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Revisiting the sphingolipid rheostat: evolving concepts in cancer therapy

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Introduction

Nearly two decades have passed since it was first proposed that regulation of the interconvertible sphingolipid metabolites, ceramide and sphingosine-1-phosphate (S1P), and their opposing signaling pathways are major determinants of cell fate, a concept referred to as the “sphingolipid rheostat”. Since then, many reports have substantiated the role of the sphingolipid rheostat in cell fate determination and in the initiation, progression, and drug sensitivity of cancer. Thus, modulation of the rheostat has emerged as a focus for treatment strategies to battle cancer. S1P regulates numerous processes important for cancer including proliferation, transformation, angiogenesis, metastasis, survival, and drug resistance. Ceramide on the other hand has been linked to cell growth arrest and cell death. With the increased understanding of sphingolipid metabolism and signaling, as well as the present focus on therapies designed to modulate the levels of sphingolipids in cancer, it is an appropriate time to re-examine the sphingolipid rheostat concept and determine how it fits within the current knowledge of sphingolipid signaling in cancer.

Sphingolipid metabolism

Sphingolipids are essential constituents of all eukaryotic membranes. They contain a sphingoid base, a fatty amino alcohol of typically 18 carbons, in mammalian cells called sphingosine. *De novo* synthesis of the sphingoid base begins with the condensation of palmitate and serine catalyzed by serine palmitoyl transferase, leading to the formation of dihydrosphingosine (sphinganine), which is then amino-acylated with a chain of 14-32 carbons to form various dihydroceramide species by a family of six (dihydro)ceramide synthases. Dihydroceramides are desaturated to form ceramides and complex sphingolipids,

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such as glycosphingolipids and sphingomyelin that are built by linking different head groups to the primary hydroxyl group of ceramides. During catabolism, both basal and signal-mediated, these head groups are hydrolyzed, regenerating ceramide. Ceramide is a bioactive lipid in its own right, and can be deacylated by ceramidases to yield sphingosine. Sphingosine, which is not an intermediate in the *de novo* biosynthetic pathway, is also a bioactive molecule and can be phosphorylated by sphingosine kinase (SphK) type 1 and 2 to sphingosine-1-phosphate (S1P), again a potent signaling molecule. S1P can be irreversibly degraded by S1P lyase (SPL) or dephosphorylated to sphingosine, which can then be re-acylated back to ceramide. It is the rapid, compartment-specific interconversion of these three metabolites with distinct effects on cell fate that forms the biochemical basis of the so-called “sphingolipid rheostat.”

The sphingolipid rheostat

In 1996, the term “sphingolipid rheostat” was proposed [1] to tie together several seminal findings demonstrating the capacity of S1P and ceramide to differentially regulate cell growth and survival by modulation of opposing signaling pathways [1-3]. This was based on the discoveries that elevation of ceramide induces cell growth arrest and apoptosis [3], whereas S1P production is required for optimal cell proliferation induced by growth factors [4] and suppresses ceramide-mediated apoptosis [1]. Insight that the “sphingolipid rheostat” coordinately regulates the levels of these sphingolipid metabolites to control cell fate emerged from inhibition of SphK leading to decreased S1P and elevated ceramide, and subsequent cell death (Figure 1). Thus, the sphingolipid rheostat appeared to be a sensing mechanism for cells to regulate their fate in part through the interconversion between S1P and ceramide.

In the years since, efforts have been made to elucidate the molecular mechanisms and signaling pathways by which these metabolites exert their effects, and to manipulate the S1P/ceramide balance to direct cells down particular paths for the development of therapeutics targeting the sphingolipid rheostat. In the process, these studies have revealed roles that S1P and ceramide play in the etiology of several debilitating human diseases, particularly cancer, and have clarified the enormous complexity of the interplay between S1P, ceramide, and sphingolipid metabolism, and how this affects complex cellular responses and biological programs. In light of these recent findings, we will revisit whether the S1P/ceramide rheostat concept adequately addresses the complex nature by which sphingolipids affect physiological processes and modulate cell fate, and, accordingly, their role in cancer.

Role of sphingolipid metabolites in cell fate and cancer Ceramide

Ceramide is a tumor suppressor, promoting intrinsic and extrinsic apoptotic pathways, autophagic cell death, and the inhibition of cell growth, and thus it is not surprising that enzymes responsible for production of ceramide are often altered in cancer resulting in reduction of ceramide accumulation [5]. Moreover, many chemotherapeutic drugs elevate ceramide, and blocking the increase in ceramide provides drug resistance. While specific molecular species of ceramide have been implicated in some of these pathways, it is unclear

whether the specific species itself is required or is merely a reflection of its compartmental- or enzyme-specific generation. For example, it was shown that in glioblastoma tumors, a metabolic shift favoring S1P at the expense of C18 ceramide may be a major contributor to angiogenesis [6]. Although there are numerous pathways affected by ceramide, only a few direct targets have been convincingly identified. The key players in ceramide regulated signaling in cancer are activation of serine/threonine protein phosphatases, such as PP1, PP2A and PP2C, protein kinase Cζ (PKCζ and inhibition of AKT (reviewed in [5]). Formation of ceramide-enriched membrane microdomains is a general mechanism by which ceramide can regulate numerous signaling pathways at the plasma membrane or in the outer mitochondrial membrane important for BAX insertion, oligomerization, pore formation and apoptosis.

S1P and its receptors

Within two years of the development of the rheostat concept, the first cell surface G-protein coupled receptor for S1P was discovered [7], followed by the identification of the other members of the S1P receptor family, designated S1PR1-5 [8]. Moreover, intracellular S1P generated by activation of SphK can readily be secreted to act in an autocrine or paracrine manner [9], a paradigm that has been coined inside-out signaling by S1P [10]. Signaling through S1PR1-5 fits nicely into the rheostat hypothesis as activation of S1PRs has been shown to promote growth, survival, motility angiogenesis, lymphangiogenesis, and metastasis, important for the pro-cancer activities of S1P [11]. For example, several S1PRs activate the pro-survival ERK and Akt signaling pathways, and S1PR3 activation initiates a signaling cascade through the mTOR pathway that counteracts ceramide-mediated autophagy [12]. Moreover, recent studies have shown that the S1P/S1PR1 axis is at the nexus between NF-κB and STAT3 and connects chronic inflammation to colitis-associated cancer [13]. S1P produced by SphK1 is essential for production of the NF-κB-regulated pro-inflammatory cytokines TNF-α and IL-6, leading to activation of the transcription factor STAT3, and consequent upregulation of its target gene S1PR1 [14]. Reciprocally, S1PR1 maintains STAT3 activation in a malicious feed-forward amplification loop important for colon cancer, lymphoma and glioblastoma [14, 15].

S1P transporters

How does S1P, which is made by intracellular SphKs exit cells to activate S1PRs? There is now ample evidence that cells export intercellular S1P into the extracellular environment both through ABC transporters as well as the major facilitator superfamily member, Spinster 2 (Spns2) [10]. The S1P secreted from tumor cells through these transporters can act in an autocrine fashion to promote the growth and motility of the tumors themselves, but more importantly on the tumor microenvironment to enhance angiogenesis and lymphangiogenesis [16], as well as affecting tumor-associated macrophages [14], and may differentially recruit immune cells such as Tregs to block anti-tumor immunity [17].

S1P intracellular targets

Though the majority of known S1P functions are attributable to its action through cell surface S1 PRs, recently several intracellular targets of relevance to cancer have been found.

The first of these is TRAF2, an essential component in the TNF- α/NF-κB signaling pathway. TRAF2 is an E3 ubiquitin ligase, and it was found that S1P bound to and stimulated its ubiquitin ligase activity [18]. In addition, SphK1 was shown to be required for the TNF-α-induced ubiquitination of RIP1 and subsequent activation of NF-κB, a pro-growth mediator. Interestingly, another group showed that SphKs were dispensable in bone marrow-derived macrophages for TNF-α-induced activation of NF-κB, though even in wild type macrophages TNF-α did not increase S1P levels, suggesting alternative mechanisms for the stimulation of TRAF2 activity [19]. S1P, produced by SphK2, was shown to bind to and inhibit histone deacetylases 1 and 2, leading to increases in histone acetylation [20]. Further support for this notion that HDACs are intracellular targets of S1P emerged from a recent study in *Drosophila*, which have no identified S1PRs, showing that increased nuclear S1P caused decreased HDAC activity and increased histone acetylation, and importantly suppressed dystrophic muscle degeneration [21]. Furthermore, the pro-drug FTY720 is also phosphorylated in the nucleus by SphK2 and FTY720-phosphate is a potent class I HDAC inhibitor [22], which might explain its potent anti-cancer effects.

Targeting S1P metabolic enzymes to modulate the sphingolipid rheostat and cancer

Recently several excellent reviews discussed how targeting specific ceramide metabolic enzymes regulates the sphingolipid rheostat to amplify tumor suppressive activities of ceramide and consequently cell fate, and highlights the usefulness of ceramide-based therapeutics for treatment of cancer [5, 23]. Therefore, we will mainly focus in this section on effects of targeting S1P metabolism.

SphK1

In many cancers, elevated levels of SphK1 are an independent predictor of mortality, and strongly correlate with poor prognosis, reduced overall survival, and advanced tumor stages [11, 24]. However, no mutations in SphKs have been identified, suggesting that it is the regulation of SphK activity, and hence a potential for S1P “cellular addiction”, that is responsible for SphK1 “oncogenic” role [25]. There is some evidence to support this notion: 1) in cell culture, expression of SphK1 and S1P levels dictate resistance to cytotoxic drugs and radiation; 2) in animal models, overexpression of SphK1 and formation of S1P leads to aggressive tumors, and inhibition of SphK1 reverses drug resistance and enhances sensitivity to radiotherapy; 3) SphK1 is overexpressed in many types of cancer and high levels of SphK1 correlate with poor outcomes in patients. Because of these observations, multiple drugs targeting SphK1 have been designed. The “first generation” inhibitors such as N,N-dimethyl-sphingosine and SK1-II, with poor potency and selectivity between SphK isoforms, as well as SK1-I which specifically targets SphK1, though with low potency, showed promise in preclinical animal models. However studies with the “second generation” of SphK1 inhibitors, such as PF-543, which are much more potent and are highly selective, have limited to no success in inducing apoptosis [26, 27], even though in all cases S1P levels were reduced and in two of these studies ceramide levels were concomitantly elevated [6, 28]. Why then, if the rheostat postulates that a reduction in S1P and rise in ceramide should increase cell death, are these SphK1 inhibitors ineffective?

There are several potential explanations for this: first, reduction of SphK1 levels due to proteasomal degradation may be critical [29]. Second, inhibitors might affect SphK1 activity in different subcellular locations and only those that affect cellular S1P vs. sphingosine and ceramide more profoundly are effective compared to those that simply inhibit SphK1. Second generation inhibitors, with their much higher specificity and potency, do not have strong effects on changing the sphingolipid rheostat and only increase ceramide levels at an order of magnitude greater concentration than their Ki values [28]. This further substantiates the notion that the sphingolipid rheostat is a critical component, not only reduction in the levels of S1P. Hence, in light of this, it might be appropriate to consider a more broad-spectrum approach to therapeutics to induce the apoptotic benefits that dictate chemotherapeutic efficiency. Third, as indicated by Abuhusain et al [6], S1P produced by cancer cells may mainly act in a paracrine manner in the tumor microenvironment that is important for angiogenesis and lymphangiogenesis but does not affect tumor growth itself. It is important to note that all of the studies reported so far with second generation SphK1 inhibitors utilized cultured cancer cells, and therefore it will be important to examine their effects in animal cancer models. As more and more SphK1 drugs are developed with fewer off target effects, establishing how perturbations in S1 P/sphingosine balance affects ceramide should become easier. These ventures should also be significantly aided with a variety of SphK1 structures that have been published [30], and with the recent development of a high-throughput assay to screen SphK inhibitors [31].

SphK2

In contrast to SphK1, the actions of SphK2 remain poorly characterized and much less is known about its biology and roles in cancer and other diseases. However, SphK2 is critical to the function of one of the few FDA approved sphingosine analog drugs, FTY-720, as it phosphorylates it to the “active” form that acts on S1 PRs (except S1PR2) [32] and inhibits histone deacetylases [22]. The difficulty in targeting SphK2 in rheostat modulation therapies is that its roles in regulating sphingolipid metabolism are not well understood. SphK2 is present in several subcellular compartments, and there are conflicting data regarding its role in cancer development. Moreover, inhibitors targeting its activity have not been as successfully developed as those for SphK1. Compound ABC294640 (SphK2 K_i = 9.8 μM) has shown promise in reducing cancer cell growth *in vitro* and in mouse models of cancer [33, 34]. However, this compound has also been linked to potential off target anti-estrogenic effects [35]. In another example, SLR080811 (SphK2 K_i = 1.3 μM) showed a reduction of the levels of S1P in cells, though it had no anti-proliferative properties, and when administered to mice, raised blood S1P levels [36], confounding evaluation of its effectiveness as a chemotherapeutic in mice cancer models. Clearly, targeting SphK2 in the treatment of disease is still in its infancy and will require significant efforts to substantially increase the potency and specificity of inhibitors. Unfortunately, unlike SphK1, little is also known of the structure of SphK2 and pharmacophore design for SphK2 is complicated by the necessity to cross secondary subcellular membrane barriers to reach the target.

S1P lyase and S1P phosphatases

As the terminal step in irreversible catabolism of all sphingolipids, SPL controls levels of S1P and other bioactive sphingolipid metabolites, and is also the link between sphingolipid and phospholipid metabolism. Indeed, deletion of SPL not only increases S1P levels but also sphingosine and ceramide, probably due to reutilization of the sphingosine backbone for ceramide synthesis. Not surprisingly, knockout mice have severely altered lipid homeostasis, aberrant S1P signaling, and inflammatory responses leading to early death [37, 38]. SPL levels are downregulated in various human cancers and inversely correlated to clinical outcomes and resistance to treatment, further supporting a role for S1P in cancer development. Several novel findings leading to mechanistic actions of SPL in tumorigenesis and chemoresistance have recently been described. Deletion of intestinal SPL promoted colon carcinogenesis through the S1P/S1PR1 axis and activation of STAT3. STAT3 in turn enhanced expression of specific miRs that target the anti-oncogenes PTEN (a lipid phosphatase that negatively regulates the PI3K/AKT pathway) and cylindromatosis (CYLD; a deubiquitinating enzyme that negatively regulates NF-κB) [39]. Interestingly, upregulation of SPL levels by consumption of sphingadienes, plant-type sphingolipids that cannot be converted to S1P, was able to enhance the metabolism of S1P attenuating STAT3 signaling, cytokine production, and tumorigenesis [39]. SPL deficiency or its inhibition has also been associated with elevated nuclear S1P levels and reduced HDAC activity that in turn induced dysregulation of Ca^{2+} homoeostasis [40], and upregulation of several S1P transporters, including multi-drug resistant proteins, contributing to chemoresistance [41].

In addition to SPL, S1P levels are also reversibly regulated by two S1P phosphatases (SGPP1 and SGPP2) that dephosphorylate S1P to sphingosine that can then be used for ceramide formation. This places S1P levels under dual control, with one pathway removing sphingolipids from the signaling pool and the other shifting the rheostat balance from the proliferative effects of S1P to the pro-apoptotic effects of sphingosine and ceramide accumulation. Similar to SPL, there is evidence that S1P phosphatase expression is downregulated in several types of cancer. Closer examinations of these studies reveals that increased S1P content in the tumors was correlated with decreased SPP2 expression and increased SphK1, supporting the notion of coordinated regulation of sphingolipid metabolism [6].

Modified rheostat paradigm: addition of the S1P/S1PR axis

The field of cancer research has embraced the concept of the sphingolipid rheostat. As more and more studies involving ceramide and S1P signaling attempt to use the rheostat model to explain their findings, and as the signaling mechanisms by which these sphingolipid metabolites exert their control on cell fate becomes more complex, and additional proteins that regulate sphingolipid metabolism are discovered, the need for a more nuanced model has become apparent. For example, although initially elevation of ceramide was linked to cell growth inhibition and reduction of tumor growth, more recent studies suggest that ceramides with different fatty acid chain lengths might play distinct functions. *De novo*-generated C18- and C16-ceramides by CerS1 and CerS6 play opposing proapoptotic and prosurvival roles in the regulation of tumor growth, respectively [42]. Whether a specific

acyl chain species is required for apoptosis, for example by N-acyl chain-specific binding to effector proteins, or whether the N-acyl chain species generated is merely a reflection of the enzyme- and/or compartment-specific generation of ceramide, has yet to be elucidated.

The molecular roles of S1P in the rheostat have become more complex. It is generally accepted that S1P is a pro-survival factor and many studies have demonstrated that S1P acts through cell surface S1PRs through “inside-out” signaling to promote cancer growth, progression and metastasis [43]. S1P does this through the autocrine promotion of tumor growth and most importantly, in a paracrine manner by enhancing angiogenesis, lymphangiogenesis, recruitment of pro-tumor immune cells, and affecting the tumor microenvironment. This suggests that the “sphingolipid rheostat” concept should be modified to include this “inside-out” signaling (Fig. 1). Furthermore, a recent study showed that S1P can also act in a feed-forward, “outside-in” signaling in a paracrine fashion. Namely, S1P acting through S1 PRs has been shown to stimulate SphK1 transcription via the transcription factor AP-1, which is composed of c-Fos and c-Jun [44]. This positive feedback loop maintains sustained activation of the SphK1-S1P axis and increased fibronectin expression leading to initiation and progression of diabetic nephropathy [44], and could also contribute to the pathogenesis of other diseases including cancer. Moreover, activation of Gq induced plasma membrane translocation of SphK1 and cross-activation of S1 PRs [45]. Hence, increased SphK1 activity and increases in S1 PR synthesis or their activations, completes this positive feedback amplification loop.

These more recent studies suggest that the enzymes of the rheostat do not just function by directly changing the fate of the sphingoid base (i.e. ceramide vs S1P) as initially conceived in the rheostat model, but also by the roles these metabolites have in myriad, often opposing, signaling pathways. While the initial rheostat model was based on the relative intracellular levels of ceramide and S1P determining signaling and cell fate, we now know that the localized production, secretion, and signaling of these metabolites has a profound effect on tumor outcomes. Our new, more nuanced understanding of the sphingolipid rheostat must be taken into consideration as we design, test, and implement new chemotherapeutics targeting this axis for cancer treatment. More work is needed to understand alterations that occur in the complex sphingolipid pathways during cancer development and progression, and their relationship to the Warburg effect and the metabolic shift from oxidative phosphorylation to the synthesis of lipids and biomass essential for increased cellular proliferation [46].

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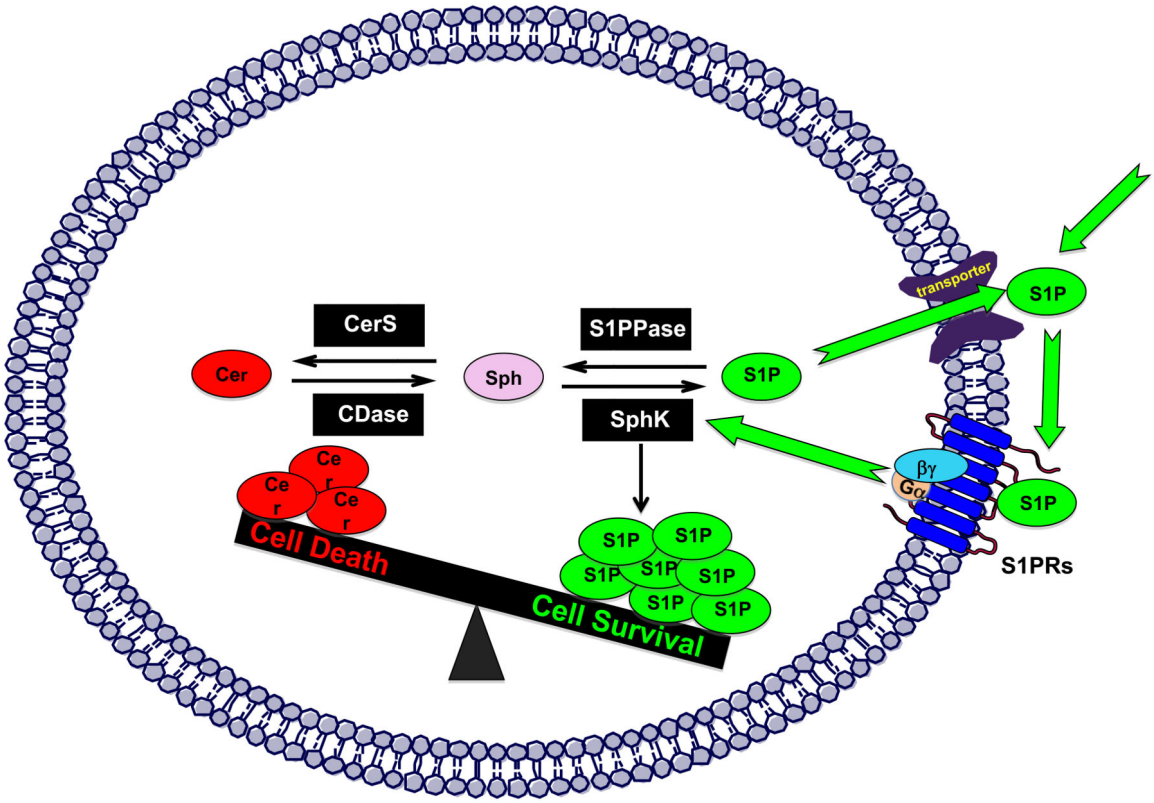

Figure 1. The updated sphingolipid rheostat

This schematic cartoon shows important enzymes that regulate the levels of S1P and ceramide and includes “inside-out” signaling by the S1P/S1PR1 axis that can influence actions of the sphingolipid rheostat. CerS, ceramide synthase; CDase, ceramidase; S1PPase, S1P phosphatase; S1 PRs, S1P receptors.