UCN-01, an anti-tumor drug, is a selective inhibitor of the conventional PKC subfamily

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Abstract **A selective PKC inhibitor, UCN-01, was shown to exhibit anti-tumor activity in vitro and in vivo. We investigated UCN-01 with respect to isozyme-specific PKC inhibition using purified recombinant or rabbit brain PKC isozymes, cPKCα, β and γ, nPKCδ, ε and η, and aPKCζ. Of the PKC isozymes examined, cPKCα was inhibited by UCN-01 most effectively (K_i = 0.44 nM), suggesting cPKCα is the prime candidate for the physiological target of UCN-01. The K_i values of UCN-01 estimated from Dixon plots for cPKC isozymes are approximately 1 nM, whereas the K_i values for nPKC isozymes are about 20 nM. Moreover, the K_i value for aPKCζ is 3.8 μM. Thus, UCN-01 discriminates between PKC subfamilies. In addition, the inhibitory effects of staurosporine, H7, and calphostin C on aPKCζ were examined and compared with those for cPKCα.**

Key words: Protein kinase C; UCN-01; PKC inhibitor

1. Introduction

Staurosporine and 7-hydroxy-staurosporine, UCN-01, were isolated from actinomyces and have been reported as potent protein kinase inhibitors [1,2]. Both inhibitors show variable cellular effects, inhibition of proliferation of several tumor cell lines, and arrest of the cell cycle [3–6]. UCN-01, however, exhibits in vivo anti-tumor effects against human tumor xenografts and murine tumors, while staurosporine has little activity against any tumor models [3]. UCN-01 shows selectivity for protein kinase C (PKC) inhibition in vitro (IC_{50}: 4.1 nM for PKC, 42 nM for protein kinase A, 45 nM for $p60^{v\text{-}src}$ protein tyrosine kinase), differing from staurosporine which is a non-selective protein kinase inhibitor and inhibits PKC with an IC_{50} of 2.7 nM [1]. Therefore, the anti-tumor activity of UCN-01 may be due to its selectivity for PKC.

At present, the mammalian PKC family consists of 11 different polypeptides divided into three subfamilies, conventional PKC (cPKC), novel PKC (nPKC), and atypical PKC (aPKC), based on their biochemical properties and structure [7,8]. We have previously demonstrated that purified nPKCδ and ε as well as cPKC are inhibited by staurosporine and UCN-01 (IC_{50} of staurosporine and UCN-01: 0.78 and 1.1 nM for cPKC, 2.0 and 8.0 nM for nPKCδ, 0.61 and 5.5 nM for nPKCε, respectively) [9]. However, the sensitivity of UCN-01 for other PKC isozymes remains to be shown.

For this report we determined the K_i values of UCN-01 for purified PKC isozymes, cPKCα, β and γ, nPKCδ, ε and η, and aPKCζ from Dixon Plots. In addition, we examined the sensitivity of aPKCζ to the so-called PKC inhibitors, staurosporine, H7 [10], and calphostin C [1], because there are few studies on the sensitivity of aPKCζ to protein kinase inhibitors.

2. Materials and methods

2.1. Preparation of PKC isozymes

cPKCα, nPKCδ, ε and η, and aPKCζ were expressed in recombinant baculovirus-infected insect cells and purified by sequential chromatography according to reported methods [11,12]. The purification of rabbit brain cPKCβ and γ has been described previously [13]. Each purified PKC samples exhibits a specific activity as follows: cPKCα, 2,500; cPKCβ, 1,600; cPKCγ, 1,100; nPKCδ, 1,200; nPKCε, 210; nPKCη, 100; aPKCζ, 240 U/mg protein (1 U = 1 nmol phosphate incorporation per minute).

2.2. PKC assay

The PKC assay was performed as previously described [14]. Four synthetic oligopeptides used as substrates were MBP_{4-14} [14], pepMARCKS [11], ε-peptide [14], and α-peptide (a cPKCα-pseudosubstrate-derived substrate peptide, STASQDVANRFARKGSLRQKNV). Each PKC isozyme in a 50 μl assay mixture (20 mM Tris-HCl, pH 7.5, 5 mM $Mg(OAc)_2$, 0.1 mM $CaCl_2$, 25 μg/ml phosphatidylserine (Avanti Polar Lipid Inc.), 10 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate (Sigma), 20 μM ATP, 0.5 μCi [γ-^{32}P]ATP (DuPont-New England Nuclear), 0.01% leupeptin and 10–50 μg/ml substrate) was incubated for 10 min at 30°C in the presence or absence of various concentrations of PKC inhibitors. UCN-01, staurosporine, and calphostin C were isolated from actinomyces or fungi as previously described [1,9]. H7 was obtained from Seikagaku Kogyo Co.

3. Results

In order to determine the physiological target of UCN-01, a compound that shows anti-tumor activity, we investigated UCN-01 with respect to isozyme-specific PKC inhibition. The K_i values estimated from Dixon plots for purified PKC isozymes are summarized in Table 1. The synthetic oligopeptide MBP_{4-14} was used as a substrate because it is a good substrate for cPKC and nPKC isozymes. However, MBP_{4-14} is a poor substrate for aPKCζ (data not shown). Thus, α-peptide was used as a substrate for aPKCζ. The K_i values for the cPKC isozymes were in the nanomolar range, consistent with the K_i value of staurosporine for cPKCα (K_i = 0.77 nM) [9]. On the other hand, the K_i values for the nPKC isozymes were about 20 nM, 12- to 56-fold higher than for cPKCs. Furthermore, the

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Abbreviations: PKC, protein kinase C; cPKC, conventional PKC; nPKC, novel PKC; aPKC, atypical PKC.

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K_i value for aPKCζ was estimated as 3.8 μM, 2,000- to 10,000- and 200-fold higher than for cPKCs and nPKCs, respectively. These results indicate that UCN-01 is highly specific for cPKC isozymes and especially for cPKCα, and that UCN-01 discriminates between PKC subfamilies.

Next we examined the sensitivity of aPKCζ to typical PKC inhibitors, staurosporine, H7, and calphostin C, and compared the results with those of cPKCα (Table 2 and Fig. 1). Two synthetic oligopeptides, ε-peptide and pepMARCKS, were used as phosphate acceptors in the PKC assay. aPKCζ turned out to be less sensitive to all three inhibitors than cPKCα. The calculated IC_{50} values of aPKCζ for staurosporine were 220 nM and 450 nM using ε-peptide and pepMARCKS, respectively, 150- and >4,500-fold higher than for cPKCα. On the other hand, our previous results indicated that staurosporine has almost the same sensitivity for cPKC and nPKC isozymes [9]. It has been demonstrated that staurosporine and UCN-01 interact with the catalytic domain of PKC to inhibit its kinase activity [1,2]. H7 also interacts with the catalytic domain of PKC and competes with ATP for binding to the enzyme [10]. The IC_{50} values of H7 for aPKCζ determined using the two substrates were 4- and 15-fold higher than those for cPKCα. Calphostin C interacts with the regulatory domain of PKC which shows significant differences among PKC subfamilies [1]. Calphostin C inhibits nPKCδ and ε with almost the same IC_{50} as that for cPKC (data not shown), while it inhibits aPKCζ with IC_{50} values of 1 or 6.5 μM, showing 5- or 30-fold higher sensitivity for cPKCα than aPKCζ.

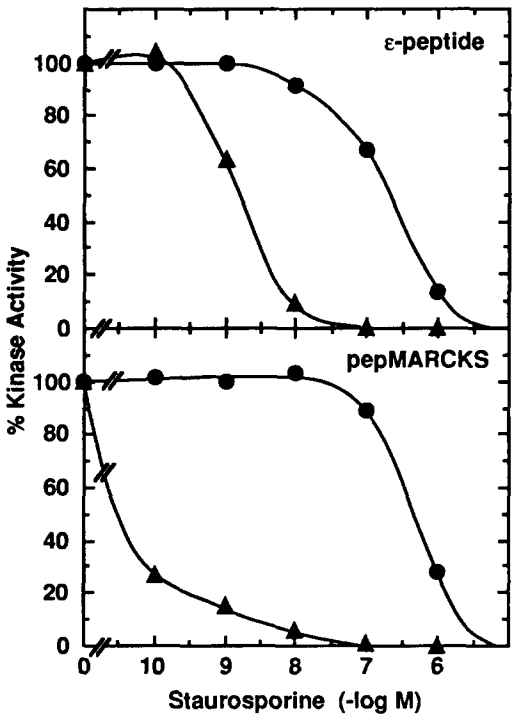

Fig. 1. Inhibition of aPKCζ (●) and cPKCα (▲) by staurosporine. Enzymatic activity was determined using two synthetic oligopeptides. Data are the means of two determinations from two or three separate experiments.

4. Discussion

To determine the K_i values of UCN-01 for PKC isozymes, first we used MBP_{4-14} as a substrate. However, MBP_{4-14} is a poor substrate for aPKCζ. Since the IC_{50} values for aPKCζ examined using four peptide substrates, MBP_{4-14}, pepMARCKS, α-peptide, and ε-peptide, showed almost the same (approximately 1–10 μM), we determined the K_i value for aPKCζ using α-peptide which is a good substrate for aPKCζ.

UCN-01 turns out to be an inhibitor that discriminates PKC subfamilies. The calculated K_i values of UCN-01 for cPKCs, nPKCs, and aPKCζ are approximately 1 nM, 20 nM, and 3.8 μM, respectively (Table 1). Among cPKC isozymes, cPKCα, which is expressed ubiquitously in a variety of cells and tissues, is inhibited by UCN-01 most effectively, suggesting that cPKCα is a potential candidate for the physiological target of UCN-01. The IC_{50} values for growth inhibition of tumor cell lines by UCN-01, however, are 20–300 nM [3,4], and 0.15–1.56 μM UCN-01 is required to arrest the cell cycle of tumor cell lines [4,6]. Therefore, nPKC isozymes as well as cPKC isozymes may play a role in the anti-tumor action of UCN-01 in vivo and in vitro. There is also the possibility that another kinase is the target for the anti-tumor action of UCN-01.

Table 1
K_i values of UCN-01 for PKC isozymes

PKC isozyme		K_i (nM)
cPKC	α	0.44
	β	1.70
	γ	0.94
nPKC	δ	19.5
	ε	24.6
	η	20.8
aPKC	ζ	3800

The values are the means of three or four separate experiments

The inhibition of aPKCζ by staurosporine is less efficient than that of cPKCα, consistent with the previous results using recombinant aPKCζ [15,16]. The IC_{50} value of staurosporine determined using aPKCζ partially purified from murine epidermis was lower (16 nM) [17], although this aPKCζ preparation was crude and its kinase activity significantly weak.

Several staurosporine-related inhibitors, K252a [14], K252b [18], Ro 32–0432 [19], Gö 6976, and Gö 6850 [20], have been reported to discriminate PKC subfamilies in the inhibition of their kinase activity. For these inhibitors, interference in the binding of ATP to the kinase has been shown to be the mechanism of inhibition [19–21]. Nonetheless, inhibition of PKC by staurosporine is not influenced by excess ATP [1] and the molecular basis for inhibition remains unknown. The IC_{50} values of H7, an ATP competitor, and calphostin C, an inhibitor interacting with the regulatory domain of PKC, for cPKCα and aPKCζ change somewhat depending on the substrate used. On the other hand, the difference in the IC_{50} values of staurosporine for cPKCα was drastic depending on which of the two substrates was used (Table 2 and Fig. 1). In addition, staurosporine shows almost the same sensitivity for cPKC and nPKC isozymes when the synthetic peptides MBP_{4-14} [9] and peptide-γ [19] are used as substrates, although there is a 100-fold selectivity for cPKCα over nPKCδ when protamine sulfate is used [15]. These results suggest that staurosporine interacts with the substrate binding domain of PKC. Taken together, the finding that the ATP-binding sites of PKC isozymes are conserved and that

Table 2
Comparison of the inhibition of aPKCζ and cPKCα by staurosporine, H7, and calphostin C

	Staurosporine IC_{50} (nM)		H7 IC_{50} (μM)		Calphostin C IC_{50} (nM)	
	ε-peptide	pepMARCKS	ε-peptide	pepMARCKS	ε-peptide	pepMARCKS
cPKCα	1.5	<0.1	24	20	200	200
aPKCζ	220	450	90	300	1000	6500
α/ζ	1/150	<1/4500	1/4	1/15	1/5	1/33

The values are averages of two or three separate experiments

H7, an ATP competitor, shows a similar sensitivity for PKC isozymes (Table 2) [14], staurosporine and its related compounds including UCN-01 may recognize a subtle differences not only in the ATP-binding domain but also in the substrate-binding site of PKC isozymes. These drugs may be useful tools with which to analyze structural differences in the catalytic domain of PKC isozymes. Further investigations using intact proteins and physiological substrates are needed to determine the sensitivity of PKC isozymes for inhibitors, because the IC_{50} value of staurosporine for cPKCα depends on the substrate used.

Calphostin C has been reported to interact with the C1 domain of PKC [7], because inhibition by calphostin C is not influenced by the concentration of phosphatidylserine or Ca^{2+} and because it inhibits the binding of phorbol esters to PKC [22]. Moreover, calphostin C dose not inhibit the kinase activity of catalytic fragment of PKC [1,22]. Since aPKCζ has a significantly different regulatory domain from cPKC and nPKC isozymes, we expected that calphostin C did not inhibit aPKCζ. However, calphostin C inhibits not only cPKC and nPKC but also aPKCζ with only a 5- to 30-fold higher IC_{50}, suggesting that calphostin C recognizes the regulatory domain of aPKCζ as well as those of cPKCs and nPKCs. cPKC and nPKC isozymes have the C1 domains with repeated cysteine-rich sequences and are dependent on phorbol esters, while aPKCζ contains only one cysteine-rich sequence and its kinase activity is independent of phorbol esters or diacylglycerols [7,8]. Recombinant aPKCζ produced by baculovirus-insect cell systems has been reported to exhibit constitutive kinase activity which is independent of phosphatidylserine and diacylglycerol [15,16], although aPKCζ purified from bovine kidney showed kinase activity dependent on phosphatidylserine [23]. Our recombinant aPKCζ also showed phosphatidylserine dependency and this dependency disappeared by limited fragmentation of aPKCζ with calpain treatment, which produced a free catalytic fragment of aPKCζ (Ueda, Y. et al., manuscript in preparation). In addition, it has been reported that phosphatidylinositol-3,4,5-P_3 and arachidonic acid activate aPKCζ [16,23,24]. The fact that the regulatory domain of aPKCζ contains a psuedosubstrate sequence also suggests the regulation of aPKCζ by binding of various lipid activators to the regulatory domain in a similar way as cPKCs and nPKCs. From these results, inhibition of aPKCζ by calphostin C is most likely due to the interaction of the inhibitor and the regulatory domain of aPKCζ which contain one cysteine rich domain.

In this report, we determined the K_i values of UCN-01 for PKC isozymes and demonstrated that UCN-01 inhibits cPKCα most effectively and that it discriminates PKC subfamilies. Furthermore, we found that aPKCζ shows less sensitivity for staurosporine than cPKCα, while H7 and calphostin C inhibit aPKCζ with a sensitivity similar to that for cPKCs and nPKCs.

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