Combination Therapy in Ischemic Stroke: Synergistic Neuroprotective Effects of Memantine and Clenbuterol

Carsten Culmsee, PhD; Vera Junker, MD; Wolfram Kremers, PharmD; Serge Thal, MD; Nikolaus Plesnila, MD; Josef Krieglstein, MD, PhD

Background and Purpose—Although excitotoxic overactivation of glutamate receptors has been identified as a major mechanism of ischemic brain damage, glutamate receptor antagonists failed in stroke trials, in most cases because of limited therapeutic windows or severe adverse effects. Therefore, we chose memantine and clenbuterol, both approved safe and efficient in their respective therapeutic categories, and examined combinations of these neuroprotectants for possible therapeutic interactions in ischemic stroke.

Methods—Combinations of the N-methyl-D-aspartate (NMDA) receptor antagonist memantine (20 mg/kg) with the β2-adrenoceptor agonist clenbuterol (0.3 to 3 mg/kg) were tested in a mouse model of permanent focal cerebral ischemia. In addition, combinations of memantine (1 to 10 nmol/L) and clenbuterol (1 to 10 nmol/L) were examined in cultured hippocampal neurons exposed to glutamate (500 μmol/L) or staurosporine (200 nmol/L).

Results—The infarct size was further reduced by combination therapy as compared with effects of the respective neuroprotectants alone. Of note, in combination with memantine, the therapeutic window of clenbuterol was significantly prolonged up to 2 hours after ischemia. Experiments in postnatal cultures of rat hippocampal neurons exposed to glutamate or staurosporine confirmed that neuroprotection by combinations of memantine and clenbuterol exceeded the effects of the individual compounds.

Conclusions—Combinations of memantine with clenbuterol extend the respective therapeutic window and provide synergistic cerebroprotective effects after stroke. (Stroke. 2004;35:1197-1202.)

Key Words: cerebral ischemia • N-methyl-D-aspartate • neurons • glutamates
neuroprotective by induction of neurotrophic growth factors such as NGF, bFGF, or TGF-β1 in vitro and in vivo.15–18

The present study in a mouse model of permanent focal cerebral ischemia and in cultured hippocampal neurons demonstrates promising therapeutic interactions of memantine and clenbuterol, including synergistic neuroprotective effects and extended therapeutic windows after ischemic stroke.

Materials and Methods

Permanent Focal Cerebral Ischemia

Male Naval Medical Research Institute (NMRI) mice (Charles River, Salzfeld, Germany) were kept under controlled light and environmental conditions (12-hour light/dark cycle, 23±1°C, 55%±5% relative humidity) and had free access to food (Altromin) and water. Permanent middle cerebral artery occlusion (MCAO) was performed in 12 to 16 animals per group (25 to 30 g) according to the method described previously.19 Briefly, after the mice were anesthetized with tribromoethanol (TBE) (350 mg/kg), a hole was drilled in the skull to expose the left MCA. The stem of the MCA and both branches were permanently occluded under visual control by electrocoagulation. Body temperature was maintained at 37±0.5°C with a heating lamp during the surgical procedure. After MCAO, rectal temperature was controlled every 30 minutes, and normothermia (37±0.5°C) was maintained by keeping the mice at an environmental temperature of 30°C for 6 hours.

In the first series of experiments memantine (20 mg/kg) was administered intraperitoneally 30 minutes before MCAO or up to 90 minutes after MCAO. Clenbuterol (0.3 to 3 mg/kg) was administered intraperitoneally before (5 hours) or after onset of ischemia (5 minutes, 1 hour, 2 hours, 3 hours, or 6 hours after MCA occlusion). Control animals received vehicle only (0.9% saline). Seven days after MCAO, the mice received 0.5 mL of a 1.5% neutral red solution intraperitoneally; 30 minutes later, the brains were removed and the unstained tissue on the brain surface was determined as infarcted area (mm²) by means of an image analyzing system (Kontron).19 Infarct areas on the cortical surface of neutral red-stained brains highly correlated with infarct volumes determined by standard volumetry on crenyl violet-stained histological sections (r=0.90, P<0.001), and such a correlation was also found 7 days after MCAO in vehicle-treated versus memantine-treated animals (infarct area: 21.2±2.36 versus 18.43±3.28, P=0.017; infarct volume: 23.79±3.35 versus 19.14±2.95; P=0.001).

Physiological Parameters

The left external carotid artery was cannulated for blood sampling, monitoring of blood gases, electrolyte status, and mean arterial blood pressure (MABP) at 15 and 30 minutes during TBE anesthesia. In addition, systolic blood pressure was measured at 2 hours and 3 hours after surgery by a tail pressure cuff to evaluate drug effects on blood pressure. As established in a separate experiment in mice, values of MABP correlated well with values of systolic blood pressure obtained with the tail pressure cuff (r=0.956, P<0.001).

Neuronal Cultures

Mixed primary cultures of hippocampal neurons and glial cells were prepared from neonatal Fischer 344 rats as described previously.17 Experiments were performed after 10 days in culture. The NMDA antagonist memantine (kind gift of Merz, Germany) and clenbuterol (kind gift of Arzneimittelwerk Dresden, Germany) were applied 1 hour or 5 hours before exposure of the cells to glutamate (500 μmol/L, 1 hour), respectively. For induction of excitotoxic cell death, medium (conditioned medium) was collected and glutamate-containing medium was added, and then exchanged again after 1 hour by the conditioned medium. Memantine and clenbuterol were present in the culture medium during and after exposure to glutamate. For induction of apoptosis, neurons were exposed to staurosporine (STS; 200 nmol/L, Sigma) for 18 hours. Cell death was determined by the trypan blue exclusion method or after staining the nuclei with the DNA-binding fluorochrome Hoechst 33258 as described previously.17,20

Statistics

All values are given as means±SD. One-way analysis of variance (ANOVA) combined with Scheffé test were used for multiple comparisons for in vitro experiments and combined with Duncan test for in vivo studies.

Results

Physiological Parameters

Physiological parameters measured at 15 and 30 minutes during TBE anesthesia are presented in Table 1. In addition, systolic blood pressure was measured at 2 hours and 3 hours in animals treated with vehicle, memantine (20 mg/kg; 15 minutes after surgery), clenbuterol (0.3 mg/kg; 2 hours after surgery), or the combination of both drugs (Table 2). There was no difference in physiological parameters between the treatment groups. Functional analysis did not reveal significant changes in general activity or exploratory behavior at 1 to 7 days after ischemia. All animals revealed slight pathological changes in the postural reflex without significant differences between groups. Moreover, all animals showed similar weight loss of 2 g within 48 hours after ischemia, followed by recovery that resulted in a gain of weight to a value exceeding the initial weight by ~1 g.

Therapeutic Interactions of Memantine and Clenbuterol in Cerebral Ischemia

On the basis of previous studies in rodent models of cerebral ischemia, we administered memantine at a dose of 20 mg/kg and clenbuterol at 0.3 mg/kg, and evaluated the respective therapeutic windows of the individual compounds in a model

<table>
<thead>
<tr>
<th>TABLE 1. Physiological Parameters During Tribromoethanol Anesthesia in Mice</th>
<th>15 Minutes</th>
<th>30 Minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.32±0.03</td>
<td>7.33±0.05</td>
</tr>
<tr>
<td>pCO₂</td>
<td>43.0±5.0</td>
<td>39.9±7.8</td>
</tr>
<tr>
<td>pO₂</td>
<td>158.2±12.3</td>
<td>155.3±4.1</td>
</tr>
<tr>
<td>Na (mmol/L)</td>
<td>142.4±1.5</td>
<td>142.1±1.8</td>
</tr>
<tr>
<td>K (mmol/L)</td>
<td>4.33±0.35</td>
<td>4.72±0.43</td>
</tr>
<tr>
<td>Ca (mmol/L)</td>
<td>1.27±0.04</td>
<td>1.26±0.04</td>
</tr>
<tr>
<td>CI (mmol/L)</td>
<td>105.8±2.2</td>
<td>107.4±1.7</td>
</tr>
<tr>
<td>MABP (mm Hg)</td>
<td>65.0±8.9</td>
<td>66.6±12.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TABLE 2. Systolic Blood Pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic Blood Pressure (mm Hg)</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Memantine</td>
</tr>
<tr>
<td>Clenbuterol</td>
</tr>
<tr>
<td>Memantine+clenbuterol</td>
</tr>
</tbody>
</table>

Memantine (20 mg/kg) was administered 15 minutes after onset of anesthesia and clenbuterol was administered 2 hours later. Control animals received vehicle only (0.9% NaCl).
Memantine (20 mg/kg) reduced the infarct size by 20% or 10% only when administered 30 minutes before or immediately after MCAO, respectively (Figure 1A). When administered at 30 or 90 minutes after onset of ischemia, memantine failed to protect brain tissue from ischemic damage (Figure 1A). Clenbuterol reduced the infarct area by 17.3% only when administered 5 hours before MCAO, whereas administration of clenbuterol immediately after ischemia or 1 to 6 hours later did not show any effect as compared with controls (Figure 1B).

The combination of memantine (20 mg/kg), administered 30 minutes before ischemia, and clenbuterol (0.3 mg/kg), administered 1 or 2 hours after MCAO, reduced the ischemic brain damage by 29% or 27.7% as compared with vehicle-treated animals (Figure 2A, B). Of note, the protective effect of the combination therapy with clenbuterol administered 1 or 2 hours after MCAO was also more pronounced than the effect achieved with memantine alone (reduction of infarct area by 15.4% or 12%, respectively; Figure 2A, 2B). The combination therapy still exposed significant cerebroprotection with clenbuterol administered 3 hours or 6 hours after ischemia as compared with vehicle controls or clenbuterol treatment alone (Figure 2C, 2D).

We next examined whether a synergistic effect of memantine and clenbuterol was also observed when clenbuterol was administered at a dose of 1 mg/kg 2 hours after onset of ischemia. In animals receiving the combination therapy, the infarct size was reduced by 23%, whereas memantine alone reduced the infarct area by 14.7% as compared with vehicle-treated controls (Figure 3A). However, further acceleration of clenbuterol doses to 3 mg/kg did not result in a reduction of the infarct size by the combination therapy as compared with controls (Figure 3B).

After optimizing the treatment regimen for clenbuterol administered intraperitoneally 2 hours after MCAO at a dose of 0.3 mg/kg, we next attempted to evaluate the therapeutic
window for memantine in the combination therapy. When administered 5 minutes after onset of ischemia, memantine exposed a similar protective effect as previously observed with pretreatment, now reducing the infarct area by 14%, and by 20% in combination with 0.3 mg/kg clenbuterol (Figure 4A). The synergistic effect of the compounds was still detectable when memantine was administered 30 minutes after onset of ischemia, with clenbuterol administered 2 hours after MCAO, whereas memantine alone had no protective effect when administered at 30 minutes after ischemia (Figure 4B). However, when administered later than 30 minutes, neither memantine alone nor the combination therapy with clenbuterol resulted in a reduction of the ischemic brain damage (Figure 4C).

Synergistic Effects of Memantine and Clenbuterol in Cultured Neurons

Using cultured hippocampal neurons, we wanted to clarify whether the combination of memantine and clenbuterol protected the cells rather than glutamate-induced excitotoxic cell death or if their synergistic activity also affected apoptotic mechanisms. In line with earlier results, both compounds reduced glutamate-induced cell death but not when administered alone. Moreover, the combination of memantine and clenbuterol further reduced glutamate toxicity in hippocampal neurons (Figure 5A). Surprisingly, a similar synergistic effect was observed when a combination of both drugs was tested against staurosporine-induced apoptosis. When administered at concentrations of 10 nmol/L, the single compounds did not have any effect on staurosporine-induced apoptosis, whereas the combination of both drugs significantly reduced the number of apoptotic nuclei (Figure 5B).

Discussion

The use of potent neuroprotectants such as the NMDA receptor antagonist memantine and even more so the β2-adrenoceptor agonist clenbuterol for stroke therapy appeared to be limited, because until now, both drugs were found inactive when applied after ischemia in experimental models of stroke. Here, we showed that combination therapy not only resulted in a further reduction of brain damage as compared with effects of the individual compounds but also significantly extended the therapeutic window. For memantine, the extension of the therapeutic window to 30 minutes after ischemia may reflect the fact that the massive release of glutamate inducing excitotoxic cell death is an early event that triggers further pathological mechanisms involved in ischemic brain damage. Although neither memantine nor clenbuterol alone was protective when applied later than 30 minutes after MCAO, the combination of both drugs...
In comparison to memantine, the extension of the therapeu-
tic window for clenbuterol in the combination therapy
was remarkable, because the therapeutic window was shifted
from 5 hours pretreatment to 2 hours after ischemia. Our
previous work in various ischemia models established a
requirement for pretreatment with clenbuterol, because the
underlying mechanism of neuroprotection, ie, the induction
of neurotrophic growth factors, had to be triggered hours
before ischemia to achieve therapeutic effects.\textsuperscript{15-18} By block-
ing glutamate toxicity, memantine probably slowed crucial
pathological mechanisms of ischemic brain damage, hence
extending the therapeutic window for drug-induced growth
factors. In addition, memantine has been reported to induce
brain-derived neurotrophic factor (BDNF) and its receptor
TrkB in brain tissue.\textsuperscript{21} Enhanced BDNF signaling could
likely act synergistically with clenbuterol-induced neurotro-
phic factors NGF, bFGF, or TGF-β.\textsuperscript{16,18}

Such synergistic effects of induced neurotrophic growth
factors may also explain why the combination of memantine
and clenbuterol exceeded neuroprotection against staurospo-
rine-induced apoptosis in cultured hippocampal neurons as
compared with the individual compounds. Notably, meman-
tine alone at a concentration of 10 μmol/L was able to protect
cultured neurons against apoptosis when applied 5 hours
before exposure to STS (not shown). Such a period of
pretreatment would be sufficient for the induction of BDNF
and TrkB receptors in cultured neurons similar to the reported
effects of memantine in brain tissue.\textsuperscript{22} Therefore, the com-
bination of memantine and clenbuterol provides enhanced
neuroprotective effects not only against glutamate-induced
excitotoxic neuronal cell death but also against apoptosis. Our
previous studies provided evidence for the anti-apoptotic
effect of clenbuterol,\textsuperscript{18,23} and clenbuterol-induced growth
factors such as NGF or TGF-β\textsubscript{1} prevented apoptosis in
cultured neurons and in animal models of cerebral
ischemia.\textsuperscript{20,23,24,25}

It is therefore likely that also in the present study ische-
mia-induced apoptotic cell death was inhibited by the β\textsubscript{2}-
adrenoceptor agonist. Similar synergistic protection against
ischemic brain damage was found when memantine was
combined with the antiapoptotic p53 inhibitor PFT\textsubscript{26}
supporting the view that comcomitant targeting of excitotoxicity
and mechanisms of apoptosis is a promising strategy for
stroke therapy. Moreover, recent data also implied that mild
hypothermia could further improve therapeutic efficiency of
such neuroprotective drug combinations.\textsuperscript{14,27,28} Like many
other NMDA-antagonists, memantine provides additional
protective effects against ischemic brain damage by induction
of hypothermia.\textsuperscript{14} Most likely, memantine-induced hypother-
mia could further exceed cerebroprotection by the combina-
tion therapy demonstrated here in normothermic animals.
Other adverse effects of NMDA receptor antagonists, such as
hypertension, were not observed in the present study; a
possible moderate stimulation of motoric activity by meman-
tine could also support recovery of stroke patients.\textsuperscript{9}

Overall, the present findings demonstrated synergistic
effects of potent neuroprotectants with different modes of
action directed against glutamate toxicity and apoptotic sig-
aling, two major pathological mechanisms involved in
stroke. Because memantine and clenbuterol together provided synergistic neuroprotection and considerable extension of the individual therapeutic windows at safe doses, this combination could be highly effective in stroke therapy.

References
Combination Therapy in Ischemic Stroke: Synergistic Neuroprotective Effects of Memantine and Clenbuterol
Carsten Culmsee, Vera Junker, Wolfram Kremers, Serge Thal, Nikolaus Plesnila and Josef Kriegstein

Stroke. 2004;35:1197-1202; originally published online April 1, 2004;
doi: 10.1161/01.STR.0000125855.17686.6d
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/35/5/1197

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/