A PEPTIDE BASED DRUG FOR THE INHIBITION OF ANGIOGENESIS

ABSTRACT

The present invention relates to a peptide based drug for the inhibition of angiogenesis. More particularly peptides based drug are derived from the sequence of lysyl oxidase (LOX) enzyme which inhibits the enzyme activity and hypoxia induced angiogenesis. The said peptides are derived from the conserved regions among the LOX isoforms. The current invention also provides a method for the treatment of angiogenesis. The present invention also provides a drug composition & a kit for the treatment of angiogenesis.

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WE CLAIM:

1.) A peptide based drug for the inhibition of angiogenesis selected from the group consisting of SEQ ID NO: 01 and SEQ ID NO: 02.

2.) The peptide based drug for the inhibition of angiogenesis as claimed in claim 1, where in angiogenesis is induced by hypoxia and copper.

3.) A drug delivery composition for the inhibition of angiogenesis consisting of one or more peptide selected from the group consisting of SEQ ID NO: 01 and SEQ ID NO: 02, and a pharmaceutically acceptable carrier.

4.) A method for the treatment of angiogenesis, where in the method consists of administering the peptide based drug as claimed in claim1 to a subject.

5.) The method as claimed in claim 4 wherein the drug is administered Sublingually, Orally, Sub – cutaneously, Topically, Intra – tumorally or Intra-vitreously.

6.) The method for the treatment of angiogenesis as claimed in claim 4, where in the angiogenesis is induced by hypoxia and copper.

7.) A kit for the treatment of angiogenesis, where in the said kit comprising of ,

a. peptides sequence having SEQ ID NO: 01 or SEQ ID NO: 02,
b. a pharmaceutically acceptable carrier,
c. Instruction manual for using said kit.

8.) The kit for the treatment of angiogenesis as claimed in claim 7, where in the angiogenesis is induced by hypoxia and copper.

9.) Use of peptide as claimed in claim 1, for the inhibition of angiogenesis.

Dated 28th Day of march '2014.

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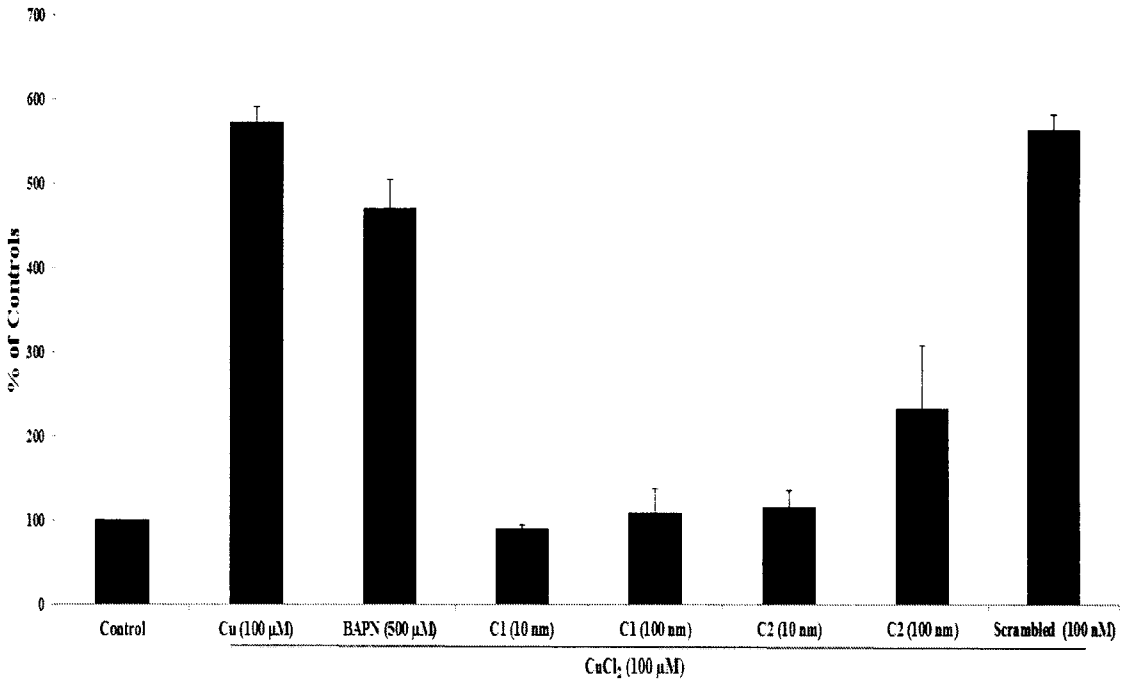

FIG: I

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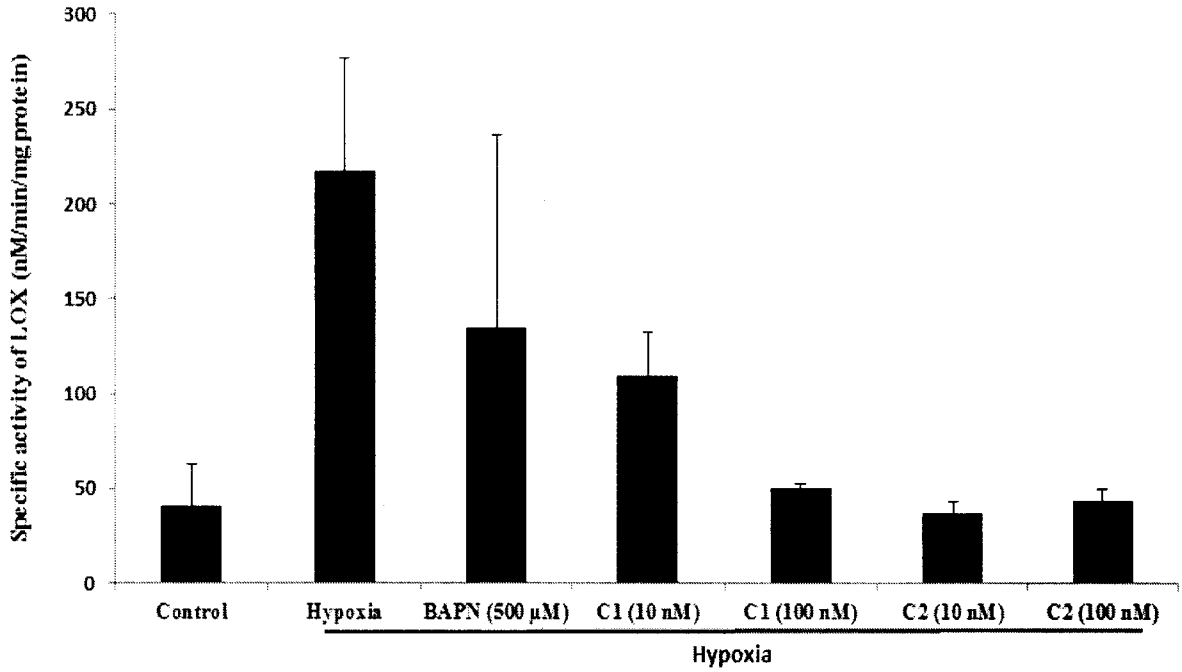

FIG: II

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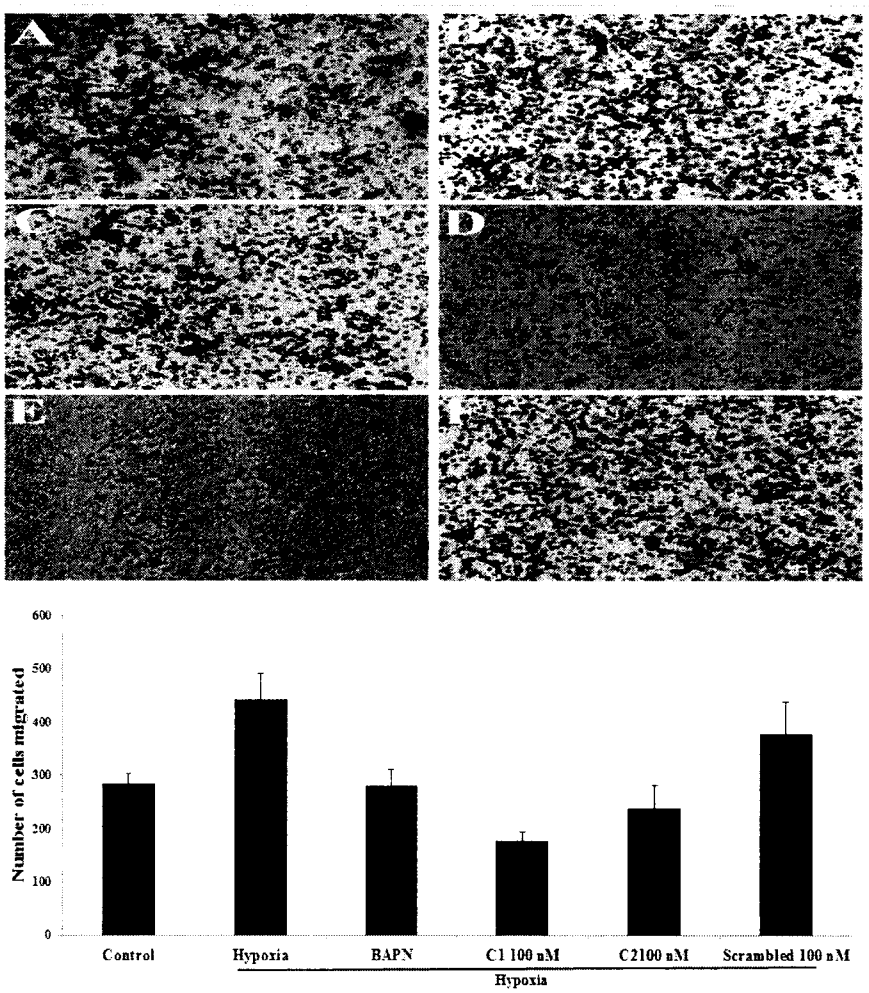

FIG: III

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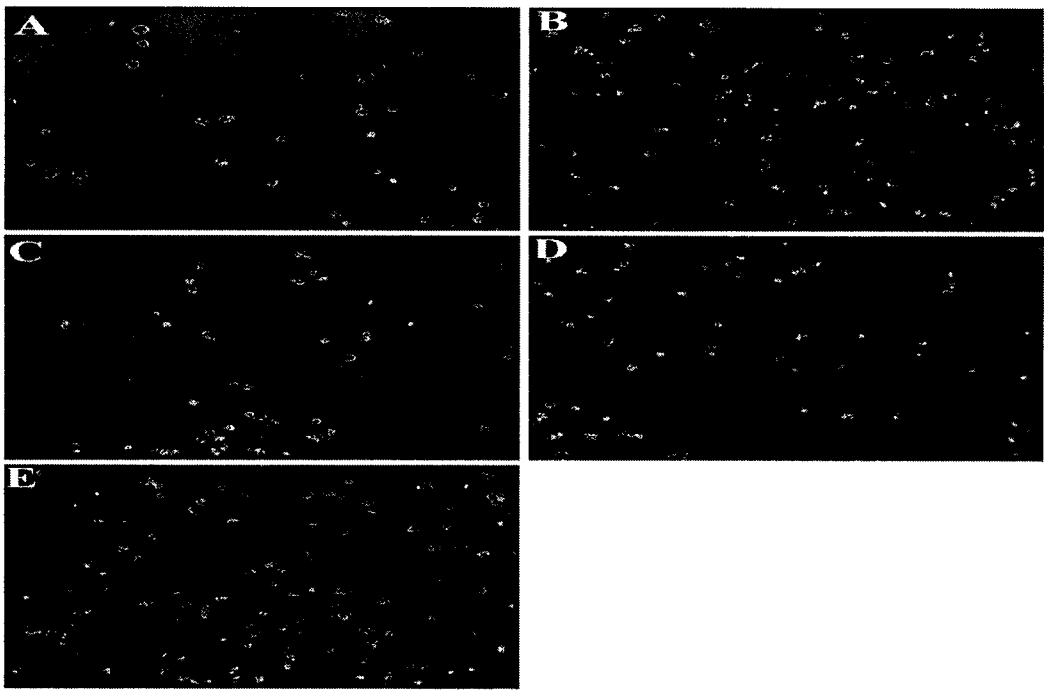

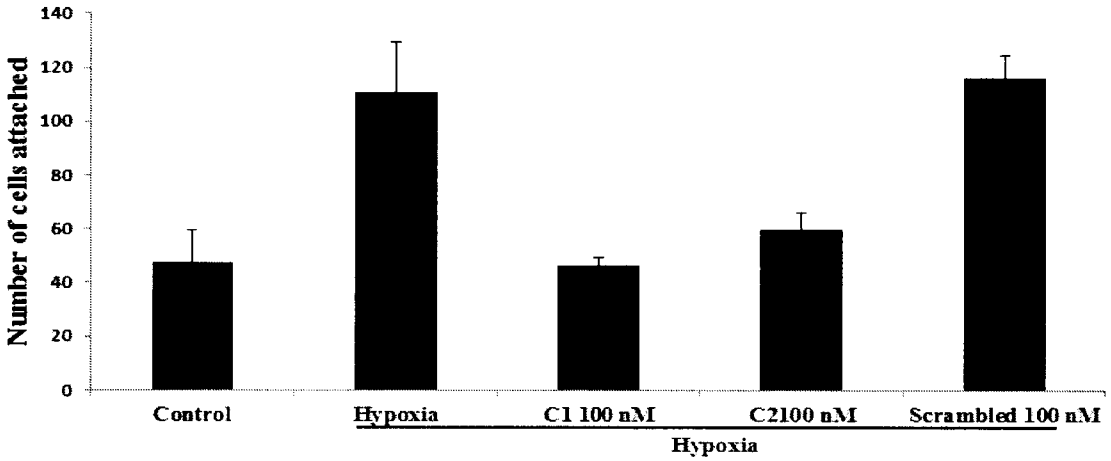

FIG: IV

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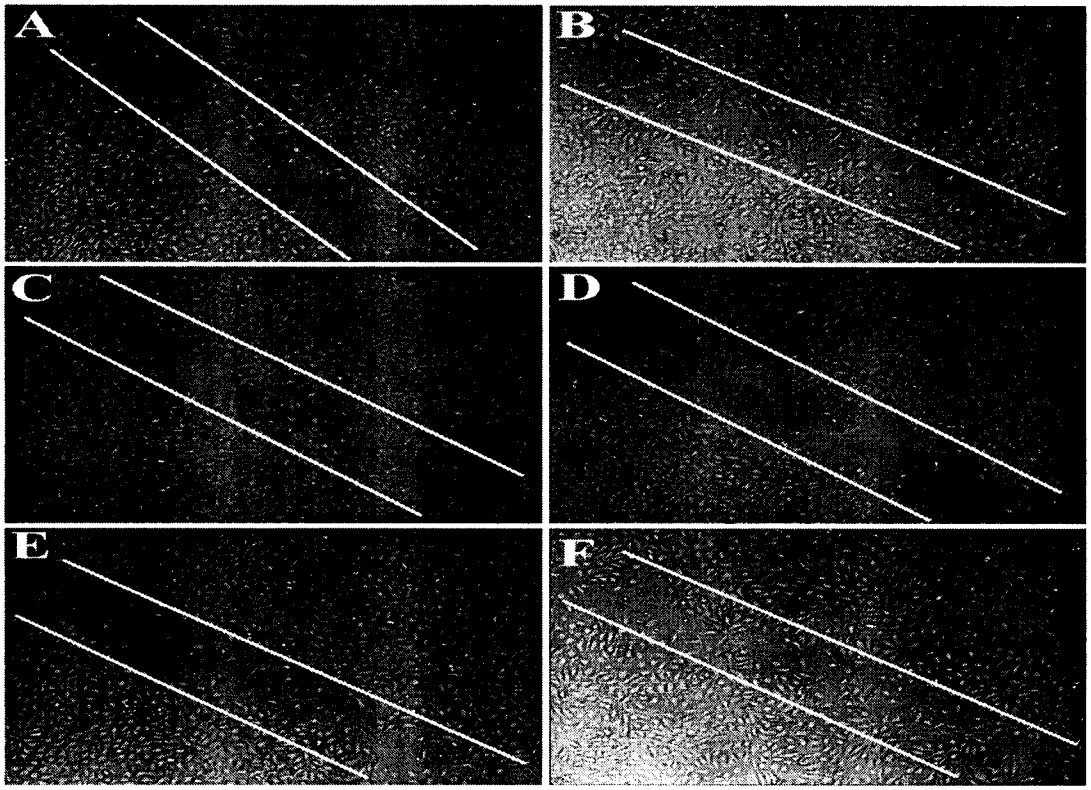

FIG: V

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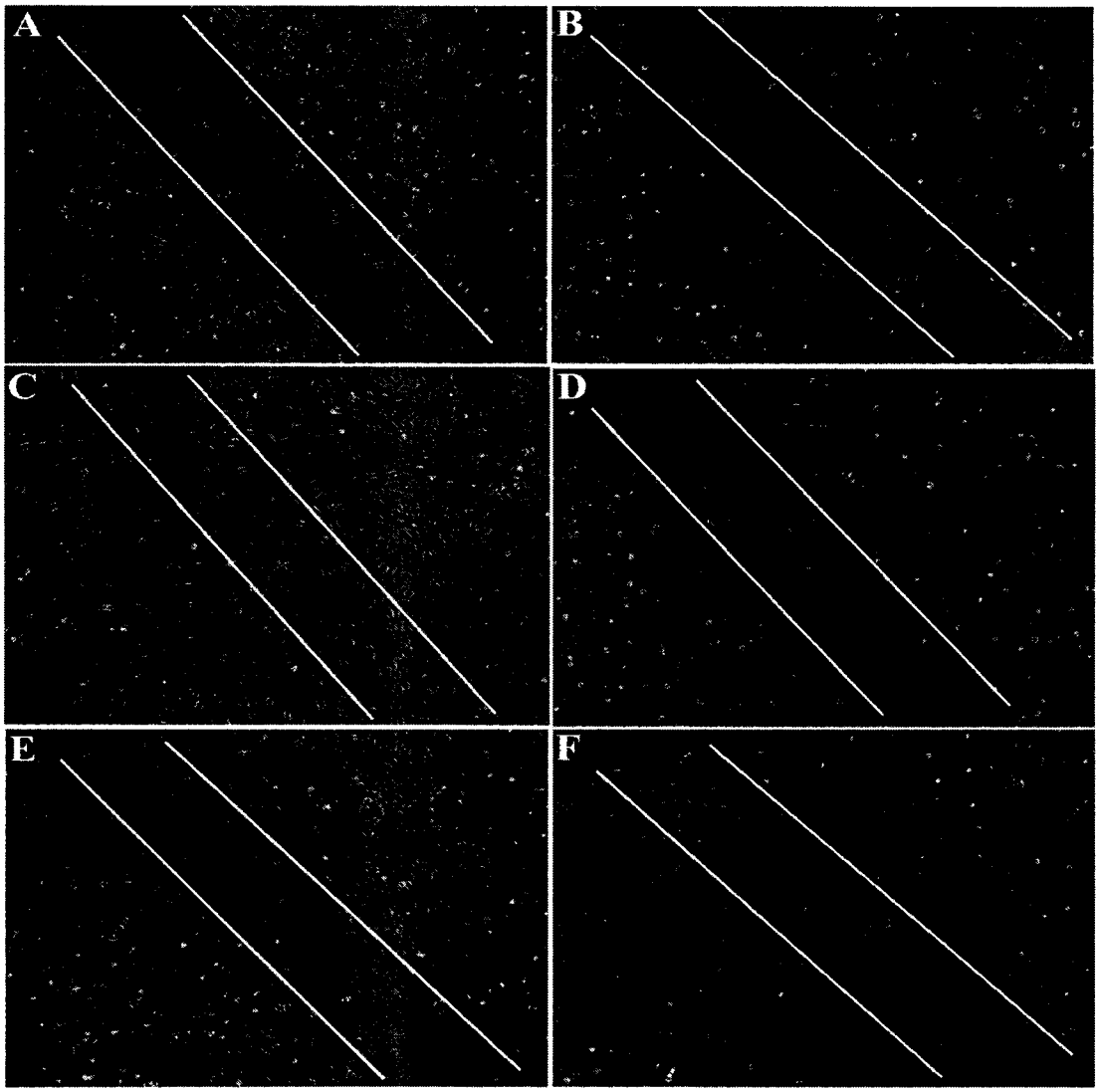

FIG: VI

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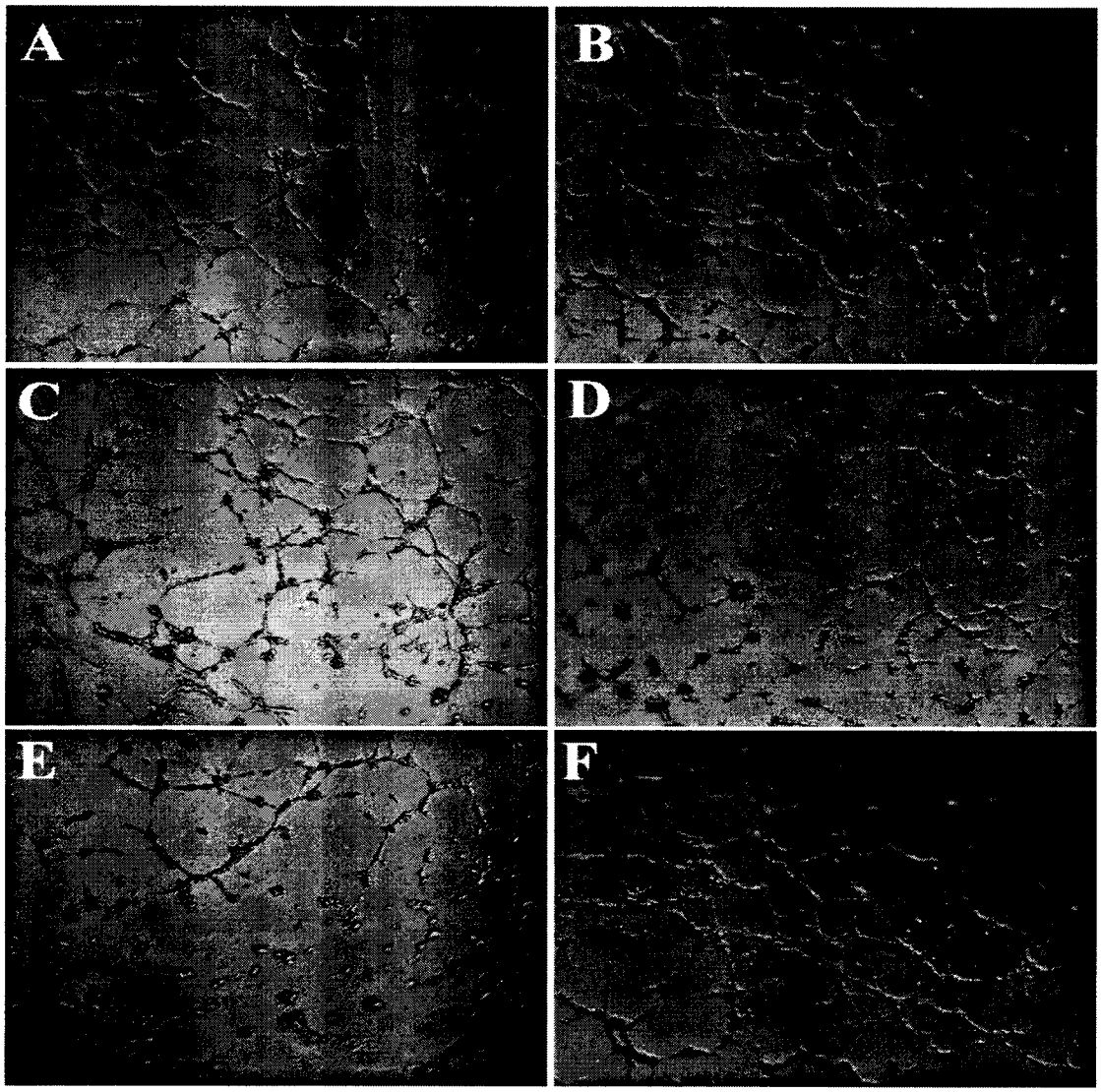

FIG: VII

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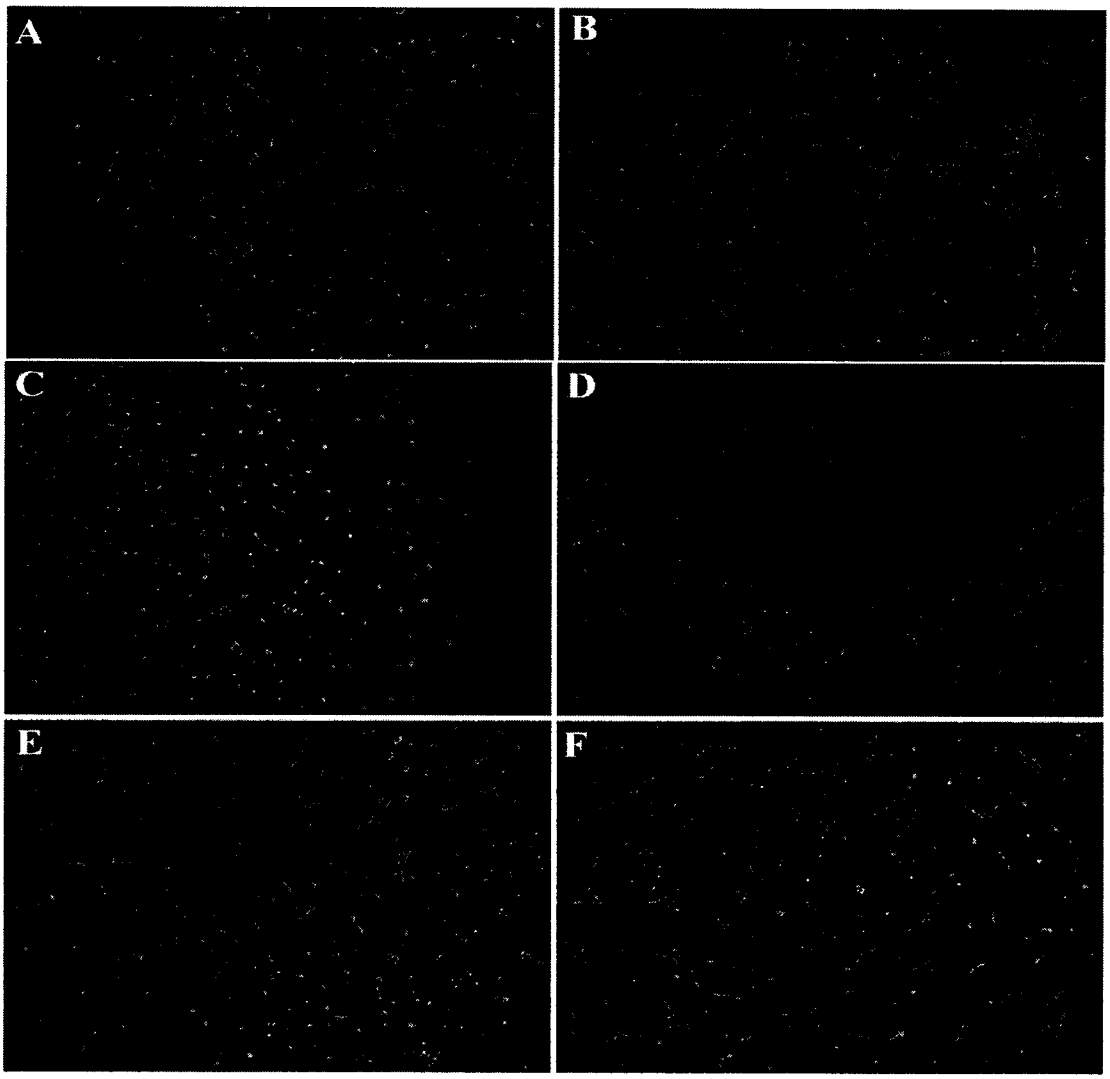

FIG: VIII

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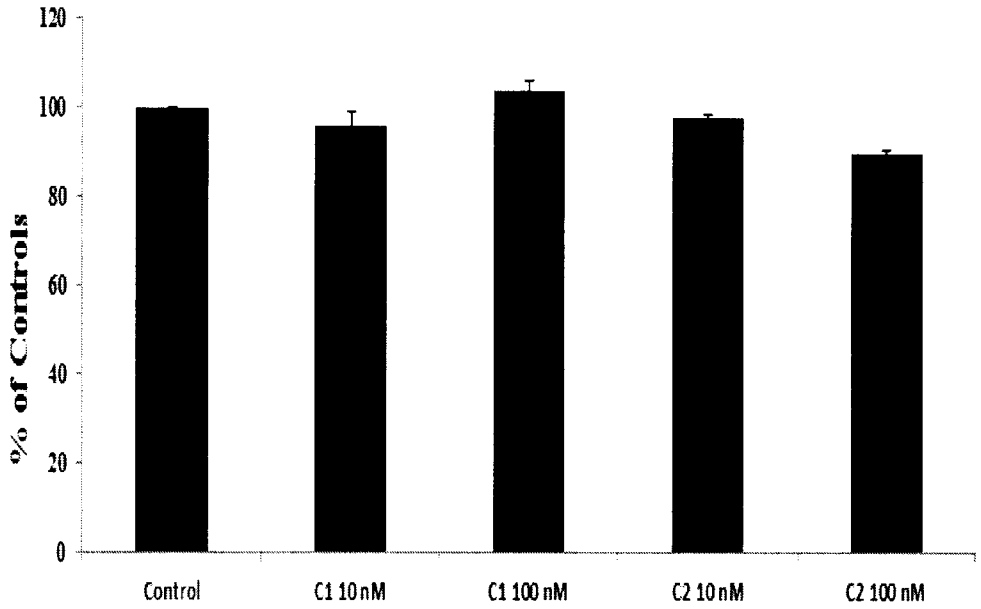

FIG: IX

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FIELD OF THE PRESENT INVENTION

The present invention relates to a peptide based drug for the inhibition of angiogenesis. More particularly peptides based drug is derived from the amino acid sequence of lysyl oxidase (LOX) enzyme which inhibits the enzyme activity and hypoxia induced angiogenesis. The said peptides are derived from the conserved regions among the LOX isoforms. The current invention also provides a method for the treatment of angiogenesis. The present invention also provides a drug composition & a kit for the treatment of angiogenesis.

BACKGROUND OF THE PRESENT INVENTION

Regulating angiogenesis has become one of the important strategies in controlling cancer since Judah Folkman reported in the year 1971 that all cancer tumors are angiogenesis-dependent. Besides cancer other processes like wound healing, pregnancy, heart diseases, diabetic retinopathy, all are associated with angiogenesis. Globally, researchers are experimenting on different strategies to control abnormal angiogenesis. They have used many molecules as targets for regulating abnormal angiogenesis. Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), nitric oxide, matrix metalloproteinase, angiostatin, endostatin etc., are among these molecules. But none developed in to an effective drug. Most of them are associated with side effects and hence there is a great demand for the identification of new targets and drug development.

Lysyl oxidase (LOX) is one of the molecules that has recently become of interest in angiogenesis and cancer research. Its expression and activity has been found to be altered in diseases related to angiogenesis like breast cancer, heart disorders and even in diabetic retinopathy. Hypoxic conditions induce the increased production or upregulation of pro-angiogenic factors and it was shown that LOX is essential for hypoxia-induced metastasis by Erler *et al.*, 2006.

OBJECT OF THE PRESENT INVENTION

The main objective of the present invention is to provide a peptide based drug for the inhibition of angiogenesis.

Another objective of the present invention is to provide a method for the treatment of angiogenesis.

Yet another objective of the present invention is to provide a drug composition for the treatment of angiogenesis.

Another objective of the present invention is to provide a kit for the treatment of angiogenesis.

Yet another objective of the current invention is to design and develop functional peptides from the amino acid sequence of LOX using computational methods.

Another objective of the current invention is to evaluate the function of these peptides in inhibiting the enzymatic activity of LOX and hypoxia induced angiogenesis.

SUMMARY OF THE PRESENT INVENTION

Accordingly the present invention provides peptides derived from LOX amino acid sequence which inhibits its enzymatic activity and hypoxia induced angiogenesis
In an embodiment of the invention, a peptide based drug for the inhibition of angiogenesis is selected from the group of sequences consisting of SEQ ID NO: 01 and SEQ ID NO: 02.
In one embodiment of the invention, angiogenesis is induced by hypoxia and copper.

In another embodiment of the invention, a drug delivery composition for the inhibition of angiogenesis is consisting of one or more peptide selected from the group consisting of SEQ ID NO: 01 and SEQ ID NO: 02, and a pharmaceutically acceptable carrier.

In yet another embodiment, A method for preparing the peptide based drug consisting of the following steps:

a. The sequences of peptides C1 and C2 were identified by multi aligning the primary sequences of all the five isoforms of LOX (Given in example1).
b. The short sequences of peptides are essentially made synthetically using FMOC chemistry and peptide synthesizers.
c. These crude peptides are purified by reverse phase chromatography using 0.1% tri fluroacetic acid and 0.1% trifluro acetic acid in acetonitrile and dried to remove solvents in a lyophilyser.
d. The mature Lysyl oxidase (mLOX) DNA insert (750bp) are cloned by infusion cloning and transformed in pQe-30Xa vector. The protein is purified using a Ni-NTA affinity column. The purity is assessed with SDS-PAGE and Immunoblotting. His tag present in the N- terminal of the purified protein is cleaved by using the factor Xa–Qiagen
e. Endothelial cells are suspended and cultured in NUNC tissue culture flask for cultivating
f. Then the cells are incubated at 37C with 5% CO2 incubator was used
g. The cells were observed for growth using zeiss microscope.
h. The cells were exposed to $CuCl_2$ (100 µM) and $CoCl_2$ (100 µM) to induce hypoxic conditions (Refer example 3)
i. The cells are treated with C peptides 10nM and 100nM concentration for12 – 15 hours.
j. The cells are subjected to viability assay by MTT (Refer example 10).
k. LOX activity is measured by amplex red assay by the method of Palamakumbura et al., 2002.
l. The cells are subjected to trans well migration (Refer example 4)
m. The cells are subjected to attachment (Refer example 5)
n. The cells are subjected to HUVECs migration. (Refer example 6)
o. The results are depicted from Fig I to Fig IX.

In still another embodiment, a method for the treatment of angiogenesis, where in the method consisting of: administering the peptides sequence having SEQ ID NO: 01 and SEQ ID NO: 02

In still another embodiment, angiogenesis is induced by hypoxia and copper.

In still another embodiment, A kit for the treatment of angiogenesis, where in the said kit comprising of,

a) peptides sequence having SEQ ID NO: 01 and SEQ ID NO: 02,
b) a pharmaceutically acceptable carrier,
c) Instruction manual for using said kit.

In still another embodiment, angiogenesis is induced by hypoxia and copper.

In still another embodiment, use of the peptide for the inhibition of angiogenesis.

BRIEF DESCRIPTION OF THE DRAWINGS:

FIG. I : Depicts the results of $CuCl_2$ induced rLOX activity. The activity of rLOX is expressed as a percentage of controls. 100 ng of rLOX was used for the experiment. C peptides co-incubated with Cu induced rLOX showed inhibition of LOX activity and they were more effective than the β-aminopropionitrile (BAPN), the known inhibitor of LOX.

FIG. II : Depicts the results of hypoxia induced LOX activity in HUVECs. $CuCl_2$ (100 µM) and Cobalt chloride ($CoCl_2$)-100 µM were used to induce hypoxic conditions. The C peptides were more effective compared to BAPN and the scrambled peptide.

FIG. III : Depicts the results of hypoxia induced HUVECs trans well migration assay. Hypoxia was induced as mentioned earlier. Hypoxia induced migration of cells, but the C peptides resisted this

migration more effectively than BAPN and scrambled peptide.

FIG. IV : Depicts the results of hypoxia induced HUVECs attachment assay. From the figure it is readily inferred that the C peptides decreased the cell attachment in hypoxic conditions.

FIG. V: Depicts the results of $CuCl_2$ induced HUVECs migration assay (scratch assay). $CuCl_2$ (100 µM) induced migration of cells but the C peptides resisted this migration to a greater extent than BAPN and scrambled peptide.

FIG. VI: Depicts the results of hypoxia induced HUVECs migration assay (scratch assay). Hypoxia was induced as mentioned earlier. Hypoxia induced migration of cells, but the C peptides resisted this migration to a greater extent than BAPN and scrambled peptide in hypoxic condition also.

FIG. VII : Depicts the results of $CuCl_2$ induced HUVECs tube formation assay. The assay was done according to the manufacturer's protocol (Millipore). $CuCl_2$ (100 µM) induced tube formation, but the C peptides reduced tube formation to a greater extent than BAPN, and the scrambled peptide did not show any effect.

FIG. VIII: Depicts the results of hypoxia induced HUVECs tube formation assay. The assay was done according to the manufacturer's protocol (Millipore). Hypoxia was induced as mentioned earlier. Hypoxia induced tube formation but the C peptides reduced tube formation. BAPN also reduced tube formation, but the scrambled peptide did not show any effect in hypoxic condition.

FIG. IX: depicts that the C peptides are nontoxic to HUVECs. This was proved from the MTT assay.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

In accordance with the objectives of the present invention, the invention provides peptides which inhibit LOX enzymatic activity and hypoxia induced angiogenesis.

C peptides inhibited $CuCl_2$ induced rLOX activity

Incubation of $CuCl_2$ at a dose of 100 µM with rLOX induced rLOX activity. Copper is one of the co-factors of LOX. Co-incubation of C peptides with this conditioned rLOX brought down its activity. BAPN, the known inhibitor of LOX, could bring down the LOX activity at 500 µM. But the peptides were more effective at very low concentrations compared to BAPN. Of the two peptides C1 was the most effective. A scrambled peptide was also included in the study which showed no effect. The results are depicted in FIG. I.

C peptides inhibited hypoxia induced LOX activity in HUVECs.

LOX is essential for hypoxia induced metastasis. We used both $CuCl_2$ and $CoCl_2$ at 100 µM to induce hypoxia in HUVEC culture. The LOX activity in the conditioned medium was measured. The results are depicted in FIG. II Under hypoxic conditions, LOX activity was significantly increased, but the C peptides significantly reduced these values. Scrambled peptides and BAPN were less effective in reducing the hypoxia induced LOX activity.

C peptides inhibited hypoxia induced HUVECs trans well migration.

The results are depicted in FIG. III. Hypoxia was induced as mentioned earlier. Cells (HUVECs) were treated with C peptides/ BAPN/ scrambled peptide and allowed to migrate through 8 microns from upper chamber to the lower chamber in 24 well plates (using Millipore trans well chambers). After 6 hours the migrated cells were fixed, stained and photographed. Migrated cells were counted using imageJ software. From FIG. III it is evident that hypoxia induced migration of cells. But co incubation with C peptides resisted this migration, and the extent of this inhibition was greater when compared with BAPN and scrambled peptide. This is further validation that the C peptides inhibit hypoxia induced HUVECs migration.

C peptides inhibited hypoxia induced HUVECs attachment.

Cells (HUVECs) were treated with C peptides/ BAPN /scrambled peptide and seeded in a gelatin coated plated under hypoxic conditions. Hypoxia was created as mentioned earlier. After 30 min the

unattached cells were removed. Attached cells were fixed, stained, photographed and counted using imageJ software. The results are depicted in FIG. IV. Hypoxia increased the number of cells attached and the C peptides decreased the number of cells attached. Scrambled peptides showed the no effect.

C peptides inhibited $CuCl_2$ induced HUVECs migration.

The results are depicted in FIG. V. Cells (HUVECs) were seeded in 12 well plates and grown to 85-95% confluence. These cells were subjected to overnight serum starvation and a scratch was made across the middle of the well using a 200 µl pipette tip. Cells were incubated with the peptides/ BAPN/ scrambled peptide in the presence of $CuCl_2$ (100 µM) for 12-15 h. The cells were observed under microscope and were photographed. It was found that at these experimental conditions, the peptides C1 and C2 inhibited $CuCl_2$ induced migration of the cells. The effect of the peptides was compared with control, BAPN (500 µM) scrambled peptide (100 nM) and $CuCl_2$ groups. The C peptides were more effective than BAPN in inhibiting cell migration. Scrambled peptide showed no effect.

C peptides inhibited hypoxia induced HUVECs migration.

Cells (HUVECs) were seeded in 12 well plates and grown to 85-95% confluence. These cells were subjected to overnight serum starvation and a scratch was made across the middle of the well using a 200 µl pipette tip. $CuCl_2$ (100 µM) and $CoCl_2$ (100 µM) was used to induce hypoxic conditions. Cells were incubated with the C peptides/ BAPN/ scrambled peptide in hypoxic condition for 12-15 h. The cells were observed under microscope and were photographed. The results are depicted in FIG. VI. It was found that at these experimental conditions the peptides C1 and C2 inhibited hypoxia induced migration of the cells. The effect of the peptides was compared with control, BAPN (500 µM) scrambled peptide (100 nM) and hypoxic groups. The C peptides were more effective than BAPN in inhibiting cell migration.

C peptides inhibited $CuCl_2$ induced tube formation.

This study was designed to investigate the anti-angiogenic effect of the C peptides in non-cancerous models of angiogenesis. Tube Formation Assay (*in vitro* Angiogenesis) provides an easy, robust system to assess angiogenesis *in vitro* and it was done by using *in vitro* angiogenesis assay kit – Millipore. HUVECs were used for this assay. Cells were incubated with the C peptides/ BAPN/ scrambled peptide in the presence of $CuCl_2$ (100 µM) in serum depleted medium and the assay was done according to manufacturer's protocol. The results are depicted in FIG VII. It was clear that $CuCl_2$ (100 µM) induced tube formation but the cells incubated with the C peptides showed fewer or weaker tubes. C peptides showed better inhibition of tubes than cells incubated with BAPN.

C peptides inhibited hypoxia induced HUVECs tube formation.

Tube formation assay was done as mentioned as earlier. $CuCl_2$ (100 µM) and $CoCl_2$ (100 µM) were used to induce hypoxic conditions. Cells were incubated with the C peptides/ BAPN/ scrambled peptide in hypoxic condition in serum depleted medium and the assay was done according to manufacturer's protocol. Tubes were obvious and present in greater numbers in hypoxic condition groups compared to the normal control groups. Inhibition of tubes was observed with peptides C1 (100 nM) and C2 (100 nM). BAPN also showed potent inhibition. Thus it was shown that the C peptides have antiangiogenic effect on non-cancerous model.

C peptides are nontoxic to HUVECs

MTT assay was done to study the toxicity of the C peptides in HUVECs. Cells were seeded in 96-well plates and grown under normal conditions. After incubating the cells with peptides (100 nM) for 4h, 20 µl of 5 mg/ml MTT reagent was added and the cells were incubated for 2-4 h at 37°C for cells to develop the formazan product. Subsequently, the MTT-containing medium was removed, and 200 µl of DMSO (Sigma) was added to solubilize the formazan. Absorbance values were determined on a

microplate reader at a wavelength of 570 nm. C peptides did not show any obvious toxic effects on normal endothelial cells (HUVECs).

EXAMPLES:

"*The following examples are given by way of illustration of the present invention and therefore should not be construed to limit the scope of the present invention*".

Example 1

Design of peptides from LOX amino acid sequence

SEQ ID:01 : C1-VAEGHKASFCLED (13 mer) in lox 315 -328

SEQ ID:02: C2-ESDYTNNVVRCD (12 mer) in lox 358 - 370

Based on Multiple Sequence Alignment - CLUSTALW, we have identified sequences from the C-terminal region of LOX family, which are conserved among the isoforms

Examples for Peptide synthesis

Peptides are procured from a commercial source. Essentially they made synthetically using FMOC chemistry and peptide synthesizers. Using this chemistry and the equipment, one can produce any given sequence. This commercial source provides custom peptides to customers from the biomedical research community, biotechnology and pharmaceutical industry with high standards of quality. Essentially this technique incorporate alpha amino acids in peptide of any desired sequence with one end attached to a solid support matrix. This approach has a flexibility of including non natural amino acids such as D isoforms, beta amino acids and can be conjugated with tags and can be modified chemically to suit the function

Example 2

Assay for C peptides that reduce $CuCl_2$ induced rLOX activity

To study the effect of peptides on $CuCl_2$ induced recombinant LOX activity, we added rLOX (100 ng) with all designed peptides at various concentrations and LOX assay was done according to the method of *Palamakumbura et al., 2002*. $CuCl_2$ at100 µM was also added to induce rLOX activity.

Expression and purification of recombinant human lysyl oxidase.

The mature Lysyl oxidase (mLOX) DNA insert (750bp) was cloned by infusion cloning and transformed in pQe-30Xa vector. The protein was purified using a Ni-NTA affinity column. The purity was assessed with SDS-PAGE and Immunoblotting. His tag present in the N- terminal of the purified protein was cleaved by using the factor Xa–Qiagen

Example 3

Assay for C peptides that reduce hypoxia induced LOX activity in HUVECs.

HUVECs (passage number between 3 and 5) were used throughout the experiment.

To assess the effect of the C peptides on LOX activity, we cultured cells to 90-100% confluence in complete growth medium (EGM, LONZA) in 12 well plates. We used $CuCl_2$ (100 µM) and $CoCl_2$ (100 µM) to induce hypoxic conditions. Overnight serum starved HUVECs were exposed to BAPN - 500 µM/ C peptides/ scrambled peptide, for 4 h under hypoxic conditions. After the incubation period, conditioned media was aspirated, centrifuged and concentrated by speed vac. Then LOX assay was done in conditioned media according to the method of *Palamakumbura et al., 2002*.

Example 4

Assay for C peptides that reduce hypoxia induced HUVECs trans well migration.

HUVECs were incubated with BAPN - 500 µM/ C peptides/ scrambled peptide, for 6 h under hypoxic conditions in serum depleted medium. We used $CuCl_2$ (100 µM) and $CoCl_2$ (100 µM) to induce hypoxic conditions. Transwell migration assay was done using multicell hanging cell culture insert (24 well, 8 µm PET) from Millipore. Placed the multicell hanging cell culture insert in 24 well plates and the treated cells were loaded in to it. Serum rich medium in the lower chamber was added and incubated at 37°C for 4-6 h. Cell were fixed and stained after incubation. Non migrant cells were wiped off with cotton swab and migrant cells were photographed and counted using imageJ software.

Example 5

Assay for C peptides that reduce hypoxia induced HUVECs attachment.

Cells were incubated with the peptides/scrambled peptide for 30 min under hypoxic conditions and seeded into gelatin coated, 24 well plates. After 30 min the floating cells were aspirated out and the attached cells in the plates were fixed and stained. They were observed under microscope and counted after taking photographs using imageJ software.

Example 6

Assay for C peptides that reduce $CuCl_2$ induced HUVECs migration.

Cells were seeded in 12 well plates and grown to 80-100% confluence. These cells were given overnight serum starvation and scratch was made through the middle of the well using a 200 µl pipette tip. Cells were incubated with the peptides/ BAPN/ Scrambled peptide in the presence of $CuCl_2$ (100 µM) for 12-15 h. The cells were observed under microscope and were photographed and analyzed.

Example 7

Assay for C peptides that reduce hypoxia induced HUVECs migration.

Cells (HUVECs) were seeded in 12 well plates and grown to 85-95% confluence. These cells were subjected to overnight serum starvation and a scratch was made across the middle of the well using a 200 µl pipette tip. $CuCl_2$ (100 µM) and $CoCl_2$ (100 µM) was used to induce hypoxic conditions. Cells were incubated with the peptides/ BAPN/ Scrambled peptide in hypoxic conditions for 12-15 h. The cells were observed under microscope and were photographed and analyzed.

Example 8

Assay for C peptides that inhibit $CuCl_2$ induced HUVECs tube formation.

This study was designed to investigate the anti-angiogenic effect of the C peptide in non-cancerous models of angiogenesis. Tube Formation Assay was done by using *in vitro* angiogenesis assay kit – Millipore. HUVECs were used for this assay. Cells were incubated with the C peptides/ BAPN/ scrambled peptide in the presence of $CuCl_2$ (100 µM) in serum depleted medium and the assay was done according to manufacturer's protocol. After 6 h of incubation the wells were photographed and analyzed.

Example 9

Assay for C peptides that inhibit hypoxia induced HUVECs tube formation.

Tube Formation Assay was done by using *in vitro* angiogenesis assay kit – Millipore. $CuCl_2$ (100 µM) and $CoCl_2$ (100 µM) were used to induce hypoxic conditions. Cells were incubated with the C peptides/ BAPN/ scrambled peptide in hypoxic conditions in serum depleted medium and the assay was done according to manufacturer's protocol. After 6h of incubation the wells were photographed and analyzed.

Example 10

MTT assay

Around 500 to 1000 cells were seeded in 96 well plates. After 24 h, these cells were exposed to C peptides and incubated for 4h. Removed the media and added 20 µl of MTT solution (5 mg/ml in