Recommendations for standards regarding preclinical neuroprotective and restorative drug development
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Abstract—The plethora of failed clinical trials with neuroprotective drugs for acute ischemic stroke have raised justifiable concerns about how best to proceed for the future development of such interventions. Preclinical testing of neuroprotective drugs is an important aspect of assessing their therapeutic potential, but guidelines concerning how to perform preclinical development of purported neuroprotective drugs for acute ischemic stroke are lacking. This conference of academicians and industry representatives was convened to suggest such guidelines for the preclinical evaluation of neuroprotective drugs and to recommend to potential clinical investigators the data they should review to reassure themselves that a particular neuroprotective drug has a reasonable chance to succeed in an appropriately designed clinical trial. Without rigorous, robust, and detailed preclinical evaluation, it is unlikely that novel neuroprotective drugs will prove to be effective when tested in large, time-consuming, and expensive clinical trials. Additionally, similar recommendations are provided for drugs with the potential to enhance recovery after acute ischemic stroke, a burgeoning new field with great potential but little currently available data. The suggestions contained in this document are meant to serve as overall guidelines that must be adapted to the individual characteristics related to particular drugs and their preclinical and clinical development needs. (Stroke. 1999;30:2752-2758.)

Key Words: neuroprotection ■ pharmacology ■ stroke
Dose of Drug Used
The application of agents that show putative efficacy in nonhuman cerebral injury models to patients has exposed significant limitations. Some pharmacological agents have been demonstrated to be successful or not at a particular dose, but in many cases dose-response curves were not generated. A drug dose effective in the mouse or rat may not be effective in large animals and/or humans. It may not be sufficient or correct to merely scale up the dose of a drug in milligrams per kilogram from the mouse to larger animals and humans. Pharmacokinetics and pharmacodynamics may vary considerably among species. In this regard, the generation of dose-response curves is critical in both small-animal and large-animal models. Some pharmacological agents may be effective at low doses, others at higher doses, and some agents may be effective in the middle dose range but not at low or high doses. Dose-response curves involving a variety of outcome measures after ischemia should be performed in several species. Another aspect to consider is that neuroprotective drug dose ranges and toxicities in animals may not overlap with those tolerated in humans. In addition, dose-response curves may be very steep in anti-ischemia efficacy studies. The blood-brain barrier, low cerebral blood flow (CBF) (ie, the drug delivery system to the tissue at risk), and plasma protein binding may present impediments for determination of appropriate dosing regimens for use in humans. Nevertheless, there should be a target concentration, a tissue level of effect identified from animal studies, or a surrogate marker that will give some indication, when the drug is given to humans, of whether there is a reasonable prospect of achieving neuroprotection in human clinical trials.

Window of Opportunity
Another factor to consider is the period of time after the ischemic event that drug initiation may be effective, that is, the window of opportunity or therapeutic time window. The window of therapeutic opportunity in animal models is not necessarily predictive of the time window in humans, but determination of relative windows is useful. There is increasing concern that the window of opportunity in humans may not be substantially longer than in smaller species. Notwithstanding these concerns, the time window for efficacy in the animal models must be clearly established. In the past, drugs such as the N-methyl-D-aspartate antagonists cerestat and selfotel demonstrated efficacy in animals for only a limited after stroke onset. This limitation may have contributed to their lack of efficacy in pivotal clinical trials. The optimal duration of treatment in animals should also be examined to guide clinical dosing because some neuroprotective strategies could have paradoxical effects on outcome according to the timing and duration of exposure.

In animal models, the time of the stroke or ischemic onset is known precisely, whereas in humans this may not be the case. In addition, in some animal models, a drug is given before the onset of ischemia or at the time of reperfusion with little attention given to administration at various times after reperfusion. In humans, however, pharmacological agents are not routinely given until hours after the ischemic event. Thus it may be inappropriate to perform expensive clinical trials in which the drug is administered, for example, at 3 hours after occlusion or after reperfusion when the only preclinical animal work available shows the drug to be effective when given at the time of arterial occlusion, reperfusion, or shortly thereafter. Thus a careful assessment of the window of opportunity, that is, the interval after the onset of ischemia or reperfusion when the drug can be successfully administered, should be determined to demonstrate whether the pharmacological agent can be effective at various times after the ischemic event. For example, it would be helpful to know if the drug was an effective neuroprotectant at 15 minutes, 1 hour, 2 hours, and longer after onset of the ischemic event in small-animal and perhaps large-animal models.

Animal Models
There are multiple models of focal ischemia involving both permanent and transient occlusion techniques. In general, for small and large animals, permanent middle cerebral artery occlusion (MCAO) models should be studied first, followed by transient (reperfusion) models. However, it must be recognized that the premature mortality rate is generally greater with permanent occlusion models. An exception to this recommendation is a situation in which the mechanism of action of the drug suggests that it is likely to be effective only with successful reperfusion. In the case of some nonhuman primates, permanent occlusion of the proximal MCA may be attended by significant mortality rates, as it is in humans. In these cases, permanent MCA occlusion may not be suitable, except under anesthesia. Permanent and transient model studies should be initially completed in rats, then possibly in cats or primates before beginning large clinical trials with humans, especially with compounds that are novel, first-in-class agents to determine if the drug is broadly effective.

Mouse models should be reserved for transgenic approaches involving mechanisms of action. Gerbil models should be avoided because many pharmacological agents act as protectants in gerbils but not in other species. There is 1 additional point concerning rat or mouse models that merits concern. Certain strains are more sensitive to MCAO and produce more sizable infarction volumes, whereas other strains produce only a small infarction with MCAO. Thus it is important to study the same strain of mouse or rat throughout the experimental stage. It is critical that when testing a potential neuroprotective agent in animal models, the experiments should be performed in a randomized and blinded fashion, as in clinical trials. Robustness of the treatment effect is an important factor that determines whether a compound will advance to clinical development.

Individual laboratories have developed ischemia models that are designed to maximize detection of neuroprotection. In some hands, the penumbral zone or potentially salvageable region may be more substantial than in others. Therefore there should be evidence of neuroprotective efficacy in 2 or more laboratories, of which at least 1 should be independent of the sponsoring company.
Physiological Monitoring

Whether one uses small or large animals, appropriate physiological monitoring is essential to maintain the animals properly and to limit variability in infarct volume size. Blood pressure, blood gases, hemoglobin, glucose, and CBF should be monitored for as long as possible, although this may be difficult in awake animals. Brain temperature is a critical variable to be monitored and maintained as constant as possible throughout the experimental protocol. This can be measured indirectly in rats with a temporalis muscle temperature probe. CBF can be measured with many techniques. Laser Doppler flowmetry is commonly used in small animals by many investigators. The laser Doppler signal can be monitored before ischemia, during ischemia, and immediately after ischemia to ensure a proper reduction of CBF. A reduction in the laser Doppler signal of ≥60% should be achieved to ensure appropriate ischemia. In large-animal models, there are other appropriate measures of CBF. In large clinical trials, minor adverse changes in these physiological variables, which may be discounted in small preclinical pharmacological studies, could have a profound influence on the trial result. Adverse events and potential drug interactions should be considered. The therapeutic index in relation to such changes should be defined.

Outcome Measures

In small-animal and large-animal models, at least 2 outcome measures should be considered: functional response and infarct volume. Outcome measures can include infarct volume, immunohistochemical analysis, neuropathology, somatosensory-evoked potentials, electroencephalography, and neurobehavior. Outcome measures must be monitored during the acute phase (1 to 3 days) and the long term (7 to 30 days) if one is to examine the long-term effects of these interventions. Acute outcome (typically reduced infarct volume) in a permanent or temporary occlusion model must be evaluated and ameliorated by drug treatment because only in acute treatment studies (typically up to 6 hours of intensive monitoring) can all the physiological variables (blood pressure, temperature, glucose) and clinical signs (signs of infection or vascular inflammation) that affect outcome be assessed comprehensively from the onset of ischemia. Only then can these variables be discounted as sources of artifact. Many preclinical studies examined outcomes only at 24 hours after the onset of ischemia. Several studies have now demonstrated that it is a necessity to follow animals for much longer time periods because if the compound is shown to be initially effective with a short survival time, initial beneficial effects may be lost over time. Long-term outcome, that is, reduced infarct volume, should be evaluated to ameliorate concerns that the drug merely slows the maturation of the histopathological process. Long-term studies present greater challenges for continuous monitoring of physiological variable and assurance of adequate pharmacological design.

Functional recovery is a major end point in clinical trials. In humans, the size of the lesion does not always correlate well with functional impairment, although this correlation has been shown to be more robust with diffusion-perfusion magnetic resonance imaging. Although it may be difficult, it is desirable to demonstrate that drugs improve functional outcome after experimental ischemia. It is challenging to measure function and behavior in brain-damaged animals, although techniques to measure these outcomes are advancing. Furthermore, rodents, for example, display extraordinary plasticity. If surrogate markers of outcome are used in clinical studies, these should be examined in animal models. Replication of improved functional outcome in at least a second species is likely to optimize the chance of success in large-scale clinical trials. Evaluation in larger species such as cats or primates is desirable rather than in rodents only. There is always concern that some pathogenic mechanisms may be disproportionately overrepresented (for example, spreading depression) or underrepresented (for example, collateral vasculature) in the rat. Moreover, as a direct consequence of small brain (and lesion) size, “impressive” volumetric tissue salvage (in percentage change) can be achieved in rodents by small shifts in the infarction boundary. Additional studies should be performed in animals in which there is postischemic reperfusion to address the issue of whether reperfusion complicates or confounds anti-ischemic efficacy demonstrated with permanent ischemia. If a drug is intended to be used solely in patients treated with thrombolysis, reperfusion models may have greater validity than permanent occlusion models.

Other Considerations: Target Populations

No animal model can exactly mimic stroke in humans. However, unless the model has relevance to stroke in humans, for example, MCAO for permanent ischemia or autologous clot embolism for thrombolysis models, questions regarding drug penetration to the ischemic tissue and pharmacokinetic and time window issues will not be answered. The age and species of the animal used also probably influence its relevance to stroke in humans. It is uncertain if benefit in young, healthy animals can be extrapolated to elderly, sick humans.

Sex Differences in Stroke

A potentially important consideration for the development of pharmacological neuroprotective drugs may involve sex differences in drug effectiveness. Recently it was demonstrated that female rats have a markedly reduced infarct volume compared with male rats with MCAO models, and ovariectomized rats have infarct volumes that are similar in size to male rats. Estrogen and perhaps even progesterone may modulate in this response, and studies demonstrated that exogenously administered estrogen reduces infarct volume size in both male and female rats. This information may lead to new therapeutic approaches to reduce infarct volume and injury. Therefore it may be important to examine neuroprotective drugs in male and female animals to separate possible sex differences. In at least 1 clinical trial involving tirilazad mesylate there were differences between men and women regarding stroke outcome (Dr Edward Hall, personal communication). The findings might be explained by differences in metabolism between men and women.
Genetic Manipulation of Enzymes in Animal Models

One new and exciting consideration concerning neuroprotection strategies involves genetic manipulation of endogenous enzymes that may play a role in the mechanism of ischemic injury. For example, mice with upregulated superoxide dismutase (an endogenous oxygen radical scavenger) demonstrate less infarction volume than wild-type mice when exposed to MCAO. Neuronal nitric oxide synthase knockout mice also demonstrate less infarct volume than wild-type mice. Additionally, this holds true for animals deficient in poly (ADP-ribose) polymerase (PARP knockout). Would the combination of the nitric oxide synthase knockout or PARP knockout with upregulated superoxide dismutase demonstrate an even greater reduction in infarct volume? The manipulation of genes such as these and others purported to be involved in mechanisms of injury or neuroprotection may lead to unusual therapeutic approaches. Information derived from these types of genetic studies may add eventually to the stroke-neuroprotective armamentarium that will develop over the next years.

Combined Pharmacological Agents

Because there are multiple mechanisms of neuronal injury after ischemia, it is appropriate to consider using pharmacological agents that affect multiple mechanisms simultaneously. This has been referred to as the “cocktail” approach. With the use of multiple neuroprotective therapies, each agent could be given either simultaneously or in rapid succession, allowing each agent to work on a different ischemic injury mechanism. For example, the combined use of an oxygen radical scavenger and excitatory amino acid antagonist may provide greater neuroprotection than either agent alone. Few prior studies evaluated multiple agent administration approaches. There is a great need for these studies in both small-animal and large-animal models of ischemia to determine whether this treatment strategy has merit. Agents that act as oxygen radical scavengers, excitatory amino acid inhibitors, inhibitors of a variety of mediators of inflammation, calcium channel–blocking agents, hypothermia, and TPA could be given simultaneously or in succession in an attempt to improve outcome. The increasing use of thrombolysis and likely benefit of neuroprotection when used in combination with TPA requires that some examination of potential interaction with thrombolytic and other agents commonly used in acute stroke should be undertaken in animals if relevant.

Recommendations to Clinicians on the Evaluation of Preclinical Data With Neuroprotective Drugs

In general terms, the ideal neuroprotective drug should demonstrate efficacy in at least 2 species, in at least 2 laboratories that use different models, is effective in both permanent and transient focal ischemia, and improves short-term and long-term histological and functional outcomes, even when administered several hours after the onset of ischemia. If the site of action is the brain, the ideal drug should achieve brain concentrations that rapidly equilibrate with plasma, whereas this is not a requirement for agents that target the vasculature such as antiadhesion molecules. There should be a consistent minimum neuroprotective concentration across different species, allowing prediction of the putative neuroprotective concentration in humans. A sigmoid rather than bell-shaped dose-response curve within the animals is particularly desirable because the latter may imply effects on physiological variables such as blood pressure that could offset neuroprotective benefits. Data to guide the duration of treatment should be available and based on the pharmacokinetic profile and purported mechanism of action. This ideal drug profile may not be attainable. The most important points for clinical investigators to assess before considering participation in a trial with a new neuroprotective agent are: (1) an adequate dose-response curve with corresponding serum levels defining at least the minimally effective and maximally tolerated doses in at least 1 species, typically the rat; (2) time window studies showing benefit when therapy is initiated at delayed time points after stroke onset in animal models; (3) adequate physiological monitoring was performed in randomized, blinded animal studies and that treatment effects are reproducible in 2 laboratories, 1 of which is independent of the sponsoring company; (4) outcome measures should include both infarct volume and functional assessment in both acute and long-term phase animal studies; (5) initial studies should be done in smaller species such as rodents subjected to permanent occlusion models, unless the mechanism of drug action suggests that reperfusion will be necessary for drug effect. In this case, clinical development probably will be linked to reperfusion therapy. A second larger species (cats, primates) should be strongly considered for further preclinical assessment for novel, first-in-class drugs; (6) the data should be published or submitted for review in a peer-reviewed journal.

Recommendations Regarding Preclinical Development of Stroke Recovery Drugs

Patients with ischemic stroke may make significant spontaneous recovery after their event. Recovery of some neurological impairments, such as speech, language, neglect, balance, and gait is common. Other neurological impairments, such as a dense visual field abnormality or hemiplegia, may not recover substantially. Recovery may relate to the size and location of the cerebral infarction as well as the initial degree of clinical deficit. The mechanisms promoting functional recovery after ischemic stroke are not entirely clear but most likely depend on functional and/or structural reorganization of the remaining intact brain. Studies in humans with stroke show that recovery may be robust for at least the first 3 months after the stroke, but further recovery may continue thereafter. This prolonged time window of opportunity to intervene on the stroke recovery process offers a substantial and currently unexploited opportunity for drug development. In animal models, several classes of compounds have already shown potential as stroke recovery–enhancing drugs. These include monoamine agonists such as amphetamines and neurotrophic growth factors. The future development of stroke recovery drugs will be dependent on the establishment...
of convenient and reproducible animal models and appropriate clinical trial design.

**Animal Models**

**Rat Models**

A standard rodent model for post–stroke recovery studies has yet to be established. The histopathology and behavioral deficits seen with global ischemia are quite distinct, and therefore the focus of these recommendations will be models of focal brain ischemia. Many of the models used for acute stroke neuroprotection studies, such as the intra-arterial suture occlusion models, result in massive hemispheric infarction and short animal survival times. For stroke recovery studies, more limited infarction must be produced, usually by direct surgical occlusion of the proximal MCA (the so-called “Tamura” method). Such surgery in rats results in considerable infarction in the dorsolateral cerebral cortex and underlying striatum with prolonged survival. The cortical areas involved subserves forelimb and hindlimb function that control sensorimotor behavior in the contralateral limbs.

Standard methods have yet to be established regarding measurements of animal behavioral deficits and their recovery after focal infarction in rodents. However, there are a number of tests of sensorimotor function that can be used. Methods to assess sensorimotor recovery of the limbs include paw-placing tasks. These tests assess the ability of the animal to place the paws on a table top in response to visual, tactile, whisker, and proprioceptive stimulation. These placing tests are highly subjective and observer dependent, and the results may be difficult to replicate. In addition, these tests depend heavily on the activation state of animals; highly agitated animals cannot be tested well with these methods. Other methods for assessing sensorimotor recovery in rats include the foot-fault test, beam walking, and beam-balance tests. These tests have been used successfully by several investigators, in particular to show the recovery-promoting effects of amphetamine treatment after cortical injury in rats. Other tests include those of spontaneous limb use, such as the cylinder test developed by Schallert and colleagues. This test assesses the ability of the animal to spontaneously use the forelimbs in rearing to explore the inner walls of a narrow glass cylinder. Normally, the animal uses each forelimb approximately half the time in exploratory movements. This balance is changed when there is unilateral brain injury, and the animal shows relatively less exploratory movements with the impaired limb. Another approach involves reaching tests developed by Kolb et al. In these tests, animals are food deprived and trained with an apparatus that assesses their ability to pick up food pellets with one limb or the other. All of the above tests can be videotaped for blind scoring of animal behavior and for archiving the data.

These behavioral tests were used in studies that show the efficacy of various classes of drugs that promote poststroke recovery in rodents. In particular, the foot-fault and beam-walking tasks demonstrated the impact of amphetamine in enhancing recovery. Paw-placing and reaching tasks were used in studies that show the efficacy of neurotrophic growth factors in enhancing stroke recovery. Generally, these behavioral methods show a marked deficit in functioning of the contralateral forelimb and hindlimb after stroke. Depending on the test used, there is a slow, steady, and incomplete recovery of function over the next month or so. Depending on the model used, amphetamines or growth factors were observed to accelerate the rate and enhance the maximal degree of recovery.

In the studies in which amphetamines were used, the administration was systemic because these small molecules are expected to cross the blood-brain barrier. In the case of growth factor treatment, studies used the intracerebral (intraventricular or intracisternal) administration because these large proteins are not expected to easily cross the blood-brain barrier.

In addition to the tests of sensorimotor abilities in rats, a number of cognitive tests are also available. The prototype test is the Morris water maze, which assesses the animal’s memory (by finding its way to a submerged platform within a large water tank). The animal navigates to this platform by means of external cues. The behavior can be learned within several trials over several days. The ability of an animal to learn and retain this information is considered an index of memory.

**Primate Models**

There may be a distinct role for primate models in the stroke recovery field. There are a number of dissimilarities between the rodent brain and that of humans and nonhuman primates that may lead to differences in response to an identical ischemic insult. Therefore it is difficult to know how to scale up dosing regimens from rodents to humans. This includes both dose and duration of drug administration. As noted above, recovery in rodent models occurs rapidly over the first few weeks after stroke. Recovery in humans with stroke may occur over a longer time, up to several months after stroke. Thus timing and duration of drug administration for humans is not easily extrapolated from rat models. A similar concern applies to drug dosage. Some drugs, for example, those that are given systemically, can be adjusted by body weight. On the other hand, other stroke recovery treatments, such as proteins and growth factors, which must be given intracerebrally, might be scaled up by brain surface area or volume. It may be difficult to make the transition from rodent models to humans without the intermediate step of primate models and the development of a noninvasive mechanism to deliver the drug to patients.

There has been more than 35 years of experience with experimental preparations of MCAO in the nonhuman primate (baboon), which allows it to be considered a standard format for fundamental studies, exploratory studies, and preclinical work in academia and industry. However, as with rodents, currently there are no standardized, well-accepted models of stroke recovery in primates, although limited experience exists with baboons. Perhaps the most extensive primate recovery work to date has been done by Nudo and colleagues in squirrel monkeys. These investigators demonstrated functional reorganization of the sensorimotor cortex in monkeys after small cortical infarcts. However, strokes were small, and there was considerable spontaneous recovery. An optimal animal model for testing stroke recovery drugs would encompass some but not complete recovery, as this would approximate the human condition.

Ideally, stroke recovery studies in primates might be done with a gyrencephalic species, similar to humans, for example,
macaque monkeys. In addition, however, there are other models in less developed primates, for example, lissencephalic brains, such as in marmosets. Behavioral studies in marmosets receiving the neuroprotective drug clomethiazole were recently reported.44

**Recommendations**
Given the current state of knowledge, the following recommendations can be made concerning the preclinical development of stroke recovery drugs.

**Rodent Models**
Putative stroke recovery drugs should be tested in rodents with models of focal cerebral infarction that permit extended recovery. Recovery of sensorimotor function of the contralateral limbs and cognitive function should be examined. The results should be independently replicated in at least 2 laboratories. Studies should be carried out in a blinded-randomized fashion. After behavioral assessments, animals should be killed and brain studies for histological analysis and infarct volume performed. Incorporating the suggestions made in the section on acute stroke treatment, such studies might also be done in models of permanent occlusion versus reperfusion and in male as well as female animals.

In stroke recovery studies, monitoring of physiological parameters during the stroke surgery is less important than it is in acute stroke studies. Rat behavioral studies should be performed for at least 1 month after infarction.

**Primate Studies**
It is reasonable to explore stroke recovery drugs that show promise in rats, subsequently in primate models. These might include established behavioral models in marmosets or squirrel monkeys as well as tests of sensorimotor and cognitive function in higher-order primates such as macaques or baboons.

**Route of Drug Administration**
The route of administration should be carefully considered in evaluating a stroke recovery drug. Many putative treatments, for example, polypeptide growth factors, might not easily cross the blood-brain barrier, and intracisternal or intraventricular administration may be necessary, although this probably is not a feasible approach for clinical development. Conversely, small molecules such as amphetamines and other monoamine agonists might cross the blood-brain barrier more easily and be appropriate for intravenous administration.

**Toxicology**
Clearly, when an effective drug and route of administration is demonstrated, careful toxicological studies in several species, including both intact animals and animals with stroke, are indicated in the drug development process.

**Dose-Response Studies**
As in the development of acute stroke treatments, careful dose-response studies are necessary for planning of future clinical trials with these agents.

**Time Window**
As in acute stroke studies, the time window of opportunity for treatment is an important variable in preclinical models that precede clinical development. The time window for administration of stroke recovery drugs is likely to be longer than that for acute stroke treatments by perhaps days after stroke onset. Such time window considerations must be carefully considered when designing clinical trials.

**Clinical Development of Stroke Recovery Drugs**
Although the preclinical assessment of stroke recovery drugs may be more difficult than that for acute stroke neuroprotective agents, the clinical development of stroke recovery drugs is likely to be easier. The time window of entry into a stroke recovery study will presumably be considerably longer than that for acute stroke studies. This prolonged time window to entry will offer investigators the opportunity to derive detailed baseline information on patients. Such information might include detailed testing of neuropsychological parameters, motor function, and functional capacities. In particular, scales such as the Functional Independence Measure, the Fugl-Meyer scale, depression rating scales, and formal neuropsychological test batteries can be given before and after treatment. Thus detailed baseline information can be obtained on every patient and compared with detailed follow-up information on each patient. In this sense, each patient can serve as his or her own control, and meaningful change scores can be computed over time. Another advantage for stroke recovery studies is that placebo responders can be more effectively excluded from these studies. Patients who are rapidly improving during the first few days after a stroke can be excluded. Recovery studies not only can use functional outcome to assess therapeutic responses but can evaluate treatment effects on quality of life.

In summary, there is currently a substantial unexplored opportunity for the development of new pharmacological agents and other treatments to enhance functional recovery after stroke. Clinical investigators must pay careful attention to and be able to critically assess the data from preclinical studies.

**Appendix**

**Writing Committee**

**Statement Contributors**
References


