Antiviral Research 59 (2003) 89–98

www.elsevier.com/locate/antiviral

Activation of latent HIV-1 expression by the potent anti-tumor promoter 12-deoxyphorbol 13-phenylacetate

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Received 19 September 2002; accepted 30 January 2003

Abstract

Agents that induce HIV-1 out of latency would be useful adjuvants for currently available anti-retroviral therapy. We report that nanomolar concentrations of 12-deoxyphorbol 13-phenylacetate (DPP), an anti-tumor-promoting phorbol ester originally isolated from a West African plant, induce the expression of HIV-1 in latently infected T cells and render them sensitive to killing by an immunotoxin targeted to the viral envelope glycoprotein. DPP also regulates an extensive series of genes under the control of protein kinase C, including several involved in T cell activation and cytoskeleton reorganization, and represses expression of the HIV-1 receptor CD4 and coreceptor CXCR4. DPP is 20–40-fold more potent than the related phorbol ester prostratin, probably due to its more lipophilic side chain structure. The combination of high potency and anti-tumor promoting activity make DPP an attractive candidate for the adjunctive therapy of persistent HIV-1 infection. Published by Elsevier B.V.

Keywords: Human immunodeficiency virus; Phorbol ester; Immunotoxin; Anti-viral compounds

1. Introduction

The ability of human immunodeficiency virus (HIV)-1 to integrate into the genome of T lymphocytes in a dormant form is a major obstacle to controlling or curing HIV-1 infection. The reservoir of latently infected T cells is established early after infection and persists for many years despite highly active anti-retroviral therapy (HAART). Viral gene expression in latently infected cells can be reactivated by a wide variety of signals including cytokines such as interleukin-2, tumor necrosis factor and macrophage colony-stimulating factor, antigens and other T cell mitogens, glucocorticoid and thyroid hormones, bacterial infections, lipopolysacchrides, and small molecules including ethanol. Consequently, if HAART is ceased, viremia rapidly reoccurs, leading to deterioration of the immune system and AIDS (Blankson et al., 2002; Chun and Fauci, 1999; Ho, 1998; Pomerantz, 2002; Sonza and Crowe, 2001).

One possible solution to the problem of HIV-1 latency is to deliberately administer agents that activate viral gene expression in the presence of HAART to prevent spreading infection by the newly synthesized virus (Chun et al., 1998). Such treatment could reduce the number of latently infected cells by causing them to be directly killed by the cytopathic action of the virus, to be recognized and destroyed by the immune system, or to express proteins that render them susceptible to targeted therapeutics such as immunotoxins.

The ideal HIV-1 inducing agent would be potent, orally available, nontoxic, active in a wide variety of latently infected cell types, and capable of penetrating anatomic sanctuaries such as the reproductive tract and central nervous system. Phorbol esters, which are commonly used to activate HIV-1 expression in latently infected cell lines grown in the laboratory, appear to meet at least some of these criteria. The phorbol esters are small, drug-like molecules that rapidly enter cells and activate protein kinase C (PKC) by acting as potent analogs of 1,2-diacylglycerol, a second messenger generated through various receptor-mediated mechanisms (Blumberg et al., 1994; Kanashiro and Khalil, 1998; Nishizuka, 1995). PKC, which is comprised of at least 12 serine/threonine-specific isozymes, can in turn induce latent HIV-1 gene expression by activating both NF-κB, which binds to the enhancer region of the HIV-1 LTR (Nabel and Baltimore, 1987), and AP-1, which can bind either to the enhancer cooperatively with NF-κB or to downstream sequence elements in concert with the CREB and ATF transcription factors (Kagnoff and Roebuck, 1999; Roebuck et al., 1996; Yang et al., 1999). PKC may also increase HIV-1 gene expression by phosphorylation of the virally

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0166-3542/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/S0166-3542(03)00034-2

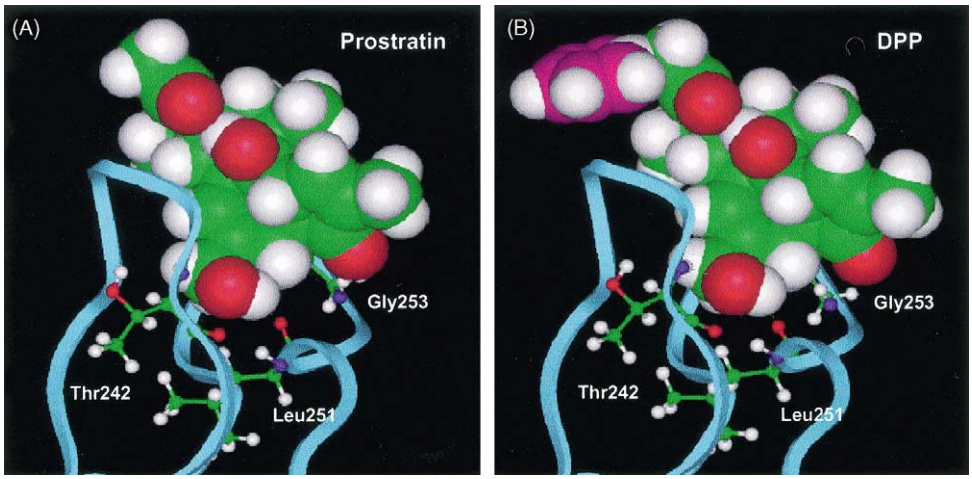

Fig. 1. Molecular modelling of (A) prostratin and (B) DPP complexed with PKC. The models are based on the atomic coordinates of phorbol 13-acetate bound to the C1b domain of PKCδ (Zhang et al., 1995) and were generated using the Dock and Discover 3 routines of Insight 2000. The phorbol esters are depicted as space filling models with the carbon atoms of the phenyl ring of DPP colored purple. The PKC backbone is shown as a ribbon and the critical ligands Thr242, Leu251, and Gly253 as stick models.

encoded TAT transcription factor and cellular TAR-binding factors (Han et al., 1992; Jakobovits et al., 1990).

Phorbol esters have generally been regarded as poor candidates for human therapeutics because of their strong tumor-promoting activity. However, clinical interest in this class of compound was kindled by the discovery that prostratin (12-deoxyphorbol 13-acetate; Fig. 1A), a nontumor promoting phorbol ester from *Pimela prostrata* (Cashmore et al., 1976) that was subsequently reisolated and identified as an anti-viral constituent of the Samoan medicinal plant *Homolanthus nutans* (Gustafson et al., 1992), can activate HIV-1 replication in latently infected cell lines (Gulakowski et al., 1997; Gustafson et al., 1992). Recent studies have shown that prostratin can also induce HIV-1 gene expression in primary cells isolated from both HIV-1 infected humans (Kulkosky et al., 2001) and SCID-hu (Thy/Liv) mice (Korin et al., 2002). Moreover, although prostratin is a mitogen in mononuclear phagocytes (Kulkosky et al., 2001), it can activate HIV-1 in quiescent T cells without causing cellular proliferation (Korin et al., 2002).

Although the preliminary results with prostratin are encouraging, its clinical potential is hampered by low potency. The concentration of prostratin required to activate HIV-1 replication in latently infected cells is 1–10 μM, which is approximately 1000-fold higher than for the widely used phorbol ester phorbol 12-myristate 13-acetate (PMA). Even at an optimal dose, prostratin induces only one-fifth as much HIV-1 expression in latently infected U1 cells as does PMA (Gulakowski et al., 1997; Kulkosky et al., 2001). Of six PKC-activating compounds studied by Bögi et al., prostratin had the poorest ability to bind to PKC and cause its translocation from the cytosol to the particulate fraction (Bogi et al., 1998).

The purpose of the present work was to develop a more potent nontumor promoting phorbol ester as a potential HIV-1 inductive therapy. We report here that 12-deoxyphorbol 13-phenylacetate (DPP; Fig. 1B), a nontumor promoting phorbol ester isolated from the West African "candle plant" *Euphorbia poissonii* (Evans and Schmidt, 1979) and the Moroccan succulent *E. resinifera* Berg. (Hergenhahn et al., 1984), induces HIV-1 gene expression in latently infected ACH-2 T cells at concentrations 20–40-fold lower than prostratin, and can be used in combination with an anti-HIV-1 envelope immunotoxin to selectively kill such cells. We also show that DPP regulates a series of PKC-sensitive genes involved in T cell activation and cytoskeleton reorganization and represses the expression of the HIV-1 receptor CD4 and coreceptor CCR5. Our results suggest that DPP may be a reasonable candidate to augment HAART for therapy of persistent HIV-1 infection.

2. Methods

2.1. Compounds

DPP, prostratin and PMA were purchased from LC Laboratories (Woborn, MA) and dissolved in DMSO. The anti-HIV-1 envelope immunotoxin 3B3:N31H/Q100eY (dsFv)-PE was expressed in bacteria and purified as described (McHugh et al., 2002).

2.2. Molecular modeling

Molecular modeling based on the atomic coordinates of phorbol 13-acetate bound to the C1b domain of PKCδ (Zhang et al., 1995) was performed using the Dock and Discover 3 routines of the Insight 2000 package (Molecular Simulations).

2.3. Cells, ELISA, and cytoxicity assays

The HIV-1-infected T cell line ACH-2 and the parental line A3.01 were obtained from the NIH AIDS Research and Reagent Reference Program (Rockville, MD) and grown in RPMI 1640 medium supplemented with 2 mM glutamine, 10% fetal bovine serum, and antibiotics. HIV-1 p24 antigen was determined with an ELISA kit (Immunodiagnostics, Bedford, MA). Cell viability was measured by the MTT (3-[4,5-dimethylthiazole-2-yl]2,4-diphenyltetrazolium bromide) oxidation procedure using Cell Proliferation Kit I (Roche Diagnostics) as described (McHugh et al., 2002).

2.4. Flow cytometry

Expression of cell surface HIV-1 envelope protein (gp120) was evaluated by flow cytometry using 3B3:N31H/Q100eY(dsFv)-PE as the primary antibody and mouse anti-PE monoclonal antibody m40-1 and FITC-conjugated goat anti-mouse IgG (Jackson Laboratories) as the secondary and tertiary reagents (McHugh et al., 2002). CD4 expression was measured using FITC-conjugated SK3 antibody and CXCR4 expression was measured using APC-conjugated 12G5 antibody (BD Biosciences, San Diego, CA). Annexin V binding was assayed with the Annexin V Apoptosis Detection Kit (Oncogene Research Products, Boston, MA). The analyses were performed on a FACS Calibur (Beckton Dickton) using CELLQUEST software.

2.5. RNA extraction and microarray analysis

A3.01 and ACH-2 cells were grown with no inducer, 500 nM DPP, 5 μM prostratin, or 50 nM PMA for 24 h. Total RNA was extracted using TriZOL (Gibco/BRL/Life Technologies) and 50 μg was reverse transcribed using SuperScript II (Gibco/BRL) in the presence of Cy3 or Cy5 labeled dUTP as described (Eisen and Brown, 1999).

Microarray slides were obtained from the Advanced Technology Center, National Cancer Institute, and contained 9984 clones from the UniGEM2 collection (Incyte Genomics). The microarrays were pre-hybridized for 1 h at 42 °C in 20 μl of 5× SSC, 0.1% SDS, 1% BSA then washed in deionized water followed by 100% isopropanol. Slides were hybridized to a mixture of the Cy3 and Cy5 labeled cDNA in 22 μl of 25% formamide, 5× SSC, 0.1% SDS for 12–16 h at 42 °C, washed (Eisen and Brown, 1999), then scanned on a GenePix 4000A scanner (Axon Instruments, Foster City, CA). The resulting images were analyzed using GenePix Pro v3.0 software (Axon Instruments) and the Cy3 and Cy5 signal intensities were normalized for each sample using the NCI mAdb database software (madb.nci.nih.gov). Data filters based on signal intensity and spot quality were used to exclude less reliable spots or insufficiently expressed genes. The complete microarray data can be obtained by e-mail to sven.bocklandt@nih.gov.

3. Results

3.1. Molecular modeling and choice of DPP

The goal of the present work was to identify an anti-tumor promoting phorbol ester that could induce latent HIV-1 gene expression more potently than prostratin. Molecular modeling, based on the X-ray structure of phorbol 13-acetate complexed with the C1b domain of PKCδ (Zhang et al., 1995), suggests that prostratin should be capable of binding to PKC with normal affinity since the critical pharmacophores O-4 and O-20 are present and capable of hydrogen bonding to PKC residues Thr242, Leu251 and Gly253 (Fig. 1A). Therefore, the low activity of this compound is probably a result of its lack of a lipophilic side chain available to interact with intracellular membranes, which is crucial for PKC activation by phorbol esters (Blumberg et al., 1994; Kanashiro and Khalil, 1998; Marquez et al., 1999; Nishizuka, 1995). Accordingly, we decided to focus our attention on DPP, in which a lipophilic aromatic ring is joined to the acetyl group at position 13 (Evans and Schmidt, 1979). As shown in Fig. 1B, molecular modeling predicts that this ring projects away from the enzyme binding site and should therefore be available to interact with membranes inside the cell. In agreement with this prediction, previous studies have shown that DPP is indeed more active than prostratin in various biological assays of PKC activation (Bogi et al., 1998). Nevertheless, DPP is nontumor-promoting, and in fact inhibits the inflammatory and tumor-promoting activities of PMA (Szallasi et al., 1992, 1993).

3.2. DPP potently activates latent HIV-1 replication

The ability of DPP to induce HIV-1 expression was investigated by measuring p24 core antigen production in ACH-2 cells, a chronically infected T cell clone that carries a single integrated provirus (Clouse et al., 1989; Folks et al., 1989). As shown in Fig. 2A, addition of nanomolar concentrations of DPP to the culture medium for 2 days increased p24 expression by 10–20-fold over background levels. The concentration of DPP required for half maximal stimulation (IC_{50}) was 4.1 ± 0.1 nM (mean ± S.E.M., $n = 4$), and full induction was reached by 62.5 nM. Prostratin increased p24 production to a similar extent, but the IC_{50} was 156 ± 56 nM (40-fold higher than for DPP), and full induction required a concentration of 1.25 μM (20-fold higher than for DPP). The kinetics of p24 induction by DPP and prostratin were very similar, with a rapid rise for the first 2 days that leveled off by day 4 and then stayed constant out to 9 days (Fig. 2B).

3.3. Induction of envelope expression and immunotoxin sensitivity

To determine whether DPP also induced expression of the HIV-1 surface envelope glycoprotein, we made use of immunotoxin 3B3:N31H/Q100eY(dsFv)-PE, in which the

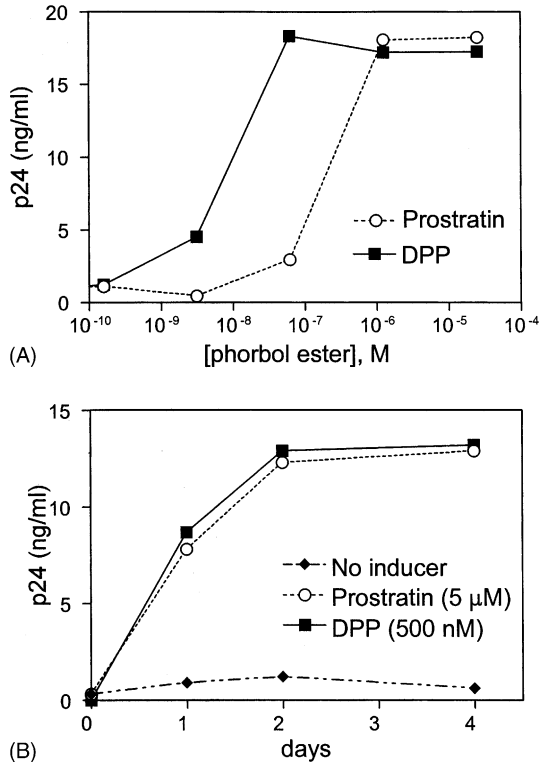

Fig. 2. Activation of latent HIV-1 expression. (A) ACH-2 cells were grown in the presence of various concentrations of prostratin or DPP for 48 h and p24 antigen production was measured by ELISA. (B) ACH-2 cells were grown with no inducer, 5 μM prostratin, or 500 nM DPP and p24 antigen was measured at the indicated times by ELISA. Results are representative of four experiments.

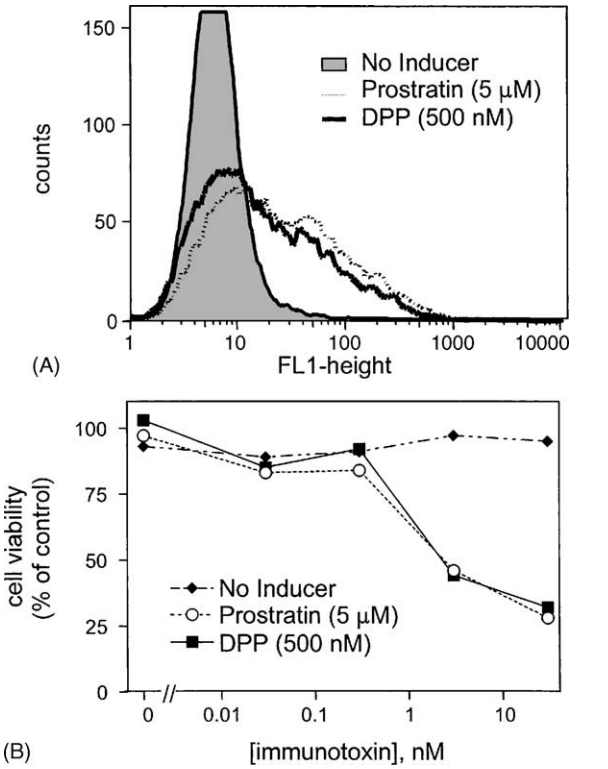

Fig. 3. Induction of envelope expression and immunotoxin sensitivity. ACH-2 cells were grown with no inducer, 5 μM prostratin, or 500 nM DPP for a total of 72 h. (A) The cells were grown without immunotoxin then stained with 3B3:N31H/Q100eY(dsFv)-PE followed by mouse anti-PE monoclonal antibody and FITC-conjugated anti-mouse IgG and analyzed by FACS. (B) The cells were grown in the presence of the indicated concentration of 3B3:N31H/Q100eY(dsFv)-PE throughout the experiment then analyzed for viability by the MTT oxidation procedure. Results are representative of three experiments.

variable region of a genetically engineered antibody that tightly binds to the CD4-binding site of gp120 is fused to *Pseudomonas* exotoxin A (McHugh et al., 2002). Flow cytometry showed that this molecule bound to the surface of ACH-2 cells grown in the presence of either DPP or prostratin but not to uninduced cells (Fig. 3A). Furthermore, cells treated with either phorbol ester were killed by the immunotoxin whereas uninduced cells remained fully viable (Fig. 3B). By contrast, A3.01 cells, the uninfected parental line of ACH-2, were not affected by the immunotoxin (data not shown). These experiments indicate that DPP induces the synthesis of late (envelope) as well as early (p24 gag) HIV-1 proteins.

To establish optimal conditions for eliminating latently infected cells by combined treatment with inducer and immunotoxin, we performed a kinetics experiment in which ACH-2 cells were grown for 1–7 days with and without DPP and with and without 3B3:N31H/Q100eY(dsFv)-PE. At each time point, samples were assayed for cell viability, Annexin V binding, and p24 production. Fig. 4 shows that cells treated with both DPP and immunotoxin showed a progressive loss of viability and increase in Annexin V binding, which is a marker for apoptosis and cell death. The maximum cell killing, which was greater than 98%, was not reached until a full week of treatment. Cells treated with immunotoxin alone showed no significant loss in viability, indicating that cell killing requires envelope expression, whereas cells treated with DPP alone displayed a partial loss of viability due to the cytopathic effects of the virus. Immunotoxin treatment reduced p24 production, but only to levels approximately 25% of those in untreated cells.

3.4. Downregulation of CD4 and CXCR4

It has been shown previously that phorbol esters, including prostratin, downregulate the cell surface expression of both the primary receptor for HIV-1, CD4, and its coreceptors CXCR4 and CCR5 (Gulakowski et al., 1997; Kulkosky et al., 2001). To determine whether DPP also has this ability, A3.01 T cells were incubated with various concentrations of DPP or prostratin then analyzed for CD4 and CXCR4 expression by FACS. Fig. 5A shows that DPP efficiently downregulated CD4 with an IC_{50} of 14 ± 3 nM (mean $\pm$ S.E.M., $n = 3$) and maximal suppression by 100 nM. Prostratin also downregulated CD4, but the IC_{50} was 330 ± 78 nM (24-fold higher than for DPP) and maximal

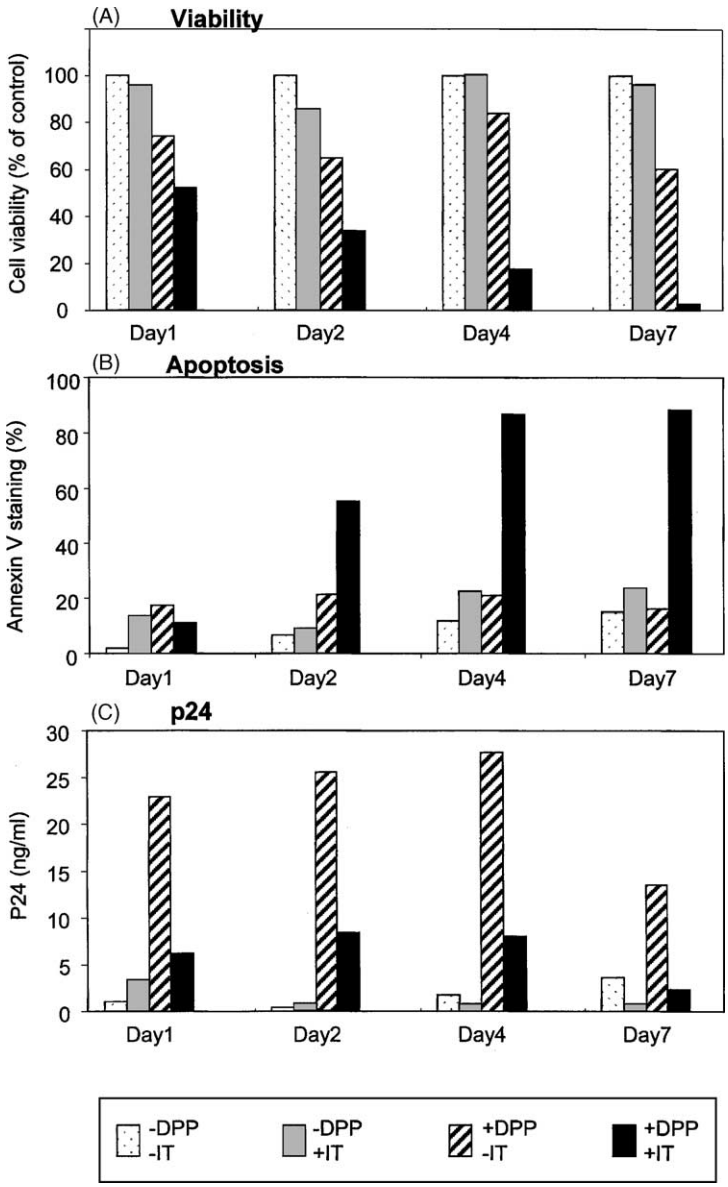

Fig. 4. Kinetics of cell killing by inducer plus immunotoxin treatment. ACH-2 cells were grown in the presence or absence of 100 nM DPP and the presence or absence of 5 nM 3B3:N31H/Q100eY(dsFv)-PE as indicated. At intervals, samples were assayed for (A) cell viability by the MTT oxidation procedure; (B) Annexin V binding by FACS; and (C) p24 production by ELISA.

effectiveness required 1 μM (10-fold higher than for DPP). Similarly, as shown in Fig. 5B, DPP potently downregulated CXCR4 expression with an IC_{50} of 2.9 ± 0.6 nM and maximal suppression by 100 nM whereas prostratin had an IC_{50} of 77 ± 17 nM (27-fold higher than for DPP) and maximal suppression at 1 μM (10-fold higher than for DPP).

3.5. Microarray analysis

To examine the full spectrum of genes regulated by DPP compared to other phorbol esters, we performed microarray analysis. A total of seven hybridizations were performed: DPP, prostratin and PMA-induced versus uninduced A3.01 cells; DPP, prostratin and PMA-induced versus uninduced ACH-2 cells; and, as a control, uninduced ACH-2 versus uninduced A3.01 cells. Using a stringent data filter, ≈5500 spots corresponding to expressed cDNA clones were identified and analyzed.

To determine whether DPP regulates a similar set of genes to prostratin and PMA, the Pearson correlations between the gene expression matrixes were calculated. The upper right quadrants of Table 1A and B show that the overall correlations between all three phorbol ester-induced gene expression patterns were ≈0.6–0.8 in both A3.01 and ACH-2 cells whereas the correlations with the control microarray pattern were less than 0.2. Because the majority of the genes on the chips are not regulated by phorbol esters, this analysis was repeated on those spots that were upregulated or

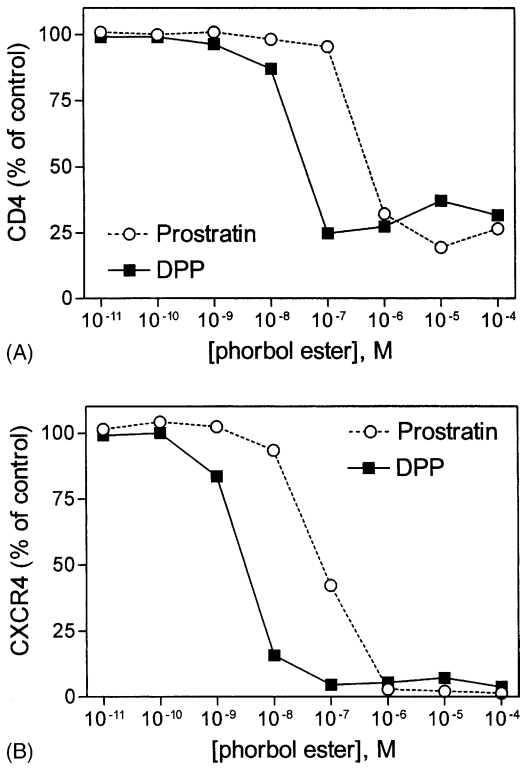

Fig. 5. Downregulation of CD4 and CXCR4. A3.01 cells were grown with the indicated concentration of prostratin or DPP for 24 h then analyzed by antibody staining and FACS. (A) CD4 was measured using a FITC-conjugated antibody. (B) CXCR4 was measured using an APC-conjugated antibody. Results are representative of three experiments.

Table 1
Correlations between phorbol ester regulated gene expression patterns

(A) A3.01

	DPP	PRO	PMA	CONT	
DPP		.780	.606	.100	All genes
PRO	.962		.697	.116	
PMA	.970	.951		.189	
CONT	.239	.064	.199		
	DPP regulated genes				

(B) ACH-2

	DPP	PRO	PMA	CONT	
DPP		.758	.781	.098	All genes
PRO	.749		.838	.060	
PMA	.945	.893		.125	
CONT	.213	.129	.192		
	DPP regulated genes				

The Pearson correlations between the microarray patterns for (A) A3.01 cells and (B) ACH-2 cells induced with DPP, prostratin (PRO), or PMA compared to uninduced cells. The control pattern (CONT) consisted of uninduced A3.01 compared to ACH-2 cells. The upper right diagonals show the results for the complete patterns, which contained 5500 cDNAs. The lower left diagonals show the results for the 27 cDNAs that were induced or repressed at least 1.75-fold by DPP.

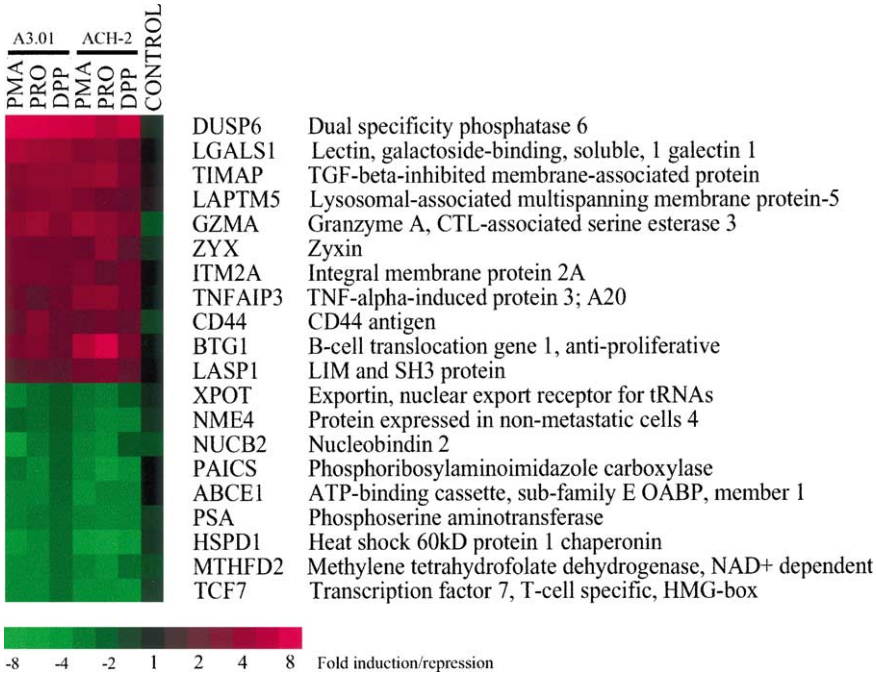

Fig. 6. Microarray analysis of DPP-regulated genes. cDNA prepared from A3.01 and ACH-2 cells induced with 50 nM PMA, 5 μM prostratin (PRO), or 500 nM DPP was labeled with Cy3 (red) and each sample was hybridized with Cy5 (green) labeled cDNA from uninduced A3.01 or ACH-2 cells. A negative control (CONTROL) consisted of uninduced Cy3 (red) labeled A3.01 cDNA hybridized with uninduced Cy5 (green) labeled ACH-2 cDNA.

downregulated at least 1.75-fold by DPP in both A3.01 and ACH-2 cells. The lower left quadrants of Table 1A and B show that the expression patterns for this subset of 27 DPP-regulated genes were correlated ≈0.8–1.0 with the corresponding patterns from prostratin and PMA-induced A3.01 and ACH-2 cells whereas the correlations to the control patterns were again small. These results show that DPP regulates a very similar set of genes as the two other phorbol esters.

To identify biological pathways influenced by DPP, we focused on those genes that were induced or repressed by at least 1.75-fold in both A3.01 and ACH-2 cells. Fig. 6 shows that, as anticipated from the correlational analysis described above, all of these loci were also induced or repressed by prostratin and PMA. As described in the Section 4, many of the upregulated genes are involved in T cell activation and cytoskeleton reorganization.

4. Discussion

In evaluating various molecules as possible adjuncts to HAART for the inductive therapy of persistent HIV-1 infection, the key considerations are potency and therapeutic ratio, short- and long-term side effects, and activity in various latently infected cell types throughout the body.

Our initial data on the potency of the anti-tumor promoting phorbol ester DPP are encouraging. DPP induced latent HIV-1 expression with an IC_{50} value of 4 nM, which was 40-fold lower than for prostratin. It also downregulated the HIV-1 receptor and coreceptor proteins CD4 and CXCR4 at lower concentrations than did prostratin. Previous results indicate that the greater potency of DPP compared to prostratin is due to its greater ability to cause intracellular translocation of PKC, apparently because of the greater lipophilicity of the aromatic side chain at position 13 (Bogi et al., 1998; Marquez et al., 1999). Molecular modeling showed that the hydrophobic phenyl group of DPP projects away from PKC in a position well poised for membrane interaction.

Like other phorbol esters, DPP and prostratin do have cytopathic side effects at sufficiently high concentrations. The IC_{50} values for the inhibition of cell growth in A3.01 lymphocytes were 46 μM for DPP and 110 μM for prostratin (data not shown), giving therapeutic ratios of HIV-1 induction to nonspecific cytotoxicity of 11,500-fold for DPP compared to 680-fold for prostratin. Although DPP and prostratin are both anti-tumor promoting (Szallasi et al., 1992, 1993), they cause skin irritation and platelet aggregation (Evans and Schmidt, 1979; Schmidt and Evans, 1980; Williamson et al., 1981), indicating that careful animal testing will be essential before human use can be considered. On the other hand, the inflammation they cause is transient compared to typical phorbol esters (Schmidt and Evans, 1980). Likewise, the fact that aqueous extracts of Euphorbiaceae have traditionally been used as herbal medications for back pain, abdominal swelling, diarrhea, yellow fever, boils, sores, gonorrhea, and circumcision wounds in Samoa, New Guinea and Indonesia (Gulakowski et al., 1997) suggests that these compounds may not be overly toxic. Although no deoxyphorbol esters have been tested as human therapeutics, clinical trials of the PKC activators bryostatin (Clamp and Jayson, 2002) and PMA (Strair et al., 2002) as anti-cancer drugs are currently in progress.

Microarray analysis showed that DPP regulates a similar set of PKC-responsive genes as do prostratin and PMA and that all of the loci most strongly induced or repressed by DPP were also upregulated or downregulated by the other two phorbol esters. Interestingly, there were a few loci that were selectively regulated by PMA but not by DPP or prostratin, which are candidates for the differential effects of these compounds on tumor promotion (data not shown); however, this will need to be verified in additional cell types.

In T cells, PKC plays a central role in signal transduction by mediating signals from the T cell receptor/CD28 complex to the transcriptional apparatus including NF-κB and AP-1, which in turn regulate HIV-1 gene expression. Given that phorbol esters are frequently used to mimic T cell activation, which leads to cell division, receptor translocation and cytokine production, it is not surprising that many of the genes we found to be induced by DPP are involved in T cell activation and cytoskeleton reorganization.

The most strongly induced gene on the arrays is DUSP6, which encodes a dual specificity phosphatase that acts as a negative feedback repressor of the MAP kinase 2 (ERK2). ERK2 is induced by phorbol esters through activation of PKC (Cobb et al., 1994), and its activation causes cellular proliferation or differentiation. LGALS1, the next most strongly induced gene on the arrays, encodes galectin-1, which is also part of the ERK2 signal transduction pathway. Galectin-1, a highly conserved beta-galactoside-binding protein, plays a crucial role in regulating T cell receptor signaling and apoptosis by ligation of glycoepitopes on T cell activation markers (Rabinovich et al., 2002).

Other induced genes on the arrays that are involved in T cell activation and turnover included ITM2A, GZMA, and TNFAIP3. ITM2A is a type II transmembrane glycoprotein know to be upregulated by T cell activation and PMA treatment (Kirchner and Bevan, 1999). GZMA, a cytotoxic T cell and natural killer cell-specific trypsin-like serine protease, is overexpressed during CD8-cell activation (Kelso et al., 2002). TNFAIP3 is a cytoplasmic zinc finger protein that is upregulated in TNF-induced NF-κB responses (Lee et al., 2000). LAPTM5, a lysosomal-associated multispanning membrane protein implicated in B cell malignancies (Seimiya et al., 2003), and BTG1, an anti-proliferative protein (Matsuda et al., 2001), are also involved in cell cycle regulation, but have not previously been analyzed in T cells.

The remaining DPP-inducible genes were all involved in cytoskeleton reorganization, which plays an important role in T cell activation by assembling an immunological synapse of antigen receptors, coreceptors, and adhesion molecules and creating a scaffold for signaling molecules. TIMAP and

CD44 bind to the cytoskeleton through ankyrin and play a role in intracellular signaling (Cao et al., 2002; Nicoll et al., 2002). PKC upregulates both the binding activity and expression level of CD44, the hyaluronan receptor (Fichter et al., 1997; Legg et al., 2002). LASP1 plays an important role in the regulation of dynamic actin-based cytoskeletal activities (Chew et al., 2000), while Zyxin binds to the mitotic spindles and adhesion plaques (Hirota et al., 2000).

In the current work, we focused on the effects of DPP on T lymphocytes because CD4+ memory T cells are a major reservoir of latent virus in HIV-1 infected individuals (Blankson et al., 2002; Chun and Fauci, 1999; Ho, 1998; Pomerantz, 2002; Sonza and Crowe, 2001). However, it is likely that DPP will also be effective in other types of latently infected cells given the ubiquity of PKC, the varied mechanisms by which it can activate HIV-1 gene transcription, and the positive results that have previously been obtained in the promonocytic U1 cell line with prostratin (Gulakowski et al., 1997) and DPP (Kim et al., 1994). Whether DPP is active in other HIV-1 reservoirs, such as NK cells, and can reach anatomic sanctuaries, such as the reproductive tract and central nervous system, remains to be examined.

Treatment of HIV-1-infected individuals with DPP in conjunction with HAART could potentially reduce the reservoirs of latently infected cells through three routes. The first is direct cell killing by the cytopathic action of HIV-1 itself. This mechanism should be quite effective in T lymphocytes, which are sensitive to viral killing, but probably less so in monocyte–macrophages and other cell types that are relatively resistant to the cytopathic effects of HIV-1 (Rosenberg and Fauci, 1989). The second mechanism is immune surveillance. Although HIV-1 induces a vigorous cellular immune response, the number of virus-specific CD8+ cytopathic T lymphocytes tends to decrease during HAART (Kalams et al., 1999; Ogg et al., 1999); therefore, it might be necessary to combine phorbol ester treatment with immune-enhancing protocols such as therapeutic vaccination. Finally, the induction of HIV-1 gene expression could make cells susceptible to targeted therapeutics such as the potent anti-HIV-1 envelope immunotoxin 3B3:N31H/Q100eY(dsFv)-PE recently developed in our laboratory (McHugh et al., 2002). A kinetics experiment showed that, in ACH-2 cells, the combination of immunotoxin and inducer is clearly more effective than either agent alone. However, the process of cell killing is rather slow, and p24 production is not completely suppressed. This emphasizes the need to combine any inductive therapy of HIV-1 with rigorous HAART to prevent infection of naïve cells by newly synthesized virus. The ability of DPP to downregulate cell surface expression of CD4 and CXCR4 will be useful in this regard by limiting spreading infection. Preliminary experiments indicate that the combination of DPP, immunotoxin and HAART reduces the latent reservoir in peripheral blood mononuclear cells from HIV-1-infected individuals.

Clinical efforts to reduce latent reservoirs of HIV-1 have thus far been largely unsuccessful. Although treatment with interleukin-2 improves CD4+ T cell counts and reduces viral reservoirs in some patients, systematic clinical studies failed to demonstrate a consistent diminution in the pool of latently infected cells or of viral rebound following cessation of therapy (Chun et al., 1999; Dybul et al., 2002). Treatment with a combination of Il-2 and a high dose of the anti-CD3 antibody OKT3 actually resulted in a depletion of CD4+ cells as well as unacceptable toxicity (Prins et al., 1999; van Praag et al., 2001); a milder, intermittent regimen of OKT3 may be more theoretically reasonable and clinically useful (Fraser et al., 2000). Attempts to improve the ability of the endogenous immune system to combat HIV-1 by structured therapy interruption have also failed to reduce viral reservoirs and may actually promote the emergence of drug- and CTL-resistant virus (Martinez-Picado et al., 2002). Cytoreductive therapy with cyclophosphamide in combination with HAART also did not reduce the cellular reservoir of the virus (Bartlett et al., 2002). Given these circumstances, together with the emerging problems of drug resistance and toxicity due to HAART, the possibility of treating persistent HIV-1 infection by inductive therapy with PKC activators such as DPP deserves further consideration.

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