Cytoprotective Properties of Plant-produced Asialoerythropoietin (asialo-rhuEPO^{P})

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Jay (Jiahua) XIE (PhD)

Associate Professor of Pharmaceutical Science
Principal Investigator of Biomanufacturing Research Institute & Technology Enterprise (BRITE)
North Carolina Central University
Durham, NC 27707, USA
Email: jxie@nccu.edu

BRITE
Unleashing the Power of Biotech at NCCU

Biomanufacturing Research Institute & Technology Enterprise (BRITE) building and initial funding

- $20.1 million funded by Golden Leaf Foundation in 2004
- Program started in 2006
- Building was completed in June 2008 with 52,000 sf facility on campus of NCCU
- $6.5 M lab equipment
- 10 tenured and tenure track faculty members with additional 30 research faculty and staff;
- ~210 students (BS, MS and PhD)

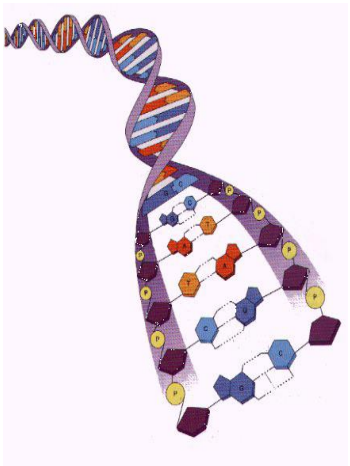
One of my responsibilities in BRITE is to use plant expression system to produce pharmaceutical proteins

Biopharmaceutical market:

- ~$106 billion in 2009
- ~$220 billion in 2016

Principle of using plants as a bioreactor:

Human genes

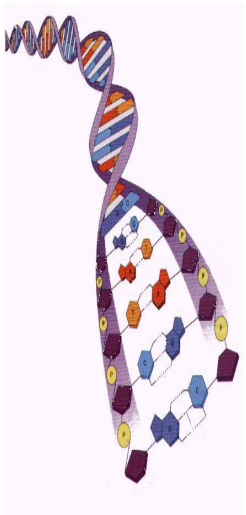


Bioreactor – transgenic plants

Pharmaceutical proteins

Various expression systems for biopharmaceuticals

Clone target gene



Animals

E. coli cells

Yeast cells

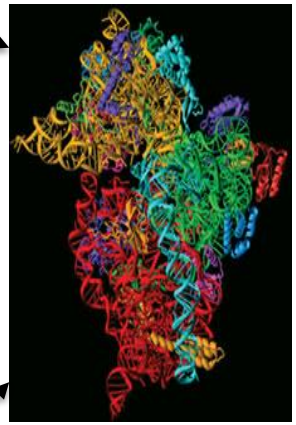
Insect cells

Mammalian cells

Plant cells

Plants

Biopharmaceuticals



- Can make complex glycan chains
- Same genetic codon as mammals
- Safe (free of human pathogens)
- Inexpensive (production cost is 50x lower than mammalian cells)
- Easy to scale-up

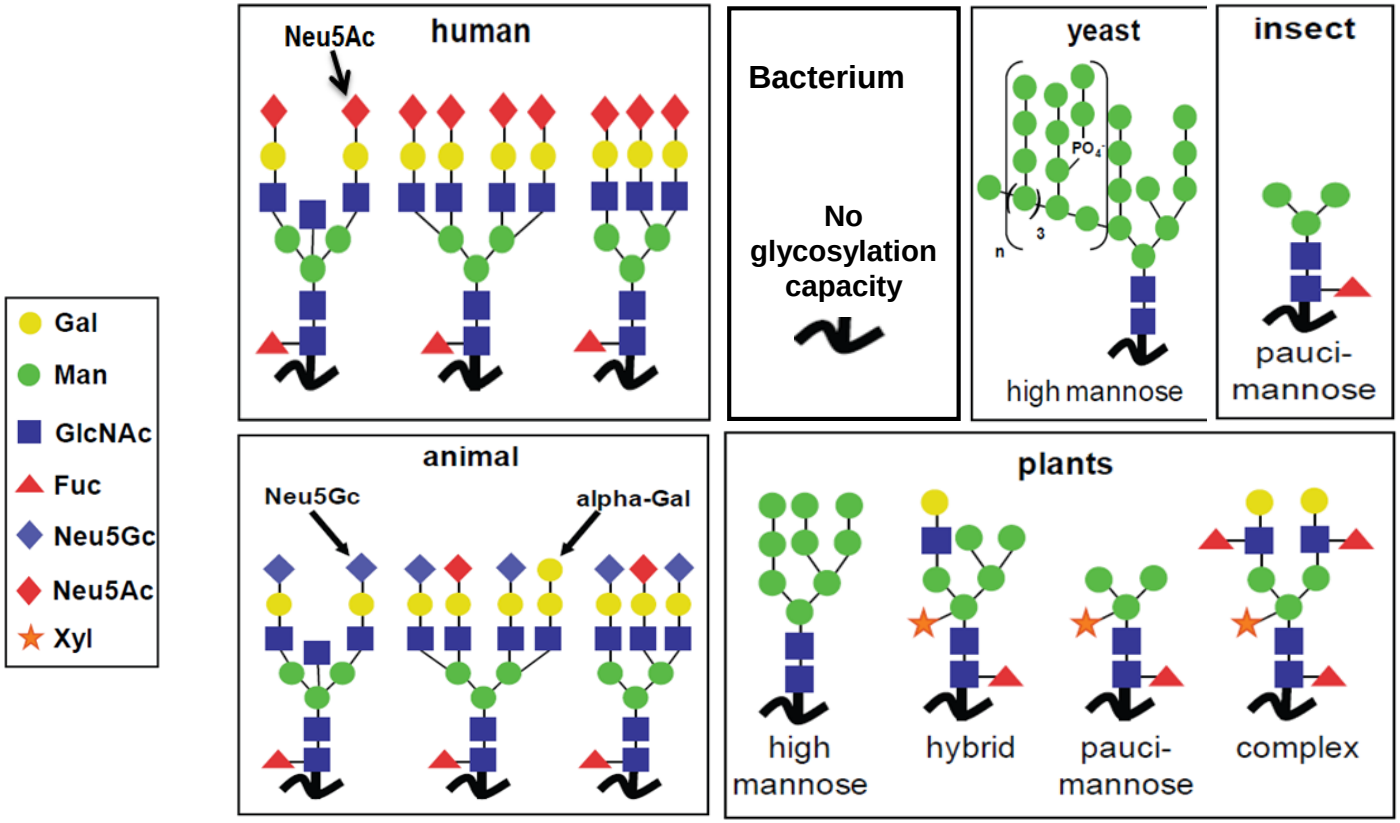
- Different glycosylation
- Very strict the USDA regulations
- Complex downstream bioprocess

BRITE
Unleashing the Power of Biotech at NCCU

What can we express in plants with some advantages?

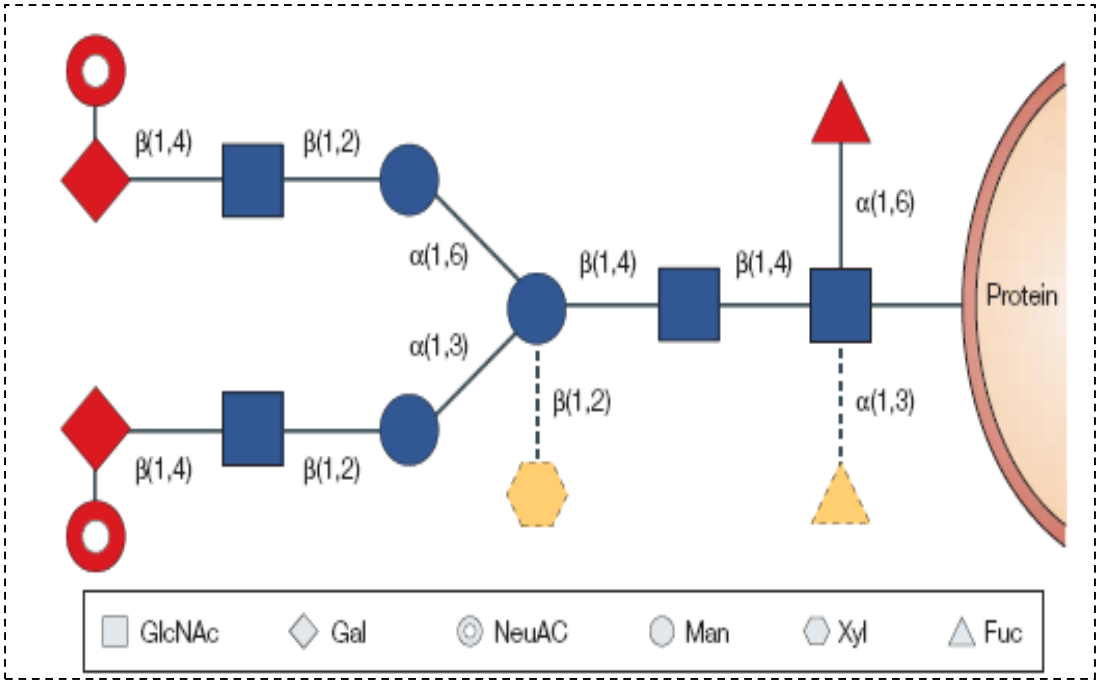
Answer - Maybe glycoproteins. Nearly 50% therapeutic and diagnostic proteins are glycoproteins and functional recombinant human glycoproteins are only produced in mammalian cells.

***N*-Glycan structures in different expression systems. Neu5Ac: *N*-acetylneuraminic acids. Neu5Gc: *N*-glycolylneuraminic acids**



Modified From Ghaderi et al., *Biotechnol Genet* Eng *Rev.* 28:147-176, 2012)

Some differences in glycan chains between plants and mammals



Blue residues: common between plants and mammals.

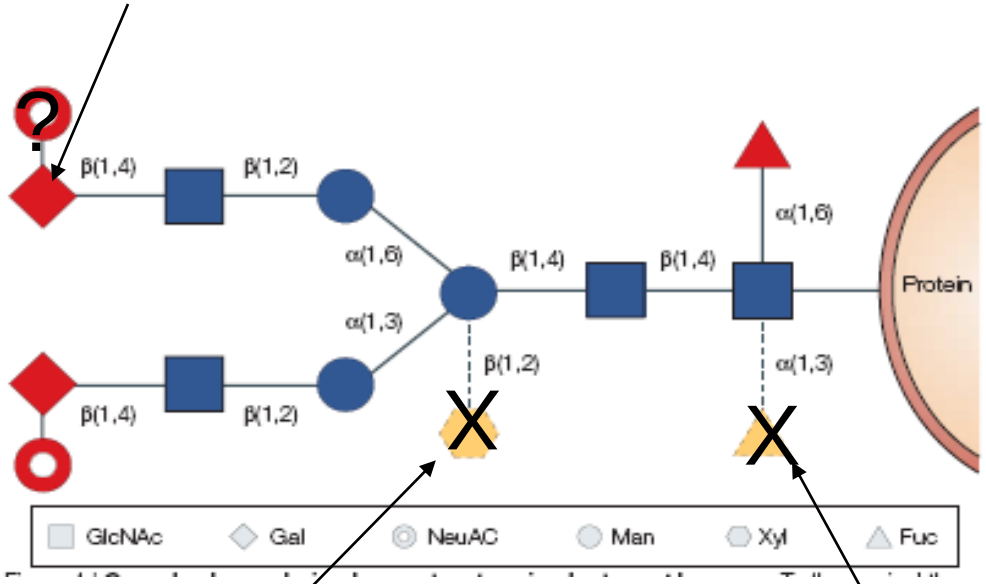
Yellow residues: unique in plants but not mammals.

Red residues: unique in mammals.

Fuc: fucose; Gal: galactose; GlcNAc: N-acetylglucosamine; Man: mannose; NeuAC: acetylneuraminic acid (sialic acid); Xyl: xylose. From Ma et al., Nat Rev Genet, 2003, 4:794-805

To “humanize” plants for glycoproteins: β(1,2) xylose and α(1,3) fucose residues must be removed whereas galactose and sialic acid residues need to be added.

Overexpression of human β1,4-galactosyltransferase (*GalT*) gene in plants could (Bakker et al., 2001):
1) produce β1,4-galactose sugar
2) suppress the addition of β1,2-xylose and α-1,3 fucose sugars



β1,2-xylosyltransferase **α1,3-fucosyltransferase**

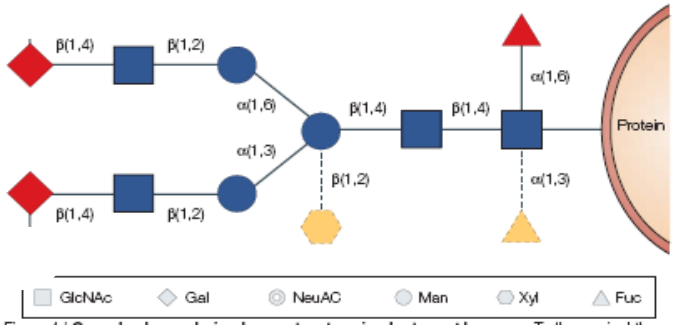
Blue residues: common to plants and humans; red residues: only in humans; yellow residues: in plants but not humans.

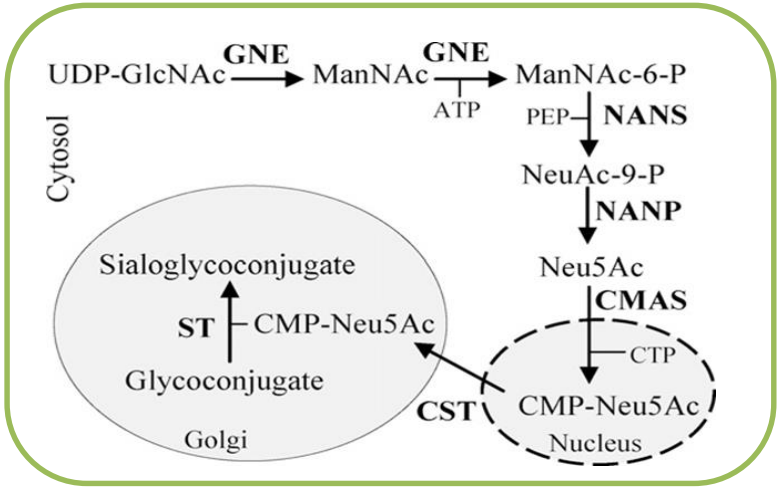
Glycoengineering of sialylation pathway is very challenged

To add sialic acids:

- **Plants lack precursor, transporter and sialyltransferase for adding sialic acid.**
- **Six more genes are needed.**

Glycoengineering plants to produce asialo-glycoproteins is relatively easy!?





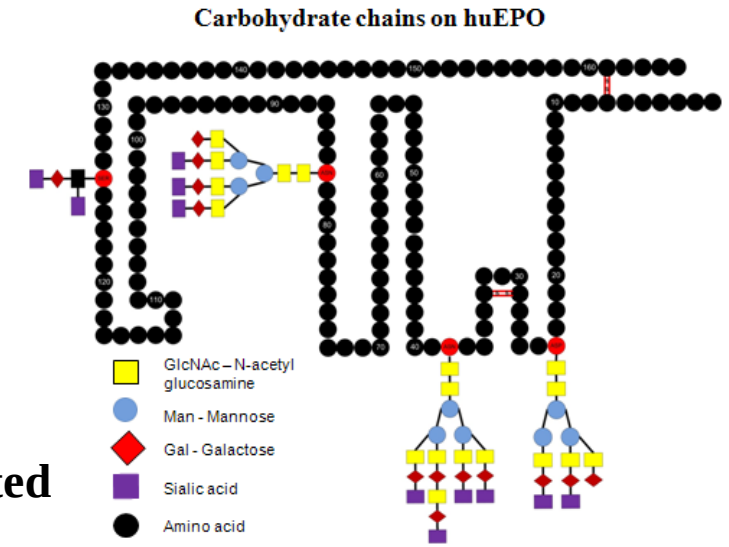
Schematic representation of the mammalian sialylation pathway from glycoconjugates (Castilho et al. 2010, J Biol Chem, 285: 15923-15930).

GNE: UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase; NANP: Neu5Ac-9-phosphate phosphatase; NANS: *N*-acetylneuraminic acid phosphate synthase gene (*NANS*); CMAS: CMP-*N*-acetylneuraminic acid synthetase; ST: α-2,6-sialyltransferase; and CST, CMP-Neu5Ac transporter.

For most glycoproteins: proper glycosylation = full biological activity
Which asialo-glycoprotein? We selected asialo-rhuEPO.

Recombinant human erythropoietin (rhuEPO)

❖ **EPO: a glyco-hormone, producing primarily in kidneys, consisting of 165 amino acids, three *N*- and one *O*-glycan chains.**



EPO has two functions:

1. <u>Erythropoietic activity</u>: Treat anemia associated from different diseases (e.g. AIDS, renal failure, etc.).

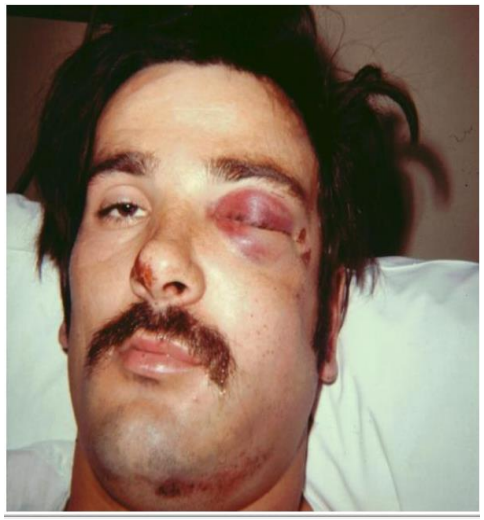
Annual market value of rhuEPO: ~$12 billion.

2. <u>Cytoprotective activities</u>: EPO and its derivatives display remarkable anti-apoptosis and tissue protection against various damages triggered by ischemia/reperfusion, hypoxia or cytotoxic agents in the brain, the heart, the kidneys and the liver.

Limitation of using rhuEPO as a cytoprotecive agent to treat tissue injury:

- Cytoprotective function of rhuEPO cannot be directly used because of its side effects caused by its erythropoietic activity.

- High doses are required for cytoprotective purposes, causing massive increase in red blood cell mass and leading to more damage.



(Side effects of rhuEPO treatment, such as thrombosis. Martin, 2006)

- **Cytoprotective EPO derivatives lacking erythropoietic activity are desired.**

Asialo-rhuEPO: enzymatically removed sialic acids from rhuEPO

Asialo-rhuEPO lacks erythropoietic function:

Table 1. Neuroprotective effects and predominating plasma half-life of enzymatic asialo-EPO and rhuEPO

	Percent protection		Plasma half-life, h		
	P-19	PC12	i.v.	i.p.	s.c.
rhuEPO	51	31 ±7	5.6	7.0	5.4
Asialo-EPO	43	34 ±4	0.023	0.5	2.5

Data from Erbayraktar et al., 2003, PNAS, 100 (11): 6741-6746. Both P-19 and PC12 are neuronal cells. i.v.:intravenous adminstration; i.p.: intraperitoneal adminstration subcutaneous adminstration.

Asialo-rhuEPO has multiple tissue-protective functions: neuro- (Erbayraktar et al., 2003), reno- (Okada et al., 2007, Transplantation, 84, 504-510) and cardio-protection (Ogino et al., 2010, JACC, 56: 1949-1958).

<u>Current situation of asialo-rhuEPO</u>:

1. No expression system available for asialo-rhuEPO production.

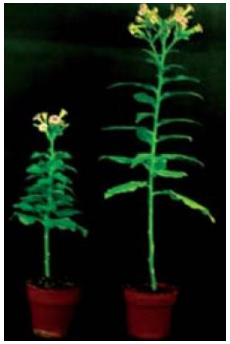
2. Too expensive using rhuEPO for asialo-rhuEPO production by enzymatically removing sialic acids.

Step 1. Can we use plants to express rhuEPO?

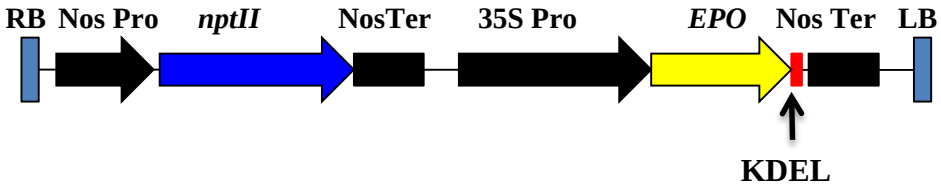
Transgenic Research **13:** 541–549, 2004.

Overexpression of human erythropoietin (EPO) affects plant morphologies: retarded vegetative growth in tobacco and male sterility in tobacco and *Arabidopsis*

Ban Yoon Cheon[1,†], Hae Jin Kim[1,†], Kyung Hee Oh[1], Sung Chul Bahn[1], Ji Hoon Ahn[1], Jang Won Choi[2], Sung Han Ok[1], Jung Myung Bae[1] & Jeong Sheop Shin[1,*]
[1]*School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Korea*
[2]*Division of Life Science, College of Natural Resources, Taegu University, Kyungsan 712-714, Korea*



Overexpressed *EPO* alone in tobacco plants



Human *EPO* gene alone in pBI121 binary vector.

Why uses tobacco plants to produce asialo-rhuEPO?

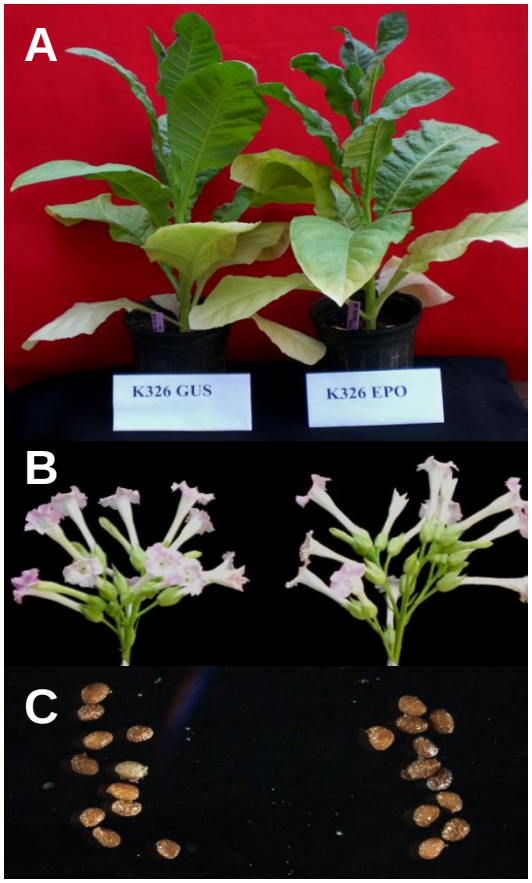
Fast growing, high biomass, well studied, easy to transform, non-food and non-feeding crop.

Musa TA et al. Plant Biotech. Rep. 2009. 3: 157-165.

All *EPO* transgenic plants are normal in growth and fertility

Table 1 Morphology, fertility and molecular analysis of human *EPO* transgenic plants and *gusA* control transgenic plants*

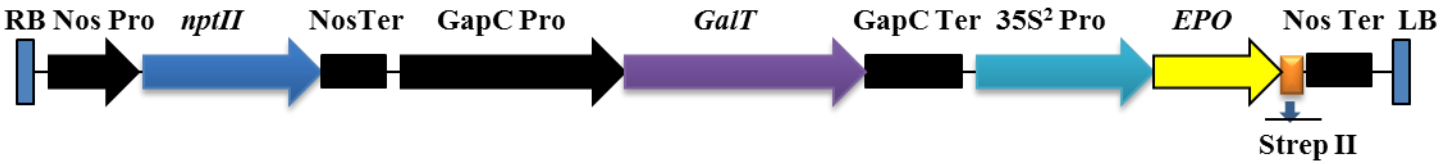
Plants	PCR for *EPO* gene	Plant height (cm)	Leaf number	Time for initial lowering (days)	Fertility
EPO1	Positive	104	27	97	Normal
EPO2	Positive	70	32	150	Normal
EPO3	Positive	102	24	93	Normal
EPO4	Positive	105	38	115	Normal
EPO5	Positive	113	37	107	Normal
EPO6	Positive	104	26	87	Normal
EPO7	Positive	99	27	85	Normal
EPO8	Positive	105	38	108	Normal
EPO9	Positive	123	39	107	Normal
EPO10	Positive	97	26	85	Normal
EPO11	Positive	100	24	84	Normal
EPO12	Positive	95	24	91	Normal
EPO13	Positive	126	34	96	Normal
EPO14	Positive	130	34	99	Normal
EPO15	Positive	105	27	91	Normal
EPO16	Positive	113	33	100	Normal
EPO17	Positive	122	30	97	Normal
EPO18	Positive	121	29	98	Normal
EPO19	Positive	115	27	95	Normal
EPO20	Positive	108	30	96	Normal
GUS1	Negative	102	25	83	Normal
GUS2	Negative	100	25	85	Normal
GUS3	Negative	100	24	88	Normal
GUS4	Negative	130	35	112	Normal
GUS5	Negative	96	25	118	Normal
GUS6	Negative	102	33	84	Normal



GUS **CEJ98**

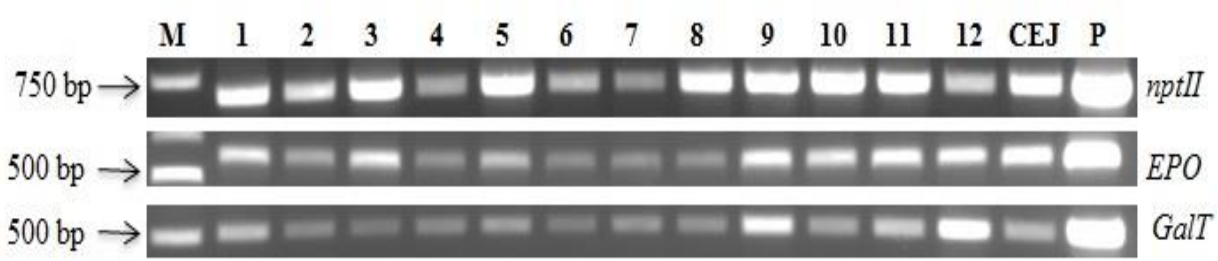
Musa et al. Plant Biotech. Rep. 2009. 3: 157-165.

Step 2. Created transgenic plants with double CaMV 35S promoter driving *EPO*



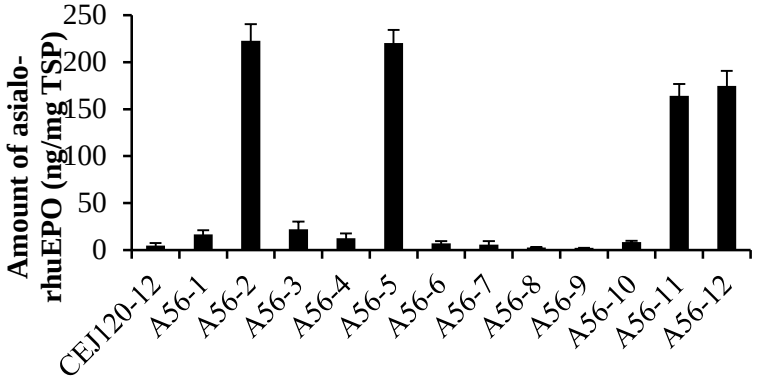
A56: The *EPO* (yellow) fused with StrepII lies downstream of CaMV 35S^2 promoter (35S^2 Pro), followed by Nos terminator (Nos Ter), whereas the *GalT* (purple) is flanked by a GapC promoter (GapC Pro) and terminator (GapC Ter).

PCR confirmed transgenes:



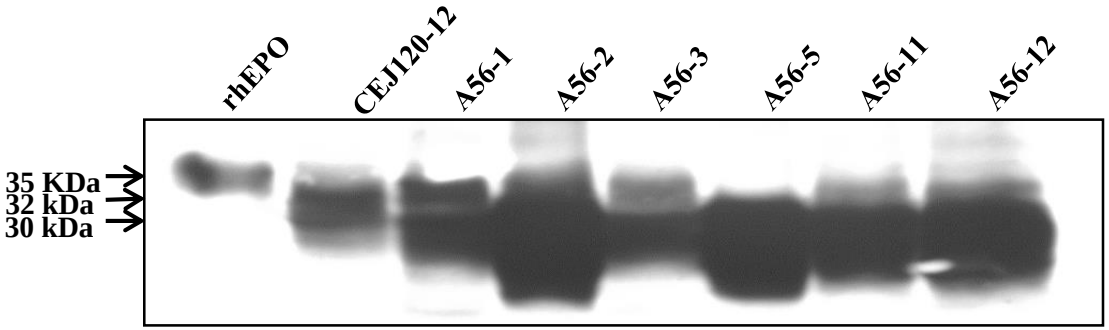
Kittur et al., PLoS One. 2013, 8(10): e76468.

Transgenic plants with asialo-rhuEPO^{P} accumulation levels: 230 ng/mg TSP



Quantification in transgenic plants A56-1 to -12 by ELISA. CEJ120-12 was used as a control.

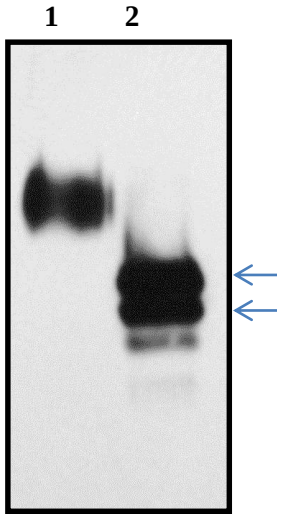
Its expression level is ~0.5 mg/kg of fresh leaves. One acre of land can produce ~4,000-6,000 kg of fresh leaves, having about 2,000-3,000 mg of asialo-rhuEPO^{P}.



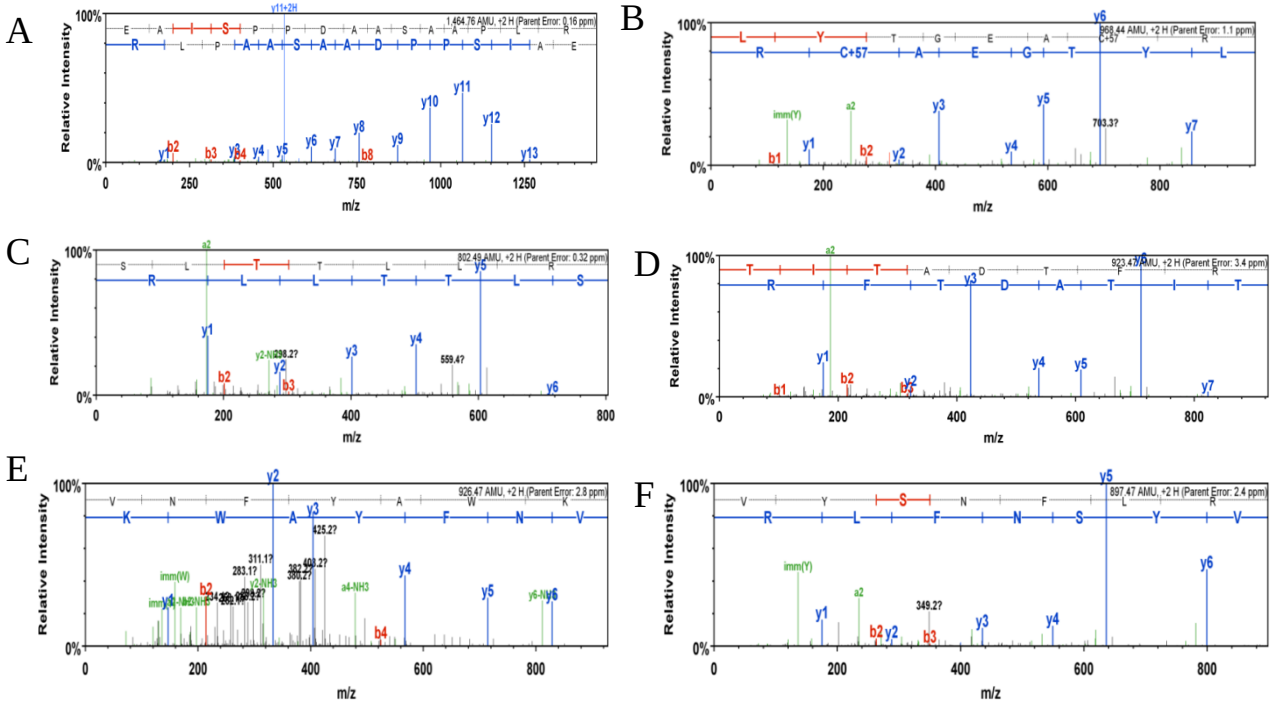
Western blot analysis of asialo-rhuEPO^{P} in selected transgenic tobacco lines (A56-1, A56-2, A56-3, A56-5, A56-11 and A56-12). Standard rhuEPO produced in CHO cells (lane 1) was used as positive control.

Kittur et al., PLoS One. 2013, 8(10): e76468.

Peptide mapping to confirm both bands are rhuEPO



Western blot analysis of asialo-rhuEPO^{P} (lane 2). Standard rhuEPO^{M} (lane 1): positive control.



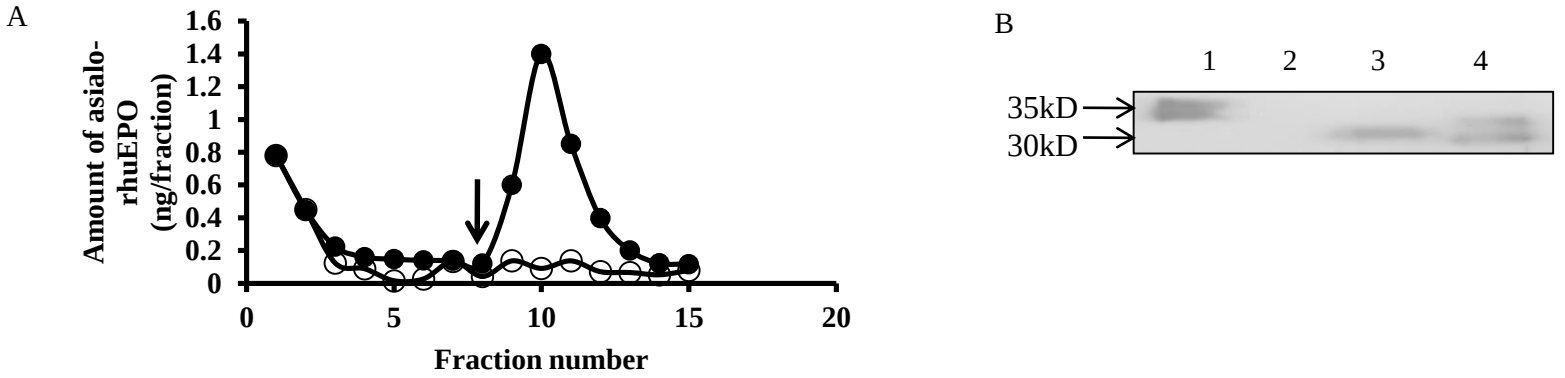
gi|119596900 (100%), 33,050.8 Da
erythropoietin, isoform CRA_b [Homo sapiens]
6 unique peptides, 6 unique spectra, 6 total spectra, 52/304 amino acids (17% coverage)

M V H V P G L W K G S E P G A E W G W G S L D S S F G K A Q A S P P P P P A H A H M Q I T A P T P G Q S R R V P G P P R
P L A A L R R T A L S S R S R T G A T A P A L L R H R A P W T A A L S S R P V G L A L H R R A S R D E G P R C E C P A W
L W L L L S L L S L P L G L P V L G A P P R L I C D S R V L E R Y L L E A K E A E N I T T G C A E H C S L N E N I T V P
D T K **V N F Y A W K** R M E V G Q Q A V E V W Q G L A L L S E A V L R G Q A L L V N S S Q P W E P L Q L H V D K A V S G L
R **S L T T L L R** A L G A Q K **E A I S P P** **D A A S A A P L R T** **I T A D T F R** K L F R **V Y S N F L R** G K L K **L Y T G E A C R**
T G D R

Kittur et al., PLoS One. 2013, 8(10): e76468.
Kittur et al., Plant Cell Rep. 2015, 34:507–516

To prove *N*-glycan chains bearing β1,4-galactose residues

A. Binding of asialo-rhuEPO^{P} to ECA (*Erythrina cristagalli agglutinin*) - agarose column



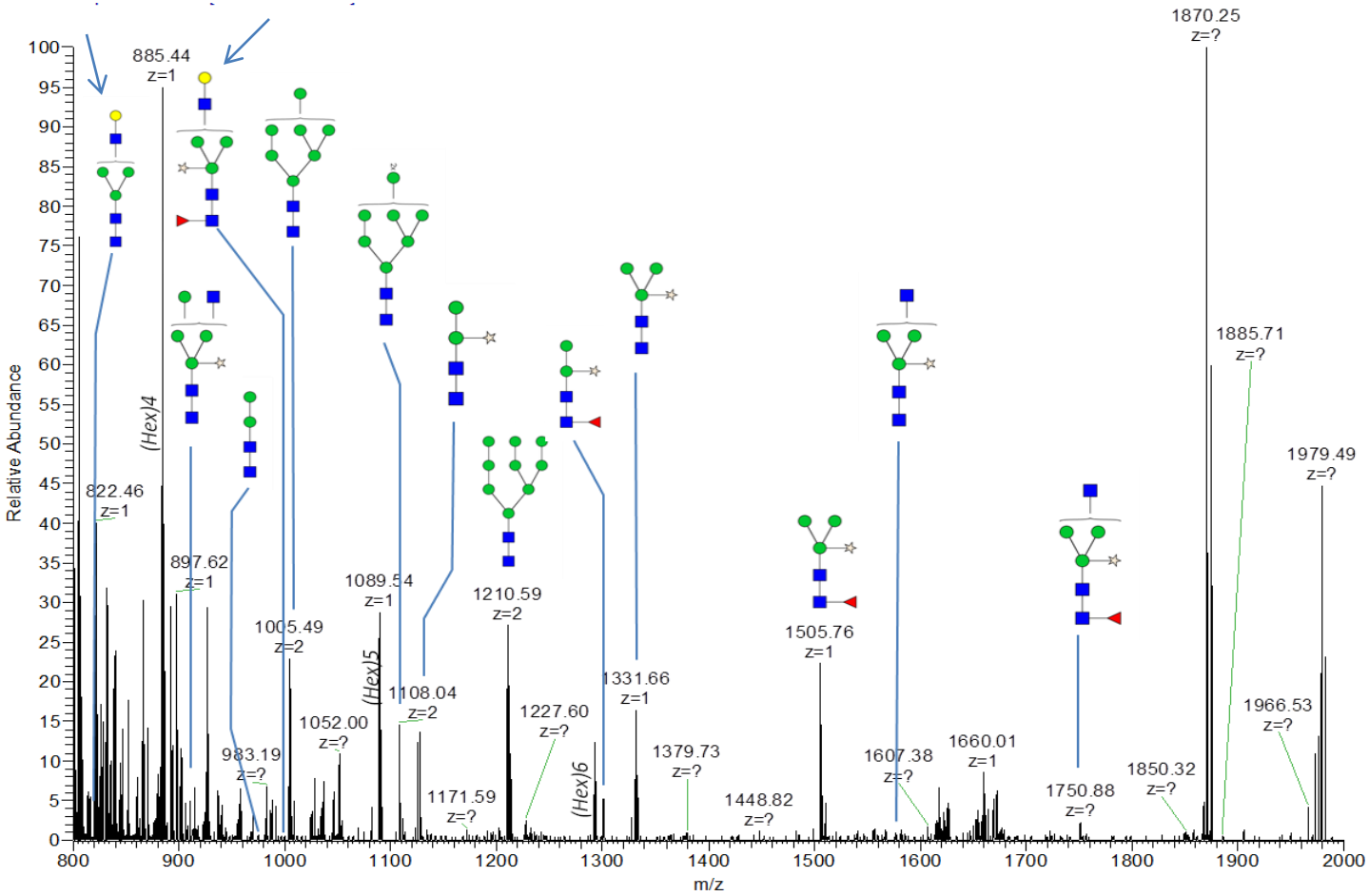
A, binding of purified asialo-rhuEPO^{P} (-●-) and asialoagalacto-EPO^{P} (-○-) to ECA - agarose column. The amount of asialo-rhuEPO determined by ELISA. B, western blot analysis of asialo-rhuEPO eluted from ECA-agarose column. Fractions (9-12) were pooled and the protein was precipitated. Lane 1, rhuEPO; lane 2, empty; lane 3, ECA-agarose fraction; and lane 4, immunoaffinity chromatography fraction.

B. Western blot analysis of GalT expression

Western blot analysis of microsomal fraction isolated from CEJ120-12 and GUS1 control plants to detect GalT. Lane 1, purified recombinant GalT; lane 2, GUS1 control plant; lane 3, CEJ120-12.

Kittur et al. Plant Cell Rep. 2012. 31: 1233-1243.

NSI-FTMS spectrum of PNGase A released and permethylated asialo-rhuEPO^{P} *N*-glycans



The schematic glycan structures of the glycans found in *N*-glycan pool of asialo-rhuEPO^{P} are shown. The structure for each peak was further verified by MS/MS analysis using total ion mapping. The symbols for the glycan structures are: filled blue square, GlcNAc; filled green circle, mannose; filled yellow circle, galactose; filled red triangle, fucose, unfilled star, xylose.

Kittur et al., PLoS One. 2013, 8(10): e76468.

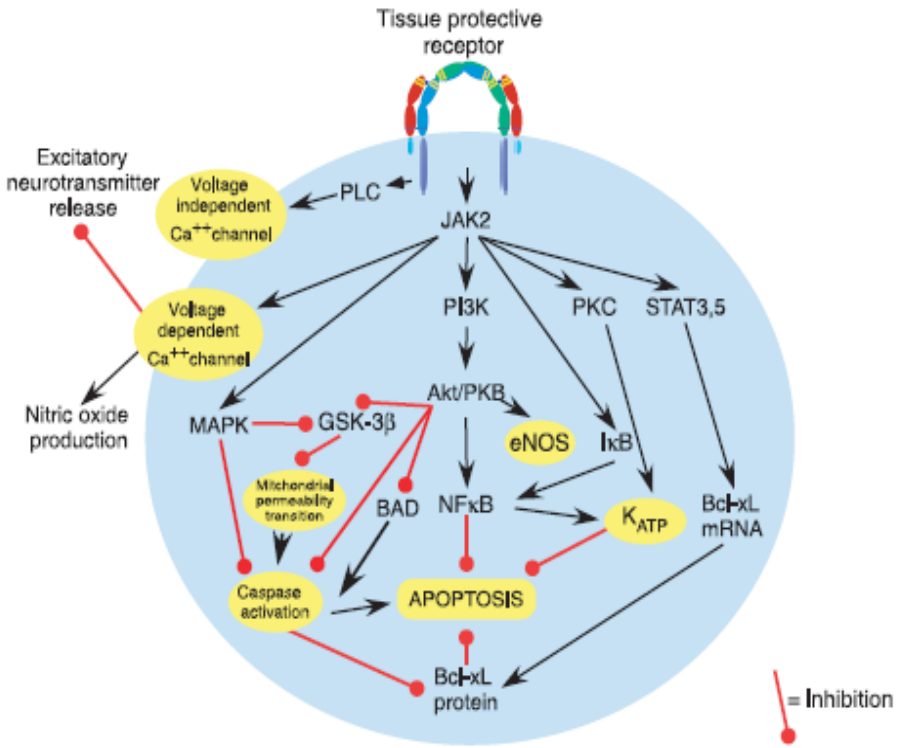
Step 3 - Establishment of an efficient purification approach from transgenic leaf tissues - ion-exchange and immunoaffinity chromatography

Steps	Volume (ml)	Total protein[a] (mg)	Total asialo-rhuEPO[P] (µg)	Total activity[b] (IU[b])	Specific activity (IU/mg)	Purification fold (x)[c]	Yield (%)[d]
Crude extract	260	178	**29**	3625	20	1	100
SP-sepharose	160	54	**15.5**	1937	36	1.8	53
IAC	24	0.02	**9.0**	1125	56250	2812	**31**

Results: 31% of recovered asialo-rhuEPO[P]

Kittur et al. (2015) Int. J. Biol. Macromolec. 72: 1111-1116

Step 4 - To study the cytoprotective properties and mechanisms of plant produce asialo-rhuEPO^{P}



Signaling pathway for rhuEPO featuring JAK2 activation and the secondary signal pathways

(Brines and Cerami, J Interl Med. 264; 405–432 2008)

- **EPO binds to the EPOR (EPO receptor)-common β-receptor complex and activate Janus Tyrosine Kinase-2 (JAK2) cascade.**
- **However, most studies supported that EPO binds to EPOR for its protective function.**
- **Activated JAK2 will further proliferate the signaling pathway of the secondary molecules, such as**
  - **STAT3/5**
  - **PI3K/AKT**
  - **MAPK**

Analysis of asialo-rhuEPO^{P} and EPOR binding affinity

Protein	Dissociation constant (K_d) nM
rhuEPO^{M}	0.12 ± 0.08
Asialo-rhuEPO^{P}	0.20 ± 0.02
Asialoagalacto-rhuEPO^{P}	0.17 ± 0.01

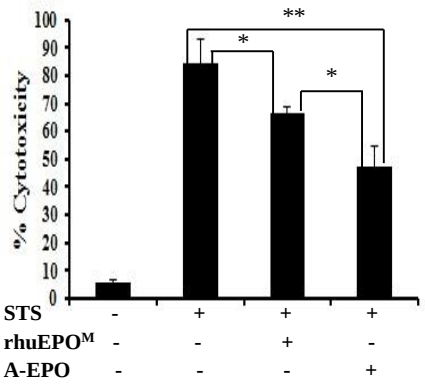
Binding isotherm of asialo-rhuEPO^{P} to EPOR. Purified asialo-rhuEPO^{P}, asialoagalacto-rhuEPO and CHO-produced rhuEPO were incubated separately with soluble EPOR receptor on ice for 15 min. The reaction mixture was then applied onto an anti-EPOR antibody coated plate. Bound EPO was detected using rabbit anti-EPO antibody.

Result: Asialo-rhuEPO^{P} displays EPOR binding affinity similar to that of rhuEPO^{M}.

Kittur et al. Plant Cell Rep. 2012. 31: 1233-1243.

Cytoprotective functions of asialo-rhuEPO^{P} in neuronal cells (N2A)

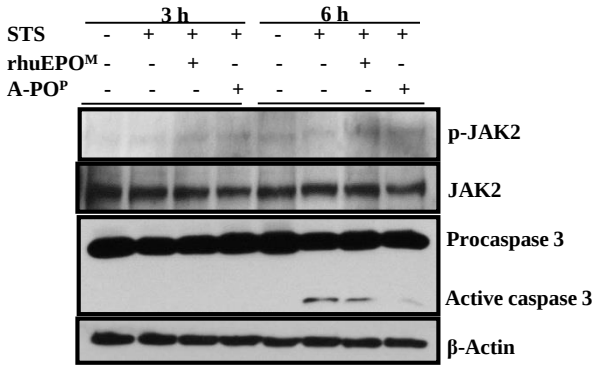
A



Cytotoxcity (%):
STS (staurosporine): 84%
STS + rhuEPO^{M}: 66% (protection 21.4%)
STS + A-EPO^{P}: 47% (protection 44.1%)

Results: asialo-rhuEPO^{P} is functionally active and has even better neuroprotective effect (~2 fold) than rhuEPO^{M}.

B



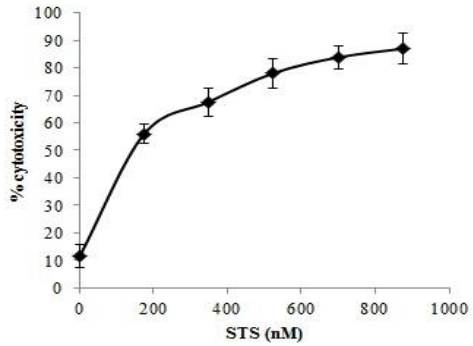
Results: asialo-rhuEPO^{P}-mediated neuroprotection is via JAK2 activation and caspase 3 inhibition.

The cytoprotective effect of asialo-rhuEPO^{P} and Western blot of STS and rhuEPO treated N2A cells to detect activated JAK2 and caspase 3. Cells treated with PBS containing 0.1% BSA (vehicle), 1 μM STS, 1 μM STS+20 U/mL asialo-rhuEPO^{P} (A-EPO) or 1 μM STS+20 U/mL rhuEPO^{M}. A. Cytotoxicity was measured by LDH assay after 12 h treatment. **: *P*<0.01; *: *P*<0.05. B. Western blot of JAK2 and caspase 3 in cell lysates prepared from cells treated for 3 and 6 h. β-actin: internal control.

Kittur et al., PLoS One. 2013, 8(10): e76468.

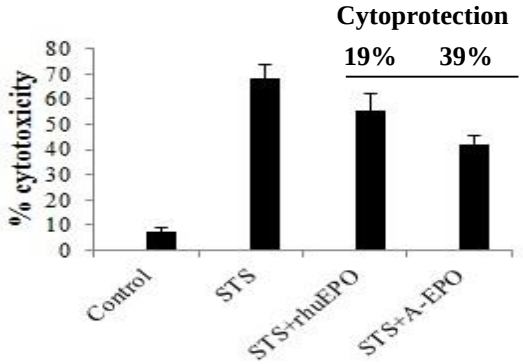
Cytoprotective functions of asialo-rhuEPO^{P} in HL1 cardiomyocytes

A



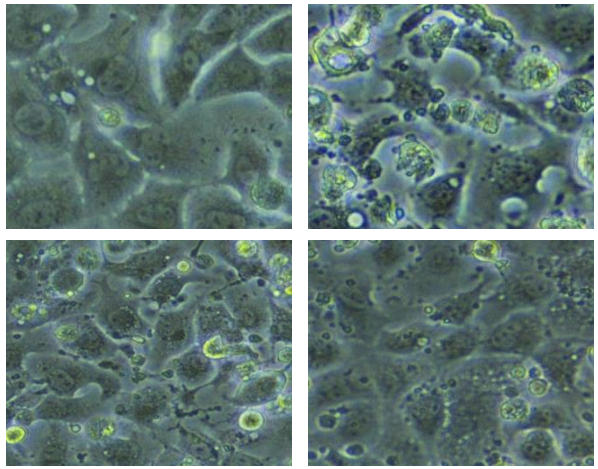
Dose response of HL1 cardiomyocytes on STS treatments. Its EC_{50} value was found to be 0.175 µM.

B



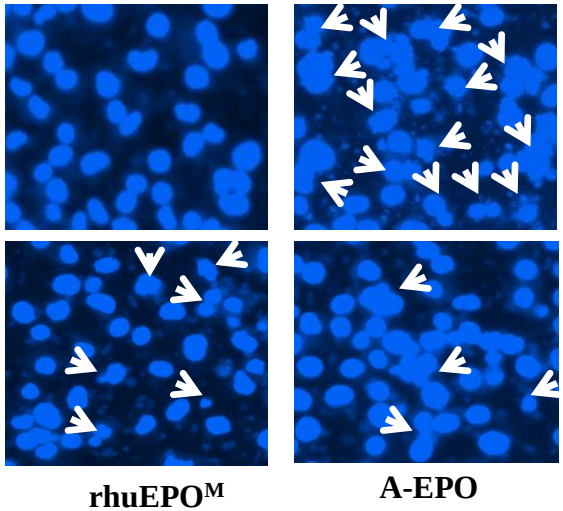
Toxicity of HL1 cardiomyocytes after treatment with STS, STS+rhuEPO^{M} (20 IU/ml) or STS+ asialo-rhuEPO^{P} (20 IU/ml).

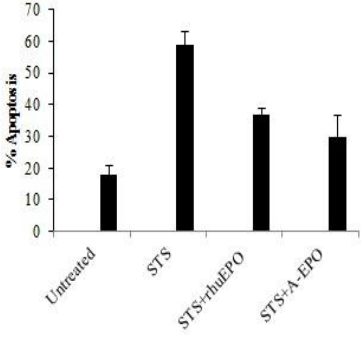
C



Morphological changes in HL1 cardiomyocytes measured using phase-contrast microscopy.

D



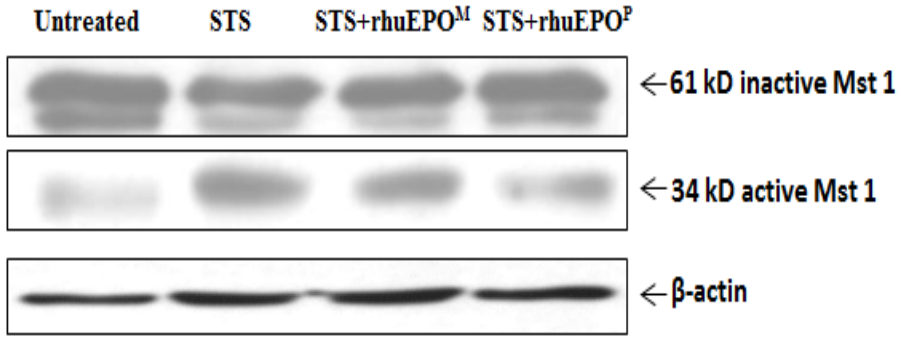


Fragmented nuclei (arrows) under different treatment conditions. Percentages of fragmented nuclei were calculated.

Kittur et al. Unpublished data

Preliminary results of its cardioprotective mechanisms: asialo-rhuEPO^{P}-mediated cardio-protection is also via JAK2 activation and caspase 3 inhibition.

Recent studies have shown that toxic- or pathologic insults induce the activation of mammalian sterile 20–like kinase 1 (Mst1) in cardiomyocytes, then to promote apoptosis (Yamamoto et al. 2003; Maejima et al. 2013).



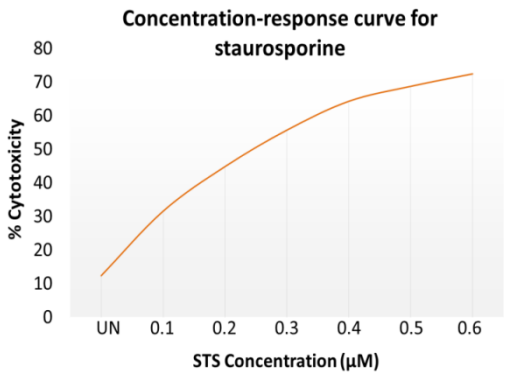
The cytoprotective effect of asialo-rhuEPO^{P} and Western blot of STS and rhuEPO treated HL-1 murine cardiomyocyte lysates to detect activated Mst1. Cells treated individually with PBS containing 0.1% BSA (vehicle control), STS, STS+rhuEPO^{M}, or STS+asialo-rhuEPO^{P} (rhuEPO^{P}). (a) Cytotoxicity was measured by LDH assay after 24 h treatment. Each experiment had six replicates. All data plotted are the average of three independent experiments ± SD. **: P<0.01; *: P<0.05. (b) Western blot of Mst1 in cell lysates prepared from cells treated for 24 h.

Kittur et al. Unpublished data

Cytoprotective functions of asialo-rhuEPO^{P} on pancreatic β-cell lines (RIN-m5F)

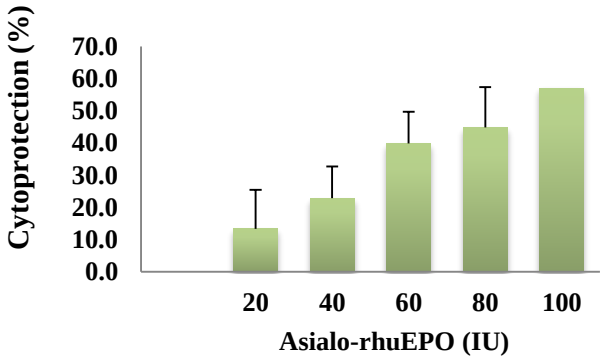
Loss of β-cell function and mass is the fundamental cause of diabetes leading to impaired insulin secretion and dysfunctional glucose homeostasis.

A



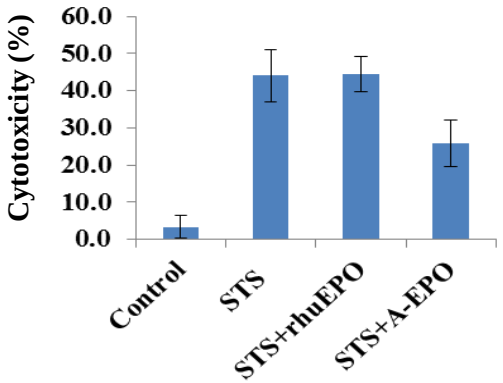
EC_{50} for STS on RIN-m5F cell line is 0.123 µM.

B



Treatments: 0.123 µM STS with 20 to 100 IU of asialo-rhuEPO^{P}. Three biological repeats were performed.

C



0.123 µM STS with 60 IU of rhuEPO or asialo-rhuEPO^{P} were used.

D

Cytoprotection of Asialo-rhuEPO^{P} on pancreatic β-cell lines (RIN-m5F)

	Control	STS	STS+ rhuEPO^{M}	STS +asialo-rhuEPO^{P}
Cytotoxicity (%)	2.5 ± 3.3	44.3 ± 8.3	45.2 ± 6.0	26.4 ± 6.7
Cytoprotection (%)	-	-	-2.9 ± 6.1	40.6 ± 8.7

Arthur et al. Unpublished data

Conclusions

1. **Successfully created transgenic tobacco plants for asialo-rhuEPO^{P} production.**
2. **Developed an efficient method to purify asialo-rhuEPO^{P} from transgenic leaves.**
3. **Confirmed that asialo-rhuEPO^{P} has broad in vitro cytoprotective functions.**

Future work

- **To study its *in vivo* neuro- and cardio-protective functions in various animal models**

  Such as, mouse model of ischemia-reperfusion injury.

- **To evaluate general pharmacological properties of asialo-rhuEPO^{P}**

  Such as, half-life, capacity to cross the blood-brain barrier (BBB), *in vivo* hematopoietic activity and immune reaction.

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Golden LEAF FOUNDATION

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Biomanufacturing Research Institute & Technology Enterprise (BRITE) building
Thank you!
Questions?
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