A Nonpromoting Phorbol from the Samoan Medicinal Plant *Homalanthus nutans* Inhibits Cell Killing by HIV-1

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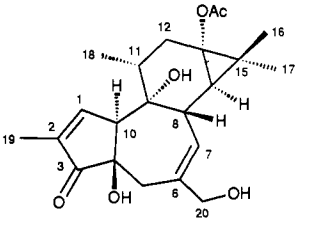
Extracts of *Homalanthus nutans*, a plant used in Samoan herbal medicine, exhibited potent activity in an in vitro, tetrazolium-based assay which detects the inhibition of the cytopathic effects of human immunodeficiency virus (HIV-1). The active constituent was identified as prostratin, a relatively polar 12-deoxyphorbol ester. Noncytotoxic concentrations of prostratin from ≥0.1 to >25 μM protected T-lymphoblastoid CEM-SS and C-8166 cells from the killing effects of HIV-1. Cytoprotective concentrations of prostratin ≥1 μM essentially stopped virus reproduction in these cell lines, as well as in the human monocytic cell line U937 and in freshly isolated human monocyte/macrophage cultures. Prostratin bound to and activated protein kinase C in vitro in CEM-SS cells and elicited other biochemical effects typical of phorbol esters in C3H10T1/2 cells; however, the compound does not appear to be a tumor promoter. In skin of CD-1 mice, high doses of prostratin induced ornithine decarboxylase only to 25–30% of the levels induced by typical phorbol esters at doses $^1/_{30}$ or less than that used for prostratin, produced kinetics of edema formation characteristic of the nonpromoting 12-deoxyphorbol 13-phenylacetate, and failed to induce the acute or chronic hyperplasias typically caused by tumor-promoting phorbols at doses of $^1/_{100}$ or less than that used for prostratin.

Introduction

The U.S. National Cancer Institute (NCI) has initiated an extensive screening program to identify potential anti-AIDS and anticancer compounds from natural sources.[1] Ongoing natural product collection projects are focusing on unusual or underexplored plant, marine, and microbial resources. As part of a collaborative effort to investigate the antiviral and anticancer properties of plants used for medicinal purposes by the indigenous people of the islands of Samoa, we evaluated a number of extracts of Samoan plants for possible anti-HIV properties.

Homalanthus nutans (Euphorbiaceae), a small indigenous tree of the primary forests of Samoa, is an important component of Samoan ethnopharmacology. Interviews with "taulasea", or Samoan healers, indicated that the leaves are used in water infusions to treat back pain and abdominal swelling, the roots to suppress diarrhea, and the stem wood to treat yellow fever.[2] In Samoa, the leaves of *H. nutans* have also been used to treat circumcision wounds.[5] Related species are used in New Guinea (*H. nervousa*) to treat boils and sores,[3] and in Indonesia *H. nutans* is used for gonorrhea.[4]

We report here that extracts of *H. nutans* have potent cytoprotective effects in human lymphocytic cells infected with the human immunodeficiency virus (HIV-1), a causative agent of the acquired immune deficiency syndrome (AIDS). Further, by tracking the HIV-1 cytopathic effect with a bioassay we have isolated and identified a pure active compound, prostratin. At noncytotoxic concentrations, this agent was found capable of preventing HIV-1 reproduction in lymphocytic and monocytoid target cells and to fully protect susceptible cells from the lytic effects of HIV-1. Prostratin is structurally similar to known tumor-promoting phorbol esters, but does not itself appear to have tumor-promoting activity.



PROSTRATIN

Results

Isolation and Identification of Prostratin. The crude organic-soluble extract of *H. nutans* was subjected to a four-step solvent partition protocol.[6] The hexane-soluble and carbon tetrachloride-soluble fractions were active in the anti-HIV assay and, as determined by TLC and ^{1}H-NMR analysis, appeared rich in lipophilic phorbol esters. The material that partitioned into chloroform (3.7 g) did not appear to contain long-chain alkyl esters of

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Table I. NMR Spectral Data for Prostratin[a]

carbon	δ ^{13}C	δ ^{1}H
1	160.6	7.50 (br s)
2	132.9	
3	209.2	
4	73.8	
5	38.7	2.53 (2 H, br s)
6	140.4	
7	130.4	5.73 (d, J = 5.4)
8	39.1	3.18 (dd, J = 5.4, 5.3)
9	76.0	
10	56.2	3.48 (dd, J = 2.5, 2.4)
11	36.6	2.13 (ddq, J = 11.5, 7.1, 6.6)
12	32.3	1.70 (dd, J = 14.7, 11.5), 2.01 (dd, J = 14.7, 7.1)
13	63.8	
14	32.8	0.88 (d, J = 5.3)
15	22.5	
16	23.2	1.09 (3 H, s)
17	15.3	1.02 (3 H, s)
18	18.8	0.98 (3 H, d, J = 6.6)
19	9.9	1.58 (3 H, 33, J = 2.9, 1.5)[b]
20	67.9	3.79 (d, J = 12.7), 3.86 (d, J = 12.7)
21	173.2	
22	21.3	1.55 (3 H, s)

[a] Spectra were obtained at 125 MHz for ^{13}C and 500 MHz for ^{1}H in C_6D_6. Coupling constants are reported in hertz. Assignments were based on homonuclear decoupling, COSY analysis, NOE enhancements, and HMQC and HMBC proton detected heteronuclear correlation experiments. [b] Long range allylic and homoallylic couplings.

phorbol by ^{1}H NMR, but was active in the anti-HIV assay. The latter sample was subjected to gel permeation chromatography (Sephadex LH-20) and reversed phase (C_{18}) HPLC to obtain a pure compound which showed striking activity in the anti-HIV screen. Throughout the isolation procedure all fractions were tested for anti-HIV activity.

The pure, compound was an optically active, white crystalline solid, mp 216–216 °C, $[\alpha]_D$ +62.6° (c 0.8, MeOH) [lit.[7] mp 217–218°, $[\alpha]_D$ +64°]. The molecular formula of $C_{22}H_{30}O_6$ was established by FAB HRMS (observed m/z 391.2088 for [MH^+], calculated m/z 391.2119 for $C_{22}H_{31}O_6$). These data, and precedents that many species of the family Euphorbiaceae produce phorbol diterpenes, suggested that the compound was a monoacetylated phorbol diterpene derivative. Characteristic ultraviolet absorbances (EtOH) at 210 (ϵ = 8900) and 236 nm (ϵ = 5900) and infrared bands ($CHCl_3$) at 3300, 1725, and 1705 cm^{-1} supported this conclusion.

Homonuclear decoupling and COSY analyses of the ^{1}H NMR spectra revealed a 12-deoxyphorbol nucleus. The ^{1}H and ^{13}C NMR resonances were carefully assigned (Table I) via several experiments, including HMQC, HMBC, and difference NOE, and found to match literature values for prostratin,[7,8] the structure of which had previously been established by X-ray crystallographic analysis.[9]

Anti-HIV Cytoprotective Activity of Prostratin. Uninfected cells and cells infected with HIV-1 were incubated with various concentrations of prostratin for 6 days. The cytoprotective and antiviral activity was then assessed using various cellular and viral endpoints.[11] Viability was estimated by the cellular metabolic reduction of the XTT tetrazolium reagent to a colored formazan[10,11] and by the cellular hydrolyiss of BCECF [bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester] to a fluorescent molecule.[11-13] Total DNA content of each well was measured using DAPI (4′,6-diamidino-2-phenylindole), which fluoresces when intercalated at A-T specific sites in chromatin.

An interesting result was obtained (Figure 1) which clearly emphasized the importance of inclusion of uninfected, drug-treated controls for proper interpretations of the assay results. Viewed in isolation, the viability data on the virus-infected CEM-SS cells (Figure 1A,B) would have indicated only a modest enhancement in cellular survival in the presence of 0.1–25 μM prostratin. However, in comparing the effects of prostratin over the same concentration range on uninfected cells, it was apparent that the agent had a growth-inhibitory effect on the control CEM-SS cells as evidenced by both viability and the DNA content assays (Figure 1A–C). Therefore, what might have appeared initially to be only a modest anticytopathic effect of prostratin in the virus-infected CEM-SS cells was actually quite profound; in the presence of a broad range of concentrations of prostratin, the number of viable cells, as indicated by both XTT–formazan production and by BCECF fluorescence, was essentially identical in the uninfected CEM-SS cell cultures and the cultures infected with HIV-1 (Figure 1). Low-power photomicrographs taken of CEM-SS cells after 6 days in culture under varying experimental conditions vividly confirmed both the antiproliferative and the anti-HIV cytopathic effects of prostratin treatment upon CEM-SS cells (Figure 2).

The effects of prostratin on HIV infection in a different lymphoblastic cell line, C-8166, are shown in Figure 1D–F. Prostratin similarly exerted potent anti-HIV cytoprotection in this cell line as measured by cellular viability (Figure 1D,E) and total DNA content (Figure 1F). Interestingly, prostratin did not cause growth inhibition (cytostasis) in this cell line, in contrast to the cytostatic effect of the compound against the CEM-SS cells.

To confirm that the direct effects of prostratin on the CEM-SS cells represented cytostasis rather than cytotoxicity, we performed recovery experiments as described in the Experimental Section. The proliferative capacity of uninfected, prostratin-treated cells was fully restored upon removal of prostratin even after 4 days of treatment (data not shown). There was no evidence of direct cytotoxicity to CEM-SS cells in the range of concentrations shown to be fully cytoprotective against HIV.

Postinfection and limited-treatment experiments (see the Experimental Section) were performed using CEM-SS cells and the XTT assay[10] to determine when in the course of HIV infection prostratin exerted its maximum protec-

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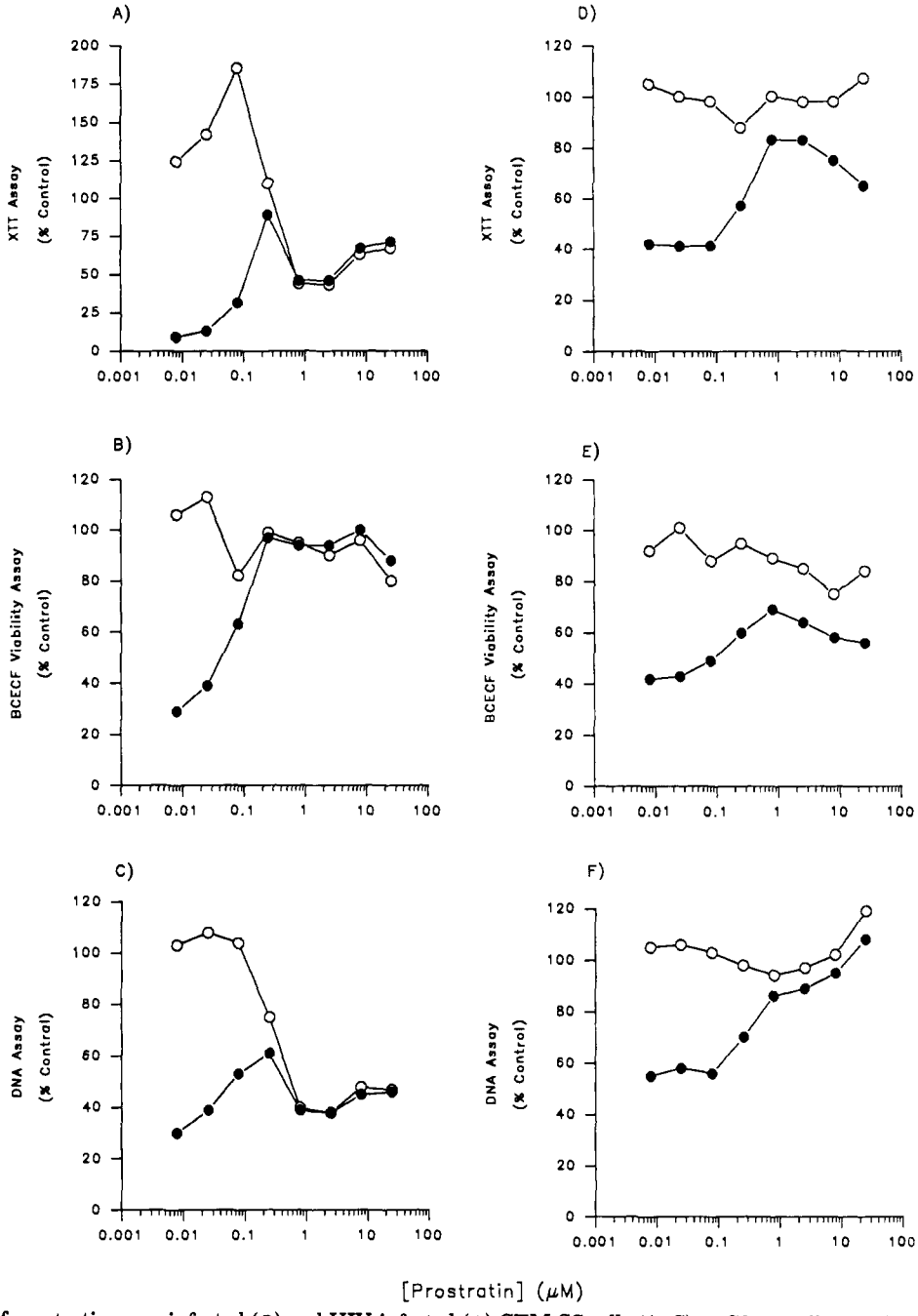

Figure 1. Effects of prostratin on uninfected (○) and HIV-infected (●) CEM-SS cells (A–C) or C8166 cells (D–F) after 6 days in culture. Viable cell numbers were estimated by the XTT-tetrazolium assay (A, D) or by the BCECF assay (B, E); graphs C and F show the total cellular DNA contents assayed by the DAPI method. Values are derived from the means of quadruplicate determinations (SEM ≤ 10%) and represent the percent of the respective uninfected, non-drug-treated control values.

tion. Postinfection experiments were carried out by infecting CEM-SS cells in the microtiter well at a viral MOI of 1.0 and then adding prostratin (25 μM final concentration) after different intervals during the 6 day incubation. Prostratin fully protected against HIV-induced cell killing of CEM-SS cells even when added as late as 24 h after infection (data not shown). However, results from limited-treatment experiments indicated the prostratin had to be continuously present during the late stages of virus infection for maximum protection. For example, freshly-infected CEM-SS cells treated continuously with prostratin (25 μM final concentration) for various intervals up to 72 h followed by incubation in prostratin-free medium showed only 50–60% of the relative viability of similarly treated cells maintained in prostratin for the entire 6-day incubation (data not shown).

Correlative assays measuring the supernatant levels of p24 viral antigen (p24), particle-associated reverse transcriptase activity (RT), and syncytium-forming units (SFU) 6 days postinfection with HIV-1 in CEM-SS and C8166 cells revealed that cytoprotective concentrations of prostratin (e.g., 0.1–25 μM) essentially abolished virus reproduction (Figure 3). With subcytoprotective concentrations of prostratin (e.g., 0.005–0.1 μM), there was a greater total accumulation of p24, RT, and SFU in the supernatants of the infected, treated cells compared to the infected, untreated controls assayed 6-days postinfection (Figure 3).

To further determine the range of action of prostratin we evaluated the effects of noncytotoxic concentrations of the agent on HIV-1 reproduction in the monocytoid cell line U937 and in freshly isolated monocyte/macrophage

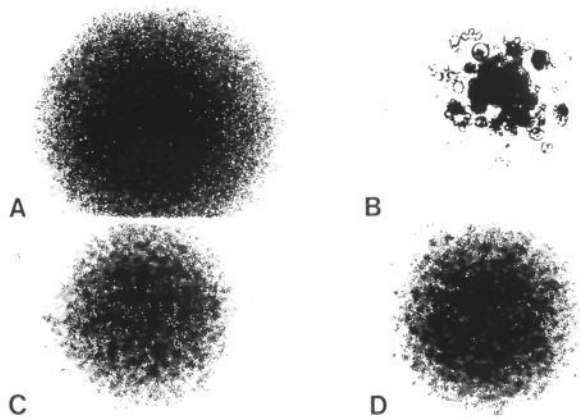

Figure 2. HIV-infected and uninfected CEM-SS cell colony morphology after 6 days in culture in the presence or absence of prostratin (25 μM). Panel A shows the uninfected, untreated control CEM-SS cells which formed a uniform pellet several hundred cell diameters thick on the bottom surface of the rounded well. Infection of CEM cells by HIV-1 resulted in dramatic morphological alterations after 6 days (panel B); the effects included giant cell (syncytia) formation, cell lysis and production of large quantities of cellular debris. In contrast, the macroscopic morphology of cell pellets of uninfected CEM cells (Panel C) and cells infected with HIV-1 (Panel D), both treated with 25 μM prostratin, were virtually identical; there was a marked decrease in the size of the cell pellets relative to the control cells in both cultures; however, the sizes of the cell pellets and the percentage of viable cells in cultures infected with HIV-1 compared to the uninfected cultures were similar after prostratin treatment, and there was no microscopic evidence of syncytia in either.

cultures. These cell types are permissive for HIV replication but are not killed by the virus. The cell line U937 supported a relatively high level of viral replication, and RT was generally the more reliable and consistent experimental indicator thereof. On the other hand, the fresh cell preparation supported a considerably lower level of viral replication which was more sensitively and reproducibly estimated by p24 production. Therefore, using supernatant RT or p24, respectively, as indices of virus replication, it was apparent that prostratin exerted a dose-dependent inhibition of HIV-1 replication in both of these acutely-infected cell culture systems (Figure 4).

An attempt also was made to evaluate the antiviral activity of prostratin in mitogen-stimulated human peripheral blood lymphocytes (PBL) using p24 as the endpoint. The compound markedly inhibited the accumulation of p24 in cultures of PHA-stimulated PHA-stimulated PBL (data not shown), however, only at concentrations which were highly cytostatic, thus making interpretation problematic, since viral replication does not occur in the quiescent PBL.

Protein Kinase C Binding and Modulation. Because prostratin is a phorbol derivative, it was of interest to see if it would bind to and activate or inhibit protein kinase C (PKC).[14,15] Interestingly, in contrast to many other phorbol derivatives, prostratin reportedly was not a tumor promoter.[16] We measured inhibition by prostratin of

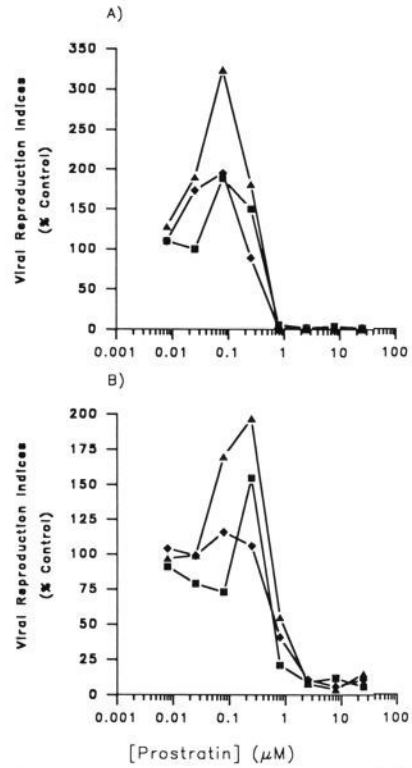

Figure 3. Effects of prostratin upon indices of HIV-1 reproduction in CEM-SS cells (A) or C8166 cells (B). Virus reproduction was estimated by reverse transcriptase activity (▲), p24 core antigen production (◆), and synthesis of infectious virions (syncytium-forming units; SFU) (■); all assays were performed on supernatants obtained from the infected cells. Values are derived from the means of quadruplicate determinations (SEM $\leq$ 10%) and represent the percent of the respective infected, non-drug-treated control values.

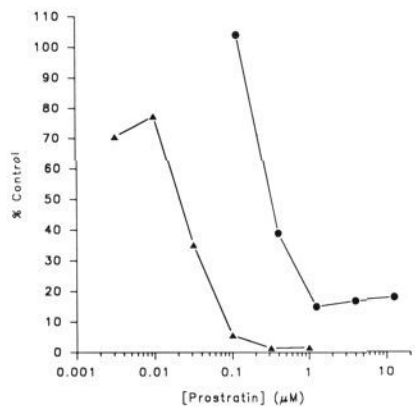

Figure 4. Effects of prostratin on HIV-replication in monocytic cells. Supernatant RT activity (▲) was used to estimate viral reproduction in untreated and prostratin-treated, HIV-1-infected U937 cells. Supernatant p24 (●) was used as an estimate of viral reproduction in untreated and prostratin-treated, HIV-1-infected monocyte/macrophage preparations. All points are graphically represented as the percentage of the respective control values for the infected, untreated cultures. Each point is the average of three determinations (SEM $\leq$ 10%).

[^{3}H]PDBu binding to PKC constituted in vitro in the presence of phosphatidylserine. The K_i for prostratin was 12.5 ± 0.4 nM (mean ± SEM; three experiments). For comparison, the K_i value for 12-deoxyphorbol 13-isobutyrate (DPIBu) was 2.1 ± 0.1 nM (mean ± range; two experiments) and the K_d for PDBu was 0.59 nM. We

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further confirmed that prostratin, like PDBu, stimulated PKC in vitro; at a concentration of 1000 nM, stimulation of PKC was about equal to that exhibited by a concentration of 100 nM PDBu.

Binding affinities of phorbol esters in cells are typically lower than those obtained with reconstituted PKC,[17] presumably reflecting the role of cellular calcium and phospholipid composition on binding.[18] In the CEM-SS cells, [^{3}H]PDBu was bound with a K_d of 4.9 ± 0.8 nM (mean ± range; two experiments). Prostratin inhibited [^{3}H]PDBu binding to the CEM cells with a K_i of 210 ± 30 nM (mean ± range; two experiments). The usual binding protocol for cells omits serum, because of reportedly variable effects of serum on binding.[17] In any case, we did not observe a substantial effect of the 10% fetal calf serum included in the assays of biological response in the CEM cells on the K_i for prostratin (K_i = 190 nM; one experiment).

Well-characterized biological responses to the phorbol esters include inhibition of binding of epidermal growth factor and release of arachidonic acid metabolites.[19] We confirmed that prostratin induced both of these responses in C3H10T1/2 cells. The half-maximally effective concentrations of prostratin were 220 and 1100 nM, respectively. The corresponding values for PDBu, determined in parallel, were 10 and 24 nM, respectively.

Since prostratin acted like a typical phorbol ester in vitro, both in PKC enzyme preparations and in intact cells, albeit with only $^1/_{20}$–$^1/_{45}$ the potency of PDBu, we examined the activity of other phorbol derivatives in the anti-HIV assay with the CEM-SS cells. The known tumor-promoting phorbol esters PMA (phorbol 12-myristate 13-acetate), at a concentration of 50 nM, and PDBu (500 nM) were similarly cytostatic and protective against HIV-1 under our experimental conditions (data not shown).

Ornithine Decarboxylase, Skin Edema, and Hyperplasia Assays. Short-chain, substituted 12-deoxyphorbol derivatives have been found to be only weakly tumor-promoting or nonpromoting,[16,20] and prostratin specifically has been reported to be devoid of tumor-promoter activity.[16] To explore this issue further, we examined the activity of prostratin when applied topically to the back skin of CD-1 mice. We measured the induction of ornithine decarboxylase (OD), since induction of this enzyme represents a well-characterized response to PKC activators. Prostratin induced OD in a dose-dependent fashion, in the range from 0.25 to 0.75 μmol/dose. For comparison, PMA reportedly caused maximal induction of OD in CD-1 mice at a dose of 0.016 μmol,[21,22] a result which we have confirmed (data not shown). We measured OD induction 5 h after prostratin treatment. This time was chosen based on preliminary time-course experiments. As was the case for PMA, maximal induction of OD by prostratin occurred at 4–6 h, although the peak of induction was broader (data not shown). The maximal level of induction by prostratin was only 25–30% of that elicited by 10 μg of PMA. This difference may reflect the broader time course found for prostratin.

In previous studies, we had reported a marked difference between the kinetics of edema formation in CD-1 mice in response to PMA (0.016 μmol/dose) and to 12-deoxyphorbol 13-phenylacetate (0.022 μmol/dose).[23] In the case of PMA, edema only began to appear at 2 h and then remained maximal until 24 h. In contrast, with the weakly promoting 12-deoxyphorbol derivative edema was maximal at 30 min and declined markedly thereafter. The pattern observed for prostratin (0.25 μmol/dose) closely resembled that for 12-deoxyphorbol 13-phenylacetate, showing the characteristic rapid onset within minutes followed by the rapid decline over a few hours (data not shown).

Perhaps the best correlate with tumor-promoting activity in the mouse skin model is the induction of hyperplasia and its maintenance upon repeated treatments.[24] Therefore, we treated the backs of CD-1 mice with prostratin at doses of 0.025, 0.075, 0.25, 0.75, and 2.20 μmol/dose. We examined the skin for hyperplasia 24, 48, and 72 h after treatment. There was no hyperplasia after 2.20 μmol prostratin, in contrast to strong hyperplasia in response to 0.016 μmol PMA (data not shown). In further experiments, animals were treated five times with prostratin at intervals of either 24 or 48 h. The dose of prostratin used was 2.2 μmol per application. We examined for hyperplasia 24 h after the last application. As was the case for the acute prostratin treatment, no hyperplasia was observed (data not shown).

Discussion

Phorbol esters elicit a wide range of biological responses in different tissues.[25-33] The reported effects of these

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compounds include inflammation, platelet activation, prostaglandin production, cell proliferation, and tumor promotion. The majority of studies have employed lipophilic diesters of the C-12 and C-13 hydroxyl groups of the parent diterpene phorbol. Representative phorbol esters studied extensively in these respects include PMA and PDBu. Comparisons of biological potency indicate that lipophilicity is an essential determinant of activity.[31,32]

Using the XTT–tetrazolium and BCECF assays we found that prostratin protected cultured T-lymphoblastoid cells from the cytopathic effects of HIV-1 (Figure 1). Virus-infected and uninfected CEM-SS cells treated with cytoprotective concentrations of prostratin grew at equivalent rates, but the overall growth rates in both groups were somewhat suppressed relative to control cells not treated with prostratin (Figure 1A–C). In the cytoprotective concentration range, the direct effect of prostratin on the CEM-SS cells was cytostatic rather than cytotoxic, as evidenced by recovery studies. The cytostatic effect of prostratin on CEM-SS cells resulted in decreased tetrazolium reduction and BCECF hydrolysis, lower optical densities relative to control cells, and a smaller cell pellet in the microtiter wells.

Microscopic observations (Figure 2) confirmed that cells cultured in the presence of prostratin grew at substantially lower rates than the respective control cells but were entirely protected from the lytic effects of the virus. Moreover, syncytia were not observed in cultures of CEM-SS infected with HIV-1 and incubated for 6 days with prostratin, while the corresponding virus-infected control cells, in the absence of prostratin, formed numerous syncytia and were lysed (Figure 2).

Interestingly, prostratin could completely protect C-8166 target cells from the killing effects of HIV, yet without showing the cytostasis seen with the CEM-SS cell line (Figure 1D,F). The C-8166 line is extremely sensitive to HIV-induced cytolysis; under our present assay conditions, prototype anti-HIV agents such as AZT or ddC required about 10-fold greater concentrations to effect a similar degree of cytoprotection in this cell line compared to the CEM-SS line (J. McMahon, et al., NCI, unpublished). Future experiments to explore possible synergistic effects of prostratin in combination with AZT, ddC, and other prototypes to modulate HIV infection and/or cytopathic effects would be of interest.

In the presence of cytoprotective concentrations of prostratin there was an essentially complete inhibition of viral reproduction in both the CEM-SS and the C-8166 cell lines. However, the cytoprotective effects may not be related directly to an effect of the agent upon HIV reproduction. Treatment of infected cells with subcytoprotective concentrations of prostratin resulted in seemingly paradoxical effects upon the 6-day total accumulation of markers of HIV reproduction in the supernatants from both the CEM-SS and the C-8166 lines compared to the corresponding infected, untreated controls (Figure 3). It is conceivable that subcytoprotective concentrations of prostratin simply prolong the ability of the infected, treated host cells to support viral replication compared to the infected, untreated host cells. It may also be pertinent that the 6-day assay time was selected as optimal for the cytopathicity endpoints. The peak and thereafter the rapid decline in the rate of viral reproduction in the infected, untreated host cells may occur much earlier than in the infected, treated cells. In a preliminary test of this hypothesis, the same experiment depicted in Figure 3 was repeated with CEM-SS cells, this time using only a 4-day total incubation. In that study the total accumulations of supernatant p24, RT, and SFU in the infected cells in the presence of subcytoprotective concentrations of prostratin was essentially the same as in the corresponding infected, untreated controls (data not shown).

Of further interest was the observation that prostratin strongly inhibited HIV reproduction in the monocytoid cell line U937. These cells support HIV replication but are not subject to the cytolytic effects that HIV causes in the lymphoblastoid lines.

Our finding that prostratin could inhibit HIV-1 reproduction in freshly isolated cells of the monocyte/macrophage lineage (Figure 4) also is of added interest. Such cells are not proliferative, yet they are permissive for HIV infection and replication and are not killed by the virus.[34] These cells may thereby play an important role in the pathogenesis of AIDS by acting as a reservoir of HIV and disseminating the virus to various tissues in vivo.[35]

Prostratin is 12-deoxyphorbol 13-acetate, a relatively polar phorbol ester. Less polar, lipophilic phorbol esters have been reported to induce a variety of responses with regard to HIV infection and expression, and such effects may be dependent upon the particular host cell line studied. For example, stimulation with PMA reportedly resulted in a reversible reduction in plasma membrane CD-4 (T4) expression[33] and decreased infection with HIV in monocytoid cells.[36] Virus production in persistently infected, undifferentiated monocyte cells reportedly was virtually eliminated when the cells were induced to differentiate with PMA.[37] However, there also have been reports of phorbol ester-mediated stimulation of viral replication in persistently infected lymphoblastic cells, resulting in renewed cytopathic effects.[38-40] In another

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report,[41] PMA was found to inhibit HIV-induced syncytium formation in MOLT-4 cells while at the same concentrations significantly enhancing p24 production. Similar to our results with prostratin, we have reported elsewhere[11] that the tumor-promoting phorbol PMA exerted a biphasic response upon indices of HIV-1 replication in the CEM-SS cell line. There have been other reports of the apparent upregulation of HIV expression in other selected cell lines (e.g., refs 42–45). However, the significance of these observations with respect to the therapeutic potential, or lack thereof, of prostratin as an antiviral or cytoprotective agent against HIV-1 is presently unknown.

Our results indicated that prostratin is a protein kinase C agonist with a potency $^1/_{20}$–$^1/_{45}$ that of PDBu. Also similar to other phorbol esters,[19] prostratin inhibited the cellular release of arachidonic acid in binding of epidermal growth factor, albeit with a potency of $^1/_{20}$–$^1/_{50}$ that of other phorbols. The cellular binding affinity of prostratin (2×10^{-7} M) was consistent with the minimum concentrations causing an inhibitory response in the XTT–tetrazolium assay. The observation that typical phorbol esters such as PDBu and PMA showed similar anti-HIV cytopathic activity to prostratin suggested that this phenomenon may represent a general response to PKC activation. However, in preliminary studies we have found that bryostatin 1, a specific PKC activator,[14] reversed the cytoprotective effects of prostratin in HIV-1-infected CEM-SS cells in a concentration-dependent fashion, and interestingly, bryostatin 1 appears to be devoid of anti-HIV activity in the primary screen (data not shown).

The inhibitory activity of prostratin in our anti-HIV assay was of particular interest since prostratin reportedly was not a tumor promoter,[16] unlike most other phorbol esters. In addition, prostratin had been found to be inactive in a cutaneous inflammation assay at a dose of 0.10 μmol/ear,[46] whereas PDBu was active at $^1/_{1500}$ of the prostratin dose.[47] All phorbol esters known to be tumor promoters are active in the latter assay. In another report, prostratin was found to have some irritant activity, but with $^1/_{34}$ the potency of PDBu,[20] in close agreement with our measurements of relative affinity for PKC.

Reports of the relative tumor-promoting activity of 12-deoxyphorbol derivatives substituted with side chains of varying hydrophobicity are also of interest. For example, the highly hydrophobic derivative 12-deoxyphorbol 13-tetradecanoate was an effective tumor promoter.[20] In contrast, compounds of intermediate hydrophobicity such as the isobutyrate, the tiglate, and the phenylacetate esters were reported to be inflammatory but only weakly promoting or nonpromoting.[16,20] Prostratin, being even less hydrophobic, might therefore be expected to be less tumor-promoting than these other compounds.

Our analysis of the short-term effects of prostratin on mouse skin support further that prostratin is a nonpromoting phorbol. The kinetics of edema formation resembled those of the weakly promoting 12-deoxyphorbol 13-phenylacetate rather than those of the potent promoter PMA. Moreover, no hyperplasia was induced in CD-1 mice by single or multiple prostratin applications even at doses 100-fold or greater than doses of PMA which induced intense hyperplasia. These negative results were not because too low doses of prostratin were used, since these same doses produced the maximal achievable induction of ornithine decarboxylase by prostratin. For PMA, it was found previously that the dose–response curves for induction of ornithine decarboxylase and for tumor promotion coincided.[22]

The previously reported promotion study with prostratin[16] left an uncertainty, in that it did not include a positive control to show that the dose examined (0.18 μmol per application in NMRI mice) was sufficient to induce other biological responses in skin. Because prostratin lies outside the range of phorbol derivatives investigated in detail, further mechanistic studies are warranted, as is further confirmation of its lack of activity under a definitive, long-term tumor promotion protocol. Nonetheless, the present results provide further confidence in the conclusion that prostratin represents a nonpromoting activator of protein kinase C which strongly inhibits the killing of human host cells in vitro by HIV. By these criteria, prostratin is unique.

The phorbol esters have previously been of interest as possible therapeutic agents on the basis of their ability to induce differentiation of a variety of cell types. A prevailing concern, however, has been their tumor-promoting activity. The potent anti-HIV cytoprotective activity and the apparent lack of tumor-promoting activity of prostratin have provided the basis for NCI's recent selection of this agent for further evaluation as a potential candidate for drug development.

Experimental Section

Ethnobotanical Techniques. Interviews concerning the use of *H. nutans* (Forster) Pax were conducted in the Samoan language with taulasea in Falealupo and Pesega vilages, Western Samoa. Bulk samples of stem wood and other parts of *H. nutans* were collected and shipped immediately to the NCI Natural Products Repository in Frederick, MD. Voucher specimens were collected at the same time, verified by taulasea, and subsequently deposited in the herbaria of Brigham Young University and Harvard University.

Bioassay-Guided Isolation and Structure Determination. Initial anti-HIV screenings of crude extracts and chromatographic fractions and the bioassay support for isolation of the pure active compounds were performed routinely by the XTT–tetrazolium assay[10] with certain modifications as previously described.[11] Large-scale separations, using high-performance liquid chromatography (HPLC), were performed initially with a Waters C-18 PrepPak 500 cartridge, and final purification was effected on a Rainin Dynamax C-18 column (1 × 25 cm). NMR spectra were recorded on a Varian VXR 500 spectrometer using $CDCl_3$ or C_6D_6 as solvent and internal standard; chemical shifts were documented in ppm relative to tetramethylsilane (TMS, $\delta = 0$). Infrared spectra were measured on a Perkin-Elmer 267 spectrometer and ultraviolet spectra were obtained with a Beckman 34 spectro-

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photometer. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. Mass spectra were recorded on a VG Micromass ZAB 2F mass spectrometer.

Isolation and Identification of Prostratin. Fresh stemwood from *H. nutans* (1.05 kg) was extracted sequentially with ethanol and CH_2Cl_2–CH_3OH (1:1) to yield 22.8 g of extract, which was separated by means of a four-step solvent partitioning scheme.[6] The $CHCl_3$-soluble fraction (3.7 g) was further separated by gel permeation through Sephadex LH-20 (4 × 140 cm) with CH_3OH–CH_2Cl_2 (1:1) into seven fractions. Fractions 2 and 3 were combined (2.3 g) and subjected to preparative HPLC using a CH_3OH–H_2O step gradient elution on C_{18} reversed-phase sorbent. The fraction that eluted with MeOH–H_2O (7:3), 95 mg, was purified further by C_{18} HPLC (MeOH–H_2O, 7:3) to provide 15 mg of prostratin, mp 215–216 °C, $[\alpha]_D$ +62.6° (*c* 0.9, CH_3OH); ^1H and ^{13}C NMR data are provided in Table I; relevant MS, IR, and UV data are provided in the Results section.

Reagents. All experimental antiviral agents and the tetrazolium reagent XTT[10] were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. Crystalline stock materials were stored at −70 °C and solubilized in 100% dimethyl sulfoxide (DMSO). Drugs were diluted in complete medium, with the final concentration of DMSO not exceeding 1% (v/v). Reference standards used in these studies included 3′-azido-3′-deoxythymidine (AZT; NSC 602670), 2′,3′-dideoxycytidine (ddC; NSC 606170), and phorbol myristate acetate (PMA; NSC 262244).

2,7-Bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF) was purchased from Molecular Probes, Inc. (Eugene, OR). The BCECF was dissolved in DMSO (1 mg/mL) immediately before use. A working solution of 2 μg/mL was prepared in Dulbecco's phosphate-buffered saline (PBS) (Gibco). DAPI (4′,6-diamidino-2-phenylindole) was purchased from Sigma Chemical Co. (St. Louis, MO); stock solutions were prepared at 100 μg/mL in distilled water by sonication, passed through a 0.45-μm filter, and stored at −20 °C. Working solutions of DAPI were prepared at 10 μg/mL in PBS containing 0.5% nonidet P-40 (NP-40; Sigma). XTT was prepared at a concentration of 1 mg/mL in serum-free RPMI 1640. Phenazine methosulfate (PMS; Sigma) was prepared at 0.153 mg/mL in PBS and stored at −20 °C. Immediately before use, XTT was dissolved at 37 °C and PMS was added to yield a final concentration of 20 μM.

Cell Lines and Virus. The human lymphocytic cell line CEM-SS[48] and the human umbilical cord blood lymphocyte cell line C-8166[49-51] were maintained in RPMI 1640 medium without phenol red and supplemented with 2 mM L-glutamine and 5% and 10% fetal bovine serum, respectively (complete medium). The monocytoid cell line U937[52] was maintained in complete medium supplemented with 5% fetal bovine serum. HIV-1, specifically the Haitian variant of HIV (HTLV-III$_{RF}$; 9×10^5–3×10^6 syncytium forming units/mL), was used throughout. Unless otherwise indicated, the infections were initiated directly in the microtiter well by adding cell-free virus supernatants diluted in complete medium to yield the desired multiplicity of infection (MOI).

Comfirmatory Assays. To further define the anti-HIV activity of the pure compound, a battery of confirmatory assays was performed concurrently as previously described.[11] Briefly, target cells were plated at a density of 10 000 CEM-SS cells/well, 50 000 C-8166 cells/well, or 5000 U937 cells/well into individual wells of round-bottomed, 96-well microtiter plates containing either dilutions of test compound or complete medium alone to provide appropriate cell and virus controls. Diluted cell-free virus supernatants were then added to yield the desired MOI (0.1–0.5). Plates were incubated for a total of 6 days, following which aliquots of supernatant fluids were removed and assayed for infectious virions, HIV core protein (p24) and reverse transcriptase activity as described.[11] Cellular viability was measured by colorimetric or fluorescent endpoints, based upon reduction of the tetrazolium salt XTT or the hydrolysis of BCECF, respectively; the DNA content of each well was determined using the fluorescent dye DAPI.[11]

Other in Vitro Assays. For recovery experiments, uninfected or HIV-infected CEM-SS cells were incubated with or without 25 μM prostratin for 1, 2, or 4 days, washed, and added to individual wells of 96-well microtiter plates (5000/well) containing complete medium with or without 25 μM prostratin. The plates were incubated for a total of 6 days and assayed for cellular viability using the XTT method.[10,11]

Other experiments were done to determine the time of exposure requirements for maximum prostratin effects and to assess preliminarily where in the virus life cycle prostratin may work. To determine the effects of a late addition of prostratin (25 μM final concentration) upon freshly infected cells, CEM-SS cells were plated at a density of 5000 cells/well in 50 μL of complete medium and infected with diluted cell-free virus (50 μL) to a final MOI of 1.0; the prostratin was then added after 0, 4, 8, 24, 48, or 72 h during the 6 day incubation to both infected and uninfected cells. Appropriate cell and virus controls were incorporated into the assay plate. After the 6 day total incubation, cellular viability was assessed using the XTT assay.[10]

To determine the ability of prostratin to abort a new infection, limited-treatment experiments were performed; CEM-SS cells were pelleted and infected in bulk with cell-free virus supernatant to a final MOI of 1.0; the infection was carried out at 22 °C for 45 min with constant agitation. After infection, cells were resuspended in complete medium with or without prostratin (25 μM final concentration); after intervals of 0, 4, 8, 24, 48, or 72 h, aliquots of cells were removed, washed free of prostratin, and plated into individual wells of a 96-well microtiter plate containing media with or without prostratin (25 μM). The plates were then incubated for a total of six days from the time of initial infection. At the end of incubation, cellular viability was assessed using the XTT assay.[10]

PHA-stimulated human peripheral blood lymphocytes were prepared according to the methods of Schinazi et al.[53] Uninfected or infected control cells and prostratin-treated, uninfected and infected cells were recultured in microculture dishes (3×10^4 cells/well/200 μL) with or without prostratin for an additional 6 days in medium containing 50 units of recombinant IL-2/mL. After incubation, virus replication was estimated by analysis of culture supernatants for the synthesis of p24 by an antigen capture ELISA (Coulter Immunology).

Fresh monocyte/macrophage cultures were prepared from blood of uninfected donors (American Red Cross) as described.[54] Briefly, cells were separated using lymphocyte separation medium (Organon Technica). Separated peripheral blood mononuclear cells (PBMC) were washed twice with Ham's balanced salt solution (HBSS) and then resuspended in HBSS plus 10% (v/v) human

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HB serum. Cells were allowed to attach in 24-well tissue culture plates for 90 min before the nonadherent cells were washed off. The adherent cells were incubated for 3 days in complete medium then washed twice with HBSS. The cultures were treated with various concentrations of prostratin for 1 h prior to infection with HIV-1 (MOI = 0.5), which was then carried out in the presence or absence of prostratin for 4 h at 37 °C. Following infection the cell monolayers were washed six times with HBSS and then reincubated for 7 days with complete medium with the appropriate concentration of prostratin. The supernatant levels of p24 were then determined as above.

Protein Kinase C Assay. Binding of [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) to PKC partially purified through the DEAE-chromatography step[55] was assayed in the presence of phosphatidylserine as described[56] except that incubation was for 5 min at 37 °C. Competition of [^{3}H]PDBu binding by prostratin was determined in the presence of 4 nM [^{3}H]PDBu. Binding of [^{3}H]PDBu to intact CEM-SS cells and competitive binding by prostratin was assayed in RPMI cell culture medium containing 0.1 mg/mL of bovine serum albumin, 25 mM Hepes, pH 7.4. Incubation was for 30 min at 37 °C, after which the cells were chilled, an aliquot was removed to determine total counts, and bound counts were determined by filtration. Nonspecific binding was determined in the presence of 5 μM nonradioactive PDBu. Activation of PKC by PDBu or prostratin was determined by the procedure of Nakadate et al.[57] except that Tris–Cl, pH 7.4, at 50 mM and 100 μg/mL of phosphatidylcholine–phosphatidylserime (4:1) were used in place of the phosphatidylserine.

C3H10T1/2 cells were grown, and [^{3}H]arachidonic acid metabolite release was determined as described previously.[19] ^{125}I-epidermal growth factor binding by C3H10T1/2 cells was assayed as described[19] using a 1-h pretreatment with phorbol ester and then a 1 h coincubation with the phorbol ester and the ^{125}I-epidermal growth factor.

Animal Studies. Female Charles River CD-1 mice, 6–8 weeks of age, were obtained from Charles River Laboratories, Wilmington, MA. The dorsal hair of each mouse was shaved 2 days before use, and only those mice showing no hair regrowth were used. Prostratin was dissolved in acetone and applied in a volume of 100 μL. Mice were killed by cervical dislocation at the times after treatment indicated in the text, and the skin was further analyzed as described below. L-[^{14}C]Ornithine was purchased from Amersham (Arlington Heights, IL) and from New England Nuclear (Boston, MA).

For analysis of ornithine decarboxylase activity, the epidermis of individual mice was separated from the dermis by brief heat treatment. The epidermal preparations of two to four mice were pooled and then homogenized for 20 s at 0.4 °C in 50 mM sodium phosphate buffer, pH 7.2, containing 0.1 mM pyridoxal phosphate and 0.1 mM EDTA,[58] using a Polytron tissue homogenizer. The supernatant fraction obtained after centrifugation at 30000*g* for 2 × 20 min at 0 °C was used for determination of enzymatic activity, quantitated by the release of CO_2 from L-[^{14}C]ornithine as described by Lichti and Gottesman.[59]

For measurement of edema, skins were removed from the mice killed by cervical dislocation; 0.6- or 1.0-cm skin punches were cut and quickly weighed. After drying for 24 h at 60 °C, the skins were reweighed. Data were expressed as the ratio of the water content (wet minus dry weight)/dry weight of each skin punch.

For examination of hyperplasia, dorsal skin was removed and fixed in 10% formalin in 0.1 M sodium phosphate buffer, pH 7.5, followed by sectioning and staining with hematoxylin–eosin. In each experiment, two mice were subjected to each treatment condition. For each mouse, three sections were examined for each of two portions of skin within the treated area.

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