

Modulation of Apoptosis in Rat Thymocytes by Analogs of Staurosporine: Lack of Direct Association with Inhibition of Protein Kinase C

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ABSTRACT

Protein kinase C (PKC) is an important constituent of the signaling pathways involved in apoptosis. The PKC inhibitor staurosporine induces apoptosis in many cell types. We characterized the role of PKC in the induction of apoptosis in immature rat thymocytes by investigating the effects of staurosporine with those of five analogs. Four of them, the indolocarbazoles CGP 41251 and UCN-01 and the bisindolylmaleimides RO 31-8220 and GF 109203X, possess high PKC-inhibitory specificity and potency, whereas one, the UCN-01 stereoisomer UCN-02, is a weak PKC inhibitor. Apoptosis was examined by flow cytometry, internucleosomal DNA cleavage, and formation of large DNA fragments. Staurosporine, UCN-01, and UCN-02 induced a concentration- and time-dependent increase in apoptosis, whereas neither CGP 41251, RO 31-8220, nor GF 109203X induced apoptosis. The mechanism of induction of

apoptosis by staurosporine, UCN-01, and UCN-02 was clearly different from the mechanism that mediates induction of apoptosis by etoposide and dexamethasone, as judged by differential effects of modulators of apoptosis. Staurosporine, UCN-01, and UCN-02 at concentrations of a hundredth to a thousandth of those at which they induced apoptosis, and RO 31-8220 inhibited apoptosis elicited by thapsigargin but not apoptosis caused by dexamethasone or etoposide. The results suggest that (i) UCN-01 and UCN-02 mimic staurosporine as inducers of thymocyte apoptosis; (ii) staurosporine, UCN-01 and UCN-02 share a biphasic effect on apoptosis in rat thymocytes, being inhibitory at low concentrations and stimulatory at high concentrations; and (iii) inhibition of PKC alone is insufficient for induction of apoptosis in thymocytes.

The serine- and threonine-specific protein kinase family PKC has been identified as a suitable target in the development of novel antineoplastic agents, because it plays a pivotal role in proliferation, differentiation, and cell death (Powis, 1995). Staurosporine, an indolo[2,3-*a*]carbazole (Fig. 1) was discovered in the course of screening extracts of the bacterium *Streptomyces* sp. for constituent alkaloids with PKC-inhibitory activity (Omura *et al.*, 1977). It is a potent, albeit nonselective, inhibitor of PKC and has become the "lead" compound among PKC inhibitors with therapeutic potential. Industrial drug development programs have furnished a variety of staurosporine analogs with higher specificity for PKC compared with that of staurosporine, exemplified by the indolocarbazoles 7-hydroxy-staurosporine (UCN-01) (Takahashi *et al.*, 1989) and *N*-benzoyl staurosporine (CGP 41251) (Meyer *et al.*, 1989) and the bisindolylmaleimides RO 31-8220 (Davis *et al.*, 1992) and GF 109203X (Toul-

lec *et al.*, 1991) (for structures, see Fig. 1). UCN-01 and CGP 41251 arrest the growth of several malignant cell types *in vitro* and possess antitumor activity *in vivo* in human tumors grown in nude mice (Meyer *et al.*, 1989; Akinaga *et al.*, 1991). Both agents are currently under clinical investigation as potential anticancer drugs. The mechanisms by which these agents exert antitumor activity are not entirely clear. Staurosporine and UCN-01, apart from inhibiting PKC, interfere in a complex fashion with the cell cycle machinery *via* the CDK system (Crissman *et al.*, 1991; Kawakami *et al.*, 1996). Both agents induce apoptosis in several cell types (for examples, see Bertrand *et al.*, 1994; Wang *et al.*, 1995; Shao *et al.*, 1997), a property that may be germane to the antineoplastic activity of this type of agent. Paradoxically, staurosporine at low concentrations can also inhibit apoptosis induced by dexamethasone in murine thymocytes (Migliorati *et al.*, 1994) or by X-radiation in rat thymocytes (Ojeda *et al.*, 1992). Induction of apoptosis by staurosporine has been linked to its PKC-inhibitory potency (Qiao *et al.*, 1996; Kobayashi *et al.*,

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ABBREVIATIONS: PKC, protein kinase C; bp, base pair(s); CAGE, conventional agarose gel electrophoresis; CDK, cyclin-dependent kinase; FIGE, field inversion gel electrophoresis; TLCK, *N*- α -tosyl-L-lysyl-chloromethyl ketone; Z-VAD.FMK, benzyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethyl ketone; Tempol, 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxy.

1997). We wished to explore this association further by testing the hypothesis that the ability to induce or inhibit apoptosis is also a characteristic of PKC-specific staurosporine analogs. For this comparison we chose CGP 41251, UCN-01, UCN-02, a UCN-01 stereoisomer and weak PKC inhibitor devoid of antineoplastic activity (Takahashi *et al.*, 1989), RO 31-8220 and GF 109203X (for structures, see Fig. 1). We investigated the effects of these agents on immature rat thymocytes, a well-defined model system for the study of apoptosis, and compared their effects with those that characterize apoptosis induced by the glucocorticoid dexamethasone, the DNA-damaging agent and topoisomerase II poison etoposide, and the calcium ATPase-inhibitor thapsigargin. Apoptosis is a major form of cell death, characterized by a series of distinct morphological and biochemical alterations (Arends and Wyllie, 1991). Prominent among these alterations are condensation and fragmentation of nuclear chromatin, compaction of cytoplasmic organelles, dilation of the endoplasmic reticulum, decrease in cell volume, and alterations to the plasma membrane resulting in the recognition and phagocytosis of apoptotic cells. The nuclear alterations in apoptosis are often associated with internucleosomal cleavage of DNA, generating "DNA ladders," which are derived from large fragments of DNA of 30–50 and 200–300 kbp (Brown *et al.*, 1993). We studied thymocyte apoptosis induced by staurosporine and its analogs by four different assays to allow both quantitative and qualitative assessment of several key features of the apoptotic phenotype: flow cytometry using the dye Hoechst 33342, DNA ladder formation, FIGE to detect large fragments and cell size. The overall aim of the investigation was 2-fold, to explore the mechanistic link between induction of apoptosis and PKC inhibition, and to improve the understanding of the mechanisms by which staurosporine analogs influence cell survival and death.

Materials and Methods

Drugs and reagents. Kinase inhibitors were provided by Kyowa Hakko (Tokyo, Japan) (UCN-01, UCN-02), Ciba-Geigy (Basel, Switzerland) (CGP 41251), and Roche Research Center (Welwyn Garden City, UK) (RO 31-8220). GF 109203X was acquired from Calbiochem-Novabiochem (Nottingham, UK), TLCK from Boehringer Mannheim UK (Lewes, UK) and Z-VAD.FMK from Enzymes Systems (Dublin, California). Other chemicals and reagents including staurosporine and thapsigargin were purchased from Sigma Chemical (Poole, UK), medium and fetal bovine serum from GIBCO Ltd. (Paisley, UK). Stock solutions of kinase inhibitors or of thapsigargin

were prepared in dimethyl sulfoxide and stored at -20° . The final concentration of dimethyl sulfoxide in the medium did not exceed 0.1%, which on its own did not elicit apoptosis in rat thymocytes.

Isolation of primary rat thymocytes. Thymocytes were isolated from male Fischer 344 rats (4–6 weeks-old, 65–85 g) as described previously (Raffray and Cohen, 1991). The cell suspension was diluted to 2×10^7 cells/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum and kept on ice until incubations were carried out. Thymocytes were incubated at 37° in an atmosphere of 95% air/5% CO_2 .

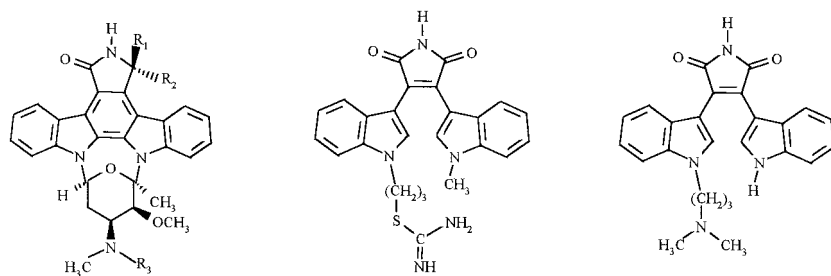
Flow cytometry. Thymocytes (1×10^6) were stained with the vital dyes Hoechst 33342 and propidium iodide (Sigma Chemical) to quantify the percentage of apoptotic and normal cells by flow cytometry (Sun *et al.*, 1992) using a Becton Dickinson fluorescence-activated cell-sorting Vantage flow cytometer. Compared with normal cells, apoptotic cells exhibit a low forward light scatter (indicative of a smaller size) and a high blue fluorescence with Hoechst 33342. These cells have been shown to be apoptotic on the basis of their ultrastructure, smaller size, and internucleosomal cleavage (Sun *et al.*, 1992). The increase in Hoechst 33342 fluorescence of apoptotic cells is caused by an increase in cell permeability.

CAGE. Thymocytes (2×10^6) were analyzed for oligonucleosomal fragmentation on 1.8% agarose gels as described by Sorensen *et al.* (1990). Electrophoresis was conducted at 20 V for 1 hr in the presence of 2% sodium dodecyl sulfate and proteinase K (1.25 mg/ml) to ensure cell lysis, and then for 3–4 hr at 100 V.

FIGE. FIGE was carried out to detect the formation of large molecular weight DNA fragments. Agarose plugs containing 1×10^6 cells were prepared and were stored at 4° until examination by FIGE as previously described by Brown *et al.* (1993). Equal portions of the plugs were loaded into the wells of a 1% NA agarose gel, and sealed with 1% agarose. The gel was run in Tris-borate-EDTA buffer (10 mM Tris-borate, 1 mM EDTA, pH 8) using a Hoeffer PC750 pulse controller.

Cell size analysis. Cell volume and diameter profiles were analyzed using a Casy 1 cell counting and sizing system (Schaefer System, Reutlingen, Germany). Cells in RPMI 1640 were diluted (typically 1:1000) with Casyton and counted in triplicate.

Percoll gradients. Thymocytes were separated on a discontinuous Percoll gradient into four fractions, (F1 to F4 in order of increasing buoyant density) as described previously (Cohen *et al.*, 1993). Thymocytes were washed and centrifuged ($200 \times g$, 5 min) and resuspended in ice-cold phosphate-buffered saline (2×10^8 cells/0.5 ml; $1 \times = 1.7$ mM KH_2PO_4 , 5 mM Na_2HPO_4 , 0.15 M NaCl, pH 7.4). Aliquots (0.5 ml) were loaded onto a gradient and centrifuged ($2000 \times g$, 10 min, 4°). Percoll gradients were calibrated with density marker beads (Pharmacia, St. Albans, Herts, UK), to ensure that the buoyant densities at the 0–60%, 60–70%, 70–80%, and 80–100% interfaces were 1.063, 1.075, 1.099, and 1.119 g/ml, respectively. Cells were collected from the first two interfaces (called fractions F1



R1, R2, R3 = H: staurosporine;
R1=OH; R2, R3 = H: UCN-01;
R1, R3 = H; R2 = OH: UCN-02;
R1, R2 = H; R3 = benzoyl: CGP41251

RO 31 8220

GF 109203X

Fig. 1. Structures of staurosporine and its analogs used in this study.

and F2, respectively), washed in RPMI 1640, and resuspended in RPMI 1640 with 10% fetal bovine serum. The F1 fraction thus obtained consists of a proliferatively enriched subpopulation, F2 contains primarily quiescent cells, and the cellular composition of these fractions has recently been characterized in detail (MacFarlane *et al.*, 1996). F1 and F2 cells were incubated for 4 hr with staurosporine (1 μM), after which the percentage of cells having undergone apoptosis was assessed by flow cytometry.

Inhibitors of apoptosis. In some experiments, the following agents were included in the incubations with rat thymocytes to study if they modulate induction of apoptosis by staurosporine and its analogs: 10 μM cycloheximide, 10 μM actinomycin D, 10 mM Tempol, 10 μM neocuproine, 100 μM TLCK, and 200 μM Z-VAD.FMK.

Results

UCN-01 and UCN-02 mimic staurosporine as inducers of thymocyte apoptosis. Rat thymocytes were incubated for up to 6 hr with staurosporine or UCN-01, UCN-02, CGP 41251, RO 31-8220, or GF 109203X, and the resulting degree of apoptosis was measured. Of these agents, staurosporine, UCN-01, and UCN-02 induced apoptosis in a time-

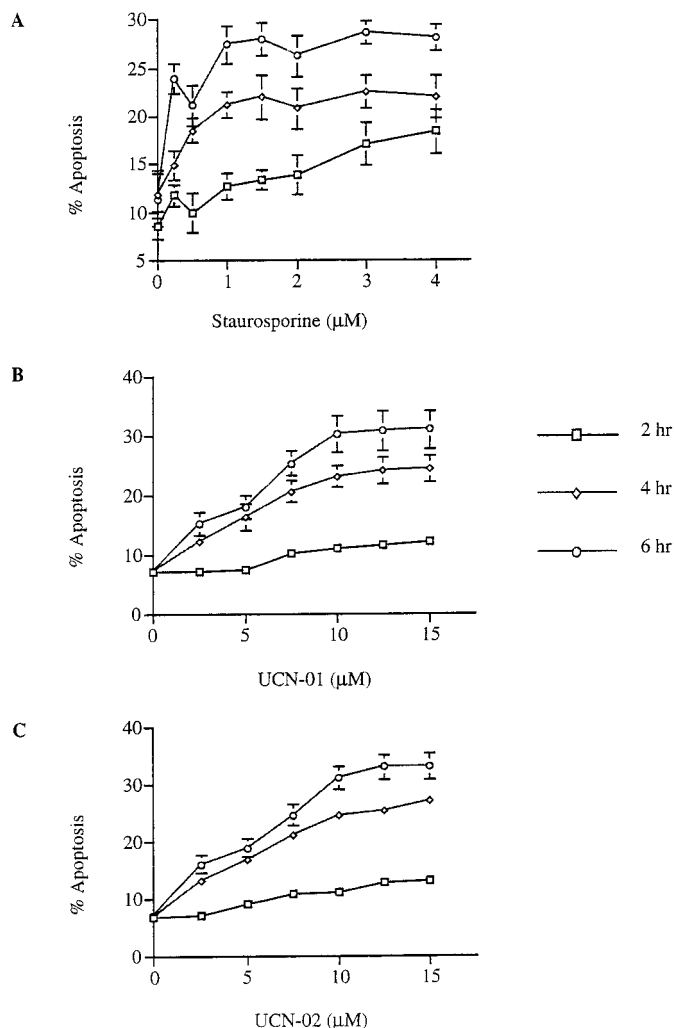


Fig. 2. Effect of staurosporine (A), UCN-01 (B), and UCN-02 (C) on apoptosis in immature rat thymocytes. Thymocytes were incubated with kinase inhibitors for 2 hr (□), 4 hr (◇) or 6 hr (○) and analyzed for staining with Hoechst 33342 by flow cytometry, as described in Materials and Methods. Data points, mean \pm standard deviation of three to nine experiments.

and concentration-dependent fashion as measured by flow cytometry (Fig. 2). This result was corroborated by increases in occurrence of internucleosomal DNA cleavage measured by CAGE (Fig. 3, lanes 2, 3, and 4, respectively), formation of large DNA fragments detected by FICE (Fig. 4, lanes 4, 5, and 6) and ultrastructural changes as judged by electron microscopy (data not shown) in cells that had been exposed to these agents for 4 hr. UCN-01 and UCN-02 were equipotent as inducers of thymocyte apoptosis, yet their potency was only a tenth of that observed with staurosporine. In contrast, neither CGP 41251, RO 31-8220, nor GF 109203X at 1 to 100 μM were able to elicit apoptosis as assessed by flow cytometry (not shown) and CAGE (Fig. 3, lanes 5, 6, and 7). Although these agents did not induce internucleosomal cleavage of DNA, it was considered possible that they generated large kbp fragments of DNA. However, using FICE, neither CGP 41251, RO 31-8220, nor GF 109203X induced such fragments (Fig. 4, lanes 1, 2, and 3). Furthermore, another PKC inhibitor chemically unrelated to staurosporine, the isoquinolinesulfonamide H-7 (up to 4 mM), also failed to induce apoptosis in thymocytes (result not shown). Ro 31-8220 and GF 109203X at 10–100 μM , although not inducing apoptosis, were cytotoxic as estimated by inclusion of propidium iodide. CGP 41251, RO 31-8220, and GF 109203X, like staurosporine and UCN-01, are potent inhibitors of PKC. Therefore, these results suggest that inhibition of PKC is not sufficient to induce apoptosis.

Effect of staurosporine, UCN-01, and UCN-02 on cell size. Cell shrinkage is a common, but poorly understood,

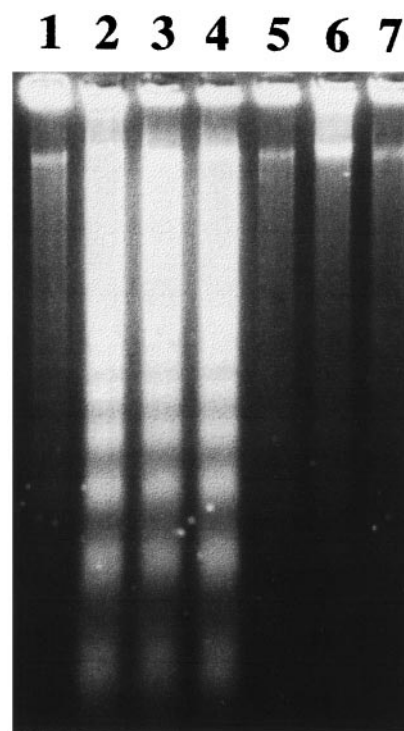


Fig. 3. Effect of staurosporine and its analogs on the occurrence of internucleosomal DNA cleavage in immature rat thymocytes measured by conventional agarose gel electrophoresis. Cells were incubated with the drugs for 4 hr. Lane 1, control cells; lane 2, staurosporine (1 μM); lane 3, UCN-01 (10 μM); lane 4, UCN-02 (10 μM); lane 5, CGP 41251 (10 μM); lane 6, RO 31-8220 (10 μM); lane 7, GF 109203X (10 μM). Experimental conditions were as described in Materials and Methods. Gels are from one experiment representative of three.

feature of apoptosis in many different cell systems (Kerr *et al.*, 1972). To establish whether staurosporine and its analogs induce cell shrinkage, thymocytes were incubated with staurosporine (1 μM), UCN-01, UCN-02, CGP 41251, RO 31-8220, and GF 109203X (all at 10 μM) for 4 hr; then, cell size was analyzed. Staurosporine, UCN-01, and UCN-02 (i.e., those agents capable of inducing apoptosis as defined above), all caused a decrease in cell diameter (Table 1). In contrast, CGP 41251, RO 31-8220, and GF 109203X (i.e., the agents that did not induce apoptosis) had no effect on cell size. The cell diameter profiles observed with staurosporine, UCN-01, and UCN-02 differed markedly from those observed in dexamethasone- and etoposide-treated thymocytes, in that they caused a distinct shift of the whole-cell size profile to the left, as shown in Fig. 5 for staurosporine. In comparison, cell size profiles from thymocytes treated with dexamethasone (Fig. 5) or etoposide (not shown) show a discrete cell population displaying smaller cell diameter and volume. This population corresponds to the percentage of cells exhibiting the apoptotic phenotype as determined by flow cytometry with Hoechst 33342, and its appearance is inhibitable by TLCK, a trypsin-like protease inhibitor (Fearnhead *et al.*, 1995a). In contrast, the shift in cell-size profiles that occurred in thymocytes treated with staurosporine, UCN-01, and UCN-02 was not inhibited by pretreatment of thymocytes for 1 hr with TLCK (50–100 μM) (results not shown).

Induction of apoptosis by staurosporine in thymocyte subpopulations. The concentration dependency of apoptosis induced by staurosporine and its analogs reached a plateau at 1 μM in the case of staurosporine and 10 μM for UCN-01 and UCN-02 (Fig. 2). The occurrence of such pla-

teaus may have been caused by different thymocytic subpopulations with marked dissimilarities in susceptibility toward the induction of apoptosis. Although the majority of thymocytes are quiescent cells residing in the G_0 phase of the cell cycle, 15–20% of the total cell population are larger, proliferatively competent cells (Bruno *et al.*, 1992). High sensitivity of this subpopulation to staurosporine-induced apoptosis with low responsiveness of the residual cell subpopulations could explain the plateau observed for the total population. To test this hypothesis, thymocytes were fractionated using Percoll centrifugation and two subpopulations, F1 and F2, of differential proliferative competence, were isolated and incubated with staurosporine (1 μM) for 4 hr. Apoptosis in the proliferatively enriched population of F1 cells increased from 6.4 ± 0.5 in control cells to 17.1 ± 1.1 in cells exposed to staurosporine. Apoptosis in the primarily quiescent population of F2 cells increased from $13 \pm 0.7\%$ in controls to 28.1 ± 1.4 in treated cells. These results do not support the notion that staurosporine caused preferential induction of apoptosis in proliferatively competent cells.

Inhibition of apoptosis induced by staurosporine, UCN-01, and UCN-02. Induction of apoptosis in rat thymocytes is modulated by many pharmacological agents, including cycloheximide, a protein synthesis inhibitor; actinomycin D, a protein translation inhibitor (Arends and Wyllie, 1991); Tempol, a cell-permeable free radical scavenger; neocuproine, a copper ion chelator (Wolfe *et al.*, 1994); TLCK (Fearnhead *et al.*, 1995b); and Z-VAD.FMK, a caspase inhibitor (Fearnhead *et al.*, 1995a). We studied the effect of these modulators on staurosporine- and UCN-01/UCN-02-induced apoptosis. Of these agents, only TLCK and Z-VAD.FMK inhibited apoptosis when they were included in incubations of thymocytes with staurosporine, UCN-01, or UCN-02. Pretreatment of thymocytes with TLCK for 1 hr followed by 4-hr coincubation with staurosporine, UCN-01, or UCN-02 decreased the percentage apoptosis measured with flow cytometry from 21.2 ± 1.4 to 10.7 ± 2.0 in the case of staurosporine, from 23.1 ± 1.7 to 7.9 ± 1.3 for UCN-01, and from 24.7 ± 1.2 to 11.1 ± 1.4 for UCN-02 ($p < 0.01$ in all three cases). The extent of inhibition by Z-VAD.FMK of apoptosis caused by these agents was variable and modest, even when pretreatment with Z-VAD.FMK was extended from 1 to 2 hr (data not shown). These results mitigate against the involvement of protein synthesis, free radicals, or metals in the mechanism by which staurosporine and its hydroxylated congeners induce apoptosis, but they implicate the action of proteases, including caspases.

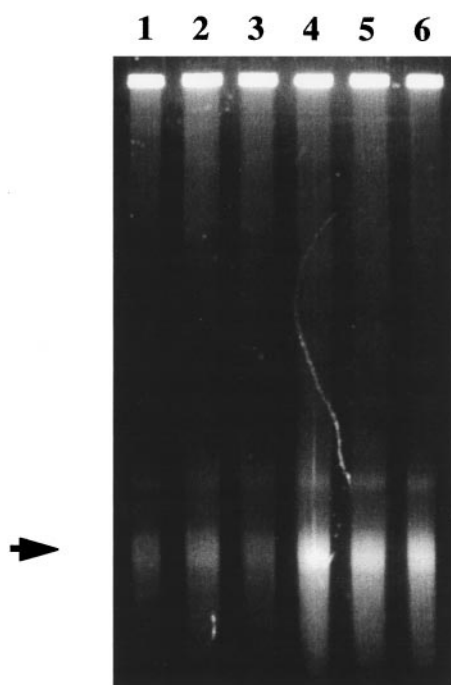


Fig. 4. Effect of staurosporine and its analogs on formation of large DNA fragments cleavage in immature rat thymocytes detected by field inversion gel electrophoresis. Cells were incubated with the drugs for 4 hr. Lane 1, CGP 41251 (10 μM); lane 2, RO 31-8220 (10 μM); lane 3, GF 109203X (10 μM); lane 4, staurosporine (1 μM); lane 5, UCN-01 (10 μM); lane 6, UCN-02 (10 μM). Experimental conditions were as described in Materials and Methods. Results are from one experiment representative of three. Arrow, distance migrated by the 48.5 kbp standard.

TABLE 1

Effect of staurosporine and its analogs on size of rat thymocytes. Cells were incubated with agents for 4 hr and mean cell diameter was estimated as described in Materials and Methods. Results are the mean of three determinations in an experiment representative of four in the case of the control cells and of two experiments for the drug-treated cells.

Treatment	Concentration	Mean cell diameter
	μM	μm
None		6.48
Staurosporine	1	5.32
UCN-01	10	5.62
UCN-02	10	5.84
CGP 41251	10	6.48
RO 31-8220	10	6.45
GF 109203X	10	6.51

Modulation of thapsigargin-induced apoptosis by staurosporine and its analogs. The role of PKC activation in apoptosis induction is paradoxical, as PKC-activating phorbol esters have been shown to induce as well as inhibit apoptosis, depending on cell type. In the light of this complexity, we addressed the question of whether kinase inhibitors of the staurosporine type can interfere with the apoptosis-inducing ability of other molecules, rather than, or in addition to, inducing apoptosis themselves. The hypothesis that was tested here is whether staurosporine and its analogs, at concentrations at which they themselves did not elicit apoptosis, could modulate apoptosis induced by dexamethasone, etoposide, or thapsigargin. These agents were chosen because they induce apoptosis *via* diverse mechanisms. Staurosporine or its analogs did not affect levels of apoptosis elicited by dexamethasone (0.1 μM) or etoposide (10 μM) (result not shown). In contrast, apoptosis induced by thapsigargin (50 nM) was inhibited by staurosporine (10 nM), UCN-01, UCN-02, RO 31-8220 (each at 100 nM), and, to some extent, GF 109203X (100 nM), when assessed by flow cytometry (Fig. 6). Likewise, estimation of DNA fragmentation by CAGE (Fig. 7) and determination of formation of large DNA fragments by FIGE (data not shown) supported the notion that staurosporine, UCN-01, UCN-02, and RO 31-8220 inhibited thapsigargin-induced apoptosis. However, GF 109203X failed to inhibit thapsigargin-induced apoptotic changes as measured by CAGE and FIGE. CGP 41251 did not affect thapsigargin-induced apoptosis as determined by any of the three methods (Fig. 7).

Discussion

The mechanism of induction of thymocyte apoptosis by staurosporine, UCN-01, and UCN-02 differs from that triggered by dexamethasone and etoposide. Staurosporine and its analogs UCN-01 and UCN-02 engendered the following biochemical changes indicative of induction of apoptotic cell death in immature rat thymocytes: (i) alteration in membrane permeability toward Hoechst 33342, (ii) formation of large DNA fragments, and (iii) occurrence of internucleosomal DNA cleavage and (intravenous) cell shrinkage. Cell shrinkage induced by staurosporine, UCN-01, and UCN-02 displayed unusual features, in that the agents caused a general shift in the thymocyte size profile,

which, together with the lack of its reversal by TLCK, suggests that this phenomenon seems to be unrelated, at least in part, to the apoptotic phenotype. We have observed a similar cell-size shift in other cell types, such as freshly isolated rat hepatocytes treated with staurosporine (Harkin ST, Cohen GM, Gescher A, unpublished observations). A further indicator of apoptosis was explored in experiments, in which the ability of staurosporine analogs to cause cleavage of the caspase substrate PKC- δ was studied. PKC- δ cleavage accompanies apoptosis in myeloid leukemia cells (Emoto *et al.*, 1995). Staurosporine, UCN-01, and UCN-02 caused cleavage of PKC- δ , whereas RO 310-8220, CGP 41251, and GF 109203X did not (results not shown), mimicking the results obtained measuring DNA fragmentation, formation of large DNA fragments, and Hoechst staining by flow cytometry.

Whereas the capability of staurosporine and UCN-01 to induce apoptosis has been described in a variety of cells (see Introduction), we show here for the first time that UCN-02 shares this property with them. Staurosporine, UCN-01, and UCN-02 were less potent than etoposide as stimulators of apoptosis, with a maximum of only $\sim 30\%$ of thymocytes being induced to undergo apoptosis (Fig. 2), compared with 60% in the case of etoposide (results not shown). This divergence highlights the relative resistance of rat thymocytes against the apoptogenicity of molecules of the staurosporine type. Whereas staurosporine and its hydroxylated congeners caused thymocytes to adopt the same apoptotic phenotype, albeit to a weaker extent, as that observed with the glucocorticoid dexamethasone and the DNA-damaging drug etoposide, the mechanisms involved in the induction of apoptosis by these agents are distinctly different. This contention is borne out by the following findings: (i) protein synthesis is a prerequisite of induction of apoptosis by etoposide and dexamethasone (Arends and Wyllie, 1991; Wolfe *et al.*, 1994), but inhibition of synthesis or translation of proteins by cycloheximide and actinomycin D failed to inhibit induction of apoptosis by staurosporine and its analogs. (ii) Metal ions and free radicals have been shown recently to play a role in the induction of apoptosis by dexamethasone and etoposide in thymocytes (Wolfe *et al.*, 1994). In contrast, neither the free radical scavenger Tempol nor the copper ion chelator neocuproine were capable of abrogating induction of apoptosis by staurosporine and its analogs. (iii) Under experimental conditions similar to those employed here, etoposide has been demonstrated previously to induce apoptosis preferentially in thymocyte subpopulations that are enriched in proliferatively competent cells (F1) compared with subpopulations of predominantly quiescent cells (F2) (Fearnhead *et al.*, 1994). In contrast, no such difference was observed in the sensitivity of these populations of thymocytes to induction of apoptosis by staurosporine, suggesting that the proliferative state of thymocytes is not an important factor in their susceptibility toward staurosporine-induced apoptosis. In summary, these results suggest that the mechanism of induction of apoptosis by staurosporine, UCN-01, and UCN-02 are clearly different from those by which two "classical" inducers of thymocyte apoptosis, a glucocorticoid and a DNA-damaging agent, exert this effect.

PKC inhibition is not sufficient for induction of apoptosis in thymocytes. It has been suggested that the capacity to inhibit PKC is a major determinant of the mechanisms by which staurosporine induces apoptosis (Qiao *et al.*,

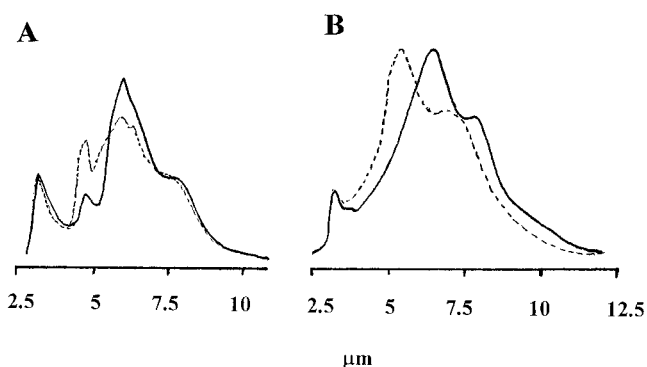


Fig. 5. Cell diameter profiles of immature rat thymocytes after incubation for 4 hr with (A) dexamethasone (0.1 μM) or (B) staurosporine (1 μM). Solid lines and broken lines, profiles of control and drug-treated cells, respectively. The profiles, which were obtained as described in Materials and Methods, are representative of four independent experiments in the case of the control cells and of two experiments for the drug treated cells.

1996; Kobayashi *et al.*, 1997). Staurosporine is a potent inhibitor of PKC enzyme activity with IC_{50} values in the low nanomolar range (Tamaoki and Nakano, 1990). Of the staurosporine congeners investigated here, UCN-01 (Tamaoki and Nakano, 1990), CGP 41251 (Meyer *et al.*, 1989), RO 31-8220 (Davis *et al.*, 1992) and GF 109203X (Toullec *et al.*, 1991) share with staurosporine the characteristic of high PKC-inhibitory potency. In addition these analogs are thought to be more specific than their parent molecule in targeting PKC (Meyer *et al.*, 1989; Tamaoki and Nakano, 1990; Toullec *et al.*, 1991; Davis *et al.*, 1992). These facts are pertinent for the interpretation of the following conclusions, which can be drawn from the results described above: (i) Of the PKC-specific staurosporine analogs, only UCN-01 was a potent inducer of apoptosis, albeit less effective than staurosporine, whereas CGP 41251, RO 31-8220, or GF 109203X failed to induce apoptosis; (ii) both stereoisomeric hydroxystaurosporines induced apoptosis to an almost identical degree, even though UCN-02 is a much weaker PKC inhibitor than UCN-01. These conclusions do not support the notion that inhibition of PKC is a pivotal arbiter of the mechanisms by which molecules of the staurosporine type induce apoptosis. It is unlikely that differences between staurosporine analogs in ability to penetrate thymocytes caused the differences in ability to induce apoptosis, for the following reasons: first, experiments by many groups (e.g., Utz *et al.*, 1994; Mahon *et al.*, 1997) including ours (Courage *et al.*, 1995; Budworth *et al.*, 1996) on biological effects of staurosporine analogs in a variety of cells in culture suggest that CGP 41251, RO 31-8220, or GF 109203X penetrate cells as staurosporine, and it is unlikely that rat thymocytes are different in this respect. Secondly, we describe above that RO 31-8220 counteracted the effect of thapsigargin at 100 nM, a concentration that is only a 100th of that which failed to induce

apoptosis (Fig. 7). Thirdly, CGP 41251 at 1 μ M significantly augmented apoptosis induced by etoposide in thymocytes (result not shown). Taken together, these considerations suggest that RO 31-8220, CGP 41251, and GF 109203X permeate the thymocyte membrane and, in that respect, behave similarly to staurosporine and its hydroxylated analogs.

Consistent with a lack of a role for PKC is the fact that in most cell types in which staurosporine triggers apoptosis, the drug concentration required to render cells apoptotic is more than a thousandfold higher than the concentration that efficiently inhibits PKC activity (Bertrand *et al.*, 1994). Further evidence against a direct link between induction of apoptosis and PKC inhibition was provided by a preliminary experiment, in which rat thymocytes treated with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate, which down-regulates PKC, exhibited essentially unchanged susceptibility toward staurosporine-induced apoptosis compared with untreated cells (results not shown). Thus, our data demonstrate that either PKC inhibition is unrelated to induction of apoptosis by molecules of the staurosporine type or, alternatively, PKC inhibition alone is insufficient for induction of apoptosis to occur, which requires additional action(s) by these agents. This inference is consistent with recent findings stating that staurosporine analogs affect multiple biochemical systems *via* influences on a variety of kinases. Claims for PKC specificity of several staurosporine analogs have not stood up to rigorous testing. For example, UCN-01, in addition to inhibiting PKC in potent manner, is a powerful modulator of CDK-activating kinases, and thus profoundly affects the CDK system (Wang *et al.*, 1995; Akiyama *et al.*, 1997). RO 31-8220 and GF 109203X are better inhibitors of

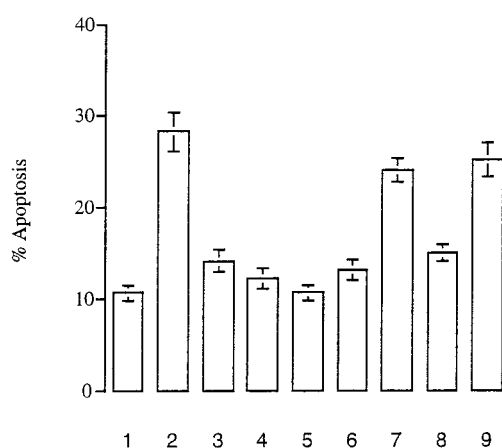


Fig. 6. Effect of staurosporine and its analogs on thapsigargin-induced apoptosis in immature rat thymocytes as shown by flow cytometry. Bar 1, untreated cells; bar 2, cells treated with thapsigargin (50 nM) only; bar 3, cells treated with thapsigargin and staurosporine (1 nM); bar 4, cells treated with thapsigargin and staurosporine (10 nM); bar 5, cells treated with thapsigargin and UCN-01 (100 nM); bar 6, cells treated with thapsigargin and UCN-02 (100 nM); bar 7, cells treated with thapsigargin and CGP 41251 (100 nM); bar 8, cells treated with thapsigargin and RO 31-8220 (100 nM); bar 9, cells treated with thapsigargin and GF 109203X (100 nM). Thymocytes were incubated with thapsigargin with or without kinase inhibitors for 4 hr and analyzed for staining with Hoechst 33342 by flow cytometry, as described in Materials and Methods. Data points, mean \pm standard deviation of three experiments. The values shown in bars 3, 4, 5, 6 and 8 were significantly different from those in bar 2 ($p < 0.01$).

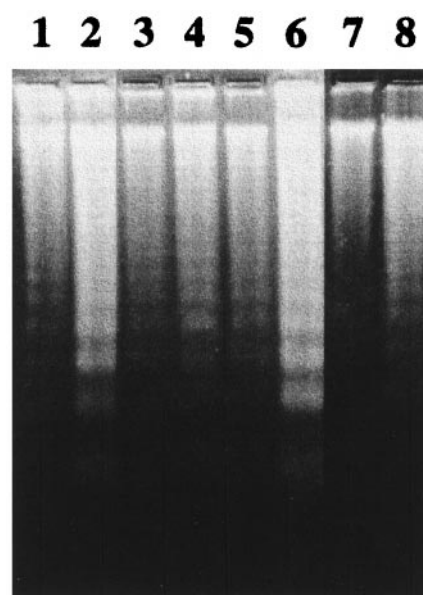


Fig. 7. Effect of staurosporine and its analogs on thapsigargin-induced apoptosis in immature rat thymocytes as detected by the occurrence of internucleosomal DNA cleavage. Thymocytes were incubated with kinase inhibitors for 4 hr, and laddering was determined as described in Materials and Methods. Lane 1, untreated cells; lane 2, cells treated with thapsigargin (50 nM) only; lane 3, cells treated with thapsigargin and staurosporine (10 nM); lane 4, cells treated with thapsigargin and UCN-01 (100 nM); lane 5, cells treated with thapsigargin and UCN-02 (100 nM); lane 6, cells treated with thapsigargin and CGP 41251 (100 nM); lane 7, cells treated with thapsigargin and RO 31-8220 (100 nM); lane 8, cells treated with thapsigargin and GF 109203X (100 nM). Gels are from one experiment representative of three.

mitogen-activated protein kinase-activated protein kinase-1 β and p70 S6 kinases than of PKC (Alessi, 1997). Taken together, these data suggest that (i) staurosporine analogs are inhibitors not only of PKC, but of a "cocktail" of kinases, (ii) the composition of this cocktail determines the nature and magnitude of their pharmacological effects, including induction of apoptosis, and their tissue- or cell-specificity, and (iii) slight alterations in the structure of the staurosporine-type molecule influence the composition of this cocktail in a dramatic fashion. It seems important to identify those enzyme constituents of the kinase cocktail affected by molecules of the staurosporine type that might mediate modulation of apoptosis. It is also possible that staurosporine congeners influence the activity of signaling components in a positive fashion or that they may be perceived by the cell as chemical stressors. In this respect, it is quite pertinent that RO 31-8220 has been found to induce activation of stress-activated protein kinase/jun kinase (Beltman *et al.*, 1996), a kinase system implicated in apoptosis in a variety of cells. It clearly constitutes a potential target for staurosporine analogs that is independent of PKC.

Inhibition of thapsigargin-induced apoptosis by staurosporine and its analogs. Staurosporine, UCN-01, and UCN-02 displayed a dual effect on thymocyte apoptosis, at high concentrations ($>1\ \mu\text{M}$) they induced apoptosis, whereas at low concentrations (10–100 nM), they inhibited thapsigargin-induced apoptosis. Intriguingly, RO 31-8220, which itself did not induce apoptosis, also inhibited thapsigargin-induced apoptosis. Staurosporine, UCN-01, UCN-02, and RO 31-8220 displayed selectivity in that they lacked the capability of interfering with induction of apoptosis by dexamethasone or etoposide. This specificity suggests that the kinase inhibitors impede an early signal transduction event unique to the apoptotic signal cascade triggered by thapsigargin, possibly related to its ability to produce a prolonged increase in cytosolic calcium levels (Thastrup *et al.*, 1989, 1990). The low concentrations of staurosporine, UCN-01, and RO 31-8220 required to antagonize thapsigargin-induced apoptosis render PKC inhibition a feasible mechanistic component of this modulation. However, this possibility seems unlikely because of the efficacy shown by UCN-02, which, in contrast to staurosporine, UCN-01, and RO 31-8220, is only a weak PKC inhibitor, and the lack of activity of CGP 41251 and GF 109203X, both of which are as potent at inhibiting PKC as UCN-01 and RO 31-8220. Likewise, a role for inhibition of specific PKC isoenzymes to explain the discrepancies is unfounded as UCN-01, CGP 41251, RO 31-8220 and GF 109203X share with each other inhibitory selectivity for conventional PKCs over novel PKCs, with little effect on atypical PKCs (Nixon, 1997). The conclusion that PKC inhibition is insufficient on its own to account for inhibition of thapsigargin-induced apoptosis by staurosporine and its analogs reflects accurately the inference drawn above with respect to induction of apoptosis by these agents.

UCN-01 and CGP 41251 are currently under clinical evaluation as potential anticancer drugs. In the light of the structural similarity between these molecules, pharmacological differences between them are intriguing, and they may eventually influence their potential therapeutic application. Of the two agents, UCN-01 is probably more cytostatic or

cytotoxic (Courage *et al.*, 1995), whereas CGP 41251 seems to possess cytotoxic drug resistance-reversing properties that are superior to those of UCN-01 (Budworth *et al.*, 1996). The work presented here now demonstrates that UCN-01 may be more potent as inducer of apoptosis than CGP 41251. It remains to be elucidated which biochemical features are responsible for the differences between these molecules. Whereas a divergent pattern of inhibitory or stimulatory specificities for kinases may be such a feature, the work presented here clearly highlights that steps unrelated to PKC inhibition determine their differential capabilities to induce apoptosis.

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