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The role of sphingolipids in AKI and the progression to CKD: potential therapuetic targets.

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THE ROLE OF SPHINGOLIPIDS IN AKI AND THE PROGRESSION TO CKD: POTENTIAL THERAPUETIC TARGETS

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DEDICATION

This thesis is dedicated to my family and friends who have always pushed me to work hard and follow my passions.

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ABSTRACT

THE ROLE OF SPHINGOLIPIDS IN AKI AND THE PROGRESSION TO CKD: POTENTIAL THERAPUETIC TARGETS

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Acute Kidney Injury (AKI) is most simply defined as a rapid decline in kidney function over a period of hours to days. There is currently a lack of effective treatment options for patients with AKI, highlighting the need to identify new therapeutic targets. Sphingolipids play a number of roles in different models of AKI, suggesting they could be promising future targets for treating kidney injury. Specifically, sphingosine-1-phosphate (S1P) and its receptors (S1PRs) have been implicated in numerous inflammatory disorders and models of AKI. The purpose of this review is to better characterize the role of S1P receptors in models of AKI and to highlight key limitations in drug development.

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KIDNEY FUNCTION

The kidneys are responsible for many fundamental physiological functions: pH balance, and electrolyte composition, filtration and elimination of metabolic and toxic wastes from the blood, regulation of the internal fluid environment to maintain proper fluid volume and tonicity, essential endocrine functions such as erythropoiesis and blood pressure regulation, and the metabolism and excretion of many drugs [1]. The kidneys are located along the posterior abdomen wall, divided into two sections: the outer cortex and the inner medulla [2]. Within these two sections are associated vasculature, nerves, lymphatic vessels and nephrons. Nephrons are the functional units of the kidney, consisting of the glomerulus, Bowman capsule, and renal tubule [2]. On average, each kidney is comprised of 1 million nephrons, accounting for nearly 25% of cardiac output [2]. Fluids flows through each successive section of the nephron, undergoing a sophisticated process of excretion and reabsorption to maintain fluid homeostasis [2].

The glomerulus is a complex capillary network responsible for ultrafiltration of blood plasma and solute clearance; the glomerular capillaries are positioned between the efferent and afferent arterioles [2]. This configuration allows for tight regulation of glomerular pressure and glomerular filtration rate (GFR). GFR is the product of capillary surface area and filtration pressure, with

the average adult GFR around 130 mL/min[2]. Despite the significance of the kidney, there is a huge gap in treatment for patients who suffer from decreased kidney function. A loss in kidney function, over a period of hours up to several days, commonly referred to as acute kidney injury (AKI), can result in a myriad of clinical manifestations and sequelae [3]. Due to the significance of the kidney, there is a push for more sensitive biomarkers and treatments for AKI.

ACUTE KIDNEY INJURY

Acute kidney injury (AKI) and its severity are defined differently amongst clinics; changes in both urine output and serum creatinine are generally used to diagnose the severity of injury. At its most basic definition, AKI is defined as a significant decrease in glomerular filtration rate (GFR) and an increase in retained waste products over a few days [3]. It has been known that a large portion of hospital inpatients experience AKI, making it one of the most common conditions linked with hospital stays. Since 2002, nearly 25% of patients admitted into the hospital develop AKI; AKI development significantly increases the patients risk of mortality compared to patients without AKI [4]. In 2012, it was reported that more than 60% of patients in intensive care suffer from some sort of AKI, which increases their mortality rate by an astounding 70% [5].

There is a long list of factors leading to the development of AKI. Complications such as hypertension, cardiovascular disease, medications, dehydration, surgery, chemotherapy and chronic kidney disease all increase one's vulnerability to AKI. Additionally, since kidney function naturally declines with age, the incidence of AKI in the older population is significantly higher [6]. Both ageing and maladaptive repair after AKI share common mechanisms that lead to increased risk of progressive chronic kidney disease. These mechanisms include tubular loss, glomerulosclerosis, senescent tubular epithelia and interstitial collagen deposition, suggesting that progressive chronic kidney

disease is comparable to excessive ageing of the kidney [7]. Due to an ever aging population, these factors have led to steady increases in hospitalized patients, particularly among the elderly [8]. Additionally, the financial burden of AKI to the United States healthcare system has been devastating, with over $1 billion going towards in hospital treatment per year [9].

Given the nature of AKI and the broad definition of when AKI starts, its frequency is difficult to monitor in a uniform manner. In 2012 alone there were over 35 different working definitions for AKI [10]. For example, both serum creatinine and blood urea nitrogen (BUN) are two nitrogenous products that can be measured to estimate changes in GFR. Serum creatinine and BUN are normally freely filtered by the kidney. When kidney filtration declines, they accumulate in circulation. Thus, when the concentrations of these products increase, a corresponding decrease in renal clearance is observed. Due to a lack on uniformity in defining AKI, there has been a push in the field to use changes in both serum creatinine and BUN, as well as other biomarkers, to more uniformly define to uniformly define AKI [8].

In an effort to create more uniform criteria for the diagnosis of AKI, the RIFLE (Risk/Injury/Failure/Loss/End-stage renal disease) and AKIN classifications (Acute Kidney Injury Network) were developed and published. The RIFLE scale classifies kidney injury by severity, with categories of risk, injury, and failure. It further classifies kidney injury outcomes as either loss of function or end stage renal disease [10]. By the RIFLE definition, AKI is diagnosed upon a doubling in patient serum creatinine from baseline and a 50% reduction in

glomerular filtration rate (GFR), with urine output dropping below 0.5 ml/kg/hr for 12 hours [10]. Renal function must decline to these levels in under 7 days and remain below these baselines for at least 12 hours. To further unify classification standards, AKIN published their guidelines in 2007 as an additional classification system. The main difference in the two classification is the fact that AKIN standards do not require estimation of baseline serum creatinine (compared to a doubling of serum creatinine from baseline under RIFLE conditions). Additionally, the AKIN classification system only diagnoses AKI after the patient achieves adequate hydration, which is difficult to track. These are important differences as the baseline serum creatinine is not always known and dehydration alone will negatively impact kidney function and can cause AKI. The levels of AKI classification in the AKIN classification system are 1, 2, or 3 based on the increase of serum creatinine within 48 hours from hydration; the risk, injury, and failure levels of RIFLE classification correspond to levels 1, 2 and 3 in the AKIN system. For example, in stage 1 of the AKIN system, AKI is defined as an increase of 0.3 mg/dl serum creatinine and a drop in urine output below 0.5 mg/kg/hr for a period of 6 hours[10]. When a patient enters the clinic, their injury is typically judged based on both RIFLE and AKIN as classifications determined by the Kidney Disease Improving Global Outcomes (KDIGO) group. The diagnosis of AKI is based on either a 0.3 mg/dl increase of serum creatinine within 2 days or a doubling of serum creatinine from estimated baseline occurring within 7 days [10].

Biomarkers of AKI

As mentioned previously, defining AKI is difficult because of the different standards held in clinics around the world. While monitoring serum creatinine is a mildly concrete scale, it has its flaws. One downside to using serum creatinine is that it does not become elevated until 24-72 hours after a renal insult with a 50% loss in nephrons [3]. For example, a 0.3 mg/dl rise in serum creatinine (approximately a 50% increase) can reflect many different phenotypes, depending on the patient. A 50% serum creatinine rise for an ICU patient with sepsis and hypotension would indicate a poor prognosis, aggressive treatment management and structural kidney damage. On the contrary, a 50% rise in serum creatinine for a patient experiencing congestive heart failure while on diuretic therapy would reflect a hemodynamic rise in creatinine, with no structural damage [9]. On the contrary, a 50% rise in serum creatinine for a patient experiencing congestive heart failure while on diuretic therapy would reflect a hemodynamic rise in creatinine, with no structural damage [11]. These are two significantly different clinical diagnoses with only minor differences in serum creatinine. Additionally, subclinical AKI is a condition where kidney injury occurs without a rise in serum creatinine. While this is less common and often a milder form of AKI, it still may result in long-term alterations in kidney function. Therefore, defining AKI solely off serum creatinine is limited because it does not account for AKI etiology, prognosis, molecular pathways or treatment responses [11]. Defining AKI by measuring BUN is another widely accepted way to monitor AKI. As with serum creatinine, BUN also has its limitations in determining GFR; the spike in BUN occurs 24 hours to days after the injury. It is also affected by

many factors besides AKI, such as race, age, body weight, metabolism, sex and protein intake [5]. In order to more effectively treat and diagnose AKI, other more sensitive biomarkers that can be detected immediately following injury are needed and must also be considered in the diagnosis.

Due to the mortality of persistent AKI, it has become more important than ever to find biomarkers of AKI in the clinic with higher specificity and sensitivity. The ideal biomarker would allow early identification of AKI in the clinic, with a variety of treatment options depending on the timeline of the injury. Due to the time dependence of BUN and serum creatinine as it relates to GFR, newer biomarkers are being investigated, including neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury marker 1 (Kim-1).

Kim-1 is a cell surface receptor in epithelial and lymphoid/myeloid cells whose expression has been shown to increase significantly during AKI and other inflammatory responses [12]. Kim-1 has a limiting role in the immune response to injury since it is known that phagocytosis of apoptotic bodies can limit the proinflammatory response [13]. Kim-1 mRNA and protein levels are low in the healthy kidney, but increase significantly in most typed of AKI. During an ischemic/toxic renal injury, the extracellular domain of Kim-1 is separated from the membrane and is excreted into the urine [12]. It has been reported that during AKI, the ectodomain shedding leads to a 100-fold increase in Kim-1 levels [12]. In models of cisplatin toxicity, Kim-1 has been a better predictor of toxicity when compared to NGAL and serum creatinine [14]. Both human and animal studies report a higher specificity from Kim-1 in detecting nephrotoxicity in

response to the chemotherapeutic cisplatin that serum creatinine [15]. Additionally, histological changes in AKI patients are closely related to rises in urinary Kim-1. In a meta-analysis consisting of 2979 patient samples, rises in Kim-1 levels relating to the diagnosis of AKI was scored at 86%, with specificity to AKI scored at 70% [12]. On the contrary, there are studies showing Kim-1 is produced by the liver in response to liver injury, limiting its specificity in diagnosing AKI [16]. The liver-type fatty acid-binding protein, which is another marker of tubular injury, is positively correlated with Kim-1 expression as early as 2-3 days following injury [16]. Despite these supporting data, larger studies for validation are still needed for Kim-1. Kim-1 has been approved by the US FDA as a biomarker for preclinical drug development and may play a significant role in clinically defining AKI in the future [17].

The discovery of NGAL as a biomarker for AKI was based on animal studies where early elevations in NGAL were documented in the urine in multiple models of AKI, including ischemic and nephrotoxic insults (see chapter 3 that discusses different causes of AKI and the corresponding animal models) in the early 2000's[18]. It was first recognized in neutrophils, but it can also function as a rapid response protein for tissue injury [19]. Despite moderate success in animal studies, NGAL as a biomarker in the clinic has mixes results in predicting AKI due to lack of specificity. For example, NGAL is a sensitive marker for kidney tissue damage and AKI, but it has also been shown to elevate in other acute and chronic inflammatory conditions[18]. Despite the lack of specificity, an assay specific for kidney secreted NGAL would greatly increase its use in the clinic.

Types and causes of AKI

There are three main classifications of AKI, namely prerenal, acute postrenal and intrinsic renal AKI, such as toxin-induced tubular injury. Intrinsic AKI represents true kidney disease, while post and pre-renal injuries are consequences of extra-renal diseases that affect GFR [20]. Prerenal injury results from decreased renal prefusion or volume depletion. Intrinsic injury results from direct injury or physical disruption to the kidneys. Postrenal injury occurs when there is insufficient urine drainage distal to the kidneys [6]. All three manifestations of AKI etiologies will be discussed here, with the heavier focus being on ischemic and nephrotoxic injury.

Pre-renal AKI is defined by conditions of normal tubular and glomerular function. In pre-renal AKI, hypoperfusion (inadequate blood supply to the kidney) results in a decrease in GFR as an adaptive technique to extra-renal insults [20]. The four main abnormalities leading to pre-renal AKI are: hypovolemia, impaired cardiac function, systemic vasodilatation and increased vascular resistance. These abnormalities can arise from a number of causes such as hemorrhages, congestive heart failure, cirrhosis, anesthesia, anaphylaxis, renal fluid loss, severe dehydration, liver disease, cardiac surgery, kidney transplants or myocardial infarction [20]. The body's normal responses to pre-renal AKI is to reabsorb sodium in an effort to increase intravascular volume and renal perfusion [4]. Therefore, the focus of pre-renal AKI treatment is aimed at restoring renal perfusion via pharmaceuticals. The majority of the time pre-renal AKI treatment focusses on correcting the cause of injury, with drugs such as angiotensin-

converting enzyme inhibitors, angiotensin receptor blockers and non-steroidal anti-inflammatory agents decreasing the glomerular filtration rate by changing the balance of vasodilatory agents in circulation [8]. Kidney function usually restores itself within a couple days, once the underlying cause has been taken care of and normal blood flow has been restored [20].

Post-renal causes of AKI are described as disrupting urinary flow, increasing intratubular pressure and thereby decreasing GFR [4]. Acute urinary tract obstructions can impair renal blood flow and trigger an inflammatory response that can decrease GFR [4]. Normally the obstruction involves both kidneys or one solitary kidney to induce significant renal failure, but there are cases where people with preexisting renal deficiencies develop AKI with the obstruction of one kidney [4]. Some more common types of urinary obstruction leading to post-renal AKI are prostatic hyperplasia/prostate cancer in males, gynecologic cancers (i.e. cervical cancer) in women, ureteral stones, papillary necrosis and neurogenic bladder to name a few [11].

Intrinsic AKI is the most common form of AKI acquired in hospitalized patients, accounting for up to 70% of cases [21]. Intrinsic forms of AKI are more difficult to classify because there are a variety of injury types that can occur based on the structural target. For simplicity, acute glomerular nephritis, acute interstitial nephritis, and acute tubular necrosis are the 3 major classifications of intrinsic AKI. These classifications are named based on insult to glomeruli, the tubules, the interstitium or the intrarenal blood vessels [4]. AKI resulting from damage to the tubules is referred to as acute tubular necrosis (ATN). Eighty to

ninety percent of the cases of acute tubular necrosis are a result of ischemic or nephrotoxic injury[22]. Both ischemic and nephrotoxic injuries involve several pathophysiological processes, including endothelial damage and vascular impairment, immune response, and tubular cell death [21]. These pathophysiological processes orchestrate AKI development in phases of initiation, extension, maintenance, and recovery [21].

During the initiation phase, the ischemic or nephrotoxic injury triggers functional damage to tubule epithelial cells. As a result, this decreases renal blood flow, causing a decrease in available cellular ATP [21]. Following ATP shortages, the inflammatory response is triggered, alongside morphological changes in epithelial cells [21]. The extension phase consists of the subsequent hypoxia and inflammatory response. Inflammatory and profibrotic cells begin infiltrating and proliferating, leading to tubule cell death and a continued decline in GFR [21]. In the maintenance phase, the decline in GFR halts as tubule cells begin dedifferentiation, migration, and proliferation [21]. Finally, renal function returns in the recovery phase. During this stage, renal epithelial cells re-establish polarity and return to normal function, while inflammatory and profibrotic cells are cleared [21]. The extent to which kidney function recovers depends heavily on which section of the nephron is injured and the extent of the injury.

The first category of ATN is ischemia reperfusion injury (IRI). IRI can induce both primary and secondary incidents, depending on the segment of the nephron most directly injured. For the most part, IRI studies focus on the last segment of the proximal tubule and the medullary thick ascending limb[22]. Both

of these segments are susceptible to ischemic events due to their location in the medulla, which is normally hypoxic [22]. The second classification of ATN is from nephrotoxic causes such as exogenous compounds like chemotherapeutics and environmental toxicants [4]. These toxins injure specific sections of the nephron depending on the mechanism and have helped additional kidney biomarkers be recognized and developed. For example, myoglobin, a toxic protein released into circulation during rhabdomyolysis, is filtered through the glomerulus and reabsorbed in the proximal tubule, where it induces death to the epithelia [4]. Historically, ATN goes through an oliguric (urine output ≤ 400 mL/24 hours) and a nonoliguric (urine output > 400 mL/day) phase for 1-2 weeks each before the recovery of renal function[4]. AKI from glomerular damage also significantly affects GFR and occurs often in cases of acute glomerulonephritis [1]. This can be a result of a number of primary renal diseases, such as idiopathic rapidly progressive glomerulonephritis, or it can result from systemic diseases such as lupus erythematosus or Wegener's granulomatosis[4]. AKI from interstitial damage often arises after an allergic reaction to a variety of medications and infections. For example, antibiotics such as sulfonamides can induce crystalluria and acute interstitial nephritis [4]. Finally, AKI from vascular tissues can occur when intrarenal vessel become damaged, leading to decreased renal perfusion and diminished GFR[4].

Chronic Kidney Disease

For many years it was generally accepted that patients who survived AKI would recover to full kidney functionality. However, recent epidemiological

studies suggest that survivor of AKI exhibit a consistently higher risk of developing Chronic Kidney Disease (CKD) and cardiovascular mortality [7]. Maladaptive repair of following AKI is a pressing issue, resulting in progressive fibrosis and tubular loss [23]. CKD is defined as a steady loss of kidney function and accumulating kidney damage, measured by albumin in urine. CKD is typically associated with interstitial fibrosis, glomerulosclerosis, and chronic inflammation[7].

Normally, CKD is diagnosed if patients progress to the failure stage of the RIFLE classification for over 3 months [10]. At this point, patients have over 75% reduction if GFR and have to be put on dialysis due to a lack of treatment options for CKD. Dialysis can slow the progression of kidney disease temporarily; these patients will eventually lose kidney function (complete loss of function for more than 4 weeks) and will develop end stage renal disease (complete loss of function for more than 3 months) [7]. Once patients reach end stage, the only survival option is a renal transplant [24].

CKD prevalence has been gradually increasing over the past 25 years, effecting older populations at alarming rates[25]. Over 60% of people aged 80 years or older have diagnosed CKD, defined by nature as a glomerular filtration rate <60 ml/min [6]. As of 2017, CKD was estimated to affect 13.4% of people globally; the CDC has recently reported over 15% of US adults have CKD, with 37 million citizens going undiagnosed. “In 2018, treating Medicare beneficiaries with CKD cost over $81.8 billion, and treating people with ESRD cost an additional $36.6 billion” [26]. That ends up being nearly $23,000 per person in

Medicare spending. Generally, CKD development is associated with old age, hypertension, diabetes, cardiovascular disease, and obesity, all of which are highly prevalent in the US population [6].

MODELS OF AKI

Due to the unmet medical need for more effective therapies for patients with AKI, it is important to understand the numerous different preclinical AKI models currently being used. The purpose of this chapter is to examine some of the different AKI animal models are being used in order to determine which models are the most appropriate in modeling human AKI. As mentioned previously, the three main classifications of AKI prerenal, acute postrenal and intrinsic renal AKI. Currently, the most common way to study AKI and renal transplantation is with ischemia reperfusion (IR) models. Renal ischemia reperfusion at its most basic definition is a temporary impairment and eventual restoration of blood and oxygen flow to the kidney, which results in a cascade of cell death, injury, inflammation and fibrosis [27]. A large variety of IR models are used to study prerenal AKI, which occurs in many clinical situations such as vessel occlusion during surgeries, postoperative hypofusion, bleeding, dehydration, shock and sepsis [27]. While there are a variety of IR models, the three most prevalent are bilateral renal clamping, unilateral renal clamping and unilateral renal clamping with a contralateral nephrectomy [28]. These IR models can be performed in a variety of ways, with variables such as ischemia time, temperature control, clamp time and animal choice having a large effect on the outcomes.

Bilateral IR induces kidney injury by blocking the blood flow to both renal arteries, which is more relevant to human studies because injured patients often have impaired blow flow to both kidneys [27]. Additionally, only bilateral clamping influences overall renal mass and leads to SCr and BUN level elevations in under 24 hours, which translates to AKI in the clinical setting [28]. One major fallback with bilateral clamping is the consistency in controlling the extent of renal injury. For example, one study found that 22-25 minutes of clamping in C57BL/6 mice will successfully induce mild to moderate injury with a full function recovery in less than a week. However, 25-30 minutes of ischemia was shown to induce severe injury, killing a large proportion of the C57BL/6 mice in under 48 hours [29]. This suggests that ischemia times must be long enough to induce tubular necrosis and meet AKI criteria, but mice mortality severely limits using bilateral IR when examining long term outcomes [30]. While long term studies with bilateral IR injury have been done, most studies indicate normal kidney morphology within 2 weeks of the injury [31]. As a result, unilateral IR models are more commonly used to study AKI beyond the initial injury phase.

Unilateral IR injury is induced by blocking the flow to one kidney, while the contralateral kidney remains functional and intact. Generally speaking, this model is used more for long term animal studies to study the mechanism of the AKI to CKD transition. For example, Zager et al. implemented 30 minutes of unilateral ischemia and made assessments up to 3 weeks later. They found prevalent necrosis in the proximal tubules at day 1 following injury and the this phenotype persisted to increased inflammation, fibrosis and histone modifications in the

week 3 assessment [32]. Similarly, Lech et al. noted significant pathological changes just days following the injury, with decreased kidney weight and corresponding fibrosis, tubular atrophy and inflammation up to 10 weeks post injury [33]. This suggests unilateral IR models have significant potential for long term studies, with a decreased risk of animal mortality due to the presence of a fully functional contralateral kidney. Additionally, longer clamping times can be utilized in this model to induce a more consistent and extensive AKI when compared to the bilateral model. The major pitfall of unilateral IR injury is the difficult in monitoring functional renal decline due to the presence of a fully functional kidney compensating after injury [27]. The third model is unilateral clamping and removal of the contralateral kidney in order to increase blood reflow to the injured kidney and avoid the compensation issue mentioned above. Compared to leaving the contralateral kidney intact, this model has been shown to have lower chances of variations along with proven ischemia for long term chronic studies of injury [28]. As seen with the unilateral model, the difficulty in achieving sufficient AKI without mortality limits this model. Skrypnyk et al. evaluated both mild and moderate induced IR induced AKI and found when the contralateral kidney was removed, only 50-60% of mice developed sufficient AKI after 24 hours [34]. Other research groups indicate this model had a 30% mortality rate in their mice population with large variations when compared to leaving the contralateral kidney intact [27]. As discussed above, there are endless models of IR induced renal injury, with varying success depending on the purpose of each experiment. The most important factors to consider when

choosing the appropriate model are the drawbacks associated with each model.

Urinary tract obstruction is common cause of renal injury that needs immediate attention to avoid interstitial fibrosis and irreversible renal injury. The unilateral ureteral obstruction (UUO) model is important as it helps monitor the transition from AKI to CKD, focusing on tubular cell injury, inflammation and fibrosis [35]. UUO is treated by relieving the obstruction, allowing urine to flow again. During UUO, the intratubular hydrostatic pressure builds up, destroying nephrons and initiating secondary ischemia downstream of the obstruction [35]. There are quite a few variations between UUO models, mainly focusing on the duration of the obstruction. Due to advances in surgical techniques, researchers are able to manipulate models by altering the timing, duration and extent of injury [36]. Acute obstruction results in AKI, however obstruction for 1-2 weeks is more common because histological features of CKD become present, allowing researchers to study fibrosis more quickly than previous models [35]. In general, male animals are most often used in UUO models because female sex organs make the procedure more difficult. There are two common variations of unilateral ureteral obstruction, complete UUO and partial UUO. In complete UUO, the ureter is ligated and completely cut, which results in interstitial inflammation, tubular dilation and fibrosis in under 7 days [37]. Complete UUO is beneficial because of the repeatability and fast progression of fibrosis, however a complete obstruction is not usually a cause of human renal injury. On the other hand, partial UUO is performed by inserting a ureter to the surrounding psoas muscle [36]. While this more closely mimics clinical obstructive nephropathy and allows

for variable degrees of obstruction, this technique is severely limited by its reproducibility. Despite some disadvantages of UUO as a model of injury, it is increasingly popular for identifying molecular mechanisms of inflammation, apoptosis and fibrosis in the progression of AKI to CKD [38].

Drug-induced nephrotoxicity is one of the largest obstacles to overcome in the clinical setting because of the kidneys specialized role in filtering substances from the blood. This makes the kidneys particularly vulnerable to damage of the tubules, interstitium, glomerulus and puts the patient at risk of chronic kidney dysfunction [39]. The kidneys are exposed to many nephrotoxicants, ranging from chemotherapeutics, pharmaceuticals, antimicrobials, drugs of abuse, environmental toxicants and natural substances, which induce injury through a variety of mechanisms [39]. The unfortunate limitation of nearly all pharmaceuticals is nephrotoxicity, emphasizing the need to develop efficient models monitoring AKI and its progression to CKD. The focus of this section is to look into some of the drug induced models of nephrotoxicity, focusing primarily on cisplatin models as an example.

Cisplatin is a commonly used chemotherapeutic for the treatment of a variety of cancers, such as lung cancer and many solid organ cancers [40]. Along with many drugs, the dose-limiting factor of cisplatin is off target nephrotoxicity when it gets transported to renal epithelial cells [40]. In order to mimic clinical scenarios of drug-induced nephrotoxicity, one recent area of focus has been on the dosing regimen in animal models of cisplatin injury. Until recently, the most common rodent model of cisplatin-induced AKI was a single high dose (20

mg/kg) of cisplatin. This results in a sharp decline in renal function in 3-4 days before the rodents must be euthanized [41]. In this model, the single high dose of cisplatin induces significant cell death, triggering cell cycle arrest, acute ER stress and apoptosis [41]. While this might mimic clinically developed AKI in severe situations, the single high dose model doesn't provide information for long term renal damage. Instead, some newer models focus on extending out the treatment window by treating mice with low doses over longer period of time. For example, the Siskind laboratory uses a repeated low-dose cisplatin (RLDC) regimen, treating mice with 7-9 mg/kg of cisplatin once weekly for 3-4 weeks [42]. This allows the mice to be aged out up to 6 months from treatment, giving better insights to progressive renal fibrosis and chronic kidney disease. When compared to the high dose model, the RLDC model is hypothesized to contribute to lower, chronic levels of ER stress and upregulated autophagy over time [41]. While this mechanism isn't completely understood, it is clear that different dosing regimens initiate different pathways of injury. Taken together, these two models provide valuable insights into the processes triggered following long term and acute treatments of cisplatin.

In addition to models varying by the dosing regimen, rodent selection is another important variable to consider in drug induced models of nephrotoxicity. For example, sex differences have been noted in both humans and mice, where females are more sensitive to cisplatin induced AKI [43]. Interestingly, female mice have been shown to be more protected from IR induced AKI, likely due to hormonal fluctuations [44]. Another important factor in nephrotoxic models is the

difference in pharmacokinetics between rodents and humans. It has been shown that the peak plasma concentration of cisplatin in mice is up to 20 times higher than in humans. Along with an increased plasma concentration, cisplatin has a much shorter half-life in mice [45]. Together, this demonstrates a quicker distribution of cisplatin in mice tissues, suggesting mice are at a higher risk of cisplatin nephrotoxicity. Finally, models of injury resulting from chemotherapeutics also have to consider the incorporation of cancer into the models of injury. Some groups have used xenograft models, subcutaneously inoculating tumors into mice before treating them with chemotherapeutics. Pabla et al. first utilized this model investigating ovarian cancer, however this model is limited because tumors are heavily affected by the microenvironment they originate in [46]. Many newer models focus on using genetically engineered mice with human cancer driver mutations in order to integrate aging and the tumor microenvironment when studying the effect of chemotherapeutics. In conclusion, there are numerous models of AKI, all of which should be tailored to mimic specific clinical situations. The focus of the next chapter is to introduce another factor, sphingolipid metabolism, that has been implicated many of the models of AKI discussed above.

SPHINGOLIPID METABOLISM

Sphingolipids are complex, bioactive lipids containing a common sphingoid base as a backbone (Figure 1) [47]. The metabolism and following sphingolipid signaling is a complex process. This chapter will highlight sphingolipid metabolism in the context of kidney function, but a more detailed overview can we found in Gault et al. [48]. For many years the kidneys have been shown to play an extensive role in sphingolipid metabolism. For example, many glycosphingolipids are uniquely expressed in renal tissues [49]. Originally it was thought that the primary role of sphingolipids was to provide structure to various cell organelles. However, more recent studies highlight the complex signaling pathways of many different bioactive sphingolipids on a variety of cellular targets [50]. Studies focusing on how these bioactive lipids effect renal physiology are of interest right now because the regulation of sphingolipid metabolism could be used as a therapeutic target in kidney injury[51].

Sphingolipid metabolism is complex because there are numerous enzyme-catalyzed reactions leading to different signaling molecules in different areas of the body and in a given cell. Ceramides are at the center of sphingolipid metabolism, which can be created from a variety of pathways including *de novo* synthesis, sphingomyelin hydrolysis, or recycling different sphingolipids [3]. In *de novo* synthesis, serine palmitoyl transferase condenses serine and palmitoyl CoA to form 3-ketosphingosine, which is the rate limiting step in *de*

novo ceramide synthesis [3]. 3-ketosphingosine then is reduced to dihydrosphingosine by 3-ketoreductase. A fatty acid is added to dihydrosphingosine is then acylated by dihydroceramide synthase, also known as ceramide synthase (CERs), to form dihydroceramide. There are six ceramide synthase isoforms that have preferences for different length fatty acids, leading to dihydroceramides (and the downstream metabolites) of different chain lengths. Dihydroceramide desaturase then desaturates dihydroceramide at the 4/5 position to form ceramide (Figure 2) [49]. Once synthesized, ceramide can be used in a variety of catabolic or anabolic reactions.

There are a number of pathways leading to more complex bioactive sphingolipids from ceramide. Ceramide can be used by sphingomyelin synthases to form sphingomyelin by attaching a phosphocholine head group to the C1 hydroxyl group of ceramide [52]. Glycosylation of ceramide, also at the C1 hydroxyl group, by adding a glucose or galactose will form the hexosylceramides, glucosylceramides, and galactosylceramides[3]. Hexosylceramides are used to synthesize even more complex gylcosphingolipids Sphingomyelins and glycosphingolipids can also be broken down to regenerate ceramide. Additionally, phosphorylation of ceramide at the C1 hydroxyl group as catalyzed by ceramide kinase forms ceramide-1-phosphate[3]. Finally, ceramide can be cleaved to form sphingosine by the action of ceramidases (acid, alkaline and neutral ceramidases). The sphingosine can then be phosphorylated by sphingosine kinases 1 or 2 to form sphingosine-1-phosphate (S1P) [53]. S1P can either be dephosphorylated by S1P phosphatases or be irreversible cleaved by

S1P lyase to form ethanolamine-1-phosphate and hexadecenal. As with all of these pathways mentioned, sphingosine can be recycled back to form ceramide by ceramide synthase enzymes or the reverse activity of ceramidases [3]. It is thought that balancing key bioactive sphingolipids can regulate several cellular processes including inflammation, the regulation of reactive oxygen species (ROS) production, and cell death (apoptosis, necrosis, etc.) [49]. These are all processes implicated development of acute kidney injury and chronic kidney disease.

S1P RECEPTORS AS POTENTIAL TARGETS

As discussed in the previous chapters, there is a clear role for sphingolipids and the progression of kidney injury. Due to the complexity of sphingolipid metabolism, there are numerous potential therapeutic targets for prevention and treatment of kidney injury. The purpose of this chapter is to focus on some of the most promising potential therapeutic targets for the prevention and treatment of renal injury, specifically the sphingosine-1-phosphate receptors (S1PRs). S1P is a natural lysophospholipid known to regulate cell migration, stress, cell growth, cytoskeletal rearrangement, apoptosis and calcium homeostasis [54]. It is present in high nanomolar concentrations in serum, where it associated with albumin and lipoproteins, and low nanomolar concentrations in cells. S1P is generated from the hydrolysis of ceramide by ceramidase enzymes, to yield sphingosine and a fatty acid. The sphingosine can be phosphorylated to produce sphingosine-1-phosphate (S1P) as catalyzed by either of two sphingosine kinase enzymes, referred to as sphingosine kinase 1 (SK1) and sphingosine kinase 2 (SK2) [55]. Once generated, S1P can interact with a number of intracellular targets [56, 57] or be secreted outside of the cell where it acts a ligand for any of the five different plasma membrane localized S1P g-coupled protein receptors (GCPRs) identified (S1PR1-5). The sphingosine

kinase-S1P-S1PR axis is drawing increasing attention as a moderator of fibrogenesis, specifically cardiac and renal fibrosis [58]. (Figure 3)

S1P has been shown to protect from the kidney from injury through a variety of pro-survival mechanisms by modulating the immune response. The activation of S1P receptors is known to induce the relocation of B and T lymphocytes away from the site of injury, potentially limiting the fibrotic response [3]. FTY720 (fingolimod), a pan agonist of S1P receptors, was approved by the FDA for the treatment of multiple sclerosis in 2010 to reduce lymphocyte egress[59]. It drew significant attention in fibrotic models of injury and has been shown to attenuate AKI in both nephrotoxic and IR models of injury. Suleimen et al. treated C57BL/6 mice with FTY720 before inducing IR injury and monitored them for 3, 5, and 7 days. They found the FTY720 treated group had an earlier recovery of renal function and hypothesized this was due to a decrease in kidney infiltrating leukocytes [60]. Perry et al. also showed that FTY720 attenuated AKI, specifically protecting proximal tubule cells, in their single high dose model of cisplatin induced AKI[61]. To further understand the effect of S1P agonists in a lymphocyte-independent mechanism, Bajwa et al. investigated IR injury in mice lacking both T and B lymphocytes. They found that mice lacking T and B lymphocytes (Rag-1 knockout mice) were only partially protected from IR injury, while treatment of FTY720 to the deficient mice resulted in significant additional protection (as presented in Figure 1 of Bajwa et al) [62]. These results suggest that S1P receptor activation is renoprotective in a mechanism independent of reduction of lymphocyte egress, namely via their activation in non-T and B-cells,

perhaps in the tubule cells [62]. Unfortunately, a number of side effects associated with FTY720 have limited its use in the further studies. The most common side effect after FTY720 administration is bradycardia, likely due to its unspecific action across all five S1P receptor types [63]. Additionally, in phase 3 clinical trials, FTY720 triggered the formation of new blood vessels in the eye and led to macular degeneration and significant vision loss [63]. While the mechanism leading to these adverse effects isn't completely understood, it further highlights the need for the development of selective S1PR agonists. The majority of pharmacological modulators currently developed lack specificity amongst the receptors, as demonstrated in Figure 4.

Additional studies to determine the specific S1PR subtype(s) involved in kidney injury and the progression to chronic kidney disease were not well understood. However, following the initial success from FTY720 in renal IR injury models, researchers focused efforts on identifying which S1PR isoform plays the major role in AKI. To further identify the specific S1PR isoform involved in kidney injury, Bajwa et al. performed kidney IP studies utilizing the first successful highly selective S1PR1 agonist, SEW287, and a S1PR1 antagonist, VPC44116. Bajwa et al., administered SEW287 or VPC44116 to the T- and B-cell deficient Rag-1 KO mice to specifically examine the impact of S1PR activation or inhibition in the absence of these lymphocytes [62]. Compared to the vehicle group with elevated serum creatine levels following IR, SEW287 significantly reduced IR injury as evidenced by the reduction in serum creatinine. However, when SEW287 and VPC44116 were treated in combination, the protection conferred by SEW287

alone was completely lost. The serum creatinine data were supported by morphologic analysis of kidney sections via H&E staining (as presented in Figure 2 in Bajwa et al.), suggesting S1PR1 activation is required for reducing tubule injury [62]. Importantly, the authors attenuated S1PR1 expression *in vivo* and *in vitro* and found that deficiency in S1PR1 enhanced injury, regardless of the presence of S1PR1 agonists[62]. In addition to IR injury models of AKI, S1PR1 has also been implicated as renoprotective in cisplatin-induced AKI. In a 2015 study, Bajwa et al. hypothesized that the protective role of FTY720 in their cisplatin-induced nephrotoxic model was due to S1PR1 activation [64]. They developed S1PR1 knock out mice and found that cisplatin induced significantly more damage in this group when compared to the control group [64]. Taken together, there is clear evidence that S1PR1 activation is renoprotective and selective S1PR1 agonists provide potential therapeutic targets in kidney injury.

When compared to S1PR1, the other S1P receptors have received less attention and there is a clear gap in knowledge. Drexler, Y., et al. identified the major roles of the five S1P receptors (Figure 5) [65], however connecting these receptors to their role in renal injury has not been fully established. Some data have been reported for S1PR2 and S1PR3, but data are lacking for S1PR4 and S1PR5. A role for S1PR2 in renal IR injury was revealed by Park et al. by treating mice with a selective S1PR2 antagonist. They found that the S1PR2 antagonist exacerbated IR injury and this was further supported by data demonstrating that knocking out S1PR2 in mice led to reduced IR injury [66]. This suggests that S1P signaling through S1PR2 negatively impacts IR injury and that S1PR2 specific

antagonists would have therapeutic potential; this is in contrast to S1PR1 which plays a protective role and therefore development of S1PR1 specific agonists for therapeutic use would be beneficial. An antagonist of S1PR3, suramin, has been shown to attenuate renal fibrosis in a UUO model of CKD by interacting with TGF-B signaling [67]. Suramin has also been shown to protect from cisplatin-induced AKI [68]. However, suramin is known to have numerous targets and its protection may be solely via S1PR3. S1PR3 has been associated to blocking the activation of killer T-cells by dendritic cells, suggesting the loss of S1PR3 reduces the inflammatory response[69]. The mechanisms of action for examples are not clearly understood, limiting their application to current drug development.

The S1P/S1PR signaling axis is not only a promising target in renal injury, as it has also been targeted to treat autoimmune disorders, COVID-19, inflammation, cancer and cardiovascular disease [70] [71]. Consequently, the drug discovery process has focused on studying the five specific S1P receptors in detail in order to develop more specific drug agonists. Until this year, only the crystal structure of an inactive S1PR1 bound to an agonist had been determined. However, Yuan et al. recently reported cryo-electron microcopy (EM) structure for both S1PR1 and S1PR5 with ligands bound, providing the ground work for identifying ligand specificity to activate these receptors [70]. The authors relied heavily on Siponimod, an orally available S1P agonist structurally similar to FYT720, to identify the binding pocket of S1PR1/5 [72]. The authors identified a conserved ligand binding pocket, composed of a hydrophobic cavity and a polar module, forming a ligand binding pocket similar to LPA receptors [70].

Additionally, a sub-pocket of the orthosteric site was identified, interacting with different side groups of established S1P agonists, such as the trimethyl domain of SEW2871 [70]. Multiple differences between the two receptors were identified, most notably in the intracellular loops (ICLs). The ICL2 region showed structural differences resulting in orientation changes around the α5 helix, which is hypothesized to play a major role in the G-protein selectivity of the receptors [70]. Taken together, this is a huge step in the right direction when it comes to identifying differences between S1P receptors, opening the door for future drug development and discovery. However, there is still a deficiency in knowledge about many specific receptor properties, which will be discussed in more detail below.

In order for any of the S1P receptors to be serious potential therapeutic targets moving forward, a lot of information still needs to be uncovered. As mentioned above, there are currently only working crystal structures for two of the receptors bound to a pharmacological agent, S1PR1 and S1PR5 [70]. This lack of structural information on actively bound S1P receptors is a huge limiting factor when it comes to identifying receptor specific agonists. For example, while S1PR1 shares many common characteristics with many identified GCPRs, some distinguishing features were first identified by Hanson et al. in 2012 when examining the agonist bound receptor [73]. They found that the N-terminus folds over the top of the bound receptor and can contribute to the binding affinity, while also forming a helical cap that limits the access to the binding pocket [73]. This is significant because it helped explain why the S1P ligands are slow to saturate

the receptor when excess ligand is present [73]. Additionally, the authors established that the volume of the hydrophobic portion of the ligand plays a vital role in downstream signaling by altering interactions with conserved polar residues [73].

All of this structural information helped provide the framework for the numerous S1PR1 agents currently being researched, but there is still a long way to go. Once there are better established crystal structures of the receptors, the specific sequence homologies of the receptors will need to be investigated further. This will be important in determining the potential of targeting individual receptors, ensuring they are different enough to selectively target. As reported by Yuan et al, both S1PR1 and S1PR5 share a highly conserved binding pocket when bound to the same agonist, highlighting the need for additional active conformations to be analyzed [70]. The main pitfall of the major commercially available drugs, such as FTY720 (fingolimod) in treating MS, is the lack of specificity, thought to contribute to the numerous adverse off target effects and failed clinical trials [59]. Assuming there will be significant enough sequence differences to target the individual receptors, either alone or in combination, there is no doubt there will be clinical applications in the near future in kidney injury research.

In addition to a sturdier structural analysis of the receptors, additional studies are needed to determine the distribution of the receptors in specific cell types. S1PR3, for example, has been shown to be highly expressed in human breast cancer cells and immune cells, but its distribution in relation to kidney

injury has not been identified [74]. In the progression of AKI to CKD, it has been well established that the S1P axis regulates the immune response by recruiting macrophages, monocytes, lymphocytes and dendritic cells in the priming stage of fibrogenesis [58]. Consequently, it is important to know how this alters the distribution of S1P receptors in the kidney in order to target them in the clinic. This will open up the potential to develop cell type specific inhibitors or modulators for S1P receptors. Despite a need to further classify differences between S1P receptors at the structural and cellular level, the S1P axis clearly has clinical implications in the treatment of kidney injury moving forward.

FIGURES

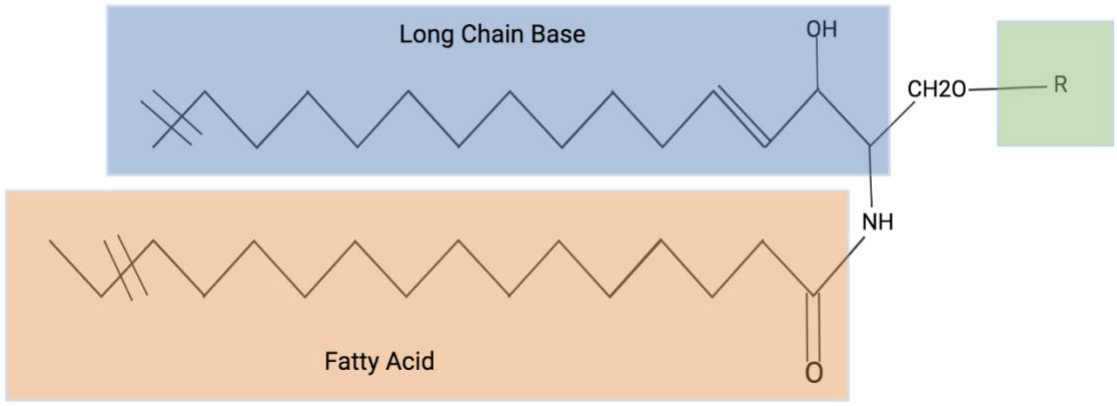

Figure 1. Sphingolipid Base Structure

The backbone of s sphingolipid contains a long chain base (blue) linked to a fatty acid (orange) via amide bond. A polar head group, R (green), is linked to the OH on C1 depending on the sphingolipid. \\ denotes the number of carbons. Figure was made using biorender.com

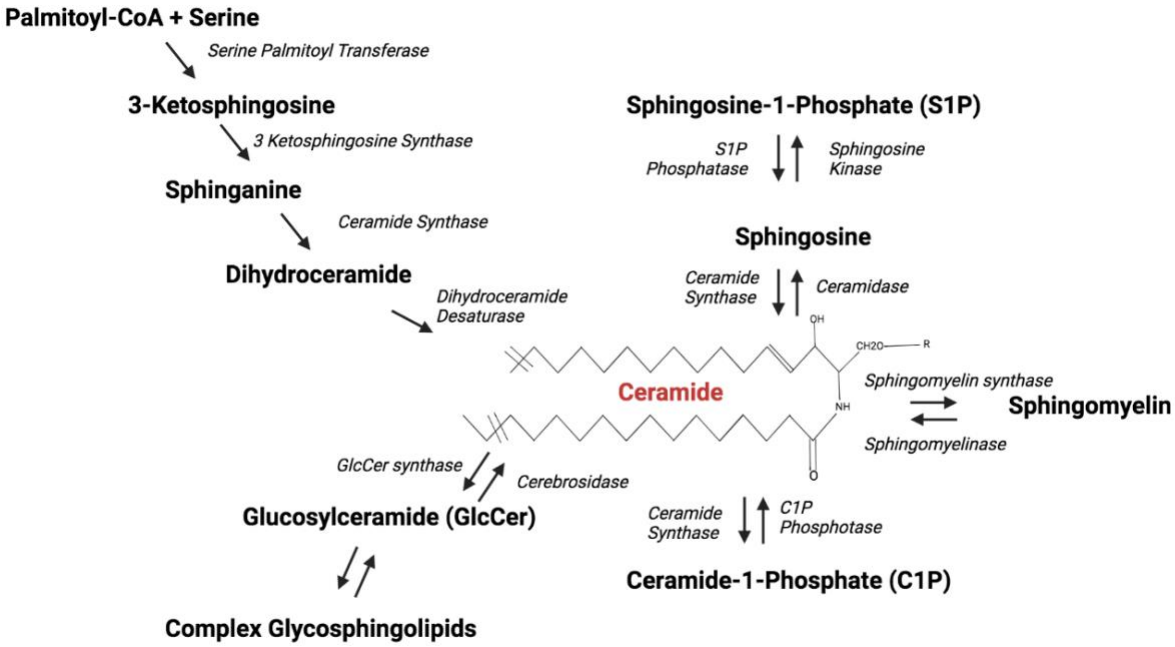

Figure 2. Sphingolipid Metabolism

Ceramide (red) is at the center of sphingolipid metabolism. The metabolizing enzymes are in *italics* and downstream sphingolipids are in **bold**. Figure was made using biorender.com

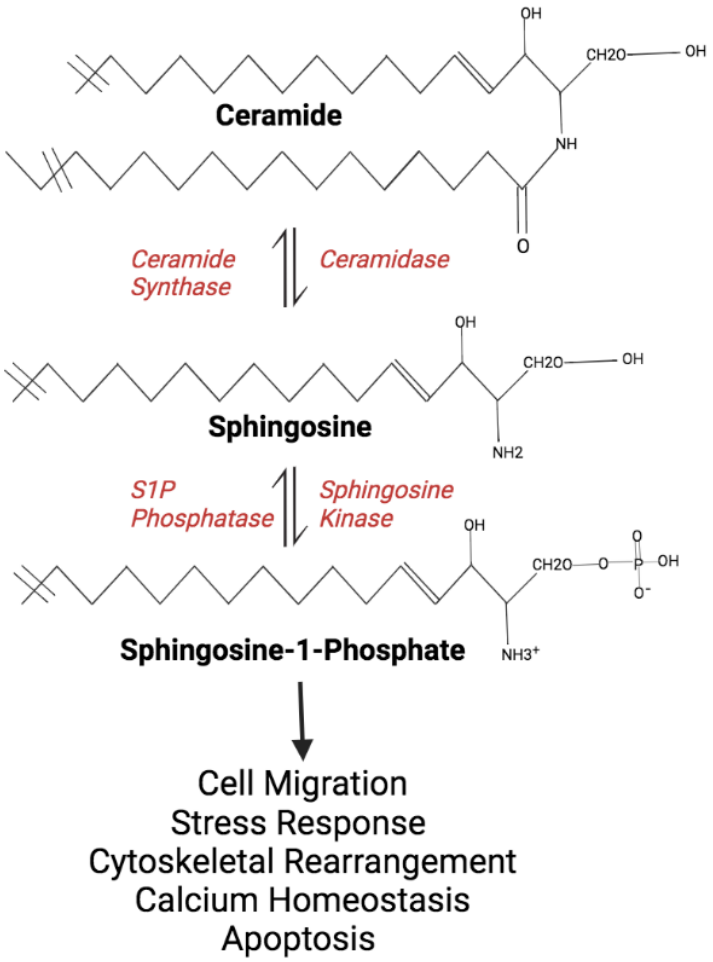

Figure 3. Sphingosine-1-Phosphate Signaling

Ceramide is broken down into sphingosine via ceramidase enzymes. Sphingosine can be phosphorylated by sphingosine kinase enzymes to from sphingosine-1-phoshate (S1P). S1P initiates cell migration, stress response, cytoskeletal rearrangement, calcium homeostasis and apoptosis [57]. Figure was made using biorender.com

S1P Receptor Modulators and their Selectivity	
Pharmacological Agent	**Selectivity**
Fingolimod (FYT720)	S1PR1,3,4,5
Siponimod (BAF312)	S1PR1,5
Ozanimod (RPC1063)	S1PR1,5
Amiselimod (MT-1303)	S1PR1,4,5
Etrasimod (APD334)	S1PR1,4,5
Ceralifimod (ONO-4641)	S1PR1,5
Ponesimod (ACT-128800)	S1PR1
VPC44116	S1PR1,3
SEW2871	S1PR1
Cenerimod	S1PR1
GSK2018682	S1PR 1,5
VPC23153	S1PR4

Figure 4. Sphingosine-1-Phosphate Receptor Modulators

The selectivity of some S1P modulators currently being evaluated to treat kidney disease, chronic inflammatory diseases, diabetes and organ failure. Adapted from McGowan, E.M., et al. [71]

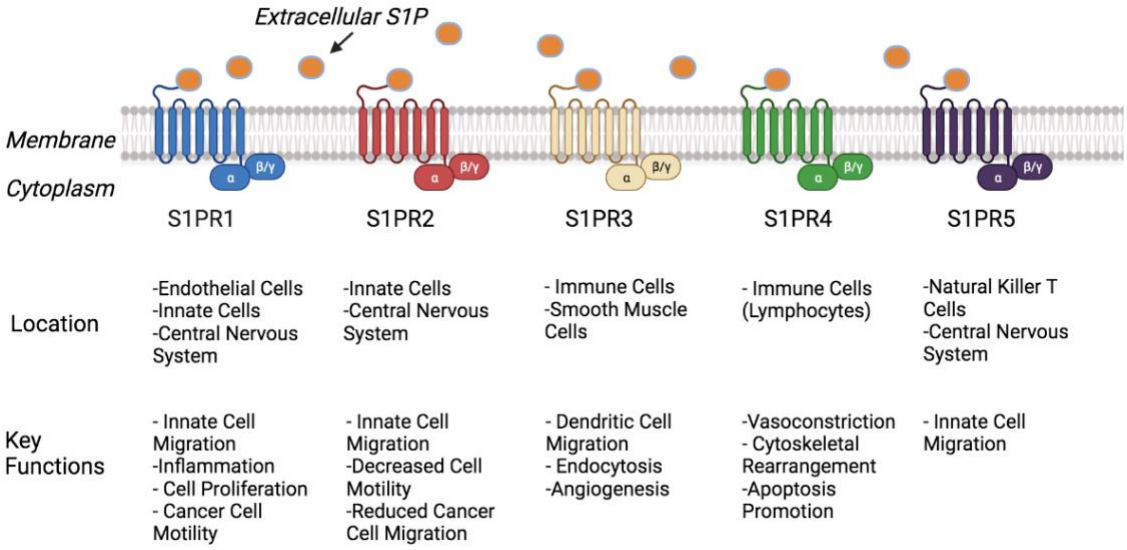

Figure 5. Location and Key Functions of the S1P Receptors

S1P Receptors are located on specific cells types, such as endothelial cells, innate cells and immune cells. Their functions vary from innate cell migration to apoptosis promotion to endocytosis. Adapted from Drexler, Y., et al [65]. Figure was made using biorender.com

SUMMARY

Acute Kidney Injury is a complex disease that is difficult to diagnose and has poor outcomes in the clinic. There are currently no treatment options for patients with AKI, highlighting the need to identify new potential therapeutic targets. While a lot of attention has been focused on specific sphingolipids or the enzymes involved in their metabolism, not enough focus has been on targeting the receptors being acted on. S1P specifically has been shown to play an important role in numerous models of AKI by signaling through five different plasma membrane g-coupled protein receptors. Of the five known S1P receptors discussed above, S1PR1 has drawn the most attention, as S1PR1 receptor activation is has been shown to be renoprotective. However, there is a clear gap in knowledge about the individual receptors, as only S1PR1 and S1PR5 have established crystal structures and individual receptor distribution is largely unknown. This has made it increasingly difficult to design drugs with receptor specificity, limiting the application for now. However, once these receptors are better characterized, targeting the S1P receptors will have huge implications in treating kidney injury.

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