REVIEW ARTICLE

# Staurosporine, a Potentially Important Gift from a Microorganism

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## I. Discovery of Staurosporine

Staurosporine (Str) was discovered in the course of screening for microbial alkaloids in 1977[1], of which stucture was elucidated to be an indolo [2,3-a] carbazole (IC) derivative in the next year[2,3], and has been shown to possess various biological activities, including hypotensive activity and inhibitory activity of platelet aggregation other than antifugal activity soon after its discovery[2,4,5]. Since TAMAOKI *et al.* reported Str to have potent inhibitory activity for protein kinase C (PKC) in 1986[6], it has been a subject of great interest and the number of Str-related reports has increased every year. In particular, in 1992, the number of papers whose titles contain Str reached over 450. Thus, Str became a very important compound, which in used frequently for various biochemical experiments, and its derivatives are potential medicines.

Various kinds of bioactve compounds of microbial origin have been discovered by screening; as a result, the number of newly discovered-substances which are non-antibiotics has been increasing every year[7]. Since 1990, the ratio of the number of non-antibiotics has exceeded 50% of the total number of secondary metabolites discovered in each year. Under the circumstances, a large number of substances have been identified with Str, because it possesses various activities[6,8]. Consequently, detailed information on biological activities of Str has accumulated. Furthermore, a number of novel IC alkaloids from microorganisms have been discovered especially by high performance liquid chromatography. Based on these reports, the essential site on the IC skeleton for expression of its activity has been made clear. Thus, the birth of IC alkaloids useful for medicine is expected in parallel with development of research on the relationship between structure and activity of these compounds.

Alkaloid is a name given to naturally occurring nitrogen-containing compounds that possess biological activities. Up to the time that microbial alkaloids, including Str, were discovered by us, the main source of alkalois other than ergot alkaloid had been higher plants.

Table 1. Taxonomic comparison of strain AM-2282 with related genera.

| Genus | Whole-cell sugar | | | Phospholipid type | Predominant menaquinone |
|---|---|---|---|---|---|
| | Ara | Gal | Rham | | |
| *Amycolata* | + | + | - | P III | MK-8 (H2, H4) |
| *Amycolatopsis* | + | + | - | P II | MK-9 (H2, H4) |
| *Nocardilopsis* | | N.D. | | P III | MK-10 (H4) |
| *Pseudonocardia* | + | + | - | P III | MK-9 (H4) |
| *Saccharopolyspora* | + | + | - | P III | MK-9 (H4) |
| *Saccharothrix* | - | + | + | P II or P IV | MK-9 (H4) |
| Strain AM-2282 | - | + | + | P II or P IV | MK-9 (H4) |

N.D.; Not detected

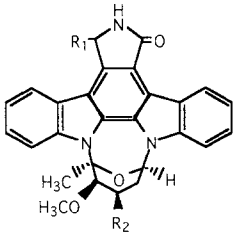
The usual strategy of screening for antibiotics missed other bioactive substances that were produced by microorganisms. Therefore, it was planned to find new substances, and then to examine their biological activities. Based on such concept, the screening of alkaoids produced by microorganisms was begun around 1973[4]. Briefly, after isolation of small amounts of new Dragendorf-positive compounds, they were examined for various biological activities using small animals or cultured cells. Several alkaloids, including pyrindicin[9], NA-337A[10], TM-64[11], quinoline-2-methanol[12], 1,3-diphenetylurea[13] and ditylomycin[14] were discovered to be produced by actinomcetes as well as Str. Furthermore, herquline[15] and neoxaline[16] were discovered by methods which had been used for detection of fungal alkaloids.

The producing strain of Str (AM-2282) was classified originally to the genus *Streptomyces*[1]. At present, it has been re-classified to the genus *Saccharothrix* by various taxonomic studies, including cultural and morphological characteristics and chemical analysis (Table 1).

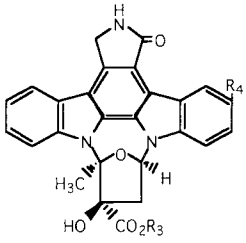
## II. Chemical Properties of Staurosporine

In some cases, prior to discovery of a target protein(s) or its biological function(s), a pharmacologically active compound is revealed to involve a target protein in cellular reactions by negative staining of the functional protein. The target protein for Str was unknown until TAMAOKI *et al.* discovered the inhibitory action of Str in 1986[6]. We are now able to confirm the effect on these biological functions as inhibition of serine/threonine or tyrosine kinases. The discovery of its biochemical activity rekindled studies on Str-sensitive protein kinases and their biological functions. Following the discovery of PKC inhibition by Str *in vitro*, tyrosine kinase inhibition was reported by NAKANO *et al.* in 1987[17]. One year later, myosin light chain (MLC) kinase inhibition was also reported[18]. After that, the inhibitory activity of Str on other kinds of protein kinases, including cdc2 kinase,

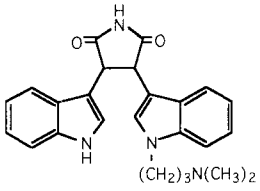
Fig. 1. Structures of Str and other IC-related compounds.



| | R1 | R2 |
|---|---|---|
| Str | H | $NHCH_3$ |
| UCN-01 | OH | $NHCH_3$ |
| UCN-01-Me | $OCH_3$ | $NHCH_3$ |
| RK-286C | H | OH |
| CGP41251 | H | $N(COPh)_2CH_3$ |



| | R3 | R4 |
|---|---|---|
| K-252a | $CH_3$ | H |
| KT5720 | $(CH_2)_5CH_3$ | H |
| KT5926 | $CH_3O(CH_2)_2$ | $CH_3$ |



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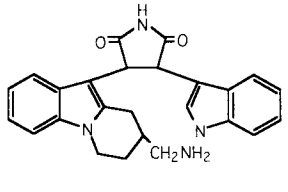


Ro31-8425

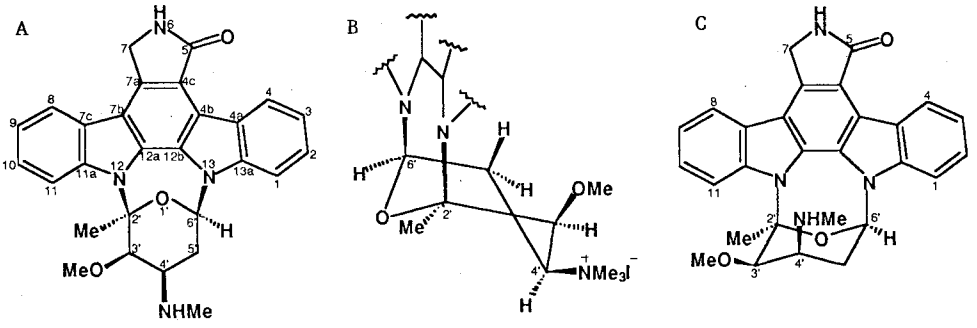
was revealed[19]. Thus, Str is recognized as a "highly potent but non-specific" inhibitor, in common with other protein kinase inhibitors which are competitive with respect to ATP, these include isoquinoline sulfonamide derivatives (H-7, H-9)[20] and IC derivatives (K-252a, KT compounds, RK compounds). Structures of Str and other IC-related compounds cited in this paper are shown in Fig. 1.

### A. Structure

As Str has highly potent and multiple biological activities, many laboratories have attempted to accomplish the total synthesis and to obatin useful derivatives. However, the absolute configuration of Str remained unclear for a long time; only the relative structure was elucidated by X-ray crystallographic analysis of its methanol solvate by FURUSAKI *et al.*[2,3]. Recently, FUNATO *et al.* determined the absolute configuration of Str by X-ray analysis of crystals of 4′-*N*-methylstaurosporine methiodide[21], because it was difficult to obtain a suitable crystal of Str itself.

As shown in Fig. 2-A and -B, the X-ray analysis of 4′-*N*-methylstaurosporine methiodide reveals the stereochemistry with 2′*S*, 3′*R*, 4′*R*, 6′*R*-configuration and a boat form conformation in the amino sugar moiety. From the results of the X-ray analysis, the absolute stereostructure

Fig. 2. Structures of Str (A and C). Dimethyl ammonium iodide derivatives (B) takes a boat conformation of the sugar moiety, but Str takes a chair form.

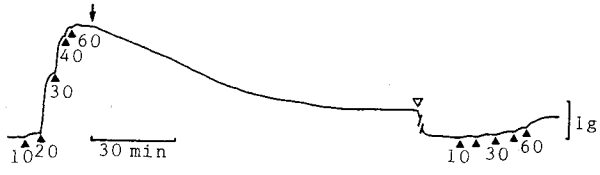



of Str was assumed to have the $2'S, 3'R, 4'R, 6'R$-configuration in a chair form of the amino sugar moiety (Fig. 2-C). It is interesting that the conformation of the amino sugar moiety of compounds 2 and 3 (Fig. 2-A) having the 4'-ammonium group is the same boat form in both solid and liquid states. This result arises a problem as to which conformation of the amino sugar moiety does protein kinase(s) require, chair or boat form. This problem is very important in the design of more potent and useful inhibitors.

## B. Hydrophobicity

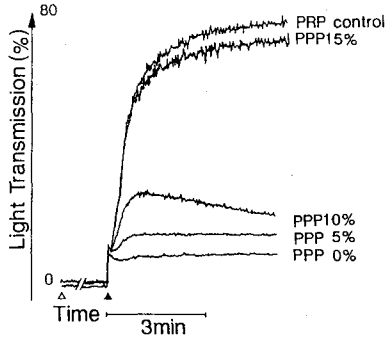
As Str is highly hydrophobic and basic, its biochemical activity may depend on experimental conditions such as the hydrophobicity and pH of the reaction mixture, and on exposure time. These conditions allow and enhance the tight binding of Str to hydrophobic and acidic components of cells or tissues. We demonstrated that the inhibitory effect on the contraction of rabbit aortic smooth muscle is sustained or even enhanced, even after repeated washing with fresh medium (Fig. 3)[22]. TAMAOKI *et al.*[6] detected that long term exposure of Str to HeLa $S_3$ cells enhances the inhibitory effect on cell growth; $IC_{50}$ values were $2.8 \times 10^{-7}$ M for 1 hour exposure and $4.1 \times 10^{-12}$ M for 72 hours exposure. Thus, when we apply it to whole cells and tissues, we have to check its membrane penetration, the distribution of Str in the cells and whether or not it reaches the target protein(s). If not, we might be misled about Str-sensitive reactions. There is a question as to why Str exhibits a low ability to inhibit platelet aggregation in platelet-rich-plasma (PRP), despite the fact that it is a highly potent inhibitor of PKC and MLC kinase *in vitro*. An $ED_{50}$ value of around 10 $\mu$M exists for platelet aggregation, where *Ki* values of 4 nM and 5 nM have been observed for PKC and MLC kinase, respectively. To study this discrepancy,

Fig. 3. Tracing of isometric tension recordings in strips of rabbit aorta.




The strips were contracted with cumulative additions of KCl (10~60 mM) and then exposed to 0.1 $\mu$M Str at the point indicated by the arrow. The effect of Str reached a plateau at 60 minutes after its addition. After repeated washing with fresh medium, the contraction of the strip remained suppressed.

Fig. 4. Influence of plasma on inhibitory activity of Str against platelet aggregation.




Platelet-poor plasma (up to 15% of total volume) was added into a reaction mixture containing washed platelets. The reaction mixtures were incubated with 1 $\mu$M Str for 3 minutes and then stimulated by 1 $\mu$M $U_{46619}$. Open and closed triangles indicate points of Str and $U_{46619}$, respectively.

we made use of guinea-pig patelets, washed and in PRP. $ED_{50}$ value for the inhibition of washed platelets was 50 nM, whereas that of platelets in PRP was 10 $\mu$M. As shown in Fig. 4, addition of plasma to the reaction mixture containing washed platelets reversed the inhibitory effect of Str (1 $\mu$M), and 15% plasma almost

Fig. 5. Inhibition patterns of Str against protein kinases.

MLC kinase (A), PKC (B) and cdc kinase (C). Reciprocal velocity is plotted *versus* 1/[ATP]. Kinase activities were assayed in the presence or absence of each concentration of Str.

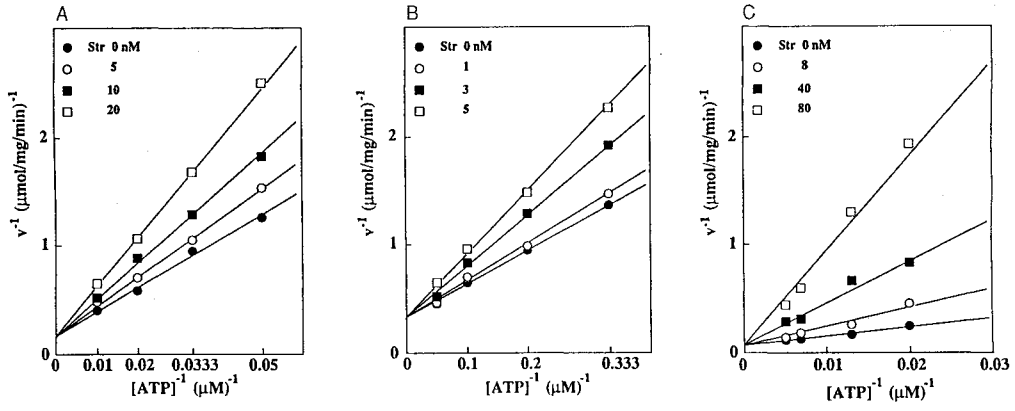



Table 2. Inhibitory effects of Str and other Indolo carbazole derivatives on several kinds of protein kinase.

| Inhibitor | Ki or IC50 value (nM) | | | | | | Reference |
|---|---|---|---|---|---|---|---|
| | PKC | PKA | PKG | PTK | MLCK | cdc2kinase | |
| Str | 2.7 | 22 | 8.5 | 6.4 | 5 | 4 | 6, 17, 18, 19, 25 |
| K-252a | 25 | 18 | 20 | - | 20 | - | 26 |
| KT5720 | >2000 | 60 | >2000 | - | >2000 | - | 27 |
| KT5926 | 72 | 1200 | 160 | - | 18 | - | 30 |
| RK-286C | *3000 | - | - | - | - | - | 29 |
| Ro31-8425 | *8 | *2800 | - | - | - | - | 33 |
| GF109203 | *10 | *2000 | - | - | - | - | 34 |
| UCN-01 | *4 | *4 | - | - | - | - | 31 |
| CGP41251 | *50 | *2400 | - | - | - | - | 32 |

-; Not tested
* indicated with IC50 values. # PKC; protein kinse C, PKA; cyclic AMP dependent protein kinase, PKG; cyclic GMP dependent protein kinase, PTK; tyrosine kinase, MCLK; myosin light chain kinase

completely abolished it. The $ED_{50}$ value changed from 50 nM to 10 $\mu$M in the reaction mixture containing 15% plasma. We attributed the difference in $ED_{50}$ values between the PRP and washed platelets to the adsorption to the plasma protein, probably due to hydrophobic interaction.

### C. Binding Domain of Str

As shown in Fig. 5, Str inhibits the activities of several protein kinases in a competitive manner with ATP, and the extents of inhibitory activities are close to each other (Table 2), suggesting that Str is a so-called "non-specific inhibitor". This supposition may result from the fact that the serine/threonine kinases, such as PKC, MLC kinase, cyclic nucleotide-dependent protein kinases and cdc2 kinase, are likely to conserve the amino acid sequence and 3-D structure of the ATP binding domain (Table 3)[23]. The ATP binding domains of the protein kinases consisit of three functional domains, phosphate anchor, catalytic loop and bottom cleft (hydrophobic) structures. These three domains could anchor the ATP-competitive inhibitor, but not always the same domain. HERBERT *et al.* demonstrated that [$^3$H] Str binds to some sites in the ATP binding domain other than that for isoquinoline sulfonamide H-7[24]. Our group using computer simulation found that H-7 possibly fits and binds to the phosphate anchor and the part of bottom cleft in the ATP binding domain of PKC (unpublished data.). These results indicate that the binding domain for Str is quite different from that of H-7. As shown in Table 3, the amino acid sequences of the phosphate anchor and catalytic loop domains seem to be comparatively well conserved among serine/threonine kianses but that of bottom cleft domain is variable. If so, it should be possible to obtain a comparatively specific inhibitor of serine/threonine kinases. In fact, some ATP competitive inhibitors were reported to be comparatively specific (Table 2). To obtain further specific and useful protein

Table 3. Sequence alignment of ATP binding sites in several serine/threonine kinases.

| Protein kinase | Phosphate Anchor | Catalytic Loop | Bottom of Cleft |
|---|---|---|---|
| | 49 57 | 165 171 | 119 125 |
| Protein kinase A | ---LGTGSFGRV--- | ---RDLKPEN--- | ---VMEYVPG--- |
| Protein kinase G | ---LGVGGFGRV--- | ---RDLKPEN--- | ---LMEACLG--- |
| Protein kinase C | ---LGKGSFGKV--- | ---RDLKLDN--- | ---VMEYVNG--- |
| MLC kinase | ---LGSGKFGAV--- | ---LDLKPEN--- | ---FMEYIEG--- |
| cdc2 kinase | ---IGEGTYGVV--- | ---RDLQPEN--- | ---VFEFLDM--- |

kinase inhibitors, we must consider the 3-D structure of the three functional domains and their characteristics.

## III. Inhibitory Activity of Staurosporine against Protein Kinases *In Vitro*

### A. Protein Kinase C and Myosin Light Chain Kinase

MLC kinase and PKC are $Ca^{2+}$-sensitive key enzymes for intracellular $Ca^{2+}$ responses of cells and tissues. The former is activated by the calmodulin-$Ca^{2+}$ complex and phosphorylates myosin light chain followed by activation of actin-myosin ATPase. The latter is activated by micellar complex of $Ca^{2+}$ and phosphatidylserine, phosphorylates various kinds of protein which have the consensus domain, X-Arg(or Lys)-X-X-Ser-X-Arg, and acts on signal transduction. The phosphate anchor and catalytic loop domains in the ATP binding sites of both PKC and MLC kinase have quite similar amino acid sequences: Leu-Gly-Lys-Gly-Ser-Phe-Gly-Lys-Val and Leu-Gly-Ser-Gly-Lys-Phe-Gly-Ala-Val, and Arg-Asp-Leu-Lys-Leu-Asp-Asn and Leu-Asp-Leu-Lys-Pro-Gln-Asn, respectively. Therefore, Str has similar *Ki* values with respect to ATP: 5 nM for MLC kinase[25] and 4 nM for PKC. Other IC derivatives, such as K-252a[26], KT-5720[27], UCN-01[28] and RK-286C[29], show a similar tendency, as described in Table 2. However, substitution of modification of the aglycone moiety modulates the specificity and inhibitory potential of the compounds. KT-5926 having substituent-$O(CH_2)_2CH_3$ at the 3 position of K-252a was found to be a MLC kinase-specific inhibitor, *i.e.* it was about 65-fold more potent than with protein kinase A[30]. Although the precise interaction mechanism of Str at the ATP binding site of both kinases is not yet known, the ATP binding domain may not require an acidic group in the aglycone moiety of Str. Substitution at the 3 and/or 9 position with the $-COOH$ group markedly lowered the inhibitory activity; *Ki* values of mono- and di-carboxy-Str for MLC kinase were 416 nM and 8 $\mu$M, respectively (unpublished data). However, substitution at the 3 and/or 9 position with a $-OH$ or $-NH_2$ group(s) did not alter the inhibitory activities against PKC and MLC kinase. On the other hand, comparatively specific inhibitors of PKC were isolated from fermentaion broths of microorganisms and characterized UCN-01-Me[31] and CGP 41251[32]. The fact that "specific compounds" were isolated or synthesized suggests that IC derivatives recognize small differences in the 3-D structure of the ATP binding site between PKC and other protein kinases, *e.g.* (protein kinase A).

### B. Protein Kinases A and G, and Other Serine/Threonine Kinases

Str inhibits two cyclic nucleotide-dependent protein kinases with *Ki* values similar to those for MLC kinase and PKC (Table 2). The inhibitory activity of Str derivatives against protein kinase A is decreased by addition of a methoxyl group at the 7 position of Str (UCN-01-Me). Also substitution of the $N^{4'}$ hydrogen with a benzoyl group (CGP 41251) lowers the inhibitory activity against protein kinase A, but increases the specificity with respect to PKC. Other IC derivatives such as K-252a and UCN-01 inhibit protein kinase A and PKC to similar extents. Bis-indolilmaleimido-related compounds, Ro 31-8425[33] and GF109203X[34] have 10~100 fold less inhibitory activity against protein kinase A than against PKC. Thus, differences in the inhibitory activity of Str against PKC (or MLC kinase) and protein kinase A may result from differences in 3-D structure and the characteristics of the ATP binding domain. If so, it should be possible to obtain specific inhibitors of protein kinase A or G.

Str at a comparatively low concentration is known to arrest non-transformed cells in the $G_1$ phase of the cell cycle (see "Blocking of cell cycle"), but the precise mechanism is unclear. Since the cdc2·cyclin family of protein kinases are involved in the onset of differentiation and mitosis, the effect of Str on these protein kinase activities was determined. Str inhibits the phosphorylation of the S-1 peptide of $H_1$ histone by $p34^{cdc2}$ kinase isolated from FM3A mouse mammary carcinoma cells,

with an $IC_{50}$ value of 4 nM[19]. Recently, we confirmed that Str inhibits cdc2·cyclin B kinase activity competitively with respect to ATP, as shown in Fig. 5. In contrast, Str inhibits the activity of CaM kinase II in a non-competitive manner with ATP, calmodulin and a phosphate acceptor ($\beta$-casein), suggesting that Str interacts with other catalytic domains distinct from the ATP binding or substrate binding sites[35]. This suggests that Str does not always bind to the ATP binding site of a serine/threonine kinase.

### C. Tyrosine Kinases

It was the first report by NAKANO *et al.* in 1988 that Str inhibits the autophosphorylation of $p60^{v\text{-}src}$ by the lysates of RSV-CEF cells with an $IC_{50}$ value of 6.4 nM[17]. The inhibitory extent was quitely similar to those of other serine/threonine kinase inhibitors. Later, several kinds of inhibition of growth factor-associated tyrosine kinase activity were reported. FUJITA-YAMAGUCHI and KATHURIA[36] demonstrated that Str inhibits the activity of insulin receptor tyrosine kinase from human placenta 10 and 100 times more potently than EGF-and IGF-associated receptor kinases, respectively; $IC_{50}$ values of 61 nM, 630 nM and 6,150 nM, respectively, were observed. Unlike the case of PKC inhibition, the inhibitory mode of insulin receptor tyrosine kinase appears to be non-competitive with ATP. SECRIST *et al.*[37] reported the inhibitory effect of submicromolar Str on platelet-derived growth factor (PDGF) receptor tyrosine kinase. Str inhibits the autophosphorylation of PDGF receptor fractions (partially purified from Swiss 3T3 cells) by 25% at 100 nM and completely inhibits at 1 $\mu$M. However, the inhibitory mechanism remains unclear. Tyrosine kinase activity of soluble epidermal growth factor receptor in a membrane fraction of A-431 cells is 10 to 100-fold less sensitive than the PDGF receptor kinase activity. They also made mention that tyrosine phosphorylation in Swiss 3T3 cells in closely correlated with supperssion of phosphoinositide phosphorylation and of tyrosine phosphorylation of a 145KD protein. Recently, YATOMI *et al.*[38] demonstrated that Str suppresses the tyrosine phosphorylation of 64, 97 and 125KD proteins in thrombin-stimulated human platelets, but inhibitory parameters ($IC_{50}$, *Ki* value) and the inhibitory mechanism were not clarified. Thus, it is clear that Str is a potent inhibitor of not only serine/threonine kinases but also tyrosine kinases.

## IV. Biological Activities of Staurosporine

### A. Relaxation of Smooth Muscle

Str and IC derivatives inhibit smooth muscle contraction, but the precise inhibitory mechanism is unknown except for the inhibition of MKC kinase[30,39]. We reported Str (5 ~ 200 nM) to inhibit both intracellular and extracellular $Ca^{2+}$ dependent contractions of rabbit aortic strips induced by various constrictors (Fig. 6)[22] and of cultured smooth muscle cells associated with MLC phosphorylation (Fig. 7)[40]. We also found that Str did not affect the transient increase in intracellular $Ca^{2+}$ in cultured smooth muscle cells evoked by $PGF_{2\alpha}$ employing intracellular $Ca^{2+}$ stores (submitted for publication). In contrast, Str induced increases of intracellular $Ca^{2+}$ in cultured smooth muscle cells[41]. Since Str inhibits 5-phosphomonoesterase activation followed by accumulation of Ins(1,4,5)P3 in aggregating platelets[42], the compound possibly enhances and sustains an increase of intracellular $Ca^{2+}$ in the agonist-induced artery, because receptor agonists may activate phospholipase C producing diacylglycerol and Ins(1,4,5)P3. However, there are many contradictory data on the involvement of Str in intracellular $Ca^{2+}$ mobility in smooth muscle. KAGEYAMA *et al.*[43] reported that low concentrations of Str inhibit the contraction of arterial smooth muscle evoked by high $K^+$ and norepinephrin through PKC inhibition and high concentrations of Str act by $Ca^{2+}$ influx inhibition. Recently, we found that Str in the range of 50 ~ 100 nM suppresses L-channel-dependent $Ca^{2+}$ influx of isolated rabbit carotid artery[44], suggesting that some portions of the inhibitory effect of Str on smooth muscle contraction by agonists are due to inhibition of the voltage-dependent $Ca^{2+}$ channel. However, the kinds of protein kinase or the existence of other Str-sensitive mechanisms that are involved in voltage-dependent channel activity are not yet known. At this time, we attribute the major portion of the inhibitory effect on smooth muscle contraction to MLC kinase inhibition, because MLC phosphorylation plays an important role in the downstream portion of the contraction pathway. Since Str inhibits a wide variety of protein kinases *in vitro*, including both serine/threonine and tyrosine kinases, and the involvement of these kinases on smooth muscle contraction has been recognized, we must determine whether the inhibitory effect of Str on the contraction is associated with a Str-sensitive mechanism(s) other than MLC kinase or PKC.

Fig. 6. Effects of Str on $PGF_{2\alpha}$ (A, C) and endothelin (END; B, D)-induced contraction of rabbit aortic strips.

The maximum contractile tension was achieved with 10~5-$\mu$M $PGF_{2\alpha}$ (A) and 1 $\mu$M END (B) in the presence of 1.2 mM external $Ca^{2+}$; (○) control; (◇) 5 nM; (△) 25 nM; (●) 50 nM; (◆) 100 nM Str (C) and (D) show rabbit aortic contractions induced with 10 $\mu$M $PGF_{2\alpha}$ and 0.1 mM END in the presence (open column) and absence (shadow column) of external $Ca^{2+}$, respectively.

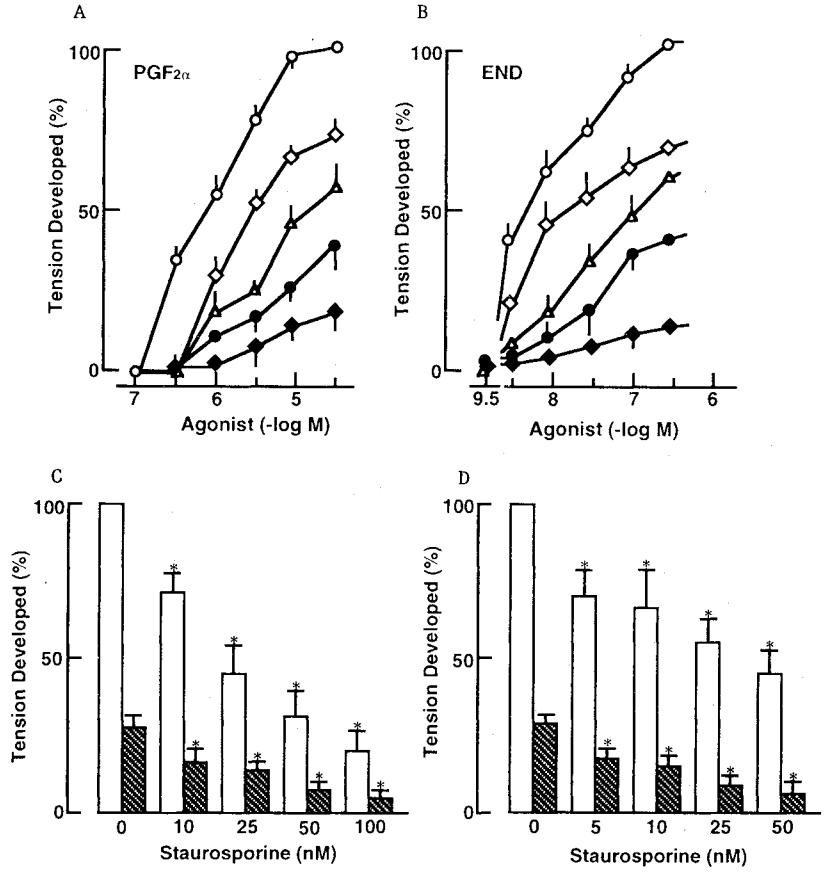
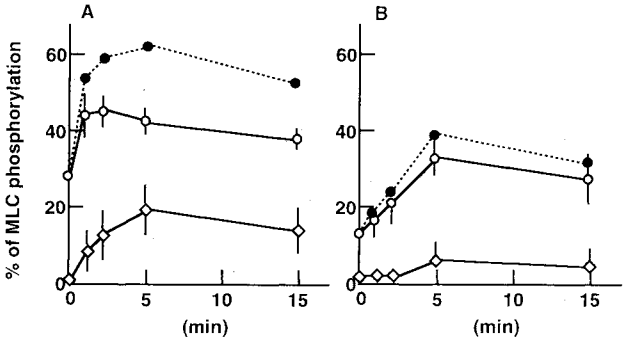



Fig. 7. MLC phosphorylation in control (A) or Str-related (B) smooth muscle cells in culture (SM-3).

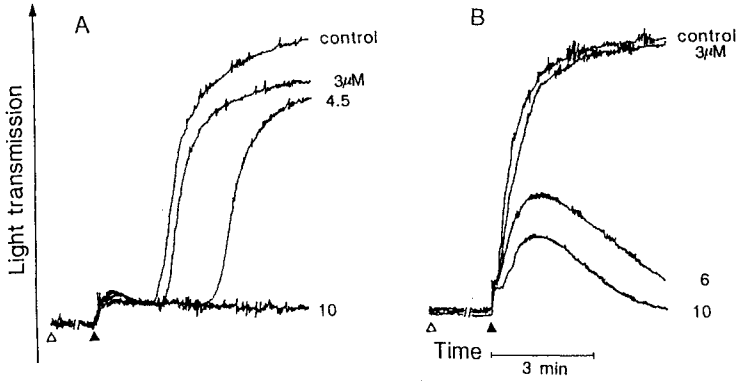
The cells were challenged with 30 $\mu$M $PGF_{2\alpha}$; (○) monophosphorylated, (◇) diphosphorylated, (●) total phosphorylated MLCs.




## B. Inhibition of Platelet Aggregation

In 1980, the inhibition of platelet aggregation by Str was suggested[5] and later this was confirmed experimentally by OKA *et al.* in 1986[8], prior to the discovery of its target protein. Str is now known to widely inhibit various types of platelet aggregatin induced by aggregants, including collagen, thrombin, $U_{46619}$, epinephrine with similar $IC_{50}$ values, except for ADP-induced aggregation, as shown in Fig. 8 and Table 4. Low concentratins of Str (~25 nM) exibit little or no effect of ADP-induced aggregation of human washed platelets, whereas this concentration strongly inhibits either 5HT release or aggregation induced by thrombin[45]. The former reaction seems not to be associated with phospholipase C activation and the latter with the formation of diacylglycerol and PKC activation, but the precise PKC-pathway involved in platelet aggergation is still unknown. High concentrations (200~400 nM) of Str do inhibit ADP-induced aggregation. As shown in Fig. 9, as well as K-252a[46], Str inhibits dose-dependently the MLC phosphorylation of platelet, parallel with aggergation. Thus, the inhibitory action of Str on platelet aggergation may be primarily due to inhibition of MLC phosphorylation. KING and RITTENHOUSE[41] proposed another effect on intracellular $Ca^{2+}$ movement in human

Fig. 8. Inhibitory effects of Str on platelet aggregation induced by collagen (A) and $U_{46619}$ (B) in guinea-pig platelet-rich plasma (PRP).



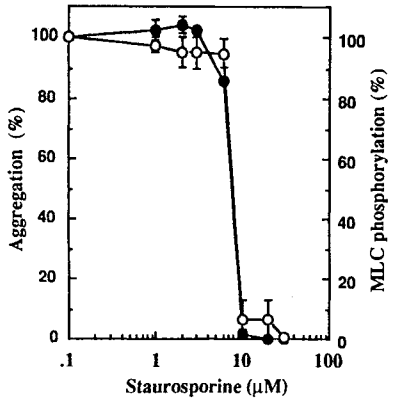

Platelets were preincubated with Str for 3 minutes before stimulation. Platelet aggregation was induced by addition of 2 μg/ml collagen or 1 μM $U_{46619}$. Open and closed triangles indicate the points of addition of Str and $U_{46619}$, respectively.

Table 4. Comparison of $IC_{50}$ values of Str against aggregation of platelets in PRP and "washed platelets" (WP).

| Agonist | IC50 value (μM) | |
|---|---|---|
| | PRP | WP |
| U46619 (1μM) | 8.1 ±0.23 | 0.15 ±0.042 |
| ADP (2μM) | 5.5 ±0.15 | 0.43 ±0.087 |
| Collagen (2μg/ml) | 5.2 ±0.16 | 0.11 ±0.016 |

Washed platelets were obtained by gel-filtration. Platelet density was adjusted to 6 x $10^8$ platelets/ml. The platelets were activated with 1μM U46619, 2μM ADP or 2μg/ml collagen. IC50 values were determined from each maximal aggregation vs. control. Each value represents the mean ±S.E. values are from four separate experiments.

Fig. 9. Inhibitory effects of Str on guinea-pig platelet aggregation and $MLC_{20}$ phosphorylation.




Platelets in PRP were stimulated by 1 μM $U_{46619}$ in the presence of various concentrations of Str. $MLC_{20}$ phosphorylation was measured by immunoblot analysis. Open and closed circles indicate the rates of platelet aggregation and $MLC_{20}$ phosphorylation, respectively. Each value represents the mean ± S.E. values from four separate experiments.

platelets exposed to thrombin. Str might induce the accumulation of Ins(1,4,5)P3 in thrombin-induced platelets, probably due to suppression of 5-phosphomonoesterase activation by PKC, thereby elevating and sustaining intracellular $Ca^{2+}$ levels. Conversely, Str strongly inhibits wheat germ aggulutinin-induced intracellular $Ca^{2+}$ transfer in a dose-dependent manner[47]. The Str effect on the $Ca^{2+}$ signal may depend on the activation pathway employed by the aggregant. Furthermore, YATOMI *et al.*[48] showed Str at 1 μM to inhibit tyrosine phosphorylation of 64, 97, and 125KD proteins in human platelets induced by thrombin. It is clear that if we can improve the specificity of Str against protein kinases, it might be possible to obtain more powerful and useful inhibitors of platelet aggregaion, hopefully being applicable to the clinic.

### C. Neurotrophic Activity

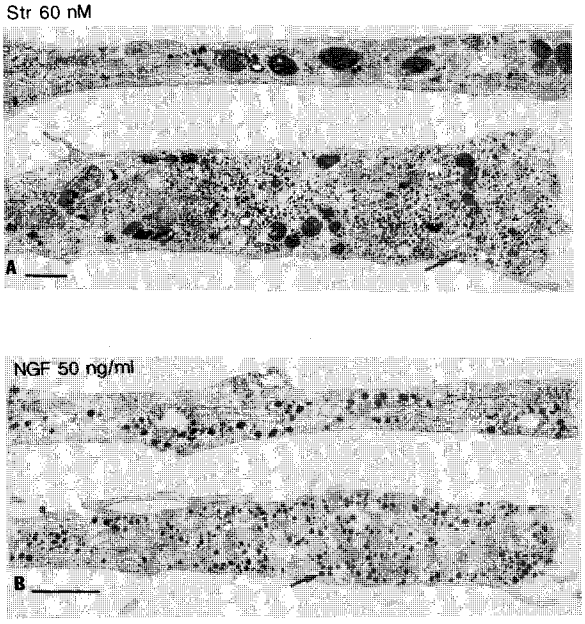
Str has dual actions on neurite outgrowth of cultured sympathetic neurons. HASHIMOTO and HAGINO[49] reported a low concentration of Str (10 nM) to inhibit almost completely the priming effect of NGF on RNA-transcription of PC12 cells, whereas higher concentrations (30 ~ 100 nM) promoted the rapid generation of neurites in a dose-dependent manner. However, the low concentration promoted neurite generation of NGF-primed PC12 cells[50]. The neurites of PC12 cells generated with comparatively high concentrations of Str have a characteristic structure similar to that induced with NGF, as shown in Fig. 10. The growth corns in cells treated with 60 nM Str (Fig. 10-A; lower picture) and 50 ng/ml NGF (Fig. 10-B; lower picture) contain numerous electron dense bodies of 90 ~ 110 nm in diameter and intermediate filaments and microtubular structures, and the neurite objects also contain filamentous structures

(Fig. 10; upper picture). Recently, OHMICHI *et al.* found that Str in the range of 10~1000 nM inhibits NGF-dependent tyrosine phosphorylation of $p140^{c\text{-}trk}$ in PC12 cells[51]. Similar results were obtained with another IC derivative, K-252a[52]. However, it is not completely established that the inhibition of these protein phosphorylations is involved in differentiation and neurite outgrowth of PC12 cells. These results indicate that Str at high concentraions could induce neurite outgrowth through a rearrangement of neurite components, thus act as a low molecular mass neurotrophic factor. Also in the presence of NGF, Str acts even at low concentrations.

We and an other group tried to elucidate the Str effect *in vivo*, using a number of experimental systems. Effects of Str on the impairment of learning in rats , in which the basal forebrain was injured with electric heating or ibotenic acid, were studied in Morris' water maje[53]. Chronic oral administration of Str (0.03~0.1 mg/kg), after the lesion reduced significantly the latent time for the goal, as compared with rats injured but not given Str (Fig. 11). However, Str treatment could not completely prevented the impairment, as did sham operated rats.

OHNO *et al.* demonstrated another effect of Str on the impairment of working memory in rats exposed to cerebral ischemia[54]. Oral administraion of Str (0.03~0.1 mg/kg) immediately after blood flow reperfusion reduced the number of errors in attempts to pass through two incorrect panels of the three panel-gates at four choice points, but administration 6 hours after ischemia did not lower the number of errors. Since Str has an inhibitory effect on the generation of $O^{2-}$ from activated neutrophils[55], the Str effect may include a protective effect on ischemic injury and neutrophil associated inflammation. Thus, Str or its derivatives may prevent an event occurring after brain hemorrhage followed by ischemia, or prevent some types of dementia.

Fig. 10. Ultrastructure of neurites of PC12 cells induced with 60 nM Str (A) or 50 ng/ml NGF (B).

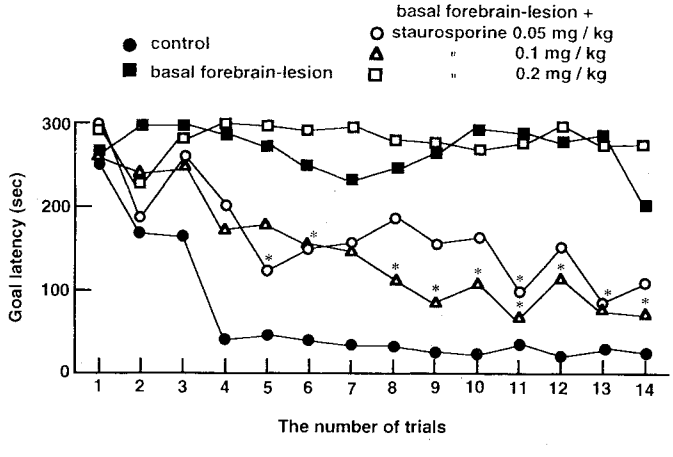



Upper picture of each panel shows neurite object and lower one growth corn containing numerous electron dense bodies (90~11 nm).

### D. Blocking of Cell Cycle

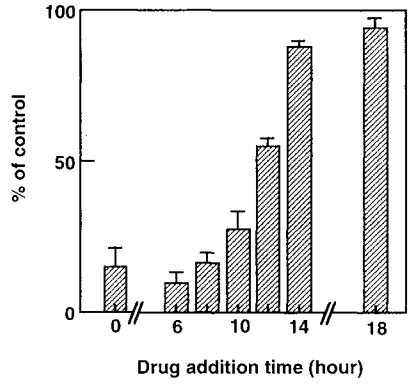
Str is now known to inhibit a variety of protein kinases *in vitro*. Among them are kinases which are related to the cell growth cycle. In 1988, we demonstrated that low concentrations of Str (10~30 ng/ml) block the transition from the $G_0$ to the S phase of cultured rabbit aortic smooth muscle cells stimulated by fetal calf serum[56]. The effective period was within 10 hours after stimulation

Fig. 11. Effect of 4 weeks of administration of Str on the basal forebrain lesion-induced amnesia in the water maze task.




Str (0.05~0.2 mg/kg) was administered for 2 weeks before the task and after each training session.

Fig. 12. Time dependence of the effect of Str on serum-induced transition from the $G_0$ to the S phase of cultured smooth muscle cells.




Str (10 ng/ml) was added at the indicated time points after serum stimulation of quiscent cells. Cell growth is indicated by the DNA synthesis index.

of cells in $G_0$ phase to enter $G_1$ phase, probably in the early or middle $G_1$ phase (Fig. 12). At that time, we did not know that Str acted on protein kinases other than PKC. Later, ABE *et al.* proposed dual actions of Str; low concentrations of Str ($1 \sim 10$ ng/ml) arrest cultured fibroblasts at the early $G_1$ phase and high concentrations ($100 \sim 200$ ng/ml) at the late $G_2$ phase[57]. It is reasonable that the most likely target protein is $p34^{cdc2}$ protein kinase in the early $G_1$ and late $G_2$ phases, rather than PKC, because Str has recently been reported to be a potent inhibitor of cdc2 kinase, and the cdc2·cyclin B activity seems to increase in the $G_1$ phase and the cdk2·cyclin A activity in the late $G_2$ phase[19]. However, we cannot exclude the possibility that Str suppersses the early $G_1$ and/or the late $G_2$-M transition through inhibition of protein kinases other than the cdc2·cyclin family as well as PKC. Thus, if we administer Str to whole animals bearing malignant cells, the result should be analyzed with great care.

### E. Antitumor Activity

Resistance to antitumor agents is a major problem in the treament of cancer. Several types of drug resistance have been characterized in cell lines made resistant to anticancer agents. One of these is the multidrug resistance (MDR) phenotype. Such MDR cells that acquire resistance to a naturally occuring drug, *e.g.*, vinca alkaloids, anthracyclines and epipodophyllotoxins generally show cross-resistance to other antitumor agents possessing different structures and different modes of action[58]. MDR is often associated with the presence of a 170KD glycoprotein, the transmembrane glycoprotein (Pgp)[59], encoded by a multidrug resistance (*mdr* 1) gene. Pgp functions as a rapid drug efflux pump transporting not only antitumor drugs but also verapamil and other $Ca^{2+}$ channel blockers, resulting in a lowered, less toxic, intracellular drug accumulation[60]. Although there exist mechanisms of MDR other than Pgp overexpression, the widespread occurrence of the expression of the *mdr* 1 gene in drug resistance suggests a clinical role in many cancers[61~63]. These observations suggest that an inhibitor of this drug efflux pump would become useful anticancer agent. A number of compounds have been reported to be able to inhibit the efflux of drugs transported by Pgp, or to be effective in reversing MDR *in vitro*, but none has so far found broad clinical application.

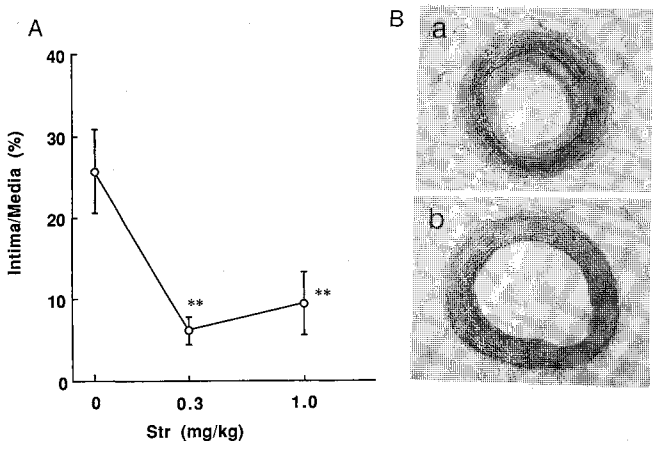
On the other hand, drug accumulation in MDR cells was found to be regulated by protein kinases, in particular *via* PKC-mediated phosphorylation of Pgp[64]. In addition, several tumor cell lines of the MDR phenotype were shown to exhibit increased PKC activity[64,65]. Therefore, an inhibitor of PKC might counter attack MDR.

Although Str had been reported to possess differentiation-inducing activity in a human neuroblastoma cell, NB-1, its mode of action remained unclear. It was considered that Str may increase the intracellular content of cyclic AMP and promote the *de novo* protein synthesis related to elongation of neurites or cell enlargement in NB-1 cells[66]. SATO *et al.* showed Str to inhibit ATP-dependent-vincristine binding to the MDR cell membrane and also azidopine photolabelling of Pgp, suggesting that Str directly binds to Pgp as well as antitumor agents and $Ca^{2+}$ channel blockers and also might be involved in the function of Pgp[67]. Recently, SAMPSON *et al.* demonstrated Str to reduce Pgp expression and modulate MDR[68].

Since str is a non-selective inhibitor of protein kinases, Str-derivatives possessing a higher degree of selectivity for PKC inhibition and PKC-mediated cellular events have been developed. These include Str-derivatives such as $CGP_{41251}$[31] and NA-382[69] inhibited PKC more selectively than Str, although they are much less inhibitory. UTZ *et al.* found that $CGP_{41251}$ not only exhibits antitumor activity, but also reverses MDR[70] after treatment of CCRF-VCR 1000 (a MDR human lymphoblastoid cell line expressing Pgp) with a combination of 500 nM adriamycin and a non-toxic concentration of 150 nM $CGP_{41251}$ ($IC_{50}$ for inhibition of cell proliferation: 420 nM $CGP_{41251}$) inhibited cell proliferation of CCRF-VCR 1000 cells by 29%. It was also shown

Fig. 13. Inhibitory effect of Str on intimal hyperplasia induced by endothelial denudation.

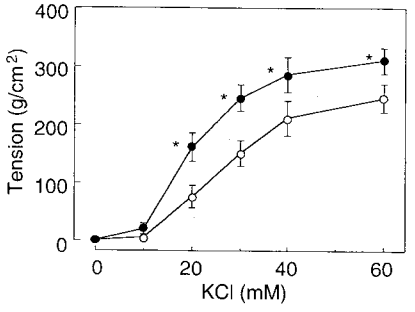
(A) Intimal media ration. (B) Histological cross-sections in the (a) absence and (b) presence of 0.3 mg/kg Str with Elastica-Van-Gieson staining.




** Significantly different ($p<0.01$) from values of control.

Fig. 14. Concentration-response of development for $K^+$.

Each curve was obtained by the cumulative addition of $K^+$ (10, 20, 30, 40 and 60 mM) to the control (○) and hyperplastic (●) artery. Each point is the mean ± S.D. of four experiments.




* Significantly different ($p<0.05$) from values for the control artery.

that treatment of CCRF-VCR 1000 cells with $CGP_{41251}$ for 10 minutes is sufficient to inhibit the efflux of rhodamine 123, a substrate of Pgp. MIYAMOTO *et al.*[71] found that NA-382 at non-cytotoxic concentration reverses effectively *in vitro* MDR of adriamycin-resistant P388 cell, without influencing drug sensitivity of sensitive P388 cell. This compound also reduced vinblastine resistance of other MDR cell lines, AH66 and K562/ADR, by inhibiting vinblastine efflux and promoting vinblastine accumulation. Although MIYAMOTO *et al.* concluded that Str derivatives interfere with the function of the drug extrusion system by their direct action on Pgp, regardless of their inhibitory activity on PKC[71], the exact mechanisim of MDR modulation by these compounds should be part of ongoing investigations. Both $CGP_{41251}$ and NA-382 might be good candidates for cancer chemotherapy of MDR.

### F. Activity against Hyperplastic Cell Growth

To reveal a Str activity against hyperplastic cell growth in whole animals, we made use of an intimal thickening artery model of rabbit induced by the baloon-endothelial injury method. Intimal thickening plaque is associated with hyperplastic smooth muscle cells responding to growth factors such as PDGF and MDGF. Oral administraion of Str (0.3～1.0 mg/kg) every days for 6 weeks after the endothelial denudation prevented in a dose-dependent manner the intimal thickening and hypersensitivity of denuded carotid artery, as shown in Figs. 13 and 14. In particular, upon stimulation with high $K^+$ or $PGF_{2a}$, the Str-treated artery exhibited normal contraction (Fig. 14) with normal levels of MLC phosphorylation, as observed in non-denuded control artery, whereas the artery bearing intimal thickening exhibited 1.5～1.8-fold extent of contraction with high levels of mono and double phosphorylations of MLC (unpublished data). This arises the possibility that Str or its derivatives might supperss intimal thickening and restenosis after angioplasty, which are the most severe events in blood circulation disease.

### References

1) ŌMURA, S.; Y. IWAI, A. HIRANO, A. NAKAGAWA, J. AWAYA, H. TSUCHIYA, Y. TAKAHASHI & R. MASUMA: A new alkaloid AM-2282 of *Streptomyces* origin. Fermentation, isolation and preliminary characterization. J. Antibiotics 30: 275～282, 1977
2) FURUSAKI, A.; N. HASHIBA, T. MATSUMOTO, A. HIRANO, Y. IWAI & S. ŌMURA: X-ray crystal structure of staurosporine: a new alkaloid from a *Streptomyces* strain. J. Chem. Soc., Chem. Commun. 1978: 800～801, 1978
3) FURUSAKI, A.; N. HASHIBA, T. MATSUMOTO, A. HIRANO, Y. IWAI & S. ŌMURA: The crystal and molecular structure of staurosporine, a new alkaloid from a *Streptomyces* strain. Bull. Chem. Soc. Jpn. 55: 3681～2685, 1982
4) ŌMURA, S. & Y. IWAI: Microbial alkaloids. Fermentation and Technology (in Japanese) 37: 223～234, 1979
5) ŌMURA, S.: Searching for new antibiotics. Farumacia (in Japanese) 16: 385～398, 1980
6) TAMAOKI, T.; H. NOMOTO, I. TAKAHASHI, Y. KATO, M. MORIMOTO & F. TOMITA: Staurosporine, a potent inhibitor of phospholipid/$Ca^{++}$ dependent protein kinase. Biochem. Biophys. Res. Commun. 135: 397～402, 1986
7) ŌMURA, S.: Trends in the search for bioactive microbial metabolites. J. Ind. Microbiol. 10: 135～156, 1992
8) OKA, S.; M. KODAMA, H. TAKEDA, N. TOMIZUKA & H. SUZUKI: Staurosporine, a potent platelet aggregation inhibitor from a *Streptomyces* species. Agric. Biol. Chem.

50: 2723～2727, 1986

9) ŌMURA. S.; H. TANAKA, J. AWAYA, Y. NARIMATSU, Y. KONDA & T. HATA: Pyrindicin, a new alkaloid from a *Strepyomyces* strain. Taxonomy, fermentaion, isolation and biological activity. Agric. Biol. Chem. 38: 899～906, 1974

10) ONDA, M.; Y. KONDA, Y. NARIMATSU, H. TANAKA, J. AWAYA & S. ŌMURA: Alkaloid from *Streptomyces* sp. NA-337. Chem. Pharm. Bull. 22: 2916～2920, 1974

11) ŌMURA, S.; Y. SUZUKI, C. KITAO, Y. TAKAHASHI & Y. KONDA: Isolation of new sulfer containing basic substance from a *Thermoactinomyces* species. J. Antibiotics 28: 609～610, 1975

12) ŌMURA, S.; Y. IWAI, Y. SUZUKI, J. AWAYA, Y. KONDA & M. ONDA: Production of quinoline-2-methanol and quinoline-2-methanol acetate by a new species of *Kitasatoa, kitasatoa griseophaeus*. J. Antibiotics 29: 797～803, 1976

13) IWAI, Y.; HIRANO, J. AWAYA, S. MATSUO & S. ŌMURA: 1,3-Diphenethylurea from *Streptomyces* sp. No. AM-2948. J. Antibiotics 31: 375～376, 1987

14) ŌMURA, S.; Y. IWAI, A. HIRANO, J. AWAYA, Y. SUZUKI & K. MATSUMOTO: A new antibiotic, AM-2504. Agric. Biol. Chem. 41: 1827～1828, 1977

15) ŌMURA, S.; A. HIRANO, Y. IWAI & R. MASUMA: Herquline, a new alkaloid produced by *Penicillium herquei*. Fermentaion, isolation and properties. J. Antibiotics 32: 786～790, 1979

16) HIRANO, A.; Y. IWAI, R. MASUMA, T. KEI & S. ŌMURA: Neoxaline, a new alkaloid produced by *Aspergillus japonicus*. Production, isolation and properties. J. Antibiotics 32: 781～785, 1979

17) NAKANO, H.; E. KOBAYASHI, I. THKAHASHI, T. TAMAOKI, Y. KUZUU & H. IBA: Staurosporine inhibits tyrosine-specific protein kinase activity of Rous sarcoma virus transforming protein p60. J. Antibiotics 40: 706～708, 1987

18) WATSON, S. P.; J. MCNALLY, L. J. SHIPMAN & P. P. GODFREY: The action of the protein kinase C inhibitor, staurosporine, on human platelets. Biochem. J. 249: 345～350, 1988

19) GADBIOS, D. M.; J. R. HAMAGUCHI, R. A. SWANK & E. M. BRADBURY: Staurosporine is a potent inhibitor of $p34^{cdc2}$ and $p34^{cdc2}$-like kinases. Biochem. Biophys. Res. Commun.184: 80～85, 1992

20) HIDAKA, H.; M. INAGAKI, S. KAWANOTO & Y. SASAKI: Isoquinoline sulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry 23: 5036～5041, 1984

21) FUNATO, N.; H. TAKAYANAGI, Y. KOUDA, Y. TODA, H. HARIGAYA, Y. IWAI & S. ŌMURA; Absolute configuration of staurosporine by X-ray analysis. Tetrahedron Lett. 35: 1251～1254, 1994

22) SASAKI, Y.; M. SETO, K. KOMATSU & S. ŌMURA: Staurosporine, a protein kinase inhibitor, attenuates intracellular $Ca^{2+}$-deoendent contractions of strips of rabbit aorta. Eur. J. Pharmacol. 202: 367～372, 1991

23) KNIGHTON, D. R.; J. ZHENG, L. F. TENEYCK, V. A. ASHIFORD, N.-G. XUONG, S. S. TAYLOR & J. M. SOWADSKI: Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. Science 253: 407～414, 1991

24) HERBERT, J. M.; E. SEBAN & J. P. MAFFRAND: Characterization of specific binding sites for [$^3$H]-staurosporine on various protein kinases. Biochem. Biophys. Res. Commun. 171: 189～195, 1990

25) SAKURADA, K.; T. IKUHARA, M. SETO & Y. SASAKI: An antibody for phosphorylated myosin light chain of smooth muscle: Application to a biochemical study. J. Biochem. 155: 18～21, 1994.

26) NAKANISHI, S.; K. YAMADA, H. KASE, S. NAKAMURA & Y. NONOMURA: K-252a, a novel microbial product, inhibits smooth muscle myosin light chain kinase. J. Biol. Chem. 263: 6215～6219, 1988

27) KASE, H.; K. IWAHASHI, S. NAKANISHI, Y. MATSUDA, K. YAMADA, M. TAKAHASHI, C. MURAKATA, A. SATO & M. KANEKO: K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. Biochem. Biophys. Res. Commun. 142: 436～440, 1987

28) TAKAHASHI, I.; Y. SAITOH, M. YOSHIDA, H. SANO, H. NAKANO, M. MORINOTO & T. TAMAOKI: UCN-01 and UCN-02, new selective inhibitors of protein kinase C. II. Purificaion, physico-chemical properties, structural determination and biological activities. J. Antibiotics 42: 571～576, 1989

29) OSADA, H.; H. TAKAHASHI, K. TSUNODA, H. KUSAKABE & K. ISONO: A new inhibitor of protein kinase C, RK-286C (4'-Demethylamino-4'-hydroxystaurosporine). J. Antibiotics 43: 163～167, 1990

30) NAKANISHI, S.; K, YAMADA, K. IWAHASHI, K. KURODA & H. KASE: KT-5926, a potent and selective inhibitor of myosin light chain kinase. Mol. Pharmacol. 37: 482～488, 1990

31) TAKAHASHI, I.; E. KOBAYASHI, H. NAKANO, C. MURAOKA, H. SAITOH, K. SUZUKI & T. TAMAOKI: Potent selective inhibition of 7-*O*-methyl UCN-01 against protein kinase C. J. Pharmacol. Exp. Therap. 255: 1218～1221, 1990

32) MEYER, T.; U. REGENASS, D. FABBRO, E. ALTERI, J. ROSEL, M. MULLER, G. CARAVATTI & A. MATTER: A derivative of staurosporine (CGP41 251) shows selectivity for protein kinase C inhibiton and *in vitro* anti-proliferative as well as *in vivo* anti-tumor activity. Int. J. Cancer 43: 851～856, 1989

33) MUID, R. E.; E. M. DALE, P. D. DAVIS, L. H. ELLIOTT, C. H. HILL, H. KUMAR, G. LAWTON, B. M. TWOMEY, J. WADSWORTH, S. E. WILKINSON & J. S. KIXON: A novel conformationally restricted protein kinase C inhibitor, Ro-31-8425, inhibits human neutrophil superoxide generation by soluble, particulate and post-receptor stimuli. FEBS Lett. 293: 169～172, 1991

34) TOULLEC, D.; P. PIANETT, H. COSTE, P. BELLEVERGUE, T. GRAND-PERRET, M. AJAK, V. BAUDET, P. BOISSIN, E. BOURSIER, F. LORIOLLE, R. DUHAMEL, D. CHARON & J. KIRILORSKY: The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. J. Biol. Chem. 266: 15771～15781, 1991

35) YANAGIHARA, N.; E. TACHIKAWA, F. IZUMI, S. YASUGAWA, H. YAMAMOTO & E. MIYAMOTO: Staurosporine: An effective inhibitor for $Ca^{2+}$/calmodulin-dependent protein kinase II. J. Neurochem. 56: 294～298, 1991

36) FUJITA-YAMAGUCHI, Y. & S. KATHURIA: Characterization

of receptor tyrosine-specific protein kinases by the use of inhibitors. Staurosporine is a 100-times more potent inhibitor of insulin receptor than IGF-I receptor. Biochem. Biophys. Res. Commun. 157: 955~962, 1988

37) SECRIST, J. P.; I. SEHGAL, G. POWIS & R. T. ABRAHAM: Preferential inhibition of the platelet-derived growth factor receptor tyrosine kinase by staurosporine. J. Biol. Chem. 265: 20394~20400, 1990

38) YATOMI, Y.; Y. OZAKI, K. SATOH & S. KUME: Anti-CD9 monoclonal antibody elicits staurosporine inhibitable phosphatidylinositol 4,5-bisphosphate bydrolysis, phosphatidylinositol 3,4-bisphosphate synthesis, and protein-tyrosine phosphorylation in human platelets. FEBS. Lett. 322: 285~290, 1993

39) LAHER, L. & J. A. BEVAN: Staurosporine, a protein kinase C inhibitor, attenuates $Ca^{2+}$-dependent stretch-induced vascular tone. Biochem. Biophys. Res. Commun. 158: 58~62, 1989

40) SASAKI, Y.; M. SETO & K. KOMATSU: Diphosphorylation of myosin light chain in smooth muscle cells in culture. FEBS. Lett. 276: 161~164, 1990

41) HIMPENS, B.; H. D. SMEDT & R. CASTEELS: Staurosporine induced $Ca^{2+}$ increase in DDT, MF-2 smooth muscle cells. Am. J. Physiol. 246 (Cell Physiol. 33): C544~C551, 1993

42) KING, W. G. & S. E. RITTENHOUSE: Inhibition of protein kinase C by staurosporine promotes elevated accumulations of inositol trisphosphates and tetrakisphosphate in human platelets exposed to thrombin. J. Biol. Chem. 246: 6070~6074, 1989

43) KAGEYAMA, M.; T. MORI, T. YANAGISAWA & N. TAIRA: Is staurosporine a specific inhibitor of protein kinase C in intact porcine coronary arteries? J. Pharmacol. Exp. Therap. 259: 1019~1026, 1991

44) ASANO, M.; K. MATSUNAGA, M. MIURA, K. M. ITO, M. SETO, K. SAKURADA, H. NAGUMO, Y. SASAKI & K. ITO: Diverse effects of staurosporine on $Ca^{2+}$ movements and contractions of vascular smooth muscles: comparison with calphostin C and wortmannin. Eur. J Pharmacol. 1995 (in press)

45) PACKHAM, M. A.; A. A. LIVNE, D. H. RUBEN & M. L. RAUD: Activation of phospholipase C and protein kinase C has little involvement in ADP-induced primary aggregation of human platelets: effects of diacylglycerols, the diacylglycerol kinase inhibitor R59022, staurosporine and oladaic acid. Biochem. J. 290: 849~856, 1993

46) YAMADA, K.; K. IWAHASHI & H. KASE: Parallel inhibition of platelet-activation factor-induced protein phosphorylation and serotonin release by K-252a, a new inhibitor of protein kinases, in rabbit platelets. Biochem. Pharmacol. 37: 1161~1166, 1988

47) YATOMI, Y.; Y. OZAKI, Y. KOIKE, K. SATOH & S. KUME: Wheat germ agglutinin-induced intracellular calcium mobilization in human platelets: Supperssion by staurosporine and resistance to cyclic AMP inhibition. Biochem. Biophys. Res. Commun. 191: 453~458, 1993

48) YATOMI, Y.; Y. OZAKI & S. KUME: Synthesis of phophatidylinositol 3,4-bisphosphate but not phosphatidylinositol 3,4,5-triphosphate is closely correlated with protein-tyrosine phosphorylation in thrombin-activated human platelets. Biochem. Biophys. Res. Commun. 186: 1480~1486, 1992

49) HASHIMOTO, S. & A. HAGINO: Blockage of nerve growth factor action in PC12h cells by staurosporine, a potent protein kinase inhibitor. J. Neurochem. 53: 1675~1685, 1989

50) IWATA, K.; Y. SASAKI & M. SHIBUKAWA: Staurosporine, a protein kinase inhibitor, potentiates neurite outgrowth and catecholamine accumulation in PC12D cells. Jap. J. Pharmacol. 46 suppl.: -65, 1988

51) OHMICHI, M.; S. J. DECKER, L. PAUG & A. R. SALTIEL: Inhibition of the cellular actions of nerve growth factor by staurosporine and K252a results from the attenuation of the activity of the *rkr* tyrosine kinase. Biochemistry 31: 4034~4039, 1992

52) BERG, M. M.; D. W. STEMBERG, L. P. PARADA & M. V. CHAO: K-252a inhibits nerve growth factor-induced *trk* proto-oncogene tyrosine phosphorylation and kinase activity. J. Biol. Chem. 267: 13~16, 1992

53) NABESHIMA, T.; S. OGAWA, H. NISHIMURA, K. FUJI, T. KAMEYAMA & Y. SASAKI: Staurosporine facillitates recovery from the basal forebrain lesion-induced impairement of leaming and defecit of cholinergic neuron in rats. J. Pharm. Exp. Ther. 257: 562~566, 1991

54) OHNO, M.; T. YAMAMOTO & S. WATANABE: Effect of staurosporine, a protein kinase C inhibitor, on impairment of working memory in rats exposed to cerebral ischemia. Eur. J. Pharmacol. 204: 113~116, 1991

55) BODWEY, J. A.; R. W. ERICKSON & J. T. CURNUTTE: Staurosporine inhibits the soluble and membrane-bound protein tyrosine kinases of human neutrophils. Biochem. Biophys. Res. Commun. 178: 423~429, 1991

56) MATSUMOTO, H. & Y. SASAKI; Staurosporine, a protein kinase C inhibitor interferes with proliferation of arterial smooth muscle cells. Biochem. Biophys. Res. Commun. 158: 105~109, 1989

57) ABE, K.; M. YOSHIDA, T. USUI, S. HORINOUCHI & T. BEPPU: Highly synchronous culture on fibroblasts from $G_2$ block caused by staurosporine, a potent inhibitor of protein kinses. Exp. Cell. Res. 192: 122~127, 1991

58) GOTTESMAN, M. M.: How cancer cells evade chemotherapy: sixteenth Richard and Hinda Rothental Foundation Award lecture. Cancer Res. 53: 747~754, 1993

59) JULIANO, R. L. & V. LING: A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochem. Biophys. Acta 455: 152~162, 1976

60) GERLACH, J. H.; J. A. ENDICOTT & A. J. JURANKA: Homology between P-glycoprotein and bacterial haemolysin transport protein suggests a model for multidrug resistance. Nature 324: 485~489, 1986

61) GOLDSTEIN, L. J.; H. GALSKY, A. FOJO, M. WILLINGHAM, S. LAI, A. GADZDAR, R. PIRKER, A. GREEN, W. CRIST, B. M. GARRETT, M. LIEBER, J. COSSMAN, M. M. GOTTESMAN & I. PASTAN: Expression of a multidrug resistance gene in human cancers. J. Nat. Cancer Inst. 81: 116~124, 1989

62) HERWEIJER, H.; P. SONNEVELD, J. BAAS & K. NOOTER: Expression of *mdr* 1 and *mdr* 3 multidrug-resistance genes in human acute and chronic leukemias and association with stimulation of drug accumulation by cyclosporine. J. Nat. Cancer Inst. 82: 1133~1140, 1990

63) NOONAN, K. E.; C. BECK, T. A. HOLZMAYER, J. E. CHIN, J. S. WUNDER, I. K. ANDRULIS, A. F. GAZDAR, C. L. WILLMAN, B. GRIFFITH, D. D. VON HOFF & I. B.

RONINSON: Quantitative analysis of MDR1 (multidrug resistance) gene expression in human tumors by polymerase chain reaction. Pro. Natl. Acad. Sci. (wash.) 87: 7160~7164, 1990

64) MA, L.; D. MARQUARDT, L. TAKEMOTO & M. S. CENTER; Analysis of P-glycoprotein phosphorylation in HL60 cells isolated for resistance to vincristine. J. Biol. Chem. 266: 5593~5599, 1991

65) LEE, S. A.; J. W. KARASZKIEWICZ & W. B. ANDERSON; Elevated level of nuclear protein kinase C in multidrug resistant MCF-7 human breast carcinoma cells. Cancer Res. 52: 3750~3759, 1992

66) MORIOKA, H.; M. ISHIHARA, H. SHIBAI & T. SUZUKI: Staurosporine-induced differentiation in a human neuroblastoma cell line, NB-1. Agric. Bio. Chem. 49: 1959~1963, 1985

67) SATO, W.; K. YUSA, M. NAITO & T. TSURUO: Staurosporine, a potent inhibitor of C-kinase, enhances drug accumulation in multidrug-resistant cells. Biochem. Biophys. Res. Commun. 173: 1251~1257, 1990

68) SAMPSON, E.; C. L. WOLFF & I. ABRAGAM: Staurosporine reduces P-glycoprotein expression and modulates multidrug resistance. Cancer Res. 68: 7~14, 1993

69) MIYAMOTO, K.; S. WAKUSAWA, K. INOKO, K. TAKAGI & M. KOYAMA: Reversal of vinblastine resistance by a staurosporine derivative, NA-382, in P388/ADR cells. Cancer Lett. 64: 177~183, 1992

70) UTZ, I.; S. HOFFER, U. REGENASS, W. HILBE, J. THALER, H. GRUNICKE & J. HOFMANN: The protein kinase C inhibitor $CGP_{41251}$, a staurosporine derivative with antitumor activity, reverses multidrug resistance. Int. J Cancer 57: 104~110, 1994

71) MIYAMOTO, K.; K. INOKO, S. WAKUSAWA, S. KAJITA, T. HASEGAWA, K. TAKAGI & M. KOYAMA: Inhibition of multidrug resistance by a new staurosporine derivative, NA-382, *in vitro* and *in vivo*. Cancer Res. 53: 1555~1559, 1993