DM199 in the Treatment of Chronic Kidney Disease

White Paper

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ABSTRACT

DiaMedica Therapeutics is developing a recombinant KLK1 protein (DM199) for treatment of Chronic Kidney Disease (CKD). Human tissue kallikrein (KLK1) is an important serine protease that plays a critical role in the regulation of microcirculation, blood pressure and vascular function. The kallikrein-kinin system (KKS) triggers a cascade of events, including the regulated release of active bradykinin in endothelial cells. This system increases nitric oxide (NO) and prostaglandin (PGI_2) to improve capillary blood flow and reduce fibrosis and inflammation. *In vivo* animal data along with clinical evidence suggests KLK1 is an effective treatment for a variety of conditions related to tissue ischemia and vascular function. Lower than normal levels of KLK1 caused by genetics or other factors have been reported to be associated with greater risk of cardiovascular and kidney disease. Here we give an overview of KLK1 based treatment options, including recombinant KLK1 (DM199), and summarize what is known about KLK1 therapy for CKD. KLK1 drug therapy (protein isolated from pig pancreas) is approved and widely used in Japan, China and Korea to treat CKD and related vascular diseases There are approximately 30 million people in the US with CKD, arising from multiple other disease states including diabetes mellitus, hypertension, lupus nephritis, glomerulonephritis, polycystic kidney disease and IgA nephropathy. This paper will discuss the rationale for using DM199 to treat patients with CKD caused by any of these diseases and highlights the need for further clinical research.

KLLK1/DM199 and Chronic Kidney Disease

DiaMedica Therapeutics has developed DM199, a recombinant form of the endogenous protein human tissue kallikrein (KLK1), as a therapeutic agent for the treatment of patients with chronic kidney disease (CKD). CKD is associated with multiple disease states, most predominantly diabetes, cardiovascular disease and high blood pressure. Other causes of CKD include rare diseases such as, Type I Diabetes, lupus nephritis, IgA nephropathy, polycystic kidney disease, nephrotic syndrome, focal segmental glomerulosclerosis, and acute kidney injury. The kallikrein-kinin system (KKS) plays a vital role in the maintenance of normal kidney function which is dysregulated in disease states as evidenced by reduced KLK1 levels in patients with CKD. DM199 is hypothesized to boost the KKS system to functionally restore KLK1 levels and therefore has potential to treat these high-risk populations.

In the United States, approximately 30 million patients have CKD, with more than 700,000 patients currently being treated for kidney

failure including approximately 500,000 dialysis patients and over 200,000 kidney transplant recipients each year. (U.S. Renal Data System Annual Data Report, 2017, Centers for Medicare & Medicaid Services). The cost to care for patients with CKD is also significant. Medicare spending for kidney disease was nearly $100 billion dollars per year in 2015, comprised of approximately $64 billion in spending for Medicare beneficiaries with CKD and $34 billion for beneficiaries with end-stage renal disease (ESRD) (United States Kidney Data System, 2017 USRDS Annual Data Report). Worldwide, approximately 300 million people are diagnosed with CKD (overall prevalence 10.8%; (Liu, 2013).

Chronic Kidney Disease and Current Therapies

Each kidney has approximately one million glomeruli composed of a collection of capillaries between two arterioles responsible for filtering blood. The pathogenesis of CKD includes both structural and hormonal processes that can alter kidney function. Typically, CKD is characterized by a gradual loss of kidney function and is sometimes accompanied by a progressive increase in urinary albumin excretion (albuminuria, considered a prognosticator). Kidney function generally declines slowly with age, but the rate of decline seen in patients with CKD depends on multiple factors, including the cause of CKD, genetic predisposition, and blood pressure.

Current drug therapies for CKD primarily target secondary disease factors that appear to cause ongoing kidney injury, including hypertension, proteinuria (abnormally high protein in the urine), and serum glucose levels. Angiotensin converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARBs) are commonly employed and considered standard of care for CKD (patients with diabetes or proteinuria). Both ACEi and ARBs affect the renin-angiotensin system (RAS). ACEi drugs block production of angiotensin and prevent the breakdown of bradykinin (BK). ARBs are antagonists of the angiotensin receptors AT1 and AT2. These therapies are intended to reduce the activity of the renin angiotensin system in the kidney, thereby improving capillary blood flow, and lowering intraglomerular pressure (Lewis et al., 1993).

ACEi breakdown of bradykinin, but in an unregulated manner. Patients with CKD are believed to have low BK, KLK1, NO and PGI_2, levels. Thus, targeting BK levels in isolation is believed to have limited benefit (Pizard et al., 2008).

Side effects of RAS-based drug treatments include elevations of serum potassium levels (hyperkalemia) and angioedema. Chronic cough also is associated with ACEi treatment and may affect up to 35% of patients taking these medications (Dicpinigaitis, 2006).

An estimated 2.5 million patients in the US have CKD and hyperkalemia (USRDS, 2011, Truven data and CDC). In six separate clinical trials, including more than 1,500 individuals with kidney insufficiency, patients were randomly assigned to ACEi or ARB treatment. Elevated serum potassium levels occurred in 3-5% of participants (Einhorn et al., 2009).

The consequences of hyperkalemia are significant in that it raises the risk of cardiac

arrhythmias or sudden cardiac death (Parham et al., 2006; Raebel, 2012).

Angioedema is another potentially serious drug related side-effect more often associated with the use of ACEi versus ARBs. This is a nonpitting edema (swelling) of the dermis and subcutaneous layers. The tongue, lips, face, and throat are most often affected and can be life threatening. However, swelling can occur in extremities, genitalia and viscera. The overall lifetime incidence of adverse events (AE) is approximately 10-15%, with ACEi-induced angioedema being responsible for 30-40% of all cases in the US (Lewis, 2013).

The notable complications of currently available therapies make the development of new, targeted therapies critical.

Tissue Kallikrein and the Kallikrein-Kinin System

KLK1 isolated from porcine pancreas has been approved for use in Japan, China and Korea to treat numerous vascular diseases including chronic kidney disease, hypertension and retinopathy. DiaMedica believes that millions of patients have been treated with porcine KLK1 marketed by companies including Bayer (Kallidinogenase), Sanwa Kagaku Kenkyusho Co., Ltd (Carnaculin®) and ChangZhou Qianhong Bio-Pharma Co., Ltd (Kallidinogenase). DiaMedica has completed enzymatic studies comparing DM199 to porcine KLK1 that demonstrated similar KLK1 enzymatic activity profiles.

Regulated Release of Bradykinin to Produce NO and PGI_2

Kallikrein proteins are generated from 15 genes found on human chromosome 19 and represent the largest protease gene cluster in the human genome (Yousef and Diamandis, 2001). These serine proteases have substrate specificity similar to that of trypsin or chymotrypsin and are known to be involved in a variety of biochemical processes. Although they have substantial structural similarity and enzymatic activity, KLK1 is the only family member that contributes to the formation of kinin peptides, such as BK and kallidin (Lys-BK). KLK1 is primarily responsible for the cleavage of low molecular weight kininogen (LMWK) to produce vasoactive kinins (Bhoola et al., 1992), which directly activate two types of BK receptors, bradykinin 1 receptor (BK1R) and bradykinin 2 receptor (BK2R) (Marceau et al., 1998; Regoli and Barabé, 1980) to release a variety of signaling molecules including NO and PGI_2 (Figure 1).

Both NO and PGI_2 stimulate the production of the second messengers cyclic GMP (cGMP) and cyclic AMP (cAMP) to mediate some of their effects. The presence of a functional endothelium is critical to the maintenance of a healthy cardiovascular system. The endothelium forms a physical barrier to circulating elements. In addition, the endothelium forms an all-encompassing metabolic barrier that extends to all areas of the body. cGMP and cAMP are believed to be co-released by endothelial cells and act in synergy. The close functional relationship between NO and PGI_2 means that if the release

of one or other of these hormones is compromised, the cardiovascular system is put under significant strain and the risk of kidney disease, heart attack and stroke increase (Mitchell et al., 2008).

Actions of BK in the Endothelium

BK receptors are widely distributed, with BK2R generally more abundant than BK1R (Marceau and Regoli, 2004). Whereas BK2Rs are constitutively expressed, BK1Rs are found at relatively low levels under normal conditions but upregulated in pro-inflammatory conditions or in BK2R knockout mice (Duka et al., 2001; Schremmer-Danninger et al., 1998; Spillmann et al., 2002). KLK1 is not to be confused with plasma kallikrein which is a different molecule associated with out-of-control bradykinin and heredity angioedema and differs from KLK1 with regard to molecular weight, biological function and immunological characteristics. It is hypothesized that BK1Rs participate in chronic phases of diseases with a strong immune component such as rheumatoid arthritis, multiple sclerosis, septic shock and diabetes (Golias et al., 2007). Based on pharmacological studies using antagonists selective for BK2R versus BK1R, it appears that many of the direct physiological actions of KLK1 are mainly mediated through BK2R receptors (Marceau and Regoli, 2004). Both the BK1R and BK2R are G-protein coupled receptors (GPCRs) that trigger a variety of intracellular second messenger systems through $G\alpha_q$ (and other $G\alpha$ variants) including activation of phospholipase C, mitogen-activated protein kinase (MAPK) and phospholipase A2 (Busse and Fleming, 1996). Fleming et al., 1995; Lal et al., 1998; Leeb-Lundberg, 2005). In vascular tissue BK2Rs, located on endothelial cells, activate endothelial nitric oxide synthase (eNOS) and increase nitric oxide (NO) thereby stimulating vasodilation and increased capillary blood flow (Amin-Hanjani et al., 2001; Emanueli et al., 2004).

Nitric Oxide formed by vascular endothelium is rapidly dispersed into the blood and binds to hemoglobin and then metabolized. Additionally, it also disperses into the vascular smooth muscle cells adjacent to the endothelium where it binds and activates guanylyl cyclase. This enzyme then catalyzes the dephosphorylation of GTP to cGMP, which acts as a second messenger for multiple cellular functions, particularly for signaling smooth muscle relaxation which often improves blood flow and reduces blood pressure.

Through these second messenger pathways KLK1 via the KKS has shown to regulate multiple physiological functions including blood glucose uptake, glycogen synthesis, blood pressure regulation, insulin sensitization (via activation of protein kinase β (AKT)), anti-oxidative stress (via $PG1_2$/eNOS), and vasodilation (via NO). Thus, it appears that KLK1 is functionally and physically positioned to release kinins (e.g. BK) to specifically enhance blood flow in inflammatory tissue via BK2R upregulation and activation.

Degradation of Kinins

Kinins are produced and destroyed locally and quickly. Residual unbound kinins are inactivated by several peptidases including

kinases I, ACE (kinases II), aminopeptidase P, and kallistatin, with ACE playing the primary role. In this way kinins should be thought of as autacoids, acting locally, rather than hormones, which act systemically. There is heterogeneity among vascular territories in the capacity for kinin production or inactivation, which impacts regulation of local blood flow. For example, kidney (peritubular capillaries, proximal tubules, glomerular capillaries) have low ACE content, contributing to a higher degree of kinin regulation of kidney blood flow (Alhenc-Gelas et al., 1989).

Direct Agonist Properties of KLK1

In addition to the enzymatic action of liberating kinins from low molecular weight kininogen, KLK1 itself is also is capable of directly activating the BK2R. Early evidence showed that KLK1 directly and independently relaxed smooth muscle tissue (rat uterine tissue) in the absence of kininogen and BK (Chao et al., 1981). *In vitro* studies using cells expression recombinant BK2Rs demonstrated that the KLK1 activity was due to direct stimulation of BK2Rs without production of BK (Biyashev et al., 2006; Hecquet et al., 2000). KLK1 activated BK2R more potently than a selection of related proteases such as trypsin or cathepsin C (Biyashev et al., 2006). The effects of KLK1 treatment were blocked by the BK2R selective antagonist, HOE 140, a bradykinin 2 agonist. KLK1 also directly displaced radiolabeled BK from BK2Rs with an IC_{50} of 3 nM and induced the redistribution of the receptor in the plasma membrane (Hecquet et al., 2002). Interestingly, the KLK1 did not cross-desensitize the receptor for subsequent activation by BK suggesting that receptor activation by KLK1 may have different downstream effects from that of BK. *In vivo* studies confirmed and extended these findings by examining the cardioprotective effect of KLK1 in kininogen-deficient rats. KLK1 treatment reduced infarct size, cardiomyocyte apoptosis and intramyocardial inflammation through kinin BK2R activation and NO formation (Chao et al., 2008). The direct mechanism of KLK1 may not operate similarly in all tissues (Charest-Morin et al., 2015), but these results indicate that the physiological role of KLK1 is not confined to the controlled release of BK. The presence of hybrid BK2R-angiotensin receptors (Abadir et al., 2006) add further complexity to a biochemical story that is still being delineated.

KLK1 and Blood Flow Regulation

The KKS plays an important role in local regulation of blood flow in organs rich in KLK1 (Madeddu et al., 2007; Regoli and Gobeil, 2015; Rhaleb et al., 2011). Tissue concentration and localization of KLK1 and other components of the KKS are consistent with its presumed role in multiple physiological processes. KLK1 is located in cardiovascular tissue (including capillary endothelial cells), kidney, pancreas, salivary glands and brain (Golias et al., 2007; Raidoo and Bhoola, 1997). KLK1 protein and mRNA are localized on endothelial and smooth muscle cells of large, medium and small blood vessels. In addition immunohistochemical studies show a distribution of BK2R in arteries and arterioles in smooth muscle cells and endothelial arterioles (Figueroa et al., 2001; Wolf et al., 1999).

Figure 1 – Biochemical and physiological pathways affected by DM199 to improve kidney function

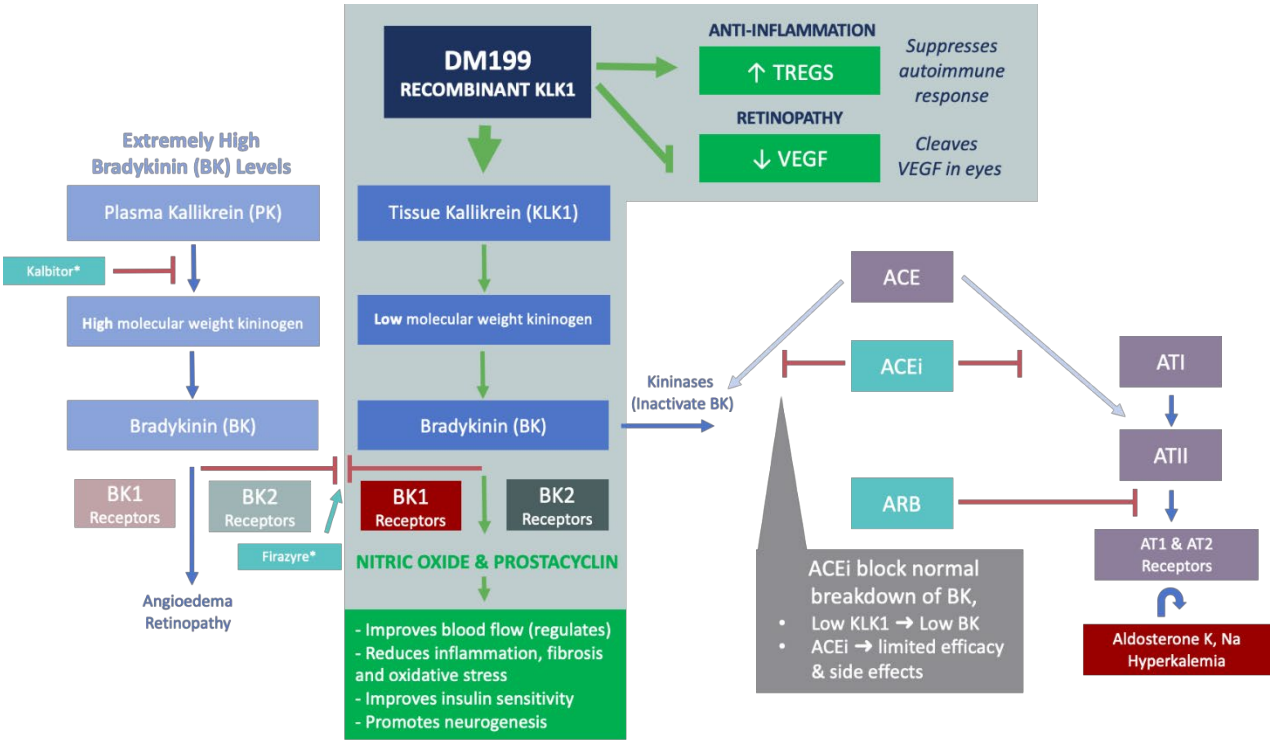

Figure 1. The primary components of the systems that regulate kidney function and control side-effects of kidney disease drug treatments are shown here. Normal, required levels of BK are generated by the action of tissue kallikrein on LMWK. DM199 replenishes deficits in tissue kallikrein. Also shown in the plasma kallikrein, which generates excessive BK almost entirely under pathological conditions such as inflammation. The drugs Kalbitor® and Firazyr® are designed to block this pathway but are also designed to avoid interfering with the tissue kallikrein system. Importantly, DM199 activates the BK pathway thought to be important for the beneficial effects of ACEi drugs. It also directly breaks down VEGF in the retina and eye to help improve diabetic retinopathy.

Kinins and BK2Rs are among the most potent vascular endothelium activators, acting on endothelial cells to trigger the release of numerous endothelial mediators promoting smooth muscle relaxation, inhibiting platelet aggregation and fibrinolysis (Alhenc-Gelas et al., 2011). The KKS is not only the endogenous vasodilatory system in the mammalian system, but is considered the most active and efficient physiological mechanism that initiates and maintains vasodilation (Regoli and Gobeil, 2015). Activation of BK receptors promotes the formation of NO (Emanueli et al., 2004), prostacyclin (PG_2; Ignarro et al., 1987) and endothelium derived hyperpolarizing factor (EDHF; Bergaya et al., 2001), all of which relax vascular smooth muscle and increase blood flow in normal physiological conditions. Finally, kinins release norepinephrine from vascular sympathetic nervous terminals thus reducing the impact of sympathetic nervous system signals that trigger vasoconstriction (Regoli et al., 2012).

KLK1 Preclinical Studies

Several pre-clinical studies demonstrate the role of KLK1 in the development of kidney injury and potential benefit of KLK1 treatment in CKD models. The KKS play a critical role in the protection of the kidney from damage resulting

from diabetes. Diabetic mice lacking the BK2R developed overt albuminuria and a marked increase in glomerular Marginal sclerosis (kidney lesions; (Kakoki et al., 2004).

In another study, diabetic mice that were KLK1 deficient showed increased albuminuria and hypertension compared with diabetic mice that expressed KLK1, suggesting the effects of KLK1 occur on the glomerular or tubular level (Bodin et al., 2009). In rodent exposed to gentamycin to induce kidney failure, treatment with KLK1 for 10 days significantly attenuated kidney failure, cortical damage, and apoptosis in the kidney. Furthermore, when gentamicin treatment was discontinued and KLK1 treatment continued, kidney histology and morphology was restored to that of control animals within two weeks (Bledsoe et al., 2006). Treatment with exogenous porcine KLK1 (isolated from the pancreas) in a Type 2 diabetic mouse model decreased albuminuria and ameliorated pathological changes including thickening of the glomerular basement membrane and loss of endothelial fenestrae (Liu et al., 2016). Additionally, gene therapy with KLK1 in a diabetic rat model showed significant decreases in blood pressure and decreased proteinuria compared to non-treated rats up to 12 weeks post-treatment (Yuan et al., 2007); (Tu et al., 2008).

Preclinical studies have also helped delineate the mechanism of KLK1 therapy in CKD. KLK1 gene therapy in a diabetic rodent model enhanced expression of PI3-Kinase and increased phosphorylation of AKT, likely due to activation of the PI3-kinase/AKT (involved in glucose transportation) pathway in the kidney. Following KLK1 treatment, activation of MAPk and phosphorylation of IL-β, essential for cell survival, was enhanced (Yuan et al., 2007). Exogenous treatment with porcine KLK1 in a diabetic mouse model increased the gene expression of tissue LMWK, KLK1, BK1 and BK2, protein expression of BK2, and kidney BK levels (Liu et al., 2016). Treatment with porcine derived KLK1 in Type I and Type 2 diabetic mouse models significantly decreased the urinary albumin to creatinine ratio, reduced structural damage, fibrosis, inflammation and oxidative stress in the kidneys. KLK1 also reduced the expression of TGF-β1 suggesting that treatment might relieve kidney fibrosis by inhibiting this pathway (Zhu et al., 2016).

Several studies suggest KLK1 treatment could reduce the risk of hyperkalemia through compared to ACEi or ARB treatment through selective modulation of the KKS (El Moghrabi et al., 2010). In rodent studies, KLK1 protects the body against post-feeding hyperkalemia by mediating the rapid modification of kidney tubular potassium transport (El Moghrabi et al., 2010). Additionally, kidney KLK1 secretion increased after acute potassium load, after potassium I.V. infusion, and after potassium-channels are blocked (El Moghrabi et al., 2010).

KLK1 levels and known predictors of CKD: Epidemiologic Evidence

Insufficient endogenous KLK1 correlates with a constellation of vascular diseases, further implicating the KKS as a crucial regulatory system for normal circulation. Diseases considered risk factors for CKD, such as hypertension and diabetes, show lower endogenous KLK1 levels (Ohira et al., 2006). In

diabetics, evidence suggests that lower endogenous levels of KLK1 are associated with disease progression. Excretion of KLK1 from urine was decreased in patients with both mild and severe (diabetic) kidney disease (Chao et al., 2014; Chiang et al., 2008; Naicker et al., 1999). Specifically, decreases in KLK1 excreted in urine were correlated with reduced glomerular filtration rates (Naicker et al., 1999).

In essential hypertension (Margolis et al., 1971) lower levels of active KLK1 have been measured in the urine compared to healthy controls. Furthermore, epidemiological studies demonstrated an inverse correlation between KLK1 urine concentrations and blood pressure in infants, children and parents (Chao et al., 2006; Margolis et al., 1971; Zinner et al., 1978).

Genetic studies have provided further evidence that KLK1 levels are linked to hypertension with deficiencies leading to elevated blood pressures and increased KLK1levels associated with decreased risk for elevated blood pressures. In humans, 10 polymorphic alleles of the *KLK1* gene generate significantly different levels of expression (Song et al., 1997).

In a Chinese Han population with essential hypertension, abnormal polymorphisms (multiple substitutions and unusual length) in the regulatory region of the *KLK1* gene were present (Hua et al., 2005), suggesting a connection between hypertension and polymorphisms in the *KLK1* gene. In African American patients with hypertensive end stage kidney disease, five *KLK1* promoter alleles were identified with unusual overlapping substitutions and length (Yu et al., 2002). The existence of natural genetic variations affecting KLK1 expression levels in patients with vascular disease further emphasize regulatory role of KLK1. Taken together, these data not only implicate the KKS as a crucial regulatory system in diseases related vascular and cardiovascular function but suggest these deficits could be related to the risk of CKD since both hypertension and cardiovascular disease are independently associated with CKD. Treating CKD patients with endogenous KLK1, like DM199, could improve disease pathology.

KLK1 Clinical Studies

DM199 offers a new approach for treating CKD, based on evidence reported for the effect of Kallidinogenase (porcine derived KLK1), currently used to treat CKD in China. Over 20 papers have been published reporting improved outcomes in patients with CKD after treatment with porcine KLK1 alone and combined with ARB treatment in unblinded clinical trials. The following is a summary of selected results from these studies.

Multiple studies investigated porcine KLK1 in combination with an ARB for its ability to stabilize and improve kidney function based on reduction of proteinuria. One study involved 68 participants with early CKD treated with either an ARB alone or an ARB + KLK1. After one month of treatment, participants receiving the combination of ARBs and KLK1 had significantly lower levels of serum cystatin (Cys C) (1.21 ± 0. 32 mg/L in ARB alone versus 1.10 ± 0.36, in the ARB + KLK1 group, mean ± SD) and improved urinary albumin excretion rate (68 ± 36 mg/24

hours in ARB alone versus 42 ± 32 in the ARB + KLK1 group, mean ± SD) (Du et al., 2012).

This was similar to an earlier study by Wu et al where the combination of KlK1 + and ARB resulted in lower urinary albumin to creatinine ratio compared to an ARB alone after 3 months of treatment (138 ± 17 mg/g in the ARB alone group versus 86 ± 16 in the ARB + KLK1 group, mean ± SD) (Wu, 2007).

Another study involved treating 90 patients for 6 months with either, ARB alone, KLK1 + ARB, or placebo. In the KLK1 + ARB group, UAER levels were 21.1 ± 9.8 µg/min compared to 70.2 ± 25.8 in the ARB alone group and 129.9 ± 36.2 in the placebo group (all values mean ± SD). Urine albumin below 30 µg/mg is considered the range of normal kidney function. β2-microglobulin, a marker of reduced glomerular filtration rate, was reduced compared to baseline and to the placebo group and ARB alone in the study (Wang et al., 2011).

KLK1 isolated from pig pancreas has also demonstrated an excellent safety profile. According to Bayer's 2012 Japan Medicine Interview Form, in an evaluation of over 5,000 patients treated with Kallidinogenase, there was 3.1% side effects in the evaluation. The main side effects include 30 cases (0.54%) of gastrointestinal disorder, 6 cases (0.11%) of rashes and 9 cases of facial hot flashes (0.16%) (Bayer, Japan Standard Commodity Classification Number 872491, 2012).

These data again suggest that KLK1 treatment including DiaMedica's recombinant KLK1, DM199, along and/or in combination with ARBs may offer a new potential treatment option for patients with CKD.

DM199 for Chronic Kidney Disease

DiaMedica Therapeutics has developed a recombinant form of KLK1, known as DM199. It has properties nearly identical to those of the porcine and human derived forms that are already available in Japan and China [including enzymatic activity]. DiaMedica Therapeutics has recently been granted FDA clearance for a Phase 1b US trial evaluating DM199 in CKD patients with Diabetes. Previously, in a series of Phase 1 clinical studies, DM199 was administered to healthy volunteers and patients with Type 2 Diabetes. These studies were designed to establish the safety and tolerability of DM199 and to characterize the pharmacokinetics after subcutaneous dosing. DM199 was safe and well tolerated in both healthy and diabetic participants at a range of doses overlapping with the proposed therapeutic doses for treating patients.

Conclusion

In conclusion, the endogenous serine protease, KLK1, plays a vital role in vascular function. Lower levels of KLK1 have been linked to multiple disease states including diabetes, hypertension, albuminuria and CKD. KLK1 potentially offers improvement in kidney function (reduced proteinuria) through reductions in fibrosis, inflammation and oxidative stress. Current treatments for CKD, most prominently ACEi drugs, owe at least some of their efficacy to their ability to enhance KKS and BK signaling. Thus, these drugs may be less effective in patients who have limited

availability of endogenous KLK1. Preclinical and clinical studies using porcine derived KLK1 protein have shown to improve the markers of disease in CKD patients. Treatment initiated in the early stages of CKD may significantly modify or potentially halt the disease trajectory. DM199 provides a novel treatment option either as a single agent or as an adjunct to current therapies. The successes with porcine KLK1 treatment in pre-clinical and initial clinical studies in patients with CKD support ongoing DM199 clinical trials in patients with CKD as a potentially promising medication to protect patients against CKD.

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