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Effect of Staurosporine Derivatives on Protein Kinase Activity and Vinblastine Accumulation in Mouse Leukaemia P388/ADR Cells

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Abstract—Inhibition by staurosporine derivatives of cyclic AMP-dependent protein kinase (A-kinase) and protein kinase C (C-kinase), and drug resistance has been investigated. The substitution of an acetyl or an ethoxycarbonyl group for the amine *N*-ethoxycarbonyl-7-oxostaurosporine moiety on the tetrahydropyran ring of staurosporine decreased inhibition of both protein kinases, but increased selectivity for C-kinase by further modification of the lactam moiety to the imide (NA-382). The activities of SF-2370 on protein kinases were decreased by decarboxylation and hydroxyalkylation. These staurosporine derivatives enhanced accumulation of vinblastine in adriamycin-resistant P388 (P388/ADR) cells in a dose-dependent manner. The potency for the drug accumulation of these compounds was correlated with their inhibitory activity on the drug efflux, but was not correlated with their activity on protein kinases. Staurosporine and NA-382, with high potency for vinblastine accumulation, inhibited the photolabelling of [^{3}H]azidopine on 140 kDa P-glycoprotein in the plasma membrane. The tetrahydrofuran compounds and NA-357, which had low potency for the drug accumulation, hardly interacted with azidopine on P-glycoprotein. Most of these compounds were highly cytotoxic by themselves, and only NA-382 was less cytotoxic among them and completely reversed the vinblastine-resistance of P388/ADR cells at a non-cytotoxic concentration. These results suggest that staurosporine derivatives can enhance drug accumulation and inhibit drug resistance through their direct action on the P-glycoprotein.

Multidrug resistance is characterized by resistance to structurally unrelated antitumour drugs and decreased accumulation and enhanced efflux of drugs resulting from the P-glycoprotein encoded by the mdr gene (Akiyama et al 1985; Kartner et al 1985; Riordan et al 1985). Many kinds of drugs including calcium-channel blockers, calmodulin inhibitors, and Rauwolfia alkaloids have been reported to interfere with the drug elimination system (Tsuruo et al 1982; Miyamoto et al 1984; Biedler & Meyers 1989). We recently reported that isoquinolinesulphonamide derivatives, which have different magnitudes of inhibitory activity on cyclic AMP-dependent protein kinase (A-kinase), promote the accumulation of vinblastine in adriamycin-resistant mouse leukaemia P388 (P388/ADR) cells by inhibiting the energy-dependent elimination of the antitumour drug from the cells and thus overcome multidrug resistance (Miyamoto et al 1990; Hagiwara et al 1991). On the other hand, Sato et al (1990) have indicated that staurosporine, which is a potent protein-kinase inhibitor, binds to the P-glycoprotein and enhances accumulation of vincristine in multidrug-resistant cells.

In this study, we studied correlation among activities of several staurosporine derivatives on protein kinases and drug resistance.

Materials and Methods

Materials

Staurosporine and SF-2370 were isolated from cultures of their producing organisms according to the previous reports (Omura et al 1977; Sezaki et al 1985). Other compounds were partially synthesized from staurosporine and SF-2370 in our laboratory and their structures are indicated in Table 1. Vinblastine was purchased from Shionogi & Co., Osaka, Japan. [^{3}H]Vinblastine (374 GBq mmol^{-1}), [^{3}H]azidopine (1·92 TBq mmol^{-1}), and [γ^{32}P]ATP (110 TBq mmol^{-1}) were obtained from Amersham International, UK.

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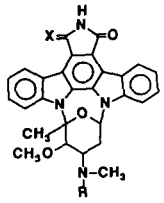
Enzyme assay

A-Kinase and protein kinase C (C-kinase) were partially purified from rabbit muscle and brain, respectively (Inagaki et al 1985). The activities of protein kinases were measured by [^{32}P]phosphate incorporation into protein substrates (Hidaka et al 1984) in the presence of various concentrations of test compound.

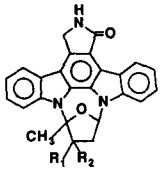
Tumour cells and culture

Parent P388 (P388/S) and P388/ADR cells were kindly supplied by the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan. Cells were passaged weekly through female BALB/c X DBA/2 (CDF_1) mice (Nippon SLC, Hamamatsu, Japan) and harvested from tumour-bearing mice 6–7 days after transplantation. Cells were suspended in RPMI 1640 medium supplemented with 10% foetal bovine serum, 20 μM 2-mercaptoethanol and 100 μg mL^{-1} kanamycin at a density of $1{\cdot}5 \times 10^5$ cells mL^{-1} and cultured with or without drugs at 37°C for 48 h in a CO_2 incubator. Cells were counted under a microscope, and the effects of drugs were expressed as the 50% growth-inhibiting concentration (IC50).

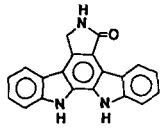
Table 1. Structures of staurosporine derivatives.



	R	X
Staurosporine	H	H,H
NA-379	$COCH_3$	H,H
NA-381	$COOCH_2CH_3$	H,H
NA-382	$COOCH_2CH_3$	O



	R_1	R_2
SF-2370	OH	$COOCH_3$
NA-332	OH	H
NA-451	OCH_2CH_2OH	H
NA-398	$OCH_2CH_2CH_2OH$	H



NA-357

Accumulation and efflux of vinblastine

Cells (2×10^6 mL^{-1}) were incubated in culture medium containing 25 mM HEPES (pH 7·4) in the presence of 10 nM [^{3}H]vinblastine (3·7 kBq mL^{-1}) with or without test compound at 37°C for 30 min. The cells were washed, dissolved in 0·5 M NaOH, and neutralized. The radioactivity was counted in a Beckman LS-5800 liquid scintillation counter after the addition of a toluene/Triton X-100 (2:1, v/v) scintillation mixture containing 0·2% PPO and 0·05% POPOP. In the efflux experiment, cells were loaded with 20 nM [^{3}H]vinblastine in a glucose-deprived Hanks solution containing 10 mM sodium azide for 30 min, then washed and cultured with or without test compound for 20 min. The radioactivity remaining in the cells was measured, and the results were expressed as the percentage of retained vinblastine in the cells to the amount after the forced accumulation.

Photoaffinity labelling

A membrane preparation (100 μg protein) by Percoll sedimentation method (Sanae et al 1989) was incubated with 74 kBq [^{3}H]azidopine for 30 min at room temperature (21°C) in the presence or absence of test drug and irradiated at 366 nm for 20 min. The sample was subjected to electrophoresis on an SDS-polyacrylamide gel. After being fixed and dried, the gel was subjected to autoradiography on a Kodak X-Omat R film (Eastman Kodak Co., Rochester, NY, USA) with an intensifying screen, at −70°C.

Results

Inhibitory activities of staurosporine derivatives on protein kinases are shown in Table 2. Staurosporine is a potent inhibitor of protein kinases (Tamaoki et al 1986). Addition of an acetyl or an ethoxycarbonyl group to the amine moiety on the tetrahydropyran ring of staurosporine (NA-379 and NA-381, respectively) decreased these activities. When the lactam moiety of NA-381 was changed to the imide (NA-382), the inhibitory activity against A-kinase was markedly decreased below that against C-kinase, and NA-382 became selective for C-kinase. Regarding derivatives of SF-2370, decarboxylation (NA-332) and further hydroxyalkylation (NA-451 and NA-398) lowered the activities. NA-357 showed only a weak activity on these protein kinases.

We examined the effects of staurosporine derivatives on accumulation of vinblastine in P388/ADR cells, which show a multidrug resistance against antitumour drugs, including vinblastine, due to decreased accumulation of drugs resulting from an energy-dependent drug elimination from the cells (Inaba & Sakurai 1979; Miyamoto et al 1990). Staurosporine and its derivatives with the tetrahydropyran ring promoted vinblastine accumulation; NA-381 and its analogue, NA-382, showed potent activity (Table 1, Fig. 1). Tetrahydrofuran derivatives, including SF-2370 and the basic structure

Table 2. Effects of staurosporine derivatives on protein kinases and vinblastine accumulation.

Compound	Protein kinase inhibition IC50 (μM) A-Kinase	C-Kinase	Vinblastine accumulation C5** (μM)
Staurosporine	0·024 ± 0·004	0·016 ± 0·003	0·96 ± 0·03
NA-379	0·15 ± 0·02	0·13 ± 0·01	0·30 ± 0·01
NA-381	15·0 ± 0·8	9·1 ± 0·6	0·053 ± 0·002
NA-382	6·5 ± 0·6	0·84 ± 0·04	0·032 ± 0·001
SF-2370	0·16 ± 0·02	0·13 ± 0·01	1·5 ± 0·1
NA-332	0·72 ± 0·07	0·62 ± 0·04	2·6 ± 0·2
NA-451	3·0 ± 0·3	2·6 ± 0·4	2·2 ± 0·1
NA-398	> 30	15 ± 0·9	1·7 ± 0·1
NA-357	> 30	> 30	2·8 ± 0·2

Each value represents the mean ± s.e. of at least two measurements in triplicate. *Concentration of the compound required to produce 50% inhibition of the enzyme activity. **Concentrations of the compound for 5-fold increase of vinblastine accumulation in P388/ADR cells obtained from Fig. 1.

Table 3. Activities of staurosporine derivatives on P388/ADR cells.

Compound	IC50 (μM)	Increase in effect of vinblastine*	
Staurosporine	0·00079 ± 0·00004	1·2 ± 0·2	(0·2 nM)
NA-379	0·076 + 0·003	1·1 ± 0·1	(20 nM)
NA-381	0·35 ± 0·01	3·2 ± 0·7	(0·1 μM)
NA-382	4·6 ± 0·3	11·2 ± 0·5**	(1·0 μM)
SF-2370	2·0 ± 0·1	1·5 ± 0·3	(0·5 μM)
NA-332	0·40 ± 0·03	1·2 ± 0·1	(0·1 μM)
NA-451	2·8 ± 0·1	2·1 ± 0·4	(0·5 μM)
NA-398	2·7 ± 0·1	2·7 ± 0·6	(0·5 μM)
NA-357	3·0 ± 0·1	1·5 ± 0·3	(1·0 μM)

Each value represents the mean ± s.e. of at least two measurements in triplicate. *Calculated by dividing the IC50 value of vinblastine in the absence of test compound by the IC50 value in the presence of test compound. Number in parentheses indicates the concentration of each compound used in the combination experiment that is about the maximum non-cytotoxic concentration. **$P < 0·01$ compared with non-treated control.

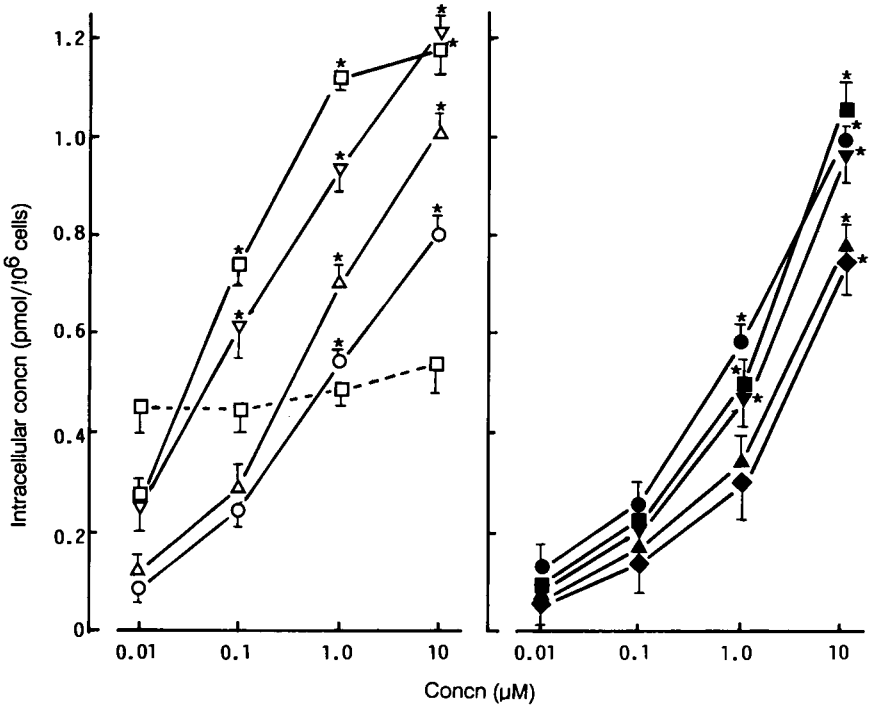


FIG. 1. Effects of staurosporine derivatives on vinblastine accumulation in P388 cells. Cells were incubated with 10 nM [^{3}H]vinblastine in the presence of varying concentrations of staurosporine (○), NA-379 (△), NA-381 (▽), NA-382 (□), SF-2370 (●), NA-332 (▲), NA-451 (▼), NA-398 (■), and NA-357 (◆) for 30 min at 37°C. P388/S cells, – – –; P388/ADR cells, ——. Each point with bar represents the mean ± s.e. of three measurements in triplicate. The amount of vinblastine after culture in the absence of test compounds for 30 min was 0·44 ± 0·05 pmol/10^6 P338/S cells and 30·9 ± 1·7 fmol/10^6 P388/ADR cells. *$P < 0·01$ compared with non-treated control.

compound NA-357, also increased vinblastine accumulation dose-dependently, but were less effective than tetrahydropyran compounds (Table 1, Fig. 1). Fig. 1 also shows that NA-382 only slightly increased vinblastine accumulation in the parent P388 cells. Staurosporine derivatives inhibited efflux of vinblastine from the cells following the order of potency for the drug accumulation (Fig. 2). It has been reported that some calcium-channel blockers, which suppress the efflux of antitumour drugs and reverse the drug resistance, competitively inhibit the binding of the antitumour drugs to P-glycoprotein overexpressed in the plasma membrane from multidrug-resistant cells (Safa et al 1986; Cornwell et al 1987). P388/ADR cells used in this study overexpressed 140 kDa protein in the plasma membrane which was immunopositive to a monoclonal antibody of P-glycoprotein C219, while the membrane protein was not detectable in the parent P388 cells (Hagiwara et al 1991). The 140 kDa P-glycoprotein was photolabelled by a calcium-channel blocker [^{3}H]azidopine; the photolabelling was selectively inhibited by vinblastine and also by staurosporine and NA-382 at 10 μM, but not by SF-2370, NA-398, or NA-357 (Fig. 3).

The combined effects of test compound at a non-cytotoxic concentration with varying concentrations of vinblastine were examined in an in-vitro culture system. Because most of the compounds, including staurosporine, were highly cytotoxic, even at the maximum non-cytotoxic concentration they increased the intracellular vinblastine level only slightly and influenced also only slightly the growth inhibitory effect

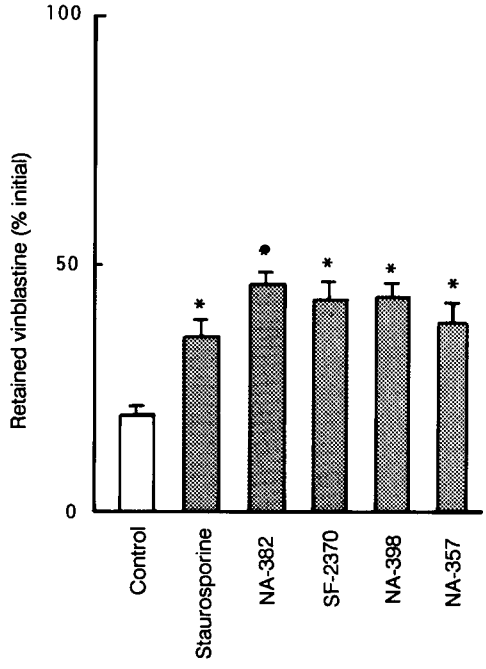


FIG. 2. Effects of staurosporine derivatives on vinblastine efflux in P388/ADR cells. After the forced accumulation of vinblastine, cells were incubated in the absence or presence of 10 μM of each compound for 20 min, and retained vinblastine in the cells was expressed as a percentage of the initial amount of vinblastine. Each column with bar represents the mean ± s.e. of three experiments in triplicate. *$P < 0·01$ compared with non-treated control.

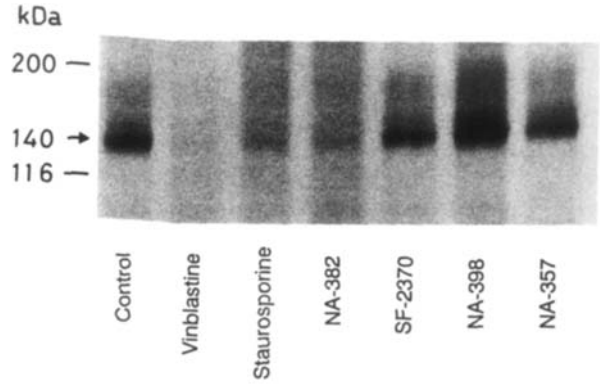


FIG. 3. Effects of staurosporine derivatives on photolabelling with 200 nM [^{3}H]azidopine on 140 kDa P-glycoprotein in membrane preparations from P388/ADR cells. Concentration of vinblastine and other compounds was 10 μM.

of vinblastine on P388/ADR cells (Table 3). As NA-382 was less cytotoxic, we could use up to 1 μM in the combined cytotoxicity test. While P388/ADR cells were resistant to vinblastine to about 10-fold numbers of the parent cells, NA-382 decreased the IC50 of VBL for P388/ADR cells ($55 \pm 2{\cdot}5$ nM) to $4{\cdot}9 \pm 0{\cdot}7$ nM and completely inhibited the drug resistance of P388/ADR cells (Table 3) without influence on the effect of vinblastine on P388/S cells (IC50: $5{\cdot}6 \pm 1{\cdot}1$ nM).

Discussion

Staurosporine is an antibiotic alkaloid having a unique structure and a potent inhibitory activity on protein kinases (Omura et al 1977; Tamaoki et al 1986). In this study, we investigated the correlation between the activities of staurosporine derivatives on protein kinases and the vinblastine resistance of P388/ADR cells. None of the compounds tested had an inhibitory activity on either A- or C-kinases greater than that of staurosporine. The accumulation of vinblastine in P388/ADR cells was greater in the presence of tetrahydropyran compounds than with tetrahydrofuran compounds, but there was no clear correlation between activities against protein kinases and drug resistance (Table 1). Most of the compounds were highly cytotoxic and at their non-cytotoxic concentration neither increased vinblastine accumulation nor potentiated the growth inhibitory effect of vinblastine. When the lactam moiety of *N*-ethoxycarbonyl-staurosporine (NA-381) was converted to the imide (NA-382), the selectivity for inhibitory activity on C-kinase was increased, and the cytotoxicity was markedly decreased, but the effect on drug accumulation was retained at the same level or greater. NA-382 at 1 μM completely restored the vinblastine resistance of P388/ADR cells.

Many investigators have indicated that multidrug-resistant tumour cells overexpress 150 to 180 kDa P-glycoproteins as a drug-elimination pump in the plasma membrane (Akiyama et al 1985; Kartner et al 1985; Riordan et al 1985). Recently, we indicated that P388/ADR cells overexpressed a 140 kDa P-glycoprotein in the plasma membrane. There is some evidence for regulation of the function of P-glycoprotein by protein kianses. For example, P-glycoprotein was phosphorylated by A- and C-kinases under conditions stimulating these kinases (Center 1985; Mellado & Horwitz 1987; Hamada et al 1987; Chambers et al 1990) and a decrease of vincristine accumulation by phorbol esters was cancelled by a C-kinase inhibitor 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (Ido et al 1986). Sato et al (1990) suggested that the function of P-glycoprotein may be regulated by phosphorylation through C-kinase because staurosporine enhanced accumulation of vincristine in multidrug-resistant cells. In this study, we have shown increased drug accumulation, and reversed drug resistance under unstimulated conditions regardless of their protein kinase-inhibitory activities. It seems that the promotion of drug accumulation may be provided by particular characteristics of these compounds rather than by their inhibitory activity on protein kinases.

It has been reported that many kinds of drugs having in common cationic and hydrophobic groups in their molecule bind to the P-glycoprotein competing with the antitumour drugs (Akiyama et al 1988; Naito & Tsuruo 1989). Staurosporine and its derivatives are antibiotic alkaloids and also have these properties. Indeed, staurosporine and NA-382 inhibited photolabelling with [^{3}H]azidopine on the protein. The tetrahydrofuran compounds, which showed low potency for drug accumulation, had low affinity for P-glycoprotein. These results suggest that staurosporine derivatives interfere with the function of the drug elimination system by their direct action on P-glycoprotein rather than by their actions on protein kinases, and low toxic compounds such as NA-382 can reverse multidrug resistance.

Acknowledgements

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