



University of Sassari

Department of Biomedical Sciences

International PhD School in Biomolecular and Biotechnological Sciences

Section: Biochemistry and Molecular Biology

Director: Leonardo A. Sechi

Cycle XXVII

***Biological evaluation of
1,3,4-oxadiazoles bis-substitute derivatives
as potential anticancer agents***

Supervisor:

Prof. Luigi Marco Bagella

PhD candidate:

Valentina Nieddu

Academic Year: 2013-2014

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ABSTRACT

Cancer is a major health problem and causes substantial morbidity and mortality worldwide.

In order to identify new potent antitumor agents with good specificity and low toxicity, a series of 12 new 1,3,4-oxadiazole bis-substitute molecules were tested on different cancer cell lines as potential antitumor candidates. All the compounds were tested in order to select the molecules with stronger and more selective cell growth inhibitory activity.

The cytotoxic effects were evaluated *in vitro* and it was found that Molecule 10 had the greatest antitumor activity on cancer cells and it exerted an antiproliferative behavior in a dose-dependent manner. Clonogenic assay revealed a visible reduction of colony formation in all cell lines.

To investigate the effects of the molecule on the cell cycle progression, flow cytometry analysis were performed identifying cell cycle arrest in G₂/M phases, therefore immunofluorescence analyses were realized using β -tubulin antibody and abnormal effects were showed on tubulin organization. To understand molecular mechanism involved in cell cycle arrest, western blot analysis were executed identifying a deregulation of p53 levels.

Same experiments were repeated in human fibroblast cells as normal control versus the respective immortalized cell lines. All the fibroblasts were less sensitive and showed a higher IC₅₀ than cancer and immortalized cells, validating that Molecule 10 has low systemic toxicity and a specific activity to cancer cells.

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1. INTRODUCTION

1.1 Cancer

Cancer is not a single pathological state but a wide group of diseases. The pathology is characterized by a series of cumulative genetic and epigenetic mutations that occur in normal cells and is described by six essential alterations in cell physiology that induce malignant growth: uncontrolled growth and division, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (Fidler 2003, Hanahan and Weinberg 2000).

Development of cancer is attributed to exogenous (chemicals, tobacco smoke, radiation and viruses) and endogenous (genetic predisposition, immunodeficiency and chronic inflammation) factors that induce changes in the genome of the cells (Zong et al 2012). Cancer can be caused by modifications leading to activation of oncogenes, thereby conferring new properties to the cells such as hyperactive growth, enhanced cell division and inhibition of programmed cell death (Sung et al 2007). Additionally, tumor suppressor genes may be inactivated, leading to disturbance of normal cell functions such as control of cell division, cell adhesion and orientation. All these properties of cancer cells are due to defects in the regulatory circuits that control proliferation and homeostasis (Brooks et al 2010, Kreeger and Lauffenburger 2010).

More recently epigenetic changes have also been related to tumor progression.

Epigenetic refers to “*Heritable changes in gene expression that are not due to any alteration in the DNA sequence*” (Holliday 1987).

Epigenetic gene regulation and genetic alteration cooperate in cancer development: cell growth and differentiation, cell cycle control, DNA repair, angiogenesis and migration (Bird 2002, Lund and van Lohuizen 2004).

DNA methylation is the most widely studied epigenetic modification. DNA methylation levels are preserved by DNMTS enzymes and are balanced in cells: overexpression of DNMTs is associated to cancer and hypomethylation induce genome instability and incorrect activation of oncogenes.

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DNA methylation contributes to a stable gene silencing mechanism and regulate gene expression and chromatin architecture in correlation with chromatin associated proteins and histone modifications (Bird 2002).

The histone N-terminal tails maintain chromatin stability and are exposed to numerous modifications: acetylation, methylation, phosphorylation, ubiquitylation, sumoylation and phosphorylation. These modifications have a crucial role in transcriptional regulation and, if deregulated, have the potential to be oncogenic (Kouzarides 2007).

Recent studies identify important correlation between change in miRNA expression and cancer. Since miRNAs regulate genes involved in transcriptional regulation, cell proliferation and apoptosis, alteration in their expression can promote tumorigenesis. miRNAs can function as tumor suppressors and oncogenes depending on their target genes (Lu et al 2005).

In the foreground, cancer is being the second leading cause of death after heart disease and stroke. According to reports from the World Health Organization (WHO), in 2012 cancer accounts for 14.1 million new cases, 8.2 million deaths and around 13 % for all deaths in 2012. It is expected that annual cancer cases will rise to 22 million within the next two decades (WHO 2014).

Tumors are widely diffused among adults and children. The most frequent types of cancer include lung, stomach, liver, colon and breast cancer (Figure 1) and are usually treated with chemotherapy, radiation therapy and surgery.

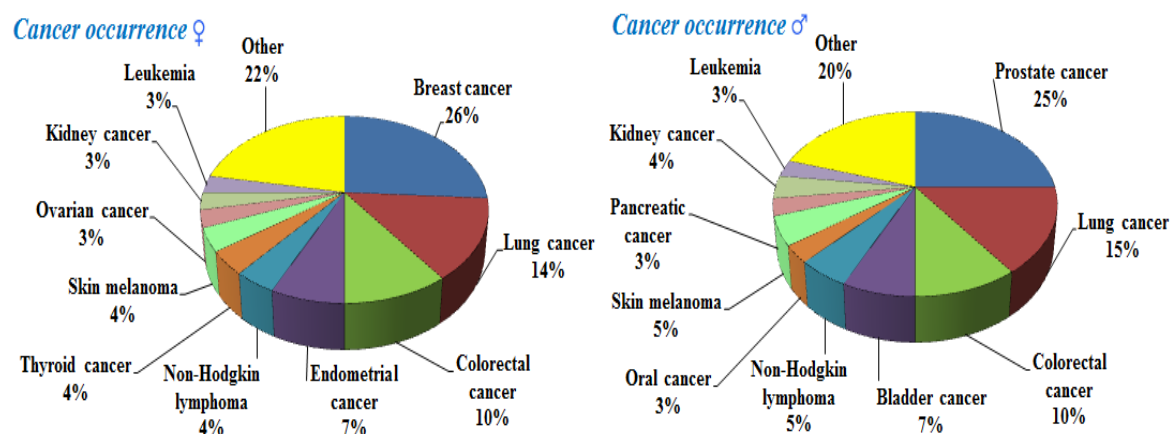


Figure 1
Cancer occurrence by sex in 2012.

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1.2 Different treatments of cancer

Until 1960, surgery and radiotherapy were the most preferred forms of treatment modality (DeVita and Chu 2008). The discovery of effective chemotherapeutic agents brought a significant revolution in the treatment of cancer malignancy (Ma and Wang 2009).

Currently, there is a wide variety of treatment options for different cancer types, depending on the location and stage of the disease.

The chemotherapeutic drugs are divided into several groups according to chemical structures and biological mechanisms, such as alkylating agents, antimetabolites, hormones and antagonist, and miscellaneous agents. Each class of drugs kills cells using different mechanism (Chabner and Roberts 2005).

Alkylating agents have been used in cancer treatment by forming a variety of inter-strand cross-linking adducts to alter DNA structure and function. The most common alkylating site is the N-7 position of guanine, but different drugs could have various alkylating sites as the N-1 and N-3 of adenine and N-3 of cytosine.

These agents are highly electrophilic compounds that attach alkyl groups to DNA by reacting at the nucleophilic sites present in the DNA bases. Thus, alkylation prevents DNA replication and RNA transcription, which results in cell death of affected cells. These agents are not phase specific but they work in all phases of the cell cycle.

Several groups of alkylating agents have been highly used and studied, to name some: nitrogen mustards, platinum compounds, ethylenimines, alkylsulfonates, triazines, piperazines, and nitrosoureas.

They are used to treat many different cancers: lung, breast and ovarian carcinomas, leukemia, lymphoma, Hodgkin disease, multiple myeloma and sarcoma (Espinosa et al 2003).

Antimetabolites are drugs, which interfere with DNA synthesis, cell division and tumor growth. They inhibit critical enzymes involved in nucleic acid synthesis or become incorporated into the nucleic acid and produce incorrect codes.

Alternatively, some antimetabolites are designed as structurally analogous of normal purines or pyrimidines that are able to compete for places in nucleotide pathways. Both mechanisms induce inhibition of DNA synthesis and cause cell death.

The main antimetabolites include pyrimidine or purine analogues and folate antagonists. Methotrexate, 5-fluorouracil, mercaptopurine, hydroxyurea, thioguanine and pentostatin are well known. They are commonly used to treat breast, ovary and intestinal cancer and leukemias (Wu 2006).

Tumors derived from sexually differentiated tissue such as prostate, breast and endometrium can be conditioned by the levels of related hormones in the body. This hormone-dependent response can be exploited chemotherapeutically to control the growth of cancer. Drugs in this category are sex hormones or hormone-like drugs that change the action or production of female or male hormones. These cancer treatment do not work in the same ways as standard chemotherapy drugs but they inhibit the cancer cells to use the hormone necessary for their grow or to prevent the body from synthesis of the hormones. Hormonal therapy includes steroids, anti-estrogens, anti-androgens, luteinizing hormone-releasing hormone (LH-RH) analogues and anti-aromatase agents (Kyle et al 2007).

The miscellaneous class contains different agents: antimetabolic, cell cycle inhibitor and antibiotic.

Antimetabolic agents prevent the function of microtubules by binding their subunits or through disruption of mitotic progression. These drugs inhibit cell mitosis by interfering with the formation of the mitotic spindle required for cell division or by binding the structural protein Tubulin, the main cellular target of these compounds, and therefore disturbing the dynamics of microtubules.

These molecules are used to treat many different types of cancer including breast, lung, myelomas, lymphomas and leukemias (Jordan and Wilson 2004).

Many clinically useful anticancer antibiotics are used in the treatment with antitumoral drugs by interfering with enzymes involved in DNA replication or by intercalating DNA to block synthesis of DNA and RNA. These drugs work in all phases of cell cycle and they are widely used for many cancers: breast, ovarian, thyroid and lung.

Examples of antibiotics include: doxorubicin, daunorubicin, mitomycin C, plicamycin, idarubicin and bleomycin (Ferrazzi et al 1991).

The development of molecules direct to the deregulated cell cycle has been evaluated as an ideal strategy for cancer therapy in recent years. These drugs aim at the abnormal expression of cyclin-dependent kinases (cdks) or at targeting the cellular checkpoints resulting in cell cycle arrest by induction of apoptosis in cancer cells (Fischer 2004).

Over the last several years, a new class of anticancer therapies has been developed and extensively tested: the inhibitors of cdks. These drugs have been tested as single agents with moderate results but, in combination with traditional cytotoxic chemotherapy, they had good results in drug resistance and cytotoxic efficacy (Dickson and Schwartz 2009). Currently, molecules under investigation include flavopiridol, seliciclib, roscovitine, puvalanol B and they have been used with a variety of human cancers, including breast and colorectal cancer, B-lymphoma, prostate and non-small cell lung cancer.

Inhibitors of the epidermal growth factor receptor (EGFR) are promising molecular targeting agents in the development of novel antitumor drugs (Baselga 2001). The EGFR is a transmembrane receptor tyrosine kinase (RTK) and its activation promotes multiple tumorigenic processes stimulating proliferation, angiogenesis and metastasis. Many different types of solid tumors show elevated levels of EGFR and its ligands are associated with aggressive cancer. This has induced to the development of different anti-EGFR mechanism directed to different components of EGFR signaling, such as inhibition of the intrinsic kinase activity of the EGFR, and inhibition of the synthesis of growth factors or their receptors (Huang and Harari 1999). It is also a promising molecular target agent in combination with radiotherapy (Lammering 2005). Actually cetuximab is licensed as a treatment for metastatic large bowel cancer and head and neck cancer. It is also being tested in research trials as a possible treatment for other types of cancer. Trastuzumab is licensed to treat breast and stomach cancer.

The reversible nature of epigenetic changes in cancer has induced to investigate in epigenetic therapies and, recently, FDA approved epigenetic drugs for cancer treatment. These drugs have been identified in the recent past and can effectively reverse DNA methylation and histone modification aberrations that occur in cancer (Yoo and Jones 2006).

DNA methylation inhibitors were the first epigenetic drugs used as cancer therapeutics. 5-azacytidine (5-aza-CR) and 5-aza2-deoxycytidine (5-aza-CdR) were approved for the treatment of all subtypes of myelodysplastic syndrome and promising results have also emerged from the treatment of other hematological malignancies. These nucleoside analogues are incorporated into the DNA of rapidly growing tumor cells during replication and they inhibit DNA methylation by blocking DNA methyltransferases (Plimack et al 2007).

Aberrant gene silencing in cancer is connected with a coincident loss of histone acetylation. Treatments with HDAC inhibitors have antitumorigenic effects including growth arrest, apoptosis and the induction of differentiation. These antiproliferative effects are mediated by the reactivation of silenced tumor suppressor genes (Carew et al 2008, Federico and Bagella 2011).

Suberoylanilide hydroxamic acid (SAHA), an HDAC inhibitor, has now been approved for use in clinic for treatment of T cell cutaneous lymphoma. Several other HDAC inhibitors, such as depsipeptide and phenylbutyrate, are currently under clinical trials (Cortez and Jones 2008). Combination treatment strategies, using DNA methylation and HDAC inhibitors together, have been observed to be more active than single treatment approaches (Cameron et al 1999).

1.3 Potential activity of oxadiazoles

The therapeutic use of anticancer drugs is complicated due to systemic toxicity, usually observed in bone marrow, gastrointestinal tract and hair and by development of resistance. Most tumors are refractory to conventional therapies currently in use and the effectiveness of the treatment is often limited by low selectivity and high toxicity (Gilchrest et al, 2009).

According to the data provided by the Food and Drug Administration (FDA), 95% of the tested drugs are not approved in the United States, and among them 70% did not pass the pre-clinical development because of its toxicity.

Therefore, the aim of new oncology studies is to explore novel drugs, therapies and new therapeutic approaches.

Scientists are focused on identifying new therapeutic targets for cancer and discovering novel, potent and selective anticancer drugs. The synthesis of new molecules as anticancer agents needs the simulation of an appropriate bioactive pharmacophore (Zhuang et al 2010) to be not only more efficient but also safer on normal cell lines, targeting only tumor cells (Kumar et al 2009).

In discovering anticancer drugs, molecules containing heterocyclic ring systems were of great importance both medicinally and industrially. Five-membered ring heterocycles containing two carbon atoms, two nitrogen atoms and one oxygen atom, known as oxadiazoles, are of considerable interest in different areas of medicinal and pesticide chemistry and also polymer and material science (Pace and Pierro 2009).

Oxadiazole rings can exist in different regioisomeric forms: two 1,2,4-isomers, 1,3,4-isomer and 1,2,5-isomer (Figure 2).

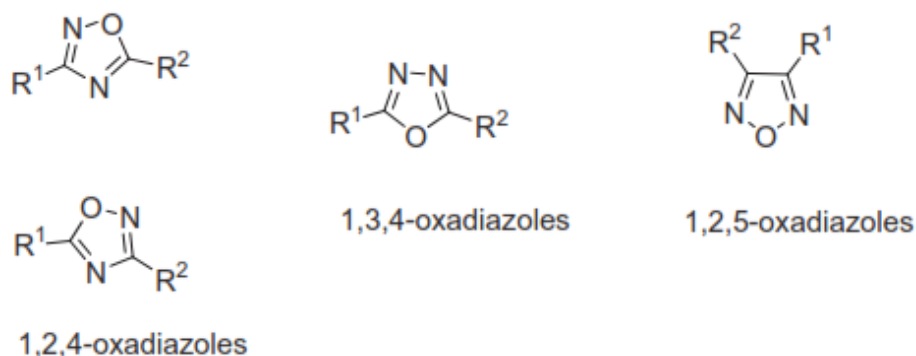


Figure 2

Regioisomeric forms of oxadiazole rings.

Over the last two decades, substituted oxadiazole have obtained plentiful attention for their broad range of biological activities and for the variety of therapeutic proprieties.

Literature indicates that these compounds possess different pharmacological and biological activities, since they act as anti-inflammatory (Nicolaidis et al 2004), antimicrobial (Gaonkar et al 2006), fungicidal (Li et al 2006) and anticancer (Abadi et al 2003, Aboraia et al 2006) agents.

The level of interest increased in the last 9 years and the number of patent applications containing oxadiazole rings has grown (Bostrom et al 2012).

Many compounds containing an oxadiazole moiety are in late-stage clinical trials: ataluren for the treatment of cystic fibrosis (Jones and Helm 2009) and raltegravir for the treatment of HIV infection (Summa et al 2008), have been directed onto the marketplace; zibotentan is in late-stage clinical trials as an anticancer agent.

Therefore, oxadiazoles are having a large impact on multiple drug discovery programs for several different purposes, including diabetes (Jones et al 2009), obesity (Lee et al 2008), inflammation (Unangst et al 1992), infection (Cottrell et al 2004) and cancer (Loetchutinat et al 2003, Szczepankiewicz et al 2001).

The rapid increase in the number of publications documenting examples of oxadiazoles activities, identify these compound as potential anticancer agents (Formagio et al 2008, Kumar et al 2008, Leteurtre et al 1994, Rostom et al 2003).

1.4 1,3,4 oxadiazole bis-substitute

The search of biologically promising new chemical compounds against cancer disease brings great attention in drug discovery.

Recent studies conducted by the group of Prof. Pinna at the Department of Chemistry and Pharmacy, University of Sassari, identified a notable role played by a new family of polycondensed heterocycles (Pau et al 2009). These compounds have a wide spectrum of pharmacological activities: anticancer, antihypertensive (Pinna et al 1996), anti-thrombotic (Cignarella et al 1989) and antiulcerative (Pinna et al 1996) properties (Figure 3).

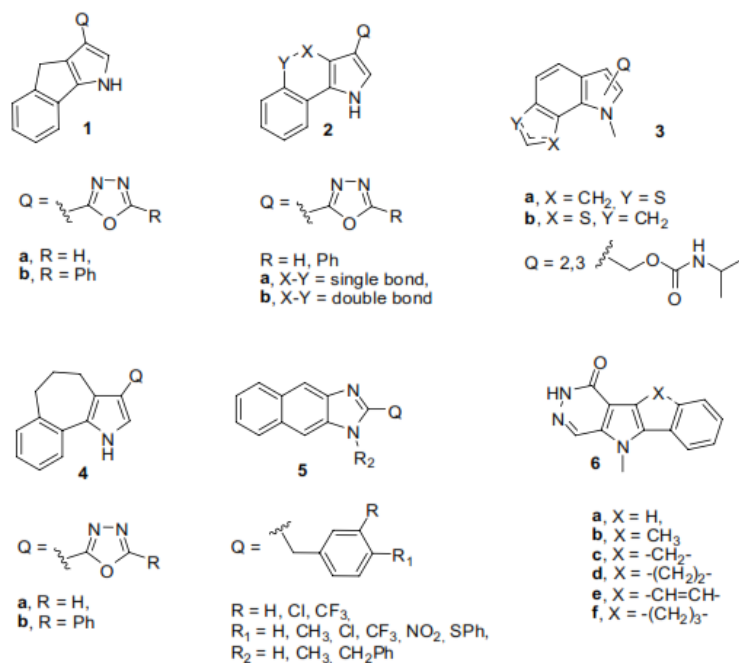


Figure 3

Chemical structure of some known molecules synthesized by the group of Prof. Pinna.

One of these compounds, containing oxadiazole motives, revealed a reasonable cytotoxic effect and its chemical structure was used as lead compound for QSAR (quantitative structure activity relationship) studies, in order to synthesize novel antiproliferative compounds as candidate cytotoxic and potential anticancer agents.

In specific, 12 molecules related to the lead compound, containing 1,3,4-oxadiazole bis-substitute moiety, were synthesized. All the compounds were analyzed by studying their antiproliferative behavior and evaluating potency, selectivity and activity in order to determine which one had the strongest and most selective inhibitory impact on tumors.

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2. AIM OF THE PROJECT

Being the second leading cause of death after heart disease cancer is drawing general attention worldwide (Thun et al 2010). Tumors are widely diffused among adults and children and, actually, chemotherapy has being the most effective treatment.

Novel approaches are urgently required for further improvements to current cancer therapies, in order to reduce the cancer death rate and mortality.

Most of the drugs currently used are not specific, leading to the many common side effects associated with cancer chemotherapy. These effects are seen in bodily systems that naturally have a rapid turnover of cells including skin, hair, gastrointestinal, and bone marrow and usually normal cells end up damaged by the chemotherapy program.

Clinical prognosis of aggressive cancers remains dismal; therefore the search for novel therapies against such tumors is warranted. The study of effective novel drugs, with low systemic toxicity, is a main field in oncology research.

A literature survey supports example of oxadiazoles as potential anticancer agents and opened up new opportunities for investigations.

The purpose of this study is to identify new potential anti-cancer drugs, containing oxadiazole motives that could be effective in inhibiting specifically tumor growth, to investigate their evaluation for drug-like properties, and to elucidate their molecular mechanisms of action.

The first objective was the identification, inside a set of compounds 1,3,4-oxadiazole bis-substitute, of molecules with a marked antiproliferative action. Subsequently, the selected compound with higher activity, Molecule 10, was tested using several cancer cell lines of varying origin as *in vitro* tumor models.

The second goal was to evaluate the cytostatic and cytotoxic effects of the molecules tested, in particular, by comparing the antiproliferative properties on primary fibroblasts to the same immortalized cell lines.

As final point, particular interest was addressed to understand the antiproliferative effects of the selected molecules and investigate the molecular pathways involved.

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3.MATERIALS AND METHODS

3.1 Cell culture medium and supplements

HeLa (cervix adenocarcinoma), Mcf7 (breast cancer), T98G (glioblastoma multiforme), RD (rhabdomyosarcoma), SH-SY5Y, Kelly, Lan1, SK-N-AS (neuroblastoma), A549, H1299 (non-small lung cancer), PC3 (prostate cancer) cell lines were grown in Dulbecco's Modified Eagle's Medium (Gibco), supplemented with 10% (v/v) Fetal Bovine Solution (FBS) (Gibco), 100 units/mL penicillin and 100 µg/mL streptomycin (Gibco). NB1, NB1 hTERT, Mrc5, Mrc5-SV1 S40, IBR3, IBR3-G SV40 cell lines were grown in DMEM supplemented, with 10% Fetal Calf Serum, 100 units/mL penicillin and 100 µg/mL streptomycin and 1% L-glutamine.

All the cell lines were incubated in a humidified incubator at 37°C with 5% CO₂.

HeLa, Mcf7, T98G, RD, A549, H1299, PC3 cell lines were obtained from ATCC; Kelly, SH-SY5Y, Lan1, SK-N-AS were provided by Prof. Arturo Sala from Brunel University; NB1, NB1-hTERT, Mrc5, Mrc5-SV1 S40, IBR3, IBR3-G SV40 were provided by Prof. Christopher Parris from Brunel University.

3.2 Proliferation assay (XTT)

The antiproliferative activity of the compounds was determined using XTT assay (Cell proliferation kit II, Roche). Cells were seeded in 96 well plates in a final volume of 100 µl culture medium for well at 37°C a plating densities ranging from 1500 to 2000 cells/well depending on the doubling time of individual cell lines. After 24 hours the culture medium was replaced with fresh medium containing the compounds in different concentrations and the cells were incubated for additional 72 hours. Then, 0,5 µl XTT electron coupling reagent and 74,5 µl medium (final volume 100 µl/well) were added in each well and incubated for 4 hours at 37°C.

The absorbance was measured using a microplate reader at the wavelength of 490 nm. Each concentration was analyzed in sestuplicate and the experiment was repeated three

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times. The drug concentration resulting in a 50% inhibition of growth (IC_{50}) was determined from the dose-response curves.

3.3 Colony formation assay

Cells were seeded into six-well plates at a concentration of 100-200 cells per well and incubated at 37°C. After 2 days the cells were treated and the medium with the compounds was refreshed every 3 days until the cells in control dishes had formed visible colonies. Next the medium was removed above the cells and the wells were washed two times with PBS. The cells were stained with 2–3 ml of a mixture of 6,0% glutaraldehyde and 0,5% crystal violet for 30 minutes. The solution was carefully removed and the wells were rinsed with tap water. The plates were left to dry in normal air at room temperature and the colonies were counted.

3.4 Immunostaining

Cells were seeded at a density 1×10^5 cells/well on 13 mm glass coverslips in a 24 well plate. After one day the cells were treated and, 24 hours later, the cells were washed and fixed using 4% paraformaldehyde (PFA) for 15 minutes. Permeabilization was performed with 0,5% Triton-X 100 for 10 minutes and the cells were blocked for 1 hour at room temperature in standard blocking buffer (5% BSA in PBS). Coverslips were incubated at 4°C overnight in primary antibody 1:200 diluted in 1% BSA in PBS. h β -tubulin (Sigma T4026) was used. Coverslips were washed with 1X PBS and then incubated for 1 hour at RT with secondary antibody (Alexa Fluor®) diluted in 1% BSA in PBS. Coverslips were then washed with 1X PBS and then incubated for 1 minute at RT with DAPI (1mg/ml) diluted in PBS and then mounted onto coverslips using a MOVIOL (Calbiochem). Images were acquired using a microscope.

3.5 Flow Cytometry Analysis

A flow cytometry analysis was carried out to determine the cell cycle distribution in treated cell lines using iodide propidium (PI, Sigma Aldrich).

Cells were plated on 6 cm dishes and after 24 hours were treated with 1 μ M of lead and Molecule 10 for different times: 2-24 hours for HeLa, T98G and RD or 24-72 hours for Mcf7, SH-SY5Y, Kelly, Lan1, SK-N-AS, A549 and H1299.

The cells were trypsinized, centrifuged for 5 minutes at 3000 rpm, washed with PBS, centrifuged for 5 minutes at 3000 rpm and fixed in 70% ice-cold ethanol and incubated at -20°C overnight. The fixed cells were centrifuged for 5 minutes at 3000 rpm and washed twice in phosphate buffered saline (PBS) and resuspended in a solution containing 20 μ g/ml RNase A and 5 μ g/ml PI in PBS overnight at 4°C in the dark and the stained nuclei were analyzed using a flow cytometry (BD Accuri C6 Flow Cytometer) by collecting 20,000 events, and the data were examined using the BD Accuri C6 software (BD Biosciences).

3.6 Growth curve

Normal fibroblast and immortalized cells were plated into 24 well plates at concentration $1,5 \times 10^4$ cells per well. After 24 hours the cells were treated with the molecules. The cells were detached and counted after 24, 48 and 72 hours with the hemocytometer.

To measure the number of viable cells in each well, the cell suspension were mixed 1:1 with 0.4% trypan blue solution in PBS.

3.7 Immunoblotting

Cells were lysated in lysis buffer (20 mM Tris HCl pH 8; 137 mM NaCl; 10% glycerol 1% Nonidet P-40; 2 mM EDTA; Protease Inhibitor Cocktails). The protein

concentration was determined by Bradford assay (Sigma Aldrich), following the manufacturer's instructions and by using BSA as a standard.

Protein extract was resolved in 8-12% SDS/PAA gel and transferred to a nitrocellulose membrane (GE Healthcare, Whatman) at 4°C and at 100V for 1 hour.

To check that the protein successfully transferred, the membrane was stained in 0,1% (v/v) Ponceau in 7% (v/v) trichloroacetic acid (TCA) until a red stain had formed on the transferred protein bands. The membrane was rinsed in TBS-T for transient staining. After the transfer, the membrane was blocked for 1 hour in 5% non-fat dry milk powder in 1% TBS-T with gentle agitation.

The membrane was exposed to primary antibody at the desired concentration in 3% non-fat dry milk in 1X TBS-T. Incubation with primary antibody took place overnight at 4°C with gentle agitation. Protein levels were detected with p53 antibody (Santa Cruz sc-126) and equal loading was controlled with the following antibody: anti-GAPDH (Santa Cruz sc-25778). Anti-rabbit and anti-mouse (1:10.000) peroxidase conjugated (Santa Cruz) and SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) were used for detection.

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4. RESULTS

4.1 Screening of 12 new potential anticancer compounds

To investigate the inhibitory effect of the new 1,3,4 oxadiazole bis-substitute molecules, cell viability was assessed in comparison with lead compound by XTT assay.

Twelve new compounds were screened for their potential anticancer activity in Mfc7, breast cancer, and HeLa, cervix adenocarcinoma, cell lines. The cells were plated in 96 well dishes at density 1500-2000 cell/w and, after 24 hours, were incubated for 72 hours with 10⁻⁵-2 μM of specific compounds or with lead compound or cultured medium (untreated cells) or DMSO (molecule's solvent used as control) or doxorubicin (a chemotherapy drug used to treat many different types of cancer).

Molecules 4-9-10-11 were found to be active at high concentration (10⁻⁵ μM) while the other molecules were not very active.

Among these compounds, at 2 μM only Molecule 10 had best activity in both tumor cell lines and inhibited cell proliferation with higher activity than lead and reference drug doxorubicin (Figure 4).

This compound showed better activity against HeLa cell line than Mcf7 cell line.

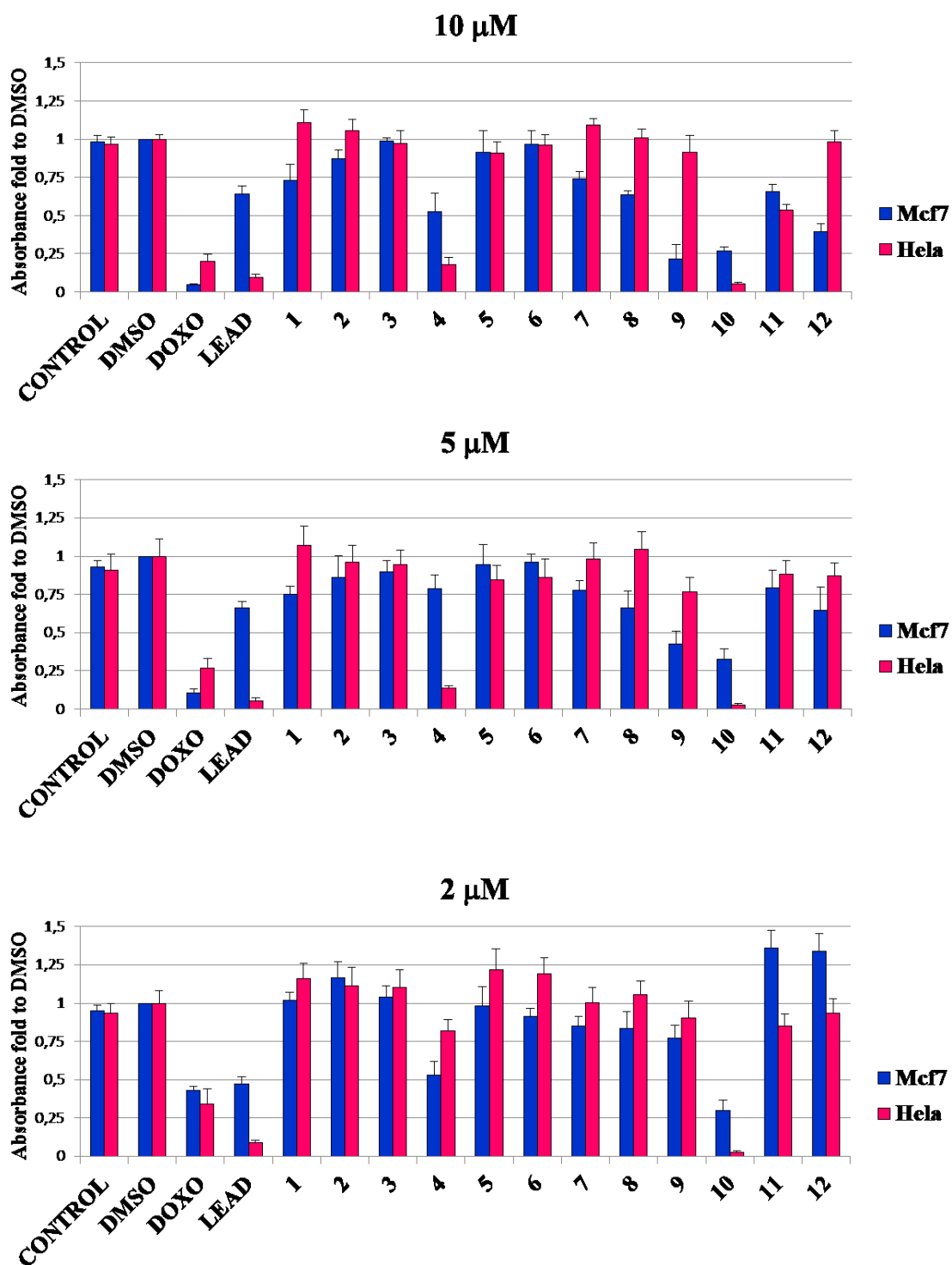


Figure 4
 Inhibition of tumor cell proliferation *in vitro*. Mcf7 and HeLa were incubated with 10-5-2 μ M of DMSO or lead or doxorubicin or different compound 1 to 12 for 72 hours.

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The antiproliferative effects of Molecule 10 were studied reducing the drug concentration from 1 to 0,02 μM . This compound exerted an antiproliferative behavior in a dose-dependent manner.

The IC_{50} value (concentration required to inhibit tumor cell proliferation by 50%) was 0,05 μM with Molecule 10 and 0,5 μM with lead for HeLa cells and 1,7 μM with Molecule 10 and 2,1 μM with lead for Mcf7.

The comparison with the lead compound showed that Molecule 10 had a higher activity in both tumor cell lines tested.

4.2 Antiproliferative effect of 1,3,4 oxadiazole bis-substitute compounds in different cell lines

Molecule 10 was thus selected for further evaluations and its activities were examined in other 9 additional different cancer cell lines: non-small lung cancer, prostate cancer, neuroblastoma, rhabdomyosarcoma and glioblastoma multiforme. All tests were carried out using different drug concentrations and all tested cell lines exhibited a cell growth inhibition in a dose-dependent manner with a stronger efficacy of Molecule 10 at very low doses, after 72 hours of treatment.

The IC_{50} for each cell line was calculated with values ranging 0,05 to 1,7 μM and a summary of all the values obtained is reported in Table 1.

Considering the IC_{50} mean values obtained for all cell lines tested it is possible to note that Molecule 10 is about 4-10 fold more efficient than lead.

	Cell line	IC ₅₀ LEAD	IC ₅₀ 10
HeLa	Cervix Adenocarcinoma	0,5 µM	0,05 µM
RD	Rhabdomyosarcoma	0,4 µM	0,06 µM
T98G	Glioblastoma multiforme	0,5 µM	0,06 µM
H1299	Non small lung cancer	0,9 µM	0,12 µM
SH-SY5Y	Neuroblastoma	0,69 µM	0,12 µM
SKNAS	Neuroblastoma	0,76 µM	0,19 µM
A549	Non small lung cancer	1 µM	0,24 µM
Kelly	Neuroblastoma	1,5 µM	0,25 µM
LAN1	Neuroblastoma	0,78 µM	0,38 µM
PC3	Prostate cancer	1,9 µM	0,67 µM
Mcf7	Breast cancer	2,1 µM	1,7 µM

Table 1

This table shows IC₅₀ values determined for a panel of 11 different cancer cell lines. Molecule 10 inhibited tumor cell proliferation in all cell lines with IC₅₀ values between 0,05 and 1,7 µM and 9 of 11 lines had concentration less than 0,4 µM.

4.3 Impact of Molecule 10 on colony formation

To assess the effect of the exposure of Molecule 10 on the ability of a single cell to grow into a colony and to measure long-term effects of the compound, colony formation experiments were performed using all the cell lines. This assay monitors a cancer cell's ability to produce a viable colony after treatment.

The experiments were performed in 6-well dishes at low density depending of the cell lines (100-200 cell/w). After 48 hours cells were exposed to lead and Molecule 10 treatments at the corresponding IC₅₀. The media was replaced every 3 days for two weeks, until cells in control dishes had formed visible colonies. Then the cells were fixed and stained with 6.0% glutaraldehyde and 0.5% crystal violet and the number of colonies were counted.

Clonal growth of all cell lines was inhibited by Molecule 10 in a dose dependent manner as shown in Figure 5.

An inhibition of colony formation up to 50-60% using Molecule 10 IC₅₀ concentration was noted and only a little fraction of seeded cells retains the capacity to produce colonies.

Conversely, lead was not able to abolish colony formation under the same conditions, but partly inhibited clonogenic growth only at the highest concentration.

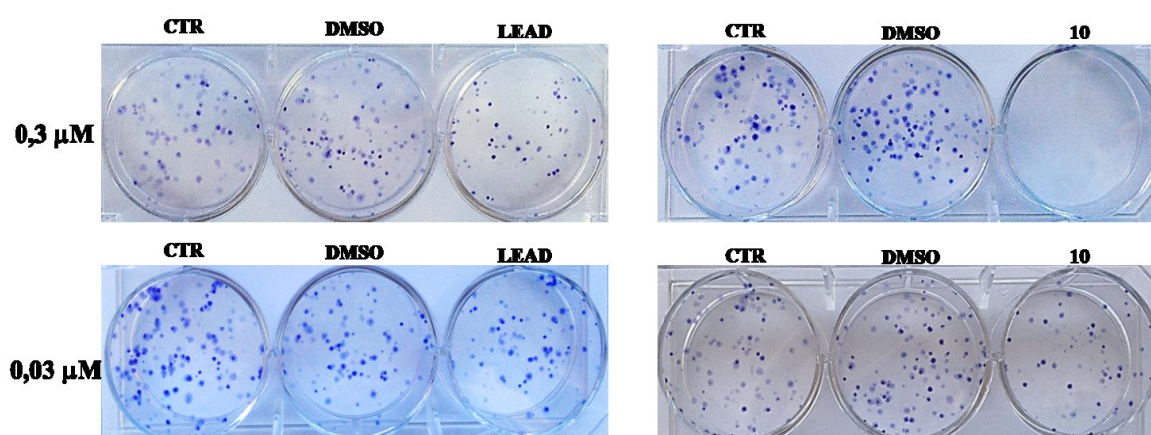


Figure 5

Colony formation assay shown the long-term effects of lead and Molecule 10 treatment of HeLa cell lines. These photographs of petri dishes are representative for all the cell lines used.

4.4 Effect of Molecule 10 on cell cycle

Most molecules that inhibit the growth of cancer cells cause alteration of cell cycle distribution. To better understand the antiproliferative activity of Molecule 10, a cell cycle analysis was performed with flow cytometry by treating cells at different time (2-72 hours) and then by subjecting them to propidium iodide staining.

After 24 hours exposure with Molecule 10, all cells resulted in a significant increase in G₂/M phase and were characterized by the presence of a decreased proportion of cells in the G₁ and S phase in comparison to untreated control, suggesting G₂/M arrest.

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Molecule 10 showed a different effect for the various cell lines.

In some cell line, as HeLa, RD and T98G, the G₂/M phase cell cycle arrest was observed earlier, 2 hours after treatment, and was in progressive increase from 2 to 10 hours (from 11% to 30% more in comparison to DMSO control); from 12 to 24 hours cells had only one pick and after 24 hours all cells were dead. A rapid arrest of cell growth was followed by cell death. Also the lead induced a G₂/M arrest but with less activity than Molecule 10 (Figure 6A).

In other cell lines, such as MCF7, PC3 and SH-SY5Y, in comparison with the DMSO control group, 24 hours treatment of Molecule 10 led to a decrease in G₁ phase (from 54,1% to 15,7%) and S phase (from 21,7% to 6,3%) and to a dramatic increase in G₂/M phase (from 24,2% to 78,1%) but this arrest remained almost unchanged for a long period of time, until 144 hours (Figure 6B).

In H1299, A549 and Lan-1 cell lines Molecule 10 induced G₂/M arrest after 24 hours with 74,6% of cell lines in this phase compared with 22,2% in DMSO. This effect changed after 48 hours of treatment with a decrease in G₂/M (42,9% with Molecule 10 in comparison to 11,8% DMSO), a decrease in G₁ phase (34,3% with Molecule 10 compared to 81% with DMSO) and 13,4% of cell population in sub-G₁, indicating a possible induction of apoptosis (Figure 6C).

This effect was not visible in the Lead, where the cell cycle was similar to DMSO.

All these results indicate that the promising antitumor activity of Molecule 10 may be attributed to the G₂/M phase arrest.

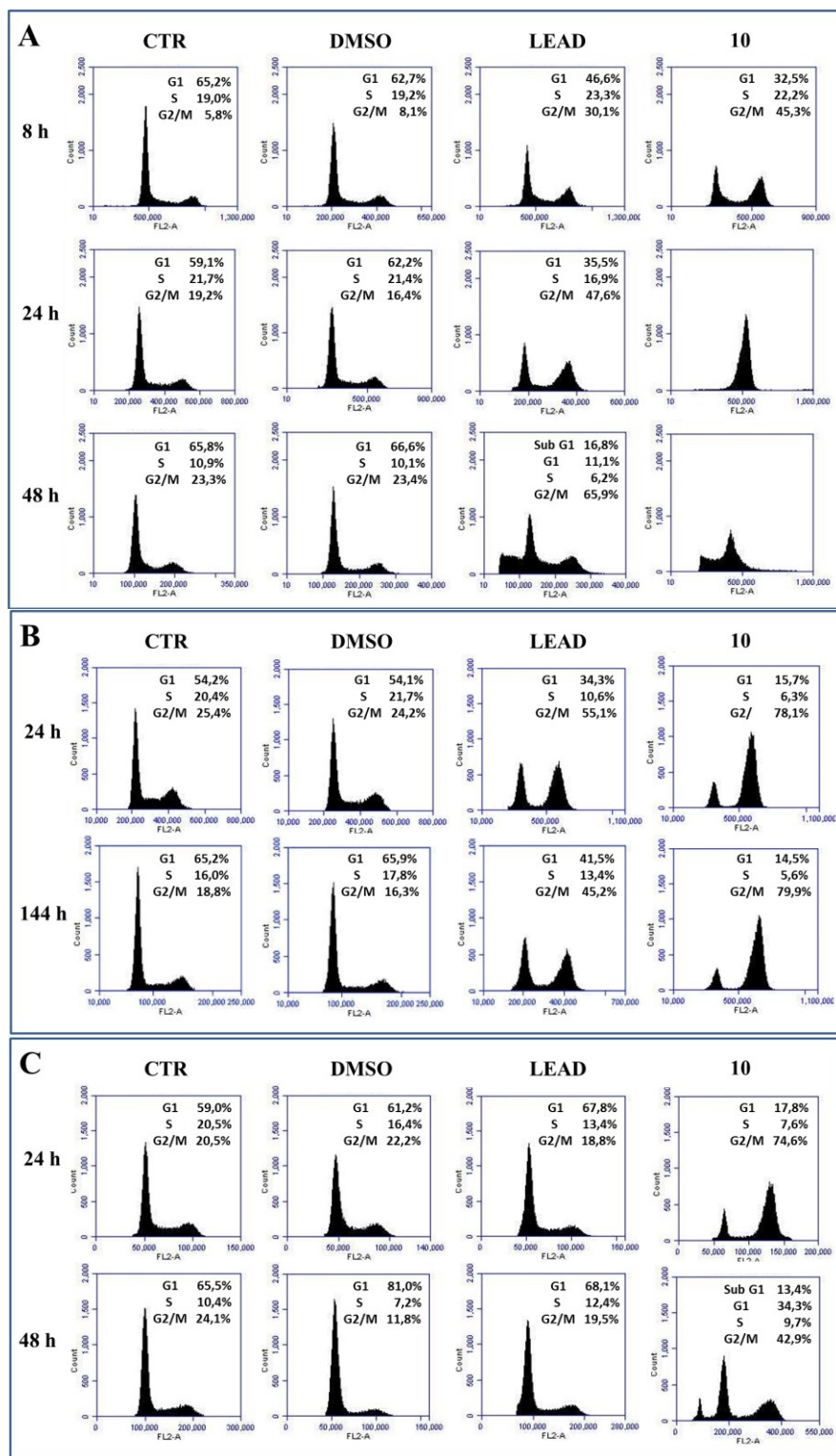


Figure 6

Cell cycle analysis of different cell lines (A: HeLa; B: Mcf7; C: H1299) treated with DMSO, lead and Molecule 10 for different hours. DNA content was determined by flow cytometry.

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In order to understand whether Molecule 10 action is reversible or irreversible, the cell lines were treated with the drug for 24 hours and the media were changed with normal cultured media. As shown in Figure 7, after replacement with fresh medium, cell cycle appeared similar to normal and control cycle (G₂/M phase: 59,5% after 24 hours Molecule 10 treatment and 31,7% after 24 hours treatment + 24 hours change medium; 28,1% after DMSO treatment and 26,4% with DMSO treatment + 24 hours change medium).

These results suggested that the G₂/M arrest caused by Molecule 10 is reversible and its effect is cytostatic and not cytotoxic.

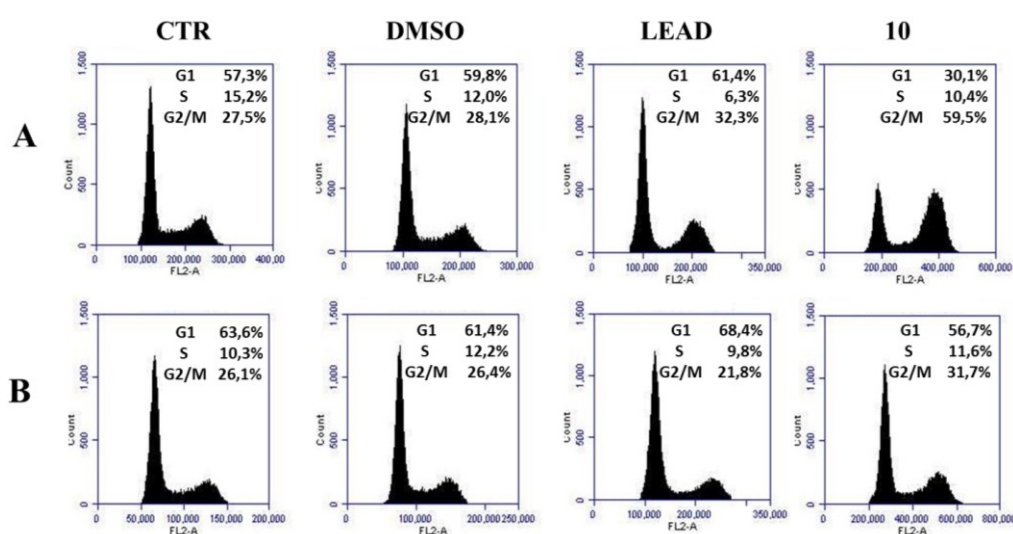


Figure 7
FACS analysis of Mcf7 cells (A) treated with different compounds for 24 hours and (B) treated for 24 hours with compounds and for 24 hours with normal medium.

4.5 Implication of Molecule 10 on tubulin organization

Since the central role of microtubules is involved during the M phase of the cell cycle, a mechanism of action of Molecule 10, might affect microtubule assembly. In order to evaluate a dynamic instability of microtubules Molecule 10 dependent, the immunofluorescence analyses using β -tubulin antibody were performed.

Cells were grown on coverslips and treated with the compounds for 24 hours, then were permeabilized, fixed, and stained with β -tubulin primary antibody, secondary

antibody and Dapi for DNA stain.

These preliminary results showed a deregulation of tubulin organization, providing useful evidences.

No changes in β -tubulin network were evident in DMSO as well as in the control.

In contrast, when cell lines were incubated with Molecule 10, different phenotypes were observed: in Mcf7 there was an abnormal distribution of β -tubulin, characterized by uneven distribution around the nuclei, induction of micronucleated cells by Molecule 10, smaller and more compact nuclei than the control.

In HeLa cell low concentrations of Molecule 10 (1 μ M for Lead and 0,1 μ M for Molecule 10) induced aberrant cell division resulting in the formation of tubulin structure without nuclei or in cell with 2 nuclei after cytokinesis and exhibited an irregular β -tubulin organization.

SH-SY5Y showed an incorrect microtubule network with an aberrant β -tubulin distribution, the loss of its elongated structure with shorter microtubule bundles distributed around the nuclei and with apoptotic and pyknotic nuclei (Figure 8).

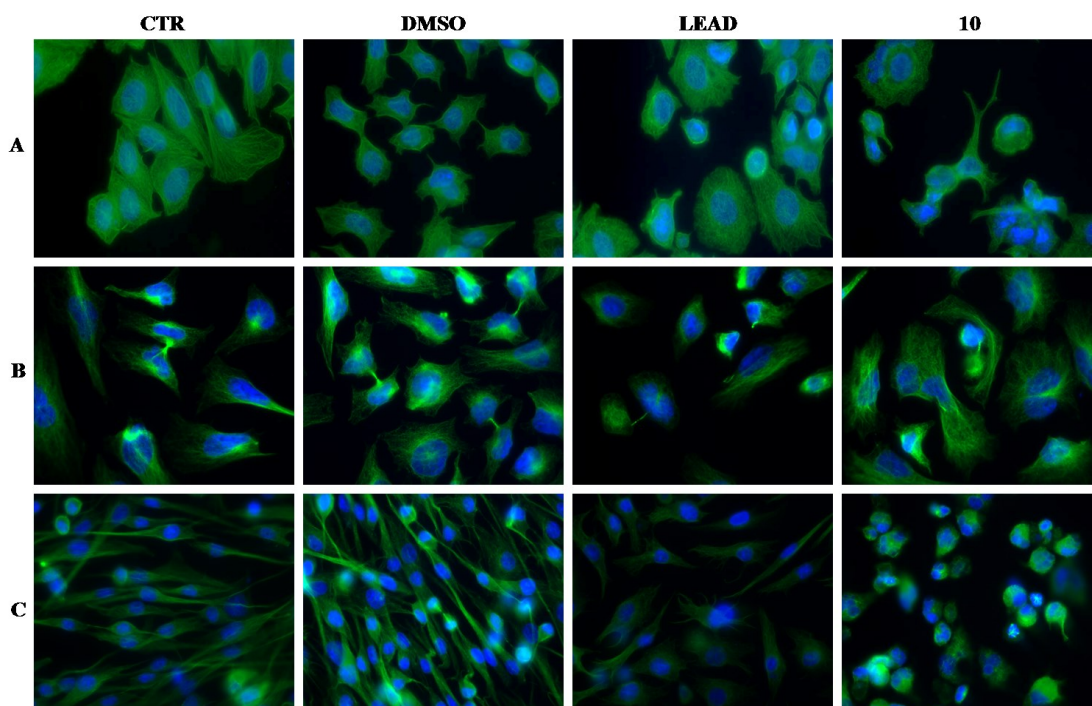


Figure 8

Immunofluorescence analysis of the expression of β -tubulin (green) and DNA (blue) in different cancer cells after incubation with Molecule 10. Representative pictures of Mcf7(A), HeLa (B) and SH-SY5Y (C) cells are shown.

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4.6 Effects of Molecule 10 on p53 levels

In order to identify molecular mechanism implicated in cell cycle arrest, p53 protein levels were analyzed by western blot and quantified by densitometric analysis using GAPDH as control. All cancer cell lines revealed a significant overexpression of this protein after Molecule 10 treatment. Particularly, as shown in Figure 9, p53 levels were 3 times higher in treated Mcf7 and A549 cells and 2 times higher in treated T98G cells compared to the respective untreated controls.

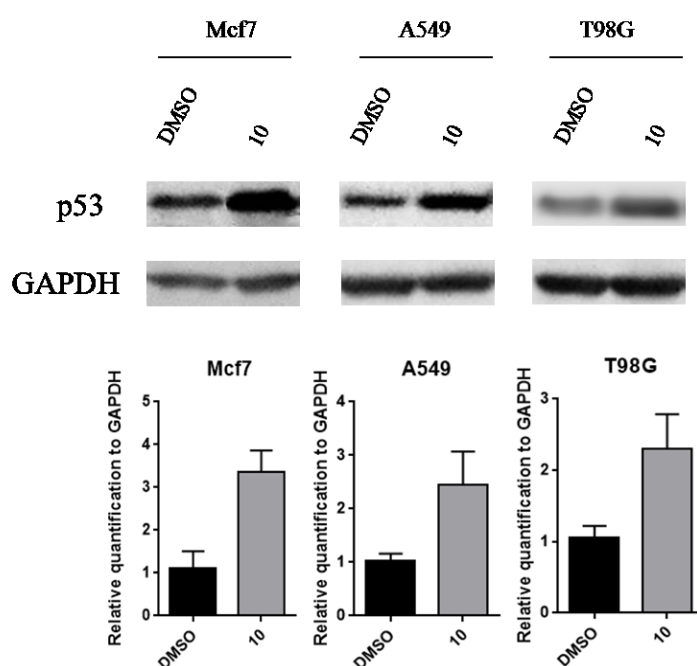


Figure 9:
Effect of Molecule 10 on p53 protein levels in Mcf7, A549 and T98G cells.

4.7 Effects of Molecule 10 on primary and immortalized cell lines

Since the toxicity and non-specific side of action of drugs represent two of the major obstacles in cancer therapy, same analysis were performed on human fibroblast cells as normal control (NB1, Mrc-5, IBR3) and on the respective immortalized cell lines (NB1 h-TERT, Mrc5 SV1 S40, IBR3-G S40).

As shown in Table 2, all the fibroblast normal cell lines were less sensitive and showed a higher IC₅₀ than immortalized and cancer cell lines. Furthermore, Molecule 10 was more active in all immortalized cell lines than in normal cell lines.

	Cell line	IC ₅₀ LEAD	IC ₅₀ 10
NB1	Primary human dermal fibroblasts	1,8 µM	1,3 µM
NB1 H-TERT	Immortalized human dermal fibroblasts	1,6 µM	0,13 µM
IBR3-G	Primary human dermal fibroblasts	1,6 µM	0,47 µM
IBR3 SV40	Immortalized human dermal fibroblasts	1,8 µM	0,21 µM
MRC5	Human lung fibroblasts	1,9 µM	0,19 µM
MRC5-SV1 S40	Immortalized human lung fibroblasts	1,7 µM	0,13 µM
WI38	Human lung fibroblast	2,1 µM	1,8 µM

Table 2

The IC₅₀ values for the synthesized compounds against human normal and immortalized cell lines.

In order to demonstrate that immortalized cells are more susceptible than fibroblast to the anti-proliferative effect of Molecule 10, fibroblast and the corresponding immortalized cells were seeded onto a 12-well plate at a starting density of 15000 cells per well. The cells were treated with DMSO and Molecule 10 and grown over a period of three days. Each day dead and alive cells were counted. The number of cells counted was reflected in a graph showing the effect of Molecule 10 on the cells treated.

Immortalized cells have the ability to grow to a higher density than primary cells: in the control, after 72 hours, primary cultures contained mean 3.5×10^4 cells/well, whereas immortalized cultures contained up to 12×10^5 cells/well.

As shown in Figure 10, following treatment with Molecule 10, a 50% reduction of cell number in the immortalized cell lines in comparison to the controls was noticed after the first 24 hours. Molecule 10 had a more dramatic effect on immortalized cells than primary cells: after 72 hours the growth of NB1 h-TERT was substantially inhibited around 82%, while the growth of NB1 was reduced to nearly 51%.

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All the three primary and immortalized cell lines were tested, obtaining similar results.

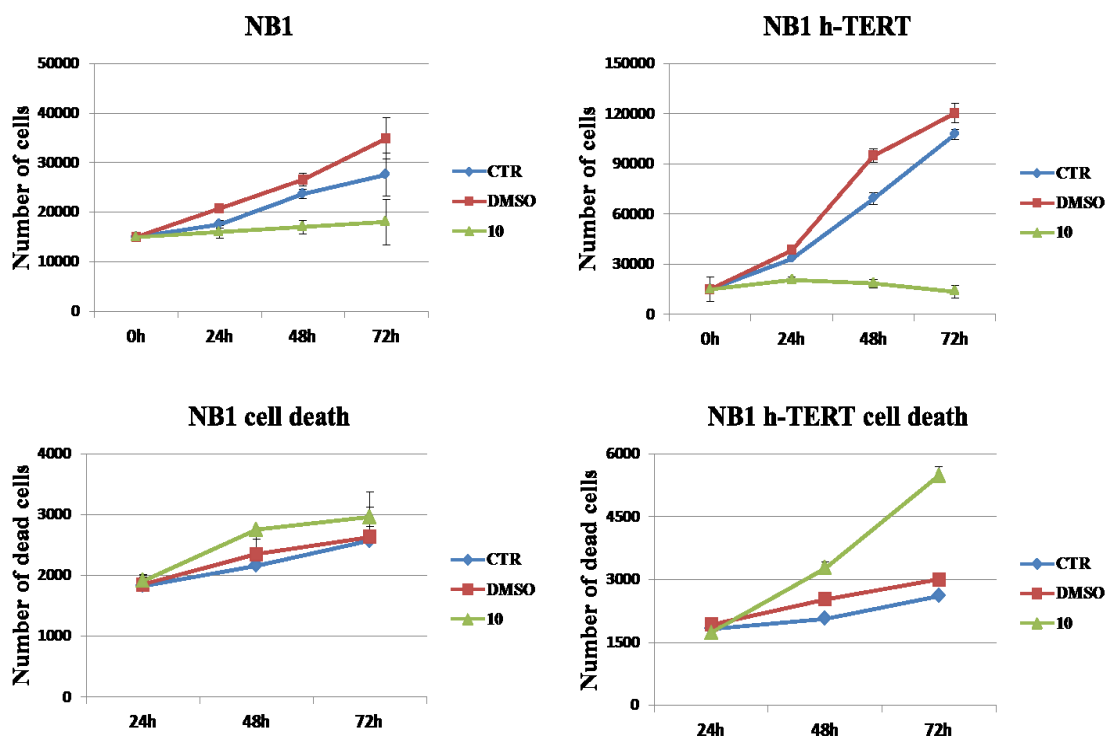


Figure 10

Effects of Molecule 10 on primary and immortalized cell lines followed by 3 days.

To confirm that fibroblast cell lines are resistant to Molecule 10 treatment, the effects of these compound were evaluated increasing the concentration of Molecule 10 (3-5-7 μM) for 72 hours.

The morphology of primary cells did not change even at very strong concentration, 7 μM and fibroblast cells appear not to be affected by these treatments (Figure 11A). On the other hand, immortalized cell lines were more sensitive and, around 30% of cells after 3 μM treatment and 50% of cells after 7 μM treatment, detached from the plate and died after 24 hours (Figure 11B). These percentages increased after 48 and 72 hours.

These data suggested the preferential antiproliferative activity of Molecule 10 on immortalized cells.

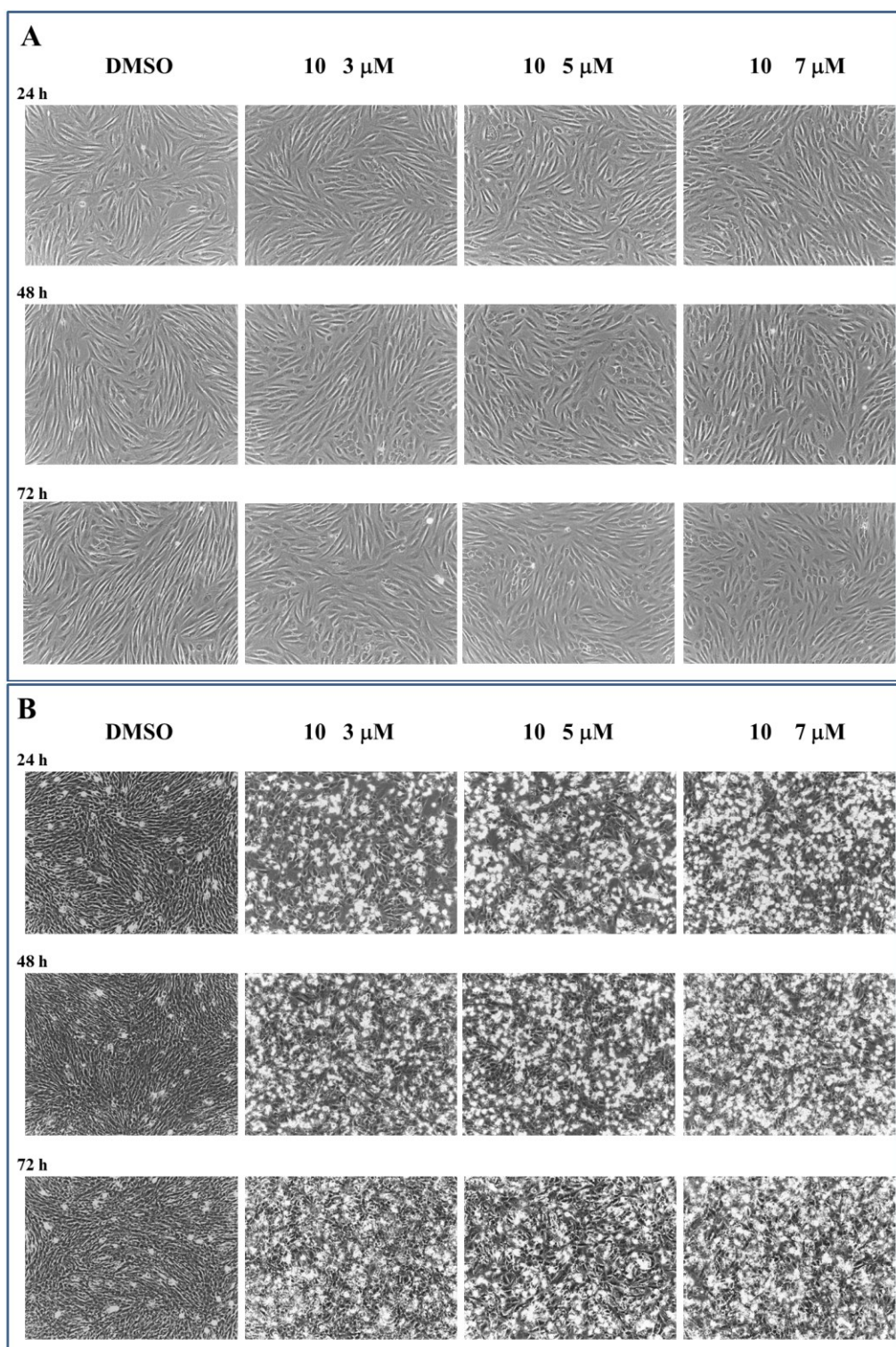


Figure 11

Representative morphological analysis of NB1, primary cells, (A) and NB1 h-TERT, immortalized cells (B), after 24-48-72 hours with increasing concentration of Molecule 10.

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In order to investigate the effects of Molecule 10 on cell cycle progression on primary and immortalized cell lines, flow cytometry analysis were performed. Interestingly, the Molecule 10 did not induce a cell cycle arrest in G₂/M phases on primary cell lines. Cells exposed to Molecule 10 for 24-48-72 hours exhibited a comparable distribution of population among all the phases of cell cycle, similar to control and DMSO. This demonstrated that the cell cycle of fibroblasts was not influenced by the treatments of Molecule 10 (Figure 12A).

As shown in Figure 12B, immortalized cells were treated using the same condition but, after 48 and 72 hours exposure to the treatment, 25% of cells showed a sub-G₁ peak and a reduction of cells in G₁ and G₂/M phases was observed.

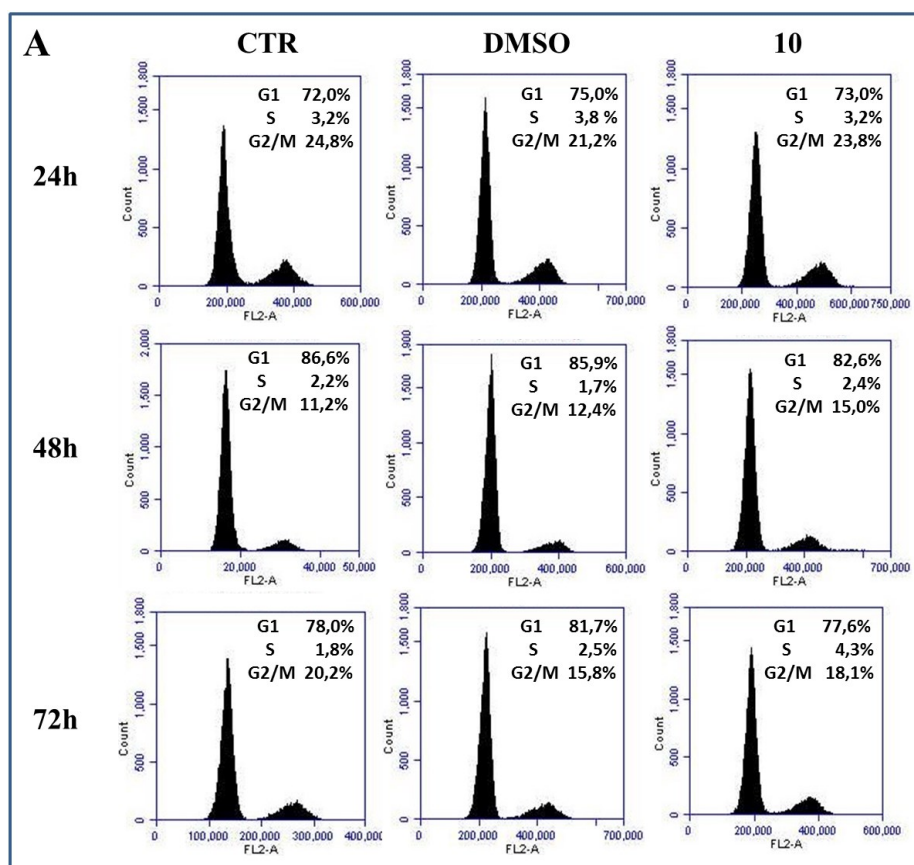


Figure 12A
Effects of Molecule 10 on cell cycle on NB1 cells

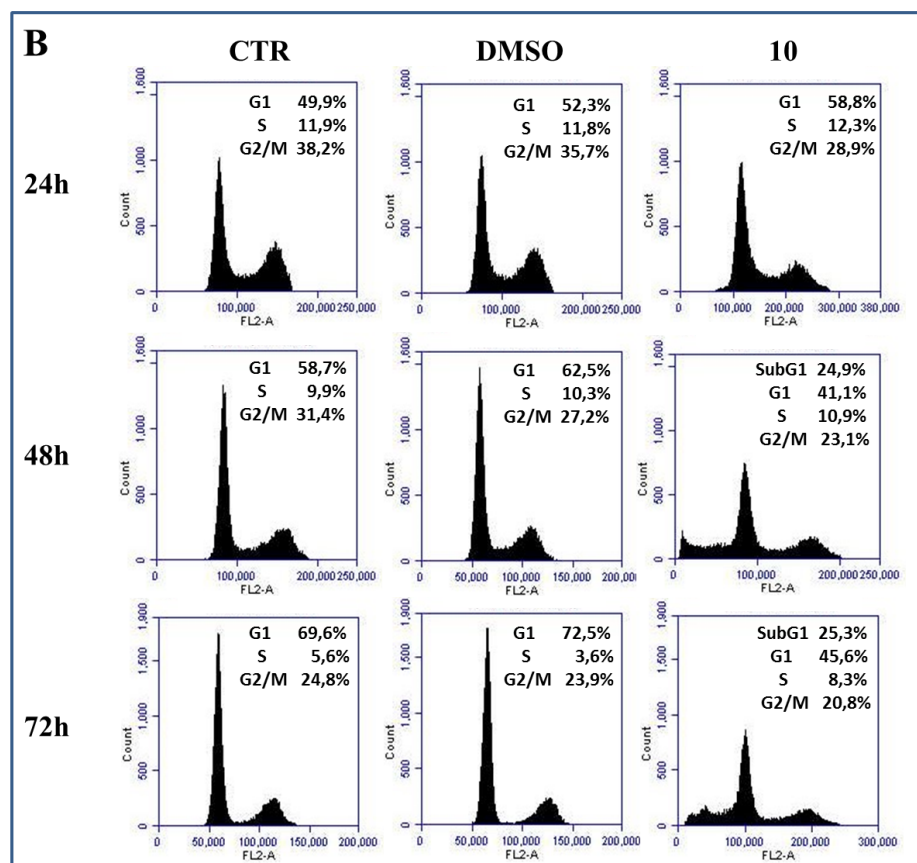


Figure 12B
Effects of Molecule 10 on cell cycle on NB1 h-TERT cells

To investigate the differences in sensitivity of Molecule 10 on p53 expression in primary and immortalized fibroblasts, cells extracts were subjected to Western blot analysis for the detection of p53.

Primary fibroblasts displayed unaltered p53 protein levels upon Molecule 10 treatment. In contrast, immortalized cells showed an increased protein level of 50% in treated versus untreated cells (Figure 13).

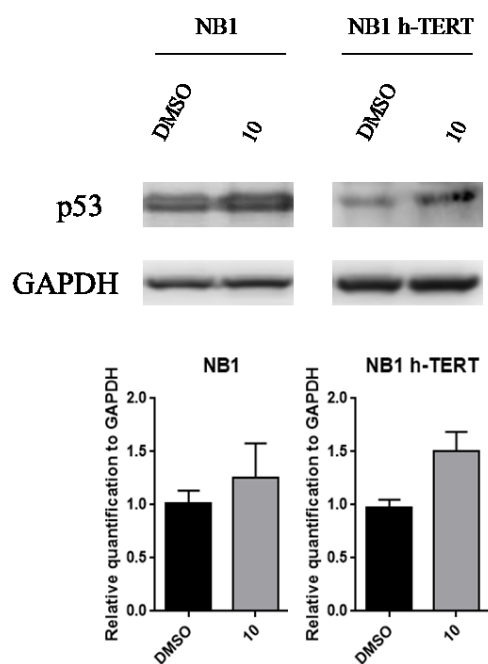


Figure 13

p53 protein expression levels on primary and immortalized cells after Molecule 10 treatment.

Together, these data indicate that normal primary cells display a low sensitivity to Molecule 10 treatment. Interestingly, Molecule 10 shows a low systemic toxicity and a specific activity for cancer cell. Based on these results, it is established that Molecule 10 has a dramatic pharmaceutical action with few toxic side effects *in vitro*.

5. DISCUSSION

Cancer is a disease characterized by uncontrolled growth and poor response rates on available treatments.

The research of biologically promising new chemical molecules against cancer disease raises important attention in drug discovery (Hoelder et al 2012). This is a complicate process involving several and sequential phases including target identification, drug design, characterization, validation, clinical candidate selection, preclinical and clinical testing (Kraljevic et al 2006).

Decades of research focused on the study of the molecular foundation of cancer have identified a new generation of promising therapies designed to target specific molecular processes that promote tumor growth and apoptosis (Baselga 2001).

Although many drug molecules have being used for the treatment of cancer, there are still a lot of difficulties in developing new drug candidates, since most of the chemotherapy molecules have the limitation of undesirable side effects and problems with resistance and intolerance. Due to the wide spectrum of activity of cytotoxic drugs and the non-specificity of treatment, many drugs can only be tolerated for short periods of time. Most side effects, such as loss of hair, nausea and vomiting, fatigue, depressed immune system, subside after the treatment but sometimes; permanent damage is caused to the kidneys, heart, lungs or reproductive system. However, benefits exceed disadvantages, and chemotherapy is the commonest form of cancer therapy.

Sometimes, tumor may be resistant to treatment with some cytotoxic drugs, and many patients, who initially respond to chemotherapy, show later a loss of response, resulting in tumor regrowth: possibly due to some genetic alteration or to the development of resistance after exposure to the drug (Groenendijk and Bernards 2014).

Even though the progress in anticancer therapies is concrete, the clinical prognosis of most malignant and aggressive cancer remain fatal, therefore the development of novel chemotherapeutic agents as more effective cancer treatments is essential. For this reason, scientists are focused on finding new therapeutic targets for cancer and on discovering novel, potent, less toxic and more specific and selective anticancer drugs.

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In recent years, oxadiazole derivatives have been identified as promising leads for the generation of new chemotherapeutic agents.

Oxadiazoles represent a broad and highly versatile class of heterocyclic compounds that have been extensively studied. Within drug discovery and development, it has been showed that compounds containing oxadiazole moiety have a wide range of biological activities and novel oxadiazole motif has been used as potential anticancer drug (Khalilullah et al 2012). Currently, the only promising example is zibotentan, which is in late-stage clinical trials as an anticancer agent (Haque et al 2014).

In this thesis, screening of twelve new molecules containing oxadiazole motif and evaluation of their mechanism of action are discussed.

Several oxadiazole compounds were analyzed, after being synthesized by the group of Prof. Pinna at the Department of Chemistry and Pharmacy, University of Sassari.

One of oxadiazole molecules, which showed important cytotoxic effects in the preliminary assay, was chosen as lead compound for QSAR studies, and it was used to synthesize 12 molecules as new potential anticancer candidates.

All the compounds were tested *in vitro* in order to evaluate the potential anticancer activities and to select the molecules with strongest and more selective cell growth inhibitory activity in different human tumor cell lines: breast cancer, cervix adenocarcinoma, non-small lung cancer, prostate cancer, neuroblastoma, rhabdomyosarcoma and glioblastoma multiforme.

In this study it has been demonstrated that Molecule 10, a new 1,3,4-oxadiazole bis-substitute compound, acts as a very promising anticancer molecule, being more effective than doxorubicin in inhibiting cancer cells growth. Specifically, Molecule 10 activity seems to be effective, rapid, and selective against all cancer cell lines tested, by causing antiproliferative activity. Moreover, Molecule 10 treatments had no effect on normal fibroblasts at the same concentrations on cancer cell lines, suggesting that this compound shows preferential cytotoxic activity on cancer cells.

In vitro response to an administered drug could be conditioned by cell culture: usually, cells grown in monolayer are more responsive to cytotoxic agents than cells grown in colonies due to the larger surface they expose to the drug, compared to the limited drug penetration in the colonies (Brown and Attardi 2005).

Our results, using colony formation assay, showed that Molecule 10 is similarly active in suppressing *in vitro* proliferation of cancer cells in both monolayer and colony cell culture conditions. This suggests that this molecule could probably perform *in vivo* anticancer activity without being conditioned by the proliferating cells in solid tumors, which is a limit of many conventional cell cycle-dependent chemotherapeutic agents (Boyer et al 1998).

Since most new drugs operate on cell cycle, the molecular and cellular target mechanisms used by Molecule 10 were studied through flow cytometry analysis and immunofluorescence. The Molecule 10 induced a strong cell cycle arrest in G₂/M phases indicating an involvement during the last phase of cell cycle and mitosis. Interestingly, no evidence on cell cycle arrest was observed in normal fibroblasts grown and treated in the same conditions, again suggesting the preferential activity of Molecule 10 on cancer cells.

Several studies have revealed that many cancer cells have shown defective G₁ checkpoint mechanism controls, leading these cells to an aberrant dependence to the G₂ checkpoint mechanisms during cell replication. In addition, mechanisms involved during G₂/M cell cycle progression are more contingent in cancer cells than in normal cells (Suganuma et al 1999). In the last years these considerations have induced to consider G₂ phase as a tactic objective for the development of anticancer drugs. Results obtained after Molecule 10 treatments allow supposing that this compound could be included in the category of antitumoral molecules.

An important cytoskeletal component, involved in mitosis, is the microtubule network. Microtubules have different roles, including cellular functions, but they are very important for chromatid alignment, formation of mitotic spindles and separation during mitosis. They are mobile as a result of alternating periods of elongation and shrinkage, and this phenomenon is known as dynamic instability. Microtubules are formed by the polymerization of a dimer of two globular proteins, alpha and beta tubulin. Fixed cells stained with β -tubulin marker indicated an alteration of filamentous β -tubulin and on tubulin organization, and it was assumed that microtubule assembly and dynamics were involved in the mechanism of action of Molecule 10.

Although a block in G₂/M stage of cell cycle has been confirmed along with, in some cases, cellular death, in order to clarify molecular mechanism involved in cell cycle arrest, expression levels of p53 protein were analyzed by western blot. p53 is a transcription factor that regulates expression of multiple genes involved in the cellular responses. It is activated by various types of stress conditions such as DNA damage, hypoxia and oncogene activation that results in p53-mediated cellular responses depending on the cell and stress, including DNA repair, cell cycle arrest and apoptosis, differentiation, senescence, inhibition of angiogenesis and metastasis (Vousden and Lu 2002).

These results showed a deregulation of p53, constituted by an overexpression on cancer and immortalized cell lines after Molecule 10 treatment, providing useful evidences and offering many suggestions to speculate on the molecular mechanisms of action of this compound, which need to be verified with targeted experiments.

The G₂/M arrest and the deregulation of p53 could be associated to apoptosis by inhibition of degradation of cell cycle regulators via p53-dependent pathways or through the initiation of apoptotic pathways.

p53 could induce Cdk-inhibitors, which preferentially targets the G₂/M specific Cdk1/Cyclin B complex. p53 transcriptionally represses Cyclin B₁ (Innocente et al 1999), Cyclin B₂, Cyclin A and Cdk1 (Badie et al 2000), leading to G₂ arrest.

Moreover, p53 could repress Topoisomerase, which is not part of the Cyclin-Cdk complexes, but it is crucial for chromosome segregation and progression into mitosis (Sandri et al 1996). Topoisomerase II is required during the G₂/M transition, where it helps to bring about the higher order compaction of chromatin to form highly condensed mitotic chromosomes (Anderson and Roberge 1996). Molecule 10 could induce p53 to cause a downregulation of topoisomerase II, by repressing its promoter, and blocking entry into mitosis.

The results of this thesis confirm that oxadiazole derivatives can be a rich source of potential anticancer drugs and clearly show that Molecule 10 is able to induce a strong antiproliferative effect, which preferentially target cancer cells.

Although further experiments are required to fully characterize the role of Molecule 10

and prove clinical evidences to substantiate the data of our experiments, based on our results we can affirm that Molecule 10 is a promising and convincing antiproliferative agent that could be considered as a good candidate to develop new therapeutic strategies against cancer.

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ACKNOWLEDGMENTS

I would like to thank my supervisor Professor Luigi Bagella for his expertise, advice and constructive criticism during my PhD.

I would like to express my gratitude to Professor Arturo Sala for giving me the opportunity to spend one year of my PhD in his laboratory.

I would also like to acknowledge Professors Pinna and Murineddu for allowing me to test their synthesized molecules.

I especially thank all the members of Sassari's and Brunel's lab for the help and support in the daily lab work and also for the laughs and nice memories.

Lastly, I would like to say a big thanks to my family for their endless support, patience and continuous encouragement.