Dissertation

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THE EFFECT OF SPECIFIC PHOSPHOLIPIDS THAT MAY HAVE A POSSIBLE ROLE IN PROPAGATING OR INHIBITING GLIOMA

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(BSc Hons)

A thesis submitted in partial fulfilment for the degree of MA (by Research) at the University of Central Lancashire

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DECLARATION

I declare that whilst registered as a candidate for the degree for which this submission is made, I have not been registered candidate for another award by any other awarding body. No material contained in this thesis has been used in any other submission for and academic award.

Signed

ABSTRACT

Cancer is one of the major causes of death in spite of a substantial increase in understanding of the molecular mechanism behind its occurrence. Glioma is the type of brain cancer which arises in the glial cells of the brain. Glioma is categorized into three areas namely: astrocytoma, oligodendroglioma and astro-oligodendroglioma (mixture of both). In our day-to-day diet, the public consume phospholipids from various sources of food products including egg, milk, soybean, offal and some vegetables. Environmental factors such as food habits could also be contributing factors of glioma. This study was designed to investigate the possible effects of dietary phospholipids in either proliferating or inhibiting the growth of glioma. In this study, the soy phospholipids, Lipoid S-100, Phospholipon ® 90H and L-α-phosphatidylcholine were tested individually on three different glioma cell lines namely 1321N1, GOS-3 and U87-MG.

Tissue culture techniques were employed to measure the activity of each phospholipid by *in vitro* studies. The ATP release by 1321N1, GOS-3 or U87-MG cell line treated with each soy derived phospholipid was measured after 48 hrs of incubation. On measurement using the ATP assay, the results obtained from 1321N1 and GOS-3 cell lines showed significant ($P < 0.05$) increases in growth on the treatment with either Lipoid S-100, Phospholipon ® 90H or L-α-phosphatidylcholine when compared with untreated cells and treated cells with 0.002% isopropyl alcohol (IPA). In contrast, Phospholipon ® 90H was found to enhance the growth of 1321N1 and GOS-3 cell lines and this effect was significantly ($P < 0.05$) larger when compared to the effects of Lipoid S-100 and L-α-phosphatidylcholine. Treatment of cells with either Lipoid S-100 or Phospholipon ® 90H showed a significant ($P < 0.05$) decrease in the growth of U87-MG cell line when compared with untreated cells and cells treated with 0.002% IPA. Following treatment with L-α-phosphatidylcholine, no effect on the growth of U87-MG cell line was observed when

compared with either untreated cells or 0.002% IPA treated cells. There was also a significant inhibition when compared with untreated cells. These results have indicated that soy derived phospholipids can enhance the growth of the low grade astrocytoma 1321N1 and GOS-3 cell lines and they do not support the growth of high grade glioblastoma U87-MG. Further experiments are required to determine the mechanism of action of soy derived phospholipids in either proliferating or inhibiting cancer cells.

Index

LIST OF ABBREVIATIONS

ECACC	European Collection of Cell Cultures
ATP	Adenosine triphosphate
FBS	Fetal Bovine Serum
DMSO	Dimethyl Sulfoxide
EMEM	Eagle's Minimal Essential Medium
DMEM	Dulbecco's Modified Eagle Medium
μM	micro molar
mM	milli molar
IPA	Isopropyl alcohol
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
PL	Phospholipids
LPL	Lysophospholipids
PIP	Phosphatidylinositol phosphate
PIP$_2$	Phosphatidylinositol bi phosphate
PIP$_3$	Phosphatidylinositol tri phosphate
SPSS	Statistical Package for the Social Sciences
SAP	Serine, amide and phosphate
SAA	Serine, amide and alcohol
SM	Sphingomyelin
NADPH	Nucleotide,adenosine, dinucleotide phosphate

ACKNOWLEDEGEMENT

1. Introduction

Currently, cancer is the major cause of death in spite of the substantial increases in understanding the molecular mechanisms behind its occurrence. Cancer is classified as a disease that occurs because of the uncontrolled division of cells and the ability of these cells to invade other normal tissues, either by direct growth into adjacent tissue (invasion) or by implantation into distant sites (metastasis) (Souhami *et al.*, 2005) . Glioma is the most common type of brain tumour and it is typically categorized as either primary or secondary. Primary tumours start in the brain, whereas secondary tumours spread to the brain from another site such as the breast or lung. The incidence of glioma has increased since the late 1970's. Every year in the U.K about 2% of new cases of glioma are diagnosed. According to the American Cancer Society in the USA, the estimated number of new cases of primary CNS malignant disorders was 16,500, with an estimated deaths of 13,000 in the year 2000 (Greenlee *et al.,* 2000). Approximately 7000 cases of malignant brain tumours are newly diagnosed every year in the USA (Kimmel *et al.,* 1987). The World Health Organisation (WHO) has classified gliomas as astrocytomas, oligodendrogliomas, and oligoastrocytomas (Table 1.1) on the basis of the phenotypical resemblance of tumour cells to normal glial cells (astrocytes or oligodendrocytes, or a combination of both). Astrocytomas are histologically graded as low grade (WHO I and II) and high grade tumours that are classified as anaplastic astrocytoma (WHO III) and glioblastoma multiforme (GBM) (WHO IV) being more malignant and aggressive (Kleihues *et al.,* 1995) (Table 1.1).

I. Astrocytomas are named for the cells where they originated from the astrocytes. These tumours can either show clear borders between normal brain tissue and the tumour (called focal) or no clear border (called diffuse). Focal astrocytomas, more common in children, are not often found in adults. Astrocytoma is the most frequent CNS tumour in people with

the Li-Fraumeni syndrome (germline mutation of the *p53* tumor suppressor gene on the short arm of chromosome 17) (MacDonald, 2002).

II. Ependymomas begin in cells called ependymal cells that are found lining certain areas of the brain and spinal cord. These cells help to repair damaged nerve tissues. They usually occur in children and young adults (Massimino, 2004).

III. Oligodendrogliomas form in oligodendrocyte cells, which produce a fatty substance called myelin that protects the nerve. More common in adults, these tumours may move to other parts of the brain or spinal cord (Uddin, 2009)

Cell lines used in the study			
Cell lines	**Diagnosis**	**Grade**	**Company**
1. 1321N1	Astrocytoma	II	ECACC,UK
2. U87-MG	Glioblastoma astrocytoma	IV	ECACC, UK
3. GOS-3	Mixed astro-oligodendroglioma	II/III	DSM2, Germany

Table 1.1: Types of Glioma cell lines used in the study with grades and company which supply them.

Although there are a number of external causes of glioma, some tumours are attributed to hereditary. The environmental factors such as diet, radiation and chemical pollution are major contributors to cancer in general. Recently, it has become clear that either non-specific drugs or anticancer drugs are able to induce apoptosis and this process is also involved in the mediation of their cytotoxic effects (Ceruti *et al.,* 2005).The regulation of apoptosis is carried out by two biochemical pathway that are extrinsic and intrinsic (Ferreira *et al.,* 2002). Some

anticancer drugs which cause cell death and apoptosis become ineffective (i.e. the cancer cells become resistant) and may even hamper the intrinsic biochemical pathway of apoptosis (Ceruti *et al.,* 2005). One area that is generally overlooked is the area of diet and nutrients as possible contributors to cancers. Epidemiology studies have suggested that diet might be important in the etiology of human cancer (Punnonen *et al.*, 1989). A past study has shown that if dietary fat intake is increased it could lead to the development of breast cancer (Punnonen *et al.*, 1989). According to the Bartch commentary in the year 1999, current evidence from experimental and human studies has summarized that the high intake of ώ-6 (Polyunsaturated fatty acid) such as linoleic acid (18:2) enhances tumorigenesis and metastasis, leading to the development of cancers such as breast, prostate and colon cancers. In contrast, ώ-3 PUFA and monounsaturated fatty acid ώ-9 employing the oleic residue have been shown to inhibit the growth of initiated cancer cells. It is therefore important to investigate whether certain dietary phospholipids can either enhance or inhibit cancer cell growth and in particular, glioma cancer cells (Bartch, 1999).

1.1 Tissue culture to study glioma growth

Tissue culture is the growth of tissue or a cell on glass or plastic surface separated from the organism or *in vitro* growth in nutrient medium typically facilitated via use of liquid (growth media) or semisolid growth media such as broth or agar. The flask or plate containing the culture is incubated, usually close to tissue normal environment. Sterile conditions are maintained to prevent contamination. Tissue culture commonly refers to the culture of animal cells and tissues (Carrel, 1992). In general, there are two types of cell culture which employ similar practical sterile techniques (Freshney, 2000)

a. **Primary cell culture** – The growth of tissue is derived from the living organisms (e.g. biopsy material). The culture consists of mixed population of cell types. Frequently, some of the cells may survive without proliferating and will therefore be

lost in the increasing population of those which are able to multiply in the conditions provided *in vitro* (Maureen, 1997).

b. **Established Cell lines-** These cells are derived from a primary culture and they can be sub- cultured in the medium indefinitely *in vitro* (Michael, 1998).

1.1.1 Media importance in tissue culture

A liquid medium is required to support the growth of the cells in tissue culture. A minute sample of tissue is either spread on or added to a culture biological medium (e.g. blood serum or tissue extract), either synthetic, or mixed origin and having the appropriate nutrients, temperature and pH for the cells for incubation. The media used for glioma cell culture are Dulbecco's Modified Eagle Medium (DMEM-500 ml) and Eagle's Minimal Essential Medium (EMEM-500ml).

1.1.2 Dulbecco's Modified Eagle Medium (DMEM) used for proliferation of cells

Dulbecco's Modified Eagle Medium (DMEM) is a basal medium that consists of vitamins, amino acids, salts, glucose and a pH indicator. DMEM is stored at 2-8°C and contains no proteins or growth promoting agents. Therefore, it requires supplementation to be a "complete" medium. It is most commonly supplemented with 5-10% Fetal Bovine Serum (FBS). Fetal bovine serum is the most widely used growth supplement for cell culture media because of its high content of embryonic growth promoting factors. When used at appropriate concentrations, it supplies many defined and undefined components that have been shown to satisfy specific metabolic requirements for the culture of cells *in vitro*. DMEM utilizes a sodium bicarbonate buffer system (3.7 g/L) and therefore requires artificial levels of CO_2 to maintain the required pH. A carbon dioxide (CO_2) level around 7-10% is optimal for incubation but many researchers can manage to successfully incubate at as low as 5% CO_2 level. A potential problem with too low CO_2 level is that the pH may

become too high. When exposed to ambient levels of CO_2, the sodium bicarbonate in the medium may cause DMEM to become basic very rapidly (Dulbecco *et al.,* 1959).

1.1.3 Eagle's Minimal Essential Medium (EMEM) used for the proliferation of cells

Eagle's Minimum Essential Medium (EMEM), developed by Harry Eagle, is one of the most widely used of all synthetic cell culture media for the cultivation of mammalian cells. EMEM has been extensively used for growing a wide variety of cells in mono-layers. It is stored at 15-30°C. The original EMEM formulation contained Earle's salts and a group of amino acids are generally referred to as essential amino acids and non-essential amino-acid. They are considered essential because in their absence, cells would not grow properly due to lack of nourishment provided. The 12 essential amino acids which may be used for the development of the medium includes L-arginine, L-cystine, L-glutamine, L-histidine, L-isoleucine, L-leucine; L-methionine, L-phenylalanine, L-threonine, L-tryptophan, L-tyrosine, and L-valine. Optional supplementation of non-essential amino acids to the formulations that incorporate either Hanks' or Eagles' salts has broadened the usefulness of this medium. The 12 non- essential amino acid optional supplementation which are useful for the growth of cells are Alanine, Cysteine, Cystine, Glutamine, Glutathione, Glycine, Histidine, Serine, Taurine, Threonine, Asparagine, Apartic Acid and Proline. EMEM contains higher concentrations of amino acids so that the medium is more similar to the protein composition of mammalian cells. EMEM also, like DMEM, requires FBS supplementation for providing growth promoting factor. Moreover, EMEM requires sodium pyruvate as a supplementation for the growth of cells since pyruvate is an intermediary organic acid metabolite in glycolysis and the first of the Embden Myerhoff pathway that can pass readily into or out of the cell. Thus, its addition to tissue culture medium provides both an energy source and a carbon skeleton for anabolic processes. Its addition may also

help in maintaining certain specialized cells and in cloning and may be necessary when the serum concentration is reduced in the medium (Culture of Animal Cells).

A variation of this EMEM is called DMEM and it contains four times higher vitamins, glucose and amino-acid which are present in the original formula. Additionally, it contains iron and phenol red. DMEM is suitable for most types of cells, including human, monkey, hamster, rat, mouse, fish and chicken cell lines (Ham *et al.,* 1979).

1.1.4 Example of composition of medium for three glioma cell lines used in the study (Figure 1.1)

    (i) **GOS-3** (Figure 1.1)**-**Dulbecco's Modified Eagle Medium (DMEM-500 ml), 10% Foetal bovine serum (FBS-50 ml), 4 mM L-glutamine (10 ml).

    (ii) **1321N1** (Figure 1.1) **-** Dulbecco's Modified Eagle Medium (DMEM-500 ml)**,** 10% Foetal bovine serum (FBS-50 ml)**,** 2 mM L-glutamine (5 ml).

    (iii) **U87-MG** (Figure 1.1)– Eagle's Minimal Essential Medium (EMEM-500ml), 10% Foetal bovine serum (FBS-50 ml), 2 mM L-glutamine (5 ml),1% Non Essential Amino Acids (NEAA- 5 ml), 1 mM Sodium Pyruvate (5 ml).
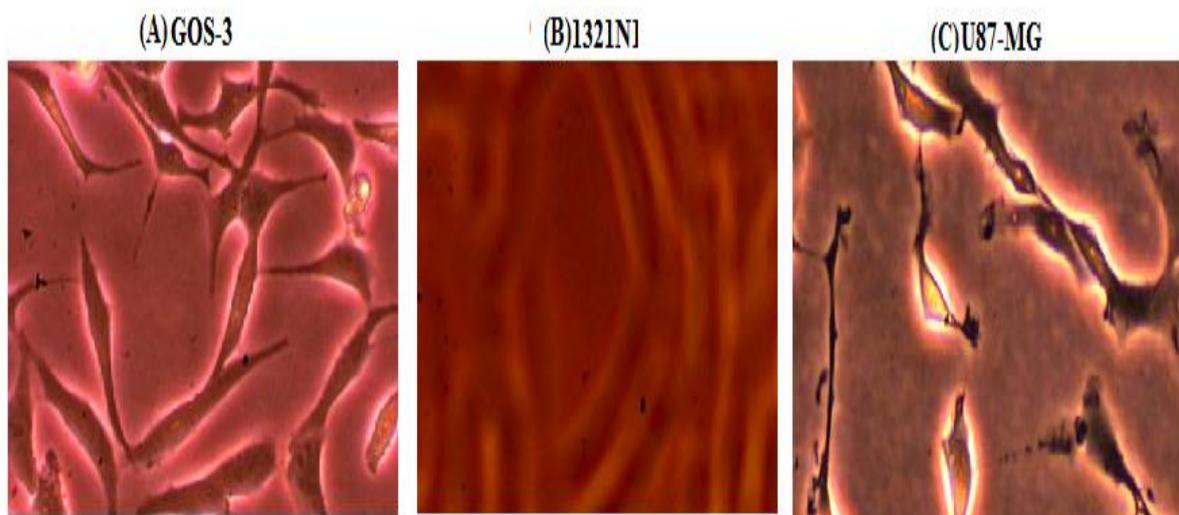
Figure 1.1: Photographs showing healthy morphological structures of (A) GOS-3 and (B) 1321N1 cell line in Dulbecco's Modified Eagle Medium (DMEM) and (C) U87-MG cell line cultured in Eagle's Minimal Essential Medium (EMEM) media.

The results are observed by a microscope, sometimes after treatment (e.g., staining) to highlight particular features. This is used to study normal and abnormal cell structure, biochemical, genetic, reproductive activity, metabolism and reactions to physical chemical and biological agents (e.g., drugs, viruses). A number of viruses are also grown in tissue cultures. The use of tissue culture technique has helped in identifying infections, enzyme deficiencies and chromosomal abnormalities as well as type of brain tumours and drug testing.

1.2 Cell viability assay to identify proliferation or inhibition of glioma cells.

Cell viability assay is an important biochemical tool in oncological research and in clinical practice to assess the tumour cell sensitivity. MTT and ATP are the assays most commonly used to identify cell viability.

1.2.1 MTT ASSAY

MTT assay is the most popular colorimetric assay in which the compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide is converted by mitochondrial

enzymes to a coloured insoluble formazan product, which is solubilised in alcohol or detergent followed by absorption measurement. The amount of colour produced is proportional to the number of live cells. This assay has been used by many investigators as a reliable method of chemosensitivity testing in malignant gliomas (Ferrari, *et al.,* 1990).

1.2.2 ATP ASSAY

The ATP assay is based on the principle of bioluminescence and measures the cellular ATP in the living cell. The cell titer glo ® luminescent assay is an ATP assay designed to measure proliferation and cytotoxicity assays. The concentration of ATP decreases as the cell undergoes either necrosis or apoptosis (Crouch, 1993). ATP assay works by the interaction of the substrate luciferin and ATP in the presence of an enzyme called luciferase which produces light. Luciferase enzyme is a high sensitivity bio detector. Its light emitting assay is very fast and accurate and rapid results are usually obtained. Luciferase functions as a biochemical signal transducer. It has a single polypeptide chain which is extracted from Photinus Pyralis and it is considered responsible for the yellow green bioluminescence of the species. The single polypeptide luciferase chain catalyzes the monooxygenation of the compound beetle luciferin (LH_2) in an ATP-dependent fashion to give oxyluciferin (OL) as shown in Figure 1.2.
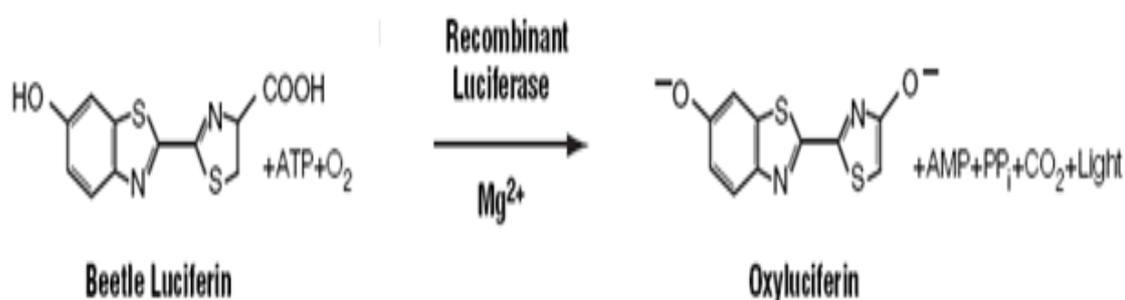
Figure 1.2: Mono-oxygenation of luciferin is catalyzed by luciferase in the presence of $Mg^{2+,}$ ATP and molecular oxygen. (Adapted from- http://www.promega.com/tbs/tb288/tb288.pdf)

The OL in the excited state might decay to its ground state by releasing a photon. The emitted photon is measured quantitatively in a luminometer. The luminescence which is obtained is directly proportional to the amount of ATP and hence the number of cells (Tisi, 2002). Luciferase is widely used as a research tool in molecular biology analysis, medicine, public health and food hygiene. The quantity of ATP in cells correlates with cell viability. The CellTiter-Glo® Reagent initiates three processes when added to cells. It lyses the cell membrane to release ATP which inhibits endogenous ATPases. This provides luciferin and luciferase which are necessary for the measurement of ATP using a bioluminescent reaction. The glow type luminescence in this assay is obtained by slow inhibition of the luciferase reaction, while at the same time stabilising the ATP, giving a constant light output which lasts for 3–5 hours (Personal Communication from Promega).

However, it must be borne in mind that there are a variety of factors known to affect ATP levels in cells (Stanley, 1986). These include:

- change of growth rate

- change of nutrients or their concentrations

- change of oxygen tension

- change of temperature

- change of pressure

- age of cells or stage of growth including stage of cell division

- density of cells

- action of agents which change cell type (tumour promoting agents)

- heavy metals and radiation

1.2.3 Comparison of the MTT and ATP assays for finding the proliferation of the cells.

MTT is a colorimetric assay and ATP is based on bioluminescence. ATP can detect 1000-2000 cells per well (in 96 well plates) whereas MTT assay cannot detect cells less than 25000 (Petty, 1995). The formazan product of MTT reduction is a crystalline precipitate that requires an additional step in the procedure to dissolve the crystals before recording absorbance readings at 570 nm. ATP assay does not show any crystal formation before measuring it luminescence. MTT is time consuming assay which requires around 2 hrs of incubation after the addition of MTT reagent. By contrast, ATP assay is not time consuming and it detects the ATP release of live cells immediately after 15 minutes incubation of plate at room temperature after addition of ATP reagent. Thus, ATP assay is a better method of quantifying cell viability.

1.3 Phospholipids

Phospholipids are part of all cell membranes of living organisms. Phospholipids play an important role in signal transduction mechanism and are arranged according to fluid mosaic pattern in biological membrane. Phospholipids are heterogeneous molecules and contain phosphoric residues, polar head groups and non-polar lipid chains (Kane, 1993) (Figure 1.3.). The polar head group contains phosphate groups, glycerol backbone, choline, ethanolamine, inositol, and hydrogen, as the head group with different chemical properties. When phospholipid molecules are placed in water, their hydrophilic heads tend to face the aqueous phase and the hydrophobic tails are forced to stick together and form a bilayer.
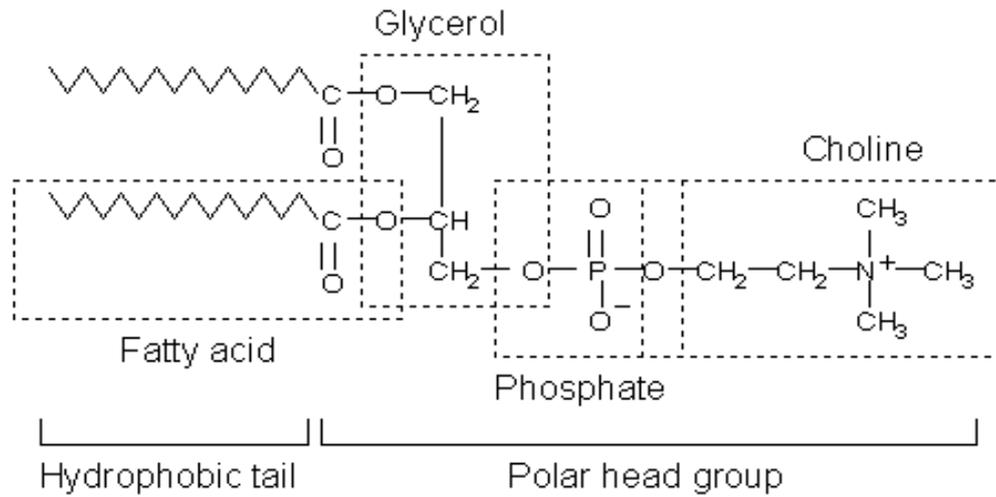
Figure 1.3: Chemical structure of a phospholipid (phosphatidylcholine).

The quantity of phospholipids in diet can help find a possible link between cancer and phospholipids. It can also help in understanding whether the dietary phospholipids would inhibit or encourage the growth of cancer cell lines. Epidemiological studies have suggested that diet may be important in both the etiology and treatment of human cancers (Punnonen *et al.,* 1989).

There are different types of phospholipids

Phosphoglycerides (PGs)

Sphingomyelin (SM)

1. Phosphatidic acid (PA)

2. Phosphatidylserine (PS)

3. Phosphatidylethanolamine (PE)

4. Phosphatidylcholine (PC)

5. Phosphatidylinositol (PI)

PGs are further subdivided into choline and non-choline group

(a) Choline group-

(1) Phosphotidylcholine-glycerol backbone

(2) Sphingomyelin- serine backbone

(b) Non-choline group-

(1) Phosphatidylserine

(2) Phosphatidylethanolamine

(3) Phosphatidylinositol

Glycerol backbone

Table 1.2: Types and subdivisions of phospholipids forming the major component of the biological membrane (Wehrmüller, 2008)

1.3.1 Fluid mosaic model of the structure of cell membrane phospholipids.

The entire living organism is made up of cell membranes. Cell membrane is made up of phospholipid bilayers arranged in a fluid mosaic pattern. A fluid mosaic model is presented for the gross organization and structure of the proteins and lipids of biological membranes. The cell membrane separates the interior of a cell from the outside environment. Fluid mosaic model was proposed in the early 1970's (Singer and Nicholson 1972). One of the most important features of this model is the idea that the phospholipid bilayer is fluid. The polar heads are directed outward and covered by at least one monolayer of non-lipid molecules on each side. The non-polar tails are directed inward (Figure 1.4) and the phospholipid molecules are free to move laterally.
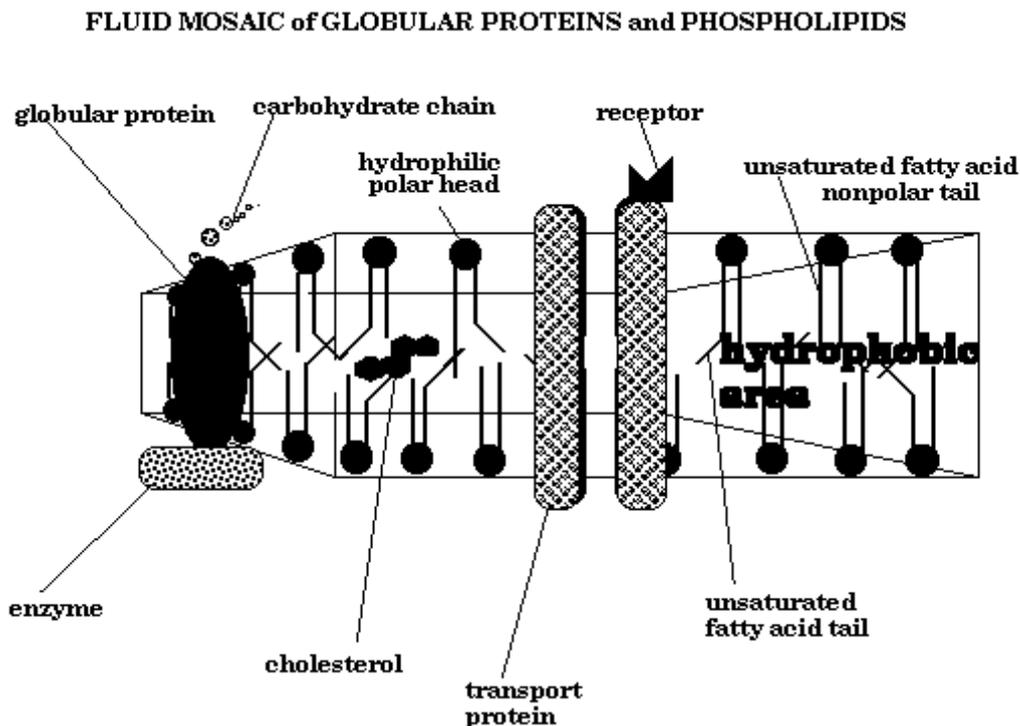
Figure 1.4: Fluid mosaic of globular proteins and phospholipid. (Adapted from-facstaff.gpc.edu/~jaliff/anacell.html)

In relation to the lateral movement of the phospholipid molecules, there is very little exchange between the two halves of the membrane bilayer (Figure 1.4). This minimal exchange, or flip flop action of the non-polar tail, allows asymmetric distribution of phospholipids.

Membrane surfaces exhibit different structural and functional characteristics on their outer and inner sides. One of the most important functions of the outer surface of the membrane lies in its interaction and communication with other cells. This is often achieved by sugar molecules present on the outer surface that act as distinguishing markers for the cell, whereas, on the other hand, the interior surface has a different composition of sugar, fats, phospholipids and protein present. Thus, the interior surface is involved in different functions. The fatty acids of the outer half of the bilayer tend to have longer, more saturated carbon chains than those of the inner half. In this model, the membrane is a mosaic of proteins embedded in a fluid phospholipid bilayer. The hydrophilic portions of the phospholipid and proteins are maximally exposed to the aqueous interface which ensures membrane stability. The fluidity of the molecule is affected by several factors. These include the type of lipid found in the membrane and the degree of unsaturation in the fatty acid chains of membrane lipids. The presence of a *cis* double bond introduces a kink into the fatty acid chain, which affects the packing of the phospholipid bilayer. The kink prevents the phospholipid molecules from being packed together too tightly, and thus contributes to the membrane fluidity. It is important to understand that in this model, both the membrane lipids and the embedded proteins are free to move. They may be mobile or fluid in nature (Nelson *et al.,* 2005)

1.3.2 Polar head groups of phospholipids

Most phospholipid head groups belong to the phosphoglycerides family, which contain glycerol joining the head and the tail. Examples of phosphoglycerides include phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylinositol (PI). They are hydrophilic (water soluble) in nature (Figure 1.4) (Wehrmuller, 2008).

1.3.3 Non-polar tail groups of phospholipids

The non-polar tail contains two fatty-acyl chains. The fatty-acyl chains are hydrophobic (water hating) in nature (Figure 1.4). The fatty-acyl chain in bio-membranes usually consisting of even numbers of carbon atoms. They may be saturated (neighbouring carbon atoms are all connected by single bonds) or unsaturated (some neighbouring carbon atoms are connected by double bonds). This antiproliferative and pro-apoptotic activity was observed on glioma cells and tissues when C18 and C20 unsaturated fatty acids like omega-3 and omega-6 were used but not when C18 and C20 saturated and monounsaturated fatty acid were used (Leaver, et *al.,* 2002).

1.3.4 Structure of saturated fatty acids and their effect on apoptosis

Saturated fatty acids have no double bonds between the carbon atoms of the fatty acid chain (Table 1.3) and are thus fully saturated with hydrogen atoms. Saturated fat is present in the dairy products, animal fats, and it is also found in trace amounts in other food products such as cotton seed oil, palm kernel oil, coconut oil, soybean oil. It is also present in some prepared foods. The saturated fatty acid palmitic has been reported to induce apoptosis of breast cancer by decreasing the mitochondrial membrane potential and causing cytochrome *c* release. Palmitate-induced breast cancer apoptosis was enhanced by the fatty acid

oxidation inhibitor etomoxir, whereas, it was reduced by fatty-acyl CoA synthase inhibitor triacsin C (Hardy et *al.*, 2003)**.**

1.3.5 Structure of unsaturated fatty acids and their effects on all signalling

Unsaturated fats have double bonds between the carbon atoms of the fatty acid chain. Unsaturated fats include monounsaturated fat (single double bond) and polyunsaturated fat (two or more double bonds) (Table 1.3). Both mono and poly unsaturated fats are predominantly present in plant products. Examples of polyunsaturated fat food sources include soybean, sunflower, fish and corn oil. Monounsaturated fat is found in high content in olives, peanuts, and canola oil. The highly unsaturated fatty acids Omega-6 and Omega-3 are involved in cell signalling in normal and transformed cells and have recently been associated with the pathways leading to tumour death due to arachidonic acid (AA) stimulating the activation of endonuleases (Leaver, *et al.,* 2002). The PUFA (arachidonic acid, gamma linolenic acid and eicosapentaenoic acid) were previously studied on glioma cells and other cancerous tissue and have been demonstrated to limit the growth of the cancer cells *in vitro* and *in vivo* (Leaver, *et al.,* 2002; Willams, J.R. *et al.,* 1998).

1.3.6 Omega – 3 (ώ-3)

The term *n−3* (also called *ω−3* or omega-3) (Table 1.3) signifies that the first double bond exists as the third carbon-carbon bond from the terminal methyl end (n) of the carbon chain. The n−3 fatty acids which are important in human nutrition include α-linolenic acid (18:3, n−3; ALA), eicosapentaenoic acid (20:5, n−3; EPA), and docosahexaenoic acid (22:6, n−3; DHA). The nutritional value of n-3 polyunsaturated fatty acids (PUFA) in the human diet is well recognised, and increased consumption of these fatty acids have been recommended (Department of Health, 1994). Several studies, however, have reported

possible anti-cancer effects of $n-3$ fatty acids particularly on breast, colon and prostate cancer (Augustsson, *et al.,* 2003; De Deckere, 1999; Caygill, et al., 1995)

1.3.7 Omega – 6(ώ-6)

The $n-6$ (also called $\omega-6$ or omega-6) (Table 1.3) signifies the family of unsaturated fatty acids which have carbon-carbon double bond in the n-6 position. The epidemiology and biochemical evidence indicates that n-6 essential fatty acid regulates tumour development (Leaver *et al.,* 2002). Many vegetable oils contain high levels of omega-6 PUFAs (Isabelle *et al.,* 2007). Some evidence suggests that cyclooxygenase inhibitors, which block the metabolism of omega-6 PUFAs, are beneficial in the prevention of colon cancer (Giardiello *et al.*, 1993) and prostate cancer (Jacobs *et al.*, 2005). The fatty acid composition of human gliomas differs from that found in non-malignant brain tissues. The content of omega-6 PUFA linoleic acid was found to be higher in glioma compared to that observed in the control samples in terms of total lipids (Martin, *et al.,* 1996). There is much experimental evidence that supports omega-6 fatty acids via eicosanoid production which may enhance breast cancer invasion and metastasis (David, 1997).

1.3.8 Omega – 9(ώ-9)

The $n-9$ fatty acids (popularly referred to as $\omega-9$ (Table 1.3) fatty acids or omega-9 fatty acids) are a family of unsaturated fatty acids which have in common a final carbon–carbon double bond in the $n-9$ position; that is, the ninth bond from the end of the fatty acid chain. They are also referred to as monounsaturated fatty acids. Oleic acid is an example of monounsaturated fatty acids ($\omega-9$). The n-9 fatty acids present in olive oil may reduce the risk of breast cancer occurrence by mechanisms that involve modification of the biosynthesis of eicosanoids from n-6 polyunsaturated fatty acids (David, 1997).

1.3.9 Fatty acid action through blood brain barrier.

The developing mammalian brain requires fatty acids, especially essential fatty acids (Dhopeshwarkar, 1973). The brain synthesizes certain linear monocarboxylic fatty acids from acetate and/or shorter fatty acids via which essential and non-essential fatty acids can enter the blood brain barrier (Dhopeshwarkar, 1973). Linear monocarboxylic fatty acids are classified as 2-5 carbons (short), 6-10 carbons (medium), or more than 10 carbons (long). Fatty acid composition of tri-glycerides and phospholipids are not readily transported to the cerebral capillaries, which are joined by tight junctions and from the anatomical basis of blood brain barrier (BBB) (Dhopeshwarkar, 1973; Bradbury, 1979). Futhermore, long chain fatty acids are generally unable to pass through BBB. Firstly, they bind tightly to plasma protein (> 99% bound). Secondly, they are almost completely ionized at pH 7.4 (> 99%) and thirdly, fatty acids do not readily flip from one side of the plasma membrane to the other (Bradbury, 1979). Palmitic acid enters the brain from blood more readily than stearic acid because palmitic acid is saturated and contains 16 carbon atoms whereas, stearic acid is also saturated but contain 18 carbon atoms. Thus, palmitic acid having short carbon chain can pass through blood brain barrier more easily compared to stearic acid (Morand *et al.,* 1981).

Chemical Formula	Common Name/ Scientific Name	Carbon Chain length	Omega double bond
Saturated fatty acid			
$CH_3(CH_2)_{10}COOH$	Lauric/ Dodecanoic acid	12	-
$CH_3(CH_2)_{12}COOH$	Myristic/ Tetradecanoic acid	14	-
$CH_3(CH_2)_{14}COOH$	Palmitic/ Hexadecanoic acid	16	-
$CH_3(CH_2)_{16}COOH$	Stearic/Octadecanoic acid	18	-
$CH_3(CH_2)_{18}COOH$	Arachidic/Eicosanoic acid	20	-
$CH_3(CH_2)_{22}COOH$	Lignoceric/Tetracosanoic acid	24	-
Unsaturated fatty acid			
$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$	Palmitoleic/ 9-Hexadecenoic Acid	16	Omega-7
$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	Oleic/9-Octadece-noic Acid	18	Omega-9
$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$	Linoleic/9,12,15-Octadecatrienoic Acid	18	Omega-6
$CH_3(CH_2)_4(CH=CHCH_2)_3CH=CH(CH_2)_3COOH$	Arachidonic/5,8,11,14-Eicosatetraenoic Acid	20	Omega-6
$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$	Alpha-Linolenic/ 9,12,15-Octadecatrienoic Acid	18	Omega-3
$CH_3(CH_2CH=CH)_6CH_2CH_2COOH$	Docosahexaenoic/ 4,7,10,13,16,19-Docosahexaenoic Acid	22	Omega-3

Table 1.3: Chemical formula for the fatty acid chain linked to the phospholipids.
(Adapted From- http://www.scientificpsychic.com/fitness/fattyacids.html)

FOOD	PALMITIC (C-16:0)	STEARIC (C-18:0)	OLEIC (C-18:1)	LINOLEIC (C-18:2)	ά-LINOLINEC (C-18:3)
Perilla oil	6	2	17	15	61
Flaxseed oil	3	7	21	16	53
Menhaden herring oil	19	4	13	1	1
Canola oil	5	2	53	22	10
Walnut oil	7	2	15	60	10
Soyabean oil	11	4	23	51	7
Milk fat	25	11	26	2	2
Beef fat	29	20	42	2	0
Palm oil	45	5	38	10	0
Olive oil	14	3	71	10	0
Corn oil	11	2	25	55	0
Sunflower seed oil	6	4	24	65	0
Borage oil	11	4	16	39	0
Evening primrose oil	6	1	11	72	1
Safflower seed oil	7	3	15	75	0

Table 1. 4: Fat constituents as % of total fat for the selected foods.
(*Note- C-18:3 mean fatty acid with an 18-carbon chain and 3- double-bonds)
(Adapted from- http://www.scientificpsychic.com/fitness/fattyacids1.html)
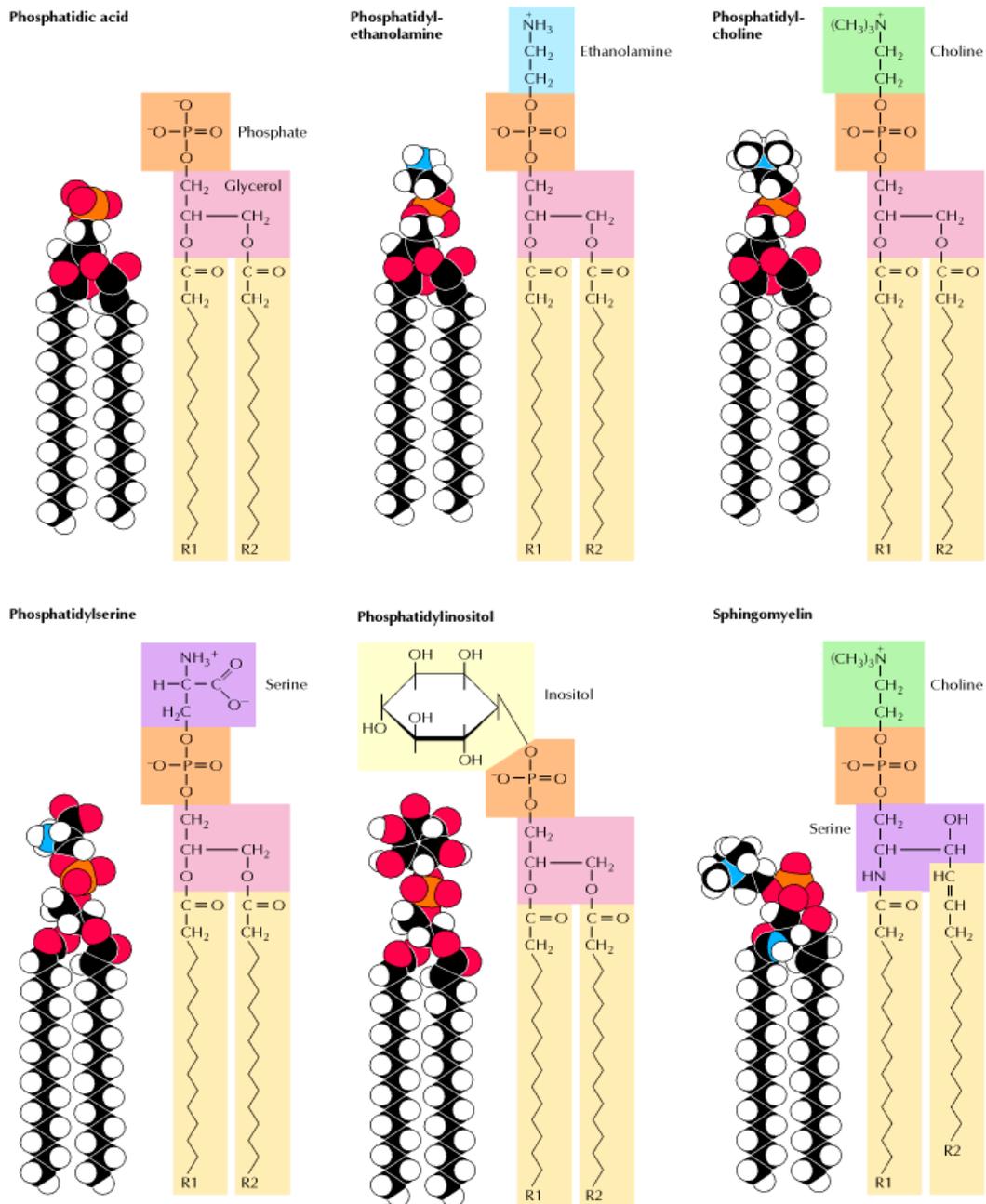
Figure 1.5: Structure of phospholipids: Phosphatidic acid (PA), Phosphatidylethanolamine (PE), Phosphatidylcholine (PC), Phosphatidylserine (PS), Phosphatidylinositol (PI) and Sphingomyelin (SM). These phospholipids have phosphate groups, polar heads and non-polar tails; designated as R1 and R2 attached to the glycerol backbone (Copper, 2000).

(Adapted from- http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=cooper.figgrp.258)
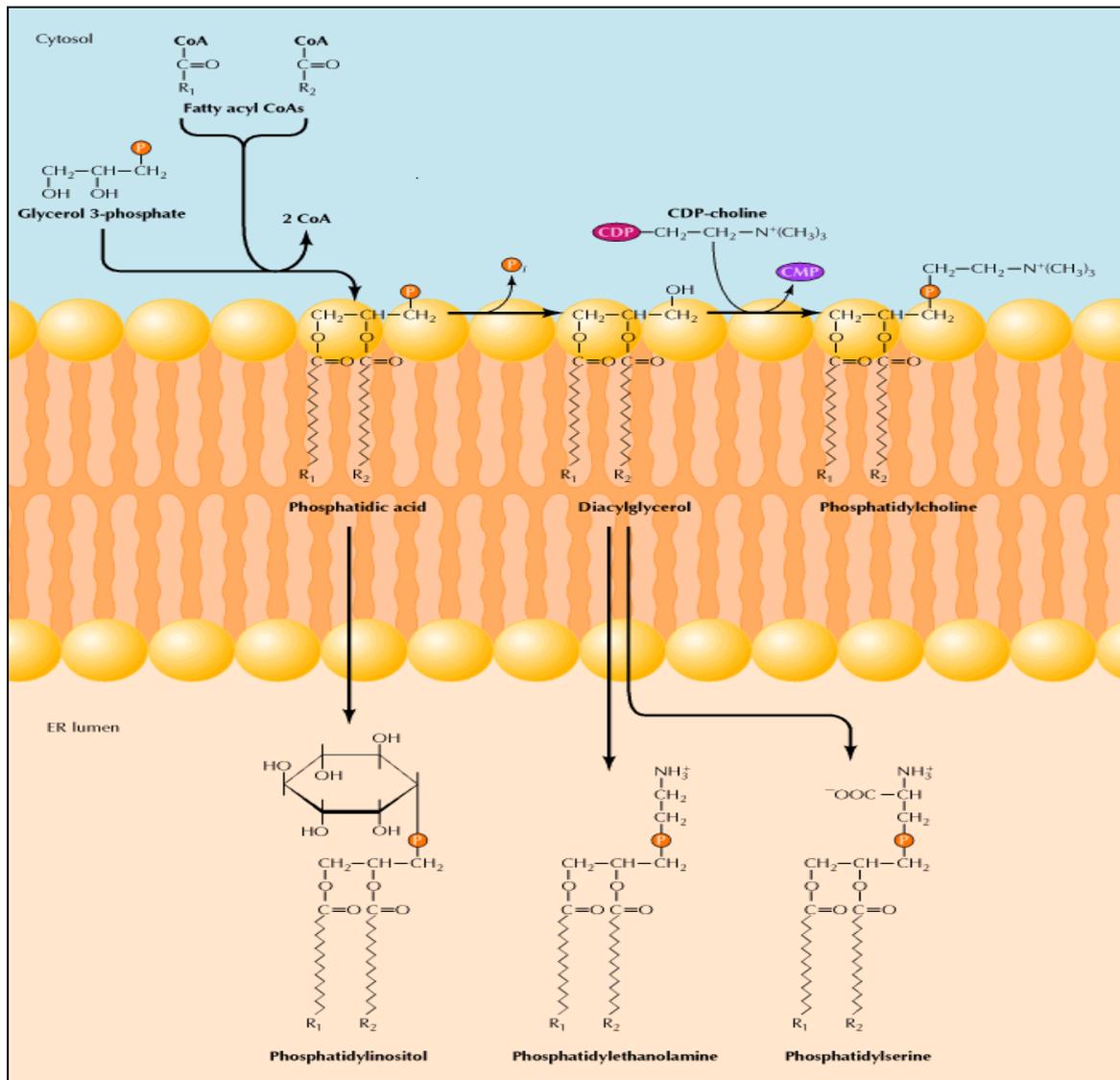
Figure 1.6: Synthesis of phospholipids: Glycerol phospholipids are synthesized in the ER membrane from cytosolic precursors. Two fatty acids linked to coenzyme A (CoA) carriers are first joined to glycerol-3-phosphate, yielding phosphatidic acid, which is simultaneously inserted into the membrane. A phosphatase then converts phosphatidic acid to diacylglycerol. The attachment of different polar head groups to diacylglycerol then results in formation of phosphatidylcholine, phosphatidylethanolamine, or phosphatidylserine. Phosphatidylinositol is formed from phosphatidic acid, rather than from diacylglycerol. (Cooper, 2000)

(Adapted from- http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=cooper.figgrp.1488)

1.3.10 Role of the phospholipids and cancers

i. Phosphatidic acid (PA)

Phosphatidic acid (Figure 1.5) is abbreviated as PA or (PtdOH) and it is a common form of phospholipid that forms the major constituent of cell membranes. It is the smallest of all the phospholipids. The saturated fatty acid is attached at carbon-1 and unsaturated fatty acid is attached at carbon-2 and the phosphate is linked at carbon-3. PA is a key intermediate in glycerolipid biosynthesis. It leads to the production of Phosphatidylethanolamine (PE), Phosphatidylcholine (PC) and Phosphatidylserine (PS) (Athenstaedt, et al., 1999).

ii. Phosphatidylethanolamine (PE)

Phosphatidylethanolamine (Figure. 1.5) (once given the trivial name 'cephalin') is usually the second most abundant phospholipid in animal and plant lipids. It comprises 20 - 50% of the total phospholipid content of the mammalian membrane. It can amount to about 20% of liver phospholipids and as much as 45% of those of the brain; higher proportions are found in mitochondria than in other organelles (Vance, 2008).

iii. Phosphatidylcholine(PC)

Phosphatidylcholine (Figure 1.5) is a phospholipid that is a major constituent of cell membranes comprising 40 to 50% of total phospholipids (Vance, 2008). PC is also known as 1,2-diacyl-sn-glycero-3-phosphocholine, PtdCho and lecithin. Phosphatidylcholine plays an important biochemical role in the maintenance and integrity of the cell-membrane. PC has also been recognized as an important signalling molecule (Exton, 1994; Billah, 1990; Kiss, 1990; Kester, 1989). Recently, it has been suggested that PC may eventually have a therapeutic role in some cancers (Semalty *et al.,* 2009). Animal studies have indicated that deficiencies in choline and PC may disrupt cell membrane signal transduction in ways that

could lead to various cancers. There is evidence suggesting that liver cancer is promoted in various animals by choline-deficient diets, and it has been shown that excess choline can protect against liver cancer in a mouse model (Lois *et al.*, 2002). In that ras transformed cell line, the biosynthesis of PC is considered to be high in cancer patients (Lenka *et al.,* 2007).

iv. Phosphatidylserine (PS)

Phosphatidylserine (abbreviated Ptd-L-Ser or PS) is a phospholipid component. Phosphatidylserine (PS) (Figure 1.5) is a quantitatively minor membrane phospholipid that makes up 2 to 10% of total phospholipids (Vance, 2008). Phosphatidylserine is an amino-phospholipid in the inner leaflet of the plasma membrane of glioma (Grace, 2000). Dietary phospholipid (PS) derived from soy lecithin has the ability to reduce the effect of both mental and physical stress which has been indicated from previous studies. PS is a popular supplement among senior athletes and is an important fat-soluble brain nutrient. It supports numerous mental functions including memory, concentration, learning and mood (Nunzi *et al.,* 1987; Nunzi *et al.,* 1989; Cohen *et al.,* 1992). PS plays an important role in cell signalling and apoptosis of the cell. PS is extremely bio available and crosses the blood-brain barrier with ease. Once in the brain, PS merges smoothly into the nerve cell membrane where it is available to facilitate cell-level energetics and homeostasis, as well as enhancing neurotransmitter production, release, and action. PS also serves as a precursor reservoir for the related phospholipids namely, Phosphatidylethanolamine (PE) and Phosphatidylcholine (PC). PS also helps to support the functions of a number of membrane proteins such as Na/K-ATPase; Ca-ATPase; Mg-ATPase (for ion transport); protein kinase C; adenylate cyclase (for processing signals that reach the cell from the environment); NADPH-cytochrome C reductase (for mitochondrial energy production) which releases the

neuro-transmitters via secretory vesicles; and receptors for NMDA and other neuro-transmitters (Kidd, 1995).

v.   Phosphatidylinositol (PI)

Phosphatidylinositol (PI) is an acidic (anionic) phospholipid that in essence consists of a phosphatidic acid backbone linked via the phosphate group to inositol (Figure 1.5). It is abbreviated as PtdIns, or PI. The inositol can be phosphorylated to form phosphatidylinositol phosphate (PIP), phosphatidylinositol bisphosphate (PIP$_2$) and phosphatidylinositol trisphosphate (PIP$_3$). PIP, PIP$_2$ and PIP$_3$ are collectively called phosphoinositides. Two phospholipid derivatives of PI are PI phosphate and PI biphosphate and they are found to be important in the signal transduction pathway located in the inner half of the plasma membrane lipid bilayer. Inhibition of phospholipid inositol metabolism can induce cancer (Nedeljkovia *et al.,* 2001). PI is especially abundant in brain tissue, where it can amount to 10% of the phospholipids. However, it is present in all tissues and cell types. There is usually less PI than PC, PE or PS in cells.

vi.   Lysophpspholipid (LPL)

The term 'lysophospholipid' (LPL) refers to any phospholipid that is missing one of its two fatty acid chain (Figure 1.5). Thus, LPL has a free alcohol in either the fatty acyl chains attached at positions 1 or 2. The derivatives of lysophospholipid (LPL) such as (serine, amide and phosphate) SAPs and (serine, amide and alcohol) SAAs, represent a novel class of cytotoxic phospholipids for prostate cancer. A number of SAPs derivatives have been designed, synthesised and evaluated for their inhibitory activity towards the growth of human prostate cancer cell lines (Gududuru *et al.,* 2004). Lysophosphatidic acids were found to be the most potent inhibitor of apoptotic cell death. The concentrations of the PC-

degradation product lyso-phosphatidylcholine were found to be decreased in cancer patients (Lenka *et al.,* 2007).

vii. Sphingomyelin(SM)

Sphingomyelin (SM) is a type of sphingo-lipids found in the membrane of animal cells especially located in myelin sheath and on axons of the nerve cells. SM consists generally of a sphingosine base with an 18-carbon chain and a double bond at position 4, attached to a phosphorylcholine fatty acid. Sphingomyelin is a group of phospholipids that on hydrolysis yield phosphoric acid, choline, sphingosine and a fatty acid. In the signal transduction pathway, extracellular inducers such as tumour necrosis factor alpha cause hydrolysis of sphigomyelin membrane, resulting in the generation of the lipid second messenger ceramide (Linardic *et al.,* 1994). There is much evidence that they support the theory that there may also be a sphingomyelin pool in the inner leaflet of the membrane (Linardic *et al.,* 1994; Zhang *et al.,* 1997). The fatty acid chains are generally long and they are saturated or either monounsaturated in nature. In bovine brain, SM is the most abundant fatty acid chains which are made of 18:0 (42%) and 24:0 (27%) carbon atoms. In contrast, in egg, SM is the dominant fatty acids with 16:0 (66%) followed by 18:0 (10%) carbon atoms (Ramstedt *et. al.*, 1999). Intestinal cells are regularly exposed to sphingolipid metabolites, i.e., ceramide and sphingolipid bases, after hydrolysis of complex sphingolipids from the diet. These metabolites are known regulators of cell growth, differentiation and death. Non-pharmacological quantities in the diet have been shown to inhibit early stages of chemically induced colon cancer in mice (Lori *et. al.,* 2003). The production of SM has been reported to be higher in lung cancer patients as compared to normal patients (Kisohara, 2001). In the 1,2-DMH (Dimethyl hydrazine) model of colonic adenocarcinoma, these investigators showed that sphingomyelin (derived from

commercially available non-fat dry milk) has inhibited colon cancer in mice (Sanchez *et. al.,* 2007).

1.4 Soy lecithin

Lecithin in a diet is rich in plant derived food which may be protective against some cancer (Kaayla, 2004). Plant diet and in particular, whole grains, legumes fruit and vegetables have been reported to be associated with a lower incidence of breast and colon cancer. In practice, lecithin is usually derived from vegetable products including soybean, peanut, cotton seed, sunflower rapeseed, corn, and ground oils. It may be isolated from marine fish, egg yolk, soy bean etc, from which it is extracted either chemically or mechanically. During the 19th century, a Scientist by the name of Maurice Gobley from France isolated a phosphorus containing lipid from egg yolk and brain and called it 'lekithos'. He showed that glycerophosphoric acid could be prepared from lecithin and later proposed the structure for the molecule based on his research (Kaayla, 2004). Lecithins are also known to be found in the cell membrane of plants and animals. Lecithin is a very common food additive. It is naturally found in many other foods, in different concentrations. It is sold as a food supplement and as an excipient in a number of medicinal formulations (Kaayla, 2004). Lecithin is a yellow brownish fatty substance composed of phosphoric acid, choline, fatty acids, glycerol, glycolipids triglycerides and phospholipids (e.g. phosphotidylcholine, phosphatidylethanolamine and phosphatidylinositol). Phospholipids help to carry out the function of the membranes and regulate biological processes such as signalling as well as being involved in metabolic and neurological diseases.

Soybean lecithin is the most commercially important vegetable lecithin. Soy is a subtropical plant native to South Eastern Asia and is mainly consumed by the Eastern style diet. It belongs to member of the pea family (Fabaceae). It grows to about 1-5 feet tall and forms

clusters of 2-4 pods each containing 2-3 beans per pods. Soy was introduced in Europe in 1700's and in U.S in the 1800's. Large scale cultivation of soy began during World War II. Currently, Midwestern U.S supplies half of the soybeans worldwide. It is used as very common food and drug additive, but it is also used as a dietary supplement. It is important to know that soy product contains putative protective factors such as oligosaccharides, protease inhibitors which are responsible for the cancer preventing effects at some sites. Soy lecithin is a combination of naturally-occurring phospholipids, which are extracted during the processing of soybean oil. The main phospholipids in soy lecithin are lyso-phospholipid, phosphatidylcholine, phosphatidylinositol, phosphatidylethanol-amine and phosphatidic acid. However, soy lecithin is sometimes used as synonym for pure phosphatidylcholine. It has a low solubility in water. There is epidemiological evidence that proves it has protective effects on colon cancer and a number of studies have shown various effects (Adlercreutz, 1998; Messina, 1994). *In vitro* studies on soy products have shown an antiproliferative effect on a wide range of cell types including those obtained from the gastrointestinal tract (Yanagihara, *et al.,* 1991). Soy lecithin provides an excellent source of choline, which is essential to every living cell in the body and is one of the main components of cell membranes. Not only is dietary choline important for the synthesis of the phospholipids in cell membranes but it is also necessary for methyl metabolism, cholinergic neurotransmission, transmembrane signalling, and lipid-cholesterol transport and metabolism (Zeisel, 2000). Without choline, the cell membranes would harden, prohibiting important nutrients from entering and leaving the cell. Soy lecithin supplementation is claimed to be beneficial for many conditions, such as: Alzheimer's disease, gallbladder disease, liver disease, bipolar disorder and high cholesterol.
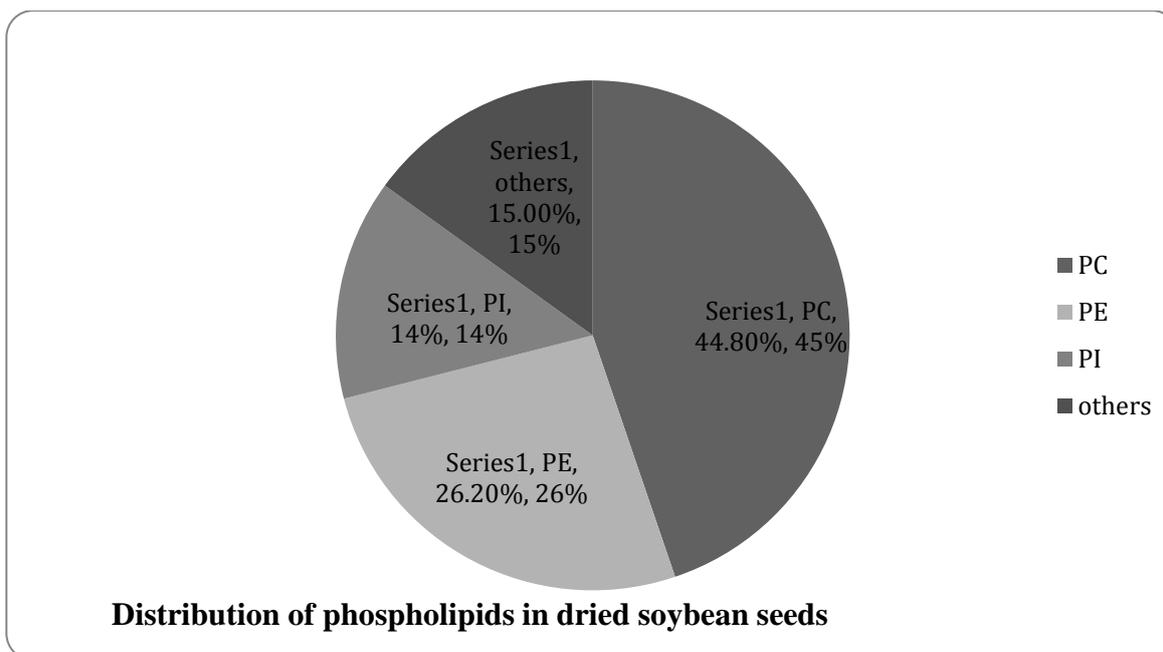
Figure 1.7: Composition of soybean lecithin: Phospholipid distribution in dried soy seeds: Phosphatidylcholine (44.8%), Phosphatidylethanolamine (26.2%), Phosphatidylinisitol (14%) and other components (15.0%) (Nyberg, 1998).

Soy lecithin is composed of three major types of phospholipids (Figure 1.7) namely: phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol along with other components (Nyberg, 1998). Compared with eggs, soybeans contain more fat (18.3 g per 100 g), but less phospholipids (1.8 g per 100 g food or 9.7 g per 100 g fat) (Souci, 2000). However, it is the best vegetarian source of phospholipids and the quantities are higher than in most other foods. Because of the high content of phospholipids in soybean which are readily available, they are often used as raw material to produce dietary supplements as well as emulsifiers for food processing (Wehrmuller, 2008).

1.4.1 Properties of lecithin

Lecithin varies in its physical properties. It changes from viscous to semi liquids to powders, depending on the free fatty acids. It may vary in colour from brown to light yellow, depending upon whether it is bleached or unbleached or on the degree of purity. When it is

exposed to air, rapid oxidation occurs, also resulting in dark yellow or brown colour (Kaayla, 2004).

1.4.2 Application in pharmaceutical formulation and technology of lecithin

Lecithins are used in a wide variety of pharmaceutical applications. They are used in food products and cosmetics. It is mainly used in pharmaceutical industries as dispersing, emulsifying, and stabilizing agents and are included in intramuscular and intravenous injections, parenteral nutrient formulation, and topical products such as cream and ointments. There is evidence that supports that phosphatidylcholine (a major component of lecithin) is important as a nutritional supplement for fetal and infant development approved by FDA-approved infant formulas (US Congress, 1980). A number of liposomes consist of lecithin as a component of the bilayer to encapsulate drug substances: their potential as novel delivery systems has been investigated (Grit *et al*., 1993). This application generally requires purified lecithin's combined in specific proportions.

1.4.3 Solubility, stability and storage conditions of lecithin

Lecithins are soluble in aliphatic and aromatic hydrocarbons, halogenated hydrocarbons, mineral oil and fatty acid. They are practically insoluble in cold vegetable and animal oils polar solvent and water. When lecithin is mixed with water it hydrates to form emulsion. Lecithin decomposes at extreme pH 4-7 and oxidizes and decomposes at temperatures ranging from 160-180° C. All lecithin grades should be stored in sealed containers protected from sunlight and oxidation (American Lecithin Company, US).

1.5 Lipoid S-100 derived from Soyabean

Lipoid S100 is a pure form of soy lecithin. For pure Lipoid S-100, the occurrence of WAXS reflections indicates (partial) crystallinity at 25 and 45°C. At 65°C, Lipoid S-100 forms a cubic liquid crystalline phase (Westesen, 1997; Anschütz, 1998). Lipoid S-100 soy lecithin is a mixture of phospholipids and these include: Phosphatidyl choline, N-Acyl-phosphatidylethanolamine, Phosphatidylethanolamine, Phosphatidylinositol and Lysophosphatidylcholine. Its wax appearance is yellow brownish in colour. It consists of fewer amounts of saturated fatty acid (Figure 1.8) (palmitic acid and stearic acid) in comparison to unsaturated fatty acids (Figure 1.8) (oleic acid, linoleic acid and linolenic acid) (Table 1.5).
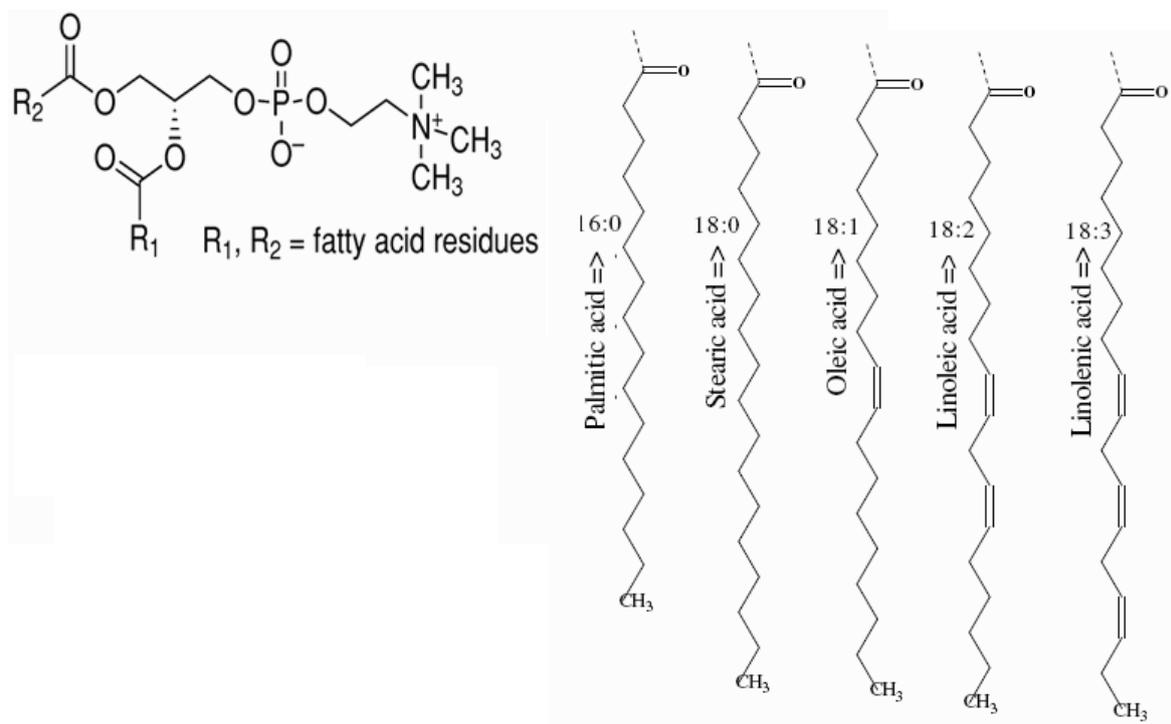
Figure 1.8: The structure of fatty acid composition of phospholipids Lipoid S-100 (Adapted from-Lipoid GmbH, Germany)**.**

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Composition of soy lecithin, Lipoid S-100

Phospholipids fraction

Phosphatidyl choline(by anhydrous weight)	94.0%
N-Acyl-phosphatidyl ethanolamine	0.5%
Phosphatidyl ethanolamine	0.1%
Phosphatidyl inositol	0.1%
Lysophosphatidyl choline	3.0%

Typical fatty acid composition in % to total fatty acids

Fatty acid	% of fat composition
Palmitic acid	12-17
Stearic acid	2-5
Oleic acid	11-15
Linoleic acid	59-70
Linolenic acid	3-7

**Approximate Molecular Weight *:* 790

**Storage *:* -20 ℃

</div>

Table 1.5: The phospholipids and the fatty acids composition in soy lecithin (Lipoid S-100) (Adapted from- Lipoid GmbH, Germany)

1.6 Phospholipon® 90H derived from Soyabean

Phospholipon® 90H is hydrogenated soy lecithin product, carrying the additive letter "H". It contains saturated materials, whereas all other fractions of phosphatidylcholine are composed of native unsaturated PC. Phospholipon® 90H contains fraction of phospholipids such as hydrogenated phosphatidylcholine and hydrogenated lysophosphatidylcholine. The component of saturated fatty acids (Figure 1.9) (palmitic acid and stearic acid) is higher in comparison to unsaturated fatty acids (Figure 1.9) (oleic acid, linoliec acid and linolenic acid) (Table 1.6). These products are pure solid lecithin fractions with a standardized content of phosphatidylcholine (PC) for oral and parenteral administration.
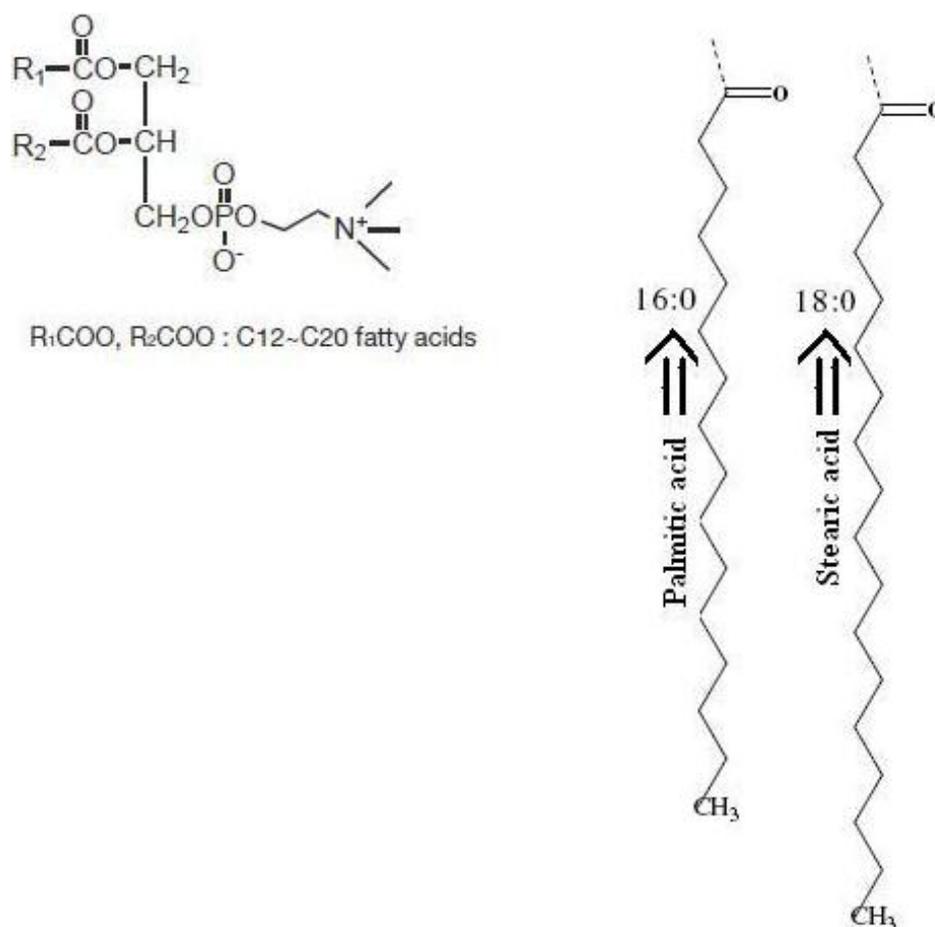
Figure 1.9: The structure of fatty acid composition of phospholipids Phospholipon® 90H (Adapted from- Phospholipid GmbH, Nattermannalle, Germany)

Composition of lecithin, hydrogenated from soy

Phospholipon®90 H

Phospholipids fraction

Hydrogenated Phosphatidylcholine   min. 90%

Hydrogenated Lysophosphatidylcholine   max. 4%

Oil/ triglycerides   max. 2%

Typical fatty acid composition in % to total fatty acids

Fatty acid	% of fat acid composition
Palmitic acid	15
Stearic acid	85

Approximate Molecular Weight- 784

- Sum of stearic acid and palmitic acid   min. 98%

- Sum of unsaturated fatty acids   max 2%

       (oleic, linoleic, linolenic acids)

Storage : Room temperature or below dry condition

Table 1.6: The phospholipids and the fatty acids composition of Phosphotidylcholine, hydrogenated from soy (Phospholipon® 90 H) Adapted from- Phospholipid GmbH, Germany (http://www.americanlecithin.com/aboutphos.html).

1.7 L-ά-Phosphatidylcholine derived from Soyabean

L-ά-Phosphatidylcholine is a pure form of soybean phospholipids. It has only PC phospholipids as the major component. L-ά-Phosphatidylcholine is not a mixture of any other phospholipid. It is a lyophilized white powder. It consists of fewer quantities of saturated fatty acids (Figure 1.10) (palmitic acid and stearic acid) in comparison to unsaturated fatty acids (Figure 1.10) (oleic acid, linoleic acid and linolenic acid) (Table 1.7). The major structural phospholipid in brain comprises of approximately 15% of total lipid; primarily localized to gray matter.
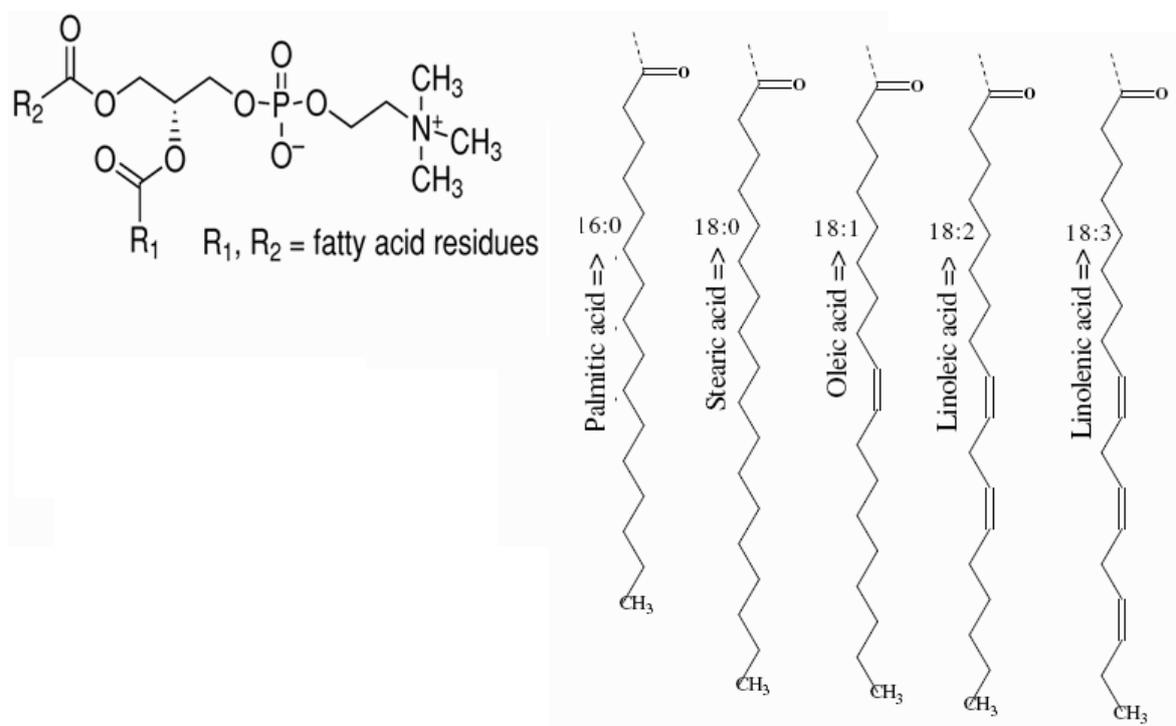
Figure 1.10: The structure of fatty acid composition of phospholipids L-ά-Phosphatidylcholine. Adapted from- Sigma Aldrich, UK.

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Composition of soy phospholipid

L-ά-Phosphatidylcholine

Synonyms: L-ά -Lecithin, 3-*sn*-Phosphatidylcholine,

Typical fatty acid composition in % to total fatty acids

Fatty acid	% of fat acid composition
Palmitic acid	13
Stearic acid	4
Oleic	10
Linoleic	64
Linolenic	6

Approximate Molecular Weight: 776

Storage Temperature: -20 °C

</div>

Table 1.7: The fatty acids composition of L-ά-Phosphatidylcholine Adapted from- Sigma Aldrich, UK (Personal Communication)

1.8 Working hypothesis

Phospholipids have been previously shown to induce different effects on cancer cells. This project was designed to findout whether specific phospholipids can either inhibit or support the growth of gliomas.

1.9 Main aim

The main aim of this study was to investigate whether there is a possible link between specific phospholipids and either inhibition or proliferation of the growth of three specific glioma cell lines.

1.9.1 Specific aims of the research:

1. To undertake a thought of literature level of the study proposed

2. To learn the techniques of tissue culture and ATP assay

3. To determine the effects of the soy phospholipids namely Lipoid S-100, Phospholipon[®] 90H and L-α-phosphatidylcholine on three different primary glioma cell lines. These include 1321N1, U87 MG and GOS-3.

4. The 1321N1, U87-MG and GOS-3 cell lines were treated with increasing concentrations of the different specific phospholipids and cell viability was determined *in vitro* using a luminescent assay (ATP assay).

5. To analyse the data and write the MSc thesis.

2.   Materials and Methods

2.1 Materials

2.1.1 Glioma cell lines

1321N1 (ECACC, UK), GOS-3 (DSM2, Germany) and U87-MG (ECACC, UK).

2.1.2 Phospholipids

- Lipoid S-100 soy lecithin (lipoid GmbH, Germany).

- Phospholipon[R] 90H Phosphotidylcholine, hydrogenated from soy (Phospholipid GmbH, Germany).

- L-ά-Phosphatidylcholine (Sigma Aldrich, UK).

2.1.3 Equipment and materials used

New Brunswick Scientific ($CO_2$81R) - water jacketed CO_2 incubator, water bath , laminar flow hood, inverted phase contrast microscopes, weighing balance, Tecan plate reader (Manufacturer: Tecan Austria GmbH, 2004 model), refrigerator, freezer (- 20°C), deep freezer (-80°C), centrifuge machine, electrical aspirator, vortex mixture, 2, 20, 200 and 1000 μl pipettes and pipette tips, motorized pipette controller, 75 cm^2/25 cm^2 culture flasks, sterile forceps, aluminium foil, 5 and 10 ml disposable plastic pipettes, 15 ml and 50 ml centrifuge tubes, Cryovials, "Mr. Frosty" freezing container and liquid nitrogen, DMSO (Sigma Aldrich, UK), 0.22 μm and 0.44 μm sterile filters (Fisher Scientific, UK), syringes(Fisher Scientific, UK), pasteur pipettes, universal bottles, 5 ml volumetric flask, glass funnel, sterile spatula and clear bottom white 96 well plates (Grenier, UK).

2.1.4 Media and supplements required for cell culture

DMEM (Dulbeco's Modified Eagle's Medium) and MEM (Minimal Essential Medium), Foetal bovine serum (FBS), trypsin, L-Glutamine, non-Essential Amino Acid (NEAA) and sodium pyruvate (All the products bought from Lonza, UK).

2.1.5 Chemicals and Reagents

- Ethanol, Methanol and Isopropanol (Fisher Scientific, UK)

- Phosphate Buffered Saline −2 tablets was dissolved in 400 ml of water and stored the solution at 4 °C. (Sigma, Aldrich, UK)

- CellTiter-Glo® luminescent Cell Viability Assay kit (Promega, UK) (Figure 2.1).
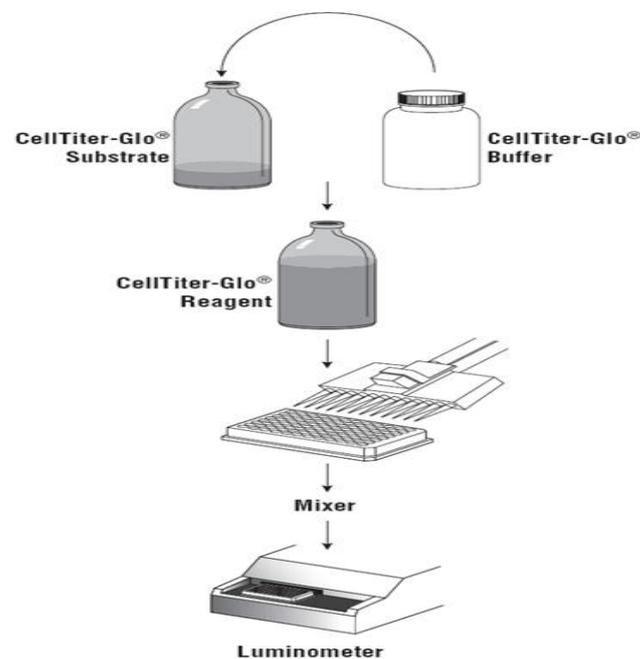
Figure 2.1: Flow diagram showing preparation and use of CellTiter-Glo® luminescent Cell Viability Assay Reagent. (Taken from-http://www.promega.com/tbs/tb288/tb288.pdf).

2.1.6 Composition of medium for the three glioma cell lines

A. 1321N1

Dulbecco's Modified Eagle Medium (DMEM-500 ml), 10% Foetal bovine serum (FBS-50 ml), 2 mM L-glutamine (5 ml)

B. U87-MG

Minimal Essential Medium (MEM-500 ml), 10% Foetal bovine serum (FBS-50 ml), 2 mM L-glutamine (5 ml),1% Non Essential Amino Acids (NEAA- 5 ml), 1 mM sodium Pyruvate (5 ml)

C. GOS-3

Dulbecco's Modified Eagle Medium (DMEM-500 ml), 10% Foetal bovine serum (FBS-50 ml), 4 mM L-glutamine (10 ml)

2.2 Methods

2.2.1 Cell culture and passaging of the Primary Glial Cells (1321N1, U87-MG and GOS-3)

Culture medium, PBS, and trypsin (sterile) were removed from the 4°C fridge freezer and subsequently placed in a water bath at 37°C for 30 min in order to equilibrate. The laminar flow hood was turned on for 10 min prior to commencement of the experiment in order to purge the air inside the cabinet. The cells were incubated at 37°C in an atmosphere of 5% CO_2 in air. Thereafter, the cells were examined under an inverted contrast microscope to note confluence and their general health. The flask was passaged when the cells had reached 70-80% confluence.

The medium was aspirated from the cultured flask and was washed with sterile PBS (5 ml if 75 cm^2 flask and 2 ml if 25 cm^2 flask were used) in order to remove any traces of serum from the cells, thus preventing the serum possibly inactivating the trypsin. Trypsin solution

(2 ml if 75 cm^2 flask and 1 ml if 25 cm^2 flask) was pipetted in the flask and incubated at 37°C in an atmosphere of 5% CO_2 in air for 3-5 min until the cells started to detach. This was confirmed by observing at intervals under an inverted microscope. The cells were left in trypsin for an appropriate length of time to avoid damage. Complete Growth Medium (2 ml) was then added to the flask to inactivate the trypsin and the cells were pipetted up and down to break down any large cell aggregates. The cell suspension was removed from the flask into a 15 ml centrifuge tube and centrifuged at 1000 r.p.m for 5 min. After centrifugation, the supernatant was aspirated and the cells were pelleted at the bottom of the centrifuge tube. Depending upon the cell pellet density, a volume of 1 ml to 2 ml of fresh medium was suspended in the centrifuge tube. The pellet cells were mixed properly in the medium. A volume of 20 μl of trypsinised cell suspension and 80 μl of tryphan blue (1:5) were mixed together and counted using the haemocytometer employing 1 ml of cell suspension. The total volume of the cell suspension present in the centrifuge tube was required to make 1 or 2 flasks. The cell suspension was divided in one or more flasks (depending on the cell density) and fresh growth medium (10 ml to 12 ml if 75 cm^2 flask and 5 ml if 25 cm^2 flask) was added to the flasks, which was then placed in a 5% CO_2 incubator to continue cell growth. In addition, the same cells were seeded in 96 well plates and approximately more than 1 million cells were frozen down in liquid nitrogen depending on the number of cells present per ml.

2.2.2 Cell Counting Method

A volume of 20 μl of cell suspension and 80 μl of tryphan blue (1:5) were pippeted into a micro centrifuge tube and mixed together. A cover slip was gently placed over the chambers of the haemocytometer, and a volume of 20 μl of cell suspension was slowly pipetted against each short side of the cover slip so that the suspension could spread into each chamber. The haemocytometer was placed onto the stage of an inverted phase contrast

microscope and focused on the centre 25 squares of one chamber. The numbers of cells in these squares were then counted. These steps were repeated for the other chambers. The average number of cells in the centre grid (1 mm^2) of each chamber was calculated. This number was multiplied by 10^4 to obtain the number of cells per 1 ml of suspension. The total number of cells was calculated by multiplying the number of cells per 1 ml by the total volume of the cell suspension.

2.2.3 Cryopreservation of cells

The cell suspension obtained during passaging enabled stocks of cells to be stored for longer periods in liquid nitrogen. This process is called cycropreservation. The process involved slowly freezing the cells down in culture medium supplemented with a cryoprotectant which helped to protect the cells from rupture due to formation of ice crystals. The most commonly used cryoprotectant is dimethyl sulphoxide (DMSO). The frozen cell suspension of 900 μl and 100 μl of DMSO contained approximately more than 1 million cells. The cell populations often survived the freezing process with high viability, using a freezing medium that consisted of the normal culture medium supplemented with 10% DMSO. However, it was common practice to increase the proportion of serum in the medium to up to 90%. The most important principle of cryopreservation involved freezing the cells slowly at a rate of 1-3°C and thawed quickly in a 37°C water bath. The vial container was frozen in a bath of isopropanol called "Mr. Frosty", which mediated a 1°C/min cooling of the cryovials in a - 80°C freezer prior to storage at a temperature below -135°C in either vapour or liquid phase nitrogen. The isopropanol employed in "Mr. Frosty" could only be used 5 times before it required replacing.

2.3 Preparation and application of each phospholipid sample.

2.3.1 Soy Lecithin and phospholipid (Lipoid S-100, Phospholipon® 90H and L-α-phosphatidylcholine)

Each phospholipid either Lipoid S-100, molecular weight 790; Phospholipon® 90H, molecular weight 784 or L-α-phosphatidylcholine, molecular weight 766 was weighed in the universal bottles according to the concentrations of 5 mM, 10 mM, 15 mM, 20 mM and 25 mM having the weights of 19.75 mg, 39.5 mg, 59.25 mg, 79 mg and 98.75 mg, respectively. For each phospholipid, these amounts were initially dissolved in 2 ml of 0.002% IPA by continuous stirring and with the brief use of a sonicator water bath. When the soy lecithin was completely dissolved in 2 ml of IPA, the 2 ml soy lecithin solution from the universal bottles was transferred into the 5 ml volumetric flasks. The universal bottle was repeatedly washed with IPA and transferred into a 5 ml volumetric flask and made up to 5 ml mark. The 5 ml stock solution from volumetric flasks was transferred to a 10 ml syringe and sterile filtered using 0.22 μm filters into another sterile universal bottles. These stock solutions were stored in sealed universal bottles in the fridge until required. Once removed from the fridge the prepared phospholipid solutions were gently warmed at 37 °C in order to ensure that the phospholipid was a complete solution, before aliquoting. A volume of 2 μl was transferred from each sterile stock solution of 5 mM, 10 mM, 15 mM, 20 mM and 25 mM. Each was dissolved in 998 μl of media to obtain the final volume of 1 ml and providing final concentrations of 10 μM, 20 μM, 30 μM, 40 μM and 50 μM. Each aliquot contained not more than 0.002% of isopropanol. A volume of 200 μl of the resultant medium of each of the five phospholipid concentrations (10 μM, 20 μM, 30 μM, 40 μM and 50 μM) was transferred in triplicate using a Gilson pipettes to 96 well plates.

2.3.2 Procedure carried out to test the effect of phospholipid on the glioma cell lines using the ATP assay.

Cell suspensions employing either 1321N1, GOS-3 or U87-MG obtained during passaging was diluted (1:10). The required volume of the cell suspension and fresh medium volume were calculated based on the number of well plates. The dilution was carried out such that 200 µl of the suspension contained 1000 cells. After the plating, the plates were incubated for 48 hrs at $37^{o}C$ in 5% CO_2 incubator. After 48 hrs, the media from 96 well plates were transferred to on sterile tissue paper and another 200 µl media containing 2 µl of the phospholipid solution were added in three replicates to give final concentration of 10 µM, 20 µM, 30 µM, 40 µM and 50 µM in the 96 well plates. Isopropanol was not more than 0.02% in all controls and phospholipid-supplemented cultures. After the supplement of the phospholipid solution, the 96-well plates were again incubated for 48 hrs at 37°C in 5% CO_2. After 48 hrs prior to the experiment, each plate was removed from incubator for 30 min. Culture medium and PBS were removed from 4°C whereas, Cell Titter Glo$^{®}$ luminescent assay covered with the aluminium foil was removed from $-20^{o}C$ and was placed in the water bath at 37°C for 30 mins to equilibrate. Thereafter, the medium from the plate was tipped off and was washed with PBS. After washing the 96 well plates, a volume of 200 µl (2:1) cell titer glo$^{®}$ luminescent assays with medium was added to each well and was allowed to remain at the room temperature for 10-15 min to equilibrate. The Cell Titer Glo luminescent assay was carried in the absence of light. Luminescence was measured on a Tecan plate reader with the appropriate settings for the plate (Figure 2.1). The software used for the luminescence assay was XFLUOR4GENIOSPRO Version: V 4.53 (Year, 2004). The measurement mode used for the assay was luminescence. The plate type used from the software was specified as GRE 96fb pdf. The temperature was stated to be 20-23 °C. The plate was then placed on a shaker and shaken gently for 2 min and allowed

to settle for 2 min. The viability measured by using Cell Titer Glo$^{®}$ (Promega) a reagent, reflects readout of the cell viability assay.

2.4 Morphology of the different cell lines

Photographs were taken of the three cell lines in either untreated control, treated with 0.002% isopropanol or with each phospholipid employing different concentrations. Figure 2.2 shows photographic examples of the three cell lines under different conditions. Other photographs are also shown in appendix of the thesis for comparison.

A. 1321N1   B. GOS-3   C. U87-MG

Untreated   Untreated   Untreated

D. 1321N1   E. GOS-3   F. U87-MG

0.002% IPA   0.002% IPA   0.002% IPA
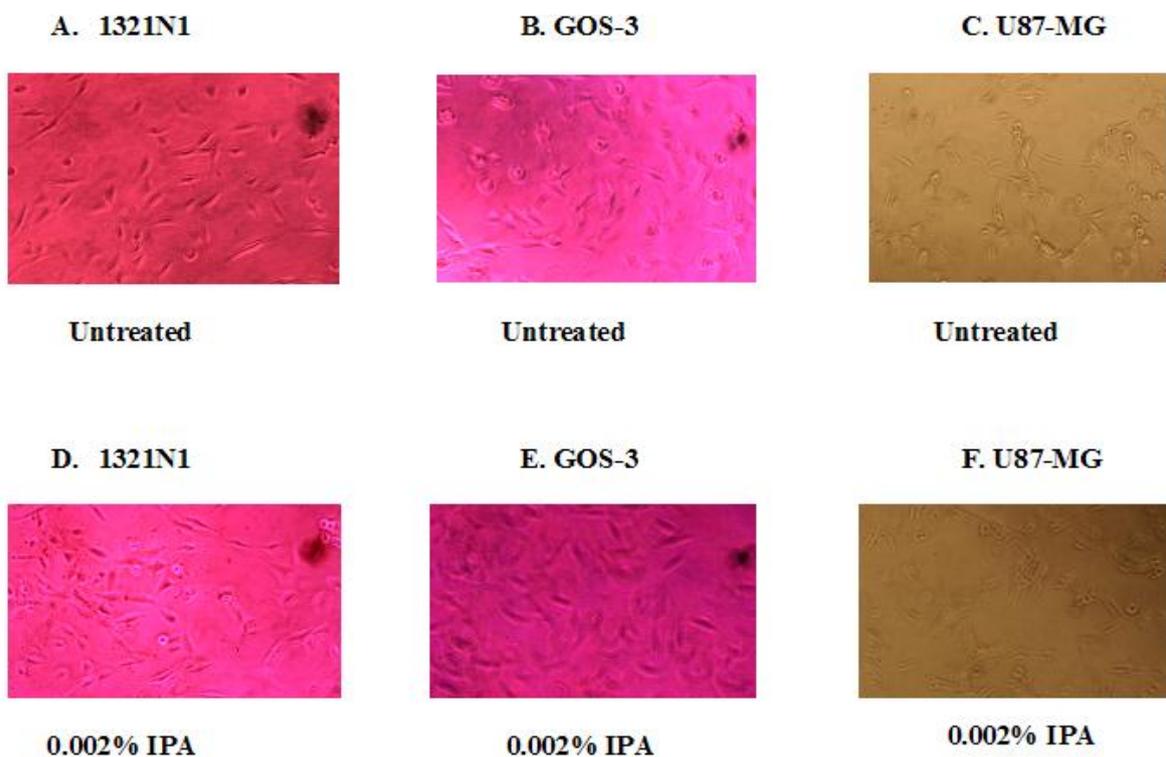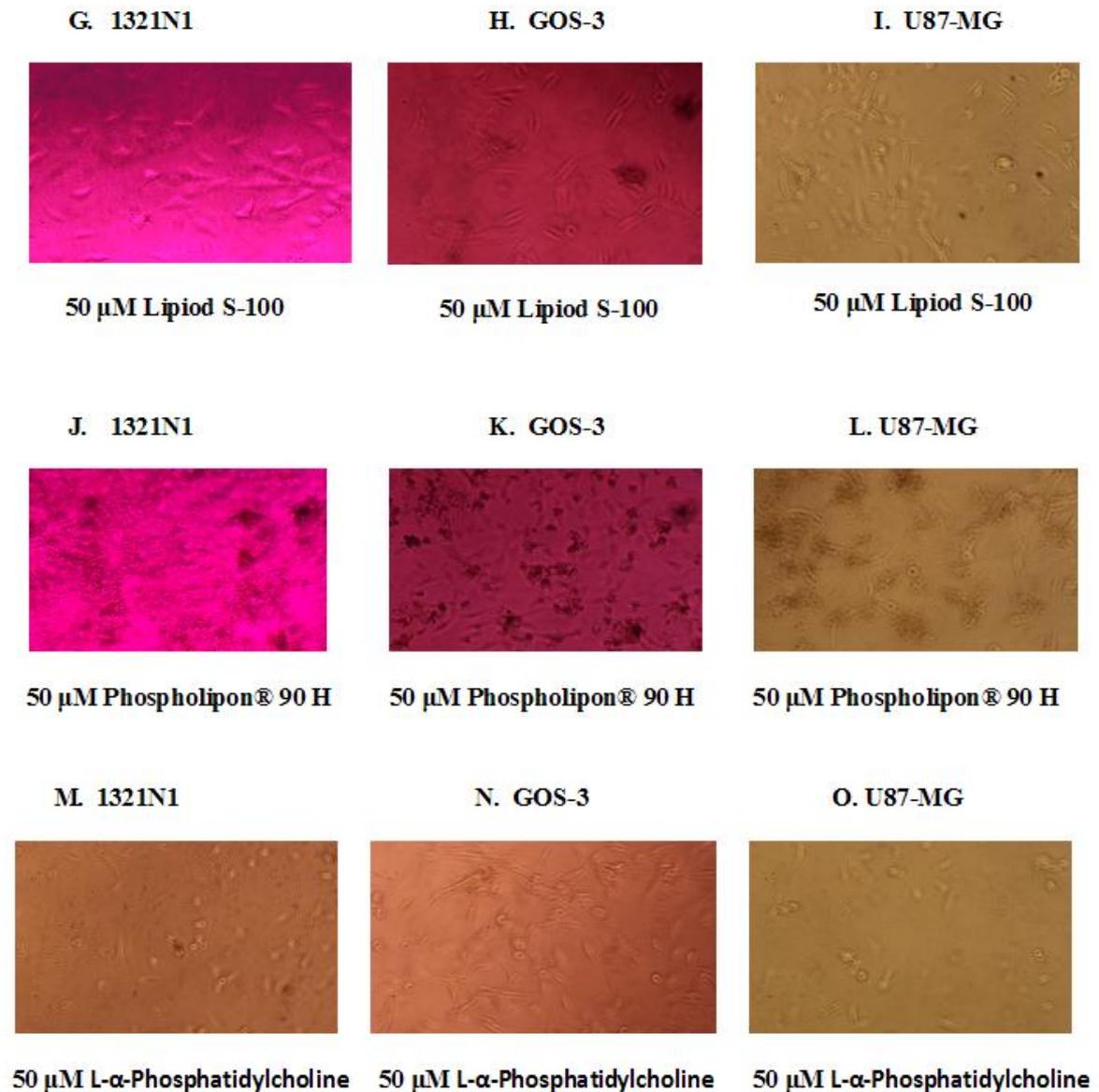
Figure 2.2: Morphology of the 1321N1, GOS-3 and U87-MG cell lines in (A-C) untreated control condition, (D-E) treated with 0.002% IPA, (G-I) treated with 50 μM Lipiod S- 100, (J-E) treated with 50 μM Phospholipon® 90H and (M-O) treated with 50 μM L-α-phosphatidylcholine. All the cell lines were incubated either alone, with 0.002% IPA or with the different concentrations of each phospholipid for 48 hours before cell viability was measures using the ATP assay. These photographs are typical of 4-5 such different experiments. Note the changes in the cell morphology comparing treated with control untreated cells.

2.5 Statistical analysis

For the cell survival ATP assay, the experiments were performed in triplicate, and the results were expressed as the mean ±Standard Deviation (SD) from the three or four independent experiments. This gave n as a repeat value 9-12 times. A statistical analysis was performed using SPSS and Microsoft Excel. All the data were evaluated for unpaired variables to compare two or more groups by paired Student's "t-test", values of *, $P < 0.05$ and **, $P < 0.01$ were considered to be statistically significant.

3. Results

3.1 Comparative study of the three different phospholipids on 1321N1, GOS-3 and U87-MG glioma cell lines

The main aim of the study was to investigate the effects of different soy derived phospholipids namely: Lipoid S-100, Phospholipon® 90H and L-α-Phosphatidylcholine, with primary glioma 1321N1, GOS - 3 and U87- MG cell lines viability under *in vitro* conditions. In order to find out the effect of the soy derived phospholipids on the glioma cells, they were treated with five different concentrations 10 μM, 20 μM, 30 μM, 40 μM and 50 μM of each phospholipid. ATP assay test was performed on each cell line after 48 hrs of treatment with phospholipids in order to determine the cell viability. Each soy derived phospholipid was dissolved in 0.002% isopropanol. In order to eliminate any direct effect of 0.002% isopropanol. It was necessary to incubate each cell line with 0.002% isopropanol as a control. The data shows that treatment of all three glial tumour cell lines (1321N1, GOS- 3 and U87- MG) with 0.002% isopropanol alone resulted in significant (*, $P < 0.05$) decreases in cell viability compared to the untreated (control) cell lines (Figures 3.1, 3.2 and 3.3). The results of the three cell lines treated with five increasing concentrations of the three different phospholipids in the study are also shown in Figures 3.1, 3.2 and 3.3for comparision.

### 3.1.1 The effects of Lipoid S-100, Phospholipon® 90H and L-α- Phosphatidylcholine on 1321N1 cell line
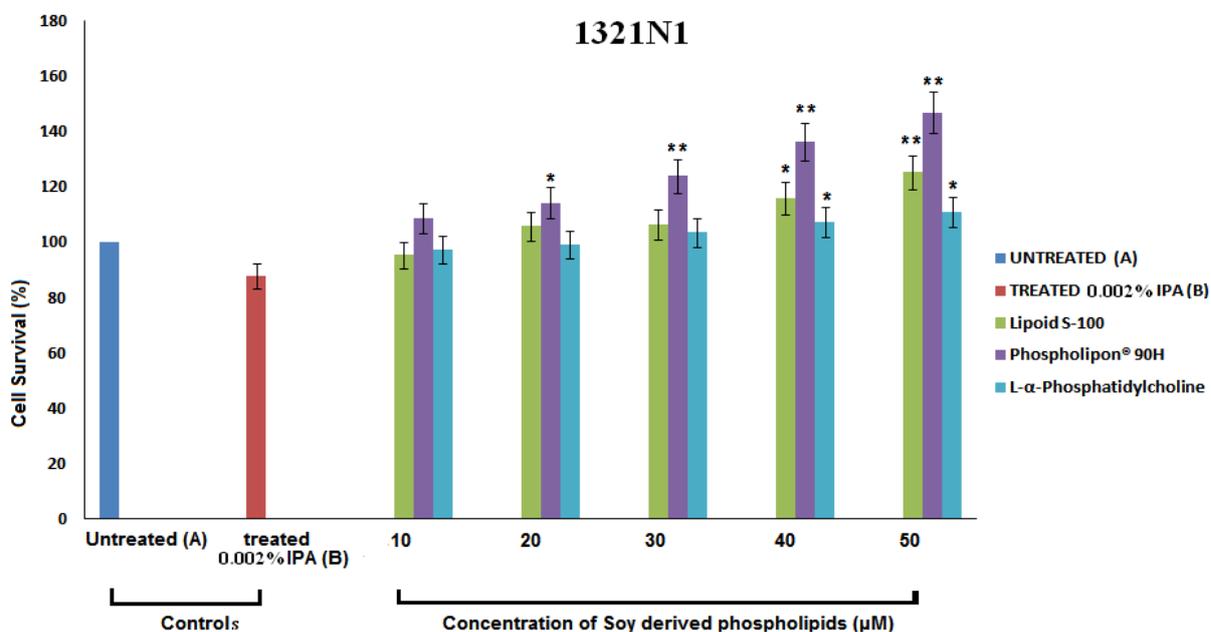
Figure 3.1: Effects of five different increasing concentrations (10 μM, 20 μM, 30 μM, 40 μM and 50 μM) of either Lipoid S-100, Phospholipon® 90H or L-α-Phosphatidylcholine on viability of the 1321N1 cell line (expressed as a percentage of control untreated cell lines). The viability of the untreated (A) and treated with 0.002% IPA (B) are also shown in the figure for comparison. The results show that Phospholipon® 90H can evoke significant ($*$, $P < 0.05$) dose dependent effects on the cell survival (proliferation) at all concentrations tested. In contrast, at 10 μM and 20 μM, Lipoid S-100 and L-α-Phosphatidylcholine had small inhibitory effects on cell viability. However, at 40 μM and 50 μM, each phospholipid enhanced the viability compared to untreated cells. Interestingly, 0.002% IPA evoked a small inhibitory effect of cell viability compared to untreated cells. The data are represented as the mean ± S.D of cell survival on treatment with the phospholipids where, n = 9-12 either with or without phospholipid dissolved in isopropanol. The results show significant increases ($*$, $P < 0.05$ and $**$, $P < 0.01$) in the survival of 1321N1 cell line with the phospholipids treatment compared to untreated cells.

The 1321N1 cells were treated with five different increasing concentrations of phospholipids and incubated for 48 hrs. After the administration of phospholipids, the cell viability was determined using Cell titer glo® luminescence assay to detect ATP release of the cell by providing luminescence to 1321N1 cells. The cells were treated with soy derived phospholipids at concentration of 10 μM, 20 μM, 30 μM, 40 μM and 50 μM and were compared with the control (1321N1 cell line treated with 0.002% IPA=B). The results show that Lipoid S-100, Phospholipon® 90H and L-α-Phosphatidylcholine an evoke significant

increases in 1321N1 cell line viability at higher concentrations when compared to 1321N1 cell line + 0.002% IPA (B) and untreated 1321N1 cell line (Figure 3.1). Lipoid S-100 showed a significant ($P < 0.05$) increase at 40 µM (*, $P < 0.05$) and 50 µM (**, $P < 0.01$). Similarly, L-α-Phosphatidylcholine showed a significant increase at 40 µM and 50 µM (*, $P < 0.05$) whereas, 1321N1 treated with Phospholipon® 90H showed a significant increased in cell viability from 20-50 µM (*, $P < 0.05$, **, $P < 0.01$) as compared to 1321N1 cell line treated with 0.002% IPA. The 1321N1 cells treated with 0.002% IPA showed only a small but significant ($P < 0.05$) inhibition compared to the untreated 1321N1 cell line (Figure 3.1).

3.1.2 The effects of Lipoid S-100, Phospholipon® 90H and L-α-Phosphatidylcholine on GOS-3 cell line.
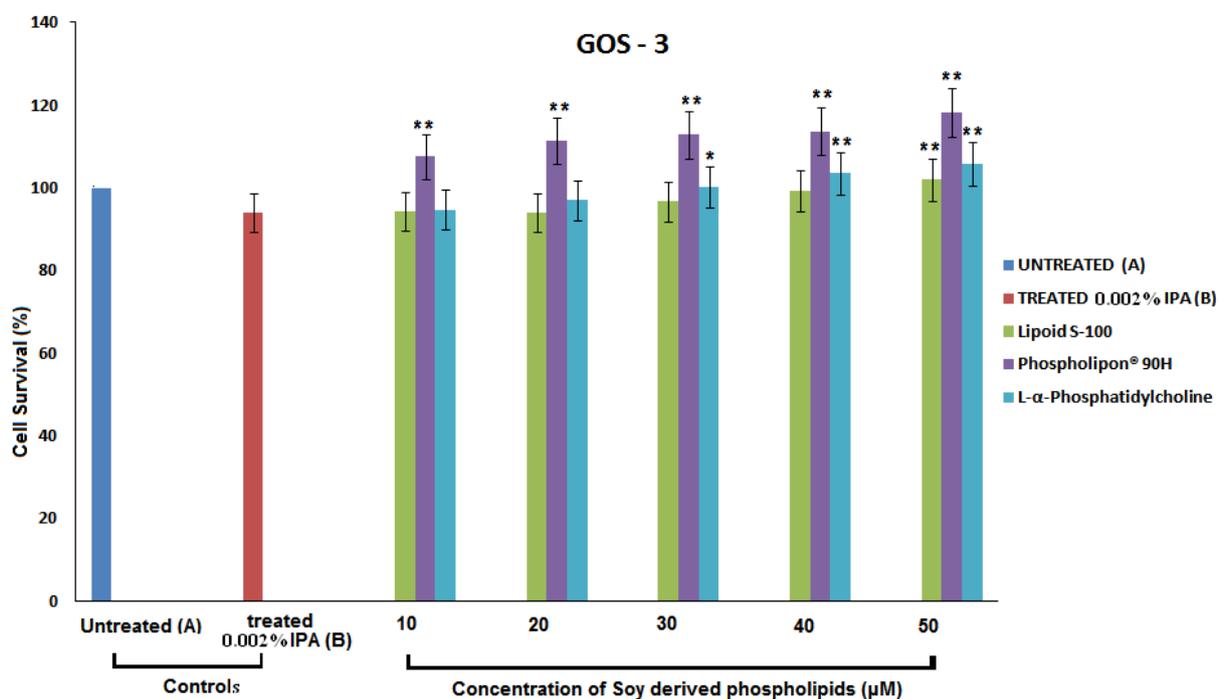
Figure 3.2: Effects of different concentration (10 μM, 20 μM, 30 μM, 40 μM 50 μM) of soy derived phospholipids: Lipoid S-100, Phospholipon® 90H or L-α-Phosphatidylcholine on the GOS-3 cells survival rate (expressed as percentage of control untreated cell lines) . The cell viability for untreated cells and the treated cell with 0.002% IPA is also shown for comparison. The result shows that Phospholipon® 90H can evoke significant (*, $P < 0.05$) increases in the cell viability at all five concentrations tested compared to untreated cells. In contrast, either Lipoid S-100 or L-α-Phosphatidylcholine seemed to evoke only small decrease in cell viability. The data are presented as the mean ± S.D of cell survival on treatment with the phospholipids where, n = 9-12 either with or without phospholipid dissolved in isopropanol. The data show significant increases compared to controls (untreated and untreated with the addition of 0.002% IPA), *, $P < 0.05$ and **, $P < 0.01$.

The GOS-3 cells were treated with increasing concentrations of phospholipids and incubated for 48 hrs. After the 48 hrs the cell viability was determined by using Cell titer glo® luminescence assay to detect ATP release of the cells by providing luminescence to the GOS-3 cells. The cells treated with soy derived phospholipids at concentration of 10 μM, 20 μM, 30 μM, 40 μM and 50 μM were compared with the untreated GOS-3 cell line either alone or in presence of 0.002% IPA(B). The results show that with Lipoid S-100, Phospholipon® 90H or L-α-Phosphatidylcholine can evoke significant (*, $P < 0.05$)

increases at higher concentrations, when compared to IPA (B) = 0.002% of isopropanol with untreated GOS-3 cell line. Lipoid S-100 showed a significant increase at 50 μM (*, P < 0.05). Phospholipon® 90H showed significant increases of the cell viability from 10-50 μM (**, P < 0.01). L-α-Phosphatidylcholine showed significant increases at 40 μM and 50 Mm (*, P < 0.01). The treated GOS-3 cell line with 0.002% IPA(B) showed only a slight inhibition compared to the untreated GOS-3 cell line, *P < 0.05 (Figure 3.2).

3.1.3 The effect of Lipoid S-100, Phospholipon® 90H and L-α-Phosphatidylcholine on U87-MG cell line.
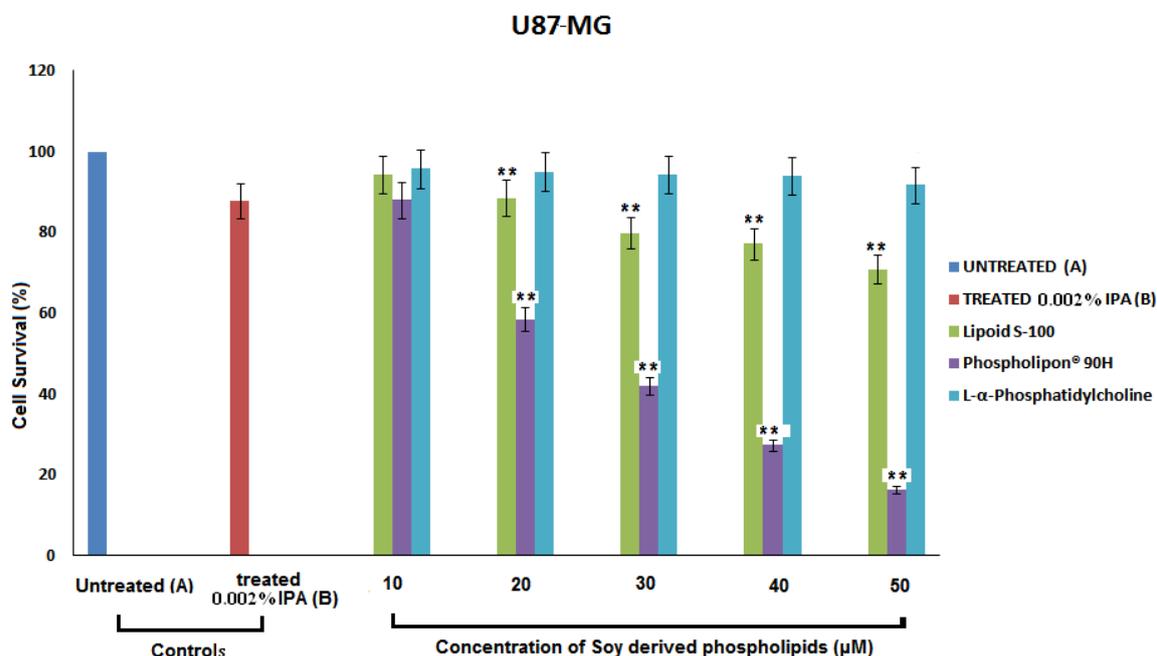
Figure 3.3: Effects of different concentrations (10 μM, 20 μM, 30 μM, 40 μM and 50 μM) of either Lipoid S-100, Phospholipon® 90H or L-α-Phosphatidylcholine on U87-MG cell survival rate compared to untreated and the cells treated with 0.002% IPA (expressed as percentage of untreated control). The results show that Phospholipon® 90H can elicit significant decreases on cell viability rate at 20-50 μM range. Similarly, Lipoid S-100 can evoke significant decreases in U87-MG cell viability rate at 20-50 μM range but the effects were less pronounced compared to Phospholipon® 90H. Surprisingly, L-α-Phosphatidylcholine had no effect on cell viability rate compared to untreated cells. Interestingly, 0.002% IPA had a small inhibitory effect on cell viability rate compared to untreated cells. The data are presented as the mean ± S.D of cell survival on treatment with the phospholipids where, n= 9-12 either with or without the phospholipids dissolved in isopropanol. The data show significant decreases in the presence of Phospholipon® 90H and L-α-Phosphatidylcholine when compared to controls (untreated and untreated + 0.002% IPA), *, P < 0.05 and **, P < 0.01.

The U87-MG cells were treated with increasing concentrations of phospholipids and incubated for 48 hrs. After the 48hrs, the cell viability was determined by using Cell titer glo® luminescence assay to detect ATP release of the live cell by giving luminescence to the U87 MG live cells. The cells treated with soy derived phospholipids at concentrations of 10 μM 20 μM, 30 μM, 40 μM and 50 μM were compared with the untreated U87-MG cell line either alone or in presence of 0.002% IPA(B). The results show that Lipoid S-100 and Phospholipon® 90H can evoke significant (*, P < 0.05) decreases in cell viability in comparison with U87-MG cell line treated with 0.002% IPA (B). However,

L-α-Phosphatidylcholine had no significant effect in comparison to U87-MG cell line treated with 0.002% IPA (B) (Figure 3.3). Lipoid S-100 evoked significant decreases at concentrations of 20-50 μM (**, P <0.01) and Phospholipon® 90H induced significant decreases at concentrations of 20 μM and 30 μM and pronounced dramatic decreases at 40 μM and 50 μM in comparison to U87-MG cell line treated with or without 0.002% IPA (B) (**, P < 0.01). L-α-Phosphatidylcholine had no significant effect. The untreated U87-MG cell line treated with 0.002% IPA (B) showed only a slight but significant inhibition compared to the untreated U87-MG cell line, *, P < 0.05 (figure 3.3)

3.2 Comparitive study of the three different cell lines in response to each phospholipids

3.2.1. The effect of soy derived Lipoid S-100 on 1321N1, GOS-3 and U87-MG.
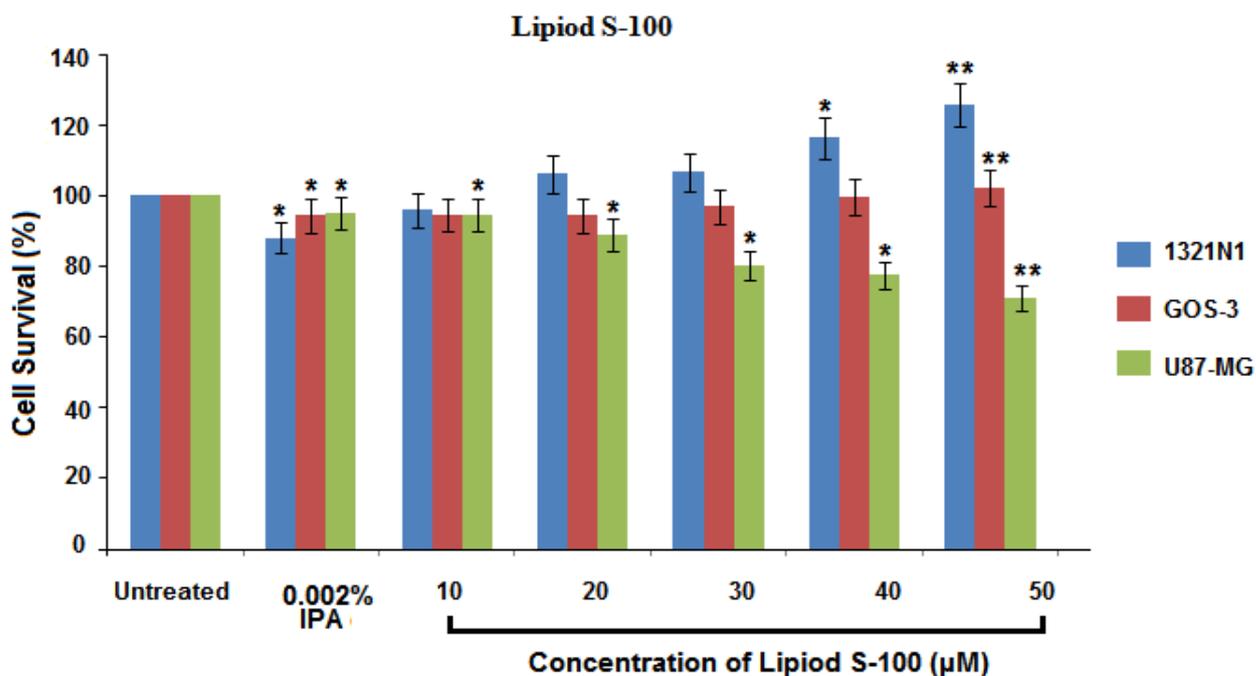
Figure 3.4: Effects of 10 μM, 20 μM, 30 μM, 40 μM and 50 μM Lipoid S-100 on cell viability employing 1321N1, GOS-3 and U87-MG for comparison cell viability (data are expressed as percentage of untreated control). The untreated and 0.002% isopropanol treated cells are also shown for comparison. The Lipoid S-100 can evoked significant (*, $P < 0.05$) decreases in U87- MG cell viability at all concentrations tested with maximal effect at 50 μM. Similarly, Lipoid S-100 can evoke small but significant decreases in GOS-3 cell viability at all concentration tested but these effects were less pronounced compared to the effect of Lipoid S-100 on U87-MG cells. In contrast, Lipoid S-100 evoked significant (*, $P < 0.05$) increase in 1321N1 cell viability at 40 μM and 50 μM compared to control untreated cells. At 20 μM and 30 μM, Lipoid S-100 evoked a small increases in cell viability compared to control untreated cells. In all three cell lines 0.002% IPA elicit significant (*, $P < 0.05$) decreases in cell viability for all the cell line compared to untreated cells. Data obtained are expressed as mean ± S.D , where n=9-12 *, $P < 0.05$ and **, $P < 0.01$ for Lipoid S-100 as compared to untreated cells.

The cell viability for the 3 different cell line was measured after the treatment with the increasing concentrations of Lipoid S-100. The results showed significant increases for 1321N1 glial tumour at 40 μM (*, $P < 0.05$) and 50 μM (**, $P < 0.01$) as compared to untreated cells. GOS-3 glial tumour showed a significant increase at 50 μM (*, $P < 0.05$) as compared to untreated GOS-3 cells. In contrast, Lipoid S-100 significantly inhibited (*, $P < 0.05$) the growth of U87-MG showing decreases from 10 μM to 50 μM (Figure 3.4).

3.2.2 The effect of soy derived Phospholipon® 90H on 1321N1, GOS-3 and U87-MG.
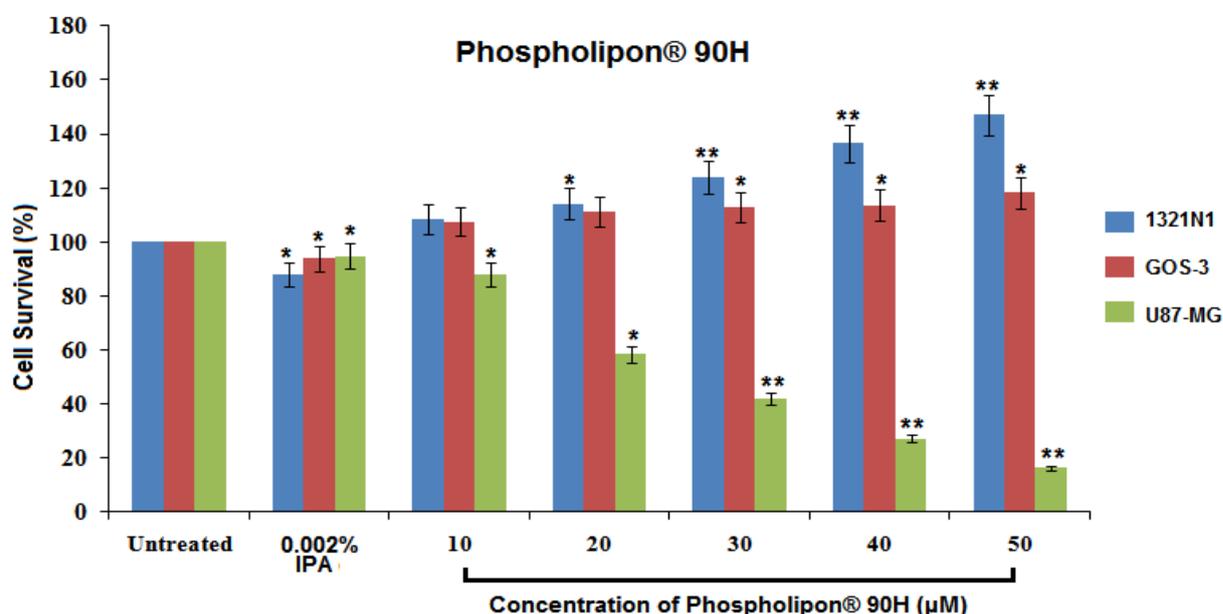
Figure 3.5 Effect of 10 μM, 20 μM, 30 μM, 40 μM and 50 μM Phospholipon® 90H on cell viability employing 1321N1, GOS-3 and U87-MG (data are expressed as percentage of untreated control). The untreated and 0.002% isopropanol treated cells are also shown for comparison. Phospholipon® 90H can evoke significant (*, $P < 0.05$) decreases in U87- MG cell viability at all concentrations tested with maximal effect at 50 μM. Similarly, Phospholipon® 90H can evoke small but significant increases in GOS-3 cell viability at 30-40 μM concentrations tested, but these effects were less pronounced compared to the effect of Phospholipon® 90H on 1321N1 cells. In contrast, Phospholipon® 90H evoked significant (*, $P < 0.05$) decreases in U87-MG cell viability at all concentrations compared to control untreated cells, Phospholipon® 90H evoked only a small increase in cell viability of 1321N1and GOS-3 compared to control untreated cells. In all three cell lines, 0.002% IPA elicited significant (*, $P < 0.05$) decreases in cell viability compared to untreated cells. Data obtained are expressed as mean ± S.D of Phospholipon® 90H, where n=9-12 and are considered significantly different if *, $P < 0.05$ and **, $P < 0.01$ as compared to untreated cells.

The soy derived phospholipid, Phospholipon® 90H at different concentrations was used to identify its effect on cell growth of 1321N1, GOS-3 and U87-MG cell lines in contrast to untreated 1321N1, GOS-3 and U87-MG cell lines. The 1321N1 cells treated with Phospholipon® 90H showed a significant increase at 20 μM (*, $P < 0.05$) and the increase was found to be from 30 μM up to 50 μM (**, $P < 0.01$). In the GOS-3 cell line, there was a moderate increase in cell survival from 30 μM to 50 μM. In contrast, Phospholipon® 90H showed moderate inhibition of U87 MG cell viability at 10 μM and showed a further extensive inhibition at 20 μM and above (*, $P < 0.05$) (Figure 3.5).

3.2.3 The effect of soy derived L-α-Phosphatidylcholine on 1321N1, GOS-3 and U87 - MG.
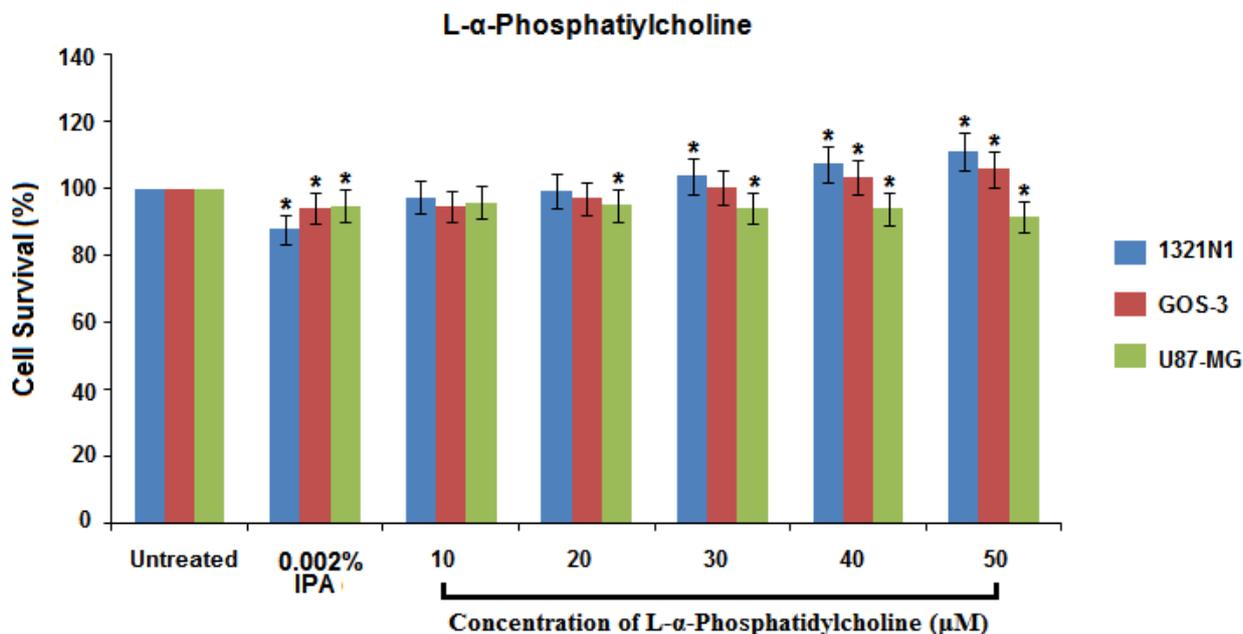
Figure 3.6: Effect of 10 μM, 20 μM, 30 μM, 40 μM and 50 μM L-α-Phosphatidylcholine on cell viability employing 1321N1, GOS-3 and U87-MG for comparison cell viability (data are expressed as percentage of untreated control). The untreated and 0.002% isopropanol treated cells are also shown for comparison. Treatment with the phospholipid shows variable effects on the three cell lines compared to the untreated cells. U87- MG cell line showed significant decreases compared to untreated cells, where as the treatment using L-α-Phosphatidylcholine on the 1321N1 cell line showed increases in cell survival at 30 μM and above, however, on the treatment using L-α-Phosphatidylcholine, GOS-3 showed an increase in cell survival at 40 μM and above compared to control (untreated). In all three cell lines 0.002% IPA elicit a significant (*, $P < 0.05$) decrease in cell viability compared to untreated cells. Data obtained are expressed as mean ± S.D, where n=9-12. L-α-Phosphatidylcholine are considered significantly different if *, $P < 0.05$ and **, $P < 0.01$ as compared to untreated cells.

Figure 3.6 shows the effect of the soy derived phospholipid, L-α-Phosphatidylcholine on 1321N1, GOS-3 and U87 MG cell line viability in comparison to the untreated cell lines for each. The 1321N1 cell line treated with L-α-Phosphatidylcholine showed no significant difference at 10 μM, while moderate increases were observed from 30 μM to 50 μM (*, $P < 0.05$). In the treated GOS-3 cell line, significant (*, $P < 0.05$) increases were observed at 40 μM and 50 μM. In contrast, U87- MG showed only slight decreases in cell viability from 20 μM to 50 μM (*, $P < 0.05$) (Figure 3.6)

4. Discussion

Phospholipids form the essential bilayers in all human membranes. Phospholipids present in the diet also play a major role in human health, especially since a number composition of phospholipids of various composition are present in certain foods. Soy derived phospholipids have phosphatidylcholine as their major composition. Soy derived phospholipids are the best source of phosphatidylcholine, as normal cells require it to maintain the integrity of the cell membrane (Wehrmüller, 2008).

Thus, this study employed three different phospholipids namely Lipoid S-100, Phospholipon[®] 90H and L-α-Phosphatidylcholine to investigate either their proliferating or inhibitory effects on three different glioma cell lines including 1321N1, GOS-3 and U87-MG compared to untreated cells and cells treated with 0.002% IPA for each cell line. The results from the study have demonstrated marked differences in the effects of increasing doses of each soy-derived phospholipid on the three glioma cell lines. The ATP analysis of the cell survival was carried out after 48 hrs following each treatment with phospholipid to measure either growth or inhibition of each the glioma cell line compared to control.

In this study, 0.002% of isopropyl alcohol (IPA) was employed to dissolve each phospholipid and as a second control, thus concentration of IPA was tested on each cell line for comparison with untreated cells. The results show that the three glioma cell lines when treated with 0.002% of IPA displayed small but significant (*, $P < 0.05$) decrease in cell viability in comparison to untreated glioma cells. This may be due to its slight toxic effect on the cell lines. The results also showed that Lipoid S-100, Phospholipon[®] 90H and L-α-Phosphatidylcholine can proliferate the growth of 1321N1 and GOS-3 but inhibited U87-MG cell lines after 48 hrs compared to untreated cells. Phospholipids have several impacts on human health. The entire cell membrane of living organisms is made up of varying composition of phospholipids. The integral phospholipids of cell membranes are involved

in maintaining the integrity of the cell membrane and cell signalling and therefore, crucial for the communication and interaction between in the body cells (Nelson *et al.,* 2005). There is much evidence that astrocytoma cell membranes have a substantial level of choline, alkylacyl-glycerophosphorylcholine compared to normal brain cells (Albert, 1977; Fan, 2006), which is in support of the present study. The soy derived phospholipids (Lipoid S-100, Phospholipon® 90H and L-α-Phosphatidylcholine) contain phosphatidylcholine as a major component with varying concentrations of fatty acids which could possibly affect the proliferation of glioma cell lines. In glioma cells, the content of omega-6 linoleic polyunsaturated fatty acid content is significantly higher compared to non-malignant brain tissue (Martin, 1996). It has been found that Lipoid S-100 has omega-6-linoleic fatty acids composition ranging from 60-70%, while L-α-Phosphatidylcholine has omega-6-linoleic fatty acids of approximately 64% attached to the glycerol backbone. The glial tumour 1321N1 and GOS-3 cell lines on treatment with increasing concentrations of Lipoid S-100 and L-α-Phosphatidylcholine evoked significant increases in the cell survival. Thus, the linoleic fatty acid and other omega-6 fatty acids attached to phosphatidylcholine could possibly serve as a nutritional source to the proliferation of 1321N1 and GOS-3 cell lines.

However, several previous studies have suggested that the essential fatty acid, linoleic acid can induce apoptosis of cancer at appropriate concentrations, without affecting the normal cells (Kwon, 2008). Therefore, U87-MG cells on treatment with either Lipoid S-100 or L-α-Phosphatidylcholine showed that apoptosis may be occurring to varying degrees. In contrast, treatment of 1321N1 and GOS-3 cell lines with the Phospholipon® 90H showed proliferative activity compared with Lipoid S-100 and L-α-Phosphatidylcholine. Phospholipon® 90H contains fatty acids, palmitic and stearic in the ratio of 15% and 85%, respectively. This phospholipid has been reported to have little difficulty passing the blood brain barrier and may serve as the source for the proliferation of glial tumours (Spector,

1988). The membrane of glioblastoma is found to have a greater amount of sphingomyelin than compared to phosphatidylcholine (Sun and Leung, 1974). However, U87-MG cell lines are epithelial glioblastoma, since, their growth can be decrease at high concentration of the phospholipids. At low concentration the phospholipids evoke either little or no effect, thus showing no significant change in the cell survival. This observation indicates that the amount of phosphatidylcholine present might be less. The U87- MG cells showed a significant decrease of cell survival on treatment with Phospholipon® 90H. However, on treatment with Lipoid S-100, there was an overall steady decrease in cell survival. On the other hand, the L-α-Phosphatidylcholine showed little or no effect compared to Phospholipon® 90H and Lipoids S-100.

Both Lipoid S-100 and Phospholipon® 90H have other phospholipid groups attached to the and they may also contribute to either growth or inhibition of the cells. A Lysolecithin molecule is reported to selectively evoke cytotoxic effect on cancer cells *in vitro* and it has no effect on the normal cells (Harmann, 1986; Hoffman 1976) whereas, L-α-Phosphatidylcholine contains the pure form of Phosphatidylcholine. The results of this study have demonstrated that differences in membrane phospholipid composition of neoplastic brain could possibly be related to either inhibition or proliferation of glioma due to the fatty acid present in the phospholipids taken exogenously.

The biochemical difference between normal and tumour cells lies in the composition of the glycolipids and phospholipids (Bergelson *et al.*, 1974; Burlakova *et a.l*, 1991; Burlakova *et al.*, 1980; Hostetler *et al.*, 1976; Spangler *et al.,* 1975). It has been reported that the difference in phospholipid content may attribute to the difference in rates of phospholipid transfer to the plasma membrane of aggressive tumours. In hepatocellular carcinoma (Palmina, 1995) cells, the activity of phosphatidylcholine activity was reported to be higher than in the control (Poortheius, 1980). Hepatocellular normal cells have the ability to

deplete the extrinsic phospholipid while the tumour cells incorporate the extrinsic phospholipid to accumulate and attenuate the growth of tumour cells. The phospholipids content varies from tumour to tumour (Palmina, 1995). Previous reports have proven that the plasma membrane of brain cells and neural cholines are elevated on the oral administration choline. This is probably caused by the release of acetylcholine in the normal brain. In the tumour cells, oral administration of phosphatidylcholine causes accumulation since there is no release of acetylcholine (Cohen, 1976; Hubrich, 1976). Many varieties of cancerous tissues contain more phosphatidylcholine with increased amounts circulating in the blood and available for use by the tumour (Nilolasev, 1972; Nordoy, 1990; Palmina, 1995).

In order to substantiate the hypothesis, it is necessary to measure the level of the different phospholipids in the cell lines, in addition to the measurement of apoptosis. These experiments may produce fundamental new insights into the action of each phospholipids on the three different glioma cell lines. Further studies are also required employing other related phospholipids.

In summary, it can be assumed from this study that 1321N1 and GOS-3 astrocytoma cell lines have a relatively high amount of phosphatidylcholine accumulation which seems to attenuate the growth of the cells. In contrast, U87-MG glioblastoma cell line has less accumulation of phosphatidylcholine which does not support the growth of U87- MG cell line.

5. Conclusions

1. The ATP assay is very sensitive and easy to measure cell death. The effects of different concentrations of the soy derived phospholipids on 1321N1, GOS-3 and U87-MG cell viability were investigated employing the ATP assay to determine either cell proliferation or cell inhibition.

2. The soy derived phospholipids, Lipoid S-100, Phospholipon® 90H and L-α-phosphatidylcholine were found to induce the growth of 1321N1 and GOS-3 cell lines at higher concentrations, when compared with untreated cells and cells treated with 0.002% IPA.

3. Lipoid S-100 and Phospholipon® 90H were found to decrease the growth of U87-MG cell line as compared to untreated U87-MG and untreated U87-MG + 0.002% IPA. In contrast, L-α-phosphatidylcholine showed no statistical significant effect on U87-MG in comparison to untreated U87-MG or cells treated with 0.002% IPA which evoked a mild inhibition in cell viability compared with the untreated U87-MG.

4. When treated with increasing concentrations (10 μM, 20 μM, 30 μM, 40 μM and 50 μM) of Lipoid S-100 and L-α-phosphatidylcholine, 1321N1 cell line showed a moderate increase in growth compared to Phospholipon®90H which showed a much larger increase in cell proliferation.

5. The GOS-3 cell line, when treated by increasing concentrations (10 μM, 20 μM, 30 μM, 40 μM and 50 μM) of Lipoid S-100 and L-α-phosphatidylcholine showed a moderate increase in growth compared to Phospholipon® 90H which showed marked increase in cell viability.

6. When treated with increasing concentrations of (10 μM, 20 μM, 30 μM, 40 μM and 50 μM) Lipoid S-100, U87-MG cell line showed a significant decrease in cell

viability. In contrast, Phospholipon® 90H showed a dramatic decrease in cell viability. L-α-phosphatidylcholine showed no significant effect on the U87-MG cell line.

7. In conclusion, the results of this study have indicated that the soy derived phospholipids Lipoid S-100, Phospholipon® 90H and L-α-phosphatidylcholine can possibly enhance the growth of low grade astrocytoma 1321N1 (grade II/III) and GOS-3 (grade III). In contrast, they seem to inhibit growth of high grade U87-MG (grade IV).

6. Scope for future study

1. The soy derived phospholipids Lipoid S-100, Phospholipon® 90H and L-α-phosphatidylcholine could possibly work as a marker to investigate the quantity phospholipids taken up by increased in the tumour and other cancer cell lines.

2. The effect of Lipoid S-100, Phospholipon® 90H and L-α-phosphatidylcholine should be tested on the normal human astrocytes.

3. The effect of Lipoid S-100, Phospholipon® 90H and L-α-phosphatidylcholine could be tested on other cancer and tumour cells.

4. The effect of other soy derived phospholipids phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol should also be tested on the glioma cell line.

5. The apoptosis of cells on the treatment with soy derived phospholipids could be analysed with the help of appropriate fluorescent assays.

6. Cytofluorimetric studies on the treatment with phospholipids will help to analyse the necrosis by identifying the morphology of tumour cells before and after treatment of the tumour cells.

7. Isolate the live cells and examine the morphological changes by the gene expression on real time PCR method.

8. Determine the mechanism of cell death by measuring cytochrome c, p53, caspase 3 activities and cytosolic calcium.

9. Measure the distribution of phospholipids in normal and glioma cell lines based on the chromatographic technique.

10. Determine the cell viability following treatment with commercial anticancer drugs with the combination of the different phospholipids on different cell lines.

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THE EFFECT OF SPECIFIC PHOSPHOLIPIDS THAT MAY HAVE POSSIBLE ROLE IN PROPAGATING AND INHIBITING GLIOMA

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AIMS

The main aim of the study was to investigate whether there is a possible link between specific phospholipids and either inhibition or proliferation of the growth of three specific glioma cell lines.

INTRODUCTION

Glioma is a type of brain tumour arising in glial cells of brain which is one of the causes of the death[1]. Every year approximately 2% of the cases in U.K are diagnosed with glioma, in spite of substantial increase in understanding of the molecular mechanism. Glioma is typically categorized as either primary or secondary brain tumour. Primary brain tumour starts in brain and secondary brain tumour spreads through other part of the body towards brain[2]. There are many types of glioma, they are categorized based on the areas in the brain (Fig 1). In our study primary glioma cells used are 1321N1 (grade II/III), GOS-3 (grade III) and U87-MG (grade IV).

Fig 1. Location and different types of brain tumour

In our day-to-day diet , we consume phospholipids from the various food products including egg, milk, soybean etc. Environmental factor such as food could also be one of the contributing factor for glioma[3]. This study was designed to investigate the possible effect of the dietary phospholipids from soy (Fig 2) either proliferating or inhibiting the growth of glioma. In this study the soy phospholipids Lipiod S-100 (lipoid GmbH, Germany), Phospholipon[R] 90H (Phospholipid GmbH, Germany) and L-á-Phosphatidylcholine (Sigma Aldrich, UK) were used to find whether soy phospholipid act as external supplemented nutrition for the growth of glioma.
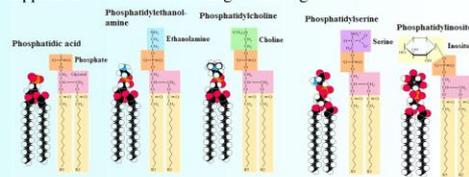
Fig 2. The structure of the soy phospholipids: Phosphatidic acid, Phosphatidylethanolamine, Phosphatidylcholine, Phosphatidylserine and Phosphatidylinositol.

METHODS

1. Preparation of phospholipid solution

- 5-25 M of the phospholipids sample was weighed in the sterile conditions.
- Each phospholipid sample weighed was dissolved in 5 ml of Isopropanol (IPA) (stock solution).
- 2 µl of the stock solution was diluted in 998 µl of cell medium providing final concentration from 10-50 µM.
- 200 µl of medium containing phospholipid solution were used to treat the cells.

2. Applying tissue culture technique

- Cells like U87-MG,1321NI and GOS-3 were cultured in 96 well plates.
- Cells were then incubated and allowed to reach a confluence of 40-50% for 48hrs.
- 10-50 µM concentration of phospholipid solution dissolved in medium were added to the cells.
- Treated cells were then incubated for 48hrs.
- Cells were then analysed by ATP assay on Teccan plate reader.
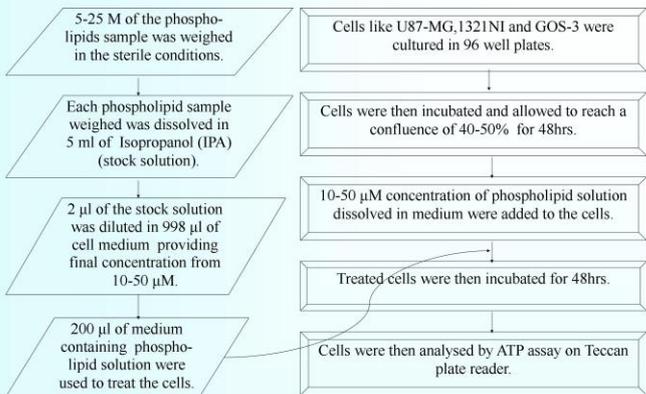
RESULTS
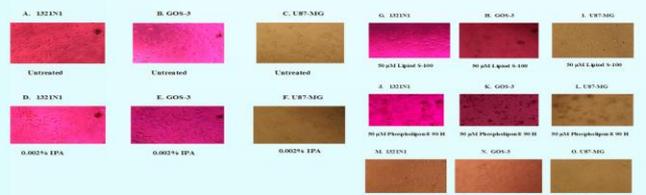
Fig 3. Morphology of the 1321N1, GOS-3 and U87-MG cell lines in (A-C) untreated condition, (D-E) treated with 0.002% IPA, (G-I) treated with 50 µM Lipoid S- 100, (J-E) treated with 50 µM Phospholipon® 90H and (M-O) treated with 50 µM L-α-phosphatidylcholine. All the cell lines were incubated either alone or with the different conditions for 48 hours before phospholipids were treated. These are typical of 4-5 such different experiments. Note the changes in the cell morphology comparing treated with control untreated cells.

**Measurement of ATP release of cells on Teccan plate reader after 48hrs of incubation .**
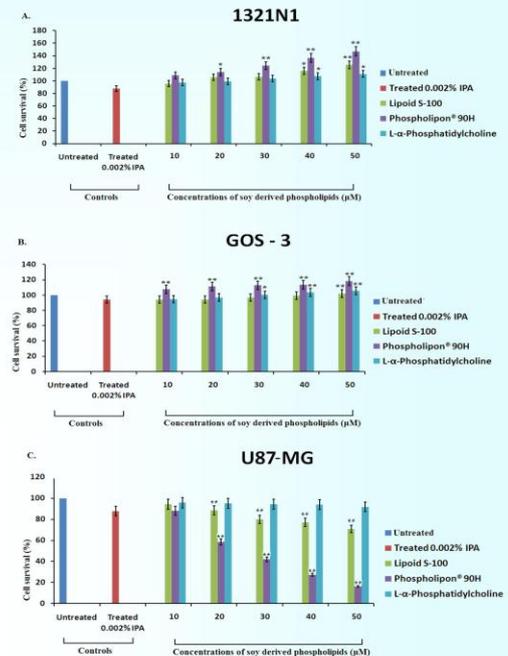
1321N1

GOS - 3

U87-MG

Fig 4. Effects of five different concentrations (10 µM, 20 µM, 30 µM, 40 µM and 50 µM) of either Lipoid S-100, Phospholipon® 90H or L-α-Phosphatidylcholine on viability of the (**A**)1321N1 cell line, (**B**) GOS-3 cell line and (**C**) U87-MG cell line (expressed as a percentage of control untreated cell lines). The viability of the untreated and treated with 0.002% IPA are also shown in the figures for comparison. Data for treated cells compared to untreated (control) cells are mean± S.D, n=9-12 (*, $P < 0.05$ and **, $P < 0.01$).

CONCLUSION AND FUTURE WORK

These results have indicated that soy derived phospholipids can enhance the growth of the low grade astrocytoma 1321N1 and GOS-3 cell lines and they do not support the growth of high grade glioblastoma U87-MG. Further experiments are required to determine the mechanism of action of soy derived phospholipids in either proliferating or inhibiting 1321N1, GOS-3 AND U87-MG.

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