

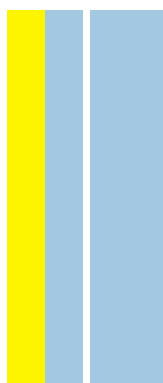
DISSERTAÇÃO DE MESTRADO
TOXICOLOGIA E CONTAMINAÇÃO AMBIENTAIS

Anticancer activity against glioblastoma cell lines by compounds present in algae, alone and in combination with anticancer drugs

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**Anticancer activity against glioblastoma cell lines by compounds
present in algae, alone and in combination with anticancer drugs**

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Abstract

Glioblastomas (GBM) are the most aggressive and lethal tumor of the central nervous system with an average life expectancy of only 1 to 2 years after diagnosis, even with the use of standard treatment of surgery, radiation, and chemotherapy. There are several anticancer drugs with alkylating properties that have been used in the therapy of malignant gliomas. Temozolomide (TMZ) is an anticancer drug widely used, sometimes in combination with ionizing radiation. However, one of the downsides of using this type of drugs in the treatment of GBM is the development of cancer drug resistance. As so, research of bioactive compounds with anticancer activity has been, in the last decades, heavily explored in terrestrial environments. However, also marine environments appear as a promising source of new bioactive compounds. Several natural compounds, existing in seaweed, have shown cytotoxic activity in *in vitro* and *in vivo* models acting through different molecular mechanisms, such as antioxidant, antiproliferative, DNA damage/DNA repair and apoptosis induction. Less explored is the ability of seaweed compounds to enhance the cytotoxic action of anticancer drugs. So, the combination of natural compounds with conventional drugs can be seen as an interesting strategy to be studied.

The aim of this work was to assess the anticancer effect of fucoxanthin (Fx) and phloroglucinol (Ph), two natural compounds present in many seaweed, either alone or in combination with TMZ, in two glioblastoma cell lines (U251 and T98G). Results showed that both cell lines are clearly different in terms of sensitivity to TMZ, in that U251 demonstrated a higher sensitivity than T98G cells. Also, Fx and Ph demonstrated cytotoxic and anti-proliferative effects against both cell lines, being the effects more evident in U251 cells. However, the mechanisms behind these effects do not appear to be the induction of cell death by apoptosis or DNA damage. The combination demonstrating the highest interest is 10 μ M TMZ + 10 μ M Fx, in U251 cells, in which was seen that Fx potentiates the TMZ action. Nevertheless, more studies are required to elucidate the mechanisms involved. In conclusion, Fx and Ph showed antiproliferative effects against glioblastoma cell lines and in the case of Fx may potentiate the activity of the alkylating drug TMZ. Fx and Ph appear as promising anti-glioblastoma agents that should be more explored.

Resumo

Glioblastomas (GBM) são dos tumores mais agressivos e letais do sistema nervoso central com uma esperança média de vida de 1 a 2 anos após diagnóstico, mesmo com o uso de tratamentos envolvendo cirurgia, radioterapia e quimioterapia. Existem diversos fármacos anticancerígenos com propriedades alquilantes que têm sido utilizados na terapia de gliomas malignos. A temozolomida (TMZ) é um fármaco anticancerígeno amplamente utilizado, por vezes em combinação com a radiação ionizante. No entanto, uma das desvantagens do uso deste tipo de fármacos no tratamento de glioblastomas é o desenvolvimento de resistência ao fármaco. Como tal, a procura de novos compostos bioativos com atividade anticancerígena tem sido, nas últimas décadas, fortemente explorada em ambientes terrestres. Contudo, também os ambientes marinhos parecem ser uma fonte promissora de novos compostos bioativos. Vários compostos naturais, existentes em algas, têm demonstrado ter atividade citotóxica em modelos *in vitro* e *in vivo* atuando através de diferentes mecanismos moleculares, através de efeitos antioxidantes, antiproliferativos, indutores de apoptose e de danos/reparação de ADN. Menos explorado tem sido a capacidade dos compostos extraídos de algas de aumentar a ação citotóxica dos fármacos anticancerígenos. Deste modo a combinação dos compostos naturais com os fármacos convencionais poderá ser uma estratégia interessante a ser estudada.

O objetivo deste trabalho foi a avaliação dos efeitos anticancerígenos da fucoxantina (Fx) e do floroglucinol (Ph), dois compostos naturais que estão presentes em algas, quer sozinhos ou em combinação com a TMZ, em duas linhagens celulares de glioblastoma (U251 e T98G). Os resultados mostraram, claramente, que as duas linhagens diferem em termos de sensibilidade à TMZ, em que as células U251 demonstraram ser mais sensíveis do que as células T98G. Além disso, a Fx e o Ph demonstraram efeitos citotóxicos e anti proliferativos em ambas as linhagens, sendo estes efeitos mais evidentes nas células U251. No entanto, os mecanismos responsáveis por estes efeitos não parecem estar relacionados com a indução de morte celular por apoptose ou por danos no ADN. A combinação que mostrou ser de maior interesse é a 10 μ M TMZ + 10 μ M Fx, nas células U251; situação em que a Fx potenciou a ação da TMZ. Apesar disto, mais estudos são necessários para ajudar a elucidar os mecanismos envolvidos. Em conclusão, a Fx e o Ph demonstraram efeitos antiproliferativos contra linhagens celulares de glioblastoma e no caso da Fx poderá potenciar a atividade do fármaco alquilante TMZ. A Fx e o Ph parecem ser agentes anticancerígenos promissores que devem ser mais explorados.

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Abbreviations

AIC – 5-aminoimidazole-4-carboxamide

BER – Base Excision Repair

BFP – 2,4-bis(4-fluorophenylacetyl)phloroglucinol

DAPI – 4',6-diamidino-2-phenylindole

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic Acid

DSB – Double Strand Breaks

EDTA – Ethylenediaminetetraacetic Acid

EGFR – Epidermal Growth Factor Receptor

FBS – Fetal Bovine Serum

FPG – Formamidopyrimidine DNA Glycosylase

Fx – Fucoxanthin

GBM – Glioblastoma

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IAP – Inhibitor of Apoptosis Proteins

LMP – Low Melting Point Agarose

MCPP – 3,6-bis(3-chlorophenylacetyl)phloroglucinol

MEM – Minimum Essential Medium Eagle

MGMT – O6-methylguanine-DNA-methyltransferase

MMR – Mismatch Repair

MPG – N-methylpurine-DNA glycosylase

MTIC – 5-(3-methyltriazene-1-yl)-imidazole-4-carboxamide

N3-meA – N3-methyladenine

N3-meG – N3-methylguanine

N7-meG – N7-methylguanine

NMP – Normal Melting Point Agarose

NO – Nitric Oxide

Pa – Pheophorbide a

PARP – Poly(ADP-ribose)

PBS – Phosphate Buffered Saline

Ph – Phloroglucinol

PFA – Paraformaldehyde

PTEN – Phosphatase and Tension Homolog

Quercetin – 3,3',4',5,7-pentahydroxyflavone

RSG – Relative Suspension Growth

ROS – Reactive Oxygen Species

SSB – Single Strand Breaks

T98G – Human Glioblastoma Anaplastic Astrocytoma Cell Line

TCGA – The Cancer Genome Atlas

TERT – Telomerase Reverse Transcriptase

TMZ – Temozolomide

TSG – Total Suspension Growth

U251 – Human Glioblastoma Astrocytoma Cell Line

VEGF – Vascular Endothelial Growth Factor

WHO – World Health Organization

XIAP – x-linked Inhibitor of Apoptosis

Chapter 1

General Introduction

1. Cancer

Cancer is a leading cause of death in developed and in development countries mainly due to the growth and aging of the population (Anand et al. 2008, Torre et al. 2015). Several factors have been identified to cause cancer, in which hereditary genetic factors are involved in 5-10 % of the cases whereas environmental factors account for around 90-95 % of all cancers (Anand et al. 2008). Infectious agents, environmental pollution, smoking tobacco, alcohol consumption, diet, obesity, radiation and physical inactivity are examples of environmental factors and lifestyle behaviors that have been associated with the development of cancer (Jemal et al. 2011, Torre et al. 2015).

Despite the enormous amount of research and rapid developments in this field, the projections suggest the increase of the world population to 7.5 billion by 2020 and the diagnosis of approximately 15 million new cancer cases (Anand et al. 2008). Nevertheless, it could be fought through prevention and awareness, in which several measures are put in place to try lower the cancer risk factors such as vaccination for viral-induced cancers, annual cancer screenings in critical age-groups, physical exercise, caloric restrictions, the consumption of vegetables, fruit, vitamins and wholegrain foods, amongst others (Anand et al. 2008). In terms of treatments, nowadays there are several strategies with different types of anticancer drugs that are in place however due to several limitations, such as cancer drug resistance, amongst others, are not very efficient in some cases (Goellner et al. 2011).

1.1. Carcinogenesis

The process of carcinogenesis starts when normal cells acquire malignancy and become abnormal cells that grow out of control. This happens due to the accumulation of DNA damage that could be due to errors in repair, replicative damage or even by chemical induction (Bertram 2000, Cohen and Arnold 2008). If the DNA damage is not properly repaired, through the intrinsic mechanisms, the cells will accumulate more and more mutations, during the replication process. These mutations can bring some survival advantages for changed cells compared with normal cells. Both genetic and epigenetic changes contribute to cancer development that present specific features, described by Hanahan and Weinberg (2011) as “hallmarks of cancer”. These hallmarks are defined as some characteristics that are shared by all types of cancer and that promote the tumor growth and spread of metastasis. The hallmarks are the following: resisting cell death, sustained proliferative signaling, inducing angiogenesis, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, avoid immune

destruction, deregulation cellular energetics, tumor-promoting inflammation and genomic instability (Hanahan and Weinberg 2011, Floor et al. 2012, You and Jones 2012).

The proliferation of cancer cells can lead to the formation of a neoplasm, known as a tumor. Not all tumors are cancerous. Benign tumors do not invade other tissues or spread to other parts of the body and do not cause major health threats. By the other hand, malignant tumors are the opposite, being very invasive and prone to metastization. This formation of metastasis happens when the cancer cells enter the bloodstream or the lymphatic system and goes to another part of the body leading to the formation of a new tumor that replaces the normal tissue (Chaffer and Weinberg 2011, Marusyk et al. 2012).

1.2. Glioblastomas

Gliomas are brain tumors develop from glial cells which are the non-neuronal cells of the brain. Several tumors can be designed as gliomas, which contain astrocytomas, oligodendrogliomas, ependymomas and glioblastomas (also recognized as glioblastoma multiforme) (Fukushima et al. 2009, Zhang et al. 2012). Glioblastomas (GBM), which are classified into grade IV astrocytoma by the World Health Organization (WHO) (Louis et al. 2007), are the main primary tumor of the central nervous system with a reduced survival time after diagnosis (1 to 2 years) even with the use of aggressive treatments involving surgery, radiotherapy and chemotherapy (Friedman et al. 2000, Jakubowicz-Gil et al. 2010). The main hallmarks of GBM include cell cycle dysregulation and uncontrolled cellular proliferation, cell survival (resistance to apoptosis), invasion and angiogenesis (Zhang et al. 2012).

In overall, signs and symptoms of glioma differ from person to person depending on the location of the glioma and could include a headache, visual loss, seizures, nausea and vomiting, cranial nerve disorders, pain, weakness, and numbness (Zeng et al. 2015). It is recognized that the interactions between environmental and genetic factors may be attributed to the development of gliomas. There are several environmental risk factors that could contribute to the development of glioma and that include alcohol consumption, allergic conditions, smoking, infectious agents and even electromagnetic radiation from cell phones. In accumulation to factors mentioned, genetic factors may also be implicated in the pathogenesis of glioma (Zeng et al. 2015).

As mentioned, nowadays several treatments are available such as surgical removal, radiotherapy, and chemotherapy, however, the survival rate is still very low. It is known that cancer drug resistance is a major problem in the treatment of GBM (Messaoudi et al. 2015).

This could be because therapies based only in one drug are less efficient in the elimination of cancer cells, not because the drug could be less effective, but due to the large genetic diversity of the tumors and that normally each drug acts by one or two pathways. As so the combination of cancer drugs against glioblastoma might be more promising since they have more probability of eliminating a bigger number of cancer cells (Jiang et al. 2014, Signore et al. 2014).

1.2.1. Molecular mechanisms involved

The Cancer Genome Atlas (TCGA) (McLendon et al. 2008) have demonstrated that GBM is a heterogeneous tumor type at the molecular level and could be sub-classified into different biologic entities, different from each other based on common genomic, epigenomic and transcriptional characteristics (Weathers and Gilbert 2014, Aldape et al. 2015). GBMs can be referred to as “primary” or “secondary” GBM depending if they arise *de novo* or if they evolve by progression from a lower-grade glioma (Cloughesy et al. 2014, Aldape et al. 2015). Notably, these two clinical demonstrations have distinct molecular hallmarks which could include telomerase reverse transcriptase (TERT) promoter mutation, amplification of the epidermal growth factor receptor (EGFR) gene and phosphatase and tension homolog (PTEN) tumor suppressor gene mutation in primary GBMs and TP53, IDH1/2 mutations in secondary GBMs (Cloughesy et al. 2014, Aldape et al. 2015). So, Chen et al. (2012) revealed a set of oncogenic pathways that are usually activated in glioblastomas such as the p53 pathway, the RB pathway, and the receptor tyrosine/kinases/RAS/phosphatidylinositol 3 kinase (RTK/RAS/PI3K) pathway. These genetic alterations will benefit cell proliferation and increase cell survival while permitting the tumor cell to escape from cell-cycle checkpoints, senescence and apoptosis (Chen et al. 2012, Cloughesy et al. 2014, Aldape et al. 2015). Beyond genetic changes, epigenetic modifications such as hypermethylation of tumor suppressor genes and pro-apoptotic genes and hypomethylation of genes that are normally silenced such as MMP9 gene (related with invasion) amongst others have been described in glioblastoma (Maleszewska and Kaminska 2013).

1.2.2. Treatment – Chemotherapy

Due to their characteristics, GBMs are very aggressive and difficult to remove by surgery, so the treatment requires the combination of chemotherapy and radiotherapy (Weathers and Gilbert 2014). The standard first-line of treatment for GBM includes the use of alkylating agents such as temozolomide (TMZ), which is widely used, sometimes in

combination with ionizing radiation (Moody and Wheelhouse 2014, Weathers and Gilbert 2014). These alkylating agents exist in the environment – like in fuel combustion or in cigarette smoke, in diet, such as in processed meat, or could exist as a product of endogenous metabolic processes – and when in low concentrations can be mutagenic and carcinogenic (Ramos et al. 2013, Roos and Kaina 2013). Due to their ability to induce extensive DNA damage and cell death, alkylating agents are used in cancer chemotherapy and have shown benefits for cancer patients, in which is seen an enhancement, when compared to patients treated with radiation alone, in the average overall survival (Thon et al. 2013, Weathers and Gilbert 2014). Alkylating agents induce a great diversity of DNA lesions due to its reactivity with different nucleophilic atoms on the DNA bases. There are different types of alkylation damage, N-alkylated adducts, which happens in large quantities (about 80 %, more abundant and more cytotoxic), such as N7-methylguanine (N7-meG), N3-methyladenine (N3-meA) and N3-methylguanine (N3-meG), and the O-alkylated adducts, which happens less (less than 10 %, less abundant but more mutagenic), and include O6-methylguanine (O6-meG) and O4-methylthymine (O4-meT) (Kondo et al. 2010, Ramos et al. 2011, Ramos et al. 2013).

TMZ was synthesized in the 1980s (Fukushima et al. 2009) and is a monofunctional SN1 type alkylating agent, which means that it can alkylate both oxygens and nitrogens in nucleic acids (Yoshimoto et al. 2012). It is one of second-generation imidazotetrazinone prodrugs that naturally converts into the active metabolite without the need for enzymatic demethylation in the liver (Parisi et al. 2015). It is administered orally and, like other cytotoxic alkylating agents, it easily penetrates the blood-brain barrier (Pan et al. 2012). TMZ when orally administered is absorbed intact and spontaneously metabolized at physiologic pH to form 5-(3-methyltriazene-1-yl)imidazole-4-carboximide (MTIC) in the organism (Friedman et al. 2000, Zhang et al. 2012). MTIC is broken down and liberates methyldiazonium cation and 5-aminoimidazole-4-carboxamide (AIC) (Figure 1). AIC is excreted through the kidneys and methyldiazonium cations, that is highly reactive, form methyl adducts mainly at N7 positions of guanine and N3 of adenine but also methylates O6 guanine residues (Friedman et al. 2000, Zhang et al. 2012).

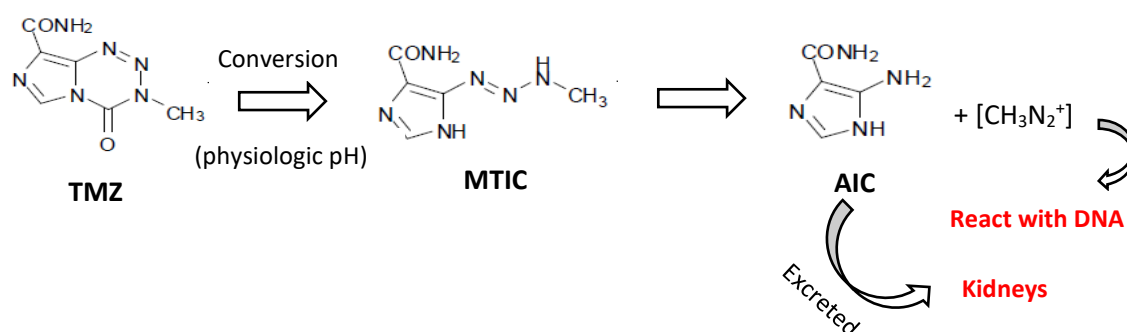


Figure 1 – Activation of TMZ. Source: Adapted from Zhang et al. (2012)

Besides the abundance of N-methylations N7-meG, N3-meA and N3-meG, these damages are repaired by the base excision repair (BER). The MPG (also known as N-methylpurine-DNA glycosylase) is a type I glycosylase, present in human cells, responsible for the removal of N-methylated bases by hydrolyzing the N-glycosidic bond leaving an apurinic/apyrimidinic site (AP site) in the DNA. Overexpression of MPG may produce an imbalance of the BER system, after treatment with alkylating agents, which may lead to an increase of the toxicity of the N-methylations (Ramos et al. 2011, Roos and Kaina 2013, Yamamoto et al. 2015).

The O6-meG are formed when the methyl groups are transferred to the 6th position of oxygen atoms of guanine. This type of damage can be repaired by O6-methylguanine-DNA methyltransferase (MGMT). However, if not repaired, during DNA replication, O6-meG mispairs with thymine instead of cytosine (Ramos et al. 2013). The post-replication mismatch repair system (MMR), can recognize the O6-methylguanine: thymine mispairs, which excises it introducing strand breaks and leaving the O6-meG, that if not repaired will pair again with thymine during next replication (Zhang et al. 2012). The action of MMR will result in the accumulation of high cytotoxic double-strand breaks (DSB) that if not repaired by the recombination repair pathway, result in cell cycle arrest and cell death (Fukushima et al. 2009).

1.2.3. Main mechanisms of resistance

Unfortunately, the treatment of GBM patients with TMZ is not always effective (Pan et al. 2012). The major problem in chemotherapy is the cancer drug resistance that is regulated by several intrinsic and extrinsic factors, such as the tumor microenvironment, the potency of the anticancer drug, the heterogeneity of cancer cells and the response of cancer cells to the drugs (Han et al. 2007). In GBM several mechanisms are involved in the

development of resistance to methylating agents including: changes in DNA repair mechanisms such as increased expression of the repair enzyme MGMT, impairment of MMR or BER pathway induced by genetic and epigenetic changes; resistance to apoptosis with low levels of Bax (pro-apoptotic protein), and increased Bcl-2 (anti-apoptotic protein), amongst others (Figure 2) (Roos et al. 2007, Kang et al. 2010, Ha et al. 2013).

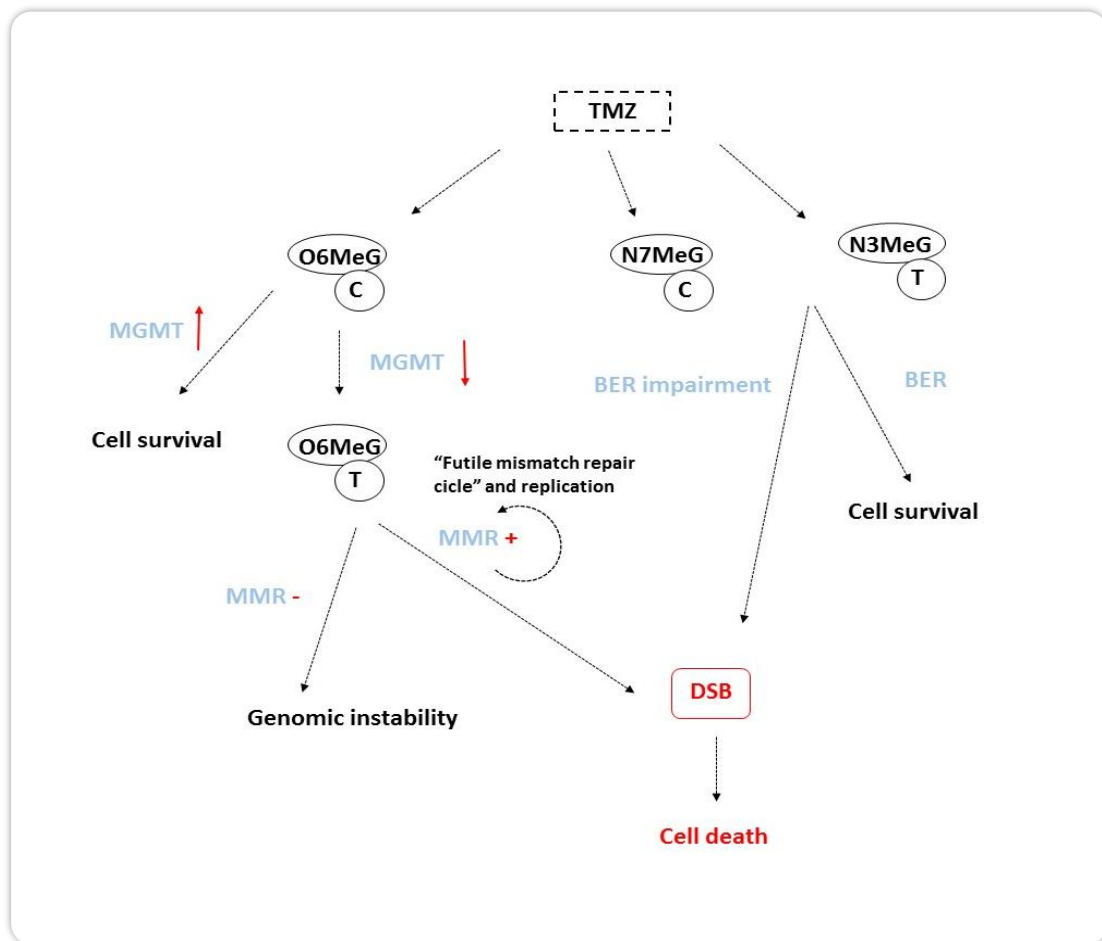


Figure 2 – Scheme of the major DNA damages induced by temozolomide (TMZ) and the cell's mechanisms of resistance. TMZ induce a diversity of DNA lesions such as N7-methylguanine (N7-meG), N3-methylguanine (N3-meG) and the O6-methylguanine (O6-meG). The O6-meG lesion can be repaired by MGMT, that when in high levels can lead to cell survival, however if the MGMT levels are low, during DNA replication, O6-meG mispairs with thymine instead of cytosine. The MMR proficient (+) can recognize the O6-meG:T mispairs, removing it and forming SB leaving the O6-meG, that if not repaired, enters in a futile mismatch repair cycle. MMR-deficient cells (-) that are unable to suffer apoptosis generate genomic instability. The N7-meG and N3-meG are quickly repaired by BER leading to cell survival, however when the BER is impaired DSB are formed. The action of the MMR and BER systems will result in the accumulation of DSB that if not repaired will lead ultimately to cell death. Source: Adapted from Roos and Kaina (2006).

MGMT - O6-methylguanine-DNA methyltransferase

MGMT is a DNA repair protein which repair alkylation damages removing the methyl group at the O6 position of guanine added by the alkylating agents. Expression of MGMT varies in accordance with species, organs, kinds of the tumor and cell lines (Fukushima et al. 2009). MGMT interposes by accepting the methyl group from DNA to an internal cysteine residue inside its active site, in an irreversible stoichiometric reaction. This reestablishes guanine in DNA but inactivates the MGMT, which is degraded through ubiquitination and suffers proteolysis (Zhang et al. 2012). No other DNA repair protein is known to act on O6-meG. Restoration of the protective mechanism requires *de novo* protein synthesis and can take up to 72 h (Payne et al. 2005). In cells, the level of MGMT expression correlates inversely with TMZ sensitivity. Depletion of MGMT enhances cellular sensitivity to O6-alkylating agents. Hypermethylation of the CpG island has been described as the essential mechanism for silencing of MGMT gene since deletion, mutation, rearrangement and mRNA instability of the MGMT gene are uncommon events (Fukushima et al. 2009). An important component of cellular resistance to the alkylating drugs is the overexpression of this protein that removes the DNA damage induced by TMZ. Conversely increased expression, through transfection with cDNA for the protein, renders cells resistant. Expression of MGMT in human tumor cells appears to exhibit greater variability than in corresponding normal tissue but is generally higher. Glioblastoma and melanoma, the tumors that are currently treated with TMZ, show the lowest levels of MGMT. However, a clear relationship between MGMT level and the response has only been shown for primary central nervous system tumors (Ma et al. 2003).

DNA-mismatch repair

Mismatch repair (MMR) system is involved in the recognition and correction of mispaired bases and insertion/deletion loops generated during DNA synthesis (Zhang et al. 2012). The MMR is activated when MGMT is depleted or suppressed by methylation of the gene promoter and cytotoxicity of TMZ is enhanced (Villano et al. 2009). So, during DNA synthesis, the MMR system recognizes O6-meG-thymine mispairs by the MutS and MutL complexes. These complexes are formed by the MSH2, MSH3 and MSH6 genes that identify small or large insertion-deletion loops and base-base mismatches (van Thuijl et al. 2015). Thymine is then removed, but due to the mispairing properties of O6-meG, thymine is reinserted during the next round of replication that will be again recognized by the MMR system and more strands are introduced. Accumulation of strand breaks can result in cell cycle arrest or apoptosis. MMR-deficient cells turn out to be tolerant to O6-meG DNA lesions

because they are incapable of suffering apoptosis in response to such lesions. Therefore, the level of MMR expression contributes to the sensitivity of cells to O6-meG which means that cells that express high levels of MMR have a higher sensitivity to O6-meG-triggered apoptosis than cells expressing lower levels (Villano et al. 2009, Roos and Kaina 2013).

Several authors have described that inactivating somatic mutations and epigenetic changes of some MMR genes grants resistance to alkylating agents in glioma *in vivo* and simultaneously accelerates mutagenesis in resistant clones. An example is MSH6 gene, when inactivated in gliomas, alkylating agents that are inducers of tumor cell death might convert to promoters of neoplastic progression. The connection with the progression of the tumor during TMZ treatment was significant reflecting the resistance conferred by MSH6 inactivation *in vitro*. The MSH6 deficit may add to the appearance of recurrent GBM during TMZ treatment. Another example is the promoter hypermethylation in MLH1 gene that has been also associated with the evolution of gliomas, namely with the development of resistance to alkylating agents (Frosina 2009, Zhang et al. 2012).

So, the cytotoxicity of TMZ is dependent on an intact MMR pathway. Therefore, genetic and epigenetic changes disturbing the correct functioning of the pathway leads to resistance to TMZ.

Base excision repair

Base excision repair (BER) is the main pathway involved in elimination and repair of modified bases (e.g. oxidized, alkylated and mismatched bases), AP sites, DNA single strand breaks (SSB) produced by reactive oxygen species (ROS), ionizing radiation and alkylating agents (Zhang et al. 2012). The main lesions induced by TMZ are N7-meG and N3-meA, however, are quickly repaired by BER. Lesion-specific glycosylases, like MPG, recognize damaged bases that hydrolytically break the N-glycosidic bond, generating an AP site. An AP endonuclease (APE-1) then cleaves the phosphodiester backbone on the 5' side of the AP site, leaving, at the DNA strand break, the 3'-OH and 5'-deoxyribose phosphate ends. The terminal residue is removed leaving a nucleotide gap. The repair can be done through a short patch BER including replacement of one nucleotide or a long patch BER involving gap filling of 2-10 nucleotides (Goellner et al. 2011, Zhang et al. 2012). At that time, the DNA polymerase β (poly β) together with XRCC1-Ligase III (Lig III) complex makes the repair process complete (Yoshimoto et al. 2012). However, it has been evidenced that the sensitivity to alkylating agents could be related to the DNA glycosylases expression, in which the expression of MPG controls the repair of the DNA damage induced

by TMZ, in GBM models, and that could lead to the development of resistance (Yoshimoto et al. 2012).

Cell death

When a cell suffers DNA damage the repair mechanisms are activated. However when that do not happen efficiently is induced the cell cycle arrest that could lead to cell death. So, DNA damage follows by cell death is based on complex enzymatic responses that could lead to apoptosis, autophagy, necrosis and other forms of cell death (Roos and Kaina 2006).

Apoptosis is a caspase-dependent programmed cell death and as so requires the activation of caspase proteases in order to cause rapid cell death that displays distinctive morphological and biochemical hallmarks, such as membrane blebbing, cell shrinkage, nuclear condensation and fragmentation, mitochondrial fragmentation, caspases activation, amongst others (Jakubowicz-Gil et al. 2013, Tait et al. 2014, Feoktistova and Leverkus 2015). Disturbances in apoptosis have a key role in pathogenesis, specifically in neurological and cardiovascular disorders, autoimmune diseases and cancer (Ouyang et al. 2012).

The role of apoptosis in the cytotoxicity of anticancer drugs, over the last few years, has become clearer. Apoptosis can occur via death receptor-independent (intrinsic or mitochondrial) or dependent (extrinsic) pathways (Lefranc et al. 2007). The mitochondrial pathway of cell death is intermediated by Bcl-2 family proteins, a set of antiapoptotic and pro-apoptotic proteins that control the path of small molecules, such as cytochrome c, which trigger caspase cascades through the mitochondrial permeability transition pore. In cells that are going to die, proapoptotic proteins such as Bax, disturb the mitochondria, leading to the release of cytochrome c to the cytosol which leads to activation of caspases and cell death (Lefranc et al. 2007, Nowsheen and Yang 2012, Jakubowicz-Gil et al. 2013). The extrinsic pathway is initiated by the activation of death receptors and includes the maturation of caspase-8. Both pathways lead to a final mutual pathway associated with activation of caspase-3 (Jakubowicz-Gil et al. 2013).

One of the main mechanisms involved in drug cancer resistance is apoptosis evasion that can occur by several mechanisms such as, reduced caspase function, impaired death receptor signaling, or disruption of the balance of pro-apoptotic and anti-apoptotic proteins, such as Bcl-2 family and p53. So dysregulations on these mechanisms could result in changes in apoptosis leading to resistance (Wong 2011, Karpel-Massler et al. 2015).

Autophagy is a caspase-independent process described by the increase of autophagic vacuoles in the cytoplasm, complemented by the degradation of Golgi apparatus and endoplasmic reticulum, which ultimately leads to the destruction of the nucleus (Jakubowicz-Gil et al. 2013). Actually, autophagy could play a pro-death or a pro-survival response depending on the cellular context. This pro-death role has been described as being activated in situations where apoptosis is inhibited, acting as another option for elimination of cancer cells (Levine and Yuan 2005, Ouyang et al. 2012). Autophagy as a pro-survival response has been described as a possible mechanism of drug cancer resistance. Several authors have demonstrated that TMZ can induce autophagy contributing for therapy resistance in glioma cell lines. (Knizhnik et al. 2013, Zanotto-Filho et al. 2015, Yan et al. 2016).

1.2.4. Main strategies to enhance the therapeutic effect of TMZ

It has been seen that, in GBM, TMZ is the anticancer drug that shows the best clinical performance. TMZ induces DNA damage and cell death, however, cancer drug resistance is still a huge problem in which new combined therapies are necessary to improve its efficacy (Zanotto-Filho et al. 2015).

One of the mechanisms involved in TMZ resistance is the high expression of MGMT, which repairs O⁶-meG lesions. As mentioned before, the MGMT-mediated repair, transferring the methyl group from guanine to an internal cysteine residue in the protein. After accepting the methyl group, it deactivates itself, making it a “suicidal protein”. Therefore, the research of drugs that modulate MGMT levels, such as O⁶-benzylguanine (O⁶-BG) or other correlated compounds, could help enhance the efficacy of chemotherapies using alkylating agents, such as TMZ (Nakada et al. 2012, Fan et al. 2013).

Another possible strategy to enhance TMZ action is through the inhibition of BER pathway. Poly(ADP-ribose) (PARP) is involved in detecting and signaling the DNA damage generated by methylating agents. PARP inhibition increases the cytotoxicity of TMZ in tumor cell lines and increases its anti-tumor effects (Payne et al. 2005, Villano et al. 2009). As noted, when O⁶-meG lesions are whether repair by MGMT or tolerated succeeding MMR disruption, N-methylpurine adducts become significant and then inhibition of BER increases TMZ therapeutic efficacy. Hence, disruption of BER by PARP inhibition offers a means to surpass resistance that regularly develops as a consequence of the selection of MMR-deficient cells during therapy (Zhang et al. 2012, Fan et al. 2013). Also, numerous inhibitors of APE activity have demonstrated to sensitize human cells to alkylating agents. One example is methoxyamine, which interrupts BER through a reaction with an aldehyde-sugar

group of the AP site, producing a steady intermediate adduct that blocks the endonuclease activity of APE. Through the inhibition of the BER, methoxyamine has demonstrated to potentiate the action of some alkylating agents in a range of cancer cell types (Fu et al. 2012).

Research in molecular profiling has led to the documentation of molecular prognostic factors in addition to the identification of molecular susceptibilities that might be targeted in the progress of new treatments in GBM (Weathers and Gilbert 2014). Therefore, in GBM, some important regulatory elements for the proper function of the cell and apoptosis, such as the Bcl-2 protein family, p53 protein, the inhibitor of apoptosis proteins (IAPs) or receptor tyrosine kinases such as the EGFR, are changed at the genetic and/or epigenetic levels modulating the expression and activity of these proteins. These alterations could be an appropriate target in GBM (Eisele and Weller 2013, Weathers and Gilbert 2014).

Another potential strategy is the inhibition of certain molecular targets related to apoptosis and autophagy resistance pathways, such as the mTOR, PI3K, and Akt. These inhibitors, such as for example, the X-linked inhibitor of apoptosis (XIAP) and rapamycin, which is an mTOR inhibitor, amongst others, could increase the level of sensitivity of cancer cells with resistance to apoptosis toward both proapoptotic and proautophagic drugs. Therefore, enhancement of therapeutic effects could be achieved with the combination of TMZ with these inhibitors as adjuvant chemotherapies (Lefranc et al. 2007).

1.3. Seaweed as source of bioactive compounds

Oceans covers more than 70 % of the planet surface and contains high biodiversity. It comprehends a varied number of animals, plants, and microorganisms, which are highly productive (Senthilkumar et al. 2013). Apropos, it is known that terrestrial and marine plants have been the most significant source of medicinal natural products (Folmer et al. 2010). More than 60 % of drugs present in the market were developed from or based on a natural compound (Harvey 2008, Molinari 2009, Harvey et al. 2015). These compounds are nowadays in use as anticancer, antibiotic, and anti-inflammatory drugs, amongst others biological activities (Folmer et al. 2010).

Seaweed comprise a wide number of species, but only a small amount is used by humans. Several applications have been described, such as in human and animal nutrition, in folk medicine, as a source of substances with pharmaceutic or industrial applications, and in agriculture as fertilizers, fungicides, and herbicides (Kılınç et al. 2013). The consumption of seaweed by humans occurs since ancient times, mainly in Asia. In Europe,

their use is more restrict but expanding (Cornish and Garbary 2010, Gambato et al. 2014). Several epidemiological studies revealed the great potential and health benefits of ingesting seaweed, such as a decrease in the number of individuals with coronary heart disease and cancer (Cornish and Garbary 2010, Senthilkumar et al. 2013, Murphy et al. 2014). Regarding the main photosynthetic pigment, cell wall component and storage products, macroalgae or seaweed, can be classified into three groups, such as green (Chlorophyta), red (Rhodophyta) or brown (Phaeophyta) seaweed (Gambato et al. 2014). All of them with high content in minerals, certain vitamins, and polysaccharides, but have also bioactive substances like polyphenols that can have certain beneficial properties (Senthilkumar et al. 2013). Seaweed normally grow in the intertidal zone, where they are constantly exposed to severe variations in temperature, oxygen concentrations, and UV radiation. However, the seaweed typically grow well, and one reason is that they produce bioactive substances that confer survival advantages. Some of these bioactive compounds are carotenoids, phlorotannins, ascorbic acid, tocopherols, amongst others that show antioxidant activity (Folmer et al. 2010).

The high number of species associated with the living conditions generate a high diversity of chemical compounds present in seaweed, many of them with interesting biological activities such as antibacterial, antiviral, immunosuppressant, anti-proliferative, anticancer, amongst others (Varshney and Singh 2013, Sharif et al. 2014). However, the search for new bioactive compounds is undergoing, looking for new activities, targets, or more interesting chemical properties worth capitalizing in pharmacokinetics/dynamics.

1.3.1. Anticancer action of seaweed compounds against glioblastoma

The anticancer activity of seaweed (crude extracts/isolated fractions/pure compounds) can be due to preventive and/or therapeutic effects. Several mechanisms of action, such as antioxidant, antiproliferative, cytotoxic, proapoptotic, antiangiogenic, antimetastatic have been proposed for the anticancer activity (Moussavou et al. 2014, Murphy et al. 2014, Zorofchian Moghadamtousi et al. 2014). The antioxidant effect is one of the most evaluated activity since oxidative stress has been closely related to development of cancer (Reuter et al. 2010). However, the effect of antioxidant agents in the survival of glioma patients are poorly investigated and two possible effects may occur. By one side, antioxidant agents could protect the normal cells against chemo/radiation damage, decreasing the side effects. By the other side, they could make the tumor cells more resistant to the cancer treatment (Lawenda et al. 2008, DeLorenze et al. 2010). So the interruption of certain signaling

cascades using antioxidants could represent a compensatory therapy against radiation-induced tissue injury, but its use should be done with precautions (Shin et al. 2014).

Anticancer activity of seaweed against GBM has been reported by several authors. Some examples are the application of aplysin, which is a bromine compound from marine organisms, in T98G cells, that demonstrated to suppress the invasion of these cells by Akt pathway inhibition (Gong et al. 2016). Lv et al. (2012) show that marchantin C and fucoidan, a macrocyclic bisbibenzyl derived from liverworts and a polysaccharide from brown seaweed, respectively, inhibit angiogenesis in T98G and THO1 cells. Also, Do et al. (2010) demonstrated that fucoidan in C6 glioma cells, inhibits the production of nitric oxide (NO) induced by tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ). Another example is the use of pheophorbide a (Pa), that is a chlorophyll-related compound from red seaweed, that show to induce G0/G1 arrest in U87 MG cells (Cho et al. 2014).

Seaweed are an important source of bioactive compounds with antioxidant activity, like carotenoids and phlorotannins. Among them, fucoxanthin and phloroglucinol have demonstrated anticancer effects in several *in vitro* and *in vivo* cancer models (Shin et al. 2014, Pádua et al. 2015).

1.3.1.1. Carotenoids

Fucoxanthin is an abundant marine carotenoid that contributes more than 10 % of the entire production of carotenoids in nature. Is an orange-colored pigment, along with chlorophylls a and c and β -carotene, present in brown seaweed (Phaeophyceae) and diatoms (Bacillariophyta) (Peng et al. 2011). Fucoxanthin has a unique molecular structure with an allenic bond and some oxygenic functional groups, such as hydroxyl, epoxy, carbonyl and carboxyl in the polyene hydrocarbon chain (Figure 3) (Dembitsky and Maoka 2007, Peng et al. 2011).

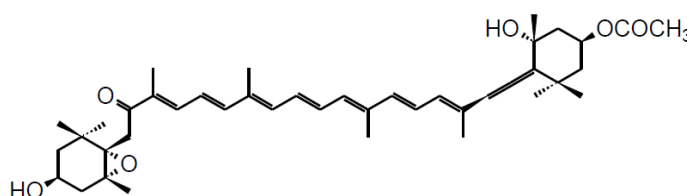


Figure 3 – Chemical structure of Fucoxanthin. Source: Kumar et al. (2013)

Fucoxanthin exhibits several health benefits, such as preventive effects on cancer through different mechanisms of action including antiproliferation, cell cycle arrest, apoptosis induction, suppression of angiogenesis amongst others (Kumar et al. 2013, Rengarajan et al. 2013). Since one of the most important characteristics of carotenoids is their ability to act as antioxidants, protecting cells and tissues from damaging effects of free radicals, fucoxanthin could attenuate the toxicity associated with the use of conventional drugs without compromising its therapeutic efficacy (Sachindra et al. 2007, Rengarajan et al. 2013).

Effects of fucoxanthin in gliomas cell lines are not yet very much explored, however, its anticancer effect in other cancers have been reported. Several authors demonstrated that fucoxanthin showed an antiproliferative effect in several cancer cell lines, such as liver cancer HepG2 cells, prostate cancer PC-3 cells, leukemia HL-60 cells and colon cancer cells HT-29 and Caco-2, amongst others (Rengarajan et al. 2013). Induction of apoptosis is a relevant strategy for cancer treatment. Fucoxanthin induces apoptosis in several cell lines such as melanoma B16F10 cells, leukemia HL-60 cells, EJ-1 human bladder cancers, in human colon cancer cell lines HCT116, Caco-2 cells, amongst others (Hosokawa et al. 1999, Hosokawa et al. 2004, Das et al. 2005, Zhang et al. 2008, Kim et al. 2010, Kim et al. 2013). Apoptosis could be induced through the activation of caspase-3 and -9 and through the downregulation of the expression of Bcl-xL and IAPs. Caspase-3 and -9 are crucial components in the mitochondrial pathway. When the caspases are activated, many cellular proteins are targeted, leading ultimately to apoptosis. Induction of cell cycle arrest by fucoxanthin is another mechanism of action related to its anticancer effect. It causes G0/G1 phase arrest of the cell cycle, in melanoma B16F10 cells, which is associated with the decrease of the expression of cyclin D1 and D2, and CDK4 followed by apoptosis (Das et al. 2005, Kim et al. 2013). Fucoxanthin also induces G1 arrest in human hepatocellular carcinoma, HepG2, and prostate cancer DU145 cell lines (Yoshiko and Hoyoku 2007). In human gastric adenocarcinoma MGC-803 cells, fucoxanthin induces cell cycle arrest in G2/M phase and apoptosis (Yu et al. 2011). Also, fucoxanthin demonstrates to be an angiogenesis inhibitor reducing the expression of the vascular endothelial growth factor (VEGF) in S180 cells and down-regulate the signal transduction by fibroblast growth factor 2 (FGF-2) and its receptor (FGFR-1) (Rengarajan et al. 2013). Lastly, fucoxanthin can also decrease oxidative DNA damage induced by H₂O₂ (Heo et al. 2008). Azqueta and Collins (2012) reviewed the effects on DNA damage induction of some carotenoids such as vitamin A, pro-vitamin A (carotenes) and non-vitamin A carotenoids (lycopene, amongst others) that were performed in several models. It was concluded that the non-vitamin A carotenoids

have a protective role against DNA damage in contrast with the pro-vitamin A carotenoids that, if used at high concentration, can induce DNA damage.

Regarding the promising data obtained until now about the effects of fucoxanthin on several cancer cell lines is important to increase the size of models used and test the effect of fucoxanthin in other cancers like GBM that could ultimately lead to the development of fucoxanthin as an anticancer agent.

1.3.1.2. Phlorotannins

Phlorotannins are tannin derivatives composed of phloroglucinol units connected to each other in diverse ways (Thomas and Kim 2011). Phloroglucinol (1,3,5-trihydroxybenzene) is a phenolic compound with an aromatic phenyl ring with three hydroxyl groups (Figure 4). Initially, phloroglucinol was isolated from *Ecklonia cava*, which is abundant on Jeju Island, Korea, but is also present in other brown seaweed.

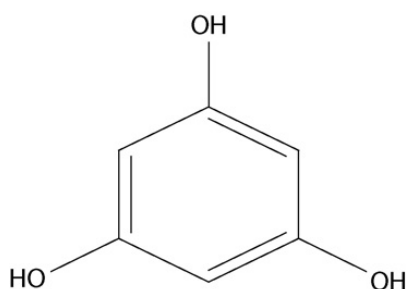


Figure 4 – Chemical structure of Phloroglucinol.
Source: Kang et al. (2010).

Ionizing radiation has been recognized to induce cell death through apoptotic pathways, which are mediated, in part, by decreased Bcl-2 expression (anti-apoptotic protein) and activation of caspase-3 and -9 (Kang et al. 2010). Phloroglucinol, *in vivo*, have demonstrated to be efficient in protect cells against radiation and extend the survival of mice exposed to lethal dose of radiation through the inhibition of mitogen-activated protein kinase kinase-4 (MKK4/SEK1), c-Jun NH2-terminal kinase (JNK) and activator protein-1 (AP-1) cascades (Kang et al. 2010, Shin et al. 2014). Phloroglucinol also down-regulates the expression of pro-apoptotic molecules such as p53 and Bax and enhances the expression of anti-apoptotic molecules, such as Bcl-2 and Bcl-X(S/L) (Ha et al. 2013). *In vitro*, phloroglucinol demonstrated to protect Chinese hamster lung fibroblast cells (V79-4) from

oxidative damage by inhibiting apoptosis induced by H₂O₂, exerting ROS scavenging activity and to enhance catalase activity (Kang et al. 2006). Also, Piao et al. (2015) showed in human HaCaT keratinocytes cells that phloroglucinol could protect against DNA damage induced by UVB through activation of the nucleotide excision repair (NER).

Besides the preventive effects, several authors have demonstrated the effects of phloroglucinol and their derivatives as a therapeutic agent in a variety of cell lines. 3,6-bis(3-chlorophenylacetyl)phloroglucinol (MCP), a phloroglucinol derivative, have anti-proliferative effect with induction of cell death in human colon cancer cells Caco-2, HCT-116 and SW480 (Huang et al. 2011). Kang et al. (2014) demonstrated that phloroglucinol induced apoptosis in human colon cancer HT-29 cells through activation of caspase-3 and -8, modifications in the Bcl-2 family and cytochrome c release. Also, it was verified that phloroglucinol is able to sensitize breast cancer MCF-7, SK-BR3 and BT549 cells to ionizing radiation as to some anticancer drugs such as cisplatin, taxol, amongst others, in which shown to inhibit PI3K/AKT and RAF-1/ERK signaling pathways. *In vivo* phloroglucinol show to stop the metastasis of breast cancer cells and extend the survival time of mice (Kim et al. 2015, Kim et al. 2015).

Effects of phloroglucinol in gliomas cell lines are not yet very much explored, however, a few studies demonstrated the *in vitro* antiproliferative activity of phloroglucinol and its derivatives in some glioma cells. According to Lu et al. (2012) the BFP (2,4-bis(4-fluorophenylacetyl)phloroglucinol), which is a phloroglucinol derivative, inhibits the proliferation of gliomas cells, such as U251, U87 and C6 cells without effect in primary human astrocytes. In U251 cells, BFP induce a sub G1 cell cycle arrest and induce apoptosis as confirmed by induction of nuclear condensation and fragmentation, nuclear shrinkage, activation of caspase-9 and caspase-3, increase in Bax protein levels without effects in the levels of Bcl-2, which may lead to an increase in the pro-apoptotic/anti-apoptotic Bcl2 ratio. So, it was shown that activation of caspases and up-regulation of pro-apoptotic protein might be involved in BFP induce cell apoptosis in human glioma (Lu et al. 2012).

In the literature, several beneficial effects of phloroglucinol and their derivatives *in vitro* and *in vivo* have been reported. However, more studies are needed to better understand the anticancer effect of phloroglucinol.

1.3.2. Interaction of natural compounds and conventional anticancer drugs

The combination of natural compounds with anticancer drugs that are already used in cancer therapy is very important as a strategy to potentiate the action of the drugs, minimizing the side effects with the reduction of the drug dose. This interaction may also bring problematic situations that should be avoided such as decreasing of the pharmacological effect or increase its toxicity in normal cells (Harvey et al. 2015). Looking at the bibliography, the interaction between natural compounds and the conventional anticancer drugs is still poorly explored, however, it seems highly relevant. Some natural compounds have been shown to have additive or synergistic effects when in combination with anticancer drugs, and in some cases also decrease the side effects of the anticancer drugs (Alekseyenko et al. 2007, Siegelin et al. 2008, Eid et al. 2012, Fujiki and Suganuma 2012).

Jakubowicz-Gil et al. (2013) studied the interaction between quercetin (3,3',4',5,7-pentahydroxyflavone) and TMZ in T98G cell line. Quercetin is a natural flavonoid found in several types of fruits and vegetables and it is known to be a powerful antioxidant which enables apoptosis of tumor cells. The results demonstrated that both quercetin and TMZ induce apoptosis through activation of caspase-3 and -9 and through the release of cytochrome c from the mitochondria to the cytoplasm which indicates that both the natural compound and the drug initiate apoptosis via an intrinsic (mitochondrial) pathway. As so, it was suggested that the combination of quercetin with TMZ was more effective than each one alone.

Another example was the study of the interaction between resveratrol and TMZ in glioma cell lines. Resveratrol (3,5,4'-trihydroxystilbene) is a polyphenol compound existing in grape skin and red wine and it was demonstrated to be an antioxidant that can penetrate the blood-brain barrier and exert antiproliferative effects, apoptosis induction or autophagy, and cell cycle arrest in several types of cancer. Lin et al. (2012) demonstrated that TMZ induces apoptosis and cytoprotective autophagy in U87 MG and GBM8401 cells, by the release of ROS and extracellular signal-regulated kinase (ERK) activation, in which RSV suppresses TMZ-induced autophagy and consequently increases the apoptosis through ROS/ERK pathway. Therefore, it was shown that the combination of RSV and TMZ have a synergistic effect, enhancing the therapeutic efficacy of TMZ.

Also, Hyun et al. (2011) show that eckol (which is a phlorotannin component) treatment improved the sensitivity of glioma stem-like cells to an anticancer drug, TMZ, and ionizing radiation. Eckol efficiently inhibits both PI3K-Akt and Ras-Raf-1-Erk pathways (which are the key signaling pathways triggered in cancer stem-like cells that contribute to

the resistance of cancer cells to ionizing radiation) in glioma stem-like cells U87MG, U373MG, X01GB, and X03AOA. Treatment with eckol produced a suppression of PI3K and Akt activities and inhibited Ras-Raf-1 interaction and Raf-1 and Erk activations in glioma cells. So eckol increases the sensitivity of glioma stem-like cells to anticancer drugs which could offer a therapeutic strategy that targets mainly cancer stem-like cells.

Information about the possible interaction of seaweed compounds with TMZ in glioblastoma is clearly missing. However, recent data showed that some seaweed compounds are able to potentiate the effects of others anticancer drugs.

One example is the fucoidan that in combination with cisplatin, tamoxifen, and paclitaxel in breast cancer may potentiate their cytotoxic activities. Zhang et al. (2013) show that the combination inhibits the cell growth with modifications in the cell cycle and induce apoptosis in MDA-MB-231 and MCF-7 cells.

Liu et al. (2013) described that the combination of fucoxanthin with cisplatin, in HepG2 cells decreased cell proliferation, when compared with cisplatin alone. Also, it was shown that the excision repair cross-complementation 1 (ERCC1) mRNA expression was reduced by fucoxanthin through the PI3K/AKT and ERK pathways, while cisplatin inhibited the thymidine phosphorylase (TP) expression through p38 pathway. So, the combination with fucoxanthin could improve cisplatin efficacy.

Although the effects of some natural compounds are already known in the treatment of GMB the interaction with anticancer drugs, namely TMZ, is underexplored and require more attention due to the unquestionable high potential of these marine compounds.

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Objectives

In view of the promising anticancer effects of fucoxanthin and phloroglucinol, two natural compounds that are present in seaweed, in several *in vitro* and *in vivo* models, the main objective of this work was to assess the anticancer effect of both compounds in glioblastoma, using the cell lines U251 and T98G. The interaction of those natural compounds with the anticancer drug, TMZ, was also evaluated. Genotoxic effects and the ability to induce cell death were some of the possible mechanisms assessed in this work.

The objectives were achieved through the performance of several techniques that allowed evaluate the following effects:

- Effects on cell viability and proliferation - This first step was achieved by using cytotoxicity and proliferation assays, such as MTT assay and cell counting using a Neubauer chamber on inverted contrast phase microscopy. This allowed to understand which compounds and which concentrations had the most interesting effects on cell viability/proliferation to continue the study.
- Effects on cell death induction - The second step was done with a nuclear condensation assay that allowed see if the compounds selected in the previous assays acts through the induction of cell death, more specifically apoptosis.
- Effects on DNA damage – This final step was performed by the standard and modified comet assay with incubation of FPG enzyme that allowed to assess strand breaks and FPG-sensitive sites, respectively. This provided us some knowledge about the ability of the tested compounds to induce DNA damage.

Chapter 2

“Anticancer effects of fucoxanthin and phloroglucinol,
alone and in combination with temozolomide, on the
U251 and T98G glioblastoma cell lines”

This Chapter compromises the draft of a manuscript to be submitted for publication:

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Anticancer effects of fucoxanthin and phloroglucinol, alone and in combination with temozolomide, on the U251 and T98G glioblastoma cell lines

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Abstract

Background: Glioblastoma (GBM) is one of the most aggressive tumors of the central nervous system (CNS) for which the most common therapy used is anticancer drugs such as temozolomide (TMZ). However, the development of resistance to TMZ is a major problem in chemotherapy, being of urgent need the development of new drugs/therapies.

Aim: Evaluation of the *in vitro* anticancer effect of two natural compounds from algae, fucoxanthin (Fx) and phloroglucinol (Ph), alone and combined with TMZ, in two glioblastoma cell lines.

Materials and methods: The natural compounds Fx (at 0.1, 1, 10 and 50 μM) and Ph (at 10, 50, 100 and 300 μM), alone or combined with TMZ, were evaluated for effects on cell viability and proliferation by MTT and cell counting, on induction of DNA damage by comet assay, and on cell death by nuclear condensation assay, in human U251 and T98G cell lines.

Results: TMZ, at 24 h, and in both cell lines, did not decrease cell viability, however, after 72 h of the initial incubation, there was a significant decrease in the proliferation of U251 cells. In this cell line was observed a slight increase of cell death, however, without visible effects in the induction of DNA damage detected by comet assay. In T98G cells, although effects on cell viability and proliferation were not observed, a significant increase of DNA damage was seen at 500 μM TMZ. Fx decreased cell viability and proliferation in U251 cells with a slight increase of cell death, however, DNA damage was not noted. In T98G cells was observed a slight decrease of the effects on cell viability and proliferation however, were not statistically significant. Even without any cytotoxic effects, induction of DNA damage was observed. Ph did not decrease cell viability, at 24 h, by cell counting, however, the metabolic activity of the cells, in the MTT decreases, mainly in U251 cells. After 48 h, was seen a decrease in the number of cells demonstrating antiproliferative effects, that could be related to the slight increase in the induction of cell death and DNA damage, in U251 cells. The combination 10 μM TMZ + 10 μM Fx, in U251 cells, decreased cell viability (assessed by cell counting), without observable effects in the MTT assay. There was also a tendency to decrease cell proliferation when compared to TMZ alone, however, the cytotoxic and antiproliferative effects were not sustained by an increase of cell death or DNA damage.

Conclusions: Generally, Fx and Ph alone inhibited cell viability and cell proliferation, more evidently in U251 cells. Cell death by apoptosis did not appear to be the mechanism responsible for this effect in the tested conditions. The combination 10 μM TMZ + 10 μM Fx

seems to be a promising combination to study, in U251 cells, in which is observed that Fx potentiated TMZ action. More studies are needed to clarify the mechanisms involved.

Keywords: Antiproliferation; cancer cell lines; cell death induction; cytotoxicity; DNA damage; fucoxanthin; phloroglucinol; temozolomide.

Abbreviations: Dimethyl sulphoxide (DMSO); Fucoxanthin (Fx); Phloroglucinol (Ph); Temozolomide (TMZ).

1. Introduction

Cancer nowadays is a principal cause of death in developing and developed countries, even with huge investments and advances in science. With the growth and aging of the population, projections suggest an increase of the new cancer cases diagnosed to approximately 15 million by 2020 (Anand et al. 2008). Glioblastomas (GBM) are classified as grade IV astrocytoma by the World Health Organization (WHO) and is the most common type of brain tumor with a weak diagnosis and a diminished survival time around 14 months post-diagnosis (Louis et al. 2007, Goellner et al. 2011, Cho et al. 2014, Hiddingh et al. 2014). Several treatments are put in place, such as chemotherapy with alkylating agents like temozolomide (TMZ), radiotherapy and surgery, however, cancer drug resistance is still a major problem in the treatment of GBM (Goellner et al. 2011).

TMZ is used in the treatment of GBM, and in some cases applied in combination with ionizing radiation, because it has the ability to induce DNA damage and consequently cell death (Ramos et al. 2013, Moody and Wheelhouse 2014, Weathers and Gilbert 2014). The main DNA damages produced by TMZ are the N7-methylguanine (N7-meG), N3-methyladenine (N3-meA) and N3-methylguanine (N3-meG), that happen more frequently, and the O6-methylguanine (O6-meG) and O4-methylthymine (O4-meT) that are less frequent however with higher cytotoxicity (Kondo et al. 2010, Ramos et al. 2011, Ramos et al. 2013). N-alkylated damages are usually repaired by the base excision repair (BER) whereas the O-alkylated damage, more specifically the O6-meG, can only be repaired by O6-methylguanine-DNA methyltransferase (MGMT). However, when O6-meG are not repaired they are converted in SB's by the mismatch repair system (MMR), becoming more toxic to the cells (Zhang et al. 2012, Ramos et al. 2013). TMZ resistance could be developed due to several mechanisms, such as, impairment of apoptotic proteins, overexpression of the DNA repair protein MGMT, damage in the MMR or BER pathways, amongst others (Roos et al. 2007, Kang et al. 2010, Goellner et al. 2011, Ha et al. 2013).

The search for new compounds with anticancer action is currently been investigated mainly in marine environments due to their exceptional biological and chemical diversity (Senthilkumar et al. 2013). Fucoxanthin (Fx) is the most abundant marine carotenoid present in micro and macroalgae (Kumar et al. 2013). Several studies showed that Fx has anticancer activity acting through a diversity of mechanisms, for instance, antiproliferative, antioxidative, DNA repair, apoptosis induction, anti-angiogenic and inhibit migration and invasion of tumor cells (Peng et al. 2011, Kim 2015). Phloroglucinol (Ph) is a phenolic compound isolated from a brown seaweed *Ecklonia cava* (Kang et al. 2010). A number of studies demonstrated that Ph and their derivatives, have cytoprotective effects against

radiation but also shown to induce antiproliferative effects, apoptosis, amongst others (Kang et al. 2010, Huang et al. 2011, Kang et al. 2014, Shin et al. 2014). However, the effect of Fx and Ph on GBM treatment and the capability of these compounds to potentiate the action of TMZ and decrease the side effects are still few explored. So, the interaction of anticancer drugs with natural compounds is an appealing strategy to study.

The aim of the present study was to assess anticancer effects of Fx and Ph, alone and in combination with the conventional anticancer drug TMZ, against the U251 and the T98G glioblastoma human cell lines. Effects on cell viability and proliferation, DNA damage and cell death induction were assessed.

2. Materials and methods

Chemicals / Reagents

TMZ, Ph, Fx, Eagle's minimum essential medium (MEM), N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), sodium bicarbonate, sodium pyruvate, trypsin solution, penicillin/streptomycin, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) and 4,6-diamidino-2-phenylindole (DAPI) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was acquired from Biochrom KG (Berlin, Germany). Ro 19-8022 was generously provided by Hoffmann-La Roche (Basel, Switzerland). Formamidopyrimidine DNA glycosylase (FPG) was acquired from New England Biolabs (Danvers, MA, USA). All other reagents and chemicals used were of analytical grade.

Cell culture

U251 and T98G cell lines were obtained from European Collection of Cell Cultures (ECACC). The cell lines were cultivated in monolayer, with MEM medium supplemented with 10 % FBS and 1 % of antibiotic solution (100 U/ml penicillin and 100 µg/ml streptomycin), 0.1 mM sodium pyruvate and 10 mM HEPES, in an incubator at 37 °C and 5 % CO₂. The medium was changed twice a week and cells were trypsinized when nearly confluent. In both cell lines were performed a preliminary test to determine the cell density to use in each assay.

Stock solutions of TMZ, Fx and Ph were prepared in DMSO and aliquots kept at -20 °C. In the day of experiments, the natural compounds and TMZ were dissolved in the culture

medium, in which the final concentration of DMSO was inferior to 0.5 % (v/v). The controls received just fresh medium with 0.5 % of DMSO (v/v), to maintain the same conditions.

Cytotoxicity and proliferation assays

To assess effects on cell viability two assays were performed: MTT assay and cell counting, using a Neubauer chamber, as described above. Cell counting by the Neubauer chamber was also used to determine effects on cell proliferation.

MTT colorimetric assay

The MTT assay was done to assess the effects of the tested compounds on cell viability. Cells were cultivated in 96-multiwell culture plate at a density of (0.4×10^5 and 0.8×10^5 cells/ml for U251 and T98G cells, respectively). Twenty-four hours after plating, the medium was discarded and cells were first treated with different concentrations of TMZ (1, 10, 100, 200, 500 μ M), for 24 h. In the negative controls, cells were incubated only with medium with 0.5 % DMSO. In the initial control ($t = 0$), MTT solution was added at 0.5 mg/ml, incubated at 37 °C for 2 h and then the MTT and medium were aspirated. The cells of the other experimental conditions were left to incubate for the remaining time of exposure (24 h). In the end of treatment, MTT solution at 0.5 mg/ml was added to all the plate and incubated for 2 h. The formazan crystals formed were dissolved in 150 μ l of DMSO:ethanol solution (1:1) (v/v), using a plate shaker for 10 min. In a microplate reader (Multiskan EX, Labsystems, USA), the absorbance (A) was read at 570 nm. The absorbance reproduces the capacity of viable cells to convert MTT to formazan crystal by mitochondrial activity and can be used as an indicator of cell viability (Vega-Avila and Pugsley 2011). The IC_{30} (concentration of TMZ that decreases the viable cells by 30 %) was determined, for each cell line, from the dose-response curves.

After choosing the IC_{30} of TMZ, cells were incubated with different concentrations of Fx (0.1, 1, 10 and 50 μ M) and Ph (10, 50, 100 and 300 μ M) either alone or in combination with the IC_{30} of TMZ, for 24 h, and the MTT was executed as described above. To assess the effects of the compounds and the drug on cell viability it was divided the value of the absorbance of each condition by the control and the results were presented as a percentage of survival in relation to the control.

Neubauer chamber cell counting

To assess the effects of Fx, Ph and TMZ alone or in combination on glioma cell viability, cell count using Neubauer chamber on inverted contrast phase microscopy was performed. In short, cells were seeded in 24-multiwell culture plates at a density of (0.6×10^5 and 0.9×10^5 cell/ml for U251 and T98G cells, respectively) and let to adhere for 24 h at 37 °C and 5 % CO₂ in a humidified incubator. After adhesion, the medium was discarded and cells were first treated with different concentrations of TMZ (1, 10, 100, 200, 500 µM) and cell counting performed, after 24 h, as described next. After determination of the IC₃₀ of TMZ for U251 cells and T98G cells, at 24 h, cells were treated with Fx (0.1, 1, 10 and 50 µM) and Ph (10, 50, 100 and 300 µM) alone or in combination with the IC₃₀ of TMZ whereas the negative controls were incubated only with medium with 0.5 % DMSO. At the beginning of incubation, for the initial control (t=0) the cells were washed, trypsinized and counted in the Neubauer chamber on inverted contrast phase microscopy (Olympus CKX41, Japan). The cells treated with the test compounds and respective controls were left to incubate for 24 h at 37 °C. After the treatment time, each condition was counted as described above and cell density was determined.

To evaluate the proliferation effects, 1/3 of the cellular suspension (300 µl) provided from the previous assay was centrifuged to ensure that the treatment was removed and the cells seeded in a new plate with fresh medium. After 48 h, the cells were counted again using the Neubauer chamber. The IC₃₀ was determined, for each cell line, from the dose-response curves. To assess the relative suspension growth (RSG), first was determined the total suspension growth (TSG) which is the ratio between the number of cells at 72 h and the number of cells at 0 h. After the RSG is determined, using the TSG of each condition divided by the TSG of the control (Azqueta et al. 2013).

For the following assays the conditions that decrease cell viability/proliferation were selected to assess cell death and genotoxic effects.

Nuclear condensation assay

To evaluate the ability of the test compounds to induce cell death it was used the nuclear condensation assay. Cells were plated at a density of (0.6×10^5 cells/ml – U251 and 0.9×10^5 cells/ml – T98G) in 24-multiwell culture plates. After 24 h of plating, the medium was discarded and cells were treated with the conditions previously selected. After 48 h of treatment, all the cells (adherent and non-adherent) were collected, centrifuged and fixed with 4 % (w/v) paraformaldehyde in PBS for 20 min at 37 °C. Cells were placed in poly-L-

lysine-treated slides using a cytospin cytocentrifuge (Thermo Scientific, USA). Each sample was circled with hydrophobic barrier pen and slides washed with PBS three times for 5 min each. Slides were incubated with 1 µg/ml of DAPI, to stain the nuclei, for 10 min protected from light. The slides were observed under a fluorescence microscope (Olympus IX71, Japan) and at least 300 cells were counted per sample. The percentage of cells with condensed nuclei was calculated from the ratio between those cells with nuclear condensation and the total number of cells (nuclei staining with DAPI) (Ramos et al. 2016).

Comet assay

The genotoxic effects of the conditions selected were evaluated by single cell electrophoresis assay, also known as, comet assay. Cells were seeded into 12-multiwell culture plates at a density of 0.75×10^5 cells/ml for U251 and 0.1×10^6 cells/ml for T98G, and let to adhere for 24 h, at 37 °C and 5 % CO₂ in a humidified incubator. After adhesion, the medium was discarded and cells were treated with the selected conditions for 24 h. After the incubation period, DNA damage (strand breaks and FPG-sensitive sites) were assessed by the standard and modified alkaline version of the comet assay (Collins et al. 2008). Briefly, cells were washed, trypsinized, centrifuged and cell density determined. Approximately 2×10^4 cells/gel were mixed in the low melting point agarose (0.5 %) and placed on a slide pre-coated with 1 % normal melting point agarose. Each experimental condition was done in duplicate (two gels in the same slide) and three slides (lysis, buffer F – FPG reaction buffer, and FPG) per condition were prepared. Slides were then incubated in lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris Base, pH 10 plus 1 % Triton X-100) at 4 °C for 1 h. After lysis, slides of the buffer F and FPG were washed three times in buffer F solution and incubated with the respective solution (buffer F or FPG) at 37°C for 30 min. After the incubation time, all the slides (lysis, buffer F and FPG) were transferred into the horizontal electrophoresis chamber with electrophoresis buffer (300 mM NaOH, 1 mM Na₂EDTA, pH >13), at 4 °C for 40 min, to unwind the DNA, followed by electrophoresis running at 25 V (1V/cm) for 20 min. The slides were washed with PBS and distilled water for 10 min each and then left to dry at room temperature overnight.

The photosensitizer Ro 19-8022 plus visible light induce oxidized guanines and was used as a positive control for FPG. Briefly, cells were plated with an approximately 1×10^5 cells/ml and after 48 h of adhesion, cells were exposed to Ro 19-8022 at different concentrations (1, 3 and 5 µM) for 5 min, on ice, under a halogen lamp (33 cm of distance). After the treatment, cells were washed with PBS, trypsinized and counted to adjust the

density to approximately 1×10^6 cells/mL. Then, the cells of positive control followed the same procedure of the comet assay as mentioned before.

For the analysis of the comets, the slides were stained with 1 µg/ml of DAPI solution for approximately 30 min and visualized in a fluorescence microscope (Olympus IX71, Japan). At least 100 random cells were analyzed per slide. Images were analyzed using the image analysis software OpenComet, to quantify the percentage of tail intensity (Gyori et al. 2014).

Statistical analysis

Analyses were performed using the GraphPad Prism v6.0 software (GraphPad Software, La Jolla, CA, USA) and results were expressed as mean \pm standard deviation (SD), from a minimum of 2 independent experiments. The results were analyzed using one-way or two-way ANOVA (depending on the experimental conditions tested) to evaluate significant differences ($p \leq 0.05$) between treatment conditions and the negative control, followed by post hoc multiple comparisons using the Bonferroni's test or the Dunnett's. The assumptions of normality and of homogeneity of variances were not tested due to the low "n", but they were assumed to occur, in line with the cell culture responses as detected in many resembling published studies and with the general good robustness of the parametric tests from departures of those basic assumptions (Rasch and Guiard 2004, Schmider et al. 2010, McKillup 2011).

3. Results

Assessment of cell viability by MTT colorimetric assay

The assessment of the effect on cell viability of the two tested natural compounds Fx and Ph, alone and in combination with the anticancer drug TMZ, was performed by the MTT colorimetric assay, using two human glioblastoma cell lines, U251 and T98G cells. All the assays were done on growing cells and the cell density used was determined previously for each cell line (data not shown). In the MTT assay, cells were incubated with a varying concentration of Fx (0.1, 1, 10 and 50 µM), Ph (10, 50, 100 and 300 µM) and TMZ (1, 10, 100, 200 and 500 µM), for 24 h, whereas the negative controls were incubated only with medium with 0.5 % DMSO.

The results from the MTT demonstrated that TMZ did not induce a significant decrease in the viability of both cell lines (Figure 1), for which it was not possible to

determine the IC₃₀. Accordingly, for the MTT assay, the concentration of TMZ used for the combination with Fx and Ph was the highest (500 μ M).

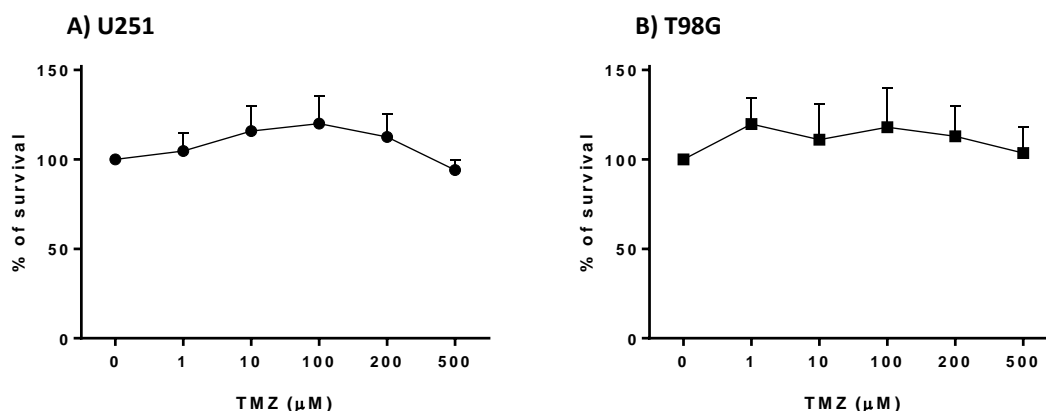


Figure 1 – Effects of Temozolomide (TMZ) on cell viability of U251 (A) and T98G (B) cells after 24 h, evaluated by MTT assay. Results are expressed as mean + SD of at least three independent experiments.

Regarding the effect of Fx on cell viability, this compound alone did not have an effect on the viability of both cell lines, except for 50 μ M, that demonstrated an inhibition of 55 % and 40 % for U251 and T98G, respectively (Figure 2A and 2B). Fx (only at 50 μ M), in combination with TMZ (500 μ M), showed an inhibition of 52 % and 38 %, for U251 and T98G, respectively, when compared with TMZ alone (Figure 2A and 2B).

In the case of Ph, it was shown a decrease of cell viability in a concentration-dependent manner in both cell lines. In U251 cells, Ph alone at 100 and 300 μ M inhibited cell viability in 28 % and 49 %, respectively. In combination with TMZ, Ph demonstrated a decrease of 23 %, 33 %, and 53 %, for 50, 100 and 300 μ M of Ph respectively, relative to the cells incubated only with TMZ (Figure 2C). The decrease in cell viability observed in combination was slightly higher than the inhibition obtained with Ph alone. In the T98G cells, the effect of Ph was lower showing a significant inhibition of 35 % at 300 μ M, while in combination with TMZ was observed a decrease in cell viability of 32 % and 45 % at 100 and 300 μ M, respectively (Figure 2D).

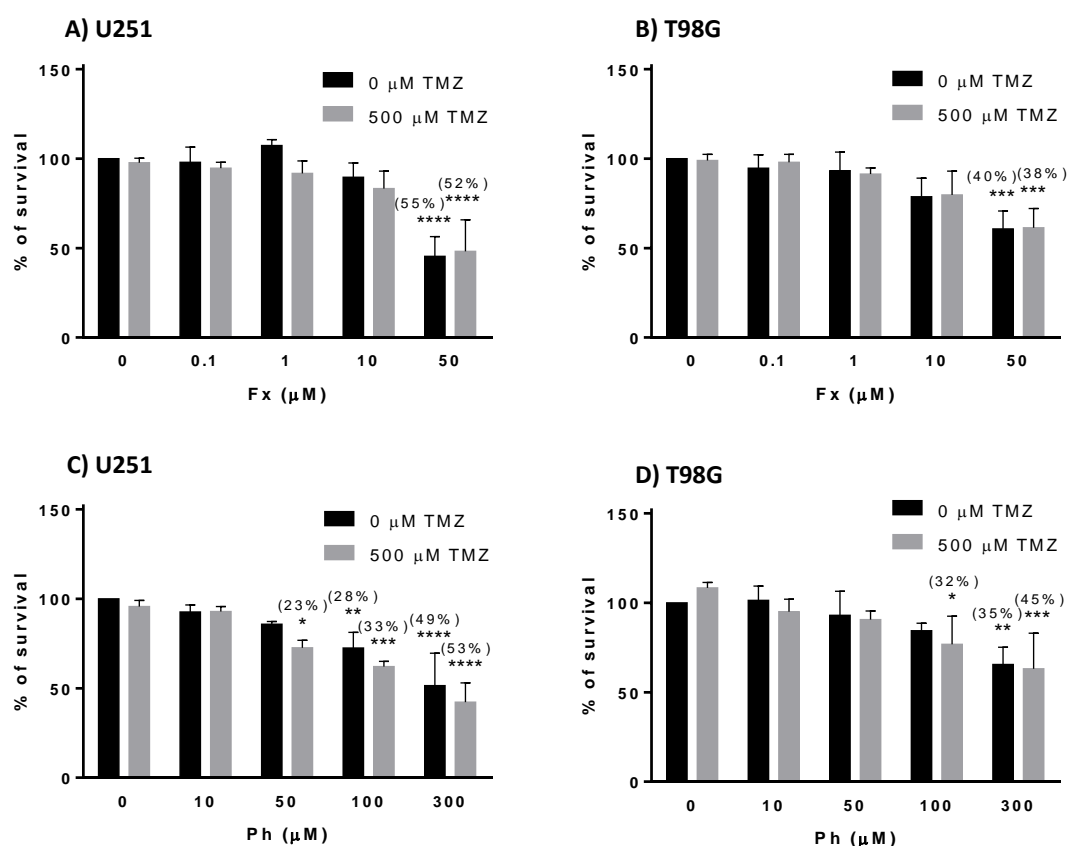


Figure 2 - Effect of Fucoxanthin (Fx) and Phloroglucinol (Ph) on the viability of U251 (A and C) and T98G (B and D) cells after 24 h, evaluated by MTT assay. Results are expressed as mean + SD of at least three independent experiments. Significant differences (* $p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$) when compared with the respective control cells (with and without TMZ) were determined by two-way ANOVA, followed by a Bonferroni multiple comparison test.

Assessment of cell viability and proliferation by cell counting using Neubauer chamber

To assess the effects of the natural compounds alone or in combination with TMZ, on cell viability and cell proliferation on glioma cells, was first determined the IC_{30} of TMZ in U251 and T98G cell lines. Cells were incubated with a different concentration of TMZ (1, 10, 100, 200 and 500 μ M) for 24 h. The results from the cytotoxicity assay, at 24 h, demonstrated that temozolomide do not present a significant decrease in the viability of both cell lines (Figure 3A and B). In the proliferation assay, 72 h after initial incubation, it was observed a significant decrease in cell proliferation of 42 %, 43 % and 54 % at 100, 200 and 500 μ M of TMZ, respectively, for the U251 cells (Figure 3C). The IC_{30} value was 10 μ M for U251 cells. However, no significant effect was observed in T98G cells after TMZ treatment (Figure 3D). So, for the combination assays with the natural compounds, T98G cells were incubated with the maximum concentration tested 500 μ M of TMZ.

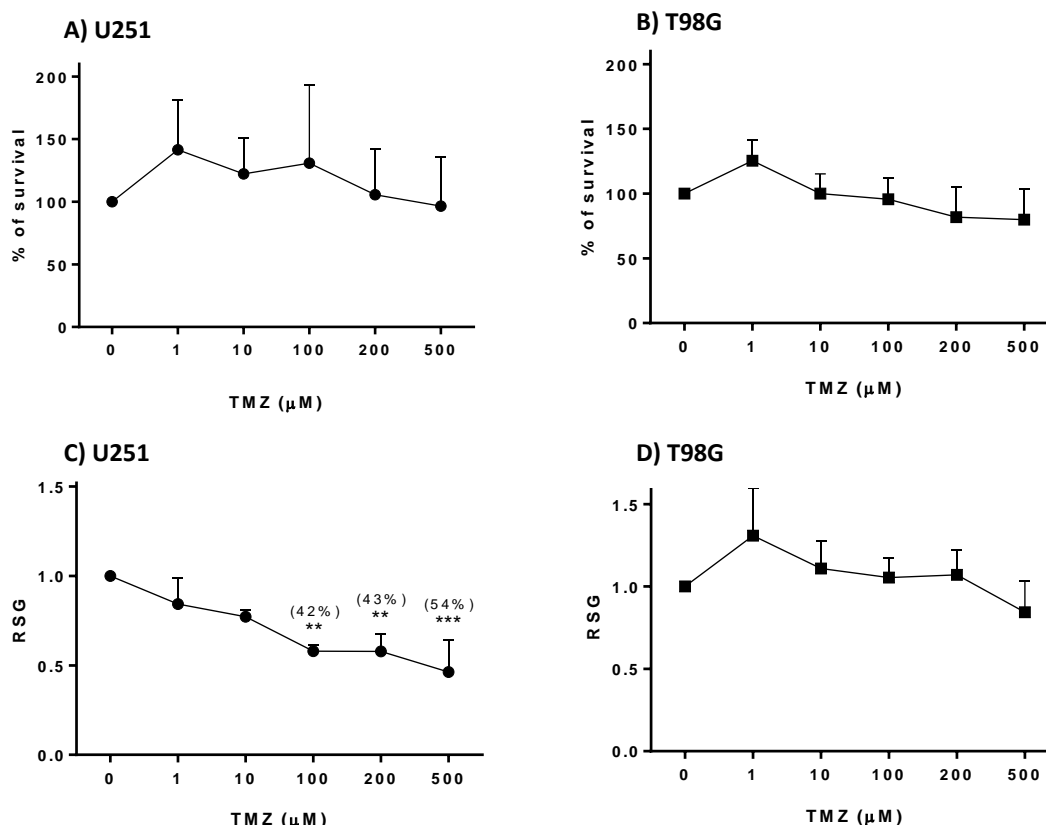


Figure 3 - Effect of Temozolomide (TMZ) on the percentage of survival, after 24 h (A and B), and on cell proliferation by Relative Suspension Growth (RSG), after 48 h (C and D) in U251 (A and C) and T98G (B and D) cells, assessed by cell counting. Results are expressed as mean + SD of at least three independent experiments. Significant differences (** $p \leq 0.01$, *** $p \leq 0.001$) when compared with control cells were determined by one-way ANOVA, followed by a Dunnett multiple comparison test.

Fx, in the cytotoxicity assay, demonstrated to decrease U251 cells' viability in 26 %, 24 % and 26 % at 0.1, 1 and 10 μM , respectively, relative to the negative control (Figure 4A). However, no effect was observed in the T98G cells (Figure 4B). Fx in combination with TMZ showed a significant decrease of 22 % only for the higher tested concentration of Fx when compared with cells incubated with TMZ alone (Figure 4A). In T98G cells, the combination (1 μM Fx + 500 μM TMZ) showed a significant inhibition of cell viability in 24 % relatively to the natural compound alone. However, no differences were observed when compared with cells treated with TMZ alone showing that the combination does not potentiate the action of TMZ (Figure 4B).

Regarding the effect of Ph, despite the graphical tendency for a diminished cellular viability, the statistical analysis does not support that the treated group differ from control, in both cell lines (Figure 4C and 4D).

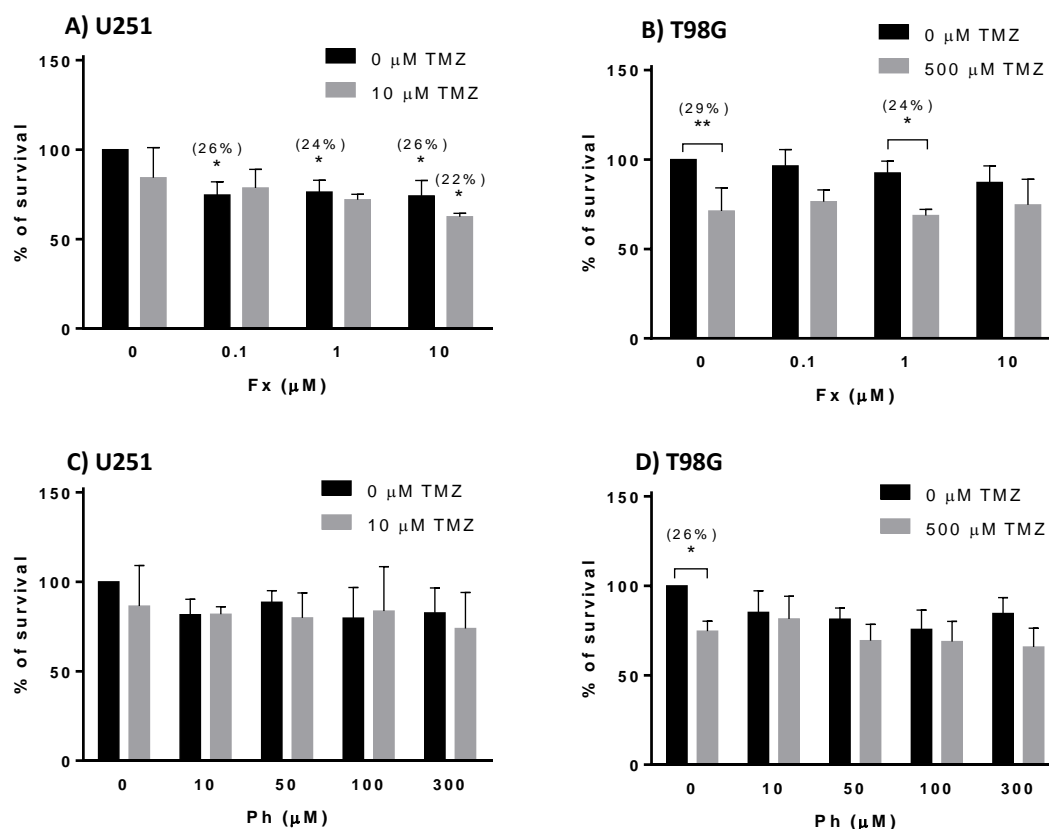


Figure 4 - Effect of Fucoxanthin (Fx) and Phloroglucinol (Ph) on the percentage of survival in U251 (A and C) and T98G (B and D) cells after 24 h, assessed by cell counting. Results are expressed as mean + SD of at least three independent experiments. Significant differences (* $p \leq 0.05$; ** $p \leq 0.01$) when compared with the respective controls were determined by two-way ANOVA, followed by a Bonferroni multiple comparison test.

Observing the results from the proliferation assay, in which the cells had 48 h in fresh medium after 24 h of incubation with the natural compounds alone or combined with TMZ, Fx inhibited cell proliferation in a dependent concentration manner. Fx at 1 and 10 μM significantly decreased the proliferation by 29 % when compared to the respective control (Figure 5A). The same effect was not seen in the T98G cells, at least statistically, even if it looked like it was lower at the highest concentration (Figure 5B).

In U251 cells, Ph decreased cell proliferation in a dose-dependent way, in which was seen a decrease of 26 % and 39 % in the RSG, at 50, 100 and 300 μM (Figure 5C). In T98G cells, Ph did not have a statistically significant effect on cell proliferation (Figure 5D). Fx and Ph in combination with TMZ did not potentiate the antiproliferative effect in both cell lines.

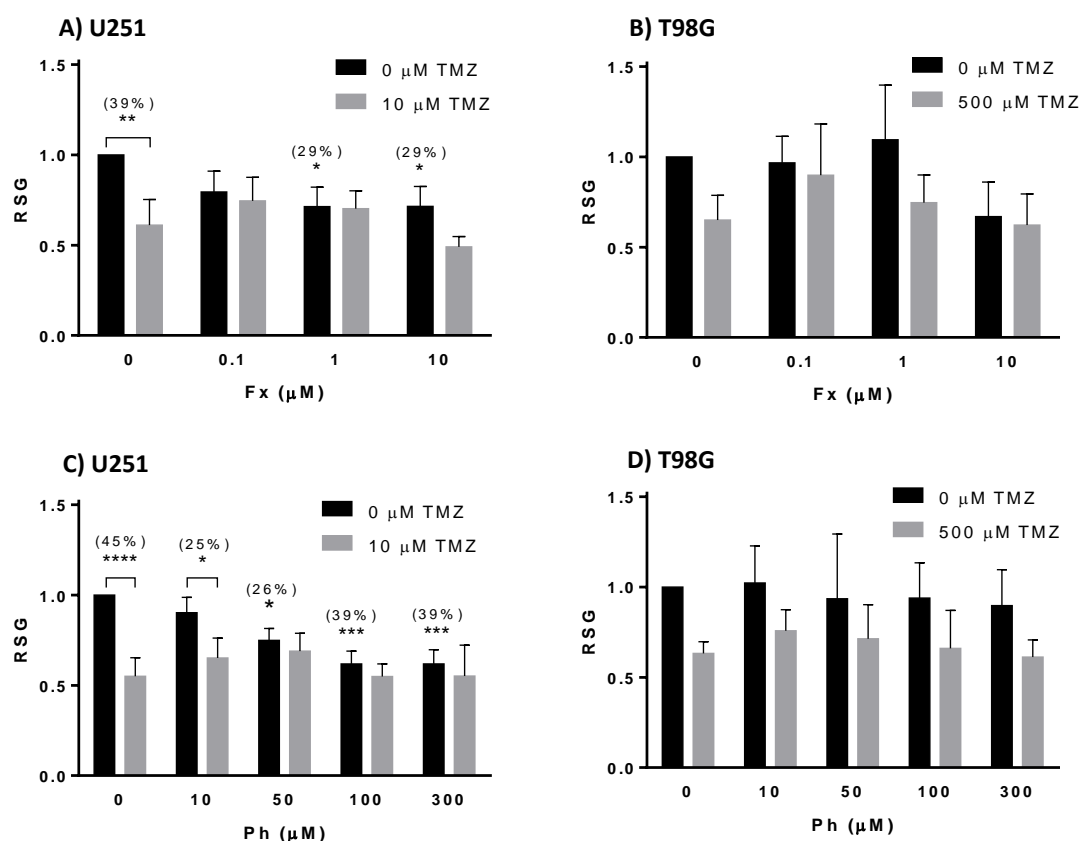


Figure 5 - Effect of Fucoxanthin (Fx) and Phloroglucinol (Ph) on cell proliferation by Relative Suspension Growth (RSG) in U251 (A and C) and T98G (B and D) cells after 48 h, assessed by cell counting. Results are expressed as mean + SD of at least three independent experiments. Significant differences (* $p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$) when compared with the respective controls were determined by two-way ANOVA, followed by a Bonferroni multiple comparison test.

After the analysis of the data from the cytotoxicity and proliferation assays, some specific conditions were chosen for each cell line (U251 and T98G) for further research. The selection was based on the significant differences obtained in those two initial assays. The select conditions that decreased cell viability/proliferation were tested in the following assays: (i) In the nuclear condensation assay was tested TMZ (10 μM), alone and in combination with Fx (0.1, 1 and 10 μM), and Ph alone (10, 50, 100 and 300 μM) in U251 cells; (ii) in T98G cells were tested TMZ (500 μM), Fx (1 and 10 μM) and Ph (300 μM). For the comet assay the tested compounds and concentrations, for the U251 cells, were TMZ (10 μM), Fx (10 μM), a combination (10 μM TMZ + 10 μM Fx) and Ph (300 μM), and in T98G was tested TMZ (500 μM), Fx (10 μM) and Ph (300 μM).

Assessment of cell death induction by nuclear condensation assay

Since the observed anti-proliferative effect induced by the compounds may be due to the induction of cell death, a nuclear condensation assay was performed after 48 h of exposure. As observed in figure 6A and B, the mean % of condensed nuclei of U251 cells incubated with TMZ and both Fx and Ph was not significantly higher than in control. Also, the combinations of TMZ with the different concentration of Fx (0.1, 1 and 10 μM), in U251 cells, did not evidence to have a significant increase in the induction of cell death. In T98G cells, only Fx at 10 μM displayed an unequivocal higher % of condensed nuclei, about 8 % greater when compared with the control (cells treated only with medium) (Figure 6C).

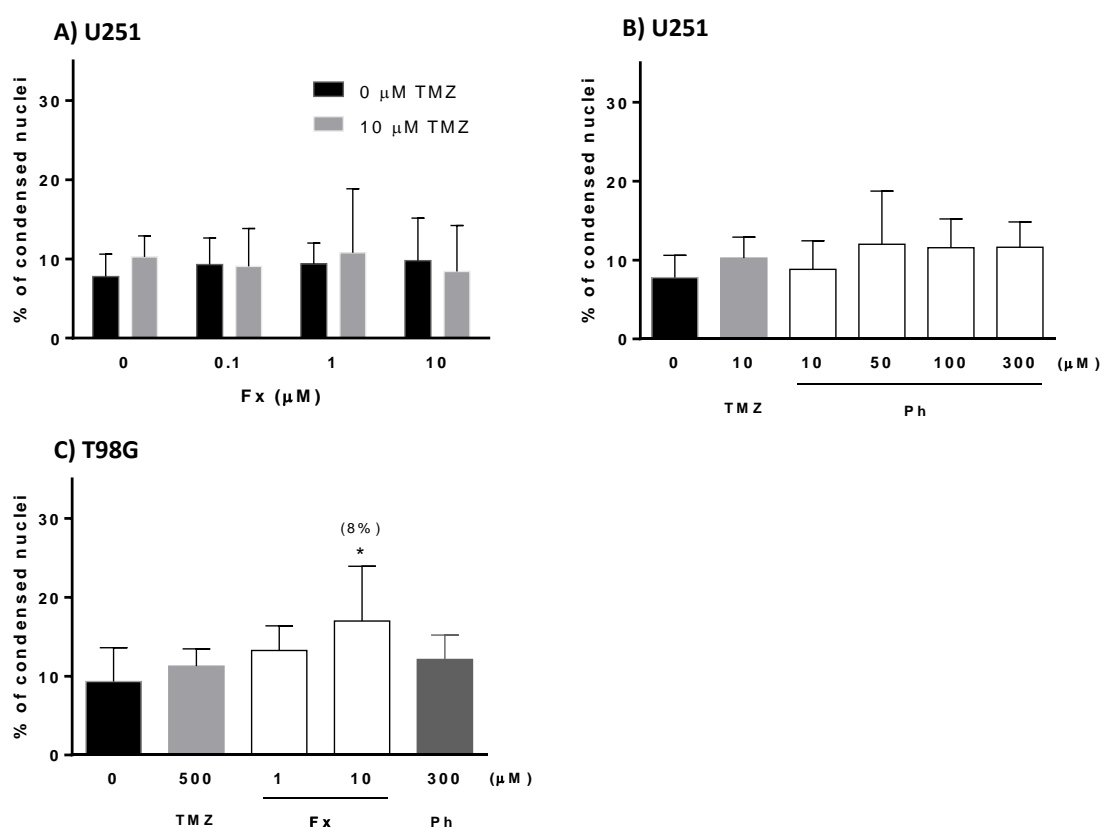


Figure 6 – Effects of Temozolomide (TMZ), Fucoxanthin (Fx) and Phloroglucinol (Ph) on the percentage of condensed nuclei in U251 (A and B) and in T98G (C) cells after 48 h, assessed by nuclear condensation assay. Results are expressed as mean + SD of at least four independent experiments. Significant differences (* $p \leq 0.05$) when compared with the respective controls were determined by two-way ANOVA (A) or by one-way ANOVA (B and C), followed by a Bonferroni multiple comparison test.

Assessment of genotoxic effects by comet assay

To start exploring the possible mechanisms involved in the anti-proliferative effect caused by the compounds, induction of DNA damage (SBs and FPG-sensitive sites) was assessed by the comet assay after 24 h of treatment. Results were expressed as the percentage of DNA in tail.

The results in figure 7A demonstrate that, at 24 h of exposure, none of the tested concentrations of the compounds and drug induce DNA damage in U251 cells. However, there seems to be an increasing trend of the FPG-sensitive sites in the combination (10 μ M TMZ + 10 μ M Fx), relatively to TMZ alone, and at 300 μ M Ph. In the T98G cells (Figure 7B and 8) only TMZ at 500 μ M induced a significant increase of SBs (black bars), relatively to the control and showed a tendency to increase FPG-sensitive sites (gray bars). Fx and Ph, significantly increased by 60 % and 57 %, respectively, the extent of the DNA damage when the FPG enzyme is added (grey bars). In both cell lines, the positive control (cells exposed to the photosensitizer Ro 19-8022), presented high levels of oxidative damages recognized by FPG (data not shown).

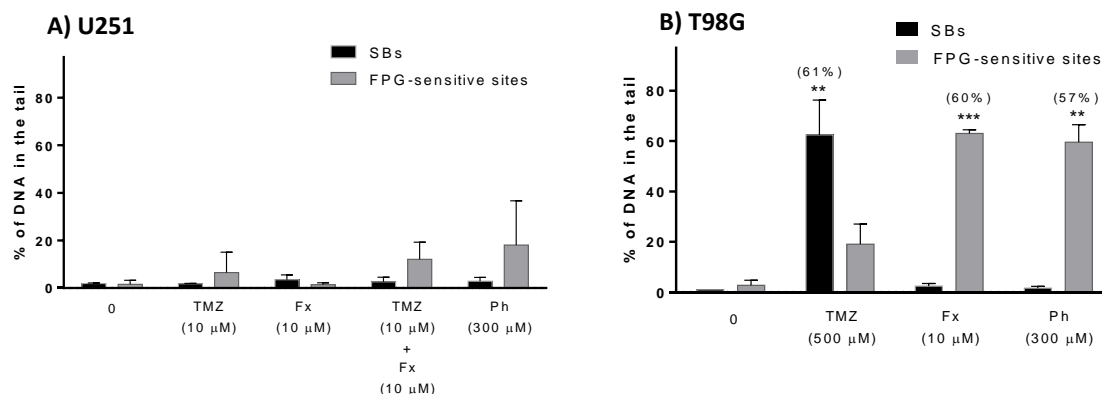


Figure 7 - Effects of temozolomide (TMZ), fucoxanthin (Fx) and phloroglucinol (Ph) on DNA damage (Strand-breaks (SBs) and FPG-sensitive sites) in U251 (A) and T98G (B) cells after 24 h, assessed by comet assay. Results are expressed as mean + SD of at least two independent experiments. Significant differences (** $p \leq 0.01$ and *** $p \leq 0.001$) when compared with the respective controls were determined by one-way ANOVA followed by a Dunnett multiple comparison test.

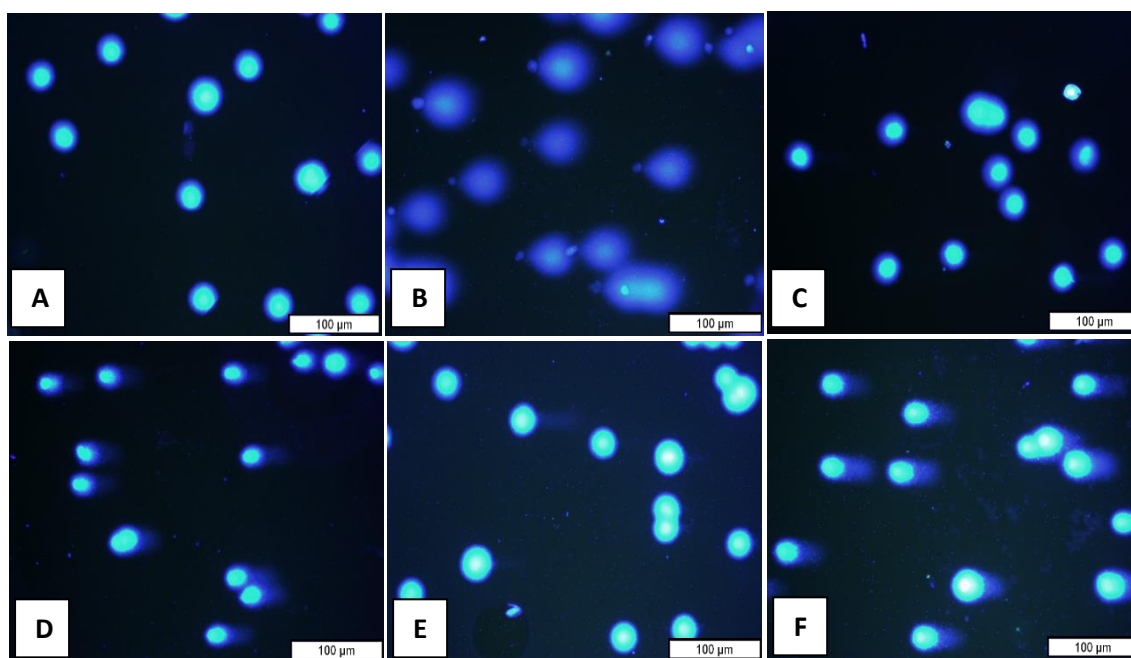


Figure 8 - Representative images of the comet assay in T98G cells treated with A) 0.5 % DMSO (negative control) (SBs); B) 3 μ M Ro19-8022 (positive control) (FPG-sensitive sites); C) 10 μ M fucoxanthin (SBs); D) 10 μ M fucoxanthin (FPG-sensitive sites); E) 300 μ M phloroglucinol (SBs); F) 300 μ M phloroglucinol (FPG-sensitive sites). Scale bar = 100 μ m.

4. Discussion

Nowadays, the standard treatment for GBM includes resection of the tumor accompanied with chemotherapy and radiotherapy (Friedman et al. 2000, Goellner et al. 2011). Alkylating agents, such as TMZ, are the most used drugs in the therapy of glioblastomas, however, drug resistance diminishes drug efficacy (Ramos et al. 2013). This makes a great necessity search for new anticancer drugs or compounds that help to improve chemotherapy (Messaoudi et al. 2015, Yang et al. 2015). The objective of this study was to assess the *in vitro* cell viability and proliferation effects of two natural compounds, Fx and Ph, present in seaweed, either alone or in combination with the conventional anticancer drug TMZ, in the U251 and T98G glioblastoma cell lines. Induction of cell death and genotoxicity (detection of DNA damage) were also assessed to start investigating the underlying mechanisms of the noted effects.

That we know of, this is the first time that Fx and Ph are described on the *in vitro* anticancer activity, either alone or in combination with TMZ, in glioblastoma cell lines. In this study, the effects on cell viability and proliferation were evaluated by the MTT assay and cell counting in U251 and T98G cells. TMZ treatment, for 24 h, did not present a decrease in cell viability/proliferation, however, after 72 h of the initial incubation, it is observed a

significant decrease, in U251 cells, suggesting that is required a longer exposure time to see the effects. This result reflects the differences in sensitivity to TMZ between both cell lines, probably mainly due to the expression of MGMT protein that was shown to be more elevated in T98G cells than in the U251, making the U251 cells more sensitive than T98G cells, as already observed by other authors (Yoshino et al. 2010, Montaldi and Sakamoto-Hojo 2013). Nevertheless, there is still some controversy relatively to the sensibility of T98G cells to TMZ (Jakubowicz-Gil et al. 2013).

TMZ induces both N-alkylated and O-alkylated damages, being the first one more abundant, however, these damages are efficiently repaired by the BER system via which they do not become cytotoxic to the cells. However, during the repair process, AP sites are formed and may accumulate as a result of the impairment of BER system that may contribute for TMZ cytotoxicity (Ramos et al. 2013, Roos and Kaina 2013, Yamamoto et al. 2015). AP sites are repair intermediates that can be detected by comet assay due to its conversion in strand breaks (SBs) in alkaline condition (Collins 2004). The comet assay is used to measure SBs, however, the specificity and sensitivity can be improved if the nucleoids are digested with specific enzymes. An example is the FPG enzyme, which recognizes specific damages in the DNA, such as 8-oxoguanine, and also alkylating damages, such as the N7-meG, converting the lesions into breaks and enhancing the quantity of DNA in the comet tail (Collins 2004). An increase of SBs in T98G cells was observed after TMZ treatment, which could be due to the detection of AP sites, as a repair intermediate during the repair process of N7meG by BER system. However, this increase does not necessarily mean a higher cytotoxicity because if the BER system is correctly working the repair process will continue. Nevertheless, the same effect is not observed in U251 cells, which may be due to the fact that both cell lines present different growth rates (the duplication times are different) (Weller et al. 1998), hence the kinetics of the repair damage will be also different. As a future perspective, a study of DNA damage over time could allow us to understand how the damage repair is processed in each cell line.

Besides the absence of DNA damage detected by comet assay after a long exposure time to TMZ, this was cytotoxic mainly for the U251 cells, in which is verified a slight increase of cell death detected by nuclear condensation assay. This may be due to the induction of O6-meG by TMZ, that in U251 cells due to low MGMT levels are not repaired and through MMR action SBs are produced and accumulated being cytotoxic for the cells (Fukushima et al. 2009, Zhang et al. 2012).

The marine carotenoid Fx decreased both cell viability and proliferation in U251 cells. In view of the slightly raised nuclear condensation, which is one of the morphological

characteristic of apoptosis (Toné et al. 2007), those decreases could be due, at least in part, to that cell death type. So, the data may indicate the capacity of Fx to induce cell death through apoptosis in U251 cells. Various studies demonstrated the ability of Fx induce apoptosis in different cell lines (Hosokawa et al. 2004, Zhang et al. 2008, Kim et al. 2010), however, this is not the only mechanism responsible for this effect. Our data do not allow the identification of the fine mechanism, but, according to the literature, Fx can also induce cell cycle arrest and other types of cell death in a variety of cell lines (Rengarajan et al. 2013). In the tested condition, Fx did not induce DNA damage detected by the comet assay in U251 cells. Although the effect of Fx on cell viability/proliferation was not statistically significant in T98G cells, it seemed to be a trend for a decrease that could be related to the induction of DNA damage recognized by FPG and a posteriorly increase of cell death. According to the literature, the increase of the DNA damage can lead to several consequences, such as: (i) cell cycle arrest with full repair of the damage and cell survival; (ii) cell cycle arrest with inefficient repair, and induction of cell death or cell cycle arrest; (iii) cell cycle arrest with inefficient repair and development of mutation, which may be lethal or constitute an adaptive advantage to sustain cell survival. Accordingly, it is opportune to point out that the cell fate after DNA induction seems to be determined by the cellular context (Tentner et al. 2012).

After an exposure of 24 h, Ph did not reveal effects in the number of viable cells, however, the metabolic activity of the cells decreases, mainly in U251 cells. After 48 h, Ph decreased the number of cells showing antiproliferative effects. This effect could be in part due to the induction of cell death, suggested by the increasing trend seen in the nuclear condensation assay, in line with the suggestive raise of the DNA damage recognized by the FPG. In T98G cells, the effect of Ph was only observed by the MTT assay at 24 h and a significant increase of DNA damage recognized by the FPG was detected. However, the proliferation of T98G cells was not affected which could mean that T98G easily repair these damages or be tolerant to them. The antiproliferative effects and cell death induction by Ph has been reported in different types of cell lines, such as colon cancer HT-29 cells and breast cancer BT549 cells, amongst others (Kang et al. 2014, Kim et al. 2015). So, since, in our study, cell death was not very evident this could indicate that the time of exposure with Ph for the nuclear condensation assay was not the most indicated being necessary the evaluation with longer exposure times (e.g. 72 h), as well as the use of other assays to evaluate cell death (e.g. annexin-V expression). However, other mechanisms such as effects on signaling pathways that control cell proliferation, cell cycle arrest and induction of other types of cell death could be involved and should be regarded in the future.

In the present study, effects on cell viability were assessed by two tests and it was observed some differences in the data of Fx and Ph when evaluated by the MTT assay or by cell counting. An example is that Fx exhibited a cytotoxic effect when evaluated by cell counting, but not with the MTT assay, whereas Ph demonstrated a higher cytotoxic effect in the MTT assay without significant effects on cell counting. The MTT assay is a colorimetric assay that measures the metabolic activity of the cells since the reduction of the MTT occurs specifically in cells that are metabolically active by the action of mitochondrial and others intracellular reductases. Alterations in that activity could result in significant changes in the MTT data in spite of the number of viable cells, which may remain constant (Vega-Avila and Pugsley 2011). Jo et al. (2015) studied the cytotoxicity induced by ethanol in glioblastoma cells (GBL-13, GBL-15, U87MG and U373 MG) using a variety of cytotoxicity assays to evaluate the accuracy of the MTT assay. The results showed that the MTT assay might miscalculate the actual cytotoxicity by presenting a higher absorbance instead of the actual reduction of the MTT by the dying cells. Several natural compounds, such as vitamin E, kaempferol, and other flavonoids, showed the ability to reduce directly the MTT, thus contributing to false negative results (Lim et al. 2015). This fact could explain the absence of results obtained with Fx in the MTT assay beside the decrease in cell counting. Regarding the Ph results, it was observed the opposite, i.e. an effect in the MTT assay - decreased the formation of formazan crystals, which reflects a lower metabolic activity, whereas the number of cells remained constant. In this case, it gives an indication that the Ph might have an effect on the mitochondrial activity therefore interfering with the assays (Kang et al. 2014). Our conclusion in respect to the use of MTT assay in the cytotoxic assays is that it may give us information about mechanisms of action when complemented with other assays, namely cell counting, neutral red or others.

Regarding the combination 10 μ M TMZ + 10 μ M Fx, it is observed a decrease in cell viability at 24 h, without affecting metabolic activity, in U251 cells. It is also visible a tendency to decrease cell proliferation when compared to TMZ alone. The cytotoxic and antiproliferative effects observed was not sustained by an increase in the number of cells with condensed nuclei; we noticed at most a suggestive increase of FPG-sensitive sites in comparison with TMZ alone. Regarding the combination, other mechanisms could have influenced the antiproliferative effects. The potentiation of the cytotoxic activity of TMZ by natural compounds was reported in other studies. Lin and collaborators demonstrated that the therapeutic efficacy of TMZ was enhanced when combined with natural compounds such as resveratrol, in U87 MG and GBM8401 cells. Resveratrol in combination with TMZ suppressed cytoprotective autophagy induced by TMZ and increases apoptosis by the ROS/ERK pathway (Lin et al. 2012). Torres et al. (2011), revealed that the combination of

THC, a cannabinoid present in marijuana, with TMZ, in glioblastoma cells, increases cytotoxic autophagy. The same study additionally showed that the inhibition of this process avoided TMZ + THC-induced cell death. Therefore, corroborating that cytotoxic autophagy activation has a critical role in the potentiation of TMZ.

In conclusion, under the assayed conditions T98G cells were more resistant to TMZ than U251 cells, and both Fx and Ph, alone, inhibited cell viability and proliferation, mainly in the U251 cells. Cell death by apoptosis does not seem to be the main mechanistic cause for the noted antiproliferative effect, and so other assays must be performed to clarify the mechanisms involved. Beside the antiproliferative effect of Fx alone, when in combination with TMZ the natural compound seems to potentiate the TMZ action against U251 cells. The data back that more studies are worth to assess the molecular mechanisms involved.

Conflict of interest

There are no conflicts of interest to report.

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Chapter 3

Conclusions and Future Perspectives

GBM is a malignant brain tumor that still does not have a cure, however, there are several drugs used in the treatment of GBM. TMZ is the most widely used drug in the treatment of GBM sometimes in combination with radiotherapy, which had some success, however, is still not a cure mainly due to the development of resistance by the cancerous cells. Several mechanisms can contribute to resistance to TMZ, such as overexpression of the repair protein MGMT, damage in the MMR or in the BER pathways, amongst others. So, several possible strategies are nowadays in the study to potentiate the action of TMZ, namely its combination with natural compounds. Some that are present in seaweed, such as carotenoids and phlorotannins, have shown promising anticancer effect. However, the interaction of these compounds with drugs, as TMZ, is still not very explored despite such research could be of major importance. The aim of this study was to evaluate the *in vitro* anticancer effect of two natural compounds present in seaweed, Fx and Ph, either alone or in combination with an anticancer drug, TMZ, in two glioblastoma cell lines, U251 and T98G.

Our results demonstrated differences in the effect of TMZ between both cell lines, showing that U251 cells are more sensitive to TMZ action than T98G which clearly reflects the genetic differences between them. Also, both, Fx and PH exhibited cytotoxicity and antiproliferative effects against both cell lines being the impacts more evident in the U251 cell line. The anti-proliferative effects observed might probably not be associated with DNA damage and cell death induction by apoptosis, since no impacts were observed by the comet and nuclear condensation assays, respectively. Consequently, other mechanisms such as cell cycle arrest and other types of cell death should be assessed. The combination 10 μ M TMZ + 10 μ M Fx seems to be the most promising one to study, in U251 cells, in which was observed that Fx potentiates TMZ action. In conclusion, Fx and Ph showed anticancer activity *in vitro* against glioblastoma cell lines, and in the case of Fx may also potentiate the action of TMZ. Accordingly, more studies are desirable to clarify what kind of mechanisms are involved.

As future perspectives in the aftermath of this work, additional approaches should be considered to confirm and improve the knowledge about the action of Fx, Ph and the combination with TMZ. In this study, effects on cell viability were determined by two assays: MTT assay and cell counting. However, differences in the results between the MTT assay and cell counting were notorious, revealing that the MTT assay could in some cases give biased results. A possible interaction between the natural compounds and the MTT assay should be tested. In this specific case, Fx should be incubated directly with the MTT solution without cells, to test if Fx has the capacity to reduce the MTT. Also, the cell counting assay presented some limitation because in this assay was performed without trypan blue. Therefore, in the future, this solution should be applied to improve the counts. We may also

speculate that the minor effects seen in the nuclear condensation assay can be due to the fact that the exposure time to the compounds was not the most appropriate. Thus, it seems worth to increase the incubation time in the nuclear condensation assay and complement it with the use of other tests, such as annexin-V and TUNEL assays. In the present study, “only” SBs, AP sites and damages recognized by FPG were analyzed by comet assay. However, the TMZ-induced DNA damage responsible for the substance’s toxicity is the O6-meG that is not detected by the comet assay, therefore, other methods and assays should be included to evaluate this specific damage. Our data from the comet assay are still preliminary in that the analysis of more replicates are still missing. Also, the study of the kinetics of DNA damage seems to be an essential step for understanding its role in the cell fate decision.

As the Dissertation project has a relatively short time to be made, some experiments were not done, yet, such as the Western blot to evaluate some molecular markers, such as MGMT, p53, caspase 3 and 9, Bcl-2, Bax, amongst others. Also, it would be of particular interest to study the effects of Fx and Ph in a normal cell line(s), viz. with astrocytes, to assess the specificity of the anticancer effect, and then move maybe to an *in vivo* model.

APPENDIX

Experimental Protocols

Protocol 1. Cell culture

Materials	Assays solutions and reagents
T25 cell culture flasks	PBS (Phosphate Buffered Saline)
Laminar flow hood	Cell culture medium e.g. MEM
Humidified, controlled temperature/CO₂ incubator	FBS (Fetal Bovine Serum)
Neubauer chamber	Antibiotics (100U/ml penicillin and 100 µg/ml streptomycin)
Inverted phase contrast microscope	Sodium bicarbonate
	Sodium pyruvate
	HEPES
	0.25 % Trypsin/EDTA solution

Assay solutions

Prepare the following assay solutions:

(A) Cell culture medium (MEM) ¹

	Final concentration	Weight / Volume
Eagle's minimum essential medium (MEM)	9.6 g/L	4.8 g
Sodium bicarbonate	2.2 g/L	1.1 g
Sodium pyruvate	110 mg/L	55 mg
HEPES	10 mM	1.192 g
FBS	10 %	50 ml
Penicillin / Streptomycin	1 %	5 ml
Ultrapure H₂O	-	445 ml

¹ Only applicable for MEM cell culture medium. Each medium has a different final composition. FBS and antibiotics % are common for all mediums.

To prepare 500 ml of medium weight the reagents into a glass container, add 445 ml of ultrapure H₂O and mix until dissolved. In the laminar flow hood, filter the solution with a filter of 0.2 µm pore. Add 50 ml of FBS and 5 ml of Penicillin / Streptomycin. Store sealed at 4 °C.

(B) PBS buffer (1X)

	Final concentration	Weight
NaCl	137 mM	8.0 g
KCl	2.7 mM	0.2 g
Na₂PO₄	10 mM	1.44 g
KH₂PO₄	2 mM	0.24 g

To prepare 1L of PBS solution dissolve the reagents in approximately 800 ml of ultrapure H₂O and adjust the pH to 7.4. Add the rest of the water for the final volume of 1L, autoclave the solution and store at 4 °C.

GENERAL GUIDELINES

*Cell maintenance*²

New culture

1. To start a cell culture, defrost an aliquot of frozen cells and mix with the appropriate cell culture medium passing them into a sterile cell culture flask with a final volume of 5 ml of warm medium. Slightly shake the flask to certify the cell suspension covers the bottom.
2. Place the flask in a humidified incubator at 37 °C and 5 % CO₂. Cells will adhere within 24 h.

² Cell manipulation must always be done under sterile conditions. Process all steps under a laminar flow hood and use aseptic manipulation techniques.

3. It is desirable to change the medium the next day in order to eliminate all traces of DMSO in the culture. The medium should be changed every two days, and cells trypsinized at least once a week when there is confluency.

Medium change

1. Before starting, always check the status and confluence of the culture under the inverted microscope.
2. In the water bath, at 37 °C, pre-heat PBS and cell culture medium.
3. Aspirate the medium from the flask and wash with 1 ml of PBS, two times. Remove the PBS and add 5 ml of cell culture medium. Place the flask in a humidified incubator at 37 °C and 5 % CO₂.

Trypsinization (Sub-culturing)

1. Once the cells reach about 80 % confluence they must be trypsinized.
2. In the water bath, at 37 °C, pre-heat the trypsin solution and PBS. Aspirate the medium and wash, two times with 1 ml PBS. Remove the PBS and add 1 ml of trypsin solution and put in the incubator at 37 °C for 5-7 min.
3. Observe in the microscope to confirm that the cells are detached. Add 4 ml of medium to annul the trypsin action and resuspend with care.
4. To perform an assay, count the cell in the Neubauer chamber to determine the cell density for a posterior plating. If not, just remove a certain amount of cell solution to a new flask (according to with the cell density) and add medium up to 5 ml. Incubate at 37 °C and 5 % CO₂.

Protocol 2. MTT colorimetric assay

Materials	Assays solutions and reagents
96-multiwell culture plates	PBS (Phosphate Buffered Saline)
Microplate reader	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) stock solution
	DMSO (dimethyl sulfoxide):ethanol (1:1) Solution

Assay solutions

Prepare the following assay solutions:

(A) MTT stock solution ³

	Final concentration	Weight
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	5 mg/ml	50 mg

To prepare 10 ml of MTT solution weight 50 mg of MTT and mix in PBS (1X). Dissolve the solution in the ultrasound bath. Make 1 ml aliquots and store at -20 °C.

(B) PBS buffer (1X)

	Final concentration	Weight
NaCl	137 mM	8.0 g
KCl	2.7 mM	0.2 g
Na₂PO₄	10 mM	1.44 g
KH₂PO₄	2 mM	0.24 g

³ MTT is carcinogenic, handle with care.

To prepare 1L of PBS solution dissolve the reagents in approximately 800 ml of ultrapure H₂O and adjust the pH to 7.4. Add the rest of the water for the final volume of 1L, autoclave the solution and store at 4 °C.

(C) DMSO:ethanol (1:1) ⁴

Mix 250 ml of dimethyl sulfoxide (DMSO) with 250 ml of pure ethanol (100 %, v/v). Store at room temperature protected from light.

PROTOCOL

Preparation of the cells ⁵

1. In the laminar flow hood, incubate (0.4×10^5 – U251 and 0.8×10^5 – T98G) in a 96-multiwell culture plate and left the cells to adhere for 24 h in a humidified incubator at 37 °C and 5 % CO₂.
2. Treat the cells as desired.

MTT

3. Add 10 µl of MTT solution (0.5 µg/ml) to the initial control (t = 0) and place in the incubator at 37 °C and 5 % CO₂ for 2 h. After 2 h aspirate gently the medium of the control cells (t = 0 h) and left to incubate in the incubator at 37 °C until the next day (for a 24 h assay).
4. After 24 h of incubation with the test compounds add 10 µl of MTT solution (0.5 µg/ml) to the remaining cells, treated cells and final control (t = 24 h), and place in the incubator at 37 °C and 5 % CO₂ for 2 h. After those 2 h aspirate the medium of all cells.
5. Protected from light, add 150 µl of DMSO:ethanol solvent to dissolve the crystals and shake gradually for 10 – 20 min to dissolve the crystals. When completed dissolved, the absorbance is measured in a microplate reader at 570 nm.

⁴ DMSO is toxic, beware of any vapors and handle with care. Use rubber or nitrile gloves for extended manipulation.

⁵ This protocol must be processed under sterile conditions.

Calculations

The percentage of cell viability is calculated by the division of the absorbance (A) of the condition by the control, multiplied by 100, as seen in the equation:

$$\% \text{ Cell viability} = (A_{\text{condition}} \times 100 / A_{\text{control}})$$

Protocol 3. Neubauer chamber cell counting (Cytotoxicity/Proliferation assay)

Materials	Assays solutions and reagents
24-multiwell culture plates	PBS (Phosphate Buffered Saline)
Neubauer chamber	0.25 % Trypsin/EDTA solution
Inverted phase contrast microscope	Cell culture medium, e.g. MEM
Eppendorf's of 1.5 ml	

Assay solutions

Prepare the following solutions:

(A) PBS buffer (1X)

	Final concentration	Weight
NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
Na₂PO₄	10 mM	1.44 g
KH₂PO₄	2 mM	0.24 g

To prepare 1L of PBS solution dissolve the reagents in approximately 800 ml of ultrapure H₂O and adjust the pH to 7.4. Add the rest of the water for the final volume of 1L, autoclave the solution and store at 4 °C.

PROTOCOL

Preparation of the cells⁶

1. In a laminar flow hood, incubate (0.6×10^5 – U251 cells and 0.9×10^5 – T98G cells) in a 24-multiwell culture plate and let cells, in a humidified incubator at 37 °C and 5 % CO₂, to adhere for 24 h.
2. Treat the cells as desired.

Cytotoxicity assay

3. Trypsinize the well of the initial control (t=0) and count the cells. Incubate the cells in a humidified incubator at 37 °C and 5 % CO₂ for 24 h.
4. In the end of the treatment wash the cells with PBS, remove it and add 250 µl of warm trypsin for 3 – 5 minutes. Stop the action of the trypsin by adding 650 µl of culture medium and resuspend the cells with care.
5. Pass 300 µL into marked Eppendorf's and centrifuge 5 min at 1500 rpm. The rest of the cellular suspension is used to count the cells in the Neubauer chamber.

Proliferation assay

6. Remove the supernatant (samples from point 5) and resuspended in 500 µL of the fresh medium which is placed in a new 24-multiwell culture plate. Let the cells incubate in a humidified incubator at 37 °C and 5 % CO₂.
7. After the 48 h, repeat the step 4 and count the cells in the Neubauer chamber.

Calculations

The percentage of cellular viability, after 24 h of incubation, is determined according to the ratio between the number of cells of each condition and the number of cells of the control multiplied by 100:

$$\% \text{ of survival} = (n^{\circ} \text{ of cell}_{\text{Condition}} / n^{\circ} \text{ of cell}_{\text{Control}}) \times 100$$

⁶ This protocol must be processed under sterile conditions.

To determine the relative suspension growth (RSG), first is determined the total suspension growth (TSG) which is the ratio between the number of cells at 72 h and the number of cells at 0 h:

$$\mathbf{TSG} = n^0 \text{ of cells (at 72 h) / } n^0 \text{ of cells (at 0 h)}$$

After the TSG is calculated the RSG using the TSG of each condition divided by the TSG of the control:

$$\mathbf{RSG} = \text{TSG}_{\text{Condition}} / \text{TSG}_{\text{Control}}$$

Protocol 4. Comet assay

Materials	Assays solutions and reagents
12-multiwell culture plates	PBS (Phosphate Buffered Saline)
Horizontal electrophoresis tank	Lysis solution, pH 10
Electric power supply	0.25 % Trypsin/EDTA solution
Fluorescence microscope	Electrophoresis Buffer (0.3 M NaOH, 1 mM EDTA, pH 13)
Microscope slides	1 % Normal Melting Point Agarose (w/v)
Coplin jar	0.5 % Low Melting Point Agarose (w/v)
Metal tray	DAPI staining solution (1 µg/ml)
Thermoblock	Buffer F

Assay solutions

Prepare the following solutions:

(B) Lysis solution, pH 10 ⁷

	Final concentration	Weight
NaCl	2.5 M	146.1 g
EDTA	0.1 M	37.22 g
Tris Base	10 mM	1.211 g

To prepare 1L of Lysis solution, weight the reagents and dissolve in approximately 900 ml of ultrapure H₂O. Adjust the pH to 10 by adding NaOH 8 M or 10 M and then add the rest of the water for the final volume of 1L. Store at 4 °C and prior to use, immediately add 1 % Triton X-100 (v/v).

⁷ This solution can be prepared and maintain for a month at 4°C without Triton X-100

(C) Electrophoresis Buffer (0.3 M NaOH, 1 mM EDTA, pH 13) ⁸

	Final concentration	Weight
NaOH	0.3 M	12.60 g
EDTA	1.0 mM	0.39 g

To prepare 1L of electrophoresis buffer dissolve the reagents in approximately 900 mL of ultrapure H₂O. Adjust the volume to 1 L and store at 4 °C.

(D) Buffer F ⁹

	Final concentration	Weight
HEPES	40.0 mM	95.32 g
KCl	0.1 M	74.56 g
EDTA	0.5 mM	1.86 g
BSA	0.2 mg/mL	2.50 g

To prepare 1L of buffer F weight the reagents and add approximately of 900 mL of ultrapure H₂O. Adjust the pH to 8 by adding KOH 10 M and adjust the volume to 1 L. A stock of 10X could be made. Store at -20°C.

(E) PBS buffer (1X)

	Final concentration	Weight
NaCl	137.0 mM	8.00 g
KCl	2.7 mM	0.20 g
Na ₂ PO ₄	10.0 mM	1.44 g
KH ₂ PO ₄	2.0 mM	0.24 g

⁸ This solution can be prepared and maintain for a week at 4°C.

⁹ This solution can be prepared and maintain for a week at 4°C. Before each use dilute the stock 10 X in ultrapure H₂O.

To prepare 1L of PBS solution dissolve the reagents in approximately 800 ml of ultrapure H₂O and adjust the pH to 7.4. Add the rest of the water for the final volume of 1L, autoclave the solution and store at 4 °C.

(F) 1 % Normal Melting Point Agarose (w/v)

Dissolve 200 mg of normal melting point (NMP) agarose in 20 ml of distilled H₂O. The solution is heated in a microwave for 1 – 2 min until is completely dissolved. Store at 4 °C and when required, melt prior to use.

(G) 0.5 % Low Melting Point Agarose (w/v)

Dissolve 100 mg of low melting point (LMP) agarose in 20 ml of PBS. The solution is heated in a microwave for 1 – 2 min until completely dissolved. Make an aliquot of 5 ml of the solution into cryotubes and store it at 4 °C. Prior to use, melt and maintain at 37 °C in a water bath.

(H) DAPI staining solution (1 µg/ml) ¹⁰

Prepare a DAPI solution of 1 µg/ml from a stock solution of 100 µg/ml. Dilute in distilled H₂O. Store at -20 °C, protected from light.

PROTOCOL

Preparation of the slides

Melt 1 % NMP agarose (w/v) and dip conventional microscope slides in this solution. Allow the slides to dry horizontally at room temperature overnight and then make a mark in the side with the agarose. Label the slides, preferably with pencil, with the appropriate assay information. Prepared slides may be stored for several days before use.

¹⁰ DAPI is carcinogenic, handle with care.

Preparation of the cells ¹¹

1. In the laminar flow hood, incubate (0.75×10^5 – U251 and 0.1×10^6 – T98G) in a 12-multiwell culture plate and let cells to adhere in a humidified incubator at 37 °C and 5 % CO₂ for 24 h.
2. Treat the cells as desired.

Comet assay

3. Place the plate on ice and wash with cold PBS, remove it and add 250 µl of warm trypsin for 3 – 5 minutes. Stop the trypsin by adding 650 µl of culture medium and resuspend the cells with care.
4. Label the tubes, place on ice and then pass everything into them. Centrifuge 5 min at 250 g with the centrifuge at 4°C.
5. The supernatant is then removed and the concentration adjusted to 10^6 cells/mL with cold PBS.
6. LMP agarose is melted and must be maintained at 37°C. Then place a metal tray on ice and identify the slides with “lysis”, “FPG” and “Buffer F”
7. In an Eppendorf, add 420 µL of LMP agarose plus 90 µL of cell suspension (from the point 5) and make circular movements to get a correct distribution of the cells in the agarose. Quickly and avoiding bubbles, apply two drops of 70 µL in each slide and cover with a 20 x 20 mm coverslip. In the end, for each sample will be 3 different slides (one for lysis, FPG and Buffer F).
8. Put the slides in the cold tray to solidify. Once solidified remove the coverslip gently and submerge them in the lysis solution previously prepare, in a Coplin jar, for at least 1h.
9. During the lysis time, put three Coplin jars with Buffer F at 4°C. Then submerge, only, the slides marked with “FPG” and “Buffer F” to do the first wash, at 4°C for 5 min. Do the other two washes consecutively with the other two Coplin jars.
10. Defrost the FPG and dilute with cold Buffer F to the desired concentration. Always maintain the enzyme on the ice. When the washes are done put the slides on a cold metal tray and add 45 µL of FPG to the slides marked “FPG” and 45 µL of Buffer F to the slides marked “Buffer F”. Cover with a 22 x 22 mm coverslip.
11. Incubate at 37°C for 30 min in a Tupperware surrounded by water pre-heated beforehand. When the incubation is complete, remove the trade and put on ice to stop the enzymatic reaction.

¹¹ This protocol must be processed under sterile conditions.

12. During the incubation time, prepare the horizontal electrophoresis tank, level it and fill with 1 L of electrophoresis buffer. Maintain the tank at 4 °C.
13. Remove the coverslips and transfer all the slides into the horizontal electrophoresis tank for 40 min at 4 °C, to allow the DNA to unwind.
14. Set the power supply to 25 V (1 V/cm) and run the electrophoresis for 20 min at 4 °C.
15. Remove the slides and immerse in cold PBS and then distilled H₂O for 10 min at 4 °C. Leave the slides to air dry.

Analysis

16. When ready for analysis, apply 30 µl of working DAPI staining solution to each sample and apply a 22 x 22 mm coverslip. From this point on, keep slides away from light.
17. Incubate for approximately 20 min at room temperature.
18. The samples are now ready to evaluate under a fluorescence microscope. A minimum of 3 independent experiments is recommended in order to produce feasible results.

Evaluation of DNA damage

The samples must be observed under a fluorescence microscope and the images analyzed by a quantitative method, by the use of an image analysis software (e.g. OpenComet). The software analysis gives us, namely % of DNA in tail, tail length and tail moment. If there is no software the comets must be assessed semi-quantitatively by manual scoring.

The samples should be analyzed by one person only and should be scored at least 100 cells per sample. Do not repeat the same areas and do not analyze cells around the edges of the gel.

Protocol 5. Nuclear condensation assay

Materials	Assays solutions and reagents
24-multiwell culture plates	PBS (Phosphate Buffered Saline)
Poly-L-Lysine microscope slides	4 % Paraformaldehyde (PFA) (w/v)
Hydrophobic barrier pen	0.25 % Trypsin/EDTA solution
Centrifuge	DAPI staining solution (1 µg/ml)
Cytocentrifuge filters	50 % Glycerol solution
Cytocentrifuge	
Fluorescence microscope	

Assay solutions

Prepare the following solutions:

(A) 4 % Paraformaldehyde (w/v) ¹²

	Final concentration	Weight
Paraformaldehyde (PFA)	4 %	4 g

Mix 4 g of PFA in PBS for a final volume of 100 ml. Dissolve the solution in a water bath at 60 °C. Keep the lid closed due to the release of vapors and stir the solution every so often. When completely dissolved, leave the solution at air temperature to cool down and adjust the pH to 7.4. Aliquot in 50 ml tubes and store at -20 °C.

¹² PFA solution is toxic, beware of vapors and handle with care.

(B) PBS buffer (1X)

	Final concentration	Weight
NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
Na ₂ PO ₄	10 mM	1.44 g
KH ₂ PO ₄	2 mM	0.24 g

To prepare 1L of PBS solution dissolve the reagents in approximately 800 ml of ultrapure H₂O and adjust the pH to 7.4. Add the rest of the water for the final volume of 1L, autoclave the solution and store at 4 °C.

(C) DAPI staining solution (1 µg/ml) ¹³

Prepare a DAPI solution of 1 µg/ml from a stock solution of 100 µg/ml. Dilute in distilled H₂O. Store at -20 °C, protected from light.

(D) 50 % Glycerol solution

Mix 5 ml of pure glycerol with 5 ml of PBS (1X) and store at 4 °C.

PROTOCOL**Preparation of the cells ¹⁴**

1. In the laminar flow hood, incubate (0.6×10^5 – U251 and 0.9×10^5 – T98G) in a 24-multiwell culture plate and let the cells adhere in a humidified incubator at 37 °C and 5 % CO₂ for 24 h.
2. Treat cells as desired.

¹³ DAPI is carcinogenic, handle with care.

¹⁴ This protocol must be processed under sterile conditions up to step 3

Nuclear condensation assay

3. Before start label the falcon tubes. Remove the medium into the Falcons, wash the cells with 1 ml of warm PBS and pass the PBS also into the falcons. Add 200 µl of warm trypsin to the cells for 3 – 5 minutes.
4. Stop the action of the trypsin with 500 µl of culture medium and resuspend the cells with care. Pass the cellular suspension into the falcon, wash the wells with 1 ml PBS and then put into the respective falcon.
5. Centrifuge the samples at 500 g for 10 min.
6. Remove the supernatant until 500 µl, resuspend the pellet with 3 ml of PBS and centrifuge the samples again at 500 g for 10 min.
7. Remove the supernatant until 500 µl, resuspend the pellet with 2 ml of PFA and let incubate at 37 °C for 15 – 20 min.
8. Add 4 ml of PBS on top of the PFA and centrifuge at 500 g for 10 min.
9. Remove the supernatant until 500 µl, collect the pellet into marked Eppendorf's and store at 4 °C or proceed with the experiment.
10. Label the poly-L-Lysine microscope slides. Prepare the filters and set the slides into the cytocentrifuge frames. Pipette 100 to 150 µl of the sample into the respective cytocentrifuge tube and cytocentrifuge at 500 rpm for 5 min. Each slide takes two samples, one on each side.
11. Remove the slides, make a circle around the samples with a hydrophobic barrier pen and wash the slides three times with PBS, for 5 min each turn.
12. Incubate with 20 µl of DAPI for 10 min in the dark, then add a 6 µl drop of 50 % glycerol and cover with a 22 x 22 mm coverslip. Store the samples at -20 °C, protected from light.

Analysis of nuclear condensation

The slides must be seen under a fluorescence microscope and counted a minimum of 300 cells per sample, in at least 3 different areas. The total number of cells and the number of cells with condensed nuclei should be counted. The percentage of cells with condensed nuclei is defined by the ratio between the number of cells presenting condensed nuclei and the total number of cells:

$$\text{\% of cells with condensed nuclei} = (\text{n}^{\circ} \text{ of cells in nuclear condensation} / \text{Total n}^{\circ} \text{ of cells})$$