Published in final edited form as:
Expert Opin Investig Drugs. 2010 October ; 19(10): 1179–1186. doi:10.1517/13543784.2010.517954.

Erythropoietin Molecules to Treat Acute Ischemic Stroke: A Translational Dilemna!

Paul A. Lapchak, Ph.D., FAHA

Abstract

Importance of the field—Since the realization that Erythropoietin (EPO) molecules have "neuroprotective" properties, they have been investigated as treatments for acute ischemic stroke (AIS), but not systematically. The results of the 2009 clinical trial showed that EPO was ineffective as a stroke treatment, and moreover, increased mortality when combined with tissue plasminogen activator (tPA). Currently, CEPO, an EPO analog is entering into a safety, tolerability and pharmacokinetic clinical trial for the treatment of AIS.

What the reader will gain—The primary aim of this article is to review the information available regarding the pharmacological and biological characteristics of EPO molecules. Secondly, based upon the translational research with EPO molecules in preclinical stroke models, a recommendation is made regarding the continued development of EPO molecules as an option to treat AIS.

Areas covered in this review—This review covers translational and clinical studies carried out over the period 1998–2010.

Take home message—EPO, CEPO and helix B peptide (HBP) EPO analogs have significant neuroprotective activity is preclinical stroke models. However, given the detrimental effect of EPO in a recent clinical trial, preclinical safety studies of EPO molecules in embolic stroke models that parallel human stroke are warranted.

Keywords

acute ischemic stroke; behavior; carbamly erythropoietin; CEPO; embolism; neuroprotection; mortality; translational science; toxicity

1. Background

Human EPO, a 34 kDa glycoprotein hormone, which promotes the maturation of erythroid progenitor cells into erythrocytes, is essential for regulating circulating levels of red blood cells. EPO was the first hematopoietic growth factor to be cloned and studied in depth [1–5]. The protein consists of a single 165 amino acid polypeptide chain with three N-glycosylation sites at asparagine residues (positions 24, 38, 83) and one serine O-glycosylation site (position 126). Studies have shown that the average carbohydrate content is approximately 40%. The oligosaccharide chains have been shown to be modified with terminal sialic acid residues with N-linked chains typically having up to 4 sialic acids per chain and O-linked chains having up to two sialic acids [2]. In addition, the mature EPO protein has eight lysine residues, in addition

Please address correspondence to Paul A. Lapchak, Ph.D., FAHA, Director of Translational Research, Cedars-Sinai Medical Center, Department of Neurology, 110 N George Burns Road, D-2091, Los Angeles, CA 90048; Office: 310-248-8188, Fax: 310-248-7568, Paul.Lapchak@cshs.org.

Declaration of interest
The author is supported by a NINDS grant *NS060685*.

to the N-terminal alanine, which provides nine primary amino groups for carbamylation [2] and the production of a molecule designated as CEPO.

The chemical process of carbamylating EPO starts with the parent molecule recombinant human EPO and a well-controlled chemical reaction in the presence of potassium cyanate resulting in the transformation of lysine residues to homocitrulline. This process known as erythropoiesis silencing was originally described by Mun and Golper[6] and Park et al.[7]. In CEPO, 8 primary lysine groups and an N-terminal amino acid are modified by carbamylation. However, CEPO can have as many as 15 carbamyl residues and there are also some isoforms of CEPO, however, the majority of isoforms do not have substantial erythropoietic activity, if any at all, assessed using a human erythroleukemia cell line (TF-1; ATCC.org ATCC No. CRL-2003) [8,9], or a human erythropoietin-dependent leukemia cell line (UT-7/EPOR; Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Cat. No. ACC 363) [10,11] in vitro assay.

2.1 EPO Molecule Responses

EPO is commonly known as an erythropoietic protein that effectively increases hemoglobin levels via the binding of the molecule to a specific EPO receptor (EPoR) located on the surface of red cell precursors in bone marrow[12–14], thereby stimulating them to transform into mature red blood cells. The binding of EPO to a transmembrane receptor (Class I cytokine receptor), initiates a series of signaling pathways and physiological responses. The homodimer receptor complex (EPoR)2 consists of an extracellular domain which contributes to ligand binding, receptor processing and transport [12,15], whereas the cytosolic regions of the receptor mediate induced mitogenesis, gene transcription, and interaction with numerous kinases, including ERK, tyrosine, PI3-, JAK-2 kinases[12,16].

Recently, Brines et al. identified portions of the EPO protein helix required for the differential erythropoietic or neuroprotective effects of the protein[15]. The structure activity information shows that EPO interacts with They propose that specific amino acid residues 58–82 of helix B confers neuroprotective activity. Moreover, an 11 amino acid sequence (or peptide) in EPO helix B, adjacent to 58–82, known as HBP, was shown to be non-erythropoietic and neuroprotective in vitro and in vivo[15]. Interestingly, the 11 amino acid sequence is not one modified by carbamylation due to the lack of lysine residues. Thus, it is proposed that the sequence may be responsible for the neuroprotective activity of EPO and CEPO[15].

Under certain circumstances, for the treatment of certain diseases or symptoms related to medical treatment, the erythropoietic activity is recognized as a benefit[17–20]. However, there may also be serious adverse effects of erythropoiesis when administered to treat conditions where increases in red blood cells are not warranted[21–25], such as ischemic stroke. In contrast to EPO, CEPO apparently does not bind to the EPoR complex and does not stimulate the production of red blood cells[26,27].

2.2 Do different receptors/signal pathways mediate the effects of CEPO?

In 2004, Brines and colleagues [28] proposed that the neuroprotective effects of EPO may be mediated, in part, by a common beta receptor (βR), a signal-transduction subunit which is shared by growth factors and cytokines (i.e. CD131). Since βR knockout mice exhibit normal erythrocyte maturation, it appears that βR is not required for erythropoiesis. The authors postulated that βR in combination with the EpoR expressed by nonhematopoietic cells constitutes a tissue-protective (i.e. neuroprotection mediating) receptor. Further to this, the authors showed that membrane proteins prepared from rat brain were greatly enriched in EpoR after passage over either EPO or CEPO columns but covalently bound in a complex with βR.

They also showed that βR co-immunoprecipitated with EpoR[28]. This suggested that a dimer of EpoR-βR may be responsible for non-hematopoietic responses to both EPO and CEPO.

Few other studies have attempted to elucidate mechanisms responsible for the neuroprotective effects of CEPO. One study[29], using mouse neural progenitor cells derived from the subventricular zone suggested that there may be an association of CEPO with the morphogen, sonic hedgehog (SHH), which mediates effects via a pathway involving SHH/Patched/Smoothened[30]. The SHH signaling pathway is usually involved in developmental processes [31–33], but can also be associated with tumorigenesis[30]. Wang et al. [29] showed that SHH may mediate CEPO-induced neurogenesis via Mash1, a downstream target of the SHH signaling pathway[29]. However, this does not appear to be unique to CEPO, since a recent study showed that EPO-induced neuroprotection was also mediated by SHH[34]. Sturm and colleagues[35] recently used human erythroleukaemic K562 cells[36] to show that both EPO and CEPO can increase the expression of frataxin, a nuclear encoded protein that may be involved in iron homeostasis in mitochondria[37]. In addition, both molecules increased frataxin in THP-1 cells[38] that do not express the classical EPO-R. This same effect of EPO was previously described using primary fibroblast cell cultures [39].

It is clear that the elucidation of pathways involved in the response(s) to EPO and CEPO are still at their rudimentary stage. Other than the observation that CEPO does not increase hematocrit [26,27,40], and identification of EPO molecule characteristics involved in EPO neuroprotection [15], little is known about unique signaling or molecular pathways involved in neuroprotection.

2.3 EPO and CEPO- Preclinical Development

A substantial literature has documented some level of neuroprotection by EPO and CEPO in multiple models (see reviews and Meta-Analyses[14,41–45]). However, it has been suggested that the efficacy and possibly safety of EPO has been overestimated[41,42] due to poor preclinical study quality.

The potential for using CEPO to treat neurodegeneration resulting from ischemia was published by Leist and colleagues[26]. The first demonstration of neuroprotection by CEPO following "ischemia" used a rodent ischemia model induced by multiple permanent occlusions. The research team found that intravenous CEPO administered up to 4 hours after a stroke reduce cortical cell death. The same research team [46], also showed that CEPO could reduce cortical cell death if CEPO treatment was delayed by 6 hours and dosing was repeated at 24 and 48 hours. More important than any effect on measured of cell death, if CEPO is to be considered as a stroke treatment, it must improve behavior. The authors also provided some preliminary evidence for improved motor function up to 28 days following the stroke. Confirmation of the neuroprotective effects of CEPO was documented by Wang et al. [40], this time using an embolic stroke model. This group also showed that delayed CEPO treatment (6-hour post-embolization) protected cortical, but not striatal neurons from embolization-induced ischemia. Another confirmation of the benefit of CEPO was documented by Lapchak et al. [47] using a translational model of multiple infarct ischemia[48], which preferentially affects cortical structures, although other brain regions such as thalamus and cerebellum are affected. CEPO administered intravenously up to 3 hours after embolization reduced behavioral deficits (primarily motor function).

Thus, experiments conducted in 3 independent laboratories, using CEPO formulated by 2 independent companies (Johnson & Johnson and Lundbeck), reduced ischemia-induced cell death or improved behavior with a therapeutic window of 3–6 hours. None of the studies documented toxicities related to CEPO administation and none of the studies attempted to combine CEPO with tPA therapy for either measures of efficacy or safety.

How does a therapeutic window of 6 hours or 3 hours in a rodent and rabbit, respectively, correlate with a therapeutic window in AIS patients. To date, there is little correlation between the therapeutic window observed for any treatment in rodents and that expected to be achieved in humans[48]. However, using correlative analysis techniques, it has recently been hypothesized[48], that a 3hour therapeutic window in the rabbit stroke model may represent a 7.3–9 hour therapeutic window in AIS patients. However, the caveat that similar PK and ADME properties of the molecule should exist in both species must be added.

3. Preclinical and Clinical Toxicity

Since there is a long history of EPO use in a clinical setting, a substantial amount of literature has been dedicated to the adverse effects of the protein. These have been reviewed previously [21,22,49,50]. An additional toxicity related to EPO administation was uncovered in the EPO stroke clinical trial conducted by Ehrenreich[51]. This will be discussed in detail in Section 5.

There is some valuable risk and benefit information in the scientific literature regarding the use of EPO to increase hemoglobin levels in chronic kidney disease patients [52,53] and in heart disease patients (congestive heart failure or ischemia) where EPO was used to maintain normal hematocrit levels (CREATE and CHOIR trials). In the trial by Besarab[54], there was no significant difference in either cerebrovascular accidents or hemorrhage-related deaths in patients on EPO-low hematocrit maintenance doses. The authors show that the mortality rate in the low-hematocrit group was higher, but not significantly higher, than the normal hematocrit group. The CREATE investigators enrolled kidney dialysis patients to be treated with EPO to attempt to normalize hemoglobin levels[53]. This trial showed no difference in adverse events when EPO was administered and the investigators concluded that normalization of anemia does not reduce the risk of cardiovascular events. The CHOIR trial by Singh et al. [52] also studied correction of anemia with EPO in kidney disease patients. Importantly, in this trial, the authors showed that there was no difference in stroke incidence between either a high hemoglobin (target 13–13.5 g/dl) or low-hemoglobin (10.5–11 g/dl) group. Thus, the administration of EPO did not promote acute ischemic strokes. However, the authors note that the hazard ratio for the high-hemoglobin group versus the low-hemoglobin group in 1.37, with a P value of 0.02.

In contrast to the study cited above, the TREAT study [55] showed that darbepoetin alfa administered to a mixed population of patients (diabetic, chronic kidney disease, and anemia) who were not undergoing dialysis, had a 5.02% incidence of stroke (101/2012), whereas the placebo group had an incidence of 2.61% (53/2026). The hazard ratio, 1.92; 95% CI, 1.38 to 2.68; $P<0.001$ for this results was significantly different. Thus, in certain patient populations, there are adverse cardiovascular events related to EPO administration when the goal is to increase or maintain hematocrit.

4. Prospects for the Use of EPO Molecules to Treat Stroke & Conclusion

In section 3.3, evidence was presented to support the possible use of EPO and CEPO to treat AIS. However, in light of the recent clinical trial results documented by Ehrenreich and colleagues [51], a translational dilemna has been created requiring researchers and clinicians to determine if the benefit of EPO therapy will outweigh the substantial “side-effects” of the drug.

Table 1 documents the criteria used by Ehrenreich and colleagues[51,56] to conduct the EPO clinical trials in AIS patients and compares the criteria to those being used to develop CEPO to treat AIS. There are only minor differences between the 2 EPO trials[51,56]. In the original trial that showed both efficacy and safety[56], a lower dose of EPO was used and an extended time to treat window was allowed. In the second trial[51], which was designed to reproduce

the original "positive" trial[56], the investigators used a more limited time to treat window and expanded the patient population to include analysis of patients receiving tPA, which is the current standard of care [51]. The novel 4-arm study design also tested the effects of combination therapy, neuroprotectant plus tPA, much like the SAINT 2 trial [57]. With negative results similar to the SAINT 2 trial, EPO was not beneficial measured on either the Scandinavian Stroke Scale (SSS) or National Institutes of Health Stroke Scale (NIHSS), when given with a mean time to treatment of 263–271 minutes (Table 2B). However, the trial was marred by major protocol violations, SAE's and the inability to reproduce the beneficial results of tPA within a 3–4.5 hours therapeutic window that has been demonstrated in many other clinical trials[58–60].

Most surprising is documentation of significantly increased mortality in the EPO-treated population compared to the placebo group (Table 2B). Prior to the publication of the Ehrenreich clinical study[51], there was no information in the literature to indicate that EPO would be lethal in stroke patients, even though there was information available concerning the general "toxicity" of EPO [21–25]. However, coincident with the clinical trial publication, Hermann and colleagues [61] reported that EPO interacted negatively with tPA to enhance membrane metalloproteinase-9 (MMP-9) activity and EPO also increased MMP-9 activity. Moreover, while EPO appeared to decrease edema, the combination of EPO and tPA significantly increased edema. Thus, this study may partially explain the negative nature of the clinical trial. A duplicate study showed similar result[62]. In the clinical study[51], the investigators tabulated the cause of death for all treatment groups. Although the patient numbers are small, in the EPO/tPA combination group, the investigators noted brain edema that was not present in any of the other 3 groups[51]. Moreover, symptomatic ICH was increased in the presence of EPO/tPA. Clearly, based upon lack of EPO efficacy, and EPO-induced toxicity whether in the absence or presence of tPA, future clinical studies to test EPO molecules should be postponed.

The second generation EPO molecule, CEPO is currently being studied for safety and efficacy (Table 1[63]). According to the clinical trial website[63], last accessed on August 18, 2010, the inclusion criteria indicate that patients will be enrolled ≤ 48 hours of a stroke. Based upon the conclusion documented in the meta-analysis of Jerndal et al[42] and Minnerup et al[41], which included numerous CEPO studies ([26,47,64], it would appear that CEPO would be most effective if administered up to 6 hours after a stroke. Thus, if CEPO is to be developed, enrollment ≤ 48 should be revised to reflect a reasonable time to treatment based upon translational studies. However, before CEPO is to be developed, safety studies in at least 2 embolic stroke models in 2 species are warranted to ensure the patient consenting to the treatment that adequate translational studies have been conducted to assure a reasonable safety margin. To date, this recommendation for logical advancement of EPO analogs to clinical trials has not been fulfilled.

5. Expert Opinion

Thus, in the aftermath of a failed randomized, placebo-controlled, double-blind trial, which included more than 500 patients, the stroke field is no closer to achieving the critical goal of developing and approving a safe and effective neuroprotective agent to treat stroke patients. The cumulative preclinical data from numerous translational studies using embolic and non-embolic stroke models suggests that EPO molecules do confer neuroprotection, reduce to some extent the volume of brain dying after an ischemic event (i.e. infarct volume), and improve a wide range of behaviors scored using diverse assessment criteria.

There is a need to straighten out the order of translational and clinical research so that we can limit casualties, both in terms of patient mortality and failed trials. It is unfortunate that a

negative interaction between EPO and tPA in patients sparked a rodent study, when that particular simple study should have been conducted to support the clinical trial or advise investigators of possible negative interactions. However, it was conducted more than 7 years after the initiation of the EPO trial[51].

In conclusion, the most recent EPO clinical data is not aligned with the preclinical efficacy data, and suggests that few AIS patients, if any, will benefit from the administration of EPO. Because of the lack of benefit in the expanded Ehrenreich trial[51], and a significantly higher mortality rate when EPO is combined with tPA, extreme caution should be used if additional EPO molecule clinical studies are to be conducted. Investigators conducting the clinical trials should arm themselves with the best preclinical translational research data possible to proceed with a well designed and conceived clinical trial so that all is not lost with another clinical failure.

Article Highlights

- Numerous EPO-type molecules have pleiotropic effects in the CNS
- EPO has been shown to be neuroprotective in preclinical animal models of stroke
- CEPO has also been shown to be neuroprotective in acute ischemic stroke models
- Receptor mediating CEPO and EPO neuroprotection require elucidation
- EPO administered to increase hematocrit significantly increases stroke incidence
- EPO administered to stroke patients in combination with tPA increases mortality
- EPO increases symptomatic hemorrhage and edema in stroke patients
- Development of EPO and analogs should be halted until adequate translational studies have been completed

Acknowledgments

Acknowledgments and Funding: This work was supported by a U01 Translational research grant NS060685 to PAL.

References

1. Egrie J. The cloning and production of recombinant human erythropoietin. Pharmacotherapy 1990 Mar-Apr;10(2 Pt 2):3S–8S. [PubMed: 2345709]
2. Browne JK, Cohen AM, Egrie JC, Lai PH, Lin FK, Strickland T, et al. Erythropoietin: gene cloning, protein structure, and biological properties. Cold Spring Harb Symp Quant Biol 1986;51(Pt 1):693–702. [PubMed: 3472747]
3. Lin FK, Suggs S, Lin CH, Browne JK, Smalling R, Egrie JC, et al. Cloning and expression of the human erythropoietin gene. Proc Natl Acad Sci U S A 1985 Nov;82(22):7580–4. [PubMed: 3865178]
4. Han F, Wu S, Xue S, Huang L, Ma X. Cloning of the human erythropoietin exons and their expression in COS-7 cells. Chin J Biotechnol 1996;12(4):227–33. [PubMed: 9187494]
5. Maouche L, Tournamille C, Hattab C, Boffa G, Cartron JP, Chretien S. Cloning of the gene encoding the human erythropoietin receptor. Blood 1991 Nov 15;78(10):2557–63. [PubMed: 1668607]
6. Mun KC, Golper TA. Impaired biological activity of erythropoietin by cyanate carbamylation. Blood Purif 2000;18(1):13–7. [PubMed: 10686438]
7. Park KD, Mun KC, Chang EJ, Park SB, Kim HC. Inhibition of erythropoietin activity by cyanate. Scand J Urol Nephrol 2004;38(1):69–72. [PubMed: 15204430]

8. Kitamura T, Tange T, Terasawa T, Chiba S, Kuwaki T, Miyagawa K, et al. Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin. J Cell Physiol 1989 Aug;140(2):323–34. [PubMed: 2663885]
9. Kitamura T, Tojo A, Kuwaki T, Chiba S, Miyazono K, Urabe A, et al. Identification and analysis of human erythropoietin receptors on a factor-dependent cell line, TF-1. Blood 1989 Feb;73(2):375–80. [PubMed: 2537111]
10. Migliaccio AR, Jiang Y, Migliaccio G, Nicolis S, Crotta S, Ronchi A, et al. Transcriptional and posttranscriptional regulation of the expression of the erythropoietin receptor gene in human erythropoietin-responsive cell lines. Blood 1993 Dec 15;82(12):3760–9. [PubMed: 8260713]
11. Miura Y, Komatsu N, Suda T. Growth and differentiation of two human megakaryoblastic cell lines; CMK and UT-7. Prog Clin Biol Res 1990;356:259–70. [PubMed: 2217443]
12. Wojchowski DM, He TC. Signal transduction in the erythropoietin receptor system. Stem Cells 1993 Sep;11(5):381–92. [PubMed: 8241949]
13. Richmond TD, Chohan M, Barber DL. Turning cells red: signal transduction mediated by erythropoietin. Trends Cell Biol 2005 Mar;15(3):146–55. [PubMed: 15752978]
14. Sharples EJ, Thiemermann C, Yaqoob MM. Novel applications of recombinant erythropoietin. Curr Opin Pharmacol 2006 Apr;6(2):184–9. [PubMed: 16483842]
15***. Brines M, Patel NS, Villa P, Brines C, Mennini T, De Paola M, et al. Nonerythropoietic, tissue-protective peptides derived from the tertiary structure of erythropoietin. Proc Natl Acad Sci U S A 2008 Aug 5;105(31):10925–30. Third generation EPO analogs to be developed. [PubMed: 18676614]
16. Lee SM, Nguyen TH, Park MH, Kim KS, Cho KJ, Moon DC, et al. EPO receptor-mediated ERK kinase and NF-kappaB activation in erythropoietin-promoted differentiation of astrocytes. Biochem Biophys Res Commun 2004 Aug 6;320(4):1087–95. [PubMed: 15249201]
17. Erslev AJ, Schuster SJ, Caro J. Erythropoietin and its clinical promise. Eur J Haematol 1989 Nov;43 (5):367–73. [PubMed: 2693128]
18. Graber SE, Krantz SB. Erythropoietin: biology and clinical use. Hematol Oncol Clin North Am 1989 Sep;3(3):369–400. [PubMed: 2561121]
19. Liberato NL, Costa A, Barosi G. Erythropoietin: biological aspects and clinical usefulness. Haematologica 1990 Jul-Aug;75(4):346–62. [PubMed: 2276680]
20. Varet B, Casadevall N, Lacombe C, Nayeaux P. Erythropoietin: physiology and clinical experience. Semin Hematol 1990 Jul;27(3 Suppl 3):25–31. [PubMed: 2198661]
21. Koide K, Arai T. [Erythropoietin--complications, side effects]. Nippon Rinsho 1991 Dec;49(Suppl): 679–87. [PubMed: 1808335]
22. Bennett WM. Side effects of erythropoietin therapy. Am J Kidney Dis 1991 Oct;18(4 Suppl 1):84–6. [PubMed: 1928085]
23. Samtleben W, Ehmer B, Lutz-Knochenhauer I, Hagmann C, Scigalla P, Gurland HJ. Side effects during recombinant human erythropoietin therapy in 2,000 ESRD patients. Contrib Nephrol 1991;88:107–16. discussion 17. [PubMed: 2040172]
24. Gretz N, Lasserre J, Kraft K, Waldherr R, Weidler B, Meisinger E, et al. Efficacy and side effects of erythropoietin used in the treatment of anemia of uremic rats. Contrib Nephrol 1988;60:236–44. [PubMed: 3345672]
25. Gretz N, Lasserre JJ, Meisinger E, Strauch M, Waldherr R, Kraft K, et al. Potential side-effects of erythropoietin. Lancet 1987 Jan 3;1(8523):46. [PubMed: 2879127]
26*. Leist M, Ghezzi P, Grasso G, Bianchi R, Villa P, Fratelli M, et al. Derivatives of erythropoietin that are tissue protective but not erythropoietic. Science 2004 Jul 9;305(5681):239–42. Second generation EPO analog to be developed for AIS. [PubMed: 15247477]
27. Kirkeby A, Torup L, Bochsen L, Kjalke M, Abel K, Theilgaard-Monch K, et al. High-dose erythropoietin alters platelet reactivity and bleeding time in rodents in contrast to the neuroprotective variant carbamyl-erythropoietin (CEPO). Thromb Haemost 2008 Apr;99(4):720–8. [PubMed: 18392330]
28. Brines M, Grasso G, Fiordaliso F, Sfacteria A, Ghezzi P, Fratelli M, et al. Erythropoietin mediates tissue protection through an erythropoietin and common beta-subunit heteroreceptor. Proc Natl Acad Sci U S A 2004 Oct 12;101(41):14907–12. [PubMed: 15456912]

29. Wang L, Zhang ZG, Gregg SR, Zhang RL, Jiao Z, LeTourneau Y, et al. The Sonic hedgehog pathway mediates carbamylated erythropoietin-enhanced proliferation and differentiation of adult neural progenitor cells. J Biol Chem 2007 Nov 2;282(44):32462–70. [PubMed: 17804404]
30. Hahn H, Wojnowski L, Miller G, Zimmer A. The patched signaling pathway in tumorigenesis and development: lessons from animal models. J Mol Med 1999 Jun;77(6):459–68. [PubMed: 10475061]
31. Park HL, Bai C, Platt KA, Matise MP, Beeghly A, Hui CC, et al. Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. Development 2000 Apr;127(8): 1593–605. [PubMed: 10725236]
32. Scotting PJ, Rex M. Transcription factors in early development of the central nervous system. Neuropathol Appl Neurobiol 1996 Dec;22(6):469–81. [PubMed: 9004232]
33. Ming JE, Roessler E, Muenke M. Human developmental disorders and the Sonic hedgehog pathway. Mol Med Today 1998 Aug;4(8):343–9. [PubMed: 9755453]
34. Ueda K, Takano H, Niitsuma Y, Hasegawa H, Uchiyama R, Oka T, et al. Sonic hedgehog is a critical mediator of erythropoietin-induced cardiac protection in mice. J Clin Invest 2010 Jun 1;120(6):2016–29. [PubMed: 20484812]
35. Sturm B, Helminger M, Steinkellner H, Heidari MM, Goldenberg H, Scheiber-Mojdehkar B. Carbamylated erythropoietin increases frataxin independent from the erythropoietin receptor. Eur J Clin Invest 2010 Jun;40(6):561–5. [PubMed: 20456483]
36. Koeffler HP, Golde DW. Human myeloid leukemia cell lines: a review. Blood 1980 Sep;56(3):344–50. [PubMed: 6996765]
37. Schapira AH. Primary and secondary defects of the mitochondrial respiratory chain. J Inherit Metab Dis 2002 May;25(3):207–14. [PubMed: 12137229]
38. Auwerx J. The human leukemia cell line, THP-1: a multifacetted model for the study of monocyte-macrophage differentiation. Experientia 1991 Jan 15;47(1):22–31. [PubMed: 1999239]
39. Acquaviva F, Castaldo I, Filla A, Giacchetti M, Marmolino D, Monticelli A, et al. Recombinant human erythropoietin increases frataxin protein expression without increasing mRNA expression. Cerebellum 2008;7(3):360–5. [PubMed: 18581197]
40. Wang Y, Zhang ZG, Rhodes K, Renzi M, Zhang RL, Kapke A, et al. Post-ischemic treatment with erythropoietin or carbamylated erythropoietin reduces infarction and improves neurological outcome in a rat model of focal cerebral ischemia. Br J Pharmacol 2007 Aug;151(8):1377–84. [PubMed: 17603558]
41. Minnerup J, Heidrich J, Rogalewski A, Schabitz WR, Wellmann J. The efficacy of erythropoietin and its analogues in animal stroke models: a meta-analysis. Stroke 2009 Sep;40(9):3113–20. [PubMed: 19542052]
42. Jerndal M, Forsberg K, Sena ES, Macleod MR, O'Collins VE, Linden T, et al. A systematic review and meta-analysis of erythropoietin in experimental stroke. J Cereb Blood Flow Metab 2010 May; 30(5):961–8. [PubMed: 20040929]
43. Ehrenreich H, Aust C, Krampe H, Jahn H, Jacob S, Herrmann M, et al. Erythropoietin: novel approaches to neuroprotection in human brain disease. Metab Brain Dis 2004 Dec;19(3–4):195–206. [PubMed: 15554415]
44. Genc S, Koroglu TF, Genc K. Erythropoietin as a novel neuroprotectant. Restorative neurology and neuroscience 2004;22(2):105–19. [PubMed: 15272145]
45. Lapchak PA. Carbamylated erythropoietin to treat neuronal injury: new development strategies. Expert Opin Investig Drugs 2008 Aug;17(8):1175–86.
46. Villa P, van Beek J, Larsen AK, Gerwien J, Christensen S, Cerami A, et al. Reduced functional deficits, neuroinflammation, and secondary tissue damage after treatment of stroke by nonerythropoietic erythropoietin derivatives. J Cereb Blood Flow Metab. 2006 Jul 12;
47. Lapchak PA, Kirkeby A, Zivin JA, Sager TN. Therapeutic window for nonerythropoietic carbamylated-erythropoietin to improve motor function following multiple infarct ischemic strokes in New Zealand white rabbits. Brain Res 2008 Oct 31;1238:208–14. [PubMed: 18761001]
48**. Lapchak, PA. Translational stroke research using a rabbit embolic stroke model: a correlative analysis hypothesis for novel therapy development; Translational Stroke Research 2010. p. 96-107.http://dx.doi.org/10.1007/s12975-010-0018-4(1Gold standard translational embolic stroke model for drug and device developement

49. Singbartl G. Adverse events of erythropoietin in long-term and in acute/short-term treatment. Clin Investig 1994;72(6 Suppl):S36–43.

50. Ryffel B. Unanticipated human toxicology of recombinant proteins. Arch Toxicol Suppl 1996;18:333–41. [PubMed: 8678809]

51**. Ehrenreich H, Weissenborn K, Prange H, Schneider D, Weimar C, Wartenberg K, et al. Recombinant human erythropoietin in the treatment of acute ischemic stroke. Stroke 2009 Dec;40 (12):e647–56. Pivotal clinical trial showing EPO toxicity in combination with tPA. [PubMed: 19834012]

52. Singh AK, Szczech L, Tang KL, Barnhart H, Sapp S, Wolfson M, et al. Correction of anemia with epoetin alfa in chronic kidney disease. N Engl J Med 2006 Nov 16;355(20):2085–98. [PubMed: 17108343]

53. Drueke TB, Locatelli F, Clyne N, Eckardt KU, Macdougall IC, Tsakiris D, et al. Normalization of hemoglobin level in patients with chronic kidney disease and anemia. N Engl J Med 2006 Nov 16;355 (20):2071–84. [PubMed: 17108342]

54. Besarab A, Bolton WK, Browne JK, Egrie JC, Nissenson AR, Okamoto DM, et al. The effects of normal as compared with low hematocrit values in patients with cardiac disease who are receiving hemodialysis and epoetin. N Engl J Med 1998 Aug 27;339(9):584–90. [PubMed: 9718377]

55**. Pfeffer MA, Burdmann EA, Chen CY, Cooper ME, de Zeeuw D, Eckardt KU, et al. A trial of darbepoetin alfa in type 2 diabetes and chronic kidney disease. N Engl J Med 2009 Nov 19;361(21): 2019–32. Toxicity related to darbepoetin administration. Doubling the incidence of stroke. [PubMed: 19880844]

56. Ehrenreich H, Hasselblatt M, Dembowski C, Cepek L, Lewczuk P, Stiefel M, et al. Erythropoietin therapy for acute stroke is both safe and beneficial. Mol Med 2002 Aug;8(8):495–505. [PubMed: 12435860]

57*. Shuaib A, Lees KR, Lyden P, Grotta J, Davalos A, Davis SM, et al. NXY-059 for the treatment of acute ischemic stroke. N Engl J Med 2007 Aug 9;357(6):562–71. Important clinical trials design study using 4 treatment groups in parallel. [PubMed: 17687131]

58. Hacke W, Kaste M, Bluhmki E, Brozman M, Davalos A, Guidetti D, et al. Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke. N Engl J Med 2008 Sep 25;359(13):1317–29. [PubMed: 18815396]

59. Del Zoppo GJ, Saver JL, Jauch EC, Adams HP Jr. Expansion of the Time Window for Treatment of Acute Ischemic Stroke With Intravenous Tissue Plasminogen Activator. A Science Advisory From the American Heart Association/American Stroke Association. Stroke. 2009 May 28;

60**. NINDS. Tissue plasminogen activator for acute ischemic stroke. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group. N Engl J Med 1995;333(24):1581–7. Important clinical trial for the only FDA-approved acute ischemic stroke treatment. [PubMed: 7477192]

61. Zechariah A, Elali A, Hermann DM. Combination of tissue-plasminogen activator with erythropoietin induces blood-brain barrier permeability, extracellular matrix disaggregation, and DNA fragmentation after focal cerebral ischemia in mice. Stroke 2010 May;41(5):1008–12. [PubMed: 20360548]

62. Jia L, Chopp M, Zhang L, Lu M, Zhang Z. Erythropoietin in Combination of Tissue Plasminogen Activator Exacerbates Brain Hemorrhage When Treatment Is Initiated 6 Hours After Stroke. Stroke. 2010 Jul 29;

63. CEPO. Clinical Trial Design. 2010. http://www.clinicaltrials.gov/ct2/show/NCT00870844

64. Villa P, van Beek J, Larsen AK, Gerwien J, Christensen S, Cerami A, et al. Reduced functional deficits, neuroinflammation, and secondary tissue damage after treatment of stroke by nonerythropoietic erythropoietin derivatives. J Cereb Blood Flow Metab 2007 Mar;27(3):552–63. [PubMed: 16835629]

Table 1

EPO Trial Design

Criteria	EPO Trial #1[56]	EPO Trial #2[51]	CEPO Trial Design[63]
Stroke Subtype	Ischemic stroke in MCA territory	Ischemic stroke in MCA territory	AIS- undefined regions or subtypes
Study Period	12/1998–6/2002	1/2003–3/2008	2/2009–10/1010
Patient # (intent to treat)	53 patients rhEPO safety (13) rhEPO (21) Placebo (19)	522 patients rhEPO (256) Placebo (266)	Unknown
Age range	< 80 years of age	≥ 18 years of age	50–90 years of age
Time to Treat	≤ 8 hours	≤ 6 hours	≤48 hours
Stroke Severity	NI	NIHSS ≥ 5	“Measurable” deficit
Drug Dose	3.3×10^4 IU/50 ml/30 min q.d. 3 days	4.0×10^4 IU/50 ml/30 min q.d. 3 days	0.6 μg/kg-50μg/kg
Major Exclusions	~ Any form of ICH ~ rhEPO allergy	~ Any form of ICH ~ rhEPO allergy ~ antibodies against EPO ~ coma (NIHSS-1a≥2)	~ Primary ICH, SAH, aneurysm ~previous EPO treatment ~Score ≥ 1 (NIHSS- 1a)Pre-stroke mRS ≥ 2
Primary Endpoints	NIHSS SSS BI mRS MRI- lesion size	NIHSS SSS BI mRS MRI- lesion size	NIHSS mRS
Secondary Measures	Serum EPO Hematocrit Hemoglobin Leukocyte counts Thrombocyte counts PTT CRP	Routine laboratory Vital signs SAE	PK Antibody formation

NIHSS- National Institutes of Health Stroke Scale; SSS- Scandinavian Stroke Scale; BI- Barthel Index; mRS- modified Rankin Scale; MRI- magnetic resonance imaging; S100β-S100 calcium binding protein β; PTT-partial prothrombin time; CRP- C-reactive protein; SAE- serious adverse events; PK-pharmacokinetics; NI- Not indicated.

Table 2

EPO Trial Results

A) EPO Trial #1[56]		
	Treatment Groups- Double-Blind	
Criteria	**EPO**	**Placebo**
Age range	39–80 (mean 68)	49–79 (mean 63)
Sex	15 males 6 female	13 male 6 female
Time to Treatment (minutes)	160–475 (mean 300)	200–465 (mean 285)
Stroke Subtype (%)		
• **cardioembolic**	42.9	52.6
• **small vessel**	14.3	14.3
• **large vessel**	19.0	23.8
• **other**	23.8	22.2
Stroke Severity		
• **SSS**	8–52 (mean 30)	6–54 (mean 30)
• **NIHSS**	3–26 (mean 11)	1–28 (mean 13)
Stroke Outcome		
• **SSS**	Improvement at day 7, 18 and 30 ($p<0.03$-$p<0.001$) **9 point increase**	Mean 30 increased to 35 on day 30
• **NIHSS**	Improvement by day 18 and 30 ($p<0.03$-$p<0.09$) **4 point decrease**	Mean 13 was stable with variability for duration
• **mRS (Rank)**		
5–6	**14%** *	37%
3–4	**33%** *	21%
2	5%*	16%
1	33%	5%
0	14%	21%
• **BI (Rank)**#	**14%**	42%
0–20	14%	5%
21–30	10%	5%
41–60	5%	5%
61–80	57%	42%
81–100		
Mortality Rate ND	ND	

B) EPO Trial #2[51]

Criteria	Treatment Groups- Double-Blind			
	EPO	**EPO/tPA**	**Placebo**	**tPA**
Age range	38–95 (mean 71.9)	20–100 (mean 66.8)	42–92 (mean 71.5)	19–95 (mean 61.2)
Sex	49 males 41 female	92 males 74 females	48 male 53 female	93 male 72 female
Time to Treatment (minutes)	42–442 (mean 271)	45–410 (mean 263)	78–485 (mean 281)	110–480 (mean 267)
Stroke Subtype (%)				
• **cardioembolic**	50	51.2	47.5	44.2
• **small vessel**	6.7	3.0	5.9	1.8
• **large vessel**	21.1	23.5	19.8	27.3
• **other**	22.2	22.3	22.2	26.7
Stroke Severity				
• **NIHSS**	4–32 (mean 13)	4–31 (mean 13.3)	14–27 (mean 11.7)	4–30 (mean 13.5)
Stroke Outcome				
• **NIHSS**	No improvement at either 30 or 90 (p>0.05)day		No improvement at either day 30 or 90 (p>0.05)	
Day 1	13.0 ± 6.4	13.3 ± 5.5	11.7 ± 5.5	13.5 ± 5.8
Day 30	10.7 ± 12	11.6 ± 12	10.3 ± 10	8.8 ± 11
Day 90	10.2 ± 12	9.5 ± 12	9.1 ± 9	7.7 ±10
• **mRS**	No improvement at either day 30 or 90 (p>0.05)		No improvement at either day 30 or 90 (p>0.05)	
Day 30	3.6 ± 1.9	3.4 + 2.0	3.6 ± 1.7	3.2 ± 2.0
Day 90	3.5 ± 3.3	3.1 ± 2.0	3.3 ± 1.8	2.9 ± 2.0
• **BI**	No change at either day 30 or 90 (p>0.05)		No change at either day 30 or 90 (p>0.05)	
Day 30	47.0 ± 43	50.8 ± 48	45.6 ± 40	54.9 ± 43
Day 90	50.9 ± 42	59.5 ± 41	52.2 ± 41	63.5 ± 41
Mortality Rate	16.4%*	16.3%*	9.0%	8.5%

* p<0.07 on day 30;

# p<0.05 on day 30;

NA- not described.

* p<0.01