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# **The natural flavonoid Luteolin induces apoptosis in colon cancer cells by dysregulating the sphingolipid rheostat**

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*To the soul of children who were victims in conflicts zones all over the world  
and most especially Syrian children who are being abused and killed since 3 years and up to this moment*

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## ABBREVIATION

<b>ACF</b>	Aberrant crypt foci
<b>ALP</b>	Alkaline phosphatase
<b>Alk-SMase</b>	Alkaline Sphingomyelinase
<b>A-SMase</b>	Acidic Sphingomyelinase
<b>BFA</b>	Brefeldin A
<b>BODIPY-C5-Cer</b>	N-(4,4-difluoro-5,7-dimethyl-bora-3a,4a-diaza-s-indacene-3-pentanoyl) sphingosine)
<b>CDase</b>	Ceramidase
<b>Cer</b>	Ceramide
<b>CRC</b>	Colorectal cancer
<b>C2-Cer</b>	N-acetyl-D-erythro-sphingosine,
<b>C6-Cer</b>	N-hexanoyl-D-erythro-sphingosine
<b>D609</b>	O-Tricyclo[5.2.1.0 <sup>2,6</sup> ] dec-9-yl dithiocarbonate potassium salt
<b>ER</b>	Endoplasmic reticulum
<b>EMEM</b>	Minimum essential medium eagle
<b>FCS</b>	Fetal Calf serum
<b>GalCer</b>	Galactosylceramide
<b>GlcCer</b>	Glucosylceramide
<b>GSLs</b>	Glycosphingolipids
<b>Gb3</b>	Globotriaosylceramide
<b>LacCer</b>	Lactosylceramide
<b>LU</b>	Luteolin
<b>LY</b>	LY294002
<b>MTT</b>	3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide
<b>N-SMase</b>	Neutral Sphingomyelinase
<b>PBS</b>	Phosphate-buffered saline
<b>PlsEtn</b>	Phosphoethanolamine plasmalogen
<b>PM</b>	Plasma membrane
<b>PNPP</b>	p-Nitrophenyl-phosphate
<b>PNP</b>	p-Nitrophenol
<b>PI3K</b>	Phosphatidylinositol-3-OH kinase
<b>SLs</b>	Sphingolipids
<b>Sph</b>	Sphingosine
<b>SM</b>	Sphingomyelin
<b>SMase</b>	Sphingomyelinase
<b>SPHKI</b>	Sphingosine kinase I
<b>SPHKII</b>	Sphingosine kinase II
<b>S1P</b>	Sphingosine-1-phosphate

## SUMMARY

Colorectal cancer is one of the most common malignancies and a leading cause of cancer death in the world. More powerful and safer therapeutic approaches are urgently needed to reduce mortality and garner better curative effects. In this regard, dietary supplements capable of preventing carcinogenesis and inhibiting the growth of colon carcinoma cells have generated intense interest. Luteolin (LU), a common dietary flavonoids, has emerged as a powerful anticancer agents able to sensitize different cancer cells, including colon cancer ones, to therapeutic-induced cytotoxicity. However, the molecular mechanisms underlying LU effects in colon cancer are largely unknown.

Sphingolipids have critical functions as signaling molecules. In particular, Ceramide (Cer) and Sphingosine-1-phosphate (S1P) are involved as key antagonist mediators in regulating crucial cell responses such as proliferation and apoptosis. Cer can act as a second messenger, and, by activating a variety of signaling pathways, is able to promote growth arrest, apoptosis, or cell differentiation. To the opposite, the sphingoid mediator S1P can act as a potent mitogen and survival factor for a variety of cell types. These two lipids together form the “sphingolipid rheostat” regulating the balance between cell growth and cell death.

The objective of our study was to investigate the effects and the molecular mechanisms of LU in colon cancer, focusing on the role of the bioactive sphingoid molecules Cer and S1P. To this purpose, we used the Caco-2 cell line, obtained from a human colon adenocarcinoma, as cell model of both colon cancer cells, and differentiated intestinal enterocytes. Indeed, in long-term culture, these cells undergo a spontaneous differentiation into polarized cells, representing so far the best available *in vitro* model of absorptive enterocytes. These two models might thus allow to distinguish the potential LU effects on colon cancer cells in comparison to their healthy counterparts.

At first, we characterized the morphological and biochemical features of both models. We found that Caco-2 cell differentiation was accompanied by the formation of “domes” across the monolayer,

known as characteristic structures of differentiated cells, and indicative of their property of absorptive epithelium. In addition, the activity of the alkaline phosphatase, a membrane-associated hydrolase was found significantly increased in differentiated Caco-2 cells compared to the tumoural cancer ones. Furthermore, we found that Caco-2 differentiation was associated with a reduction of the phospholipids/protein ratio, and whereas phosphatidylcholine was the most abundant phospholipid species of tumoural cells, phosphatidylethanolamine prevailed in the differentiated ones. Moreover, phosphatidylethanolamine plasmalogen, a quantitative relevant species in the cancer cells, exhibited a marked decrease with intestinal differentiation.

As far as the sphingolipid composition concerns, we first demonstrated that Caco-2 differentiation was associated to an increase in the total amount of sphingolipids, including Sphingomyelin (SM), the major component and precursor of both Cer and S1P, and above all ceramide. Since the SM hydrolysis, triggered by Sphingomyelinases (SMases), has been implicated in colon tumourigenesis, we then evaluated whether changes in the activity of the known different SMases (the neutral (N-SMase) and alkaline (Alk-SMase) enzymes) occur with cell differentiation. We found that the Alk-SMase activity which was barely detectable in tumoural cancer cells, significantly increased in differentiated ones. The high level of this enzyme is consistent with its presence in the apical brush border, characteristic of intestinal cells. Interestingly a significant increase in the N-SMase was observed in the differentiated Caco-2 cells suggesting a role for this enzyme in intestinal cells. Prompted by these data indicating that the SM hydrolytic potential is enhanced in differentiated Caco-2 cells, we then investigated SM metabolism in both tumoural and differentiated cells. We found that the tumoural cells were much more rapid in the production of Cer from SM than the differentiated ones, and that Cer turnover was present and rapid in both cell types. Inhibition of N-SMase activity, the most abundant enzyme, resulted in no variation of Cer formation in both types of cells, suggesting that the lysosomal acid SMase (A-SMase) is involved in SM degradation. After this characterization, we then evaluated the effect of LU on the tumoural and intestinal cells. Interestingly, we found that

the flavonoid exhibited a potent cytotoxic effect on tumoural cells, by inducing apoptosis, without affecting the viability of differentiated cells.

Instead, we found that LU induces an increase in intracellular Cer level with a more pronounced trend in tumoural cells. Based on these results we examined whether Cer is involved in the mechanism of LU-induced apoptosis. Notably we obtained that conditions leading to enhance the Cer content in colon cancer cells, as treatment with short-chain Cer analogues and inhibitor of sphingomyelin synthase (SM-synthase) were succeeded by inducing apoptosis in tumoural cells, thus mimicking the LU effect.

Furthermore, we evaluated the possible effect of LU on the formation of Cer and S1P in tumoural Caco-2 cells. Pulse experiments showed that treatment with LU induced a dose-dependent increase in Cer, paralleled by a concomitant decrease of both SM and glycosphingolipids synthesis. This result suggested that LU most probably acts by reducing the availability of Cer as a substrate for both SM-synthase and glucosylceramide synthase enzymes localized in the Golgi complex, possibly by inducing a defect in the common vesicular route responsible for complex SLs biosynthetic process. Fluorescent studies using a BODIPY-C5-CER, a ceramide analogue known to mimic the ER-Golgi traffic of natural Cer in living cells, supported the presence of an aberrant traffic of Cer during LU treatment. Furthermore, pulse experiments using treatment with Brefeldin A, known to induce a retrograde merging of Golgi membranes with the ER, demonstrated that LU was not more able to exert alterations of Cer metabolism, thus indicating that the ER-Golgi traffic of Cer is the site of LU action.

In parallel, different protein kinases, including Akt have been shown to regulate ER to Golgi traffic. In addition, a study in our laboratory reported for the first time a putative role of PI3K /Akt pathway in the regulation of the vesicle-mediated movements of Cer in the ER-Golgi district. Prompted by these findings, pulse experiments using LY294002, a representative PI3K inhibitor, showed that, LY294002 and subsequently the inhibition of PI3K/Akt had the same effect on the Cer metabolism observed during LU treatment. Furthermore, we obtained that LU strongly inhibited the Akt

phosphorylation as a downstream response to PI3K inhibition. Taken together, it emerges that blockade of PI3K by LU and subsequently the downregulation of PI3K/Akt pathway is considered at least one of the mechanisms responsible for the effect of this flavonoid on Cer trafficking observed in our tumoural cell model

Further analyses revealed that LU was able to alter not only Cer content but also to decrease the endogenous S1P level inducing thereby a shift of the “sphingolipid balance” to the side of death. Based on this result, we demonstrated for the first time that LU was able to act as inhibitor of Sphingosine kinase (SPHK) activity in tumoural cells, especially SPHKII, the up-regulated isoform in our cancer cell model, exhibiting only a modest effect on SPHKI.

Furthermore, in order to gain deeper insights into the role of the “Sphingolipid rheostat” in mediating the effect of LU, we attempted to push the balance towards S1P with addition of exogenous S1P. The results demonstrated that S1P conferred a significant resistance of colon cancer cells to the cytotoxic effect of LU. The mechanism by which S1P acts as LU-antagonist was proved to be mainly by the up-regulation of the PI3K/Akt pathway, which is able to rescue colon cancer cells from the apoptotic effect of the LU-induced increase of Cer.

Taken together, our results demonstrate, for the first time, that the dietary natural flavonoid LU induces apoptosis in colon cancer cells by targeting the “sphingolipid rheostat”, and directing the balance in favor of Cer. As the balance between Cer and S1P appears to be an important target for development of new and effective therapeutic strategies against tumour progression, LU could be a novel antitumoural agent not only in colon cancer but possibly in the treatment of other solid tumours.



# INTRODUCTION

## 1. Colorectal Cancer

### 1.1. Colorectal cancer features

*Colorectal cancer (CRC) is one of the leading tumours worldwide and it is considered among the big killers, together with lung, prostate and breast cancer. In the recent years, many efforts have been made to develop a convincing therapy to treat this frequent disease: adjuvant chemotherapy was demonstrated to be effective, chiefly in stage III patients, and surgery was optimized in order to achieve the best results with a low morbidity. Furthermore, several new target-oriented drugs are under evaluation and some of them (cetuximab and bevacizumab) have already exhibited a good activity/efficacy, mainly in combination with chemotherapy. The development of updated recommendations for the best management of these patients is crucial in order to obtain the best results, not only in clinical research but also in every-day practice.*

#### 1.1.1. Epidemiological data: Incidence, mortality and survival

Cancers of the colon and rectum are the third most common tumour type worldwide and the fourth most common cause of death in both sexes [Ferlay et al., 2010]. In term of **incidence**, cancer of the colon is more frequent than rectal cancer: the ratio of colon to rectum cases is 2:1 or more in industrialized countries (rather more in females), while in non-industrialized countries rates are generally similar. Moreover, the geographic distribution of CRC is different: indeed, more of 63% of the cases occur in developed countries with a Western culture and its rates increase with industrialization and urbanization. The incidence rate varies up to 10-fold between countries with the highest rates and those with the lowest rates. It ranges from more than 40% people in the United States, Australia, New Zealand, and Western Europe to less than 5% in Africa and some parts of Asia where it remains relatively uncommon [Ferlay., et al 2010] (Fig.1). In Europe around 250,000 new colon cases are diagnosed each year, accounting for around 9% of all the malignancies. The incidence

is slightly higher in Western and Northern Europe than in Southern and Eastern Europe. In general, this incidence increases in countries where the overall risk of large bowel disease is low, while in countries with high incidence rates there have been either stabilizations or decreases in incidence, particularly in younger age groups. In CRC, the greatest increases in incidence are observed in Asia, as well as in countries of Eastern Europe. In Western Europe and Oceania, the overall (age-adjusted) rates remain fairly constant. In the USA, since the mid-1980s a decline in CRC incidence was observed in both sexes, but not in the black population [Parkin *et al.*, 2001]. In Italy [Grande *et al.*, 2007], the annual incidence rates were estimated to increase throughout the period 1970-2010 from 30 to 70 per 100,000 for men, and to stabilize from the end of the 1990s at around 38 per 100,000 for women. The estimated numbers of annual new diagnosis and deaths, for the year 2005, were 46,000 and 16,000 respectively; 58% of both were related to men. About 70% of patients with CRC are over 65 years of age. CRC is rare under the age of 45 years (2 per 100,000/year). In the age group 45-54 years CRC incidence is about 20 per 100,000/year and thereafter it increases at a much higher rate (55 per 100,000/year for aged 55-64, 150 for aged 65-74 and >250 per 100,000/year for those older than 75 years of age) [Parkin *et al.*, 2001].

Worldwide *mortality* attributable to colorectal cancer is approximately half that of the incidence. It is estimated that about 600,000 deaths from CRC still occur worldwide annually, making this disease the fourth most common cause of death from cancer. As observed for incidence, mortality rates are generally lower in women than in men. Less variability in mortality rates was observed worldwide (six fold in men, five fold in women), with the highest mortality rates in both sexes estimated in Central and Eastern Europe (20.3 per 100,000 for male, 12.1 per 100,000 for female), and the lowest in Middle Africa (3.5 and 2.7, respectively) [Ferlay *et al.*, 2010]. However, it is generally difficult to interpret temporal changes in mortality as they are influenced by trends over time in incidence and survival. The incidence rate may be a more appropriate indicator of trends in disease occurrence.

CRC incidence is unaffected by changes in treatment and survival, although it has been shown to be influenced by improved diagnostic techniques and screening programs (**Fig.1**).

The *survival* of CRC patients is highly dependent upon stage of disease at diagnosis, and typically ranges from a 90% 5-year survival rate for cancers detected at the localized stage, 70% for regional, to 10% for people diagnosed for distant metastatic cancer [*Ries et al., 2008, Jemal et al., 2004*]. In general, the earlier the stage at diagnosis, the higher the chance of survival. In addition, 5-year relative survival decreased with age from 63% to 49% from the youngest (15-45 years) to the oldest age group of patients (75 years and over) [*Labianca et al 2010*]. Since the 1960s, survival for colon cancer at all stages increased substantially. The relative improvement in 5-year survival over this period has been better in countries with high life expectancy and good access to modern specialized health care. However, enormous disparities in colon cancer survival exist globally and even within regions [*Jackson-Thompson et al., 2006*]. For example, in the USA, the survival of patients diagnosed with CRC, in 2000-2002, was 65.5% while in Europe was 56.2% [*Verdecchia et al., 2007*]. This variation is not easily explained, but most of the marked global and regional disparity in survival is likely due to differences in access to diagnostic and treatment services.

### **1.1.2. Aetiological and risk factors**

Several risk factors are associated with the incidence of colorectal cancer, including those that an individual cannot control include age and hereditary factors. In addition, a substantial number of environmental and lifestyle risk factors may play an important role in the development of this type of cancer.

## Non-modifiable risk factors

- Age

The probability of colorectal cancer diagnosis increases progressively after the age of 40, rising sharply after age 50: more than 90% of colorectal cancer cases occur in people aged 50 or older. The incidence rate is more than 50 times higher in persons aged 60 to 79 years than in those younger than 40 years. However, colorectal cancer appears to be increasing among younger persons. In fact, in the United States, CRC is now one of the 10 most commonly diagnosed cancers among men and women aged 20 to 49 years [Jackson-Thompson et al., 2006].

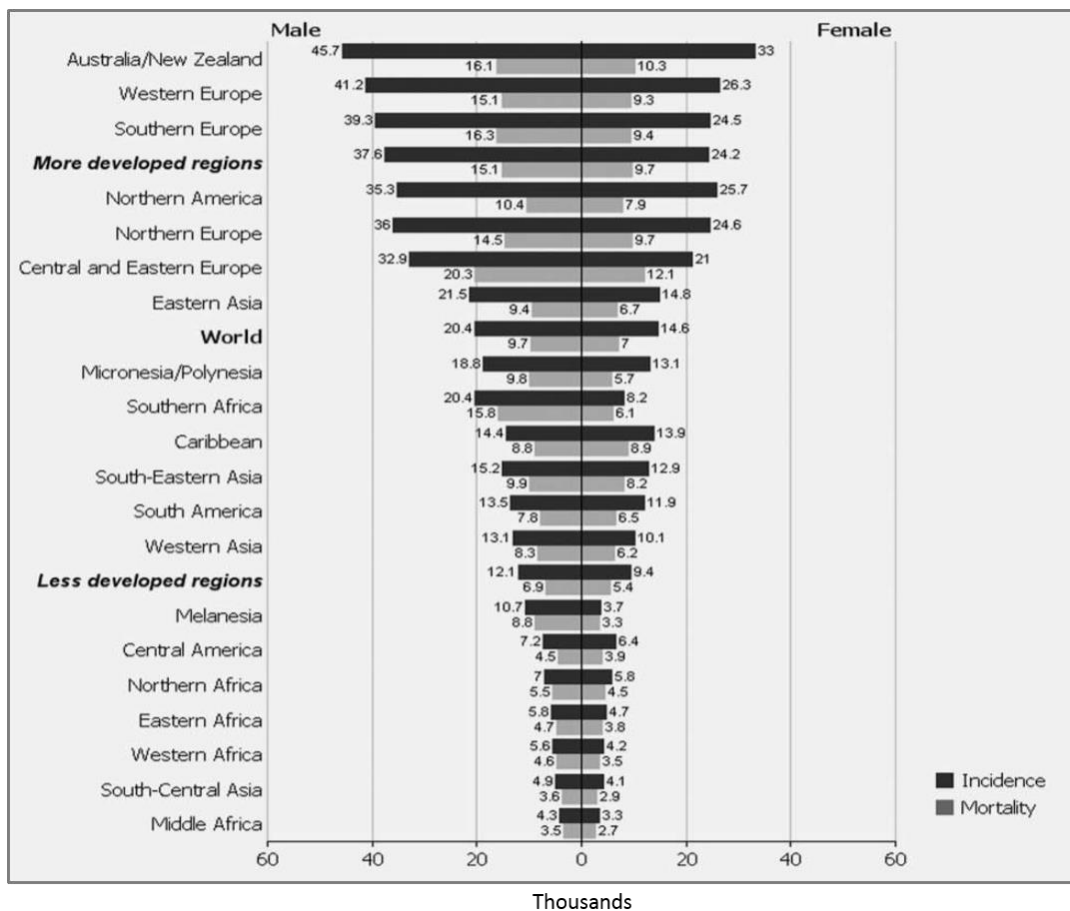


Figure 1. Estimated age-standardized incidence and mortality rates for colorectal cancer [Labianca et al., 2010]

- Genetic factors

Approximately 5 to 10% of colorectal cancers are a consequence of recognized hereditary conditions. The most common inherited conditions are familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) called Lynch syndrome [*Jeter et al., 2006*]. Genes responsible for these forms of inherited CRC have been identified.

**Familial adenomatous polyposis FAP** was the first recognized and best-characterized colonic polyposis syndrome. It is a highly penetrant autosomal dominant disorder caused by germline mutations of the Adenomatous Polyposis Coli (APC) tumor suppressor gene [*Bodmer et al., 1987, Kinzler et al., 1991*]. FAP accounts for less than 1% of all CRC. Clinically, patients with FAP present hundreds to thousands of colorectal adenomatous polyps, usually in the second decade of life. The lifetime risk of CRC approaches 100% and patients with FAP present also the risk of extra-colonic manifestations such as cutaneous lesions, osteomas, dental anomalies, congenital hypertrophy of the retinal pigment epithelium, desmoid tumors, and extracolonic cancers (liver, pancreas, gastric and small bowel, thyroid, and central nervous system).

Attenuated FAP (AFAP) is a less aggressive form of the disease; it is characterized by delayed age of onset and fewer colorectal adenomatous polyps. Extra-colonic manifestations are less common in attenuated FAP.

**Hereditary Non Polyposis Colorectal Cancer (Lynch syndrome)** is an autosomal dominant condition caused by germline mutations in DNA mismatch repair (MMR) genes, *MLH1* (MutL homolog 1), *MSH2* (MutS homolog 2), *PMS2* (postmeiotic segregation 2) and *MSH6* (MutS homolog 6) genes [*Papadopoulos et al., 1994*]. Bi-allelic inactivation of any of these MMR genes results in defective DNA repair and in the subsequent accumulation of repetitive short nucleotide sequences called microsatellites. Recently, germline deletions in the *TACSTD1* gene (a gene directly upstream of *MSH2*), which encodes the epithelial cell adhesion molecule (Ep-CAM), has been identified as the causative mutation familialHNPCC [*Ligtenberg et al., 2009*]. The risk of developing cancer in

HNPCC patients and families depending on the gene mutation. For example, families with *MSH2* mutations have more extracolonic cancers than *MLH1* mutation carriers. Families that harbor *MSH6* mutations develop CRC at a more advanced age, and have a higher risk of developing endometrial cancers [*Vasen et al., 2007*]. HNPCC is the most common hereditary CRC syndrome, accounting for 2-3% of all CRC cases. The hallmark of HNPCC is the presence of microsatellite instability (MSI). Patients with HNPCC develop CRC at a younger age than the general population, have a predilection for proximal colon cancers (70-85% of colon cancers are right sided), and are at a higher risk for synchronous CRCs. Patients are at a higher risk of developing extra-colonic tumors including endometrial, ovarian, gastric, small bowel, pancreatic, hepatobiliary, skin, brain, and urethral tumors. The cumulative lifetime risk of an extra-colonic malignancy in females and males is 47% and 27%, respectively.

- Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a term used to describe two diseases: ulcerative colitis and Crohn disease. Ulcerative colitis causes inflammation of the mucosa of the colon and rectum; Crohn disease causes inflammation of the full thickness of the bowel wall and may involve any part of the digestive tract from the mouth to the anus. These conditions increase an individual's overall risk of developing colorectal cancer. The relative risk of colorectal cancer in patients with inflammatory bowel disease has been estimated between 4-to 20-fold [*Bethesda et al., 2006*].

- Adenomatous Polyps

Neoplastic polyps of the colorectum, namely tubular and villous adenomas, are precursor lesions of colorectal cancer [*Janout et al., 2001*]. The lifetime risk of developing a colorectal adenoma is nearly 19% in the U.S. population. Nearly 95% of sporadic colorectal cancers develop from these adenomas. An individual with a history of adenomas has an increased risk of developing colorectal cancer, than individuals with no previous history of adenomas. A long latency period, estimated at 5 to 10 years, is usually required for the development of malignancy from adenomas. Detection and removal of an

adenoma prior to malignant transformation may reduce the risk of colorectal cancer. However, complete removal of adenomatous polyps or localized carcinoma is associated with an increased likelihood of future development of cancer elsewhere in the colon and rectum [*de Jong et al., 2005*].

### **Modifiable risk factors**

Colorectal cancer is widely considered to be an environmental disease, with “environmental” defined broadly to include a wide range of often ill-defined cultural, social, and lifestyle factors. As such, colorectal cancer is one of the major cancers for which modifiable causes may be readily identified, and a large proportion of cases theoretically preventable [*Johnson et al., 2007*]. Some of the evidence of environmental risk comes from studies of migrants and their offspring, among migrants from low-risk to high-risk countries, incidence rates of colorectal cancer tend to increase toward those typical of the population of the host country. For example, among offspring of Southern Europe migrants to Australia and Japanese migrants to Hawaii, the risk of colorectal cancer is increased in comparison with that of populations of the country of origin. In fact, CRC incidence in the offspring of Japanese migrants to the United States, approaches or surpasses that in the white population, and it is three or four times higher than among the Japanese in Japan. Apart from migration, some other geographic factors can influence in the incidence of CRC, one of them is the urban residence. The incidence is consistently higher among urban residents. For this reason, current residence in an urban area is a stronger predictor of risk than is an urban location of birth [*Janout et al., 2001*]. This excess incidence in urban areas is more apparent among men than women and for colon cancer than for rectal cancer.

- Cigarette Smoking

The association between tobacco cigarette smoking and lung cancer is well established, but smoking is also extremely harmful to the colon and rectum. Evidence shows that 12% of colorectal cancer deaths are attributed to smoking [Zisman *et al.*, 2006]. The carcinogens found in tobacco, increase cancer growth in the colon and rectum and increase the risk of being diagnosed with this cancer. Cigarette smoking is important for both formation and growth rate of adenomatous polyps, the recognized precursor lesions of colorectal cancer [Botteri *et al.*, 2008]. Larger polyps found in the colon and rectum are associated with long-term smoking. Evidence also demonstrates an earlier average age of onset incidence of colorectal cancer among smokers.

- Heavy Alcohol Consumption

As with smoking, the regular consumption of alcohol may be associated with increased risk of developing colorectal cancer. Alcohol consumption is a risk factor in the onset of colorectal cancer at a younger age [Zisman *et al.*, 2006, Tsong *et al.*, 2007] as well as a disproportionate increase of tumors in the distal colon [Bazensky *et al.*, 2007]. Reactive metabolites of alcohol such as acetaldehyde can be carcinogenic [Po *et al.*, 2004]. There is also an interaction with smoking [Zisman *et al.*, 2006]. Tobacco may induce specific mutations in DNA that are less efficiently repaired in the presence of alcohol. Alcohol may also function as a solvent, enhancing penetration of other carcinogenic molecules into mucosal cells. Additionally, the effects of alcohol may be mediated through the production of prostaglandins, lipid peroxidation, and the generation of free radical oxygen species. Lastly, high consumers of alcohol may have diets low in essential nutrients, making tissues susceptible to carcinogenesis.



- Physical Activity and Obesity

Several lifestyle-related factors have been linked to colorectal cancer. Two modifiable and interrelated risk factors, physical inactivity and excess body weight, are reported to account for about a fourth to a third of colorectal cancers. There is abundant evidence that higher overall levels of physical activity are associated with a lower risk of colorectal cancer, including evidence of a dose-response effect, with frequency and intensity of physical activity inversely associated with risk [*Lee et al., 2007*]. Regular physical activity and a healthy diet can help decrease the risk of colorectal cancer, although the evidence is stronger for colonic than for rectal disease. The biologic mechanisms potentially responsible for the association between reduced physical activity and colorectal cancer are beginning to be elucidated. Sustained moderate physical activity raises the metabolic rate and increases maximal oxygen uptake. In the long term, regular periods of such activity increase the body's metabolic efficiency and capacity, as well as reducing blood pressure and insulin resistance. In addition, physical activity increases gut motility. The lack of physical activity in daily routines also can be attributed to the increased incidence of obesity in men and women, another factor associated with colorectal cancer [*Campbell et al., 2007*]. Several biologic correlates of being overweight or obese, notably increased circulating estrogens and decreased insulin sensitivity, are believed to influence cancer risk, and are particularly associated with excess abdominal adiposity independent of overall body adiposity. However, the increased risk associated with overweight and obesity does not seem to result merely from increased energy intake; it may reflect differences in metabolic efficiency. Studies suggest that individuals who use energy more efficiently may be at a lower risk of colorectal cancer.

- Nutritional practices

It has been estimated that nutrition could account for more than one third of cancer deaths, and that dietary factors are responsible for 70% to 90% of all cases ( for more details see part II: diet in colon cancer as a double edged sword).

### 1.1.3. Molecular pathways in colon cancer

Colorectal cancer is a heterogeneous disease with different molecular pathways leading to different phenotypes. Genetic and epigenetic alterations act to deregulate conserved signaling pathways involved in cellular metabolism, proliferation, differentiation, survival, and apoptosis. Understanding the molecular basis of colorectal carcinogenesis has important ramifications in both prognosis and treatment of CRC. To date, three distinct molecular pathways have been recognized. These are the Chromosomal Instability (CIN) pathway, Microsatellite Instability (MSI) pathway, and the CpG Island Methylator Phenotype (CIMP) pathway. These pathways are not mutually exclusive, with some tumors exhibiting features of more than one pathway.

#### Chromosomal instability pathway

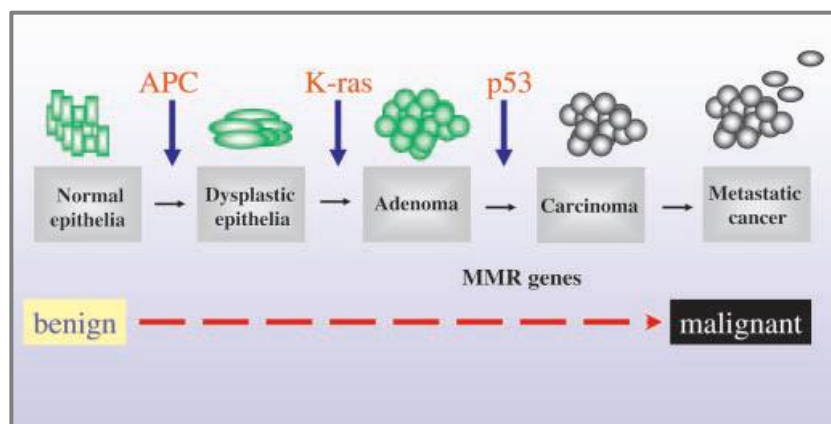
Chromosomal instability is the most common cause of genomic instability in CRC. It accounts for 65–70% of sporadic CRC. It is characterized by gain or loss of whole chromosomes or chromosomal regions harboring genes integral for the process of colorectal carcinogenesis. CIN results from defects in chromosome segregation with subsequent aneuploidy, telomere dysfunction, or defects in the DNA damage response mechanisms [*Pino et al., 2010*]. The consequence is an imbalance in chromosome number (aneuploidy), chromosomal genomic amplifications, and a high frequency of LOH.

A genetic model describing the transition from healthy colonic epithelia through increasingly dysplastic adenoma to malignant cancer has been proposed that identifies a number of key oncogenes and tumor suppressor genes, the progressive acquisition of activating or loss of function mutations in which drives the adenoma to carcinoma transition (**Fig.2**). Of the genes characterized to date, inactivation of the tumor suppressor genes APC and p53 and activation of the oncogene Kirsten-ras

(K-ras) are thought to be particularly important determinants of tumor initiation and progression [Fearon et al., 1990].

- The K-ras oncogene

The *K-ras* proto-oncogene is mutated in 30-60% of CRC and large adenomas [Wang et al., 2006]. It has been proposed that activated K-ras may play an important role in the transition from adenoma to carcinoma through activation of downstream targets including *BCL-2*, *H2AFZ*, *RAP1B*, *TBX19*, *E2F4*, and *MMP1*. The *K-ras* gene product, a 21 kDa membrane bound protein involved in signal transduction, is activated in response to extracellular signals. The mutated protein is locked in the active form due to impaired GTPase activity, which hydrolyses GTP to GDP. Most activating mutations are found in codons 12 and 13 of exon 1. Ras activation affects multiple cellular pathways that control cellular growth, differentiation, survival, apoptosis, cytoskeleton organization, cell motility, proliferation, and inflammation.



**Figure 2. A model of the genetic changes required for progression from adenoma to carcinoma in the development of colorectal cancer.** The proposed order of mutations in APC, K-ras, p53, and the DNA MMR genes is illustrated [Al sohaili et al., 2012]

- The APC and MCC genes

Allelic loss of chromosome 5q has been reported in 20-50% of sporadic CRC. Two important genes are located on the long arm of chromosome 5; these are the *APC* and the Mutated in Colorectal Cancer

(MCC) genes. Somatic *APC* mutations are seen in 60-80% of CRC as well as in a large percentage of colorectal precursor lesions (adenomas), indicating that *APC* mutation is an early event in the process of colorectal tumorigenesis [Powell et al., 1992]. *APC* was described as the “gatekeeper” gene regulating the entry of epithelial cells into the adenoma-carcinoma progression. It belongs to the canonical Wnt/ wingless pathway that plays important role for both initiation and progression of CRC and represents a “final common pathway”, as other signaling pathways converge and interact with this pathway [Behrens et al., 2005]. The MCC gene is located on 5q21. It is commonly silenced in colorectal cancers through promoter hypermethylation [Kohonen-Corish et al., 2007]. MCC has been identified as one of the “driver genes” of colorectal carcinogenesis in a mouse model. It is a cell cycle regulatory protein that induces cell cycle arrest in response to DNA damage [Pangon et al., 2010]. In addition, a recent study suggested that MCC could also inhibit Wnt/ $\beta$ -catenin signal transduction independent of *APC*.

- Mutations in p53

The p53 is a transcription factor with tumor suppressor activity that binds to a specific DNA sequence and activates a number of genes involved in cell cycle arrest, apoptosis, senescence, autophagy, and cellular metabolism. In addition, p53 functions as a transcription factor, exerting cell cycle control by binding to specific recognition sequences in variety of genes including p21, Bax, and Bcl-2 in response to DNA damage or other cellular stress. The p53 facilitates the cellular adaptation in response to different cellular stresses including DNA damage by mutagens, oncogenic stimulation, hypoxia, and telomere erosion. The p53 gene, localized on the short arm of chromosome 17, is mutated in up to 70% of colorectal cancers [Vogelstein et al., 1988]. The p53 are proposed to be relatively late events in the development of colorectal tumors, with the loss of p53-mediated pathways of apoptosis considered to be an important determinant of progression from adenoma to malignant tumor.

### **Microsatellite instability pathway (MSI)**

Microsatellites are short repeat nucleotide sequences that are spread out over the whole genome and are prone to errors during replication due to their repetitive manner. The DNA mismatch repair (MMR) system recognizes and repairs base-pair mismatches that occur during DNA replication. Instability of microsatellites is a reflection of the inability of the MMR system to correct these errors and is recognized by frameshift mutations in the microsatellite repeats. The discovery of MSI in 1993, its linkage to HNPCC, and the subsequent cloning of MMR genes have led to the recognition of MSI as an alternative pathway in colorectal carcinogenesis. Germline mutation in MMR genes results in HNPCC, while somatic mutation or hypermethylation silencing of MMR genes accounts for about 15% of sporadic CRC. Microsatellite instability tumors have fewer mutations in K-Ras and p53 [Soreide *et al.*, 2006].

Mutation in the polyadenine tract of transforming growth factor b type II receptor (*TGFbRII*) inactivates gene function [Markowitz *et al.*, 1995] and has been observed in 90% of CRC with MSI. TGFb-II signaling inhibits cellular proliferation, and therefore alterations in the gene function represent a possible mechanism in MSI carcinogenesis. There is a large list of genes containing coding repeats that are susceptible to mutations in the presence of defective MMR function. It includes genes involved in DNA repair (e.g. *RAD50*, *MSH3*, *MSH6*, *BLM*, *MBD4*, and *MLH3*), apoptosis (e.g. *APAF1*, *BAX*, *BCL-10*, and *Caspase 5*), signal transduction (e.g. *TGFbRII*, *ACTRII*, *IGF1R*, and *WISP-3*), cell cycle (*PTEN* and *RIZ*), and the transcription factor TCF-4 [Iacopetta *et al.*, 2010].

### CpG Island Methylator Phenotype pathway

CpG Island Methylator Phenotype (CIMP) refers to the presence of concomitant hypermethylation of multiple genes. Five markers have been chosen to serve as markers for CIMP, such as CACNA1G, IGF2, NEUROG1, RUNX3, and SOCS1. CIMP positivity is defined by methylation of at least three markers [Weisenberger *et al.*, 2006] CIMP-high CRC accounts for 15-20% of sporadic CRC and has distinct characteristics. It is more common in females, older patients, and proximal location (right colon).

## 1.2. Diet in colon cancer as a double-edge sword

*Before the 20th century, colorectal cancer was relatively uncommon, however the incidence has risen dramatically especially in the last fifty years. Moreover, the majority of CRC continues to occur in industrialized countries. Several risk factors, already discussed above, have been proposed it has been estimated that nutrition could account for more than one third of cancer deaths [Doll et al., 1981], and that dietary factors are responsible for 70% to 90% of all cases.*

### 1.2.1. Western diet as a colon cancer promoter

Nutritional patterns collectively termed the “Western diet”, including high-fat and cholesterol, high-protein, high-sugar, and excess salt intake, as well as frequent consumption of processed and ‘fast foods’, promote the incidence of colorectal cancer.

There is evidence from both case-control and cohort studies that consumption of processed or red meat, especially when cooked at high temperatures by methods such as frying, grilling or broiling, is associated with increased risk of colorectal cancer. Indeed, some studies including the Nurses’ Health Study show that colorectal carcinogenesis could involve the secretion of insulin as a response to red and processed meats and thus subsequent activation of insulin and insulin growth factor-1 receptors, may lead to increased cell proliferation and reduced apoptosis [Giovannucci et al., 1995]. Furthermore, cooking meat at high temperatures may lead to the formation of mutagenic and carcinogenic heterocyclic amines (Haem) through the interaction of muscle creatinine with amino acids [Sugimura et al., 2004] as well as the formation of N-nitroso compounds [Cross et al., 2004]. Fish and poultry are alternative sources of protein and have been shown to reduce the risk of CRC and adenoma [Willett et al., 1990]. Possible mechanisms may involve more efficient methylation due to high methionine content in these foods or the presence of n-3 polyunsaturated fatty acids (PUFA), especially from oily fish.

Other studies have demonstrated an increase in the risk of colorectal cancer with increased total energy intake [Satia-Abouta *et al.*, 2003]. Dietary lipids provide a rich source of energy and diets high in lipids, especially animal fat, may increase the risk of colorectal cancer [Hamer *et al.*, 2008]. Different types of fats may play different roles in colorectal carcinogenesis via different mechanisms such as upregulation of apoptosis, inhibition of interleukin 1 and tumour necrosis factor- $\alpha$  synthesis, COX-2 inhibition and modulation of the redox environment in the colonocytes [Vanamala *et al.*, 2008].

### 1.2.2. Dietary components in colon cancer clinical

Almost 25 centuries ago, Hippocrates, the father of medicine, proclaimed “Let food be thy medicine and medicine be thy food”. In fact, exploring the association between diet and health continues until our days. The elucidation of some targets for dietary components is fundamental to the development of effective prevention strategies and approaches. Accumulating preclinical studies indicate that dietary constituents including those involved with differentiation/proliferation and inflammation can influence various genetic and epigenetic events associated with colon cancer (Table 1).

Bioactive food component	Protocol ID	Subjects (patients with)	Trial type	Phase
Inulin	NCT00319007	FAP*	Prevention	II
N-3 FAs	NCT00168987	Gastroenterological tumors	Supportive care	IV
Vitamin D/Ca	NCT00153816	Removed large bowel adenomas	Prevention	II / III
Selenium	NCT0078897	Adenomatous colorectal polyps	Prevention	III
Curcumin	NCT00295035	Metastatic colon cancer	Treatment	III
	NCT00118989	Resected adenomatous colonic polyps	Prevention	II
	NCT00248053	FAP	Prevention	II
Resveratrol	NCT00256334	Colon cancer	Biomarker	I / II

\* FAP, familial adenomatous polyposis: <http://www.cancer.gov/clinicaltrials>.

**Table 1. Colon cancer trials with bioactive dietary components [Young *et al.*, 2007]**



- Calcium and vitamin D

Garland *et al.* proposed that lower levels of vitamin D could account for the increase in mortality from colon cancer in higher latitudes and epidemiological studies showed that deaths from colorectal cancer have been found to be higher in areas with less sunlight. Populations consuming higher amounts of fresh fish, shellfish, calcium and vitamin D have lower incidence of colorectal cancer and may even have the lowest incidence of both colon and rectal cancer in Europe and North America. In addition, evidence reported that The active form of vitamin D, 1,25-dihydroxyvitamin D<sub>3</sub> (Calcitriol) which is formed by hydroxylating the pro-vitamins in the liver and kidneys, inhibits the accumulation of  $\beta$ -catenin by facilitating its degradation. The reduced  $\beta$ -catenin causes a decrease in its transcriptional activity that stimulates the expression of  $\beta$ -catenin/TCF4-responsive oncogenes such as c-myc. These findings are further supported by the data demonstrating that Calcitriol significantly decreases the expression of c-myc gene in the nucleus of SW837 cells. Thus, it is likely that the active form of vitamin D suppresses the oncogenic function of c-myc and thus inhibits the formation of ACF. The relation between vitamin D and colorectal cancer risk is also suggested in the Women's Health Initiative. The Women's Health Initiative was a clinical trial examining the effect of a diet low in fat and high in fruit, vegetables, and grains in preventing breast and colorectal cancers and heart disease in healthy postmenopausal women. Findings revealed a significant inverse trend with lower baseline levels of serum 1,25-dihydroxyvitamin D<sub>3</sub> being associated with an increased risk of colorectal cancer [*Tangpricha et al., 2001*].

Calcium was found to have protective effect on colorectal cancer risk in some prospective studies Data from the HPFS and NHS cohorts showed that total, dietary and supplemented calcium reduced the risk of distal colon but not proximal cancer. Most of the risk reduction was achieved by calcium intake of 700-800 mg/day. An analysis of 10 cohort studies showed 22% reduction in the risk of colorectal cancer in those with higher intake of calcium [*McCullough et al., 2003*].

- Sodium-butyrate

Links between inflammation and colorectal cancer have been demonstrated by the increased risk of developing colon cancer in patients with inflammatory bowel diseases as well as the effectiveness of anti-inflammatory drugs to reduce intestinal tumors. In any case, it is not terribly surprising that consumption of dietary components with anti-inflammatory activity has been associated with the reduced risk of developing colorectal cancer. The inhibition of inflammatory processes by such diverse food components as butyrate is beginning to identify key events that are essential for the transformation of normal epithelial colonic cells into a neoplasm. Experimentally, the anti-tumorigenic properties of fiber, which may include non-digestible oligosaccharides such as inulin and oligofructose, appear to relate to butyrate formation by colonic bacteria [*Lupton et al., 2004*]. The preventive and anti-inflammatory activities of butyrate have been associated with its ability to modulate transforming growth factor (TGF)- $\beta$  signaling, IFN- $\gamma$ -mediated apoptosis, and the expression of intestinal muc2 gene that is responsible for mucin synthesis in model systems.

**TGF- $\beta$**  is recognized as an antiinflammatory cytokine that is expressed in the gut epithelium and thus serves as an important negative regulator of the proliferation of colonocytes. Recently, Nguyen et al [*Nguyen et al., 2006*] demonstrated that Na-butyrate selectively enhanced TGF- $\beta$ -induced phosphorylation of Smad3 in gut epithelial cells. Smad3 protein is normally maintained in an unphosphorylated, inactive state in the cytoplasm, and thus, the response is to change the normal regulatory control. When TGF- $\beta$  binds and activates its receptor complex, Smad3 is activated by phosphorylation and forms, a heterodimeric complex with Smad4 and Smad2. This complex translocates into nucleus and regulates transcription of tumor suppressor genes such a p27 or oncogenes such as myc. This effect is further magnified by the supplementation of Na-butyrate and the apoptotic effect of butyrate on colonocytes may be in part explained by the regulation of phosphorylation of Smad3 in TGF- $\beta$  signaling.

Butyrate was also shown to sensitize colon cancer cells to apoptosis induced by **IFN- $\gamma$** , a cytokine produced by activated T lymphocytes and natural killer cells. Studies reported that butyrate enhances IFN- $\gamma$ -induced apoptosis through the inactivation of STAT1 transcription factor in colon cancer cells [*Biswas S et al., 2006*].

The ability of butyrate to inhibit **MUC2 gene** expression may be important. MUC2 gene is a differentiation marker of the secretory goblet cell lineage that predominantly synthesizes mucins. In vitro studies demonstrate that the treatment of HT29 colon cancer cells with Na-butyrate induces a marked reduction in MUC2 mRNA levels as a function of time. Perturbations in goblet cell function via inactivation of the MUC2 gene may be associated with the decreased development of intestinal tumors [*Augenlicht et al., 2003*].

- (n-3) Fatty acids

Another bioactive food component that produces anti-inflammatory effects is (n-3) Fatty acids ((n-3) FAs). Epidemiological and preclinical studies suggest that the consumption of a diet high in (n-3) FAs reduces the risk of colon cancer. The (n-3) FAs existing in foods include  $\alpha$ -linolenic acid in plants such as olive, walnut, and canola as well as eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) occurring in some fish. The cancer preventive effects of (n-3) FAs appear to be primarily attributed to its anti-inflammatory functions. Inhibition of IL-1 and TNF- $\alpha$  synthesis is a common finding when (n-3) FAs are provided. In addition, it has been shown that supplementation of (n-3) FAs to healthy humans suppresses the capacity of monocytes to synthesize TNF- $\alpha$ . It is likely that the (n-3) FA-mediated decrease in TNF- $\alpha$  brings about increased activity of caspase-3 as well as a decreased activity of NF $\kappa$ B, which induces the apoptosis of colon cancer cells. However, the recent observations that (n-3) FAs can suppress the expression of HER-2/neu transgenic mice suggest that these fatty acids may have multiple biological effects [*Yee et al., 2005*].

- Selenium

An inverse association between Selenium supplementation and the risk of colorectal cancer was observed in several studies. Selenium supplementation by way of brewer's yeast supplementation was associated with up to 50% reduction in the incidence of colorectal cancer [*Jacobs et al., 2004, Reid et al., 2006*].

- Phytochemicals

Polyphenols are a class of chemicals known for their numerous benefits especially their antioxidant effects inhibition of cellular proliferation, induction of cell cycle arrest, interaction with apoptotic pathways and antiangiogenic and antimetastatic properties. The most important dietary sources of polyphenols are fruits, vegetables, seeds, and beverages such as fruit juice, green tea, coffee, cocoa drinks, red wine, and beer. The chemoprotective role of polyphenols against cancer has been extensively studied. Evidence from case-control studies, cell culture and animal studies have shown a protective role against colorectal malignancy [*Araújo et al., 2011; Johnson et al., 2007*].

**Curcumin**. This polyphenol is a curcuminoid found in turmeric spice that has antioxidant, anti-inflammatory and antitumour properties. A study reported that Curcumin is able to inhibit human cancer cell growth by suppressing gene expression of epidermal growth factor receptor through reducing the activity of the transcription factor Egr-1 [*Chen et al., 2006*]. Furthermore, Curcumin has been revealed to possess anti-metastatic properties; Su et al [*Su et al., 2006*] showed that Curcumin is able to inhibit cell migration of human colon cancer colo-205 cells through the inhibition of nuclear factor kappa B/p65 and downregulates cyclooxygenase-2 and matrix metalloproteinase-2 expressions. In addition, it was able to reduce the number and size of ileal and rectal adenomas in patients with familial adenomatous polyposis [*Cruz-Correa et al., 2006*].

**Resveratrol**. Resveratrol is a natural phenol smaller in size than polyphenols. It is found in the skin of grapes and red wine and has been shown to inhibit metastasis by reducing hypoxia inducible factor-

1 $\alpha$  and MMP-9 expression in colonocytes [*Wu et al., 2008*] as well as inhibiting Wnt signalling and  $\beta$ -catenin localisation [*Hope et al., 2008*].

### 1.3. Caco-2 cells as tumour and healthy models

In the 1970s, Caco-2 cell line was established from human colon adenocarcinoma with the aim of performing studies on cancer mechanisms [Fogh *et al.*, 1977]. Only a decade later, due to the difficulties encountered in obtaining intestinal differentiated cell lines in culture from normal tissue, attention was directed to the specific intestinal properties of some gastrointestinal tumour cell lines. In most cell lines, only a partial differentiation was induced by cell treatment with synthetic or natural factors added to the medium [Pinto *et al.*, 1983]. However, the Caco-2 cell line exhibited spontaneous enterocytic differentiation in long-term culture. The first studies on the Caco-2 cell line demonstrated that these cells, upon differentiation, express several morphological and biochemical characteristics of small intestinal enterocytes [Pinto *et al.*, 1983]. These cells grow in monolayer, show a cylindrical polarized morphology, with microvilli on the apical side, tight junctions between adjacent cells, and also express disaccharidases and peptidases typically found on the apical membrane in normal small intestinal villus cells, (i.e. sucrase-isomaltase, lactase, aminopeptidase N, dipeptidylpeptidase IV). The functional polarity of the cell monolayer is further indicated by the fact that, after confluence, Caco-2 cells form domes on impermeable substrates, and transport ions and water toward the basolateral surface [Pinto *et al.*, 1983]. Among other intestinal properties that were shown to be expressed in Caco-2 cells upon differentiation, there is the expression of polarized membrane receptors for growth factors as well as several transport activities on both apical and basolateral membranes. In fact, it has been shown that Caco-2 cells express the epidermal growth factor (EGF) receptor and its affinity, level of expression and related cellular response are strongly modulated by the degree of cell differentiation [Kuvada *et al.*, 1999]. It was also shown that the vitamin D receptor is present both in undifferentiated and differentiated cells, with 2.6 times more binding sites in the latter than in the former [Giuliano *et al.*, 1991].

In addition, specific intestinal carriers for sugars, amino acids, di- and tripeptides, vitamins, bile acids, micronutrients including heavy metals and nucleosides have been identified and functionally characterized in Caco-2 cells [*Hidalgo and Li, 1996*]. Moreover, the functional activity of the Na<sup>+</sup>/H<sup>+</sup> transporter NHE3 gradually increases (up to 80%) from day 3 to day 22 post confluence [*Janecki et al., 1999*]. An additional study by Mailleau and coworkers [*Mailleau et al., 1998*], showed that the expression of the Cl<sup>-</sup> channel present on the apical membrane domain of epithelial cells (CFTR) and known also as a transporter of ATP-binding cassettes (ABC), increased from day 1 up to day 24 of culture. In the same study the activity of the Na<sup>+</sup>/glucose transporter SGLT1, was shown to rapidly increase with differentiation [*Mailleau et al., 1998*]. An increase in SGLT1 expression with time in culture had originally been shown to be associated with a parallel increase in the facilitated sugar transporters GLUT2 (glucose) and GLUT5 (fructose). Another member of the ABC family is the apical efflux pump P-gp, a trans-membrane protein encoded by the multidrug resistance (MDR1) gene, responsible for the apical efflux of several substances, including many drugs. Differentiated Caco-2 cells have an elevated activity of P-gp, higher than the human intestine in vivo rendering these cells able to be used as a model for intestinal bioavailability [*Goto et al., 2003*]. Furthermore, among the amino acid transport systems, the Na-dependent acidic amino acid transporter XAG-, resulting from the expression of the EAAT1 protein, has been shown to raise steadily between day 8 and day 17 from seeding [*Mordrelle et al., 2000*].

The biochemical and functional properties exhibited by the Caco-2 cell line at different times in culture, as well as their stability in terms of morphology, growth characteristics and differentiated features have been the rationale for the use of these cells as a model of both colon cancer tumor and intestinal barrier in a very great number of studies.

## 2. Flavonoids and Luteolin

*Epidemiological studies have shown that a diet rich in plant-derived foods is consistently associated with a reduced risk of developing colon cancer. Although the compounds responsible for this preventive effect are not fully identify, evidence suggests that flavonoids may be good candidates. In fact, for thousands of years several plants and spices containing flavonoid derivatives have found application as disease preventive and therapeutic agents in traditional medicine in Asia. In addition, the potential health benefits of a particular food plant, plant tissue or herb appears to mirror its flavonoid composition. However, the mechanism through which flavonoids mediate their protective effects in diets rich in these foodstuffs is not completely understood.*

### 2.1 Flavonoids

#### 2.1.1 Structure

Flavonoids are a large group of polyphenolic compounds, constituted by 5000 structurally unique and distinct molecules identified in the plant kingdom. Chemically, the common structure of these low molecular weight substances consists of two fused six-membered rings , an aromatic A-ring and a heterocyclic C-ring, connected through a carbon-carbon bridge to an aromatic B-ring. Based on their molecular backbone structure and hydroxyl group, flavonoids could be further divided into several subgroups as shown in **Fig.3: flavones** (2-phenylchromen-4-one); e.g., apigenin, luteolin, and tangeretin; **flavonols** (3-hydroxy-2-phenylchromen-4-one), e.g., quercetin, kaempferol, myricetin and rhamnazin; **flavanones** (2, 3-dihydro-2-phenylchromen-4-one), e.g., hesperetin, naringenin, and eriodictyol; **flavanols** [2-phenyl-3,4-dihydro-2H-chromen (flavan)-3-ol, flavan-4-ol, flavan-3,4-diol], e.g., catechins and epicatechins; **flavanonols** (3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one), e.g., silibinin, taxifolin, and dihydrokaempferol; **isoflavones** (3-phenylchromen-4-one), e.g., genistein, daidzein, and glycitein, and anthocyanidins (2-phenylchromenylium), e.g., cyanidin, delphinidin, and malvidin [*Ververidis et al., 2007*].



### 2.1.2. Source and biological activities

Flavonoids are recognized in plants, primarily, as the pigments responsible for the colors of leaves, especially in autumn [Brouillard *et al.*, 1988]. Moreover, as components of edible plants and plant foodstuffs, flavonoids constitute an integral part of the human diet [Herrmann *et al.*, 1976, Harborne *et al.*, 2000]. Indeed, flavonoids are widely distributed in fruits, vegetables, nuts, seeds, herbs, spices flowers, as well as in beverages such as red wine and teas.

Many efforts have been made to determine the dietary intake of flavonoids, however, the formation of flavonoids in plants is influenced by numerous factors including light, plant genetics, environmental conditions, germination, degree of ripeness, and processing and storage, as well as species variety [Bravo *et al.*, 1998]. For example, cherry tomatoes contain six times more quercetin per gram fresh weight than normal size varieties of tomatoes [Duthie *et al.*, 2000]. This is likely due to the fact that this flavonol is synthesized and stored in the skin of the tomato, and smaller varieties have a higher skin to volume ratio. In addition to factors affecting content, there has been a lack of agreement on an appropriate method to analyze the different types of flavonoids. As a consequence, information in the literature on content of flavonoids in plant foods is incomplete and often contradictory.

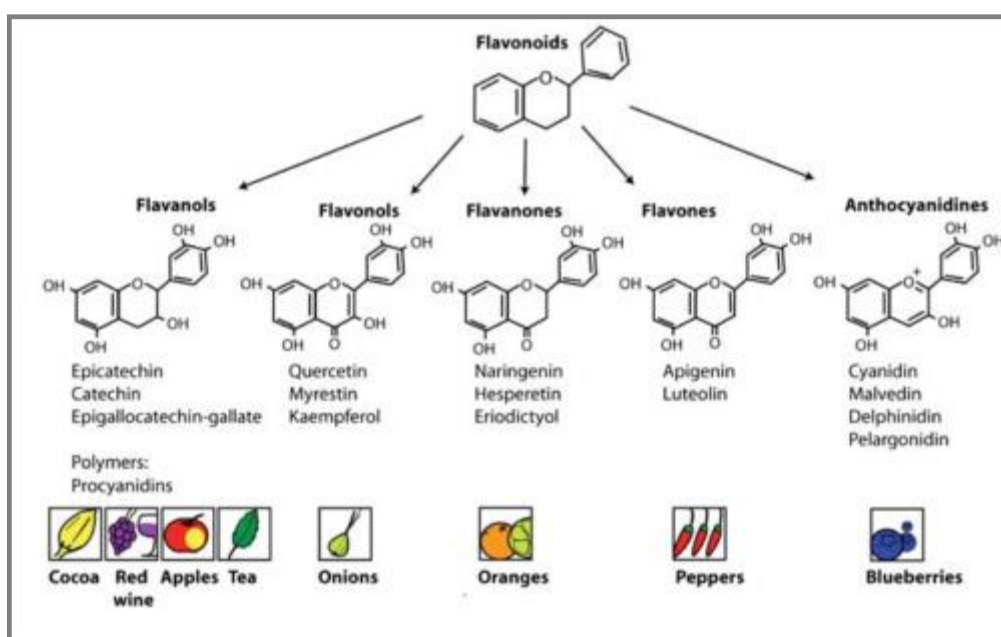


Figure 3. Molecular backbone structures of flavonoids and its subgroups [Kanaswami *et al.*, 2005]

Therefore, estimation of dietary intake of flavonoids is difficult and only a few data about it are available. Investigations in different populations have revealed that mean total flavonoid intake is usually in the order of 100-200 mg/day, flavonoles (with quercetin and kaempferol as main components) contribute 5-20 mg/day, flavones only about 1 mg; up to 90 % of the flavone fraction may be apigenin [Herrmann *et al.*, 1976].

Growing evidence highlights flavonoids as compounds with potential biological, pharmacological, and medicinal properties [Cody *et al.*, 1986 and 1988]. Indeed, flavonoids are reported, in addition to their free radical scavenging activity, to have multiple beneficial effects including vasodilatory, antiinflammatory, antibacterial, immune-stimulating, antiallergic, antiviral, and estrogenic effects, as well as to be inhibitors of phospholipase A2, cyclooxygenase, and lipoxygenase, glutathione reductase and xanthine oxidase.

Furthermore, an impressive body of information suggests that flavonoids could represent promising agents in anti-cancer research thanks to their ability to block the cell cycle, to induce apoptosis [Kuntz *et al.*,1999], to disrupt mitotic spindle formation [Beutler *et al.*, 1998], and to inhibit angiogenesis [Mojzisa *et al.*, 2008] (Fig.4). However, because the chemistry of the flavonoids seems to critically influence their activities, each member of this family must have been individually investigated for its potential applications in anti-cancer therapy.

In our study, we focused on Luteolin (LU); LU structure, properties and functional roles are described in the next section.

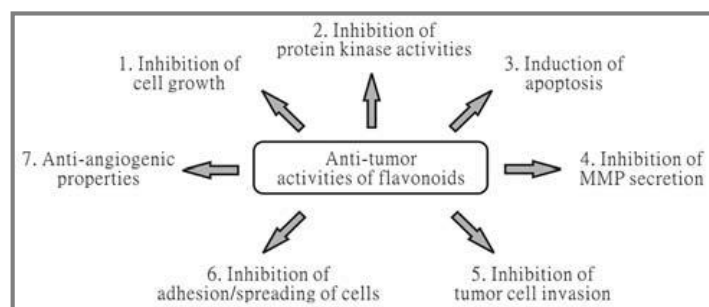
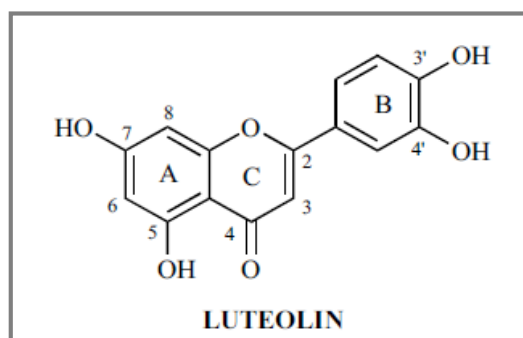


Figure 4. Anti-tumor activities of flavonoids [Kanadaswami *et al.*, 2005]

## 2.2. Luteolin

### 2.2.1. Structure and derivatives

Belonging to the flavone group of flavonoids, LU has a C<sub>6</sub>-C<sub>3</sub>-C<sub>6</sub> backbone and possesses two benzene rings, a third, oxygen-containing ring, and a 2–3 carbon double bond. LU also possesses hydroxyl groups at carbons 5, 7, 3', and 4' positions (**Fig. 5**) [Ross *et al.*, 2002]. The hydroxyl moieties and 2-3 double bond are important structure features in LU that are associated with its biochemical and biological activities [Chan *et al.*, 2003]. As for other flavonoids, LU may be present in plants as an aglycone (without any sugars bound to it) and as glycosides (with one or several sugars bound to it). Most glycosides of LU are O-glycosides, i.e., the sugar moieties are bound to the aglycone through one or several free hydroxyl (OH) groups. These glycosides usually have sugar moieties at positions 5, 7, 3' and 4'. Cynaroside (LU-7-O-glucoside) and scolymoside (LU-7-O-rutinoside) are classical examples of LU-7-O-glycosides. Sugars can also be bound to LU through a C-C bond, forming C-glycosides. LU-8-C-glucoside (orientin) and LU-6-C-glucoside (isoorientin) are the most common C-glycosides of LU. Glucose is the most frequent sugar found in LU glycosides; apiose, rhamnose, rutinose, galactose, arabinose, glucuronic acid and xylose are other sugars commonly found in LU glycosides. The glycoside is hydrolyzed to free LU prior to absorption [Hempel *et al.*, 1999].



**Figure 5. Chemical structure of Luteolin**

**A and B:** two aromatic rings with hydroxyl groups at 5, 7, 3' and 4' positions. **C:** chromenone ring with 2-3 carbon double bond

### 2.2.2. Sources

LU and its glycosides have been identified in many edible plants as carrots (*Daucus carota*), peppers (*Capsicum annuum*), celery (*Apium graveolens*), olive oil (*Olea europaea*), peppermint (*Mentha x piperita*), thyme (*Thymus vulgaris*), rosemary (*Rosmarinus officinalis*), oregano (*Origanum vulgare*), lettuce (*Lactuca sativa*), Perilla leaves (*Perilla frutescens*), pomegranate (*Punica granatum*), artichoke (*Cynara scolymus*), chocolate (*Theobroma cacao*), rooibos tea (*Aspalathus linearis*), turnip (*Brassica napus*), capers (*Capparis spinosa*) and cucumber (*Cucumis sativus*). It has also been identified in lemon, beets, Brussels sprouts, cabbage, cauliflower, chives, fennel, horseradish, parsley, spinach and green tea. It has been reported that the LU intake is about 0.1 mg/day [López-Lázaro *et al.*, 2009].

### 2.2.3. Bioavailability and metabolism

In order to exert some of its biological activities in an *in vivo* setting, the bioavailability of LU needs to be sufficiently high and its metabolism sufficiently low. Otherwise, many activities of LU observed *in vitro* will not be relevant *in vivo*. Since LU is a common dietary constituent and since the oral route is the preferred route of administration for most drugs, it is important to know the bioavailability and metabolism of this flavonoid after oral ingestion.

Shimoi *et al.* investigated the intestinal absorption of LU and LU-7-O-beta-glucoside in rats [Shimoi *et al.*, 1998]. The absorption analysis demonstrated that LU was converted to glucuronides during passing through the intestinal mucosa and that LU-7-O-beta-glucoside was absorbed after hydrolysis to LU. Free LU, its conjugates and methylated conjugates were found to be present in plasma. This suggests that some molecules of LU can escape the intestinal conjugation and the hepatic sulfation/methylation. In agreement, the presence of free LU and its monoglucuronide were observed in human serum after ingestion of this flavonoid. Free LU was absorbed more efficiently from the duodeno-jejunum than from the ileum, and LU in propyleneglycol was absorbed more rapidly than

that in carboxymethyl cellulose. The plasma concentration of LU and its conjugates reached the highest level 15 min and 30 min after dosing with LU in propyleneglycol, respectively. The ratio of LU to LU monoglucuronide in plasma of LPS-treated rats increased suggesting that the plasma concentrations of free LU can increase in some pathological processes such as inflammation [Shimoi *et al.*, 2001].

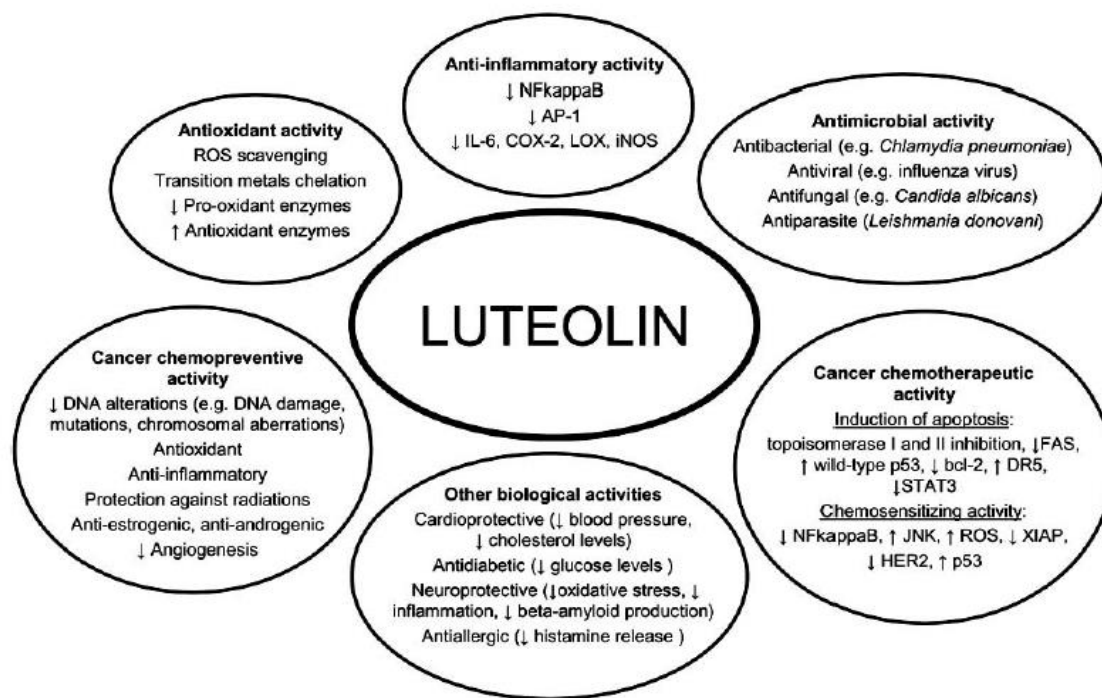
In accordance with the observations reported above, Zhou and coworkers [Zhou *et al.*, 2008] demonstrated that LU is absorbed passively in the intestine of rats and that its absorption is more efficient in the jejunum and duodenum than in the colon and ileum. The bioavailability of LU in peanut hull extract (PHE) was significantly greater than that of pure LU; after oral administration of 14.3 mg/kg LU or 92.3 mg/kg of PHE (with 14.3 mg/kg LU), they observed that the maximum plasma concentration of LU PHE (*C* max) was about 8-fold higher than pure LU. The time to reach the maximum plasma concentration was about 1 hour for LU and 30 minutes for PHE. The half-life of LU was about 5 hours for LU and 3 hours for PHE [Zhou *et al.*, 2008].

Taken together, these reports firmly indicate that LU can be absorbed after oral administration. Although most LU found in plasma is in the form of glucuronide and sulfate conjugates, low concentrations of free LU can be achieved in plasma after oral ingestion of this flavonoid. Moreover, the plasma concentrations of LU depend on the form in which LU is ingested; the maximum concentrations of LU are achieved after 1-2 h, and LU remains in the plasma for several hours [Zhou *et al.*, 2008].

Intriguingly, Jin-Feng Qiu and colleagues recently created a water-soluble LU to render the use of LU clinically available without obstacles, as its poor water solubility. The authors used monomethoxy poly(ethylene glycol)-poly( $\epsilon$ -caprolactone) (MPEG-PCL) micelles to encapsulate LU by a self-assembly method. Encapsulation of LU in MPEG-PCL micelles improved the half-life and peak concentration of LU when the drug was intravenously administered at a dose of 30 mg/kg in rats. In addition, LU/MPEG-PCL micelles could slowly release LU *in vitro* maintaining its cytotoxicity on 4T1 breast cancer cells and C-26 colon carcinoma cells [Qiu *et al.*, 2013].

## 2.2.4 Biological activities

Preclinical studies have shown that this flavone possesses a variety of pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial and anticancer activities (**Fig.6**)



**Figure 6.** *Biological activities and possible mechanisms of action of luteolin [Lopez-Lazaro et al., 2007]*

- Antioxidant, anti-inflammatory and other anti-biological effects

Previous studies demonstrated a strong relationship between the molecular structures of flavonoids and their antioxidant activities [Rice-Evans et al., 1996 and 2001]. In particular, flavonoids display two classical antioxidant structural features that are the presence of a B-ring catechol group, which serves to donate hydrogen/electron and subsequently to stabilize a radical species and the presence of a C2-C3 double bond in conjugation with an oxo-group at C4 which serves to bind transition metal ions such as iron and copper. Because LU fulfills these two structural requirements, it is not surprising that LU possesses antioxidant properties. Indeed, several studies demonstrated the capacity of LU to scavenge reactive oxygen and nitrogen species, in various types of cells as murine leukemia L1210

cells [Horváthová *et al.*, 2003], human HMB 2 melanoma cells [Horváthová *et al.*, 2005] and in hepG2 hepatic cells [Kanazawa *et al.*, 2006]. Furthermore, LU is able to chelate transition metals that may induce oxidative damage by suppressing the Fenton reaction [Mira *et al.*, 2002, Cheng *et al.*, 2000] and its capable to inhibit/ prooxidant enzymes and to induce the activity of antioxidant enzymes [Choi *et al.*, 2008, Sadik *et al.*, 2003]. The antioxidant activity of LU has not only been observed *in vitro* but also *in vivo*. Indeed, the treatment of heroin-dependent mice with LU and verbascoside limited the oxidative stress status and the damage to DNA, proteins and lipids in brain [Qiusheng *et al.*, 2005].

At micromolar concentrations, LU also displays specific anti-inflammatory effects *in vitro* and *in vivo* that are only partly explained by its antioxidant capacities. The anti-inflammatory activity includes suppression of pro-inflammatory cytokines expressions, reduction of various pro-inflammatory enzymes activities such as cyclooxygenase (COX) [Harris *et al.*, 2006, Hu *et al.*, 2004], lipoxygenases (LOX) [Sadik *et al.*, 2003] and inducible nitric oxide synthase (iNOS) [Kim *et al.*, 2006, Hu *et al.*, 2004]. LU also exhibited a high inhibitory activity against both thromboxane and leukotriene synthesis. Furthermore, several works have shown that LU inhibits NF-kappa B activity, causing a subsequent downexpression of pro-inflammatory cytokines, chemokines and enzymes as TNF, IL-1, IL-6, IL8, COX-2, iNOS [Kim *et al.*, 2006]. Up to now, LU is the most potent flavonoids in the inhibition of TNF-alpha release from macrophages, and is able to block lipopolysaccharide (LPS)-induced activation of NF-kappa B, as well as NF-kappa B-driven gene expression [Harris *et al.*, 2006]. Another mechanism involved in the anti-inflammatory activity of LU is the inhibition of the activator protein 1 (AP-1), a transcriptional regulator composed of members of the Fos and Jun families, that controls the expression of the inflammatory cytokine IL-6 together with NF-kappa B [Harris *et al.*, 2006].

Choi and colleagues have observed that, in mice, the administration of water supplemented with LU decreased IL-6 levels in plasma and IL-6 production in hippocampus [Choi et al., 2008] suggesting that LU reduced LPS-induced IL-6 production *in vitro* and *in vivo* by inhibiting the JNK and AP-1 signaling pathway.

An *in vivo* study reported that LU, after parenteral and oral application, significantly inhibited the scratching behavior associated with allergic cutaneous anaphylaxis in animal models of irritant and allergic contact dermatitis inflammation [Baolin et al., 2005]. LU has also shown anti-allergic activity *in vitro* and *in vivo* through different mechanisms including inhibition of histamine release [Ueda et al., 2002, Kimata et al., 2000]. Moreover, numerous studies have reported that LU, its glycosides and plants containing it, showed antibacterial activity [Lv. et al., 2008, Zhu et al., 2004]; as on the course of acute Chlamydia pneumoniae infection *in vivo* [Törmäkangas et al., 2005], analysis of lung tissue revealed that LU histologically suppressed inflammation, the development of C.Pneumoniae-specific antibodies and the presence of chlamydia in lung. In addition, LU was reported as influenza virus neuraminidase inhibitor playing therefore the role of a natural molecule with antiviral properties [Liu. et al., 2008]. An antifungal activity was also attributed to LU by the fact that it showed activity against Epidermophyton floccosum, Trichophyton rubrum and Trichophyton mentagrophytes [De Campos et al., 2005].

Moreover, experimental data indicated that LU may prevent cardiovascular disease by reducing blood pressure and cholesterol levels [Ichimura et al., 2006], diabetes by reducing glucose levels [Cunha et al., 2008, Zarzuelo et al., 1996] and neurodegenerative disease by reducing oxidative stress, inflammation and beta-amyloid production [Rezai-Zadeh et al., 2008]. Due to its protective role, it is not surprising that plants rich in Luteolin have been used for many years in traditional medicine to treat infectious diseases.



- Anticancer activity

#### Cancer preventive activity

Several *in vivo* studies suggest that LU has also a cancer preventive potential. In a 20-methylcholanyrene-induced fibrosarcoma rodent model, a LU-rich diet administration significantly suppressed tumor incidence by reducing lipid peroxides and cytochrome P450, increasing the activity of glutathione-S-transferase (GST), and suppressing DNA synthesis [Elangovan *et al.*, 1994]. Furthermore, in a murine skin carcinogenesis model, topical applications of LU prior to 12-tetradecanoylphorbol 13-acetate (TPA) treatment resulted in a significant reduction in tumor incidence and multiplicity, due to the inhibiting of the inflammatory response and the scavenging reactive oxygen radicals by this flavonoid [Ueda *et al.*, 2003].

Several mechanisms participate in the cancer preventive activity of LU. Numerous studies have shown that LU can prevent the DNA alterations induced by different carcinogenic agents both *in vitro* and *in vivo* [Cai *et al.*, 1997]. Taj and coworkers studied the chemopreventive activity of LU against mutagenicity induced by deep-fried fish and mutton extracts in rats; they found that LU pre-treatment efficiently protected bone marrow cells against micronuclei and chromosome aberrations induced by these mutagens [Taj *et al.*, 1996]. In addition, a growing body of research suggests that the anti-inflammatory and antioxidant properties of this flavonoid, play a crucial role in its cancer preventive activity, since it is recognized that inflammatory diseases and the high levels of ROS increase the risk of developing many types of cancer [Mantovani *et al.*, 2008]. Evidence also associated the cancer preventive activity of LU with its capacity to inhibit glycolysis and ATP production in cancer cells *in vitro*, an essential process to both generate energy and proliferate during tumor development [Du GJ *et al.*, 2008].

## Cancer therapeutic activity

Besides its cancer preventive effects, accumulating data of research supports the idea that LU could be considered as a cancer therapeutic agent. Its anticancer property is associated to multiple mechanisms such as inducing apoptosis, suppressing cancer cell proliferation, angiogenesis metastasis and chemo-sensitizing also a variety of cancer cells during cancer therapy.

### **Inhibition of cancer cell proliferation**

Like many others flavonoids, LU is able to inhibit the proliferation of cancer cells derived from nearly all types of cancers mainly through regulating the cell cycle. It was reported that, in human gastric and prostate cancer, and in melanoma cells, LU is able to arrest in G1 phase the cell cycle by up-regulating the cyclin-dependent kinases (CDK) inhibitors or by inhibiting directly the CDK2 activity [*Kobayashi et al., 2002, Casagrande et al., 2001*]. In addition, the inhibitory effect of LU on cancer cell proliferation is partly achieved through blocking the proliferation signaling pathways induced by several growth factors including epidermal growth factor (EGF), platelet derived growth factor (PDGF), insulin-like growth factor (IGF), and fibroblast growth factor (FGF). Indeed, it was reported that LU inhibited insulin-like growth factor 1 (IGF-1) induced activation of IGF-1R and AKT in prostate cancer PC-3 and DU145 cells. Thus, inhibition of AKT by LU resulted in decreased phosphorylation of its downstream targets, including p70S6K1, GSK-3beta and FKHR/FKHRL1. In addition, the same study showed that LU was able to suppress prostate tumor growth in vivo through suppressing IGF-1R/AKT signaling [*Fang et al., 2007*]. Similarly, LU inhibits PDGF-induced proliferation by inhibiting PDGF receptor phosphorylation in vascular smooth muscle cells [*Kim et al., 2005*]. In addition LU can act, not only by affecting the receptor activity, but also by directly targeting the downstream pathways that are involved in cell proliferation. For example, protein kinase C, a family of serine/threonine protein kinases that regulates growth factor response and cell proliferation, differentiation and apoptosis, can be inhibited in a concentration-dependent manner by LU intact cells [*Weinstein et al., 1997*].

## Induction of apoptosis

Numerous studies have demonstrated that LU can induce apoptosis in a variety of cancer cells lines. Although the mechanisms underlying LU-induced apoptosis are complex, they can be generalized as processes either enhancing apoptosis or decreasing the survival signaling in cancer cells.

In cervical and prostate cancer cells, it has been reported that LU is a potent activator of apoptosis by up-regulating the expression of the death receptor 5 (DR5) [*Horinaka et al., 2005*], a downstream gene of the p53 tumor-suppressor family, frequently inactivated to promote tumor growth *in vitro* and *in vivo*. Moreover LU is able to induce the activation and the accumulation of wild-type p53, consequently causing apoptosis, that occurred out of the G2/M phase of the cell cycle [*Plaumann et al., 1996*]. LU induce apoptosis also cleaving Bcl-2 family members in human leukemia HL-60 cells. Indeed, LU could decrease the mitochondrial membrane potential, is able to trigger cytochrome c release to cytosol, and subsequently induce the processing of procaspase-9 and procaspase-3, which are followed by the cleavage of poly-(ADP-ribose) polymerase (PARP) and DNA fragmentation factor (DFF-45). The cleavage of the pro-apoptotic Bcl-2 proteins, such as Bad and Bax to produce their truncated and active forms, and the cleavage of the anti-apoptotic Bcl-2 proteins, such as Bcl-2 and Bcl-XL, into their potent pro-apoptotic fragments were also detected [*Cheng et al., 2005*].

Selvendiran and colleagues reported that LU targeted the signal transducer and activator of transcription 3 (STAT3) through dual pathways; the ubiquitin-dependent degradation in Tyr (705)-phosphorylated STAT3 and the gradual down-regulation in Ser (727)-phosphorylated STAT3 through inactivation of CDK5, thereby triggering apoptosis via up-regulation of Fas/CD95 in human hepatoma cells [*Selvendiran et al., 2006*]. The possible role of topoisomerases in the putative anticancer and carcinogenic properties of LU was discussed and data suggest that topo II $\beta$  has an important function in LU-induced cell growth inhibition and cell death in K562 leukemia cells [*Lopez-Lazaro et al., 2007*].

On the other hand, LU shifts the cell survival and death balance to the side of death by suppressing cell survival pathways such as PI3K/AKT, NF-kappa B, and MAPKs in different cancer cells including epidermoid carcinoma, leukemia and heptoma.

### **Anti-angiogenesis**

Accumulating data indicates that LU is a potent angiogenesis inhibitor *in vitro* and *in vivo* at concentrations in the low micromolar range. It was reported that LU inhibited vascular endothelial growth factor (VEGF)-induced angiogenesis in the rabbit corneal assay and in human umbilical vein endothelial cells (HUVECs). In the former *in vivo* model LU was able to inhibit VEGF-induced neovascularization whereas in the latter model, it was found that LU exerts anti-survival and antimitotic effects. LU was able to inhibit VEGF-induced PI3K activity and to abolish the activation of AKT, a downstream target of PI3K, conveying the survival downstream signals. With regard to its antimitotic activity, LU inhibited VEGF-induced phosphorylation of p70 S6 kinase (S6K), a downstream effector of PI3K responsible for G(1) progression [*Bagli et al., 2004*].

### **Anti-metastasis**

Evidence suggested that LU may prevent the processes of invasion and metastasis by inhibiting for instance, matrix metalloproteinases (MMPs), namely MMP-2 and -9, which are involved in several stages of metastasis, and the Focal adhesion kinase (FAK) activity, since it is well known that FAK phosphorylation is associated with increased invasive potential [*Ende et al., 2004, Huang et al., 1999*]. Additionally, LU blocks critical signal transduction pathways for migration and metastasis in cancer cells such as the EGFR-signaling pathway [*Lee et al., 2004*].

### Chemosensitizer in cancer therapy

LU has been tested with other anticancer agents for its anticancer cell properties, and has sensitized different drug-induced cytotoxicity in a variety of cancer cells. Shi and coworkers reported, in a xenograft mouse model, that LU enhanced the cancer therapeutic activity of cisplatin via p53

stabilization and accumulation [Shi et al., 2007]. Moreover, the pretreatment with a non-cytotoxic concentration of LU significantly sensitized the apoptosis induced by the TNF-related apoptosis-inducing ligand (TRAIL), TRAIL, in both TRAIL-sensitive (HeLa) and TRAIL-resistant cancer cells (CNE1, HT29, and HepG2). Such sensitization is achieved through enhanced caspase-8 activation and caspase-3 maturation. Furthermore, LU sensitizes TRAIL-induced apoptosis through ubiquitination and proteasomal degradation of the protein level of X-linked inhibitor of apoptosis protein (XIAP), therefore downregulating its expression. Moreover, LU inhibited PKC activity that known to stabilize XIAP, and thus sensitizing TRAIL-induced apoptosis [Shi et al., 2005, Horinaka et al., 2005]. In other study, Shi and colleagues demonstrated that the pretreatment with LU greatly sensitized TNF alpha-induced apoptotic cell death in a number of human cancer cell lines, including colorectal cancer COLO205, HCT116 cells and cervical cancer HeLa cells via the inhibition of nuclear transcription factor-kappa B (NF-kappaB) activity and c-Jun N-terminal kinase (JNK), and therefore suppressing the expression of NF-kappaB-targeted antiapoptotic genes, including A20 and cellular inhibitor of apoptosis protein-1 (c-IAP1) [Shi et al., 2004]. In addition, in nude mice with xenografted SKOV3ip1-induced tumors, LU significantly inhibited HER2 expression and tumor growth in a dose-dependent manner, and rapamycin further enhanced the effect of LU with a concomitant p21 inhibition [Chiang et al., 2007].

#### 2.2.5. Luteolin and colon tumorigenesis

Emerging pieces of evidence recently documented that LU can exert anticancer effects in colon cancer. In particular, three studies by Manju et al showed that LU exerts chemopreventive effects against 1,2 dimethylhydrazine-induced colon carcinogenesis in rats; this effect was due to unclear mechanisms and, LU antioxidant properties have been related to this anticarcinogenic activity [Manju et al. 2005 and 2007]. Furthermore, in an *in vitro* study, Lim. and colleagues revealed that LU downregulates the activation of the PI3K/AKT and ERK1/2 pathways via a reduction in IGF-IR signaling in HT-29 cells; this may be one of the unclear mechanisms responsible for the LU-induced

apoptosis and cell cycle arrest [*Lim et al., 2007*]. In addition, LU induces growth arrest in HCT-15 colon cancer cells through involvement of Wnt/ $\beta$ -catenin/GSK-3 $\beta$  signaling. Moreover, a very recent study suggests that LU inhibits azoxymethane-induced colorectal cancer through activation of Nrf2 signaling [*Pandurangan et al., 2014*].

Notwithstanding these studies implicate LU as an anti-cancer effector in colon cancer, but little is known about the mechanisms by which LU exerts these effects.

### 3. Sphingolipids

#### 3.1. Overview on Sphingolipids

Sphingolipids (SLs) represent a major class of lipids that are ubiquitous constituents of membranes in eukaryotes. They were discovered by Thudichum in 1876 as structural constituents of biological membrane. However, in the last 20 years growing evidence highlights a pivotal role of SLs, and particularly some of them, as bioactive molecules able to regulate different cell properties and involved in the modulation of key cell functions. Concerning their chemical structures, SLs are amphipathic molecules composed by a hydrophilic (or polar head) and a hydrophobic (or tail) portion. Ceramide (Cer) is the central building block of all complex SLs and is responsible for their insertion into the outer layer of the plasma membrane (PM). Cer is constituted by a long chain amino alcohol like the 2-amino-1,3-dihydroxyoctadec-4-ene (Sphingosine, Sph), connected to a fatty acid by an amide linkage. Through its primary alcoholic residue, a variety of charged, neutral, phosphorylated, or glycosylated moieties, representing the polar head group, are attached to Cer further forming complex SLs [Merrill *et al.*, 2007]. In particular, the most abundant mammalian SL called sphingomyelin (SM), a phosphorylcholine residue is attached to Cer, whereas in glycosphingolipids (GSLs) the polar group is represented by an oligosaccharide chains, that may contain up to 15-20 saccharidic units, most frequently glucose, galactose, N-acetylglucosamine, N-acetylgalactosamine [Yu *et al.*, 2007]. The GSLs containing one or more sialic acid residues in the carbohydrate chain are referred as gangliosides. Furthermore, both the sphingoid base, Sph and Cer may exist in the phosphorylated form in correspondence with the carbon in position 1 (C-1) giving rise to sphingosine-1-phosphate (S1P) and ceramide-1-phosphate (C1P), respectively.

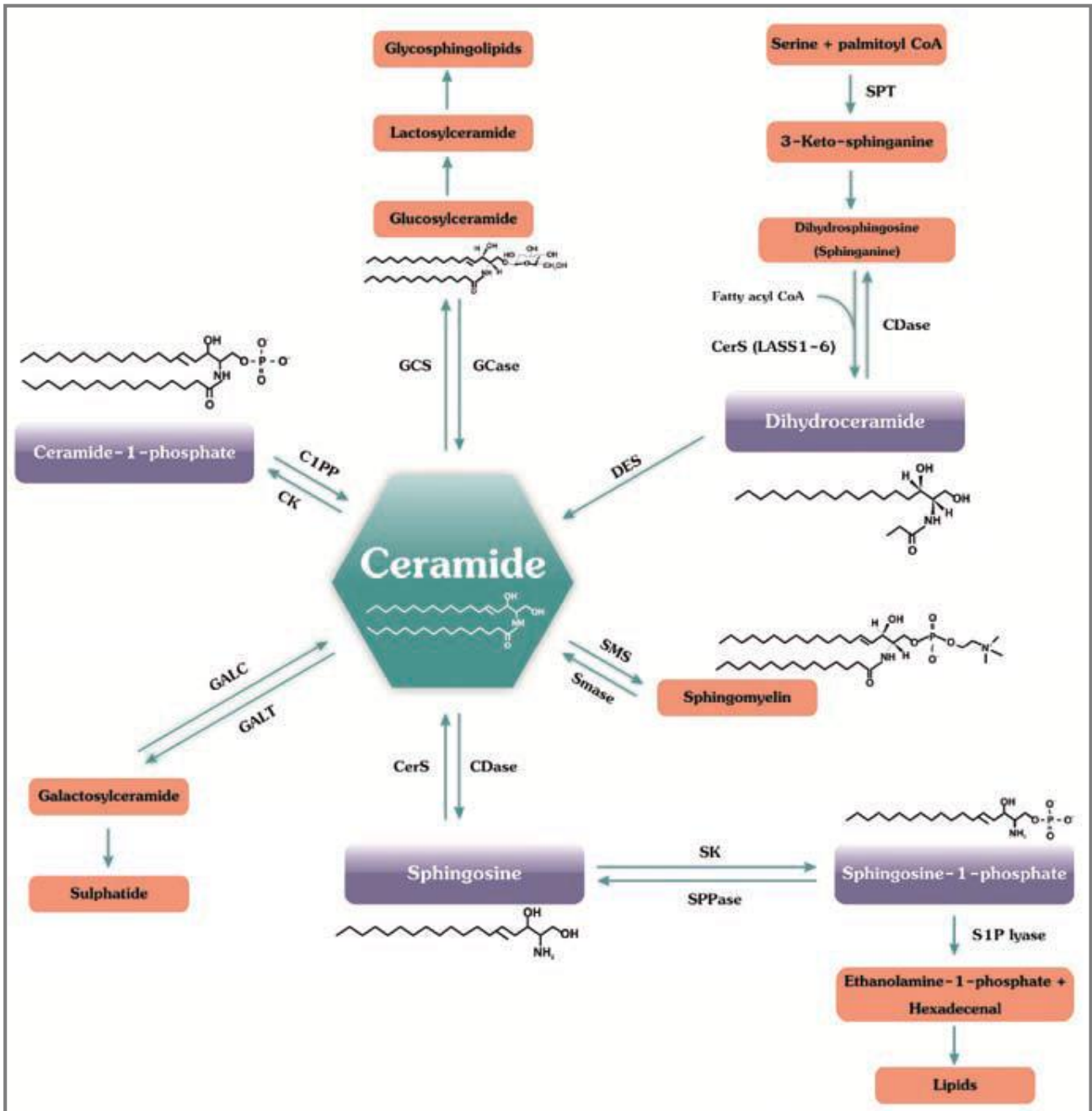
The hydrophilic and hydrophobic moieties result in both polar and non-polar regions giving the molecules an amphipathic character which accounts for their tendency to aggregate into membranous structures [Feizi *et al.*, 1985]. The hydrophobic portion is embedded in the lipid core of biological membranes, whereas the hydrophilic portion is protruding into the extracellular milieu. The Cer

backbone confers some physical-chemical properties to the SLs that differs from those of other membrane lipids. In fact, the amidic linkage, with the contemporary presence in the same molecule of a hydrogen bond donor and an acceptor group (the carbonyl oxygen and the amidic hydrogen), allows the formation of a network of hydrogen bonds. The presence of hydrogen linkages considerably stabilizes the SL segregation in specific membrane areas, which appear enriched with this lipid family and for this reason they have been defined “SL-rich membrane domains” [*Pascher et al., 1976*]. In addition, the numerous hydrogen bonds in these domains confer rigidity and resistance to the membrane allowing their differentiation by physical-chemical properties from the remaining membrane. They are ubiquitously present in different organelle membranes and particularly abundant in the plasma membrane . In particular, SM and GSLs represent the major SLs, displaying an asymmetric or polarized distribution, and play important roles in the regulation of membrane fluidity and sub-domain structures [*Hakomori et al., 1990*]. GSLs are clearly significant contributors to the structure of the outer leaflet of most eukaryotic cell membranes and show a specific distribution on the membrane for each cell type, which can vary with differentiation and neoplastic transformation.

### 3.1.1. Sphingolipid metabolism

Despite the diversity of structure and function of SLs, common biosynthetic and catabolic pathways govern their metabolism and content. Cer constitutes the center of SL metabolism that can be produced in at least three distinct ways. First, it can be originated from the de novo pathway, second, through the hydrolysis of complex SLs and finally, through the hydrolysis of complex SLs (**Fig 7**) [*Adan-Gokbulut et al., 2013*].





**Figure.7. Schematic representation of sphingolipid metabolism [Adan-Gokbulut et al., 2013]**  
**SPT**, serine-palmitoyl transferase; **KSR**, ketosphinganine reductase; **CS**, ceramide synthase; **DES**, dihydroceramide desaturase; **GCS**, glucosylceramidesynthase; **GlcCDase**, glucosylceramidase; **CGT**, ceramide galactosyl transferase; **GalCDase**, galactosylceramidase; **C1PP**, ceramide-1-phosphate phosphatase; **CK**, ceramide kinase; **SMS**, sphingomyelin synthase; **PC**, phosphatidylcholine; **DAG**, diacylglycerol; **SMase**, spingomyelinase; **SK**, sphingosine kinase; **S1PP**, S1P phosphatase.

### **De novo pathway**

The *de novo* biosynthetic pathway of SLs starts at the cytosolic face of the endoplasmic reticulum (ER), and potentially in ER-associated membranes, such as the perinuclear and mitochondria-associated membranes, where enzyme activities, responsible for the reaction sequence leading to Cer, are localized [Michel et al., 1997]. This pathway begins with the condensation of the amino acid L-serine with a fatty acyl coenzyme A (CoA), usually palmitoyl CoA and in less extent stearoyl CoA, catalyzed by serine palmitoyl transferase to generate 3-keto-dihydrosphingosine [Nagiec et al., 1994, Weiss et al., 1997]. On its turn, 3-keto-dihydrosphingosine is reduced by a NADPH-dependent oxidoreductase, called the 3-ketosphinganine reductase, to form dihydrosphingosine (sphinganine) [Stoffel et al., 1968]. Sphinganine is subsequently the substrate of the dihydro(dh)-Cer synthase (CerS), which uses an acyl donor (often palmitoyl-CoA, but also stearoyl-CoA) to bind sphinganine to a fatty acid forming dh-Cer [Rother et al., 1992, Merrill et al., 1986]. It is worth that CerS is able to catalyse the N-acylation not only of dh-Sph but also of Sph derived from complex SL catabolism, thus being involved in the efficient recycling of this long chain base.

Six mammalian genes that encode Cer synthase have recently been identified and recognized as members of the LASS family (Longevity Assurance Genes). Each of these genes specifically synthesizes one of the several Cer species that differ in the fatty acyl chain length CoAs. For example, LASS1 is involved in the production of C18Cer, LASS2 of C22Cer, LASS3 of C18/C24Cer, LASS4 of C20Cer, LASS5 and LASS6 of C16Cer and C14/C16Cer respectively [Levy et al., 2010, Pewzner-Jung et al., 2006]. The major part of the dh-Cer pool is then desaturated by the dihydroCer desaturase (DES) generating a 4,5-*trans*-double bond to form Cer [Geeraert et al., 1997, Michel et al., 1997, Mikami et al., 1998].

Then, The neo-synthesized Cer and that formed from Sph recycling can either directly reach the PM or be used as common precursor in the GSLs and SM biosynthesis. The formation of complex SLs requires a mechanism of Cer transport from the ER, site of its biosynthesis, to the Golgi apparatus.

The synthesis of SM occurs via addition of phosphocholine group to the hydroxyl group in position 1 of the sphingoid base of the Cer. This reaction is catalyzed by SM synthase (SMS). To date, several studies suggest the existence of two different enzymatic SMS isoforms: SMS1, localized in the luminal side of the *cis/medial* Golgi apparatus and SMS2, primarily localized to the PM [Hannun *et al.*, 2008, Huitema *et al.*, 2004]. Experimental evidence has shown that about 90% of the *de novo* synthesis of SM occurs in the *cis/medial* Golgi, and only a small percentage occurs at the level of the PM. The localization of SMS2 at the PM suggests its role as a regulator of SM and Cer levels for signalling pathways and signal transduction.

Regarding the biosynthesis of GSLs, different membrane-bound glycosyltransferases are responsible for the sequential addition of sugar residues to the hydroxyl group in position 1 of the Cer, through  $\beta$ -glycosidic bond, leading to the growth of the oligosaccharide chain. Glucosylceramide (GlcCer) is the first glycosylated product, obtained from Cer and UDP-glucose, through a reaction catalyzed by GlcCer synthase (GCS), enzyme localized at the cytosolic leaflet of the Golgi apparatus [Jeckel *et al.*, 1992]. Therefore, GlcCer biosynthesis requires an efficient transport mechanism of Cer from the cytoplasmic side of the ER to the cytoplasmic side of the *cis*-Golgi. Once synthesized, GlcCer reach directly the PM, presumably transported in a vesicular system, or it can be translocated by a flippase enzyme to the luminal side of the Golgi where it is further glycosylated by other glycosyltransferases located in this cellular district to generate more complex GSLs such as a gangliosides. In the biosynthesis of gangliosides, glycosyltransferases act by associating to GlcCer individual saccharide units, following a precise sequential order [Ichikawa *et al.*, 1998, Degroote *et al.*, 2004]. Galactosylceramide (GalCer), the common precursor for galacto-series of GSLS found in vertebrates, is formed by the addition of a galactose moiety from UDP-Gal to GlcCer catalysed by galactosyltransferase.

Cer can also undergo phosphorylation of the hydroxyl group of the carbon in position 1 by the Cer kinase (CK), with the consequent formation of Cer-1-phosphate (C1P) [Hannun *et al.*, 2008]. The subcellular localization of this enzyme has not been definitely identified, but it seems to be at the

level of the plasma membrane, the Golgi apparatus, and the cytoplasm [*Van Overloop et al., 2006*]. SLs reach their final destination at the plasma membrane mainly following the vesicular flow of exocytosis from the ER or the Golgi apparatus to the PM.

### **Catabolism**

Another important point of regulation of SLs composition is the lipidic degradation that occurs mostly in the acidic compartments of the cells, the lysosomes, where SLs are transported from the plasma membrane by the endocytic vesicular flow through the early and late endosomal compartment to be catabolyzed.

Lysosomal glycosidases sequentially cleave off the sugar residues from the non-reducing end of their glycolipid substrates. The resulting monosaccharides, sialic acids, fatty acids and sphingoid bases can leave the lysosome and can be used within recovery processes called “salvage pathway” (discussed below) or can be further degraded.

The intralysosomal degradation of most, if not all, GSLs requires, besides exoglycohydrolases, effector protein molecules named “sphingolipid activator proteins (SAPs, or saposines)” [*Huwiler et al., 2000*]. The sequence of sugar removal from gangliosides within the lysosomes is as follows: from GM1, galactose is removed by a  $\beta$ -galactosidase, working in the presence of either the GM2-AP or SAP-B [*Wilkening et al., 2000*], to produce GM2. The resulting ganglioside GM2 is cleaved to ganglioside GM3 and Nacetyl-galactosamine only by the  $\beta$ -hexosaminidase isoenzymes Hex A which requires the GM2-AP, an activator that is essential for the *in vivo* degradation of the GM2 gangliosides. In some cells and animals, sialic acid is removed from GM1 and GM2 by a specific sialidase (GM1-and GM2-sialidase) producing the corresponding a-sialo derivatives GA1 and GA2, that, by the action of  $\beta$ -galactosidase and  $\beta$ -hexosaminidase or only  $\beta$ -hexosaminidase respectively, are converted to Lactosylceramide (LacCer).

LacCer is produced also directly from GM3 by the action of a sialidase, which cleaves this ganglioside into LacCer and sialic acid in a reaction stimulated by SAP-B [*Fingerhut et al., 1992*]. LacCer is then degraded to Cer by the sequential action of a  $\beta$ -galactosidase (in the presence of either SAP-B or -C) and  $\beta$  glucosidase [*Zschoche et al., 1994*].

For the *non-glycosylated* SLs, like Cer and SM, non-lysosomal degradation steps are known which apparently do not need the assistance of an activator protein. The SM degradation is catalyzed by sphingomyelinase (SMase) that generates Cer and phosphorylcholine from the cleavage of the phosphodiester bond. To date, three classes of this enzyme have been described and distinguished according to their subcellular localization and the optimum pH, in acidic, neutral, and alkaline SMase. The acidic sphingomyelinase (A-SMase) represents multiple enzymatic forms that exist in different cellular and extracellular compartments. A common protein precursor (pro-ASMase) is differentially trafficked to form lysosomal aSMase (L-SMase) or secretory A-SMase (S-SMase) [*Schissel et al., 1998, Tabas et al., 1999*]. While L-SMase resides in the endo-lysosomal compartment, S-SMase is an extracellular enzyme. An additional form of A-SMase has been reported in close association with the outer leaflet of the PM where it can form Cer-rich platforms [*Stancevic and Kolesnick, 2010*]. The neutral SMase (N-SMase) has various subcellular locations including the inner leaflet of the plasma membrane, ER, Golgi, and even the nucleus [*Marchesini et al., 2004, Clarke et al., 2006*]. The alkaline SMase (Alk-SMase) isoform has now been re-named NPP7 because of its similarity to the nucleotide-pyrophosphatase/phosphodiesterase (NPP) family of enzymes. In addition to its role in SM digestion, a potential implication of this enzyme in cell signaling processes has also been suggested. In particular, Alk-SMase has been shown to inhibit cell proliferation in HT-29 colon carcinoma cells [*Duan et al., 2006*].

Cer is degraded by ceramidases (CDases); three isoforms of CDases have been identified and classified by their pH optimal as acidic, neutral and alkaline CDases. These CDases are located at the plasma membrane, lysosome, and ER/Golgi complex, respectively [*Mao et al 2008*]. These enzymes hydrolytically cleave Cer in fatty acid and Sph. Cer-derived Sph can be recycled or undergo

phosphorylation in position C1 with the generation of S1P by SPHK. S1P can be metabolized through the irreversible cleavage in position C2-C3, to hexadecenal and phosphoethanolamine in a reaction catalyzed by the S1P lyase enzyme, located on the ER cytosolic side [*Serra et al., 2010*]. Notably, this step is the only exit pathway from the SL pathways. S1P can also be dephosphorylated back to Sph through a reaction catalyzed by either lipid phosphate, or S1P specific phosphatases [*Le Stunff et al., 2002 and 2007*].

The “salvage pathway” is a recovery process by which the products of the SLs catabolism and in particular sph can be re-used for a new cycle of SLs metabolism [*Hannun et al., 2008*]. Cer-derived Sph reaches the ER where it is N-acylated to regenerate again the Cer as a precursor for the biosynthesis of complex SLs. The Sph recycling process is an energy advantage to cells and, in different cell types, may constitute the principal pathway of Cer and complex SLs synthesis [*Tettamanti et al., 2003*].

### **3.1.2. Sphingolipids: actors of signalling and regulatory pathways**

SLs were once considered only structural components of the cellular membranes; however, the variations found in their chemical structures allow them to play diverse roles in cellular metabolism. Indeed, SLs physico-chemical properties enable them to fulfil and regulate a wide spectrum of relevant biological functions including molecular sorting, cell-cell interaction and intracellular transport. Indeed, membrane SLs through their hydrophilic portion are able to recognize and interact with elements of the extracellular microenvironment (protein or oligosaccharide component of other cells), allowing the transfer of the information in the intracellular compartment. Furthermore, different stimuli applied to the cells (UV radiation, chemotherapy, growth factors, etc.) generate metabolic reactions that lead to the production of different intermediate of SL metabolism, Cer, Sph, S1P, and C1P acting as chief bioactive mediators involved, as intra- or extra-cellular messengers, in the regulation of crucial processes as cell growth, death, migration and senescence. Indeed, the

regulatory and signalling functions of individual SLs provide an explanation for the structural diversity of this class of molecules and their intricate pathways of metabolism. Cer and S1P are the best studied “bioactive” SLs, and they exert opposite effects in many systems, with Cer usually inhibiting proliferation and promoting apoptosis, and S1P stimulating growth and suppressing apoptosis.

### Ceramide: tumor-suppressor lipid

Besides its role as the precursor of complex SLs, Cer is a signaling molecule capable of regulating many fundamental cellular functions. It is referred to as a “*tumor suppressor lipid*”, since it powerfully potentiates signaling events that drive apoptosis, cell cycle arrest, and autophagic responses. In the typical cancer cell, Cer levels and signaling are usually suppressed by overexpression of Cer-metabolizing enzymes or downregulation of Cer-generating enzymes. Moreover, chemotherapeutic drugs as well as radiotherapy can increase intracellular Cer levels, and exogenously treating cancer cells with short-chain Cer leads to anticancer effects. Evidence strongly suggests that the upregulation of Cer levels is a promising anticancer strategy [*Ogretmen and Hannun, 2004*].

- Ceramide in cell cycle arrest and differentiation

In the regulatory functions of cellular processes such as cell cycle arrest and differentiation, different studies have shown that Cer is upstream of cell cycle regulators. In fact, Cer interacts with protein kinases and phosphatases that regulate important signaling pathways in cancer such as Akt, protein kinase C (PKC), or MAP kinases [*Ogretmen and Hannun, 2004*].

Accumulating studies showed that Cer could modulate the Ras–Raf–MAPK pathway via specific interactions with the kinase suppressor of RAS (KSR) and c-Raf; Cer stimulates the autophosphorylation of KSR, resulting in the transactivation of c-Raf, thus regulating the Ras–Raf–MAPK pathway [*Kolch et al., 2000*]. More recently, the CA3 domain of KSR was identified as the

binding site for Cer [Yin *et al.*, 2009]. Interestingly, inhibition of Cer generation prevented KSR translocation to GSLs-enriched plasma membranes and, thus, reduced its activation. Additionally, c-Raf itself was also identified as a Cer-binding protein. Cer produced via IL-1 $\beta$  stimulation not only binds to c-Raf, but also increased the activity of this mitogen-activated protein kinase [Huwiler *et al.*, 1996].

Long-chain Cer activate protein phosphatase-1 (PP1) and protein phosphatase-2A (PP-2A), which are known as Cer-activated protein phosphatases (CAPPs) [Chalfant *et al.*, 1999]. The connection between PP1 and Cer in regulating the retinoblastoma protein (RB) has been documented [Lee *et al.*, 2000, Chalfant *et al.*, 2002]. RB plays a critical role in cell cycle regulation, and Cer has been shown to dephosphorylate RB, leading to growth arrest in cancer cells. However, whether direct interaction between PP1 and Cer is involved in the regulation of PP1 activity is unclear.

PP2A is a tumor suppressor in cancer, and its activation regulates various downstream oncoproteins [Janssens *et al.*, 2005, Westermarck *et al.*, 2008]. In particular, the naturally occurring D-erythro isoform of Cer was the most potent at increasing PP2A activity and this effect was demonstrated with Cer containing short and long fatty acids chain lengths [Dobrowsky *et al.*, 1993, Wolff *et al.*, 1994]. Cer regulates PP2A activity via binding to its biological inhibitor (SET/I2PP2A), which controls PP2A activity and its downstream targets, such as proto-oncogene c-Myc, retinoblastoma protein, cyclin-dependent kinases (CDKs) and Bcl-2 family members [Ogretmen *et al.*, 2004, Mukhopadhyay *et al.*, 2009], thus describing a novel mechanism for regulating PP2A-dependent antiproliferative roles of Cer.

Further studies also indicated that the activation of protein kinase zeta (PKC-zeta, an atypical form of PKC) by Cer is involved in the inactivation of Akt within the structured membrane micro-domains, leading to growth arrest in vascular smooth muscle cells [Fox *et al.*, 2007]. An important *in vivo* example of Cer-mediated growth arrest was observed with the use of Cer-coated balloon catheters, which caused growth arrest of vascular smooth muscle-cells (VSMC) after stretch injury *in vivo*.



Mechanistically, this growth arrest of VSMC was linked to Cer-induced Akt inhibition, which was mediated through PKC-zeta [*Bourbon et al., 2000*].

The idea that Cer is a regulator of cell differentiation has been recognized since the discovery that vitamin D3-induced differentiation of HL-60 and U037 human leukemia cells. This differentiation resulted in a progressive increase in the hydrolysis of SM by N-SMase, resulting in the elevation of Cer, which induced monocytic, but not neutrophilic or macrophage-type, differentiation of these cells [*Okazaki et al., 1989*]. In neuronal cell lines, Cer mimics nerve growth factor function, and induces differentiation of Purkinje cells, and hippocampal neurons [*Dobrowsky et al., 1994*]. In addition, Cer induces differentiation in Neuro2a cells via upregulation of N-SMase, as previously reported in our laboratory [*Riboni et al., 1995*].

- Ceramide in cell death (Apoptosis)

Apoptosis can be induced by various factors including chemotherapeutic agents, CD95, tumor necrosis factor-1, growth factor withdrawal, hypoxia, or DNA damage. Many of these mediators of apoptosis are regulators of Cer generation, suggesting a pivotal role of Cer in apoptosis [Pettus et al., 2002]. Moreover, different studies indicate that endogenous Cer levels increase in cells before triggering the apoptotic cascade [*Dbaibo et al., 1997*].

Additionally, increasing the endogenous levels of Cer with inhibitors of Cer metabolism enzymes or by over-expression of Cer generating enzymes results in apoptosis [*Abe et al., 1995; Bielawska et al 1996*]. For example, in leukemia cells, expression of bacterial SMase, which generates Cer from intracellular pools of SM, caused a significant increase in Cer levels and induced apoptosis [*Zhang et al., 1997*]. In addition, ionizing radiation activates A-SMase for Cer generation. Importantly, human lymphoblasts and mice deficient in SMase are resistant to high doses of radiation, suggesting an active role of Cer in apoptosis regulation [*Santana et al., 1996*]. On the other hand, inhibitors of the *de novo* pathway, such as fumonisin B1 [*Plo et al., 1999*], prevent apoptosis, providing evidence for the role of Cer generation in mediating apoptosis.

One of the mechanisms by which Cer regulates apoptosis is through the induction of Fas capping, which involves the lateral segregation of cross-linked Fas ligand with its surface receptor at the SM-enriched plasma membrane of Jurkat T lymphocytes, necessary for its optimal function in cell killing [Cremesti *et al.*, 2001]. On the other hand, cells that are resistant to Cer and CD95/Fas-induced apoptosis have defective mitochondrial apoptosis [Raisova *et al.*, 2000], indicating that perturbations of ceramide-CD95/Fas signaling can result in the development of resistance to cell death in human cancer cells.

Another target for Cer was also identified as cathepsin D, an aspartic protease [Heinrich *et al.*, 1999]. Procathepsin D can be secreted into the tumor microenvironment, leading to the degradation of extracellular matrix proteins and contributing to tumor metastasis and growth [Abbott *et al.*, 2010]. Interestingly, the interaction between Cer and cathepsin D induced autocatalytic proteolysis, leading to the enzymatically active form of cathepsin D. Functionally, cathepsin D has been implicated in mediating apoptosis via IFN- $\gamma$ , Fas and TNF- $\alpha$  [Deiss *et al.*, 1996], as well as chemotherapy agents [Abbott *et al.*, 2010]. An equally wide array of studies have investigated the relationship between Cer and the Bcl-2 proteins family, particularly Bcl-2 itself, Bcl-x<sub>L</sub>, and Bax. For example, in C6 glioma cells, etoposide induces Cer formation by N-SMase activation, which then increases the Bax/Bcl-2 ratio and in A549 cells, exogenous and endogenous Cer produced from gemcitabine treatment enhance the expression of proapoptotic Bcl-x and caspase 9 splice variants, demonstrating regulation of Bcl-2 family protein expression levels and splicing patterns by the bioactive lipid. The connection between Cer and Bax has also been reported by several studies. One study has shown that Bax overexpression does not affect Cer production in response to etoposide, yet Cer treatment in prostate and colorectal cancer cell lines induces apoptosis only when Bax is overexpressed suggesting a link between the lipid and the protein [Sawada *et al.*, 2000].

- Ceramide in cell senescence

The relationship between Cer and senescence came with the observation that Cer levels increased significantly as human fibroblasts entered the senescent phase [*Venable et al., 1995*]. This was supported by the fact that fibroblasts treated with Cer recapitulated the morphologic and biochemical changes of senescence such as activation of retinoblastoma, regulation of cyclin-dependent kinases, or inhibition of growth factor signaling. Mechanistically, these changes induced by ceramide occur through inhibition of phospholipase D, which leads to the reduction of diacylglycerol (DAG) generation, and results in the failure to translocate and activate PKC to the membrane, a critical response in transducing mitogenic stimuli.

Senescence is also regulated by alterations in telomere length, which is maintained by telomerase, one of the down-stream targets of Cer signaling. One Study showed that Cer also mediates telomerase-independent rapid shortening of telomere length in A549 cells [*Sundararaj et al., 2004*]. Cer-mediated telomere shortening was linked to the inhibition of an unexpected role of a nuclear form of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in telomere/binding and protection function. Taken together, these results support that Cer play important roles in the regulation of senescence and aging.

### **Sphingosine-1-phosphate: tumor-promoter lipid**

The bioactive SL metabolite S1P is an important lipid mediator that was implicated in many biological processes. S1P was detected in many organisms such as plants, yeast, worms, flies, and mammals. More than two decades have elapsed since it was first suggested that S1P can regulate cell growth [*Zhang et al., 1991*].

In addition, different studies, performed in both cell cultures and animals, demonstrated that S1P can regulate different physiological and pathological processes strictly related to cancer and inflammation, favoring cell survival, migration, and angiogenesis [*Spiegel et al., 2002, Hannun et al., 2008*]. S1P can exert its bioactive properties in the intracellular milieu, acting as a second messenger, and mainly in the extracellular milieu, where, after secretion from cells, can bind five specific cell surface receptors (S1P1-5) coupled to different G-proteins and thereby activating several signal transduction pathways that control cell behaviour [*Payne et al., 2002*].

S1P has emerged as an onco-promoter molecule. Interestingly, the administration of S1P was shown to promote estrogen-dependent tumorigenesis of MCF-7 human breast cancer cells [*Nava et al., 2002*]. Johnson and colleagues using siRNAs to inhibit S1P phosphatase 1 (S1PP1), which converts S1P to Sph, observed an increase in the intracellular and extracellular levels of S1P rendering MCF-7 cells resistant to the cytotoxic actions of TNF $\alpha$  and daunorubicin [*Johnson et al., 2003*]. In many cell types, the administration of S1P results in the inhibition of different forms of cellular apoptosis, including the Cer-mediated one [*Cuvillier et al., 1996*], through the induction of pro-survival proteins expression (Bcl-2, MCL1), stimulation of the DNA replication, and inhibition of pro-apoptotic proteins expression (Bad, Bax). In addition, exogenous S1P is able to block the translocation of Bax to the mitochondria, which is required for the release of mitochondrial cytochrome c and the consequent caspase activation [*Pyne et al., 2010*]. Furthermore, S1P binding to S1P3 receptor enhances cell survival by suppression of Bax expression and activation of endothelial NOS, PI3K and Akt [*Strub et al., 2010*].

An increasing number of evidence demonstrated that S1P acts as a potent pro-angiogenic factor, inducing the migration of endothelial cells and promoting the formation of new blood vessels. The mechanisms of S1P-mediated neovascularization involve the migration of endothelial cells through the activation of S1P receptors, and downstream regulation of the Rho family of small GTPases, which in turn regulate cell motility and remodelling of the cytoskeleton [*Kluk et al., 2002*]. The main S1P receptor involved in the process of vascular maturation is S1P1, which is required for the

stabilization of nascent blood vessels during embryonic development, indeed its knockout in mouse resulted in a lethal vascular defect [Liu *et al.*, 2000]. As S1P is also secreted extracellularly, modulation of SPHK and S1P in tumour cells provides a potential mechanism for recruiting endothelial cells and promoting blood-vessel formation/angiogenesis [Ogretmen *et al.*, 2004]. Indeed, combining S1P with other pro-angiogenic factors, such as basic fibroblast growth factor or VEGF, produced synergistic enhancement of neovascularization in tissue samples of mouse aortic rings, an *ex vivo* model of angiogenesis [Wu *et al.*, 2003].

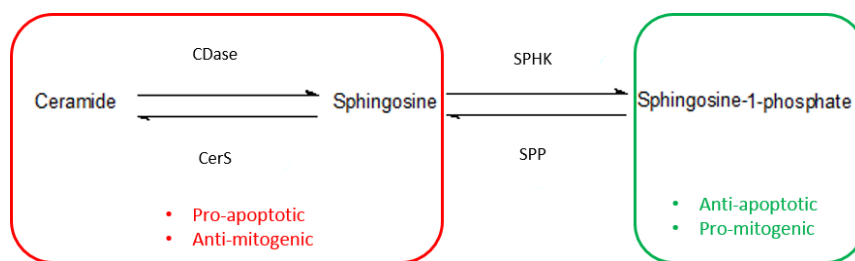
S1P also stimulates the motility of cancer cells through S1P1 or S1P3. By contrast, S1P can inhibit cancer cell motility through S1P2-dependent regulation of Rho. Thus, the specific effect of S1P is partly determined by the predominance of the receptor subtypes expressed. For example, S1P stimulates the migration of gastric tumour cells that exclusively expressing S1P3 and inhibits the motility of others that predominantly express S1PR2 [Yamashita *et al.*, 2006]. Similarly, the inhibition of melanoma cell migration is through S1P2 and involves the inhibition of Rac, activation of Rho, and the subsequent tyrosine phosphorylation of focal adhesion kinase and paxillin as well as the increment of stress fiber formation [Arikawa *et al.*, 2003, Yamamura *et al.*, 2000]. On the other hand, S1P stimulates the migration of fibrosarcoma cells through a S1P1–Rac1–CDCI dependent pathway involving the tyrosine phosphorylation of membrane type matrix metalloproteinase 1 (MT1-MMP), a zinc-dependent proteolytic enzyme involved in degradation of the extracellular matrix, facilitating cell migration [Fisher *et al.*, 2006, Nyalendo *et al.*, 2007]. Furthermore, ovarian cancer cell invasion induced by S1P involves S1PR1 or S1PR3 and calcium mobilization [Park *et al.*, 2007]. In the context of its extracellular actions, S1P functions as a second messenger in cellular proliferation, cell survival and suppression of apoptosis. Furthermore, a variety of growth factors and cytokines, including PDGF, EGF, TNF-K, and nerve growth factor, which are well known inducers of cellular proliferation and/or differentiation, also activate SPHK and thereby increasing cellular S1P levels [Meyer *et al.*, 1999, Xia *et al.*, 1999]. The intracellular targets of S1P remain much more elusive. Interestingly, microinjection of S1P into fibroblasts increases calcium mobilization from

internal stores, and DNA synthesis, thus enhancing proliferation and survival [Van Brocklyn et al., 1998]. The S1P role as an intracellular messenger has been also suggested by some indirect evidences, such as the PDGF-induced translocation of SK to the nuclear membrane with the concomitant increase in the nucleus-associated SPHK activity. This implies that S1P may have a role in the nucleus, and it was suggested that it may be involved in cell cycle progression, although no direct evidence for this has yet appeared. S1P has also been shown to activate ERK and inhibit c-Jun N-terminal kinase (JNK) activation which is significant, since the balance of ERK and JNK activation has been implicated in the control of apoptosis [Payne et al., 2002].

Overall, and in contrast to the well-established actions of S1P through its receptors, the intracellular roles of S1P are poorly documented, and the identification of its direct intracellular targets remains elusive.

### 3.1.3. Sphingolipids rheostat

Since S1P and Cer elicit opposing cellular effects, e.g. proliferation and survival versus growth arrest and apoptosis, these two lipids together gave birth to the cellular “sphingolipid rheostat” that has been proved to be a critical balance determining the survival or death of mammalian cells [Cuvillier et al., 1996] (Fig.8).



**Figure 8. Sphingolipid Rheostat**

The rheostat model is currently based on the assumption that Cer induces apoptosis. This is believed to be due to the differential regulation by Cer and S1P of members of the ERK and stress activated

protein kinase families and caspase proteases [*Cuvillier et al., 1996, Coroneos et al., 1996, Pyne et al., 1996*]. Importantly, the rheostat model for controlling the Cer/S1P balance and cell fate does not rely upon whether S1P acts intracellularly or by binding to its specific receptor

A key piece of evidence to support the rheostat hypothesis is that Cer-mediated programmed cell death is suppressed by the addition of S1P. Indeed, Cer-induced JNK activation and apoptosis were prevented by the addition of S1P. Furthermore, the PKC-mediated activation of SPHK and the concomitant increase in S1P levels were shown to have a similar inhibitory effect on Cer-mediated apoptosis. Subsequently, Fas ligation or C2-ceramide-mediated caspase-3 and -7 activation and resulting poly(ADP-ribose) polymerase cleavage were shown to be blocked by PKC activation and attenuated by the addition of exogenous S1P [*Cuvillier et al., 1998*].

Further support for the rheostat model comes from studies showing that growth factors and cytokines tend to differentially regulate SMase/CDase and SPHK activities and, consequently, Cer and S1P levels. For example, alkaline membrane-associated CDase is up-regulated by growth factors, including PDGF, FGF, IGF and EGF, in a tyrosine kinase-dependent manner, with a concomitant increase in SMase activity [*Edsall et al., 1997*].

Furthermore, manipulation of intracellular S1P levels can also affect the levels of Cer and Sph. For example, NIH 3T3 cells transfected with SPHK1a exhibit a substantial decrease in their ceramide content [*Kohama et al., 1998*]. This correlates with increased survival upon serum depletion, which usually induces apoptosis [*Olivera et al., 1999*]. Therefore, the concomitant removal of Cer upon SPHK overexpression may prevent Cer-induced apoptosis. Additionally, the subsequently formed S1P may promote cell survival by inducing mitogenic signalling pathways that counteract the effects of Cer.

### 3.1.4. Sphingolipid Signaling in Cancer Therapy

Understanding the intrinsic mechanisms of action for Cer and S1P can open doors to new therapies to battle cancer. It has been well established that increases in intracellular Cer will promote apoptosis. Thus, finding ways to intrinsically elevate Cer in cancer cells is desirable. Conversely, S1P has been shown to promote cancer pathogenesis, thus, suppression of its generation/accumulation could suppress tumor growth.

#### Ceramide Metabolism in Cancer Therapeutics

Increasing endogenous Cer has been suggested as an effective method to regulate cancer cell growth. To this end, certain chemotherapeutic agents have been identified to impact Cer metabolism by promoting Cer synthesis *de novo*, by activating SMases and/or by blocking GlcCer formation. In each case, the result is an enhanced Cer-governed cytotoxic response. [Futerman and Hannun, 2004; Ogretmen and Hannun, 2004]. In this regard, different studies have shown that the effects of paclitaxel are linked to the *de novo* synthesis of Cer in MDA-MB-468 and MCF-7 breast cancer cells and that paclitaxel-dependent cytotoxicity was abrogated by blocking Cer production with L-cycloserine, an inhibitor of Cer synthesis [Charles *et al.*, 2001]. Moreover, the co-administration of paclitaxel with exogenous ceramide substantially inhibits cell proliferation and elicits apoptosis in a synergistic fashion in Jurkat T cells [Myrick *et al.*, 1999] and Tu138 head and neck squamous cell cancer [Mehta *et al.*, 2000]. In addition, triphenylethylene antiestrogens, such as tamoxifen was observed to block conversion of Cer to GlcCer and its activity is independent of estrogen receptor status [Cabot *et al.*, 1996]. DT388-GM-CSF, a fusion toxin consisting of a truncated diphtheria toxin linked to human granulocyte–macrophage colony-stimulating factor (GM-CSF), is toxic to acute myeloid leukemia progenitors bearing the GM-CSF receptor but not to normal marrow cell progenitors. DT388-GM-CSF also is a potent agonist of Cer formation in vincristine-resistant HL-60



cells HL-60/VCR cells. In these cells, Cer is generated via hydrolysis of cellular SM, suggesting a SMase-governed response [Frankel AE et al., 1998].

Additionally, small molecule inhibitors of the SL pathway able to induce the accumulation of Cer were used in some cancers. For example, B13, an inhibitor of acid CDase was used in a metastatic colon cancer mouse model and a prostate cancer xenograft model [Selzner et al., 2001]. In both cases, B13 caused the accumulation of Cer and resulted in prevention of tumor growth. Another effective approach to increase Cer accumulation in cancer cells was the inhibition of SM synthase, or acid CDase [Meng et al., 2004].

Furthermore, the use of Cer analogues or mimetics could also promote apoptotic pathways in cancer cells. In fact, many studies report that exogenous treatment with Cer induces cell death, and/or growth arrest [Bielawska et al., 2008]. These findings were supported with *in vivo* studies, in which treatment with recently developed exogenous Cer (ceramidoids) inhibited cancer cell growth, and decreased tumor progression in HNSCC and other cancer models [Bielawska et al., 2008]. It should also be noted that treatment of cells with exogenous Cer may result in the generation of endogenous long chain Cer via the Sph recycling pathway, which can be blocked by FB1, and not by myriocin. This alternative pathway for the generation of endogenous Cer seems to be important for the regulation of telomerase and c-Myc in A549 cells [Sultan et al., 2006]. Furthermore some dietary compounds has been implicated in the alteration of Cer metabolism, and thereby leading to cell death. Indeed, treatment of prostate and lung cancer cells with  $\gamma$ -tocopherol ( $\gamma$ T), the main dietary form of vitamin E, inhibited cell proliferation and induced apoptosis [Jiang et al., 2004], which were concomitant with the accumulation of dihydroceramides. 1,25-Dihydroxyvitamin D3 stimulates hydrolysis of SM in leukemia cells [Okazaki et al., 1989] and in keratinocytes [Geilen et al., 1996]. It also inhibits the growth of prostate adenocarcinoma cells, but it is not known whether Cer is involved in growth inhibition [Getzenberg et al., 1996].

## S1P as a target for anti Cancer therapeutic

Accumulating data strongly suggest that S1P may present an important target for anti-cancer therapeutics. Indeed, the use of a monoclonal antibody that binds S1P with high affinity and specificity was observed to reduce tumor progression effects in various murine xenograft and allograft models and to prevent S1P-induced cell proliferation, release of pro-angiogenic cytokines, and protection of tumor cells from apoptosis by S1P [Visentin *et al.*, 2006].

Furthermore, another anti-cancer therapeutic strategy has been to use inhibitors of SPHK1. In fact, preliminary studies *in situ* and in animal models indicate that SPHK1 inhibitors prevent cancer cell proliferation and tumor growth [French *et al.*, 2006] and inhibition of S1PR1 and S1PR3 receptors with their antagonists may inhibit cancer cell growth [Davis *et al.*, 2005].

Additional experimental evidence supporting the pro-survival roles of S1P/S1PR axis was obtained by employing the potent immunosuppressive agent FTY720, which is known to engage with S1PRs and down-regulate S1P1 [Brinkmann *et al.*, 2004]. FTY20 is phosphorylated *in vivo* to FTY720-P possibly by SPHK2 [Paugh *et al.*, 2003], which then induces sequestration of lymphocytes in lymph tissues by engaging S1PRs with high affinity and specificity [Brinkmann *et al.*, 2004]. These data implicate that S1PRs play important roles in immunosuppression. More importantly, treatment with FTY720 inhibits angiogenesis and tumor vascularization, and mediates cell death, suggesting that it might be exploited as an anti-cancer therapeutic agent [LaMontagne *et al.*, 2006].

## 3.2. Sphingolipids and colon tumorigenesis

Although sphingolipids contribute only to a small proportion of the total cellular lipid pool, their metabolism alteration may be a trigger for pathogenesis of many diseases. Furthermore, accumulating evidence suggests that alterations of the sphingolipid metabolism, assumption of dietary sphingolipids, or modification of endogenous enzymatic activity could have a significant impact on colon carcinogenesis [Koizumi et al., 1981, Van Blitterswijk et al., 1984].

### 3.2.1. Overview of the sphingolipids roles in colon cancer cells

In this section, I will report the effects of exogenous SLs in colon cancer cells, the effect of specific targeting of SL metabolism and the alteration of SL metabolism by chemotherapeutic drugs (**Fig.9**)

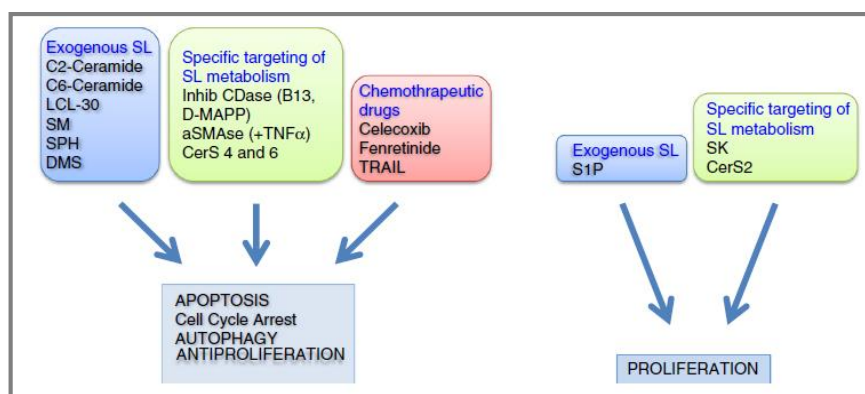


Figure 9. Summary of the sphingolipids effects in colon cancer cells [Hannun et al., 2013]

### Effects of adding exogenous sphingolipids

- Sphingomyelin

SM metabolism seems to play an important role in response to therapy, such as the chemotherapeutic daunorubicin, and to H<sub>2</sub>O<sub>2</sub>, heat, and ionizing radiation. All these events induced the formation of Cer from SM hydrolysis through the activation of the *de novo* and the salvage pathways. The resulting Cer is responsible for initiating the apoptotic response. Of note, it has been reported that the exogenous administration of SM increased 5-fluorouracil (5-FU) and doxorubicin sensitivity by 100-

300% in HCT15 and MOSER colon cancer cell lines. However, this effect was not achieved in other colon cancer cell lines such as HT29, LoVo and WiDr, nor in HUVEC [Modrak *et al.*, 2000], suggesting that these different responses may be due to alterations in SM/Cer metabolism in the non-responsive cells. Indeed, HT29 cells, cultured with gradually increasing concentrations of colchicine (an antimitotic agent), displayed an increased resistance to different chemotherapeutic agents, and showed higher levels of GlcCer and GalCer, confirming that specific changes in SL levels [Kok *et al.*, 2000].

- Ceramide and its analogs

It has been previously reported that SL bases and Cer induced apoptosis in colon cancer cells and could be potential mediators for the protective role of more complex dietary SLs in CRC carcinogenesis [Ahn *et al.*, 2002]. In SW403 cells, it has been demonstrated that the administration of two short-chain Cer analogs (C2 and C6-ceramides) and of two inhibitors of CDases (D-MAPP and B13) caused cell death, via activation of caspase-3 and cytochrome c release. In addition, C2-ceramide was able to induce not only apoptosis but also cell cycle arrest at the G2/M phase, causing an accumulation of cells in the S phase [Ahn *et al.*, 2002]. Furthermore, C2-ceramide was implicated in mediating macroautophagy by increasing proteolysis and accumulation of autophagic vacuoles in HT29 cells. Indeed, studies demonstrated that Cer was able to reverse 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 Cer for the first time in control of a major lysosomal pathway [Scarlatti *et al.*, 2004]. In addition, in different colon cancer cells (LoVo, HT29 and HCT15) the administration of C6-ceramide in combination with P-glycoprotein antagonists (tamoxifen, cyclosporine A, VX-170 and verapamil), that inhibit the conversion of Cer to GlcCer, increased cytotoxicity via caspase activation, PARP cleavage, DNA fragmentation, cell cycle arrest, mitochondrial membrane permeability and expression of p53 [Morad *et al.*, 2013]. Furthermore, it has been observed that in HT29 and HCT-116, two colon cancer cell lines, Sph, sphinganine and C2-

ceramide, but not C2-dihydroceramide, were able to inhibit cell growth and to induce cell death in a time and dose-dependent manner [*Ahn et al., 2010*].

Of interest, the administration of 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) [*Sweeney et al., 1996*]. Moreover, treatment of SW403 with a cationic long chain Cer [ $\omega$ -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 [*Dindo et al., 2006*].

It is important to remark that colon cancer cells present differential expression of certain proteins in response to Cer. 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 and caspase-10) and growth arrest (PCNA) [*Fillet et al., 2005*]. In addition, it was reported that resistance to TRAIL (TNF-Related-Apoptosis-Inducing-Ligand) in colon cancer cells was associated with defects in Cer signalling. In fact, analyzing two colon-cancer cell lines, SW480 and SW620, isolated from the same patient (primary and subsequent metastasis, respectively) with different sensitivity to this pathway, it has been observed that Cer were comparable in both cell lines. However the TRAIL-responder SW480 cells contained a higher percentage of C16 and C18-ceramide and lower C24-ceramide than the TRAIL-resistant SW620 cells. Only in SW480 cells C16-ceramide levels raised upon TRAIL-treatment, and this increase was parallel with caspase-3/-7 activation. Interestingly, combination of C6-ceramide with TRAIL resulted in apoptosis in SW620 cells. These results suggest that Cer plays a role in promoting TRAIL-mediated apoptosis and that TRAIL-resistant cancer may benefit from combination therapy with Cer or agents that enhance Cer accumulation [*Voelkel-Johnson et al., 2005*]. Later studies identified CerS6, which preferentially generates C16-ceramide, as a protein able to influence susceptibility to TRAIL [*White-Gilbertson et al., 2009*].

- Sphingosine-1-phosphate

S1P is another SL that has an impact on colorectal carcinogenesis. Thamilselvan and coworkers reported that S1P, as in other types of cancer, stimulates proliferation of colon cancer cells by activating p38 and ERK/MAP kinases [*Thamilselvan et al., 2002*]. 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 [*Visentin et al., 2006*].

These results, together with the effect of S1P antibodies in reducing tumor progression in murine xenografts and allografts [*Visentin et al., 2006*], suggest the bioactive lipid S1P as a target for this type of cancer therapy

### Effects of targeting sphingolipids metabolism and its enzymes

Increasing Cer levels by blocking CDases was highly effective in inducing apoptosis and preventing growth of colon cancer. Indeed, B13, a CDase inhibitor, appears to be specific for treatment of primary and metastatic colon cancer cells, since normal liver cells were resistant to treatment, as no increase of ceramide content or apoptosis were observed [*Selzner et al., 2001*]. Veldman and colleagues [*Veldman et al., 1998*] evaluated the effect of increased Cer levels in HT29rev cells, by either incubating the cells with bacterial SMase (b-SMase) or adding C2-ceramide. Treatment with C2-ceramide resulted in a rapid accumulation of this compound within the cells and in induction of apoptosis, whereas b-SMase treatment did not induce apoptosis despite hydrolyzing cellular SM and increasing Cer levels. Indeed, the b-SMase-generated Cer was converted to more complex SLs. Even after the use of inhibitors to block this conversion, and thereby inducing an accumulation of Cer 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. Moreover, Cer generated by

the addition of exogenous A-SMase contributed to tumor necrosis factor alpha (TNF $\alpha$ ) mediated apoptosis in HT29 [Colell *et al.*, 2002]. In a complementary study, TNF $\alpha$  induced apoptosis in a time and dose-dependent manner, but downregulation of A-SMase prevented TNF-stimulated apoptosis [Jaiswal *et al.*, 2004]. In addition, it was observed that SPHK and N-SMase contributed to the regulation of cell sensitivity upon treatment with oxaliplatin by controlling Cer formation, and affecting the Akt pathway; indeed, treatment with oxaliplatin increased C16, C24 and C24:1-ceramides in sensitive colon cancer cells but not in resistant ones due to the activity of N-SMase instead of A-SMase. It was further observed that silencing SPHK isoforms, which already performed a high activity and protein expression in resistant cells, upon oxaliplatin treatment, caspase activity and cellular Cer formation increased, leading to cell death. Moreover, in these experimental conditions the Akt pathway was affected too: Akt phosphorylation decreased and p53 and p21 protein levels increased.

### Effects of an alteration in sphingolipids metabolism induced by chemotherapeutic agents

It has been reported that Camptothecin a topoisomerase I inhibitor used in first line treatment of solid CRC and in second line for 5-FU resistant patients, was able to increase Cer levels, through the activation of serine palmitoyltransferase and CerS, which subsequently induced growth inhibition via caspase-3 activation in a p53-independent manner [Chauvier *et al.*, 2002].

Regarding Fenretinide, a synthetic retinoid, it has been observed that it was able to induce an increase of Cer levels in neuroblastoma cells and in colon cancer cell lines (HT29 and LoVo). This increase in Cer was associated to an increase of cell death by a combination of apoptosis and necrosis in a p53 and caspase-independent manner. Importantly, some 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 Cer metabolism [Wang *et al.*, 2008, Rahmaniyan *et al.*, 2011]. It was reported that 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. 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 [Schiffmann *et al.*, 2010]. Furthermore, the use of RNAi for CerS6 specifically decreased 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 Cer-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 [Martin *et al.*, 2005].

## 2.2. Overview of dietary sphingolipids roles in colon cancer models

*In this section*, I will report the distribution of SLs in the diet and their effects in chemically-induced colon cancer models such as 1,2 dimethylhydrazine (DMH) and azoxymethane (AOM), inherited models (APC<sup>Min/+</sup> mouse) and also colon cancer xenografts.



## Sphingolipids in the diet

Sphingolipids form a natural part of a normal diet, indeed the daily dietary intake of all SLs in adult human is estimated about 300-400mg [Nilsson *et al.*, 2003]. Fruit and vegetable products provide only about 50 mg of SLs per day. Especially rich in SLs are dairy products, particularly eggs and milk. Some SLs, except SM and gangliosides, are present in fruit and plants (cucumbers, grapes, broccoli, black bean, and wheat). Another major source of SLs, mainly SM, are animal-origin tissues like poultry (chicken, turkey), beef, pork, and fish (salmon, catfish) [Blank *et al.*, 1992]. Human milk is the only source of sphingolipids for neonates and it consists of SM, LacCer, GlcCer, and gangliosides (GM1, GM3, and GD3). Even though most of the SLs that enter the gastrointestinal system are hydrolyzed in the small intestine, a small percentage also enters the large intestine. In particular, SM is digested and absorbed mainly in the small intestine. Animal studies proved that consumed SM is digested only partially and it is a slow process [Nyberg *et al.*, 1997]. On the other hand, in human, more than 80% of SM can be digested, and the rest is excreted with feces [Ohlsson *et al.*, 2010]. SM is resistant to digestion by pancreatic enzymes [Duan *et al.*, 2009]. Other SLs as Sph and dihydrosphingosine are quickly absorbed in the small intestine and further metabolized to free fatty acids, mainly palmitate, and in the lesser extent to Cer.

## 1,2 dimethylhydrazine-induced colon cancer model

It is important to remark that dietary SLs showed both chemotherapeutic and chemopreventive effects in DMH-treated animals. As demonstrated by studies performed by Lemonnier and colleagues, DMH-treated mice, receiving SM before or after tumour initiation, induced a decrease in the formation and proliferation of tumours in the lower half of the colonic crypts, associated with a concomitant increase in the number of apoptotic cells [Lemonnier *et al.*, 2003]. Furthermore, Dillehay and coworkers showed that CF1 female mice treated with DMH and fed with a diet very

low in SL (less than 0.005% weight), when supplemented with milk SM exhibited a significant reduction in the number of aberrant crypt foci with respect to the control group. Indeed, tumour incidence in the control group was 47% while in mice supplemented with SM was 20% [Dillehay *et al.*, 1994]. These data were confirmed subsequently, in a follow up study in which SM supplementation caused a reduction in ACF and in the number of aberrant crypts per focus. Unfortunately, after 40 weeks of treatment, any difference in tumor incidence was found [Schmelz *et al.*, 1996]. Nevertheless, in both studies the mice fed with a diet rich in SL and SM, showed tumour with a low grade (adenocarcinoma and adenoma respectively), suggesting that dietary SM may suppress the appearance or advancement to more malignant tumors. Moreover, GlcCer, LacCer and ganglioside GD3 found in induced at least a 40% decrease in the number of ACF (comparable with the reduction accomplished by SM), accompanied by a decrease of proliferation in the colonic crypts, but any variation in the apoptotic cell number per crypt was observed [Schmelz *et al.*, 1996]. To better understand the effects of dietary SLs in colon cancer, Schmelz and colleagues [Schmelz *et al.*, 1997] studied the effects of chemically synthesized SLs. CF1 female mice treated with DMH were fed with a diet low in SL 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 about the 50% for milk SM and synthetic SM and 70% for synthetic dihydroSM, confirming the role of SM in suppressing ACF formation and suggesting that the potency of dihydroSM did not depend on the 4,5-trans bond of the SL backbone. Since most SLs are digested in the small intestine, an analog of Cer (Cer- $\beta$ -glucuronide), was specifically designed to be delivered to the colon [Schmelz *et al.*, 1999]. Cer- $\beta$ -glucuronide induced in the lower half of the colonic crypts a decrease in the number of ACF (about 40%) and reduced the number of proliferative cells (about 20%).

Finally, since plant SLs differ structurally from mammals, their effect in colon cancer has also been evaluated by one study. It has been reported that soy-GlcCer, added to a diet low in SL, 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 [*Symolon et al., 2004*].

### Azoxymethane-induced colon cancer model

Many studies in AOM-treated animals demonstrated a chemotherapeutic as well as a chemopreventive effect of dietary SLs. Rats treated with 35 mg/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 of proliferative cells in the base of the crypt, without affect immunity system [*Exon et al., 2003*].

Since a very high percentage of colon cancer tumors present mutations in p53, especially in the advanced stages [*Baker et al., 1990*], the role of p53 in the effects of dietary SLs on colon cancer was evaluated. In fact, p53 wild type and p53<sup>+/-</sup> mice were treated with AOM and fed with a diet containing 0.1% SM for 4 weeks. The short-term results indicated that p53 status did not modify the effects of SLs on proliferation and apoptosis. Moreover, after 33-38 weeks of treatment, SM administration produced no significant effects on either tumour incidence or size in both genotypes. However, both tumour incidence and size trended lower with dietary SL, demonstrating that the effect of dietary SM in colon cancer was not p53-dependent [*Hu et al., 2008*].

Sprague-Dawley rats treated with AOM were utilized to indirectly evaluate the effects of SLs in colon cancer using ursodeoxycholic acid (UDCA). This compound was previously shown to exert an antiproliferative and proapoptotic effect in HT29 cells, accompanied by a rapid increase in Alk-SMase activity. AOM treated rats showed that UDCA administration 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 N-SMase and Alk-SMase [*Andersson et al., 2008*].

## Adenomatous polyposis coli gene APC Min/+ mice colon cancer model

Orally administered SLs are able not only to decrease of ACF and tumours formation 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<sup>Min/+</sup> mouse. APC<sup>Min/+</sup> mice treated with Enigmol, a sphingoid base analog that cannot be phosphorylated by SK1 and is poorly N-acetylated, showed a decrease of in the number of tumours, without effects on body weight and on liver and kidney. [Symolon *et al.*, 2011].

The APC<sup>Min/+</sup> mouse model has been used also to evaluate the effects of plant SLs on colon cancer. The data obtained showed that a diet supplemented with 0.1% GlcCer reduced the number of adenomas by 70%. In the APC<sup>Min/+</sup> mice intestinal mucosal cells, the administration of Soy GlcCer was able to affect the expression of at least 96 genes in a dose-dependent manner [Symolon *et al.*, 2004].

## Human xenografts

Human xenografts were mostly used to evaluate in colon cancer the effects of SLs alone or in combination with anti-cancer drugs. Nojiri and colleagues reported in a human colon cancer xenograft a drastic decrease in tumour volume after treatment with a drug that increases the levels of ganglioside GM3 [Nojiri *et al.*, 2002]. Moreover, it has been demonstrated in human xenografts that the administration of SM in presence of 5-fluorouracil or irinotecan treatment strongly reduced tumour growth with respect the untreated control. Any toxic effect on hematopoietic, hepatic and renal cells was observed [Modrak *et al.*, 2000; Modrak *et al.*, 2002].

Furthermore, Schiffmann and colleagues have demonstrated in HCT116 human xenograft that Celecoxib was able to inhibit tumour growth. This effect may be probably due to by an increase in the levels of C16:0-ceramide in stomach, small intestine, and tumor tissues, with any detectable differences in other tissues, such as brain, lung, heart and testes [Schiffmann *et al.*, 2008].

### 2.3. Sphingolipids enzymes in colon cancer

Several studies suggest an association between colon carcinoma and changes in SLs enzymes. These alterations in enzyme expression and activity suggest that colon cancer may be associated with decreased Cer and increased S1P levels.

#### Alkaline SMase

It has been shown that in healthy tissue exists a gradient in the expression and activity of Alk-SMase from the ascending colon to the rectum. On the other hand, in colon cancer cells Alk-SMase activity is strongly reduced and it is almost undetectable in adenocarcinomas [*Hertervig et al., 1997*]. Moreover, it has been observed that the expression of Alk-SMase changed in response to dietary factors and to two chemical agents, UDCA and 5-aminosalicylic acid, both showing chemopreventive effects against colon cancer. It has been documented that the UDCA treatment increased the activity of Alk-SMase in intestinal mucosa and inhibited the development of colon carcinoma [*Cheng et al., 1999*]. In an in vitro study it has been observed that the increase in Alk-SMase activity UDCA-induced is accompanied by a decrease in cell proliferation and caspase activation in HT29 cells [*Andersson et al., 2003 and 2008*]. Furthermore, it has been reported that 5-aminosalicylic acid administration is able to increase Alk-SMase levels in the colonic mucosa after ingestion [*Rubin et al., 2005*]. The decrease of Alk-SMase activity detected in untreated colon cancer cells reduces the hydrolysis of SM and therefore reduces Cer generation [*Duan et al., 2006*]. Since Cer can activate anti-proliferative and apoptotic pathways, lack of Cer in colon cancer may contribute to colon cancer development.

## Sphingosine kinase

Sphingosine Kinases 1 and 2 can phosphorylate Sph to form S1P, which in turn exerts proliferative effects in different cells models. Regarding intestinal cells, the expression of these two isoforms was shown in both small intestine and colon [Fukuda *et al.*, 2003]. Moreover, SPHK1 expression is increased in human colon cancer compared to normal colon mucosa [Kawamori *et al.*, 2006]. In particular, adenomas show a higher expression of SPHK1 with respect to normal mucosa, and metastatic colon cancer has higher SPHK1 expression than non-metastatic cancer. Kohno and colleagues showed that in APC<sup>Min/+</sup> mouse model, SPHK1 expression is required for small intestinal tumor cell proliferation. Indeed deletion of the SPHK1 gene suppressed adenoma size but not their incidence. Moreover, in polyps the inhibition of SPHK1 expression significantly reduced the epithelial cell proliferation, suggesting that SPHK1 may regulate adenoma progression [Kohno *et al.*, 2006]. In addition, Kawamori and coworkers demonstrated that SPHK1 and S1P levels were significantly elevated in colon cancer tissues compared to healthy mucosa. Additionally, SK1<sup>-/-</sup> mice subjected to AOM/DSS treatment presented not only significantly fewer ACF but also a reduction in colon cancer development [Kawamori *et al.*, 2009].

## S1P lyase and S1P phosphatase

In colon cells S1P levels are controlled not only by SPHKs, but also by enzymes involved in its metabolism, such as S1P lyase and S1P phosphatase. In this regard, APC<sup>Min/+</sup> mice show not only high levels of Sph and S1P in general, but also decreased expression and activity of S1P lyase in adenomas when compared to intestinal epithelium [Oskouian *et al.*, 2006]. Furthermore, studies on colon cancer patients demonstrated a downexpression of S1P lyase and S1P phosphatase, indicating that the breakdown of S1P may be blocked in colon cancer [Oskouian *et al.*, 2007].

## Glucosylceramide synthase

Glucosylceramide synthase catalyzes the formation of GSLs. *In vitro* studies performed on colon cancer cells demonstrated that cells resistant to drug treatment presented higher levels of glucosylceramide synthase compared to healthy controls [Kovbasnjuk *et al.*, 2005]. On the other hand, in colon cancer cells, the inhibition of glucosylceramide synthase activity resulted in an enhanced apoptosis [Uchida *et al.*, 2002].

## AIM OF THE WORK

Colorectal cancer represents a challenging problem in oncology and globally [Ferlay *et al.*, 2010]. Although surgical resection is curative in early stage diseases, currently used chemotherapies for advanced colon cancer are mainly palliative. Unfortunately, drug toxicity and the development of resistance to therapeutic agents are also potential obstacles to the successful treatment of this disorder. Hence, one of the tasks debated for the management of colorectal cancer is to decline the cytotoxic chemotherapy problem by finding novel natural agents that possess cancer-preventive, and/or growth inhibitory activity against cancer.

Almost 25 centuries ago, Hippocrates, the father of medicine, proclaimed “Let food be thy medicine and medicine be thy food.” In fact, diet plays a well-recognized key role in the etiology of colon cancer [Wiseman *et al.*, 2008], and dietary compounds are receiving increasing attention for prevention and/or alternative treatment of this cancer. Long-known preventive effect of plant-based diets on tumorigenesis and other chronic diseases is well documented. Indeed, plant materials might contain phytochemicals that have been extensively studied for their protective properties as antioxidant and anti-inflammatory activities. Moreover, it has been suggested that many of them could exert antimitotic and antitumorigenic effects, thereby offering anticancer protection to individuals consuming such diets [Thakur *et al.*, 2014]. Importantly, LU, a member of flavonoids family, has emerged to possess anticancer properties associated to multiple mechanisms such as inducing apoptosis, suppressing cancer cell proliferation, angiogenesis, and metastasis. *In vitro* studies reported that LU arrests the cell cycle at the G<sub>1</sub> phase in human gastric cancer cells [Matsuoka *et al.*, 1993], and induces apoptosis in human myeloid leukemia cells [Ko *et al.*, 2002]. In addition, a potent preventive effect via topical application of LU was also observed for the antitumour promotion of skin papillomas in mice [Ueda *et al.*, 2003]. In colon cancer, studies by Manju and coworkers [Manju *et al.* 2005 and 2007] showed that LU exerts preventive effects against 1,2 dimethyl-



hydrazine-induced colon carcinogenesis in rats. Despite these promising findings, little is known about the molecular mechanisms underlying the cancer preventive and therapeutic activities of LU.

Accumulating literature indicates that the bioactive sphingolipid Cer plays an important role in mediating pro-apoptotic responses via various mechanisms in human cancer cells, and that it is a major player in the mechanism of action of many chemotherapeutic drugs [Ogretmen *et al.*, 2004]. Indeed, a number of clinically important cytotoxic agents appear to be effective because of their ability to induce specific changes in sphingolipid levels, especially by promoting an increase in Cer content, thus resulting in enhanced Cer-governed cytotoxic responses.

Therefore, on these premises the identification of natural and, especially, dietary compounds, exhibiting antitumour effects by altering SL metabolism, could represent a critical start for new approaches that could imply natural molecules as antitumoural agents and/or as chemosensitizer to elicit enhanced drug sensitivity and to overcome the problem of drug toxicity.

On these bases, the aim of this PhD project was to investigate the sensitivity of human colon cancer cells and their normal counterpart toward the effect of LU, and to understand its molecular mechanism focusing on the ability of LU to target the Cer metabolism as a hallmark of its antitumour effect. In parallel, we aimed to extend our attention also to SIP, the physiological antagonist of Cer, thus focusing on the cellular “sphingolipid rheostat”, a regulating balance crucial in the survival or death of mammalian cells [Cuvillier *et al.*, 1996].

As experimental model, we used the Caco-2 cell line. These cells were obtained from a human colon adenocarcinoma and can be grown in culture as 1) undifferentiated, representative of a colon tumour model, and 2) fully differentiated intestinal cells, differentiated in culture into an enterocyte-like phenotype, which represents so far the best available *in vitro* model of absorptive enterocytes [Pinto *M et al.*, 1983]. These two models might thus allow to distinguish the potential LU effects on colon cancer cells in comparison to their healthy counterparts.

## MATERIALS AND METHODS

### Materials

All reagents were of the highest purity available. Chloroform was further distilled before use. EMEM, L-glutamine, streptomycin, penicillin, amphotericin-B, sodium pyruvate, aprotinin, leupeptin, pepstatin, bestatin, BrefeldinA (BFA), fatty acid free bovine serum albumin (BSA), N-acetyl-D-erythro-sphingosine (C2-Ceramide), N-hexanoyl-D-erythro-sphingosine (C6-Ceramide), O-Tricyclo[5.2.1.0<sup>2,6</sup>]dec-9-yl dithiocarbonate potassium salt (D609), Luteolin (LU), 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide (MTT), p-nitrophenyl-phosphate (PNPP), p-nitrophenol (PNP), Hoechst 33342, bovine brain sphingomyelin, Kodak Biomax film and other common chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). N-(4,4-difluoro-5,7-dimethyl-bora-3a,4a-diaza-s-indacene-3-pentanoyl) sphingosine (BODIPY-C5-Cer) was from Molecular Probes Europe (Leiden, The Netherlands). D-erythro dihydrosphingosine and sphingosine-1-phosphate (S1P) were purchased from Enzo Life Sciences (Farmingdale, NY, USA). Fetal calf serum (FCS) was from EuroClone (Pero, Milan, Italy). D-erythro-[<sup>3</sup>H]-sphingosine ([<sup>3</sup>H]-Sph) (20.05 Ci/mmol) and [Choline-Methyl-<sup>14</sup>C] bovine brain sphingomyelin ([<sup>14</sup>C]-SM) (52 mCi/mmol), Ultima Gold were from PerkinElmer Life Sciences (Boston, MA, USA). [<sup>3</sup>H]-dihydrosphingosine, D-erythro ([<sup>3</sup>H]-DHSph) (1 mCi/ml) was from American Radiolabeled Chemicals (St Louis, MO, USA). LY294002 was from Vinci-Biochem (Firenze, Italy). The Primary antibody recognizing phospho-Akt (Ser-473) were from Cell Signaling Technology, Inc. (Danvers, MA). Goat anti-mouse horseradish peroxidase-linked secondary antibody was from Thermo Fisher Scientific (Waltham, MA, USA). The primary rabbit anti-GAPDH antibody and Goat anti-rabbit horseradish peroxidase-linked secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). High performance thin layer chromatography (HPTLC) silica gel plates and all solvents were from Merck (Darmstadt, Germany). SuperSignal West Pico and West Femto Maximum Sensitivity Chemiluminescent Substrate and

bovine serum albumin (BSA) fraction V were purchased from Pierce Chemical Co (Rockford, IL, USA).

## Methods

### 4.1. Cell culture

Caco-2 human colon carcinoma cell line (BS TCL 87) was obtained from the Istituto Zooprofilattico Sperimentale della Lombardia e dell' Emilia (Brescia, Italy), cultured in a humidified atmosphere at 37° C with 5% CO<sub>2</sub> and routinely maintained in EMEM containing: 15% (v/v) heat-inactivated fetal calf serum, 1mM sodium pyruvate, 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml amphotericin B. The culture medium was usually changed every 48 hours. Cells were maintained by serial passages in 75 cm<sup>2</sup> culture flasks. At reaching confluence, subculture was prepared using a 0.025% trypsin–0.01% EDTA solution. Cells were re-suspended in their culture medium, counted by trypan blue dye exclusion test using a Neubauer emocytometer, and then re-plated ( $16 \times 10^3$  cells/cm<sup>2</sup>) to allow cell propagation.

Since Caco-2 cells have the capacity to spontaneously differentiate, *in vitro* over confluence, into cells possessing the morphology and function of enterocytes [*Pinto M et al., 1983*], we used in our experiments:

- *Tumoural Caco-2 cells* which resemble to those found in tumour tissues, at 2d day after confluence
- *Differentiated Caco-2 cells* which lose the tumourigenic phenotype and show characteristics of healthy enterocytes, at 10-day after confluence.

Both cell models were plated for experiments at the opportune density (12500 cells/cm<sup>2</sup>) in tissue plate dishes or in 24 well plates (8 and 1.5 cm<sup>2</sup>, respectively).

## 4.2. Enzyme assays

SMases and ALP activity were assayed using as enzyme source cell homogenate prepared as follows: cells were washed three times with ice-cold PBS, harvested and centrifuged 10000 x g for 5 minutes, at 4°C. After centrifugation, cells were re-suspended in 10 mM Tris-Cl pH 7.4 containing as protease inhibitors 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 0.5 µg/ml leupeptin and 0.5 µg/ml pepstatin, in order to obtain 2 µg/µl of cell protein as a final concentration. The cellular suspension was then sonicated 10 seconds at 4°C for three times. Protein concentration was determined by the Lowry method [Lowry *et al.*, 1951].

### 4.2.1. ALP activity

ALP activity assay was based on p-nitrophenol (PNP) production starting from p-nitrophenyl-phosphate (PNPP) [Messer *et al.*, 1966]. The reaction mixture consisting of 7 mM PNPP, 100 mM Tris-Cl pH 7.4 and 5 mM MgCl<sub>2</sub> in a volume of 0.5 ml was prepared and added to 30 µg of cell homogenate. Standard solutions of PNP ranging from 0 to 50 nmoles were prepared and diluted to 0.5 ml with the reaction mixture without PNPP. After an incubation period of 30 minutes at 37°C in the dark, the reaction was stopped by adding 1 ml NaOH 0.1 N. The absorbance was measured at 410 nm, and enzyme activity was calculated according to a standard curve. Protein content was determined according to Lowry [Lowry *et al.*, 1951].

### 4.2.2. Alk-SMase and N-SMase activity

SMase activities were assayed using experimental conditions known to selectively favour N-SMase or Alk-SMase with minor modifications as follows.

The N-SMase incubation mixture contained 50 mM Tris-Cl pH 7.4, 10 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 250 µM bovine brain SM, 3.8 µM [<sup>14</sup>C-choline]-SM (0.005µCi) and 20µg of cell protein in a final volume of 25 µl.

The Alk-SMase incubation mixture contained 50 mM Tris-Cl pH 9.0, 2 mM EDTA, 10 mM taurocholate, 0.15 mM NaCl, 250  $\mu$ M bovine brain SM, 15.4  $\mu$ M [ $^{14}$ C-choline]-SM (0.02  $\mu$ Ci) and 20  $\mu$ g of cell protein in a final volume of 25  $\mu$ l.

After 30 minutes of incubation at 37°C, the reactions were stopped by adding 375  $\mu$ l of chloroform/methanol 2:1 (v/v). After mixture, 50  $\mu$ l distilled H<sub>2</sub>O were added and phases were separated by centrifugation at 1000 x g for 5 minutes, at 4°C. A biphasic solution was obtained, the upper aqueous phase rich in labeled choline was counted for radioactivity by liquid scintillation counting.

#### 4.2.3. SPHKs activity

2d Cells were washed with ice-cold PBS and harvested in SPHK buffer (containing 20 mM Tris-Cl pH 7.4, 1 mM EDTA, 0.5 mM deoxypyridoxine, 15 mM NaF, 1 mM  $\beta$ -mercaptoethanol, 1 mM sodium orthovanadate (Na<sub>3</sub>VO<sub>4</sub>), 40 mM  $\beta$ -glycerophosphate, 0.4 mM phenylmethylsulfonyl fluoride (PMSF), 10% glycerol, and complete protease inhibitors as 9.7  $\mu$ M bestatin, 1  $\mu$ M pepstatin, 10  $\mu$ g/ml aprotinin and 10 $\mu$ g/ml trypsin inhibitor) and disrupted by freeze-thawing procedure. Protein content was determined according to Lowry [Lowry *et al.*, 1951]. SPHKs activity was assayed using experimental conditions known to selectively favor SPHKI or SPHKII activity.

For the SPHKI activity, 20  $\mu$ g of proteins were incubated in 50  $\mu$ l of reaction mixture containing 2mM ATP, 20mM MgCl<sub>2</sub>, 25 $\mu$ M D-erythro-sphingosine and 0.2  $\mu$ Ci [ $^3$ H]-D-erythro-sphingosine as 0.25% triton X-100 micelles and SPHK buffer supplemented with 0.5% Triton X 100 and different concentration of LU or vehicle (0,07% DMSO).

For the SPHKII activity, 20  $\mu$ g of proteins were incubated in 50  $\mu$ l of reaction mixture containing 2 mM ATP, 20 mM MgCl<sub>2</sub>, 25  $\mu$ M D-erythro-sphingosine and 0.2  $\mu$ Ci [ $^3$ H]-D-erythro-sphingosine as BSA-complex and SPHK buffer supplemented with 200 mM KCl and different concentrations of LU or vehicle (0,07% DMSO).

The mixture was incubated at 37°C for 30 minutes. The reaction was terminated by the addition of 100 µl of chloroform/methanol 1:2 (v/v). Subsequently, 66 µl of chloroform and 17.3 µl H<sub>2</sub>O/NH<sub>3</sub> 10:3 (v/v) were added, and samples were centrifuged at 8500 × g for 5 minutes at 4°C. 120 µl of the aqueous phase was transferred to new glass tube. Subsequently, 100 µl of chloroform and 15 µl of H<sub>2</sub>O/methanol/HCl were added to the aqueous phase and the two phases were separated by centrifugation at 8500 x g for 5 minutes, at 4°C. The organic phase was then transferred to a new glass tube whereas the aqueous phase was washed with 100 µl chloroform and centrifuged as described above. The second organic phase was transferred and added to the first one. The two-combined phases were dried under nitrogen stream and resuspended in chloroform/methanol/HCl 100: 200:1 (v/v/v). Lipids of the organic phase were then resolved on HPTLC using 1-butanol/acetic acid/water 3:1:1.(v/v/v) as solvent system and visualized by autoradiography.

### 4.3. Phospholipid content and composition

2d and 10d Cells were washed three times in ice-cold PBS and harvested with 500 µl methanol, and 1 ml chloroform was then added. After mixing for 10 minutes, samples were centrifuged at 10.000 x g for 15 minutes, at 4°C. The supernatant was collected, and the precipitate was re-extracted with 500 µl chloroform/methanol 2:1 (v/v), mixed and centrifuged as described above. The second supernatant was added to the first, and the two combined supernatants were used as total lipid extract (TLE) [*Riboni et al., 2000*]. An aliquot of the TLE was used to determine the phospholipid content according to the Bartlett assay, a colorimetric assay based on the determination of liberated inorganic phosphate [*Bartlett et al 1958*].

To determine the distribution of different phospholipids, an aliquot of the TLE was submitted to two-dimensional HPTLC using as a solvent systems chloroform/methanol/acetic acid/water 30:20:2:1 (v/v/v/v), for the first run, and chloroform/methanol/acetone/acetic acid/water 10:2:4:2:1 (v/v) for the

second run. The two runs were separated by an exposure of the plate to HCl vapors for 15 minutes. At the end of the second run, the HPTLC plate was sprayed with the copper acetate reagent and incubated at 120°C for 2-3 minutes. The relative abundance of each spot was quantified by densitometric scanning of the HPTLC plate.

#### 4.4. Cell treatments

Stock solutions were prepared by dissolving molecules as follows:

**LU** : 35 mM in DMSO.

**D609** : 50 mM were prepared in distilled H<sub>2</sub>O.

**C2-Cer:** 5 mM stock solutions were prepared in absolute ethanol.

**C6-Cer:** 1 mM stock solutions were used as 1:1 complex with fatty acid-free BSA, due to its precipitation from aqueous media, particularly those lacking serum. Briefly, 50 mM C6-cer stock solutions in absolute ethanol were diluted with 1 mM fatty acid-free BSA/Tris-Cl pH 7.4 at 1 mM as a final concentration.

**BFA** : 17.8 mM stock solutions were prepared in absolute ethanol.

**S1P** : 100 μM stock solutions were dissolved in fatty acid free bovine serum albumin (4 mg/ml in PBS) and incubated for 30 minutes at 37°C with constant stirring before use.

**LY294002** : 32.5mM stock solutions were prepared in DMSO.

**Hoechst 33342:** 4mM were prepared in distilled H<sub>2</sub>O.

Stock solutions were then diluted in fresh medium at the desired concentrations and administered to cells for the indicated period of time. In parallel, untreated cells were also incubated with vehicles as controls. In all cases, ethanol and DMSO final concentration never exceeded 0.1 and 0.57 % (v/v) respectively, concentrations that do not affect cell survival.



#### **4.5. Determination of cell viability: MTT assay**

Caco-2 cells were seeded at 12500 cells/cm<sup>2</sup> in 24-well plates. At 2-day or 10-day after confluence, Caco-2 cells were treated with different agents for the indicated periods of time. The medium was then replaced by MTT dissolved in fresh medium (0.8 mg/ml) for 4 hours. The formazan crystals formed in viable cells were then solubilized in iso-propanol/formic acid 95:5 (v/v) for 10 minutes with constant stirring to ameliorate the lysis process. The absorbance at 570nm was measured using a microplate reader (Wallack Multilabel Counter, Perkin Elmer, Boston, MA, USA).

#### **4.6. Fluorescence studies**

2d or 10d Caco-2 cells were plated on glass coverslips at 12500 cells/cm<sup>2</sup> in 35 mm dishes.

##### **4.6.1. Analysis of cell apoptosis: Hoechst staining**

2d or 10d Caco-2 cells were incubated with 200 µM LU or 300 µM D609 in culture medium. After 24 hours of treatment, cells were incubated with 10 µM Hoechst 33342 dye in the dark for 15 minutes at 37°C.

##### **4.6.2. Analysis of the intracellular distribution of BODIPY-C5-Cer**

2d Caco-2 cells were treated for 90 minutes at 37°C in culture medium in the presence of vehicle (0,57% DMSO) or 20 and 200 µM of LU. After treatment, cells were incubated with 2.5 µM BODIPY-C5-Cer (as 1:1 complex with fatty acid-free BSA) in KRH solution, containing 25 mM HEPES, 125 mM NaCl, 5 mM KCl, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 6 mM glucose and 2 mM CaCl<sub>2</sub>, at 4°C for 30 minutes as described previously [*Pagano et al., 1991*]. After loading, cells were washed twice in KRH solution and incubated for 30 minutes at 37°C in the appropriate EMEM conditioned medium containing 0.17 mg of fatty acid-free BSA/ml.

In both cases, cells were then washed three times in PBS for 5 minutes with constant stirring and fixed with 0.5% glutaraldehyde solution in PBS for 10 minutes at 4°C. After been fixed, the specimens were immediately observed and analyzed by a fluorescence microscope (Olympus BX-50) equipped with a fast high-resolution CCD camera (Colorview 12) and an image analytical software (Analysis from Soft Imaging System GmbH).

#### 4.7. Phospho-Akt immunoblotting

2d Caco-2 cells were incubated with 20 and 200µM LU or 0.5 and 0.2-1µM S1P for the indicated period of times in culture medium. At the end, cells were washed with ice cold PBS for three times and lysed with Akt buffer containing 20 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM sodium pyrophosphate (Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>), 1 mM PMSF in the presence of proteases inhibitors (2 µg/ml Pepstatin , 2 µg/ml Aprotinin, and 2 µg/ml leupeptin). After 20 minutes of constant stirring at 4°C, solubilized proteins were centrifuged at 8000 x g for 10 minutes at 4°C. A small aliquot of the supernatant was analysed for the protein content with the Comassie Blue-based assay [*Sedmak et al 1977*] and the remaining part was denatured by the addition of Sample Buffer 4X containing 250 mM Tris-Cl pH 6.8, 40% glycerol (v/v), 8% SDS (w/v), 0.4 M DTT and 0.02% bromophenol blue (w/v) and by heating for 5 minutes at 100°C. Supernatants were subjected to SDS-PAGE on 10% of polyacrylamide gel and transferred onto nitrocellulose membranes. Membranes were then blocked, under constant stirring, for 1 hour at room temperature in Tris-buffered saline (25mM Tris-Cl pH 7.4, 154 mM NaCl), containing 0.1% Tween 20 and 5% skim milk. Once blocked, membranes were incubated overnight at 4 °C with a primary antibody against phospho-Akt (Ser473) (1:1000) and then with goat anti-mouse IgG (H+L) peroxidase-conjugated (1:2000) as a secondary antibody for 1 hour at room temperature.

Signals were detected via the enhanced chemiluminescence method using SuperSignal West Pico Maximum sensitivity Chemiluminescent Substrate, and membranes were exposed to Kodak Biomax films. The relative abundance of each protein band was analysed via densitometry scanning of the exposed films. Immunoblots were probed with an antibody for GAPDH as a protein loading control.

## **4.8. Pulse/chase experiments with radiolabeled sphingosine**

### **4.8.1. Pulse/chase protocols**

Metabolic studies were performed on Caco-2 cells plated at 12500 cells/cm<sup>2</sup> and grown as a monolayer on 35 mm dishes in culture medium. At the time of the experiments, the medium pulse was prepared by adding [<sup>3</sup>H]-Sph to fresh medium containing 15% FCS, to obtain 0.55 µCi/ml as a final concentration. Cells were rinsed twice with fresh culture medium and incubated in 0.9 ml of medium pulse.

**[<sup>3</sup>H]-Ssphingosine metabolism:** Caco-2 cells were fed with 0.55 µCi/ml of [<sup>3</sup>H]-Sph in culture medium in presence or absence of 20 and 200 µM LU or 20 µM LY294002. After 2 hours of pulse, cells and media were collected and processed as described below. In order to evaluate the effect of BFA on Sph metabolism, the cells were pre-incubated for 30 minutes at 37°C with 1 µg/ml BFA and then pulsed with [<sup>3</sup>H]-Sph as described above in presence of BFA. After 2 hours of pulse, cells were washed twice with PBS at 4 °C, harvested, and total lipids were extracted and processed as described below.

**Sphingolipid content** Total sphingolipids cells were labeled at equilibrium by incubating cells with 0.55 µCi/ml of [<sup>3</sup>H]-Sph in culture medium for 6 hours. At the end of incubation, the plates were washed twice with fresh medium and chased for 22 hours culture medium. [<sup>3</sup>H]-Sph-labeled cells were then stimulated with in culture medium in presence or absence of 20 or 200 µM LU, as indicated.

After 24 hours treatment, cells were washed twice with PBS at 4 °C, harvested, and submitted to lipid extraction, partitioning and alkaline methanolysis, as described below.

#### 4.8.2. Extraction and partial purification of cellular sphingolipids

At the end of the pulse or pulse/chase period, cells were rinsed twice with cold PBS, harvested and total lipids were extracted with chloroform/methanol as previously reported [*Riboni et al., 2000*].

The TLE was partitioned by adding 260 µl 0.1 M NH<sub>4</sub>OH. After mixing for 5 minutes, the two phases were separated by centrifugation at 8500 x g for 10 minutes, at 4°C. The upper phase, containing the polar sphingoid molecules (including S1P and gangliosides) was evaporated to dryness under a nitrogen stream, re-suspended in 100 µl chloroform/methanol/HCl 100:100.1 (v/v) and counted for radioactivity by liquid scintillation. The lower phase (LP), containing the apolar sphingoid molecules (including Cer, Sph, SM and neutral glycosphingolipids) was subjected to a mild alkaline methanolysis in order to remove the glycerophospholipids [*Riboni et al., 2000*]. In particular, after evaporation to dryness under a nitrogen stream, the LP was re-dissolved in 50µl chloroform. After the addition of 50 µl 0.2 N methanolic KOH, the sample was vortexed and incubated at 37°C for 1 hour. The reaction was then stopped by neutralization with 60µl 0.2N methanolic HCl. Then, 90 µl methanol and 350 µl chloroform were added. After mixing, the phases were separated by the addition of 95 µl water and centrifuged at 8500 x g for 5 minutes at 4°C. The aqueous phase was removed and the lower phase was evaporated to dryness under a nitrogen stream, re-suspended with 300 µl chloroform/methanol 2:1 (v/v) and counted for radioactivity by liquid scintillation.

#### **4.8.3. Analysis of the pulse medium: identification and quantification of extracellular S1P and tritiated water**

Extracellular S1P was extracted from pulse medium under appropriate conditions able to recover about 90% of S1P [Anelli V *et al* 2005]. In particular, a two-step partitioning, in alkaline and acidic conditions, respectively, was performed. Briefly, the medium was centrifuged at 8000 x g for 10 minutes at 4°C to remove dislodged cells. Aliquots (400 µl) of the supernatant were added to 750 µl of cold chloroform/methanol/ HCl 100:200:1 (v/v/v). After mixing at 4°C and after the addition of 500 µl of chloroform and 130 µl of 3.5 N NH<sub>4</sub>OH, the phases were separated by centrifugation at 10000 x g for 5 minutes at 4°C. The upper aqueous phase, containing volatile metabolites and extracellular S1P, was further partitioned by adding 750 µl of chloroform and 40 µl of 37% HCl. After mixing and centrifugation as described above, the acidic organic phase, containing extracellular S1P, was evaporated under a nitrogen stream, re-suspended in chloroform/methanol/HCl 100:100:1 (v/v/v.), and analyzed by HPTLC. An aliquot of the alkaline aqueous phase was evaporated under a nitrogen stream and analyzed to its [<sup>3</sup>H]-water content, the product of S1P degradation.

#### **4.8.4. Protein content**

The final pellet obtained after lipid extraction, containing cellular proteins, was dried under a nitrogen stream and digested overnight with 50 µl 1 N NaOH. After dilution with 450 µl of distilled water and vigorous mixing, aliquots were submitted to protein determination using the Lowry method [Lowry *et al.*, 1951].

#### **4.8.5. Separation and quantification of radiolabeled sphingolipids**

The radiolabeled sphingolipids recovered in the methanolysed organic or aqueous phase were subjected to high performance thin layer chromatography (HPTLC) on silica gel plates developed in a chromatographic chamber by the use of appropriate solvent systems as follows.

Sphingolipids contained in the organic phase were separated using chloroform/methanol/water 110:40:6 (v/v/v). A standard mix composed of tritiated Cer, GlcCer, LacCer, Sph, and SM was chromatographed on the same plate and used as internal standard.

The fractions containing cellular and extracellular S1P were separated with n-butanol/acetic acid/water 3:1:1 (v/v/v) as solvent system. Standard [<sup>3</sup>H]-S1P was chromatographed on the same plate and used as internal standard.

To detect and quantify incorporation of the radiolabeled into different sphingolipids separated by HPTLC, the plates were submitted to digital autoradiography (Beta-Imager 2000, Biospace, Paris, FR). The different spots of sphingolipids were quantified by the beta vision analysis software (Biospace, Paris, FR).

#### **4.9. Protein assays**

The determination of the protein content was performed using the Comassie blue-based assay [*Sedmak et al 1977*] or the Lowry assay [*Lowry et al 1951*]. In both cases bovine serum albumin (BSA) was used as standard. The protein content was obtained by spectrophotometric reading at 595 nm for the Bradford method and 750 nm for the Lowry assay.

#### **4.10. Statistical analysis**

Results are expressed as mean value  $\pm$  SD for at least three independent experiments. Statistical analysis of the data was performed by the Student's t test. A *p* value  $< 0.05$  was considered significant. Data were analyzed using StatMate software, version 4.0 (GraphPad).

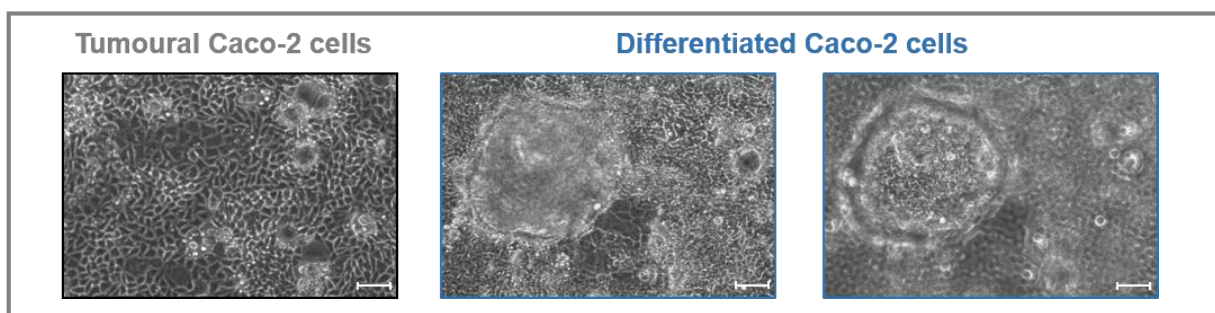
# RESULTS

## 5.1. Characterization of tumoural and differentiated CaCo-2 cells

It has been previously reported that intestinal cell differentiation can be mimicked *in vitro* using Caco-2 cell cultures. Although cancerous in origin, these cells undergo a gradual differentiation process that takes place spontaneously once confluence has been reached [Pinto *et al.*, 1983]. In these conditions Caco-2 cells can be used as a model of the healthy intestinal epithelium. Thus, in the present study, we used proliferating Caco-2 cells, at 2-day after confluence as tumoural cells, as well as differentiated, 10-day post confluence Caco-2 cells.

### 5.1.1. Morphological and biochemical features of differentiation

In order to assess if a spontaneously differentiation occurred at 10 day after confluence, we analysed some morphological and biochemical markers recognised as typical of colonic cell differentiation. Images acquired by phase-contrast microscopy showed obvious morphological differences between the tumoural and the differentiated Caco-2 cell models.

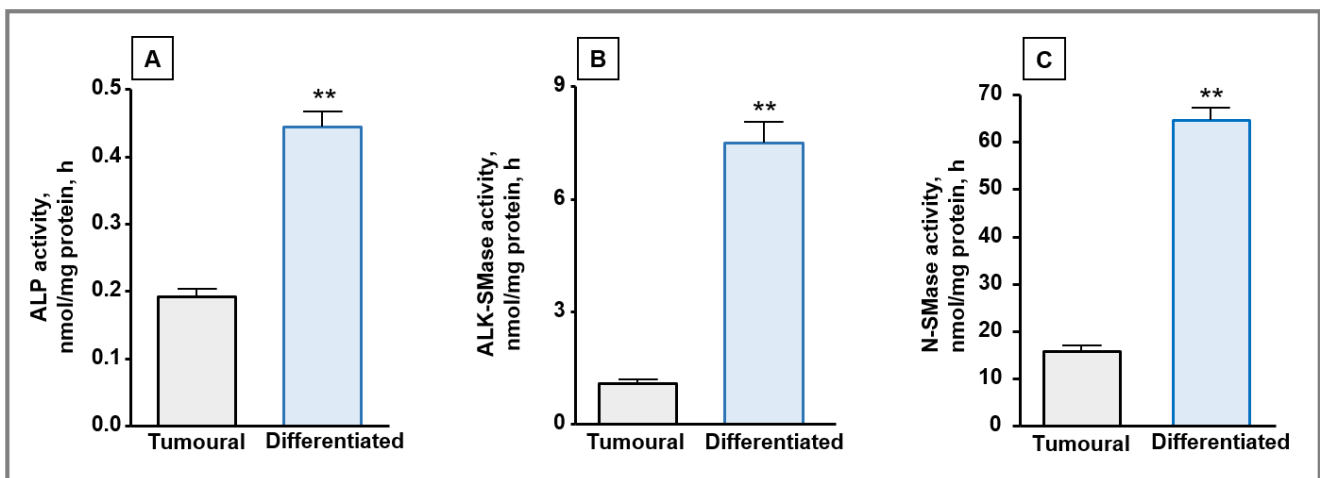


**Figure 10. Morphological features of tumoural and differentiated Caco-2 cells.** Representative microscopic images of tumoural and differentiated Caco-2 cells are shown. Images were viewed on a contrast phase microscope (magnification, 10X; scale bar, 100  $\mu$ m).

At 2-day after confluence, cells grew as monolayers with a typical epithelial morphology. On the other hand, after 10 days of confluence, Caco-2 cells underwent differentiation exhibiting the formation of “domes” across the differentiated cell monolayers (**Fig.10**). Dome formation is a

subsequence of cell polarity and an index of the ability to transport fluid from the apical surface to the basolateral one. These structures are the result of a fluid accumulation between the lower side of the transporting Caco-2 cell monolayer and the plastic of the tissue culture plate [Ramond *et al.*, 1985].

In addition, it has reported that upon reaching confluence, Caco-2 cells spontaneously assemble a brush border and express brush-border membrane-associated hydrolases, typical of a differentiated biochemical phenotype [Chung *et al.*, 1985]. Among these hydrolases, we studied alkaline phosphatase (ALP), whose induction is significantly correlated with cell differentiation [Matsumoto *et al.*, 1990]. Our results demonstrated that in differentiated Caco-2 cells, ALP specific activity was more than twice-fold higher than that of tumoural Caco-2 cells ( $0.44 \pm 0.04$  nmol, mg protein<sup>-1</sup>, hour<sup>-1</sup> and  $0.19 \pm 0.02$  nmol, mg protein<sup>-1</sup>, hour<sup>-1</sup>, respectively) (Fig. 11A).



**Figure 11. Brush-border enzymes activity in tumoural and differentiated Caco-2 cells.** The activities of alkaline phosphatase (ALP) (A), alkaline sphingomyelinase (Alk-SMase) (B) and neutral sphingomyelinase (N-SMase) (C) were assayed in both tumoural (gray) and differentiated cells (blue) using cell homogenates as enzyme source. Data are expressed as nmol/mg prot, h. Values are the mean ± SD of at least three independent experiments. Asterisks represent significant differences between tumoural (gray) and differentiated cells (blue) (\*\* p < 0.01, Student's *t* test).

We further investigated the alkaline-sphingomyelinase (Alk-SMase), a brush-border enzyme whose activity has been previously found decreased in human colorectal carcinoma with respect to normal tissue [Hertervig *et al.*, 1997, 1999]. Based on this data, we hypothesized that Alk-SMase activity

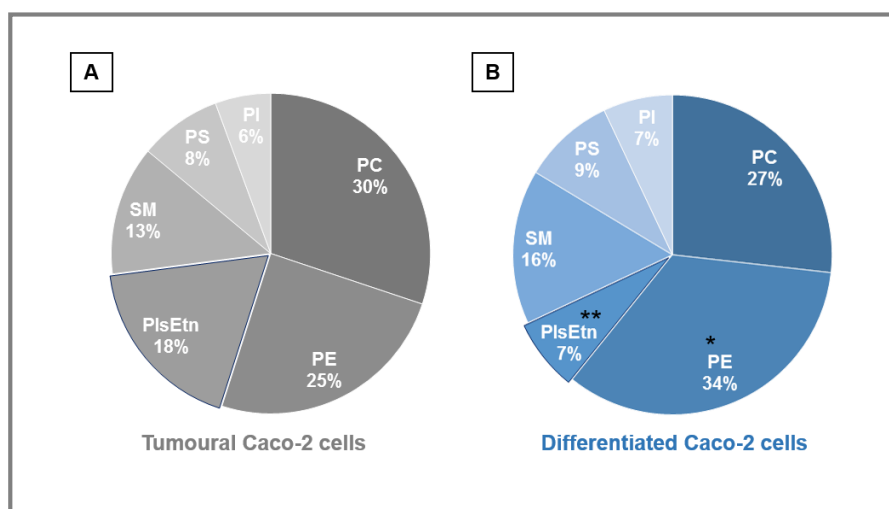


may be restored when Caco-2 cells lose their tumourigenic phenotypes once differentiated *in vitro*. Indeed, as shown in **Fig. 11B**, the Alk-SMase activity markedly increased, about 6.86-fold in differentiated Caco-2 cells, when compared to the tumoural ones.

In addition, we investigated the neutral-sphingomyelinase (N-SMase), taking into account that this enzyme is also a plasma-membrane enzyme form and that some studies demonstrated its implication during neuroblastoma Neuro2a cell differentiation [*Riboni et al., 1995*]. Our data in **Fig. 11C**, show that the N-SMase activity significantly increased, about 4-fold, in differentiated Caco2 cells, with respect to tumoural Caco-2 cells.

### 5.1.2. Phospholipid distribution

Similar to the significant changes occurred in enzyme markers, the differentiation of Caco-2 cells was associated with changes of cell phospholipid profiles. We found that the total phospholipid/protein ratio exhibited a 27% reduction during differentiation (from  $145.9 \pm 4.56$  nmol P, mg protein<sup>-1</sup> to  $106.8 \pm 5.15$  nmol P, mg protein<sup>-1</sup>). The same phospholipid classes were present in both models, but with a different pattern. Differentiated Caco-2 cells were richer in PE (+37%), PS (+12%), PI (+25%) and SM (+20%) and poorer in PC (-11%) compared with tumoural Caco-2 cells. Notably, phosphatidylethanolamine plasmalogen, a quantitative relevant species in the tumoural cells [*Phipps et al., 1995*], exhibited a marked decrease (-60%) during intestinal differentiation (**Fig. 12A-B**).

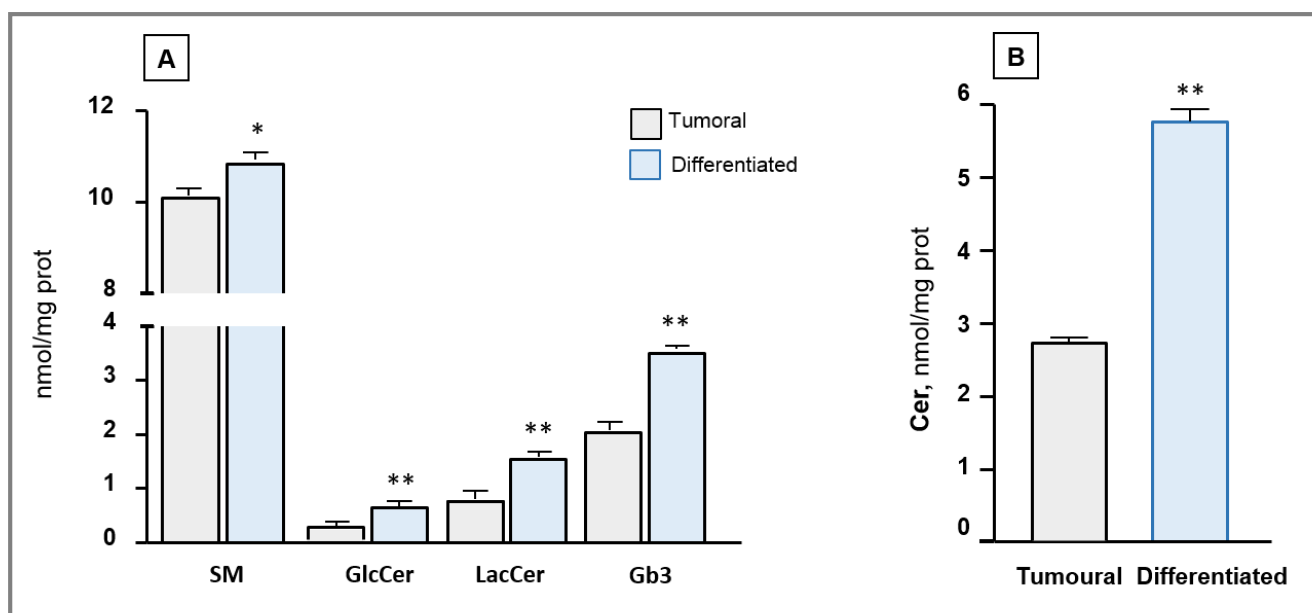


**Figure 12. Phospholipids content in tumoural and differentiated Caco-2 cells.** Tumoural (A) and differentiated (B) Caco-2 cells were harvested and submitted to lipid extraction as described in “Material and Methods” section. Phospholipids were separated by a two-dimensional HPTLC on silica gel plates. Panels show the content of phospholipids in both cell models, and underline the content of phosphoethanolamine plasmalogen (PlsEtn). Data are expressed as percentage of at least three independent experiments. Asterisks represent significant differences between tumoural (gray) and differentiated cells (blue) (\*  $p < 0.05$  and \*\*  $p < 0.01$ , student’s t test ).

### 5.1.3. Sphingolipid profile and ceramide level

Because previous data in literature showed that colon cancer specimens exhibited approximately half the levels of Cer when compared with respective healthy colon mucosa obtained from the same patient [Selzner M et al 2001], we wondered if a change in the endogenous level of Cer could be present along with Caco-2 cell differentiation. To this purpose, we first evaluated the content of total SLs in both cell models. We obtained that this value in tumoural cells is about  $15.71 \pm 0.50$  nmol, mg protein<sup>-1</sup> and significantly increased in differentiated cells to  $22.40 \pm 1.21$  nmol, mg protein<sup>-1</sup>. Moreover, significant changes in SM and neutral GSLs were observed; as shown in Fig. 13A, their content increased with intestinal differentiation.

Concerning the content of endogenous Cer, its levels were twofold with respect to tumoural cells (Fig. 13B). This result indicates that Cer content is inversely associated with malignant progression of Caco-2 cells.



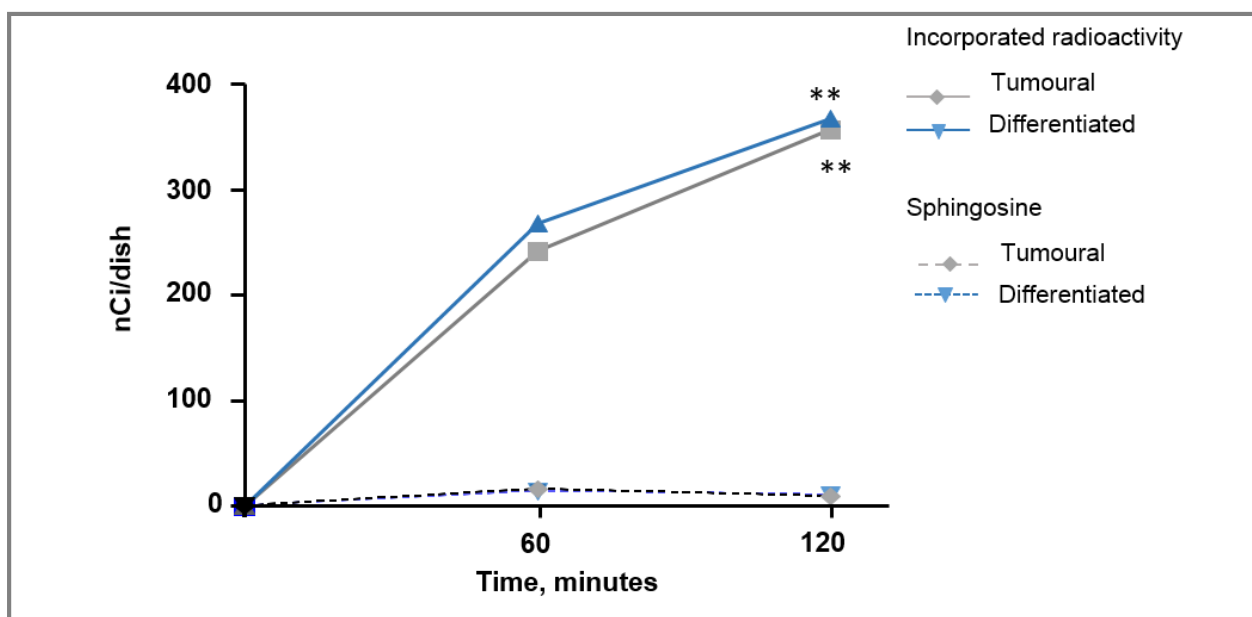
**Figure 13. Sphingolipids content in Caco-2 cell models.** Tumoural (gray) and differentiated (blue) Caco-2 cells were pulsed with 0.55  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]-Sphingosine for 6 hours, and then chased for 48 hours. At the end of chase, cells were harvested and submitted to lipid extraction and quantification as described in “Material and Methods” section. **Panel A** shows the complex sphingolipids content. **Panel B** shows the endogenous ceramide (Cer) level. Data are expressed as nmol/mg protein. Values are the mean  $\pm$  S.D. of at least three independent experiments. Asterisks represent significant differences between tumoural (gray) and differentiated cells (blue) (\*\*  $p < 0.01$ , Student’s  $t$  test)

#### 5.1.4. Metabolic studies in Caco-2 cell models

The metabolic fate of radiolabeled Sph and SM were then investigated in our cell models.

- Metabolism of tritiated Sphingosine in tumoural and differentiated Caco-2 cells

As shown in **Fig. 14**, after administration of [ $^3\text{H}$ ]-Sph for 60 and 120 minutes, both tumoural and differentiated Caco-2 cells rapidly incorporated and metabolized [ $^3\text{H}$ ]-Sph in a time-dependent fashion. The total incorporated radioactivity, present in the TLE, as well as tritiated water released in the culture medium, were slightly higher in differentiated than tumoural Caco-2 cells and markedly increased with pulse time in both cell types. Conversely, [ $^3\text{H}$ ]-Sph diminished with pulse time and represented only a minor portion of total incorporated radioactivity at all the investigated times. Infact, at 120 minutes pulse, its amount accounted for less than 3% of the total incorporated radioactivity in both cell types.

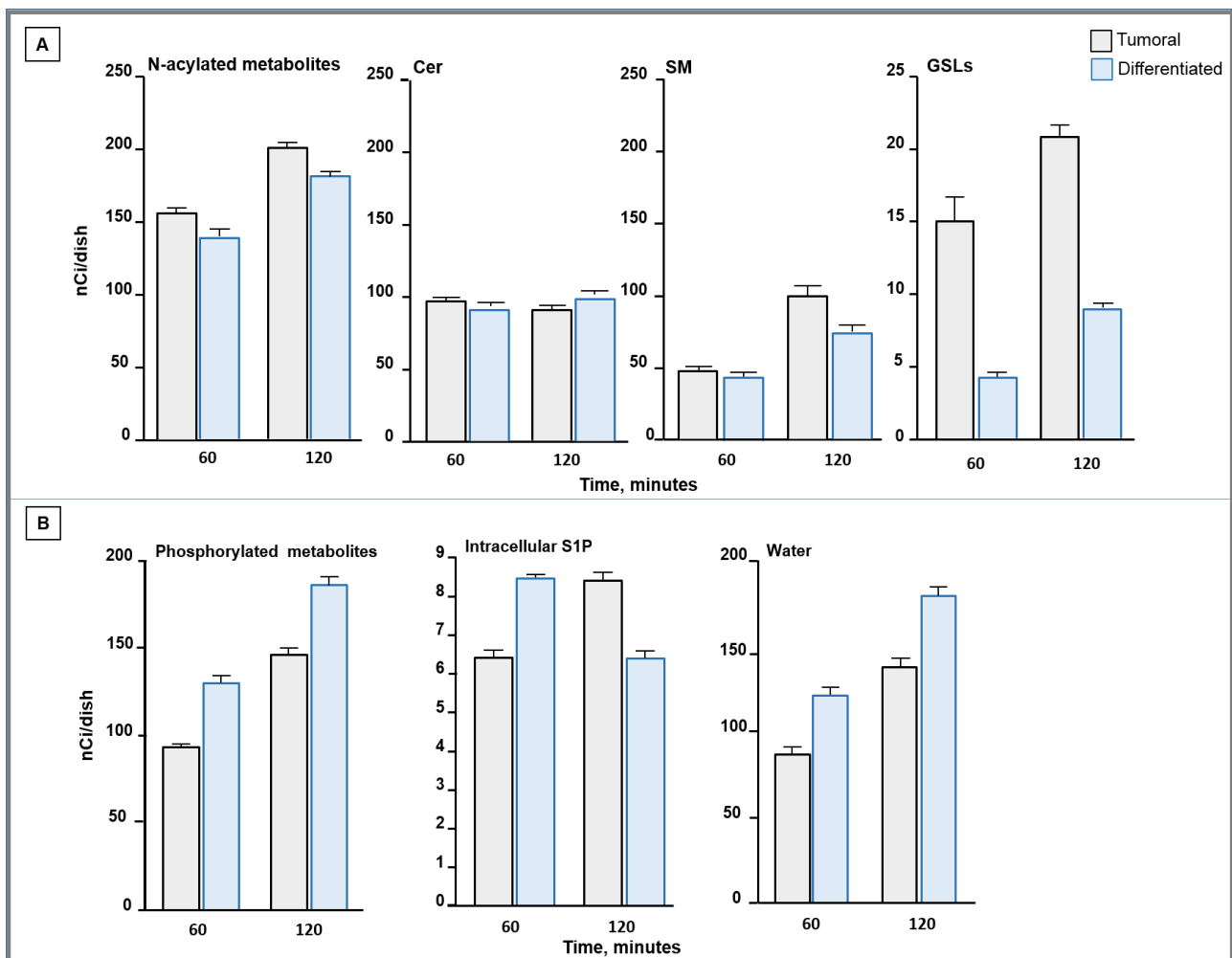


**Figure 14. Uptake of [<sup>3</sup>H]-Sphingosine by Caco-2 cell models.** Tumoural and differentiated Caco-2 cells were pulsed with 0.55  $\mu$ Ci/ml [<sup>3</sup>H]-Sphingosine for 60 and 120 minutes, as indicated, in EMEM containing 15% FCS. Incorporated radioactivity was calculated as the sum of the radioactivity present in the cell lipid extract and the radioactivity present in the culture medium. Data are expressed as nCi/dish. Values are the mean of at least three independent experiments. Asterisks represent significant differences between cells at different times of pulse (\*\*  $p < 0.01$ , Student's  $t$  test).

Most of the [<sup>3</sup>H]-Sph taken up was metabolically processed, either by degradation (assessed as [<sup>3</sup>H]-water released into the culture medium) or by N-acylation, represented by the production of Cer and its use for the synthesis of complex SLs (GSLs and SM). **Fig. 15A** shows that in both cell models the radioactivity representative of the Sph N-acylation process increased in a time dependent fashion and was higher in tumoural cells with respect to the differentiated ones. With increasing pulse times, both cell types were able to rapidly N-acylate exogenous Sph and efficiently produce Cer and to maintain its level constant from 60 up to 120 minutes of pulse. Throughout pulse time, Cer utilization for the biosynthesis of complex SLs was higher in tumoural cells.

Concerning the process of Sph phosphorylation, as shown in **Fig. 15B**, in both cell models, this radioactivity increased over pulse time. Of relevance, Sph-phosphorylated radioactivity was higher in differentiated Caco-2 cells, accounting for about 45% of the total incorporated radioactivity after both 60 and 120 minutes of pulse, whereas in tumoural Caco-2 cells it was about 35%. Moreover,

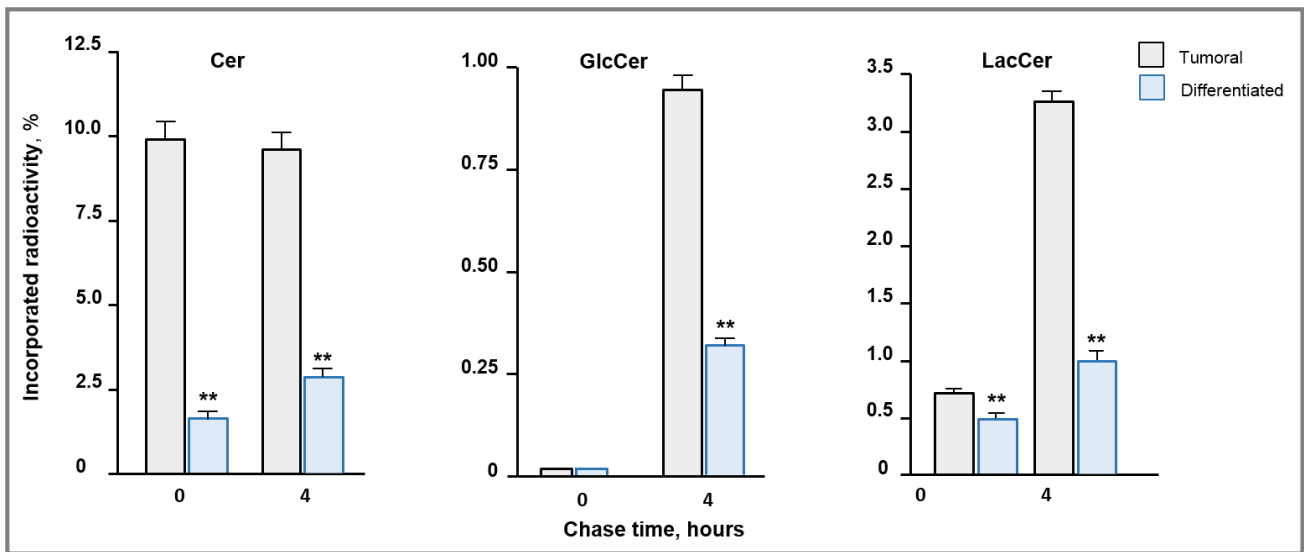
tritiated water represented the bulk of Sph phosphorylation-associated radioactivity (accounting for more than 95%) and raised with the increase of the pulse times suggesting that both cell models are able to efficiently synthesize and degrade S1P. Interestingly, at 120 minutes of pulse, the radioactivity associated to intracellular S1P was found significantly higher in tumoural cells than that of differentiated cells.



**Figure 15. Incorporation of radioactivity into N-acylation and phosphorylation pathways.** Tumoural (gray) and differentiated (blue) Caco-2 cells were pulsed with 0.55  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]-sphingosine for 60-120 minutes in EMEM containing 15% FCS. **Panel A** shows the radioactivity incorporated into N-acylated compounds, represented by Cer, SM and GSLs. **Panel B** shows the radioactivity incorporated into phosphorylated metabolites, represented by S1P and water. Data are expressed as nCi/dish. Values are the mean  $\pm$  SD of at least three independent.

- Metabolism of tritiated SM in both tumoural and differentiated Caco-2 cells

Concerning the feeding experiments with [Sph-<sup>3</sup>H]-SM, after 2 hours of pulse with 0.625 μM [Sph-<sup>3</sup>H]-SM, followed or not by 4 hours chase, we found that both of cell models incorporated the same radioactivity and were able to produce [<sup>3</sup>H]-Cer. Surprisingly, Cer formation was about 10-fold higher in the tumoural Caco-2 cells than in the differentiated ones. Conversely, other [<sup>3</sup>H]-metabolites produced during [Sph-<sup>3</sup>H]-SM metabolism (mainly GlcCer and LacCer) were markedly low in differentiated and tumoural CaCo-2 cells. After chase, a pronounced increase in their content were observed in tumoural cells when compared to the differentiated ones, suggesting a more rapid Cer turnover present in tumoural model (**Fig. 16**).

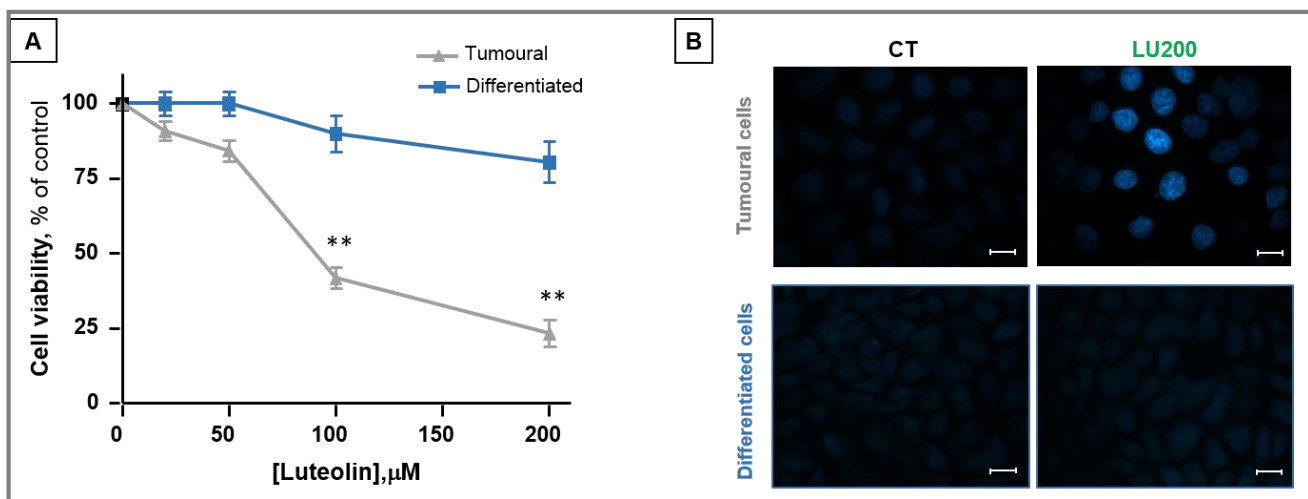


**Figure 16. Metabolism of exogenous SM in Caco-2 cell models.** Cells were pulsed with 0.6 μCi/ml of [<sup>3</sup>H-Sph] SM for 2 hours followed or not by 4 hours chase. The radioactivity incorporated into Cer, GlcCer, and LacCer was measured. Data are expressed as % of incorporated radioactivity. Values are the means ± SD of three experiments in duplicate. Asterisks represent significant differences between tumoural (gray) and differentiated cells (blue) (\*\* p < 0.01, Student's *t* test).

As previously showed, the activity of N-SMase increased was elevated in differentiated cells, we administered [Sph-<sup>3</sup>H]-SM, in presence of GW4869, that blocks the N-Smase activity . We obtained that [<sup>3</sup>H]-Cer formation was not affected by this inhibitor, thus indicating that SM degradation is most probably processed in the lysosomes by the A-Smase (*data not shown*).

## 5.2. Effect of LU on tumoural and differentiated Caco-2 cell viability

Once characterized, we then assessed the sensitivity of both Caco-2 cell models to LU. Cells were treated with different concentrations ranging from 20 to 200  $\mu\text{M}$  of LU for 48 hours. As illustrated in **Fig. 17A**, LU induced a dose-dependent reduction in tumoural cells viability. In particular, at 100 and 200  $\mu\text{M}$ , LU exhibited a potent cytotoxic effect, as the cell viability decreased by 58% and 77%, respectively. Conversely, cell viability of differentiated Caco-2 did not significantly change with LU at doses 20-100  $\mu\text{M}$ . The highest LU concentration (200  $\mu\text{M}$ ) had only a weak effect on differentiated cell survival, as a decrease of less than 20% was observed.



**Figure 17. Effect of Luteolin on the survival of tumoural and differentiated Caco-2- cells.** **Panel A**, tumoural and differentiated cells viability was assessed after treatment with different concentrations of Luteolin. Data are expressed as % of cell viability with respect to control cells treated with vehicle (0.57% DMSO) (100%). Values are mean  $\pm$  SD of three independent experiments Asterisks represent significant differences between LU-treated cells and vehicle treated cells (\*\*  $p < 0.01$ , Student's  $t$  test). **Panel B**, representative images of Tumoural and differentiated Caco-2 cells treated with vehicle (0.57% DMSO) or 200  $\mu\text{M}$  Luteolin (LU200) and stained with Hoechst 33342 (magnification, 100X; scale bar, 10  $\mu\text{m}$ ).

It was reported that LU induces apoptosis in a number of cell types [Wang *et al.*, 2013, George *et al.*, 2013]. Therefore, in order to ascertain whether the cytotoxic effect of LU was caused by apoptosis, we examined chromatin condensation (a hallmark of apoptotic cell death) using Hoechst 33324 dye. As shown in **Fig. 17B**, control cells displayed uniformly light blue nuclei, meanwhile, with 200  $\mu\text{M}$

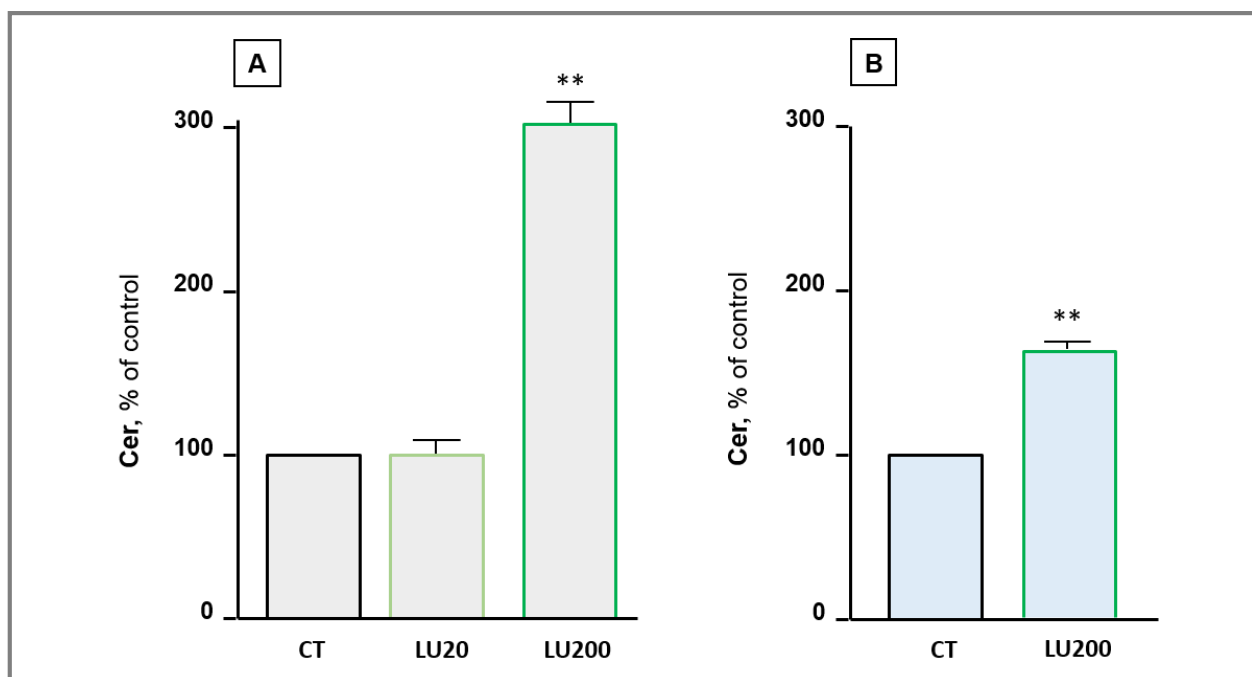
LU treatment, tumoural cells exhibited nuclei with brilliant blue staining, indicating chromatin condensation. To the opposite, and of relevance, no significant change in nuclear fluorescence was observed in differentiated cells treated with 200  $\mu\text{M}$  LU for 48 hours, with respect to the control ones. The impressive effect of LU observed in cultured human colon cancer paralleled with its innocuous effect on the differentiated model, prompted us to better understand how LU induces apoptosis in tumoural cells.

### 5.3. Role of Cer as mediator of LU toxicity in tumoural Caco-2 cells

#### 5.3.1. Change in Cer levels during LU treatment in Caco-2 cells

Accumulating data in literature indicate that Cer plays an important role as a tumour suppressor lipid by inducing anti-proliferative and pro-apoptotic responses and, therefore, acting as an endogenous mediator in the activity of different chemotherapeutic drugs [*Hannun et al., 1996, Pettus et al., 2002*]. On these premises, we investigated the possible involvement of Cer as a mediator for the apoptotic activity of LU in tumoural Caco-2 cells. We thus measured intracellular Cer level after 24 hours of LU treatment (20 and 200  $\mu\text{M}$ ), when no signs of toxicity was observed (Trypan blue analyses revealed that the large majority of treated cells were vital) in tumoural and differentiated cells. **Fig. 18** shows that in tumoural cells, the endogenous Cer levels at 20  $\mu\text{M}$  of LU was similar to that of control cells. Instead, we found that 200  $\mu\text{M}$  of LU induces an increase in intracellular Cer level with a more pronounced trend in tumoural cells. Notably, at 200 $\mu\text{M}$  of LU an increase corresponding to 3- and 1.6-fold with respect to control cells was reached in tumoural and differentiated cells, respectively.





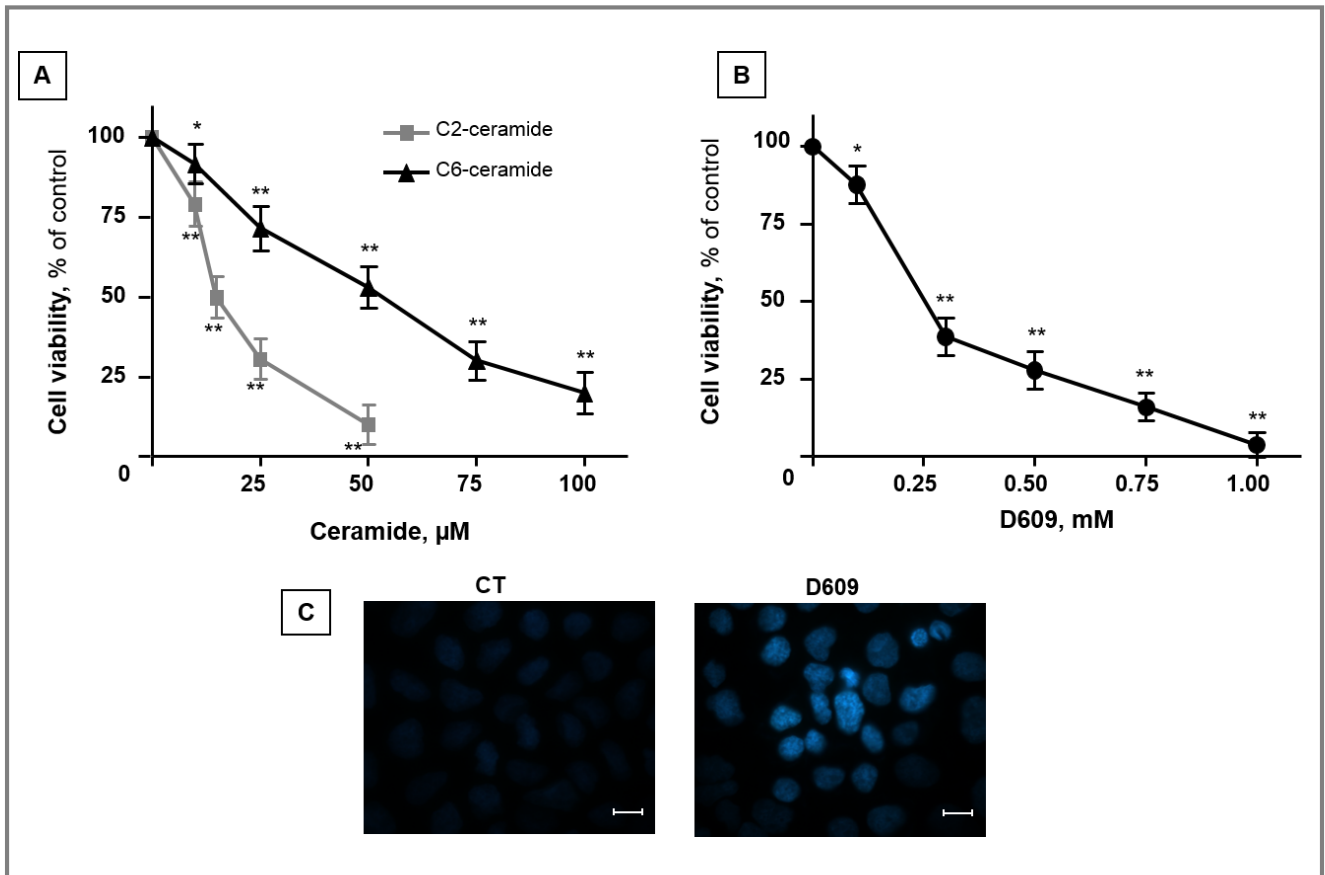
**Figure 18. Effect of Luteolin on Ceramide levels in tumoural and differentiated Caco-2 cells.** Both Caco-2 cell models were pulsed with [<sup>3</sup>H]-Sphingosine for 6 hours, chased for 22 hours, and treated with 20 μM (LU20), or 200 μM (LU200) of Luteolin or vehicle (0.57%DMSO) (CT). Panels show the endogenous Ceramide content expressed as % with respect to CT cells in tumoural (A) and differentiated (B) cells. Data are the mean ± SD of at least three independent experiments. Asterisks represent significant differences between LU-treated cells and vehicle treated cells (\*\* p < 0.01, Student's *t* test).

### 5.3.2. Effect of induced increase of intracellular Cer on tumoural Caco-2 cell

#### viability

To obtain further evidence on the role of Cer in mediating the LU toxicity, we examined if an increase in the Cer level was able to mimic the apoptotic effect of LU. To this purpose, tumoural cells were incubated with various concentrations of cell-permeable ceramides (C2 and C6-Cer), or D609, an inhibitor of sphingomyelin synthase. All these treatments are known to effectively increase the cellular Cer level [Milhas D et al 2012]. At the end of treatments, our results revealed that both Cer analogues were toxic in a dose-dependent manner (Fig. 19A). Specifically, C2-Cer showed a noticeably pronounced toxicity at 50 μM associated with a cell survival of 10%, meanwhile, under the same conditions, C6-Cer decreased the cell viability by only 47%. This result may be interpreted as that the potency of the cytotoxic effect of the different ceramides was inversely related to the length

of their N-acyl chain. In addition, the results obtained demonstrated that D609 was able to induce a dose-dependent reduction of cell viability too (**Fig. 19B**).



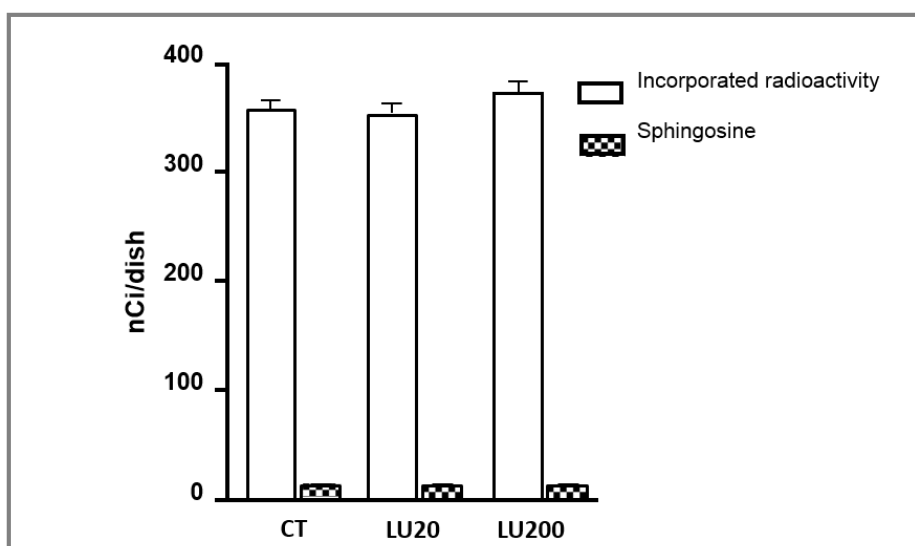
**Figure 19. Effect of Ceramide and D609 on Tumoural cell viability.** Cell viability was assessed after cell exposure to different concentrations of C2 and C6 Ceramide (**A**) or D609 (**B**) for 24 and 48 hours, respectively. Data are expressed as % of cell viability with respect to control cells. Values are the mean  $\pm$  SD of three independent experiments. Asterisks represent significant differences between treated cells and CT cells (\*  $p < 0.05$  and \*\*  $p < 0.01$ , Student's  $t$  test). (**C**) Representative images of tumoural cells incubated in presence or absence (CT) of 0.3 mM D609 (D609) for 24 hours and stained with Hoechst 33342 (magnification, 100X; scale bar, 10  $\mu\text{m}$ ).

In parallel, Hoechst staining was assessed and showed chromatin condensation indicating that these treatments caused cell apoptosis (**Fig. 19C**).

#### 5.4. Effect of LU on Sph metabolism in tumoural Caco-2 cells

To obtain information on the effect of LU on Cer metabolism, we performed short time pulse experiment by feeding cells with [<sup>3</sup>H]-Sph, as the metabolic precursor of Cer, taking into account that tumoural Caco-2 cells are able to rapidly incorporated exogenously administered [<sup>3</sup>H]-Sph as reported above.

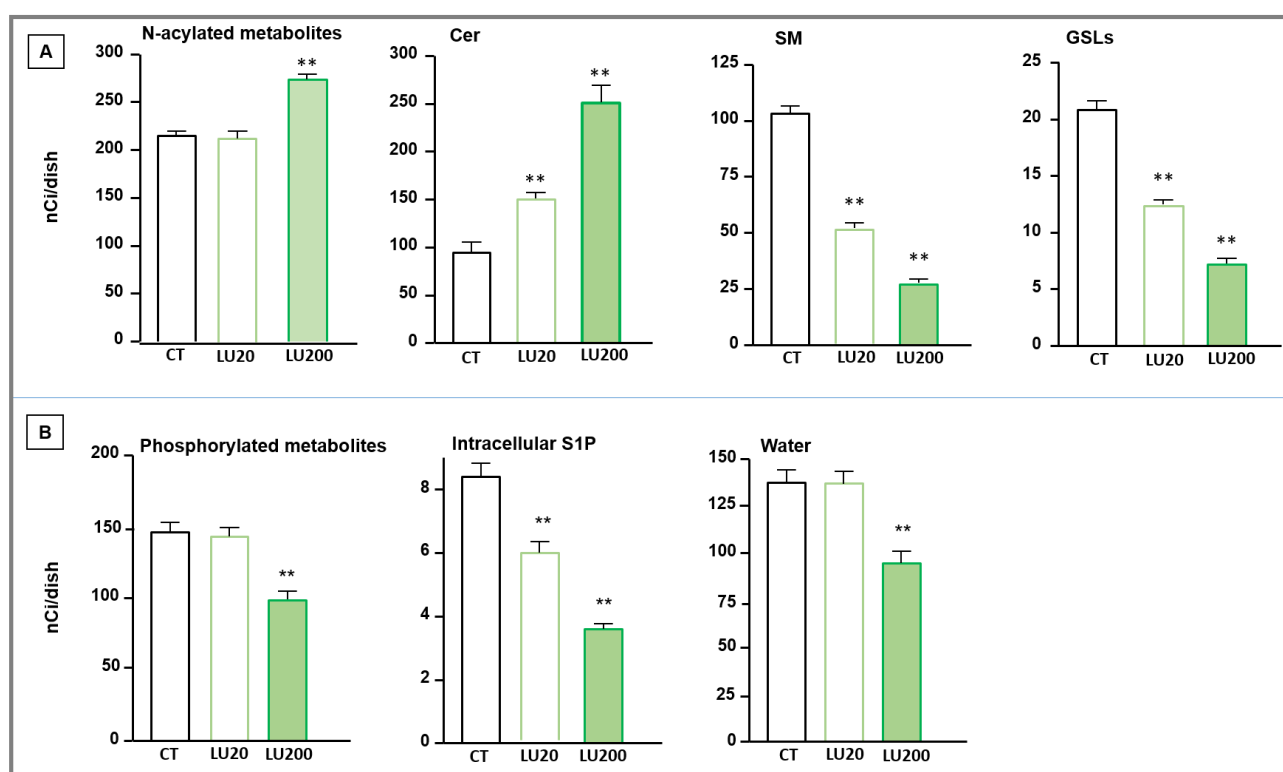
After a 120 minutes pulse, the radioactivity present in TLE, as well as that of tritiated water (produced by complete Sph degradation) was found very similar in control and LU-treated cells. Furthermore, [<sup>3</sup>H]-Sph represented a minor portion of the incorporated radioactivity, accounting for less than 5% of the total incorporated radioactivity in all cases (**Fig. 20**).



**Figure 20.** *Uptake of [<sup>3</sup>H]-Sphingosine by Tumoural Caco-2 cells after LU treatment.* Cells were pulsed with 0.55  $\mu$ Ci/ml [<sup>3</sup>H]-sphingosine for 2 hours in EMEM supplemented with 15% FCS in the presence of 20  $\mu$ M (LU20) and 200  $\mu$ M (LU200) of Luteolin, or vehicle (CT, 0.57% DMSO). Incorporated radioactivity is the sum of the radioactivity present in the cell lipid extract and present in the culture medium. Data are expressed as nCi/dish. Values are the mean  $\pm$  SD of at least three independent experiments

When analyzing organic metabolites, we first focused on the Sph N-acylation products. As shown in **Fig. 21A**, the radioactivity incorporated into N-acylated compounds (sum of Cer, SM and GSLs) was very similar in control and LU-treated cells at the non-toxic dose (20  $\mu$ M LU). Of note, a significant increase about  $35 \pm 2.56$  % was observed at the toxic dose of LU with respect to control cells.

However, both treatments with LU at 20 and 200  $\mu\text{M}$  modified the distribution of radioactivity among N-acylated compounds. Indeed, as shown in **Fig. 21A**, [ $^3\text{H}$ ]-Cer level increased in a dose-dependent manner after LU treatment, accounting for an increase of about  $65 \pm 4.7\%$  and  $163 \pm 17.5\%$  in 20 and 200  $\mu\text{M}$  LU concentrations, respectively, with respect to control cells. In parallel, LU induced a significant decrease of the radioactivity associated to complex SLs. In fact, the SM radioactivity was  $49 \pm 5.4\%$  and  $71 \pm 3.3\%$  less at 20 and 200  $\mu\text{M}$  LU, respectively than in control. Concerning the GSLs, their content decreased in a dose-dependent fashion, by about  $41 \pm 4.7\%$  and  $67 \pm 6.8\%$ . Taken together, these results demonstrate that LU inhibits the utilization of newly synthesized Cer for the biosynthesis of complex SLs.



**Figure 21. Incorporation of radioactivity into N-acylation and phosphorylation pathways.** Tumoural Caco-2 cells were pulsed with  $0.55 \mu\text{Ci/ml}$  of [ $^3\text{H}$ ]-sphingosine for 2 hours in EMEM supplemented with 15% FCS in presence of vehicle (CT), 20  $\mu\text{M}$  (LU20), or 200  $\mu\text{M}$  (LU200). **Panel A** shows the radioactivity incorporated into N-acylated compounds, represented by Cer, SM and GSLs. **Panel B** shows the radioactivity incorporated into phosphorylated compounds, represented by S1P and water. Data are the mean  $\pm$  SD of at least three independent experiments. Asterisks represent significant differences between LU-treated cells and vehicle treated cells (\*\*  $p < 0.01$ , Student's  $t$  test)

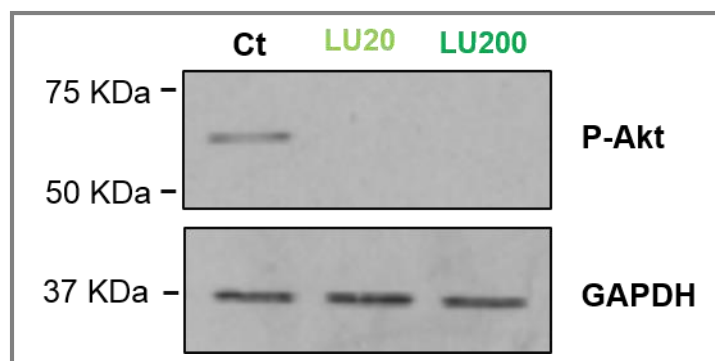
We then analyzed the Sph phosphorylation process, including S1P production and degradation, measured as tritiated water. Of relevance, the increase of the N-acylated radioactivity observed at the toxic dose of LU (200  $\mu\text{M}$ ) was accompanied by a marked decrease of Sph-phosphorylation-associated radioactivity, corresponding to  $32 \pm 1.7$  % of the control. Interestingly, tritiated water which represented the bulk of Sph-phosphorylation-associated radioactivity had a similar trend in LU-treated cells (20  $\mu\text{M}$ ) with respect to control cells, whereas it was  $31 \pm 1.9$  % lower in LU-treated cells (200  $\mu\text{M}$ ) than in control ones (**Fig. 21B**). Moreover, the radioactivity associated to extracellular S1P production represented less than 0.3 % of the Sph phosphorylation process in both cell types. Interestingly, even if the radioactivity associated to intracellular S1P was markedly lower than tritiated water, its content decreased in a dose dependent fashion after LU treatment (**Fig. 21B**).

## **5.5. Accumulation of Cer in the endoplasmic reticulum in LU-treated cells: possible impairment of Cer trafficking**

The results obtained in our lab that reported a strong link between the PI3K/AKT pathway and the Endoplasmic Reticulum (ER) to Golgi Traffic of Cer, [*Giussani et al., 2009*], coupled with more recent data that demonstrated the capacity of LU to inhibit PI3K activity in different cell types [*Kim et al., 2013, Lim et al., 2012*], prompted us to speculate that, in LU treated cells, Cer accumulation in the ER might be due to an aberrant export of Cer to the Golgi apparatus.

### **5.5.1. Effect of LU on p-AKT expression**

Based on these data, we first investigated the capacity of LU to influence the phosphorylation of Akt in tumoural Caco-2 cells, as a downstream effect of an inhibition of PI3K activity. Immunoblot analyses demonstrated that LU markedly reduced the Akt activation. Indeed, as shown in **Fig. 22**, phospho-Akt was undetectable, not only at toxic doses, but surprisingly also at 20  $\mu\text{M}$  LU.

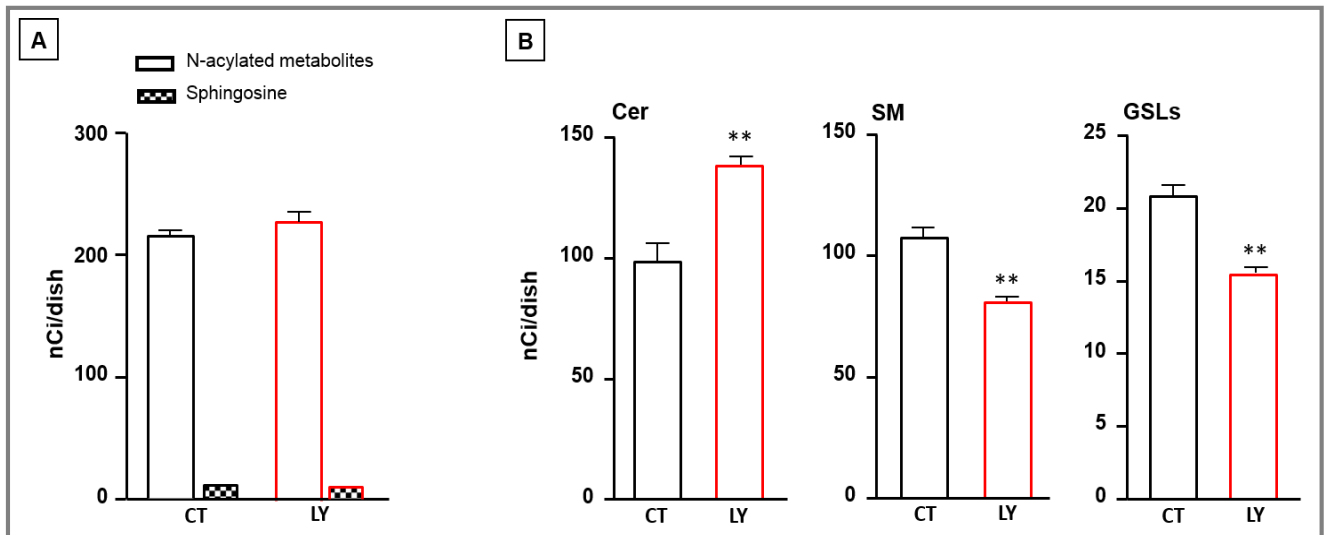


**Figure 22.** *Effect of Luteolin on the expression of p-AKT protein in tumoural Caco-2 cells.* Cells were treated with vehicle (CT, 0.57% DMSO) or 20  $\mu$ M (LU20), 200  $\mu$ M (LU200) Luteolin for 30 minutes. Cells were then lysed and aliquots containing 30  $\mu$ g of proteins were analyzed by immunoblotting with anti-pAKT and anti-GAPDH antibodies. The immunoblots shown are representative of one out of three similar experiments.

### 5.5.2. Effect of PI3K/Akt inhibition on [ $^3$ H]-Sph metabolism

In light of the efficient inhibition of Akt phosphorylation by LU, we investigate if the inhibition PI3K, through LY294002 (LY), could have the similar effect observed after LU treatment on Cer metabolism. To this purpose, we performed a short time pulse experiment by feeding cells with [ $^3$ H]-Sph in the presence or absence of 20  $\mu$ M LY, a dose used to inhibit efficiently the Akt phosphorylation in Caco-2 cells. We first obtained that treatment with LY did not alter the [ $^3$ H]-Sph uptake: the incorporated radioactivity was very similar in both control and treated cells. Furthermore, residual intracellular [ $^3$ H]-Sph represented about only the 3% of the incorporated radioactivity, suggesting that cells were able to rapidly metabolize Sph in both cases (**Fig. 23A**).

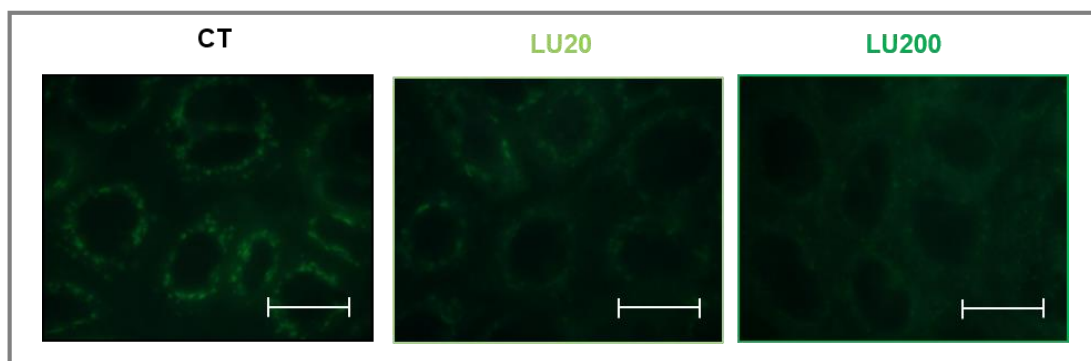
The radioactivity associated to the Sph N-acylated metabolites was very similar in control and LY-treated cells. Of note, treatment with LY markedly modified the radioactivity distribution among the different N-acylated compounds (**Fig. 23B**). In fact, the [ $^3$ H]-Cer levels were about  $40 \pm 1.8$  % higher in treated cells, whereas the radioactivity associated to SM and GSLs were  $24 \pm 0.8$ % and  $21 \pm 1.7$ % lower than in control cells, respectively. Thus, similar to the effect of LU on the alteration of Cer metabolism, the inhibition of the PI3K/Akt pathway promotes a decrease in the metabolic utilization of Cer for the biosynthesis of complex SLs in tumoural Caco-2 cells.



**Figure 23. Incorporation of radioactivity into *N*-acylated metabolites prior and after treatment with LY294002.** Tumoural Caco-2 cells were pulsed with 0.55 mCi/ml [<sup>3</sup>H]-Sphingosine for 2 hours in EMEM containing 15% FCS in the presence of 20mM LY294002 (LY, red) or vehicle (CT, 0.57% DMSO). **Panel A** shows the radioactivity incorporated into *N*-acylated metabolites (sum of Cer, SM and GSLs) and the radioactivity associated to intracellular Sph. **Panel B** shows the distribution of radioactivity into *N*-acylated compounds. Data are expressed as nCi/dish. Values are the mean ± SD of at least three independent experiments. Asterisks represent significant differences between LY-treated cells and vehicle treated cells (\*\*  $p < 0.01$ , Student's *t* test).

### 5.5.3. Effect of LU on the intracellular distribution of BODIPY-C5-Cer

Prompted by these results, we investigated the effect of LU on the ER-Golgi traffic of Cer. To this purpose, we qualitatively examined the transport of Cer using BODIPY-C5-Cer, a Cer analogue extensively utilized as a fluorescent tool able to mimic the ER-Golgi traffic of natural Cer in living cells [Ktistakis *et al.*, 1995; Pagano *et al.*, 1991]. Indeed, after administration to cells, BODIPY-C5-Cer is readily incorporated into the plasma membrane even at 4°C and, after movement across the plasma membrane, fluorescently labels intracellular membranes, particularly the ER. Upon warming the cells at 37°C, a distribution of BODIPY-C5-Cer occurs, as it is efficiently transported from the ER to the Golgi. Tumoural Caco-2 cells were treated with 20 and 200 μM of LU for 90 minutes prior to loading with BODIPY-C5-Cer. Then, the transport of this fluorescent analogue to the Golgi apparatus was monitored, the results showed that in control cells, the fluorescence appeared in compact perinuclear regions, corresponding to the Golgi apparatus, indicating that BODIPY-C5-Cer exited the ER network (Fig. 24).



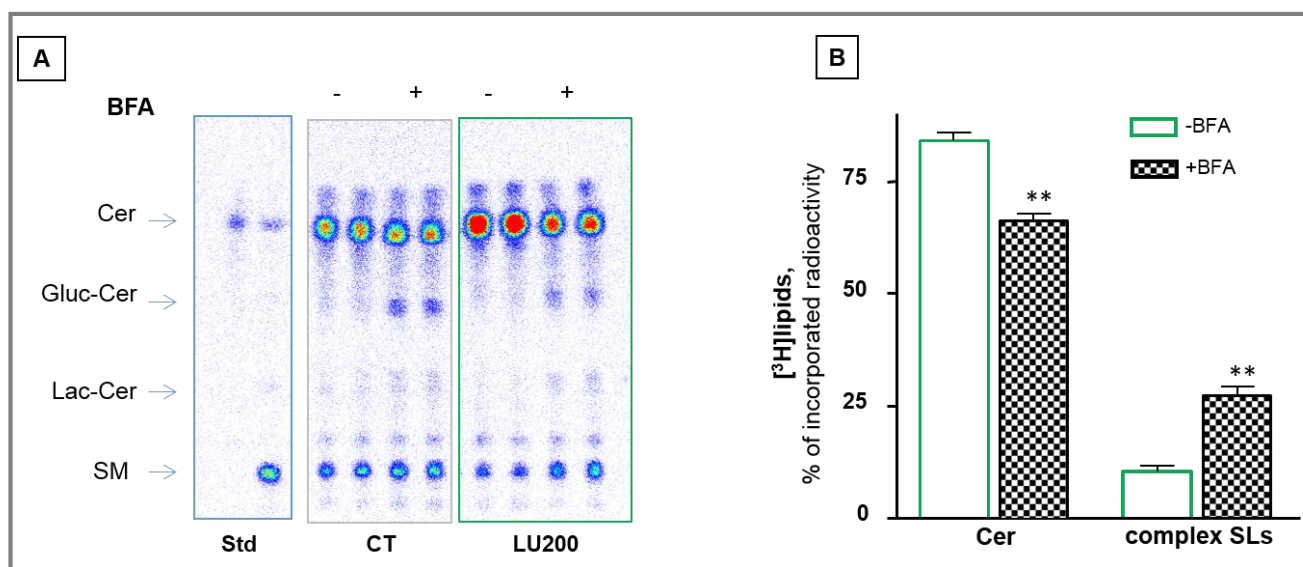
**Figure 24.** *Effect of Luteolin on BODIPY-C5-Cer distribution in Tumoural Caco-2 cells.* Cells were pretreated with 20  $\mu\text{M}$  (LU20) or 200  $\mu\text{M}$  (LU200) Luteolin or 0.57% DMSO (CT) for 90 minutes. Cells were then incubated with 2.5  $\mu\text{M}$  of BODIPY-C5-Cer for 30 minutes at 4°C. Then, cells were further incubated, in the presence or absence of LU, at 37°C for 30 minutes in order to allow the intracellular distribution of the fluorescent ceramide. Cells were fixed and examined by fluorescence microscopy (magnification, 100X; scale bar, 10  $\mu\text{m}$ ).

In contrast, the LU treatment reduced the accumulation of intracellular fluorescence in the Golgi region in a dose-dependent manner. This fluorescence was observed in a diffuse reticular ER network, suggesting that the transport to the Golgi apparatus was inhibited.

#### 5.5.4. Effect of LU on complex SLs biosynthesis from [ $^3\text{H}$ ]-Sph in BFA-treated cells

To further confirm the putative role of LU in inhibiting the Cer export from the ER, we next investigated the effect BFA treatment on [ $^3\text{H}$ ]-Sph metabolism in LU-treated tumoural cells. The rationale of this experiment resides in the fact that BFA is a macrolide able to merge the ER and Golgi compartments, thus making the ER-accumulated Cer accessible for the biosynthesis of complex SLs in the Golgi [Chardin *et al.*, 1999]. As shown in **Fig. 25A-B**, BFA treatment was found effective in reducing the increased Cer levels observed during LU treatment as well as in restoring the biosynthesis of complex SLs. Thus, in the presence of BFA, LU was unable to impair the metabolic utilization of Cer.

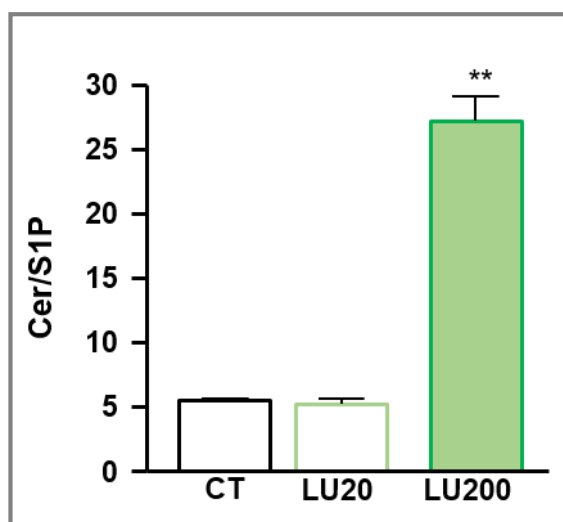




**Figure 25. Effect of Luteolin on  $[^3\text{H}]$ -Sphingosine metabolism in BFA-treated Caco-2 cells.** Tumoural Caco-2 cells were pretreated for 30 minutes at 37°C with 1  $\mu\text{g}/\text{ml}$  Brefeldin A (BFA) or not, as indicated and then pulsed with 0.55  $\mu\text{Ci}/\text{ml}$   $[^3\text{H}]$ -Sphingosine in the absence (CT) or presence of 200  $\mu\text{M}$  Luteolin (LU200) for 2 hours at 37°C. At the end, cell lipids were extracted and separated by HPTLC. **Panel A** shows a representative autoradiographic image of sphingolipids in the organic phase. **Panel B** shows the radioactivity incorporated into Cer and complex sphingolipids in LU treated cells in the presence (+BFA) or absence (-BFA) of BFA. Data are the mean  $\pm$  SD of at least three independent experiments. Asterisks represent significant differences between BFA-treated cells and CT cells (\*\*  $p < 0.01$ , Student's  $t$  test)

## 5.6. Effect of Luteolin on sphingolipids rheostat in tumoural Caco-2 cells

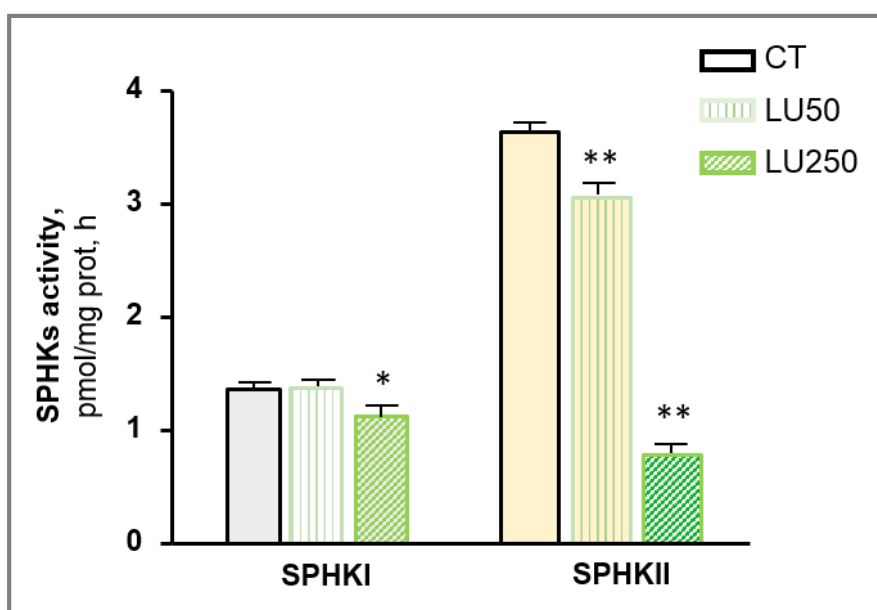
As reported above, LU induced a decrease of the radioactivity associated to intracellular S1P after short pulse time. We therefore analyzed LU-treated cells, for their S1P content. We found that S1P level was decreased by about 40 % at 200  $\mu\text{M}$  if compared to the control level, whereas no significant change was observed at LU 20  $\mu\text{M}$  (*data not shown*). Furthermore, this decrease in S1P content at 200  $\mu\text{M}$  of LU, coupled with the concomitant increase of Cer, resulted also in an intracellular Cer/S1P ratio significantly higher (about 5-fold) with respect to the control cells. The marked increase of the Cer/S1P ratio led us to speculate that this could be a reason of the cytotoxic effect exerted by the highest LU dose (200  $\mu\text{M}$ ) in tumoural Caco-2 cells (**Fig. 26**).



**Figure 26. Ceramide and intracellular S1P ratio in tumoural Caco-2 cells.** Caco-2 cells were pulsed with 0.55  $\mu\text{Ci/ml}$  of [ $^3\text{H}$ ]-sphingosine for 6 hours, chased for 22 hours, and treated with 20  $\mu\text{M}$  (LU20), or 200  $\mu\text{M}$  (LU200) of Luteolin or vehicle (0.57%DMSO) (CT). At the end of chase, cells were harvested and submitted to lipid extraction and quantification as described in “Material and Methods”. Histograms represent the ratio between ceramide and intracellular S1P-associated radioactivity. Data are the mean  $\pm$  SD of at least three independent experiments Asterisks represent significant differences between LU-treated cells and CT cells (\*\*  $p < 0.01$ , Student’s  $t$  test).

### 5.7. Effect of LU on Sphingosine kinases activity in tumoural Caco-2 cells

The decrease of the S1P content under LU treatment led us to hypothesize that this data could be consequent to the inhibition of sphingosine kinases (SPHKs) activity. On this base, we assessed the effect of LU on the *in vitro* enzyme activity of the two SPHKs recognized isoforms (SPHKI and SPHKII) by using experimental conditions able to selectively favor the activity of each enzyme. Using the homogenate from tumoural cells as control, the measured SPHKI activity was  $1.36 \pm 0.07$  pmol, mg protein $^{-1}$ , min $^{-1}$  and that of SPHKII  $3.6 \pm 0.6$  pmol, mg protein $^{-1}$ , min $^{-1}$ . By adding different doses of LU (50  $\mu\text{M}$  and 250  $\mu\text{M}$ ) to the enzyme assay, a dose-dependent inhibition of the SPHKII enzyme activity was observed; 50  $\mu\text{M}$  of LU yielded 16 % inhibition, whereas at high dose of LU (250  $\mu\text{M}$ ), the activity of SPHKII was strongly reduced, about 79%, with respect to the control cells. In parallel, the activity of SPHKI shows a decrease of only 17% with no change at 50  $\mu\text{M}$  of LU. These results suggested that SPHKII is the active isoform and the important target of LU (**Fig. 27**).



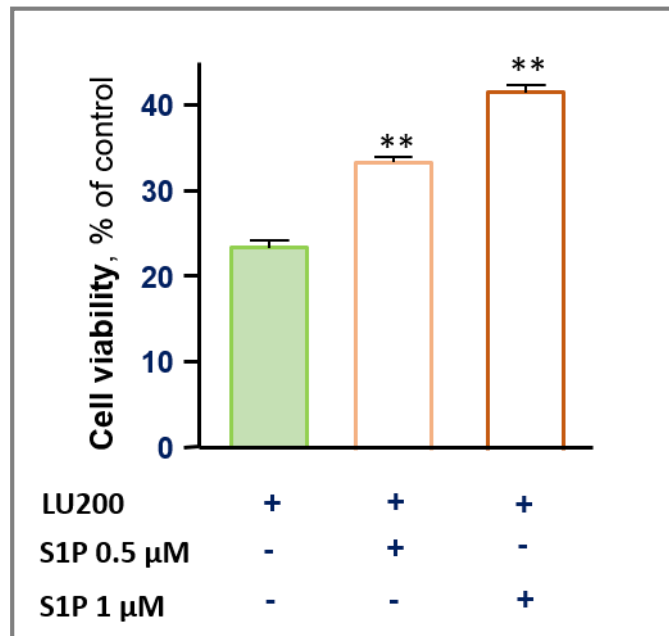
**Figure 27.** *Effect of LU on SPHKs in vitro activities in Caco-2 cells.* SPHKI and SPHKII activities were assayed using tumoural cell homogenate as enzyme source and under buffer conditions favoring SPHKI or SPHKII activity. 50  $\mu$ M (LU50), 250  $\mu$ M (LU250) of Luteolin or vehicle (CT, 0.07% DMSO) were added to the appropriate SK Buffer. Data are expressed as pmol/mg protein, h. Values are the mean  $\pm$  SD of at least three independent experiments. Asterisks represent significant differences between LU-treated cells and CT cells (\*\*  $p < 0.01$ , Student's  $t$  test).

## 5.8. S1P acts as a Cer antagonist in favouring tumoural Caco-2 cell survival against LU toxicity

### 5.8.1. Role of S1P in LU-induced toxicity in tumoural Caco-2 cells

The evidence that LU induces a decrease in the intracellular S1P content stimulated us to analyse whether exogenous S1P administration might be able to modulate tumoural Caco-2 cell death by preventing the cytotoxic effect of LU. To this purpose, we incubated 200  $\mu$ M LU-treated cells in the absence or presence of extracellular S1P (0.5-1  $\mu$ M) for 48 hours. Interestingly, as shown in **Fig. 28**, the co-treatment of S1P and LU rendered tumoural Caco-2 cells resistant to the toxic effect of the flavonoid. In fact, exogenous S1P was able to reduce the cytotoxic effect of LU by inducing a 1.5 and

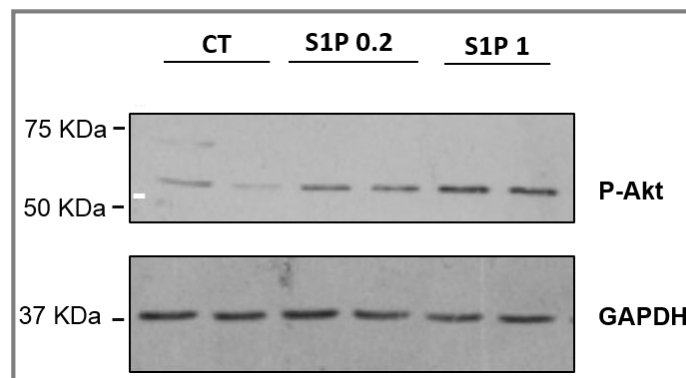
2-fold increase in viable cell number at 0.5 and 1  $\mu\text{M}$  respectively, compared to cells treated only with LU.



**Figure 28. Effect of exogenous S1P on LU-induced apoptosis in Caco-2 cells.** Tumoural Caco-2 cells were exposed to 0.5-1  $\mu\text{M}$  S1P, 200  $\mu\text{M}$  LU, separately or in combination, as indicated. Cell viability was measured by MTT assay after 48 hours of treatment. Results are expressed as percentage of viable cells with respect to vehicle-treated cells (100%). Data are mean  $\pm$  SEM of at least three independent experiments. Asterisks represent significant differences between S1P-treated cells and LU-treated cells (\*\*  $p < 0.01$ , Student's  $t$  test).

### 5.8.2. Effect of S1P on phospho-Akt expression in tumoural Caco-2 cells

Finally, we investigated the possible mechanism by which S1P elicits a protective effects against LU-induced apoptosis in tumoural Caco-2 cells. Immunoblot analyses demonstrated that S1P was able to stimulate Akt activation. Indeed, as shown in **Fig. 29**, phospho-Akt level was about 1.5 and 2.5 fold higher in 0.2 and 1  $\mu$ M S1P-treated cells, with respect to the control ones.



**Figure 29.** *Effect of S1P on the expression of p-AKT in Caco-2 cells.* Tumoural Caco-2 cells were incubated in presence or absence of 0.2 and 1.0  $\mu$ M S1P for 30 minutes, respectively. Cells were then lysed and aliquots containing 30  $\mu$ g of proteins were analyzed by immunoblotting with anti-p-AKT and anti-GAPDH antibodies. The immunoblottings shown are representative of one out of three similar experiments.

## DISCUSSION

For an antitumoural agent, it is important to efficiently kill cancer cells but not normal ones. An understanding of the sensitivity of normal and cancer colon cells toward an antitumoural agent is crucial because it represents a determinant factor for its selective biological activities. Thus, one of the purposes of this study was to examine the effect of LU on cell toxicity in fully differentiated Caco-2 cells, spontaneously differentiated to represent the normal enterocytes, and the undifferentiated Caco-2 cells, that represent the tumour model.

Differentiation in our cell model was monitored by a number of morphological and biochemical markers. Among which is the development of cell polarity with dome formation [Ramond *et al.*, 1985], a result of fluid transport by the cell monolayer, implying an establishment of the intercellular junctions, characteristic of epithelial cells. In addition, another well-known biochemical marker observed, is the cell-assembling brush border a characteristic of absorptive enterocytes related to an increase in the ALP activity a brush border membrane-associated hydrolases [Matsumoto *et al.*, 1990, Hauri *et al.*, 1985].

We further focused on SMases, a class of Cer-generating enzymes. We identified that the activities of both Alk-SMase and N-SMase are differentiation-related; indeed these activities significantly increased in differentiated cells, in parallel with alkaline phosphatase. As it concerns the Alk-SMase, our results are in agreement with the knowledge that differentiation might be a prerequisite for enterocytes to express a functionally active enzyme, due to its localization in the apical surface of the cells, and that this activity is reduced when the differentiation of enterocytes is defective, thus in the absence of the brush border. In fact, previous studies demonstrated a significant decrease of Alk-SMase activity in human colorectal adenomas, carcinomas, in familial adenomatous polyposis [Hertervig *et al.*, 1996 and 1999], and also in long-standing ulcerative mutation colitis [Sjöqvist *et al.*, 2002], thus supporting the increase of its activity in the differentiated healthy model and its lack in the tumour one. In addition, Duan and colleagues [Duan *et al.*, 2004] reported a mutation of Alk-

SMase in HT29 colon cancer cells. Altogether, the Alk-SMase appears as a novel marker of intestinal differentiation conferring to the intestinal cells the capacity to hydrolyze, and thus digest, dietary sphingomyelin. Furthermore, we found for the first time a significant increase of the N-SMase activity in the differentiated Caco-2 cells. This finding prompts us to hypothesize a key role of this enzyme in the intestinal differentiation. Despite the role of this enzyme in intestinal cells remains to be identified, it is worth noting that a study by our laboratory reported an increase in the activity of the N-SMase as a source to a Cer pool, which mediates the differentiation of Neuro2a cells [Riboni *et al.*, 1995]. Other studies reporting an increase in the activity of N-SMase enzyme with cell differentiation [Spence *et al.*, 1978] support this hypothesis.

Further experiments focused on the effect of Luteolin in the two cell models. Intriguingly, the results of these experiments demonstrated a differential susceptibility to LU-induced cell apoptosis, based on the differentiation degree of Caco-2 cells. Indeed, we found that when administered in the range 20-200  $\mu\text{M}$ , this polyphenol is able to exert an apoptotic and cytotoxic effect on colon cancer cells, but was ineffective in the normal counterpart. Notably, LU was used in the range of the known physiological concentrations ( $\leq 200 \mu\text{M}$ ), that are those reached by dietary polyphenols in the gastrointestinal tract [Scalbert *et al.*, 2000]. In addition, and of interest, we found that LU induces an increase of the intracellular Cer level, with a more pronounced trend in undifferentiated cells. Intriguingly, the endogenous basal level of Cer in differentiated cell was found two-fold higher than that of the cancer ones. This result supports a previous study showing a decrease in Cer content in colon cancer specimens with respect to normal colon mucosa obtained in the same patient [Selzner *et al.*, 2001]. Overall it appears that the tumour intestinal cells are equipped in maintaining a low Cer level, and are particularly sensitive to the apoptotic effect of Cer when an increase of their Cer content occurs, as that induced by LU.

Accumulating literature indicates that the bioactive SL Cer plays an important role in mediating apoptotic responses via various mechanisms in human cancer cells and that it is a major player in the mechanism of action of many chemotherapeutic drugs [Ogretmen *et al.*, 2004, Reynolds

*et al., 2004*]. As it concerns colon cancer, it was reported that Cer resulting from *de novo* pathway or catabolism modulation acts as a second messenger of the antitumor drugs camptothecin and homocamptothecin in HT29 cells [*D. Chauvier et al., 2002*]. Furthermore, supplementation of SM, the well-known Cer precursor, has been shown not only to reduce aberrant colonic crypts and adenocarcinomas in CF1 mice [*Dillehay et al., 1994, Schmelz, et al., 1996*], but also to enhance 5-fluorocil efficacy in colonic tumor xenografts [*Modrak et al., 2002*]. Based on these findings, we examined whether Cer is involved in the mechanism of LU-induced apoptosis. Notably we obtained that conditions leading to enhance the Cer content in colon cancer cells, thus mimicking the effect of LU on the endogenous Cer level, were successful in inducing cell apoptosis. In fact, the treatments with either exogenous short-chain Cer analogues (C2-Cer and C6-Cer) or an inhibitor of SM synthase (D609) were followed by the induction of cell death. This effect was found dose-depend, and exhibited characteristic features of apoptosis, including chromatin condensation and nuclear fragmentation. These results are consistent with previous findings reporting cell death in SW403 colon cancer cells, upon treatment with C2- and C6-ceramides and two inhibitors of Cdases, which are treatments inducing an accumulation of the intracellular ceramide [*Selzner et al., 2001*]. Altogether, our results point out for the first time the evidence that LU leads to apoptosis in colon cancer cells via a mechanism that involves Cer accumulation.

It is well documented that mechanisms utilized by some anti-cancer drugs result in increased generation and/or accumulation of endogenous Cer either via the activation of the *de novo* pathway, or by an increased activity of SMases [*Ogretmen et al., 2004*]. However, this appears not to be the case with LU. In fact, we report that in colon cancer cells, LU actually impairs the vesicular ER to Golgi transport of Cer, which results in its accumulation, parallel to the decrease of both SM and GSLs. Different lines of evidence supported this conclusion. First, by using short pulse experimental approaches with tritiated Sph as Cer precursor, the pathway responsible for the LU-dependent Cer increase was found to involve a reduced utilization of Cer for the biosynthesis of complex SLs, i.e. SM and GSLs. A crucial step in the biosynthesis of complex SLs is the transport of Cer from ER,



where the enzymes of Cer biosynthesis are localized, to the Golgi complex and where consequently the biosynthesis of SM and GlcCer occurs. Several pieces of evidence, obtained in both mammalian cells and yeast [Funato *et al.*, 2001, Viani *et al.*, 2003], support that besides the CERT-mediated transport that exclusively acts on SM biosynthetic process, neo-synthesized Cer in the ER appears to move to Golgi, through a biosynthetic vesicular route functional to Cer utilization for both SM and GlcCer biosynthesis. In agreement, the formation of transport vesicles involved in anterograde, as well as retrograde trafficking was extensively studied at the molecular level [Aridor and Balch *et al.*, 1996, Bednarek *et al.*, 1996]. The concomitant decrease of both SM and GSLs in LU-treated cells prompted us to hypothesize that LU most probably acts by reducing the availability of Cer as a substrate for both SM-synthase and GlcCer-synthase enzymes in the Golgi complex, possibly by inducing a defect in the common vesicular route responsible for complex SL biosynthetic processes. Very convincing support to this hypothesis was obtained by the intracellular distribution of BODIPY-C<sub>5</sub>-Cer, a fluorescent probe able to mimic the ER-Golgi trafficking of Cer in cells [Ktistakis *et al.*, 1995, Pagano *et al.*, 2007]. Indeed, a significant ER deposit of fluorescence during LU treatment was observed, whereas in control cells BODIPY-C<sub>5</sub>-CER fluorescence exited from the ER to accumulate in the Golgi. Furthermore, the treatment with BFA, a fungal macrocyclic lactone known to induce a retrograde merging of the *cis*-Golgi membranes with the ER [Lippincott *et al.*, 1989; Klausner *et al.*, 1992], rendered Cer metabolism insensitive to the LU treatment, thus definitely demonstrating that LU is able to impair the ER-Golgi translocation of Cer. In addition, the results obtained with BFA demonstrated that the Golgi ultrastructure was not altered in LU-treated cells. This is in agreement with some findings that reported little perturbation of the Golgi under conditions of Cer accumulation [Maceyka *et al.*, 1997], whereas another study suggests that Sph, generated from the hydrolysis of ceramide, causes Golgi fragmentation [Hu *et al.*, 2005]. We can also conclude that the *cis*-Golgi represents the major subcellular site for both GlcCer and SM biosynthesis in colon cancer cells, as occurs in many cell types [Schweizer *et al.*, 1994]. Taken together, these results demonstrate that LU is able to modulate the intracellular traffic of Cer between the ER and the Golgi apparatus.

Consequently, newly synthesized Cer accumulates in the ER, and this appears to act as a mediator of the apoptotic effect of LU in colon cancer cells.

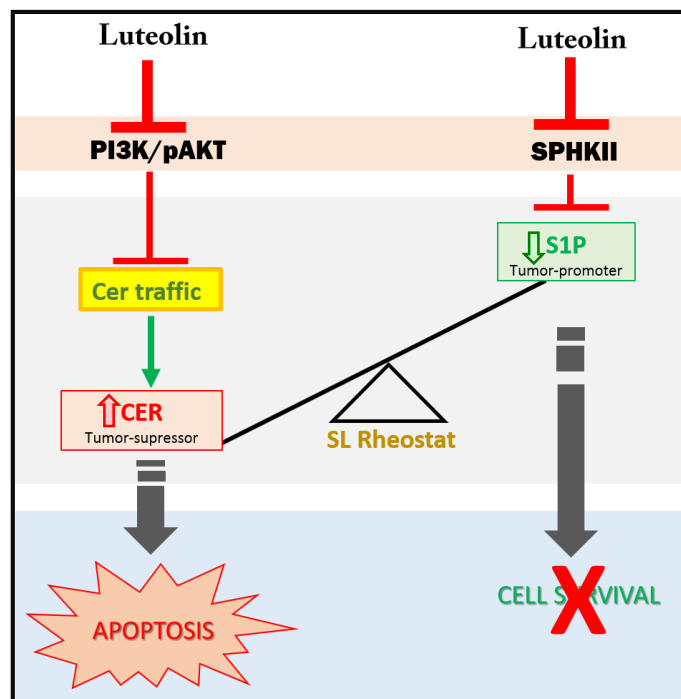
Protein phosphorylation, known to modulate many membrane traffic processes throughout the cells, has also been implicated in the transport between the ER and Golgi, and different protein kinases, including Akt, have been shown to regulate ER to Golgi traffic [Du *et al.*, 2006]. In addition, a study in our laboratory reported a putative role of PI3K/Akt pathway in the regulation of the vesicle-mediated movements of Cer in the ER-Golgi district; in C6 glioma cells. Infact, it was observed that inhibition of PI3K, and consequently of Akt, strongly induced Cer accumulation, and this was accompanied by a decrease in the biosynthesis of complex SLs [Giussani *et al.*, 2009]. Prompted by these findings, and aiming to examine whether PI3K/Akt exhibits the same effects on Cer metabolism on colon cancer cells too, LY294002, a well-recognized PI3K inhibitor that blocks ATP binding to the p110 $\alpha$  PI3K catalytic domain [Vlahos *et al.*, 1994], was administered to Caco2 cells. The results demonstrated that LY294002 is able to exert an impairment in the Cer metabolism in tumoural cells, thus mimicking the effect observed after LU treatment. Interestingly, we obtained that in our cell model LU acts as a potent inhibitor of Akt phosphorylation, as a downstream response of PI3K inhibition. In this context it is interesting to note that Quercetin, the lead compound on which LY294002 was identified as PI3K inhibitor [Vlahos *et al.*, 1994], possesses the same chemical core structure of some flavonoids, including LU, and is able to inhibit PI3K activity by binding to its ATP binding pocket [Matter *et al.*, 1992]. This result supports previous studies that showed the inhibition of PI3K, and down-regulation of its downstream component pAkt, as the primary target of LU in colon cancer cells [Lim *et al.*, 2012]. Thus, from this set of data, it emerges that the inhibition of PI3K by LU, and subsequently the down-regulation of the PI3K/Akt pathway, represents one of the mechanisms responsible for the effect of this flavonoid on Cer trafficking in our cancer cell model.

Despite Cer is anti-proliferative, S1P is its well-recognized counter-player. Indeed S1P has been implicated in the promotion of cellular proliferation and survival [Spiegel *et al.*, 2002]. Importantly, our data showed that the exposure of Caco-2 cancer cell to the toxic dose of LU not only

increases the intracellular level of Cer, but also decreases the endogenous S1P level, inducing thereby a shift of the “SL balance” to the side of Cer, thus promoting cell death. SPHKs have been reported as key regulating factors in the sphingolipid rheostat, able to modulate the balance of proliferative and apoptotic signals through the conversion of Cer and Sph to S1P [*Hait et al., 2006*]. Of relevance, we demonstrated for the first time that LU was able to act as inhibitor of SPHK activity in colon cancer cells, especially SPHKII, the up-regulated isoform in our cancer cell model, exhibiting only a modest effect on SPHKI. Thus, it emerges an additional, relevant effect of LU in Caco-2 cells that is a potent inhibition of SPHKII activity, resulting in a decreased S1P content.

In order to gain deeper insights into the role of the “SL balance” in mediating the effect of LU, we attempted to push the balance between Cer and S1P towards S1P by adding S1P to colon cancer cells. The results demonstrated that S1P conferred a significant resistance of colon cancer cells to the cytotoxic effect of LU. The mechanism by which S1P acts as LU-antagonist was proved to be mainly by the up-regulation of the PI3K/Akt pathway, which is able to rescue colon cancer cells from the apoptotic effect of the LU-induced increase of Cer.

To sum up, as shown in **Fig. 30**, the results of this study provide novel informations about LU as an antitumour agent in colon cancer cells, and the mechanisms underlying its apoptotic effect on colon cancer cells. These mechanisms involve two major targets that converge on the “SL rheostat” shifting it to the side of death. First, our data demonstrate LU as an efficient inhibitor of PI3K. This effect leads to a down-regulation of PI3K/Akt activity, which on its turn impairs Cer traffic. As consequence, endogenous Cer accumulated, and acts as mediator of the apoptotic effect of LU. Furthermore, we identified a further target of LU. Indeed we obtained evidence that LU can act as a potent inhibitor of SPHKII, and this results in the decrease of endogenous S1P, favoring the SL balance to orient toward the cell death effect. In addition, we revealed that the effect of LU on Cer and S1P are strictly interconnected. Indeed, we found that S1P is able to promote cell survival by activating the PI3K/Akt signaling pathway, thus antagonizing the LU effect.



**Figure 30.** *Schematic representation of the molecular mechanisms underlying the pro-apoptotic action of Luteolin in colon cancer cells.*

Taken together, the results of this research demonstrate, for the first time, that the dietary natural flavonoid LU is able to induce apoptosis in colon cancer cells by targeting the “SL rheostat”, and directing the balance in favor of Cer. As the balance between Cer and S1P appears to be an important target for development of new and effective therapeutic strategies against tumor progression, LU could be a novel antitumoural, natural agent not only in colon cancer but also, possibly in the treatment of other solid tumours.

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