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# Inhibition of nerve growth factor-induced neurite outgrowth of PC12 cells by a protein kinase inhibitor which does not permeate the cell membrane

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K-252a, a protein kinase inhibitor of microbial origin, has proven to be a specific inhibitor of nerve growth factor. In this study, the effects of K-252b, the 9-carboxylic acid derivative of K-252a, on nerve growth factor-induced neurite outgrowth in PC12 cells was examined. K-252b is hydrophilic and does not permeate the cell membrane of PC12 cells, whereas K-252a clearly does. K-252b is, however, as potent as K-252a itself in inhibiting the nerve growth factor-induced neurite outgrowth. These results can be interpreted to suggest that effects of K-252b may be through surface-bound/anchored K-252b-sensitive molecules on PC12 cells.

Nerve growth factor; Neurite outgrowth; PC12; K-252a; K-252b; Ecto-protein kinase

## 1. INTRODUCTION

Nerve growth factor (NGF) is required for the survival and development of the sympathetic and certain of the sensory neurons [1]. The details of the action are not clear as yet, although they have been extensively studied using a pheochromocytoma cell line PC12 that differentiates in response to NGF. There is evidence, however, that changes in intracellular phosphorylation are involved in the signal transduction pathways utilized by NGF [2–4].

K-252a, an alkaloid isolated from the culture broth of *Nocardiopsis* sp., potently inhibits protein kinases in vitro including protein kinase C and cyclic nucleotide-dependent kinases [5,6] as well as other enzymes [7–9]. On the other hand, K-252a has proven to be a specific inhibitor of the effects of NGF on PC12 cells [10–15]. The exact site of K-252a action has not yet been determined, but it is suggested that it may involve a very early stage in the cascade of events resulting from NGF binding to its receptor [10,13–15]. When the site of K-252a action is discovered, it should put us one step closer to understanding the mechanism of action of NGF.

Since Ehrlich et al. [16] showed the existence of protein kinases localized at the external surface of the plasma membrane (ecto-protein kinase) in neuronal cells, it was of interest to determine if the site of K-252a action is such an ecto-protein kinase. K-252b used in this study is a compound isolated from the same microbial culture broth as K-252a and has a very similar structure [17] (Fig. 1). K-252b is almost equipotent to K-252a in inhibiting several species of protein kinase activities in vitro [6]. K-252b is a hydrophilic compound with a free carboxylic acid residue and, therefore, expected not to pass the cell membrane freely in contrast to K-252a.

In this paper, we examined the cell membrane permeability of K-252b and the effects of K-252b on NGF-induced neurite outgrowth in PC12 cells.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

7 S NGF was purchased from Wako Pure Chemical Industries, Osaka, Japan. Dibutyryl cyclic-AMP and fluorescein diacetate were obtained from Sigma Chemical Co. K-252a and K-252b were isolated from the culture broth of *Nocardiopsis* sp. as described [5,17]. All other chemicals were of reagent grade.

### 2.2. *Addition of drugs*

Stock solutions of K-252a (2 mM) and K-252b (2 mM) were prepared in dimethylsulfoxide and stored at −20°C in the dark. A stock solution of fluorescein diacetate was prepared by dissolving 5 mg/ml in 2-methoxyethanol. The stock solutions were diluted with culture medium or PBS just before use and added to the culture medium. The final concentrations of the solvents were 0.01%, a concentration that had no damage on cell growth and differentiation.

### 2.3. *Cell membrane permeability*

The amount of K-252a and K-252b was determined by measuring their intrinsic fluorescence. PC12 cells were grown as monolayers in culture flasks at 37°C in 5% $CO_2$ as described [4]. The confluent cell monolayer in 150 $cm^2$ culture flasks was washed twice with Locke's

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*Abbreviations:* NGF, nerve growth factor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; dibutyryl cAMP, *N*-2′-dibutyryladenosine 3′,5′-cyclic monophosphate.

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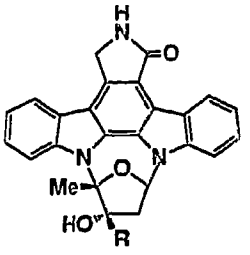


K-252a R=$CO_2Me$
K-252b R=$CO_2H$

Fig. 1. Structures of K-252a and K-252b.

solution [18] and incubated at 37°C for the indicated time in 5% $CO_2$ in the identical solution in the presence or absence of different concentrations of K-252a or K-252b. After incubation, the medium was subjected to the determination of the extracellular amounts of the compounds. For the determination of intracellular levels of K-252a and K-252b, the cells were scraped off and collected from the culture dish in the Locke's solution. The collected cells were washed by centrifugation. The pellets were taken up in a 50 mM HEPES buffer, pH 7.4, and sonicated (Ultrasonic Disruptor Model UR-200P, Tomy Seiko Co., Tokyo, Japan; 5 s blasts with 10 s intervals) for 20 s and subsequently cleared by centrifugation (19 000 × *g* for 10 min) from particulate material; the amount of each compound in the supernatant corresponds to that in the cytosol. The K-252a or K-252b present in the above medium (representing material not taken up) and in the cytosolic fraction (representing incorporated material) was extracted with $CHCl_3$/MeOH (9:1) after adjusting to pH 3.0 with HCl; more than 90% of K-252a or K-252b was recovered by this extraction. The extracts were concentrated in vacuo, dissolved in 2 ml of 5% dimethylsulfoxide in 50 mM HEPES buffer, pH 7.4, and subjected to fluorescence measurement. Fluorescein diacetate, a nonpolar ester, was used as a positive control to examine membrane permeability, since this compound easily crosses the cell membranes and is hydrolyzed by intracellular esterases to produce a greenish yellow fluorescence [18].

2.4. *Fluorescence measurements*

Fluorescence measurements were carried out at room temperature using a Hitachi F3000 fluorescence spectrometer. Excitation was at 290 nm for K-252a and K-252b and 480 nm for fluorescein diacetate, and emission intensity was measured from 300–550 nm and 450–700 nm, respectively. Molar fluorescence intensity of K-252b is 2.3-fold higher than that of K-252a.

## 3. RESULTS

### 3.1. *Cell membrane permeability*

PC12 cells were incubated with fluorescein diacetate at concentrations ranging from 0.1 to 15 μg/ml. As shown in Fig. 2, fluorescein diacetate was incorporated into PC12 cells in a dose-dependent manner under these experimental conditions. Under the same conditions, when incubated at concentrations ranging from 50 to 200 nM, K-252a is also incorporated by PC12 cells in a dose-dependent manner (Fig. 3A). For determining the time course of K-252a incorporation, cells were harvested at various times after the incubation with K-252a. K-252a was rapidly accumulated in the cell cytoplasm as early as 15 min after the exposure of the cells to 300 nM K-252a (Fig. 4); the amount of K-252a taken

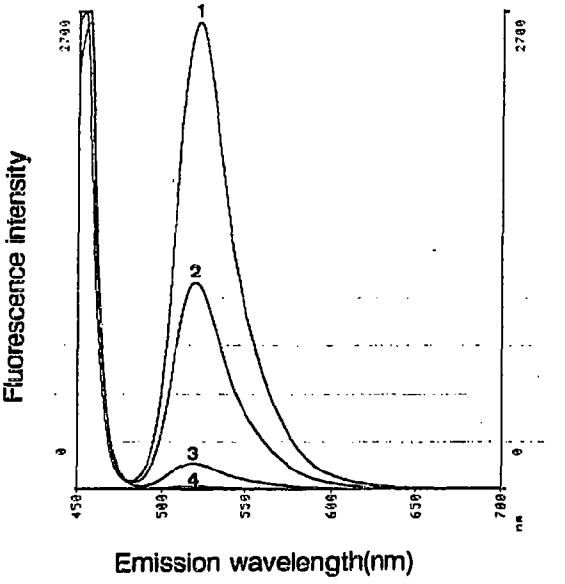


Fig. 2. Incorporation of fluorescein diacetate into PC12 cells. PC12 cells were incubated for 1 h at 37°C in the presence of different concentrations of fluorescein diacetate: (1) 15 μg/ml; (2) 5 μg/ml; (3) 1 μg/ml; (4) 0.1 μg/ml. The incorporated amount of fluorescein diacetate was determined according to the method described in Materials and Methods. For fluorescence measurement, excitation was at 480 nm, and emission intensity was measured from 450–700 nm.

up after 8 h corresponds to 10% of the initial amount. By contrast, a hydrophilic compound K-252b is hardly incorporated into the cells even when incubated at 300 nM (Fig. 3B). Taking a higher molar fluorescent intensity of K-252b (2.3-fold) than that of K-252a into consideration, we can conclude that K-252b is not easily taken up by PC12 cells in contrast to K-252a. It was established by thin-layer chromatography that there had been no decomposition or chemical alterations of K-252a and K-252b, which are present in the cytosolic fraction and extracellular medium (data not shown).

### 3.2. *Effects of K-252a and K-252b on NGF-induced neurite outgrowth*

K-252a, at a concentration of 300 nM, completely inhibited the formation of neurites by PC12 cells treated with 200 ng/ml of NGF (Fig. 5). The inhibition by K-252a of neurite outgrowth was only partial at 100 nM or lower concentrations; at 300 nM there was loss of cells because of its cytotoxicity. K-252b also completely inhibited the NGF-induced neurite outgrowth at 300 nM. At concentrations of 100, 50, 12.5 nM, there was no difference between K-252a and K-252b in their inhibition of the neurite formation (Fig. 5). K-252a and K-252b, therefore, inhibited NGF-induced neurite outgrowth with an equal potency. It is noteworthy, however, that K-252b showed no cytotoxic damage on PC12 cells even at 300 nM in contrast to K-252a (Fig. 5). K-252b was without effect on dibutyryl cAMP-induced neurite elongation in PC12 cells (data not shown).

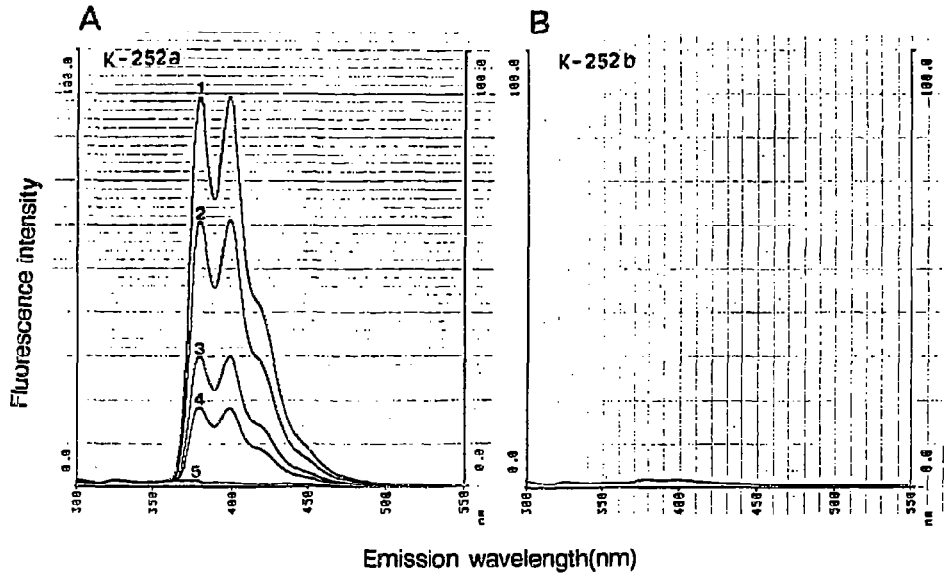


Fig. 3. Incorporation of K-252a and K-252b into PC12 cells. PC12 cells were incubated for 1 h at 37°C in the presence of different concentrations of K-252a (A) or K-252b (B). The incorporated amount of K-252a or K-252b was determined according to the method described in Materials and Methods. For fluorescence measurement, excitation was at 290 nm, and emission intensity was measured from 350–550 nm: (A) 300 nM (1), 200 nM (2), 100 nM (3), 50 nM (4), or 0 nM (5, control) of K-252a was used. (B) 300 nM of K-252b was used.

## 4. DISCUSSION

Recent data have demonstrated that the product of the tyrosine kinase *trk* protooncogene binds NGF and is a component of the high affinity NGF receptor [19,20], but the signal transduction pathway utilized by NGF is still somewhat uncertain. The availability of specific inhibitor of NGF actions would be very useful to clarify this point and K-252a proved to be such an inhibitor [10–15]. Indeed, K-252a selectively blocks NGF-induced alterations, without affecting NGF binding to its receptor, in PC12 cells [10–14] and dorsal root ganglia from chick embryos [21] and human fetus [22], while sparing, or in some cases augmenting, similar events caused by other agents [10,23]. However, K-252a was also found to be toxic to cells at concentrations not much higher than those required for NGF blockage [10]. Furthermore, effects of K-252a on PC12 cells and primary neurons have been observed in the absence of NGF [10,24].

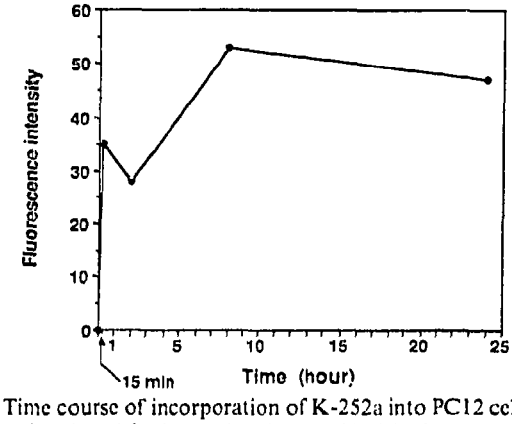


Fig. 4. Time course of incorporation of K-252a into PC12 cells. PC12 cells were incubated for increasing times at 37°C in the presence of 300 nM of K-252a. Cells were harvested at various times, and the incorporated amount of K-252a was determined according to the method described under Materials and Methods. For fluorescence measurement, excitation was at 290 nm, and emission intensity was measured at 402 nm.

K-252b is a hydrophilic compound and has been shown to be equipotent to K-252a itself in inhibiting several species of protein kinase activities in vitro, but with a slightly different substrate specificity than that of K-252a [6]. As presented here, K-252b is as effective as K-252a in blocking NGF-induced neurite outgrowth by PC12 cells, although K-252b does not easily permeate the PC12 cell membranes in contrast to K-252a. The precise mechanism for the inhibition by K-252b is still a matter of speculation and study, but there are several possible meechanisms that might explain how K-252b could inhibit the NGF action. It is possible, for example, that a critical kinase is simply more sensitive to K-252b than to K-252a. Another possibility is that effects of K-252b may be through the surface-bound/anchored molecule such as an ecto-protein kinase, a surface-located protein kinase. An ecto-protein kinase has been shown to be present in a variety of cells [16,25–30] and is particularly interesting in view of its possible role as regulator of transduction of external stimuli. On the other hand, there are several papers describing the intriguing observation that K-252b inhibits several other cellular responses. K-252b inhibits phorbol ester-induced long-term potentiation, which has received much attention as a possible neural basis for learning and memory, in rat hippocampal slices [31], and it also inhibits functional synapse formation between cultured neurons of rat cerebral cortex [32]. There is no evidence that an ecto-protein kinase is involved in synaptic plasti-

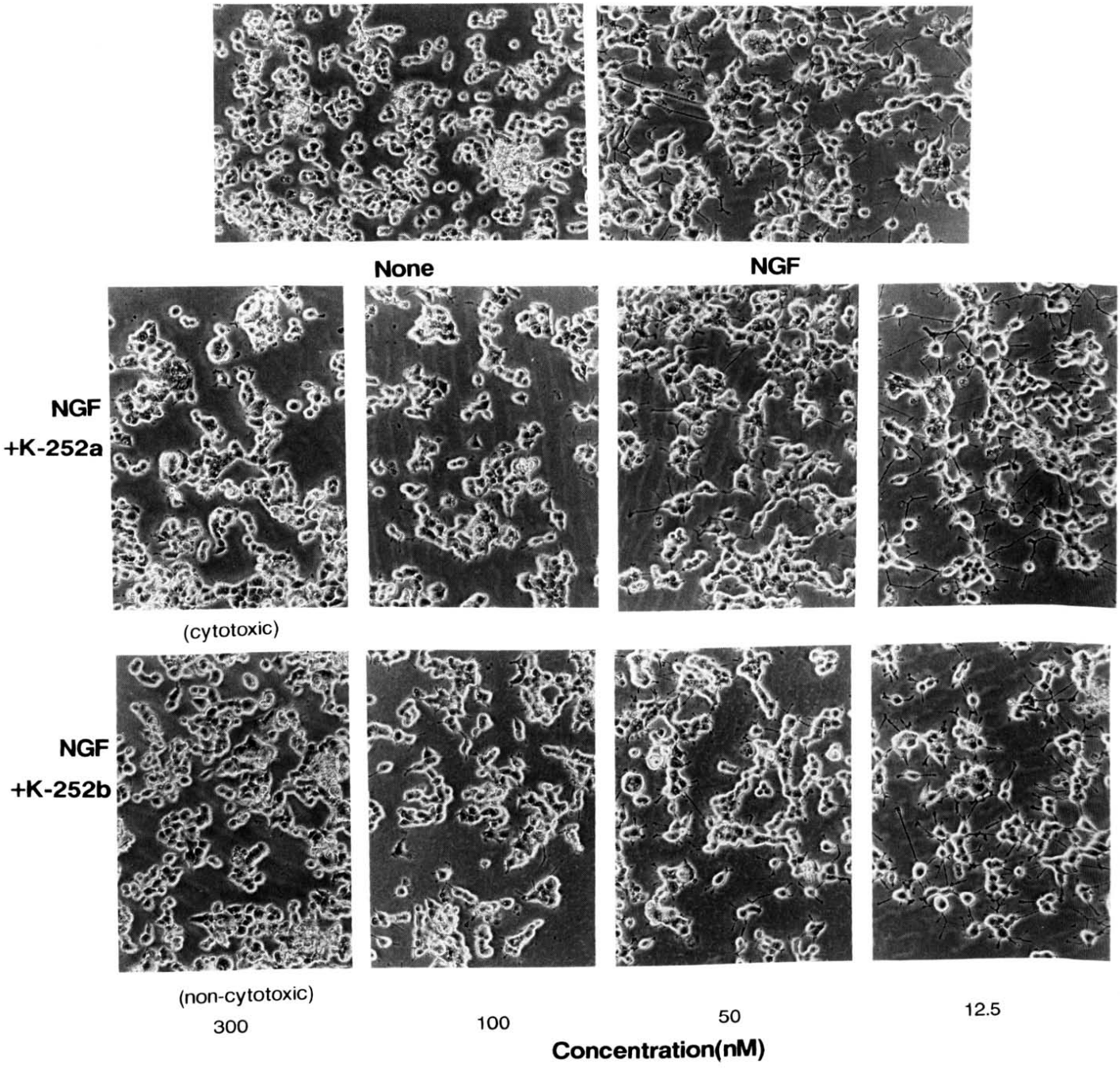


Fig. 5. Effects of K-252a and K-252b on NGF-induced neurite outgrowth. PC12 cells were treated with 7 S NGF (200 ng/ml) and the indicated amount of K-252a or K-252b for 72 h.

city and formation, but, if so, K-252b might provide a new tool for the dissection and study of ecto-protein kinase-requiring processes.

K-252b did not show general toxicity at concentrations up to 10 $\mu$M against PC12 cells (data not shown) and should become a useful tool for inhibiting NGF action in vivo. The lack of cytotoxicity of K-252b can be explained by its properties of low membrane permeability. Indeed, Knüsel and Hefti [33] showed that K-252b exhibited selective NGF antagonistic effects on cholinergic neurons of basal forebrain without showing cytotoxicity.

In conclusion, K-252b is as potent as K-252a in inhibiting NGF-induced neurite outgrowth by PC12 cells and, due to its low toxicity, should become a useful tool to clarify a signal transduction pathway utilized by NGF in both in vitro and in vivo.

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