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Toward a Multimodal Neuroprotective Treatment of Stroke

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Background and Purpose—Stroke remains a common medical problem with importance attributable to the demographic changes in industrialized societies.

Summary of Review—After years of setbacks, acute stroke therapy has finally emerged, including thrombolysis with tissue plasminogen activator (t-PA). However, t-PA treatment is limited by a narrow time window and side effects, so that only 3% of all stroke patients receive thrombolysis. Unimodal targeting of key events in stroke pathophysiology was not effective in providing long-term benefits, leading to negative results in previous clinical neuroprotective stroke trials. A successful future stroke therapy should approach multiple pathophysiological mechanisms besides revascularization at once, including reduction of t-PA–related side effects, prevention of cell death, stimulation of neuroregeneration, and plasticity.

Conclusions—Strategies targeting these processes include multiple combination therapies as well as treatment with multimodal drugs that interact with these mechanisms. Here, we review such combination approaches, and outline how this concept could be developed into future stroke treatment. **(*Stroke*. 2006;37:1129-1136.)**

Key Words: apoptosis ■ clinical trials ■ excitotoxicity ■ growth factors ■ inflammation ■ neuroprotection ■ stroke

Despite tremendous efforts in stroke research and significant improvements in stroke care within the last decade, therapy is still insufficient. The only approved treatment for ischemic stroke is intravenous recombinant tissue plasminogen activator (t-PA; alteplase) that effectively recanalizes the occluded vessel and improves neurological outcome.[1] Because of the narrow time window of 3 hours and the potentially dangerous side effects of hemorrhagic complications, only 3% of all stroke patients receive t-PA.[2] The remaining 97% of patients receive no specific therapy, exemplifying stroke as an undertreated disease demanding an improvement of the existing therapy and a vigorous search for new therapies.

The identification of the pathophysiological key events that contribute to the development of infarction led to studies mainly targeting single mechanisms of injury. Such controlled and defined experimental studies produced promising pilot results and led to numerous randomized and placebo-controlled pivotal trials that failed to show a significant therapeutic benefit. Although the dampened enthusiasm for neuroprotection was recently encouraged by the results of the Stroke-Acute-Ischemic NXY-Treatment (SAINT) I trial,[3] overall information from previous trials suggests that an approach targeting a single mechanism alone will likely have only a limited effect on stroke outcome. Based on the complex pathophysiological cascade associated with acute ischemic stroke, a multimodal approach targeting an array of key mechanisms appears to be a key future approach to enhance therapy. We review the status of this concept based on the existing work and outline how stroke treatment could be developed.

Status of Combination Therapy: Experimental and Clinical Studies

The experience that drugs acting against a single mechanism failed in acute stroke trials led to the idea that a combination of different drugs could be promising to modulate or stop the dynamics of ischemic pathophysiology and to achieve synergistic effects. Indeed, there is solid preclinical evidence indicating that combinations of neuroprotective agents synergistically decrease infarct volume, improve neurological outcome, and extend the therapeutic time window. The combinations of channel blockers, such as glutamate receptor antagonists plus the β2-agonist clenbuterol[4] or the γ-aminobutyric acid agonist muscimol,[5,6] as well as caffeine with ethanol,[7] showed additive effects on outcome parameters in animal studies. Combinations of similar-acting classes of drugs, such as antioxidants and free-radical scavengers,[8–13] or other neuroprotective agents, such as magnesium,[11,12] apparative approaches such as hypo-

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TABLE 1. Combination Treatments (experimental studies)

Agents	Result of Clinical Trials/Stroke Model	References
Growth factors		
Insulin-like growth factor-1 (IGF-1) derivatives (G2MePE) and caffeinol	Beneficial effects on lesion size/rat	46
bFGF and citicoline	Synergistic effects of low-dose combination/rat	23
bFGF with caspase inhibitors (z-VAD.FMK, z-DEVD.FMK)	Reduced infarct volume, and reduced neurological deficit, extended time window/mice	22
Brain-derived neurotrophic factor (BDNF) and mild hypothermia	Synergistic effect in attenuating striatal glutamate release; reduced early infarct size/rat	47
Free radical scavengers, antioxidants		
Free radical scavengers PBN and S-PBN	Reduced infarct volume/rat	8
NO synthase inhibitor LNA and antioxidant/superoxide scavenger DtBHB	Reduced infarct volume/rat	9
Antioxidants U-74389G and U-101033E	Less deficit, reduced infarct volume/rat	10
Antioxidant tirilazad and magnesium	Benefit on infarct size/rat	11
Antioxidant tirilazad, magnesium, and mild hypothermia	Increased benefit/rat	12
Free radical scavenger EPC-K1 and heparin	Reduced hemorrhage, reduced infarct volume, improvement of function/rabbit	13
Channel blockers		
γ-Aminobutyric acid-A agonist muscimol with glutamate antagonist MK-801	Beneficial effects/rat	5, 6
N-methyl-D-aspartate receptor antagonist memantine and β2-agonist clenbuterol	Reduced infarct size, prolonged therapeutic window/rat	4
Mild hypothermia		
Caffeine, ethanol, and mild hypothermia	Increased effects/rat	18
Mild hypothermia and bcl-2 overexpression (mitochondrial membrane protein	Extended time window for gene therapy due to mild hypothermia/rat	17
Anti-inflammatory agent minocycline and mild hypothermia	No additive effect/rat	14
Mild hypothermia with barbiturate methohexital (burst suppression)	No additional effect/rat	15
Other mechanisms		
Lubeluzole and hemodilution with diaspirin cross-linked hemoglobin (DCLHb)	Augmented benefit/rat	19
Cyclosporine-A and methylprednisolone	Partial and transient effects due to combination/rat	20
Caffeine and ethanol	Reduced lesion size/rat	7
Isoflurane and z-VAD-fmk (nonspecific caspase inhibitor)	Effects on infarct size and neurological function/rat	21

thermia,[12,14–18] heparin,[13] or other mechanisms[19–21] were also able to synergistically enhance efficacy compared with each single compound (Table 1).

Four main principles of combination approaches could be differentiated. Most promising will be a combination of 2 drugs with different neuroprotective mechanisms. One example for that approach is the combination of memantine, which reduces excitotoxicity and free radical formation, and the β2-adrenoceptor agonist clenbuterol, which induces nerve growth factor synthesis. A combination of both further reduces infarct size compared with the effects of each drug alone. Furthermore, in combination with memantine, the therapeutic window of clenbuterol was significantly prolonged.[4] Expanding the therapeutic time window is another target for combination therapies;[17] for example, treatment with a subthreshold dose of basic fibroblast growth factor (bFGF) extended the therapeutic window for the caspase inhibitor *N*-benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp-(OMe)-fluoro-methylketone (z-DEVD.FMK) from 1 to 3 hours after reperfusion.[22] The third approach focuses the combination of individually ineffective drugs or drugs that are ineffective at low dosages, exerting a protective effect only in combination. For instance, the low-dose combination of bFGF and citicoline reduced the infarct volume after temporary experimental focal cerebral ischemia, whereas bFGF and citicoline alone did not.[23] These findings are relevant because reduced dosages might accessorily alleviate the possibility of side effects. Finally, combining thrombolysis with neuroprotection could enhance the efficacy of both drugs[24–33] and may represent a near future development (Table 2). A few attempts in clinical trials showed that such combinations are feasible and safe (t-PA+lubeluzole or clomethiazole); however, additive benefits were not observed.[34–37] Some substances such as free-radical scavengers demonstrated a reduction of thrombolytic side effects.[13] These substances may enhance the effect of thrombolysis, even when a neuroprotective effect for the individual drug alone could not be shown. Interesting is the well-tolerated combination of caffeine plus low-dose ethanol (caffeinol),[7] which resulted in a reduction of infarct volume and did not interfere with or complicate thrombolysis.[18,38]

Promising Candidates for Combination Therapies

Growth Factors Featuring Hematopoietic Factors

Growth factors are naturally occurring substances in mammalian organisms with a broad array of activity, essential for

TABLE 2. Combinations Using t-PA

Agents	Result of Clinical Trials/Stroke Model	References
Clinical studies		
Caffeine and ethanol (caffeinol) (thrombolysis encouraged)	Pilot study: safe	38
t-PA with clomethiazole	Pilot study: safe and feasible/patients with acute stroke	37
t-PA with lubeluzole	Pilot study: safe and feasible/patients with acute stroke	34, 35
Experimental studies		
Fibrinolytic therapies		
t-PA with glutamate antagonist MK-801	Combination more effective than t-PA alone in reducing neurologic damage/rabbit	24
t-PA with nimodipine	No benefit/rabbit	24
t-PA with anti-CD 18 monoclonal antibodies	Significant improvement of neurologic outcome/rabbit	25
t-PA with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor antagonist YM872	Additional effect on infarct size/rat	26
Mild hypothermia with t-PA	No additional effect due to combination/rat	16
Urokinase (UK) and batroxobin (DF-521)	No higher risk on bleedings but no effects due to combination/rat	27
Amino acid receptor antagonist dizocilpine and alteplase	Significant effects/rat	28
Topiramate and urokinase	Benefit on infarct volume/rat	29, 30
Citicoline and urokinase	Significantly more protection due to combination/rat	31
N-methyl-D-aspartate antagonist eliprodil with t-PA	Improved outcome/rat	32
t-PA with 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[*f*]quinoxaline	Not significant effect/rat	33
Selective thrombin inhibitor argatroban and free radical scavenger edaravone (MCI-186)	Increased survival ratio/gerbil	84

survival, proliferation, maturation, and growth of developing cells and organs, including neuronal networks and structures. In addition to their role in coordinating development, growth factors are expressed and released in response to various brain insults such as cerebral ischemia. Many of these factors, such as brain-derived neurotrophic factor, bFGF, insulin-like growth factor-1, vascular endothelial growth factor, glial cell line–derived neurotrophic factor, and osteogenic protein-1, display excellent neuroprotective effects by blocking key events of the ischemic cascade and trophic effects by inducing regeneration and reorganization.[39–45] In addition, combinations of growth factors with drugs synergistically enhance such multiple effects: growth factors combined with caffeinol[46] or antiapoptotic drugs such as caspase inhibitors[22] or hypothermia[47] all have synergistic effects on neurological outcome, lesion volume, and mortality compared with each compound alone. Despite their endogenous origin, the use of growth factors for clinical purposes appears to be limited by major adverse effects and by their poor blood–brain barrier penetration related to their proteinaceous nature. Indeed, the only growth factor clinically tested for the treatment of acute ischemic stroke in a larger phase II study was bFGF, which failed because of hemorrhagic complications.[48]

Hematopoietic factors are growth factors stimulating and controlling development and maturation of red and white blood cells. For more than a decade in clinical practice, recent research uncovered a previously unknown but nevertheless fundamental role of these factors in the brain. The best known member of this family is erythropoietin (EPO), a critical modulator of erythroid production acting through the specific EPO receptor. EPO was recently shown to be neuroprotective in vitro and in vivo after various brain insults and to have pleiotropic effects including antiexcitotoxic and antiapoptotic as well as angiogenic and neurogenic effects.[49–51] Interestingly, a small pilot study demonstrated beneficial effects for the treatment in acute human stroke.[52] A carbamylated EPO variant without affinity to EPO receptor and without hematopoietic activity was neuroprotective after various cerebral insults including stroke, strengthening the hypothesis of a previously unknown function of this factor in the central nervous system (CNS).[53] Because of this specificity within the brain, carbamylated EPO variant might be better suited than EPO as candidate for stroke drug development.

The counterpart of EPO in the hematopoietic system is granulocyte colony-stimulating factor (G-CSF), which stimulates differentiation of the neutrophilic lineage of hematopoietic cells, commonly used to treat neutropenia.[54] Like EPO, G-CSF exerts its effects via the specific G-CSF receptor, stimulates the growth of neutrophil granulocyte precursors,[55] and regulates the survival of mature neutrophils[56] by inhibition of apoptosis.[57] Similar to EPO, G-CSF has a broad and fundamental presence within the CNS. Focal cerebral ischemia induced a massive upregulation of G-CSF and its receptor (>100-fold),[58] more than reported for any other gene including EPO. The action of G-CSF after an acute ischemic injury appears specifically mediated through the G-CSF receptor predominantly expressed by neurons, particularly in the peri-infarct zone, suggesting an autocrine adaptive system in neurons at risk. Supporting this system by exogenous systemic administration of G-CSF causes robust neuroprotection in different stroke models with a time window for therapy of ≥2 hours and potentially longer.[58–60] Exogenously

administrated G-CSF passes the intact blood–brain barrier.[58] The pleiotropic effects of G-CSF include potent antiexcitatory effects[59] and, even more important, strong antiapoptotic activity by evoking 3 independent antiapoptotic pathways in neurons.[58] G-CSF signaling and its role in suppressing apoptosis in neurons parallels its function on cells of the hematopoietic lineage.[61] In addition, G-CSF has potent anti-inflammatory effects in both systemic and CNS infections by acting as a immunomodulator.[62] In addition to its efficient interaction in acute stroke pathology, G-CSF enhances brain recovery after stroke.[58] G-CSF improved sensorimotor function correlating with increased neuronal progenitor activation in the periphery of the ischemic lesion (cortex and corpus callosum) and enhanced neurogenesis in the dentate gyrus. G-CSF increased the number of newly generated neurons under ischemic conditions but also in nonischemic sham animals. G-CSF may therefore enhance structural repair and function even in healthy subjects or at long intervals after stroke.

Hematopoietic factor signaling appears to be a novel protective system in the brain, counteracting key mechanisms in acute stroke pathology as well as regulating and enhancing stroke recovery, including the formation of new neurons. For therapeutic purposes, factors such as EPO or G-CSF fulfill the criteria of a novel type of stroke drugs. Multimodality concentrated in 1 compound, together with the ability to penetrate the blood–brain barrier, and a well-documented history of favorable safety make these drugs ideal candidates for stroke therapy. A randomized, multicenter, placebo-controlled phase IIa trial with G-CSF is currently ongoing to establish the safety of this protein in acute stroke patients.[63]

Free Radical Scavengers Featuring NXY-059

During the ischemic cascade, highly reactive free oxygen radicals that occur mainly in the reperfusion period of ischemia cause an excessive activation of excitatory amino acid receptors. Free radicals also act as toxic triggers for inflammation and apoptosis that further damage the ischemic brain.[64] Thus, scavenging free radicals reflects a plausible pathophysiologically oriented neuroprotective approach, and indeed, free radical scavengers were shown in numerous experimental studies to be neuroprotective.[65] NXY-059 is a nitrone-based free-radical trapping agent and the first neuroprotective agent preclinically developed in adherence to the recommendations of the Stroke Therapy Academic Industry Roundtable (STAIR) group. The recent results of the phase III study (SAINT I) suggested a reasonable treatment effect.[3] In this study, NXY-059 showed a favorable shift in the primary outcome parameter (modified Rankin Scale) at day 90, whereas it had no significant effect on neurological outcome (National Institutes of Health Stroke Scale) and functions of daily living (Barthel Index). Indeed, this study seems to provide the first proof-of-principle for the concept discussed here, in that NXY-059 cotreatment significantly reduced rtPA-related hemorrhages. A second NXY-059 trial (SAINT II) to confirm these encouraging results is ongoing, and the sample size was recently increased to add more power to the trial.

Because of its nature of scavenging free radicals that typically occur as reperfusion-associated phenomenon, NXY-059 would be a perfect candidate to be combined with t-PA. This combination could substantially reduce thrombolysis-related side effects and potentially increase the therapeutic time window for recanalization.

Citicoline

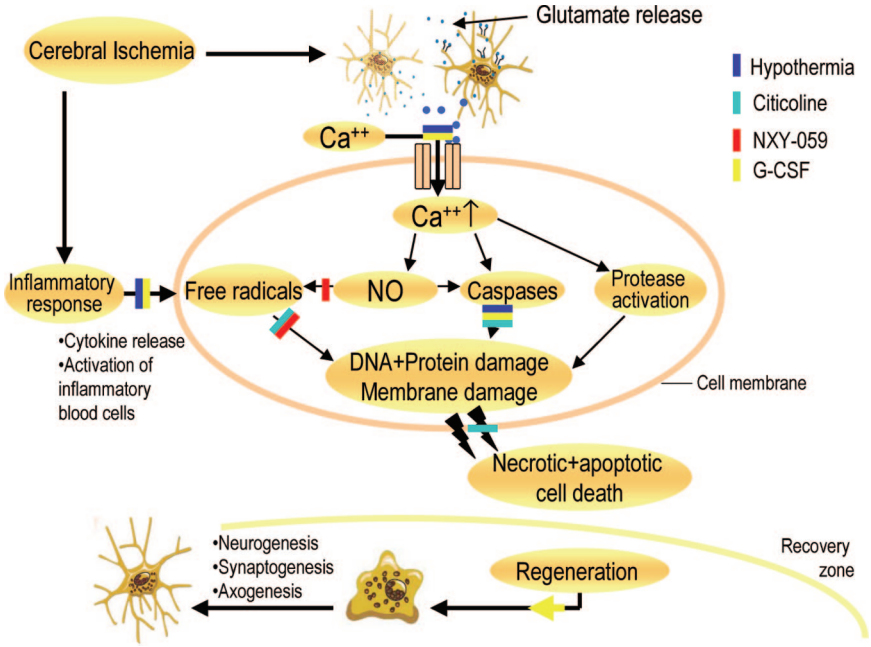
Citicoline, a naturally occurring endogenous compound, is a phosphatidylcholine precursor serving as intermediate in the synthesis of membrane lipids. Although the robust neuroprotective effects of citicoline are documented in several stroke models,[66,67] the mechanisms are less well studied, and are at current thought to be related to a membrane-stabilizing function, to reduction of free radical release, to reduction of apoptosis, and to recovery-enhancing effects.[68,69] There is evidence from randomized, placebo-controlled trials that citicoline improves neurological outcome after human stroke.[70,71] However, the clinical phase III trial failed to show an efficacy in the primary end point functional neurological outcome.[72] However, a meta-analysis based on all available trials indicates that there might be an effect for the subgroup of moderate to severe ischemic strokes.[73] Therefore, another phase III trial is under way.

Because of its good tolerability, citicoline represents an interesting candidate for a combination approach. As indeed indicated from preclinical studies, combinations of citicoline with either growth factors or thrombolytic drugs were able to exert true synergistic effects, hereby reducing mortality and infarct volume.[23,31]

Hypothermia

Hypothermia has a well-documented history of neuroprotection in numerous experimental studies and was recently shown to improve outcome after global ischemia in patients with cardiac arrest.[74] The situation after focal ischemia in humans is currently unclear, although data from case series and a small pilot study suggest that moderate hypothermia (33°C) could potentially improve neurological outcome in patients with malignant middle cerebral artery infarction.[75] The mechanisms of neuroprotection achieved by hypothermia are manifold and simply include the temperature-dependent reduction of cerebral metabolism, the decrease in cellular metabolism, and facilitation of postischemic glucose utilization.[76] However, hypothermia also interacts with several key events in the ischemic cascade, including prevention of the blood–brain barrier breakdown and hereby reducing edema formation and intracerebral pressure increase.[77] Hypothermia furthermore reduces free radical formation,[78] exerts antiexcitotoxic effects by suppressing elevations of intracellular calcium, inhibits the release of excitotoxic amino acids, reduces intracellular acidosis,[79] and has antiapoptotic functions.[80]

Hypothermia could be an ideal combination to other neuroprotectants or to thrombolysis, as indicated from several experimental studies (Table 2). In combination with thrombolysis, hypothermia may prevent the toxic effects of t-PA on the reperfused brain.[80] However, because of the intensive apparative requirements to perform hypothermia, it may be reserved for the small subgroup of large middle cerebral artery infarctions. For



Interaction within ischemic pathophysiology of the currently most promising candidates for a multimodal neuroprotective approach. Stroke onset triggers an array of mechanisms, including depolarization of presynaptic neurons, glutamate release, activation of postsynaptic glutamate receptors, and subsequent intracellular calcium increase with subsequent activation of apoptosis and toxic radical release. The ischemia-induced inflammation then further maintains these processes. Particularly, hypothermia (blue bar) and G-CSF treatment (yellow bar) interact with multiple mechanisms of the ischemic cascade. The green bar within the cell membrane indicates the membrane-stabilizing function of citicoline. After passing through the acute phase of the stroke, reorganization processes such as neurogenesis, axogenesis, and synaptogenesis are induced by recovery-inducing and enhancing factors such as G-CSF (yellow arrow).

the interaction within ischemic pathophysiology of these promising candidates see the Figure.

Developing Combination Therapy Into Future

Based on such prerequisites, near future development of combination therapy will likely focus on the combination of t-PA with a neuroprotectant to enhance thrombolytic therapy. However, significant improvements in stroke therapy may crucially depend on the development of additional strategies in the hyperacute phase before thrombolysis and thereafter.

Combinations With Thrombolysis

The most efficient synergy probably arises from the combination of 2 different mechanisms of therapy such as thrombolysis and tissue-protecting strategies. In contrast to controlled experimental studies in which synergistic effects on lesion volume and neurological outcome could clearly be demonstrated, the proof of concept in clinical studies is lacking. Currently, besides t-PA, no other proven, effective stroke drugs are available, making the situation even more difficult. Combining an ineffective neuroprotectant with thrombolysis to synergistically improve outcome appears not very promising and was indeed not shown to be effective so far.[34–36,81–83] Combination trials in humans with the goal to achieve synergistic effects should be postponed until an effective drug besides t-PA can be identified. Drugs that complement thrombolysis should ideally inactivate or block t-PA–related side effects such as hemorrhage or reperfusion injury. The most promising candidate appears to be the free radical scavenger NXY-059, which could complement thrombolysis, probably even when the positive effect for the drug alone would be minor. The rationale is that a specific effect accounting for the synergy would particularly be activated after thrombolysis and does not occur to a great extent without that treatment. Other interesting candidates currently not known to interfere with thrombolysis could therefore ideally be combined with t-PA, including the hematopoietic factors G-CSF and EPO as well as citicoline. Citicoline could be interesting because of its membrane-stabilizing and free radical–scavenging functions.

Extending the Therapeutic Time Window

Extending the time window for treatment initiation is an intriguing idea, implying that ultra-early application of a drug or a condition (hypothermia) expands the time window for further treatment such as thrombolysis or neuroprotection. Drugs that fulfill this possibility must be safe and easy to administer. Potential candidates attributable to their good tolerability are citicoline and also magnesium. Particularly magnesium has multimodal neuroprotective actions as shown by a number of experimental studies. Early administration of magnesium in the field (FAST MAG) was indeed shown to be feasible and safe in a preliminary study and is being continued in an ongoing trial. The results of this study will not only show the specific efficacy of magnesium in this context, but it may rather strengthen the conceptual basis of prehospital initiation of treatment and could trigger subsequent trials with other drugs. Another potent substance for ultra early application could be G-CSF because of its multimodal action, good tolerability, and easy applicability. The safety of G-CSF in acute ischemic stroke is currently studied in a multicenter randomized phase II trial.[63] Despite a number of positive experimental studies, hypothermia appears because of the devices needed to implement not to be a good choice for extending the time window in the clinical situation.

Re-Engineering the Brain After Stroke

In addition to the initial treatment (thrombolysis, neuroprotection), brain structures and function may be re-engineered. This process should not be delayed too long, probably days to a few weeks after stroke. Perfect candidates are drugs with trophic and regenerative effects such as growth factors, particularly the well-tolerated hematopoietic factors G-CSF

and EPO, as well as neurotransmitters with restorative function such as amphetamine or l-dopa. Although several experimental and clinical pilot studies suggested beneficial effects for some drugs (eg, amphetamine), no large multicenter phase III trial has been conducted so far. This challenges the development of recovery-enhancing drugs because of discordance regarding end points and meaningful clinical improvements of neurological function. However, future development in this field will reinforce the experimentally driven concept that a specific pharmacological support of recovery complemented by physical training can further enhance recovery. The long-term goal would be to link recovery-enhancing therapies in a defined fashion to the already established acute stroke treatment. The potential of stem cells in that context needs to be determined because several studies showed beneficial effects after cell transplantation in chronic experimental stroke.

What Could Be the Next Steps for Further Development?

In the future, when one of the candidate drugs will have been shown to be effective in a clinical trial, further development toward a combination approach should be driven by STAIR-guided experimental studies. The following approaches should be considered when a new drug will be added to t-PA. (1) In vitro data should demonstrate lack of interference between t-PA and the candidate drug. (2) The time window of the ideal application of the second drug in relation to t-PA must be defined. Extending the time window of t-PA efficacy is an important goal. (3) Dose response studies need to be performed. (4) The candidate drug should be tested in an embolic stroke model with thrombolysis (preferably in 2 different species). (5) The outcome parameters such as infarct volume and functional neurological outcome need to be defined and tested, particularly to differentiate between effects of the single drugs versus the combination treatment.

Once these criteria are tested, the concept could enter clinical development. The next step would be a clinical feasibility and safety trial (phase IIa) with ≈10 to 15 patients included per arm randomized to different doses of the combination versus t-PA. An alternative to this classic design represents an adaptive treatment allocations across a broader range of doses, requiring, however, more subjects to be recruited. After assessing safety, the particular combination then can proceed to phase IIb with ≈200 to 300 patients per treatment arm, which will give further safety information. At this stage, some hints of efficacy could be expected, although a typical phase IIb trial will be underpowered for the detection of clinical efficacy end points. However, defining the end points for a phase IIb-III trial might be one of the most difficult tasks. Besides relying on clinical outcome markers, surrogate markers could be important to demonstrate that a specific combination reduces negative effects of thrombolysis. A significant enlargement of the therapeutic time window for effective t-PA treatment might be sufficient to move toward a pivotal phase III trial.

Conclusion

Developing combination therapy appears to be fundamental to improve future stroke treatment. This concept consists of a combination of drugs or a single drug that blocks different key mechanisms associated with ischemic pathophysiology. The near future will likely focus on combinations with thrombolysis to reduce thrombolytic side effects and to achieve additional protection. Further strategies include the extention of the therapeutic time window by ultra-early application of drugs in the field as well as the linkage of recovery-enhancing drugs to acute stroke treatment.

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