Glibenclamide Is Superior to Decompressive Craniectomy in a Rat Model of Malignant Stroke * Online-Only Data Supplement (Methods)
J. Marc Simard, Natalia Tsymbalyuk, Orest Tsymbalyuk, Svetlana Ivanova, Vladimir Yurovsky and Volodymyr Gerzanich

Stroke 2010;41;531-537; originally published online Jan 21, 2010;
DOI: 10.1161/STROKEAHA.109.572644

Stroke is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
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Glibenclamide Is Superior to Decompressive Craniectomy in a Rat Model of Malignant Stroke

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Background and Purpose—Treating patients with malignant cerebral infarctions remains a major unsolved problem in medicine. Decompressive craniectomy (DC) improves the bleak outlook but is suboptimal. Using a rat model of severe ischemia/reperfusion with very high mortality due to malignant cerebral edema, we tested the hypothesis that blocking of sulfonylurea receptor 1–regulated NC$_{Ca-ATP}$ channels with glibenclamide would compare favorably to DC when reperfusion and treatment were begun 6 hours after onset of ischemia.

Methods—Male Wistar rats underwent filament occlusion of the middle cerebral artery to reduce laser Doppler flowmetry perfusion signals by $>75\%$, with filament removal plus treatment 6 hours later. In rats treated with vehicle versus glibenclamide (10 $\mu$g/kg IP plus 200 ng/h SC), we compared mortality, neurologic function, and brain swelling at 24 hours. In rats treated with DC versus glibenclamide, we compared neurologic function for 2 weeks and histologic outcomes.

Results—Compared with vehicle, glibenclamide treatment reduced 24-hour mortality from 67% to 5% and reduced hemispheric swelling at 24 hours from 21% to 8%. DC eliminated 24-hour mortality, but neurologic function during the next 2 weeks was significantly better with glibenclamide compared with DC. Watershed cortex and deep white matter were significantly better preserved with glibenclamide compared with DC.

Conclusions—In a rat model of severe ischemia/reperfusion, with reperfusion and treatment beginning 6 hours after onset of ischemia, glibenclamide is as effective as DC in preventing death from malignant cerebral edema but is superior to DC in preserving neurologic function and the integrity of watershed cortex and deep white matter. *(Stroke. 2010;41:531-537.)*

Key Words: cerebral ischemia ■ malignant cerebral edema ■ white matter ■ glibenclamide ■ decompressive craniectomy ■ sulfonylurea receptor 1—regulated NC$_{Ca-ATP}$ channels

The treatment of patients with malignant cerebral infarctions remains 1 of the major unsolved problems in medicine. Malignant infarction occurs in 10% to 15% of stroke victims and is characterized by the formation of malignant cerebral edema. It is the syndrome of a large stroke that causes progressive edema, which results in tissue swelling and compromises arterial inflow to surrounding tissues, culminating in further ischemic damage, enlargement of the infarct, herniation, and death. Despite the best available medical management, the prognosis for these patients is poor, with case fatality rates as high as 60% to 80%.1,2 Until recently, a large cerebral infarction was regarded chiefly as an untreatable condition with fatal outcome.

Decompressive craniectomy (DC) has improved the bleak outlook for malignant infarctions. In a meta-analysis of 3 prospective trials, DC was found to significantly reduce poor outcome and case fatality in patients who were randomized within 48 hours of stroke onset.3 However, factors such as limited eligibility for surgery among patients who are gravely ill and have important comorbidities and the reduced efficacy of DC in patients >60 years of age4 continue to drive the search for nonsurgical treatments for malignant cerebral edema.

Emerging evidence indicates that, after cerebral ischemia, newly upregulated sulfonylurea receptor 1 (SUR1)–regulated NC$_{Ca-ATP}$ channels play a leading role in oncotic swelling (cytotoxic edema) and oncotic death of neurons and astrocytes and in the formation of space-occupying ionic and vasogenic edema caused by microvascular dysfunction.5,6 In several rat models of stroke, blocking of these channels by the sulfonylurea inhibitor, glibenclamide, confirmed the critical role of brain swelling in determining both infarct volume and mortality.

We studied a rat model of severe ischemia with delayed reperfusion that mimicked the late presentation and high mortality often encountered in patients with stroke. Low-dose glibenclamide, administered 6 hours after onset of ischemia at the time of filament withdrawal, reduced brain swelling,
essentially eliminated mortality, and was associated with surprisingly good neurologic function that was durable out to 2 weeks. DC performed at the same time also eliminated mortality\(^7\)\(^{-9}\) but yielded less favorable neurologic scores and worse damage to the watershed cortex and deep white matter. Our findings suggest that glibenclamide may be preferable to DC in the treatment of malignant cerebral edema.

Methods

General
Surgical procedures were approved by the institutional animal care and use committee of the University of Maryland. We studied 3 series of rats (male Wistar, 225 to 325 g), including 2 for preclinical stroke trials. In series 1 treatments (vehicle versus glibenclamide), all included rats (21 vehicle and 22 glibenclamide treated) were used for measurement of neurologic function at 24 hours or at death, if sooner; a subgroup of rats (12 vehicle and 10 glibenclamide treated) was used for measurement of hemispheric swelling at 24 hours or at death, if sooner; another subgroup of rats (8 vehicle and 10 glibenclamide treated) was used for repeated measurement of neurologic function over the course of 2 weeks or at death, if sooner, with 5 of the glibenclamide-treated rats used for histology at 2 weeks. In series 2 treatment (DC), all 10 included rats were used for repeated measurements of neurologic function over the course of 2 weeks, with 5 rats used for histology at 2 weeks. Animals in series 3 were untreated and were used for measurement of laser Doppler flowmetry (LDF) perfusion signals during reperfusion or were euthanized for pathology, immunohistochemistry, or in situ hybridization. Details consistent with good laboratory practice, including inclusion/exclusion criteria, methods for allocation to treatment group, etc, are provided in the supplemental Data Supplement (which includes an addition to the Methods section, a table, and a figure), available online at http://stroke.ahajournals.org.

Rat Model of Malignant Cerebral Edema
Procedures for the 6-hour-long middle cerebral artery (MCA) occlusion via the external carotid artery and for monitoring LDF perfusion signals for \(>75\%\) reduction have been described,\(^6\) with additional details provided in the Data Supplement.

Treatments
A loading dose of glibenclamide (10 \(\mu g/kg\) IP) was given 15 minutes before removing the occluder at 6 hours. After the occluder was removed, a mini-osmotic pump (model 2001, 1.0 \(\mu L/h\); Durect Corp, Cupertino, Calif) was implanted subcutaneously for continuous infusion of glibenclamide (200 ng/h SC for 24 hours or 7 days). Controls were administered vehicle (normal saline plus dimethyl sulfoxide) in the same way. Solutions of glibenclamide (Sigma, St. Louis) were prepared as described.\(^6\)

DC was performed immediately after removing the occluder at 6 hours. The craniectomy plus dural opening extended from coronal to lambdoidal sutures and from the sagittal suture to the zygoma (\(\approx 7 \times 9\) mm).

Outcome Measures
Neurologic function was assessed according to the neuroscore\(^10\) and the modified Garcia score.\(^11\) For hemispheric swelling, area measurements of involved versus noninvolved hemispheres were made on 5 consecutive 2-mm coronal brain sections.

Analysis of Preclinical Data
Parametric data (swelling at 24 hours, serial weights) were analyzed by Student \(t\) test or a repeated-measures 1-way ANOVA with Bonferroni comparisons. Mortality was analyzed with a \(2 \times 2\) contingency table and Fisher exact test. Nonparametric data (neuroscores and Garcia scores) were analyzed with the Mann-Whitney \(U\) test for the 24-hour data or, for repeated measurements, by first rank-transforming the combined data, with tied ranks replaced by the average rank, and a repeated-measures 2-way ANOVA with Bonferroni comparisons.

Histology, Immunohistochemistry, and In Situ Hybridization
To stain myelin, we used eriochrome cyanin-R (Sigma, St. Louis). To perform terminal deoxynucleotidyl transferase dUTP nick end-labeling, we used the in situ cell death detection kit, fluorescein (No. 1684795, Roche Molecular Biochemicals). For immunolabeling, we used primary antibodies directed against SUR1 (SC-5789, Sigma), von Willebrand factor (F5320, Sigma), oligodendrocyte-specific protein (ab7474, Abcam), oligodendrocyte transcription factor 2 (Olig2, IBL), and specificity protein 1 (Sp1, clone PEP2; Santa Cruz). For in situ hybridization of mRNA for SUR1, we used digoxigenin-labeled probes (sense, 5-GCCCGGGCACCCTGCTGGCTCTGTGTGTCCT-TCCGCGCCTGGGCATCG-3') designed and supplied by GeneDetect.\(^5\)\(^6\)

Results

Temporary Thrombosis of the MCA
Occlusion of the origin of the MCA for 6 hours, which yielded a reduction in LDF perfusion signal by \(>75\%\), was associated with thrombosis of the trunk and distal branches of the MCA (Figure 1A).\(^12\) However, within 3 hours of remov-
ing the occluder, MCA vessels had cleared by spontaneous thrombolysis (not shown).

Thrombolysis of major MCA branches does not necessarily imply reperfusion, because microemboli could still occlude more distal branches. We therefore measured the time course of reperfusion of the MCA territory in 6 rats by LDF during and after removal of the occluder. In some cases, reperfusion occurred rapidly, whereas in others, reperfusion occurred in a stepwise fashion over the course of 1 to 2 hours (Figure 1B). In the 6 rats examined by LDF, there was never failure to reperfuse, as judged by LDF perfusion signals, making this model truly 1 of reperfusion and not of permanent occlusion. This insult of 6-hour ischemia/reperfusion (I/R) resulted in massive infarcts that characteristically involved the entire MCA territory, with a minimal mass effect from swelling at early times (Figure 1C).13

SUR1 Upregulation

Brains were examined for SUR1 expression after 6 hours of ischemia, with or without 3-hour reperfusion. Immunolabeling showed prominent upregulation of SUR1 throughout the MCA territory, which was confirmed by immunoblot (Figure 2A), consistent with previous detailed observations of progressive upregulation of SUR1 with time in a similar model of malignant cerebral edema.5 In situ hybridization of mRNA for Abcc8, the gene that encodes SUR1, confirmed transcriptional upregulation of SUR1 (Figures 2B and 2C).

At higher magnification, SUR1 immunolabeling was identified in large neuron-like cells as well as in capillaries (Figure 2D), corroborating previous observations made in other models of stroke.5,6 In addition, we also observed that SUR1 upregulation in capillaries after 6 hours of ischemia continued to increase during 3 hours of reperfusion, in terms of both protein and mRNA (Figures 2E and 2F).

SUR1 was also upregulated in white matter, which has not been previously reported. SUR1 expression was prominent in the corpus callosum and in en face white matter bundles in the putamen (Figure 3A). In situ hybridization of mRNA for Abcc8 confirmed transcriptional upregulation of SUR1 (Figure 3B). Colabeling for oligodendrocyte-specific protein showed that SUR1 was upregulated in oligodendrocytes but not in axons (Figures 3C and 3D).

Sp1 Transcription Factor

The promoter region Abcc8, the gene that encodes SUR1, contains multiple consensus sequences for binding Sp1, and Sp1 has been established as a key transcriptional regulator of SUR1 expression.14,15 The association between Abcc8 and Sp1 has been validated for neurons and other central nervous system cells in vivo after ischemic injury.5 This finding was confirmed in the current study, which showed that oligodendrocytes as well as microvascular endothelial cells exhibit prominent upregulation and nuclear translocation of Sp1 after severe I/R (Figures 3E and 3F). Together, these findings reaffirm that Sp1 forms part of the transcriptional program responsible for upregulating SUR1/Abcc8 in multiple cell types after cerebral I/R.

Effect of Glibenclamide at 24 Hours

We tested the effect of glibenclamide, the potent, highly selective blocker of SUR1.16 Rats were subjected to 6-hour I/R and at 6 hours received either vehicle or glibenclamide at the same formulation, dose, and route of administration as in our previous study.5 Low-dose glibenclamide was previously reported to have no significant effect on systolic blood pressure5 and to be minimally hypoglycemicogenetic.5,6 In the current study, we found that glibenclamide had no effect on LDF perfusion signals, blood gases, or serum chemistry, with...
only a small effect on glucose at 1 hour but not at 24 hours (see Data Supplement).

Functional outcome was evaluated at 24 hours according to the 9-point neuroscore (0 for no apparent deficit, 8 for death). Neuroscores at 24 hours were significantly different between groups \((P<0.0001)\), with median scores of 8 versus 2 for vehicle- versus glibenclamide-treated rats, respectively (Figure 4A). In the same rats, 24-hour mortality (neuroscore 8) was 14 of 21 (67\%) in the control group versus 1 of 22 (5\%) in the glibenclamide-treated group \((P<0.0001; \text{Figure 4B})\).

A subset of rats from this series was examined for hemispheric swelling at 24 hours or at death, if earlier. The brains of glibenclamide-treated rats showed significantly less swelling of the infarcted hemisphere compared with those of vehicle-treated controls \((21.3\pm2.2\% \text{ vs } 7.9\pm1.1\%, P<0.0001; \text{Figures 4C and 4D})\).

### Effect of Glibenclamide Versus DC During 2 Weeks

Other rats from this series were followed up until death or up to 2 weeks. Only 1 of 8 vehicle-treated rats not euthanized at 24 hours survived for 2 weeks, making the overall mortality in this model \(\approx 90\%\). By contrast, 10 of 10 glibenclamide-treated rats survived the entire 2 weeks.

Owing to high mortality in vehicle-treated rats, further comparisons with glibenclamide-treated rats beyond 24 hours were not feasible. An additional group of 10 rats was subjected to the same ischemic insult (6-hour I/R) and was treated with DC at 6 hours. The outcome of these rats over the course of 2 weeks was compared with the outcome of glibenclamide-treated rats that were followed up for 2 weeks.

Individual neuroscores for the 2 treatment groups showed that DC and glibenclamide were equally effective in essentially eliminating mortality: no rat in either group died (Figure 5A). The fact that DC eliminated mortality confirmed that the cause of death in this model was brain swelling. The
fact that glibenclamide was as effective as DC in eliminating mortality confirmed that a critical mechanism of action of glibenclamide was to reduce brain swelling, consistent with the 24-hour measurements of hemispheric swelling reported earlier.

Neuroscores were better for glibenclamide-treated rats compared with DC-treated rats (Figure 5A). All animals treated with glibenclamide reached a neuroscore of 0 by the end of 1 week, whereas none of the DC-treated rats achieved this score after 2 weeks. By the end of 2 weeks, DC-treated rats exhibited upper motor neuron deficits not evident in the glibenclamide-treated group.

The difference between treatment groups was borne out by the Garcia scores (Figure 5B). After 7 to 10 days of recovery, glibenclamide-treated rats all showed stable hemisensory neglect to contralateral whisker stimulation but otherwise appeared remarkably unaffected, with no overt motor deficit. DC-treated rats all exhibited the same hemisensory deficit but in addition showed difficulty with the beam walk or did not achieve symmetry of locomotion.

We recorded body weight as an objective measure of well-being. Glibenclamide-treated rats showed a brief interruption in weight gain after the ischemic insult but then recovered progressive weight gain at the expected pace (Figure 5C). DC-treated rats showed no weight gain during the 2-week period of observation (Figure 5C).

**Effect of Glibenclamide Versus DC on Tissue Sparing**

Histologic examination showed that all animals regardless of treatment exhibited large areas of necrosis involving the MCA territory. However, glibenclamide-treated rats showed significantly better preservation of the medial cortex (anterior cerebral artery territory and watershed cortex), which comprise hindlimb and forelimb areas of the cortex (Figures 6A and 6B). Measurement of these cortical areas in coronal sections showed significantly larger values in glibenclamide-treated rats compared with DC-treated rats ($P<0.01$; Figure 6C).

Eriochrome staining for white matter revealed a striking difference between glibenclamide- and DC-treated rats. In DC-treated rats, deep white matter of the lateral corpus callosum, lateral to the lateral ventricle, was usually destroyed, whereas in glibenclamide-treated rats, this deep white matter was spared (Figures 6A and 6B, arrows). Measurement of the thickness of the deep white matter in this region showed that it was significantly greater in glibenclamide-treated rats than in DC-treated rats ($P<0.001$; Figure 6D).

**Discussion**

Most rodent models of stroke focus on the outcome measure of lesion size and strive to achieve low mortality. This narrow focus does not reflect the wide range of severity of stroke that can afflict humans, because it deliberately omits study of the most severe strokes that result in death. In humans, DC is used exclusively to save life, but surprisingly, studies of DC in rodent models have usually used relatively modest ischemia associated with mortality rates far below the 60% to 80% encountered in humans with malignant infarcts who are candidates for DC. In the present study, rats were subjected to 6-hour ischemia with a reduction in LDF perfusion signals by >75%. The occluder was withdrawn at 6 hours, but actual reperfusion occurred as much as 1 to 2 hours later. This model of severe I/R was associated with 67% to 90% mortality, closely approximating the high case fatality rates associated with malignant infarcts in humans. DC performed at 6 hours in this model was highly effective in reducing mortality, reflecting the experience with DC in humans. As noted by the Stroke Therapy Academic Industry Roundtable, the closer that animal studies link directly to...
facilitate hemorrhagic transformation. From a physiologic standpoint, preventing swelling is preferable to decompressing the already-swollen brain. To our knowledge, this is the first report to demonstrate that a pharmacologic treatment can aid in preserving white matter after severe ischemic injury. SUR1, the regulatory subunit of the SUR1-regulated NC_{Ca-ATP} channel, was upregulated in oligodendrocytes. This channel has previously been implicated in cell death of neurons, astrocytes, and endothelial cells and may also be involved in the death of oligodendrocytes. The human brain has a higher proportion of white matter than the rodent brain, making it important that stroke treatments protect white matter as well as neurons. As noted by the Stroke Therapy Academic Industry Roundtable, it is unlikely that any treatment that targets only neurons and that does not salvage white matter would have widespread clinical relevance. In summary, we have shown that in a model of severe I/R with reperfusion and treatment started 6 hours after onset of ischemia, glibenclamide is as effective as DC in preventing death from malignant cerebral edema but is superior to DC in preserving neurologic function and in maintaining the integrity of watershed cortex and deep white matter.

Acknowledgments
This work was supported by grants to J.M.S. from the National Heart, Lung, and Blood Institute (HL082517) and the National Institute of Neurological Disorders and Stroke (NS061808, NS060801).

Disclosures
J.M.S. holds a US patent (No. 7 285 574), “A novel non-selective cation channel in neural cells and methods for treating brain swelling,” is a member of the scientific advisory board, and holds shares in Remedy Pharmaceuticals. No support, direct or indirect, was provided to J.M.S. or for this project by Remedy Pharmaceuticals. The other authors report no disclosures.

References


Online-Only Data Supplement (Methods)

Good Laboratory Practice

We endeavored to adhere to the major aspects of good laboratory practice, as follows. With regard to animal species, strain, substrain, and source, male Wistar rats (225 to 325 g) were obtained from Harlan Laboratories (Indianapolis, Ind.).

For sample size calculation, power analysis (power and precision) was performed to determine the sample size required for a power of 80% when comparing the proportions of death in 2 groups. For proportions of 0.7 versus 0.2 (N = 0.05, 2-tailed), which are similar to values reported for a less severe model of malignant cerebral edema without reperfusion, a sample size of 15 would be required.

With respect to inclusion and exclusion criteria, animals were excluded before allocation if they did not achieve a sustained reduction in LDF perfusion signal >75% during the 30-minute period of observation or if the occluder could not be withdrawn intact at 6 hours. Of 70 rats that met the criteria for LDF perfusion signals in series 1 and 2 combined, 4 of 25, 8 of 30, and 5 of 15 were excluded after allocation from the vehicle-, glibenclamide-, and DC-treated groups, respectively, for premature death occurring within 5 hours of reperfusion1 attributed to anesthetia or surgery (0, 1, and 2); subarachnoid or subdural hemorrhage found at necropsy (3, 5, and 1); and miscellaneous causes unrelated to cerebral ischemia (1, 2, and 2).

Randomization was performed by coin toss. For allocation concealment, allocation was assigned daily by 1 investigator who did not perform the experiments (V.G.), with allocation assignment determined either randomly or purposely to balance group allocation. A different investigator who was blinded to the treatment and who did not evaluate outcome (N.T.) performed all of the surgical procedures for MCA occlusion and to implant the mini-osmotic pumps. A different investigator (O.T.) loaded the mini-osmotic pumps and performed the DC. The investigator who made daily allocations (V.G.) also coded the treatment syringes and minipumps and was the only person with knowledge of the treatment. We did not report on animals excluded from analysis.

For the blinded assessment of outcome, neuroscores at 24 hours (vehicle vs glibenclamide) were determined by 2 investigators (V.Y. and O.T.) who were blinded to treatment. Serial neurologic function (neuroscores and Garcia scores) and weights for 2 weeks (glibenclamide vs DC) were determined by 2 independent investigators (V.G. and O.T.) who could not be blinded because of obvious differences in physical appearance (presence of a subcutaneous min-osmotic pump vs cranectomy) and because glibenclamide-treated rats from series 1 were processed before DC-treated rats from series 2. Differences in scoring between investigators were settled by choosing the worse of the 2 scores.

In this rat model of malignant cerebral edema, rats that had been fasted overnight (to prevent severe xylazine-induced hyperglycemia) were anesthetized (ketamine, 60 mg/kg and xylazine, 7.5 mg/kg, IP) and allowed to spontaneously ventilate air supplemented with O2. Temperature was maintained at 37°C with a heating pad regulated by a rectal temperature probe (Harvard Apparatus, Holliston, Mass.). Perfusion signals were measured by LDF (Moor Instruments, Axminster, UK), with the probe affixed to the thinned skull 2 mm caudal and 4 mm lateral to bregma with the use of 0-cyanoacrylate adhesive. Details of the procedures used to manufacture filament occluders and to achieve MCA occlusion via the external carotid artery stump have been described elsewhere.4 LDF perfusion signals were monitored for 30 minutes after MCA occlusion, after which rats were allowed to recover from anesthesia. After 5½ hours of ischemia, the rat was reanesthetized, and at 6 hours, the occluder was removed, the external carotid artery stump was ligated, and flow was restored in the internal carotid artery. LDF perfusion signals were not normally measured at reperfusion, except in a separate group of 6 rats not used for outcome measures. LDF perfusion signals (supplemental Figure I), as well as arterial blood gases, hematocrit, electrolytes and glucose (i-STAT, Heska Corp, Ft. Collins, Colo; supplemental Table I), were not appreciably affected by the doses of glibenclamide used.

For outcome measures, neurologic function was assessed by the neuroscore5 and the more comprehensive modified Garcia score.6,7 The neuroscore, including death, ranges from 0 to 8 (0, normal; 1, forelimb flexion; 2, shoulder abduction; 3, reduced resistance; 4, spontaneous movements; 5, circling only; 6, walk only with stim-
vation; 7, unresponsive; 8, death). Mortality (neuroscore = 8) was assigned when a rat died (unwitnessed) from stroke without exhibiting exclusion criteria, as determined at necropsy. The Garcia score, ranging from 0 to 15, tests spontaneous activity, symmetry of movement, floor walking, beam walking, and response to vibrissae touch, with each measure earning an individual score of 0 to 3.

In 1 subgroup, neuroscores were determined at 24 hours. In another subgroup, serial neuroscores as well as Garcia scores were evaluated on days 1, 3, 7, 10, and 14. At the time designated for scoring, if a rat was unresponsive (neuroscore = 7) and if it exhibited agonal respiration, it was considered preterminal because such rats never survived more than a few hours. A rat in this condition was euthanized and was assigned a neuroscore of 8 for the next designated scoring time (eg, neuroscores of 7 and 8 for days 1 and 2, respectively).

References
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