Method of treatment of Hypoxia Inducible Factor (HIF)-related conditions

DESCRIPTION

FIELD OF THE INVENTION

The present invention relates to methods of treatment of Hypoxia Inducible Factor (HIF)-related conditions, and in particular to methods of treatment of HIF-related conditions comprising the administration of a composition comprising transferrins.

BACKGROUND OF THE INVENTION

Protecting cells, and specially neurons, from damage caused by various factors, including stroke, neurodegenerative disease, traumatic injury, etc., is important for longterm recovery of cell or neuronal function. Therapeutic treatment of injured cells or neurons by single agents has advantages, but is often not sufficient to mobilize the complexity of molecules needed to help in restoring complete function.

Physiological response to protect neurons or other cells from hypoxic or ischemic events, or from oxidation, is often considered to be mediated by expression of genes that are up-regulated through the Hypoxia Inducible Factor (HIF) signaling pathway, a key regulatory pathway that is responsive to cellular insults. In the brain, upregulation of neuroprotective molecules is believed to be a critical factor in protecting cells from irreparable damage. However, few available drugs are sufficiently able to

prevent, restore or reduce damage to neurons and other tissues. Additionally they are often toxic, have short half-lives, or both. For example, the international patent application WO2006/20727 proposes the use of deferoxamine as neuroprotector agent against the harmful effects of reperfusion; however, the administration of deferoxamine poses problems due to its reduced half-life in plasma.

Transferrins are iron-binding blood plasma glycoproteins that control the level of free iron in biological fluids. Transferrins function as the main transporter of iron in the circulation where it exists in an iron-free apo-transferrin (ApoTf) form, as monoferric transferrin, or as diferric holo-transferrin (HoloTf). Typically, iron is bound to 30% of all transferrin binding sites in circulation. The neuroprotection function of ApoTf but not HoloTf has been disclosed by Chen-Roetling *et al.* (Chen-Roetling J., Chen L., and Regan R.F. *Neuropharmacology*, 2011; 60(2-3): 423-431.), suggesting that ApoTf may mitigate the neurotoxicity of hemoglobin after intracerebral hemorrhage.

The present inventors have found that it may be possible to boost the neuroprotective properties of transferrin administration in patients by combining it with other iron chelating agents or with another iron-binding plasma protein, such as Apolactoferrin, which has been shown to increase HIF-1α protein levels in some tissues and have effects on plasma EPO levels (Zakharova E.T. *et al.* Biometals (2012) 25:1247-1259). Molecules with iron chelating abilities have been suggested to be HIF pathway activators by blocking the activity of prolyl hydroxylases.

Therefore, the present invention refers to a method of treatment of Hypoxia Inducible Factor (HIF)-related conditions comprising the administration of a composition comprising transferrin. The present invention also refers to a method of treatment of Hypoxia Inducible Factor (HIF)-related conditions wherein the administered composition further comprises a combination of a transferrin and an iron chelator.

As used herein, the term "transferrin" and its plural refers to ApoTf alone, or HoloTf alone or a mixture thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is further described with reference to the following drawings, in which:

Figure 1 shows that compositions of majority ApoTf and majority HoloTf induce HIF-1α protein under normoxic and hypoxic conditions 6 hrs post treatment.

Figure 2 shows that compositions of majority ApoTf and majority HoloTf induce HIF-1α protein under normoxic conditions 24 hrs post treatment.

Figure 3 shows that mixtures of ApoTf and HoloTf induce HIF-1α protein 6 hrs post treatment.

Figure 4A shows mRNA expression levels of Glut1 under normoxic and hypoxic conditions in the presence of HSA,

Apo-transferrin or Holo-transferrin.

Figure 4B shows mRNA expression levels of VEGF under normoxic and hypoxic conditions in the presence of HSA, Apo-transferrin or Holo-transferrin.

Figure 5A shows *in vitro* or *in vivo* toxicity of compositions comprising either majority HoloTf or majority ApoTf.

Figure 5B shows Modified Bederson and General Behavioral scores for rats intravenously treated with drug comprising majority of ApoTf or HoloTf.

Figure 6A shows Scatter plot of the infarct volume of ApoTf (385 mg/kg, IV) or saline treatment in transient Middle Cerebral Artery occlusion (MCAo) rat model.

Figure 6B shows triphenyltetrazolium chloride (TTC) stained Coronal sections from a representative control and ApoTf treated rat.

Figure 7 shows protection of neuronal cells from the toxic effects of Abeta(1-42) by mixtures comprising mostly ApoTf and HoloTf.

Figure 8A shows treatment of SH-SY5Y neuronal cells with 4 mg/ml of majority ApoTf and with a combination of majority ApoTf and DFO or IOX2.

Figure 8B shows treatment of SH-SY5Y neuronal cells with 4 mg/ml of the indicated protein and with a combination of the indicated protein and 10uM M30 plus.

Figure 8C shows treatment of SH-SY5Y neuronal cells with 4 mg/ml of the indicated protein and with a combination of the indicated protein and 200uM DFO.

Figure 9A shows mRNA expression levels of Glut1 in response to majority Apotransferrin and DFO or IOX2 combinations.

Figure 9B shows mRNA expression levels of VEGF in response to majority Apotransferrin and DFO or IOX2 combinations.

Figure 10A shows HIF-1α levels after treatment of Primary human renal proximal tubule epithelial (RPTEC) cells with 4 mg/mL majority Apo-transferrin, majority Holo-transferrin or various mixtures of each for 6hrs under normal oxygen levels.

Figure 10B shows HIF-1α levels after treatment of primary cortical epithelial (HRCE) cells with 4 mg/mL majority Apo-transferrin, majority Holo-transferrin or various mixtures of each for 6hrs under normal oxygen levels.

Figure 11A shows viability of primary human renal proximal tubule epithelial (RPTEC) or cortical epithelial (HRCE) cells when treated with majority ApoTf or DFO for 48 hours.

Figure 11B shows viability of RPTEC or HRCE cells when treated for 72hrs with 4mg/mL of majority ApoTf,

majority HoloTf, mixtures of transferrin.

Figure 12 shows caspase 3/7 activation within human primary kidney cells in the presence of majority ApoTf or DFO.

Figure 13A shows HIF-1α levels after treatment of lung cell line NCI-H1650 with 4 mg/mL majority Apo-transferrin, majority Holo-transferrin or pd-Transferrin for 6hrs under normal oxygen levels.

Figure 13B shows HIF-1α levels after treatment of primary hepatocyte cells with 4 mg/mL majority Apo-transferrin, majority Holo-transferrin or pd-Transferrin for 6hrs under normal oxygen levels.

Figure 14A shows viability of human lung cell line, NCI-H1650, when treated for 72 hours with 4mg/mL of majority ApoTf, majority HoloTf, or pd- transferrin.

Figure 14B shows viability of primary human hepatocytes when treated for 72 hours with 4mg/mL of majority ApoTf, majority HoloTf, or pd- transferrin.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect, the present invention refers to a method of treatment of Hypoxia Inducible Factor (HIF)-related conditions comprising the administration of a composition comprising transferrin. Preferably, transferrin is recombinant, plasmaderived or chemically synthesized transferrin.

When transferrin is recombinant, it can be obtained according to any technique known in the art of protein expression, production and purification. For example, nucleic acid sequence of transferrin can be inserted in any vector suitable for expression in the elected host cell, e.g. bacteria (*Escherichia coli, Bacilus subtilis, Salmonella typhimurium, Pseudomonas, Streptomyces* and *Staphylococcus*), yeast (*Saccharomyces, Pichia* or *Kuyveromyces* genus), insect cells (*Bombyx mori, Mamestra brassicae, Spodoptera frugiperda, Trichoplusia ni* or *Drosophila melanogaster*) or mammalian cells (HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS-1), human hepatocellular carcinoma cells (e.g., Hep G2), human adenovirus transformed 293 cells, mouse L-929 cells, HaK hamster cell lines, murine 3T3 cells derived from Swiss, Balb-c or NIH mice, CV-1 cell line, cell strains derived from in vitro culture of primary tissue or primary explants).

It is contemplated that plasma derived transferrin is isolated from a suitable fraction of plasma. In a preferred embodiment transferrin is isolated from fraction IV, and most preferably fraction IV-I or fraction IV-IV, of the Cohn fractionation process. In another preferred embodiment, transferrin derives from a waste fraction of a method of purifying alpha$_1$-proteinase inhibitor (A1PI). Preferably said purifying method is as follows:

(a) removing a portion of contaminating proteins from the aqueous solution by precipitation in order to obtain a purified solution containing A1PI;

(b) passing the purified solution through an anion

exchange resin so that A1PI binds to the anion exchange resin;

(c) eluting A1PI from the anion exchange resin to obtain an eluted solution containing A1PI;

(d) passing the eluted solution through a cation exchange resin;

(e) collecting a flow-through from the cation exchange resin that contains A1PI; and

(f) contacting the eluted solution of step (c) or the flow-through of step (e) with a hydrophobic adsorbent of at least one HIC medium.

In the most preferred embodiment, the aqueous solution used in the method of purifying (A1PI) mentioned above is blood, plasma or a plasma derived fraction.

In a further embodiment transferrin comprises at least one post translational modification, preferably pegylation, glycosylation, polysialylation or combination thereof.

In one embodiment, transferrin used in the method of treatment of the present invention is a full length transferrin with the amino acid sequence set forth in SEQ ID NO: 1. Further embodiments encompass the use in the method of treatment of the present invention of transferrin derivatives with at least 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98% or 99% homology or similarity with SEQ ID NO: 1 as long as 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% or 100% of the iron chelating activity of the wild-type transferrin is preserved. A person skilled in the art would readily recognize that the differences in homology between transferrin and the amino acid sequence SEQ ID NO:

1 can be due to conservative and/or non-conservative amino acid substitutions that do not affect the iron chelating function.

In another embodiment, it is contemplated that a fragment of the wild-type transferrin (SEQ ID NO: 1) is used in the method of treatment of the present invention. A person skilled in the art would readily select a suitable fragment of the wild-type transferrin so that it keeps the iron chelating activity of the wild-type transferrin.

In an additional embodiment, transferrin is modified to increase its iron binding affinity. A person skilled in the art would envisage that the residues or zones to be modified can be determined by several techniques know in the art as, for example, site directed mutagenesis, alanine screening, crystallography or analysis of deletions and/or insertions.

It is contemplated that transferrin used in the method of treatment of the present invention is in the form of a protein conjugate or a fusion protein in order to, for example, extend its half-life in blood, wherein transferrin is conjugated or fused to any other protein, protein fragment, protein domain or peptide. In a preferred embodiment, transferrin is conjugated or fused to a full-length, fragment, domain or peptide of serum albumins (as for example, bovine, from rabbits or from humans), keyhole limpet hemocyanin, immunoglobulin molecules (including Fc domain of immunoglobulins), thyroglobulin, ovalbumin, tetanus toxoid, or a toxoid from other pathogenic bacteria, such as diphtheria, E. coli,

cholera, or H. pylori, or an attenuated toxin derivative, cytokines, chemokines, glucagon-like peptide-1, exendin-4 or XTEN.

The transferrin used in the method of the present invention can be Holo-Tf alone, Apo-Tf alone, or a mixture of ApoTf and HoloTf. In a preferred embodiment, the transferrin used in the method of the present invention is a mixture of ApoTf and HoloTf and, preferably, said mixture has a percentage ratio between 99:1 and 70:30 (ApoTf:HoloTf), even more preferably, said mixture has a proportion or percentage ratio that is comparable to that of a fraction obtained or purified from biological fluids. In the most preferred embodiment the mixture of ApoTf and HoloTf used in the method of the present invention has a proportion or percentage ratio of ApoTf and HoloTf as the proportion or percentage ratio of ApoTf and HoloTf present in human blood or plasma.

In the method of the present invention, the composition can further comprise an iron chelator. In a preferred embodiment, the iron chelator is selected from the group comprising M30, deferoxamine (DFO), deferasirox, deferiprone, deferitrin, L1NA11, CP363, CP502, IOX2 ethylenediaminetetraacetic acid (EDTA) or combinations thereof. In the most preferred embodiment, the iron chelator is deferoxamine (DFO).

In the method of the present invention, treatment of Hypoxia Inducible Factor (HIF)-related condition comprises treatment of organs with a composition comprising transferrin in preparation for transplantation into a human. In a preferred embodiment, organs are selected from

the group comprising kidney, liver, lung, and heart.

Moreover, in the method of the present invention, treatment of Hypoxia Inducible Factor (HIF)-related condition comprises administration of a composition comprising transferrin to organ transplant recipients prior to or after the transplantation. In a preferred embodiment, the method of the present invention comprises administration of a composition comprising transferrin to organ transplant recipients prior to or after the transplantation, and where transplant organs have been previously treated with a composition comprising transferrin in preparation for transplantation into a human.

In another aspect, the present invention refers to a method of treatment of Hypoxia Inducible Factor (HIF)-related condition, wherein said condition comprises treatment of ischemia in a human comprising the administration to said human of a composition comprising transferrin. In a preferred embodiment, said ischemia is due to cardiac arrest, thrombotic clots, traumatic injury or stroke.

In yet another aspect, the present invention refers to a method of treatment of Hypoxia Inducible Factor (HIF)-related condition, wherein said treatment is a presurgical administration to a human patient in cases when ischemia or oxygen deprivation of tissues/organs is observed or anticipated.

While the invention will now be described in connection with certain preferred embodiments in the

following examples so that aspects thereof may be more fully understood and appreciated, it is not intended to limit the invention to these particular embodiments. On the contrary, it is intended to cover all alternatives, modifications and equivalents as may be included within the scope of the invention as defined by the appended claims. Thus, the following examples which include preferred embodiments will serve to illustrate the practice of this invention, it being understood that the particulars shown are by way of example and for purposes of illustrative discussion of preferred embodiments of the present invention only and are presented in the cause of providing what is believed to be the most useful and readily understood description of formulation procedures as well as of the principles and conceptual aspects of the invention.

EXAMPLES

Experiments performed in the following examples were treated with transferrin comprising either the Apo- or Holo- forms. A broad variety of transferrin mixtures were tested; the relative percentages of ApoTf and HoloTf comprising majority ApoTf, majority HoloTf, pdTf and specifically defined transferrin mixtures are highlighted in Table 1 below.

Table 1:

	Majority Apo	Majority Holo	Mix 1	Mix 2	Mix 3	pdTf
% ApoTf	98	30	95	64	33	68
% HoloTf	2	70	5	36	67	32

Example 1. Compositions comprising majority ApoTf and majority HoloTf induce HIF-1α protein under normoxic and hypoxic conditions after 6 hours of treatment.

Human neuroblastoma SH-SY5Y cell line cells, cultured in serum free media were treated with plasma derived ApoTf and HoloTf (in both cases at a concentration of 1mg/mL and 4mg/mL) for 6 hours under normoxia (21% oxygen) and hypoxia conditions (1% oxygen). As controls, untreated cells or cells treated with human serum albumin (HSA) at a concentration of 1mg/mL or 4mg/mL, are used. After 6hrs intracellular proteins were harvested and tested for HIF-1α protein levels by ELISA.

As shown in Figure 1, a significant increase in HIF-1α cellular protein levels occurred under both normoxic and hypoxic conditions and for the two concentrations tested for ApoTf. Regarding HoloTf a significant increase in the cellular protein levels of HIF-1α was observed for both normoxic and hypoxic conditions when cells were treated with the 1mg/mL concentration and for normoxic condition when cells were treated with the 4mg/mL concentration. When cells were treated with the 4mg/mL concentration of HoloTf a tendency towards an increase in the cellular protein levels of HIF-1α was seen.

Example 2. Compositions comprising majority ApoTf and majority HoloTf induce HIF-1α protein under normoxic conditions after 24 hours of treatment.

Experiment performed in example 1 was repeated but performing treatments for 24 hours and only under normoxic

conditions. After 24 hrs intracellular proteins were harvested and tested for HIF-1α protein levels by ELISA. Figure 2 shows the results obtained for this experiment. As can be seen in said figure, ApoTf increased cellular protein levels of HIF-1α in both concentrations tested. For HoloTf a significant increase of cellular protein levels of HIF-1α was observed when treatment was performed using a concentration of 4mg/mL and a tendency towards an increase of said protein was seen when using the 1mg/mL concentration.

Example 3. Mixtures of ApoTf and HoloTf induce HIF-1α protein after 6 hours of treatment.

After 6 hrs, intracellular proteins were harvested and tested for HIF-1α protein levels by ELISA. As shown in Figure 3, an increase of HIF-1α cellular protein levels after 6 hours treatment with plasma derived majority ApoTf, majority HoloTf or mixtures thereof under normoxic conditions was observed. As also can be seen in said figure, all mixtures of ApoTf and HoloTf upregulate HIF-1α protein in SH-SY5Y neuronal cells.

Example 4. mRNA expression levels of Glut1 and VEGF under normoxic and hypoxic conditions in the presence of HSA, Apo-transferrin or Holo-transferrin.

The stabilization of, and increase in, HIF-1α protein typically leads to an upregulation of HIF-related genes (increase in the transcription of genes targeted by HIF), i.e. genes that have HIF binding sites in their transcriptional regulatory elements. Two well

characterized genes that are activated by HIF-1α protein are Glut1 receptor and VEGF. Therefore, in order to analyze mRNA expression changes in each of these HIF target genes SH-SY5Y cell line cells were cultured and treated with majority ApoTf or majority HoloTf at a concentration of 1mg/mL and 4mg/mL under normoxic (21% oxygen) or hypoxic (1% oxygen) conditions for 6 hours. As a negative controls, cells were treated with HSA (1mg/mL or 4mg/mL) or were left untreated. After 6hrs, intracellular mRNA was harvested and tested for Glut1 and VEGF expression levels by qPCR. Expression results were calculated relative to the expression of the corresponding transcript seen in untreated cells. Figures 4A and 4B show the expression results obtained for Glut1 receptor and VEGF, respectively. Values in the figures are shown as Relative Gene Expression, with the target gene (Glut1 or VEGF) normalized for housekeeper (beta-actin) expression. As can be directly derived from said figures, under hypoxic conditions, expression of both Glut1 (Fig. 4A) and VEGF (Fig. 4B) were significantly increased when treated with Apo-transferrin relative to HSA controls. Interestingly, under normoxic conditions, Holo-transferrin, but not Apo-transferrin, increased expression of only Glut1.

Example 5. Mixtures of ApoTf and HoloTf do not show toxicity *in vitro* or *in vivo*.

Since it has been reported that HoloTf is toxic to cells *in vivo* and *in vitro*, toxicity of various compositions containing majority ApoTf, majority HoloTf or mixtures of ApoTf + HoloTf was tested in SH-SY5Y cells. SH-SY5Y cells were treated with the indicated

concentrations of 4mg/mL Tf (as indicated in Figure 5A), M30 or DFO for 72 hours. After 72 hours, cells were subjected to a Cell Titer Glow viability assay. Control cells, untreated cells, were set to a value of 100% viable. The average viability and standard deviations are shown for each treatment condition. No toxic effects were seen with any composition containing majority ApoTf and, surprisingly, no toxicity or detrimental effects of majority HoloTf were observed.

Interestingly, neither compositions of majority ApoTf nor majority HoloTf showed significant differences in these behavioral criteria, suggesting that there were no detrimental effects of HoloTf in vivo. Figure 5B shows modified Bederson and General Behavioral scores for rats intravenously treated with drug comprising majority of ApoTf or HoloTf. *In vivo* neurological function was assessed by a modified Bederson score (Bederson et al., 1986b; Crumrine et al., 2011) using the following definitions:

Score 0: No apparent neurological deficits;

Score 1: Body torsion present;

Score 2: Body torsion with right side weakness;

Score 3: Body torsion, right side weakness with circling behavior; and

Score 4: Seizure Activity.

General behavioral scores of rats were developed by the CALS personnel for the purpose of monitoring recovery of animals following surgical procedures (standard CALS post-operative care). A numerical value was assigned to the predetermined behavioral observations.

Score 0: Behavior consistent with a normal naïve rat (i.e. no ipsilateral deficit);

Score 1: Bright/active/responsive; the rat spontaneously moves and explores his cage, responds to external stimuli, explores the top of the cage;

Score 2: Quiet/alert/responsive; reserved behavior but will respond to external stimulus, tends not to rear or explore the top of the cage;

Score 3: Depressed behavior: tends not to move unless prodded, quickly returns to a somnolent state, little to no interest in external stimuli;

Score 4: Unresponsive: remains in a prostrate position even when prodded; and

Score 5: Seizure activity requiring euthanasia.

Example 6. *In vivo* cellular protection by transferrin.

The MCAo (Middle Cerebral Artery occlusion) rat model of brain stroke was used to assess cellular protection by transferrin. Stroke was surgically induced to 16 rats by using the MCAo technique. 8 rats were treated by injection of saline solution in the brain and the other 8 by injection of ApoTf in the brain. Figures 6A and 6B shows that a significant decrease in the volume of the infracted area was observed in the rats treated with a mixture comprising a majority of ApoTf when compared with control rats (treated with saline solution). Figure 6A shows a scatter plot of the infarct volume of ApoTf (385 mg/kg, IV) or saline treatment in transient MCAo; and Figure 6B shows TTC stained Coronal sections from a representative control and ApoTf treated rat.

Example 7. ApoTf and HoloTf protect SH-SY5Y from Abeta 1-42 toxicity.

Upregulation of the HIF pathway is known to play a protective role in a number of neurodegenerative diseases, including pathologies that result in destruction of nerve cells and neurons. Since treatment of SH-SY5Y upregulates HIF, treatment of cells with Apo- or Holo-transferrin should provide a protective effect on cells subjected to substances known to induce neurodegeneration. Figure 7 highlights data assessing whether majorities of Apo- and Holo-transferrin could protect SH-SY5Y cells from the toxic effects of the known neurodegenerative toxin oligomerized Abeta 1-42 peptide (Figure 7). SH-SY5Y neuronal cells cultured in growth media were treated with 4 mg/mL Apo-transferrin or Holo-transferrin for 24hrs under normal oxygen levels. After 24hrs, cells were treated with oligomerized Abeta1-42 peptide for an additional 72 hours. Following treatment with oligomerized Abeta1-42, cells were subjected to a ApoGlo caspase 3/7 activation assay. Control cells, untreated cells, were set to a normalized value of 1. The average caspase induction, relative to control cells, and standard deviations are shown for each treatment condition. Interestingly, these data show that both majority ApoTf and HoloTf protect SH-SY5Y cells from Abeta induced toxicity. These data also further confirm lack of inherent toxicity with either ApoTf or HoloTf.

Example 8. Synergystic effect with small molecule HIF activators and ApoTf/HoloTf mixtures.

Transferrin may act synergistically with other HIF

activating small molecules, such as other iron chelators or enzyme inhibitors. This could allow lower levels of these small molecules to be administered, eliciting fewer side effects but retaining high therapeutic levels. To determine whether Apotransferrin increases the potency of the iron chelator, DFO, and the phd2 inhibitor IOX2; SH-SY5Y neuronal cells cultured in serum free media were treated with 4 mg/mL of the indicated proteins in the presence or absence of small molecule drug under normal oxygen levels. The results of the experiment are shown in Figures 8A, 8B, and 8C. The data shown in Figure 8A relates to treatment of cells with a combination of DFO or IOX2, at the indicated concentrations, plus 4mg/mL protein. $CoCl_2$ was used as an experimental positive control. The data shown in Figure 8B relates to treatment of cells with a combination of 10uM M30 plus/minus 4mg/mL protein. The data shown in Figure 8C relates to treatment of cells with a combination of 200uM DFO plus/minus 4mg/mL protein. After 6hrs intracellular proteins were harvested and tested for HIF-1α protein levels by ELISA. Data are shown in pg/mL with standard deviation.

Example 9. mRNA expression levels of Glut1 and VEGF in response to majority Apotransferrin and DFO or IOX2 combinations.

In addition, mRNA expression levels of Glut1 and VEGF in response to majority Apotransferrin and DFO or IOX2 combinations were determined. SH-SY5Y neuronal cells cultured in serum free media were treated with 4 mg/mL human serum albumin or majority Apotransferrin under normal oxygen levels. Where indicated, either 200uM DFO or 1uM IOX2 were co-treated with the HSA and majority

Apotransferrin. After 6hr treatments, intracellular mRNA was harvested and tested for Glut1 and VEGF expression levels by qPCR. Values are shown as Relative Gene Expression, with the target gene (Glut1 or VEGF) normalized for housekeeper (beta-actin) expression. Standard deviations are shown. Figures 9A and 9B show that Glut1 and VEGF mRNA levels increase synergistically and additively with the addition of both Apotransferrin and small molecule activators of the HIF pathway.

Example 10. Compositions of majority ApoTf and majority HoloTf induce HIF-1α protein in human primary kidney cells.

It is well-known in the art that many small molecules used for the treatment of conditions related or provoked by hypoxia are toxic and have numerous side effects, e.g. DFO. One of the most apparent side effects of said small molecules is kidney toxicity. Therefore, in order to assess whether transferrin and/or mixtures increase HIF-1α levels in primary kidney cells; human primary kidney cells, both primary human renal proximal tubule epithelial (RPTEC) or cortical epithelial cells (HRCE) were obtained. Primary human renal proximal tubule epithelial (RPTEC) or primary cortical epithelial (HRCE) cells cultured in serum free media were treated with 4 mg/mL majority Apotransferrin, majority Holo-transferrin or various mixtures of each for 6hrs under normal oxygen levels. After 6hrs intracellular proteins were harvested and tested for HIF-1α protein levels by ELISA. Figures 10A and 10B reveal that HIF-1α levels are induced with transferrin composed of mixtures of Apo-transferrin and Holo-

transferrin in RPTEC and HRCE, respectively.

Example 11. Viability of human primary kidney cells in the presence of Transferrins or DFO, including Caspase 3/7 activation within human primary kidney cells in the presence of majority ApoTf or DFO.

Considering the anticipated safety profile of a human plasma protein, toxicity of DFO and transferrins (majority Apo, majority Holo and mixtures) was assessed in primary human kidney cells. The renal proximal tubule epithelial (RPTEC) or cortical epithelial (HRCE) cells were treated with the indicated concentrations of majority ApoTf or DFO for 48 hours (Figure 11A); and RPTEC or HRCE cells were treated for 72hrs with 4mg/mL of majority ApoTf, majority HoloTf, mixtures of transferrin (Figure 11B). After 48 or 72 hours, cells were subjected to a Cell Titer Glow viability assay. Control cells, untreated cells, were set to a value of 100% viable. The average viability and standard deviations are shown for each treatment condition. Figures 11A and 11B shows that while DFO had significant toxicity, none of the transferrin molecules showed any detrimental effects on these primary kidney cells.

In order to assess caspase 3/7 activation within human primary kidney cells in the presence of ApoTf or DFO; RPTE or HRC cells were treated with the indicated concentrations of ApoTf or DFO for 48 hours. After 48 hours, cells were subjected to a ApoGlo caspase 3/7 activation assay. Control cells, untreated cells, were set to a normalized value of 1. The average caspase activity, relative to control cells, and standard deviations are

shown in Figure 12 for each treatment condition.

Example 12. No upregulation of HIF was observed in primary human hepatocytes or NCI-H1650, a lung cell line.

As detailed above, both plasma derived Apo-transferrin and Holo-transferrin increase the cellular levels of HIF-1α, in the human neuronal cell line SH-SY5Y. In addition to neuronal cells, liver and lung organ transplants may also benefit from induction of HIF signaling. Hence, in order to assess the same; effect of transferrins on HIF-1α levels in primary hepatocytes and a lung cell line (NCI-H1650) was determined. The lung cell line NCI-H1650 or primary hepatocyte cells cultured in serum free media were treated with 4 mg/mL majority Apo-transferrin, majority Holotransferrin or pd-Transferrin for 6hrs under normal oxygen levels. After 6hrs intracellular proteins were harvested and tested for HIF-1α protein levels by ELISA. The data, as highlighted in Figures 13A and 13B, shows that HIF-1α levels are not induced with transferrin or mixtures of Apo-transferrin and Holo-transferrin in NCIH1650 or primary hepatocytes.

Example 13. Viability of NCI-H1650 and human primary hepatocytes in the presence of Transferrins.

Given the anticipated safety profile of a human plasma protein, toxicity of transferrins (majority Apo, majority Holo and pd-transferrin) in NCI-H1650 and primary human hepatocyte cells was assessed. The human lung cell line, NCI-H1650, and primary human hepatocytes were treated for 72 hours with 4mg/mL of majority ApoTf,

majority HoloTf, or pd- transferrin. After 72 hours, cells were subjected to a Cell Titer Glow viability assay. Control cells, untreated cells, were set to a value of 100% viable. The average viability and standard deviations are shown in Figures 14A and 14B for each treatment condition. The data shows that no toxicity was observed with compositions containing either majority HoloTf or majority ApoTf in lung cells, NCI-H1650, or primary hepatocytes.

CONCLUSIONS:

The experiments performed in the human neuronal cell line SH-SY5Y showed that both plasma derived Apo-transferrin and Holo-transferrin increased the cellular levels of HIF-1α. The increase in HIF-1α levels occurred under both normoxic and hypoxic conditions. Administration of Apo-transferrin to cells under normal oxygen conditions raised the levels of HIF-1α to a similar level of that seen when cells were exposed to a hypoxic environment. Exposure of SH-SY5Y cells to Apo-transferrin in normoxic conditions for longer periods increased the level of HIF-1α to a greater extent than shorter time. The human serum albumin negative controls had no effect on HIF-1α levels.

Various mixtures of ApoTf and HoloTf all upregulated HIF-1α protein in SH-SY5Y neuronal cells and primary kidney cells.

No upregulation of HIF-1α was observed in primary human hepatocytes, or NCI-H1650, a lung cell line.

Various mixtures of ApoTf and HoloTf all upregulated HIF-1α target genes in SH-SY5Y neuronal cells.

No toxicity was observed with compositions containing either majority HoloTf or majority ApoTf in any cell type (neuronal, lung, kidney or hepatocyte) or *in vivo*.

In vivo treatment of rats in a neurological stress model of ischemia-reperfusion showed that transferrin (composed of mostly ApoTf) protects rat cells from infarct.

Mixtures comprising mostly of ApoTf or HoloTf protected neuronal cells from the toxic effects of Abeta (1-42) oligomer.

Only mixtures composed of majority ApoTf had synergistic effects with M30 or DFO, and these synergistic activities only occurred in SH-SY5Y neuronal cells.

CLAIMS

1. A method of treating a HIF-related pathological condition, comprising:

a. diagnosing for a HIF-related pathological condition in a patient in need thereof, and

b. administering to the patient a composition comprising therapeutically effective amounts of transferrin.

2. The method of claim 1, wherein the composition comprises therapeutically-effective amounts of transferrin and either an iron chelator or PHD2 enzyme inhibitor.

3. The method of claim 1, wherein the HIF-related pathological condition is associated with organ transplantation in a transplant recipient.

4. The method of claim 3, wherein the organ has been treated with a composition comprising transferrin in preparation for transplantation into the recipient.

5. The method of claim 4, where the organ has been treated with a composition comprising transferrin and iron chelator or PHD2 enzyme inhibitor in preparation for transplantation into the recipient.

6. The method of claim 1, wherein the condition is associated with ischemia or oxygen deprivation in a patient prior to surgery.

7. The method of claim 1, wherein the condition is ischemia due to cardiac arrest, thrombotic clots, or traumatic

injury.

8. The method of claim 1, where interruption of blood flow occurs during a surgical intervention.

9. The method of claim 1, wherein the condition is due to a neurodegenerative disease selected from the group consisting of Alzheimer's disease, Parkinson's disease, Huntington's disease and Amylotrophic Lateral Sclerosis.

10. The method of claim 1, wherein said transferrin is recombinant.

11. The method of claim 1, wherein said transferrin is modified by pegylation, glycosylation, polysialylation, or other physical modifications to extend plasma half-life of the protein, including covalent fusion to domains that extend half-life in blood, such as the Fc domain of immunoglobulin, albumin, glucagon-like peptide-1, exendin-4, XTEN, etc.

12. The method of claim 1, wherein said transferrin is a protein conjugate between full length transferrin or fragments of transferrin with any other protein, protein fragment, or peptide.

13. The method of claim 1, wherein said transferrin is modified to increase metal binding affinity.

14. The method of claim 1, wherein said transferrin are derivatives of transferrin comprising more than 50% similarity.

15. The method of claim 1, wherein said transferrin is

holo-transferrin alone.

16. The method of claim 1, wherein said transferrin is apo-transferrin alone.

17. The method of claim 1, wherein said transferrin is a mixture of apotransferrin and holo-transferrin, in a ratio of 99% Apo-Tf:1% Holo-Tf to 30% Apo- Tf:70% Holo-Tf, or the proportion that is most comparable to a fraction obtained or purified from biological fluids.

18. The method of claim 1, wherein said composition further comprises an iron chelator.

19. The method of claim 1, wherein said iron chelator is M30, deferoxamine (DFO), Deferasirox, deferiprone, deferitrin, L1NA11, CP363, CP502, or Ethylenediaminetetraacetic acid (EDTA).

20. The method of claim 1, wherein said PHD2 enzyme inhibitor is IOX2, IOX3, dimethyloxallyl glycine or other 2-oxoglutarate binding site molecules.

21. A pharmaceutical composition comprising transferrin to attain a blood concentration of at least 25uM and up to 250uM, but most preferably 175uM, and 500- 6000mg, but most preferably 1000mg, of deferoxamine (DFO).