Translational Stroke Research of the Combination of Thrombolysis and Antioxidant Therapy

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Abstract—Stroke is an enormous public health problem with an imperative need for more effective therapy. Recombinant tissue plasminogen activator is the only licensed drug for acute stroke, but its efficacy may be limited by the toxicity of the compound and by reperfusion injury. The coadministration of neuroprotective drugs could augment the value of thrombolytic therapy, but the evidence in support of this approach is scarce. The use of the free radical trapping NXY-059, either with or without recombinant tissue plasminogen activator, was not successful in Phase III studies. However, these results could reflect its weak antioxidant capacity, poor blood–brain barrier penetration, and lack of synergism with recombinant tissue plasminogen activator as well as the overly broad treatment window used in the reported trials. This article contends that further translational research should explore newer antioxidant drugs in combination with thrombolytic agents, but only if the combination yields additive or synergistic effects in preclinical thromboembolic models or in biomarker-assisted Phase II studies. Edaravone and novel nitrones endowed with a better pharmacokinetic profile or multitarget and thrombolytic activity are discussed as well as the latest research data on uric acid, a strong endogenous antioxidant in blood that is early consumed after acute stroke. The coadministration of uric acid and recombinant tissue plasminogen activator has shown to provide synergistic neuroprotection in experimental thromboembolic models and to lessen several biomarkers of oxidative stress in patients with acute stroke. The clinical efficacy of uric acid is currently under investigation in a Phase III trial that follows current recommendations of also evaluating surrogate biomarkers of treatment effects. (Stroke. 2011;42:00-00.)

Key Words: acute stroke ■ clinical trials ■ neuroprotection ■ thrombolysis

Stroke is a major cause of morbidity and disability in industrialized countries and it is predicted that the overall costs of stroke care will account for 6.2% of the total burden of illness in 2020. Therefore, there is an imperative need for effective preventive therapy, early critical care, and rehabilitation patients with stroke. Recombinant tissue plasminogen activator (rtPA) is the most effective treatment in human stroke. Currently, rtPA is approved for use within 3 hours of stroke onset, and the drug is also beneficial in selected patients treated within 4.5 hours of clinical onset but not beyond. Regrettably, numerous neuroprotective drugs were unable to demonstrate beneficial effects in Phase II/III clinical trials despite previous encouraging preclinical results.

The wealth of negative results obtained in previous clinical trials could suggest that effective neuroprotection is not feasible in human stroke. However, we believe that before reaching this overcast and nihilistic conclusion, it is mandatory to re-examine whether neuroprotection failed because most of the previous trials allowed an excessive delay to the onset of treatment and excluded or limited the coadministration of thrombolytic therapy. The lack of combination treatment with thrombolytic agents might have hindered the access of the neuroprotective agent to the ischemic brain in adequate drug concentrations. The delayed treatment onset may have resulted in the exhaustion of the mechanism of damage aimed by the neuroprotectant and/or the lack of salvageable penumbral tissue in the treated patients, yet the truth of these assumptions can only be revealed if newer combination trials designed according to these criteria are able to provide neuroprotection. However, which of the many drug candidates should be assessed in these trials in combination with thrombolytic therapy? This article sustains that a sensible strategy is to prioritize the study of newer antioxidant drugs. However, to prevent the replication of previous failures, it is also emphasized that before launching any antioxidant candidate into a costly translational research planning, the new agent must show recognizable additive or synergistic effects with thrombolytic agents in preclinical thromboembolic models or in Phase II studies.

Arterial Recanalization Is Mandatory but May Not Be Sufficient

The main goal of acute stroke therapy is the recanalization and adequate reperfusion of a symptomatic occluded vessel, which may occur too late spontaneously and only in 1 of 4 strokes. The rates of recanalization increase to 46.2%, 63.2%, 67.5%,
and 83.6\% after intravenous, intra-arterial, combined intravenous/intra-arterial, or mechanical thrombolysis,\textsuperscript{8} and these approaches are correlated with improved outcome.\textsuperscript{8} The rates of adequate reperfusion after thrombolytic therapy are less well known and marked discrepancies may exist between the rate of recanalization and the magnitude of the final treatment effects,\textsuperscript{9} suggesting that in some instances, reperfusion may be insufficient to block the ischemic cascade.\textsuperscript{10} Untimely reperfusion may be injurious and facilitate the development of cerebral edema, brain hemorrhage, or both in experimental conditions.\textsuperscript{11} In human stroke, the relevance of reperfusion injury is less well established, but brain perfusion MRI studies suggest that 40\% to 50\% of the patients show hyperperfusion within hours of stroke onset.\textsuperscript{12} The 10-fold increase of cerebral bleeding encountered in thrombolysed patients could also be due in part to reperfusion injury.

### Oxidative Stress: A Key Mechanism of Reperfusion Injury

The ischemic brain is particularly vulnerable to oxidative stress compared with other organs as the result of its high consumption of oxygen, its rich content of iron and unsaturated lipids, and its relatively low antioxidant capacity.\textsuperscript{13} During ischemia/reperfusion, there is a surge in production of superoxide, nitric oxide, and peroxynitrate for at least 6 to 12 hours\textsuperscript{14} that facilitates a vicious cycle that impinges on mitochondrial dysfunction, excitotoxicity, lipid peroxidation, and inflammation.\textsuperscript{15} As a result, there is an increased expression of proinflammatory cytokines, endothelial adhesion molecules, and matrix metalloproteinases (MMPs), further increasing oxidative stress and tissue damage.\textsuperscript{15,16} During reperfusion, oxidative stress reaches higher peaks and has a more sustained duration than other pathogenic mechanisms of ischemic cell death, further supporting the research of antioxidant therapies in reperfused individuals. rtPA-induced reperfusion may also result in additional hurdles due to the purported neurotoxicity of the drug.\textsuperscript{17} Indeed, although intravascular rtPA promotes fibrinolysis, extravascular rtPA interacts with gluatamate receptors,\textsuperscript{18} low-density lipoprotein-receptor-related protein, and latent platelet-derived growth factor-CC\textsuperscript{19} that may lead to expression of MMP-9, degradation of the extracellular matrix, edema, hemorrhage, and cellular death.

### Stroke Biomarkers of Oxidative Stress and rtPA Effects

Over the last years, a growing number of biomarkers has been assessed to refine the diagnosis and outcome of acute stroke or serve as surrogate indicators of treatment effects.\textsuperscript{20–24} However, most available studies are relatively small, do not have adequate control groups, use nonstandardized methods, and are unable to demonstrate independent predictive power to a validated clinical model,\textsuperscript{25} yet some biomarkers are promising surrogate indicators of treatment effects and could be used in Phase II studies to monitor treatment effects.\textsuperscript{26} Acute MMP-9 concentrations have been related with rtPA use\textsuperscript{27} and also with blood–brain barrier disruption,\textsuperscript{27} hemorrhagic complications,\textsuperscript{28} lesion growth,\textsuperscript{29} and poor long-term outcome\textsuperscript{30} in rtPA-treated patients.

### Thrombolysis in Combination With Antioxidant Therapy: Where Are We?

Several antioxidant therapies have shown neuroprotection in experimental models of brain ischemia.\textsuperscript{31} These antioxidants pertain to major classes that include inhibitors of free radical production, free radical scavengers, and boosters of free radical degradation, as summarized in the Table. However, only few of these agents have been assessed in combination with

#### Table. Antioxidant Drugs With Neuroprotective Effects in Experimental Conditions

| Antioxidant | Species | Middle Cerebral Artery Occlusion | Less Than Lesion Volume |
|-------------|---------|----------------------------------|------------------------|
| Inhibition of free radical production |
| Allopurinol | Rat | P | 24%–69% |
| Oxyurinol | Rat | P | 30% |
| NS-398 | Rat/mouse | P/2 h | 16%–29% |
| Nimesulide | Rat | 1 h | 0%–59% |
| Free radical scavengers |
| Lipoic acid | Mouse | 45 min | 38% |
| N-acetylcysteine | Rat | 30 min/2 h | 0%–67% |
| Glutathione monoethyl ester | Rat | 2 h | 65% |
| Tirilizid | Rat | P/2 h | 25%–40% |
| Uric acid | Rat | 2 h/TEO | 55%–80% |
| Melatonin | Rat | P/2 h | 30%–54% |
| A phenyl-tert-butylnitro nitrone (PBN) | Rat | P/2 h | 0%–65% |
| S-PBN | Rat | P | 35% |
| NXY-059 (Gerovive) | Rat | P/2 h | 0%–80% |
| Stilbazulanyl nitrite | Rat | 2 h | 42%–72% |
| Vitamin E | Rat/mouse | P/4 h | 45%–55% |
| Vitamin C | Mouse | P/45 min | 0% |
| Dehydroascorbic acid | Mouse | P/45 min | 50%–90% |
| EPC-K1 | Rat | PCO | 0%–40% |
| Resveratrol | Rat | 2 h | 48% |
| Oxresveratrol | Rat | 2 h | 36%–44% |
| Edaravone | Rat | P/PCO | 0%–77% |
| Increase of free radical degradation |
| Superoxide dismutase | Rat | P/2 h | 21%–57% |
| TEMPO | Rat | 1 h | 0%–50% |
| M40401 | Rat | 1.30 h | 0%–73% |
| AEOL 10113 | Rat/mouse | 1.30 h | 0%–70% |
| AEOL 10150 | Rat/mouse | 1 h/1.30 h | 25%–43% |
| EUK-134 | Rat | P | 86%–90% |
| Ebselen | Rat | P/2 h | 0%–37% |
| FeTMPyP | Rat | 2 h | 0%–38% |
| FeTPPSS | Rat | 2 h | 0%–62% |

Modified from Margail I, Plotkine M, Lerouet D. Antioxidant strategies in the treatment of stroke. Free Radic Biol Med. 2005;39:429–443 with permission. P indicates permanent ischemia; PCO, photochemical occlusion; TEO, thromboembolic occlusion.
thrombolytic therapy, and still fewer have demonstrated additive or synergistic effects, as we further discuss subsequently.

Nitrones and Thrombolysis in Combination
The administration of the free radical trapping NXY-059 (Cerovive), either with or without rtPA, was a major clinical failure despite what has been considered a modern trial well founded on extensive preclinical data. Nonetheless, it should be stressed that experiments with this compound using the rabbit small clot embolic stroke model had anticipated that NXY-059G was neuroprotective if it was administered 5 minutes after embolization only. The coadministration of NXY-059G increased the safety of rtPA given up to 1 hour of embolization but without statistically significant synergistic effects. In correspondence with these results, the Stroke-Acute Ischemic NXY Treatment (SAINT) I trial suggested a decreased incidence of rtPA-related hemorrhagic complications, but the pooled analysis of SAINT-I and SAINT-II trials did not identify a better stroke outcome in patients receiving the combination therapy at a time delay of approximately 4 hours.

The clinical efficacy of NXY-059 may have also be limited by its reduced ability to penetrate the blood–brain barrier and its restricted radical trapping capacity. Contrarily, novel chain-breaking antioxidants with low oxidation potentials and high lipophilicity properties such as azulenyl nitrones overcome these limitations. Currently, stilbazulenyl nitrene has shown neuroprotective effects in transient ischemia in rats, but the drug remains to be assessed in combination with rtPA. The use of nitrones with multitarget properties is another therapeutic strategy that would deserve translational research. Thus, 2,3,5,6-tetramethylpyrazine is a nitrene that can be synthesized from Ligusticum wallicchii (Chuanxiong), 1 of the 50 fundamental herbs used in traditional Chinese medicine and which has been used traditionally for the treatment of stroke. Recently, the administration of 2,3,5,6-tetramethylpyrazine 1 hour after the intraluminal occlusion of the middle cerebral artery in the rat showed a reduction of 56% of the infarction volume and a 71% inhibition of venous thrombus formation. These findings justify additional study of the application of synthetic 2,3,5,6-tetramethylpyrazine in acute stroke.

Edaravone and Thrombolysis in Combination
The clinical efficacy and toxicology of edaravone have been extensively reviewed. The drug readily crosses the blood–brain barrier; scavenges hydroxyl, peroxyl, and peroxide radicals; and in experimental conditions inhibits important pathogenic mechanisms such as delayed neuronal death, microglia-induced neurotoxicity, long-term inflammation, lipo-oxygenase, oxidation of low-density lipoproteins, expression of vascular endothelial growth factor, aquaporin-4, and MMP-9. In the rabbit small clot embolic stroke model, edaravone resulted in decreased behavioral deficits when administered up to 3 hours postembolization, although the drug did not result in synergistic effects when used in combination with rtPA.

The use of edaravone in acute stroke was approved in Japan in 2001, and it has been associated with lower serum levels of MMP-9 when it is given within 12 to 36 hours of stroke onset. In combination with rtPA, 1 study reported a lower incidence of brain edema and white matter tissue injury in edaravone-treated patients. Additional studies are required to verify whether edaravone improves the benefits of rtPA, but an ongoing Phase IIa clinical trial of this agent has specifically excluded these patients.

Uric Acid and Thrombolysis in Combination
Uric acid (UA) is the major antioxidant in blood, and its concentration is almost 10-fold higher than other antioxidants. The properties of UA include scavenging of hydroxyl radicals, hydrogen peroxide, and peroxynitrite; suppression of the Fenton reaction; chelation of transition metals; and prevention of lipid peroxidation. During brain ischemia, adenosine 5’-triphosphate is degraded to adenine and xanthine, resulting in increased generation of UA in the brain. Administration of UA up to 1 hour after middle cerebral artery occlusion and reperfusion is neuroprotective in rats. UA is also neuroprotective in thromboembolic models in rats, in which UA is given 20 minutes after the injection of thrombi in the middle cerebral artery and rtPA is given at 3 hours of reperfusion. The combination of UA and rtPA shows synergistic effects compared with either treatment alone, and the effects include reduced tyrosine nitrination, less neutrophil infiltration in the brain, reduced infarct volume, and improved behavior.

In patients, there is a rapid consumption of UA after stroke and more remarkable after recanalization. Higher UA levels at stroke admission are associated with better outcome and less infarction growth at follow-up, and these effects have been found in patients treated or not with thrombolytic therapy. In patients receiving rtPA, lower UA levels are associated with a greater incidence of malignant middle cerebral artery infarctions and/or hemorrhagic transformation of the infarction. In a Phase II placebo-controlled trial, the combination of UA and rtPA was safe and prevented an early fall of circulating UA levels. UA also prevented an increment of malondialdehyde and active (α)-MMP-9 in rtPA-treated patients, and these biomarkers were found inversely correlated with stroke outcome at 3 months. These preclinical and clinical findings have boosted an ongoing Phase Ib/3, randomized, placebo-controlled trial that assesses in 420 patients with stroke whether the combination of UA and rtPA given up to 4.5 hours after stroke onset is beneficial. The study also assesses the time course of several oxidative stress biomarkers such as MMP-9 or malondialdehyde and the treatment response obtained in relation to the course of multiparametric imaging findings.

Conclusions
A wealth of preclinical and clinical data indicate that oxidative stress plays a major role after brain ischemia and still more so after rtPA-induced recanalization and reperfusion. Therefore, further translational research of the combination of antioxidant and thrombolytic agents seems justified. The negative results of the combination of NXY-059 and rtPA could reflect the proper limitations of this nitrone (weak
antioxidant capacity, poor blood–brain barrier penetration, lack of synergism with rtPA and the broad treatment delay that was allowed in Phase III trials, outlasting the very short time window of efficacy observed in preclinical thromboembolic models. However, this should not thwart new attempts to improve stroke outcome with the modulation of oxidative stress. Newer nitrones with a better pharmacokinetic profile, or with multitarget and thrombolytic activity, deserve further study. The available evidence does not support synergistic effects between edaravone and rtPA, and this combination was excluded in an ongoing study aimed to confirm the efficacy of edaravone. The administration of UA is currently being assessed with the aim of boosting an endogenous antioxidant mechanism, which is only active in humans and higher primates. Current evidence suggests that UA is an adaptive system that is early consumed after stroke and that the combined administration of UA and rtPA offers synergistic neuroprotection in experimental models including thromboembolic stroke. In patients with acute stroke, this approach was safe and lessened several biomarkers of oxidative stress in a Phase II study, and its efficacy is currently evaluated in a proof-of-concept clinical trial due to be completed in 2012.

Disclosures
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