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The Non-peptide Neuroprotective Agent SR 57746A Interacts with Neurotrophin-3 to Induce Differentiation in the PC12 Cell-line

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Abstract

SR 57746A (1-(2β-naphthylethyl)-4-(3-trifluoromethylphenyl) - 1,2,5,6 - tetrahydropyridine hydrochloride) is a neuroprotective compound which potentiates nerve-growth factor (NGF)-induced differentiation in PC12 cells. We have evaluated the interaction of SR 57746A with the other members of the neurotrophin family in this cell-line.

In contrast with NGF, neurotrophin-3 did not increase the differentiation of PC12 cells. However, the association of SR 57746A with neurotrophin-3 significantly increased neurite outgrowth. No significant activity on neurite outgrowth was observed with brain-derived neurotrophic factor or neurotrophin-4, either alone or combined with SR 57746A.

These results indicate that as well as potentiating the effect of NGF SR 57746A enables neurotrophin-3, which alone is inactive, to increase the differentiation of these cells.

The concept of target-derived neurotrophic factors originated with the discovery of nerve-growth factor (NGF), now known to be essential for the development of several different types of neurone, including peripheral sensory neurones and central cholinergic neurones (Thoenen 1987). In recent years many other endogenous proteins have been reported to have neurotrophic properties; some (members of the neurotrophin family) are structurally related to NGF whereas many others, including some cytokines and numerous growth factors, are structurally unrelated. Although these factors were initially characterized as playing an essential role during the development of the nervous system, more recent studies have shown they can also influence the survival of adult neurones subjected to traumatic insults. In the context of potential therapeutic use the most interesting aspect of neurotrophic factors is their capacity to minimize experimentally-induced degeneration of neurones in a variety of lesion models (Mattson & Scheff 1994). Unfortunately, the protein nature of the neurotrophic factors renders their clinical use highly problematic, because of poor passage through biological barriers and a high propensity to metabolic degradation (Hefti 1994). Nevertheless, a substance which could enhance the effects of one or more neurotrophic factors after oral administration would clearly be a good candidate for the treatment of neurodegenerative diseases.

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SR 57746A (1-(2β-naphthylethyl)-4-(3-trifluoromethylphenyl) - 1,2,5,6 - tetrahydropyridine hydrochloride) is an orally-active, non-peptide which has been found to have neurotrophic effects in a variety of NGF-dependent experimental systems both in-vitro and in-vivo, a profile of activity which confers on the compound considerable potential for the treatment of neurodegenerative conditions. In-vitro SR 57746A increases survival and neurite outgrowth in foetal septal cultures, enhances some effects of NGF in PC12 cells, and increases the synthesis of NGF mRNA and protein in astrocytoma and fibroblast cell-lines (Fournier et al 1992; Pradines et al 1995). The neuroprotective effects of the compound were confirmed in-vivo after oral administration in four distinct models of neurodegeneration: transient global ischaemia (four-vessel occlusion); septo-hippocampal lesion produced by injection of vincristine into the medial septum; sciatic nerve crushing; and acrylamide-induced peripheral neuropathy (Fournier et al 1993).

The phaeochromocytoma PC12 cell-line has been extensively used as a model for the analysis of the effects NGF-related processes (Greene & Tischler 1976). These cells are derived from the neural crest and respond to NGF with dramatic biological and

morphological changes that result in differentiation into a phenotype similar to that of mature sympathetic neurones (Gunning et al 1981). In our initial studies with SR 57746A in this cell-line we observed that the compound by itself was unable to modify the survival or differentiation of PC12 cells.

Surprisingly, however, it potentiated the effect of NGF on differentiation, without modifying NGF-induced cell survival (Fournier et al 1992). Later studies showed that SR 57746A also enhanced the effect of NGF on the expression of choline acetyl transferase and acetylcholinesterase in these cells, and induced a rapid redistribution of F-actin (Pradines et al 1995). We have extended our studies of the effects of SR 57746A in PC12 cells to include possible interactions with other members of the neurotrophin family, i.e. brain-derived neurotrophic factor (BDNF), neurotrophin-3 and neurotrophin-4, in an attempt to characterize further the mechanism of action of the compound.

Materials and Methods

The PC12 cell-line was purchased from American Type Culture Collection (Rockville, USA), and routinely grown in RPMI-1640 medium containing 10% foetal calf serum, 5% horse serum, glutamine (2%) and gentamycin (0·5%). The assays were performed in 96-microwell plates. Before cells were plated the wells were coated with poly-L-lysine. The plating density was 3×10^5 cells cm^{-2} in serum-free N1 medium. Cells were cultured in a humidified 37°C incubator in an atmosphere of 95% air–5% CO_2. SR 57746A (batch 95-05) was dissolved in dimethylsulphoxide (DMSO) and diluted as required in culture medium. NGF, neurotrophin-3, neurotrophin-4 and BDNF (Tebu, France) were dissolved and diluted as required in culture medium. The culture medium was the serum-free N1 medium to which were added DMSO (1%) and bovine serum albumin (0·1%).

For studies of neurite outgrowth the cells were plated in wells containing the culture medium or the test compounds and maintained for two days under these conditions. The cells were then fixed with a mixture of glutaraldehyde and paraformaldehyde in cacodylate buffer. Five microscopic areas were scored per well, corresponding to 100–200 cells, and neurite outgrowth was assessed by counting the percentage of cells bearing at least one neurite equal to or longer than the diameter of one cell. This was performed by a person unaware of the treatment the cells had undergone. For evaluation of cell survival, after four days in culture the medium was removed and replaced with a tetrazolium salt [3 bromo-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium] dissolved in culture medium (0·15 mg mL^{-1}). The cells were incubated for 4 h at 37°C. The mitochondrial succinodehydrogenases of living cells reduced the tetrazolium salt to formazan blue; after dissolution in DMSO the optical density was measured at 540 nm (Manthorpe et al 1988). Assays were performed in 10 wells per group. The statistical difference between groups was performed using the Kruskal-Wallis test. All experiments were repeated at least three times.

Results

In the serum-free N1 medium the PC12 cells were rounded and poorly differentiated. As previously described (Fournier et al 1992; Pradines et al 1995), the optimum concentration of NGF (1 nM) increased the differentiation of these cells; SR 57746A alone was inactive whereas when the compound was incubated with 1 nM NGF it induced significant potentiation of the activity of the neurotrophin (Table 1). In contrast with NGF, neurotrophin-3 (5, 50 or 500 ng mL^{-1}) did not increase the differentiation of the PC12 cells. However, the association of 250 nM SR57746A with each of these concentrations of neurotrophin-3 significantly increased the neurite outgrowth of PC12 cells (Table 2). When associated with 50 ng mL^{-1} neurotrophin-3, 25 nM SR 57746A was inactive on the neurite outgrowth of PC12 cells, but SR 57746A at concentrations of 250 nM and 2·5 μM significantly ($P < 0·01$) increased the neuronal differentiation of these cells, by 65% and 102% respectively (Figure 1). This activity was dependent on the concentration of SR 57746A ($r^2 = 0·991$; type 1 regression).

No significant activity on neurite outgrowth of PC12 cells was observed with neurotrophin-4 or BDNF either alone or combined with SR 57746A (Table 3). Whereas NGF increased the survival of PC12 cells, BDNF, neurotrophin-3, neurotrophin-4 or SR 57746A did not. Furthermore, co-administration with SR 57746A did not alter the effects of the neurotrophins on PC12 cell survival (results not shown).

Discussion

These results indicate that, as well as potentiating the effect of NGF on the differentiation of PC12 cells, SR 57746A enables neurotrophin-3, which alone is inactive, to increase the differentiation of these cells. In contrast with this interaction with neurotrophin-3, SR 57746A had no capacity to

Table 1. Activity of SR 57746A and NGF alone or in combination on neurite outgrowth of PC12 cells.

Treatment	Neurite outgrowth (% ± s.e.m.)
Control	5·5 ± 0·8
SR 57746A (250 nM)	4·3 ± 0·9
Nerve-growth factor (1 nM)	13·3 ± 1·9*
Nerve-growth factor (1 nM) + SR 57746A (250 nM)	23·6 ± 2·8*†

*$P < 0.01$, significantly different from result for control; †$P < 0.05$, significantly different from result for NGF alone.

Table 2. Activity of SR 57746A and neurotrophin-3 alone or in combination on neurite outgrowth of PC12 cells.

Treatment	Neurite outgrowth (% ± s.e.m.)
Control	8·2 ± 0·6
SR 57746A (250 nM)	9·7 ± 1·0
Neurotrophin-3 (5 ng mL^{-1})	8·6 ± 1·1
Neurotrophin-3 (50 ng mL^{-1})	9·2 ± 1·0
Neurotrophin-3 (500 ng mL^{-1})	10·0 ± 1·3
Neurotrophin-3 (5 ng mL^{-1}) + SR 57746A (250 nM)	14·1 ± 1·7*
Neurotrophin-3 (50 ng mL^{-1}) + SR 57746A (250 nM)	14·1 ± 1·4*
Neurotrophin-3 (500 ng mL^{-1}) + SR 57746A (250 nM)	13·4 ± 1·4*

*$P < 0.01$, significantly different from result for control.

induce an effect of BDNF or neurotrophin-4 on PC12 cell differentiation. The effects of NGF on the differentiation of PC12 cells have been shown to be produced via the stimulation of its receptor, trkA (Kaplan & Stephens 1994). The absence of effect of neurotrophin-3, BDNF or neurotrophin-4 alone on PC12 cell differentiation is consistent with the absence of their preferred receptors (trkB for BDNF and neurotrophin-4, and trkC for neurotrophin-3) on these cells. Neurotrophin-3, however, does have a low capacity to stimulate the trkA receptor, and a higher capacity to stimulate a subtype of the trkA receptor ($trkA_{II}$), which is present in relatively high concentrations in PC12 cells (Clary & Reichardt 1994). A critical parameter involved in the differentiating effect of neurotrophin-3 in PC12 cells seems to be the relative extent of stimulation of trkA and p75 receptors in the cells. Neurotrophin-3 can induce differentiation in mutant PC12 cells when they are deficient in the p75 receptor (Benedetti et al 1993), and trkA-mediated effects of NGF are enhanced in PC12 cells when binding to p75 receptors is prevented, for example by antibodies to the low-affinity receptor (Kahle et al 1994). Conversely, a trkA-mediated modulation of p75-related intracellular events has also been suggested (Dobrowsky et al 1995). One manner in which SR 57746A could unmask an effect of neurotrophin-3 could, therefore, be by changing the relative effects of neurotrophin-3 on trkA- (or $trkA_{II}$-) and p75-mediated events, for example by reducing the extent of p75 receptor stimulation or by facilitation of trkA-mediated events. SR 57746A has been found to

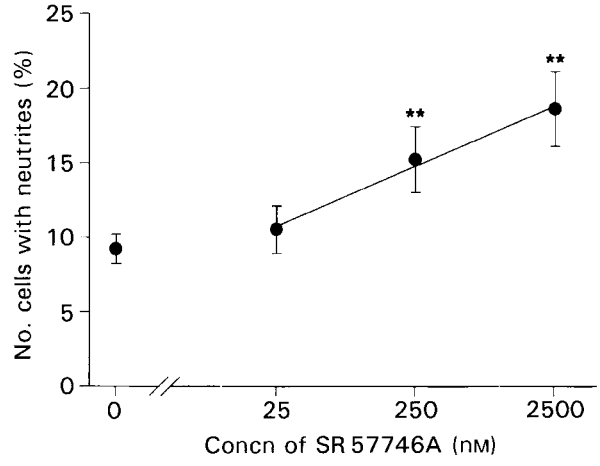

Figure 1. Activity on neurite outgrowth of PC12 cells of different concentrations of SR 57746A combined with 50 ng mL^{-1} neurotrophin-3. The neurite outgrowth (percent of neurite-bearing cells ± s.e.m.) was not significantly modified by neurotrophin-3 (50 ng mL^{-1}) alone or combined with 25 nM SR 57746A. The combination of neurotrophin-3 with 250 nM or 2·5 μM SR 57746A induced significant differentiation of PC12 cells (**$P < 0.01$ compared with the control group). The activity of SR 57746A on neurotrophin-3 (50 ng mL^{-1}) was dependent on the concentration of SR 57746A ($r^2 = 0.991$; type 1 regression).

Table 3. Activity of neurotrophin-4 and brain-derived neurotrophic factor (BDNF), either alone or in combination with SR 57746A, on neurite outgrowth of PC12 cells.

Treatment	Neurite outgrowth (% ± s.e.m.)
Control	9·9 ± 0·7
SR 57746A (250 nM)	12·4 ± 1·2
Neurotrophin-4 (5 ng mL^{-1})	9·7 ± 1·7
Neurotrophin-4 (50 ng mL^{-1})	10·4 ± 2·4
Neurotrophin-4 (500 ng mL^{-1})	11·3 ± 1·7
Neurotrophin-4 (5 ng mL^{-1}) + SR 57746A (250 nM)	14·2 ± 2·2
Neurotrophin-4 (50 ng mL^{-1}) + SR 57746A (250 nM)	12·6 ± 2·2
Neurotrophin-4 (500 ng mL^{-1}) + SR 57746A (250 nM)	10·5 ± 1·1
BDNF (5 ng mL^{-1})	11·7 ± 2·3
BDNF (50 ng mL^{-1})	10·6 ± 2·7
BDNF (500 ng mL^{-1})	15·4 ± 3·4
BDNF (5 ng mL^{-1}) + SR 57746A (250 nM)	10·8 ± 2·1
BDNF (50 ng mL^{-1}) + SR 57746A (250 nM)	12·2 ± 2·6
BDNF (500 ng mL^{-1}) + SR 57746A (250 nM)	13·3 ± 1·8

modify some intracellular events in PC12 cells in the absence of exogenous neurotrophic factor (e.g. redistribution of F-actin (Pradines et al 1995)), suggesting that the enhancement of the effects of NGF and neurotrophin-3 by SR 57746A might be mediated by stimulation of an intracellular event which, while alone is insufficient to lead to cell differentiation, could prime the transduction pathway leading to neurite elongation, so that it requires less stimulation to produce its full effects.

Several different experimental conditions have already been characterized under which neurotrophin-3 can increase the differentiation of PC12 cells, or the differentiating effect of NGF is enhanced. Molecules that have been reported as potentiating the effects of NGF on PC12 cell differentiation include ciliary neurotrophic factor (Zhong et al 1994), somatostatin (Ferriero et al 1994), and interleukin-6 (Sterneck et al 1996). However, the mechanisms underlying their potentiation of NGF have not yet been fully elucidated, and their capacity to interact with neurotrophin-3 in PC12 cells has not been described. Incubation with the protein kinase inhibitor K 252b has been shown to unmask an effect of neurotrophin-3 on PC12 cell differentiation (Knüsel et al 1992). K 252b also potentiated trk autophosphorylation by neurotrophin-3 in cells with the trkA receptor, but not in cells transfected with the trkB or trkC receptors (Maroney et al 1997). The effect of SR 57746A would not seem to be derived from the same mechanism as that of K 252b, however, because K 252b concomitantly inhibited the differentiating effect of NGF on PC12 cells (Knüsel et al 1992) whereas SR 57746A potentiates this effect. It has been suggested that K 252b might increase the effects of neurotrophin-3 by modifying the trkA receptor structure in a manner that facilitates its interaction with neurotrophin-3 (but might reduce that with NGF) (Knüsel et al 1992). A similar effect of SR 57746A might occur, but in this instance the modification would also be expected to enhance the interaction of NGF with trkA. SR 57746A might, therefore, be a useful tool for study of the processes underlying the enhancement of NGF- and neurotrophin-3-induced effects in PC12 cells.

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