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INHIBITORY ACTIVITY AND SELECTIVITY OF STAUROSPORINE DERIVATIVES TOWARDS PROTEIN KINASE C

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Abstract: The synthesis and *in vitro* protein kinase C (PKC) inhibition of a series of staurosporine derivatives is described. Essential for activity is a free NH of the lactam portion of the molecule. A large variety of substituents is tolerated at the secondary amine, although in most cases these modifications lead to a decrease in activity. Acylation of the methylamino group leads generally to the most selective derivatives with respect to other serine/threonine and tyrosine kinases.

The multifunctional serine/threonine-specific protein kinase C (PKC), the major receptor for a number of tumor promoting agents utilizes diacylglycerol (DAG) and/or other lipids in combination with calcium to modulate various cellular functions[1-9]. Recent molecular cloning revealed that PKC consists of a family of related but distinct enzymes[1-5]. The PKC subtypes can be classified into 3 groups comprising the conventional calcium-dependent PKC subtypes (cPKCs: α-, βI-, βII- and γ-PKC), the non-conventional calcium-independent PKC isoforms (nPKCs: δ-, ε-, η- and θ-PKC) and the atypical calcium-independent and DAG or phorbol ester unresponsive PKC subtypes (aPKCs: ζ- and λ-PKC). The finding that tumor promoting phorbol esters such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) were able to replace the endogenous activator DAG in the stimulation of PKC has provided insights into the role of this enzyme family in the regulation of a variety of cellular processes such as exocytosis, gene expression, proliferation, differentiation and tumor promotion[1-9]. The various PKC subtypes show distinct enzymological properties, differential tissue expression with specific subcellular localization and different modes of cellular regulation[1-9]. These features combined with the finding that more than one subtype of PKC is usually expressed in a single cell type have led to the impression that each member of the PKC family plays discrete roles in the processing of various physiological and pathological responses to extracellular stimuli.

The scenarios of multistep carcinogenesis suggest close cooperativity between oncoproteins and PKC in regulating cellular growth and neoplastic transformation. Various oncogenes increase DAG levels and activation of PKC by TPA or overexpression of a specific PKC isoform enhances the transforming potenrial of various oncogenes[1-9]. These findings, indicate that deregulated PKC activity contributes to the uncontrolled proliferation of tumor cells making PKC an attractive target for the development of antitumor agents.

Staurosporine (1), an alkaloid first isolated by Omura *et al.*[10] and found almost a decade later to be a very potent inhibitor of PKC by Tamaoki *et al.*[11] was the lead structure for the present study. However, staurosporine is completely unselective with respect to inhibition of other protein kinases including tyrosine-specific protein kinases[11] (see also table I). In the last decade a large number of alkaloids have been isolated and reported to contain an identical or closely related indolecarbazole moiety as staurosporine or to differ in the "glycosidic" part of the molecule. These include K-252a-d[12-14], UCN-01[15,16], and its methyl ether[17], TAN-999 and TAN-1030A[18], RK-286C[19,20], 7-oxo-staurosporine RK-1409[21,22] and RK-1409B[23]. In order to

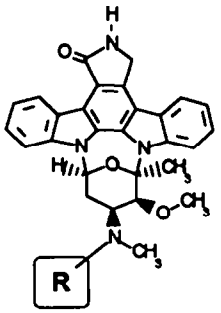
improve the selectivity of this very potent and interesting class of compounds we have synthesized derivatives of staurosporine and tested them on a a variety of of serine/threonine- or tyrosine-specific protein kinases.

Chemistry: The staurosporine derivatives shown in tables I and II have been synthesized by standard alkylation and acylation reactions starting from staurosporine, and most of them were purified by chromatography on silica gel. Accordingly, alkyl derivatives **2-5** and **7** were obtained by reaction of staurosporine with the corresponding alkyl iodides and bromides in N,N-dimethylformamide (DMF) in the presence of N,N-diisopropylethylamine. N-Phenyl-staurosporine (**6**) was prepared by reaction of **1** with a 1 N phenyl-diazonium chloride solution in dioxane for one hour, and the acetic acid derivative **8** was obtained by hydrolysis of ester **7** with sodium hydroxide in methanol. Reaction of staurosporine with neat acrylonitrile at 140°C (sealed tube) for 44 h gave the 2-cyanoethyl derivative **9**, and reaction of **1** with 1,2-epoxyhexane at 110°C in ethanol (sealed tube) for 24 h yielded the 2-hydroxy-1-hexyl derivative **10**. Most of the acylation reactions at the secondary amine of staurosporine were performed with the corresponding acid chlorides in chloroform in the presence of one equivalent of N,N-diisopropylethylamine (**12**, **14**, **20-30**, table I). Acetic anhydride, trifluoroacetic anhydride, succinic anhydride, and di-*tert*-butyl dicarbonate were used to prepare **11**, **15**, **18**, and **19**, respectively. The thiourea **13** was obtained by reaction of **1** with methyl isothiocyanate, and **16** was obtained by coupling **1** with the BOC-glycine using DCC and HOBT. Cleavage of the BOC group in **16** with HCl/ethyl acetate gave glycyl derivative **17**, and the carboxylic acid **31** was generated by hydrolysis of ester **30** with sodium hydroxide in methanol. Alkylation of the lactam nitrogen of N-benzoyl-staurosporine (**20**) with sodium hydride and benzyl bromide or methyl bromoacetate gave **32** and **33**, respectively (table II). The synthesis of the oxygenated products shown in table III was achieved by oxidation of the 7-position in N-BOC-staurosporine (**19**) or N-benzoyl-staurosporine (**20**) with chromium-trioxide pyridine complex[24] in dichloromethane to give **34** and **40**, respectively. Deprotection of **34** using trifluoroacetic acid in dichloromethane gave 7-oxo-staurosporine (**35**) which was then reduced with sodium borohydride in dichloromethane/isopropanol to give a mixture of all four possible isomeric hydroxy compounds **36-39**. These could be separated by preparative HPLC on a Lichrosorb Si-60 column (16x250 mm) using a mixture of dichloromethane/isopropanol saturated with water as eluent. By comparison of the ^{1}H-NMR data and the optical rotations of **36** and **37** with those reported by Takahashi et al.[16] it was concluded that **36** corresponds to UCN-01 and **37** to UCN-02, respectively. All the compounds shown in tables I to III were characterized by their ^{1}H-NMR, FAB-MS and IR spectra.

Results and Discussion: As already mentioned, staurosporine is an unselective protein kinase inhibitor with the exception of the protein tyrosine kinase activity of the EGF receptor (EGF-R) which is somewhat less sensitive. The same is true for all the alkyl derivatives listed in table I (entries **1** to **10**). It is also evident from the data that the alkylation of the secondary amine is accompanied with a loss of inhibitory activity towards PKC and with only a marginally improved selectivity. The only exception is the quaternary ammonium salt **4** which is about equipotent to **1** and shows a moderate selectivity with respect to cAMP-dependent protein kinase (PKA).

Table I (entries **11** to **31**) summarizes our results obtained with acyl substituents at the secondary amine. Again a drop in PKC activity can be observed but in some examples the concomitant loss of activity on the other kinases is much more pronounced, which results in an overall increase in selectivity. One such compound is N-benzoyl-staurosporine (**20**) which shows good selectivity for PKC as compared to all kinases tested except for phosphorylase kinase (PPK), an observation made with all the staurosporine derivatives presented in this study. PPK seems to have very similar structural requirements as PKC for this class of compounds. In order to further improve the potency and/or selectivity of **20**, derivatives **21-31** were synthesized. No additional improvement could be achieved, except for the dinitro derivative **28** which is completely inactive on PKA and the EGF-R but at the same time is 20 times less potent on PKC than staurosporine.

Table I: Inhibition of protein kinases by alkyl and acyl derivatives of staurosporine (1), IC_{50} [μM]:



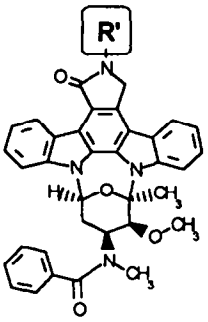
Comp.	R	PKC	PKA	PPK	S6-K	PTK	c-src
1	H (Staurosporine)	0.006	0.015	0.003	0.005	0.1	0.35
2	Et	0.10	1.6	0.07		0.33	0.24
3	Me, Me	0.032	0.068	0.021	0.05	0.019	
4	Et, Me	0.014	0.27	0.018		0.043	
5	Benzyl	0.70	0.48	0.10		0.91	
6	Phenyl	0.63	0.055	0.015			
7	Carbomethoxymethyl	0.26	3.2	0.44		1.3	
8	Carboxymethyl	0.046	0.14	0.015		0.031	
9	2-Cyanoethyl	0.19	1.4	0.024		1.1	
10	2-Hydroxy-1-hexyl	0.14	0.36	0.05		1.3	
11	Acetyl	0.075	1.5	0.01		2.6	
12	Methoxycarbonyl	0.18	0.48	0.04		2.4	
13	N-Methyl-thiocarbamoyl	0.041	1.6	0.01	0.9	12.5	
14	Methylsulfonyl	0.081	1.0	0.019		1.1	
15	Trifluoracetyl	0.13	17.0	0.01	0.1	6.4	
16	BOC-glycyl	0.064	2.25	0.12	0.2	3.5	0.16
17	Glycyl	0.014	0.36	0.051		0.22	
18	Succinoyl	0.04	0.7	0.062		0.16	0.2
19	BOC	0.35	28	0.42	1.0	13.5	
20	Benzoyl	0.050	2.4	0.048	5	1.9	0.8
21	Cyclohexanoyl	0.56	1.57	0.08			
22	Nicotinoyl	0.031	0.38	0.012		8.18	
23	2-Pyrazinoyl	0.036	0.75	0.020		8.18	
24	2-Chlorobenzoyl	0.23	1.5	0.078		4.7	
25	3-Chlorobenzoyl	0.57	6.8	1.9		4.6	
26	4-Chlorobenzoyl	0.35	2.6	0.32		7.0	
27	3-Nitrobenzoyl	0.063	2.5	0.06	0.1	4.8	
28	3,5-Dinitrobenzoyl	0.125	> 100	0.37	0.2	>70	
29	4-Methoxybenzoyl	0.27	1.5	0.13		3.6	
30	Methyl 4-terephthaloyl	0.315	3.67	0.053		7.4	
31	4-Terephthaloyl	0.062	10.0	0.03	0.1	0.11	

In order to explore the influence of a substituent on the lactam nitrogen of **20**, the two derivatives **32** and **33** shown in table II were synthesized. Surprisingly these two compounds were completely devoid of any inhibitory activity towards all the protein kinases. This suggests that the lactam portion of staurosporine could be involved in a hydrogen bond which is essential for binding and/or that there is no space to accommodate a substituent in this position.

7-Hydroxy-staurosporine (**36**) (UCN-01)[15] and N-benzoyl-staurosporine (**20**) (CGP41251)[25] have been reported to be remarkably selective PKC inhibitors. Unexpectedly, the combination of the two structural modifications which by themselves lead to an increase in selectivity, did not give rise to more selective compounds (table III). On the contrary **40** and also **41** were both less

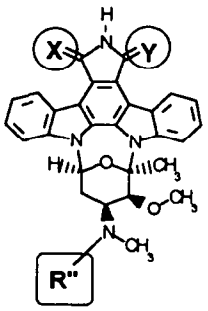
selective than UCN-01 or CGP41251. 7-Oxo-staurosporine (**35**) behaved similar to staurosporine in the protein kinase assays. On the other hand the four isomeric hydroxy-staurosporines **36-39** gave confusing results: they were about equipotent on PKA but varied quite strongly in their PKC inhibitory activity. Therefore **36** and **38** were selective for PKC with respect to PKA while **37** showed high selectivity for phosphorylase kinase.

Table II: Inhibition of protein kinases by 6-substituted N-benzoyl-staurosporine (**20**), IC_{50} [μM]:



Comp.	R	PKC	PKA	PPK	S6-K	PTK
32	Benzyl	> 100	> 100	~ 100	> 100	> 100
33	CH_2COOCH_3	> 100	> 100	~ 100	> 100	> 100

Table III: Inhibition of protein kinases by oxo- and hydroxy-derivatives of staurosporine (**1**), IC_{50} [μM]:



Comp.	R"	X	Y	PKC	PKA	PPK	EGF-R	c-src
34	BOC	O	O	1.5	>100	5.25		
35	H	O	O	0.009	0.026	0.005	0.2	0.8
36[1)]	H	O	H,OH	0.013	1.15	0.004	0.017	0.6
37[2)]	H	O	OH,H	0.255	1.85	0.006		
38[3)]	H	H,OH	O	0.096	2.25	0.022	0.26	1.8
39[4)]	H	OH, H	O	0.235	3.3	0.17		
40	Benzoyl	O	O	0.095	0.75	0.27		
41	Benzoyl	O	H,OH	0.030	0.3	0.035		

1) $[\alpha]_D$ = + 140° (c = 0.1, MeOH)
2) $[\alpha]_D$ = - 20° (c = 0.1, MeOH)
3) $[\alpha]_D$ = + 55° (c = 0.1, MeOH)
4) $[\alpha]_D$ = - 75° (c = 0.1, MeOH)

Kinetic Analysis: The exact mode of action of staurosporine is not completely understood[11,26-28]. We therefore decided to study one of our most selective staurosporine derivatives in more detail. As illustrated in figure 1, BOC-glycyl-staurosporine (16) at concentrations of up to 300 µM did not inhibit the binding of [^{3}H]-phorbol dibutyrate, but it was an apparent competitive inhibitor with respect to the phosphate donor ATP (Ki = 45 nM, figure 2). This clearly shows that 16 interacts with the catalytic domain of PKC and it is in line with the finding that 16 inhibited the phosphorylation of protamine sulfate by PKC, an exogenous substrate which is phosphorylated in the absence of lipid activators (data not shown).

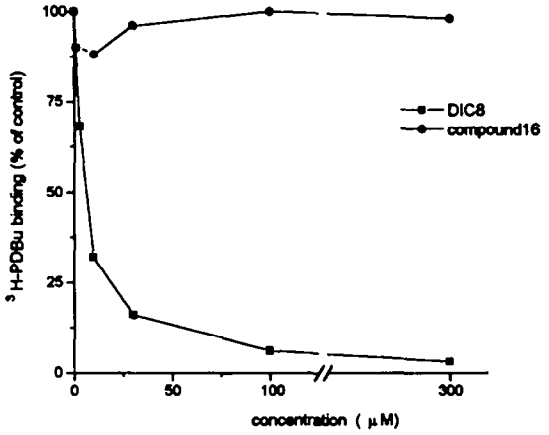


Figure 1: Effect of BOC-glycyl-staurosporine (16) on ^{3}[H]-PDBu binding to purified PKC

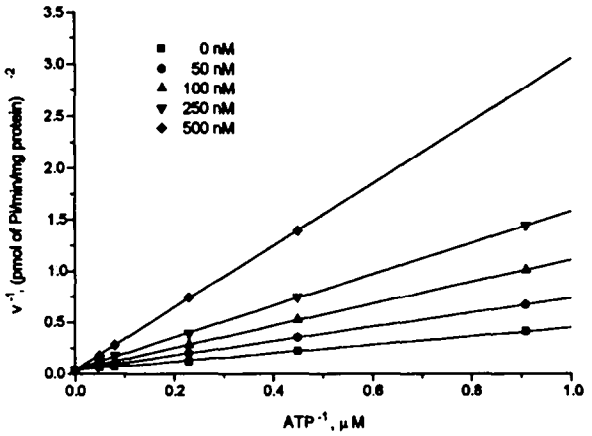


Figure 2: Inhibition of PKC activity by BOC-glycyl-staurosporine (16)

In summary, it is possible to obtain a remarkable degree of selectivity for inhibition of PKC by staurosporine derivatives although in most cases the increase in selectivity is paralleled by a more or less pronounced decrease in potency. While a wide range of substituents at the secondary amine and oxygenation at the 7-position are tolerated, the attachment of a substituent at the lactam nitrogen atom abolishes completely the protein kinase inhibitory activity. Selective inhibitors of PKC like the staurosporine derivatives presented in this study, may contribute to better understand the role(s) of PKC in signal transduction and may eventually lead to the development of therapeutically useful compounds.

Methods and Abbreviations: The enzyme assays for protein kinase C (PKC), cAMP-dependent protein kinase (PKA), phosphorylase kinase (PPK), S6 kinase (S6-K), and the protein tyrosine kinase of the epidermal growth factor receptor (PTK) were performed as described by Meyer et al.[25] and c-src was assayed as described by Geissler et al.[29].

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References:

1) Asaoka, Y., Nakamura, S., Yoshida, K., Nishizuka, Y. (1992) *TIBS* **17**, 414-418
2) Nishizuka, Y. (1988) *Nature* **334**, 661-665
3) Bell, R.M., Burns, D.J., (1991) *J. Biol Chem* **256**, 4661-4664
4) Huang, K.-P. (1989) *Trends in Neur. Sci.* **12**, 425-431.
5) Parker, P.J. (1992) In " Protein kinase C: current concepts and future perspectives" (Lester, D. and Epand, R., eds) pp.3-25. Ellis Horwood Series in Biochemistry and Biotechnology, Chichester, West Sussex, England.
6) Houslay, M.D. (1991) *Eur. J. Biochem.* **195**, 9-27
7) Nishizuka, Y. (1986) *Science* **233**, 305-312.
8) Jaken, S. (1992) In " Protein kinase C: current concepts and future perspectives" (Lester, D. and Epand, R., eds) pp.237-255. Ellis Horwood Series in Biochemistry and Biotechnology, Chichester, West Sussex, England.
9) Borner, C., Fabbro, D. (1992)In " Protein kinase C: current concepts and future perspectives" (Lester, D. and Epand, R., eds) pp.29 354. Ellis Horwood Series in Biochemistry and Biotechnology, Chichester, West Sussex, England
10) Omura, S.; Iwai, Y.; Hirano, A.; Nakagawa, A.; Awaya, J.; Tsuchiya, H ; Takahashi, Y ; Masuma R., *J. Antibiot.* **1977**, *30*, 275-281.
11) Tamaoki, T.; Nomoto, H.; Takahashi, I.; Kato, Y.; Morimoto, M.; Tomita, F.; *Biochem. Biophys Res. Commun.* **1986**, *135*, 397-402.
12) Kase, H.; Iwahashi, K.; Matsuda, Y.; *J. Antibiot.* **1986**, *39*, 1059-1065.
13) Nakanishi, S.; Matsuda, Y.; Iwahashi, K.; Kase, H ; *J. Antibiot.* **1986**, *39*, 1066-1071.
14) Yasuzawa, T.; Iida, T.; Yoshida, M.; Hirayama, N.; Takahashi, M.; Shirahata, K.; Sano, H.; *J. Antibiot.* **1986**, *39*, 1072-1078
15) Takahashi, I.; Kobayashi, E.; Asano, K.; Yoshida, M ; Nakano, H.; *J. Antibiot.* **1987**, *40*, 1782-1783.
16) Takahashi, I.; Saitoh, Y.; Yoshida, M.; Sano, H.; Nakano, H.; Morimoto, M.; Tamaoki, T.; *J. Antibiot.* **1989**, *42*, 571-576.
17) Takahashi, I.; Kobayashi, E ; Nakano, H.; Murakata, C.; Saitoh, H ; Suzuki, K.; Tamaoki, T ; *J. Pharmacol. Exp. Ther.* **1990**, *255*, 1218-1221.
18) Tanida, S.; Takizawa, M.; Takahashi, T.; Tsubotani, S.; Harada, S.; *J. Antibiot* **1989**, *42*, 1619-1630.
19) Osada, H.; Takahashi, H.; Tsunoda, K.; Kusakabe, H.; Isono, K.; *J. Antibiot.* **1990**, *43*, 163-167.
20) Takahashi, H.; Osada, H.; Uramoto, M.; Isono, K.; *J. Antibiot.* **1990**, *43*, 168-173.
21) Osada, H., Koshino, H.; Kudo, T.; Onose, R.; Isono, K.; *J. Antibiot.* **1992**, *45*, 189-194.
22) Koshino, H.; Osada, H.; Isono, K.; *J. Antibiot.* **1992**, *45*, 195-198
23) Koshino, H.; Osada, H.; Amano, S ; Onose, R.; Isono, K.; *J. Antibiot.* **1992**, *45*, 1428-1432.
24) Fieser & Fieser, *Reagents for Organic Synthesis*, Vol. **1**, Wiley,1967, 145.
25) Meyer, T.; Regenass, U., Fabbro, D., Alteri, E.; Rösel, J.; Müller, M.; Caravatti, G.; Matter, A.; *Int. J. Cancer*, **1989**, *43*, 851-856.
26) Nakadate, T.; Jeng, A.Y.; Blumberg, P.M.; *Biochem. Pharmacol.* **1988**, *37*, 1541-1545.
27) Rüegg, U.T ; Burgess, G.M.; *TiPS* **1989**, *10*, 218-220.
28) Ward, N.E.; O'Brian C.A.; *Mol. Pharmacol.* **1992**, *41*, 387-392.
29) Geissler, J.F.; Traxler, P.; Regenass, U.; Murray, B.J.; Rösel, J.; Meyer, T.; McGlynn, E.; Storni, A.; Lydon, N.B.; *J. Biol Chem* **1990**, *36*, 22255-22261

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