Translational vehicles for neuroprotection

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Abstract
This review will analyse particular criteria in the analysis of stroke diagnosis and treatment, which are pivotal for the successful translation of experimental data from the laboratory to humans.

Introduction
A thrombo-embolic stroke results in the formation of three compartments in the brain: the core, the penumbra and the unaffected tissue. The core is an area or volume of tissue that has severely compromised CBF (cerebral blood flow) and will infarct very rapidly, showing early and extensive damage. The penumbra surrounds the core area, is hypoperfused, but remains viable and can be salvaged. As an operations definition, if the penumbra is reperefused it will escape infarction. The rest of the brain is largely unaffected, even though the CBF may be reduced as in oligaemia.

Neurovascular imaging
The importance of imaging in the development of stroke treatments, such as thrombolysis, cannot be underplayed. Imaging has provided a surrogate for translating the animal observations of intravenous thrombolysis treatment to successful clinical intervention.

Different imaging modalities are available that allow the investigation of the various aspects of the neurovascular unit. Neurovascular imaging is particularly helpful to look at both the vasculature and the parenchyma. For research purposes, the perfusion/diffusion mismatch can be used to study the properties of the ischaemic penumbra [1].

The approach to the CT (computed tomography) scan is to exclude any patients with haemorrhage or mass lesions from thrombolysis treatment. The focus is on vascular lesions as well as on early ischaemic changes.

Thrombolysis
MRI (magnetic resonance imaging) modalities can be employed to demonstrate the success of thrombolysis. The perfusion weighted/diffusion weighted mismatch, accompanied by large artery occlusion, provides a viable clinical image, which can show the effectiveness of thrombolysis on a case-by-case basis. The perfusion/diffusion mismatch can be recovered if either the obstructed artery is opened or the collateral flow is improved [2]. This pattern suggests that if the ischaemic tissue is reperefused, because of successful lytic intervention, there will be improved clinical outcome.

Currently, thrombolytic treatment is most commonly carried out using recombinant tPA (tissue plasminogen activator) also called altepase [3]. The reason that some patients receiving thrombolysis do not show improved recovery is because tPA opens arteries with up to 50% efficiency, depending on which artery is occluded [4]. In more detail, following intravenous intervention, only 12% of patients with a carotid T occlusion achieve reperefusion, 40% with a middle cerebral artery (MCA) occlusion, 30% with an M1 occlusion, and approx. 50% with an M2 occlusion.

A fundamental issue to do with thrombolysis is whether or not the efficacy studies, as analysed in the meta-analysis, can be translated from efficacy to effectiveness within the community. To assess the effectiveness of thrombolysis in actual clinical practice, the CASES (Canadian Alteplase for Stroke Effectiveness Study) was carried out [5]. The outcome was matched to the randomized trials in an open-label register, prospectively collected with ascertainment. The study was part of the process for licensing tPA for acute ischaemic stroke in Canada.

All the risk factors were analysed to establish predictors of good outcomes or predictors of increased risk of haemorrhage, thus stratifying patient risk. It was revealed that the CASES outcome was dependent on several factors, such as serum glucose and age. Those over the age of 70 years had a markedly decreased chance of good recovery [mRS (modified Rankin Scale) of 0–2] at 32%. Additionally, those over the age of 80 years had only a 25% chance of good recovery. Despite this, there was no increase in the risk of haemorrhage in those over the age of 80 years. There was also evidence that increased serum glucose levels reduced the chances of good outcome.

In addition, the ASPECTS (Alberta Stroke Program early CT score) [6] was a strong predictor of the CASES outcome. The ASPECTS is a ten-point score that allows us to look at the grey/white differential in much the same way as cardiologists have used the ST segment on the ECG (electrocardiogram).
In the CASES, those who had an ASPECTS of 4 and above were likely to achieve some benefit. However, those scoring 4 or less were unlikely to see any improved outcome following thrombolysis.

We are, therefore, able to come up with rules of prediction for the outcome of thrombolysis treatment. These include the NIHSS (National Institutes of Health Stroke Scale) score, the ASPECTS, age and serum glucose. Moreover, the two variables that predict symptomatic intracranial haemorrhage, in addition to protocol violation, include serum glucose and the onset-to-needle time.

In conclusion, the opening of occluded arteries, as a surrogate for determining the dose and duration requirements of thrombolysis, has been invaluable [7].

**Spontaneous intracranial haemorrhage**

Similarly to ischaemic stroke, spontaneous intracranial haemorrhage is also now managed within the first several hours. One of the surprises of seeing patients acutely and performing CT within an hour or two of the onset is the realization that haemorrhages actually grow over time. In fact, there is often quite marked haematoma growth seen between 2 and 8 h following symptom onset [8].

Increases in the haematoma volume are strongly correlated to outcome and mortality [9]. Therefore the growth of the haematoma can be used as a surrogate for demonstrating, on a case-by-case basis and between groups, the effect of thrombostatic drugs. Although surgical treatments have been unsuccessful in improving the chance of survival, there still remains some question as to whether superficial haemorrhages could be eventually managed with early surgery.

The outcome of the recent international STICH (surgical trial in intracerebral haemorrhage) was disappointingly negative [10]. Although the patients in the STICH trial were treated late, for therapies with thrombostatic drugs, like with thrombolytics, treatment should be given within the first several hours following the onset of haemorrhage.

Thrombostatic treatment with activated recombinant FVIIa (Factor VIIa) enhances haemostasis at the site of bleeding, without systematic activation of the coagulation cascade [11]. In the same way that tPA is activated by fibrin, FVIIa is activated in the presence of tissue factor [12]. Tissue factor is a lipoprotein that is constitutively expressed on the plasma membrane of leucocytes, subendothelial tissue and platelets. The activation of tissue factor initiates the formation of thrombin on activated platelets [13]. Thrombin, a serine proteinase, converts fibrinogen into fibrin, thus producing a stable clot that is resistant to fibrinolysis.

The efficacy of FVIIa was assessed during the NovoSeven Phase II trial [11]. This was a multicentre, randomized, double-blind trial in which 399 patients were assigned to placebo or one of three doses of FVIIa. Patients had to be imaged within 3 h of onset and treated within 60 min of the CT scan. They had a follow-up scan at 24 h and were seen in follow-up at 90 days.

The study included those with spontaneous intracerebral haemorrhage and cerebellar haemorrhage. Those with no time of onset or with haemorrhage secondary to a lesion such as infarction, tumour, aneurysm, arteriovenous malformations, or trauma were excluded. Additionally, those with risk of bleeding because of known oral anticoagulant use, thrombocytopenia or coagulopathy were also excluded.

The study showed that of those receiving placebo there was a 30% increase in the volume of intracerebral haemorrhage from baseline. However, the patients receiving FVIIa reduced the haemorrhage size to 16% from baseline for those receiving 40 µg/kg \( (P = 0.07) \), 14% for those receiving 80 µg/kg \( (P = 0.05) \), and 11% for those receiving 160 µg/kg \( (P = 0.02) \). The infusion of FVIIa, therefore, achieved somewhere between a 45 and 62% relative reduction in the size of growth of the haematoma. This is associated with a dramatic reduction in mortality decreasing from 28% down to approx. 18% for those receiving treatment. Importantly, there was an increase in those recovering to a mRS of 0–1 from 8% for the placebo group to 24% for those receiving 160 µg/kg FVIIa.

Overall, the efficacy of FVIIa is comparable with that seen with thrombolysis. Importantly, the surrogate of seeing reductions in haemorrhage size, following active intervention, allows the imaging to guide which dose of FVIIa treatment does not carry risk.

**Animal models of neuroprotection**

Traditionally, the aim of the experimental studies of neuroprotection is to reduce the infarct volume following permanent or transient MCA occlusion, by the administration of neuroprotective drugs. Alternatively, a neuroprotective agent can allow longer intervals of ischaemia to be sustained, therefore increasing the tissue window for reperfusion.

Most of the neuroprotection work comes from *in vitro* experiments with cell culture and hippocampal slices, subjected to oxygen and glucose deprivation, otherwise cultured under relatively high oxygen conditions (all reviewed in [14]). Drugs, such as glutamate receptor antagonists and specifically NMDA (N-methyl-D-aspartate) receptor antagonists, can prevent cell death in both cell cultures and brain slices, as measured by propidium iodide staining [15].

In reality, the *in vitro* work shows cytoprotection, which has rarely been demonstrated in the global model of cerebral ischaemia. Global ischaemia results in a selective and delayed cell death in the CA1 pyramidal neurons of the hippocampus [16]. As a result, protecting the sensitive CA1 cells from global ischaemia should infer cytoprotection. However, the CA1 neurons are very hard to rescue from global ischaemia with pharmacological as opposed to physiological intervention. Thus much of the protection seen in global models, for example by the NMDA receptor antagonists, is the manifestation of temporary hypothermia, which is a side effect of the drug [17].

Another question that remains to be answered is whether the pharmacologically observed protection in the *in vitro* models is truly translated to *in vivo* models of focal cerebral ischaemia, as assessed by a reduction in the infarct size. In focal models, reduction of infarct volume may possibly...
result in subtly improved CBF because of either vasodilation or protection with drugs that are active at the vasculature [18]. For example, pre-treating rats or mice with simvastatin improves their tolerance to models of ischaemia, but also dramatically improves CBF [19]. Therefore the intervention reduces the severity of the insult, hence explaining the observed improvement in the outcome.

Overall, in models of global ischaemia, pharmacological protection has been accounted for by subtle changes in temperature. In focal ischaemia, reductions in infarct volume have probably been a result of subtle changes in CBF.

**Mechanisms of neuroprotection**

The best neuroprotectant would always be the rapid return of oxygen and glucose to physiological levels and the clearing of products of anaerobic metabolism, such as lactate.

Intracellular influx of water during ischaemia will cause osmotic lysis, whereas, the cytoplasmic build-up of high calcium concentrations will lead to deleterious secondary events (reviewed in [20]). In particular, phospholipase A2 is activated by calcium, resulting in the breakdown of arachidonic acid, the production of free radicals, lipid peroxidation and ultimately breakdown of cellular membranes. Attempts to block calcium flux through voltage-gated calcium channels [21], NMDA receptors [18] or TRP (transient receptor potential) channels have been reported [22]. Although these approaches have been effective *in vitro* and have been capable *in vivo* of reducing infarct volume, in the wake of global ischaemia, CA1 neurons could not be protected.

A novel method to confer neuroprotection was recently published that prevented NO (nitric oxide) toxicity. This was achieved by dissociating the interaction between the NMDA receptor and the scaffolding protein, PSD-95 (postsynaptic density 95) [23]. The TAT-NR2B9C protein was used, which disrupts the NMDA receptor–PSD-95 interaction, thereby preventing the downstream formation of NO and the subsequent injury to the mitochondria.

Similarly, agents that block either the release of cytochrome c from the mitochondria or the subsequent activation of caspase 3, which would otherwise lead to the activation of DNase, can all prevent ischaemic cell death [24].

To determine whether the neuroprotective compounds have the pharmacological action seen *in vitro*, in an *in vivo* situation we need *in vivo* molecular markers. Consequently, the mechanism of action would be confirmed for compounds that block the production of NO, the synthesis of free radicals or the activation of caspases.

If we can, therefore, correlate drug-induced mechanistic change and demonstrate that this results in the improvement of histological outcome, then it would be possible to translate what is observed *in vitro* to an *in vivo* setting. Subsequently, we can use a similar imaging bridge to determine whether or not this mechanism is active in clinical stroke and inhibited by the use of neuroprotective agents.

**NXY-059**

The nitrone PBN (α-phenyl-N-t-butylnitrone) and the recently developed compound from Astra Zeneca, NXY-059 (Cerovive™), are purported to act as free radical spin traps (for a review, see [25]). Free radicals are a result of intracellular NO formation and of the reperfusion cascade. Therefore, by blocking the post-ischaemic burst of radicals in both pathways, neuronal cell death should be prevented.

PBN, initially used to quantify free radicals [26], was redesigned so it could be infused intravenously to inhibit the deleterious effects of free radicals by trapping them *in situ*. NXY-059 is structurally related to PBN and although it is more water-soluble, it exhibits poor penetration across the blood–brain barrier [27]. The suggested neuroprotective mechanism of NXY-059 is to prevent the increase of free radical formation, thereby blocking the ischaemic cascade. NXY-059 reacts with free radicals, forming non-reactive adducts.

Significantly, the agent has demonstrated protection in *in vivo* models of ischaemia [28,29]. In particular, transient and permanent focal ischaemia not only in rat, but also in marmosets (sub-human primates), demonstrated that NXY-059 administration caused marked reduction in the infarct volume, following permanent MCA occlusion [30]. The drug was effective even if it was given 4 h following MCA occlusion [31].

The time course of NXY-059 is similar to the effects of reperfusion agents, such as tPA. The best effect is observed immediately and up to 4 h following permanent occlusion.

However, focal models of ischaemia in rats have revealed that significantly different concentrations of NXY-059 are required for the same protective effect in permanent compared with temporary MCA occlusion [29]. Following temporary MCA occlusion, doses of NXY-059 of 10 mg/kg per h, were sufficient to produce significant reduction in infarct volume. However, in the permanent rat models, doses as high as 50 mg/kg per h had to be used to attain similar protection. This implies a different mechanism in temporary and permanent focal ischaemia.

Importantly, NXY-059 has been demonstrated to be safe in toxicology studies and so far has exhibited no side effects in the clinical trials [32]. Recently, the results of the SAINT (Stroke–Acute Ischaemic NXY Treatment)–I trial were published. This was a phase III clinical trial, which showed that NXY-059 treatment results in statistically significant improvement of the primary outcome [33]. Those achieving an mRS of 0, 1 and 2 went from 43% in the placebo group to 45% in the treated group, i.e. number needed to treat = 50.

Critically, the same study revealed that the drug was not effective in an additive sense, when co-administered with tPA. Taking also into account that NXY-059 is not permeable across the blood–brain barrier [27], it can be suggested that, similarly to tPA, its protective effects might be related to improving CBF, particularly when high concentrations are used. This would imply that the drug might be working in permanent ischaemia by improving CBF, while in transient
focal ischaemia by inhibiting breakdown in the blood–brain barrier.

A surrogate for the prevention of blood–brain barrier breakdown is the reduction in haemorrhages in those patients getting tPA together with NXY-059. In keeping with this, the SAINT-I study showed that this group of patients had a statistically significant reduction in the number of haemorrhages. This effect of NXY-059 may well be mediated by protecting the endothelium from radically induced damage and also by inhibiting MMPs (matrix metalloproteinases), thus leading to a reduction in haemorrhage.

The drug is not truly neuroprotective as it does not reach the target organ, i.e. the brain, but may protect the brain from haemorrhagic side effects of tPA and may also improve CBF in permanent ischaemia. Therefore NXY-059 might truly be acting in the blood–endothelial interface.

A second phase III trial is under way and this should confirm the effect seen in the SAINT-I study. If the results are actually replicated, then we will have an agent that will be useful in combination with tPA, in limiting reperfusion haemorrhage.

**Molecular imaging**

It would be an exciting breakthrough if we had an agent, such as NXY-059, that could be given along with tPA to inhibit the disruption or injury to endothelial-type junctions. The protective action of NXY-059 on the vasculature can be established by using molecular markers, such as the activation of MMPs. However, to really demonstrate that the drug is working *in vivo* by blocking free radicals, it would be necessary to measure oxidative or nitrosative stress in the brain. Free radicals can be visualized in the brain with imaging modalities, such as ESR or proton/electron double resonance imaging.

The free radical-trapping capacity of NXY-059 can be determined with microdialysis and HPLC experiments. Critically, these studies would determine whether the drug is eventually reaching the brain parenchyma. The ultimate hope would be that infusion of NXY-059 would reduce the free radical signal, especially following focal cerebral ischaemia.

Even if this could not be done in the brain, it would be useful to demonstrate the free radical-trapping properties of NXY-059 in other body areas. For example, in mice that are treated with lipopolysaccharide (an inducer of inducible NO synthase), NO can be visualized in the abdomen using ESR. Therefore a similar model could be employed to demonstrate that the NO concentration in the abdomen can be knocked down with NXY-059.

This notion, of using molecular imaging to visualize the *in vivo* mechanism of action of NXY-059, should also be applied to other neuroprotective compounds. In particular, it would be interesting to detect a reduction of NO formation in the brain following administration of the TAT-NR2B9C protein. Additionally, *in vivo* imaging of caspase activity with bioluminescent surrogates would be useful to demonstrate the inhibition of caspases.

Furthermore, the inhibition of both lactate accumulation and acidosis by iron channel blockers can be imaged using magnetic resonance spectroscopy. Imaging the lactate signal could eventually provide a surrogate for neuroprotection. During ischaemia, lactate accumulates and, following reperfusion, in a normothermic animal the formation of lactate remains unabated (reviewed in [39]). However, following reperfusion, in a hypothermic animal the lactate clears out. Therefore, the lactate signal is a surrogate, which can be titrated and may be useful in human neuroprotection, if high field imaging was made available.

The concept of molecular labelling is therefore very important, particularly if we can image a signal that contributes to the maturation of the lesion. The use of paramagnetic iron oxide in conjunction with MRI demonstrated macrophage infiltration in tissue that will infarct. This leads to potentially causal relationships between the growth of the injury and the inflammatory process. Therefore the inflammatory cascade can constitute an imaging surrogate of ischaemic injury.

The inflammatory injury can be inhibited with anti-inflammatory agents, such as the interleukin-1 receptor antagonist. The inflammatory process can be imaged by tagging gadolinium to mimetics that recognize the activation of E- or P-selectin. In more detail, mimetics of the sialyl-Lewis antigen, which are tagged to gadolinium, can recognize P-selectin expression on endothelial cells. Eventually, minutes after infusion of sialyl-Lewis/gadolinium, there is marked increase in uptake in endothelial compartments, representing tissue that is undergoing active infarction.

In conclusion, imaging selective surrogates that allow visualization of the ischaemic pathways mentioned above, i.e. free radicals and lactate, or the activation of caspases and inflammation, would be pivotal for the concept of bridging biomarkers. Such biomarkers would allow one to extrapolate *in vivo* neuroprotection to the animal models, hopefully, beyond to human phase II proof-of-concept neuroprotection studies.

**Translating neuroprotection**

The contribution of imaging in the development of both thrombolytic and thrombostatic treatments has been invaluable. Although thrombolysis has been efficacious in animal models and stroke patients, numerous attempts to treat stroke with neuroprotective agents have massively failed up to now. The successful translation of future neuroprotective studies from animal experiments to humans would depend on meeting eight criteria.

The first rule is to ensure that the cellular basis *in vitro* is truly extrapolated to *in vivo*, by using global models to study cytoprotection and focal models to demonstrate organotypic protection. Secondly, the effect must not be the result of physiological bias such as decreasing temperature to protect cells and improving CBF to protect the organ. Thirdly, the pharmacological activity *in vitro* has to be matched with appropriate *in vivo* levels, together with demonstrable tissue.
availability. Fourthly, the drug must be given late but in a logical temporal sequence, when the mechanism being inhibited is active. However, the mechanism must anticipate the evolution of injury rather than the result of the injury.

Fifthly, the outcome must be indefatigable survival, with both grey and white matter protection leading to improved behavioural outcome. Sixthly, the drugs must work in intracerebral haemorrhage, i.e. they can be given before CT scanning to optimize the time at which these drugs are delivered. Finally, there must be monitoring in in vivo animal models with bridging biomarkers, which must lead to imaging surrogates in phase II studies.

References

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