European Journal of Biotechnology and Bioscience
Online ISSN: 2321-9122
www.biosciencejournals.com
Volume 3; Issue 10; October 2015; Page No. 45-51

A review-potential angiogenesis targets and frontiers in cancer research: anthrax toxin's receptors

Sugandha Bhatia

Department of Biotechnology, Lovely Professional University, Jalandhar-Delhi G.T. Road (NH-1), Phagwara, Punjab, India.
Academy of Scientific and Innovative Research (AcSIR), CSIR-IGIB, Delhi, India.

Abstract

The role of cognate host receptors of anthrax toxin binding protein, Tumor Endothelial Marker-8 (TEM-8) and Capillary Morphogenesis Gene-2 (CMG-2) are being delineated from entry portals of anthrax toxin proteins to intercellular site to being regulators in angiogenesis. *In vitro* and *in vivo* model systems had documented their upregulation on endothelial cells undergoing angiogenesis or blood vessel growth. Inhibiting angiogenesis (formation of new blood vessels), which are feeding and supporting tumors are also one of the current targets of anti-cancer therapies. TEM-8 and CMG-2 are highly upregulated during angiogenesis and bind differentially to extracellular matrix proteins including collagen I, collagen VI, collage XIV and laminin. TEM-8 and CMG-2 regulate endothelial cell migration, proliferation and tubule formation which make them apparently good targets as anti-angiogenic and anti-tumorigenic therapeutics. This intriguing finding has also led to detailed studies on the strategies of designing modified forms of anthrax toxin proteins (Protective Antigen, Lethal Factor, Edema Factor) which impairs the normal cellular activities to combat tumor endothelium specifically.

Keywords: Angiogenesis, anti-tumorigenic, anthrax toxin receptors (ANTXR1 and ANTXR2), extracellular matrix, TEM-8 (tumor endothelial marker-8), CMG-2 (capillary morphogenesis gene-2).

1. Introduction

It is well anticipated that tumor and the blood vessels supporting pathological angiogenesis differentially express many genes as compared to normal functional blood vessels and tissues. Among which, a vast panel of 'tumor endothelial markers' (TEM 1 to TEM 9) are over-expressed and highlighted in various malignant tumor endothelium as compared to normal endothelium (Mehran *et al.*2014; St Croix *et al.,* 2000) [47, 59]. One of the increased expression is also of TEM-8, which was also subsequently discovered to be the first receptor for anthrax toxin and in that context named as anthrax toxin receptor 1 (ANTXR1). *In vitro* screening of endothelial cells undergoing capillary tube formation in 3D collagen matrices upregulate many genes involved in basement matrix assembly, growth factors, endothelial cells differentiation markers and capillary morphogenesis gene-2 (CMG-2) (Bell *et al.,* 2001) [2]. This protein, termed as anthrax toxin receptor 2 (ANTXR2) was later identified by both Collier and Leppla group as a major receptor for anthrax toxin mediating lethality *in vivo* (Scobie *et al.,* 2003) [56]. Thus, both of the anthrax toxin receptors, CMG-2 and TEM-8, has potential role in the vasculature formation during angiogenesis.

2. Anthrax toxin Proteins

Bacillus anthracis is a gram-positive, rod-shaped, spore forming and capsule forming bacteria which secrets virulence factors that are the main causative agents of anthrax toxicity (Mock and Fouet, 2001) [48]. *B. anthracis* can enter through the wounded skin, gastrointestinal tract or by inhalation in the respiratory tract. The spores are taken up by macrophages (present adjacent to the epithelial route) and are germinated when macrophages reached to the lymph nodes (Weiner and Hanna, 2003) [49]. Anthrax secretes tripartite toxin, where PA (protective antigen) acts as the binding (B) domain and LF (lethal factor) and EF (edema factor) act as active (A) domains individually to form the binary toxins: lethal toxin (LeTx) and edema toxin (EdTx), respectively (Singh *et al.,* 1999) [8]. These tripartite proteins of the anthax toxin- LF, EF and PA are major virulent agents in infected hosts and the second major virulence factor in *B. anthracis* is its poly-γ-D-glutamic acid capsule (Collier and Young, 2003) [20].

PA recognizes the von Willebrand Factor A (vWA) domain of the two cell surface receptors, TEM-8 and CMG-2. Receptor-bound 83 kDa PA protein is proteolytically processed by host furin protein present in cell membrane, resulting in the dissociation of a 20 kDa fragment of PA from its N terminus. The remaining receptor bound 63kDa PA heptamerizes, orchestrating the formation of a prepore that can bind up to 3 molecules of LF or EF and instigating internalization of the complex into clathrin coated endosomes. There are implications that various signaling mechanism are recruited after the toxin binding to the receptor to mediate its internalization which includes src-like kinase (SLK) signaling, Wnt signaling, and PI3 kinase signaling (Abrami *et al.,* 2008, 2010; Wei *et al.,* 2006) [3, 62]. Src-like kinases, src and fyn are predicted to phosphorylate tyrosine residues at the intercellular sites of TEM-8 or CMG-2 receptors after the binding of the PA subunit of anthrax toxin. It's further demonstrated that Src dependent of the TEM-8 and CMG-2 is required for their subsequent ubiquitination, which turns on the next event that is clathrin-mediated endocytosis of the receptors and internalization of anthrax toxin (Abrami *et al.,* 2010) [2, 4]. Endocytosis is also mediated by toxin-induced ubiquitination of anthrax receptors by the E3 ligase Cbl (Abrami *et al.,* 2006) [6]. Role of heterotetrameric adaptor protein AP-1 in internalization of the clathrin coated endosomes is also been documented (Abrami *et al.,* 2010) [2, 4]. After internalization, the low pH environment of the endocytic vesicle causes a conformational rearrangement of

the PA prepore structure to pore formation by extending into the membrane structure of vesicle (Blaustein *et al.,* 1989) [10]. This acidic pH also mediates unfolding of LF and EF toxin proteins which drives their translocation in the intercellular milieu (Krantz *et al.,* 2005) [37].

LF is a 90-KDa zinc-dependent metalloprotease that cleaves the members of mitogen-activated protein kinase kinases family (Klimpel *et al.,* 1994 [35]; Duesbery *et al.,* 1998) [26]. Inactivation of these major MEKs *i.e*., extracellular signal regulated kinases, c-Jun N-terminal kinases and p38 MAPKs impairs various important cellular processes task such as cell division, cell differentiation, cellular response to different types of stress (Cargnello and Roux, 2011) [16] and LF also directly activates Nlrp1b inflammasome and caspase-1 dependent apoptotic machinery (Levin *et al.,* 2008; Kirby 2004) [39, 34] as illustrated in Figure 1.

EF (89kDa) is a calmodulin-dependent adenylate cyclase which amplifies the level of cAMP (Robertson *et al.,* 1988) [53]. Activity of EF is regulated in a biphasic manner by intracellular level of Ca^{++}. Resting or cellular levels of Ca^{++} activates the EF *via* calmodulin, whereas excessive levels of Ca^{2++} reduce its activity due to competitive inhibition by Mg^{++} ion in the active site (Tang and Guo, 2009). EF is considered to be associated with the perinuclear later endosomal membrane, thereby generating a cAMP gradient decreasing from the nucleus to plasma membrane (Guidi-Rontani *et al.,* 2000; Zornetta *et al.,* 2010; Dal Molin *et al.,* 2006) [30, 66, 22] which is in contrast to the endogenous host adenylate cyclases generated a cAMP gradient (in decreased orientation from plasma membrane to nucleus) (Dal Molin *et al.,* 2006; 2007) [22]. The intracellular actions of EF and LF had cytotoxic effect on the host cells and ultimately lead to loss of cellular viability. The disease is mostly lethal because of high bacterial load and release of tripartite proteins in high amount in the host sera leading to multi-organ and immune system failure and ultimately death of the host (Turk, 2007) [61].

3. Anthrax Toxin Receptors TEM-8 and CMG-2

Both TEM-8 and CMG-2 are type one transmembrane proteins, which contains an extracellular metal ion dependent adhesion site (MIDAS) for PA binding in a von Willebrand Factor A (vWA) domain of approximately 200 amino acids. vWA domains also facilitate interactions of protein with its cognate ligands reported such as collagen VI, collagen XIV and integrins. Three isoforms of TEM8 (of length 333, 368 and 564 amino acids) and four isoforms of CMG-2 (of length 322, 386, 488 and 489 amino acids) are generated by mRNA splice variants. The short isoforms are predicted to be secretory proteins as they are devoid of transmembrane region and cannot internalize PA moieties and the long isoforms of TEM-8 and CMG-2 shared 40% amino acid identity throughout their sequence and 60% amino acid identity within their vWA domains (Scobie *et al.,* 2003; Bradley *et al.,* 2001; Carson-Walter *et al.,* 2001; Liu *et al.,* 2003) [56, 14, 17], 40, 43]. The structure of the long isoforms of TEM-8 and CMG-2 has been plotted using Protter, a web based tool (Omasits *et al.,* 2014) [50] (Figure 2) highlighting the different residues involved in PA binding and various other post translational modifications reported till date such as phosphorylation, ubiquitination and palmitoylation. The cytoplasmic tails of TEM-8 and CMG-2 have been demonstrated as important in regulating the half-life of the receptors at the plasma membrane. Palmitoylation of cytoplasmic cysteine residues has been documented to increase the half-life of the receptors by preventing their premature clearance from the membrane (Abrami *et al.,* 2006) [6]. Both receptors also contain tyrosine residues, which after PA binding gets phosphorylated and is responsible for toxin internalization (Abrami *et al.,* 2010) [2, 4]. However, truncation studies of the TEM-8 receptor had postulated the relevance of extracellular and transmembrane region for PA binding, processing, oligomer formation, and translocation of lethal toxin into the cytosol (Liu *et al.,* 2003) [40, 43]. In the absence of anthrax toxin, TEM-8 resides in intracellular endosomes as well as in the cell membrane and is recycled between endosomes and the cell membrane via process regulated by Rab11 GTPase (Gu *et al.,* 2010) [29]. CMG2 has been confirmed as the major anthrax toxin receptor *in vivo* as *CMG2*$^{-/-}$ mice were completely resistant to B. anthracis spore infection (Liu *et al.,* 2009). The PA has 11-fold higher affinity for CMG2 than for TEM8. The K_d of the vWA domain of CMG-2-PA interaction is 170 pm indicating a 1000-fold tighter interaction than binding of vWA domains to endogenous ligands. This dissociation rate constant also reflected stable interaction time of CMG2.PA monomer complex for upto 30 h suggesting its possible therapeutic role where soluble forms of CMG-2 can acts as decoy receptors for anthrax toxicity and to succumb potential tumor (Wigelsworth *et al.,* 2004 [38, 65]; Lacy *et al.,* 2004) [38, 65].

4. Expression studies of TEM-8 and CMG-2

Upregulated expression of TEM-8 and CMG-2 receptors is reported during angiogenesis; tubule formation *in vitro* and in endothelial cells lining tumor blood vessels (Bell *et al.,* 2001 [2]; St Croix *et al.,* 2000) [59]. TEM-8 is temporally regulated and induced during chick embryogenesis (Herrmann *et al.,* 2010) [31]. Both TEM-8 and CMG-2 proteins expression were localized to epithelial lining of the mouse organs that also constitute anthrax toxins site of entry – skin, lung, and small intestine. Different isoforms of TEM8 were detected by western blot in mouse heart, skin, small intestine, ovary, testis, spleen, liver, lung, brain and kidney (Bonuccelli *et al.,* 2005) [12, 32] whereas no such comparative data of TEM8 expression determination in different human tissues is till date being validated. However, preferential expression of TEM-8 protein was observed in the blood vessels of colon cancer tissues as compared to patient-matched normal colonic mucosa. Its increased expression was also detected in endothelial cells of human tumors, including bladder, esophageal, and lung (Nanda *et al.,* 2004) [49]. CMG-2 mRNA expression has been reported in many human tissues, including heart, skeletal muscle, colon, spleen, kidney, liver, small intestine, placenta, and lung (Scobie *et al.,* 2003) [56]. Immunohistochemical localization studies had determined expression of CMG-2 in the mouse vascular endothelium and epithelial cells lining skin, colon, and lung. In both normal and malignant breast tissue, CMG-2 expression co-localizes with Collagen IV (Reeves *et al.,* 2009) [51]. Apart from both being common anthrax toxin receptor, no *in vivo* or *in vitro* striking studies of CMG-2 and TEM-8 with each other on the co-expression and interaction pattern has been postulated.

5. Indigenous role of TEM-8 and CMG-2

CMG-2 influences the endothelial cell proliferation and tubule formation in cell culture studies observed, with little effect on the migration of endothelial cells. Studies using RNA interference had interpreted that decreased CMG-2 expression

in human umbilical vein endothelial cells (HUVECs) results in reduced cellular proliferation and capillary tubules formation in Collagen I gels, whereas there was no effect on the cellular migration demonstrated using cellular scratch wound assay. However, overexpression of CMG-2 causes increased cellular proliferation and tubule network formation in these HUVEC cells (Reeves *et al.,* 2009) [51].

Evidence for the role of TEM-8 has emerged to be a key regulator in maintaining endothelial cell shape and migration. Overexpression of TEM-8 in endothelial cells results in its migration three times faster than the regular rate and enhances its adhesion to Collagen I thereby inhibiting the adhesion of endothelial cells to gelatin or collagen I (Hotchkiss *et al.,* 2005) [32]. Comparatively, TEM-8 knockdown by ribozyme transgenes in immortalized human umbilical cord endothelial cells (HECVs) exhibited reduced migration and tubule formation in comparison with control HECVs (Rmali *et al.,* 2005) [52]. Role of TEM-8 in regulating cell spreading is also being demonstrated in HEK293 cells. HEK293 cells adherence onto a PA coated surface plates resulted in wider cell spreading and cell body extension via TEM-8. TEM-8 interaction with the actin cytoskeleton was also observed and its inhibition by a cytoskeleton- disrupting drug, cytochalasin D localize TEM-8 and actin at the base of lamellipodia and during cell spreading, actin filaments along with TEM-8 colocalizes into the lamellipodia (Werner *et al.,* 2006) [64]. CHO cells, being of non-endothelial origin, transfection of full length TEM-8, a transmembrane/vWA construct and the vWA domain alone resulted in 'tubules' formation in 24 hours within these cells in Matrigel assay (Rmali *et al.,* 2005) [52]. The mice generated by knocking down the TEM-8 receptor expression by Liu and Cullen group were all normal in growth and development with the exception of excess extracellular matrix deposition such as collagen type I, type VI in many tissues. Tumor growth was also delayed in TEM-8$^{-/-}$ knockout mice when B16 melanoma cells were implanted. Interestingly, the defects in the physiological angiogenesis were not observed in TEM-8$^{-/-}$ mice (Cullen *et al.,* 2009; Liu *et al.,* 2009) [21] whereas recombinant soluble forms of the TEM-8 receptor have anti-angiogenic effects *in vitro* (Hotchkiss *et al.,* 2005) [32] ascertaining TEM-8 can be a better target for pathological angiogenic therapeutics.

6. Strategies to combat tumor using biological anthrax toxin molecules

In a number of studies, native and mutant forms of Anthrax toxin molecules (PA & LF) have been investigated as anti-angiogenic and tumoricidal agents. PA has been used to facilitate the specific delivery of anthrax and other fused toxin proteins from Diphtheria, Pseudomonas using TEM-8 & CMG-2 receptors. PA combined with FP59 (consists of the first 254 amino acids of *B. anthracis* lethal toxin for PA binding and ADP ribosylation domain of Pseudomonas exotoxin A), a fusion protein induces 50 fold more tumor cell death than PA and LF combination (Arora *et al.,* 1993) [9]. Direct tumoricidal and anti-angiogenic effects of LeTx have been reported due to MAPK inhibition by LF which disrupts the further signal cascade and was earlier considered as the main inhibitory mechanism for tumor growth (Duesbery *et al.,* 2001; Koo *et al.,* 2002; Huang *et al.,* 2008) [25, 36, 33]. During *in vitro* studies, many tumors had exhibited constitutive MAPK signaling rendering them more susceptible to LeTx induced toxicity. For example, in melanoma cells, a point mutation in B-RAF makes it constitutively active without stimulation by growth factors resulting in enhanced MAPK activity (Abi-Habib *et al.,* 2005; Alfano *et al.,* 2008) [1, 6].

The mutants of PA for its selective targeting of tumor cells had also being designed. These mutations are at site recognized by furin for cleavage and is replaced by sites recognized by the proteases which are overexpressed in tumor cells eg matrix metalloproteinase (MMP) or urokinase plasminogen activator (uPA). In vitro, these combinations of mutated PA molecules had represented potent anti-angiogenic and tumoricidal activity and reduced animal toxicity when injected in mice compared with administration of PA molecules mutated at the sites recognized by Furin protease. It has been observed that administration of FP59 along with the mutant of PA that showed more prefer in binding to TEM-8 receptor than CMG-2 achieves more sepecific tumoricidal activity (Liu *et al*., 2005; 2008; Chen *et al.,* 2007) [1] emphasizing TEM-8 receptor also as better target for tumoricidial strategies. Significantly, the toxicity to the injected animal was also reduced and the immune response generated to this altered PA protein was approximately 6-fold lower than that by native PA (Liu *et al.,* 2008). Lethal toxin designed specifically for targeting tumor vasculature by substituting furin cleavge site with matrix metalloproteinases also inhibited the endothelial cell invasion. Also, endothelial cells interaction with extracellular matrix molecules such as collagen I, collagen IV and gelatin was reduced, thereby promoting its inability to form blood- vessel like tubules (Alfano *et al.,* 2009) [7].

Further, a form of PA is also been designed with three amino acids mutated PA^{SSSR}, (R164S, K165S,K166S, R167R) which is resistant to cleavage by endogenous furin-like proteases, and remains bound to the anthrax toxin receptors for an extended period of time without being internalized. The blockade of anthrax receptors inhibited vascular endothelium growth factor (VEGF) and serum-induced human microvascular endothelial cell (HMVEC) migration with no effect on endothelial cell proliferation. In the experiment performed using corneal micropocket assay, PA^{SSSR} inhibited angiogenesis in vivo and inhibits Lewis Lung carcinoma growth. PA^{SSSR} can be considered as nice candidate for reducing angiogenesis and tumor growth as it excludes use of toxin LF n EF molecules on endothelial or tumor cells (Rogers *et al.,* 2007; Gordon *et al.,* 1995) [54, 28].

As evidence for its role as anti-angiogenic, it been reported that intravitreal injection of LeTx can perturbed the mouse retinal vasculature development (Bromberg-White *et al.,* 2009) [15]. In vivo models of the anaplastic thyroid carcinoma (ATC) tumors from mice treated with LeTx displayed reduced endothelial cell recruitment and tumor vascularization. PA mutated to recognize MMP proteases induced necrosis of tumor cells within eighteen hours after a single dose of toxin proteins injection without affecting the vasculature of these tumors (Alfano *et al.,* 2010) [8]. In immunocompromised mice, intratumoral injections of LeTx had inhibited tumor growth along with reduction in the number of blood vessels supporting the tumors at concentrations that even do not result in animal toxicity (Duesbery *et al.,* 2001) [25]. A recombinant soluble TEM-8 receptor and Anti-TEM8 monoclonal antibodies designed had selectively blocked tumor growth and tumor angiogenesis (Hotchkiss *et al.,* 2005 [32]; Chaudhary *et al.,* 2012) [18]. Anti-tumorigenic molecules targeting TEM-8 will represent tumor- selective agents as comparative studies of both

mouse and human tissues had reported the higher expression of TEM-8 within tumor vessels as compared to other cells within the tumor environment and normal tissues (St Croix *et al.,* 2000; Carson-Walter *et al.,* 2001; Nanda *et al.,* 2004) [59, 17, 49]. In various pre-clinical models, anti-angiogenic TEM-8 targeted therapeutics designed include a fusion protein combining anti-TEM-8 antibody with truncated tissue factor, which disrupts the colorectal tumor vasculature and promotes local thrombosis, thereby reducing colorectal tumor volume (Fernando *et al.,* 2009) [27]. DNA vaccine targeted to TEM-8 also showed signs of anti-angiogenic and inhibited tumor growth in mice (Ruan *et al.,* 2009) [55]. TEM-8-Fc is a monoclonal antibody with anti-angiogenic effect that inhibits metastasis of some tumors and had showed remarkably reduced blood vessels density (Duan *et al.,* 2007).

7. Conclusion

It is now well perceived that TEM-8 and CMG-2 is highly upregulated during angiogenesis and specific targeting of these receptors can avert pathological angiogenesis and tumor growth. Both receptors promote tubule formation of the blood vessels where TEM-8 mediates endothelial cell migration and CMG-2 influences endothelial cell proliferation. These receptors bind to extracellular matrix proteins, such as collagen, integrins to regulate the tubule development. However, despite being toxin receptors and important tumor marker, the exact downstream signal transduction mechanisms by which the anthrax receptors influence angiogenic responses are not been documented. The natural ligands for these receptors in vivo is also not been discovered till date. Further, open challenges which need to be addressed are the understanding of the diverse mechanism which impairs the cell death by lethal toxin apart from MAPKK pathway inhibition. Lethal toxin and its mutant being represented as novice strategy for tumoricidal therapy but its unwanted toxicity and immunogenic responses developed to the delivered patients' needs to be assess cautiously before it is being implemented as therapeutic cure against tumor and pathological angiogenesis.

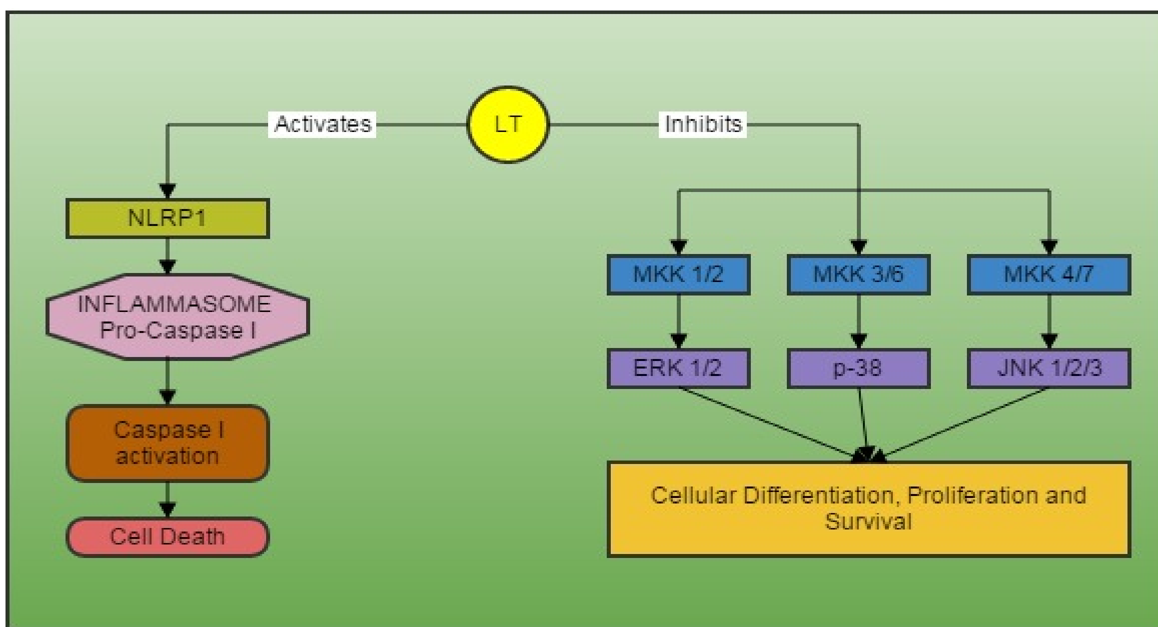

Fig 1: Targets of Lethal Toxin: The Lethal Toxin (LT) after internalization inactivates various MKKs, targeting the downstream signal of the MAPK signaling pathways which are essential for cellular differentiation, proliferation and survival and on another side, LT induces the Nlrp1 activation of the inflammasome subsequently activating the apoptotic machinery. Both the mechanisms impaired the normal cellular activities resulting in cell death.

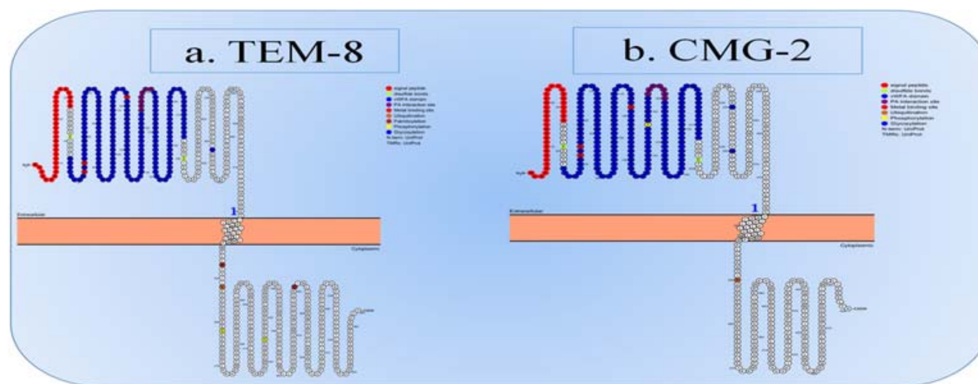

Fig 2: Predicted membrane Topology of TEM-8 and CMG-2 demonstrating experimentally verified PA interaction site and other observed post translational modifications (ubiquitination, palmitoylation, phosphorylation, glycosylation) at their specific residues. Image is generated using Protter with UniProt accession: Q9H6X2 for TEM-8 and P58335 for CMG-2.

Acknowledgments
This review was prepared as part of the course work curriculum at Council of Scientific and Industrial Research (CSIR)-Institute of Genomics and Integrative Biology. The author thanks Dr. Yogendra Singh and Dr. Lalit Kumar Singh for critical reading and providing valuable suggestions in the manuscript.

References

1. Abi-Habib RJ, Urieto JO, Liu S, Leppla SH, Duesbery NS, Frankel AE. BRAF status and mitogen- activated protein/extracellular signal-regulated kinase kinase 1/2 activity indicate sensitivity of melanoma cells to anthrax lethal toxin. Mol Cancer Ther 2005; 4:1303-1310.
2. Abrami L, Bischofberger M, Kunz B, Groux R, van der Goot FG. Endocytosis of the anthrax toxin is mediated by clathrin, actin and unconventional adaptors. PLoS Pathog 2010a; 6:e1000792. (Mentioned Year with alphabets in the text, since both have same year i.e., 2010a)
3. Abrami L, Kunz B, Deuquet J, Bafico A, Davidson G, van der Goot FG. Functional interactions between anthrax toxin receptors and the WNT signalling protein LRP6. Cell Microbiol 2008; 10:2509-2519.
4. Abrami L, Kunz B, van der Goot FG. Anthrax toxin triggers the activation of src-like kinases to mediate its own uptake. Proc Natl Acad Sci USA 2010b; 107:1020-1024. (Mentioned Year with alphabets in the text, since both have same year i.e., 2010b)
5. Abrami L, Leppla SH, Van Der Goot FG. Receptor palmitoylation and ubiquitination regulate anthrax toxin endocytosis, J Cell Biol. 2006; 172:309-320.
6. Alfano RW, Leppla SH, Liu S, Bugge TH, Herlyn M, Smalley KS *et al.* Cytotoxicity of the matrix metalloproteinase-activated anthrax lethal toxin is dependent on gelatinase expression and B-RAF status in human melanoma cells. Mol Cancer Ther 2008; 7:1218-1226.
7. Alfano RW, Leppla SH, Liu S, Bugge TH, Meininger CJ, Lairmore TC *et al.* Matrix metalloproteinase-activated anthrax lethal toxin inhibits endothelial invasion and neovasculature formation during in vitro morphogenesis. Mol Cancer Res. 2009; 7:452-461.
8. Alfano RW, Leppla SH, Liu S, Bugge TH, Ortiz JM, Lairmore TCet *et al.* Inhibition of tumor angiogenesis by the matrix metalloproteinase- activated anthrax lethal toxin in an orthotopic model of anaplastic thyroid carcinoma. Mol Cancer Ther 2010; 9:190-201.
9. Arora N, Leppla SH. Residues 1–254 of anthrax toxin lethal factor are sufficient to cause cellular uptake of fused polypeptides, J Biol Chem. 1993; 268:3334-3341.
10. Blaustein RO, Koehler TM, Collier RJ, Finkelstein A. Anthrax toxin: channel-forming activity of protective antigen in planar phospholipid bilayers. Proc. Natl. Acad. Sci. USA 1989; 86:2209-2213.
11. Bell SE, Mavila A, Salazar R, Bayless KJ, Kanagala S, Maxwell SA *et al.* Differential gene expression during capillary morphogenesis in 3D collagen matrices: regulated expression of genes involved in basement membrane matrix assembly, cell cycle progression, cellular differentiation and G-protein signaling, J Cell Sci. 2001; 114:2755-2773.
12. Bonuccelli G, Sotgia F, Frank PG, Williams TM, de Almeida CJ, Tanowitz HB *et al.* ATR/TEM8 is highly expressed in epithelial cells lining *Bacillus anthracis* three sites of entry: implications for the pathogenesis of anthrax infection, Am J Physiol Cell Physiol. 2005; 288:1402-1410.
13. Bradley KA, Mogridge J, Jonah G, Rainey A, Batty S, Young JA. Binding of anthrax toxin to its receptor is similar to alpha integrin-ligand interactions, J Biol Chem. 2003; 278:49342-49347. (Mention in the text)
14. Bradley KA, Mogridge J, Mourez M, Collier RJ, Young JA. Identification of the cellular receptor for anthrax toxin. Nature 2001; 414:225-229.
15. Bromberg-White JL, Boguslawski E, Duesbery NS. Perturbation of mouse retinal vascular morphogenesis by anthrax lethal toxin. PLoS One 2009; 4:e6956.
16. Cargnello M, Roux PP. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev. 2011; 75:50-83. doi 10.1128/MMBR.00031-10.
17. Carson-Walter EB, Watkins DN, Nanda A, Vogelstein B, Kinzler KW, St Croix B. Cell surface tumor endothelial markers are conserved in mice and humans. Cancer Res. 2001; 61:6649-6655.
18. Chaudhary A, Hilton MB, Seaman S, Haines DC, Stevenson S, Lemotte PK *et al.* TEM8/ANTXR1 blockade inhibits pathological angiogenesis and potentiates tumoricidal responses against multiple cancer types. Cancer Cell 2012; 21:212-226. doi 10.1016/j.ccr.2012.01.004.
19. Chen KH, Liu S, Bankston LA, Liddington RC, Leppla SH. Selection of anthrax toxin protective antigen variants that discriminate between the cellular receptors TEM8 and CMG2 and achieve targeting of tumor cells, J Biol Chem. 2007; 282:9834-9845.
20. Collier RJ, Young JA. Anthrax toxin. Annu Rev Cell Dev Biol 2003; 19:45-70. doi 10.1146/annurev. cellbio.19.111301.140655
21. Cullen M, Seaman S, Chaudhary A, Yang MY, Hilton MB, Logsdon D *et al.* Host-Derived Tumor Endothelial Marker 8 Promotes the Growth of Melanoma. Cancer Res. 2009; 69:6021-6026. doi: 10.1158/0008-5472.
22. Dal Molin F, Tonello F, Ladant D, Zornetta I, Zamparo I, Di Benedetto G *et al.* Cell entry and cAMP imaging of anthrax edema toxin, EMBO J. 2006; 25:5405-5413. doi 10.1038/sj.emboj.7601408
23. Dal Molin F, Zornetta I, Puhar A, Tonello F, Zaccolo M, Montecucco C. camp imaging of cells treated with pertussis toxin, cholera toxin, and anthrax edema toxin. Biochem Biophys Res Commun 2008; 376:429-433. doi 10.1016/j.bbrc.2008.09.012
24. Duan HF, Hu XW, Chen JL, Gao LH, Xi YY, Lu Y *et al.* Antitumor activities of TEM8-Fc: an engineered antibody-like molecule targeting tumor endothelial marker 8, J Natl Cancer Inst. 2007; 99:1551-1555.
25. Duesbery NS, Resau J, Webb CP, Koochekpour S, Koo HM, Leppla SH *et al.* Suppression of ras-mediated transformation and inhibition of tumor growth and angiogenesis by anthrax lethal factor, a proteolytic inhibitor of multiple MEK pathways. Proc Natl Acad Sci USA. 2001; 98:4089-4094.

26. Duesbery NS, Webb CP, Leppla SH, Gordon VM, Klimpel KR, Copeland TD *et al.* Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. Science 1998; 280:734-737.
27. Fernando S, Fletcher BS. Targeting tumor endothelial marker 8 in the tumor vasculature of colorectal carcinomas in mice. Cancer Res. 2009; 69:5126-5132.
28. Gordon VM, Klimpel KR, Arora N, Henderson MA, Leppla SH. Proteolytic activation of bacterial toxins by eukaryotic cells is performed by furin and by additional cellular proteases. Infect Immun 1995; 63:82-87.
29. Gu J, Faundez V, Werner E. Endosomal recycling regulates anthrax toxin receptor 1/tumor endothelial marker 8-dependent cell spreading. Exp Cell Res. 2010; 316:1946-1957. doi 10.1016/j.yexcr.2010.03.026.
30. Guidi-Rontani C, Weber-Levy M, Mock M, Cabiaux V. Translocation of *Bacillus anthracis* lethal and oedema factors across endosome membranes. Cell Microbiol 2000; 2:259-264. doi 10.1046/j.1462-5822.2000.00057.x
31. Herrmann D, Ferrer-Vaquer A, Lahsnig C, Firnberg N, Leibbrandt A, Neubuser A. Expression and regulation of ANTXR1 in the chick embryo. Dev Dyn 2010; 239:680-687.
32. Hotchkiss KA, Basile CM, Spring SC, Bonuccelli G, Lisanti MP, Terman BI. TEM8 expression stimulates endothelial cell adhesion and migration by regulating cell-matrix interactions on collagen. Exp Cell Res. 2005; 305:133-144.
33. Huang D, Ding Y, Luo WM, Bender S, Qian CN, Kort E *et al.* Inhibition of MAPK kinase signaling pathways suppressed renal cell carcinoma growth and angiogenesis in vivo. Cancer Res. 2008; 68:81-88.
34. Kirby JE. Anthrax lethal toxin induces human endothelial cell apoptosis. Infect Immun. 2004; 72:430-439.
35. Klimpel KR, Arora N, Leppla SH. Anthrax toxin lethal factor contains a zinc metalloprotease consensus sequence which is required for lethal toxin activity. Mol Microbiol 1994; 13:1093-1100.
36. Koo HM, VanBrocklin M, McWilliams MJ, Leppla SH, Duesbery NS, Woude GF. Apoptosis and melanogenesis in human melanoma cells induced by anthrax lethal factor inactivation of mitogen- activated protein kinase kinase. Proc Natl Acad Sci USA 2002; 99:3052-3057.
37. Krantz BA, Melnyk RA, Zhang S, Juris SJ, Lacy DB, Wu Z *et al.* A phenylalanine clamp catalyzes protein translocation through the anthrax toxin pore. Science 2005; 309:777-781.
38. Lacy DB, Wigelsworth DJ, Scobie HM, Young JA, Collier RJ. Crystal structure of the von Willebrand factor a domain of human capillary morphogenesis protein 2 an anthrax toxin receptor. Proc Natl Acad Sci USA 2004; 101:6367-6372.
39. Levin TC, Wickliffe KE, Leppla SH, Moayeri M. Heat shock inhibits caspase-1 activity while also preventing its inflammasome-mediated activation by anthrax lethal toxin. Cell Microbiol 2008; 10:2434-2446.
40. Liu S, Aaronson H, Mitola DJ, Leppla SH, Bugge TH. Potent antitumor activity of a urokinase- activated engineered anthrax toxin. Proc Natl Acad Sci USA 2003a; 100:657-662. (Mentioned Year with alphabets in the text, since both have same year i.e., 2003a)
41. Liu S, Bugge TH, Leppla SH. Targeting of tumor cells by cell surface urokinase plasminogen activator-dependent anthrax toxin, J Biol Chem. 2001; 276:17976-17984.. (Mention in the text)
42. Liu S, Crown D, Miller-Randolph S, Moayeri M, Wang H, Hu H *et al.* Capillary morphogenesis protein-2 is the major receptor mediating lethality of anthrax toxin in vivo. Proc Natl Acad Sci USA 2009; 106:12424-12429. doi 10.1073/pnas.0905409106.
43. Liu S, Leppla SH. Cell surface tumor endothelium marker 8 cytoplasmic tail-independent anthrax toxin binding, proteolytic processing, oligomer formation, and internalization, J Biol Chem. 2003b; 278:5227-5234. (Mentioned Year with alphabets in the text, since both have same year i.e., 2003b)
44. Liu S, Netzel-Arnett S, Birkedal-Hansen H, Leppla SH. Tumor cell-selective cytotoxicity of matrix metalloproteinase-activated anthrax toxin. Cancer Res. 2000; 60:6061-6067.
45. Liu S, Redeye V, Kuremsky JG, Kuhnen M, Molinolo A, Bugge TH *et al.* Intermolecular complementation achieves high-specificity tumor targeting by anthrax toxin. Nat Biotechnol. 2005; 23:725-730.
46. Liu S, Wang H, Currie BM, Molinolo A, Leung HJ, Moayeri M *et al.* Matrix metalloproteinase-activated anthrax lethal toxin demonstrates high potency in targeting tumor vasculature, J Biol Chem. 2008; 283:529-540.
47. Mehran R, Nilsson M, Khajavi M, Du Z Cascone T, Wu HK, Cortes A *et al.* Tumor endothelial markers define novel subsets of cancer-specific circulating endothelial cells associated with antitumor efficacy. Cancer Res. 2014; 10:2731-2741. doi 10.1158/0008-5472.
48. Mock M, Fouet A. Anthrax. Annu Rev Microbiol 2001; 55:647-671.
49. Nanda A, Carson-Walter EB, Seaman S, Barber TD, Stampfl J, Singh S *et al.* TEM8 interacts with the cleaved C5 domain of collagen alpha 3(VI). Cancer Res. 2004; 64:817-820.
50. Omasits U, Ahrens CH, Müller S, Wollscheid B. Protter interactive protein feature visualization and integration with experimental proteomic data. Bioinformatics 2014; 30:884-6. doi 10.1093/bioinformatics/btt607.
51. Reeves CV, Dufraine J, Young JA, Kitajewski J. Anthrax toxin receptor 2 is expressed in murine and tumor vasculature and functions in endothelial proliferation and morphogenesis. Oncogene 2009; 29:789-801. doi 10.1038/onc.2009.383.
52. Rmali KA, Puntis MC, Jiang WG. TEM-8 and tubule formation in endothelial cells, its potential role of its vW/TM domains. Biochem Biophys Res Commun. 2005; 334:231-238.
53. Robertson DL, Tippetts MT, Leppla SH. Nucleotide sequence of the *Bacillus anthracis* edema factor gene (cya) a calmodulin-dependent adenylate cyclase. Gene 1988; 73:363-371.
54. Rogers MS, Christensen KA, Birsner AE, Short SM, Wigelsworth DJ, Collier RJ *et al.* Mutant anthrax toxin B moiety (protective antigen) inhibits angiogenesis and tumor growth. Cancer Res 2007; 67:9980-9985.
55. Ruan Z, Yang Z, Wang Y, Wang H, Chen Y, Shang X *et al.* DNA vaccine against tumor endothelial marker 8

inhibits tumor angiogenesis and growth, J Immunother. 2009; 32:486-491.
56. Scobie HM, Rainey GJ, Bradley KA, Young JA. Human capillary morphogenesis protein 2 functions as an anthrax toxin receptor. Proc Natl Acad Sci USA 2003; 100:5170-5174.
57. Scobie HM, Thomas D, Marlett JM, Destito G, Wigelsworth DJ, Collier RJ *et al.* A soluble receptor decoy protects rats against anthrax lethal toxin challenge, J Infect Dis. 2005; 192:1047-1051.
58. Singh Y, Klimpel KR, Goel S, Swain PK, Leppla SH. Oligomerization of anthrax toxin protective antigen and binding of lethal factor during endocytic uptake into mammalian cells. Infect Immun 1999; 67:1853-1859.
59. St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E *et al.* Genes expressed in human tumor endothelium. Science 2000; 289:1197-1202.
60. Tang WJ, Guo Q. The adenylyl cyclase activity of anthrax edema factor. Mol Aspects Med 2009; 30:423-430. Doi 10.1016/j.mam.2009.06.001
61. Turk BE. Manipulation of host signalling pathways by anthrax toxins, Biochem J. 2007; 402:405-417. doi 10.1042/BJ20061891
62. Wei W, Lu Q, Chaudry GJ, Leppla SH, Cohen SN. The LDL receptor-related protein LRP6 mediates internalization and lethality of anthrax toxin. Cell 2006; 124:1141-1154.
63. Weiner MA, Hanna PC. Macrophage-mediated germination of *Bacillus anthracis* endospores requires the gerH operon. Infect Immun. 2003; 7:3954-3959.
64. Werner E, Kowalczyk AP, Faundez V. Anthrax toxin receptor 1/tumor endothelium marker 8 mediates cell spreading by coupling extracellular ligands to the actin cytoskeleton, J Biol Chem. 2006; 281:23227-23236.
65. Wigelsworth DJ, Krantz BA, Christensen KA, Lacy DB, Juris SJ, Collier RJ. Binding stoichiometry and kinetics of the interaction of a human anthrax toxin receptor, CMG2, with protective antigen, J Biol Chem. 2004; 279:23349-23356.
66. Zornetta I, Brandi L, Janowiak B, Dal Molin F, Tonello F, Collier RJ *et al.* Imaging the cell entry of the anthrax oedema and lethal toxins with fluorescent protein chimeras. Cell Microbiol 2010; 12:1435-1445. doi 10.1111/j.1462-5822.2010.01480.x.