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SYNTHESIS AND BIOLOGICAL ACTIVITY OF THE NEURONAL CALCIUM CHANNEL BLOCKER 2-AMINO-1-(2,5-DIMETHOXYPHENYL)-5-TRIFLUOROMETHYL BENZIMIDAZOLE (NS-649).

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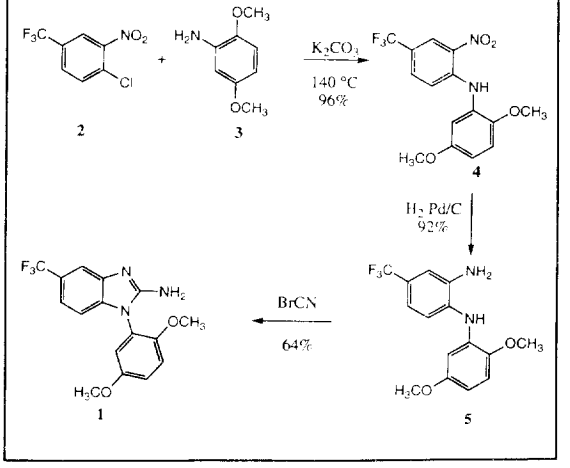
Abstract: The substituted benzimidazole, NS-649, has been shown to be a blocker of neuronal calcium channels in patch-clamp studies. NS-649 dose-dependently inhibited release of D-aspartate from cerebellar granule neurons. The neuroprotective effect of this compound in *in vitro* and *in vivo* models of ischemia was demonstrated.

Neuronal death after ischemia is associated with an increase in the cytoplasmic concentration of free calcium (for a review see ref. 1). It has been shown that ischemia causes excessive release of glutamate,[2,3] and glutamate induced neurotoxicity is - at least partly - dependent on the presence of extracellular calcium.[4,5] This relationship is an integral part of the excitotoxic hypothesis[6] of neuronal cell death. When neurons are deprived of their energy supply (e.g. after a stroke), calcium ions diffuse into the cells, which are challenged with calcium influx via various routes, eventually leading to toxic Ca^{2+} levels. All of these routes are potential targets for pharmacological intervention aimed at neuroprotection. Voltage sensitive calcium channels (VSCCs) are such targets, not only since they mediate calcium influx in response to membrane depolarization, but also because activation of VSCCs in the synaptic boutons is a crucial factor in release of neurotransmitters like glutamate.[7] Since the NMDA-subtype of glutamate receptors is well known to be a major route for calcium influx,[5] opening of VSCCs could be regarded as a trigger of glutamate induced neurotoxicity. We report here the synthesis and pharmacological characterization of a substituted benzimidazole. The compound, NS-649, is shown to be a blocker of neuronal calcium channels and of depolarization-induced D-aspartate release. Furthermore, we have demonstrated the neuroprotective effect of this compound in an *in vitro* and an *in vivo* model of ischemia. A compound with this pharmacological profile may have potential in ameliorating the pathological damage after a stroke.

Scheme 1



The synthesis of NS-649 (**1**) is outlined in Scheme 1 (for details see ref. 8). The nucleophilic displacement of chlorine from **2** by **3** was achieved in the presence of potassium carbonate and a minimal amount of *N*-methyl-pyrrolidone (NMP) at 140 °C (0.7 volumes of NMP relative to **2**). After dilution with water and filtration, a 96 % yield of **4** was obtained. Hydrogenation in ethanol over 5 % palladium on charcoal, resulted in a 92 % yield of pure **5**. Cyclization with cyanogen bromide in *N,N*-dimethylformamide afforded, after an extractive workup and treatment with activated carbon, NS-649 as white crystals with analytical and spectral data in accordance with structure **1** (see ref. 9).

Using the whole cell patch-clamp method,[10] the effect of NS-649 on VSCCs was tested on embryonic chick dorsal root ganglion (DRG) cells as well as mouse cerebellar granule neurons. Figure 1A shows typical recordings of VSCC mediated currents from DRG-cells. NS-649 dose-dependently depressed the currents and the block was partially reversible. In Figure 1B the full dose-response curve is presented. An IC_{50}-value of 32 μM was estimated. Chick DRG-cells express predominantly ω-conotoxin GVIa sensitive (N-type) VSCCs and a minor fraction of dihydropyridine sensitive (L-type) VSCCs.[11,12] NS-649 showed no selectivity for either subtype in DRG-cells, and the compound equipotently blocked calcium currents in cerebellar granule neurons (data not shown), which have been reported to express L, N, P, Q and R subtypes[13]. However, NS-649 (30 μM) showed no effect in Ca^{2+}-induced contractions in potassium-depolarized guinea pig taenia coli (data not shown, see ref. 11 for experimental details), indicating lack of effect on calcium channels in smooth muscles. Hence, NS-649 is a non-selective modulator of neuronal high-threshold VSCCs.

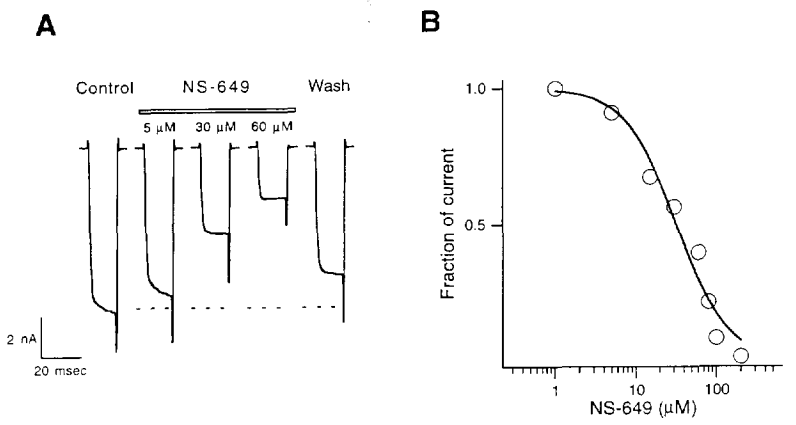


Figure 1. (**A**) Typical recordings of calcium channel mediated currents with 10 mM Ba^{2+} as the charge carrier in the presence of increasing concentrations of NS-649. The currents were activated by 20 msec depolarizing voltage steps to +10 mV elicited every 15 sec from a holding potential of -70 mV. The control trace was recorded after 4 min of stable recording, whereas the traces showing the effects of the different concentrations of NS-649 were taken after 6 min equilibration at each concentration.
(**B**) Effect of NS-649 on calcium currents from DRG-cells. The data are normalized with respect to the control trace, and fitted to the Hill-equation. The IC_{50}-value was calculated from the fit parameters.

As mentioned before, an important physiological function of VSCCs is the regulation of neurotransmitter release, a process dependent on influx of calcium into the synaptic terminal[7]. Cultured mouse cerebellar granule neurons[14,15] were incubated with tritiated D-aspartate, a non-metabolizable glutamate analog.[16] As a model for excitatory neurotransmission, release of D-aspartate was monitored essentially as described by Drejer *et al.*[17] It can be seen from Figure 2A, that NS-649 dose-dependently reduced the high-potassium induced release of D-aspartate (IC_{50} ~ 2.4 μM). In order to evaluate the effect of NS-649 on neuronal degeneration caused by energy depletion, cultured cerebellar granule neurons were exposed to the compound and the viability of the cells was monitored after incubation with sodium azide, an inhibitor of oxidative phosphorylation,[18] as previously described.[19] As seen in Figure 2B, NS-649 (30 μM) largely prevented neuronal death in this model of anoxia.

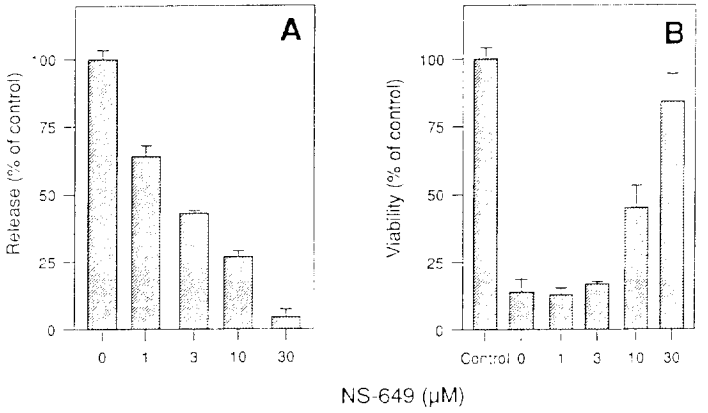


Figure 2. (**A**) Effect of NS-649 on high-potassium (30 mM) evoked release of ^{3}H-D-aspartate from cultured cerebellar granule neurons. (**B**) Effect of NS-649 on neurodegeneration induced by energy depletion. Cerebellar granule neurons were incubated with different concentrations of NS-649 or with vehicle (control and 0 μM) and subsequently with 10 mM sodium azide or vehicle (control). After one hour the viability of the cultures was determined as the ability to reduce the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl- tetrazolium bromide, see ref. 19 for details). The data in both graphs are mean of three experiments ±SEM.

The mouse middle cerebral artery occlusion (MCAO) model was used to evaluate the anti-ischemic effect of NS-649 *in vivo*. As shown in Figure 3, the NS-649 treatment resulted in a significant ($p<0.01$) ≈30% reduction of infarct volume in this model of focal cerebral ischemia. In conclusion, we have described the synthesis and the neuroprotective effect of the substituted benzimidazole NS-649. The compound was found to be a non-selective blocker of neuronal calcium channels, an effective inhibitor of the release of D-aspartate from neurons. Furthermore, NS-649 protected cultured neurons from degeneration caused by energy depletion, and the compound significantly reduced infarct volume in the mouse MCAO-model.

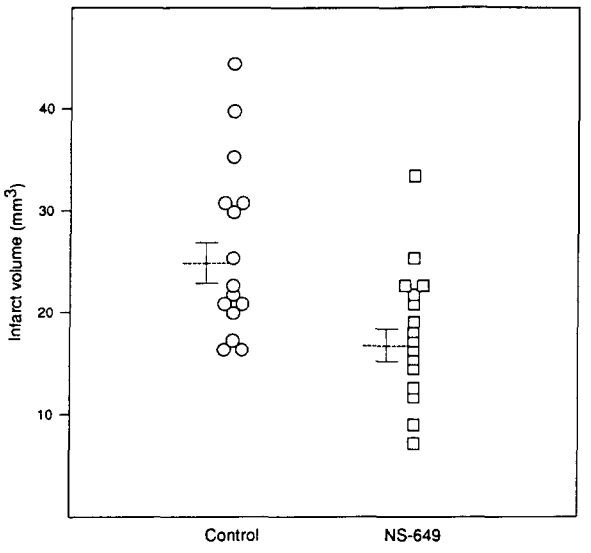


Figure 3: Effect of NS-649 in the mouse MCAO-model. Infarct volume from individual animals as well as mean ±SEM are shown. NS-649 (50 mg/kg, i.p.) was administrated at 0.5 h and 6 h post-occlusion and once a day for the following two days. The control group was injected with the vehicle (7% Tween 80) at the same time intervals (for details, see ref. 11).

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9. Spectral data: EI MS: m/z = 337 (100 %), 322 (17 %), 318 (10 %), 306 (23 %). ^{1}H NMR ($CDCl_3$): δ 7.69 (s, 1), 7.29 (d, 8.4 Hz, 1), 7.10 (d, 9.3 Hz, 1), 7.06 (dd, 9.3 Hz, 3.0 Hz, 1), 6.96 (d, 8.4 Hz, 1), 6.93 (d, 3.0 Hz, 1), 5.2 (s, broad, 2), 3.80 (s, 3), 3.72 (s, 3).
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