Review paper

Biological activity of ceramides and other sphingolipids

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Abstract

Sphingolipids are a large group of lipids which play a key role in the cellular life cycle. In addition to structural functions (they are constituents of cell membranes), they are known to be involved in processes of intercellular recognition and signal transmission. Ceramides, which are sphingolipid metabolites, take part in signal transduction and initiation of a range of processes which affect the cell life. Depending on the external stimulus and cell type, the processes may include inhibition of proliferation, initiation of differentiation or apoptosis. For example, studies have shown that an increased concentration of endogenous ceramides caused by activation of the membrane receptor CD95 activates a number of processes triggering programmed cell death. At the same time, study results have demonstrated that due to their specific structure ceramides are able to interact directly with a number of key enzymes and activate them. What is more, not only endogenous ceramides have the capacity to elicit a specific biological response. Exogenous ceramides and their structural analogues are also able to affect the cellular life cycle, which makes them potentially therapeutic substances.

Key words: ceramides, apoptosis, sphingolipids, sphingomyelin cycle.

Introduction

Sphingolipids are a very important group of substances which play key functions in the regulation of cell life cycle processes. Three lipids belonging to the class of sphingolipids are implicated in regulating processes occurring in mammalian cells. They are: sphingosine, ceramide (Cer) and sphingosine-1-phosphate (S1P). The latter two have opposite functions, which is why the Cer/S1P balance is a determinant of the cell fate [1]. The intracellular concentration of both substances is regulated in the so-called sphingomyelin cycle in which, in specific conditions, ceramides are released by sphingomyelinases (i.e. enzymes which catalyze the breakdown of sphingomyelin) [2]. Ceramides, which are secondary signal molecules, are necessary in the cell life cycle – not only in the skin but also in other organs, such as the liver [3]. Ceramides act as intermediaries inducing exogenous agents to elicit different types of biological response in cells, such as inhibition of proliferation or initiation of differentiation or apoptosis processes [3]. The role of ceramides in the life cycle of different types of cells has been thoroughly elucidated [4]. Thanks to their ability to interact directly with endogenous enzymes, the lipids usually activate paths leading to the cell death. It should be stressed, though, that their activity depends strictly on the cell type [1]. Aside from typical biophysical properties, due to their unique chemical structure and the property of self-organization in water, ceramides are the basic building blocks of the intercellular cement of the stratum corneum. Appropriate ceramide content and proportions between different ceramide groups in the cement determine the functioning of the epidermal barrier.

Ceramides – chemical structure and nomenclature

Regarding the chemical structure, ceramides are formed through an amide bond between two permanent constituents: fatty acid and amine alcohol (sphingoid base) [5-8]. A group of amine alcohols which take part in the formation of ceramides includes sphingosine, phytosphingosine, 6-hydroxysphingosine and dihydrosphingosine [6, 7]. The group of fatty acids includes α-hydroxy acids, ω-hydroxy acids and acids not containing hydroxyl groups. Fatty acid chains can vary in length. Mammalian cells usually contain acids with hydrocarbon chains that

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are 16-24 carbon atoms long (C16-C24). The structure of ceramides present in the stratum corneum is slightly different, though. They are usually built up of fatty acids that do not contain hydroxyl groups and are between 16 and 30 carbon atoms long (C16-C30). Some ceramides in the stratum corneum have a unique structure which is not seen in any other tissues [6, 8, 9]. One example is O-acyl ceramides incorporating a long-chain fatty acid (C28-C32) with a terminal hydroxyl group which may either be unbound or attached via an ester bond to linoleic acid or α-hydroxy acid. ω-hydroxy ceramides, as opposed to other ceramides, are capable of creating covalent bonds with proteins. The property determines the creation of compact stratum corneum. Disorders in the process of bond development between the intercellular cement and corneocyte envelope loosen the structure of the stratum corneum, thus promoting transepidermal water loss and penetration of substances of exogenous origin [8, 10-12]. A total of eleven ceramide groups have been identified in human epidermis. Studies investigating their chemical structures have contributed to the development of new nomenclature [5-8, 13]. Under the new guidelines, digits which used to serve as a basis for distinguishing ceramides in terms of polarity were replaced with letters of the alphabet designating different elements of ceramide structure. The first letter in the ceramide symbol indicates the type of sphingoid base, and the second – the type of acid building the ceramide structure. Symbols of three long-chain ceramides have an additional prefix designating the fatty acid bound at the ω position. In accordance with new nomenclature guidelines, Table 1 lists symbols of all ceramides present in the human intercellular cement of the stratum corneum.

Intercellular synthesis of ceramides

De novo synthesis of ceramides occurs in cells and tissues throughout the body. The synthesis site is endoplasmic reticulum (ER), the primary substrates being serine and palmitoyl-CoA [1-4]. The process begins with the condensation of the substrates catalyzed by an appropriate transferase (serine palmitoyl transferase, SPT), producing 3-ketosphinganine. The compound, after being reduced to sphinganine, is converted into dihydrosphingosine by dihydroceramide synthase. Another enzyme in the synthesis path is dihydroceramide desaturase which is responsible for creating a double bond at the C4 position. The process leads to the formation of an appropriate ceramide which is then transported to the Golgi apparatus where it undergoes further transformations [2, 3, 14]. Because ceramides synthesized in ER are poorly soluble in cytosol, they are transported actively, by means of ceramide transport proteins (CERT).

Another source of endogenous ceramides is the breakdown of sphingomyelin. The process directly increases ceramide concentration in cells. Sphingomyelin breakdown is catalyzed by the following enzymes: acid lysosomal sphingomyelinase (A-SMase), a membrane-associated neutral sphingomyelinase dependent on the concentration of Mg^{2+} cations, and Mg^{2+}-independent sphingomyelinase (N-SMase) [14, 15]. Ceramides thus produced can affect specific cell life functions or be discharged into the intercellular space in the form of sugar derivatives. The release of ceramides in this process occurs through the activation of specific proteins by exogenous agents (chemical substances or physical factors). The process in which the concentration of sphingomyelin-derived intracellular ceramides is elevated and the sphingomyelin structure is restored by ceramides is referred to as the sphingomyelin cycle, and it occurs in the Golgi apparatus.

Ceramides produced in the cell can undergo a range of transformations resulting in various metabolites. Hydrolysis causes removal of the fatty acid and formation of sphingosine which is then further phosphorylated by the activity of sphingosine kinase. The process gives rise to the above-mentioned sphingosine-1-phosphate (S1P) which plays a major role in the transmission of information in cells [1]. Ceramides can also undergo phosphorylation mediated by ceramide kinase. The result is ceramide-1-phosphate (C1P) which is in equilibrium with the original substrate. Similarly to S1P, it is a mitogenic factor affecting cell viability and playing a key role in inflammatory processes [1, 3, 14].

Production of epidermal ceramides

The synthesis of ceramides building up the intercellular cement of the stratum corneum takes place during the process of keratinocyte differentiation and is always

Table 1. Names of ceramides according to the new nomenclature [7]

Fatty acid	Sphingoid base			
	Phytosphingosine (P)	Sphingosine (S)	6-hydroxy-sphingosine (H)	Dihydrosphingosine (DS)
Omega hydroxy acid bound by ester binding (EO)	EOP	EOS	EOH	EODS(?)
α-Hydroxy acid (A)	AP	AS	AH	ADS
Acid without hydroxyl groups in the structure (N)	NP	NS	NH	NDS

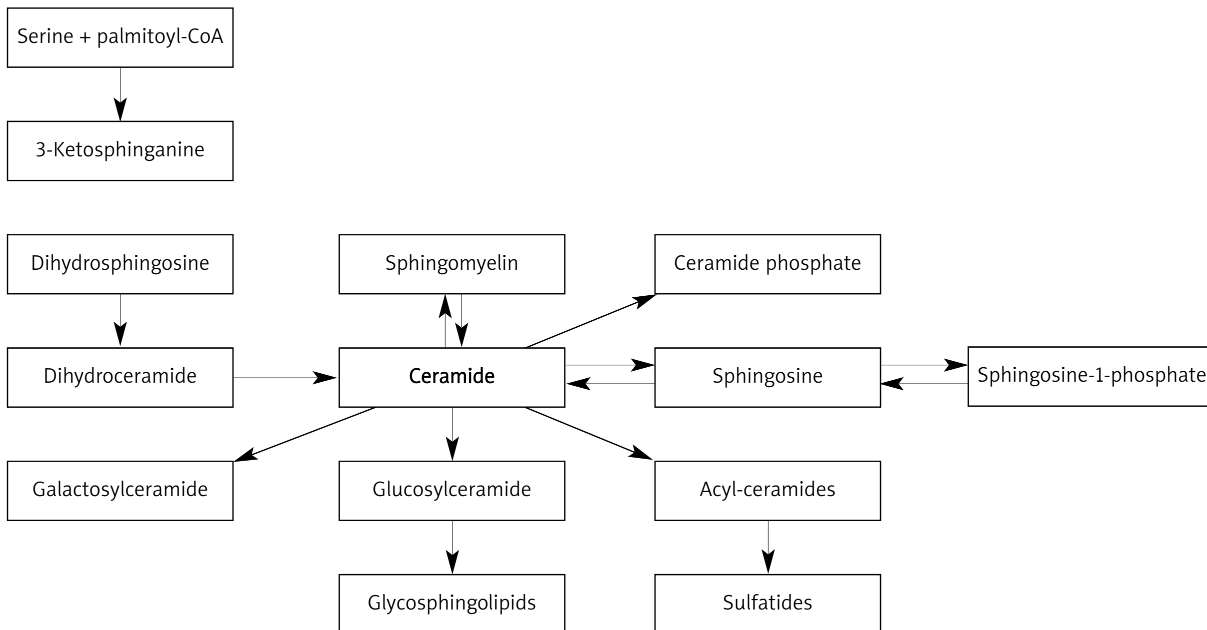

Figure 1. Diagram illustrating *de novo* synthesis and metabolism of ceramide [14]

of "de novo" type (Figure 1) [1, 3, 5, 15]. During the process of formation of different ceramides which are structural elements of the intercellular cement, their precursors are modified by appropriate enzymes at the 1-hydroxy position, creating glycoceramides or sphingomyelin through the transfer of phosphocholine from phosphatidylcholine. Experiments have shown that stratum corneum ceramides are mainly derived from glycoceramides and only a small number have their origin in the breakdown of sphingomyelin [1, 4]. Glycoceramides have also been confirmed as precursors of all ceramides belonging to 9 groups, while Cer NS- and Cer AS-class ceramides have been shown to arise from SM. Ceramide precursors formed in the process, together with other lipids, are packed in lamellar bodies (Odland bodies). The exocytosis of lamellar bodies between the stratum granulosum and stratum corneum activates the release of precursors into the intercellular space. As the next stage, the precursors give rise to lamellar layers of the intercellular cement.

Sphingomyelin cycle

The sphingomyelin cycle was verified in a study using HL-60, a human leukaemia cell line, which was incubated with 1α,25-dihydroxyvitamin D3, achieving reversible hydrolysis of sphingomyelin catalyzed by neutral sphingomyelinase (N-SMase) [16]. Increased concentrations of intracellular ceramides activated the process of transfer of the phosphocholine group from phosphatidylcholine to ceramides, thus restoring the original concentration of sphingomyelin. The activation of the sphingomyelin cycle requires an external stimulus such as interleukin-1β, monoclonal antibodies anti-FAS/APO-1, TNF-α, interferon γ or analogue of vitamin D_3 – calcipotriol [16]. Some of the substances activate sphingomyelinase initiating the process of ceramide release from sphingomyelin. A direct elevation of ceramide concentration elicits a specific cellular response. Typically, it is reduction of proliferation, activation of the process of differentiation or induction of apoptosis.

Ceramides and their structural analogues versus cell response

The current state of knowledge makes it possible to quite precisely define pathways leading to gradual programmed cell death. Since studies confirmed that ceramides produce an effect similar to the activity of TNF-α, a hypothesis was put forward that endogenous ceramides are involved in signal transmission within the cell – for example inducing apoptosis. At the same time, it was found that only ceramides with a particular structure and some of their analogues were capable of triggering any biological effect [1, 15, 16]. The biological activity of endogenous ceramides was corroborated by using exogenous bacterial sphingomyelinase which caused ceramide release from cell membrane lipids and induced programmed cell death through their mediation. At the same time, a number of studies have demonstrated ceramides to be the main factors at play in cell response induced by the activation of the cell membrane receptor

CD95 (FasR) which, following multiple transformations, stimulates acid sphingomyelinase (A-SMase) to release ceramides from sphingomyelin [1]. The best proof of the link between receptor CD95 and endogenous ceramides are studies in which murine hepatocytes from A-SMase-depleted mice were stimulated with CD95 antigen without elevating the concentration of endogenous ceramides and, as a consequence, triggering off the process of apoptosis [4].

Studies have demonstrated endogenous ceramides to be capable of direct interactions with some enzymes. Their primary targets are kinases (CAPK, or ceramide activated protein kinase, also known as KSR, kinase suppressor of Ras) belonging to the class of proline-directed kinases, isoforms of protein kinase C – PKC ζ, protein phosphatases (CAPP) including class 2A protein serine-threonine phosphatases and mitogen activated protein kinase (MAPK) and SAPK/JNK [1, 2, 14]. It has recently been established that ceramides decrease catalytic activity and suppress the translocation of PKCα from cytosol to the inner surface of cell membrane, thus affecting cell growth and phosphatidylcholine metabolism [1, 4, 17]. Ceramides activate effector proteins such as Rb, NF-κ and AP-1 (transcription factor), apoptosis antagonist Bcl-2, phospholipase D and cathepsin D, and interfere with the splicing of caspase-9 and $Bcl\text{-}X_L$ as well as reducing the activity of telomerases and inducing phosphorylation processes. An important element of ceramide involvement in the activation of apoptosis-associated processes is the impact ceramides have on mitochondrial functioning and, in particular, on processes relating to the respiratory chain. On the one hand, ceramides increase the concentration of hydrogen peroxide. On the other, they are responsible for overproduction of reactive oxygen species (ROS) which are activators of programmed cell death [16-18]. In addition, ceramides take part in the creation of channels in the outer mitochondrial membrane, triggering the release of cytochrome c into the cytoplasm and activation of processes leading to the activation of caspases which ultimately effect cell death.

A number of studies into the properties of ceramides and their analogues have shown that it is not only endogenous ceramides that elicit specific biological response in cells. One example is the study by Pillai *et al.* investigating the effects of exogenous ceramides and sphingomyelin on the proliferation and differentiation of HL60 cells and immortalized keratinocytes HaCaT [19]. The study examined two short-chain ceramides C2 and C6, natural ceramides 3 and 6b, and sphingomyelin (SM). Pillai *et al.* demonstrated both similarities and dissimilarities in biological effects exerted by exogenous ceramides and their precursors, depending on the type of cell line. Whereas short-chain ceramides exerted a similar effect of inhibiting the proliferation process in the HL-60 and HaCaT lines, sphingomyelin – a precursor of endogenous ceramides – stimulated the proliferation of keratinocytes and had no influence on the proliferation of leukaemia cells [19]. Opposite results were obtained for cell differentiation: the short-chain ceramide C2 stimulated the differentiation process regardless of the type of cell line, while sphingomyelin stimulated the differentiation of HL 60 cells and inhibited the differentiation of HaCaT-line keratinocytes. Opposite effects produced by ceramides and sphingomyelin were elucidated in a study with 1,25-dihydroxyvitamin D, the active form of vitamin D which stimulates the process of differentiation. Pillai *et al.* proved, for example, that the inhibition of the process of keratinocyte differentiation by exogenous sphingomyelin might be a consequence of intracellular accumulation or disturbed synthesis of lipids in the cell membrane and an increase in membrane fluidity [19].

Another study by same author sought to examine the effect of ceramides C2 and C6, neoceramide and pseudoceramide in combination with vitamin D metabolite (1,25-dihydroxyvitamin D_3 – 1,25D) and its precursor (25-dihydroxyvitamin D_3 – 25D) on neonatal skin keratinocytes [20].

Similarly to the previous study, an analysis was performed to determine the level of transglutaminase-1 and the activity of DNA synthesis in thymidine test. Vitamin D

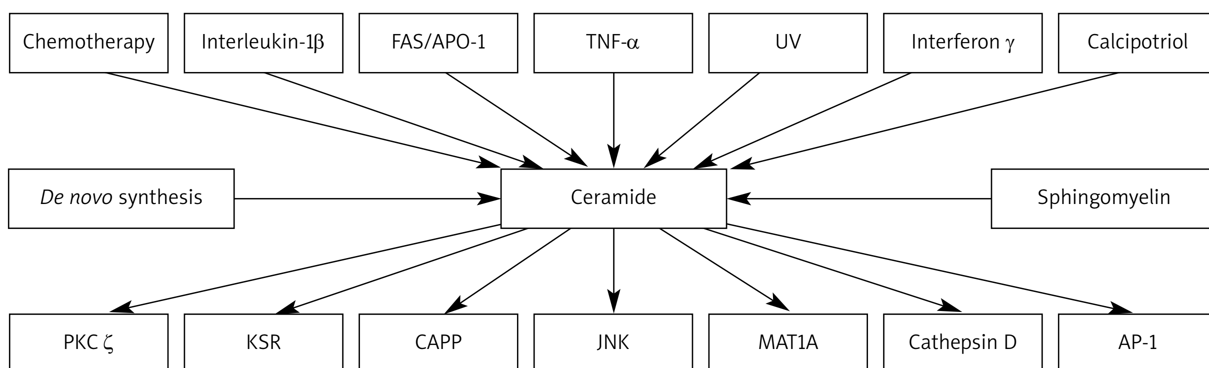

Figure 2. Ceramide as a second messenger in the process of cell growth [4]

is regarded as an inducer of the process of differentiation. Ceramide C2 at a concentration of 10 μM failed to bring about any significant changes in cell functioning, similarly to low concentrations of 1.25D and 25D. Only high concentrations of these substances (1,000 nM) brought about inhibition of keratinocyte growth. The combination of derivatives of vitamin D metabolite with ceramide C2 suppressed cell growth [20]. Ceramide analogues, in turn, exhibited various inhibitory activities. Pseudoceramide was found to be much more active than short-chain ceramides or neoceramides. Ceramide C2 reduced DNA synthesis by 13%, while neoceramide C2 – by 19%, C6 – by 15%, pseudoceramide C2 – by 38% and C6 – by 49%. In combination with 1.25D both neoceramides and pseudoceramides had a synergistic effect. In order to confirm the activation of the process of differentiation, the level of marker TG-1 under the influence of ceramides C2 and C6 was assessed. Ceramide C2 was found to be much more active (TG-1 elevation by ca. 75%) than the C6 derivative (TG-1 elevation by ca. 25%) [20].

The experiment conducted by Takeda *et al.* investigating short-chain ceramides C2 and C6 in interaction with human HaCaT keratinocytes showed that only the latter of the structural analogues of ceramides induced apoptosis [21]. A mechanism accounting for the ceramide's action on cells was proposed. According to Takeda, after entering the cell, the short-chain ceramide C6 is hydrolyzed releasing free sphingosine which is then used in the sphingomyelin cycle for the synthesis of long-chain ceramides. At the same time, results of studies by Takeda *et al.* confirmed that epidermal cells had a selective response to exogenous ceramides, depending on the length of the fatty acid chain.

In a study of HaCaT cells, Uchida *et al.* investigated the effects produced by two ceramides: short-chain C2 and natural C18 as well as two pseudoceramides: PC-104 and Bio 391, on biological processes in keratinocytes [22].

Results of the study show that pseudoceramides have a much lesser effect on the inhibition of keratinocyte

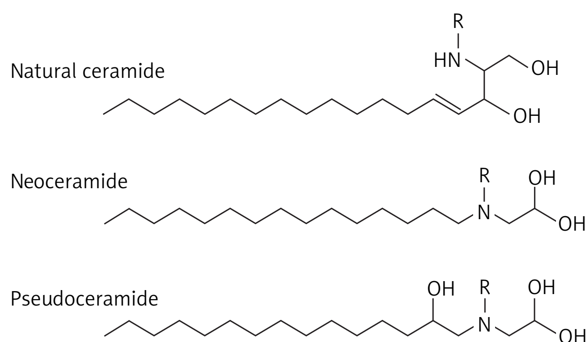

Figure 3. Ceramides used in the study including keratinocytes derived from neonatal skin. The "R" group denotes chain C2 or C6 [20]

growth. Like in other studies, ceramide C2 inhibited cell growth and increased the secretion of LDH (lactate dehydrogenase), a marker of cytotoxicity and advanced apoptotic state. Natural ceramide C18 played a part in reducing the mitochondrial membrane potential indicating the process of apoptosis. Pseudoceramides PC-104 and Bio 391, in turn, failed to reduce the membrane potential or induce the secretion of lactate dehydrogenase, which shows that both structural analogues of natural ceramides lack biological activity [21].

An important insight into how the short-chain ceramide C2 affects the HaCaT cell line and melanoma (MRC-5) cells was provided by studies conducted by Kolettas *et al.* [23]. Similarly to other cell lines, the ceramide was responsible for the induction of apoptosis, while concentration and intubation time differed depending on cell line type – it was necessary to use a higher concentration and longer intubation time for MRC-5 cancer cells. It was also proven that an overexpression of gene encoding the Bcl-2 protein efficiently lowered the probability of induction of apoptosis for both cell lines. Literature reports demonstrate that an overexpression of the Bcl-2 gene

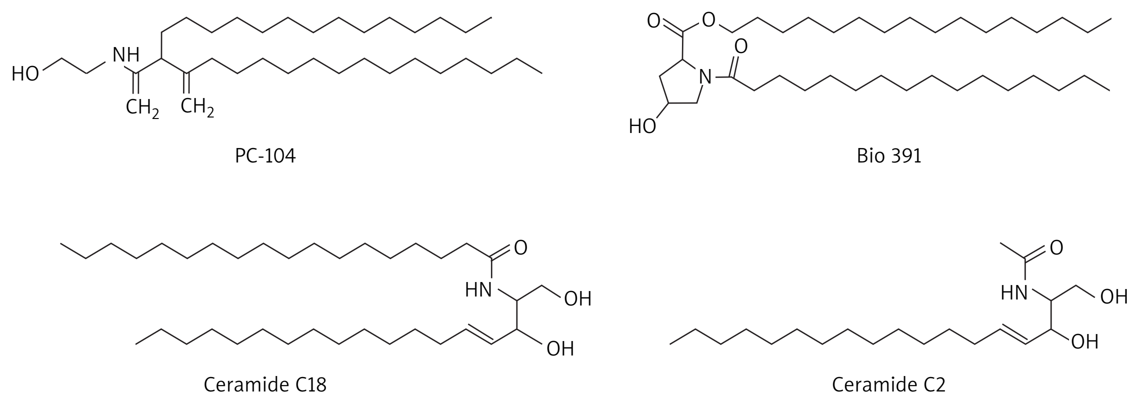

Figure 4. Ceramides and pseudoceramides used in the study by Uchida *et al.* [22]

inhibits the release of cytochrome c into the cytoplasm, thus preventing the process of apoptosis [24].

Studies also confirm that ceramidases have a significant effect on the concentration of endogenous ceramides. Reduced activity of these enzymes increases the concentration of endogenous ceramides as a result of blocking the regulation process responsible for the balance between sphingosine and ceramides. It is one of three methods of increasing the concentration of intracellular ceramides. The other two methods are: the supply of exogenous sphingomyelinase and the inhibition of glucosylceramide synthase [23, 25]. The study again revealed certain differences in the activity of substances (synthetic analogues of ceramides) depending on the cell line. D-e-MAPP ((1S,2R)-2-N-myristoylamino-1-phenyl-1-propanol) turned out to be an active inhibitor of alkaline ceramidase in leukaemia cells HL-60. For keratinocytes HaCaT, however, the inhibition of the enzyme was marginal in importance. In addition, D-e-MAPP was less active than its structural analogue D-NMAPPD ((1R,2R)-2-N-myristoylamino-1-(4-nitrophenyl)-1,3-propandiol). The research thus proves that there are methods of interfering with ceramide metabolism, which suggests that, for example, ceramidase inhibitors could be a useful weapon in combating hyperproliferative skin diseases.

From the viewpoint of practical applications of structural analogues of ceramides, important insights seem to have been provided by Bektas *et al.* in a study investigating the response of HaCaT cells following incubation with three different synthetic ceramides [26]. According to the authors, two out of three substances under study could be used as an alternative to vitamin D_3 and its analogues which are used in the treatment of psoriasis, however have an adverse effect on calcium balance. Structural analogues of ceramides were also found to inhibit the proliferation of keratinocytes and induce their programmed death. What is more, they were found to be much more fast-acting than interferon γ and K252 – the specific factor inhibiting the activity of kinases.

Summary

The current state of knowledge makes it possible to determine in detail the role of ceramides in cell life. Endogenous ceramides are not only structural components of cell membranes or intercellular cement of the stratum corneum, but they are also key factors involved in the regulation of many biological processes. Undoubtedly one of the most important functions of endogenous ceramides is their involvement in the transmission of signals triggering off the apoptosis process. Endogenous ceramides, either synthesized de novo or arising from the process of sphingomyelin breakdown, interact – either directly or indirectly – with key enzymes determining cell survival. Another important factor is that exogenous ceramides and their structural analogues are able to influence cell life processes. They produce specific cell responses depending on the type of ceramide and type of cells used in investigations. However, the majority of studies resulted in a similar effect, i.e. stimulation of the apoptosis process. The finding has given rise to discussions about possible uses of ceramides and their analogues in cancer or cardiac treatment [26]. Even though ceramides have been known for a long time, there are ongoing studies on their practical applications. Both natural ceramides and their synthetic analogues are the focus of research interest. According to literature reports, synthetic lipids can be an alternative to antiproliferative substances currently used in skin pathologies of hyperproliferative origin [26].

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