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Preclinical development of new anticancer strategies based on the telomeric G-quadruplex ligands in colorectal cancer advanced models

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Environment and Public Health

In the third millennium, cancer became the major problem for the worldwide health. Independently of the socio-economic status of the countries, is a multifactorial phenomenon. The factors that most impact on this disease, as for the human health in general, are often related to the environment that surrounds us. The industrial expansion leads to ever-increasing pollution and every day airborne toxic agents contribute to the ozone hole which favors an increase in ionizing radiation. Every day highly toxic waste extremely harmful to the nature and to human health are released into the environment. Many of these waste products are mutagens and, like ionizing radiation, cause genetic mutations and contribute to the onset of cancer in humans.

The western countries are highly affected by cancer, even if the cancer burden is on the rise also in under-developed countries, with a projected 81-100% increase by 2030. The pollution, one of the main problems of the modernity, is associated with very different kind of tumors such as lung and nasopharyngeal cancer (Ding et al., 2015). The management of environmental resources is in fact a matter of increasing importance in modern times for its consequences on public health. The most affected natural resources are water, air and the atmosphere in general, with dramatic consequences on human health and increasing cancer cases.

Water pollutants are organic, inorganic, biological and radiological substances as a direct result of human activity, particularly during industrial processes. Industrial waste that is released by industries are of different types. For example, the caustic soda and chlorine factories release heavy metals such as cadmium, a dangerous risk factor for cancer (Huff et al., 2007; Hartwing et al., 2013), and other heavy metals such as chromium, copper, lead, nickel, zinc and mercury that can go up to the food chain (Kim et al., 2015). Another heavy metal well known as a cancer risk factor is arsenic (Smith et al., 1992). Water polluted by arsenic for domestic use brings increased risk in developing lung, bladder, breast and skin cancer (Mendez et al., 2017; Khanjani et al., 2017; Gamboa-Loira et al., 2017). In 2001 the Environmental Protection Agency (EPA) for municipal water established as maximum concentration of arsenic <10μg / l. However, recent studies have nevertheless shown that exposure to water for domestic use with these concentrations of arsenic is associated with a higher incidence of skin cancer raising more and more public attention on this problem (Mayer et al., 2016). Other sources of water pollution are fertilizers and herbicides used in intensive agricultural production. Glyphosate is the most famous commercially successful herbicide of all time since 2016. Used since 1974, only recently the International Agency for Research on Cancer (IARC) in March 2015 decided to reclassify the compound as category 2A (probably carcinogenic to humans), following consensus opinion of the scientific community (Davoren et al.,

2018). However, the conclusion of the IARC was not confirmed by the EU assessment or the recent WHO / FAO joint evaluation, both using additional evidence (Portier et al., 2016; Tarazona et al., 2017). In fact, current and official opinions of the competent international regulatory and health bodies remain divided on the carcinogenic status as a human carcinogen (Andreotti et al., 2018). In any case, the current case of glyphosate can be considered paradigmatic of the management of environmental resources and public health.

Air pollution levels are dangerously high in many parts of the world, although their association with human diseases has long been known (Pope et al., 2002; Raaschou-Nielsen et al., 2013). The World Health Organization estimates that 90% of the world's population breathes air containing high levels of pollutants and that about 7 million people die each year from exposure to fine particles in polluted air that penetrate deep into the lungs and into the system cardiovascular, as evidenced by the 4.2 million deaths in 2016 due to air pollution. The most dangerous pollutants for public health include nitrogen dioxide (NO2), sulfur dioxide (SO2) and well-documented particles with a diameter of less than 10 and 2.5 microns (PM10 and PM2.5). These particles are able to penetrate deep into the lungs and enter the bloodstream causing cardiovascular, cerebrovascular and respiratory impacts. In 2013 they were classified as lung cancer cause by the WHO International Agency for Research on Cancer (IARC) and new evidences constantly correlate these air pollutants with lung cancer (Zhou et al., 2017).

Another alarming environmental phenomenon is the ozone hole caused mainly by the release of chlorofluorocarbons (CFC), chemical compounds that are released by gases composed of chlorine, fluorine and carbon, used for spray cans, in refrigerating circuits of refrigerators and air conditioners of air and as foaming agents for the manufacture of materials such as expanded polystyrene. These compounds introduce chlorine into the atmosphere causing depletion in the ozone that normally filters UV rays allowing life on Earth. This phenomenon has long been recognized as causing an increase in the incidence of tumors due to gene mutations caused by UV rays (Jones et al., 1987), in particular in skin cancers (Narayanan et al., 2010).

Global policies are striving to manage the environment and the resources that allow life. As recently stated by Dr. Maria Neira, Director of the Department of Public Health, WHO's social and environmental determinants of health, "We are seeing an acceleration of political interest in this global challenge to public health". However, diseases related to the environment, and in particular cancer, are constantly increasing, with emerging evidence also correlating colorectal cancer to environmental pollution (El-Tawil, 2010; López-Abente et al., 2012). Among the most dangerous pollutants there are persistent organic pollutants (POP) which include organochlorine pesticides

(OCPs) and polychlorinated biphenyls (PCBs), resistant to environmental degradation. POPs bioaccumulate in food and adipose tissue of living organisms and then are released from adipocytes to circulation through lipolysis. The chronic exposure to low-dose is associated with an increased risk of colorectal polyps and cancer (Lee et al., 2018).

Even prolonged exposure to pesticides may be a potential risk factor for colon cancer. (Francis et al., 2018). Alavanja and colleagues studied the effects of 50 commonly used pesticides in 56,813 pesticide applicators and found a potential relationship between exposure to chlorpyrifos and aldicarb with the incidence of colorectal cancer (Lee et al., 2007). Moreover, a meta-analysis study suggested that aldicarb could increase colon cancer risk, imazethapyr may promote cancer risk in the proximal colon region, and colorectal cancer risk is probably enhanced by exposure to pesticides such as pendimethalin, chlorpyrifos, chlordane, or toxaphene (Alexander et al., 2012). Recently, exploring the massive number of modern xenobiotics, dangerous chemicals to which human organism is exposed through nutrition, environmentalsand drugs, a research group analysed 6000 human-made compounds and found that 16.3% of those chemicals were pesticides, of which less than 1% had been investigated in the context of cancer (Guha et al., 2016). For these reasons it is necessary reducing pesticide residue levels in the environment and food production to safeguard global human health.

It is therefore increasingly necessary to develop new therapies that can have effective effects on cancer, a world-wide disease with more and more risk factors which contribute to its emergence.

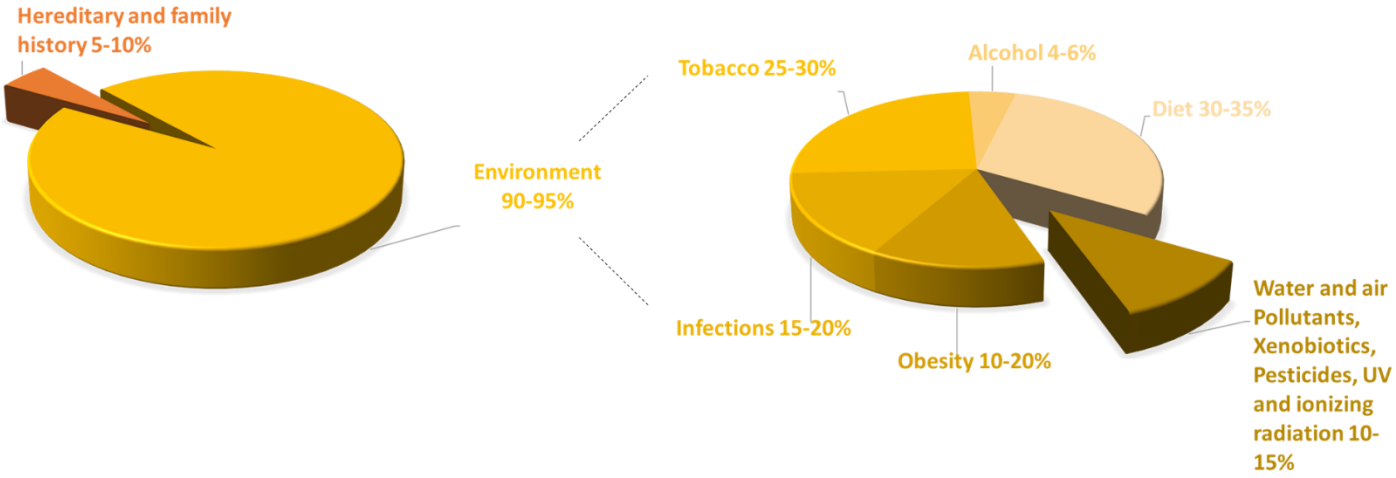


Figure 1. Factor risks of cancer.

Cancer Biogenesis

Cancer development is a multi-stages carcinogenesis process and this concept was proposed for the first time in 1947 by Berenblum and Shubik and supported by many later studies (I. Berenblum and P. Shubik. 1947). In these studies all the main characteristics of carcinogenesis have been described, starting from its gene mutation etiology. In particular, DNA mutations can occur in two different classes of genes that regulate cellular homeostasis: oncogenes and tumor suppressors (Weinber, 1994; Vogelstein and Kinzler, 1998).

Oncogenes and oncosuppressors

The mutations that occur in the DNA sequences of the so-called oncogenes are often 'gain-of-function' mutations that make the transcribed protein more active than normal counterpart. In fact, oncogenes are the positive regulators of carcinogenesis and encode for the proteins that regulate the cell growth such as growth factors, protein kinases involved in signal transduction such as RAS GTPases, tyrosine kinase receptors such as VEGFR (Vascular Endothelial Growth Factor Receptor) and transcription factors. In normal cells they are called proto-oncogenes and, when they undergo mutations, they are activated becoming oncogenes leading to neoplastic development (Bishop, 1983). Molecular studies on viral oncogenes and their products have led to the discovery of physiological proto-oncogenes, involved in viral transformations of cellular genomes, such as *c-Myc*, *c-Myb* and *C-H-RAS* (Rapp, 1983; Antohi, et al. 1987). A large number of mutations in specific oncogenes such as *RAS* and *MYC* have been associated for a long time with different types of cancer, such as *RAS* in the case of pancreatic, colorectal and lung cancer or *MYC* in the case of melanoma, breast cancer and Burkitt's lymphoma (Bishop, 1987; Marina et al., 2006; Prior et al., 2012).

While oncogenes are considered as positive regulators, tumor suppressor are the negative regulators of carcinogenesis. These genes normally act by regulating cell proliferation and the progression through the cell cycle, finally inhibiting tumor development. In many tumors, mutations in these genes cause the so-called 'loss-of-function', removing negative regulators of cell proliferation and contributing to the abnormal proliferation of tumor cells (Perkins and Stern, 1997). Among the variety of tumor suppressor are the most widely studied *Rb* (Retinoblastoma) and the cell master regulator *p53* (Farnebo et al., 2010). The proteins encoded by these two genes inhibit the cell cycle by blocking the transcription of the genes necessary for the transition from the G1 phase to the S phase. Retinoblastoma tumor development is principally based on *Rb* gene inactivation through

chromosomal mutations and, in general, mutations in the *Rb* gene lead to the loss of normal cell cycle control inhibitors because of its role as a major G1 checkpoint, with activity in blocking S-phase entry and cell growth (Giacinti and Giordano, 2006). In general, loss of protein Rb (pRb) functions may induce cell cycle deregulation and so lead to a malignant phenotype and this effect is usually coupled to genetic changes that cause the loss of the apoptotic signal and enhances the neoplastic transformation.

P53 is the genomic guardian with a major role in genomic stability and cellular balance. In normal cells, this gene is able to warn DNA damage and repair it when is possible. When the DNA damage repair is not possible, *p53* induces apoptosis (Kastan, 1997). The other activities of *p53* are the regulation of cell cycle, in the checkpoint in the transition from the G1 phase to the S phase and the induction of cell differentiation. Mutations in *p53* lead to a loss of function that causes the repression of apoptosis, the promotion of cell division through the release of the G1/S checkpoint and avoids cell differentiation with subsequent neoplastic development (Curtis, 1993). These mutations represent the main genetic changes occurring in about 50% of the human cancer cases.
Most mutations (75%) in *p53* are missense replacements of which 30% corresponds to the substitution of the highly mutable CpG dinucleotides in codons encoding the residues that have an essential role in the chemical structure that allows the interaction of *p53* with specific sequences of DNA (Hainaut and Hollstein, 2000). The other mutations in *p53* include frameshift insertions and deletions (9%), non-sense mutations (7%), silent mutations (5%) and other infrequent alterations (Olivier et al., 2002). Most mutations in *p53* mainly involve the DNA-binding domain (DBD-DNA binding domain) from the amino acid 102 to the amino acid 292 (Muller and Voudsen, 2013). In particular, there are several hotspots, which are the most susceptible to mutations. These hotspots identify their respective mutants, such as Y220, G245, R248, R249, R273, R282. The most frequent mutants can be divided into mutants for contact with DNA, such as R248W and R273H, and in conformational mutants such as R175H, Y220C, G245S, R248Q, R249S, R282W (Joerger and Fersht, 2010).

Despite several studies during the years, about the roles and regulation of *p53* remains a lot to learn. The challenge is to convert the knowledge about *p53* into clinical application. Traditional strategies have been directed towards *p53* mutants with the aim of identifying molecular compounds to eliminate *p53* mutant or to restore its tumor suppressive function. However, the new technologies and the growing understanding of *p53* activity are leading to important preclinical progress. As it is emerging, tumor cells with mutated *p53* activate genes and secondary pathways to survive, thus

providing alternative molecules to be targeted by new therapies. Indeed, targeting these new genes is a promising cancer strategy (Zhao et al., 2017; Kastenhuber and Lowe, 2017).

Cancer development

Currently tumor development is described with a three-phase model in which initiation, promotion and tumor progression is distinguished. In the first phase of initiation in the cell occur irreversible somatic mutations with genetic and epigenetic alterations that reduces the sensitivity of the cells to tissue growth constraints. This transformed population produces minimal or no observable effect on the morphology of the tissue and, without additional stimuli, will not form a tumor.

In the next phase of promotion, transformed cells can remain in a quiescent state or receive stimuli that promote uncontrolled proliferation such as wounding or inflammation (Balkwill et al., 2005). The primary mechanisms for tumor promotion are an increase in the evolutionary rate followed by an increase in local blood flow that alters the adaptive landscape.

In the third phase of development towards tumor progression, the origin of tumor heterogeneity occurs with the formation of more malignant cell subpopulations. In this phase, gene amplification events, point mutations, translocations and chromosomal rearrangements make the cells more aggressive and potentially more resistant to drugs. The cells that reach this high rate of mutations begin to proliferate rapidly increasing the size of the tumor and, consequently, even the most malignant subpopulations increase. The progression phase of carcinogenesis is dominated by cellular adaptations to stringent microenvironmental conditions including hypoxia and acidosis that causes normal cell death. Indeed, the phenotype emerging has a potent proliferative advantage based on the creation of an acidic environment (through upregulated glycolysis) that is toxic to its competitors but not itself. This phenomenon causes a large die in normal cells and promote the cancer invasion (Vincent and Gatenby, 2008).

Differently from normal cells, in the tumor cells the set of genetic mutations and the stages of cancer development are distinguishable in well-defined features called hallmarks of cancer.

Hallmarks of cancer

The genetic alterations caused by mutations in oncogenes and oncosuppressors involve a series of different events that promote the development of the tumor. In 2000 the complexity of these events was first classified in six essential alterations in the cellular physiology which collectively induce malignant growth in all types of tumors. These so-called hallmarks (Figure 2) of cancer are: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000).

The first of the six capabilities defined by Hanahan and Weinberg is the acquired growth signals because of the prevalence of dominant oncogenes that have been found to modulate it. Cancer cells can achieve this autonomy through the alteration of extracellular growth signals, transcellular transducers of those signals and intracellular downstream pathway. The surface receptors which transduce growth-stimulatory signals are involved in cancer progression too. Receptor overexpression may enable the cancer cell to become hyper-responsive to ambient levels of growth factors that normally would not trigger proliferation (Aaronson et al., 1991). These receptors often carry tyrosine kinase activities in their cytoplasmic domains and are overexpressed in many cancers. An example of growth factors receptors overexpression is the case of Epidermal Growth Factor Receptor (EGFR) that is often upregulated in colorectal and breast cancer (Normanno et al., 2006; Sigismund et al., 2018). The most complex mechanisms of acquired growth system autonomy derive from alterations in pathways that regulate cell-growth such as the SOS-RAS-RAF-MAPK.

The insensitivity to antigrowth signals is the second aspect of tumors. In fact, normal cells have multiple antiproliferative signals that control homeostasis maintaining the quiescence and inhibiting the growth while cancer cells are insensitive to antigrowth signals. Pathways that enable cells to evade these signals are associated with the cell cycle clock, in particular with the components governing the transit of the cell through the G1 phase of its growth cycle. Cells, on the basis of received signals, decide when to proliferate, to be quiescent, or to enter into a postmitotic state. Anti-growth signals can block proliferation forcing out of the active proliferative cycle into the quiescent (G0) state. At the molecular level, the master protein that regulate the antiproliferative signals is the previously described Retinoblastoma protein. The hypophosphorylation of pRb causes the block of proliferation involving the sequester of E2F transcription factors that controls the gene expression necessary for progression from G1 to S phase (Weinberg, 1995).

Evading apoptosis is another ability that elicit cancer cells to expand in uncontrolled rate. Apoptosis is regulated by the Bcl-2 family of proteins, whose can be proapoptotic (Bax, Bak, Bid, Bim) or

antiapoptotic (Bcl-2, Bcl-XL, Bcl-W) (Yip and Reed, 2008), and the equilibrium between these two classes ensures appropriate regulation of programmed cell death during development and maintains homeostasis. In particular, increased expression of pro-survival Bcl-2 proteins is found in many cancer types. This upregulation can occur through a variety of mechanisms including chromosomal translocation, gene amplification, increased gene expression or protein stability with various mechanisms. Targeting the pro-survival Bcl-2 proteins could help to eliminate pre-cancerous lesions or early-stage tumors (Campbell and Tait, 2018; Reed, 2018).

Besides mutations in Bcl-2 family proteins, tumor cells become resistant to apoptosis through a variety of strategies such as the loss of function of *p53* tumor suppressor gene, the well-known proapoptotic regulator previously described (Shen and White, 2001). p53 tumor suppressor protein, in response to DNA damage, can elicit apoptosis by upregulating expression of proapoptotic Bax protein that in turn stimulates mitochondria to release cytochrome C (Gao et al., 2001) inducing a series of biochemical reactions that result in caspase activation and subsequent cell death (Jiang and Wang, 2004).

Cancer cells have the ability to generate macroscopic tumors thanks to the unlimited replicative potential based on protection of telomere ends of chromosome. Normal cells can pass through only a limited number of successive cell growth/division cycles progressively shortening before entering senescence, the irreversible nonproliferative but viable state, and then crisis, which leads to cell death. Accordingly, the length of telomeres dictates how many successive cell growth/division cycles can pass through before telomeres are largely eroded, lost their protective functions and triggering crisis. Instead, cancer cells reactivate Telomerase, the DNA polymerase that adds telomer repeat segment to the ends of telomeric DNA. This enzyme is normally absent in normal cells (except the germinal lines) but expressed in many types of cancer cells. The Telomerase activity make cells resistant to senescence, crisis and successive apoptosis. These observations led to search for a possible antitumoral therapeutic strategy based on the targeting Telomerase in order to inhibit its activity leading to telomere shortening and activation of programmed cell death (Shay and Wright, 2002).

Another crucial mechanism is the oxygen and nutrient supplying that reach the tumor, necessary for cellular functions and growth. Tumor cells are able to stimulate the vessels growth, in an organogenesis similar way, involving soluble factors and their receptors exposed on the surface of the endothelial cells. Integrins and adhesion molecules which mediate the cell-cell association and the formation of the cell-matrix also play critical roles. The first inducers of angiogenesis are vascular endothelial growth factor (VEGF), a highly specific mitogen for endothelial cells, and

fibroblast growth factors (FGF1/2) (Hoeben et al., 2004). Many tumors increased expression of VEGF and/or FGFs compared to their normal tissue counterparts. In this way, the formation of new vessels allows on the one hand the arrival of oxygen and nutrients but also permits cancer cells to enter in the bloodstream and spread into other organs. Currently, in many types of cancer such as colorectal, lung or breast cancer, an important anti-cancer strategy is directed towards the process of angiogenesis with drugs such as the monoclonal antibody Bevacizumab (Maione et al., 2017; Tampellini et al., 2016; Bareschino et al., 2011).

Cancer cell spread is facilitated by deep angiogenesis in tumor core and cause the distant settlements of tumor cells. Metastases that are resistant to conventional therapy are the major cause of death from cancer. The capability to invade and metastasize enables cancer cells to escape the primary tumor mass and colonize new organs where nutrients and space are not limited until they cause the organ collapse. Moreover, metastases are heterogeneous populations very difficult to treat. However, the growth and survival of metastases depend on interactions between tumor cells and host homeostatic mechanisms. Targeting these interactions, in addition to the tumor cells, can produce synergistic therapeutic effects against existing metastases (Fidler and Kripke, 2015).

A decade later their first hallmark classification, Hanahan and Weinberg published an updating review with two emerging hallmarks: reprogramming energy metabolism and evading immune response, and two enabling characteristics: genome instability and tumor-promoting inflammation (Hanahan and Weinberg, 2011).

The genomic instability is consequent to multiple stressors, including the failure of the DNA replication process and/or defects in the mechanisms of DNA damage repair, and elicit new genetic alterations giving to neoplastic cells a selective advantage in the different subclones allowing dominance on normal tissues. The multistep tumorigenic process can be defined as the succession of clonal expansions triggered by the acquisition of selective genetic alterations that affect the inactivation of tumor suppressor genes, the hyperactivation of oncogenes and also the alteration of epigenetic mechanisms such as DNA methylation and modifications histone (Jones and Baylin, 2007). The accumulation of mutations rate depends on the grade of genome instability that can lead to increased sensitivity to mutagens. Furthermore, the accumulation of mutations can be accelerated by compromising the surveillance systems that normally control genomic integrity and force the genetically damaged cells to senescence or apoptosis (Jackson and Bartek, 2009). Impairment in Homologous Recombination (HR), a crucial DNA repair mechanism, favors genomic instability and several studies recognized that somatic mutations or alterations in genes involved in HR are present in a wide variety of tumors (Lord et al., 2016). However, HR deficiency also represents a

therapeutic target. In fact, HR-defective tumors are hypersensitive to chemotherapeutics (Cisplatin and Trabectedin) and to PARP inhibitors, molecules targeting the base excision repair pathway (Helleday et al., 2008; Livraghi et al., 2015) with a well-known specificity against tumors defective of BRCA2, a master gene involved in Homologous recombination mechanism (Bryant et al., 2005; Farmer et al., 2005). So, targeting genomic instability represent a possible therapeutic strategy.

Inflammation is known to contribute to cancer progression and characterizes many types of cancer that are infiltrated by both the innate and the adaptive immune cells (Mantovani et al., 2008; Barnes and Amir, 2017). Cancer progression can be evaluated through several markers as a prognostic value, such as the neutrophil-to-lymphocyte ratio (NLR), the lymphocyte-to-monocyte ratio (LMR) and the Glasgow prognostic score (GPS), and the recent the systemic inflammatory score (SIS), based on the combination of the LMR and the serum albumin concentration associated with clinical outcomes in patients with various types of cancer, including colorectal cancer (Shibutani et al., 2013; Shibutani et al., 2017; Suzuki et al., 2018). The immune responses occur as a physiological mechanism that recognizes the tumor antigens as unknown and attacks them in an attempt to eradicate the tumor. Nevertheless, inflammation can contribute to multiple functions by providing active molecules for the tumor microenvironment such as growth factors, survival factors, proangiogenic factors and enzymes that modify the extracellular matrix that facilitate angiogenesis, invasion and metastases; moreover, inflammation induces signals that lead to the activation of the epithelial-to-mesenchymal transition (EMT), the series of events through which epithelial tumor cells lose many of their characteristics and acquire a mesenchymal phenotype that gives it a spindle or stellate shape, and a high mobility that allows the migration in the surrounding interstitium by means of different mechanisms based on the emission of phylopodia and pseudopodia (Thiery and Sleeman, 2003; Friedl, 2004; De Nardo et al., 2010). In addition, inflammatory cells may release chemicals, particularly reactive oxygen species, which are actively mutagenic to neighbouring tumor cells, accelerating their genetic evolution to more malignant states (Grivennikov et al., 2010).

On one side, immune system can permit the cancer development through tumor-promoting inflammation, on the other side cancer evade and destruct the immune response (Vinaya et al., 2015). Highly immunogenic tumor cells can evade the immune response by disabling the components of the immune system. For example, cancer cells can paralyze infiltrating CTLs and NK cells secreting TGF-b or other immunosuppressive factors (Yang et al., 2010; Shields et al., 2010). For example, the IFN-γ produced by lymphocytes infiltrating the tumor may induce the upregulation of the PD-L1 immuno-inhibitory molecule on malignant cells (Tauble et al., 2012). In

addition, cellular immune system computations such as regulatory T cells (T-regs) and myeloid-derived suppressor cells (MDSCs) may also suppress cytotoxic responses of lymphocytes (Mougiakakos et al., 2010; Umansky et al., 2016). On this, studies of novel anticancer strategies are focused on targeting these cells (Beatty and Gladney, 2015; Shitara et al., 2018). In addition, tumor cells bind CTLA-4 and PD-1 inhibitory antigens associated with cytotoxic T lymphocytes and activate them by inhibiting their signals and consequently avoiding the immune response. Although not all tumors respond to immunotherapies, therapeutic monoclonal antibodies that interrupt these inhibitory signals are showing long-term survival benefits in several types of cancer, particularly in melanoma (Fellner, 2012; Topalin et al., 2012). In particular, the cytotoxic T-lymphocyte T antigen (CTLA-4) is a negative regulator of T cell activation. The inhibition of negative regulation by binding with CTLA-4 has been shown to promote stimulation of adaptive immunity and enhancement of T cell activation. Therefore, monoclonal antibodies are directed toward CTLA-4 including ipilimumab, approved in clinic in 2011, and tremelimumab, evaluated extensively in melanoma, are currently considered as new therapeutic strategies to increase anti-tumor immunity response (Grosso et al., 2013; Zhang et al., 2018).

The uncontrolled proliferation of tumor cells is also permitted by a deregulated metabolism with respect to the physiological conditions of normal cells adopting a different metabolic profile (Warburg et al., 1924). In fact, normal cells, in aerobic conditions, obtain energy by processing glucose in pyruvate through glycolysis in the cytosol and in carbon dioxide in the mitochondria. Under anaerobic conditions, glycolysis is favored and relatively little pyruvate is sent to the mitochondria that consume oxygen. What is described by the Warburg effect, on the other hand, is an abnormal metabolism in cancer cells that, even in the presence of oxygen, limit glucose metabolism largely to so-called 'aerobic' glycolysis. This reprogramming of the energy metabolism leads to a lower efficiency in the production of ATP which is however compensated by the tumor cells through a markedly higher use of glucose also thanks to the increase of glucose transporters, in particular GLUT1, which increases the import of glucose into the cytoplasm. In addition, also oncogenes such as *RAS* and *MYC* and tumor suppressors such as *p53* are associated with glycolytic transport (De Bernardis et al., 2008; De Bernardis et al., 2016; Danhier et al., 2017). Glycolysis dependence increases in hypoxic conditions that occur in many types of cancer. Under conditions of low or no oxygen availability, tumor cells increase the production of glucose transporters and enzymes necessary for the glycolytic pathway. Therefore, both RAS oncoprotein and hypoxic conditions can independently increase the levels of transcription factors of HIF1α and HIF2α, the transcription factors directly involved in tumor proliferation under hypoxic conditions. Therapeutic

strategies for inhibition of HIF1α have been studied for a long time (Powis et al., 2004; Semenza, 2010a; Semenza, 2010b; Masoud et al., 2015).

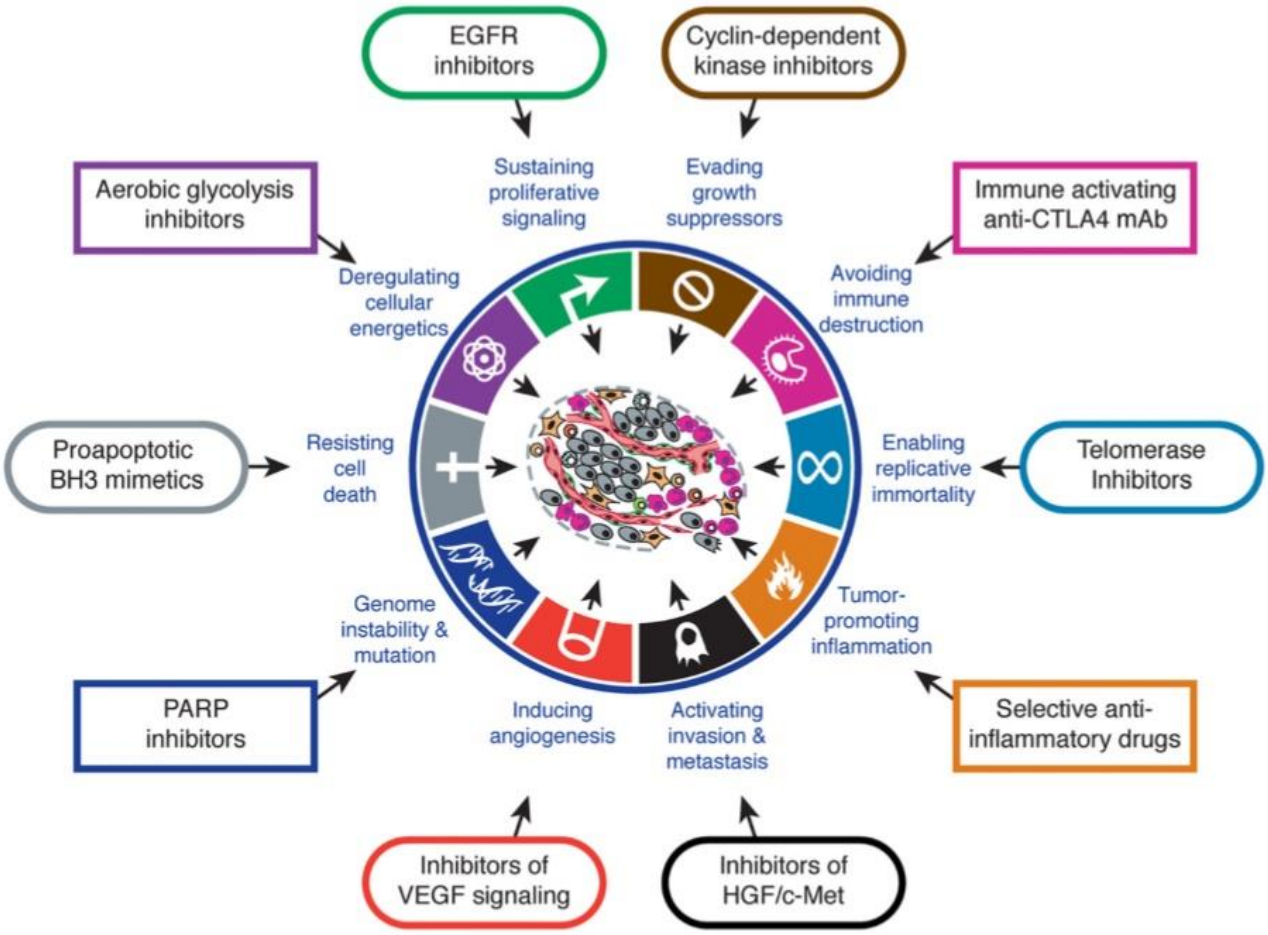


Figure 2. Hallmarks of Cancer and the respective therapeutic targeting (Hanahan and Weinberg, 2011).

Epigenetics and cancer

In addition to gene alterations, in recent years epigenetics has also emerged as a hallmark of cancer. The term "epigenetics" was coined by Conrad Waddington in 1942 to describe changes in cellular phenotype that are independent of changes in DNA sequence (Waddington, 1942). These changes are reversible and include key processes of DNA methylation, histone modifications, nucleosome remodelling, and alterations in noncoding RNA profiles. Alterations in these processes can lead to impaired gene function and cellular neoplastic transformation. Epigenetic modifications come first genetic changes and usually occur at early stage in neoplastic development. Recent technological advances offer a better understanding of the underlying epigenetic alterations during carcinogenesis and provide insight into the discovery of putative epigenetic biomarkers for detection, prognosis, risk assessment, and disease monitoring (Dawson et al., 2012; Kanwal et al., 2015). In general, tumor epigenome is characterized by extensive hypomethylation of DNA and CpG island density (CpG island methylator phenotype [CIMP]) and involves simultaneous hypermethylation of numerous CpG islands near to the promoter regions of a wide of genes. Hypomethylation events have a significant role in tumorigenesis and occur in genomic sequences that also include retrotransposons, poor promoters in CpG and introns (Jones and Baylin, 2002; Weisenberger et al., 2015). In colorectal cancer, CIMP is defined as a subset of colorectal tumors with an exceptionally high frequency of methylation of 'type C' loci, defined as loci methylated in cancer but not in normal tissue (Toyota et al., 1999).

DNA hypomethylation increases genomic instability and activates the proto-oncogenes, while site-specific hypermethylation contributes to tumorigenesis by silencing both oncosuppressor genes directly and other classes of genes indirectly by silencing their transcription factors. Epigenetic markers are widely known also for colorectal cancer (Wilson et al., 2007; Verma and Kumar, 2017), for example the methylation of *GATA-4* and *GATA-5*, (Akiyama et al., 2003), *ADAMTS1*, *MAL*, and *MGMT* promoter observed in both benign and malignant tumors compared with normal mucosa samples (Ahlquist et al., 2008), and, for early detection of colorectal cancer, a combination of hypermethylated markers such as *APC*, *MGMT*, *RASSF2A*, and *WIF-1* (Lee et al., 2009).

Studies on chromatin in tumor cells have highlighted also others epigenetic modifications such as Histone acetylation, a well-studied post-translational histone modification that is controlled by the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). HDACs remove the acetyl groups reversing chromatin acetylation and alter the transcription of oncogenes and tumor suppressor genes. In particular, loss of acetylation causes gene repression. In fact, HDAC enzymes are overexpressed in many tumors (Audia and Campbell., 2016) and for this

reason the therapeutic potential of HDAC inhibitors (HDACi) is emerged as a therapeutic strategy for cancer treatment (Li and Seto, 2016).

A controversial factor beyond genetics

An interesting recent study finds that the genetic alterations are not the unique responsible factors for cancer development. In fact, in 2015 Tomasetti and Vogelstein wrote a controversial article attributing to genetic alterations, in addition to genetic inheritance and environmental factors, a third responsible cause: the case (Tomasetti and Vogelstein, 2015). In particular, authors explain their theory with a stochastic effect associated with the number of stem cell divisions for each tissue during the course of human life. The number of stem cells in each tissue, and their rate of proliferation, can be certainly influenced by genetic and environmental factors such as those affecting stature or weight, and the likelihood of accumulating genetic mutations is directly proportional to the number of cell divisions. The stochastic effects of DNA replication can therefore be estimated numerically, distinct from external environmental factors and often more important than hereditary factors. With this study, therefore, Tomasetti and Vogelstein argue that for many tumors greater attention should be paid to early diagnosis rather than prevention. Subsequently, several articles questioning this theory were published. In fact, this controversial study has raised several objections, one of which concerns the issue of prevention. Gotay and colleagues (School of Population and Public Health, University of British Columbia, Vancouver) argue that cancer-related cancer prevention, and the types of cancer that most cause death in developed countries, such as lung cancer or to the colon, they are highly preventable. Many types of cancer are instead considered by Tomasetti and Vogelstein as non-preventable tumors, but in reality they have well-known risk factors such as the use of tobacco or alcohol in esophageal cancer or exposure to UV rays in melanoma. Gotay and collaborators also argue that the variability in cancer incidence is well documented, so the theory that these tumors mainly originate from random mutations in stem cells does not seem to be compatible with known data. Moreover, the number of cell divisions in stem cells are not simply caused by time or case but are influenced by factors such as obesity, environmental pollution, infections and inflammation (Doles et al., 2012; Chia-Lung et al., 2013). Potter and Ross (Prentice Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA) argue that the correlation between number of cell divisions of stem cells and the incidence of cancer in the United States is interesting but involves only 31 cancer histotypes. Tumors such as lymphoma, stomach cancer, breast, prostate, cervix, kidney, endometrium, bladder, which are the most common not only in the United States but also in the rest

of the world, are excluded from the study, so their incidence rates are not explained by the number of stem cell divisions. For almost all types of cancer, there are variations in incidence in the world between the western and eastern countries and in particular the poorest and the richest. In fact, the environment and the lifestyle are of fundamental impact on cancer and in particular on colorectal cancer.

Despite the critical issue, the theory of Tomasetti and Vogelstein continues to be interesting and controversial at the same time.

Colorectal Cancer

Statistics

Colorectal cancer is one of the three most prevalent cancer in the world. In particular, in United States occur about 1,36 millions of new cases and almost 700,000 death per year (Brenner et al., 2014; Brody, 2015). Beside the uncontrollable genetic factor, several lifestyle factors play an important role and are responsible of approximately 90% of colorectal cancer occurrence. Indeed, the incidence of this kind of cancer is very inconsistent over the world, with the highest rates in Europe, New Zealand, United States and Australia, and the lowest rates in Africa and South Asia. In 2012, a study showed a large disparity of colorectal cancer occurrence depending on socioeconomic status with an increased risk for the lowest socioeconomic status compared to the highest one due to the highest prevalence of adverse health behaviors such as unhealthy diet, alcohol consumption, smoking, obesity and absence of physical activity (Doubeni et al., 2012; Turati et al., 2017). In Europe, the individual countries have different cancer statistics but the homogenization of diet and lifestyles across the countries makes the cancer rates closer (Ferndandez et al., 2005; Malvezzi et al., 2018). In particular, colorectal cancer is a disease of the modern age: migrant studies showed that colorectal cancer mortality tends to change faster than for other cancers following lifestyle and diet modification (Kolonel et al., 2006; Brody et al., 2006). An important impact on survival for colorectal cancer is the organized screening prevention above 50 years of age that is very effective in reducing the incidence and the mortality such as also the combination of drugs, nutritional supplements, diet and exercise (Issa et al., 2017; Basu et al., 2018). The number of cancer survivors continues to increase because of both advances in early detection by colonoscopy and treatment and the aging and growth of the population (Miller et al., 2016; Siegel et al., 2017).

In Italy, the Italian Association of Medical Oncology (AIOM) in collaboration with Italian Association of Tumor Registers (AIRTUM) record every year the data about Italian cancer statistics. In Italy, every day 1.000 people receive a new diagnosis of malignant tumor and in 2018 have been diagnosed just over 373,000 new cases of malignancy, of which about 194,000 in men and 178,000 in women. Also in Italy, colorectal cancer is one of the three most predominant tumor among males (15%) and males (13%). The latest data available supplied by the National Institute of Statistics indicate that in 2015 tumors are the second leading cause of death (29% of all deaths) after cardio-circulatory diseases (37%). Indeed, among the total 600,000 death, just over 178,232 death was tumor related. Every day more than 485 people die due to cancer in Italy. The leading cause of death in the population is the lung cancer (19%), the first cause of death among men, while

breast cancer (17%) is the leading cause of death in women. The second leading cancer with the same impact on men and women is colorectal cancer (11% and 12% respectively). An encouraging data for Italy is the 5-year survival of the Italian Cancer Register pool (54% for men and 63% for women) which is lower than in the United States (69% in both sexes) and of Australian Cancer Registries (66% and 68%) but the highest at the European level as reported by EUROCARE-5, the European Cancer Registry Based Study on Survival and Care of Cancer. In fact, in Italy the 5-year survival, currently 65% for both sexes, has increased compared to that of cases diagnosed in the previous five-year-olds for men (54% in 2005-2009 compared to 51% in 2000-2004, 46% in 1995-1999 and 39% in 1990-1994) and women (respectively 63% vs 60%, 58% and 55%). This overall positive result has been reported for the other most frequent cancer sites including female breast (87%) and prostate (91%).

Clinical features

People who are already ill with inflammatory bowel disease, such as the most common forms of Ulcerative Colitis and Chron's Disease and live in an inflamed colon for a long period of time, have a higher risk of developing colorectal cancer and this depends both by the degree of inflammation and by the time it has lasted (Bernstein et al., 2001; Yashiro et al., 2014). It has been estimated that 18% of patients with a history of about 30 years of ulcerative colitis disease develop colorectal cancer. However, there is some evidence showing that in these patients the risk to develop cancer can be decreased thanks to better management of the disease through the use of drugs, control of the inflammation state and use of screening to detect precancerous lesions (Jess et al., 2012; Nguyen et al., 2012). The diabetic patients have also an increased risk of developing colorectal cancer (Campbell et al., 2010; Zhu et al., 2017) being the risk estimated 27% higher in type 2 diabetes patients and stronger in men than in women (Luo et al., 2012; Gonzalez et al., 2017). In general, colorectal cancer patients with diabetes appear to have a slightly lower survival rate than non-diabetic patients (Mills et al., 2013).

About 5% of patients with colorectal cancer have a well-defined genetic syndrome. The most common of these is Lynch syndrome or HNPCC (hereditary non-polyposis colorectal cancer). Approximately one in 35 patients has Lynch syndrome (Hampel et al., 2008; Kanth et al., 2017). Although individuals with this syndrome are predisposed to various disease such as stomach, ovary or endometrium cancer, the risk of developing colorectal cancer is the highest (Carethers and Stoffel, 2015; Singh and Resnick, 2017). For this reason, a growing interest in improving methods

of identifying Lynch syndrome among cancer patients is increasing to prevention opportunities (Kravochuck and Church, 2017).

Familial Adenomatous Polyposis (FAP) is the second most common genetic syndrome that predisposes to colorectal cancer and is characterized by the development of hundreds or thousands of colorectal polyps, which have a high tendency to undergo malignant transformation in affected individuals (Jasperson et al., 2010; Wells et al., 2017). Without any intervention, the risk of developing cancer is about 100% at 40 years of age (Galiatsatos and Foulkes, 2006). FAP is a cancer syndrome caused by a germline mutation in the adenomatous polyposis coli (*APC*) gene. Among associated lesions in FAP, desmoid tumors represent a common possible life-threatening condition that requires special attention. They are rare tumors occurring with a particularly high incidence in FAP, especially after surgery. In agreement with Knudson's 'two-hit' theory, the inactivation of the residual *APC* gene in FAP is a critical step in the development of both colorectal cancer and desmoids. (De Marchis et al., 2017).

People having a colorectal cancer have 2-3 times greater risk to develop the same disease than those who have no history of this disease in the family. Indeed, about 20% of colorectal cancer patients have a close parent that had the same diagnosis, but an early diagnosis is associated with a greater survival of the disease due to greater awareness (Morris et al., 2013).

Stages and progression

To establish the appropriate therapies for patients is important to define the cancer staging and grading to predict the clinical behaviour of malignancies following the international accepted criteria known as tumor-node-metastasis (TNM), a common system used in the clinic. Cure and survival rate depend on the stage of the CRC. In particular, it is possible to distinguish the phases according to the size and local growth of primary tumor called ‘stage T’, the involvement of the lymph nodes called ‘stage N’, the presence of distant metastases called ‘stage M’, and the genetic mutation of *APC*, *KRAS*, *BRAF* and *p53* (Telloni, 2017; Charlton et al., 2017). To describe the cancer stage exists also the Surveillance Epidemiology and End Results (SEER) method, used mainly for the statistical analysis of tumor registries. The SEER system provides the cancer staging based on the cancer invasiveness. When the primary tumor has not yet begun to invade the walls of the colon or rectum is called ‘in situ’. When the cancer has grown in the walls of the colon or rectum but has not yet spread through the walls of neighbouring tissues is called ‘local’. When

cancer has spread through the wall of the colon or rectum and has invaded nearby tissues, or has spread through bloodstream to nearby lymph nodes, is called ‘distant’.

Colorectal cancer begins with the transformation of the intestinal epithelial cells (IECs) which lose their normal functions, becoming hyper-proliferative. This formation, called adenoma, can growth and become cancerous with the ability to invade the submucosa and the colon, reflecting in cytologic and histologic dysplasia (Terzic´ et al., 2010; Lockhead et al., 2014).

Beyond the genetic modifications which regulate the so called ‘adenoma-carcinoma-sequence’, chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) occur during colorectal cancer biogenesis. Hyper-proliferation and tumor development are based on these mechanisms.

About 10% of CRC cases are hereditary, and up to 90% are sporadic (without family history or genetic predisposition). Chromosomal instability is observed in about 70% of all sporadic cancers and is characterized by an accumulation of mutations. The first mutation that occurs in development of colorectal cancer is within APC gene, which affects chromosome segregation during cell division. Subsequent mutations occur in *KRAS* oncogene, which affects cell growth, differentiation, motility and survival; *BRAF*, with effects on growth and apoptosis. Over time, these mutations can cause a loss of function of the *p53* gene (Pino et al., 2010; Kang et al., 2011; Bateman, 2014; Simon, 2016).

Microsatellite instability is caused by the disruption of DNA repair genes and results in a not uniform replication of repetitive DNA sequences in short and noncoding regions (microsatellites) and increased susceptibility to additional genetic mutations (O’Brien et al., 2006; Boland et al., 2010). Indeed, MSI is associated with germline mutations in DNA mismatch repair genes as well as sporadic mutations due to aberrant methylation of promoter regions (associated with CpG island methylator phenotype) of oncosuppressor genes. Nowadays, mutation in *KRAS*, *BRAF* and microsatellite instability represent the validated biomarkers in colorectal cancer.

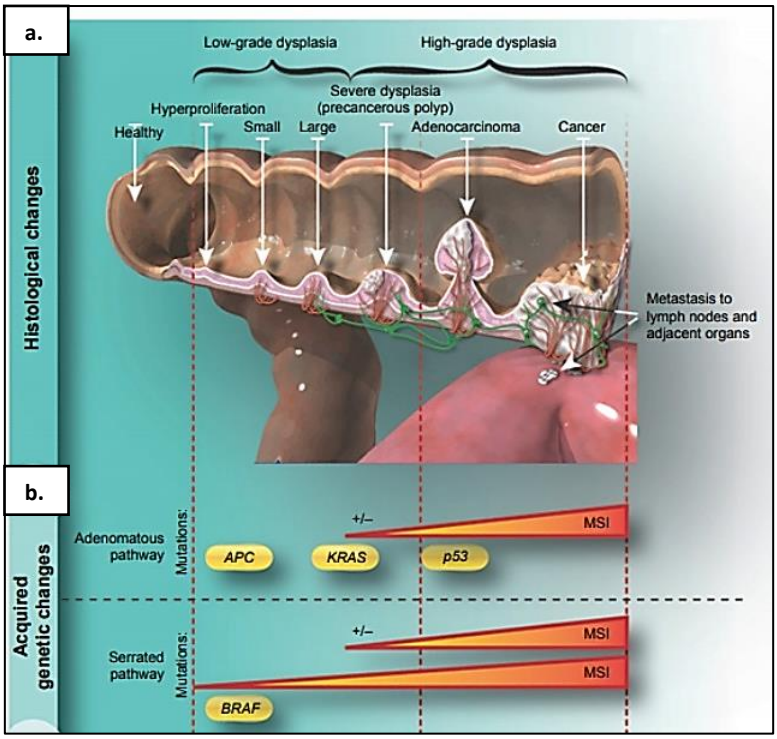


Figure 3. Colorectal cancer development. a) Histological changes through staging and b) the respective genetic alterations (Simon, 2016).

KRAS and colorectal cancer

The first description of the adeno-carcinomas was presented in 1978 by Day and Morson. They described the carcinogenesis of colorectal cancer from initial precursors to invasive carcinoma in hereditary cases and in sporadic cases (Day et al., 1978). Then, Vogelstein's group identified the genetic alterations responsible for the development of colorectal cancer, in particular on *APC*, *p53* and *KRAS* genes (Vogelstein et al., 1988). The *RAS* gene was initially identified as a homologue of the viral oncogene of Kirsten's sarcoma in the rat (Chang et al., 1982). *KRAS* mutation is found in 30% of all types of human cancer and is commonly associated with poor prognosis for the lack of effective therapies for treatment of *KRAS* mutant tumors (Haigis et al., 2017). The KRAS protein, also called p21, is a member of the Ras protein superfamily located on human chromosome 12, and is encoded by 189 amino acids; contains four coding exons and a non-coding 5 'exon (Malumbers et Barbacid, 2003). P21 is a GTP/GDP-binding membrane protein and is widely expressed in most human cells. As a small GTPase, it is involved in the intracellular signal transduction and is responsible for the activation of the EGFR signal. The exchange between the active state in which it binds GTP and the inactive state in which it binds GDP is finely controlled by the GTPase proteins and by the exchange factors of the nucleotide guanine (Vigil et al., 2010). Under physiologically

normal conditions, the upstream signal activates *KRAS* wild type by promoting the exchange of the bond from GDP to GTP. This process is transient due to GAP-mediated GTP hydrolysis and is altered when the *KRAS* status is mutated.

The *KRAS* mutation is present in about 35-45% of colorectal cancer cases (Karapetis et al., 2008), and codons 12 and 13 are two hotspots representing the 95% of mutations, with approximately 80% mutations in codon 12 and 15% mutations in codon 13. The remaining 5% of mutations occur in codons 61, 146 and 145 (Forbes et al., 2005). These mutations damage the intrinsic activity of *KRAS* and hinder GAP from promoting the hydrolysis of GTP causing *KRAS* accumulation in the constitutively active GTP-binding form (Schubbert et al., 2007; Boutin et al., 2017). For this reason, *KRAS* mutations play a critical role in the tumorigenesis of tumors such as colorectal, pancreatic, thyroid and lung cancer.

The predictive value of *KRAS* for the anti-EGFR (epidermal growth factor receptor) antibody response was validated for the first time in 2006 by a study of Lièvre and collaborators in which patients with mutated *KRAS* showed no signs of response to treatment with anti-EGFR antibody and had a lower overall survival (OS) compared to patients with wild-type *KRAS* who received the same treatment (Lièvre et al., 2006). The humanized monoclonal antibodies, such as cetuximab (Erbitux) and panitumumab (Vectibix), are directed against the extracellular domain of the EGFR. Cetuximab is a chimeric human-mouse IgG1 monoclonal that was approved by the Food and Drug Administration (FDA) in 2004, while panitumumab is a human monoclonal IgG2 k antibody that was approved by the FDA in 2007. These antibodies block ligand binding and lead to inhibition of the downstream RAS-RAF-MEK-ERK signalling pathway. Indeed, testing *RAS* status is mandatory before treatment with anti-EGFR therapy, while *EGFR* status is not predictive for the efficacy of treatment (Cong and Du, 2012). Analysis including *KRAS* exons 2, 3 and 4 (codons 12, 13, 59, 61, 117 and 146), NRAS exons 2, 3 and 4 (codons 12, 13, 59, 61 and 117) and *BRAF V600E* have to be assessed before the initiation of therapy with anti-EGFR (Tol et al., 2009; Van Cutsem et al., 2016). The use of the anti-EGFR antibodies-based treatment is adopted in the so called 'personalized treatment' for the metastatic stage of colorectal cancer (mCRC) with *KRAS*, *NRAS* and *BRAF* genes not mutated (Kerapetis et al., 2008; Freeman et al., 2008; Cohn et al., 2011; Stintzing et al., 2014). Furthermore, *EGFR* is over-expressed just in about 80% of colorectal carcinomas (Sartore-Bianchi et al., 2007).

However, cetuximab can potentiate the antitumoral effects of the classic chemotherapy in patients who do not carry mutations in *KRAS*. Furthermore, in patients with mutated *KRAS* the combination of oxaliplatin with anti-EGFR antibody showed a harmful effect with a lower overall survival

compared to single chemotherapy (Douillard et al., 2013). For patients with tumors that have mutations in *KRAS* the only drug available is bevacizumab that can be combined with different treatment scheduling such as FOLFOX (Saltz et al., 2008). FOLFOX is one of the common drugs combination used for the colorectal cancer treatments and is also known as Oxaliplatin de Gramont or OxMdG. FOLFOX is composed of: FOL - Folinic Acid; F - Fluorouracil (5-FU); OX - Oxaliplatin. Folinic acid is a B group vitamin to be taken with Fluorouracil as it makes chemotherapy even more active against cancerous cells. Bevacizumab can also be used in combination with FOLFIRI (Fuchs et al., 2008) which is composed of: FOL - Folinic acid (Leucovorin); F - Fluorouracil; IRI - Irinotecan, a topoisomerase inhibitor. Last combination of bevacizumab is with FOLFOXIRI, which is a combination of 5-Fluorouracil, Leucovorin, Oxaliplatin and Irinotecan. In a carefully selected population, young and in the best medical conditions, the combination of bevacizumab with FOLFOXIRI has a better efficacy of bevacizumab combined with FOLFIRI (Cremolini et al., 2015).

Because the restriction of FDA and EMA in 2008 for the use of anti-EGFRs to *KRAS* exon 2 wild-type patients, translational research has been focusing on development of new anticancer drugs against *KRAS*, but the picomolar affinity of RAS for GTP and the millimolar cellular concentrations of GTP failed the discover of new small molecules able to antagonize RAS-GTP binding (Pabke et al., 2017). Currently, many different approaches are under investigation to inhibit *KRAS* expression (Figure 4). In particular, one of the investigated strategy adopting G-quadruplex ligands to stabilize the G-quadruplex structure that forms in *KRAS* gene promoter sequence inhibiting its translation (Porru, Pompili et al., 2018).

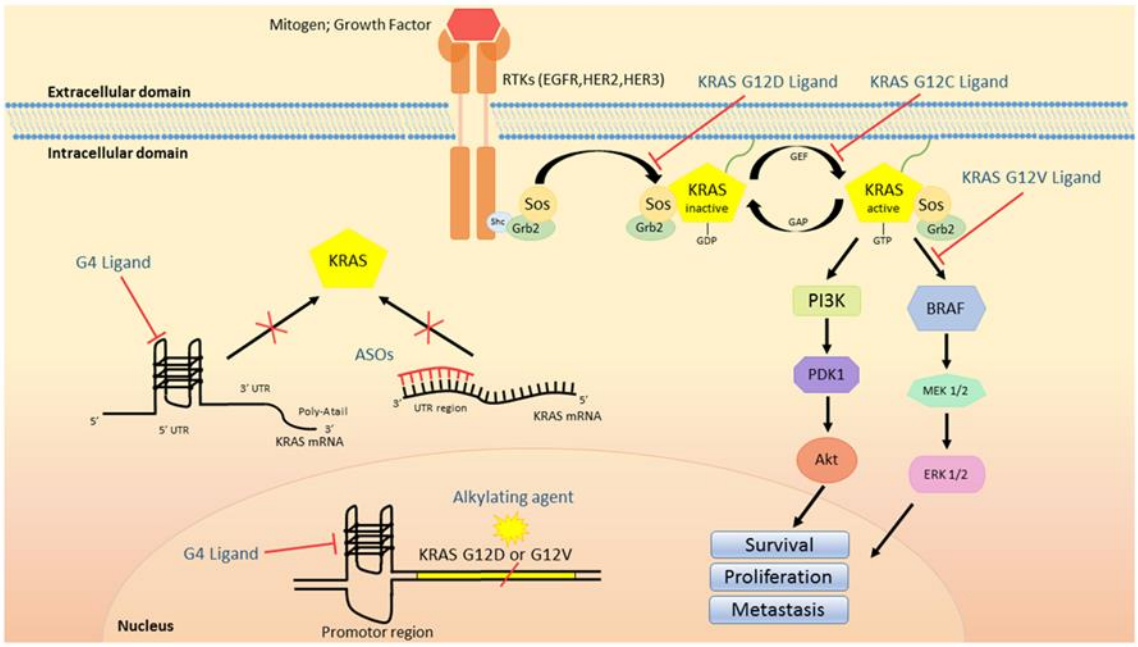


Figure 4. The schematic representation of the new different strategies against *KRAS*. ASOs: Antisense Oligonucleotides; GAP: GTP-ase Activating Proteins; GEF: Guanine Nucleotide Exchange Factor; G4: G-quadruplex; RTKs: Receptor Tyrosine Kinases (Porru, Pompili et al., 2018).

G-Quadruplex

In the last twenty years the interest about G-quadruplex structures has grown a lot and now represents one of the most interesting research field (O'Hagan et al., 2018). Indeed, the G-quadruplex structures have been finally shown associated with genome instability, genetic diseases and cancer progression (Hänsel-Hertsch et al., 2017) and considered as new possible therapeutic targets against cancer. In fact, many molecules have been developed to interact and stabilize the G-quadruplexes with the aim of identifying new potential anticancer therapies (Balasubramanian et al., 2011; Muller et al., 2014). But what the G-quadruplexes are? The first hypothesis that GC-rich sequences DNA could form higher-order structures was postulated in the 1910 by Bang's study, based on the observation that guanylic acid forms a gel at high concentrations (Bang et al., 1910). Fifty years later, the molecular basis for the association was subsequently determined by fibre diffraction before and by biophysical studies after (Gellert et al., 1977; Howard et al., 1977). Gellert and Howard observed that the higher-order structures was the tetrameric structures using the concept that the Hoogsteen hydrogen-bonded guanine G-quadruplex is the basic structural motif. In the late 1980s, the demonstration that conserved DNA sequences repeats of telomeres form G-quadruplex structures *in vitro* led to the hypothesis that they could be biologically relevant (Henderson et al, 1987; Sen et al., 1988; Williamson et al., 1989). In 2001 Shaffitzel's group demonstrated that telomeres adopt a G-quadruplex configuration *in vivo* by immunostaining of Stylonychia macronuclei with antibodies raised against G-quadruplex structures with telomere sequences (Shaffitzel et al., 2001), and the following studies described these non-canonical DNA secondary structures not only at the chromosome ends but also in the 5′- UTR of transcribed genes, splicing sites and particularly in the promoters of a wide range of cancer-related genes as *KRAS*, *HIF1-α*, *KIT*, *MYC*, *BCL-2* or *VEGFR-2* (Siddiqui-Jain et al., 2002; Neidle et al., 2010; Rhodes et al., 2014; Salvati et el., 2014 Hänsel-Hertsch et al., 2016). It appeared immediately clear that the stabilization of the G-quadruplex structures in these promoters results in the down-regulation of the oncogenes, underlying the importance to develop new anticancer strategies based on these G-quadruplex ligands (Neidle et al., 2009; Collie et al., 2011; Rhodes et al., 2015). In fact, these compounds exhibit a selective antitumoral activity towards the cancer cell both *in vitro* and in mouse models. Some of these molecules like Quarfloxin, able to indirectly affect MYC transcription (Bidzinska et al., 2013) and its related drug CX-5461, a potent small-molecule rRNA synthesis inhibitor specific for patients with *BRCA1/2* deficient tumors, are now in clinical trial for the evaluation of safety, tolerability and efficacy in patients with solid tumors (Hong et al., 2017).

A new relevant aspect about G-quadruplex has emerged a few years ago with the observation of Biffi's group that demonstrated that immortalized tumor cells have a greater number of G-quadruplexes than normal cells and also in patient-derived tissues they observed the same phenomenon in human cancers of liver and stomach as compared to background non-neoplastic tissue (Biffi et al., 2013; Biffi et al., 2014). The evidence of this big difference in the number of G-quadruplex foci between cancer cells and normal cells, probably because of the loss of chromatin integrity (Tseng et al., 2018), can provide both important new aspects on the molecular mechanisms underlying carcinogenesis and a new direction for early diagnosis and drug development in personalized medicine and biomedical research (Hänsel-Hertsch et al., 2017). In fact, while a first *in silico* analyses identified more than 300,000 sites with G-quadruplex forming potential in the human genome (Huppert and Balasubramanian, 2005), Chambers et al. have recently detected more than 700,000 G-quadruplex structures genome wide by new G-quadruplex-seq approaches (Chambers et al., 2015).

The molecular structure

Guanine-rich ssDNA can adopt secondary structures known as G-quadruplex under physiological-like conditions (Lipps and Rhodes, 2009). The putative G-quadruplex forming sequences (PQS) contain motifs of guanines called 'G-tracts' and the typical G-quadruplex structure contains at least three guanines stacks ($G \geq 3NxG \geq 3NxG \geq 3NxG \geq 3$) because of the structures with only two stacks of guanines are possible but have very low stability. The G-quartets (Figure 5b) are joined by π-π bonds which are stable planar arrangements of four Guanine residues bound by hydrogen bonds according to the pairing non-canonical of Hoogsteen (Figure 5a) (Parkinson et al., 2002).

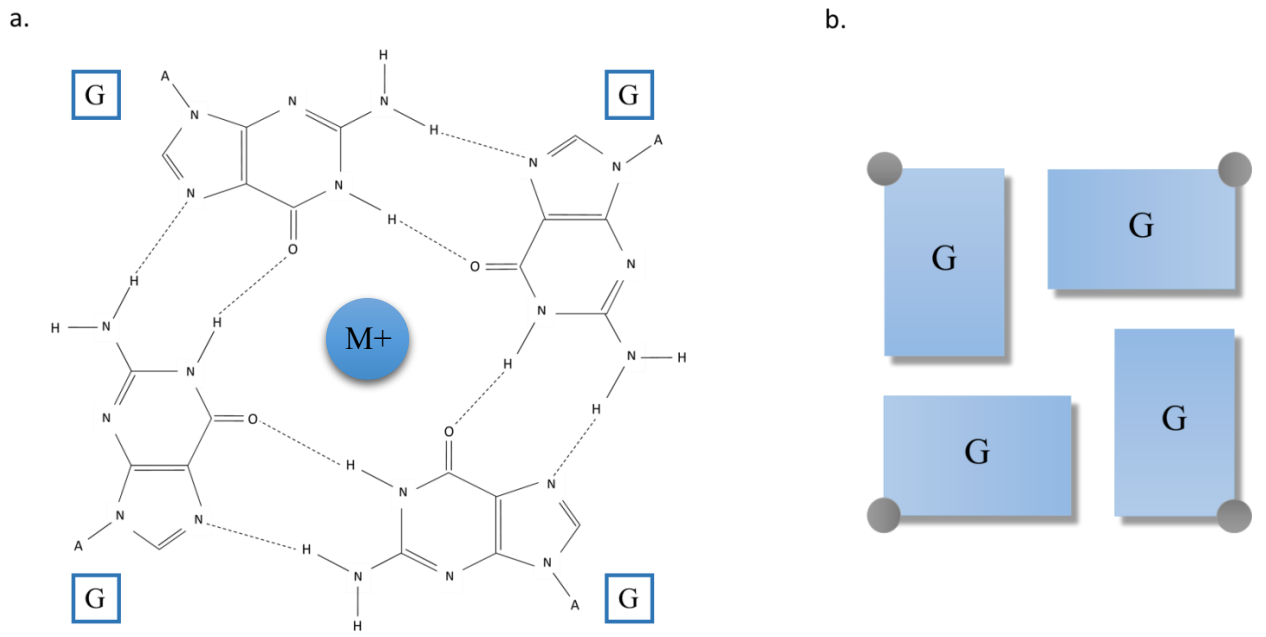


Figure 5. Basic of the G-Quadruplex structure viewed as a tetrameric alignment viewed from above. a) Chemical form. Hoogsteen base pair between adjacent Guanines are shown as dotted lines. b) Schematic form. Individuals Guanines are represented as rectangles and the attached sugars as circles. M+: Metal alkalin ions.

G-quadruplex structures can be classified into various group whose complexity depends on variable parameters such as the lenght and sequence composition of the total G-quadruplex motif, the number of oligonucleotide chains (unimolecular, bimolecular, tetramolecular), the glycosidic conformation, the size and the type of the loops between the guanines, the nature of the binding factors such as cations or presence of ligand and the molecular encumbrance. (Dai et al., 2008; Bochman et al., 2012; Du et al., 2013).

There are different topological variants possible for the four strands. All four strands may be parallel, three parallel and one in the opposite direction (antiparallel), or there may be two in one direction and two in the other, either with the parallel pairs adjacent to each other or opposite each other. A shorthand has arisen which describes all the arrangements with at least one antiparallel strand as ‘antiparallel’, although this does not give a full description of the structures.

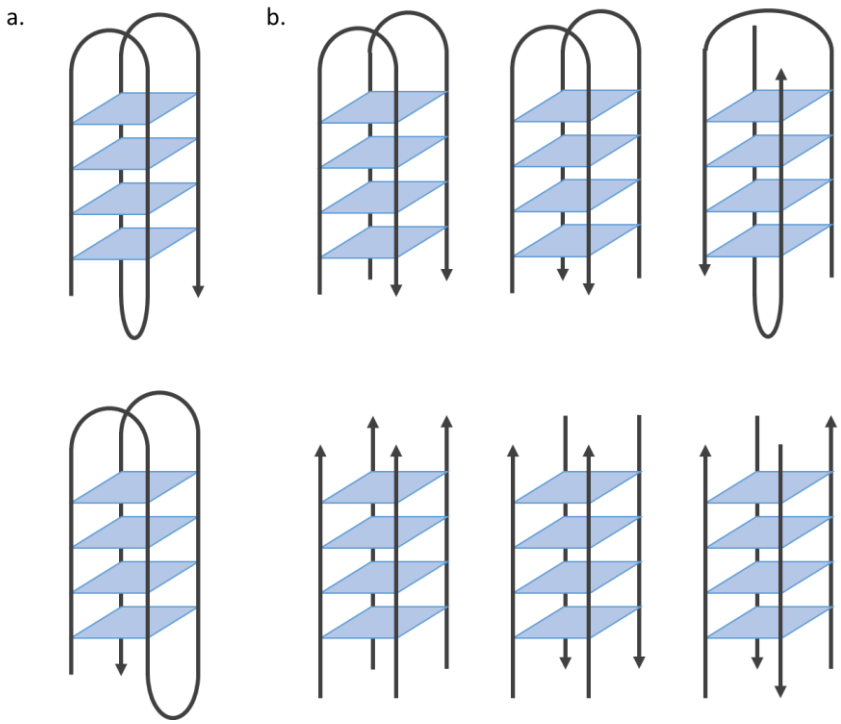


Figure 6. A schematic diagrams of intramolecular (a) and intermolecular (b) G-quadruplex DNA structures. The arrowheads indicate the direction of the DNA strands. The intermolecular structures shown have two (upper) or four (lower) strands.

At molecular level, the different directionality of the strands relates to the conformational state of the glycosidic bond between the guanine base and the sugar. In fact, the guanosine involved in the formation of tetrads can present the sugar in *syn* or *anti* conformation (Figure 7), unlike the bases present in the right-handed double helical structure of B-form DNA (B-DNA) described by Watson and Crick in the 1953 (Watson and Crick, 1953). Moreover, the guanines adopt the *anti* conformation in parallel G-quadruplex, whereas in antiparallel intramolecular structures the *anti* and *syn* confomation alternate.

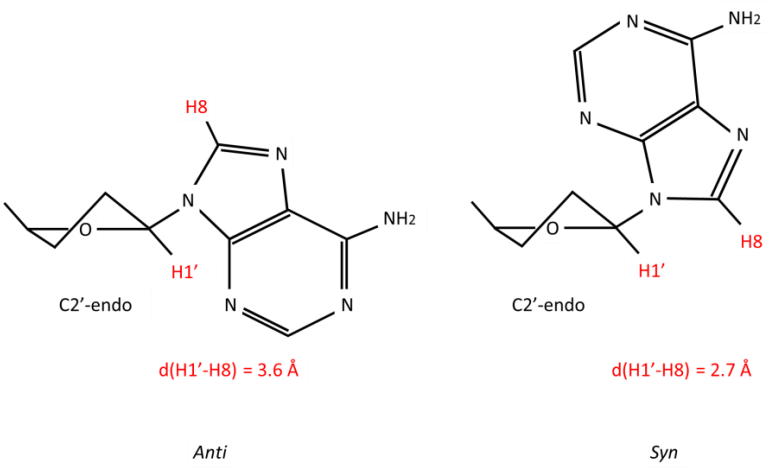


Figure 7. The bond between the base and sugar can rotate. The two conformation that can be adopted: *anti* and *syn*.

G-quadruplex structures are held together by sequences of varying lengths forming single-chain loops arranged outside the core. The single strand involved in the intramolecular structure has the generic sequence GnXnGnXGnXGn, where 'n' is the number of guanine residues present in each stretch involved directly in the formation of tetrads and X can be any residue (guanine included) which then constitute the connection loops. The connection loops can be divided into four main families (Figure 8) distinguished in: loops defined "edge wise" that connect two adjacent antiparallel filaments; diagonal loops that bind two opposite antiparallel filaments; loops called "double chain reversal" that join two parallel adjacent strands; "V-shaped" loops that connect the angles of two tetrads of overlapped guanine, in which a connection is missing (Phan et al., 2006).

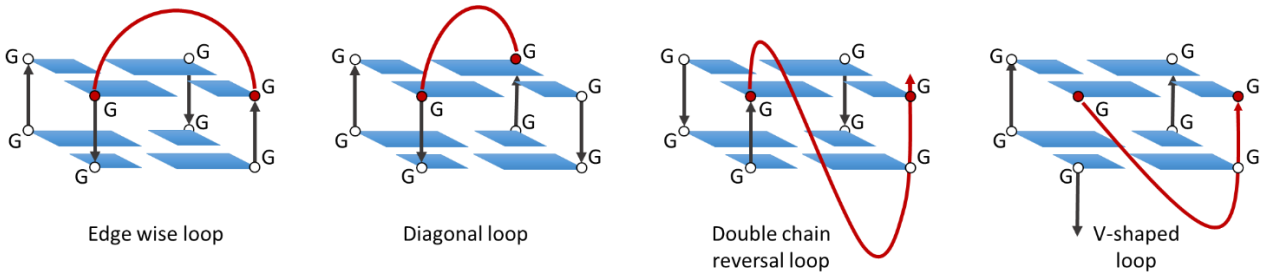


Figure 8. The different possible G-quadruplex connection loops.

Another important factor of stability of the G-quadruplex structures is the presence of cations such as K+, Na+, Rb+, Cs+ and Li+ (Williamson et al., 2004). The main stabilizing factor are K+ or Na+, which neutralize the electrostatic repulsion of the electronegative carbonyl oxygens of the guanine bases (Balasubramanian end Neidle, 2009; Bochman et al., 2012) but, because of their different dimensions, they are differently positioned: Na+ ions are arranged on the plane identified by the tetrad of guanine while K+ ions, more cumbersome, are positioned in the space between two tetrads. So, the Na+ solution and K+ solution containing crystal permit the formation of structures very different from each other in terms of strand orientations, loop topologies and glycosidic conformations of guanines (Wang et al., 1993; Parkinson et al., 2002). Because in mammalian cells K+ is much more abundant than Na+ (140mM K +, 5-15mM Na +), the G-quadruplex formed structures are stabilized mainly by the K+ ions and are considered to be biologically more relevant (Patel, 2002; Luu et al., 2006; Ambrus et al., 2006; Xu et al., 2006), although the Na+ structure was reported more than twenty years ago (Wang and Patel, 1993).

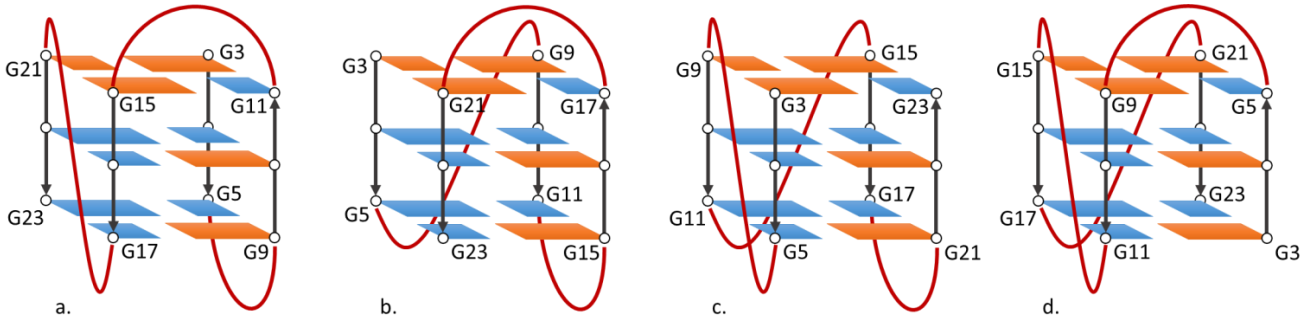


Figure 9. Schematic structures of possible intramolecular human telomeric (3+1) G-quadruplexes- a) Form 2 observed for the d[TAGGG(TTAGGG)3TT] sequence in K+ solution (Phan et al., 2006). b) Form 1 observed for the d[TAGGG(TTAGGG)3] sequence in K+ solution (Luu et al., 2006). c) and d) Models of intramolecular (3+1) G-quadruplexes with two double-chain-reversal loops. Loops are red; *anti* and *syn* guanines are coloured orange and blue respectively.

Form 2 (Figure 9a) differs from Form 1 (Figure 9b) by loop arrangements: the double-chain-reversal loop is formed by the third linker instead of the first linker. The folding topology of Form 2 is similar to the (3+1) G-quadruplex formed by the four-repeat Tetrahymena telomeric sequence (Wang and Patel, 1994) and by a variant sequence of four G-tracts from the human BCL-2 promoter (Dai et al., 2006). The three-repeat human telomeric sequence adopts a (3+1) G-quadruplex with yet another loop arrangement (Zhang et al., 2005).

Telomeres and G-quadruplex

Telomeres are small DNA portion at the ends of the eukaryotic chromosomes. They are composed of a double-stranded region and a single-stranded G-rich 3′ overhang and are essential to protect chromosomes from degradation and end-to-end fusions, recognized as DSBs (Zakian, 2012). In most telomeric DNAs, guanines and cytosines are distributed asymmetrically between the two DNA strands, with the G-rich strand running 5′ to 3′ from the centromere to the telomere. The name 'telomeres' was coined by Herman J. Muller in 1938 from Greece telos= end and meros= part (Muller HJ, 2013) The Remaking of Chromosomes The collecting Net-Woods Hole 13, (1938), 181-98.). Their importance for chromosome stability was discovered by the 'Nobel prize' Barbara McClintock in mais and Drosophila models (McClintock, 1938; McClintock, 1941). Telomerase, the telomere terminal transferase enzyme, was first discovered in 1985 in the single cell organism, Tetrahymena (Greider et al., 1985) and, a decade later, was described as an almost universal marker in advanced human cancers (Kim et al., 1994; Shay et al., 1997). In 1997 the catalytic protein component was isolated first in yeast (Lingner et al., 1997) and shortly thereafter in humans (Nakamura et al., 1997; Meyerson et al., 1997).

Telomerase is highly regulated in normal transit amplifying stem-like cells and expressed in most human tumors (80-85%), maintaining the length of telomeres (Kim et al., 1994) and, in combination of oncogenic changes, the cells escape senescence and become immortal (Figure 10) making the inhibition of telomerase one of the older attractive target for cancer therapeutics (Hurley, 2002).

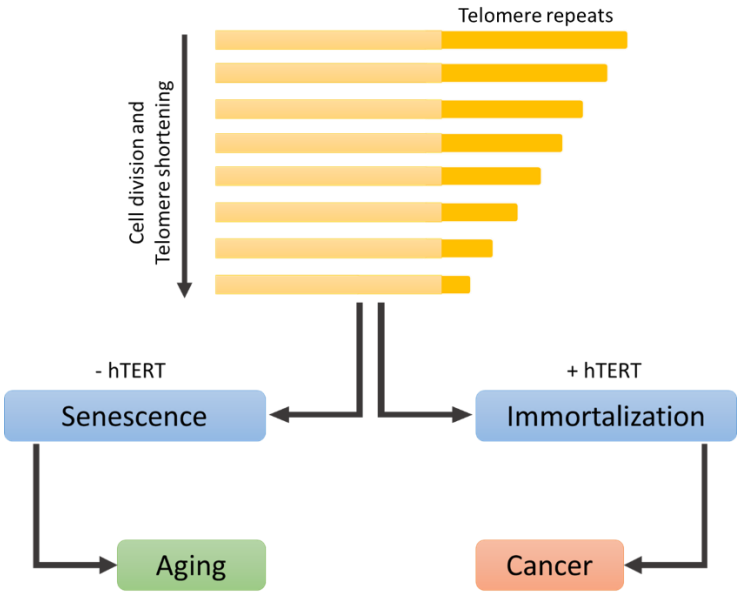


Figure 10. In all normal somatic cells, the telomeres progressively shortening with increased cell divisions. In absence of a mechanism to maintain the length of the telomeres, cells undergo replicative senescence (aging). On the other hand, the expression of the catalytic subunit TERT of the telomerase enzyme is just sufficient to maintain telomere length and immortalize normal cells. In the addition of oncogenic changes, this event brings the cells becoming malignant.

Human telomeric DNA, 4–14 kb long (de Lange et al., 1990), is composed of (TTAGGG/ CCCTAA)n repeats, with a 100–200 nt G-rich strand overhang at 30 ends (Makarovet al., 1997; McElligott et al., 1997; Wright et al., 1997). The telomere repeats are G-rich DNA sequences and display high propensity to adopt G-quadruplex configurations (Parkinson et al., 2002). Dai et al. observed that while the hybrid-type G-quadruplexes appear to be the major conformations in K+ solution, human telomeric sequences are always in equilibrium between Hybrid-1 and Hybrid-2 structures (Figure 8a, 8b), which is largely determined by the 3′-flanking sequence. Furthermore, both hybrid-type G-quadruplexes suggest a direct means for multimer formation with effective packing in the human telomeric sequence and provide important implications for drug targeting of G-quadruplexes in human telomeres (Dai et al., 2007). Moreover, the telomere repeats are bounded by the proteins of the Shelterin complex able to protect chromosome ends from degradation and fusion. The G-quadruplex telomeric structures can interfere with telomere replication leading to fragile and shorter telomeres. Based on these observations, the compounds which can stabilize these structures induce telomere dysfunction not only in cells with telomerase activity but also in ALT+ cells during the first recombination phase needed for the alternative lengthening of telomeres.

Recent observations provide direct evidence for ATRX protein playing a role in aiding replication, and the knowledge that ATRX targets the G-quadruplexes suggest that these structures could cause the replication fork stalling in alternative telomeres lengthening mechanism. In fact, mutations in human *ATRX* have recently been identified in tumors that maintain their telomeres by this pathway telomerase-independent involving homologous recombination (Shay et al., 2012). ATRX dysfunction induced replication defects and increased the number of DNA damage response mechanisms at telomeres in mouse embryonic stem cells (Clynes et al., 2014). This observation taken together with the knowledge that ATRX targets G-quadruplexes (Hegyi, 2015), suggest that G-quadruplexes cause the replication fork stalling. Evidence for genome instability arising from defective replication has also come from studies in which the human subtelomeric minisatellite CBE1, which forms G-quadruplex structure in vitro (Piazza et al., 2012), was inserted into the genome of Pif1 deficient yeast (Ribeyre et al., 2009; Lopes et al., 2011). Based on this evidence, the treatment with G-quadruplex-stabilizing compound induces telomere dysfunction and in particular for the telomeric G-quadruplex the study regarding the 3' telomeric overhang in physiologic conditions have identified this conformation as possible target for anti-cancer therapy (Tahara et al., 2006; Salvati et al., 2007; Rodriguez et al., 2008; Hänsel et al., 2013).

G-quadruplex in transcription and translation

G-quadruplexes sequences are prevalent within regulatory regions of the genome. Good evidence has been provided to support the hypothesis that G-quadruplexes exist in human cells, play a role in human diseases and can be targeted with ligands to modulate biological functions (Lam et al., 2013; Zizza et al., 2016). The visualization of the regulation of G-quadruplex structures is an important observation because unresolved G-quadruplex structures are likely to be an obstacle for DNA replication and telomere elongation. G-quadruplex structures have been showed to be involved in certain mechanisms such as homologous recombination, recombination between sister chromatids, telomeric DNA transactions, gene transcription and mRNA translation. Genome-wide studies in vertebrates have recently identified a consensus G-rich motif potentially able to form G-quadruplexes in most replication origins (Mendoza et al., 2016). Several studies have been conducted to understand the frequency with G-quadruplex structures occur in DNA and the role that these structures could have. A first large-scale analysis on 1% of the human genome (Cadoret et al, 2008) showed that most efficient origins of replication were strongly associated with CpG islands (CGI) and transcriptional regulatory elements. A genome-wide association between origins of

replication and potential G-quadruplex formation has also been found in human cells (Besnard et al, 2012). The hypothesis that G-quadruplex structures can regulate origin activity has been reinforced by the observation that the human origin recognition complex, the landing platform of the pre-replication complex, binds preferentially to G-quadruplex motifs on G-rich RNA or single stranded DNA (Hoshina et al, 2013).

The analyses of the putative sequences G-quadruplex forming structures started with a bioinformatics analyses that identified ~370,000 regions in the human genome with G-quadruplex forming potential (Todd et al., 2005; Huppert and Balasubramanian, 2005) and with a more recently study, which used novel approach with a new algorithm high-resolution sequencing– based methods (Bedrat et al., 2016) detected 716,310 distinct G-quadruplex structures genome wide (Chambers et al., 2015). Chambers et al. observed a high G-quadruplex density in functional regions, such as 5′ untranslated (5'UTR) regions and splicing sites, as well as in genes previously not predicted to contain these structures (such as *BRCA2*). G-quadruplex formation was significantly associated with oncogenes, tumor suppressors and somatic copy number alterations related to cancer development (Zack et al., 2013). Moreover, Chambers's analyses revealed also the position within occurred these putative G-quadruplex structures. Surprisingly, the data showed that the sequence with G-quadruplex-forming potential are not distributed randomly in the genome but are often localized near the replication origins. Indeed, the majority of the 250,000 human replication origins are close to G-quadruplex motifs (Besnard et al., 2012; Valton et al., 2014; Fragkos et al., 2015), suggesting that the formation of stable G-quadruplex structures participates in the replication mechanism. Additionally, the sequences with G-quadruplex-forming potential were identified also in the promoter regions of numerous genes, arguing for a potential role in transcription regulation (Fry, 2013; Kim, 2017). So, G-quadruplexes may influence transcription in both positive and negative ways (Figure 11). First, depending on which DNA strand encodes the G-quadruplex motif, the structure could either inhibit transcription (if the motif is on the template strand, blocking the transcription machinery) or enhance transcription (if the motif is on the non-template strand, maintaining the transcribed strand in a single-stranded conformation). Second, proteins bound to the G-quadruplex structures (for example, transcriptional enhancers versus repressors) could also affect transcription (Qin and Hurley, 2008).

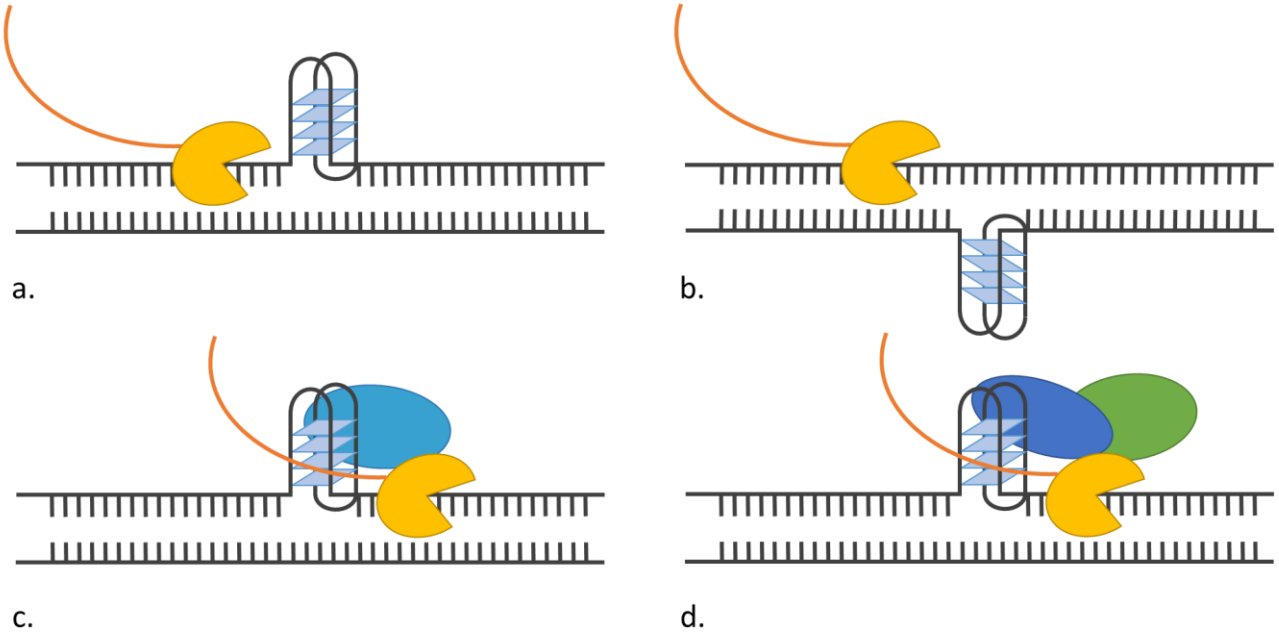


Figure 11. Possible roles of G-quadruplex structures during transcription by RNA polymerase (yellow) (Bochman et al., 2012). a) Blocking transcription by inhibiting polymerase (yellow) due to the formation of a stable G-quadruplex. b) Facilitating transcription by a stable G-quadruplex in the complementary strand enhances transcription. c) Stimulating transcription by recruiting proteins (blue) that recruit or stimulate polymerase. d) Blocking transcription via the recruitment of G-quadruplex binding proteins (blue and green), which directly or indirectly repress transcription.

G-quadruplex-forming sequences are also found in several transcriptional regulatory regions of important oncogenes such as *KRAS*, *MYC*, *BCL-2* and *HIF1α* (Lemarteleur et al., 2004; Kumari et al., 2015; Sengupta et al., 2017). One of the most studied is the system of G-quadruplex involved *KRAS* transcription. As previous described, *KRAS* gene is located in the locus 12p12.1 and encodes for a 21 kDa protein which is anchored to the inner surface of the plasma membrane and acts as a molecular switch that, through the RAF-MAP kinase pathway, transmits to the nucleus survival and cell death signals (Adjei, 2001). Mutation in *KRAS* gene locks the protein in the active state that constitutively transmits to the nucleus mitogenic signals (Smit et al., 1988).

The human and mouse *KRAS* gene contains a sequence within promoter called NHPPE (nuclease hypersensitive polypurine–polypyrimidine element) that is essential for transcription (Hoffman et al., 1990). This sequence is located between nt -327/-296 in human, positions relative to the exon 0/intron 1 boundary (Figure 12). The well-studied feature of this sequence is the ability to assume an unusual DNA structure attributed to an intramolecular parallel G-quadruplex, consisting of three G-tetrads and three loops. Because of the polypurine–polypyrimidine nature of these duplex sequences, which contains four or more runs of clusters of three or more guanines on the purine-rich strand, they often show a single-stranded character and hence are hypersensitive to nucleases.

The secondary DNA structures may inactivate transcription while for the transcriptional activation is required the conversion to the duplex region. It is evident that the molecules designed to stabilize the G-quadruplex structures prevent this transition and inactivate the gene expression.

Cogoi and co-workers (Cogoi and Xodo, 2006) demonstrated that *KRAS* G-quadruplex arrests DNA polymerase and that G-quadruplex ligand TMPyP4 stabilize G-quadruplex structure in the promoter inhibiting the gene expression. In fact, they demonstrated that the G-rich strand of NHPPE, located within the proximal promoter region of *KRAS*, is able to form a G-quadruplex structure that seems to be involved in the mechanism of transcription regulation. Indeed, the human and the mouse *KRAS* NHPPE G-rich strands display melting temperature of 64 and 73°C, respectively, as well as a K^+-dependent capacity to arrest DNA polymerase. The G-quadruplex ligand TMPyP4, a cationic porphyrin, stacks to the external G-tetrads of the *KRAS* quadruplexes, increasing the Tm by approximately 20°C. These findings raise the intriguing question that the G-quadruplex formed within the NHPPE of *KRAS* may be involved in the regulation of transcription.

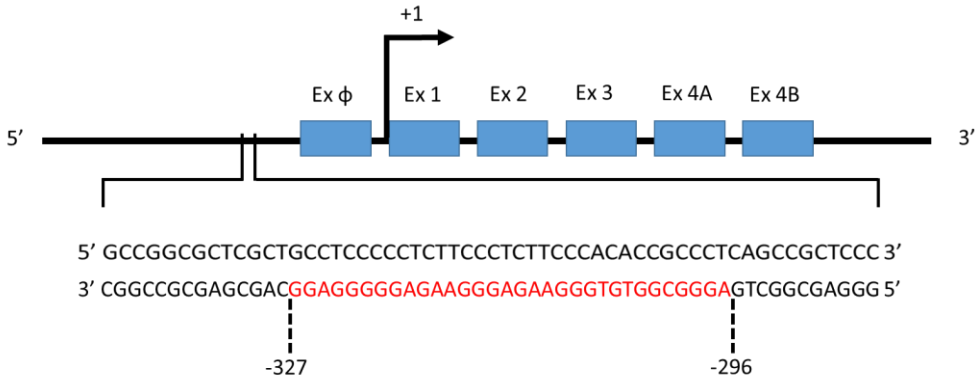


Figure 12. Sequence of NHPPE contained in the human *KRAS* gene. Position relative to exon0/intron 1 boundary are shown.

Furthermore, the same authors proposed a model in which the NHPPE of *KRAS* exists in equilibrium between two form: the favouring transcription and the folded quadruplex which inhibits transcription. This mechanism, which is probably adopted by other growth-related genes, provides useful hints for the rational design of anticancer drugs against the *KRAS* oncogene. Moreover, the stabilization of the *KRAS* G-quadruplex with the cationic porphyrin TMPyP4 resulted in a strong inhibition of *KRAS* transcription shedding light on new molecular strategies to suppress the expression of *KRAS* in tumor cells (Cogoi and Xodo, 2006).

In addition to promoter gene sequences, G-quadruplex structures were also identified in the introns of certain genes localized nearby to the splice acceptor sites of the adjacent exons. The treatment with G-quadruplex ligand can affect the activity of the splicing machinery causing intron retention events that could be responsible for the synthesis of immature RNA transcript that, in several cases, are rapidly degraded (Didiot et al., 2008; Marcel et al., 2011).

Nowadays, we can consider in expansion the therapeutic significance of G-quadruplex structures in 5'-UTR regions and introns due to their ability to affect the expression of target genes by regulating the transcriptional or the translational processes, respectively (Balasubramanian et al., 2011; Bugaut and Balasubramanian, 2012).

G-quadruplex in viruses

The putative G-quadruplex forming sequences are involved also in many human infectious diseases. Indeed, these sequences have been found not only in human genome but also in pathogenic vectors such as bacteria and viruses (Beaume et al., 2013; Metifiot et al., 2014; Perrone et al., 2017). Despite the steps forward in the antiviral research, viral infections are still one of the major problems for human health which cause millions of deaths every year. Actually, the majority of viruses still lack a specific and effective vaccine because of their ability to be drug-resistant. To reach important benefits on a global scale it is still necessary to develop new therapeutic approaches for the management of viral diseases. To this purpose, recent data showed that the G-quadruplex ligands have not only an encouraging anti-cancer activity but also an innovative mechanism of action in antiviral treatment and valuable tools to better understand virus mechanisms. The antiviral activity of G-quadruplex ligand is mediated by an increased interaction between the molecule and the loop of the viral G-quadruplex structure observed through NMR (Nuclear magnetic resonance) spectroscopy (De Nicola et al., 2016). As demonstrated in Herpes virus simplex type 1 (HSV1), the number of viral G-quadruplexes increases consistently during viral replication (Artusi et al., 2016), representing an important point for the selective activity of the G-quadruplex ligands (Table 1). Moreover, viruses such as HIV, the herpes or papilloma virus family, undergo latency. These latent viruses are characterized by the fact that the current therapies can target the viral proteins but fail to remove the latent virus, instead following the G-quadruplex-mediated approach could be possible eradicate the so far incurable latent virus (Piekna-Przybylska et al., 2017). An important aspect about the use of these therapeutic approaches could be the tolerability in the immunocompromised or elderly patients. While the antiviral research still need to improve the understanding of the viral G-quadruplexes architecture and the selective activity of the ligands toward the virus versus cells, the application of G-quadruplex ligands as antiviral agents is expanding in the research and pharmacological application of these molecules as a new frontier in the clinical management (Ruggiero et al., 2018).

Virus	Genome	n° G4s	Compounds
HIV	(+)ssRNA 9.75 kb	12 (Perrone et al. 2013)	B19 TMPyP4 PIPER (Piekna-Przybylska et al. 2017) (Perrone et al. 2013)
EBV	dsDNA 172 kb	13 (Biswas et al. 2016)	B19 Pyridostatin PhenDC3 (Lista et al. 2017) (Murat et al. 2014) (Norseen et al. 2009)
HCV	(+)ssRNA 9.6 kb	2 (Wang et al. 2016)	TMPyP4 (Wang et al. 2016)
HBV	Partially circular dsDNA 3.2 kb	1 (Biswas et al. 2017)	B19 Pyridostatin (Biswas et al. 2017)
EBOV	(-)ssRNA 18.9 kb	1 (Wang et al. 2016)	TMPyP4 (Wang et al. 2016)

Table 1. Some examples of the G-quadruplex ligands able to bind the viruses G-quadruplex structure.

G-Quadruplex Ligands

The uniqueness of the structure and the particular topology of G-quadruplex provide a specific recognition target to small molecules through various binding modes (Figure 13) in an analogous double-helical DNA intercalators manner (Ou et al., 2008; Zhang et al., 2014; Xiong et al., 2015). The compounds designed to bind G-quadruplex should usually follow several principles to be specific and selective. They can stack with the G-quartet through π–π interactions. Ligands with an extended planar aromatic system, which are similar to a G-quartet in size and shape, facilitate stacking on the G-quartet. This aromatic system can be a rigid flat or twisted surface. The compounds can also interact with the loops and grooves of the G-quadruplex or with the negative electrostatic center of the G-quadruplex by electrostatic interaction with the cationic center of the aromatic core (Yan et al., 2013).

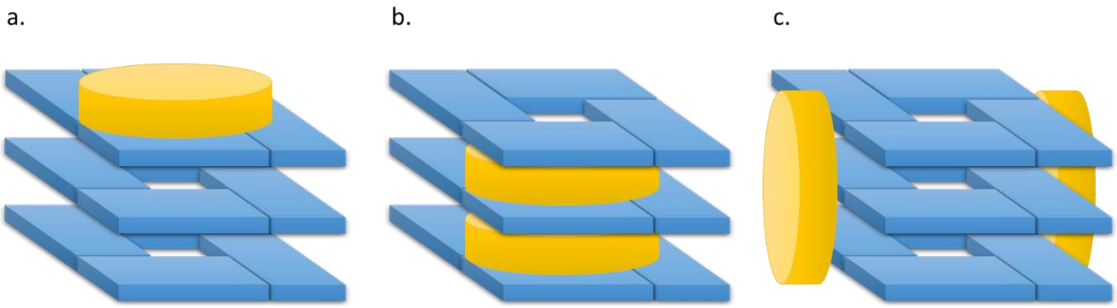


Figure 13. A schematic representation of the recognition modes of G-quadruplex bimolecular structures by different ligands. a) External stacking mode on the surface of the terminal quartet. b) Intercalating mode between the stacks of G-tetrads. c) Groove binding mode.

Through targeting G-quadruplexes, small-molecule ligands play pivotal roles in the regulation of the transcription of oncogene promoters containing G-quadruplex. Several groups of compounds, including cationic porphyrin (TMPyP4), acridine (BSU6039), polycyclic acridine (RHPS4), and N,N'-bis-[2-(1piperidino)ethyl]-3,4,9,10-perylenetetracarboxylic diimide (PIPER), have been identified as interacting specifically with G-quadruplexes.

Despite several studies and promising results obtained about the anticancer efficacy of these G-quadruplex ligands in preclinical experiments, only two compounds entered in Phase II clinical trials: Quarfloxin, a fluoroquinolone derivative compound developed by Hurley's research group (Drygin et al., 2009; Brooks et al., 2010), and its derivate CX-5461. Quarfloxin (Figure 14a), also

known as CX-3543, is a fluoroquinolone derivative that exhibits cytotoxic activity *in vitro*, thanks to its capability to induce apoptosis in cancer cells and showed therapeutic efficacy , *in vivo* tumor models. At molecular level, Quarfloxin disrupts nucleolin/G-quadruplex complexes on rDNA, accumulates in cancer cells, in particular in nucleoli causing their redistribution by inhibition of rRNA synthesis, and activates the p53-mediated response which starts the apoptosis process. Moreover, Quarfloxin selectively inhibits rRNA synthesis by interfering with the elongation step of PolI transcription. Surprisingly, telomere dysfunction has not been observed in cells under Quarfloxin treatment (Drygin et al., 2009). Despite Quarfloxin was the first ligand that reached Phase II clinical trials, it was withdrawn due to bioavailability related problems (Yao and Zhang, 2018). CX-5461 (Figure 14b), the Quarfloxin derivate, is now in advanced Phase I clinical trial, in particular for the treatment of patients with *BRCA1/2* deficient tumors (Canadian trial, NCT02719977, opened May 2016). CX-5461 is an inhibitor of rRNA synthesis and acts inhibiting Polymerase I-driven transcription of rRNA and is a strong G-quadruplex stabilizer. The pathway of non-homologous end joining event, and in particular BRCA proteins, are required to repair the CX-5461-induced DNA damage. The cancer cells with defective NHEJ fail to repair the DNA damage and it leads to lethality. For this reason, this new compound has specific toxicity towards *BRCA* deficient tumors (Xu et al., 2017).

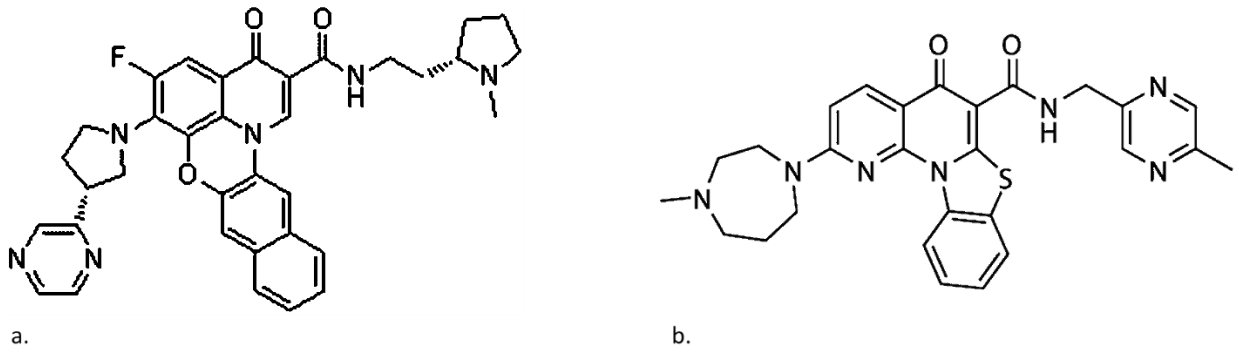


Figure 14. The chemical structure of Quarfloxin (a.) and its derivate CX-5461 (b.).

The G-quadruplex ligands were initially synthesized to interfere with telomerase activity (Sun et al., 1997). Surprisingly, several groups observed that these compounds were also effective in cells defective for telomerase, suggesting that in addition to its telomerase inhibitory properties, these

compounds interfere with telomere replication. Indeed, in subsequent studies it has been shown that G-quadruplex ligands display antitumor effect also through the alteration of telomeric chromatin, observed following the activation of the γ-H2AX damage foci and the interaction with the G-quadruplex present in the promotor sequence gene. These results have provided the first evidences to understand these molecules as true interactors of telomeres and G-quadruplex structures in the whole DNA (Rizzo et al., 2009; Salvati et al., 2012; Gauthier et al., 2012).

As previously described, these ligands are able to bind and stabilize the G-quadruplex architecture through intercalation. In fact, the first compound studied in this activity was Ethidium bromide (Figure 15), a tricyclic compound able to interact with Guanine-rich sequences in double, triple and quadruple DNA molecules (Guo et al., 1992; Koeppel et al., 2001).

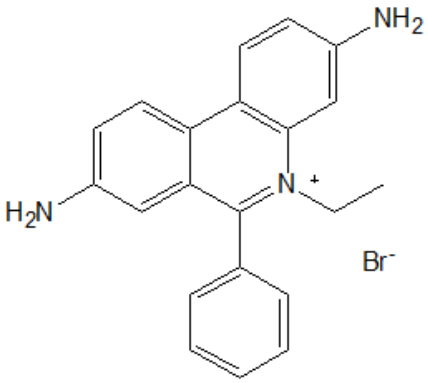


Figure 15. Chemical structure of Ethidium Bromide.

Ethidium bromide belongs to the class of tricyclic molecules that also include fluoroenones and anthraquinones (Figure 16 a and b respectively). The fluorenone-based compounds were the first G-quadruplex binders described to exhibit a high broad range of telomerase inhibitory activity with a general reduction in cellular cytotoxicity in comparison with analogous anthraquinone-based compounds which have a central ring of six carbon atoms (Perry et al., 1999). In fact, the different cytotoxic activities are due to the difference in molecular structure.

The fluorenone architecture is based on three rings with five carbon atoms that alters the relative angular dispositions of the two aminoalkylamide chains. For this different orientation of the side chains (compared to the chromophore), it's been possible to observe through molecular modelling that the disubstituted 2.7 fluorenones bind the G-quadruplexes only by distorting the geometry of

the structure, an expensive phenomenon from an energy point of view. Therefore, the binding energies between fluorenones and G-quadruplex structures are greater than those observed with anthraquinones (Kim et al., 2003).

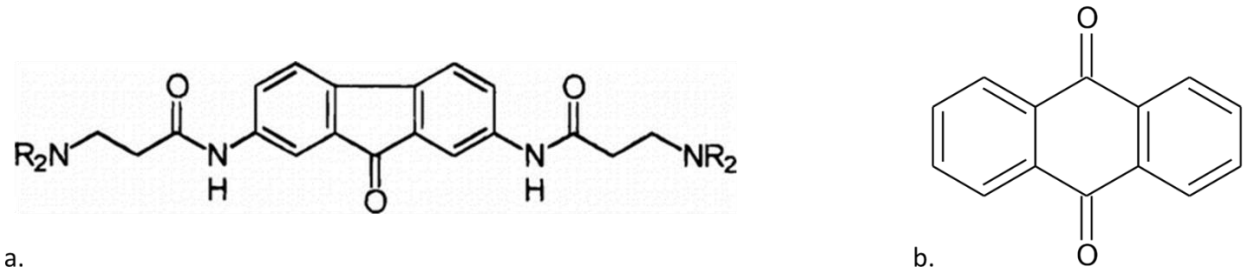


Figure 16. Chemical structures of fluoroenone (a.) and anthraquinone (b.).

Beyond the tricyclic molecules, the G-quadruplex ligands can also be classified into polycyclic molecules such as Porphyrins and Perilenes. Through these molecules it has been possible to start investigating the ability of these compounds not only as telomerase inhibitors but also as ligands of G-quadruplex structures present in DNA sequences other than the telomere. In particular, porphyrins include TMPyP2 and TMPyP4 (Figure 17 b and a respectively). TMPyP4, as previously described, can bind the G-quadruplex structure present in the *KRAS* oncogene promoter. Moreover, compared to TMPyP2, TMPyP4 binds and stabilizes parallel and antiparallel G-quadruplex structures exhibiting greater antitumor efficacy not only in positive telomerase cells, where it inhibits the action of telomerase, but also in negative telomerase cells that possess an alternative mechanism of telomere lengthening (ALT) (Wheelhouse et al., 1998; Han and Hurley, 2000; Kim et al., 2003). However, both molecules, TMPyP4 and TMPyP2, were also tested on the *C-MYC* oncogene promoter sequence showing a good stabilization capacity of the G-quadruplex structure (Siddiqui-Jain et al., 2002).

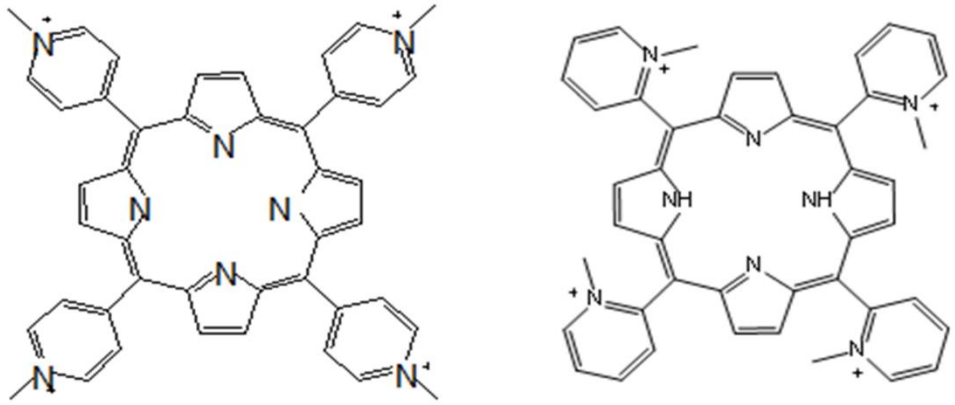


Figure 17. Chemical structure of porfirines TMPyP4 (a) and TMPyP2 (b).

The perylene molecule, fluorescent in nature, consists of two naphthalene molecules connected by a carbon-carbon bond at the 1 and 8 positions on both molecules and all of the carbon atoms in perylene are sp_2 hybridized. These derivatives interact with G-quadruplex structures with high affinity. One of the first molecule designed and synthesized based on these molecular features was PIPER (Figure 18), a perylene with two positive charges in the side chain. This molecule, fundamental in the development of the following G-quadruplex ligands, have a good selectivity towards the G-quadruplex structure interacting with the quartets of guanine (Federoff et al., 1998). Perylene-derivate molecules are still synthetized and studied in mechanism that regulate cancer such as the *HIF-1α* and *Stat3* signalling pathways for therapeutic application potentials (Hu et al., 2012; Chen et al., 2014; Vasimalla et al., 2017).

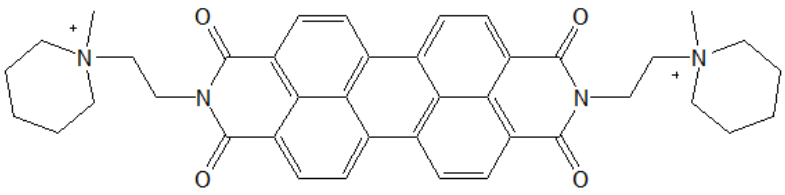


Figure 18. Chemical structure of perylene compound PIPER.

Another polycycle molecule is Telomestatin (Figure 19) that was first isolated from bacteria *Streptomyces Anulatus 3533-SV4* (Shin-ya et al., 2001). Telomestatin is a macrocyclic chemical compound that interact with the 3' telomeric overhang showing the most potent ability to inhibit telomerase activity in cancer cells. Indeed, Telomestatin induces the formation of G-quadruplex structure in the telomeric regions, causing a decrease in the activity of the telomerase and in the shelterin protein of POT1 and TRF2 from their sites (Gomez et al., 2006). The treatment with Telomestatin is also associated with an increase of DNA damage response at telomere and also in additional DNA targets as show the gamma-H2AX foci, suggesting that Telomestatin interact also with no-telomeric G-quadruplex even in absence of monovalent cations (Kim et al., 2002). In fact, Miyazaki et al. have been demonstrated that Telomestatin have a strong antitumoral efficacy in Glioma Stem Cell treatment identifying a novel molecular target. In particular, Telomestatin impairs the migration potential of GSCs, reduce the size of intracranial GSC-derived mouse tumors and, of notice, is able to bind the G-quadruplex structure in the *C-MYB* promoter, identified as a new molecular target of this compound (Miyazaki et al., 2012).

The synthesis of the all possible stereoisomers of Telomestatin have been already achieved. In particular, the S-stereoisomer have been demonstrated to inhibit telomerase activity four time stronger than the natural product R-telomestatin (Doi et al., 2011).

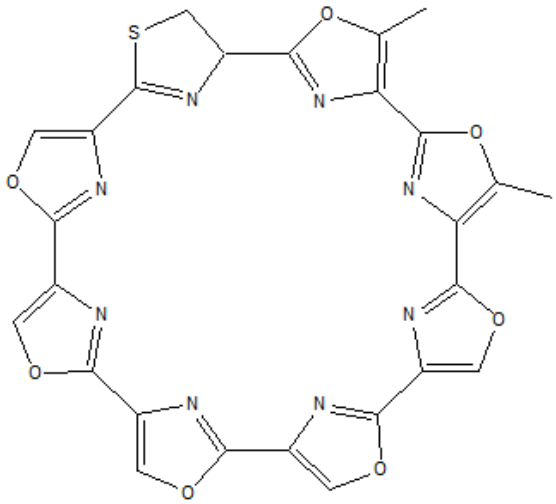


Figure 19. Chemical structure of Telomestatin.

Another class of polycyclic ligands is that of Acridines. These molecules are composed of three rings with a nitrogen atom in the central ring which gives it aromaticity. Acridine derivatives can have two or three substituents in the aromatic nucleus which can increase the selectivity for the G-quadruplex structures and decrease the affinity for the double-stranded DNA. One of the best studied acridine derivatives is BRACO-19 (Figure 20). This compound has been described to interact with telomeres stabilizing the telomeric G-quadruplex (Read et al., 2001) and display antitumor efficacy through inhibiting telomerase activity (Gowan et al., 2002; Harrison et al., 2003). BRACO-19 have been reported having a strong antitumor effect in different cancer histotype such as glioma and human uterus carcinoma. In vitro, human uterus carcinoma BRACO-19 treatment causes drastic decrease of nuclear human telomerase reverse transcriptase (hTERT), induces senescence and telomere shortening. Immunostaining of xenograft tissues showed that this response was paralleled by loss of nuclear hTERT protein expression and an increase in atypical mitoses indicative of telomere dysfunction. Cytoplasmic hTERT expression and its colocalization with ubiquitin was observed suggesting that hTERT is bound to ubiquitin and targeted for enhanced degradation upon BRACO-19 treatment (Burger et al., 2005). Recently, Zhou's group observed that BRACO-19 treatment is tumor-cell specific resulting in growth inhibition and telomerase activity inhibition in high grade brain tumor cells. Moreover, the growth suppression induced by BRACO-19 triggers the production of DNA damage response occurred at telomeres and causes its uncapping as demonstrated the anaphase bridges. As normally associated with telomere uncapping, during BRACO-19 treatment occurred also the delocalization of TRF2 and POT1 from their sites (Zhou et al., 2016).

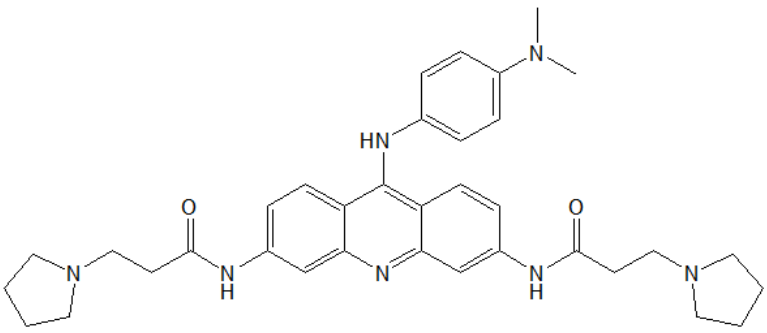


Figure 20. Chemical structure of the 3,6,9 trisubstituted acridine derivative BRACO-19.

Based on the promising results on BRACO-19, the novel pentacyclic acridine RHPS4 (3,11-difluoro-6,8,13-trimethyl-8H-quino[4,3,2-kl]acridinium methosulfate) with an increased selectivity towards quadruplex of DNA, has been synthetized (Gowan et al., 2001; Heald et al., 2002). In the host laboratory RHPS4 (Figure 21) has been extensively studied and other than an anti-telomerase activity, the telomeric dysfunction and apoptosis in tumor cells of different histotype under RHPS4 treatment have been observed, even in those cells in which oncosuppressor *p53* is mutated (Leonetti et al., 2004). Indeed, RHPS4 induces the formation of telomere damage foci and, mediated by the ATR kinase, causes a strong increasing of the phosphorylation of the histone H2AX (Salvati et al., 2007) which colocalize with telomere protein TRF1 forming the so-called Telomere Dysfunction Induced foci (TIF) (Takai et al., 2003). The others damage response factors whose phosphorylation occurs are RAD17 and 53BP1. Moreover, whereas high DNA damage is already present, such as in tumor cells, RHPS4 is able to induce the loss of POT1 from the telomeric shelterin complex. The antitumor efficacy of RHPS4 have been also investigated *in vivo*, through experiments in mouse models (Salvati et al., 2007; Leonetti et al., 2008). These studies have not revealed toxicity of the drug, except a brief change in blood pressure just after the administration of the dose, identified as a reversible hypotension. In fact, the effective dose for antitumor treatment in nude mice did not result in death due to toxicity or weight loss of the animal during treatment. Moreover, the histological analyses of the organs explanted at the end of the treatments did not show any lesions or morphological alterations in the examined tissues. However, the differences in function between human and mouse myocardium put a limitation in the study of cardiac effects in pharmacology through the use of mouse models (Hasenfuss, 1998). For these reasons, in view of clinical application in humans, cardiac effects of RHPS4 have been studied in "cavia porcellus" (Guinea pig). In this animal model, during RHPS4 treatment it has been observed a significant decrease in the QTcB interval, the time required for the ventricular myocardium to depolarize and repolarize. To investigate the molecular basis of this event, the study continued by verifying with which of the 54 most important human pharmacological receptors (Cerep ExpresS Profile Screen) the RHPS4 interacted. The study demonstrated that the compound interacted significantly with the β2 adrenergic receptor and the muscarinic M1, M2 and M3 receptors (Iachettini et al., 2013). Inconclusion, despite its promising antitumor efficacy shown *in vitro* and *in vivo*, these results predicted the possible complications in the use of RHPS4 in the humans, thus making it necessary to study new G-quadruplex ligands for clinical development.

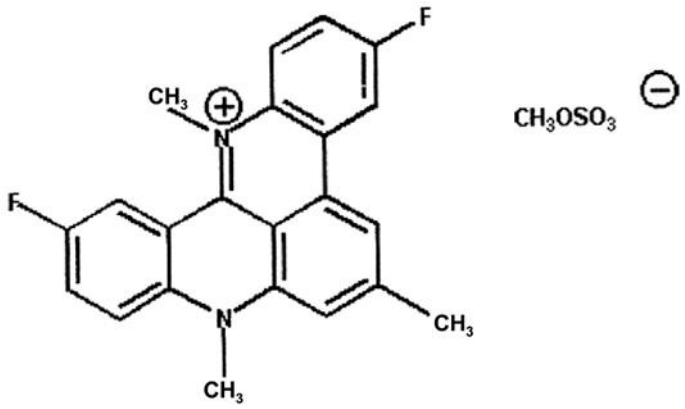


Figure 21. Chemical structure of RHPS4.

EMICORON

Recently, in the Chemistry Department at La Sapienza University in Rome, has been synthetized a new G-quadruplex ligand from two initial molecules: PPL3C and CORON (Figure 22 a and b respectively), two well studied molecules able to bound G-quadruplex structures (Rosu et al., 2002; Mazzitelli et al., 2006; Rosu et al., 2008; Casagrande et al., 2009; Casagrande et al., 2011). The chimeric molecule obtained by these two molecules is EMICORON (N,N'-bis[2-(1piperidino) -ethyl] -1- (1-piperidinyl) 6- [2-(1-piperidino)-ethyl] -benzo[ghi]perylene-3,4:9,10 tetracarboxylic diimide) (Figure 21 c). At molecular level, EMICORON presents one more aromatic ring with respect to simple perylene diimides, the unique structural features such as the aromatic core able to give effective π- π interactions with three of the four G-quadruplex grooves and one piperidinyl group bound to the perylene bay area. This bulky group avoids excessive auto-aggregation and consequent precipitation typical of polycyclic aromatic compounds in aqueous solution. At the same time gives a good selectivity for quadruplex structures with respect to duplex DNA but with the advantage of higher binding constants than the previously reported compounds, both with respect to N-cyclic bay-monosubstituted perylene diimide PPL3C and to coronene derivative CORON (Franceschin et al., 2012).

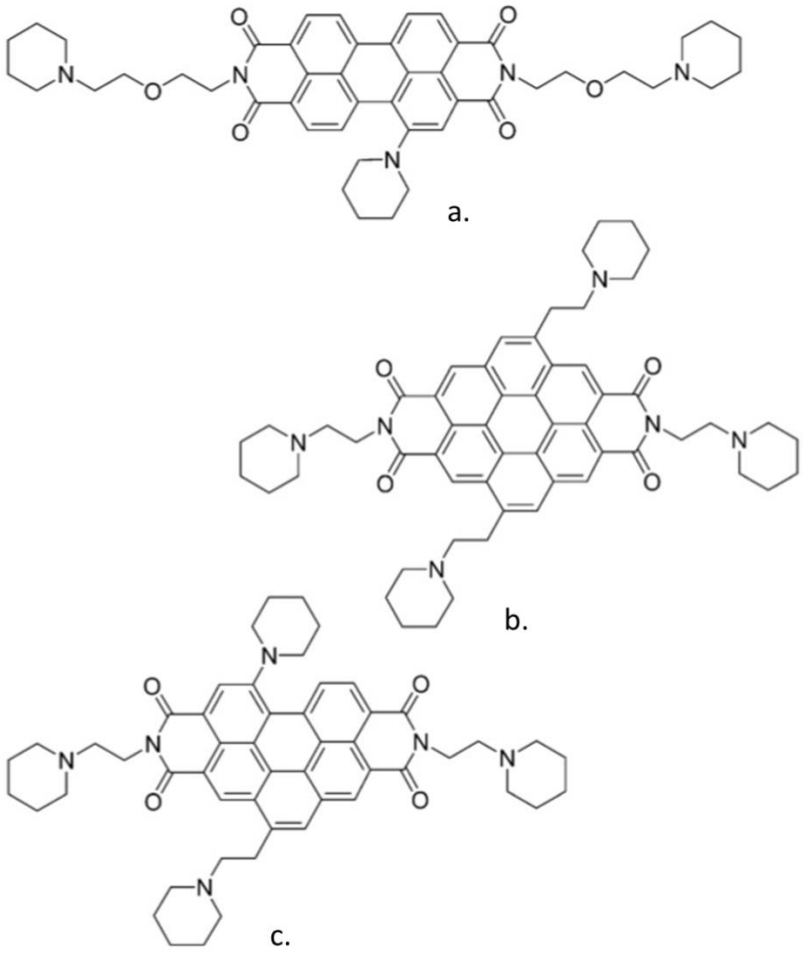


Figure 22. Chemical structure of a) PPL3C, b) CORON and c) EMICORON.

Typical of the hydrosoluble coronene, EMICORON is a G-quadruplex ligand having antitumoral effect. In particular, this compound has the ability to inhibit the growth both of the tumor cells with telomerase activity and in telomerase-negative ALT+ cells (Pompili et al., 2017). The antitumoral activity is displayed also in a potent DNA damage response characterized by a significant increase in the percentage of the cells positive for the formation of several telomeric foci (colocalizing with TRF1) containing phosphorylated H2AX and 53BP1, which are the markers for interphase telomers and DNA double-strand break respectively. TIFs are typical of the telomere deprotection occurring during cellular senescence or upon the loss of telomeric proteins (van Steensel et al., 1997; van Steensel et al., 1998; D'Adda et al., 2003; Hockemeyeret al., 2005 Biroccio et al., 2006;). Indeed, the effect of the compound involve three shelterin complex proteins: POT1 which is delocalized from telomeres, whereas both TR1 and TRF2 remain associated to the TTAGGG repeats. So, EMICORON is able not only to limit the growth of transformed/tumoral cells but also able, as others G-quadruplex ligands, to uncap telomeres (Salvati et al., 2010; Burger et al., 2005) The data

obtained from NCI Drug Screen Program, by comparing different tumor histotype, provide the evidence that EMICORON is highly active on all 52 human tumor cell lines tested with the capability to inhibit the 50% of cell growth (IC50) always below the 1mm concentration. Moreover, these data provide evidences that the mutated state of the main oncosuppressor *p53* makes cells more sensitive to the treatment with EMICORON.

Beyond the effect on telomeres, EMICORON can bind G-quadruplex on the sequence in promoter with G-quadruplex forming potential. The host laboratory demonstrated that EMICORON can bind and stabilize the G-rich sequences located within the promoters of *C-MYC* and *BCL2* genes. Consistently with these results, EMICORON inhibits the mRNA expression of both genes and this transcriptional inhibition was found to correlate with a reduction in protein level of about 50% for both the targets (Micheli et al., 2016) in melanoma cells which express high levels of both oncogenes (Biroccio et al., 2001). Based on these results, it is clear that EMICORON exerts antiproliferative activity not only by causing telomere uncapping but also by inhibiting cell proliferation via down-regulation of oncogenes expression and their relative signalling pathways. In fact, among the different targets of EMICORON, the host laboratory identified also the cancer stem cell CD133 surface marker that contains G-quadruplex forming sequence within the introns 3 and 7 of its gene (Zizza et al., 2016). The expression of *CD133*, associated with tumorigenicity and progression, confers aggressiveness to the cancer stem cells and correlates with poor prognosis and metastatic dissemination in tumors of different histotypes (Kashihara et al., 2014). The hosting group evaluated the effect of EMICORON on two patient-derived colon cancer stem cells (CSCs), characterized by the expression of the surface markers *CD133* and resistant to Oxaliplatin and 5-Fluorouracil, two common drugs used in the treatment of the colorectal cancer (CRC). The results showed that EMICORON down-regulated the expression of *CD133*, reduced the survival of the cells of about 50% and inhibited the size of CSC tumorspheres. These results provided additional evidences about the antitumoral efficacy of EMICORON for its ability to affect the stemness properties of patient-derived colon CSCs by reducing the expression of CD133 marker.

The hosting group has identified the maximum tolerated dose (MTD) in healthy mice treated by oral gavage with the dose of 400mg/kg and for therapeutic purpose decided to fractionate this dose by giving 15mg/kg/day in mice. This scheduling was well tolerated as histological analysis confirmed that the treatment was free of adverse effects as showed no differences in the major organs compared to the organs of mice treated only with the vehicle. Moreover, the average metabolome of treated mice was similar to that of untreated mice. In addition, it has been demonstrated that EMICORON can reach similar blood levels in humans as in mice by the bone

marrow colony-forming unit granulocyte-macrophage assay (CFU-GM) which compares the toxic effects of the drugs on the bone marrow of mice and humans. Finally, it has been demonstrated that EMICORON, has a strong and selective antitumor efficacy *in vivo* in advanced mouse model such as PDXs and orthotopic implant of colorectal cancer (Porru et al., 2015).

Preclinical Mouse Models

Preclinical models are indispensable for the study of cancer and the development of new anticancer drugs. Despite the *in vitro* cellular systems provide important informations on the efficacy of drugs, they fail to simulate the complexity of a complete tissue or organism and cannot provide conclusive indications regarding both the efficacy and possible toxicity of a new drug. Furthermore, although many therapies show favorable antitumor responses in preclinical experiments, only 5% of new antineoplastic compounds have shown efficacy in clinical studies. Many factors are responsible for this high failure rate, including the lack of predictive preclinical cancer models (Hutchinson et al., 2011). Animal models are a useful system that can be used in different stages of drug development in order to integrate their complexity, their physiological relevance and the predictability to clinical response. The use of animals in scientific research is a very heated topic in the ethical debate. In 1959 the British academics Russell and Burch of the "University Federation of Animal Welfare" defined the 3R principle by providing a framework to address this dilemma (Russell WMS and Burch RL, 1959). The 3R, important from a legal, ethical and scientific point of view are ‘replacement’: when possible, is better adopt methods that replace the use of animals with cell cultures, human volunteers or scientific models for scientific purposes. ‘Refinement’: adopt methods that can alleviate or minimize suffering and stress and that can improve the welfare of the animals used in the experimentation. ‘Reduction’: adopt methods that allow researchers to obtain a comparable amount of information from a few animals, or more information from the same number of animals.

In 2010, the European Parliament issued Directive 2010/63 / EU to regulate and standardize animal testing in all European countries and firmly subscribe to the 3R principle. In Italy, in implementation of this European directive, the legislative decree n. 26 of 4 March 2014 was issued. This decree, with respect to the European directive, places serious rules on scientific research and introduces greater restrictions on the use of animals for scientific research. The decree provides for example that from 1 January 2017 organ xenografts and *in vivo* studies on the effects of drugs of abuse will be banned.

Preclinical models have to recapitulate key features of human tumors to confirm the therapeutic efficacy of a compound to predict the clinical outcome (Maider et al., 2018). Cancer studies conducted *in vivo* on animals are determinant for the identification of new therapeutic targets, the development of new compounds, their usefulness in combination with chemotherapy or radiotherapy and the study of tumor resistance to drug treatment. The versatility of these models therefore offers benefits regarding the possible translationality of preclinical results to clinical

practice (Teicher et al., 2006). For the preclinical models of human tumors are used different mouse strains which differ in the degree of immunodeficiency, necessary to avoid the rejection of the implant and for the growth of human tumors. The first mutation in mice giving immunodeficiency were found in 1966 on chromosome 11. This mutation makes the mice both atymic and therefore missing T cells. This mutation in the *FOXN1* gene also renders mice without hairs, so these mice are called Nude. Subsequently, various genetic mutations have been described allowing the development of mouse strains with greater immunodeficiency degrees than Nude mice. The strain of SCID (Severe Combined Immunodeficiency) mice presenting the recessive SCID mutation on chromosome 16 are free of T and B lymphocytes despite having thymus, lymph nodes, splenic follicles and a normal number of NK cells. Another strain used, more immunodepressed, is that of NOD / SCID (non-obese diabetes / SCID) mice. These mice have an additional mutation that causes the lack of β2-Microglobulin and also lack mature lymphocytes, class I MHC and NK cell activity. A more advanced animal models as recipients for xenotransplantation are NOD/SCID/gamma(c)(null) mice double homozygous for the severe combined immunodeficiency (SCID) mutation and interleukin-2Rgamma (IL-2Rgamma) allelic mutation (gamma(c)(null)) generated by 8 backcross matings of C57BL/6J-gamma(c)(null) mice and NOD/Shi-scid mice. In these mice the interferon-gamma production from dendritic cells from the NOD/SCID/gamma(c)(null) spleen is significantly suppressed and immunological dysfunctions such as cytokine production capability and the functional incompetence of T, B, and NK cells, lead to the high engraftment levels of xenograft in NOD/SCID/gamma(c)(null) mice (Ito et al., 2002). Immunocompetent mice are instead used in experiments involving the use of murine and not human cells. In this case the most used syngenic strains are the C57BL / 6 and BALB / c, different from each other for the different Th1 / Th2 response. The detailed knowledge of the respective mouse strain is therefore important to select the most appropriate variant for tumor growth studies or for experiments where an immune response is required (Wartha et al., 2014).

There are many animal models used in cancer translational research. There are heterotopic xenograft models in which the tumor cells are implanted in a different site than the organ of origin; the orthotopic xenograft model, in which the tumor cells are implanted in the origin organ, and a third model provides for the use of genetically engineered mice. The so-called xenograft model consists of the implant of human cancer cell lines in immunocompromised mice that can be inoculated subcutaneous, intramuscularly or intraperitoneal. Tumor growth can be followed and monitored through the use of a caliper or by using bioimaging techniques to compare the growth of a control tumor with the growth of a tumor that is treated with new drugs.

An alternative to the more classic xenograft model is the patient-derived xenograft model (PDXs) (Porru, Pompili et al., 2016). In this model a small fragment of tumor comes directly from the patient and is immediately implanted subcutaneously in immunodeficient animals and subsequently maintained *in vivo* by mouse-to-mouse transitions (Tentler et al., 2012; Hidalgo et al., 2014). PDXs have emerged as an important platform for translational research and preclinical testing since the first study date back to the 1980s when Fiebig and colleagues published a high degree of correlation between clinical response and cytotoxic agents in lung cancer patients and in their corresponding PDXs models (Fiebig et al., 1985). PDXs models are used to address the therapeutic efficacy of new treatment targets, novel combinations of therapies, optimal schedules according to tolerability and sensitivity and primary and secondary resistance. Several studies suggest that PDXs predict clinical response to therapy better than traditional xenografts (Voskoglou-Nomikos et al., 2003; Hidalgo et al., 2011; Echeverria et al., 2018). In fact, the advantages of these models are that there is no clonal selection of tumor cells; genetic profile, histopathologic features and heterogeneity of the tumor are maintained, reflecting the patient's clinical situation; initial tumor stroma is maintained to support tissue growth and homeostasis and is maintained also the mechanisms of resistance to treatment (Bertotti et al., 2011; Bruna et al., 2016). Concerning the resistance-drug mechanisms, Bertotti et al. demonstrated the value of large PDXs cohorts in exploring mechanisms of resistance to cetuximab in colorectal cancer, evaluating alternative targeted-treatments and thereby creating an evidence-based rationale for clinical trials (Bertotti et al., 2015). Moreover, they demonstrated that PDXs tumors retained the morphology and genomic features of their original counterparts and responded to cetuximab (anti-EGFR antibody) in the same way as was observed in the clinic representing powerful tools in predicting the response to anticancer therapy and supporting the role of this model in mimicking the response of this disease in humans (Sartore-Bianchi et al., 2016). In the perspective of personalized medicine, PDXs offer a route toward personalized treatment acting as an ‘avatar’ model, in which PDXs obtained from a patient enrolled in a clinical trial could be treated with the same therapy administered to the patient, thus permitting to identify biomarkers of sensitivity or resistance to treatments (Bousquet et al., 2014).

In recent years several studies of PDXs models have emerged but the size of the resources and the processes for creating and characterizing PDXs models is quite variable. Because of the crucial information about tumors, host strains, transplant and quality assurance processes which are often inconsistently presented in both the scientific literature and in database resources, limiting the ability of researchers to find relevant models and associated data, Meehan et al. have generated a draft PDXs-Minimal Information standard to describe and it serve as the starting point for community wide adoption. The PDXs-MI consists of four modules that reflect the process of

generating, validating and using a PDXs model: Clinical, Model Creation, Model Quality Assurance, Model Study (Meehan et al., 2017).

The treatment of PDXs bearing mice with novel drug permits to identify an accurate therapy avoiding treatment failures and to elucidate the resistance mechanism. For these reasons, nowadays PDXs is considered one of the most advanced mouse model for translational cancer research purpose.

Aim

G-quadruplex ligand EMICORON has been demonstrated to have a strong antitumor activity in colorectal model both *in vitro* and *in vivo*. Moreover, preliminary data showed that EMICORON can synergize with chemotherapeutics commonly used in clinical treatment of colorectal cancer in a specific scheduling manner. On the basis of these data, we decided to verify the ability of EMICORON to potentiate the chemotherapy in advanced colorectal cancer models. Preliminarily, we verified the efficacy of the combination of topoisomerase I inhibitor plus EMICORON in 3D colorectal cancer cell line culture system, and then we confirmed the data in a colorectal cancer xenograft. Then, we obtained three colorectal cancer PDXs (*KRAS* mutated) at F2 generation in which we verified the antitumor efficacy of EMICORON treatment. Furthermore, to confirm in advanced colorectal cancer model the therapeutic efficacy of EMICORON in combination with chemotherapy, we treated PDXs with with EMICORON in combination with chemotehrapy regimen adopted for clinical management of *KRAS* mutated colorectal cancer . Then, to confirm the EMICORON antitumoral efficacy against *KRAS* mutated tumors, we investigated its molecular activity on *KRAS* gene expression.

Materials and Methods

Cell lines

HT29, HCT116, (American Type Culture. Manassas, VA, USA) were maintained with DMEM medium (Euroclone, Milan, Italy) supplemented with 10% FBS (Hy-Clone, Logan, UT, USA), 5% L-glutamine (Euroclone, Milan, Italy), 5% Penicillin and streptomycin (Euroclone, Milan, Italy). HT29 LUC2 (PerkinElmer, Waltham, MA, USA) were maintained in McCoys medium supplemented with Heat inactivated 10% FBS. All cells were cultured in adhesion condition at 37°C, 5% CO2 and 3% O2.

Drugs

The drugs used for the experiments are N,N-Bis[2-(1-piperidino)-ethyl]-1-(1piperidinyl)-6-[2-(1-piperidino)-ethyl]-benzo [ghi] perylene-3,4:9,10-tetracarboxylic diimide (EMICORON) synthesized at Chemistry Department of La Sapienza University in Rome (Franceschin et al., 2012); 7-etil-10-idrossicamptotecina SN-38 (Alexis, Florence, Italy), Irinotecan active metabolite; clinical-grade Irinotecan (CAMPTO, Pfizer, Borgo S Michele, LT, Italy); 5-Fluorouracil (Teva Italia SRL, Milan, Italy).

Multicellular tumor spheroids

For multicellular-spheroid experiments, HT29-LUC2 colon cancer cells were seeded at the density of 1500 cells/well into a 96-wells plate coated with 50 µl of agarose in 200 µl of McCoys medium. After 72 hours, the spheroids were treated with SN-38 at 1.5 µM for 2 hours, EMICORON at 1.5 µM for 96 hours and with the combination SN-38 -> EMICORON. The luminescence intensity of spheroids was analysed by the IVIS imaging system 200 series (PerkinElmer) and data were acquired and analysed using the living image software version 3.0 (PerkinElmer).

In vivo experiments.

CD-1 male nude (nu/nu) mice, 6-8 weeks old were purchased from Charles River Laboratories (Calco, Italy), SCID, NOD-SCID and NSG from Harlan Laboratories (S. Peter at Natisone, Italy). All animal procedures were approved by the ethics committee of the Regina Elena National Cancer Institute (CE/534/12) and were in compliance with the national and international directives (D.L. March 4, 2014, no. 26; directive 2010/63/EU of the European Parliament and of the council; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 2011). The animals were euthanized for ethical reasons when tumors reached a mean of 2.5-3 cm^3 or when they became moribund during the observation period (the time of euthanization was recorded as the time of death).

PDXs

PDXs models were developed from the biopsy of three patients at the IRCSS Regina Elena National Cancer Institute in Rome, by implanting the tumor fragments directly into CD1-nude, SCID, NOD-SCID or NSG mice. PDXs were maintained by mouse-to-mouse passages and for all experiments we used F2 generation tumors. The F0 and F1 generation tumor was explanted from the sacrificed mouse, cut into fragments of about 25-30 mm^3 and implanted again into immunocompromised mice subcutaneously in Matrigel (Corning Cat. No. 354234). When the tumor reached about 300 mm^3 the mice were divided into the various treatment groups. Tumor growth was assessed by using the caliper 3 times a week and the tumor volume was determined with the formula (a x b2) / 2, where a and b are respectively the largest and minor tumor size.

Antitumoral activity

Antitumor efficacy of treatments was calculated using the following end points: percent tumor volume inhibition (TVI%), calculated as [1-(mean tumor volume of the treated group / mean tumor volume of the control group] x 100; tumor growth delay, evaluated as T-C, where T and C are the median times for treated and control tumors, respectively, to reach the same size (i.e. 1000 mm^3); disease stabilization, defined as the maintenance for at least two weeks or three weeks of the same tumor volume as the start of treatment; complete response, defined as the disappearance of tumor as evaluated by palpability, for at least three weeks in the course of treatment; percent increase in lifespan (ILS%), calculated by dividing the median survival time (in days) of treated mice by the median survival time (in days) of control mice, subtracting the resulting value from 1, and

multiplying that value by 100. Stable disease, defined as the maintenance for at least two or three weeks of the same tumor volume as the start of treatment. Complete response, defined as the disappearance of tumor as evaluated by palpability, for at least three weeks.

Histological and immunohistochemical studies

To verify the maintenance of the same characteristics between the patient's tumors and the tumors grown in mice, the tumors were explanted, stored in formalin and cut into 4-µm sections on SuperFrost Plus slides (Menzel-Gläser, Braunschweig, Germany) to be included in paraffin. The sections were stained with Haematoxylin and Eosin and analysed under an optical microscope. Immunohistochemical analyses were performed using the following antibodies: anti-cytokeratin 20 (CK20 clone Ks20.8, Dako) and anti-CDX2 (CDX2, clone CDX2-88, Biocare Medical)

Next Generation Sequencing

For molecular analysis, 3 or 4 whole 5µm thick paraffin sections were harvested by macrodissection of circled tumor areas. DNA extraction was performed by using the QIAamp DNA kit (QIAGEN, Milan, Italy). DNA quantification was conducted through Qubit 3.0 Fluorometer by using Qubit® dsDNA HS assay kit (Life Technologies, Invitrogen). Ion Torrent Next Generation Sequencing (NGS) technology needs only minute quantities of DNA. Ten ng of purified genomic DNA were used for library preparation with the Oncomine™ Solid Tumor DNA Kit (Life Technologies) to sequence hotspot mutations in 22 genes (*EGFR, ALK, ERBB2, ERBB4, FGFR1, FGFR2, FGFR3, MET, DDR2, KRAS, PIK3CA, BRAF, AKT1, PTEN, NRAS, MAP2K1, STK11, NOTCH1, CTNNB1, SMAD4, FBXW7, TP53*).

Library Emulation PCR and Chip 520 loading was performed by the Ion Chef™ instrument (Thermo Fisher scientific) with Ion 520™/ 530™ Kit-Chef (Thermo Fisher). Chips sequencing was performed on an Ion TorrentTM Ion S5 sequencer. Results were analysed using the torrent suite software v3.6.2 (Life Technologies) and Ion Reporter Software 5.4. Only mutations reported in the COSMIC (Sanger Institute Catalogue of Somatic Mutations in Cancer) database (http://www.sanger.ac.uk/cosmic) were taken into account.

Western blot analyses

HCT116 cells (200.000) were placed in 60mm plate and the whole cell extracts were fractionated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane using a transfer apparatus according to the manufacturer's protocols (Bio-Rad). After incubation with 5% non-fat milk in T-TBS (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 1 hour, the membrane was washed once with T-TBS and incubated with antibodies against Actin (Mouse, Sigma Aldrich) (1:20.000) and KRAS (Mouse, Santa Cruz Biotechnology) (1:1000) at 4 °C overnight. Membranes were washed three times for 10 min and incubated with a 1:3000 dilution of anti-mouse antibodies for 1 hour. Blots were washed with T-TBS three times and developed with the ECL system (Amersham Biosciences) according to the manufacturer's protocols.

RNA extraction, RT-PCR and Real-time PCR.

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. 500 ng of total RNA was used for cDNA synthesis following the QuantiTect Reverse Transcription Kit (Qiagen) protocol with the a first step of amplification at 42°C for 40 min followed by one cycle of denaturation at 95°C for 3 min, and then the reaction was stored at 4°C. Real-time PCR was conducted with following steps: 50° for 2', a first denaturation step at 95°C for 10', 41 cycles of denaturation at 95°C for 15" and primer annealing and extension at 60°C for 1', and then the reaction was stored at 4°C. Real-time PCR was carried out using an 7900HT Fast Real-Time PCR System (Applied Biosystems). The geometric mean of housekeeping gene Actin was used as an internal control to normalize the variability in expression levels. The primer sequences: Actin FW 5'- AGCACTGTGTTGGCGTACAG – 3', RV 5'- TCCCTGGAGAAGAGCTACGA; KRAS FW 5'- ACACAAAACAGGCTCAGGACT -3', RV 5'- TGTCGGATCTCCCTCACCAA -3'. Expression data were normalized to the geometric mean of housekeeping gene Actin to control the variability in expression levels and were analysed using the 2 -ΔΔCT method described by Livak and Schmittgen (Livak et al., 2001).

Statistical analysis

The Student's t-test (not paired, two-tailed) was used to compare statistical differences. Survival curves of mice were processed using the Kaplan-Meier method, and statistical differences between groups were assessed by logarithmic ratios with Yates correction (Primer of Biostatistics software, McGraw-Hill, New York, NY, USA). The differences are considered statistically significant when $p < 0.05$.

Results

EMICORON potentiates the antitumor efficacy of chemotherapy in multicellular tumor spheroids of colorectal cancer cell line HT29*luc*

In 2003 Abbott first stressed the necessity to adopt new *in vitro* models for the development of new therapeutic strategies to be used before switching to animal models (Abbott; 2003). Nowadays, multicellular tumor spheroids (MCTSs) are probably the most appropriate approach for 3D culturing and one of the most promising *in vitro* system to test new therapy, to predict clinical efficacy and to investigate differentiation, migration and invasion processes of tumor cells (Kunz-Schughart et al.; 2004). Indeed, tumor cells that grown as 3D structure resemble many aspects of the pathophysiological situation in human solid tumors miming the expression of antigens, pH and oxygen gradients within its microenvironment, penetration rate of growth factors and distribution of proliferating or quiescent cells. For these reasons, MCT models are much closer to clinical expression profile than 2D cultures and can acquire some type of clinically relevant such as drug transport and binding, and multicellular resistance to apoptosis-inducing drugs that may mimic also the chemoresistance found in solid tumors (Friedrich et al.; 2009). Moreover, with increasing size, oxygen or nutrient gradients are observed as well as a potential accumulation of catabolites in central regions, highly reminiscent of poorly vascularized areas in solid tumors (Carlsson et al.; 1988). All these features affect the binding and bioactivity of drug candidates which often lose efficacy in the 3D pathophysiological environment. Supported by these considerations, we adopted MCT system culture as a valuable model to provide more comprehensive assessment of tumor response to EMICORON treatment. Preliminary experiments performed *in vitro* on 2D HT29 established colon cancer cells demonstrated that EMICORON has a synergistic effect with the conventional chemotherapeutics adopted in colorectal cancer clinical treatment such as SN-38, in particular in SN-38 followed by EMICORON scheduling (Figure 23 a and b). On the basis of these data, our aim was to evaluate the cytotoxic activity of the combination of topoisomerase I inhibitor SN-38, the active metabolite of Irinotecan, followed by EMICORON in the three-dimensional multicellular tumor spheroids.

Cells were seeded 1500 per well in a 96 well plate. After 72 hours, HT29*luc* 3D spheroids formed were treated respectively with 1.5µM SN-38 alone for two hours, 1.5µM EMICORON alone for 96 hours, and with the combination 1.5µM SN38 -> 1,5µM EMICORON. After SN-38 treatment the medium was changed with fresh medium and cells were allowed to grown until the end of treatment. The LUC expression (Figure 23 c), analysed at the end of treatments by the IVIS imaging

system, showed the photons emitted. In particular, untreated spheroids emitted a mean of $9{,}79\times10^6$ photons, SN-38 treated spheroids emitted a mean of $3{,}5\times10^6$ photons, EMICORON treated spheroids emitted $6{,}65\times10^6$ photons, SN-38 plus EMICORON spheroids treated emitted $1{,}36\times10^6$ photons (Figure 23 d.). This data demonstrated a decrease of proliferation in cells treated with the combination SN-38 -> EMICORON about 87%, while the decrease of proliferation causes by EMICORON alone and SN-38 alone was about 33% and 64% respectively, showing a significant decrease of proliferation in cells treated with the combination SN38 -> EMICORON compared to the drugs given alone. Then, we proceeded to investigate the efficacy of this combination *in vivo* in a colorectal cancer xenograft.

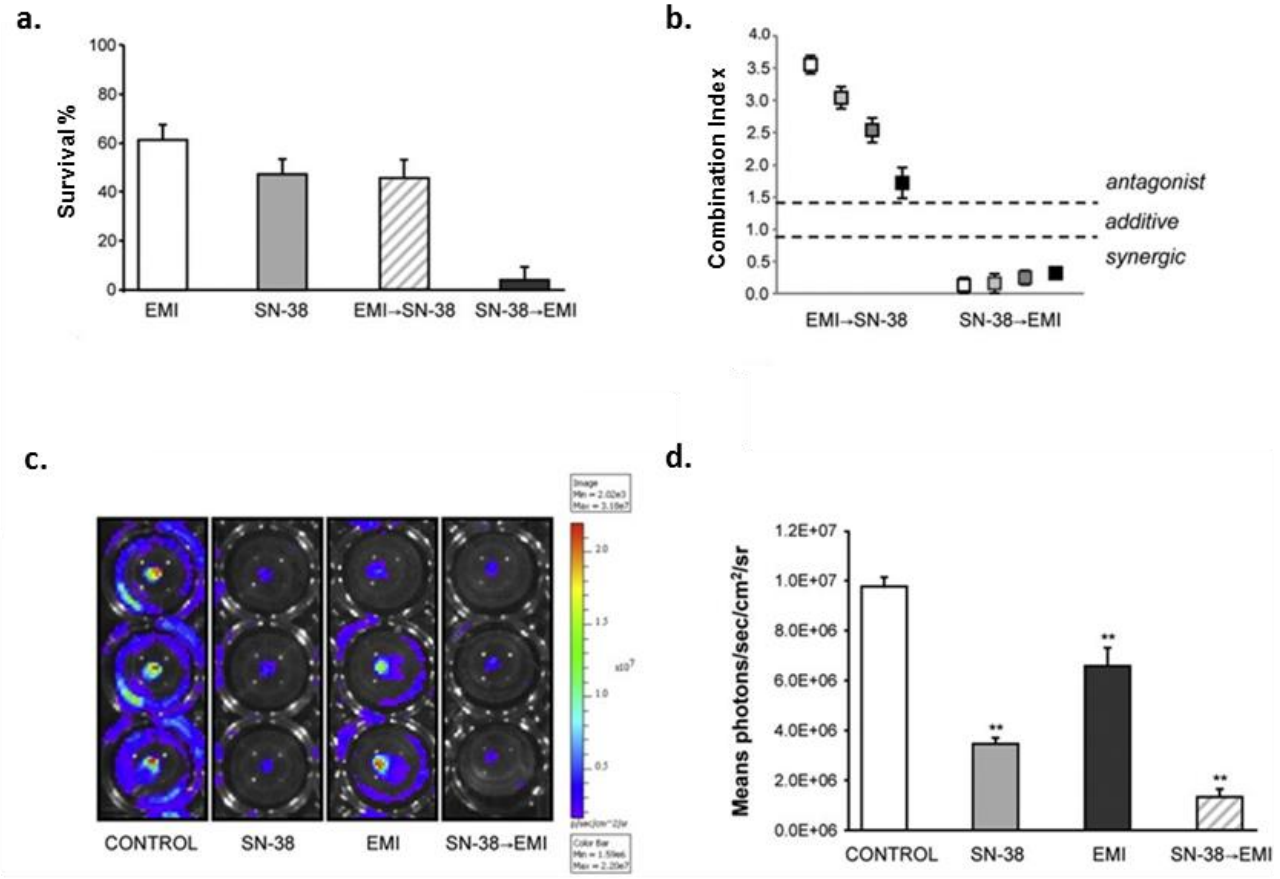


Figure 23. Cytotoxic activity of EMICORON in combination with SN-38. a) HT29 cells were treated with SN-38 alone or in combination with EMICORON by giving two different sequences of drug administration. b) Combination index is plotted at 50% (white squares), 75% (light grey squares) 90% (dark grey squares) and 95% (black squares) fraction killed. HT29*luc* 3D spheroids were treated with SN-38 or EMICORON alone or with the combination SN-38/EMICORON. c) The LUC expression was analysed by the IVIS imaging system. d) Histogram reports the means ± SD of three independent experiments.

EMICORON potentiates the antitumor efficacy of chemotherapy in HT29 xenograft models

Based on results obtained in colorectal cancer spheroids showing the strong synergistic interaction of SN-38 and EMICORON, we evaluated the antitumor activity of this combination in mice bearing HT29 xenograft. In particular, for the *in vivo* experiments we used Irinotecan, the SN-38 corresponding drug used in clinical treatment.

Nude mice were injected intramuscularly with $3x10^6$ HT29 cells/mouse. After 4 days, when the tumors were grown to about $250mm^3$, we separated the mice in four groups with six mice for each group. Control group was treated with only vehicle, Irinotecan was given intraperitoneally at 15mg/kg/day for 5 consecutive days, EMICORON was given *per os* at 15mg/kg/day for 20 consecutive days and in the combination Irinotecan (days 4-8) was followed by EMICORON (days 9-28). In order to verify the efficacy of the combination, mice were administered with two cycles of treatment by giving Irinotecan 15mg/Kg (days 4-8; 24-28), EMICORON 15mg/Kg (days 4-18; 24-38) and the combination Irinotecan (days 4-8; 24-28). EMICORON (days 9-22; 29-43). The mice treated with one cycle of combination Irinotecan -> EMICORON showed a decreased tumor growth in comparison to the treatment with the drugs alone (Figure 24 a). Consistently, in the mice treated with two cycles of treatment we observed a stronger tumor growth than those received one cycle of treatment (Figure 24 b). In particular, we observed a more marked tumor volume reduction and a delay of tumor re-growth compared to the group receiving only one cycle of treatment. In fact, as reported in Table 2, after one cycle of treatment the tumor volume was significantly (P = 0.0007) reduced of 79% in Irinotecan/EMICORON combination (group c) with respect to the 68% observed after Irinotecan treatment (group a) and of 33% (P b 0.0001) observed after EMICORON treatment (group b). Moreover, after combination treatment a delay of tumor regrowth of 19 days and the stabilization of the disease in one out of six mice treated was reported. The improvement of the response of HT29 xenografts when two cycles of treatment were administered, it was evident by the prolongation of the antitumoral effect which persisted for 26 days and significantly improved (P = 0.008) in comparison to Irinotecan given alone (13 days). Moreover, while in the group treated with Irinotecan alone two out of six mice showed stabilization of disease or complete response, the combination produced an improvement both in the number of mice with stable disease (two out of six mice) and in mice showing the complete response (three out of six mice) with tumors being not almost detectable. Although a relapse and progression of tumor growth were subsequently observed, the combination treatment resulted in an impressive increase of overall survival of mice of 114% significantly different (P = 0.005) compared to 68% observed after treatment with Irinotecan alone.

These results demonstrate the ability of EMICORON to potentiates the Irinotecan efficacy towards colorectal cancer xenograft confirming the data obtained in 3D model.

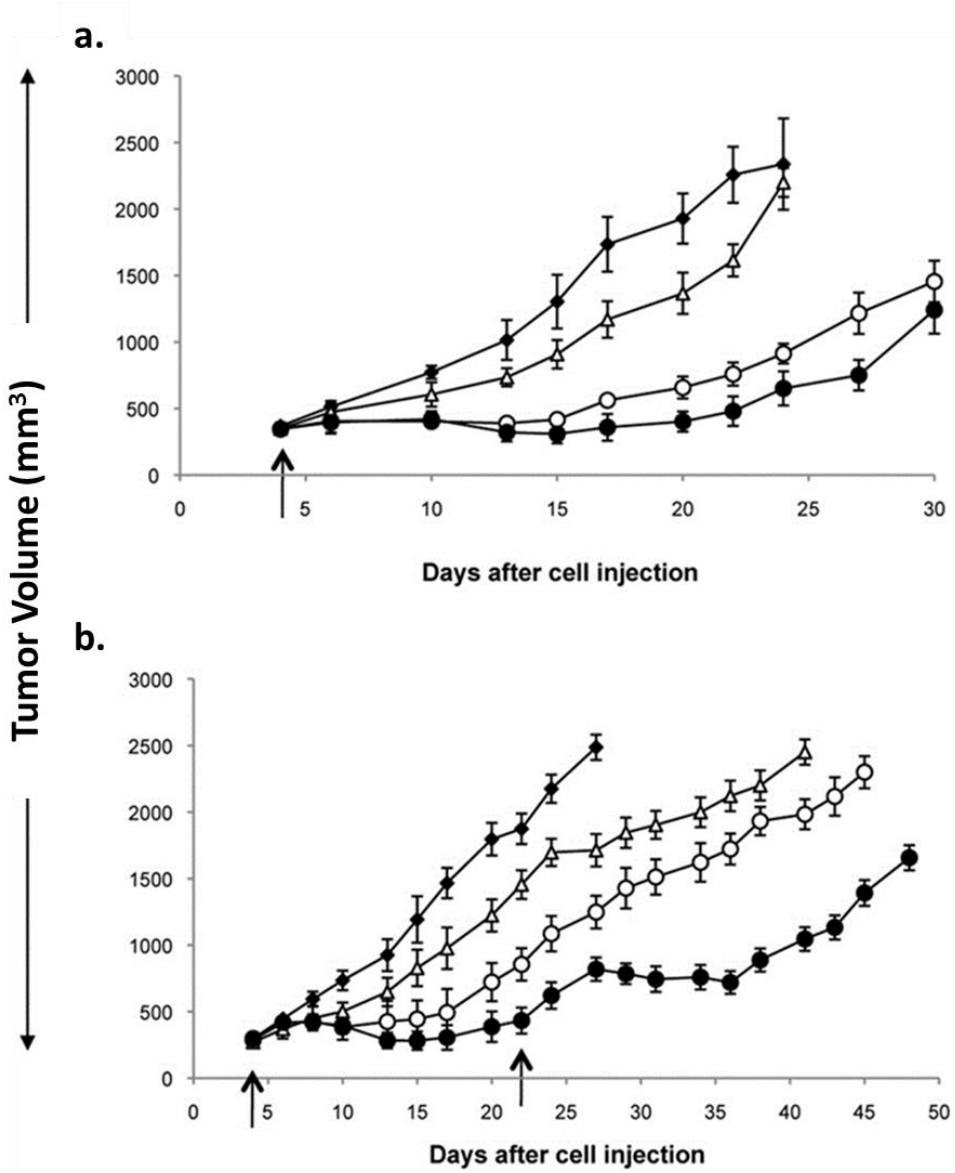


Figure 24. Antitumor activity of EMICORON in combination with Irinotecan against colorectal tumor-bearing mice. Mice were treated with Irinotecan for five consecutive days (○), with EMICORON for 20 consecutive days (Δ) or with the combination (●). Untreated mice (•) were given with vehicle for the same time of treatment. Mice were treated with one (a) or with two (b) cycles of treatment. Arrows indicate the start of each cycle of treatment. Tumor volume (mm^3) ± SD is reported.

Table 2. Therapeutic efficacy of IRINOTECAN and EMICORON combination after one cycle and two cycles of treatment on HT29 colon cancer xenografts.

Treatment groups[#]	TVI* (%)	T-C [§] (days)	Stable disease∞/ mice treated	Complete response[♣]/ mice treated	ILS[&](%)
a. IRN (one cycle)	68	13	0/6[♦]	0/6	69
b. EMI (one cycle)	33	3	0/6[♦]	0/6	19
c. IRN → EMI (one cycle)	79	19	1/6[♦]	0/6	85
d. IRN (two cycles)	55	10	1/6[φ]	1/6	68
e. EMI (two cycles)	36	2	0/6[φ]	0/6	21
f. IRN → EMI (two cycles)	78	26	2/6[φ]	3/6	114

[#] HT29 tumor bearing-mice were treated as follows: a. IRINOTECAN (IRN) at 15mg/kg/day i.p. for 5 consecutive days (days 4–8; b. EMICORON (EMI) at 15 mg/kg/day o.s. for 20 consecutive days (days 4–23); c. IRN at 15mg/kg/day i.p. for 5 consecutive days (days 4–8) and EMI at 15 mg/kg/day o.s. for 20 consecutive days (days 9–28); d. IRN at 15mg/kg/day i.p. from days 4 to 8 and from days 24 to 28; e. EMI at 15mg/kg/day o.s. from days 4 to 18 and from 24 to 38; f. IRN at 15 mg/kg/day i.p. from days 4 to 8 and from days 24 to 28 and EMI at 15mg/kg/day o.s. from days 9 to 22 and from 29 to 43. *Tumor volume inhibition was calculated at the nadir of the effect. [§]Calculated as the median times for treated (T) and control (C) tumors to reach the same size (1000 mm^3). ∞Stable disease was defined as the maintenance for at least two weeks[♦] or three weeks[φ] of the same tumor volume as the start of treatment. [♣]Complete response was defined as the disappearance of tumor as evaluated by palpability, for at least three weeks during the treatment. [&]Increase in lifespan (ILS) of treated mice was calculated comparing their median survival time (MST) with those of untreated mice.

EMICORON has a strong antitumor activity in three PDXs models of colorectal cancer

Patient Derived Xenografts models are based on the implant of tumor fragments directly derived from patient in immunocompromised mice. The fragment is implanted generally subcutaneously on the lower back of immunodeficient mice. PDXs are mainly used to study the antitumor effects of new drugs or combination of new drugs plus common regimens adopted in clinical treatment. The heterogeneity of PDXs and the reliability in maintaining the same characteristics of the patient's original tumor makes the model representative of patient providing indications about sensitivity or resistance to new treatments (Porru, Pompili et al., 2016).

Once implanted subcutaneously, the tumor is grown and maintained by mouse-to-mouse passages. The first-passage in mice, within the first fragment is implanted, are identified as F0 generation. When the tumor volume reaches a considerable size (300-500 mm^3) the mice were sacrificed and the PDXs are explanted and sectioned into small pieces of about 15-20 mm^3 that are kept in Matrigel and implanted in a second generation of mice called F1. From generation F1, through the same passages, is obtained the generation F2 that is usually used to conduct the experiments. Indeed, the number of mice is increased with each generation in order to have more animals to use for experiments (Porru, Pompili et al., 2018).

In our experiments, we utilized the F2 generation of three colorectal cancer PDXs (Figure 26 a, b and c) obtained from patients of National Cancer Institute Regina Elena in Roma. Before starting treatments with EMICORON we evaluated whether the three PDXs grown in mice retaining the same characteristics as the human tumors. The morphological stability of PDXs was investigated by histological analysis based on Haematoxylin and Eosin (H&E) staining and immunohistochemical analysis of CDX2 and CK-20 epithelial markers, specific for colon adenocarcinoma (Figure 25 d)

For histological analysis the tumor sections of about 4 μm were cut to the microtome, included in paraffin and stained with Haematoxylin and Eosin. This type of analysis is a two-color histological staining based on the different pH value of the various tissues and the various organelles that make up the cell (Fischer et al., 2008). Haematoxylin and Eosin stains are used for recognizing various tissue types and the morphologic changes occurring in tumor development. Haematoxylin is a plant substance isolated from a wood extract (Haematoxylum campechianum) with a deep blue-purple color that stains nucleic acids. The true colorant is Hematein, the product of oxidation, which is the chromophore, is anionic and has no particular affinity with nucleic acids. Eosin is a slightly acidic pink dye, bind proteins non-specifically and colors the cytoplasm, connective tissue and

intracellular substance.

In a typical tissue, the bichromic staining of the cells shows the nuclei and the various acid components of the cytoplasm (*e.g.* ribosomes) colored blue while the cytoplasm and the extracellular matrix exhibit varying degrees of pink coloring. The coloration with Haematoxylin and Eosin highlights that the tumors implanted in mice maintain the same morphological characteristics of those of the patients (Figures 25 d), confirming the morphological stability of the tumor.

Then, we analysed the expression levels of CDX2 and CK20 epithelial markers by immunohistochemical analysis (Kim et al., 2013).

CK-20 expression is almost entirely confined to the gastric and intestinal epithelium (Moll et al., 1992). Cytokeratin CK-20 is an acid cytokeratin generally associated with a basic cytokeratin such as CK-7 (Campbell et al., 2001). The groups of cytokeratins expressed by an epithelial cell depend mainly on the type of epithelium to which the cell belongs and from the stage of development and this classification is also valid for malignant tumors of epithelia (carcinomas) (Chu et al., 2000). Therefore, the study with immunohistochemical techniques of the expressed cytokeratins profile allows to identify the epithelium that has transformed into carcinoma and the consequent characterization of the type of tumor in surgical pathology (Walid et al., 2009).

CDX2 (caudal type homeobox transcription factor 2) is a nuclear homeobox transcription factor that belongs to the caudal-related family of *CDX* homeobox genes. The gene encoding *CDX2* is a nonclustered hexapeptide located on chromosome 13q12-13 with a crucial role for axial patterning of the alimentary tract during embryonic development and is involved in the processes of intestinal cell proliferation, differentiation, adhesion, and apoptosis (Silberg et al., 2002). CDX2 is considered specific for enterocytes and has been used for the diagnosis of primary and metastatic colorectal adenocarcinoma. Its expression has been reported to be organ specific and is normally expressed throughout embryonic and postnatal life within the nuclei of epithelial cells of the alimentary tract from the proximal duodenum to the distal rectum (Werling et al., 2003; Reda et al., 2011). In transgenic mice in which the expression of CDX2 in the gastric mucosa is induced, the appearance of intestinal metaplasia in the gastric epithelium is further evidence of the key role of CDX2 in intestinal differentiation. CDX-2 is therefore a marker of neoplasia of colorectal-intestinal origin (Sen et al., 2015). Analysis of the expression levels of CK-20 and CDX2 markers (Figure 25 d) shows that the tumor grown in animals maintains the characteristics of undifferentiated adenocarcinoma observed in the patient.

Furthermore, Next Generation Sequencing analysis (NGS) confirmed in all PDXs the identical mutation in the *KRAS* gene of the patient's tumor (data not shown). NGS is used for biological and medical research emerged as a standard technology for multiple high-throughput molecular profiling assays. Among these are transcriptome sequencing (RNA-Seq), whole-genome and whole-exome sequencing (WGS/WXS) for instance for genome-wide association studies (GWAS), chromatin immunoprecipitation or methylated DNA immunoprecipitation followed by sequencing (ChIP-Seq or MeDIP-Seq), as well as a multitude of more specialized protocols such as CLIP-Seq, ATAC-Seq, or FAIRE-Seq (Kulkarni et al., 2017). In cancer research NGS broadly describes those technologies that share the ability to massively parallel sequence millions of DNA templates in order to individuate the mutations in tumor genome in a tumor-specific panel representing the most mutated genes such as *KRAS* in colorectal cancer (Meldrum et al., 2011).

In agreement with the individual variability of the mice in terms of tumor appearance, we decided to consider each mouse as a different patient and start treatment when each tumor reached the volume of 250-300 mm^3 and follow individually the responses to the treatment. Once we verified that the PDXs had preserved the morphological characteristics with respect to the patients' tumors, we started the treatments with the EMICORON. Animals were treated orally by gavage with EMICORON 15mg/Kg/day for seven consecutive days. As shown in Figure 26 e, at the nadir of effect, EMICORON showed a marked antitumor activity in particular in PDX1, with a mean inhibition of about 60% in comparison to control tumors. In the others PDX models we observed a strong but lower decrease in tumor volume. In particular, in PDX2 the inhibition of tumor growth was about 50%, while, interestingly, PDX3 was less responsive to treatment. In particular, one mouse showed complete response, two mice partial response followed by a rapid disease progression and no-response was elicited in one mouse (Figure 26). This result may reflect the selection of EMICORON resistant fraction during mouse-to-mouse passages and suggest to investigate the changes in gene expression after treatment to predict drivers of tumor resistance to G-quadruplex ligands.

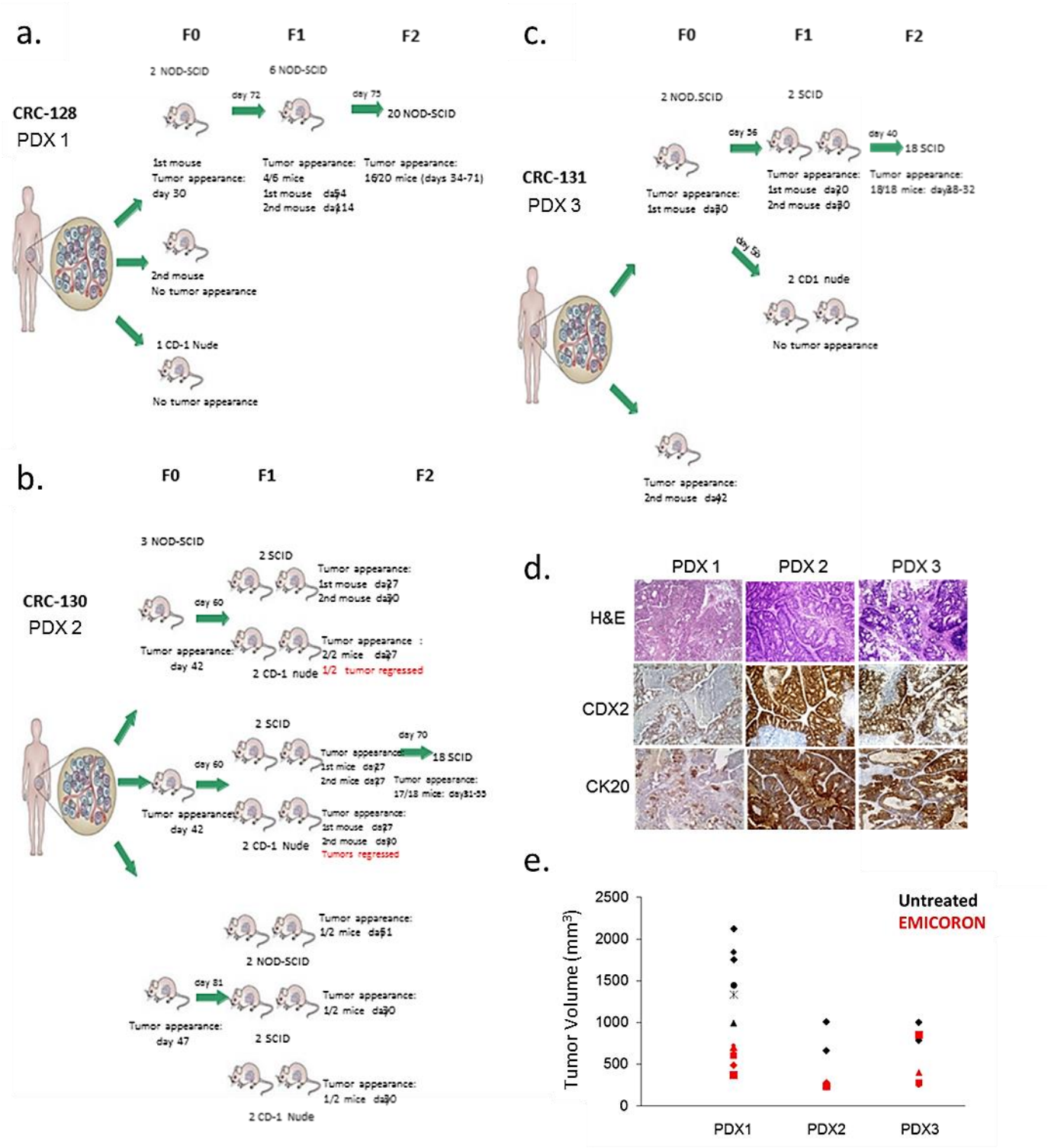


Figure 25. Take rate obtained by the implantation of the three tumor fragments from patients with colorectal cancer in different strains of immunodeficient mice (a, b and c). d) Morphological analysis of the three PDXs after staining with Haematoxylin - Eosin and the CDX2 and CK20 markers. e) Antitumor effect of EMICORON treatment on three PDXs models. Data are representative of the maximum effect.

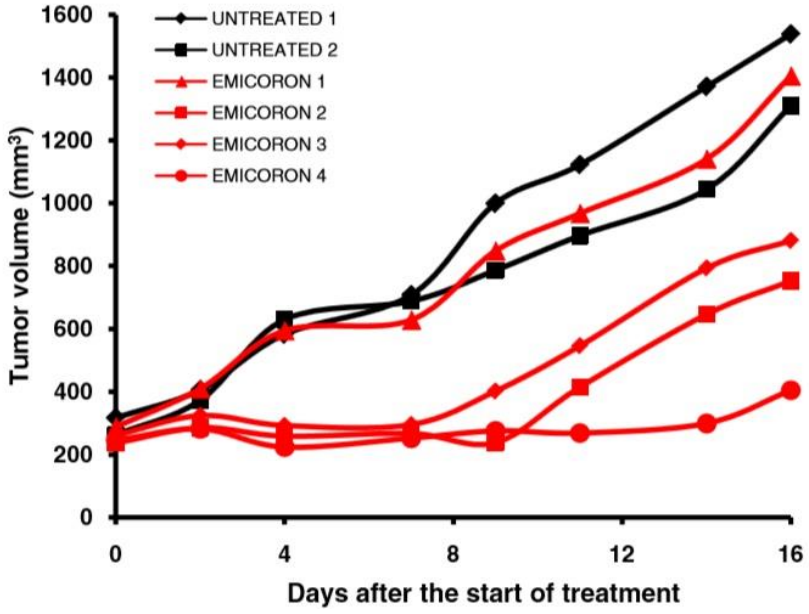


Figure 26. NOD-SCID mice at passage F2 were treated with EMICORON *per os* at 15mg/day for 15 consecutive days, starting with a tumor volume of 300 mm^3 was evident. Curves represent untreated (black) or EMICORON-treated mice (red).

EMICORON potentiates the activity of the FOLFIRI regimen

The efficacy of EMICORON demonstrated in combination with Irinotecan in the xenograft model of colorectal cancer and in the three PDXs models suggested us to verify the activity of the EMICORON in combination with the treatment regimen FOLFIRI in one of the three PDXs models developed, in particular the CRC131.

FOLFIRI, one of the common chemotherapy regimens used in the treatment of advanced-stage and metastatic colorectal cancer, became the standard first-line chemotherapy for colorectal cancer in the U.S. and Europe in 2000. This clinical treatment consists of every two weeks cycle of Irinotecan (180mg/m² IV over 90 minutes) concurrently with Leucovorin calcium (calcium folinate), a vitamin B derivative that increases the cytotoxicity of 5-Fluorouracil, (400mg/m² [or 2 x 250mg/m²] intravenous over 120 minutes) followed by two types of 5-Fluorouracil administration (400–500 mg/m² intravenous bolus and then 2400–3000mg/m² intravenous infusion over 46 hours).

For these experiments we proceeded as before: once verified the same morphology of PDXs respect to the patients' tumors, we started the treatments in two group of mice with FOLFIRI and FOLFIRI -> EMICORON at the same time we started the treatment with EMICORON in the first group of mice when each tumor reached the volume of 250-300 mm^3 and follow individually the responses to the treatment, and in agreement with the individual variability of the mice in terms of tumor appearance, we decided to consider each mouse as a different patient. One group of mice followed FOLFIRI regimen and were treated with Irinotecan, given intraperitoneally at 15mg/kg/day for 5 consecutive days, followed by 5-Fluorouracil (19mg/kg/day) given intraperitoneally concurrently with Leucovorin (20mg/kg/day) for 5 consecutive days. The group under combination treatment was treated with FOLFIRI regimen and after two days of interruption with EMICORON, given by oral gavage at 15mg/kg/day for 7 consecutive days.

During the first 10 days after the end of the treatments we measured the tumor volume to calculate the fold increase compared to the mean of controls (Figure 27). In the FOLFIRI treated group, after three weeks of the end of treatment we observed in 3/5 mice the stabilization of the disease and in 1/5 the complete tumor regression (Table 3). In the group treated with the combination FOLFIRI plus EMICORON during the first 10 days of treatment only one tumor restarted to grow, while in the following two weeks we observed in 2/5 mice the stabilization of the disease and in 3/5 mice a complete tumor regression. In the tumors treated with EMICORON, after three weeks of the end of treatment, we observed in 4/5 mice the progression of the disease. These results suggest that the EMICORON potentiates the antitumor efficacy of the standard chemotherapy used for clinical treatment of colon cancer.

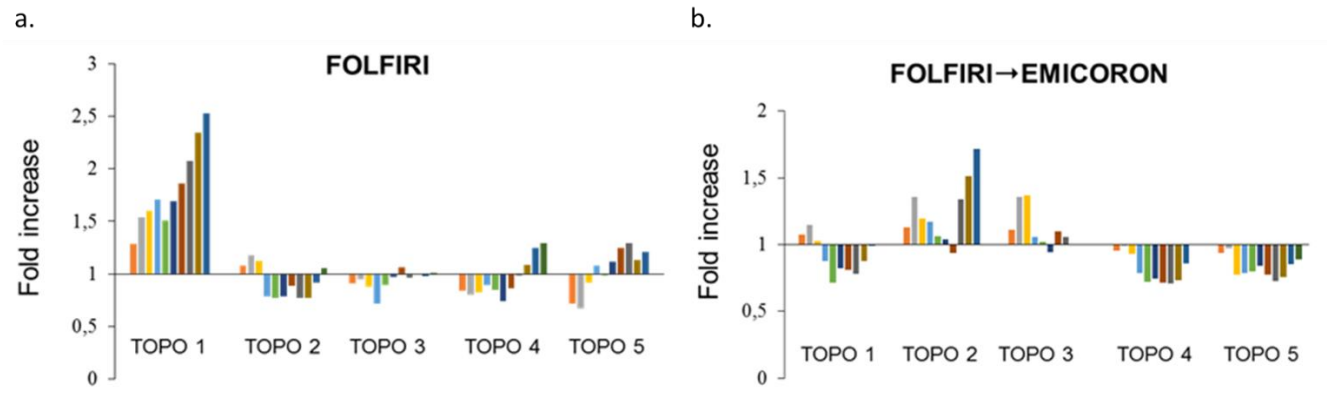


Figure 27. Histograms related to the treatment with the FOLFIRI regimen alone and in combination with EMICORON. a) Mice treated with FOLFIRI regimen (Irinotecan -> 5'-Fluorouracil + Leucovorin). b) Mice were treated with the combination of FOLFIRI regimen plus EMICORON. The data represent the fold increase of tumor volume compared to the mean of controls in the first 10 days after the end of treatment.

Table 3. Therapeutic efficacy of FOLFIRI regimen in comparison with FOLFIRI plus EMICORON in CRC-131 PDX model.

Treatment groups	Stable disease∞/ mice treated	Complete response♣/ mice treated	Progressionφ/ mice treated
EMICORON	1/5	0/5	4/5
FOLFIRI	3/5	1/5	1/5
FOLFIRI plus EMICORON	2/5	3/5	0/5

∞Stable disease was defined as the maintenance for at least three weeks of the same tumor volume as the start of treatment. ♣Complete response was defined as the disappearance of tumor as evaluated by palpability, for at least three weeks during the treatment. φ Progression was defined as the constant growth of tumor evaluated by tumor calibration during the three weeks in the course of treatment.

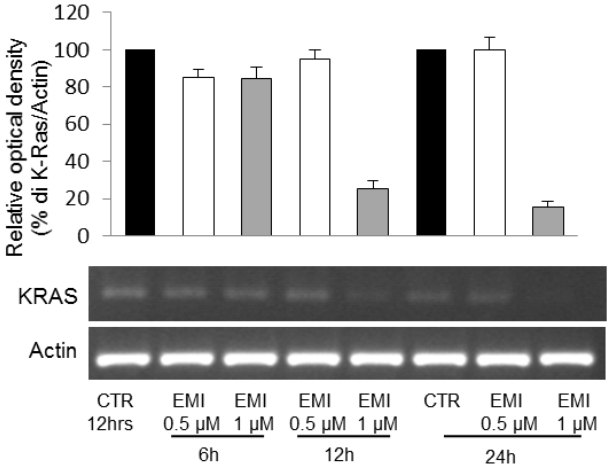
EMICORON downregulates the expression of *KRAS* oncogene

Based on evidence that the *KRAS* promoter contains guanine-rich sequences capable of forming G-quadruplex structures and on the evidence that has already shown, as previously described, that G-quadruplex ligands can bind *KRAS* and downregulate it, we conducted an *in vitro* experiment on HCT116 colon cancer cells in order to verify the downregulation of the transcription and/or translation of *KRAS* gene.

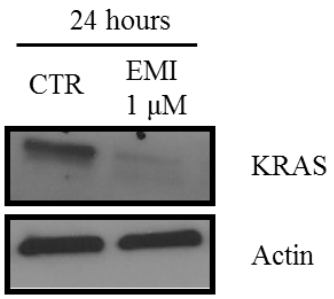
HCT116 cells were placed in 60 mm plates, treated with EMICORON at 0.5 and 1μM and harvested at 6, 12 or 24 hours and, after extracting total RNA and reverse-transcribing, a Quantitative Real Time PCR assay was performed. The qRT-PCR products show *KRAS* downregulation at 12 and 24 hours of treatment in particular with EMICORON at 1μM (Figure 28 a). Then we assessed the expression of the protein in Western Blot. The cells were treated with EMICORON at 1μM and as shown in Figure 28 b there was also a strong downregulation of the protein, confirming the downregulation of the gene transcription.

To test the effect, for a longer time, of EMICORON in *KRAS* expression, experiments of qPCR Real Time was conducted. As shown in the histogram (Figure 28 c), the downregulation of the transcription at 24 hours of treatment was confirmed with about 30% of inhibition, and at 48 hours with about 40% of inhibition, while at 72 hours of treatment the gene transcription is resumed. This data supports even more the effectiveness of EMICORON to bind G-quadruplex structures in *KRAS* oncogene.

a.



b.



c.

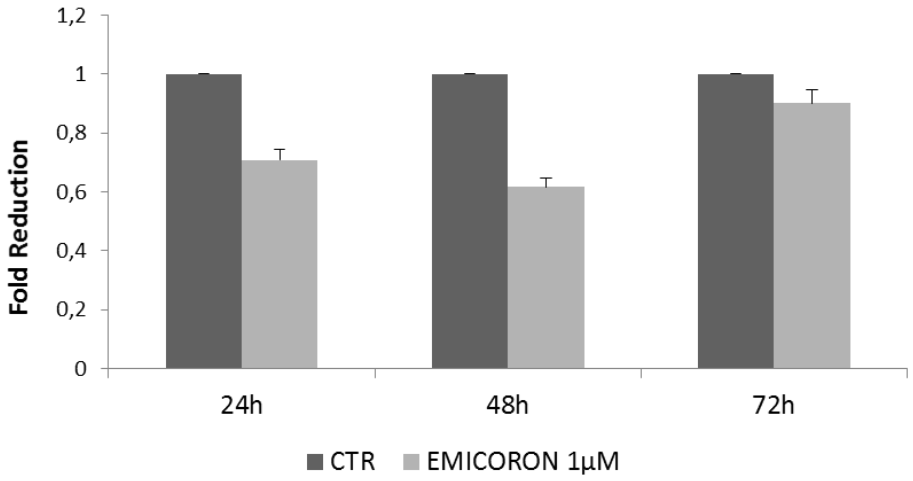


Figure 28. EMICORON effects on the expression of *KRAS* in HCT116 colon cancer cells. a) qRT-PCR products were run in TAE1X agarose 1% gel and the relative optical densities of *KRAS* are represented by histogram. A representative picture of PCR products is shown. Densitometry was performed with ImageJ software version 1.40. b) KRAS protein levels were detected by Western blot at 24 hours of treatment. c) Histogram shows EMICORON effects at 24, 48 and 72 hours on *KRAS* expression analysed by qPCR Real Time. Histograms show the mean values ± SD.

Discussion

The use of advanced preclinical tumor models allows to generate the growth conditions closest to the clinical situation and to study the antitumor efficacy of new drugs or new therapeutic combinations. In this doctoral project has been studied the antitumor efficacy of the G-quadruplex ligands EMICORON on three-dimensional *in vitro* models, xenograft models and PDXs models, the more advanced mouse model. First, we confirmed the ability of EMICORON to potentiate the activity of the chemotherapeutic SN-38, active metabolite of Irinotecan, in tumor spheroids of a stabilized tumor cell line. This three-dimensional model is the *in vitro* model with features closer to human solid tumors. The SN38-> EMICORON combination showed a synergistic effect, confirming the *in vitro* data obtained on 2D models. To investigate this effect in *in vivo* study, the mice were injected with a stabilized colon cancer cell line. In this model we combined EMICORON with Irinotecan, a drug commonly used in the clinic for the treatment of colorectal cancer. The results obtained show that EMICORON potentiates the activity of the chemotherapy with a strong antitumor efficacy.

In order to study the efficacy of EMICORON in an experimental model predictive of clinical response in humans, we tested its antitumor efficacy in PDXs models of colorectal cancer. The PDXs, which consist of implanting a patient-derived tumor fragment directly into immunosuppressed mice, are the living models closest to the clinic because they retain the same characteristics of the tumor as resistance/sensitivity to drugs, genetic, molecular and histological heterogeneity of the patient's tumor also through several passages of tumor mouse-to-mouse. These characteristics cannot be found instead in the classic stabilized cell lines that *in vivo* adapt to grow outside their natural tumor microenvironment and this involves morphological and genetic changes. In the project we investigated the efficacy of EMICORON in three PDXs that retained the same characteristics of the tumors of origin. The results obtained demonstrate that the single treatment with EMICORON exhibits a marked antitumor activity even if, as the results obtained by the treatment of PDX3 demonstrated, EMICORON can meet the phenomenon of drug resistance, thus highlighting that PDXs represent a useful model in studying intratumor heterogeneity which is one of the main reasons for the failure of treatment and disease progression. However, the combination with the FOLFIRI regimen on the same PDXs model compared to individual treatments has shown to have a better antitumor efficacy, as evidenced by the marked inhibition of tumor growth, by the stabilization of the disease and by the significant improvement of the survival.

Patient with metastatic colon cancer which carrying *KRAS* mutation, which is present in about 45% of colon cancer patient, do not benefit of therapy with the epidermal growth factor receptor monoclonal antibodies (EGFR). Since all PDXs models evaluated carried the *KRAS* mutation, we decided to verify *in vitro* the ability of the EMICORON to downregulate the expression of this oncogene. The results obtained show that EMICORON is able to inhibit the expression of the oncoprotein by stabilizing the G-quadruplex structures present in the gene promoter, suggesting this mechanism at the basis of the antitumor efficacy shown in the treatment of mutated *KRAS* tumors and suggesting the adoption of EMICORON for the treatment of EGFR inhibitor-resistant tumors. However, despite the marked antitumor efficacy shown in the more advanced in *in vitro* and *in vivo* models and the likely selectivity in mutated *KRAS* tumors, these results provide a compelling argument that the integration of EMICORON in standard chemotherapeutic regimens could be a highly valuable strategy for the treatment of human colon cancer adopting the G-quadruplex ligand in combinations with the conventional treatment regimens. In fact, we do not suggest the use of EMICORON as monotherapy but rather as a new possible molecular target drug to be associated with the standard chemotherapeutics, particularly in presence of resistance to the most commonly drugs used for clinical management of colon cancer patients.

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Abbreviations

ALT: Alternative Lenghtening of Telomeres

APC: Adenomatous Polyposis Coli

BRAF: v-Raf Murine Sarcoma Viral Oncogene Homolog B

BRCA: Breast Related Cancer Antigens

CI: Combination Index

CIMP: CpG Island Methylator Phenotype

CIN: Chromosomal Instability

CTLA-4: Cytotoxic T-Lymphocyte Antigen 4

DSB: Double strand breaks

EGFR: Epidermal growth factor receptor

EMI: EMICORON

EMT: Epithelial-Mesenchymal Transition

FAP: Familial Adenomatous Polyposis

GAP: GTP-ase Activating Proteins

GTP/GDP: Guanosine Tri/Di-phosphate

HAT: Histone acetyltransferase

HDACs: Histone deacetylase

HNPCC: Hereditary Non-Polyposis Colorectal Cancer

hTERT: Human Telomerase Reverse Transcriptase

IC: Inhibitory Concentration

IRN: Irinotecan

KRAS: Kirsten's Sarcoma in the rat

mCRC: Metastatic Stage of Colorectal Cancer (mCRC)

MCTs: Multicellular Tumor Spheroids

MSI: Microsatellite Instability

NGS: Next Generation Sequencing Analysis

NHPPE: Nuclease Hypersensitive Polypurine–Polypyrimidine Element

PD-L1: Programmed Death-Ligand 1

PDXs: Patient Derived Xenografts

POT1: Protection of Telomere Protein 1

PQS: Putative Quadruplex Sequences

Rb: Retinoblastoma

RHPS4: pentacyclic acridine RHPS4 (3,11-difluoro-6,8,13-trimethyl-8H-quino[4,3,2-kl]acridinium methosulfate)

TIF: Telomere Induced Foci

TNM: Tumor-Node-Metastasis

TRF1: Telomere Repeat Factor 1

TRF2: Telomere Repeat Factor 2

VEGFR: Vascular Endothelial Growth Factor Receptor