Inhibition of Ocular Tumor and Endothelial Cell Growth with a $TEAD4_{216}$ Peptide Fragment

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Abstract: *Purpose:* Transcriptional enhancer factor 1-related (RTEF-1) also known as TEAD4 is expressed in ocular vascular endothelial cells and plays a role in the control of VEGF expression. Alternative processing of TEAD4 hnRNA results in different proteins able to stimulate or inhibit VEGF gene transcription. The purpose of this study is to test whether short peptide fragments (STY-RMR), representing functional domains of the inhibitory $TEAD4_{216}$ isoform, can inhibit tumor as well as endothelial cell proliferation.

Experimental Design: Cell proliferation was assessed using a colorimetric assay in cell lines incubated with STY-RMR, the amount of secreted VEGF within media was determined both in treated and control cell lines.

Results: Significant dose dependent inhibition of cell proliferation was observed. Maximal inhibition of ocular melanoma (Mel 202 and Mel 207) cell proliferation was observed at a dose of 30 mg/100ml of STY-RMR (87% and 60% inhibition, respectively). At the same dose, more than 50% inhibition was observed in retinoblastoma and breast cancer cells ($P < 0.001$). Significant inhibition of primate ocular endothelial cell proliferation (42% at 30 mg/100 ml ($p < 0.001$), and retinal pigment epithelial cells showed also a 75% inhibition ($p = 0.007$). Secreted VEGF was decreased in the media of all tested cell lines that had been exposed to STY-RMR.

Conclusion: Functional short peptide domains derived from the $TEAD4_{216}$ isoform may prove to be useful for treatment of ocular tumors and other VEGF dependent neovascular disease.

Inhibition of proliferation and VEGF production within ocular endothelial cells indicate the potential of this agent to treat age-related macular degeneration (ARMD) and diabetic retinopathy (DR).

Keywords: Ocular tumors, Age-related macular degeneration (ARMD), Diabetic retinopathy, STY-RMR, Endothelial cell.

INTRODUCTION

Solid tumors depend on the formation of new blood vessels from preexisting vessels to supply them with nutrients and oxygen in order to grow beyond a size of 1–2 mm^3 [1]. In addition to the need for an expanding vascular network, evidence suggests that some tumor cell proliferation can be directly influenced by VEGF auto-regulation. Angiogenesis is a complex multistep process which starts with vascular endothelial growth factor (VEGF) induced vasodilatation and increased vascular permeability of pre-existing capillaries, or post-capillary venules [2].

Transcriptional enhancer factor 1-related (TEAD4) is present within ocular vascular endothelial cells and plays a role in the control of the transcription of the VEGF gene [3] Full-length TEAD4 is known to stimulate cell proliferation *in vitro*. In cancer biology, internal hypoxic conditions are a common feature of solid tumors [4-6], gene transcripts associated with cancer metastasis are up regulated under hypoxic conditions and hypoxic gene signatures are associated with poorer prognosis [7]. Hypoxia-induced genes like VEGF-A are set on initiating tumor vascularization [8]. TEAD4 plays an important role in transcriptional regulation of angiogenic genes in hypoxic endothelial cells and independent of HIF-1α [9]. However, other studies suggest that TEAD4 act via HIF-1 as a key regulator of angiogenesis in response to hypoxia [10].

We have previously shown that various TEAD4 domain is the only domain present in the inhibitor $TEAD4_{216}$ and absent in the stimulator $TEAD4_{148}$, therefore we hypothesized that this small fragment of

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26 amino acids may mediate this inhibitory effect. This STY domain was linked to a cell penetrating peptide motif (RMR) to test the potential of this agent to inhibit tumor cell proliferation i*n vitro*. We have previously demonstrated isoforms (generated through alternative hnRNA splicing) may be stimulatory ($TEAD4_{148}$) or inhibitory ($TEAD4_{216}$) to cell proliferation. A Ser-Thr-Tyr (STY) that a variety of human tumors expressed isoforms of TEAD4, therefore our purpose was to test whether a short peptide fragment, representing functional domains of the 651 $TEAD4_{216}$ isoform, can inhibit tumor and endothelial cell proliferation.

METHODS

A 26 amino acid sequence corresponding to a Ser-Thr-Tyr domain within $TEAD4_{216}$ (Figure **1**), linked to a 10 amino acid cell importation signal (RMR) was synthesized (GenScript NJ). Human ocular melanoma cells (Mel 270, Mel 202; a kind gift from Dr Bruce Ksander), retinoblastoma cells (Y79; ATTC, MD), primate retina/choroid ocular endothelial cells (RF/6A; ATCC, MD) and human retinal pigment epithelial cells (ARPE19; ATCC, MD), (CRL 1500 breast cancer cell line; ATCC, MD) were plated into 96 well plates and cultured for 24 h. Recombinant STY-RMR peptide was added to the cell culture media at various concentrations (10 to 30 mg /100ml).

Cell proliferation was assessed at 72 hours using a colorimetric XTT assay (Roche Diagnostics, Indianapolis, and CN, USA). Cell proliferation was expressed as a percentage and compared with untreated control cell growth (n = 3).

The amount of VEGF within media was determined by VEGF ELISA (R&D Systems, MN, USA) and compared between STY-RMR treated and controls (n = 3).

RESULTS

To test whether the STY domain alone (which is present within the $TEAD4_{216}$ repressor isoform but absent from the $TEAD4_{148}$ enhancer isoform) can mediate repressor activity we synthesized STY linked to a cell penetrating peptide derived from tat (RMR) (Gen Script NJ) (Figure **1**).

Transcription enhancer factor 1-related (TEAD4) is a member of the TEAD DNA binding domain family, and it is present within ocular vascular endothelial cells and plays a role in the control of VEGF expression. We have demonstrated that a variety of human tumors expressed isoforms of TEAD4 (Figure **2**).

Breast cancer cells (CRL 1500) showed a very significant inhibition of 87% (P= 0.000002) at (30 ug/ 100 ul) compared with untreated controls, the

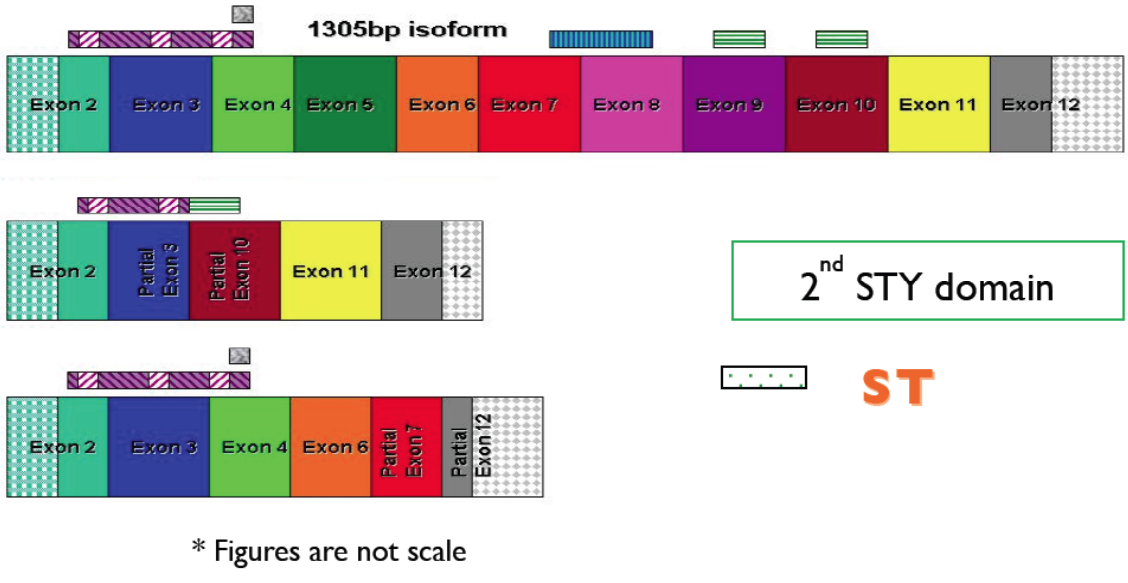

Figure 1: The second STY domain is present within the $TEAD4_{216}$ repressor isoform but absent from the $TEAD4_{148}$ enhancer isoform. To test whether the STY domain alone can mediate repressor activity we synthesized STY linked to a cell penetrating peptide derived from tat (RMR). The STY domain is 26 amino acids and the RMR is 10 amino acids in length, making the STY-RMR peptide 36 amino acids long.

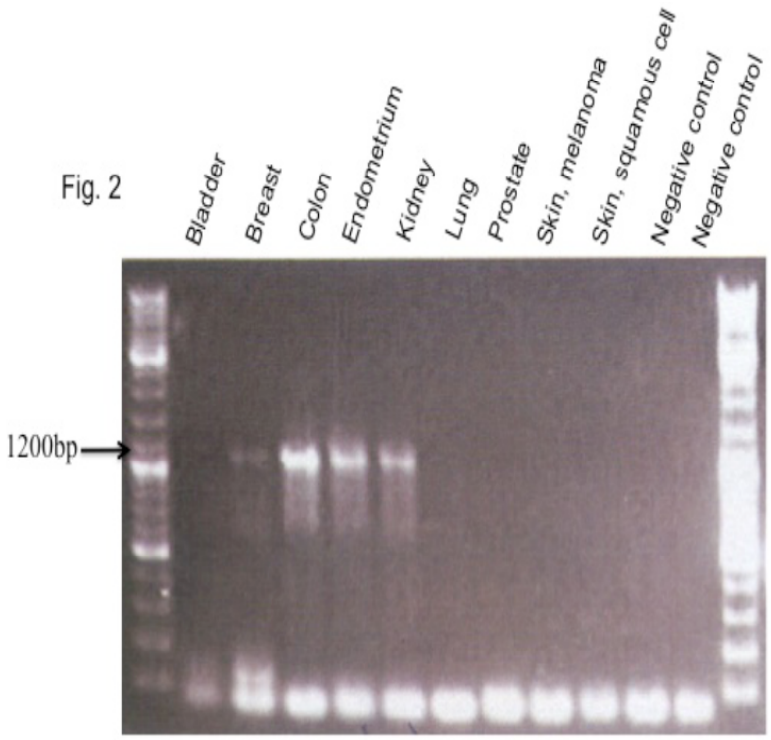

Figure 2: Variety of human tumors expressed isoforms of TEAD4.

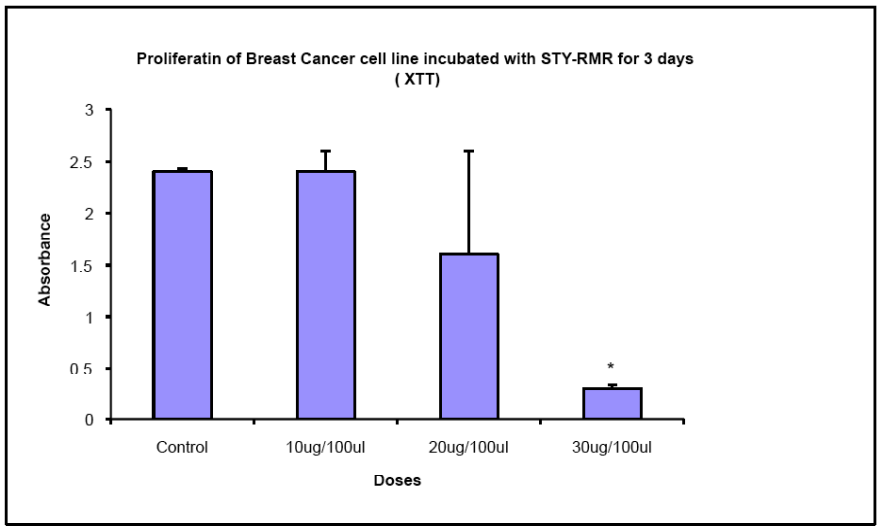

Figure 3: Breast cancer cells (CRL 1500) showed a very significant inhibition of 87% (P= 0.000002) at (30 ug/ 100 ul) compared with untreated controls, the mean results of 3 independent experiments are shown (±SEM).

mean results of 3 independent experiments are shown (±SEM) in (Figure **3**), and the proliferation of retinoblastoma cells is also inhibited by STY-RMR peptide in a dose dependent manner. We observed 85% inhibition at 30 mg /100ml which is statistically different from control experiments in the absence of STY-RMR (P= 0. 00003). Mean results of 3 independent experiments are shown (±SEM) (Figure **4**). A scrambled sequence of STY-RMR was tested as a control and it did not show any significant inhibition in Y79 retinoblastoma cells (Figure **5**).

STY-RMR can inhibit cell proliferation in two different ocular melanoma cell lines. Significant inhibition (P= 0.006) of 60% below control was also observed in Mel270 incubated at the same dose and inhibition was dose dependent (Figure **6a**) and 87% inhibition was observed at 30 ug /100ml for the Mel 202

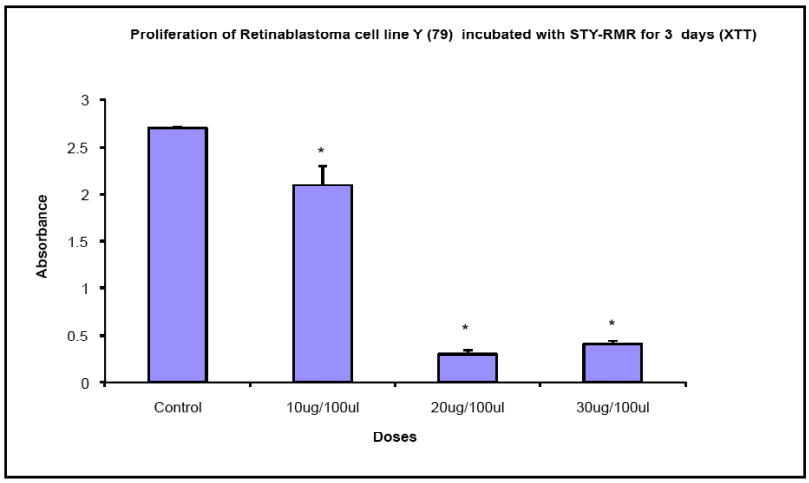

Figure 4: We observed 85% inhibition in proliferation of retinoblastoma at 30 ug /100ml which is statistically different from control experiments in the absence of STY-RMR (P= 0. 00003). Mean results of 3 independent experiments are shown (±SEM).

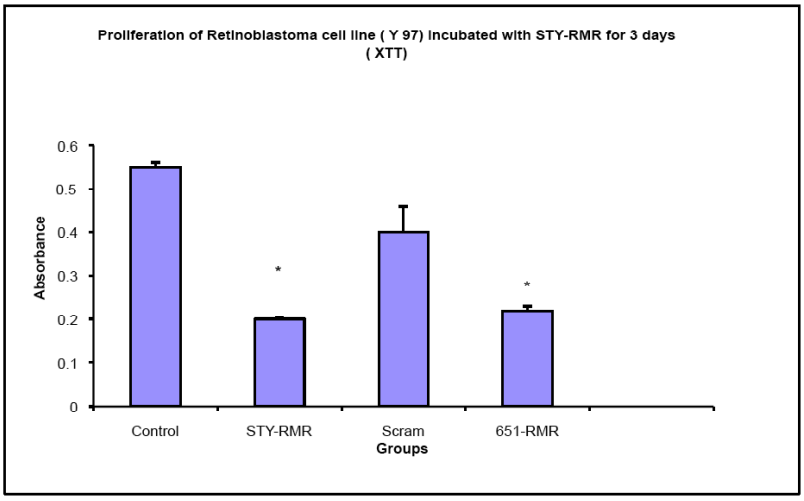

Figure 5: Scrambled version was tested as a control and it did not show any significant inhibition in retinoblastoma (Y79).

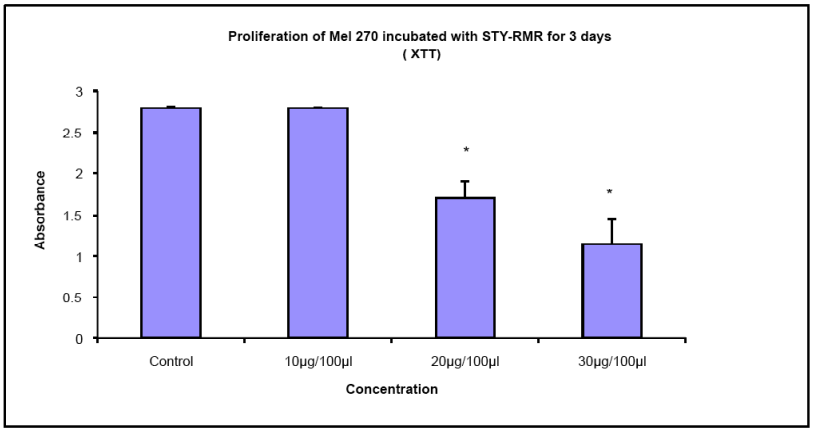

Figure 6(a): Significant inhibition (P= 0.006) of 60% below control was also observed in Mel 270 incubated at the same dose and inhibition was dose dependent.

cells mean results of 3 independent experiments are shown (±SEM) (p = 0. 001) (Figure **6b**).

STY-RMR peptide can inhibit proliferation of ocular vascular endothelial (RF/6A) and retinal pigment

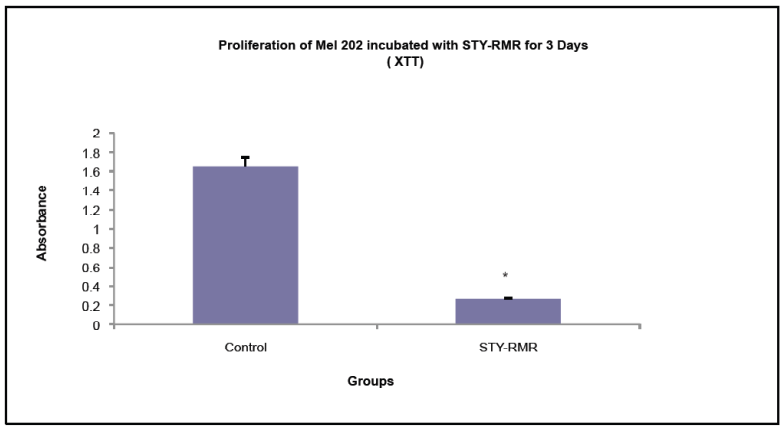

Figure 6(b): 87% inhibition was observed at 30 ug /100ml for the Mel 202 cells mean results of 3 independent experiments are shown (±SEM) (p = 0. 001).

epithelial cells (ARPE-19) (Figure **7a**). A (42%, p= 0 .001) inhibition of RF-6A (Figure **7b**) and 75% inhibition of ARPE-19 cells was observed with 30 mg /100ml of STY-RMR (P = 0.007). A dose dependent response was also observed as inhibition of cell proliferation was obtained with 3ug/100ul of treatment.

ELISA indicates that STY-RMR treatment is able to decrease secreted VEGF levels in ocular melanoma cell line 270. By STY-RMR peptide. We observed 81% reduction in VEGF levels (p= 0.01) at 30 mg / 100ml treatment (Figure **8**).

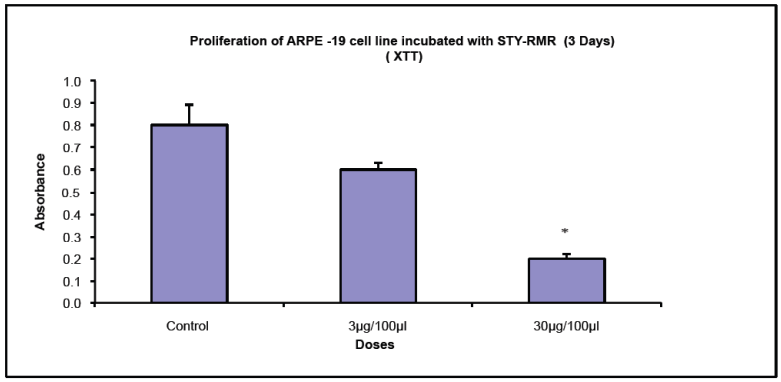

Figure 7(a): STY-RMR peptide can inhibit proliferation of ocular vascular endothelial (RF/6A) and retinal pigment epithelial cells (ARPE-19).

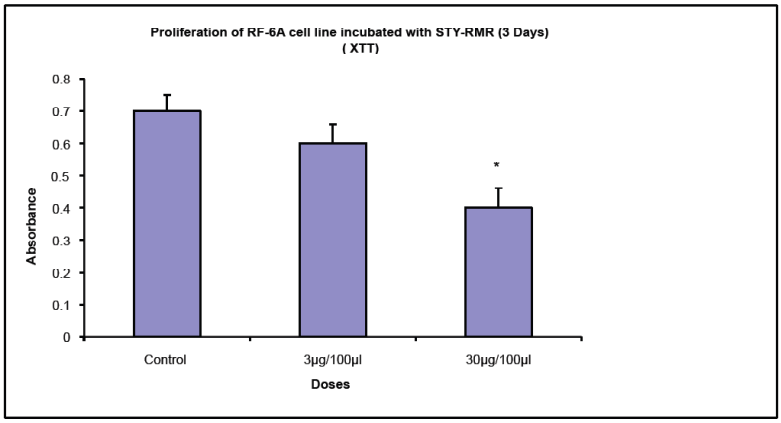

Figure 7(b): A (42%, p= 0 .001) inhibition of RF-6A and 75% inhibition of ARPE-19 cells was observed with 30 mg /100ml of STY-RMR (P = 0.007). A dose dependent response was also observed as inhibition of cell proliferation was obtained with 3ug/100ul of treatment.

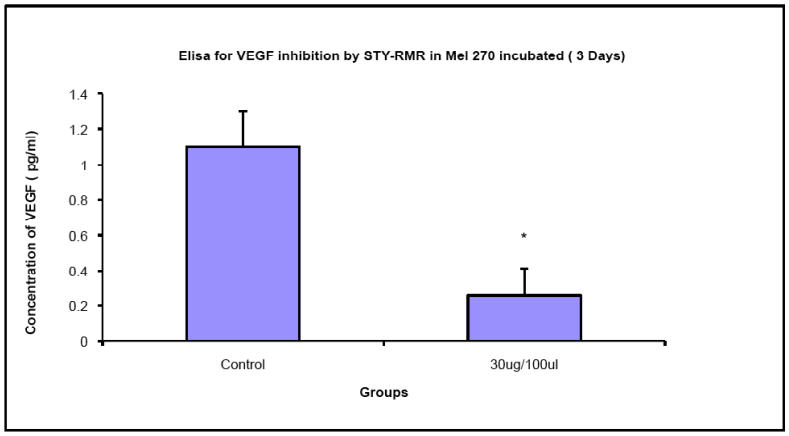

Figure 8: ELISA indicates that STY-RMR treatment is able to decrease secreted VEGF levels in ocular melanoma cell line 270. by STY-RMR peptide. We observed 81% reduction in VEGF levels (p= 0.01) at 30 mg / 100ml treatment.

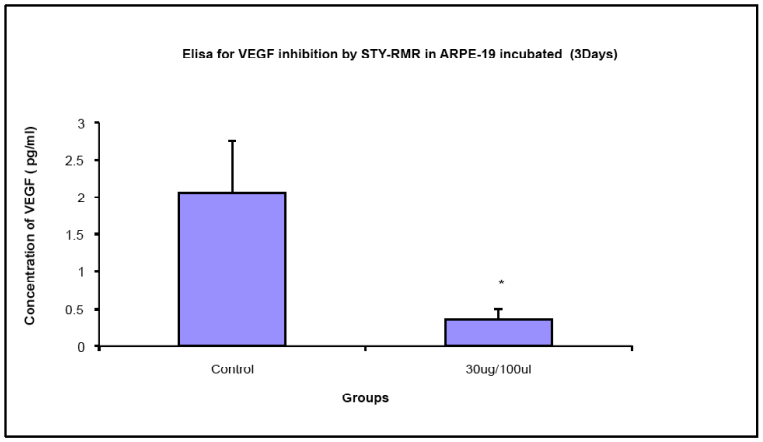

Figure 9: VEGF levels is inhibited in ARPE19 cells by STY-RMR peptide. An 88% reduction compared to control was observed after treatment with 30 mg /100ml (p= 0.05).

VEGF levels is inhibited in ARPE19 cells by STY-RMR peptide (Figure **9**). An 88% reduction compared to control was observed after treatment with 30 mg /100ml (p= 0.05).

Levels of secreted VEGF is lower in ocular retinal-choroid derived vascular endothelial cells after treatment with STY-RMR peptide. We observed 80% inhibition of VEGF at 30mg/100ml treatment (p= 0.03) in (Figure **10**).

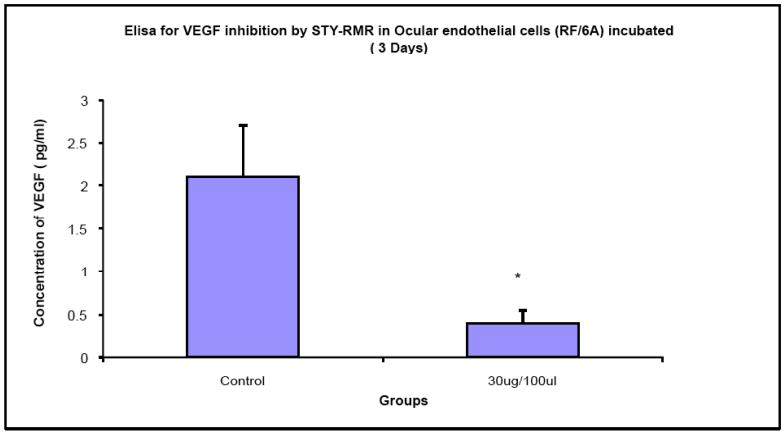

Figure 10: Levels of secreted VEGF is lower in ocular retinal-choroidal derived vascular endothelial cells after treatment with STY-RMR peptide. We observed 80% at 30 mg /100ml treatment (p= 0.03).

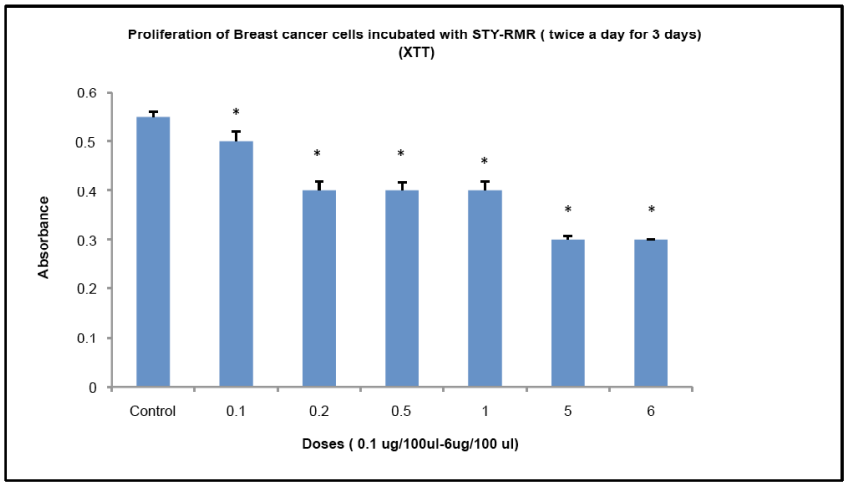

Figure 11: Significant inhibition in the proliferation of cancer breast cell due to VEGF reduction by STY-RMR incubated cancer.

We further tested the potential of this agent to inhibit a breast tumor cell line at a lower range of daily doses, we focused on breast cancer to optimize conditions of delivery and efficacy.

A significant inhibitory effect could be achieved by much lower daily doses twice a day for 3 days (Figure **11**), since breast cancer cell (CRL 1500) had 9% inhibition at 0.1 ug/ 100ul (p= 0.02), and 27% inhibition at 0.2, 0.5, and 1 ug/100 ul) (p= 0.02, 0.017, 0.02) respectively, however this inhibition was increased to 50% at (5 and 6 ug/100ul) with much higher significance (p= 0.00002, 0.0005), indicating that this factor may have similar metronomic effect observed for chemotherapeutic agents which can inhibit tumors either by high one shot or daily small doses due to thrompospondin release which is known to be an endogenous anti-angiogenic factor, however we cannot assume that the inhibitory effect of STY-RMR given once is a cytotoxic one at the highest dose despite the observation of a potent cell death induction at (30ug/ 100 ul) since we have also observed a significant VEGF reduction at the previous dose.

This inhibition in breast tumor cells was also associated with decreased VEGF levels by STY-RMR 78% (p= 0.02) even at low concentration (0.1ug/100ul) (Figure **12**).

DISCUSSION

Transcription enhancer factor 1-related (TEAD4) is a member of the TEAD DNA binding domain family, and it is present within ocular vascular endothelial cells and plays a role in the control of VEGF expression [3], it has been previously suggested that members of the TEAD family might play a role in mammary tumor genesis [11]. We have demonstrated that a variety of

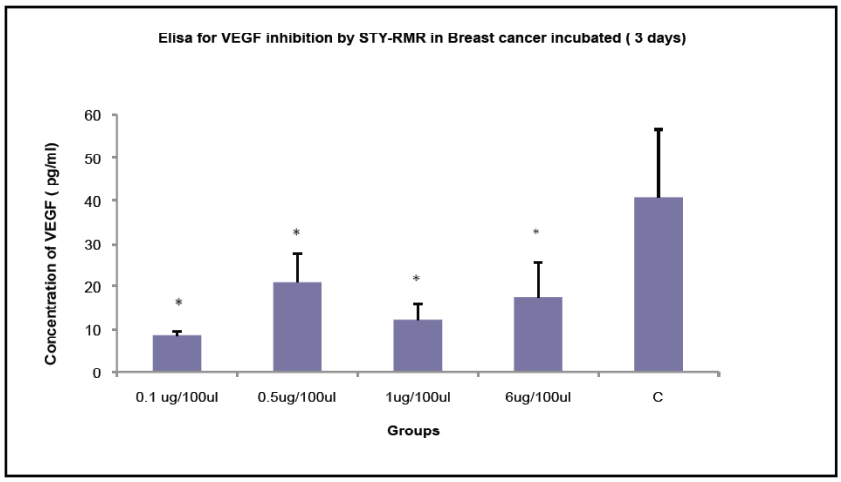

Figure 12: Significant inhibition of VEGF by STY-RMR incubated cancer breast cell.

human tumors expressed isoforms of TEAD4, therefore we were motivated to test the therapeutic potential of such isoforms to treat cancer.

The full-length TEAD4 is known to stimulate cell proliferation *in vitro*, and we have shown that various TEAD4 isoforms (generated through alternative hnRNA splicing) may be stimulatory or inhibitory to cell proliferation. A Ser-Thr-Tyr (STY) domain is the only domain present in the inhibitor $TEAD4_{216}$ isoform while absent in the stimulator $TEAD4_{148}$, therefore we hypothesized that this small fragment of 26 amino acids mediates this inhibitory effect. This STY domain was linked to a cell penetrating peptide motif (RMR) to test the potential of this agent to inhibit tumor cell proliferation *in vitro*.

The activity of several anti-angiogenic proteins such as Endostatin is mimicked by a 27-amino-acid peptide [12] and it is recently developed as Fc-Endostatin which showed similar inhibition activity to this tested peptide [13] indicating that short functional domains have therapeutic potential.

Ocular melanoma and retinoblastoma (Rb) are intraocular tumors whose growth are influenced by concomitant expression of VEGF, it has been reported that the number of ocular micrometastasis is correlated with the levels of VEGF in the serum [14]. Avastin is known to suppress the growth and hepatic metastasis of uveal melanoma through VEGF inhibition therefore VEGF can be used as a prognostic factor for ocular melanoma [15] but not for retinoblastoma [16]. RF-6A (a retinal endothelial cell line) and ARPE-19 (a retinal pigment epithelium cell line) are ocular derived cell lines whose growth are VEGF-dependent [17].

Significant inhibition of cell proliferation was observed with this STY-RMR in several tumor cell lines. Dose dependent responses were noted, however the range of this inhibitory effect is tumor type and /or cell line specific We tested the potential of this agent to inhibit tumor cell lines at a lower range of daily doses, a very significant inhibitory effect could be achieved by much lower daily doses for 3 days indicating that this factor may have similar metronomic effect observed for chemotherapeutic agents which can inhibit tumors either by high single dose or daily small doses such as that observed with thrompospondin release [18-20].

An inhibitory effect on cell proliferation was noted with the STY-RMR peptide in human Y79 retinoblastoma cells a suspension cell line. the effect was lost when the cells were exposed to the scrambled peptide at the same dose indicating that the sequence order of the STY domain is critical to the function. The efficacy of STY-RMR to inhibit tumor cells which are growing in suspension suggests this compound may prove to be therapeutic on metastatic cells in the circulation or non-solid cancers such as leukemia.

We further questioned whether STY-RMR inhibits the levels of VEGF in the same studied cell lines (ocular melanoma, RF-6A, ARPE-19, and breast cancer). We observed a significant decrease of VEGF production within different cell lines which indicates the potential of this agent to treat angiogenic diseases.

CONCLUSION

We have demonstrated that a small peptide which contains the STY motif of the human TEAD4 protein, when linked with a cell penetration motif, is able to inhibit cell proliferation rates in a number of cell lines germane to human disease. This effect is associated with a decreased rate of VEGF synthesis and may prove therapeutically useful.

FUNDING

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