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Interactions of vitamin D analogue CB1093, TNFα and ceramide on breast cancer cell apoptosis

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Abstract

Mechanisms by which vitamin D analogues promote apoptosis in tumour cells are unclear. In this study we have examined possible interactions between the synthetic vitamin D analogue CB1093 and two other known mediators of apoptosis, TNFα and ceramide, in MCF-7, T47D and Hs578T breast cancer cells. These studies indicated that cytosolic phospholipase A_2 ($cPLA_2$) is involved in CB1093 as well as TNFα-mediated cell death. CB1093 promoted both TNFα and ceramide-induced c-PLA_2 activation, which was inversely related to loss of cell viability in MCF-7 and Hs578T cells. TNFα alone (5–20 ng/ml) failed to induce cytotoxicity and activation of $cPLA_2$ in T47D cells. However, pretreatment of these cells with CB1093 potentiated C_2-ceramide-induced $cPLA_2$ activation and cell death. Treatment with CB1093 alone induced loss of cell viability and DNA fragmentation in all three cell lines by 5 days and these effects were accompanied by activation of $cPLA_2$. Furthermore, co-treatment with the $cPLA_2$ inhibitor $AACOCF_3$ led to partial protection against loss of cell viability induced by CB1093 in Hs578T and T47D cells as well as MCF-7 cells. The broad-spectrum caspase inhibitor z-VAD-fmk prevented TNFα but not C_2-ceramide and CB1093-mediated release of arachidonic acid and cell death in MCF-7 cells. These results indicate that CB1093 potentiates responsiveness of breast cancer cells to TNFα and suggest that ceramide and/or $cPLA_2$ might be involved as downstream effectors in vitamin D-mediated caspase-independent cell death. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Vitamin D analogues; $cPLA_2$; TNFα; Ceramide; Apoptosis; Human breast cancer cells

1. Introduction

The active hormonal form of vitamin D_3, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), regulates diverse biological functions in many cell types via binding to its nuclear receptor VDR (Bikle, 1997; Haussler et al., 1998). The observation that a high proportion of breast tumour biopsy specimens contain receptors for $1,25(OH)_2D_3$, and that the growth of cultured human breast cancer cells is inhibited in the presence of this vitamin D metabolite, led to the suggestion that vitamin D derivatives might have potential as therapeutic agents in this and other malignancies (Colston, 1997). Synthetic vitamin D analogues have been developed which retain the ability to control cell proliferation and differentiation but display reduced calcaemic activity relative to $1,25(OH)_2D_3$ in vivo (Binderup and Godtfedsen, 1997; Bouillon et al., 1995). In addition, a number of these compounds have been tested for their effectiveness in inhibiting the growth of breast cancer cells in vitro (Mathiasen et al., 1993; Elstner et al., 1995) and have been shown to cause striking tumour regression in animal models of breast cancer (Colston et al., 1992; Danielsson et al., 1997; Welsh et al., 1998). Several studies have suggested that vitamin D analogues may induce active cell death (apoptosis) in breast cancer cells (Danielsson et al., 1997; James et al., 1995; Simboli-Campbell et al., 1996). The failure of cells to undergo apoptosis is a major determining factor in the development of many types of tumour. A better

Abbreviations: AA, arachidonic acid; $AACOCF_3$, arachidonyl trifluoromethylketone; CB1093, 20-epi-22(S)-ethoxy-23yne-24α,26α,27α-trihomo-1a,25-dihydroxyvitamin D3; $cPLA_2$, cytosolic phospholipase A2; TNFα, tumour necrosis factor alpha; z-VAD-fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

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understanding of the signalling mechanisms involved in apoptosis mediated by novel vitamin D analogues is likely to be of value in determining their potential in breast cancer therapy.

Mechanisms by which vitamin D analogues promote apoptosis could involve increased expression of genes that stimulate cell death or suppression of survival signals. In the present study we have examined a possible involvement of TNFα, a known inducer of apoptosis, in a common pathway leading to vitamin D-mediated cell death in breast cancer cells. TNFα is a potent cytokine which demonstrates anti-tumour activity both in vivo and in vitro and this cytokine induces apoptosis in a variety of tumour cell lines. Of the two TNF receptors, TNF-RI (p55) and TNF-RII (p75), the former is thought to promote cytotoxic effects (Schall et al., 1990; Smith et al., 1990). TNF-RI possesses an intracellular 'death domain' consisting of 80 amino acid residues, which is homologous to the 48 kD Fas transmembrane protein. Studies have shown that the 'death domain' of TNF-RI specifically interacts with adapter proteins (TRADD/FADD), and the newly formed complex can interact with and activate caspase 8/FLICE leading to a cascade of caspase activation and cell death (Chinnaaiyan et al., 1995). However, recent papers have demonstrated that TNFα induces apoptosis and necrosis by caspase-dependent and -independent mechanisms (Slowik et al., 1997). The sphingomyelin pathway mediates signalling for apoptosis induced by several agents including TNFα (Wiegmann et al., 1994). Activation of sphingomyelinases hydrolyses sphingomyelin to generate ceramide, which acts as a second messenger to activate apoptosis (Obeid et al., 1993).

Recently it has been demonstrated that activation of cytosolic phospholipase A_2 ($cPLA_2$ or PLA_2-IV isoform) is also involved in TNFα-mediated apoptosis in several cell lines including MCF-7 breast cancer cells (Voelkel-Johnson et al., 1996; Wissing et al., 1997). $cPLA_2$ is involved in arachidonic acid (AA) release from its cellular storage in the phospholipid pool and plays an important role in cellular signalling (Qiu et al., 1998). $cPLA_2$ is a high molecular weight enzyme (85 kDa) which does not share homology with the other small molecular weight phospholipase isoforms (Kashiwagi et al., 1998). $cPLA_2$ is normally located in the cytoplasm and translocates to the cell membrane on activation by increased intracellular calcium levels or phosphorylation (Wiegmann et al., 1994). We have recently presented evidence that activation of $cPLA_2$ may be a feature of apoptosis induced by vitamin D analogues in MCF-7 breast cancer cells (Pirianov et al., 1999). In order to further define the cellular and molecular mechanisms involved in vitamin D-induced apoptosis, we have assessed the effects of the vitamin D analogue CB1093 on TNFα-induced apoptosis in different breast cancer cell lines (MCF-7, T47D and Hs578T) cells. We now report that vitamin D analogues potentiate responsiveness of breast cancer cells to TNFα and suggest that ceramide and/or $cPLA_2$ might be involved in vitamin D-mediated caspase-independent cell death.

2. Materials and methods

2.1. Reagents

CB1093(20-epi-22(S)-ethoxy-23yne-24α,26a,27α-trihomo-lα,25-dihydroxyvitamin D_3) was a gift from Dr Lise Binderup, Leo Pharmaceutical Products, Denmark. The vitamin D analogue was dissolved in ethanol and stored at -20°C. For use in experiments the ethanol concentration did not exceed 0.1% in tissue culture medium. Human recombinant TNFα, C_2-ceramide, a broad spectrum caspase inhibitor *N*-benzyloxycarbonyl - Val - Ala - Asp - fluoromethylketone (z-VAD-fmk) and the $cPLA_2$ inhibitor arachidonyl trifluoromethylketone ($AACOCF_3$) were purchased from Calbiochem-UK. [3H-*methyl*]-thymidine (80.0 Ci/mmol) was purchased from Amersham International (UK) and 5.6.8.9.11.12.14.15-[^{3}H]-arachidonic acid (240 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Tissue culture media (RPMI-1640 and DMEM) were obtained from Life Technologies (Paisley, Scotland).

2.2. Cell cultures

MCF-7 and T47D human breast cancer cell lines (Danish Cancer Society) and Hs578T human breast cancer cells (European Tissue Culture Collection) were maintained in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 5% foetal calf serum (FCS).

2.3. Cell viability and DNA fragmentation assays

2.3.1. Neutral red assay

Neutral red is a vital dye that accumulates in the lysosomes of living, uninjured cells. Cytotoxicity of TNFα or ceramide in control and CB1093 pretreated cultures was determined by the method of Rocker et al. (1994). Briefly, cells were seeded into 24 well plates (Nunc, Oxford UK) at a density of 2×10^4 cells/well and left to adhere for 4–5 h, after which medium containing 100 nM CB1093 with or without inhibitors of interest was added. Following a preincubation period of 48 h with the vitamin D analogue, fresh medium containing the analogues with and without rhTNFα was added and cultures were incubated for a

further 18–24 h. In other experiments cells were switched to serum-free medium (RPMI-1640, bovine serum albumin 0.5 mg/ml and transferrin 0.01 mg/ml) and C_2-ceramide was added. At the end of the incubation period, medium was removed and cells were incubated with neutral red solution (Sigma, Poole, UK, 40 μg/ml in phenol red and serum-free DMEM) for 2 h at 37°C. After removal of the neutral red solution, wells were rinsed once with 1 ml 4% formal saline containing 0.5% $CaC1_2$. Plates were inverted on paper towel to drain and 200 μl of elution fluid (1%, of acetic acid in 50% ethanol) was added. Following incubation at room temperature for 30 min with gentle shaking, absorbance at 550 nm was determined using a Titertek plate reader. Cytotoxic effect of TNFα was calculated as previously described (Rocker et al., 1994).

2.3.2. MTS assay

Cell viability was determined by MTS dye-reduction measuring mitochondrial respiratory function (Cory et al., 1991). Cells were plated in 96 well microtitre plates (2×10^3 cells/well) and treated with inhibitors and compounds of interest for various lengths of time. Cells were incubated with MTS dye (20 μl/well) for 2 h, and solubilzed with 10% SDS at 18°C for 16 h. Absorbance was read in a Titertek plate reader at 492 nm.

2.3.3. DNA fragmentation assay

Cells were incubated with [^{3}H]-thymidine (1 μCi/ml) for 24 h to label DNA and then washed before exposure to the indicated agents. Cells were lysed and [^{3}H]thymidine incorporated into both soluble and unfragmented DNA was determined by liquid scintillation counting using the formula % fragmented DNA = 100 (fragmented/fragmented + intact chromatin) as previously described (Duke and Cohen, 1992).

2.4. Activation of $cPLA_2$

Measurement of [^{3}H]arachidonic acid (AA) release from MCF-7, Hs578T and T47D breast cancer cells was determined as described by Wu et al. (1998) with some modifications. Briefly, cells were seeded into 24 well plates and treated with 100 nM CB1093 or ethanol vehicle and incubated at 37°C for 48 h. After 30 h of treatment, cells were labeled with 0.3 μCi/ml 5.6.8.9.11.12.14.15-[^{3}H]AA for the final 18 h of treatment. The unincorporated [^{3}H]AA was then removed by washing three times with serum free medium. Fresh medium was added containing graded concentrations of rhTNFα (0.1–20 ng/ml), or vehicle for 22 h. In some experiments cells were switched to serum-free medium and C_2-ceramide (0.5–20 μM) was added for 5 h. In experiments using $AACOCF_3$, or z-VAD-fmk inhibitors, cells were first pretreated for 3 h with the inhibitors and then exposed to the drugs. Media were removed, centrifuged at 2000 ***g*** for 5 min and 0.5 ml of supernatant processed for liquid scintillation counting. Cells were removed by trypsinization, centrifuged at 2000 ***g*** and cell pellets solubilized in 0.5 M sodium hydroxide and 0.5 ml assessed for radioactivity. The percentage of release of [^{3}H]AA was calculated by the formula $[S/(S+P)] \times 100$ where S and P represent radioactivity detected in 0.5 ml of supernatant and solubilized cell pellet respectively. Non-enzymatic release of [^{3}H]AA from prelabelled cells, frozen at −80°C and thawed at room temperature was less than 10% in all experiments. Results were expressed as a percent of [^{3}H]AA release in non-treated cultures (accepted as 100%).

2.5. Statistical analysis

Statistical analysis was performed using unpaired Student's t-test or ANOVA using Statview-4 software package (Apple Macintosh). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Comparison of cytotoxic effects of TNFα and potentiation by CB1093 in three breast cancer cell lines

Initial studies showed that MCF-7 cells were responsive to induction of apoptosis by TNFα. Hs578T cells were less responsive (13% vs 49% loss of cell viability after 24 h incubation with 10 ng/ml TNFα, Fig. 1). T47D cells did not demonstrate loss of cell viability in

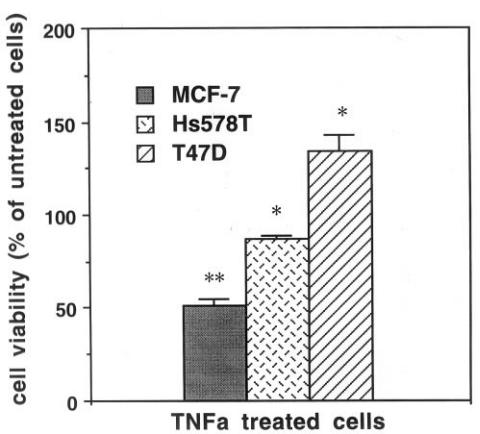

Fig. 1. Differential effects of TNFα in three human breast cancer cell lines. MCF-7, Hs578T and T47D cells were seeded into 96 well microtitre plates (2×10^3 cells/well) and allowed to attach for 18 h. Medium was removed and replaced with fresh medium containing 10 ng/ml rhTNFα. After a further 24 h incubation, cell viability was determined by MTS dye reduction assay as described in Section 2. Results are the mean ± SD of three replicate estimations. The difference between cultures treated with TNFα and vehicle treated controls was significant at $^*P < 0.05$, $^{**}P < 0.001$.

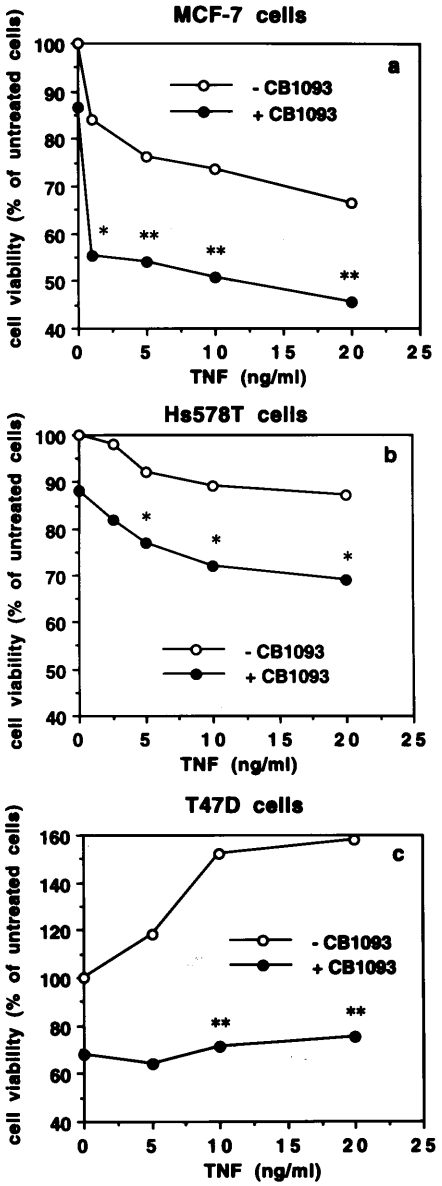

Fig. 2. CB1093 promotes cytotoxic effect of TNFα in human breast cancer cells. MCF-7 (a) Hs578T (b) and T47D (c) breast cancer cells were pretreated with ethanol vehicle or 100 nM CB1093 for 48 h, washed and posttreated with TNFα (0–20 ng/ml) for a further 24 h. Cell viability was determined by MTS (a,b) and neutral red assay (c) as described in Section 2. The difference between cultures treated with TNFα alone and TNFα plus CB1093 was found to be significant at $^{*}P < 0.05$, $^{**}P < 0.001$.

response to 10 ng/ml TNFα at 24 h of treatment (Fig. 1) but a weak mitogenic effect was observed. Incubation of cells for a longer time (up to 72 h) or with a higher dose of the cytokine also failed to induce cell death (data not shown). In both MCF-7 and Hs578T cells, effects of TNFα were augmented in cultures pretreated for 48 h with the vitamin D analogue CB1093. Cells primed with the vitamin D analogue showed increased loss of cell viability (Fig. 2a and b) and DNA fragmentation (data not shown) in response to increasing concentrations of TNFα. Although T47D cells were found to be resistant to the cytotoxic actions of TNFα, loss of cell viability was seen in cultures treated with CB1093 but this effect was not enhanced with TNFα co-treatment (Fig. 2c).

3.2. Potentiation by CB1093 of TNFα-induced cytotoxicity is associated with activation of cytosolic phospholipase A_2

In some cell types, TNFα-mediated killing is accompanied by activation of $cPLA_2$. AA generated as a result of $c.PLA_2$ activation has been implicated in a signal transduction pathway resulting in cell death (Wissing et al., 1997; Voelkel-Johnson et al., 1996). We have previously demonstrated that preincubation of MCF-7 cells with CB1093 promotes effects of TNFα on both cell viability and $cPLA_2$ activation as assessed by [^{3}H]AA release. To further evaluate the relationship between $cPLA_2$ activation and potentiation of TNFα effects by vitamin D analogues, Hs578T cells were primed with CB1093 (100 nM for 48 h). After labeling with [^{3}H]AA (0.3 μCi/ml) for the last 18 h of this incubation, cells were washed and preincubated with $AACOCF_3$ for 3h. Finally, cells were treated with TNFα (0–20 ng/ml) for a further 18 h. A modest dose-related increase in the activation of $cPLA_2$, as assessed by [^{3}H]AA release, was observed with TNFα treatment which was potentiated when cells were pretreated with CB1093 (Fig. 3a). The activation of $cPLA_2$ and its enhancement by CB1093 was inversely related to cell viability (Fig. 2b). Cotreatment of cells with $AACOCF_3$, a specific $cP1A_2$ inhibitor limited the effectiveness of CB1093 to promote TNFα-induced activation of $cPLA_2$ (Fig. 3a). As described above, T47D cells were found to be resistant to the cytotoxic actions of TNFα: loss of cell viability was seen in cultures treated with CB1093 alone but this effect was not enhanced with TNFα co-treatment (Fig. 2c). Incubation of T47D cells with CB1093 alone also promoted $cPLA_2$ activation, which was not potentiated by addition of increasing concentrations of TNFα up to 20 ng/ml (Fig. 3b).

3.3. CB1093 promotes C_2-ceramide-induced DNA fragmentation and $cPLA_2$ activation

A possible mechanism by which vitamin D analogues may potentiate TNFα-induced cytotoxicity in responsive cells is by promotion of a common apoptotic pathway. Generation of ceramide through hydrolysis of sphingomyelin appears to play a role in TNFα-induced apoptosis. We next determined if pretreatment with the vitamin D analogue CB1093 could modulate this step in the TNFα signalling pathway. Our preliminary studies with MCF-7 cells showed that treatment with cell permeable C_2-ceramide leads to a time-dependent acti-

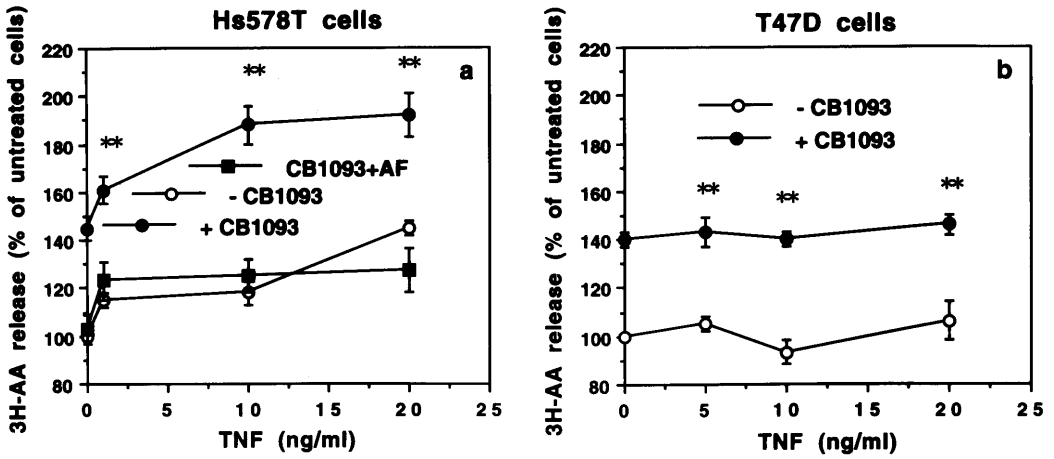

Fig. 3. Effects of CB1093 and TNFα on $cPLA_2$ activation in Hs578T and T47D breast cancer cells. (a) Hs578T cells were pretreated with CB1093 (100 nM for 48 h) and labelled with [^{3}H]AA (0.3 μCi/ml) for the last 18 h, washed and posttreated with or without 5 μM $AACOCF_3$ (AF) for 3 h and finally exposed to TNFα (0–20 ng/ml) for further 18 h. $cPLA_2$ activation was assessed by [^{3}H]AA release as described in Section 2. (b) T47D cells were primed with CB1093 (100 nM for 48 h), labelled with [^{3}H]AA (0.3 μCi/ml) for the last 18 h, washed and posttreated with TNFα (0–20 ng/ml) for 18 h. $cPIA_2$ activation was assessed by [^{3}H]AA release as described in Section 2 The difference between cultures treated with TNFα alone and TNFα plus CB1093 was found to be significant at $^{**}P < 0.001$. Results are the mean ± SD of three replicate estimations and are representative of three separate experiments.

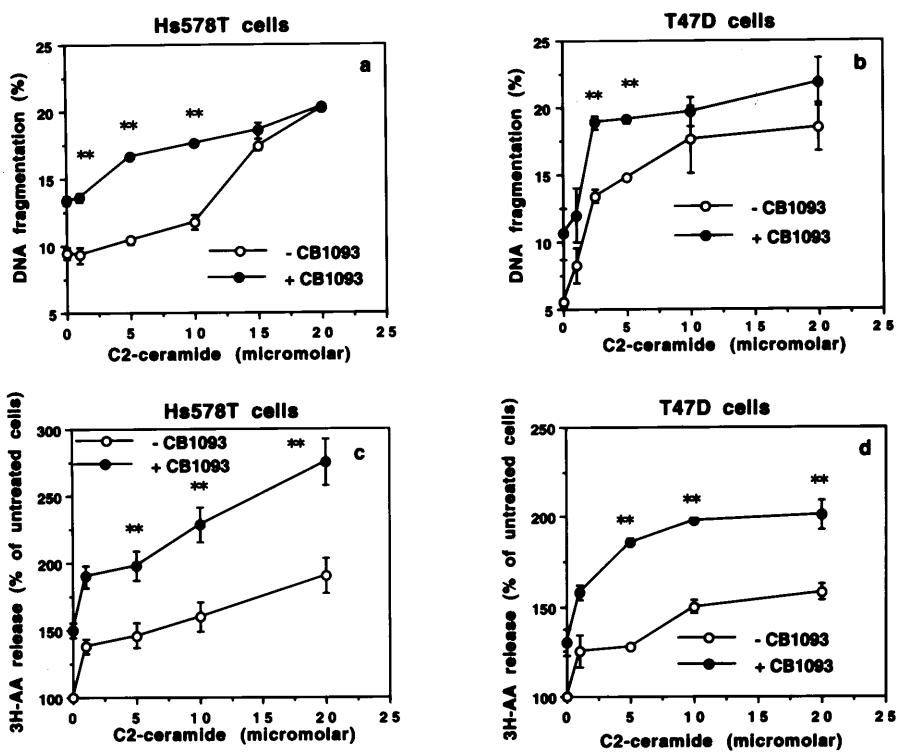

Fig. 4. CB1093 potentiates C_2-ceramide-induced apoptosis associated with $cPLA_2$ activation in breast cancer cells. Hs578T (a) and T47D cells (b) were labelled with [^{3}H]thymidine (0.5 μCi/ml for 24 h), washed twice and treated with CB1093 (100 nM for 48 h), then switched to serum-free medium and exposed to C_2-ceramide (0–20 μM) for a further 24 h. DNA fragmentation was then determined as described in Section 2. Hs578T (c) and T47D (d) cells were pretreated with CB1093 (100 nM for 48 h) and labelled with [^{3}H]AA (0.3 μCi/ml) for the last 18 h of incubation. Cells were then washed and then exposed to C_2-ceramide (0–20 μM) in serum-free medium for a further 6 h. $cPLA_2$ activation was assessed by [^{3}H]AA release as described in Section 2. Results are the mean ± SD of three replicate estimations and are representative of three separate experiments ($^{**}P < 0.001$).

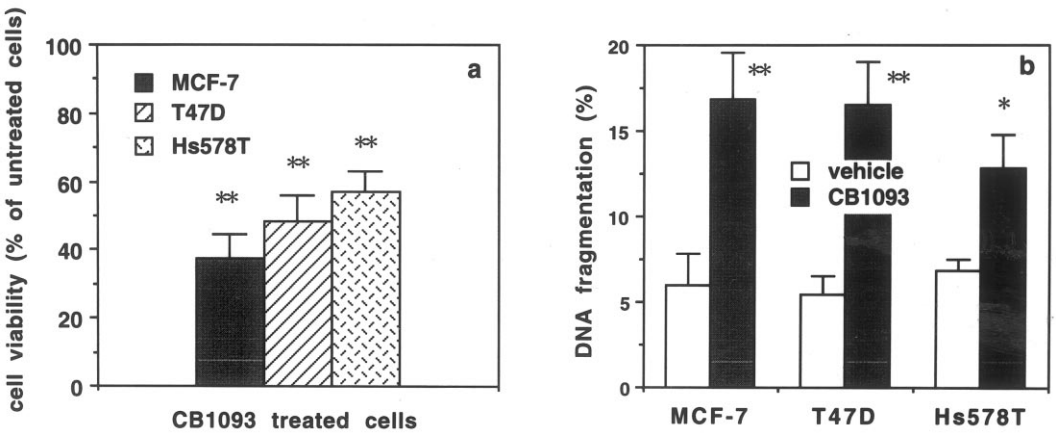

Fig. 5. CB1093 induces cell death in breast cancer cells (a) MCF-7, T47D and Hs578T cells were treated with CB1093 (100 nM for 5 days). Cell viability was determined by MTS assay (MCF-7 and Hs578T cells) or neutral red assay (T47D cells) as described in Section 2. (b) MCF-7, T47D and Hs578T cells were labelled with [^{3}H]thymidine (0.5 μCi/ml), washed twice and treated with CB1093 (100 nM for 5 days) and assessed for DNA fragmentation as described in Section 2. Statistical comparison between CB1093 treated and untreated cells (vehicle) was found to be significant at $*P < 0.05$ and $**P < 0.001$. Results are the mean ± SD of three replicate estimations and are representative of three separate experiments.

vation of $cPLA_2$ which is more rapid than that seen with TNFα, being evident at 6 h after cells are exposed to C_2-ceramide (Pirianov et al., 1999). Furthermore, pretreatment of MCF-7 cells with CB1093 for 48 h was associated with enhanced $cPLA_2$ activation in response to C_2-ceramide. To further investigate potential interactions between vitamin D analogues and ceramide, Hs578T and T47D cells were pretreated with CB1093 (100 nM for 48 h) and exposed to C_2-ceramide (1–20 μM for 24 h) before quantitation of DNA fragmentation. CB1093 treatment augmented the level of C_2-ceramide-induced intranucleosomal DNA fragmentation in both cell lines (Fig. 4a and b). In addition pretreatment of both Hs578T and T47D cells with CB1093 for 48 h was associated with enhanced $cPLA_2$ activation in response to C_2-ceramide (Fig. 4c and d).

3.4. Loss of cell viability in breast cancer cells treated with CB1093 alone is associated with $cPLA_2$ activation and is caspase-independent

We and others previously have shown that vitamin D analogues induce cell death in MCF-7 cells (Danielsson et al., 1997; James et al., 1995; Simboli-Campbell et al., 1996). We have now extended these initial observations to demonstrate that vitamin D analogues can activate programmed cell death in T47D cells, which express mutant p53, and Hs578T cells, which are oestrogen receptor negative breast cancer cells. Results clearly demonstrate that CB1093 induces a loss of cell viability (Fig. 5a) associated with DNA fragmentation (Fig. 5b) in all three breast cancer cells by 5 days of treatment.

To investigate whether caspases are involved in either the potentiation of TNFα-induced cytotoxicity or apoptosis-induced by CB1093 alone, MCF-7 cells were pretreated with a broad-spectrum caspase inhibitor (z-VAD-fmk, 50 μM) for 3 h and CB1093 (100 nM) was added for a further 48 h. Cultures were then exposed to TNFα (20 ng/ml) or vehicle for a further 24 h. Results showed that TNFα-induced loss of cell viability was substantially inhibited (about 90%) in the presence of z-VAD-fmk. However, the inhibitor was only partially effective in preventing loss of cell viability in response to TNFα in cells which were primed with CB1093, indicating that the mechanisms by which CB1093 potentiates TNFα-induced cell death may be caspase-independent (Fig. 6a). Since it has been suggested that $cPLA_2$ activation is caspase-dependent in MCF-7 cells, the role of caspase inhibitor z-VAD-fmk in CB1093, TNFα and C_2-ceramide-induced release of [^{3}H]AA was investigated by pretreating cells with 50 μM of z-VAD-fmk. Results demonstrate that TNFα induces $cPLA_2$ activation in a caspase-dependent manner, while CB1093 or C_2-ceramide-induced release of [^{3}H]AA appears to be caspase-independent in MCF-7 cells (Fig. 6b). In additional experiments, MCF-7 cells were incubated with 50 μM zVAD-fmk for 3 h and then co-treated with 100 nM CB1093 alone for up to 6 days. No protection against CB1093-induced loss of cell viability was observed in cultures co-treated with the caspase inhibitor (Fig. 6c).

Our previous studies have suggested that CB1093-induced apoptosis in MCF-7 cells is associated with $cPLA_2$ activation. In order to determine if activation of $cPLA_2$ could be involved in the induction of apoptosis mediated by vitamin D analogues in other breast cancer cells, activity of this enzyme was determined in Hs578T and T47D cells treated from 1 to 4 days with 100 nM CB1093. Results show that treatment with CB1093 alone increases $cPLA_2$ activity after 2–3 days of treat-

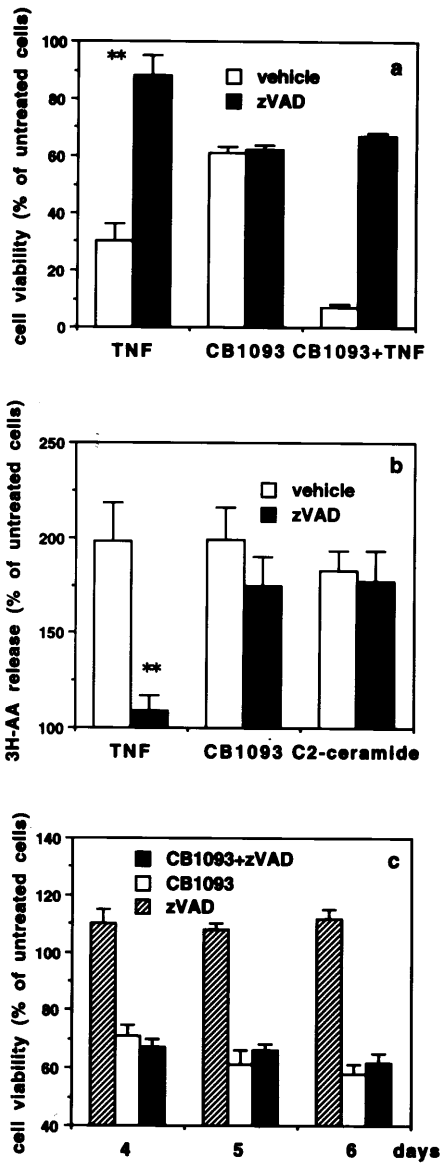

Fig. 6. Effect of z-VAD-fmk on MCF-7 cell viability and $cPLA_2$ activation in response to TNFα, C_2-ceramide or CB1093. (a) MCF-7 cells were preincubated with or without z-VAD-fmk (50 μM for 3 h), primed with CB1093 (100 nM for 48 h), then cultured in the presence or absence of z-VAD-fmk (50 μM for 3 h) and finally exposed to TNFα (20 ng/ml for 24 h). Cell viability was assessed by MTS assay as described in Section 2. (b) MCF-7 cells were preincubated with or without z-VAD-fmk (50 μM for 3 h) then treated with CB1093 (100 nM for 48 h) and labelled with [^{3}H]AA (0.3 μCi/ml) for the last 18 h. Cells were then washed and posttreated with or without z-VAD-fmk (50 μM for 3 h) and finally exposed to TNFα (10 ng/ ml for 18 h) or C_2-ceramide (10 μM in serum free medium for 6 h). $cPLA_2$ activation was assessed by [^{3}H]AA release as described in Section 2. Statistical comparison (a, b) between TNFα alone and TNFα plus z-VAD-fmk was found to be significant at $^{**}P < 0.001$. (c) MCF-7 cells were preincubated with or without z-VAD-fmk (50 μM for 3 h) and then co-treated with CB1093 (100 nM for 6 days). These agents were renewed in fresh medium every 48 h for 6 days. Cell viability was assessed by MTS assay as described in Section 2. Statistical comparison between CB1093 plus z-VAD-fmk and CB1093 alone was found to be not significant at any time point. Results are the mean ± SD of three replicate estimations and are representative of three separate experiments.

ment in both cell lines, and that this activation is prevented by the specific $cPLA_2$ inhibitor $AACOCF_3$ (Fig. 7a and b). Furthermore, coincubation of Hs578T and T47D cells with CB1093 and $AACOCF_3$ led to partial protection against loss of cell viability induced by CB1093 alone (Fig. 7c and d). This protection was evident from 72 h of treatment, the time at which $cPLA_2$ activation was observed. These findings suggest that activation of $cPLA_2$ may be a common feature in vitamin D induced apoptosis in breast cancer cells.

4. Discussion

The novel vitamin D analogue CB1093 has been shown to display potent antitumour effects in vivo and in vitro (Danielsson et al., 1997). The mechanisms by which vitamin D analogues induce active cell death in breast cancer cells remain to be elucidated but may involve suppression of cell survival signals and/or induction of genes that stimulate apoptosis. We have previously demonstrated that pretreatment of MCF-7 breast cancer cells with synthetic analogues of vitamin D potentiates responsiveness to TNFα-induced cytotoxicity (Pirianov et al., 1999). Our present findings indicate that TNFα exerts differential effects on breast cancer cells. Thus MCF-7 cells are responsive to induction of apoptosis by TNFα, Hs578T cells demonstrate reduced responsiveness and T47D cells are resistant to the cytotoxic effects of the cytokine. Furthermore, the vitamin D analogue was demonstrated to potentiate the cytotoxic effects of TNFα in Hs578T cells and modified responsiveness of T47D cells. Possible mechanisms by which vitamin D analogues might enhance responsiveness of cells to TNFα include increased signal recognition, decreased expression of proteins that mediate TNFα resistance or the promotion of a common apoptotic pathway. In a previous study we were unable to observe either substantial increases in the expression TNF-RI or endogenous production of TNFα by MCF-7 cells (Pirianov et al., 1999).

Several intracellular pathways have been demonstrated to be involved in TNFα-initiated cytotoxic processes, including free radical generation (Chang et al., 1992) and activation of the caspase system (Henkart, 1996). In addition, TNFα-mediated apoptosis in MCF-7 cells has been reported to be associated with increased ceramide production (Dbaido et al., 1997). Furthermore, it has been documented that inability of TNFα to induce ceramide formation and $cPLA_2$ activation leads to resistance to TNFα-mediated cell death in MCF-7 cells (Cai et al., 1997). Our present findings indicate that resistance of T47D cells to TNFα-induced apoptosis is also associated with impaired activation of $cPLA_2$

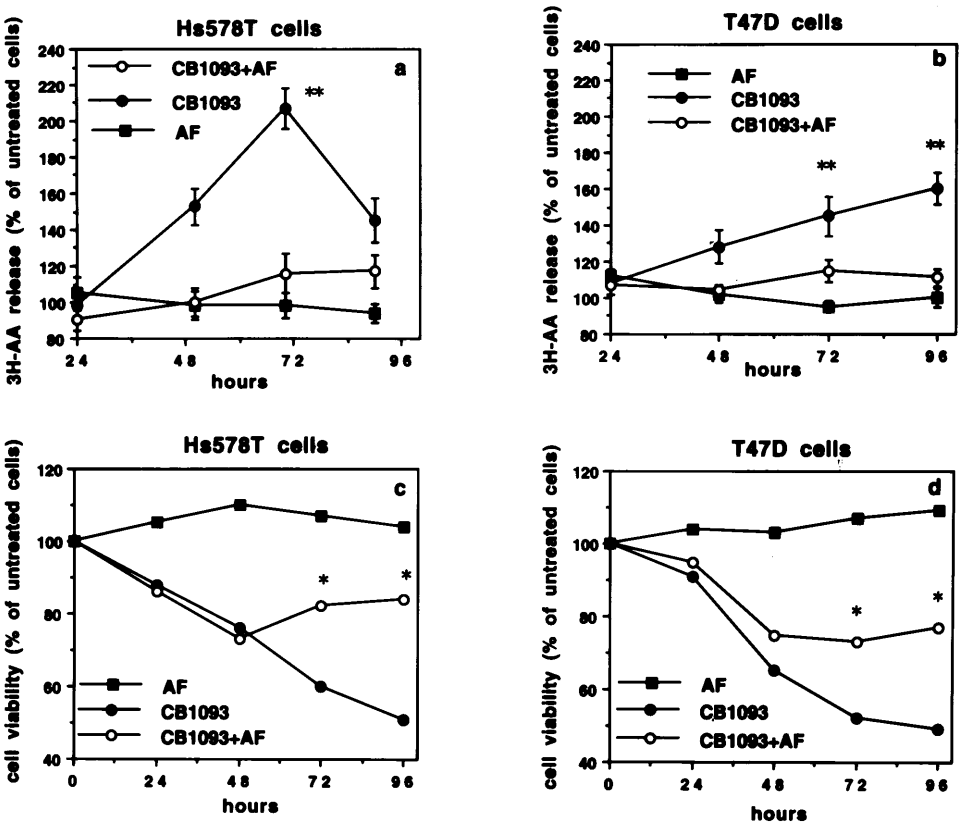

Fig. 7. CB1093 promotes release of AA and the cPLA$_2$ inhibitor AACOCF$_3$ partially prevents CB1093-mediated loss of cell viability in breast cancer cells. Hs578T (a) and T47D cells (b) were labelled with [^{3}H]AA (0.3 μCi/ml) for 18 h, washed and posttreated with or without 5 μM AACOCF$_3$ (AF) for 3 h and then treated with CB1093 (100 nM for 96 h). cPLA$_2$ activation was assessed by [^{3}H]AA release as described in Section 2. Statistical comparison between CB1093 treated and CB1093 untreated cells was found to be significant at $^{**}P < 0.001$. Replicate cultures (c, d) were assessed for cell viability determined by MTS assay as described in Section 2. Statistical comparison between CB1093 alone and CB1093 plus AACOCF$_3$ was found to be significant at $^{*}P < 0.05$. Results are the mean ± SD of three replicate estimations and are representative of three separate experiments.

in response to the cytokine. However, T47D cells retain the capacity to undergo apoptosis and activate cPLA$_2$ in response to exogenous ceramide, indicating a block in TNFα signalling which may lead to impaired ceramide generation. Hs578T cells show reduced responsiveness to TNFα in comparison with MCF-7 cells, and cPLA$_2$ activation was only observed at higher concentrations of the cytokine (20 ng/ml). Vitamin D analogues increase responsiveness of MCF-7, Hs578T and T47D cells to exogenous C$_2$-ceramide in terms of loss of cell viability, DNA fragmentation and ceramide-induced activation of cPLA$_2$. For all three cellular functions the dose response curve to C$_2$-ceramide was shifted to the left in CB1093-primed cells, suggesting that treatment with the vitamin D analogue increases levels of intracellular ceramide. Our present studies demonstrate that C$_2$-ceramide activates cPLA$_2$ in a dose-related manner, and suggest that cPLA$_2$ activation is downstream of ceramide generation in the apoptotic pathway. The process which leads to cPLA$_2$ activation during apoptosis remains unclear but may involve sustained phosphorylation by ceramide-activated protein kinase or mitogenic-activated protein kinases or transient increases in intracellular calcium (Obeid et al., 1993).

The role of caspases in cPLA$_2$ activation is controversial, with evidence that caspases can activate cPLA$_2$ in WEHI cells (Wissing et al., 1997) or alternatively in HeLa cells, inactivate by cleavage at the active centre of the enzyme (Luschen et al., 1998). However, the recent identification of multiple cleavage sites for caspases in the cPLA$_2$ molecule may indicate the potential generation of a variety of cleavage products, which may have different roles in apoptosis (Luschen et al., 1998). Using a broad-spectrum caspase inhibitor (z-VAD-fmk), we have demonstrated that TNFα-induced cPLA$_2$ activation is caspase-dependent in MCF-7 cells. However, our data show that z-VAD-fmk does not prevent CB1093 or ceramide-induced cPLA$_2$ activation and loss of MCF-7 cell viability. It appears that release of AA, as a second messenger, is likely be caspase-dependent in TNFα signalling and caspase-independent in vitamin D and ceramide signalling. Several very recent findings have

demonstrated that TNFα induces cell death by both caspase-dependent and -independent pathways and that ceramide and JNK activation may be involved in the latter pathways (Jones et al., 1999; Roulston et al., 1998). Furthermore, we found that Hs578T and T47D cells, which were unresponsive to the cytotoxic effects of TNFα, maintained sensitivity to ceramide-induced apoptosis that was potentiated by CB1093. Therefore we suggest that vitamin D analogues potentiate TNFα-induced cell death by enhanced accumulation of intracellular ceramide, which acts as a downstream effector of caspase-independent pathways.

In addition to promoting TNFα and ceramide-induced apoptosis, the vitamin D analogue CB1093 alone induces DNA fragmentation and loss of cell viability in all three breast cancer cell lines. Our results show that vitamin D analogues induce cell death in breast cancer cells by mechanisms that require neither activation of any known caspases; nor functional p53. Furthermore, treatment with CB1093 alone increases $cPLA_2$ activity in T47D, Hs578T and MCF-7 cells. Co-incubation of cells with CB1093 and inhibitors of $cPLA_2$ in each cell line led to partial protection against loss of cell viability induced by CB1093 alone, suggesting that activation of $cPLA_2$ may play a role in vitamin D-induced apoptosis in breast cancer cells.

In summary, the results of our study indicate that synthetic analogues of vitamin D increase responsiveness of three different breast cancer cells to TNFα-induced cell death. Our findings suggest that the accumulation of intracellular ceramide could be the point of convergence in TNFα and vitamin D signalling with $cPLA_2$ activation and release of AA serving as one common effector of apoptosis. Further studies are required to elucidate the differential role of caspase activation in these processes.

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