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REVIEW

Pharmacological approaches to acute ischaemic stroke: reperfusion certainly, neuroprotection possibly

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Stroke is a major cause of both death and disability. However, there are no pharmacological treatments used in most countries other than recombinant tissue plasminogen activator, a thrombolytic, and this is only used in about 4% of patients presenting after an acute ischaemic stroke. One novel thrombolytic (desmoteplase) has just been reported to have failed in a Phase IIb/III trial, but other thrombolytics and reperfusion agents remain in development. The picture with neuroprotectant agents, that is compounds that act to preserve neurones following an acute cerebral ischaemic insult, is even more bleak. Despite the development of over 1000 compounds, many proving effective in animal models of stroke, none has demonstrated efficacy in patients in the over 100 clinical trials conducted. This includes NXY-059, which was developed in accordance with the guidelines proposed by an academic-industry roundtable group (STAIR). This review examines the available data on compounds currently in development. It also proposes that the failure of translation between efficacy in preclinical models and patients is likely to terminate most current neuroprotective drug development. It is suggested that animal models must be made more representative of the patient condition (with other co-morbid conditions) and suggests that since stroke is primarily a cardiovascular disease with a neurological outcome, more research on the neurovascular unit would be valuable. New approaches on neuroinflammation, neurorestoration and neurorepair are also likely to gain prominence in the search for new drugs to treat this major clinical problem.
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Abbreviations: AMPA, (RS)-α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid; MCA, middle cerebral artery; NMDA, *N*-methyl-D-aspartate; NXY-059, disodium 2,4-disulphophenyl-N-tert-butylnitrone; rt-PA, recombinant tissue plasminogen activator; STAIR, Stroke Therapy Academic Industry Roundtable

The burden of stroke

There are many excellent reviews on the economic and social consequences of stroke (Payne *et al*., 2002) and this will only be covered very briefly here to emphasize how vital it is that effective therapy be developed quickly.

Stroke is the third major cause of death in the major industrialized countries after cardiovascular disease and cancer. The overall incidence of stroke is predicted to increase over the next decade by 12% but by around 20% in low-income families (Editorial, 2005). More than 30% of the stroke survivors will have severe disability, and calculations suggest that over 50 million healthy life-years will be lost by 2015 (Editorial, 2005). The burden of stroke to the individual, the family of stroke victims and society as a whole is clearly enormous.

These figures, however, do not give any insight to the consequences to the individual stroke patient. Saver (2006) undertook the task of calculating the impact a stroke has on nervous tissue. His calculations are sobering. Patients experiencing a typical large-vessel acute ischaemic stroke will lose 120 million neurones, 830 billion synapses and 714 km of myelinated fibres each hour. Compared with the normal rate of neurone loss during aging, the ischaemic brain will age 3.6 years for every hour the stroke goes untreated. Little wonder that the phrase 'time is brain' was coined to emphasize the need to try and treat a stroke rapidly.

The large majority (85%) of strokes in the western world are ischaemic, that is, a stroke resulting from an occlusion of a major cerebral artery, commonly the middle cerebral artery (MCA) by a thrombus or embolism. The other strokes are

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haemorrhagic, where a blood vessel bursts either in the brain or on its surface. These figures differ in China where haemorrhagic strokes are more common (Zhang *et al.*, 2003b).

Some risk factors can be mitigated. For example, the use of antihypertensives to lower blood pressure and statins to treat hyperlipidaemia has proven value. Furthermore, changing lifestyle (stopping smoking, decreasing body weight) undoubtedly decreases the risk of suffering a stroke.

However, providing effective pharmacological treatment immediately following an acute stroke to lessen the cerebral damage remains an elusive goal. Only one drug is approved for clinical use in most countries and that is the thrombolytic agent recombinant tissue plasminogen activator (rt-PA; alteplase) and this is only given to 4–5% of patients for reasons discussed below. The need to develop an effective treatment for stroke, therefore, remains paramount. This review deals only with acute pharmacological treatments, therefore mechanical approaches such as the MERCI retriever are not reviewed, but details of this therapy can be found elsewhere (Molina and Saver, 2005).

The classification of thrombolytics and neuroprotectants

Experimental drugs developed to treat acute ischaemic stroke have tended to be grouped by investigators into two distinct groups: thrombolytics and neuroprotectants. The former group encompasses compounds that restore blood flow, while the latter term covers drugs that act to preserve neurones (see Green and Ashwood, 2005).

Although this classification has proved useful, it has weaknesses and limitations. For example, drugs are now being developed that restore blood flow by mechanisms other than thrombolysis. Furthermore, if a drug restoring blood flow also preserves cerebral tissue compromised by the ischaemia, why should this compound not also be called a neuroprotective agent?

The idea of neuroprotection evolved in response to the idea that drugs could be developed that would interfere with the biochemical mechanisms that follow an ischaemic insult and thereby prevent cell death. Over the last 25 years, neurochemists have provided a great deal of information on the chain of events that follows an ischaemic episode (the so-called 'ischaemic cascade'; see Figure 1). Many compounds that act on one or more of these mechanisms have been developed and some are shown in Figure 1 and these compounds (and many others) do indeed prevent ischaemia-induced cell death in the brain of experimental animals. Such compounds have generally been referred to as neuroprotectants.

However, recent research suggesting that drugs working on the neurovascular unit (Lo *et al.*, 2003; Abbott *et al.*, 2006) may also prove to be useful in protecting neurones following an experimentally induced stroke (del Zoppo, 2006), further weakens the division of neuroprotection and thrombolysis, since these drugs by acting primarily on the vasculature are nevertheless providing neuroprotection.

Therefore, while I will continue (for ease of discussion) to use these terms for the purposes of grouping experimental compounds in this review, I do question whether we can continue to use this simplistic classification in the future.

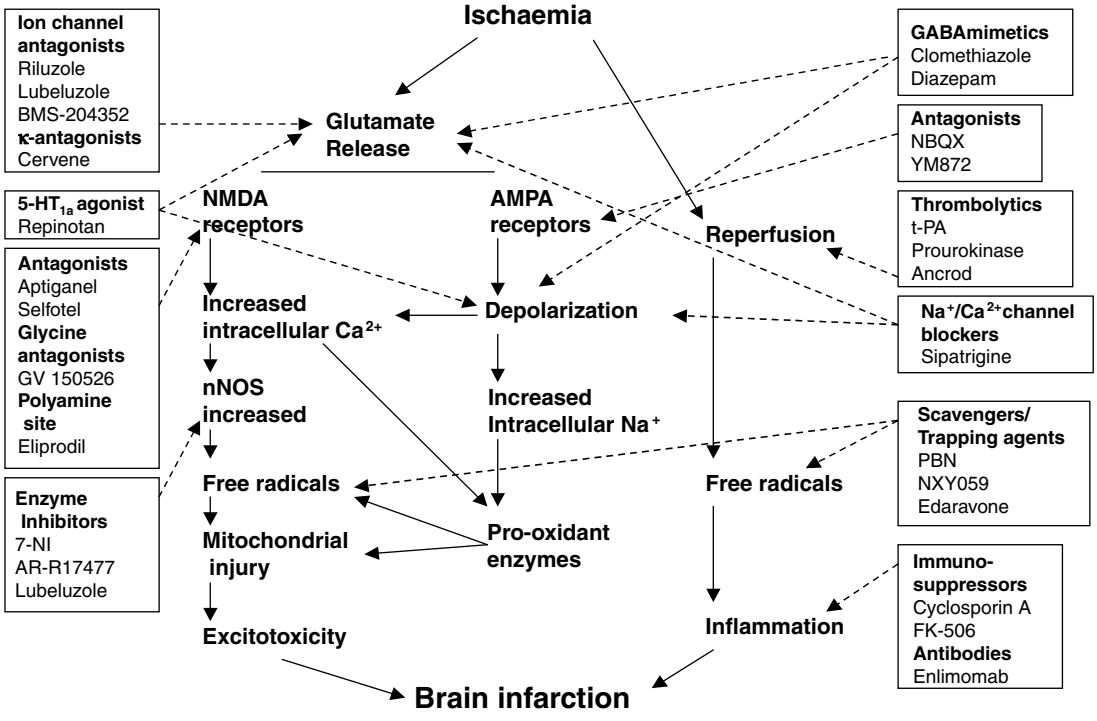

Figure 1 A simplified diagram of the mechanisms involved in the 'ischaemic cascade' together with some of the compounds developed to interfere with the mechanisms involved in the cascade and thereby provide neuroprotection. Reproduced from (Green *et al.*, 2003b) with permission of Elsevier Ltd.

Thrombolytics and other antiaggregation compounds

Thrombolytics

As stated above, the only compound licensed in most countries for clinical use in acute ischaemic stroke is rt-PA (alteplase), a drug initially in use for the treatment of myocardial infarction. It was approved on the basis of two Phase III trials, which studied its efficacy when given within 3 h of onset of the symptom (NINDS t-PA Stroke Study Group, 1995). It is also effective in a rat thromboembolic stroke model when given within 3 h, but not at later times (Brinker *et al*. (1999a) and at present clinical trials have also failed to demonstrate unequivocal benefit using later times in stroke patients (Hacke *et al*., 1998). This immediately limits the usefulness of the drug, as drug administration within 3 h of diagnosis is a challenge in many countries and this challenge is further increased by the need for a computed tomography scan to confirm that the stroke is ischaemic and not haemorrhagic, a condition that would be exacerbated by a thrombolytic drug.

A further reason for the low use of rt-PA in stroke is that the compound increases the incidence of cerebral haemorrhage (Wardlaw *et al*., 1997), a problem that can be mimicked in experimental animals (Brinker *et al*., 1999b; Asahi *et al*., 2000) and which appears to be associated with the drug increasing the levels of matrix metalloproteinase-9 in the endothelium (Lapchak *et al*., 2000; Pfefferkorn and Rosenberg, 2003).

Looking to the future, desmoteplase is another thrombolytic in development. This is a genetically engineered version of a protein in the saliva of Vampire Bats (Schleuning, 2001). Because desmoteplase has a requirement for fibrin as a cofactor, it is more potent than rt-PA in the presence of fibrin (Bringmann *et al*., 1995). It has been suggested that this characteristic will give desmoteplase the ability to dissolve clots without increasing systemic clotting and thereby increases the haemorrhagic transformation, a problem that occurs with rt-PA. Although this hypothesis was not supported by a preclinical study (Montoney *et al*., 1995), it appears that appropriate dosing in patients might assist in achieving this goal (Hacke *et al*., 2005).

Some preclinical evidence suggests that if rt-PA reaches the extracellular space in the CNS it can induce neurotoxicity (Kaur *et al*., 2004), and recent studies suggest that this problem is not seen with desmoteplase (Liberatore *et al*., 2003; Reddrop *et al*., 2005; Lopez-Atalaya *et al*., 2007). However, there is no evidence has been reported that rt-PA does induce neurotoxicity clinically.

The first Phase IIb/III trial (DIAS-2) has recently completed the investigation of two dose levels (90 and 125 $\mu g\,kg^{-1}$) given 3–9 h post-onset of stroke symptoms. The trial was not large (186 patients) and a press announcement from the companies developing the compound on 31st May 2007 (www.frx.com/news/PressRelease.aspx?ID = 1009782) reported no difference between the placebo- and drug-treated group (data were also presented at the European Stroke Conference in Glasgow). It is difficult to further evaluate these data until more information is available, but it is clearly a major setback in the development of this new thrombolytic.

Antiaggregation compounds and other perfusion-enhancing compounds

The monoclonal antibody abciximab is an antiaggregation compound that binds to the glycoprotein IIb/IIIa receptor on the surface of platelets, and which promotes fibrinolysis by preventing the platelets from sticking together, thereby inhibiting clot formation (Tam *et al*., 1998). It is in current clinical use for the restoration of coronary blood flow, often being given in combination with thrombolytics.

Preclinical studies on abciximab to investigate its possible use in stroke cannot be performed, because it is not active in rodents (Sassoli *et al*., 2001). Therefore, an analogue 7E3 $F(ab')_2$ has been used in rat thromboembolic stroke models and has provided encouraging data. Shuaib *et al*. (2002) reported that both rt-PA (10 or 20 $mg\,kg^{-1}$) and 7E3 $F(ab')_2$ (6 $mg\,kg^{-1}$) reduced infarct size as the two drugs did in combination. However, the cerebral haemorrhage rate increased following administration of either drug with the highest incidence of haemorrhage occurring in the group given the high-dose combination. Zhang *et al*. (2003a) also observed a decrease in infarct size in rats treated with a combination of 7E3 $F(ab')_2$ and rt-PA, but found no reduction in infarct size when the drugs were administered individually. A further study by this group found that administration of tenectaplase to rats together with 7E3 $F(ab')_2$ also resulted in a decrease in infarct size when both compounds were given at 4 h (Zhang *et al*., 2004).

Although the recent company press release announced the termination of the Phase III trial of abciximab in stroke, following a recommendation by the independent safety and efficacy monitoring committee, a new trial is now reported to be starting.

The problem of increased haemorrhage rate seen following rt-PA administration does not seem to be obviated by using other types of approach to increase blood flow in the brain of stroke patients. For example, intravenous administration of the defibrinogenating compound ancrod also increased cerebral haemorrhage rate in a Phase III trial, when it was given within 3 h of stroke onset, although it also produced a favourable neurological response compared with placebo (Sherman *et al*., 2000). A Phase III trial of the compound given up to 6 h after stroke onset was negative but a new trial, also examining the effect of administration within 6 h, began in September 2005.

Heparin may be beneficial if given within 3 h, but again increases the incidence of cerebral haemorrhage (Camerlingo *et al*., 2005). Plasmin and microplasmin, a truncated form of plasmin, are finbrinolytic agents that may be free of the problem of increasing cerebral haemorrhage rate, which again makes them candidates for development as therapeutic agents for stroke (Molina and Saver, 2005).

Finally, there are indications that early aspirin can be of some value to reduce the early recurrence, with no excess of subsequent haemorrhagic stroke, although the absolute benefit in terms of patient numbers is small (Chen *et al*., 2000) and aspirin may thus confer modest benefit if given within 48 h of stroke (Sherman, 2004).

Neuroprotective agents

Introduction

Despite the fact that the thrombolytic rt-PA has demonstrated efficacy in treating acute ischaemic stroke patients, the short time window, risk of haemorrhage and requirement for a computed tomography scan mean that few patients actually receive the drug and other approaches to treating stoke have been pursued vigorously. The increasing knowledge of the mechanisms involved in producing cell death following a stroke obtained in the 1980s and beyond produced a buoyant hope that interfering with the mechanisms involved in this so-called 'ischaemic cascade' or pathway of 'excitotoxic cell death' (Figure 1) would lead to lessening the neuronal damage that would normally occur. However, not a single one of the many compounds developed to produce neuroprotection, usually by interfering with one of the mechanisms shown, has achieved clinical success, despite many of them providing clear evidence of efficacy in animal models (Green *et al*., 2003a), a recent calculation has suggested that the number of experimental compounds that have failed at some stage of development now numbers over 1000 (O'Collins *et al*., 2006). During the late 1990s, this situation was considered so severe that a group of scientists from both academia and industry convened to produce guidelines to be used for preclinical drug development with the suggestion that no compound should progress to clinical development unless it fulfilled the guidelines. The group was called the Stroke Therapy Academic Industry Roundtable (STAIR) and the published guidelines (Stroke Therapy Academic Industry Roundtable, 1999) are now often referred to as the STAIR criteria. These have been modified by both the STAIR group itself (Stroke Therapy Academic Industry Roundtable, 2001) and others (see Table 1; Green *et al*., 2003a). However, it is now reasonable to question them in the light of recent results (see later).

Table 1 Outline of the recommendations of the Stroke Therapy Academic Industry Roundtable (1999) as modified by Green *et al.* (2003a) that should be met before a compound is progressed to clinical trial

STAIR recommendations

- Adequate dose–response plus serum concentrations measured, thereby defining minimally and maximally effective doses
- Time window studies confirming efficacy. Time and duration of drug administration appropriate to the mechanism of action and appropriate to the proposed clinical protocol
- Physiological monitoring of animals undertaken
- Randomized, blinded studies; reproducible effects (one independent)
- Infarct volume measured, compound provides subcortical as well as cortical protection.
- Histological protection of $>70\%$ in both transient and permanent focal ischaemia when drug is given 15–30 min post occlusion
- Attenuates white matter damage in brain
- Functional tests used, short and long term assessment
- Small rodent studied with permanent MCAO. Must show efficacy in permanent MCAO models; compound is efficacious as monotherapy
- Larger species used for novel, first-in-class compound
- Studies published in peer-reviewed journal

Many of the compounds developed in the early days of neuroprotective research belonged to only one class, the glutamate antagonists; however, there have also been a substantial number of compounds synthesized and evaluated that act at other sites on the ischaemic cascade (see O'Collins *et al*., 2006).

In the following sections, I will only consider compounds that have failed if the information gained gives understanding of future research directions. Otherwise only compounds in continued development will be discussed.

Drugs altering glutamate function

Because a pathological increase in glutamate was one of the early changes to be shown to occur after an episode of cerebral ischaemia, many compounds interfering with glutamate function were developed during the late 1980s and early 1990s. These were predominantly *N*-methyl-D-aspartate (NMDA) receptor subtype antagonists, but some (RS)-α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) receptor antagonists were investigated and also a glycine modulator site antagonist. It seems reasonable in retrospect to suggest that a major problem with glutamate antagonism as a mechanism suitable for therapeutic intervention target is that pathological glutamate release is an event occurring very early in the ischaemic cascade (Figure 1). This presumably makes it necessary to give such drugs very quickly after the ischaemic insult (Dirnagl *et al*., 1999; Green *et al*., 2003b; Hoyte *et al*., 2004), and rapid admission is often a major clinical problem. However, other problems that also arose with some of these drugs included poor pharmacokinetics, poor brain penetration and a failure to protect subcortical structures.

There may now be only one NMDA antagonist in development. This is traxoprodil (CP-101 606), which has NR2B receptor subtype selectivity (Chenard *et al*., 1995; Wang and Shuaib, 2005). It is an effective glutamate antagonist in a primary cortical neurone preparation (Menniti, 1997), but there is little information on its *in vivo* activity. It reduces damage after cortical compression induced brain ischaemia (Kundrotiene *et al*., 2004) and is neuroprotective when given 15 min prior to MCA occlusion (MCAO) in the cat (Di *et al*., 1997). It has also been reported to be an effective neuroprotectant when given 2 h after clot insertion in a rat thromboembolic stroke model, decreasing infarct volume dose dependently (Yang *et al*., 2003). There has been little recently published information about traxoprodil apart from a clinical pharmacokinetic study and a clinical investigation in severe traumatic brain injury, which did not reveal unequivocal evidence of efficacy (Yurkewicz *et al*., 2005). Its development for stroke may therefore have been terminated.

It is well established that magnesium 'gates' the NMDA receptor channel and that reducing the Mg^{2+} concentration *in vitro* enhances NMDA-induced electrophysiological responses (Nowak *et al*., 1984). Magnesium also reduces presynaptic glutamate release (Lin *et al*., 2002). A dose of magnesium sulphate is neuroprotective in both transient (Marinov *et al*., 1996; Schmid-Elsaesser *et al*., 1999) and

permanent (Izumi *et al*., 1991) MCAO models of stroke and also in an embolic stroke model (Yang *et al*., 2000).

Following the pilot studies on the effect of magnesium in stroke patients, a full clinical multicentre trial was undertaken in almost 2400 patients. Although no reduction in death or disability was detected, the study did observe a possible benefit in lacunar strokes (Muir *et al*., 2004). According to the investigators, this finding proposed was worthy of further investigation, even though the result was the opposite to what had been hypothesized before the trial took place.

Gavestinel is a compound that acts at the glycine-regulatory site of the NMDA receptor, thereby antagonizing the effect of pathological glutamate function (Di Fabio *et al*., 1998). The failure of the compound in a Phase III trial (Sacco *et al*., 2001) has probably prevented any further development of compounds with this mode of action.

AMPA antagonists have also received significant interest as neuroprotectants. However, compounds that have been developed have suffered from various safety problems, including sedation, visual disturbance, and memory impairment (Weiser, 2005), and the development of YM 872, the only AMPA antagonist to have been in recent clinical investigation, has been terminated.

5-HT$_{1A}$ agonists

Repinotan is a potent 5-HT$_{1A}$ receptor agonist that was suggested to act as a neuroprotectant by inhibiting neuronal firing in he dorsal raphé nuclei, thereby leading to the inhibition of excessive ischaemia-induced glutamate release. It may also produce hyperpolarization and thus reduce anoxia-induced depolarization.

In animal models of stroke, repinotan was reported to be effective in both transient and permanent MCAO models even when given up to 5 h after the occlusion (Mauler and Horváth, 2005).

A Phase III clinical trial was initiated and then modified and reclassified as a Phase IIb study with an inclusion time window of 4.5 h. However, the company terminated development in December 2004 due to the drugs failure to meet efficacy endpoints.

Piclozotan (SUN N4057) is another potent 5-HT1A agonist that has been reported to be an effective neuroprotective agent when given immediately following the start of a transient MCAO (Kamei *et al*., 2001). These authors also stated the compound to be in clinical development (Phase IIb) for stroke, but the lack of subsequent communication suggests that its development has been terminated.

Citicoline

Citicoline or cytidine-5′ diphosphocholine (CDP-choline), has been in clinical use in Europe and Japan for many years for a variety of degenerative neurological disorders. It is an intermediate in the biosynthesis of phosphotidylcholine, which is of major importance in regulating cell membrane integrity.

The interest in citicoline as a neuroprotectant is based on the fact that phosphotidylcholine is broken down to free fatty acids during ischaemia, thereby generating free radicals that in turn induce cell damage (Phillis and Regan, 2004). Citicoline, by reducing lipid metabolism following ischaemia, reduces the levels of free fatty acids (Trovarelli *et al*., 1981; Rao *et al*., 1999) and, therefore, decreases free radical production (Adibhatla and Hatcher, 2002). There is good evidence for citicoline being effective as a neuroprotectant in models of acute ischaemic stroke. It has been shown to reduce neurological deficits a rat global ischaemia model (Kakihana *et al*., 1988) and a rat transient focal ischaemia model (Sobrado *et al*., 2003). Citicoline also significantly reduced infarct size in a rat thromboembolic focal ischaemia model (Shuaib *et al*., 2000).

A total of four Phase III studies on citicoline have been conducted. However, all examined small numbers of patients with variable stroke severity and dosing varied across the studies (500–2000 mg). While the trials showed trends for improvement, conclusions were difficult because of either small cohort size or possible inappropriate primary outcome measures. Dávalos *et al*. (2002) performed a meta-analysis on the data from the four trials (total patient numbers 1372: 583 placebo; 789 citicoline) and this suggested a beneficial effect of the drug ($P = 0.034$) with the greatest effect at the highest dose. A further Phase III trial was initiated in 2006.

Metal chelation

Metal ions are vital factors in controlling for enzymes, co-factors and cellular transporters, including matrix metalloproteinases, calpain and Cu/Zn superoxide dismutase, whose disruption have been proposed to be intimately associated with cell death following acute cerebral ischaemia. Disruption in metal ion homoeostasis has also been suggested to be involved in various chronic neurodegenerative conditions such as Parkinson's and Alzheimer's Diseases (Angel *et al*., 2002). The probability that disturbance in metal ion regulation could be associated with cerebral ischaemia is indicated by the observation that disturbances in zinc homoeostasis was associated with cerebral cell death following transient focal ischaemia (Koh *et al*., 1996), and this has resulted in the development of DP-b99. This is a derivative of BAPTA, a compound that chelates divalent metal ions including zinc, calcium, iron and copper (Angel *et al*., 2002). This chelating action may explain its beneficial effect of DP-b99 in inhibiting the oxidative stress-induced increases in calpain *in vitro* (Friedman *et al*., 2004) and reduction in ischaemia-induced matrix metalloproteinase activation in the brain of rats subjected to MCAO (Angel *et al*., 2004). The compound has also been reported to decrease infarct size in a rat MCAO model in an abstract publication (Angel *et al*., 2004). The compound appears to be well tolerated in healthy volunteers (Rosenberg *et al*., 2005) and a recent press release from the company reported efficacy in a Phase IIb clinical trial. No initiation of a Phase III trial has been announced.

Another metal chelator that decreases infarct volume in a rat transient focal ischaemia model is PAN-811, which was originally developed as an anticancer drug, due to its ability to chelate iron, but which also modulates calcium homoeostasis and, therefore, presumably reduces free radical produc-

tion (Lu *et al*., 2004). The compound decreased infarct size by a modest 35% in a transient MCAO model and has a narrow dose range (Lu *et al*., 2004), making it unlikely that it will progress into clinical development. However, these data indicate that metal chelation may be a viable mechanistic approach to neuroprotection.

Arundic acid

Arundic acid (ONO-2506) is an astrocyte-modulating compound that inhibits the synthesis of the protein S-100β in cultured astrocytes and inhibits the increase in the concentration of S-100β in the colony-stimulating factor and plasma of rats subjected to transient or permanent MCAO (Tateishi *et al*., 2002). S-100β has been implicated in producing cell death through its activation of several intracellular signalling pathways (Asano *et al*., 2005). The compound also has actions on $GABA_A$ receptors, glutamate transporters and lipopolysaccharide-inducible nitric oxide synthase expression in cultured astrocyte preparations (Asano *et al*., 2005).

Arundic acid has been reported to have a very long therapeutic time window of 24 h in transient and 48 h in permanent MCAO. This has been proposed to be due to its effects on both S100β and the other diverse mechanisms outlined above (Asano *et al*., 2005). Other studies, including a neuroprotective effect on primates subjected to permanent MCAO were reviewed by Asano *et al*. (2005).

In May 2005, Ono Pharmaceuticals reported that the Phase II clinical study of the drug for acute stroke initiated in North America would be terminated following a futility analysis by an independent board of advisors. However, a new trial has recently been initiated in this continent and the study in Japan is continuing (see http://www.strokecenter.org/trials).

Albumin

Administration of albumin administration decreases infarct size in rats subjected to both transient (Huh *et al*., 1998) and permanent MCAO (Liu *et al*., 2001). The time window for administration in transient ischaemia is 4 h after occlusion (Belayev *et al*., 2001). It is proposed that only part of the neuroprotective effect of albumin is via haemodilution (Huh *et al*., 1998), as several other mechanisms can have also been proposed to be involved (Huh *et al*., 1998; Belayev *et al*., 2001). While a small open study suggested increased cardiopulmonary adverse events in stroke patients when albumin was given within 24 h of stroke onset (Koch *et al*., 2004), a subsequent Phase I trial indicated that albumin was well tolerated (Ginsberg *et al*., 2005) and a Phase III study was started in late 2006 with completion projected to be in 2010.

Cytokines

Cytokines, particularly the tumour necrosis factor (TNF)α and IL-1β (Figure 2), are the major factors involved in the inflammatory response of the brain to injury (Barone and Parsons, 2000; Allan and Rothwell, 2001), and both TNFα antibodies (Barone *et al*., 1997) and IL-1β antibodies (Relton and Rothwell, 1992) have been shown to inhibit ischaemia-induced cerebral damage. Although a recombinant IL-1α antibody has recently been found to be well tolerated in a Phase II study in stroke patients (Emsley *et al*., 2005), there is a problem in developing compounds antagonizing the action of TNFα in stroke, because TNFα can also assist cell survival (Hallenbeck, 2002; Heldmann *et al*., 2005). Nevertheless, efforts are now being made to produce nonpeptide anti-TNFα compounds in addition to the protein-based compounds. These approaches include targeting enzymes involved in the biosynthesis of TNFα. Another approach is to produce a compound targeting the protease (TNFα converting enzyme or TACE), which acts on membrane-bound TNFα and thus generates soluble TNFα (Lovering and Zhang, 2005). Some compounds with TACE inhibitory activity are neuroprotective in animal stroke models. However, they also inhibit matrix metalloproteinases, key enzymes involved in blood brain barrier damage, so their mechanism of neuroprotective action remains unclear (Lovering and Zhang, 2005).

A cytokine that has distinct possibilities for clinical development is erythropoietin or a close congener. Erythropoietin not only is a primary stimulator of red blood cell formation but also appears to have functions in the brain (Masuda *et al*., 1993). *In vitro*, it protects cortical neurones from glutamate-induced neurotxicity and *in vivo* is neuroprotetive in the gerbil global ischaemia model (Sakanaka *et al*., 1998).

A clinical trial of erythropoietin in stroke patients suggested efficacy when it was given in three doses over 3 days (Ehrenreich *et al*., 2002). However, concerns over safety of the compound in non-anaemic patients have led to the development of related compounds that retain the neuroprotective properties but lacking the blood forming properties of erythropoietin (see Torup, 2007). One such compound is carbamylated erythropoietin and Wang *et al*. (2007) have recently examined both erythropoietin and carbamylated erythropoietin in rats using an embolic stroke model and found that all neuroprotective doses of erythropoietin increased the haematocrit. However, a low dose of the latter could produce effective neuroprotection without any haematological effects.

It has been suggested that erythropoietin and carbamylated erythropoietin might provide efficacy through several mechanisms of action including antiapoptotic effects and stimulation of angiogenesis and neurogenenesis (Torup, 2007), which may enhance the chances of success for this new approach to stroke therapy.

Free radical scavengers and trapping agents

The fact that there is clear evidence that free radicals are involved in the production of cerebral tissue damage following an ischaemic insult and also in the damage produced by reperfusion (Love, 1999; Chan, 2001) has resulted in the development of several compounds that have been designed to remove free radicals and, therefore, hopefully to lessen ischaemia-induced damage.

Ebselen is a selenium compound with glutathione peroxidase-like activity, which may act as a mimic for this

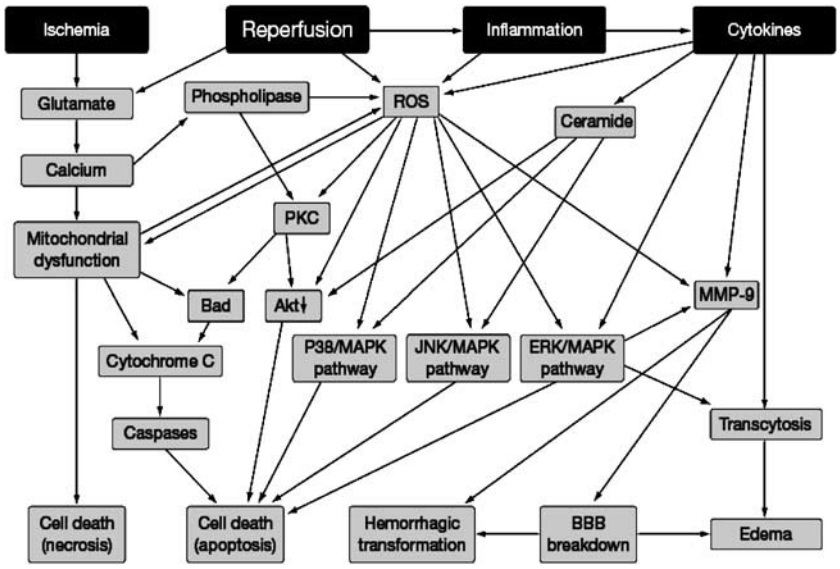

Figure 2 Some of the pathways proposed to be involved in cell death and cell survival following an acute cerebral ischaemia. This figure is simplified and illustrates only some of the pathways. Some pathways can initiate cell death or cell survival depending on the time after the onset of ischaemia and the severity of the ischaemia. There is also a lack of agreement among investigators as to the specific role of some of these signalling systems and the figure is used here merely to illustrate possible targets for pharmacological intervention. Figure adapted from Green and Shuaib (2006) with permission of Elsevier Ltd.

enzyme rather than being a free radical scavenger (Noguchi *et al.*, 1992). It is neuroprotective in rat models of transient ischaemia but not permanent focal ischaemia (Salom *et al.*, 2004). The small clinical studies performed failed to provide clear evidence for efficacy in stroke and development was terminated (see Green and Ashwood, 2005).

The lazaroid compound tirilazad was also ineffective in rat permanent ischaemia models (Xue *et al.*, 1992) and also failed to demonstrate efficacy in transient ischaemia models when given several hours after the insult. Several clinical trials failed to demonstrate the efficacy (see Green and Ashwood, 2005).

Available preclinical data on the effects of edaravone, a hydroxyl radical scavenger, in stroke models are sparse, although it has been examined in a variety of other disease models. In cerebral ischaemia models, nothing has been published on dose–response data and most studies gave the compound almost immediately after the ischaemic insult. The compound also failed to protect subcortical structures (see Green and Ashwood, 2005). Published clinical data are also limited. Nevertheless, the compound has been approved by the regulatory authority in Japan to treat stroke patients apparently on the basis of a single placebo-controlled study in a small number of patients. It is not known whether the compound will be developed outside Japan.

Disodium 2,4-disulphophenyl-N-tert-butylnitrone (NXY-059)

The development of the neuroprotectant disodium 2,4-disulphophenyl-*N*-tert-butylnitrone (NXY-059) will be considered in some detail, because this compound was, until recently, thought to be the most likely neuroprotective agent to succeed in the clinic and demonstrate unequivocal evidence of efficacy in stroke patients. Its recent failure therefore provides both lessons and questions on the future of neuroprotectant drug development.

NXY-059 is a nitrone-derived compound with free radical trapping properties (Maples *et al.*, 2001; Green *et al.*, 2003b; Williams *et al.*, 2007). Because NXY-059 was in development at the time of the publication of the STAIR criteria, its development programme was focussed on following the guidelines (Table 1; Stroke Therapy Academic Industry Roundtable, 1999). Consequently, it has become the first compound to have been developed in accordance with the criteria (Lees *et al.*, 2001; Green and Ashwood, 2005; Shuaib, 2006).

The compound has been shown to produce clear dose-dependent neuroprotection in rats in both transient (Kuroda *et al.*, 1999; Sydserff *et al.*, 2002) and permanent (Zhao *et al.*, 2001; Sydserff *et al.*, 2002) MCAO models of stroke, including subcortical protection (Figure 3; Sydserff *et al.*, 2002) and in rat (Wang and Shuaib, 2004) and rabbit (Lapchak *et al.*, 2002) embolic stroke models. NXY-059 also has a wide window of opportunity producing statistically significant neuroprotection, when given at 4 h after permanent focal ischaemia (Sydserff *et al.*, 2002) and 5 h after transient ischaemia (Kuroda *et al.*, 1999). In a primate model of permanent focal ischaemia, it lessened both the motor deficits in the paretic arm of marmosets and also spatial hemineglect, even when given 4 h after permanent MCAO (Marshall *et al.*, 2001, 2003). Thus, the compound provided clear evidence for producing functional improvement, in addition to the histological evidence for it decreasing infarct size.

Overall, therefore NXY-059 could be claimed to have met all the STAIR criteria (Table 1). In particular, it is worth emphasizing that the window of therapeutic opportunity (greater than 4 h) and the fact that NXY-059 had a maximal

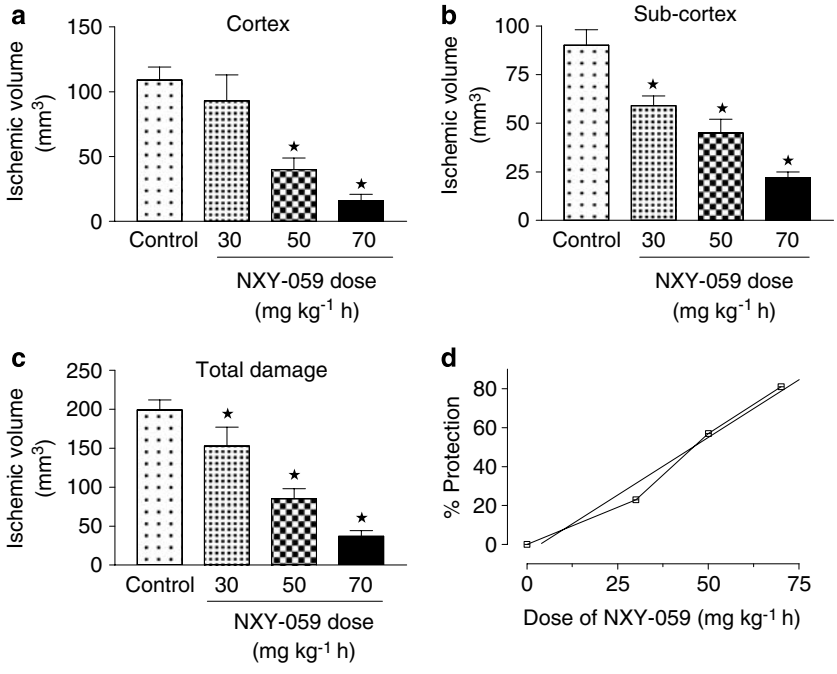

Figure 3 The effect of increasing doses of NXY-059 on the volume of ischaemic damage in the (**a**) cortex, (**b**) subcortex and (**c**) total brain volume, together with the (**d**) dose–response versus neuroprotection. Reproduced from Sydserff *et al.* (2002) with permission of Nature Publishing Group.

effective neuroprotection in rats at a plasma unbound concentration of 24 μmol l^{-1} in rat transient focal ischaemia and 140 μmol l^{-1} in rat permanent focal ischaemia models allowed direct translation to the design of the clinical trial, as NXY-059 was well tolerated in stroke patients at a plasma unbound concentration of 260 μmol l^{-1} (Lees *et al.*, 2003).

The subsequent Phase III trial design was such that the plasma concentration of drug in the patients exceeded the concentration known to be maximally effective in rodent models and the time window of inclusion matched that known to be effective in both rodent and primate models. This is the first time that this has been achieved (Green and Ashwood, 2005).

The first Phase III trial was in 1700 patients (SAINT I) and the drug was found to significantly reduce disability (using the modified Rankin score) within 6 h of stroke onset without apparent tolerability or safety issues (Lees *et al.*, 2006). NXY-059 did not improve neurological function as measured by the NIHSS score (Lees *et al.*, 2006). However, the companion SAINT II study, which had a patient size of 3200 patients, failed to confirm efficacy (Shuaib *et al.*, 2007).

This failure has naturally resulted in others proposing reasons for the negative outcome and it is worth reviewing these suggestions briefly. Proctor and Tamborello (2007) have proposed that the positive SAINT I result was due to an impurity in the drug substance not present in the SAINT II investigation. This hypothesis is based on the fact that a hydroxylamine derivative has been shown to be present in samples of another nitrone (phenylbutyl nitrone) and is a potent radical scavenger (Atamna *et al.*, 2000). However, this proposal has been rapidly refuted by the investigators (Lees *et al.*, 2007), and even cursory examination of the original *in vitro* study on the hydroxylamine (Atamna *et al.*, 2000) indicates that the amount of impurity that has to be present in the drug substance is likely to be quite enormous to produce a pharmacological effect, which renders this suggestion untenable. In addition, a paper has appeared questioning the validity of the claim that NXY-059 did meet the STAIR criteria and criticizing some of the preclinical data (Savitz, 2007). However, a survey by a recent independent review group (O'Collins *et al.*, 2006) did conclude that the work on NXY-059 did satisfy the original STAIR criteria and Feuerstein *et al.* (2007) suggested the clinical trial had been well founded on extensive preclinical data. It should also be emphasized that the major studies on the time window and dose dependence were performed with appropriate blinding and allocation concealment.

The clinical failure of the nitrone NXY-059 must cast a shadow over the further development of another nitrone-derived compound, stilbazulenyl nitrone, in transient MCAO. Stilbazulenyl nitrone is an effective neuroprotectant in transient ischaemia in rats (Ginsberg *et al.*, 2003). A major claim for the value of this compound over NXY-059 is its greater lipophilicity and potency. However, while it does have greater penetration of cerebral tissue (Ley *et al.*, 2005) than NXY-059 (Dehouck *et al.*, 2002; Green *et al.*, 2006), this increase is modest, and its importance remains unclear as there is some evidence that NXY-059 may be acting at the level of the endothelium (Culot *et al.*, 2006; Hainsworth *et al.*, 2007). This supports earlier proposal that NXY-059 might be acting at the microvasculature and enhances recent proposals that neuroprotection may be achieved by the drugs acting on the neurovascular unit (Abbott *et al.*, 2006; del Zoppo, 2006). An action at this site is also likely to explain the neuroprotective effect of the nitrone 2-sulphophenyl-*N*-tert-butylnitrone, as this also has poor

cerebral penetration. Given the suggestion that even the brain penetrating nitrone *N*-tert-butylnitrone may be acting outside the brain to produce its neuroprotective action in rats (Gido *et al*., 2000); one wonders if all nitrones therefore act at the same site in animals to produce neuroprotection. In any event, the failure of NXY-059 must surely make any company wary of developing any other nitrone compound.

Neuroprotection research: the current situation

Problems of animal models

I have previously argued strongly in favour of the use of animal models in preclinical stroke medicine research (Green *et al*., 2003a; Green and Shuaib, 2006). In support of the arguments, it was pointed out that most compounds that failed in the clinic did not replicate in the patients either the drug exposure required for efficacy in the respective animal models or the time window, or both. Furthermore, the fact that many of the physiological factors that influence the severity of ischaemic damage in animal models have the same effect on stroke outcome in patients seemed to indicate cross-species validity (Green *et al*., 2003a). This view was supported by studies in marmosets, in which it is observed that focal ischaemia results not only in motor problems in the contralateral arm, but also spatial hemineglect and both of these problems have exact clinical correlates (Marshall and Ridley, 1996; Marshall *et al*., 2001).

However, the failure of NXY-059 in the recent large Phase III study (Shuaib *et al*., 2007) does lead to major questions as to the value of animal models, at least as utilized at present. NXY-059 was not only developed in accordance with the STAIR criteria (Lees *et al*., 2001; Green and Ashwood, 2005; Shuaib, 2006), its development circumvented the major weaknesses of many earlier trials as listed above. Specifically, the plasma levels in patients were considerably in excess of those required for a maximum neuroprotective effect in rats, the time window for treatment initiation was equivalent to that reported to be required in rats and, in the marmoset model, the drug had been shown to improve both the use of the paretic arm and lessen spatial hemineglect, two problems with exact clinical correlates.

Given the likelihood that current neuroprotective drugs in development have also used the same or similar stroke models as those used in the NXY-059 development programme, one has to question their chances of clinical success.

Although there are several animal models of acute ischaemic stroke available, they are mostly closely related with modest modifications sometimes introduced by the scientists using them (Green and Cross, 1997; Traystman, 2003). Basically, most models induce an occlusion of the MCA because most human strokes result from an occlusion of this artery (Mohr *et al*., 1986). If this occlusion is temporary, then the model mimics the problem of both ischaemia and reperfusion, whereas permanent occlusion presumably produces the problem of long-term vessel blockade, as often occurs in humans (Ringelstein *et al*., 1992).

However, even before the clinical failure of NXY-059 was known, some stroke researchers questioned the value of the models and the data generated by them. For example Carney (2005) said: 'For stroke, the [animal] models and the clinical condition are extremely different, which is reflected in the failure of molecules in the human condition'. This view was supported in another article published around the same time (Kaste, 2005), which was discussed further in succeeding articles (Donnan and Davis, 2005; Fisher and Tatlisumak, 2005).

A major criticism of the current animal models is that they nearly all utilize young healthy animals. In contrast, stoke patients are usually elderly, with a variety of other clinical problems such as hypertension, myocardial infarction and diabetes. With the exception of hypertension, which has been modelled by examining the effects of vessel occlusion in spontaneously hypertensive rats (Zhao *et al*., 2001), few of the other problems are routinely included in the animal models. Both Davis *et al*. (1995) and Schaller (2007) have examined older animals and data suggest that neuroprotective efficacy is reduced in these animals.

The question, therefore, arises as to whether the predictive value of animal models of stroke would improve if other comorbid conditions were included, and if so why? Until such modelling is performed, it can be shown that compounds like NXY-059 fail to provide neuroprotection we cannot know. However, it does seem reasonable in the light of current knowledge to suppose that other comorbid conditions may alter cerebrovascular functions such as the structure of the blood–brain barrier or the neuroimmune system. The future of neuroprotection research may, therefore, depend on the development of better animal models.

Another factor generally omitted in animal studies is white matter protection. It was noted as a weakness in preclinical studies some time ago (Muir and Grosset, 1999), but the requirement for stroke models to be performed in large animals to reliably measure this parameter means that little progress has been made since.

Animal models and future experimental design

One point that should be considered in all future studies is the design and size of primary pharmacodynamic trials. The CAMARADES collaboration (http://www.camarades.info/index.htm) is now proposing that all investigators conduct animal studies with full regard to random allocation, blinding and allocation concealment, and they further suggest, on the basis of their re-evaluation of existing preclinical publications, that failure to meet such methodology generally results in neuroprotective efficacy being overestimated. Although such techniques were not in regular use over 10 years ago when most of the preclinical work was performed on NXY-059 (for such is the time-line for drug development), the major studies on the time window and dose dependence were undertaken with appropriate blinding and allocation concealment. Furthermore, the constancy of the positive preclinical neuroprotection outcome data suggest that there must be a far more fundamental problem in translating the effect of drugs in animal models to that in human subjects, and one which all investigators must now confront if drugs are in future to be clinically successful.

The CAMARADES inspection of published data also indicates that the cohort size of the animal studies is

generally not large (often 6–12 animals per group). Meta-analysis of preclinical studies can now being performed by systematic reviews of stroke compounds tested in preclinical stroke models (see http://www.camarades.info/index.htm). These reviews are performed using Cochrane Collaboration approaches (as used in clinical studies but applied to animal investigations) to evaluate more clearly the predictability and transferability of animal models to the clinical situation. One certainly suspects that experimental group size is, at present, generally not sufficient to provide good statistical power to many studies and that single high-quality studies are rare, a problem that has been discussed in detail elsewhere (Perel *et al*., 2007). This weakness in experimental design is apparent in both academic and pharmaceutical company studies. Given the enormous costs of clinical development of stroke drugs, it is obvious that patient studies should only commence following statistically compelling data from the relatively cheap preclinical studies. Equally pertinent is the argument that no patient should be exposed to the novel compounds without very persuasive preclinical data being available. All these considerations must be encompassed in the planning of research in the future.

The use of combination therapy

It has been suggested by several investigators (for example Wagner and Jauch, 2004) that combining a thrombolytic plus a neuroprotectant might offer advantages such as enhancing the degree of clinical improvement, or extending the treatment window for rt-PA, or both. However several preclinical studies have failed to demonstrate enhancement of the effect of rt-PA and a neuroprotectant compared with the neuroprotectant alone (Lapchak *et al*., 2002; Yang *et al*., 2003). Lu *et al*. (2005) have recently presented evidence as to why it is complicated to demonstrate the value of combination treatments in animals and thereby prove synergy rather than addition. They suggested that factorial designs must be used with group size of sufficient number to allow confidence in the result obtained. A final point is that assessment must be made to ensure that the presence of the neuroprotectant does not alter the thrombolytic properties of the thrombolytic (see Mutch *et al*., 2008).

Stroke therapy research: the future

The failure of NXY-059, a compound developed with close regard to the best currently established guidelines of preclinical and clinical methodology may result in the pharmaceutical industry switching strategies. Few companies are going to feel the urge to continue developing drugs that interfere with biochemical mechanisms known to be involved with the ischaemic cascade and burden themselves with the huge costs of clinical trials in the light of so many failures.

Several approaches to the treatment of stroke are now being evaluated, which involve either biopharmaceuticals or initial biopharmaceutical approaches with the aim of finally producing small molecules from the data obtained. Furthermore, I would suggest that we will see increasing research on related approaches which result in neurorestoration or neurorepair.

Growth factors

The studies that have examined growth factors as neuroprotectants in animal stroke models have recently been reviewed critically by Ren and Finkelstein (2005). Studies have included the effect of basic fibroblast growth factor, osteogenic protein-1 (OP-1), vascular endothelial growth factor and granulocyte colony-stimulating factor. While Ren and Finkelstein (2005) suggested that such compounds might have more applicability in assisting long term recovery rather than acute treatment, fibroblast growth factor is neuroprotective if given soon after an acute MCAO (Ellsworth *et al*., 2003).

Cell survival/cell death pathways

Considerable knowledge is now being gained about the mechanisms responsible for controlling cell death and cell survival. The pathways involved are complex (Figure 2), but one can be sure that research activity will increase in an effort to develop compounds that will modify the pathways. There are, however, major problems to be overcome, as some of the factors shown can lead to either cell survival or cell death depending on the duration or severity of the ischaemic insult or the isoform being targeted (see for example Resnick and Fennell, 2004). There is also controversy about the role of some of the pathways and the way that they interact (Martindale and Holbrook, 2002). While manipulation of these pathways may prove valuable following a stroke, we remain ignorant at present as to how we might 'switch on' or 'switch off' such mechanisms in the damaged brain without disrupting homoeostasis in many other regions of the body where such intervention might prove problematic.

Conclusions

The continued development of drug to induce reperfusion following an acute ischaemic will continue, as this is the only approach with proven therapeutic benefit at present. Success with compounds presently in Phase III and in preclinical development will have to encompass not only improved functional outcomes compared with placebo, but also a longer therapeutic window than rt-PA. The new compounds will also have to possess a satisfactory adverse event profile, particularly a low incidence of haemorrhagic transformation, a problem that has limited acceptance of rt-PA by clinicians.

The lack of success with the neuroprotectant approach to therapy is going to markedly inhibit future research and development. NXY-059 adhered to the STAIR criteria in its development programme and failed. New ideas are therefore desperately needed. Perhaps new animal models mimicking more closely the morbidity problems of the stroke patient might be the way forward. At present, any novel compounds in development have presumably gone through similar

screening techniques *in vivo* to those employed in the development of NXY-059, so it is hard to imagine they will enjoy any greater clinical success than NXY-059.

Another major point raised by the NXY-059 clinical programme is that more than one large clinical trial will be required by any regulatory body to prove efficacy. And when one says large, this really means large in a way not contemplated by many earlier trials. The total patient population of the two NXY-059 trials (SAINT 1 and SAINT 2) was around 5000 patients. This is a huge investment for any company to make.

I have little doubt research in the pharmaceutical industry will continue, because the clinical need is great and the commercial rewards are strong. However, research may not continue with neuroprotection as such, but rather on neuro-restoration and neurorepair as we get to understand more about the molecular mechanisms involved in such approaches.

Conflict of interest

The author is a former employee of AstraZeneca Pharmaceuticals.

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