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Sphingolipids in colon cancer

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Abstract

Colorectal cancer is one of the major causes of death in the western world. Despite increasing knowledge of the molecular signaling pathways implicated in colon cancer, therapeutic outcomes are still only moderately successful. Sphingolipids, a family of N-acyl linked lipids, have not only structural functions but are also implicated in important biological functions. Ceramide, sphingosine and sphingosine-1-phosphate are the most important bioactive lipids, and they regulate several key cellular functions. Accumulating evidence suggests that many cancers present alterations in sphingolipids and their metabolizing enzymes. The aim of this review is to discuss the emerging roles of sphingolipids, both endogenous and dietary, in colon cancer and the interaction of sphingolipids with WNT/β-catenin pathway, one of the most important signaling cascades that regulate development and homeostasis in intestine

Keywords

Colorectal cancer; Sphingolipids; Dietary; Ceramide; Sphingosine; Sphingosine-1-Phosphate

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Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in western countries. The most recent data published by the World Health Organization reported an incidence of 25.0 per 100,000 women and 34.1 per 100,000 men in the USA for the year 2008. They reported, for the same year, 7.7 deaths per 100,000 women and 9.9 per 100,000 men [1]. Estimates made by the American Cancer Society for the year 2013 predicted 142,820 new cases and 50,830 deaths for both sexes [2]. Age remains a fundamental risk factor but modifiable factors such as obesity, physical inactivity, a diet high in red or processed meat, alcohol consumption, long-term smoking, and possibly very low intake of fruits and vegetables may also play an important role in colon cancer incidence. Despite the fact that sporadic colon cancer is the most common diagnosis, heredity can also be an important factor involved in colon cancer occurrence [3]. The very well described familial adenomatous polyposis (FAP) and its attenuated form (AFAP) are due to mutations in the adenomatous polyposis coli (APC) gene [4, 5]. Studies have also reported an association of colon cancer with specific hereditary syndrome such as Lynch Syndrome or MUTYH bi-allelic mutation [6–8]. Moreover, familial history of colon cancer, independently of any identifiable syndrome, is also an increased risk factor for CRC [9].

The intestinal epithelium is regenerated throughout life with intense proliferation occurring in the crypts of Lieberkühn, which contain stem cells and their progeny [10]. The progeny or transit-amplifying cells divide 4–5 times in the crypt before they migrate and differentiate completely into specialized intestinal epithelial cell types. The homeostasis of intestinal epithelium is based on a tight regulatory balance between self-renewal at the bottom of the crypt and differentiation [11]. It is disruption of this tightly regulated balance, through a series of known mutations that leads to intestinal tumorigenesis. Sporadic CRC is one of the best studied malignancies and the various stages of the disease have been well defined [12]. It is believed that CRC occurs as a result of the activation of oncogenes coupled with the inactivation of tumor suppressor genes. Mutations in several genes are required (WNT/β-catenin pathway, K-Ras, Smad4, p53) and although these genetic alterations occur in a preferred sequence, the accumulation of changes determines the tumor properties [11]. Aberrant crypt foci (ACF) is the smallest identifiable lesion and is considered the putative premalignant lesion for colon cancer [13]. ACF may present different histologic features ranging from hyperplasia to dysplasia, and their involvement in CRC is not totally understood. The most common ACF is associated with hyperplastic crypts, and generally presents mutations in K-Ras although it rarely develops malignant carcinoma. However, the dysplastic ACF presents mutations in APC and is frequently associated with CRC [14]. Adenomas progress into carcinomas and malignancy coincides with inactivation of the p53 gene in 50% of the tumors. In general it is believed that CRC evolves through a series of restriction points, at which only those cells acquiring the correct mutational advantages can expand.

Sphingolipids constitute a family of lipids that are evolutionarily conserved among all eukaryotes. For many years, these molecules were considered structural components of biologic membranes, however studies over more than two decades have disclosed important biologic activities of many sphingolipids. Sphingolipids such as sphingosine (SPH), ceramide, sphingosine-1-phosphate (S1P) have been implicated as modulators of physiologic and pathophysiologic processes such as cell growth, cell death, autophagy, angiogenesis, cell adhesion, differentiation, migration, senescence, intracellular trafficking, stress [15–17] and inflammatory responses [18]. Ceramide and S1P often exert opposing functions in the cell; as ceramide has been shown to mediate cell cycle arrest and cell death in response to cell stress [19, 20], S1P has been shown to promote cell survival and proliferation [21]. The flux between these various metabolites is tightly controlled through

several enzymes which are critical in regulating the levels and function of these bioactive sphingolipids.

Alterations in bioactive sphingolipids and their metabolism have been linked to several human diseases including cancer [22–24]. Accumulating evidence also suggests that alterations of the sphingolipid pathway, both by addition of dietary sphingolipids or modification of endogenous enzymatic activity, may play a role in colon cancer development which will be the subject of discussion in this review.

Part I: Sphingolipids, a class of bioactive lipids

Briefly, ceramide is considered the central hub of sphingolipid metabolism. The *de novo* pathway of ceramide synthesis starts with the condensation of serine and palmitoyl-CoA catalyzed by serine palmitoyl transferase to generate 3-keto-dihydrosphingosine [25]. 3-keto-dihydrosphingosine is reduced to form dyhydrosphingosine (sphinganine), that is then N-acylated by (dihydro)ceramide synthases (CerS) to produce dihydroceramide by the action of one of six (dihydro)ceramide synthases [26]. Ceramide is then generated as the product of dihydroceramide desaturase. Ceramide can be converted to sphingomyelin (SM) by sphingomyelin synthase, and SM can be hydrolyzed into ceramide by the activity of several different sphingomyelinases (SMase) characterized by the pH required for optimal enzymatic activity [27–29]. Ceramide can also be glycosylated by glucosylceramide synthase (GCS) to generate glucosylceramide [30]. In addition, ceramide can be further hydrolyzed to produce SPH through the action of several different ceramidases (CDase), also characterized by the pH required for optimal activity. SPH can in turn be phosphorylated by sphingosine kinases 1 and 2 (SK1 and 2) to form S1P or reacylated back to ceramide [31]. S1P can be metabolized back to SPH by S1P phosphatases, whereas S1P lyase (SPL) irreversibly cleaves S1P to generate ethanolamine phosphate and hexadecenal resulting in the only exit pathway from metabolism [32, 33].

Alterations in bioactive sphingolipids and their enzymes have been linked to several different pathologies including: Faber disease [lack of acid CDase (aCDase) activity], Fabry disease [deficiency in alpha-galactosidase A (GLA)], Niemann-Pick disease [lack of acid SMase (aSMase) activity], Gaucher disease (lack of β-glucosidase), Tay-Sachs disease, Sanhoff disease and GM2A deficiency (accumulation of GM2 gangliosides), (reviewed in [34]). Other diseases such as Alzheimer's [35], atherosclerosis [36], cystic fibrosis [37] and cancer have also been linked to alterations of sphingoipids. In addition, sphingoipids have been associated with cancer progression and response to cancer treatment, including resistance to chemotherapeutic drugs. Indeed, several cancer models have shown significant alterations in enzymes implicated in ceramide generation [aSMase, neutral SMase (nSMAse), CerS] and degradation [aCDase and neutral CDase (nCDase)], resulting in loss of ceramide [38]. In addition, high levels of S1P have been detected in several cancers as a result of alteration of enzymes implicated in its phosphorylation and catabolism [39]. Although this review is focused on the role of sphingoipids in colon cancer, it is important to note that sphingoipids may also play a significant role in other cancers including prostate cancer, breast cancer and head and neck cancer (reviewed in [24]).

1.1. Metabolism of sphingoipids in the colon

Sphingoipids are enriched in the apical membrane of the polarized cells found throughout the intestinal tract. They are considered essential for structural integrity, and it is believed they may act as receptors for toxins, viruses, and bacteria [40]. In particular, the small intestine is characterized by an unusually high content of sphingolipids, comprising 40% of the lipids localized in the apical membrane [41]. The metabolism of dietary SM begins with the release of cholecystokinin from endocrine cells in the intestine, which then stimulates

the release of alkaline SMase (alk-SMase) from the gallbladder as well as the secretion of trypsin from the pancreas. This trypsin cleaves the alk-SMase bound in the intestinal mucosa to be released into the lumen for enhanced enzymatic activity [42]. Alk-SMase hydrolyzes SM into ceramide within the intestinal lumen, and this ceramide is further hydrolyzed by the membrane bound nCDase to form SPH. SPH is absorbed inside the enterocytes by a diffusion mechanism and can be phosphorylated and converted to S1P by the action of SK1 and SK2. Inside the enterocytes, S1P, as mentioned above, can be degraded by SPL or synthesized back to ceramide and other complex sphingolipids [32].

1.1.1 Alkaline SMase—In several studies, alk-SMase has been implicated in the development of colon cancer [43, 44]. In normal tissue, a gradient in the expression and activity of alk-SMase from the ascending colon to the rectum has been shown, while in CRC this activity is decreased by 75% with almost undetectable levels in adenoarcinomas [45]. Measurement of alk-SMase activity in CRC patients revealed that the decreased activity in adenomas is not directly linked to mutations in the APC gene [43]. Interestingly this was also observed in patients with chronic colitis [46]. The expression of alk-SMase has been reported to be changed in response to dietary factors and also in response to two chemical agents. The first, ursodeoxycholic acid (UDCA, present in bile), increased the activity of alk-SMase in intestinal mucosa and inhibited the development of colon carcinoma [47]. This finding has been confirmed *in vitro* in HT29 cells, where treatment with UDCA increased alk-SMase activity which was associated with a decrease in cell proliferation and caspase activation [48, 49]. The second factor associated with increasing alk-SMase activity is 5-aminosalicylic acid, an anti-inflammatory drug that has been shown to increase alk-SMase levels in the colonic mucosa after ingestion [50]. Both agents have also shown chemopreventive effects against colon cancer [50, 51]. The decrease of alk-SMase activity detected in colon cancer reduces the hydrolysis of SM and therefore reduces ceramide generation [52]. Since ceramide can inhibit proliferative and anti-apoptotic pathways and also activate anti-proliferative and apoptotic pathways, lack of ceramide in colon cancer may contribute to colon cancer development.

1.1.2. Sphingosine kinase—As discussed above, SK1 and 2 can phosphorylate SPH to form S1P, which exerts proliferative effects in colon cancer cells. Expression of SK1 and 2 have been shown in both the small intestine and in the colon [53]. SK1 has been shown to be over-expressed in human colon cancer compared to normal colon mucosa [54]. In particular, adenomas have higher expression of SK1 when compared to normal mucosa, and metastatic colon cancer has higher SK1 expression than non-metastatic cancer [39]. Kohno *et al* showed that SK1 expression was required for small intestinal tumor cell proliferation in $APC^{Min/+}$, mouse model for intestinal neoplasia, since deletion of the SK1 gene in these mice suppressed adenoma size but not their incidence. This study also demonstrated that epithelial cell proliferation in the polyps was significantly attenuated suggesting that SK1 may regulate adenoma progression [55]. Interestingly, deletion of S1P receptors 2 and 3, and the loss of an allele in receptor 1 did not recapitulate the SK1 deletion. Moreover, while tissue SPH content was elevated in the adenomas of $APC^{Min/+}$ $SK1^{-/-}$ as expected, S1P levels were not significantly altered. In another colon cancer murine model of colitis associated cancer induced with azoxymethane (AOM) and dextran sulfate sodium (DSS), SK1 and S1P were significantly elevated in colon cancer tissues compared to normal mucosa [39]. Additionally, $SK1^{-/-}$ mice subjected to AOM/DSS treatment presented not only significantly fewer ACF but also a reduction in colon cancer development [39]. Interestingly, $SK2^{-/-}$ mice have been shown to develop tumors similar to WT mice upon administration of AOM/DSS [56]. These results suggest that decreased SK1, but not SK2 activity, decreases both proliferation and colon cancer formation, further suggesting a

pivotal role for bioactive sphingolipids (possibly a combination of effects of S1P and ceramide) in CRC.

1.1.3. S-1-P Lyase (SPL) and S-1-P phosphatase—The levels of S1P in the colon are not only controlled by SKs, but also by enzymes that control its metabolism, and these include SPL and S1P phosphatase. S1P can be metabolized back to SPH by S1P phosphatases or by type 2 phosphatidate phosphohydrolases. In addition, S1P can also be degraded in an irreversible manner by SPL. In the apex of the intestinal villi, there is a strong catabolism of S1P due to high expression of SPL. As proliferation in the intestinal epithelium occurs at the bottom of the crypt, controlling the levels of S1P by SPL at the top of the crypt may be a mechanism to prevent mitogenic signaling and promote the rapid cell turnover characteristic of this tissue [10]. It is important to note that S1P has been also implicated in live cell extrusion in colon epithelium, which has been suggested to be a tumor-supressive mechanism that prevents the accumulation of excess epithelial cells and maintains homeostatic cell number by matching the number of dividing cells with the number of dying cells [57]. $APC^{Min/+}$ mice have not only high levels of SPH and S1P in general, but also show decreased expression and activity of SPL in adenomas when compared to intestinal epithelium [58]. In colon cancer patients, the expression of SPL (and also S1P phosphatase) has been shown to be downregulated, indicating that breakdown of S1P may be blocked in colon cancer [59].

1.1.4. Neutral ceramidase—nCDase is a key enzyme in regulating the balance between ceramides, SPH and S1P. In the intestine this enzyme is expressed in the plasma membrane of intestinal epithelial cells, and includes a transmembrane domain and an extracellular catalytic domain [60]. Intestinal nCDase was initially purified in rat intestine mucosa [61] and in human gastrointestinal tract through the analysis of ileostomy contents [62]. Like alk-SMase, nCDase distribution is higher in the small intestine, peaking at the jejunum, and decreasing in the colon [63, 64]. $nCDase^{-/-}$ mice are viable and do not present any severe pathology [65]. While in general sphingolipids levels were similar to those in control mice, the levels of C16:0 ceramide were significantly increased in the intestinal tract. Also, after feeding the $nCDase^{-/-}$ animals a diet supplemented with 0.1% milk SM, the amount of ceramide in feces was much higher than in the control mice, suggesting defective intestinal digestion of dietary sphingolipids [65]. The role of nCDase in the development of colon cancer has yet to be elucidated.

1.1.5. Glucosylceramide synthase—GCS catalyzes the formation of glycosphingolipids. There is evidence suggesting that conversion of ceramide into glycosphingolipids is related to cancer invasiveness, metastasis and drug resistance. Ceramide glycosylation, through GCS, allows cellular escape from ceramide-induced programed cell death in cancer cell lines [66]. It is been shown that glucosylceramide accumulates in adriamycin-resistant breast carcinoma cells, in vinblastine-resistant epithelioid carcinoma cells, and in tumor specimens from patients showing poor response to chemotherapy [67]. This multidrug resistance can be increased by overexpression of GCS, and also can be reversed in human cancer breast cells by GSC gene targeting with siRNA [68]. In vitro, colon cancer cells resistant to drug treatment exhibited high levels of expression of GCS [69], while inhibition of this enzyme resulted in enhanced apoptosis [70].

Altogether, these studies suggest that alterations in the metabolism of sphingolipids may play a critical role in susceptibility to colon cancer. Several studies suggest an association between colon carcinoma and changes in sphingolipids enzymes as shown in Figure 1. These alterations in enzyme expression and activity suggest that colon cancer may be associated with decreased ceramide and increased S1P levels.

Part II. Dietary sphingolipids in colon cancer

Sphingolipids form a natural part of a normal diet, although there is no known nutritional requirement for them [71]. Even though most of the sphingolipids that enter the gastrointestinal system are hydrolyzed in the small intestine, a small percentage also enters the large intestine. The effect of dietary sphingolipids in colon cancer has been actively studied both *in vitro* and *in vivo*. Evidence of the importance of dietary sphingolipids in CRC was first described by Dudeja *et* al. [72], where they found differences in both sphingolipid composition and sphingolipid enzyme activities between tumor and normal tissue. In particular, their data demonstrated that mice treated with the pro-carcinogen 1.2-Dimethylhydrazine (DMH) displayed an increase in SM levels in colonic tissues even before the appearance of adenomas, and this was accompanied by a reduction of SMase. DMH treatment also decreased glyco-sphingolipids and induced changes in the glyco-sphingolipid pattern [73]. These early discoveries, together with evidence suggesting that high-fat diet increases the risk of colon cancer, provided a basis to investigate the effect of dietary sphingolipid in colon cancer.

2.1 Effects of sphingolipids in colon cancer cells

Sphingoid bases and ceramide have been shown to induce apoptosis in colon cancer cells and suggested as potential mediators of the protective role of more complex dietary sphingolipids in CRC development [74]. Also, the interplay between ceramides of various chain lengths seems to be crucial for cancer progression. Ceramide production and its biological function are highly dependent on the length of the covalently linked fatty acid controlled by the six isoforms of mammalian CerS (1 to 6). They differ in their tissue expression pattern and in their substrate specificity resulting in the production of ceramides with different N-acyl side chains. For example, cyclooxygenase-2 (COX-2) inhibitor (celecoxib) mediates its antiproliferative effects, in part, by selectively activating CerS6 in human colon carcinoma in HCT116 cells, generating an increase of C16:0 ceramide. Overexpressing CerS 2, 4 or 6 in colon and breast cancer cells demonstrated that overexpression of either CerS4 or 6 increased generation of long chain C16:0, C18:0 and C20:0-ceramides, yet the overexpression of CerS2 had no effect on ceramide production. The upregulation of CerS4 or 6 led to the inhibition of cell proliferation and induction of apoptosis, while upregulation of CerS2 increased cell proliferation [75].

It is important to remark that colon cancer cells present differential expression of certain proteins in response to ceramide. In particular, HCT116 cells treated with C6-ceramide showed 43 proteins differentially expressed compared to untreated cells, and many of these proteins are implicated in different cellular process such as apoptosis (caspase-8, caspase-10) and growth arrest (PCNA) [76].

Thus altered sphingolipid composition has been shown in colon cancer cells [77, 78]. Specific sphingolipids (and bioactive sphingolipid metabolites) together with changes in the expression and/or function of sphingolipid-metabolizing enzymes, could impact therapeutic response. Here we will review the effects of exogenous sphingolipids in colon cancer cells, effect of specific targeting of sphingolipid metabolism and alteration of sphingolipid metabolism by chemotherapeutic drugs (Figure 2).

2.1.1 Effects of adding exogenous sphingolipids—Cell death, via activation of caspase-3 and cytochrome c release in SW403 cells has been demonstrated to occur upon treatment with two short-chain ceramide analogues (C2 and C6-ceramide) and two inhibitors of CDases (D-MAPP and B13). Also, the cytotoxic effects of C6-ceramide in colon cancer cells (LoVo, HT29 and HCT15) can be magnified when combined with different P-glycoprotein antagonists such as tamoxifen, cyclosporine A, VX-170 and verapamil (that

inhibit conversion of ceramide to glucosylceramide) with an increase in caspase activation, PARP cleavage, DNA fragmentation, cell cycle arrest, increased mitochondrial membrane permeability and enhanced expression of p53 [79]. In another study, SPH, sphinganine and C2-ceramide [74], but not C2-dihydroceramide, inhibited growth and caused death in colon cancer cells (HT29 and HCT-116) in a time and dose-dependent manner. In addition, C2-ceramide also induced apoptosis and cell cycle arrest at the G2/M phase causing an accumulation of cells in the S phase [74]. The 4,5-trans double bond of ceramide was necessary for the apoptotic effects of C2-ceramide, but not for the sphingoid bases [80]. C2-ceramide has been implicated in apoptosis and cell cycle arrest, as well as in mediating macroautophagy by increasing proteolysis and accumulation of autophagic vacuoles in HT29 cells. In this case ceramide reversed the interleukin 13-dependent inhibition of macroautophagy by interfering with the activation of Akt and stimulating the expression of the autophagy gene product beclin-1, implicating ceramide for the first time in control of a major lysosomal pathway [81]. Of particular interest is that SPH and its methylated derivative N,N-dimethylsphingosine (DMS), a non-specific SK inhibitor, induced apoptosis specifically in colon cancer cells (HT29, HRT18, MNK74 and COLO205) but not in primary cultures [HUVEC (human umbilical vein endothelial cells) or mesangial cells] [82]. Treatment of SW403 with a cationic long chain ceramide [ω-pyridinium bromide D-erythro-C16-ceramide (LCL-30)], targeting negatively charged mitochondria, induced cell death with mitochondrial accumulation and subsequent release of cytochrome c and activation of caspase-3 and caspase-9 [83]. Sphingoid bases prepared from plants also decreased cell viability in SW403 in a dose-dependent manner, similar to that of cells treated with SPH, and its effects were, at least in part, mediated by activation of caspase-3 [84].

In cancer cells, including colon cancer cells, S1P has been shown to stimulate proliferation by activating p38 and ERK MAP kinases [85]. In particular, S1P activated invasion, proliferation and protection from cytotoxic agents in HT29 and interestingly, addition of anti-S1P monoclonal antibody reversed all these processes by increasing activation of caspase-3 [85]. These results, together with the effect of S1P antibodies reducing tumor progression in murine xenografts and allografts, suggest the bioactive lipid S1P as a target for cancer therapy [86].

SM metabolism seems to play an important role in response to therapy, as some chemotherapeutic drugs (daunorubicin), H_2O_2, heat, and ionizing radiation induce formation of ceramide from SM hydrolysis as well as from activation of the *de novo* and salvage pathways. The resulting ceramide has been shown to be responsible for initiating the apoptotic response. Exogenous administration of SM to colon cancer cells (HCT15 and MOSER) increased 5- fluorouracil (5-FU) and doxorubicin sensitivity by 100–300%, however this effect was not achieved in other colon cancer cell lines such as HT29, Lovo and WiDr, nor in HUVEC [87], suggesting that these different responses to co-treatment with SM may be due to alterations in SM/ceramide metabolism in the non-responsive cells. To study this possibility, HT29 cells cultured with gradually increasing concentrations of colchicine (an antimitotic agent) not only displayed an increased resistance to colchicine, doxorubicine, etoposide, vincristine and taxol, but also showed higher levels of glucosylceramide and galactosylceramide, confirming that specific changes in sphingolipid levels could be associated with different responses to therapeutic agents [88].

TRAIL (TNF-Related Apoptosis-Inducing Ligand) is a member of the tumor necrosis factor superfamily that selectively induces apoptosis in malignant cells, although not all cancer cells are susceptible [89]. Resistance to TRAIL has been associated with defects in ceramide signaling in colon cancer cells. To investigate whether TRAIL resistance was due to low ceramide levels or defects in sphingolipid metabolism, two colon cancer cells (SW480 and SW620) from the same patient (primary and subsequent metastasis respectively) with

different sensitivity to this pathway were analyzed. While the overall levels of ceramide were comparable in both cell lines, SW480 cells, which respond to TRAIL, contained a higher percentage of C16 and C18-ceramide and lower C24-ceramide than the TRAIL-resistant SW620 cells. Upon TRAIL treatment mainly C16-ceramide was increased in SW480 cells but not SW620, parallel with caspase-3/7 activation. Interestingly, combination of C6-ceramide with TRAIL resulted in apoptosis in SW620 cells. These results suggest that ceramide plays a role in promoting TRAIL-mediated apoptosis and that TRAIL-resistant cancers may benefit from combination therapy with ceramide or agents that enhance ceramide accumulation [90]. Later studies identified CerS6, which preferentially generates C16-ceramide, as a protein able to influence susceptibility to TRAIL [91].

2.1.2 Specific targeting of sphingolipid metabolism and its effect on colon cancer cells—Modification of sphingolipids affects metabolism and apoptosis in colon cancer cells. The CDase inhibitor B13 proved to be specific for colon cancer cells since normal liver cells were resistant to treatment with no increase of ceramide or apoptosis [92]. The effect of increased ceramide levels in HT29rev cells by either incubating the cells with bacterial SMase (bSMase) or adding C2-ceramide was evaluated by Veldman *et al.* [93]. Treatment with C2-ceramide resulted in rapid accumulation of the compound by the cells and in induction of apoptosis, whereas bSMase treatment did not induce apoptosis despite hydrolyzing cellular SM and increasing ceramide levels. The bSMase-generated ceramide, however, was converted to more complex sphingolipids. Even after the use of inhibitors to block this conversion into more complex lipids, thereby inducing an accumulation of ceramide in the cell, apoptosis was not detected. These results suggested that C2-ceramide is able to reach putative intracellular targets involved in the propagation of the apoptotic signal, but not ceramide generated by aSMase [93]. Ceramide generated by the addition of exogenous aSMase contributes to tumor necrosis factor alpha (TNFα) mediated apoptosis in HT29 [94]. In a complementary study TNFα induced apoptosis in a time and dose-dependent manner, but downregulation of aSMase prevented TNF-stimulated apoptosis [95]. In addition, the relationship between SK, cellular ceramide concentration, and chemosensitivity was investigated in nine colon cancer cell lines. SK1 and SK2 activity and protein expression were the highest in RKO cells and the lowest in HCT116 [96]. HCT116 were sensitive to the effects of oxaliplatin, while RKO were the most resistant. Treatment of HCT116 with oxaliplatin increased C16, C24 and C24:1-ceramides, but not in RKO. Pretreatment of HCT116 with an inhibitor of nSMase suppressed the increase in ceramide levels and activation of caspases, suggesting that ceramide formation was due to the activation of nSMase, instead of aSMase. In contrast, treatment of RKO with SK1 and SK2 siRNA suppressed cell viability, increased caspase activity and cellular ceramide formation upon oxaliplatin treatment. Another pathway demonstrated to be affected by SK1 and SK2 silencing is the AKT pathway, decreasing its phosphorylation and increasing p53 and p21 protein levels in response to oxaliplatin. These studies indicated that SK and nSMase contribute to the regulation of chemosensitivity controlling ceramide formation and affecting the AKT pathway [96].

2.1.3. Alteration of sphingolipid metabolism by chemotherapeutic drugs—CerS6 is a key enzyme implicated in the response to chemotherapy. Treatment of HCT116 cells with celecoxib, which induces apoptosis and inhibits proliferation in cancer cells, led to a significant increase in the levels of C16-ceramide, with a concomitant increase of CerS6 activity [97]. siRNA against CerS6 showed that this enzyme was responsible for the increase of C16:0-ceramide and also partially protected the cells from the cytotoxic effects of celecoxib [97]. These results were confirmed *in vivo* using HCT-116 xenografts. In another study, HT29 treated with the topoisomerase I inhibitor camptothecin (first line treatment of solid CRC, and in second line for 5-FU resistant patients) increased the levels of ceramide

through activation of serine-palmitoyltransferase and CerS activities, and this ceramide induced growth inhibition via caspase-3 activation in a p53-independent manner [98].

Fenretinide, a synthetic retinoid, induced an increase of ceramide levels in neuroblastoma cells and also in colon cancer cells (HT29 and LoVo). This increase in ceramide was associated with an increase of cell death by a combination of apoptosis and necrosis in a p53 and caspase-independent manner. Interestingly, this effect can be enhanced in combination with safingol, an inhibitor of protein kinase C and SK [99]. Importantly, recent studies showed that fenretinide directly inhibits dihydroceramide desaturase in cells, raising the possibility that many of the actions of this compound may be mediated by its ability to alter ceramide metabolism [100, 101].

Using RNAi for CerS6 resulted in a specific decrease in C16-ceramide and protected SW480 cell against TRAIL-mediated apoptosis, while increasing CerS6 expression sensitized SW620 cell to TRAIL. Another way to circumvent CRC cell resistance to TRAIL-mediated apoptosis is by using COX-2 inhibitors to manipulate lipid metabolism. COX-2 inhibition sensitizes human colon carcinoma cells to TRAIL-induced apoptosis by inducing clustering of the TRAIL receptor DR5 at the cell surface and redistribution of the death-inducing signaling complex components into cholesterol-rich and ceramide-rich domains known as caveolae. It is believed that this mechanism enhances the initiation of receptor-mediated signal transduction. This process requires accumulation of arachidonic acid and sequential activation of aSMase for the generation of ceramide within the outer leaflet of the plasma membrane [102].

2.2. Effects of dietary sphingolipids in different colon cancer models

The effects of dietary sphingolipids in colon cancer have been studied in different animal models. Here we will review the effects of sphingolipids in chemically-induced colon cancer models such as DHM and AOM, inherited models ($APC^{Min/+}$ mouse), and also colon cancer xenografts.

2.2.1. DMH-induced colon cancer model—Many studies involving dietary sphingolipids (SM, Glucosylceramide, lactosylceramide, ganglioside GD3 extracted from milk, synthetic SM, synthetic dyhydroSM, ceramide analogs, plant sphingolipids) in DMH-treated animals demonstrated a chemotherapeutic as well as a chemopreventive effect. Dillehay *et al.* showed that CF1 female mice treated with DMH and fed with AIN76A, a diet very low in sphingolipid content, (comprising less than 0.005% weight) supplemented with milk SM exhibited a significant reduction in the number of ACF when compared with the control group. Tumor incidence in the control group was 47% while in mice supplemented with SM the incidence was reduced to 20% [103]. In a follow up study that included a longer time course, SM supplementation also caused a reduction in ACF and the number of aberrant crypts per focus. However, after 40 weeks of treatment, there was no difference in tumor incidence. What was remarkable in this instance was that while all the tumors found in animals fed with AIN76A were adenocarcinomas, at least 31% of the tumors in mice fed with a diet supplemented with SM were adenomas, suggesting that dietary SM may also suppress the appearance or advancement to more malignant tumors [104]. In an effort to better understand the effects of dietary sphingolipids in colon cancer, Schmeltz *et al.* [105] studied the effects of chemically synthesized sphingolipids. CF1 female mice treated with DMH were fed the AIN76A diet supplemented with either SM from milk, N-palmitoylsphingomyelin, or N-palmitoyldihydrosphingomyelin for 4 weeks. The number of ACF in the SM-fed groups was significantly lower than in the control group by 54%, 52%, and 70% for milk SM, synthetic SM, and synthetic dihydroSM, respectively. This study confirmed that SM suppressed ACF formation, and that the potency of

dihydrosphingomyelin did not depend on the 4,5-trans bond of the sphingolipid backbone. Since most sphingolipids are digested in the small intestine, an analogue of ceramide (Cer-β-glucuronide) was specifically designed to be delivered to the colon [106]. Cer-β-glucuronide induced not only a 37% decrease in the number of ACF but also reduced the number of BrDU-positive cells (20%) in the lower half of the colonic crypts [106]. Other sphingolipids from milk were also evaluated. Glucosylceramide, lactosylceramide, or ganglioside GD3 induced at least a 40% decrease in the number of ACF (comparable with the reduction accomplished by SM), and was accompanied by a decrease of proliferation in the colonic crypts. Interestingly, there was no difference in the number of apoptotic cells per crypt among the different groups and the controls [106]. It is important to remark that dietary sphingolipids showed both chemotherapeutic and chemopreventive effects as demonstrated by studies performed by Lemonnier *et al.* where they showed DMH-treated mice receiving SM before or after tumor initiation induced a similar decrease in the number of tumors formed and in proliferation in the lower half of the colonic crypts, as well as a similar increase in the number of apoptotic cells [107]. Finally, since plant sphingolipids differ structurally from mammals, their effect in colon cancer has also been evaluated. Soy glucosylceramide (GlcCer), added to the AIN76A diet, decreased colonic cell proliferation in the upper half of the crypts by 50%, reduced the number of ACF and also reduced the number of adenomas [108].

2.2.2 AOM-induced colon cancer model—The effect of sphingolipids in diet was also tested in animals treated with AOM. Rats treated with 35mg/kg of SM orally during the 6 weeks following AOM treatment decreased the number of ACF, specifically in the proximal end of the colon, and also induced a decrease in proliferation in the base of the crypt. This treatment did not affect any of the immune functions tested (antibody formation, delayed type hypersensitivity or natural killer cell cytotoxicity) [109].

Tumor suppressor gene p53 plays an important role in maintaining intestinal homeostasis in the colon by regulating DNA repair and apoptosis. In fact, a very high percentage of colon cancer tumors present mutations in p53, especially in the advanced stages [110]. To evaluate the role of p53 in the effects of dietary sphingolipids on colon cancer, p53 wild type and p53+/− mice were treated with AOM and fed a diet containing 0.1% SM for 4 weeks. The short-term results indicated that p53 status did not modify the effects of sphingolipids on proliferation and apoptosis. When mice were euthanized 33–38 weeks later, SM administration produced no significant effects on either tumor incidence or size in both genotypes (24% *vs.* 38.1%). However, both tumor incidence and size trended lower with dietary sphingolipid [111], demonstrating that the effect of dietary SM in colon cancer was not p53-dependent.

Sprague-Dawley rats treated with AOM were utilized to indirectly evaluate the effects of sphingolipids in colon cancer using UDCA. This compound was previously shown to exert an antiproliferative and proapoptotic effect in HT29 cells, and this was accompanied by a rapid increase in alk-SMase activity. AOM treated rats showed that UDCA induced a reduction in the number of ACF containing three or more crypts. While AOM treatment by itself reduced mucosal alk-SMase activity, treatment with UDCA increased the activity of both colonic nSMase and aSMase [49].

2.2.3. APC$^{Min/+}$ mice colon cancer model—Orally administered sphingolipids not only showed a decrease of ACF and tumors in colon cancer models chemically induced with either DHM or AOM, but their effect has also been described in inherited models such as the APC$^{Min/+}$ mouse. APC$^{Min/+}$ mice treated with Enigmol, a sphingoid base analogue ((2S,3S, 5S)-2-amino-3,5-dihydroxyoctadecane) that cannot be phosphorylated by SK1 and is poorly N-acetylated, showed a decrease of 50% in the number of tumors when administered orally

at 0.025% (w/w) of the diet. At these doses, Enigmol had no adverse effects on body weight on any of the liver and kidney biomarkers, even though higher doses could cause dehydration [112].

The effects of plant sphingolipids in colon cancer were also studied in $APC^{Min/+}$ mouse, where a diet supplemented with 0.1% GlcCer reduced the number of adenomas by 70%. The effects of sphingolipids from plants on gene expression in the intestinal mucosal cells on $APC^{Min/+}$ mice were analyzed using Affymetrix Gene Chip microarrays. Soy GlcCer affected the expression of at least 96 genes by ≥2 fold in a dose-dependent manner, increasing 32 and decreasing 64. As an example, HIF1α and TCF4, two relevant genes implicated in cancer, showed a significant decrease in mRNA levels that was also confirmed by RT-PCR [108].

2.2.4. Human xenografts—Human xenografts have been used in mice to evaluate the effects of sphingolipids in colon cancer. In particular, xenografts have been mostly used to demonstrate that sphingolipids can enhance and/or mediate the efficacy of anti-cancer drugs. A human colon cancer xenograft HCT116 showed a drastic decrease in tumor volume when treated with a drug that increases the levels of ganglioside GM3 (glycosphingolipid) [113]. SM has been shown to increase the effect of 5-FU (standard first-line chemotherapy for colon cancer) in HT29 human xenografts. In this study, SM was delivered intravenously (10mg/day per 7 days) to ensure it readily reached the tumor. Neither 5FU nor SM alone had an effect on tumor growth; however, when used in combination tumor growth was reduced approximately 250% compared with the untreated control group [87]. In a follow up study, mice bearing HT29, HCT15 and GW-39 xenografts also showed an increase in the tumor response to 5-FU or irinotecan treatment when combined with exogenous SM. This effect was attributed to a reversal of the apoptotic attenuation, without inducing significant hematopoietic, hepatic or renal toxicity [114].

Celecoxib, an inhibitor of COX-2 that induces pro-apoptotic and growth inhibiting effects in cancer cells and activates the sphingolipids pathway was used to treat HCT116 human xenograft in order to confirm results obtained *in vitro* (in more detail below). This compound inhibited tumor growth [115] accompanied by an increase in the levels of C16:0-ceramide in stomach, small intestine, and tumor tissues with no detectable differences in other tissues (brain, lung, heart and testes) [97].

Part III. Sphingolipids in WNT/β-catenin pathway

Wnt pathway mutations are frequent in colon cancer. The central protein in this pathway is β-catenin, a cytoplasmic/nuclear protein that is regulated by phosphorylation and by proteolysis [116]. Axin, a tumor suppressor protein, acts as the scaffold of this complex interacting directly with β-catenin, APC (another suppressor protein), CK1 and GSK3α and/or β. When this pathway is not activated, CK1 and GSK3α/β sequentially phosphorylate β-catenin at Ser/Thr residues near the N-terminus. Once β-catenin is phosphorylated, it is recognized by β-TrCP, a component of E3 ubiquitin ligase complex, whereby β-catenin is ubiquitinated and targeted for destruction by the proteasome. Upon activation, the destruction complex is disassembled and, as a consequence, stable non-phosphorylated β-catenin accumulates and translocates to the nucleus, where it binds to the LEF/TCF transcription factors promoting the transcription of genes that include cyclin D1 and c-myc [117]. Mutations in the APC gene cause FAP, a hereditary cancer syndrome that leads to formation of colon adenomas in early adulthood. Also, most cases of CRC result in acquired loss of both APC alleles. In general, loss of APC function leads to inappropriate stabilization of β-catenin that induces its mobilization to the nucleus. Mutations in APC are not the only important genetic mutations; other mutations in other proteins of the WNT/β-catenin

pathway occur, including point mutations that remove the regulatory N-terminal Ser/Thr residues in β-catenin or mutations in Axin2 [116].

The relationship between sphingolipids and the WNT/β-catenin pathway has been described in different systems. In *Drosophila*, downregulation of its unique ceramide synthase protein led to a decrease in the activity of the WNT/β-catenin pathway [118]. Also, ASAH1 knockdown in human adrenal cortical cells not only led to a decrease in cell proliferation, but also a decrease of the levels of β-catenin, together with a decrease of PCNA and cyclin B2 [119]. Another enzyme of sphingolipid metabolism, GCS, upregulated MDR1 (multi drug resistance transporter) expression in the regulation of cancer drug resistance thorough β-catenin signaling by diminishing its degradation and increasing its translocation to the nucleus [120]. In breast cancer cells, cell confluence induced up-regulation of nSMase2. This up-regulation increased ceramide levels and induced dephosphorylation of β-catenin on threonine41/serine45, and this effect was prevented using siRNA for nSMase2. Furthermore, addition of long or very long chain ceramides induced dephosphorylation of β-catenin. The phosphatase implicated in this dephosphorylation was identified as PP1cγ as determined by using phosphatase inhibitors and siRNA [121]. On the other hand, and to counter the ceramide effects, S1P in osteoblasts activated the PI3K/AKT/GSK3β pathway leading to the promotion of nuclear translocation of β-catenin [122].

The effects of sphingolipids in the WNT/β-catenin pathway is particularly important in colon cancer cells, since β-catenin is one of the most important proteins regulating development and homeostasis [123]. One of the most used animal models for colon cancer is the $APC^{Min/+}$ mice (truncated APC gene product), which are particularly useful not only to study the effect of dietary sphingolipids in adenomas but also to determine their effect in the WNT/β-catenin pathway. Dietary sphingolipids induced not only a decrease in the number of adenomas in this model, but also resulted in redistribution of β-catenin. In this study, mice were fed AIN76A alone, or AIN76A supplemented with either sphingolipids isolated from milk, supplemented with ceramide, or supplemented with a mix of sphingolipids and ceramide to ensure all the metabolites reach all regions of the intestine. After 65 days, the control mice presented an average of 55.8±4.6 adenomas/mouse, while the other groups displayed a decrease of at least 40% with a maximum reduction of 50% in the mice fed a mixture of ceramide and more complex sphingolipids. While β-catenin distribution in normal mice was mostly restricted to the membrane of the intestinal epithelium, in $APC^{Min/+}$ mice β-catenin localization was more diffuse throughout the cell. The animals fed the sphingolipid enriched diet not only presented a major reduction in the number of adenomas, but β-catenin was localized to the cellular membranes with a distribution similar to wild type mice [124]. These results were confirmed *in vitro* using two colon cancer cell lines with APC mutations [125]. While β-catenin distribution was both cytoplasmic and nuclear in untreated cells, after SPH or bovine ceramide treatment, cells presented an overall reduction in the levels of β-catenin in both the cytoplasm and nucleus [124]. The effect of sphingolipids on the redistribution of β-catenin was also observed in CF1 mice treated with DMH. Mice treated with DMH showed an elevated expression of β-catenin levels with both membrane and cytosolic distribution in cells in the lumen and in the colonic crypt. DMH mice treated with 0.05% SM showed reduction and redistribution of β-catenin to plasma membrane of cells lining the colonic lumen in a manner similar to the untreated mice. The mechanism behind sphingolipid reduction and redistribution of β-catenin is not very well known; however, the mRNA expression levels of β-catenin in the colonic mucosa of mice treated with DMH after AIN76A diet with or without SM supplements showed no differences, indicating a post-transcriptional or post-translational modulation [125].

Enigmol (sphingoid base analogue) reduced the number of adenomas by 50% in $APC^{Min/+}$ mice when administered orally. The same study showed *in vitro* treatment with Enigmol

demonstrated 50% growth inhibition in 57 out of 60 different cancer cell lines tested [112]. In particular, HT29 cells treated with Enigmol showed cell death associated with caspase-3 activation. HT29 cells exhibit increased of nuclear and cytosolic β-catenin due to a truncation of the APC gene. After treatment with Enigmol or SPH, β-catenin nuclear and cytoplasmic expression was mostly diminished as demonstrated by immunofluorescence and Western blot, and redistributed to cell-to-cell boundaries considered a normal location for β-catenin. The sphingoid base compound not only decreased the total levels of β-catenin, but also increased the levels of the inactivated p-S33/S37/T41 β-catenin, probably by activating CK-1α and GSK-3β, as these kinases were also activated after Enigmol treatment [112].

Summary

In spite of the advances in the understanding of the mechanisms that induce CRC, treatment outcomes have not significantly improved. Over 90% of CRC patients present a deregulation of the WNT/β-catenin signaling pathway, with 30–70% of those patients involving APC mutations [126]. Since these mutations have been associated with colon cancer initiation, WNT/β-catenin has been considered a valid target for chemotherapy. There is increasing evidence that alteration in sphingolipid metabolism can modulate susceptibility to intestinal tumorigenesis and that these effects seem to be mediated, at least in part, by normalizing the levels and distribution of β-catenin. Indeed, dietary sphingolipids have both chemopreventive and chemotherapeutic effects in colon cancer animal models, and modulation of the enzymes implicated in sphingolipid metabolism seem to change the susceptibility to colon cancer formation as shown in the $SK1^{-/-}$ model following AOM/DSS treatment [39]. In addition, *in vitro* alteration of both sphingolipids and their metabolism impact proliferation, apoptosis, autophagy and other important biologic functions in colon cancer cells. Research into the *in vivo* and *in vitro* effects of sphingolipids have shown that they are mediated in part by modulation of the β-catenin pathway, underlining the importance of sphingolipids in CRC. Therefore, when taken together, these studies provide a rationale for investigating sphingolipid metabolism as a possible target in both the chemoprevention and chemotherapy of CRC.

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Abbreviations

CRC	colorectal cancer
FAP	Familial Adenomatous Polyposis
AFAP	Attenuated Familial Adenomatous Polyposis
APC	Adenomatous Polyposis Coli
K-Ras	Kirsten rat sarcoma viral oncogene homolog
ACF	aberrant crypt foci
S1P	Sphingosine-1-Phosphate
CerS	Ceramide Synthase
SM	Sphingomyelin
SMase	Sphingomyleinase

GCS	glucosylceramide synthase
SPH	sphingosine
CDase	Ceramidase
SK	Sphingosine Kinase
GLA	Alpha-Galactosidase A
SPL	Sphingosine-1-Phosphate Lyase
aCDase	acid Ceramidase
aSMase	acid Sphingomyelinase
nSMase	neutral Sphingomyelinase
nCDase	neutral Ceramidase
alk-SMase	Alkaline Sphingomyelinase
UDCA	Ursodeoxycholic Acid
AOM	Azoxymethane
DMH	1.2-Dimethylhydrazine
HUVEC	human umbilical vein endothelial cells
DSS	Dextran sulfate sodium
BrdU	Bromodeoxyuridine
DNA	Deoxyribonucleic acid
mRNA	messenger ribonucleic acid
GlcCer	Glucosylceramide
RT-PCR	Reverse Transcriptase- Polymerase Chain Reaction
5-FU	5-fluorouracil
COX-2	cyclooxygenase-2
DMS	N, N-Dimethylsphingosine
ERK	Extracellular-signal-regulated kinase
bSMase	bacterial Sphingomyelinase
TNF	Tumor Necrosis Factor
PCNA	Proliferating cell nuclear antigen
siRNA	small interference ribonucleic acid
TRAIL	TNF-Related Apoptosis-Inducing Ligand
CK1	Casein Kinase 1
GSK3	Glycogen Synthase Kinase 3
β-TrCP	Beta –Transducin Repeat Containing Protein
LEF/TCF	Lymphoid Enhancer Factor/T-Cell Factor
PP1c	Protein phosphatase 1c
MDR1	Multi drug resistance transporter

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Highlights

1. Role of sphingolipids in colon cancer.
2. Endogenous and dietary effect of sphingolipids in colon cancer, and
3. Interaction of sphingolipids with WNT/β-catenin pathway in colon.

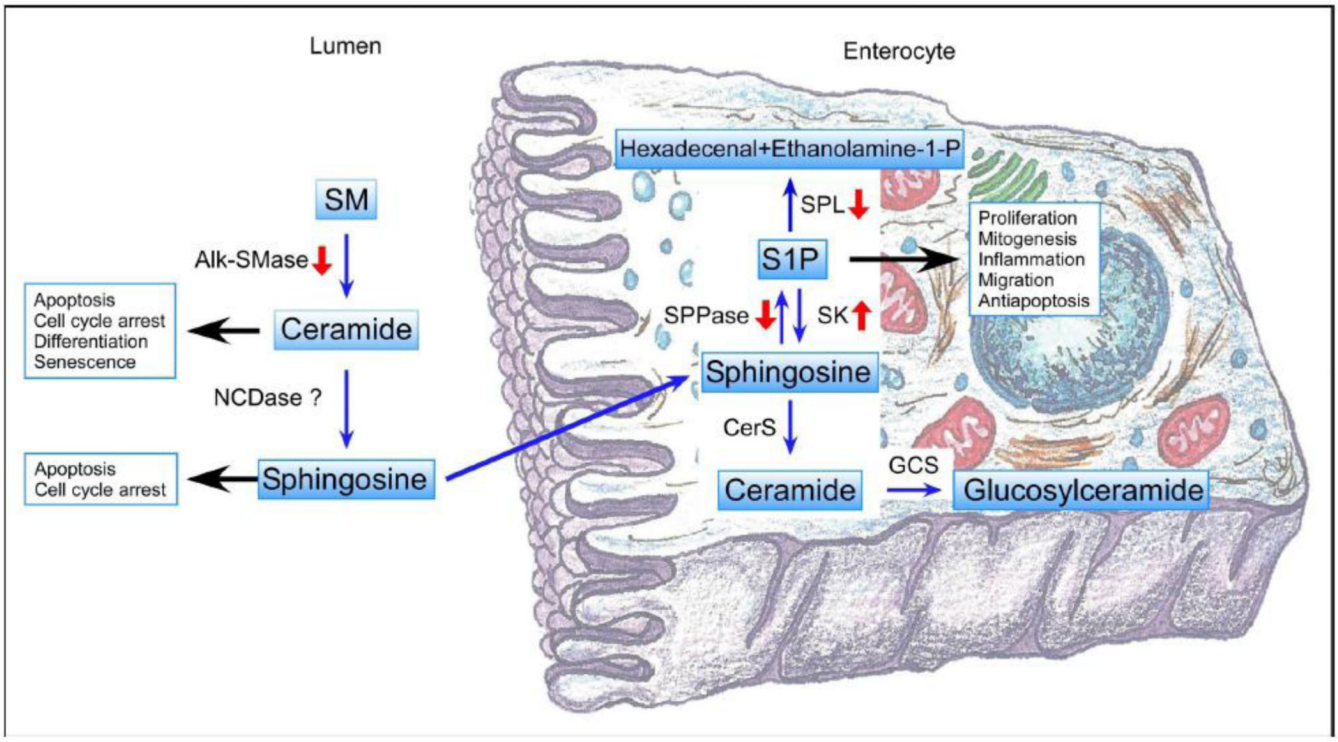

Figure 1.

Summary of the changes in sphingolipid metabolic enzyme described in colon cancer, along with an overview of the major roles in cell homeostasis. Figure based on figure 9 in Kono et. al. [65], with permission. SM: Sphingomyelin; alk-SMase: alkaline-Sphingomyelinase; nCDase: neutral Ceramidase; SPL: Sphingosine-1-P Lyase; S1P: Sphingosine-1-Phosphate; SPPase: Sphingosine-1-P Phosphatase; SK: Sphingosine Kinase; CerS: Ceramide Synthase and GCS: Glucosylceramide Synthase.

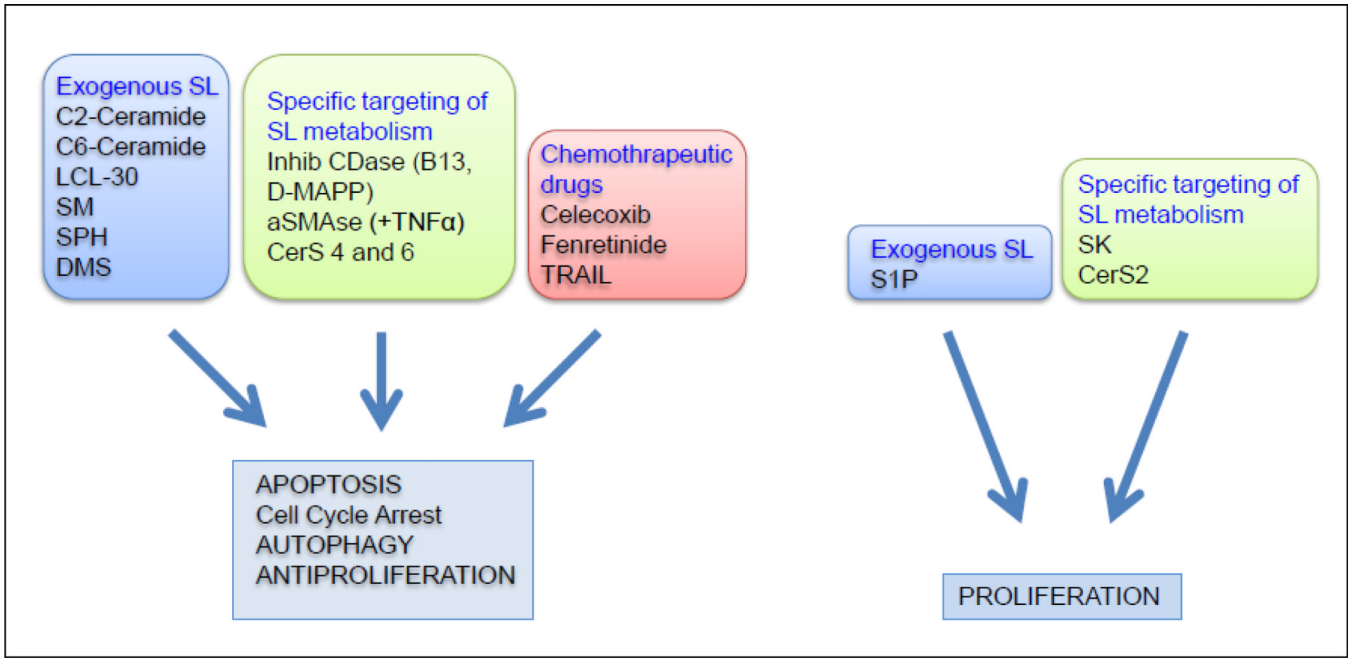

Figure 2.

Summary of the effects of sphingolipids in colon cancer cells. LCL-30: ω-pyridinium bromide D-erythro-C16-ceramide; SM: Sphingomyelin; SPH: Sphingosine; DMS: N,N-dimethylsphingosine; CDase: Ceramidase; aSMase: acid Sphingomyelinase; TNF-α: Tumor Necrosis Factor alfa; CerS: Ceramide Synthase; TRAIL: TNF-Related Apoptosis-Inducing Ligand; S1P: Sphingosine 1 Phosphate; SK: Sphingosine Kinase.