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Report

Effect of novel modulators of protein kinase C activity upon chemotherapy-induced differentiation and apoptosis in myeloid leukemic cells

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Modulation of protein kinase C (PKC) activity has been demonstrated to either prevent or enhance drug-induced apoptosis in various tissue types. We tested four novel modulators of PKC activity in comparison to the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for the capability to affect differentiation, cell cycle progression and apoptosis in the human myeloid leukemia cell lines U937 and HL-60. Farnesyl thiotriazole (FTT) and *N*-(*n*-heptyl)-5-chloro-1-naphthalenesulfonamide (SC-10) are both direct activators of PKC, whereas 6-(2-(4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl)ethyl)-7-methyl–5H-thiazolo [3,2-*a*]pyrimidin-5-one (R59022) and [3-[2-[4-(bis-(4-fluorophenyl) methylene]piperidin-1-yl)ethyl]-2,3-dihydro-2-thioxo-4(1H)-quinazolinone (R59949) are diacyl glycerol kinase inhibitors that activate PKC by enhancing the levels of the endogenous ligand diacyl glycerol. U937 cells displayed a slight reduction in the number of cells in G_2/M cell cycle phase after exposure to FTT, SC-10, R59022 and R59949, respectively. In contrast, HL-60 cells demonstrated a largely unaltered cell cycle distribution. Whereas TPA treatment resulted in a strong induction of $p21^{WAF/CIP1}$, c-Fos and c-Jun levels, neither one of the novel PKC activators altered expression of these proteins. Consequently, we tested the ability of the activators to cause membrane translocation of PKC. While TPA treatment resulted in translocation of the PKC isoforms α, δ and ε, SC-10 and FTT failed to induce alterations in the PKC content of the membrane and cytosolic fractions, respectively. Expression of the β_2-integrin CD11c that is induced during TPA-mediated differentiation remained unaltered after exposure to SC-10 and was partly reduced after treatment with FTT. To further investigate the effect of these activators upon apoptosis in leukemic cells, HL-60 and U937 cells were treated with 1-β-D-arabinofuranosylcytosine (Ara-C) or etoposide (VP-16). Whereas TPA strongly reduced apoptosis in Ara-C- or VP-16-treated U937 cells, little if any reduction was observed after pretreatment with either FTT, SC-10, R59022 or R59949, respectively, in these cells. In contrast, TPA enhanced apoptosis in Ara-C- or VP-16-treated HL-60 cells. Interestingly, FTT and SC-10 demonstrated a protective effect in Ara-C-treated HL-60 cells. Taken together, these data suggest that the novel PKC activators FTT, SC-10, R59022 and R59949 exhibit modest biological effects upon leukemic blast cells, and are not capable of enhancing the apoptotic response of these cells to cytotoxic drugs. [© 2002 Lippincott Williams & Wilkins.]

Key words: Acute myeloid leukemia, apoptosis, chemotherapy, differentiation, protein kinase C.

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Introduction

Protein kinase C (PKC) is a family of serine/threonine kinases implicated in important cellular responses such as differentiation, cell cycle progression and apoptosis.[1–3]

The analysis of these biological functions has been facilitated by the availability of modulators of PKC kinase activity. The most widely characterized PKC modulators are phorbol esters. These compounds mimic endogenous diacyl glycerol and bind to the Cys region of most PKC isoforms. Subsequently, the PKC protein undergoes a conformational change, thus liberating the kinase domain from binding to the pseudosubstrate domain located in the regulatory region. Substrates are phosphorylated in the presence of various cofactors such as Ca^{2+} and phospholipids. As a result of phorbol ester or diacyl glycerol binding, PKC proteins show increased affinity to lipophilic structures and can translocate to membranes. According to the structure and biochemical properties, PKCs can be divided into conventional or calcium-dependent (α, β_I, β_{II} and γ), novel or calcium-independent (δ, ε, η and θ) and atypical (λ/ι, μ and ζ) isoenzymes

Specific isoforms have been demonstrated to be involved in signal transduction pathways relevant for

clonal expansion or malignant transformation. In this context, induced expression of PKCε in NIH 3T3 cells contributes to neoplastic transformation, as demonstrated by tumor formation in nude mice.[4] Overexpression of PKCι but not PKCζ in K562 cells was associated with resistance to drug-induced apoptosis.[5] In factor-dependent human TF-1 cells, overexpression of PKCε extends cell survival in the absence of cytokines and induces *bcl-2* protein expression.[6] A direct role for PKC in apoptosis has been demonstrated for several isoforms, most notably for PKCδ which can be cleaved and activated by caspase-3.[3,7] Moreover, overexpression of the catalytic domain, but not the full-length protein or a kinase-dead mutant of PKCδ, induces apoptosis in HeLa cells.[7] PKCδ associates constitutively with and phosphorylates the DNA-dependent protein kinase catalytic subunit. Thereby, the function of DNA-dependent protein kinase to form complexes with DNA and to phosphorylate its downstream target, p53, is inhibited. In this context, modulation of PKC signaling pathways by selective inhibition or activation of cofactor-dependent molecules or downstream kinases is an attractive concept to enhance the cytotoxic effects of chemotherapeutic compounds. Inhibitors of Raf-1 kinase, MEK1/2, Ras as well as certain antisense oligonucleotide molecules have entered early clinical trials. Bryostatin-1 is a PKC-activating substance with properties distinct from phorbol ester. It demonstrates selectivity for activating, translocating and down-regulating certain isoforms such as PKCδ.[8] Apart from its differentiation-inducing effect on leukemic cells *in vitro*, synergistic effects with chemotherapeutic drugs such as 1-β-D-arabinofuranosylcytosine (Ara-C)[9] have been described. A number of phase I and phase II clinical trials have been published in recent years, however, demonstrating only minimal antitumor activity in indolent non-Hodgkin's lymphoma, colorectal carcinoma, melanoma, multiple myeloma or renal cell carcinoma.[10–14] In contrast, some clinical responses were observed in patients with chronic lymphocytic leukemia.[15]

Whereas byrostatin-1 failed to meet the expectations raised by previous *in vitro* studies, distinct activators of PKC should be investigated for this purpose. Thus, in the present study we evaluated four modulators of PKC activity in leukemic cell lines. SC-10 [*N*-(*n*-heptyl)-5-chloro-1-naphthalenesulfonamide], a naphthalenesulfonamide PKC activator, and farnesyl thiotriazole (FTT), a farnesylcysteine derivative, are both direct activators of PKC activity. In addition, we tested two compounds that enhance PKC activity by inhibition of diacyl glycerol kinase. Here, 6-(2-(4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl)ethyl)-7-methyl–5H-thiazolo[3,2-*a*]pyrimidin-5-one (R59022) prevents the conversion of diacyl glycerol to phosphatidic acid by diacyl glycerol kinase (DGK) isoforms, whereas [3-[2-[4-(bis-(4-fluorophenyl)methylene]piperidin-1-yl)ethyl]-2,3-dihydro-2-thioxo-4(1H)-quinazolinone (R59949) more selectively inhibits the DGK α isoform.[16–20]

Material and methods

Cell lines and reagents

Human U937 and HL-60 myeloid leukemic cells (ATCC, Rockville, MD) were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS) (BioWhittaker, Verviers, Belgium) supplemented with 2 mM L-glutamine without antibiotics. The cells were incubated in a humidified atmosphere (37°C, 5% CO_2) and treated with various concentrations of the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Sigma, Deisenhofen, Germany), the diacylglycerol kinase inhibitors R59022 and R59949, the direct PKC activators FTT and SC-10, as well as Bryostatin-1 (Biomol, Hamburg, Germany), the etoposide VP-16 (Bristol, München, Germany) and Ara-C (Cell Pharm, Hannover, Germany) for the times indicated.

Flow cytometry

For cell cycle analysis, untreated and stimulated U937 and HL-60 cells (10^6 cells) were fixed with 70% (w/v) ice-cold ethanol overnight. Adherent cells were removed from the plastic surface of the culture flask with a rubber policeman. After two washes with ice-cold PBS, the fixed cells were resuspended in 1 ml of PBS containing 40 μg/ml propidium iodide (Sigma) and 500 U/ml RNase A (Boehringer Mannheim, Mannheim, Germany). Following incubation for 30 min in the dark the cells were analyzed on a Epics XL-MCL flow cytometer (Coulter, Kredfeld, Germany) using System II software. To identify apoptotic populations, objects with fluorescence values below 20% of that of the G_1 peak were gated out. As previously described, the propidium iodide fluorescence signal peak versus the integral was used to discriminate G_2/M cells from G_0/G_1 doublets.[21]

For analysis of cell surface markers, 10^6 cells were washed with PBS and resuspended in 1 ml PBS with 2% FCS. Aliquots of 100 μl of cells were incubated at 4°C with R-phycoerythrin (PE)-conjugated anti-CD11c

or PE-conjugated anti-CD4 (Coulter) for 10 min in the dark. After centrifugation the cells were resuspended in 500 μl of PBS with 2% FCS and flow cytometry was performed.

Statistical considerations

Kruskal–Wallis one-way analysis of variance on Ranks was used to determine the statistical significance of treatment results. The pairwise multiple comparison procedure was performed according to Dunn's method.

Immunoblot analysis

Untreated and stimulated U937 cells were washed 3 times in ice-cold PBS and lysed in a buffer containing 25 mM Tris–HCl (pH 7.6), 1 mM Na_3VO_4, 10 mM NaF and 10 mM EDTA with the addition of 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonylfluoride. The cells were swollen on ice for 30 min, homogenized with 30 strokes of a homogenizer type S and 1% (v/v) NP-40 was added. The homogenates were centrifuged at 15 000 *g* for 15 min at 4°C. Protein concentration was adjusted using a colorimetric assay. Controls for equal loading of protein were performed using an antibody against β-actin. Protein samples were subjected to SDS–PAGE and transferred to a PVDF membrane (Millipore, Schwalbach, Germany). The transfer buffer contained 25 mM Tris–HCl, 192 mM glycine, 0.037 (w/v) SDS and 20% (v/v) methanol. The membranes were blocked with PBS containing 5% dried milk and 0.05% Tween 20 (PBS/Tween). After washing 4 times with PBS/Tween, the membranes were incubated with appropriate antibodies anti-p21, anti-c-Fos, anti-c-Jun, anti-β-actin, anti-PKCα, anti-PKCδ and anti-PKCε, all from Santa Cruz Biotechnology (Santa Cruz, CA). Bound antibodies were decorated with peroxidase-conjugated secondary antibody (donkey anti-rabbit IgG, sheep anti-mouse: Amersham, Freiburg, Germany; rabbit anti-goat: Dako, Hamburg, Germany; diluted 1:2000 in blocking solution) for 1 h at room temperature and visualized by autoradiography using the ECL detection kit (Amersham).

Results

Establishment of non-toxic concentrations of FTT, SC-10, R59022 and R59949 in U937 cells

A dose–response experiment for FTT, SC-10, R59022 and R59949 was performed to establish a non-toxic concentration range. U937 cells were incubated with increasing concentrations of these PKC modulators for 72 h. The cells were ethanol-fixed, stained with propidium iodide and tested for the appearance of a sub-G_1 DNA peak, which has previously been established as a marker of late apoptosis/necrosis in U937 cells.[21] Whereas 100 μM of R59949 demonstrated approximately 30% of dead cells, R59022, FTT and SC-10 resulted in cell death in more than 85% of U937 cells (Figure 1). Most likely, secondary metabolic effects may be responsible for the observed cytotoxicity at the high doses. Previous work has demonstrated biological effects of these compounds at concentrations of 10 μM in various cell systems. Thus, a concentration of 10 μM was used for all subsequent experiments.

Effects of FTT, SC-10, R59022 and R59949 on cell cycle distribution of HL-60 and U937 cells

Both U937 and HL-60 are rapidly proliferating myeloid leukemic cell lines. It has been demonstrated previously that PKC activators such as phorbol ester significantly reduce the proliferation rate and alter cell cycle distribution. Consequently, we tested the cell cycle response of these cells to both diacylglycerol kinase inhibitors and direct PKC activators. Whereas the known and statistically significant effects of TPA on cell cycle distribution

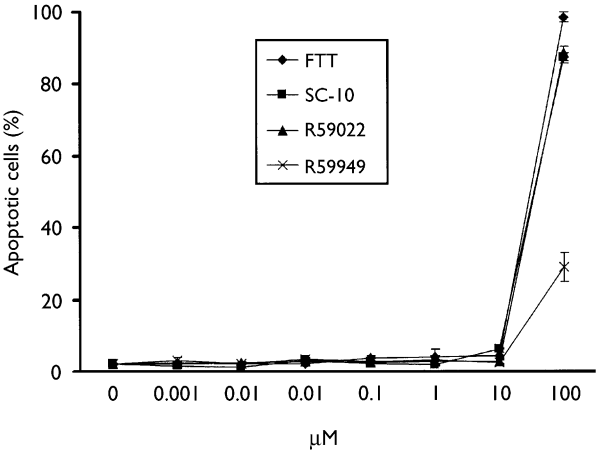

Figure 1. Analysis of toxicity of various concentrations of the PKC modulating compounds FTT, SC-10, R59022, and R59949 in U937 cells. Cells were treated with the indicated concentrations for 3 days. Apoptotic cells were quantified using propidium iodide staining of fixed cells by flow cytometry. The columns represent the means of at least three independent experiments $\pm$ SE measuring sub-G_1 (hypodiploid) DNA.

in both cells lines could be reproduced, there was little if any difference between FTT-, SC-10-, R59022- or R59949-treated and untreated control cells. Only the diminution of the G_2/M phase fraction in FTT-, SC-10-, R59022- or R59949-treated U937, but not HL-60 cells reached statistical significance (Figure 2A and B).

Expression of p21, c-Fos and c-Jun

Progression through the cell cycle is regulated by a complex network of proteins, among them the retinoblastoma protein Rb. The control of Rb function is achieved by phosphorylation and dephosphorylation on serine and threonine residues. Phosphorylation is regulated by the cyclin D-dependent kinases CDK4 and CDK6 which are in turn regulated by interaction with CDK inhibitors, among them members of the $p21^{WAF/CIP1}$ family (p21, p27 and p57). Expression of $p21^{WAF/CIP1}$ is necessary for cell cycle exit and entry into a quiescent G_0 cell-cycle state. Furthermore, differentiation of myeloid cells into monocyte/macrophage-like cells requires the induction of early response genes such as c-*fos* and c-*jun*. In this context we analyzed the cellular content of $p21^{WAF/CIP1}$ as well as c-Fos and c-Jun protein by Western blotting. Whereas TPA treatment resulted in strong expression of $p21^{WAF/CIP1}$, c-Fos and c-Jun, respectively, FTT, SC-10, R59022 and R59949 exposure for 3 days demonstrated little if any change in the expression of $p21^{WAF/CIP1}$, c-Fos and c-Jun, respectively (Figure 3A).

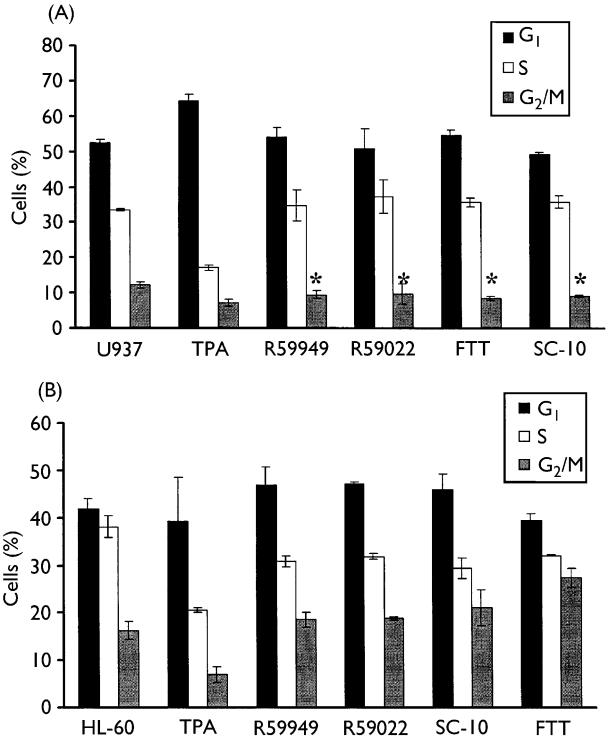

Figure 2. (A) Analysis of alteration in cell cycle distribution of U937 cells after treatment with PKC-activating compounds. Cells were stimulated with 10 μM of FTT, SC-10, R59022 and R59949 or 5 nM TPA, respectively, for 3 days. Quantification of G_1, S and G_2/M phase cells was performed using propidium iodide staining of fixed cells by flow cytometry. The columns represent the means of at least three independent experiments $\pm$ SE. *Statistically significant difference compared to untreated U937 cells ($p < 0.05$). (B) Analysis of alteration in cell cycle distribution of HL-60 cells after treatment with PKC activating compounds. Cells were stimulated with 10 μM of FTT, SC-10, R59022 and R59949 or 5 nM TPA, respectively, for 3 days. Quantification of G_1, S and G_2/M phase cells was performed using propidium iodide staining of fixed cells by flow cytometry.

FTT, SC-10, R59022 and R59949 are not involved in membrane relocalization of PKC isoforms

Translocation of PKC molecules to different subcellular compartments such as the membrane fraction is a hallmark of PKC activation and has been widely used as a surrogate marker for kinase activity. Treatment of myeloid leukemic cells with phorbol ester or macrocyclic lactons like Bryostatin-1 is associated with a rapid PKC redistribution and subsequent down-regulation of the activated kinase after 72 h.[22] Previous work has demonstrated that incubation of U937 cells with 5 nM TPA for 72 h leads to cessation of proliferation, adhesion of cells to matrix and expression of a monocyte/macrophase-like phenotype.[21,23] This effect was accompanied by a translocation of PKCα, δ and ε, respectively (Figure 3B). In contrast, treatment of U937 cells with 10 μM of the direct PKC activators FTT and SC-10 did not alter the morphology of the cells (data not shown). Moreover, exposure to FTT and SC-10 did not result in membrane translocation of these PKC isoforms (Figure 3B).

Expression of CD4 and CD11c

Reflecting their origin from the monocyte/macrophage system, differentiation of U937 cells requires the attachment of cells to matrix, enabling the interaction of integrin receptors to their respective ligands. Furthermore, differentiation is accompanied

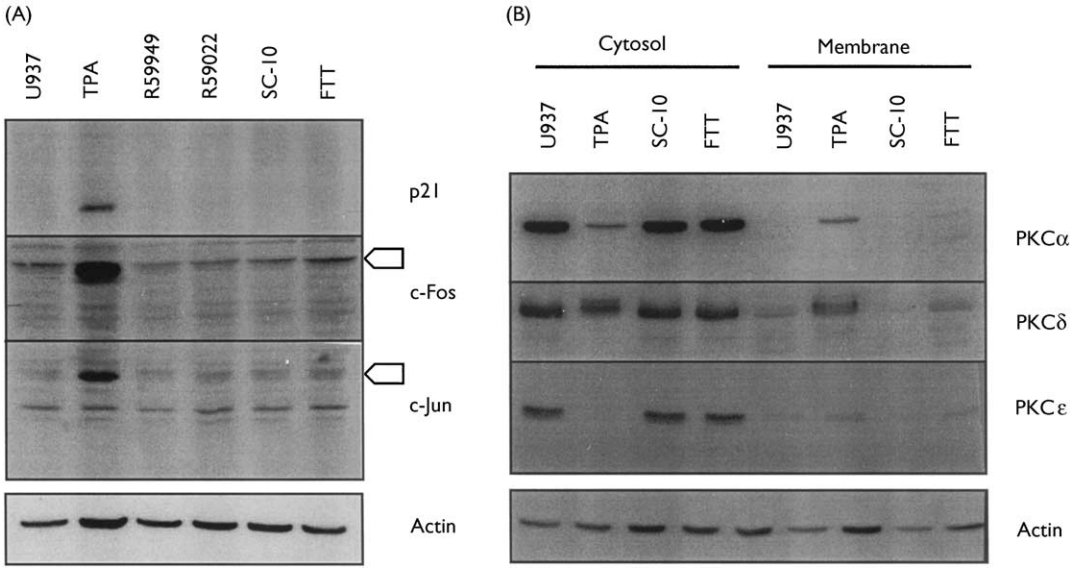

Figure 3. (A) Protein expression levels of the differentiation and cell cycle regulating protein $p21^{WAF1/CIP1}$, c-Fos and c-Jun in U937 cells. Cells were treated with 10 μM of FTT, SC-10, R59022 or R59949, respectively, for 72 h. The actin blot indicates equal loading of proteins. A representative set of blots is presented. (B) Subcellular distribution of PKC isoforms in U937 cells. Cells were treated with 10 μM of FTT and SC-10 or 5 nM of TPA for 72 h. Cytosolic and membrane protein fraction were evaluated for the expression of PKCα, PKCδ and PKCε. The actin blot indicates equal loading of proteins.

by down-regulation of certain markers such as the B cell epitope CD4. Concomitant with the failure of SC-10- and FTT-treated U937 cells to undergo differentiation, these cells failed to down-regulate CD4 and up-regulate CD11c, respectively (Figure 4). In contrast, a small but statistically significant down-regulation of CD11c was observed after exposure to FTT.

Effect of PKC modulators on the induction of apoptosis after stimulation with cytotoxic compounds

Exposure to PKC activating agents has been shown to enhance or inhibit the response of leukemic cells to cytotoxic compounds.[24,25] Leukemic cells are highly sensitive to chemotherapeutic agents like Ara-C or the topoisomerase II inhibitor VP-16. We were interested to test the ability of PKC activators to enhance or suppress the induction of apoptosis in these cells. U937 or HL-60 cells were preincubated with 5 nM TPA or 10 μM FTT, SC-10, R59022 and R59949, respectively, for 1 h. Subsequently, the cells were challenged with 68 μM VP-16 or 1 μM Ara-C (Figure 5A–D). Both VP-16 and Ara-C proved to be strong inducers of cell death. A trend towards enhancement of apoptosis was observed in HL-60 cells treated with VP-16 following a preincubation with TPA or either one of the DAG kinase inhibitors (Figure 5B). Similarly, in Ara-C-treated HL-60 cells preincubation with TPA, but not with the other PKC modulators, enhanced apoptosis. This effect, however, was less than additive (Figure 5D). Interestingly, and in contrast to the effect of TPA exposure, SC-10 and FTT inhibited the Ara-C-induced apoptosis, which reached statistical significance in the case of SC-10. In contrast, no significant effect of either FTT, SC-10, R59022 and R59949 on VP-16- or Ara-C-treated U937 cells was observed, whereas TPA reduced apoptosis in both instances as demonstrated previously (Figure 5A and C).

Discussion

The modulation of pathways involved in the execution of apoptotic signals is an attractive therapeutic concept. It is therefore important to dissect signaling pathways in malignant cells to find key targets suitable to pharmacological intervention. Kinases are interesting candidates, since they are involved in virtually all such pathways and are subject to various types of regulation. However, selective kinase activators in contrast to inhibitors are still rare. In this context, the availability of phorbol esters as strong activators of the PKC family of serine/threonine kinases has sparked preclinical research

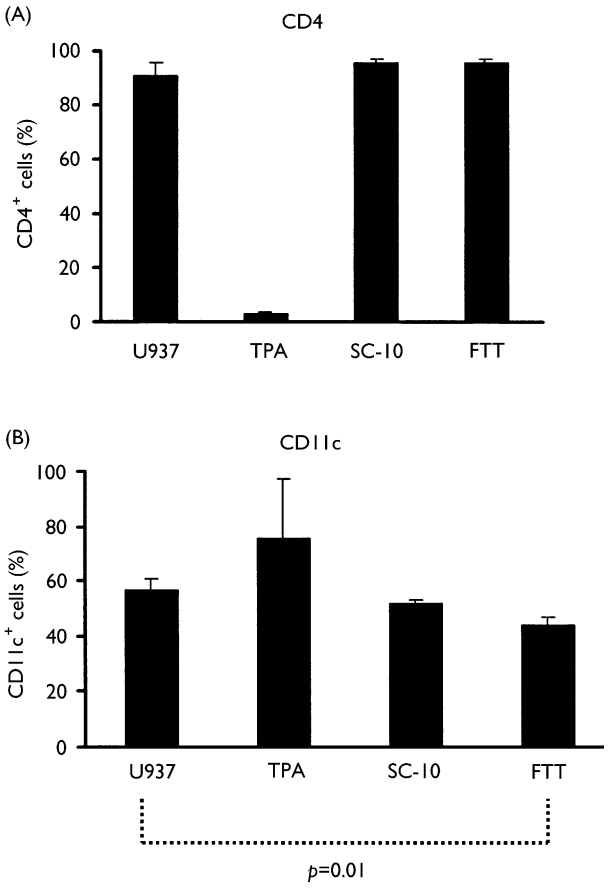

Figure 4. Expression of (A) CD4 and (B) CD11c as differentiation markers in U937 cells. Cells were treated with 5 nM TPA, 10 μM SC-10 or 10 μM FTT for 72 h. Expression of surface markers was determined by flow cytometry. All data represent the mean + SD of two independent experiments.

particularly in the context of hematological malignancies. Phorbol esters, however, are not suitable for therapeutic application due to their intrinsic tumor-promoting activity in certain experimental models. Thus, different compounds have been synthesized, either acting as direct PKC activators like SC-10 and FTT or enhancing the level of DAG, the physiologic activator of PKC. Another PKC-activating compound with properties distinct from phorbol ester, Bryostatin-1, entered clinical trials several years ago, but has failed to live up to the expectations raised by preclinical investigations.[10–12,14,15]

It is thus interesting to note that only marginal effects could be observed in two widely used myeloid cell lines. Whereas the strong PKC activator TPA, used as positive control, demonstrated the known effects upon differentiation, apoptosis, subcellular translocation and modulation of cell surface markers as well as signaling proteins,[21–23,26] the two diacylglycerol kinase inhibitors R59022 and R59949 did not demonstrate any measurable biological alterations in these cells. Although inhibition of diacylglycerol phosphorylation by R59022 and R59949 has been proven in several cellular systems,[27–29] there is only little information available about the biological responses following this DAG kinase inhibition in hematopoetic cells. In previous work, enhanced adhesion following short-term treatment with 10 μM R59949 was demonstrated in peripheral blood neutrophils, whereas the response of blast cells was not tested.[30] In our experiments, neither increased adherence to matrix nor enhanced expression of the β_2 integrin CD11c was detectable in leukemic cells at identical concentrations of the drug.

Previous reports have shown that TPA treatment induces apoptosis in U937 and HL-60 cells.[21,31,32] Furthermore, different PKC activators increase apoptosis after Ara-C pretreatment.[33] In contrast, activation of PKC by TPA and phosphatidylserine inhibited daunorubicin-induced neutral sphingomyelinase activation, sphingomyelin hydrolysis, ceramide generation, and apoptosis.[34] Moreover, activation of PKC prevents induction of apoptosis by geranylgeraniol in HL-60 cells.[35] Thus, PKC emerges as a potentially critical regulator of both pro- and anti-apoptotic pathways.

Whereas phorbol ester pretreatment clearly diminished the pro-apoptotic effects of the cytotoxic compounds VP-16 and Ara-C in U937 cells, it enhanced the cell death-promoting activities of VP-16 and Ara-C in HL-60 cells. These findings are in contrast to previously published data demonstrating protection from VP-16-induced apoptosis by TPA in HL-60 cells.[24] Activation of PKC isoforms most likely occurs with different kinetics in distinct cell lines. In addition, membrane translocation and down-regulation of the active kinase may be differentially regulated in different leukemic clones. Thus, apart from different doses of the chemotherapeutic agent, the timing of PKC activation in relationship to the onset of cytotoxic stimulation may be critical in determining potentially contrasting effects such as protection and enhancement of apoptosis. In this context, it is possible to define 'windows of opportunity' for incubation with PKC agonists like TPA and Bryostatin-1 in relationship to chemotherapy treatment that enables a diverse outcome of a given combination of compounds.

Interestingly, a minor apoptosis-reducing effect was observed after preincubation of HL-60 cells with SC-10 and FTT followed by VP-16 that was statistically significant for SC-10. Moreover, the expression of the β_2 integrin CD11c was significantly reduced after

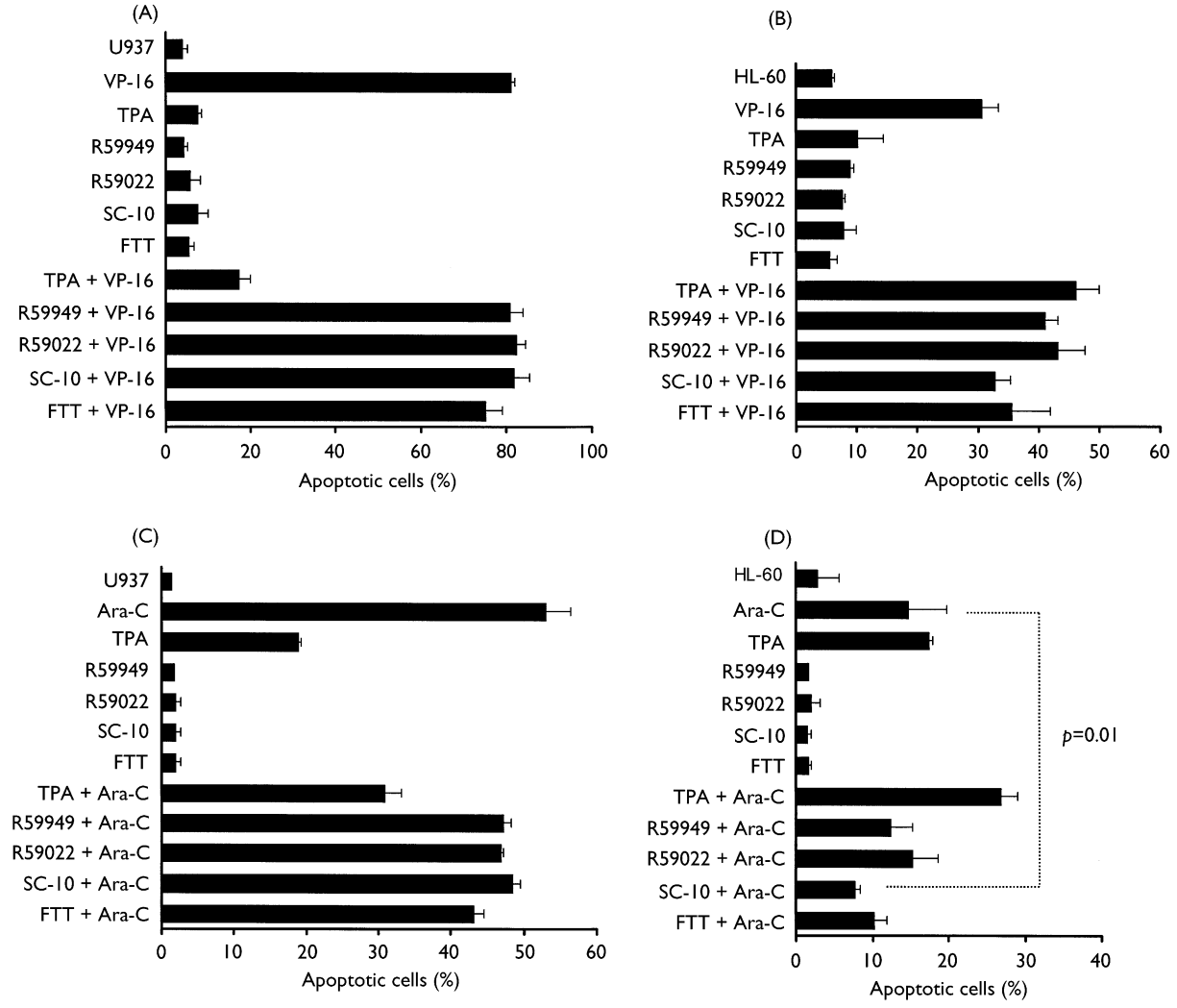

Figure 5. Modulation of chemotherapy-induced apoptosis after preincubation with PKC-activating compounds. In all experiments, cells were pretreated with 10 μM of FTT, SC-10, R59022 and R59949, or 5 nM TPA for 24 h. Apoptotic cells were quantified using propidium iodide staining of fixed cells by flow cytometry. The columns represent the means of at least three independent experiments $\pm$ SE measuring sub-G_1 (hypodiploid) DNA. (A) U937 cells were exposed to 68 μM VP-16 for 4 h following preincubation. (B) HL-60 cells were exposed to 68 μM VP-16 for 4 h following preincubation. (C) U937 cells were exposed to 1 μM Ara-C for 24 h following preincubation. (D) HL-60 cells were exposed to 1 μM Ara-C for 24 h following preincubation.

FTT exposure. Thus, SC-10 and FTT demonstrate effects that are at least in part in contrast to the biological responses observed after TPA-mediated PKC activation.

We have demonstrated that the novel PKC-targeted compounds FTT, SC-10, R59022 and R59949 have only minimal activity in the leukemic cell lines U937 and HL-60, which are among the most widely used leukemic cell lines. Apart from Bryostatin-1, which is not an isoform-selective PKC modulator, no other PKC-activating compound has been developed for clinical use. Recent data concerning the role of specific PKC isoforms in apoptosis underscores the importance of PKC kinases. In particular, PKCδ has been shown to be critically involved in the induction and regulation of apoptosis.[3,36,37] Thus, the synthesis of new isoenzyme-selective PKC activators would be of considerable interest for the elucidation of PKC function as well as for evaluation of synergistic effects with known chemotherapeutic substances.

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