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Thérapie ciblée des glioblastomes via l'internalisation d'une toxine grâce à des biopolymères dirigés à la surface des cellules cancéreuses

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Glioblastoma targeted therapy approaches based on toxin internalization *via* cell surface directed biopolymers

by
Anne-Chloé DHEZ

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Prof. Rodolfo IPPOLITI
Dr. José COURTY

Abstract

Targeted cancer therapies are drugs designed to interfere with specific molecules necessary for tumor growth and progression. Traditional cytotoxic chemotherapies usually kill rapidly dividing cells in the body by interfering with cell division. A primary goal of targeted therapies is to fight cancer cells with more precision and potentially fewer side effects.

Antibody-based therapy for cancer has become established over the past 15 years and is now one of the most successful and important targeted strategies. In some cases, monoclonal antibodies are conjugated to radio-isotopes or toxins (immunotoxin) to allow specific delivery of these cytotoxic agents to the intended cancer cell target. Furthermore targeted therapies may be based also on the use of targeting molecules other than antibodies, such as peptides, growth factors, and also nucleic acids.

Indeed, in this work we studied a multi targeting strategy to deliver toxic substances (protein toxin or its gene) to cancer cells (glioblastoma).

Our group published a paper describing the use of PDZ protein domain of hCASK (serine kinase calcium/calmodulin-dependent of MAGUK family) and to exploit the ability of this protein to bind to the C-terminus of hCD98 in the extracellular space. CD98 is an interesting target because it is overexpressed in different types of tumors (Giansanti F S. D., 2015). hCASK-PDZ was genetically fused to the toxin saporin and this chimeric toxin proved to be active on glioblastoma cells *in vitro*.

Other cell killing agents were designed to recognize and bind specifically nucleolin (NCL). This multifunctional protein is overexpressed on the surface of activated endothelial and tumor cells. In this context, compounds targeting NCL, such an aptamer, and a multivalent pseudopeptide, have been developed and investigated for cancer therapy.

The aptamer against NCL, NCL-APT also known as AS1411 (Antisoma, UK), is a US Food and Drug Administration (FDA)-approved NCL targeting agent. It binds to NCL on the cell surface, preferentially gets internalized, and inhibits cancer cell growth sparing normal cells (Bates PJ L. D., 2009).

In parallel, our group, recently developed a multivalent synthetic pseudopeptide N6L that selectively binds to nucleolin (Destouches D. P. N.-K., 2011). N6L strongly inhibits breast

cancer growth by inducing apoptosis of tumor cells and is currently in preparation for phase II clinical trials (IPP-204106). We demonstrated the anti-proliferative effect of N6L on human glioblastoma cells in primary culture prepared from post-surgical specimens (Benedetti E, 2015).

The overexpression of NCL on glioblastoma cell surface and the recognized selectivity of AS1411 and N6L prompted us to study a way to increase the efficiency of these ligands binding them Saporin coding gene or the protein toxin Saporin-S6, a type 1 RIP (Ribosome-Inactivating Protein) widely studied because of its potential therapeutic application in a variety of human diseases as toxic moiety of a conjugate.

The characterization of the toxic activity of AS1411 linked to saporin gene (APT-SAP) and of NCL linked to saporin protein (SAP-N6L) is therefore described. Both these researches are under evaluation for publication.

All the described targeted approaches, notwithstanding some problems, look promising and need further research, but confirm the fact that exploiting targets to deliver toxic substances is the future of therapy for cancer forms that are difficult to beat with conventional therapies.

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List of Abbreviations

AMP : Anti-Microbial Peptides
ARF : Alternative Reading Frame
BBB : Blood Brain Barrier
CNS : Central Nervous System
CPP : Cell Penetrating Peptides
CSC : Cancer Stem Cells
CT : ChemoTherapy
DMEM : Dulbecco's Modified Eagle's Medium
DMSO : dimethylsulfoxide
DNA : DeoxyriboNucleic Acid
EDTA : ethylene diamine tetraacetate
EF : Elongation Factor
EGFR : Epidermal Growth Factor Receptor
ELISA : Enzyme Linked Immuno Sorbent Assay
ER : Endoplasmic Reticulum
ERAD : Endoplasmic Reticulum -Associated protein Degradation
FBS : fetal bovine serum
FDA : Food and Drug Administration
GBM : Glioblastoma Multiforme
GFP : Green Fluorescent Protein
HBSS : Hank's Balanced Salt Solution
HGP : Human Genome Project
HIV : Human Immunodeficiency Virus
ICGC : International Cancer Genome Consortium
IDH1/2 : Isocitrate DeHydrogenase
IL : InterLeukin
IMDM : Iscove's Modified Dulbecco's Medium
i.p. : intraperitoneal
IPTG : IsoPropyl β -D-1-ThioGalactopyranoside
ITs : ImmunoToxins
kDa : kilo Dalton
LRP : Lipoprotein Receptor-related Protein
mAb : monoclonal Antibody
MAPK : Mitogen-Activated Protein Kinases
Mdm2 : Mouse double minute 2 homolog
MEM : Minimum Essential Medium Eagle
MGMT : O6-MethylGuanin-DNA-MethylTransferase

MHC-II : Major HistoCompatibility
miRNA : microRNA
MNP: iron oxide nanoparticles
mTOR : mammalian Target Of Rapamycin
MW : Molecular Weight
NCL : NuCleolin
O/N : OverNight
PDZ : initialism combining the first letters of the first three proteins discovered to share the domain : post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)
PFS : Progression-Free Survival
pI : isoelectric point
PI3K : Phosphatidylinositol-3-Kinases
PMSF : phenylmethanesulphonylfluoride
PNAG : PolyNucleotide:Adenosine Glycosylase
PTEN : Phosphatase and TENsin homolog
PVDF : polyvinylidene difluoride
RGD/NGR : arginine glycine aspartic /asparagine glycine arginine
RIP : Ribosome-Inactivating Protein
RNA : RiboNucleic Acid
RTK : Receptor Tyrosine Kinase
SDS : sodium dodecylsulfate
SELEX : Systematic Evolution of Ligands By Exponential Enrichment
SRL : Sarcin Ricin Loop
Tat : Transactivating transcriptional activator
TCGA : The Cancer Genome Atlas
TGF β : Transforming Growth Factor beta
TMZ : TeMoZolomide
UPR : Unfolded Protein Response
WT : Wild Type
WHO : World Health Organization

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Introduction

Therapy of cancer

Generality and current treatment

Cancer is the second leading cause of deaths all over the world. Globally 7.6 million deaths are caused by cancer which represents 13% of all global deaths (Jemal A, 2011). Surgery, chemotherapy, and irradiation are the mainstream therapeutic approaches for cancer. However, the chemotherapy success is limited due to lack of selectivity for tumor cells over normal cells resulting in insufficient drug concentrations in tumors, systemic toxicity and the appearance of drug-resistant tumor cells (Xu G, 2001).

Targeted therapies

What is it?

In theory

Recently targeted therapy is gaining importance due to its specificity towards cancer cells while sparing toxicity to off-target cells. There is an old adage that cancer is a hundred diseases masquerading into one. In support of this, Hanahan and Weinberg have defined several hallmarks of cancer, common to most, if not all, cancers (Hanahan D, 2011). Application of improved DNA sequencing technologies developed during the Human Genome Project (HGP) has confirmed and extended this adage, revealing the fact that even within a single cancer group or subgroup, each cancer has a unique genetic make-up. Indeed, recent findings from The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC) have further verified that although each cancer seems to be unique in its repertoire of genetic mutations, a range of signaling pathways are frequently affected within particular cancer types (Alexandrov LB, 2013). As we learned to understand many of the mechanisms which drive cancer development and progression, a paradigmatic change for new treatment options occurred. Instead of focusing mostly on unspecific cytotoxic therapeutics, new substances directed against cancer-driving molecules made their way to the clinic. The idea behind this concept of « targeted cancer therapies » is in theory

quite simple: if we are able to bring cancer-driving molecules under control, we might be able to stop cancer development and progression. Targeted therapy is sometimes called precision medicine, as specific targets allow cancer to be treated and possibly normal cells to be skipped. This different approach of therapy has significantly changed the treatment of cancer over the past years.

Which types of targeted therapies are available?

Many targeted cancer therapies have been approved by the Food and Drug Administration (FDA) to treat specific types of cancer. These therapies include hormone therapies, signal transduction inhibitors, gene expression modulators, apoptosis inducers, angiogenesis inhibitors, immunotherapies, and toxin delivery molecules.

Antibody-based therapy

Antibody-based therapy for cancer has become established over the past 15 years and is now one of the most successful and important strategies for treating patients with haematological malignancies and solid tumours. The fundamental basis of antibody-based therapy of tumours dates back to the original observations of antigen expression by tumour cells through serological techniques in the 1960s (Rettig WJ, 1989). The definition of cell surface antigens that are expressed by human cancers has revealed a broad array of targets that are overexpressed, mutated or selectively expressed compared with normal tissues (Van den Eynde BJ, 1997).

Unconjugated antibodies

The killing of tumour cells using monoclonal antibodies (mAbs) can result from direct action of the antibody. Unlabelled antibodies cause tumour cells to die through the recruitment and activation of effector cells by antibody-dependent cell-mediated cytotoxicity, by complement-dependent cytotoxicity, by the blocking of receptor-ligand interactions, or by induction of apoptosis.

Conjugated antibodies

Some unlabelled antibodies do not induce cell death by themselves and instead deliver radioisotopes, toxins, enzymes, or drugs to tumour sites. Specific targeting of cytotoxic agents to tumour cells has the potential to reach high concentrations at tumour sites, without the dose-limiting side-effects of systemic administration.

Alternatives to antibody

Although antibody-mediated therapy is highly specific and results in fewer side effects, potential immunogenicity and high cost of production may limit its clinical applications. To overcome these obstacles, specific drug delivery systems have recently been explored, like oligonucleotide aptamer-based targeted therapeutics or synthetic peptide.

Both peptide-and aptamer- targeting ligands are « man-made », in that they are designed and synthesized in the laboratory. Whether designed by selection from a library of sequences, by engineered evolution, or by rational design the ultimate sequence of a targeting peptide or aptamer is chosen by the researcher and is chemically synthesized, thus making production of large quantities relatively economical. Plus, both peptides and aptamers can achieve specificity and affinities that rival much larger molecules, such as full proteins and antibodies, making them ideal for use as targeting ligands.

Aptamer-based therapy

The emergence of aptamers created an attractive alternative to antibodies (Nimjee SM, 2005). In 1990 two revolutionary studies presented the method of *in vitro* generation of high-affinity molecules against selected targets when Tuerk and Gold succeeded in selecting RNA ligands against T4 DNA polymerase (Tuerk C, 1990); and Ellington and Szostak against organic dyes (Ellington AD, 1990). The first group coined the moniker SELEX (for Systematic Evolution of Ligands by EXponential enrichment), while the second introduced the term Aptamer (from the Latin *aptus* - fit, and Greek *meros* - part), now defined as single-stranded

nucleic acids, RNA or DNA molecules of 20-100 bases long capable of spontaneously folding into 3D structures and selectively binding to their cognate targets.

Currently, there are over 4,000 published articles referenced in the PubMed database that include the term « aptamer ». In the last decade, many aptamers targeting cell surface biomarkers have been developed through the advancement of both the protein- and/or cell-based SELEX technologies. SELEX technology allowed for rapid interrogation of large synthetic libraries and drastically broadened the spectrum of targets, which now includes not only toxic and non-immunogenic molecules, but also many synthetic and natural materials, and small compounds (Song KM, 2012).

Similar to antibodies in binding affinity (nanomolar to picomolar range binding constants), aptamers are less immunogenic, smaller (10-50 kDa), and can be used in a variety of environmental conditions, aptamers interact with their targets by recognizing a specific three-dimensional structure and are thus termed « chemical antibodies ». Due to their small size and oligonucleotide properties, aptamers offer several advantages over protein antibodies in both their extensive clinical applicability and a less challenging industrial synthesis process.

Owing to these advantages, these aptamers have been extensively studied for diagnosis and/or treatment of hematological malignancies (Nimjee SM, 2005) (Parekh P, 2013), lung (Esposito CL, 2011), liver (Wang FB, 2013), breast (Zhang, 2012), ovarian (Van Simaey D, 2010), brain (Kang D, 2012), colorectal (Sefah K, 2010), and pancreatic cancers (Dua, 2013), as well as for identification and characterization of CSCs (Shigdar S, 2013).

Peptide-based therapy

Peptide therapeutics is a promising field for emerging anti-cancer agents. Current research focuses on developing peptides that can (Kalyanaraman B, 2002) serve as tumor targeting moieties and (Naumov GN, 2003) permeabilize membranes with cytotoxic consequences. Peptides that could be developed into therapies to treat cancer can be organized into three major groups. In the first group are those peptides, usually naturally occurring or derived from a known protein, which have inherent membrane binding capacity, can form pores or

disrupt membranes, and through this mode of action are cytotoxic. Anti-microbial peptides (AMPs), like cecropins and magainins, are part of this first group as are “pore-forming” peptides derived from the Bcl-2 family of apoptosis mediators. In the second group are cell penetrating peptides (CPPs), such as the transactivating transcriptional activator (Tat) from HIV (human immunodeficiency virus), which function like a « Trojan horse » and internalize tethered cargo. The problems associated with most CPPs, like Tat, are related to the absence of tumor cell specificity, requiring modifications or fusion with a tumor-specific ligand for optimal anti-cancer effectiveness. The third group is comprised of tumor targeting peptides (TTPs) that are specific for tumor-related surface markers, such as membrane receptors, and can be used to deliver cytotoxic cargo (i.e. drugs or a cytotoxic peptide) specifically to tumor tissue or vasculature. Examples of these are peptides with RGD (arginine/glycine/aspartic acid) or NGR (asparagine/glycine/arginine) motifs that bind to integrins or cell surface associated molecules or receptors frequently overexpressed on tumor cells or tumor-associated blood vessels (Boohaker RJ, 2012). CPPs may directly penetrate membranes or like TTPs employ a biologically active mechanism like endocytosis for internalization of cargo. To produce the ideal therapeutic peptide, one would need to integrate the essential characteristics from each of the peptides in these groups: tumor targeting, cell membrane penetrating and death- inducing.

During last years, a large number of cell-binding peptides and peptides with potential therapeutic activities have been identified. However, the use of natural peptides as therapeutic drugs has been largely hampered by their short half-life in blood. Moreover, they have generally a poor bioavailability in tissues and organs. Obviously, blood clearance of peptides must be minimized in comparison with the rate of extravasation at the target sites. In order to be used as drugs, peptides have to be chemically modified. In this respect, the introduction of d-amino acids, or pseudo amino acids (pseudo-peptide), and peptide cyclization are the most common strategies to increase peptide stability (Adessi C, 2002). Thus, peptides analogs should be screened by a set of *in vitro* and *in vivo* assays to identify the most suitable agents for clinical use. Recent progress in chemical synthesis and design are expected to facilitate the design of protease-resistant peptides with improved bioavailability (Adessi C, 2002). As « chemical antibodies », pseudopeptide represent an

excellent alternative to replace or supplement protein antibodies, which have been extensively used in the clinic.

To resume, « chemical antibodies » aptamer as well pseudopeptide have many advantages over traditional antibodies : (i) they can penetrate tissues faster and more efficiently due to their significantly lower molecular weight, (ii) they are virtually nonimmunogenic *in vivo*. More importantly, a well-established synthesis protocol and chemical modification technology lead to (iv) rapid, large-scale synthesis and modification capacity that includes a variety of functional moieties, (v) low structural variation during chemical synthesis; and (vi) they have lower production costs.

Biomarker

Individualized therapy is paramount for improving of cancer treatment. The development of rationalized and individualized therapy is reliant on the identification of the specific biomarkers, validation of the biomarkers to identify the therapeutic targets, and drug development against the identified targets.

Clinically, it is estimated that about 60% of cancer-targeting drugs, target cell surface biomarkers, (Yildirim MA, 2007) making them attractive for disease treatment. Cell surface biomarkers are functionally important molecules involved in many biological processes, such as signal transduction, cell adhesion and migration, cell–cell interactions, and communication between the intra- and extra-cellular environments. An abnormal expression of cell surface biomarkers is often related to tumorigenesis (Josic D, 2008).

In this project, we focused on glioblastoma (GBM) because it is the most common and lethal primary malignancy of the central nervous system (CNS). Despite promising results in preclinical trials, many of targeted therapies have provided limited or no therapeutic efficacy in human clinical trials. Thus, although survival of patients with GBM continues to slowly improve, treatment of GBM remains extremely challenging. Continued research and development of new molecular targeted, based on a detailed understanding of molecular

pathogenesis, can reasonably be expected to lead to increased survival and more favorable prognosis of patients with GBM.

Over the past few year, the two biomarkers CD98 and nucleolin, proteins overexpressed in cell surface of glioblastoma cells (Takeuchi H, 2008) (Yanagida O, 2001) (Galzio R, 2012) have gained in importance into the challenge to find new therapeutic target for glioblastoma.

Tumors of central nervous system

Generality

The central nervous system (CNS) consists of neurons and neuroglial cells. There are four types of neuroglial cells: astrocytes, oligodendrocytes, ependymal cells and microglia. CNS tumors are among the most heterogeneous types of tumors. Until 2007, they were classified according to the 2007 World Health Organization (WHO) classification which includes over 130 subtypes on basis of morphology and biological behavior. This classification system grades CNS tumors into relatively benign, so-called low- grade tumors (WHO grade I or II) and malignant: high-grade tumors (WHO grade III or IV).

The current update (2016 CNS WHO) thus breaks with the century-old principle of diagnosis based entirely on microscopy by incorporating molecular parameters into the classification of CNS tumor entities (Louis DN P. A.-B., 2016). The use of « integrated » (Louis DN P. A.-B., 2014) phenotypic and genotypic parameters for CNS tumor classification adds a level of objectivity that has been missing from some aspects of the diagnostic process in the past. It is hoped that this additional objectivity will yield more biologically homogeneous and narrowly defined diagnostic entities than in prior classifications, which in turn should lead to greater diagnostic accuracy as well as improved patient management and more accurate determinations of prognosis and treatment response.

Gliomas

Tumors that originate from glial cells, the supportive tissue of the brain and spinal cord, are called gliomas. After meningiomas, which are benign tumors arising from the meninges of the brain, gliomas are the most common type of tumor arising from the central nervous system. They constitute about 28% of all benign and malignant primary tumors, i.e. tumors formed *de novo* and not originating from a preceding tumor, and 80% of all primary malignant tumors in the CNS. Glioma account for 29% of all primary brain and CNS tumors (Dolecek TA, 2012). Astrocytomas are the most common common glioma subtype accounting for approximately 75% of malignant brain tumours. Astrocytomas are

categorized in a four-grade scale established by the World Health Organization based on their morphological and pathological features (Louis DN O. H., 2007).

Glioblastoma multiforme

Grade IV astrocytomas are called glioblastoma multiforme (GBM). It is the most common type of primary brain tumor in adults, constituting about 73% of all astrocytomas, and also the most aggressive with a median survival of only 15 months despite multimodal treatment with surgery, radiation and chemotherapy. Histologically, glioblastoma are marked by increased cellularity and mitotic activity, necrosis, and vascular proliferation (Omuro A, 2013). Because the cells of this cancer vary in size and shape (*ie* are pleomorphic), the term multiforme has been proposed. The GBM is characterized by uncontrolled proliferation, massive angiogenesis, high genomic instability, and apoptosis resistance to chemo-radiotherapy, features that are useful prognostic factors for overall survival. Many more, these neoplasms are highly invasive and infiltrate brain parenchyma (functional tissue) that makes them difficult to resect surgically.

Subclassification

GBMs can be divided into primary GBM (or *de novo*) and secondary GBM (Rich JN B. D., 2004).

- Primary tumors comprise approximately 90% of GBM cases and are diagnosed without clinical or histological evidence of a previous low-grade neoplasia. They grow very quickly in older patients between 60 and 70 years and are characterized by a brief medical history (two-thirds of patients, less than 3 months).
- The secondary GBMs represent the minority of cases (<10%), derived from a low-grade tumor that undergoes a process of progression to high grade tumor. The median time to progression from a LGG to GBM is 5.3 years, from anaplastic astrocytoma to GBM is 1.4 years. The secondary GBMs typically occur in younger adult subjects with a mean age at diagnosis of 45 years and the time of progression from low to high grade ranges from months to decades (Ohgaki H, 2009).

Invasion

A hallmark of glioblastoma is its high invasive potential. Within the brain, glioblastoma demonstrates specific patterns of invasion that are distinct from systemic tumours that have metastasized to the brain. Glioblastoma cells have the potential to invade throughout the brain as single cells preferentially traveling along anatomically defined brain regions known as Scherer's secondary structures: white matter tracts, blood vessels and subpial glial space (Cuddapah VA, 2014). Glioblastoma tumours rarely metastasize out of the central nervous system (Bellail AC, 2004). At the cellular level, three key processes enable invasion: 1) adhesion to the brain parenchyma, 2) cellular motility *via* actin/myosin contractile motions, and 3) degradation of extracellular matrix components (Lefranc F, 2005).

Incidence

The worldwide incidence rate of primary malignant brain and other CNS tumors in 2012, age-adjusted using the world standard population, was 3.4 per 100,000. Incidence rates by gender were 3.9 per 100,000 in males and 3.0 per 100,000 in females. This represented an estimated 139,608 males and 116,605 females who were diagnosed worldwide with a primary malignant brain tumor in 2012, an overall total of 256,213 individuals. Sex, however, seems to have no meaning in terms of outcomes (Walid, 2008).

The incidence rates were higher in more developed countries (5.1 per 100,000) than in less developed countries (3.0 per 100,000) (IARC). The incidence of malignant brain tumors rose steadily over the last quarter of a century in both adults and children (Siegel RL, 2016). A proportion of this trend may be due to improved neuro-imaging techniques and access to medical care (Schwartzbaum JA, 2006). Familial gene mutations, immune disease, and high dose ionizing irradiation are known risk factors of brain tumors but are likely responsible for a minority of cases. Epidemiological studies and geographic variability in case numbers suggest that the etiology of brain tumors may be associated with environmental factors and exposure to carcinogens (Schwartzbaum JA, 2006) (Wiemels J, 2010).

Genetic alterations

Gliomagenesis is an extremely unstable genetic process with activation of oncogenes, inactivation of tumor suppressor genes and many different genetic mutations. In fact, malignant gliomas held the greatest range of genetic abnormalities among tumors. These multiple alterations affecting several signaling pathways, result in enhanced cellular proliferation and apoptosis resistance (Sathornsumetee S, 2007).

Primary GBM arise rapidly, within less than 3 months, without evidence of a pre-existing low-grade lesions. However, the analysis of mutations indicates that the same genetic pathways are affected in both primary and secondary GBMs. So, the same genetic pathways could be targeted in both sub-types, but the frequency of specific genetic mutations may differ in the two GBMs types.

Since 2008, a key discovery for GBM classification was the observation of a high frequency of mutations (10%) in the isocitrate dehydrogenase genes (IDH1/2). Subsequently, IDH1 mutations were found to match closely with the secondary GBM subgroup and were extremely common in low grade astrocytomas and oligodendrogliomas (Parsons DW, 2008), (Yan H P. D.-H., 2009). Methylation alterations epigenetically affecting gene expression have come to light over the last few years as an important factor influencing gene pathways in cancer. Widespread hypomethylation of the cancer genome occurs concomitantly with hypermethylation of promoter CpG islands resulting in gene silencing. Specifically, methylation of the O-6-methylguanine-DNA methyltransferase (MGMT) gene promoter results in defective DNA repair and is associated with improved response to alkylating chemotherapy agents in GBM (Hegi ME, 2005).

Prognostic and predictive

The characterization of malignant tumors, often takes place through molecular analysis and detection of cancer biomarkers. In oncology, they are two classes: prognostic and predictive. The firsts indicate the possible outcome of the disease, independently of the treatment received; conversely, the predictive inform about the expected outcome after application of treatment protocols. Therefore, the predictive markers help in choosing between two or more treatment options and are important for personalized therapy (targeted therapy).

Currently the widely used molecular markers to characterized GBM, evaluated in the clinical routine of brain tumors are the following: mutations of isocitrate dehydrogenase 1 and 2 (IDH1/2) gene, the methylation status of promoter of the MGMT gene, and the co-deletion of chromosomes 1p and 19q (**figure 1**).

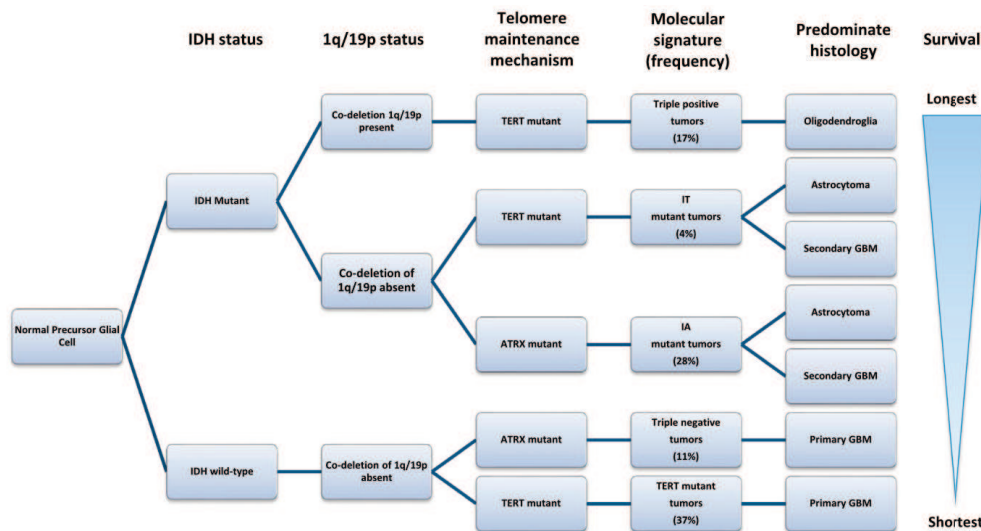


Figure 1 : **Schematic Representation of Combined Findings from** (Jiao Y, 2012), (Killela PJ, 2013), (Brat DJ & Members of the Cancer Biomarker Reporting Committee, 2015)(Eckel-Passow JE, 2015) **and synthetised by** (Foote MB, 2015).

Characteristic molecular signature groups are determined based on the tumor status of IDH and 1q/19p combined with cancer telomere maintenance strategy (TERT or ATRX mutation conferring telomerase or ALT telomere maintenance, respectively). Each subgroup is associated with the predominate histology most frequently witnessed with the respective genetic signature as well as relative survival. Acronyms in the schematic include IDH (isocitrate dehydrogenase), TERT (telomerase reverse transcriptase), and ATRX (alpha-thalassemia/mental retardation syndrome X-linked) as well as the subgroups “IT” (IDH and TERT) and “IA” (IDH and ATRX). Triple-positive tumors refer to gliomas with IDH and TERT mutations and with co-deletion of 1q/19p. Triple-negative tumors refer to gliomas wild-type for IDH, TERT and without with co-deletion of 1q/19p. Frequency estimates are derived from Eckel- Passow et al. (2015).

Current standard of care and treatment

In 1920s the neurosurgeon Walter Dandy decided to remove the entire affected hemisphere from two patients with extensive glioblastoma. Despite these radical surgeries, both patients died by development of new glioblastomas in the contralateral hemispheres (Gardner,

1933). Unfortunately, almost a century later, this catastrophic panorama has not substantially changed. Its aggressive growth and inexorable recurrence made glioblastoma extremely lethal (reviewed in (Siebzehnrubl FA, 2011)).

The current treatment strategy for GBM patients consists of maximal surgical resection, radiotherapy and chemotherapy (Stupp R W. D., 2005). Due to the infiltrative character of these tumors a complete surgical resection is typically impossible. However, a gross total resection of more than 97% is correlated with increased survival. Maximal resection with preservation of neurological function is the main goal of neurosurgeons in the management of GBM. Many evidences indicate that extensive resection, as long as neurological functioning is maintained, increases effectiveness of adjunct chemo- and radiotherapy and are associated with better outcomes (Lamborn KR, 2004) (Stummer W N. U., 2011). Maximum resection has been recently improved due to advanced imaging modalities including functional MRI, intraoperative stereotactic navigation or fluorescent tumor markers (Stummer W & Group., 2006)

Currently the chemotherapy employed after resection is the temozolomide (TMZ), an oral DNA alkylating agent with good penetration of the BBB (blood Brain Barrier). Unfortunately TMZ does not provide a cure for GBM and only marginally extends life. Therefore, new treatment strategies for GBM are need (Cimini A I. R., 2011).

Therapies under investigation : molecularly targeted therapies

Due to recent progress in genomics, several aberrantly activated pathways and mutated genes have been implicated in the pathogenesis and malignant progression of GBM (Bai RY, 2011) (Sathornsumetee S, 2007) (Van Meir EG, 2004). These findings have inspired the investigation of molecularly targeted therapies designed to target tumor-specific recurrent genetic alterations as a novel approach to treating GBM. Many mutations occur in receptor tyrosine kinases (RTKs) or components of their downstream signaling pathways, making them potential targets for drug development and evaluation in clinical trials (Patel M, 2012). The majority of growth factor receptors are transmembrane glycoprotein RTKs with

extracellular ligand-binding domains and intracellular kinase domains. Activation of these RTKs triggers a cascade of downstream signaling events, and inappropriate activation of these signaling pathways is thought to drive tumor growth, survival, invasion into normal brain, and secretion of angiogenic factors (Sathornsumetee S, 2007). Thus, inhibition of these pathways and their downstream intracellular signaling components is the goal of molecularly targeted approaches to treatment of GBM.

In clinical trials, targeted agents are generally studied as single agent therapies in recurrent GBM or used in combination with RT and TMZ for treatment of newly diagnosed GBM. (Day SE, 2012) Overall, the results of multiple clinical trials with various molecularly targeted agents have demonstrated only modest therapeutic benefit.

Mechanisms of resistance of GBMs to chemotherapy

Normal cells, when submitted to external stress, induce cell cycle arrest, DNA repair, or apoptosis whereas GBM cells have lost several safeguard mechanisms and evade these controls by mutating specific regulators of cell cycle arrest and apoptosis (Maher EA, 2001). In addition, uncontrolled invasiveness and critical location makes the handling of primary gliomas a great challenge in oncology. Whereas efficient chemotherapeutic treatments have been developed for other cancers, none of them is currently effective for most GBMs, despite improved knowledge about genetic alterations and the understanding of molecular mechanisms involved in the development of brain tumors. Several factors may explain the resistance of glioma to drugs and the delay in developing efficient therapies against brain tumors.

Contributions to the mechanism of drug resistance by the genetic background

The role of *TP53*, *RB* and mitogenic pathways has been dissected on the molecular level by studying genetically engineered mice models in order to define possible routes that lead to the development of gliomas. Only one or two elements of the growth

factors/Ras/Raf/MEK/ERK, PI3K/PTEN/AKT/mTOR, p53/ARF/MDM2, and p16/Rb/cyclinD/CDK4 pathways are targeted in these animal models.

The finding that different genetic alterations of the p53, RB and mitogenic pathways can generate malignant gliomas indicates that combinatorial molecular therapies simultaneously targeting crucial cellular pathways might be more successful than those disrupting a single pathway. However, all current chemotherapies that are targeting only one or two pathways are ineffective against gliomas.

Epigenetic and genetic alterations renders GBMs resistant to chemotherapy

In contrast to findings obtained from animal models, many more genes and pathways are affected in human gliomas due to a most severe mutator phenotype that causes to the accumulation of epigenetic and genetic alterations (Merlo, 2003). DNA methylation and histone deacetylation have a central role in the control of gene expression including transcriptional repression of tumor suppressor genes. Studies on the involvement of methylation in carcinogenesis have established this epigenetic modification as a third pathway to the loss of function and as one potential hit. Such findings show that there are multiple mechanisms that may lead to the generation of high-grade gliomas. In this perspective, DNA methylation and deacetylation become targets for cancer therapy. Understanding the roles of hypermethylation and histone deacetylation in cancer (Lund AH, 2004) has clear implications on the therapeutic use of agents targeting the DNA methylation machinery and HDACs (Johnstone, 2002) (Suzuki T, 2005).

Blood-brain barrier : an obstacle to drug delivery

Drug delivery is a major obstacle for successful treatment of malignant gliomas because of the BBB (Nagane M, 1999). The BBB is a dynamic interface between the blood and the brain, located on the endothelial cells of the blood capillaries of the brain, it eliminates (toxic) substances from the endothelial compartment and supplies the brain with nutrients and other compounds. Thus, it restricts pharmacological accessibility, thereby limiting the

accumulation of many drugs in the brain. The design of a targeted therapy for glioma treatment should take into account high selectivity for tumor cells relative to normal cells, efficient delivery into the brain parenchyma and limited toxicity (Lesniak MS, 2001). In the other hand, it's interesting to highlight that tumor-driven angiogenesis and inflammation are often accompanied by increased capillary permeability. So BBB disruption in GBM characterized by a mild leakage of the blood-brain barrier without significant neoangiogenesis (unchanged cerebral blood volume) might be an opportunity since it might offer an entrance for targeted drug therapeutics into the brain tumor. Indeed this type of leakage could permit and justify the use of a big molecule such as proteins as treatment (Huhndorf M, 2016).

Innovative targets for GBM therapy :

CD98 and Nucleolin

CD98

Structure

The glycoprotein-associated integrin hCD98 (also known as 4F2) is a cell surface heterodimer formed by covalent linkage of the CD98 heavy chain with several light chains (LAT1, LAT2, y1LAT1, y1LAT2, xCT, and asc-1), forming an amino acid transporter (Chillarón J, 2001). CD98 has a single trans-membrane domain formed by amino acids 82–104, the cytoplasmic portion formed by the amino acids 1–81 and the extracellular domain formed by amino acids 105–529. The human CD98 C-terminus contains a potential binding domain for PDZ, thus suggesting that CD98 may be associated with extracellular proteins containing PDZ domains. This region is pointed out as hCD98 and binds the class II PDZ domain in the extracellular space (amino acids 520–529) (Yan Y, 2007).

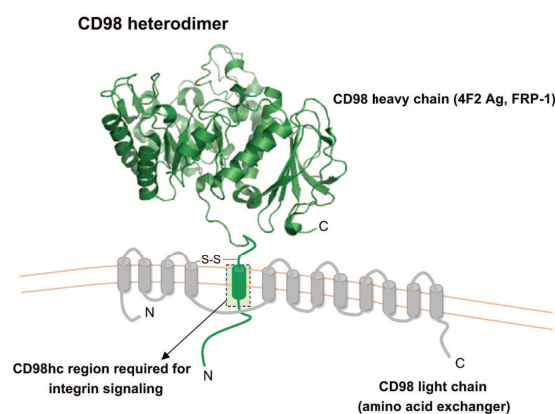


Figure 2: **Schematic illustration of CD98** (Cantor JM, 2012)

The CD98 heavy chain (known as CH98hc, 4F2 Ag or FRP-1) is encoded by the and SLC3A2 gene in humans. CD98hc is a type II transmembrane protein with a large, heavily glycosylated extracellular domain, and a short transmembrane domain and cytoplasmic tail. The extracellular, or ecto-, domain was crystallized, and a ribbon representation of the structure is shown (Fort J, 2007). The CD98 heterodimer is formed by disulfide bonds between the membrane-proximal section of the CD98hc extracellular domain and any one of at least six possible CD98 light chains (the amino acid transporters LAT-1 or LAT-2, etc.).

Roles

hCD98 was originally identified on the surface of leukocytes, where it was thought to play a role in the immune response by directing leukocyte migration, intercellular interactions and signal transduction. Since then, CD98 is expressed in many cells types, including renal tubules in the gastrointestinal tract, and polarized epithelial cells, (Rossier G, 1999) (Merlin D, 2001) (Dave MH, 2004) but very high expression of CD98 has been reported in different types of tumors. The light chain LAT1 conjugated with CD98 has also been identified in glioma cells and it is expressed at lower levels in the blood-brain barrier (Yanagida O, 2001) (Takeuchi H, 2008) (Kobayashi K, 2008) (Okubo S, 2010). Furthermore several studies indicate the important role of LAT1 and CD98hc (heavy chain) in malignant transformation and carcinogenesis so that the complex LAT1-CD98hc may represent a unique target for intervention on cancer. The CD98 is widely expressed in rapid growing cells, where it can also modulate oncogenic transformation and cell fusion. LAT1 was found with high expression levels in rat tumors (*Rattus norvegicus*), such as gliomas (C6), hepatoma (dRLh-84), and hepatocellular carcinoma (FAA- HTC1), while the normal liver does not express LAT1. High levels of LAT1 were also found in other human cancer cell lines derived from stomach, melanoma, and small cell lung (Kanai Y, 1998). The CD98 is believed to be used in the regulation of amino acid transport and it appears deactivated after the G1 phase, when cells are activated and proliferation is enabled. Instead the complex is up- regulated in tumor cells that are in rapid division and proliferation making within these essential amino acids in order to grow and differentiate (Nawashiro H, 2002).

Targeting CD98 as a cancer therapy *via* PDZ domain

The extracellular domain of the glycoprotein- associated integrin hCD98 protrudes into the basolateral extracellular space of the intestine and contains a PDZ class II-binding domain (GLLLRFPYAA, amino acids 520–529).

PDZ domain

PDZ domains consist of 80–90 amino acids comprising six beta-strands (betaA to betaF) and two alpha-helices, A and B, compactly arranged into a globular structure. Peptide binding of the ligand takes place in an elongated surface groove as an antiparallel beta-strand interacting with the betaB strand and the B helix (Jemth P, 2007). The structure of PDZ domains allows binding to a free carboxylate group at the end of a peptide through a carboxylate-binding loop between the betaA and the betaB strands. They recognize and bind short peptide sequences located at the C-terminus of a protein with K_d in the range 1–50 mM (Gianni S, 2006). The primary function of PDZ domains is to recognize and bind specific ~5-amino acid motifs that occur at the COOH terminus of target proteins. Interestingly, prediction of the molecular orientation of hCD98 has suggested that the C-terminal tail is extracellular (Teixeira S, 1987), indicating that hCD98 may associate with extracellular PDZ domain- containing proteins. One possible protein that may bind to the C terminus of hCD98 in the extracellular space is the MAGUK family protein hCASK (Cohen AR, 1998). This protein is the human homolog of *Caenorhabditis elegans* LIN-2 and *Drosophila* camguk and is widely expressed in human tissues (Nix SL, 2000)(Sanford JL, 2004). It has a unique domain composition, including an N-terminal region with homology to the calcium/calmodulin-dependent protein kinase, followed by the three characteristic MAGUK domains (PDZ class II consensus domain, SH3 and GUK) (Daniels DL, 1998).

In the present study, we hypothesized that hCASK might represent a natural ligand to CD98 and accordingly investigated the expression of hCASK in glioma and the possibility that hCASK interacts with the extracellular C-terminus of CD98 in a PDZ-dependent manner.

For these reasons we decided to verify if PDZ domains may be used to target CD98 in human glioblastoma cells as a model system, and we used as a toxic domain the Ribosome-Inactivating Protein (RIP) saporin produced by *Saponaria officinalis* L., a toxin widely studied for the production of immunotoxins (conjugates of an antibody or its fragments and a toxin (Flavell DJ C. S., 1991) (Flavell DJ N. A., 1997) (Anselmo AC, 2014) and targeted toxins (Ippoliti R L. E., 1996) (Giansanti F D. L., 2010) (Lombardi A, 2010) (Cimini A M. S., 2012).

Nucleolin

Nucleolin (NCL), first described by Orick et al. in 1973, is a major, highly conserved protein found in all eukaryotes. It is an abundant nucleolar phosphoprotein of eukaryotic cells and is known to be involved in several cellular processes as well as its central role in ribosomal biogenesis (Lapeyre B, 1987) (Bourbon HM L. B., 1988) (Bourbon HM A. F., 1990) (Srivastava M F. P., 1989) (Ginisty H A. F., 1998). Nucleolin is one of the best studied nucleolar protein and represents up to 5% of the total proteins in the nucleolus. Beside that, nucleolin expression can also be detected in other cellular compartments including, cytoplasm and cell surface (Lapeyre B, 1987) (Hovanessian AG P.-D. F., 2000). Nucleolin is a highly mobile multifunctional protein that can bind to DNA, RNA and proteins and is found to be enriched in exponentially growing and cancerous surface cells (Lapeyre B, 1987) (Derenzini M, 1995).

Structure

The nucleolin protein consists of three different structural domains consisting of a highly acidic and highly phosphorylated N-terminal domain (Ginisty H S. H., 1999), one central domain consisting of four central RNA-binding domains (RBDs), and a glycine/arginine rich C-terminal domain (GAR) (**figure 3**).

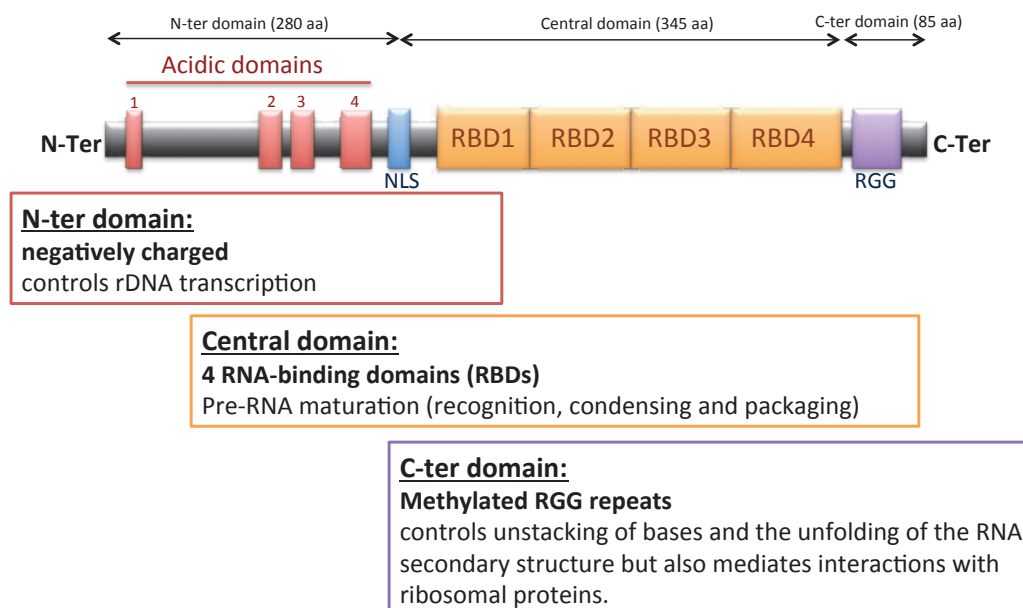


Figure 3: Schematic view of different domains and their functions in nucleolin

Roles

The most characteristic feature of nucleolin is its multifunctionality. Localization of nucleolin within the nucleolus, and its transient interaction with pre-ribosomes and rRNA suggest its prominent role in ribosomal biogenesis. For simplicity, these functions are summarized in

Table 1.

In actively growing and dividing cells, nucleolin may also be found at very high levels in the cell membrane (Srivastava M P. H., 1999) (Hovanessian AG S. C., 2010) (Huang Y, 2006). Since nucleolin was found on the surface of cells that were actively growing and dividing, it became an attractive protein to study as a potential cancer biomarker.

Location	Interaction	Proposed Function		
Surface	Phosphatidylserine	Macrophage receptor	Hirano, K. & al.2005	
	CD43	Macrophage receptor	Hirano, K. & al.2005	
	Factor J	Inhibition of complement activation	Larrucea, S. & al. 1999	
	uPAR	Induce translocation of receptor/ligand and stimulate mitogenic response	Stepanova, V. & al. 2008	
	MyH9	Translocation of nucleolin within surface and cytoplasm with	Huang, Y. & al. 2006	
	Endostatin	Receptor and surface -> cytoplasmic ->nuclear shuttling	Shi, H. & al. 2007	
	Laminin-1	Neurite growth and maintenance	Kibbey, M. & al. 1995	
	Tipa	Receptor and surface -> cytoplasmic ->nuclear shuttling	Suganuma, M. & al. 2012	
	Viral receptor	Coxsackie B, Hepatitis, HIV, AVV-2	Callebaut, C. & al. 2001	
	PTN	Angiogenesis stimulation	Koutsoumpa, M. & al. 2012	
	ErbB	Receptor dimerization and phosphorylation	Di Segni, A. & al. 2008	
	Integrin $\alpha_v\beta_3$	Migration stimulation	Koutsoumpa, M. & al. 2013	
	Hepatocyte growth factor	Receptor and inducer of proliferation stimulation	Tate, A. & al. 2001	
	LDL receptor	Low affinity receptor of HepG2 cells	Semenkovich, C. & al. 1990	
	L-Selectin	Receptor and inducer of proliferation stimulation	Harms, G. & al. 2001	
	Cytoplasm	bcl-2 ARE	mRNA stabilization	Ishimaru, D. & al. 2010
bcl-xl ARE		mRNA stabilization	Zhang, J. & al. 2008	
MMP-9		mRNA stabilization	Fahling, M. & al. 2005	
APP 3'UTR		Cytoplasmic RNA stability	Zaidi, S. & al. 1995	
Granzyme A		Degradation protein of Granzyme A	Pasternack, M. & al. 1991	
TGF- β receptor 1		Cytoplasmic??nuclear transport	Chandra, M. & al. 2012	
YB-1		IL-2 mRNA stabilization	Chen, C. & al. 2000	
Midkine		Anti-apoptotic cytoplasm ->nuclear transport	Take, M. & al. 1994	
PRMT5		Nucleolin methylation	Teng, Y. & al. 2007	
HdM2		Inhibition of p53 ubiquitination	Saxena, A. & al. 2006	
Nucleus		LR1 complex	Immunoglobulin switch recombination, c-myc and EBNA-1 transcription	Hanakahi, L. & al. 1997
		Histone H1 and H2AX	Chromatin decondensation	Erard, M. & al. 1988
	MDC1	Accumulation at DSB in DNA repair	Kobayashi, J. & al. 2012	
	Rad51	Recombinational DNA repair	De, A. & al. 2006	
	p53	Inhibition of DNA replication and repair during stress	Takagi, M. & al. 2005	
	hRPA	Inhibition of chromosomal replication during stress	Daniely, Y. & al. 2000	
	Viral proteins	Transcription factor	Grinstein, E. & al. 2002	
	VEGF promoter	Transcriptional activator	Uribe, D. & al. 2011	
	Krüppel-like-factor-2	Transcriptional promoter	Huddleson, J. & al. 2006	
	Rb	G1 phase transcriptional repressor of HPV18 oncogenes	Grinstein, E. & al. 2006	
	Insulin-receptor substrate- 1	Nuclear -> cytoplasmic shuttling during differentiation	Burks, D. & al. 1998	
	A- and C- Myb	Repressor of Myb transcriptional regulation	Ying, G. & al. 2000	
	YB-1	DNA repair	Gaudreault, I. & al. 2004	
	Telomerase	Telomerase nucleolar localization	Khurts, S. & al. 2004	
	PCNA	During stress inhibits NER	Yang, C. & al. 2009	
	PRMT5	Nucleolin methylation	Teng, Y. & al. 2007	
Drosha	MicroRNA biogenesis	Pickering, B. F. & al. 2011		

Table 1: Nucleolin interacting proteins and their functions

Targeting nucleolin as a cancer therapy

The successful elimination and inhibition of tumor growth by direct nucleolin targeting or through the use of nucleolin ligand-linked drug delivery systems further underscores nucleolin's potential as a cancer therapy. Nucleolin is an attractive target for cancer therapy, as it appears to have low off-target effects primarily because of its restrictive expression on the cell surface of cancer cells and cancer associated cells.

Nucleolin serves as a target in pre-clinical trials include F3 peptide, aptamer, and LNA-aptamers linked to various drug carriers for delivery of microRNAs, radionuclides and doxorubicin (Prickett WM, 2011) (Hu Q, 2013) (Kim JK, 2012) (Kanwar JR, 2011) (Fang X, 2010).

Aptamers : AS1411

Aptamers, ssDNA/RNA molecules, targeting nucleolin have recently come to the forefront in the translational and clinical search for potential therapeutics in neoplasms. A novel class of aptamers called GROs for G-rich oligonucleotides, with a strong secondary structure, binds nucleolin and inhibits growth of tumors in prostate, breast, and cervical carcinoma models (Xu X, 2001). The most successful nucleolin, GRO, AS1411, has shown anti-proliferative activity in almost every cancer cell type tested, and thus appears to have a broad therapeutic potential (Bates PJ L. D., 2009); AS1411 moved from Phase I clinical trials, in a variety of advanced solid tumors, into two Phase II clinical trials for acute myeloid leukemia and renal cell carcinoma. Based on published articles and meeting reports (Bates PJ L. D., 2009) (D. Laber, 2004) (D. Laber B. T., 2007) (Rosenberg JE, 2014), the clinical results for AS1411 can be summarized thus: (i) AS1411 has been tested in over 100 patients and is well tolerated with no evidence of causing any severe side effects with continuous infusion dosing of up to 40 mg/kg/day for 7 days, (ii) the overall response rate is low but at least 7 patients—3 with renal cell carcinoma and 4 with acute myeloid leukemia—have had long-lasting clinical responses where their cancers disappeared or shrank substantially, and (iii) steady-state plasma concentrations of AS1411 have been in the range of 1-6 μ M or less during clinical trials. AS1411 still has some potential for clinical impact due to its excellent

safety profile and ability to induce durable responses in some patients with intractable cancers. However, the suboptimal pharmacology and low potency of this unmodified DNA may limit its future development in unselected cancer patients.

An alternative approach would be to use the targeting properties of AS1411 to deliver drugs selectively to cancer cells. The increasing number of publications referring to AS1411 is largely driven by the possibility of using this aptamer to direct attached molecules or particles into malignant cells (**figure 4**), which is an attractive topic in cancer research.

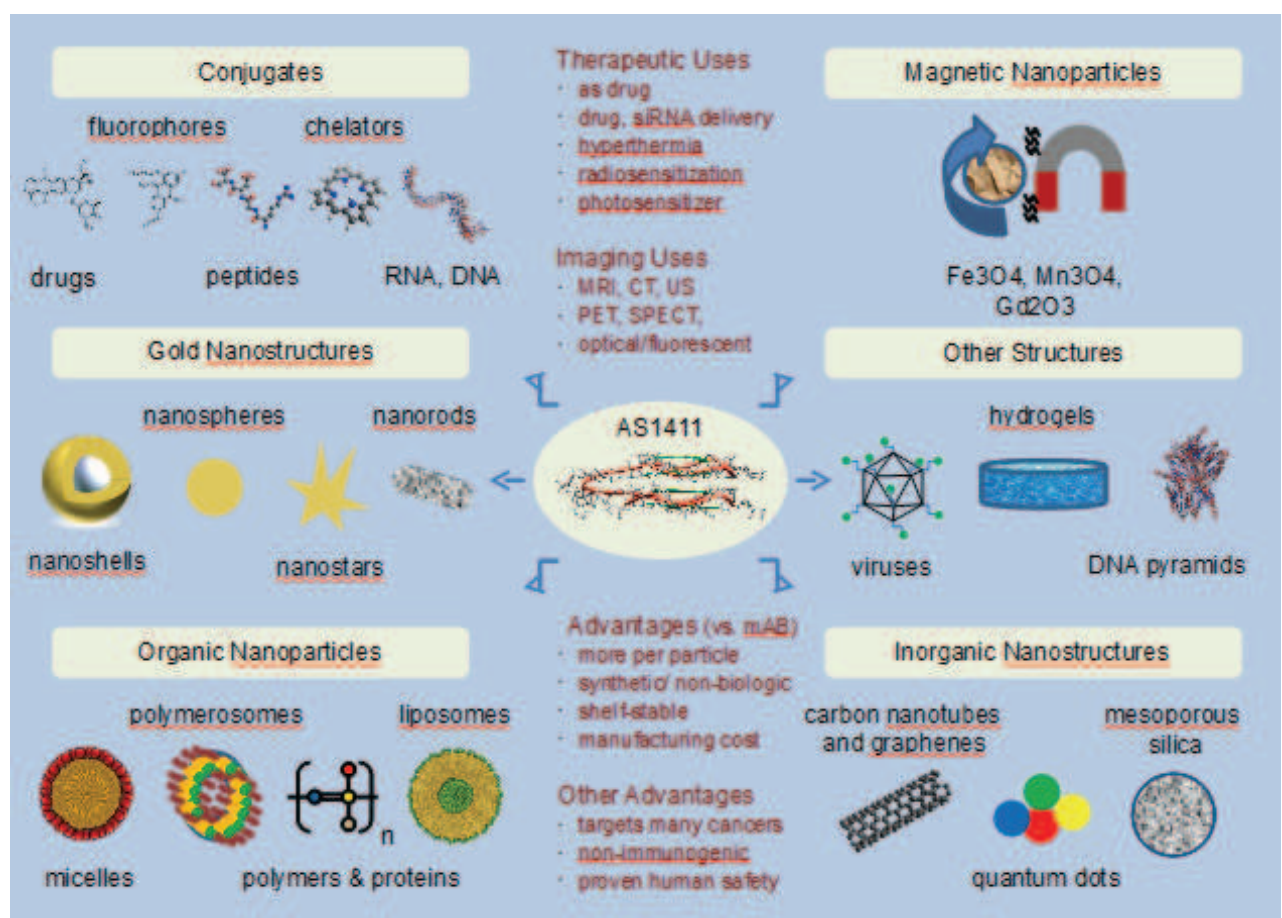


Figure 4: **Uses and advantages of AS1411 as a cancer-targeting agent** (Bates PJ R.-R. E., 2016)

Illustration showing the variety of structures that AS1411 has been conjugated to for the purposes of cancer targeting. The bullet points (red text) indicate potential clinical applications for AS1411-conjugated materials and anticipated advantages of using AS1411 compared to monoclonal antibodies (mAB) or alternative targeting agents (e.g. other aptamers, small molecules, peptides, and proteins).

For small molecule chemotherapy drugs, there is some evidence that delivery *via* AS1411-conjugated particles can circumvent drug-resistance pumps (Liao ZX, 2015), pass the blood-brain barrier (Gao H, 2012), and reduce toxicity to normal tissues that are usually adversely affected by free drug (Liao ZX, 2015) (Li X, 2015). In summation, there is strong evidence that AS1411 is an effective targeting ligand that can be used to deliver wide-ranging cargoes to cancer cells.

Pseudopeptide : NucANTs (Nucleolin ANTagonists)

Initially, nucleolin was targeted by anti-HIV pentameric pseudopeptide HB-19, which binds irreversibly to the RGG C-terminal domain of nucleolin and is currently being tested as a potential therapeutic in cancer beyond its original purpose in HIV infection. To improve the activity of HB-19, pseudopeptides called NucAnt (Nucleolin Antagonist) have been synthesized. Nucant pseudopeptides are composed of a short polypeptide or template, rich in lysine residues. At any lysine residue, one pseudotriptide $\text{Lys}\psi(\text{CH}_2\text{N})\text{-Pro-Arg}$ (Destouches D P. N.-K., 2011) is covalently anchored, and the number of pseudotriptides determines the valence of binding of the antagonist to nucleolin surface. Among them, NucAnt 3 or N3, is pentavalent, while NucAnt 7 or N7, NucAnt 6 and NucAnt 6L are hexavalent, (Meng GZ, 2011), differing each other for amino acid composition of the template. Hexavalent NucAnt 6L or N6L (patented by Immupharma WO 2009 141687 A1), used in this work, is a presents all the amino acids of the pseudotriptide in the L configuration. Furthermore, N6L is the only compound that assumes a three-dimensional alpha elix 310 conformation among all the NucAnt pseudopeptides (**figure 5**). N6L is also that which presents the greatest blocking activity on the surface nucleolin (Destouches D P. N.-K., 2011).

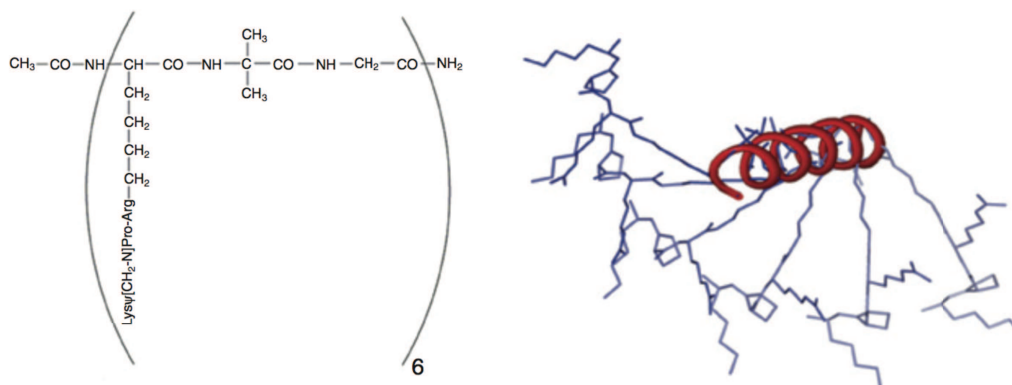


Figure 5: Representation of semi-developed formula and 3D structure of N6L

N6L is built on a 3_{10} helical matrix composed of 6 repeats of Lys-Aib-Gly. Six reduced peptide bond pseudotripeptides Lys[CH₂N]-Pro-Arg are grafted onto the lysine residues of the matrix. α -Aminoisobutyric acid (Aib) is an achiral residue, which contains a pair of methyl groups at the C α carbon atom. Ψ symbolizes the reduced form between residus K and P.

Previous study reported that the N6L suppressed both tumor growth and angiogenesis (Destouches D. E. K.-K., 2008). Currently, N6L is under evaluation in a phase II clinical trial (Krust B, 2011). We demonstrated the effects of N6L on human glioblastoma cells in primary culture prepared from post-surgical specimens (Benedetti E, 2015). The results obtained indicated a anti-proliferative effect of N6L and point towards its possible use as adjuvant agent to the standard therapeutic protocols presently utilized for glioblastoma.

In a recent study, N6L was used for the first time as a targeting agent (Sader & P, 2015). Indeed, in this paper, they have developed iron oxide nanoparticles (MNP) functionalized by the multivalent pseudopeptide N6L that target breast tumor by binding to nucleolin and glycosaminoglycans. They shown that N6L linked to the MNP induces the targeting of tumor cells but has no effect on their growth, emphasizing the use of N6L, as the tumor targeting ligand of MNP. In light of these results, the next step will be the improvement of the amount of MNP-N6L that reaches the tumor by interaction with cell-surface glycosaminoglycans and nucleolin, thereby constituting a potentially promising targeting system of theranostic applications for imaging and magnetic hyperthermia, and to multifunctionalize the MNP with N6L as the targeting agent in conjunction with anticancer drugs for targeted drug delivery.

Aptamer AS1411 and pseudopeptide N6L are already in clinical trial for their own antitumoral activity after binding to overexpressed NCL in cancer cells. To increase efficiency, in this study we was looking for exploit them and their specific binding capacity to deliver one ribosome inactivating protein (RIP) to glioblastoma cells.

Ribosome-inactivating proteins (RIPs)

From plant defense to tumor attack

The history of RIPs began when the interest of the scientific community turned to the toxicity of plant poisons. Firstly discovered over a century ago in the castor oil plant *Ricinus communis* after the characterization of ricin, RIPs have become of great scientific interest due to their importance in human health, either as pathogens or as potential therapeutics. The term RIPs was introduced by Stirpe to define plant proteins that inactivate animal ribosomes, in a period when the details of their enzymatic activity and structure were still unknown (Stirpe F B. L., 1986). Widely distributed in nature, RIPs have been found predominantly in plants, bacteria and fungi, often in multiple isoforms (Stirpe, 2013). Most of them are produced by plants, where their physiological role is still controversial. It has been hypothesized that plants accumulate RIPs in some of their tissues as a defensive mechanism against biotic and abiotic stresses (Nielsen KL, 2001) (Polito L B. M., 2013)). Well-known examples of plant-derived RIPs include ricin, abrin, ebulin, nigrin, saporin, trichosanthin and volkensin.

Classification of RIPs

Plant RIPs are currently classified into three groups based on their physical properties and the presence or absence of a lectin-like chain (**figure 6**).

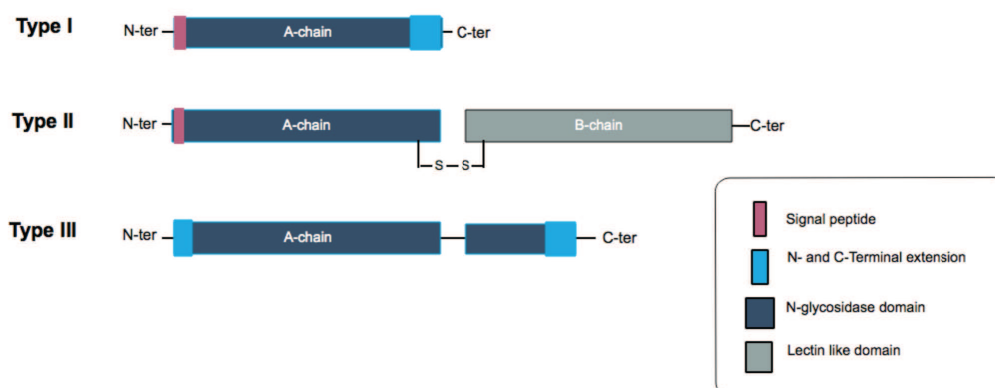


Figure 6: Schematic representation showing a comparison between primary structure of the three RIP groups.

Type 1 RIPs

The majority of RIPs discovered so far actually belong to type 1 and are preferentially distributed within particular plant families such as *Caryophyllaceae*, *Cucurbitaceae* and *Euphorbiaceae* (Stirpe, 2004). Notably examples of type 1 RIPs are pokeweed antiviral protein (PAP, (from *Phytolacca Americana*)), saporin (from *Saponaria officinalis* L.), dianthin (from *Dianthus caryophyllus*), momordin (from *Momordica charantia*) and gelonin (from *Gelonium multiflorum*). Type I RIPs are monomeric proteins with a relative molecular mass of between 27 - 32 kDa. Most of them are synthesized as pre-proteins composed of a signal peptide, the mature protein and a C-terminal extension, as demonstrated by different studies on protein and DNA sequences (Nielsen KL, 2001). It is likely that the synthesis of type 1 RIPs follows the secretory pathway, so that these cytotoxic proteins are segregated into the vacuoles or other extra-cytoplasmic compartment, but detailed localization studies are available only for a few type 1 RIPs (Van Damme EJ, 2002).

Type I RIPs are much less toxic to intact cells and whole animals than type II RIPs, by a factor of 10^2 - 10^4 (Olsnes S, 1981). This is due to the absence of a lectin subunit and consequently they are less capable of binding to cells and entering them. However, in cell-free systems, type I and type II RIPs are similarly effective in inhibiting protein synthesis (Foà-Tomasi L, 1982).

Type 2 RIPs

Type 2 RIPs can be composed of two or four polypeptide chains, with an approximate molecular weight of 60 kDa or 120 kDa, respectively. At least one chain possess enzymatic activity and is therefore called A-chain (A, active). The A-chain is linked by disulphide bonds and other non-covalent bonds to a galactose-specific lectin B-chain. The majority of type 2 RIPs known are heterodimers composed of one A-chain linked to a B-chain, like abrin, modeccin, ricin, volkensin and stenodactylin.

Most of the knowledge about type 2 RIPs biosynthesis has been obtained by studies on ricin in castor bean seeds. Ricin, and probably most of type 2 RIPs, is synthesized as a preprotein formed by a signal peptide, the mature A-chain and the mature B-chain linked by a 12-residue linker peptide. The pre-proRIP is co-translationally translocated into the endoplasmic

reticulum (ER) lumen, where the signal peptide is cleaved and four exposed asparagine residues are *N*-glycosylated. The formation of disulfide bonds between A-chain and B-chain, and also within the B-chain, occurs in the ER. The pro-RIP is subsequently transported *via* the ER and the Golgi complex into protein storage vacuoles, where the excision of the internal 12-residue linker yields mature protein. This maturation step implies that the protein becomes active only after its arrival in the storage compartment, probably a mechanism to prevent unwanted activation of the toxin in the cytoplasm (Lord J.M., 1994.) Several non-toxic type 2 RIPs have been described. Despite strong anti-ribosomal molecular activity *in vitro*, non-toxic type 2 RIPs lack the high toxicity in cultured animals cells and *in vivo* rodents, maybe because of individual changes in the high-affinity sugar binding sites of the B-chains, which alter their intracellular trafficking (Ferrerias JM C. L., 2011).

Type 3 RIPs

Type 3 RIPs are a group that comprises two proteins characterized only from maize and barley (b-32 and JIP60, respectively). Type 3 RIPs are synthesized as single- chain inactive precursors (proRIPs) that require proteolytic processing events to produce two noncovalently linked chains equivalent to a type 1 RIP. The absence of a signal peptide in the gene encoding type 3 RIPs indicates that these proteins are synthesized on free-polysomes in the cytoplasm. The function of the extra domains in the type 3 RIPs is not known (Van Damme EJ, 2002).

Biological activities of RIPs

RIPs are officially classified as rRNA *N*-glycosylases (EC 3.2.2.22). They recognize a specific and highly conserved region in the large subunit of rRNA and cleave a specific *N*-glycosidic bond between an adenine and the nucleotide on the rRNA (**figure 7**). The first description of the mechanism underlying RIP-induced ribosomal damage was described by Endo and co-workers. Using ricin and rat liver ribosomes as substrate, they showed that the adenine cleavage was highly selective and that the specific adenine removed (A4324) lies in a highly conserved sequence, GAGA, that is present in a universally conserved loop (termed

sarcin/ricin loop, SRL) located at the top of a stem region in the 28S rRNA (Endo, 1987). The SRL is important for binding and GTPase activation of the translational GTPases, which include the elongation factor 1 (EF1) and the elongation factor 2 (EF2), by the ribosome. The irreversible removal of this adenine from GAGA sequence prevents the binding of EF2 to ribosomes and affects both the EF1- and EF2-dependent GTPase activities with subsequent arrest of protein synthesis at the translocation step, thus inhibiting irreversibly cellular protein synthesis.

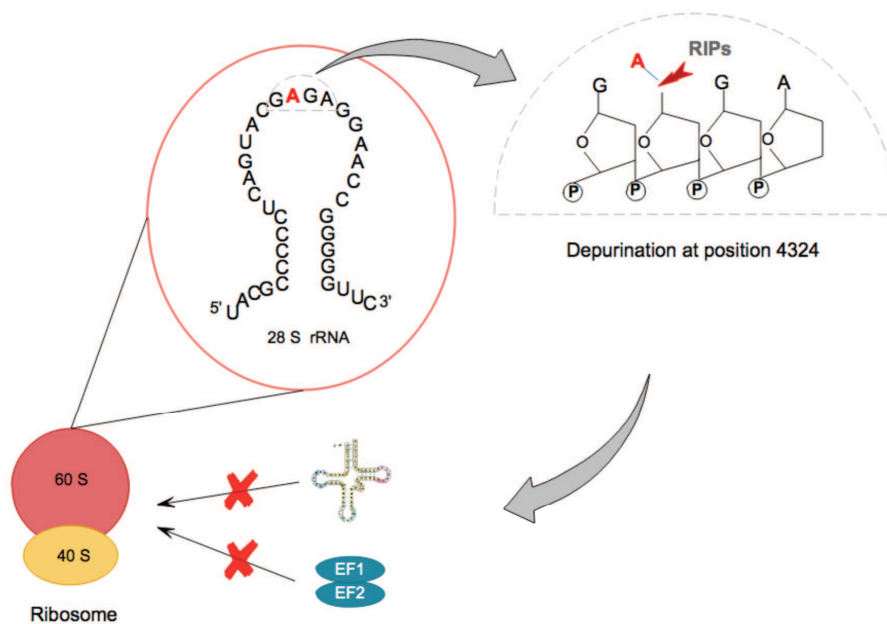


Figure 7 : Schematic representation of the biochemical action of RIPs such as ricin and saporin

The enzymatic activity is directed at removing an adenine on the large ribosomal subunit, which results in failure of binding of elongation factor-2 and cessation of protein synthesis by altered ribosome

RIPs also show in vitro N-glycosylase activity on different substrates, such as mRNA, tRNA, DNA and poly(A) (Barbieri L V. P., 1997) (Bolognesi A P. L., 2002), and some RIPs have also shown activity against poly(ADP-ribosyl)ated proteins (Barbieri L B. M., 2003). For this reason, the enzymatic activity of RIPs has been defined as polynucleotide:adenosine glycosylase (PNAG) (Bolognesi A P. L., 2002).

Even if all RIPs share a common activity on 28S rRNA, it is becoming clear that they do not share a single common pathway for the induction of apoptosis, instead, it is likely that RIPs are able to induce multiple cell death pathways in different cell types. In addition to the

inhibition of translation, alternative mechanisms were proposed to explain how RIPs induce apoptosis, such as (a) the ribotoxic stress response; (b) ER-stress and the activation of unfolded protein response (UPR) genes; (c) interactions with anti-oxidant proteins and the production of reactive oxygen species. All these mechanisms could cooperate in RIP-induced apoptosis at different levels and in different ways depending on cell type.

RIPs employment in experimental and clinical medicine

Immunotoxins and chimeric toxins

Generality

Since it is not possible to define a therapeutic window for these toxins, directing these agents to tumors with appropriate ligands is necessary. Most of the interest in RIPs in the biomedical field has been the possibility of directing their high cytotoxicity in a selective manner to deplete populations of undesired cells. This was achieved by linking them to molecules, in particular monoclonal antibodies (mAbs), but also lectins, hormones, growth factors, to form “immunotoxins” (ITs) or other cell-binding conjugates capable of selective killing of unwanted cells. To date, RIP-based ITs have been employed to treat cancer and also autoimmune disorders (Madhumathi J, 2012).

The term immunotoxin is generally referred to a toxin targeted by an antibody, while toxins linked to other carriers are commonly referred to as “chimeric toxins” or “conjugates”. After the IT targeting moiety binds to the target cell surface, the payload is internalized to the endocytic compartment. Processing and trafficking of these molecules is target- and toxin-specific, but converge in the delivery of the toxic cargo to appropriate cellular compartment.

The first saporin-containing ITs were constructed by Thorpe and colleagues in 1985, and they coupled the toxin to a monoclonal anti-Thy 1.1 antibody and its F(ab')₂ fragment (Thorpe PE B. A., 1985). At that time, the anti-tumour effects of saporin-containing ITs were being exploited by other research groups (Stirpe F D. M., 1987), (Glennie MJ, 1987).

Saporin

Structure

Many type 1 RIPs have been studied. However, the best characterized and most widely utilized type 1 RIPs are the saporins. *Saponaria* RIPs, were first purified and partially characterised by Stirpe *et al* (1983) from the seeds of *Saponaria officinalis* (soapwort) and, to a lesser extent, from the leaves. Saporin-S6 belongs to a multigene family of proteins that includes more than nine different isoforms isolated from various plant tissues, such as leaf, root, and seed (Ferrerias JM B. L., 1993). All isoforms differ from each other in both their chemico-physical and biological properties, but they all have a molecular weight of approximately 30 kDa. Saporin-6 (designated according to the original protein fraction), was present in surprisingly high amounts in seeds; 7% of the total seed protein.

The mature form of saporin-S6 is 253 amino acids long. The sequence was determined in 1990 and almost 10% of the amino acids in saporin-S6 are lysine residues, a condition that confers to the protein an extremely high pI (approximately 10) (Maras B, 1990). No neutral sugars are present in the saporin-S6 molecule, despite the presence of glycosylation sites in the pro-saporin C-terminal extension sequence that is cleaved to form the mature protein. The 2.0 Å resolution crystal structure of saporin-S6 showed that this protein contains two main domains: a predominantly β -stranded N-terminal domain and an α -helix-rich C-terminal domain (**figure 8**). The N-terminal domain shows a high similarity to that of other RIPs. The C-terminal region includes a two stranded antiparallel β -sheet element connected by a short loop between the β 7 and β 8 strands; this structural motif is shorter than many other RIPs and may contribute to an increased accessibility to the substrate (Savino C F. L., 1998) (Savino C F. L., 2000).

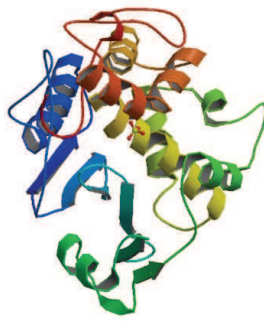


Figure 8: **The 2.0 Å resolution crystal structure of saporin-S6** (Savino C F. L., 2000)

Saporin-S6 is extremely resistant to high temperature, to denaturation by urea or guanidine and to attack by proteolytic enzymes (Santanché S, 1997) and it is also very stable in response to chemical modifications such as those necessary for derivatization and conjugation procedures (Bolognesi A T. P., 1992).

Cellular interaction

Endocytosis

As we show before, the cellular interaction of type 1 RIPs has been examined in many studies with inconclusive results. Particularly controversial is the debate regarding the mechanism of saporin-S6 endocytosis. It was initially suggested that saporin-S6, like all type 1 RIPs, enters cells through passive mechanisms such as fluid phase pinocytosis. Saporin-S6 uptake by cells was described to occur by a mechanism that does not depend on specific binding sites (Battelli MG M. V., 1992). However, the observations that some cell types show a moderate resistance to saporin-S6 cytotoxicity and that some organs are more sensitive to saporin-S6 intoxication led some researchers to search for a possible receptor. Receptor-mediated endocytosis through the α_2 -macroglobulin receptor, also called low-density lipoprotein receptor-related protein (LRP), was proposed as the binding mechanism for saporin-S6 (Cavallaro U, 1995). A discrepancy was reported between the level of LRP and saporin-S6 cytotoxicity; i.e., LRP-positive or -negative cell lines showed similar sensitivities towards saporin-S6, suggesting a receptor-independent endocytosis mechanism (Bagga S H.

M., 2003,). Recently, electron microscopy experiments were performed, indicating that saporin-S6 endocytosis by HeLa cells mainly occurs through non-coated vesicles (Bolognesi A P. L., 2012).

Intracellular trafficking

After initial internalization, saporin-S6 should reach its cytosolic targets to exert its cytotoxic activity. Most information on the intracellular trafficking of RIP was obtained via studies of the endocytic pathway of ricin. Once it enters the cell, ricin travels backward from the Golgi complex to the endoplasmic reticulum (ER), where the separated A-chain exploits the ER-associated degradation pathway to enter the cytosol (Wesche J, 1999). A comparison between the endocytosis of ricin and saporin-S6 by Vero or HeLa cells was performed using immunofluorescence and treatment with brefeldin A or chloroquine, indicating that the type 1 RIP follows a Golgi-independent pathway to the cytosol and does not require a low pH for membrane translocation (Vago R, 2005). Chloroquine and monensin have been used to raise the pH of the endosomal compartment to evaluate the intracellular routing of saporin-S6 in a CD30⁺ cell line (Battelli MG B. A., 1998;) and in a prostatic cancer cell line (Ippoliti R G. P., 1998). Because these substances had no effect on the cytotoxicity induced by saporin-S6, unlike that induced by ricin, the intracellular transport of saporin-S6 to the cytosol should not involve lysosomes or the Golgi cisternae. In addition, the translocation mechanism should be low-pH independent (Battelli MG B. A., 1998;) (Ippoliti R G. P., 1998). There is evidence of saporin-S6 localization within the ER, Golgi apparatus and nucleus in HeLa cells, indicating that saporin-S6 can reach various intracellular compartments, possibly by more than one pathway. Saporin-S6 was found localized intracellularly within 20 minutes of exposure. Double immunofluorescence analysis performed by confocal microscopy showed that approximately 30% of saporin-S6 colocalized with the ER marker BiP, and approximately 7% co-localized with the Golgi apparatus, which was marked with GM130 (Bolognesi A P. L., 2012). Using transmission electron microscopy, after 20 min of incubation, gold-saporin-S6 molecules were seen mainly localized on the plasma membrane and in clear vesicles and vacuoles. Single gold particles were observed in sub-plasmalemmal clear vacuoles and free in the cytoplasm. After 40 min, saporin-S6 molecules were clustered in late endosomes and lysosomes in proximity to the nucleus and close to the rER cistern. After 60 min of incubation, the nuclear localization of saporin-S6 was observed in approximately 10% of

HeLa cells, with a gold labeling intensity ranging from moderate (8% of cells containing <10 gold particles/nucleus) to intense (2% of HeLa cells showing >10 gold particles/nucleus). This study was the first report of saporin-S6 detection in cell nuclei. It must be underlined that these results were obtained with HeLa cells and they cannot automatically be extended to any other cell type.

Mechanisms of intoxication and cell death

Initially, the toxic effects of saporin-S6 were attributed to the inhibition of protein synthesis, but the observation that RIPs also depurinate DNA and other nucleic acids opened new perspectives about the mechanism of cytotoxicity. The first descriptions that saporin-S6 is able to kill cells via apoptosis were reported in 1996 (Bolognesi A T. P., 1996,). In 2008, it was reported that the onset of apoptosis is independent of protein synthesis inhibition (Sikriwal D, 2008). The capability of saporin-S6 to induce apoptosis results not just from its ability to block protein synthesis, as different mutants lacking RIP activity can induce DNA fragmentation and apoptosis (Bagga S S. D., 2003).

Different hypothesis have been done to try to clarify : Cell death by saporin-S6 may be induced by various cell injuries depending of cells type. For example, in the U87 glioblastoma cell line, the activation of ERK1/2 has been observed to forego caspase 8 or 9 activation after saporin-S6 treatment. This ERK1/2 activation might induce a cell cycle arrest in G1 phase with a decrease in D1 cyclin levels. Furthermore, the inhibition of protein synthesis could activate p53 (Cimini A M. S., 2012). It is likely that the cell damage induced by saporin-S6 represents a cell type-dependent multi-direction pathway in which apoptosis seems to be the main detectable effect in several different cell lines.

« The perfect moiety »

Because its ability to induce cell death by more than one pathway and thus provide more difficulty to tumor cells to acquire a resistant phenotype to saporin-induced cell death. And because saporin-S6 is extremely resistant to high temperature, to denaturation by urea or guanidine and to attack by proteolytic enzymes. Saporin-S6 is also very stable in response to

chemical modifications such as those necessary for derivatization and conjugation procedure.

Altogether, these characteristics make it an ideal candidate as the toxin component in immunotoxins and chimeric toxins for therapeutic use

The project summary

Monoclonal antibodies, aptamers and peptide can have direct toxicity but to increase or add toxicity on this targeting agent, one toxic molecule (plant and bacterial biological toxins, drugs...) could be linked to them. The toxin should not affect cells that lack the target for the targeting agent —i.e., the vast majority of cells in the body. We focussed our attention to the study of delivery systems directed towards cancer membrane markers, particularly nucleolin and CD98, using as a payload moiety the plant toxin saporin or its gene.

We described the use of PDZ protein domain of hCASK (serine kinase calcium/calmodulin-dependent of MAGUK family) to deliver saporin exploiting the ability of hCASK to bind to the C-terminus of hCD98 in the extracellular space (Giansanti F., 2015). hCASK-PDZ was genetically fused to the toxin saporin and this chimeric toxin proved to be active on glioblastoma cells *in vitro*.

We also characterized the presence of nucleolin on the surface of glioblastoma cells (Benedetti, 2015) and exploited selectivity of Aptamer AS1411 and pseudopeptide N6L towards this marker to deliver saporin gene or protein. Both molecules are already in clinical trial for their own antitumoral activity after binding to overexpressed NCL in cancer cells, thus suggesting that increasing their efficiency may be useful to their therapeutic action.

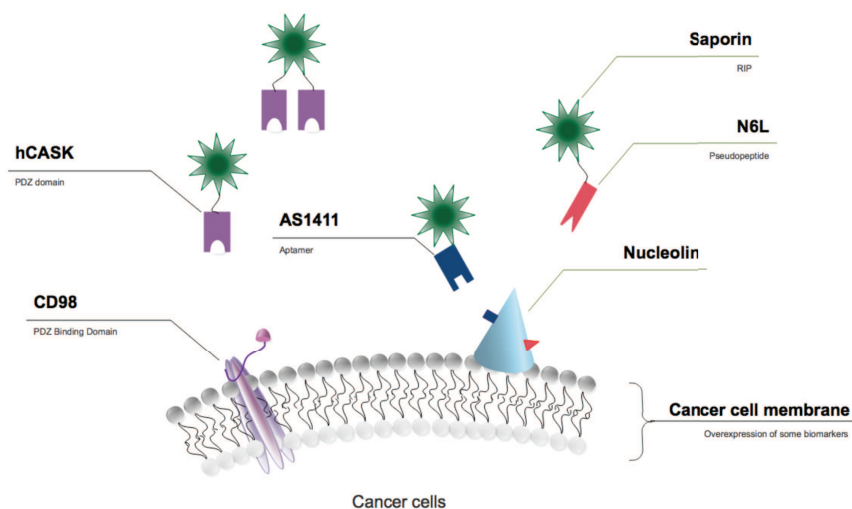


Figure 9 : Schematic representation of glioblastoma cell membrane with overexpression of different biomarkers and different approaches to target them and permit internalization of one RIP (saporin).

Materials and Methods

Triton X-100, dimethylsulfoxide (DMSO), sodium dodecylsulfate (SDS), Tween 20, bovine serum albumin (BSA), Nonidet P40, sodium deoxycolate, ethylene diamine tetraacetate (EDTA), phenylmethanesulphonylfluoride (PMSF), NaCl, polyvinylidene difluoride (PVDF) sheets, glycerol, acetone, fluorescein-labeled anti-rabbit and anti-mouse IgG antibodies, fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Medium Eagle (MEM), Iscove's Modified Dulbecco's Medium (IMDM), Hank's Balanced Salt Solution (HBSS), Penicillin/Streptomycin, trypsin, L-glutamine, PBS, Trypan Blue, Poli-L-Lysine were all purchased from Sigma Chemical CO (St. Louis, CO, USA). Micro-BCA kit was purchased Pierce Biotechnology (Rockford, IL, USA). 5'-Bromo-2'-deoxyuridine labeling and detection kit and Cell Death detection ELISA kit was purchased from Roche, Penzberg, Germany, while Cell Titer One Solution Cell Proliferation Assay was required to Promega, Madison, USA. N6L (2mM 5% D-Mannitol), where synthesized and provided by José Courty. Primary antibodies anti Pro-Caspase 3, 8, 9, antibodies anti β -actin, anti LC3, anti P62 were purchased from Sigma Aldrich, (St.Louis, USA). Horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG secondary antibodies were from S.Cruz Biotechnology (Santa Cruz, CA, USA). Anti-gp273 antibody was obtained from Department of Evolutionary Biology, University of Siena, Siena, Italy. Acrylamide, molecular weights standard, blocking solution, Enhanced chemiluminescence (ECL) Bio-Rad Laboratories, (Hercules, CA, USA).

Cell cultures

U87-MG (human glioblastoma), MDA-MB-231 (human breast cancer), Caco-2 (human colorectal cancer), HeLa (human cervical cancer) and 3T3 (Mouse fibroblast) cell lines were purchased from ATCC (American Type Culture Collection, US) and GL15 (kindly provided by Prof.ssa Cristina Limatola, Department of Physiology and Pharmacology, Faculty of Pharmacy and Medicine, University of Rome "La Sapienza") were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated FBS, 0.1 mg/ml penicillin/streptomycin and 2mM Lglutamine.

U87-LUC cells were kindly provided by Dr.David Gillepsie (huntsman Cancer Institute Salt Lake City, UTAH 84010 and were cultured in DMEM supplemented with 10% FBS, 0.1 mg/ml penicillin/streptomycin.

Primary cells of Glioblastoma (C10414) were obtained as previously described (Galzio R, 2012)

OCI-AML2 (human, peripheral blood, leukemia, acute myeloid), OCI-AML3 (acute myeloid leukemia cells) carry an NPM1 gene mutation (type A) cells were kindly provided by Dr. Federici Luca (University of Chieti) And were grown in Minimum Essential Medium Eagle (MEM) Alpha Modifications supplemented with 20% heat inactivated FBS, 0.1 mg/ml penicillin/streptomycin and 2mM Lglutamine

HL60 (Human promyelocytic leukemia cells) cells line cells were kindly provided by Dr. Federici Luca (University of Chieti) and were grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% heat inactivated FBS, 0.1 mg/ml penicillin/streptomycin. The cell cultures were incubated at 37°C in humidified 95% air 5% CO₂ atmosphere.

NRM6 nucleolin-negative cells (mouse fibroblasts transfected with the gene for EGFRvIII) were cultured in DMEM supplemented with 10% FBS, 0.1 mg/ml penicillin/streptomycin.

Neurospheres

Cancer neural stem cells were isolated from biopsy of patients with diagnosis of Grade IV glioma (glioblastoma multiforme), generous gift from Prof. Galzio of the Department of Neurosurgery, S. Salvatore Hospital, L'Aquila, Italy. All samples were classified according to the World Health Organization guidelines (WHO). Glioblastoma cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 0.1 mg/ml penicillin, and 0.1 mg/ml streptomycin and incubated at 37°C in humidified 95% air 5% CO₂ atmosphere. Thereafter, U87 the culture medium was replaced with serum-free neural stem cell medium, Dulbecco's Modified Eagle's Medium-F12 (DMEM-F12; Sigma), containing 20ng/ml of both recombinant human epidermal growth factor (EGF) and fibroblast growth factor-basic (b-FGF; PeproTech, Hamburg, Germany) until several tumorsphere were visible under microscopy. After several passages primary tumorspheres were dissociated and single cells were seeded at 1.10⁴/well. The secondary spheres derived from single cells of primary tumorspheres were analyzed under phase contrast microscopy Leica DMIL and fluorescence microscopy Zeiss Axioplan 2, with Leica DFC 350 FX camera.

Competition assay

In order to assess the specificity of recognition of hCASK chimeras a competition experiment on U87-MG cells was performed. Cell viability assay on U87-MG cells treated with h(CASK)₂-

SAP or hCASK-SAP at their respective IC_{50} concentrations in the presence of anti CD98 polyclonal antibody (Santa Cruz, CA).

To test the specificity of APT-SAP and APT towards nucleolin expressed on glioblastoma cells, we performed a competition assay in which cells were exposed to the DNA constructs in the presence of an excess anti-nucleolin polyclonal antibody (gp273).

To test the specificity of N6L or SAP-N6L towards nucleolin expressed on glioblastoma cells, we performed a competition assay in which cells were exposed to N6L alone or the conjugate in the presence of an excess anti-nucleolin monoclonal antibody (MS3 from Santa Cruz).

Western blot analysis

For Western blotting, cell lysates in ice-cold RIPA buffer (phosphate buffer saline pH 7.4 containing 0,5% sodium deoxycolate, 1% Nonidet P-40, 0,1% SDS, 5 mM EDTA, 100 mM sodium fluoride, 2 mM sodium pyrophosphate, 1 mM PMSF, 2 mM ortovanadate, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 10 μ g/ml pepstatin) were centrifuged and the supernatants were assayed for protein content. Lysates from control and treated sub-confluent cells (40 μ g total proteins per sample) were run on 12-15% polyacrylamide SDS denaturing gels. Protein bands were transferred into PVDF sheets by wet electrophoretic transfer. Non-specific binding sites were blocked with blocking solution: 5% (w/v) non-fat dry milk in Tris-buffer saline (TBS). Membranes were incubated with the primary antibody at the dilution reported in the manufacturer's datasheets overnight (in incubating solution: 1% (w/v) non-fat dry milk in TBS), followed by washes with Tris-buffer saline containing 0.05% (v/v) Tween-20 (TBS-T). Then membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG secondary (1:10,000, in incubating solution) for 1 h. After extensive washings with TBS-T immunoreactive bands were visualized by enhanced chemiluminescence (ECL), according to the manufacturer's instructions. Then, blot analyses were performed using Image J.

Apoptosis measurements

At expiration of the incubation the determination of cytoplasmic histone-associated DNA fragments was performed by using the Cell Death Detection ELISA Kit (Roche®), following the

manufacturer's instructions. Briefly, the assay is based on the quantitative sandwich-enzyme-immunoassay-principle using mouse monoclonal antibodies directed against DNA and histones, respectively. The absorbance peak at 405 nm was measured in a spectrophotometric microplate reader. The results are expressed as percentage of absorbance, resulting from the activity of peroxidase conjugated anti-DNA antibody complexed with mono- and oligo-nucleosomes released into the cytoplasm of treated cells, compared with the control untreated cells. Statistical analysis of the data was obtained by two-tailed P value calculation with the paired t-test.

Purification of hCASK-SAP and (hCASK)₂-SAP chimeras

The pellet of 1 L of culture of induced bacteria was resuspended in a 20mM phosphate buffer pH 6.5 supplemented with proteases inhibitors (1 mg/ml pepstatin, 1 mg/ml leupeptin, 10 mg/ml aprotinin, and 15 mg/ml PMSF) and 1 mg/ml DNase. The suspension was sonicated and then centrifuged at 7,000g for 30min at 4°C, to eliminate cells debris. The supernatant was acidified at pH 5 with diluted acetic acid, to precipitate contaminant proteins with acidic isoelectric point. This solution was centrifuged at 7,500g for 60 min at 4°C. This second supernatant was recovered, neutralized at pH 6.5 and dialyzed for 48–72 h, against 20 mM phosphate buffer pH 6.5 and filtered with 0.22 μm filters before chromatographic separation using an AKTA prime plus GE Healthcare FPLC-apparatus. For His-tagged proteins an HisTrap HP column, prepacked with Ni Sepharose High Performance (GE Healthcare, USA), was used. The column was equilibrated with 10 column volumes (CV) of binding buffer (20 mM phosphate buffer, 0,5 M NaCl, 50 mM imidazole, pH 7,4). After the sample was loaded, the column was washed with binding buffer. The elution was done with a continuous linear gradient from 0% to 70% elution buffer (20 mM phosphate Buffer, 0,5 M NaCl, 1 M imidazole, pH 7,4). The column equilibration and the elution were obtained using a constant flux of 1 ml/min. The eluted protein fractions were collected and successively analyzed by SDS-PAGE and Western blotting using anti His-Tag (Ab Cam, UK) or anti Saporin antibodies (Sigma Aldrich, USA). Once verified the presence and the purity of the protein, fractions containing it have been collected and dialyzed against PBS for the protein hCASK or DMEM medium for the chimera hCASK-SAP or (hCASK)₂-SAP at 4°C for 12–24h with changes of buffer every 6–8 h. The concentration of proteins were estimated by UV spectroscopy

based on the theoretical extinction coefficient at 280 nm (<http://web.expasy.org/protparam/>). The chromatographic purification of Saporin from seeds was performed by ion-exchange chromatography on Resource S (Amersham Pharmacia) column in 20 mM phosphate buffer pH 6.5 and eluting with a linear NaCl salt gradient [Savino et al., 1998].

Biochemical characterization of hCASK-SAP and (hCASK)₂-SAP

Affinity measurements

The affinity of binding to target peptide sequences was assayed for both the fusion proteins by titration of the ligand binding site with N-terminal dansylated peptides. As a control we used the recombinant free PDZ domain of hCASK. A dansyl- peptide corresponding to the human sequence (RFPYAA) and one corresponding to the rat C-terminal sequence (QFPFVA) of CD98 were purchased from ProteoGenix SaS (Shiltigheim, France). The experiment was performed recording the fluorescence spectra of dansylated peptides (Exc. | 340nm, emission between 450 and 650nm) mixing increasing amounts of free peptide with fixed protein (final concentrations 40 mM for the peptide, 1 mM for the protein). The binding of the peptide to the PDZ produced a quenching of the fluorescence signal revealed as a hyperbolic dependence of the maximum fluorescence (540 nm), while the free peptide or the unrecognized peptide gave a linear dependence. Data were fitted according to a single site ligand binding or when not possible to a linear regression equation using the software Grafit 5.0.1 (Erithacus Software Limited).

RIP activity on isolated ribosomes

Both hCASK-SAP and (hCASK)₂-SAP were assayed for their ability to release adenine from isolated ribosomes according to the following protocol. Ribosomes from yeast (*S. cerevisiae*) were prepared according to standard procedures (Battaner and Vasquez, 1971). Enzymatic reaction was obtained mixing proteins and ribosomes in a total volume of 200 µl at 37°C for 1 h. Negative control was performed mixing ribosomes with only PBS. Control with standards of adenine was performed mixing ribosomes with progressive amounts of adenine (Sigma) in PBS. Reaction was stopped adding 1 volume of cold ethanol and putting the samples 15 min at -80°C (Zamboni et al., 1989). After 20min of centrifugation, the supernatant was

recovered and dried with a vacuum centrifuge. Pellet was suspended in MilliQ water (Millipore), loaded on 100,000Da cut-off Microcon tubes (Millipore) and flow through was recovered. Analysis were based on Yang method (Yang et al., 2007) using Waters Acquity UPLC I-Class system with PDA detector (Waters, MA), connected to a Waters Empower 3 software and equipped with Waters Acquity UPLC BEH C18 column (50 2.1mm i.d., particle size 1.7 mm). The column temperature was maintained at 25°C and the sample manager temperature was set at 10°C. Samples were separated using a gradient mobile phase consisting of 0.5mM acetic acid (A) and acetonitrile (B). The gradient condition was: 0–5 min, 0–5% B; 5–6 min, 5–100% B; washing column with 100% B for 1min and isocratic reconditioning the column with 100% A for 3 min. The flow rate was set at 0.25ml/min and the injection volume was 1ml. The peaks were detected at 254 nm. The area corresponding to the peak revealed from free standard adenine (min. 1.7–1.9) was integrated using the instruments software. A calibration curve was obtained running free adenine standards. hCASK-SAP and (hCASK)₂-SAP and saporin from seeds were tested. The amounts of adenine released by each toxin was extrapolated from the calibration curve. The data are reported as pmoles of adenine released/nmole of toxin present in the incubation mixture.

Aptamer-saporin, DNA constructs

APT-SAP vector DNA and the single strands corresponding to AS1411 (Apt) were purchased from Eurofins MWG Operon. The nucleotide sequence of AS1411 (5'-d(GGTGGTGGTGGTTGTGGTGGTGGTGG)-3') was placed at the 5' terminus of a synthetic construct containing the CMV promoter, saporin gene, a chimeric intron derived from pCI vector, and a poly-adenylation signal at the 3', subcloned into pCMV6 mammalian expression vector using SmaI restriction sites. APT-SAP was amplified in *E. coli* DH5 α cells, extracted using QIAGEN midi-prep plasmid extraction kit, and quantified by ethidium bromide staining on agarose gel, using standard DNA as a reference. APT-SAP was used as full double stranded plasmid (APT-SAP). AS1441 (Apt) was used either as a single strand (APT_s) DNA or reconstituted with the complementary strand (APT_m). Before cell toxicity tests all constructs were denatured at 95 °C for 10 minutes and then slowly cooled down to room temperature in 10 mM Tris/HCl buffer pH 7.5 containing 100 mM NaCl in the presence of 5 mM KCl. The DNA thus obtained was immediately used.

Cell cycle analysis

Cell cycle analyses were performed by flow cytometry (Nicoletti et al., 1991; with minor modifications). Control and treated cells were detached at 96 h, washed twice with ice-cold PBS 1× and fixed in 70% ethanol at 4 °C for 30 min. Then, the fixed cells were again washed twice with ice-cold PBS, stained with 50µg/mL propidium iodide, in the presence of 25 µg/mL RNase A and incubated for 30 min in the dark. Cell cycle phase-distribution was analyzed by flow cytometry collecting data from 10,000 events per sample with a Becton Dickinson FACScan system equipped with Modfit LT for Mac V3.0 cell cycle and Cell Quest analysis software.

Purification of Saporin from *Saponaria officinalis* seeds and synthesis of Saporin-N6L conjugate

Saporin was purified from *Saponaria officinalis* seeds as previously described (Savino C F. L., 1998). The toxin was then derivatized by introducing a disulfide bridge by N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP ThermoFisher Scientific) as previously described (Ippoliti R L. E., 1995) and chemically conjugated to N6L-SH. N6L was synthesized, as previously described, using the solid phase peptide methodology (Destouches D P. N.-K., 2011). To achieve covalent functionalization with a disulfide bond, a cysteine- modified N6L pseudopeptide (N6L-SH) was synthesized to introduce a single free thiol function. Saporin from seeds in Borate Buffer pH9 reacted with SDPD (N-succinimidyl 3-(2-pyridyldithio)) ratio [SAP:SDPD] [1:2], 1h at room temperature protected from light. Equilibration of a desalting column with hPBS, and buffer exchange the SDPD-modified Saporin to remove reaction by products and excess nonreacted SDPD reagent was done. The cross-linking reaction was carried out by addition of N6L-SH to the solution [SAP:N6L-SH] [1:5] and incubation of the mixture 2h at room temperature (following the kinetic of reaction at 343nm). Purification of the N6L-Sap conjugate was achieved by gel filtration column (resin Sephadex G-75 (GE Healthcare)) to remove free Saporin and free N6L-SH in hPBS (flux= 1mL/min). Chemical characterization by gel SDS-PAGE in reducing and unreducing condition to confirm the fact that the SDPD reagent produce disulfide-containing linkages that can be cleaved with reducing agents.

We tested two others chemical cross-linkers: Sulfo-EMCS (N-ε-maleimidocaproyloxysulfosuccinimide ester) (ThermoFisher Scientific) and SM (PEG)₂ (succinimidyl-[(N-maleimidopropionamido)-diethyleneglycol] ester) (ThermoFisher Scientific). These cross-

linkers produce non cleavable bridge between saporine and N6L-SH. Reaction was doing according manufacturer's protocol and the purification by following the same protocol used for SPDP cross-linker.

Synthesis of FITC -SAP-N6L conjugate and TexasRED -Transferrin conjugate

Saporin from seeds in Borate Buffer pH9 reacted with FITC (28 mM in DMSO) with a ratio [SAP:FITC] [1:5], 1h at room temperature protected from light. Passage through a desalting column was necessary to separate Sap-FITC from excess non reacted FITC. Separation of Saporin and Sap-FITC was done by a Resource S columns in phosphate buffer 10 mM and 1M NaCl gradient. Sap-FITC and N6L-SH were linked as previously described.

Transferrin was dissolved in borate buffer (pH 9) and reacted with Texas red with a ratio [transferrin:Texas] [1:5] 1h at room temperature protected from light. To remove non reacted Texas red passage through a desalting column was done.

Immunofluorescence

Cells growth on poly-L-lysine coated coverslips for 24h. Cells were incubated with SAP-FITC, N6L- SAP-FITC or TexasRED-Transferrin one hour at 4°C and different times at 37°C (0, 30min and 1h). Then cells were washed twice with PBS, fixed for 10 min at RT in 4% paraformaldehyde in PBS and permeabilized in PBS containing 0.1% Triton X-100 for 10 min at RT. Nonspecific binding sites were blocked for 30 minutes with 3% BSA in PBS. Cells were then incubated with mouse anti-lamp1 (1:20) or (mouse) anti-TGN (1:200) or (mouse) anti-golgin (1:200) in PBS containing 3% BSA overnight at 4°C. After extensive washings with PBS the cells were incubated with AlexaFluor 488 and 546 (mouse) secondary antibodies (1:2000 in PBS containing 3% BSA) 1h at RT. After extensive washings with PBS, coverslips were finally mounted in Vectashield mounting medium containing DAPI and observed and photographed under confocal Laser scanning microscope (Sarastro 2000).

Intracranial implantation of U87-LUC glioma cells in mice

Orthotopic implant of glioblastoma cells was performed as previously described (Benedetti E, 2015) a human GBM U87MG subline, stably transfected with the firefly luciferase (U87-LUC cells) kindly provided by Dr. David Gillespie (Huntsman Cancer Institute Salt Lake City, UTAH 84010) and cultured in DMEM supplemented with 10% FBS, 2mM L-glutamine, 50

UI/ml Penicillin-streptomycin until confluence and, was used for establishing intracranial xenograft GBM tumors. U87-LUC cells were injected (1.5×10^5 in 3 μ L HBSS) into the right striatum (coordinates: 1mm lateral to the middle line and depth 3,5mm) through a burr hole in the skull using a 10- μ L Hamilton syringe to deliver tumor cells to a intraparenchymal depth of 4-week-old female BALB/c-nu/nu athymic mice (Charles River Laboratories France) anesthetized with a ketamine/xylazine cocktail solution. Tumor growth was monitored and measured *via* bioluminescence imaging *in vivo*. Animals were daily monitored for cachexia (evaluated by body weight waste), behavior, and survival, when the animals lost 20% body weight were euthanized. All experimental procedures were approved by the Institution Animal Care and Uses Committee at the University of L'Aquila. Mice were monitored until signs of morbidity and euthanized to determine the effects of the treatment on overall survival. Animals injected with U87-LUC cells were weekly anesthetized and ip injected with 100 mg/kg body weight of D-luciferin, 5 minutes before photon recording by the Aequoria 2D luminescence imaging system (Hamamatsu Photonics, Naka-ku, Japan). The bioluminescent signal was quantified by measuring the amount of highlighted pixel in the regions of interest shaped around each site of photon emission.

Drug treatment of mice

SAP-N6L and Saporin were prepared in sterile vehicle (PBS) at a concentration of 0,75 μ g/ml. The mice were treated once by week with 0,5 mg/kg body weight of SAP-N6L, Saporin and vehicle from day 2 post-cell inoculation for 4 weeks and the drug was administered by intraperitoneal injection (ip). The study consisted of 14 vehicle-treated control mice, 8 SAP-N6L and 7 Saporin-treated mice.

Expression and purification of cysteine introduced recombinant saporin

Site-directed mutagenesis

We used the sap-6 isomer, a 253 amino acid long protein, to introduce cysteine into its sequence. We designed sense and antisense oligos with the programm Snap gene view. We introduced the cysteine mutation at the position 14 or 158 of the sap-6 gene, utilizing polymerase chain reaction. Alanine at position 14 or 158 were replaced by cysteine.

Constructions	Vectors	Primers 5'-->3'
SapA14C	pET11	CTAGTAAATCCGACCTGCGGTCAATACTCATCTTT AAAAGATGAGTATTGACCGCAGGTCGGATTACTAG
SapA158C	pET11	GCAGTGAACAAGAAGTGCCGTGTGGTTAAAAAC GTTTTAACCACACGGCACTTCTTGTTCACTGC

DNA amplification was performed in DH5 α Competent Cells and DNA extraction was performed by miniprep kit (QIAprep Spin Miniprep) following manufacturer's protocol. Sequencing was performed by a commercial sequencing laboratory by using SI forward primer.

Transformation by Heat Shock, and Extraction of Plasmid DNA

We used BL21 (DE3) pLysS E. coli strains for the production. BL21 (DE3) pLysS E. coli strain yields high protein expression of the recombinant saporin. pET11d plasmid was transformed into BL21(DE3)pLysS Competent Cells (Novagen) following manufacturer's protocol. Respective transformations were plated into LB-agar plates containing 50 ng/mL ampicillin and Chloramphenicol 50 ng/mL and incubated O/N at 37°C incubator. Four colonies for SAP-WT and each mutant were picked and incubated O/N at 37°C with convenient antibiotics.

Extraction and purification of SapWT, SapA14C and SapA158C

When the OD₆₀₀ value is 0.6 to 0.8, protein expression was induced by adding 1 mM IPTG to 12 L culture. Incubation was continued at 25°C under rigorous shaking for 4 hours. Cells were harvested at 10,000 \times g for 30 minutes.

Recombinant SAP WT and different mutants SAP A14C and SAP A158C were purified from the pellet of a 12 L of culture of induced bacteria (BL21 SAP (pET11)) resuspended in 20 mM Phosphate buffer pH 6.5 supplemented with protease inhibitors (Pepstatin 1 mg/mL, Leupeptin 1 mg/mL, Aprotinin 10 mg/mL, PMSF 15 mg/mL) and DNase 1 mg/mL and by 2 mM DTT (to prevent dimerization via disulfide bond forming between the engineered cysteine residues). The suspension was sonicated and then centrifuged at 7,000 g for 30 min at +4 °C, to eliminate cells debris. The supernatant was acidified at pH 5 with diluted acetic acid, to precipitate contaminant proteins with an acidic isoelectric point. This solution was centrifuged at 7,500 g for 60 min at +4°C. This second supernatant was recovered, neutralized at pH 6.5 and dialyzed for 48–72 h, against 20 mM Phosphate buffer pH 6.5 and filtered through 0.22 mm filters before chromatographic separation.

Next saporin and different mutants were purified by ion exchange chromatography on Sepharose Fast Flow CM (Amersham Biosciences/GE Healthcare) and eluted with a linear NaCl gradient. The purified Saporin fractions were then analyzed by SDS-PAGE.

Cross-linking between N6L-SH and recombinant saporin

Saporin recombinant (SAPre) in phosphate buffer 0,1M (g/mol), pH 6.5 was reacted with 80 fold molar excess of DTNB (Ellman's Reagent; (5,5-dithio-bis-(2-nitrobenzoic acid)) for 1h at room temperature to avoid dimerization of SAPre. The SAPre/DTNB mix was injected to gel-filtration chromatography and eluted with acetate buffer 20mM, pH 4.5. N6L-SH was reacted with TCEP (Tris(2-carboxyethyl)phosphine hydrochloride) [N6L:TCEP] [1:100] to avoid oligomerization in acetate buffer 20mM, pH 4.5.

N6L-SH was added to SAPre-TNB in a molar ratio [SAP:N6L] [1:100] and the reaction performed at 37°C overnight under vacuum condition (addition of liquid nitrogen).

Reaction was stopped with addition 500 molar excess of iodoacetamide.

Statistical analysis :

Statistical significance was determined by the ANOVA unpaired t test, using GraphPad Prism 7.0 software (GraphPad, San Diego, CA). Comparisons of the mean values among groups were performed by means of ANOVA and unpaired Student t-test. Each experiment included at least triplicate measurements for each condition tested. All results are expressed as mean \pm SE from at least three independent experiments. Values of p less than 0.05 were accepted as significant (*p < 0.05, **p < 0.01, ***p < 0.001).

Results

Part I

CD98 as a tool for Targeting cancer Cells : Using a PDZ domain in the engineering and production of a saporin chimeric toxin

(Giansanti F. &al. , 2015)

Background

In this study, we have studied a PDZ protein domain as a possible tool for cellular targeting of the ribosome inactivating protein Saporin, exploiting the ability of PDZ domains to recognize and bind short peptide sequences located at the C-terminus of a cognate protein. We have focused our attention on the PDZ domain from hCASK (Human calcium/calmodulin-dependent serine protein kinase) that binds extracellular CD98 in epithelial cells, being this antigen recognized as a marker for several human tumors and particularly considered a negative prognostic marker for human glioblastoma.

We designed and produced recombinant chimeric toxins made up of the PDZ-hCASK sequence linked to Saporin SO6 as monovalent (hCASK-SAP) or bivalent toxin (containing two PDZ domains) (hCASK)₂-SAP and assayed them for their cytotoxicity on human glioblastoma cells lines (GL15 and U87-MG). hCASK-SAP and (hCASK)₂-SAP were toxic towards glioblastoma cells inducing cell death *via* apoptosis and their action resulted in higher toxicity when co-administered with Saponin, a known enhancer of Saporin toxicity in chimeric constructs (Bachran D, 2011) (Weng A T. M.-B.-O., 2012). The results obtained in this work represent the proof of concept, for the first time, that a PDZ-mediated targeting system can be used to deliver toxic molecules to cancer cells, potentially representing an alternative to the use of antibodies or growth factors in the constructions of new anticancer molecules.

Results

Expression, purification and biochemical characterization of chimeric toxins

A 3D model of chimeric hCASK-SAP and (hCASK)₂-SAP chimeric toxins is reported in **figure 10**. A single PDZ domain from hCASK was linked through a (G₄S)₃ peptide at the C-terminus of Saporin to obtain hCASK-SAP, while two consecutive PDZ domains (each separated by a (G₄S)₃ peptide) were linked to Saporin in the (hCASK)₂-SAP chimera.

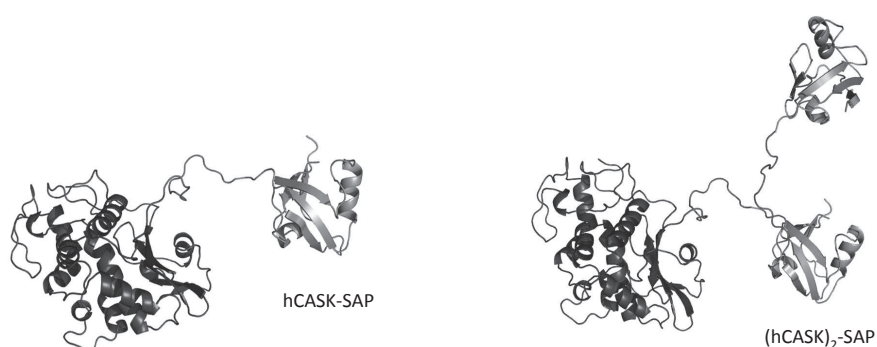


Figure 10 : **Cartoon representation of the 3D models of hCASK-SAP (at the top) and (hCASK)₂-SAP (at the bottom) chimeric toxins is shown**

The models were built with the program COOT [Emsley and Cowtan, 2004] starting from the available crystal structures of Saporin (PDB: 1QI7, in dark grey) and hCASK PDZ (PDB: 1KWA, in light grey). The crystal structures were linked by one and two (G₄S)₃ peptides in the case of hCASK-SAP and (hCASK)₂-SAP, respectively, and the resulting models have been subjected to geometry idealization. The figure was prepared using PyMol [The PyMOL Molecular Graphics System, Version 0.99rc6 Schrödinger, LLC].

The expression of hCASK single domain (9 kDa) from pET28, was performed in a E.coli BL21 (DE3) strain as a control of the production of recombinant proteins carrying this particular domain. Moreover the expression of hCASK-SAP (39 kDa) and (hCASK)₂-SAP (48 kDa) from pET11a in E.coli Rosetta GamiTM B pLysS(DE3) or E. coli BL21 (DE3) respectively, was obtained. After setting the culture/induction conditions (induction at 30°C for 4h after administration of 1mM IPTG) the screening of recombinants clones was performed and the best ones selected for scale-up protein expression.

The biological activity of both hCASK-SAP and (hCASK)₂-SAP was tested as the ability to bind the target sequence of C-terminal CD98, using dansylated synthetic peptides.

figures 11 and 12 report the fluorescence spectra of the dansyl-peptides after mixing with PDZ-containing hCASK, hCASK-SAP and (hCASK)₂-SAP. The interaction caused a decrease in fluorescence that is fitted by a single site binding equation only in the case of the human peptide.

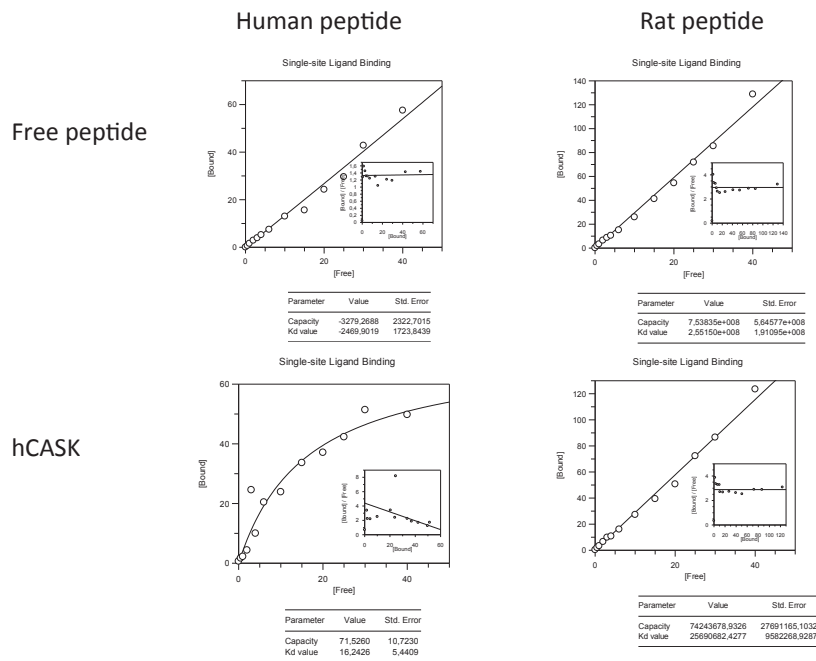


Figure 11: Fluorescence assay titration of the binding of human CD98 C-terminal peptide (dansyl derivative) with hCASK.

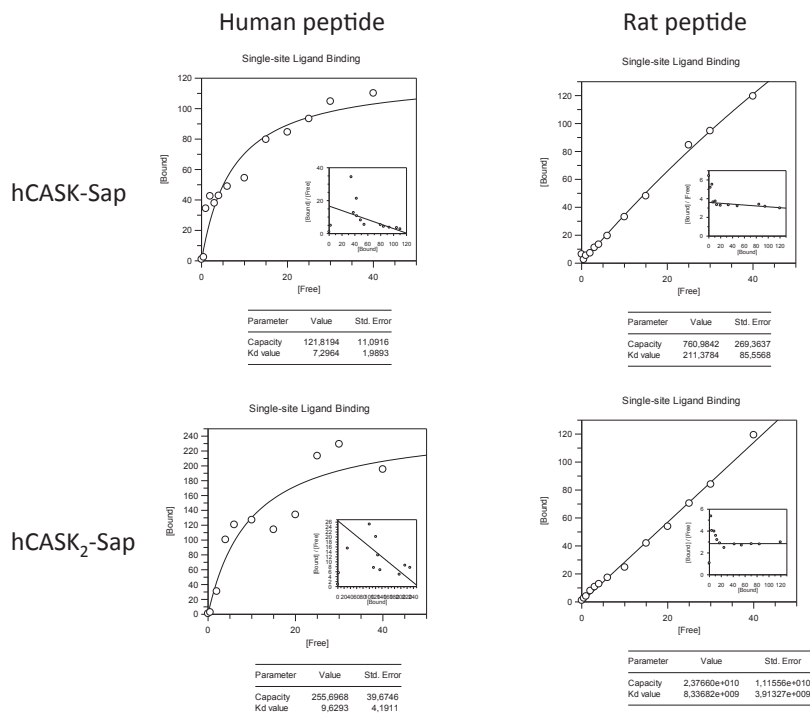


Figure 12: Fluorescence assay titration of the binding of human CD98 C-terminal peptide (dansyl derivative) with hCASK-SAP and (hCASK)₂-SAP

As reported in **Table 2** both hCASK-SAP and (hCASK)₂-SAP retained the affinity towards the human peptide sequence of C-terminal CD98, if compared with the free PDZ from hCASK. They all showed affinity in the order of micromolar (Kd ranging from 6 to 15 μM). Notably none of the above tested proteins showed significant affinity towards a peptide derived from the rat CD98 C-terminal sequence.

Type of protein	Kd (μM)
hCASK-PDZ	16,2 ± 5
hCASK-SAP	7,3 ± 2
(hCASK) ₂ -SAP	9,6 ± 4

Table 2: Kd values calculated from fitting curves; according to a single site ligand binding equation

Furthermore we tested the ability of the same proteins in releasing adenine from isolated yeast ribosomes. The test was done following the release of adenine by HPLC chromatography.

Figure 13 reports the chromatogram of standard solutions of adenine, where the peak at about 1.8 minutes represents the signal of the released adenine. **Figure 14** reports the chromatograms of samples of ribosomes incubated with known concentrations of saporin, hCASK-SAP and (hCASK)₂-SAP.

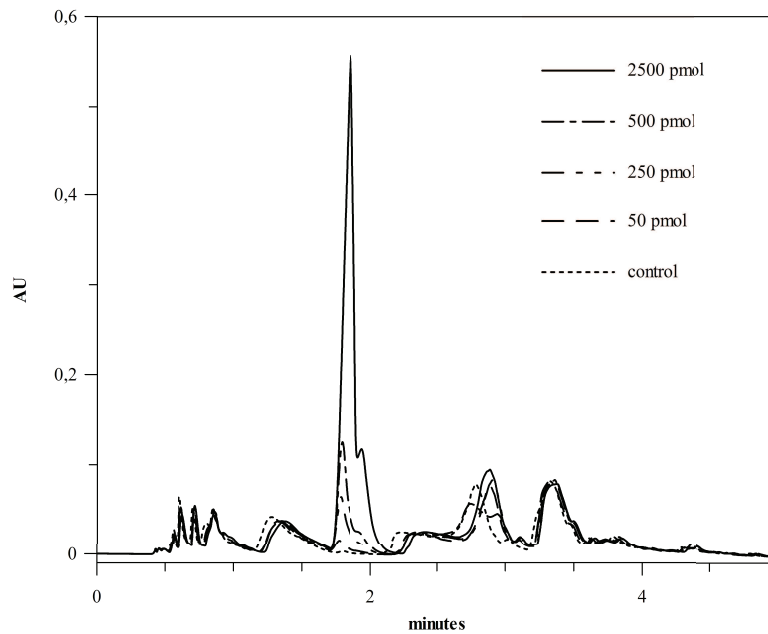


Figure 13: Reverse-phase chromatography of adenine standard

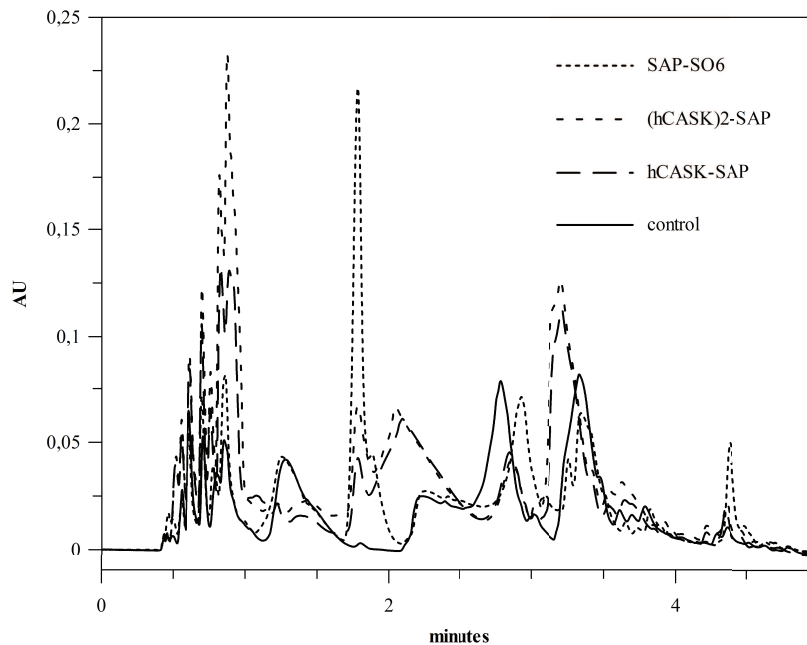


Figure 14: Reverse-phase chromatography of adenine release from yeast ribosomes incubated with known concentrations of saporin, hCASK-SAP and (hCASK)₂-SAP

As reported in **Table 3**, the enzyme activity of hCASK-SAP and (hCASK)₂-SAP is comparable to that of seed saporin in the conditions used.

Type of protein	Picomoles of adenine released/nanomole of protein
SAP-S06	220.7
hCASK-SAP	215.3
(hCASK) ₂ -SAP	224.3

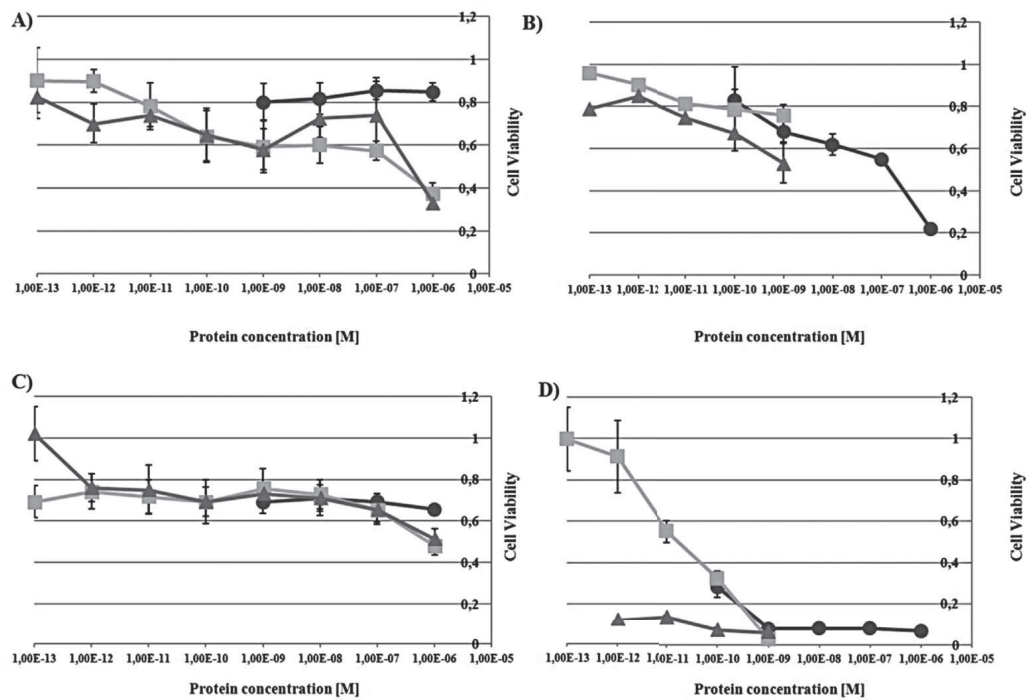
Table 3: **Adenine release from yeast ribosomes calculated from integration of UPLC chromatographic analysis**

The effect of (hCASK- SAP and (hCASK)₂-SAP) on cell growth

In vitro inhibition is significantly increased compared to Saporin alone in U87-MG cells but not in GL15

To evaluate the biological activity the two chimeric toxins (hCASK- SAP and (hCASK)₂-SAP) were tested on two glioblastoma cell lines (U87-MG and GL15). **Figure 15** shows the MTS cells viability assay obtained for both chimeras or Saporin purified from seeds of *Saponaria officinalis* at reported concentrations (10^{-7} – 10^{-13} M) and in the presence or absence of Saponin [2 mg/ml] as “coadjuvant”. hCASK-SAP induces cell death on both U87-MG and GL15 with a similar IC₅₀ ($>10^{-8}$ M) and the addition of the second PDZ domain on (hCASK)₂-SAP significantly increases the toxicity of this chimera on U87-MG cells but not in GL15 cells. These results may be partially attributed to the intrinsic lower activity of Saporin on GL15 cells, but also to different intracellular routes followed by the two toxins (Cimini A M. S., 2012). In order to explore this possibility, we studied the effect of the addition of Saponin SA1641 from *Saponinum album* (an extract obtained from the roots of the plant *Gypsophila paniculata* L.). This substance has been already reported to greatly potentiate the toxicity of Saporin-based chimeras (Weng A., 2012) by acting on the intracellular transport of this toxin, and its possible role in the endosomal escape of Saporin has been suggested. The addition of SA1641 induces a strong increase of Saporin and Saporin chimeras effects in U87-MG cells,

as the IC₅₀ values were lowered of at least three orders of magnitude. In GL15 cells we did not observe any significant effect of SA1641, neither on Saporin nor on the two chimeras.



(E)

	IC ₅₀ (M)		
	hCASK-SAP	(hCASK) ₂ -SAP	SAP-SO6
U87 – SPN	>>10 ⁻⁸	5 × 10 ⁻⁹	5 × 10 ⁻⁷
U87 + SPN	5 × 10 ⁻¹⁰	<<10 ⁻¹²	<10 ⁻¹¹
GL15 – SPN	10 ⁻⁷	5 × 10 ⁻⁶	>10 ⁻⁶
GL15 + SPN	10 ⁻⁷	10 ⁻⁶	>10 ⁻⁶

Figure 15: **MTS Cell viability assay** on GL15 (A and C) or U87-MG (B and D) cells treated with Saporin purified from seeds of *Saponaria officinalis* (●) SAP-SO6 or with the two chimera toxins (■) hCASK-SAP and (▲) (hCASK)₂-SAP in absence (a) or presence (b) of Saporin SA1641 [2 mg/ml]. In both cases the proliferation was calculated using control cells as 100% of viability. The corresponding IC₅₀ was calculated from fitting curves obtained with the software Graphit and reported in (E).

Specificity of action of hCASK-SAP and (hCASK)₂-SAP on U87-MG cells

In order to assess the specificity of recognition of hCASK chimeras a competition experiment on U87-MG cells (sensitive to toxic action of both chimeras) was performed. In the case of

hCASK-SAP the experiment was performed in the presence of SA1641, a condition necessary to get a measurable IC_{50} for this toxin on U87-MG cells.

As shown in **figure 16** the addition of an excess of antibody anti-CD98 competes with the toxicity of both hCASK-SAP and $(hCASK)_2$ -SAP. Indeed, we treated cells with fixed concentration of chimeras corresponding to their IC_{50} and with different concentration of antibody anti-CD98. We didn't observe the expected effect of chimeras on cells (in theory 50% of the ratio survival/death) confirming the competition for the same binding site (*i.e.*CD98).

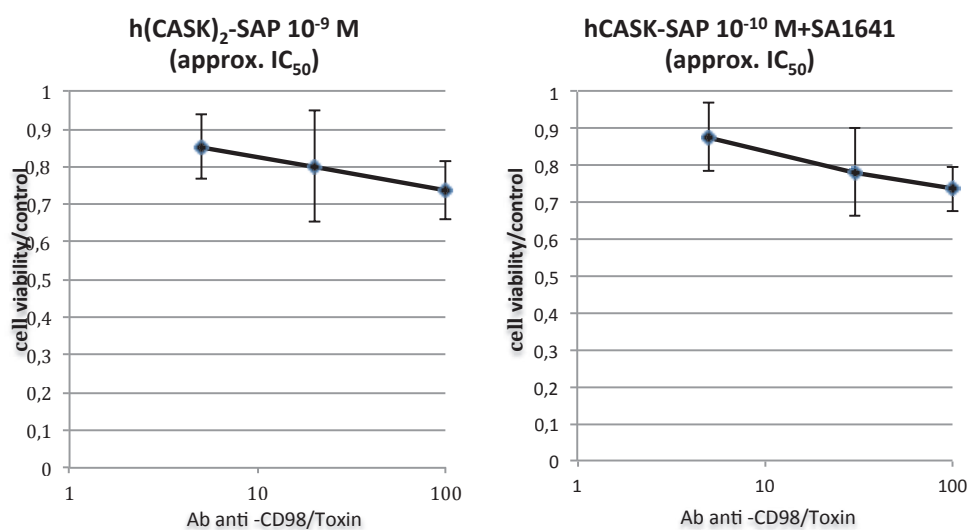


Figure 16 : **Competition assay**

Cell viability assay on U87-MG cells treated with $(hCASK)_2$ -SAP or hCASK-SAP β SA1641 at their respective IC_{50} concentrations (with 2 mg/ml SA1641 for hCASK-SAP) in the presence of anti CD98 polyclonal antibody.

Mechanism of action of hCASK-SAP and $(hCASK)_2$ -SAP in GB cells

hCASK-SAP and $(hCASK)_2$ -SAP induce apoptosis in U87-MG cells *in vitro* dependently of number of hCASK and in low level in GL15 cells without correlation of hCASK number.

In order to establish the pro-apoptotic potential of the two chimeric- toxins (hCASK-SAP and $(hCASK)_2$ -SAP) the levels of cytoplasmic nucleosomes after 96 h of treatment both on U87-MG and GL15 cell lines were evaluated. In **figure 17** it is evident that the $(hCASK)_2$ -SAP toxin is more effective than hCASK-SAP towards U87-MG cell line **(A)(B)**. It is worth noting that

both chimeric toxins exhibit significant ability to induce apoptosis in comparison with free saporin at doses that are one or two orders of magnitude lower in U87-MG cells, while the two toxins have low and similar pro-apoptotic effects on GL15 (C) cells in comparison to the free toxin.

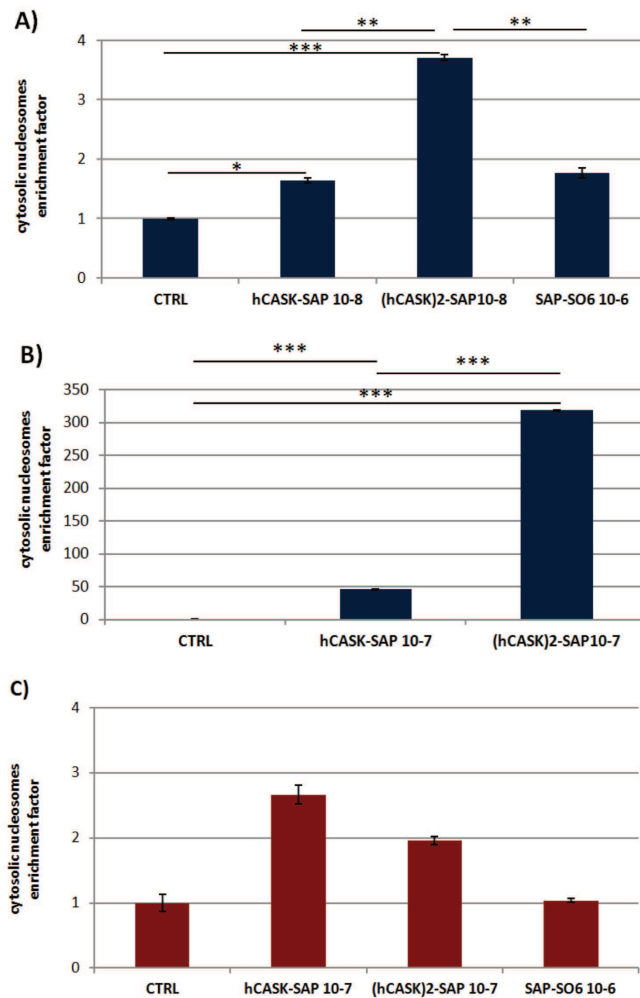


Figure 17 : **Detection of nucleosomes fragmentation in cytoplasmic fractions** Apoptosis was detected with Cell Death Detection ELISA kit. Cells U87-MG and GL15 cells treated for 96 h with the two chimeric toxins hCASK-SAP, (hCASK)2-SAP at concentration corresponding to IC₅₀ reported in Table I (Panel A for U87 cell line, Panel C for GL15 cell line). Cell lysate from adherent was subjected to cell death ELISA assay to detect nucleosomes fragmentation (N = 6 data are mean ±SE). Apoptosis measured on Panel B shows the net apoptosis augmentation for U87 cell lines, depending on the increase of the concentration of the constructs (10⁻⁷ M) and/or form increasing number of hCASK domains. In the figures are reported in the ordinate the enrichment of cytosolic nucleosomes compared to the control cells untreated. (* P < 0.1; ** P < 0.01; *** P < 0.0001).

Discussion

In this part of work we engineered a PDZ protein domain as possible tool for cellular targeting of the ribosome inactivating protein Saporin, with the aim of proposing proof of concept for designing new systems to target cancer cells. We wished to exploit the ability of PDZ domains to recognize and bind short peptide sequences usually found at the C-terminus of cognate proteins in cellular protein-protein interaction phenomena, such as organization of transport, localization, sorting and spatial arrangement of proteins.

We linked PDZ domain of hCASK to Saporin, a well-studied toxin that has been used for the production of chimeric immunotoxins (Flavell DJ C. S., 1991) (Lombardi A, 2010) and so far is one of the most useful tools in the neurobiology research (Lappi DA, 2004). We produced fusions of one or two hCASK-PDZ domains with Saporin and tested it on two human glioblastoma model cell lines (GL15 and U87-MG). Both hCASK-SAP chimeras (carrying one or two PDZ domains), maintain the biological properties of the original macromolecular components (hCASK-PDZ and saporin) and induce cell cytotoxicity and cell death by apoptotic mechanisms in a dose-dependent and/or time dependent manner, with increasing activity as a function of the number of PDZ domains. A significant increase in toxicity was observed upon addition of a second hCASK domain to Saporin, (hCASK)₂-SAP, driving IC₅₀ value down to 10⁻⁹ M, allowing this latter fusion to be two orders of magnitude more effective than the free Saporin toxin. Surprisingly, even though GL15 cells express CD98 on the cell surface, we didn't observe significant different toxicities for both hCASK-Saporin fusions if compared to free Saporin, being their IC₅₀ around 10⁻⁷ M. The therapeutic compound Saponin SA1641, known to increase the toxicity of Saporin-based chimeras in many cell lines (Weng A T. M.-B.-S.-O., 2012) drastically increased the toxicity of Saporin and h(CASK)₂-SAP in U87-MG cells (IC₅₀ <10⁻¹¹ M and <<10⁻¹² M respectively), and allowed hCASK-SAP to have an IC₅₀ of 10⁻¹⁰ M. On the other hand, SA1641 did not produce any increase of toxicity in GL15 cells for any of the toxins studied.

These results in U87-MG cells may be indicative of intracellular delivery passing through the endosomal transport, where Saponin SA1641 can exert its potent release of Saporin, but

also indicate that intracellular delivery mediated by a single hCASK domain may not be efficient in releasing Saporin moiety inside the cell cytoplasm. In fact the addition of a second PDZ domain, greatly increases the toxicity of Saporin, in U87-MG cells.

In GL15 cells, the cellular response seems to be mediated by Saporin internalization mechanisms rather than hCASK delivery, since we couldn't observe differences between the toxins even in the presence of SA1641, suggesting in these cells an intracellular delivery that differs from that obtained in U87-MG cells. We previously did observe a differential sensitivity of GL15 cells to Saporin and its transferrin-conjugates (Cimini A M. S., 2012), partially due to the presence in this cell line of a mutated p53, a phenomenon that has been interpreted as the need of a functional DNA damage sensor system to allow Saporin toxicity to be exerted (Cimini A M. S., 2012).

The observation that a second hCASK domain may increase the toxic potential of Saporin, is confirmed by the induction of apoptosis, since in U87-MG cells (differentially sensitive to Saporin and hCASK fusions) we observed a clear increase of apoptotic cell death as a function of the number of hCASK domains both at 10^{-8} M and 10^{-7} M concentrations. Again the pro-apoptotic effect on GL15 cells of the two hCASK-containing chimeras is reduced and similar.

We tested the specificity of the toxic effect on U87-MG cells by competing the toxin activity with an excess anti CD98 antibody, thus confirming that the intracellular delivery of hCASK-SAP and h(CASK)₂-SAP is dependent on the binding of the PDZ domain of hCASK to the membrane receptor. This experiment is significant to establish that this protein-protein interactions have enough selectivity to be proposed for the delivery of toxic molecules to tissues over-expressing CD98.

Thus, the results obtained suggest that increasing the number of PDZ domains (hCASK) may be a strategy to obtain efficient delivery of toxic cargos in cancer cells, exploiting their selectivity to CD98. Further combination of PDZ domains, with other selectivity, could be in principle used to engineer multivalent targeting systems, allowing the creation of chimeric molecules able to recognize different targets on the surface of cancer cells. This multi-target approach may be useful to efficiently kill cancer cells in a heterogeneous population, where

the lower expression of a single antigen may be limiting to have good therapeutic effects upon treatment in vivo with targeted toxin (Flavell DJ N. A., 1997).

Since to our knowledge this is the first description of the use of a PDZ domain in drug targeting, this strategy could open new perspectives in the creation of innovative drugs in the next future.

Part II.a

Nucleolin as a tool for Targeting Cancer Cells :

a) Using an Aptamer in the engineering and production of a saporin chimeric toxin

b) Using a pseudopeptide in the engineering and production of a saporin chimeric toxin

- 1 . Saporin from seeds of *Saponaria officinalis*

- 2 . Recombinant saporin

a) Using an Aptamer in the engineering and production of a saporin chimeric toxin

Background

AS1411 is a first-in-class anticancer agent, currently in phase II clinical trial (Bates PJ L. D., 2009) (Rizzieri D, 2010) (Rosenberg JE, 2014). It is a G-quadruplex-forming oligodeoxynucleotide that binds to nucleolin, but its mechanism of action is not completely understood (Reyes-Reyes EM T. Y., 2010). This molecule and its related analogues can inhibit proliferation and induce cell death in many types of cancer cells but have little effect on normal cells (Bates PJ K. J., 1999) (Xu X, 2001). Biological effects of this class of molecules in cancer cells include cell cycle arrest, inhibition of NF- κ B signaling, induction of tumor suppressor gene expression, and reduction of bcl-2 expression (Girvan AC, 2006) (Teng Y, 2007).

Because of the the ability of AS1411 to bind to nucleolin and to subsequently enter the cytosol, several groups have shown that AS1411 is an excellent targeting vehicle to deliver drugs and/or dyes to cancer cells (Malik MT, 2015) (Guo J, 2011) (Trinh TL, 2015).

In this study we describe the use of the aptamer AS1411 (APT) sequence as part of an expression plasmid, targeting nucleolin to deliver the gene of the ribosome inactivating protein saporin to glioblastoma cancer cells. We constructed an expression cassette including AS1411 at the 5' and saporin gene (codon optimized for the expression in mammalian cells) at the 3' (APT-SAP), and showed the effects of this DNA construct on glioblastoma cell lines growth.

This new approach of toxic gene delivery may be suggested as a specific and reliable system to treat glioblastoma, a cancer form that is currently having very poor prognosis due to low sensitivity to chemotherapy and with a rapid recurrence after surgery.

Results

Characterization of U87-MG cells and primary cells lines for the presence of membrane nucleolin

We have previously determined the presence of nucleolin on cells surface by immunolocalization using polyclonal antibodies (gp 273) raised against the glycosilated membrane nucleolin form (see, methods) As shown in **figure 18** U87-MG cell line (panel A) and particularly primary glioblastoma cells (panel B) show a clear membrane localization of nucleolin. In the course of experiments we have also used a cell line (NRM6) that is completely negative for nucleolin staining (panel C).

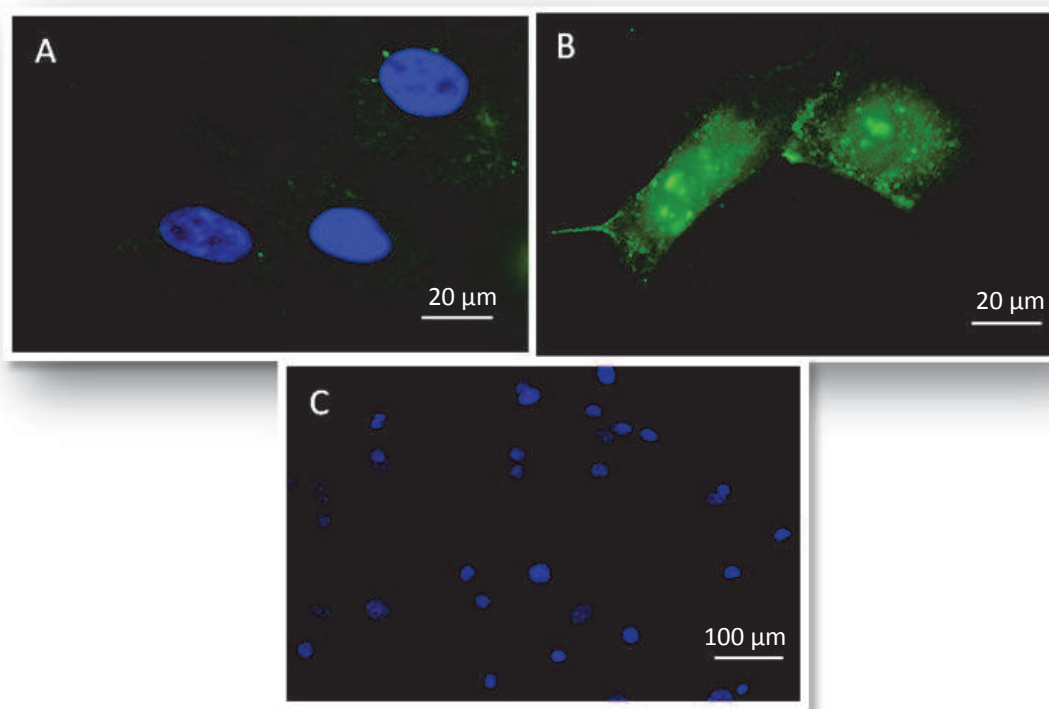


Figure 18: **Immunofluorescence staining with anti-nucleolin**

Imunofluorescence on U87-MG cells (A), primary glioblastoma cells (B) and NRM6 cells line (mouse fibroblasts) after incubation with antibody anti-nucleolin

On the basis of these observations, we have treated cells with DNA constructs as described below.

Different DNA constructs used in this study

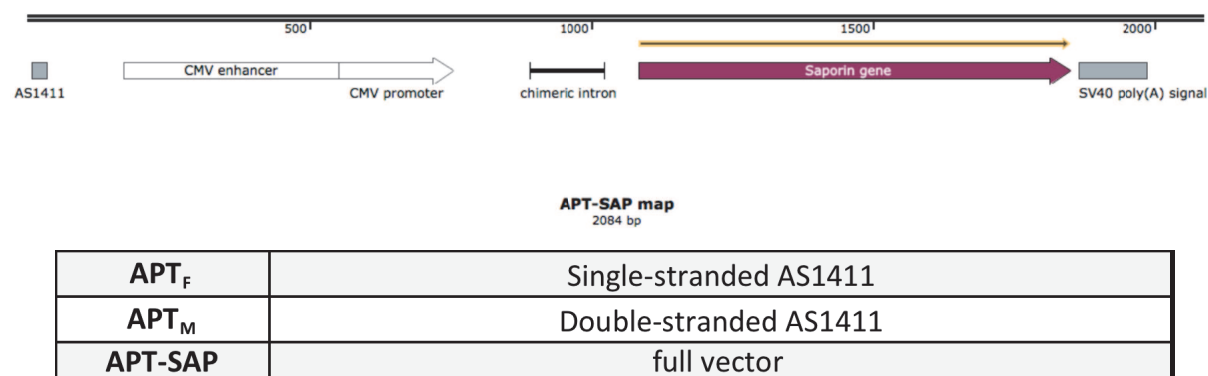


Figure 19: **Map of the construct APT-SAP and resume of different conformations used for treatment**

The effect of APT and APT-SAP on cell growth

Inhibition *in vitro* is significantly increased compared to APT alone in GB cells

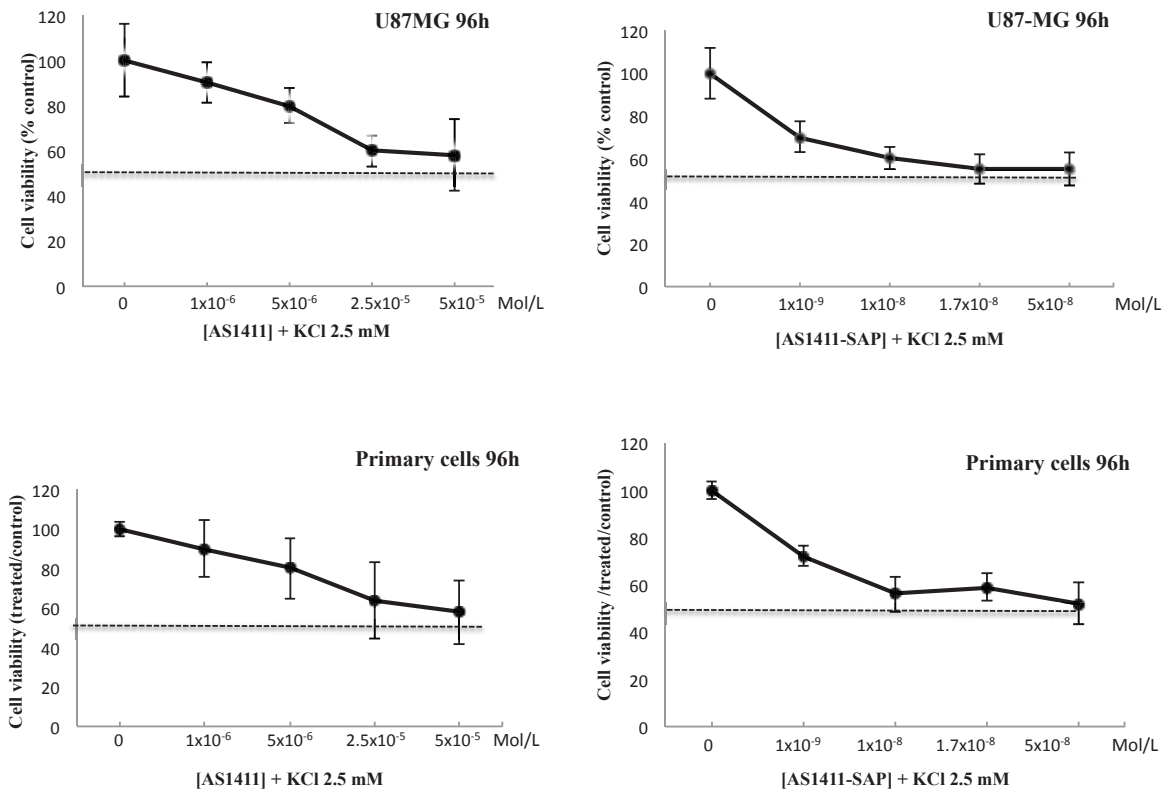
APT-SAP (pCMV6-APT-SAP) was purified by gel electrophoresis on agarose before the use. In preliminary experiments the treatment with AS1411 (APT) and APT-SAP was performed at different concentration range for 96 hours. The results show (**figure 20 (a)**) a clear activity of APT-SAP in contrast with the faint effect on cell viability of AS1411 (APT) in the low concentration range (1-100 nM). The cytotoxic effect, at maximum concentration, is evident for APT-SAP (50% inhibition of cell viability, 17 nM U87-MG cell line and 20 nM in glioblastoma primary cells) compared to the one of APT (50% of inhibition of cell viability at 25 μ M in U87-MG and the same effect at 50 μ M in primary cells).

Since APT-SAP is a double stranded DNA, we made a test comparing APT (AS1411 as single stranded DNA) with that of a mixture of APT and its complementary strand (APT_M).

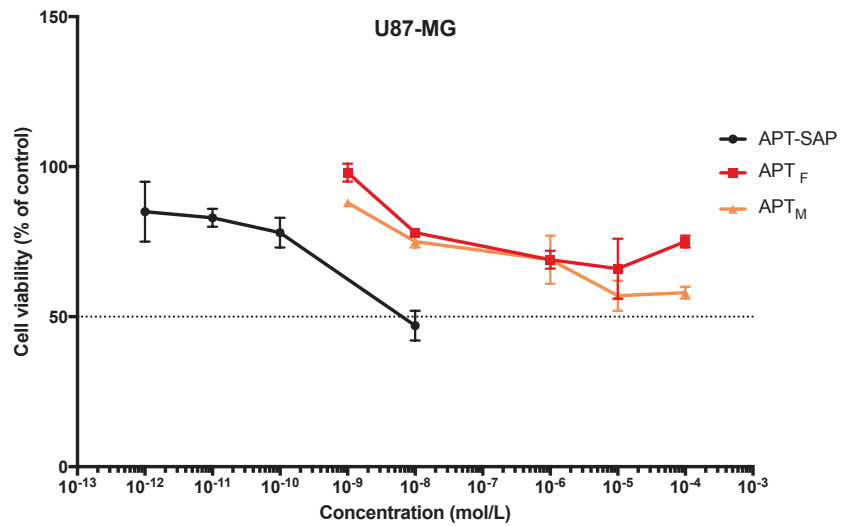
APT is still active at the same extent if tested in the presence of the complementary strand (APT_M), suggesting that the formation of G-quadruplex structures may not depend strictly on a single stranded initial conformation (**figure 20 (b)**).

Toxicity induced by APT-SAP and APT alone is evident also observing microscopy images taken after 96 h incubation (**figure 20 (c)**).

(a)



(b)



(c)

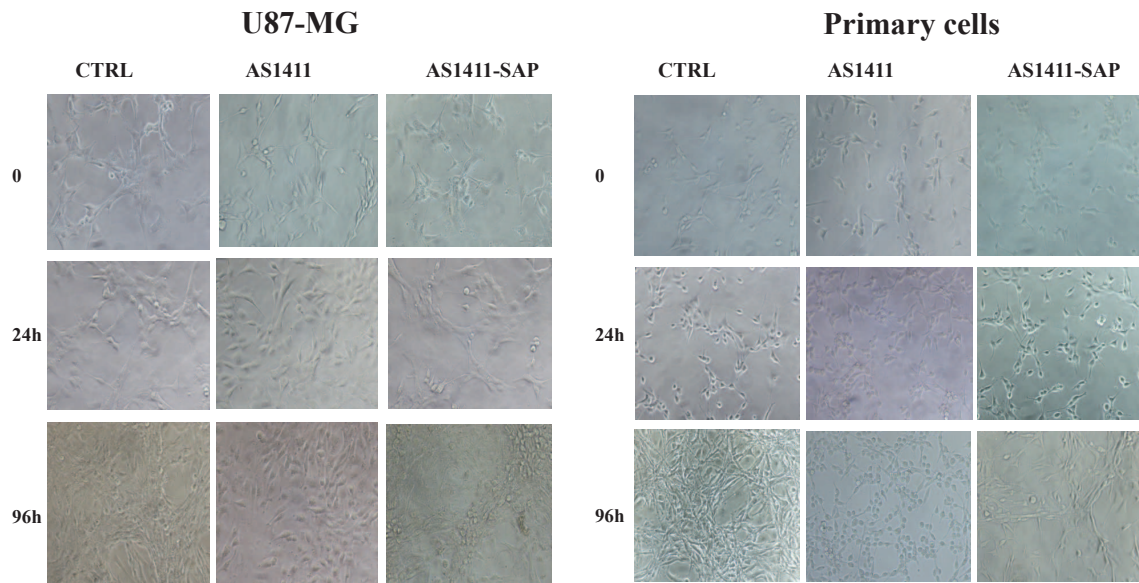


Figure 20: (a) **Dose response curve showing IC_{50}** of APT (AS1411) or APT-SAP on U87-MG cells and in glioblastomas primary cells. (b) **Dose response curve showing IC_{50}** of APT-SAP(a), or APT (AS1411) in a single strand conformation (APT_F) or in a double strand conformation (APT_M) on U87-MG cells (c) **Representative images** observed with an optical microscope of glioblastoma cell line (U87-MG) and primary cells at t0, t24h and t96h after treatment with APT (AS1411) or APT-SAP at their respective IC_{50} (U87-MG : APT 25 μ M, APT-SAP 17nM ; primary cells : APT 50 μ M, APT-SAP 20nM).

Specificity of action of APT-SAP

APT-SAP acts *via* nucleolin to target cancer cells

To test the specificity of APT-SAP and APT towards nucleolin expressed on glioblastoma cells, we performed a competition assay in which cells were exposed to the DNA constructs in the presence of an excess anti-nucleolin polyclonal antibody (gp273). As shown in **figure 21**, the presence of the antibody competes with the activity of both APT-SAP (10 nM) and APT_F but not surprisingly with APT_M (both at 50 μ M).

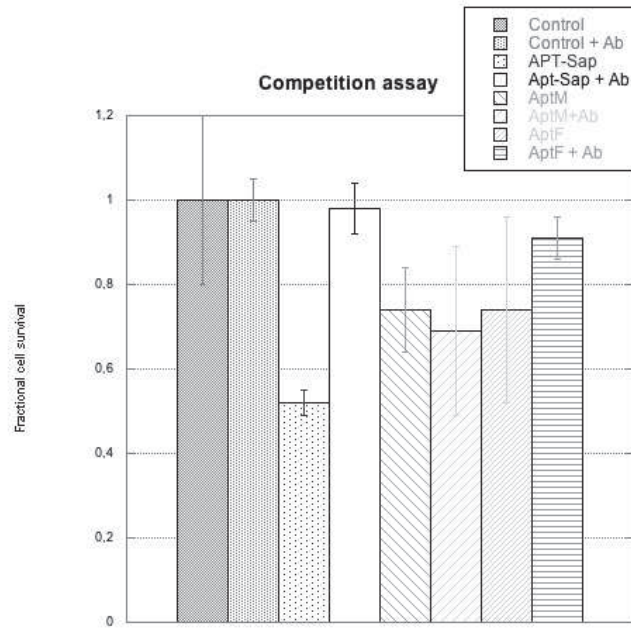


Figure 21: **Competition assay**

Toxicity of APT-SAP and APT (F or M) in the absence or in the presence of polyclonal anti-nucleolin antibody (gp273)

Furthermore to test the selectivity toward nucleolin-membrane positive cells, we also made a cytotoxicity assay on nucleolin-negative cells (NR6M, mouse fibroblasts). As shown in **figure 22**, NR6M cells are completely unaffected by the treatment with APT-SAP or APT, under conditions that were already found to inhibit cell growth for glioblastoma cells.

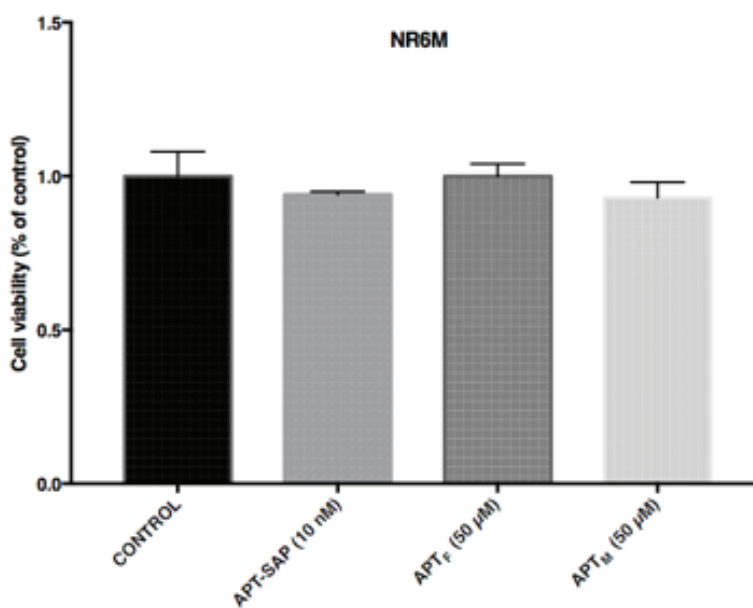
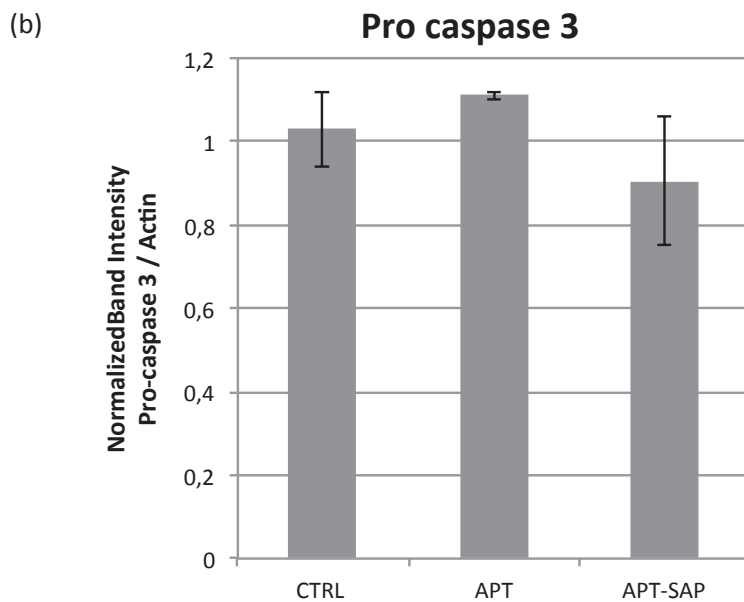
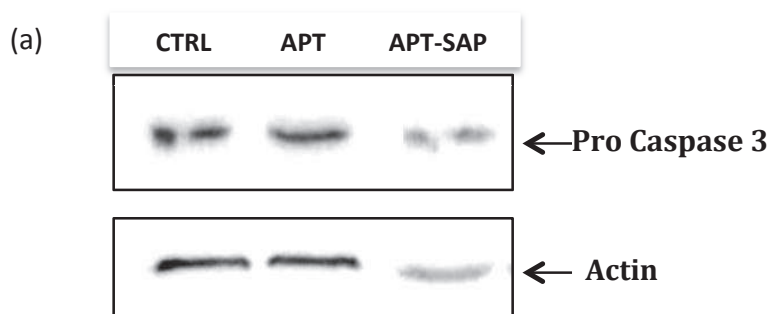


Figure 22: **Cell viability of NR6M cells treated with the various constructs.**

Mechanism of action

APT-SAP does not induce apoptosis in glioblastoma cells *in vitro*

To this aim, induction of apoptosis was measured after 96h exposure of cells to APT and APT-SAP, by measuring the activation of pro-caspase 3 and by the quantification of histone-complexed DNA fragments in cytosol of treated cells and. U87-MG cells appear to be not affected by apoptosis (**figure 23(a), 23(b) and 23(c)**).



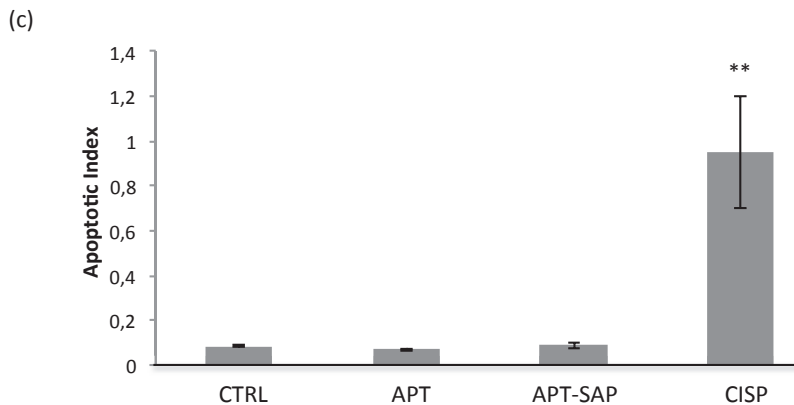


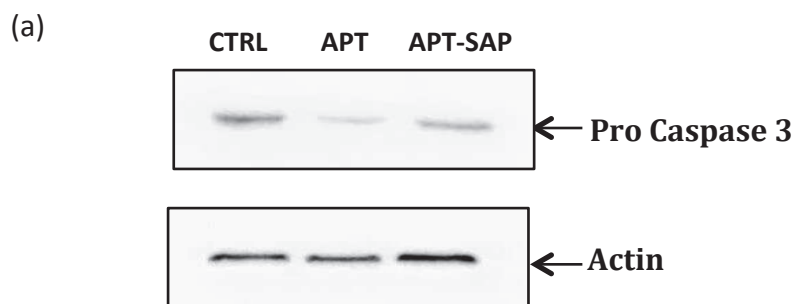
Figure 23: Detection of apoptosis markers after treatment with APT-SAP and APT on U87 glioblastoma cells.

(a) Western blotting analysis of pro-caspase3 activation and (b) its quantification.

(c) Detection of nucleosomes fragmentation in cytoplasmic fractions. Apoptosis was detected with Cell Death Detection ELISA kit. Cells U87-MG treated for 96 h with APT-SAP or APT. Cis-platin (CISP) was used as a positive control.

Data show a non-significant increase in the percentage of treated cells undergoing apoptosis respect to control cells, suggesting that cell growth impairments could also be due to other events rather than apoptotic mechanisms in U87-MG cells.

A similar analysis was performed on primary glioblastoma cells, as reported in **figure 24** (a),(b) and (c). In this case a slight increase of caspase3 activation was observed upon treatment with APT-SAP but this result does not correlate with nucleosome fragmentation, thus suggesting taht also in this case apoptotic cell death may not be preminent.



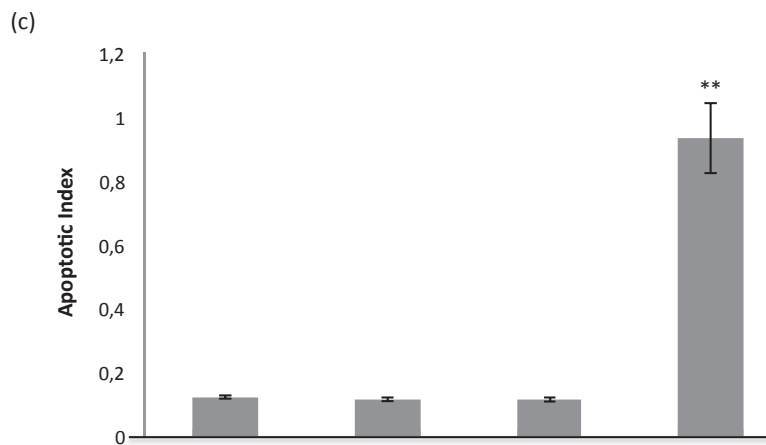
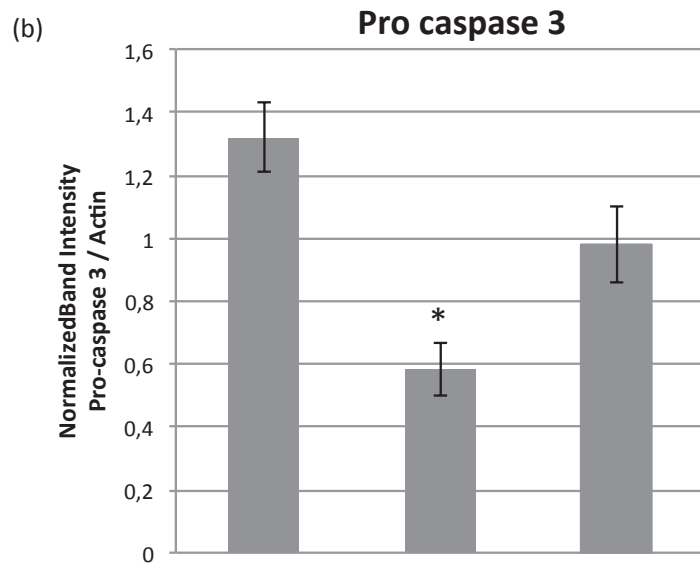


Figure 24: **Detection of apoptosis markers after treatment with APT-SAP and APT on primary glioblastoma cells.**

(a) Western blotting analysis of pro-caspase3 activation and (b) its quantification.

(c) Detection of nucleosomes fragmentation in cytoplasmic fractions. Apoptosis was detected with Cell Death Detection ELISA kit. Cells U87-MG treated for 96 h with APT-SAP or APT. Cis-platin (CISP) was used as a positive control.

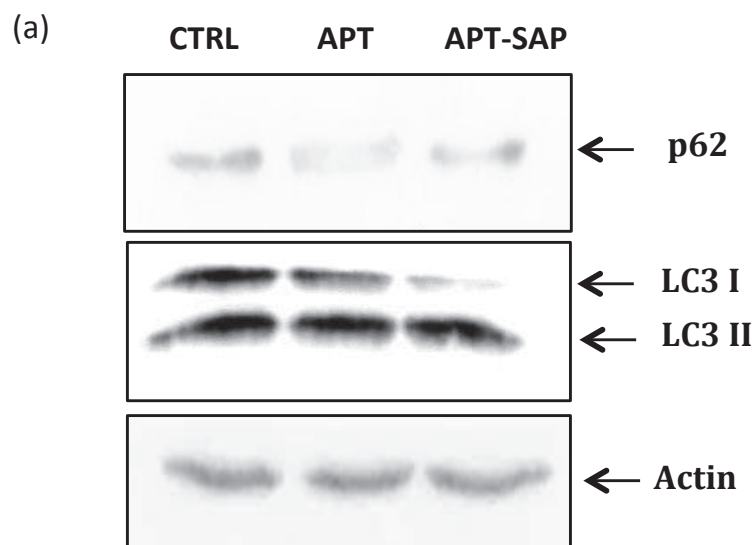
APT and APT-SAP treatment induces autophagic cell death activity

Starting from the observations obtained for cell viability and apoptosis we investigated the intracellular pathways leading to cytotoxic effects of APT and APT-SAP in U87-MG and primary GB.

In order to verify whether cell growth inhibition could be due to other events rather than apoptotic mechanisms in U87-MG cells we evaluated the Microtubule-associated protein 1A/1B-light chain 3 (LC3) expression after treatment with APT and APT-SAP. Detecting LC3 by immunoblotting or immunofluorescence has become a reliable method for monitoring autophagy and autophagy-related processes. Cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes. Autophagosomes fuse with lysosomes to form autolysosomes, and intra-autophagosomal components are degraded by lysosomal hydrolases. The ratio LC3II/LC3I may indicate autophagic activity (Tanida I, 2004).

At the same time another recognized marker for detection of autophagy is the decrease of p62 (Bjørkøy G, 2009) . Both these markers were analyzed.

The analysis of value of LC3 II/LC3I in U87 cells shows an increase of this ratio in the treated cells (APT and APT-SAP) with respect to the control cells (**figure 25(b)**) suggesting the presence of autophagic activity after 96h of treatment (see also **figure 25 (a)**) . Furthermore after the same treatments, U87 cells showed a marked decrease of p62 (**see figure 25 (c)**).



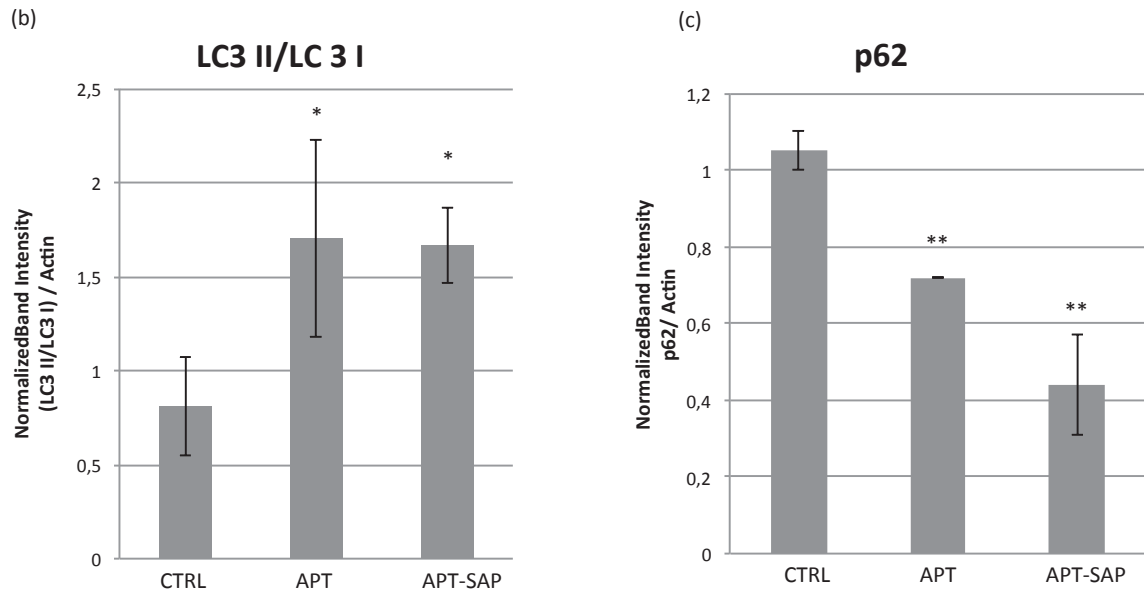
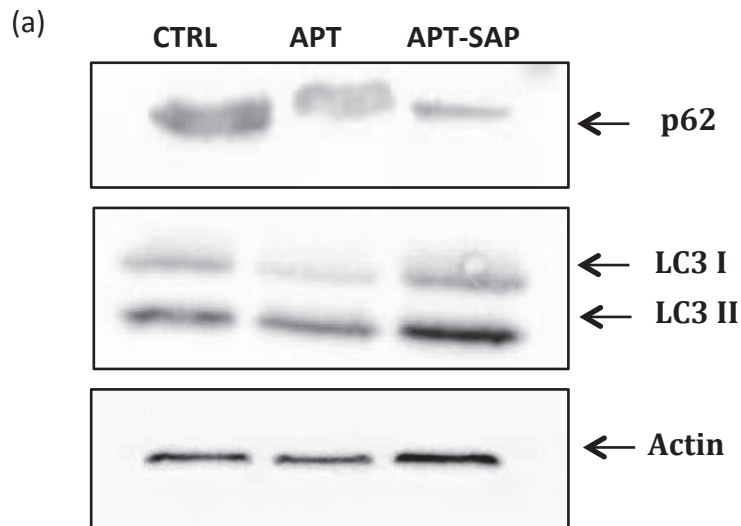


Figure 25: **Western-Blotting analysis**

LC3 and p62 immunoblotting analysis of U87-MG cell line after 96h of treatment with APT-SAP or APT (a) and relative densitometric analysis (b) and (c) respectively

Similarly the analysis was done on primary GB, as shown in **figure 26 (a), 26(b) and 26(c)**. The results obtained are similar to those of U87, suggesting autophagy also for primary GB.



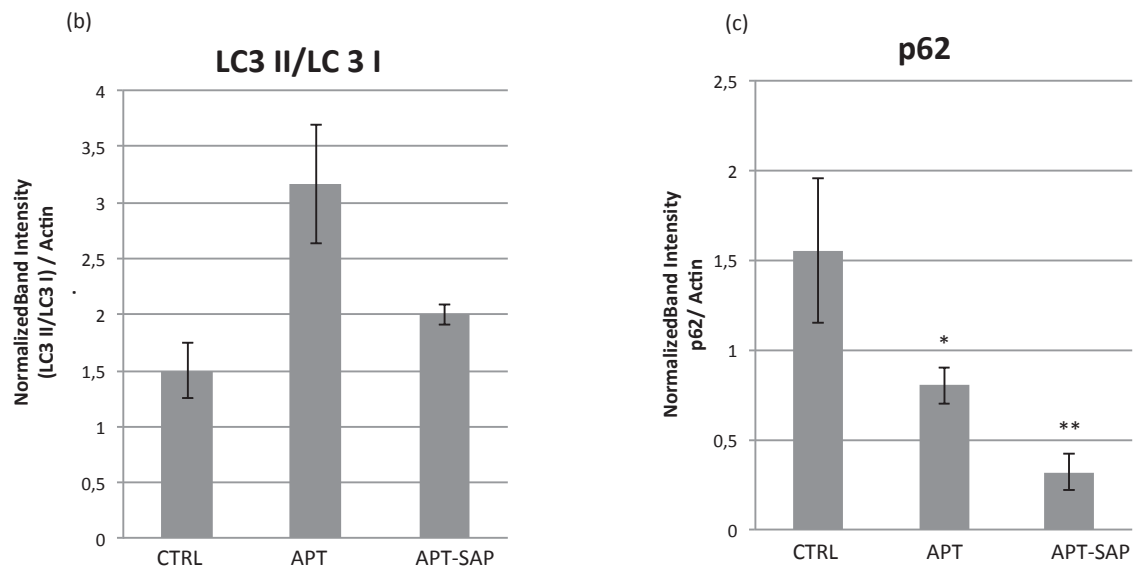


Figure 26: **Western-Blotting analysis**

LC3 and p62 immunoblotting analysis of U87-MG cell line after 96h of treatment with APT-SAP or APT (a) and relative densitometric analysis (b) and (c) respectively.

Discussion

The toxic gene therapy is an attractive mode of gene therapy that is particularly optimal for the treatment of cancer. The success of this therapy mainly lies in (1) the successful delivery of the toxic genes selectively to tumor cells; and (2) effective killing of the tumor cells by the expressed gene products. To address the challenge of the drug potency, herein, we constructed an expression cassette including the aptamer AS1411 at the 5' and saporin gene (codon optimized for the expression in mammalian cells) at the 3' (APT-SAP), and showed the effects of this DNA construct on GB cells lines (U87-MG) and primary GB cells growth. A marked decrease in cell viability could be observed in glioblastoma cells. The cytotoxic effect in U87 cells is greater for APT-SAP than APT, being the 50% inhibition of cell viability achieved at the concentration of 17 nM and more than 25 μ M respectively. On primary GB cells IC_{50} values of 20 nM and 50 μ M were measured for APT-SAP and APT respectively. Competition assay and the non effect on viability of our construct in cell lines negatives for nucleolin expression (NRM6) in cell surface confirmed the fact that APT-SAP and APT act *via* the nucleolin. The investigation of intracellular pathways related to cytotoxic effects of APT and APT-SAP in U87-MG cell lines show a non-activation of apoptotic cell death but highlights the presence of autophagic processes that could cause the block in the G1 phase of the cell cycle in treated cells respect the control cells as reported by Vitaliy Kaminskyy et al. (Kaminskyy V, 2011). The increase of ratio LC3 II/LC3 I as well as the decrease of p62 reveal an autophagy activated process.

Considering the data shown in this study, it is conceivable that the AS1411 moiety of the AS1411-drug-conjugated compounds will enhance the drug uptake/delivery exponentially and that doing so might circumvent the need for increased nucleolin expression to manifest maximal sensitivity.

Part II.b

Nucleolin as a tool for Targeting Cancer Cells :

a) Using an Aptamer in the engineering and production of a saporin chimeric toxin

b) Using a pseudopeptide in the engineering and production of a saporin chimeric toxin

1 . Saporin from seeds of *Saponaria officinalis*

2 . Recombinant saporin

b) Using a pseudopeptide in the engineering and production of a saporin chimeric toxin

1. Saporin from seeds of *Saponaria officinalis*

Background

In this work we exploited the overexpression of cell surface nucleolin as the target for N6L. Previous study reported that N6L suppressed both tumor growth and angiogenesis (Destouches D. E. K.-K., 2008). We demonstrated the effects of N6L on human glioblastoma cells in primary culture prepared from post-surgical specimens (Benedetti E, 2015). The results obtained indicated an anti-proliferative effect of N6L and point towards its possible use as adjuvant agent to the standard therapeutic protocols presently utilized for glioblastoma.

To increase efficiency of N6L we decided to bind to the protein toxin Saporin by chemical cross-linking.

Results

To obtain the conjugate SAP-N6L, we tested different chemical cross-linker with the aim of working with the most specific and toxic conjugate again cells with nucleolin overexpressed in cell surface (**figure 27**).

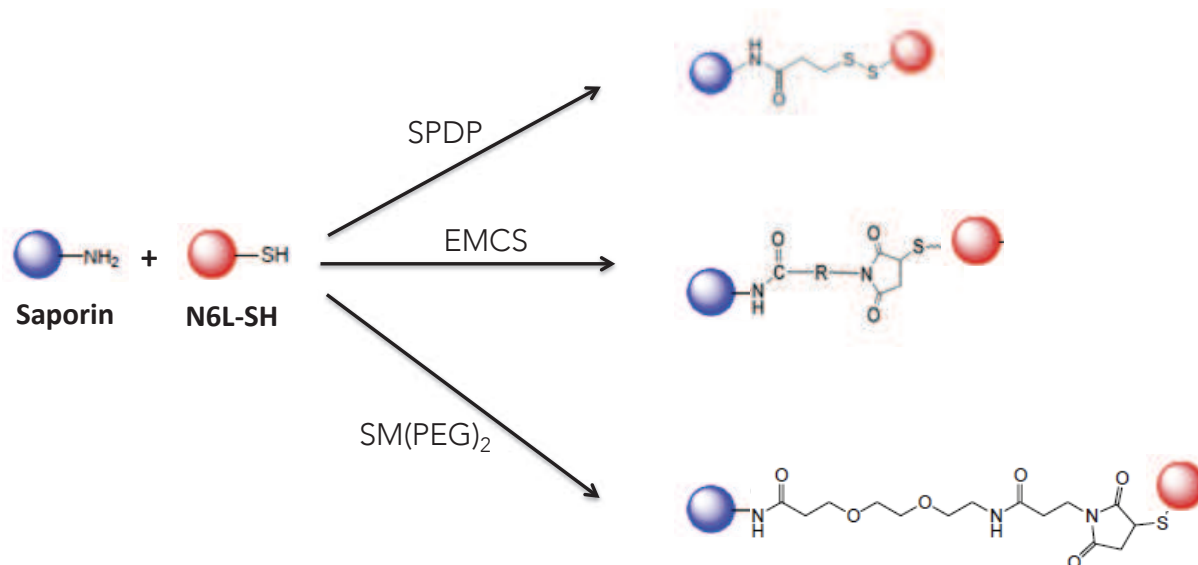


Figure 27 : Different chemical cross-linker used to bind Saporin and N6L

Purification of SAP-N6L after reaction with cross linker

For convenience we present here purification steps after reaction with SDPD cross-linker, but the purification protocol is the same for all different cross -linkers. After derivatization of saporin to introduce SH groups, we can follow the kinetic reaction when we add N6L-SH in the mixture. After this reaction, we eliminate free N6L-SH by gel filtration (elution profile in **figure 28 (a)**).

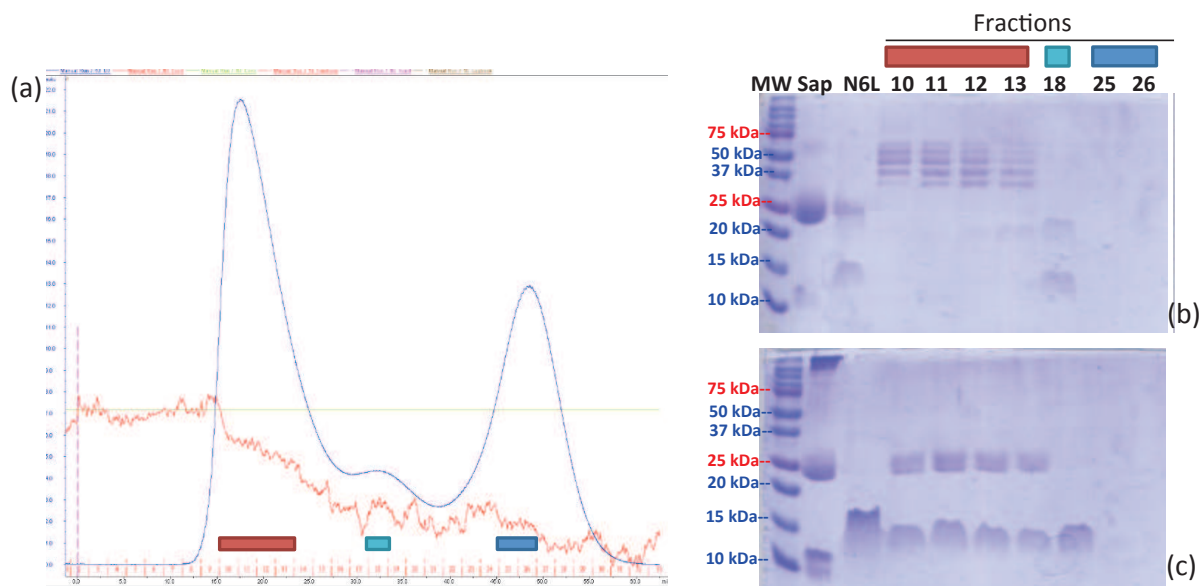


Figure 28 : Purification of SAP-N6L after chemical cross-linking

(a) Sample elution profile obtained after passage of reaction mixture (Pyridyldithiol-activated-saporin + N6L-SH) through Sephadex G-75 gel filtration column (GE Healthcare). SDS-PAGE analysis of fractions obtained in the absence (b) or in the presence (c) of β -mercaptoethanol in the sample. The samples were stained by Coomassie-blue. Sap corresponds to saporin from seeds and N6L is N6L-SH. Fractions 10, 11, 12 and 13 correspond to the first peak, fraction 18 to the second peak and fractions 25 and 26 to the third one.

The conjugate SAP-N6L exists in different conformations and is dissociated under reducing condition

We observed that under non-reducing conditions SAP-N6L is not represented by a single species (**figure 28 (b)**). Indeed SAP-N6L is a mixture of 5 different complexes (three in majority), ranging between 28 kDa and 50kDa, suggesting the linking of different number of N6L molecules to saporin. This result is not unexpected as chemical modification of saporin with the crosslinker SPDP is likely to occur on different amino groups (lysines). Under reducing conditions the link between Saporin and N6L is sensible to reducing agents (**figure 28 (c)**). So, it is supposed that when SAP-N6L will be in reducing environment (cytoplasm) disulfide bonds between Saporin and N6L can be broken and saporin and N6L could act separately.

Comparison between different cross-linkers

We have proceeded cross-linking with these three different cross-linker and we have tested them on glioblastoma (GB) cells line and primary cells to choose the more active. As we show in **figure 29**, SAP-N6L conjugate linked with SPDP (so with a disulfide bridge) is clearly the most toxic. This could be easily explained by the fact that as the link between Saporin and N6L is sensible to reducing agents, in cytoplasm saporin could act separately from N6L and *vice versa*.

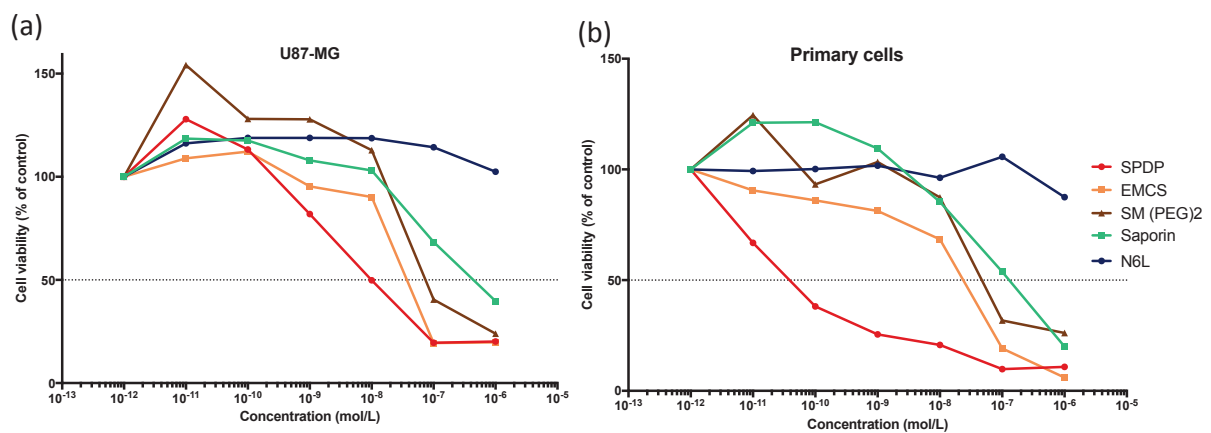


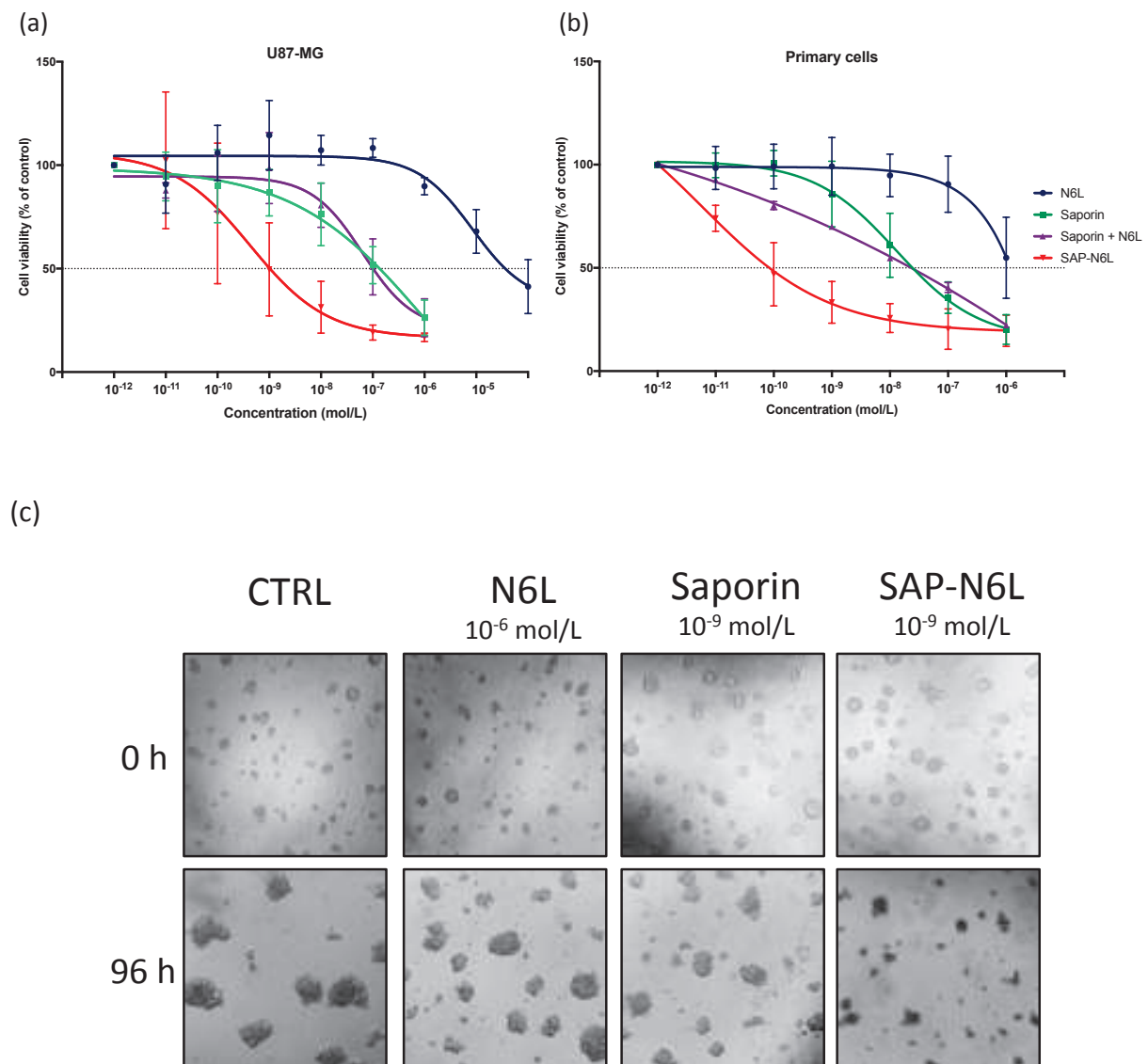
Figure 29 : **Dose response curve showing IC₅₀** of N6L (in blue), Saporin (in green) and SAP-N6L with different cross-linkers (SPDP in red, EMCS in orange and SM(PEG)₂ in brown) in (a) glioblastoma cells line (U87-MG) and (b) glioblastoma primary cells (C10414). Cells (2×10^3 / well) were seeded in 96-well plates in a final volume of 200 μ l of medium (5% of FBS) containing appropriate concentrations for 96h. Viability was evaluated using a colorimetric assay based on MTS reduction. (N = 3 data are mean)

The effect of SAP-N6L on cell growth

Inhibition *in vitro* is significantly increased compared to N6L and Saporin alone in GB cells. The toxic effects of N6L, Saporin and SAP-N6L on GB cells were studied using a GB cell line (U87-MG) and a primary culture derived from surgical specimen. As shown in **figure 30**, SAP-N6L decreases cell viability drastically if compared to N6L or saporin alone. U87-MG cells **(a)** are sensitive to SAP-N6L with an IC₅₀ in the nanomolar range and the effect is more impressive in primary GB cells **(b)**, where IC₅₀ of SAP-N6L is one log lower. We observed that

toxicity of SAP-N6L is 1000 fold more efficient than saporin alone in glioblastoma primary cells and 100 fold more in U87-MG. And if we consider N6L alone, SAP-N6L is 10000 fold more efficient in primary cells and more than 1000 fold in U87-MG cell line.

We further performed toxicity experiments on culture system composed of free-floating clusters of U87-MG stem cells (neurospheres). We have tested SAP-N6L on these neurospheres to understand if the toxic conjugate is able to penetrate inside these 3D-clusters and furthermore to target cancer stem cells. In **figure 30 (c)**, we can note that after 96h of treatment with SAP-N6L, neurospheres are smaller than in control and seem to be released from the initial mass. Concerning cells viability we can observe that it is significantly decreased after treatment with SAP-N6L in comparison to N6L and saporin alone. Moreover, we obtained IC₅₀ similar to those obtained on adherent U87-MG cells (**figure 30 (d)**).



(d)

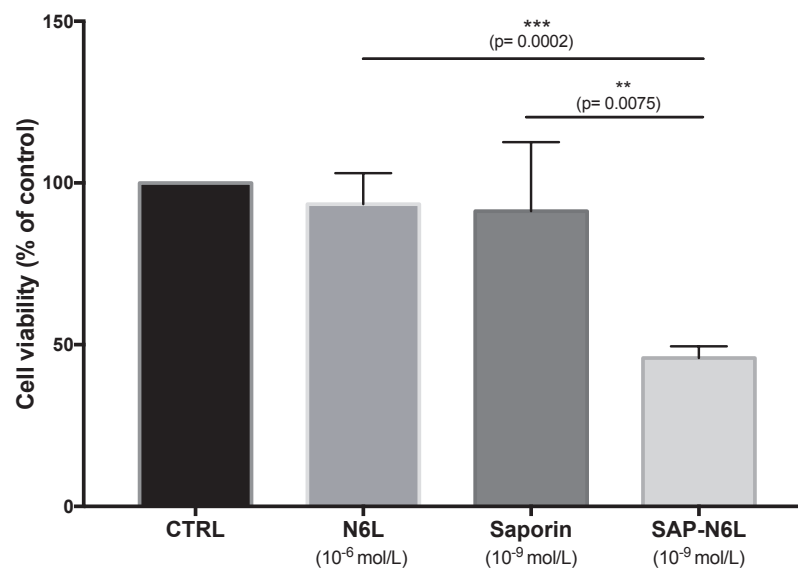
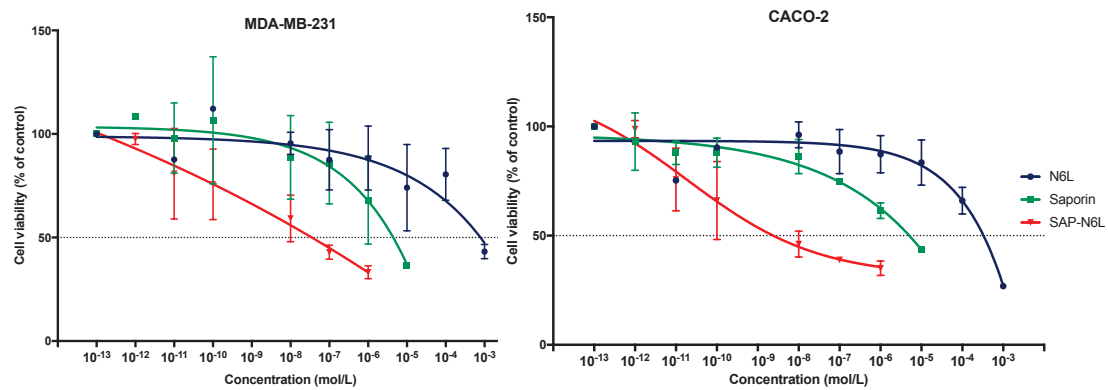


Figure 30: **The effect of Sap-N6L on GB cell growth inhibition *in vitro*** is significantly increased compared to N6L and Saporin alone or with Saporin and N6L not cross-linked Dose response curve showing IC₅₀ of N6L, Saporin, Saporin and N6L without cross-linking and SAP-N6L in (a) glioblastoma cells line (U87-MG) and (b) glioblastoma primary cells (C10414). Viability was evaluated using a colorimetric assay based on MTS reduction. (N = 9 data are mean ±SE).(c) Contrast phase microscopy of glioblastoma neurospheres before and after treatment with N6L, Saporin and SAP-N6L. Cells were seeded at concentration 1 × 10⁴ / well in 96-well plates in a final volume of 200 μl of medium. When neurospheres started to be formed, we treated with 200 μl of medium containing appropriate concentrations. (d) Histogram showing viability of cells from neurospheres after manual disruption after 96h of treatment with N6L, Saporin and SAP-N6L.

The effect of SAP-N6L cell growth inhibition *in vitro* is significantly increased compared to N6L and Saporin alone also in cancer cells other than glioblastoma

For other human cancer types overexpressing nucleolin, such as adenocarcinoma (mammary gland, cervix and colon tissue) as well as in acute myeloid leukemia, we showed (**figure 31, Table 4**) that SAP-N6L is more active than N6L or saporin alone. These results indicate that SAP-N6L could be efficient in killing different kind of cancer cells.

(a)



(b)

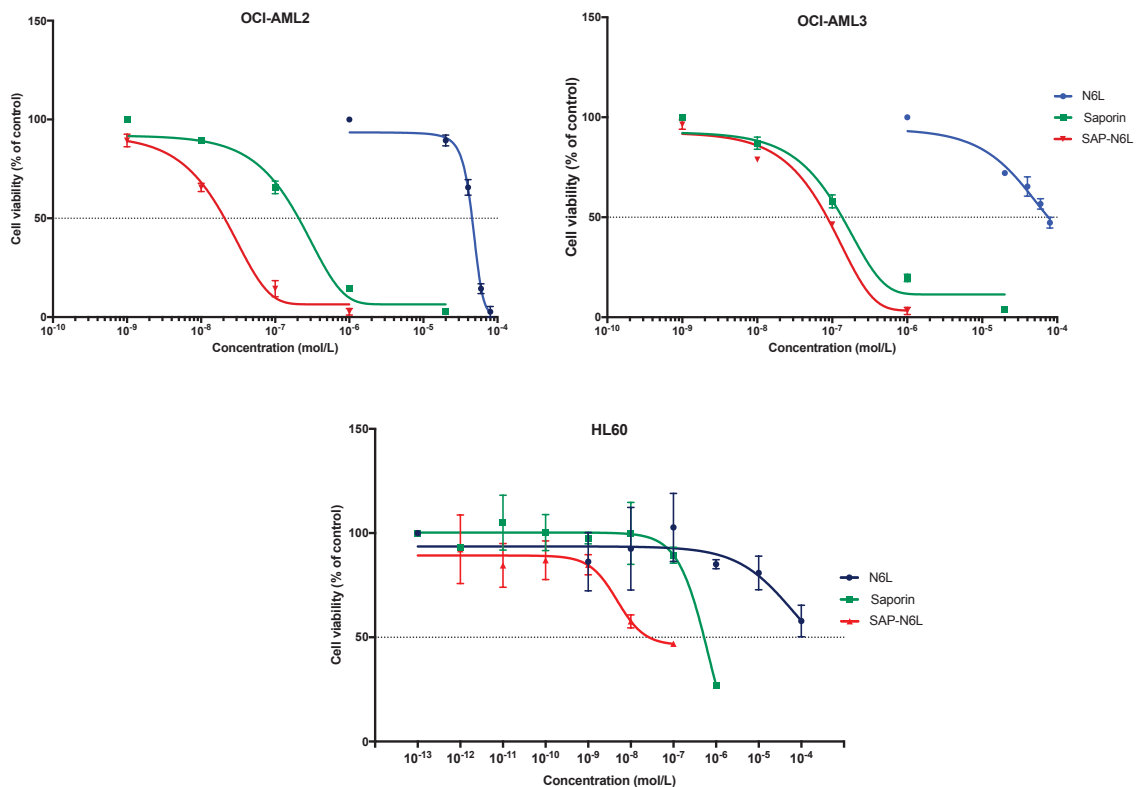


Figure 31: N6L, Saporin and SAP-N6L exert distinct inhibitory effects on different types of tumor cells

(a) Dose response curve showing IC₅₀ of N6L, Saporin and SAP-N6L in human breast adenocarcinoma cells line (c) (MDA-MB-231) and (d) in colorectal adenocarcinoma cells line (Caco-2). Cells (2×10^3 / well) were seeded in 96-well plates in a final volume of 200 μ l of medium (5% of FBS) containing appropriate concentrations for 96h.

(b) Dose response curve showing IC₅₀ of N6L, Saporin and SAP-N6L in OCI-AML2 (human, peripheral blood, leukemia, acute myeloid), OCI-AML3 (acute myeloid leukemia cells) carry an NPM1 gene mutation (type A), HL60 (Human promyelocytic leukemia cells) cells line. Cells (2×10^3 / well) were seeded in 96-well plates in a final volume of 200 μ l of medium (20% of FBS) containing appropriate concentrations for 96h. Viability was evaluated using a colorimetric assay based on MTS reduction.

Mouse embryonic fibroblasts 3T3, known to be particularly resistant to free saporin, were then used as a control to verify the potency of SAP-N6L. Interestingly we observed that 3T3 cells are resistant to saporin (more than 85% of cell viability at 100 nM, similarly to HeLa cells; **figure 32**) and significantly less sensitive to SAP-N6L ($IC_{50}=100$ nM) than primary human GB cells ($IC_{50}=0.1$ nM).

In 3T3 cell line (as well as in HeLa cells) we have the confirmation that the toxicity of SAP-N6L is due to saporin, since the addition of N6L ligand induces potentiation of toxicity suggesting the role of N6L as a carrier of the toxin.

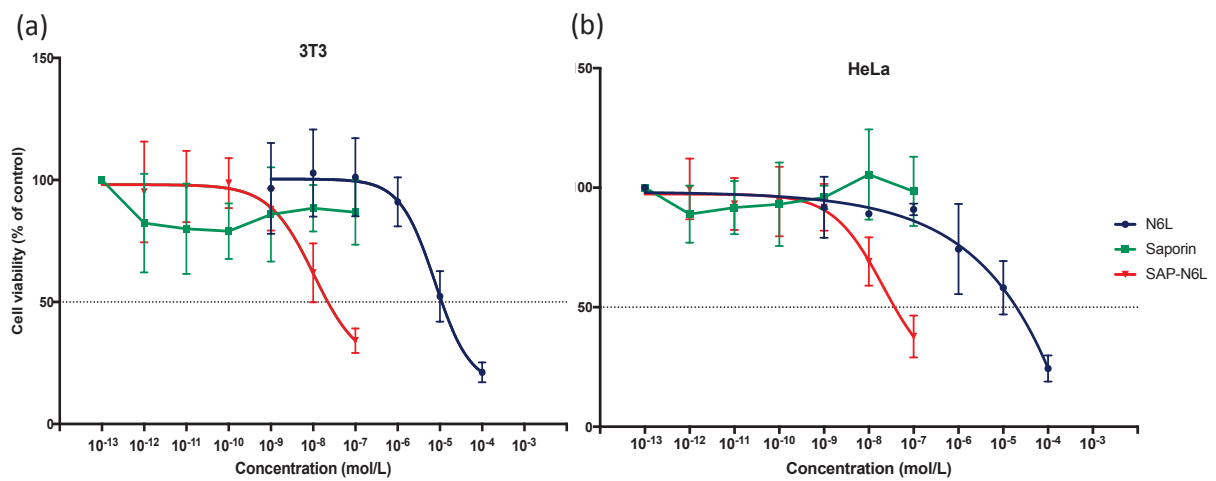


Figure 32: Some cell line reveal a resistance to the saporin

Dose response curve showing IC_{50} of N6L, Saporin and SAP-N6L in (a) 3T3 and (b) HeLa cells line. Cells (2×10^3 / well) were seeded in 96-well plates in a final volume of 200 μ l of medium (5% of FBS) containing appropriate concentrations for 96h. Viability was evaluated using a colorimetric assay based on MTS reduction. (N = 9 data are mean \pm SE)

Organism	Tissue	Disease		IC_{50} (mol.L ⁻¹)		
				N6L	Saporin	SAP-N6L
human	brain	Glioblastoma	U87-MG	5×10^{-5}	1×10^{-7}	1×10^{-9}
	cervix	adenocarcinoma	Primary cells	1×10^{-6}	3×10^{-8}	1×10^{-10}
			HeLa	4×10^{-5}	-	8×10^{-8}
	mammary gland	adenocarcinoma	MDA-MB-231	1×10^{-3}	8×10^{-6}	5×10^{-8}
	Colon		Caco-2	8×10^{-4}	7×10^{-6}	3×10^{-9}
	peripheral blood	acute myeloid leukemia	OCI-AML2	8×10^{-5}	5×10^{-7}	3×10^{-8}
			OCI-AML3	1×10^{-4}	8×10^{-6}	1×10^{-7}
mouse	embryo	-	HL60	$>1 \times 10^{-4}$	8×10^{-6}	8×10^{-8}
			NIH/3T3	3×10^{-5}	-	3×10^{-8}

Table 4 : Resume of IC_{50} for different cell lines

Mechanism of action of SAP-N6L

SAP-N6L acts *via* the NCL to target cancer cells

Response specificity assay was performed to understand if our conjugate bind cells *via* the the NCL . We treated glioblastoma cells with SAP-N6L and with or without antibody anti-NCL, and we quantified the cell viability with MTS assay. As shown in **figure 33**, the efficiency of SAP-N6L is significantly decreased (as well for N6L) in the presence of antibody anti-NCL as a competitor. So this result confirms that SAP-N6L and antibody anti-NCL are in competition for the same binding site. Saporin toxicity is not affected significantly in presence of antibody confirming data concerning SAP-N6L and its specific binding to NCL.

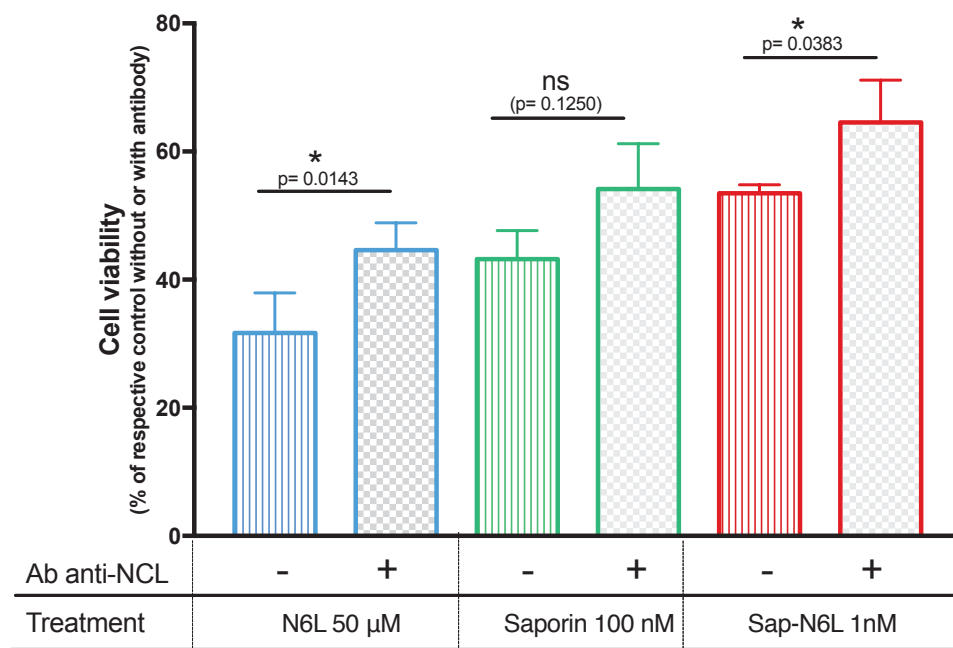


Figure 33: **Cytotoxicity of SAP-N6L in competition with Antibody Anti-NCL in U87-MG**

Cells viability after treatment of glioblastoma cell line (U87-MG) and SAP-N6L with antibody anti-NCL to understand if there is a competition between both for the same binding site. Data are percentage relative to respective control for each condition (with or without antibody); p values shown are the results of the ANOVA for SAP-N6L treatment condition versus SAP-N6L + antibody (N = 5 data are mean \pm SE).

SAP-N6L induces apoptosis in glioblastoma cells *in vitro*

As shown in **figure 34**, after treatment with SAP-N6L, N6L or Saporin, the quantity of nucleosomes fragmentation in cytoplasmic fractions, was measured to detect the cell death pathway. We observed that after treatment with 1nM of N6L, Saporin or SAP-N6L the quantity of fragmented nucleosomes (effect corresponding to an early event in apoptosis) is significantly increased in SAP-N6L treated cells.

To confirm this apoptotic pathway, that seems to be the most probable cause of cell death, we proceeded to Western-blot experiments (**figure 35 (a)(b) and (c)**). Activation of different caspases was confirmed by Western blotting using respectively a specific anti- procaspase 3,8 and 9 antibody supporting the hypothesis of apoptotic cell death.

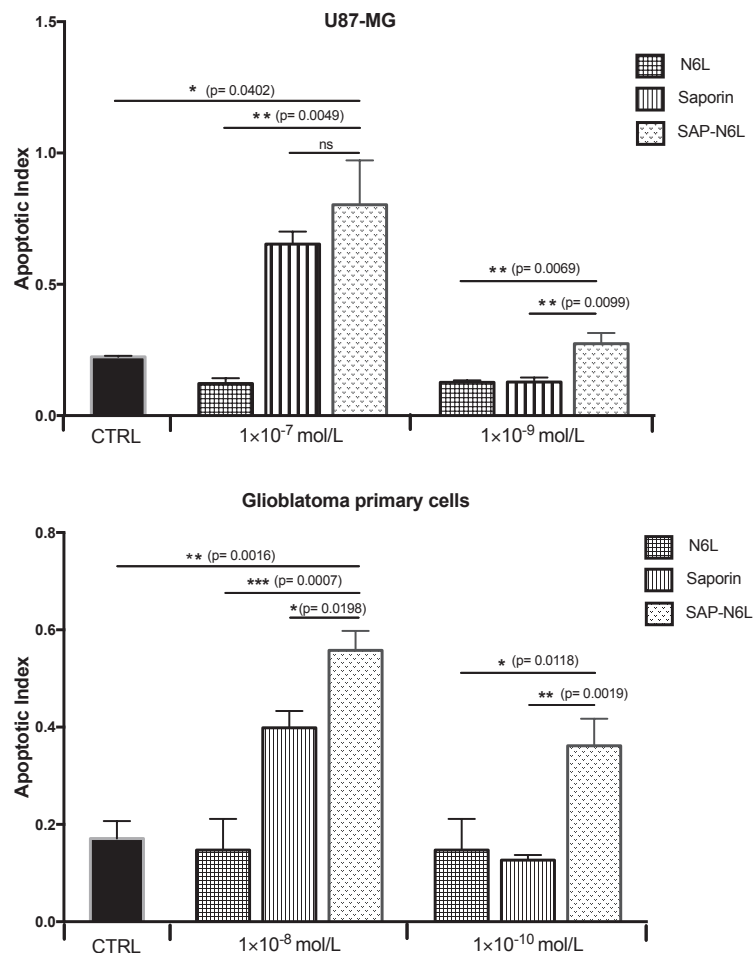
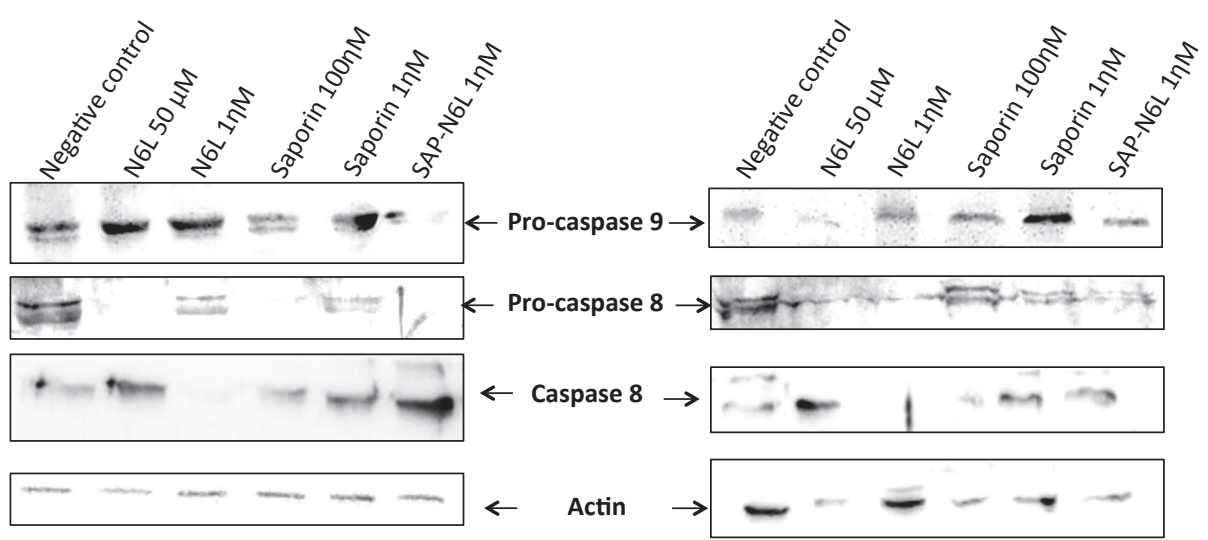
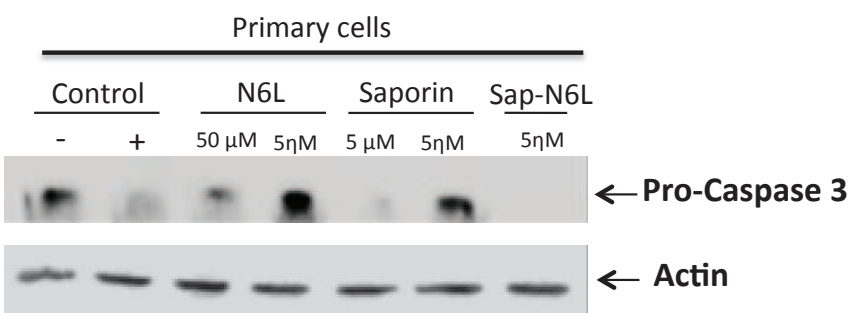
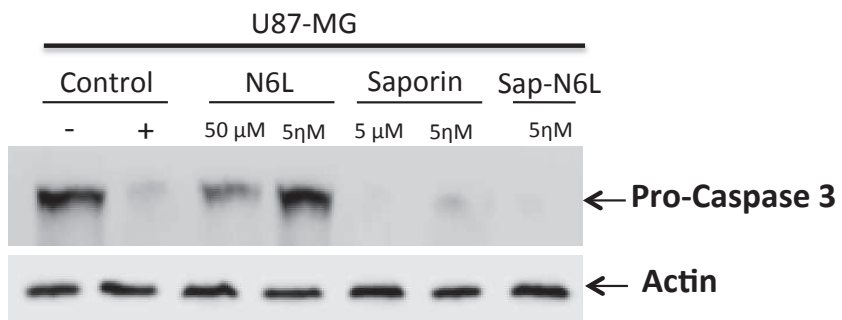


Figure 34: **Detection of nucleosomes fragmentation in cytoplasmic fractions**

Apoptosis was detected with Cell Death Detection ELISA kit. Cells were treated with different doses corresponding to IC₅₀ of each for 96 h. Cell lysate from adherent and floating cells was subjected to cell death ELISA assay to detect nucleosomes fragmentation (N = 6 data are mean ±SE). p values shown are the results of ANOVA test.



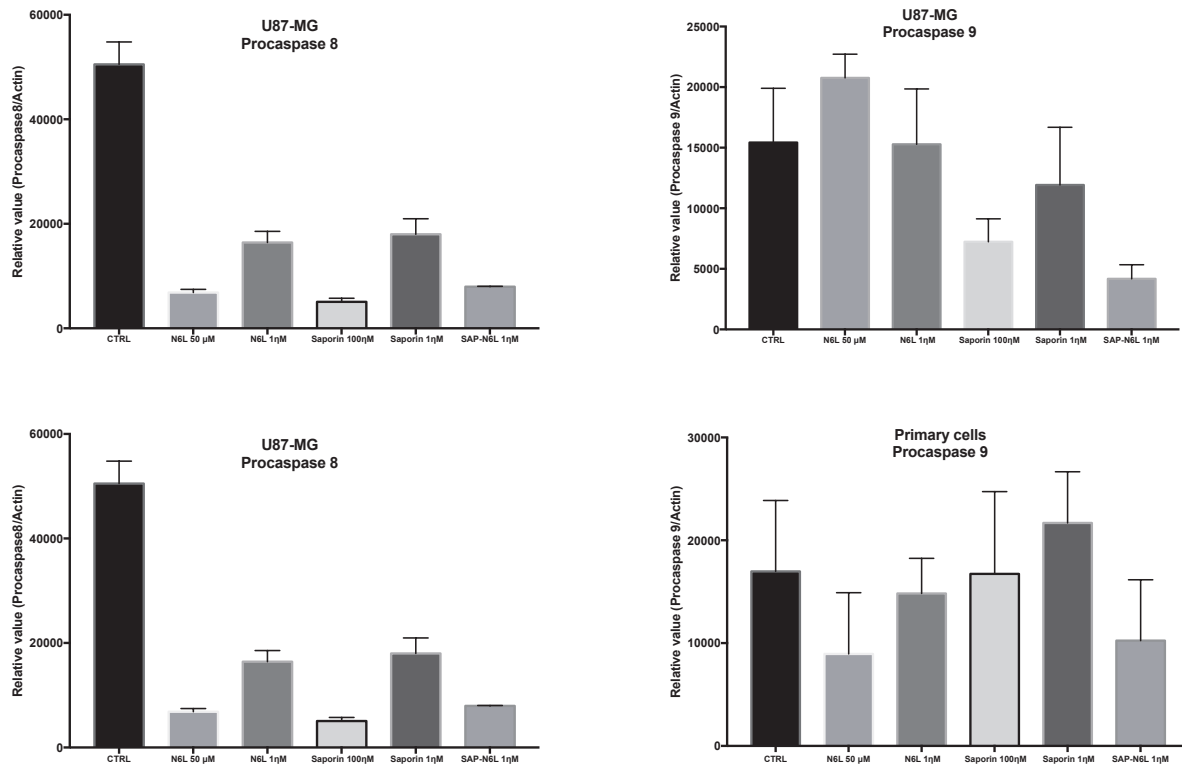


Figure 35: **Western Blot analysis**

Western Blot analysis of pro-caspase 3,8 and 9 * in cell lysates from glioblastoma cells (U87-MG and primary cells) treated with indicated concentrations of N6L, Saporin or SAP-N6L. Cis-platin was used as the positive control. Bar graph showing the data of procaspase 8 and procaspase 9 normalized by β -actin ($N = 3$ data are mean \pm SE)

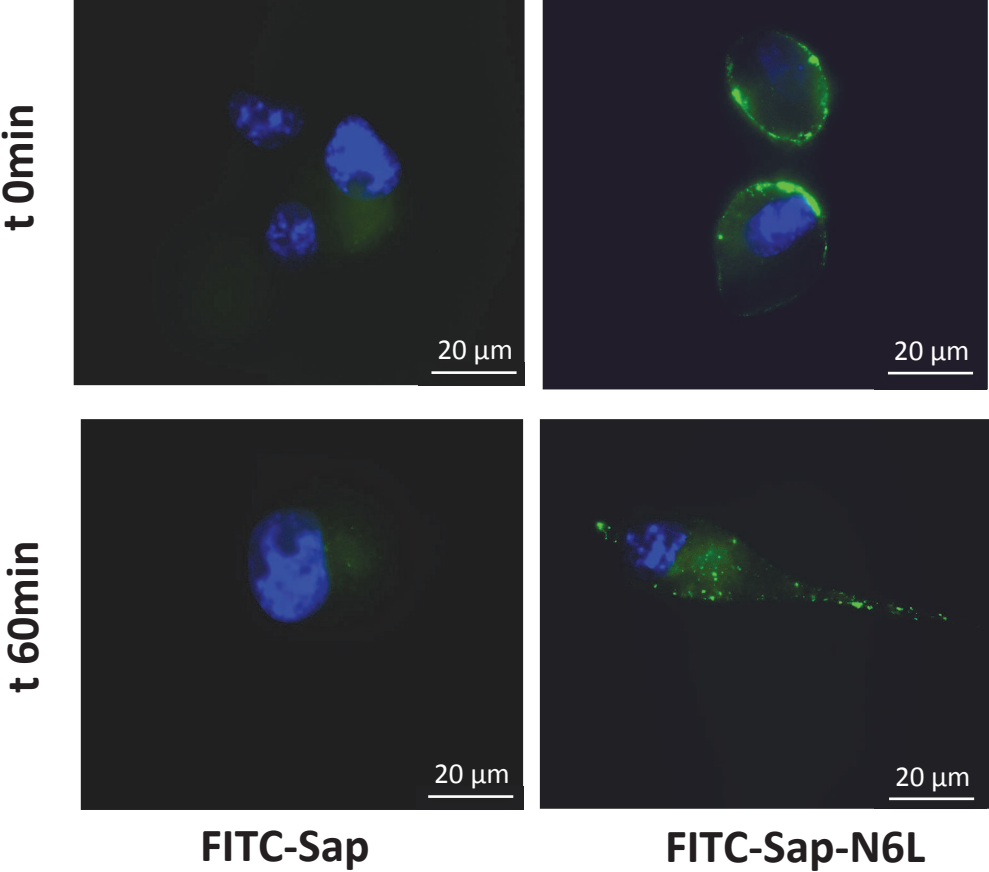
Internalization of SAP-N6L

Fluorescence microscopy assay showed (see **figure 36(a)**) that N6L is necessary for the internalization of saporin in U87-MG cells. After incubation of U87-MG cells, with 0,4 μ M FITC-Sap or SAP-N6L. We observed that FITC-Sap does not enter inside cells. On the contrary, at t0 FITC-SAP-N6L, is present on cell surface membrane but after 1h at 37°C, we can notice that the conjugate is inside cells and seems to be present in vesicles.

To understand which internalization pathway SAP-N6L is following, we did some colocalization experiments (**figure 36(b)(c)**). These complementary experiments have shown that the internalization pathway is different from the one taken by transferrin (endocytosis through coated pits) although SAP-N6L's pathway seems to be vesicular too. In addition, we

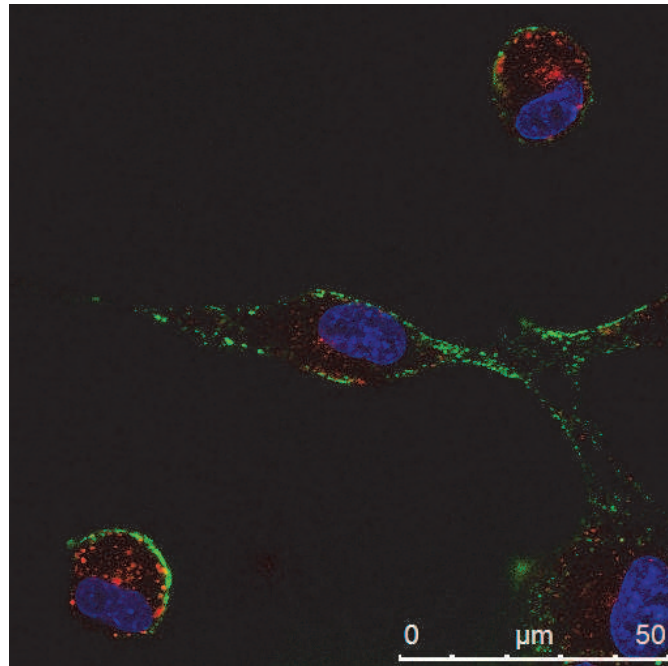
tried to obtain some colocalization with some markers of endocytic pathway (antibody anti-Lamp1, ant-TGN and anti-golgin; but we didn't find any significant colocalization.

(a)

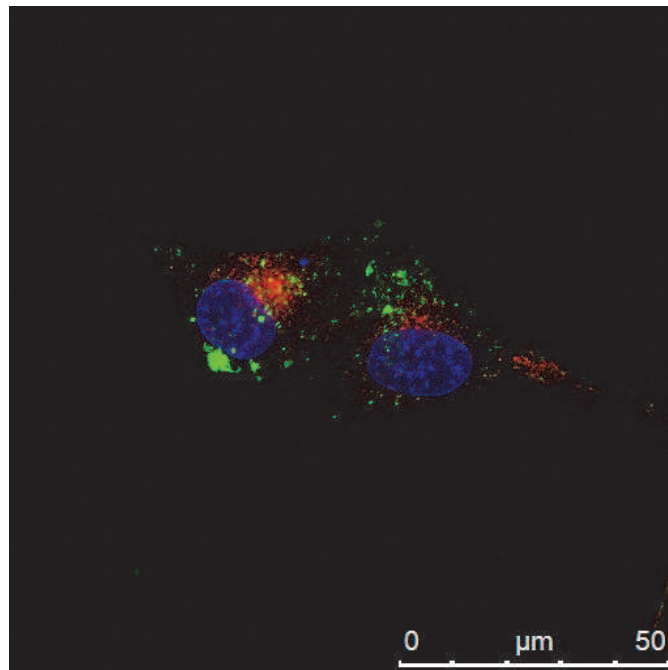


(b)

t 0min



t 60min



FITC-Sap-N6L
TexasRed-transferrin

(c)

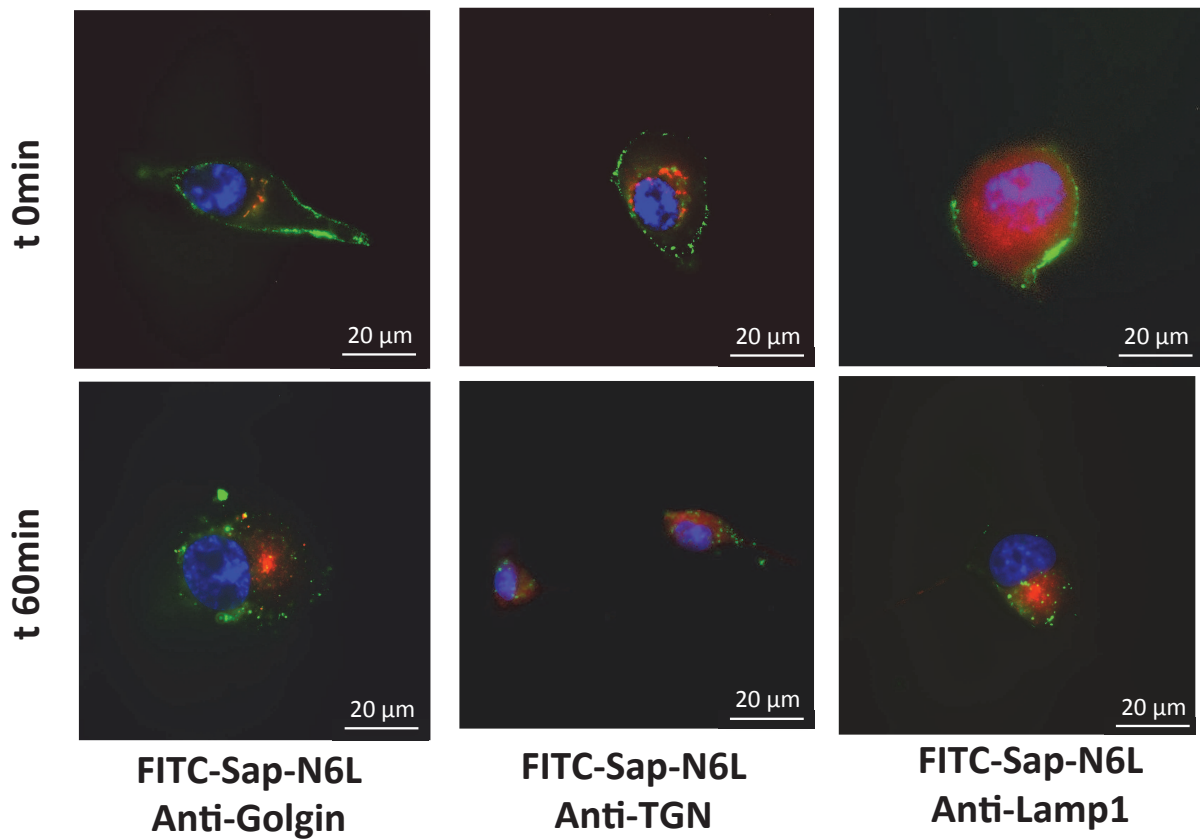


Figure 36: **Internalization of SAP-N6L**

(a) Fluorescence microscopy was used to follow internalization of Sap-N6L in U87-MG cells. Cells were incubated with 0,4 μM of saporin or Sap-N6L both labelled with FITC.

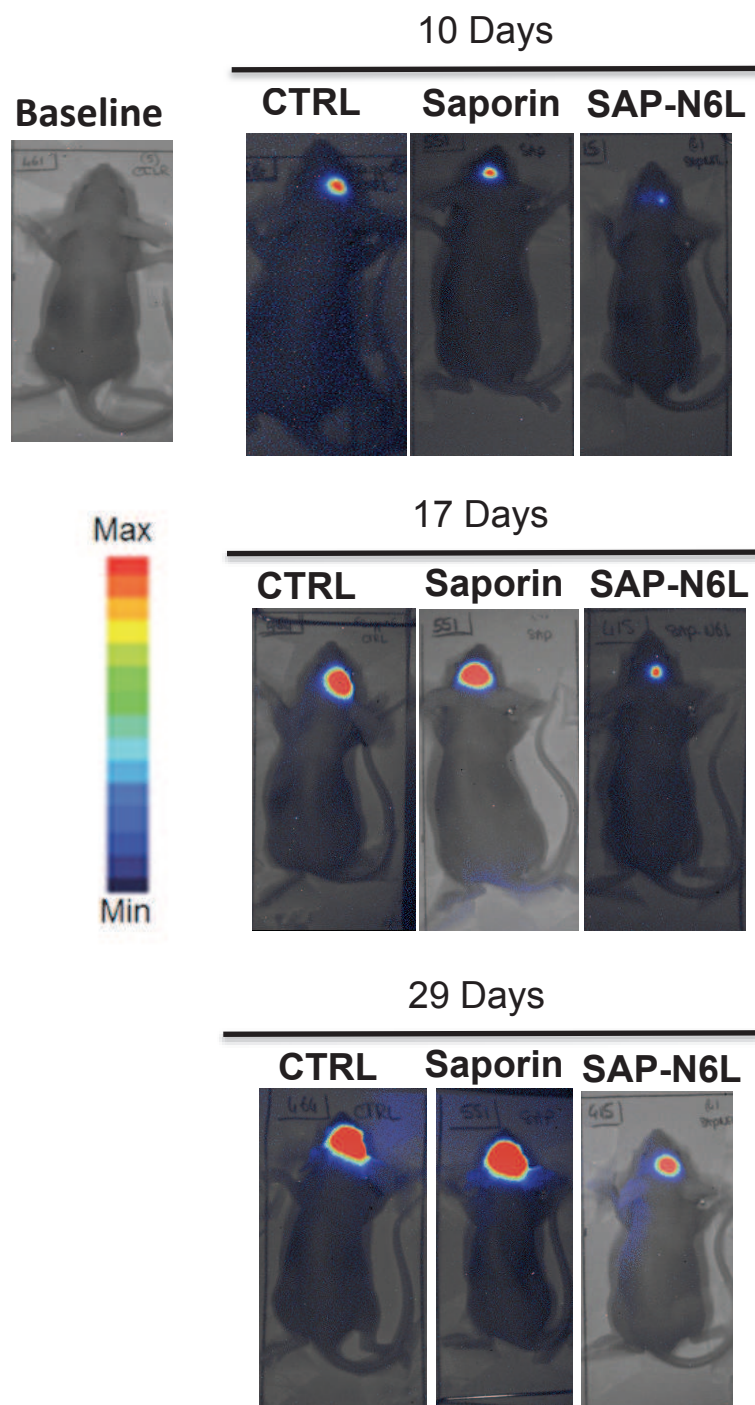
(b) Confocal laser scanning microscopic analysis after different times of incubation of U87-MG cells with 0,4 μM Sap-N6L (FITC labelled) and with 0,2 μM of Transferrin conjugated (Texas Red labelled).

(c) Immunofluorescence on U87-MG cells after incubation with 0,4 μM of FITC-Sap-N6L and different antibody anti-golgin, anti-TGN and anti-Lamp1.

***In vivo* effects of N6L**

Finally, *in vivo* studies were performed on BALB/c-nu/nu athymic mice injected intracranially with U87-LUC cells; the bioluminescence data, analyzed weekly, show a significant decrease of tumor growth in SAP-N6L systemically treated mice (**figure 37 (a)(b)**). In addition, the survival function shows that N6L is able to increase mice survival (**figure 37 (c)**).

(a)



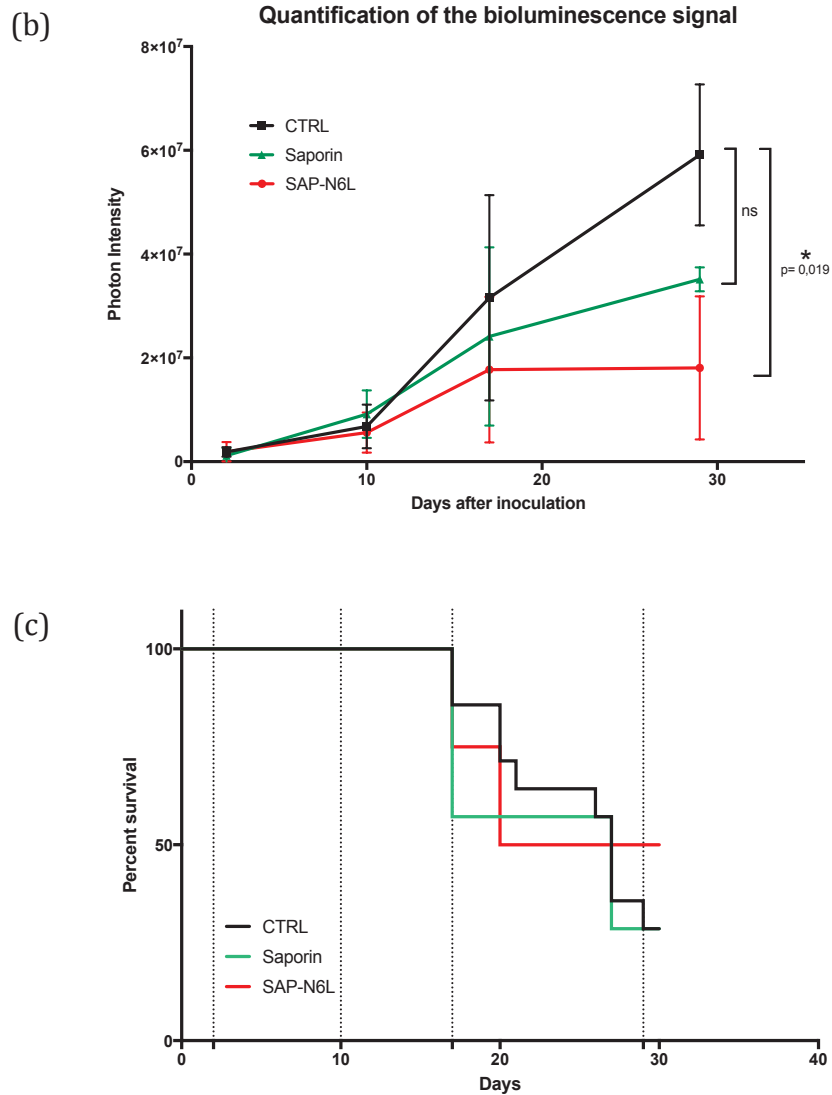


Figure 37: *In vivo* studies performed on BALB/c-nu/nu athymic mice injected intracranially with U87-LUC cells and treated with 0,5mg/kg of Saporin or SAP-N6L once by week from day 2 post-cell inoculation for 4 weeks

(a) representative image of bioluminescence in Control, Saporin and Sap-N6L treated mice is reported. (b) quantification of the bioluminescence signal recorded at the indicated times. p values shown are the results of the ANOVA for control group versus treated groups. (c) The survival function obtained by Kaplan-Meier survival plot of different groups.

Discussion

In this study, we investigated the biological effect of one antagonist of nucleolin (N6L) linked to a toxin *via* disulfide bridge. The multivalent pseudopeptide N6L targets a nucleolin-receptor complex overexpressed selectively at the cell surface of tumor cells, and mediates lethal effects to cancer cells after internalization, and antitumor activities. By using a tumor-specific cell internalization moiety (N6L) conjugated to a toxin (saporin, SAP), we allow this molecule to target cancer cells specifically. For that purpose, we obtained an active conjugate (SAP-N6L) that works with a two-step process: specific binding *via* N6L and cytotoxicity *via* saporin.

We observed that toxic activity of SAP-N6L is 1000 fold more efficient than saporin alone in glioblastoma primary cells and 100 fold more in the model glioblastoma cell line U87-MG. Since glioblastoma is known to be sensitive to N6L due to the presence of high amounts of nucleolin on the cell membrane, we wondered whether other human cancer cell lines were sensitive to SAP-N6L. We observed some differences among the different cancer cells tested in IC_{50} for SAP-N6L that may be associated either with different levels of cell surface NCL, or with different NCL partners on the surface of the different tumor cells. It was recently shown that targeting cell surface NCL is effective in inhibiting glioma cell migration only in cells that express $\alpha v \beta 3$ integrin (Koutsoumpa M P. C., 2013) These data favor the notion that the amounts of cell surface NCL differing among the different cancer cells determine the effectiveness of cell surface NCL targeting molecules, but knowing that N6L binds NCL but with a lower affinity also another nucleoprotein (nucleophosmin), we can explain why we observed a good activity of SAP-N6L in intestinal Caco-2 cells despite the fact that these cells don't express a big amount of NCL on cell surface. (Dean P, 2011 Jun;). Furthermore we tested N6L-SAP also on leukemic cells carrying WT (OCI-AML2) and heterozygous mutant (OCI-AML3) nucleophosmin, showing a slight difference in toxicity of N6L-SAP suggesting also in this case the interaction with this potential target, different from NCL.

We observed a great reduced activity of N6L and SAP-N6L in 3T3 mouse fibroblasts, as expected for a non human cell line of non tumour origin (even though hNCL and mNCL share large sequence homology) and furthermore, since saporin itself is poorly active on this cell line, we had a confirmation that N6L can drive the saporin toxin moiety inside the cells.

As it was already shown (Olivieri F, 1996), 3T3 and HeLa cells seem to be more resistant to saporin than other cells, as demonstrated by the fact that the inhibition on cells occurs in high concentration (> 100 nmol/L), suggesting that the RIP internalization is not mediated by a specific receptor. Saporin extracted from seeds has been shown to bind to alpha2-macroglobulin receptor, also called low-density lipoprotein receptor-related protein (LRP). LRP is expressed in many tissues and cell types, particularly in fibroblasts, monocytes and hepatocytes, and is responsible for the uptake and clearance of macromolecular complexes between proteinases and alpha2-macroglobulin (Cavallaro U, 1995). But recent studies show that cell binding and internalization activities of saporin to various cell lines does not appear to be only through LRP (Shveta Baggaa & , 2003). The mechanism of cell entry of type 1 RIPs have been examined in various studies, but some questions remain unanswered. A comparison between the endocytosis of ricin (type 2 RIP) and saporin indicated that the type 1 RIP follows a Golgi-independent pathway to the cytosol and does not require a low pH for membrane translocation which allows the internalization of the toxin without receptor binding (Vago R, 2005) (Bolognesi A P. L., 2012). The latter observation suggests that type 1 RIPs may also be able to follow the intracellular route of misfolded proteins without being degraded by the proteasome. Anyway, because of the lack of membrane binding subunit, type I RIPs are not very toxic to cells unless an action is taken to allow toxin internalization to the cytosol. With immunofluorescence assay, we confirmed that without N6L, saporin is not present inside cells. So SAP-N6L is efficient and carry saporin inside the cytoplasm.

To confirm that SAP-N6L enters the cells *via* nucleolin, we underwent competition experiments. Indeed we observed that SAP-N6L and antibody anti NCL are in competition for the same binding site, because there is a significative difference between cells' response after treatment with SAP-N6L alone or in the presence of this antibody.

It is still not clear how N6L is internalized after binding to nucleolin. In our study, we considered that SAP-N6L is internalized *via* the N6L and we tried to understand which pathway could be followed. This issue was investigated by looking for colocalization with different markers of endocytosis and retrograde transport pathway. SAP-N6L labelled with FITC didn't show any colocalization with these markers (LAMP1, TGN, Golgin) although it was revealed that SAP-N6L is present inside vesicles. As we know that transferrin enters the cell through clathrin-mediated endocytosis (Mayle KM, 2012) and it has been shown for example that the endostatin is internalized by this pathway after binding to nucleolin (Song N, 2012),

we hypothesized that SAP-N6L could take the same way. But FITC-SAP-N6L shows no colocalization with Transferrin. Therefore, SAP-N6L appears to employ an internalization mechanism, independent of conventional clathrin mediated-endocytosis. This alternative route could be caveolin –mediated endocytosis; in fact caveolae are the most common reported non-clathrin-coated plasma membrane buds or flotillin dependent endocytosis and it has been shown that nucleolin localizes to lipid rafts and associates with flotillin (Chen X, 2011).

Next step was to understand how the conjugate acts when it is inside cells. As saporin and N6L are binding through a disulfide bond, when SAP-N6L arrives in the cytoplasm, it can be disrupted because of reducing environment. Our results demonstrated that SAP-N6L induces cell death with concentrations in the low nanomolar range. At these concentrations, N6L acts like a targeting agent because it has no biological effect in that concentration range, so we hypothesized that toxicity comes only from saporin.

Saporin can induce cell death by more than one pathway and this behaviour may render more difficult to tumor cells to acquire a resistant phenotype to saporin-induced cell death. So mechanism of intoxication and cell death induced by saporin is not so clear in general, but apoptosis seems to be the main detectable effect in several different cell lines. We observed that SAP-N6L induces nucleosomes fragmentation in cytoplasmic fractions with results comparable to those obtained with free saporin used 1000 fold more concentrated. So saporin linked to N6L is 1000 fold more efficient to induce apoptosis than saporin alone. Activation of different caspases was confirmed by Western blotting using respectively a specific anti- procaspase 3, 8 and 9.

Finally, *in vivo* studies were performed on BALB/c-nu/nu athymic mice injected intracranially with U87-LUC cells; the bioluminescence data, analyzed weekly, show a significant decrease of tumor growth in SAP-N6L treated mice. In addition, the survival function shows that N6L is able to increase mice survival. In fact in SAP-N6L treated group, we can observe that at the end of experiment 50% mice of this group are still alive if compared to only 28,5% in both control and saporin groups. These results are a good starting point and but demand further study, including the use of implantable osmotic pumps for a direct local administration.

Part II.b

Nucleolin as a tool for Targeting Cancer

Cells :

a) Using an Aptamer in the engineering and production of a saporin chimeric toxin

b) Using a pseudopeptide in the engineering and production of a saporin chimeric toxin

1 . Saporin from seeds of *Saponaria officinalis*

2 . Recombinant saporin

2. Recombinant Saporin

Background

SAP-N6L was produced by using SDPD N-succinimidyl 3(2-pyridyl)dithio)-propionate to cross-link N6L-SH and native saporin. Lysine residues in saporin were first derivatized by the cross-linking agent and then coupled to the cysteine residue of N6L, resulting in heterogeneous molecule that we found difficult to characterize. Indeed, as saporin has 24 lysine residues, the result is a mixture containing various molar ratios of cross-linker/saporin. It is not known which lysines in saporin are modified or whether there is preference for modification of some lysines. Therefore, we sought to synthesize a homogeneous chemical conjugate which would make it possible to conduct reproducible efficacy and toxicity studies for potential drug development.

Here we report the synthesis and characterization of a homogeneous chemical conjugate produced from a recombinant saporin with different mutations to introduce cysteine residue (**figure 38**). We chose to introduce a Cys in the saporin isoform SAP-6 because native saporin does not contain any cysteine residues. Therefore, recombinant saporin containing a cysteine codon added at the position 14 or 158 (instead Alanine residue) was cloned and expressed in E.coli BL21 (DE3). We used this expression system although we know the intrinsic toxicity against host bacteria, a phenomenon reported to limit the expression of saporin-based chimeras (Giansanti F D. L., 2010) (Lombardi A, 2010).

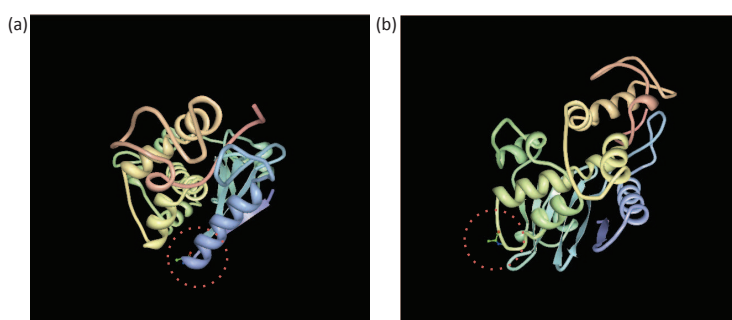


Figure 38: Localisation of different mutations in 3D structure of saporin
Red circle represents Alanine 14 (a) and Alanine 158 (b)

Results

Expression, purification of recombinant saporin WT and mutants

After setting the culture/induction conditions (induction at 25°C for 4h after administration of 1mM IPTG) the screening of recombinants clones was performed and the best ones selected for scale-up protein expression (red boxes in **figure 39**).

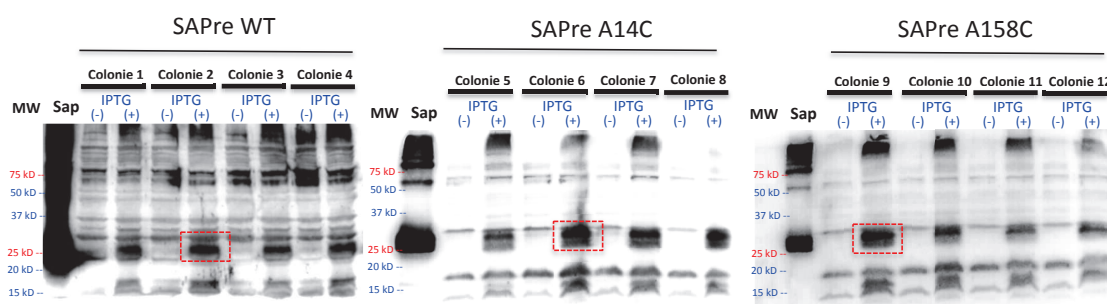


Figure 39: **Screening of different colonies after IPTG induction** by Western Blotting using anti- saporin antibody

Samples correspond to E. coli BL21 cell lysate of different colonies with recombinant plasmid of Sap WT, Sap A14C and Sap A158C before and after induction (4 h) by 1 mM IPTG.

The pellet of 12 liters of culture of induced bacteria was resuspended in different buffers and after several step (described in Materials and Methods), the final supernatant was loaded in ion exchange column. The elution was done with a continuous linear salt gradient from 0% to 30% of NaCl 1M.

Recombinant Saporin WT (SAPre WT)

As shown in **figure 40** SAPre WT was particularly present in the fractions (black circles) corresponding to about 200mM of NaCl.

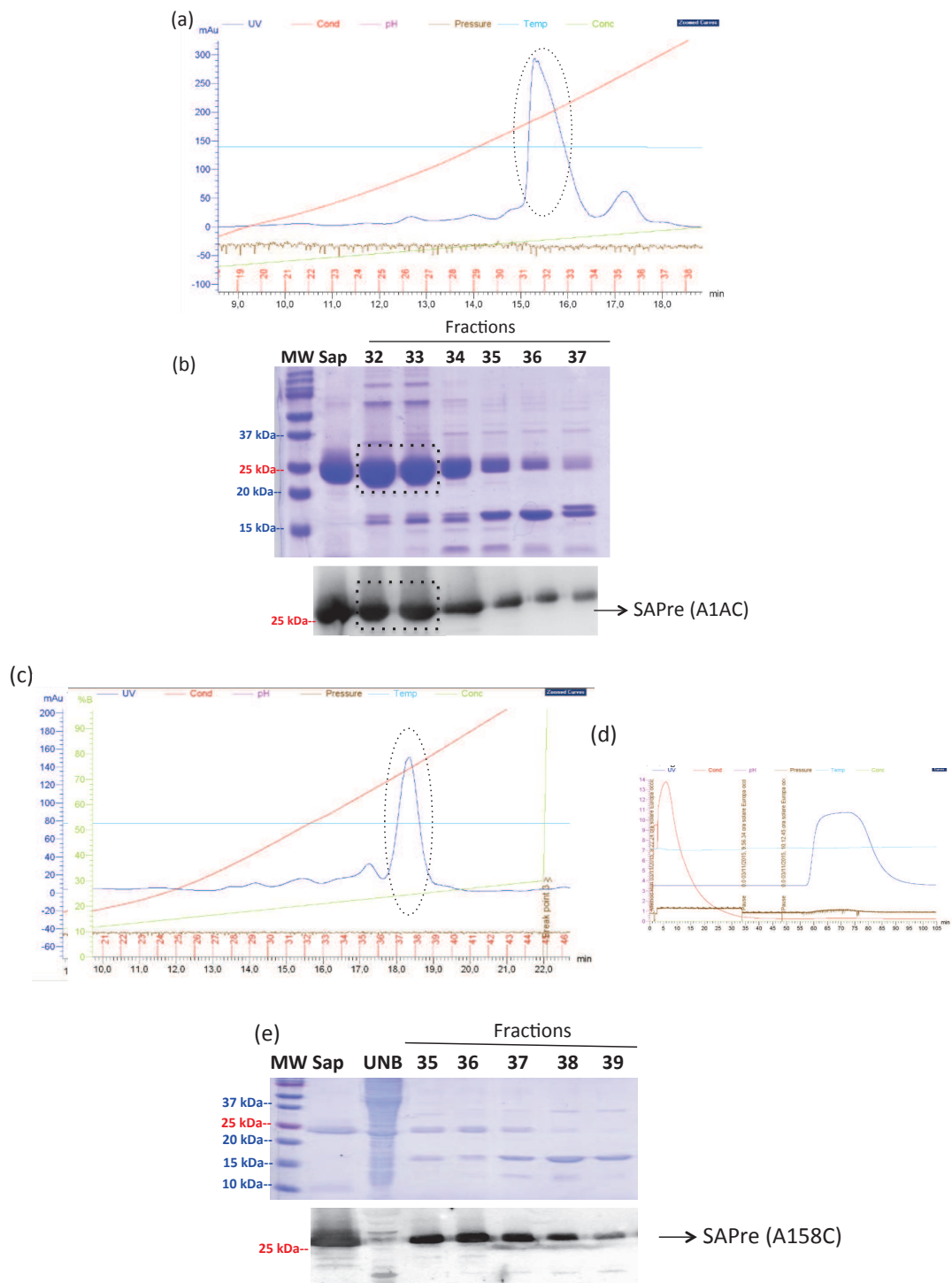


Figure 41: Purification of recombinant saporin A158C from 12 liters of bacterial culture

(a) ion-exchange chromatographic purification of recombinant saporin A158C. Fraction marked by the circle (37) corresponds to a 220 mM NaCl concentration in the linear gradient used for elution. Fractions 35, 36, 37, 38 and 39 are reported in the SDS-PAGE and blot analysis (antibody anti-saporin). (b) SDS-PAGE analysis of fractions obtained from the ion-exchange chromatography. Sap correspond to saporin from seeds, UNB correspond to unbinding fraction during loading of sample in column (c). The samples were stained either by Coomassie-blue or analysed by western blotting using a polyclonal anti-SAP serum.

***In vitro* evaluation of toxicity**

The effect of SAPre (WT, A14C and A158C) in GB cell growth inhibition *in vitro* is similar compared to natural Saporin

To evaluate the biological activity the three recombinant toxins (SAPre WT, SAPre A14C and SAPre WT A158C) were tested on U87-MG. **Figure 42** shows the MTS cell viability assay obtained for all or Saporin purified from seeds of *Saponaria officinalis* at reported concentrations (10^{-6} – 10^{-12} M). Recombinant saporin (WT and A14C mutant) induce cell death on U87-MG cells with a similar IC_{50} around 10^{-8} M, while A158C mutant performs slightly better. So the toxicity is well conserved and the mutations didn't affect this activity.

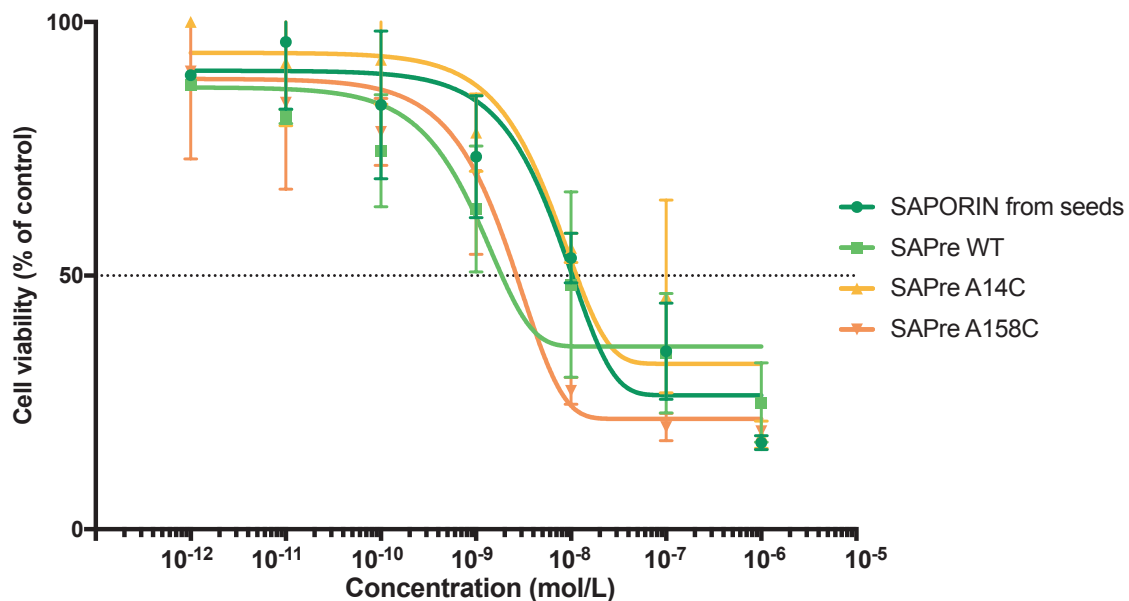


Figure 42: **Dose response curve showing IC_{50}** of Saporin from seeds (in dark green), recombinant saporin WT (in light green), and two different mutants of recombinant saporin : SAPreA14C (in yellow) and SAPreA158C (in orange) in glioblastoma cells line (U87-MG). Cells (2×10^3 / well) were seeded in 96-well plates in a final volume of 200 μ l of medium (5% of FBS) containing appropriate concentrations for 96h. Viability was evaluated using a colorimetric assay based on MTS reduction. (N = 3 data are mean).

Cross-linking between SAPre and N6L-SH

SAPre and N6L-SH were linked via a disulfide bridge. In an effort to minimize SAPre dimerization and N6L-SH oligomerisation, they were pre-treated respectively with DTNB and TCEP. Then excess was removed by gel filtration. After reaction overnight between SAPre and N6L-SH in hypoxic conditions, N6L unreacted was removed by dialysis. We tried to separate SAPre-N6L and Sapre unreacted by gel filtration chromatography but without success given the very small size difference between both (4 kDa). So we decided to work with the mix directly after reaction assuming that we have free saporin inside.

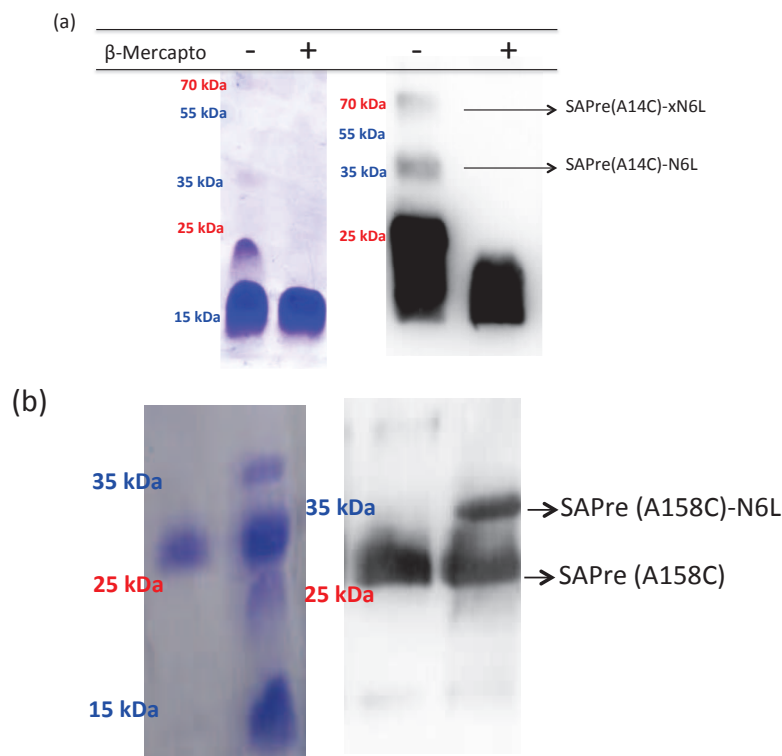


Figure 43: **SAP-N6L conjugate with mutated recombinant saporin**

SDS-PAGE and Western-Blot analysis after reaction between recombinant saporin (a) SAPre A14C, (b) SAPre A158C and N6L-SH and dialysis to eliminate free N6L-SH. Samples were analyzed on a 15% SDS-PAGE. The figure shows coomassie brilliant blue staining of SDS gel and Western Blot with antibody anti-saporin. (a) lane legends are 1: mix after reaction (SAPre A14C+ N6L-SH) in non reducing conditions, 2 : mix after reaction (SAPre A14C+ N6L-SH) under reducing conditions. (b) lane legends are 1: saporin from seed as a control, 2 : mix after reaction (SAPre A158C+ N6L-SH) in non reducing conditions.

The effect of SAPre-N6L cell growth inhibition *in vitro* is not increased if compared to and Saporin alone in GB cell lines.

Figure 44 shows the MTS cell viability assay obtained for both chimeras or Saporin purified from seeds of *Saponaria officinalis* at reported concentrations (10^{-6} – 10^{-11} M)

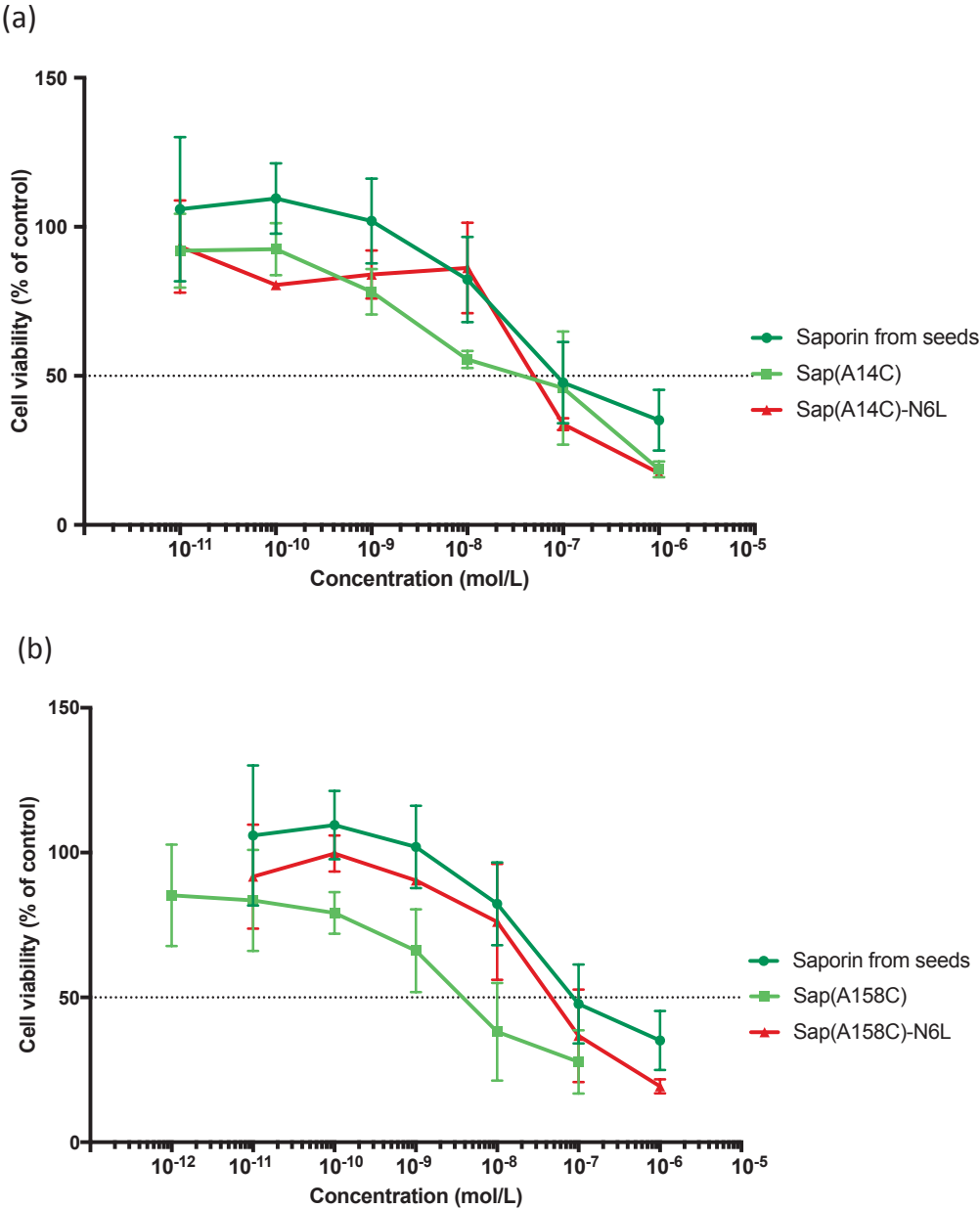


Figure 44: **SAP-N6L conjugate with recombinant mutated saporin**
 Dose response curve showing IC₅₀ of Saporin from seeds (in dark green), recombinant saporin Sap(A14C) (a) or Sap(A158C) (b) (in light green), and Sap(A14C)-N6L (a) or Sap(A158C)-N6L (b) (in red) in glioblastoma cells line (U87-MG).

Discussion

The results obtained suggest that our conjugate SAP-N6L with a ratio [1 :1] is not efficient to kill cancer cells specifically. Indeed, with this conjugate we didn't obtain any increase of toxicity compared to saporin alone. In first part of this study we was working with an heterogenous conjugate constituted with one saporin and different numbers of N6L (1 until 9). Our experiments do not permit us to conclude if it was one N6L in different sites in saporin, or if the N6L structure itself as a cluster (**figure 28(b)**). Indeed as the chemical cross-linker acts on Lysine residues and as saporin has 24 lysine residues, the result is a mixture containing various molar ratios of cross-linker/saporin.

The other possibility to explain this inactivity could be that as N6L is a very small peptide, even if we chose residue in surface of the saporin (**figure 38**) to insert SH group (*via* substitution of Alanine by Cysteine), maybe this two position do not allow N6L to bind cell surface nucleolin and permit internalization of Saporin.

Increasing the number of mutations and so far the number of N6L (under control by mutagenesis) for one saporin may be a strategy to obtain efficient delivery of toxic cargos in cancer cells.

Further experiments with the N6L attached in a wider variety of positions could help elucidate some of these unknowns.

Conclusion and perspectives

In this PhD work we investigated three different tools for cellular targeting of the ribosome inactivating protein : Saporin. It's a toxin widely studied for the production of immunotoxins (conjugates of an antibody) (Flavell DJ C. S., 1991) (Flavell DJ N. A., 1997) (Anselmo AC, 2014) and targeted toxins (Ippoliti R L. E., 1996) (Giansanti F, 2010) (Lombardi A, 2010) (Cimini A M. S., 2012). As possible carriers for toxic molecules, we tested PDZ domain, AS1411 aptamer and the pseudopeptide NucANT (N6L).

We focused on GBM model because it is actually one of the most dangerous cancers and it remains incurable. The failure of conventional oncologic treatment to selectively eradicate glioblastoma cells has prompted investigators to look for new and more targeted therapeutic options, as well as for improved prognostic biomarkers. However, the most challenges for chemotherapeutic drugs are the blood brain barrier (BBB) and non-specific delivery to non-tumor tissues. In particular, the relatively weak enhanced permeability and retention (EPR) effect of brain tumors compared with peripheral tumors results in the low delivery efficiency to the brain tumor tissues. In recent decades, with the advantages of passive targeting strategy, nano-drug delivery systems have been paid increasing attention to delivering drugs to the exact foci through the interactions between targeting moieties and ligand of protein highly expressed in specific cell type in glioma tissues to improve pharmacokinetics/pharmacodynamics (Luo Z, 2017).

More and more biomarkers continue to be identified in GBM patients. Developing new drugs to treat malignant glioma is urgently needed and in this study we present two new biomarker ; CD98 and nucleolin, and three possibility to target them (PDZ domain, aptamer and pseudopeptide).

This work was aimed at studying the possibility to kill cancer cells, in particular glioblastoma, with specific peptides targeting surface antigens, such as CD98 and nucleolin, and driving a toxic molecule (saporin) inside the tumor. Such peptides were linked to the toxin using either chemical cross-linking or genetic engineering approaches. The results overall demonstrate that this approach may give interesting results if the toxin is delivered to the cancer surface by a multivalent system (more than a single peptide) and that engineering the complexes may possibly help to solve this issue. Indeed, we observed that drug targeting using monomeric protein systems are less efficient than polymeric ones. It has been

demonstrated in a recent review that a conjugation strategy that results in a more heterogeneous mixture is not a drawback (Behrens CR, 2014). Actually some experiments concerning antibody drug conjugates led to the conclusion that the optimal loading was two to four drugs per antibody to maximize potency while avoiding rapid clearance from circulation. While the cysteine and lysine attachment methods can be adjusted to give an average drug loading of two to four per antibody, the resulting mixture will still be heterogeneous and contain species with both less and more drug loading than desired. ADC produced by chemical crosslinking approved by FDA (Adcetris[®], marketed by Seattle Genetics and Millennium/Takeda, and Kadcyra[®], marketed by Genentech and Roche) are so heterogeneous conjugates.

Furthermore we demonstrated that the delivery of the toxin gene using an aptamer-based carrier, may be suggested as an alternative approach to kill cancer cells by an enzyme activity revealed only inside the target cell. The use of multiple peptides to deliver the gene of the toxin may be the future developments of this research.

Publications

- Giansanti F, Sabatini D, Pennacchio MR, Scotti S, Angelucci F, **Dhez AC**, Antonosante A, Cimini A, Giordano A, Ippoliti R., PDZ Domain in the Engineering and Production of a Saporin Chimeric Toxin as a Tool for targeting Cancer Cells. *J Cell Biochem.* 2015
- Benedetti E., Antonosante A., d'Angelo M., Cristiano L., Galzio R., Destouches D., Florio T.M., **Dhez AC.**, Cinque B, Fidoamore A., Rosati F, Cifone MG, Ippoliti R., Giordano A., Courty J., Cimini A.,, Nucleolin antagonist NucAnt 6L triggers autophagic cell death in human glioblastoma primary cells., *Oncotarget* ,2015
- Benedetti E, d'Angelo M, Ammazalorso A, Gravina G, Laezza C, Antonosante A, Panella G, Cinque B, Cristiano L, **Dhez AC**, Astarita C, Galzio R, Cifone MG, Ippoliti R, Amoroso R, Di Cesare E, Giordano A, Cimini A. PPAR α Antagonist AA452 Triggers Metabolic Reprogramming and Increases Sensitivity to Radiation Therapy in Human Glioblastoma Primary Cells, *J Cell Physiol*, 2016
- De Cola A. , Franceschini M., Di Matteo A., Colotti G., Celani R., Clemente E., Ippoliti R, **Dhez AC.**, De Laurenzi V., Courty J. and Federici L. N6L pseudopeptide interferes with nucleophosmin protein-protein interactions and sensitizes leukemic cells to chemotherapy. (under submission in April 2017)
- **Dhez Anne-Chloé**, Benedetti Elisabetta, Antonosante Andrea, Panella Gloria, Ranieri Brigida, Florio Tiziana Marilena, Cristiano Loredana, Giansanti Francesco, Di Leandro Luana, d'Angelo Michele, De Cola Antonella, Federici Luca, Courty José, Cimini Annamaria, Ippoliti Rodolfo., Targeted therapy of human glioblastoma via delivery of a toxin through a peptide directed to cell surface nucleolin. (under submission in April 2017)

Spoken productions

- Poster presentation in 14th International Congress on Targeted Anticancer Therapies Washington on March 2016
- Poster presentation in 29th annual conference of Italian Association of Cell Cultures in L'Aquila on November 2016

Bibliography

Adessi C, S. C. (2002, May). Converting a peptide into a drug: strategies to improve stability and bioavailability. *Curr Med Chem.* , ;9(9):963-78.

Ahluwalia MS, G. C. (2010). Progress on antiangiogenic therapy for patients with malignant glioma. *J Oncol.* , 689018.

Alewine C, H. R. (2015 , Feb). Advances in anticancer immunotoxin therapy. *Oncologist.* , ;20(2):176-85.

Alexandrov LB, N.-Z. S.-D. (2013, Aug 22). Signatures of mutational processes in human cancer. *Nature.* , 500(7463):415-21.

Anselmo AC, M. S. (2014, Sep 28). An overview of clinical and commercial impact of drug delivery systems. *J Control Release.* , 190:15-28.

Bachran D, S. S. (2011). The endocytic uptake pathways of targeted toxins are influenced by synergistically acting Gypsophila saponins. *Mol Pharm.* , 8(6):2262-72.

Bagga S, H. M. (2003,). Cytotoxicity of ribosome-inactivating protein saporin is not mediated through alpha2-macroglobulin receptor. *FEBS Lett.* , 541, 16–20.

Bagga S, S. D. (2003, Feb). The cytotoxic activity of ribosome-inactivating protein saporin-6 is attributed to its rRNA N-glycosidase and internucleosomal DNA fragmentation activities. *J Biol Chem.* , 14;278(7):4813-20.

Bai RY, S. V. (2011, Jun). Molecular targeting of glioblastoma: Drug discovery and therapies. *Trends Mol Med.* , 17(6):301-12.

Balss J, M. J. (2008, Dec). Analysis of the IDH1 codon 132 mutation in brain tumors. *Acta Neuropathol.* , 116(6):597-602.

Barbieri L, B. M. (1982). Reduction of ricine and other plant toxins by thiol:protein disulfide oxidoreductases. *Arch. Biochem. Biophys.* , 216, 380–383 .

Barbieri L, B. M. (2003). Ribosome-inactivating proteins depurinate poly(ADP-ribosyl)ated poly(ADP-ribose) polymerase and have transforming activity for 3T3 fibroblasts. . *FEBS Lett.* , 538, 178–182.

Barbieri L, V. P. (1997). Polynucleotide: Adenosine glycosidase activity of ribosome-inactivating proteins: Effect on DNA, RNA and poly(A). *Nucleic Acids Res.* 25, 518–522.

Batchelor TT, S. A. (2007, Jan). AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients. *Cancer Cell.* , 11(1):83-95.

Bates PJ, K. J. (1999, Sep). Antiproliferative activity of G-rich oligonucleotides correlates with protein binding. *J Biol Chem.* , 274(37):26369-77.

Bates PJ, L. D. (2009, Jun). Discovery and development of the G-rich oligonucleotide AS1411 as a novel treatment for cancer. *Exp Mol Pathol.* , ;86(3):151-64.

Bates PJ, R.-R. E. (2016, Dec 20.). G-quadruplex oligonucleotide AS1411 as a cancer-targeting agent: Uses and mechanisms. *Biochim Biophys Acta.* , S0304-4165(16)30509-8.

Battelli MG, B. A. (1998). Different sensitivity of CD30+ cell lines to Ber-H2/saporin-S6 immunotoxin. *J Drug Target* , 5(3):181-91.

Battelli MG, M. V. (1992, Jul). High sensitivity of cultured human trophoblasts to ribosome-inactivating proteins. *Exp Cell Res.*, 201(1):109-12.

Behrens C and Liu B (2014) Methods for site specific drg conjugation to antibodies. *mAbs*, 6:1,46-53.

Belenguer P, C.-F. M. (1990 , Jul). Mitosis-specific phosphorylation of nucleolin by p34cdc2 protein kinase. *Mol Cell Biol* , 10(7):3607-18.

Bellail AC, H. S. (2004, Jun). Microregional extracellular matrix heterogeneity in brain modulates glioma cell invasion. *Int J Biochem Cell Biol*, 36(6):1046-69.

- Bellisola G, F. G.** (2004, May 1). Reductive activation of ricin and ricin A-chain immunotoxins by protein disulfide isomerase and thioredoxin reductase. *Biochem Pharmacol.* , ;67(9):1721-31.
- Benedetti E, A. A.** (2015, Dec). Nucleolin antagonist triggers autophagic cell death in human glioblastoma primary cells and decreased in vivo tumor growth in orthotopic brain tumor model. *Oncotarget.*
- Benny O, P. P.** (2009). Novel technologies for antiangiogenic drug delivery in the brain. . *Cell Adh Migr.* , 3(2):224-9.
- Bhatt P, d. C.** (2012, Feb);). Specific domains of nucleolin interact with Hdm2 and antagonize Hdm2-mediated p53 ubiquitination. *FEBS J.* , 279(3):370-83.
- Bjørkøy G, Lamark T, Pankiv S, Øvervatn A, Brech A, Johansen T.** Monitoring autophagic degradation of p62/SQSTM1. *Methods Enzymol.* 2009;452:181-97.
- Biernat W, K. P.** (1997, Feb). Amplification and overexpression of MDM2 in primary (de novo) glioblastomas. *J Neuropathol Exp Neurol.* ;56(2):180-5.
- Blythman HE, C. P.** (1981, Mar). Immunotoxins: hybrid molecules of monoclonal antibodies and a toxin subunit specifically kill tumour cells. *Nature.* , 12;290(5802):145-6.
- Bolognesi A, P. L.** (2004). Immunotoxins and other conjugates: pre-clinical studies. *Mini Rev Med Chem.*
- Bolognesi A, P. L.** (2012). Endocytosis and intracellular localisation of type 1 ribosome-inactivating protein saporin-s6. *J Biol Regul Homeost Agents.* , 26(1):97-109.
- Bolognesi A, P. L.** (2002). Ribosome-inactivating and adenine polynucleotide glycosylase activities in *Mirabilis jalapa* L. tissues. *Biol. Chem.* , 13709–13716.
- Bolognesi A, T. P.** (1992 , Sep). A comparison of anti-lymphocyte immunotoxins containing different ribosome-inactivating proteins and antibodies. *Clin Exp Immunol.* , 89(3):341-6.
- Bolognesi A, T. P.** (1996). Induction of apoptosis by ribosome-inactivating proteins and related immunotoxins. *Int. J. Cancer* , 68, 349–355.
- Boohaker RJ, L. M.** (2012). The Use of Therapeutic Peptides to Target and to Kill Cancer Cells. *Current medicinal chemistry* , 19(22):3794-3804.
- Borthakur G.** (2013, Aug). Precision 're'arming of CD33 antibodies. *Blood.* , 22;122(8):1334.
- Bourbon HM, A. F.** (1990, Apr). Nucleolin gene organization in rodents: highly conserved sequences within three of the 13 introns. *Gene.* , 88(2):187-96.
- Bourbon HM, L. B.** (1988, Apr). Structure of the mouse nucleolin gene. The complete sequence reveals that each RNA binding domain is encoded by two independent exons. *J Mol Biol.* , 200(4):627-38.
- Bouvet P, D. J.** (1998, Jul 24). Nucleolin interacts with several ribosomal proteins through its RGG domain. *J Biol Chem.* , 273(30):19025-9.
- Brat DJ, C. P., & Members of the Cancer Biomarker Reporting Committee, C. o.** (2015, Sep). Template for Reporting Results of Biomarker Testing of Specimens From Patients With Tumors of the Central Nervous System. *Arch Pathol Lab Med.*;139(9):1087-93.
- Brown PD, K. S., & N0177., N. C.** (2008). Phase I/II trial of erlotinib and temozolomide with radiation therapy in the treatment of newly diagnosed glioblastoma multiforme: North Central Cancer Treatment Group Study N0177. *J Clin Oncol.* , 1;26(34):5603-9.
- Bugler B, C.-F. M.** (1982, Nov).Detection and localization of a class of proteins immunologically related to a 100-kDa nucleolar protein. . *Eur J Biochem.* , 128(2-3):475-80.
- Burks DJ, W. J.** (1998, Nov 20). IRS pleckstrin homology domains bind to acidic motifs in proteins. *J Biol Chem.* ;273(47):31061-7.

- Caizergues-Ferrer** M, M. P. (1989, Mar). Nucleolin from *Xenopus laevis*: cDNA cloning and expression during development. *Genes Dev.*;3(3):324-33.
- Callebaut** C, N. S. (2001, Mar 15). Inhibition of HIV infection by the cytokine midkine. *Virology.* ;281(2):248-64.
- Cantor** JM, G. M. (2012, Mar). CD98 at the crossroads of adaptive immunity and cancer. *J Cell Sci.* , 15;125(Pt 6):1373-82.
- Carpentier** M, M. W. (2005, Apr). Nucleolin undergoes partial N- and O-glycosylations in the extranuclear cell compartment. *Biochemistry.* , 19;44(15):5804-15.
- Carzaniga**, R. S.-S. (1994). Cellular and subcellular distribution of saporins, type I ribosome-inactivating proteins, in soapwort (*Saponaria officinalis* L.). *Planta,* , 194, 461–470.
- Cavallaro** U, N. A. (1995, Aug 15;). Alpha 2-macroglobulin receptor mediates binding and cytotoxicity of plant ribosome-inactivating proteins. *Eur J Biochem.* , 232(1):165-71.
- Chakravarti** A, W. M.-F. (2013, Apr). RTOG 0211: a phase 1/2 study of radiation therapy with concurrent gefitinib for newly diagnosed glioblastoma patients. . *Int J Radiat Oncol Biol Phys.* 1;85(5):1206-11.
- Chamberlain** MC, G. M. (2007, Mar). Early necrosis following concurrent Temodar and radiotherapy in patients with glioblastoma. *J Neurooncol.* , 82(1):81-3.
- Chandra** M, Z. S. (2012 , Jun). Nuclear translocation of type I transforming growth factor β receptor confers a novel function in RNA processing. *Mol Cell Biol.* , 32(12):2183-95.
- Chatzidakis** I, M. C. (2010). T cells as sources and targets of TNF: implications for immunity and autoimmunity. . *Curr Dir Autoimmun.*, 11:105-18.
- Chaudhry** IH, O. D. (2001, Oct). Vascular endothelial growth factor expression correlates with tumour grade and vascularity in gliomas. *Histopathology.* ;39(4):409-15.
- Chen** J, G. K. (2012, May 11;). Interactions of nucleolin and ribosomal protein L26 (RPL26) in translational control of human p53 mRNA. *J Biol Chem.* , 287(20):16467-76.
- Chen** L, V. Z. (2014). Predicting the likelihood of an isocitrate dehydrogenase 1 or 2 mutation in diagnoses of infiltrative glioma. *Neurooncology* , 16:1478–1483.
- Chen** X, S. S. (2011 , Jan). Nucleolin-mediated cellular trafficking of DNA nanoparticle is lipid raft and microtubule dependent and can be modulated by glucocorticoid. . *Mol Ther.* , 19(1):93-102.
- Chen** ZY, W. Y. (2014). Advance of molecular imaging technology and targeted imaging agent in imaging and therapy. *Biomed Res Int.* , 819324.
- Chillarón** J, R. R. (2001, Dec). Heteromeric amino acid transporters: biochemistry, genetics, and physiology. *Am J Physiol Renal Physiol.*, 281(6):F995-1018.
- Christian** S., P. J. (2003). Nucleolin expressed at the cell surface is a marker of endothelial cells in angiogenic blood vessels. *J. Cell Biol.* 163 , 871–878.
- Cimini** A, I. R. (2011). Innovative Therapies against Human Glioblastoma Multiforme. *ISRN Oncol.* ;2011:787490.
- Cimini** A, M. S. (2012, Mar). Distinct cellular responses induced by saporin and a transferrin-saporin conjugate in two different human glioblastoma cell lines. *J Cell Physiol* , 227(3):939-51.
- Clarke** J, B. N. (2010, Mar). Recent advances in therapy for glioblastoma. *Arch Neurol.* , ;67(3):279-83.
- Cloughesy** TF, W. P. (2006, Aug). Phase II trial of tipifarnib in patients with recurrent malignant glioma either receiving or not receiving enzyme-inducing antiepileptic drugs: a North American Brain Tumor Consortium Study. *J Clin Oncol.* , 1;24(22):3651-6.

Cloughesy TF, Y. K. (2008, Jan). Antitumor activity of rapamycin in a Phase I trial for patients with recurrent PTEN-deficient glioblastoma. *PLoS Med.* , 22;5(1):e8.

Cohen AR, W. D. (1998, Jul). Human CASK/LIN-2 binds syndecan-2 and protein 4.1 and localizes to the basolateral membrane of epithelial cells. *J Cell Biol.* , 142(1):129-38.

Cohen BA, C. P. (1998, Nov). An artificial cell-cycle inhibitor isolated from a combinatorial library. *Proc Natl Acad Sci U S A.* , 24;95(24):14272-7.

Colas P, C. B. (1996, Apr). Genetic selection of peptide aptamers that recognize and inhibit cyclin-dependent kinase 2. *Nature.*

Combs SE, H. S.-E. (2006 , May). Treatment of primary glioblastoma multiforme with cetuximab, radiotherapy and temozolomide (GERT)--phase I/II trial: study protocol. *BMC Cancer.*, 18;6:133.

Cuddapah VA, R. S. (2014, Jul). A neurocentric perspective on glioma invasion. *Nat Rev Neurosci.* , 5(7):455-65.

D. Laber, B. T. (2007). Pharmacokinetics of the anti-nucleolin aptamer AS1411 in a phase I study. *Molecular Cancer Therapeutics*, 6 , C147-C147.

D. Laber, M. C. (2004). A phase I study of AGRO100 in advanced cancer. *ASCO Annual Meeting Proceedings* , pp. 3112.

Daniels DL, C. A. (1998 , Apr). Crystal structure of the hCASK PDZ domain reveals the structural basis of class II PDZ domain target recognition. *Nat Struct Biol.* , 5(4):317-25.

Daniely Y, B. J. (2000, May 15). Formation of a complex between nucleolin and replication protein A after cell stress prevents initiation of DNA replication. *J Cell Biol.*, 149(4):799-810.

Daniely Y, D. D. (2002, Aug). Stress-dependent nucleolin mobilization mediated by p53-nucleolin complex formation. *Mol Cell Biol.*, 22(16):6014-22.

Dave MH, S. N. (2004). Expression of heteromeric amino acid transporters along the murine intestine. *J Physiol.* , 15;558(Pt 2):597-610.

Day SE, W. A. (2012, Jul). Clinical trials of small molecule inhibitors in high-grade glioma. *Neurosurg Clin N Am.* , 23(3):407-16.

Dean P, K. B. (2011 Jun). Cell-surface nucleolin is sequestered into EPEC microcolonies and may play a role during infection. *Microbiology.* , 157(Pt 6):1761-7.

Derenzini M, S. V.-V. (1995, Jul). Quantitative changes of the two major AgNOR proteins, nucleolin and protein B23, related to stimulation of rDNA transcription. *Exp Cell Res.*, 219(1):276-82.

Destouches D, E. K.-K. (2008, Jun 18). Suppression of tumor growth and angiogenesis by a specific antagonist of the cell-surface expressed nucleolin. *PLoS One.* ;3(6):e2518.

Destouches D, P. N.-K. (2011). A simple approach to cancer therapy afforded by multivalent pseudopeptides that target cell-surface nucleoproteins. *Cancer Res.* , 1;71(9):3296-305.

Di Segni A., F. K.-K. (2008). Identification of nucleolin as new ErbB receptors-interacting protein. *PLoS One* 3 , e2310.

Dixit, S. (2014, May). Immunotherapy for high-grade glioma. *Future Oncol.* , 10(6):911-5.

Dolecek TA, P. J. (2012, Nov). CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2005-2009. *Neuro Oncol.*

Dresemann, G. (2005). Imatinib and hydroxyurea in pretreated progressive glioblastoma multiforme: a patient series. *Ann Oncol.* , 16(10):1702-8.

Dua, P. (2013). Alkalinephosphatase ALPPL-2 is a novel pancreatic carcinoma-associated protein. *Cancer Res* , 73: 1934–1945.

Dumler I, S. V. (1999 , Dec 16). Urokinase-induced mitogenesis is mediated by casein kinase 2 and nucleolin. *Curr Biol.* 9(24):1468-76.

Dvorak, H. (2002, Nov). Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. *J Clin Oncol.*, 1;20(21):4368-80.

Eckel-Passow JE, L. D. (2015, Jun). Glioma Groups Based on 1p/19q, IDH, and TERT Promoter Mutations in Tumors. *N Engl J Med.*, 25;372(26):2499-508.

Ehtesham M, K. P. (2002, Dec). Induction of glioblastoma apoptosis using neural stem cell-mediated delivery of tumor necrosis factor-related apoptosis-inducing ligand. *Cancer Res.*, 15;62(24):7170-4.

El Khoury D, D. D.-K.-B. (2010 Jun). Targeting surface nucleolin with a multivalent pseudopeptide delays development of spontaneous melanoma in RET transgenic mice. *BMC Cancer.*, 10.1186/1471-2407-10-325.

Ellington AD, S. J. (1990, Aug 30). In vitro selection of RNA molecules that bind specific ligands. *Nature*, 346(6287):818-22.

Endo, Y. M. (1987). The mechanism of action of ricin and related toxic lectins on eukaryotic ribosomes. The site and the characteristics of the modification in 28 S ribosomal RNA caused by the toxins. *J Biol Chem*, 262, 5908–5912.

Erard MS, B. P.-F. (1988, Aug 15). A major nucleolar protein, nucleolin, induces chromatin decondensation by binding to histone H1. *Eur J Biochem.*, 175(3):525-30.

Esposito CL, P. D. (2011). A neutralizing RNA aptamer against EGFR causes selective apoptotic cell death. *PLoS ONE* 6: e24071.

Fähling M, S. A. (2005, Oct 15). Role of nucleolin in posttranscriptional control of MMP-9 expression. *Biochim Biophys Acta.*;1731(1):32-40.

Fang SH, Y. N. (1993, Sep). The self-cleaving activity of nucleolin determines its molecular dynamics in relation to cell proliferation. *Exp Cell Res.*, 208(1):48-53.

Fang X, T. W. (2010, Jan) Aptamers generated from cell-SELEX for molecular medicine: a chemical biology approach. *Acc Chem Res.*, 19;43(1):48-57.

Ferrara, N. (2009). VEGF-A: a critical regulator of blood vessel growth. *Eur Cytokine Netw.*, 20(4):158-63.

Ferreras JM, B. L. (1993, Oct 19). Distribution and properties of major ribosome-inactivating proteins (28 S rRNA N-glycosidases) of the plant *Saponaria officinalis* L. (Caryophyllaceae). *Biochim Biophys Acta.*, 1216(1):31-42.

Ferreras JM, C. L. (2011, May). Use of ribosome-inactivating proteins from *sambucus* for the construction of immunotoxins and conjugates for cancer therapy. *Toxins (Basel).*, 3(5):420-41.

Flavell DJ, C. S. (1991, Aug;). Characteristics and performance of a bispecific F(ab'gamma)2 antibody for delivering saporin to a CD7+ human acute T-cell leukaemia cell line. *Br J Cancer.*, 64(2):274-80.

Flavell DJ, N. A. (1997, Nov). Systemic therapy with 3BIT, a triple combination cocktail of anti-CD19, -CD22, and -CD38-saporin immunotoxins, is curative of human B-cell lymphoma in severe combined immunodeficient mice. *Cancer Res.*, 1;57(21):4824-9.

Foà-Tomasi L, C.-F. G. (1982). Effect of ribosome-inactivating proteins on virus-infected cells. Inhibition of virus multiplication and of protein synthesis. *Arch Virol.*, 71(4):323-32.

Fogal V, S. K. (2009). Cell surface nucleolin antagonist causes endothelial cell apoptosis and normalization of tumor vasculature. *Angiogenesis.*, 12(1):91-100.

Folkman, J. (1971). Nov Tumor angiogenesis: therapeutic implications. *N Engl J Med.*, 18;285(21):1182-6.

- Foote** MB, P. N. (2015, Jul). Genetic Classification of Gliomas: Refining Histopathology. *Cancer Cell.* , 13;28(1):9-11.
- Fort** J, d. I.-C.-O.-R. (2007, Oct 26). The structure of human 4F2hc ectodomain provides a model for homodimerization and electrostatic interaction with plasma membrane. *J Biol Chem.* , ;282(43):31444-52. .
- Frankel** AD, P. C. (1988). Cellular uptake of the tat protein from human immunodeficiency virus. . *Cell.* , 23;55(6):1189-93.
- Fu** Y, C. Y. (2009, Dec 15). The heparin binding motif of endostatin mediates its interaction with receptor nucleolin. . *Biochemistry.* , ;48(49):11655-63.
- Fu** Z, F. C. (2005). Proteomic evidence for roles for nucleolin and poly[ADP-ribosyl] transferase in drug resistance. . *J Proteome Res.* , 4(5):1583-91. .
- Fukai** J, N. K. (2008, Oct;). Antitumor activity of cetuximab against malignant glioma cells overexpressing EGFR deletion mutant variant III. . *Cancer Sci.* , 99(10):2062-9.
- Gaudreault** I, G. D. (2004, Jan). Print 2004. YB-1 promotes strand separation in vitro of duplex DNA containing either mispaired bases or cisplatin modifications, exhibits endonucleolytic activities and binds several DNA repair proteins. . *Nucleic Acids Res.* , 12;32(1):316-27.
- Galzio** R, R. F. (2012, Feb;). Glycosylated nucleolin as marker for human gliomas. . *J Cell Biochem.* , 113(2):571-9.
- Gao H, Q. J. (2012 , Jul). Precise glioma targeting of and penetration by aptamer and peptide dual-functioned nanoparticles. *Biomaterials.* , 33(20):5115-23.
- Gardner**, W. (1933). Removal of the right cerebral hemisphere for infiltrating glioma. *J. Am. Med. Assoc.* , 101: 823–826,.
- Gas** N, E. M. (1985). Immunolocalization of the 100 kDa nucleolar protein during the mitotic cycle in CHO cells. *Biol Cell.*;53(3):209-18.
- Gaspar**(1994).Supratentorialmalignantglioma:patternsofrecurrenceandimplicationsforextern al beam local treatment. . *Int J Radiat Oncol Biol Phys* , 24: 55±57.
- Geng** L, S. E. (2006, Jan). STI571 (Gleevec) improves tumor growth delay and survival in irradiated mouse models of glioblastoma. . *Int J Radiat Oncol Biol Phys.* , 1;64(1):263-71.
- Ghisolfi** L, J. G. (1992, Feb 15). The glycine-rich domain of nucleolin has an unusual supersecondary structure responsible for its RNA-helix-destabilizing properties. *J Biol Chem.* , 267(5):2955-9.
- Gianni** S, W. T.-A. (2006, Dec;). Demonstration of long-range interactions in a PDZ domain by NMR, kinetics, and protein engineering. *Structure.* , 14(12):1801-9.
- Giansanti** F, D. L. (2010, Feb). Engineering a switchable toxin: the potential use of PDZ domains in the expression, targeting and activation of modified saporin variants. *Protein Eng Des Sel.* , 23(2):61-8.
- Giansanti** F., S. D. (2015, Jul). PDZ Domain in the Engineering and Production of a Saporin Chimeric Toxin as a Tool for targeting Cancer Cells. *J Cell Biochem.* , 116(7):1256-66.
- Ginisty** H, A. F. (1998, Mar 2). Nucleolin functions in the first step of ribosomal RNA processing. . *EMBO J.* , 17(5):1476-86.
- Ginisty** H, S. H. (1999, Mar;). Structure and functions of nucleolin. . *J Cell Sci.* , 112 (Pt 6):761-72.
- Girvan** AC, T. Y. (2006). AGRO100 inhibits activation of nuclear factor-kappaB (NF-kappaB) by forming a complex with NF-kappaB essential modulator (NEMO) and nucleolin. *Mol Cancer Ther.* 5(7):1790-9.

Glennie MJ, M. H. (1987, Jul). Emergence of immunoglobulin variants following treatment of a B cell leukemia with an immunotoxin composed of antiidiotypic antibody and saporin. *J Exp Med.* , 166(1):43-62.

Goldmacher VS, T. N. (1986, Apr). Evidence that pinocytosis in lymphoid cells has a low capacity. *J Cell Biol.* , 102(4):1312-9.

González V, G. K. (2009, Aug). Identification and characterization of nucleolin as a c-myc G-quadruplex-binding protein. *J Biol Chem* , 28;284(35):23622-35.

Guo J, G. X. (2011, Nov). Aptamer-functionalized PEG-PLGA nanoparticles for enhanced anti-glioma drug delivery. *Biomaterials.* , 32(31):8010-20.

Grinstein E, S. Y. (2006, Aug 4). Cell cycle-controlled interaction of nucleolin with the retinoblastoma protein and cancerous cell transformation. *J Biol Chem* , 281(31):22223-35.

Gupta AK, G. M. (2005, Jun). Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications. *Biomaterials.* , 26(18):3995-4021.

Haas-Kogan DA, P. M. (2005, Jun). Epidermal growth factor receptor, protein kinase B/Akt, and glioma response to erlotinib. *J Natl Cancer Inst.* , 15;97(12):880-7.

Hainsworth J, S. K. (2012, Apr). Phase II study of concurrent radiation therapy, temozolomide, and bevacizumab followed by bevacizumab/everolimus as first-line treatment for patients with glioblastoma. *Clin Adv Hematol Oncol.* ,;10(4):240-6.

Hanahan D, W. R. (2011, Mar). Hallmarks of cancer: the next generation. *Cell.* , 4;144(5):646-74.

Hanakahi LA, B. Z. (2000, Dec 19). The C-terminal domain of nucleolin accelerates nucleic acid annealing. *Biochemistry.* , 39(50):15493-9.

Hasselbalch B, L. U. (2010, Oct). Cetuximab insufficiently inhibits glioma cell growth due to persistent EGFR downstream signaling. *Cancer Invest.* , 28(8):775-87.

Hawasli AH, B. S.-T. (2013, Dec). Magnetic resonance imaging-guided focused laser interstitial thermal therapy for intracranial lesions: single-institution series. *Neurosurgery.* , 73(6):1007-17.

Hayes RL, K. M. (1995, Sep). Improved long term survival after intracavitary interleukin-2 and lymphokine-activated killer cells for adults with recurrent malignant glioma. *Cancer.* , 1;76(5):840-52.

Hegi ME, D. A. (2005 , Mar). MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med.* , 10;352(10):997-1003.

Heine MA, R. M. (1993, Nov). The Gly/Arg-rich (GAR) domain of *Xenopus* nucleolin facilitates in vitro nucleic acid binding and in vivo nucleolar localization. *Mol Biol Cell.* , 4(11):1189-204.

Hirano K, M. Y. (2005, Nov 25). A multifunctional shuttling protein nucleolin is a macrophage receptor for apoptotic cells. *J Biol Chem.* , 280(47):39284-93.

Hoja-Łukowicz D, P. M. (2009, Sep). The new face of nucleolin in human melanoma. *Cancer Immunol Immunother.* , 58(9):1471-80.

Hovanesian AG, P.-D. F. (2000, Dec 15). The cell-surface-expressed nucleolin is associated with the actin cytoskeleton. *Exp Cell Res.* , 261(2):312-28.

Hovanesian AG, S. C. (2010, Dec 23;). Surface expressed nucleolin is constantly induced in tumor cells to mediate calcium-dependent ligand internalization. *PLoS One.* , 5(12):e15787.

Hu Q, G. G. (2013, Jan). 34(4):1135-45. F3 peptide-functionalized PEG-PLA nanoparticles co-administrated with tLyp-1 peptide for anti-glioma drug delivery. *Biomaterials.*

Huang Y, S. H. (2006, May). The angiogenic function of nucleolin is mediated by vascular endothelial growth factor and nonmuscle myosin. *Blood.* , 1;107(9):3564-71.

Huddleson JP, A. N. (2006, Jun 2). Up-regulation of the KLF2 transcription factor by fluid shear stress requires nucleolin. *J Biol Chem.* ;281(22):15121-8.

Huhndorf M, M. A. (2016, Dec 22) Alterations of the Blood-Brain Barrier and Regional Perfusion in Tumor Development: MRI Insights from a Rat C6 Glioma Model. *PLoS One.*, 11(12):e0168174.

Hurt MR, M. J.-P. (1992, Jan). Amplification of epidermal growth factor receptor gene in gliomas: histopathology and prognosis. *J Neuropathol Exp Neurol* , 51(1):84-90.

IARC, C. B. (s.d.). Cancer Incidence and Mortality Worldwide:. *GLOBOCAN 2012* .

Ippoliti R, G. P. (1998, Aug). The effect of monensin and chloroquine on the endocytosis and toxicity of chimeric toxins. *Cell Mol Life Sci.* , 4(8):866-75. 5.

Ippoliti R, L. E. (1995, Sep). A chimeric saporin-transferrin conjugate compared to ricin toxin: role of the carrier in intracellular transport and toxicity. *FASEB J.* (12):1220-5.

Ippoliti R, L. E. (1996). A saporin-insulin conjugate: synthesis and biochemical characterization. *Nat Toxins.* , 4(4):156-62.

Ivanov AA, K. F. (2013). Targeting protein-protein interactions as an anticancer strategy. . 34(7):393-400.

Jain RK, d. T. (2007). Angiogenesis in brain tumours. . *Nat Rev Neurosci.* , (8):610-22.

Jemal A, B. F. (2011). Global cancer statistics. . *CA Cancer J Clin.* , 61(2):69-90.

Jemth P, G. S. (2007, Jul). PDZ domains: folding and binding. *Biochemistry.* , 46(30):8701-8.

Jiao Y, K. P.-S. (2012, Jul). Frequent ATRX, CIC, FUBP1 and IDH1 mutations refine the classification of malignant gliomas. . *Oncotarget.* , 3(7):709-22.

Johnstone, R. (2002, Apr). Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. . *Nat Rev Drug Discov.* , 1(4):287-99.

Josic D, C. J. (2008 , Apr). Membrane proteins as diagnostic biomarkers and targets for new therapies. *Curr Opin Mol Ther.* , 10(2):116-23.

Kalyanaraman B, J. J. (2002, May-Jun). Doxorubicin-induced apoptosis: implications in cardiotoxicity. *Mol Cell Biochem.* ;234-235(1-2):119-24.

Kaminsky V, A. A. (2011, Jan). A quantitative assay for the monitoring of autophagosome accumulation in different phases of the cell cycle. *Autophagy.* , 7(1):83-90.

Kanai Y, S. H. (1998 , Sep 11). Expression cloning and characterization of a transporter for large neutral amino acids activated by the heavy chain of 4F2 antigen (CD98). *J Biol Chem.* , 273(37):23629-32.

Kanally CW, D. D. (2010, Jan). Clinical applications of a peptide-based vaccine for glioblastoma. *Neurosurg Clin N Am.*

Kang D, W. J. (2012). Selection of DNA aptamers against glioblastoma cells with high affinity and specificity. *PLoS ONE* 7

Kang R, Z. H. (2011, Apr;) The Beclin 1 network regulates autophagy and apoptosis. . *Cell Death Differ.* , 18(4):571-80.

Kanwar JR, R. K. (2011, Dec). Chimeric aptamers in cancer cell-targeted drug delivery. . *Crit Rev Biochem Mol Biol.* , ;46(6):459-77.

Kawakami K, N. O. (2006 ,Jul 7). Targeted anticancer immunotoxins and cytotoxic agents with direct killing moieties. *ScientificWorldJournal.* , ;6:781-90.

Khurts S, M. K. (2004, Dec 3;). Nucleolin interacts with telomerase. *J Biol Chem.* , 279(49):51508-15.

Kibbey MC, J. B. (1995 , Oct). A 110-kD nuclear shuttling protein, nucleolin, binds to the neurite-promoting IKVAV site of laminin-1. *J Neurosci Res.* , 15;42(3):314-22.

Killela PJ, R. Z. (2013, Apr 9). TERT promoter mutations occur frequently in gliomas and a subset of tumors derived from cells with low rates of self-renewal. *Proc Natl Acad Sci U S A.* , ;110(15):6021-6

Kim JK, C. K. (2012, Jan). Molecular imaging of a cancer-targeting theragnostics probe using a nucleolin aptamer- and microRNA-221 molecular beacon-conjugated nanoparticle. *Biomaterials.* , 33(1):207-17

Kirson ED, D. V.-S. (2007, Jun). Alternating electric fields arrest cell proliferation in animal tumor models and human brain tumors. *Proc Natl Acad Sci U S A.* , 12;104(24):10152-7.

Kleihues P, C. W. (2000). Pathology & genetics of tumours of the nervous systems. *IARC Press.* , pp 29–39.

Kleihues P, L. D. (2002, Mar). The WHO classification of tumors of the nervous system. *J Neuropathol Exp Neurol.* , 215-25;

Kleihues P, O. H. (1999, Jan). Primary and secondary glioblastomas: from concept to clinical diagnosis. *Neuro Oncol.* , 1(1):44-51.

Kleihues P. Glioblastoma. Kleihues P, C. W. (2000). Pathology & genetics of tumours of the nervous systems. *IARC Press.* , pp 29–39.

Kleinman HK, W. B. (1991, Nov 1). Identification of a 110-kDa nonintegrin cell surface laminin-binding protein which recognizes an A chain neurite-promoting peptide. *Arch Biochem Biophys.* , ;290(2):320-5.

Knobbe CB, R. J. (2004, Mar). Hypermethylation and transcriptional downregulation of the carboxyl-terminal modulator protein gene in glioblastomas *J Natl Cancer Inst.* , 17;96(6):483-6.

Kobayashi J, F. H. (2012). Nucleolin participates in DNA double-strand break-induced damage response through MDC1-dependent pathway. *PLoS One.* , 7(11):e49245.

Kobayashi K, O. A. (2008). Enhanced tumor growth elicited by L-type amino acid transporter 1 in human malignant glioma cells. *Neurosurgery.* , 62(2):493-503.

Kool M, K. J. (2008, Aug). Integrated genomics identifies five medulloblastoma subtypes with distinct genetic profiles, pathway signatures and clinicopathological features. *PLoS One.* , 28;3(8):e3088.

Koutsoumpa M, D. G. (2012, Mar 16). Pleiotrophin expression and role in physiological angiogenesis in vivo: potential involvement of nucleolin. *Vasc Cell.*

Koutsoumpa M, P. C. (2013, Jan 4;). Interplay between $\alpha\beta3$ integrin and nucleolin regulates human endothelial and glioma cell migration. *J Biol Chem.* , 288(1):343-54.

Kreisl TN, K. L. (2009, Feb). Phase II trial of single-agent bevacizumab followed by bevacizumab plus irinotecan at tumor progression in recurrent glioblastoma. *J Clin Oncol.* , 10;27(5):740-5.

Kreisl TN, M. K. (2012, Dec). A phase I/II trial of vandetanib for patients with recurrent malignant glioma. *Neuro Oncol.* , 14(12):1519-26.

Kreitman RJ, (1999, Oct;). Immunotoxins in cancer therapy. *Curr Opin Immunol.* , 11(5):570-8.

Krolick KA, V. C. (1980, Sep;). Selective killing of normal or neoplastic B cells by antibodies coupled to the A chain of ricin. *Proc Natl Acad Sci U S A.* , 77(9):5419-23.

Krust B, E. K. (2011, Aug 3). Targeting surface nucleolin with multivalent HB-19 and related Nucant pseudopeptides results in distinct inhibitory mechanisms depending on the malignant tumor cell type. *BMC Cancer.*

Krust B, E. K. (2011). Targeting surface nucleolin with multivalent HB-19 and related Nucant pseudopeptides results in distinct inhibitory mechanisms depending on the malignant tumor cell type. *BMC Cancer*. , 11:333, 10.1186/1471-2407-11-333.

Laks DR, V. K. (2010, Sep;). Brain tumor stem cells as therapeutic targets in models of glioma. *Yonsei Med J.* , 51(5):633-40.

Lamborn KR, C. S. (2004, Jul). Prognostic factors for survival of patients with glioblastoma: recursive partitioning analysis. *Neuro Oncol.* , 6(3):227-35.

Lapeyre B, B. H. (1987 , Mar). Nucleolin, the major nucleolar protein of growing eukaryotic cells: an unusual protein structure revealed by the nucleotide sequence. *Proc Natl Acad Sci U S A.* , 84(6):1472-6.

Lappi DA, W. R. (2004). Immunotoxins and neuropeptide-toxin conjugates experimental applications. *Mini Rev Med Chem.* , 4(5):585-95.

Larrucea S, C. R.-R.-T. (1999 , Dec). Internalization of factor J and cellular signalization after factor J-cell interaction. *Biochem Biophys Res Commun.* , 266(1):51-7.

Lefranc F, B. J. (2005, Apr). Possible future issues in the treatment of glioblastomas: special emphasis on cell migration and the resistance of migrating glioblastoma cells to apoptosis. *J Clin Oncol.* , 1;23(10):2411-22.

Lesniak MS, L. R. (2001, May). Drug delivery to tumors of the central nervous system. *Curr Neurol Neurosci Rep.* , ;1(3):210-6.

Li X, Y. Y. (2015 , Jan). Targeted delivery of anticancer drugs by aptamer AS1411 mediated Pluronic F127/cyclodextrin-linked polymer composite micelles. *Nanomedicine.* , 11(1):175-84.

Li YM, H. W. (2010, Nov). Targeted toxins in brain tumor therapy. *Toxins (Basel).* , 2(11):2645-62.

Liao ZX, C. E. (2015, Jun). An AS1411 aptamer-conjugated liposomal system containing a bubble-generating agent for tumor-specific chemotherapy that overcomes multidrug resistance. *J Control Release.* , 28;208:42-51.

Lombardi A, B. S. (2010, Jan). *Pichia pastoris* as a host for secretion of toxic saporin chimeras. *FASEB J.* , 24(1):253-65.

Lord J.M., L. R. (1994.). Ricin: structure, mode of action, and some current applications. *FASEB J.* , 201–208.

Losfeld ME, K. D. (2009 , Jan). The cell surface expressed nucleolin is a glycoprotein that triggers calcium entry into mammalian cells. *Exp Cell Res.* , 15;315(2):357-69.

Louis DN, O. H. (2007, Aug). The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol.* , 114(2):97-109.

Louis DN, P. A.-B. (2014, Sep). International Society Of Neuropathology--Haarlem consensus guidelines for nervous system tumor classification and grading. International Society Of Neuropathology. *Brain Pathol.* ;24(5):429-35.

Louis DN, P. A.-B. (2016, Jun). The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. 1. *Acta Neuropathol.* , 131(6):803-20.

Lund AH, v. L. (2004 , Oct). Epigenetics and cancer. *Genes Dev.* , 1;18(19):2315-35.

Luo Z, Y. Z. (2017, Mar 15). Precise glioblastoma targeting by AS1411 aptamer-functionalized poly (l-γ-glutamylglutamine)-paclitaxel nanoconjugates. *J Colloid Interface Sci.* , 490:783-796.

MacKay S, W. D. (2014). Developing trends in aptamer-based biosensor devices and their applications. *IEEE Trans Biomed Circuits Syst.*

Madan S, G. P. (1992). Interaction of gelonin with macrophages: Effect of lysosomotropic amines. *Exp. Cell Res.* , 198, 52–58 .

Madhumathi J, V. R. (2012, Jun);. Therapeutic targets and recent advances in protein immunotoxins. *Curr Opin Microbiol.* , 15(3):300-9.

Maher EA, F. F. (2001, Jun). Malignant glioma: genetics and biology of a grave matter. *Genes Dev.* , 15(11):1311-33.

Maira SM, G. I. (2001, Oct). Carboxyl-terminal modulator protein (CTMP), a negative regulator of PKB/Akt and v-Akt at the plasma membrane. *Science.* , 294(5541):374-80.

Malik MT, O. M.-R. (2015 , Sep 8). AS1411-conjugated gold nanospheres and their potential for breast cancer therapy. *Oncotarget.* ;6(26):22270-81.

Maras B, I. R. (1990, Aug). The amino acid sequence of a ribosome-inactivating protein from *Saponaria officinalis* seeds. *Biochem Int.* , 21(5):831-8.

Mascini M, P. I. (2012, Feb). Nucleic acid and peptide aptamers: fundamentals and bioanalytical aspects. *Angew Chem Int Ed Engl.* , 51(6):1316-32.

Mayle KM, L. A. (2012). The intracellular trafficking pathway of transferrin. *Biochim Biophys Acta.* , 1820(3):264-81.

Mellinghoff IK, C. T. (2007, Jan). PTEN-mediated resistance to epidermal growth factor receptor kinase inhibitors. *Clin Cancer Res.* , 15;13(2 Pt 1):378-81.

Meng GZ, X. S. (2011, Jan). Downregulation of cell-surface-expressed nucleolin inhibits the growth of hepatocellular carcinoma cells in vitro 33(1):23-7.

Merlin D, S. S. (2001 , Oct 19). CD98-mediated links between amino acid transport and beta 1 integrin distribution in polarized columnar epithelia. *J Biol Chem.* , ;276(42):39282-9.

Merlo, A. (2003, Jul). Genes and pathways driving glioblastomas in humans and murine disease models. *Neurosurg Rev.* , 26(3):145-58.

Miletic H, N. S. (2009, Apr). Anti-VEGF therapies for malignant glioma: treatment effects and escape mechanisms. *Expert Opin Ther Targets.* , 13(4):455-68.

Minota S, J. W. (1991, Apr 1). Autoantibodies to nucleolin in systemic lupus erythematosus and other diseases. *J Immunol.* , ;146(7):2249-52.

Mitchell DA, F. P. (2008 , Apr). Immunotherapy of malignant brain tumors. *Immunol Rev.* , 222:70-100.

Miyazaki H, B. M. (1980, Dec). Preparation of antibody (IgG)-ricin A-chain conjugate and its biologic activity. *Gan.* , 71(6):766-74.

Modena P, B. F. (2012, Nov). Predictors of outcome in an AIEOP series of childhood ependymomas: a multifactorial analysis. *Neuro Oncol.* , 14(11):1346-56.

Moolten F, Z. S. (1976). Immunotherapy of experimental animal tumors with antitumor antibodies conjugated to diphtheria toxin or ricin. *Ann N Y Acad Sci.* , 277(00):690-9.

Morimoto H, O. A. (2007, Jul-Aug). Differential expression of protein phosphatase type 1 isotypes and nucleolin during cell cycle arrest. *Cell Biochem Funct.* , 25(4):369-75.

Morokoff A, N. W. (2015, Aug;). Molecular subtypes, stem cells and heterogeneity: Implications for personalised therapy in glioma. *J Clin Neurosci.* , 22(8):1219-26. .

Mourmouras V, C. G. (2009, Jun;). Nucleolin protein expression in cutaneous melanocytic lesions. *J Cutan Pathol.* , 36(6):637-46.

Nagane M, H. H. (1999, Feb). Causes of drug resistance and novel therapeutic opportunities for the treatment of glioblastoma. *Drug Resist Updat.* 2(1):30-37.

Naumov GN, T. J. (2003 , Dec;). Ineffectiveness of doxorubicin treatment on solitary dormant mammary carcinoma cells or late-developing metastases. *Breast Cancer Res Treat.* , 82(3):199-206.

Nawashiro H, O. N. (2002, Mar;). The role of CD98 in astrocytic neoplasms. *Hum Cell.*, 15(1):25-31.

Nduom EK, H. C. (2012). Glioblastoma cancer stem-like cells: implications for pathogenesis and treatment. *Cancer J.* , 18(1):100-6.

Neyns B, S. J. (2009, Sep). Stratified phase II trial of cetuximab in patients with recurrent high-grade glioma. *Ann Oncol.* , 20(9):1596-603.

Nielsen KL, I. C. (2001, Sep). Differential activity and structure of highly similar peroxidases. Spectroscopic, crystallographic, and enzymatic analyses of lignifying *Arabidopsis thaliana* peroxidase A2 and horseradish peroxidase A2. *Biochemistry.* , 18;40(37):11013-21.

Nilsson U, J. R. (2010 , May). Attenuation of tumor growth by formation of antiproliferative glycosaminoglycans correlates with low acetylation of histone H3. *Cancer Res.* , 1;70(9):3771-9.

Nimjee SM, R. C. (2005). Aptamers: an emerging class of therapeutics. . *Annu Rev Med.* , ;56:555-83.

Nisole S, S. E. (2002, Jun 7). The anti-HIV pentameric pseudopeptide HB-19 binds the C-terminal end of nucleolin and prevents anchorage of virus particles in the plasma membrane of target cells. *J Biol Chem.*, 277(23):20877-86.

Nix SL, C. A. (2000 , Dec 29). hCASK and hDlg associate in epithelia, and their src homology 3 and guanylate kinase domains participate in both intramolecular and intermolecular interactions. *J Biol Chem.* , 275(52):41192-200.

Nobusawa S, W. T. (2009, Oct). IDH1 mutations as molecular signature and predictive factor of secondary glioblastomas. *Clin Cancer Res.* , 1;15(19):6002-7.

Ohgaki H, K. P. (2009, Dec;). Genetic alterations and signaling pathways in the evolution of gliomas. *Cancer Sci* , 00(12):2235-41.

Ohgaki H, K. P. (2013, Feb). The definition of primary and secondary glioblastoma. . *Clin Cancer Res.* , 15;19(4):764-72.

Okubo S, Z. H. (2010). Correlation of L-methyl-11C-methionine (MET) uptake with L-type amino acid transporter 1 in human gliomas. *J Neurooncol.* , 99(2):217-25.

Olivieri F, P. V.-C. (1996, Nov). A systemic antiviral resistance-inducing protein isolated from *Clerodendrum inerme* Gaertn. is a polynucleotide:adenosine glycosidase (ribosome-inactivating protein). *FEBS Lett.* , 4;396(2-3):132-4.

Olsnes S, P. A. (1981). Chimeric toxins. . *Pharmacol Ther.* , 15(3):355-81.

Omuro A, D. L. (2013, Nov). Glioblastoma and other malignant gliomas: a clinical review. *JAMA.* , 6;310(17):1842-50.

Ostman, A. (2004, Aug). PDGF receptors-mediators of autocrine tumor growth and regulators of tumor vasculature and stroma. *Cytokine Growth Factor Rev.* , 15(4):275-86.

Parekh P, K. S. (2013). Immunotherapy of CD30-expressing lymphoma using a highly stable ssDNA aptamer. *Biomaterials* , 34: 8909–8917.

Parsons DW, J. S. (2008 , 2008). An integrated genomic analysis of human glioblastoma multiforme. *Science.* , 26;321(5897):1807-12.

Pastan I, O. M. (2011, Jun). Immunotoxins with decreased immunogenicity and improved activity. . *Leuk Lymphoma.*, 2:87-90.

Pasternack MS, B. K. (1991, Aug 5). Granzyme A binding to target cell proteins. Granzyme A binds to and cleaves nucleolin in vitro. *J Biol Chem.* , 266(22):14703-8.

Patel M, V. M. (2012, Sep). Molecular targeted therapy in recurrent glioblastoma: current challenges and future directions. *Expert Opin Investig Drugs.* , 21(9):1247-66.

Peereboom DM, S. D. (2010, May). Phase II trial of erlotinib with temozolomide and radiation in patients with newly diagnosed glioblastoma multiforme. *J Neurooncol.* , 98(1):93-9.

Peng L, L. J. (2010, Jul 15). High levels of nucleolar expression of nucleolin are associated with better prognosis in patients with stage II pancreatic ductal adenocarcinoma. *Clin Cancer Res.* ;16(14):3734-42.

Perry J, O. M. (2012). Novel therapies in glioblastoma. *Neurol Res Int.*

Pickering BF, Y. D. (2011, Dec 23). Nucleolin protein interacts with microprocessor complex to affect biogenesis of microRNAs 15a and 16. *J Biol Chem.*, 286(51):44095-103.

Pinho MC, P. P.-C. (2014, Jan). Low incidence of pseudoprogression by imaging in newly diagnosed glioblastoma patients treated with cediranib in combination with chemoradiation. *Oncologist* , 19(1):75-81.

Polito L, B. M. (2011). Immunotoxins and other conjugates containing saporin-s6 for cancer therapy. . *Toxins (Basel).*, 697-720.

Polito L, B. M. (2013, Jul 25). Protein synthesis inhibition activity by strawberry tissue protein extracts during plant life cycle and under biotic and abiotic stresses. *Int J Mol Sci.* , 14(8):15532-45.

Polito L, B. M. (2009, May). Saporin induces multiple death pathways in lymphoma cells with different intensity and timing as compared to ricin. *Int J Biochem Cell Biol.* , 41(5):1055-61. doi: 10.1016/j.biocel.2008.09.021.

Prados MD, L. K. (2006, Jan). Phase 1 study of erlotinib HCl alone and combined with temozolomide in patients with stable or recurrent malignant glioma. *Neuro Oncol.* , ;8(1):67-78.

Prados MD, C. S.-R.-K. (2009, Feb). Phase II study of erlotinib plus temozolomide during and after radiation therapy in patients with newly diagnosed glioblastoma multiforme or gliosarcoma. *J Clin Oncol.* , 1;27(4):579-84.

Presta LG, C. H. (1997, Oct). Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. *Cancer Res.* , 15;57(20):4593-9.

Prickett WM, V. R. (2011, Nov 11). Vascular targeted single-walled carbon nanotubes for near-infrared light therapy of cancer. *Nanotechnology.*

Qin Z, L. B. (2011, Oct 15). Antibodies against nucleolin in recipients of organ transplants. *Transplantation.* , 92(7):829-35.

Qiu J, B. K. (1999, May 10). A 110-kDa nuclear shuttle protein, nucleolin, specifically binds to adeno-associated virus type 2 (AAV-2) capsid. *Virology.* , 257(2):373-82.

Rahmathulla G, H. E.-S. (2013). Bevacizumab in high-grade gliomas: a review of its uses, toxicity assessment, and future treatment challenges. *Onco Targets* , 6:371-89.

Raizer JJ, A. L., & Consortium., N. A. (2010, Jan). A phase II trial of erlotinib in patients with recurrent malignant gliomas and nonprogressive glioblastoma multiforme postradiation therapy. *Neuro Oncol.* , 12(1):95-103.

Rapak A, F. P. (1997, Apr 15). Retrograde transport of mutant ricin to the endoplasmic reticulum with subsequent translocation to cytosol. *Proc Natl Acad Sci U S A.* , 94(8):3783-8.

Raymond E, B. A. (2008, Oct). Phase II study of imatinib in patients with recurrent gliomas of various histologies: a European Organisation for Research and Treatment of Cancer Brain Tumor Group Study. *J Clin Oncol.* , 1;26(28):4659-65.

Razis E, S. P.-F. (2009 , Oct). Phase II study of neoadjuvant imatinib in glioblastoma: evaluation of clinical and molecular effects of the treatment. *Clin Cancer Res.*, 1;15(19):6258-66.

Reardon DA, E. M. (2005, Dec). Phase II study of imatinib mesylate plus hydroxyurea in adults with recurrent glioblastoma multiforme. *J Clin Oncol.* , 20;23(36):9359-68.

Reardon DA, F. K.-R. (2008, Dec). Randomized phase II study of cilengitide, an integrin-targeting arginine-glycine-aspartic acid peptide, in recurrent glioblastoma multiforme. *J Clin Oncol.* , 1;26(34):5610-7.

Reardon DA, V. J. (2011, Dec). Phase I study of sunitinib and irinotecan for patients with recurrent malignant glioma. *J Neurooncol.*, 105(3):621-7.

Refsnes K, O. S. (1974, Jun 10). On the toxic proteins abrin and ricin. Studies of their binding to and entry into Ehrlich ascites cells. *J Biol Chem.* , 249(11):3557-62.

Rettig WJ, O. L. (1989). Immunogenetics of human cell surface differentiation. *Annu Rev Immunol.* ;7:481-511.

Reyes-Reyes EM, A. S. (2008, Jul). Cell-surface nucleolin is a signal transducing P-selectin binding protein for human colon carcinoma cells. *Exp Cell Res.* , 1;314(11-12):2212-23.

Reyes-Reyes EM, T. Y. (2010, Nov). A new paradigm for aptamer therapeutic AS1411 action: uptake by macropinocytosis and its stimulation by a nucleolin-dependent mechanism. *Cancer Res.* 1;70(21):8617-29.

Rich JN, B. D. (2004, May). Development of novel targeted therapies in the treatment of malignant glioma. *Nat Rev Drug Discov.* , 3(5):430-46.

Rich JN, R. D.-U. (2004). Phase II trial of gefitinib in recurrent glioblastoma. *J Clin Oncol.* , 1;22(1):133-42.

Ridley L, R. R. (2008, Oct;). Multifactorial analysis of predictors of outcome in pediatric intracranial ependymoma. *Neuro Oncol.* , 10(5):675-89.

Riina HA, K. J. (2010 , Mar). Balloon-assisted superselective intra-arterial cerebral infusion of bevacizumab for malignant brainstem glioma. A technical note. *Interv Neuroradiol.* , ;16(1):71-6.

Rizzieri D, S.-G. K. (2010). Long term outcomes of responders in a randomized, controlled Phase II trial of aptamer AS1411 in AML. *J Clin Oncol.* , 28:15S.

RO, D. (2011, Feb;). Cancer immunotherapy. . *Cancer Biother Radiopharm.* , 26(1):1-64.

Rosenberg JE, B. R. (2014, Feb). A phase II trial of AS1411 (a novel nucleolin-targeted DNA aptamer) in metastatic renal cell carcinoma. *Invest New Drugs.* , 32(1):178-87.

Rossier G, M. C. (1999). LAT2, a new basolateral 4F2hc/CD98-associated amino acid transporter of kidney and intestine. *J Biol Chem.* , 3;274(49):34948-54.

Rustamzadeh E, L. C. (2003 , Oct;). Targeting the over-expressed urokinase-type plasminogen activator receptor on glioblastoma multiforme. *J Neurooncol.* , 65(1):63-75.

Sader,M., & P. C.-E. (2015). Functionalization of Iron Oxide Magnetic Nanoparticles with the Multivalent Pseudopeptide N6I for Breast Tumor Targeting. *J Nanomed Nanotechnol.*

Sadler K, T. J. (2002, May). Peptide dendrimers: applications and synthesis. *J Biotechnol.* , 90(3-4):195-229.

Said EA, C. J. (2005, Sep). Pleiotrophin inhibits HIV infection by binding the cell surface-expressed nucleolin. *FEBS J.* , 272(18):4646-59.

Samuels Y, W. Z. (2004, Apr). High frequency of mutations of the PIK3CA gene in human cancers. *Science.* , 23;304(5670):554.

Sanford JL, M. T.-F. (2004). CASK and Dlg form a PDZ protein complex at the mammalian neuromuscular junction. *Muscle Nerve.*, 30(2):164-71.

Sansal I, S. W. (2004, Jul). The biology and clinical relevance of the PTEN tumor suppressor pathway. *J Clin Oncol.* , 15;22(14):2954-63.

Santanché S, B. A. (1997, May). The unusual stability of saporin, a candidate for the synthesis of immunotoxins. *Biochem Biophys Res Commun.*, 8;234(1):129-32.

- Sathornsumetee S, R. D.** (2007, Jul). Molecularly targeted therapy for malignant glioma. *Cancer.*, 1;110(1):13-24.
- Savino C, F. L.** (1998, Jul 1). Crystallization and preliminary X-ray study of saporin, a ribosome-inactivating protein from *Saponaria officinalis*. *Acta Crystallogr D Biol Crystallogr.* , 54(Pt 4):636-8.
- Savino C, F. L.** (2000, Mar). The crystal structure of saporin SO6 from *Saponaria officinalis* and its interaction with the ribosome. *FEBS Lett.* , 31;470(3):239-43.
- Saxena A, R. C.** (2006 , Nov 23). Nucleolin inhibits Hdm2 by multiple pathways leading to p53 stabilization. *Oncogene.* ;25(55):7274-88.
- Scaringi C, M. G.** (2012 , Oct;). Integrin inhibitor cilengitide for the treatment of glioblastoma: a brief overview of current clinical results. *Anticancer Res.* , 32(10):4213-23.
- Schlegel J, M. A.** (1994, Jan). Amplification of the epidermal-growth-factor-receptor gene correlates with different growth behaviour in human glioblastoma. *Int J Cancer.* , 2;56(1):72-7.
- Schmidt NO, W. M.** (1999). Levels of vascular endothelial growth factor, hepatocyte growth factor/scatter factor and basic fibroblast growth factor in human gliomas and their relation to angiogenesis. *Int J Cancer.* , 19;84(1):10-8.
- Schwartzbaum JA, F. J.** (2006, Sep). Epidemiology and molecular pathology of glioma. *Nat Clin Pract Neurol.* , 2(9):494-503.
- Sebolt-Leopold JS, H. R.** (2004). Targeting the mitogen-activated protein kinase cascade to treat cancer. *Nat Rev Cancer.* , 4(12):937-47.
- Sefah K, M. L.-C.** (2010). DNA aptamers as molecular probes for colorectal cancer study. *PLoS ONE* , 5: e14269.
- Semenkovich CF, O. R.** (1990, Oct). A protein partially expressed on the surface of HepG2 cells that binds lipoproteins specifically is nucleolin. *Biochemistry*, 29(41):9708-13.
- Sengupta TK, B. S.** (2004, Mar 19). Identification of nucleolin as an AU-rich element binding protein involved in bcl-2 mRNA stabilization. *J Biol Chem*;279(12):10855-63.
- Sheng J, Z. W.** (2010, Apr 20). Identification of biomarkers for cervical cancer in peripheral blood lymphocytes using oligonucleotide microarrays. *Chin Med J* , ;123(8):1000-5.
- Shi H, H. Y.** (2007 , Oct 15). Nucleolin is a receptor that mediates antiangiogenic and antitumor activity of endostatin. *Blood.* ;110(8):2899-906.
- Shibata Y, M. T.** (2002, Oct;). Nuclear targeting by the growth factor midkine. *Mol Cell Biol.* , 22(19):6788-96.
- Shigdar S, Q. L.** (2013). RNA aptamers targeting cancer stem cell marker. *Cancer Lett* , CD133. 330: 84–95.
- Shveta Baggaa, M. H.** (2003). Cytotoxicity of ribosome-inactivating protein saporin is not mediated through K2-macroglobulin receptor. *FEBS Letters.*
- Siebzehnrubl FA, V.-M. V.** (2011). Isolation and characterization of adult neural stem cells. . *Methods Mol Biol.* , 750:61-77.
- Siegel RL, M. K.** (2016). Cancer statistics, 2016. . *CA Cancer J Clin.*
- Sikriwal D, G. P.** (2008). Ribosome inactivating protein saporin induces apoptosis through mitochondrial cascade, independent of translation inhibition. *Int J Biochem Cell Biol.* , 40(12):2880-8.
- Singh SK, C. I.** (2003). Identification of a cancer stem cell in human brain tumors. *Cancer Res* , 63: 5821-5828.
- Sloan AE, A. M.-P.** (2013, Jun;). Results of the NeuroBlate System first-in-humans Phase I clinical trial for recurrent glioblastoma: clinical article. *J Neurosurg.* , 118(6):1202-19.

- Song** KM, L. S. (2012). Aptamers and their biological applications. *Sensors (Basel)* , 12(1):612-31.
- Song** N, D. Y. (2012, Dec). The nuclear translocation of endostatin is mediated by its receptor nucleolin in endothelial cells. *Angiogenesis* , 15(4):697-711.
- Srivastava** M, F. P. (1989, Jun 19). Cloning and sequencing of the human nucleolin cDNA. *FEBS Lett.* , 250(1):99-105.
- Srivastava** M, P. H. (1999 , Nov). Molecular dissection of nucleolin's role in growth and cell proliferation: new insights. *FASEB J.* , 13(14):1911-22.
- Stirpe** F, B. L. (1986, Jan 20;). Ribosome-inactivating proteins up to date. *FEBS Lett.* , 195(1-2):1-8.
- Stepanova** V, L. T. (2008, Jul). Nuclear translocation of urokinase-type plasminogen activator. *Blood* , 1;112(1):100-10.
- Stirpe** F, D. M. (1987). Hepatotoxicity of immunotoxins made with saporin, a ribosome-inactivating protein from *Saponaria officinalis*. . *Virchows Arch B Cell Pathol Incl Mol Pathol.* , ;53(5):259-71.
- Stirpe**, F. (2013, Jun). Ribosome-inactivating proteins: from toxins to useful proteins. *Toxicol.* , 1;67:12-6.
- Stirpe**, F. (2004 , Sep). Ribosome-inactivating proteins. . *Toxicol.* , 15;44(4):371-83. .
- Storck S, S. M. (2007). Functions of the histone chaperone nucleolin in diseases. *Subcell Biochem.* , 41:125-44.
- Stummer** W, N. U. (2011, Jun;). Favorable outcome in the elderly cohort treated by concomitant temozolomide radiochemotherapy in a multicentric phase II safety study of 5-ALA. *J Neurooncol.* , 103(2):361-70.
- Stummer** W, P. U., & Group., A.-G. S. (2006, May). Fluorescence-guided surgery with 5-aminolevulinic acid for resection of malignant glioma: a randomised controlled multicentre phase III trial. *Lancet Oncol.* , 7(5):392-401.
- Stupp** R. (2013). Cilengitide combined with standard treatment for patients with newly diagnosed glioblastoma and methylated O6-methylguanine-DNA methyltransferase (MGMT) gene promoter: Key results of the multicenter, randomized, open-label, controlled, phase III CENTRIC study. *2013 ASCO*.
- Stupp** R, W. D. (2005, Jun) The role of radio- and chemotherapy in glioblastoma. *Onkologie.* , 28(6-7):315-7.
- Suganuma** M, W. T. (2012, Sep). Human gastric cancer development with TNF- α -inducing protein secreted from *Helicobacter pylori*. *Cancer Lett.* , 28;322(2):133-8.
- Suzuki** T, N. Y. (2005, Feb). Novel inhibitors of human histone deacetylases: design, synthesis, enzyme inhibition, and cancer cell growth inhibition of SAHA-based non-hydroxamates. *J Med Chem* , 24;48(4):1019-32.
- Szeitner** Z, A. J. (2014, Dec). Is less more? Lessons from aptamer selection strategies. *J Pharm Biomed Anal.* , ;101:58-65.
- Takagi** M, A. M. (2005, Oct 7) Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. *Cell* , ;123(1):49-63.
- Takeuchi** H, K. T. (2008 , Apr;). CD98 immunoreactivity in multinucleated giant cells of glioblastomas: an immunohistochemical double labeling study. *Neuropathology.* , 28(2):127-31.
- Tanida** I, U. T. (2004, Dec;). LC3 conjugation system in mammalian autophagy. . *Int J Biochem Cell Biol.* , 36(12):2503-18.

- Tayyari** F, M. D. (2011, Aug). Identification of nucleolin as a cellular receptor for human respiratory syncytial virus. *Nat Med.* , 14;17(9):1132-5.
- Tazzari** PL, B. L. (1988). An immunotoxin containing a rat IgM monoclonal antibody (Campath 1) and saporin 6: effect on T lymphocytes and hemopoietic cells. *Cancer Immunol Immunother.*, 26(3):231-6.
- Teixeira** S, D. G. (1987, Jul). Primary structure of the human 4F2 antigen heavy chain predicts a transmembrane protein with a cytoplasmic NH₂ terminus. *J Biol Chem.*, 15;262(20):9574-80.
- Teng** Y, G. A. (2007, Nov). AS1411 alters the localization of a complex containing protein arginine methyltransferase 5 and nucleolin. *Cancer Res.* , 67(21):10491-500.
- Thomas** AA, E. M. (2012). Immunotherapy for the treatment of glioblastoma. *Cancer J.* , ;18(1):59-68.
- Thorpe** PE, B. A. (1985). An immunotoxin composed of monoclonal anti-Thy 1.1 antibody and a ribosome-inactivating protein from *Saponaria officinalis*: potent antitumor effects in vitro and in vivo. *J Natl Cancer Inst.* , 75(1):151-9.
- Thorpe** PE, C. A. (1981, Jan). Abrogation of the non-specific toxicity of abrin conjugated to anti-lymphocyte globulin. *Clin Exp Immunol.*, 43(1):195-200.
- Thorpe** PE, R. W. (1982). The preparation and cytotoxic properties of antibody-toxin conjugates. *Immunol Rev.* , 62:119-58.
- Torchilin** VP. (2008, Mar) Tat peptide-mediated intracellular delivery of pharmaceutical nanocarriers. *Adv Drug Deliv Rev.* , 1;60(4-5):548-58.
- Tran** M, V. C. (2013, Jan) Production of unique immunotoxin cancer therapeutics in algal chloroplasts. *Proc Natl Acad Sci U S A.* , 110(1):E15-22.
- Trinh** TL, Z. G. (2015 , Nov 2). A Synthetic Aptamer-Drug Adduct for Targeted Liver Cancer Therapy. *PLoS One.* , 10(11):e0136673.
- Tuerk** C, G. L. (1990) Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science.* , 249(4968):505-10.
- Turck** N, L. O.-A. (2006, Feb). Effect of laminin-1 on intestinal cell differentiation involves inhibition of nuclear nucleolin. *J Cell Physiol.* , 206(2):545-55.
- Tuteja** N, H. N. (1995, Jul 28). Human DNA helicase IV is nucleolin, an RNA helicase modulated by phosphorylation. *Gene.* , 160(2):143-8.
- Uhm** JH, B. K. (2011, Jun). Phase II evaluation of gefitinib in patients with newly diagnosed Grade 4 astrocytoma: Mayo/North Central Cancer Treatment Group Study N0074. *Int J Radiat Oncol Biol Phys.* , 1;80(2):347-53.
- Uribe** DJ, G. K. (2011, May) Heterogeneous nuclear ribonucleoprotein K and nucleolin as transcriptional activators of the vascular endothelial growth factor promoter through interaction with secondary DNA structures. *Biochemistry.*, 10;50(18):3796-806.
- Uzawa** T, T. S. (2013, Mar). Expansion of the aptamer library from a "natural soup" to an "unnatural soup". *Chem Commun (Camb).* , 4;49(18):1786-95.
- Vago** R, M. C. (2005, Oct). Saporin and ricin A chain follow different intracellular routes to enter the cytosol of intoxicated cells. *FEBS J.* , 272(19):4983-95.
- Vallera** DA, L. C.-M. (2002 , Apr). Targeting urokinase-type plasminogen activator receptor on human glioblastoma tumors with diphtheria toxin fusion protein DTAT. *J Natl Cancer Inst.* , 17;94(8):597-606.
- Van Damme** EJ, H. B. (2002, Oct).Two distinct jacalin-related lectins with a different specificity and subcellular location are major vegetative storage proteins in the bark of the black mulberry tree. *Plant Physiol.* , 130(2):757-69.

- Van den Bent** MJ, B. A. (2009, Mar). Randomized phase II trial of erlotinib versus temozolomide or carmustine in recurrent glioblastoma: EORTC brain tumor group study 26034. *J Clin Oncol.* , 10;27(8):1268-74.
- Van den Eynde** BJ, v. d. (1997, Oct). T cell defined tumor antigens. *Curr Opin Immunol.* , 9(5):684-93.
- Van Gool S**, M. W. (2009, Oct). Dendritic cell therapy of high-grade gliomas. *Brain Pathol.* , 19(4):694-712.
- Van Meir** EG, B. A. (2004, Apr). Emerging molecular therapies for brain tumors. *Semin Oncol.* , 31(2 Suppl 4):38-46.
- Van Simaey**s D, L.-C. D. (2010). Study of the molecular recognition of aptamers selected through ovarian cancer cell-. *SELEX. PLoS ONE* , 5: e13770.
- Vauleon** E, A. T. (2010). Overview of cellular immunotherapy for patients with glioblastoma. *Clin Dev Immunol.* , pii: 689171.
- Verhaak** RG, H. K., & Network., C. G. (2010 , Jan). Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell.* , 19;17(1):98-110.
- Vivanco** I, S. C. (2002, Jul). The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer.* , 2(7):489-501.
- Walid**, M.(2008). Prognostic factors for long-term survival after glioblastoma. *Perm J.* , 12(4):45-8.
- Wan** X, H. B. (2007, Mar). Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism. *Oncogene.* , 22;26(13):1932-40.
- Wang** D, L. Q. (1997, Nov-Dec). Generation and characterization of an anti-CD19 single-chain Fv immunotoxin composed of C-terminal disulfide-linked dgRTA. *Bioconjug Chem.* ,:878-84.
- Wang** FB, R. Y. (2013). Recognition and capture of metastatic hepatocellular carcinoma cells using aptamer-conjugated quantum dots and magnetic particles. *Biomaterials* , 34: 3816–3827.
- Watanabe** T, T. H. (2010, Jun). Nucleolin as cell surface receptor for tumor necrosis factor-alpha inducing protein: a carcinogenic factor of Helicobacter pylori. *J Cancer Res Clin Oncol.* , 136(6):911-21.
- Watne** K, H. B. (1990) Reversible oedema and necrosis after irradiation of the brain. Diagnostic procedures and clinical manifestations. . *Acta Oncol.* , ;29(7):891-5.
- Weng** A, T. M.-B.-O. (2012, Nov). Saponins modulate the intracellular trafficking of protein toxins. *J Control Release.* , 28;164(1):74-86.
- Weng** A, T. M.-B.-S.-O. (2012, Jun). The toxin component of targeted anti-tumor toxins determines their efficacy increase by saponins. *Mol Oncol.* , 6(3):323-32.
- Wesche** J, R. A. (1999, Nov 26). Dependence of ricin toxicity on translocation of the toxin A-chain from the endoplasmic reticulum to the cytosol. *J Biol Chem.* , 274(48):34443-9.
- Weyergang** A, S. P. (2011, Sep). Photochemical internalization of tumor-targeted protein toxins. *Lasers Surg Med.* , 43(7):721-33.
- Wick** W, W. M. (2014, Jul). MGMT testing--the challenges for biomarker-based glioma treatment. *Nat Rev Neurol.* , 10(7):372-85.
- Wiemels** J, W. M. (2010, Sep). Epidemiology and etiology of meningioma. *J Neurooncol.* , 99(3):307-14.
- Wilson** TA, K. M. (2014, May). Glioblastoma multiforme: State of the art and future therapeutics. *Surg Neurol Int.* , 8;5:64. doi: 10.4103/2152-7806.132138.

- Wirsching** HG, W. M. (2016 , Oct). The Role of Molecular Diagnostics in the Management of Patients with Gliomas. *Curr Treat Options Oncol.* , 17(10):51. .
- Xiang** D, S. S. (2015 , Jan). Nucleic acid aptamer-guided cancer therapeutics and diagnostics: the next generation of cancer medicine. *Theranostics.* , 1;5(1):23-42.
- Xing** H, H. K. (2014, May) DNA Aptamer Technology for Personalized Medicine.*Curr Opin Chem Eng.* , 1;4:79-87.
- Xu** G, M. H. (2001, Nov). Strategies for enzyme/prodrug cancer therapy. *Clin Cancer Res.* , ;7(11):3314-24.
- Xu** X, H. F. (2001, Nov 16). Inhibition of DNA replication and induction of S phase cell cycle arrest by G-rich oligonucleotides. *J Biol Chem.*;276(46):43221-30.
- Yan** H, B. D. (2009 , Dec). Mutant metabolic enzymes are at the origin of gliomas. *Cancer Res.* , 15;69(24):9157-9.
- Yan** H, P. D.-H. (2009). IDH1 and IDH2 mutations in gliomas. *N Engl J Med.* , 19;360(8):765-73.
- Yan** Y, V. S. (2007) Extracellular interaction between hCD98 and the PDZ class II domain of hCASK in intestinal epithelia. *J Membr Biol.* , 215(1):15-26.
- Yanagida** O, K. Y. (2001, Oct). Human L-type amino acid transporter 1 (LAT1): characterization of function and expression in tumor cell lines. *Biochim Biophys Acta.* , 1;1514(2):291-302.
- Yang** C, K. M. (2009). Nucleolin Binds to the Proliferating Cell Nuclear Antigen and Inhibits Nucleotide Excision Repair. *Mol Cell Pharmacol.* ;1(3):130-137.
- Yang** TH, T. W. (1994, Sep). Purification and characterization of nucleolin and its identification as a transcription repressor. *Mol Cell Biol.* , 14(9):6068-74.
- Ye** M, H. J. (2012). Generating aptamers by cell-SELEX for applications in molecular medicine. *Int J Mol Sci.*;13(3):3341-53.
- Yildirim** MA, G. K. (2007, Oct). Drug-target network. *Nat Biotechnol.* , 25(10):1119-26.
- Youle** RJ, N. D. (1980, Sep). Anti-Thy 1.2 monoclonal antibody linked to ricin is a potent cell-type-specific toxin. *Proc Natl Acad Sci U S A.* , 77(9):5483-6.
- Zaidi** SH, M. J. (1995, Jul 21). Nucleolin and heterogeneous nuclear ribonucleoprotein C proteins specifically interact with the 3'-untranslated region of amyloid protein precursor mRNA. *J Biol Chem.* , 270(29):17292-8.
- Zhang** J, T. G. (2008, Feb 15) Nucleolin stabilizes Bcl-X L messenger RNA in response to UVA irradiation. *Cancer Res.* , 68(4):1046-54.
- Zhang**, K. S. (2012). A novel aptamer developed for breast cancer cell internalization. *ChemMedChem* , 7: 79–84.
- Zhao** DX, L. M., & Writing Group of the Cardiac Surgery, C. A. (2009, Jan). Routine intraoperative completion angiography after coronary artery bypass grafting and 1-stop hybrid revascularization results from a fully integrated hybrid catheterization laboratory/operating room. *J Am Coll Cardiol.* , 20;53(3):232-41.
- Zhou** G, S. M. (1997, Dec 5). Nucleolin is a protein kinase C-zeta substrate. Connection between cell surface signaling and nucleus in PC12 cells. *J Biol Chem.* , 272(49):31130-7.
- Zhu** Y, L. D. (1999, Jun). Nucleolin, defective for MPF phosphorylation, localizes normally during mitosis and nucleologenesis. *Histochem Cell Biol.* , 111(6):477-87.
- Zhu** Y, P. L. (2002, Aug). The molecular and genetic basis of neurological tumours. *Nat Rev Cancer* , 2(8):616-26.