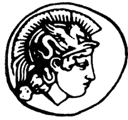

PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 73 (2000) 105–112

The Journal of
Steroid Biochemistry
&
Molecular Biology

www.elsevier.com/locate/jsbmb

# Synergistic anticancer activity of 1,25-dihydroxyvitamin $D_3$ and immune cytokines: the involvement of reactive oxygen species

Ruth Koren[a,b,*], Dafna Rocker[b], Ofira Kotestiano[b], Uri A. Liberman[a,b], Amiram Ravid[a]

[a]*Department of Physiology and Pharmacology, The Basil and Gerald Felsenstein Medical Research Center, Rabin Medical Center, Petah Tikva, Israel*

[b]*Sackler Faculty of Medicine, The Basil and Gerald Felsenstein Medical Research Center, Rabin Medical Center, Tel-Aviv University, Tel-Aviv, Israel*

Received 3 September 1999; accepted 31 January 2000

**Abstract**

It was previously shown that 1,25-dihydroxyvitamin $D_3$ (1,25$(OH)_2D_3$) enhances the cytotoxic activity of tumor necrosis factor α (TNFα), doxorubicin and menadione. A feature shared by these anticancer agents is the involvement of reactive oxygen species (ROS) in their action. In this work we found that 1,25$(OH)_2D_3$ acted synergistically with interleukin 1 β (IL-1β) or interleukin 6 (IL-6) to inhibit the proliferation of MCF-7 breast cancer cells. The extent of the synergism was maximal at 1 nM, a concentration at which 1,25$(OH)_2D_3$, acting singly, only marginally reduced the cell number. The thiol antioxidant, *N*-acetylcysteine (NAC) abolished the synergism between IL-1β or IL-6 and 1,25$(OH)_2D_3$, but had only a small protective effect when the cytokines acted alone. NAC and reduced glutathione (GSH) protected MCF-7 cells from cytotoxicity induced both by TNFα alone and by TNFα and 1,25$(OH)_2D_3$. A two-day exposure to TNFα caused a $27.7 \pm 3.1\%$ (mean $\pm$ SEM) reduction in GSH content. This effect increased to $46.4 \pm 5.5\%$ by co-treatment with 1,25$(OH)_2D_3$ which did not affect GSH levels on it own. We conclude that 1,25$(OH)_2D_3$ can act synergistically with anticancer cytokines present in the tumor milieu and that ROS plays a mediatory role in this interaction. © 2000 Elsevier Science Ltd. All rights reserved.

*Keywords:* Calcitriol; Vitamin D; Tumor necrosis factor; Interleukin 1; Interleukin 6; Reactive oxygen species

## 1. Introduction

Recent epidemiological studies provide evidence supporting the claim that the hormonal form of vitamin D, 1,25-dihydroxyvitamin D (1,25$(OH)_2D$), may have a role in the host anticancer defense activity. Examples of such studies include the following: (1) Studies of polymorphism in the vitamin D receptor gene indicate a linkage between prostate cancer incidence and the expression of a specific receptor allele [1–3]; (2) Presence of vitamin D receptors in tumor cells has been associated with slower tumor progression in breast cancer patients [4]; (3) Inverse correlation between tumor incidence and progression on the one hand and serum levels of vitamin D metabolites or vitamin D intake on the other hand, have been reported for colorectal, prostate and breast cancer [5]. Intriguingly, some studies report a significant correlation with the serum levels of 25-hydroxyvitamin D, the biologically inert parent compound of 1,25$(OH)_2D_3$ [6,7]. A possible explanation for this could be that 1,25$(OH)_2D$ is produced from its precursor 25 hydroxyvitamin D in

* Corresponding author. Laboratory for Calcium Regulating Hormones, Felsenstein Medical Research Center, Beilinson Campus, Petah, Tikva 49100, Israel. Fax: +972-3-9211478.

*E-mail address:* rkoren@post.tau.ac.il (R. Koren).

0960-0760/00/$ - see front matter © 2000 Elsevier Science Ltd. All rights reserved.
PII: S0960-0760(00)00068-6

the tumor milieu by tumor-associated macrophages or, in some cases, by the tumor cells themselves [8–11].

The anticancer potential of vitamin D in vivo was also demonstrated in animal studies [5] and in preliminary clinical trials [12,13] with natural and synthetic vitamin D receptor agonists. Many researchers attribute the in vivo anticancer activity of active vitamin D metabolites and analogs to their ability to inhibit tumor cell proliferation, to induce differentiation and, in some cases, apoptosis [5]. Furthermore, our recent studies showed that treatment with $1,25(OH)_2D_3$ sensitizes cancer cells to the tumoricidal activity of the immune cytokine, tumor necrosis factor (TNF) [14,15], in addition to its direct effect on tumor cell proliferation and viability. This activity may contribute to the anticancer potential of the hormone, since both the hormone and the cytokine may be both produced whenever immune cells infiltrate incipient or progressing tumors [8–11,16,17].

Two other cytokines with potential anticancer activity that are also present in the tumor milieu are interleukin 1 (IL-1) and interleukin 6 (IL-6). IL-1 which is produced by tumor-associated leukocytes and some tumor cells [16–19] inhibits proliferation and induces death of various cancer cells [20–22]. On the other hand, IL-6 was shown to have both stimulatory and inhibitory effects on cells from different origins [23]. In the case of breast cancer cells, this cytokine was shown to inhibit proliferation [24].

We have reported recently that cells treated with $1,25(OH)_2D_3$ are more susceptible not only to the cytotoxic action of TNF$\alpha$ but also to that of the anticancer drug doxorubicin and the cytotoxic quinone, menadione [25]. A common feature shared by these three agents is the involvement of reactive oxygen species (ROS) in their cytotoxic action, which is due, at least partially, to the increased rate of superoxide generation by various metabolic pathways [26–32]. IL-1 seems to resemble TNF$\alpha$ in this respect [33,34]. Although IL-6 was shown to increase superoxide production by neutrophils [35] and hepatocytes [36], to our knowledge there is no evidence regarding a similar action in other cell types. Our previous results suggest that ROS have a role in the potentiation of doxorubicin action by $1,25(OH)_2D_3$ and that the effect of the hormone is associated with decreased expression of the antioxidant enzyme Cu/Zn superoxide dismutase [25].

The present study aims to expand these findings by addressing the following questions: (1), Does $1,25(OH)_2D_3$ enhance the cytostatic/cytotoxic activity of other cytokines with a presumed role in the anticancer activity of the immune system? (2) To what extent are ROS involved in the combined anticancer activity of $1,25(OH)_2D_3$ and such cytokines?

## 2. Materials and methods

### 2.1. Materials

Tissue culture media were purchased from Biological Industries, Beit Haemek, Israel. Tissue culture dishes were from Corning Glass Work, Corning, NY, USA. $1,25(OH)_2D_3$ was obtained from Hoffman-LaRoche, Nutley, NJ, USA (the generous gift of Dr. M. Uskokovic is recognized). Human recombinant TNF$\alpha$ and IL-1$\beta$ were obtained from PeproTech, Rocky Hill, NJ, USA. IL-6 was from Interpharm Laboratories, Ness Ziona, Israel. Crystal violet (CV) was from Edward Gurr, London, UK. *N*-Acetylcysteine (NAC) and reduced glutathione (GSH) were purchased from Sigma, St. Louis, MO, USA. All other reagents were of analytical grade.

### 2.2. Cell culture

MCF-7 human breast cancer cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/l glucose and supplemented with 10% fetal calf serum and antibiotics. Cells were sub-cultured twice weekly. For the assessment of the cytostatic/cytotoxic activity of cytokines 3000 or 10,000 cells/well were plated in 96-well microtiter plates. For determination of acid soluble thiols: $3 \times 10^5$ cells were plated in a 60-mm petri dish. Cultures were treated with $1,25(OH)_2D_3$ and/or cytokines for 24–48 h after seeding. The $1,25(OH)_2D_3$ vehicle ethanol was added to control cultures, and its concentration was kept at or below 0.06%.

### 2.3. Monitoring of cell number

Cells were quantified by CV staining as described in [37]. In brief, after removal of culture medium, cells were incubated with a CV solution (1% w/v in 20% ethanol) for 15 min at room temperature and rinsed in water thoroughly. After drying, the dye was extracted with 70% ethanol and its absorbance determined at 550 nm using a microplate reader. In a preliminary experiment we found a linear relationship ($r = 0.999$) between cell number and CV staining in the range of 2000–100,000 cells/well. The cells inoculated at a density of 2000–10,000 cells/well entered the proliferative phase following a lag of 48 h. Proliferation continued for at least four additional days for cells seeded at 2000–5000 cells/well and for three additional days for cells seeded at 5000–10,000 cells/well.

### 2.4. Calculations

The inhibitory effect of each agent added to the cultures was calculated as follows: Inhibition (%) = [1 −

(CV staining in treated wells/CV staining in control wells)] × 100. The theoretical additive inhibitory effect of the agents a and b was calculated as described in [37,38] using the following equation:

$$I_{ab} = 100 \times [1 - (1 - I_a/100) \times (1 - I_b/100)]$$

where $I_{ab}$ is the calculated additive inhibitory effect expressed as % inhibition. $I_a$ and $I_b$ are the measured inhibitory effects (%) of each agent acting alone as compared with that of the control cultures. This equation was derived assuming the inhibitory agents act independently on the same target population.

### *2.5. Determination of low molecular weight thiols*

Cells were removed from culture dishes by incubation in PBS containing 5 mM EDTA (PBS-EDTA) at 4°C. The acid soluble fraction, extracted with 2% sulfosalicylic acid in PBS-EDTA and cleared by centrifugation (16,000 × *g* for 1 min), was used to determine non-protein sulfhydryl groups. Four aliquots of different volumes of the acid extract from each culture dish were transferred to 96-well microtiter plates and adjusted to pH 8 with Tris buffer (1 M). Quantification of total sulfhydryl groups was performed by the method of Ellman [39]. Data from each culture dish were analyzed by linear regression and the *r* values were never less then 0.98. Data were expressed as glutathione equivalents (nmol/mg protein) using a glutathione standard curve. Protein content was determined by the method of Lowry et al. [40] adapted to 96 microtiter plates.

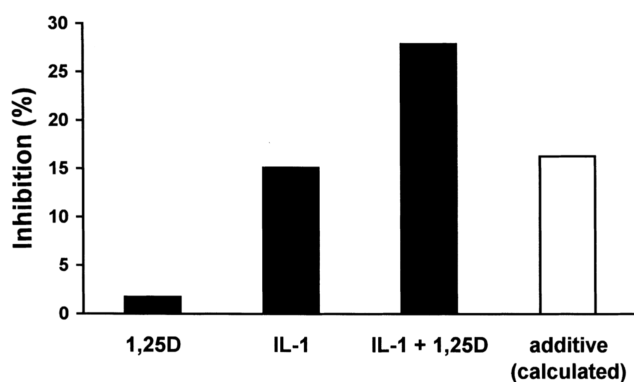


Fig. 1. The effect of $1,25(OH)_2D_3$ and IL-1β on MCF-7 cell cultures. MCF-7 cells were seeded in 96-well microtiter plates (3000 cells/well). 24 h later cells were treated with $1,25(OH)_2D_3$ (10 nM), IL-1β (0.1 ng/ml) or both. Cells were stained with CV 96 h later. This experiment is one of the 14 independent experiments conducted in the same protocol. The data are expressed as percent inhibition compared with cultures treated with the vehicle ethanol. Each bar represents the average of five replicate cultures. The open bar denotes the calculated additive inhibitory effect.

## 3. Results

MCF-7 cells are a well studied target for the action of vitamin D receptor agonists, and the synergistic interaction between the immune cytokine TNF and $1,25(OH)_2D_3$ was first established using this model [14]. Our first aim was to examine whether $1,25(OH)_2D_3$ also affects the antiproliferative activity of the two other cytokines, IL-1β and IL-6. The data presented in Figs. 1–3 indicate that this is indeed the case. Fig. 1 illustrates the interaction between $1,25(OH)_2D_3$ and a suboptimal concentration (0.1 ng/ml) of IL-1β which inhibits MCF-7 cell proliferation by ~15%. A four-day treatment with $1,25(OH)_2D_3$ (10 nM) alone resulted in a slight reduction in cell number

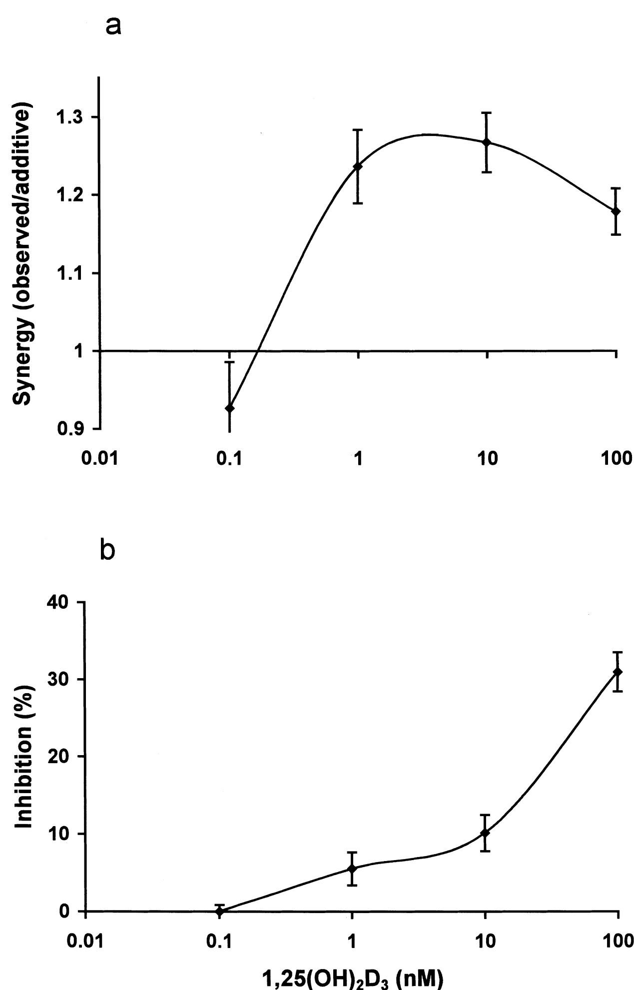


Fig. 2. The dose response of the effect of $1,25(OH)_2D_3$ on MCF-7 cells in the presence or absence of IL-1β. Cells were seeded and treated as described in the legend to Fig. 1, $1,25(OH)_2D_3$ at various concentrations was added alone or together with IL-1β (0.3 ng/ml). Synergy (Fig. 2(a)) is expressed as the ratio between the observed and the calculated additive inhibitory effect. The inhibitory effect of $1,25(OH)_2D_3$ alone (Fig. 2(b)) was calculated in respect to vehicle-treated cultures. Each data point represents the mean ± SEM of five independent experiments each performed with five replicate cultures.

(2–10% in different experiments). The actual antiproliferative effect of the cytokine–hormone combination was more pronounced than that would be expected had IL-1β and $1,25(OH)_2D_3$ acted in a strictly additive manner. (The theoretical additive inhibitory effect was calculated as described in [37,38]). The extent of synergism between the hormone and cytokine was expressed as the ratio between the observed inhibitory effect of the hormone–cytokine combination and the calculated additive inhibitory effect (denoted as synergistic ratio — SR). In 13 out of 14 independent experiments, similar to those shown in Fig. 1, we found synergistic interaction between IL-1β and $1,25(OH)_2D_3$ ($SR = 1.51 \pm 0.12$, mean ± SEM, was significantly different from 1, $p < 1 \times 10^{-4}$, Student's *t*-test). The extent of the synergism was dependent on the concentration of $1,25(OH)_2D_3$, attaining maximum between 1 and 10 nM (Fig. 2(a)). It is interesting that the cytostatic/apoptotic [5,41] action of $1,25(OH)_2D_3$ by itself was notable only at much higher concentrations of the hormone (Fig. 2(b)). At a concentration of 1 nM, $1,25(OH)_2D_3$ only marginally reduced the cell number in culture and the effect increased dose dependently at least up to a concentration of 100 nM. These findings may suggest that different mechanisms underlie the effects of $1,25(OH)_2D_3$ acting singly or in combination with IL-1β.

$1,25(OH)_2D_3$ acted synergistically also with IL-6 to reduce MCF-7 cell number in culture (Fig. 3). The effect was reproducible in four independent experiments. (SR values averaged $2.88 \pm 0.57$, mean ± SEM, $p < 0.05$).

The second objective of this work was to assess the involvement of ROS in the combined action of the cytokines and $1,25(OH)_2D_3$. This issue was addressed by examining the protective effect of the thiol antioxidant *N*-acetylcysteine (NAC). NAC is thought to act by raising intracellular concentrations of glutathione and by direct scavenging of ROS [42,43]. Preliminary experiments showed that NAC was inhibitory by itself to MCF-7 cell proliferation at concentrations higher then 10 mM. Therefore, higher concentrations were avoided in subsequent experiments.

We first examined the effect of NAC on the synergistic interaction between $1,25(OH)_2D_3$ and the cytokines, IL-1β and IL-6. The results of one out of four independent experiments with similar results performed with IL-1β and out of three with IL-6 are shown in Fig. 4. Whereas NAC had only a small protective effect against IL-1β and IL-6 activities when acting on their own, it completely prevented the synergistic interaction between the cytokines and the hormone. These results strongly suggest a mediatory role for ROS in the interaction between $1,25(OH)_2D_3$ and these cytokines.

It has been reported that TNFα induces apoptosis in MCF-7 cells in a ROS-dependent process [44]. The antioxidants NAC and GSH were employed to assess the role of ROS in the interaction between $1,25(OH)_2D_3$ and this cytokine. In accordance with our previous findings described in detail in [14], cotreatment of cells with $1,25(OH)_2D_3$ and TNFα markedly enhanced the cytotoxic potency of the cytokine (Figs. 5 and 6). Incubation of cells with various concentrations of TNFα in the presence of NAC for 48 h resulted in partial inhibition of TNFα toxicity both in the presence or absence of $1,25(OH)_2D_3$ (Fig. 5(a)). Similar results were obtained when GSH was used as the antioxidant (Fig. 5(b)).

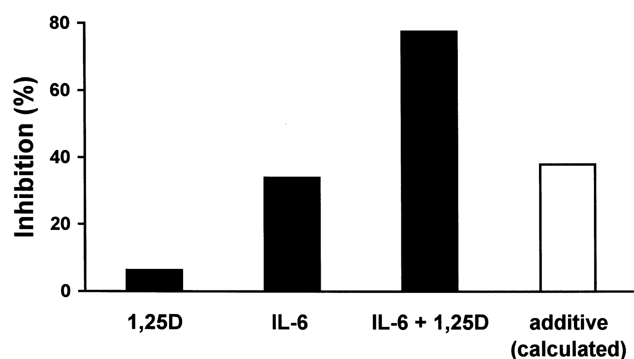


Fig. 3. The effect of $1,25(OH)_2D_3$ and IL-6 on MCF-7 cell cultures. The cells were seeded and treated as described in the legend to Fig. 1 except that the cytokine added was IL-6 (25 ng/ml) and CV staining was performed 48 h after treatment. Data are expressed as in Fig. 1. This experiment is representative of four independent experiments. Each bar represents the average of five replicate cultures.

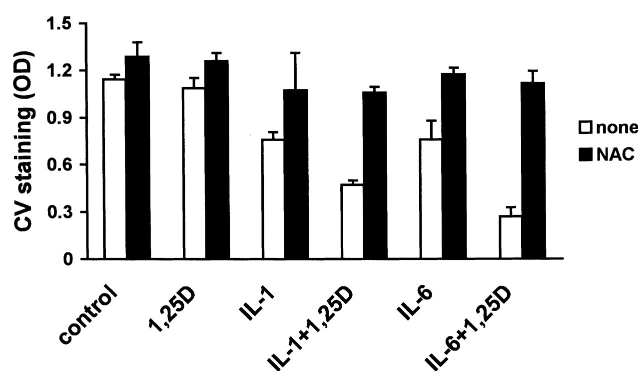


Fig. 4. The effect of NAC on the cytostatic/toxic activities of IL-1β, IL-6 alone and together with $1,25(OH)_2D_3$. Cells were seeded, treated and harvested as described in the legend to Fig. 3. $1,25(OH)_2D_3$ (10 nM), IL-1β (1 ng/ml), IL-6 (25 ng/ml) and NAC (10 mM) were added 24 h after seeding. The experiment represents one out of four similar experiments performed with IL-1β and one out of three with IL-6. Each bar represents the mean ± STD of five replicate cultures. (In these experiments cultures treated with both cytokines were monitored in parallel. Since the optimal time for observing the effect of IL-6 is 48 h, the incubation time with IL-1β was shortened as well (compare with Fig. 1) and consequently its concentration was raised to 1 ng/ml).

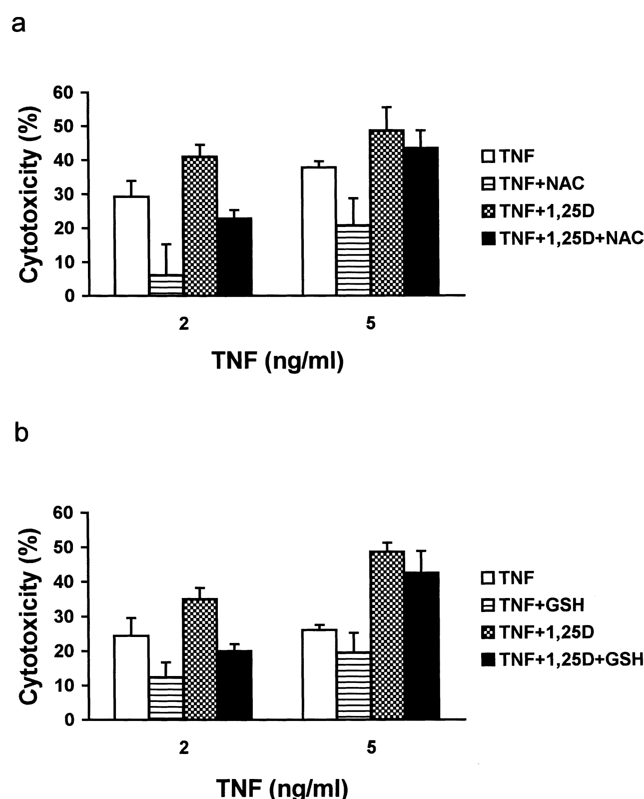


Fig. 5. The effect of NAC and GSH on the cytotoxic activity of TNF alone and together with $1,25(OH)_2D_3$. Cells were seeded in 96-well microtiter plates ($10^4$ cells/well). 24 h later cells were treated with TNF, NAC (10 mM, Fig. 5(a)), GSH (15 mM, Fig. 5(b)) and $1,25(OH)_2D_3$ (100 nM). This experiment is representative of five or four experiments performed with NAC or GSH, respectively. Data, presented as % cytotoxicity, are the mean ± STD of three replicate cultures. Cytotoxicity was calculated with respect to parallel cultures containing all additives except TNF.

From the results shown in Figs. 5 and 6 it seems that the antioxidants exert a weaker protective effect against the combined cytotoxic activity of TNFα and $1,25(OH)_2D_3$ than against that of TNFα alone. A plausible explanation for this observation is that both the cytotoxic activity of TNFα and its enhancement by $1,25(OH)_2D_3$ are ROS-dependent and that the reduced protective effect of the antioxidants after hormone treatment is due to their limited capacity to cope with the increased oxidative challenge. The results shown in Fig. 6 bear out this assumption. We pooled the data from five and four independent experiments performed with NAC and GSH, respectively. In these experiments we determined the protective effects of NAC and GSH on the cytotoxic activity of different TNFα concentrations in the presence or absence of $1,25(OH)_2D_3$ (100 nM). We found a highly significant inverse correlation between the protective effects of the thiol antioxidants and the extent of cytotoxicity. Moreover, the regression lines describing the data derived from cultures treated with TNFα alone or with TNFα and $1,25(OH)_2D_3$ are indistinguishable. We conclude from these experiments that the protective effect of the antioxidants was determined independently by the severity of the cytotoxic effect, whether increased cell damage was due to higher TNFα concentration or addition of $1,25(OH)_2D_3$. We further infer that as with TNFα on its own, ROS has a mediatory role in the enhanced cytotoxic activity of the cytokine in the presence of $1,25(OH)_2D_3$.

The involvement of ROS in the interplay between $1,25(OH)_2D_3$ and the three cytokines, TNFα, IL-1β

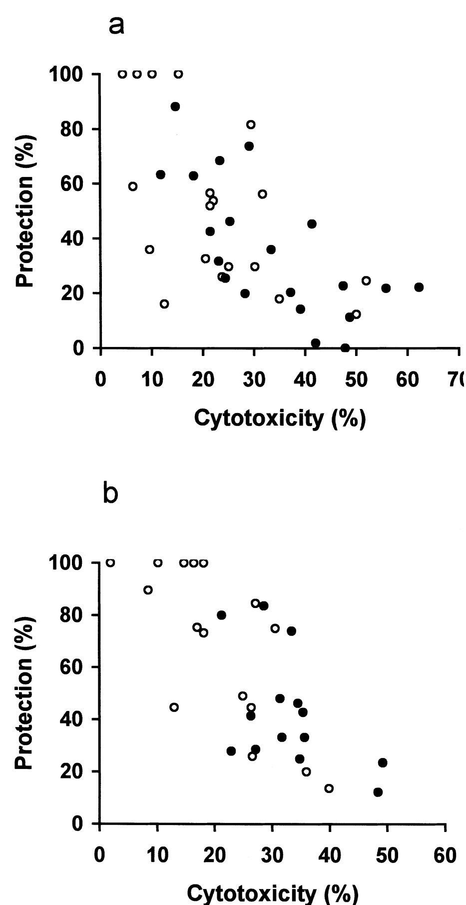


Fig. 6. Correlation between the protective effect of thiol antioxidants and cytotoxicity induced by TNF and $1,25(OH)_2D_3$. Data were pooled from experiments identical to those described in Fig. 5(a) and (b) for the correlations in Fig. 6(a) and (b), respectively. The concentration range of TNF was 0.5–10 ng/ml for Fig. 6(a) and 0.5–5 ng/ml for Fig. 6(b). "Protection" is the extent of reduction in cytotoxicity as a result of the addition of the antioxidant (NAC in Fig. 6(a) and GSH in Fig. 6(b)). The correlation coefficients were $r = 0.61$ ($p = 9 \times 10^{-6}$) for Fig. 6(a) and $r = 0.75$ ($p = 1.4 \times 10^{-7}$) for Fig. 6(b). The regression lines were indistinguishable whether calculated from assays containing TNF alone (○) or TNF with $1,25(OH)_2D_3$ (●). The linear regression equations were: for Fig. 6(a), cultures containing TNF alone, $y = -1.25(\pm 0.4)x + 77(\pm 29)$; cultures containing TNF and $1,25(OH)_2D_3$, $y = -1.25(\pm 0.3)x + 77(\pm 20)$. For Fig. 6(b), cultures containing TNF alone, $y = -2.0(\pm 0.5)x + 112(\pm 20)$; cultures containing TNF and $1,25(OH)_2D_3$, $y = -1.9(\pm 0.6)x + 103(\pm 21)$.

and IL-6, was inferred from the protective action of thiol antioxidants. The data in Table 1 provide direct evidence to support this notion. The major low molecular weight thiol compound in mammalian cells is the tripeptide glutathione [45]. This holds true also in MCF-7 cells, as found in preliminary experiments in which the glutathione synthesis inhibitor buthionine sulfoximine reduced the low molecular weight thiol pool by 90% (data not shown). It has been reported earlier that TNFα caused a decrease in the cellular content of acid soluble thiols in various experimental systems [28,46,47]. This decrease is considered a marker of oxidative stress [48].

We exposed MCF-7 cells to TNFα at a subtoxic concentration (0.5 ng/ml) for 48 h, both in the presence and in the absence of $1,25(OH)_2D_3$ (100 nM), and determined their acid soluble thiol content. Results obtained from three independent experiments (Table 1) can be summarized as follows: $1,25(OH)_2D_3$ by itself did not cause any significant change in the basal cellular level of low molecular weight, reduced thiols. (The inter-experimental variation in the size of the basal reduced thiol pool probably reflects the differences in culture cell density, since we have found a significant inverse correlation between these two parameters (data not shown)). Treatment with TNFα alone decreased the level of acid soluble thiols by $27.7 \pm 3.1\%$ (mean ± SEM, $p = 1.6 \times 10^{-4}$, ANOVA). Reduction in thiol levels by $46.4 \pm 5.5\%$ (mean ± SEM) was obtained after combined treatment with TNFα and $1,25(OH)_2D_3$ (100 nM) ($p = 4.3 \times 10^{-6}$, ANOVA, compared with TNFα alone). In parallel cultures, no reduction in cell number or viability (as judged by uptake of the vital dye neutral red) was observed. In summary, reduction in the cellular content of reduced glutathione is a direct evidence that treatment with $1,25(OH)_2D_3$ increased the oxidative stress induced by TNFα in MCF-7 cells.

Table 1
The effect of TNFα and $1,25(OH)_2D_3$ on the intracellular level of low molecular weight thiols[a]

| Experiment | Treatment | Glutathione equivalents (nmol/mg protein) | |
|---|---|---|---|
| | | None | $1,25(OH)_2D_3$ |
| 1 | None | $71.8 \pm 7.6$ | $81.9 \pm 9.1$ |
| | TNFα | $52.6 \pm 2.5$ (26.7%)[b] | $34.8 \pm 6.9$ (57.5%) |
| 2 | None | $53.9 \pm 7.3$ | $46.6 \pm 6.5$ |
| | TNFα | $35.8 \pm 5.4$ (33.6%) | $26.9 \pm 2.1$ (42.3%) |
| 3 | None | $39.2 \pm 5.4$ | $33.4 \pm 5.0$ |
| | TNFα | $30.2 \pm 0.4$ (22.9%) | $20.2 \pm 1.9$ (39.5%) |

[a] MCF-7 cells were incubated with TNF (0.5 ng/ml) in the presence or absence of $1,25(OH)_2D_3$ (100 nM) for 48 h. Data are expressed as the mean ± STD of triplicate (experiment 1 and 2) or five replicate (experiment 3) cultures. The effect of $1,25(OH)_2D_3$ alone on the reduced thiol levels was not significant. The effect of TNF was significant ($p = 1.6 \times 10^{-4}$, two way ANOVA) and the difference between cultures co-treated with TNF and $1,25(OH)_2D_3$ as compared with cultures treated with TNF alone was also significant ($p = 4.3 \times 10^{-6}$, two way ANOVA).

[b] Reduction in thiol content is presented as percent of thiol content in the respective control cultures not treated with TNF.

## 4. Discussion

The evidence presented above lends credence to the notion that the hormonal form of vitamin D, $1,25(OH)_2D_3$, may take part in the action of immune host defense mechanisms that limit tumor growth. $1,25(OH)_2D_3$ may be produced in the vicinity of tumor cells by tumor-associated macrophages that, together with other tumor infiltrating leukocytes, secrete cytokines with anticancer potential. The results of this study, combined with previous findings [14,15], indicate that the anticancer potential of the hormone and such cytokines, acting in concert, is considerably greater than that predicted assuming independent and additive actions. In addition, the $ED_{50}$ for $1,25(OH)_2D_3$ in its synergistic interaction with the cytokines is one or more orders of magnitude smaller than that for its antiproliferative effect on its own (Fig. 2). Stated differently, hormone concentrations that do not on their own affect cell number or viability, can markedly enhance the cytotoxic/static effect of the immune cytokines. It is possible that these two different actions of $1,25(OH)_2D_3$ involve different VDR target genes. It is also noteworthy, that due to local production of $1,25(OH)_2D_3$ that may take place within the tumor milieu, the concentrations of the hormone in the vicinity of cancer cells may significantly exceed the circulating levels. This may be particularly true in cases, such as breast carcinoma, where infiltrating-activated macrophages comprise a substantial component of the tumor cell mass [49,50].

It is noteworthy that pretreatment of cancer cells with $1,25(OH)_2D_3$ selectively increased their susceptibility to some anticancer agents. Whereas $1,25(OH)_2D_3$ enhanced the activity of TNFα, IL-1β and IL-6, the anticancer drug doxorubicin and another cytotoxic quinone, menadione [25], it did not affect the sensitivity of cancer cells to the killing activity of lymphokine-activated killer cells, to the antiprolifeative activity of interferon α [15] or to the cytotoxic activity of topoisomerase inhibitor etoposide [25]. A distinctive feature shared by doxorubicin and menadione is the involvement of ROS in their cytotoxic mode of action [31,32]. Moreover, exposure to antioxidant practically abolished the synergism between $1,25(OH)_2D_3$ and doxorubicin [25]. The findings of this work are consistent with the notion that ROS have a mediatory role also in the interplay between $1,25(OH)_2D_3$ and the immune cytokines. One line of evidence leading to this

conclusion is the ability of thiol antioxidants to inhibit cytotoxicity that is induced by the combination of $1,25(OH)_2D_3$ and the immune cytokines. In the case of IL-1β and IL-6, the effect of NAC on the action of the cytokines on their own is small, but the synergistic interaction with $1,25(OH)_2D_3$ is abrogated by the addition of the antioxidant. A plausible interpretation of these data is that a ROS dependent mechanism plays only a minor role in the cytostatic/toxic activity of IL-1β and IL-6 on their own, but this mechanism is selectively enhanced by $1,25(OH)_2D_3$. Whereas there are some reports demonstrating increased superoxide production in cells exposed to IL-1 [33,34], the evidence for IL-6 is much less conclusive [35,36]. In the case of TNFα we found that antioxidants affected the action of TNFα alone and its enhancement following treatment with $1,25(OH)_2D_3$ to a similar extent. Taking into account also the previous information that the major pathway leading to TNF-induced apoptosis in MCF-7 cells is ROS-dependent [44], we infer that the efficiency of this pathway is enhanced by $1,25(OH)_2D_3$. Direct evidence for the involvement of ROS in the interaction between $1,25(OH)_2D_3$ and TNFα is the increased oxidative stress experienced by cells exposed to TNFα in the presence of $1,25(OH)_2D_3$. This increased oxidative stress was manifested by reduction of GSH content.

The ROS-dependent interaction between $1,25(OH)_2D_3$ and the immune cytokines could be due to two different mechanisms: (1) a hormone-dependent increase in intracellular ROS formation that in turn mediates the action of the cytokine; (2) a hormone dependent increase in the toxicity of ROS produced intracellularly, possibly due to reduced effectiveness of cellular defense or repair mechanisms. Recently obtained evidence supports the second alternative, however, without excluding the first one. We found that treatment of MCF-7 cells with $1,25(OH)_2D_3$ reduced the expression of Cu/Zn superoxide dismutase [25], one of the major enzymes responsible for cellular defense against ROS. Lower levels of this enzyme result in impaired ability of cancer cells to cope with superoxides. In conclusion, this study sheds new light on the role that endogenous-active vitamin D metabolites may play in the host defense mechanism against tumors. Additionally, this work provides a theoretical basis for applications of vitamin D derivatives in cancer therapy, both as sole agents or combined with immunotherapy, radiotherapy and ROS dependent chemotherapy.

## Acknowledgements

This research was supported by The Israel Science Foundation administered by The Israel Academy of Sciences and Humanities (research grant No. 684/931).

## References

[1] S.A. Ingles, G.A. Coetzee, R.K. Ross, B.E. Henderson, L.N. Kolonel, L. Crocitto, W. Wang, R.W. Haile, Association of prostate cancer with vitamin D receptor haplotypes in African-Americans, Cancer Res. 58 (1998) 1620–1623.

[2] J.A. Taylor, A. Hirvonen, M. Watson, G. Pittman, J.L. Mohler, D.A. Bell, Association of prostate cancer with vitamin D receptor gene polymorphism, Cancer Res. 56 (1996) 4108–4210.

[3] J. Ma, M.J. Stampfer, P.H. Gann, H.L. Hough, E. Giovannucci, K.T. Kelsey, C.H. Hennekens, D.J. Hunter, Vitamin D receptor polymorphisms, circulating vitamin D metabolites, and risk of prostate cancer in United States physicians, Cancer Epidemiol. Biomarkers Prev. 7 (1998) 385–390.

[4] K.W. Colston, U. Berger, R.C. Coombes, Possible role for vitamin D in controlling breast cancer cell proliferation, Lancet 1 (1989) 188–191.

[5] G.P. Studzinski, D.C. Moore, Sunlight — can it prevent as well as cause cancer?, Cancer Res. 55 (1995) 4014–4022.

[6] A.R. Glass, J.W. Kikendall, L.H. Sobin, P.E. Bowen, Serum 25-hydroxyvitamin D concentrations in colonic neoplasia, Horm. Metab. Res. 25 (1993) 397–398.

[7] C.F. Garland, G.W. Comstock, F.C. Garland, K.J. Helsing, E.K. Shaw, E.D. Gorham, Serum 25-hydroxyvitamin D and colon cancer: eight-year prospective study, Lancet 2 (1989) 1176–1178.

[8] A. Dusso, J. Finch, J. Delmez, N. Rapp, S. Lopez-Hilker, A. Brown, E. Slatopolsky, Extrarenal production of calcitriol, Kidney Int. Suppl. 29 (1990) S36–40.

[9] H. Reichel, H.P. Koeffler, A.W. Norman, Production of 1 alpha, 25-dihydroxyvitamin $D_3$ by hematopoietic cells, Prog. Clin. Biol. Res. 332 (1990) 81–97.

[10] E.B. Mawer, M.E. Hayes, S.E. Heys, M. Davies, A. White, M.F. Stewart, G.N. Smith, Constitutive synthesis of 1,25-dihydroxyvitamin $D_3$ by a human small cell lung cancer cell line, J. Clin. Endocrinol. Metab. 79 (1994) 554–560.

[11] G.G. Schwartz, L.W. Whitlatch, T.C. Chen, B.L. Lokeshwar, M.F. Holick, Human prostate cells synthesize 1,25-dihydroxyvitamin $D_3$ from 25-hydroxyvitamin $D_3$, Cancer Epidemiol. Biomarkers Prev. 7 (1998) 391–395.

[12] T. Gulliford, J. English, K.W. Colston, P. Menday, S. Moller, R.C. Coombes, A phase I study of the vitamin D analogue EB 1089 in patients with advanced breast and colorectal cancer, Br. J. Cancer 78 (1998) 6–13.

[13] C. Gross, T. Stamey, S. Hancock, D. Feldman, Treatment of early recurrent prostate cancer with 1,25-dihydroxyvitamin $D_3$ (calcitriol), J. Urol. 159 (1998) 2035–2039.

[14] D. Rocker, A. Ravid, U.A Liberman, O. Garach-Jehoshua, R. Koren, 1,25-Dihydroxyvitamin $D_3$ potentiates the cytotoxic effect of TNF on human breast cancer cells, Mol. Cell. Endocrinol. 106 (1994) 157–162.

[15] R. Yacobi, R. Koren, U.A. Liberman, C. Rotem, L. Wasserman, A. Ravid, 1,25-dihydroxyvitamin $D_3$ increases the sensitivity of human renal carcinoma cells to tumor necrosis factor alpha but not to interferon alpha or lymphokine-activated killer cells, J. Endocrinol. 149 (1996) 327–333.

[16] C. O'Sullivan, C.E. Lewis, Tumour-associated leucocytes: friends or foes in breast carcinoma, J. Pathol. 172 (1994) 229–235.

[17] R.D. Leek, A.L. Harris, C.E. Lewis, Cytokine networks in

solid human tumors: regulation of angiogenesis, J. Leukoc. Biol. 56 (1994) 423–435.

[18] J.M. Watson, J.L. Sensintaffar, J.S. Berek, O. Martinez-Maza, Constitutive production of interleukin 6 by ovarian cancer cell lines and by primary ovarian tumor cultures, Cancer Res. 50 (1990) 6959–6965.

[19] C.B. Siegall, G. Schwab, R.P. Nordan, D.J. FitzGerald, I. Pastan, Expression of the interleukin-6 receptor and interleukin 6 in prostate carcinoma cells, Cancer Res. 50 (1990) 7786–7788.

[20] K. Onozaki, K. Matsushima, B.B. Aggarwal, J.J. Oppenheim, Human interleukin 1 is a cytocidal factor for several tumor cell lines, J. Immunol. 135 (1985) 3962–3968.

[21] L.B. Lachman, C.A. Dinarello, N.D. Llansa, I.J. Fidler, Natural and recombinant human interleukin-1 beta is cytotoxic for human melanoma cells, J. Immunol. 136 (1986) 3098–3102.

[22] M.K. Sgagias, A. Kasid, D.N. Danforth, Interleukin-1 alpha and tumor necrosis factor-alpha (TNF alpha) inhibit growth and induce TNF messenger RNA in MCF-7 human breast cancer cells, Mol. Endocrinol. 5 (1991) 1740–1747.

[23] S. Akira, T. Taga, T. Kishimoto, Interleukin-6 in biology and medicine, Adv. Immunol. 54 (1993) 1–78.

[24] D.N. Danforth, M.K. Sgagias, Interleukin-1 alpha and interleukin-6 act additively to inhibit growth of MCF-7 breast cancer cells in vitro, Cancer Res. 53 (1993) 1538–1545.

[25] A. Ravid, D. Rocker, A. Machlenkin, C. Rotem, A. Hochman, G. Kessler-Icekson, U.A. Liberman, R. Koren, 1,25-dihydroxyvitamin $D_3$ enhances the susceptibility of breast cancer cells to doxorubicin-induced oxidative damage, Cancer Res. 59 (1999) 862–867.

[26] N. Yamauchi, H. Kuriyama, N. Watanabe, H. Neda, M. Maeda, Y. Niitsu, Intracellular hydroxyl radical production induced by recombinant human tumor necrosis factor and its implication in the killing of tumor cells in vitro, Cancer Res. 49 (1989) 1671–1675.

[27] N. Matthews, M.L. Neale, S.K. Jackson, J.M. Stark, Tumour cell killing by tumour necrosis factor: inhibition by anaerobic conditions, free-radical scavengers and inhibitors of arachidonate metabolism, Immunology 62 (1987) 153–155.

[28] R.J. Zimmerman, A. Chan, S.A. Leadon, Oxidative damage in murine tumor cells treated in vitro by recombinant human tumor necrosis factor, Cancer Res. 49 (1989) 1644–1648.

[29] R.J. Zimmerman, B.J. Marafino, A. Chan, P. Landre, J.L Winkelhake, The role of oxidant injury in tumor cell sensitivity to recombinant human tumor necrosis factor in vivo, J. Immunol. 142 (1989) 1405–1409.

[30] N. Yamauchi, N. Watanabe, H. Kuriyama, H. Neda, M. Maeda, T. Himeno, Y. Tsu, Y. Niitsu, Suppressive effects of intracellular glutathione on hydroxyl radical production induced by tumor necrosis factor, Int. J. Cancer 46 (1990) 884–888.

[31] J.W. Lown, H.H. Chen, J.A. Plambeck, E.M. Acton, Further studies on the generation of reactive oxygen species from activated anthracyclines and the relationship to cytotoxic action and cardiotoxic effects, Biochem. Pharmacol. 31 (1982) 575–581.

[32] E.O. Ngo, T.P. Sun, J.Y. Chang, C.C. Wang, K.H. Chi, A.L. Cheng, L.M. Nutter, Menadione-induced DNA damage in a human tumor cell line, Biochem. Pharmacol. 42 (1991) 1961–1968.

[33] B. Meier, H.H. Radeke, S. Selle, M. Younes, H. Sies, K. Resch, G.G. Habermehl, Human fibroblasts release reactive oxygen species in response to interleukin-1 or tumour necrosis factor-alpha, Biochem. J. 263 (1989) 539–545.

[34] K. Fukuda, M. Takayama, M. Ueno, M. Oh, S. Asada, F. Kumano, S. Tanaka, Hyaluronic acid inhibits interleukin-1-induced superoxide anion in bovine chondrocytes, Inflamm. Res. 46 (1997) 114–117.

[35] P.G. Mullen, A.C. Windsor, C.J. Walsh, A.A. Fowler III, H.J. Sugerman, Tumor necrosis factor-alpha and interleukin-6 selectively regulate neutrophil function in vitro, J. Surg. Res. 58 (1995) 124–130.

[36] A. Nussler, J.C. Drapier, L. Renia, S. Pied, F. Miltgen, M. Gentilini, D. Mazier, L-arginine-dependent destruction of intrahepatic malaria parasites in response to tumor necrosis factor and/or interleukin-6 stimulation, Eur. J. Immunol. 21 (1991) 227–230.

[37] N. Saati, A. Ravid, U.A. Liberman, R. Koren, 1,25-dihydroxyvitamin $D_3$ and agents that increase intracellular adenosine $3',5'$-monophosphate synergistically inhibit fibroblast proliferation, in vitro, Cell. Dev. Biol. Anim. 33 (1997) 310–314.

[38] A. Ravid, R. Koren, R. Narinsky, C. Rotem, A. Novogrodsky, U.A. Liberman, 1,25-dihydroxyvitamin $D_3$ and agents that increase intracellular adenosine $3',5'$-monophosphate synergistically inhibit the mitogenic stimulation of human lymphocytes, J. Clin. Endocrinol. Metab. 70 (1990) 1687–1692.

[39] G.L. Ellman, Tissue sulfhydryl groups, Arch. Biochem. Biophys. 82 (1959) 70–77.

[40] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.

[41] C.J. Narvaez, J. Welsh, Differential effects of 1,25-dihydroxyvitamin $D_3$ and tetradecanoylphorbol acetate on cell cycle and apoptosis of MCF-7 cells and a vitamin $D_3$-resistant variant, Endocrinology 138 (1997) 4690–4698.

[42] A.F. Junod, L. Jornot, G. Grichting, Comparative study on the selenium and *N*-acetylcysteine-related effects on the toxic actions of hyperoxia, paraquat and the enzyme reaction hypoxanthine-xanthine oxidase in cultured endothelial cells, Agents Actions 22 (1987) 176–183.

[43] O.I. Aruoma, B. Halliwell, B.M. Hoet, J. Butler, The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide and hypochlorous acid, Free Radic. Biol. Med. 6 (1989) 593–597.

[44] B. Liu, N. Andrieu-Abadie, T. Levade, P. Zhang, L.M. Obeid, Y.A. Hannun, Glutathione regulation of neutral sphingomyelinase in tumor necrosis factor-alpha-induced cell death, J. Biol. Chem. 273 (1998) 11313–11320.

[45] E.A. Bump, J.M. Brown, Role of glutathione in the radiation response of mammalian cells in vitro and in vivo, Pharmacol. Ther. 47 (1990) 117–136.

[46] G.M. Adamson, R.E. Billings, Tumor necrosis factor induced oxidative stress in isolated mouse hepatocytes, Arch. Biochem. Biophys. 294 (1992) 223–229.

[47] Y. Ishii, C.A. Partridge, P.J. Del Reccaio, A.B. Malik, Tumor necrosis factor-α-mediated decrease in glutathione increases the sensitivity of pulmonary vascular endothelial cells to $H_2O_2$, J. Clin. Invest. 89 (1991) 794–802.

[48] J.D. Adams, J.R. Mitchell, Plasma glutathione and glutathione disulfide in the rat: regulation and response to oxidative stress, J. Pharmacol. Exp. Ther. 227 (1983) 749–754.

[49] R.J. Steele, M. Brown, O. Eremin, Characterisation of macrophages infiltrating human mammary carcinomas, Br. J. Cancer 51 (1985) 135–138.

[50] P.M. Kelly, R.S. Davison, E. Bliss, J.O. McGee, Macrophages in human breast disease: a quantitative immunohistochemical study, Br. J. Cancer 57 (1988) 174–177.