INVOLVEMENT OF THE RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS (RAGE) IN PROGRESSION OF PANCREATIC CANCER

Nancy Azizian MS

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INVOLVEMENT OF THE RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS (RAGE) IN PROGRESSION OF PANCREATIC CANCER

by

Nancy Azizian, MS

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IN VolVEMENT OF THE ReCEPTOR FOR ADVANCED GLYCATION END PRODUCTS (RAGE) IN PROGRESSION OF PANCREATIC CANCER

A

Dissertation

Presented to the Faculty of

The University of Texas

MD Anderson Cancer Center UTHealth

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Nancy Azizian, MS

Houston, Texas

August, 2017
IN Volvement of the Receptor for Advanced Glycation End Products (RAGE) in Progression of Pancreatic Cancer

Nancy Azizian, MS
Advisory Professor: Craig D. Logsdon, Ph.D.

Abstract. Oncogenic KRAS is central to several cancer types including pancreatic ductal adenocarcinoma (PDAC), but has been determined to be “undruggable”. Recent studies have indicated that oncogenic KRAS is not constitutively active but relies on a feed-forward stimulatory mechanism involving inflammation. In the current study we investigated the mechanisms by which, the receptor for advanced glycation end products (RAGE) affects and maintains KRAS activity. We observed that RAGE levels were elevated and there was a shift in the levels of specific isoforms upon inflammation in pancreatic cells and PDAC. Furthermore, RAGE agonists were found to increase Ras activity and downstream signaling in a time- and dose-dependent manner. Likewise, inhibition of RAGE decreased Ras signaling activity and downstream signals in multiple PDAC cell lines. In vivo, inhibition of RAGE activity using an antagonist inhibited tumor progression and increased survival rate. These data indicate that RAGE plays a central role in maintaining the activity of oncogenic KRAS and supporting tumor growth. These data raises the possibility of new approaches to inhibit the carcinogenic actions of KRAS indirectly by blocking the mechanisms through which, RAGE maintains its activity.
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<td>RAGE</td>
<td>Receptor for Advanced Glycated End products</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High Mobility Group Box 1</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<tr>
<td>NF-kB</td>
<td>Nuclear Factor kappa-light-chain-enhancer of activated B cells</td>
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<td>SP1</td>
<td>Specificity Protein 1</td>
</tr>
<tr>
<td>AP1</td>
<td>Activator Protein 1</td>
</tr>
<tr>
<td>Egr1</td>
<td>Early Growth Response 1</td>
</tr>
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<td>TTF1</td>
<td>Thyroid Transcription Factor 1</td>
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<td>HIF1</td>
<td>Hypoxia-inducible Factor 1</td>
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<td>NMD</td>
<td>Nonsense-Mediated mRNA Degradation</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>esRAGE</td>
<td>endogenous soluble RAGE</td>
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<tr>
<td>sRAGE</td>
<td>soluble RAGE</td>
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<td>ADAM10</td>
<td>A Disintegrin and Metalloproteinase domain-containing protein 10</td>
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<td>MMP9</td>
<td>Metalloprotease 9</td>
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<tr>
<td>CML</td>
<td>Carboxy-methyl-lysine</td>
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<tr>
<td>AGE</td>
<td>Advanced Glycation End product</td>
</tr>
<tr>
<td>MG</td>
<td>Methylglyoxal</td>
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<tr>
<td>CEL</td>
<td>Carboxy-ethyl lysine</td>
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<tr>
<td>MOLD</td>
<td>Methylglyoxal-lysine dimer</td>
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<td>GOLD</td>
<td>Glyoxal-lysine dimer</td>
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<td>GST</td>
<td>Glutathione S-transferases</td>
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<td>High Mobility Group A</td>
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<td>High Mobility Group Nucleosome-binding</td>
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<td>Toll-like Receptors</td>
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<td>Mitogen-activated Protein kinase</td>
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<td>SAPK/JNK</td>
<td>Stress-activated Protein Kinases/c-Jun N-terminal Kinases</td>
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<td>IL</td>
<td>Interleukin</td>
</tr>
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<td>ICAM</td>
<td>Intercellular Adhesion Molecule</td>
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<td>VCAM</td>
<td>Vascular Cell Adhesion Molecule</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>TNFa</td>
<td>Tumor Necrosis Factor α</td>
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<tr>
<td>IFNγ</td>
<td>Interferon γ</td>
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<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
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<td>BBB</td>
<td>Blood Brain Barrier</td>
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<td>Aβ</td>
<td>Amyloid Beta</td>
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<td>ISF</td>
<td>Interstitial Fluid</td>
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<td>LRP1</td>
<td>Lipoprotein Receptor related Protein 1</td>
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<td>APP</td>
<td>Amyloid Protein Precursor</td>
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<td>Human Umbilical Vein Cells</td>
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<td>OA</td>
<td>Osteoarthritis</td>
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<td>IDH</td>
<td>Isocitrate Dehydrogenase</td>
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<td>GAPDH</td>
<td>Glyceraldehyde-3-Phosphate Dehydrogenase</td>
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<td>Erk</td>
<td>Extracellular signal Regulated Kinases</td>
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<td>Rapidly accelerated fibrosarcoma</td>
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<td>mDia1</td>
<td>mammalian Diaphanous related formin 1</td>
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<td>eukaryotic Initiation Factor 2α</td>
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<td>COX2</td>
<td>Cyclooxygenase 2</td>
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<td>Nicotinamide adenine dinucleotide phosphate (NADPH)-Oxidase</td>
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<td>Superoxide dismutase</td>
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<td>GSH</td>
<td>Glutathione</td>
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<td>C-C Motif Chemokine Ligand 2</td>
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<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<td>PMN</td>
<td>Polymorphonuclear leukocytes</td>
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<td>ECM</td>
<td>Extracellular Matrix</td>
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<td>PI3K</td>
<td>Phospho-Inositide 3-Kinase</td>
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<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
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<td>Definition</td>
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<tr>
<td>Treg cells</td>
<td>T regulatory cells</td>
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<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor β</td>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<td>AT1R</td>
<td>Angiotensin II Type 1 Receptor</td>
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<td>Smad2</td>
<td>Small body size mothers against decapentaplegic</td>
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<td>ROCK</td>
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<td>JAK2</td>
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<td>Macrophage-1 antigen</td>
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<td>M-CSF</td>
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<td>9, 10 Dimethyl 1, 2 Benzantracene</td>
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<td>TPA</td>
<td>12-O-Tetradecanoylphorbol 13 acetate</td>
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<td>MDSCs</td>
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<td>DAMP</td>
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<td>Immunohistochemistry</td>
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<tr>
<td>PDAC</td>
<td>Pancreatic Ductal Adenocarcinoma</td>
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<tr>
<td>PanIN</td>
<td>Pancreatic Intraepithelial Neoplasia</td>
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<td>HE staining</td>
<td>Hematoxylin Eosin staining</td>
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<td>DNA Damage Response</td>
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<td>IR</td>
<td>Ionizing Radiation</td>
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<td>Tumor Microenvironment</td>
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**Introduction:** RAGE (Receptor for Advanced Glycation End products) is a Pattern Recognition Receptor (PRR), and a member of the immunoglobulin (Ig) superfamily of type I cell surface receptors that has been identified as a major protein involved in the exacerbation of multiple chronic pathologies including diabetes, Alzheimer’s disease, sepsis, most inflammatory diseases, neurodegeneration, and cancer (232). Under normal conditions, RAGE is expressed at low levels in all tissues and cell types and at high levels in lung and skin. However, under conditions of disease, the protein expression is elevated in multiple compartments including, the endothelia, neurons, and smooth muscle cells.

The nature of the contribution of RAGE to cancer is unclear. RAGE plays key roles in processes involved in wound healing, inflammation and angiogenesis, which in turn provide a background role for RAGE in cancer progression. RAGE is involved in cancer initiation. Ablation of RAGE in genetic mouse models of pancreatic cancer has shown to delay malignancy (214). RAGE is involved in a multitude of signaling activities, which regulate cellular functions such as migration and invasion, cell motility, cell proliferation, and survival (234).

In a tissue dependent context, interactions between RAGE and its ligands define its underlying role in disease progression. Proteins of the S100 family, nucleic acids, HMGB1, and amyloid β molecules are among the multitude of RAGE interacting ligands (235). A variety of tumors including gastric, colorectal, prostate, lung, bladder, glioma, and pancreatic cancers show increased expression of RAGE. Direct involvement of RAGE in cancer progression through engagement with its ligands, labels the receptor as a potential target of cancer therapies.
Growing evidence suggests that RAGE plays a major role in the progression of pancreatic cancer (214). Studies have shown a positive correlation between the RAGE levels and the metastatic characteristics of several human pancreatic cancer cell lines (211). RAGE ligands such as S100P and S100A6 are shown to prominently express in human pancreatic cancer tumors, when compared to the control tissues (211). Logsdon and colleagues demonstrated that blocking S100P with the RAGE antagonistic peptide, or the S100P binding drug, cromolyn, reduced viability, growth, and the invasive characteristics of human cancer cell lines. Moreover, treatment of the mice with these drugs reduced tumor growth and metastasis in the orthotopic mouse model of pancreatic cancer (230).

In a KRAS mouse model of pancreatic ductal adenocarcinoma, RAGE ablation (KRAS\textsuperscript{LSL.G12D/+; RAGE\textsuperscript{-/-}; PdxCre}) caused a significant decrease in the number of pancreatic intraepithelial neoplasia (PanIN) lesions, reduced the tumor burden, and increased the median survival in the RAGE \textsuperscript{-/-} mice when compared with the wild type control mice (214). Mechanistically, RAGE was shown to promote cell survival by inhibiting apoptosis and promoting autophagy (206).

RAGE plays an important role in resistance to chemo- and radiotherapy through the release of HMGB1 from necrotic cells, which causes a sustained activation of RAGE and cell survival through NF-kB signaling (231). Most importantly, RAGE is expressed in many cells that form the tumor microenvironment (TME). T cells, endothelial cells, fibroblasts, and myeloid-derived suppressor cells are among tumor microenvironment components with high expression of RAGE (232). Based on its role in activating signaling pathways involved in cancer
promotion, as well as its expression in cancer and stromal cells, RAGE may prove as a promising target for pancreatic cancer.

In this study, we examine the hypothesis that RAGE plays a role in promotion of pancreatic cancer by activating the major signaling pathways downstream of KRAS activity. We aim to investigate: 1) whether RAGE is involved in KRAS activation and downstream signaling including Erk and NF-kB, 2) whether RAGE has a role in PDAC tumor progression and survival, utilizing an orthotopic mouse model, and 3) the mechanisms by which, RAGE inhibition may reduce tumor growth.

We utilize known RAGE ligands such as methylglyoxal (MG), HMGB1, and nucleic acids to examine if RAGE-ligand binding in pancreatic cancer cells will lead to sustained activation of pathways downstream of, or convergent with KRAS activation. Additionally, we will discuss RAGE structure, localization, signaling and its potential roles in cancer initiation and maintenance. We will examine whether RAGE is expressed in pancreatic acinar cells, and if increased expression of RAGE will stimulate activation of KRAS and its downstream signaling. Furthermore, we will test whether inhibition of RAGE reduces the levels of multiple cancer related signaling pathways and reduces pancreatic tumor growth and prolongs survival in mouse model of pancreatic cancer.
Chapter 1. Receptor for Advanced Glycation End Product

1.1 RAGE Structure

RAGE comprises three domains: an extracellular domain, responsible for ligand binding, a single transmembrane domain, and a cytoplasmic domain involved in signal transduction. The RAGE ectodomain contains three Ig molecules; the N-terminus V-type Ig domain, followed by C1 and C2 C-type domains (Figure 1). The V and the adjacent C1 domains compose the positively charged functional unit involved in ligand binding. With the exception of a few ligands, which bind with some affinity to the C2 domain, most ligands bind to the V domain. The negatively charged cytosolic domain responsible for downstream signaling is mainly unstructured, with no homology with any known signaling domain, has no enzymatic activity, and is subject to shedding by protease cleavage (1).
Figure 1. Receptor for advanced glycation end-products (RAGE) structure. RAGE contains three domains; the C-terminal intracellular (IC) domain, the transmembrane (TM) domain, and the extracellular (EC) N-terminal domain. Additionally the first 22 N-terminal amino acids are designated as the signaling peptide (SP).

The human RAGE gene maps to chromosome 6p21.3, located in a region between the Classes II and III genes of the Major Histocompatibility Complex (MHC). The gene is 1.7 kb long, is composed of 11 exons, and codes for a protein of 404 amino acids and has an apparent molecular mass of ~55 Kd. The gene has been described in mammals but not in other species. NF-kB, SP1, AP1 and 2, HIF1, Egr1 (Early growth response 1), and TTF1 (thyroid transcription factor 1) all bind to the 5’-UTR region of RAGE promoter to activate RAGE transcription (6). As discussed in detail below, binding of NF-kB to RAGE promoter involves a positive feedback mechanism, by which, the de novo synthesis of NF-kB transcript will maintain an active pool of the protein as well as the downstream pathway involved (151). Similarly, under conditions of systemic hypoxia HIF1 binding to RAGE promoter causes the stimulation of neuronal RAGE. This increased RAGE activity is part of the neuro protective-neuro regenerative response following ischemic insults, as RAGE deficient mice exhibit increased brain damage when subjected to hypoxia/ischemia (233).

The gene is also regulated epigenetically through cytosine methylation of its promoter at AP2 and SP1 binding sites (9). The 3’-UTR of RAGE transcript contains a sequence targeted by miR-30, binding to which, will culminate in transcriptional downregulation (10).

1.2 RAGE Isoforms
Full-length RAGE contains an N-terminal V-type domain, involved in ligand binding, and two C-type domains, a helical transmembrane domain, and a C-terminal cytoplasmic domain, required for signal transduction. A RAGE variant with a deletion of the V-type domain is unable to interact with the ligands, and therefore cannot take part in downstream activation. On the other hand, RAGE variants without the C-terminal domain function as molecular decoys, competing with the full-length protein for ligand binding, but incapable of conducting signal transduction (7). RAGE molecules homodimerize through formation of a disulfide bond between cysteine residues 259 and 301 of C2 domain.

In addition to the full length RAGE (fl RAGE), 19 naturally occurring splice variants termed RAGE_v1 to RAGE_v19 have been detected at the transcript or protein level (8). A mechanism involved in regulating splicing of RAGE is the binding of HNRNPH (heterogeneous nuclear ribonucleoprotein H) to the G-rich cis elements (11). Such regulation of RAGE alternative splicing has important clinical values due to a balancing act between the expression of full RAGE (assumed to have cytotoxic roles), and endogenous soluble RAGE (considered to prohibit the signaling that culminate in RAGE-related disorders).

The majority of these splice variants are unstable at the transcript or protein level. Approximately 50% of the splice variants in human are reportedly targeted for NMD (nonsense-mediated mRNA degradation) (1). Vollmar and colleagues have reported the detection of 6 distinct RAGE isoforms in mouse pancreata, compared with 3 isoforms in lung. Differential expression of these isoforms, occurs upon induction of pancreatitis (via cerulein), or hyperglycemia (Streptozotocin-STZ) (122).
The soluble RAGE isoforms, sRAGE, comprise two variants; esRAGE (endogenous or RAGE_v1), which is produced through alternative splicing at exon 9, and the cleaved RAGE (cRAGE), which is produced by proteolytic cleavage of the extracellular (ectodomain) domain of the full length protein by ectodomain shedding (236). Ectodomain shedding is mediated by metalloproteinases such as MM9 or ADAM10. RAGE shedding releases the soluble ectodomain (sRAGE), which subsequently acts as an inhibitor of RAGE signaling. ADAM10 is the responsible metalloproteinase involved in RAGE shedding. The clinical value of RAGE shedding is underscored by reports that show that an increase in levels of the shed protein (sRAGE) is correlated with better prognosis in diseases such as cancer, diabetes and inflammatory states (15). HMGB1 binding to RAGE, resulting in cellular activation induces receptor shedding, which in turn results in production of sRAGE, a potent RAGE inhibitor. Proteolytic cleavage is also promoted by other factors such as increased concentration of Ca$^{2+}$ (2).

Soluble isoforms contain the V and C domains but lack the transmembrane and cytosolic regions. The soluble isoforms do not participate in signaling pathway, but mainly act as molecular decoys that scavenge the ligands in the extracellular space, decreasing the concentration of the available ligand pool (15). In general, RAGE alternative splicing in humans is cell specific; in lung and aortic smooth muscle cells the full length variant is the predominant isoform, while in the endothelial cells esRAGE is the prevalently detected transcript (13, 14). The ratio of full length to soluble RAGE protein is tissue specific and varies between 0.59 to 1.79; indeed the change in full length/soluble RAGE can be used as a diagnostic tool in many diseases (15).
The V domain of RAGE (amino acids 23-119) is located farthest from the plasma membrane, while the C2 domain (amino acids 234-325) is located the most proximal. The V and C1 domains (amino acids 120-233) are joined together to form a single functional unit, VC1. The VC1 and C2 domains are linked through an amorphous stretch of several amino acids, which functions as a flexible linker between the two entities. The V domain contains a very high number of arginine and lysine residues, which endow the domain with a net positive charge. An analysis of the existing RAGE structures shows that arginine and lysine form a large positively charged patch, located on one side of the VC1 domain. In contrast, the C2 domain is composed of mainly negatively charged amino acids. RAGE ligands are characterized by a predominantly negative net charge distributed over the entire molecule or on a single domain, and reported to bind to the V or VC1 tandem domains (16-21). As RAGE recognizes different classes of ligands, the differential binding modes derived from ligand-receptor interactions may serve the basis for ligand specificity in different compartments. Indeed, strong electrostatic interactions, and subsequent formation of a tight receptor-ligand complex lead to sustained activation of the downstream signaling pathways, which is addressed in this study.

While most interactions with ligands happen at the V domain, the C1 domain plays a role at either recognizing or stabilizing the V domain binding. In this light, the negatively charged ligands display a preference for the oppositely charged VC1 domain, while being repelled from the C2 domain due to its highly negative charge. The electrostatic interaction between the receptor and its ligands is key to formation of a stable complex.
As a member of the pattern recognition receptors, RAGE recognizes a multitude of ligands, which in its turn complicates the nature of signaling involved. RAGE is able to bind a variety of ligand classes owing to the positively charged nature of the V-domain that is responsible for ligand-binding acts as an electrostatic trap for negatively charged ligands (13,14). The receptor-ligand binding elicits immediate downstream signaling. This response is dependent on, and influenced by several factors including the identity and concentration of the ligands, surface concentration of the receptor itself, and the presence of other ligands and co-receptors. The intensity of the elicited signal is also dependent on the ligand affinity and the duration of the signaling, as well as the half-life of the interacting components.

RAGE is also post-translationally modified by glycosylation, phosphorylation, proteolysis, and disulfide bond formation. Disulfide bonds form between the cysteine residues in the Ig domain. RAGE V1 domain contains two glycosylation sites (4, 5). Proteolytic cleavage of RAGE can produce the soluble ectodomain, C-terminal, and the transmembrane domains. While the soluble ectodomain is localized to the intracellular vesicles or the extracellular space, the C-terminal domain is localized to cytoplasm or nucleus (2, 3).

RAGE can assemble into multimeric structures in the plasma membrane in the absence of ligands. Crystal structure of the receptor reveals that the VC1 domains are arranged in a parallel position, with a side-by-side contact. Both soluble and transmembrane isoforms can oligomerize through interaction of their multiple V domains. Although oligomerization may happen in the absence of ligands, ligand binding shifts the equilibrium towards a more stable, higher order oligomeric structure, with an increased affinity for the extracellular ligands (18, 22). Increased RAGE levels at the cell surface upon activation may promote further assembly
due to a positive feedback loop, by stabilizing the ligand-receptor binding assemblies, and sustaining the RAGE expression. Subsequently, the increased protein levels at the cell surface will promote preassembly, and thereby sustained activation of downstream pathways.

1.3 RAGE Ligands

The ability of RAGE to recognize and bind to multiple ligands in different cell types creates a complex and often pleiotropic signaling response. Almost all extracellular ligands bind to the V domain, and this binding induces downstream signaling cascades. AGEs, HMGB1, S100 proteins, Collagens I and IV, and Amyloid β (Aβ) peptides, phosphatidylserine, C3a, and immunoglobulin light chains are among the ligands interacting with RAGE, and involved in downstream signaling (12).

1.3a. AGE

RAGE was identified in a search for receptors of AGEs (advanced glycation end products). AGEs are a class of unstable, reactive compounds, which accumulate during diabetes and aging. AGE is produced from a non-enzymatic reaction between reducing sugars and aldehydes and proteins, lipids, and nucleic acids. Differential mechanisms of AGE production deem it as a rather heterogeneous group of molecules.

Four pathways may contribute to AGE formation: Maillard reaction, glucose oxidation and lipid peroxidation, and polyol pathway. Formation of AGEs through Maillard reaction occurs in three steps: First, glucose carbonyl group is attached non-enzymatically to the free amino acid (lysine or arginine) of the protein, DNA, or lipid compound to form a Schiff base. This step is rapid, reversible, and depends on glucose concentration. In the second step, the Schiff base undergoes a slow chemical rearrangement to form ketoamine (Amadori) products. This step
takes place more slowly but is still reversible. In the third irreversible step, Amadori products will undergo further rearrangement to produce protein adducts and crosslinks (23, 24).

AGEs can also form through glucose autooxidation, or lipid peroxidation to produce dicarbonyl derivatives. Dicarbonyl derivatives such as glyoxal or methylglyoxal (MG) can interact with monoacids to produce AGEs (25). In polyol pathway, glucose is first converted to sorbitol by aldose reductase, followed by conversion to fructose by sorbitol dehydrogenase. Fructose metabolites are then converted to α-oxaldehyde derivatives, and interact with monoacids to produce AGEs (26).

Carboxy-methyl-lysine (CML) is the most abundantly studied AGE molecule, and is detected in tissues with highly damaged proteins. It is produced by oxidative degradation of Amadori products or direct conjugation of glyoxal moiety to lysine residue (27, 28). Dicarbonyl compounds such as glyoxal and MG form through oxidative degradation or autooxidation of Amadori products. Due to their highly reactive nature, these AGEs can produce protein crosslinks. Among other AGE molecules, carboxy-ethyl lysine (CEL), methylglyoxal derived hydroimidazolones, and pyrraline form protein adducts, while glyoxal and methylglyoxal lysine dimers, GOLD and MOLD form protein crosslinks (23, 29).

AGEs can accumulate in body through food ingestion or endogenous production. The endogenous production of AGE molecules is increased in diseases such as diabetes (29). Environmental factors such as diet and smoking also contribute to AGE accumulation (30). Several mechanisms exist to remove accumulated AGEs in body. Glutathione dependent glyoxalase GLO1 and GLO2 are two enzymes responsible for AGE detoxification. These enzymes catalyze glyoxal, MG, and other oxoaldehydes to the less toxic D-lactate through a
mechanism that involves reduced glutathione (GST). Another mechanism that detects and
d Breaks the Amadori products involves fructosamine kinases. These enzymes phosphorylate
and destabilize the Amadori products causing their breakdown (31).

AGE products exert their function through their involvement in protein turnover,
extracellular matrix metabolism, and protein crosslinks. However, these molecules also serve
as ligands for RAGE. Binding in the most part occurs through interaction with the RAGE V1
domain.

1.3b. HMGB1

HMGB1 is a known RAGE ligand, which plays an important role in angiogenesis and
increased inflammation (38-42). HMG (High Mobility Group) proteins are a group of highly
basic, non-histone proteins involved in chromatin architecture. HMG proteins are subdivided
into three groups: HMGA, HMGB, and HMGN. HMGB1 (amphoterin) is a highly conserved 30
Kd protein with both intra- and extracellular functions. The protein binds avidly to DNA and
heparin. As a part of nucleosome architecture, HMGB1 stabilizes nucleosomal structure and
facilitates transcription (34-36). HMGB1 can be passively released by necrotic cells but not the
apoptotic cells (37). It can however be secreted actively by monocytes, macrophages, and
endothelial cells.

HMGB1 null cells have highly reduced capacity to induce inflammation. As a cytosolic
protein, HMGB1 plays a role in autophagy. Several modifications including acetylation,
methylation, phosphorylation, and ADP ribosylation are required for HMGB1 translocation
from nucleus to cytoplasm, and eventual release in the extracellular space (43). Once in
extracellular space, HMGB1 mediates immune and inflammatory responses through binding to RAGE and several receptors of TLR family of proteins (44).

In vitro experiments show that HMGB1 interaction with RAGE occurs through residues 150-183 of HMGB1 (45). In RAGE, binding occurs in VC1 domain (46). HMGB1 binding to RAGE occurs at nanomolar concentrations, and this binding is partly responsible for the localization of HMGB1 to the cell surface.

HMGB1-RAGE interaction is shown to be responsible for cell motility, migration and invasion of tumor cells in diverse group of cancers, including lung cancer (237) and hepatocellular carcinoma (238). Migratory properties of other cells such as immune cells (granulocytes, monocytes, and dendritic cells), endothelial and smooth muscle cells, and stem cells are also attributed to this interaction (47-53).

In vivo, HMGB1 is involved in MAP kinase and JNK signaling pathways. HMGB1 signaling through the toll like receptors TLR2, and TLR4 results in upregulation of NF-kB pathway. As a result, production of several cytokines (IL1B, IL6, IL8, MCP1), and adhesion molecules such as ICAM and VCAM is increased (54). In endothelial cells and macrophages, positive feedback mechanisms involved in increased TNFa production, culminate in HMGB1 enhanced secretion (42).

HMGB1 binding to RAGE is augmented through CpG DNA. However, its ability to activate RAGE may be stemming from its binding to a variety of cytokines and LPS, which results in increased expression of interferon gamma (IFNγ) in peripheral blood mononuclear cells (PBMC) (53).
1.3c. Amyloid Beta (Aβ)

RAGE is the major receptor to transport Aβ peptides across the blood brain barrier (BBB) from blood to brain (56). Several factors are involved in tight regulation of Aβ concentration within the interstitial fluid (ISF) of a normal brain; 1- production of the Aβ peptides from their precursor proteins (APP), 2- enzymatic degradation within the brain, 3- rapid clearance across the BBB by lipoprotein receptor related protein 1 (LRP1), and 4- influx into the brain across the BBB via RAGE (57, 58, 59). Continuous removal of Aβ peptides from brain through Aβ peptide metabolism, or transport across the blood brain barrier is essential to prevent neurotoxicity. Endothelial cells of mice or patients with Alzheimer’s disease (AD) show an increased RAGE expression, along with reduced LRP1 levels.

Aβ peptides (~4.5 Kd) are the proteolytic products of amyloid protein precursor (APP) (60), and exists in different forms; monomers, soluble oligomers, or insoluble aggregate (12). These peptides (the most common of which are Aβ 40 and Aβ 42) are produced by a variety of cells. The circulating chaperone-bound fraction in plasma, is in equilibrium with the free unbound fraction (61). Circulating Aβ enters brain on the luminal surface of brain vessels in a RAGE dependent manner (62, 63, 64). RAGE-Aβ interactions are dependent on the oligomerization state of the amyloid peptides (16). Amyloid β oligomers bind to RAGE through their interaction with V domain, while the Aβ aggregates do so through binding to the C1 domain (16).

Soluble Aβ binds RAGE at nanomolar concentrations. The resulting pathology is due to suppression of cerebral blood flow, as well as inflammatory and NF-κB-mediated apoptotic responses in the endothelia (62, 65).
1.3d. S100/calgranulin Proteins

S100 proteins include a group of 24 members belonging to the EF-hand Ca\(^{2+}\) binding family of proteins expressed exclusively in vertebrates (66). S100 proteins are involved in regulation of calcium homeostasis, cell growth and differentiation, as well as cytoskeleton and energy metabolism (67-69). The majority of S100 proteins function as dimers (66). The coding gene cluster is located on human chromosome 1q21 and is designated as S100a family of genes.

In S100 proteins, the two Ca\(^{2+}\) binding loops of the EF-hand motif are flanked by α-helices. The N-terminal loop is non-canonical and binds Ca\(^{2+}\) less avidly that the C-terminal loop (70).

Ca\(^{2+}\) binding causes a conformational change within the hydrophobic binding domain, allowing the protein dimer to bind two target proteins. (71). S100 proteins are expressed in various cell types including macrophages, lymphocytes, and dendritic cells (72). Several S100 proteins bind negative regulatory domain of p53, while some bind to the tetramerization domain of p53, and therefore have a role in oligomerization (73).

Individual S100 proteins are overexpressed in a variety of inflammatory pathologies such as rheumatoid arthritis, systemic autoimmune disease and chronic inflammatory bowel disease (74, 75).

Almost all secreted S100 proteins act through RAGE (76-77). The S100 protein ligands interact differentially with RAGE; whereas S100B exclusively bind to V domain, S100A6 binds to the V and C2 domains, and S100A12 interacts with V and C1 domains. RAGE glycosylation has been shown to modulate the S100-RAGE interaction (60).
Not all S100 proteins act as RAGE ligands. In general, non-RAGE ligands display more affinity for Zn\(^{2+}\), have altered or different Ca\(^{2+}\) binding clefts, and have minimal or no oligomerization capacity. S100A2, A3, A5, A10, A14, A16, G, and Z are among the non-RAGE ligand proteins.

**S100A8/A9** express in keratinocytes, epithelial cells, and cells of myeloid origins (78-80). These proteins can heterodimerize (in the absence of calcium), or heterotetramerize in the presence of calcium. Their functions involve myeloid differentiation, inflammatory response, and anti-microbial activities (78, 81). Their role in cancer promotion has been shown for gastric, prostate, and colorectal cancers (82-84). In tumor cells, the heterooligomers have been shown to promote cell growth via Erk and p38 MAP kinase pathways in a RAGE dependent manner (85). However in human umbilical aortic cells (HUVEC), RAGE dependent signaling has been observed only after prior treatment of the cells with AGE molecules (86). Additionally, CML modified S100A8/A9 molecules in intestinal tissues are shown to activate the NF-κB inflammatory response in a RAGE dependent manner (87), suggesting an interplay among RAGE, AGES, and S100 proteins.

**S100A4** is expressed in neurons and chondrocytes (77). Its role in tumor progression and development is confirmed in mouse knockout models (88-89). The protein is implicated in MMP13 (metalloproteinase 13) upregulation in a RAGE and calcium dependent manner (90).

**S100A6** is found in multitude of organs including spleen, brain, lung, muscles, and kidney (91). Upon calcium binding S1006 is shown to translocate from cytoplasm to nucleus. The protein is overexpressed in several malignancies including colorectal, lung, gastric, and pancreatic cancers (92-94). At the molecular level, S100A6 was shown to interact with several
proteins including tropomyosin, annexins II and XI, p53 (95), and RAGE (20). Binding to RAGE occurs through C2 domain, and is strictly calcium dependent.

**S100A7** binds calcium and zinc with low affinity. Calcium binding does not cause large conformational changes in the protein (96). The protein plays a role in several malignancies including ductal and invasive breast cancer and lung squamous cell carcinoma (97-99), and stimulates lymphocytes, monocytes, and granulocytes in a RAGE dependent manner (100).

**S100A11** is expressed in a variety of cells, but in higher amounts in lung and smooth muscle tissues (101). The molecule binds to annexins A1 and A2, p53, the repair protein Rad54B, and RAGE. S100A11 is suggested to play a dual role in cancer promotion; in breast, prostate, and pancreatic cancer, it has a role in tumor progression (93, 102), while in bladder and renal carcinomas it is known to act as a tumor suppressor (103). S100A11 role in modulation of osteoarthritis (OA) occurs through interaction with RAGE (104). In chondrocytes S100A11-RAGE interaction results in hypertrophy (104, 105).

**S100A12** is expressed in neutrophils, monocytes, and lymphocytes (106). The protein is translocated from cytosol to membrane in the presence of increased calcium concentration. At the molecular level S100A12 interacts with NADP⁺-dependent isocitrate dehydrogenase (IDH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), annexin V, and RAGE (107). The protein is suggested to bind RAGE within the V and C1 domains (60, 108).

**S100A13** has been detected at the transcript level in many tissues including spleen, heart, brain, kidney, and ovary. In addition to calcium the molecule binds to copper with micromolar affinity (109). Experiments in endothelial cells, suggest that translocation of S100A13 from nucleus to cytoplasm may occur through its interactions with RAGE (110).
**S100P** was first identified in placenta, but is now known to express in normal cells and organs including prostate, leukocytes, esophagus, and stomach (111, 112). S100P is present in numerous tumors including ovarian, gastric, colorectal, breast, pancreatic, and prostate cancers (112, 113). Its direct interaction with RAGE was shown in BxPC3 (pancreatic cancer cells), and SW480 (colon cancer cells) through immunoprecipitation experiments (113, 114, 115). In these cells S100P was shown to trigger MAP kinase and NF-kB activities in a RAGE dependent manner.

**S100B** is expressed in the astrocytes of the human brain cortex, melanocytes, and myeloid dendritic cells. Along with S100A1 and S100A6, S100B is most abundantly detected in brain cells. S100B protein binds to both regulatory and tetramerization domains of p53 (73). The protein binding to RAGE occurs in the V and C1 domains, and its oligomerization increases the binding affinity (20, 18). RAGE-S100B interactions activates the intracellular formation of ROS species, and upregulates the PI3K/AKT and NF-kB pathways resulting in cell proliferation (114, 116,117). S100B activation of RAGE upregulates TNFa and IL1β production in microglia, and stimulates AP1 transcription through JNK pathway (12).

**1.3e. Nucleic Acids**

RAGE is reported to bind nucleic acids in a sequence nonspecific manner (118, 119) within their phosphate backbone. This interaction occurs in the extracellular matrix, with an affinity range of nanomolar to micromolar, and promotes nucleic acid uptake in the cell. The RAGE-DNA complexes have been detected in early and late endosomal compartments, where nucleic acid specific TLRs, TLR8, and TLR9 are located. Through a direct interaction with, and activation of these TLRs, RAGE plays a role in the promotion of inflammatory response.
1.4 RAGE mediated Cell Signaling

RAGE-ligand-binding results in activation of distinct signaling pathways, including Ras, Rac-1, members of the mitogen-activated protein (MAP) kinase family (ERK, p38 and SAPK/JNK) and nuclear factor NF-κB (2,15,19–23). Many of these are critical pathways in the regulation of cellular survival, growth, migration and invasion.

1.4a. Ras

Engagement of RAGE with its ligands has been shown to trigger Ras activation pathway in different cell lines. In rat pulmonary artery smooth muscle, AGE-RAGE interactions were reported to induce cellular oxidative stress through generation of reactive oxygen intermediates (239). Schmidt and colleagues demonstrated that the resulting oxidative stress triggers Ras/MAP kinase pathway, causing NF-κB nuclear translocation (128). In pulmonary epithelial cells, cigarette smoke extract can induce RAGE expression. Hoidal and colleagues have demonstrated that this ligand-induced expression of RAGE mediates Ras activation, nuclear translocation of NF-κB, and cytokine secretion (240).

In a more recent study, immunoprecipitation experiments in pancreatic cancer cells demonstrated a direct interaction between RAGE and KRAS, the Ras gene mutated in most lung and pancreatic cancers. The interaction was significantly increased in the KRAS $^{G12D}$ mutants, and conditions such as hypoxia, also augmented the interaction, suggesting a direct role for RAGE in regulating KRAS signaling. RAGE mediated activation of the KRAS-Raf-Mek-Erk and KRAS-PI3K-AKT pathways is responsible for HIF1α signaling activity in pancreatic tumors (206). Due to its pleiotropic nature, Ras has proven a difficult therapeutic target. Therefore,
targeting RAGE as the major molecular effector may provide a more efficient tool in preventing Ras-related molecular signaling.

1.4b. mDia1

A yeast two-hybrid screen has identified mammalian Diaphanous related formin 1 (mDia1) as the interacting partner of RAGE C-terminal domain (ctRAGE) (123). The 140 kDa adaptor protein is encoded by the gene Drf1/Diaph1 located on chromosome 5q31 and belongs to the formin family of actin and tubulin polymerization proteins. The protein is characterized by two proline rich formin homology (FH) domains FH1 and FH2. Arginine 5 and glutamine 6 residues of ctRAGE are essential for interaction with mDia1 (124). Increased RAGE expression is shown to upregulate mDia1 (125). Many ctRAGE dependent signaling pathways such as Rac1 (Ras-related C3 botulinum toxin substrate 1)/ Cdc42 (cell division control protein 42), Egr1 (early growth response protein 1), AKT, and GSK3β (glycogen synthase kinase 3B) are regulated through mDia1 (123-126).

In aortic SMC (smooth muscle cells) S100B and S100A12 reportedly bind to RAGE, inducing translocation of Src to membrane via mDia1, and its subsequent phosphorylation. This Src activation is responsible for many downstream signaling events involving p38, Erk, STAT3 (signal transducers and activators of transcription 3), and NF-κB activation, as well as phosphorylation of Caveolin 1 (127). Importantly, blocking Src activity reduces the effects of S100B on cell migration and inflammation, suggesting a direct involvement of Src in RAGE dependent signaling.
1.4c. NF-kB Pathway

NF-kB (nuclear factor k light chain enhancer of activated B cells) is the key link between inflammation and immune response. RAGE mediated activation of NF-kB depends on, and happens through the classical MAP kinase pathway (128). In mammalian cells, NF-kB consists of 5 proteins: p65 (RelA), RelB, c-Rel, p50 (NF-kB1), and p52 (NF-kB2), which associate to form homo- and hetero- dimers. All family proteins contain a Rel homology domain (RHD), which is essential for dimerization (129). In its inactive form NF-kB is sequestered by IκB (inhibitor of kB) in the cytoplasm. IκB phosphorylation by IKK (IκB kinase) complex releases NF-kB, which is then translocated to nucleus (130). NF-kB is the first known signal transduction molecule activated by AGEs. Upon RAGE stimulation, NF-kB is translocated to the nucleus and binds to RAGE promoter region to enhance its activity (131). NF-kB also binds to the Glo1 gene promoter, whose enzymatic activity is involved in breaking AGE molecules, and inhibits its expression.

1.4d. Mitogen Activated Protein Kinases

Upon stimulation, RAGE can activate MAP (mitogen activated protein) kinase signaling likely by its direct activation of Ras activity. These pathways converge on IKK to activate NF-kB. In rat pulmonary artery smooth muscle cells suppression of RAGE activity via employing RAGE antibody and sRAGE, or inhibition of Ras by its inhibitor farnesyl thiosalicylic acid, prevents activation of NF-kB and MAP kinase protein ERK (extracellular signal regulated kinase) (128). Additionally, CtRAGE may promote MAP kinase activity by binding to ERK through its putative ERK binding domain located within amino acids 362-375 (132). In human monocytic leukemia cells, another MAP kinase protein, p38, is required for induction of NF-kB by CML AGEs (133).
In chondrocytes, AGE-RAGE interaction is reported to cause endoplasmic reticulum (ER) stress, as evidenced by phosphorylation of eukaryotic initiation factor 2α (eIF2α) and induction of biomarkers of ER stress. This in turn results in activation of p38 MAP kinase and NF-κB pathways, followed by increased expression of COX2 (cyclooxygenase 2) (134). In this light, the involvement of RAGE in cartilage degradation associates with osteoarthritis. In mouse aortic endothelial cells, induction of VCAM1 (vascular cell adhesion molecule 1) by RAGE ligands occurs through JNK (c Jun N-terminal kinase). In mouse microglia BV2 cells, JNK activation via S100B, leads to transcriptional activation of activator protein 1 (AP1), which together with NF-κB upregulates IL1β, TNFα, and COX2 (135,116). Thus in response to RAGE activation, three classical MAP kinase related pathways can be stimulated.

1.4e. Oxidative Stress Pathways

RAGE activation can induce oxidative stress either directly by activating NOX (nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase) pathway, downregulating the activity of superoxide dismutase (SOD), and catalase, or indirectly by reducing antioxidant mechanisms such as those involving GSH and ascorbate (31, 32). GSH reduction can further downregulate GLO1 function in detoxifying glyoxal derived AGEs (33).

1.5 RAGE expression in different cell types

RAGE is expressed during embryonic development, with the highest expression in brain tissues. However, this expression is eventually decreased and under physiological conditions RAGE expression is low in most tissues with the exception of lung tissues but is increased upon inflammatory response and in disease state. Immune cells such as neutrophils,
monocytes, macrophages, and leukocytes are responsible for secretion of most RAGE ligands, thus playing a crucial role in the onset of inflammatory response (120). While increased concentration of ligands may play a role in downregulating many receptors, RAGE molecules are upregulated in a ligand rich environment (121). RAGE are responsible for cell migration, autophagy, proliferation and apoptosis in a context dependent manner. RAGE bind to different ligand types and are activated in different tissues. RAGE ability to recognize multitude of ligands leads to the complications involved in signaling pathway (245).

1.5a. Endothelial Cells

Endothelial cells of the vasculature lining are known to activate RAGE on inflammation and injury. For example, cardiovascular injury leads to the release of ligands such as S100B (175) and HMGB1 into the circulation, which in turn bind to the receptors expressed on the endothelia, activating MAP kinase and NF-\(k\)B pathways (176-8). This primes the endothelial cells to release cytokines such as TNFa, MCP1, and IL6, and stimulate the endothelium for leukocyte adhesion. Among the target genes activated by NF-\(k\)B pathway are those involved in leukocyte adhesion such as ICAM1 and VCAM1 (179-81). Interaction of RAGE with AGE ligands in endothelial cells activates the Rho signaling pathway, leading to cytoskeletal reorganization, which results in increased vascular permeability required for transmigration of leukocytes (182). Direct interaction of RAGE with the leukocyte adhesion molecule Mac1 in acute inflammation controls leukocyte recruitment.

1.5b. SMC

Binding of the RAGE molecules expressed in smooth muscle cells to AGE and S100 proteins mediates the arterial damages caused in pathologies such as diabetes, atherosclerosis, and
vascular injury (183). The underlying mechanism in these cases is activation of MAP kinase pathway, leading to expression of MMP2, the metalloprotease involved in vascular SMC migration (184). In aortic SMC, the interaction of ligands such as S100B with RAGE, induces translocation of c-Src to plasma membrane through mDia1, followed by phosphorylation of c-Src (127), Rac1 activation, Akt phosphorylation and ultimately cell migration (185).

1.5c. CNS

RAGE is expressed in all CNS cell types including neurons, microglia, and astrocytes (186, 117). During development neurons express high levels of RAGE, but this expression decreases after birth and stays at low levels in brain neurons (4). S100B is a known neurotrophic factor, the expression of which, in cultured mouse neuroblastoma cells is accompanied by cell survival (117). The pro-survival effects of S100B are due to NF-κB activation, and the subsequent production of anti-apoptotic molecules such as Bcl2 (117). However, under conditions of inflammation or infection, increased RAGE activity causes activation of ERK pathway, and accumulation of ROS products, causing cell death. Indeed, in patients with Alzheimer disease, a pronounced increase in RAGE levels is detected in neurons, astrocytes, microglia, and endothelia of brain tissues (discussed below) (187). Transportation of Aβ molecules through the brain blood barrier is mediated by RAGE, where it binds to RAGE in neurons and microglia. The resulting engagement of NF-κB pathway through MAP kinase signaling causes neurotoxicity and loss of brain function (188).

1.5d. Monocytes and Macrophages

Monocytes in the blood are derived from the myeloid progenitors in the bone marrow. During inflammation, or in response to injury monocytes can infiltrate the affected tissues and
differentiate to macrophages. Monocytes express both soluble sRAGE and membrane-bound RAGE, which is involved in activation and differentiation of monocytes (4). AGE-RAGE interaction triggers the release of proinflammatory cytokines such as CCL2, IL1B, and TNFa. Macrophages are present in all tissues, and are activated by proinflammatory cytokines on pathogen recognition. Once activated, macrophages differentiate into one of the two subtypes: M1 and M2. M1 (classically activated macrophages) induce T helper type 1(Th1) response, and trigger proinflammatory responses characterized by cytokine release. Alternatively activated macrophages (M2), can be induced by IL4 and IL13, have an immunoregulatory role, and are involved in tissue remodeling activities such vascularization in the aftermath of infection. Tumor associate macrophages (TAMs), a population of macrophages associated with pro- and anti- tumoral response in tumor stroma display characteristic M2 phenotypes exemplified by angiogenesis, metastasis, promotion of tumor growth, and immunosuppression (189). Like M1 macrophages, M2 macrophages express RAGE on their surface. Cellular responses due to RAGE activation are dependent on the ligand and the state of differentiation of monocytes/macrophages. For example, many tumor derived activities such as vascularization and increased level of VEGF are characterized by HMGB1-mediated RAGE activation. (189). Indeed, IL10 production in M2 macrophages occurs through HMGB1-RAGE signaling (190).

1.5e. Granulocytes

Granulocytes are circulating polymorphonuclear leukocytes (PMN), derived from myeloid cells, involved in protection against pathogens and parasites. They are characterized by their granular morphology and high contents of cytotoxic and antimicrobial molecules (191).
Granulocytes are classified into neutrophils, basophils, and eosenophils on the basis of their histological staining, and function in several chronic inflammatory diseases of skin, CNS, lung, and intestine. Importantly, granulocytes are among the first leukocytes recruited from the bloodstream to the infected tissues.

Neutrophils are a subset of granulocytes with phagocytic properties. RAGE-AGE interaction on the surface of neutrophils results in an increased phagocytic response. AGE induced hyperactivation of neutrophils caused by hyperglycemia is a contributing factor in chronic inflammation of diabetes. RAGE interaction with Mac1/β2 integrin (CD11b/CD18) on neutrophil surface mediates the recruitment of neutrophils to intestinal epithelia (192). Neutrophils infiltrating the tissues undergoing inflammation interact with the components of extracellular matrix (ECM). RAGE dependent binding to AGE-collagen results in activation of PI3K, but not ERK or p38 pathways (193).

1.5f. Dendritic Cells (DCs)

In response to microbial agents, dendritic cells undergo a process referred to as maturation. Maturing DCs are responsible for productive activation of naïve T cells in lymph nodes through upregulating the expression of membrane components such as MHC class I and II molecules, as well as CD40, CD80, and CD86 (197). RAGE expression by maturing DCs is required for migration of these cells to draining lymph nodes (197). Expression of ligands such as HMGB1 in DCs committed to maturation in peripheral tissues causes RAGE activation. Migration of DCs to a secondary lymphoid organ, will subsequently initiate the clonal expansion of antigen specific T cells. Ablation of RAGE disrupts this function, abrogating the T cell dependent initiating immune responses.
DC migration depends on MAP kinase, p38 and NF-kB pathways, which are initiated by the original maturing stimuli. However, activation of RAGE by HMGB1 bypasses/sustains the need for these events (197).

1.5g. T Cells

T cells are key components of the adaptive immunity. Upon antigen recognition, T cells can expand and differentiate into regulator and effector subtypes, on the basis of expression of cell surface markers, such as CD8 and CD4. In general, T cells show lower expression of RAGE compared with other leukocytes. However, the involvement of RAGE in adaptive immune response was highlighted in a series of experiments where RAGE blockade in a model of multiple sclerosis impaired the recruitment of T effector cells in the CNS. In the mouse model of type I diabetes, differentiation of T effector cells, depended on RAGE (194). RAGE is expressed on CD4, CD8, and CD44 T effector cells (195). Activation of RAGE by HMGB1 in DCs promotes differentiation into Th1 effector cells (49). RAGE plays a role in immunosuppression through activation of Tregs. Tregs (CD4+CD25+CD127−) isolated from healthy donors show more surface expression of RAGE when compared to the conventional T cells (CD4+CD25−CD127−) (196).

1.6 Effects of RAGE on cellular functions

1.6a. Cell migration

RAGE is involved in the proliferation and migration of vascular smooth muscle cells, which play an important role in the development of atherosclerosis. RAGE regulates smooth muscle cell migration by phosphorylation and inhibition of GSK3β (125). Alternatively, RAGE plays a role in upregulating TGFβ (transforming growth factor β) signaling. In rat mesangial cells,
upregulation of TGFβ signaling by RAGE-AGE depends on accumulation of ROS (reactive oxygen species), and activation of AT1R (angiotensin II type 1 receptor). Indeed, phosphorylation of Smad2, a major component of TGFβ signaling is blocked by antioxidant N-acetyl cystein (a ROS blocking agent), or candesartan (an AT1R inhibitor) (136). TGFβ also promotes interaction between its receptor, TGFβR and several members of Ras family of proteins, including RhoA (Ras homolog) and ROCK (Rho associated coiled coil containing protein kinase), involved in stabilizing actin assembly. The RAGE-TGFβ-ROCK interaction plays an important role in development of atherosclerosis (137).

1.6b. Cell Mass and proliferation

Dysregulation of cell mass triggered by RAGE activation is known as a major cause of diseases such as cancer and atherosclerosis. RAGE effects on cell proliferation and apoptosis is dependent on the cell types and pathways involved. One major determinant factor is the concentration of RAGE ligands. For example nanomolar concentrations of S100B in N18 neuroblastoma and C6 glioblastoma cells under conditions of serum starvation promote cell survival in a Bcl2 (B cell lymphoma 2) dependent manner. In contrast, RAGE hyperactivation caused by micromolar concentrations of S100B induce apoptosis as suggested by increased caspase 3 activity, and cytochrome C release (117). Involvement of RAGE in apoptotic events is mediated through p38 MAP kinase and JNK pathways. Egr1 is an important target of RAGE-JNK pathway. In hepatic cells, following ischemia/reperfusion (I/R) injury, Egr1-JNK is upregulated in a RAGE dependent manner (138).

RAGE effects on cell proliferation stems from its ability to activate the three canonical MAP kinase pathways, p38, JNK, and ERK. In vascular smooth muscle cells 1-10 ug/ml of AGE-BSA
was shown to induce ERK2 and increase cell proliferation, while increased concentration of 20 ug/ml had an inhibitory effects (139). In contrast, no such effects were observed in vascular endothelial cells, pointing to cell- and dose- specificity of the interactions. RAGE-MAP kinase interactions are a major culprit in tumor cell proliferation (140).

RAGE interaction with components of JAK/STAT pathway has implications in cell proliferation. Treatment of rat kidney interstitial fibroblast cells with AGE-BSA is shown to induce mitogenesis and collagen deposition. These proliferative effects are mediated by RAGE-JAK2-STAT3 interaction and CDK4 (cyclin dependent 4), and result in p21 reduction (141). In aortic smooth muscles, S100B induces cell proliferation in a JAK2 dependent manner, and CML induction of proliferation in vascular smooth muscle cells is mediated by STAT3 (142).

1.6c. Autophagy

Autophagy is a process in which, cytoplasmic proteins or organelles are sequestered and degraded by lysosomal components. Basal autophagy under normal nutrient conditions is maintained by hVps34 (homolog of vacuolar protein sorting 34), in a process that involves the formation of hVps34-beclin1-autophagosome complex (143). Under condition of nutrient starvation however, mTORC1 (mechanistic target of rapamycin complex 1) plays a role in induction of autophagy. Induced autophagy plays a major role in RAGE mediated survival. A recent study shows that RAGE induced autophagy plays a role in early metastasis in pancreatic cancers. Autophagy in tumor cells is mediated by hVps34 but not the mTORC1 pathway (144).

Another regulator of autophagy is AMPK (AMP activated protein kinase) pathway. In mouse cortical cells, treatment with Amyloid β facilitates the formation of autophagic
vacuoles through activation of AMPK pathway (145), which also works to downregulate mTORC1 pathway.

### 1.7 RAGE and Diseases

#### 1.7a. Inflammation

RAGE signaling plays a key role in cellular response to inflammation. Under acute conditions, a transient upregulation and release of RAGE ligands induces a rapid innate response. However, accumulation of these ligands along with sustained downstream response in chronic condition result in tissue dysfunction.

Increased RAGE expression has been linked to a variety of inflammatory diseases including rheumatoid-and osteo-arthritis, vascular dysfunctions, inflammatory bowel and renal diseases, and complications associated with late stages of diabetes (146). The pleiotropic effects of RAGE signaling in most part is related to its expression patterns. RAGE expression has been detected in most cells of immune system including macrophages and monocytes, DC (dendritic cells), neutrophils, and B and T lymphocytes (147-149). RAGE is also expressed in endothelial cells, and plays a role as a leukocyte receptor through its direct interaction with B2 integrin Mac1. Activation of NF-κB by RAGE has a major role in upregulation of the innate and adaptive systems (32). Additionally, numerous RAGE ligands accumulate and trigger NF-κB pathway in the areas of tissue damage and inflammation (150). Sustained RAGE-NF-κB interactions causes the de novo synthesis of p65 transcripts, resulting in maintenance of
active NF-kB pool, overriding the negative feedback mechanisms (151). The long lasting production of NF-kB has been reported in inflammatory bowel disease.

Elevated levels of AGE products observed in diabetes, result in an increase in RAGE expression in blood vessel endothelia and the smooth muscle cells of the vasculature, and subsequent invasion of the circulating immune cells (198). RAGE activation is directly connected to the sustained inflammatory response in chronic inflammatory diseases such as rheumatoid arthritis (163), inflammatory renal disorders (199), atherosclerosis (200), and neuroinflammation (201).

1.7b. RAGE and Atherosclerosis

Atherosclerosis is a chronic inflammatory disease of arteries. RAGE involvement in atherosclerosis stems from its expression in a variety of cells implicated in disease progression including vascular and endothelial cells, monocytes, macrophages, and lymphocytes. RAGE binding to its ligands in injured vasculature promotes generation of ROS (reactive oxygen species) (1), which would in turn activate NF-kB pathway and ultimate production of TNFa and b, interleukins, and IFNG (154-156). Therefore atherogenetic modulations in vascular cells are a direct outcome of RAGE-ligand binding. RAGE-AGE binding on endothelial cells causes a reduction in thrombomodulin activities, transforming the endothelial cells to pro-coagulant surfaces. Expression of adhesion molecules such as ICAM, VCAM, and E-selectin via NF-kB pathway is a direct result of RAGE-AGE interactions (154). Infiltration of monocytes into the subendothelial space causes differentiation of these cells into macrophages, which accumulate in the cell walls, accelerating the formation of fatty streak formation (157). In
smooth muscle cells RAGE-AGE interaction results in cell proliferation, chemotactic migration, and fibrogenesis (158, 159).

1.7c. RAGE and Alzheimer’s disease

Alzheimer disease (AD) is a neurodegenerative disease associated with decline in cognitive functions. During the progressive stage of AD, overexpression of RAGE is detected in amyloid plaques, microglia, and neurons. Binding of Aβ and AGE molecules to RAGE is concomitant with an increase in NF-κB activity and release of related cytokines such as TNFα, IL1 and IL6. RAGE activation on ligand binding has been shown to increase BACE1 expression responsible for promoting Aβ production in brain (160). RAGE activation causes neurodegeneration via mitochondrial dysfunction. This occurs through the uptake and targeting of Aβ in the mitochondria, which subsequently, reduces cytochrome C oxidase, a major mitochondrial respiratory enzyme (161). Activation of RAGE by Aβ causes the upregulation of macrophage colony stimulating factor (M-CSF) in neurons, accompanied by release of proinflammatory cytokines in microglia (162). RAGE-Aβ interaction at the luminal membrane of BBB (brain blood barrier) is suggested to involve transport of circulating Aβ through BBB.

1.7d. Rage and Arthritis

RAGE and its ligands are present at an increased level in focal degenerated cartilages in osteoarthritis (OA), as well as infiltrating lymphocytes and macrophages in rheumatoid arthritis (RA) (163). RAGE-AGE interaction on chondrocytes enhances MMP1 production, and proteoglycan release by the cells (164).
1.7e. RAGE in cancer

RAGE expression has been reported in several human tumors including lung, prostate, and colorectal, pancreas, prostate, brain, breast, and ovarian cancers, as well as melanoma and lymphoma. (165). In its capacity to induce inflammatory responses, RAGE can have an impact in creating the proper microenvironment for neoplastic progression. In a mouse model of skin cancer, RAGE −/− mice were resistant to DMBA (9, 10 dimethyl 1, 2 benzanthracene)/TPA (12-O-tetradecanoylphorbol 13 acetate) induced carcinogenesis, and on occasions where tumors were produced, they were smaller in size, less advanced, and more differentiated (148). More importantly, this study showed a positive feedback involved in the increased production of RAGE ligands S100A8, and S100A9 in the epithelial cells. In bone marrow transplantation experiments, RAGE expression on immune cells but not the keratinocytes was responsible for the recruitment of innate immune cells and subsequent induction of epidermal hyperplasia (148). A common finding in several mouse studies implicates RAGE in development and recruitment of MDSCs (myeloid derived suppressor cells). In one study S100A8/S100A9 were found to bind RAGE on MDSCs and stimulate their migration via NF-kB signaling pathway (166).

Both RAGE and TLRs (toll like receptors) play a crucial role in innate immunity through recognizing and interacting with exogenous microbial particles, PAMPs (pathogen associated molecular patterns), as well as the exogenous molecules produced and released in inflammatory response, DAMPs (damage associated molecular patterns) (168). RAGE interaction with TLRs is involved in amplification of the inflammatory response. RAGE and TLRs share several ligands including LPS (lipopolyssacharides), Amyloid β, and HMGB1.
Additionally, RAGE interacts with the adaptor molecules TIRAP and MyD88, both of which are also utilized by TLRs (169-172).

A study by Hudson and colleagues showed that ectopic expression of RAGE in breast cancer cells increases MEK-epithelial to mesenchymal transition signaling and promotes lung metastasis. In the same study, treatment of tumor-xenograft mice with the amide compound FPS-ZM1, inhibited tumor growth, angiogenesis, and inflammatory cell recruitment (209). In lung cancer cells, RAGE expression contributed to the activation of PI3K/AKT and KRAS/RAF1 pathways (241). Interestingly, ablation of RAGE in tumor xenograft model of lung cancer showed significant reduction of tumor growth (241).

In a recent report, RAGE interaction with proteinase 3 (PR3) on the cell surface of prostate cancer cells was shown to mediate homing of the prostate cancer cells to the bone marrow, and the increased migratory capability and tumor cell motility involved activation of Erk and JNK pathways (242).

### 1.8 RAGE inhibitors

RAGE activity can be targeted indirectly by interfering with downstream signaling pathways, or binding to RAGE ligands (sRAGE, esRAGE), or directly by blocking the ligand-RAGE interface (RAP, FPS-ZM1, anti-RAGE blocking antibodies), or interference with RAGE synthesis (RAGE-AGE aptamers) (210).

### 1.8a. FPS-ZM1
FPS-ZM1 was originally identified in a screening of the second generation of a group of tertiary amide compounds capable of blocking RAGE activity (173). FPS-ZM1 was subsequently shown to block RAGE activity at the BBB and brain, as mediated by amyloid β accumulation. Transcriptional activity of BACE1, a key enzyme involved in Aβ production, and mediated in a RAGE-NF-kB dependent manner, is reduced by FPS-ZM1 by at least 2 folds (173). Additionally, FPS-ZM1 can block binding to RAGE, of other ligands such as AGE, HMGB1, and S100B. FPS-ZM1 inhibitory effects result from direct binding to RAGE V domain (Ki 25, 148, and 230nM) against Aβ40, HMGB1 and S100B.

1.8b. RAP (RAGE Antagonist Peptide)

RAP was synthesized using three sequences corresponding to regions of S100P with presumable interactions with RAGE. In subsequent experiments, RAP was shown to reduce NF-kB activation in vitro, and reduced the growth and metastasis of pancreatic tumors (211).

1.8c. RAGE blocking antibodies

RAGE neutralizing antibodies have been recently shown to abrogate the Erk signaling activity in a RAGE dependent manner. Additionally, in a mouse model of uremia, RAGE neutralizing antibody was shown to block progression of atherosclerosis (215). Ganju and colleagues reported a dramatic regression of growth and metastasis in a breast cancer mouse model, where mice were treated with RAGE neutralizing antibodies. Mechanistically, the antibody blocked, and interfered with interaction of RAGE with its ligand S100A7 (213). Most currently available blocking antibodies recognize the extracellular domains of the protein, indicating a requirement for structural integrity of protein for proper function.
1.8d. RAGE Aptamers

DNA aptamers raised against RAGE, can reportedly bind to the V domain and significantly interfere with RAGE-AGE interactions. Treatment with RAGE aptamer was shown to improve albuminuria, inflammation, and nephropathy in a rat model of diabetes (212).

1.8e. sRAGE

sRAGE is a heterogeneous sub-population of RAGE molecules, that can be generated through proteolytic activity of MMP9 or ADAM10 on the full length RAGE. sRAGE can alternatively be formed by alternative splicing of RAGE transcript (esRAGE). The absence of the transmembrane and C-terminal domains, render sRAGE incapable of signaling activities. In turn, sRAGE acts as molecular decoy competing with RAGE for ligands.
Chapter 2. Materials and Methods

Reagents

FPS-ZM1 was purchased from MilliporeSigma (St. Louis, MO). The antibodies against RAGE N- and C- termini were purchased from Abcam (Cambridge, MA). Antibody against the extracellular domain of RAGE was purchased from R&D systems (Minneapolis, MN). Total and phospho- Erk (T202/Y204), total and phospho- JNK (T183/Y185), phospho-p38 (T180/Y182), and phospho-H2AX (S139) were from CST (Danvers, MA). Phospho-IkBα antibody was from Santa Cruz (Dallas, TX). IkBα antibody, and all secondary antibodies, including the conjugate Alexa Fluor antibodies were purchased from ThermoFisher Scientific. Ras activation assay kit was purchased from MilliporeSigma. Recombinant HMGB1 was purchased from Biolegend (San Diego, CA). Sequence for the optimal mouse stimulatory CpG primer, ODN 1826, was adopted from Sirois et al. (118): T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*T, where the asterisk indicates a phosphorothioate linkage. Sequence for random DNA: GGTCGTTCCATT TTACTCCAC was adopted from (243).

All human pancreatic cancer cell lines used in this study were obtained from ATCC. KPC (KRAS^{LSL,G12D/+}; p53^{R172H/+}; PdxCre^{Tg/+}) murine PDAC primary cell line is described extensively by Hingorani et al (207).

Histology, Immunohistochemistry, and Immunofluorescence

Tissues were fixed in 10% formalin (neutral buffered), paraffin embedded and sectioned. Hematoxylin and Eosin (HE) staining was performed according to the established protocols. Briefly, following paraffin removal by xylene, and three ethanol changes (100%, 95%, and
80%), tissues were hydrated in water and stained with Hematoxylin for 1 minute. Following a brief wash with water, and 90% ethanol, the tissues were stained with ethanol-based Eosin, and dehydrated in changes of ethanol (90%, 100%), and xylene.

For immunohistochemical (IHC) analysis of the tissues, samples were hydrated as above. Antigen retrieval was performed using the retrieval buffer (Agilent, Santa Clara CA) for 25-30 minutes at 100C, and the endogenous peroxidase activity was quenched by incubating sections in 3% hydrogen peroxide for 15-30 minutes. Epitope labeling was performed using the Elite Vectastain ABC kit (Vector Laboratories, Burlingame CA) according to manufacturer’s protocol. Primary antibodies against the middle region, or N- and C-terminal domains of RAGE (Abcam) were diluted 1:200 (or otherwise as recommended by the supplier). Primary antibody incubations were done at 4C for 10-12 hours. The secondary antibodies used for the IHC studies were biotinylated. Signal detection was performed using the peroxidase substrate DAB (Agilent, Santa Clara CA).

Immunofluorescence detection of RAGE in tissue samples was performed as described for IHC, except the endogenous peroxidase quenching was not performed. All Images were captured using an inverted Nikon Eclipse Ti2 microscope, except for images taken for figure 5, which were captured by an Olympus FV 1000 laser confocal microscope.

Ras activation assay

Ras activation assay was performed essentially as per manufacturer’s recommendation. Briefly, following treatment, acini were pelleted at 1000xg. The supernatant was discarded and cells were resuspended in ice-cold Mg²⁺ lysis buffer. Cells were lysed by repeated pipetting. Lysates were cleared by centrifugation at 14,000xg at 4C. A fraction of the cleared
lysate was removed as input. To the remaining lysate, 10 µl of the Raf1 RBD (Ras binding 
domain)-conjugated agarose was added. Reaction was incubated for 50 minutes at 4C with 
gentle agitation. Beads were spun down, and washed in lysis buffer, and resuspended in 2x 
loading buffer and boiled prior to fractionation on SDS gel.

**Animals**

6-8 week old female C57BL/6 Wild Type (WT) mice used for the *in vivo* studies were 
purchased from Taconic Biosciences. All animal experiments were approved by IACUC 
(Institutional Animal Care and Use Committee), and performed at the veterinary facilities of 
The University of Texas MD Anderson Cancer Center in accordance with institutional 
guidelines.

**Acinar cell isolation**

Mouse acini were isolated as previously described (222). Briefly, pancreatic compartments 
were isolated and washed twice in HBSS buffer. The tissues were then sliced into small pieces 
using a scalpel. The excised tissue pieces were collected in HBSS buffer and centrifuged at 4C 
at 450xg for 2 minutes. The tissues were subsequently resuspended in collagenase IV buffer 
(10 mM HEPES, 200 U/ml Collagenase IV, 0.25 mg/ml trypsin) and incubated at 37C for 25-30 
minutes with triturating every 5 minutes. Collagenase was neutralized using 10 ml washing 
buffer (HBSS, 5% FBS, 10 mM HEPES). Samples were then centrifuged at 4C for 3 minutes, and 
washed subsequently with 10 ml of washing buffer three times. Digested tissues were then 
passed through a 100 µm filter to remove the undigested debris, and resuspended in 
Waymouth media containing 1% FBS, 0.25 mg/ml trypsin. The acini were recovered at 37C for 
one hour before processing for the experiments.
Protein extraction and Western blot analysis

Tissue samples were homogenized in lysis buffer (25 mM Tris HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol) using a polytron homogenizer. Homogenates were incubated on ice for 20-40 minutes and subsequently centrifuged at 14,000 RPM at 4C. The resulting supernatants were transferred to new tubes, and protein concentrations were determined using BCA assay (ThermoFisher Scientific). 40-50 µg protein was fractionated on SDS-PAGE, and transferred to nitrocellulose membranes, and subsequently blocked (in 5% dry milk). The membranes were incubated in primary antibody overnight. The blots were incubated in peroxidase-conjugated secondary antibodies for 1 hour at room temperature, and the signals were visualized using chemiluminescence (BioRad).

Orthotopic transplantation of tumor model

KPC primary cells stably expressing the luciferase reporter were grown in RPMI medium, supplemented with 10% FBS. Sub-confluent cultures of cells were harvested and washed in PBS, and suspended in PBS as single cells. Isoflurane was used as general anesthesia.

Mice abdominal cavities were excised through a ~1 cm-wide transverse laparotomy. 0.25x10⁶ KPC cells were injected in the pancreas tail. Mice were injected the analgesic buprenorphine hydrochloride at 0.1 mg/kg post surgically. Tumor volume was monitored by whole-body fluorescence imaging on a weekly basis, beginning three days after surgery. Imaging was performed using the D-luciferin Bioluminescence Substrate (BioGold), which was administered through i.p. injection (150 mg/kg). In vivo imaging was conducted with the IVIS Spectrum In Vivo Imaging System (Perkin Elmer).
Intraperitoneal injection of the therapeutic FPS-ZM1, was started one week after the surgery. Experimental mice were injected FPS-ZM1 at a concentration of 5mg/kg/day, while control mice were injected 4% DMSO (vehicle). Mice were euthanized on displaying sign of morbidity. All mice that had displayed a signal of at least $10^7$ photons following the surgery were included in the study. Control (untreated mice) and treatment groups, had 13 and 9 mice per group.

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Chapter 3. Results

3a. Pancreatic compartment expresses RAGE. During embryonic development, RAGE is constitutively expressed in all tissues, specifically lung (7). However, this expression is lowered in adults, and with the exception of lung most tissues express little RAGE. To investigate the role of RAGE in pancreatic pathologies, we sought to determine RAGE localization in the pancreatic compartment. Acinar cells isolated from WT mice showed the presence of different RAGE variants as previously described by Vollmar and colleagues (Figure 2A) (122). Expression of RAGE in pancreatic acinar cells makes it a bona fide compartment for studying the RAGE effects and its mediated pathways. To understand the mechanisms by which, ligand binding would stimulate RAGE, isolated acini were treated with methylglyoxal (MG), and Erk and JNK activation were analyzed. Methylglyoxal is a potent glycating agent and the precursor to the arginine derived AGE products hydroimidazolone and imidazopurionone (202). Binding at nanomolar concentrations of methylglyoxal-BSA to V1 domain of RAGE is reported to result in signal transduction (203). Treatment of acinar cells with MG for 30 minutes elicited a moderate Erk signaling, which was diminished by 1 hour (Figure 2B). During the one hour methylglyoxal treatment RAGE expression pattern was not altered (Figure 2A). MEK and its downstream effector, Erk are required for transforming activities of Ras, and Ras function is facilitated through the activity of Raf and its downstream effectors, including Erk.
Figure 2. Primary acinar cells display increased JNK and MAPK activities in response to treatment with methylglyoxal (MG). Primary acinar cells isolated from WT mouse pancreata were treated with 2.5 mM methylglyoxal following 1 hour of recovery. A) Expression of RAGE in primary acinar cells. Cells were treated or not with methylglyoxal for 60 minutes and lysates from the treated and untreated acini were harvested and analyzed by western blot. B) Time course analysis of response to MG in acinar cells. Isolated acini were treated with 2.5 mM methylglyoxal as indicated. Total protein and phosphorylated forms of Erk 1/2, and JNK were detected by western blots 30 or 60 minutes after treatment with methylglyoxal. Untreated samples were collected at 60 minutes in the time course. C) Ras activity assay in MG treated cells. Cells were left untreated, or treated for 7 minutes (with EGF), or 30 minutes (with MG) to stimulate Ras activity before being harvested. Proteins were extracted and subjected to Raf1 resin binding. The experiments were repeated at least three times.
A Ras pulldown assay was conducted to capture the endogenous pool of activated Ras. Cells were treated (or not) with MG and harvested 30 minutes following the treatment. Lysates from the treated and untreated groups were subjected to Raf1 binding pulldown. MG potently activated Ras to the levels comparable with EGF (Figure 2C). These experiments clearly show that pancreatic acinar compartment expresses RAGE, and that RAGE is involved in the activation of Ras and its downstream signaling, including Erk and JNK pathways.

3b. Differential RAGE expression in pancreas reflects the disease state. Vollmar and his colleagues have reported the detection of six RAGE isoforms in mouse pancreatic tissues (122). Induction of acute pancreatitis or diabetes via administration of cerulein or streptozotocin (STZ) respectively, resulted in a differential expression of RAGE isoforms. Moreover, the detected low molecular species were not degradation products (122).

To assess RAGE expression in normal tissues, and tissues undergoing pathology, i.e. chronic pancreatitis or pancreatic cancer, we utilized antibodies raised against epitopes within different regions of the molecule. Western blot analysis of tissue samples collected from WT mice or mice undergoing pathology (chronic pancreatitis or pancreatic cancer), revealed a distinct expression pattern. In all tissue samples a 50 kDa band corresponding to the apparent molecular mass of the predominant RAGE isoform was detected. However, an additional lower molecular weight species (~ 25 kDa) detected in the lysates from tissues undergoing pathology was constantly absent in the lysates from WT samples (Figure 3A).
Figure 3. SDS-PAGE analysis of RAGE expression in pancreatic tissues. RAGE expression was detected in tissues using antibodies raised against different regions of protein. Protein lysates were obtained from pancreatic tissues of WT mice, or mice undergoing chronic pancreatitis or pancreatic cancer (pathology). A) RAGE detection using an antibody against the entire extracellular domain of RAGE in samples isolated from WT mice, or mice with pancreatic cancer. B) Ponceau S staining of the membrane shown in A. C) and D) RAGE detection using an antibody against the N-terminal domain (B), or C-terminal domain (C) of RAGE (samples in B and C are identical). Lysates produced from the lung tissues of RA⁻/⁻ (RAGE knockout) mice ran as negative control.

It is highly unlikely that the detected species is a nonspecific signal, or due to protein degradation, as this pattern is consistently present in all samples with disease state (chronic
pancreatitis and cancer), and is detected utilizing at least two different antibodies raised against different epitopes. This differential protein expression may be due to the presence of different RAGE isoforms as suggested by Vollmar and colleagues. Interestingly, RAGE expression matches the pathological state of tissues; the appearance of the low molecular species is accompanied by increased level of p22phox, a protein implicated in pancreatic malignancies (Figure 3C) (204). Lysates from lung and liver tissues isolated from RA −/− mice were used as a negative control. The appearance of multiple bands in the western blot analysis in those samples may be due to incomplete knockout of the gene in the organs as observed by Arnold and colleagues (244).

Immunohistochemical (IHC) analysis of pancreatic tissues depicts a pattern in which RAGE is localized to both membrane and cytoplasmic compartments. RAGE was readily detected in the stroma of cancerous tissues. In tissues undergoing pathology, RAGE was detected in a pattern that suggests it is present in infiltrating immune cells as well (Figure 4A). The nature of these infiltrating immune cells will be further explored using pan-leukocyte antibody (CD45), as well as antibodies against neutrophils (Gr-1), and macrophages (F4/80).

Immunofluorescent staining of the mouse tissue samples showed a punctate localization of the protein in the cytoplasm, with minor localization in the nucleus. However, in samples from cancer tissues, RAGE was mainly localized to the cellular membranes and nuclear compartments (Figure 4B).
Figure 4. RAGE localization by immunohistochemistry and immunofluorescence. Tissue samples from WT mice, or mice undergoing pathology (chronic pancreatitis or cancer) were stained with antibodies against RAGE extracellular domain. A) Immunohistochemical analysis of WT tissues and tissues with chronic pancreatitis or cancer. Control samples were stained with secondary antibody. Scale bar is 200 µm. B) Immunofluorescence analysis of WT and cancer tissues. Arrows point to the stromal colocalization of RAGE and the nuclear signal. Scale bar is 50 µm.

Ras-MEK signaling was recently found to engage Chk1, a member of phosphatidylinositol 3-Kinase family, to enable survival upon DNA damage, irrespective of p53 mutation status (226).
Figure 5. RAGE and H2AX-Gamma foci colocalize in nucleus. Immunofluorescence analysis of pancreatic tissues using an antibody against the RAGE C-terminal domain (red), or H2AX-Gamma (green), and DAPI staining of the nuclei (blue). Arrow points to the nuclei, in which colocalization is visible.

Chk1 is responsible for coordinating the DNA damage and cell cycle checkpoint responses (227). Mechanistically, Ras-MEK signaling drives Chk1 expression by promoting cancer cell growth and genotoxic stress, which in turn upregulate Chk1 to mediate a response to the consequent DNA damage (226). As pancreatic tissues undergoing pancreatitis or progression into cancer display increased accumulation of DNA damage foci (228), we sought to test whether involvement of RAGE in upregulation of Ras pathway could indeed play a role in an increased DNA damage response. To this end, we used H2AX phosphorylation as a readout and biomarker of DNA damage.
Interestingly, using the antibody raised against RAGE cytosolic domain, a distinct nuclear localization was detected (Figure 5). Immunofluorescence (IF) analysis of these samples showed colocalization of the RAGE signal with the nuclear phospho-H2AX, a marker of DNA damage.

Taken together, RAGE shows a ubiquitous and distinct localization pattern in pancreatic tissues undergoing pathology. In WT tissues, RAGE is mainly localized to the cytoplasmic compartment with a punctate distribution. In the course of disease progression, RAGE is mainly localized to the areas surrounding the nucleus and is present in the nuclei of PanIN lesions, as well as infiltrating cells in the stroma. As mutant Ras is functionally dependent on DNA damage response pathways (226), the observed redistribution in tissues undergoing pathology may have implications for its interactions with Ras and its downstream signaling.

3c. A specific RAGE inhibitor, FPS-ZM1, blocks activation of downstream molecular signaling.

Our observation that RAGE variant(s) localize to nuclear damage foci prompted us to investigate its possible role in DNA damage response (DDR) and signaling. A variety of genotoxic agents including etoposide, hydroxyurea (HU), ionizing radiation (IR), mitomycin C (MMC), and UV have been reported to activate Erk, a known RAGE effector in mouse and human cell lines (205). While the underlying mechanisms responsible for Erk activation in DDR are poorly understood, current evidence points to the involvement of the MAP kinase protein, MEK. In turn, Erk facilitates activation of ATM and ATR, the two major kinases of DDR (226).
Erk activation is dependent on the cell type and genotoxic agent involved. However, in all cases Erk activation occurs with fast kinetics, reaching a plateau in one hour. Erk activity reportedly modulates checkpoint activation as well as re-initiation of cell cycle following DNA damage induced cell cycle arrest (226). Given our data showing colocalization of RAGE and H2AX signals, we used RAGE inhibitor FPS-ZM1, to seek whether blocking RAGE activity could indeed cause inactivation of Erk pathway. FPS-ZM1 competes for ligand access by binding to RAGE V domain. In KPC (KRAS\textsuperscript{L53G12D}/+; p53\textsuperscript{R172H}+/+; PdxCre) mouse primary cells, 20 µM FPS-ZM1 inhibited Erk and NF-kB activities in the presence and absence of two known RAGE ligands, CpG primer, and HMGB1 (Figure 6A). Interestingly, treating the cells with a RAGE-specific neutralizing antibody yielded identical results, showing direct RAGE involvement in Erk

![Figure 6. RAGE inhibitor, FPS-ZM1, suppresses NF-kB and Erk activities in cells expressing oncogenic KRAS, but does not suppress DNA damage signaling. A) Erk and NF-kB signaling in response to FPS-ZM1. Murine primary KPC cells were grown in media containing 5% FBS for 16 hours, and treated or not with 20 µM FPS-ZM1 for 4 hours.](image-url)
hours before treatment with CpG B primer (1 µM), HMGB1 (1 µg/ml), control Igg (10 µg/ml), or RAGE-blocking antibody (10 µg/ml) for 2 hours. 8) FPS-ZM1 effects on KPC cells in the presence or absence of etoposide. KPC cell were treated with 40 µM FPS-ZM1 in the presence and absence of 100 µM etoposide for 16 hours (The figure is a representative of at least 3 separate experiments).

and NF-kB1 signaling pathways (Figure 6A). In these experiments the basal level of phosphorylated Erk was not increased above the background using the RAGE-specific ligands. The presence of 5% serum in the media, which was needed for the growth, may have contributed to higher basal activity and low level of stimulation by CpG B and HMGB1 ligands. Further experiments are needed to address the high basal Erk phosphorylation.

Treatment of KPC cells with etoposide increased the basal Erk activity, while inducing a large DNA damage response as evidenced by an increased level of phospho-H2AX. Administration of 40 µM FPS-ZM1 completely abrogated Erk activity in the absence of DNA damage, and brought it to basal levels in cells treated with 100 µM etoposide. However, it did not change DDR as evidenced by high levels of H2AX phosphorylation (Figure 6B). It is important to note that original studies were conducted with 40 µM or higher concentrations of FPS-ZM1. However to avoid toxicity, and due to the observation that concentrations as low as 20 µM showed inhibitory effects, all subsequent studies were done at 20 µM concentration of the inhibitor.

Together, these data show direct involvement of RAGE in Erk and NF-kB signaling following stimulation with RAGE ligands HMGB1 and CpG, as well as treatment with DNA damage agent, etoposide, and that RAGE inhibitor, FPS-ZM1, can block these pathways.
3d. FPS-ZM1 downregulates methylglyoxal-induced activation of MAP kinase pathway. Our experiments with primary acini indicated that methylglyoxal could act as an agonist to activate MAP kinase and JNK pathways (Figure 2). We repeated these experiments in KPC cells to investigate whether RAGE was directly responsible for downstream activation. KPC cells were grown in low serum media, and treated with 2.5 mM methylglyoxal for up to two hours, in the

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**Figure 7.** MAP kinase activity in KPC mouse cells is abrogated upon treatment with FPS-ZM1. A) KPC cells were grown in media containing 5% serum for 12-16 hours, and treated with 2.5 mM methylglyoxal for indicated time points in the presence or absence of 40 µM FPS-ZM1 (The figure is a representative of at least three different experiments).
presence or absence of 40 μM RAGE inhibitor. FPS-ZM1 abrogated Erk activity at earlier time points, while p38 activation was significantly diminished in the presence of RAGE antagonist at all time points (Figure 7A). Earlier experiments using two known RAGE ligands, CpG B nucleotides and HMGB1, showed a direct involvement of RAGE in regulating Erk activity, as ligand binding was blocked using a neutralizing/blocking antibody against RAGE. In the presence of RAGE specific antibody, but not the IgG control, Erk and NF-kB signals were robustly diminished (Figure 6A).

This result confirms that upon engagement with AGE ligands, RAGE activation upregulates the components of MAP kinase pathways. More importantly, FPS-ZM1 can directly interfere with this binding to reduce the activation of MAP kinase components. Together, these results demonstrate that pancreas is a bona fide compartment for RAGE expression and signaling, and that methylglyoxal is capable of activating MAP kinase signaling. Erk and p38 activation occur directly via RAGE, and as a specific RAGE inhibitor, FPS-ZM1, downregulates their activities.

3e. FPS-ZM1 reduces the Erk and NF-kB signaling in human pancreatic cancer cells. We used established human pancreatic cancer cell lines (Figure 8A), to investigate whether FPS-ZM1 could block RAGE induced signaling pathways in a manner observed in KPC cells. Treatment with increasing concentrations of FPS-ZM1 blocked NF-kB activity in the KPC and human cancer cells alike. The Erk signaling response was, however, less ubiquitous. In Panc1 cells, treatment with 20 μM FPS-ZM1 caused Erk activation above the basal levels, while lowering the ligand induced activity. The ligand induced activity was fully blocked by RAGE blocking antibody (Figure 8B). As in KPC cells, Erk response was lowered to basal levels in Capan2 cells,
while AsPC1 and BxPC3 cells were unresponsive to treatment doses used. In Capan2 cells, DDR was also reduced in response to FPS-ZM1 treatment (Figure 8C). Taken together, RAGE inhibitor FPS-ZM1, can reduce NF-kB and Erk signaling pathways downstream of RAGE in wide variety of cells.

<table>
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<tr>
<th>Cell line</th>
<th>Kras</th>
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<td>WT</td>
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<td></td>
<td></td>
<td>Homozygous deletion of exon 5</td>
<td>Homozygous deletion</td>
<td>100 Thr</td>
</tr>
<tr>
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<td>WT</td>
<td>Homozygous deletion</td>
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A)
Figure 8. FPS-ZM1 reduces the Erk and NF-kB signaling in human pancreatic cancer cells. A) Table of the established human pancreatic cancer cell lines used in this section. The genotype of the most common mutations in these cancer cell lines is indicated (229). B) Human Panc1 pancreatic cancer cells were grown in serum free media for 16 hours, and subsequently treated (or not) with 20 µM FPS-ZM1 for 4 hours before treatment with Random DNA (1µM), CpG primer (1 µM), HMGB1 (1 µg/ml), control IgG or blocking antibody (10 µg/ml) for 2 hours. C) Cells were serum starved for 16 hours, treated in the presence or absence of 35 or 70 µM FPS-ZM1, and harvested and processed as for Panc1 cells.

3f. FPS-ZM1 impairs tumor progression in an in vivo mouse model. FPS-ZM1 is used in treatment of Alzheimer’s disease by blocking the amyloid β ligands (173). The tertiary amide derivative binds to the V domain of RAGE, blocking ligand access. Its use as an anticancer drug however, was only recently reported (209); in an orthotopic model of breast cancer, intraperitoneal administration of 1 mg/kg, twice per week of FPS-ZM1 was shown to block the growth of both primary and metastatic breast cancer cells (209).

Given the ability of the drug in blocking the activity of MAP kinase proteins in both human and murine primary cancer cells, we used an orthotopic mouse model to test the drug efficiency in vivo. An unpublished communication had made use of two FPS-ZM1 concentrations, 1 and 5 mg/kg/day in treatment of the mice in an orthotopic model of PDAC. We surmised that a higher dose of the drug may be necessary due to the enriched stromal nature and invasiveness of PDAC. Syngeneic C57BL/6 mice were injected with KPC cells, and disease progression was monitored in the untreated control mice and mice treated via intraperitoneal injection of 5mg/kg/d of FPS-ZM1. FPS-ZM1 blocked PDAC progression, and increased the survival rate by ~ 1.6 fold.
Figure 9. Association of drug treatment with survival in orthotopic xenografts. Kaplan-Meier survival curve. 0.25x10^6 KPC cells were surgically implanted in mice pancreata. Tumor development was monitored by whole body imaging starting 3 days after xenograft implantation and continued on a weekly basis. Treatment started 7 days after surgery. 5mg/kg/day FPS-ZM1 was administered via intraperitoneal injection. Mice were sacrificed on displaying signs of morbidity.
Examining the tissues samples isolated from control untreated mice, and mice treated with FPS-ZM1 showed a differential distribution of RAGE in these tissues (Figure 10). In control mice RAGE was mainly localized to the cell and nuclear membranes, while in FPS-ZM1 treated mice, it showed a general and scattered cytoplasmic localization. As only membrane bound RAGE is functionally active, these data may indicate a major role for FPS-ZM1 in regulating the activation of downstream pathways. In untreated tissues, RAGE may be engaged in a fully active membrane-bound conformation, capable of stimulating Erk and NF-κB activation.
(Figure 6). However, treatment with the drug, may induce protein shedding (or expression of the soluble isoform (sRAGE), which in turn, will give rise to the production of an inactive product (sRAGE), whose major role is to scavenge and remove the available pool of ligands.

These results establish a role for FPS-ZM1 as a bona fide RAGE inhibitor, capable of blocking the ability of the receptor to activate MAP kinase related pathways both in vitro and in a tumor dependent manner.
Chapter 4. Discussion

RAGE is a pattern recognition receptor, engaging multitude of ligands due to its pleiotropic nature. RAGE-ligand interactions in turn trigger the activation of several downstream signaling pathways including MAP kinase, NF-kB, JNK, and JAK/STAT pathways, all of which are implicated in a variety of cancers.

While the mechanisms underlying the involvement of RAGE in disease progression is less understood, an emerging picture alludes to the multi-ligand nature of RAGE activation. RAGE was first identified as a receptor for the advanced glycation end products (AGE). However, its expression in a multitude of cells of innate immunity, and its involvement in differentiation and maturation of myeloid cells, shed a new light on its role in regulating the immune response. S100 family of proteins, HMGB1, amyloid β, and nucleic acids are among the ligands that have been shown to engage RAGE and elicit proinflammatory responses downstream of prototypical pathways such as MAP kinase, and NF-kB. Molecular studies demonstrate a correlation between prolonged inflammatory responses and cancer development (219). RAGE overexpression has been detected in most types of solid tumors with inflammatory microenvironment (148).

RAGE deficient mice were recently shown to have diminished propensity toward breast cancer. Furthermore, administration of neutralizing RAGE antibodies or sRAGE inhibited tumor progression and metastasis in an S100A7 transgenic mouse model of breast cancer (213). In an orthotopic model of mouse breast cancer, RAGE played a role in driving metastasis, and FPS-ZM1 was shown to block the metastatic and invasive nature of the
disease through reducing angiogenesis and recruitment of inflammatory cells in the tumor microenvironment (TME) (209).

In pancreatic ductal adenocarcinoma (PDAC), a prototypical example of epithelial derived cancers, RAGE mediated signaling is also involved in amplification and sustenance of pro-tumor inflammatory responses. Loss of RAGE function in a KRAS$^{G12D}$ mouse model of PDAC prolongs survival by inhibiting the formation of high grade PanIN (pancreatic intra-epithelial neoplasia) lesions, as well as progression to PDAC (214). Additionally, RAGE knockdown in human and mouse pancreatic cancer cell lines enhances the anticancer activity of chemotherapeutics (217).

Our data indicates that inhibiting RAGE through the use of its specific inhibitor, FPS-ZM1, may abrogate RAGE function via changing its cellular distribution. This may be accomplished through either proteolytic shedding, which renders the protein soluble, and thereby inactive, or by way of inducing the expression of the soluble form (sRAGE), which function as a molecular decoy capable of binding to, and removing the *bona fide* RAGE ligands.

Mutations of *KRAS* occur in ~98% of pancreatic cancers. The KRAS proto-oncogene encodes a GTPase, which cycles between its GTP-bound active and GDP-bound inactive states (223). The constitutive activity caused by KRAS mutations, and stimulation of downstream signaling pathways are the major driving force behind many of the phenotypic hallmarks of cancer such as reprogrammed metabolism, increased proliferation, metastasis, altered TME, suppression and evasion from immune response (224).

In PDAC, KRAS mutation is an early event, since ~90% of low grade PanIN lesions are shown to contain the mutation (216). Subsequent inactivation, and accumulation of
mutations in genes such as p16, TP53, and SMAD4, contribute to the development and maintenance of KRAS driven event (225). The inevitable role of KRAS in cancer progression has prompted several studies to identify pharmacological approaches to directly inhibit KRAS. However, currently no successful strategies are available.

RAGE is present at low levels in most tissues, but its expression is increased in pathology related contexts. We show that pancreas is the bona fide compartment expressing RAGE, and demonstrate that methylglyoxal, can activate Erk signaling and NF-kB activity in a RAGE-dependent manner. More importantly, this activation is mediated through Ras, as shown through Ras increased GTPase activity in methylglyoxal treated acini.

While the nature of RAGE-ligand interaction in pancreatic malignancies is not well understood, recent studies have shown an important role for the members of S100 family of proteins. Expression of several S100 ligands is increased in pancreatic ductal adenocarcinoma tissues. Additionally, HMGB1 molecules released from necrotic cells are known to increase RAGE activity.

To investigate KRAS-derived signaling pancreatic malignancies through RAGE activation, we used methylglyoxal. Methylglyoxal is a highly reactive intermediate of glucose metabolism and a precursor of AGE products. Treatment of KPC primary mouse cells, harboring the KRAS \(^{G12D}\) mutation, with methylglyoxal elicited robust activation of MAP kinase pathways in a dose and time dependent manner, and RAGE antagonist, FPS-ZM1 inhibited MAP kinase activity, pointing to a direct role for RAGE in downstream responses.

We found that the RAGE C-terminal domain is translocated to the nucleus, and colocalizes with the DNA damage foci (as assayed by H2AX-Gamma staining). In response to DDR, Erk was
reportedly activated (128). To examine whether RAGE nuclear localization is related to DDR, KPC mouse cells, expressing the KRAS$^{G12D}$ were treated with the DNA double strand break inducing reagent, etoposide, and Erk activation was accessed. RAGE could partially drive the DNA damage dependent upregulation of Erk, as the signal was abated to basal levels through treatment with the RAGE antagonist, FPS-ZM1.

FPS-ZM1 has been demonstrated to function as a specific inhibitor of RAGE. The drug was originally designed and used in treatment of Alzheimer’s disease. However, a recent study reports its successful application in suppression of breast cancer and metastasis. Interestingly, the drug has minimal toxic side effects, at doses as high as 500 mg/kg in mouse experiments (173). FPS-ZM1 has been recently shown to inhibit cancer progression and metastatic properties in an orthotopic model of breast cancer.

Here we show that the specific RAGE antagonist, FPS-ZM1, can impair tumor progression, and extend the survival rate in an orthotopic model of PDAC. Analysis of the samples isolated from treated and untreated mice shows high levels of soluble RAGE (sRAGE), not present in the samples isolated from wild type mice. Both treated and untreated mice show tumor growth, indicating that the drug may not inhibit tumor cell viability or proliferation. However, treated mice have a survival rate that is about twice higher than that of the untreated mice. Our in vitro data indicate that this resistance to local progression, may in part, be due to blocking RAGE direct engagement with its corresponding ligand, and abrogation of downstream MAP kinase signaling.

Recently, chemotherapy-related upregulation of RAGE was shown to contribute to drug resistance in pancreatic cancer, and RAGE knockdown in human and mouse pancreatic cancer
cells enhanced the anticancer activity of several chemotherapeutic drugs such as gemcitabine (144). In this light, current data encourages the use of small molecule inhibitor, FPS-ZM1, in treatment of multiple malignancies, alone or in combinatorial therapies. Genetic ablation of RAGE was also shown to prevent accumulation of Treg and myeloid derived suppressor cells (MDSC) in spleen, suggesting RAGE involvement in regulation of immune response in tumor microenvironment (220). Pancreatic cancer cells express a number of ligands, such as PDL1 (programmed death 1 ligand), that are aimed at inactivating the cytotoxic T cells in the local tumor microenvironment. However, the use of checkpoint inhibitors such as PD1 or cytotoxic T lymphocyte antigen 4 (CTLA4) in single therapies has failed to give any objective response to patients with pancreatic ductal adenocarcinoma. Developing strategies to combine the use of immunotherapeutics such checkpoint inhibitors with the pharmacological intervention of FPS-ZM1 may prove rather effective. Lack of toxicity, which can afford higher doses of the drug in clinical settings may prove useful in retarding tumor progression.

Due to its success in impairment of tumor progression in breast and pancreatic cancers, FPS-ZM1 should be afforded a clinical trial for cancer stricken patients.

As a pattern recognition receptor, RAGE is involved in several cellular functions. Figure 11 summarizes some aspects of this study including cellular localization, activation of MAP kinase and NF-κB pathways, and inhibitory effects on cell signaling downstream of Ras pathway.
Figure 11. **RAGE is a pattern recognition receptor involved in different cellular functions.** Full length, membrane bound RAGE engages its ligands at its extracellular domain. RAGE cytoplasmic domain engages components of MAP kinase and NF-kB pathways. Upon stimulation, RAGE multimerizes through its C and V1 domains. Multimers form a functional unit, stabilizing the receptor assemblies. RAGE regulation occurs through proteolytic cleavage at C terminus. The cytoplasmic domain may stay in the cytoplasm, or translocate to nucleus. Nuclear localization of the C-terminal domain is not clear. Upon binding to ligands, RAGE can also undergo phagocytosis, and is compartmentalized in the endosomes. In endosomes, RAGE can interacts with endosomal components of TLR family and participate in production of inflammatory cytokines through engaging ligands such as HMGB1 and nucleic acids. Alternatively, it may translocate to endoplasmic reticulum to participate in ER-endosome trafficking. Nuclear localization of RAGE is less understood, but may occur due to DNA damage response.
Chapter 5. Concluding Remarks

In 2016, more than 53,000 patients were estimated to be diagnosed with PDAC (208). The disease is still considered incurable, with a one year survival rate of 20%, and five year rate of 7% for all stages of pancreatic cancer combined. The high mortality rate is due for the most part, to lack of major clinically validated screening procedures at the early stages, the invasive nature of the disease, and a high rate of metastasis. Currently, tumor resection is considered the method of choice, even though less than 20% of diagnosed patients are eligible. Most affected patients die of metastasis to lung, liver, and peritoneum (208). A complete understanding of the disease will be therefore needed for full diagnostic and interventional applications.

RAGE is a multi-ligand binding receptor, with a broad expression in many cell types. The nature of the ligands and their concentration, co-expression of other ligands, and the cell type, are all major determinants in cellular responses elicited by RAGE.

Increased levels of RAGE have been detected in several malignancies including pancreatic, oral, breast, osteosarcoma, colorectal, ovarian, and prostate cancers (209, and references therein).

RAGE is involved in plethora of signaling pathways, most important of which is the mitogen activated protein kinase (MAPK). Convergence of MAP kinase and NF-κB pathways is responsible for the release and subsequent activation of NF-κB. Indeed, suppression of RAGE activity by an antagonist or sRAGE has been shown to block both downstream pathways.
Pleiotropic involvement of RAGE in pathologies such as inflammation, vascular disease, diabetes, and cancer makes the protein a valuable therapeutic target. In this study, we aim at targeting RAGE using a commercially available amide derivative, FPS-ZM1, which is proven to work in ameliorating symptoms of Alzheimer disease by blocking amyloid β aggregates. Our study offers promising results in PDAC outcome: FPS-ZM1 retards tumorigenicity in mice with xenograft implantation, as a result of which, the animals show higher survival rates, and increased resistance to cancer progression. We demonstrate that these effects may be due to blockade of MAP kinase and NF-κB activities.
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Vita

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