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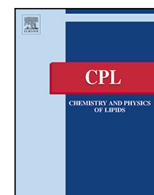

Inhibitors of Ceramidases

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ABSTRACT

The topic of ceramidases has experienced an enormous boost during the last few years. Ceramidases catalyze the degradation of ceramide to sphingosine and fatty acids. Ceramide is not only the central hub of sphingolipid biosynthesis and degradation, it is also a key molecule in sphingolipid signaling, promoting differentiation or apoptosis. Acid ceramidase inhibition sensitizes certain types of cancer to chemo- and radio-therapy and this is suggestive of a role of acid ceramidase inhibitors as chemo-sensitizers which can act synergistically with chemo-therapeutic drugs. In this review, we summarize the development of ceramide analogues as first-generation ceramidase inhibitors together with data on their activity in cells and disease models. Furthermore, we describe the recent developments that have led to highly potent second-generation ceramidase inhibitors that act at nanomolar concentrations. In the third part, various assays of ceramidases are described and their relevance for accurately measuring ceramidase activities and for the development of novel inhibitors is highlighted. Besides potential clinical implications, the recent improvements in ceramidase inhibition and assaying may help to better understand the mechanisms of ceramide biology.

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1. Introduction

Sphingolipids represent a family of diverse bioactive molecules that are part of a highly integrated metabolic network. In this network, ceramide occupies a central position in biosynthesis, catabolism and as a precursor of complex sphingolipids, thereby currently considered as a metabolic hub of sphingolipid metabolism. Ceramide can be generated by three different pathways; *de novo biosynthesis* pathway, degradation or hydrolytic pathway, and salvage pathway (Fig. 1). Details on sphingolipid metabolism are well covered in the literature and are briefly discussed in this review; interested readers are recommended to refer to reviews (Pralhada Rao et al., 2013; Bartke and Hannun, 2009; Don et al., 2014; Gault et al., 2010; Hannun and Obeid, 2008). The *de novo biosynthesis* of ceramide begins on the cytosolic surface of the endoplasmic reticulum with the condensation of serine and palmitoyl-coenzyme A (CoA) to produce 3-ketodihydrosphingosine through the action of serine palmitoyltransferase (SPT). In turn, 3-ketodihydrosphingosine is reduced to dihydrosphingosine (sphinganine), followed by *N*-acylation to produce dihydroceramide (dhCer), the reaction catalyzed by one of six different dihydroceramide synthase (CerS1-6). After its formation, dhCer is desaturated by dihydroceramide desaturase (DES) through introduction of a 4,5-*trans*-double bond on the sphingoid backbone, to form ceramide in the last step of *de novo biosynthesis* pathway. The second major pathway of ceramide generation is the “degradation or hydrolysis of sphingomyelin”. This pathway, which occurs mainly in the plasma membrane and mitochondria, involves the hydrolysis of phosphodiester bonds in sphingomyelin by one of several sphingomyelinases (SMases) to provide ceramide and phosphorylcholine. In addition to the above described pathways, ceramides can also be generated by “a *salvage/recycle* pathway”. In this pathway, sphingosine is recycled by re-acylation to form ceramide through the action of CerS1-6. Importantly, sphingosine utilized in this pathway is derived from the lysosomal degradation of complex sphingolipids, mainly sphingomyelin and glycosphingolipids. In this pathway, SMases and other hydrolases provide ceramide, which is then degraded by acid ceramidase to form sphingosine.

Once generated, various different headgroups can be added to ceramide to form different classes of complex sphingolipids. Alternatively, ceramide can be hydrolyzed through the action of ceramidases (CDases) to liberate sphingosine, which, in turn, can be phosphorylated by sphingosine kinases to generate sphingosine-1-phosphate (S1P).

Regulation of the balance among sphingolipids metabolites “sphingolipid homeostasis” is of fundamental importance for

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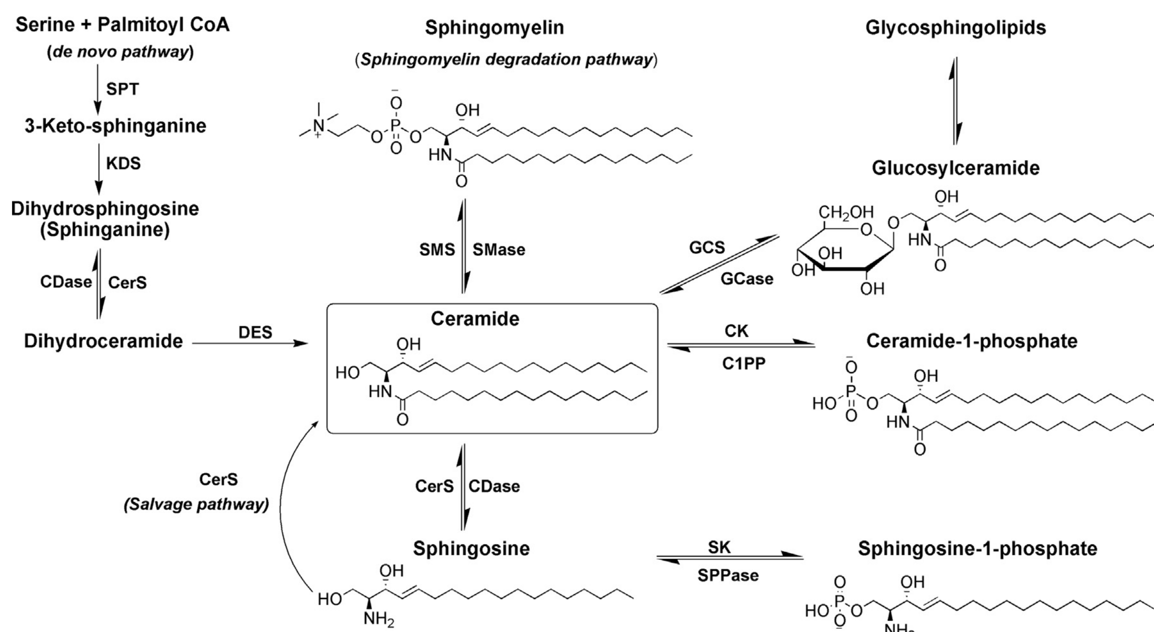

Fig. 1. Scheme of bioactive sphingolipid metabolism. Ceramide can be produced by *de novo* biosynthesis pathway, sphingomyelin degradation pathway, and salvage pathway. SPT, serine palmitoyltransferase; KDS, 3-ketodihydrosphingosine reductase; DES, dihydroceramide desaturase; CK, ceramide kinase; C1PP, ceramide-1-phosphate phosphatase; SMS, sphingomyelin synthase; SMase, sphingomyelinase; GCS, glucosyl-ceramide synthase; GCase, glucosyl ceramidase; CDase, ceramidase; CerS, ceramide synthase; SPPase, S1P phosphatase; SK, sphingosine kinase.

controlling of cellular responses and for cell homeostasis. Several studies revealed that alteration of sphingolipid homeostasis may lead to the onset and/or progression of diseases such as Alzheimer's disease, cancer, type 2 diabetes, sphingolipidoses, chronic inflammation and cardiovascular diseases (Young et al., 2012; Segui et al., 2006; Kolter and Sandhoff, 2006; Truman et al., 2014). Within the family of sphingolipids, ceramide, sphingosine and S1P are considered as the main bioactive effectors. While ceramide has emerged as a tumor-suppressor lipid mediating various cellular responses such as necrosis (Hetz et al., 2002), differentiation (Okazaki et al., 1989), senescence (Venable et al., 1995) and apoptosis (Obeid et al., 1993), the counterpart S1P is a tumor-promoting lipid regulating proliferation, cell growth, angiogenesis, cell migration, cell survival, and inflammation (Maceyka et al., 2012; Hla, 2004; Kunkel and Maceyka, 2013). Therefore, the dynamic equilibrium between the intracellular ceramide and S1P, described as "ceramide/S1P rheostat", is considered to be a critical factor that determines the cell survival or death (Cuvillier et al., 1996; Maceyka et al., 2002; Taniguchi et al., 2012; Lavieu et al., 2007; Taha et al., 2006; Van Brocklyn and Williams, 2012). Since ceramide metabolism is the only pathway for S1P generation through the action of CDases, CDases have been suggested to play critical roles in regulating the ceramide/S1P rheostat, and thereby controlling cellular responses regulated by these bioactive lipids. Therefore, CDases may be important target for development of novel therapeutic compounds for treatment of diseases such as cancer, obesity, Farber disease, and chronic inflammation (see below).

2. Ceramidases and their role in regulating cellular responses

Ceramidases are a heterogeneous family of *N*-acylsphingosine amidohydrolase enzymes that catalyze the cleavage of ceramides into sphingosine and fatty acids. Details on ceramidases and their role in regulating cellular responses have been recently reviewed (Saied and Arenz, 2014; Canals et al., 2011; Mao and Obeid, 2008). Presently, there are five different human ceramidases encoded by five distinct genes. Depending on their pH optima, they can be classified into acid ceramidase (aCDase/*ASAH1*); neutral ceramidase (nCDase/*ASAH2*); alkaline ceramidase 1 (ACER1/*ASAH3*); alkaline ceramidase 2 (ACER2/*ASAH3L*); and alkaline ceramidase 3 (ACER3). These ceramidases reside within different cellular compartments and display different substrate specificities. aCDase is localized in the lysosomes from which a portion is secreted extracellularly (Bernardo et al., 1995; Ferlinz et al., 2001). It has an optimal pH of 4.5 (He et al., 2003) and favors medium chain unsaturated ceramides as substrates (Momoi et al., 1982). nCDase is localized to the outer leaflet of the plasma membrane (PM) (Hwang et al., 2005) as a type II integral membrane protein (Tani et al., 2003) or it is secreted into the intestinal lumen (Olsson et al., 2004). The nCDase has a broad optimal pH, ranging from 7 to 9, and favors the natural D-*erythro*-ceramide isomer as a substrate over other isomers of ceramide (El Bawab et al., 2000, 1999). ACER1 is localized to the ER and prefers very long chain unsaturated ceramides as substrates (Sun et al., 2008; Mao et al., 2003). ACER2 is a Golgi ceramidase that uses long or very long chain ceramides as substrates (Sun et al., 2010; Xu et al., 2006). ACER3 is localizes to both the ER and Golgi apparatus and prefers long-chain (but not very long) unsaturated ceramides as substrates (Hu et al., 2010; Mao et al., 2001). Noteworthy, most of these ceramidases showed to catalyze the hydrolysis of ceramide (ceramidase activity) and the reverse reaction of catalyzing the ceramide formation through a CoA-independent mechanism. In contrast to CerS activity, the reverse activity of ceramidases uses a free fatty acid and sphingosine as substrates. This concept was first proposed by Gatt (1963). However, this early study did not use highly purified enzyme, thus the physiological significance was not elucidated for many years. Later, this intriguing finding was confirmed by several studies using cloned acid, neutral and alkaline CDases (Mao et al., 2003, 2000; El Bawab et al., 2001; Tani et al., 2000; Okino et al., 2003, 1998; Mao and Xu, 2000; Kita et al., 2000; Galadari et al., 2009). However, it is still unclear which activity the enzymes prefer in cells. In a recent report, Novgorodov et al. showed that the reversed activity of nCDase is a key element of ceramide formation in liver mitochondria *in vivo*. Obviously, ceramide formation occurs from coupled activities of a

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