Neuroprotection evolved as a concept as a direct result of the gradual unraveling of the complexities of the ischemic cascade. This was a process that began at about the time that Olney first proposed a neurotoxic role for glutamate under ischemic conditions and continues with the description of a seemingly never ending series of interrelated pathways.1 The common feature of the cascade was its inexorable passage to cell death unless single or multiple components could be attenuated. The sequence of events is now well known and includes early energy failure with depletion of adenosine triphosphate, glutamate release, and its effects on the postsynaptic NMDA receptors, disruption of sodium and calcium ion homeostasis, free radical generation from mitochondria, which then may destroy lipid membranes, inflammatory components, endogenous thrombolysis, and the triggering of both necrotic and apoptotic cell death.2 Each of these pathways (and many others) have been explored using pharmacological probes to observe neurochemical and other outcomes. In most instances, blockade of these pathways has led to protection of cellular function. The properties of these probes have now been exploited to develop “neuroprotectants.” These have been shown to protect cells as well as volumes of tissue from ischemia and improve clinical outcomes in animal models of stroke.3 4

Neuroprotection has been demonstrated at various levels ranging from individual cells, usually in culture, brain slices in which the neurovascular unit is more likely to remain intact, and, finally, in vivo in animal models of cerebral ischemia.5 These have ranged from rodents to primates. Neuroprotective compounds have been shown to be effective at single and multiple sites within the cascade. This has been achieved by a single action with a single compound, multiple actions by combinations of compounds, or by compounds with multiple actions. Importantly, this cascade, which was so brilliantly unraveled, is a cascade largely confined to the gray matter of animal models under ischemic stress. It is not the cascade of white matter that occupies only 10% of rodent brain, but approximately 50% of human brain and is domi-
nated by ion channel dysfunction. It may not even be the cascade of human gray matter, although there is indirect evidence that glutamate neurotoxicity may be involved in human ischemic stroke from studies of temporally related changes in the serum. Similarly, microdialysis techniques have been used to show elevations of excitotoxic compounds within the extracellular space of patients with massive cerebral infarction.

The animal model gray matter neurochemical ischemic cascade does represent, however, the accumulated knowledge of 30 years of research. Indeed, each component of the cascade has been validated many times over in different laboratories and using different approaches. To laboratory investigators, there seems little doubt that the majority of neuroprotectants is effective in protecting the brain from ischemia. This has been demonstrated many times with different classes of compounds and using the outcome measures of a reduction in infarct volume and improved neurological scores in a number of animal models. The apparently simple matter of translating this into the human paradigm then, however, went horribly wrong.

**Difficulties in Translation to Humans**

The early clinical trials were optimistically small without adequate attention to sample size. A variety of compounds that had been shown to have neuroprotective properties were tested but with surprisingly long therapeutic time windows. Calcium channel blockers were tested quite early and showed promise but were later discounted. Fortunately, the conduct of clinical trials gradually became more sophisticated and used larger sample sizes and shorter therapeutic time windows. However, repeated clinical trial failures of categories of compounds ranging from NMDA antagonists through to antinflammatory agents heightened concern among academic and industry leaders, so much so that a series of Stroke Therapy Academic Round Table (STAIR) conferences between academic and industry leaders were organized to identify factors, which may be impeding an apparently straightforward translational research task. Many of the problems of translation were perceived to be in the preclinical arena. Specifically, there was a need to improve preclinical methodologies so that clinical researchers could be more confident in the efficacy in animal models of the compounds being subjected to clinical trial. Clinical trial issues were also thought to be important, not the least of which were sample size and therapeutic time window considerations. Indeed, new levels of sophistication in clinical trials were reached with the introduction of adaptive design techniques and shift analyses of clinical outcomes. This led to increasing levels of confidence in the results of phase III clinical trials and hope that success was just around the corner. Unfortunately, this was not to be the case.

**The Turning Point**

The compound NXY-059 is a free radical scavenger and was considered to be one of the most likely compounds to be successfully translated into the human stroke paradigm. It had undergone one of the most rigorous preclinical testing regimes to date. A series of investigators in different laboratories had shown that NXY-059 was neuroprotective in rodent models of cerebral ischemia and similar efficacy was demonstrated in marmosets. Pharmacokinetic studies were performed with serum levels achieved in clinical trial that were at least equivalent to those showing neuroprotection in animal models. Indeed, it was felt by many investigators that NXY-059, if clinically successful, would be a validation of the STAIR criteria. However, it must be recognized that this program certainly had its limitations, which have been highlighted by others. In particular, some evidence that NXY-059 was a reasonable free radical scavenger by the demonstration of IC50 oxygen radical inhibition in vitro and in vivo systems would have been helpful. Furthermore, there was no replicated evidence that NXY-059 was effective beyond 3 hours and behavioral improvement beyond 1 week had not been reported in rodents. In marmosets, although NXY-059 did improve arm weakness, it did not significantly reduce infarct volumes.

For the clinical program, there was a mismatch of time windows with the preclinical evidence (3 hours). In the SAINT I trial, intravenous NXY-059 was given within 6 hours of stroke onset for a total of 72 hours in 1722 patients. Despite this, outcomes were reported as being positive based on a shift analysis of the modified Rankin scale of primary end points. The issues of concern were the finding of a negative secondary outcome measure of change in neurological score (National Institutes of Health Stroke Scale), the lack of a positive relationship between time of onset of therapy and clinical outcome, and whether the shift analysis technique may produce results that are statistically significant but not clinically meaningful. The relationship between time of therapy and clinical outcome is clearly seen with the intravenous tissue plasminogen activator, the only licensed compound for acute ischemic stroke, and has provided additional evidence for the “time is brain” concept. Given the disappointments of the past, the sponsors and investigators quite rightly insisted on a second phase III trial, this time conducted with a sample size of 3200 patients but an otherwise similar protocol. Although the full results have not as yet been published at the time of this writing, the results were presented at the International Stroke Congress in February 2007 and were completely negative. A reasonable interpretation of the results of both trials combined is that the first positive result was due to the play of chance. After more than 20 trials of neuroprotection, it would be expected that approximately one would be positive when an α is set at 0.05 and no adjustments made for the equivalent of multiple comparisons.

Clearly, a point has been reached in neuroprotection research where we cannot continue to do “business as usual.” Either translational research in neuroprotection should stop altogether or we approach the problem in quite a different way. I would favor the latter option for a number of reasons. First, it is hard to deny the biological plausibility of the neuroprotective concept garnered from 30 years of accumulated knowledge about the ischemic cascade and our ability to attenuate its progression using a variety of pharmacological tools at different levels. In other words, despite a number of general shortcomings to this research, which are discussed...
shortly, it seems a reasonable assumption that neuroprotection is effective in animal models of stroke. Second, there is enough evidence from other body organs in humans from the transplantation literature, including kidney, liver, and heart, that strategies may be put in place to prolong cellular life under hypoxic and/or ischemic conditions. Hence, it would seem reasonable to pursue the aim of prolonging in vivo brain cell life in much the same way. Before doing so, however, some hard questions need to be asked.

The Hard Questions
These are outlined in Table 1 and each are addressed in turn.

Are We Selecting Drugs That Really Work in Animal Models?
Probably not. The main reason for this is a clear lack of rigor in preclinical testing.33 In a series of studies, we have identified problems such as marked publication bias, a lack of randomization of animals before intervention, and a lack of blinding for both induction therapy and outcome measures randomized of animals before intervention, and a lack of blinding for both induction therapy and outcome measures and a lack of the use of internal controls.34,35 Indeed, there is bline association between diminishing effect size for outcomes such as volume of infarction and neurological scores with the quality of studies as defined by a 10-point score based on the STAIR criteria, which we have developed to quantify the experimental rigor with which animal experiments have been conducted35 (Table 2). Furthermore, given that the average sample size for these experiments is usually 5 to 6 animals, with no real regard for the variance of the population, the power is usually only approximately 50% (Table 3). In other words, the probability of repeating a positive study and obtaining the same result is only approximately 50%. Interestingly, after undertaking a reanalysis of the preclinical NXY-059 and data using our quality score, the overall mean was only 4.5 out of the possible score of 10. Hence, NXY-059, among others, may not be the most ideal compound to move forward into human studies.

Are Rodent and Human Cells Similar in their Response to Hypoxia and/or Ischemia?
Based on the sequencing that has now been achieved for the human and mouse genome, some comparisons can be made between the 2. Expressed simply, it appears that although the mouse genome is approximately 14% smaller than the human genome (2.5 GB compared with 2.9 GB), approximately 90% of the gene order has been conserved in both species. Even more surprisingly, the proportion of mouse genes without any homolog currently detectable in the human genome seems to be less than 1%. However, the generation of proteins from these genes, or proteomics, seems to differ significantly between species. For most categories of proteins, the mouse has a similar percentage compared with humans. However, there are significant differences in some areas such as the translational apparatus category, protein, and other metabolic processes.36 This may suggest that there may be important differences in the way the human and rodents cells respond to ischemic stress. At least this hypothesis needs to be tested more directly.

Is There Any Evidence That Neuroprotectants Are Effective in Human Cells or Brain Tissue?
As a corollary to the second point, there seems to be little or no direct evidence to support the view that when placed under hypoxic and/or ischemic conditions that human cells or brain tissue may be protected from either necrotic or apoptotic cell death. Some limited evidence comes from the study of Werth et al who used cellular swelling as an outcome measure in human neocortical slices and showed that MK801 attenuated the effects of oxygen–glucose deprivation.37 For the most promising candidate neuroprotectants, this would seem to be a fundamental requirement to reassure investigators that translational success was likely before progressing to in vivo studies in humans.

Is There Any Evidence That Neuroprotectants That Have Been Subjected to Human Trial Actually Reach the Target Tissue, the Ischemic Penumbra?
The only evidence that any of the neuroprotectants have actually crossed the blood–brain barrier in trials of neuroprotection is purely clinical. In a number of the earlier studies

### Table 1. Neuroprotection: The Hard Questions

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<td>Are we selecting drugs that really work in animal models?</td>
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<td>Are rat cells the same as human cells?</td>
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<td>3.</td>
<td>Is there any evidence that neuroprotectants are effective in human brain tissue?</td>
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<td>4.</td>
<td>Is there any evidence that neuroprotectants reach their target in humans?</td>
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<td>Are the human proof of concept models of cerebral ischemia ideal?</td>
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### Table 2. A Score for Experiments Using Animal Models of Stroke

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<td>Blinded assessment of outcome</td>
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<td>6.</td>
<td>Anesthetic without neuroprotective activity</td>
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<td>Appropriate animal model (aged, diabetic, or hypertensive)</td>
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<td>Compliance with animal welfare regulations</td>
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involving NMDA antagonists, side effects such as somnolence and visual hallucinations would suggest that penetration deep into the brain had occurred. Beyond this, however, there has been no documentation that the blood–brain barrier has been crossed in adequate concentrations or reached its therapeutic target, the ischemic penumbra. Indeed, NXY-059 crossed the blood–brain barrier very poorly under normal conditions in rodents.19

Are Human Proof of Concept Studies Ideal? Most proof of concept studies involving neuroprotectants have been of the standard phase II type. Often, therapeutic windows are quite long, well beyond the 3-hour target proven to be effective with thrombolysis. Two important principles need to be considered. First, the most powerful effect of neuroprotectants in animal models is when the test compound is preloaded or given very early after the onset of ischemia.35 For the latter, there are novel clinical circumstances in which the therapeutic target, the ischemic penumbra. Indeed, it would seem reasonable that clinical trials for neuroprotectants should be enriched with patients who have significant penumbral presence as the prime target of therapy.

By addressing these questions, a logical road map for a new approach to translational research in neuroprotection can be developed (Figure).

A New Road Map for Translational Research in Neuroprotection Better Proof of Efficacy in Animal Models When screening drugs for efficacy, we must expect a much higher level of evidence than we have in the past. Better preclinical trial design with attention to the quality issues outlined earlier will give investigators more confidence that the purported neuroprotectant is definitely effective in animal models. The most important issues to address are adequacy of sample size based on power calculations whereby a power of at least 80% is generated as we would expect in clinical trials. Similarly, full randomization and blinding should be standard practice, and it would be useful to run internal controls with reliable neuroprotection strategies such as hypothermia to be assured that either success or failure of a given experiment was not due to technical errors. Furthermore, it may be useful to undertake meta-analyses to summarize the data available and establish their strengths and limitations.35,40 In other words, there should be little difference between the principles of the conduct of experiments in animal models and clinical trials of stroke. Given that there is an expectation in clinical trials to adhere to certain standards of reporting such as outlined in the CONSORT statement, a similar set of standards needs to be developed for the animal experimental literature.41

This focus on better proof of efficacy in animal models from among the myriad of existing compounds that are said to reduce infarct volumes and improve clinical outcomes should not detract from the ongoing efforts to identify new targets of therapy. However, as these new targets are identified and appropriate neuroprotectants developed (not the subject of this road map per se), they should be subject to the same rigorous approach to translational research outlined in this article.

In Vitro Efficacy in Human Tissue This approach is not new but has been underused because of the apparent success of neuroprotection in animal models and the expectation that this would readily translate in the human paradigm. However, in this new environment of repeated failures of translation, the importance of establishing efficacy in this model has been magnified. There are well-established models in which tissue is subjected to hypoxic or ischemic stress using preparations such as cell culture or brain slices. The tissue is placed in a sealed chamber from which oxygen is removed and/or glucose as an energy source is deprived. These are known as oxygen–glucose deprivation models or oxygen deprivation and are almost universally conducted using cell lines or slices from animals rather than humans with a few exceptions such as the study of Werth et al mentioned earlier.37 The use of cultures of neurons, astrocytes, or oligodendrocytes may allow a more cell-specific response to ischemia to be investigated in this system.42 Although studies with individual cell lines may help to establish the subcellular mechanism of neuroprotection, some caution needs to be exercised in case phenotypic change in cell structure has occurred during culture. Conversely, when brain slices are used, the integrity of the neurovascular unit is more likely to be retained.43 This is an important issue because it is unclear as to which component of the neurovascular unit (or interaction between the 3 components of glial cell, neuron, and blood vessel) is responsible for the neuroprotective effect seen with some compounds or even other indirect effects.

The tissue for brain slices may be readily obtained during standard surgical procedures such as temporal lobectomy for patients with temporal lobe epilepsy, removal of benign tumors or arterial and venous malformations, surgical drainage of intracerebral hematomas, or even hemicraniectomy for ischemic stroke. Obviously, the tissue removed should be as far from the pathology as possible to be assured that it is neurochemically intact.

In Vivo Studies of the Distribution of Neuroprotectants in the Human Brain Given the uncertainty as to whether many neuroprotectants cross the blood–brain barrier in humans, it would seem to be another very logical step to be assured that this occurs before
embarking on later phase human studies. As mentioned earlier, this may be particularly important for compounds such as NXY-059 in which there is uncertainty about the ability of the compound to cross the blood–brain barrier under normal conditions in the rodent. The simplest way to achieve this is to use positron emission tomographic technology. By labeling potential neuroprotectants with positron emitters such as C11, its in vivo distribution can be readily determined together with its brain pharmacodynamics. Although C11 is the most attractive positron emitter to use in these circumstances because it rarely alters the chemical properties of the labeled compound, other positron emitters such as F-18 may be more appropriate in some instances. This is a heavier isotope and may, therefore, alter the chemical characteristics of the labeled compound, although probably not enough to alter its blood–brain barrier-crossing properties. These studies do not reduce the need for standard pharmacokinetic studies to study the peripheral behavior of relevant compounds, but compliment them to provide critical information about drug concentrations at target sites.

Having established that the putative neuroprotectant enters the brain in adequate concentrations, it is also important to determine as to whether the principal target, the ischemic penumbra, is reached. Here, MR with diffusion-weighted/perfusion-weighted imaging mismatch to identify the extent and location of the penumbra together with positron emission tomography to determine the distribution of the neuroprotectant would be a useful approach. Once evidence that the neuroprotectant is likely to reach its target is provided, proof of concept studies in humans could begin.

Efficacy in Novel Human Models of Cerebral Ischemia

Proof of Concept

As mentioned earlier, neuroprotectants have been shown to be most effective when either given within minutes after the onset of cerebral ischemia or they have been preloaded. The former is difficult to achieve in humans, but the latter model may be developed in a number of different circumstances.

The first of these is in patients who present when transient ischemic attack or minor stroke. It has been more recently shown that the risk of recurrent stroke within the first month is approximately 13% and in some subsets as high as 8% within the first 2 days. Hence, if the agent was introduced early after the onset of the initial clinical event, a “preloading” of a trial neuroprotectant could occur. Outcome events could then be a composite of the disability rating on the modified Rankin Scale of new stroke events and the volume of additional ischemic events (symptomatic and asymptomatic) seen on initial compared with repeated MR diffusion-weighted imaging at 30 days.

The second is in patients who are undergoing procedures whereby the likelihood of a stroke event occurring in the perioperative period is enhanced. Obvious examples include carotid endarterectomy, cardiac bypass surgery, and carotid artery stenting. In each of these, the perioperative stroke risk is on the order of 3% to 7%. Again, outcome measures could include a combination of clinical disability scores and imaging volumes of infarction. The advantage of incorporating imaging outcome measures in both of these study designs is to reduce the sample size requirements.

Phase II and Phase III Studies

By the time this stage is reached, the investigators should have gained confidence from the previous proof of concept studies that their putative neuroprotectant really is effective in human tissue. Hence, the likelihood of producing positive phase II and III results in appropriately designed trials is higher than would be expected from the number of negative studies to date.

Phase II Trials

Here, the principle of selecting patients who are appropriate for the compound being tested is important. As mentioned earlier, the concept of the ischemic cascade has been built on 30 years of research in which this sequence of neurochemical events within gray matter has been documented. The cascade in white matter is less well studied and, rather than being dominated by glutamate excitotoxicity, is more governed by ion channel dysfunction. Hence, although some neuroprotectants may have mechanisms of action restricted to the gray matter cascade, others may be only effective in white matter and some in both. Because most neuroprotectants developed so far have been tested predominantly in gray matter models of cerebral ischemia, the most appropriate target in humans is the ischemic penumbra in this compartment. Not surprisingly, approximately 50% of the ischemic brain volume in standard trials of neuroprotection is in white matter. Hence, in phase II trials involving these compounds, enriched populations using imaging techniques such as MR or CT perfusion should be used to identify patients with appropriate penumbral patterns. This approach has already been used in trials of thrombolysis with some success, although the principle was one of reperfusion rather than neuroprotection per se. The primary outcome measure in these penumbra-defined populations should be infarct core growth based on diffusion-weighted and T2 images for which sample size estimates have been published.8

Phase III Trials

The general principles of phase III studies of neuroprotection should be that an appropriately short time therapeutic window is used based on the time window established in animal models. They should be adequately powered (power of at least 80%) and based on an expectation that absolute benefits may be quite small, probably on the order of 3% to 5%. Correspondingly, outcome measures should be reasonably sensitive to small absolute benefits. Hence, the move toward shift analyses of changes in categories of the modified Rankin Scale rather than dichotomous outcomes in which large amounts of clinically meaningful data remain unused is encouraging. Similarly, adaptive design techniques in which doses are adjusted based on clinical response should allow a smooth transition from phase II to phase III studies.

Conclusions

Because we have reached a point in neuroprotection research at which either this avenue is closed altogether or new
strategies are developed, this road map offers a way forward. By adopting an incremental approach with an emphasis on scientific rigor in selecting putative neuroprotectants in animal models, demonstrating that neuroprotection occurs at a cellular and tissue level in humans, that the neuroprotectants reach the target ischemic penumbra, and that the neuroprotectant is likely to be effective in phase II and III acute stroke trials based on their efficacy in novel models of human cerebral ischemia, successful translation from bench to bedside is more likely to be successful.

Acknowledgments

My early mentors, including Peter Bladin, Austin Doyle, Jack Whisnant, Tachi Yanagihara, and Bob Ackerman, must bear some responsibility for my interest in stroke in general and this area in particular. Throughout my career, most of my work has been done in collaboration with Stephen Davis without whom much of this would not have been possible. Discussions with my colleagues, David Howells and Malcolm Macleod, ignited my interest in animal model collaboration with Stephen Davis without whom much of this would generally. The road map is theirs as much as mine. I thank all my colleagues and research staff for their ongoing contribution to this team effort.

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G.A.D. has provided advice to a number of pharmaceutical companies as a member of scientific advisory boards and has received honoraria for various presentations at international scientific meetings.

References


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