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Biological activity of anti-miR-221 Peptide  
Nucleic Acids and relative building blocks

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## Preface

The study of new molecules able to selectively and stably interact with DNA and RNA is a field of great interest in consideration all the possible applications in medicine. In the last years, many studies have been conducted aimed to the explanation of epigenetic modifications (DNA methylation, histone modification and expression of non-coding RNA molecules, as microRNA) important in the mechanisms of gene regulation; in fact this is one of the main steps for understanding the biology in both human conditions, normal and pathological, and for the development of new bioactive molecules. RNA has been the preferred target of long-standing studies aimed at the discovery of molecules able to block gene expression in a sequence selective way.

During the past few years, molecular biologists have discovered hundreds of gene sequences that encode small RNA molecules, the microRNAs (miRNAs), 21 to 25 nucleotides in length, which are involved in the post-transcriptional regulation of gene expression. Indeed, there are hints that the level of some miRNAs is altered in cancer and in other pathologies; for instance, miRNAs regulate cancer-promoting genes, playing a pivotal role in cancer onset and progression. Briefly, microRNAs recognizing a target sequence in the 3'UTR of the mRNA target, and based on the partial or total complementarity of the sequences, they bind to mRNA leading to the inhibition of translation or degradation of the mRNA, respectively. Furthermore, in the pathogenesis of tumors, miRs play a double role; in fact, if it interferes with an mRNA coding for a tumor-suppressor protein, it become "oncogenes", applying itself as a target for anticancer therapy.

Synthetic oligonucleotides or analogues acting as competitors by binding to miRNA have been proposed as novel potential drugs. Among the most efficient molecules proposed for these applications are the PNAs. Peptide nucleic acids (PNAs) are oligonucleotide analogues with a polyamide backbone and are very promising tools for binding RNA, since they have a higher affinity for RNA than DNA, are stable to nucleases and are very specific. Their use as therapeutic agents have been proposed since early studies and recent advancements in cellular delivery systems; anti-gene and antisense strategies make them good candidates for drug development.

In this contest, we have selected the miR-221 as target for the ours novel engineered PNAs; miR-221 have been shown to be overexpressed in many different types of tumor, as glioblastoma, hepatocarcinoma, prostate and breast cancer; moreover, it had been shown to be associated to the suppressor of p27<sup>kip1</sup>, a cell cycle inhibitor and tumor suppressor protein. In fact, it had been found that in the 3'UTR of p27<sup>kip1</sup> mRNA there are at least two binding sites for miR-221.

The major limit of the PNAs technology is the low cellular uptake, in particular on eukaryotic cells; in order to solve this problem, several approaches have been proposed; our PNAs presents modifications of the backbone with positive charged groups; in fact it has been demonstrated that these modifications are able to enhance cellular uptake and consequently PNA efficiency. Another modification that we have insert in the PNAs employed in our experiments is to link to PNAs a polyarginine (R) tails, based on the observation that this cell-membrane penetrating oligopeptides are able to facilitate uptake of conjugated molecules.

The anti-miR-221 PNAs (PNA-a221 and Rpep-PNA-a221) proposed in this PhD thesis were synthetized by Prof. Corradini, Prof. Marchelli and collaborators at University of Parma, Department of Organic and Industrial Chemistry. They first designed the synthesis of new class of uracil dimers by substitution of amide linker on C(5) with more flexible methylamine linker, obtaining the 5-methylamino uracil. This reaction intermediate had been used for the synthesis of the 5-methylazidouracil PNA monomer. The R8 peptide was linked to the N-term of PNA, following the synthesis on solid phase.

The results presented in this PhD Thesis clearly show that: (a) the polyarginine-PNA conjugated anti-miR-221 is efficient internalized in the target cell lines without any transfection reagents; (b) targeting miR-221 by PNA resulted in a lowering of level of miR-221 and up-regulation of p27<sup>kip1</sup> mRNA and protein in breast cancer MDA-MB-231 cell line. On the other hand, protocols of medicinal chemistry finalized to the design and the production of the final form of bioactive molecules, generate intermediates during chemical synthesis that, at least in theory, might retain effects on biological function. Therefore, in order to maximize the production of bioactive agents, all the synthetic intermediates deserve attention at least in a first screening.

In our case, the uracil dimers derivatives, employed during the synthesis of PNA-anti-miR-221 were considered; in addition we also considered the C(5) uracil

modified monomers, used as starting molecules for the synthesis of dimers. As a first explorative investigation, we analyzed the possible antitumor activity of their analogs, possibly associated, as found in several other antitumor agents, with activation of terminal erythroid differentiation. We have first evaluated the antiproliferative and induction of erythroid differentiation activities of dimers; our results are published in Accetta *et al.* (2009) and briefly described in the *Introduction Part 1* of this PhD Thesis. We have demonstrated for the first time that this kind of uracil derivatives can be considered to be a new class of erythroid differentiation inducers.

Following this initial results, we have analyzed also the C(5) uracil derivative monomers; we present *the Results Part 1* of this thesis. We have focused our attention in particular on Compound 9, which between all, has proved to be a potent erythroid inducer on K562 cell line. In this respect, it's widely demonstrated that inducers of K562 erythroid differentiation are often in erythroid cell isolated from beta-thalassemia patients.

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PART 1

*URACIL DERIVATIVES SHOWING  
ANTIPROLIFERATIVE AND ERYTHROID DIFFERENTIATION*



## - INTRODUCTION -

### 1.1 Pyrimidine

Pyrimidine is a heterocyclic aromatic organic compound similar to benzene and pyridine, containing two nitrogen atoms at positions 1 and 3 of the six-member ring, as shows in Figure 1 (Gilchrist et al., 1997). Three nucleobases found in nucleic acids, cytosine (C), thymine (T), and uracil (U), are pyrimidine derivatives. In DNA and RNA, these bases form hydrogen bonds with their complementary purines. Thus, in DNA, the purines adenine (A) and guanine (G) pair up with the pyrimidines thymine (T) and cytosine (C), respectively.

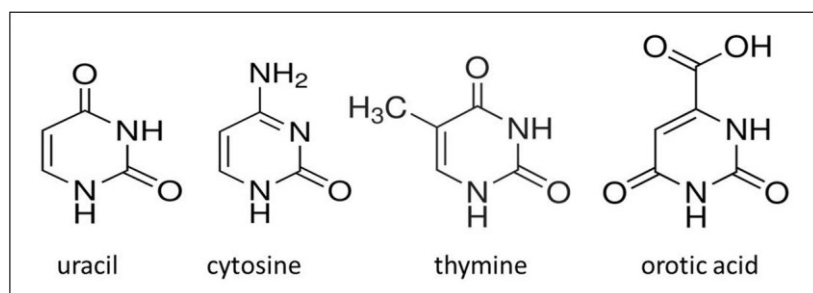


Figure 1. Chemical structure of natural pyrimidine

The pyrimidine system is a very important pharmacophore core of naturally occurring and synthetic bioactive compounds (Lagoja et al., 2005); the potential of pyrimidine compounds is linked to the possibility of being used as antagonists in the biosynthetic pathways of pyrimidine nucleobases or in other important processes, by competing for the same binding sites of naturally occurring compounds.

#### 1.1.1 Biological activity of pyrimidine derivatives

A series of modified pyrimidines were and are currently used as drugs. For example, fluoropyrimidine drugs such as 5-fluorouracil (5-FU) and capecitabine are a mainstay in the treatment of numerous solid tumors, alone or as part of combination

therapies. Despite the high interpatient pharmacokinetic variability and the cytotoxic effect, 5-FU is currently used in cancer therapeutics, in particular for colorectal and gastric cancer (Mercier et al., 2006).

Azidothymidine (AZT) was the first applied drug for HIV treatment. AZT is phosphorylated by intracellular kinases to AZT-5'-triphosphate (AZT-5'-3P), which is the active metabolite. AZT-5'-3P is able to interfere with the RNA-dependent-DNA-polymerase (reverse transcriptase), acts as viral inhibitor. It can also be incorporated into the growing viral DNA, acting as a terminator signal.

Another example, bacimethrin (4-amino-5-hydroxymethyl-2-methoxypyrimidine) is known as the simplest pyrimidine antibiotics, acting as antagonist on bacterial thiamine biosynthesis (Reddick et al., 2001).

In literature it is also reported that uracil derivatives are potentially biologically active compounds. For examples, oxime libraries based on dimeric uracil derivatives have been proposed for the development of uracil DNA glycosylase (UNG) inhibitors (Jiang et al., 2005). As described by Maruyama *et al.*, 1,3-disubstituted uracil derivatives have shown an antiviral activity against HIV-1 and human cytomegalovirus (HCMV), probably interacting with the amino acid residues of HIV-1 reverse transcriptase (Maruyama et al., 2007).

Another class of molecules, obtained by Isobe *et al.*, the N(1)- and N(3)-uracil derivatives, have shown anti-inflammatory activities via inhibition of the picryl chloride-induced contact hypersensitivity reaction (CHR) in mice. These activities were essentially equipotent with that of tacrolimus, a strong immunosuppressant. (Isobe et al., 2003). Another pyrimidine analogs, 1-Benzyl derivatives of 5-(arylamino)-uracil, exhibit promising inhibitory activity against HIV-1 in CEM-SS culture, and activity anti-EBV in AKATA cell culture. (Mikhail et al., 2010).

### 1.1.2 C(5)-substituted uracil derivatives

The pyrimidine bases of RNA are uracil (U) and cytosine (C), while thymine (T) and C are used for DNA. The C(5) position of C and U is unsubstituted, whereas the C(5) of T is substituted with a Me group. Miller *et al.* hypothesized that various C(5)-substituted uracil derivatives were formed during chemical evolution, and that C(5)-substituted U derivatives may have played important roles in the transition from an

“RNA world” to a “DNA-RNA-protein world” (Robertson et al., 1995). Several C(5) uracil derivatives have been described as cytostatic and antiviral compounds; Gazivoda *et al.*, have evaluated the antitumoral and antiviral activities of a new class of C(5) aryl, alkenyl, and alkynyl substituted uracil derivatives. In particular the 5-(phenylethynyl)uracil-2,3-di-O-benzylated l-ascorbic acid derivative have shown selective inhibitory effect toward all tumor cell lines except for cervical carcinoma (HeLa), pancreatic carcinoma (MiaPaCa-2), laryngeal carcinoma (Hep-2), and colon carcinoma (SW 620), and no cytotoxicity to normal human fibroblast (WI 38); while 5-propynyl substituted uracil derivative of l-ascorbic acid have shown inhibitory activity against vesicular stomatitis virus, Coxsackie B4 virus and Sindbis viruses (Gazivoda et al., 2007).

Following our general project, aimed at the synthesis of oligonucleotide analogues, in particular PNA, with modifications able to improve their binding activity (Sforza et al., 2007; Corradini et al., 2007. Corradini, Marchelli and collaborators have designed some uracil dimers, using C(5) modified uracil derivatives, connected with a spacer through the 5-positions (Figure 2).

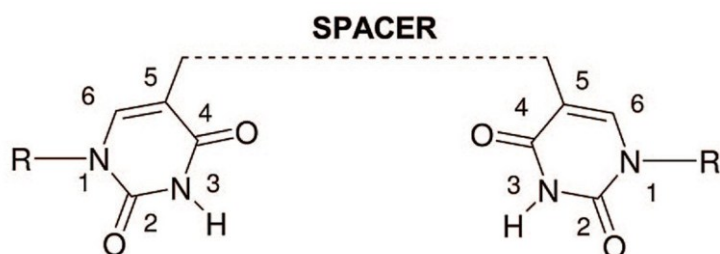


Figure 2. General scheme of uracil dimers

### 1.1.3 Synthesis of 5-carboxamido uracil dimers

N1-Alkylated uracil derivatives were synthesized from isoorotic acid (uracil-5-carboxylic acid) or thymine. The carboxylic acid derivatives were condensed with diamines in order to produce dimeric compounds or with monoamines in order to obtain reference monomeric compounds. Some of the derivatives, in particular the uracil dimers, were the first to be tested in our laboratories for the erythroid differentiation activity. In the retrosynthetic design, Prof. Corradini and his

collaborators have used as starting material either isoorotic acid (5-uracilcarboxylic acid) or thymine. As a reference compound, isoorotic acid methyl ester was synthesized by reaction with  $\text{SOCl}_2$  in methanol. Several derivatives were obtained with the purpose of varying the group at N1, the type (monomeric or dimeric) of the amine residue and the size and rigidity of the spacer, in order to produce chemical diversity. Direct alkylation of isoorotic acid with reactive substrates, such as methyl or allyl halides lead to dialkylated products at both nitrogen atoms; therefore, regioselective mono-alkylation of uracil was performed using temporary protection of the carboxylic and carbonyl oxygens with trimethylsilyl groups, through reaction with hexamethyldisilazane (HMDS) in a 3:1 excess and in the presence of trimethylchlorosilane (TMS-Cl), as shown in Figure 3.

Reaction of thymine with more hindered long chain primary haloalkanes lead to selective monoalkylation at N(1) (Figure 4), thus affording the substrate 4 suitable for the synthesis of uracil derivatives with a C8 alkyl chain at N(1). Oxidation of methyl group of 4 with  $\text{K}_2\text{S}_2\text{O}_8$  in the presence of copper(II) leads to the uracil-5-carboxaldehyde which was then oxidized by reaction with sodium chlorite to the corresponding carboxylic acid 5 bearing a C8 alkyl chain at N1 position.

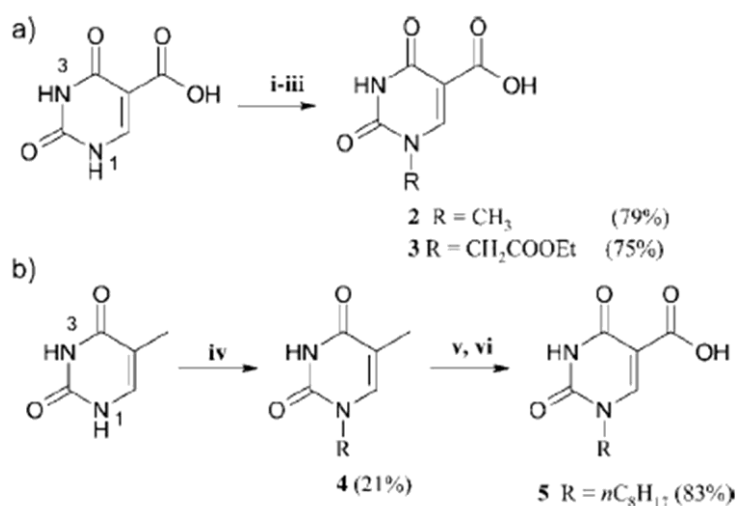


Figure 3. Synthesis of N1-alkylated isoorotic acid derivatives (a) via regioselective alkylation of Isoorotic acid ((i) HMDS, TMS-Cl, reflux 4 h; (ii)  $\text{CH}_3\text{I}$  or ethyl bromoacetate in excess, reflux, 18 h; (iii)  $\text{H}_2\text{O}/\text{CH}_3\text{COOH}$ , room temp, 20 min.) and (b) via oxidation of thymine derivatives ((iv)  $[\text{Br}-n\text{-C}_8\text{H}_{17}, \text{NaH}, \text{DMF}, 80\text{ }^\circ\text{C}, 4\text{ h}]$ ; (v) 2,6-lutidine,  $\text{K}_2\text{S}_2\text{O}_8$ ,  $\text{CuSO}_4$ ,  $\text{H}_2\text{O}/\text{AcCN}$ ,  $80\text{ }^\circ\text{C}$ , 1.5 h; (vi)  $\text{NaClO}_2$ ,  $\text{NaH}_2\text{PO}_4$ ; t-BuOH/THF, r.t., 24 h)

Several other commercially available diamines were used as linkers for the reaction with the carboxylic derivatives. Butylamine was used for generating reference monomeric compound **10** (Figure 4), and benzylamine was used for generating compound **12**, both containing a uracil moiety with a carboxamide group at C(5) (Figure 4). The first series of derivatives, containing either a methyl or an ethoxycarbonylmethyl group at N1, was synthesized using reaction with thionyl chloride to generate the corresponding acyl chloride, followed by reaction with the corresponding amine in pyridine (Figure 4a). Since the yields obtained with this method were not optimal (25-30%), mainly because of loss of product during workup, a second series of derivatives was obtained by activation of the carboxylic moiety of **5** (Figure 3) with fluoride using 2,4,6-trifluoro-1,3,5-triazine as fluorinating agent, thus providing the stable intermediate **11** which could be isolated. Subsequent reaction of the acyl fluoride **11** with the corresponding diamine or monoamine in acetonitrile gave the compounds **12-17** (Figure 4b).

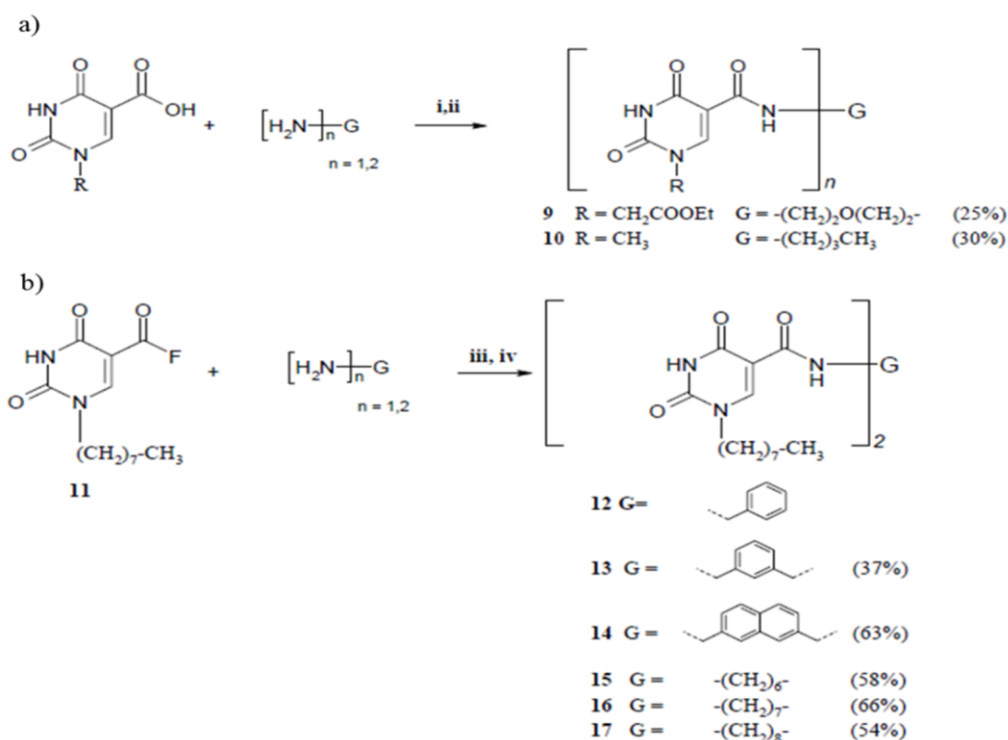


Figure 4. Synthesis of monomeric and dimeric uracil derivatives: (a) **12** and **13** via acyl chloride ((i) SOCl<sub>2</sub>, DMF, 70-80 °C, 2 h; (ii) (H<sub>2</sub>N)<sub>n</sub>-G, Py, 2 h); (b) **19-23** via acyl fluoride ((iii) H<sub>2</sub>N-G-NH<sub>2</sub> or H<sub>2</sub>N-G-NH<sub>2</sub> · 2HCl, DIEA, AcCN, 80 °C, 7 h; (iv) 1 M HCl, 0.5 h)



We have recently reported a novel class of C(5) linked N(1) alkylated uracil dimers rationally designed to potentially interact with adenine in biological systems: these pyrimidine derivatives have shown antiproliferative and erythroid differentiation activities toward human chronic myelogenous leukemia K562 cells (Accetta et al., 2009). Results are described in the following *Section 1.3*.

## **1.2 Terminal differentiation therapy of human cancer**

Cancer morbidity and mortality continue to be major problems worldwide, despite significant improvements and innovations in the diagnosis, prevention and therapy of specific cancers, delimiting longevity and the quality of human life. Although the precise reason for the lack of greater efficacy of current therapies is not known, it may relate to the fact that cancer is not caused by a single genetic change, but instead represents the combination of common and distinct multi-step changes in specific neoplasms.

Cancer is a process in which changes in regulating circuits are produced, such as proliferation control, the balance between cellular survival and programmed cellular death (apoptosis), the communication with neighboring cells and extracellular matrix, angiogenesis, and, finally, migration of the tumoral cell, invasion and metastatic dissemination. This process implies the progressive development of a more malign phenotype with an increase of genetic alteration involving genes at several levels of expression during long periods of time. These genetic changes uncouple the normal balance between multiplication and cellular differentiation with an increase in the rate of proliferating cells; it results in the formation of new abnormal cellular products which block final differentiation of cells leading to accumulation of immature cells (Sell et al., 2006). Moreover, the progeny of mutated cell is maintained in a self-renewing tissue stem cell and its immediate progeny or due to cellular components that display stem cell like properties known as *cancer stem cells* (CSCs), that grow into cancer. Failure to achieve complete and safe eradication of cancer is due to the presence of quiescent population of CSCs (Garg, 2009).

Once cancer is diagnosed, a variety of treatment options are considered, mostly depending on tumor type, extent, and location of the cancer lesion. The most

commonly used therapeutic modalities are radiation therapy and chemotherapy. These therapies have proven successful for some tumors; nevertheless, even if classic chemotherapeutical agents have been very important, the mechanism of action of these drugs depends on the cytodestruction of the neoplastic cells, and their beneficial effect are normally accompanied by a notable morbidity, cytotoxicity and drug resistance (both single and multidrug) (Marchel et al., 2006).

The knowledge of the mechanisms involved in differentiation and malignant transformation has allowed the search of alternative routes for anticancer therapy that does not imply cellular death. Example of these alternative routes of cancer therapy are radiation therapy, immunotherapy, differentiation therapy and angiogenesis inhibition therapy (Fisher et al., 1985, 1986; Rosen, 2000).

The objective of these multiple treatment protocols is to eliminate the problems associated with cancer cell resistance to a particular drug or class of drugs and to diminish toxicity associated with high-dose chemotherapy.

### **1.2.1 Cell differentiation**

Cancer begins when a normal cell, which replicates only slightly or not at all, suffers a somatic mutation in a gene and starts to proliferate uncontrollably. Everything indicates that several successive mutations are necessary to change a normal cell into invading carcinoma (Burnet M., 1957) and demonstrates the monoclonal origin of tumors (cellular clone is produced from mutated cell) in which each one of its components reproduces the mutations.

Differentiation program is a complex multistep developmental process of cell specialization that follows the *determination* (set of progressive restrictions in cell developmental potentials) of a genetic program specific for cell lineage. Development of the differentiation program includes the cellular type and that distinguish the specialized cells, such as muscle cells, nerve and skin. Terminal differentiation is the end stage of this process where the cells irreversibly lose their proliferative capacity and which represents a form of negative control of growing. Regulating molecules interact to produce the correct balance between cellular multiplication and differentiation during embryogenesis and the normal behavior of an adult.

In cancer, neoplastic cells exhibit defects in their ability to differentiate, and the development of these defects in the process of differentiation appears to be an intimate part of the transformation process. Compared with normal cells, tumor cells are less differentiated, which is one of their important biological characteristics. This “undifferentiated state” of tumors is the direct consequence of the “uncontrolled cell proliferation state”: cells are blocked in a stage in which they retain infinite proliferative capability. This hallmark has suggested a novel and potentially less toxic form of cancer therapy involves the use of agents, alone or in combination, that modify the state of differentiation and growth of cancer cells: the differentiation therapy (Fischer et al., 1985).

### **1.2.2 The differentiation therapy**

Because normal terminal differentiation often results in non-proliferating cells that often undergo apoptosis as they complete their normal life span, it was plausible to develop strategies to activate normal pathways of differentiation in premalignant and malignant cells using physiological or pharmacological agents that can bypass the epigenetic and genetic abnormalities that abrogate differentiation. This approach is called *differentiation therapy*.

Differentiation therapy is based on the concept that cancer cells are normal cells that have been arrested at an immature or less differentiated state, lack the ability to control their own growth, and so multiply at an abnormal fast rate. Differentiation therapy aims to force the cancer cell to resume the process of maturation, so as to prevent, suppress or reverse the malignant phenotype by inducing differentiation with the associated growth arrest, senescence and apoptosis (Figure 5). Although differentiation therapy does not destroy the cancer cell, it restrains their growth and allows the application of more conventional therapy (such as chemotherapy) to eradicate the malignant cells.

Even if only partially successful, differentiation therapy can convert malignant tumors into benign tumors. The mechanism by which differentiation inducing agents cause phenotypic changes in tumor cells is believed to involve the selective activation of defined sets of genes that negatively control cell proliferation and the suppression of genes facilitating expression of the cancer state (Jiang et al., 1994).

By defining the spectrum of genes that are modified as a consequence of induction or irreversible growth arrest, terminal cell differentiation and loss of tumorigenic potential, it should be possible to identify potentially important target genes and molecules for therapeutic intervention in cancer.

The first differentiation agent found to be successful was all-trans-retinoic acid (ATRA) in the treatment of acute promyelocytic leukemia (APL); APL, a distinct subtype of acute myelogenous leukemia (AML), results from the arrest of the maturation of hematopoietic progenitors at the promyelocyte stage. It has been shown that APL is associated with a reciprocal chromosomal translocation, involving chromosomes 15 and 17, which fuses the gene encoding the retinoic acid receptor  $\alpha$  (RAR $\alpha$ ) and the promyelocytic leukemia (PML) gene. The introduction of all-trans retinoic acid (ATRA) in 1987 changed the treatment paradigm of APL (Huang et al., 1988). Huang and collaborators demonstrated for the first time a complete remission of APL following ATRA treatment. Several studies conducted during the early 1990s found that APL patients receiving induction therapy consisting of ATRA followed by chemotherapy fared significantly better than patients treated with chemotherapy alone.

In addition, ATRA therapy has significant and potentially fatal adverse effects, known as retinoic acid syndrome, which consists of elevated white blood cell (WBC) counts, fever, respiratory distress, interstitial pulmonary infiltration, pleural effusion, and weight gain. Among patients in remission following treatment with ATRA alone, the incidence of retinoic acid syndrome is approximately 25%, (Vahdat et al., 1994) whereas that in patients treated with a combination of ATRA plus chemotherapy is reduced to 5%. (Sanz et al., 1999). Based on the results of these studies, the combination of ATRA plus chemotherapy became the standard approach for treating newly diagnosed APL. Most APL patients are actually treated first with ATRA; this compound induces a complete remission in about 70% of cases. ATRA is considered the “prototype” of differentiation therapy agents.

Another example of terminal differentiation induction regarding the therapy of melanoma. Cutaneous malignant melanoma is the fifth most common cancer in the United States and it is also the most frequent cause of death from malignancy in young Caucasian females (Meier et al., 1998).

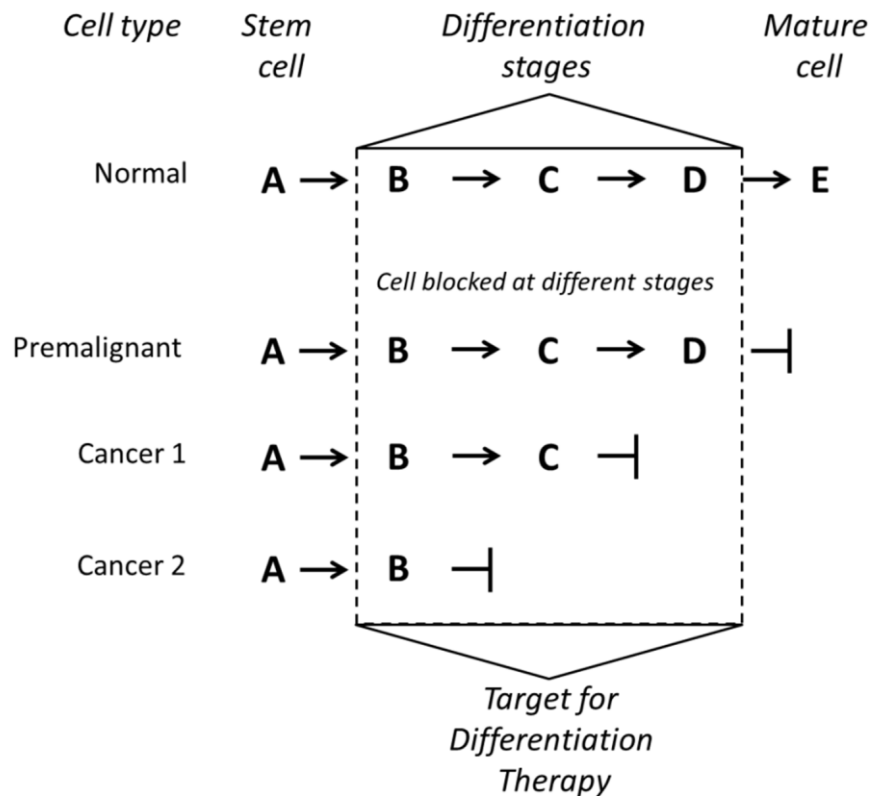


Figure 5. Targets for differentiation therapy. In a normal differentiation pathway, a hypothetical stem cell designated A is required to undergo a series of progressive changes in gene expression and resultant phenotypic changes represented by the letters B, C and D before reaching the terminally differentiated mature cell stage designated E. Cancer development is associated with aberrant differentiation. This can be represented by a block in differentiation that can occur already in premalignant cells. The scheme shows three possible blocks at late stages of the differentiation pathway (stage D) and at early stages of the pathway (stage B)

Although the focus of intense research, the molecular changes regulating melanoma development and progression remain to be defined, since melanomas are extremely heterogeneous in their genetic background. Numerous studies confirm that melanoma cell exhibits characteristics similar to those observed in de-differentiated cells. Jiang and Fisher (1993) have demonstrated that the treatment of metastatic human melanoma cells with a combination of human fibroblast interferon (IFN-b) and antileukemic compound mezerein (MEZ) results in a loss of tumorigenic potential, irreversible growth arrest, antigenic modulation, enhanced melanin synthesis, profound changes in gene expression and terminal cell differentiation. The mechanism by which IFNs cause growth arrest and differentiation remains to be clearly define; however, many evidence suggested that the signaling pathway for

IFNs (Jag/STAT) may interact with other major pathways in order to elicit effect on tumor growth, such as MAPK, PKA and PKC pathways (Weber et al., 1998).

In the literature is also reported, by more than three decades, the terminal differentiation effect of suberoylanilide hydroxamic acid, also named SAHA (*Vorinostat*).

SAHA is an HDAC inhibitor and it is currently used for the treatment of cutaneous T cell lymphoma. But, early indications about the differentiating effect of SAHA are older than three decades ago, when Breslow and collaborators (1991), showed that, if used at lower concentrations of toxic, SAHA caused growth arrest and differentiation in murine erythroleukemia MELC cells (Breslow et al., 1991). This drug is also used in clinical trials against both hematological and solid tumors (Marks PA., 2007).

A variety of anticancer agents (Table 1), both natural and synthetic, have been found to induce terminal differentiation and/or apoptosis rather than by cytotoxic action *in vitro* in various cell lines, including murine erythroleukemia cells, human myeloid leukemia cells and murine and human embryonal carcinoma cells (Leszczyniecka et al., 2001).

Table 1. Differentiation-inducing agents and their suggested mechanism of action (*Lotan R., 2002*)

Differentiation-inducing agent	Mechanism of action
Short-chain fatty acids (butyrate and derivatives)	Inhibit histone deacetylase, thereby enhancing gene expression, alter the methylation and phosphorylation of nuclear proteins and alter gene transcription
Aromatic fatty acids (phenylbutyrate, phenylacetate)	Inhibition of protein prenylation critical for signal transduction; activation of peroxisome proliferator-activated receptor, a transcription factor related to the steroid nuclear receptor superfamily; hypomethylation of DNA; depletion of circulating glutamine which is required for tumour growth
Trichostain A, SAHA	Inhibit histone deacetylase, thereby modulating gene expression
PPAR ligands	Activate nuclear receptors (PPARs) and modulate gene transcription
Haxamethylenebisacetamide	Modulate cell cycle and differentiation related genes
Vitamin A analogues (retinoids)	Activate nuclear receptors (RARs and RXRs) and modulate gene transcription
Vitamin D3 analogues	Activate nuclear receptors (VDR) and modulate gene transcription
Cytokines (TNF- $\alpha$ interleukins)	Act via cell surface receptors to induce signal transduction pathways resulting in alterations in gene expression
Interferons	Activate transcription factors (Stats) and thereby alter gene transcription
5-Aza-2-deoxycytidine	Decreases DNA hypermethylation and thereby restores expression of silenced genes
Staurosporin	Protein kinase C inhibition
8-Choloro-cyclic adenosine monophosphate (8-Cl-cAMP)	Downregulates RI and upregulates the RIIB subunit of protein kinase A
Actinomycin D	DNA intercalation
Cytosine arabinoside; cyclophosphamides; aclacinomycin (at subtoxic concentrations)	Inhibition of DNA synthesis

Some of these molecules, such as trichostatin A and cytosine arabinoside, together with other described in the following section, are classified as chemotherapeutic thanks to their high cytotoxic effect; but in addition, when used at sub-toxic concentrations, they are able to induce also the terminal erythroid differentiation.

### **1.2.3 Antitumor agents acting through induction of erythroid terminal differentiation**

In the literature there are numerous publications concerning the study of anticancer agents known as inducers of terminal differentiation. In particular, we want to focus our attention on anticancer possessing also erythro-differentiating ability. It is possible to divide these molecules in different categories, such as (a) cytotoxic agents, (b) HDAC inhibitors, (c) DNA binding drugs (DBD) and (d) from natural world. The same rationale that has led to the successful application of combination of cytotoxic agents with non-overlapping toxicities and distinct mechanisms of action can be applied to differentiation-inducing agents.

#### **1.2.3.1 *Cytotoxic agents***

Cytarabine, or cytosine arabinoside (AraC), is a chemotherapy agent used mainly in the treatment of cancers of white blood cells such as acute myeloid leukemia (AML) and non-Hodgkin lymphoma. AraC interferes with the synthesis of DNA: inhibiting both DNA and RNA polymerases, leads tumor cells unable to duplicate themselves. Myleran is an alkylating agents used for CML therapy and after bone marrow transplantation; and vinblastine, is currently used for lymphoma, testicular, breast and bladder cancer therapy. These molecules terminate actively cycling progenitors and perturb cellular growth to trigger rapid erythroid-regeneration kinetics and formation of mature red blood cells to contain hemoglobin (Galanello et al., 1988).

### 1.2.3.2 Hydroxyurea

The Multicenter Study of Hydroxyurea was initiated in 1992 to establish the first drug treatment for Sickle Cell Disease (SCD). Hydroxyurea (HU) is a ribonuclease reductase inhibitor that produces blockade of DNA synthesis and cell death. This compound also alters growth rates and gene expression in mammalian cells (Adunyah et al., 1995). One of the first studies showing a clear effect of HU on erythroid differentiation was published in 1993 by Fibach and his collaborators, in which HU was found to have multiple effects on human peripheral blood-derived progenitor cell: an increase in the proportion of fetal hemoglobin (HbF) produced; an inhibition of cell proliferation; and an increase in hemoglobin (Hb) content per cell. Several reports confirmed the HbF augmenting effect of HU both *in vitro* and *in vivo* (Wang et al., 2002; Fucharoen et al., 2004; Siriboon et al., 1996; Loukopoulos et al., 2005; Loutradi et al., 2000; Zargari et al., 2004), as summarized in Table 2. For example, Yavarian *et al.* (Yavarian et al., 2004) reported the treatment with HU of 133 patients with transfusion-dependent  $\beta$ -thalassemia. After one year of treatment these patients were classified into: good responders (61%) who shifted from monthly blood transfusion dependency to a stable transfusion-free condition; moderate responders (23%) who remained transfusion dependent but at longer intervals (6 months or more), and non-responders, who remained at the same level of transfusion dependency.

Table 2. Clinical trials employing Hydroxyurea as *In vivo* inducer of HbF (from Gambari e Fibach, *medicinal chemistry of fetal hemoglobin inducer for treatment of beta-thalassemia*, *Curr Med Chem*, 2007, 14:199-212)

References	Genotype/Phenotype <sup>(a)</sup>	N° of patients treated <sup>(b)</sup>	N° of patients responding to the treatment <sup>(c)</sup>
Hoppe et al., 1999	$\beta$ -thalassemia intermedia	5	3 (60%)
de Paula et al., 2003	$\beta$ -thalassemia intermedia	7	3 (43%)
de Paula et al., 2003	$\beta$ -thalassemia major	4	1 (25%)
Bradai et al., 2003	$\beta$ -thalassemia intermedia	2	2 (100%)
Bradai et al., 2003	$\beta$ -thalassemia major	5	5 (100%)
Alebouyeh et al., 2004	$\beta$ -thalassemia major	36	25 (69%)
Yavarian et al., 2004	$\beta$ -thalassemia major	133	81 (61%)
Dixit et al., 2005	H- $\beta$ -thalassemia intermedia	37	26 (70%)
Karimi et al., 2005	$\beta$ -thalassemia intermedia	166	83 (50%)
Singer et al., 2005	HbE/ $\beta^0$ -thalassemia	42	20 (50%)



### 1.2.3.3 *Histone deacetylases Inhibitors (HDACi)*

Gene expression is controlled by alterations in chromatin structure produced by acetylation or deacetylation of histone tails, resulting in gene activation or repression, respectively. Histone deacetylase (HDAC) enzymes produce deacetylation of histone tails, causing chromatin condensation and transcriptional silencing.

Several findings suggest that inhibition of the activity of histone deacetylases (HDACs) is associated with an increased expression of the  $\gamma$ -globin genes. Several inhibitors, such as sodium butyrate, trichostatin A, adicypin, and scriptaid, used at low doses, are employed in colorectal cancer therapy. Moreover, they have been shown to induce HbF synthesis *in vitro*; butyrate also induced HbF in humans (Perrine et al., 1993; McCaffrey et al., 1997; Pace et al., 2003; Cao et al., 2004; Johnson et al., 2001; Pace et al., 2005). Mechanistically, the HDAC inhibitors bind to a central zinc atom in HDACs to block enzymatic deacetylation of histone H3 and H4, leading to an hyperacetylation of  $\epsilon$ -amino groups of lysine residues in histones. This in turn causes a decreased association of basic core histone proteins with the DNA, rendering certain genes more accessible to the transcriptional machinery (Cao et al., 2004).

### 1.2.3.4 *DNA-binding drugs*

The DNA-binding drugs (DBDs) are molecules able to interact with the major groove of DNA are expected to inhibit complex formation between transcription factors and target DNA elements (Gambari et al., 2003). Several DBDs are or have been used in therapy. Chromomycin and mithramycin (MTH) were used in different kinds of cancer, such as ovarian, stomach, bladder, prostate cancer and hepatoma and they are also employed in the treatment of hypercalcemia; MTH is also used in CML and AML therapy. Tallimustine and tallimustine analogues are anticancer and antiviral agents. Many reports demonstrated that some DBDs display DNA sequence selectivity, and that even similar DBDs differ with respect to stability of their complexes with DNA. Our group has demonstrated that tallimustine (Baraldi et al., 2000; Bianchi et al., 2001; Chiarabelli et al., 2003) and some cisplatin analogues (well-known alkylating agent used for testicular, ovarian, lung, breast, stomach, prostate cancers and also for neuroblastoma, melanoma and sarcoma) (Bianchi et

al., 2000) as well as the GC-rich binders chromomycin and MTH (Bianchi et al., 1999) are powerful inducers of differentiation of K562 cells, suggesting that the expression of crucial genes involved in erythroid differentiation of these cells are influenced by DBDs. Several DBDs, such as tallimustine, MTH and cisplatin, increase of fetal hemoglobin (HbF) production in erythroid precursor cells from normal human subjects. The extent of induction was found to be higher than that of hydroxyurea (HU). Since, among the DBDs studied, MTH displayed the lowest cytotoxicity, we compared it to HU on fetal hemoglobin production by thalassemic erythroid precursors (Fibach et al., 2003). The results demonstrated that in cultures derived from 12 patients, mithramycin increased HbF production in all cases, while HU was not effective in two cases and was toxic in one. In the majority of cases the activity of mithramycin was higher than hydroxyurea. In all cases, HU strongly inhibited cell proliferation, while, at concentrations able to induce HbF production, mithramycin had minimal effect on cell growth.

#### 1.2.3.5 *Erythro-differentiating agents from natural world*

Several reviews and papers have been published on the possible use of extracts from medicinal plants for biomedical purposes including therapeutic strategies for the treatment of a number of diseases such as dyslipidemia (Alder et al., 2003) and atherosclerosis (Wang et al., 1999), hepatitis (Luper et al., 1998), inflammatory diseases (Nakhai et al., 2007), osteoporosis (Xie et al., 2005), bacterial and virus infections (Khan et al., 2005). In the case of cancer, only few examples are available, as rapamycin and its analogues everolimus and resveratrol, that are briefly described.

Rapamycin (Figure 6a) is a lipophilic macrolide, isolated from a strain of *Streptomyces hygroscopicus* found in a soil from Easter Island, that possesses immunosuppressive, antifungal and anti-tumor properties. This molecule is also approved by the U.S. Food and Drug Administration as an immunosuppressive agent for preventing rejection in patients receiving organ transplantation. The rapamycin, employed in combined therapy with doxorubicin, drive AKT-positive lymphomas into remission in mice. Our group has demonstrated that rapamycin, tested on the human leukemia K562 cell line and the two-phase liquid culture of human erythroid

progenitors isolated from normal donors and patients with beta-thalassemia, has the ability to induce terminal erythroid differentiation and induction of HbF levels. The interest in rapamycin as an HbF-inducer is related to the fact that its effect is not associated with cytotoxicity and cell growth inhibition, in contrast to other inducers.

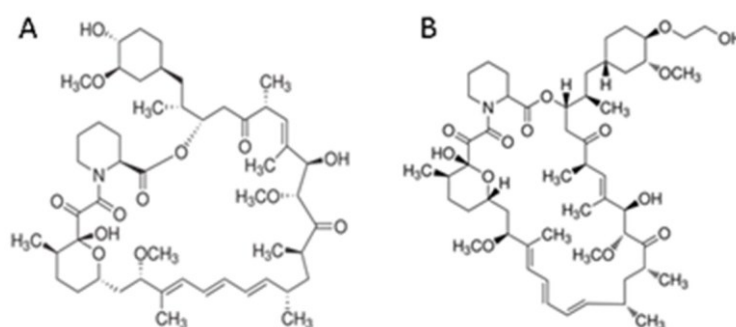


Figure 6. Chemical structure of Rapamycin (A) and Everolimus (B)

Several rapamycin-like molecules have been described, exhibiting better characteristics than rapamycin. For instance, everolimus (Figure 6b) is an immunosuppressive macrolide bearing a stable 2-hydroxyethyl chain substitution at position 40 on the rapamycin structure (Dumont et al., 2004; Augustine et al., 2004). Clinical experience, largely limited to its use in kidney transplant patients, indicates that the administration of everolimus is associated with low rates of acute rejection and a tolerable safety profile. Everolimus is a mTOR inhibitor and it acts by the block of proliferation. Recent observations in heart transplant patients suggest that the anti-proliferative effects of this compound may prevent allograft vasculopathy. Zuccato et al., (Zuccato et al., 2007) have determined the effects of everolimus on the erythroid differentiation of K562 cells and on the  $\gamma$ -globin mRNA accumulation in cultures of erythroid precursors isolated from  $\beta$ -thalassaemia patients.

Resveratrol, 3,5,4'-trihydroxystilbene (Figure 7), is a natural phytoalexin present in large quantity in red wine, preferentially in the skin of grapes (Jeandet et al., 1991).

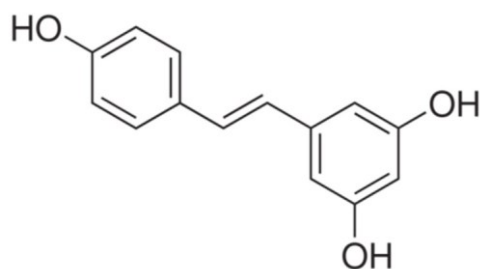


Figure 7. Chemical structure of Resveratrol

Actually, resveratrol is used in support of conventional anticancer chemotherapy in skin cancer, breast and prostate cancer. Rodrigue *et al.* (Rodrigue *et al.*, 2001) found that resveratrol possesses similar properties to HU toward erythroid differentiation. They firmly demonstrated that resveratrol induces differentiation of K562 cells and augmentation of HbF in erythroid precursor cells isolated from eight sickle cell patients.

In our laboratories, when erythroid precursor cells from normal subjects were treated with increasing concentrations of resveratrol and analysis of accumulation of globin mRNA sequences was performed by quantitative RT-PCR, a clear increase in accumulation of  $\gamma$ -globin mRNA content was found. Increase in accumulation of  $\alpha$ -globin and  $\beta$ -globin mRNA was much lower. Taken together these data strongly indicate resveratrol as a strong inducer of HbF and a selective stimulator of the expression in  $\gamma$ -globin genes (Bianchi *et al.*, 2009).

The potential of agents that stimulate cell differentiation to serve for cancer therapy has been studied extensively *in vitro* and in animal models. Such agents can suppress growth and enhance differentiation, which may also lead to apoptosis. Only a few of the numerous differentiation inducing agents shown in Table 1 have been examined in clinical trials and even for those that have been investigated, most trials were phase I or II. The only definitive demonstration of the efficacy of differentiation therapy is the treatment of acute promyelocytic leukemia patients with ATRA.

#### 1.2.4 K562 cell line as useful experimental model for erythroid differentiation

Erythroleukemic K562 cell line was isolated and characterized by Lozzio and Lozzio (Lozzio et al., 1975) from a patient with chronic myelogenous leukemia in blast crisis. K562 cell line has been extensively employed as a very useful in vitro model to study the molecular mechanism(s) regulating the expression of embryonic and fetal human globin genes (Rutherford et al., 1981). K562 cells exhibit a low proportion of hemoglobin-synthesizing cells under standard cell growth conditions, but are able to undergo terminal erythroid differentiation when treated with a variety of compounds, including short fatty acids, 5-azacytidine (Gambari et al., 2007), mithramycin and chromomycin (Bianchi et al., 1999; Fibach et al., 2003), cisplatin and cisplatin analogs (Bianchi et al., 2000), tallimustine (Bianchi et al., 2001; Gambari and Fibach, 2007), rapamycin (Fibach et al., 2006), everolimus (Zuccato et al., 2007), psoralens (Lampronti et al., 2003) and resveratrol (Bianchi et al., 2009). Following erythroid induction, increase of expression of  $\epsilon$  and  $\gamma$  globin genes is observed, leading to accumulation of Hb Portland ( $\zeta_2\gamma_2$ ) and Hb Gower 1 ( $\zeta_2\epsilon_2$ ) (Gambari et al., 2007).

Several antitumor drugs were demonstrated to induce erythroid differentiation of K562 cells. Some of us have recently demonstrated that DNA binding drugs (DBDs) exhibiting antitumor activity are powerful inducers of differentiation of K562 cells, suggesting that the expression of crucial genes involved in terminal erythroid differentiation of these cells is influenced by DBDs.

Thus, this experimental cell system appears to be suitable for the screening of molecules able to inhibit cell growth by acting on the activation of terminal differentiation pathways.

K562 cells grow in culture as single, undifferentiated, cells in suspension, with low production of hemoglobins. When stimulated by various agents, they respond within few days with a significant increase in the production of hemoglobins and  $\gamma$ -globin mRNA (Bianchi et al., 1999).

### **1.3 “New uracil dimers showing erythroid differentiation inducing activities”**

As described in *section 1.1.3* and reported by Accetta *et al.* (2009), a series of uracil dimers has been synthesized starting from uracil, thymine or 5-carboxyuracil (isoorotic acid), and tested for antiproliferative and erythroid differentiation activity.

#### **1.3.1 Screening of anti-proliferative and differentiation inducing properties**

We first determined for all the synthesized molecules the effects on cell proliferation (Table 3). To this aim, K562 cells were cultured in the presence of increasing concentrations of compounds and cell number per milliliter was determined after 3, 4, and 5 days. These time points were selected because between days 3 and 5 untreated control K562 cells are on the log phase of cell growth.

In order to determine the effects on the erythroid differentiation, cells were seeded at an initial concentration of  $3 \times 10^4$  cells/ml and the proportion of benzidine-positive cells was determined after 4-7 days of cell culture using a solution containing 0.2% benzidine in 0.5M glacial acetic acid (10% H<sub>2</sub>O<sub>2</sub>) (Bianchi *et al.*, 2001). Benzidine positivity indicates the presence of intracellular hemoglobin. All methods will be better described in “Materials and Methods” chapter. Table 3 indicates the antiproliferative effects (IC<sub>50</sub> values) and the erythroid induction ability (% of benzidine-positive cells) of all the tested compounds. The best erythroid induction ability was displayed by compound **14**. The data shown in Table 3 were obtained using concentrations of compounds approaching those giving 50% of inhibition of cell growth (these concentrations were chosen to better compare the potential erythroid inducing activity in experimental conditions leading, for most of the compounds tested, to similar effects on cell proliferation rate). In addition, it should be noted that compound **14** was able to induce erythroid differentiation of K562 cells after 6 days cell culture even if added at concentrations lower than that shown in Table 3 (an average of  $52 \pm 4.5$  % of benzidine-positive cells was obtained in four independent experiments with 400  $\mu$ M compound **14**).

Table 3. Antiproliferative Effects (IC<sub>50</sub>) and Percentage of Benzidine-Positive Cells after Treatment with uracil compounds and concentration used

Compound	IC <sub>50</sub> (μM)	%B+ cells	concentration (μM)
<b>1</b>	> 800	5 ± 3.4	800
<b>5</b>	247 ± 33	30 ± 5.8	300
<b>9</b>	> 800	1 ± 0.8	800
<b>10</b>	536 ± 45	5 ± 2.3	800
<b>12</b>	75 ± 7.3	5 ± 3.3	100
<b>13</b>	247 ± 23	40 ± 8.4	200
<b>14</b>	517 ± 63	78 ± 7.3	600
<b>15</b>	600 ± 85	40 ± 5.5	600
<b>16</b>	220 ± 35	50 ± 6.8	600
<b>17</b>	420 ± 93	45 ± 3.3	600

Results are presented as average ± SD of three independent experiments performed). The IC<sub>50</sub> was calculated as the concentration of compounds necessary to decrease cell number (after 4 days culture period) at 50% of the values obtained in control untreated K562 cell cultures. The % of benzidine-positive (hemoglobin-containing) cells was determined after 6 days induction period at concentrations of the tested compounds indicated in the right column.

### 1.3.2 Conclusions

In conclusion, in this paper, we have demonstrated for the first time that this kind of uracil derivatives can be considered to be a new class of erythroid differentiation inducers, and that dimeric derivatives with suitable spacers have the best performing characteristics: low cytotoxicity and higher differentiating ability. Furthermore, the best results were obtained with the compound bearing a naphthalene linker (compound **14**), which avoids collapse of the uracil moieties, indicating that a possible recognition of complementary functionalities (such as adenine derivatives) could be implicated in the induction of biological properties.

These findings can be the starting point for the quest for more effective and specific drugs for the induction of terminal erythroid differentiation, ultimately leading to new insights in the treatment of neoplastic diseases with molecules acting by inducing differentiation rather than by exerting cytotoxic effects. In addition, these molecules might be of interest for the experimental treatment of β-thalassemic erythroid cells for which the induction of γ-globin mRNA could be very beneficial. (Fibach et al., 2003). In this respect it has been demonstrated that inducers of K562 erythroid differentiation are often able to induce fetal hemoglobin production in erythroid cells isolated from β-thalassemia patients (Gambari et al., 2007).

#### **1.4 “C(5) modified uracil derivatives showing antiproliferative and erythroid differentiation inducing activities on human chronic myelogenous leukemia K562 cells”**

As just described, we have recently reported a novel class of C(5) linked N(1) alkylated uracil dimers rationally designed to potentially interact with adenine in biological systems: these pyrimidine derivatives have shown antiproliferative and erythroid differentiation activities toward human chronic myelogenous leukemia K562 cells (Accetta et al., 2009). Following these results, in this thesis we would present some biological data obtained from a series of C(5) uracil monomers. These monomers were used, as previously described, as intermediates for the synthesis reactions.

##### **1.4.1 Chemical synthesis of uracil monomers**

Compounds **1**, **2** and **4** were purchased from Sigma-Aldrich and tested without further purification. Synthesis of compounds **5**, **8**, **9**, **10** and **11** is described in section 1.1.3 of this thesis and reported in Accetta et al. (2009). All syntheses started from uracil, thymine or 5-carboxyuracil (isoorotic acid). Also full synthetic procedures and characterization of compounds **3**, **12**, **13**, **14**, **15**, **16**, **17** and **18** are reported section 1.1.3 (Accetta et al., 2010).

The C(5) position of pyrimidine nucleosides can be easily modified with a variety of different residues. Substitution at this position is often used for introducing substituents on DNA oligomers with reporter groups. In a general project, aimed at molecular engineering of peptide nucleic acid (PNA) derivatives (A. Accetta, PhD thesis, 2010), a set of N(1) and/or N(3) alkyl-C(5) modified uracil derivatives were prepared. Since these compounds are structurally analogs of modified nucleosides, they could be considered as potential drugs *per se*. This set of compounds was chosen for screening since: i) it contains compounds alkylated at N(1) and N(3) positions or both; ii) it contains a series of C(5) derivatives with different functional groups; iii) it has lipophilic groups in both the N(1), N(3), or C(5) region of uracil; iv) it contains derivatives with intact uracil (and thymine) hydrogen bond pattern or lacking of the N(3) hydrogen.



Briefly, modification of uracil at both N(1) and C(5) positions was obtained in several ways. For the more reactive uracil and isoorotic acid, and in the presence of small electrophiles such as methyl iodide, the regioselective alkylation at N(1) can be obtained by temporary protection of the N(3) with trimethylsilyl (Accetta et al., 2009) or benzoyl groups.

The latter method was used in the synthesis of **7** from **6**. Azidomethyl derivative **3** was obtained by reaction of uracil with formaldehyde, thus introducing a 5-hydroxymethyl derivative which could be further elaborated to azide by nucleophilic substitution (via a chloromethyl intermediate). Alkylation of **3** with  $\alpha$ -bromoacetic acid derivatives led to compounds **15** and **16**. The latter was converted to **17**, bearing an amino group through Staudinger reduction. The amide derivative **18** was then obtained by reaction of the amino compounds with 2-naphthalenecarboxylic acid after activation of the latter with HBTU. Regioselective alkylation at N(1) was obtained also by exploiting the higher reactivity of the N(1) position in thymine and iodouracil toward sterically hindered electrophiles, which allowed direct synthesis of compounds **9**, according to Coutouli-Argyropoulou and Zachariadou, (Coutouli-Argyropoulou et al., 2005) and **13**. The N(1), N(3)- doubly alkylated compound **12** was obtained as a side product of thymine alkylation. Oxidation of thymine methyl group led to the N(1)- alkylated-5-carboxylic derivative **10** (Accetta et al., 2009), which was then converted into the carboxamide derivative **11** via HBTU activation and reaction with benzylamine. Hydrolysis of **13** with TFA provided derivative **14**, bearing a polar substituent in the N(1) position.

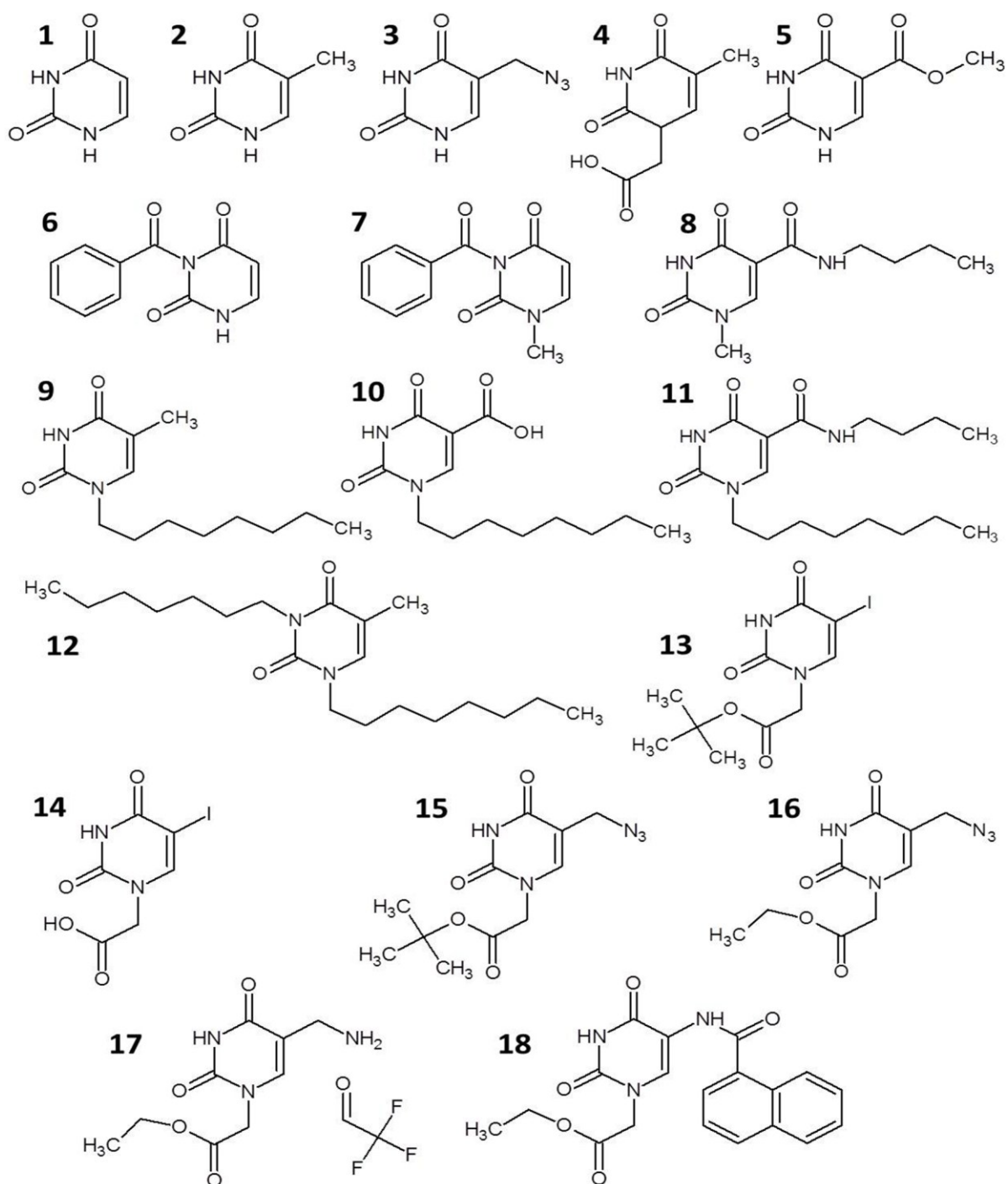
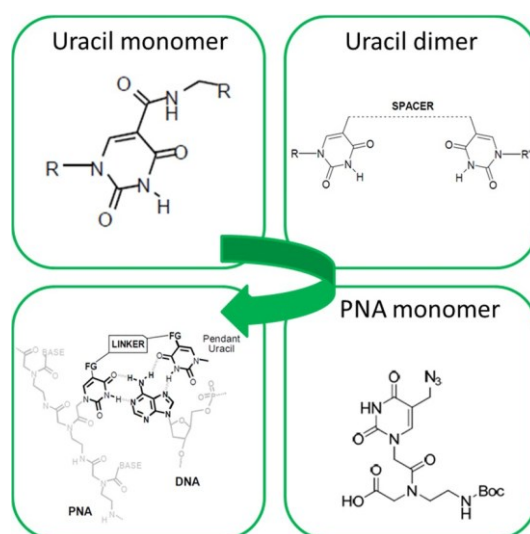


Figure 8. Chemical structures of uracil monomers tested. Alternative name: 1, uracil; 2, thymine (5-methyluracil); 3, 5-azidomethyluracil; 4, (thymine-1-yl)acetic acid (1-(carboxymethyl)thymine); 5, methyl 5-uracilcarboxylate (methyl isoorotate); 6, 3-benzoyluracil; 7, 3-benzoyl-1-methyluracil; 8 N-butyl-1-methyl-5-uracilcarboxamide; 9, 1-octylthymine; 10, 1-octyl-5-uracilcarboxylic acid (1-octylisoorotic acid); 11, N-benzyl-1-octyl-5-uracilcarboxamide; 12, 1,3-dioctylthymine; 13, t-butyl (5-iodouracil-1-yl)acetate; 14, (5-iodouracil-1-yl)acetic acid; 15, t-butyl (5-azidomethyluracil-1-yl)acetate; 16, ethyl (5-azidomethyluracil-1-yl)acetate; 17, ethyl (5-aminomethyluracil-1-yl)acetate; 18, ethyl [5-(N-(2-naphthylcarboxyl)aminomethyl)uracil-1-yl]acetate

## 1.5 Aim and thesis outlook

Recently we have reported a novel class of C(5) linked N(1)-alkylated uracil dimers rationally designed to potentially interact with adenine in biological system. These pyrimidine derivatives have shown antiproliferative and erythroid differentiation activities toward human chronic myelogenous leukemia K562 cells (Accetta et al., 2009). In this thesis we report the screening study on a set of different modified C(5) uracil derivatives; many of them are reaction intermediates for the synthesis of modified uracil dimers. We have evaluated their antiproliferative effect in connection with erythroid differentiation pathways; we would like to propose these molecules as a new class of drug candidates for the treatment of chronic myelogenous leukemia.

As pointed out from the title of this thesis, we analyzed the biological activity, in particular on erythroid differentiation pathway, of uracil modified monomer used as intermediates of reaction during the synthesis of uracil dimers. These uracil dimers have previously showed interesting biological properties probably in connection with their ability to cooperatively interact with adenine. Using the same geometry, Corradini and his collaborators have synthesized the PNA monomer containing these novel modified dimeric uracils. This new nucleobases performed better than thymine for the adenine recognition on complementary DNA, conferring better selectivity and affinity. These “engineering” uracil were used to the specific design of anti-miR-221 PNAs. The biological activities of these anti-miR-221 PNA were evaluated in a breast cancer cell model, and the results are reported in the *Part 2 of this PhD thesis*.



## - MATERIALS AND METHODS -

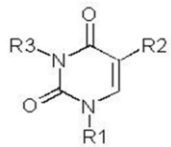
### 2.1 Cell culture: human erythroleukemic K562 cell line

The human K562 cell line, isolated from a patient with chronic myelogenous leukemia in blast crisis and characterized by Lozzio and Lozzio (Lozzio et al., 1975), were obtained from the American Type Culture Collection (Rockville, Md., USA). K562 were maintained in a humidified atmosphere of 5% CO<sub>2</sub>/air at 37 °C in suspension culture using Roswell Park Memorial Institute 1640 medium (RPMI 1640) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Analytical de Mori, Milan, Italy), 50 units/ml penicillin and 50 mg/ml streptomycin (Bianchi et al., 2001).

### 2.2 C(5) modified uracil monomers

For the experiments regarding erythroid differentiation in K562 cell line, we used a serie of uracil monomers and dimers synthesized by Prof. Roberto Corradini and his collaborators, in particular Alessandro Accetta, at University of Parma, Dept. of Organic and Industrial Chemistry. The methods of design and synthesis are well described in Accetta et al., 2009. Here, we report the chemical structure of compounds tested (Table 4).

Table 4. Chemical structure of compounds tested



Compound	R1	R2	R3
1	-H	-H	-H
2	-H	-CH <sub>3</sub>	-H
3	-H	-CH <sub>2</sub> N <sub>3</sub>	-H
4	-CH <sub>2</sub> COOH	CH <sub>3</sub>	-H
5	-H	-COOCH <sub>3</sub>	-H
6	-H	-H	-COPh
7	-CH <sub>3</sub>	-H	-COPh
8	-CH <sub>3</sub>	-CONH <i>n</i> C <sub>4</sub> H <sub>9</sub>	-H
9	<i>n</i> C <sub>8</sub> H <sub>17</sub>	-CH <sub>3</sub>	-H
10	<i>n</i> C <sub>8</sub> H <sub>17</sub>	-COOH	-H
11	<i>n</i> C <sub>8</sub> H <sub>17</sub>	-CONHCH <sub>2</sub> Ph	-H
12	<i>n</i> C <sub>8</sub> H <sub>17</sub>	-CH <sub>3</sub>	<i>n</i> C <sub>8</sub> H <sub>17</sub>
13	-CH <sub>2</sub> COO <i>t</i> Bu	-I	-H
14	-CH <sub>2</sub> COOH	-I	-H
15	-CH <sub>2</sub> COO <i>t</i> Bu	-CH <sub>2</sub> N <sub>3</sub>	-H
16	-CH <sub>2</sub> COOEt	-CH <sub>2</sub> N <sub>3</sub>	-H
17	-CH <sub>2</sub> COOEt	-CH <sub>2</sub> NH <sub>2</sub>	-H
18	-CH <sub>2</sub> COOEt	-CH <sub>2</sub> NHCO(2-naphthyl)	-H

### **2.3 Antiproliferative activity**

We first determined for all the uracil-derived compounds the effects on cell proliferation. To this aim, K562 cells were seeded at an initial concentration of  $3 \times 10^4$  cells/ml and cultured in the presence of increasing concentrations of compounds. Non-treated cells were considered as control. Cell number/ ml was determined after 3, 4, and 5 days, using a ZF Coulter Counter (Coulter Electronics, Hialeah, FL, USA). These time points were selected because between days 3 and 5 untreated control K562 cells are on the log phase of cell growth (Bianchi et al., 2001). The  $IC_{50}$  value (concentration of compound required to inhibit proliferation by 50%) was calculated on three independent experiments.

### **2.4 Benzidine assay**

In order to determine the effects on the erythroid differentiation of uracil-derivatives monomers, K562 cells were seeded at an initial concentration of  $3 \times 10^4$  cells/ml and the proportion of benzidine- positive cells was determined after 4- 7 days of cell treatment with the compounds, using a solution containing 0.2% benzidine in 0.5 M glacial acetic acid (10%  $H_2O_2$ ) as previously described (Bianchi et al, 2001). The percentage of benzidine-positive cells (blue-cells) was calculated on total cells and indicates the presence of intracellular hemoglobin.

### **2.5 Transfection of K562 cells with fluorescence protein genes under the $\gamma$ -globin and the $\beta$ -globin gene promoters**

K562 cells were stably transfected with the pCCL.Prom $\beta$ .HcRed1.Prom $\gamma$ .EGFP, containing the green and red fluorescence protein (FP) genes under the control of the  $\gamma$ -globin and  $\beta$ -globin gene promoters, respectively (Guerrini et al., 2009; Lampronti et al., 2009). In this system, increases in the green and red signals are consistent with  $\gamma$ -globin and  $\beta$ -globin gene promoter driven activity, respectively. To determine the activity of chemical compounds in inducing the expression of  $\gamma$ -globin and  $\beta$ -globin genes, cells were seeded at  $8 \times 10^3$  cells/ml and treated with the appropriate concentration of the chemical inducer. After 5 days of culture, cells were

assayed for fluorescent proteins expression. First of all they were analyzed under a fluorescence inverted microscope, using filters suitable for both green and red FPs. The fluorescence intensity was then determined by fluorescence-activated cell sorting (FACS) analysis.

## 2.6 RNA isolation

The total cellular RNA was extracted by *TRIZOL*® *Reagent* (Sigma-Aldrich, St.Louis, Missouri, USA). All reagents and materials used were RNase-free. After 6 days of treatment with the compounds, the cells were centrifuged at 1,200 rpm for 10 minutes at 4°C, washed in 1X PBS, re-centrifuged and then lysed with 1 ml *TRIZOL*® *Reagent*. The homogenate was incubated 5 minutes at room temperature and 200 µl of chloroform were added; the samples were shaken vigorously for 15 second, incubated for 5 minutes at room temperature and finally centrifuged at 12,000 rpm for 15 minutes at 4°C. The aqueous phase was transferred into a clean tube and 500 µl of isopropanol was added. The RNAs were and incubated for 10 minute at room temperature (25°C). After that, the samples were centrifuged at 12,000 rpm for 10 minutes at 4°C; the isolated RNA was precipitated in 2 volumes of absolute ethanol and stored at -80°C, washed once with cold 75% ethanol, dried and dissolved in 10 µl of diethylpyrocarbonate (DEPC)-treated water before use (Sambrook et al., 1989) and conserved at -80°C.

### 2.6.1 RNA quantification

The concentration of RNA should be determined by measuring the absorbance at 260 nm ( $A_{260}$ ) in a spectrophotometer. The concentration is obtained by the equation:

$$\text{g/ml} = \text{OD} \times 40 \times \text{DIL}$$

where OD is the value read from the instrument, 40 is the correction coefficient for reading the RNA at the spectrophotometer (according to the Lambert-Beer law) and DIL is the dilution factor. An absorbance of 1 unit at 260 nm corresponds to 40 µg of

RNA per ml ( $A_{260} = 1 = 40 \mu\text{g/ml}$ ). This relation is valid only for measurements in water.

### 2.6.2 RNA electrophoresis on agarose gel

The 1% agarose gel is prepared by dissolving 1 g of agarose powder in 100 ml of TAE 1x (obtained by dilution from 50x TAE = 2 M Tris-HCl, 0.05 M EDTA pH = 8.0 and 5.71% acid Acetic 99.8%); EtBr 10 g/ml was added to the solution.

### 2.7 Reverse transcription reaction- Random Hexamer

Reverse transcription of 1  $\mu\text{g}$  of total RNA was performed using with the *TaqMan® Reverse Transcription Reagents PCR kit* (Applied Biosystems). RNA was incubated with 2,5  $\mu\text{M}$  *Random Hexamers* at 25°C for 10 minutes and then immediately cooled to 4°C. After that, the RT reaction solution, prepared considering a final volume of 50  $\mu\text{l}$ , progressively adding the following reagents: 1X *TaqMan RT Buffer* (10X), 5,5 mM *MgCl<sub>2</sub>* (25 mM), *deoxyNTPs Mixture*, 0,4 U/ $\mu\text{l}$  *RNase Inhibitor* and 1,25 U/ $\mu\text{l}$  *MultiScribe™ Reverse Transcriptase* (50 U/ $\mu\text{l}$ ) was added. To perform the reverse transcription reaction, the samples were incubated for 30 minutes at 48°C, to allow the extension, and at 95°C for 5 minute to inactivate the enzyme. After thermal cycling, cDNA were stored at -80°C.

### 2.8 Real-Time Quantitative Polymerase Chain Reaction

The *quantitative real-time polymerase chain reaction* (qPCR) assays of transcripts were carried out using gene-specific double fluorescently labelled probes (Applied Biosystems, Warrington Cheshire, UK) in a 7700 Sequence Detection System, version 1.6.3. To evaluate the erythroid induction of uracil derivatives in K562 cell line, the following primer and probe sequences were used (Table 5). The kit for quantitative qRT-PCR for  $\zeta$ -globin mRNA and  $\epsilon$ -globin mRNA was from Applied Biosystems ( $\zeta$ -globin mRNA: Hs00923579\_m1;  $\epsilon$ -globin mRNA: Hs00362216\_m1). The fluorescent reporter and the quencher were 6-carboxyfluorescein (FAM) and 6-

carboxy-N,N,N',N'-tetramethylrhodamine (TAMRA), respectively. The reference sequences were 18S ribosomal RNA, where the probe was fluorescently labelled with VIC TM (Applied Biosystems).

Table 5. Sequences of primer and probe used for qPCR gene expression analysis

Primers & Probes	Sequences
$\alpha$ -globin fw	5'-TCCCCACCACCAAGACCTAC-3'
$\alpha$ -globin rev	5'-CCTTAACCTGGGCAGAGCC-3'
$\alpha$ -globin probe	5'-FAM-TCCCGCACTTCGACCTGAGCCA-TAMRA-3'
$\gamma$ -globin fw	5'-TGGCAAGAAGGTGCTGACTTC-3'
$\gamma$ -globin rev	5'-TCACTCAGCTGGGCAAAGG-3'
$\gamma$ -globin probe	5'-FAM-TGGGAGATGCCATAAAGCACCTGG-TAMRA-3'
glycophorin A fw	5'-CGGTATTCGCCGACTGATAAA-3'
glycophorin A rev	5'-AAAGGCAGTCTGTGTCAGGT-3'
glycophorin A probe	5'-FAM-AAAGCCATCTGATGTAAAACCTCTTCCCCT-TAMRA-3'
transferrin receptor fw	5'-TCAGAGCGTCGGGATATCG-3' transferrin
receptor rev	5'-TGAAGTCCACACAGAAGAACA-3'
transferrin receptor probe	5'-FAM-TGGCGGCTCGGGACGGA-TAMRA-3'

The n-fold increase was determined as published elsewhere (Fibach et al., 2003; Lampronti et al., 2003) by comparing the 'threshold' cycle ( $C_T$ ) value relative to the amplification of the cDNA to be quantified with that of the amplified reference 18S ribosomal RNA (or U6 RNA) sequences ( $\Delta C_T$  value). In order to quantify the increase in the levels of expression of a target gene in different PCR products, the differences between  $\Delta C_T$  values were considered ( $\Delta\Delta C_T$ ). The  $\Delta\Delta C_T$  represents the negative exponent in the equation  $2^{-\Delta\Delta C_T}$ , quantifying the n-fold increase in the expression of the target gene in several samples, taking the levels of the 18S RNA (or U6 RNA) sequences as unchanged.

## 2.9 High Performance Liquid Chromatography

The hemoglobin contents in K562 cells were evaluated using High Performance Liquid Chromatography (HPLC). Cells were treated with the compounds for 6 days; after, cells were harvested, washed once with PBS, and the pellets were lysed in water. After incubation on ice for 15 min and spinning for 5 min at 14,000 rpm in a microcentrifuge, the supernatant was collected and hemoglobin proteins were separated by cation-exchange HPLC, using a



Beckman Coulter instrument System Gold 126 Solvent Module-166 Detector and a 3.5 x 0.46 cm column packed with porous (100-nm pore size) 5-mm microparticulate polyaspartic acid-silica, Poly CAT A (Poly LC, Columbia MD, USA) (Ching-Nan et al., 1993). Samples were eluted in a solvent gradient using aqueous sodium chloride-BisTris-KCN buffers, and detection was performed at 415 nm. The gradient was made up of mobile phase A (20 mM Bis Tris, 2 mM KCN, pH 6.98) and mobile phase B (20 mM Bis Tris, 2 mM KCN, 200 mM NaCl, pH 6.57), with a flow rate of 1.7 ml/min. Elution of hemoglobins was performed by increasing buffer B from 11% to 40% and to 100% at 8 and 12 min, respectively, and then decreasing to 11% buffer B for the least 8 min before application of next sample. The peak areas were used for quantification of individual hemoglobin peaks, utilizing standard controls of HbA and HbF (Analytical Control Systems, Fishers IN, USA) (Fibach et al., 2003).

## **2.10 FACS analysis**

For the determination of fluorescence intensity by FACScan (Becton Dickinson, Franklin Lakes, NJ, USA), cells were harvested and washed. Then  $1 \times 10^4$  cells were analyzed by the CellQuest™ version 3.3 software (Becton Dickinson, Franklin Lakes, NJ, USA), using the fl1 channel to detect green fluorescence and fl3 channel to detect red fluorescence. The results were expressed as median fold, i.e. the ratio between the median fluorescence intensity values obtained in the presence and absence of treatment, respectively. A graphic presentation of data was finally obtained by histograms, showing the number of cells versus the expressed fluorescence intensity (Salvatori et al., 2009).

## **2.11 Measurement of apoptosis**

### **2.11.1 *DeadEnd™ Colorimetric TUNEL System***

Apoptotic cells were detected by the DeadEnd™ Colorimetric TUNEL System (Promega Italia, Milan, Italy) according to the manufacturer's instructions. The DeadEnd™ Colorimetric TUNEL System end-labels the fragmented DNA of apoptotic

cells using a modified TUNEL (TdT-mediated dUTP Nick-End Labeling) assay. Biotinylated nucleotide is incorporated at the 3'-OH DNA ends using the enzyme Terminal Deoxynucleotidyl Transferase, Recombinant (rTdT). Horseradish-peroxidase-labeled streptavidin (Streptavidin HRP) is then bound to these biotinylated nucleotides, which are detected using the peroxidase substrate, hydrogen peroxide, and the stable chromogen, diaminobenzidine (DAB). Using this procedure, apoptotic nuclei are stained dark brown.

K562 cells were treated with the increasing concentrations of compounds; after 5 days, (when differentiated) the cells were rinsed twice with PBS solution and fixed for 25 min in 4% paraformaldehyde at room temperature. Measurement of apoptosis was calculated as a percentage of apoptotic nuclei (dark brown nuclei) versus total nuclei, evaluated in three independent experiments. A dark brown DAB signal indicates positive staining, while shades of blue-green to greenish tan indicate a non-reactive cell. The positive-control was the treatment with DNA-se 1 (Lampronti et al., 2009).

#### 2.11.2 Annexin V/PI release assay

Apoptosis was also detected with Annexin V/PI release assay. The ApoAlert Annexin V Apoptosis Kit provides a simple and effective method to detect one of the earliest events in apoptosis (the externalization of phosphatidylserine) in living cells. This assay takes advantage of the fact that phosphatidylserine (PS) is translocated from the inner (cytoplasmic) leaflet of the plasma membrane to the outer (cell surface) leaflet soon after the induction of apoptosis, and that the annexin V protein has a strong, specific affinity for PS. PS on the outer leaflet is available to bind labeled annexin V, providing the basis for a simple staining assay.

Cells were treated with the compounds and, after 72 hours, cells were collected, centrifuged for 5 min at 1,500 rpm. Cells were rinsed with Binding Buffer and then Annexin V was added and the samples were incubated at room temperature for 15 min in the dark, according to the manufacturer's instructions. For the analysis we have used FACS Calibur Becton Dickinson Immunocytometry System (Becton Dickinson, San Jose, CA, USA) (Viola et al., 2008).

### 2.11.3 *Cell cycle analysis by FACS*

Cell cycle analysis was performed by flow cytometry of propidium iodide-treated cells, using the FACS Calibur Becton Dickinson Immunocytometry System (Becton Dickinson, San Jose, CA, USA). PI, a DNA intercalating fluorescent compound, can directly correlate the fluorescence with the amount of cell DNA content. As the DNA content of cells duplicates during the S phase of the cell cycle, the relative amount of cells in the G<sub>0</sub> phase and G<sub>1</sub> phase (before S phase), in the S phase, and in the G<sub>2</sub> phase and M phase (after S phase) can be determined, as the fluorescence of cells in the G<sub>2</sub>/M phase will be twice as high as that of cells in the G<sub>0</sub>/G<sub>1</sub> phase.

K562 were seeded at initial concentration of  $1 \times 10^5$  cell/ml in 12-well plate and treated with the compounds. After 72 hours, the cells were collected, washed with PBS and centrifuged at 1,500 rpm for 5 minutes. The supernatant was eliminated and the cells were stained in the dark for 30 minutes with propidium iodide (50  $\mu$ g/ml), NP40 and RNase (10  $\mu$ g/ $\mu$ l). After incubation, cells were washed twice with PBS, centrifuged at 1,500 rpm for 5 minutes and analyzed (Chui et al., 2010).

## - RESULTS -

### 3.1 Antiproliferative and erythroid differentiating activities of the uracil monomers

We first determined for all the synthesized molecules the effects on cell proliferation and on erythroid differentiation. To this aim, K562 cells were cultured in the presence of increasing concentrations of compounds; after 3, 4 and 5 days of treatment cell number/ml was determined by Coulter Counter and IC<sub>50</sub> value (concentration of compound able to inhibit by 50% the cells growth) and after 5, 6 and 7 days of treatment we evaluated, using benzidine assay, the erythroid differentiation (% B+ cells), as reported in “Materials and Methods” Chapter.

Table 6. Antiproliferative activity (IC<sub>50</sub>)<sup>a</sup> and induction of erythroid differentiation<sup>b</sup> (% B+ cells) of uracil derived-compounds on erythroleukemia K562 cells line.

Compound	IC <sub>50</sub>	% B+ cells	Concentration (μM)
1	> 400 μM	1.1 ± 0.8	400
2	> 400 μM	1.3 ± 0.6	400
3	120.05 ± 4.7 μM	4.1 ± 1.2	100
4	> 400 μM	2.0 ± 0.9	400
5	> 800 μM	7.3 ± 1.9	800
6	350.7 ± 15.9 μM	1.1 ± 0.5	400
7	650.6 ± 12.4 μM	1.5 ± 0.8	800
8	488 ± 68 μM	12 ± 1.9	400
9	<b>29.4 ± 1.2 μM</b>	<b>98 ± 3.6</b>	<b>50</b>
10	268 ± 15.5 μM	25 ± 2.9	200
11	66.54 ± 13 μM	2.2 ± 1.0	100
12	29.7 ± 1.4 μM	2.1 ± 0.9	50
13	> 400 μM	1.2 ± 0.2	400
14	> 400 μM	1.6 ± 0.8	400
15	721.3 ± 20.9 μM	2.3 ± 0.6	800
16	764.2 ± 25.6 μM	2.1 ± 0.8	800
17	> 800 μM	2.0 ± 0.9	800
18	115 ± 16 μM	30 ± 4.9	200

<sup>a</sup> Results are presented as average ± S.D. (three independent experiments performed). <sup>b</sup> Values are expressed as average ± S.D. (three independent experiments performed) after 6 days of treatment at concentrations close to those needed to obtain 50% inhibition of cell growth after 3 day culture period (and reported of the far right column)

As shown in Table 6, it appears evident that the most active compounds with respect to antiproliferative effects ( $IC_{50}$  values lower than  $150 \mu\text{M}$ ) are compounds **3**, **9**, **11**, **12** and **18**; compound **9** being the most active molecule ( $IC_{50} = 29.4 \pm 1.2 \mu\text{M}$ ). However, among these molecules, only compound **9** is a very effective inducer of erythroid differentiation of K562 cells (Table 6). In fact, compounds **9** and **12** have similar  $IC_{50}$  ( $29.4 \pm 1.2 \mu\text{M}$  and  $29.7 \pm 1.4 \mu\text{M}$ , respectively), but when we analyze the proportion of benzidine-positive cells,  $50 \mu\text{M}$  compound **9** display over 90% hemoglobin-expressing cells, while  $50 \mu\text{M}$  compound **12** shown only about 2% of benzidine-positive cells. As far as the other compounds, only **8**, **10** and **18** are able to induce differentiation ( $12 \pm 1.9$ ,  $25 \pm 2.9$ ,  $30 \pm 4.9 \mu\text{M}$ , respectively) but at very high concentrations ( $400 \mu\text{M}$  and  $200 \mu\text{M}$ , respectively). When the same compounds were added at higher concentrations, no further increase in the proportion of benzidine-positive cells was obtained (data not shown). According with these results, compound **9** was analyzed in more detail.

### **3.2 Effects of compound 9 on proliferation and erythroid differentiation of K562 cells**

The analysis of the effects of compound **9** on the K562 cellular system is shown in Figure 9. In the experiment shown in Figure 9a, K562 cells were cultured for 6-7 days in the absence or in the presence of increasing concentrations ( $10$ ,  $30$ ,  $50 \mu\text{M}$ ) of compound **9**. As it is clearly evident, a concentration-dependent inhibition of cell growth was observed. Figure 9c reports the effect on the proliferation of K562 cells after 3 days of treatment with compound **9** at different concentrations.  $IC_{50}$  was obtained at about  $30 \mu\text{M}$ . Figure 9d shows the percentages of differentiation induced by compound **9** at different concentrations ( $10$ ,  $30$  and  $50 \mu\text{M}$ ).

We like to underline that more than 60% benzidine-positive cells are detectable after only 3 days of induction, demonstrating that the inducing effects of compound **9** was very high, and it can be considered among the most powerful inducer of K562 differentiation (see also Table 7). Despite the fact that there is no linear relationship between proportion of differentiated cells and cells with lower proliferation efficiency, the data summarized in Figure 9 c and d, demonstrate that

30  $\mu\text{M}$  compound **9** is a concentration sufficient to (a) inhibit cell growth and (b) induce differentiation of the majority of treated K562 cells.

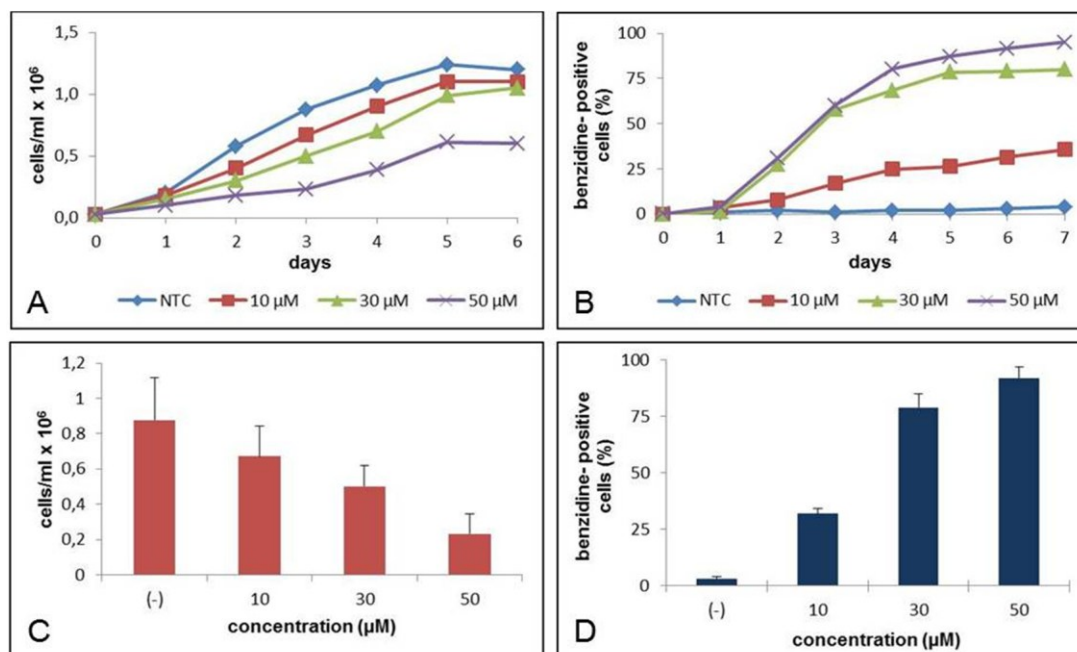


Figure 9. Effect of compound **9** on the proliferation (A) and erythroid differentiation of K562 cells (B). The effect on the proliferation of K562 after 3 days of treatment with compd **9** at different concentration (C) and % of benzidine-positive cells after 6 days of treatment (D). The values in treated cultures represented in C) were compared with untreated control cultures (taken as 100%)

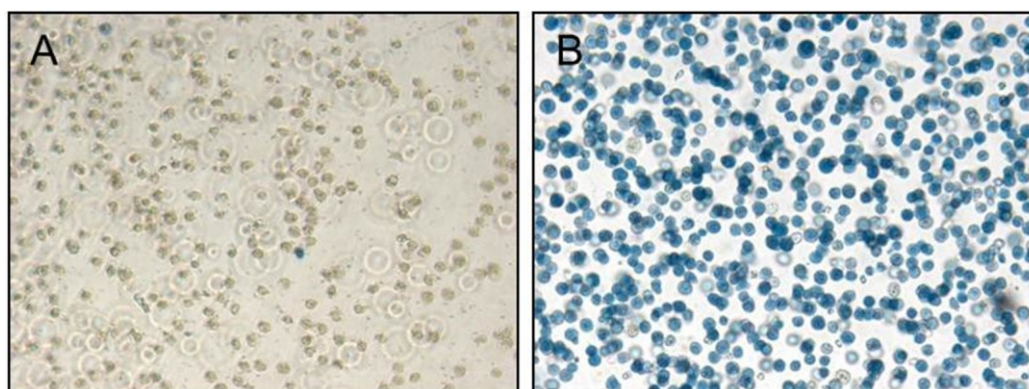


Figure 10. K562 untreated cells (A) and treated with 30  $\mu\text{M}$  compound **9** (B). The positive-benzidine cells are blue, depending from the accumulation of hemoglobin

Further experiments are required to verify whether decrease of cell growth is a pre-requisite for differentiation or is the cause of this feature. Figure 10 shows a microscopic analysis of K562 cells cultured taken after 6 days of K562 cell cultured in the absence or in the presence of 30  $\mu$ M compound **9**. In these assays, the blue-color of benzidine-stained cells is compatible with high accumulation of hemoglobin (Bianchi et al., 2001).

In a second set of experiments, the biological activity of compound **9** was compared to that of 5-fluorouracil (5-FU), extensively employed as anti-tumor agent in leukemias (Mini et al., 1990; Schilsky et al., 1996). Interestingly, as far as the effects on cell proliferation, similar  $IC_{50}$  values were obtained when compound **9** and 5-FU were employed.

Table 7. Antiproliferative effects ( $IC_{50}$ )<sup>a</sup> and induction of erythroid differentiation<sup>b</sup> (% B+ cells) of uracil, compound **9** and 5-fluorouracil on K562 cell line.

Compound	$IC_{50}$	% B+ cells (30 $\mu$ M)
Uracil	> 500 $\mu$ M	1.2 $\pm$ 0.2
Compound <b>9</b>	17.52 $\pm$ 2.88 $\mu$ M	92.3 $\pm$ 2.1
5-Fluorouracil	17.73 $\pm$ 2.23 $\mu$ M	3.1 $\pm$ 0.6

<sup>a</sup> Results are presented as average  $\pm$  S.D. (three independent experiments performed) of concentration needed to obtain 50% inhibition of cell growth after 3 days culture period.

<sup>b</sup> Values are expressed as average  $\pm$  S.D. (three independent experiments performed) after 6 days of treatment.

On the other hand the effects of compound **9** and 5-FU on erythroid differentiation were found to be very different, being only compound **9** able to activate the full program of differentiation. As expected, uracil, in agreement with the first set of results depicted in Table 6, did not show any effects on K562 with respect to inhibition of cell proliferation and induction of erythroid differentiation (Table 7).

### 3.3 Effects of compound **9** on the transcriptional activity of the $\gamma$ -globin and the $\beta$ -globin gene promoters of K562 cells

To determine the effects of compound **9** on the transcription of the globin genes, we used a clonal K562 cell population stably transfected with a reporter construct, carrying the genes for the green (EGFP) and red (RFP) fluorescent proteins (FP) genes, under the control of the  $\gamma$ -globin and the  $\beta$ -globin promoters, respectively (Guerrini et al., 2009). To compare the relative effect of compound **9** on the transcriptional activity of  $\gamma$ -globin and  $\beta$ -globin promoters, the experiment depicted in Figure 11 was performed. Cells were treated with 30  $\mu$ M compound **9**, isolated after 5 days and employed in FACS analysis. This assay shows that compound **9** enhanced EGFP by  $2.10 \pm 0.63$ , whereas the increase in RFP was  $1.39 \pm 0.10$  fold, indicating an effect of compound **9** on the transcription directed by both the  $\gamma$ -globin and  $\beta$ -globin gene promoters. On the contrary, as recently published by our research group, other erythroid differentiation inducers have a stimulatory selectivity for the  $\gamma$ -globin or the  $\beta$ -globin gene promoter. (Guerrini et al., 2009).

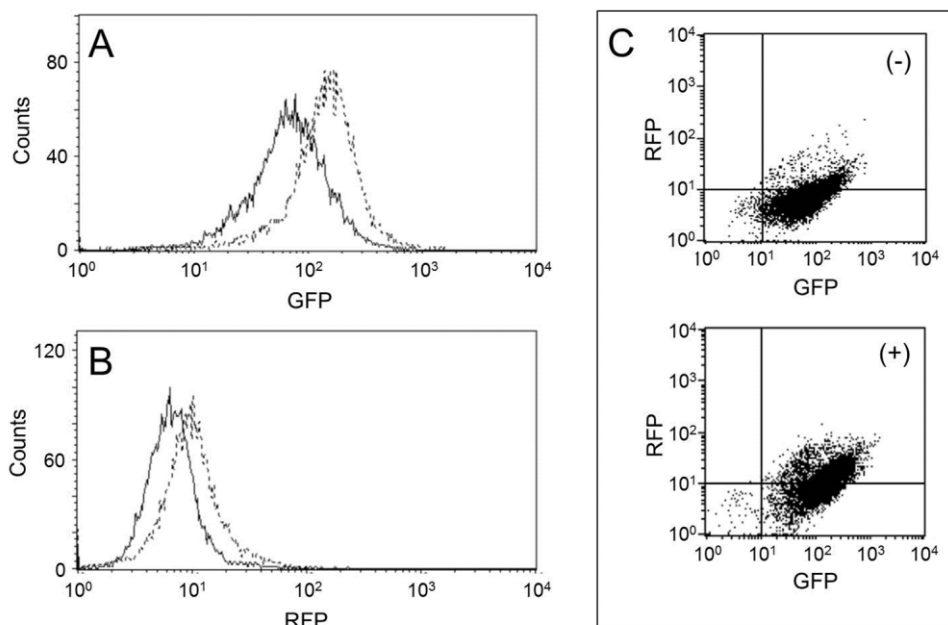


Figure 11. Representative example of FACS analysis of a K562 cell clone containing the reporter construct pCCL.Prom $\beta$ .HcRed1.Prom $\gamma$ .EGFP, untreated or treated with compound **9** 30  $\mu$ M for 5 days. **(A,B)** Histogram plots obtained from untreated (solid lines) or treated (dotted lines) cells, showing the relationship between number of cells and intensity of expressed fluorescence, green **(A)** or red **(B)**, respectively. **(C)** Dot plots obtained from untreated (-, upper panel) or treated (+, lower panel) cells, showing the cell population distribution as a function of the two different fluorescences



### 3.4 Effects of compound 9 on biochemical parameters associated to the activation of the K562 erythroid phenotype

The effects of compound **9** on the transcription of  $\alpha$ -globin mRNA and  $\gamma$ -globin mRNA were analyzed by qRT-PCR amplifications. Cells were treated with compounds and after 6 days total RNA was extracted by TriReagent assay, reverse transcribed into cDNA and amplified by Real Time PCR, using gene-specific double fluorescence labeled probes in an ABI Prism 7700 Sequence Detection System version 1.7.3 (Applied Biosystem, Monza, Italy). As reported in Figure 12a, the treatment with increasing concentration of compound **9** (10, 30 and 50  $\mu$ M) stimulates a sharp accumulation of  $\alpha$ -globin mRNA and  $\gamma$ -globin mRNA. More in detail, treatment with 10  $\mu$ M induce an increase of 8,88 times and 8,90 times for  $\alpha$ -globin mRNA and  $\gamma$ -globin mRNA, respectively; treatment with 30  $\mu$ M induce an accumulation of  $\alpha$ - and  $\gamma$ -globin mRNA of 14.22 and 16.22 greater than negative control (untreated cells); finally treatment with 50  $\mu$ M compound **9** is able to induce an increase of 17.03 times and 16.45 times for  $\alpha$ - and  $\gamma$ -globin mRNA.

We have also compared the globins accumulation of compound **9** with 100  $\mu$ M of compounds **10** and **11** (Figure 12b), which induce erythroid differentiation on K562 cell model to a much lower extent (Table 6).

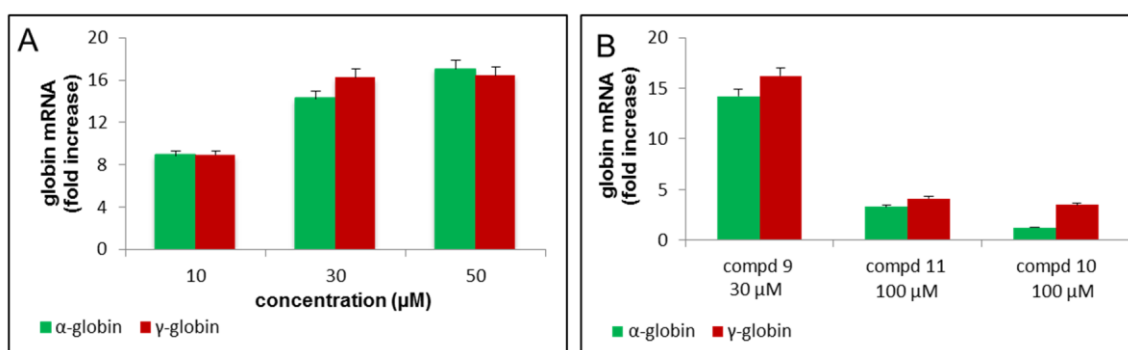


Figure 12. (A) Accumulation of  $\alpha$ -globin mRNA and  $\gamma$ -globin in K562 cells treated for 6 days with 30  $\mu$ M compound **9**. Results are presented as fold increase of  $\alpha$ -globin and  $\gamma$ -globin mRNAs with respect to untreated cells. (B) Effect of compounds **9**, **11** and **10** on accumulation of  $\alpha$ -globin and  $\gamma$ -globin mRNA. The results of untreated cells were taken as 1. Results represent the average  $\pm$ S.D. of three independent experiments

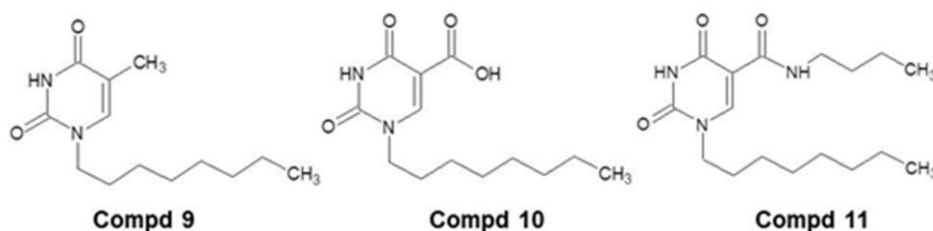


Figure 13. Comparison with chemical structures of compound **9**, **10** and **11**

Following the preliminary results (Table 6) compounds **10** and **11** induce only slight increase of accumulation of  $\alpha$ -globin (1.24 and 3.29, respectively) and  $\gamma$ -globin mRNAs (3.51 and 4.11, respectively). Further qRT-PCR data demonstrated that also the embryonic-type  $\zeta$ - and  $\varepsilon$ -globin mRNAs are induced by compound **9** (Figure 14). In addition, the effects of compound **9** on other genes involved in the expression of erythroid program. To this aim the mRNA coding glycophorin A (Watanabe et al., 1985; Kohmura et al., 2004; Ida et al., 2009) and transferrin receptor (Gambari et al., 1986; Harigae et al., 2006) were studied by qRT-PCR.

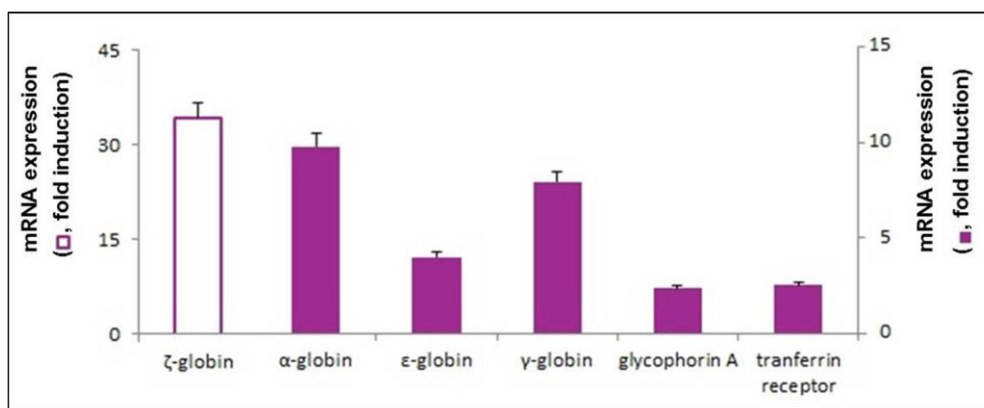


Figure 14. Accumulation (fold increase in respect to control uninduced cells) of the  $\alpha$ -like  $\zeta$ - and  $\alpha$ -globin mRNAs, the  $\beta$ -like  $\varepsilon$ - and  $\gamma$ -globin mRNA, the glycophorin A and transferrin receptor mRNAs in K562 cells treated for 6 days with 30  $\mu$ M compound **9**. Results represent the average  $\pm$ S.D. of three independent experiments

The results obtained are shown in Figure 14 and demonstrate that both relative amounts of glycophorin A and transferrin receptor mRNAs increase following treatment with compound **9**, confirms the induction of erythroid pathway by this compound.

### 3.5 Effect of compound 9 on hemoglobin accumulation measured by HPLC

After analyzing globins gene expression, we investigated, using HPLC technology, the accumulation of hemoglobin in K562 cells treated with the compound 9. Cells were treated for 6 days with compound 9, harvested, washed with PBS and lysed with water and incubation on ice for 15 minutes. After centrifugation, the supernatant was collected and hemoglobin proteins were separated by cation-exchange HPLC, using a Beckman Coulter instrument System Gold 126 Solvent Module-166 Detector and a 3.5 x 0.46 cm column packed with porous (100-nm pore size) 5-mm microparticulate polyaspartic acid-silica, Poly CAT A (Poly LC, Columbia MD, USA).

Fully in agreement with HPLC analyses, showing that this molecule induces a sharp increase of embryonic and fetal hemoglobins (Figure 15). At the retention time of 0.30 minutes we find the peak of embryonic hemoglobins, Gower 1 ( $\zeta_2\epsilon_2$ ) and Portland ( $\zeta_2\gamma_2$ ), constituting 100% of the sample composition. Treating cells with the compound 9, we can see a decrease to 76.19% of the embryonic hemoglobins, but an increase to 23.81% of fetal hemoglobin (retention time 2.208 minutes).

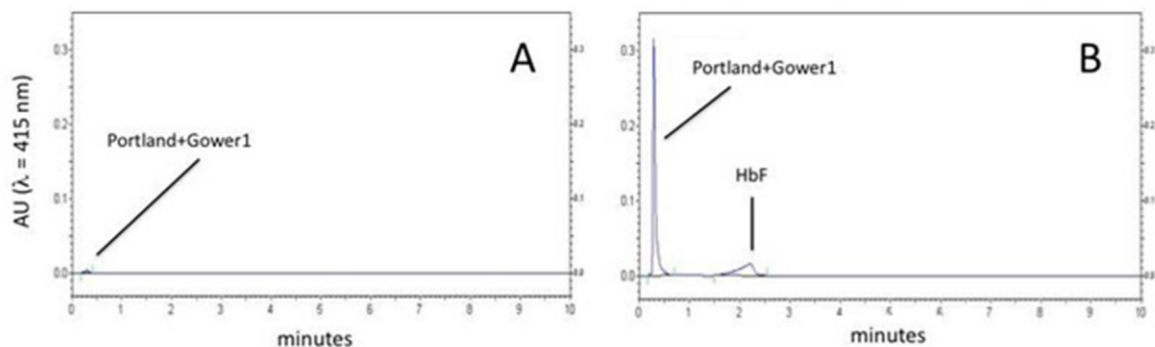


Figure 15. HPLC analysis of K562 cellular lysates: chromatogram A shows the hemoglobin profile (Hb Portland and Hb Gower1) in untreated cells, chromatogram B presents the increased production of Hb Portland/Gower1 and HbF in K562 cells treated with compound 9

### 3.6 The induction of erythroid differentiation of compound 9 is not associated with the activation of the apoptotic pathway

Since several inducers of erythroid differentiation are also strong inducers of apoptosis of K562 cells (for instance mithramycin and psoralens) (Bianchi et al., 2001; Lampronti et al., 2003) we performed a series of experiments to detect the

apoptotic induction of compound **9** in K562 cells. Cell cycle analysis was done by flow cytometry of propidium iodide-treated cells, using the FACS Calibur Becton Dickinson Immunocytometry System (Becton Dickinson, San Jose, CA, USA) (Figure 16). FACS analysis demonstrates no major alteration of cell cycle parameters and no accumulation of sub-G1 cells (an hallmark of apoptosis). As shown in Figure 16, there are no significant changes in the profile of the cell cycle; in fact G0/G1 phase is always about 39%, both in negative control and in treatments with 30 and 50  $\mu\text{M}$  compound **9**.

About apoptotic phase, the values change from 4.1% to 7.4% and finally to 10.2% (negative cells, 30 and 50  $\mu\text{M}$  compound **9**, respectively) of total cells; this variation is likely to be induced by the toxicity of the compound at used concentrations.

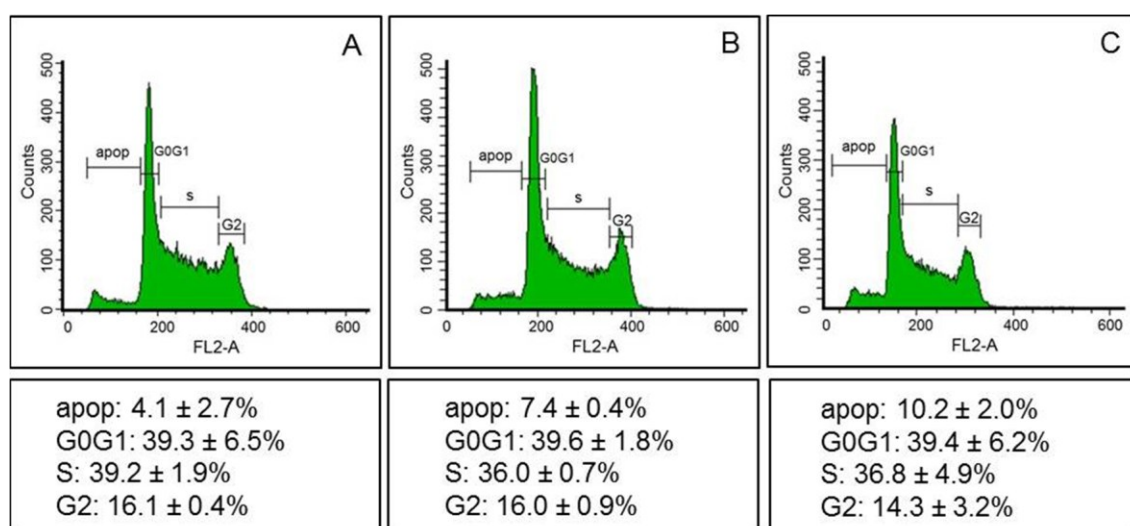


Figure 16. Analysis of cell cycle using a fluorescence-activated cell sorter (FACS). **A**: untreated K562 cells; **B,C**: K562 cells treated with 30  $\mu\text{M}$  (**B**) and 50  $\mu\text{M}$  (**C**) compound **9**. Similar data were reproducibly obtained in three independent experiments

Apoptosis was not evident also when *Annexin V-FITC* staining was performed. Cells were treated with the compounds for 72 hours, collected, centrifuged and rinsed with Binding Buffer; then, annexin V was added and the samples were incubated at room temperature for 15 min in the dark, according to the manufacturer's instructions. For the analysis we have used FACS Calibur Becton Dickinson Immunocytometry System (Becton Dickinson, San Jose, CA, USA) (Viola et al., 2008) (Table 8).

Finally, we have detected apoptosis using the colorimetric *DeadEnd TUNEL* assay (Figure 17). K562 cells were treated with the increasing concentrations of compounds; after 5 days, in according to the manufacturer's instructions, K562 were rinse with PBS and fixed with 4% paraformaldehyde at room temperature.

Table 8. Apoptosis of K562 cells detected by flow cytometry after annexin V–FITC staining. For this analysis the cells were treated with annexin V and PI, 30  $\mu$ M and 50  $\mu$ M compound 9.

Treatment	PI-positive (necrosis)	PI & Annexin positive	PI & Annexin negative	Annexin-positive (apoptosis)
NTC	0.0	0.04	99.91	0.05
NTC + Annexin	0.0	0.07	99.58	0.35
NTC + PI	0.39	0.04	99.53	0.03
NTC + Annexin & PI	0.13	0.18	98.88	0.80
Compd 9 30 $\mu$ M	0.59	1.44	96.96	1.00
Compd 9 50 $\mu$ M	1.31	0.66	97.52	0.51

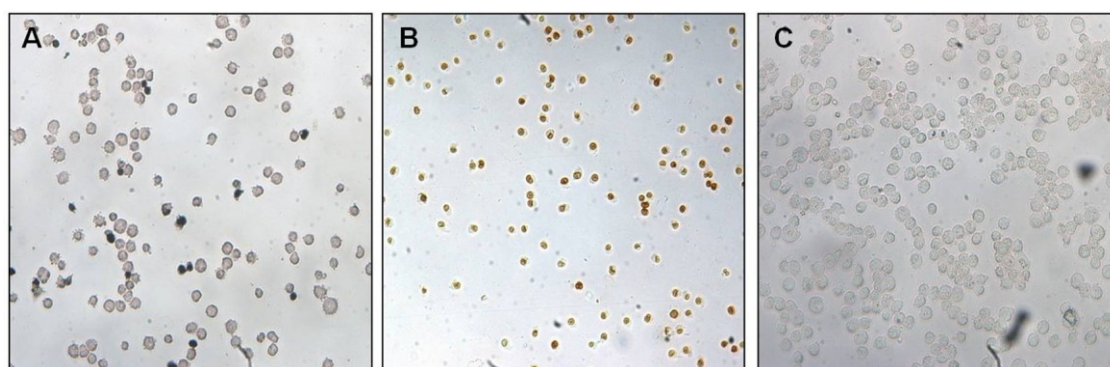


Figure 17. Detection of apoptosis by DeadEnd TUNEL assay in K562 cell line (A), treated with DNase I (1U/ml) (B), 30  $\mu$ M compound 9 (C). Brown color reaction, visible only in the treatment with the DNase I, indicates apoptotic cells. Cells were photographed at a magnification of 20X

Measurement of apoptosis was calculated as a % of apoptotic nuclei (dark brown nuclei) versus total nuclei, evaluated in three independent experiments. As shown in Figure 20 the treatment with 30  $\mu$ M of compound 9 not induce apoptosis. For positive control we have used DNase I, a well-known apoptotic inducer.

The results reported in *Part 1* of thesis are already published in the following paper:

Brogna E, Lampronti I, Breveglieri G, Accetta A, Corradini R, Manicardi A, Borgatti M, Canella A, Multineddu C, Marchelli R, Gambari R., 2011. **C(5) modified uracil derivatives showing antiproliferative and erythroid differentiation inducing activities on human chronic myelogenous leukemia K562 cells.** Eur J Pharmacol. 672(1-3):30-37

## - DISCUSSION AND CONCLUSIONS (1) -

The biological activity of the derivatives tested in this study can lead to the following alternative effects: (a) a strong antiproliferative effect linked to an high level of erythroid differentiation activity and (b) an antiproliferative effect independent from the activation of the erythroid program (Table 6). The highest antiproliferative effect and erythroid induction ability was exhibited by compound **9**, a thymine derivative bearing a *n*-octyl chain on nitrogen N(1), whereas thymine (compound **2**) did not show any effect, suggesting the importance of the linear alkyl chain in position N(1). The insertion of the alkyl chain in both N(1) and N(3) positions of thymine (compound **12**) brought a loss of erythroid induction activity with retention of antiproliferative effect, suggesting the fundamental role of hydrogen in position N(3) of thymine for the erythroid induction activity. Appreciable, even if in some cases much lower, erythroid differentiation activity was shown by derivatives **8**, **10** and **18**. Activity of compound **10** can be rationalized considering its structural similarities to compound **9** (methyl group substituted by COOH); compounds **8** and **18** show the same pattern, when properly oriented (Brognara et al., 2011); therefore, they can be considered as “C-nucleoside” equivalent (Lu et al., 2009) of compound **9** with exchange of the role of N(1) and C(5), which leads to N(3)-H in the same relative orientation with respect to the lipophilic group as in thymine derivatives. In conclusion we found a simple thymine derivative, N(1)-octyl-thymine that exhibit strong antiproliferative activity, high ability to induce terminal erythroid differentiation without activation of the apoptotic pathway. To our knowledge this compound should be considered among the most efficient inducers of erythroid differentiation of K562 cells.

However, this work should be considered as the starting point for the development of more effective and specific drugs for the induction of terminal erythroid differentiation, with the aim of leading new insights in the treatment of neoplastic diseases with molecules acting by inducing differentiation rather than simply by exerting cytotoxic and/or antiproliferative effects. Compound **9** might be of some interest since strongly inhibits cell growth (the activity is similar to that displayed by 5-fluorouracil), but at the same time does induce erythroid differentiation. In this respect, the development of new compounds against chronic myelogenous leukaemia cell lines might be of great interest, since it has been

already demonstrated that synergistic effects of molecules inducing differentiation can be useful for the efficient treatment of chronic myelogenous leukaemia (Rabizadeh et al., 2007, Jakubowska et al., 2008).

In addition, and more importantly, Imatinib mesylate-resistant human chronic myelogenous leukemia cell lines can be treated with erythroid inducers, as recently demonstrated using the phytoalexin resveratrol (Puissant et al., 2008). It should be interesting to determine the activity of compound **9** in Imatinib-resistant cell lines, since the effects of resveratrol as erythroid-inducing agent are significantly different from those displayed by compound **9** in respect to induction of globin mRNAs (Bianchi et al., 2009) and activation of apoptosis and cell-cycle alterations (Puissant et al., 2008).

On the other hand, molecules similar to compound **9** might be of interest for the experimental treatment of  $\alpha$ -thalassemic erythroid cells, for which the induction of  $\alpha$ -globin mRNA could be very beneficial (Fibach et al., 2003; Gambari et al., 2007). In this respect it has been demonstrated that inducers of K562 erythroid differentiation are often able to induce fetal hemoglobin production in erythroid cells isolated from  $\alpha$ -thalassemia patients (Gambari et al., 2007).

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## PART 2

### *MODIFIED PNAs INTERFERING WITH miR-221 IN HUMAN BREAST CANCER CELL LINES*



## - INTRODUCTION -

The double helix of DNA is Nature's simple and elegant solution to the problem of storing, retrieving, and communicating the genetic information of a living organism. DNA has many important characteristics that allow it to perform these functions. Two of the most important properties are the specificity and the reversible nature of the hydrogen bonding between complementary nucleobases, properties which allow the strands of the double helix to be unwound and then rewound in exactly the same configuration.

The field of life science realized early on the important implications of these traits. If specific, single strands of DNA could be synthesized, then the base sequences of genes could be studied and manipulated using these defined molecules. Synthetic oligonucleotides are now indispensable tools for life scientists, with many applications in molecular biology, genetic diagnostics, and most likely also soon in medicine.

### **1.1 Peptide nucleic acids**

Peptide nucleic acid (PNA) is an artificially synthesized polymer similar to DNA or RNA invented by Peter E. Nielsen, Michael Egholm, Rolf H. Berg and Ole Buchardt (Univ. Copenhagen) in 1991 (Nielsen et al., 1991). As shown in Figure 1 While DNA and RNA have a deoxyribose and ribose sugar backbone, respectively, PNA's backbone is composed of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds. The various purine and pyrimidine bases are linked to the backbone by methylene carbonyl bonds (Egholm et al., 1993).

PNA was originally designed as a ligand for the recognition of double stranded DNA (Nielsen et al., 1999). The concept was to mimic an oligonucleotide binding to double stranded DNA via Hoogsteen base pairing in the major groove. Thus the nucleobases of DNA were retained, but the deoxyribose phosphodiester backbone of DNA was replaced by a neutral pseudo-peptide backbone. Nielsen and his colleagues could not imagine all the possible properties and applications that could be developed based on the neutral backbone. With many properties that set them



apart from traditional DNA analogs, PNAs have added a new dimension to synthetic DNA analogs and mimics in molecular biology, diagnostics, and therapeutics.

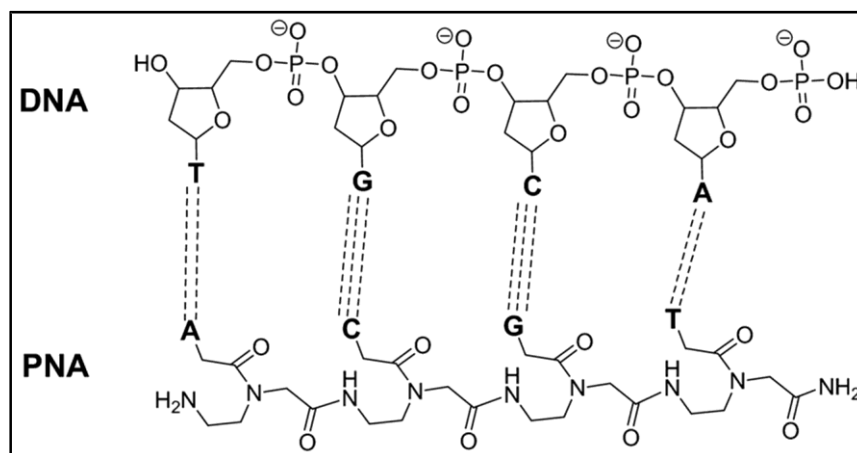


Figure 1. DNA and PNA chemical structures

### 1.1.1 PNAs Structure and Properties

The PNAs have been introduced by Nielsen *et al.* (1999); PNAs are synthetic DNA analogs in which the phosphodiester backbone is replaced by repetitive units of N-(2-aminoethyl) glycine to which the purine and pyrimidine bases are attached via a methyl carbonyl linker (Figure 1). The procedures for PNA synthesis are similar to those employed for peptide synthesis, using standard solid-phase manual or automated synthesis. The PNA molecules can routinely be labeled with biotin or fluorophores. A subsequent generation of PNAs could involve modification of the N-(2-aminoethyl) glycine backbone (PNA analogs) or chimeric architecture, like PNA-peptide chimeras or PNA-DNA chimeras developed in order to improve the solubility and the cellular uptake of PNAs or to exhibit new biological properties (Koch *et al.*, 1995). The synthetic backbone provides PNA with unique hybridization characteristics. Unlike DNA and RNA, the PNA backbone is not charged. Consequently, there is no electrostatic repulsion when PNAs hybridize to its target nucleic acid sequence, giving a higher stability to the PNA-DNA or PNA-RNA duplexes than the natural homo- or heteroduplexes. This greater stability is reflected by a higher thermal melting temperature ( $T_m$ ), as compared to the corresponding DNA-DNA or DNA-RNA duplexes (Jensen *et al.*, 1997). The unnatural backbone of

PNAs also means that PNAs are not degraded by nucleases or proteases. For instance, incubation of PNAs with S1 nuclease or DNase I has no effect on PNA (Demidov et al., 1993). Owing to this resistance to the enzyme degradation, the lifetime of PNAs is extended both *in vivo* and *in vitro*. Also, PNAs are not recognized by polymerases and therefore cannot be directly used as primers or be copied.

### 1.1.2 Strand invasion and PNAs applications

PNAs hybridize to complementary DNA or RNA in a sequence-dependent manner, according to the Watson–Crick hydrogen bonding scheme. In contrast to DNA, PNA can bind in either parallel or antiparallel manner. However, the antiparallel binding is favored over the parallel one. Structural information on the PNA binding modes have been obtained by nuclear magnetic resonance and by X-ray crystallography (Rasmussen et al., 1997).

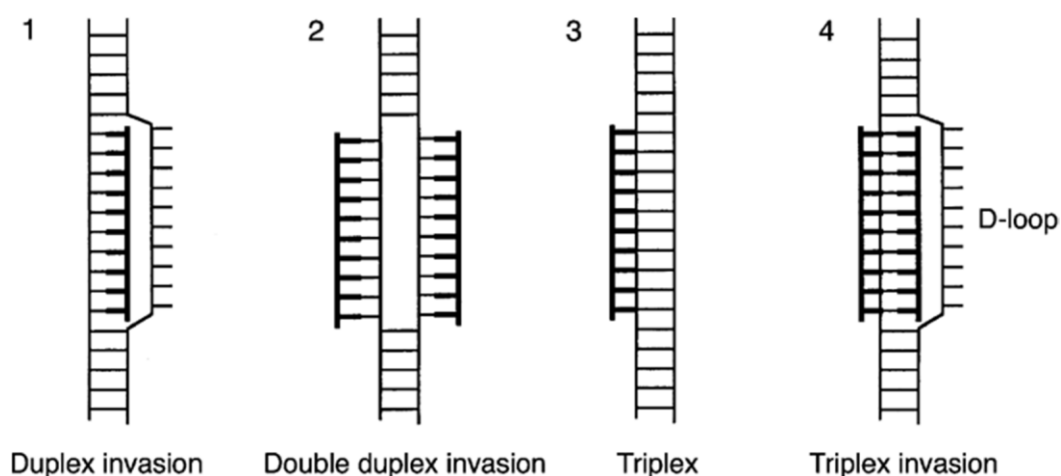


Figure 2. Scheme of PNA binding modes for targeting double-stranded DNA. PNA oligomers are drawn in bold. (1) Standard duplex invasion complex formed with some homopurine PNAs. (2) Double-duplex invasion complex, very stable but only possible with PNAs containing modified nucleobases. (3) Conventional triple helical structure (triplex) formed with cytosine-rich homopyrimidine PNAs binding to complementary homopurine DNA targets. (4) Stable triplex invasion complex, leading to the displacement of the second DNA strand into a 'D-loop'.

PNA probes can bind to either single-stranded DNA or RNA, or to double-stranded DNA (Figure 2). Homopyrimidine PNAs with a minimum of 10-mers, as well as PNAs containing a high proportion of pyrimidine residues, bind to complementary

DNA sequences to form highly stable (PNA)<sub>2</sub>–DNA triplex helices displaying T<sub>m</sub> over 70°C. In these triplexes, one PNA strand hybridizes to DNA through standard Watson–Crick base pairing rules, while the other PNA strand binds to DNA through Hoogsteen hydrogen bonds. The resulting structure is called P-loops. The stability of these triple helices is so high that homopyrimidine PNA targeted to purine tracts of dsDNA invades the duplex by displacing one of the DNA strands (Nielsen et al., 1999). The efficiency of this strand invasion can be further enhanced by using two homopyrimidine PNA oligomers connected via a flexible linker or by the presence of nonstandard nucleobases in the PNA molecule.

Finally, PNA–DNA hybridization is significantly more affected by base mismatches than DNA–DNA hybridization. Using a 15-mer PNA, all possible single mismatch combinations were tested in both PNA/DNA duplexes and corresponding DNA/DNA duplexes. In the PNA/DNA duplexes, the average T<sub>m</sub> was 15°C, whereas it was 11°C in the corresponding DNA/DNA duplexes (Giesen et al., 1998; Nielsen et al., 1999). Similar results were obtained for PNA/RNA duplexes. This high level of discrimination at single base level indicates that short PNA probes could offer high specificity, and thus allow the further development of several PNA-based strategies for molecular investigations and diagnosis.

### **1.1.3 PNAs as bioactive molecules for gene expression control**

A very important point suggesting PNAs as appealing molecules in the field of artificial regulation of gene expression, both *in vitro* and *in vivo*, is that they are resistant to both nucleases and proteases (Borgatti et al., 2003; Calabretta et al., 2011). Borgatti *et al.* have reported that PNAs and PNA–DNA chimeras, unlike DNA molecules, are stable in cellular extracts and serum; in their studies, these authors also determined the resistance of double-stranded decoy molecules based on PNA–DNA chimeras to exonucleases (both 3′→5′ and 5′→3′ exonucleases), endonucleases and 5′-phosphatases (Borgatti et al., 2003). Finally, they used liposomes as protective agents in experimental conditions in which the DNA-based molecules were found to be unstable (high concentrations of enzymes, cellular extracts or serum). The results obtained demonstrated that PNA-based molecules are more resistant than DNA-based decoys to exo- and endo-nucleases, serum and cellular extracts. In

addition, the resistance of PNA-based molecules in the presence of high concentrations of serum and cellular extracts was increased after complexation to cationic liposomes. These studies are in agreement with other published reports demonstrating PNAs as very stable molecules in cell culture conditions and, more importantly, in biological fluids (Demidov et al., 1994; Shiraishi et al., 2008; Gambari et al., 2010). Moreover, because PNA oligomers lack of a repetitively charged backbone, they do not interact with polyanion-binding proteins, which may complicate the actions of nucleotide analogues when used as gene-targeting agents (Stein et al., 1999). Accordingly, PNAs have been proposed as excellent candidates for *in vitro* and *in vivo* modulation of gene expression, as shown in Figure 3.

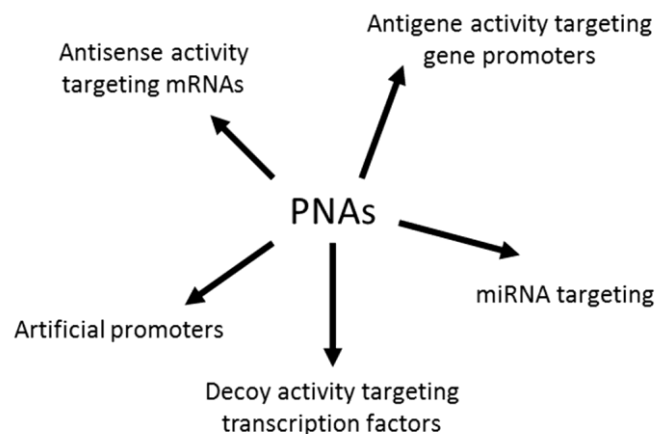


Figure 3. Biological activity of PNAs derived from the most recent papers available in the literature

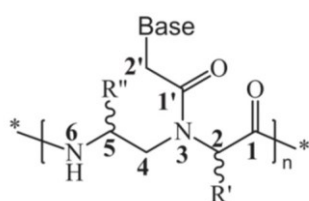
PNAs are able to target RNA molecules mimicking the antisense approach firmly recognized as a very important experimental strategy to inhibit the biological activity of target mRNAs. For instance, Shiraishi *et al.* used antisense PNAs targeting the 5'-or 3'-splice sites in intron 2 or the 3'-splice site of intron 3 of *mdm2* pre-mRNA in order to inhibit RNA splicing and expression of the *mdm2* human cancer gene in choriocarcinoma (JAR) cells. Treatment of JAR cells with bioactive PNAs resulted in a reduction in the level of MDM2 protein and a concomitant increase in the level of tumor suppressor p53. In addition, a combination of this PNA with the DNA damaging agent camptothecin inhibited cell growth more than camptothecin alone. The interesting conclusion was that antisense targeting of splice junctions of *mdm2* pre-

mRNA may be a powerful method to evaluate the cellular function of MDM2 splice variants as well as a promising approach for discovery of *mdm2* targeted anticancer drugs (Shiraishi et al., 2010). PNAs were found to be very effective in targeting promoters (by triple-helix formation or following strand invasion). In this specific case, they act as inhibitors of transcription, but also as transcriptional activators (Mollegaard et al., 1994; Tonelli et al., 2005). Finally, despite the fact that PNA–PNA and PNA–DNA hybrids exhibit low binding efficiency to transcription factors (TFs) and therefore cannot be proposed as efficient TFs decoy molecules (Mischiati et al., 1999). PNA–DNA chimeras have been described as reagents of great interest in gene therapy, as they are able to stably interact with TFs inhibiting TF-dependent effects (Borgatti et al., 2003; Gambari et al., 2004; Borgatti et al., 2004). This was studied by our research group and was demonstrated for NF- $\kappa$ B and Sp1, for which we designed bioactive double stranded PNA–DNA–PNA chimeras. However, as for many large macromolecules, therapeutic applications of unmodified PNAs remain limited by their low cellular uptake and poor ability to reach their intracellular target. In this respect, PNAs can be modified in order to achieve better performances in terms of cellular permeation, higher affinity, and specificity for the target DNA and RNA sequences (Wojciechowski et al., 2007; Manicardi et al., 2010; Zhou et al., 2006; Shiraishi et al., 2011) In any case, their affinity for RNAs make these reagents of great interest in all the experimental approaches involving RNA targeting.

#### **1.1.4 Modified PNAs and PNAs delivery**

It's important to understand the effect of PNAs on intact cells and problems related to its delivery into the cell. The cellular uptake of this unique nucleic acid analogs is very low in eukaryotic cells (Rasmussen et al., 2006): this is the major limit in the use of PNAs for the alteration of gene expression and, for this reason, it is still considered to be the major challenge that must be overcome before it can be used as therapeutic drug (Arghya et al., 2000). In order to remove this drawback, several approaches have been considered, for example, cellular microinjection and delivery of unmodified PNAs. Moreover, the use of cationic liposomes and/or microspheres is not indicated, in consideration of the low binding efficiency of PNAs to these structures (Fabbri et al., 2011).

In this respect, we have proposed to use PNA-DNA hybrids or PNA-DNA chimeras to dock the PNAs to cationic liposomes (Nastruzzi et al., 2000; Borgatti et al., 2002; Cortesi et al., 2004). However, the use of cationic liposomes and microspheres might be toxic to target cells, especially if for the experiments primary cells were employed. Another possible strategy consists to link PNAs to polylysine (K) or a polyarginine (R) tails, based on the observation that this cell-membrane penetrating oligopeptides are able to facilitate uptake of conjugated molecules (Abes et al., 2008). Since this discovery, many other modifications of the original PNA backbones have been proposed in order to improve performances in term of affinity and specificity. Modification of the PNA backbone with positively charged groups (Figure 4) has also been demonstrated to enhance cellular uptake and consequently PNA efficiency (Zhou et al., 2003, 2006; Corradini et al., 2007).



R' = R'' = H : standard achiral PNA  
 R' = side chain, R'' = H : acyclic chiral PNA substituted at position 2  
 R' = H, R'' = side chain : acyclic chiral PNA substituted at position 5

Figure 4. Structure of backbone modified PNA

Although the steric requirements for binding RNA have not been extensively studied so far, the availability of different chemical strategies to design and synthesize PNA analogues is the basis for the development of new peptide nucleic acids (PNAs) specifically aimed at targeting RNA, to be used for miR targeting (Section 1.2.6).

In the last few years several research groups have been involved in the synthesis and in the studies of the binding properties of PNAs with a chiral constrained backbone obtained by insertion of stereogenic centers either at the C2 (alpha) or C5 (gamma) position of the monomer. The insertion of one chiral monomeric unit in a PNA strand has resulted in increased DNA binding affinity, when the side chain was positively charged (e.g. lysine or arginine). Therefore, the affinity

of chiral PNAs for complementary DNA emerged to be a contribution of different factors: electrostatic interactions, steric hindrance and, most interestingly, enantioselectivity with a preference for the D-configuration at the 2 position of the monomer forms (Menchise et al., 2003). The 2D-lysine "chiral box" PNA showed also increased sequence selectivity, both in terms of direction control and of recognition of a single base mismatch (Sforza et al., 2000). In fact, this type of structures was found ideal for targeting point mutations in genes of diagnostic interest as published by our collaborators Corradini *et al.*, 2004, and Tedeschi *et al.*, 2005. Recently, Ly and co-workers reported the synthesis and uptake properties of  $\gamma$ GPNA, in which the PNA backbone had a homo-arginine side chain at the 5-position (or  $\gamma$  position) (Sahu et al., 2009), showing an excellent cellular uptake. More recently, chiral PNAs with L- or D-stereocenters either at the 2- or the 5-positions of the monomer or with both stereocenters simultaneously present have also been synthesized (Sforza et al., 2007, Manicardi et al., 2010); the biological activities of these modified PNAs are describe in *Results section of Part 2*.

Substitution at both C(2) and C(5) carbons of the PNA backbone with amino acid side chains leads to ambivalent structures having properties of DNA or RNA mimic on one side and peptide mimics on the other side, thus allowing recognition by specific receptors, as shown very recently by a short PNA mimicking the function of a nuclear localization peptide (NLS) (Sforza et al., 2010). Thus, and to obtain PNAs with both peptide properties and RNA binding ability. This strategy can be used to further improve the efficiency of PNAs for miR targeting. In fact, the use of peptides as carriers represents a "Achille's heel" of the potential PNA-based drug candidates, since the peptide part might be subjected to enzymatic degradation, whereas the incorporation of the peptide signal into the PNA backbone does not lead to enzymatic degradation, even in the presence of highly active proteases.

PNAs bearing modified nucleobases able to induce additional interactions providing high improvement in RNA and DNA binding affinities have also been described (Wojciechowski et al., 2009). Combination of modified nucleobases and backbone modification with C(2) or C(5) modified residues was found to be the best approach in order to achieve strand invasion into mixed DNA sequences (Ishizuka et al., 2009, Chenna et al., 2008), a strategy which could also be very fruitful in challenging double-stranded miRs.

### 1.1.5 Synthesis of Modified peptide nucleic acids monomers

The peptide nucleic acids (PNAs) used in our experiments were synthesized by Prof. Roberto Corradini and his groups (University of Parma, Dept. of Organic and Industrial Chemistry) (Manicardi et al., 2010). They first designed the synthesis of new class of dimers by substitution of amide linker on C(5) with more flexible methyl amino linker, shows in Figure 5; this modification should depress part of self-aggregation of dimmers in favor of solubility since secondary amine is protonate in physiological conditions.

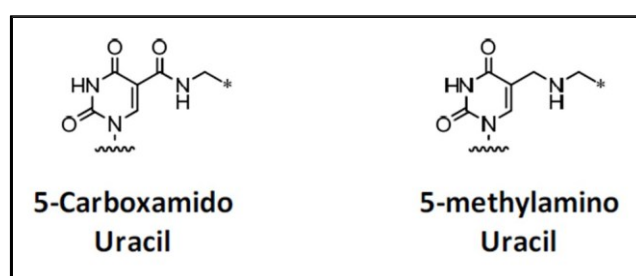


Figura 5. 5-Carboxiamido-Uracil and 5-methylamido Uracil structures

Synthesis of these dimers was well described in *section 1.1.3 of Part 1*; briefly, it was designed by reaction of 5-formyl-uracils with different diamine linker through reductive amination reaction. The synthesis of 5-formyl-uracils was carried out by oxidation of N(1)-alkylated thymine or by formylation under Vielsmeyer-Haack conditions of N(1)-alkylated-N(3)-benzoylated uracil. The synthesis of 5-methylazidouracil PNA monomers is described below in two phases: synthesis of 5-methylazidouracil (Figure 6) and its incorporation into Boc-amino-ethyl-glycine backbone (Figure 7). The synthesis of 5-methylazidouracil started from the regioselective hydroxymethylation of C(5) of uracil to afford 5-hydroxymethyluracil **20**, that was transformed to the alkyl chloride **21** using concentrated HCl. Nucleophilic substitution of **21** with sodium azide gives the desired product **22**. This last reaction was carried out at low temperature (0°C) in order to avoid self-reaction of **21** on its N(1) or N(3), which would form polymeric compounds, thus lowering the yield. 5-methyluracil was then regioselectively alkylated on N(1) by bromoacetic acid esters to give **23** and **24**, leading to only 49% yield due to formation of the bis-alkylated side



product. Ester deprotection of **23** and **24** using appropriate conditions (basic hydrolysis for **24** and acid cleavage for **23**) leads to compound **25**. 5-methylazidouracil acetic acid **25** was activated with DCC/DhBtOH and linked to the Boc-aeg-OEt backbone to afford the ethyl ester PNA monomer **26**. The hydrolysis of ethyl ester of **26** afforded the desired PNA monomer ready to use for the solid phase synthesis of oligomers (Accetta A., 2010).

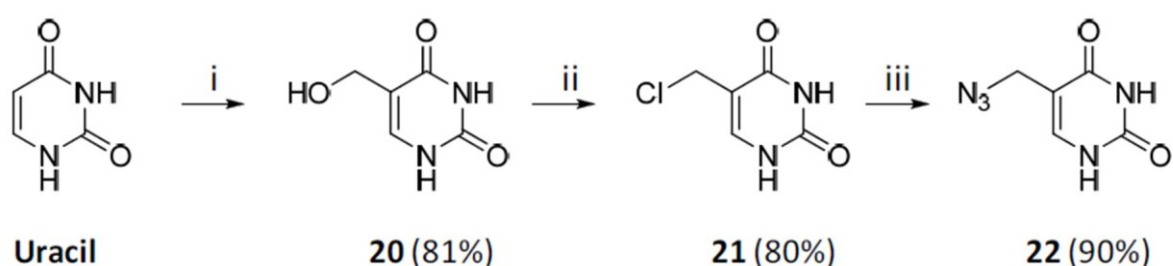


Figure 6. Synthesis of 5-azidomethyluracil. (i) [CH<sub>2</sub>O, Et<sub>3</sub>N, water, 60°C, overnight]. (ii) [HCl 37%, 4H, rt]. (iii) [NaN<sub>3</sub>, DMF, 0°C, 1h]

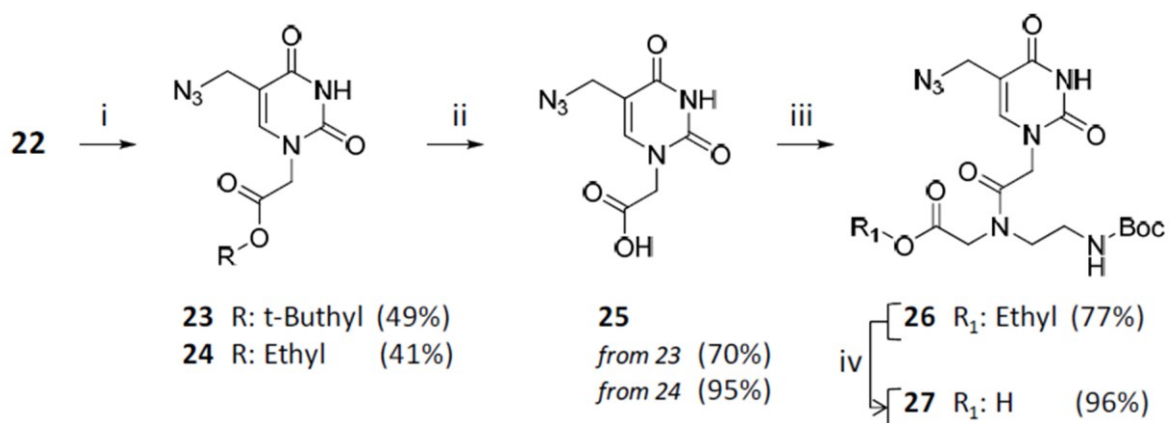


Figure 7. Synthesis of 5-methylazidouracil Boc-PNA monomer. (i) [BrCH<sub>2</sub>COOR, K<sub>2</sub>CO<sub>3</sub>, DMF, rt, overnight]. (ii) [(a) TFA/DCM; (b) NaOH, water/MeOH for 24h]. (iii) [DCC, DhBtOH, DIEA, DMF]. (iv) [NaOH, water/MeOH]

### 1.1.6 Synthesis of PNAs oligomers based on engineered uracil derivatives

Chemical modification of Peptide Nucleic Acids (PNA) is being studied extensively as an approach for the development of improved probes for nucleic acids recognition and for therapy based on gene regulation (Tonelli et al., 2005). The synthesis of modified PNA oligomers often requires optimization and slightly modification of the standard procedures in order to ensure better yields and purity and was performed using different strategies: the synthesis of PNA oligomers containing *Mod-1* and *Mod-2* was performed by hybrid Boc/Fmoc chemistry, while modified monomers (*Mod-3*) were Fmoc protected at the aminoethyl nitrose (Figure 8). The PNA oligomer containing *Mod-2* showed interesting recognition properties towards complementary DNA. The amide group directly linked to the C(5) position of uracil (Figure 9a) was tolerated and did not lead to a dramatic drops in stability of the PNA:DNA duplex.

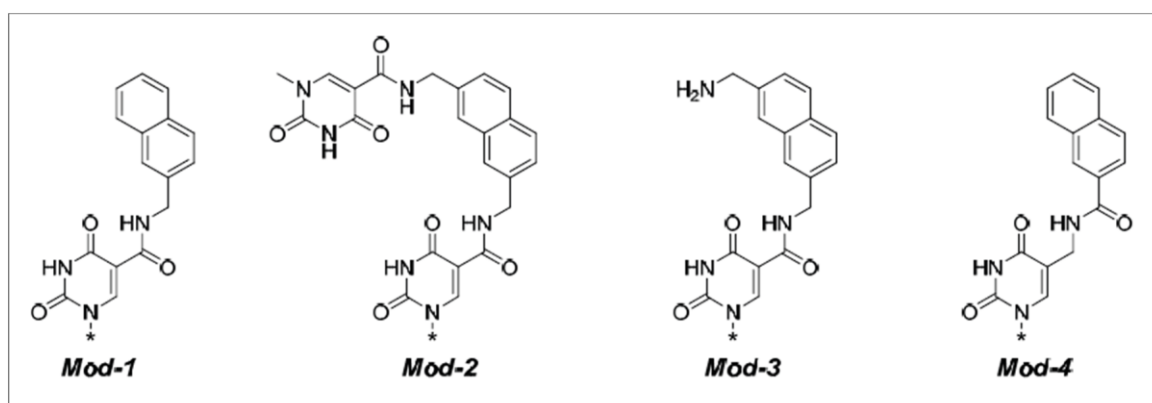


Figure 8. Modification incorporate into PNA oligomers employed

However, we explored the possibility to use derivatives with a substituent showing electronic effects more similar to those of thymine by simply reversing the amide (*Mod-4*) on the C(5) position of uracil (Figure 9b). PNA oligomers containing *Mod-4* were synthesized using an innovative strategy by means of an azide modified precursor monomer of uracil which could be further modified by solid phase synthesis. This strategy can allow faster and simpler synthesis of oligomers, since it avoids the necessity to synthesize PNA monomers containing hindered and complex modified bases.

Furthermore, the modification of the base on solid phase could avoid problems of steric hindrance in coupling steps, since the modified base is built step by step. The 5-(azidomethyl)uracil-PNA-monomer (Figure 10) was chosen as the common precursor monomer.

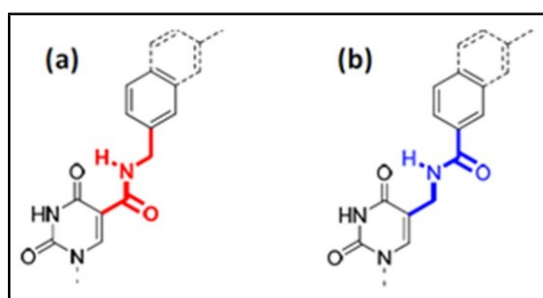


Figure 9. (a) Carboxamido derivatives; (b) Reversed Amide, methylamido derivatives

The azide group was chosen because it is easily transformed into an amino group under mild conditions, such as those used in Staudinger's reduction, which can be performed also on solid phase; the amino group thus obtained can be used in reactions with activated carboxylic acids to form a new amide bond on the C(5) position of uracil.

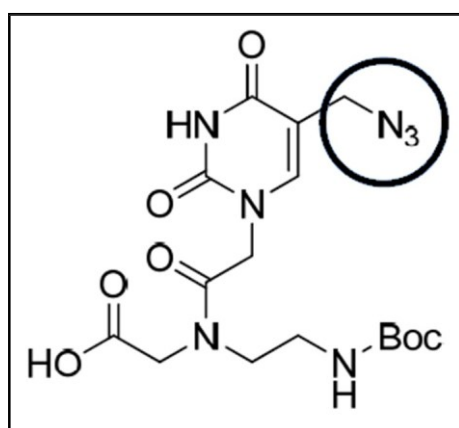


Figure 10. 5-(azidomethyl)-PNA monomer. The azide group was chosen as hidden and protected amino group

In conclusion, these findings are not only important for improving of the stability and selectivity of PNA oligomers, but it is important since these engineered PNAs were specifically designed in order to precisely interact with specific sites on complementary DNA or RNA. Inversion of the amide group of Mod-1 to Mod-4 resulted to be detrimental for stability, but not for selectivity; however, in this case the different fluorescence response of naphthalene in PNA PR AAUN3 with full-match and mismatched DNA opens the possibility to develop fluorescent probes for DNA recognition.

## **1.2 Targeting microRNAs involved in human diseases**

### **1.2.1 The microRNA**

The identification of all epigenetic modifications involved in gene expression is one of the major steps forward for understanding human biology in both normal and pathological conditions. This field is referred to as epigenomics, and it is defined as epigenetic changes (i.e. DNA methylation, histone modification and expression of noncoding RNAs such as microRNAs) on a genomic scale (Costa F., 2010). In this context, microRNAs play a pivotal role.

MicroRNAs (miRNAs, miRs) are a family of small (19–25 nucleotides in length) noncoding RNAs that regulate gene expression by sequence-selective targeting of mRNAs, leading to a translational repression or mRNA degradation, depending on the degree of complementarity between miRNAs and the target mRNA sequences (He et al., 2010; Krol et al., 2010). Since their discovery and first characterization in 1993 by Ambros V., Lee R. and Feinbaum R., the number of microRNA sequences deposited in the miRBase databases is continuously growing (Kozomara et al., 2010) and the microRNAs are proving to have an important role in the cellular regulatory pathways.

### **1.2.2 MicroRNA biological relevance: involvement in the gene expression control**

Considering that a single miRNA can target several mRNAs and a single mRNA might contain in the 3'-UTR sequence several signals for miRNA recognition, it is calculated that at least 10-40% of human mRNAs are a target for microRNAs (Tsai et al., 2010). In fact, a single miRNA might bind to as many as 200 gene targets and that these targets can be diverse in their function; they include transcription factors, secreted factors, receptor and transporters. So, miRs potentially control the expression of about one-third of human mRNAs (Kerscher et al., 2006).

Therefore, a great interest is concentrated on the identification of validated targets of microRNAs. This specific field of microRNA research has confirmed that the complex networks constituted by miRNAs and RNA targets coding for structural and regulatory proteins lead to the control of highly regulated biological functions, such as differentiation (Masaki et al., 2007), cell cycle (Wang et al., 2009) and apoptosis (Subramanian et al., 2010).

Since a single 3'UTR of a given mRNA contains signal sequences for several microRNAs, which microRNA should be targeted in order to achieve alteration of the expression of the gene should be experimentally evaluated. With respect to the possible effects of the expression of other mRNA targets, it should be clearly stated that alteration of a single microRNA might retain multiple effects.

On the other hand, miRNAs usually bind to their targets with incomplete complementarity; for this reason, the identification of gene targets with only a simple BLAST search is impossible. However, current bioinformatics approaches have taken advantage of the fact that miRNAs within families have highest homology at the 5' end of the mature miRNA, which is crucial for the stability and proper loading of the miRNA into the miRISC complex (Schwartz et al., 2003).

### **1.2.3 Biogenesis of microRNAs**

Some miRNAs are encoded by unique genes (intergenic miRNAs) (Berezikov et al., 2007) and others are embedded into the intronic regions of protein-coding genes (intragenic miRNAs) (Hinske et al., 2010). The transcription by RNA polymerase II of these miR genes gives rise to long primary miRNAs (pri-miRNAs)

with typical stem-loop structures. These are rapidly processed by the nuclear RNase endonuclease-III Drosha, which, removing the branches, gives rise to precursor miRNAs (pre-miRNA) of around 60-100 nucleotides in length (Figure 11). In both cases of intergenic miRNAs and intragenic miRNAs, the pre-miRNAs are transported from the nucleus to the cytoplasm by exportin-5. In the cytoplasm, pre-miRNAs are further processed by another RNase endonuclease-III (Dicer) to generate mature miRNAs around 22-nt long, which generate the RNA-induced silencing complex RISC.

#### **1.2.4 MicroRNA and gene regulation**

Structurally, miRNAs are small non-coding regulatory RNAs; these small RNAs post-transcriptionally repress gene expression by recognizing complementary target sites most often in the 3' untranslated region (UTR) of target messenger RNAs (mRNAs) (Bagga et al., 2005). However, animal miRNAs may also target 5'UTR and coding regions of mRNAs, as documented by experiments involving both artificial and natural mRNAs and also by bioinformatic predictions (Orom et al., 2008).

MicroRNAs silence the expression of the target mRNAs, either by mRNA cleavage or by translation repression. Nevertheless, it has been described that miRNAs can also increase the expression of a target mRNA (Vasudevan et al., 2007). Perfect miRNA:mRNA complementarity leads to cleavage of the mRNA by AGO2; this is the small interfering RNA (siRNA) pathway, which while important experimentally, is not thought to occur with endogenous mammalian miRs. Instead, the imperfect pairing with mRNA causes a down-regulation of translation, even if the mechanism by which this occurs is still not clear. Whatever the precise nature of the mechanism, affected mRNAs accumulate in granular cytoplasmic "P-bodies" along with RISC proteins (Pillai et al., 2005). Generally, mRNA abundance is ultimately also reduced (Hendrickson et al., 2009). This is important, as it means that the impact of miR activity can be assessed (at least to some extent) by measures of mRNA abundance such as expression profiling.

The basic mechanism leading to alteration of gene expression is based on the recruitment of mature miRNA at the level of the RISC silencing complex. This process occurs in the cytoplasm, where the pre-miRNA hairpin is cleaved by the

RNase III enzyme Dicer, which interacts with the 3' end of the hairpin and cuts away the loop joining the 3' and 5' arms, yielding an imperfect miRNA/ miRNA duplex. One of the strands is incorporated into the RISC, where it binds to target mRNA sequence. Perfect or near perfect base pairing with the target RNA promotes cleavage of the RNA (Choe et al., 2011). It is proposed that in the case of partially complementary microRNAs, in order to recognize their targets, nucleotides 2-7 of the miRNA (the 'seed sequence') are important (Cuccato et al., 2011).

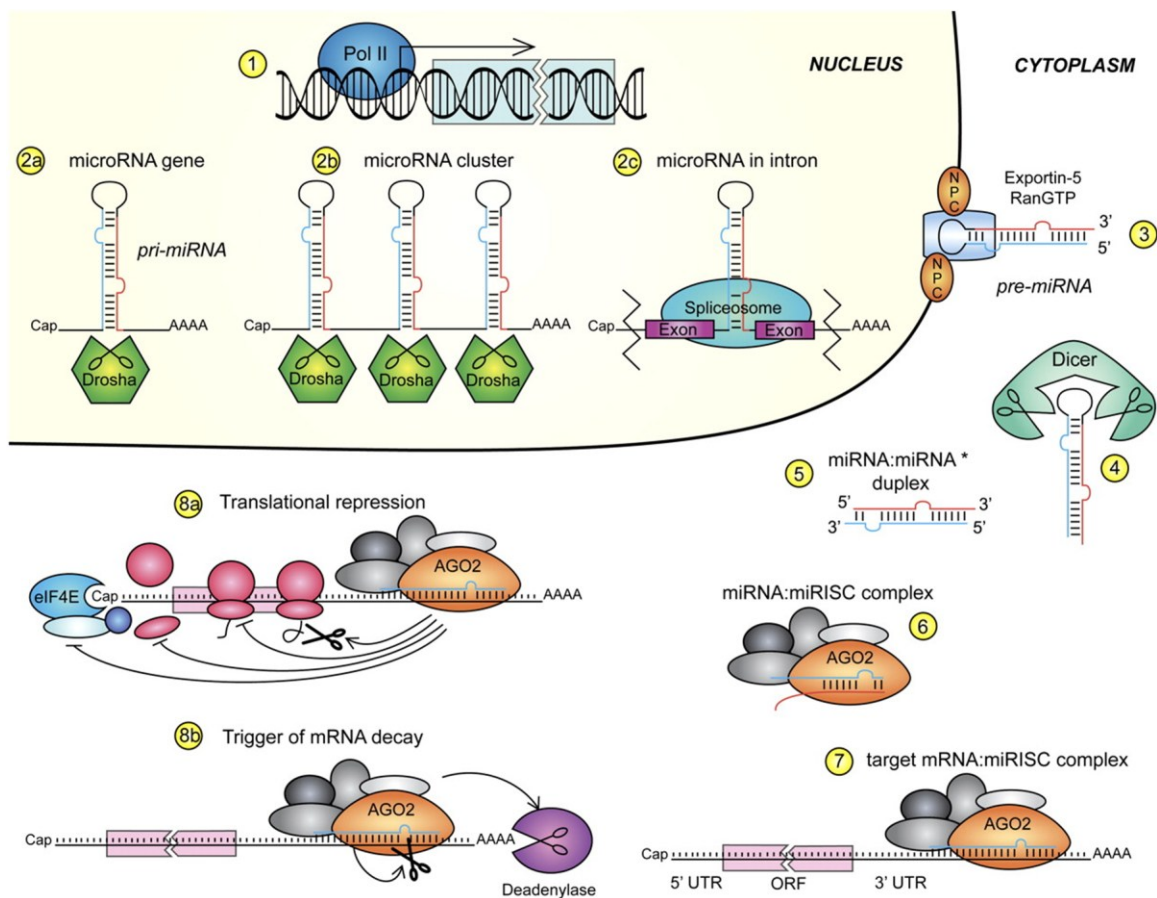


Figure 11. Biogenesis and action of miRNAs. (Step 1) miRNAs are transcribed mainly by polymerase II. miRNA transcribed from genes (step 2a), polycistronic clusters (step 2b), or the intronic region (step 2c), called pri-miRNAs, and in the first two cases are then processed by the type III RNase Drosha. (Step 3) The newly formed stem-loop structure, pre-miRNA, is recognized by the XPO5, Ran-GTP complex, and is transported to the cytoplasm through the NPC. Dicer cleaves the loop (step 4), leaving a double-strand fragment, the miRNA:miRNA\* duplex (step 5). The blue strand is the future mature miRNA, while the red strand is its antisense miRNA\*. The duplex is then unwound and loaded into the miRISC complex (step 6) where it recognizes and anneals to its mRNA target 3' UTR (step 7). The messenger RNA:miRISC complex mediates translational repression (step 8a) and mRNA decay (step 8b). *From Araldi E., Schipani E., Genes Dev, 2010; 24:1075-1080*

This is the key process permitting mature miRNAs to exert their effects in gene regulation. The final effect of miRNAs activity is the inhibition of the synthesis of the protein(s) encoded by the target mRNA(s). This has of course important biological implications depending on the role of the protein in the cellular network. Since a single 3'UTR of a given mRNA contains signal sequences for several microRNAs, applied biological studies are needed to determine which microRNA should be targeted to achieve alteration of gene expression. Possible effects on the expression of other mRNA targets should be considered. An alteration of a single microRNA may exhibit multiple effects, possibly in combination with the targeting activity of other miRNAs, enabling the achievement of strong biological effect (Hemida et al., 2010; Bader et al., 2010).

### **1.2.5 MicroRNA and cancer**

Like transcription factors, miRNAs regulate diverse cellular pathway and are widely believed to regulate most biological processes. Genetic (deletion, amplification or translocation) or epigenetic (DNA methylation, histone modification and expression of non-coding microRNAs) alterations had been demonstrated to be associated with human pathologies. A recent study showed that about 50% of annotated human microRNAs are located in areas of the genome, known as “fragile sites”, that are associated with cancer; for this reason, miRNAs play a pivotal role in cancer development and progression (Calin et al., 2004).

As well as guided alterations of miRNAs have been suggested as a novel approach to develop innovative therapeutic protocols. MicroRNA therapeutics appears as a novel field in which miRNA activity is the major target of the intervention (Hemida et al., 2010; Sibley et al., 2010). Interestingly, microRNAs play a double role in cancer, behaving both as oncogenes or tumor suppressor genes (Figure 12).

In general, miRs promoting cancer targets mRNA coding for tumor-suppression proteins, while microRNAs exhibiting tumor-suppression properties usually target mRNAs coding oncoproteins (Table 2). MicroRNAs which have been demonstrated to play a crucial role in the initiation and progression of human cancer are defined as oncogenic miRNAs (oncomiRs). The oncomiR expression profiling of human malignancies has also identified a number of diagnostic and prognostic



cancer signals; table 1 show representative examples of oncomiRs (Cho et al., 2007; Lowery et al., 2008). Mir-15a and miR-16 are severely down-regulated in 70% of patients with chronic lymphocytic leukemia (CLL) and induce apoptosis by targeting antiapoptotic gene B cell lymphoma 2 (BCL2) mRNA (Cimmino et al., 2005), which is a key player in many types of human cancers including leukemias, lymphomas, and carcinomas (Sanchez et al., 2003).

Emerging evidences suggests that miR let-7 family may play a critical role in lung cancer development. Takamizawa *et al.* (2004) observed that the expression levels of let-7 were frequently reduced in both in vitro and in vivo lung cancer studies. Also Johnson *et al.* (2005) showed that lung tumor tissues display significantly reduced levels of let-7 and significantly increased levels of RAS protein relative to normal lung tissue, suggesting let-7 regulation of RAS as a mechanism for lung oncogenesis. MiR-let7 is not down-regulated in lung cancer, but also in breast, gastric, ovary and colon cancer, while it is overexpressed in AML disease. Colorectal neoplasia is also characterized by alterations in miRNAs expression. MiR-145 and miR-143 are frequently reduced at the adenomatous and cancer stages of colorectal neoplasia. However, it was also described that the levels of pre-miR-143 and pre-miR-145 are not altered in precancerous and neoplastic colorectal tissue, suggesting that post-transcriptional control is the cause for the reduced mature miRNA levels (Michael et al., 2003).

The role of miR-26a in carcinogenesis appears to be a complicated one, in the sense that both oncogenic and tumor suppressive effects were reported in cancers such as glioblastoma and hepatocellular carcinoma, respectively. A reduced expression of miR-26a is observed in hepatocellular carcinoma (HCC) cells; in a recent study published by Chen *et al.* (2011), tumors had reduced levels of miR-26 expression, as compared with paired non-cancerous tissues, which indicated that the level of miR-26 expression was also associated with hepatocellular carcinoma. The same miR is, instead, overexpressed in breast cancer and glioblastoma (Huse et al., 2009).

An opposite effect on tumor development is displayed by other miRNAs; miR-200 family is deeply involved in EMT process and is deregulated in breast cancer (Korpal et al., 2008). miR-10b is highly expressed in metastatic breast cancer cells and positively regulates cell migration and invasion (Ma et al., 2007).

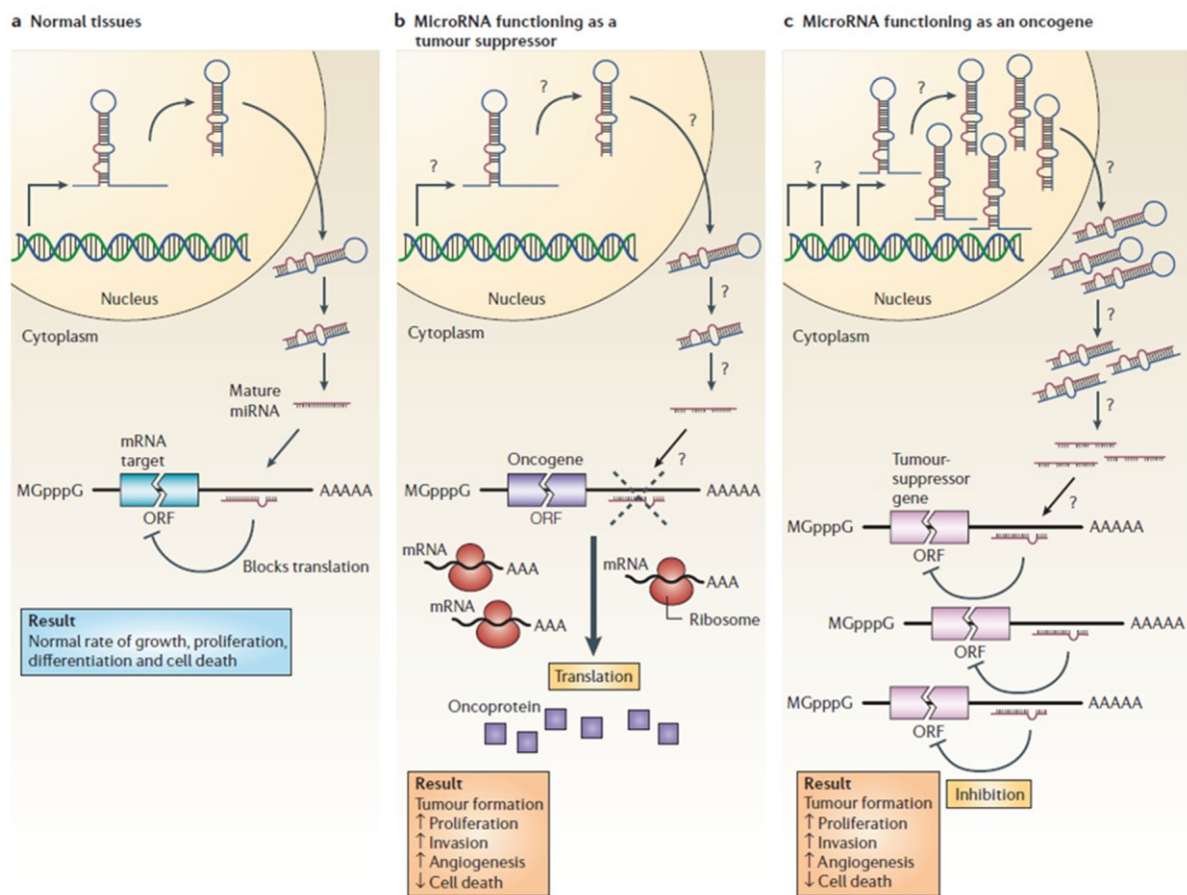


Figure 12. MicroRNAs can function as tumor suppressors and oncogenes.

**(a)** In normal tissues, proper miRNA transcription, processing and binding through complementary sequences on the target mRNA results in the repression of target-gene expression through a block in protein translation or altered mRNA stability (not shown). The overall result is normal rates of cellular growth, proliferation, differentiation and cell death. **(b)** The reduction or deletion of a miRNA that functions as a tumor suppressor leads to tumor formation. A reduction in or elimination of mature miRNA levels can occur because of defects at any stage of miRNA biogenesis (indicated by question marks) and ultimately leads to the inappropriate expression of the miRNA-target oncoprotein (purple squares). The overall outcome might involve increased proliferation, invasiveness or angiogenesis, decreased levels of apoptosis, or undifferentiated or de-differentiated tissue, ultimately leading to tumor formation. **(c)** The amplification or overexpression of a miRNA that has an oncogenic role would also result in tumor formation. In this situation, increased amounts of a miRNA, which might be produced at inappropriate times or in the wrong tissues, would eliminate the expression of a miRNA-target tumor-suppressor gene (pink) and lead to cancer progression. Increased levels of mature miRNA might occur because of amplification of the miRNA gene, a constitutively active promoter, increased efficiency in miRNA processing or increased stability of the miRNA (indicated by question marks). ORF, open reading frame.

From Kerscher E., *Oncomirs-microRNAs with a role in cancer*, 2006, *nature reviews*, 6:259-269

Table 1. MiRNAs exhibiting oncogene function in cancer (oncomiRs). (*Gambari et al., 2011*)

MicroRNA	Disease	Biological effects	Target mRNA/pathway	Reference
miR-182	Melanoma	Promotion of melanoma metastasis	MITF and FOXO3	Segura, 2009
miR-221; miR-222	Atypical teratoid/ rhabdoid tumors (ATRT)	Inhibition of the tumor suppressor and inhibitor of cell cycle p27Kip1	p27Kip1	Sredni, 2010
miR-221; miR-222	Glioblastoma	Inhibition of cell apoptosis	PUMA	Zhang CZ, 2010
miR-21	Breast, colon, pancreas, lung, prostate, liver, and stomach cancer; chronic lymphocytic leukemia; glioblastoma	Stimulation of cellular proliferation, apoptosis, and migration; action on mitochondrial apoptosis tumor-suppressive pathways	PTEN, PDCD4, TPM1, P53 and TGF- $\beta$	Volinia, 2006; Frankel, 2008; Calin, 2005; Garzon, 2008; Chan JA, 2005; Papagiannakopoulos, 2008
miR-122a	Hepatocellular carcinoma	Cell cycle regulation, DNA repair, carcinogenetic process leading to HCC development	CCNG1	Gramantieri, 2007
miR-214	Ovarian cancer	Stimulation of cell survival and cisplatin resistance	PTEN	Yang H, 2008
miR-132; miR-212	Pancreatic adenocarcinoma (PDAC)	Stimulation of cell proliferation via b2 adrenergic pathway	Rb1	Park JK, 2011
miR-375	Gastric cancer	Promotion of carcinogenesis	JAK2 and PDK1	Xu Y, 2011
miR-23b*	Renal cancer cells	Down-regulation of POX (tumor suppressor), increase of HIF signaling	POX	Liu W, 2010
miR-301	Breast cancer	Promotion of growth, proliferation, invasion, and metastases	FOXF2, BBC3, and PTEN	Shi W, 2011
miR-675	Colorectal cancer (CRC)	Overexpression of H19 (oncofetal non-coding RNA) in cancer tissues	RB	Tsang WP, 2010
miR-296	Brain tumors	Promotion of angiogenesis	HGS	Wurdinger, 2008
miR-10b	Human esophageal cancer cells	Promotion of migration and invasion	KLF4	Tian Y, 2010
miR-378	Breast carcinoma	Enhancement of cell survival; reduction of caspase-3 activity; promotion of tumor growth and angiogenesis	Sufu and Fus-1	Lee DY, 2007
miR-372; miR-373	Testicular tumors	Promotion of tumorigenesis in cooperation with RAS	LATS2	Voorhoeve, 2007

Abbreviations: MITF, microphthalmia-associated transcription factor-M; FOXO3, forkhead box O3; p27Kip1, cyclin-dependent kinase inhibitor 1B; PUMA, BCL2 binding component 3; PTEN, PDCD4, phosphatase and tensin homolog; TPM1, tropomyosin 1; P53, tumor protein p53; TGF- $\beta$ , transforming growth factor, beta 1; CCNG1, cyclin G1; Rb1, retinoblastoma tumor suppressor; JAK2; PDK1, 30-phosphoinositide dependent protein kinase-1; E2F1, E2F transcription factor 1; Bim, BCL2-like 11 (apoptosis facilitator); CDH1, cadherin 1, type 1, E-cadherin (epithelial); POX, proline oxidase; FOXF2, forkhead box F2; BBC3, BCL2 binding component 3; RB, retinoblastoma 1; HGS, hepatocyte growth factor-regulated tyrosine kinase substrate; KLF4, Kruppel-like factor 4; Sufu, suppressor of fused homolog (Drosophila); Fus-1, tumor suppressor candidate 2; and LATS2, large tumor suppressor, homolog 2 (Drosophila)

In human testicular germ cell tumors two miRNAs were reported to be oncogenic, miR-372 and miR-373; they down-regulate p53-mediated CDK inhibition through direct inhibition of the Large Tumor Suppressor Homolog 2 (LATS2), and permitted the proliferation and tumorigenesis of primary human cells which have both oncogenic RAS and active wild-type p53 (Voorhoeve et al., 2006). In several types of lymphomas, including Burkitt's lymphoma, the expression of miR-155 is increased compared to normal cells. Mir-155 is located in the conserved region of the BIC gene and expression of BIC/miR-155 is elevated in Hodgkin and Burkitt lymphoma (Eis et al., 2005).

Table 2 MiRNAs exhibiting tumor suppressor functions (*Gambari et al., 2011*)

MicroRNA	Disease	Biological effects	Target mRNA/pathway	Reference
miR198	Hepatocellular carcinoma	Inhibition of migration and invasion	HGF/c-MET	Tan S., 2011
miR-449	Gastric cancer	Inhibition of cell proliferation	GMNN, MET, CCNE2 and SIRT1	Bou Kheir T, 2011
miR-181b	Chronic lymphocytic leukemia	Inhibition of disease progression	Mcl-1 and Bcl-2	Visone R, 2011
miR-193b	Breast cancer	Alteration of ERalfa signaling, such as steroid synthesis and down-regulation of the ERalfa receptor	AKR1C2, AKR1C1, YWHAZ (14-3-3 family protein)	Leivonen, 2011
miR-218	Gastric cancer	Suppression of tumor metastasis	ROBO1	Tie J, 2010
miR-126	Non-small cell lung cancer cells	Tumor suppressor genes and involved in development, cell proliferation and cell death, cell migration and blood vessel formation	EGFL7	Sun Y, 2010
miR-15a; miR-16-1	Chronic lymphocytic leukemia	Induction of apoptosis and decrease tumorigenicity	Bcl-2	Calin, 2008
miR-145; miR-133a; miR-133b	Esophageal squamous cell carcinoma	Inhibition of cell proliferation and cell invasion	FSCN1	Kano M, 2010
miR-1	Head and neck squamous cell carcinoma (HNSCC)	Inhibition of cell proliferation, invasion, and migration and promotion of apoptosis and cell cycle arrest	TAGLN2	Nohata N, 2011
miR-205	Human prostate	Reduction of cell migration/invasion through down-regulation of protein kinase Ce	CHN1, ErbB3, E2F1, E2F5, ZEB2, PRKCE	Gandellini P, 2009
miR-101	Neuroblastoma; bladder transitional cell carcinoma (TCC)	Inhibition of proliferation and clonogenic growth; alteration of global chromatin structure	MYCN EZH2, the catalytic subunit of PRC2	Friedman, 2009
miR-204	Neuroblastoma	Stimulation of increased sensitivity to cisplatin treatment and promotion of cell survival	TrkB	Ryan, 2011

Abbreviations: HGF, hepatocyte growth factor (hepatopoietin A; scatter factor); c-MET, met proto-oncogene (hepatocyte growth factor receptor); GMNN, geminin, DNA replication inhibitor; CCNE2, cyclin E2; SIRT1, sirtuin 1; Mcl-1, myeloid cell leukemia sequence 1 (BCL2-related); BCL2, B-cell CLL/lymphoma 2; AKR1C1, aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase); AKR1C2, aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III); YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide; ROBO1, Robo1 receptor; EGFL7, EGF-like-domain, multiple 7; FSCN1, fascin homolog 1, actin-bundling protein (*Strongylocentrotus purpuratus*); TAGLN2, transgelin 2; CHN1, chimerin (chimaerin) 1; ErbB3, v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian); E2F1, E2F transcription factor 1; E2F5, E2F transcription factor 5, p130-binding; ZEB2, zinc finger E-box binding homeobox 2; PRKCE, protein kinase C, epsilon; MYCN, v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian); EZH2, enhancer of zeste homolog 2; PRC2, polycomb repressive complex 2; and TrkB, neurotrophic tyrosine kinase, receptor, type 2

Finally, also miR-17-92 cluster was found overexpressed in lung cancer (Hayashita et al., 2005). In conclusion, miRNAs are deeply involved in tumor onset and progression so that therapeutic strategies involving miRNA silencing have been proposed. Since miRNAs can behave as tumor suppressor genes, miRNA replacement therapy has been also proposed as a possible therapy of cancer (Bader et al., 2010). However, it remained unclear if the reduced miRNA expression was the cause or the consequence of malignant transformation.

### *1.2.5.1 MicroRNA in breast cancer*

Over the past few years, miRNA profiling studies have led to the identification of miRNAs that are aberrantly expressed in human breast cancer. The function of only a handful of these miRNAs in breast cancer has been investigated. As in other cancers, some miRNAs can function as tumor suppressors and other miRNAs as oncogenes. Thus, tumor formation may arise from a reduction or deletion of a tumor suppressor miRNA and/or amplification or overexpression of an oncogenic miRNA. In addition, tumor metastasis may be promoted by enhanced expression of prometastatic and/or down regulation of antimetastatic miRNAs. The functions of these miRNAs in breast tumor progression and metastasis are discussed below.

### *1.2.5.2 Tumor suppressor miRNA in breast cancer*

Among all, recent findings have focused attention especially on some miRs that inhibit the breast cancer formation and/or metastases. MiR-206 is up-regulated in estrogen receptor (ER)  $\alpha$ -negative breast cancer; it was recently shown to inhibit the expression of ESR1 mRNA through two binding site in the ESR1 3'-UTR (Adams et al., 2007). Another study showed that miR-206 expression decreased in ER $\alpha$ -positive human breast cancer tissues and that miR-206 suppresses ESR1 expression and inhibits growth of MCF-7 breast cancer cells (Leivonen et al., 2009). The tumor suppressive role for miR-206 in breast cancer was further substantiated by the recent demonstration that miR-206, miR-335 and miR-126 are potently down-regulated in metastatic breast cancer cells compared to parental cells. Importantly, restoring the expression of these three miRNAs reduced their invasive capacity (Tavazoie et al., 2008).

MiR-125a and miR-125b are down-regulated in HER2-amplified and HER2-overexpressing breast cancers. These two miRNAs are potential tumor suppressors and their overexpression in SKBR3 cells (a HER2-dependent human breast cancer cell line) suppresses HER2 and HER3 mRNA and protein levels, leading to a reduction in anchorage-dependent growth, cell motility, and invasiveness (Scott et al., 2007).

Invasion and metastasis are the hallmarks of malignant tumor progression. Increasing evidence indicates that activation of the embryonic program “epithelial-

mesenchymal transition" (EMT) promotes these processes by allowing detachment of cells from each other, thereby increasing tumor cell mobility and dissemination. In recent years, it has become evident that aberrant activation of EMT is responsible for the malignant transformation of many human cancers. EMT is activated by EMT-inducing transcriptional repressor, including ZFH family transcription factors. Recently, ZEB1, a ZFH-family member, was shown to be a crucial EMT activator in human cancers, including breast cancer (Aigner et al., 2007). The miR-200 family of miRNAs has been shown to be crucial inducers of an epithelial phenotype by suppressing the expression of the EMT inducers ZEB1 and ZEB2: over expression of miR-200 family members in mesenchymal cells initiated mesenchymal-to-epithelial transition and, by additional analysis, decrease in miRNA-200 family miRNAs was associated with highly aggressive, metaplastic breast tumors (Gregory et al., 2008), indicating that miR-200 family was strongly associated with the epithelial phenotype. MiR-34a is one of several miRNAs that are down regulated in multiple cancers and has been shown to be transcriptionally regulated by p53. In the context of breast cancer, only one study has shown that miR-34a levels were lower in triple negative and mesenchymal breast cancer cell lines compared with normal epithelial lines and HER-2+ lines (Kato et al., 2009).

MiR-31 was recently shown to prevent metastasis at multiple steps by inhibiting the expression of prometastatic genes. MiR-31 is expressed in normal breast cells and its abundance was shown to be dependent on the metastatic state of the tumor. It is moderately decreased in non-metastatic breast cancer cell lines and is almost undetectable in metastatic mouse and human breast cancer cell lines. Importantly, the authors demonstrated that introducing miR-31 in metastatic breast cancer cells suppressed metastasis-related functions (motility and invasion) in vitro and metastasis in vivo (Valastyan et al., 2009).

#### 1.2.5.3 Oncogenic miRNA in breast cancer

MiR-21 was one of the first oncogenic miRs to be characterized, being up-regulated in numerous tumors, a finding initially made in glioblastoma (Chan et al., 2005) and soon afterward in breast cancer (Iorio et al., 2005). Si *et al.* (2007) found that antagonizing miR-21 caused apoptosis in MCF-7 cells, which was associated

with lower expression of Bcl-2 protein in the anti-miR-21-transfected MCF-7 cells and also in tumors derived from these cells, suggesting that Bcl-2 may be an indirect target of miR-21.

MiR-155 is over-expressed in a number of human malignancies, including breast cancer. A recent study has shown that miR-155 is up-regulated in normal mouse mammary gland epithelial cells (NMuMG cells) by the TGF- $\beta$ /Smad4 pathway and mediates TGF- $\beta$ - induced EMT and cell invasion (Kong et al., 2008). They further demonstrated that miR-155 is associated with cancer invasiveness in human primary breast carcinoma by showing that miR-155 is highly expressed in invasive tumors but not in noninvasive cancer tissues. These results implicate miR-155 in EMT and invasion as observed in NMuMG cells and suggest that miR-155 may play a critical role in breast cancer metastasis.

The miR-10 family consists of miR-10a and miR-10b. They are up-regulated in numerous tumors, including brain, colon and liver cancer (Volinia et al., 2006; Gaur et al., 2007; Varnholt et al., 2008). MiR-10a binds to several mRNAs via their 5'UTRs, inducing up-regulation and overexpression aids RAS-driven formation in cell culture (Orom et al., 2008). Unlike miR-155, miR-10b was highly expressed only in metastatic cancer cells; in fact, miR-10b overexpression initiates invasion and metastasis in a murine model of breast cancer (Ma et al., 2007); the same study reports an association between miR-10b expression and metastasis in human tumors.

MiRNAs of the miR-373/520c family were identified as prometastatic using a forward genetic screen involving overexpression of almost 450 miRNAs in a non-metastatic human MCF-7 breast cancer cell line (Kato et al., 2009).

### **1.2.6 Antisense strategy and miR targeting**

The most widely used approach to alter processing or targeting functions of microRNA is the antisense strategies, which have been reported in several papers in which microRNAs have been targeted by anti-miR molecules. This antisense strategy appears to be more specific in respect to targeting of transcription factors or miRNA promoters, since it affects single microRNAs or microRNA families, while targeting transcription factors or transcription factors binding sites is expected to have deep

effects on the whole transcriptome, due to the fact that a single transcription factor is able to bind and regulate several genes. In addition, recent reports suggest that miRNAs can be packaged in exosome fractions, followed by release of exosome/miRNAs from producing cells into body fluids. This last feature has important diagnostic/prognostic implications.

Given the role of miRNAs in epigenetic regulation of gene expression, miRNAs have been proposed as possible candidates for drug targeting with the objective of interfering with biological functions, altering the expression of the mRNAs specifically regulated by the targeted miRNAs (Kota et al., 2010). The number of known microRNAs which regulate gene expression is continuously growing, with 1,527 sequences present to date in the miRbase for humans (as available on January 30th, 2012 at [www.mirbase.org](http://www.mirbase.org)). An increasing number of reports have shown that targeting of microRNA biogenesis has a deep impact on specific phenotypes and even on pathological conditions. It has been demonstrated that miRNAs can be antagonized in vivo by oligonucleotides composed of highly affine nucleotide mimics (Elmen et al., 2008). Up to now synthetic oligonucleotides have been used for targeting microRNAs, although with several problems, including delivery and stability. Only one of the two strands of miR (termed guiding strand) is incorporated into the miRISC complex, and is therefore the ideal target for oligonucleotides designed to inhibit miR functions. Though long mRNA containing multiple target sites could serve as scavenger for miR (called RNA sponge), RNA molecules are not very effective as anti-miR, since they would recreate a dsRNA similar to the miR precursor.

Therefore, several modified oligonucleotide analogs have been designed in order to bind to the guiding strand by Watson–Crick base pairing and prevent further processing. In principle, all derivatives which were proved to be effective in the so called antisense strategy can also be used to target miR. High stability of the complex formed by the anti-miR agent and the target miRNA is therefore one of the major points. Stability of anti- miR under physiological condition is another important issue; good cellular delivery is also necessary in order to achieve miR inhibition. RNAs are sensitive to both chemical and enzymatic degradation, due to the presence of the 2'-OH group, which can be converted to an internal nucleophile and thus represents RNA "Achille's heel"; therefore, alkylation of the 2'-oxygen in RNA has the effect of producing more stable derivatives with good affinity for complementary miR. Commercially available antago-miRs mainly belong to this class, in particular



with methyl and methoxyethyl as alkyl groups; 2'-deoxy-2'-fluoro derivatives have also been used. Locked nucleic acids (LNAs) are RNA analogs in which a methylene bridge has been introduced between the 2'-oxygen and the C(4)-carbon, thus creating a further ring which strongly constrain the furanoside in a C3'-endo-like (i.e. RNA-like) conformation, more effective for RNA recognition. Usually, oligomers containing LNA used in antisense studies have alternated LNA–DNA monomers, whereas those used as anti-miRs are partially made of LNA monomers inserted in a strand composed of 2'-O-methyl RNA units. For example, Elmen *et al.* (2008) had demonstrated that antagonism on microRNA-122 in mice, treated with LNA-anti-miR, leads to up-regulation of a large set of predicted target mRNA in the liver. More drastic change in the backbone of oligonucleotides derivatives are represented by peptide nucleic acid (PNAs).

#### 1.2.6.1 MiR targeting by PNAs

PNAs are very promising tools for RNA recognition, since they have a higher affinity for RNA than for DNA (Nielsen *et al.*, 2004), are more specific and are resistant to DNAses and proteases (Demidov *et al.*, 1994). Despite the PNAs possess many advantages for their application as an anti-miR strategy, the literature are reporter only few jobs. One of the reasons is the lack of cellular permeation by simple unmodified PNA, or segregation in lysosomes of some PNA-peptide conjugates, which can prevent the access to the target miRNA. As described in *Section 1.1.4 of Part 2*, some changes have been tested to bypass these limits. The first example of targeting microRNAs using PNA-based molecules is provided by miR-122. Fabani and his collaborators demonstrated, using PNAs and PNA-peptide conjugates, that these oligonucleotide analogs, in particular K-PNA-K<sub>3</sub>, evaluated for the first time in microRNA inhibition, are more effective than standard 2'-O-methyl oligonucleotides in binding and inhibiting microRNA action (Fabani *et al.*, 2009). In a parallel work, Oh *et al.* (2009) described the effectiveness of miR targeting by PNA-peptide conjugates, using a series of cell penetrating peptides (CPP) as carriers, including R6 pen, Tat, a four Lys sequence, and transportan ( Oh, *et al.*, 2009). PNAs were found to be more effective than LNAs and 2'-OMe oligonucleotides. Furthermore PNAs were found to be more resistant to degradation than LNAs, even

if stored at room temperature, suggesting better performances of the former class as candidate drugs. More recently, a PNA targeted against miR-155 has been used in cellular systems and in mice (Fabani et al., 2010). In this study, the induction of miR-155 by bacterial lipopolysaccharide (LPS) was reduced by using a PNA matching the miR target and linked to four lysine residues.

In a recent study we have evaluated the activity of a PNA targeting miR-210, which is associated to hypoxia, and is modulated during erythroid differentiation, in leukemic K562 cells (Fabbri et al., 2010). The major conclusions of our study were that a PNA against miR-210 conjugated with a polyarginine peptide (R-pep-PNA-a210): (a) is efficiently internalized within the target cells; (b) strongly inhibits miR-210 activity; (c) deeply alters the expression of raptor and  $\gamma$ -globin genes. Unlike commercially available antagomiRs, which need continuous administrations, a single administration of R-pep-PNA-a210 was sufficient to obtain the biological effects.

Following the results obtained from inhibition of miR-210, we have synthesized a R<sub>8</sub>-PNA-anti-miR-221; the microRNA-221 is of great interest because it has been found to be associated with cancer, since it is able to target the 3'UTR of onco-suppressor mRNA p27<sup>kip1</sup>, as below described.

### **1.3 Cell cycle deregulation in human breast cancer**

#### **1.3.1 The cell cycle**

As “cell cycle” we define the process by which a cell correctly divides into two daughter cells and is central to the understanding of all life (Nurse, 2000). It is in fact largely accepted that is essential for their survival that the two daughter cells receive a full complement of all the organelles and a copy of the genome correctly duplicated. A typical eukaryotic cell cycle is illustrated by human cells in culture, which divide approximately every 24 hours. The cell cycle comprises a series of tightly controlled events that drive the replication of DNA and cell division. It is divided into several phases: preparation for (G1 phase), and execution of, DNA synthesis (S phase), a second gap phase (G2), and mitosis (M). Quiescence (G0) is a

biochemically distinct state from which cells can re-enter the cell cycle and go on to DNA replication and mitosis. To ensure that DNA faithfully replicates and that the replicated chromosomes correctly segregates into the two newly divided cells, in all eukaryotic cells, cell cycle progression is stringently controlled (Heichman et al., 1994; Wuarin et al., 1996). In particular several mechanisms ensure that S phase is completed before mitosis begins and that M phase started only if the DNA has been faithfully replicated. This is possible since two Gap phases (the G1 separating the M and S phases, and the G2 between the S and M phases) are present in somatic cells and dictate the timing of cell division during which the control mechanisms principally act. However, in embryonic cells, that need to fast replicate, the two Gap phases seem to be absent. In living organisms the cells are usually in a state of quiescence called G0 and can re-enter into the cell cycle after stimuli derived from the local microenvironment (for example growth factors stimulation). This process was been reproduced in *in vitro* experiments, about 35 years ago, by Arthur Pardee (1974), that was able to demonstrate that, in a normal cell, growth factors are necessary to initiate and maintain the transition from early to late G1 phase since to the so called “restriction point” or G1 phase checkpoint experiments. The beginning of the G1 phase is the only part of the cell cycle that seems to be dependent on growth factors stimulation. When cells are stimulated by growth factors to enter the cycle from G0, they generally require continuous mitogen stimulation to be driven to the restriction point, after which mitogens can be withdrawn and cells will enter S phase and complete the cycle in their absence. Once the restriction point has been passed, and cell is irreversibly committed to complete cell division, the cell enter S phase during which the DNA is duplicated. At this point, in the G2 phase, before the Mitosis (M phase) could start, the cell control that DNA has been faithful duplicated checks the internal signaling events necessary for a successful division. As cells exit mitosis, the cell cycle is reset, allowing the establishment of a new, competent replication state in G0 or G1 phases.

### **1.3.2 Cyclins and CDKs**

The transitions between these phases of the cell cycle are orderly regulated by Cyclin-Dependent Kinases (CDK) activity (Figure 13). CDKs are serine and threonine

kinases, and their actions are dependent on associations with their activating subunits, cyclins (Hunt, 1991; Sherr, 1994). Cyclin abundance is regulated by protein synthesis and degradation; the activity of CDKs is therefore regulated to a large degree by the presence of different cyclins. Cyclin specificity can be achieved in various ways: cyclins are expressed or are present at stable levels at different times; they are differentially sensitive to cell-cycle-regulated inhibitors; they are differentially restricted to specific subcellular locations; or they bind specifically to only some phosphorylation targets. Cyclins are regulated at the level of protein degradation by ubiquitin-mediated proteolysis. Proteins that are tagged with a chain of ubiquitin molecules are degraded by the 26S proteasome. The sensitivity of different cyclins to different ubiquitin ligases constitutes an important mechanism for cyclin specificity in controlling the cell-cycle engine.

Restriction point control is mediated by two families of enzymes, the cyclin D- and E- dependent kinases. The D-type cyclins (D1, D2, and D3) (Matsushime et al.; Xiong et al.; Motokura et al., 1991) interact combinatorially with two distinct catalytic partners: CDK4 and CDK6 (Meyerson et al., 1994). Whereas CDK4 and CDK6 are relatively long-lived proteins, the D-type cyclins are unstable, and their induction, synthesis, and assembly with their catalytic partners all depend upon persistent mitogenic signaling. D-type cyclins are usually absent from cell cycles that proceed independently of extrinsic mitogenic signals. Conversely, constitutive activation of the cyclin pathway can reduce or overcome certain requirements for cell proliferation and thereby contribute to oncogenic transformation (Sherr et al., 1996).

The mitogen-dependent accumulation of the cyclin D-dependent kinases triggers the phosphorylation of the Retinoblastoma protein (Rb), thereby helping to cancel its growth-repressive functions (Ewen et al., 1993). Rb represses the transcription of genes whose products are required for DNA synthesis by binding transcription factors such as the E2Fs. Apart from a battery of genes that regulate DNA transcription, E2Fs induce the cyclin E and A genes. Cyclin E enters into a complex with its catalytic partner CDK2 (Koff et al., 1991; Lew et al., 1991) and collaborates with the cyclin D-dependent kinases to complete Rb phosphorylation (Lundberg et al., 1998). This shift in Rb phosphorylation from mitogen-dependent cyclin D-CDK4/6 complexes to mitogen-independent cyclin E-CDK2 accounts in part for the loss of dependency on extracellular growth factors at the restriction point. The

activity of the cyclin E-CDK2 complex peaks at the G1-S transition, after which cyclin E is degraded and replaced by cyclin A.

Other levels of CDKs regulation are represented by phosphorylation and dephosphorylation of CDKs catalytic activity. The cyclin-CDK complexes can be kept in an inactive state by phosphorylation on one, or sometimes two, residues in the ATP-binding site of the CDK. CDK proteins generally remain at constant levels throughout the cell cycle, while cyclins and post-translational modifiers (including kinases and phosphatases) undergo periodic fluctuation to regulate DNA syntheses and cell division (Caldon et al., 2006). The sequential accumulation of different cyclins allows the formation of specific cyclin-CDK complexes that target substrates involved in transitions between the cell-cycle phases. During G1 phase the predominant cyclin-CDK complexes are cyclin D-CDK4/6 and cyclin E-CDK2, followed by cyclin A-CDK2 during S phase, then cyclin A-CDK1 and cyclin B-CDK1 during G2 and mitosis. Although control of the G2/M transition is implicated in events in cancer resulting in chromosomal aberrations, the G1/S transition encompasses many of the important cell-cycle events that may be specifically altered in breast cancer, including the actions of the oncogenes/ tumor suppressors cyclin E, cyclin D1, and p27<sup>kip1</sup>.

#### *1.3.2.1 Cyclin D1-CDK4/6*

Cyclin D1 is a critical modulator of the G1/S transition through Rb phosphorylation and p21/ p27 titration. Its levels and activity are regulated to integrate extracellular signaling with the cell-cycle machinery. During G1, cyclin D1 is controlled by a number of mitogenic factors, acting on its transcription and translation as well as the stability, localization and CDK complex association of the cyclin D1 protein. Cyclin D1 is excluded from the nucleus during S phase and the protein is rapidly degraded, through phosphorylation by GSK-3b (Sherr et al., 1999).

CDK4 and CDK6 display much more restricted substrate preferences than the other CDKs; their principal known substrates are the product of the retinoblastoma gene, pRb, and other members of the pocket protein family, which, in their non-phosphorylated forms, inhibit the E2F-mediated transcription of genes essential for DNA synthesis. The importance of pRb as a CDK4/6 substrate is illustrated by the

observation that cyclin D1 is not required for G1 phase progression in cells lacking pRb. In breast cancer, a non-functional Rb pathway, characterized by loss of Rb or lack of concordance between Rb phosphorylation and cell proliferation, is associated with tamoxifen- resistance (Lehn et al., 2011). Furthermore, cyclin D1 expression is required for tamoxifen-induced cell cycle progression, suggesting a role for CDK4/6 activity.

#### 1.3.2.2 Cyclin E-CDK2

The two E-type cyclins, cyclins E1 and E2 (collectively referred to as cyclin E), are closely related and often co-expressed. During G1 phase, CDK2 is activated through binding cyclin E, and then, via phosphorylation of target proteins, facilitates the first steps of S phase. One critical target for cyclin E-CDK2 is pRb, but in addition to promoting S phase entry through pRb phosphorylation, cyclin E-CDK2 also phosphorylates a number of proteins with a more direct role in DNA replication, including NPAT, which facilitates histone synthesis, and components of the pre-replication complex (Hwang et al., 2005). The activity of cyclin E-CDK2 is finely regulated through protein-protein interactions, phosphorylation events and degradation, but a primary level of control is through the periodic expression of cyclin E. Cyclin E1 is a well-known E2F target gene and cyclin E2 is also likely to be E2F-regulated. During G1 phase, pRb phosphorylation by cyclin D1- CDK4/6 allows the release of E2F, which in turn drives cyclin E expression to reach a maximum near the G1/S phase boundary (Sherr et al., 2005; Hwang et al., 2005). As the cyclin E-CDK2 complex is activated, it phosphorylates pRb, providing a positive feedback loop for cyclin E transcription.

A secondary level of control of cyclin E-CDK2 is through the CDK inhibitors p21 and p27. These proteins bind to cyclin E-CDK2, profoundly inhibiting kinase activity and hence stabilizing cyclin E. The availability of p21 and p27 to inhibit cyclin E-CDK2 can be modulated not only through alterations in their overall abundance but also through their sequestration by cyclin D1-CDK4/6 and by cytoplasmic relocalisation (Sherr et al., 1999; Coqueret et al., 2002). The cyclin E-CDK2 complex also positively regulates its own activity by phosphorylating p27, which is then targeted for degradation.

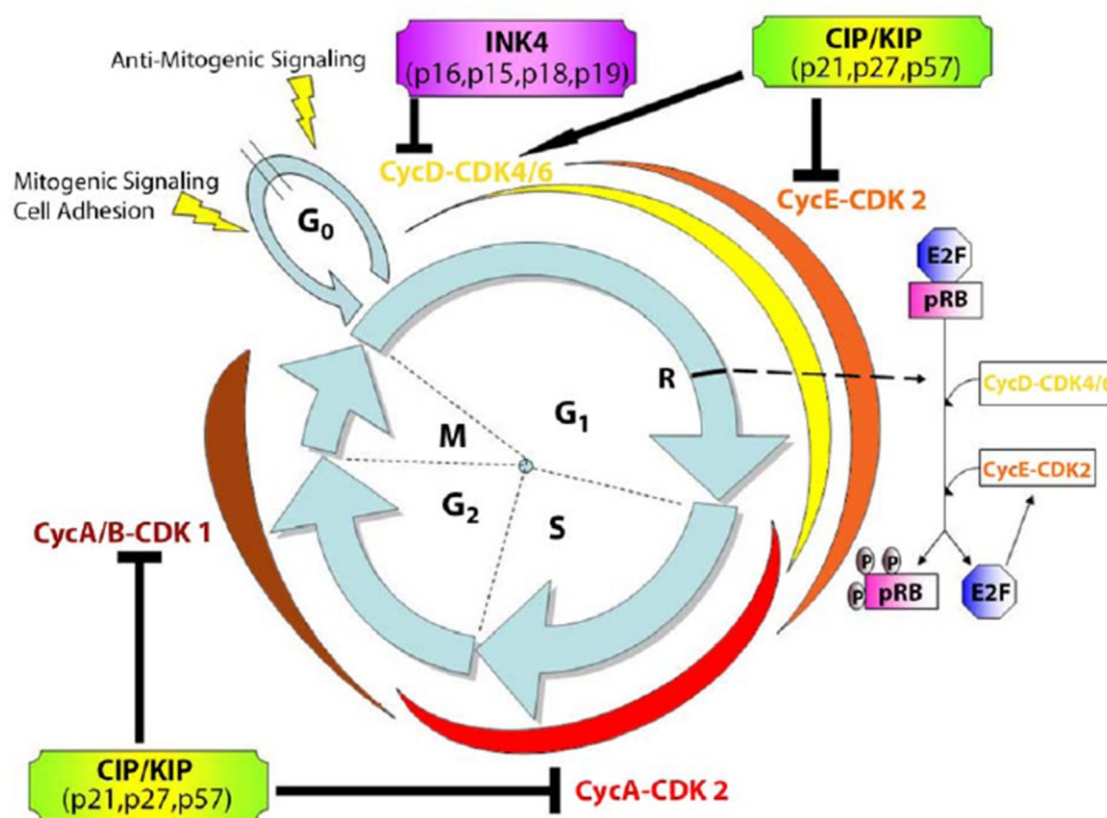


Figure 13. Schematic representation of cell cycle regulation. Mitogenic stimuli promote cell cycle progression from G<sub>0</sub> to G<sub>1</sub> inducing the expression of D type cyclins and lowering the expression of p27. Sequential activation of cyclin E-CDK2, cyclin A-CDK2, cyclin A-CDK1 and cyclin B-CDK1 allow the cells to pass through the restriction point (R) and to complete the mitotic division. The passage through the R point is due to the inactivation of the pRB protein by CDKs-dependent phosphorylation

### 1.3.3 Cyclin Kinase Inhibitors

The CKIs, also called CDK inhibitors, can be divided into 2 families on the basis of sequence: the INK4 (CDK4 inhibitor) family and the Kip/Cip (kinase inhibitor protein) family (Hunter et al., 1994; Sherr et al., 1995; Harper et al., 1996).

The INK4 proteins are almost entirely composed of ankyrin repeats (Serrano et al., 1993), a putative protein-protein interaction motif. This family comprises four members: p15<sup>INK4b</sup>, p16<sup>INK4a</sup>, p18<sup>INK4c</sup> and p19<sup>INK4d</sup>. In humans, INK4a and INK4b are closely linked on the short arm of chromosome 9, whereas INK4c maps to chromosome 1 and INK4d maps to chromosome 19. These inhibitors are specific for the CDK4 and CDK6 kinases (Hirai et al., 1995) that bind the D-type cyclins (Matsushime et al., 1992). These kinase complexes have been most closely linked to

the control of G1 phase, especially with regard to the decision to proliferate or not according to the presence of growth factors, and to cell size control. The INK4 proteins are also able to inhibit CDK4/6 in a complex with cyclin D, and it will be interesting to see whether this bears any relation to the inhibitory mechanisms employed by the Kip/Cip family.

The Kip/Cip family comprises three proteins; p21<sup>Cip1/Waf1/Sdi1</sup>, p27<sup>Kip1</sup> and p57<sup>Kip2</sup>. These inhibitors show tissue specific distribution patterns; for example, p27<sup>Kip1</sup> and p57<sup>Kip2</sup> has been detected in some specialized tissue, as neurons, skeletal muscle and lung during their development, while these inhibitors are not expressed in proliferating cell. Their expression regulation is also differently regulated following specific stimuli. Indeed p57<sup>Kip2</sup> is imprinted (Matsuoka et al., 1996), and have been implicated in cell differentiation (Elledge et al., 1996), and in the response to stress. p21 transcription is up-regulated in response to DNA damage by wild type but not mutant p53, and p27<sup>Kip1</sup> was initially identified as the factor responsible for inhibiting proliferation in contact-inhibited and TGFβ-treated cells. Until now the Kip/Cip family had been thought specific for the cyclin-CDK complexes (for the G1 and S phase kinases). However, there is a report showing that p21, a DNA-damage-inducible cell-cycle inhibitor, acts also as an inhibitor of the SAPK (kinases stress-activated protein), group of mammalian MAP, such as JNK, suggested that p21 may participate in regulating signaling cascades that are activated by cellular stresses such as DNA damage. The first one requires that p21 bind both the CDK and the cyclin. In the second type of interaction, p21 bind only the cyclin or the CDK.

#### **1.3.4 Cyclin Kinase Inhibitor p27<sup>Kip1</sup>**

Protein p27<sup>Kip1</sup> was first identified as a CDK2-inhibitory activity detected in contact inhibited or TGF-beta treated cells (Koff et al., 1993; Polyak et al., 1994). In fact p27<sup>Kip1</sup> has a crucial role in the G1-S transition, by interacting with and inhibiting cyclin E-CDK2 and cyclin A-CDK2 activity, thus blocking cell cycle progression. In early G1 p27<sup>Kip1</sup> promotes cyclin D-CDK4/6 complex assembly and nuclear import, increasing cyclin D stability, all without inhibiting CDK4 kinase activity. In proliferating cells, p27<sup>Kip1</sup> is primarily associated with cyclin D-CDK4/6 complexes, but these



complexes are catalytically active, whereas in G1 arrested cells p27<sup>kip1</sup> preferentially binds and inhibits Cyclin E-CDK2.

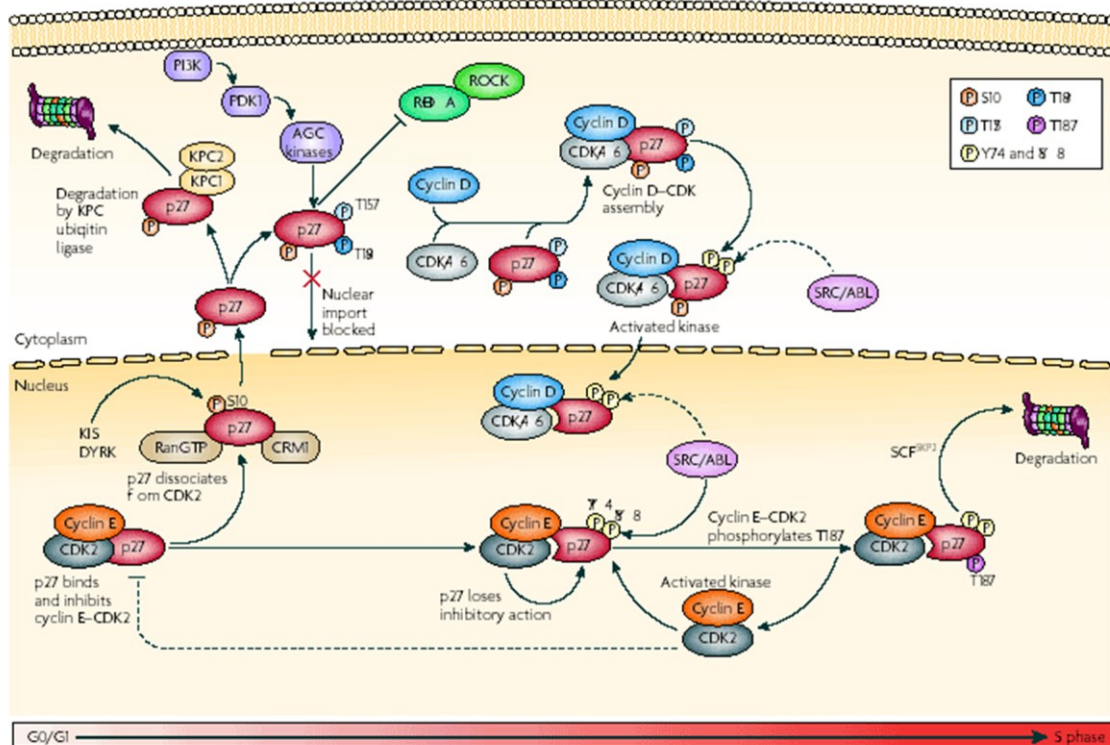


Figure 14. p27<sup>kip1</sup> pathway during eukaryotic cell cycle

The sequestration of p27<sup>kip1</sup> by cyclin D-CDK4/6 complexes effectively frees CDK2 from inhibition and allows both CDK4/6 and CDK2 to remain active. In this way mitogen induction of cyclin D expression determines cell cycle progression both by activating CDK4 and by sequestering p27<sup>kip1</sup> thus favoring cyclin E-CDK2 activation. Activation of cyclin E by E2F, after hyper-phosphorylation of pRb, enables the formation of the active cyclin E-CDK2 complex. This is accelerated by the continued sequestration of Cip/Kip proteins into complexes with assembling cyclin D-CDK complexes. Cyclin E-CDK2 completes the phosphorylation of Rb, further enabling activation of E2F responsive genes, including cyclin A. Once cyclin E-CDK2 is activated, p27<sup>kip1</sup> is rapidly degraded, contributing to the irreversibility of passage through the restriction point. If cells are persistently stimulated with mitogens, cyclin D-dependent kinase activity remains high in the subsequent cell cycles, p27<sup>kip1</sup> levels

stay low, and virtually all of the p27<sup>kip1</sup> can be found in complexes with the cyclin D-CDK4/6. When mitogens are withdrawn, cyclin D is rapidly degraded, and the pool of previously sequestered Cip/Kip proteins are mobilized to inhibit cyclin E-CDK2, thereby arresting progression usually within a single cycle. Multiple extracellular stimuli regulate p27<sup>kip1</sup> abundance, which functions as a sensor of external signals to cell cycle regulation. In normal cells p27<sup>kip1</sup> is expressed at high levels in quiescence phase, whereas it decreases rapidly after mitogen triggering and cell cycle re-entry.

#### 1.3.4.1 Regulation of p27<sup>kip1</sup>

In normal cells, the amount of p27<sup>kip1</sup> protein is high during G0 phase but decrease rapidly on re-entry of cells into G1 phase (Nourse et al., 1994). This rapid removal of p27<sup>kip1</sup> at the G0/G1 transition is required for effective progression of the cell cycle to S phase. The abundance of p27<sup>kip1</sup> is thought to be controlled by multiple mechanisms that operate at the level of its synthesis (transcription, translation), degradation and localization (Millard et al., 2000; Connor et al., 2003). In particular, a change in the subcellular localization of p27<sup>kip1</sup> induced by the exposure of quiescent cells to growth stimuli is crucial for the down-regulation of this protein at the G0/G1 transition. In G0 phase, p27<sup>kip1</sup> accumulates in the nucleus, where it inhibits the cyclin-CDK complexes. However, p27<sup>kip1</sup> undergoes rapid translocation from the nucleus into the cytoplasm by yet unspecified mechanism that seems to involve phosphorylation; the translocation of p27<sup>kip1</sup> from the nucleus to the cytoplasm is followed by its degradation by ubiquitin-proteasome pathway (Kamura et al., 2004).

Beside modulation of its expression levels, subcellular localization also plays a pivotal role in governing p27<sup>kip1</sup> function; it is widely accepted that to act as cell cycle inhibitor p27<sup>kip1</sup> must be located in the nucleus, whereas its cytoplasmic sequestration allows cell cycle progression (Belletti et al., 2005). In fact, to inhibit Cyclin E-CDK2, p27<sup>kip1</sup> needs to be imported into the nucleus. Nuclear import depends on the presence of a nuclear localization signal (NLS) localized at the C-terminus of the protein (Zeng et al., 2000).

As cells progress along the cell cycle, p27<sup>kip1</sup> shuttles between nucleus and cytoplasm. The cytoplasmic redistribution of p27<sup>kip1</sup> induced by mitogenic stimulation is dependent on the phosphorylation of a specific serine residue (S10) (Rodier et al.,

2001), that promotes its nuclear export to the cytoplasm and overcomes p27-induced growth arrest. Other phosphorylation sites of p27<sup>kip1</sup>, such as Thr 157 and Thr 198 in the human protein, have been implicated in the cytoplasmic translocation and localization of p27<sup>kip1</sup>; this residue is located in the nuclear localization signal (NLS) of p27<sup>kip1</sup>, and its phosphorylation prevents the nuclear localization of p27<sup>kip1</sup>. Also the Thr 198 is phosphorylated, but it's largely restricted to the cytoplasm in cell exposed to growth stimuli, and the nuclear export of p27<sup>kip1</sup> during G1 phase is delayed by mutation of this residue to Ala (Kossatz et al., 2006). Phosphorylation of p27<sup>kip1</sup> on Thr 198 thus appears to be required for the timely exit of p27<sup>kip1</sup> from the nucleus after mitogenic stimulation and seems to be determinant of p27<sup>kip1</sup> stability, preventing ubiquitylation and proteasomal turnover. As described for p27<sup>kip1</sup> cytoplasmic localization, also p27<sup>kip1</sup> sequestration into higher order complexes with cyclin D-CDK4 is important for regulating p27<sup>kip1</sup> functions. Various growth signaling pathways stimulate the assembly of these heterotrimeric complexes, containing p27 with cyclin Ds and CDK4/6. Indeed, the MEK/ERK pathway which induces cyclin D transcription, favors the assembly of the cycling D-CDK4, and sequesters p27<sup>kip1</sup> in inactive cytoplasmic complexes (Cheng et al., 1998). Also the proto-oncogene c-Myc, by increasing the expression levels of cyclin D and cyclin E, is responsible for p27<sup>kip1</sup> sequestration (Bouchard et al., 1999), and this molecular event appears essential for Myc-induced cell cycle progression. More importantly, p27<sup>kip1</sup> sequestration seems to be one of the relevant mechanisms to human cancerogenesis; in fact, many of the molecules important for the proliferation, differentiation, survival and migration, commonly classified as oncogenes, including Bcr-Abl, Ras, PI3K and STAT, have the ability to modulate and/or down-regulate p27<sup>kip1</sup>, so that its expression may be a powerful prognostic marker since it may represent the readout of multiple different signals transduction pathways known to be involved in the onset and/or the development of human tumors.

#### *1.3.4.2 p27<sup>kip1</sup> deregulation in breast cancer*

As previously described, p27<sup>kip1</sup> is a key regulator of G1-to-S phase progression of cell cycle; it acts as cell cycle inhibitor and prevents premature activation of cyclin E-CDK2 in G1 and promotes the assembly and activation of D-

type cyclin-CDKs. While the p27<sup>kip1</sup> gene is rarely mutated in human breast cancers, the action of p27<sup>kip1</sup> is impaired in breast and other human cancers through accelerated p27<sup>kip1</sup> proteolysis, sequestration by cyclin D-CDKs, and by p27<sup>kip1</sup> mislocalization in tumor cell cytoplasm. P27<sup>kip1</sup> mRNA levels are constant throughout the cell cycle (Hengst et al., 1996). P27<sup>kip1</sup> level and activity increase in response to many stimuli, as differentiation signals (Chen et al., 1996), loss of adhesion to extracellular matrix (Watanabe et al., 1996) and signaling by growth-inhibitory factors (Koff et al., 1993). On exit from quiescence, p27<sup>kip1</sup> protein level fall rapidly thanks to a dramatic decrease in p27<sup>kip1</sup> translation and activation of ubiquitin-mediated p27<sup>kip1</sup> proteolysis. p27<sup>kip1</sup> proteolysis is excessively activated in many human cancer, in particular in breast cancer. This is mediated by oncogenic activation of multiple receptor tyrosine kinase and signal transduction pathways. Ras may play an important role in modulation of p27<sup>kip1</sup> loss. Transfection of dominant negative Ras allele increased p27<sup>kip1</sup> accumulation, and caused G1 arrest, suggesting that Ras is required for p27<sup>kip1</sup> degradation at G1 to S phase entrance (Takuwa N et al., 1997).

On the contrary, overexpression of mitogen-activated protein kinase (MAPK) in fibroblasts increased p27<sup>kip1</sup> degradation; in fact, p27<sup>kip1</sup> contains several MAPK consensus sites, and MAPK can phosphorylate p27<sup>kip1</sup>, leading to a reduction of its ability to bind CDK2. (Kawada et al., 1997). The regulation of p27<sup>kip1</sup> degradation appears to be linked to phosphorylation of p27<sup>kip1</sup> by cyclin E/CDK2 (Montagnoli et al., 1999) and its export from the nucleus (Tomoda et al., 1999). Reduced levels of p27<sup>kip1</sup> protein, caused by an accelerated proteolysis, have been observed in up to 60% of human carcinomas, including lung, prostate, breast, colon, and ovarian carcinoma and in melanoma, glioma, and certain lymphomas; moreover, p27<sup>kip1</sup> loss is usually associated with high grade tumor. p27<sup>kip1</sup> also plays an important role in cellular differentiation and development. Moeller *et al.*, (2003) have demonstrated that p27<sup>kip1</sup> knockout mice manifest altered differentiation and multiorgan hyperplasia, suggesting a key role for p27<sup>kip1</sup> in terminal differentiation and a strong relation between reduce p27<sup>kip1</sup> levels and tumor grade in many human cancers (Moeller et al., 2003). Malignant cells often show anchorage-independence, property key in invasive and metastatic. It has long demonstrated that the abrogation of p27<sup>kip1</sup> expression by antisense constructs in mammary carcinoma cells not only increased the proliferative fraction of tumor spheroids, rendering them more susceptible to cytotoxic drugs, but also reduced intercellular adhesion. Thus, loss of p27<sup>kip1</sup> in

human cancers may promote tumorigenesis by abrogating the cell cycle arrest induced by loss of cell–cell contact or loss of contact with the tissue substratum, facilitating the acquisition of tumor cell motility and metastasis (Croix et al., 1996). Loss of p27<sup>kip1</sup> is also an indicator of poor patient outcome in a majority of breast cancer studies.

Some research groups have reported p27<sup>kip1</sup> studies in primary breast cancers, identified p27<sup>kip1</sup> as an independent prognostic indicator (Alkarain et al., 2004). Although p27<sup>kip1</sup> is localized exclusively into the nucleus of normal breast epithelial cells, tumors that retain abundant p27<sup>kip1</sup> often show protein mislocalization in the cytoplasm away from nuclear cyclin-CDK targets. Cytoplasmic localization would prevent p27<sup>kip1</sup> from binding and inhibiting nuclear cyclin-CDK targets. This mislocalization of p27<sup>kip1</sup> has been seen in up to 40% of primary human breast cancers. For each level of nuclear p27<sup>kip1</sup> staining (high or low), when p27<sup>kip1</sup> was present in cytoplasm rather than in the nucleus alone, patient survival was worse. These observations raise the possibility that consideration of the presence or absence of cytoplasmic p27<sup>kip1</sup> may add to the prognostic significance of p27<sup>kip1</sup> levels in human breast cancers.

In addition to the well-known mechanisms of degradation, phosphorylation and cellular localization of p27<sup>kip1</sup>, in recent years has been investigating the possible role of miRNAs in the deregulation that occurs in cancer development. It was found that several miRNAs are directly involved in human cancers, including lung, brain, liver and breast.

### **1.3.5 Regulation of cell cycle factors by microRNAs in human cancer**

Cell cycle regulators frequently act as oncogenes or tumor suppressors. One of the major cell cycle pathways, regulated by retinoblastoma (pRb) protein, is altered in almost every human cancer (Malumbres et al., 2001). pRb itself is abnormally down-regulated by the overexpression of the miR-106a in different human cancers (Volinia et al., 2006).

Also P130/RBL2, another member of pRb family, is controlled by miR-290 cluster and by miR-17-92 cluster. The cyclins and CDKs are also targeted by microRNA. In particular, CDK6 mRNA is down-regulated by miR-34a, miR-124 and

miR-127 (He et al., 2007). Some other oncogenic miRNAs may exert their function through the inhibition of cell cycle inhibitors such as members of the INK4 or Cip/Kip families. For example, p16<sup>INK4a</sup> is controlled by miR-24, a miRNA that is down-regulated during replicative senescence.

p21<sup>Cip1</sup> is a direct target of miR-106b, which is overexpressed in multiple tumor types and plays a critical role in cell proliferation by regulating the G1-to-S cell cycle transition (Ivanovska et al., 2008).

The p27<sup>Kip1</sup> protein, with a relevant role as tumor suppressor in human cancer, is mostly controlled at the post-transcriptional level. When some alterations occur, they predispose cells to tumorigenesis upon exposure to carcinogenesis. MiR-221 and miR-222 can function as oncogenes in human tumors by binding to target sites in the 3'UTR of p27<sup>Kip1</sup> (Figure 16) inhibiting its translation (Le Sage et al., 2007) mRNA transcript is directly target by miR-221 in glioblastoma (Gillies et al., 2007) and prostate cancer cells (Galardi et al., 2007). The physiological up-regulation of miR-221 and miR-222 coordinates competency for initiation of S phase with growth factor signaling pathways that stimulate cell proliferation. Therefore, disruption of miRNAs expression that target cell cycle proteins, could ultimately lead to the progression of the malignant phenotype in human tumors (Figure 15).

Despite the fact that in this PhD Thesis we focused our attention on breast cancer cellular model systems, we like to underline that p27<sup>Kip1</sup>/miR-221 are deeply involved in other tumors for which PNA-based treatments are expected to be very appealing, as just described, glioblastoma and prostate cancer.

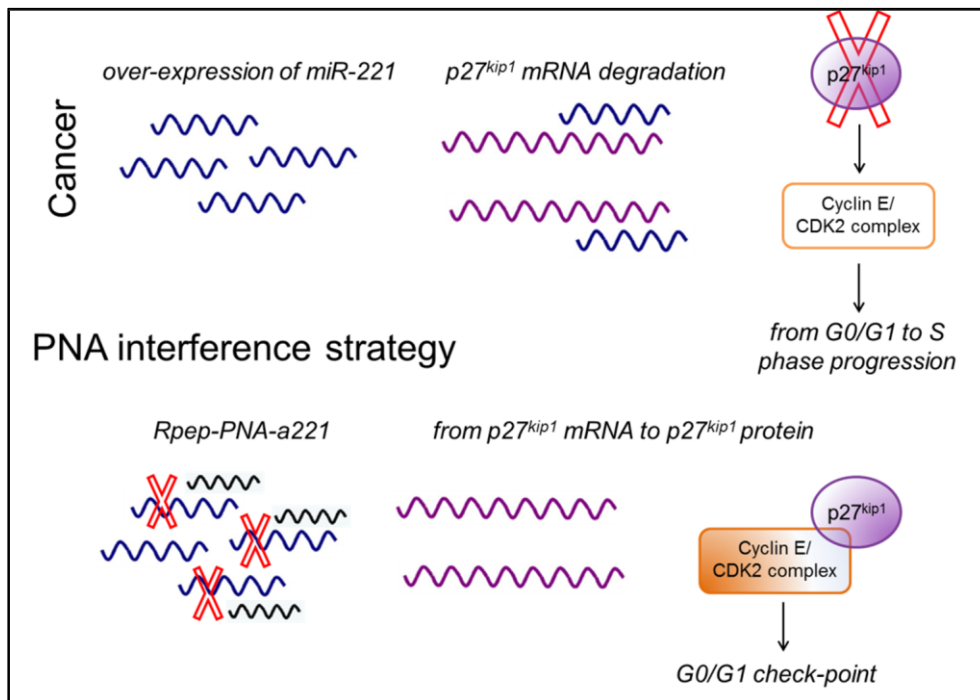


Figure 15. PNAs interference against miR-221 strategy

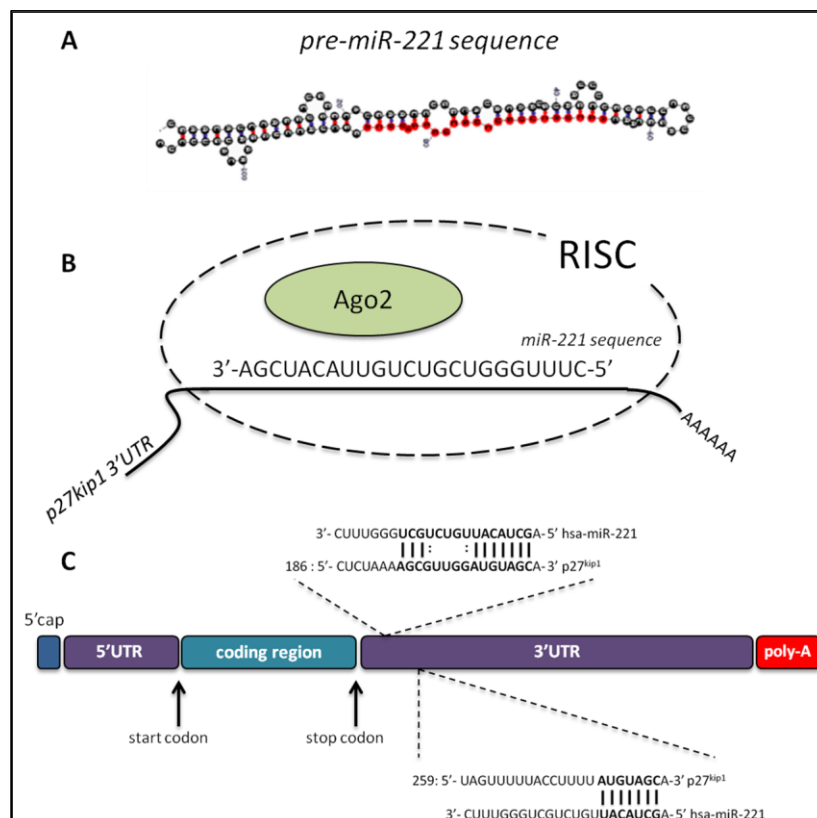


Figure 16. Structure of pre-miR-221 (A), schematic representation of RISC complex with miR-221 and 3'UTR of  $p27^{kip1}$  (B) and putative miR-221 sites of interaction with 3'UTR of human  $p27^{kip1}$  mRNA (C)

#### **1.4 Human breast cancer MDA-MB-231 and MCF-7 as experimental model to study the miR-221 modulation after treatment with PNAs**

The human breast cancer cell lines (MCF-7 and MDA-MB-231) were purchased from ATCC and grown in Dulbecco's Modified Eagle Medium (D-MEM, Gibco), containing 10% fetal bovine serum (FBS, Celbio, Milano, Italy) and 2 mM L-Glutamine (Sigma-Aldrich) and maintained at 37°C in humidified atmosphere of 5% CO<sub>2</sub>/air.

MDA-MB-231 (Figure 17a) was obtained from a patient in 1973 (Cailleau et al., 1974) at M. D. Anderson Cancer Center. With epithelial-like morphology, the MDA-MB-231 breast cancer cells appear phenotypically as spindle shaped cells. *In vitro*, the MDA-MB-231 cell line has an invasive phenotype. It has abundant activity in both the Boyden chamber chemoinvasion and chemotaxis assay. The MDA-MB-231 cell line is also able to grow on agarose, an indicator of transformation and tumorigenicity, and displays a relatively high colony forming efficiency.

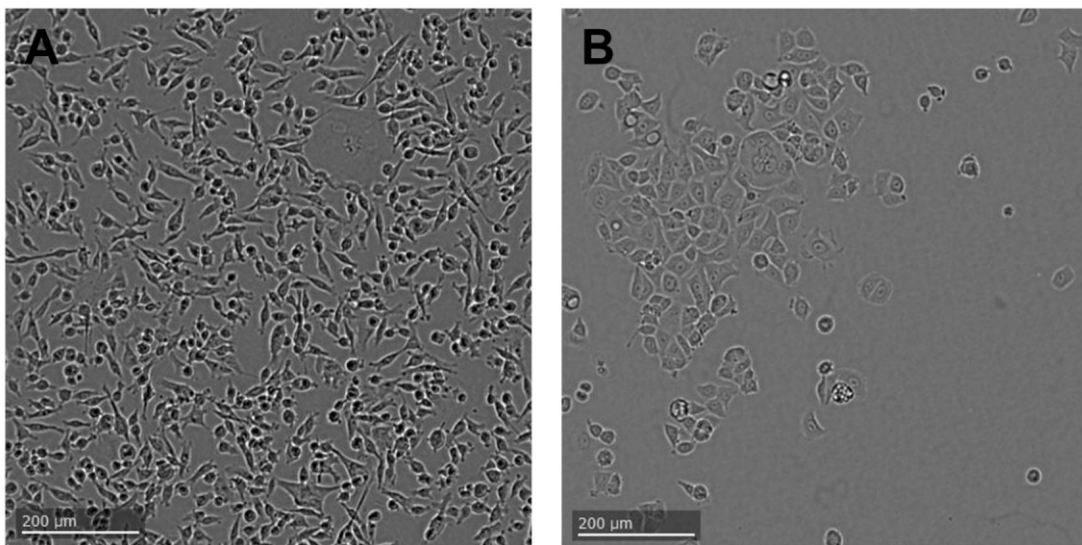


Figure 17. Pictures of (A) human breast cancer cell lines MDA-MB-231 (Phenotype: Basal Breast Carcinoma; triple negative: HER2-, Estrogen Receptor-, Progesterone Receptor -) and (B) MCF-7 (Phenotype: Luminal Breast Carcinoma; Estrogen Receptor +, HER2-), taken with optical microscope

MCF-7 (Figure 17b) is a human breast cancer cell line that was first isolated in 1970 (Soule et al., 1973) from the malignant adenocarcinoma breast tissue of a 69-year old woman. MCF-7 is the acronym of Michigan Cancer Foundation- 7, referring



to the institute in Detroit where the cell line was established. MCF-7 cells are useful for *in vitro* breast cancer studies because the cell line has retained several ideal characteristics particular to the mammary epithelium. These include the ability for MCF-7 cells to process estrogen via estrogen receptors. MCF-7 cells are also sensitive to cytokeratin. When grown *in vitro*, the cell line is capable of forming domes and the epithelial like cells grow in monolayers. Growth can also be inhibited using tumor necrosis factor alpha (TNF alpha).

These two cell lines are widely used as an experimental model for the study of breast cancer. Estrogen is involved in several physiological and pathological effects through estrogen receptor (ER)-mediated transcriptional gene regulation. Several lines of evidence have suggested that estrogen receptor  $\alpha$  (ER $\alpha$ )-negative breast tumor is highly aggressive and unresponsive to hormonal therapy. MDA-MB-231 are ER $\alpha$ -negative human breast cancer cell lines, that shows high level of miR-221/miR-222; the over-expression of miR-221/222, in fact, suppress the expression on ER $\alpha$  at the post-transcriptional level, conferring estrogen-independent growth; moreover, it also suppresses the expression of different tumor suppressor, such as CDKN1B (p27<sup>kip1</sup>), PTEN, TIMP3, FOXO3, promoting high proliferation (Di Leva et al., 2010). In a recent work, le Sage *et al.*, (2007) have examined several breast and glioblastoma cancer cell lines for miR-221&222 expression. To explore the effect of miR-221&222 antagomiRs on cellular proliferation they have selected two cell lines that showed endogenous expression of miR-221&222 (U87 and MDA-MB-231), and two negative cell lines (MCF-7 and HeLa). Interestingly, treatment of U87 and MDA-MB-231 with both antagomiR-221&222 resulted in a clear proliferation arrest phenotype, which was accompanied by a significant reduction in detectable miRNA levels. However, no significant effect on proliferation was observed in treated MCF-7 and HeLa cell lines. Interestingly, when p27<sup>Kip1</sup> levels were examined by immunostaining, only the cells containing miR-221&222 (U87 and MDA-MB-231) showed increased levels of the cell cycle inhibitor p27<sup>Kip1</sup> after administration of the antagomiRs. These results demonstrate the high expression of miR-221 is directly related to the uncontrolled proliferation and indirectly related to the down-regulation of tumor-suppressor p27<sup>kip1</sup>, suggesting the importance of their genetic interaction in the development and tumor progression (le Sage, 2007). More recently, Lee *et al.*, (2011) have identified one such estrogen down-regulated microRNA, miR-34b, as an onco-suppressor that targets cyclin D1 and JAG-1 in a ER-positive/wild-type p53

breast cancer cell line (MCF-7) as well as in ovarian and endometrial cells, but not in ER-negative or mutant p53 breast cancer cell lines (T47D, MBA-MB-361 and MDA-MB-435).

In according with our purpose, namely to determine the biological activity of PNAs against miR-221 and the consequent effect on p27<sup>kip1</sup>, we decided to use these two cell lines, in particular the MDA-MB-231, in which miR-221 is up-regulated and p27<sup>kip1</sup> down-regulated.

## - MATERIALS AND METHODS -

### 2.1 Human breast cancer cell lines

The human breast cancer cell lines (MCF-7 and MDA-MB-231) were purchased from ATCC and grown in Dulbecco's Modified Eagle Medium (D-MEM, Life Technologies Italia, Monza, MB), containing 10% fetal bovine serum (FBS, Celbio, Milano, Italy) and 2 mM L-Glutamine (Sigma-Aldrich) and maintained at 37°C in humidified atmosphere of 5% CO<sub>2</sub>/air.

### 2.2 Anti-miR-221 Peptide Nucleic Acids

The peptide nucleic acids (PNAs) used in our experiments were synthesized by Prof. Roberto Corradini and his collaborators (University of Parma, Dept. of Organic and Industrial Chemistry) with standard manual Boc-based chemistry using commercially available monomers (ASM, Hannover, Germany) with HBTU/DIPEA coupling as described elsewhere (Manicardi et al., 2010). All the PNAs (Table 3) were synthesized using MBHA resin loaded with Boc-PNA-G(Z)-OH as first monomer. The R8 tail of Rpep-PNA-a221 was introduced after the 2-(2-(Fmoc amino) ethoxy) acetic acid; AEEA, spacer (Applied Biosystems, Foster City, CA, USA) was introduced using the same coupling procedures. 5(6)-carboxyfluorescein (Sigma-Aldrich) was introduced using DIC/DhBtOH coupling. The synthesis of peptide FI-Rpep was performed using standard manual Fmoc based chemistry with HBTU/DIPEA coupling on a Rink amide resin loaded with Fmoc-Arg(Pmc)-OH as first monomer.

Table 3. Properties of PNA-a221 and Rpep-PNA-a221

Name	Sequence	Molecular Weight
<b>PNA-a221</b>	H-AAACCCAGCAGACAATGT-NH <sub>2</sub>	4881 Da
<b>FI-PNA-a221</b>	FI-AEEA-AAACCCAGCAGACAATGT-NH <sub>2</sub>	5368 Da
<b>Rpep-PNA-a221</b>	H-RRRRRRRR-AAACCCAGCAGACAATGT-NH <sub>2</sub>	6129 Da
<b>FI-Rpep-PNA-a221</b>	FI-AEEA-RRRRRRRR-AAACCCAGCAGACAATGT-NH <sub>2</sub>	6635 Da

### **2.3 PNAs antiproliferative activity on MDA-MB-231 and MCF-7 cell lines**

Cells ( $2 \times 10^4$  cells/ml) were seeded in 24-well plate. After 24 hours, media was changed and replaced with experimental media containing different concentration of PNAs-a221. Untreated cells were taken as growth control. Cells were growth for 72 hours in a humidified incubator in 5% CO<sub>2</sub> at 37°C. After this incubation, medium was removed, cell wash with PBS (Phosphate Buffered Saline), trypsinized for 5 minute at 37°C and counted by a ZF Coulter Counter. The IC<sub>50</sub> value is means  $\pm$  SD from three independent experiments.

### **2.4 Cellular Uptake of PNAs**

The first step necessary for obtaining anti-miR221 activity in cells is the effective cellular uptake of PNAs. For this reason, we have performed a series of experiments using both MDA-MB-231 and MCF-7 cell lines.

#### *2.4.1 Fluorescence activated cell sorting*

Since different PNA structures could be affected by proteases present in serum, leading to different cellular uptake, the first experiments have been conducted in the presence or in the absence of serum. Presence of DNase and proteinase activity in serum has been reported and this can lead to partial degradation of the carrier polyarginine peptide, whereas the PNA unnatural backbone was reported to be very stable to degradation in human serum (Demidov et al., 1994).

MDA-MB-231 and MCF-7 cells were seeded, respectively, at  $1,5 \times 10^5$  and  $2 \times 10^5$  cell/ml in 6-well plate. When cells were adherent, we treated them with different concentration of fluorescein-labeled PNAs. After 24 hours of incubation, cells were washed once with PBS, trypsinized and analyzed by fluorescence-activated cell sorting analysis (FACS).

### 2.4.2 BioStation IM technology

The Nikon BioStation IM (Figure 18) is a cell incubator (providing consistent environmental control of temperature, humidity and gas concentration) and monitoring system that allows to conduct live cell imaging. The BioStation IM incorporates a motorized inverted microscope, an incubation chamber, and a high-sensitivity cooled CCD camera into a single package. Being an all-in-one, complete solution from one vendor, IM overcomes stability and compatibility issues experienced in multi-vendor systems.

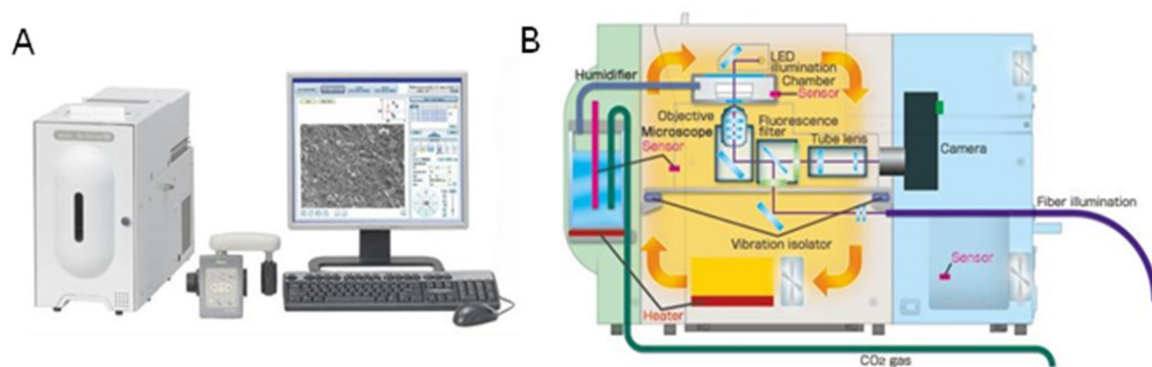


Figure 18. Biostation IM technology (A) and total integrated system (B); The BioStation IM incorporates a motorized inverted microscope, an incubation chamber, and a high-sensitivity cooled CCD camera into a single package

Cells are easily maintained at the appropriate temperature, humidity and CO<sub>2</sub> (or O<sub>2</sub>) levels with BioStation IM. 5% CO<sub>2</sub> (or O<sub>2</sub>), either from a premixed tank or the optional CO<sub>2</sub> (or O<sub>2</sub>) mixer is humidified by bubbling through a bottle of water located at the front of the instrument. Sensors located throughout the instruments keep the temperature of the sample at 37°C.

Cells were seeded at  $2 \times 10^5$  cell/ml, 300  $\mu$ l in each well in a  $\mu$ -Dish Hi-Q<sup>4</sup> and monitored for 24 hours. The images were taken every 30 minutes of incubation.

## 2.5 RNA isolation

MDA-MB-231 and MCF-7 cells were seeded at initial concentration of  $3 \times 10^4$  or  $2 \times 10^4$  cell/ml in a 12-well plate; when cells were adherents, treated for 48 and 96 hours with PNAs. After the incubation, cells were washed with PBS 1x and trypsinized. The total cellular RNA was isolated by *TRIZOL® Reagent* (Sigma-Aldrich, St.Louis, Missouri, USA), as well described in *Chapter “Materials and Methods” of Part 1* of this PhD Thesis, quantified by measuring the absorbance at 260 nm ( $A_{260}$ ) in a spectrophotometer, and quality controlled by electrophoresis on 1% agarose gel.

## 2.6 Reverse transcription reaction (RT-PCR)

### 2.6.1 RT with Random Primer

Reverse transcription of 1 µg of total RNA was performed using with the *TaqMan® Reverse Transcription Reagents PCR kit* (Applied Biosystems). RNA was incubated with 2,5 µM *Random Hexamers* at 25°C for 10 minutes and then immediately cooled to 4°C. After that, the RT reaction solution, prepared considering a final volume of 50 µl, progressively adding the following reagents: 1X *TaqMan RT Buffer* (10X), 5,5 mM *MgCl<sub>2</sub>* (25 mM), deoxyNTPs *Mixture*, 0,4 U/µl *RNase Inhibitor* and 1,25 U/µl *MultiScribe™ Reverse Transcriptase* (50 U/µl) was added. To perform the reverse transcription reaction, the samples were incubated for 30 minutes at 48°C, to allow the extension, and at 95°C for 5 minute to inactivate the enzyme. After thermal cycling, cDNA were stored at -80°C.

### 2.6.2 RT for microRNA

Reverse transcription of 1µg of total RNA was performed using with the *TaqMan® MicroRNA Assay* (Applied Biosystems). For each RNA samples, miR-221 and U6 RT Primers were added. The RT Master Mix reaction solution was prepared adding the following reagents: *Reverse Transcription Buffer* (10X), 1mM *dNTPs* (100mM), 0,25 U/µl *RNase Inhibitor* (20 U/µl), 3,33 U/ µl *MultiScribe™ Reverse Transcriptase* (50 U/µl). To perform the reverse transcription reaction, the samples

were incubated for 30 minutes at 16°C, 30 minutes at 42°C to allow the extension, and at 85°C for 5 minute to inactivate the enzyme. After thermal cycling, cDNA were stored at -80°C.

## **2.7 Real-Time PCR**

The *quantitative real-time polymerase chain reaction* (qPCR) assays of transcripts were carried out using gene-specific double fluorescently labeled probes (Applied Biosystems, Warrington Cheshire, UK) in a 7700 Sequence Detection System, version 1.6.3. The primer and probe used to assay p27<sup>kip1</sup> (Assay ID Details Hs00153277\_m1) were purchased from Applied Biosystems (Applied Biosystems, Foster City, CA, USA). Relative expression was calculated using the comparative cycle threshold method and as reference genes the endogenous control human 18S rRNA. To perform the qPCR, the samples were firstly incubated for 2 min at 50°C and for 10 min at 95°C. The amplification consists by 40 cycles of 15 seconds at 95°C and 1 min at 60°C.

For has-miR-221 (Assay ID: 000524), hsa-let-7c (Assay ID: 000379) and has-miR-200c (Assay ID: 002300) quantification using real-time qPCR reagents, the primers and probes were obtained from Applied Biosystems, and reactions were performed in duplicate according to manufacturer's protocols in a 7700 Sequence Detection System version 1.7 (Applied Biosystems, Foster City, CA, USA). Relative expression was calculated the comparative cycle threshold method and as reference genes the endogenous control human U6 RNA. To perform the qPCR reactions the samples were firstly incubated at 95°C for 10 minutes; the amplification was performed for 40 cycles of 15 seconds at 95°C and 1 min at 60°C.

## **2.8 Nuclear and cytoplasmic proteins extraction**

MDA-231 cells were plated in T-75 flask cell-cultured and treated with PNA-a221 and Rpep-PNA-a221 2 µM for 96 and 120 hours. After incubation, cells were washed with PBS 1x and collected by centrifugation at 2,000 rpm for 5 minutes. To obtain nuclear and cytoplasmic extracts we used the *Margilene Nuclear Extraction Kit* (G-Biosciences, St Louis, MO, USA). To each cellular pellet, resuspended in 1ml

PBS 1X, were add 500  $\mu$ l of *Complete Hypotonic Lysis Buffer*. Samples were incubated 10 minutes on ice. After incubation, 50  $\mu$ l of *Detergent Solution* was added, samples vortexed and centrifuged 5 minutes at 3,000 rpm at 4°C. The supernatant was removed from nuclear pellet. This supernatant contains the cytoplasmic extracts that were stored at -80°C until quantification. We add to nuclear pellet, 500  $\mu$ l of *Complete Wash Solution*, and centrifuge samples for 5 minutes at 3,000 rpm at 4°C. After removing supernatant, *Extraction Buffer 1* (one-half pellet volume) and *Extraction Buffer 2* (one-half pellet volume) were added to the samples, then vortexed and incubated on ice for 30 minutes. Each sample was vortexed momentarily at high speed every 10 minutes. Finally, nuclear pellets were centrifuged 30 minutes at 12,000 rpm at 4°C. The supernatants, containing nuclear extracts, were collected and stored at -80°C until quantification.

### 2.8.1 Protein extracts quantification- Bradford Assay

The Bradford Reagent (Bio-Rad Laboratories, MI, Italy) was used to determine the concentration of proteins in nuclear and cytoplasm extracted, obtained from MDA-231 cells treated with PNA anti-miR-221. To 1 ml of Bradford Reagent were added 3  $\mu$ l of each samples, mixed and analyzed at spectrophotometer to determine the absorption at 595 nm. To normalize the quantification, a standard curve was prepared using BSA (bovine serum albumin) as standard protein.

## 2.9 Western Blot Assay

Twenty  $\mu$ g of nuclear and cytoplasmic extracts were denatured for 5 min at 98°C in 1X SDS sample buffer (62,5 mM Tris-HCl pH 6.8, 2% SDS, 50 mM Dithiotreitol (DTT), 0.01% bromophenol blue, 10% glicerol) and loaded on 12,5% SDS-PAGE gel in Tris-glycine Buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). A biotinylated protein ladder (size range of 9-200 kDa) (Cell Signaling, Euroclone S.p.A., Pero, Milano, Italy) was used as standard to determine molecular weight. The electrotransfer to 20 microns nitrocellulose membrane (Pierce, Euroclone S.p.A., Pero, Milano, Italy) was performed over-night at 360 mA and 4°C in electrotransfer buffer (25 mM Tris, 192 mM Glycine, 5% methanol). The membrane were prestained



in Ponceau S Solution (Sigma, St.Louis, MO, USA) to verify the transfer, washed with 25 ml TBS (10 mM Tris-HCl pH 7.4, 150 mM NaCl) for 10 min at room temperature and incubated in 25 ml of blocking buffer for 2 h at room temperature. The membranes were washed three times for 5 min each with 25 ml of TBS/T (TBS, 0.1% Tween-20) and incubated with primary rabbit monoclonal antibody (1:1000) (Cell Signaling, Euroclone S.p.A., Pero, Milan, Italy) in 15 ml primary antibody dilution buffer with gentle agitation over-night at 4°C. The day after, the membrane were washed three times for 5 min each with 20 ml of TBS/T and incubated in 15 ml of blocking buffer, in gentle agitation for 2 h at room temperature, with an appropriate HRP-conjugated secondary antibody (1:2000) and an HRP-conjugated anti-biotin antibody (1:1000) used to detect biotinylated protein marker. Finally, after three washes each with 20 ml of TBS/T for 5 min, the membranes were incubated with 10 ml LumiGLO<sup>®</sup> (0.5 ml 20X LumiGLO<sup>®</sup>, 0.5 ml 20X Peroxide and 9.0 ml Milli-Q water) (Cell Signaling, Euroclone S.p.A., Pero, Milan, Italy) in gentle agitation for 5 min at room temperature and exposed to x-ray film (Pierce, Euroclone S.p.A., Pero, Milan, Italy). As necessary, after stripping procedure using the Restore<sup>™</sup> Western Blot Stripping Buffer (Pierce, Euroclone S.p.A., Pero, Milan, Italy) membranes were reprobated with primary and secondary antibodies. X-ray films for chemiluminescent blots were analysed by Gel Doc 2000 (Bio-Rad Laboratoires, Milan, Italy) using Quantity One program to elaborate the intensity data of our specific protein targets. Ponceau S staining was used as normalization control, but others marker proteins were taken as reference too and specifically reported. p27<sup>kip1</sup> and  $\beta$ -actin Rabbit mAb and were purchase from Cell Signaling (Euroclone, MI, Italy).

## - RESULTS-

### 3.1 Uptake and biostability of PNAs in breast cancer cell lines

The first step necessary for obtaining anti-miR activity in cells is the effective cellular uptake. We have recently demonstrated that Rpep-PNA-a210 was efficiently internalized within leukemic K562 cells, unlike PNA-a210, and this was accompanied by a pronounced anti-miR activity of the former and no activity for the latter (Fabbri et al., 2011).

#### 3.1.1 Treatment with trypsin

Trypsin is an enzyme belonging to the class of hydrolase, which catalyzes the cleavage with specificity for arginine and lysine. Possessing Rpep-PNA-a221 a tag of 8 arginine, as a preliminary experiment, we checked if the PNAs could be degraded by treatment with trypsin. We have pre-incubated 2  $\mu$ M PNA-a221 and Rpep-PNA-a221 for 10 minutes, at room temperature, in the dark with 2  $\mu$ l and 4  $\mu$ l of 0,05%Trypsin-EDTA. After incubation, PNAs were added to cells and after 24 hours of incubation, cells were washed once with PBS, trypsinized and analyzed by fluorescence-activated cell sorting analysis (FACS).

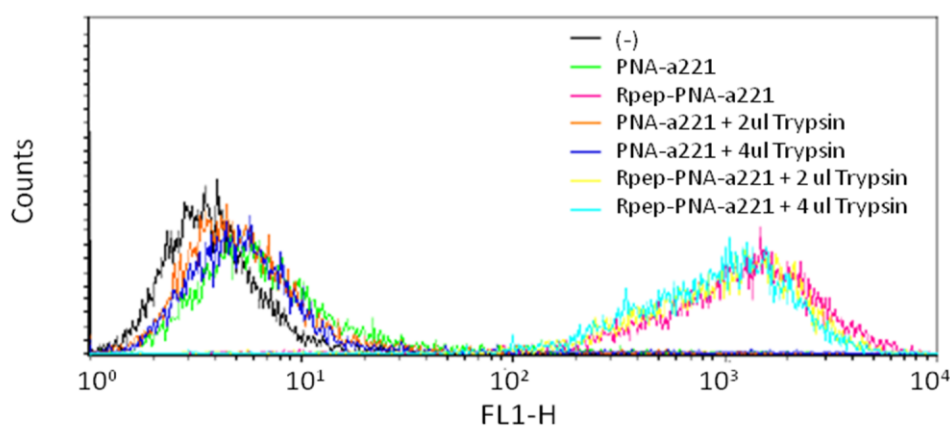


Figure 19. FACS analysis showing the uptake of FI-PNA-a221 and FI-Rpep-PNA-a221 pre-incubated with different volumes (2  $\mu$ l and 4  $\mu$ l) of trypsin-EDTA

As shown in Figure 19, the treatment with 2  $\mu$ l or 4  $\mu$ l of trypsin does not cause any variation in the cellular uptake, neither for FI-PNA-a221 nor for FI-Rpep-PNA-a221; in fact, the FI-PNA-a221 remains not internalized by the cells (comparable with untreated cells with FL-1 value from  $10^0$  to  $10^1$ ), while the FI-Rpep-PNA-a221 is effectively absorbed (FL-1 value  $> 10^2$ ).

### 3.1.2 Culture and treatment w/ or w/o FBS

Since different PNA structures could be affected by proteases present in serum, leading to different cellular uptake, these experiments have been conducted in the presence or in the absence of serum. Cells were cultured in a complete medium (D-MEM + FBS 10%) or only with D-MEM, and treated with FI-PNAa221 and FI-Rpep-PNA-a221 (1 $\mu$ M and 2  $\mu$ M). Figure 20 reports the results obtained from FACS analysis. The FI-PNA-a221, both in presence or absence of serum (Figure 19a and 19c) shows low internalization; these results are in agreement with previous results obtained with PNA-a210 (Manicardi et al., 2010). On the contrary the R<sub>8</sub>-conjugated FI-PNA-a221 (Figure 20b and 20d) is efficiently internalized, better in presence of serum (Figure 20d).

For this experiment we used K562 cell line, since it was a useful and simple in-vitro experimental cellular model. Cells were seeded at concentration of  $2 \times 10^5$  cell/ml in 12-well plate, treated with increasing concentrations PNAs and cultured for 12 hours in the presence or absence of FBS. We can conclude that the serum does not interfere with the cellular uptake, indeed, is able to improve cell viability. Following these preliminary data, we set up uptake a series of experiments to investigate the uptake of PNAs in our cellular models, MDA-MB-231 and MCF-7 cells line.

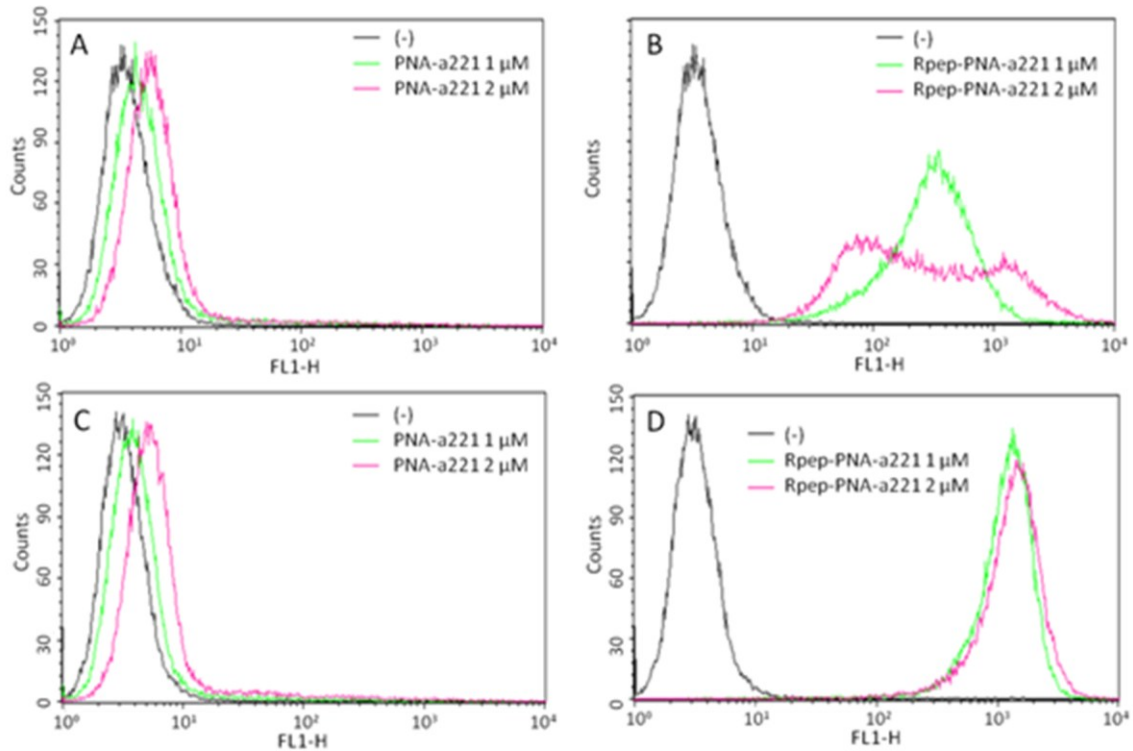


Figure 20. Cellular uptake analysis (FACS) showing cellular uptake of (A) PNA-a221 and (B) Rpep-PNA-a221 cultured without FBS, (C) PNA-a221 and (D) Rpep-PNA-a221 cultured in the presence of FBS

### 3.1.3 Cellular uptake in MDA-MB-231 and MCF-7

Following our preliminary results regarding the cellular uptake, MDA-MB-231 and MCF-7 were incubated for 24 hours with FI-PNA-a221 and FI-Rpep-PNA-a221 at 1  $\mu$ M, 2  $\mu$ M and 4  $\mu$ M concentrations. After incubation, cells were washed with PBS, trypsinized and analyzed by fluorescence-activated cell sorting analysis (FACS), obtaining the results shown in Figure 21.

Analyzing these results, in both our cellular models, MDA-MB-231 and MCF-7, the PNAs with R<sub>8</sub>-peptide is better internalized by cells than other. We can also observe that MDA-MB-231 and MCF-7 respond in a different way to the treatment with FI-Rpep-PNA-a221: although the proportion of positive cells (area under the curve, M1 value) is comparable for both cell lines, the two samples are differently distributed. In Table 4 are reported the statistic values of the analysis.

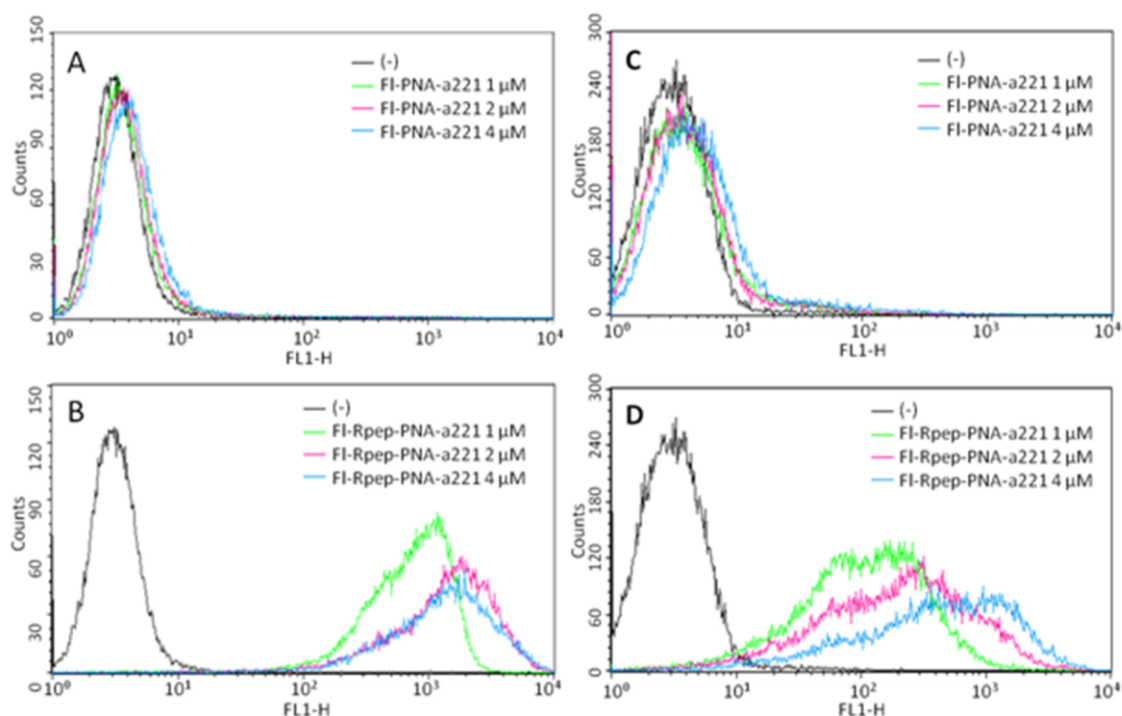


Figure 21. FACS analysis showing the cellular delivery of FI-PNA-a221 and Rpep-PNA-a221 in MDA-MB-231 (A,B) and MCF-7 (C,D) after 24 hours incubation at different concentration

Table 4. Statistical analysis of Rpep-PNA-a221 uptake in MDA-MB-231 and MCF-7 by FACS

FI-Rpep-PNA-a221	MDA-MB-231		MCF-7	
	M1	M2	M1	M2
1 $\mu\text{M}$	99.88%	34.12%	98.01%	0.66%
2 $\mu\text{M}$	99.97%	67.56%	98.56%	9.14%
4 $\mu\text{M}$	99.99%	64.39%	99.44%	26.60%

M1: value (%) of fluorescent cells ( $>10^1$ ) calculated on total cell analyzed (cells Gated)

M2: value (%) of fluorescent cells ( $>10^3$ ) calculated on total cell analyzed (cells Gated)

It can be observed that, in both cell lines, the M1 value, which represents the percentage of cells with fluorescence  $> 10^1$ , shows no major changes; in fact, the values remain around 98-99%; this means that all cells analyzed have internalized the FI-Rpep-PNA-a221. On the contrary, the M2 value, which is referred to strongly fluorescent cells, that is indicative of a higher uptake, is very different between the two cell lines, with values that reach approximately to 67% in MDA-MB-231 cells, while, in MCF-7 cells, only with 4  $\mu\text{M}$  PNA, arrive to 26.60%. This analysis could indicate a different cellular response to this specific treatment with the PNA anti-miR221, considering also the different phenotype that these cells present.

### 3.1.4 Cellular uptake by BioStation IM

We have also performed a series of experiments using the Biostation IM technology. Cells were seeded at  $2 \times 10^5$  cell/ml, 300  $\mu$ l in each well in a  $\mu$ -Dish Hi-Q<sup>4</sup> and monitored for 24 hours. The images were taken every 30 minutes of incubation, as well described in Chapter “*Materials and Methods*”.

In Figure 22, there are report pictures of untreated MDA-MB-231, treated with 2  $\mu$ M FI-PNA-a221 and 2  $\mu$ M FI-Rpep-PNA-a221 taken after 24 hours of incubation. As shown, while the FI-PNA-a221 remains largely outside the cells (white arrows, Figure 22b), FI-Rpep-PNA-a221 is absorbed by cells (yellow arrows, Figure 22c). Further investigations, using fluorescence microscopy, are needed to discriminate whether the accumulation in the cytoplasm or nucleus.

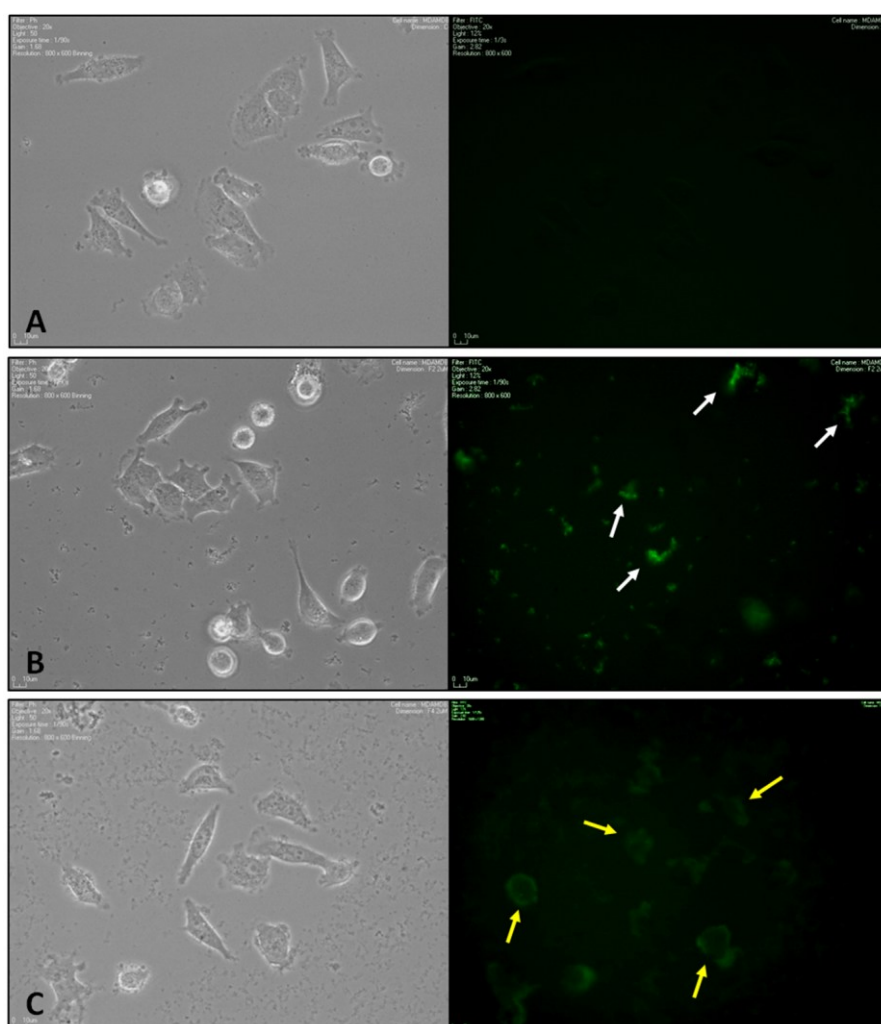


Figure 22. Visible and fluorescent 20X magnification photographs of MDA-MB-231 cells untreated (A), treated with FI-PNA-a221 (B) and FI-PNA-a221-Rpep (C)

### 3.2 Antiproliferative activity of PNA-a221 and Rpep-PNA-a221

Having demonstrated that the Rpep-PNA-a221 is better internalized into cells if compared to the PNA-a221, and in order to determine the concentrations of PNAs to be employed for in vitro studies on MDA-MB-231 cells, we determined the  $IC_{50}$  value. For this reason, MDA-MB-231 and MCF-7 cells ( $2 \times 10^4$  cells/ml) were seeded in 24-well plate; after 24 hours the media was changed and replaced with experimental media containing different concentration of PNAs. Cells were growth for 72 hours and after this incubation, medium was removed, cell wash with PBS and trypsinized for 5 minute at  $37^\circ C$  and counted by a *ZF Coulter Counter*. As reported in Figure 23, the treatment with PNAs anti-miR-221 do not affect the MDA-MB-231 and MCF-7 cell proliferation; in fact the  $IC_{50}$  is  $> 10 \mu M$ ; interesting, the concentrations used for the next experiments ( $2 \mu M$ ), do not affect cells growth. Results represent the average  $\pm$  S.D. of three independent experiments.

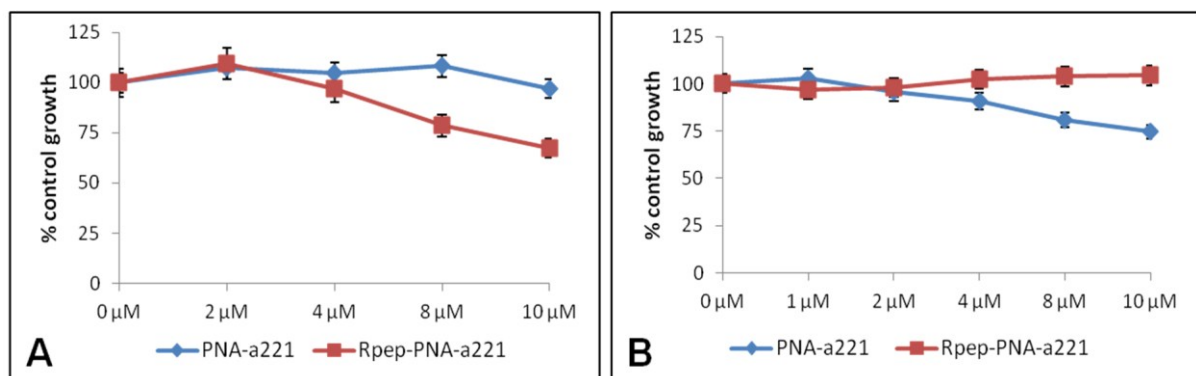


Figure 23. Effect of PNA-a221 and Rpep-PNA-a221 on MDA-MB-231 (A) and MCF-7 (B) proliferation. Cells were treated with different concentrations of PNAs and Counted with Coulter Counter. Results represent the average  $\pm$  S.D. of three independent experiments

We have recently published (Fabbri et al., 2011) the antiproliferative activity of R-pep, composed by 8 arginine. The  $IC_{50}$  value, calculated on K562 cells treatment, was  $> 10 \mu M$ , suggesting, also for  $R_8$ , no cytotoxic activity at the used concentrations.

### 3.3 Rpep-PNA inhibitory effects on miR-221 analyzed by qPCR assay

We initially evaluated the accumulation of miR-221 in standard condition, without any treatment. The cells were cultured for 48 and 96 hours and total RNA was isolated. The over-expression of miR-221 in estrogen receptor (ER)-negative breast cancer (MDA-MB-231) is well known in literature, and it has been recently reported by Zhao *et al.*, (2008); we already confirmed that miR-221 is about 4 times more expressed in MDA-MB-231 cells (invasive phenotype) than in MCF-7, suggesting a possible correlation with expression of miR-221 and cancer grade. (Figure 24).

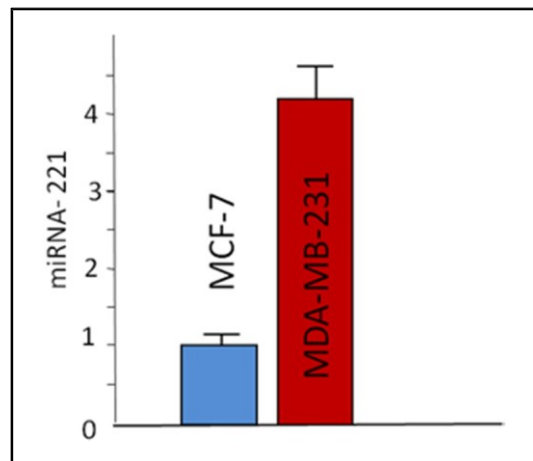


Figure 24. Accumulation of miR-221 in MCF-7 and MDA-MB-231 cell lines in normal culture conditions

Following these results, we decided to proceed with the analysis of inhibition of mir-221 only in MDA-MB-231 cell line, which expresses miR-221 at higher levels. Considering the data obtained by FACS analysis regarding the cellular uptake, preliminary experiments were prepared by treating the cells with increasing concentrations (0.5, 1 and 2  $\mu$ M) of PNAs to identify the best concentration that express an anti-miR-221 activity; the inhibitory activity on miR-221 is dose-dependent. In addition, the concentration of 2  $\mu$ M of Rpep-PNA-a221 prominently inhibits the accumulation of miR-221, both after 48 hours and 96 hours (Figure 25). Anti-miR-221 activity was evaluated by RT-PCR analysis. MDA-MB-231 cells were treated with 2  $\mu$ M PNAs; after 48 and 96 hours of incubation the total RNA was



isolated. As shown in Figure 25, the expression of miR-221 is significantly inhibited by treatment with Rpep-PNA; in particular, the Ct value (cycle threshold) change from 20.03 to 24.56 at 48 hours and from 20.29 to 23.46 at 96 hours.

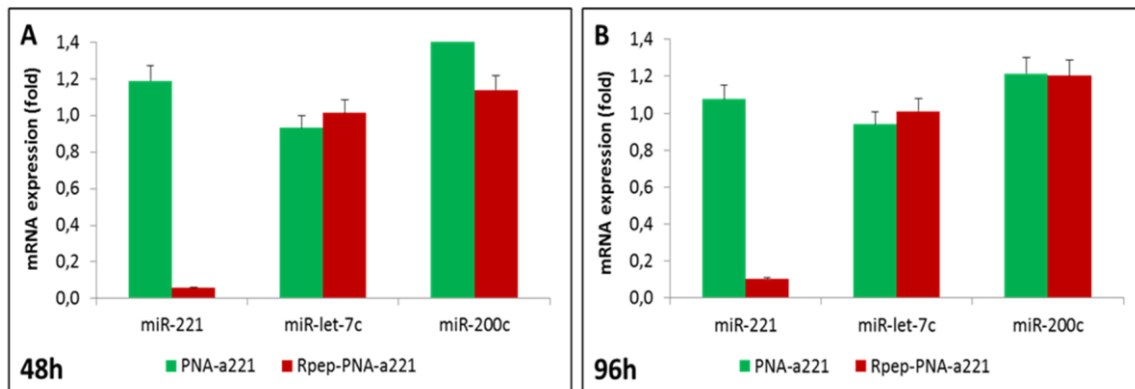


Figure 25. Accumulation of miR-221, miR-let-7c and miR-200c in MDA-MB-231 cells treated for 48 (A) and 96 (B) hours with 2  $\mu$ M PNA-a221 and Rpep-PNA-a221. qRT-PCR amplifications were performed on RNA from untreated or treated cells using primers amplifying U6 RNA as reference gene. Results are presented as fold increase of miR-221, miR-let-7c and miR-200c mRNAs with respect to untreated cells. The results of untreated cells were taken as 1. Results represent the average  $\pm$  S.D. of three independent experiments

Increasing of Ct value indicates a decrease in the accumulation of miR-221, indicating an efficient inhibition activity of Rpep-PNA-a221. It's can also note that the activities of the Rpep-PNA is specific for miR-221; in fact, Figure 23 shows that this effects are restricted to miR-221, since, despite the fact that some alteration of miR content occurs, no suppression of accumulation of miR-let-7c and miR-200c has been obtained. These data demonstrate the specificity on the PNAs synthetized.

### 3.4 Modulation of p27<sup>kip1</sup> by treatment with anti-miR-221 PNAs

It' s well known in the literature, as more completely described in the chapter "Introduction" of this PhD Thesis, that in the 3'UTR region of p27<sup>kip1</sup> mRNA are presents at least two sites recognized by miR-221(Galardi et al., 2007) Since microRNA-221 is able to target the onco-suppressor p27<sup>kip1</sup> mRNA, involved in cell cycle control, we have prepared a series of experiments to understand whether the inhibition activity of miR-221 induced by PNAs could influence the expression of

p27<sup>kip1</sup>. Firstly, we have investigated the basal expression of p27<sup>kip1</sup> by RT-qPCR in untreated cells. As shown in Figure 26, MCF-7 cells present higher level of accumulation of p27<sup>kip1</sup> mRNA than MDA-MB-231 cells.

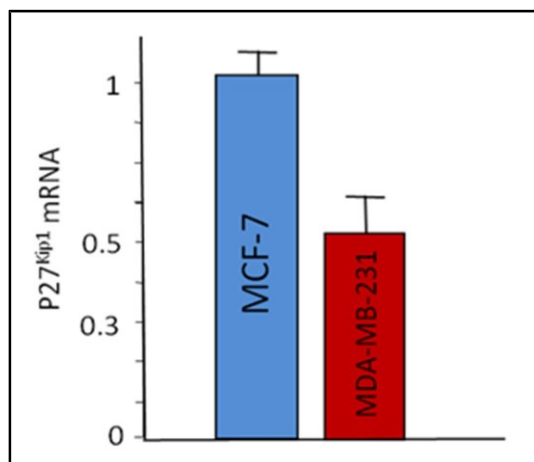


Figure 26. Expression of p27<sup>kip1</sup> mRNA in MCF-7 and MDA-MB-231 cell lines in normal culture conditions

### 3.4.1 Accumulation of p27<sup>kip1</sup> mRNA

To analyze the p27<sup>kip1</sup> mRNA accumulation, MDA-MB-231 cells were treated with 2  $\mu$ M PNAs for 48 and 96 hours; after incubation, total RNA was isolated by Trizol® Reagent, following manufacture's instruction. We perform qPCR using CDKN1B probe and primers (Applied Biosystems, Foster City, CA, USA). Relative expression was calculated using the comparative cycle threshold method and as reference genes the endogenous control human 18S rRNA.

Results shown in Figure 27 indicate that no change of p27<sup>kip1</sup> mRNA content occurs in MDA-MB-231 cells treated with PNA-a221, whereas significant increase of presence of p27<sup>kip1</sup> mRNA is observed with the Rpep-PNA-a221, better after 96 hours than 48 hours. This result is strongly in agreement with that obtained using the commercially available anti-miR against miR-221. These results about the expression of mRNA, have suggested us to investigate whether this variation in mRNA level could have a visible effect on the p27<sup>kip1</sup> protein content. For this aim, we performed a series of Western Blot assay, using antibodies against p27<sup>kip1</sup> and  $\beta$ -actin (Figure 28a).

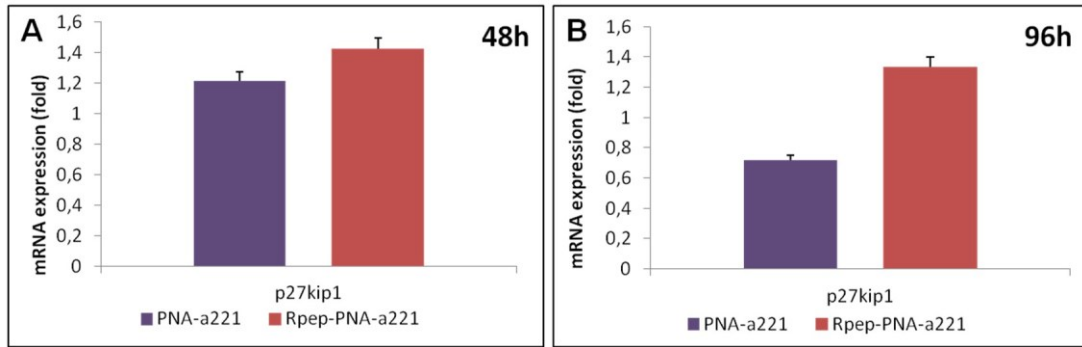


Figure 27. p27<sup>kip1</sup> mRNA content in MDA-MB-231 cell treated with 2  $\mu$ M PNA-a221 and Rpep-PNA-a221 for 48 hours (A) and 96 hours (B). qRT-PCR amplifications were performed on RNA from untreated or treated cells using primer amplifying 18S rRNA as reference gene

### 3.4.2 Western Blot analysis of p27<sup>kip1</sup>

Cytoplasmic protein extracts were obtained from MDA-MB-231 cells after 96 hours of PNAs treatment. It's possible to notice a visible increase in the intensity of the spot of p27<sup>kip1</sup> with the Rpep-PNA-a221 treatment, if compared with untreated cells. Figure 27b shows the relative intensity of the p27<sup>kip1</sup> spots, obtained from densitometric analysis of the films. The treatment with PNA-a221 is able to increase the accumulation of p27<sup>kip1</sup> from 0.44% to 1.20%, while the relative intensity of the accumulation of p27<sup>kip1</sup> increases up to 1.87% with the treatment with Rpep-PNA-a221. All the values are normalized on  $\beta$ -actin protein expression, taken as reference protein.

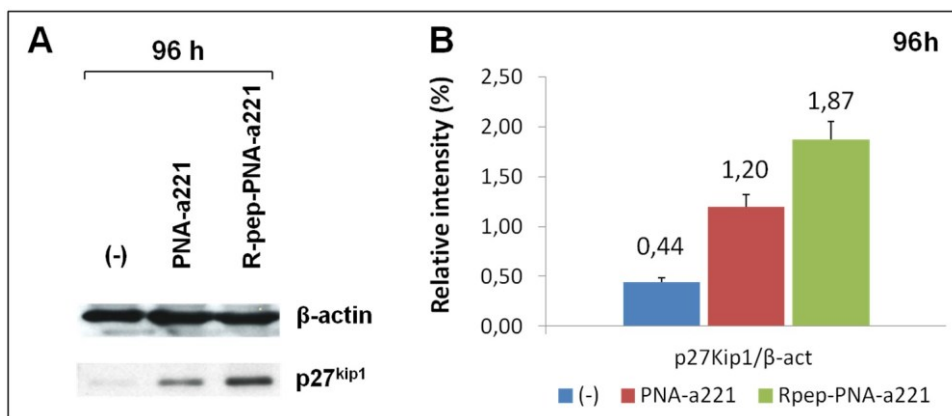


Figure 28. (A) Western Blot analysis performed on MDA-MB-231 cells treated with 2  $\mu$ M PNAs for 96 hours. (B) Graph generated by densitometric analysis of the films and the normalized intensity values represent mean and standard deviation from three independent experiments. Ponceau S staining was used as normalization control

## - DISCUSSION AND CONCLUSIONS (2) -

MicroRNA-221 is deeply involved in cancer, and it was found up-regulated in glioma, hepatocellular carcinoma, pancreatic adenocarcinoma, melanoma, chronic lymphocytic leukemia, thyroid papillary carcinoma. In breast cancer, miR-221 was found to be up-regulated in breast cancer cell lines and primary tumor cell cultures exhibiting high metastatic potential. Taken together, miR-221 should be considered as an oncomiR and, for this reason, a strong candidate for miRNA-therapeutics based on antagomiR molecules. As far as possible target molecules of miR-221, many of them have been firmly established, such as DVL2, PUMA, PTEN, p27<sup>Kip1</sup>. In the context of breast tumors, of great interest is the study published by Farace et al., identifying p27<sup>Kip1</sup> mRNA as a possible target of miR-221. This finding is very intriguing, since p27<sup>Kip1</sup> has been proposed as a tumor suppressor gene, which is down-regulated in several types of tumors. These data support the concept that targeting miR-221 with antagomiR molecules might lead to an increased expression of the tumor-suppressor p27<sup>Kip1</sup>, bringing novel treatment options in cancer treatment.

The major conclusions of this study is that a PNA against miR-221 is efficiently internalized within target cells only if linked to an arginine-rich peptide, strongly inhibits miR-221 activity and deeply alters the expression of p27<sup>Kip1</sup> gene. Unlike commercially available anti-miR, which needs continuous administrations, a single administration of Rpep-PNA-a221 is sufficient to obtain the biological effects without the need for transfection reagents. Interestingly, modifications allowing efficient uptake by target cells (Results, Figure 20 B and D) are necessary to obtain the biological activity, since PNA-a221, despite being able to hybridize to the target nucleotide sequence (Results, Figure 24) is not internalized (Results, Figure 20 A and C) and displays a very low activity on p27<sup>Kip1</sup> (Results, Figure 26 and 27). Therefore, efficient delivery strategies are necessary and, in this respect, we would like to underline that the delivery of Rpep-PNA-a221 needs no transfection reagents (such as lipofectin, lipofectamine and similar reagents) which, on the contrary, are required when RNA or DNA-based analogues are used.

From a theoretical point of view, these studies fully support the concept that p27<sup>Kip1</sup> mRNA might be considered among possible targets of miR-221. In fact, in the presence of Rpep-PNA-a221 we observed effects MDA-MB-231 cells compatible with a decrease of miR-221 (it should be underlined that the effects of Rpep-PNA-a221 might be based on binding to mature miR-221, but also to pre-miR sequences), an increase of p27<sup>Kip1</sup>.

From a general point of view, our results allow to propose PNA-based molecules as very promising reagents to modulate the biological activity of microRNAs. These data should encourage further research on PNA analogues to increase efficiency of delivery, stability and change of intracellular distribution in view of the selected miR targets, such as mature miR, pre-miR or primary miRNA (pri-miR) sequences. Despite the fact that in this paper we focused our attention on breast cancer cellular model systems, we like to underline that p27<sup>Kip1</sup>/miR-221 are deeply involved in other tumors for which PNA-based treatments are expected to be very appealing. One example in glioblastoma, which expresses high levels of miR-221 and down regulated p27<sup>Kip1</sup> that should be considered the major onco-suppressor protein in this type of tumors. Lu and Zhang (2009) have first demonstrated that miR221/222 promote malignant progression of glioblastoma through activation of the Akt pathway and inhibition of p27<sup>Kip1</sup>; in a second manuscript, the same group reported that co-suppression of miR-221/222 cluster suppresses human glioma cell growth by targeting p27<sup>Kip1</sup> in vitro and in vivo (Zhang et al., 2009). Interestingly, modified PNAs can be easily delivered to brain tissues originating glioblastoma, as demonstrated by Pession *et al.*, using PNA-based molecules targeting the NMYC mRNA (Pession et al., 2005). Our data strongly suggest that PNAs targeting miR-221 and restoring p27<sup>Kip1</sup> levels might be considered for experimental therapy of gliomas.

Based on these findings, future research activity should be focused on the development of more effective PNA-based anti-microRNA molecules. In this respect, because the use of peptides as carriers might represent a problem for potential PNA-based drug candidates (as the peptide portion might be subject to enzymatic degradation), the incorporation of positively charged peptides with the PNA backbone (and not at the end) would protect the molecule from proteolysis, allowing highly efficient uptake, and thus persevering activity against targeted microRNAs.

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## - GENERAL CONCLUSIONS -

The general conclusion of this PhD Thesis is related to the biomedical applications of the final product of PNA synthesis (the PNA itself) and the intermediate molecules obtained during the synthetic activity. In all the chemical synthesis approach of any pharmaceutical laboratory several molecules are produced, which are usually not considered for biological assays and technology transfer.

The studies presented in this PhD Thesis teach that even intermediate synthetic molecules deserve attention in respect to possible biological effects on cells relevant to human pathologies. In our study we screened a set of C(5) uracil derivatives monomers, that were employed during the PNA synthesis, for activity on differentiated functions in K562 cells, a model system mimicking some features of chronic myelogenous leukemia (CML) (Gambari et al., 2007). We found that the highest antiproliferative effect and erythroid induction ability was exhibited by compound **9**, a thymine derivative bearing a n-octyl chain on nitrogen N(1), whereas thymine (compound **2**) did not show any effect, suggesting the importance of the linear alkyl chain in N(1) position. We have also compared compound **9** with other two similar molecules, compound **10** (1-octyl-5-uracilcarboxylic acid) and compound **11** (N-benzyl-1-octyl-5-uracilcarboxamide); these derivatives present the alkyl chain in N1 position but different groups in C(5) position. Compound **10** has an appreciable but much lower erythroid differentiation activity, suggesting the importance of C(5) position. Compound **9**, furthermore, exhibits induce erythroid terminal differentiation without activation of apoptotic pathway.

The interest in the context on anti-tumor differentiation therapy is related to the fact that, when compared to known erythroid differentiation antitumor inducers (such as for instance cytosine arabinoside, mithramycin, resveratrol), the lead compound we were able to identify is the most active agent. Therefore these molecules in our opinion deserves further research activity in order to define its possible application, for instance in the control of proliferation/differentiation of CML primary cells resistance to the commonly employed Imatinib (Gleevec®) therapy.

As far as the final product of the synthetic strategy (a PNA recognizing miR-221 and able to be internalized in target tumor cells thanks to a linked Arg-8 peptide), the results here presented allow to conclude that (a) it is internalized at high efficiency into target tumor cells; (b) inhibits the miR-221 hybridization availability and (c) has important effects on biological functions regulated by miR-221 (i.e. expression of the p27<sup>Kip1</sup> mRNA/protein).

These results are in our opinion of interest, considering on one hand the role of miR-221 in cancer and, on the other hand, the role of p27<sup>Kip1</sup>.

As far as miR-221, it was found to be up-regulated in several tumors; in breast cancer miR-221 is up-regulated in breast cancer cell line (such as MDA-MB-231) and in metastatic tumors. Taken together, miR-221 should be considered as an oncomiR and, for this reason, of great interest as molecular target of anticancer therapy.

In the context of breast cancer, it was identified p27<sup>Kip1</sup> mRNA as possible target of miR-221. The cyclin-kinase inhibitor p27<sup>Kip1</sup> is a tumor-suppressor protein, involved in the control of cell cycle during the G0/G1 check-point transition: the loss or decrease of p27<sup>Kip1</sup>, together with others, is one of contributory causes of the “*proliferating state*” of invasive cancer, which remains in this growth phase without arrive to differentiation. It is of great interest since it was found to be down-regulated in several type of tumors.

In conclusion, we have identify two reagents of possible interest for the development of anti-cancer protocols: N(1)-octyl-thymine for the treatment of CML cells and Rpep-PNA-a221 for possible use in the treatment of breast cancer cells.



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