Antiproliferative and Apoptosis-Inducing Effects of Lipophilic Vitamins on Human Melanoma A375 Cells in Vitro

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The effects of six lipophilic vitamins: tretinoin (ATRA), vitamin D3 (VD3), VE, VK1, VK3, and VK5 on cell proliferation and apoptosis in human A375 melanoma cells were investigated. VD3, VK1, and VK3 were found to inhibit cell proliferation significantly at concentration ranges of 10–100 μmol/L (p<0.01), while the other vitamins did not show inhibitory effects at 100 μmol/L. VK5 showed the strongest effects with IC50 values of less than 10 μmol/L. Dacarbazine slightly inhibited the proliferation of A375 cells at a concentration range of 25–100 μmol/L, but the effects were not statistically significant. VK3 and VK5 increased annexin-V positive apoptotic cells, as well as activating caspase-3, in A375 cells. Our findings showed that VD3, VK3, and VK5 inhibited the growth of dacarbazine resistant human melanoma cells, while ATRA, VE, and VK1 had little effect on the cell growth. The effects of VK5 and VK1 were observed at concentrations lower than 10 μmol/L, which are suggested to have resulted from apoptosis-induction in the melanoma cells.

Key words lipophilic vitamin; A375 human melanoma cell; growth inhibition; apoptosis; caspase-3

In patients with metastatic melanoma, systemic therapies are known to be ineffective because of the high resistance of melanoma cells to various anticancer therapies. Single-agent dacarbazine remains the standard chemotherapy with response rates ranging from 11 to 25%, while complete responses are rare and of short durations (three to six months). The median survival time for patients with metastatic melanoma is less than one year. At present, no treatment options are available for patients with advanced melanoma which provides either a sufficient response rate or significant prolongation of overall survival. The lack of efficacious treatments for advanced melanoma makes new approaches necessary.

Lipophilic vitamins have been examined for their anticancer properties by many researchers. For instance, the differentiation-inducing efficacy of tretinoin (ATRA) against clinical promyelocytic leukemia, the antiproliferative and apoptosis inducing efficacy of vitamin D3 (VD3) in breast cancer and colorectal cancer, the apoptosis-inducing and chemosensitivity-enhancing effects of VE succinate in bladder cancer cells, and the antitumor effects of VK2, VK3, and VK5 against hepatocellular carcinoma have been reported. While some reports suggest that ATRA, VD3, VE, and VE succinate may have anti-cancer efficacy against melanoma, few studies have been conducted to examine the effects of VK derivatives on human melanoma cells.

A375 human melanoma cells are amelanotic but are highly metastatic, and thus in the present study we systematically investigated the antiproliferative and apoptosis-inducing effects of six lipophilic vitamins including the three VK derivatives: VK3, VK5, and VK1 on A375 cells in vitro.

MATERIALS AND METHODS

Reagents Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, and ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco BRL Co. (Grand Island, NY, U.S.A.). Dacarbazine and lipophilic vitamins all-trans-retinoic acid (ATRA), cholecalciferol (VD3), Δα-alpha-tocopherol (VE), phytomonadione (VK1), 2-methyl-1,4-naphtoquinone (VK3), and 4-amino-2-methyl-1-naphthol hydrochloride (VK4) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dacarbazine stock solutions were made at a concentration of 10 mmol/L with ethanol and diluted to working concentrations before use. Test-compound solutions were made at a concentration of 5 mmol/L with ethanol and also diluted to working concentrations before use. An annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit I from BD PharMingen (San Diego, CA, U.S.A.) was used.

Cell Culture Human melanoma cell line A375 cells (ATCC No. CRL-1619) and mouse melanoma cell line B16F10 cells (ATCC No. CRL-6475) were obtained from the American Type Cell Culture Collection (Manassas, VA, U.S.A.). Cells of both lines were cultured in the same way as follows: cultured in DMEM supplemented with 10% fetal bovine serum, 100000 IU/L penicillin, and 100 μg/mL streptomycin at 37°C in a humidified incubator with 5% CO2. For our experimental studies, the cells were grown to 90% confluence, harvested with 0.025% trypsin and 0.05% EDTA in a phosphate-buffered saline (Gibco BRL Co.), plated at a desired density, and allowed to re-equilibrate for 24 h before any treatment. All experiments were conducted in DMEM containing 10% fetal bovine serum, 100000 IU/L penicillin, and 100 μg/mL streptomycin.

Cell Proliferation Assay Proliferation of the A375 cells and the B16F10 cells was tested by WST-8 assay procedures (Promega, Madison, WI, U.S.A.). Briefly, they were plated at a density of 1×104 cells/well in 100 μL of medium in 96 flat-bottom wells of a microtiter plate, with the exception of the medium control wells. After incubation for 3 h at 37°C in a humidified incubator with 5% CO2, the microtiter plates were added with final agent concentrations of 0.1, 1, 10, or 100 μmol/L, and the plate was incubated for an additional 24, 48, or 72 h at 37°C in a humidified incubator with 5% CO2. Two microliters of Cell Counting Kit-8 reagent solution was added to each well, and the plate was incubated for another 2 h. Cell growth was determined by measuring the optical density at 450 nm ab-
sorbance (ref. 630 nm).

**Analysis of Apoptotic Cells**  After the A375 cells (2×10^5/mL) were incubated in the presence of the indicated concentrations of the agents for 72 h in 5% CO_2/air at 37°C, 1 mL of this cell suspension was placed in a 1.5 mL tube, and the cells were washed twice in cold phosphate-buffered saline (pH 7.2). Cells in each tube were resuspended with 89 μL of phosphate-buffered saline containing 1% fetal bovine serum, and 1 μL of annexin V-FITC solution and 10 μL of propidium iodide (PI) solution were added to the tube (total volume was 100μL). Then, the cells were incubated for 15 min at room temperature in the dark. Subsequently, 400μL of binding buffer was added to each tube, and the cells were analyzed by flow cytometry within 1 h after staining. The data were analyzed with a FACSCant flow cytometer (Becton Dickinson) using FACS Diva software (Becton Dickinson). The fluorescence intensity of the FITC was detected on FITC channel (515—545 nm) of the FACSCant. PI fluorescence was detected on PerCP-Cy5.5 channel (more than 670 nm). Forward- and side-scatters were used to gate the cell population of the A375 cells. A minimum of 30000 events of the cells ungated were collected per sample.

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**Fig. 1. Effect of Lipophilic Vitamins on Cell Viability of A375 Cells in Vitro**

Cells were treated with serial concentrations of VA (ATRA), VD_3, VE, VK_1, VK_3, and VK_5 for 24 (A), 48 (B), and 72 (C) h, respectively. Then, cell viability was determined by WST-8 assay. Data are expressed as percent of viable cells relative to the viability of control cells treated with the vehicle alone (n=5, mean±S.E.). Asterisk indicates a significant difference between the control cells and the lipophilic vitamin-treated cells (*p<0.01).
Caspase-3/7 Activity Assay  A375 cells were incubated with the test agents for 4, 12, and 24 h, respectively. Thereafter, 100 μL of Caspase-Glo 3/7 (Promega, Madison, WI, U.S.A.) reagent was added to each sample, and the cells were incubated for 30 min and assayed according to the manufacturer's instructions.

Statistical Analysis  Comparison of the data in multiple groups was carried out by Bonferroni/Dunn multiple comparison. These analyses were performed with Graph Pad Prism 4.0. In each case, two-sided p values less than 0.05 were considered to be significant.

RESULTS

Effects of Lipophilic Vitamins on A375 Cell Proliferation in Vitro  The effects of the six lipophilic vitamins ATRA, VD₃, VE, VK₃, VK₁, and VK₅ on the cell proliferation of human A375 melanoma cells in vitro were examined by the WST-8 assay procedure. The percentages of viable A375 cells incubated in the presence of VD₃, VK₅, and VK₅ for 24, 48 and 72 h are shown in Fig. 1. VD₃ inhibited the cell proliferation significantly at 100 μmol/L (p < 0.01), while at lower concentrations (0.1—10 μmol/L), it did not exhibit any significant effect after 24 h culture. When the cells were cultured for 48 or 72 h in the presence of 50 μmol/L of VD₃, cell viability also decreased significantly (p < 0.01).

VK₃ and VK₅ inhibited the cell proliferation significantly at a concentration range of 10—100 μmol/L after 24—72 h of culture (p < 0.01). On the other hand, the other vitamins did not show any inhibitory effects even at concentrations of 100 μmol/L. Thus, VK₅ and VK₅ exhibited the strongest effects with IC₅₀ values of less than 10 μmol/L.

Since A375 cells are amelanotic, we also examined the effects of VK₃ and VK₅ on in vitro proliferation of mouse eumelanotic melanoma B16F10 cells (Fig. 2). The cells were incubated for 72 h in the presence of serial concentrations of VK₃ and VK₅ in vitro, and the cell proliferation was examined by the WST-8 assay procedure. Both of these vitamins inhibited the cell proliferation dose-dependently, and the effects of VK₅ were somewhat stronger than those on the proliferation of human melanoma A375 cells (Fig. 1C).

Next, we examined the morphological changes of A375 cells treated with VK derivatives. The cells were incubated in the absence or presence of 5 or 100 μmol/L of VK₃ or VK₅, and the cell morphology was observed by light microscopy (Fig. 3). The untreated (control) cells grew by attaching to the bottom of the plate with long and narrow shapes. The cells treated with 5 μmol/L of the VK derivatives for 24 h...

Fig. 2. Effect of Lipophilic Vitamins on Cell Viability of Mouse Melanoma B16F10 Cells in Vitro

Cells were treated with serial concentrations of VK₃ (circle) and VK₅ (triangle) for 72 h, and cell viability was determined by WST-8 assay. Data are expressed as percent of viable cells relative to the viability of control cells treated with the vehicle alone (n = 3, mean ± S.E.).

Fig. 3. Morphological Changes of A375 Cells Treated with VK Derivatives

Cells were incubated in the presence of the vehicle alone (A), 5 μmol/L VK₃ (B), 100 μmol/L VK₅ (C), 5 μmol/L VK₅ (D), and 100 μmol/L VK₅ (E), respectively, for 24 h and cell morphology was observed by light microscope.
showed little morphological change, as compared to the morphology of the control cells. However, the cells treated with 100 μmol/L VK₅ or VK₆ for 24 h detached from the bottom of the plate and assumed a round cell shape (Figs. 3C, E). B16F10 cells treated with 100 μmol/L VK₃ or VK₆ also showed similar morphological changes (data not shown). A375 cells treated with ATRA, VD₃, VE, or VK₆ did not show any apparent morphological changes as compared to the morphology of the control cells (data not shown).

Dacarbazine is used clinically for the treatment of metastatic melanoma. The agent appeared to inhibit the proliferation of A375 cells in vitro at a concentration range of 25—100 μmol/L, but the effects were not statistically significant (Fig. 4A). Then, we examined the co-administered effects of VD₃, VK₃, and VK₅ with dacarbazine on the proliferation of A375 cells. The cells were cultured in the presence of vitamins with or without 25 μmol/L dacarbazine, and the percentage of viable cells was estimated by WST-8 assay procedures (Fig. 4B). Dacarbazine co-administration tended to increase the antiproliferative efficacy of these vitamins, and a statistically significant additive effect was observed with dacarbazine plus 5 μmol/L VK₃, as compared to the effect of VK₃ alone (p<0.05) (Fig. 4B).

VK₃ and VK₅ induce Apoptosis in A375 Cells As described above, VK₃ and VK₅ showed the strongest effects on the proliferation of A375 in vitro at concentrations of less than 10 μmol/L. Therefore, we examined the apoptosis inducing effects of VK₃ and VK₅, in addition to the effects of VD₃ and dacarbazine, by estimating annexin-V positive apoptotic cells with flow cytometry, as well as determining caspase-3/7 activity, in the agent-treated cells.

Typical dot plot data of flow cytometric analysis of A375 cells treated with VK₃, VK₅, VD₃, or dacarbazine (DTIC) followed by staining with annexin V/PI are shown in Fig. 5A. A375 cells incubated with 5 or 100 μmol/L of the VK derivatives for 24 h showed that annexin-V positive and PI negative cells (apoptotic cells) increased after treatment with ATRA, VD₃, VE, and VK₁, have reported that the IC₅₀ value of dacarbazine against the growth of A375 cells was higher than 548 μmol/L. Thus, A375 cells appear to be resistant to the suppressive effects of dacarbazine. Accordingly, the data presented above showed that VD₃, VK₃, and VK₅ suppress A375 cell proliferation in vitro at a concentration range of 10—100 μmol/L, while other lipophilic vitamins, ATRA, VE, and VK₁, have little suppressive efficacy against the proliferation of A375 cells. It has been reported that the IC₅₀ value of dacarbazine against the growth of A375 cells in vitro was higher than 548 μmol/L. Thus, A375 cells appear to be resistant to the suppressive effects of dacarbazine. According to the data of the present study, together with the observations of other researchers, the suppressive effects of VD₃, VK₃, and VK₅ against the proliferation of A375 cells in vitro are more than five times stronger than that of dacarbazine. In particular, VK₃ and VK₅ showed significant antiproliferative efficacies at a concentration range of less than 10 μmol/L. The antiproliferative effects of these two vitamins

DISCUSSION

A375 is a highly metastatic human amelanotic cell line, and we therefore chose it to examine the suppressive efficacies of lipophilic vitamin derivatives, which in turn may reflect a clinical response. The data presented above showed that VD₃, VK₃, and VK₅ suppress A375 cell proliferation in vitro at a concentration range of 10—100 μmol/L, while other lipophilic vitamins, ATRA, VE, and VK₁, have little suppressive efficacy against the proliferation of A375 cells. It has been reported that the IC₅₀ value of dacarbazine against the growth of A375 cells in vitro was higher than 548 μmol/L. Thus, A375 cells appear to be resistant to the suppressive effects of dacarbazine. According to the data of the present study, together with the observations of other researchers, the suppressive effects of VD₃, VK₃, and VK₅ against the proliferation of A375 cells in vitro are more than five times stronger than that of dacarbazine. In particular, VK₃ and VK₅ showed significant antiproliferative efficacies at a concentration range of less than 10 μmol/L. The antiproliferative effects of these two vitamins
Fig. 5. Flow Cytometric Analysis of Apoptotic A375 Cells Treated with Lyophilic Vitamins

(A) Typical dot plot data for apoptotic A375 cells treated with VK₃, VK₅, VD₃, or dacarbazine (DTIC) for 24 h, stained with annexin V (abscissa)/PI (ordinate), and analyzed by flow cytometry. (B, C) Quantitative determination of apoptotic A375 cells treated with VD₃, VK₃, VK₅, or dacarbazine using annexin V/PI staining followed by flow cytometry. Cells were cultured in the presence of the indicated concentrations of the agents for 4 h (A) and 24 h (B), respectively, and the percentages of apoptotic cells were calculated (n=4, mean±S.E.). Asterisk indicates a significant difference between control- and VK₃ or VK₅ treated cells (*p<0.001).
were also observed in mouse eumelanotic B16F10 melanoma cells. The effects of VK₃ on B16F10 cells were rather stronger than those of the agent on A375 cells. Thus, the efficacies of these vitamins appear not to be specific to amelanotic A375 cells. Our findings also suggest that VK₃ and VK₅ influenced cell morphology, and inhibited A375 cell growth by inducing apoptosis, whereas the suppressive mechanisms of VD₃ and dacarbazine are suggested to be different from those of VK derivatives.

Lipophilic vitamins have been examined for their anticancer properties by many researchers.³⁻¹¹ ATRA is clinically used for the treatment of promyelocytic leukemia,³ however, the drug showed little suppressive efficacy against A375 melanoma cells in the present study. The apoptosis-inducing effects of VE succinate in bladder cancer cells,⁷ human promyelocytic leukemia HL-60 cells,¹⁰ and EL4 thymic lymphoma⁷ have been previously reported, however, in the present study it was found that VE has less efficacy against A375 cell growth. The anticancer efficacies of VD derivatives have been suggested in several cancer models including melanoma,⁴⁻⁶,¹⁰ and the results of the present study support these observations, though VD₃ concentration inhibiting A375 proliferation was relatively high (100μmol/L). Our data suggested that 20μmol/L VD₃ increased apoptotic cells, whereas a higher concentration (50μmol/L) had little effect on inducing apoptosis in A375 cell culture (Fig. 5). It has been reported that VD₃ induces differentiation in cancer cells,¹⁰ and thus VD₃ at higher concentration (50μmol/L) is suggested to induce differentiation in A357 cells in the present study. Alternative explanation would be that the higher concentration of VD₃ resulted in cell disruption and subsequent increase in number of cell debris instead of apoptotic bodies (Fig. 5A). The antitumor effects of VK₂, VK₃, and VK₅ against colorectal cancer¹⁹ and hepatocellular carcinoma⁸ models have been reported. Moreover, VK₂ and VK₅ have been shown to have apoptosis-inducing effects in human tumorigenic cells.¹⁹,²⁰ VK₂ was reported to induce apoptosis in a cell line established from a patient with myelodysplastic syndrome in blastic transformation,²¹ while VK₂, VK₃,

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Fig. 6. Activation of Caspase-3/7 in A375 Cells Induced by VD₃, VK₃, VK₅, and/or Dacarbazine (DTIC) Treatment for 4 (A), 12 (B) and 24h (C), Respectively

Data are expressed as percent caspase-3/7 activity relative to the activity of control cells treated with vehicle alone (n=4, mean±S.E.). Asterisks indicate significant differences between control- and VK₃, VK₅, or combined drug-treated cells (*p<0.05, **p<0.01, ***p<0.001).
and VK₃ exerted antitumor effects on established colorectal cancer in mice by inducing apoptotic death of tumor cells.²⁹ Few studies, however, have been conducted to examine the effects of VK derivatives on human melanoma cells. Thus, the present study is the first to show, to the best of our knowledge, that the VK derivatives VK₅ and VK₇ suppress the proliferation of and induce apoptosis in human melanoma cells.

While the mechanisms of VK₅ and VK₇ anti-melanoma actions were not clarified in the present study, Shah et al. reported that ubiquitin ligase inhibition by menadione attenuates hypoxia and Ras/mitogen-activated protein kinase (MAPK) signaling, which results in blockade of melanoma tumorigenesis.²² On the other hand, Chowdhury et al. suggested that menadione enhanced arsenic induced apoptosis in malignant melanoma cells through ROS generation, p38 signaling and p53 activation.²³ Thus, blockades of the above cellular signaling cascades in combination are suggested to be part of the underlying mechanisms of anti-melanoma effects of VK derivatives observed in the present study.

Among the VK derivatives, phytodienone (VK₇) and menatetrenone (VK₃) have been clinically used for the treatment of osteoporosis. These vitamins are used through intravenous injection, as well as oral administration, at doses of between 10—20 mg, and the blood concentration in humans after a 10 mg intravenous administration can reach approximately 1—10 μmol/L without serious side effects.²⁴,²⁵ Thus, although rapid intravenous administration of VK may be associated with side-effects including facial flushing, chest constriction, and cyanosis, and VK concentrations used in the present study in vitro may be achievable in clinical cases via intravenous administration.

Dacarbazine is used as a standard chemotherapy for treatment of patients with metastatic melanoma; however, systemic therapies with dacarbazine alone are known to be ineffective in many cases because of high resistance of melanoma cells to various anticancer therapies including dacarbazine.¹⁵ Thus, at present there are no treatment options available for patients with advanced melanoma providing either sufficient response rates or a significant prolongation of overall survival. The antiproliferative mechanism(s) of dacarbazine against cancer cells is mainly related to the methylation of O(6)-guanine, mediated by a methyldiazonium ion, a highly reactive derivative of the two compounds.²⁶ Our present data suggest that A375 cells also exhibit resistance to the growth-suppressive effect of dacarbazine (Fig. 4A), for the IC₅₀ value of the drug against the in vitro proliferation of the cells was higher than 100 μmol/L. Moreover, dacarbazine did not induce apoptosis in A375 cells in the present study (Figs. 5, 6). In addition, dacarbazine did not promote significantly the apoptosis-inducing effects of these vitamins (Figs. 1, 5B,C). However, co-administration of VD₃, VK₅, and VK₇ with dacarbazine tended to enhance the suppressive efficacies on cell viability, and a statistically significant effect was observed by the combination of VK₅ and dacarbazine, as compared to the effect of dacarbazine alone (Fig. 4B). VK₃ induced apoptosis in A375 cells (Figs. 5, 6), and thus the action mechanisms of VK₃ and dacarbazine are suggested to be different.

The present study, in conclusion, showed that VD₃, VK₅, and VK₇ inhibited the growth of dacarbazine resistant human melanoma cells, while ATRA, VE, and VK₇ had little effect on the cell growth. The effects of VK₅ and VK₇ were observed at concentrations lower than 10 μmol/L, which are suggested to have resulted from apoptosis-induction in the melanoma cells.

REFERENCES


