Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN

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COORDINATORE Prof. PAOLO PINTON

Emerging strategies for selective targeting in therapeutic approaches against gliomas

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ABSTRACT

Glioblastoma multiforme (GBM) is a lethal malignant primary brain tumor in adulthood, characterized by several genetic alterations and cellular heterogeneity (Louis DN, 2016).
The standard treatment consists of a combination of surgery, radiotherapy and chemotherapy. Despite the aggressive treatments, a definitive therapy is not available at present, and the mean patient survival time reaches only 14.6 months (Wilson TA, 2014).
Temozolomide (TMZ), is an alkylating agent currently used as first line therapy in combination with radiotherapy (Zhang J, 2012). Considering drug-resistance issues, as of now, TMZ used alone or in combination with radiotherapy can only increase the lifetime expectancy of GBM patients. The enhanced activity of a DNA repair enzyme, the O(6)-methylguanine-DNA-methyltransferase (MGMT), represents one of the main mechanisms of drug resistance in glioma, since it repairs TMZ-induced DNA lesions (Fan CH, 2013).
Gliomagenesis is attributed to many molecular changes involving both genetic and epigenetic mechanisms; thus, modifications in the apoptotic pathways may not only contribute to the develop of the tumor, but also to the resistance towards classical genotoxic approaches of therapy (Adamson C, 2009).
Further, microRNAs, a class of small non-coding RNAs, play a pivotal role in the development of a malignant phenotype of glioma cells, cell survival, proliferation, tumor angiogenesis and metastasis. Tumorigenesis occurs as the result of imbalances between oncomiRNAs and tumor-suppressor miRNAs, both acting as gene regulators at post-trascriptional level by either repressing translation or degradating the target mRNA. For instance, the oncomiRNAs microRNA-155 and microRNA-221, are significantly elevated in GBM, downregulating multiple genes associated with cancer cell proliferation, apoptosis, invasiveness and drug resistance (Liu Q, 2015; Xie Q, 2014; Zhang CZ, 2010; Shea A, 2016).
Therefore, new therapeutic targets and tools should be developed based on a better understanding of the molecular pathogenesis of glioma. In particular, it would be interesting to design new therapeutic approaches that enhance currently available treatments and/or limit tumor growth, as well as reducing resistance to chemoterapeutic drugs.

The aim of this PhD thesis was to develop novel possible therapeutic interventions inhibiting the growth of the tumor cells, as well as inducing apoptotic cell death by sensitizing glioblastoma cells to temozolomide treatment and/or potentiating its activity. In light of this aim, human U251 and TMZ-resistant T98G glioma cells were studied combining: (i) the treatment with temozolomide and corilagin, an interesting tannin extracted from plants of the *Phillantus* family, and (ii) temozolomide with anti-miR-221 and anti-miR-155 PNAs. Corilagin (COR) is known to exhibit antioxidant, anti-inflammatory and antitumor activity. It was reported to induce biological effects by interfering with the anti-apoptotic NF-kB transcription factor (Gambari R, 2012; Dong XR, 2010) and to induce cancer cell apoptosis (Jia L, 2013, Ming Y, 2013; Gu Y, 2016). Therefore, corilagin could potentially work as an active compound for treating glioblastoma. Firstly, by docking studies, it was demonstrated that corilagin (COR) is able to suppress the level of NF-kB by preventing its ability to bind with DNA. Upon COR treatment, U251 and T98G glioma cells showed a reduction in their proliferation, but most importantly showed increased apoptosis. Interestingly, when using T98G glioma cells, these showed resistance to TMZ treatment, and when co-treated with TMZ plus COR, they reached a level of apoptosis higher than that with temozolomide or corilagin alone. This is probably related to the fact that corilagin, but mainly corilagin with temozolomide (COR + TMZ), decrease the expression of MGMT mRNA and induced caspase-3 (CASP-3) activation; moreover, corilagin demonstrated to be active at inhibiting cell migration, particularly when combined with temozolomide.

As regards to targeting microRNAs to modulate cancer behaviour, peptide nucleic acids (PNAs) against miR-155 and miR-221 were also considered by this thesis. PNAs are synthetic nucleic acid analogues wherein the negatively charged sugar-phosphate backbone is replaced with charge-neutral amide linkages; they are considered ideal candidates for application as antisense therapeutics that block expression of complementary mRNA (Nielsen PE, 1991; Larsen HJ, 1999). MiR-155 and miR-221 were selected from a long list of microRNAs dysregulated in GBM, since they were predicted to target caspase-3 mRNA, an important factor involved in the apoptosis pathway. The results obtained from the Bio-Plex analysis and from the Caspase 3/7 assay, after treatment of glioma cells with PNAs R8-PNA-a155 and R8-PNA-a221, confirmed our hypothesis that CASP-3 is a target gene of miR-155 and miR-221. More recently, within our group, it was demonstrated that R8-PNA-a221 has pro-apoptotic effects in glioma cells (Brognara E, 2016); thus, also the

effect of PNAs R8-PNA-a155 was investigated on both U251 and T98G cells. The obtained data showed that both PNAs are selective in targeting their relative microRNA and they are able to induce apoptosis in both the glioma cell line models under study.

Since oncomiRNAs promote cancer cell growth and survival, we investigated whether, in TMZ-resistant T98G glioblastoma cells, combining temozolomide with PNAs could have a greater effect than single treatments. Co-administration of R8-PNA-a221 or R8-PNA-a155 induced apoptosis of TMZ-treated T98G cells at a level higher than that obtained following singular administration of R8-PNA-a221 or R8-PNA-a155; similar was the effect observed on apoptosis involving caspase-3 activation.

In conclusion, the results reported in this PhD thesis demonstrate that apoptosis induction, could be an efficient way to modulate the progression of cancer and the drug resistance. With this research, we showed that using natural compounds or synthetic agents, such as corilagin and PNAs, respectively, could constitute an efficient and powerful strategy to render resistant-glioma cells more susceptible towards therapy-induced apoptosis, as well as improving the efficacy of the first-line drug temozolomide treatment.

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INTRODUCTION

1. BRAIN TUMORS: GLIOMA AND GLIOBLASTOMA

1.1 GLIAL CELLS

Neuroglia or glial cells, are non-neuronal cells that maintain homeostasis, form myelin, and provide support and protection for neurons in the central nervous system (CNS). Glial cells are distinct according to their size in microglial and in macroglial cells.

Microglial cells are the resident macrophages of the CNS, in fact they can transform into a special type of macrophage that can clear up the neuronal debris monitoring the health of neurons by detecting injuries. These mononuclear cells are small, distributed throughout the brain and function as key immune effector cells (Figure 1) (Hambardzumyan D, 2016).

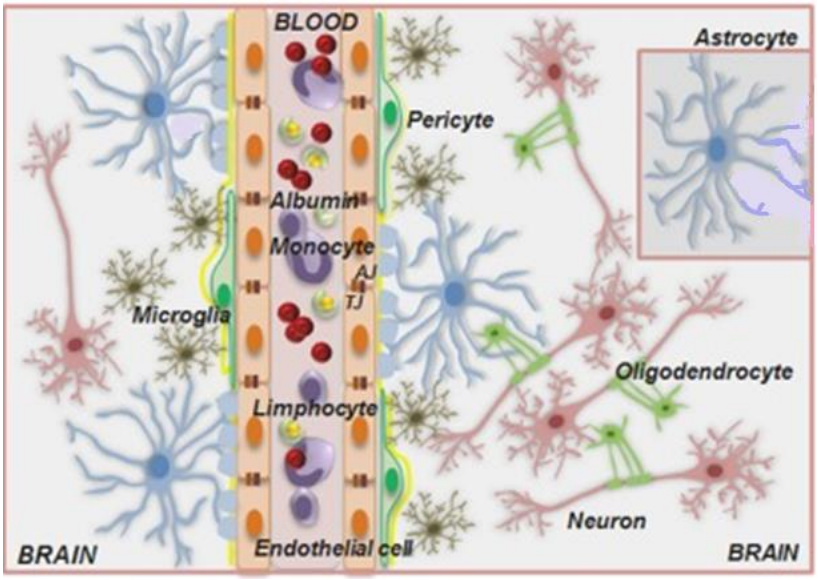

Figure 1. Bood-brain barrier and cells of the SNC. The BBB is composed by endothelial cells (orange) and their tight junctions (TJ), located more apically, and adherens junctions (AJ), located underneath, as well as by astrocyte end-feet (light blue spots), perivascular microglia (greenish-brown), pericytes (light-green), and the basement membrane (yellow). Integrity of all BBB elements is essential for neuron (pink) function and for oligodendrocytes (solid-green) to exert protection of axon function and promotion of neuronal survival. Circulation of UCB in the blood (red) is provided by its binding to human serum albumin (grey); microglial cells (brown) have the main role of immune effectors (Brites D, 2012).

The macroglial cells can be distinguished in astrocytes, oligodendrocytes, and ependymal cells (Figure 2). Astrocytes are the most abundant cell type in the neuroglia, are delicate, star-shaped branching glial cells. Their numerous radiating processes cling to neurons and their synaptic endings. These astrocytes cover nearly all the capillaries in the CNS. They support and brace the neurons and anchor them to their nutrient supply lines. They also play an important role in making exchanges between capillaries and neurons. Astrocytes also regulate the external chemical environment of neurons by removing excess ions and recycling neurotransmitters released during synaptic transmission (Gourine AV, 2010).

Oligodendrocytes, have fewer processes compared to astrocytes; more precisely the interfascicular type, have the function of coating myelin and wrapping tightly the axons of neurons, forming the so-called insulating myelin sheath that allows the isolation of axons with a better propagation of the electrical signals (Baumann N, 2001).

Finally, the ependymal cells delimit the ventricles of the central nervous system forming a permeable barrier between the cerebrospinal fluid (CSF) and underlying cells and by the beat of their eyelashes promote circulation of cerebrospinal fluid (Carlén M, 2009).

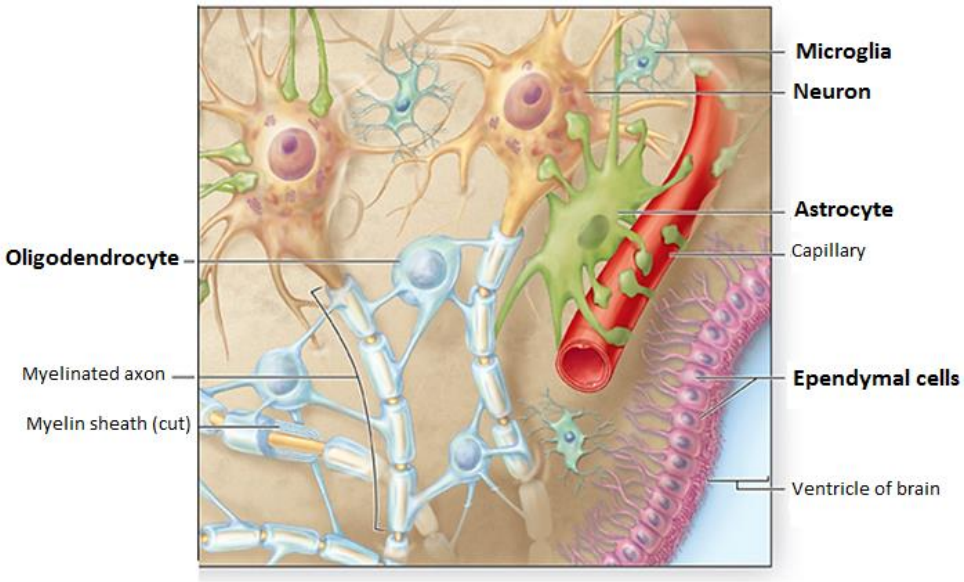

Figure 2. Representation of glial cells in the nervous tissue.

1.2 GLIOMA

Glioma is among the most malignant cancers that occurs in adulthood and that arises from adult neural stem cells or multipotent neural progenitor cells that persist in proliferative niches in the human CNS, may also arise from more differentiated lineages within the brain, including neuron-glial-positive oligodendrocyte precursor cells, astrocytes and even mature neurons.

Despite their genetic differences, and possibly divergent cells of origin, all malignant gliomas share aggressive invasiveness: the cells actively migrate through the tortuous extracellular spaces of the brain, which leads to the formation of distant satellite tumours. Even if gliomas are exceedingly adept at infiltrating organs, only 0.4–2% metastasize outside the brain. This is in stark contrast to small-cell lung carcinoma, mammary ductal carcinoma, prostate cancer and colorectal cancers, which characteristically metastasize beyond the original organ. (Cuddapah VA, 2014).

Based on the kind of glial cells involved, glioma can be distinguished in different types.

- The astrocytoma originates from astrocytes and it is the most common brain tumor that can develop at any age. Astrocytoma may be focal, when the tumor is quite distinct from the surrounding healthy brain tissue, or spread when the cancer cells are scattered in healthy brain tissue. The astrocytoma is usually localized to the cerebellum, to the brain stem and to the telencephalon. From the macroscopic point of view, astrocytoma presents itself as a mass characterized by cells with fibrillar and eosinophilic cytoplasm and with the elongated and roundish nucleus. Glioblastoma (GBM) is the most common astrocytoma malignant tumor (Pedersen CL, 2013).

- Oligodendroglioma originates from oligodendrocytes. The oligodendroglioma is a rare brain tumor that occurs mainly in the cortex and in white matter of the cerebral hemispheres. It develops mainly around 40 years. These tumors appear as masses with not defined limits in the brain parenchyma. The cells are small and show a small nucleus surrounded by a halo of clear cytoplasm (Chowdhary S, 2006).

- Ependymoma affects the ependymal cells. This is a rare cancer that can appear at any age but mainly develops before age 10, or around 45 years old and can affect

both the brain and the spinal cord. It is a tumor that presents a well differentiated and organized cell population that often organize themselves into cords or papillae. The cells are rounded of medium size; they have a rounded nucleus and a normo-chromatin cytoplasm (Asaid M, 2015).

- Mixed glioma affects simultaneously oligodendrocytes and astrocytes and is called also oligo-astrocytoma. It is a very rare tumor that usually affects the brain and develops in men of adulthood. The pathological anatomy of this type of brain tumor is represent by the presence of both astrocytoma cells and oligodendroglioma cells (Olson JD, 2000).

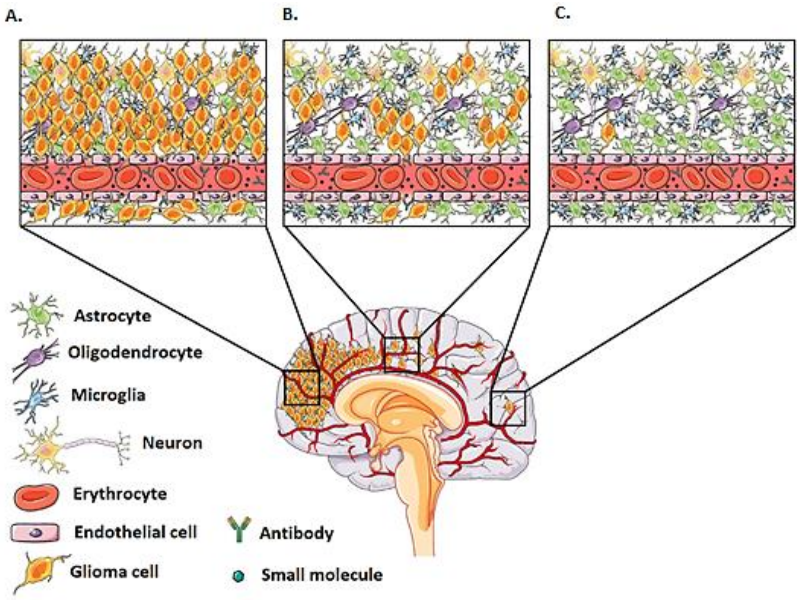

Figure 3. Different stage of glioma development in brain. In glioma the blood-brain barrier (BBB) can be compromised (A) slightly damaged (B) or completely intact (C).

1.2.1 Incidence and epidemiology of glioma

Brain tumors, in Europe, have an incidence of 5 on 100.000 inhabitants/year without significant differences between European nationalities; they represent 2% of all cancer deaths, but the incidence is increasing in these last years (AIOM 2016). Every year, more than 22,000 Americans are diagnosed with a malignant glioma. In children, brain tumors cause one-quarter of all cancer deaths (Ostrom QT, 2015). As shown in Figure 4, pilocytic astrocytoma is the most common glioma in children, while glioblastoma is the most

common in adults and the incidence increases with advancing age; this is probably due to the length of time required for malignant transformation, the necessity of many genetic alterations prior to the onset of clinical disease, and/or diminished immune surveillance (Ostrom QT, 2014).

Current therapy for these primary brain cancers is inadequate, and approximately 95% of patients succumb to the disease within 5 years of diagnosis.

It is not simple to decree the incidence rate of glioma in the world population because its impact varies significantly according to many variables, such as the histological type, the age at diagnosis, the gender, the race and the country of origin. Considering the age at which the diagnosis is confirmed, it was possible to determine that 4.67-5.73 per 100000 people develop glioma, but these data do not allow to obtain a realistic incidence rate because very often the diagnosis is not confirmed in older people, and many of these patients are not integrated and taken into account (Ostrom QT, 2014).

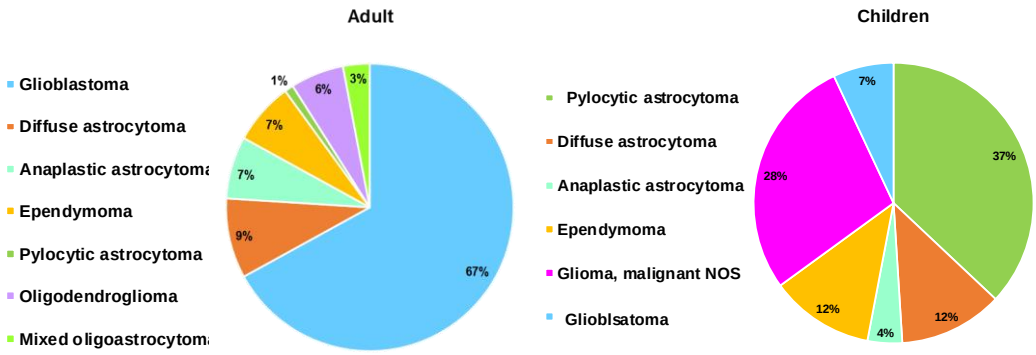

Figure 4. Percentages of glioma by histologic type in adults (left) and children (right)

As regards the spread of glioma in the population, studies show that occurs more frequently in male than in female, except in the case of pilocytic astrocytoma which affects both sexes with an equal incidence. In addition to gender, also the race is a discriminating factor in the development of glioma and this is confirmed by a higher incidence of this type of brain tumor in non-Hispanic white people than other races (Ohgaki H, 2005).

The onset of glioma has been associated with many risk factors, but only a few proved the effective correlation to the tumor development. A risk factor is a condition that can contribute to the pathogenesis and development of a disease and in the case of glioma, only exposure to high doses of ionizing radiations (IRs) is considered a factor that impacts

sharply on the origin of brain tumor. This is considered a potential dose-dependent risk factor, but appears to be accepted only from the epidemiological literature of tumors and not in the scientific literature of radiation. This can derive by the belief that brain cells can established radio resistance due to their high differentiation or from the limited number of studies conducted on the wide range of radiation doses. Despite that, exposure to high doses of ionizing radiation is the main risk factor, most accredited association to the development of glioma (Ostrom QT, 2015).

Another factor that is not yet considered risk but which is the object of many studies is the prolonged use of the mobile phone. A study on the trends of developing glioma in the various risk scenarios in the nordic countries has not revealed any clear change. Despite this, the connection between onset of glioma and prolonged use of the mobile phone remains a particularly interesting topic (Deltour I, 2012).

Other possible associations with glioma were studied in several years, arriving to consider certain professional working risk factors, but with unsatisfactory results from the scientific point of view. Initially it was thought that work situations that precluded the use and the proximity of pesticides or chlorinated solvents could be a risky situation and related to the onset of glioma. These theories, however, are not confirmed yet and no studies show relationship between exposure to chemicals and the development of brain tumor (Ohgaki H, 2005).

Recent epidemiological research has placed attention on factors that have the ability to reduce the risk of developing glioma. For example, a study shows that in the presence of allergies with symptoms like asthma, hay fever, eczema there is a 40% reduction of the progression of glioma (Wigertz A, 2007). Other analysis revealed that the risk of glioma decreases with the increase in the number of allergies types or based on the age of diagnosis of allergy, or according to the type of brain tissue affected by the tumor. They have proved encouraging data regarding the reduction of oligodendroglioma. These data are, however, recruited because there is no evidence that confirm them satisfactorily (Ostrom QT, 2014). In addition, inherited genetic predisposition to glioma has long been suspected because of increased familial risk; Li–Fraumeni syndrome, caused by a constitutive loss of-function mutation in the TP53 gene, is the familial tumor syndrome most frequently associated with glioma. However, numerous other rare Mendelian disorders increase risk of glioma, including neurofibromatosis 1 and neurofibromatosis 2, tuberous sclerosis, Lynch syndrome, and melanoma-neural system tumor syndrome

(Ostrom QT, 2015; Florian IS, 2013). Recently, Bainbridge et al. (2015), demonstrated that rare loss-of-function mutations in the POT1 gene are associated with greatly increased risk of glioma in families. Many gliomas contain acquired TERT promoter mutations which also appear to be driver mutations in a large proportion of gliomas (Killela PJ, 2013). Inherited genetic variants in eight different gene regions have been consistently associated with glioma risk via GWAS, including those in/near TERC, TERT, EGFR, CCDC26, CDKN2B, PHLDB1, TP53, and RTEL1 (Walsh KM, 2016). Therefore, all these findings suggest that glioma is due to the potential interaction between inherited genetic variants, somatic alterations in glial tumors and environmental exposures.

1.2.2 Classification of malignant glioma

The identification of distinct genetic and epigenetic profiles in different types of gliomas has revealed novel diagnostic, prognostic, and predictive molecular biomarkers for refinement of glioma classification and improved prediction of therapy response and outcome. Therefore, the new (2016) World Health Organization (WHO) classification of tumors of the central nervous system breaks with the traditional principle of diagnosis based on histologic criteria only and incorporates molecular markers (Louis DN, 2016). This will involve a multilayered approach combining histologic features and molecular information in an “integrated diagnosis”.

Markers with prognostic and predictive value are MGMT expression, 1p/19q codeletion and IDH1 and IDH2 mutations. Elevated MGMT (O-6-methylguanine-DNA methyltransferase) gene expression confers resistance to alkylating agents like temozolomide and detecting its expression levels is crucial to indicate the option of alkylating agents or to select a second line targeted therapy; the deletion of 1p and 19q strongly characterizes oligodendroglioma, and, not only, confers positive prognosis but it is also predictive and indicative for specific chemotherapy; IDH1/IDH2 were found to be mutated particularly in oligoastrocytoma, oligodendroglioma, diffuse astrocytoma and secondary glioblastoma, thus they represent predictive markers for these kind of gliomas (AIOM, 2016).

The past 2007 World Health Organization (WHO) classification of astrocytoma depended on cellular morphology to determine tumor grade and it was divided into four classes, identified by the first four roman numerals, depending on their severity:

- Grade I: tumor masses are easily distinguishable from the surrounding healthy tissues and total removal leads to recovery;
- Grade II: tumors are low-grade malignancies that may follow long clinical courses due to tumor infiltration in healthy brain mass. This type is more difficult to cure and often cannot be completely resected; usually patients with this degree of tumor survive more than 5 years (Louis DN, 2007).
- Grade III: tumors exhibit aggressive behavior characterized by increased anaplasia, the cells assume the form of cellular undifferentiated elements. Due to this biological pattern, these tumors have often a quick progression which causes a reduction of about 3 years of survival (Louis DN, 2007).
- Grade IV: tumors also known as glioblastoma multiforme (GBM), exhibit more advanced features of malignancy, including vascular proliferation and necrosis, often refractory to radiotherapy or chemotherapy (Altieri R, 2014).

Benign tumors are considered of Grade I and II and are characterized by slow growth and localized in circumscribed areas of the brain; while all malignant tumors are reputed of Grade III and IV and expand rapidly to invade surrounding tissue regions.

It is not to exclude the possibility that a tumor of Grade I or II can be transformed into a tumor of higher grade of severity, such as the glioblastoma multiforme, characterized by a high mortality and difficult to be treated.

A recent chapter in the book of Masui K. provided an overview of the molecular diagnostics of diffusely infiltrating gliomas according to the revised WHO classification of 2016 with the major glioma entities defined by histologic features and molecular marker profiles (Masui K, 2016). With the new classification, raises the need to standardize the terminology in as practical a manner as possible; CNS tumor diagnoses should consist of a histopathological name followed by the genetic features, with the genetic features following a comma and as adjectives (Louis DN, 2016). The major glioma entities are now defined by histologic features and molecular marker profiles. If molecular testing cannot be performed, i.e., due to limited tissue availability, low tumor cell content, inconclusive testing results, or other circumstances impeding molecular testing, classification based on histology alone followed by the term “not otherwise specified (NOS)” is recommended. On the light of this, the new 2106 WHO classification, contains a number of newly recognized entities, variants and patterns such as IDH-wildtype and IDH-mutant glioblastoma (entities), epithelioid glioblastoma (variant), glioblastoma with primitive

neuronal component (pattern). Moreover, it has added newly recognized neoplasms, and has deleted some entities, variants and patterns that no longer have diagnostic and/or biological relevance. The most notable changes involve diffuse gliomas (Figure 5), which now include the WHO grade II and grade III astrocytic tumors, the grade II and III oligodendrogliomas, the grade IV glioblastomas, as well as the related diffuse gliomas of childhood, basing not only on their growth pattern and behaviors, but also on the shared genetic driver mutations in the IDH1 and IDH2 genes.

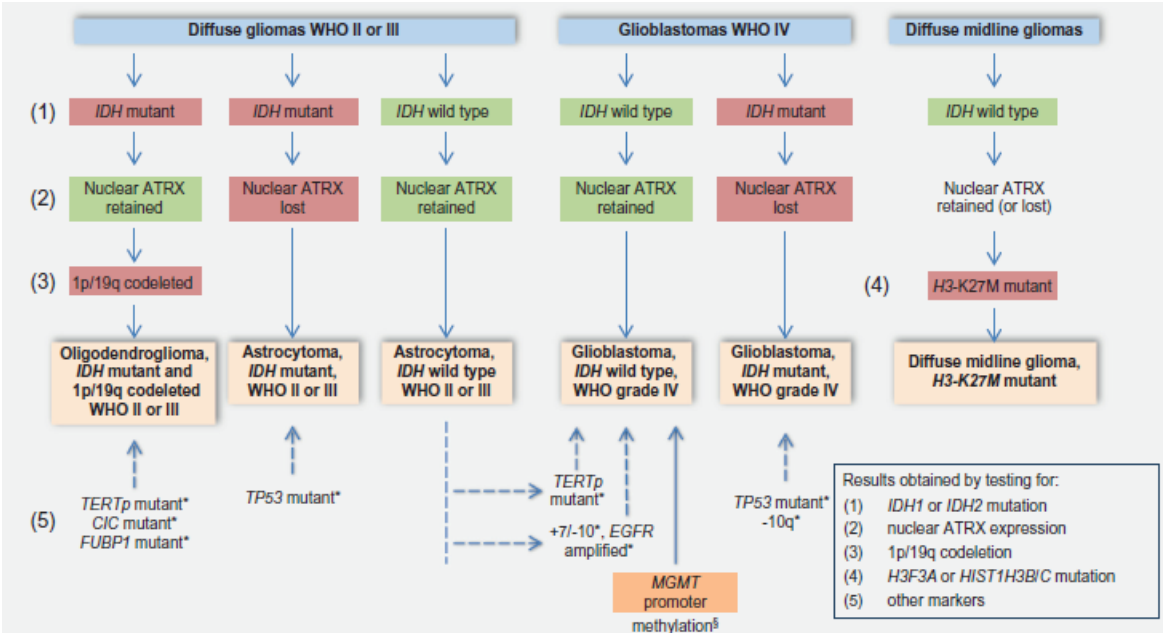

Figure 5. Molecular diagnostics of diffuse gliomas based on genetic aberrations considered as diagnostic biomarkers, namely IDH1 or IDH2 mutation, loss of nuclear ATRX expression, 1p/19q co-deletion, and H3-K27M mutation. The biomarker patterns that are typical for the most common diffuse glioma entities are shown, and are represented also the selected chromosomal and genetic alterations that may serve as additional diagnostic markers. O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation does not represent a diagnostic marker but it is important as predictive marker for response to alkylating agent chemotherapy (Masui K, 2016).

The WHO grade II diffuse astrocytomas and WHO grade III anaplastic astrocytomas are now each divided into IDH-mutant, IDH-wildtype and NOS categories. Also the classification of glioblastomas received some modifications; they are divided into (1) glioblastoma, IDH-wildtype (about 90 % of cases), which generally corresponds with the clinically defined primary or *de novo* glioblastoma and mainly affects patients over 55 years of age (Ohgaki H, 2013); (2) glioblastoma, IDH-mutant (about 10 % of cases), which corresponds to secondary glioblastoma with a history of prior lower grade diffuse glioma

and preferentially arises in younger patients (Ohgaki H, 2013); and (3) glioblastoma, NOS, that comprehends those tumors for which full IDH evaluation cannot be performed (Louis DN, 2016). This new approach allows for a clearer definition and more precise classification of biologically and clinically distinct glioma entities. In addition, it is possible to develop a personalized treatment of glioma patients according to predictive molecular biomarkers and tumor-specific genetic or epigenetic alterations.

1.3. GLIOBLASTOMA MULTIFORME

Glioblastoma multiforme (GBM) is a rare cancer defined by the International Classification of Oncological (ICD-O) with the code 9440/3. It is the most common and malignant tumor of the glial neoplasms, representing 60-70% of all malignant gliomas, and it belongs to the class of astrocytomas (Fritz A, 2014). GBM is more frequently localized in the subcortical white matter of the cerebral hemisphere, affecting front-lateral and temporal-lateral areas. The most typical presentation, however, is the bilateral that involves both hemispheres creating a butterfly appearance in the *corpus callosum* (Preusser M, 2006). They are distinguished in primary glioblastoma, which is manifesting *de novo*, without the presence of any previous pathology, and in secondary glioblastoma, which instead develop from an aggravation of astrocytoma already present in the brain.

Primary and secondary GBM evolve from different genetic precursor and distinct genetic alterations (Figure 6). Common in primary glioblastoma are the overexpression of epidermal growth factor receptor (EGFR), phosphatase and tensin homolog (PTEN) and mutations and loss of chromosome 10, whereas IDH1 mutations, TP53 mutations and 19p loss are usually found in secondary GBM (Wilson TA, 2014). Since IDH1 mutations occur most of all in young patients and in secondary GBM (only 5% in primary GBM), it is currently considered a molecular marker for secondary GBM, although indistinguishable from primary GBM from a pathological perspective (Nobusawa S, 2009).

Considering the vast differences in gene expression in glioblastoma, it was possible to identify 4 glioblastoma subtypes: classical, neural, proneural, and mesenchymal (Verhaak RG, 2010). Having an expression pattern between the mesenchymal and proneural subtypes, the most controversial subtype is the neural. Aberrations and gene expression of EGFR, NF1, and PDGFRA/IDH1 characterize the classical, mesenchymal, and proneural subtypes, respectively (Weathers SS, 2016).

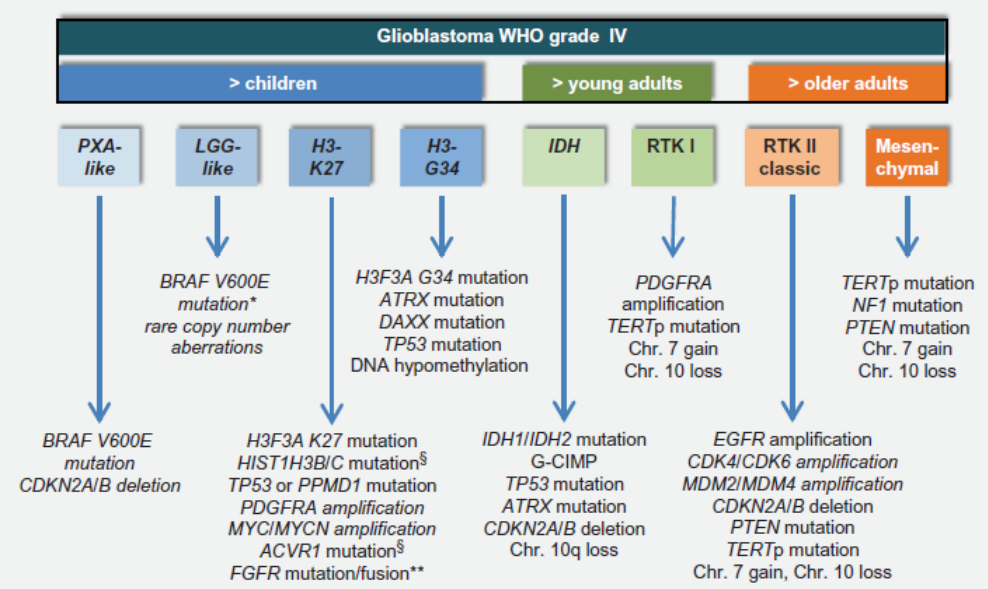

Figure 6. Glioblastoma subgroups in children and adults as defined by distinct DNA methylation profiles and most commonly associated genetic alterations (Sturm D, 2012; Korshunov A, 2015). The four subgroups on the left side are predominant in children, among which "PXA-like" and "LGG-like" glioblastoma subgroups are associated with more favorable outcome, while the "H3-K27" subgroup associates with poor outcome. The "IDH" and "RTK I" subgroups typically manifest in young adults, while the "RTK II (classic)" and "mesenchymal subgroups" mostly develop in elderly patients (>50 years of age). The "H3-K27" subgroup includes diffuse midline gliomas in the thalamus, pons, and spinal cord. LGG-like, low-grade glioma-like molecular profile; PXA-like, pleomorphic xanthoastrocytoma-like molecular profile; RTK, receptor tyrosine kinase; TERTp, TERT promoter (Masui K, 2016).

From macroscopic point of view, GBM is presented as a large mass with nuanced borderlines due to the extreme ability of tumors to invade neighboring areas.The mass appears of greyish color with reddish or black patches respectively due to bleeding or necrosis of the tumor parenchyma (Lemée JM, 2015). Glioblastoma multiforme is characterized by poorly differentiated cells with an intense microvascular activity due to an accentuated and focalized proliferation of endothelial cells which form numerous rolled up vases, some of which are thrombosed (Dimberg A, 2014).

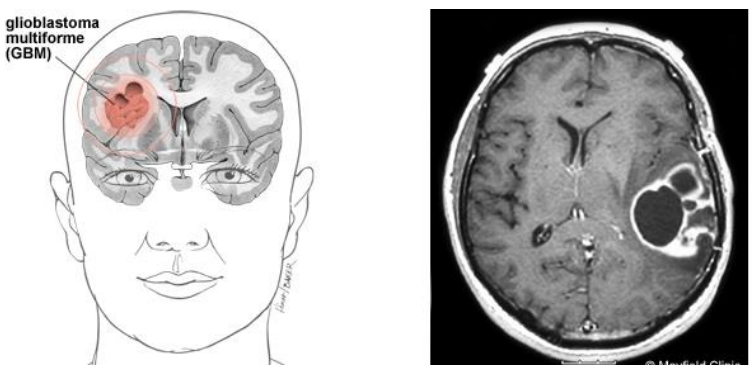

Figure 7. Illustration and MRI of a glioblastoma tumor in the parietal lobe (from Mayfield Brain & Spine)

GBM tumor tissue is characterized by cellular heterogeneity and many hypotheses were proposed to explain how heterogeneous tumor cell subpopulations are generated in glioblastoma (Figure 8).

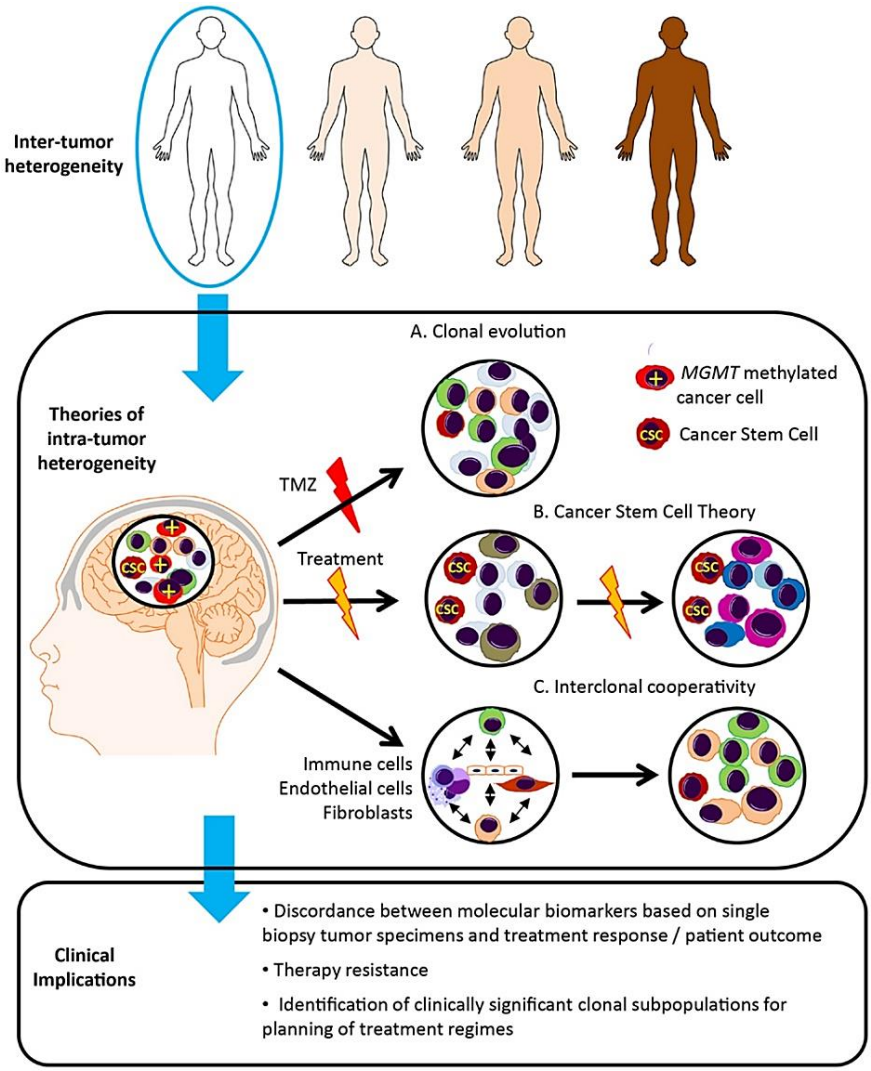

Figure 8. Representation of the evolution of cellular heterogeneity in glioblastoma.

According to Darwin's theory (Figure 8A), the cells acquire genetic changes due to clonal evolution, pharmacological and natural selection and will be transmitted to other cells with the proliferation. The selection and proliferation of cells, which carry "driver mutation", allow the progression of tumor and the development of resistance to therapy (Parker NR, 2014).

An alternative supposition is the cancer stem cell theory (Figure 8B), which admits the

presence of tumor cells that possess the ability to self-renew. The continuous proliferation of these cells and the generation of clones with genetic variables profiles, are the cause of cell heterogeneity (Sampetrean O, 2013).

Another assumption is recently developed, called interclonal cooperativity (Figure 8C): different factors, as immune infiltrate, aberrant microvascular proliferation, hypoxia or stromal factors (like microglial cells and reactive astrocytes) co-operate with genetic subpopulations of tumor cells to generate a favorable microenvironment. The microenvironment created is responsible for disease progression and malignant phenotype (Lyons JG, 2008).

Currently there are not definitive data to support any of these theories, however it is believed that brain tumors, such as GBM, initially are monoclonal and, due to increased degree of proliferation, acquire genotypic and phenotypic heterogeneity (Parker NR, 2014).

1.3.1 Epidemiology and etiology

Glioblastoma is the most common and aggressive cancer of the Central Nervous System and represents approximately 70% of all glial tumors. The average annual incidence rate of GBM is about 3.19 persons per 100000 population (Gallego O, 2015).

Regarding distribution between genders, it is 40 % more common in males than in females (Wen PY, 2008), but also differences of race are constant, with higher rates of incidence in individuals of white race and non-Hispanic ethnicity. The GBM can occur at any age but usually affects adults who are between 45 and 70 years old, in fact the average age at which it is most often diagnosed is 65 years old, with patients being 10–15 years younger in secondary compared to primary GBM. The advanced age and the poor available treatments negatively affect the prognosis. In fact, it is shown that the same treatment applied in younger patients, not only is more efficient, but also presents lower toxicity (Kumthekar PU, 2014).

Due to the high aggressiveness of the tumor, the median survival is very low, from 1 year (17.7%) to 15 months, with a 42.4% at 6 months (Thakkar JP, 2014; Ohgaki H, 2005).

The risk factors associated with the onset of GBM are unknown to date. The only one that has a causal association, except for hereditary syndromes such as neurofibromatosis, tuberous sclerosis, Li Fraumeni syndrome, Turcot syndrome and Cowden syndrome, is exposure to ionizing radiations (IRs). Recent studies showed that the differentiated cells isolated from a tumor resection of GMB after exposure to IRs acquire a stem-like

phenotype with a consequent increased tumorigenicity (Dahan P, 2014).

Other factors, such as exposure to electromagnetic fields or to N-nitrosamines and polyvinyl chloride, are alleged to be risk factor, and need experimental studies.

1.3.2 Clinical diagnosis

The symptoms of glioblastoma depend on the size and location of the cancer but generally are nonspecific and, in most cases, they appear when the disease is at an advanced stage. The most common symptoms are sub-acute headache, nausea or vomiting, seizures, focal neurological deficit, confusion, personality changes or combinations of these symptoms; GBM patients present also papilledema caused by excessive dilation of cerebral vessels. They are caused by increased pressure in the brain that are given by the tumor mass accretion, which prevents the normal flow of cerebrospinal fluid, or are given by the formation of an edema in the areas surrounding the tumors. In the advanced stages of cancer more severe symptoms, such as aphasia, convulsive seizures and in some cases epileptic fits, are present (Louis DN, 2007).

In front of a suspected case of glioblastoma, after the reconstruction of the patient's medical history, the diagnostic neurological examinations include tests to assess the patient’s mental state and cognitive abilities and tests for vision, hearing, balance, coordination, strength and reflexes to evaluate neuromuscular disorders. The presence of a glioblastoma is confirmed by specific analysis such as brain magnetic resonance imaging scan (MRI) with or without dye, TAC and biopsy of the tumor like stereotactic needle biopsy or part of operation of removed brain cancer (Shiroishi MS, 2016).

1.3.3 Therapy of GBM

The GBM is the most common malignant primary brain tumor in adults and invariably carries a very poor prognosis. In recent years, the knowledge about glioma tumor biology has grown significantly and led to the identification of molecular prognostic factors and molecular vulnerabilities that could be potentially targeted in the development of novel treatments in gliomas.

Over the years, new solutions were proposed as potential therapies for brain cancer, but until now none of these represent a definitive treatment.

The identification of the 4 subtypes of glioblastoma (classical, neural, proneural, and mesenchymal) has highlighted the challenge that different therapeutic approaches may be

needed depending on the subtype; nevertheless, and considering intratumoral cellular heterogeneity, the therapeutic solution is not easy to find (Figure 9) (Weathers SS, 2016). Moreover, the presence of blood-brain barrier represents another obstacle for drug delivery and efficiency (Stavrovskaya AA, 2015).

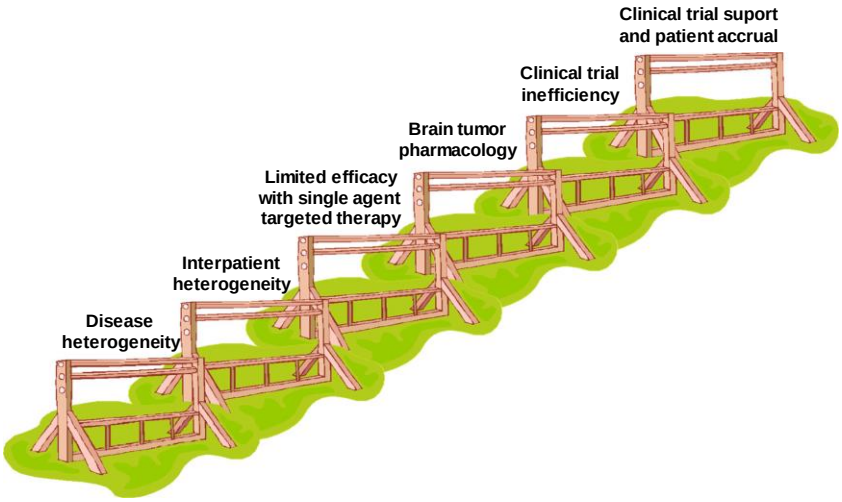

Figure 9. Challenges in the development of personalized, targeted therapeutics in glioblastoma (Weathers SS, 2016)

The first approach is surgery, to totally remove, in the best of cases, the tumor mass. Although it does not represent a definitive cure, surgery is indeed still considered the preferred treatment as it provides the opportunity to greatly lengthen the life expectancy after diagnosis. With the increasing development of pharmaceutical chemistry, the researchers attempted to associate with surgical resection the chemotherapy, slowing down tumor progression and prolonging survival. It was thought to approach GBM also by bombarding the tumor with ionizing radiation in order to upset the balance instituted within the tumor cell. Radiotherapy was associated also to chemotherapy to try to obtain a synergistic effect. These three clinical approaches represent cardinal therapies as specific anti-cancer treatment for glioblastoma (Alifieris C, 2015); in fact, upon initial diagnosis of glioblastoma multiforme (GBM), standard treatment consists of maximal surgical resection, radiotherapy, and concomitant and adjuvant chemotherapy with temozolomide (Sathornsumetee S, 2008). For patients older than 70 years, less aggressive therapy is sometimes employed, using radiation or temozolomide alone (Zarnett OJ, 2015).

At the same time, many studies are conducted to overcome tumor heterogeneity which may require the development of novel polytherapeutic strategies. Combination therapy may be

superior to monotherapy, and it has to be rationally considered combinatorial strategies utilizing immunotherapy, radiotherapy, targeted therapy, antiangiogenic therapy, and/or chemotherapy (Weathers SS, 2016).

In addition to specific anticancer treatments, symptomatic therapies are usually combined with the aim to reduce symptoms such as epilepsy (anticonvulsant) and headache (corticosteroids) given by the increased intracranial pressure caused by edema (Kerrigan S, 2011; Le Rhun É, 2015).

Surgery

Surgical removal of tumor mass, where possible, is the best approach for eliminating the tumor. The operation itself is very complex and dangerous, because glioblastoma is characterized by a high number of cells and particularly by a high infiltrating power, which leads to a dispersion of cancer cells into healthy tissue. This makes impossible to remove completely the tumor, because of the risk to damage a brain healthy area and loose some fundamental cognitive functions (Talibi SS, 2014). Surgical resection is also not always possible, because the tumor may be in brain areas not easily accessible by the neurosurgeon. In this regard, over the last decade, innovative surgical techniques have been proposed: fluorescence-guided surgery, intra-operative Magnetic Resonance Imaging, neuronavigation with/without diffusion tensor imaging and intra-operative ultrasound, which allow a more defined vision of the tumor and therefore the possibility to remove in a more specific way greater tumor area (Talibi SS, 2014).

Radiation therapy

The radiation therapy is a medical discipline mainly used in the treatment of tumor alone or in combination with chemotherapy. It is used after surgical treatment to eliminate the cancer cells that may have escaped or simply for killing neoplastic cells that are in areas rather difficult to reach, and the radiation becomes the first and most important therapeutic treatment (Gallego O, 2015). This technique uses ionizing radiations that through a beam of photons excites electrons of tissues, and ionizes the atoms of the DNA molecule. The ionization of DNA molecules causes the malfunction of genes that induces cell death. The efficacy of radiotherapy is largely demonstrated; important is the study conducted by Stupp R. (2005), in which it was confirmed that radiotherapy addicted to surgical resection increased survival in a range from 3 to 12 months depending on the case, and that the two-

year survival rate increases to 26.5 % with radiotherapy plus temozolomide instead of 10.4 % with radiotherapy alone (Stupp R, 2005; Delgado-López PD, 2016)

Chemotherapy

Chemotherapy is a therapeutic treatment based on the use of specific drugs that have the ability to kill selectively neoplastic cells and to preserve the health of the normal ones. This requirement, however, is difficult to realize, because there are no still anticancer drugs able to have an exclusive effect on the tumor mass without presenting side effects on the organism.

In glioblastoma, temozolomide is the first-line anticancer drug; current standard therapy consists of external beam, involved-field cranial radiation therapy plus concomitant daily temozolomide, followed by six cycles of adjuvant temozolomide (Stupp R, 2009).

Treatment options at recurrence include surgical resection with or without the placement of carmustine wafers, re-irradiation and chemotherapeutics such as nitrosoureas, lomustine and carmustine (Venur VA, 2015); the antiangiogenic bevacizumab (Gilbert MR, 2014) and cilegitide (Stupp R, 2014) were enrolled in phase III studies, but the results did not permit their enrolment in therapy.

1.3.3.1 Temozolomide and TMZ-resistance

In clinical studies, temozolomide has demonstrated reproducible linear pharmacokinetics with approximately 100% bioavailability, noncumulative minimal myelosuppression that is rapidly reversible, and antitumor activity against a variety of solid tumors in both children and adults, including gliomas in which it is the leading chemotherapeutic agent. TMZ has some characteristics like small size and lipophilic proprieties that allow it to cross the blood-brain barrier (BBB) (Agarwala SS, 2000) and represents a new class of second-generation imidazotetrazine prodrugs.

As demonstrated by the reaction shown in Figure 10, temozolomide does not require hepatic metabolism for activation, due to the spontaneous conversion under physiological conditions, to the active alkylating agent imidazole-4-carboxamide component (MTIC). The spontaneous conversion, pH-dependent, to the reactive methylating agent MTIC is initiated by the effect of water at the highly electropositive C^4 position of temozolomide. This activity opens the ring, releases CO_2, and generates MTIC. MTIC degrades to the methyldiazonium cation, which transfers the methyl group to DNA and to the final product

4-amino-imidazole-5-carboxamide (AIC), which is excreted by the kidneys (Friedman HS, 2000).

The methylation of DNA and subsequent formation of O6-MeG (O6-methylguanine) followed by arrest of the cell cycle at G2/M phase seem to be the principal mechanisms responsible for the cytotoxicity of temozolomide to malignant cells (Zhang J, 2012).

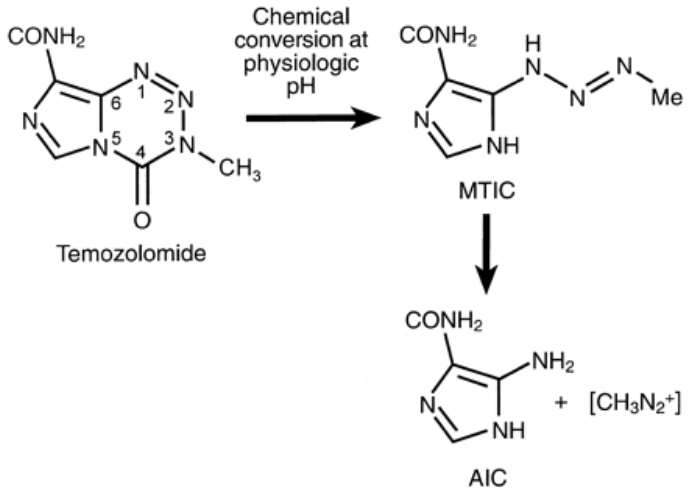

Figure 10. Mechanism of activation of TMZ to the imidazole-4-carboxamide component (MTIC) and subsequent inactivation to 4-amino-imidazole-5-carboxamide (AIC) under physiological conditions.

Temozolomide is usually used at the beginning of the glioblastoma treatment at a daily dose of 75 mg/m^2 body surface area. TMZ may be associated to radiotherapy, in fact, normally the first approach is a treatment that involves chemotherapy with radiation therapy: a daily dose of 75 mg/m^2 given 7 days per week from the first to the last day of radiotherapy, for at most 49 days. After a 4-week break administering six cycles of adjuvant oral temozolomide (150-200 mg/m^2) for 5 days every 28 days (Stupp R, 2009). This well-known protocol provides the best outcome in terms of overall survival, in fact, combining TMZ with radiotherapy improved therapeutic results in patients with GBM. In particular, two-year survival time of patients was increased from 10.4% (radiotherapy alone) to 26.5% (radiotherapy + TMZ) (Minniti G, 2009).

Nevertheless, the therapeutic efficacy is often compromised by insurgence of TMZ-resistance. Various studies show in fact that the limited efficacy of TMZ is correlated to high levels of O^6-methylguanine DNA methyltransferase (MGMT), an enzyme that has the ability to repair damaged DNA in cancer cells. MGMT is a protein of 22 kDa which

transfers an alkyl group from DNA onto a cysteine residue of the enzyme, reducing unmethylated guanine, and preventing formation of interstrand DNA crosslinks; it is able to remove not only the methyl, but also ethyl, isopropyl and butyl groups in position O^6 of guanine, promoting DNA repairing (Park CK, 2012). The high activity of MGMT thus leads to a lower effect of TMZ on the tumor cells with the possible emergence of a resistant phenotype; or probably, given the GBM cellular heterogeneity, is more correct to talk about different degree of cell sensitivity to the drug. Several genes and proteins can affect sensitivity of GBM cells to TMZ. However, methylation of the MGMT gene is currently considered as one of the most important factors for predicting sensitivity of GBM cells to treatment with alkylating agents. Since methylation "switches off" the promoter in the MGMT gene, many studies demonstrated that efficacy of TMZ was higher in patients containing methylated respect to unmethylated promoter in the MGMT gene; moreover, also the upregulation of MGMT is associated to decreased efficacy of TMZ (Stavrovskaya AA, 2016). Therefore, the research is presently active in identifying alkylating drugs alternative to temozolomide or MGMT pseudosubstrates to bypass or potentiate TMZ activity, particularly on patients with unmethylated MGMT gene.

In addition to MGMT, other mechanisms of DNA repair, such as MMR and BER, contributes to reduced efficacy of temozolomide on cancer cells. DNA mismatch repair (MMR) is a system that corrects the insertion of wrong nucleotide bases in the process of DNA synthesis. This system intervenes in the absence of MGMT and remove new DNA strands containing pairing O^6-MG/T causing cell cycle arrest and apoptosis of the cells. Mutations in the protein complex MMR can cause the failure to recognize and correct O^6-MG, allowing DNA replication and the completion of cell cycle (Zhang J, 2012). The alteration of the MMR system could therefore also affect the efficacy of TMZ treatment.

Temozolomide also showed a cytotoxic effect on glioblastoma cancer cells when the base excision repair (BER) system results mutated. In fact, the BER pathway has the function of removing the methylation to N^3 or N^7 of guanine caused by TMZ, inactivating TMZ effect on cancer cells (Zhang J, 2012).

The resistance of GBM cells to treatment with TMZ has proved to be also correlated to overexpression of EGFR. The epidermal growth factor receptor (EGFR) plays a crucial role in the development of cancer because it influences cell proliferation, angiogenesis, migration and resistance to chemotherapy. The pathway mediated by EGFR is highly activated in glioblastoma cancer causing inhibition of the apoptotic process (Ekstrand AJ,

1991). The alteration of the EGFR gene, that causes the amplification of the signal mediated by the respective receptor, is responsible therefore also of the decreased efficiency of TMZ on cancer cells.

Moreover, a connection between the altered expression of the p53 tumor suppressor protein and the development of resistance to TMZ treatment exists in glioblastoma cells. P53 is a protein encoded by the TP53 gene with the function of blocking permanently or temporary cell growth in stress situations by inhibiting the activity of Mdm2 oncoprotein (Burton EC, 2002). Mdm2 is an oncogene that is often amplified in glioblastoma. Its overexpression may be caused by a loss of function of the p53 protein due to a mutation of the TP53 gene, or by a mutation of the PTEN gene whose normal function would be to inhibit PI3K and the transcription of Mdm2, leading to the promotion of tumor cell growth and a resistance to chemo and radiotherapy (Mayo LD, 2002).

In a recent review (2016), Stavrovskaya AA., describes other genes and proteins found to be involved in developing resistance of GBM to TMZ, including IDH1/2 (isocitrate dehydrogenase 1 and 2), YB-1 (Y-box Binding Protein-1), MELK (Maternal Embryonic Leucine zipper Kinase), MVP/LRP (major vault protein/lung resistance protein), MDR1 (multidrug resistance 1), and genes encoding other ABC transporters as well as Akt3 kinase.

Important in the TMZ-resistance is also the activity of some epigenetic regulators such as microRNAs. It was found that expression of these small non-coding RNAs in tumors substantially changed compared to normal body tissues. Some miRNAs are involved in controlling sensitivity of GBM cells to chemotherapy, particularly to TMZ. For instance, miR-21, which is overexpressed in GBM cells, was shown to inhibit TMZ-induced apoptosis, while its downregulation was associated to the sensitization of glioma cells to temozolomide treatment (Wong ST, 2012). Conversely, miR-200a is highly expressed in glioma cell cultures that are more sensitive to TMZ and its upregulation is correlated to inhibition of MGMT activity (Wang Q, 2012).

Also miR-181d overexpression was associated to reduction of MGMT mRNA levels and conferred pro-apoptotic sensitivity to temozolomide in different GBM cell lines including T98G that are resistant to TMZ (Cabrini G, 2015). On the contrary, miR-221/222 are usually overexpressed in glioblastoma and their downregulation through antimiR PNAs treatments, in combination with TMZ, showed to induce high level of apoptosis in TMZ-resistant T98G glioma cells (Brognara E, 2016).

Concluding, there are multiple and various molecular mechanisms that could determine resistance of GBM cells to TMZ, and successes in molecular and genetic studies on this rare and aggressive disease opens a path towards the develop of individualized treatments for patients with glioblastoma.

1.3.3.2 Emerging therapies

Gliomas notoriously develop drug resistance and/or upregulate compensatory pathways in response to monotherapies; hence, use of these agents as adjuvants or as a part of a drug cocktail may meet greater clinical success.

Moreover, due to the molecular complexity and pathological abnormal heterogeneity that characterizes the GBM, very often the classical therapeutic approaches fails. Therefore, there is a need to discover new possible therapeutic advances that allow a satisfactory molecular targeted therapy.

Unlike the classical cure, targeted therapy does not have an effect on nonspecific mechanisms, but acts on the mechanisms that cause transformation of cells from normal to cancerous. More precisely, such therapy modulates the expression of particular types of genes, called oncogenes or tumor suppressor genes. This is made possible by significant advances in the understanding of genesis of gliomas at the molecular level, identifying an increasing number of gene mutations responsible for the transformation of the cell in the tumor.

The molecular targets implicated in the pathogenesis of GBM are involved in different signaling pathways. The tumor protein p53, encoded by *TP53*, a tumor suppressor gene, is a transcription factor that has many functions, such as, repairing damaged DNA or starting apoptotic process if the damage is extensive. Mutations in the *TP53* gene make the protein unable to bind to complementary sequences of DNA and therefore unable to regulate cell activity (Appin CL, 2015).

Another interesting molecular target is the epidermal growth factor receptor, EGFR, considered a proto-oncogene. When activated by its ligand, the receptor with its tyrosine kinase activity initiates a cascade of phosphorylation through which allows controlling growth and proliferation of cell. Mutations of the EGFR cause a constant activation of the receptor that leads to an uncontrolled cell division (Appin CL, 2015).

Another signaling pathway affects the mechanisms of angiogenesis that allow the vascularization of the growing tumor. Vascular endothelial growth factor, VEGF, is a

protein that by binding to the respective receptor increases vascular permeability and causes vasodilation. The overproduction of VEGF leads to increased vascularization of tumor cells. Anti-VEGF therapies may use monoclonal antibodies with the task of inhibiting the tyrosine kinase involved in his specific receptors; Aflibercept is a new agent that binds to VEGF and inhibits angiogenesis factors (Macarulla T, 2014; Wang H, 2015). Also bevacizumab is a monoclonal antibody that binds and neutralizes the VEGF ligand; it has been clinically tested for recurrent GBM and proved to be beneficial in terms of progression-free survival as an adjuvant to radiotherapy and temozolomide (Stupp R, 2009).

An interesting turn in the treatment of glioblastoma occurred with the proposal to use a new class of small non-coding RNA molecules, called microRNAs. These small molecules have demonstrated the ability to regulate the expression of specific molecular targets involved in processes crucial for the development, differentiation, and cell division. Thus, through rather recent studies and researches, are synthesized molecules, called PNAs, with the ability to bind the target nucleic acid to modulate the expression of specific molecules and finally obtaining a positive action for the cell (Malcher J, 2014). Nowadays microRNAs and PNAs represent potential effective therapies that could be applied to the treatment of many cancers, including the glioblastoma.

Finally, as already mentioned, many evidences suggest that combined treatments lead to better therapeutic results than single treatments. Therefore, in the recent scientific literature it is possible to find studies on therapeutic approaches that combine drugs already designated for GBM (i.e. TMZ and cilegitide in Verschaeve V, 2011) or new therapeutic approaches aim at, for example, sensitizing GMB cells to TMZ such as quercetin treatments; Hu J et al. (2016) showed that nanoliposomes containing quercetin and TMZ were rapidly taken up by the U87 glioma cell and enhanced TMZ effects, suggesting also a novel and effective nanocarrier for enhancing drug delivery to brain tumors.

Interesting is also the study on RIST, characterized from the combination of rapamycin, irinotecan, sunitinib and temozolomide; cell co-treatment with these compounds achieved significant anti-tumorigenic activity (reduced cell growth and induction of apoptosis) avoiding high-dose chemotherapy (Nonnenmacher L, 2015).

2. MicroRNA

MicroRNAs (miRNAs, miRs) are endogenous small non-coding single-stranded RNAs; they are about 21 - 25 nucleotides long and highly conserved across species. They work by repressing translation or causing degradation of the mRNAs target; thus, they repress gene expression post-transcriptionally. MiRNAs are involved in complex regulatory networks and each miRNA can regulate different genes; in eukaryotes they are fundamental to normal cellular function and their altered expression and activity is implicated in a variety of pathological processes.

2.1 History of MicroRNAs

In 1993, Ambros and colleagues, presented the first report on microRNAs. At that time, he was conducting a study on lin-4, a gene acting early in *Chaenorabditis elegans* larval development to affect the timing of various developmental events. Animals with lin-4 loss-of-function mutations are missing some adult structures, but a suppressor mutation in the gene lin-14 was able to revert the null-lin-4 mutation phenotype. Then, they discovered that lin-4 could negatively regulates lin-14 and that it did not encode a protein but it generated many RNA fragments including two very small lin-4 transcripts of only 61 nt and 22 nt in length. In 1992 it was reached the conclusion that lin-4 transcripts were complementary to a repeated sequence in the 3'UTR of the lin-14 gene (Lee R, 1993).

The second miRNA to be discovered, in 2000, was let-7. Likewise lin-4, let-7 is a heterochronic gene of *C. elegans*; Reinhart and Ruvkun et al. (2000) reported that it was a 21-nt RNA controlling the L4-to-adult transition of larval development. Let-7 is complementary to the untranslated region 3'UTR of genes lin-14, lin-28, lin-41, lin-42 and therefore is responsible for regulating the expression of these genes. Unlike lin-4, the let-7 sequence was found to be conserved across species from flies to humans, suggesting ubiquitous distribution of miRNAs and this fact triggered a revolution in the research of a new class of small ncRNAs, called miRNAs.

The term miRNA was formally introduced in 2001, when it was found that microRNAs probably could act on more than one target sequence and that they are excised from longer transcripts that contain stem-loop structures about 30 base pairs in length. Moreover, it was discovered that interaction domains are only 6 to 10 base pairs long and the base-paired structures are frequently imperfect and interspersed by G/U wobbles and less stable, non-

Watson-Crick base pairs such as G/A, C/U, A/A and U/U (Lagos-Quintana M, 2001). Since then, microRNAs have been largely studied and currently, thousands of miRNAs have been identified in humans and other species. A platform was necessary to organize this large number of miRNAs discovered and that would be discovered in the future with a universally accepted nomenclature, and also for getting information regarding their position on the sequence of complementary mRNA. The “miRBASE” database is the central miRNA sequence repository that continues to expand (Cammaerts S, 2015). To date, it contains 28645 entries (www.mirbase.org) and, although the functions of most of these miRNAs remain to be discovered, research on this area continues to expand and grow rapidly.

2.2 Biogenesis

The gene sequences that code for microRNAs may be localized in single genes, in intergenic regions, or in intronic sequences of host genes. Recent studies have confirmed that not only intergenic microRNAs are transcribed as independent units, but also the intronic gene sequences can be transcribed independently from the host gene transcription (Ramalingam P, 2014). MiRNAs biogenesis (Figure 11) starts in the nucleus with the transcription by RNA polymerase II to long primary transcripts, called pri-miRNAs; they are capped (5’end) and polyadenylated (3’end), approximately of 70 nucleotides with the typical hairpin structure (via intramolecular base pairing). Pri-miRNAs are rapidly processed by the microprocessor complex: RNAse endonuclease III Drosha and DGCR8 (DiGeorge syndrome critical region gene 8). DGCR8 contains a binding domain for RNA that is used to bind pri-miRNA allowing the catalytic domain of Drosha to cut the RNA at about eleven nucleotides from the hairpin base. The resulting product is the pre-miRNA, a 60-120 nucleotide long miRNA precursor that carries a hydroxyl to the 3'end and a phosphate group at the 5'end. In both cases of intergenic miRNAs and intragenic miRNAs, the pre-miRNAs are transported from the nucleus to the cytoplasm by Exportin-5 in presence of RanGTP (GTP-binding nuclear protein Ran), which provides the necessary energy for the transport. In the cytoplasm happens the final cleavage: the RNAse III enzyme Dicer interacts with the 5' and 3'ends of the hairpin and cuts the extremities in staggered manner, eliminating the stem-loop and producing a mature double stranded miRNA around 22 nucleotide long.

Thereafter, one strand is normally degraded and the other is incorporated into the RNA-

induced silencing complex (RISC). Here inside, miRNAs interact selectively with Argonaute proteins, a riboproteic complex responsible for the selection process of the filament guide and for the degradation of the complementary strand. Finally, within RISC, the mature single-stranded miRNA interacts with the target mRNA, most frequently with the 3' untranslated region (Cammaerts S, 2015; Shea A, 2016).

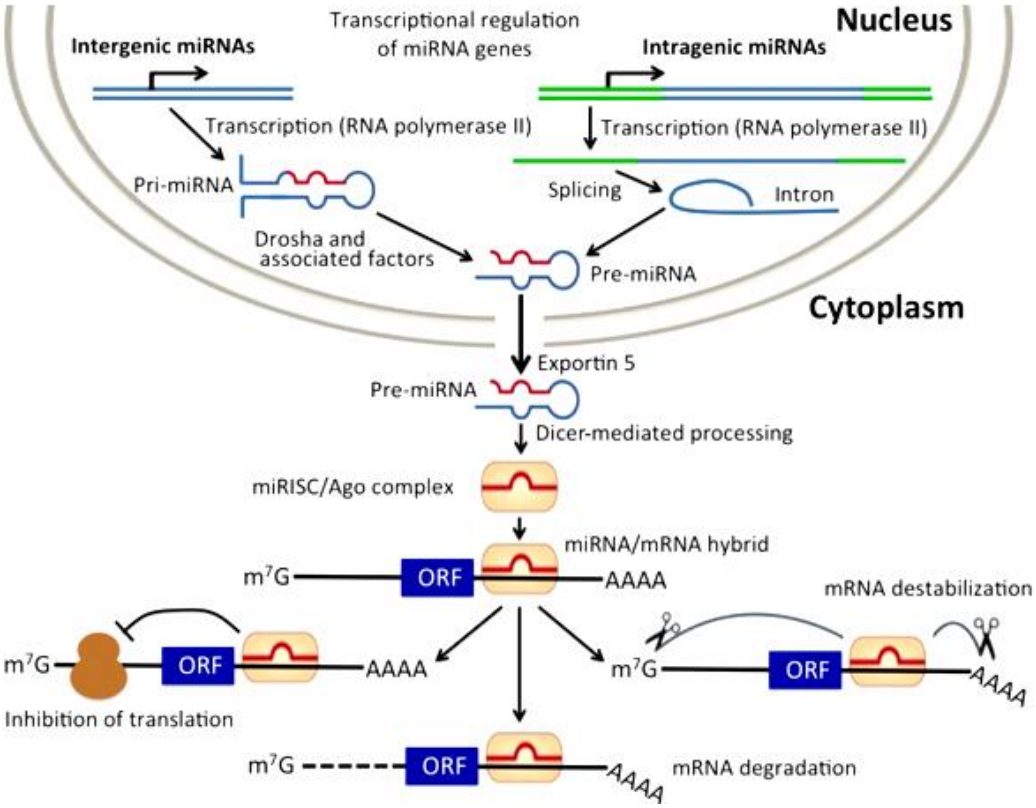

Figure 11. MicroRNA biogenesis (from Chandan K. Sen, 2015)

2.3 Gene regulation

The primary action of a miRNA is to target the cognate mRNA by base pairing. Each miRNA is predicted to individually regulate hundreds to thousands of mRNAs depending on cell type, context and binding of cofactors. The consequence may be mRNA cleavage and degradation or translation repression, according to the extent of complementarity. Moreover, specific mRNAs can be modulated by numerous different miRNAs, suggesting the possibility of cooperative repression imposed by multiple miRNAs.

There are three main feature in the mechanism of miRNA-mRNA matching: the presence of a seed region containing 2-8 nts that precisely match their complementary sequences

within the 3'untranslated region of the mRNA target; the mismatches and bulges may not be present in the region where the miRNA associates with Ago, but they could be in the miRNA-mRNA duplex; the universal existence of complementarity between the 3' half of the miRNA (typically at 13-16 nts) and the 3'UTR of the mRNA to stabilize the duplex.

Although most of the miRNAs demonstrated to bind the 3'UTR of their target transcripts, recent studies have suggested that there are other regions of the mRNA that can be targeted, 5'UTR, promoter and open reading frames (Chandan K. Sen, 2015; Almeida MI, 2011).

Basically, the alteration of gene expression is based on the recruitment of mature miRNA at the level of the RISC silencing complex. One of the miRNA strands, cut in the cytoplasm and incorporated into the RISC, binds the mRNA target sequence. In case of perfect complementarity, it occurs mRNA cleavage, commonly seen in plants; on the contrary, imperfect complementarity leads to a reduction or an inhibition of protein synthesis by translational repression; indeed, animal miRNAs recognize their target mRNAs through partial base pairing, especially within the 'seed' sequence at nucleotides 2–7 or 2–8 of the miRNA (Iwakawa HO, 2015). This evidence has important biological implications depending on the role played by the repressed protein in the cellular network.

2.4 Biological relevance

Since microRNAs have been discovered, it has been investigated their possible functional roles in the regulation of cellular processes. As previously said, the small requirement of complementarity (7-8 nts) makes possible for a single mRNA to be target of several miRNAs and it is estimated that miRNAs regulate up to one-third of human genes, participating in various biological process like cell proliferation, differentiation, cell cycle regulation, apoptosis, hematopoiesis and hypoxia (Shea A, 2016). For this reason, bioinformatics analysis is very useful to predict the binding site between miRNA and target mRNA, and several databases like TargetScan and miRTarbase are available and constantly updated.

MiRNAs play a key role in various physiological processes, such as the differentiation and maintenance of tissue identity (Shalaby T, 2014); the regulation of the PTEN-AKT-FOXO1 pathway and receptor editing during B cell maturation (Coffre M, 2016); in the hematologic context miRNAs behave like epigenetic regulators, as they possess key biochemical and biological properties that can provide both stability and alterability to the

epigenetic program (Roden C, 2016). Nevertheless, it is easily deduced the implication of miRNAs also in various pathological conditions. In cardiovascular diseases by miRNA microarray analysis, more than 12 miRNAs were found to be deregulated during heart failure and cardiac hypertrophy (Van Rooij, 2006). All the inflammatory conditions are associated to altered microRNAs expression, for example in autoimmune diseases like psoriasis, rheumatoid arthritis and lupus erythematosus (Almeida MI, 2011). MiRNAs have a considerable role also in diabetes mellitus and neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Ghelany HS, 2012). They are involved not only in the disease development, but also in the etiology suggesting that miRNAs could be potential diagnostic markers.

The altered expression of microRNAs has been demonstrated to have an important role in cancer and more than 50% of the human miRNAs encoding genes are found in fragile chromosomal sites associated with tumor (Calin GA, 2004). Different mechanisms are responsible of miRNAs alteration, such as altered transcriptional regulation, abnormal miRNA processing, defects in localization of miRNAs, gene mutation, epigenetic factors and chromosomal changes (Visone R, 2009). Usually, the consequence of this, is the upregulation of oncogenes and/or the downregulation of tumor suppressor, supporting tumorigenesis (Croce CM, 2005) (Figure 12).

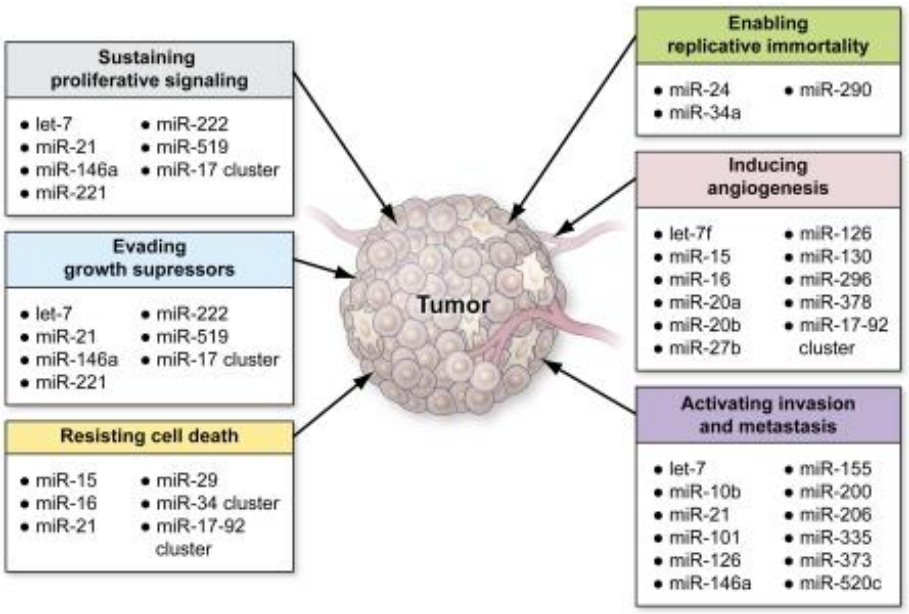

Figure 12. Aberrant miRNA expression affects signaling pathways to enhance tumorigenesis. Representative miRNAs that have been shown to act as oncogenes or tumor suppressor genes to affect the 6 common hallmarks of cancer (Ross SA, 2011).

Besides their functional effects on tumor cell signaling pathways, microRNAs have been shown to exhibit tissue specific expression patterns (Liu H, 2009), suggesting that they have potential utility as clinical biomarkers. Circulating microRNAs have been identified in several different body fluids, where they are very stable: serum, plasma, saliva, and urine (Weber JA, 2010). Therefore, their profile can be used to identify disease types, since elevated circulating microRNAs are significantly associated with disease-associated genetic variants.

2.5 MiRNAs in cancer

The first evidence of the involvement of microRNAs in human cancer derived from studies on chronic lymphocitic leukemia (CLL), in an attempt to identify tumour suppressors at chromosome 13q14, frequently deleted in CLL. Prof. Croce's group reported that rather than containing a protein coding tumour suppressor gene, this region contains two microRNA genes, miR-15a and miR-16-1, expressed in the same polycistronic RNA. This result firstly evidenced that microRNAs could be involved in the pathogenesis of human cancer as the deletion of chromosome 13q14 caused the loss of these two microRNAs (Calin GA, 2002).

Starting from this discovery, the same group studied all the known microRNA genes and found many of them located in chromosomal loci prone to deletions or amplifications, as was found in many different human tumours (Calin GA, 2004).

Research on microRNAs in cancer grew rapidly and showed the involvement of microRNAs in many human tumors (Lin S, 2015). From the comparison of a normal human genome map to an altered one, it was demonstrated that more than 50% of the genes that encode miRNAs are located in "fragile site". These areas are regions rather sensitive to chromosomal mutations which can cause the suppression of particular genes, known as tumor suppressor, or the de-repression of oncogenes with the consequent onset of the tumor. From those data can be seen that microRNAs play a rather important role in the development and progression of tumors (Calin GA, 2004). Lu J et al. (2005) identified a global downregulation of miRNAs expression in cancer in comparison with normal tissue, which could be linked with the poor differentiation of tumor cells. On the other hand, many other miRNAs result to be upregulated, some of them playing oncogenic roles (Di Leva G, 2014).

MicroRNAs can play a dual action in cancer; they can act as oncomiRNA/metastamiRNA

or as tumor suppressor miRNA, affecting the main hallmarks of cancer such as sustaining proliferation, evading growth suppressor, resisting cell death, inducing angiogenesis, activating invasion and metastasis (Figure 13).

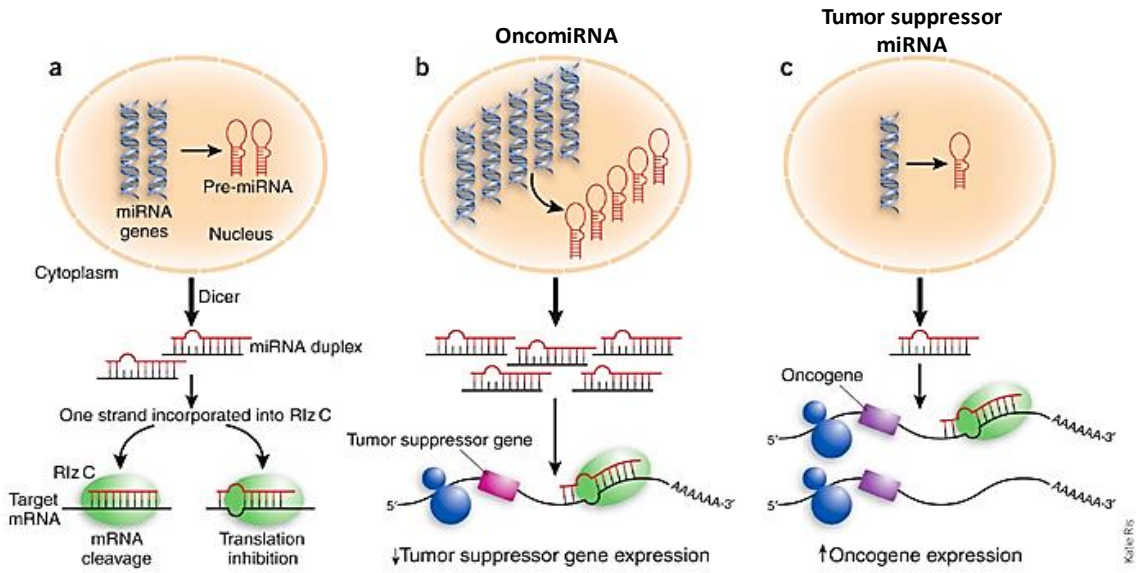

Figure 13. MicroRNAs as oncogenes or tumour suppressor genes. (a) pri-miRNAs are processed into pre-miRNAs in the nucleus. Pre-miRNAs are exported to the cytoplasm and are processed by the enzyme Dicer to yield miRNAs. These miRNAs operate by either cleaving mRNA or inhibiting translation in concert with RISC (RNA-induced silencing complex). (b) Overexpression of oncomiRNAs can decrease expression of the target, such as a tumor suppressor gene. (c) Underexpression of tumor suppressor miRNAs can result in increased expression of a target such as an oncogene (Caldas C, 2005).

Moreover, it is important to stress that miRNAs property are tissue-specific, so their expression can lead to different outcomes depending on which of their target are present in the cellular context. Therefore, to define miRNAs as oncogenic or tumor suppressor it should be specified the type of cell or tissue in which they are (Garzon R, 2010). Through the literature we can also see that certain miRNAs have been shown to be oncogenic in one scenario, but tumor suppressive in another. Diversity of effects is not a surprise given the large number of genes that can be influenced by a single miRNA (Svoronos AA, 2016).

2.5.1 Tumor suppressor miRNAs

Tumor suppressor miRNAs (miRsupp) act to impede cancer cell proliferation through targeting oncogenes post-transcriptionally and they have been found to be often downregulated in tumors.

Liu G et al. (2015), demonstrated that miR-18a has a protective role in colorectal carcinoma where it binds TBP-like 1 gene and reduces proliferation, invasion and migration of the cancer cells.

MiR-148a was shown to be significantly downregulated in renal cell carcinoma (RCC) tissues and cell lines; this is associated with large tumor size and lymph node metastasis. It was seen that overexpression of miR-148a significantly inhibited RCC cell proliferation, colony formation, migration and invasion and *in vivo* studies revealed that high levels of miR-148a suppress RCC xenograft tumor growth; as confirm of this, is the fact that AKT2 is a direct target and it is negatively correlated with miR-148a expression (Cao H, 2016).

Another example of tumor suppressor miRNA is miR-124-3p which is involved in the regulation of gastrulation and neural development in brain, and it targets some important genes, such as RAC1, the androgen receptor, SPHK1, ROCK2 and EZH2. Recently, in breast cancer it was found that miR-124-3p is constitutively downregulated and it can suppress the proliferation and invasion of breast cancer cells. Mechanistic studies revealed that miR-124-3p directly binds the CBL 3'-UTR and inhibits CBL (Cbl proto-oncogene, E3 ubiquitin protein ligase) expression; this could be a reason why the downregulation of miR-124-3p can promote the development of breast cancer (Wang Y, 2016).

The family of miR-181 is involved in the regulation of myeloid cells development and the expression of these miRNAs causes uncontrolled progression and proliferation of myeloid cells that leads to chronic lymphocytic leukemia (Su R, 2015).

The decreased expression of miR-101 instead has been shown to have an effect on the growing of neuroblastoma. Normally this miRNA inhibits excessive cell proliferation by hitting the proto-oncogene MYCN, but decreased expression of miR-101 causes excessive levels of MYCN causing an uncontrolled division and massive cell growth that leads to the development of neuroblastoma (Buechner J, 2011).

2.5.2 OncomiRNAs

Oncogenic miRNAs (oncomiRs) are responsible of the down regulation of tumor suppressor genes. Usually, they are upregulated and play a causal role in the onset and progression of human cancer.

In Table 1 are reported some of the numerous miRNAs that have shown increased expression in correlation to the development of tumors.

Table 1. MiRNAs exhibiting oncogenic functions (Gambari R, 2016).

microRNA	**Disease**	**Biological effect**	**Target mRNA/pathway**
miR-10b	Human esophageal cancer cells, gastric carcinoma	Promotion of migration and invasion	KLF4
miR-21	Breast, colon, pancreatic, lung, prostate, liver and stomach cancer, chronic lymphocytic leukemia; acute myeloid leukaemia, glioblastoma, neuroblastoma	Stimulation of cellular proliferation; action on mitochondrial apoptosis tumor-supressive pathways, resisting cell death	PTEN, TPM1, PDCD4, p63, RECK, p53, TGF-β
miR-23b	Renal cancer cells	Downregulation of POX (tumor suppressor), increase in HIF signaling	POX
miR-27a	Prostate cancer	Increase in the expression of AR target genes and prostate cancer cell growth	PHB
miR-100	Myeloid leukemia, glioma	Promotion of cell differentiation, survival and apoptosis	RBSP3, ATM
miR-125b	B-cell leukemia	Induction of cell differentiation and transformation	MAP3K11, ARID3B
miR-132 miR-212	Pancreatic adenocarcinoma (PDAC)	Stimulation of cell proliferation via the β2 adrenergic pathway	Rb1
miR-155	Lymphoma, leukemia, breast, colon, lung, pancreatic, thyroid brain cancer, diffuse large B-cell lymphoma (DLBCL)	Causes the constitutive activation of signal transducer and activator of transcription 3, sustaining proliferative signaling, resistance of cell death, activation invasion, migration and metastasis	SOCS1, RhoA, FOXO3a, VHL
miR-17	Neuroblastoma	Marked increase of *in vitro* and *in vivo* tumorigenesis	p21, BIM
miR-182	Melanoma	Promotion of melanoma metastases	MITF, FOXO3
miR-214	Ovarian cancer	Stimulation of cell survival and cisplatin resistance	PTEN
miR-221 miR-222	Atypical teratoid/rhabdoid tumors (ATRT), osteosarcoma, glioma, breast cancer, follicular thyroid carcinoma (FTC), digestive system carcinoma	Decrease of cell cycle inhibitor $p27^{Kip1}$, tumor development and progression by regulating proliferative signaling pathways, altering telomere and telomerase activity, avoiding cell death from tumor suppressors, autophagy and apoptosis, monitoring angiogenesis, supporting epithelial-mesenchymal transition, and even controlling cell-specific function within the microenvironment	$p27^{Kip1}$, PTEN, KIT, TRPS1, PUMA, PTPμ, FOXO3, PIK3R1, TIMP3, TIMP2, DDIT4, MDM2, ERα, SOCS3, OCS1, HDAC6, ANGPTL2, BBC3, BMF, RECK, PDLIM2, RelA, $p57^{Kip2}$

miR-296	Brain tumors	Promotion of angiogenesis	HGS
miR-301	Breast cancer	Promotion of growth, proliferation, invasion and metastases	FOXF2, BBC3, PTEN
miR-372 miR-373	Testicular tumors	Promotion of tumorigenesis in cooperation with RAS	LATS2
miR-375	Gastric cancer	Promotion of carcinogenesis	JAK2, PDK1
miR-378	Breast carcinoma	Ehnancement of cell survival; reduction of caspase-3 activity; promotion of growth and angiogenesis	Sufu, Fus-1
miR-519a	Hepatocellular carcinoma, breast cancer	Promotion of tumor growth, proliferation; inhibition of apoptosis; tamoxifen resistance	PTEN/PI3K/ AKT/FOXF2
miR-675	Colorectal cancer	Overexpression of H19 (oncofetal non-coding RNA) in cancer tissues	RB
miR-1908	Glioblastoma	Promotion of anchorage independent growth *in vitro*, increasing of tumor forming potential *in vivo*	PTEN

Among these, miR-21 results involved in the pathogenesis of many neoplastic diseases including glioblastoma, head and neck cancer, ovarian cancer, B-cell lymphoma, hepatocellular carcinoma, cervical cancer, and lung cancer. Functional studies in cancer cell lines indicate that miR-21 plays an important role in the oncogenic process, it is regulated by STAT3 and NF-κB transcription factors, which are both constitutively activated in a variety of cancers; as a consequence, cells are characterized by high proliferation, low apoptosis, high invasion, and metastatic potential (Pfeffer SR, 2015). It was found that miR-21 promotes the cell invasion by regulating multiple genes, such as PTEN, RECK and MARCKS (Giunti L, 2015) and suppressing the expression of the tumor suppressors, IGFBP3 and FBX011 (Pfeffer SR, 2015).

MiR10b was identified to promote tumor metastasis in breast cancer, invasiveness in nasopharyngeal carcinoma, glioma, acute myeloid leukemia, esophageal cancer, colon cancer, neurofibromatosis type 1, and in pancreatic cancer is associated with invasive behavior and poor prognosis. Indeed, it was found that miR-10b decreased the expression of homeobox D10 (HOXD10), a member of the HOX genes family, resulting in increased expression of RhoC, a HOXD10 target and a metastasis promoter (Hawa Z, 2016).

Recently it was demonstrated that both in cell lines and tissue samples, miR-96 promotes growth and invasiveness of prostate cancer acting on TGF-β/mTOR signaling and promoting bone metastasis (Siu MK, 2015). PTEN is another tumor suppressor gene

usually downregulated by miRNAs; for example, miR-1908, overexpressed in glioblastoma, binds this gene increasing proliferation, migration and invasion. As well, miR-19a acts as oncomiRNA in myeloma by targeting PTEN; Zhang X. et al. (2016) demonstrated that elevated expression of miR-19a (after transfection) was associated to upregulation of BCL-2 and MDR mRNA and protein, two of the main proteins related to apoptosis and drug resistance. This evidence may provide miR-19a/PTEN/AKT axis as a potential therapeutic target for the treatment of myeloma.

Other oncomiRNAs, miR-301 and miR-378, have an increased expression in association with the development of breast cancer and for this reason are considered as prognostic factors for tumor development: miR-301 has shown to promote proliferation, invasion and cellular migration and also the ability to establish resistance to the chemotherapy with tamoxifen (Shi W, 2011); while, miR-378 demonstrated to inhibit the expression of ERRγ and GABPA, two PGC-1β partners that cause a metabolic change from oxidative to glycolytic bioenergetics pathway (Eichner LJ, 2010).

MiR-155 and miR-221, as shown in Table 1, are oncomiRs with many tumorigenic effects on different types of tumors, and in this thesis it was focused the attention particularly on them.

MiR-221

MiR-221 belongs to the cluster miR-221-222; it is located in tandem on human chromosome Xp11 and it is transcribed as a single RNA precursor with RNA polymerase II. The miR-221/222 cluster is highly conserved in vertebrates, including human, mouse and rat and they have the same seed sequence. Its expression is upregulated by NF-κB, angiotensin II, HMGB1, microphthalmia-associated transcription factor (MITF), HOXB7/pBX2, and it is downregulated by promyelocytic leukemia zinc finger (PLZF) and a repressive complex composed of estrogen receptor α (ERα) and two nuclear receptor corepressors, NCOR1 and NCOR2 (Shimono Y, 2015). MiR-221 is involved in many physiological and pathological pathways. It participates to the lung branching morphogenesis and epithelial cell development; studies *in vivo* and *in vitro* showed that enforced expression of miR-221 with LPS, increased the release of pro-inflammatory cytokines TNF-α and IL-6 and enhanced the activation of NF-κB and MAPKs. However, it was seen that restoration of A20 (regulator of NF-κB and MAPKs signaling), its direct target, abolished the stimulatory effect of miR-221 on production of pro-inflammatory

cytokines (Zhao D, 2016). MiR-221 (together with miR-222) mostly function as oncogene in human epithelial tumors, but it acts also as tumor suppressor in some tumors, such as erythroleukemia. MiR-221 and miR-222 (Figure 13) regulate cell cycle progression, apoptosis, cell migration and stemness by targeting cell cycle inhibitors CDKN1B ($p27^{Kip1}$) in glioblastoma, breast cancer, lung and pancreatic cancer, and also CDKN1C ($p57^{Kip2}$) in hepatocellular carcinoma. In 2010, PUMA was identified as a novel target of miR-221/222 in human epithelial cancers (Zhang C, 2010), but it influences apoptosis binding also PTEN and Bim. Other studied targets are FOXO3, c-Kit, TIMP3, ERα and DNA methyltransferase DNMT3b (Garofalo M, 2012).

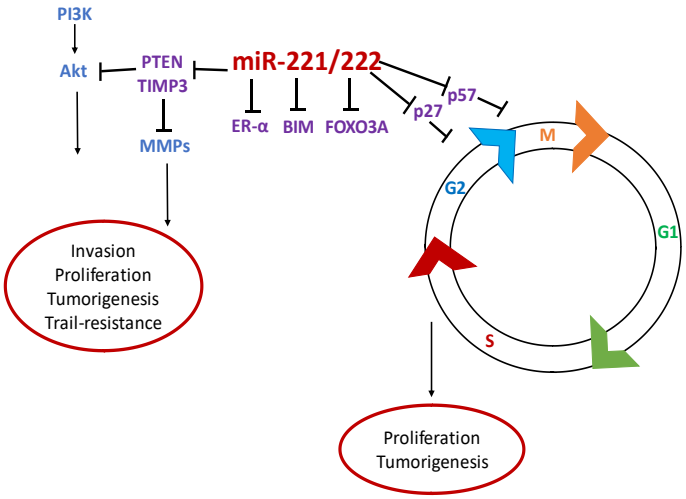

Figure 13. Representation of the main targets of miR-221/222 in cancer.

Moreover, miR-221 is a positive regulator of EMT (epithelial-mesenchymal transition). The EMT is a strong process in tumor invasion, metastasis and tumorigenesis that describes the molecular reprogramming and phenotype changes occurring in cells that transit from polarized immotile epithelial cells to motile mesenchymal cells. In this context, Lambertini E et al. (2012) showed that Slug can transcriptionally control miR-221.

MiR-155

MiR-155 was the first miRNA assigned (2005) to have an oncogenic role (Palumbo S, 2014); it is encoded by an exon of the noncoding RNA known as the B-cell integration cluster (BIC), which is located on chromosome 21q21. From miRBase we can see that miR-155 sequence is conserved between human, mouse, chicken and other species.

Initially, it was found accumulated in Burkitt's lymphoma and in human B cell lymphoma patients (Zhou H, 2016). In a transgenic mouse model, Costinean et al. (2006), demonstrated that when it is overexpressed it leds to the development of B-cell leukemia and high-grade lymphoma tumor (Costinean S, 2006). In many studies it was found that miR-155 regulates Th2 immune responses and gene signaling, therefore overexpression of miR-155 is involved in the development of asthma and activation of allergy-promoting cells (Zhou H, 2016).

In glioma, miR-155 is overexpressed and it is involved in chemo-resistance behavior as consequence of nuclear factor NF-κB activation (Schliesser MG, 2016); moreover, targeting Max interactor-1 (MXI1), an antagonist of c-Myc that is involved in brain tumor progression, it promotes glioma cell proliferation (Zhou J, 2013). Recently, it has also been shown that high miR-155 expression was associated with poor prognosis in patients with acute myelogeneous leukemia (AML) and in HL-60 cells it was seen that targeting miR-155 upregulated PUMA and downregulated Slug expression, having as results the decrease of HL-60 cell growth, impaired colony formation and increase of apoptosis by 45%. These results suggest a way to have antileukemic activities in AML through a novel mechanism resulting in inhibition of Slug expression and increase of PUMA expression by targeting miR-155 (Liang H, 2016). As in other type of cancer, miR-155 takes part in a sequence of bioprocesses that contribute to the development of breast cancer drug resistance, including repression of FOXO3a, enhancement of epithelial-to-mesenchymal transition (EMT) and mitogen-activated protein kinase (MAPK) signaling, reduction of RhoA, and it affects the length of telomeres (Yu DD, 2015). As like as other miRNAs, miR-155 can also act as tumor-suppressor: in Caski cervical cancer cells, it suppresses EGF-induced EMT decreasing migration/invasion, inhibiting cell proliferation and enhancing the chemosensitivity to DDP (cis-diamminedichloroplatinum) in humans (Lei C, 2012). The dysregulation of miR-155 has been strongly implicated also in Helicobacter pylori-related gastric disease, inflammatory bowel disease and colorectal cancer through the modulation of several targets such as FOXO3a, c-myc, TP53INP and the pro-apoptotic Tspan14, Lpin1 and Pmaip1 (Wan J, 2016).

2.5.3 MetastamiRNAs

In addition to miRsupp and oncomiR, there is the class of metastamiRNA which includes miRNAs mostly implicated in cell migration and metastasis.

In breast cancer, miR-10b targets directly HOXD10 facilitating overexpression of the pro-metastatic gene RHOC (Iorio MV, 2011); while miR-126 and miR-335 were found to act as negative regulators of tumor invasion and metastasis, they target SOX4 (SRY-box containing transcription factor), MERTK (c-Mer tyrosine Kinase) and TNC (tenascin C) inhibiting cell invasion *in vitro* and metastasis *in vivo* (Tavazoie SF, 2008). MiR-34 is involved into the network mediated by p53, it is lost in several tumor types and it inhibits migration and invasion by downregulation of MET expression in human HCC cells (Li N, 2009). Neo-angiogenesis is a crucial step during metastatic process and miRNAs can influence it. In endothelial cells miR-210 is a promoter of this process because it represses directly endothelial ligand Epharin A3; miR-16 and miR-15b, reduced by hypoxic signals, target VEGF supporting the angiogenetic process. On the other hand, miR-221/222 repress proliferative and angiogenetic properties of c-Kit (Iorio MV, 2011).

2.6 MicroRNAs in Glioblastoma

Glioblastoma is the most common and lethal cancer of the adult brain and, in order to find new approaches for its treatment, many recent studies identified microRNAs as important regulators of glioblastoma. As already cited, miRNAs, in relation to cancer pathobiology, impact many of the hallmarks of cancer: they contribute to resistance to cell death, sustain proliferative signaling, induction of angiogenesis, activation of invasion and metastasis and development of drug resistance.

Many studies were conducted to identify the expression profiles of microRNAs involved in glioma and new strategies to fight again this insidious disease. Table 2 contains miRNAs, collected in Sheas's review (2016), that are overexpressed and underexpressed in glioma and the main pathways in which they are involved.

Table 2. Overexpressed or underexpressed microRNAs involved in glioblastoma (Shea A, 2016)

miRNA	**mRNA targets**	**Expressionchange with GBM/ poorprognosis**	**Role(s) in cancerprogression**
let-7	NRAS, KRAS, CCND1	Decrease	Proliferation, apoptosis, migration, invasion, chemoresistance
miR-7	EGFR, RAF1, PI3K, FAK, IRS2	Decrease	Survival, proliferation, apoptosis, invasion, angiogenesis
miR-9/miR-9*	SOX2, PTCH1, FOXP1, CAMTA1	Increase;decrease	Proliferation, stemness, chemoresistance

miR-10a/b	BCL2L11, TFAP2C, CDKN2A, CDKN1A, CSMD, HOXD10, E2F1	Increase	Proliferation, apoptosis, migration, invasion, stemness
miR-15a		Increase	
miR-15b	NRP2, CCND1	Decrease	Proliferation, apoptosis, invasion, angiogenesis
miR-16	BMI1, NFKB1, BCL2, ZYX	Decrease;increase	Proliferation, apoptosis, migration, invasion, angiogenesis
miR-17	CAMTA1, PTEN, MDM2	Increase	Survival, proliferation, migration, invasion, stemness, chemoresistance, stress response
miR 17–92 cluster	CTFG	Increase	Proliferation, apoptosis, stemness
miR-18a/18a*	NEO1, DLL3, CTGF, SMAD3	Increase	Proliferation, apoptosis, migration, invasion, stemness
miR-19a/b	PTEN	Increase	Survival, proliferation
miR-20a	TIMP2	Increase	Invasion
miR-21	PDCD4, IGFB3, FBXO11, ANP32A, SMARCA4, LRRFIP1, HNRPK, TP63, RECK, TIMP3, TPM1, FASLG, SPRY2	Increase	Survival, proliferation, apoptosis, migration, invasion, chemoresistance
miR-23b	PTK2B	Decrease	Migration, invasion
miR-24	ST7L	Increase	Proliferation, apoptosis, invasion
miR-25	CDKN1C, NEFL, MDM2, TSC1	Increase; decrease	Viability, proliferation, invasion
miR-26a	PTEN, ATM	Increase; decrease	Radioresistance
miR-26b	EPHA2	Decrease	Proliferation, migration, invasion, vasculogenic mimicry
miR-27a	FOXO3a	Increase; decrease with higher grade	Proliferation, apoptosis, invasion
miR-27b		Increase	Proliferation, apoptosis, invasion
miR-28		Increase	
miR-29a/b	MCL1, PDPN	Increase; decrease	Proliferation, apoptosis, invasion
miR-30a	SOCS3, SEPT7	Increase	Proliferation, apoptosis, invasion, stemness
miR-31	FIH1, RDX, TRADD	Decrease; increase	Proliferation, apoptosis, migration, invasion, stemness, angiogenesis, chemoresistance
miR-32	MDM2, TSC1	Decrease	Survival, proliferation
miR-34a	MET, NOTCH1, NOTCH2, CCND1, CDK6, RICTOR, SIRT1	Decrease	Survival, proliferation, apoptosis, migration, invasion, stemness
miR-92a	BCL2L11	Increase	Proliferation, apoptosis

miR-92b	DKK3, NLK	Increase	Proliferation, apoptosis, invasion
miR-93	ITGB3	Increase	Survival, proliferation, angiogenesis, stemness
miR-95		Decrease	
miR-100	SMRT/NCOR2, ATM	Decrease; increase	Proliferation, apoptosis, radioresistance
miR-101	KLF6	Decrease	Proliferation, apoptosis, invasion, migration
miR-106a	SLC2A3, TIMP2	Decrease; increase in GSCs	Proliferation, invasion, metabolism
miR-107	SALL4, NOTCH2, CDK6	Decrease	Proliferation, apoptosis, invasion
miR-124	PIM3, NRAS, SOS1, PPP1R3L, RRAS, NRAS, SNAI2, MAPK14, TEAD1, SERP1, LAMB1, CDK4, IQGAP1	Decrease	Proliferation, apoptosis, migration, invasion, stemness, angiogenesis, chemoresistance, radioresistance, stress response
miR-125a	NRG1, PDPN	Decrease	Proliferation, apoptosis, invasion, migration
miR-125b	LIN28, BAK1, MAPK14, CDK6, CDC25A, BMF, MAZ, E2F2	Decrease; increase	Survival, proliferation, apoptosis, invasion, stemness, angiogenesis, chemoresistance
miR-126		Decrease	
miR-128	P70S6K1, SUZ12, BMI1, PDGFRα, EGFR, E2F3a, WEE1, MSI1	Decrease	Proliferation, apoptosis, angiogenesis, stemness, radioresistance
miR-130a		Decrease	Chemoresistance
miR-130b	MST1, SAV1	Increase	Stemness
miR-132		Increase; decrease	
miR-135b	ADAM12, SMAD5, GSK3β	Decrease	Proliferation, migration, stemness, radioresistance
miR-136	AEG1, BCL2	Decrease	Apoptosis, chemoresistance
miR-137	RTVP1, PTGS2, MSI1	Decrease	Proliferation, apoptosis, migration, invasion, stemness
miR-139	ELTD1, MCL1	Decrease	Proliferation, apoptosis, chemoresistance
miR-140		Increase	
miR-143	HK2, RAS	Decrease; increase	Migration, invasion, angiogenesis, chemoresistance, stemness, glycolysis
miR-145	CTGF, ABCG2	Decrease; increase	Proliferation, migration, invasion
miR-146	NOTCH1	Decrease; increase	Proliferation
miR-148a	FIH1	Increase	Proliferation, stemness,

			angiogenesis
miR-152	KLF4, MMP3, XIST	Decrease	Proliferation, apoptosis, migration, invasion, stemness
miR-153	BCL2, MCL1, IRS1	Decrease	Viability, proliferation, apoptosis, stemness
miR-155	GABRA 1, EAG1, MAPK13, MAPK14, FOXO3a, MXI1	Increase	Proliferation, apoptosis, invasion, chemoresistance
miR-181	FOS, KPNA4, MGMT, RAP1B, BCL2, NOTCH2, MGMT, KRAS, BCL2, MDM2	Decrease	Proliferation, apoptosis, invasion, stemness, chemoresistance, radioresistance
miR-182	BCL2L12, HIF2A, MET, CYLD, LRRC4	Increase	Proliferation, apoptosis, invasion, angiogenesis, chemoresistance, stemness
miR-184	FOXO3, SND1	Decrease	Proliferation, invasion, chemoresistance
miR-193	SMAD3	Increase	Proliferation
miR-195	CCND1, CCNE1	Decrease; increase in TMZ-resistant GBM	Proliferation, chemoresistance
miR-196	NFKBIA	Increase	Proliferation, apoptosis
miR-200a/b	MGMT	Decrease; increase	Chemoresistance
miR-203	SNAI2, PLD2	Decrease	Chemoresistance
miR-205	VEGFA, LRP1	Decrease	Proliferation, apoptosis, invasion, migration
miR-210	HIF3A	Increase	Survival, chemoresistance
miR-218	LEF1, IKBKB, BIM1, ECOP, CDK6	Decrease	Survival, proliferation, apoptosis, migration, invasion, stemness
miR-221/222	PTEN, PUMA, CDKN1B, CDKN1C, PTPRM, MGMT, SEMA3B, TIMP3, GJA1	Increase; decrease	Viability, proliferation, apoptosis, migration, invasion, chemoresistance, radioresistance
miR-296-3p	EAG1	Decrease	Proliferation, chemoresistance
miR-302/367 cluster	CXCR4		Invasion, chemoresistance, stemness
miR-320	E2F1	Decrease	Proliferation, migration
miR-323	IGFR1	Decrease; increase	Proliferation, apoptosis, migration
miR-326	NOB1	Decrease	Proliferation, apoptosis
miR-328	SRFP1	Decrease; increased in low grade GBM	Proliferation, invasion
miR-329	E2F1	Decrease; increase	Survival, proliferation, apoptosis
miR-330	SH3GL2	Increase; decrease	Proliferation, apoptosis, migration, invasion
miR-331-3p	NRP2, HER2	Decrease	Proliferation, apoptosis, migration,

			invasion, angiogenesis
miR-335	DAAM1, PAX6	Increase	Survival, proliferation, apoptosis, invasion, stemness
miR-340	PLAT, ROCK1, CDK6, CCND1, CCND2	Decrease	Proliferation, apoptosis, migration, invasion, stemness
miR-363	BIM, CASP3	Increase	Survival
miR-372	PHLPP2	Increase	Proliferation, apoptosisinvasion
miR-377	SP1	Decrease	Proliferation, invasion
miR-378		Decrease	Migration, invasion
miR-381	LLRC4	Increase	Proliferation
miR-410	MET	Decrease	Proliferation, invasion
miR-451	CAB39	Decrease	Proliferation, invasion, apoptosis, stemness
miR-455-3p	SMAD2	Increase	Chemoresistance
miR-483-5p	ERK1	Decrease	Proliferation
miR-487b		Decrease	Proliferation, apoptosis
miR-491-3p	IGFBP2, CDK6	Decrease	Proliferation, invasion, stemness
miR-491-5p	BCL2L1, EGFR, CDK6, MMP9	Decrease	Proliferation, invasion, stemness
miR-513	LRP6	Decrease	Proliferation
miR-582-5p	CASP3, CASP9	Increase	Proliferation, apoptosis
miR-603	MGMT, WIF1, CTNNBIP1	Increase	Proliferation, chemoresistance
miR-655	SENP6	Increase	Invasion
miR-663	PIK3CD	Decrease	Proliferation, invasion
miR-873	IGF2BP1, BCL2	Decrease; increase	Proliferation, apoptosis, migration, invasion, chemoresistance
miR-874		Decrease	

MiR-21 is one of the most studied miRNA because it can influence numerous biological processes. In addition to the promotion of cell cycle progression, it is involved in the promotion of invasion and metastasis and resistance to chemotherapeutics through the downregulation of numerous targets such as PDCD4 (Chen Y, 2008), TIMP3 (Gabriely G, 2008), IGFBP3 (Yang CH, 2014) and LRRFIP1 (Li Y, 2009). Moreover, miR-21 has anti-apoptotic activity by targeting the signaling pathways of P53 and TGF-β as well as the mitochondrial apoptotic pathway; supporting this evidence is also the fact that the reduction of miR-21 has induced apoptosis through the activation of caspase 3 and 9 in

glioma cells (Zhou X, 2010).

MiR-218 is usually low expressed in glioma and it has pro-apototic activity. It was shown that exogenous administration of miR-218 can repress its target CDK6, inhibiting cell proliferation and inducing apoptosis; the sensitization to apoptosis occurs also through the regulation of epidermal growth factor receptor coamplified and overexpressed protein (ECOP), which can suppress the transcriptional activity of NF-κB (Xia H, 2013).

Lin J et al. (2012) demonstrate, in an orthotopic human glioma mouse model, that inhibition of miR-10b diminished the growth, invasiveness, and angiogenicity of glioma cells in the brain, significantly prolonging the survival of glioma-bearing mice.

MiR-10b, together with miR-21, were detected at high levels in body fluids, such as serum and/or cerebrospinal fluid, of a limited number of patients with GBM; while, miR-205 has also been identified as a potential biomarker due to its significantly lower levels in the serum of GBM patients as compared to control. Further, patients with advanced pathological grade demonstrated longer overall survival times when serum miR-205 levels were elevated (Yue X, 2016). Therefore, expression profiles of miRNAs are useful for predicting GBM patient survival and, potentially, for identifying efficacious therapeutic targets. MiR-10b is the first microRNA investigated in a clinical trial with the aim to demonstrate if its expression level could be a prognostic and diagnostic marker in primary glioma samples (Rolle K, 2015).

All these findings suggest a miRNA-based therapy that could provide a progress in cancer treatment.

2.6.1 The role of miR-221 and miR-155 in glioblastoma oncogenesis

MiR-221

Mir-221 is one of the most expressed oncomiRNA studied in glioma, it has been reported to promote oncogenesis *in vitro* and *in vivo* by regulating the STAT3/Akt pathway and by regulating post-transcriptionally the tumor suppressor $p27^{kip1}$. Indeed, in U251 human glioblastoma cells, treated with antisense miR-221, the cell growth was slowed down by reducing the G1 to S shift in the cell cycle (Zhang C, 2010).

MiR-221 also promotes cell survival also through targeting of p53-upregulated modulator of apoptosis (PUMA), which normally binds Bcl-2 and Bcl-xl inducing apoptosis (Zhang CZ, 2010). Another specific target affected by miR-221 (and miR-222) is the protein PTEN, which acts by suppressing the action of PI3K-AKT-mTOR, controlling in this way

the survival, proliferation, energy metabolism and cellular architecture. Overexpression of miR-221 led to cell survival and resistance towards the most common therapies: carmustine (BCNU), temozolomide (TMZ) and radiation. This is due to the targeting of PTEN, that can suppress the PI3-K/Akt signaling axis; the targeting of DNM3 gene, a member of the Dynamin family, a GTPase that is recently revealed to correlate with malignant diseases; and the activation of Akt independent of PTEN status (Xie Q, 2014; Yang JK, 2016; Li W, 2014).

Zhang C et al. (2012) demonstrated that high levels of miR-221/222 expression in gliomas confer highly aggressive invasion and poorer overall survival by targeting TIMP3 and act as prognostic factors for glioma patients.

Recently, Semaphorin 3B (SEMA3B) gene was identified as another target of miR-221. SEMA3B regulates neuronal migration and acts as a tumor suppressor gene; its expression level in glioma cells was found lower than normal glia cells and an inverse correlation between miR-221 and SEMA3B was also demonstrated (Cai G, 2015). Invasiveness in glioblastoma is also due to the binding of miR-221 with the protein tyrosine phosphatase PTPμ, that in normal condition suppresses cell migration (Quintavalle C, 2012).

These data therefore suggest that in order to limit the development and proliferation of tumor cells, it should be reduced the activity of miR-221 (and miR-222) by inducing in this way the natural tumor suppressor which are nothing more than the targets of such miRNAs.

MiR-155

MiR-155, together with miR-21, in a microarray profile, was found to be upregulated in primary and secondary glioblastoma, keeping tumour cells in an inappropriate primitive and proliferative developmental state (D'Urso P, 2012). Starting from Labrakais's finding of a link between the expression of GABA receptors and the growth of glioma cells, it was demonstrated that GABRA1 is a direct target of miR-155 and the upregulation of the miRNA is associated to a downregulation of the GABA receptor, making tumour cells unresponsive to GABA cell proliferation inhibition and promoting cellular growth in malignant tumours (Labrakakis C, 1998; D'Urso P, 2012). This evidence suggested the availment of RNA-silencing approaches against miR-155 with the aim to control proliferation and signaling pathways regulated by GABA-A receptor (Poltronieri P, 2013).

To confirm this, miR-155 was found higher expressed in glioma tissues than the matched non-cancerous brains and, similarly, in glioma cell lines like U251 MG, U373 MG and U87 MG respect to normal astrocytes. Moreover, in an intracranial glioma model established by directly injecting U87 MG cells in the fore-brain of mice, it was demonstrated that the downregulation of miR-155 (after treatment with miR-155 inhibitor) reduced the growth of intracranial glioma xenografts and prolonged the survival time of the mice. This recent study conducted by Yan Z et al. (2015), also demonstrated that miR-155 is able to activate Wnt/β-catenin signaling, a very important molecular pathway that contributes to many aspects of glioma, by targeting HBP1.

In a panel of glioma cells, transfected with miR-155 inhibitor and miR-155 mimics, miR-155 was found to enhance the proliferative and invasive ability of glioma cells by regulating FOXO3a gene. FOXO3a is a negative regulator of Akt signaling and it was reported that its inactivation in glioma cells led to the downregulation of Bim, a pro-apoptotic protein (Ling N, 2013).

Overall, these evidence prove that miR-155 could be an interesting target for miRNA-antisense therapy.

2.7 MiRNAs in drug resistance in glioma

Currently, GBM is treated with cytoreductive surgery followed by concurrent radiation and chemotherapy with cytotoxic drug like temozolomide. The response to the therapy is very variable across patients and, the nonspecific cell targeting, causes many side effects, including development of drug resistance. This is due to the heterogeneity of tumor, caused by factors such as differences in genotypes and microRNA profile.

From a study on TMZ-sensitive GBM cell lines (Ujifuku K, 2010) and another on primary GBM tumors (Slaby O, 2010) emerged that miR-195, miR-455-3p, miR-10a and miR-21 were upregulated in resistant cells; while miR-221/222, miR-181b/c and miR-128 were downregulated in GBM. Knockdown of miR-195 and downregulation of miR-181b/c had a strong correlation with responsiveness to TMZ treatment, suggesting that their presence could be used as predictive marker for response to TMZ therapy (Ujifuku K, 2010; Slaby O, 2010).

MiR-21, which is usually upregulated and implicated in many carcinogenic pathway, inhibits the activity of TMZ on apoptosis, through the downregulation of pro-apoptotic proteins (Bax and CASP-3) and upregulation of anti-apoptotic Bcl-2. Furthermore, miR-21

was found involved in the resistance also to other chemotherapeutics such as sunitinib, paclitaxel, doxorubicin and VM-26 (Shi L, 2010; Shea A, 2016).

Recently, Xie Q et al. (2014) studied the role of miR-221 in carmustine-resistant glioma cells and they demonstrated that miR-221 induces cell proliferation and carmustine resistance by targeting PI3K/Akt signaling axis and downregulating PTEN. Moreover, miR-221/222 overexpression produces an increase in the sensitivity to TMZ by targeting MGMT, a suicide cellular DNA repair enzyme; but MGMT downregulation caused by the high levels of these miRs becomes chronic, rendering the cells unable to repair DNA damage (Quintavalle C, 2013).

The dysregulation of microRNAs can also affect the radiosensitivity of glioma cells. Again, miR-21 plays a critical role by modulating a tumor suppressor network and phosphoinositide kinase PI3K/Akt pathway; also miR-221 and miR-93 were found associated to radioresistance (Table 3) (Rolle K, 2015).

Table 3. microRNAs involved in drug resistance and radioresistance in glioblastoma (Shea A, 2016; Rolle K, 2015)

Chemoresistance	**Radioresistance**
miR-138; miR-203; miR-30b/c; let-7; miR-9; miR-31; miR-124; miR-143; miR-155; miR-210; miR-873; miR-21; miR-125b-2; miR-95; miR-455-3p; miR-10a; miR-181d; miR-195; miR-221/222; miR-423-5p; miR-93	let 7 family; miR-221/222; miR-425; miR-93; miR-181; miR-26a; miR-100; miR-124; miR-128

2.8 Targeting or mimicking miRNAs: therapeutic strategies

The increasing knowledge on microRNAs, their role in carcinogenesis and their ability to target several genes within the same pathway or even multiple oncogenic pathways, suggest their targeting as a novel therapeutic approach with the aim of countering the progression of the disease and improving the rate of patient survival.

Importantly, microRNAs can function as either oncogenes or tumor suppressors; thus, there are two possible different approaches: the inhibition of oncomiRs expression, and the restoration of miRsupp expression

In the first case, overexpressed intracellular miRNAs can be inhibited by synthetic antagomiRNAs: antisense single-stranded oligonucleotides complementary to mature endogenous miRNAs. Antisense oligonucleotides work as competitive inhibitors of miRNAs, presumably by annealing to the mature miRNA guide strand and inducing

degradation or stoichiometric duplex formation.

There are some fundamental features that have to be respected to gain an efficient inhibition of the target miRNA: high binding affinity, high resistance to nuclease degradation, low toxicity and specific efficient delivery. The structure of oligonucleotides permits to adapt these factors by modification of the sugar, the nucleobase, nucleotides linkages and/or the addiction of nonnucleotide modifiers. Therefore, several modified oligonucleotide analogues have been designed in order to bind to the guiding strand by Watson-Crick base pairing and prevent further processing.

Some of the artificial nucleic acids include peptide nucleic acid (PNA), locked nucleic acid (LNA), glycol nucleic acid (GNA) and therose nucleic acid (TNA), the difference respect to naturally occurring DNA or RNA is in the backbone structure of the molecule (Jolly P, 2016).

Locked nucleic acids (LNAs) are currently the most used class of antagomiRs; a methylene bridge connects the 2'-O atom and the 4'-C atom locking the ribose ring (Nana-SinkamSP, 2013). In this way they display a high hybridization affinity toward complementary single-stranded RNA and complementary single- or double-stranded DNA. LNA oligonucleotides also show high nuclease resistance, good aqueous solubility and low toxicity (Garzon R, 2010; Shea A, 2016). There are many studies on the use of LNAs directed against miR-122; in African green monkeys, LNA-antagomiR-122 showed to reach the cytoplasm of hepatocytes and inhibit miR-122 reducing plasma cholesterol levels without toxicity (Elmèn J, 2008). In hepatitis C virus, miR-122 is necessary for the virus replication and its correspondent LNA was able to suppress viremia and improve HCV-induced liver pathology in chimpanzees (Lanford RE, 2010). The studies on this LNA-modified antisense oligonucleotide against miR-122, allowed Santaris to start the first clinical trial on LNA-anti-miR in human, named Miravirsen. The results obtained from Phase I and II studies demonstrated that patients had a dose-dependent reduction in HCV levels and no adverse side effects (Janssen HL, 2013).

But, as deductible, since microRNAs can influence various pathways, the long term effects of antagomiRNAs could represent a limit. For example, miR-122 is also a tumor suppressor and its downregulation is associated with reduced patient survival (Wang G, 2014). Thus, anti-miRNA approach has to consider that the effects could have bad consequence in other pathways. Other obstacles in the antisense nucleotides therapy could be the low cellular uptake, keeping the treatments in the liver and the delivery across the

blood-brain barrier.

A good alternative to antagomiRs, are miRNA sponges. They were developed by Ebert MS et al. (2007) modifying an mRNA with multiple tandem binding sites for an endogenous miRNA with which it can stably interact and prevent the association with its endogenous targets. The sponge's binding sites are specific to the miRNA's seed region, thus a single sponge can block all miRNA family members that contain the same seed sequence (Ebert MS, 2010). An example for this class of anti-miRNA treatment is represented from a lentivirus-mediated sponge against miR-23b in glioblastoma, which showed to reduce angiogenesis, migration and invasion of GBM cells (Chen L, 2014).

On the other hand, miRNA replacement therapy has the purpose to restore or amplify a loss of function, such as a tumor suppressor activity. They are synthetic miRNA mimics, formed by double-stranded RNA molecules that have identical sequences to their naturally occurring equivalents. For instance, in a recent study, U251 and U87 GBM cells were transiently transfected with miR-22 mimic (with lipofectamine) using, as antisense oligonucleotides, the miR-22 stem loop primer for complementary DNA; the upregulation of the miRNA inhibited the expression of epidermal growth factor receptor (EGFR), matrix metallopeptidase 9 (MMP9) and, particularly, miR-22 mimics decreased expression of SIRT1 protein, demonstrated to be a novel target of this microRNA. The final effect has been the inhibition of cell proliferation, migration, and invasion of both the glioma cell lines (Chen H, 2016).

Interestingly, a Phase I study on miRNA mimics exists already. MiRNA Therapeutics has conducted a clinical trial on miR-34 with a liposome-formulated mimic of miR-34, MRX34, on unresectable primary liver cancer or advanced metastatic cancer with liver involvement. However, the study has been terminated before its ending because of five immune related serious adverse events (clinicaltrials.gov).

As mentioned, one of the obstacle in miRNA therapy is the delivery of the molecule; therefore, there are many strategies being investigated: nanoparticles, adenovirus-associated vectors, mesenchimal stem cells.

Lee HK et al. (2013) used mesenchymal stem cells (MSCs) to deliver miR-124 and miR-145 mimics; they have been shown to migrate to experimental glioma, via gap junction-dependent and independent processes, and to exert anti-tumor effects by binding their targets SCP-1 and Sox2. The results of this study suggests that MSCs can deliver synthetic exogenous miRNA mimics both to glioma cells *in vitro* and *in vivo*.

Xue HY et al. (2016) developed a strategy to deliver miR-375 into hepatocellular carcinoma cells by assembling miR-375 mimics on the surface of gold nanoparticles. They showed that in this way there is a good cellular uptake and release of intact miR-375 that expressed its tumor suppressor activity. Moreover, gold nanoparticles demonstrated to be biocompatible, non-toxic, easily uptaken and they protect miRNA from degradation, suggesting that they could be an attractive platform for nucleic acid delivery in cancer treatment.

All these findings clarify the importance of microRNAs not only in the pathogenesis, but also in the possibility of treatment from diseases such as cancer. Obviously, the application of miRNA-based treatments is still far and needs more studies. The delivery is a big obstacle, because it has to be efficient and specific; just think of glioblastoma where the drug has to cross the blood-brain barrier to be accumulated in the brain tumor.

To guarantee their specificity is also not easy, since a single microRNA can target more than one mRNA affecting various pathways, and the risk is to obtain not only the desired effect, but also other effects that could be deleterious. Moreover, tumors often respond differently to the same therapy, thus there is the need of more individualized treatments. MicroRNA profiling could be used as a critical factor for evaluating response to treatment and also to realize personalized treatments.

3. PROGRAMMED CELL DEATH

Each multicellular organism is characterized by continuous mitotic events that lead to the generation of new cells from progenitor cells and fundamental is the balance between procreation and elimination of cells themselves.

Programmed cell death (PCD) is a normal physiological process and it is possible to distinguish three different types of programmed cell deaths: apoptosis, autophagy and necrosis (Figure 14). Recently, it was identified another type of PCD, necroptosis, which is defined as a regulated form of necrotic cell death (Dillon CP, 2016).

Kerr JF, in 1972, explained that apoptosis is a process occurring spontaneously or from external stimuli to the cell, causing structural changes, such as nuclear and cytoplasmic condensation. Apoptosis regulates both the maintenance of adult tissues and the embryonic development. For example, the differentiation of fingers and toes in a developing human embryo occurs because cells between the fingers apoptose and the result is that the digits are separate. In adults, programmed cell death balances the turnover of lots of tissues such as the bone marrow and intestine, where billions of cells die every hour. Moreover, whether some cells became damaged or abnormal and potentially dangerous, through PCD activation they can be eliminated. Different types of insults can lead to apoptosis such as virus, thus preventing the production of new virus particles and limiting spread of the virus in the host organism, or DNA damage carrying mutation that might develop to cancer (Dexter RM, 1995). For these reasons failure in the normal process of PCD results in various human diseases such as cancer, immunological and developmental disorders and neurodegenerative diseases (Fuchs Y, 2011).

Autophagy, also called programmed cell death type II to distinguish from the type I apoptosis, is the process that incorporates the components of the cell into lysosomes to be degraded. This is an important process involved in cell turnover in order to eliminate all the damaged or pathogen organelles. Unlike necrosis, autophagy is a cellular mechanism that can trigger physiologically leading advantages to the cell. Nonetheless, increasing evidence reveals that autophagic dysfunction is associated with human diseases, such as cancer (Lin L, 2015).

Necrosis is an uncontrolled and passive process that can be mediated by direct damage to cell membrane or interference with the energy supply; it results from a variety of accidental and severe insults (toxin, physical stimuli, ischemia). Even if it shares with

apoptosis some biochemical networks, this process causes a rapid and uncontrolled swelling of the cell that result in the cell explosion with the consequent release of its intracellular content into the extracellular environment (Elmore S, 2007).

Necroptosis is a programmed necrotic cell death which is thought to be induced by apoptotic death stimuli, such as TNF-α and Fas ligand; it seems to have a pathway caspase-independent and shares some biological aspects with both apoptosis and necrosis (Dasgupta A, 2016).

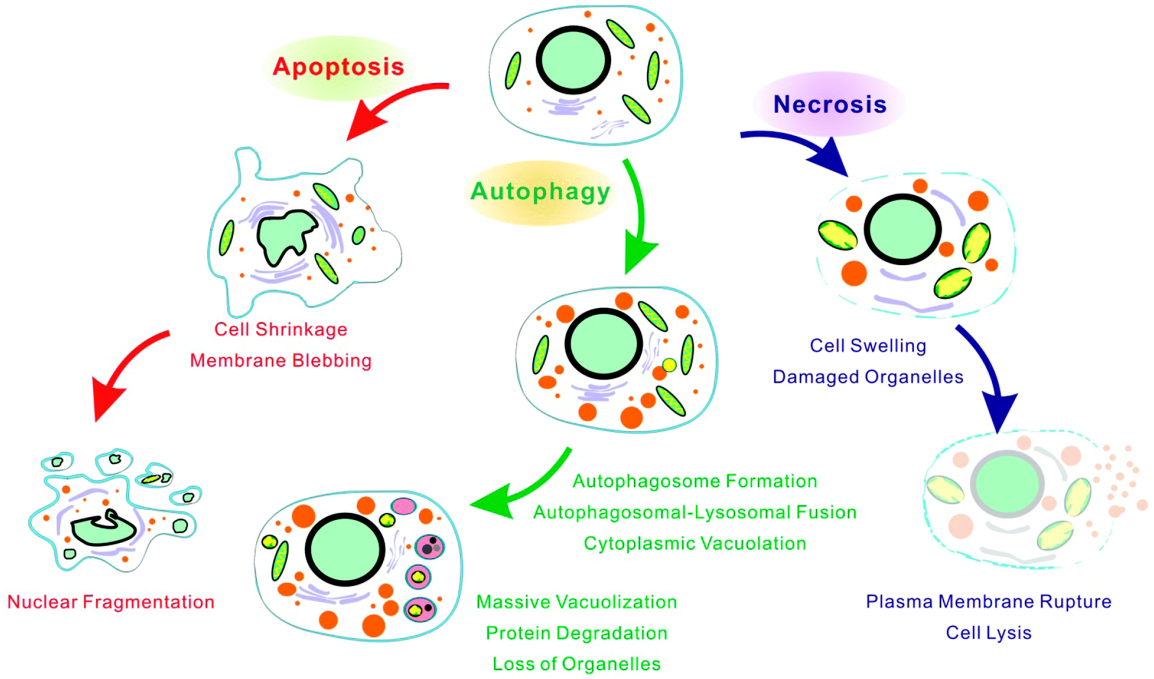

Figure 14. The three major pathways of cell death. Cells can be directed to different programmed cell death mechanisms depending on several factors. In the left, the apoptosis pathway is represented with the characteristic cellular shrinkage and formation of the apoptotic bodies without leakage of contents. In the middle, autophagy is illustrated with the appearance of vacuoles, the autophagosome, and its fusion with the lysosome, which ends in organelle digestion. In the right, the necrotic pathway shows the cytosol and organelle swelling and rupture of plasma membrane with subsequent leakage of cellular contents

3.1 Apoptosis

Apoptosis, as a programmed cell death (PCD), is essential for normal cell mechanisms. The word “Apoptosis” derives from Greek language “απόπτωσις” and means "falling off”, like leaves from a tree (Duque-Parra JE, 2005). A cell that is undergoing apoptosis is easily distinguished morphologically if compared to a healthy one. The apoptotic cell is characterized by a spherical shape due to the digestion of proteins forming the cytoskeleton and by a reduced volume caused instead by the condensation of the cell itself, which leads

to the expulsion of the water. A subsequent change within the cell is DNA fragmentation. The fragmentation occurs in two stages, firstly the DNA is broken into large fragments and subsequently these pieces are reduced into small fragments of a nucleosome size. In addition, the cell has a marked chromatin condensation, which gives origin to the pyknotic nuclei. The next morphological change is given by the disgregation of the cell into small vesicles, the apoptotic bodies, through a process called blebbing. These vesicles then are absorbed and destroyed by the surrounding phagocytes, leaving no trace of the cell (Kerr JF, 1972). Along with morphological alteration, the apoptotic process also involves biochemical changes which can be classified in three major categories including activation of caspases, DNA protein breakdown and membrane changes leading to the phagocytic cells recognition (Elmore S, 2007).

The mechanisms of apoptosis are very complex and involve a cascade of energy-requiring molecular events. Past research indicates that there are two main apoptotic pathways: the extrinsic or death receptor pathway, and the intrinsic or mitochondrial pathway (Goldar S, 2015). An additional pathway also operates during T-cell-mediated cytotoxicity involving perforin-granzyme-mediated cell killing. The perforin/granzyme pathway can induce apoptosis via either granzyme B or granzyme A (Trapani JA, 2002). All the three pathways then converge on the same terminal execution phase, which is initiated by the cleavage and activation of effector caspase-3 and -7, resulting in the morphological changes explained above (degradation of cytoplasmic, cytoskeletal and nuclear proteins, fragmentation of nuclear DNA, membrane blebbing, expression of cell-surface ligands for phagocytosis, and finally uptake by phagocytic cells).

Understanding the mechanisms of apoptosis is crucial and helps in understanding whether disordered apoptosis is behind the pathogenic condition. This in turn, may help in the development of drugs that target certain apoptotic genes or pathways.

3.1.1 The extrinsic death receptor pathway

As suggested by its name, the extrinsic pathway begins with the attachment of extracellular ligands, for example, tumor necrosis factor (TNF), Fas ligand (Fas-L), and TNF-related apoptosis-inducing ligand (TRAIL) to the extracellular domain of their transmembrane death receptors (Jin Z, 2005). The death domain transmits the death signal from the surface to the intracellular signaling pathway; the receptor–ligand binding leads to the recruitment of the procaspase-8 enzyme to the death inducing signaling complex (DISC) and

consequently activation of pro-caspase 8 to caspase-8, an initiator caspase starting apoptosis by cleavage of other downstream or executioner caspases (Jin Z, 2005).

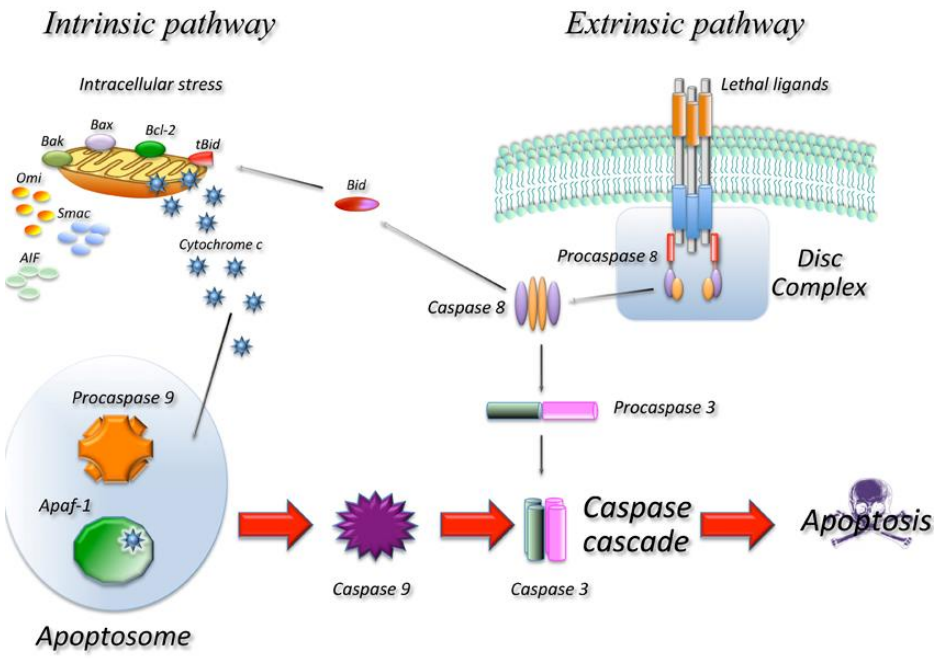

Figure 15. Schematic representation of the main molecular pathways leading to apoptosis: intrinsic and extrinsic pathway (Favaloro B, 2012).

3.1.2 The intrinsic mitochondrial apoptosis pathway

As its name implies, the intrinsic pathway is initiated within the cell as a consequence of intracellular stimuli such as DNA damage, growth factor depletion, hypoxia, accumulation of unfolded proteins, cytotoxic drugs or severe oxidative stress. This apoptotic pathway is also defined mitochondrial apoptosis; in fact, cellular stressors cause change in the outer mitochondrial membrane permeability and the release into the cytosol of pro-apoptotic molecules as cytochrome-c (Danial NN, 2004). Regulation of the intrinsic pathway is strictly controlled by a group of proteins belonging to the Bcl-2 family. All the Bcl-2 members are situated on the outer layer of the mitochondrial membrane as dimers; they control the permeability of the membrane by acting as ion channels or by creating pores. Bcl-2 proteins can be divided between pro-apoptotic proteins (Bad, Bax, Bak and Bim) and anti-apoptotic proteins (Bcl-2, Bcl-xl and Bcl-w) which act, respectively, by promoting or inhibiting the release of cytochrome-c (Wong RS, 2011). Other apoptotic factors are released by mitochondria including apoptosis inducing factor (AIF), second mitochondria-

derived activator of caspase (Smac) and direct IAP Binding protein with Low pI (DIABLO). Subsequently, in the cytosol, cytochrome c interacts with apoptosis protease-activating factor 1 (Apaf-1) and forms a complex recognized as the apoptosome (Goldar S, 2015). Apoptosome leads to activation of initiator caspase (usually caspase-9), which in turn activates executioner caspase-3 and initiates a caspase cascade, which eventually leads to demolition of the cell (Jin Z, 2005). Also Smac/DIABLO promote apoptosis through caspase activation, by negatively modulating the inhibitors of apoptosis proteins (IAPs) (Du C, 2000).

3.2 Caspases

Caspases are key players in the mechanism of apoptosis as they are both the initiators and executioners. They are protagonists in both intrinsic and extrinsic pathway of apoptosis.

Caspases are recognized as protease enzymes that have the ability to hydrolyze proteins at the level of specific amino acids. Caspases present a cysteine in their catalytic site and are able to break selectively the peptide bond at the aspartate residues. In humans, were found 11 different caspases, each with a different action: 6 are involved in the apoptotic process, whereas the other 5 have the function of cytokine activation.

Caspases involved in apoptosis can be distinguished in initiator caspases (caspase-2, 8, 9, 10) and in effector caspases (caspase-3, 6, 7).

The initiator caspases are found in the cytoplasm in the form of pro-caspase and are the inactive form that have a pro-domain. They are located upstream of the apoptotic process and can be turned into dimers through the recognition of their domains from specific molecules. Internal signals that come from the way of mitochondrial apoptosis, recognize the CARD (Caspase activation and recruitment domain) domain of the initiators, while DED domains are activated by other molecules that derive from external signals. This type of caspases plays a mediating role in the process of programmed cell death, as it is capable of activating other caspases that can be initiators or effector, without causing directly the apoptotic effect.

Also the effector caspases are in the cytoplasm in an inactive form, in their whole structure. These caspases are activated by other initiators caspases through proteolytic cleavage of their structure causing the effect of apoptosis. Being the main process when enzymatic cascade of caspase is initiated, a positive feedback ensures that the apoptosis is successful (Pop C, 2009).

Caspase-3 is the best executor caspase thanks to its ability to cleavage a large number of nuclear proteins associated with apoptosis, like cytochrome c/dATP-inducible cleavage of fodrin, DNA fragmentation factor 45 (DFF45)/inhibitor of caspase-activated DNase (ICAD), receptor-interacting protein (RIP), X-linked inhibitor of apoptosis protein (X-IAP), signal transducer and activator of transcription-1 (STAT1), but not for cleavage of poly (ADP-ribose) polymerase (PARP) or lamin A. Instead, effector caspases 6 and 7 are not considered very important in the last stages of the apoptotic process, because their reduction does not cause severe impacts on the process itself (Slee EA, 2001).

The various pathways demonstrated that the caspase's cascade are triggered during the programmed cell death making possible the inactivation of target molecules involved in peculiar processes to the cell, through a complicated series of enzymatic reactions that result in the death of the cell.

3.3 Phosphatidylserine (PS)

An event occurring in early stages of apoptosis is the exposure of the phosphatidylserine protein from the inner surface of the cell membrane, where it is usually maintained, by an aminophospholipid translocase to the outer layers of the membrane. This is one of the changes occurring in phospholipid distribution which take place during the apoptotic process. In particular, the phosphatidylserine translocation is a marker for the early recognition of dead cell by macrophages, which results in phagocytosis without release of pro-inflammatory cellular components. Therefore, PS is a good candidate for being an 'eat me' signal; this specific marker of apoptosis can readily be detected through its affinity for Annexin V (Figure 16) in the presence of calcium ions, representing the basis of a common staining assay for recognition of apoptotic cells (Hengartner MO, 2001).

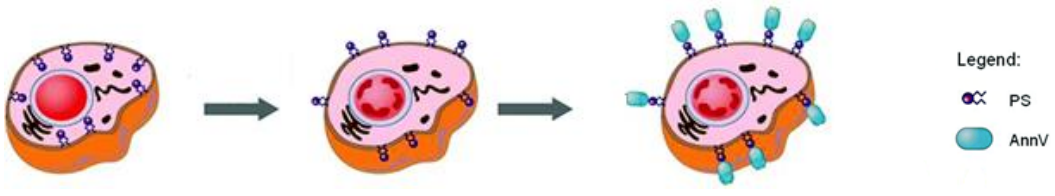

Figure 16. Externalization of phosphatidylserine from apoptotic cell and binding with Annexin V.

3.4 The role of apoptosis in cancer

In cancer, there is a loss of balance between cell division and cell death and, since apoptosis is a biological phenomenon involved in maintaining tissue homeostasis, it is not surprising that alterations of apoptosis play an important role in cancer development. Evasion of cell death is one of the essential changes in a cell that is transformed in a malignant one. Apoptotic pathways can be altered by cancer cells transcriptionally, translationally, and post translationally (Goldar S, 2015). As reported by Wong RS et al. (2011), apoptotic evasion can be acquired in many ways including impaired balance of pro-apoptotic and anti-apoptotic proteins, reduced caspase functionality or altered signalling of death receptors.

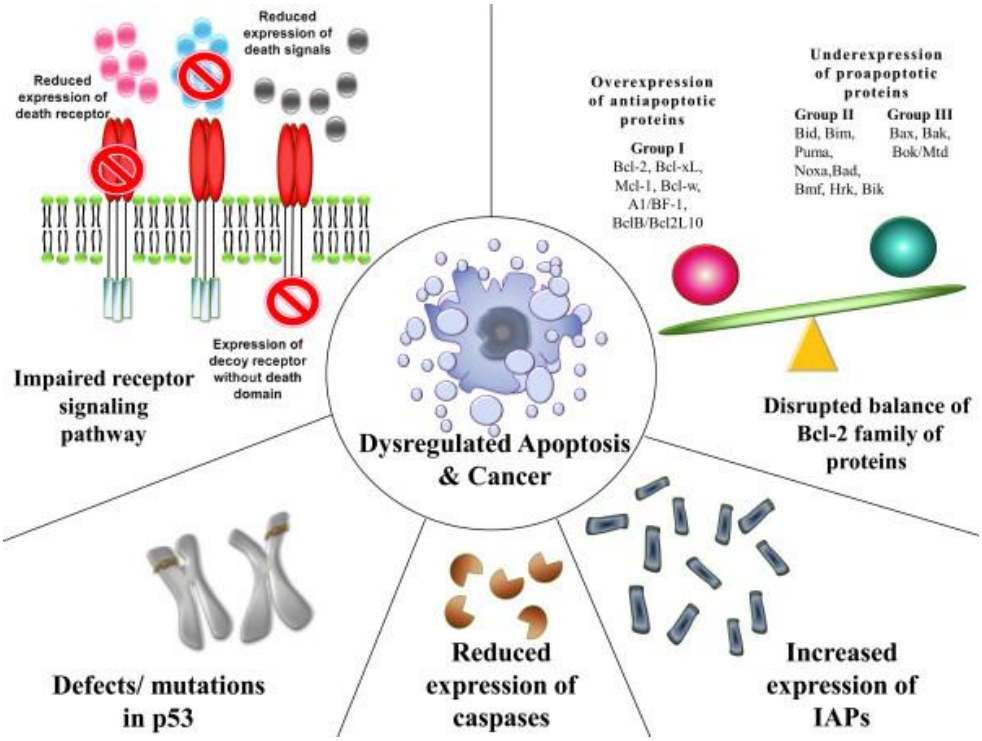

Figure 17. The involvement of apoptosis in cancer onset. Alteration occurring at different levels can led to dysregulated apoptosis and subsequent cell transformation (Wong RS, 2011).

The imbalance of pro- and anti-apoptotic proteins can derive from downregulation of some pro-apoptotic proteins or overexpression of the anti-apoptotic ones or even the combination of both. The impaired expression of pro-apoptotic players such as p53 protein, the so called ‘guardian of genome’, plays a central role in many tumors. In fact, since it is involved in the control of cell cycle, gene amplification, DNA recombination and

senescence, it was found that p53 alteration is associated with more than 50% of human cancers and it has been identified as one of the most targetable molecules for developing anticancer treatments (Kim SH, 2011).

Important for cancer cell survival is the disequilibrium between proteins of the BCL-2 family. For example, over-expression of anti-apoptotic Bcl-xL protein was correlated with poor prognosis and resistance to cancer therapeutics (Minn AJ, 1995); Bcl-2 overexpression has been reported to led to inhibition of TRAIL-induced apoptosis in neuroblastoma, glioblastoma and breast carcinoma cells (Fulda S, 2002). Another dysregulated factor can be abnormal expression of IAPs which are endogenous inhibitors of caspases, triggering their degradation and counteracting their activity.

Among the changes conferring apoptosis evasion, many evidences exist proving the involvement of miRNAs in the alteration of the apoptotic process which is a key step in miRNA-mediated promotion of cancer onset and drug resistance (Figure 18). MiRNAs have been established to act as anti-apoptotic or pro-apoptotic regulators by targeting different mRNAs involved in the apoptotic pathways.

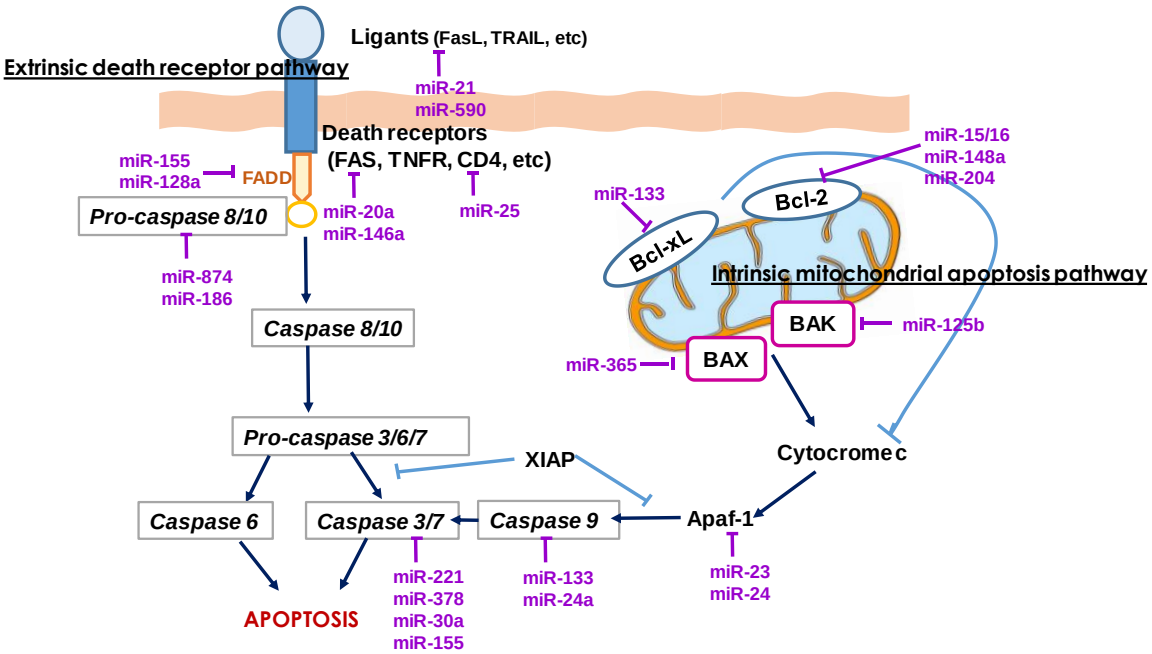

Figure 18. Genes and some miRNAs involved in the regulation of the intrinsic and extrinsic apoptosis pathway.

MiR-15 and miR-16 negatively regulate Bcl-2 and they are frequently deleted or down-regulated in the majority of chronic lymphocytic leukemias (CLL). Transfection of miR-15 and miR-16 as a cluster in a leukemic cell line model, reduced Bcl-2 expression and resulted in the activation of the intrinsic apoptosis pathway (Cimmino A, 2005). Important in cancer is also the role of the oncomiR-221, which is upregulated in many cancers. In human glioma cells, apoptosis is blocked by targeted inhibition of pro-apototic PUMA gene by miR-221 (Zhang CZ, 2010); moreover, it targets caspase-3 repressing its expression and contributing to a lower apoptotic rate in different cancer cell lines, thus supporting the selection of more aggressive cancer cells (Ergun S, 2014). Another example of microRNA active in the apoptotic pathway is miR-155; in activated macrophages the expression of miR-155 was associated to reduction of caspase-3 mRNA and suppression of apoptosis (De Santis R, 2016).

In this context the antisense therapy could represent a potential strategy aiming to the re-sensitization of cancer cells toward the programmed cell death.

3.5 The role of NF-κB

The nuclear factor κB (NF-κB) comprises a family of transcription factors involved in the regulation of a wide variety of biological processes. Well-known is its role in the regulation of immune responses and inflammation, but there are many evidences that underline its role also in oncogenesis (Di Donato JA, 2012; Dolcet X, 2005; Bours V, 2000).

NF-κB is an ubiquitously expressed transcription factor sequestered in the cytoplasm of many cell types by inhibitory proteins named IκB. In response to a large variety of stimuli (i.e. pro-inflammatory cytokines, infectious agents, pro-apoptotic stimuli), the IκB inhibitor is rapidly phosphorylated and degraded, thus allowing NF-κB nuclear translocation, DNA binding to specific recognition sequences in promoters, and transcription of the target genes (Karin M, 1999). The nature of NF-κB activating stimuli reflects its main functions.

NF-κB also regulates the expression of genes involved in important processes that have a key role in the development and progression of cancer such as proliferation, migration and apoptosis. Pro-apoptotic and pro-inflammatory stimuli, oxidative stress, cytotoxic drugs and ionizing radiations can activate NF-κB (Hellweg CE, 2015; Das KC, 1997).

NF-κB may activate the transcription of several genes involved in the suppression of cell

death by both intrinsic and extrinsic pathways (Figure 19). Essentially, NF-κB has an anti-apoptotic role since it may target anti-apoptotic genes coding for TNF receptor-associated factor (TRAF 1 and TRAF 2), cIAPs, A20, IEX-1L, Bfl-1/A1, Bcl-xL and it also interferes with p53 transcriptional activity (Bours V, 2000).

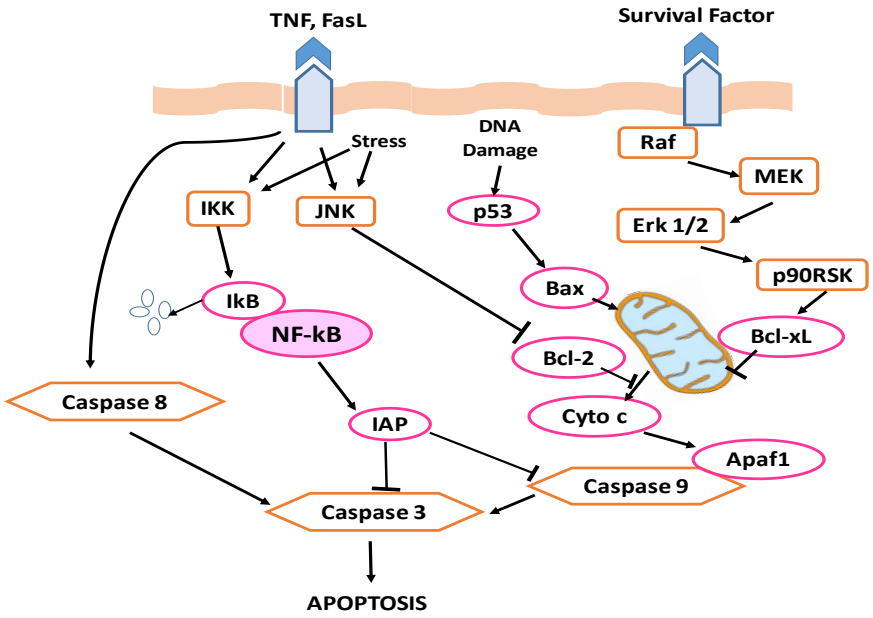

Figure 19. The NF-κB cascade and its interaction with pro- and anti- apoptotic pathways.

For instance, inducing the expression of the Inhibitors of Apoptosis (IAPs), resulted in the suppression of apoptosis by direct inhibition of effector caspases (caspases 3, 6, 7, 9); NF-κB is frequently observed to be constitutively activated in various neoplasms such as breast, prostate and ovarian cancer and it participates in aggressive and resistant phenotypes; even in glioblastoma onset and progression it plays an important role, and it was found to contribute to the resistance to alkylating agents through abrogating the apoptotic signal initiated by genotoxic stress (Bredel M, 2006).

3.6 Targeting apoptosis in glioblastoma

In recent years, a heightened interest has been shown to understand underlying mechanisms of apoptosis to strategize therapeutic interventions.

Modifications in the apoptotic pathways may significantly contribute to gliomagenesis and also to the development of chemoresistance. Many studies were conducted on the alterations in the molecular pathways of apoptosis in glioblastoma cell to render these cells

more susceptible towards therapy-induced apoptosis. The main effort is to overcome the drug-resistance that very often increases the threshold of therapeutic efficacy.

As in other pathological conditions, in glioblastoma several key regulatory elements of apoptosis are altered, including the p53 protein, the BCL-2 protein family, the inhibitor of apoptosis proteins (IAPs) or receptor tyrosine kinases like the epidermal growth factor receptor (EGFR), and these represent potential factors on which it could be possible to act therapeutically (Eisele G, 2013).

For example, compound p53R3 demonstrated to enforce DNA binding of mutant or wild-type p53 restoring its pro-apoptotic function and it enhanced the cell surface expression of receptors for TNF-related apoptosis-inducing ligand (TRAIL) sensitizing glioma cells to TRAIL-induced apoptosis (Weinmann L, 2008). Gossypol, a polyphenolic compound derived from the cotton plant, showed to bind several members of BCL-2 anti-apoptotic protein family, inducing autophagic cell death and cytochrome c release (Voss V, 2010); the results were so interesting that a phase I study (NCT00390403, data await publication) investigated the effects of gossypol when administered with temozolomide with or without radiation therapy in glioblastoma patients.

Very recently it was found that icaritin, a flavonoid extracted from *Epimedi herba*, significantly induced both caspase-dependent apoptosis and autophagy in human U87 GBM cell line (Li Z, 2016). Also the combination of different drugs is continuously studied with the aim to potentiate the anti-tumor effects or sensitize the chemoresistant cells; Wang Y et al. (2015) showed that the flavonoid hispidulin enhanced the anti-tumor activity of TMZ in glioblastoma by both inhibiting cell proliferation and inducing cell apoptosis, probably by downregulating Bcl-2 protein. Also the combination of radiotherapy and chloroquine seems to be interesting, in fact it enhanced the U87 glioma initiating cells apoptosis, as demonstrated by the enhanced levels of caspase-3 and reduced levels of Bcl-2 (Ye H, 2016).

As already discussed, microRNA exert an important role in the modulation of apoptosis and they represent interesting potential targets for novel therapeutic strategies. Chan et al (2005) found that knock-down of miR-21 in cultured glioblastoma cells triggered activation of caspases and lead to increased apoptotic cell death. Downregulation of miR-21 also inhibited cell growth *in vivo* and displayed synergism with s-TRAIL towards cytotoxicity, leading to caspase-activity increase (Corsten MF, 2007). Accordingly, U251 cells treated with antisense-miR-21 showed a decreased expression of Bcl-2 and activation

of caspase 3 and 9 (Zhou X, 2010).

Silencing of miR-155, which is overexpressed and involved in chemoresistance in glioblastoma, enhanced the chemosensitivity to taxol treatment in human U251 glioma cells inducing apoptosis activation (Chen L, 2012). Similarly, anti-miR-221/222 enhanced temozolomide effect in human glioma cells inducing expression of BAX, Apaf-1 and cleaved-caspase-3; moreover, BCL-2 was found to be downregulated and p27, CASP-3, PTEN and TIMP3 upregulated after antisense targeting of miR-221/222 (Zhang CZ, 2009).

Researches in the scientific literature and in TargetScan allowed us to select miR-155 and miR-221 as the most interesting miRs targeting caspase-3 and overexpressed in glioblastoma (Figure 51 in Results section), as potential targets for an apoptosis-inducing antisense therapy.

In recent years, numerous studies have focused on the role of NF-κB in cancer and apoptosis, and in its targeting as a possible treatment of cancer.

Zanotto-Filho A et al. (2011) observed that genetic silencing, using siRNA to knockdown NF-κB -p65, or pharmaceutical inhibition of the NF-κB pathway efficiently restores the chemosensitivity of glioma cells and induces their apoptosis.

A recent study showed that saponin 1, a triterpenoid saponin extracted from *Anemone taipaiensis*, induces apoptosis of glioblastoma cells by the down-regulation and inactivation of NF-κB and by the increase of the cellular content of pro-apoptotic Bax protein which led to the activation of caspase-9 and caspase-3 (Li J, 2013). This link between inhibition of NF-κB and induction of apoptosis was also demonstrated by Kiekow's study in which a quercetin derivative (a flavonoid) demonstrated a powerful activity against glioma cells by inhibiting the nuclear translocation of NF-κB and by activating the caspase-3-dependent apoptotic pathway (Kiekow CJ, 2016).

4. NATURAL PRODUCTS AS POTENTIAL DRUGS

Throughout human evolution, natural products have exercised an enormous impact on medicine and health. For thousands of years compounds derived from natural sources such as plants, animals or micro-organisms constituted the only treatment for diseases and injuries. The oldest medical text (2600 BC) came from Mesopotamia and described about 1000 plants; furthermore, in Egypt, the *Ebers Papyru (*1550 BC), listed more than 700 natural agents and about 800 prescriptions; in addition, the *Corpus Hippocraticum* (Hippocrates, 460-377 BC) described more than 400 natural agents along with their uses. Moreover, the Traditional Chinese Medicine, which still find its application in today's medicine, provides a lot of information about natural products and their uses.

Despite the wide use of medicinal plants in both Orient and Occident, these plants effective active components remained unknown until the eighteenth and nineteenth centuries. It is around this time (1805) that the German pharmacist Friedrich Wilhelm Sertürner isolated morphine from opium; this is the first pure naturally-derived medicine and the first compound to be commercialized by Merck (1826). On this knowledge, the field started to prefer compound synthesis over their isolation from natural sources. In fact, a large number of well-known natural compounds were identified, analyzed and synthesized, allowing chemists to reduce the cost of drug production (Ji HF, 2009).

The study of the structures of natural compounds allowed chemists to modify them by suppressing, or enhancing, particular characteristics such as stability, solubility or efficiency; this led to the creation of many compounds over the years.

The past few years have seen a renewed interest in the use of natural compounds. Many factors interplay in the pathogenesis of many diseases and the use of a selective compound against a single target often fails to yield the desired effect; thus there is a greater need to identify combined treatments, particularly in cancer therapy. Combined treatments strategy could bring many advantages such as preventing or reducing drug resistance, along with increasing the effect of the main treatment.

On this evidence, researchers' focus moved towards naturally occurring bioactive compounds, and particularly on the more traditional uses of plants and the analysis of the effects of extracts obtained from medicinal plants; the latter, is one of the most interesting approaches for the identification of these compounds. For example, quercetin, a flavonoid present in many fruits and vegetables, can interact directly with several ABC transporter

proteins to inhibit drug efflux; further, it has a chemosensitizer activity that enhances the cytotoxic effects of chemotherapeutic drugs on many drug resistant cells. Lv and colleagues (2016) demonstrated that nanoparticles encapsulating chemotherapeutic doxorubicin and chemosensitizer quercetin can increase the intracellular concentration of doxorubicin and reduce its resistance in MCF-7/ADR breast cancer cells. Moreover, anthocyanins are flavonoids which show different beneficial properties such as reverse multidrug resistance by targeting ABC transmembrane protein and, in colon cancer cells they have been shown to induce apoptosis by activating caspase-3 (Lin BW, 2016). Essential oils extracted from fennel, cumin and clove have antioxidant property and were found to alleviate cyclophosphamide-induced hepatotoxicity in cancer treatment in mice (Sheweita SA, 2016).

In conclusion, the support from nature could be very interesting and efficient for new therapeutic strategies. There are many plants and natural compounds that continue to be studied for their promising activities, one of these is corilagin which is the object of this thesis.

4.1 CORILAGIN

Corilagin, β-1-O-galloyl-3,6-(R)-hexahydroxydiphenoyl-D-glucose ($C_{27}H_{22}O_{18}$), is a polyphenol found in the herbal preparations of the Traditional Chinese Medicine (Figure 20).

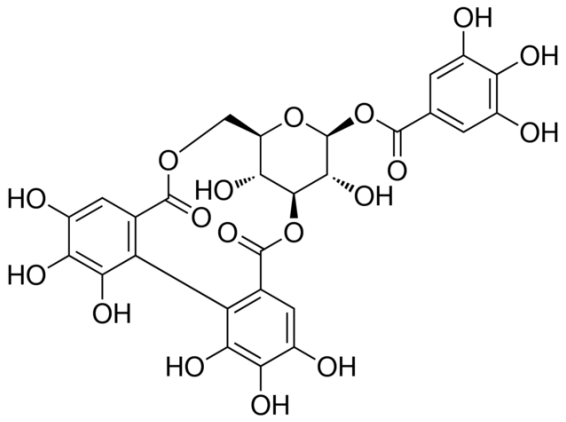

Figure 20. Corilagin chemical structure.

Corilagin was isolated in 1951 from the pods of the legume Caesaplina coriaria (Dividivi), hence the name of the molecule (Schmidt OT, 1951). Corilagin is a hydrolyzable tannin with a molecular weight of 634,45 g/mol, consisting of one glucose unit esterified with single hexahydroxydiphenic acid and gallic acid moieties.

Several plants contain gallotannin corilagin: *Terminalia chebula* and *T. catappa* (Kinoshita S, 2007), *Anogeissus latifolia (Combretaceae), Acer Spp. (Aceraceae), Sapium japonicum, Ricinus communis, Aleurites cordata (Euphorbiaceae)* (Gambari R, 2012*), Dimocarpus longan (Sapindaceae)*; particularly rich in this compound is the family of *Phillanthaceae* and most of all *Phillanthus urinaria, P. niruri* (Zheng ZZ, 2016). and *P. eblica*. (Mao X, 2016) (Figure 21).

Figure 21. *Phyllanthus* species containing corilagin.

All the three above mentioned species have Asian origin but are widely found in all tropical and subtropical regions of the world including America, China and India.

There are more than 1000 species in the genus *Phyllanthus* and many of them are used as traditional medicines. The plant extracts have been used since ancient times, for treating hepatic, urinary and sexual disorders as well as hypertension, diabetes, and other common ailments (Sarin B, 2014). Modern day scientific investigations have confirmed pharmacognostic properties of *Phyllanthus* herbs. In the traditional Ayurvedic medicine, *P. niruri* has been used for the treatment of gallbladder and kidney stones, viral infections, fever, diabetes, liver and gastrointestinal diseases. (Zheng ZZ, 2016). *P. emblica* fruits (*Fructus Phyllanthi*) are historically used in China for the treatment of liver and digestive diseases (Mao X, 2016); the boiled water extracts from the whole plant of *P. urinaria* has

been reported to induce apoptosis in human cancer cell lines and it has anti-angiogenic effects in mice with lung carcinoma (Hau DKP, 2009).
In recent years the interest toward corilagin has grown and, as emerged from the current literature, many properties have been attributed to it.

4.2 Corilagin properties

Antioxidant activity. The oxidative stress is one of the main characteristic of Alzheimer's disease; the accumulation of free radicals leads to excessive lipid peroxidation and neuronal degeneration in certain brain regions. Corilagin extracted from *P. ussufiensis,* showed antioxidant activity similar, or even higher, to positive controls, like BHA (butylhydroxyl anisol) and α-tocopherol, on several reactive oxygen species such as hydrogen peroxide, DPPH, hydroxyl radicals and superoxide anion (Chung SK, 2003).
Reactive oxygen species (ROS)-mediated stress in microglia *in vivo* could result in neuronal injury. In a study on tert-butyl hydroperoxide (TBHP)-induced injury in cultured N9 murine microglial cells, corilagin has been shown to attenuate this oxidative stress and its protective effects may be ascribed to its antioxidant and antiapoptotic properties (Chen Y, 2011).
Moreover, studies have demonstrated that corilagin has also Nitric Oxide (NO) scavenging activity (Kumaran A, 2006; Yuandani, 2016); this is of interest as during infections and inflammations, formation of NO is elevated and may lead to some undesired effects like renal dysfunction and tumor growth.

Anti-inflammatory activity. In the scientific literature we can find many publications about the role of corilagin on inflammation and, probably, its anti-inflammatory activity could be related to its ability to affect transcription factor nuclear factor κB (NF-κB) and the consequent release of pro-inflammatory cytokines (Zhao L, 2008).
YJ Guo and collegues (2010) studied the effect of corilagin in herpes simplex virus (HSV)-1 infected microglials, both *in vivo* and *in vitro,* showing a reduced release of cytokines and the induction of apoptosis via death receptor pathway, mitochondrially gated pathway and endoplasmic reticulum pathway; these results suggest a possible use of corilagin in viral encephalitis (Guo YJ, 2010).
EMSA (Electrophoretic Mobility Shift Assay) studies demonstrated that corilagin is active in inhibiting NF-kappaB/DNA interactions; 400 nM corilagin fully suppresses the

interactions between the p50/p65 heterodimer and target DNA (Figure 22A). This evidence constituted the starting point for Gambari's research which aimed to investigate the possible role of this gallotannin on inflammation in Cystic Fibrosis (CF). TNF-α was used to increase the expression of IL-8 (cytokine highly expressed in CF) in IB3-1 bronchial CF epithelial cells; 24 hours of treatment with corilagin showed significant reduction of the expression of IL-8 mRNA (38 ± 4.2 μM) (Figure 22B) and IL-8 protein (Figure 22C). without having cytotoxic effects on the cell viability (Gambari R, 2012).

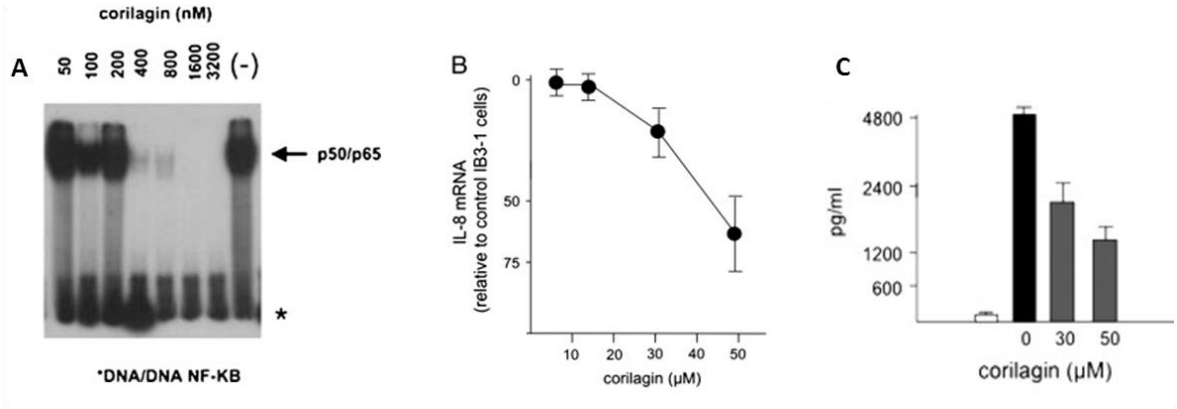

Figure 21. Corilagin effects on IB3-1 cells: (A) Inhibition of NF-kappaB/DNA interactions (EMSA). (B) Inhibition of mRNA IL-8 accumulation. (C) Inhibition of IL-8 secretion (Gambari R, 2012).

Corilagin showed protection against neuroinflammation caused by aggregation and deposition of beta-amyloid peptides (Aβ) in Alzheimer's disease: it reduced mediators such as tumor necrosis factor-α, nitric oxide, and prostaglandin E2; it further downregulated cyclooxygenase-2 and inducible nitric oxide synthase expression in PC12 cells. Further, in this study (Youn K, 2016), pretreatment with corilagin attenuated caspase-3 activity and suppressed the degradation of inhibitor of κB (IκB)-α and subsequent activation of NF-κB.

In a mouse model with radiation-induced brain injury (RIBI), corilagin inhibited microglial activation, downregulated the expression of inflammatory cytokines like TNF-α and IL-1β and inhibited the irradiation-induced activation of NF-κB pathways by upregulating p-STAT3 expression (Tong F, 2015). In rats with cholestasis associated oxidative stress and inflammation, corilagin improved their living conditions, decreased bilirubin level and, as demonstrated by immunohistochemistry, suppressesed NF-κB translocation (Jin F, 2013).

Hepatoprotective activity. *P. urinaria* extracts, particularly corilagin and gallic acid, may protect the hepatocytes from acetaminophen induced hepatotoxicity. This study was conducted on mice, administered intraperitoneally with a lethal dose of acetaminophen and an oral administration of the plant extract. This resulted in the reduction of the cytochrome P450 CYP2E1 protein level and enzymatic activity (Hau DKP, 2009).

Recently it was shown that corilagin exerts its protective effects on hemorrhagic shock-induced liver injury, via the Akt-dependent pathway: the hemorrhagic shock induced in male Sprague-Dawley rats has as response an increase of hepatic enzymes activity and of pro-inflammatory citokynes; these parameters were significantly attenuated in corilagin-treated rats and hepatic phospho-Akt expression was also higher than in vehicle-treated rats, confirming the hepatoprotective activity of corilagin also through this possible mechanism (Liu FC, 2016).

Antitumor activity. Looking at the literature of these last years, we can see an increase of the publications about the role of corilagin in cancer.

One of the first studies on this topic was conducted in 2010 from Fong's group; fifteen athymic nude mice were injected subcutaneously with the human hepatocellular carcinoma Hep3B cells and when tumour size reached a mean volume of about 200 mm^3 they were divided in 3 groups. They administrated corilagin intraperitoneally for a continuous period of 7 days at a concentration of 7.5 and 15 mg/kg body weight/day. The result was that in mice treated with 15 mg/kg, the tumor size was one-fourth of that found in the control vehicle group and all the mice survived after the treatment period (Hau DK, 2010).

Corilagin demonstrated clear inhibition also of ovarian cancer cell growth (the IC_{50} were 27 μM for SKOv3ip and 28 μM for Hey cells) with low toxicity against normal ovarian cells (the IC_{50} was 160 μM for OSE cells) and *in vivo* inhibition of the growth of SKOv3ip xenograft tumors. It was shown that in these cells corilagin induced cell cycle arrest at the G2/M stage and enhanced apoptosis; moreover, it inhibited TGF-β secretion into the culture supernatant of all tested ovarian cancer cell lines and blocked the TGF-β-induced stabilization of Snail (Figure 23A). Consequently, this influenced proteins involved in TGF-β pathway like p-AKT, p-ERK and pSmad2 associated to cell cycle regulation, cell migration anti-apoptotic activity. TGF-β plays an important role in ovarian cancer epithelial-mesenchymal transition (EMT) and metastasis, therefore, corilagin, with an unclear mechanism, blocking TGF-β, inhibits EMT and metastasis diffusion (Jia L, 2013).

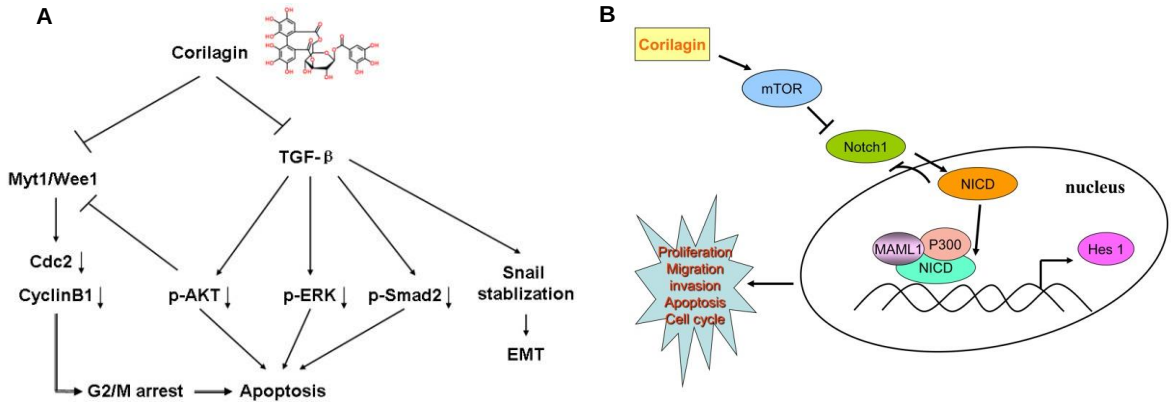

Figure 23. (A) Pathways potentially affected by corilagin through inhibition of TGF-β in ovarian cancer. (B) Molecular mechanisms by corilagin through Notch signaling pathway in the suppressesion of cholangiocarcinoma progression.

The arrest at the G2/M stage of cell cycle by corilagin (40 μM) was demonstrated also in hepatocellular carcinoma cell lines; a molecular explanation may be provided by western blotting results which showed that cdc2 and cyclin B1 expression, synthesized and accumulated to promote cell cycle progression from the G2 to M phase, was significantly decreased, and that $p21^{Cip1}$, a cyclin-dependent kinase inhibitor, was increased in a corilagin-dependent manner. Then it was observed morphological changes typical of apoptotic cells, infact corilagin was able to decrease p-Akt and increase p-p53, both involved in cell cycle arrest and apoptosis (Ming Y, 2013).

Very recently, Gu Y et al. (2016) confirmed the role of corilagin in inducing apoptosis (it inhibited the mRNA level of bcl-2 and promoted caspase 3 associated with apoptotic gene detection), reducing cell growth and arresting cell cycle at the G2/M stage in cholangiocarcinoma cells.

It is known that corilagin has several mechanisms in a tumor, such as MAPK, NF-κB and TGF-β1 signaling. Gu, in his research, studied the involvement of corilagin in the regulation of Notch - mTOR pathway. It is a signaling pathway evolutionarily conserved and plays an important role in cell fate determination, proliferation, differentiation and survival; both *in vitro* and *in vivo* corilagin demonstrated to repress the expression of Notch1 and mTOR (Figure 23B), suggesting another way through which the tannin may act (Gu Y, 2016).

Nuclear factor κB (NF-κB) is a nuclear transcription factor that regulates expression of a large number of genes that are critical for the regulation of apoptosis, viral replication, tumorigenesis, inflammation, and various autoimmune diseases. In glioma, high NF-κB expression is strongly correlated with rapid tumor progression and poor patient survival rates, thus inhibiting the expression of NF-κB could induce apoptosis of tumor. Corilagin induced apoptosis and inhibited the cell proliferation of U251 glioblastoma cells and their stem-like cells; as in other cell lines, it arrested dose dependently the cell cycle (in G2/M phase in U251 and in S phase in U251 stem-like cells) but, more interestingly, increasing corilagin concentrations increased the IKBα expression in cytoplasm of U251 cells and U251 stem-like cells, but the p65 expression in nucleus was decreased. Thereby the analyzed natural active ingredient, inhibits NF-κB signaling pathway and induces tumor cell apoptosis (Yang WT, 2016).

Other activities. In methicillin resistant *Staphilococcus aureus* corilagin, added to the colture medium, reduced the MIC of β-lactams by inhibiting activity of the penicillin binding protein 2 (PBP2a), one of the responsible of their resistance. Therefore, corilagin potentiated the activity of β-lactams (Shiota S, 2004), but it has also antibiotic effect against *E. coli* and *C. albicans* where dose-dependently disrupted their membrane permeability (Li N, 2013).

The protective activity of corilagin is further demonstrated at gastric level: it was shown that gastric lesion ethanol-induced in mice can be reduced, as well as with omeprazole treatment, with a protective effect higher than 80%; then it was evidenced a reduction of oxidative damage and neutrophil infiltration in gastric tissues from ulcerated mice after intraduodenally administration of methanol extract of *P. niruri* (Klein-Júnior LC, 2016).

Starting from the knowledge that in several ethno-medicines *P. niruri* is used also for disease associated to pain, in 2013, Moreira demonstrated the anti-nociceptive activity of corilagin in mouse models. The inhibition of NO production, pro-inflammatory mediators release and the interaction with the glutamatergic system, make corilagin a possible novel peripheral analgesic for the control of pain (Moreira J, 2013).

Corilagin has proved useful also in cigarette smoke (CS) induced alterations; CS is able to damage airway epithelium integrity by causing cellular junctions loss through oxidative damage and NF*κ*B activation. Calu-3 bronchial epithelial cells were used as model for this study, they were pretreated with corilagin and exposed to CS; the polyphenol not only

reduced NF-κB activation, as it is already known, but it was able to prevent the loss of Cx40 (protein present in cellular gap junctions), most likely thanks to its free radical quenching properties, and prevent the formation of Cx40-4HNE-protein adducts (Valacchi G, 2015). The inactivation of oxidative stress, proinflammatory cytokine release and NF-κB and TGF-β1 signaling due to corilagin has been revealed interesting for idiopathic pulmonary fibrosis too, since it attenuated bleomycin-induced epithelial injury and fibrosis (Wang Z, 2014).

As we could learn, corilagin has multiple beneficial activities. Although it seems a promising new drug, it needs of more detailed explanations on its bioavailability and permeability because it has relatively high hydrophilicity and large molecular weight; its chemical characteristics could represent a difficulty, for example, for crossing the blood-brain barrier. About that, Mao's group studied the transport of corilagin and its two hydrolysates gallic acid (GA) and ellagic acid (EA) through intestinal epithelial Caco-2 cell monolayer, demonstrating that the transport of corilagin is via passive diffusion combined with protein mediated transport. The Caco-2 cells were incubated at the concentration of 1.00 mg/ml from 30 minutes to 180 minutes and the transport percentages and transport rates of the compounds were increased in a nonlinear manner with the time and concentration; probably they may be transported across the Caco-2 cell monolayers through protein mediated pathways. There are various transporters expressed in the membranes such as P-gp, MRPs, OATP and SGLT1; incubation of corilagin, GA and EA with inhibitors of these proteins permitted to see that the transport of corilagin and GA were increased when they were incubated with verapamil and indomethacin suggesting that the possible transport pathway may be P-gp mediated for the absorption and MRPs mediated for the efflux in Caco-2 cells (Mao X, 2016).

As exposed above, in the medical practice, it is growing the use of combined treatments to reduce drug resistance, increase drug activity or to enhance chemosensitivity to a drug.
Corilagin has proved interesting in this sense; it showed to potentiate the activity of beta-lactams against methicillin-resistant *S. aureus* (Shimizu M, 2001; Shiota S, 2004) and in 2014, the collaboration between Gambari and Chui's group, demonstrated that corilagin sensitize Hep3B hepatoma cells to cisplatin and doxorubicin. Concentration of corilagin that has insignificant anti-proliferative effect on the Hep3B hepatoma cells, if added

simultaneously with various concentrations of either cisplatin or doxorubicin, could significantly potentiate the cytotoxicity of both chemotherapeutic drugs, reducing their IC_{50} values (Gambari R, 2014). This means that corilagin could be tested on other drug-resistant cells to investigate whether it would enhance the activity of the main treatment for that disease and, if the results would be positive also in *in vivo* studies, it could be an innovative development in the multi-drug resistance. For example, it would be very important in cancer treatment because, corilagin in combination with lower dosages of the anticancer chemotherapeutic standard drugs, could permit to obtain an increment in the anticancer effect reducing side effects associated to high dosage and drug-resistance.

5. BIOMOLECULES FOR miRNA THERAPEUTIC: PNAs

The development of biomolecules for the control of miRNA activity is the result of many years' projects aimed at controlling gene expression by targeting mRNA.

Peptide nucleic acids (PNAs) are DNA analogues in which the sugar-phosphate backbone has been replaced by N-(2-aminoethyl)glycine units. These molecules were described for the first time by Nielsen et al. in 1991 at the Copenhagen University (Nielsen PE, 1991). In a PNA, the four naturally occurring nucleobases (adenine, cytosine, guanine, thymine), are connected to the central amine of the peptide backbone by a methylene bridge and a carbonyl group.

Originally, PNA was designed as a ligand for the recognition of double stranded DNA (Nielsen PE, 1999). The aim was to mimic an oligonucleotide binding to double stranded DNA via Hoogsteen base pairing in the major groove. Thus, as shown in Figure 24, the nucleobases of DNA were retained, but the deoxyribose phosphodiester backbone of DNA was replaced by a neutral pseudo-peptide backbone. Many properties and applications of PNA were discovered; because of its unique physico-chemical and biochemical properties, which cannot be achievable with naturally occurring oligonucleotides, PNAs are nowadays particularly interesting for scientists of many sectors, such as diagnostic, chemical, biological and even medical.

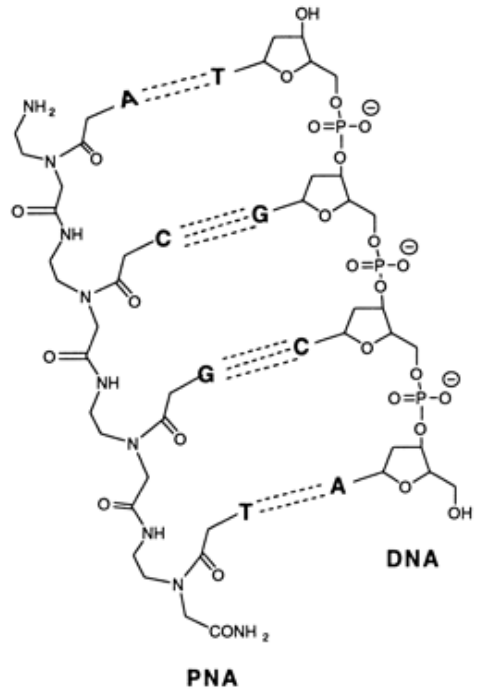

Figure 24. Comparison of PNA and DNA structures.

5.1 Structure and properties of PNAs

The structure of the PNA, is very similar to the structure of nucleic acids, in fact consists of a skeleton of repeating units of N-(2-aminoethyl)-glycine linked together by peptide bonds. In this carbonaceous body, that is acyclic, achiral and neutral, purine (adenine and guanine) and pyrimidine bases (cytosine and thymidine) are linked by methylene-carbonyl bonds. The substantial difference between PNA and DNA, or its analogues, is the absence of pentose sugars and phosphate groups. It is important to remember that formally, PNAs are nor nucleic acids neither peptides, whereas in practical application they can be defined as a type of peptide-like molecules (Nielsen PE, 1999).

The structural modification changes the negative charges of the DNA sugar-phosphate backbone to a neutral charge of the peptide-like backbone. Despite this, PNAs are structurally like any peptide with a C-terminus at the right end position and a N-terminus at the left end position, therefore, they are capable of sequence-specific and efficient hybridization with complementary DNA and RNA, forming double helices with Watson-Crick base pairing (Egholm M, 1993). They are also able to generate triple helix formation with double stranded DNA and perform strand invasion. This is because of the reduced electrostatic repulsion between PNA and DNA than the one existing between DNA and DNA, due to the absence of charge (Nielsen PE, 1999). Moreover, PNAs can recognize RNA with a higher affinity than DNA, and they are specific and resistant to DNAses and proteases. Demidov and collegues (1993) demonstrated that incubation of PNAs with S1 nuclease or DNAseI has no effect on PNA. Due to PNAs resistance to enzymatic degradation, their lifetime is prolonged, and since they are not recognized by polymerases, PNAs cannot be used as primers or be copied. PNAs are resistant to acidic pH while in basic conditions a N-acyl transfer reaction could occur (Christensen L, 1994). Generally, they display good aqueous solubility which could be impaired by some phosphate buffer. This could depend on the sequence composition since solubility problem are more frequent for PNA longer than 12 units and rich in purine, particularly guanine. To overcome this limit, many chemical modifications have been applied (Nielsen PE, 1999).

5.2 PNAs binding

Conversely to natural nucleic acids, PNAs have the ability to bind the complementary sequence, which is located on the double-stranded DNA, with a parallel or antiparallel orientation. Antiparallel means that the PNA N-terminus faces the nucleic acid 3’ terminus

while its C-terminus binds to the 5' terminus of the nucleic acid and, usually, this hybridization is preferred because it is more stable. The parallel orientation is followed by the Hoogsteen PNA strand binding, during triplex formation (Nielsen PE, 1999). This creates the possibility for PNAs to bind two DNA tracts of opposite sequence.

The PNA binding modes have been investigated by nuclear magnetic resonance and by X-ray crystallography. From these studies, it emerged that PNAs can adopt both A- and B-type structures (as in Figure 25) when associating with RNA and DNA, respectively; in addition, PNA–PNA duplexes form an unusual helix conformation, called P-type and are characterized by a large pitch of 18 base pairs (Nielsen PE, 1999). Therefore, PNAs can bind either the single-stranded DNA or RNA or the double-stranded DNA, but the way in which the PNA interacts with the target DNA depends on bases composition of the sequence involved.

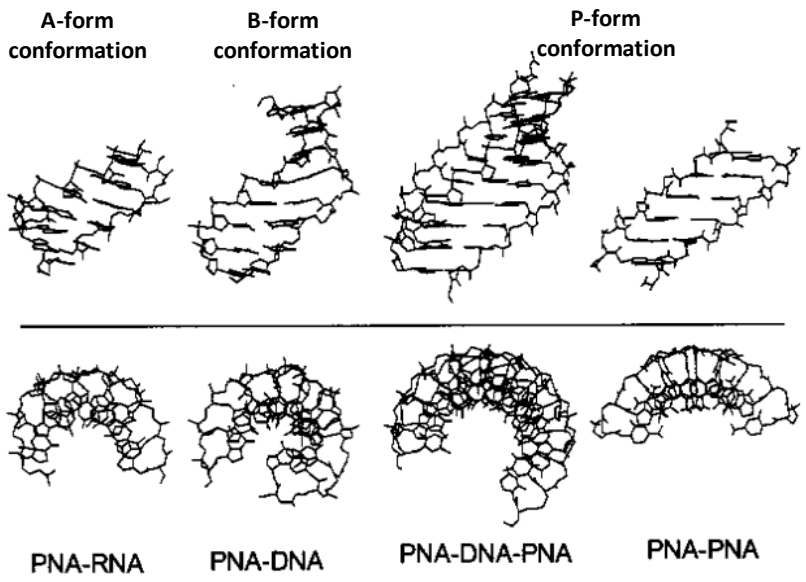

Figure 25. Structures of various PNA complexes (Nielsen PE, 1999).

Polypyrimidine PNAs are able to form stable adducts with complementary polypurine tracts of DNA, through the formation of PNA:DNA:PNA triplexes. The base pairing in these complexes occurs via Watson-Crick and Hoogsteen hydrogen bonds and the stability of these structures enables PNA to perform strand invasion with the formation of a loop structure, called P-loop (Figure 26B), a property which is uniquely shown by PNAs.

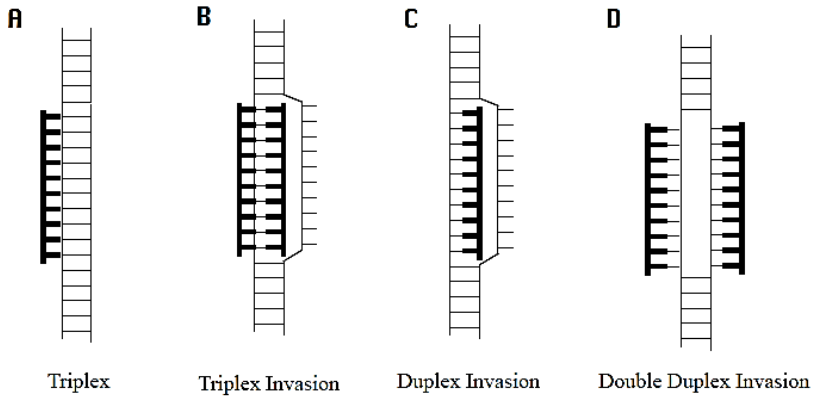

Figure 26. PNA-DNA binding modes (Nielsen PE, 2001).

Other ways of interaction between PNA and double stranded DNA have been discovered. The standard duplex invasion (Figure 26C) consists of the formation of a duplex PNA:DNA that is limited to the PNA high in purines, which forms a very stable complex. Instead, a high cytosine content in the PNA is necessary for classical triplex formation which occurs with a single PNA Hoogsteen strand; the triplex PNA:DNA:DNA is the kind of complex studied for the creation of PNA (Figure 26A) and it is not very stable. Another type of invasion is created using pseudo-complementary PNA containing diaminopurine-thiouracil base pairs. These form the double duplex invasion that turned out to be very stable (Figure 26D) (Nielsen PE, 2000).

PNA hybridization with single strand DNA creates DNA-PNA duplexes even more stable and more sensitive to sequence mismatch than natural DNA-DNA. Comparing the melting temperatures (Tm) between natural nucleic acid complexes and PNA-nucleic acid hybrids, it was determined the increased binding affinity and discrimination between fully complementary and mismatched hybrids proper of PNAs. Using a 15-mer PNA, all possible single mismatch combinations were tested in both PNA:DNA duplexes and corresponding DNA:DNA duplexes. In the PNA:DNA duplexes, the average Tm was 15°C, as opposed to 11°C in the corresponding DNA:DNA duplexes (Giesen U, 1998; Nielsen PE; 1999). Similar results were obtained for PNA:RNA duplexes. This discrimination indicates that short PNA probes could offer high specificity, allowing the further development of several PNA-based strategies for molecular investigations and diagnosis.

5.3 Synthesis of PNAs

The peptide nucleic acids used during my PhD course were synthetized by Professor Roberto Corradini and his research group at the University of Parma (Department of organic and Industrial chemistry). They synthesized two peptide nucleic acids containing three adjacent modified chiral monomers containing modification to C-2 or C-5. The resulting chiral PNAs showed DNA hybridization abilities strongly dependent on the stereochemistry (Manicardi A, 2010).

As regards to the monomers that carry out the modification on the C-5 of the uracil dimers, the amide function has been replaced with a less rigid methyl amino linker. This modification should depress in part the self-aggregation of the molecule into dimers and enhance its solubility because under physiological conditions the amine is protonated. These C-5 modified dimers were synthesized by reacting 5-formyl-uracils with different amines trough reductive amination reaction. The synthesis of 5-formyl-uracils is given by the oxidation of N(1)-alkylated-thymine or by formylation of N(1)-alkylated-N(3)-benzoylated uracil under Vielsemeyer-Haack conditions (Accetta A, 2009).

As shown in Figure 27, the monomers of 5-methylazidouracil are synthesized starting from regioselective hydroxymethylation of C-5 of uracil molecule. The 5-hydroxymethyl uracil generated (20), using concentrated HCl, is transformed into the corresponding alkyl chloride (21). Finally, by nucleophilic substitution of the chloride with sodium azide the desired product is generated (22); this last reaction is carried out at low temperature (0°C) in order to avoid the alkyl chloride self-reaction on its N(1) or N(3) that would lead to polymeric compounds formation, reducing the yeld. The 5-metilazidouracil is then embedded into a Boc-amino-ethyl-glycine structure (Figure 28). To do this, the 5-methylazidouracil is regioselective alkylated on N(1) by reacting with bromoacetic esters. The resulting ester (23 or 24) is deprotonated by basic or acid hydrolysis, depending on ester previously formed, and generates the 5-metilazidouracil acetic acid (25). This acid is activated with DCC/DhBtOH and linked to the Boc-aeg-OEt backbone to get the ethyl ester PNA monomer (26). With the hydrolysis of the ethyl ester is obtained the desired PNA monomer (27) that can be used for the synthesis of the oligomers on the solid phase.

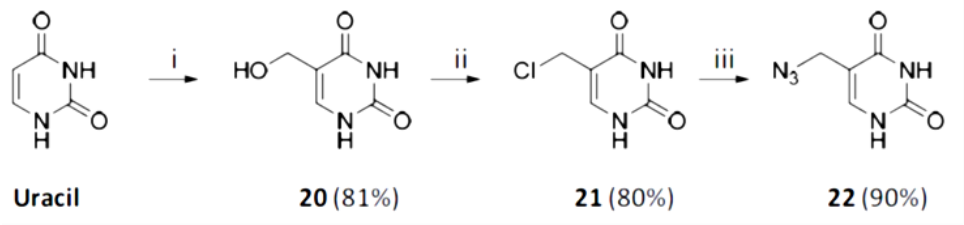

Figure 27. Synthesis of 5-azidomethyluracil. (i) CH2O, Et3N, water, 60°C, overnight; (ii) HCl 37%, 4H, rt; (iii) NaN3, DMF, 0°C, 1h.

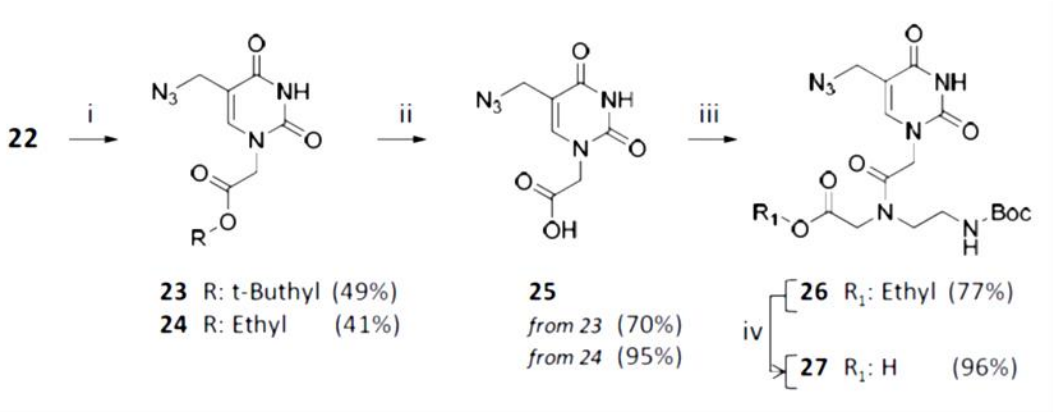

Figure 28. Synthesis of 5-methylazidouracil Boc-PNA monomer. (i) BrCH2COOR, K2CO3, DMF, rt, overnight; (ii) a. TFA/DCM; b. NaOH, water/MeOH for 24h; (iii) DCC, DhBtOH, DIEA, DMF; (iv) NaOH, water/MeOH.

5.3.1 Synthesis of anti-miR-221 and anti-miR-155 PNAs

PNAs used for this PhD project (R8-PNA-a221 and R8-PNA-a155) (Figure 29), are linked to an octaarginin tail (R8). To obtain fluorescently labeled PNA, a 2-[2-(Fmoc-amino)ethoxy)ethoxy]acetic acid [AEEA] spacer was linked to the N-terminus of PNA and then 5(6)-carboxyfluorescein was introduced using DIC/DhBtOH coupling. This peptide was chosen among other possible carriers since octa- and nona-arginine are found to be the most efficient at cellular internalization (Wender PA, 2000; Fuchs SM, 2004) and also at cellular internalization of drugs (Kirschberg TA, 2003) and PNAs (Brognara E, 2014); moreover, R8 can accumulate in some tumors to a higher degree than other cell-penetrating peptides (CPP) (Nakase I, 2012).

PNAs purification was performed by RP-HPLC and then they were characterized by UPLC-MS (Brognara E, 2014).

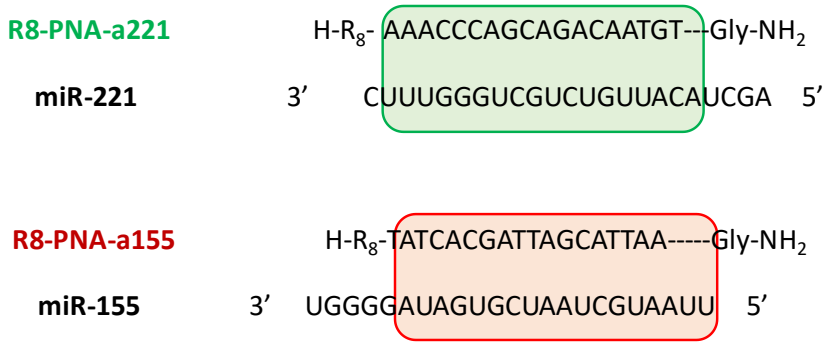

Figure 29. PNAs used in this PhD thesis and synthesized by Prof. Corradini's group. Sequences of R8-PNA-a221 and R8-PNA-a155 and binding site in their relative microRNA.

5.4 PNAs biological properties and applications

PNA is a versatile synthetic pseudopeptide with many applications.

Since the peptide backbone of PNAs is not easily recognized by nucleases and proteases, it is found to be biostable in human serum, bacterial cell extracts, nuclear mouse ascites and tumor cell extracts (Demidov VV, 1994).

Due to its superior properties than other DNA mimics, PNAs have attracted wide attention among chemists and biologists and now they are studied not only as antisense and antigene technologies, but also as nucleic acid biosensors, tools for genome mapping, within hybridization techniques for genetic detection, and modulation of PCR analyses or FISH technology (Figure 30) (Sharma C, 2017).

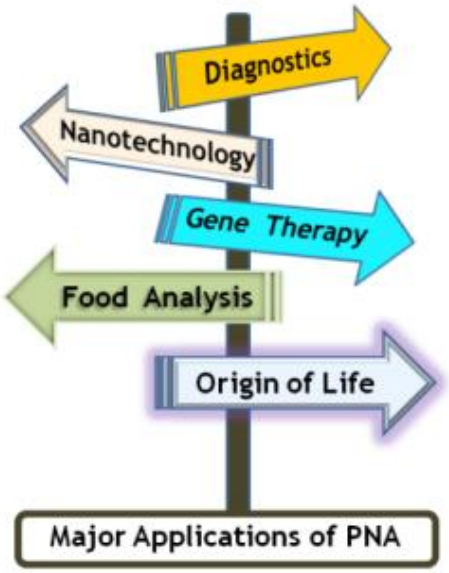

Figure 30. Major applications of PNAs (Sharma C, 2017).

In the last years PNAs have been demonstrated to perform modulation of gene expression through different mechanisms, they have the potential to inhibit replication, induce transcription arrest and translation arrest. Many studies explained the antisense and antigene properties of PNAs. An antigene PNA binds to the complementary sequence in DNA, while antisense PNAs hybridize to the specific mRNA and inhibit translation (Nielsen PE, 2010). Application of PNAs as antisense reagents was first demonstrated in 1992. The nuclear microinjection of a 15-mer PNA targeting the translation start region of SV40 large T antigen mRNA inhibited transcription in cell extracts. This inhibition was both sequence-specific and dose-dependent.

Moreover, PNAs are unable to elicit an antibody response when injected alone, therefore they are non-immunogenic (Upadhyay A, 2008).

These properties place PNAs at a great potential advantage as a therapeutic compound.

In this PhD thesis, the attention is drawn to PNA antisense approach. Through the hybridization with target mRNA and subsequent formation of PNA-mRNA complex, PNAs can inhibit the expression of a specific target protein (Dean DA, 2000). Furthermore, PNAs, when targeting specific splice sites in pre-mRNA are able to modulate the splicing process in many ways, depending on the targeted sequences. Thus, they could act in the correction of aberrant splicing or induction of alternative splicing site with restoration of a functional protein; in addition, they could also induce exon skipping, inhibiting a mature protein expression (Shiraishi T, 2008 and 2010).

Recently, it has been shown that specific RNA targeting using antisense PNA molecules can efficiently modulate gene expression in *P. falciparum*. In fact, by conjugating antisense PNA with octa-D-lysine CPP, Kolevzo et al. (2014) were able to downregulate the gene expression of the malaria virus, suggesting an alternative approach to silence the essential viral genes and produce antimalarial activity avoiding the frequent drug resistance.

Moreover, PNAs can act as anti-genes by binding directly to DNA. For instance, to achieve nuclear delivery, the classical PNA structure has been modified by linking its NH_2 terminus to a nuclear localization signal (NLS) peptide; thus, this PNA-NLS was synthesized to target MYCN proto-oncogene in human neuroblastoma cells. The complementarity with MYCN gene sequence, forms a PNA-DNA complex which interferes with the RNA polymerase II activity and MYCN transcription protein (Tonelli R, 2005).

In addition, PNAs find their application as decoy agents as they have been proved to inhibit transcription factor (TF) activity in the form of PNA-DNA-PNA (PDP) chimeras; these are PNA-DNA covalently bonded hybrids. In this instance, TF is recognized by the oligopeptide and binds to it. As a result, the real target is not bound by the transcription factor and the mRNA is not transcribed, causing expression failure of the corresponding protein (Borgatti M, 2003).

The major limitation of PNAs biological use is constituted by their poor cellular uptake. If issues related to uptake or cellular delivery are solved, PNA holds favorable biological properties for the development of gene-based therapeutic drugs. Nonetheless, its biological properties are proving to be beneficial in developing diagnostic tools and in biochemistry.

5.5 PNAs as anti-miRNA strategy

The issue of targeting miRNAs appears to be among the most relevant ones in the field of applied biomedicine. MiRNAs show an altered expression in pathological conditions such as cancer, and they seem to be potential targets for new therapeutic approaches.

PNAs are very promising tools due to their specificity and stability in biological fluids, as are resistant to nucleases and proteases action; further, they exhibit higher affinity for RNA than DNA, and can form very stable PNA:RNA duplexes, which can efficiently disrupt dsRNA duplex (Nielsen PE, 2014). MicroRNAs are very short, usually only 21-23 bases long, so anti-miRNA oligonucleotides are usually designed to be a perfect reverse complement intended to base pair with the full miRNA. It has been demonstrated that through antisense strategy the PNA can bind directly the microRNA repressing its expression (Ray A, 2000). Such antisense strategy is considered very specific, as it seems to discriminate the binding to a microRNA promoter rather than to transcription factors. In fact, by inhibiting the promoter, PNA can prevent the expression of a specific microRNA; these effects can be enhanced by inhibiting transcription factors, due to these factors' ability to bind to more genes resulting in loss of multiple miRNAs expression.

However, many oligopeptides and analogues were synthesized with the aim to silence the over-expression of specific microRNAs. The first example of targeting microRNAs using PNA-based molecules is provided by Fabani et al. with miR-122, they used PNAs and PNA-peptide conjugated to cell penetrating peptide (R6-Penetratin); further, 4 lysines residue was used to target miRNA-122 obtaining compounds of superior binding and anti-miR-122 activity, compared to 2′-O-methyl oligonucleotides (Fabani MM, 2008). In

addition, PNA delivery has been demonstrated linking PNAs to polylysine or polyarginine tails, based on the observation that this cell membrane penetrating oligopeptides are able to facilitate uptake of conjugated molecules (Torres AG, 2011).

Since the beginning, the main limit in the use of PNAs for gene expression modulation is the cellular uptake. To overcome this drawback, many modifications and several approaches have been evaluated. In 2010, in our group, it was tested the activity of an octa-arginine-coniugated PNA against miR-210 (Rpep-PNA-a210) in human erythroleukemia K562 cell line. Rpep-PNA-a210 showed to strongly inhibit miR-210 without the need of transfection agents. Conversely, without polyarginine, and despite displaying hybridization abilities, PNA-a210 could not be internalized by the cells and reach its target. Moreover, this study, demonstrated that unlike commercially available antagomiRs, which need continuous administrations, a single administration of Rpep-PNA-a210 was sufficient to obtain the biological effects (Fabbri E, 2011). Following these results, it was synthesized a PNA against miR-221 linked to an octa-arginine tail (R8-PNA-a221). It was tested on MDA-MB-231 breast cancer cells, confirming the efficient internalization without use of transfecting agents and strong inhibition of miR-221 expression. As a consequence, the expression of $p27^{kip1}$, a direct target of miR-221, was increased (Brognara E, 2012).

Similar interesting results were observed also in glioblastoma cell lines, where miR-221 was found to be express at high levels (refer to microRNA, chapter 2). Delivery systems of PNAs across the blood-brain barrier have been previously described (Suzuki T, 2004) and uptake of PNAs within neuronal cells has been demonstrated to be more efficient than other cell types (Sethi D, 2012); therefore, the possibility that PNAs against miR-221 participate in restoring levels of miR-221 downregulated genes could be an important starting point for the development of therapeutic strategies using PNAs targeting miR-221. In a recent study, Brognara et al., tested the uptake and biological effects of the R8-PNA-a221 *versus* PNA-a221, as well as its effects without R8; further, this study considered R8-PNA-a221 with mutations in the nucleotide sequence (R8-PNA-a221-MUT), as well as comparing R8-PNA-a221 biological effects to R8-PNA-a222, as miR-221 and miR-222 belong to the same cluster. The main results of this study were that R8-PNA-a221 can be well internalized leading to inhibition of miR-221 without inhibitory effects on miR-222. The specificity of the effect was sustained by the finding that the mutant R8-PNA-a221-MUT was inactive at inhibiting miR-221. Moreover, only R8-PNA-a221 was able to increase the expression of $p27^{kip1}$ and TIMP-3 and to induce apoptosis in glioblastoma cells

(Brognara E, 2014).

The efficiency of PNAs can be improved also with association to delivery systems such as mesoporous silica nanoparticles (MSNPs). Bertucci et al. (2015) demonstrated that a multi-functional nanosystem in which MSNPs are loaded with anti-cancer drug temozolomide (TMZ) and decorated on the surface with R8-PNA-a221, can be rapidly internalized into glioma cells. The advantages are that TMZ is slowly delivered in cell cytoplasm as well as the PNA is delivered and released into the cells. Results showed an increase of apoptosis in TMZ-resistant T98G glioma cells, suggesting the advantages of combined therapies (Bertucci A, 2015).

In another study conducted this year by our group on glioma cells, it was found that the combined treatment of both R8-PNA-a221 and R8-PNA-a222 induced higher pro-apoptotic effects respect to single PNAs treatments. In addition, the treatment of TMZ-resistant T98G cells with R8-PNA-a221 and R8-PNA-a222 reversed temozolomide-dependent cell resistence to apoptosis; whereas, when R8-PNA-a221 and R8-PNA-a222 were co-administered with temozolomide, apoptosis was induced at a level higher than that obtained following singular administration of these molecules, i.e. a decrease of TMZ resistance (Brognara E, 2016).

Some studies are oriented toward development of techniques able to achieve specific tropism of the antisense-drugs. Cheng et al. (2015) tested a new platform of anti-miR delivery with tropism for the acid cancer microenvironment. They linked an anti-miR-155 PNA to a peptide with a low pH-induced transmembrane structure (pHLIP) producing a construct able to deliver the anti-miR across plasma menbranes under low pH conditions.

Overall these evidences support the concept that anti-miR strategy could lead to therapeutic relevant inhibition of mRNA dependent effects and that PNA-based anti-miR molecules are very promising reagents to regulate tumor cell growth.

AIM OF THE THESIS

Glioblastoma multiforme (GBM) is a devastating malignant brain tumor and, despite aggressive treatments such as surgical resection, chemotherapy and radiation therapy, the life expectancy is about of 18 months. Although common clinical presentations and histology, it has been clearly demonstrated that GMB is a highly anaplastic and morphologically highly heterogeneous tumor. In fact, the difficulty in treating GBM can be due to a variety of cells forming the tumor. Some cells may respond well to certain therapies, while others may not be affected at all. This is the reason why the treatment plan for glioblastoma may combine several approaches. Therefore, it is required the design of new therapeutic modalities, especially those that enhance currently available treatments and/or limit tumor growth, also reducing the chemoterapeutic drug resistance.

The study of gene expression profiles has contributed significantly to the identification of the molecular changes to be considered among the causes of tumorigenesis, and provides a number of potential biomarkers for diagnosis, prognosis and prediction of response to therapy. These studies allow also the identification of new molecular targets for the development of innovative chemotherapy drugs. Among these, O(6)-Methylguanine-DNA-methyltransferase (MGMT) is a DNA repair protein involved in drug resistance particularly towards temozolomide (TMZ), which is the first-line chemotherapeutic alkylating agent for glioblastoma therapy. Indeed, low levels of functional MGMT have been correlated with success of treatment, while high levels bring about failure of therapy (Sharm S, 2009; McNamara MG, 2013). Other possible interesting targets are: vascular endothelial growth factor (VEGF) involved in angiogenesis and hallmark of the neovascularization in gliomas; phosphatase and tensin homolog (PTEN), a tumor suppressor gene involved in the regulation of cell proliferation, apoptosis and tumor invasion (McNamara MG, 2013).

Growing evidences showed that also Nuclear Factor KappaB (NF-κB) has an important role in carcinogenesis, including gliomagenesis (Sai K, 2014); it regulates the expression of genes involved in many processes of cancer development and progression, such as cell proliferation, migration and apoptosis (Dolcet X, 2005; Napetschnig J, 2013).

Besides the already mentioned MGMT, there are many molecular mechanisms underlying the resistance of GBM cells to TMZ, including both genetic and epigenetic mechanisms.

To enhance the benefit of TMZ in the treatment of glioblastomas, effective combination strategies are needed to sensitize glioblastoma cells to TMZ. In this regard, there are natural molecules that seem to be very interesting (Yang L, 2015; Wang Y, 2015). Among these, corilagin, a gallotannin extracted from plants of the *Phillantus* family, resulted of particular interest as it exhibited antioxidant, anti-inflammatory, hepatoprotective and antitumor activity. Recently, our group demonstrated in *in vitro* EMSA experiments that corilagin inhibits NF-kappaB/DNA interactions. Therefore, since NF-κB has an important role in apoptosis and in cancer, molecules which have a potential to inhibit NF-κB could be considered potential anticancer agents. Moreover, it was shown that corilagin inhibits cell growth, induces cell cycle arrest in G2/M phase and induces apoptosis in different types of tumor cells. Studies conducted on this compound found that these anticancer effects are due to the intervention of corilagin on pathways such as MAPK, NF-κB and TGF-β1 (Jia L, 2013; Ming Y, 2013; Gu Y, 2016).

These findings led us to further investigate the role of corilagin in glioblastoma as anticancer drug. In fact, the main aim of this PhD thesis was to investigate different treatments to attenuate resistance and increase the efficacy of TMZ treatment in a specific type of glioblastoma model, T98G cells, which are resistant to temozolomide. In particular, it was investigated the possible advantages deriving from combined treatments with TMZ to identify the eventual existence of additive antitumor effects or synergistic mechanisms. Among the potential chemosensitizers, the natural compound corilagin, was selected as an ideal agent for GBM therapy. This study was designed to determine whether corilagin could potentiate the antitumor activity of TMZ in glioblastoma, through the inhibition of cell growth and migration and through the induction of apoptosis by activating caspase-3 pathway.

Other important factors to be considered in glioblastoma therapy are microRNAs. These are small non-coding RNA molecules, 21 to 25 nucleotides in lenght, that are involved in the post-trascriptional regulation of gene expression. Many experimental evidences showed strong correlation between the altered expression of microRNAs and the onset and progression of tumors. According to these studies, tumorigenesis occurs as the result of imbalances between oncomiRNAs and tumor suppressor miRNAs. More precisely, oncomiRs are tumor suppressor gene downregulators that are up-regulated in some cancers, and tumor suppressor miRs target oncogenes and are usually downregulated (Hata A, 2015). Among many miRNAs, miR-221 and miR-155, both oncomiRs, are

overexpressed in many types of human cancer. In glioblastoma, miR-221 was found to target genes inhibiting apoptosis and enhancing glioma cell migration such as PTEN, PUMA, $p27^{kip1}$, MGMT and TIMP3, suggesting its involvement in the viability and proliferation of cancer cells. In addition, miR-221 has been associated to chemo and radioresistance (Shea A, 2016). Similarly, miR-155 enhances glioma cell proliferation by hitting GABA-A receptor expression (D'Urso PI, 2012), and plays a role in glioma cells migration and invasiveness (Ling N, 2013). Therefore, targeting miRNAs could be a powerful way for developing selective and individual therapies.

The inhibition of miRNA activity can be achieved using miRNA inhibitors and oligomers, including oligonucleotide analogues (miRNA antisense therapy), miRNA sponges or through miRNA masking (Gambari R, 2016). In particular, peptide nucleic acids (PNAs) belong to the antisense strategy to force the reduction of miRNAs expression. They are oligonucleotide analogues with a polyamide backbone stable to nucleases and with high affinity and specificity for binding RNA. In our lab, Brognara et al. (2012) studied the effect of a polyarginine-PNA conjugate targeted against miR-221 in MDA-MB-231 breast cancer cells. Targeting miR-221 by PNA resulted in lowering of the hybridization levels of miR-221 and upregulation of $p27^{Kip1}$ gene expression. In another recent study (Brognara E, 2016), using an octaarginine (R8)-coniugated-PNA-a221 we demonstrated the downregulation of miR-221 expression and the consequent induction of apoptosis in glioblastoma cell lines. Moreover, co-administration of both antimiR-221 and antimiR-222 increased pro-apoptotic effects.

Finally, in TMZ-resistant T98G cells the co-administration of R8-PNA-a221, R8-PNA-a222 and TMZ induced apoptosis at a level higher than that obtained following singular administration of the PNAs (Brognara E, 2016). Taken together these findings suggested us the possibility of the existence of similar dynamics between other PNAs targeting oncomiR, such as miR-155, and temozolomide in glioblastoma. Another reason why we selected miR-221 and miR-155, among all miRNAs that are dysregulated in glioblastoma, is that they seem to target the 3'UTR region of caspase-3 mRNA, an important factor occurring during apoptosis process.

Therefore, the second purpose of this PhD thesis was aimed at sensitizing T98G glioblastoma cells to temozolomide, but through PNAs treatments. Firstly, we wanted to identify miRNA targeting caspase-3 by comparing miRNAs highly expressed in tissues from glioma patients, miRNAs validated in gliomas for their oncogenic properties and

mRNAs putatively interacting with the 3'UTR of caspase-3 mRNA, which is deeply involved in activation of the apoptotic pathway. From this research, miR-155 and miR-221 were selected and it was assessed the anti-miRNA properties of PNAs sequence specific for the targeting of miR-155 and miR-221 in glioblastoma cell lines. Since PNAs treatments induced apoptosis, we wanted to demonstrate if the combined treatment with PNAs and TMZ permitted to obtain a highest apoptotic and anti-proliferative effect, aiming to reverse the drug-resistance phenotype in the temozolomide-resistant T98G glioma cell line.

Running parallel to the innovative strategies of the co-administration of chemotherapeutic drug and anti-tumor natural compound, and the combination of antisense miRNA based therapy with chemotherapeutic drug, this study also aimed to investigate the involvement of caspase-3 as a direct target of the two analysed miRNAs, miR-221 and miR-155.

Finally, with these findings, we hope to identify new therapeutic approaches based on the enhancement of conventional therapy (corilagin plus temozolomide), as well as a specific and individual therapy based on the use of antisense approach (R8-PNA-a155/R8-PNA-a221 plus temozolomide).

MATERIAL AND METHODS

1. HUMAN GLIOMA CELL LINES

Glioblastoma, the most malignant and frequent form of primary brain tumor, is characterized by different population and subpopulation of cells. Cell cultures represent an important biological model that have facilitated cancer research. In this thesis T98G and U251 are used as glioblastoma cell models to assess, in *in vitro* studies, the effects of different substances on specific aspects of the cancer cell life involved in glioma.

T98G: Human Glioblastoma cell line

T98G is a human cell line (Sigma Aldrich, St. Louis, Missouri, USA) derived from a glioblastoma multiforme tumor of a 61-year-old caucasian male.

These cells are characterized by an arrest of their cell cycle in the G1 phase, as for normal cells, but what differs the T98G cells are the proprieties of immortality and anchoring (GH Stein, 1979).

T98G cells grow in adhesion (Figure 31), they were cultured in humidified atmosphere of 5% CO_2/air in RPMI-1640 medium (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS, Celbio, Milan, Italy), 100U/ml penicillin and 100mg/ml streptomycin. The subcultivation ratio was 1:10 and the medium was renewed 2 to 3 times per week.

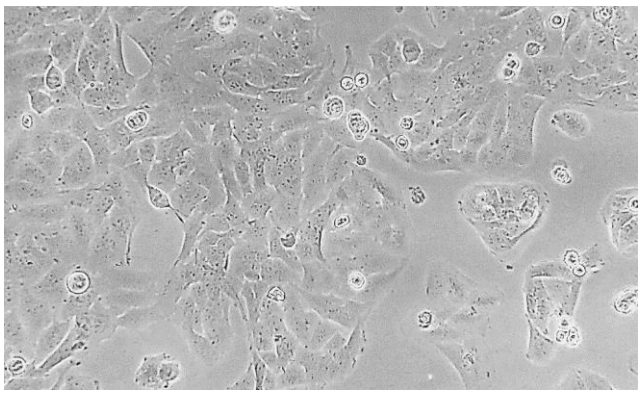

Figure 31. T98G glioblastoma cells.

U251: Human Glioblastoma astrocytoma cell line

U251 glioma cells (Sigma Aldrich, St. Louis, Missouri, USA) represent an important biological model to study genetic aberrations and molecular pathways in GBM.

The U251 cell line was established at the Wallenberg laboratory, Uppsala University, in Sweden, more than 40 years ago from a male patient with malignant astrocytoma (Torsvik A, 2014).

U251 cells in culture condition adhere to the surface of the flask and show an elongated shape with branches (Figure 32). They were cultured in humidified atmosphere of 5% CO_2/air in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100U/ml penicillin and 100mg/ml streptomycin. The subcultivation ratio was 1:5 and the medium was renewed 2 times per week.

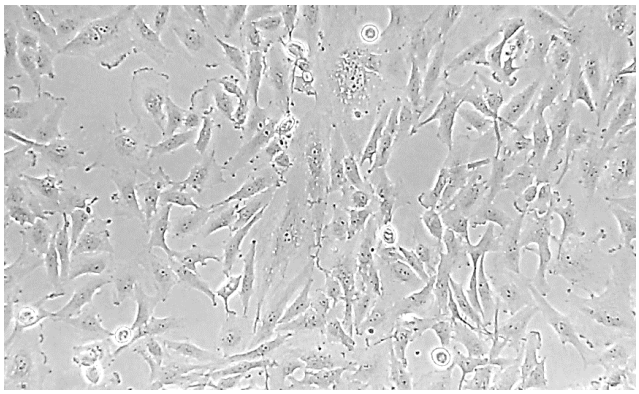

Figure 32. U251 glioblastoma cells.

2. CELL TREATMENTS

For our experiments the glioblastoma cells were seeded when they reached about 80% of confluence in the culture flask; for the 48-hour treatments they were plated onto a 12-well plate at a density of 3,5 x 10^4 cells/well for T98G cells and 4,5 x 10^4 cells/well for U251 cells in 700 µl medium (final volume). The seed was a bit lower for the 72 hour treatments: 2,5 x 10^4 cells/well for T98G cells and 3,5 x 10^4 cells/well for U251 cells.

The treatments were performed the day after seeding. After having removed the medium, rinsed the cells with DPBS (Lonza, Verviers, Belgium) to remove any trace of debris and replace the medium with a fresh one, U251 and T98G cells were treated with the compounds in analysis.

- **Corilagin**: a polyphenol, specifically a hydrolyzable tannin, was purchased from Sigma Aldrich as a white powder. It has a molecular weight of 634.45 g/mol and it is dissolved in DMSO and MeOH (1:1) to obtain a solution concentrated 10 mM. Glioblastoma cells were treated with increasing concentration of corilagin (COR): 10, 25, 35, 40, 50, 75, 100 µM to identify the IC_{50} (concentration of the compound that inhibits the cell growth of 50% respect to untreated cells) and to study the pro-apoptotic effect. Then, for the co-treatment studies, it was selected 35 µM COR that is lower than the IC_{50} value.

- **Temozolomide:** an antitumor prodrug used in advanced gliomas therapy (Von Neubeck C, 2015). The pure powder of temozolomide (Sigma Aldrich) is dissolved in 10% MeOH/DMSO solvent to obtain a concentrated solution of 100 mM.
  U251 and T98G cells were treated with increasing concentration of the drug (50, 100, 200, 400, 600 µM) to confirm the TMZ- resistance of T98G cells. Then, for the combined treatment experiments, T98G cells were treated with TMZ 400 µM.

- **Anti-miRNAs R8-PNA-a155 and R8-PNA-a221:** peptide nucleic acids (Table 4) were synthetized by Prof. Roberto Corradini and his collaborators (University of Parma, Dept. of Organic and Industrial Chemistry) with standard manual Boc-based chemistry using commercially available monomers (ASM, Hannover, Germany) with HBTU/DIPEA coupling (Brognara E, 2014).

Table 4. Characteristics of PNAs antimiR-221 and antimiR-155 used in our experiments

Name	Sequence	Concentration
R8-PNA-a221	H-R8-AAACCCAGCAGACAATGT-Gly-NH_2	558.1 µM
R8-PNA-a155	H-R8-TATCACGATTAGCATTAA -Gly-NH_2	916µM

The PNAs are suspended in a solution of ultrapure water + 10% MeOH and the working solution are diluted in ultrapure water (Sigma Aldrich).
For the initial experiments, different concentration (0.5, 1, 2, 4 µM) of the anti-miRNA PNAs were used to treat U251 and T98G glioma cells. These allowed to

select the proper concentrations for the further studies: 2 µM for R8-PNA-a221 and 4 µM for R8-PNA-a155.

- **Combined treatment with corilagin and temozolomide**: the two compounds were added to the cells cultured in plate at the same time, 24 hours after seeding as the single treatments. Corilagin was used at concentration of 35 µM, while TMZ was 400 µM concentrated. In each experiment the combined treatment was compared to untreated cells and cells treated with COR and TMZ alone.

- **Combined treatment with anti-miRNA PNAs and temozolomide**: U251 and T98G cells were treated at the same time with 2 µM R8-PNA-a221 and 400 µM TMZ or 4 µM R8-PNA-a155 and 400 µM TMZ. In each experiment the combined treatment was compared to untreated cells and cells treated with PNAs and TMZ alone.

3. ANTI-PROLIFERATIVE ACTIVITY

Adherent glioma cells, U251 and T98G, were cultured as described above (Cell treatments, section 2). Initially, it was studied the anti-proliferative effect and cells were treated with increasing concentrations of the compounds (TMZ, COR, R8-PNA-a221 and R8-PNA-a155) to identify the IC_{50}. After a defined treatment time (24, 48 or 72 hours), cells were washed with sterile phosphate-buffered saline 1X (DPBS) and trypsinized with Trypsin/EDTA. After few minutes in incubator at 37°C, cells were detached from the bottom of the well and the trypsin was neutralized with an equal volume of FBS and resuspended in fresh medium. Cells were mixed thoroughly by pipetting, then 50 µL of suspended cells were picked up, diluted in 5 ml of physiological solution (Vetrotecnica, Padova, Italy) and counted in dedicated plastic cups. Cell growth was monitored as cell number per ml, using a Z2 Beckman Coulter Counter (Beckman Coulter, Pasadena, California, USA). Non-treated cells were considered as control.

Glioblastoma cells were counted in each experiment, also when they were treated with the combination of TMZ + COR or TMZ + R8-PNA-a221/ R8-PNA-a155, to evaluate the inhibition of cell growth or any cytotoxic effect.

3.1 Cell Viability

Cell viability was analyzed for T98G glioma cells after combined treatment with corilagin and temozolomide using the Muse™ Count & Viability Reagent. This assay allows the quantitative analysis of cell count and viability on the cytofluorimeter Muse™ Cell Analyzer; it is based on differentially stained viable and non-viable cells based on their permeability to the two DNA binding dyes present in the reagent. A DNA-binding dye in the reagent stains cells that have lost their membrane integrity, allowing the dye to stain the nucleus of dead and dying cells, discriminating viable (live cells that do not stain) from non-viable (dead or dying cells that stain) cells. While, a membrane-permeant DNA staining dye stains all cells with a nucleus discriminating cells with a nucleus from debris and non-nucleated cells. Therefore, data are displayed in two dot plots.

T98G cells, after treatment for 48 hours with 35 µM COR, 400 µM TMZ and COR (35 µM) plus TMZ (400 µM) in a 12 well plate, were detached and resuspended in fresh medium. 10 µl of cell suspension corresponding to each sample were added to 190 µl of Count & Viability Reagent in a sample tube; after vortexing, the T98G cells were allowed to stain for 5 minutes and then the assay was performed. Samples were analyzed after having performed the adjust setting with the negative control.

4. ANALYSIS OF APOPTOSIS

Muse cell Analyzer

Muse cell analyzer (EDM Millipore Corporation, Merck KGaA, Darmstadt, Germany) is a small instrument which mechanism is based on flow cytometry and it is able to deliver an accurate, precise and quantitative cell analysis.

A cell suspension, previously treated with a fluorescent compound, is aspired by a capillary that carries the cells in a measuring area where they are invested by a laser light focused. Under the laser light, cells generate a signal which is detected by a sensor that send amplified data to an analyzer that represents them graphically.

4.1 Annexin V and Death cell assay

The Muse™ Annexin V & Dead Cell Assay was used for the quantitative analysis of live, early apoptosis, late apoptosis and death cells through the detection of the binding between Annexin V and Phosphatidylserine on the external membrane of apoptotic cells.

Among the physiological changes that occur during apoptotic process, there are:

externalization of phosphatidylserine (PS), that is a membrane component normally localized in the cytoplasmatic face of the cell membrane, to the cell surface; and, in late stages, loss of membrane integrity. Annexin V is a calcium-dependent phospholipid-binding protein conjugated with fluorochromes molecules that has a high affinity for PS and when it binds it on the external membrane (Annexin V- PS) we have the detection of the apoptotic cell population. In the kit reagent there is also a dead cell marker used as an indicator of cell membrane structural integrity, so it can recognise only late apoptotic cells, dead cells and debris.

For optimal throughput, final cell concentrations should be between 1 x 10^5 and 5 x 10^5 cells/ml, although apoptosis can be detected in cultures with as few as 1 x 10^4 cells/ml.

After 48 hours of incubation with the treatments, cells were trypsinized, washed and resuspended in fresh medium. Treated cells and untreated controls (50 µl) were incubated with 50 µl of the Muse™ Annexin-V & Dead Cell Reagent and after 15 minutes in the dark at room temperature, the assay was performed with the Muse cell analyzer.

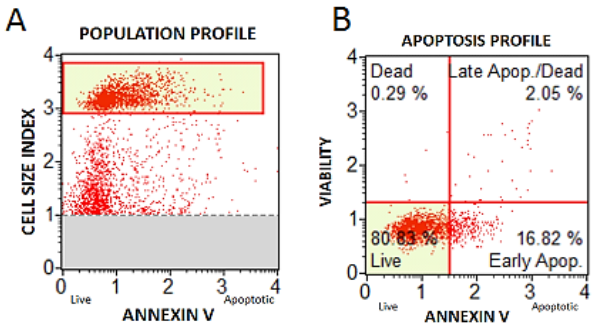

Figure 33. Representative results displayed with optional dot plots. The first plot in (A) shows Annexin V vs Cell Size and the second plot in (B) shows Annexin V vs Viability. The gated cell population in the first quadrant (A) is represented in the second graph as percentage of live, early apoptotic, late apoptotic/dead or dead according to their binding with Annexin V and thus to their apoptotic profile.

Samples were analyzed after having performed the adjust setting with the negative control; four populations of cells can be distinguished in this assay (Figure 33B):

- non-apoptotic cells: represent viable cells, so there are no markers of apoptosis. Annexin V-PE (-) and death cell marker (-);
- early apoptotic cells: cells are in the early stages of apoptosis. Is present therefore the marker for apoptosis, Annexin V-PE (+) but not death cell marker (-);
- late stage apoptotic and dead cells: cells are in the late stages of apoptosis or dead by

apoptotic mechanisms. There are both markers, Annexin V-PE (+) and dead cell marker (+);

- mostly nuclear debris: cells which have died by necrosis and not for apoptotic pathway. This is the only death cell marker (+) and not Annexin V-PE (-).

4.2 Caspase 3/7 assay

The apoptotic status of the glioma cells was investigated on caspase 3/7 activation, cellular plasma membrane permeabilization and cell death, after 48 hours of treatment.

The caspases are cysteine proteases that play a central role in propagating the process of programmed cell death in response to pro-apoptotic signals. While some caspases primarily act to initiate intracellular event cascade, other called effector caspases act further downstream and direct cellular breakdown through cleavage of structural proteins.

The Caspase 3/7 kit permits to recognize cells in various stages of apoptosis based on the activity of the executioner caspases 3 and 7.

The Muse^{TM} Caspase 3/7 reagent is cell membrane permeable and non-toxic to the cell; it contains a DNA binding dye that is linked to an amino acid sequence Asp-Glu-Val-Asp (DEVD) substrate. Cleavage by active caspase 3/7 of the DEVD peptide substrate in the cell results in release of the dye, translocation to the nucleus and binding of the dye to DNA with high fluorescence emission. With this reagent, we detect early and late stages of apoptosis.

A dead cell marker, 7-AAD, is also included in the assay as an indicator of cell membrane structural integrity and cell death. It is excluded from live, healthy cells, as well as early apoptotic cells, but permeates later stage apoptotic and dead cells.

As previously described for Annexin V assay, the cells were analyzed after their resuspension in fresh medium.

50 µl of cell suspension for each sample, corresponding to about 1 x 10^5 cells/ml, was centrifuged (1200 rpm x 5 min.) and resuspended in 25 µl of 1X Assay Buffer BA.

The Muse^{TM} Caspase-3/7 Reagent working solution was prepared by diluting the stock solution 1:8 in 1X PBS, following protocol instruction by manufacturer, in the moment of use. Then 2,5 µl were distributed to each sample and, after pipetting up and down, samples were incubated for 30 minutes at 37°C in the incubator in the dark.

Meanwhile the Muse^{TM} Caspase 7-AAD working solution was prepared by adding 1 µl of Muse^{TM} Caspase 7-AAD stock solution to 74 µl of 1X Assay Buffer BA.

Once termed the incubation, 75 µl of the second solution were added to each sample and, after 5 minutes at room temperature protected from the light, the assay was run using the Muse cell analyzer.

With this assay four population of cells can be distinguished (Figure 34B):

• (LL) Live cells: Caspase-3/7(–) and 7-AAD(–)

• (LR) Apoptotic cells exhibiting Caspase-3/7 activity: Caspase-3/7 (+) and 7-AAD(–)

• (UR) Late Apoptotic/Dead cells: Caspase-3/7(+) and 7-AAD(+)

• (UL) Necrotic cells: Caspase-3/7(–) and 7-AAD(+)

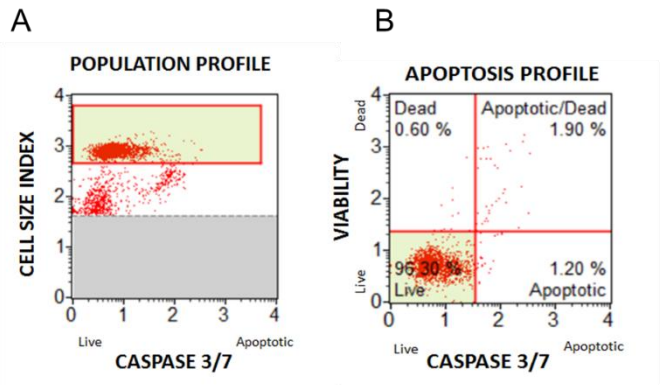

Figure 34. Representative results displayed with optional dot plots. The first plot in (A) shows Caspase 3/7 vs Cell Size and the second plot in (B) shows Caspase 3/7 vs Viability. The gated cell population in the first quadrant (A) is represented in the second graph as percentage of live, apoptotic, apoptotic/dead or dead according to their apoptotic profile.

4.3 Fluorometric Tunel Assay

T98G apoptotic cells were detected and quantified using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) that measures the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTPa3'-OH DNA ends using Terminal Deoxynucleotidyl Transferase (TdT), wich forms a polymeric tail.

T98G cells were cultured in poly-L-lysine-coated culture slides; about 1 x 10^4 cells were seeded for each chamber of the slide. After 24 hours they were treated with the compounds (400 µM TMZ, 35 µM COR, 35 µM COR+ 400 µM TMZ) and 48 hours later, fluorometric tunnel assay was performed according to the manufacturer's protocol. Briefly, T98G cells were fixed with 4% formaldehyde in PBS (25 min, 4°C) and permeabilized with 0,2% Triton X-100 in PBS (5 min). After an equilibration step (10 min, RT), TdT reaction mix, containing equilibration buffer, nucleotide mix and Terminal Deoxynucleotidyl

Transferase, was added to each sample for an incubation of 60 minutes at 37°C, protected from the light. Then, the reaction was stopped immerging the slide in 2xSSC (15 min) and the samples were stained with propidium iodide solution. Each step of the protocol was followed by PBS washings. Finally, the fluorescein-12-dUTP-labeled DNA that identify the apoptotic cells, could be visualized by fluorescence microscope Nikon Eclipse 80i and images could be captured with a camera connected to the microscope.

5. SCRATCH WOUND ASSAY: effect on cell migration

T98G cells were seeded in a 24-well plate at a confluence of 80%. 24 hours after seeding a vertical wound was created in the T98G cell monolayer using a 200 µL pipette tip. After washing 3 times with PBS to eliminate all the cellular debris, it was replaced fresh medium and T98G cells were treated with 35 µM COR, 400 µM TMZ and the two drugs together. Images were captured with a camera connected to the microscope Nikon Eclipse TS100 at designated times (0, 16, 24 and 48 hours) to assess the rate of gap closure compared with untreated cells.

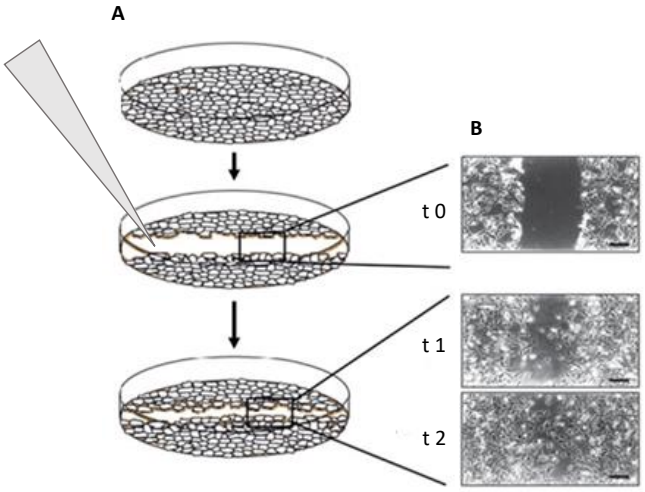

Figure 35. Representation of a scratch wound assay. (A) On a cell layer it is created a scratch using a pipette tip and then the cell migration is monitored. (B) Example of cell invasion at the time of the scratch (t 0), after some hours (t 1) and before the closure of the scratch (t 2).

6. RNA TOTAL EXTRACTION

Extracting total RNA is an important process that has the aim to evaluate gene or miRNA expression after cell specific treatments. Total RNA was isolated with Trizol Reagent (Sigma Aldrich), a mixture of guanidine thiocyanate and phenol in a monophasic solution,

which has the ability to separate effectively RNA to DNA and proteins, preserving intact the nucleic acid. Cells were separated from supernatant after a 4000 rpm centrifugation for 4 minutes at room temperature.

From manufacturers instruction it was used 1 ml of Trizol Reagent to lyse 5-10 x 10^6 cells by repeated pipetting, then to ensure complete dissociation of nucleoprotein complexes, the samples incubated for 3 minutes at room temperature. Thus, 200 µl of Chloroform per ml of Trizol Reagent, were added, shaked vigorously for 15 seconds. After waiting 3 minutes, the resulting mixture was centrifuged at 12000 rpm for 10 minutes at 4°C, to separate protein, the red organic phase, DNA in interphase, and RNA, the colorless upper aqueous phase. Then, 500 µl of 2-propanol were added to the aqueous phase.

Samples were mixed, by inverting the tube at least seven time. The samples were left for 10 minutes at room temperature, and then they were centrifuged at 12000 rpm for 10 minutes at 4°C to precipitate RNA. The pellet of RNA was washed with 500 µl of 75% EtOH, was centrifuged at 12000 rpm for 15 minutes at 4°C, dried and dissolved in nuclease-free water. Samples were stored at -80°C until the execution of qualitative and quantitative assays.

Also the extraction of microRNAs was performed following this protocol of total RNA extraction.

6.1 RNA electrophoresis on agarose gel

Qualitative analysis of RNA confirms the effective extraction and the integrity of RNA itself, providing a visual check of the extracted RNA quality as the absence of DNA or protein contaminations. Electrophoresis is a methodology through which molecules having an electric charge move with different speeds, depending on their charge and size, in an electrical field separating from each other.

The gel used in this technique was a 1% agarose gel able to separate nucleic acid fragments from 500 bp to 20000 bp through its ability to retain between the meshes of the gel the larger fragments, impeding the race, and instead leaving migrate the smaller fragments, which then will be observed in the lower part of the gel.

The 1% agarose gel was prepared by dissolving 1 g of agarose powder in 100 ml of TAE 1X. TAE 1X is a buffer solution, obtained by dilution from 50X TAE = 2 M Tris-HCl, 0.05 M EDTA pH=8 and 5.71% acid acetic 99.8%, and allows to maintain stable pH and concentration of ions in the gel, facilitating electrical conduction and uniform movement of

the nucleic acids fragments during electrophoresis. EtBr 10 g/ml was added to the solution; intercalating to the nitrogenous bases of acid nucleic makes visible migration of fragments in the UV light. RNA is negatively charged so when the electric field of 80-90 V is applied, samples migrate toward the positive pole. When the run ended the gel was observed and photographed in UV light using UV trans illuminator, Gel Doc 2000 (Biorad, Hercules, CA, USA).

6.2 RNA quantification

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A260) in spectrophotometer. The concentration is obtained by the equation:

$$g/ml = OD \times 40 \times DIL$$

where OD is the value read from the instrument, 40 is the correction coefficient for reading the RNA at the spectrophotometer (according to the Lambert-Beer law) and DIL is the dilution factor. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml (A260 = 1 = 40 µg/ml). This relation is valid only for measurements in water.

7. MicroRNA QUANTIFICATION

7.1 Reverse Transcription of microRNAs

The Reverse Transcription is a technique that allows to obtain a molecule of complementary DNA (cDNA) from a RNA template. Reverse transcription of 300 ng of total RNA was performed using the TaqMan MicroRNA Assay kit (Life Sciences, Life Technologies, CA, USA) that use a stem-looped small RNA-specific RT primer for reverse transcription.

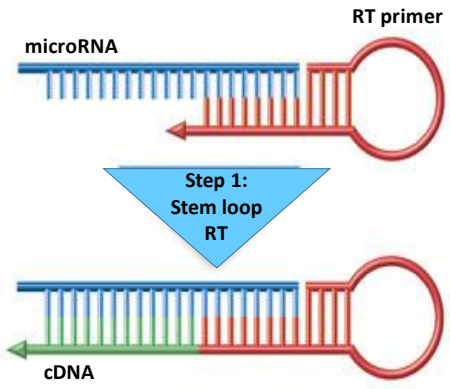

Figure 36. Representation of Reverse Transcription reaction.

For each sample, 300 ng of total RNA was diluted with nuclease-free water to a final volume of 18.32 µl. The resulting solution was added with 6 µl of miRNA-specific primers 50 nM mix (Applied Biosystem, Monza, Italy). The mixture of water, RNA and primers was incubated at 16°C for 30 minutes in the Bio-Rad MyCycler PCR (BioRad, Hercules, CA, USA) in order to allow the template-primers pairing. Then, the RT Master Mix was prepared combining the following reagents: dNTPs 100mM, RNase Inhibitor 20 U/µl, 10X Reverse Transcription Buffer, MultiScribe Reverse Transcriptase 50 U/µl. Each sample was added with 5.68 µl of the RT Master Mix reaching a final volume of 30 µl.
Reverse Transcription was performed incubating the final mixture at 42°C for 30 minutes, during which the reaction run, followed by 5 minutes at 85°C to inactivate the enzyme. The cDNAs obtained were stored at -80°C and analyzed by RT-PCR within few days.

7.2 Real-Time Quantitative PCR of microRNAs

The Reverse transcription followed by polymerase chain reaction is the best method to amplify and detect the amount of mRNA and therefore to quantify the expression of a specific gene of interest. It allows to quickly obtain a million copies of the cDNA of interest from extremely reduced initial quantities.

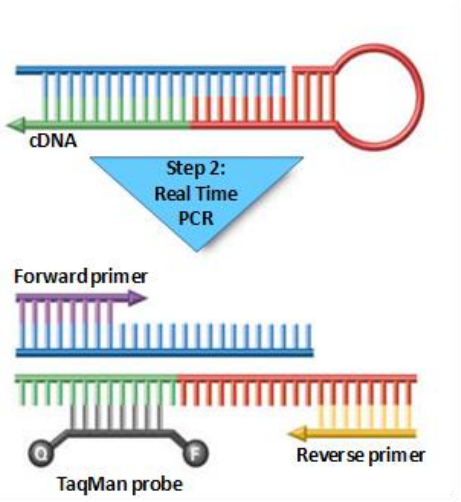

Figure 37. Representation of the reaction of RT-qPCR. Polimerization: A fluorescent reporter (F) dye and a quencher (Q) are attached to the 5' and 3' ends of a TaqMan probe, respectively. Strand Displacement: when the probe is intact, the reporter dye emission is quenched. Cleavage: during each extension cycle, the DNA polymerase cleaves the reporter dye from the probe. Polymerization Completed: once separated from the quencher, the reporter dye emits its characteristics fluorescent.

The PCR process generally consists of a series of changes of temperature that are repeated 49 times, during which the cDNA pairs with the probes and is amplified, thereby obtaining a million copies of the cDNA of interest. The amplification protocol consists of:

- 95°C for 10 minutes, which allows the denaturation and separation of the nucleic acids double chain;
- 95°C for 15 seconds, that allows the annealing and binding of the probes with the DNA template;
- 60°C for 1 hour, that facilitates the polymerization carried out by the DNA polymerase.

Evaluation of mature miRNAs levels variation in transfected cells was performed by using TaqMan MicroRNA Assays kit.

For each sample, 44 µl of Master Mix 2X were added to 6 µl of cDNA (obtained from the previous Reverse Transcription reaction). Master Mix 2X is composed of TaqMan Universal PCR Master Mix 2X, No AmpErase UNG and nuclease-free water. Specific reverse PCR probes for miRNAs of interest (miR-155, miR-221) were used normalizing their expression with respect to snRNA-U6 (primers and porobes were purchased from Applied Biosystems). After vortexing the samples, 25 µl of each mixture was plated in duplicate in Multiplate PCR Plates 96-well. To eliminate any air bubbles, the plate was centrifuged at 1500 rpm for 2 minutes at room temperature, then the plate was positioned in the suitable instrument, CFX touch Real-Time PCR Detection System and the amplification protocol described above was started.

7.3 ΔΔCt normalization model

ΔΔCt normalization model is a relative quantification method in which the normalization is performed using a reference gene. This model involves the comparison of Ct (threshold cycle) values between treated and non-treated samples. Ct values obtained, both the treated samples and the control (untreated), are normalized to an adequate endogenous miRNA housekeeping. In practical terms, the Delta Delta Ct is given from mathematical operation in which the housekeeping gene Ct are subtracted from the target gene Ct value. Each Ct obtained is compared to the ΔCt of the control sample.

$$\Delta Ct = Ct \text{ target gene} - Ct \text{ housekeeping gene}$$

$$\Delta\Delta Ct = \Delta Ct \text{ treated sample} - \Delta Ct \text{ control sample}$$

$$\text{Fold difference} = 2^{-\Delta\Delta Ct}$$

The fold difference allowed to determine the relative expression of the target gene in the treated sample with respect to the control sample.

8. TARGET GENE mRNAs QUANTIFICATION

8.1 Random RT

In this case, the reaction was performed using random hexamers as primers in order to obtain reverse transcription of the entire transcriptome. Reverse transcription was performed using TaqMan Reverse Transcription Reagents PCR kit (Applied Biosystems). 500 ng of total RNA were added to RNAse free water reaching 19.25 µl, then, after adding 1 µl of RNAse inhibitor to each sample, they were incubated at 60°C for 5 minutes in the Bio-Rad MyCycler Thermal Cycler PCR (Biorad). Subsequently, RNA samples were incubated with 2.5 µl of random hexamers for 10 minutes at room temperature and then immediately cooled. The RT reaction solution was prepared using the following reagents: TaqMan RT Buffer (10X), $MgCl_2$ (25 mM), deoxyNTPs Mixture, RNAse inhibitor and MultiScribe Reverse Transcriptase (50 U/µl). 27.25 µl of RT reaction solution were added to each sample, reaching a final volume of 50 µl, and then incubated at 48°C for 30 minutes and at 100°C for 5 minutes. After thermal cycling, cDNAs were stored at -80°C.

8.2 Real-Time qPCR of target genes

Evaluation of gene expression modulation in treated cells was performed with the quantitative real-time polymerase chain reaction (RT-qPCR) assay.

The target genes investigated in this PhD thesis were: PTEN, PUMA, $p27^{kip1}$, BCL-2, VEGF, CASP-3 and MGMT.

8.2.1 TaqMan RT-qPCR assay

The quantitative RT-qPCR assay of PTEN, PUMA, $p27^{kip1}$ and MGMT transcripts were carried out using gene-specific double fluorescently labeled probes (Applied Biosystems, Warrington Cheshire, UK). For each sample, that was prepared in duplicate, 34 µl of Master Mix 2X were added to 2 µl of cDNA (obtained from previous reverse transcription reaction), along with 14 µl of nuclease-free water. The Master Mix is composed of PCR probes, also containing the specific primers for reverse-transcripted mRNA to amplify, TaqMan Universal PCR Master Mix (Applied Biosystem, Monza, Italy) and nuclease-free water. Specific reverse PCR probes for mRNAs of interest (PTEN, PUMA, $p27^{kip1}$,

MGMT) were used normalizing their expression with respect to RPL13A.

As for miRNA RT-qPCR assay, after vortexing the samples, 25 µl of each mixture was plated in duplicate in specific PCR plates 96-well, and, after centrifuging (1500 rpm for 2 minutes RT) to eliminate air bubbles, the plate was positioned in the CFX touch Real-Time PCR Detection System (Biorad, Hercules, CA, USA) and started the following amplification protocol:

- 2 minutes at 50°C to activate the enzyme
- 10 minutes at 95°C to perform a first denaturation of cDNA
- 15 seconds at 95°C to separate the double chain created at each previous cycle
- 1 minute at 60°C to obtain the annealing and elongation

The two initial steps were performed only at the beginning of the reaction while the two last steps were repeated 45 times. Relative expression was calculated using the comparative cycle threshold method (ΔΔCt) and as reference genes the endogenous controls GAPDH and RPL13A.

8.2.2 SYBR Green RT-qPCR assay

The evaluation of caspase-3 (CASP-3) and VEGF transcripts were made through RT-qPCR SYBR Green assay and RPL13A was used to normalized their expression.

Each sample was prepared in duplicate, 44 µl of Mix 2X were added to 6 µl of cDNA. The Mix is composed of: Syber Green Super mix, forward and reverse primers (50 ng/µl) specific for the genes to investigate and nuclease free water.

Reverse and forward primers for CASP-3 cDNA were purchased by IDT (Integated DNA Technologies), while reverse and forward primers for VEGF and RPL13A were purchased by Sigma-Aldrich.

As previously described, after vortexing the samples, 25 µl of each mixture were plated and then, after centrifuging, the plate was positioned in the CFX touch Real-Time PCR Detection System. In this case, the protocol run was:

- 3 minutes at 95°C
- 10 seconds at 96°C
- 30 seconds at 60°C

This cycle was repeated 45 times. The ΔΔCt method was used to calculate the relative expression of caspase-3 and VEGF using as reference gene the endogenous control RPL13A.

9. CASPASE-3 QUANTIFICATION: Bio-Plex Pro RBM Apoptosis Assays

Caspase-3 was analyzed using Bio-Plex Pro RBM Apoptosis Assays (Bio-Rad Laboratories, Hercules, CA), that is essentially an immunoassay formatted on magnetic beads. Glioblastoma cells were seeded in 6 well plates, the day after they were treated with the compounds and after 72 hours total cell extracts were prepared. According to the manufacturer's recommendations, cells were washed with cold PBS, centrifuged and the pellet was suspended in LDB buffer by pipetting up and down; after 8 fast thermal shock cycles, using dry ice, the suspension was centrifuged at 4°C for 10 minutes at 10000 x g. Carefully the supernatant was removed and transferred in new tubes. Protein quantification was performed using BCA protein assay (Thermo Scientific); and finally for the analysis with Bio-Plex Pro RBM Apoptosis Panel 3, samples were diluted to final concentration of 500 µg/ml with LDB. As described by the manufacturer, after the preparation of the standard with a serial dilution 1:3, the assay was performed. Briefely, 10 µl of blocker was added to all the 96 wells of the plate and then 30 µl of the standard, control, sample or blank were added to the appropriate well of the plate. After having vortexed, the capture beads were added to all wells and the reaction was incubated on shaker at 850 rpm for 1 hour at RT, protected from the light. Then, the plate was washed three times with 1X assay buffer. The detection antibodies (40 µl) were added to each well and followed a second incubation (as the first one). Afterwars 20 µl of diluted streptavidin-PE (SA-PE) was added and incubated for 30 minutes at RT at 850 rpm. After three washing of the wells with 1X assay buffer, the beads were resuspended in 100 µl 1X assay buffer and incubated for 30 seconds. At the end, the seal was removed and the plate was red at low PMT with Bio-Plex 200.

RESULTS

Combined treatment with corilagin and temozolomide in glioblastoma cells

Glioblastoma multiforme is a devastating malignant brain tumor, a disease that still lack an effective treatment strategy.

To find new therapeutic approaches we investigated the efficacy of combinatorial treatment in the two GBM cellular models U251 and T98G. In particular, we were interested in the effect of the co-administration of the natural molecule corilagin (COR) with the temozolomide (TMZ) chemotherapeutic drug, one of the most used chemotherapy drug for the treatment of glioma.

1. COMPUTATIONAL STUDIES

Following the hypothesis of a possible interaction with NF-κB, docking studies were performed to identify a possible binding mode for corilagin at the DNA binding site of NF-κB. This research activity was made possible thanks to a collaboration with Dr. Giuseppe Marzaro and Prof. Adriana Chilin, Department of Pharmaceutical and Pharmacological Sciences (University of Padova). As shown in Figure 38A and summarized in Figure 38B, corilagin can establish several H-bonds and a arene-cation interaction with the positive charged Lys and Arg, that are aminoacid residues abundant in the DNA-binding region of NF-κB. In this way the interaction between the transcription factor (NF-κB) and the nucleic acid (DNA) is impaired. These results correlate with our previous EMSA experimental results (Gambari R, 2012) demonstrating that corilagin (COR) is able to suppress the level of NF-κB by preventing its ability to bind with DNA. This possible mechanism of action of corilagin is clearly different to that proposed for temozolomide, which has been shown to methylate the DNA (Zhang J, 2012).

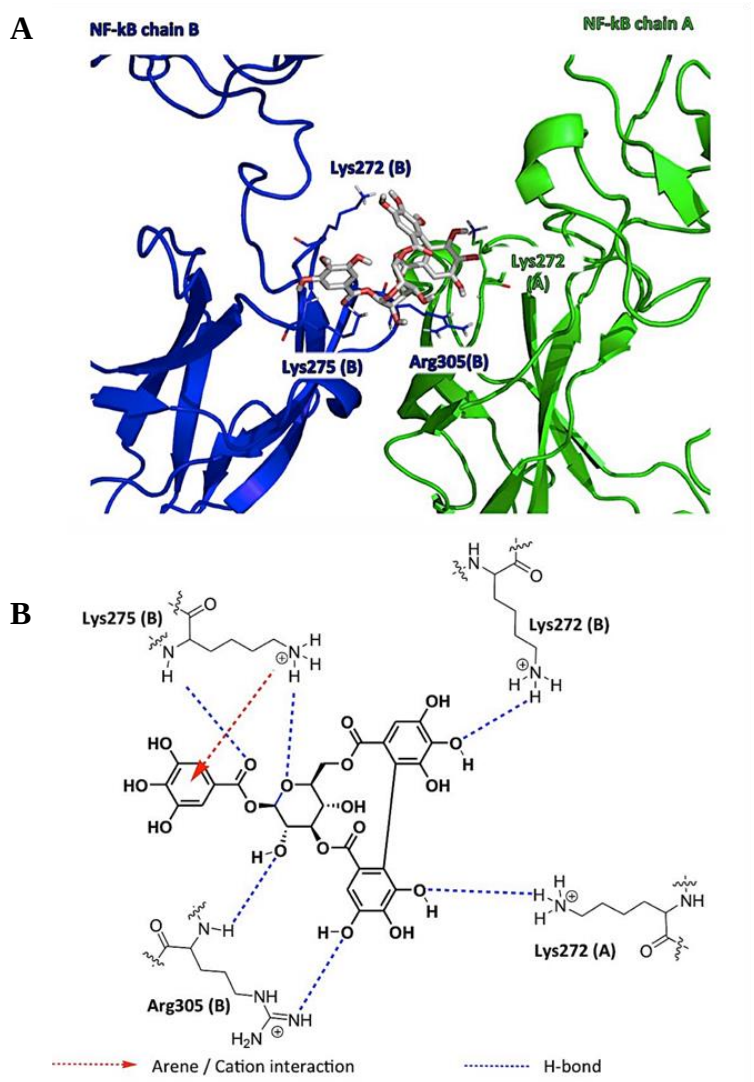

Figure 38. (A) Proposed binding site of corilagin in the DNA-binding region of NF-κB: detail of the amino acid residues involved in the proposed binding mode of corilagin (atom colored stick) with NF-κB (green and blue cartoons). (B) Schematic representation of the interactions revealed by molecular docking simulation between corilagin and NF-κB.

2. PROLIFERATION OF *IN VITRO* GLIOBLASTOMA CELLS

In order to perform a suitable treatment on the different tumor cellular models employed, the glioblastoma U251 and T98G cell lines, it was necessary to assess the anti-proliferative and cytotoxic activity of corilagin, the investigated compound in this thesis, and temozolomide.

Anti-proliferative activity of temozolomide

We first wanted to confirm the known effect of the single treatment with temozolomide on U251, a TMZ-sensitive cell line, and T98G, a TMZ-resistant cell line. For this purpose, we

treated the cells with different concentrations of TMZ for 48 hours and determined the cell number per ml using the Z2 Beckman Coulter Counter. As expected (Figure 39), T98G cells resulted resistant to TMZ treatment; in fact, only high concentrations (about 400 µM) of TMZ caused a reduction of cell growth after comparison to untreated cells (-), and the IC_{50} value (drug concentration able to inhibit of 50% the cell growth with respect to untreated cells) was obtained at about 560 µM of TMZ, a concentration 12 fold higher than the one found in the TMZ-sensitive U251 cells. In fact, TMZ treatment in U251 cells resulted in a decrease in proliferation at a concentration of 10 µM compared with untreated cells (-), and the IC_{50} was found to be at about 44 µM.

These results confirm the anti-proliferative activity of TMZ in U251 cells and the resistance of T98G cells to this compound.

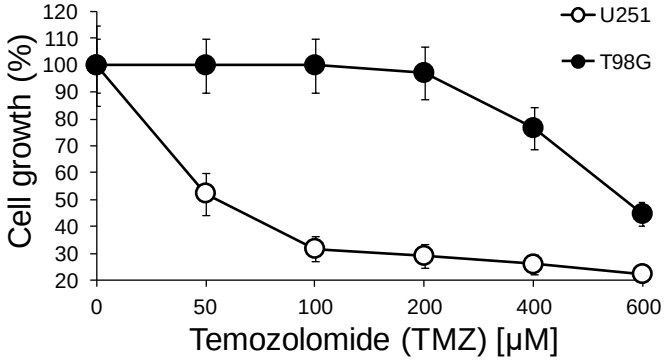

Figure 39. Effect of increasing concentrations of TMZ on proliferation of T98G and U251 glioblastoma cells. Cells were cultured for 48 hours in the presence of TMZ. The number of cells/ml counted and the percentage of cell growth efficiency was evaluated with respect to untreated control cells.

The concentration of TMZ used in the following experiments (400 µM) was determined on the basis of these results, which are also the demonstration that T98G cells are resistant to TMZ treatment. This evidence was the starting point for my project.

Anti-proliferative activity of corilagin

In order to determine the effect of corilagin on the glioblastoma cell lines T98G and U251 cells we evaluated its effects on cell proliferation and its IC_{50} was identified on both the glioblastoma cell lines T98G and U251. To define the conditions of treatment of corilagin, cells were cultured with increasing concentrations (10, 25, 35, 40, 50, 75, 100 µM) of COR at three time points 24, 48 and 72 hours. As shown in Figure 40, COR displayed a dose and

time-dependent anti-proliferative effect on both U251 (Figure 40A) and T98G (Figure 40B) cell lines. In fact, after 24-hour treatment, U251 cells growth was found to be similar to the untreated cells up to the 50 µM concentration, then from 75 µM there is a cytotoxic effect. In T98G cells, cell growth is maintained around 80% respect to the untreated cells also at high concentrations.

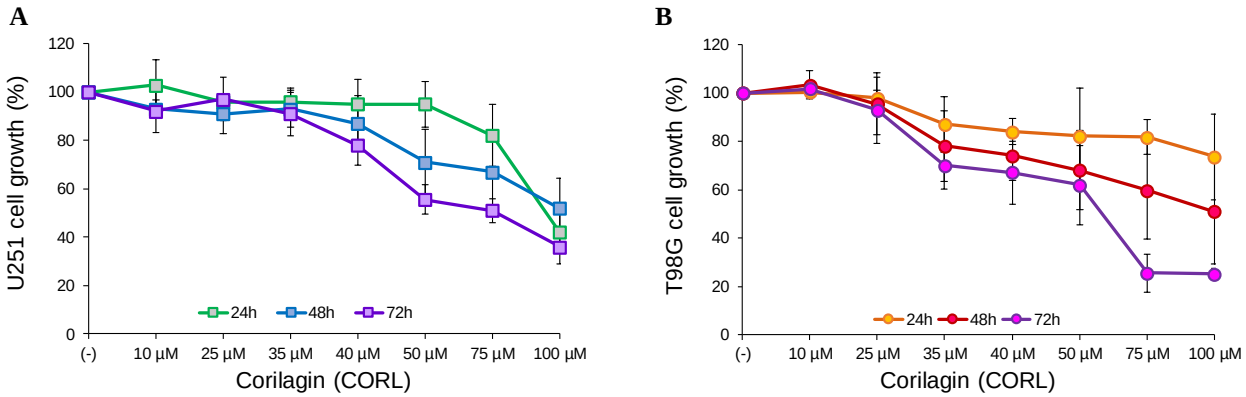

Figure 40. Effects of increasing concentrations of corilagin on U251 (A) and T98G (B) cells proliferation. Cells were cultured for 24, 48 and 72 hours in the presence of COR and then number of cells/ml was counted. Results are expressed as 'Cell growth (%)' which was calculated considering the cell/ml value of non-treated cells as the 100%. Results are expressed as average ± S.D (five independent experiments performed).

After 2 days of treatment with corilagin both glioblastoma cell lines showed a reduction in cell growth of about 30% compared to untreated controls at a concentration of 50 µM of COR. After 72 hours of treatment the number of T98G and U251 cells was strongly reduced starting from 50 µM concentration, and the anti-proliferative effect is more evident in T98G cells where with COR 75 µM, their growth was drastically reduced by 80% compared to untreated cells. The IC_{50} values (Table 5) at 48 hours was found to be 85 ± 9.14 µM for U251 cells and 94 ± 4.51 µM for T98G cells.

These results suggest that both cell lines are sensitive to corilagin treatment. For following experiments and our purpose to investigate the effects of the combined treatment of corilagin and temozolomide (COR + TMZ), we selected a concentration lower than the one inducing the 50% of cell growth inhibition. Thus, 35 µM COR was used for the next studies.

Table 5. IC_{50} values calculated after 24, 48 and 72-hour treatment of U251 and T98G glioblastoma cells with increasing concentrations of corilagin.

	U251 IC_{50}	**T98G** IC_{50}
24 h	86 ± 6.56 µM	125 ± 8.23 µM
48 h	85 ± 9.14 µM	94 ± 4.51 µM
72 h	78 ± 5.34 µM	61,2 ± 3.14 µM

Anti-proliferative effect of combined treatment (COR+TMZ)

As already stated, the aim of this project was to investigate whether corilagin has anticancer effect on TMZ-resistant T98G glioblastoma cells and whether it could be able to sensitize these cells to the TMZ treatment. We performed the following experiments on combined treatment only on T98G cells with 48 hours of incubation. Since 35 µM corilagin could only exert a very low anti-proliferative effect on the T98G cells and since the 400 µM high concentration of temozolomide had no significant effect on the proliferation of this cell line, we performed experiments of 48-hour treatment with single COR (35 µM) and TMZ (400 µM) administrations and with the combination of them. Then, the cell number/ml was determined with a Z2 Beckman Coulter Counter and the percentage of cell growth was calculated with respect to untreated control cells (-). The histograms depicted in Figure 41 show that 48-hour treatment with corilagin and temozolomide alone did not lead to any statistically significant anti-proliferative effect, but the combination of them significantly reduces cell growth of about 40% with respect to untreated cells. Therefore, corilagin could significantly potentiate the cytotoxicity of the TMZ chemotherapeutic drug, reducing its IC_{50} values.

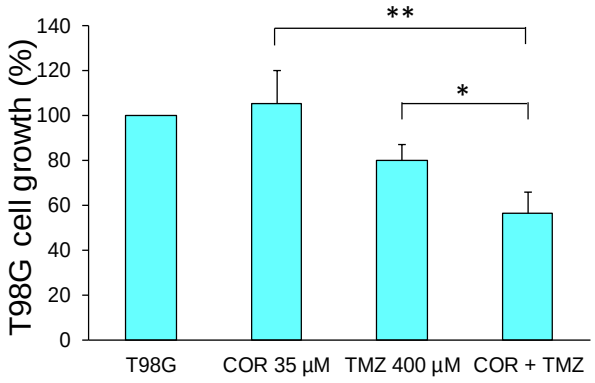

Figure 41. Effects of 35 µM COR, 400 µM TMZ and COR (35 µM) plus TMZ (400 µM) on the proliferation of T98G cells. Cell growth was evaluated after 48-hour treatment. The data represent the average ± SD from three independent experiments and statistical significance was determined by one-way ANOVA; (*) $P<0.05$ comparing COR + TMZ to TMZ and (**) $P<0.01$ compared to COR.

2.1 T98G glioblastoma cells viability

In order to verify the viability of the T98G glioblastoma cells exposed to a combined treatment in term of membrane integrity, a viability assay was performed.

When Muse Count and Viability assay was performed, as shown in Figure 42, it can be observed that the cells treated with 35 µM COR, 400 µM TMZ and COR (35 µM) plus TMZ (400 µM) for 48 hours, do not exhibit significant increase of cytotoxicity. However, after a combined treatment of T98G cells, an increase of proportion of cells that are either dead or dying (after comparison with untreated cells or cells treated with single compounds administration) was detectable.

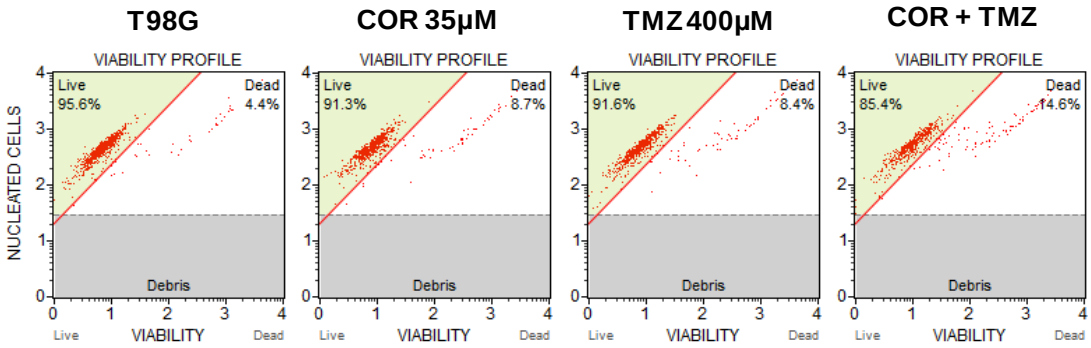

Figure 42. A representative example of the viabiliy assay performed on T98G cells treated with 35 µM COR, 400 µM TMZ or 35 µM COR + 400 µM TMZ. After 48 hours, cells were detached, incubated with the DNA-binding dyes of the Muse viability assay and then analyzed. Cells are recognized as live or dead based on their membrane integrity.

3. INDUCTION OF APOPTOSIS IN GLIOBLASTOMA CELLS

3.1 Effects of corilagin on U251 and T98G glioblastoma cells

Corilagin, as previously described in this thesis, is a novel interesting compound for anti-cancer therapy because, among its activities, it is also capable of inducing apoptosis in cancer cells with low toxicity against normal cells (Jia L, 2013; Ming Y, 2013; Yang WT, 2016). Furthermore, corilagin is able to interact with the anti-apoptotic factor NF-κB (Figure 38) inhibiting its functions (Gambari R, 2012). Therefore, we examined whether corilagin induces apoptosis in our glioblastoma cell models U251 and T98G cells. To this aim the cells were treated for 48 hours with increasing concentrations of corilagin, then Annexin V assay was performed using the Muse Cell Analyzer. The assay is based on the evaluation of the exposure of phosphatidylserine on the cell membrane, an event occurring in cells during the apoptotic process.

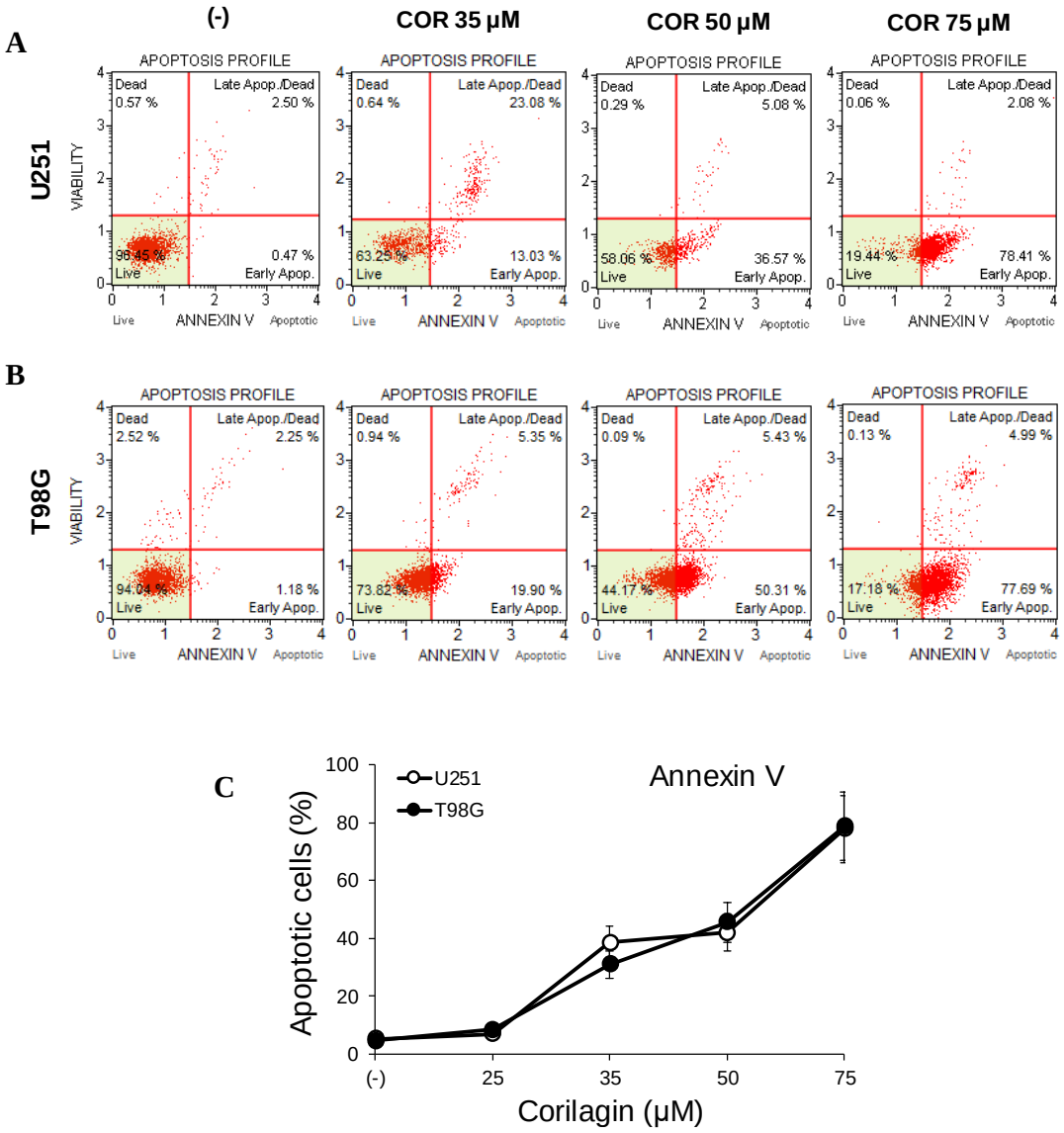

Figure 43. Effect of increasing concentrations (25 to 75 µM) of corilagin on apoptosis of U251 (A) and T98G (B) cells. Representative example of the increase of apoptotic cells after 48-hour treatment are shown in panels A and B. (C) Summary of the results obtained in three independent experiments. The effects of the treatments described in (A) and (B) are reported for U251 (white symbols) and T98G (back symbols); the data represent the sum of the percentage of early and late apoptotic cells (average ± SD, n = 3). The apoptosic phenotype was determined using the Annexin and Dead cell kit with Muse.

Figure 43 sustains the pro-apoptotic effect of corilagin; the number of cells, most of all in early apoptosis, increases with the increasing of corilagin concentration, on both the glioblastoma cell lines. The percentage of apoptotic cells was around 80% at the concentration of 75 µM. This interesting evidence, prompted us to focus the studies on T98G TMZ-resistant cells.

3.2 Co-treatment of T98G glioma cells with corilagin and temozolomide

As shown in Figure 39, the T98G cell line is resistant to temozolomide (TMZ) and a very low increase of apoptosis (Figure 44) was observed when these cells were treated with 400 µM TMZ.

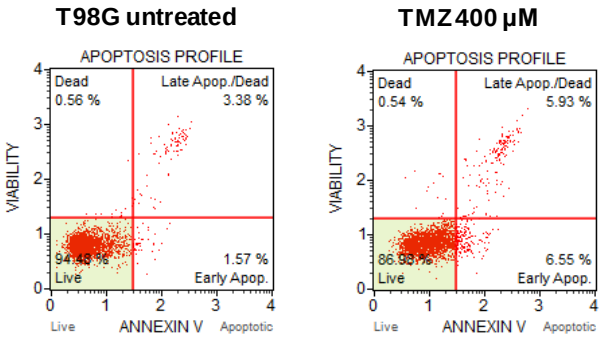

Figure 44. Lack of efficient pro-apoptotic effects of TMZ on the T98G cell line. T98G cells were cultured for 48 h without or with TMZ and then the induction of apoptosis determined by Annexin V assay.

One of the hypothesized mechanism that cause drug resistance of T98G cells is the mechanism of cell repair that commonly protect both normal and cancer cells (Zhang J, 2012). Since TMZ is a chemotherapy drug used in glioblastoma and neuroblastoma therapy, the choice and use of appropriately combined treatment might be an important step to overcome temozolomide tolerance. Therefore, corilagin with its antiproliferative and pro-apoptotic effects, represented to us a possible candidate for the study of combined treatments in T98G glioblastoma cells.

Annexin V assay

In order to determine whether a combined treatment of TMZ and COR on T98G cells could be an efficient treatment against glioblastoma we investigated their combined effect on cell apoptosis. For the following experiments 35 µM corilagin was chosen, since no effect was seen at lower concentrations. T98G were cultured for 48 and 72 hours in the presence of 35 µM COR, 400 µM TMZ and with the combination of these drugs.

The results obtained by the Annexin V assay show that co-administration of corilagin and temozolomide induced apoptosis of TMZ-resistant T98G cells at a level higher (39.64 ± 9.50) than that obtained following single administration of COR (26.76 ± 9.07) and TMZ (13.20 ± 2.52) (Figure 45A). The effect after 48 or 72 hours of treatment resulted similar and most of the cells were in early apoptosis. As shown in the representative example

reported in Figure 45B, the percentage of apoptotic cells with the combined treatment was usually higher than the sum of apoptotic cells of each single treatment. The results obtained suggest that COR and TMZ possibly act in a synergistic effect in inducing apoptosis of T98G cells.

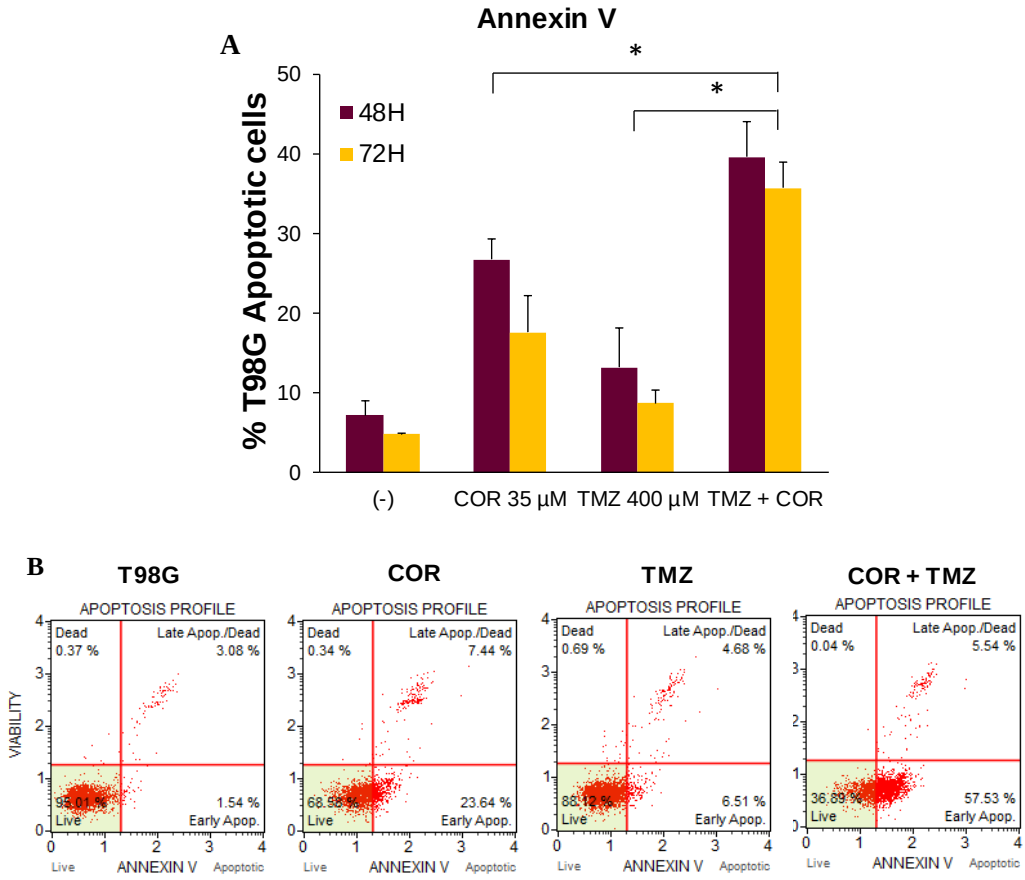

Figure 45. (A) Pro-apoptotic effect on T98G cells treated with 35 µM COR, 400 µM TMZ and COR (35 µM) plus TMZ (400 µM) after 48 and 72 hours. The data represent the average ± SD from five independent experiments, and include cells in early and late apoptosis. Statistical significance was determined by one-way ANOVA, (**) P<0.01 for COR+TMZ compared to single treatments (B) Representative example of Annexin V assay performed on T98G cells treated for 48 hours as described above.

Table 6. Effect of COR (35 µM), TMZ (400 µM) and COR + TMZ after 48 and 72 hours of treatment. The percentage of the cell populations indicated in Annexin V assay are reported. Total Apoptotic % includes the percentage of cells in early and late apoptosis. Results are expressed as average of five independent experiments performed.

	48 h			**72 h**		
	Live %	**Total apoptotic %**	**Δ**	**Live %**	**Total apoptotic %**	**Δ**
T98G	92.83	7.17		95.14	4.86	
COR	73.24	26.76	*19.59*	82.39	17.61	*12.75*
TMZ	86.80	13.20	*6.13*	91.27	8.73	*3.87*
COR + TMZ	60.36	39.64	*32.47*	64.24	35.76	*30.90*

Caspase 3/7 assay

The pro-apoptotic activity and the possible synergism between COR and TMZ were further verified by Caspase 3/7 assay, based on another marker of apoptosis. These analyses were conducted on the same plates used for the Annexin V assay, so it was possible to compare the results. Figure 46 shows that, after 48 hours, the combination of TMZ with 35 µM corilagin induces a pro-apoptotic effect (about 21%) that is higher than that caused by the single addition of COR (about 14%) or TMZ (about 10%).

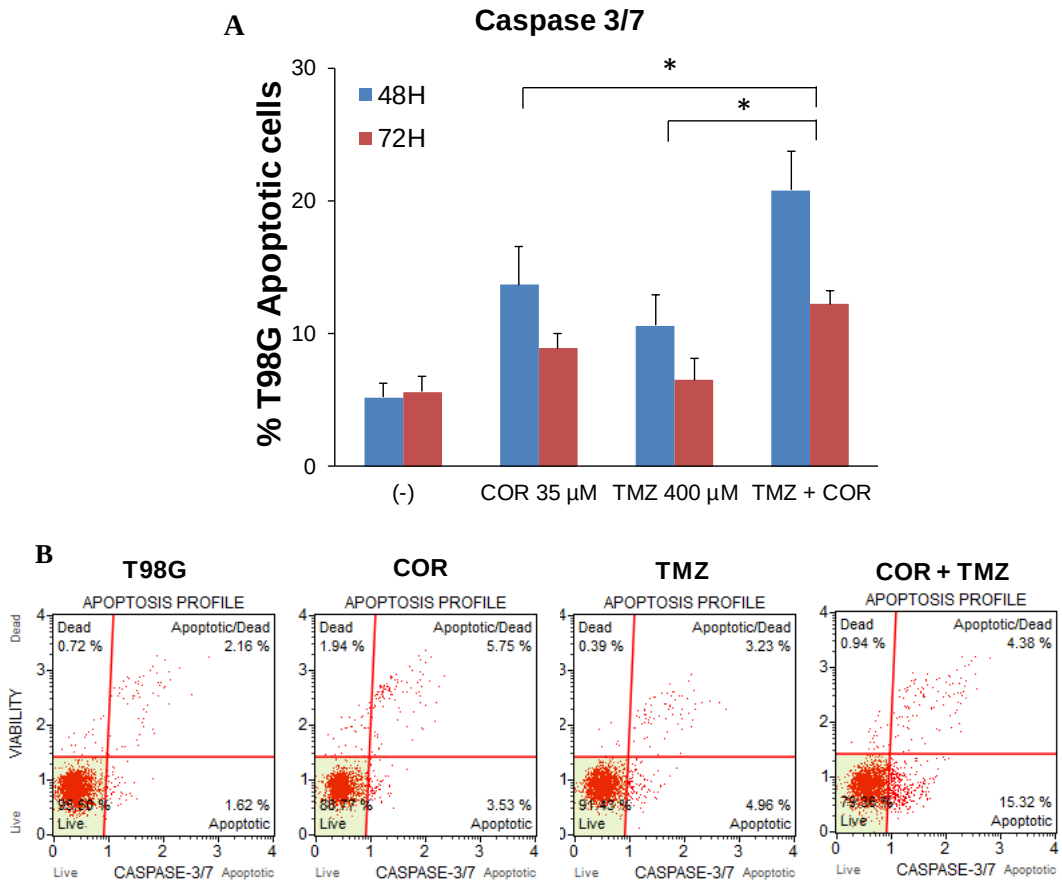

Figure 46. (A) Pro-apoptotic effect on T98G cells treated with 35 µM COR, 400 µM TMZ and the combined treatment after 48 and 72 hours. The data represent the average ± SD from five independent experiments and include cells in apoptosis and late apoptosis/dead. Statistical significance was determined by one-way ANOVA, (*) P<0.05 for COR+TMZ compared to single treatments (B) Representative example of Caspase 3/7 assay performed on T98G cells treated for 48 hours as described above.

Table 7. Effect of COR (35 µM), TMZ (400 µM) and COR + TMZ after 48 and 72 hours of treatment. The percentage of the cells with a positive casapse 3/7 are reported. Total Apoptotic % includes the percentage of apoptotic cells and apoptotic/dead cells. Results are expressed as average of five independent experiments performed.

	48 h			72 h		
	Live %	**Total Caspase-3/7 reactive cells %**	**Δ**	**Live %**	**Total Caspase-3/7 reactive cells %**	**Δ**
T98G	94.82	5.18		94.48	5.52	
COR	86.30	13.70	*8.52*	91.10	8.90	*3.38*
TMZ	89.37	10.63	*5.45*	93.56	6.44	*0.92*
COR + TMZ	79.17	20.83	*15.65*	87.78	12.22	*6.70*

The data obtained by the Caspase 3/7 assay confirm a possible synergistic effect of COR and TMZ to induce the apoptotic pathway when co-administered to T98G cells.

TUNEL assay

The comprehension of how a compound acts on a specific pathway is very important to sustain its possible use for a therapeutic treatment. Therefore, fluorometric TUNEL assay was also performed to study corilagin activity and its effect when combined with TMZ. TUNEL assay has been designed to detect apoptotic cells that undergo extensive DNA degradation during the late stages of apoptosis.

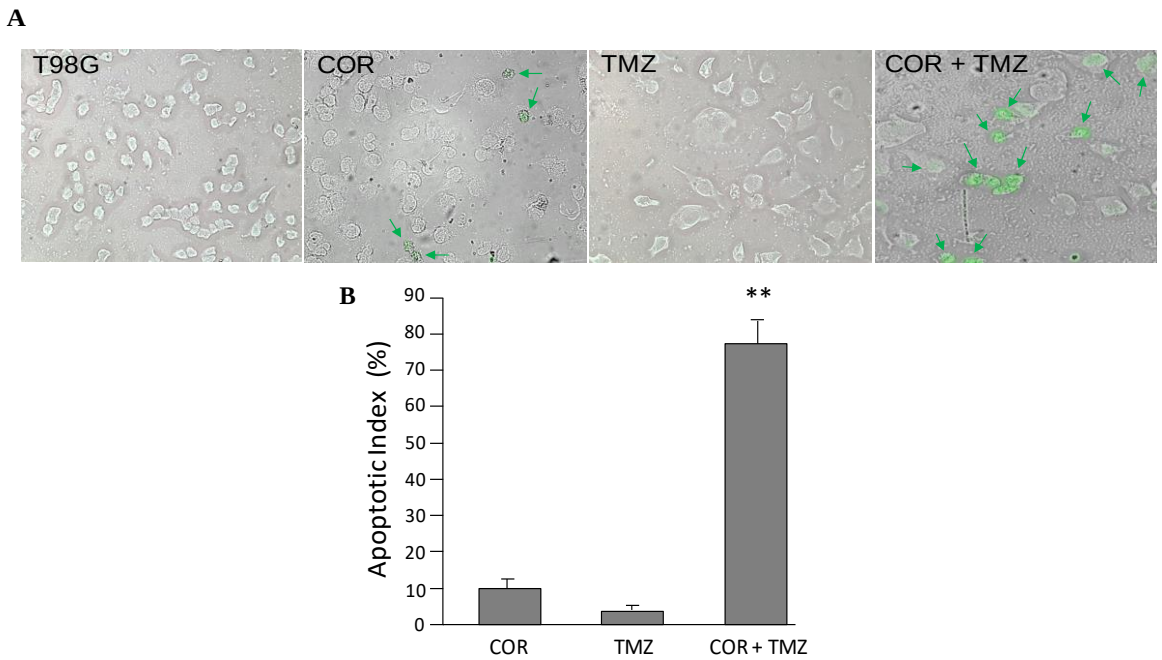

Figure 47. TUNEL Staining of T98G glioblastoma cells. (A) Representative example. Green arrows indicate the apoptotic cells. (B) The apoptotic index was estimated as follows: (number of TUNEL-positive cells)/(total number of epithelial cells) × 100. The data represent the average of three independent experiments ± S.D and statistical significance was determined by one-way ANOVA; (**) P<0.05 comparing COR + TMZ to TMZ and COR.

After 48 hours of treatment, T98G cells were fixed with formaldehyde and the assay was performed. The images were taken with a fluorescence microscopy and, as it is shown in Figure 47, corilagin (35 µM) did not induce a significant apoptotic effect, but suffering of the cells can be appreciated. Their morphology is different from untreated T98G cells or cells treated with TMZ, indicating that probably they are activating some phases of apoptosis. On the contrary, in the cell populations treated with both COR and TMZ, cells positive to the apoptosis assay (about 80%) are clearly detectable. With respect to untreated T98G cells or cells exposed to single treatments, the number of cells is lower and their morphology resembles that of apoptotic cells.

4. MODULATION OF GENES INVOLVED IN GLIOBLASTOMA PROGRESSION

The interesting results obtained from the analysis on cell proliferation and apoptosis prompted us to investigate the role of corilagin in the modulation of some genes involved in key pathways leading to cancer development and progression.

After 48-hour treatment of the TMZ-resistant T98G cells with 35 µM corilagin, total RNA was extracted and reverted using random hexamers. The RT-qPCR analysis was performed using specific primers and probes, and RPL13A was chosen as reference gene. Data were normalized with respect to untreated sample and the ΔΔCt method was used to calculated genes relative expression.

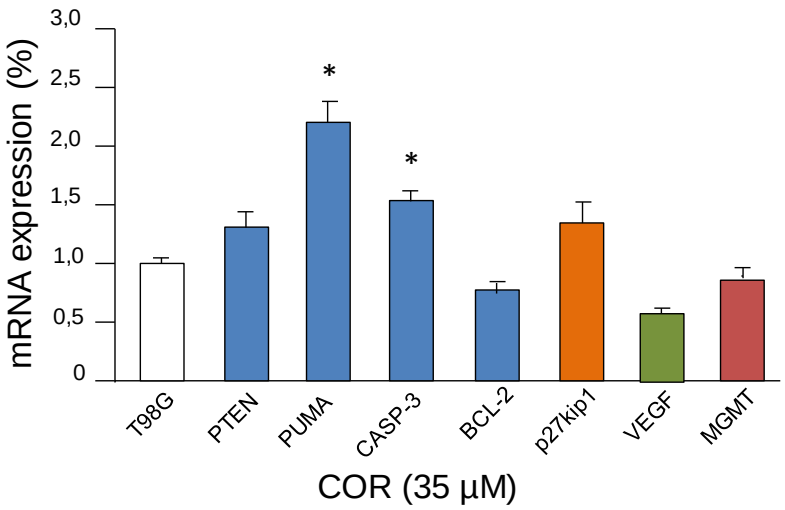

Figure 48. RT-qPCR amplifications were performed on RNA from untreated or treated with 35 µM COR T98G cells using primers amplifying RPL13A as reference gene. Results are presented as fold changes of PTEN, $p27^{kip1}$, PUMA, CASP-3, BCL-2 and VEGF mRNA content with respect to the untreated cells. The data represent the average ± SD from three independent experiments and statistical significance was determined by one-way ANOVA, (*) $P<0.05$ compared to untreated T98G cells.

The mRNA of the $p27^{kip1}$ protein, associated to cell cycle, and the mRNAs of pro-apoptotic genes, such as PTEN, PUMA and caspase-3 were found to be increased with respect to the untreated T98G cells. Conversely, the expression of the anti-apoptotic BCL-2 mRNA and the angiogenetic VEGF mRNA were found decreased after corilagin treatment (Figure 48).

4.1 Co-treatment of corilagin with temozolomide: effects on MGMT

Many applied studies are at present aimed at rendering glioblastoma cells more susceptible towards therapy-induced apoptosis. However, interventions are, as already pointed out, complicated by resistance towards therapeutic agents. The alkylating cytostatic drug TMZ constitutes, in combination with radiotherapy, the current therapy for glioblastoma. However, its activity may be counteracted in the tumors by expression of the DNA repair enzyme MGMT, which repairs the temozolomide-induced DNA lesion.

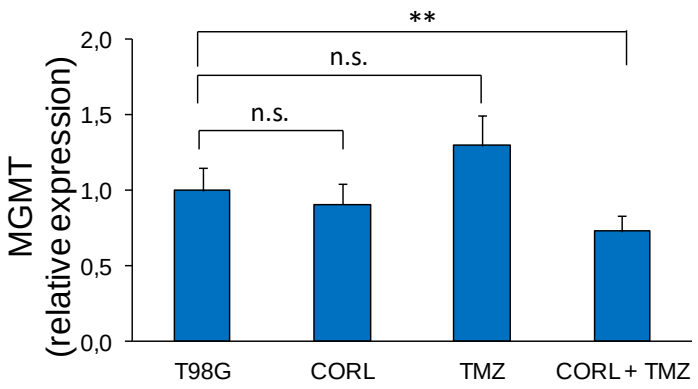

Figure 49. Expression of MGMT mRNA in T98G glioma cells treated for 48 h with 35 µM COR, 400 µM TMZ and the combined treatment. RT-qPCR was performed and the ΔΔCt method was used considering RPL13A gene as reference and normalization of data was obtained in comparison to untreated samples. The data represent the average ± SD from three independent experiments.

Therefore, it seemed interesting to verify whether corilagin could decrease the expression of MGMT in T98G TMZ-resistant cells. As shown in Figure 49, after 48-hour treatment, corilagin slightly decreased MGMT mRNA expression. The downregulation of the repairing gene was even higher when COR is co-administered with TMZ. This result suggests a possible sensitization of T98G cells by corilagin in the response to temozolomide treatment.

5. INHIBITION OF CELL MIGRATION

Cell migration is a fundamental process in the development and maintenance of multicellular organisms, and it is also involved in immune response. Error during this process have serious consequence, including the tumor formation and metastasis. Therefore, we assessed the effect of corilagin on the ability of TMZ-resistant T98G cells to migrate by scratch assays. Yue G et al. (2016) already reported the effect of corilagin on cholangiocarcinoma cells by scratch test; but here, the aim was also to verify if corilagin together with TMZ could have a higher inhibitory effect on cancer cell migration. We created a gap called "scratch", using a 200-µl pipette tip on a confluent T98G monolayer cells. After having removed the detached cells, they were treated with 35 µM COR, 400 µM TMZ and co-treated with both drugs. Pictures were captured at the beginning and at regular intervals during cell migration to evaluate the closure of the scratch.

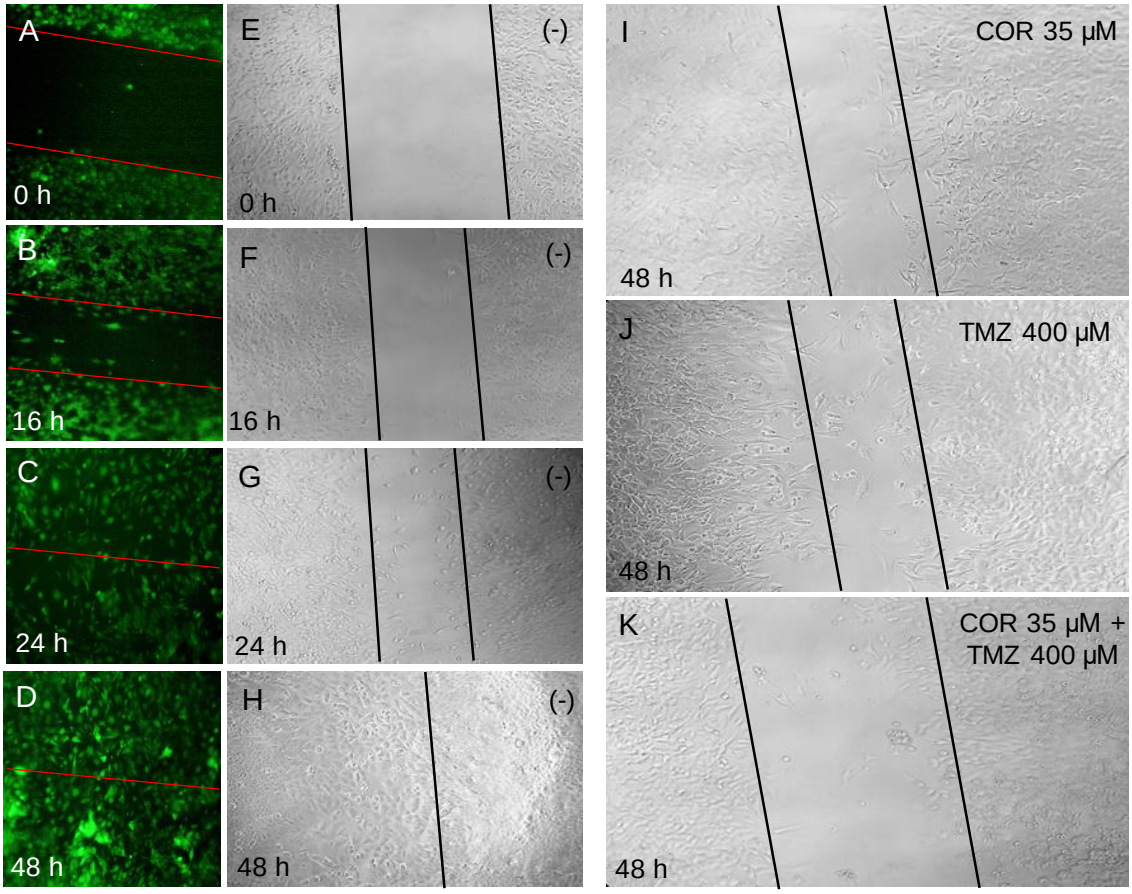

Figure 50. Corilagin decreases cell migration *in vitro*. Alterations in TMZ-resistant T98G cell migration following treatment with corilagin and temozolomide were assessed using a wound scratch healing assay. (A-D) Wound scratch healing assay assessed using CellTracker fluorescent dye and the microscope Nikon Eclipse 80i. T98G untreated cells were monitored for 48 hours until the scratch has been closed. (E-H). Cell migration of untreated T98G cells (-) after 16 hours (F), 24 hours (G) and 48 hours (H) after the scratch. After 48 hours of treatment with COR (I) and TMZ (J) the scratch is still opened, but the co-treatment (K) strongly inhibited the cell invasion of the scratch.

The lines drawn in the pictures (Figure 50) define the area of the gap and allow to follow the migration of the cells during the time. Untreated T98G cells (Figure 50 A-D and E-H), completely close the scratch within 48 hours; on the contrary cells treated with single administration of COR (Figure 50I) and TMZ (Figure 50J), migrate but cannot invade the whole gap within 48 hours. Interestingly with the combined treatment (Figure 50K) the gap is after 48 hours still large and similar to that created at the beginning of the experiment.

Combined treatment with temozolomide and anti-miR-155 or anti-miR-221 PNAs in glioblastoma cells

1. IDENTIFICATION OF microRNA TARGETS

In order to identify possible miRNA targets within sequences of mRNAs coding proteins important for the control of the apoptotic pathway, we compared three sets of miRNAs: (a) miRNAs highly expressed in tissues from glioblastoma patients; (b) miRNAs validated in glioblastoma for their oncogenic properties and (c) miRNAs putatively interacting with the 3'UTR of caspase-3 mRNA, which is deeply involved in activation of the apoptotic pathway. The comparison of the three lists (reported in Table 8) is shown in Figure 51, depicting the Vienn diagram obtained. As clearly evident only three microRNA sequences were common within the three sets, miR-155, miR-221 and miR-30a. Interestingly, miR-221 was already found to exhibit anti-apoptotic effects on gliomas, and miR-221 targeting with antisense peptide nucleic acid (PNA) was able to induce apoptosis and reverse TMZ-resistance (Brognara E, 2014). Similarly, miR-155 was demonstrated to be involved in the sensitization of glioma cell lines to anti-tumor drugs. On the contrary, no report is to our knowledge available on the possible involvement of miR-30a to activation of drug resistance in gliomas. For these reasons, we focused our attention on targeting miR-155 and miR-221.

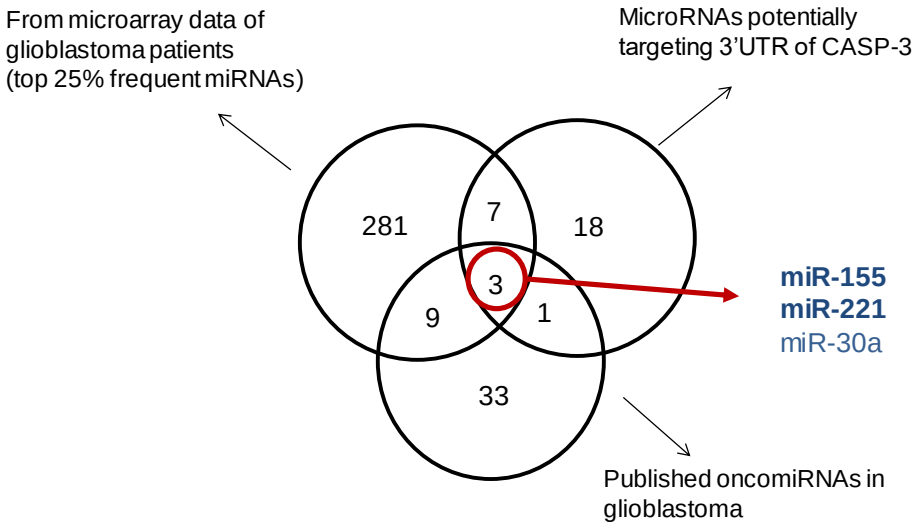

Figure 51. Venn diagram showing the relationship between the most frequent over-expressed miRNAs in glioblastoma patients, glioblastoma oncomiRNAs published in PubMed and miRNAs involved in glioblastoma potentially targeting CASP-3 mRNA selected from a research in miRNA databases.

Table 8. List of three sets of microRNAs analysed. The list of miRNAs highly expressed in glioblastoma patients were from Khalil et al. (2016)

SET OF miRNAs	miRNAs LIST					
miRNAs from microarray data of glioma patients	let-7a-5p	miR-1285-3p	miR-204-5p	miR-3194-5p	miR-3907	miR-564
	miR-103a-3p	miR-1288	miR-20a-5p	miR-3195	miR-3911	miR-566
	miR-10b-3p	miR-1290	miR-210	miR-3196	miR-3917	miR-572
	let-7e-5p	miR-1299	miR-212-3p	miR-3198	miR-3925-5p	miR-574-3p
	let-7c	miR-1305	miR-21-3p	miR-3202	miR-3926	miR-574-5p
	miR-1237-3p	miR-1306-3p	miR-21-5p	miR-320a	miR-3934-5p	miR-575
	let-7i-5p	miR-130a-3p	miR-221-3p	miR-320b	miR-3935	miR-584-5p
	let-7f-5p	miR-130b-3p	miR-222-3p	miR-320c	miR-3937	miR-601
	let-7g-5p	miR-1321	miR-223-3p	miR-320d	miR-3945	miR-602
	miR-1229-3p	miR-134	miR-22-3p	miR-320e	miR-423-5p	miR-605
	miR-1234-3p	miR-135a-3p	miR-2276	miR-324-3p	miR-424-3p	miR-610
	let-7b-5p	miR-138-2-3p	miR-2277-3p	miR-324-5p	miR-4253	miR-622
	miR-1180	miR-139-3p	miR-23a-3p	miR-330-3p	miR-425-3p	miR-623
	miR-1202	miR-140-3p	miR-23a-5p	miR-331-3p	miR-4257	miR-630
	miR-1182	miR-140-5p	miR-23b-3p	miR-33b-3p	miR-4259	miR-631
	let-7d-5p	miR-145-5p	miR-24-3p	miR-342-3p	miR-4261	miR-636
	miR-1183	miR-1469	miR-25-3p	miR-345-5p	miR-4267	miR-638
	miR-100-5p	miR-1471	miR-26a-5p	miR-34a-5p	miR-4269	miR-642b-3p
	miR-1207-5p	miR-148a-3p	miR-26b-5p	miR-3605-5p	miR-4270	miR-648
	miR-1208	miR-149-3p	miR-27a-3p	miR-3610	miR-4271	miR-650
	miR-1228-3p	miR-149-5p	miR-27b-3p	miR-3614-5p	miR-4281	miR-659-3p
	miR-1226-5p	miR-150-3p	miR-2861	miR-361-5p	miR-4284	miR-662
	miR-10b-5p	miR-151a-3p	miR-29a-3p	miR-3620-3p	miR-4286	miR-663a
	miR-1225-3p	miR-151a-5p	miR-29b-3p	miR-3621	miR-4291	miR-664a-5p
	miR-107	miR-1539	miR-29c-3p	miR-3622b-5p	miR-4294	miR-665
	miR-106b-5p	miR-155-5p	miR-30a-3p	miR-3646	miR-4298	miR-671-5p
	miR-1224-5p	miR-15a-5p	miR-30a-5p	miR-3648	miR-4299	miR-711
	miR-1181	miR-15b-5p	miR-30b-5p	miR-3651	miR-4304	miR-718
	miR-1225-5p	miR-16-5p	miR-30c-2-3p	miR-3652	miR-4306	miR-720
	miR-1238-3p	miR-17-5p	miR-30c-5p	miR-3654	miR-4313	miR-760
	miR-124-3p	miR-181a-5p	miR-30d-5p	miR-3656	miR-4314	miR-762
	miR-1246	miR-181b-5p	miR-30e-5p	miR-3659	miR-4322	miR-765
	miR-1249	miR-181c-5p	miR-3121-3p	miR-365a-3p	miR-4327	miR-766-3p
	miR-125a-3p	miR-181d	miR-3125	miR-365b-5p	miR-451a	miR-770-5p
	miR-125a-5p	miR-1825	miR-3127-5p	miR-3660	miR-483-5p	miR-874
	miR-125b-2-3p	miR-187-5p	miR-3131	miR-3663-3p	miR-486-5p	miR-877-3p
	miR-125b-5p	miR-188-5p	miR-3132	miR-3663-5p	miR-494	miR-877-5p
	miR-1260a	miR-191-3p	miR-3137	miR-3665	miR-497-5p	miR-887
	miR-1260b	miR-1914-3p	miR-3138	miR-3667-5p	miR-498	miR-892b
	miR-126-3p	miR-1915-3p	miR-3141	miR-3676-3p	miR-500a-5p	miR-92a-3p
	miR-1268a	miR-193b-3p	miR-3147	miR-3679-5p	miR-501-5p	miR-92b-3p
	miR-1273c	miR-193b-5p	miR-3154	miR-3680-3p	miR-513a-5p	miR-93-5p
	miR-1273d	miR-195-5p	miR-3156-5p	miR-3682-3p	miR-513b	miR-936
	miR-1273e	miR-1972	miR-3161	miR-3692-5p	miR-513c-5p	miR-939-5p
	miR-1274a	miR-1973	miR-3162-5p	miR-370	miR-514b-5p	miR-9-3p
	miR-1274b	miR-197-3p	miR-3173-3p	miR-3713	miR-520b	miR-940
	miR-1275	miR-198	miR-3174	miR-371a-5p	miR-520e	miR-9-5p
	miR-128	miR-19a-3p	miR-3180-3p	miR-373-5p	miR-548q	miR-99a-5p
	miR-1280	miR-19b-3p	miR-3188	miR-378_v17.0	miR-550a-5p	miR-99b-3p
	miR-1281	miR-202-3p	miR-3190-3p	miR-378b	miR-557	miR-99b-5p

Published oncomiRNAs in glioma	miR-10a	miR-25
	miR-10b-3p	miR-26a
	miR-125b	miR-27b
	miR-130b-3p	miR-29a
	miR-143	miR-30a-5p
	miR-145	miR-31
	miR-148a-3p	miR-323a
	miR-155-5p	miR-323b
	miR-15a-5p	miR-328
	miR-16-1	miR-335
	miR-16-2	miR-363
	miR-17-5p	miR-381
	miR-182	miR-455-3p
	miR-18a	miR-582-5p
	miR-193a	miR-603
	miR-195-5p	miR-873
	miR-196b	miR-9
	miR-19a	miR-92a
	miR-19b	miR-92b
	miR-200a	miR-93
	miR-200b	miR-21-3p
	miR-20a-5p	miR-210
	miR-20b	miR-221-3p

miRNAs potentially targeting 3'UTR of CASP-3	let-7a-5p	miR-382-5p
	let-7g-5p	miR-421
	miR-128-3p	miR-4666b
	miR-138-5p	miR-5011-3p
	miR-155-5p	miR-548
	miR-196a-5p	miR-562
	miR-221-3p	miR-582-5p
	miR-26b-5p	miR-6076
	miR-30a-5p	miR-6124
	miR-30b-5p	miR-651-3p
	miR-30d-5p	miR-6797-3p
	miR-363-3p	miR-885-5p
	miR-3680-3p	miR-888-5p
	miR-374a-5p	miR-30e-5p
	miR-375	

2. EXPRESSION OF microRNA-155 AND microRNA-221 IN TUMOR CELLS

MiR-221 and miR-155 are usually overexpressed in many tumors including glioblastoma. Their downregulation, using the antagomiRNAs strategy, was associated to reduced cancer cells growth and migration, inhibition of cell cycle progression, induction of apoptosis and chemosensitization of tumors, including drug-resistant tumor clones (Brognara E, 2014; He S, 2015; Meng W, 2012).

Firstly, the content of miR-155 and miR-221 in U251 and T98G glioblastoma cell lines was analyzed, in order to quantify their basal expression levels in untreated cells. The RT-qPCR results obtained indicate that both the analyzed miRNAs are highly expressed in

U251 cells with respect to T98G cells (Figure 52). However, we can conclude that in both glioma cell lines the extent of the expression of these miRNAs is sufficient to consider these molecules as potential targets for therapeutic interventions.

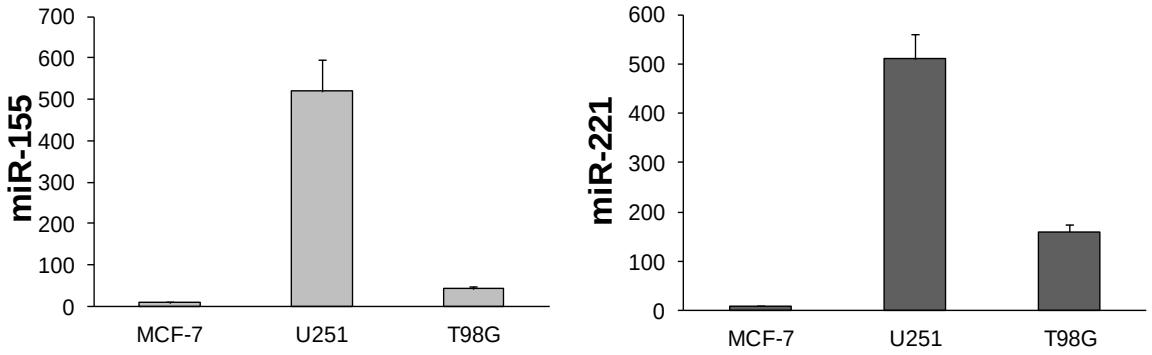

Figure 52. Relative expression of miR-221 and miR-155 in MCF-7 breast cancer cells, U251 and T98G glioma cells in normal culture condition. Internal RT-qPCR control was the U6 snRNA. The data are presented as fold content with respect to the level of miR-155 and miR-221 in MCF-7 cells.

3. IDENTIFICATION OF THE BINDING SITES OF miR-155 AND miR-221 ON CASP-3 mRNA

The sequence examination of the 3'UTR region of caspase-3 mRNA allowed the identification and localization of the sites complementary to the miR-155 and miR-221 seed region (see scheme reported in Figure 53).

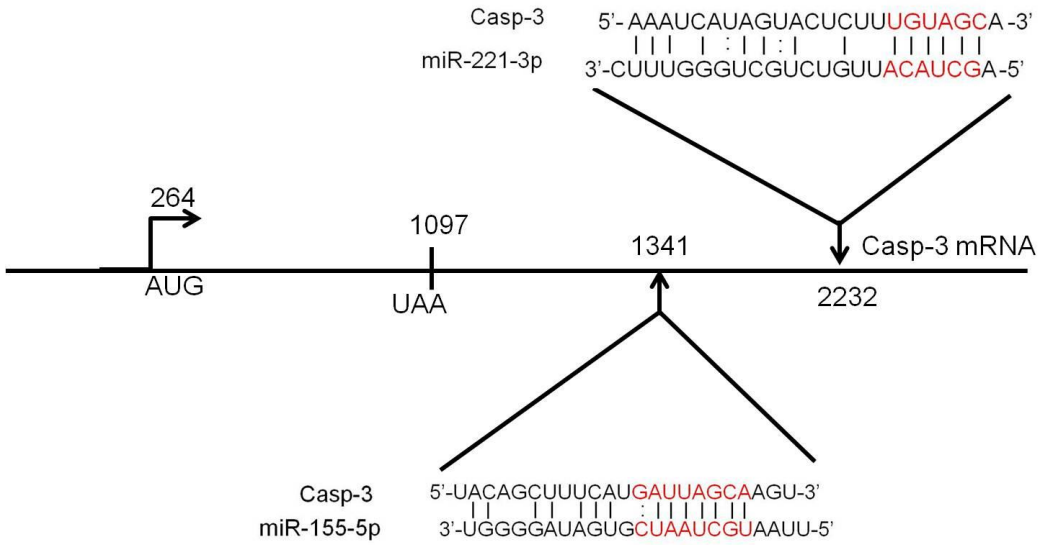

Figure 53. Representation of the 3' UTR region of Caspase-3 mRNA and complementary binding site of the miR-155 and miR-221 seed region.

To verify the biological effect of these miRNAs on caspase-3 gene expression, a loss of function experiment was performed by treating U251 and T98G glioma cells with R8-PNA-a155 and R8-PNA-a221. Before performing these experiments, the activity and specificity of these PNAs on their target miRNAs was evaluated (chapter 4).

4. R8-PNA-a155 AND R8-PNA-a221 INHIBITORY EFFECTS ON miR-155 AND miR-221

The effect of the employed PNAs with putative anti-miR activity was evaluated on miR-155 and miR-221. Glioma cells were seeded and, after 24 hours R8-PNA-a155 and R8-PNA-a221 were added to reach the concentration of 1, 2 and 4 µM. After further 48 hours of incubation, total RNA was isolated and then retro-transcribed using specific primers for miR-155 and miR-221, using U6 snRNA as endogenous reference control. The relative hybridization of the employed molecular probes to the miRNAs was quantified by RT-qPCR analysis and data were normalized with respect to miR-155 and miR-221 content in untreated cells using the comparative cycle threshold method. The inhibitory activity on the two miRNAs is dose-dependent. As shown in Figure 54, the expression of miR-155 and miR-221 in our glioblastoma cell models is significantly inhibited by treatment with R8-PNA-a155 and R8-PNA-a221, respectively; in particular, treatment with PNAs is very evident at concentration of 4 µM for both the PNAs, but also at 2 µM the effect is significant.

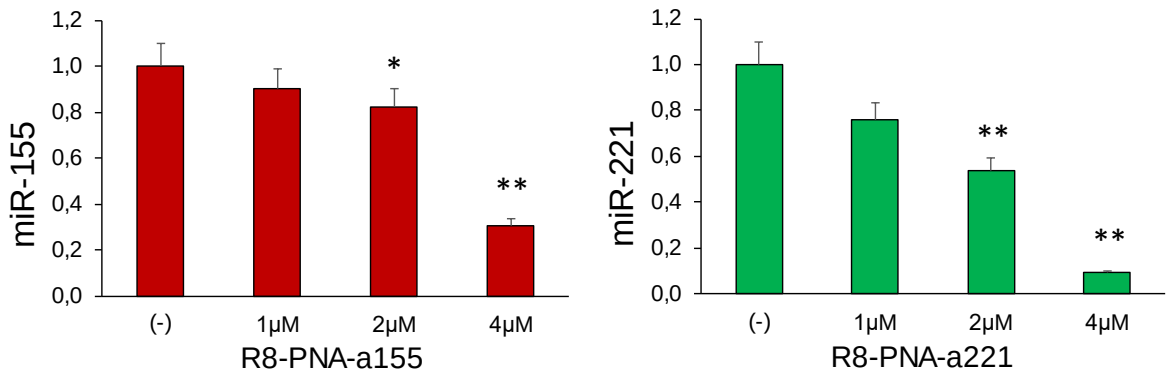

Figure 54. Relative expression of miR-155 and miR-221 in glioblastoma cells, after 48-hour treatment with increasing concentration of R8-PNA-a155 and R8-PNA-a221. Internal RT-qPCR control was U6 snRNA. The data represent the average ± SD from three independent experiments and statistical significance was determined by one-way ANOVA, (**) $P<0.01$ and (*) $P<0.05$ compared to untreated cells (-).

4.1 Specificity of R8-PNA-a155 and R8-PNA-a221 on respective miRNAs

The specificity of the treatment of U251 and T98G cells with the R8-PNA-a155 and R8-PNA-a221 was verified (a) using mutated PNAs and (b) comparing their effects on the expression of both miRNA targets after treatment. Figure 55, shows that specific inhibitory effects are obtained with both R8-PNAs, used at a concentration of 2 µM (R8-PNA-a221) and 4 µM (R8-PNA-a155). In fact, miR-155 and miR-221 specific hybridization signal was strongly reduced only when RNAs were isolated from U251 and T98G glioma cells cultured for 48 hours in the presence of R8-PNA-a155 and R8-PNA-a221, while no effects were observed with their mutated molecules R8-PNA-a155-MUT and R8-PNA-a221-MUT, which differ from R8-PNA-a155 and R8-PNA-a221 for 4 nucleotides in the miRs sequence.

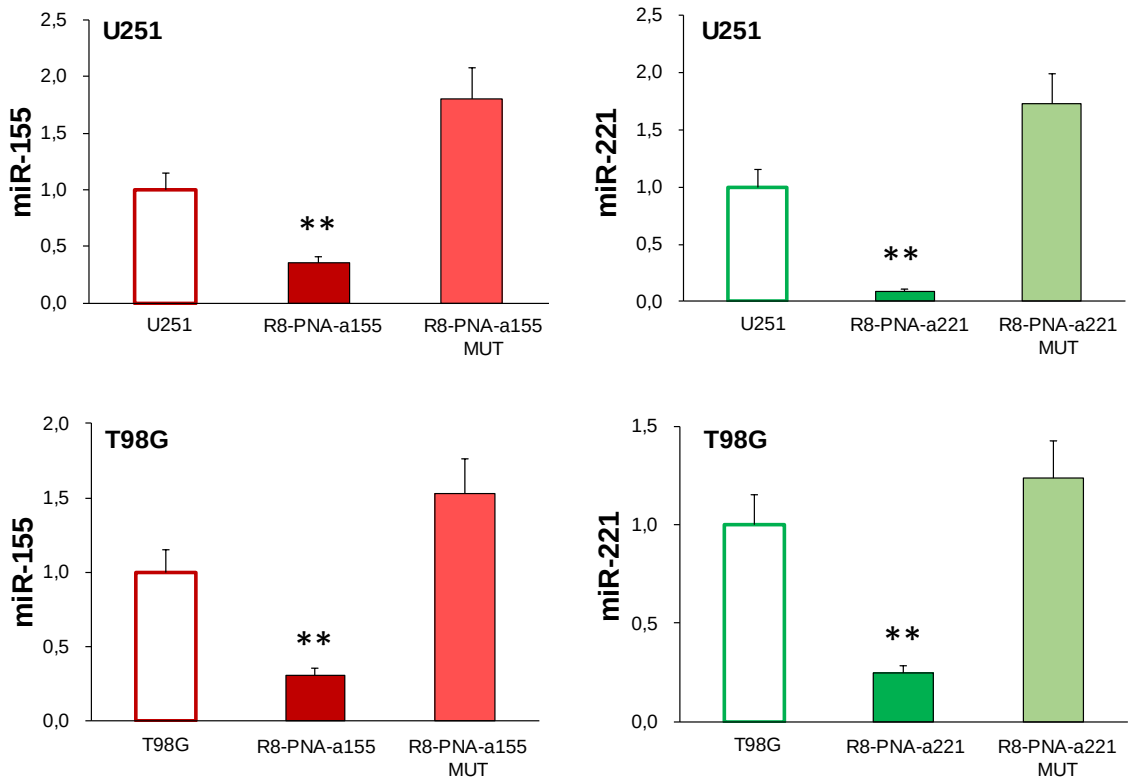

Figure 55. Accumulation of miR-155 (red) and miR-221 (green) in U251 (above) and T98G (below) glioma cells. Cells were treated with 4 µM R8-PNA-a155 and 2 µM R8-PNA-a221 and the relative mutated molecules (MUT) with the same concentrations. The downregulation of miR-155 is visible only for treatment with R8-PNA-a155; the same result was obtained for miR-221. The data represent the average ± SD from three independent experiments and statistical significance was determined by one-way ANOVA, (**) P<0.01 compared to untreated cells (U251 and T98G).

Furthermore, after 48 hours of treatment, despite the fact that some alteration of miRNA content occurs, no significant suppression of accumulation of miR let-7c (a microRNA expressed in GBM, often used as reference gene) has been obtained (Figure 56A-B).

The specificity of PNAs was further verified in T98G glioma cells. The effect of R8-PNA-a155 on miR-221 and the effect of R8-PNA-a221 on miR-155 is negligible when compared to the effect of each PNA on their specific miRNA (Figure 57A-B). Altogether, the data shown in Figures 56-57 demonstrate the specificity of the biological effects of the PNAs synthesized.

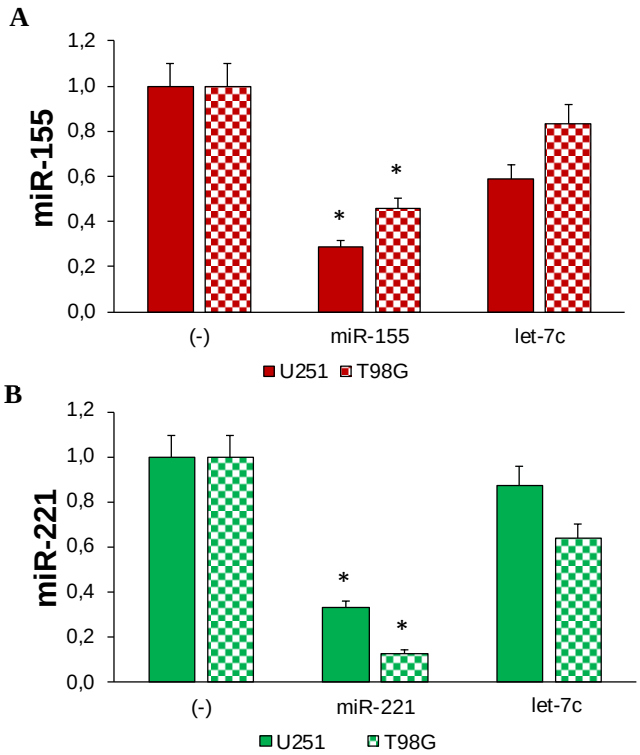

Figure 56. Accumulation of miR-155, miR-221 and let-7c in U251 and T98G cells treated for 48 hours with 4 µM R8-PNA-a155 (A) and 2 µM R8-PNA-a221 (B). qRT-PCR amplifications were performed on RNA from untreated or treated cells using primers amplifying U6 RNA as reference gene. Results are presented as fold increase of miR-155, miR-221 and let-7c mRNAs with respect to untreated cells, taken as 1. The data represent the average ± SD from three independent experiments and statistical significance was determined by one-way ANOVA, (*) $P<0.05$ compared to untreated cells (-).

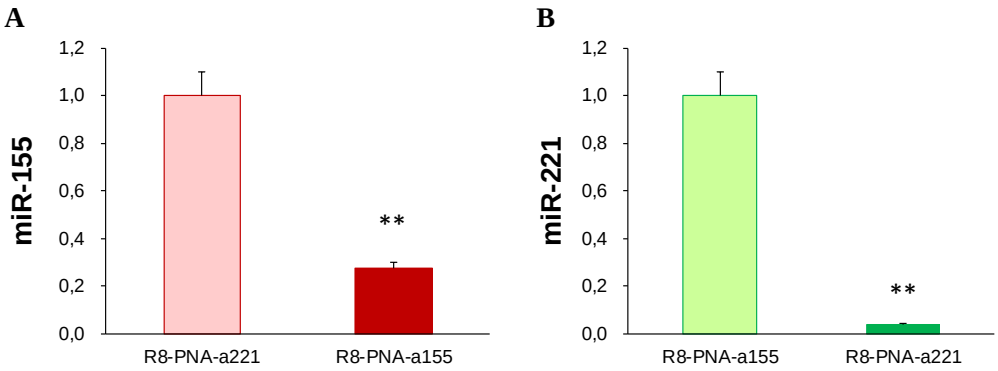

Figure 57. Accumulation of miR-155 (A) and miR-221 (B) in T98G cells treated for 48 hours with 4 μM R8-PNA-a155 and 2 μM R8-PNA-a221. qRT-PCR amplifications were performed on RNA from untreated or treated cells using primers amplifying U6 RNA as reference sequences. Results are presented as fold content of miR-155 and miR-221 mRNAs with respect to cells treated with R8-PNA-a221 (A) and R8-PNA-a155 (B), taken as 1. The data represent the average ± SD from three independent experiments and statistical significance was determined by one-way ANOVA, (**) $P<0.01$ compared to the aspecific PNA treatment.

5. CASPASE-3 AS TARGET FOR miR-155 AND miR-221 IN GLIOBLASTOMA

In order to verify the possible involvement of miR-155 and miR-221 on CASP-3 gene expression, caspase-3 was quantified in PNAs-treated cells using a specific and quantitative Bio-PlexPro RBM Apoptosis Assays.

Since U251 cells express higher level of miR-155 and miR-221 and since an effect on apoptosis is visible also with low PNAs concentrations, they were treated with R8-PNA-a155 and R8-PNA-a221 at the concentration of 2 μM. Conversely, T98G glioblastoma cells express a lower accumulation of these miRNAs and they were treated with 4 μM of the two PNAs. The incubation with the PNAs was performed for 72 hours, after which the total cell extracts were prepared. Bio-Plex apoptosis assay is an immunoassay based on magnetic beads, and the one that we used was specific for caspase-3. The data obtained were analyzed with Bio-Plex Manager software and, as shown in Figure 58, it was found that treatment with PNAs induces increase of the cytoplasmatic content of caspase-3 protein in both cell lines, with the exception of R8-PNA-a155 in U251 cells, that most likely was used at too low concentration. This means that CASP-3 is a direct target of miR-221 and miR-155, since their downregulation by the respective PNAs is associated to an increase of caspase-3 protein expression in our glioblastoma cellular models systems.

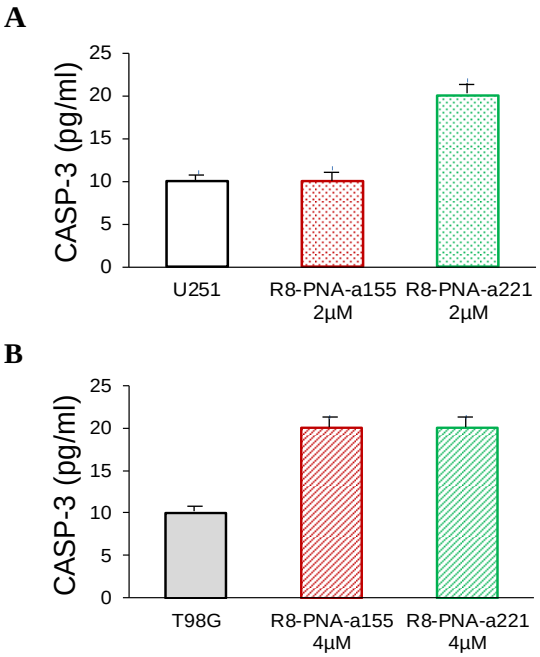

Figure 58. Cytoplasmic content of caspase-3 protein in U251 (A) and T98G (B) after treatment for 72 hours with R8-PNA-a 221 and R8-PNA-a 155 at the indicated concentrations. The concentration (pg/ml) of CASP-3 protein is compared to the quantity in the untreated cells.

These results are also supported by using the simple technology represented by the Muse Casapse 3/7 assay in order to study the caspase 3/7 biological activity in treated cells. More precisely, from this assay, after 48 hours of antisense treatment with anti-miR-155 and anti-miR-221 PNAs resulted that the percentage of caspase 3/7 positive cells was higher than 45% in U251 glioma cells and above 30 % in T98G glioma cells (Figure 59).

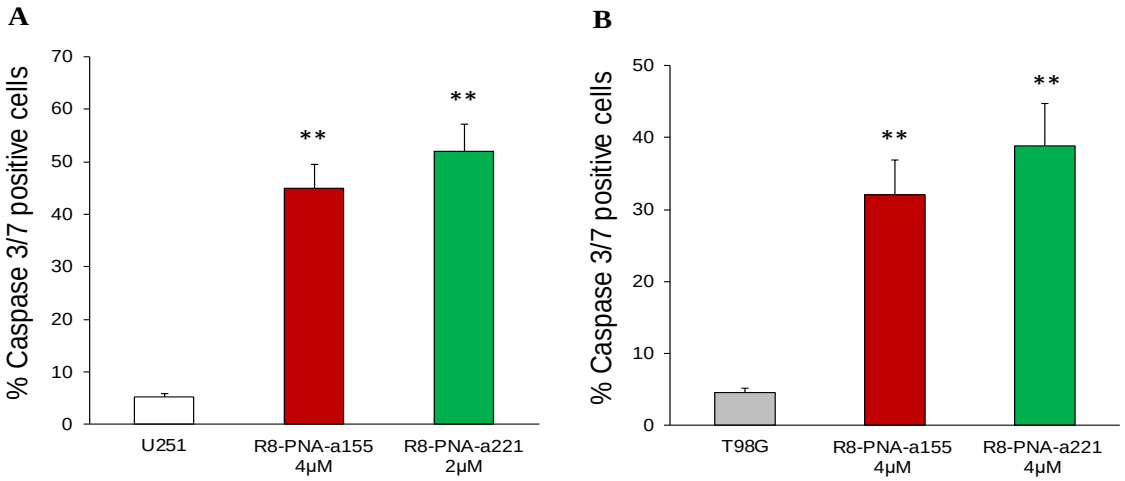

Figure 59. Effects of the PNAs R8-PNA-a155 and R8-PNA-a221 after 48 hours of treatment, on the modulation of Caspase 3/7 activity in U251 and T98G glioma cells. (A) Percentage of U251 cells with caspase 3/7 activated. (B) Percentage of T98G cells exhibiting caspase 3/7 activity. Results represent the average ± S.D of three independent experiments. Statistical significance was determined by one-way ANOVA, (**) P<0.01 compared to untreated glioblastoma cells (U251 and T98G).

6. EFFECTS OF PNAs anti-miR-155 and anti-miR-221 ON GLIOBLASTOMA CELL GROWTH

6.1 R8-PNA-a155 and R8-PNA-a221 anti-proliferative activity

After proving the specificities of the two PNAs we were intrigued to examine their effects on tumor cell growth. U251 and T98G glioblastoma cell lines were cultured in the presence of R8-PNA-a155 and R8-PNA-a221 and the respective mutated PNAs for 48 hours. After this incubation, medium was removed, cells were washed with PBS and trypsinized and then counted by a Z2 Beckman Coulter Counter. As reported in Figure 60, the treatment with singularly administered PNAs and PNAs-MUT does not affect the U251 and T98G cell proliferation, suggesting no cytotoxic activity at the used concentrations.

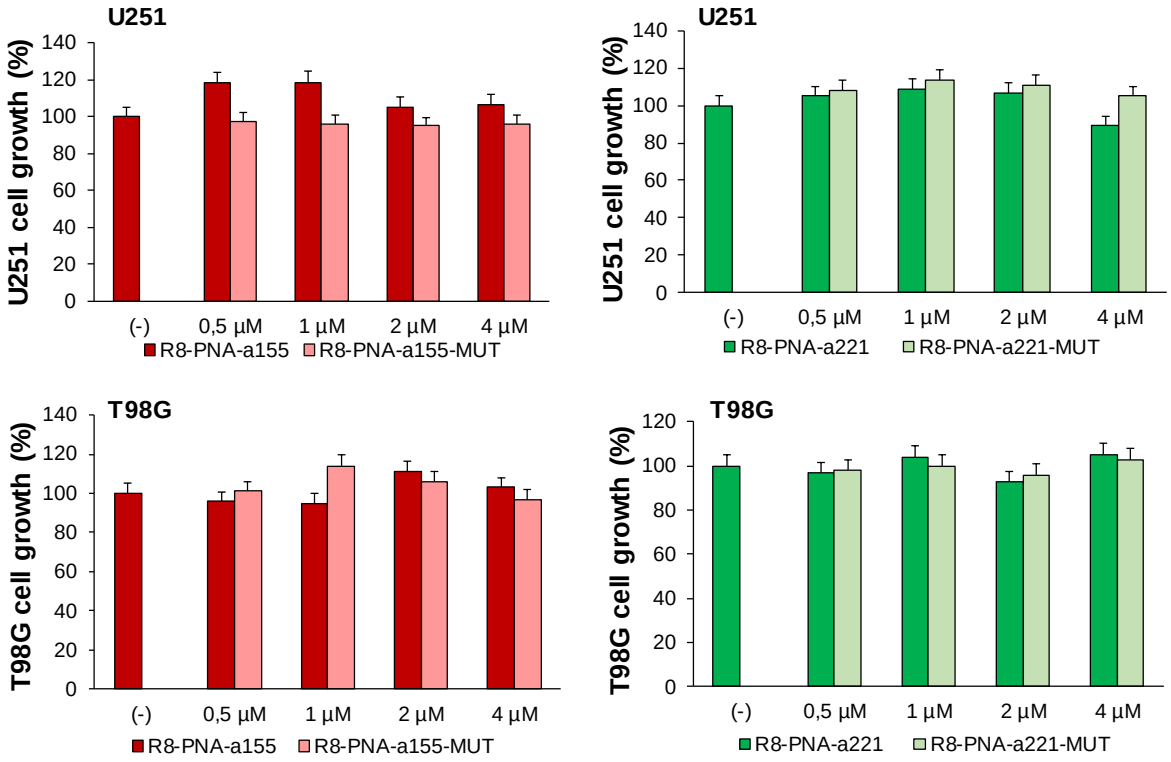

Figure 60. Effect of R8-PNA-a155 and R8-PNA-a221 compared with effect of R8-PNA-a155-MUT and R8-PNA-a221-MUT on U251 and T98G proliferation. Cells were treated with different concentration of PNAs and modified PNAs and counted with coulter counter. Results represent the average ± SD of three independent experiments.

6.2 Combined treatment with R8-PNA-a155 or R8-PNA-a221 and TMZ on T98G glioblastoma cells anti-proliferative activity

In order to identify a possible therapeutic interest of the downregulation of the selected miRNAs and the treatment with cytotoxic alkylating effect of TMZ, we treated our glioblastoma cellular model T98G (TMZ-resistant) with both R8-PNA-a155 or R8-PNA-a221 and TMZ.

T98G cells were seeded, after 24 hours the culture medium was changed and new medium was added together with the tested molecules: R8-PNA-a155 (4 µM), R8-PNA-a221 (2µM) and 400 µM temozolomide. T98G cells were exposed to single treatments as well as to combined treatments. After 48 hours of incubation, cells were counted as previously described.

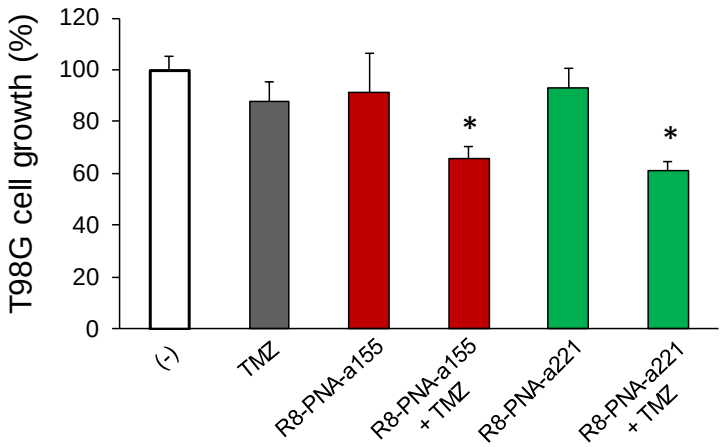

Figure 61. Effect of single and/or combined treatment with 4 µM R8-PNA-a155, 2 µM R8-PNA-a221 and 400 µM TMZ on TMZ-resistant T98G glioma cells. After 48-hour treatment the number of cell/ml were counted and the percentage of cell growth derived respect to untreated cells. The data represent the average ± SD from three independent experiments and statistical significance was determined by one-way ANOVA, (*) $P<0.05$ compared to TMZ and PNAs single treatments.

Figure 61 shows that the co-treatment TMZ plus R8-PNA-a155 or R8-PNA-a221 reduces T98G cell growth of about 40% with respect to untreated cells (-); conversely single treatments do not inhibit cell proliferation as already demonstrated. This preliminary result was the basis for the following experiments focusing on the effects of combined treatments on apoptosis.

7. INDUCTION OF APOPTOSIS OF GLIOBLASTOMA CELLS

7.1 Effect of R8-PNA-a155 and R8-PNA-a221 on U251 and T98G glioblastoma cells

Even if we did not see any significant short-term anti-proliferative effects, the altered morphology of the cells treated with the highest concentrations of PNAs (data not shown), suggested to analyze the apoptotic state of T98G and U251 glioma cells.

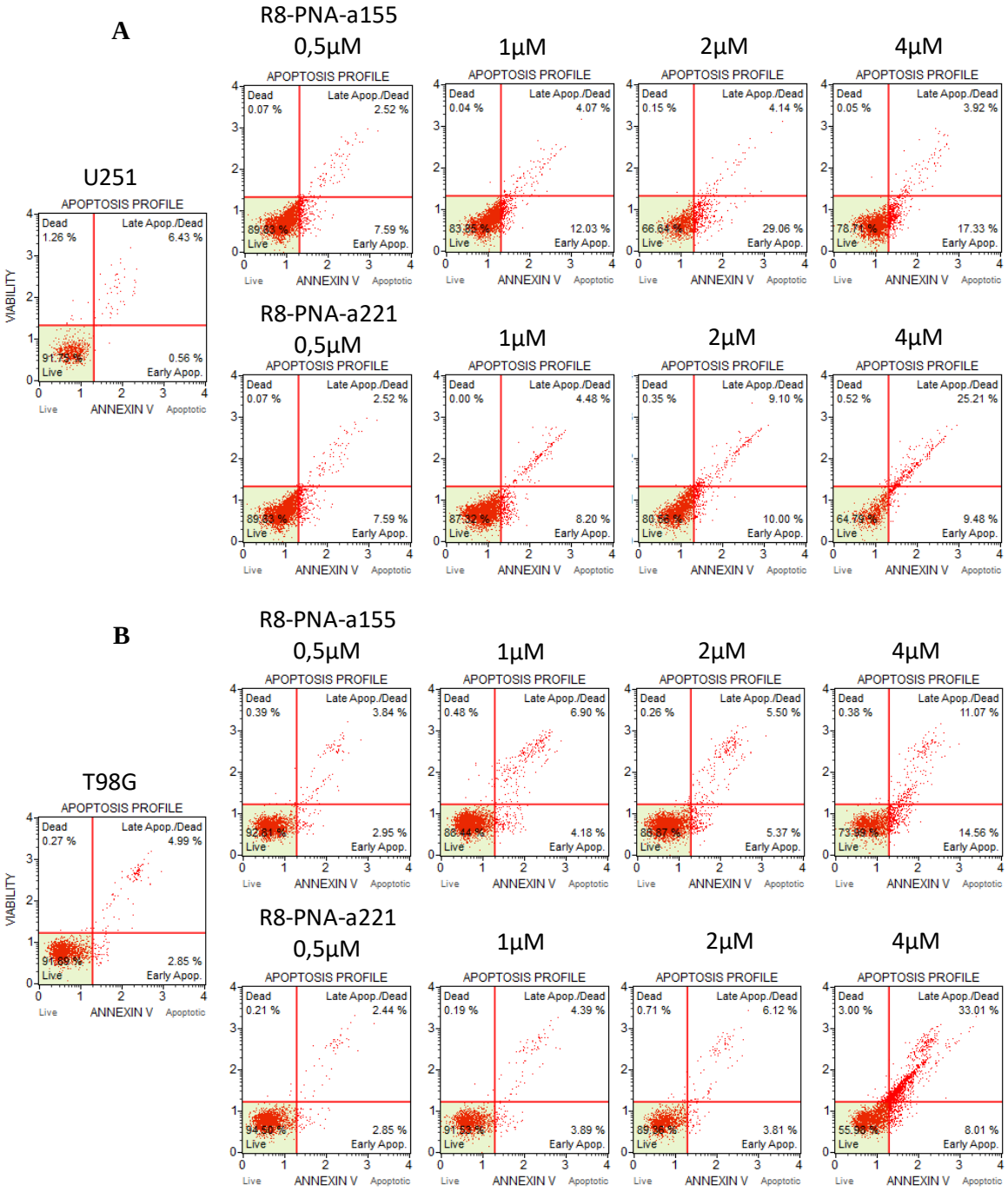

Figure 62. Representative experiment of Annexin V assay performed on U251 (A) and T98G (B) cells treated for 48 hours with increasing concentration of the PNAs R8-PNA-a155 and R8-PNA-a221.

Thus, after 48 hours of treatment with increasing concentration of R8-PNA-a155 and R8-PNA-a221, Annexin V assay was performed using Muse cell analyzer.

As shown in the representative scatter plots on Figure 62, treatment with PNAs induced apoptosis in both the cell lines; the percentage of apoptotic cells is higher with the increase of the concentration used of the PNAs. More precisely, 4 µM R8-PNA-a155 induces apoptotis in more than 20% of both U251 and T98G cells, and 2 µM R8-PNA-a221 leads to apoptosis more than 30% of U251 and T98G cells (Figure 63). To verify whether this effect is sequence-specific, Annexin V assay was performed also after treatment with R8-PNA-a155-MUT and R8-PNA-a221-MUT. The histograms (Figure 63) demonstrate that the mutated molecule of PNAs have no effect in inducing apoptosis.

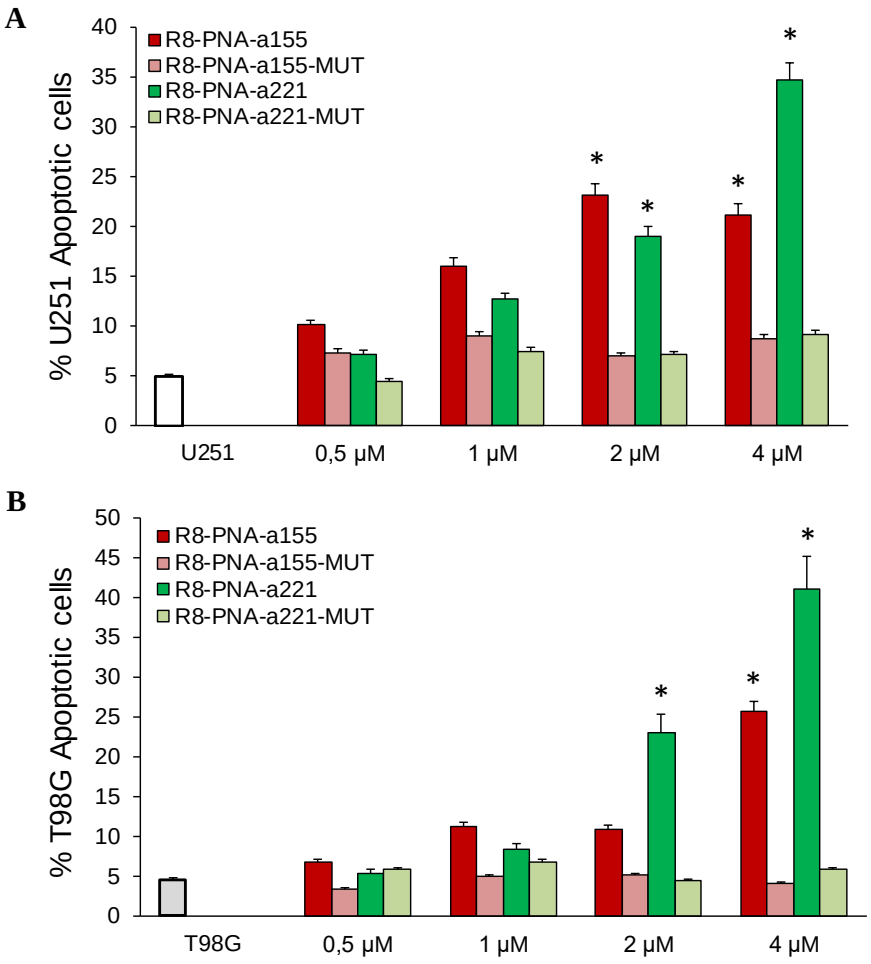

Figure 63. Pro-apoptotic effect of increasing concentrations of R8-PNA-a155 and R8-PNA-a221 treatments on U251 (A) and T98G (B) cell line after 48 hours. Effect of PNAs is compared with the effect of the same concentration of their mutated form, R8-PNA-a155-MUT and R8-PNA-a221-MUT. Results obtained are expressed as the average of three single experiments ± S.D, and include cells in early and late apoptosis. Statistical significance was determined by one-way ANOVA, (*) $P<0.05$ compared to untreated cells.

The induction of apoptosis in cancer cells could be the basis of antitumor therapeutic approach. In our specific case, patients with glioblastoma have low survival chance, in relation to the fact that it is very difficult to cure the patients due to the development of resistance of tumor cells to chemotherapeutics. Therefore, finding novel therapeutic strategies is an important key step. From the results obtained using R8-PNA-a155 and R8-PNA-a221 in the apoptosis study, the idea to evaluate the induction of apoptosis by PNAs also in combination with temozolomide was followed.

7.2 Effect on apoptosis of the co-treatment with R8-PNA-a155 or R8-PNA-a221 and temozolomide on T98G glioblastoma cells

As already reported, T98G is a glioblastoma cell model resistant to temozolomide treatments, as demonstrated at the beginning of the results chapter (see Figure 39). Therefore, we focused our experiments of combined treatments based on PNAs and TMZ only on this cell line. T98G cells were cultured as described before and the treatment with R8-PNA-a155, R8-PNA-a221 and TMZ, alone and in combination, was done.

After the incubation of 48 hours, Annexin V assay was performed with Muse instrument. The obtained results show that the fluorescence emission, due to the presence of phosphatidylserine on the cell membrane which is an apoptosis index, is considerably higher in the co-treatment compared with single treatments, as shown in Figures 64-65.

As we can see from Table 9, in each sample most of the apoptotic cells are in late apoptosis, and the pro-apoptotic effect of the co-treatment leads to an apoptotic level major than the sum of single treatments, reaching about 30% of apoptotic T98G cells treated with R8-PNA-a155 plus TMZ and around 43% with R8-PNA-a221 plus TMZ.

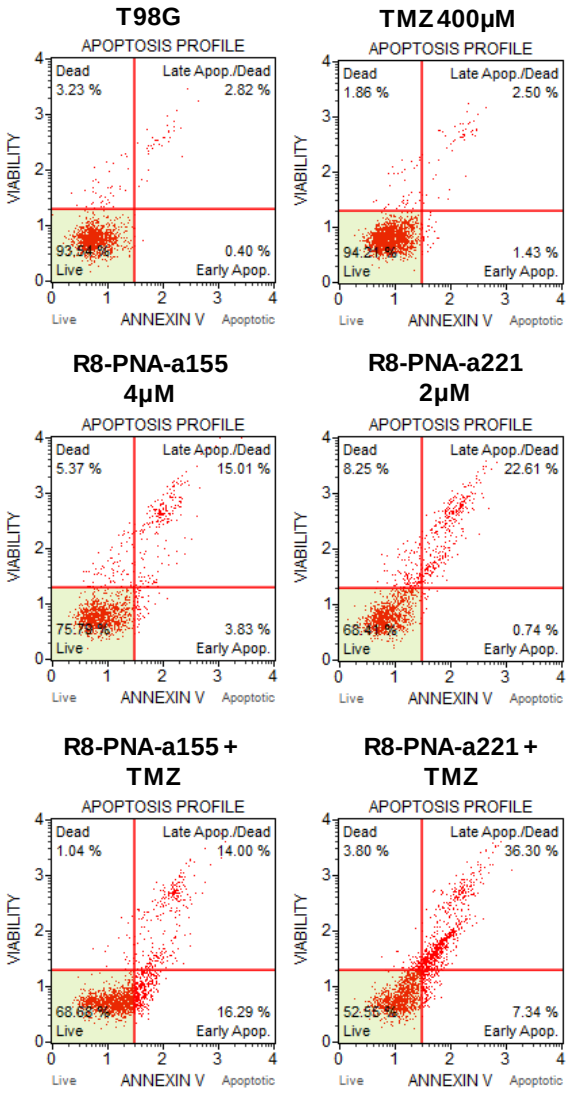

Figure 64. Representative example of scatter plots obtained from Annexin V assay performed on TMZ-resistant T98G cells treated with 4 µM R8-PNA-a155 and 2 µM R8-PNA-a221 with/without 400 µM TMZ for 48 hours.

Table 9. Pro-apoptotic effect of R8-PNA-a155, R8-PNA-a221 and temozolomide (4 µM, 2 µM and 400 µM, respectively) in T98G glioma cells for 48 hours. The percentage of the cell populations indicated in Annexin V assay are reported. Data are expressed as average of three independent experiments.

	(-)	**TMZ 400µM**	**R8-PNA-a155 4µM**	**R8-PNA-a155+TMZ**	**R8-PNA-a221 2µM**	**R8-PNA-a221+TMZ**
Live %	93,54	94,21	75,79	68,68	68,41	52,55
Early Apop %	0,40	1,43	3,83	16,29	0,74	7,34
Late Apop %	2,82	2,50	15,01	14,00	22,61	36,30
Total Apop %	3,23	3,93	18,84	30,28	23,34	43,65

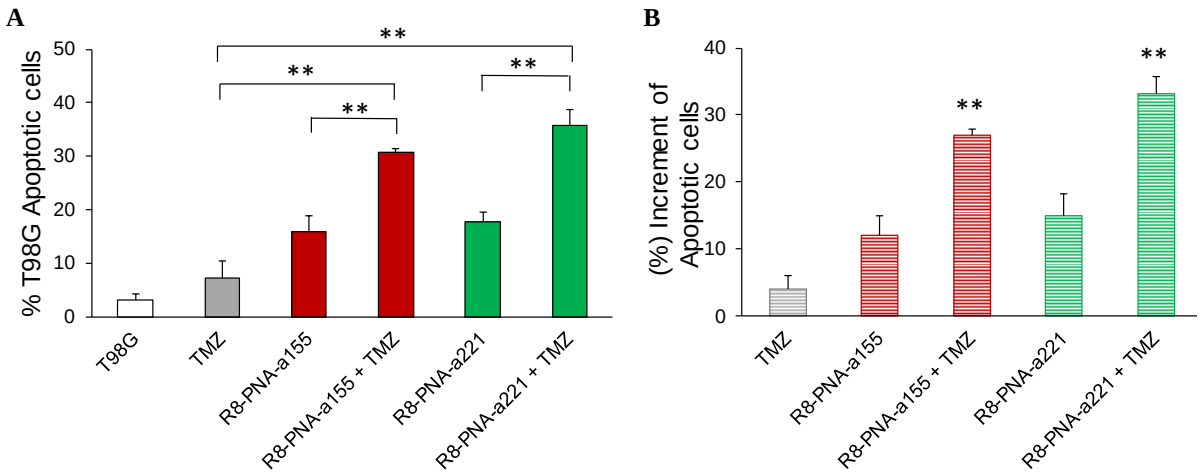

Figure 65. Effects of 4 µM R8-PNA-a155 and 2 µM R8-PNA-a221 with/without 400 µM TMZ on apoptosis in T98G cell line after 48-hour treatment. (A) Percentage of total Annexin V positive cells. (B) Increase of the percentage of apoptosis in treated cells with respect to T98G untreated cells. Results are expressed as average of three independent experiments ± S.D and include cells in early apoptosis and in late apoptosis. Statistical significance was determined by one-way ANOVA, (**) P<0.01 comparing the combined treatment with TMZ and (*) P<0.05 when the co-treatment is compared to PNA anti-miR single treatments

7.2.1 Modulation of caspase 3/7

Since the results obtained using the Annexin V assay on the combined treatment PNAs plus TMZ were considered very interesting, the effect on the activation of caspases was also investigated. In particular, after 48 hours of treatment with R8-PNA-a155, R8-PNA-a221, TMZ and the combination of them, samples were tested to Annexin V in parallel with Caspase 3/7 assay using the Muse cell analyzer. In this way it was possible to compare the results.

Interestingly, the obtained results indicated that PNAs, and particularly the co-treatment with TMZ, activate the effector caspases, inducing high levels of apoptosis in T98G glioblastoma cells. Scatter plots in Figure 66 is a representative example and shows that there seems to be an additive and synergic effect between R8-PNA-a155 and R8-PNA-a221 in combination with TMZ in the induction of apoptosis through the activation of caspase 3 and 7.

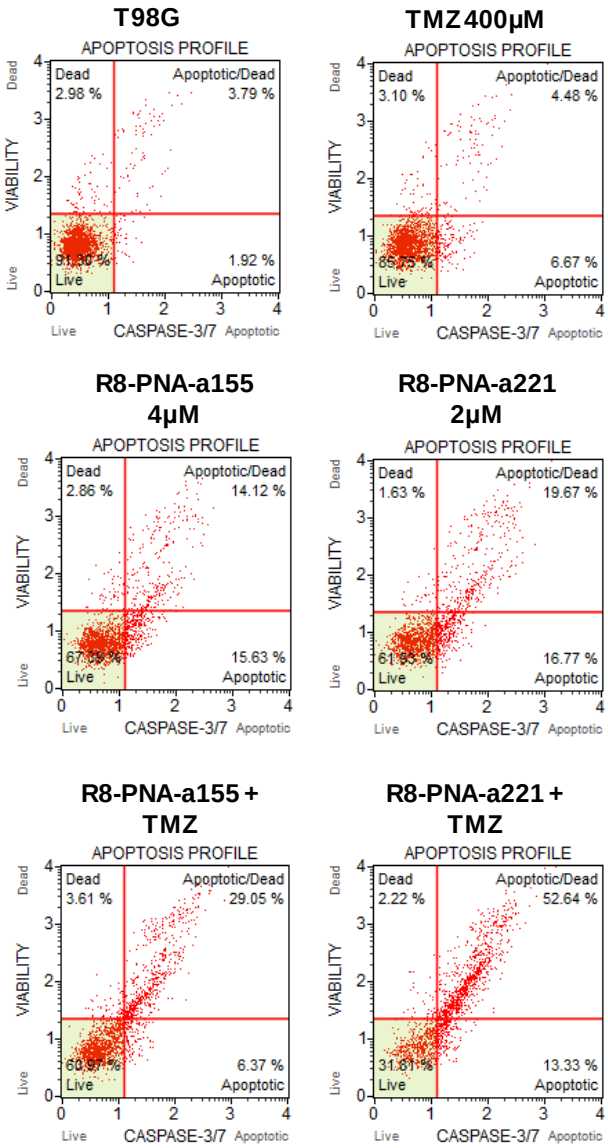

Figure 66. Representative example of Caspase 3/7 assay performed on T98G cells treated for 48 hours with 4 µM R8-PNA-a155 and 2 µM R8-PNA-a221 in association with 400µM TMZ or alone.

Table 10. Pro-apoptotic effect activating caspase 3/7 of R8-PNA-a155, R8-PNA-a221 and temozolomide (4 µM, 2 µM and 400 µM, respectively) in T98G glioma cells after 48-hour treatment. The percentage of the cell populations indicated in Caspase 3/7 assay are reported. Data are expressed as average of three independent experiments.

	(-)	**TMZ 400µM**	**R8-PNA-a155 4µM**	**R8-PNA-a155+TMZ**	**R8-PNA-a221 2µM**	**R8-PNA-a221+TMZ**
Live %	94,09	85,72	78,63	64,67	62,83	39,24
Apoptotic%	1,81	7,49	11,37	21,89	4,94	8,38
Apop / Dead %	2,46	3,57	8,25	12,50	28,31	46,42
Total Apop %	4,28	11,06	19,62	34,39	33,25	54,80

Indeed, the percentage of apoptotic cells increases from 11% with TMZ to about 34% when combined with R8-PNA-a155 and to 55% in combination with R8-PNA-a221 (Figure 67A). In term of increment of apoptosis with respect to T98G untreated cells (-), this means that the co-treatment R8-PNA-a155 plus TMZ caused around 30% of increment and R8-PNA-a221 plus TMZ presented about a 50% of apoptotic cells more than negative control (-) (Figure 67B). Therefore, the data show that co-treatments induce a higher apoptosis than the sum of apoptotic cells obtained by the individual treatments.

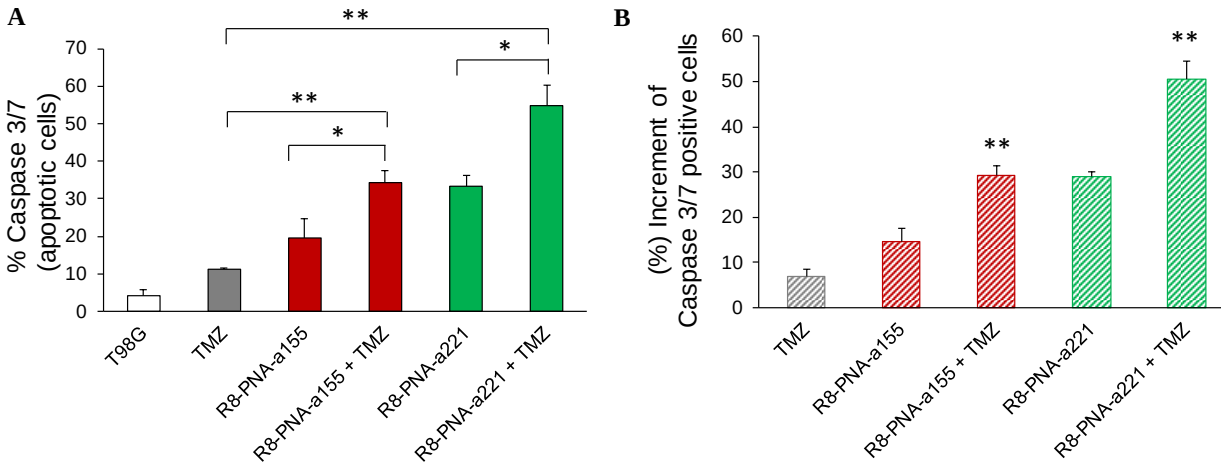

Figure 67. Effects of 4 µM R8-PNA-a155 and 2 µM R8-PNA-a221 with/without 400 µM TMZ on caspase 3 and 7 in T98G cells after 48-hour treatment. (A) Percentage of total Caspase 3/7 reactive cells. (B) Increment of the percentage of apoptosis in treated cells with respect to T98G untreated cells. Results are expressed as average of three independent experiments ± S.D and include cells in apoptosis and in late apoptosis/dead. Statistical significance was determined by one-way ANOVA, (**) P<0.01 comparing the combined treatment with TMZ and (*) P<0.05 when the co-treatment is compared to PNAs antimiRs single treatments

7.3 Effect on apoptosis of the combined treatment with R8-PNA-a155, R8-PNA-a221 and temozolomide on T98G glioblastoma cells

In order to identify a potential therapeutic strategy based on combined treatments, the effect of TMZ on T98G cells co-treated with R8-PNA-a155 and R8-PNA-a221 was investigated. In these experiments, for both PNAs R8-PNA-a155 and R8-PNA-a221, a sub-optimal concentration of 2 µM was used.

As shown in Figures 68-69, in Annexin V assay (Figure 68) as well as in Caspase 3/7 assay (Figure 69), the levels reached of apoptosis induction by the co-administration of 2 µM R8-PNA-a155, 2 µM R8-PNA-a221 and TMZ to T98G cells are higher than those obtained by the treatment with of 2 µM R8-PNA-a155 plus 2 µM R8-PNA-a221.

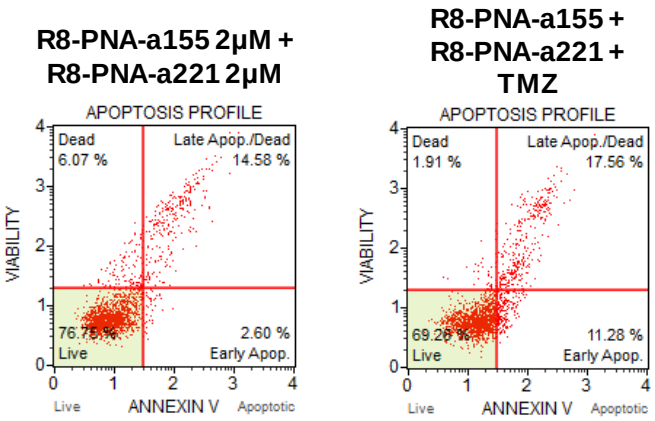

Figure 68. Representative example of scatter plots obtained from Annexin V assay performed on TMZ-resistant T98G cells co-treated with 2 µM R8-PNA-a155 and 2 µM R8-PNA-a221 with/without 400 µM TMZ for 48 hours.

Table 11. Pro-apoptotic effect of combined treatment with R8-PNA-a155, R8-PNA-a221 and temozolomide (4 µM, 2 µM and 400 µM, respectively) in T98G glioma cells for 48 hours. The percentage of the cell populations indicated in Annexin V assay are reported. Data are expressed as average of three independent experiments.

	(-)	**TMZ 400µM**	**R8-PNA-a221 + R8-PNA-a155**	**R8-PNA-a155 + R8-PNA-a221 +TMZ**
Live %	93.54	94.21	76.75	69.26
Early Apop %	0.40	1.43	2.60	11.28
Late Apop %	2.82	2.50	14.58	17.56
Total Apop %	3.23	3.93	17.17	28.84
Δ	-	0.70	13.94	25.61

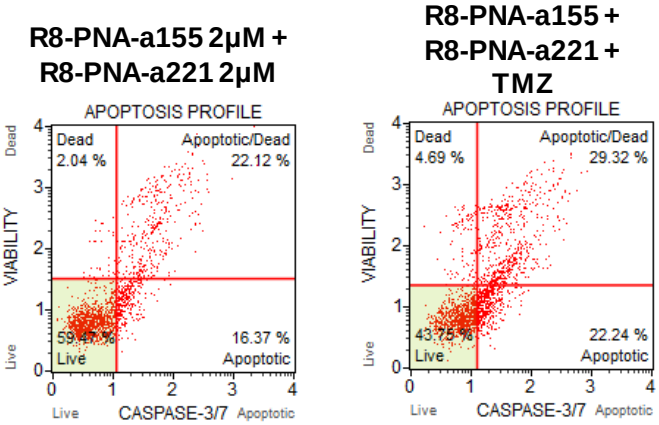

Figure 69. Representative example of Caspase 3/7 assay performed on T98G cells co-treated for 48 hours with 2 µM R8-PNA-a155 and 2 µM R8-PNA-a221 in association with 400µM TMZ or alone.

Table 12. Pro-apoptotic effect activating caspase 3/7 of R8-PNA-a155, R8-PNA-a221 and temozolomide (4 μM, 2 μM and 400 μM, respectively) and the combination of them, in T98G glioma cells after 48-hour treatment. The percentage of the cell populations indicated in Caspase 3/7 assay are reported. Data are expressed as average of three independent experiments.

	(-)	**TMZ 400μM**	**R8-PNA-a221 + R8-PNA-a155**	**R8-PNA-a155 + R8-PNA-a221+TMZ**
Live %	94.09	85.72	59.47	43.75
Apoptotic%	1.81	7.49	16.37	22.24
Apop / Dead %	2.46	3.57	22.12	29.32
Total Apop %	4.28	11.06	38.49	51.56
Δ	-	6.78	34.21	47.28

Combined treatment with corilagin and anti-miR-221 PNA in glioblastoma cells

Since corilagin was found to be very interesting in inducing apoptosis of our glioblastoma cell models, U251 and T98G, we started to study to verify possible interest in treating these cell lines with both corilagin and the most active PNA, i.e. PNA-a221.

1. ANTI-PROLIFERATIVE EFFECT OF COMBINED TREATMENT WITH CORILAGIN AND R8-PNA-a221

Glioblastoma cells were incubated for 48 hours with 35 μM corilagin and/or 2 μM R8-PNA-a221. The anti-proliferative effect was evaluated by counting the cells with Z2 Beckman Coulter Counter. The percentage of cell growth is calculated respect to the number of cells in the untreated sample; thus, as shown in the graphs in Figure 70, the co-treatment with COR and TMZ reduces the cell growth at higher levels than the single compounds. Moreover, the effect on T98G cells is more evident than in U251 cells.

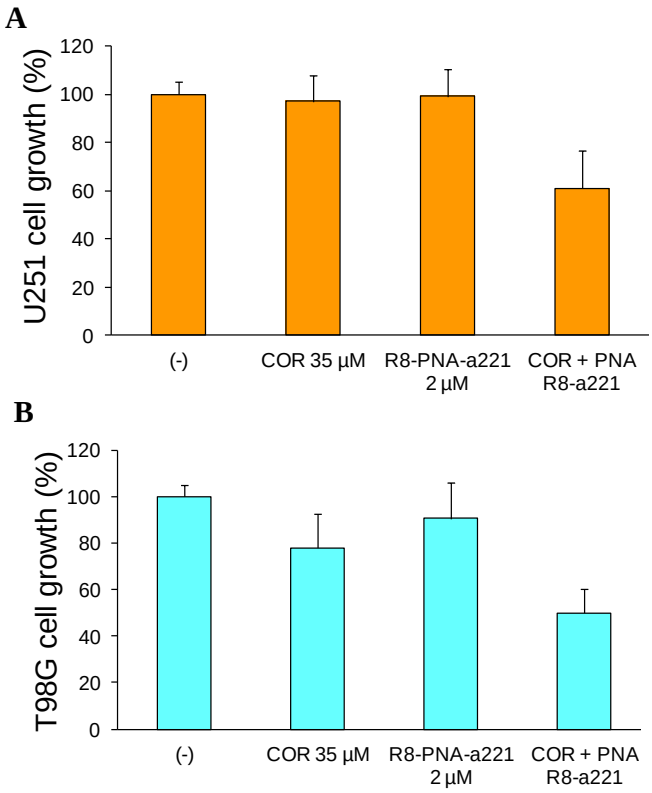

Figure 70. Anti-proliferative effect of COR (35 μM), R8-PNA-a221 (2 μM) and 35 μM COR plus 2 μM R8-PNA-a221. U251 (A) and T98G (B) were treated for 48 hours, then cells were counted and compared to untreated cells. Data represent the average ± S.D of three independent experiments.

2. PRO-APOPTOTIC EFFECT OF CORILAGIN WITH R8-PNA-a221 ON GLIOBLASTOMA CELLS

We proved in the first two parts of this thesis, that both corilagin and PNA anti-miR-221 play a pro-apoptotic effect when singly used on both U251 and T98G cell lines. Therefore, we further investigated whether a combined therapy with this two compounds could have a synergistic effect on the treatment of glioblastoma.

In order to verify if treating the cells simultaneously with corilagin and PNA could enhance the pro-apoptotic effect of single treatments, U251 and T98G were treated with the compounds for 48 hours. The effect on apoptosis was performed with Muse Annexin V assay. As shown in Figure 71, the combination of COR with 2 μM R8-PNA-a221 induced higher pro-apoptotic effects than COR or PNA alone, supporting a possible synergistic effect of these two molecules on T98G (Figure 71A) and U251 (Figure 71B) glioma cells.

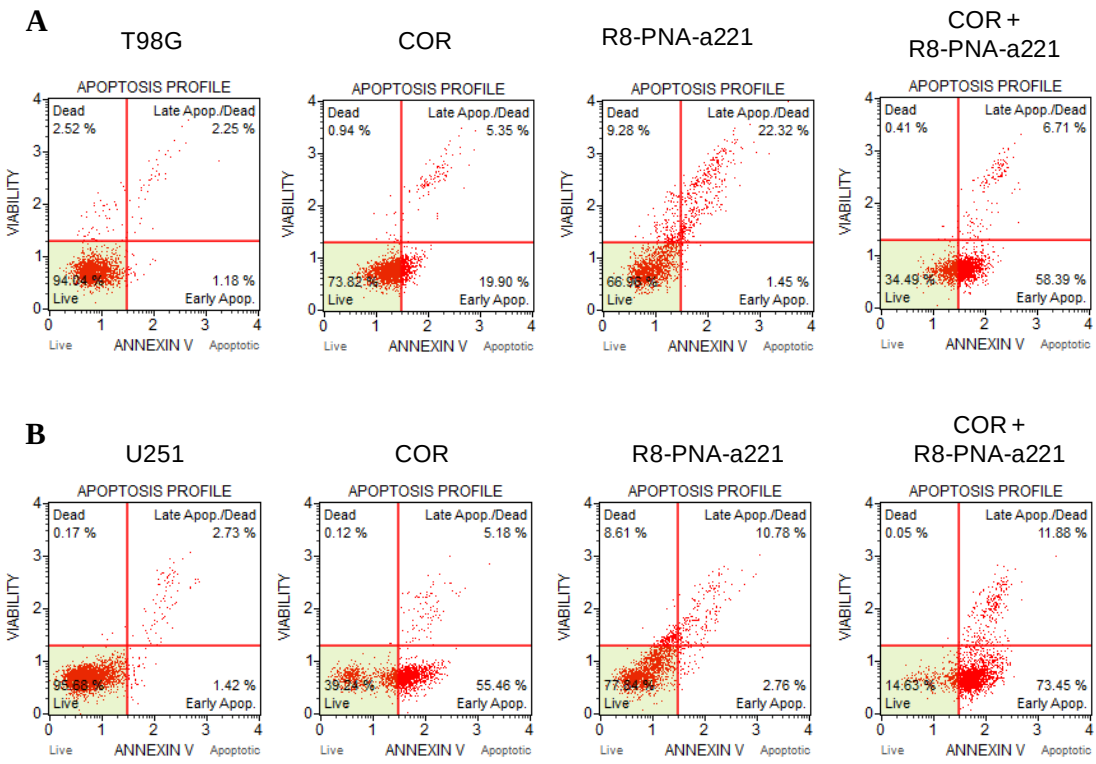

Figure 71. Representative example of scatter plots obtained from Annexin V assay performed on (a) TMZ-resistant T98G cells and (B) U251 glioma cells treated with 2 μM R8-PNA-a221 with/without 35 μM COR for 48 hours.

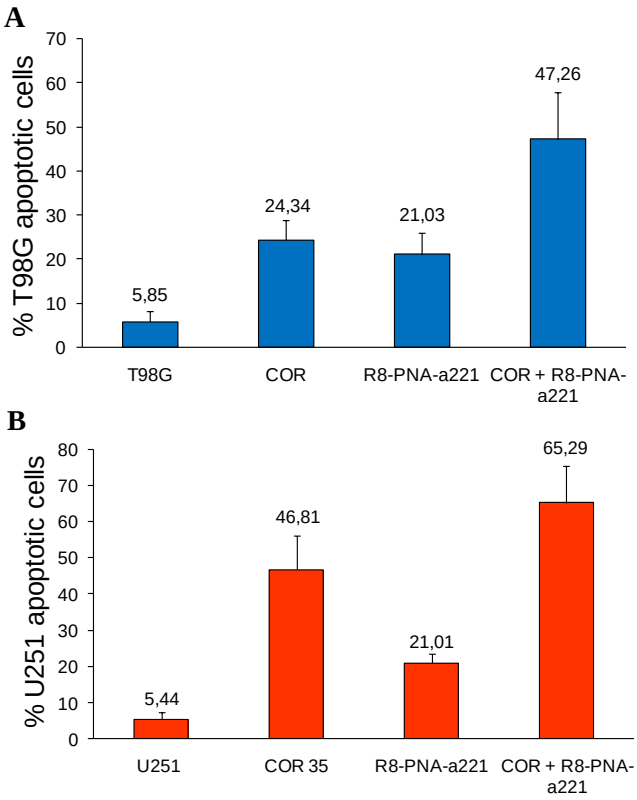

Figure 72. Effects on apoptosis in T98G (A) and U251 (B) glioma cells after 48-hour treatment with 2 μM R8-PNA-a221 with/without 35 μM COR. Percentage of total Annexin V positive cells. Results are expressed as average of three independent experiments ± S.D and include cells in early and in late apoptosis.

In COR-treated U251 and T98G cells a significant increase of early apoptotic cells can be observed, while in R8-PNA-a221-treatment, cells are prevalently in late apoptosis. The combination of the two treatments confirms a synergism between COR and R8-PNA-a221, since it shows an increase of both early and late apoptosis. The full data set is showed in Figure 72A-B.

DISCUSSION AND CONCLUSIONS

Glioblastoma multiforme (GBM) is a lethal malignant tumor and one of the most common gliomas. The current clinical treatment consists of a combination of surgery (oncotomy), radiotherapy, and chemotherapy. However, no curative treatment is available at present, and even in the presence of such a radical treatment strategy, the mean patient survival time reaches only 14.6 months (Wilson TA, 2014).

Temozolomide (TMZ), usually combined with radiotherapy, is an imidazotetrazine alkylating agent currently used as first line therapy for gliomas treatment due to its DNA-damaging effect (Zhang J, 2012). However, drug-resistance occurs often and it is only able to cause an increase of the life-time expectancy. One of the main mechanisms of drug resistance in glioma, is the enhanced activity of DNA repair enzyme, O(6)-methylguanine-DNA-methyltransferase (MGMT) which repairs TMZ-induced DNA lesions, O6-meG, by transferring the alkyl group from guanine to a cysteine residue (Fan CH, 2013).

Therefore, an urgent need does exist for novel drugs for treating gliomas, also in combination with temozolomide, in order to develop effective strategies to overcome resistance. For example, Ursolic acid (UA) is a naturally derived pentacyclic triterpene acid that exerts anticancer effects, and is able to cross the blood-brain barrier. Recently, in TMZ-resistant GBM cell lines it was demonstrated that the combined treatment of TMZ and UA, synergistically enhanced cytotoxicity and senescence in TMZ-resistant GBM cells, and this effect was also correlated with the downregulation of MGMT (Zhu Z, 2016). Many other strategies have been studied in these last years to enhance TMZ treatment in glioblastoma, such as the use of deferiprone (DFP), an orally administered iron chelator that, when combined with TMZ, significantly reduced cell viability, produced cell cycle arrest at G2/M phase, and enhanced apoptosis (Alexiou GA, 2016). Another example is based on the inhibition of the gap junction protein connexin 43 (Cx43), which was seen to be associated with TMZ resistance and showed to sensitize human MGMT-deficient and TMZ-resistant GBM cells to TMZ treatment (Murphy SF; 2016). As a final example, resveratrol was found to be able to enhance glioblastoma-initiating cells to temozolomide-induced apoptosis, suggesting a new combination strategy (Li H, 2016).

Since corilagin (COR), a gallotannin extracted from plants of the *Phillantus* family, among

others, exhibited antioxidant, anti-inflammatory and antitumor activity, in particular inducing cancer cell apoptosis (Jia L, 2013, Ming Y, 2013; Gu Y, 2016), we were interested to learn more about its potential anticancer effects in glioblastoma. Our interest towards this compound is also supported by the observation that corilagin can inhibit the interaction between NF-κB with its DNA target binding site.

Docking studies performed by Dr. Marzaro G. and Prof. Chilin A. from University of Padova, confirmed that corilagin is able to bind NF-κB, therefore supporting the finding of an inhibitory effect of corilagin on NF-κB/DNA interactions. The results obtained in this PhD thesis showed that (a) corilagin inhibits cell growth of U251 glioma cells through activation of the apoptotic pathway, in fact, after 48 hours of treatment, concentration lower than the $IC_{50,}$ induced apoptosis in about 40% of treated U251 cells. In addition, (b) corilagin showed to be active also on temozolomide-resistant T98G glioblastoma cells, on which the effects were dose-dependent and the induction of apoptosis appreciable starting from 35 μM COR, while for anti-proliferative effect higher concentrations were found to be necessary.

With these results, and considering that corilagin probably acts through the inactivation of NF-κB pathway, we further studied whether corilagin sensitize T98G glioblastoma cells to TMZ, in order to enhance the benefit of TMZ in the treatment of glioblastoma.

Our results show that (d) when T98G glioma cells were treated with both temozolomide and corilagin, a higher level of pro-apoptotic and anti-proliferative effects were obtained. This is probably related to the fact that (e) corilagin, but most of all, corilagin with temozolomide decrease the expression of MGMT mRNA. Moreover (f) the combined treatment (COR + TMZ) induces caspase-3 activation more than the single treatments. Interestingly, (g) corilagin is active also in the inhibition of cell migration; in fact, with respect to T98G untreated glioma cells, the proliferation and invasiveness ability were strongly inhibited when they were treated with corilagin and temozolomide together.

In conclusion the present study focused on the effects of corilagin on temozolomide resistant T98G cells, demonstrating in particular, the efficacy in inducing high levels of apoptosis and inhibition of tumor cell growth *in vitro*; and the gain in susceptibility of these resistant cell line to the effects of temozolomide. These results corroborate our hypothesis that corilagin is a potential drug against glioblastoma. Further explorations on the efficacy of corilagin in glioblastoma *in vivo* are warranted.

Beside this possible therapeutic strategy, we also investigated the efficacy of targeting

oncomiRNAs by PNAs in glioblastoma cancer cells. MicroRNA (miRNA) therapeutics in cancer is based on targeting or mimicking miRNAs involved in cancer onset, progression, angiogenesis, epithelial-mesenchymal transition, metastasis. This strategy has been proposed since several years and is based on the well-recognized fact that miRNAs have a key role in the post-transcriptional control of gene expression by sequence-selective targeting of mRNAs and are key players in several biological functions and pathological processes, including cancer.

The oncogene, microRNA-155 and microRNA-221, are significantly elevated in GBM, downregulating multiple genes associated with cancer cell proliferation, apoptosis, invasiveness and drug resistance (Liu Q, 2015; Xie Q, 2014; Zhang CZ, 2010; Shea A, 2016). Therefore, they could be specific target for treatments against gliomas and their downregulation could enhance chemosensitivity to anticancer drugs. It was demonstrated that the treatment of glioma cells with anti-miR-155 combined with taxol or temozolomide, decreased cell growth and significantly enhanced apoptosis, suggesting an increase in the chemosensitivity to taxol and temozolomide when combined with miR-155 inhibitor (Meng W, 2012; Liu Q, 2015). Likewise, co-suppression of miR-221 is associated with inhibition of cell growth and activation of apoptosis through the increased expression of its target PUMA (Zhang CZ, 2010); moreover, the downregulation of miR-221/222 demonstrated to sensitize glioma cells to temozolomide regulating apoptosis (Chen L, 2012). Recently, our group demonstrated that a PNA targeting the miR-221 can be internalized by glioma cells when it is linked to an octaarginine tail (R8), leading to inhibition of miR-221 and activation of the apoptotic pathway (Brognara E, 2014). In the present thesis it was investigated not only the activity of R8-PNA-a221, but also of R8-PNA-a155 on glioblastoma cell lines.

Our interest towards these two microRNAs was also based on the fact that from a research aimed at finding apoptosis targets, from a huge number of microRNAs involved in glioblastoma, miR-155 and miR-221 were found to target caspase-3 mRNA. This is an important effector caspase during the apoptosis process and, by an immunoassay performed with Bio-Plex, we verified that when glioma cells are treated with PNAs against miR-221 and miR-155 the cytoplasmatic content of CASP-3 increased. This evidence was supported also by Caspase 3/7 assays that showed an increasing of cells with caspase 3/7 activity in association with an increase of PNAs concentration.

In our glioblastoma cellular model systems, U251 and T98G cells, both the PNAs showed

selective and specific activity recognizing only their respective miRNA target sequence with only a little alteration of the content of another unrelated miRNA (miR-let-7c). The specific activity of R8-PNA-a155 and R8-PNA-a221 was demonstrated also in comparison to their mutated form, where 4 nucleotides in the PNA sequences were changed. Only R8-PNA-a155 and R8-PNA-a221 were able to downregulate miR-155 and miR-221 mRNA accumulation, respectively. Moreover, both PNAs induced apoptosis in U251 and TMZ-resistant GBM cells in a dose-dependent manner, whereas no effect was obtained with R8-PNA-a155-MUT and R8-PNA-a221-MUT.

As oncomirs promote growth of cancer cells and support survival during chemotherapy, thus microRNA-silencing therapies could be a valuable approach to be associated with anticancer drugs and chemotherapy treatments. Therefore, in our TMZ-resistant glioblastoma cell model, we investigated whether combining temozolomide with PNAs could have more effect than the single treatments. The result is that 48 hours of co-treatment with R8-PNA-a155 or R8-PNA-a221 and TMZ leads to an apoptotic level higher than the sum of single treatments, reaching about 30% of apoptotic T98G cells with R8-PNA-a155 plus TMZ and around 43% with R8-PNA-a221 plus TMZ. Also the cell growth was considerably reduced when cells were treated with both PNAs and TMZ, suggesting that the combined treatment reverses the resistance of the cells to apoptosis induced by temozolomide.

Our results support the concept that anti-miRNA strategy led to therapeutic inhibition of miRNA dependent effects and that PNA-based anti-miRNA molecules are very promising reagents to regulate tumor cell growth. The obtained data confirm also that miRNA therapeutics can be successfully combined with chemical treatments to obtain high effects with lower doses of reagents, respect to the ones necessary to have an effect as single treatment. Further research on PNA analogues to increase efficiency of delivery, stability and control of intracellular distribution for specific targets are further steps for the selection of best candidate drugs.

Since R8-PNA-a155 or R8-PNA-a221 act in a synergistic fashion with TMZ to modulate cancer cell growth and apoptosis, we wandered if a combined treatment between the two PNAs and corilagin would have the same effect. And as hypothesized, our initial study shows that co-treatment of R8-PNA-a221 and coriligin has a synergistic effect on apoptosis. Currently we are exploring further to understand the mechanisms underlying this effect.

The scientific literature proves that inducing apoptosis is one of the major route for chemotherapeutic agents to eradicate cancer cells. The findings exposed in this PhD thesis demonstrate that there are different approaches to reach this scope and that the combined therapy, in the specific case using natural compounds, as corilagin, or synthetic agents, as PNAs, could be an efficient strategy to sensitize resistant-glioma cells and to improve the efficacy of the first-line drug temozolomide treatment.

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ABBREVIATIONS

µg	Micrograms
µl	Microliters
µM	Micromolar
7-AAD	7-Aminoactinomycin D
ADP-Ribose	Adenosine Diphosphate Ribose
AIC	4-amino-5-imidazole-carboxamide
AIF	Apoptosis Inducing Factor
AML	Acute myelogeneous leukemia
APAF-1	Apoptotic protease activating factor-1
Bak	BCL2-antagonist/killer
Bax	BCL2-associated X protein
BBB	Blood-brain barrier
BCL-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BCNU	Carmustine
BER	Base Excision Repair
Bim	Bcl-2-like protein 11
bp	Pair of bases
BrCH2COOR	Bromoacetic esters
CARD	Caspase activation and recruitment domain
CASP-3	Caspase - 3
CBL	Cbl proto-oncogene, E3 ubiquitin protein ligase
CCDC26	Coiled-Coil Domain Containing 26 or CCDC26 Long Non-Coding RNA
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27Kip1)
CDKN1C	Cyclin-dependent kinase inhibitor 1C (p57Kip2)
CDKN2B	Cyclin Dependent Kinase Inhibitor 2B
cDNA	Complementary DNA
CF	Cystic Fibrosis
CH2O	Formaldehyde
CLL	Chronic Lymphocytic Leukemia

CNS	Central Nervous System
CO2	Carbon dioxide
COR	Corilagin
Ct	Threshold cycle
Cyt-c	Cytochrome-c
dATP	Deoxyadenosine triphosphate
DCC/DhBtOH	N,N'-Dicyclohexylcarbodiimide/3-Hydroxy-1,2,3 benzotriazin-4(3H)-one
DD	Death Domain
DDP	Cis-diamminedichloroplatinum
DED	Death Effector Domain
DEVD	N-Acetyl-Asp-Glu-Val-Asp sequence
DFF45	DNA fragmentation factor, 45kDa, alpha polypeptide
DFP	Deferiprone
DGCR8	DiGeorge syndrome critical region gene 8
DIABLO	Direct IAP Binding protein with Low pI
DIEA	N,N-Diisopropylethylamine
DISC	Death Inducing Signalling Complex
DMF	N,N-dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNM3	Dynamin 3
DNMT3b	DNA Methyltransferase 3 Beta
DPBS	Dulbecco's Phosphate-Buffered Saline
ECOP	EGFR-coamplified and overexpressed protein
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial–mesenchymal transition
ERRγ	Estrogen-related receptor gamma
ERα	Estrogen receptor α
Et3N	Triethylamine
EZH2	Enhancer of zeste 2 polycomb repressive complex 2 subunit
FADD	Fas-Associated protein with Death Domain

Fas	Fas (TNF receptor superfamily member 6)
FasL	Fas ligand (TNF superfamily, member 6)
FBS	Fetal Bovine Serum
FBX011	F-Box only protein 11
FOXO3	Forkhead box O3
GABPA	GA binding protein transcription factor
GBM	Glioblastoma Multiform
GNA	Glycol nucleic acid
GWAS	Genome-wide association study
HBP1	HMG-Box Transcription Factor 1
HBTU/DIPEA	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate /diisopropylethylamine
HCl	Hydrochloric acid
HMGB1	High Mobility Group Box 1
HOXB7/pBX2	Homeobox B7/PBX Homeobox 2
HOXD10	Homeobox-leucine zipper protein
IAPs	Inhibitors of apoptosis proteins
IC50	half maximal inhibitory concentration
ICAD	Inhibitor of Caspase Activated DNase
ICD-O	International Classification of Disease for Oncology
IDH-1	Isocitrate dehydrogenase 1
IDH-2	Isocitrate dehydrogenase 2
IGFBP3	Insulin-like growth factor-binding protein 3,
K2CO3	Potassium carbonate
LNA	Locked Nucleic Acid
LRRFIP1	Leucine Rich Repeat Interacting Protein 1
MAPKs	Mitogen-activated protein kinase
MARCKS	Myristoylated alanine-rich C-kinase substrate
MDM2	Mouse double minute 2 homolog
MDR1	Multidrug resistance 1
MELK	Maternal Embryonic Leucine zipper Kinase
MeOH	Methanol
MERTK	c-Mer tyrosine Kinase

MET	MET proto-oncogene
MGMT	O-6-methylguanine-DNA methyltransferase
MiRNA, MiR	MicroRNA
MITF	Microphthalmia-associated transcription factor
ml	Milliliters
MMP9	Matrix metallopeptidase 9
MMR	DNA mismatch repair
MRI	Magnetic Resonance Imaging
MSCs	Mesenchymal stem cells
mRNA	Messenger RNA
MTIC	5-(3-methyl-1-triazeno)imidazole-4-carboxamide
mTOR	Mammalian target of rapamycin
MVP/LRP	Major vault protein/lung resistance protein
MXI1	Max interactor-1
MYCN	v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog
NaN3	Sodium azide
NaOH	Sodium hydroxide
NCOR1	Nuclear receptor corepressor 1
NCOR2	Nuclear receptor corepressor 2
NF-κB	Nuclear factor κB
Notch1	Notch Homolog 1, Translocation-Associated (Drosophila)
nts	Nucleotides
ODN or Oligo-dT	Oligodeoxynucleotide
p27Kip1	Cyclin-dependent kinase inhibitor 1B
p53	Cellular tumor antigen p53
p57Kip2	Cyclin-dependent kinase inhibitor 1C
PARP	Poly ADP ribose polymerase
PCD	Programmed cell death
PDCD4	Programmed cell death protein 4
PDGFRA	Platelet-derived growth factor receptor alpha
PGC-1β	Peroxisome proliferator-activated receptor γ coactivator 1β
PHLDB1	Pleckstrin Homology Like Domain Family B Member 1

PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PI3K-AKT-mTOR	Phosphatidylinositol-4,5-bisphosphate 3-kinase/ Protein kinase B/ mammalian target of rapamycin
PLZF	Promyelocytic leukemia zinc finger
Pmaip1	Phorbol-12-Myristate-13-Acetate-Induced Protein 1
PNA	Peptide nucleic acids
PS	Phosphatidylserine
PTEN	Phosphatase and tensin homolog
PUMA	BCL2 binding component 3
RAC1	Ras-Related C3 Botulinum Toxin Substrate 1
RANGTP	GTP-binding nuclear protein Ran
RCC	Renal cell carcinoma
RECK	Reversion-inducing cysteine-rich protein with Kazal motifs
RHOC	Rho-related GTP-binding protein RhoC
RIP	Receptor-interacting protein
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
ROCK1	Rho-associated protein kinase 1
ROCK2	Rho Associated Coiled-Coil Containing Protein Kinase 2
ROCK2	Rho-associated protein kinase 2
RPL13A	Ribosomal Protein L13a
rpm	Revolutions per minute
RPMI-1640	Roswell Park Memorial Institute-1640
rt	Room temperature
RT	Reverse Transcription
RTEL1	Regulator Of Telomere Elongation Helicase 1
RT-qPCR	Real-Time quantitative Polymerase Chain Reaction
SCP1	Stress-activated protein kinase
SEMA3B	Semaphorin 3B
siRNA	Short interfering RNA
SIRT1	NAD-dependent protein deacetylase sirtuin-1

SMAC/DIABLO	Second mitochondria-derived activator of Caspases/Diablo homolog
SNAI2	Snail Family Zinc Finger 1
snRNA	Small nuclear RNA
SOCS1	Suppressor of cytokine signaling 1
SOX4	SRY-box containing transcription factor
SOX4	Transcription factor SOX-4
SPHK1	Sphingosine Kinase 1
STAT1	Signal Transducer and Activator of Transcription-1
TAE	Tris-acetate-EDTA
TBHP	Tert-butyl hydroperoxide
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TFA/DCM	Trifluoroacetic Acid/ Dichloromethane
TGF-β	Transforming growth factor b1
TIMP3	Tissue Inhibitor of Metalloproteinases 3
TNA	Therose nucleic acid
TMZ	Temozolomide
TNC	Tenascin
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
TP53	Cellular tumor antigen p53
TP53INP	Tumor Protein P53 Inducible Nuclear Protein 1
TRADD	Tumor necrosis factor Receptor type 1-Associated DEATH Domain protein
TRAF6	TNF receptor-associated factor 6
TRAIL	TNF-related apoptosis-inducing ligand
Tspan14	Transmembrane 4 Superfamily Member 14
UTR	Untranslated region
UA	Ursolic acid
UV	Ultra Violet
VEGF	Vascular endothelial growth factor
Vf	Final volume

WHO	World Health Organization
Wnt/β	Protein Wnt/β-Catenin
X-IAP	X-linked inhibitor of apoptosis
YB-1	Y-box Binding Protein-1
ΔΔCt	Delta delta threshold cycle

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PUBLICATIONS

Int J Oncol. 2016 Mar;48(3):1029-38. doi: 10.3892/ijo.2015.3308. Epub 2015 Dec 24.

High levels of apoptosis are induced in human glioma cell lines by co-administration of peptide nucleic acids targeting miR-221 and miR-222.

Brognara E, Fabbri E, Montagner G, Gasparello J, Manicardi A, Corradini R, Bianchi N, Finotti A, Breveglieri G, Borgatti M, Lampronti I, **Milani R**, Dechecchi MC, Cabrini G, Gambari R.

Abstract

The biological activity of a combined treatment of U251, U373 and T98G glioma cell lines with two anti-miR PNAs, directed against miR-221 and miR-222 and conjugated with an ocataarginine tail (R8-PNA-a221 and R8-PNA-a222) for efficient cellular delivery, was determined. Apoptosis was analyzed, and the effect of the combined treatment of glioma cells with either or both PNAs on the reversion of drug-resistance phenotype was assessed in the temozolomide-resistant T98G glioma cell line. Selectivity of PNA/miRNA interactions was studied by surface plasmon resonance (SPR)-based Biacore analysis. Specificity of the PNA effects at the cellular level was analyzed by RT-qPCR. These experiments support the concept that the effects of R8-PNA-a221 and R8-PNA-a222 are specific. The studies on apoptosis confirmed that the R8-PNA-a221 induces apoptosis and demonstrated the pro-apoptotic effects of R8-PNA-a222. Remarkably, increased pro-apoptotic effects were obtained with the co-administration of both anti-miR-221 and anti-miR-222 PNAs. In addition, co-administration of R8-PNA-a221 and R8-PNA-a222 induced apoptosis of TMZ-treated T98G cells at a level higher than that obtained following singular administration of R8-PNA-a221 or R8-PNA-a222.

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Phloridzin derivatives inhibiting pro-inflammatory cytokine expression in human cystic fibrosis IB3-1 cells.

Milani R, Marcellini A, Montagner G, Baldisserotto A, Manfredini S, Gambari R, Lampronti I.

Abstract

Cystic Fibrosis (CF) is the most diffuse autosomal recessive genetic disease affecting Caucasians. A persistent recruitment of neutrophils in the bronchi of CF patients contributes to exacerbate the airway tissue damage, suggesting that modulation of chemokine expression may be an important target for the patient's well being thus the identification of innovative anti-inflammatory drugs is considered a longterm goal to prevent progressive tissue deterioration. Phloridzin, isolated from Malus domestica by a selective molecular imprinting extraction, and its structural analogues, Phloridzin heptapropionate (F1) and Phloridzin tetrapropionate (F2), were initially investigated because of their ability to reduce IL-6 and IL-8 expression in human CF bronchial epithelial cells (IB3-1) stimulated with TNF-α. Release of these cytokines by CF cells was shown to be controlled by the Transcription Factor (TF) NF-kB. The results of the present investigation show that of all the derivatives tested, Phloridzin tetrapropionate (F2) is the most interesting and has greatest potential as it demonstrates inhibitory effects on the expression and production of different cytokines involved in CF inflammation processes, including RANTES, VEGF, GM-CSF, IL-12, G-CSF, MIP-1b, IL-17, IL-10 and IP-10, without any correlated anti-proliferative and pro-apoptotic effects.

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Design, synthesis and biological activity of a novel Rutin analogue with improved lipid soluble properties.

Baldisserotto A, Vertuani S, Bino A, De Lucia D, Lampronti I, **Milani R**, Gambari R, Manfredini S.

Abstract

Recent interest in flavonoids has increased greatly due to their biological and pharmacological activities. Flavonoids, consist of a large group of low molecular weight polyphenolic substances, naturally occurring in fruits, vegetables, tea, and wine, and are an integral part of the human diet. Rutin is a common dietary flavonoid that is widely consumed worldwide from plant-derived beverages and foods as traditional and folk medicine remedy as well. Rutin exhibit important pharmacological activities, including anti-oxidation, anti-inflammation, anti-diabetic, anti-adipogenic, neuroprotective and hormone therapy. Here, we present the synthesis, antimicrobial, antiproliferative and pro-apoptotic effect on human leukemic K562 cells of compound R2, a new semi-synthetic derivative of Rutin as compared to Rutin itself. The new derivative was also included in finished topical formulations to evaluate a potential application to the dermatology field in view of the antioxidant/antimicrobial/antiinflammatory properties. Stability studies were performed by HPLC; PCL assay and ORAC tests were used to determine the antioxidant activity. R2 presented an antioxidant activity very close to that of the parent Rutin while bearing much better lipophilic character. Regarding antiproliferative effects on the human K562 cell line, R2 was found to be more effective than parent Rutin. Preliminary experiments demonstrated that R2 inhibits NF-kB activity and promotes cellular apoptosis.

ABSTRACTS

21nd World Congress on Advances in Oncology and 19th International Symposium on Molecular Medicine (Athens, Grece, 2016)

Possible detection of Autologous Blood Transfusion (ABT) based on circulating plasma microRNAs involved in erythroid differentiation and fetal hemoglobin induction

Alessia Finotti, Nicola Lamberti, Jessica Gasparello, Nicoletta Bianchi, Enrica Fabbri, Lucia Carmela Cosenza, Roberta Milani, Ilaria Lampronti, Francesca Dalla Corte, Roberto Reverberi, Fabio Manfredini, Roberto Gambari

Detection of Autologous Blood Transfusions (ABT) is a key issue in the field of anti-doping for the performance enhancing effects of this prohibited method and the consequent unfair use in sport. Unfortunately, at present no direct detection method of ABT is available. In the last years, after the implementation of the Athlete's Biological Passport, scientists have searched novel parameters, combined biomarkers using mathematical approaches and tested new techniques as 'omics' technologies to detect ABT. The present study was performed to determine whether miRNA associated to erythroid phenotype, fetal haemoglobin production and regulation of gamma-globin genes are modulated following ABT. From 6 healthy subjects 500 ml of blood was withdrawn (T2) and then infused after 35 days (T5). Blood samples for microarray analysis of miRNAs were taken 5 days before (T1) and 10 days after blood withdrawal (T3), at day of infusion (T5), and at 3 days (T6) and 15 days (T8) after infusion. For three subjects the withdrawn blood was stored at -80°C, for three at +4°C before infusion. The global microRNAs profiling was performed for a total of 39 RNA samples (extracted from plasma), using the Agilent Human microRNA microarray v.21.0 (#G4872A). This chip represents 2549 microRNAs, sourced from the Sanger miRBase database (Release 21). Microarray results were analyzed using the GeneSpring GX 13 software (Agilent Technologies). Differentially expressed miRNAs were selected following determination of the fold-change analysis, taking T1 as reference sample. Both up-regulated and down-regulated miRNAs were considered. The first set of microRNAs analyzed was constituted by miRNAs related to erythroid differentiation, HbF

production and transcriptional regulation of gamma-globin genes. The results obtained allows to conclude that (a) a person-to-person variability is present when the different ABT subjects were analyzed; (b) miRNAs whose expression was found modified following ABT were miR-766-3p (upregulated), miR-191-3p (upregulated) and miR-16-5p (down-regulated). These results support the concept that miRNA analysis might be considered for detection of ABT when serum samples in analytical test finalized to detection of doping.

Anti-inflammatory activity of novel 4,6,4'-trimethylangelicin's analogues: effects on the NF-kB activity and IL-8 expression in Cystic Fibrosis IB3-1 cells

Ilaria Lampronti, Roberta Milani, Maria Giulia Manzione, Roberto Gambar, Giulio Cabrini, Giovanni Marzaro, Adriana Chilin

Psoralens are well-known furocoumarins belonging to the class of photosensitizers used for their activity in the treatment of various chronic inflammatory skin diseases. They are characterized by a differently substituted tricyclic aromatic skeleton, derived from condensation of a coumarin nucleus with a furan ring1. Recently, it was established that 4,6,4'-trimethyl-angelicin (TMA) is a strong inhibitor of the expression of the IL-8 gene in bronchial epithelial cells in which the inflammatory response has been challenged with P. aeruginosa2, the most common bacterium found in the airways of patients affected by Cystic Fibrosis (CF); moreover TMA, in addition to its anti-inflammatory and CFTR potentiator activities, also displays CFTR corrector properties3. These findings suggested us to analyze new synthetic derivatives of TMA in order to evaluate their biological activities on human bronchial epithelial CF IB3-1 cells. In inflammatory processes that involve CF patients NF-κB transcription factor plays a crucial role. In fact, the expression of many genes encoding for cytokines, chemokines, adhesion molecules, and other proteins involved in inflammation, is regulated by NF-κB. Analogues able to inhibit NF-κB/DNA interaction at lower concentration than TMA were selected to investigate their biological activity on IB3-1 cells induced with TNF-α. In this biological system NF-κB IL-8 gene expression was investigated. Some analogues showed similar activity (33-38% of inhibition of IL-8 expression) to the lead compound TMA. Other analogues displayed higher activities, in particular the most interesting compounds showing relevant anti-inflammatory effects are nine and they were found to cause 56-83% reduction of IL-8 mRNA expression at low concentrations (1-10 μM), without changes in cell proliferation pattern, demonstrating their potential interest for a possible anti-inflammatory therapy of CF.

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High levels of apoptosis induced in human glioma cell lines by combined treatment with antagomiR PNAs and pre-miRNA molecules

Enrica Fabbri, Eleonora Brognara, Giulia Montagner, Jessica Gasparello, Nicoletta Bianchi, Alessia Finotti, Giulia Breveglieri, Monica Borgatti, Ilaria Lampronti, Roberta Milani, Giulio Cabrini and Roberto Gambari

Peptide nucleic acids (PNAs) are artificially synthesized RNA or DNA analogues with a N-(2-aminoethyl)glycine unit backbone without the sugar-phosphate. These molecules are very interesting because of their capability of forming Watson-Crick double helices despite a radical structural change with respect to DNA and RNA. They can hybridize efficiently to complementary DNA- and RNA-specific sequences. For this reason, they have been proposed as useful tools for the alteration of gene expression. Forced interactions between PNAs and microRNAs have been recently found to alter biological functions. MicroRNAs are non-coding RNA molecules, around 22 nucleotides long, with a regulatory activity on gene translation or a generation of cleaved target RNA transcripts. Among the possible microRNA targets involved in cancer, the cluster miR-221/222 plays a very important role and is responsible for strong anti-apoptotic effects. On the other hand, miR-124-3p has been demonstrated to be a strong pro-apoptotic molecule. In order to induce apoptosis, the human glioma cell lines U251 and T98G were treated with pre-miR-124-3p together with PNAs targeting miR-221 and miR-222 (R8-PNA-a221 and R8-PNA-a222, bearing a oligoarginine peptide R8 to facilitate cell uptake). The effects of this combined treatment were analyzed looking at (a) the rate of cell growth, (b) caspase 3/7 cascade activation and (c) annexin 5 assay. The results obtained clearly indicate a potentiation of the pro-apoptotic effects using a combined treatment of glioma cell lines with pre-miR-124-3p and PNA-base antagomiR-221 and antagomiR-222. In conclusion, combined treatments with pre-miRNA and antagomiR molecules may be taken into consideration in order to achieve an efficient outcome in miRNA therapeutics of cancer diseases including gliomas.

Induction of apoptosis of human glioma cell lines: Effects of combined treatment with corilagin and temozolomide

Roberta Milani, Ilaria Lampronti, Enrica Fabbri, Eleonora Brognara, Nicoletta Bianchi, Alessia Finotti, Chung-Hin Chui, Monica Borgatti and Roberto Gambari

Corilagin (beta-1-O-galloyl-3,6-(R)-hexahydroxydiphenoyl-d-glucose), a gallotannin identified in several plants, including Phyllanthus urinaria, has been shown to exhibit versatile medicinal activities. In the present paper, we report experiments aiming at determining the effects of corilagin on nuclear factor kappaB (NF-κB) binding to DNA target and on apoptosis by administering corilagin to two glioma cell lines (U251 and T98G), one of which (T98G) is resistant to temozolomide (TMZ). The data obtained in experiments based on electrophoretic mobility shift assay and in molecular docking simulations demonstrate that corilagin binds to NF-κB and inhibits NF-κB/DNA interactions. The effects on apoptosis were evaluated using the MUSETM instrument and annexin-5 and caspase 3/7 assay kits. The results obtained indicate that corilagin inhibits cell proliferation and induces apoptosis in U251 and T98G glioma cell lines. The effects on cell growth indicate a 60 and 100 μM IC50 of corilagin on U251 and T98G cell lines, respectively. By contrast, dramatic differences were found using TMZ (IC50 75 μM for U251 and 500 μM for T98G). Therefore, corilagin is also effective on the TMZ-resistant T98G glioma cells. This is sustained by further experiments based on the two apoptosis assays and showing that both U251 and T98G cell lines express apoptotic markers when treated with 50 (U251) and 75 (T98G) μM corilagin. Finally, T98G glioma cells treated with sub-optimal concentrations of corilagin (50 μM) expressed high apoptotic levels when they were co-treated with TMZ.