Cerebrovascular characterization of clazosentan, the first nonpeptide endothelin receptor antagonist shown to be clinically effective for the treatment of cerebral vasospasm. Part II: Effect on endothelin$_B$ receptor–mediated relaxation

**HARTMUT VATTER, M.D., MICHAEL ZIMMERMANN, M.D., PH.D., VERONIKA TESANOVIC, ANDREAS RAABE, M.D., PH.D., VOLKER SEIFERT, M.D., PH.D., AND LOTHAR SCHILLING, M.D., PH.D.**

Department of Neurosurgery, Johann Wolfgang Goethe University, Frankfurt am Main; Department of Neurosurgery, Ruprecht Karls University, Heidelberg; and Faculty of Clinical Medicine, University Hospital Mannheim, Germany

Object. The disturbed balance between nitric oxide and endothelin (ET)–1 in the cerebrovasculature seems to play a major role in the development of cerebral vasospasm after subarachnoid hemorrhage. Endothelin-1 represents the contractile part in this balance. In addition to the prevailing ET$_A$ receptor–dependent contractile effect, ET-1 also has ET$_B$ receptor–mediated vasodilatory attributes. The aim of the present study was to define the actual selectivity of clazosentan, the first putative highly ET$_B$ receptor–selective antagonist clinically proven to be effective in the treatment of vasospasm in the cerebrovasculature.

Methods. Rat basilar artery ring segments with endothelial function were used for the measurement of isometric force. Concentration effect curves were constructed by cumulative application of sarafotoxin S6c, ET-1, or big ET-1 in the presence or absence of clazosentan (10$^{-6}$ to 10$^{-4}$ M) after a precontraction was induced by prostaglandin F$_2\alpha$. The inhibition by clazosentan was estimated by the value of the affinity constant (pA$_2$).

The relaxation induced by sarafotoxin S6c, ET-1, and big ET-1 was inhibited in a competitive manner by clazosentan, yielding pA$_2$ values of 7.1, 6.7, and 6.5, respectively. The selectivity to the ET$_B$ receptor in the cerebrovascular system was approximately two logarithmic units.

Conclusions. The present investigation shows a competitive inhibition of ET$_B$ receptor–mediated relaxation in cerebral vessels by clazosentan in therapeutically relevant concentrations. Thus, additional clinical trials should be undertaken to evaluate clazosentan concentrations in cerebrospinal fluid. Furthermore, the present data may be taken to describe the pharmacological properties for an ET receptor antagonist specifically tailored for the treatment of pathological conditions of impaired cerebral blood flow.

**KEY WORDS** • cerebral vasospasm • rat basilar artery • clazosentan • endothelin • sarafotoxin S6c • endothelin$_B$ receptor

Mechanisms contributing to the development of cerebral vasospasm after SAH have not been completely uncovered despite recent scientific progress in this area. The disturbance of the networklike interaction between NO and ET-1 in the cerebrovasculature, however, is assumed to play a major role in the pathophysiological sequence leading to cerebral vasospasm. In this network ET-1 represents the contractile partner. It is a 21–amino acid polypeptide that was first isolated from porcine endothelial cells. The vasomotor effects of ET-1 are mediated by activation of specific receptors known as ET$_A$ and ET$_B$. Stimulation of the ET$_B$ receptor located on smooth-muscle cells induces vasoconstriction, whereas vasorelaxation or dilation is mediated by the ET$_A$ receptor on the endothelium. This receptor has been named ET$_B$ to distinguish it from a smooth-muscle subtype of the ET$_A$ receptor found in some peripheral blood vessels. Activation of the latter receptor, which has been named ET$_A$, mediates contraction. Both the ET$_A$ and ET$_B$ receptors are encoded by the same gene, and therefore they may differ in localization and function rather than in molecular configuration.

The presence of a contractile ET$_B$ receptor has not yet been proved in the cerebrovasculature under physiological conditions, but evidence has been presented in favor of its expression in cerebral vessels after experimental stroke and SAH. The existence of the vasodilatory ET$_A$ receptor in the cerebrovasculature, however, is not a subject of debate. The vasodilator effect is coupled to NO production, but it is often masked in cerebral arteries because of the simultaneously occurring activation of functionally predominant ET$_A$ receptor–mediated contraction. Accordingly, interference with the ET system for the treatment of cerebral vasospasm after SAH should have as its goal the prevention of ET$_A$ receptor–mediated vasoconstriction without impairment of the ET$_B$ receptor–dependent dilatory effect. Therefore, a highly ET$_B$ receptor–selective antagonist seems to be the most suitable approach for this indication.
Characterization of clazosentan on the ET_{B} receptor

Clazosentan (previously known as Ro 61-1790) is a selective ET_{B} receptor antagonist with a high affinity to the ET_{B} receptor\(^1\) (also see Part I of our study\(^2\)), although some affinity to the ET_{A} receptor has been considered, based on findings of competition binding assays.\(^3\) Clazosentan represents the first pharmacological compound to be successful in the prevention and reversal of cerebral vasospasm in an initial clinical trial including 32 patients.\(^4\) This remarkable effect of clazosentan on cerebral vasospasm is most probably due to its high affinity to the cerebrovascular ET_{B} receptor, but a potential interaction with the cerebrovascular ET_{A} receptor may also occur. Such an interaction may at least partly attenuate the therapeutic effect and may thus represent an undesired side effect due to the pharmacological properties of the antagonist.

The aim of the present study was, therefore, to characterize the interaction of clazosentan with ET_{B} receptor–mediated relaxation in the cerebrovasculature. This study is important because it will offer a description of the actual receptor selectivity of clazosentan in its target organ and may help improve our basic knowledge, leading to well-tailored compounds for the treatment of cerebral vasospasm after SAH.

Materials and Methods

Tissue Preparation

The experiments were performed using BA ring segments from male Sprague-Dawley rats, each of which weighed between 250 and 350 g. The procedure followed to obtain the BA segments is described in detail in Part I\(^1\) and will only be briefly summarized here. The animals were anesthetized with CO\(_2\) and then killed by exsanguination from the external carotid arteries. The brain together with the cerebral vessels were excised and immersed in cold modified Krebs–Högestätt solution. The BA was dissected meticulously with the aid of a binocular microscope and cut into four-ring segments, each measuring 2 mm in length. The segments were transferred to an organ bath and mounted over two U-shaped stainless-steel wires (70 μm diameter) for the measurement of isometric force. The organ baths were filled with modified Krebs–Högestätt solution and continuously bubbled with a humidified gas mixture of 95% O\(_2\)/5% CO\(_2\), resulting in a pH of approximately 7.35 at the end of the adaptation procedure.

Experimental Protocol

After preparation of the segments, the temperature in the organ baths was gradually increased to 37°C. During this adaptation period of 60 minutes, the segments were repeatedly stretched to adjust a resting tension of 3 to 4 mN. Contraction of segments was induced by 124 mM K\(^+\) Krebs solution (Krebs–Högestätt solution with an equimolar exchange of NaCl by KCl); this was used as a reference contraction following the adaptation period. Segments reaching less than 2.5 mN in the reference contraction were discarded. The functional integrity of the endothelium was tested by application of acetylcholine (10–5 M) after a precontraction had been induced with 5-hydroxtryptamine (10–5 M). Segments yielding a relaxation of more than 30% of the precontraction were considered to possess a functionally intact endothelium (E\(_{\text{relax}}\)), as demonstrated previously by correlation of functional data with electron microscopy investigations.\(^5\) Only E\(_{\text{relax}}\) segments were included in the present experiments. Concentration effect curves for the ET_{B} receptor were therefore studied under the aid of a binocular microscope and cut into four-ring segments, each measuring 2 mm in length. The segments were transferred to an organ bath and mounted over two U-shaped stainless-steel wires (70 μm diameter) for the measurement of isometric force. The organ baths were filled with modified Krebs–Högestätt solution and continuously bubbled with a humidified gas mixture of 95% O\(_2\)/5% CO\(_2\), resulting in a pH of approximately 7.35 at the end of the adaptation procedure.

Effect of Clazosentan on Relaxation of BA Segments

Induced by Sarafotoxin S6c

Cumulative application of sarafotoxin S6c on precontracted segments resulted in a dose-dependent relaxation (Fig. 2). In the presence of low concentrations of clazosentan (10\(^{-7}\) and 10\(^{-6}\) M), no significant changes in the CEC occurred (Table 1). In the presence of higher concentrations of clazosentan (10\(^{-6}\) and 10\(^{-5}\) M), however, the CEC of sarafotoxin S6c–induced relaxation was shifted to the right without a significant effect on the E_{max} or slope (Table 1 and Fig. 1). This observation is compatible with a competitive inhibition of ET_{B} receptor–mediated relaxation by clazosentan. The pA\(_{\text{2}}\) value was estimated to be 7.1, which is approximately 63-fold lower than the respective value obtained for the ET_{A} receptor–mediated contraction in E–BA segments (see Part I\(^1\)).
Effect of Clazosentan on Relaxation of BA Segments Induced by ET-1 and Big ET-1

In the absence of clazosentan ET-1–induced a small transient relaxation only in precontracted segments. Therefore, a reliable pD₂ value for this relaxation could not be calculated. In the presence of high concentrations of clazosentan (10⁻⁷ M), however, application of ET-1 resulted in a reproducible biphasic response of vessel tone consisting of a peak and a plateau (Fig. 1A). In either case the maximum relaxant effect (Eₘₐₓ) was virtually identical and comparable with the Eₘₐₓ values yielded by sarafotoxin S6c (Tables 1 and 2). The CEC for ET-1–induced relaxation in the presence of clazosentan (10⁻⁷ M) was significantly shifted to the right by a factor of 6.1 compared with the CEC in the presence of 10⁻⁷ M clazosentan (Fig. 3). The slopes of these CECs did not differ significantly, which was compatible with a competitive inhibition of relaxation by clazosentan in the higher concentration (10⁻⁶ M). Assuming that ET₁ receptor–dependent relaxation is unmasked but not yet markedly inhibited by 10⁻⁷ M clazosentan, a pA₂ of 6.7 could be estimated by the shift between both these CECs determined using the method of van Rossum. In contrast to ET-1, a significant relaxation for big ET-1 was observed in the absence of clazosentan in accordance with previous results. In the presence of 10⁻⁶ M clazosentan, however, the CEC was significantly shifted to the right compared with the control (Fig. 4); however, neither effect reached statistical significance. In the presence of clazosentan (10⁻⁷ M) the CEC for big ET-1–induced relaxation was shifted to the right and the Eₘₐₓ was markedly enhanced compared with the control curve (Fig. 4). These observations further support the assumption that the ET₃ receptor–mediated relaxation is unmasked but not or only slightly inhibited in the presence of 10⁻⁷ M clazosentan, although it is inhibited in the presence of higher concentrations. The shift in CECs for big ET-1–induced relaxation caused by 10⁻⁶ M clazosentan was 4.3 compared with the CEC obtained in the presence of 10⁻⁷ M clazosentan. The slopes of these CECs showed a slight tendency to increase with higher concentrations of the antagonist; however, this trend was not significant. The pA₂, which was estimated in
Characterization of clazosentan on the ET<sub>B</sub> receptor

The same manner as for ET-1, that is, by a comparison of CECs in the presence of 10<sup>-7</sup> M and 10<sup>-6</sup> M clazosentan, had a value of 6.5, which was very similar to the value found for ET-1.

**Discussion**

The present data indicate a competitive inhibition of ET<sub>A</sub> receptor–mediated relaxation by clazosentan in its potential target organ, the cerebrovasculature. Although assessed as a highly selective high-affinity ET<sub>A</sub> receptor antagonist, its inhibitory effect on the ET<sub>A</sub> receptor was observed in a concentration range that may be reached in the plasma of patients during treatment of cerebral vasospasm (V Breu, personal communication, 2004). This action could thus attenuate the antivasospastic efficacy of clazosentan. Therefore, the relevance of its interaction with the ET<sub>A</sub> receptor justifies basic and clinical investigations to optimize the therapeutic regimen for this compound.

In the present investigation, the inhibitory effect of clazosentan on the ET<sub>A</sub> receptor–mediated relaxation of the rat BA was comparable to findings of functional studies on the ET<sub>A</sub> receptor–mediated contraction of the isolated rat trachea and to those of competition binding assays. In those studies, pA<sub>2</sub> values between 6.7 and 5.5 were obtained. The ET<sub>A</sub> receptor selectivity of clazosentan was derived from the markedly higher affinity of this compound at the ET<sub>A</sub> receptor site—approximately 100 to 15,000-fold higher than that found at the ET<sub>B</sub> receptor. This wide range of affinity/selectivity difference may be caused by different structures of ET receptors in different tissues and a variable second messenger coupling of receptor subtypes. The present data demonstrate an ET<sub>A</sub> receptor selectivity that is little less than two logarithmic units, underlining the need to use the specific target organ for the characterization of a potentially useful drug.

To separate out the contraction- and relaxation-inducing actions of ET-1, activation of the ET<sub>B</sub> receptor must be antagonized as described previously. In the present study, this was achieved with clazosentan, resulting in a biphasic response of the precontracted arteries to applications of ET-1 and big ET-1. The first part of this response is a relaxation, which in most instances was not sustained. This relaxation appears to be due to activation of ET<sub>B</sub> receptors on the endothelial cells and release of NO because it can be abolished by the ET<sub>B</sub> receptor antagonist BQ788 and by an inhibitor of NO synthase, respectively. The second part of the response is a partial or complete recovery of tone or even a further contraction. The mechanisms underlying the second part of the response seem to be more complex, and several components may be involved including the following: 1) a spontaneous attenuation of the relaxant response due to consumption of L-arginine; 2) a tachyphylaxis-like response of smooth muscle soluble guanylyl cyclase, and 3) an increasing activation of the ET<sub>B</sub> receptor with increasing concentrations of ET-1 or big ET-1. Therefore, we chose to analyze the peak value of the initial relaxation exclusively because this appears to reflect ET<sub>B</sub> receptor activation most reliably.

Under physiological conditions, the ET-1–induced constriction of cerebral blood vessels is mediated by activation of the ET<sub>B</sub> receptor, in contrast to some peripheral vascular beds where an ET<sub>B</sub> receptor–dependent vasoconstriction was shown. Under pathological conditions, however, an ET<sub>B</sub> receptor–mediated component may emerge in cerebral arteries. In a previously published study, experimental cerebral vasospasm could be attenuated by long-term treatment with selective ET<sub>B</sub> receptor antagonists. This effect was attributed by the authors to an inhibition of the ET<sub>B</sub> receptor–mediated release of ET-1. This hypothesis obviously does not presuppose the presence of a contractile ET<sub>B</sub> receptor in the cerebrovasculature. More recently, evidence for an up-regulation of the level of ET<sub>B</sub> receptors in the cerebrovasculature was presented based on gene expression studies performed after experimental SAH. Nevertheless, the

### Table 1

**Effect of clazosentan on relaxation of rat BA segments induced by sarafotoxin S6c**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pD&lt;sub&gt;2&lt;/sub&gt;</th>
<th>E&lt;sub&gt;max&lt;/sub&gt; (%)</th>
<th>Shift Compared w/ Control at EC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Slope Around EC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>control (9 observations)</td>
<td>9.95 ± 0.07</td>
<td>72 ± 9</td>
<td>--</td>
<td>0.37 ± 0.06</td>
</tr>
<tr>
<td>clazosentan (M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10&lt;sup&gt;-8&lt;/sup&gt; (5 observations)</td>
<td>9.77 ± 0.04</td>
<td>71 ± 14</td>
<td>1.4</td>
<td>0.45 ± 0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.36 ± 0.03</td>
</tr>
<tr>
<td>10&lt;sup&gt;-7&lt;/sup&gt; (7 observations)</td>
<td>10.02 ± 0.10</td>
<td>68 ± 6</td>
<td>0.8</td>
<td>0.35 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.57 ± 0.10</td>
</tr>
<tr>
<td>10&lt;sup&gt;-6&lt;/sup&gt; (9 observations)</td>
<td>9.58 ± 0.08†</td>
<td>83 ± 6</td>
<td>2.4</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10&lt;sup&gt;-5&lt;/sup&gt; (8 observations)</td>
<td>8.77 ± 0.11†</td>
<td>84 ± 4</td>
<td>15.2</td>
<td>--</td>
</tr>
</tbody>
</table>

* The pA<sub>2</sub> for sarafotoxin S6c is estimated at 7.1. Most values are expressed as means ± SEMs; — = not applicable.
† p < 0.05 compared with control.

### Table 2

**Effect of clazosentan on relaxation of rat BA segments induced by ET-1 and big ET-1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pD&lt;sub&gt;2&lt;/sub&gt;</th>
<th>E&lt;sub&gt;max&lt;/sub&gt; (%)</th>
<th>Slope of CECs Around EC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1 (E+) control (6 observations)</td>
<td></td>
<td></td>
<td>--</td>
</tr>
<tr>
<td>clazosentan (M)</td>
<td></td>
<td></td>
<td>24 ± 10</td>
</tr>
<tr>
<td>10&lt;sup&gt;-7&lt;/sup&gt; (7 observations)</td>
<td>10.07 ± 0.28</td>
<td>85 ± 5†</td>
<td>28 ± 5</td>
</tr>
<tr>
<td>10&lt;sup&gt;-6&lt;/sup&gt; (7 observations)</td>
<td>9.28 ± 0.43</td>
<td>85 ± 8†</td>
<td>42 ± 15</td>
</tr>
<tr>
<td>big ET-1 (E+) control (6 observations)</td>
<td></td>
<td></td>
<td>25 ± 4</td>
</tr>
<tr>
<td>clazosentan (M)</td>
<td></td>
<td></td>
<td>75 ± 10</td>
</tr>
<tr>
<td>10&lt;sup&gt;-7&lt;/sup&gt; (7 observations)</td>
<td>10.73 ± 0.37</td>
<td>110 ± 10</td>
<td>32 ± 5</td>
</tr>
<tr>
<td>10&lt;sup&gt;-6&lt;/sup&gt; (7 observations)</td>
<td>9.52 ± 0.36</td>
<td>8.89 ± 0.641</td>
<td>48 ± 15</td>
</tr>
</tbody>
</table>

* Data are shown for segments with endothelial function (E+). Values are expressed as SEMs.
† p < 0.05 compared with control.

J. Neurosurg. / Volume 102 / June, 2005
The functional meaning of this observation is not yet clear, because application of the selective ET_\text{B} receptor agonist sarafotoxin S6c did not elicit contraction in rat cerebral arteries isolated after experimental SAH.

In experimental cerebral ischemia in rats administration of an ET_\text{B} receptor antagonist increased the infarction volume, and in ET_\text{B} receptor–deficient animals neuronal damage was augmented. These data may be taken to indicate a neuroprotective action on the part of the ET_\text{B} receptor after cerebral ischemia. This neuroprotection has been related to an avascular effect—ET_\text{B1} receptor–mediated vasodilation—resulting in maintenance or improvement of CBF. Nevertheless, there is also evidence for an upregulation of ET_\text{B} expression and a contractile effect of sarafotoxin S6c in the deendothelialized rat middle cerebral artery after ischemia–reperfusion injury. Such a contractile effect cannot be observed in segments carrying an intact endothelium (unpublished observations), however, thus challenging the concept of an ET_\text{B1} receptor–mediated increase in vascular tone. Therefore, treatment of impaired CBF under pathological conditions should inhibit ET_\text{B} receptor–mediated contraction without impairing ET_\text{A} receptor–dependent vasodilatory action. Accordingly, a successful prevention or treatment of experimentally induced cerebral vasospasm following SAH was widely observed by selective ET_\text{A} receptor antagonists such as clazosentan and by mixed receptor antagonists displaying a high affinity to the ET_\text{A} receptor.

The plasma concentrations of clazosentan in patients treated for the prevention or reversal of cerebral vasospasm were in the range of 5 × 10^{-7} to 10^{-6} M (V Breu, personal communication, 2004). The present results demonstrate that in this concentration range an ET_\text{A} receptor antagonistic effect of clazosentan has to be expected. If this holds true, the therapeutic efficacy of clazosentan may be limited due to partial inhibition of the relaxation induced by stimulation of the ET_\text{A} receptor. The cerebrovascular effects of ET-1, however, are thought to be elicited from the adventitial side of the cerebral blood vessels, and an increase in the concentration of ET-1 in CSF has been related to the development of cerebral vasospasm in some clinical studies. Accordingly, the penetration of clazosentan into the CSF and its actual concentration in this compartment appear to be important issues. Previous investigations have provided evidence in favor of the ability of clazosentan to cross the blood–brain barrier. In view of the efficacy of clazosentan to prevent and even reverse cerebral vasospasm in patients, our findings indicate a level of clazosentan in the CSF that is sufficient for inhibition of the ET_\text{A} receptor. Whether clazosentan reaches its optimal concentration in the CSF cannot be judged yet. Nevertheless, once data on the actual clazosentan content in the CSF become available, the data of the present study will provide the basis to assess and possibly optimize dosing to the maximal therapeutic effect attainable.
Characterization of clazosentan on the ET_{B} receptor

Conclusions

The present investigation provides evidence for a potential inhibition of ET_{B} receptor–mediated relaxation by therapeutically relevant concentrations of clazosentan in the cerebrovasculature. This antagonism occurs in addition to the prevailing inhibitory effect of clazosentan on ET_{A} receptor–mediated contraction. Therefore, the concentration range of this antagonist, in which the desired inhibition of the contraction is achieved without attenuation of vasodilatation, may be narrower than expected. Evaluation of clazosentan’s concentration in the CSF, which would reflect the effective concentration at the receptor site more reliably than its plasma concentration, is thus warranted. These data may be useful to adapt the dosage to the optimal level to be derived from the present study. Furthermore, the present data may also be worthwhile to describe the pharmacological properties of an ET receptor antagonist to be specifically tailored for the treatment of conditions of impaired CBF under pathological conditions.

Disclosure

The present investigations were exclusively funded by the Kuratorium ZNS and by funds from the Department of Neurosurgery of the Johann Wolfgang Goethe University, Frankfurt am Main, Germany. Clazosentan was kindly provided by Volker Breu, Ph.D. (Actelion Pharmaceuticals Ltd., Allschwil, Switzerland) without any further financial support of the study.

Acknowledgment

The authors gratefully acknowledge M. Eberhardt for excellent technical assistance in editing the manuscript and for performing the data analysis.

References

ing severe aneurysmal subarachnoid hemorrhage—a randomized, double-blind, placebo-controlled, multicenter, phase IIa study. J Neurosurg (In press, 2005)


Manuscript received September 29, 2004. Accepted in final form February 18, 2005.

This study was funded by Kuratorium ZNS Grant No. 2001009 to Dr. Zimmermann.

Address reprint requests to: Hartmut Vatter, M.D., Schleusenweg 2–16, D-60528 Frankfurt am Main, Germany. email: H.Vatter@em.uni-frankfurt.de.