms underlying the radioadaptive response are largely unknown. NF- κ B is reported to play a central role in the response through multiple pathways (16). In addition, Orlowski demonstrated that elevated basal NF- κ B activity in certain cancers has been linked with tumour resistance to chemotherapy and radiation (30), and the established radioresistant cells in this study were consistent with this tendency. Although not all experiments show an enhanced radiosensitivity due to NF- κ B inhibition (11, 32), NF- κ B seems to be a key molecule to overcome radioresistance. It seems quite promising for a future strategy that VK-2 derivatives showed a growth inhibitory effect in radioresistant clones.

In conclusion, a radioresistant tumour model was originally established from colon cancer cell lines through NF- κ B activation, and it could be a useful tool for evaluating anti-tumour agents. Newly synthesized VK2 derivatives (MQ-1, MQ-2 and MQ-3) seemed to be potential anti-tumour agents in various cancers and radioresistant cancers. The efficacy of those compounds was related to the generation of reactive oxygen species. These findings together might pave the way for the treatment of radioresistant or recurrent cancers.

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