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Novel indolocarbazole protein kinase C inhibitors prevent reactivation of HIV-1 in latently infected cells

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Summary

Suppression of human immunodeficiency virus-1 (HIV-1) reactivation in latently infected cells by protein kinase C (PKC) inhibitors has been described. Based on an initial finding with the indolocarbazole inhibitor Gö 6976 we have examined several members of this new class of potent and specific PKC inhibitors with respect to their ability to prevent the PKC-mediated induction of HIV-1 replication in the latently infected U1 cell line. Two of these compounds strongly inhibited not only PMA-induced release of p24-antigen and infectious virus particles into the supernatant (50% inhibition at 0.04–0.35 μM) but also TNF-α-mediated HIV-1 reactivation in the same concentration range. Significant lower toxicities compared to Gö 6976 were observed for the new compounds, with 50% cytotoxic concentrations at 5.2 μM for Gö 7775 and 3.4 μM for Gö 7716. This resulted in selectivity indices which were 10–20-times higher compared to the reference compound Gö 6976 and were comparable to those of registered anti-AIDS drugs. No anti-HIV-1 activity was observed for a closely related indolocarbazole analogue with no inhibitory activity in the PKC in vitro enzyme assay. This study demonstrates the important role of PKC in reactivation of HIV-1 in latently infected cells and points to the potential of indolocarbazoles to preserve the latent state of HIV-1 infection.

Antiviral; HIV-1; Indolocarbazoles; Latency; Protein kinase C inhibitors

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Introduction

The course of the HIV-1 infection is characterized by a short peak of viremia followed by a long and variable period of latent or persistent infection with no symptoms of disease (Curran et al., 1985). During this asymptomatic phase various cell types have been shown to be infected (Zagury et al., 1986; Ho et al., 1986; Gartner et al., 1986; Nicholson et al., 1986) and integrated proviral DNA as well as non-integrated virus genomes can be detected in patient's peripheral blood mononuclear cells (PBMC) (Bukrinsky et al., 1991). Recent reports also show that high numbers of latently infected $CD4^+$ T lymphocytes and macrophages are present in the lymphoid system throughout the infection which may represent a significant reservoir for virus dissemination (Embretson et al., 1993; Pantaleo et al., 1993). These observations support the assumption that a latent or persistent infection prevails in the asymptomatic phase with a shift to productive infection during full-blown AIDS. Latently infected cell lines with integrated proviral DNA have been developed, which allow to study reactivation of HIV-1 by external signals (Clouse et al., 1989; Laurence et al., 1990; Kinter et al., 1990).

A number of stimulating agents including different cytokines such as tumor necrosis factor α (TNF-α) (Kinter et al., 1990; Folks et al., 1989; Duh et al., 1989; Poli et al., 1990) have been shown to trigger reactivation of latent HIV-1 in vitro. Virus expression depends on the activation state of the host cell and probably involves the activation of cellular PKC since stimulation of latently infected cells with PMA, a known activator of PKC, induces virus replication in a concentration-dependent manner (Laurence et al., 1990; Kinter et al., 1990). The effect of PKC on the viral genome was suggested to be mediated by modulation of host cell transcription factors, e.g. NF-κB (Ghosh and Baltimore, 1990), and of the viral *trans*-activator protein *tat* (Jakobovits et al., 1990). Moreover, it has been shown that PKC also modulates the activity of a cellular TAR RNA binding factor (Han et al., 1992).

Recently, the anti-HIV-1 activity of Gö 6976, a new PKC inhibitor belonging to the class of non-glycosidic indolocarbazoles, has been described (Qatsha et al., 1993). In the attempt to further evaluate this novel inhibitory approach we have compared several related compounds with regard to their in vitro protein kinase inhibition as well as their antiviral activities. Due to a considerably lower toxicity their selectivity indices are 10–20-times higher compared to Gö 6976. Our study indicates that indolocarbazoles may lead to the development of antiviral drugs with the potential to inhibit provirus reactivation in early infection.

Materials and Methods

Reagents Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma Chemical Co. (Munich, Germany) and stored at 1 mM in ethanol at $-20°C$.

Recombinant TNF-α obtained from Genzyme (Munich, Germany) was stored at 20000 U/ml in phosphate-buffered saline at −70°C. The protein kinase inhibitor H7 (1-[5-isoquinolinesulphonyl]-2-methylpiperazine dihydrochloride, Seikagaku Inc., Japan) was stored at 10 mM as aqueous solution at 4°C. Indolocarbazole PKC inhibitors were synthesized as described in a patent application (Kleinschroth et al.). The four indolocarbazole compounds were dissolved at 10 mM in 100% DMSO and aliquots stored at 4°C. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) was obtained from Sigma, dissolved at 7.5 mg/ml in phosphate-buffered saline and stored at 4°C.

Protein kinase assays In vitro enzyme assays for the various protein kinases were performed as described by Qatsha et al. (1993).

Cell culture Latently HIV-1 infected U1 cells (kindly provided by Dr. G. Poli, NIH (Kinter et al., 1990)) and MT2 cells (Miyoshi et al., 1981) were grown in RPMI-1640 standard medium (SM) supplemented with 1 mM L-glutamine, 50 U penicillin per ml, 50 μg streptomycin per ml and 10% heat-inactivated fetal calf serum (Seromed, Berlin, Germany, Lot No. 6R07) at 37°C in a humified atmosphere (5% CO_2). Throughout the experiments cells were kept at a density of 5×10^5 cells per ml.

HIV-1 induction procedure To activate the expression of HIV-1, U1 cells were incubated at 37°C in SM containing 10 nM PMA or in SM containing TNF-α (100 U/ml). To avoid depletion of endogeneous PKC by prolonged activation with PMA, treatment was stopped after 15 min by three wash cycles with SM. In order to block HIV-1 induction, PKC inhibitors were added to the cells at various concentrations 1 h ahead of PMA- or TNF-α-treatment and were present throughout the experiment. The final concentration of DMSO in the culture was 0.001%. Forty-eight h after PKC stimulation culture supernatants were harvested, cleared of cellular debris and either used directly for syncytium assay or stored at −20°C for p24 ELISA.

Syncytium assay The ability of indolocarbazole derivatives to prevent HIV-1 replication from latently infected U1 cells upon stimulation of endogeneous PKC was determined by microtiter syncytium formation assay. Infectious virus particles were pelleted from 1 ml fresh culture supernatant (20000 × g, 90 min, 4°C) from PMA- and TNF-α-treated U1 cells with or without previous treatment with PKC inhibitors. Virus pellets were resuspended in 100 μl of SM and serially diluted in 96-well microtiter plates (flat bottom, Falcon). A total of 1.25×10^4 freshly prepared MT2 cells (Traunecker et al, 1989) were seeded into each well and microscopically scored for syncytium formation at day 3 to 4 after infection.

p24 ELISA Cell- free supernatants from PMA- and TNF-α-activated U1 cells pretreated with or without drugs were collected for the determination of p24

production by antigen capture ELISA (NEN-DuPont, Dreieich, Germany) following the instructions of the Manufacturer.

Presentation of data For each parameter tested, decrease of virus replication upon inhibition of endogeneous PKC was presented as percent of the untreated, PMA- or TNF-α-activated controls. The effect of each compound was evaluated by the 50% effective virus inhibitory concentration (IC_{50}), 50% cytotoxic concentration (CC_{50}), and selectivity index ($SI = CC_{50}/IC_{50}$).

Cytotoxicity assay The cytotoxicity of PKC inhibitors was measured by the MTT assay, as described previously (Mosman, 1983). Activated U1 cells were treated with or without PKC inhibitors in the presence or absence of stimulating agents as described above (see HIV-1 induction procedure). Cells were seeded in triplicate at 5×10^4 cells per well in 96-well microtiter plates (flat bottom, Falcon) and incubated for 48 h at 37°C (5% CO_2). Cytotoxicity of PKC inhibitors was expressed as percent of viable cells in the drug-free controls.

Results

In vitro inhibition of purified protein kinases

Following the discovery of Gö 6976 as a potent and selective PKC inhibitor with antiviral activity, we have tested a large number of indolocarbazoles for in vitro inhibition of four different serine/threonine protein kinases and of tyrosine kinase activity from mouse B cells. This resulted in the identification of two novel potent PKC inhibitors (Gö 7775 and Gö 7716) which inhibit PKC in vitro with IC_{50} values of 44 nM and 32 nM concentrations, respectively (Table 1). Both compounds were inactive in the tyrosine kinase assay but differed with respect to their selectivity against serine/threonine kinases. Gö 7775 inhibited all four serine/threonine protein kinases with almost equal potency, whereas Gö 7716 showed significant selectivity at least over cAMP-dependent protein kinase. Gö 7922 was included into the study as an inactive indolocarbazole analogue which was obtained by methylation of the lactame nitrogen of Gö 6976. For comparison, Table 1 also contains the IC_{50} values for H7 in four of the in vitro enzyme assays. These data show that the indolocarbazole derivatives are up to 1000-times more potent and clearly more selective for PKC than the prototype inhibitor H7.

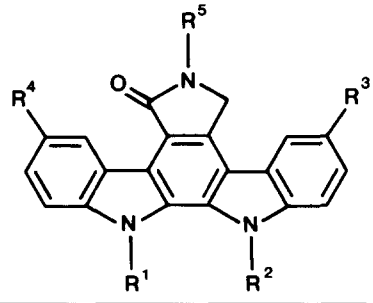
Inhibition of PMA-induced HIV-1 reactivation

Activation of PKC in U1 cells with PMA led to the reactivation of the integrated latent HIV-1 genome. Virus expression resulted in a strong expression of virus-specific proteins as well as in the release of infectious virus particles into the culture supernatant. The inhibitory capacity of the novel indolocarbazole PKC inhibitors was tested in a concentration range from 3 nM

TABLE 1

Chemical structure of indolocarbazole PKC inhibitors and inhibition of protein kinases in vitro

Inhibition of protein kinases was determined in five different in vitro enzyme assays: PKC (C), cAMP dependent protein kinase (A), cGMP dependent protein kinase (G), myosin light-chain kinase (M) and tyrosin kinase activity (T). IC_{50} value is defined as the inhibitor concentration which reduces the enzyme activity to 50% of the control value. Data are mean values of two or three independent experiments.



Inhibitor	$R^1(R^2)$	$R^2(R^1)$	R^3,R^4	R^5	Inhibition of protein kinases IC_{50} (μM) C	A	G	M	T
Gö 7775	H	$-(CH_2)_2CN$	-OH	H	0.044	0.48	0.043	0.43	>10
Gö 7716	$-CH_3$	$-CH_2CHF_2$	H	H	0.032	2.4	0.71	0.44	>10
Gö 6976	$-CH_3$	$-(CH_2)_2CN$	H	H	0.020	>100	6.2	5.8	>10
Gö 7922	$-CH_3$	$-(CH_2)_2CN$	H	$-CH_3$	>30	>30	>30	>10	>10
H7	–	–	–	–	20	36	7.0	420	ND[a]

[a]ND, not determined.

to 10 μM. The results from one representative experiment with the two novel active indolocarbazole compounds (Gö 7775 and Gö 7716), Gö 6976, H7 and an inactive analogue (Gö 7922) are shown in Fig. 1. The active compounds exhibited concentration-dependent inhibition of HIV-1 p24 release and, in parallel, of production of infectious virus particles. More than 80% inhibition at non-toxic concentrations was achieved at 1 μM for Gö 7775 (90% for p24 and 84% for infectious particles, respectively) and at 0.1 μM for both Gö 7716 (98% and 99%, respectively) and for Gö 6976 (86% and 79%, respectively). In contrast, equally effective inhibition of HIV-1 expression with the control inhibitor H7 was only seen at a concentration of 5 μM (87% for p24 and 93% for infectious particles). The inactive analogue Gö 7922 showed no inhibition of either p24 production or number of syncytium forming units at non-toxic concentrations. Table 2 summarizes mean IC_{50} values for the production of p24 and infectious virus particles from two to three independent experiments. For the two most active compounds Gö 7716 and Gö 6976 IC_{50} values for inhibition of p24 release are 0.08 μM and 0.04 μM, respectively, and 0.04 μM for both compounds for the inhibition of release of infectious particles.

Inhibition of TNF-α-induced HIV-1 reactivation

Gö 7716 was additionally tested for inhibition of HIV-1 expression induced

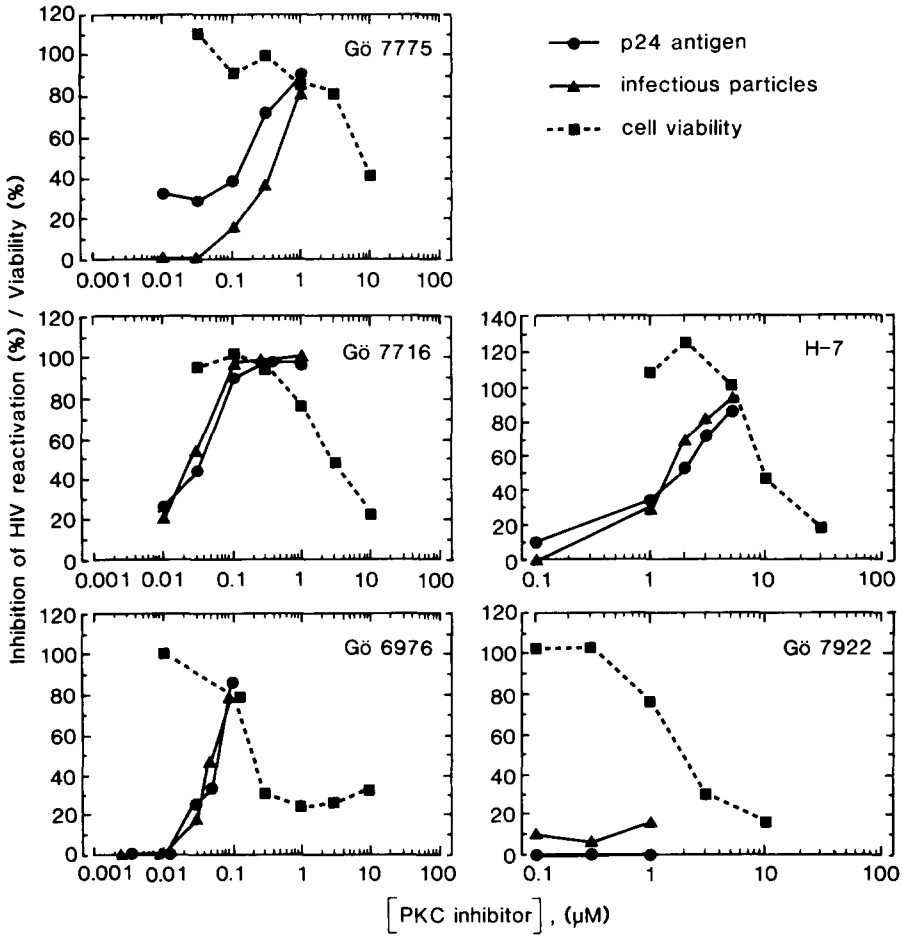


Fig. 1. Concentration response relationship for HIV-1 inhibition and cytotoxicity of PKC inhibitors in PMA-activated U1 cells. Inhibition of HIV-1 was measured by p24 ELISA (●) and syncytium assay (▲). Cytotoxicity was measured by MTT assay (■). U1 cells were seeded into 96-well plates at 5×10^4 per well. Culture supernatants were harvested and cells assayed for viability 48 h after activation of PKC. Inhibitory effects refer to values obtained for control cultures without drugs. Control values (= 100%) for p24 antigen and infectious virus particles in the supernatant and for cell viability were 66 ng/ml, 724 syncytium forming units per ml and 0.295 OD, respectively. Results shown are averages of duplicates for p24 and syncytium assays and triplicates for MTT assay of one representative experiment. Comparable results were obtained in three independent experiments.

in U1 cells by the physiologic mediator TNF-α. To determine virus inhibition in this system, again release of both p24 and infectious virus particles into the culture supernatant was measured. As shown for one representative experiment in Fig. 2 effective inhibition of more than 80% for p24 release was achieved with drug concentrations lower than 0.3 μM. Mean IC_{50} values of 0.03 μM for release of p24 and of 0.02 μM for production of infectious virus were determined from two independent experiments (see also Table 2). Gö 6976 and

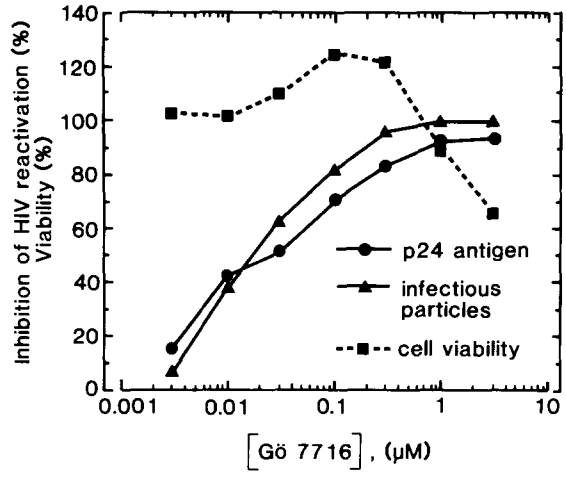


Fig. 2. Concentration response relationship for HIV-1 inhibition and cytotoxicity of Gö 7716 in TNF-α-activated U1 cells. Inhibition of HIV-1 was measured by p24 ELISA (●) and syncytium assay (▲). Cytotoxicity was measured by MTT assay (■). U1 cells were seeded into 96-well plates at 5×10^4 per well. Culture supernatants were harvested and cells assayed for viability 48 h after stimulation with TNF-α. Inhibitory effects refer to values obtained for control cultures without drug. Control values (= 100%) for p24 antigen and infectious virus particles in the supernatant and for cell viability were 24 ng/ml, 352 syncytium forming units per ml and 0.283 OD, respectively. Results shown are averages of duplicates for p24 and syncytium assays and triplicates for MTT assay. One representative experiment is shown. Two to three independent experiments were performed with comparable results.

H7 were included into this part of the study at standard concentrations of 0.1 μM and 2 μM, respectively. For these two inhibitors mean inhibition values of 79% and 78% for the release of p24 were determined, respectively.

TABLE 2

Antiviral and cytotoxic activities of PKC inhibitors

U1 cells were pretreated with PKC inhibitors for 60 min prior to stimulation with either PMA (10 nM) or TNFalpha (100 U/ml). CC_{50}, 50% viable cells; IC_{50}, 50% inhibition; SI, selectivity index (CC_{50}/IC_{50}). Data are mean values of two to three independent experiments.

Inhibitor	Cytotoxicity	p24 Antigen		Infectious particles	
	CC_{50} (μM)	IC_{50} (μM)	SI	IC_{50} (μM)	SI
Stimulation with PMA					
Gö 7775	5.2	0.23	23	0.35	15
Gö 7716	3.4	0.08	42	0.04	85
Gö 6976	0.18	0.04	4	0.04	5
Gö 7922	1.9	>>1	–	>>1	–
H7	8.4	2	4	1.6	5
Stimulation with TNF-α					
Gö 7716	2.7	0.03	93	0.02	159

Cytotoxicity of PKC inhibitors

The effect of the novel indolocarbazole PKC inhibitors on the viability of U1 cells was determined by the MTT assay in comparison with Gö 6976. The results for the individual inhibitors are shown in Fig. 1. Data are expressed as percent viable cells compared to the drug-free stimulated control. Considerable differences in cytotoxicity were observed among these compounds. Gö 6976 showed a rather steep concentration-dependent decline of cellular viability. At a concentration of 0.1 μM 79% of cells were metabolically active whereas at 0.3 μM viability decreased to 31%. A CC_{50} value of 0.2 μM was obtained from these results (Table 2). Gö 7775, Gö 7716 and H7 were considerably less cytotoxic. Toxicity of Gö 7775 showed a plateau between 0.03 μM and 3 μM concentration with more than 80% viable cells. CC_{50} values of 5.2 μM and 3.4 μM for Gö 7775 and Gö 7716, respectively, and of 8.4 μM for H7 were obtained (Table 2). No difference in cytotoxicity of the compounds was observed between PMA- or TNF-α-stimulated and unstimulated cells (data not shown).

Discussion

HIV-1 infection is characterized by a long phase of clinical latency with no detectable virus in the periphery and active HIV disease in the lymphoid organs (Embretson et al., 1993; Pantaleo et al., 1993). The development of chemotherapeutic agents with the potential to preserve this state of infection would be of great value. Transcriptional activation of HIV-1 from latently infected U1 cells has previously been suggested to be mediated by PKC (Kinter et al., 1990; Qatsha et al., 1993). We have evaluated new PKC inhibitors in this cellular system, which are of considerable potency and selectivity in protein kinase assays. These compounds suppressed the production of infectious virus very efficiently in a concentration-dependent manner as evidenced in a syncytium formation assay. Effective inhibition was achieved in the nanomolar range, concentrations which are well below those generally observed for enzyme inhibitors. Very similar dose response curves were obtained with Gö 7716 and Gö 6976 both in the PKC in vitro enzyme assay and in the cellular virus inhibition assay. This suggests that inhibition of PKC activation prevents virus reactivation in latently infected cells. However, it cannot be excluded that the inhibition of protein kinases other than PKC also contributed to this effect. Further experiments will have to confirm these findings in primary infected cells.

In the present study we also attempted to investigate the mechanism of antiviral activity of PKC inhibitors by measuring the suppression of p24 antigen production from U1 cells. As shown in Fig. 1, inhibition of p24 release was strongly correlated with the inhibition of production of infectious virus as evidenced by syncytium formation with almost identical IC_{50} values (Table 2). Therefore, we conclude that the observed antiviral activity cannot be attributed to the production of defective virus particles but rather to a block at an earlier

stage of virus production. These data are consistent with the observations of Qatsha et al. (1993) who reported on the inhibition of proviral transcription and protein synthesis after treatment of cells with Gö 6976. Furthermore, inhibition of HIV-1 expression was not caused by reduction of cell proliferation because cell numbers in stimulated cultures differed from unstimulated cultures by less than 15% (data not shown).

In the in vivo pathogenesis of HIV-1 infection, the cytokine TNF-α very likely plays an important role (reviewed in Matsuyama et al., 1991). Increased serum levels in AIDS patients (Lahdevirta et al. 1988; Reddy et al., 1988) as well as increased spontaneous secretion of this cytokine by monocytes from HIV-1 infected individuals have been described (Roux-Lombard et al., 1989). Stimulation of cells with TNF-α has been reported to lead to an increased binding of NF-κB to the HIV-LTR and to the induction of HIV-1 replication (Duh et al., 1989; Osborn et al., 1989). The involvement of PKC in TNF-α-mediated signal transduction is still a matter of some debate. However, this seems to be cell type specific and evidence exists that, in monocytic cells, TNF-α signalling is indeed mediated through PKC (Schütze et al., 1990). Using the inhibitor Gö 7716 we clearly showed that TNF-α-mediated HIV-1 activation can be inhibited to an even greater extent compared to PMA-mediated activation. Thus, our data support and extend previous findings (Schütze et al., 1990) that TNF-α-mediated signalling in monocytic cells may involve PKC.

The most significant differences between the new compounds tested and our reference substance Gö 6976 were observed with regard to their toxicities. In our assay system Gö 7716 was tolerated by the cells in concentrations up to 30-times higher compared to Gö 6976. Also toxicity was not correlated to the selectivity of the compounds for an individual protein kinase. Moreover, it appears not to be related to the enzyme inhibition, since Gö 7922, a compound with no inhibitory activity against any protein kinase tested, proved to be equally toxic compared to the effective compounds.

Due to its considerably lower cellular toxicity the highest selectivity indices of the compounds tested were found for Gö 7716. Of note, these indices are a factor two higher in TNF-α stimulated cells compared to PMA-stimulated cells and are in the same range as for the dideoxynucleoside DDI, a registered antiviral drug used in AIDS-patients (De Clercq, 1991). Thus, indolocarbazoles may provide a promising chemical structure for the development of drugs against HIV-1 reactivation. Such therapeutic agents would offer the opportunity to treat early asymptomatic infections in which the reverse transcriptase inhibitor azidothymidine was shown to be ineffective (Aboulker and Swart, 1993).

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