Discussion
A comprehensive overview of glioma development and progression suggests that the process is well orchestrated by intrinsic properties of the glioma cells, including microenvironmental factors [Okada et al., 2009]. The glioma microenvironment is composed of tumor cells, endothelial cells, intermingling glia, neurons, a variety of leukocyte subsets, extracellular matrix fibers, and soluble mediators [Hanahan et al., 2000; Coussens et al., 2002; Liotta et al., 2001]. An intricate interplay between the cellular and extracellular components determines the success or failure of tumor progression. Despite significant multimodality in innovative therapeutic intervention, glioma continues to show its worst prognosis and restricts the victim’s life to 12-18 months after diagnosis which emphasizes the need for development of novel therapeutic strategies for this challenging tumor [Groves et al., 2002]. These tumors exhibit extremely high levels of neovascularization, which may contribute to their extremely aggressive behavior with the induction and creation of a neovasculature, allowing tumors to overcome the limitations in nutrient and oxygen delivery that develop when the tumor reaches a size of ≈ 1 to 2 mm in diameter [Hanahan et al., 1996; Folkman et al., 2006]. To progress, these tumors stimulate the formation of new blood through processes driven primarily by various proangiogenic factors such as VEGF, MMPs, Integrin and EGFR [Hanahan et al., 1996].

However, during the progression of gliomas conventional cytotoxic chemotherapies control them by blocking the expansion and proliferation steps, but leave angiogenesis and invasion unchecked. The long term exposure of chemotherapy has significant side effects including interstitial lung diseases, mouth ulcer, cardiovascular diseases and tumor lysis syndrome. Considering these limitations, search for molecule or compounds which possess non toxic antiangiogenic activity is always welcome.

In the present study N’ N’-ethylnitrosourea (ENU) a chemical carcinogenic agent, was injected intraperitoneally in 3-5 days old rat pups. After 5-6 months full blown of tumor was found in 100% of animals injected with ENU. Previous study [Mukherjee et al., 2004; Slikker et al., 2004] have shown that administration of ENU create the genomic instability along with mutations in p53 during the process of gliomagenesis. N-ethyl-N-nitrosourea (ENU) is a synthetic alkylating compound which is toxic and carcinogenic to the cells It is a potent mutagen, and primarily affects glial cells. It induces random point mutations in the brain compartment. In brain parenchyma compartment ENU causes the alkylation in O6 position of guanine, by transfers its ethyl group to oxygen and nitrogen reactive sites of the nucleotides resulting transformation from G:C to A:T shift in brain due to the deficiency of the DNA repairing enzyme AGAT in brain tissue [Slikker et al., 2004].
Table 2.2: Reactive sites of ENU alkylation (Noveroske et al., 2000)

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Reactive sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>N1, N3, and N7</td>
</tr>
<tr>
<td>Thymine</td>
<td>O2, O4, and N3</td>
</tr>
<tr>
<td>Guanine</td>
<td>O6, N3, and N7</td>
</tr>
<tr>
<td>Cytosine</td>
<td>O2 and N3</td>
</tr>
</tbody>
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The ethylated nucleotide is not recognized correctly during DNA replication which results in mispairing to a non-complementary nucleotide.

**Fig. 23: Mechanism of action of ENU.** A) Alkylation of thymine results in the formation of O4-Ethylthymine which is recognized as cytosine and mispairs with guanine. B) Mispairing leads to the corresponding base exchange during DNA replication (Noveroske et al., 2000).
In glioma angiogenic studies, one of the prime objectives is to identify and purify the cells in the process of angiogenesis in glioma microenvironment, and, consequently measurement of their expression of phenotypic marker has been one of the main tools for such analysis. A modified method was applied to separate the ECs from other cell types. The brain tissues were minced and proteolytically digested, after which cells were separated out through couple of gradient density centrifugations and the isolated ECs were characterized with phenotypic markers CD31 and CD34.

CD31 has been used as a target to select EC because of its abundance and specific expression on the membranes of this cell [Beijnum et al., 2008]. Flowcytometry was used to establish the purity of EC by their phenotypic Abs [Beijnum et al., 2008]. CD31 expression acts as a cell adhesion and signaling molecule at the endothelial cell junctions where they solely hold in trans-homophilic binding [Kim et al., 2000; Gladson et al., 1996]. The interaction between CD31 and integrin αvβ3 occurs on the same cell surface [Wong et al., 2000] regulating the endothelial cell migration and adhesion. In our experiment ENU induction causes significant (P<0.001) increase of the level of phenotypic markers (CD31) as compared to normal group, but after treatment with T11TS, the level gradually decreases with the consecutive doses and in the third dose it was brought back to near normal levels CD31 (35.33± 1.525) (Fig.1). Interestingly, our in situ immunofluorescence result (Fig.2) analysis revealed a significant difference in the expression of CD31 in endothelial cell linings which was abundant in highly vascularized malignant gliomas but after T11TS administration with three consecutive doses the expression of CD31 sharply decreased and was brought back to the normal level in the ET3 group. This may be indicative of the fact that inhibition of CD31 expression in ENU group after T11TS administration hinders the cell-cell contact and trans-homophilic binding efficacy of endothelial cells.

**Down regulation of CD31 Expression done by Flowcytometry and In situ immunofluorescence methods convincingly proved T11TS mediated glioma angiogenic regression and was the basis of the entire study. The results obtained from the above study provide strong evidence that T11TS therapy exerts it effects on glioma angiogenesis progression of glioma associated brain endothelial cells by down regulation of phenotypic marker like CD31.**

Encouraged by CD31 findings we used CD34 as another endothelial cell phenotypic marker because during formation of vessels its expression maintains the proliferating stalk cell phenotype and simultaneously, they signal to adjacent EC via Delta-like ligand (DLL)-Notch interactions [Gerhardt et al., 2003; Hellstrom et al., 2007]. In line with these recent findings present study revealed that (Fig. 1) ENU induction causes the significant (P<0.001) increase of the CD34 marker in glioma associated EC that supported the above document and hint that the expression was high because of the high EC
proliferation during glioma. While in 1\textsuperscript{st} dose of T11TS significant down regulation of CD34 was observed and similar expression was observed in 2\textsuperscript{nd} dose and in 3\textsuperscript{rd} dose. This reversion of CD34 expression and proliferation of ECs cells supported that T11TS either directly or through molecular effector mechanism imposes breaks in the cell-cell contact and help in clearing of glioma from brain tissue. Recent evidence has indicated that CD34 acts as an anti-adhesive molecule during lumen formation in developing blood vessels, by maintaining or promoting the separation between contralateral apical endothelial cell surfaces in a luminal tube. Although the function of CD34 in resting endothelial cells and in tip cells during angiogenesis was not addressed in the present study, we speculate that the proposed anti-adhesive property of CD34 may also play a role in maintaining vascular lumina in resting tissues, as well as in allowing the migration of tip cells and the movement and probing of their filopodia through the tissue matrix.

Like CD31, down regulation CD34 expression after T11TS administration in glioma induced rat measured by Flowcytometry method was another convincing evidence of T11TS mediated glioma angiogenic regression. The results obtained from the above study provide convincing evidence that T11TS therapy exerts its effects on glioma angiogenesis progression of glioma associated brain endothelial cells by down regulation of phenotypic marker like CD34.

In histological findings (Fig.2) there was evidence of copious angiogenic vessels with the tumor growth in rat brain after 5 months of ENU treatment in 3 to 5 days old rats pups. But after the 1\textsuperscript{st} dose of T11TS the microvessels were clearly disrupted and the ologodendroglial cells had the nuclei extruding from the cells indicating cell death. After the 2\textsuperscript{nd} dose of T11TS administration, the ET2 group showed lesser number of disrupted microvessles. Also immune cells were seen attached to the disrupted oligodendroglial cells offering the kiss of death. In the ET3 group the brain matrix was cleared up of the residual angiogenic vessels and the disrupted oligodendroglial cells. Only very few scattered out hollow tubes were visible.

To decipher the involved mechanistic approach in T11TS induced tumor angiogenic regression, we evaluated the comparative estimation of Integrin αv, MMP-2, MMP-9, TIMP-1 and TIMP-2 expression in glioma associated brain endothelial cells. Adhesion and migration of tumor cells are essential steps in tumor cell invasion and metastasis. Both processes require a co-ordinated expression of adhesion molecules and proteolytic enzymes. The role of integrins in cell migration and invasion is one of their most studied functions in tumor biology by directly binding to the components of the extracellular matrix and providing the traction necessary for cell motility and invasion.
Numerous studies have shown that glioblastoma commonly displays enhanced expression of several integrins along with their ECM ligands such as αvβ3 and αvβ5 (tenascin and vitronectin receptors), α5β1 (fibronectin receptor), α2β1 (collagens receptor), and α3β1, α6β4, and α6β1 (laminins receptors) [Bellail et al., 2004]. Some are focused on the αv integrin family because integrins like αvβ3 and αvβ5 are markers of glioblastoma malignancy [Bello et al., 2001] and influence a variety of processes in glioblastoma progression in vivo, including proliferation, apoptosis, and angiogenesis. In this way, integrins maintain the integrity of different organs and tissues by preventing cells from surviving in an improper environment. In line with this we checked the expression of integrin αv in in vivo model and found that in glioma condition expression of Integrin αv was significantly (P<0.001) increased compared to normal while T11TS administration in glioma induced rats triggers significant (P<0.001) down regulation of proangiogenic integrin αv expression in brain ECs as evident from the flow cytometry and immunoblot studies (Fig. 8). This result hints that high expression of Integrin in quiescent ECs help to degrade and remodel the ECM during their invasion but T11TS administration prevent the degradation of the ECM by modulating the expression of integrin αv expression in brain. Furthermore, our in situ immunoflorescence imaging studies strongly support the above results of integrin αv (Fig.9) and showed multiple endothelial cells adhering to each other in ENU group, but the T11TS treated group showed fewer Integrin αv stained endothelial cells with disruption of the vasculature and with the break in endothelial adhesion.

Previously it had been reported that αvβ3 enhance MMP-2 expression, resulting in an increased invasiveness [Pietsch et al., 1997]. MMP-2 plays a central role in the glioma angiogenic progression. In earlier studies, the extracellular matrix was viewed as a barrier to endothelial cell invasion, whereas secreted MMPs remove this barrier and allow endothelial cell migration. In in vitro system exogenous application of pro- MMP-2 in endothelial cells induces morphologic change like tube formation. During vessel formation sprouting of endothelial is expected to encounter a variety of extracellular matrices. Early events during the angiogenic response include detachment of endothelial cells from their underlying basement membrane and interaction with the provisional matrix formed by leakage of fibrinogen from the vascular compartment. After traversing these matrices, one can envision the tip of the endothelial sprout contacting interstitial extracellular matrix composed principally of type I, Type II collagen and as well as fibronectin. Moreover, MMP-2 association with αvβ3 Integrin is important for EC migration in glioma and disruption of this complex result in inhibition of angiogenesis [Stiletto et al., 2001; Oaring et al., 1999]. Consistent with these reports we estimated the MMP-2 expression pattern in glioma group and glioma+T11TS groups. Our results showed that MMP-2 expressions was significantly increased in ENU group (Fig.3) when compared to
the normal group but after T11TS administration it gradually comes back to near normal level after the 3rd dose of T11TS administration ECs evident from the flow cytometry, zymography and immunoblot studies.

Inspired from the outcome of MMP-2 expression pattern our next goal was to study the cell invasion process by the madrigal assay. Since MMP2 is a proinvasive protein, T11TS mediated reduction of MMP2 levels in the glioma group would be expected to result in decreased cell invasiveness. Our in vitro matrigel (Fig.4) assay demonstrated that the invasive capacity of the ECs of ENU group dramatically increased (P<0.001) but were down regulated in a stepwise manner after administration of T11TS. This evidence suggests that after administration of T11TS in vivo it inhibits cell invasion and thereby hinders metastasis. T11TS induced inhibition of Integrin αv expression in brain endothelial cells might be a mechanistic factor triggering downregulation of MMP-2 in endothelial cells via blocking interaction between Integrin αv and MMP-2.

MMPs expression is regulated at several levels including transcriptional level by growth factors and at the enzymatic level by activation of the latent form by the tissue inhibitors of metalloproteinase (TIMPs). The TIMPs are well-studied inhibitors of MMPs and consist of a family of four structurally related proteins (TIMP-1–4). Among the TIMP family, TIMP2 is a 21-kDa non-glycosylated protein that forms latent interaction with and activated forms of the enzyme 72-kDa type IV collagenase [Goldberg et al., 1989]. Our findings suggested that T11TS treatment in ENU group resulted in down regulation of MMP-2 expression by recruiting active TIMP-2 (Fig. 5.1&2) on ECs. This hints at the disruption of the MMP-2/Integrin complex and recruitment of active TIMP-2 thereby, inhibiting angiogenesis in glioma bearing rats. TIMPs inhibit MMP activity in two different mechanisms: MMP dependent and MMP-independent mechanism. In MMP-independent mechanism TIMP-2 inhibits fibroblast growth factor- and vascular endothelial growth factor (VEGF) as well as dephosphorylation of focal contact molecules such as focal adhesion kinase (FAK) and paxillin associated with cytoskeletal changes. Focal adhesions are typically composed of integrins and various cytoplasmic proteins that link the cytoskeleton to the ECM [Dejana et al., 1999]. FAK is a nonreceptor protein tyrosine kinase that is localized at focal adhesions and plays a significant role in regulating EC interactions with the ECM [Webb et al., 2002; Zachary et al., 1992]. Phosphorylation of FAK triggers downstream signaling events, including phosphorylation of paxillin to regulate Rho-family GTPases, which promote actin dynamics associated with cell motility [Parsons et al., 2003; Ilic et al., 1997]. While, MMP dependent mechanism involves interaction of the amino-terminal cysteine residue with the zinc atom at the MMP active site [Junseo et al., 2004].
We also investigated the effect of T11TS on MMP-9 and TIMP-1 mediated glioma cell invasion on endothelial cells because MMP-9 has been implicated in invasion, tumor angiogenesis, in studies using several models of tumor. Like Geltatinase A, expression of Progelatinase-B is controlled primarily at the level of gene expression, its transcription being activated by mitogens and inflammatory mediators [Cornelius et al., 1995]. Furthermore, MMP-9 is not activated by MT-MMPs, but is activated more promiscuously by plasmin, stromelysin-1 and gelatinase-A. To determine whether T11TS administration could inhibit MMP-9 expression in glioma associated brain ECs we studied expression of MMP9. Our findings revealed significant expression (P<0.001) of MMP-9 in ENU group of ECs compared to normal group which suggest high expression of MMP-9 (Fig. 6.1&2) in glioma bearing animals promotes cell invasion and triggering the angiogenic switch as a paracrine regulator of tumor progression. However, T11TS administration in glioma bearing rats significantly attenuated MMP-9 expression in brain ECs which implicates that T11TS hinders brain endothelial cell migration and invasion thereby abrogating a crucial step of angiogenesis. TIMP-1 inhibits tumor invasion and metastasis in experimental models [Schultz et al., 1988; Tsuchiya et al., 1993]. TIMP-1 has complex roles in physiological and pathological tissue remodeling [Gomez et al., 1997; Jiang et al., 2002]. The most widely studied function of TIMP-1 is as an inhibitor of MMP-9. Conversely, it acts as a mitogen of various non-malignant and malignant cell types in vitro [Hayakawa et al., 1992; Bertaux et al., 1991; Luparello et al., 1999]. TIMP-1 inhibits angiogenesis in experimental in vitro and in vivo models. TIMP-1-mediated inhibition of HDMVEC migration in vitro through MMP-dependent stimulation of VE-cadherin and MMP-independent stimulation of PTEN with subsequent dephosphorylation of FAK and cytoskeletal remodeling [Akahane et al., 2004]. Downregulation of TIMP1 in brain ECs of ENU group hints at promotion of endothelial cell migration during gliomagenesis. The upregulation of TIMP1 after T11TS administration in all three doses indirectly suggest that T11TS help in the recruitment of TIMP-1(Fig. 7.1&2) and its binding to MMP-9 thereby hindering ECs migration in T11TS treated ENU group of animals.

The positive outcome of such an in-vivo study might form a strong evidence for elucidation of T11TS as an anti-invasive agent in glioma. The novel finding in this work points towards effective down regulation of Integrin αv, MMP-2, MMP-9 and up-regulation of TIMP-1 and TIMP-2 by administration of T11TS in ENU group. This provides a broad hint of the mechanistic approach whereby T11TS inhibits angiogenesis which may be a key event leading to glioma regression in a rat model.
In glioma angiogenesis another area of grave concern among oncologists is the inflammatory status of brain tumor. Generally, glioma which participates in tissue inflammation must have utilized the aberrant cytokine signaling generated by inflammatory cells during early lesion implantation at a foreign site. Although the complete roles of inflammatory cells such as macrophages and their secreted cytokines are not delineated, several evidences have suggested that these cells are important for such kind of diseases primarily through the production of soluble factors. Tumor initiation, growth, and progression not only depend on malignant traits of the tumor cells but are strongly influenced by the tumor microenvironment. The tumor stroma consist of various cell types such as inflammatory cells namely macrophage cells, T-cells, endothelial cells and fibroblast cells, which can promote or inhibit tumor growth. During the last few decades, many tumor suppressor genes and oncogenes have been identified, and recognized as important tool to fight cancer. Two particular important processes that contribute to pathology of most types of tumors are angiogenesis and inflammation. There is a link between inflammation and angiogenesis because inflammation is a defensive reaction of living tissue to injury which involves vascular response and establishment of vascular response generates new blood vessel formation [Ping Wu et al., 2012]. Depending on the tumor type, the tumor microenvironment/stroma contains various cells are generally considered to promote tumor growth and invasion by provision of growth factors, matrix remodeling, and induction of angiogenesis [Egeblad et al., 2010; Schafer et al., 2008; Kalluri et al., 2006]. However, persistence of inflammation can lead to severe tissue damage and contribute to the acute endothelial activation. In many inflammatory conditions it had been found that the inflammatory cells namely monocytes/macrophages, lymphocytes, and microglia fully participate in the angiogenic process by secreting pro- and anti-inflammatory cytokines, that could control endothelial cell proliferation, their survival and apoptosis, as well as their migration and activation [Lingen et al., 2001], which, when persistent, results in capillary sprouting.

The processes of activation of endothelial cells can be divided into two phases, one immediate type I activation phase which is independent of de novo gene expression, and is mediated by signaling through G-protein coupled receptors e.g. the histamine receptor. Binding of histamine to its receptor activates endothelial nitric oxide synthase (NOS3) leading to increased synthesis of NO, an important vasodilator which increases local blood flow. Whereas, Type II activation results by delayed type of activation phase this requires de novo gene expression by endothelial cells [Teixeira et al., 1993]. In the latter case-endothelial cell activation is more sustained mediated by inflammatory cytokines TNF-α and IL-1β which bind to their cognate receptors on endothelial cells, TNF receptor 1 (TNFR1) and IL-1β receptor. Binding of ligand allows for recruitment of an adaptor complex which in turn recruits and
activates the inhibitor of κB (IκB) kinase (Iκκ) complex [Hayden et al., 2008]. Active Iκκ phosphorylates IκB-α which leads to its polyubiquitination and rapid degradation by the proteasome. This releases heterodimers of the NF-κB subunits p50 and p65 (Rel-A), allowing them to accumulate in the nucleus and to induce transcription of a large set of target genes, including pro-inflammatory cytokines, genes coding for adhesion molecules and anti-apoptotic genes. This pathway is called the canonical nuclear factor κB (NF-κB) pathway.

Much like tumors in other organs, gliomas often contain an inflammatory component, which mostly consists of activated microglia. However, infiltration of other myeloid and lymphoid cells has also been described in glioma. In the glioma microenvironment inflammatory cells are believed to contribute to glioma evolution and development by providing growth factors, cytokines and chemokines which stimulate tumor cell proliferation, invasion and angiogenesis [Watters et al., 2005; Sciume et al., 2010]. Furthermore, skewing of microglia and/or brain infiltrating macrophages towards a "M2" like phenotype has been reported to correlate with the histological grade [Komohara et al., 2008]. These inflammatory cells, as well as cancer initiating cells, are likely to create a microenvironment which is strongly immuno-suppressive, allowing the glioma cells to evade anti-cancer immune responses [Wei et al., 2010]. In 2002 Baker and his colleges demonstrated that products of inflammatory cells like VEGF-A in vitro condition induces the secretion of TNF-α, IL-6, IL-8 and IFN-γ from HUVEC as well as ICAM1 expression on endothelial, and down regulate an inflammatory inhibitor IL-10 [Baker et al., 2002]. This kind of shift in glioma microenvironment further increases the brain tumor angiogenesis and decreases the anti-tumor response of the immune system and creates potential difficulties with therapy designed to destroy the tumor.

Encouraged by our recent findings [Singh et al., 2014; Bhattacharya et al., 2013] we examined the effect of T11TS on the expression of the inflammatory mediators TNF-α, IL-6, IL-8 and NF-κB in rat brain endothelial cells and in microglial cells. In inflammation and angiogenesis, TNF-α plays a dual role: it controls both tissue destruction and recovery, and these roles are determined by the context in which this cytokine acts [Fajardo et al., 1992]. With regard to cancer development and progression, animal experiments have shown differing roles for a cytokine depending on the dosage. For example, high concentrations of TNF-α impair angiogenesis [Fajardo et al., 1992]. In support of this conclusion TNF-α protein expression were measured by Flow cytometric and ELISA assay before and after T11TS treatments (Fig. 14). Biologically TNF-α acts as a trimmer which binds to two distinct cell surface receptors such as p55 TNF receptor (TNFR-1) & p75 receptor (TNFR-2). Our results analysis have shown that TNF-α expression in ENU group was significantly (P<0.001) upregulated compared to normal group in microglia cells. This finding may provide a mechanistic explanation for
Figure 21: The relationship between tumor growth and progression, inflammation and angiogenesis. Growth of solid tumors results in most cases in a tumor microenvironment and the release of various growth factors and cytokines, which together drive inflammation and angiogenesis in tumor stroma. At the same time, inflammatory cells and blood vessels support tumor growth and progression.
the role of TNF-α during glioma since high levels of TNF-α are found in chronic wounds [Leibovich et al., 1987]. However, following T11TS administration we have found that expression of TNF-α protein was decreased. Within cellular microenvironment the present study demonstrated for the first time that T11TS could decrease the expression of TNF-α in glioma induced animals hints that and supported the previous observation normal wound healing requires some involvement of inflammatory cytokines but abnormal wound healing may result from increased amounts of these mediators.

Inflammation induces the genetic instability in the tumor microenvironment [Colotta et al., 2009]. Mantovani et al in 2008 have reported that inflammation and cancer are a linked with two pathways called extrinsic and intrinsic. In the later pathway, modification of gene leads to the appearance of inflammation-related programs that guide the formation of an inflammatory microenvironment. Among the programmers NF-κB is one of the major molecules which contribute to tumor progression in vivo [Karin et al., 2005; Wolf et al., 2010] while inhibition of NF-κB expression led to the decreased tumor burden and decreased numbers of different types of metastases [Connelly et al., 2011]. In addition there is growing body of evidence that NF-κB can be activated in cells by a variety of stimuli ranging from inflammatory cytokines to Chemokines [Mercurio et al., 1999] and is involved in inflammation and unusual angiogenesis [Xie et al., 2010]. So NF-κB functioned as a bridging molecule between angiogenic tumor growth and inflammation in inflammatory cells and tumor cells.

The present study (Fig. 15) demonstrated that in ENU group NF-κB expression was significantly (P<0.001) increased compared to normal brain ECs, while, T11TS administration in glioma bearing rats down regulate expressions of NF-κB in glioma associated brain ECs. Tanabe et al 2010 have revealed that binding of TNF-α on TNF-R phosphorylate subunits of NF-κB at Ser 536 and Ser 468 leading to its nuclear translocation and transcription of genes such as those for the proinflammatory cytokines interleukin IL-6, and TNF-α [Li Q et al., 2002; Hayden et al., 2004]. Accumulating results strongly suggest that T11TS repairs the expression pattern of regulatory molecules TNF-α and NF-κB concomitantly responsible for angiogenesis and inflammation in glioma induced animals.

Moreover, activation of NF-κB acts as pleiotropic regulator of many genes, including those encoding adhesion molecules and chemokines which act as angiogenic factors. Interleukin (IL)-8 is a member of the chemokine family and promoter of the IL-8 gene contains potential binding sites for the transcription factor NF-κB [Patterson et al., 1995]. Transcriptional stimulation of the IL-8 gene by the potent pro-inflammatory cytokines IL-1 or TNF-α act in autocrine and as well as paracrine manner in tumor cell, stromal cells and endothelial cells involved in angiogenesis [Yoshida et al., 1997], tumor growth, and metastasis [Inoue et al., 2000; Singh et al., 1994; Ishiko et al., 1995]. Furthermore, earlier data clearly demonstrate that over expression of IL-8 in high grade of glioma induced the expression
of survival-associated genes and matrix degraded proteins such as MMPs, depend on the concentration of IL-8 which further enhances endothelial cell migration, proliferation and survival [Charalambous et al., 2005]. Recently we have shown that [Singh et al., 2014] in glioma associated brain ECs expression of MMP 2 and 9 was high but T11TS induction further decrease the expression and as well as endothelial cell migration in glioma bearing rat. Further we extended these studies to show that in glioma associated ECs there were any significant (P< 0.001) changes of expression of IL8 in disease control and also the T11TS treated animals confirmed. While, our FCAS assay (Fig.16) have shown similar results like ELISA that glioma associated brain ECs constitutively over expressed IL-8 receptor (CXCR1) in ENU group. Binding of IL-8 to its receptors like CXCR1 and CXCR2 [Murdoch et al., 1999; Salcedo et al., 2000] in HUVEC have been shown to play a major role in endothelial cell proliferation [Li A et al., 2003], migration and promote the angiogenesis through its auto as well as paracrine manner. However, the results obtained from FACS and ELISA assays revealed that in T11TS treated animals significant (P<0.001) down regulation occurred in IL-8 receptors as well as secretion of IL-8 compared to ENU group. This indicated that T11TS inhibits the expression of pro-angiogenic cytokine IL-8 via inhibition of the pro-inflammatory pathway TNF-α /NF-κB. It therefore seems that T11TS acts in a feedback loop to inhibit continued pro-inflammatory cytokine production of IL-8. Inhibition of IL-8 have shown that reduction of angiogenic and inflammatory change directly suppresses EC survival and proliferation [Li A et al., 2003].

IL-6 is another potent cytokine which is produced at the site of inflammation and plays a key role during inflammatory processes, and participates as stimulator for the production of other pro-angiogenic and inflammatory proteins. Endothelial cells generally do not express IL-6 receptors but they are thought to respond to IL-6 through trans-signalling network [Romano et al., 1997]. Recent in vitro evidence has suggested that soluble IL-6 receptor (sIL-6Rs) are alternatively spliced version of the receptor and exist in the serum which bind to free IL-6 and forming complex called IL-6/sIL-6R complex or transignalling pathway. Cleavage of receptor occurred via two different ways either IL8 or TNF-α. This complex then binds to the gp130 receptor, a transmembrane glycoprotein expressed by endothelial cells. Growing lines of evidence have indicated that inhibition of phosphorylation of NF-κB at both Ser 536 and Ser 468 by wedelolactone significantly suppressed IL-6 secretion in glioma cells. Recent studies from our laboratory had shown that in vivo T11TS administration inhibits the expression of the IL-6 glioma associated ECs. Flowcytometry and ELISA (Fig.17) studies revealed that the IL-6 cytokine expressions were differently modulated in normal, glioma bearing animals and glioma+T11TS treated animals. In glioma associated brain ECs significant (P<0.001) difference in IL-6 expression was observed, compared to normal group. Following T11TS administration seemed to
down regulate the IL-6 expression in glioma associated brain ECs compared to the ENU group. These results depict that T11TS administration in glioma triggers the inhibition of the transsignalling pathway (via soluble receptors) through down regulation of IL8 or TNF-α.

Tedgui et al in 2001 have demonstrated that vascular inflammation can be restricted by anti-inflammatory mechanism by maintaining the integrity and homeostasis of the vascular wall. Anti-inflammatory mechanisms in the vascular system involve anti-inflammatory external signals together with intracellular mediators that modify primary transcription factors IkB/nuclear factor-kB which play central role in the regulation of inflammatory mediators. In brain tumor microenvironment microglia act as a major cytokine source to secret the pro as well as anti inflammatory or angiogenic cytokine and their balance of local cytokine environment is determined by interacting cells. Previous reports have documented that IL-10 significantly blunts inflammation and decrease leukocyte migration via downregulation of adhesion molecules ICAM-1 and VCAM-1 [Henke et al., 2000; Santucci et al., 1996] through decreasing the TNF-α level [Downing et al., 1998]. Moreover, some other experiments revealed that IL-10 act as anti-inflammatory agent in macrophages in vitro system against proinflammatroy cytokines (TNF-α and IL-1). However, the effects of IL-10 and IL-4 on vascular cells may vary according to the origin of the cells and the signaling pathways induced by the proinflammatory stimuli. Since aberrant angiogenesis is the result of significant pathogenic component of tumor and chronic inflammation, we investigated the effect of T11TS in IL-4 and IL-10 secretion derived from macrophages in tumor microenvironment. In 1999 Stearns et al have shown that in tumor cell line IL-10 inhibit the matrix metalloproteinase secretion while up regulating TIMP-1 expression. Recently we have demonstrated that T11TS therapy inhibits glioma angiogenesis with inhibition of endothelial cell migration by down regulation of pro-angiogenic component MMP-2 and MMP-9 and upregulates the expression of TIMP-1 & -2 in glioma associated brain endothelial cells [Singh et al., 2014]. Accumulating data hints that additional studies were required to resolve the role of T11TS in tumor growth and metastasis. FACS and ELISA assays were used to determine IL-4 and IL-10 of the cytokine level in all five groups. The data obtained from these above assays revealed that in glioma group expression of IL-10 was decreased compared to normal group. However, T11TS administration upregulates IL-10 (Fig) expression levels significantly (P<0.001) in ENU group compared to normal control group. High level of IL-10 after T11TS administration glioma group possibly decreased TNF-α production in glioma associated endothelial cells and in turn facilitated cell-cell adhesion (VECAM-1) inhibition. In vivo mice model transfer IL-10 cDNA results in significant decrease in endothelial NF-kB activation and in cellular adhesion expression of ICAM-1 and VCAM-1 had already been documented [Mallat et al., 2000].
The present finding of IL-4 by FACS and ELISA assays indicated that in metastatic condition expression of IL-4 was low (Fig. 19) compared to normal. Others have shown that anti-inflammatory cytokines IL-12 and IL-4 appear at lower level in patient’s sample serum at 57–80% compared to controls group [Albulescu et al., 2013]. Furthermore, our statistical analysis (t-test) suggests that T11TS administration in glioma bearing rats enhance IL-4 expression compared to ENU group. The earliest studies of IL-4 in macrophages showed that it acted as an anti-inflammatory agent when administered concurrently or shortly after an inflammatory stimulus, and was capable of downregulating the production of inflammatory cytokines such as TNF-α [Hart et al., 1989].

Inspired from the outcome of pro-inflammatory and anti-inflammatory cytokines results our next goal was to study the expression pattern of cell adhesion molecules. During inflammatory process traffic of leukocytes across the endothelial layer through the cell-cell junctions is required. Vascular Endothelial Cadherin (VE-cadherin/CD144), cell adhesion molecules which are equally important regulators of angiogenesis apart from Integrin. CD144 form intercellular junction between endothelial cells, which give the endothelium the ability to control the passage of solutes/macromolecules and circulating cells (leukocytes) [Simionescu et al., 1991] and endothelial surface polarity [Muller et al., 1986] and thereby protects the underlying tissue from damage in pathological condition such as inflammation and tumor development. However, during inflammation and angiogenesis redistribution of VE cadherin regulate the initiation and maturation of newly formed vessels. In endothelial cells stabilization of VE-cadherin is maintained by its intracellular partner’s beta-catenin. Differential expression of catenins is thought to play an important role in the regulation of VE-cadherin function [Lampugnani et al., 1997]. Majority of these studies are based on in vitro experiments with HUVECs. Research evidence elucidating the implications of vasculature break induction in vivo in glioma associated brain endothelial cells on control of glioma angiogenesis is limited. We have attempted to delineate the vasculature break in endothelial adhesion and proteins involved in T11TS induced regression of glioma angiogenesis. The findings unearthed that (Fig. 10) there were significant (P<0.001) over expression of CD144 in brain ECs in ENU group compared to normal. Over expression of CD144 initiate the migration of leukocytes across the endothelial layer through the cell-cell junctions and simultaneously, the initiation of angiogenesis occurs when the continuity of the endothelial layer is interrupted due to the loosening of the cell-cell contacts enabling the endothelial cells to migrate to the free area [Ozawa et al., 1998; Allport et al., 2000]. Our in situ immunofluorescence staining of glioma induced brain sections (Fig. 11) studies revealed that in ENU group cells contacted with each other which maintained the morphological integrity and quiescence of the newly formed vessel which further suggests that VE-cadherin may be a key player in this process.
Following T11TS administration in glioma bearing rats the expression of VE-cadherin was concomitantly down regulated in glioma associated brain ECs proving that T11TS deformed the morphological integrity and decreased cellular proliferation rate as is evident in the (ET1 and ET2) slide of insitu immunoflorescence. However, after noting the significant(P<0.001) changes in VE-cadherin expression in T11TS therapy we further evaluated the effects of T11TS on the expression level of beta-catenin in normal, glioma and Glioma+T11TS treated animals. Our results obtained in this study of glioma induced rat model indicate that the T11TS significantly inhibits the expression level of beta-catenin in glioma associated brain ECs compared to ENU which was confirmed by western blot and flow cytometry Figure12. Previous experiments have revealed that treatment with curcumin concomitantly declined the cytosol as well as a nuclear protein expression of beta-catenin in HUVECs cell culture medium. In line with this our data strongly pointed that T11TS therapy not only inhibited the VE-cadherin expression but it can also downregulated the cytoplasmic bound form of Beta-catenin. Collectively, these results obtained from CD144 and Beta-catenin experiments confirmed that T11TS therapy causes the disruption of the vasculature and break in endothelial adhesion in glioma induced rat.

CD44 a transmembrane glycoprotein, found in a wide variety of tissues including the brain tissue. Expressions of CD44 are associated with a high rate of cell division and allow tumor cells to colonize through its ligands hyaluronan in tumor micro environments. Like collagen, Hyaluronan (HA) is another abundant component of the ECM in brain compartment and plays an essential role during glioma invasion through the proteolytic modification [Bellail et al., 2004; Kim et al., 2005a; Kim et al., 2005b; Bignami et al., 1992]. During glioma growth certain matrix molecules including HA are synthesized in the brain micro-environment [Wiranowska et al., 1994; Knott et al., 1998; Mahesparan et al., 2003] and provides hydrated spaces for glioma cell migration and invasion for the consolidation of neovassles. Accumulating, results evidence that in numerous pathological conditions including tumor invasion and inflammation, an injury-associated increase in the production of HA [Dahl et al., 1985; Day et al., 2005; Edward et al., 2007]. It was previously reported that the standard form of CD44 exists in three isoforms: a cytoplasmic tail, a transmembrane region and an extracellular large domain [Griffioen et al., 1997]. The extracellular domain of CD44 contains a docking site for ECM components and responsible for binding of HA [Porta et al., 2003]. Under regulatory condition CD44 is over expressed in tumor cells and is cleaved by membrane-associated metalloproteinase (MMPs). This shedding of CD44 plays a critical role in efficient efficient cell detachment from the HA substrate and enhance tumor cell migration and invasion [Okamoto et al., 1999; & 2001; Murakami et al., 2003; Murai et al., 2004; Okada et al., 1996].
Expression of CD44 at the mRNA and protein level in ECs was investigated in vitro model and provided the evidence that the molecule is involved in tumor angiogenesis. Distribution of CD44 on endothelial cells is controversial because few studies have reported that endothelial cells do not expressed this marker while recent other studies have showed that after additional activation with tumor necrosis factor-α (TNF-α) EC expressed the CD44 molecules on their membrane in tumor angiogenic condition which might be of advantage for leukocyte infiltration in inflamed tissue and is therefore able to contribute directly to endothelial cell activation. We quantitatively studied endothelial CD44 expression in glioma induced brain endothelial cells and also in T11TS treated animals. Flow cytometric measurements indicate a high expression of CD44 on glioma associated brain endothelial cells. Western blot analysis of EC lysates, prepared from all five groups reveal and suggesting similar reversion shifts of the expression of CD44 as in FACS from ENU to ET1, ET2, & ET3 groups. This difference is probably caused by angiogenic factor produced by tumor cell which might lead to homotypic CD44 adhesion (involving HA) to EC and facilitated extravasation requires for metastatic spread. While, administration of T11TS causes the break in homotypic cell adhesion to ECs.