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DNA Polymerase Gamma Inhibition by Vitamin K3 Induced Mitochondria-mediated Cytotoxicity in Human Cancer Cells

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Summary

Among the vitamin K compounds, vitamin K3 (VK3) exhibits distinct cytotoxic activity in cancer cells and is thought to affect redox cycling; however, the underlying mechanisms remain unclear. Here we demonstrated that VK3 selectively inhibited DNA polymerase gamma (pol γ), the key enzyme responsible for mitochondrial DNA (mtDNA) replication and repair. VK3 at 30 μM inhibited pol γ by more than 80%, caused impairment of mtDNA replication and repair, and induced a significant increase of reactive oxygen species (ROS), leading to apoptosis. At a lower concentration (3 μM), VK3 did not cause a significant increase of ROS, but was able to effectively inhibit cell proliferation, which could be reversed by supplementing glycolytic substrates. The cytotoxic action of VK3 was independent of p53 tumor suppressor gene status. Interestingly, VK3 only inhibited pol γ but did not affect other DNA polymerases including human pol α, pol β, pol δ, and pol ε. Vitamins K1 and K2 exhibited no inhibitory effect on any of the DNA polymerases tested. These data together suggest that the inhibition of pol γ by VK3 is relatively specific, and that this compound seems to exert its anticancer activity by two possible mechanisms in a concentration-dependent manner: (1) induction of ROS-mediated cell death at high

concentrations and (2) inhibition of cell proliferation at lower concentrations likely through the suppression of mitochondrial respiratory function. These findings may explain various cytotoxic actions induced by VK3, and may pave the way for the further use of VK3.

Keyword: Vitamin K3, Colorectal Cancer, DNA polymerase gamma, Mitochondria, Superoxide,

Abbreviation: nDNA, nuclear DNA; mtDNA, mitochondrial DNA; VK, vitamin K; HIV, human immunodeficiency virus; HE, PBS, phosphate-buffered saline; Dihydroethidine; c-DCF, 5-carboxy-2', 7'-dichlorodihydrofluorescein diacetate; pol, DNA polymerase; ROS, reactive oxygen species; ND1, NADH dehydrogenase 1; SOD, superoxide dismutase; MTT, 3-(4,5-dimethyl thiazol-2)-2,5-diphenyltetrazolium bromide; FITC, fluorescein isothiocyanate; PI, propidium iodide; NAC, *N*-acetylcysteine.

Introduction

Vitamin K (VK) is a family of fat-soluble compounds that includes phylloquinone (VK1), menaquinone (VK2), and menadione (VK3). The best-known member of the vitamin K family is phylloquinone, which is found in many higher plants and algae, with the highest concentrations in green leafy vegetables. Menaquinones are produced by bacteria and menadione is a synthetic analogue that acts as a provitamin (turns into a vitamin in the body). Vitamin K has two important characteristics: it is a critical factor in blood coagulation (clotting) and it inhibits cancerous cell growth (1).

Although the anti-tumor effects of vitamin K compounds have been under investigation since 1947 (2), it is unfortunate that more progress has not been made. The reason may be that the proposed theories were unconvincing to explain the difference in anti-tumor actions between VK1, VK2, and VK3. The most popular theory is that vitamin K compounds cause oxidative damage to malignant tumor cells, and other theories include the induction of cell death and inhibition of the cell cycle of the malignant cell so that it cannot continue growing (1). In general, although VK3 has a higher cytotoxic effect than VK1 and VK2, it is still uncertain how VK3 induces higher cytotoxicity in malignant cells.

DNA polymerases (pols) are indispensable for maintaining the integrity of the genome, both through faithful replication of DNA and by repairing damage to DNA. Among the 16 highly specialized mammalian polymerases, 15 are involved in maintaining nuclear genetic information. Replication and maintenenance of the mitochondrial genome relies on unique polymerase, pol γ (3). Pol γ is responsible for all aspects of mitochondrial DNA (mtDNA) synthesis, including all replication, recombination of the mitochondrial genome, and repair of mtDNA damage. It has been reported that mtDNA mutations or impairments of mtDNA coded protein syntheses may lead to superoxide production (4-6), and the superoxide itself is the major cause of both nuclear and mitochondrial DNA damage. Thus, it remains a matter of investigation whether pol γ plays a crucial role in the process of repair after mtDNA damage, or whether inhibition of pol γ leads to anti-tumor effects.

Here, we show novel mechanisms for cytotoxic actions of VK3 that it selectively inhibits pol γ activities in various human cancer cells. Superoxide production and cytotoxicity showed two different fashions according to the concentrations of VK3. These findings may integrate several reported cytotoxic actions of VK3 and pave the way for the further use of VK3.

Materials and Methods

Cell Lines and Chemicals. HCT116 colon carcinoma cell lines with wild-type p53 (HCT116 p53+/+) and their isogenic derivatives that lack p53 (HCT116 p53–/–) were kindly gifted by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore). The cells were maintained in McCoy's 5A medium containing 10% fetal bovine serum (FBS) (normal medium), or in McCoy's 5A- based enriched medium in several experiments, adding 10% FBS, 2 mM sodium pyruvate, 50 μg/ml uridine to the former normal medium at 37 °C with 5% CO_2. The immortalized human bronchial epithelial cells (BEAS-2B) described previously (7) were cultured in supplemented Keratinocyte-SFM medium (Invitrogen). Immortalized human B cells (PSC B cell) were obtained from the Health Science Research Resources Bank (Osaka, Japan) and were cultured in RPMI-1640 medium (8). Other cells, such as HCT15, SW620, H1299, A549, MCF-7, HeLa, HepG2, PANC-1, LNCaP, PC3, DU145, Raji, HL60, and MRC-5, were obtained from ATCC and cultured in RPMI-1640 medium. Vitamin K compounds (VK1, VK2, and VK3), rotenone, antimycin A, and *N*-acetylcysteine (NAC) were purchased from Sigma (St. Louis, MO). Nucleotides and chemically synthesized DNA template-primers such as poly (dA), poly (rA), and oligo $(dT)_{12\text{-}18}$ and [^{3}H]-dTTP

(deoxythymidine triphosphate, 43 Ci/mmol) were purchased from GE Healthcare Biosciences (Buckinghamshire, UK). Dihydroethidine (HE) and 5-carboxy-2', 7'-dichlorodihydrofluorescein diacetate (c-DCF) and Mitotracker green were obtained from Molecular Probes (Eugene, OR). All other reagents were of analytical grade and purchased from Nakalai Tesque. Ltd. (Kyoto, Japan).

Enzymes. Pol α was purified from calf thymus by immuno-affinity column chromatography as described by Tamai *et al*. (9). Recombinant rat pol β was purified from *E. coli* JMpβ5 as described by Date *et al*. (10). The human pol γ catalytic gene was cloned into pFastBac. Histidine-tagged enzyme was expressed and purified as described by Umeda *et al.* (11). Human pols δ and ε were purified by the nuclear fractionation of human peripheral blood cancer cells (Molt-4) using the second subunit of pol δ and ε-conjugated affinity column chromatography, respectively (12). Recombinant human pols η and ι tagged with His_6 at their C-terminal were expressed in SF9 insect cells using the baculovirus expression system, and were purified as described previously (13, 14). A truncated form of pol κ (i.e., hDINB1DC) with 6 x His-tags attached at the C-terminal was overproduced using the BAC-to-BAC Baculovirus Expression System kit (GIBCO BRL) and purified as described previously (15).

Recombinant human His-pol λ was overexpressed and purified according to a method described previously (16).

DNA polymerase assays. The reaction mixtures for pol α, pol β, plant pols and prokaryotic pols were described previously (17, 18). Those for pol γ, and pols δ and ε were as described by Umeda *et al*. (11) and Ogawa *et al*. (19), respectively. The reaction mixtures for pols η, ι and κ were the same as that for pol α, and the reaction mixture for pol λ was the same as that for pol β. For pols (i.e., DNA-dependent pols), poly (dA)/oligo $(dT)_{12\text{-}18}$ (A/T = 2/1) and dTTP were used as the DNA template-primer and nucleotide (i.e., deoxyribonucleotide triphosphates, dNTP) substrate, respectively. For RNA-dependent pol γ, poly (rA)/oligo $(dT)_{12\text{-}18}$ (A/T = 2/1) and dTTP were used as the template-primer and nucleotide substrate, respectively.

VK compounds were dissolved in distilled dimethyl sulfoxide (DMSO). Aliquots of 4 μl of sonicated samples were mixed with 16 μl of each enzyme (final amount 0.05 units) in 50 mM Tris-HCl (pH7.5) containing 1 mM dithiothreitol, 50 % glycerol and 0.1 mM EDTA, and kept at 0 °C for 10 min. These inhibitor-enzyme mixtures (8 μl) were added to 16 μl of each of the enzyme standard reaction mixtures, and incubation was carried out at 37 °C for 60 min.

Activity without the inhibitor was considered 100 %, and the remaining activity at each concentration of the inhibitor was determined relative to this value. One unit of pol activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of dNTP (i.e., dTTP) into synthetic DNA template-primers in 60 min at 37 °C under the normal reaction conditions for each enzyme (17, 18).

Isolation of mitochondria. Mitochondria were isolated from control and treated cells as described previously (20). Then, cells were harvested, and re-suspended in 3 volumes of isolation buffer (10 mM Tris–HCl, pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 70 mM sucrose, 210 mM mannitol, and protease inhibitors). After incubating in an ice-bath for 15 min, the cell suspension was homogenized with 15 strokes.

Measurement of DNA polymerase γ inhibitory activity in the mitochondrial extract from human cancer cells. Both HCT116 p53+/+ and HCT116 p53–/– cells were treated with 0, 3, and 30 μM of VK3 for 24 hr and then stored at -80 °C. Using the isolated mitochondrial fraction, the pol γ activity of 2 μg of the extract from the mitochondrial fraction was assayed as described previously (17, 18).

Evaluation of mitochondrial mass in each cell. To investigate morphological effect to

mitochondria by VK3, cells were stained by Mitotracker green (Molecular Probes) and mitochondrial mass volumes per cell were measured by a flow cytometer as described previously (21).

Analyses of cellular superoxide and hydrogen peroxide. Cellular superoxide and hydrogen peroxide were measured by flow cytometer analyses using HE and c-DCF (4). 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 in 50 μM at 37 °C for 60 min.

Western blot analysis. Protein lysates were prepared from the control and VK3-treated cells, and separated by electrophoresis on 8%-15% SDS-PAGE. Amounts of the pol γ in the mitochondrial fraction were evaluated by using its polyclonal antibodies (Ab-3, NeoMarkers). Antibodies for superoxide dismutase 1 (SOD1, Calbiochem), SOD2 (Calbiochem), and β-actin (Sigma) were used (22). Antibodies for cytochrome C (Santa Cruz Biotechnology) and cytochrome oxidase subunit IV (COX IV, Molecular Probes) were purchased for the evaluation of mitochondrial specific proteins. The mitochondrial Hsp60 protein (antibody

N-20, Santa Cruz Biotechnology) was blotted to ensure equal loading of mitochondrial protein (10). Those 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).

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 hrs) as described previously (4). For a longer term survival analysis, a colony-forming assay was performed. VK compounds were added about 18 hr (overnight) after seeding, and cells were incubated for 10-14 days. Fixation and staining were according to previously described methods (23, 24)

Mitochondrial DNA fragmentation analysis. Mitochondrial DNA fragmentation was evaluated by the following method. The mitochondrial pellets were then digested in buffer containing 10 mM Tris-HCl, pH 7.8, 10 mM NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate, and 0.1 mg/ml proteinase K for 30 min. For analysis, 10 μg of purified mtDNA was loaded onto a 2 % agarose gel containing 10 μg/ml ethidium bromide and electrophoresed with DNA molecular weight markers (*λ-Hind* III, and *ϕX-174 Hae* III) (Takara, Tokyo,

Japan) in 0.5 x Tris borate-EDTA buffer (18 mM Tris-HCl pH 8.0, 18 mM boric acid, and 1 mM EDTA) at 100 V for 1 h. After treatment with RNase A (final concentration, 100 μg/ml) overnight, mtDNA bands were visualized under ultraviolet light and photographed.

Sequencing of mitochondrial DNA. Direct mitochondrial sequencing of multiple regions (ND1, ND4, and Cytochrome b) was performed according to the previously described methods (6). Briefly, primer sequences were as follows: 5'AACATACCCATGGCCAACCT3' sense and 5'GGCAGGAGTAATCAGAGGTG3' antisense for NDI (#3304-3836), 5'GACTCCCTAAAGCCCATGTCG3' sense and 5'TTGATCAGGAGAACGTGGTTAC3' antisense for ND4 (#11403-11927), and 5'AGTCCCACCCTCACACGATTC3' sense and 5'ACTGGTTGTCCTCCGATTCAGG30 for cytochrome b (#15260-15774). The annealing temperatures were 55°C for NDI, 58°C for ND4, and 58°C for cytochrome b. The sequencing chromatograms were further evaluated manually, excluding the unstable first 20-30 portions. Complete replacement of nucleotides was defined as a point mutation. Any nucleotide position with two or more significant peaks of mixed nucleotide signals (heteroplasmy) was estimated for the percentage of each nucleotide, based on the area under the curve of the corresponding nucleotide peak. Cases of

heteroplasmy accounting for less than 30% of the total base signal were considered insignificant and were not scored. Only those with greater than 30% heteroplasmic signal were counted.

Assessment of apoptosis by annexin-V and PI staining. Cells were stained with annexin-V-FITC for exposure of phosphatidylserine on the cell surface as an indicator of apoptosis, following the manufacturer's instructions (BD Biosciences) (25).

Results

Effects of vitamin K compounds on DNA polymerase activity. First, the inhibition of the activities of mammalian pols by VKs was investigated. In the three VK compounds tested, 30 μM of VK3 indicated pol γ inhibitory activity, but the same concentration of VK1 and VK2 did not influence the activity of all pols tested including pol γ. VK3 selectively inhibited pol γ activity, and did not suppress the activities of other mammalian pols such as pols α, β, δ, ε, η, ι, κ, and λ (Table 1). Pol γ is the sole pol in animal mitochondria. Biochemical and genetic evidence document a key role for pol γ in mitochondrial DNA replication. Since pol γ has not only DNA-dependent pol activity but also RNA-dependent pol activity (26), the influence of both activities by VK3 was investigated. The inhibitory activity of VK3 on RNA-dependent pol γ was as strong as that on DNA-dependent pol γ, with 50 % inhibition observed at doses of 6.8 and 6.0 μM, respectively (Table 1). When activated DNA (i.e., DNA digested by bovine DNase I) was used as the template-primer instead of the synthesized DNA template-primer such as poly(dA)/oligo(dT)$_{12\text{-}18}$, the mode of inhibition by these compounds did not change (data not shown). These results suggested that longer isoprenoid chains of the benzquinone ring in VK weaken the inhibitory effect on pol γ. VK compounds had no inhibitory effect on

fish (cherry salmon, *Oncorhynchus masou*) pols α and δ, insect (Fruit fly, *Drosophila melanogaster*) pols α, δ and ε, plant (cauliflower inflorescence) pol I (α-like), pol II (β-like) and pol λ, or prokaryotic pols such as the Klenow fragment of *E. coli* pol I, *Taq* pol and T4 pol (data not shown).

Inhibitory effect of vitamin K compounds on the activities of other DNA metabolic enzymes. VK compounds did not inhibit the activities of other DNA metabolic enzymes such as calf primase of pol α, calf terminal deoxynucleotidyltransferase, HIV-1 reverse transcriptase, T7 RNA polymerase, T4 polynucleotide kinase and bovine deoxyribonuclease I (data not shown). These results could suggest that VK3 selectively inhibited the activity of mitochondrial pol γ in the pols and DNA metabolic enzymes tested.

Effects of vitamin K compounds on DNA polymerase γ inhibitory activity and growth inhibition in human cancer cells. Pol activity in the mitochondrial extract of VK compound-treated cells was investigated (Fig. 1A). The pol in the mitochondria fraction is pol γ only, and 2 μg of the extract of human colon cells, HCT116, with or without the p53 gene, had approximately 0.11 and 0.12 units of pol γ activity, respectively. The pol γ activity of VK3-treated cells was significantly lower than that of non-treated cells, and enzyme activity

was decreased 26.8-28.8 % of 3 μM and 70.6-70.8 % of 30 μM. The inhibitory effect of VK3 seemed to be independent of p53. On the other hand, VK1- and VK2-treated cells had almost no influence. To investigate the underlying mechanisms of actions of VK3, mitochondrial volume were evaluated by flow cytometer using Mitotracker green. As shown in the Fig 1B, mitochondrial mass per each cell first enlarged and then decreased within 8hr after exposure to 30 μM of VK3, whereas the mitochondrial mass did not changed by 3 μM of VK3. The results indicated that VK3 induced destructive mitochondria targeted damage in the dose of 30 μM. This phenomenon was further supported the quantitative changes of pol γ. As shown in the Fig. 1C, by a dose of 30 μM of VK3, the amount of DNA polymerase γ decreased in a time dependent manner, whereas those did not change by lesser doses (3, 10 μM) of VK3, which meant that 30 μM of VK3 clearly decreased or damaged pol γ in the mitochondria. Moreover, with the treatment of 30 μM of VK3, the mitochondrial specific proteins such as SOD2, cytochrome C, and COX IV, decreased within 6 hr. On the contrary, SOD1 in the cytoplasm seemed to be unchanged. Cytochrome C seemed to release from the mitochondrion to the cytoplasm within 3 hr and then induced apoptosis. These data together, the treatment of

30 μM of VK3 induced certain damage to the mitochondria, but not to the cytoplasm. (Fig. 1D).

As shown in Fig. 2, VK3 with a dose of 3 μM had a potent growth inhibitory effect on HCT116 p53 +/+ and p53–/– cells in a colony forming assay, whereas VK1 and VK2 did not prevent cell growth in the same concentration. To clarify whether the inhibitory effect by VK3 might be observed in other types of tumor, cytotoxic effects by VK compounds were investigated by MTT assays (Table 2). Interestingly, VK3 was the strongest inhibitor of various human cancer cells and the concentration required for IC_{50} was ranging from 6.0 μM to 12 μM (median, 7 μM), whereas VK3 showed lesser inhibitory effects in human normal cells (ranging from 10 μM to 26 μM, median: 13 μM). Since the IC_{50} values of VK3 were 6.0 μM for human pol γ activities (Table 1), the IC_{50} values for cell survival by both colony-forming assay and that of those MTT assays were well consistent with the IC_{50} values *in vitro* for the enzyme.

ROS-generating effect of vitamin K compounds. To assess intracellular events in VK-treated cells, the amount of superoxide was firstly investigated. With 30 μM of VK3, the superoxide level increased more than 30-fold in p53+/+ cells and more than 40-fold in p53–/–

cells in 24 hr, whereas the same dose of VK1 and VK2 induced a minimum increase of superoxide generation (less than 2-fold) (Fig. 3A). Sequential evaluation by 30 μM of VK3 showed that superoxide generation was observed in time-dependent manner in both p53+/+ and p53−/− cells (Fig. 3B). With 3 μM of VK3 for 24 hr incubation, an increase of superoxide by VK3 was similar to those by rotenone and by antimycin A (data not shown), whereas with 30 μM of VK3 was quite different from those results; therefore, it was possible that the cytotoxic effect with 3 μM of VK3 was due to redox cycling modulation without large amounts of superoxide generation. Next, the amounts of hydrogen peroxide were assessed. The amounts of hydrogen peroxide were slightly elevated by VK3 (3-fold in p53+/+ cells, and 4-fold in p53−/− cells) in the same incubation time (Fig. 3C), but they were not increased by VK1 or VK2 in both cells (data not shown). To test whether the mode of these free radical generations was consistent with other types of tumor, the same experiment was performed using several human cancer and normal cells (Table 3). As a result, superoxide increased ranging from 11-fold to 40-fold (median, 20-fold) in cancer cells, whereas hydrogen peroxide did lesser (range: 3- to 16-fold, median: 10-fold). The tendency in normal cells was almost similar with those in cancer cells.

Effect of mtDNA repair through the inhibition of pol γ by VK3. The amount of superoxide generated by 30 μM of VK3 seemed to be quite different from that by 3 μM of VK3. As for the mechanism producing a large amount of superoxide, a possible theory has not been proposed previously; therefore, we hypothesized that VK3 may suppress mtDNA replication or repair abilities of pol γ and that those suppression may lead to superoxide production, with the reasons that pol γ enzymatic activity was dramatically decreased by 30 μM of VK3 (Fig. 1A). To address this hypothesis, a status of mtDNA, especially a length of mtDNA was first studied by direct electroporesis of mtDNA, with the result that no fragmentation or deletion was seen in the treatment by VK3 (0, 3, 30 μM for 24 hr) (Fig. 4A). Next, an occurrence of point mutation or heteroplasmy was assessed by direct sequencing in several mtDNA-coded regions. As a result, the frequency of heteroplasmy greatly increased in multiple regions, consisting of a mitochondrial respiratory complex (Fig. 4B and 4C). On the other hand, point mutations were rarely observed (Fig. 4C), seemingly due to experimental design performing within 24 hr. These results together indicated that the treatment with 30 μM of VK3 decreased pol γ mtDNA repair activity, in which the impairment of mtDNA repair might lead to greater amounts of superoxide production.

A link of the pol γ inhibition by VK3 and induction of apoptotic cell death through superoxide generation. To clarify whether superoxide generation was the main factor in VK3-induced cytotoxicity, annexin-V and PI double-staining apoptosis assays were performed. The proportion of the apoptosis increased in a dose-dependent manner of VK3, and the induction of apoptosis was dramatically suppressed by the ROS scavenger N-acetylcysteine (NAC) (Fig. 5A). Superoxide-mediated apoptosis by 30 μM of VK3 occurred already at 18 hr, which may be independent of p53 (Fig. 5B).

Cell survival by glycolytic pathway after VK3 cytotoxicity. It has been widely reported that cell-depleted mtDNA could produce lower amounts of ATP by glycolysis and survive in a uridine- and sodium pyruvate-containing medium (27). Those cells lacked the ability of oxidative phosphorylation because of defective mitochondrial respiratory function and were called "ρ0 cells". We then hypothesized that if the cytotoxic mechanism of VK3 may be caused by mitochondrial respiratory dysfunction, cells could be rescued by glycolytic energy production in enriched medium. As a result, 3 μM of VK3 showed high cytotoxicity in both p53+/+ and p53–/– cells, and cells exposed to the same dose of VK3 were successfully rescued by enriched medium (Fig. 6A); however, cells could not survive when 30 μM of VK3

was exposed in the same condition (Fig. 6B). The difference clearly illustrated that VK3 has at least two different cytotoxic actions according to VK3 concentration. These results illustrated that VK3, at a concentration of 3 μM, inhibits mitochondrial respiratory function and induced cytotoxicity in human cancer cells. Based on these observations, a model was proposed by illustrating a novel mechanism of VK3 by the inhibition of pol γ activity leading to superoxide generation and mitochondrial respiratory dysfunction (Fig. 7).

Discussion

The major reason why VK3 has not been widely developed as an anti-tumor agent may be that the mechanism of toxicity in normal cells has not been clarified. However, it is important to note that most previous investigations have been concerned with cells, such as hypatocytes, cardiomyocytes, and neurons, which contain greater amounts of mitochondria, and that cancer cells are also believed to upregulate their mitochondrial biogenesis and to contain larger amounts of mitochondria compared to their normal cell counterparts (21, 28-29). As for the mechanisms of the cytotoxic effects of VK3, there are several interesting theories demonstrating that VK3 decreases intracellular thiols (30), modifies intracellular Ca^{2+} homeostasis (30), and causes cell death (31). Interestingly, those findings were closely related to mitochondrial dysfunctions, and they might be initiated in mitochondria. Thus, those reports suggested that VK3 penetrates the mitochondrial membrane and has several effects on the components of mitochondria. In this report, we firstly identified the inhibitory effect of VK3 on pol γ, which resides in the matrix of mitochondria. As shown in the table 2, there was a tendency that the IC_{50}s of cancer cells seemed to be lesser than those of normal cells, however, it was quite difficult to conclude those differences to be not significant mainly

because types of normal cells were limited. However, it might be possible that there is a small difference among the poly γ activity or amount between cancer and normal cells. As far as we investigated, there are no reports demonstrating that the cancerous pol γ is different from normal one. On the contrary, our group previously reported that numbers of mitochondrion in cancer cells are larger than normal counterparts (21). Because each mitochondrion contains mtDNA and pol γ, it could be speculated that amount of pol γ in cancer cells and effects of VK3 might be larger than those of in normal cells. These difference might be an explanation for the prescribed tendency. These findings also implied that VK3 penetrates both inner and outer mitochondrial membranes; therefore, these findings may be a key to integrate several reported VK3 actions at mitochondria, and pave the way to the biological effects of VK3.

Historically, the effectiveness of VK3 against cancer was believed to be due to oxidative stress via redox cycling of the quinone to produce ROS, such as the superoxide radical and hydrogen peroxide (32). From our results subjected to both human cancer and normal cells, the major ROS induced by VK3 was the superoxide. When a lesser dose of VK3 (3 μM) was applied, the increase of superoxide production was quite small and similar to the results by mitochondrial respiratory chain complex inhibitors. On the other hand, the mechanism of

superoxide generation by a higher dose of VK3 (30 μM) seemed different. Notably, the two different actions were well consistent with the magnitude of inhibition of pol γ activity *in vitro* and *in vivo* (Fig. 1A). It was shown that irreversibly greater amounts of superoxide production were caused by the impairment of the synthesis of components of the respiratory chain complex. From the literature, when the coding regions of pol γ were mutated, it was reported that mtDNA might induce mutations in experimental models (33, 34), and in clinical observation (35). In our results, the majority of nucleotides changes were heteroplasmies, whereas the proportion of point mutation was quite small (Fig. 4C). As a single cell contains 100-1,000 mitochondria and mitochondria carry 1-10 copies of the mitochondrial genome, it is reasonable that after short-term inhibition (24 hr) of pol γ activities, errors of mtDNA repair might be induced to various extents in each mitochondrion. Those changes were mixed and might be detected as heteroplasmic change.

As far as we investigated, VK3 is the second compound with an inhibitory effect on pol γ activities. Highly active antiretroviral therapy (HAART) against HIV, such as zidovudine (3′-azido-2′, 3′-deoxythymidine; AZT) and other nucleoside analogues, was first reported as having an effect leading to mitochondrial dysfunctions, such as cardiac dysfunction, hepatic

failure, skeletal myopathy, and lactic acidosis (36). Notably, those side effects induced by HAART had quite similar characteristics, manifested by mitochondrial dysfunction. On the other hand, it has been reported that HAART is effective against AIDS-related malignancies (37). In these points, VK3 and HAART have similar effects and characteristics. Martin and colleagues (38) demonstrated mtDNA mutations in five of 26 patients undergoing NRTI therapy in which novel mtDNA sequence variations were found to arise within individuals over a relatively brief period (average treatment duration was 24 months). In addition to these reports, although the chemical structure of VK3 is not similar to that of AZT, their actions were quite similar.

Carr and colleagues energetically investigated the growth inhibitory effects of their originally synthesized vitamin K-related compounds (39). From their reports, they demonstrated that the potency of growth inhibitory action correlated with the decreasing length of the side chain. VK-related quinoid compounds with short side chains or without a side chain like VK3 seemed to have greater cytotoxicity against hepatoma cells. Phase I study using the VK3-related compound was conducted and the adverse effects seemed acceptable (40). Based on these observations, VK3 and its related compounds have the possibility to be

applied as an anti-tumor agent. Further investigations are expected in addition to these findings.

In conclusion, novel mechanisms were identified in that VK3 selectively suppressed pol γ activities leading to two different cytotoxic actions that were shifted in a VK3 concentration-dependent manner. These findings may explain various cytotoxic actions by VK3, and may pave the way for the further use of VK3

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Table 1

IC_{50} values of vitamin K compounds for the activities of various DNA polymerases and other DNA metabolic enzymes

Enzyme	IC_{50} values of vitamin K (μ M)		
	VK1	VK2	VK3
--- Mammalian DNA polymerases ---			
Calf DNA polymerase α	>200	>200	>200
Rat DNA polymerase β	>200	>200	>200
Human DNA polymerase γ			
DNA-dependent pol activity	>200	>200	6.0
RNA-dependent pol activity	>200	>200	6.8
Human DNA polymerase δ	>200	>200	>200
Human DNA polymerase ε	>200	>200	>200
Human DNA polymerase η	>200	>200	>200
Human DNA polymerase ι	>200	>200	>200

Human DNA polymerase κ	>200	>200	>200
Human DNA polymerase λ	>200	>200	>200

These compounds were incubated with each enzyme (0.05 units). Enzymatic activity was measured as described in the text. Enzymatic activity in the absence of compounds was taken as 100 %.

Table 2

IC_{50} values of growth inhibitory effect of vitamin K compounds evaluating by MTT assays in human cancer and normal cells.

		IC_{50} (μM)		
Cell Type	Tissue origin	VK1	VK2	VK3
HCT116 p53+/+	Colon	>100	>100	6
HCT116 p53-/-	Colon	>100	>100	6
HCT15	Colon	>100	>100	10
SW620	Colon	>100	90	5
H1299	Lung	>100	28	10
A549	Lung	>100	55	12
MCF-7	Breast	>100	25	8
HeLa	Uterus	>100	>100	6
HepG2	Liver	>100	>100	8

PANC-1	Pancreas	>100	>100	6
LNCaP	Prostate	>100	85	7
PC3	Prostate	>100	>100	6
DU145	Prostate	>100	>100	10
Raji	B cell lymphoma	>100	>100	8
HL60	Leukemia	>100	>100	6
	Median	>100	100 (SD: 7)	7 (SD: 0.5)
BEAS	Normal bronchial epithelial cells	>100	>100	10
PSC B cell	Normal B cell	>100	>100	13
MRC-5	Normal fibroblast	>100	>100	26
	Median	>100	>100	13 (SD: 4.9)

Table 3

Superoxide and hydrogen peroxide generation after exposure to VK3 in human cancer and normal cells.

		Superoxide	Hydrogen Peroxide
		(-fold, VK3 30μM / Control)	
HCT116 p53+/+	Colon	24	3
HCT15	Colon	35	7
SW620	Colon	40	16
H1299	Lung	13	10
MCF-7	Breast	11	9
PANC-1	Pancreas	13	11
PC3	Prostate	14	12
Raji	B cell lymphoma	20	3
HL60	Leukemia	25	12

	Median	20 (SD: 3.5)	10 (SD: 1.3)
BEAS	Normal bronchial epithelial cells	14	4
PSC B cell	Normal B cell	19	13
	Median	17 (SD: 2.5)	9 (SD: 4.5)

FIGURE LEGENDS

Fig.1. A, Total DNA polymerase activity of the extract of mitochondria fraction from HCT116 (p53+/+ and p53–/–) cells incubated with or without vitamin K compounds for 24 hr. One unit of pol activity is defined as the amount that catalyzes the incorporation of 1 nmol of dexyribonucleoside triphosphates (i.e., dTTP) into synthetic template-primers (i.e., poly (dA)/oligo $(dT)_{12\text{-}18}$, A/T = 2/1) at 37 ºC in 60 min. Enzymatic activity in the absence of compound was taken as 100 %. Data are shown as the means ± SEM for three independent experiments. B, Sequential mitochondrial mass changes after VK3 treatments. Each bar indicated a median size of mitochondrial mass out of 10,000 cells stained by Mitotracker green. The mitochondrial mass enlarged by a higher dose (30 μM) of VK3 and then decreased within 8hr, whereas those did not change by a lesser dose (3 μM) of VK3. C, Western blots analysis of the DNA polymerase γ after 0, 3, and 6 hr incubation with 3, 10, and 30 μM of VK3. By a dose of 30 μM of VK3, the amount of DNA polymerase γ decreased in a time dependent manner, whereas those did not change by lesser doses (3, 10 μM) of VK3. Band of Hsp60 showed equal amounts of mitochondrial protein loaded. D, Western blot analyses of mitochondria specific proteins after 30 μM of VK3. With the treatment of 30 μM of VK3, the

mitochondria specific proteins such as SOD2, cytochrome C, and COX IV, decreased within 6 hr. On the contrary, SOD1 in the cytoplasm seemed to be unchanged. Cytochrome C seemed to release from mitochodria to cytoplasm within 6 hr.

Fig. 2. A, Growth inhibitory effect of vitamin K compounds (3 μM) in both HCT116 p53+/+ and p53–/– cells. Equal amounts of DMSO with vitamin K compounds were added as a control. B, Numbers of colonies after 14 days' incubation with those vitamin K compounds were counted.

Fig. 3. A, Superoxide generation after exposure to VK1, VK2, and VK3 in both HCT116 p53+/+ and p53–/– cells. B, Time-dependent increase of superoxide generation by 30 μM of VK3. C, Hydrogen peroxide generation after exposure to VK3 (3, 30 μM) was evaluated.

Fig. 4. A, Mitochondrial DNA fragmentation analysis by direct electrophoresis of mtDNA. Samples and DNA markers were electrophoresed after the indicated VK3 treatments of both HCT116 p53+/+ and p53-/- cells for 24 hr. (control, 3, and 30 μM). No fragmentations or deletions were observed in those conditions. B, Example of direct sequencing analysis. In the ND1 portion, typical heteroplasmic changes were frequently observed in the sample treated by 30 μM of VK3 for 24 hr (arrows), compared to the control sample. C, Frequencies of point

mutation or heteroplasmy after 3, or 30 μM of VK3 in HCT116 p53+/+ cells. Averaged numbers of mutation or heteroplasmy in 3 independent experiments are shown.

Fig. 5. A, VK3 induced ROS-mediated apoptosis in HCT116 p53+/+ cells. Cells were incubated with various concentrations [(1) 3 μM, (2) 10 μM, and (3) 30 μM] of VK3 alone, and with an antioxidant of NAC [(4) VK3 30 μM + NAC 1 mM] for 24 hr at 37 ºC. B, Time-dependent increase of apoptosis by 30 μM of VK3 (control, 1, 4, 18, and 24 hr) in HCT116 p53+/+ cells and p53–/– cells.

Fig. 6. Cell growth inhibition by VK3 and cell proliferation by glycolysis. A, The 3 μM of VK3 showed growth inhibition in both HCT116 p53+/+ and p53–/– cells; however, it was successfully recovered by adding glycolytic substrates. B, On the other hand, 30 μM of VK3 also showed cell growth inhibition, which was not rescued in the same condition.

Fig. 7. A schema of the mechanisms of VK3-induced growth inhibition and cytotoxicity in human cancer cells.

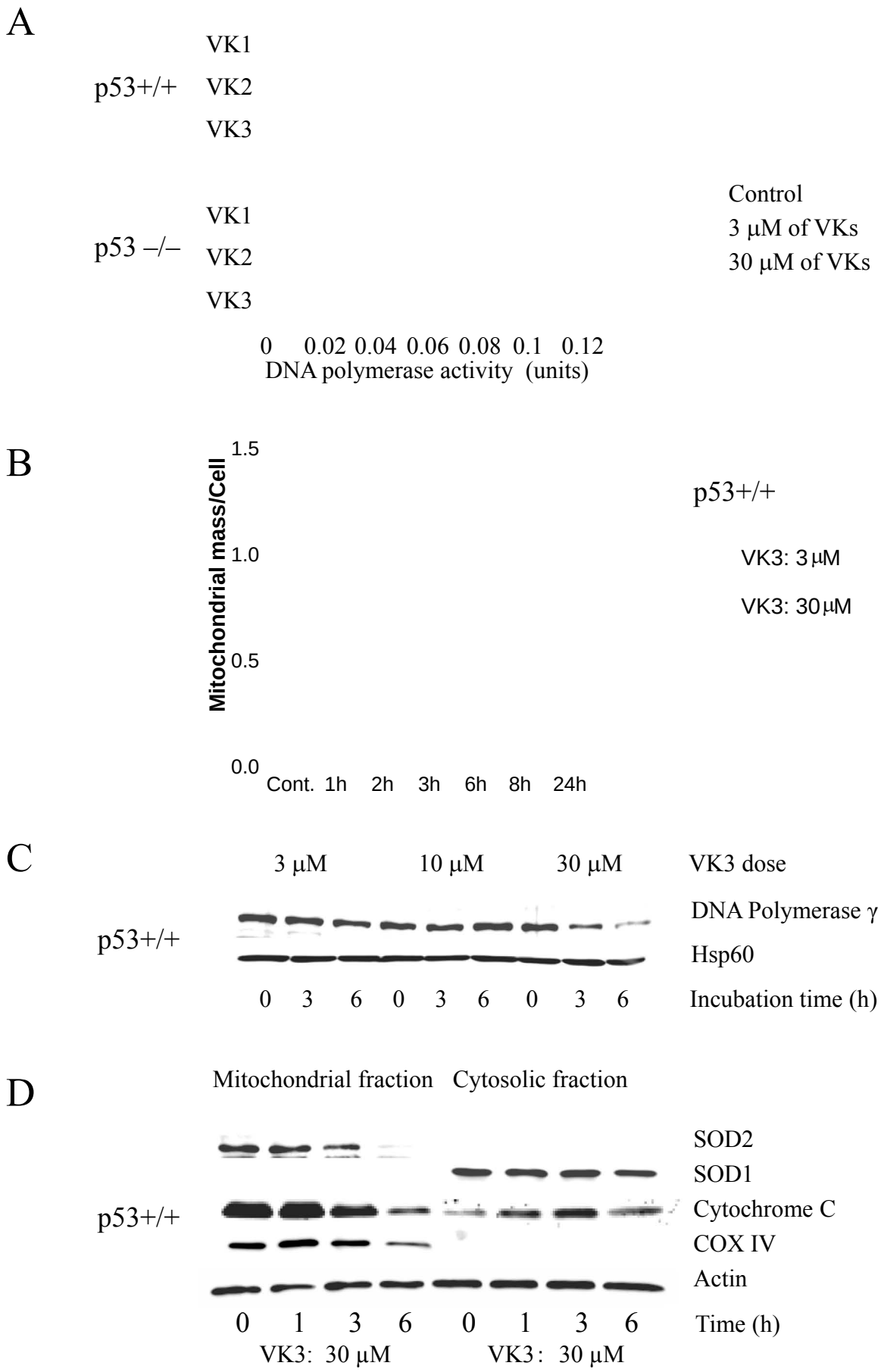


Fig. 1. Sasaki R. *et al.*

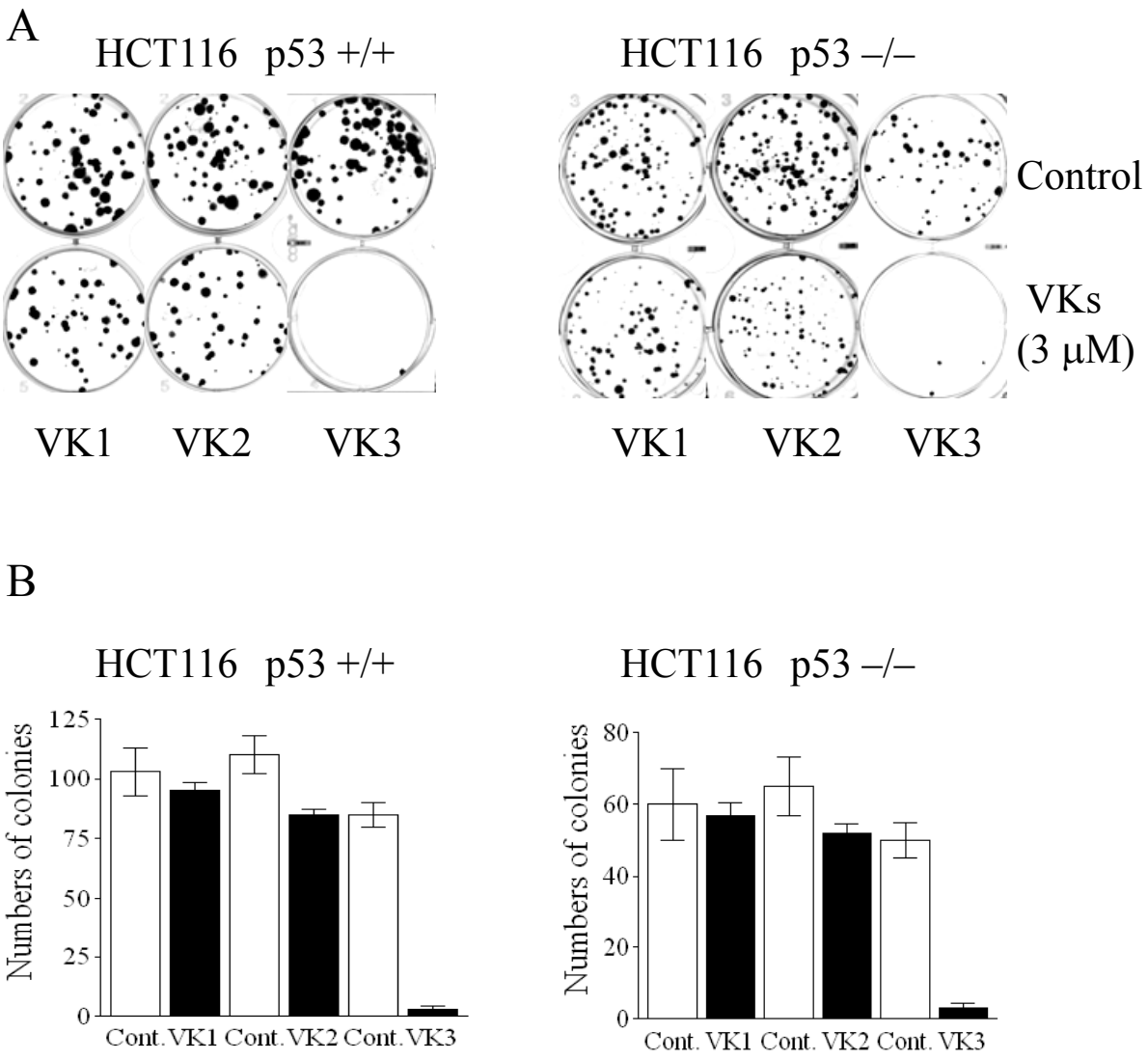


Fig. 2. Sasaki R. *et al.*

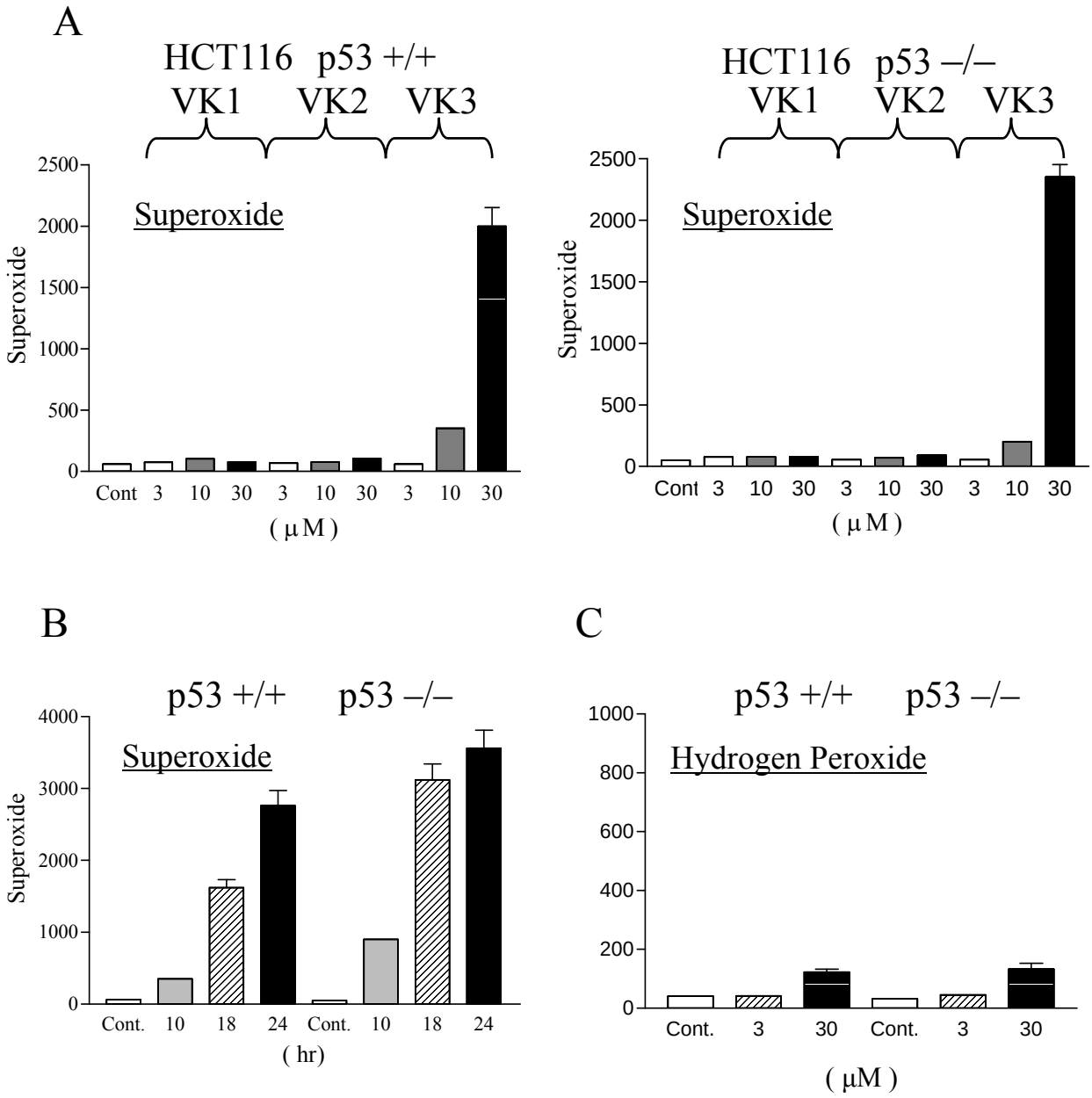
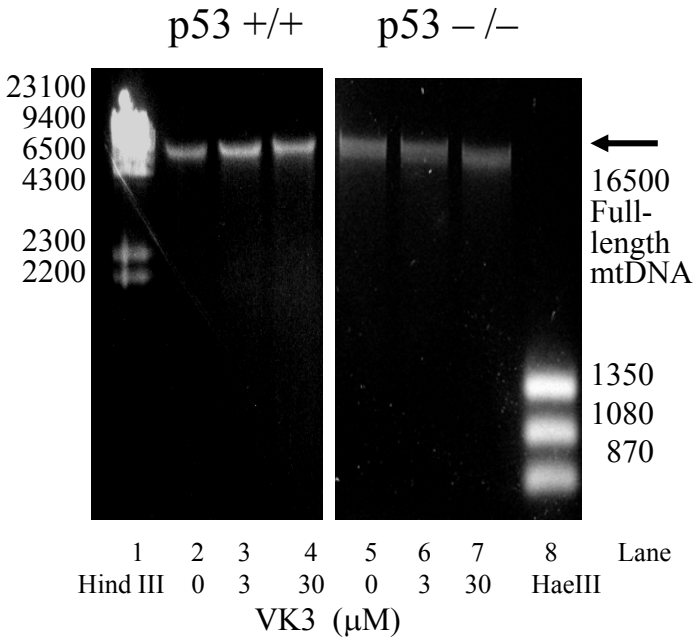


Fig. 3 Sasaki R. *et al.*

A



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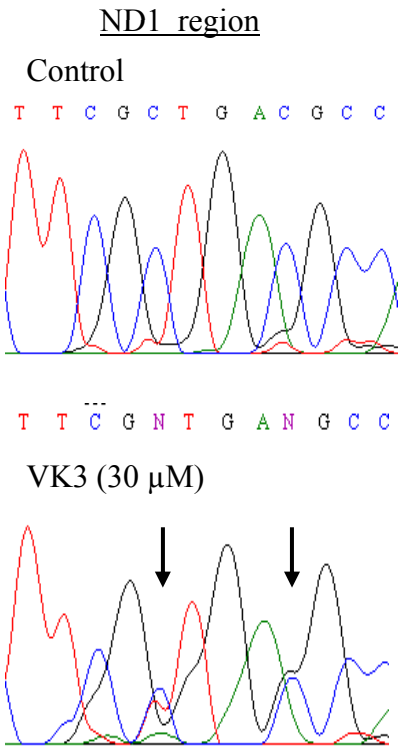

C

Portion	Cont.	VK3 Concentration			
		3 μM		30 μM	
		M *	HP #	M *	HP #
ND1	0	1	7	0	28
ND4	0	0	5	0	35
Cyt b	0	0	1	0	21

*Mutation, # Heteroplasmy

Fig. 4. Sasaki R. *et al.*

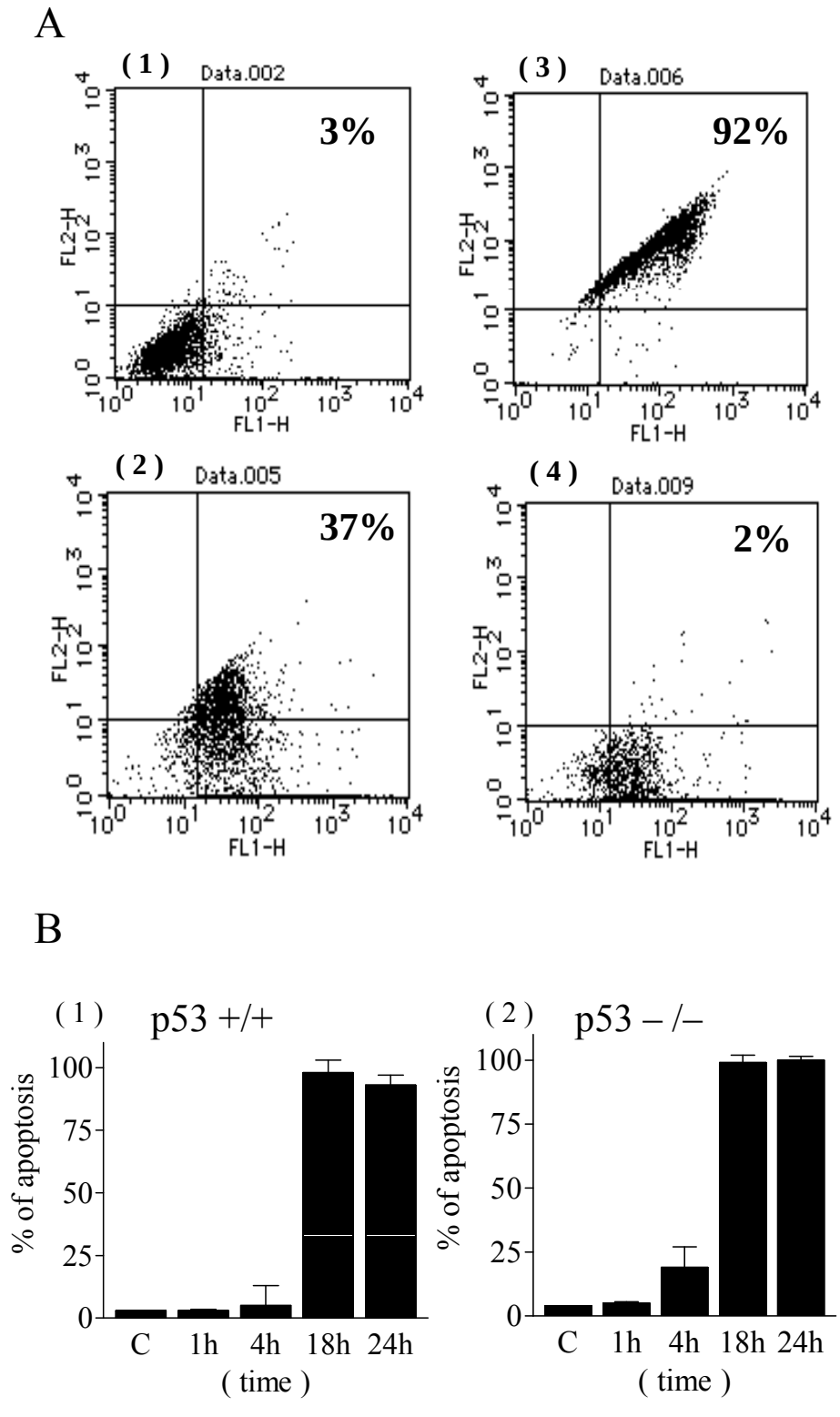

Fig. 5. Sasaki R. *et al.*

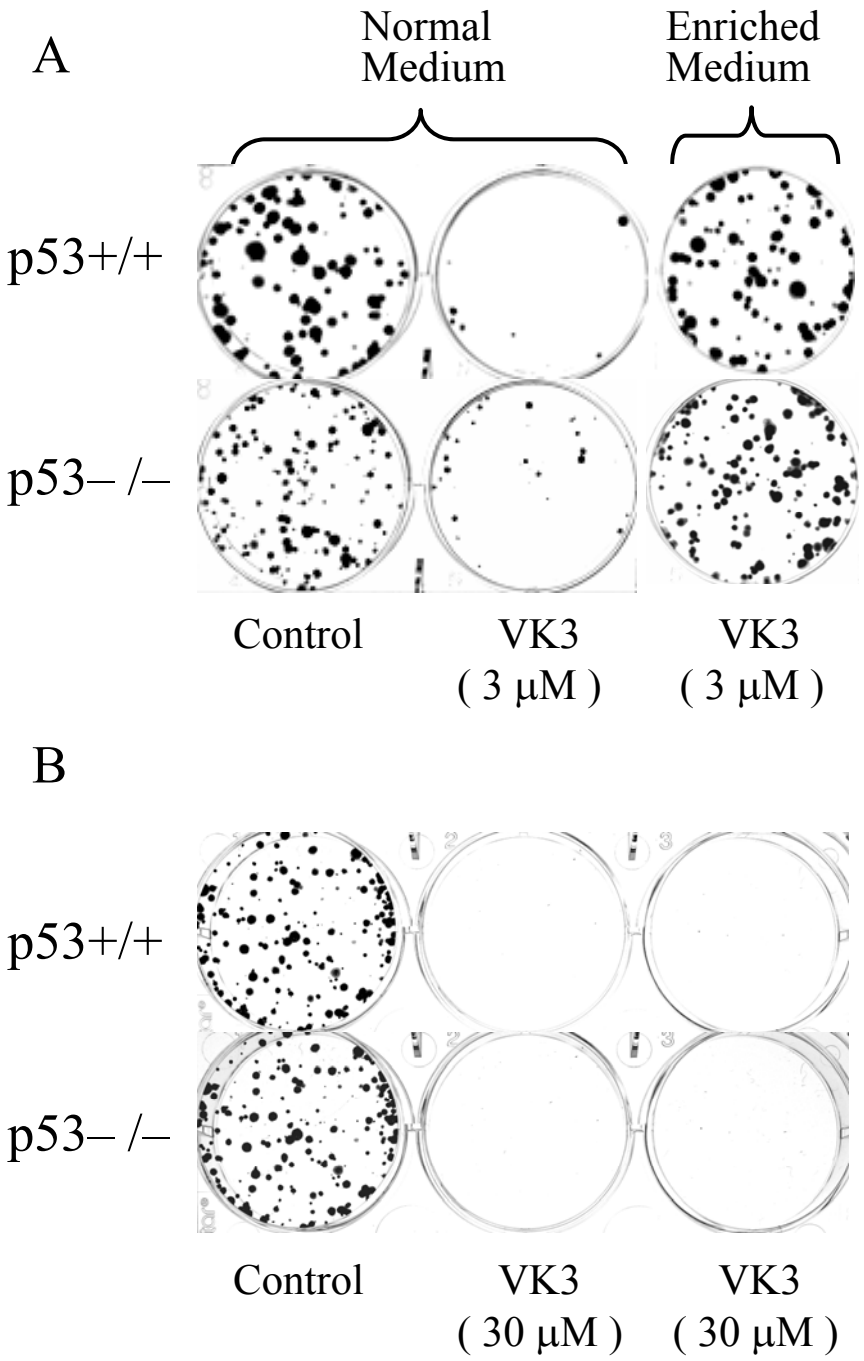

Fig. 6, Sasaki R. *et al.*

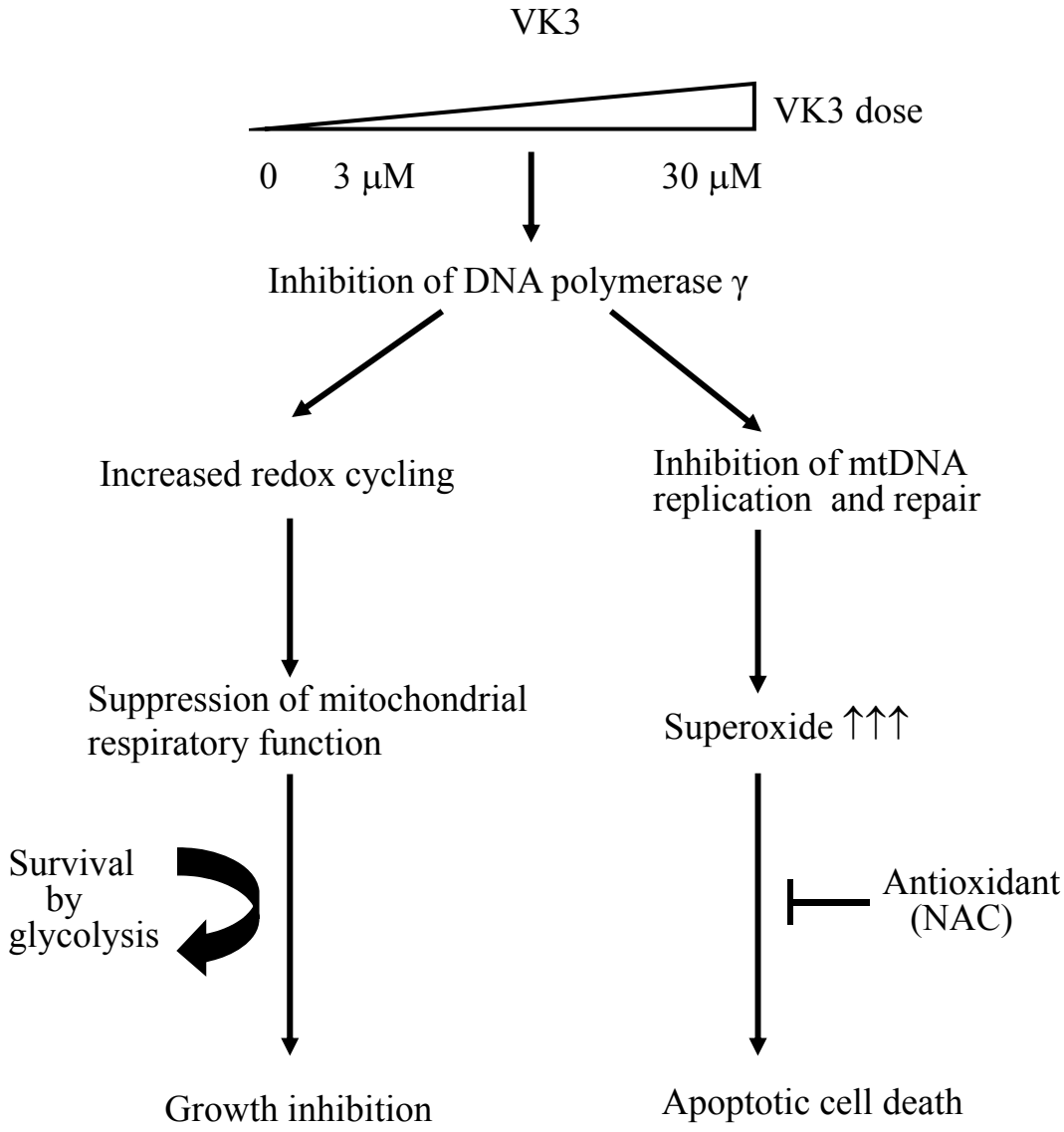

Fig. 7, Sasaki R. *et al.*