

# Synergistic Antileukemic Activity of Carnosic Acid-Rich Rosemary Extract and the 19-nor Gemini Vitamin D Analogue in a Mouse Model of Systemic Acute Myeloid Leukemia

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## Key Words

Acute myeloid leukemia · Carnosic acid · Differentiation · Hypercalcemia · Rosemary extract · Syngeneic mouse model · Vitamin D

## Abstract

**Objective:** Differentiation therapy with the hormonal form of vitamin D, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (1,25D<sub>3</sub>), is a promising approach to treatment of acute myeloid leukemia (AML); however, 1,25D<sub>3</sub> induces hypercalcemia at pharmacologically active doses. We investigated the in vitro and in vivo antileukemic efficacy of combined treatment with non-toxic doses of a low-calcemic 1,25D<sub>3</sub> analogue, 1,25-dihydroxy-21(3-hydroxy-3-methyl-butyl)-19-nor-cholecalciferol (19-nor-Gemini; Ro27-5646), and rosemary plant agents in a mouse model of AML. **Methods:** Proliferation and differentiation of WEHI-3B D<sup>-</sup> (WEHI) murine myelomonocytic leukemia cells in vitro were determined by standard assays. Reactive oxygen species, glutathione and protein expression levels were measured by flow cytometry, enzymatic assay and Western blotting, respectively. Systemic AML was developed by intravenous injection of WEHI cells in syngeneic

Balb/c mice. **Results:** 19-nor-Gemini had a higher potency than its parent compounds, Gemini (Ro27-2310) and 1,25D<sub>3</sub>, in the induction of differentiation (EC<sub>50</sub> = 0.059 ± 0.011, 0.275 ± 0.093 and 0.652 ± 0.085 nM, respectively) and growth arrest (IC<sub>50</sub> = 0.072 ± 0.018, 0.165 ± 0.061 and 0.895 ± 0.144 nM, respectively) in WEHI cells in vitro, and lower in vivo toxicity. Combined treatment of leukemia-bearing mice with 19-nor-Gemini (injected intraperitoneally) and standardized rosemary extract (mixed with food) resulted in a synergistic increase in survival (from 42.2 ± 2.5 days in untreated mice to 66.5 ± 4.2 days, n = 3) and normalization of white blood cell and differential counts. This was consistent with strong cooperative antiproliferative and differentiation effects of low concentrations of 19-nor-Gemini or 1,25D<sub>3</sub> combined with rosemary extract or its major polyphenolic component, carnosic acid, as well as with the antioxidant action of rosemary agents and vitamin D derivatives in WEHI cell cultures. **Conclusion:** Combined effectiveness of 1,25D<sub>3</sub> analogues and rosemary agents against mouse AML warrants further exploration of this therapeutic approach in translational models of human leukemia.

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## Introduction

A characteristic feature of acute myeloid leukemia (AML) is a defect in the capacity of myeloid progenitors to mature into non-replicating adult cells. Despite recent advancements in our understanding of the etiology and pathophysiology of AML, therapy of this disorder remains a tremendous challenge and it continues to have the lowest survival rate of all leukemias. One of the major obstacles in the management of AML is that the current chemotherapeutic drugs are highly toxic and poorly tolerated, particularly by older patients [1]. Differentiation therapy is a relatively novel and targeted approach to AML treatment, based on the induction of leukemic cells to mature beyond the differentiation block, and thus to restore the normal cellular phenotype without cytodestruction. The physiologically active form of vitamin D<sub>3</sub>, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (1,25D<sub>3</sub>), has the ability to induce differentiation, growth arrest and apoptosis in a wide variety of cancer cell types [2, 3]. However, the hypercalcemic effect at pharmacologically effective doses has greatly limited its clinical usefulness. Attempts to circumvent this adverse reaction have included the use of high intermittent doses of 1,25D<sub>3</sub> that can be administered to patients with prostate and other cancers without apparent toxicity [4]. Further, a number of vitamin D analogues with lower calcemic effects have been synthesized, which retain the differentiation and antiproliferative activities of the parent compound in myeloid leukemia cells and other cancer cell lines [5–8].

One of the important principles in integrative cancer treatment, which is used in conventional chemotherapy, is that single agents, though likely to show modest activity when used alone, display synergistic effects when combined with other agents. Thus, most chemotherapy regimens employ multiple agents with different modes of action. For example, standard treatment of AML generally includes a synthetic pyrimidine nucleoside cytarabine (Ara-C), which functions as an antimetabolite and DNA polymerase inhibitor, in combination with an anthracycline agent (idarubicin or daunorubicin), which acts as topoisomerase II inhibitor and DNA intercalator [1]. In analogy, the anticancer effects of combinations of vitamin D derivatives (deltanoids) with other agents have been extensively studied in cultures of leukemic cells and other cancer cell types [9–12], animal models of cancer [13, 14] and clinical trials [15–17]. These studies are especially important since combination treatment may allow lower doses of deltanoids, thereby further limiting calcemic toxicity of these compounds.

Of the various agents tested in combination with deltanoids, natural plant-derived compounds, such as polyphenolic antioxidants, represent a group of relatively safe compounds which constitute a part of the diet or herbal medicinal remedies. For example, several plant polyphenolic antioxidants, e.g. curcumin derived from turmeric and silibinin from milk thistle, have been shown to markedly potentiate the differentiation effects of 1,25D<sub>3</sub> in leukemic cell cultures [18–20]. We have recently shown that carnolic acid, the major polyphenolic antioxidant of the rosemary plant (*Rosmarinus officinalis*), synergistically potentiates the differentiating and antiproliferative effects of 1,25D<sub>3</sub> and its low-calcemic analogue, 1,25-dihydroxy-16-ene-5,6-trans-cholecalciferol (Ro25-4020) in human myeloblastic (HL60) and promonocytic (U937) leukemia cells as well as WEHI-3B D- (WEHI) murine myelomonocytic leukemia cells [14, 20–23]. Similar potentiating effects in both human and murine leukemic cells were obtained with the ethanolic extract of rosemary leaves containing ~30% carnolic acid [14].

In this study, we employed a recently established syngeneic mouse model of systemic AML to explore if under clinically relevant experimental conditions combinations of a low-calcemic vitamin D analogue of the Gemini family [24], 1,25-dihydroxy-21(3-hydroxy-3-methyl-butyl)-19-nor-cholecalciferol (19-nor-Gemini; Ro27-5646), and polyphenol-rich rosemary preparations have therapeutic potential in human-like disseminated leukemia. Indeed, the combined treatment with rosemary extract and this deltanoid produced a dramatic prolongation of life in our systemic AML model. Considering the essential similarity of WEHI and human leukemia cells with respect to the enhanced antiproliferative and differentiating action of rosemary polyphenol-deltanoid combinations [14], our *in vivo* data provide the basis for initial steps towards human translational research of such combinations in myeloid leukemia and eventually other types of cancer.

## Materials and Methods

### Materials

The deltanoids, 1,25D<sub>3</sub>, 1,25-dihydroxy-21-(3-hydroxy-3-methylbutyl)-cholecalciferol (Gemini) and 19-nor Gemini, were prepared at BioXell, Nutley, N.J., USA. Carnolic acid was purchased from Alexis Biochemicals (Läufelfingen, Switzerland). A dried ethanolic extract of rosemary leaves (rosemary extract; lot No. LR-06-1-3/A) containing 33.9% (w/w) carnolic acid and 5.16% (w/w) carnolicol as the major polyphenols, was provided by Lycopodium Natural Products Industries (Beer Sheva, Israel). Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse F4/80 antibody was purchased from eBioscience (San Diego, Calif., USA).

Other reagents were obtained from Sigma (St. Louis, Mo., USA). Stock solutions of carnosic acid (10 mM), rosemary extract (10 mM equivalent of carnosic acid), 1,25D<sub>3</sub>, Gemini and 19-nor-Gemini (0.2–1.0 mM) were prepared in absolute ethanol. The precise concentrations of 1,25D<sub>3</sub> and Gemini analogues in stock solutions were verified spectrophotometrically at 264 nm ( $\epsilon = 19,000$ ) and 262 nm ( $\epsilon = 24,960$ ), respectively [14, 25].

#### *WEHI-3B D- Cell Culture and Treatments*

WEHI-3B D- murine myelomonocytic leukemia cells were obtained from Dr. A. Sartorelli (Yale University School of Medicine, New Haven, Conn., USA). Cells were maintained at 37°C in McCoy's 5a medium supplemented with heat-inactivated 10% fetal calf serum and 1% penicillin-streptomycin (Biological Industries, Beit Haemek, Israel) in 95% air/5% CO<sub>2</sub>. For in vitro experiments, cells were seeded in 6-well culture plates at  $5.0 \times 10^3$  cells/ml or  $2.5 \times 10^4$  cells/ml and incubated with test agents for 96 or 48 h, respectively. Control samples were treated with vehicle (0.2% ethanol), which did not significantly affect the parameters tested.

#### *Proliferation and Differentiation Assays*

Cell number and viability were estimated on the basis of the trypan blue exclusion assay by counting cells in a Vi-Cell XR cell viability analyzer (Beckman Coulter, Miami, Fla., USA). The expression of F4/80 (murine monocyte/macrophage differentiation marker) was assessed by flow cytometry in  $1 \times 10^6$  cells labeled with 0.3  $\mu$ g anti-F4/80-FITC, as described previously [14]. Analysis was performed on a Cytomics FC500 flow cytometer equipped with CXP software (Beckman Coulter). Cell differentiation was also measured by 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-stimulated superoxide production using the nitroblue tetrazolium reduction assay, and by cytochemical determination of the cytoplasmic enzyme monocyte-specific esterase (MSE) activity, as described previously [14, 22].

#### *Measurement of Intracellular Reactive Oxygen Species by Flow Cytometry*

Determination of intracellular reactive oxygen species (ROS) was performed as described previously [21] using the oxidation-sensitive fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA). Intracellular peroxides oxidize this probe to a highly fluorescent compound DCF. Cells ( $5 \times 10^5$ ) were harvested, washed with HEPES-buffered HBSS and loaded with 5  $\mu$ M DCFH-DA for 15 min at 37°C in a shaking water bath. The green fluorescence intensity was then analyzed on a Cytomics FC500 flow cytometer. For each analysis 10,000 events were recorded.

#### *Total Cellular Glutathione Assay*

Cells ( $2 \times 10^6$ ) were collected, washed with ice-cold PBS and resuspended in 200  $\mu$ l 5% 5-sulfosalicylic acid. After 15 min on ice with intermittent vortexing, the suspension was centrifuged at 16,000 g for 5 min to remove protein precipitates. Total glutathione was determined in the supernatants by the glutathione reductase recycling assay as described previously [21].

#### *Preparation of Whole Cell Extracts and Immunoblotting*

Whole cell extracts were prepared as described previously [22]. Briefly, cells were solubilized in 1 $\times$  lysis buffer (Cell Signaling, Danvers, Mass., USA) and mixed with SDS sample buffer. Protein concentration was determined by a Bio-Rad protein assay

using bovine serum albumin as the standard. Samples (50  $\mu$ g of protein) were subjected to SDS-PAGE on a 10% gel and transferred to nitrocellulose membranes (Whatman, Dassel, Germany). The membranes were blocked with 5% defatted milk, blotted overnight with goat anti-NQO1 (C-19) and then with a horseradish-linked secondary antibody for 1 h. The protein bands were visualized using a Western Lightning<sup>®</sup> Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, Mass., USA). The blots were stripped and successively reprobed for the constitutively present protein, calreticulin, which served as the loading control. The optical density (OD) of each band was quantitated using an ImageMaster VDS-CL imaging system (Amersham Pharmacia Biotech, Piscataway, N.J., USA).

#### *Experimental Animals*

Experiments were carried out in the animal facility of the Soroka University Medical Center (Beer-Sheva, Israel) in accordance with the Ben-Gurion University Institutional Animal Care and Use Committee-approved protocol. Six- to 8-week-old female Balb/c mice were purchased from Harlan Laboratories (Jerusalem, Israel). Mice were housed in sterilized cages and provided with autoclaved water and a standard powdered rodent diet ad libitum. Experimental protocols were initiated following a 7-day acclimatization period.

#### *Administration of Vitamin D Derivatives*

Stock solutions of 1,25D<sub>3</sub>, Gemini and 19-nor-Gemini in ethanol were diluted in ice-cold sterile PBS. The vehicle contained PBS and appropriate concentrations of ethanol. The final ethanol concentration in the injection volume did not exceed 0.1%. Deltanoids or vehicle were injected intraperitoneally in 100  $\mu$ l PBS, three times a week (Sunday, Tuesday and Thursday), as described previously [14].

#### *Cell Inoculation and Treatment Protocol*

Mice were inoculated in the lateral tail vein with  $2 \times 10^6$  WEHI cells suspended in 100  $\mu$ l sterile PBS and randomly assigned to four groups (6 or 10 animals per group; see Statistical Analyses). All treatments began 7 days after inoculation. The control group (group 1) received intraperitoneal injections of the deltanoid vehicle (0.05% ethanol in PBS, 3 times per week); group 2 received intraperitoneal injections of 0.1  $\mu$ g 19-nor-Gemini in 0.05% ethanol/PBS, 3 times per week; group 3 received 4% (w/w) rosemary extract mixed with food, ad libitum, and the combination group (group 4) was treated with both 0.1  $\mu$ g 19-nor-Gemini (3 times per week) and 4% (w/w) rosemary extract mixed with food.

#### *Animal Survival Assay*

The control and treated leukemia-bearing mice were monitored for up to 15 weeks. Animal body weight, appearance and behavior were assessed daily. To determine white blood cell (WBC) number and differential counts, peripheral blood samples were collected weekly from the retro-orbital sinus under isoflurane anesthesia. Moribund animals were humanely sacrificed by the institutionally approved method (CO<sub>2</sub> suffocation). The bone marrow was removed from both femurs, smeared on glass slides and subjected to cytological analysis. Overall animal survival was estimated by the Kaplan-Meier analysis [26]. All hematological analyses were performed using standard procedures in the Hematology Laboratory of the Soroka University Medical Center.

### Evaluation of *in vivo* Toxicity

Toxicity of deltanoids and rosemary extract was estimated in pilot experiments in healthy mice by clinical measures (weight loss, changes in appearance and behavior, lethargy and death) and blood chemistry tests (serum calcium, albumin, total bilirubin, creatinine and urea), as described previously [14]. In animal survival assays, blood chemistry was monitored every 2 weeks in the retro-orbital samples from 5 mice randomly selected from each treatment group. The biochemical tests were performed by automatic analysis in the Biochemistry Laboratory of the Soroka University Medical Center.

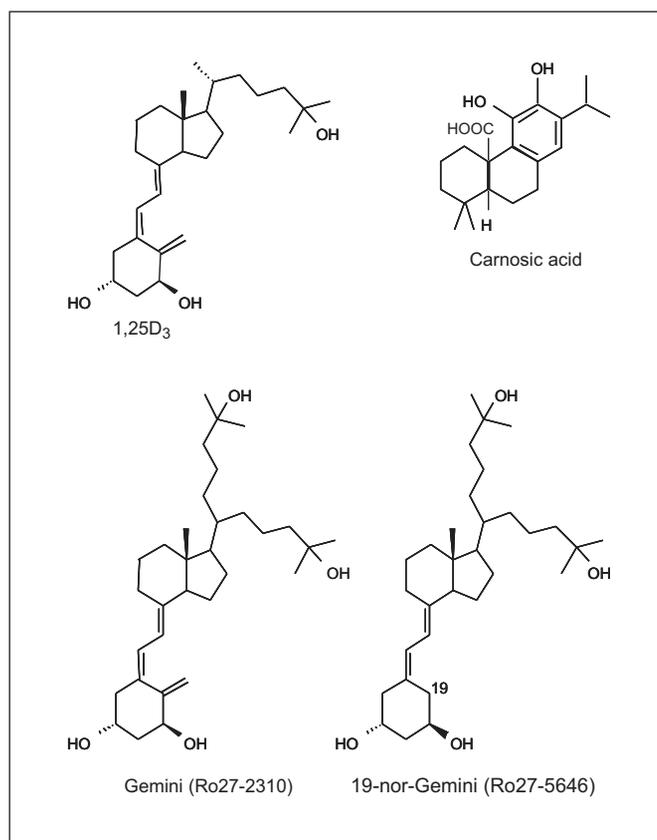
### Statistical Analyses

*In vitro* experiments were repeated at least three times. Dose-response curves for deltanoids in cell proliferation and differentiation assays were generated by nonlinear regression analysis. Two compounds (A and B) were considered to show synergy in the particular experiment if the effect of their combination (AB) was larger than the sum of their individual effects ( $AB > A + B$ ), the data being compared after subtraction of the respective control values from A, B and AB [22]. Statistically significant differences between different treatments were estimated by unpaired, two-tailed Student's *t* test. Animal survival experiments were repeated three times in groups of 6 mice (two experiments) and 10 mice (one experiment). The log-rank (Mantel-Cox) test was used to determine significant differences between survival curves generated by the Kaplan-Meier method. All statistical analyses were performed using a GraphPad Prism 3.0 program (GraphPad Software, San Diego, Calif., USA).  $p < 0.05$  was considered statistically significant.

## Results

### *19-nor-Gemini Is a More Potent and Less Toxic Deltanoid than Its Parent Compounds, Gemini or 1,25D<sub>3</sub>*

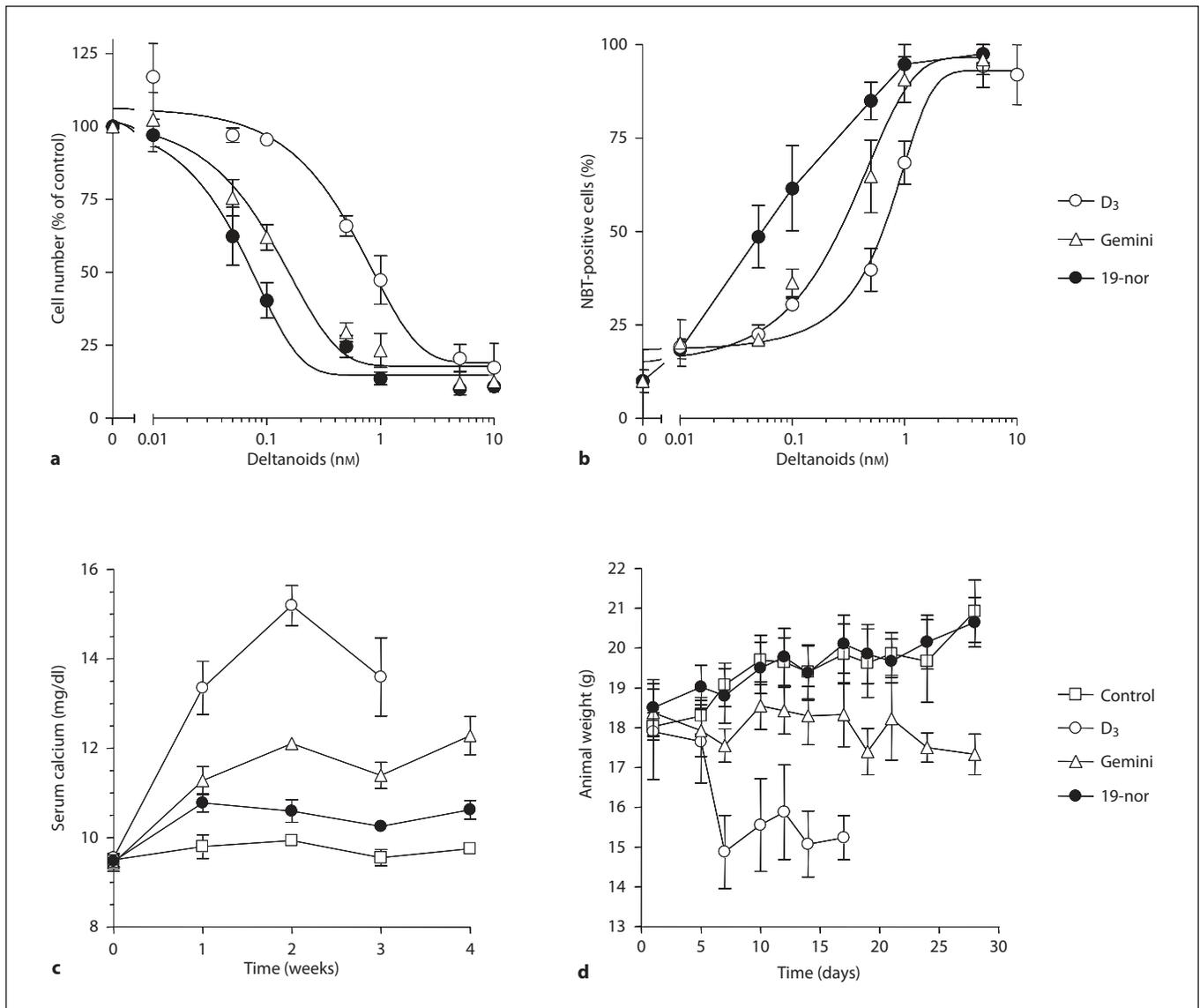
In order to select a potent and low-toxic deltanoid for the *in vivo* study, we first compared the differentiation and antiproliferative effects of 1,25D<sub>3</sub> and its two analogues of the Gemini series with two identical side chains at carbon 20 [24], Gemini and its 19-nor derivative 19-nor-Gemini (fig. 1), in WEHI cell culture. Cells were grown in the presence of increasing concentrations of either compound for 96 h followed by measurement of a general myeloid differentiation marker (superoxide production) and cell proliferation. As shown in figure 2a, b, the order of potencies was: 19-nor-Gemini > Gemini > 1,25D<sub>3</sub> for both the induction of differentiation ( $EC_{50} = 0.059 \pm 0.011, 0.275 \pm 0.093$  and  $0.652 \pm 0.085$  nM, respectively) and cell growth inhibition ( $IC_{50} = 0.072 \pm 0.018, 0.165 \pm 0.061$  and  $0.895 \pm 0.144$  nM, respectively). No significant decrease in cell viability compared to vehicle-treated control cells ( $\geq 95\%$  viable cells) was ob-



**Fig. 1.** Structures of the rosemary polyphenol, carnosic acid and deltanoids used in this study. Carnosic acid, the polyphenolic diterpene with antioxidant properties derived from the plant *R. officinalis* L. (rosemary). 1,25D<sub>3</sub> = Parent vitamin D compound, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>; Gemini (Ro27-2310) = the vitamin D analogue which contains two side chains, combining a C-20-normal with a C-20-epi side chain [1,25-dihydroxy-21-(3-hydroxy-3-methylbutyl)-cholecalciferol]; 19-nor Gemini (Ro27-5646) = its derivative with deletion of C-19 [1,25-dihydroxy-21-(3-hydroxy-3-methyl-butyl)-19-nor-cholecalciferol].

served even at the highest concentrations of deltanoids used (data not shown).

Preparatory to the *in vivo* testing, we established in a pilot experiment that the maximal tolerated dose for intraperitoneal 19-nor-Gemini in healthy mice was 0.1  $\mu$ g/mouse, i.e. approximately 5  $\mu$ g/kg (data not shown). To directly compare the calcemic effects of Gemini, 19-nor-Gemini and 1,25D<sub>3</sub>, four groups of mice were intraperitoneally injected with vehicle (control) or deltanoids at an equal dose of 0.1  $\mu$ g/mouse, for 4 weeks. As shown in figure 2c, mice that received 19-nor-Gemini had borderline serum calcium levels which did not significantly exceed the normal values for Balb/c mice (8.5–10.5 mg/dl). Fur-

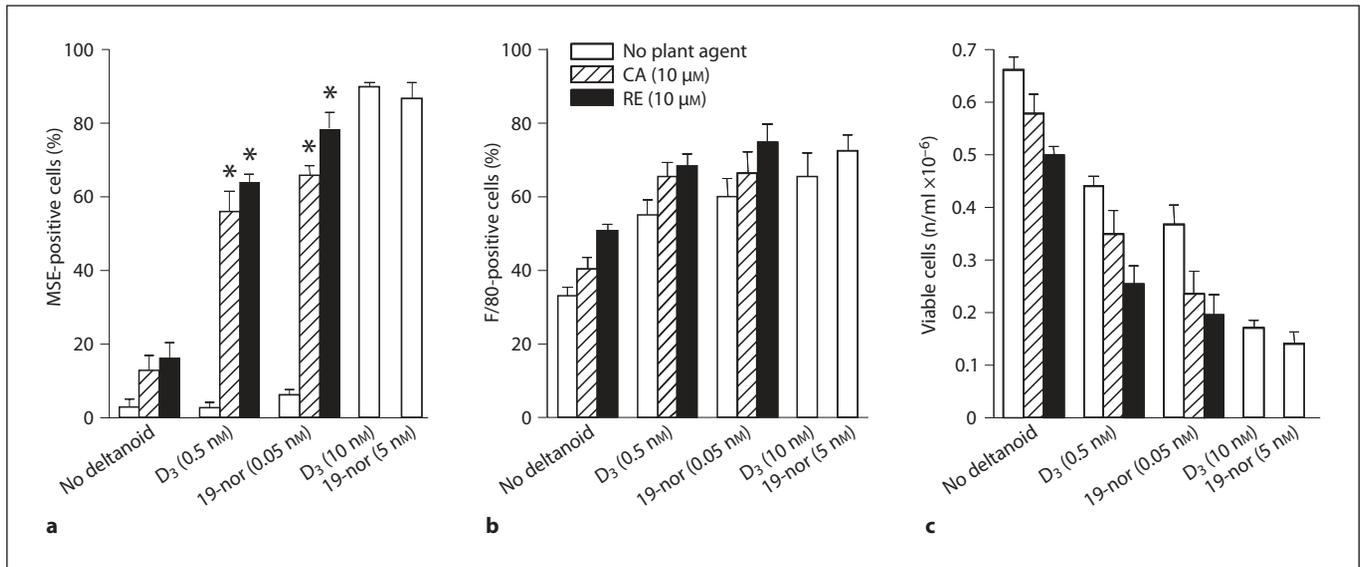


**Fig. 2.** 19-nor-Gemini is a more potent antiproliferative and differentiation agent than 1,25D<sub>3</sub> or Ro27-2310, with lower calcemic activity. **a, b** WEHI cells ( $5 \times 10^3$ /ml) were incubated with 1,25D<sub>3</sub> (D<sub>3</sub>), Gemini or 19-nor-Gemini (19-nor) for 96 h. Means  $\pm$  SE of at least three independent experiments performed in duplicate. **a** Viable cell counts. The data are expressed as percent of cell numbers in control cultures incubated with vehicle alone (0.1% ethanol). The average control cell number at 96 h was  $624,000 \pm 34,000$  cells/ml. **b** TPA ( $2 \mu\text{M}$ )-stimulated nitroblue tetrazolium

(NBT) reduction (myeloid differentiation marker). The percentage of cells containing black formazan deposits was evaluated by counting 200 cells. **c, d** Effects of deltanoids on serum calcium and animal weight gain. Healthy Balb/c mice were intraperitoneally injected with 1,25D<sub>3</sub>, Gemini, 19-nor (all at  $0.1 \mu\text{g}$ ) or vehicle (0.1% ethanol), 3 times per week. **c** Serum calcium levels were measured weekly. **d** Mice from the experiment shown in **c** were weighed 3 times per week. Means  $\pm$  SE of one experiment performed in groups of 10 animals.

thermore, the body weight gain of the 19-nor-Gemini-injected animals was not significantly different from that of the control group (fig. 2d). In contrast, animals treated with  $0.1 \mu\text{g}$  1,25D<sub>3</sub> showed severe hypercalcemia (fig. 2c) and dramatic weight loss (fig. 2d), and were sacrificed

during week 3 due to their moribund state. Gemini demonstrated intermediate toxicity (fig. 2c, d). On the basis of the above data, 19-nor-Gemini was chosen for the further in vitro and in vivo experiments as the most effective and less toxic of the three tested deltanoids.



**Fig. 3.** Carnosic acid (CA) and rosemary extract (RE) cooperate with 1,25D<sub>3</sub> (D<sub>3</sub>) and 19-nor-Gemini (19-nor) in the induction of differentiation (**a**, **b**) and inhibition of proliferation (**c**) in WEHI cells. Cells (5 × 10<sup>3</sup>/ml) were incubated with the indicated compounds for 96 h. Differentiation was assessed by MSE (**a**) and

F4/80 (**b**) assays. **c** Viable cell counts. Means ± SE of four to seven independent experiments performed in duplicate. Synergistic effects of the deltanoid-carnosic acid and deltanoid-rosemary extract combinations on MSE expression were statistically significant (\* p < 0.001).

#### *19-nor-Gemini and Rosemary Preparations Cooperate in the Induction of Differentiation and Inhibition of Proliferation in WEHI Cells*

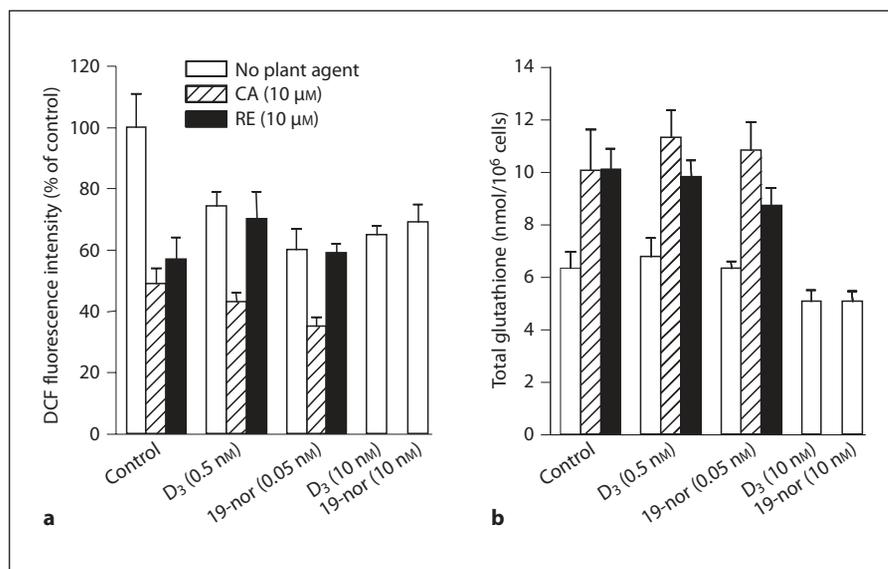
The ability of 19-nor-Gemini to cooperate with rosemary preparations in the differentiation of WEHI cells was examined by the expression of specific monocyte/macrophage differentiation markers (MSE and F4/80). 1,25D<sub>3</sub> was used as the positive control. Control cells and those treated with single agents, such as approximately isoeffective concentrations of 1,25D<sub>3</sub> and 19-nor-Gemini (0.5 and 0.05 nM, respectively; fig. 2a, b) and 10 μM carnosic acid or an equivalent concentration of rosemary extract, exhibited low MSE levels (fig. 3a). However, the combined treatments resulted in a marked synergistic increase in MSE expression. F4/80 marker levels in control WEHI cells were slightly above 30% and were further elevated by single agents, whereas the combinations produced nearly additive effects (fig. 3b). The combined treatments did not produce significant cytotoxicity (cells retained 92 ± 5% viability under all experimental conditions; data not shown). However, at least an additive substantial growth-inhibitory effect was observed (fig. 3c). Rosemary extract, either alone or in combination with deltanoids, was slightly more potent than carnosic acid in both cell differentiation and growth assays (fig. 3). It is possible that

minor components of rosemary extract, e.g. carnosol, can contribute to the biological effects of the extract along with carnosic acid. Importantly, the combinations of deltanoids at low concentrations with rosemary agents were almost as effective as much higher doses of 19-nor-Gemini (5 nM) or 1,25D<sub>3</sub> (10 nM) alone (fig. 3) which exhibited maximal effects in dose-response tests (fig. 2a, b).

#### *Antioxidant Effects of Rosemary-Derived Agents and Deltanoids in WEHI Cells*

Rosemary extract and purified polyphenols demonstrate strong antioxidant effects in various biologic systems [27]. We therefore investigated whether cooperative differentiation and antiproliferative effects of rosemary agents and deltanoids are associated with changes in the cellular redox state. As shown in figure 4a, WEHI cells incubated with carnosic acid or rosemary extract alone for 48 h displayed lower intracellular ROS levels than control cells, as indicated by a lower DCF fluorescence intensity. Interestingly, 1,25D and Ro27-5646 alone, irrespective of the concentration applied, produced a similar antioxidant effect. Combined treatments did not demonstrate cooperative reduction in ROS levels although the combinations containing carnosic acid had stronger antioxidant effects than those containing rosemary extract (fig. 4a).

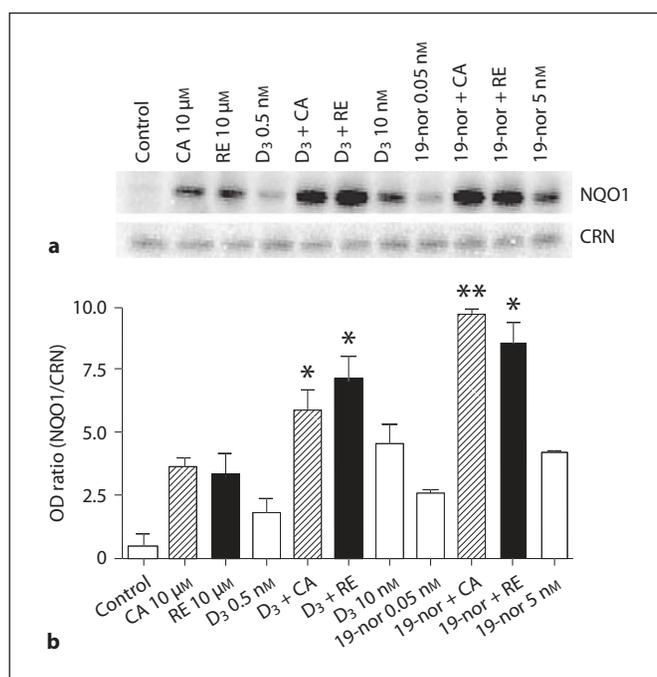
**Fig. 4.** Antioxidant effects of rosemary-derived agents and deltanoids in WEHI cells. Cells ( $2.5 \times 10^4$ /ml) were incubated with carnosic acid (CA), rosemary extract (RE), 1,25D<sub>3</sub> (D<sub>3</sub>), 19-nor Gemini (19-nor) or their combinations for 48 h. **a** Intracellular levels of ROS determined by DCF fluorescence. **b** Total cellular glutathione content. Means  $\pm$  SE of four independent experiments performed in duplicate.



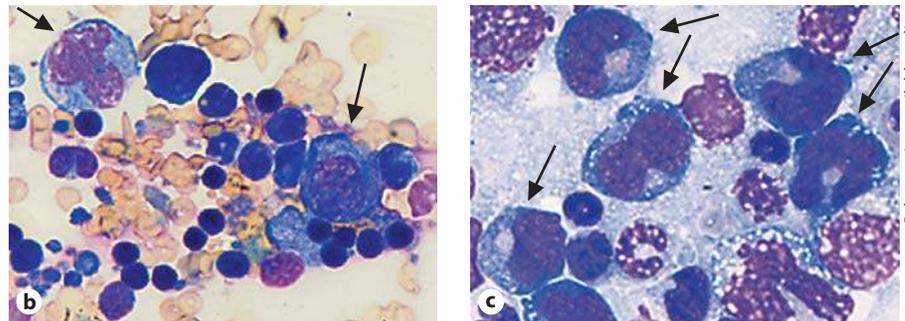
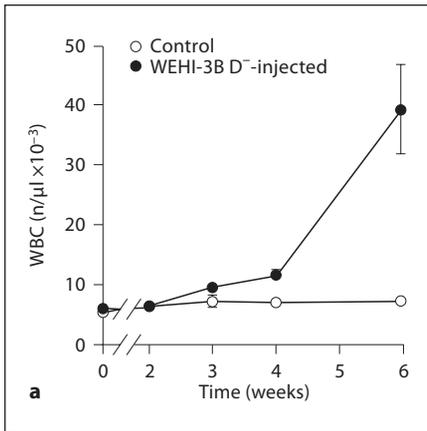
The levels of glutathione, the major cellular antioxidant, were elevated by carnosic acid and rosemary extract, but not by deltanoids while no appreciable cooperativity between the two types of agents was observed (fig. 4b). Further, we found that cell treatment with both the rosemary agents and deltanoids resulted in the induction of NADP(H)-quinone oxidoreductase (fig. 5), the antioxidant and phase II detoxifying enzyme known to be induced by various phytochemicals via activation of the antioxidant response element transcription system [28]. In contrast to the lack of cooperativity in the reduction in ROS levels and glutathione elevation, significant synergistic effects of drug combinations on NADP(H)-quinone oxidoreductase induction were observed (fig. 5). Taken together, these data suggest that rosemary agents and deltanoids may affect the cellular redox status by distinct mechanisms and can cooperate at least in some of the redox-regulating pathways, leading to the establishment of reducing conditions within the cell.

#### Establishment of a Syngeneic Mouse Model of Systemic AML

We adapted a syngeneic mouse model of systemic AML in which a disseminated disease was initiated by the injection of WEHI cells into the lateral tail vein [29]. Balb/c mice injected with  $2 \times 10^6$  cells i.v. presented clinical symptoms of AML, such as loss of appetite and apathy, which appeared with a significant delay of 25–35 days after inoculation. Within 2–4 days after appearance of these first symptoms, the animals became moribund

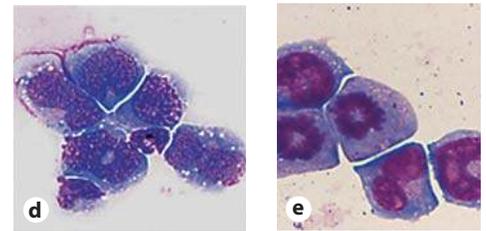


**Fig. 5.** Induction of NADP(H)-quinone oxidoreductase (NQO1) by rosemary-derived agents and deltanoids in WEHI cells. Cells ( $2.5 \times 10^4$ /ml) were incubated for 48 h with carnosic acid (CA), rosemary extract (RE), 1,25D<sub>3</sub> (D<sub>3</sub>), 19-nor Gemini (19-nor) or their combinations. **a** NQO1 protein levels. Membranes were reblotted for calreticulin (CRN) to demonstrate similar protein loading. **b** Quantitative representation of NQO1 protein expression. Average OD ratios (NQO1 vs. CRN) of three similar experiments are shown (means  $\pm$  SE). Statistically significant differences in synergistic effects of the deltanoid/carnosic acid and deltanoid/rosemary extract combinations were found (\*  $p < 0.05$  and \*\*  $p < 0.01$ ).



Color version available online

**Fig. 6.** Systemic leukemia induced by intravenous inoculation of WEHI cells in syngeneic Balb/c mice. Six animals were injected with  $2 \times 10^6$  WEHI cells in the lateral tail vein and monitored for 6 weeks. **a** WBC counts in retro-orbital blood samples. **b–d** Wright-Giemsa-stained smears of peripheral blood (**b**) and bone marrow (**c**) were taken from a sacrificed moribund mouse, 41 days after cell inoculation. Large blast cells are indicated by arrows. Leukemic blasts clustered in the bone marrow (**d**) and cultured WEHI cells (**e**) display similar size and morphology.  $\times 1000$ .



**Table 1.** Cooperative effects of 19-nor-Gemini (19-nor) and rosemary extract (RE) on the survival of leukemia-bearing animals

Experiment No.	Median survival time, days				p value
	control	19-nor (0.1 μg)	RE (4%)	19-nor + RE <sup>1</sup>	
1 (n = 6)	47	52.5	54	67 (42.5)	0.0180
2 (n = 6)	40.5	51	46.5	73.5 (81.5)	0.0365
3 (n = 10)	39	43	43.5	59 (51.3)	0.0104

Median survival times were determined in three experiments by the Kaplan-Meier analysis (fig. 7a). n = Number of mice per treatment group. Significant differences in the median survival times between the 19-nor + RE treatment group and the untreated control group (log-rank test) are shown.

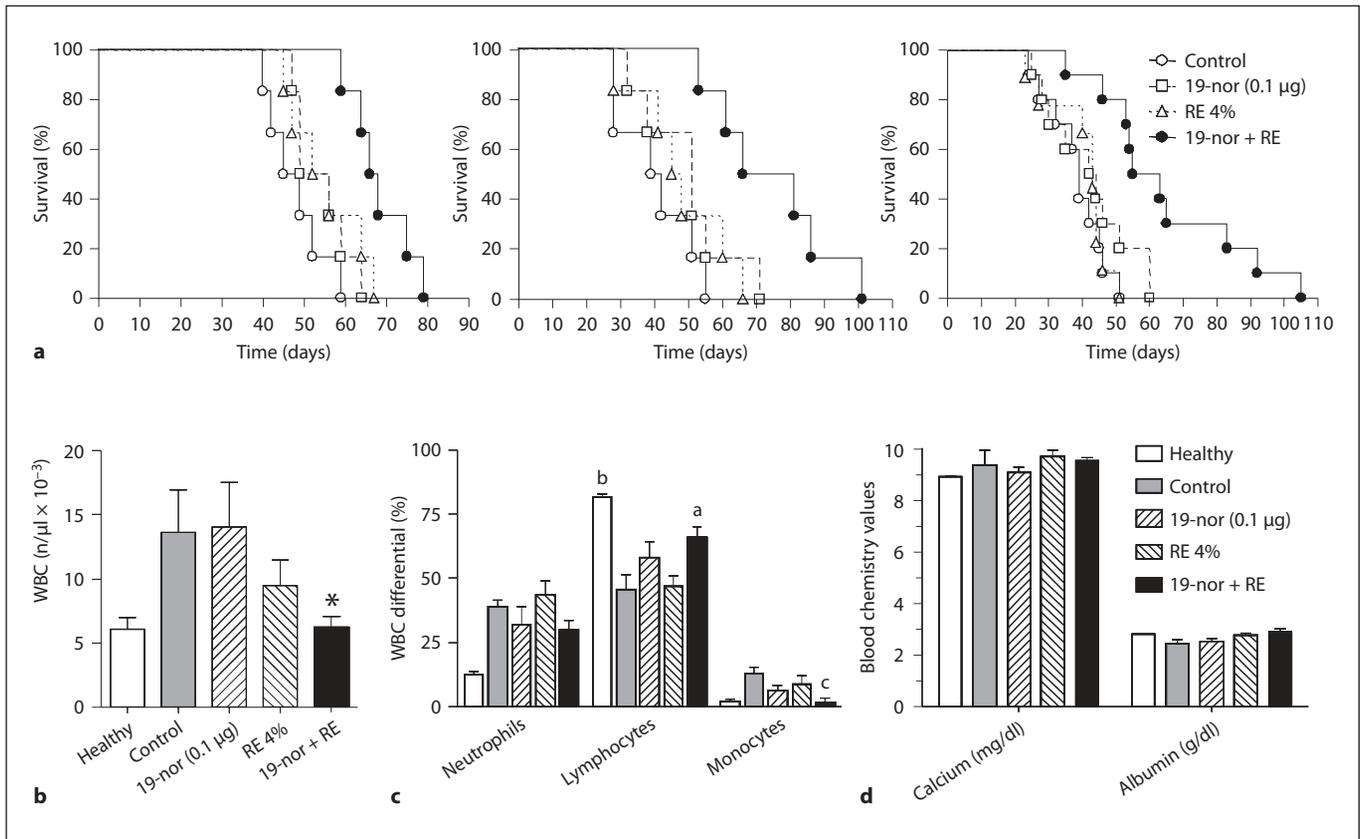
<sup>1</sup> The percent increase in the animal life span in the 19-nor + RE treatment group compared with untreated control mice is indicated in parentheses.

and were sacrificed due to progressively poor conditions, such as hind limb paralysis, lateral recumbency and/or lethargy. The median survival of leukemia-bearing mice was  $43.0 \pm 1.2$  days (n = 22). AML was diagnosed by leukocytosis ( $\geq 10,000$  WBC/ $\mu$ l; fig. 6a) and the appearance of large proportions of blast-like cells in both peripheral blood (fig. 6b) and bone marrow (fig. 6c). The

blast cells detected in leukemic specimens (fig. 6b, c) were clearly distinguished from host WBCs by the size and morphology but were similar to WEHI cells growing in liquid culture (compare fig. 6d, e). Necropsy revealed the enlargement of the spleen and frequent leukemia cell deposits in lymphatic nodes, liver and ovary (data not shown).

#### Combined Antileukemic Effects of 19-nor-Gemini and Rosemary Extract

To determine the antileukemic activity of 19-nor-Gemini and rosemary extract, alone and in combination, three independent *in vivo* experiments were performed in groups of 6–10 animals (fig. 7a; table 1). Mice inoculated intravenously with WEHI cells were treated with 0.1 μg 19-nor-Gemini, 4% (w/w) rosemary extract or their combination, as described. To approximate the treatment protocol to the clinical situation, all treatments started 7 days after cell inoculation in order to ensure homing of the leukemic cells to the bone marrow prior to interventions. At this time point, the WEHI blast cells represented 2–5% of the total nucleated bone marrow cells, as detected on Wright-Giemsa-stained smears (data not shown). As demonstrated in figure 7a, treatment with 19-nor-Gemini or rosemary extract alone had only a slight effect on median survival compared with control mice.



**Fig. 7.** Combined antileukemic effect of rosemary extract (RE) and 19-nor-Gemini (19-nor) in a mouse model of systemic AML. **a** Balb/c mice were inoculated with  $2 \times 10^6$  WEHI cells i.v. and treated with 19-nor-Gemini (0.1  $\mu\text{g}$ ) or rosemary extract (4% w/w, mixed with food) either alone or in combination, or vehicle (0.05% ethanol, i.p.). Groups of 6 (experiments 1 and 2) and 10 (experiment 3) mice were used. Animal survival was estimated by the Kaplan-Meier analysis. WBC number (**b**) and differential count

(**c**) as well as blood chemistry parameters (**d**) were determined on day 38 in retro-orbital blood samples of 6 mice randomly selected from each treatment group (experiment 3). Means  $\pm$  SE. **b** \*  $p < 0.05$  vs. untreated control. **c** <sup>a</sup>  $p < 0.05$  and <sup>b</sup>  $p < 0.01$ : statistically significant differences in neutrophil/lymphocyte ratios obtained for combination-treated mice ( $0.44 \pm 0.05$ ) vs. untreated controls ( $0.85 \pm 0.11$ ), and healthy animals ( $0.15 \pm 0.02$ ) vs. untreated controls ( $0.85 \pm 0.11$ ); <sup>c</sup>  $p < 0.05$ : vs. untreated control.

However, the combined treatment markedly (by 42.5–81.5%) extended the animal life span in a synergistic manner (see median survival data in table 1). This effect was associated with the improvement in both the WBC and differential counts. Thus, when measured 38 days after WEHI cell inoculation (experiment 3), significantly lower total WBC numbers were detected in mice receiving combined treatment compared with the control mice (fig. 7b). Further, the WBC differential count of control mice and those treated with single agents displayed a relatively large myeloid component with minimal morphologic dyspoiesis (fig. 7c), consistent with mouse myeloproliferative conditions, e.g. nonlymphoid (myeloid) leukemias [30]. However, the combined treatment significantly decreased neutrophil/lymphocyte ratios and

monocyte counts (fig. 7c). Importantly, the above WBC parameters in the combination group were close to those determined in the healthy mice which did not receive either leukemic cell injections or any other treatment (fig. 7b, c). While synergizing with 19-nor-Gemini in the antileukemic action, rosemary extract did not increase the calcemic effect of the deltanoid (fig. 7d). Serum albumin levels in control or treated mice were not significantly different from those in the intact healthy animals (fig. 7d), which indicates the absence of liver toxicity in these experiments. Collectively, these results, along with our previous data obtained in a leukemic tumor model [14], strongly suggest that combinations of rosemary antioxidants and deltanoids may be considered as a promising effective and low-toxic therapy of AML.

## Discussion

Here we characterized the combined *in vitro* and *in vivo* antileukemic activity of the low-calcemic deltanoid, 19-nor-Gemini, and rosemary phenolic preparations. As determined in the *in vitro* assays, this deltanoid demonstrated a much greater differentiation capacity and antiproliferative effect in WEHI cells compared with its parent compounds Gemini and 1,25D<sub>3</sub> (fig. 2a, b), and strongly cooperated in these activities with both purified carnosic acid and carnosic acid-rich rosemary extract. Thus, being moderately (~1.5-fold) less calcemic but about 10-fold more potent than 1,25D<sub>3</sub>, this analogue would exert superior anticancer activity in clinical situations, especially in combination with other agents.

We have recently shown that another low-calcemic deltanoid (Ro25-4020) and rosemary extract cooperatively inhibit the growth of WEHI cell-derived leukemic tumors in Balb/c mice [14]. Although such tumor is reminiscent of chloroma, a human solid leukemic tumor, it does not fully model the disseminated AML. Thus, here we translated these findings to a more clinically relevant mouse model of systemic AML. Our data show for the first time that rosemary extract and 19-nor-Gemini synergistically prolonged the survival of leukemia-bearing Balb/c mice. This action was consistent with significantly improved hematologic parameters, such as WBC count and the neutrophil/lymphocyte ratio, in the combination-treated animals. It should be noted, however, that while markedly delaying the development of systemic leukemia, the 19-nor-Gemini/rosemary extract combination did not completely eliminate the pathologic process, so that leukemia-bearing mice eventually died of AML. Therefore, there is an ultimate need for the further optimization of the treatment protocol and/or utilization of different/novel combinations of deltanoids and a variety of plant polyphenols.

The molecular mechanism of the synergistic activity of 1,25D<sub>3</sub> and plant polyphenolic antioxidants against myeloid leukemia cells has been extensively studied in our and other laboratories [7, 18–23] (see also Danilenko and Studzinski [31] for a recent review). We have shown that in human HL60 cells, 1,25D<sub>3</sub> and carnosic acid cooperate in an antioxidant effect and elevation of glutathione levels, whereas glutathione depletion markedly suppressed the differentiation effects of both 1,25D<sub>3</sub> alone and, particularly, its combination with the polyphenol [21]. The current study in murine AML cells further supports these findings by demonstrating that both rosemary agents and deltanoids have antioxidant actions and

can cooperate in at least some of the redox-regulating cellular pathways, e.g. the induction of the antioxidant enzyme NADP(H)-quinone oxidoreductase.

The nature of the link between the antioxidant effect of rosemary agents and their ability to sensitize leukemic cells to deltanoids still needs further elucidation. However, recent findings suggest that generation of intracellular reducing conditions promotes cell differentiation in leukemia, possibly via modulation of certain redox-sensitive signal transduction and transcription systems, such as signaling kinase pathways and the transcription factors nuclear factor  $\kappa$ B (NF $\kappa$ B) and activating protein-1 (AP-1) [32, 33]. For instance, potentiation of deltanoid-induced HL60 cell differentiation by the polyphenolic antioxidant curcumin was associated with inhibition of NF $\kappa$ B [18]. Also, different plant antioxidants which are capable of reducing NF $\kappa$ B activity were found to sensitize cancer cells to apoptosis due to inhibition of NF $\kappa$ B-dependent transcription of 'survival genes' that provide resistance to apoptosis [34, 35]. We have found that polyphenols potentiate 1,25D<sub>3</sub>-induced activation of mitogen-activated protein kinase (extracellular signal-regulated kinase and c-Jun N-terminal kinase) pathways, which target AP-1 [20, 21]. Further, both AP-1 binding to its cognate DNA response element and transcriptional activity were markedly augmented by 1,25D<sub>3</sub>-polyphenol combinations [20, 21]. In addition, we have recently reported that competitive inhibition of AP-1 by a double-stranded decoy oligodeoxynucleotide markedly decreased the differentiation response of HL60 cells to 1,25D<sub>3</sub> [36].

Up-regulation of AP-1 [37] and another transcription factor, Wilms' tumor suppressor 1 (WT1) [38], in solid tumor cells has been shown to elevate the expression of vitamin D receptor (VDR) and, thereby, to enhance the antiproliferative effect of 1,25D<sub>3</sub>. In leukemia cells, elevation of VDR levels by 1,25D<sub>3</sub> and carnosic acid [22] correlated with the activation of AP-1 [20, 21], whereas WT1 was shown to be down-regulated by 1,25D<sub>3</sub> and other inducers of myeloid differentiation [39] as well as by curcumin [40]. Therefore, the precise roles of AP-1, WT1 and VDR in the enhanced antileukemic effects of 1,25D<sub>3</sub>-polyphenol combinations remain to be elucidated.

Taken together, the results presented here clearly demonstrate that a vitamin D derivative and a plant polyphenolic preparation can strongly cooperate against systemic AML. Therefore, at least some polyphenols with differentiation-enhancing activity, which also possess antitumor properties of their own [41–43], can be em-

ployed in the deltanoid-based, low-toxic combination differentiation therapy of at least some types of myeloid leukemias. Further, both deltanoids [44] and various phytochemicals [45] have been shown to suppress carcinogenesis and, thus, their cooperation may account for the chemopreventive effect of vitamin D and polyphenol-rich diets.

Syngeneic mouse models are widely employed in transgenial leukemia studies [46, 47]. Moreover, mouse AML has already been shown to respond to deltanoid therapy in syngeneic models [14, 48–50]. The attractive advantages of these models include their relatively low costs and the use of mice with non-compromised immune system. We have demonstrated that murine and human leukemic cells respond to combined deltanoid-polyphenol treatments in a very similar manner [14],

which suggests that our current study would provide useful information for potential clinical applications of such combinations.

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