Journal of Cellular Biochemistry 97:198–206 (2006)

Anticancer Effects of the Novel 1α, 25-Dihydroxyvitamin D3 Hybrid Analog QW1624F2-2 in Human Neuroblastoma

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Abstract Vitamin D3 analogs are potential anti-cancer agents with theoretically wide therapeutic index, but there have been limited studies directed towards human neuroblastoma. The antiproliferative ability of the novel vitamin D3 hybrid analog QW-1624F2-2 (QW, 1-hydroxymethyl-16-ene-24, 24-F2-26, 27-bishomo-25-hydroxyvitamin D3) was examined in two human neuroblastoma-derived cell-lines. Analog QW inhibited cell-cycle progression of IMR5 cells with accumulation in G1 phase. QW induced the differentiation of CHP134 as evidenced by increased neurite length. These effects were accompanied by decreased expression of *MYCN* in both the cell-lines treated with QW. Furthermore, QW inhibited the migration of CHP134 cells in matrigel invasion assays, indicating its anti-invasive ability. In athymic nude mice, we found that QW was less calcemic than EB1089 (1α, 25-dihydroxy-22, 24-diene-24, 26,27-trishomovitamin D3). Systemic administration of QW in a mouse xenotransplantation model revealed that it is more effective than EB1089 in suppressing the growth of CHP134 flank tumors. In summary, the low-calcemic hybrid analog QW showed significant anti-tumor activity in vivo and thus exhibits potential as a novel cancer therapeutic. J. Cell. Biochem. 97: 198–206, 2006. © 2005 Wiley-Liss, Inc.

Key words: low-calcemic; vitamin D3 analog; anticancer; neuroblastoma

Neuroblastoma is a childhood tumor of the peripheral nervous system, with primary tumors commonly arising throughout the sympathetic system including the adrenal medulla. Fifty percent of the children with neuroblastoma have metastatic disease at diagnosis, and survival in this cohort is less than one-third [Kushner and Cheung, 1998; Sidell, 1998; Reynolds and Seeger, 2000]. Patients with neuroblastoma present with a heterogeneous mode of disease defined as low, intermediate, and high-risk groups. The low-risk group is characterized by histologically differentiated localized tumors that lack *MYCN* amplification. The high-risk group is

Abbreviations used: VDR, Vitamin D receptor; RA, Retinoic acid; MTT, 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; QW, 1-hydroxymethyl-16-ene-24,24-F2-26,27-bishomo-25- hydroxyvitamin D3; EB 1089, 1α,25-dihydroxy-22,24-diene-24,26,27-trishomovitamin D3.

Grant sponsor: NIH; Grant number: CA 93547.

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Received 9 February 2005; Accepted 27 July 2005
DOI 10.1002/jcb.20629

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characterized by metastatic tumors with poorly differentiated cells and often (40–50%) containing amplification of the *MYCN* proto-oncogene with a poor response to chemo- and radiation-therapy [Maris and Matthay, 1999]. In spite of multiagent therapy and bone marrow transplantation, most high-risk patients die due to disease. In previous studies, Matthay et al. [1999] and Reynolds and Lemons, [2001] used 13-*cis* RA to prevent tumor recurrence as part of a maintenance therapy approach. Studies by Weinstein et al. [2003] suggest that despite the improved survival rates achieved with 13-*cis* RA, patients develop RA resistance. Development of agents with fewer side effects that can also be effective in retinoid-resistant neuroblastoma will have high therapeutic value.

A combination of retinoids and vitamin D3 analogs have been shown to inhibit the proliferation of neuroblastoma cell-lines with down regulation of N-Myc and C-Myc [Wang et al., 2000; Stio et al., 2001]. Vitamin D3 analogs have been shown to be effective in several human cancers [Norman et al., 1990; Ettinger and DeLuca, 1996; Moore et al., 1999]. However, due to the fact these agents often induced dose-limiting hypercalcemia, clinical utility is limited. Among the recent analogs, Peleg et al. [1995] found that 24 oxa, 20-epiD, and 3-epiD are potent in inducing cell death. Mathiasen et al. [1993] and Colston et al. [1992] found that EB1089 (1α, 25-dihydroxy-22, 24-diene-24,26,27-trishomovitamin D3) was effective in several malignant cells. OCT (22-oxa-1, 25-dihydroxyvitamin D3) exhibits significant anticancer activity without inducing hypercalcemia [Abe-Hashimoto et al., 1993]. Posner [2002] and Kensler et al. [2000] found that QW (does not contain the natural 1-hydroxyl group) was effective in inhibiting the proliferation of skin, prostate, and breast cancer cells. QW has been shown to exhibit [Posner et al., 1998, 2004; Peleg and Posner, 2003] 80–100 times less calcemic activity than calcitriol and also non-genotoxic. In view of these observations, this study was aimed at examining the anticancer efficacy of QW in human neuroblastoma.

MATERIALS AND METHODS

Cell Culture and Compounds

Human neuroblastoma cell lines (IMR-5, CHP-134, SH-SY5Y, and NB-69) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 1% OPI, 100 U/ml penicillin, and 100 μg/ml streptomycin. Earlier [Sivak et al., 1997], it was shown that IMR-5 and CHP-134 contain amplification of *MYCN* locus. The cell-lines used in the current study were kept at less than 20 passages. EB1089 was a gift from Leo Pharmaceuticals, Denmark. Vitamin D3 analogs were dissolved in absolute ethanol at 10^{-3} M as stock solution, and stored at −20°C protected from light. The concentration of the analogs was determined by UV absorbance measurements using their molar extinction coefficient at 264 nm. Dilutions were made in the culture medium for various treatments.

Western Blot Analysis

Cells growing in mid-log phase were washed with PBS and transferred to lysis buffer (HEPES pH 7.6: 25 mM; Triton X100: 0.1%; NaCl: 300 mM; β-glycerophosphate: 20 mM; $MgCl_2$: 1.5 mM; EDTA: 0.2 mM; DTT: 0.2 mM; sodium orthovanadate: 0.2 mM; sodium fluoride: 10 mM; benzamidine:1 mM; leupeptin: 2 μg/ml; aprotinin: 4 μg/ml, and PMSF: 500 μM), and centrifuged at 10,000*g* for 30 min at 4°C. The protein concentration in the supernatant was determined by bicinchoninic acid method (Pierce). Total protein (75 μg) from the whole cell lysate was electrophoresed in 10% SDS–PAGE gels and transferred to nitrocellulose membrane. The blots were blocked with 5% non-fat dry milk in Tris-buffered saline and probed with N-Myc antibodies (gift from Dr. Ikegaki, Emory University, Atlanta). Specific antibody binding was detected by horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibodies and visualized with ECL reagent (Amersham Pharmacia Biotech).

Cell Viability Analysis

The effect of QW on the proliferation of neuroblastoma cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method [Mosmann, 1983] according to manufacturer's instructions (Promega). Briefly, 1×10^3 cells were seeded in 96-well plates and treated with either vehicle only (ethanol 0.1%) or 7.5 μM QW for 6 days (two times 24 h and 72 h). MTT reagent (10 μl/well) was added and incubated at 37°C for 3–4 h and the absorbance of the samples was measured at 490 nm. Each experiment was repeated three times in triplicate samples.

Cell Cycle Analysis. CHP 134 and IMR5 cells (4×10^5) were seeded in 100mm plates. After 24 h, the cells were treated with ethanol or 7.5 μM QW and fresh medium containing QW was added after 72 h. Cells were harvested for cell-cycle analysis on day 6. The cells were washed with PBS, fixed in 70% ethanol, stored at $-70°C$, and stained with propidium iodide. Modfit program was used to determine the distribution of cells in different phases of the cell cycle.

Differentiation and Neurite growth. Control and QW-treated CHP134 cells were photographed on day 6, and 20 random fields were chosen for quantification of neurite length. The mean neurite length was measured from 150 cells and expressed as mean ± SE. *P*-value was calculated using student's *t*-test.

Cell Migration Assays

CHP134 and IMR5 cells (2×10^5) were seeded in six well-plates and allowed to adhere overnight. Next day, the cells were treated with 7.5 μM of EB1089 or QW. After 48 h, the cells were trypsinized and in vitro migration of the cells was measured by the invasion of cells through matrigel-coated (Collaborative Research, Boston, MA) transwell inserts (Costar, Cambridge, MA). Briefly, transwell inserts with an 8-μm pore size were coated with a final concentration of 0.7 mg/ml of matrigel, cells were trypsinized, and 200 μl of cell suspension ($\sim 3 \times 10^5$ cells/ml) were added in triplicate wells. After a 48 h incubation period, the cells that passed through the filter into the lower wells were quantified by computer-assisted image analysis with the Image-Pro Discovery program.

In Vivo Toxicity Experiments and Serum Calcium Determination

Three groups of athymic nude mice (6 animals/group) were injected (i.p) with vehicle alone, QW, or EB1089 (0.5 μg/kg) everyday for 2 weeks. Body weight was monitored every alternate day and serum calcium was determined at the end of 1- and 2 weeks. Serum calcium was estimated using a calcium assay kit from Sigma.

Mouse Xenograft Studies

The effect of EB1089 and QW on the growth of CHP134 flank xenograft tumors was assessed in female athymic nude mice. CHP134 cells (1.5×10^7 cells/mouse) growing in log phase were harvested and mixed with matrigel on ice and injected subcutaneously, and the tumors were allowed to grow up to $\sim 0.2 cm^3$ (3–4 weeks). Three groups of athymic nude mice (6/group) were injected everyday with the vehicle alone, or with EB1089 or QW (0.5 μg/kg body weight) for up to 4–5 weeks. The body weight and tumor volume were measured 3 times/week. The tumor volume was calculated as (Length (mm)) $\times$ $(\text{Width})^2/2$. Tumor tissues were isolated for histopathological analysis (H&E staining).

RESULTS

Effect of QW on Survival of NB Cells

The antiproliferative ability of QW was examined in CHP134, IMR5 (*MYCN*-amplified), and SH-SY5Y and NB69 (*MYCN* non-amplified) cell-lines. At lower concentrations (1–2 μM), QW did not show growth inhibition in MTT assays. We observed about 25% decrease in proliferation of CHP134 and IMR5 when exposed to 7.5 μM concentration of QW for 6 days. However, these concentrations were ineffective ($<10\%$ inhibition) in SH-SY5Y and NB 69 cells (data not shown). It is possible that N-*myc* amplified cell-lines (CHP134 and IMR5) may be more sensitive to QW. Because CHP134 and IMR5 cells exhibited consistently higher sensitivity to QW, further studies were performed with these cell lines.

Effects of QW on Cell Cycle and Differentiation

To assess the ability of QW to inhibit cell-cycle-progression, CHP134 and IMR5 cells were treated with 7.5 μM QW for 6 days, and then analyzed by flow cytometry. QW caused accumulation of IMR5 cells in the G1 phase of the cell-cycle (Fig. 1A, Table I). Morphological appearance of differentiation was not observed in IMR5 cells treated with QW. The cell-cycle-arrest was only marginal in CHP134 cells. However, we observed morphological appearance of neurite outgrowth in CHP134 cells exposed to QW (Fig. 1B). Quantification of neurite length suggests that QW induces significant increase (~80–90%) in the mean neurite length compared to control (Fig. 1C).

QW Down-Regulates N-Myc

Next, we tested the effect of QW in modulating N-Myc expression. Treatment with 7.5 μM QW for 6 days decreased N-Myc (Fig. 2). C-Myc

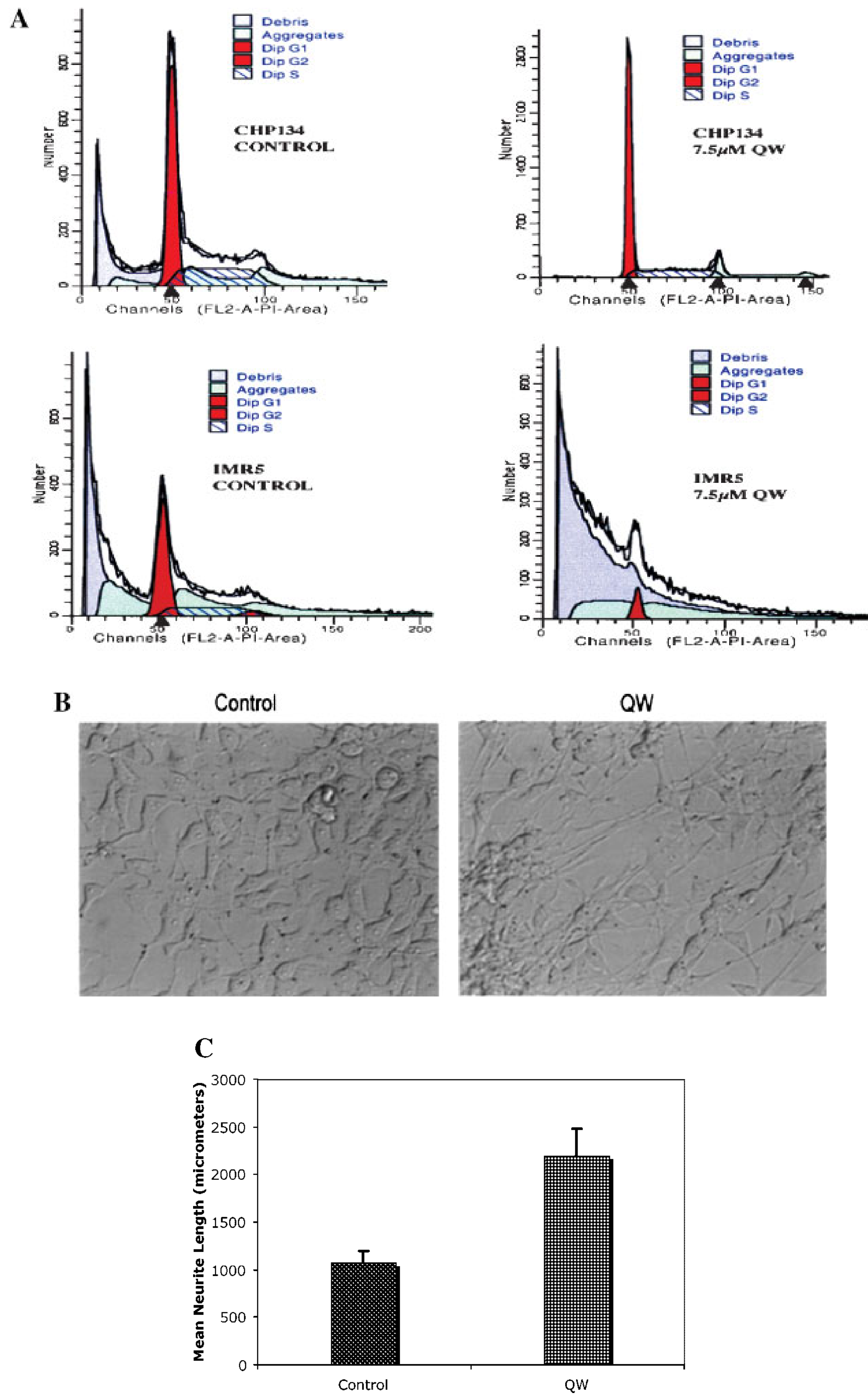


Fig. 1.

TABLE I. Cell-Cycle Analysis of CHP134 and IMR5 Cells Treated With 7.5μM QW for 6 Days (24 h and 72 h). Cells Were Stained With Propidium Iodide and Subjected to FACS Analysis. The Distribution of Cells in Different Phases of the Cell Cycle is Indicated. The cv Values for the Samples Were in the Range of 2.4–6

Neuroblastoma cell-lines	G1 (%)		G2 (%)		S (%)	
	Control	QW	Control	QW	Control	QW
CHP134	56	68	0	0	39	32
IMR5	63	97	5.5	0	31	3.0

expression was not altered in SH-SY5Y and NB 69 cells treated with QW (data not shown). These results suggest that QW may be acting specifically on neuroblastoma cells that express high N-Myc. Additional studies in multiple neuroblastoma cell-lines are necessary before this hypothesis can be confirmed.

QW Inhibits the Migration of Neuroblastoma Cells in Matrigel Invasion Assay

The ability of QW to inhibit cell migration was assayed in vitro using transwell chambers. After a 48 h incubation period, cells that passed through the filter into the lower wells were quantified and expressed as a percentage of the sum of cells in the upper and lower wells. Cells on the lower side of the membrane were fixed, stained with Hema-3, and photographed. The invasive potential of the CHP134 and IMR5 cells treated with QW (7.5 μM) was compared with cells treated with EB1089 (7.5 μM). CHP134 (Fig. 3A, top panel) and IMR5 cells (Fig. 3A, bottom panel) treated with QW that invaded through the transwell inserts were considerably less compared to untreated cells. Anti-invasive potential of QW was similar and comparable to EB1089. Quantitative assessment of random fields indicated 37% of CHP134 and 30% of IMR5 cells treated with QW invaded through matrigel compared to control cells. CHP134 and IMR5 cells treated with analog EB1089 invaded 27% and 20%, respectively. These results suggest that EB 1089 might be moderately more effective than QW in inhibiting the migration of neuroblastoma cells. In contrast, the in vivo studies revealed that EB1089 was calcemic at > 0.5 μg/kg body weight [Binderup, Personal communication] while QW at these concentrations did not exhibit calcemic activity (Table II).

Effect of QW on Serum Calcium

Previous studies [Posner, 2002] indicated that QW is less calcemic compared to many of the currently available vitamin D3 analogs. The in vivo effects of QW were assessed by monitoring serum calcium and body weight after administering QW and EB1089 (0.5 μg/kg body weight). EB1089 was selected as a reference based on its proven in vivo anti-tumor ability in other cancers [Zhang et al., 2005]. These results (Table II) suggest that EB1089 causes mild hypercalcemia with an insignificant decrease in body weight (data not shown). In contrast, the body weight and serum calcium remained near normal over a period of 2 weeks in QW administered group. The results are in agreement with other in vivo studies using a maximum dose of 0.5 μg/kg body weight of EB1089 [Dr.Lise Binderup, Leo Pharmaceuticals, personal communication]. Therefore, high concentration of EB1089 could not be used for in vivo studies as it may cause calcemic side effects at > 0.5 μg/kg. However, we found that higher doses of QW (4.0 μg/kg) injected every day up to 2 weeks did not cause hypercalcemia suggesting that high doses of QW may not cause adverse side effects (data not shown).

Fig. 1. Effect of QW on cell cycle and differentiation. **A**: The growth inhibitory effect of QW on CHP134 and IMR cells was determined by cell cycle analysis. Cells (4×10^5) growing in log phase were treated with QW (7.5 μM) for 6 days (two treatments at 24 h and 72 h). The cells were washed with PBS twice, fixed in 70% alcohol, stained with propidium iodide, and subjected to FACS analysis. **B**: Differentiation of CHP134 cells: After 6 days of exposure to 7.5 μM QW (24 h and 72 h), control and treated cells were photographed at 20× magnification. **C**: Neurite measurement: Neurite length was calculated from 150 cells selected from randomly chosen fields of control and QW treated CHP134 cells. Mean neurite length is expressed as mm (μm) ± SE. Control 1072 ± 123 μm; QW:2195 ± 277 μm. The *P*-value (<0.004).

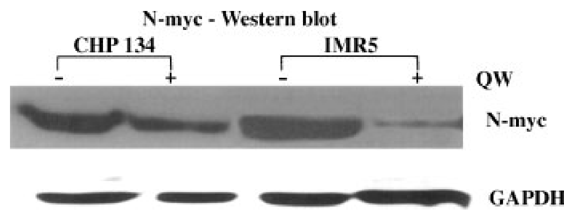


Fig. 2. QW down-regulates N-Myc expression: CHP134 and IMR5 cells were exposed to (7.5 μM) QW for 6 days and the cell lysates (75 μg protein) were subjected to SDS–PAGE and Western blot analysis and probed with N-Myc monoclonal antibodies.

Effect of QW on CHP134 Xenograft Tumor Growth

The antitumor effect of QW was assessed in athymic nude mice bearing CHP134 s.c tumors. Cells (1.5×10^7 cells/mouse) were injected and when the tumor reached a size of 0.2cm^3 (~3 weeks), six mice in each group received i.p injections of either vehicle alone, or EB1089 or QW. The body weight, and the tumor volume were recorded 3 times/week starting from the first injection of the vitamin D3 analogs. All mice injected with the EB1089 or QW showed tumor growth inhibition beginning on day 10 after the first injection, and the inhibitory effects continued for 20 days, at which time ~60–75% of inhibition was observed in EB1089 and QW treated groups (Fig. 4A,B). The photographs (Fig. 4A) were taken after 20 days after the first injection of EB1089 or QW. Figure 4B shows the growth of the tumor expressed as volume of the tumor at different time points (day 15 and 20). The two data points were selected as maximum growth inhibition was observed with EB1089 during this period. The tumor volume in EB1089-treated mice was similar to control group after 5 weeks (data not shown) indicating that the effect of EB1089 may be transient. In contrast, we observed significant inhibition of tumor growth beyond 6 weeks in QW-treated mice. Analysis of tumors (H&E staining) suggest that QW-treated tumors

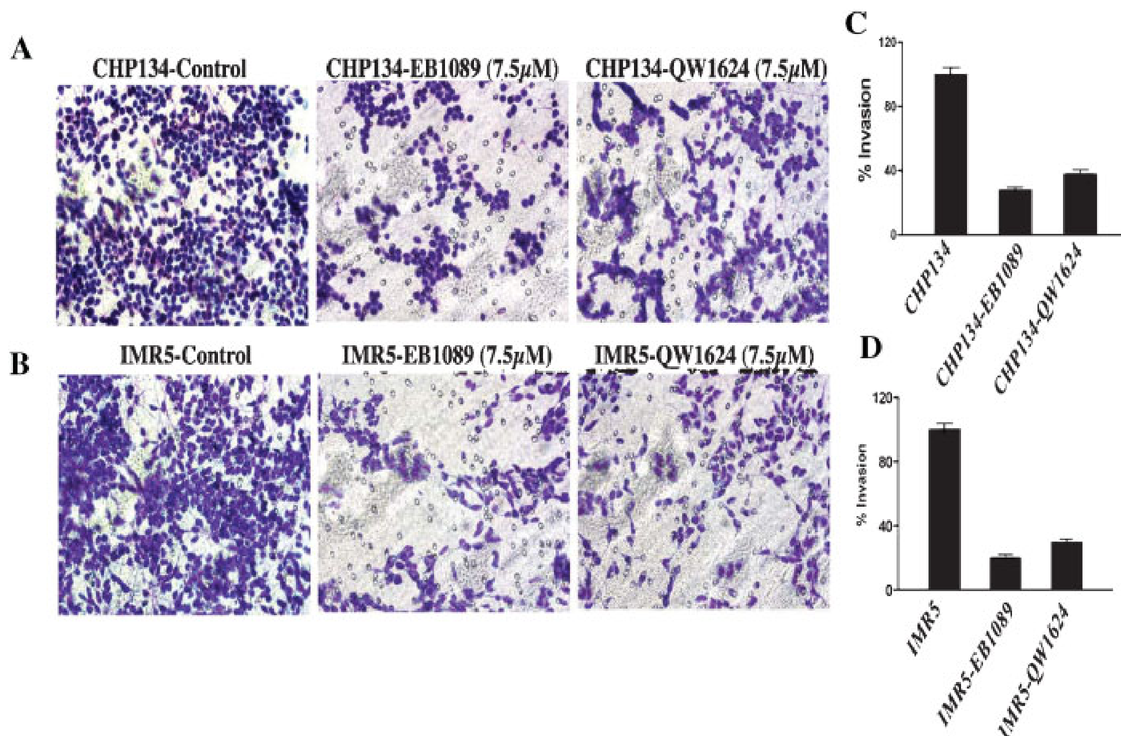
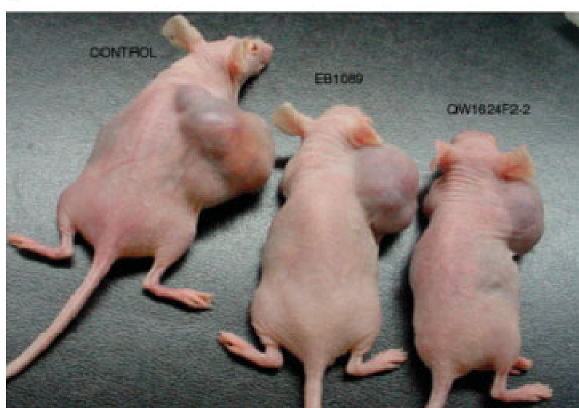


Fig. 3. Effect of QW on invasion of neuroblastoma cells in matrigels: QW inhibits the invasion of neuroblastoma cells in matrigel invasion assay. ~3×10^6 cells of CHP134 and IMR5 cells were layered on matrigel-coated transwell inserts (8-μm pores) and allowed to invade for 48 h. Invasion was calculated as a percentage of total cells. The filters were stained with Hema-3 (**A**) and invasion was quantified as described in Materials and Methods (**B**). Data shown are mean ± SD of three different experiments from each clone ($P < 0.001$). CHP134 (A) and IMR5 (B) cells treated with 7.5 μM EB1089 and QW. After 48 h, 3×10^5 cells were allowed to invade for 24 h through transwell inserts (8-μm pores) coated with matrigel. The cells that invaded through the membrane were stained, counted, and photographed under a light microscope at 20× magnification. Invasion was quantified by computer-assisted image analysis with the Image-Pro Discovery program by counting five random fields.

TABLE II. Effect of QW and EB1089 (0.5 μg/kg Body Weight) on Serum Calcium Levels in Athymic Nude Mice. The Compounds Were Administered (i.p) Every Day for 2 Weeks and Serum Calcium Concentration was Determined

Treatment group	Serum calcium (mg/dl) 1 week	Serum calcium (mg/dl) 2 weeks
Control	8.9 ± 0.4	9.3 ± 0.09
EB1089	12 ± 0.5	12.7 ± 1.6
QW	9.7 ± 0.12	8.4 ± 0.6

A



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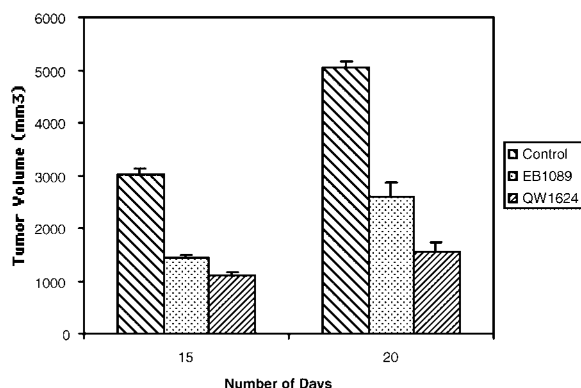

Fig. 4. QW inhibits CHP134 flank xenograft tumors. CHP134 cells were injected (1.5×10^7 cells/mouse) subcutaneously into athymic nude mice and the tumors were allowed to grow up to $0.2 cm^3$ (3–4 weeks). The mice (6/group) were injected (i.p) everyday with the vehicle alone, or EB1089 or QW (0.5 μg/kg body weight) up to 5 weeks. The tumor growth was measured 3 times/week using a Vernier calipers. The tumor volume was calculated $(\text{Length} \times \text{Width})^2/2$. **A**: It shows the photograph demonstrating the effect of QW and EB1089 on the growth of CHP134 cells grown as flank xenografts. The photographs were taken on day 20 after the first injection of the vitamin D3 analogs. **B**: It shows the tumor volume on day 15 and day 20 after the initial injection of the vitamin D3 compounds (*P*-values for all the data points were < 0.001).

contain more necrotic areas compared to control (data not shown).

DISCUSSION

Because dose-limiting hypercalcemia of the vitamin D analogs limits the therapeutic potential, identification of low-calcemic analogs is critical in vitamin D3-mediated anticancer therapies. Among the recently discovered vitamin D3 analogs EB1089, OCT, QW, and Ro-26-9228 might have clinical utility. The effect of these newer compounds in human neuroblastoma has not been examined. In the current study, we used 7.5 μM QW to achieve significant inhibition. This is a high concentration compared to many other studies [Guyton et al., 2001; Gumireddy et al., 2003] using much lower doses. But earlier investigators [Hershberger et al., 2001] have utilized up to 5 μM calcitriol in PC-3 prostate cancer cells in combination with paclitaxel. Significant accumulation of IMR5 cells in G1 phase of the cell-cycle was associated with down-regulation of N-Myc. Earlier, we have shown [Gumireddy et al., 2003] that 20-epi vitamin D3 and KH1060 decreased the expression of C-Myc in SH-SY5Y, NB69, and SK-N-AS cells. In addition, we also observed a decrease in N-Myc in IMR5 and NGP cells exposed to these analogs. However, the N-Myc expression was not altered in CHP134 cells treated with 20-epiD3 or KH1060. In this report, we show that QW is effective in decreasing N-Myc expression in CHP134 and IMR5 cells suggesting that N-Myc might be a potential target of QW, and this possibility needs further confirmation. Lack of response in NB69 and SH-SY5Y cell-lines to QW could be due to higher expression of 25-hydroxyvitamin D-24-hydroxylase that converts QW to biologically inactive metabolites. It has been shown earlier [Ly et al., 1999] that inhibitors of 24-hydroxylase increase the half-life of 25-hydroxyvitamin D. QW caused an increase in the number of neurite bearing cells and also the length of the neurites suggesting that long time exposure to QW might induce efficient differentiation that is comparable to RA-induced differentiation. Earlier [Blutt et al., 1997; James et al., 1997], it has been shown that some of the vitamin D3 analogs and retinoids exert synergistic effects in prostate cancer and leukemic cells. Synergistic inhibition of proliferation and decrease in c-Myc was observed by Stio et al. [2001], when SH-SY5Y cells were treated

with 9cisRA and vitamin D3. Our results show that QW down-regulates N-Myc in CHP134 and IMR cells. It is possible that in combination studies with retinoids, much lower concentrations of QW might be effective in neuroblastoma cells.

Complications that arise due to metastasis contribute to significant mortality in children with high-risk neuroblastoma. Koli and Keski-Oja [2000] have observed significant inhibition of the invasive capacity of MDA-MB-231 breast carcinoma cells in presence of vitamin D3 compounds. We found that QW inhibits CHP134 and IMR5 cell migration in transwell chambers indicating that QW is as effective as EB1089. But the low calcemic activity of QW offers distinct advantages over EB1089 and other vitamin D3 analogs.

In vivo experiments that monitor serum calcium suggest that QW is less calcemic than EB1089. In these studies, QW was administered i.p every day for 2 weeks without any adverse effects. The body weight of the mice remained normal and serum calcium levels did not cause any side effects. The in vivo efficacy data suggest that QW is more effective than EB1089 in suppressing CHP134 tumors. Its antitumor efficacy combined with non-calcemic effects (at 8 times higher dose than EB1089) suggest that higher concentrations of QW could be used. The differences in the effectiveness between the two analogs could be due to differential catabolism or differential binding of QW and EB1089 to vitamin D binding protein (DBP) or VDR. It is possible that EB1089 might be rapidly metabolized into inactive products whereas QW or its metabolites might be effective for longer periods of time. H&E staining showed more necrosis in QW treated tumors. We did not observe any apoptosis or morphological appearance of differentiation in these tumors. Analysis for markers of differentiation will identify whether QW-induced in vitro differentiation could also be observed in vivo. In combination treatments, QW might sensitize the neuroblastoma cells to other antitumor agents such as paclitaxel as observed by Hershberger et al. [2001] in prostate cancer cells. Additional studies demonstrating its antitumor efficacy in other *MYCN* amplified neuroblastoma cell-lines are essential with this new analog.

ACKNOWLEDGMENTS

We thank the Leo Pharmaceutical Co., Denmark for a generous gift of EB1089.

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