

**A combinatorial preclinical *in vitro* strategy against  
human glioblastoma cells – specific targeting of  
protein kinases, histone deacetylases and glycolysis.**

**Inauguraldissertation**

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

und

der Université Louis Pasteur Strasbourg

von

**Viviane Egler**

aus Frankreich

Genève 2009

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät  
auf Antrag von:

Prof. M. A. Rüegg, Biozentrum, Basel (Switzerland)  
Prof. K. Takeda, Faculty of Pharmacy, ULP Strasbourg (France)  
Prof. A. Merlo, Kantonsspital Basel (Switzerland)  
Prof. G. Thomas, Genome Research Institute, Cincinnati (US)  
Dr. B. Hemmings, FMI, Basel (Switzerland)

Basel, den 17. Juni 2005

Prof. Dr. H.-J. Wirz  
Dekan

## **Acknowledgements**

I am grateful to Prof Adrian Merlo and Prof Kenneth Takeda for introducing me into the field of cancer, for supporting and motivating me in this project and to supervise my thesis at the universities of Basel and Strasbourg. I also thank Dr Roland Imber, for his technical support and advice and Dr George Thomas who introduced me into the field of energy regulation and cell growth. I am also grateful to Dr Jean-Louis Boulay for reading the manuscript and to Prof Markus Rüegg for reporting this work to the biological faculty of the University of Basel. I express also my thanks to my family and friends who always encouraged me.

This work has been supported by the Swiss National Science Foundation (31-67123.01), by Oncosuisse (01338-02-2003) and by Novartis Ltd, Oncology (Basel). The compounds, AEE788, STI571 (Glivec), RAD001, LAQ824 and EPO906 have been kindly provided by Novartis Ltd.

SUMMARY	4
PREAMBULE	5
INTRODUCTION	8
1. CNS development and cellular origin of gliomas	9
1.1. Precursors of CNS tumors	9
1.2. Grading and classification of gliomas	9
1.3. Genetic alterations at the origin of gliomas	10
1.3.1. Secondary GBM	11
1.3.2. Primary GBM	12
2. General tumor biology	13
2.1. Suppression of the natural induction of apoptosis	13
2.2. Uncontrolled proliferation	14
2.3. Sustained angiogenesis	15
2.4. Unchecked migratory and infiltration capacities	16
2.5. Benefit of bad telomeres	17
2.6. High aerobic glycolysis	18
3. Targeted chemotherapy against GBMs	20
3.1. Mechanisms of resistance of GBMs to chemotherapy	21
3.2. Choice of drugs and combinatorial strategy	25
COMBINATORIAL STRATEGY FOR TREATMENT OF GBM: RESULTS OF PRECLINICAL <i>IN VITRO</i> TUMOR MODEL	38
1. Effects of “biologicals” on human glioma cell lines after single and combined treatment (AEE788, Glivec, RAD001)	39
2. Effects of the “cytotoxics” HDACs inhibitors (HDIs) and EPO906A on human glioma cell lines	42
3. Indomethacin enhances HDACs inhibitors-induced apoptosis	44
4. Effects of the glycolytic inhibitor 2-deoxy-D-glucose (2-DG) on human cancer cell lines	46
5. RAD001 delays 2-deoxy-D-glucose-induced apoptosis in human glioma cell lines	50
6. 2-deoxy-D-glucose combined with HDACS inhibitors causes a synergistic induction of apoptosis in human glioma cell lines by inhibiting p21 upregulation	54
CONCLUSION AND DISCUSSION	63
ANNEXS	80
MATERIALS AND METHODS	90
ABBREVIATIONS	95
BIBLIOGRAPHY	97
FIGURES	109
Curriculum Vitae	

## Summary

Effective treatment of malignant gliomas remains one of the great challenges in neurooncology. The incidence of malignant gliomas is 5 to 10 per 100.000 persons, and the outcome is always fatal with a mean survival of less than one year. Tumor location and invasiveness into normal brain tissues are the two main obstacles for surgical cure. Resistance to conventional chemo- and radiotherapy is a consequence of high genetic instability leading to countless mutations. The presence of the blood-brain barrier makes drug targeting difficult. Nevertheless, mechanisms of gliomagenesis are becoming better understood. Among the key cellular pathways, those controlling apoptosis, proliferation, repair and invasion are most frequently disrupted due to alterations in *TP53*, *p16/p14*, *RB*, *PTEN*, *EGFR* and *PDGFR*. In addition, many regulatory genes are epigenetically silenced by methylation and acetylation. A cardinal feature of malignant gliomas, as well as many other cancers, is a high rate of ATP consumption via upregulation of glycolysis. All these pathways represent potential targets for interference with specific drugs.

Recently, small lipo-soluble molecules were designed as a new class of drugs more specifically targeting those altered cancer pathways. These drugs were applied to human GBM cells for induction of apoptosis as a readout of drug efficacy. Single applications of the PKIs, AEE788, Glivec and RADOO1 or the glycolytic inhibitor, 2-DG, were not able to induce cell death, but only a cytostatic effect at clinically meaningful dose ranges. In contrast, cytotoxic drugs like the microtubule inhibitor EPO906 or HDAC inhibitors (HDIs) like trichostatin A, sodium butyrate and LAQ824 were able to efficiently trigger apoptosis, but are known to lead to toxicity with increased dose ranges in mice and humans. We therefore tested combinations of low doses of cytotoxic drugs with cytostatic drugs and indomethacin. Since p21 was shown to be re-expressed upon HDIs and to protect cancer cells against apoptotic stimuli, 2-DG was used as a sensitizer for HDIs-induced apoptosis. 2-DG strongly inhibits protein expression. Synergistic induction of apoptosis was observed in glioma and other cancer cell lines upon this combined treatment. Thus, drug combination that inflict a cytotoxic stress to cancer cells and simultaneously impair the p21-regulated response of the stressed cells to halt the cell cycle machinery may be a new way to treat cancer in the future.

## PREAMBULE

**Therapeutic resistance of GBMs.** Cancer remains the second most fatal disorder in developed countries after cardiovascular disease. In France, an increase of 60% of cancer cases was noticed over the last twenty years. Among all cancers, glioblastoma multiforme (GBM) is the most frequent and malignant human brain tumor. GBMs are very aggressive, highly invasive and neurologically destructive and are considered to be among the deadliest of human cancers, with median survival ranging from 9 to 12 months. For example, from the 715 newly diagnosed cases of glioblastoma that occurred in the resident population of the Canton of Zurich in Switzerland between 1980 and 1994, 42.4% died at 6 months, 17.7% at 1 year, and 3.3% at 2 years (Ohgaki, Dessen et al. 2004). Importantly, these statistics have not changed significantly over the past two decades, despite treatment efforts underlined by technological advances in neurosurgery, radiation therapy and drug development. This is in part a consequence of the location of these tumors in relatively inaccessible tissues, harboring critical neurological functions, and of the presence of the blood-brain barrier which is a major obstacle for drug treatment. Since twenty years, the explosion of knowledge in cancer biology, basic science and drug development has led to efficient progress in the treatment of a number of common human cancers, including those of breast, lung or prostate. However, these therapies are ineffective in the treatment of GBMs. By now, human malignant gliomas are well characterized, demonstrated by the creation of several murine glioma models, but in these models only one or two pathways are targeted whereas in human gliomas, many additional genes and pathways are altered to promote the development of the tumor (Merlo 2003).

**History of brain tumor surgery.** The history of treating brain tumors dates from the middle of the 19<sup>th</sup> century and parallels advances in modern surgical techniques. Cranial surgery, however, is much older, although the underlying diseases remain speculative. A fascinating archaeological discovery was made in 1997, near Ensisheim (Alsace), which gives evidence for stone-age cranial surgery. The skull of a man showed a double trepanation practiced 7000 years ago (Alt, Jeunesse et al. 1997). The case described in Nature review “*is exceptional for several reasons: it appears to be the oldest healed neurosurgical operation known worldwide, its technical realization testifies to the high craftsmanship and well-founded anatomical*

*knowledge of the surgeon, and the success of the unusual trepanations is established by the long survival of the "patient."* The first modern brain tumor surgery was performed in 1884 by Rickman Godlee, but, because of technical difficulties, the initial enthusiasm for surgery soon dropped. Surgical techniques improved with Harvey Cushing in 1926 where the first histological grading of tumors was developed and showed a correlation with the clinical outcome. Radiation therapy was introduced soon thereafter, but the median survival was only increased for several months. In the eighties, chemotherapy was developed for leukemia and solid tumors, and agents that could pass the blood-brain barrier were added to the treatment protocols for brain tumors. Nitrosourea-based chemotherapy could increase median survival in combination with surgery and radiotherapy for a few weeks, but patients cannot be cured with any cytotoxic agent currently available. Recently, radio-chemotherapy has prolonged mean survival from 12 to 14 months in younger GBM patients with a good performance score and also increased the rate of patients surviving 24 months - a modest effect with regard to costs and effort (Stupp, Mason et al. 2005). Progress in molecular and cellular biology will eventually lead to a better understanding of the biology of human gliomas which will permit the development of more specific and effective therapies (Maher, Furnari et al. 2001).

## INTRODUCTION

# 1. Central nervous system (CNS) development and cellular origin of gliomas

## 1.1. Precursors of CNS tumors

In vertebrates, two types of progenitor cells arise from the embryonic neural tube (neuroectoderm): neuronal-restricted progenitor cells and glial-restricted progenitor cells, for the basis of all cell types of the central nervous system (CNS), including neurons, astrocytes and oligodendrocytes. Astrocytes perform diverse functions in the CNS, including regulation of neuronal growth and survival, guiding cell migration and axonal growth during development, promoting synapse formation and modulating synaptic transmission, and orchestrating inflammatory and immune responses during brain injury and infection (Maher, Furnari et al. 2001; Wechsler-Reya and Scott 2001; Zhu and Parada 2002).

At the end of the development, neurons become post-mitotic and only a smaller fraction of stem cells remain, whereas glial cells retain the ability to proliferate throughout life. It is therefore not surprising that most CNS tumors are of glial origin. These tumors are called gliomas, and include tumors composed predominantly of astrocytes (astrocytomas), oligodendrocytes (oligodendroglioma) or a mixture of various glial cells (oligoastrocytomas) (Annex 1)

## 1.2. Grading and classification of gliomas

The World Health Organization (WHO) grading system classifies gliomas into grade I to IV based on their degree of malignancy, as determined by histopathological criteria. In the CNS, grade I gliomas generally develop in a benign way and are circumscribed, whereas grade II-IV gliomas are malignant and diffusely infiltrate

throughout the brain. Astrocytoma are the most common CNS neoplasm, representing more than 60% of all primary brain tumors. Glioblastoma multiforme (GBM) is the most malignant form of infiltrating astrocytoma (WHO grade IV astrocytoma) and one of the most aggressive human cancers, with a very short median survival of 8 to 10 months.

Grade IV astrocytoma can be divided into two subclasses based on clinical characteristics: primary and secondary GBMs. Primary GBMs, present in older patients, are very aggressive, highly invasive and arise from a *de novo* process, in the absence of a pre-existing low-grade lesion. Secondary GBMs are usually observed in younger patients and develop progressively from low-grade astrocytoma over a period of 5 to 10 years. Genetic studies of GBM indicate that there are common, but also distinct genetic pathways that lead to these two subtypes (Maher, Furnari et al. 2001; Wechsler-Reya and Scott 2001; Zhu and Parada 2002) (Annex 1).

### 1.3. Genetic alterations at the origin of gliomas

The median survival from the time that a GBM is established is statistically the same for primary and secondary GBM, which reflects equal capacities to proliferate, invade and resist to all therapeutic modalities. The same genetic pathways are targeted in both sub-types, but the frequency of specific genetic mutations may differ in the two GBMs types. Microvascular proliferation and necrosis are found in primary as well as in secondary GBM, and both are composed of highly infiltrative and less well-differentiated cells than low-grade astrocytomas. It has been proposed that in secondary GBM, astrocytic tumor cells in low-grade gliomas undergo dedifferentiation during progression to GBM, possibly because of the accumulation of genetic mutations in key pathways regulating glial cell differentiation. A similar mechanism occurs in primary GBM, or as an alternative, primary GBM represents malignant transformation of more immature glial precursor cells, such as the pluripotent neural stem cells or the glial progenitor cells (Maher, Furnari et al. 2001; Wechsler-Reya and Scott 2001; Zhu and Parada 2002).

### 1.3.1. Secondary GBM

The presence of some genetic alterations in both low and high-grade astrocytomas indicate that these common mutations are involved in early phases of tumor formation. For example, the tumor suppressor protein p53, which regulates cell cycle progression and apoptosis, fits to this hypothesis. Indeed, patients with Li-Fraumeni syndrome carry a germ line mutation in the *TP53* gene (which encodes p53 in humans) and are predisposed to the development of various tumors, including astrocytomas. Mutations occurring in the course of gliomagenesis are found with the same frequency in all grades of astrocytomas (more than 60% for p53). The early occurrence of *TP53* mutation point to the need for astrocytoma cells to evade apoptosis in order to migrate and survive in a non-adequate environment. Although the study of human astrocytomas shows an association of *TP53* mutations with early tumor stages, observations in knock out or transgenic mice indicate that the loss of *TP53* alone is not sufficient to initiate astrocytoma formation, and that additional genetic events are required (Maher, Furnari et al. 2001; Wechsler-Reya and Scott 2001; Zhu and Parada 2002).

Downstream of the growth factor receptors are two major pathways, the Ras and the PI3K/PTEN pathways. The small GTP-binding protein, Ras, is an important downstream effector of the growth factor receptor tyrosine kinases (GF-RTK), involved in transmission of survival and growth signals. The GF-RTK-RAS signaling cascade is frequently targeted in human cancers rendering cancer cells independent of exogenous growth factors (Kinzler and Vogelstein 1996). Among them, platelet-derived growth-factor PDGF and its receptor are both highly co-expressed in every grade of astrocytomas, indicating that the cells establish an autocrine stimulatory loop. Neurofibromatosis type 1 (NF-1) is a familial cancer syndrome in which patients develop multiple tumors of the CNS. The gene *NFI* encodes for a protein that shares homology with the GAP family (RAS GTPase-activating protein). Loss of both *NFI* copies has been shown in these tumors, negatively regulating Ras. Activation of the RAS-mediated MAPK is found in astrocytomas. Transgenic mice models overexpressing oncogenic *Ras* in astrocytes lead to development of astrocytoma. Concerning the PI3K/PTEN pathway, mutations in the *PI3KCA* gene have also been detected in glioblastomas and other forms of brain tumors. These mutations lead to

increased lipid kinase activity (Samuels, Wang et al. 2004). In addition, the suppressor tumor gene *PTEN* is mutated or deleted in more than 30% of primary GBMs. These mutations lead to increased AKT activity, promoting growth and survival (Knobbe, Merlo et al. 2002). These observations support the hypothesis that p53 and the growth factor signaling pathways are involved in the initiation of low-grade astrocytoma development.

Tumor progression is caused by additional genetic alterations of genes involved in the regulation of cell cycle progression that lead to uncontrolled proliferation and high mitotic activity, the hallmark of high-grade astrocytomas (Ishii, Maier et al. 1999). The tumor suppressor gene *Rb* is the key regulator of the G1/S-phase checkpoint. RB is regulated by cyclin-dependent kinases (CDK) and CDK inhibitors (CKI) which are frequently mutated in GBM: loss of p16<sup>INK4A</sup> and p14<sup>ARF</sup> (both are encoded by the same gene, *CDKN2A*, but use an alternative reading frame) is detected in 40 to 57% of GBMs, *CDK4* amplification and loss of *RB* are identified in 14 to 33% of GBMs. In total, 80% of GBMs harbour mutations in the INK4A/CDK4/RB pathway, whereas such mutations are rare in low-grade astrocytomas (Labuhn, Jones et al. 2001). The remaining 20% GBMs show mutations in other players of this pathway, such as the overexpression of Cyclin D1 and CDK6. Studies in mouse models support the idea that all these mutations are important for the progression of astrocytomas, but not for tumor initiation (Maher, Furnari et al. 2001; Wechsler-Reya and Scott 2001; Zhu and Parada 2002) (Annex 1, 2).

### 1.3.2. Primary GBM

Primary GBM arise rapidly, within less than 3 months, without evidence of a pre-existing low-grade lesion. However, the analysis of mutations indicates that the same genetic pathways are affected in both primary and secondary GBMs, namely p53, the growth factor pathway and the cell cycle regulatory pathway. In primary GBMs, both p53 and RB pathways are simultaneously affected by the homologous deletion of the *CDKN2A* locus, which encodes for p16<sup>INK4A</sup> (involved in RB regulation) and p14<sup>ARF</sup>. ARF stabilizes p53 by antagonizing MDM2 (mouse double minute 2) which targets p53 for ubiquitin-mediated degradation. The amplification of the epidermal growth

factor receptor (*EGFR*) gene, which promotes growth of the tumor, is found in 40% of primary GBMs, but is rare in secondary GBMs. The loss of the long arm of chromosome 10 is the most common genetic alteration associated with GBMs. Several genes are located in this region, including the tumor suppressor *PTEN* which is deleted or mutated in more than 30% of primary GBMs, and only in 4% of secondary GBMs. The *PTEN* protein functions as a protein and lipid phosphatase for which the main substrate is the phosphatidylinositol(3,4,5)-triphosphate (PIP3), a PI3K product. PI3K has been found to be mutated in GBMs (Samuels, Wang et al. 2004). *PTEN* acts as a negative regulator of the PI3K-AKT survival pathway since AKT activity is enhanced in *PTEN* mutated/deleted GBMs, promoting growth and cell survival (Vivanco and Sawyers 2002; Zhu and Parada 2002; Sansal and Sellers 2004). The carboxyl-terminal modulator protein (CTMP) is another negative regulatory component of the pathway controlling PKB activity (Maira, Galetic et al. 2001). The gene coding CTMP has been found to be epigenetically silenced in GBMs (Knobbe, Reifenberger et al. 2004).

Recent observations support a plastic model in which development of primary GBMs can arise in two ways: primary GBM can develop from transformation of adult neural stem cells localized in the subventricular zones of the brain or can dedifferentiate from astrocytes. Glial progenitor cells can develop into neural stem cells in response to exogenous fibroblast growth factor 2 (FGF2), astrocytes can be de-differentiated into neural stem cells in response to epidermal growth factor (EGF) signaling and the loss of INK4A/ARF. Thereafter, amplification of the *EGFR* or mutations in *PTEN* can lead to primary GBM (Maher, Furnari et al. 2001; Wechsler-Reya and Scott 2001; Zhu and Parada 2002) (Annex 1, 2).

## 2. General tumor biology and specific aspects for GBMs

### 2.1. Suppression of the natural induction of apoptosis

The increase of a tumor mass arise through the loss of the equilibrium between cellular proliferation and cell death. The apoptotic index correlates with tumor proliferation and is negatively associated with survival. Tumor cell proliferation is associated with loss of normal induction of apoptosis. In normal cells, DNA damage, induced by irradiation, drugs or environmental toxic agents, induces cell death. One key sensor of DNA damage is the nuclear protein TP53 (Vogelstein, Lane et al. 2000). This transcription factor and tumor suppressor integrates numerous signals that are crucial for the control of life and death of the cell (Lane and Fischer 2004; Levine, Finlay et al. 2004). The protein p53 is activated, in part, by various signals that induce its dissociation from its inhibitor, MDM2. Indeed, p53 is phosphorylated by various kinases in response to DNA-damaging stimuli. Once activated, p53 binds to specific sequences in the DNA and initiates the transcription of many genes involved in genetic stability, cell-cycle inhibition and apoptosis. Concerning the cell cycle inhibition, p53 induces or maintains growth arrest through increased expression of cell-cycle regulators, such as p21, an inhibitor of Cyclin Dependent Kinases (CDK), allowing repair of DNA damage (el-Deiry, Tokino et al. 1993; Harper, Adami et al. 1993). Mutated cells are normally eliminated by apoptosis. However, if mutations generate relative resistance to apoptosis, such as mutations in *p53* or *HDM2* genes, the cell-intrinsic threshold to induce apoptosis is considerably elevated (Van Meir, Roemer et al. 1995). Consecutive tumor cell populations are selected due to mutations that convey a growth advantage. This clonal evolution of cancer is the Darwinian principle for tumorigenesis (Nowell 1976). In most cases, the loss of p53 function and activation of AKT (by mutation or deletion in the PTEN gene or PI3K encoding gene) occurring in GBMs contribute to the suppression of apoptosis in the presence of apoptotic stimuli and to an increased proliferation (Vivanco and Sawyers 2002). Mutations/deletions in *TPp53* gene occur with a frequency of 75% in human glioblastoma cell lines (Ishii, Maier et al. 1999). Studies done in GBM patients show that the mutations in the p53 gene do not markedly affect the survival of GBM patients, but suggest that in gliomas, the p53 mutation may contribute not only to tumorigenesis (as an early event) but also to progression to malignancy (as a late event) (Shiraishi, Tada et al. 2002).

## 2.2. Uncontrolled proliferation

Normal cells are not able to proliferate in the absence of stimulatory signals whereas tumor cells generate their own growth signals, which reduce their dependence on the stimulation from the surrounding normal tissue. Many known oncogenes act by mimicking normal growth signaling and many cancer cells acquire the ability to synthesize growth factors to which they are responsive, creating a positive feedback loop, either by autocrine or paracrine stimulation. Two illustrative examples are the production of the platelet-derived growth factor (PDGF) and transforming growth factor (TGF $\beta$ ) by glioblastomas (Fontana, Bodmer et al. 1991; Lokker, Sullivan et al. 2002). In addition, growth factor receptors, which often carry tyrosine kinase activity, are overexpressed in many cancers, including glioblastomas that overexpress the epidermal growth factor receptor (EGFR) (Barker, Simmons et al. 2001). Other alterations may elicit ligand-independent signaling. For example, the truncated version of EGFR lacks part of its cytoplasmic domain, resulting in a constitutively active receptor and activation of the PI3K/AKT (Vivanco and Sawyers 2002) and the MAPK pathways (Hunter 2000). More downstream, other components in the signaling pathways can be mutated. Cell cycle regulatory proteins are often disrupted in order to allow proliferation and avoid the block at the G1 checkpoint (Molinari 2000; Kastan and Bartek 2004). Disruption of the cell cycle machinery also contributes to glioblastoma development. Changes in the genes encoding cyclins, CDKs, CDKs inhibitors (Hunter and Pines 1994; MacLachlan, Sang et al. 1995; Fischer and Gianella-Borradori 2003), and the retinoblastoma protein (RB) (Knudson, Hethcote et al. 1975) have been observed at high frequency in glioblastomas. RB is regulating the progression through G1 by sequestering the E2F transcription factor responsible for the transcription of proteins needed for the transition in S phase and the replication (Hanahan and Weinberg 2000). Historically, the RB gene gave rise to the concept of the two-step model to inactivate a tumor suppressor gene (Knudson 1996) by loss of heterozygosity to inactivate the wild-type allele (Cavenee, Scrabble et al. 1991) .

## 2.3 Sustained angiogenesis

Oxygen and nutrients supplied by the vasculature are crucial for cell function and survival. Angiogenesis, the formation of new blood vessels, is a discrete step in tumor progression that is required for expansion of tumor mass (Bouck, Stellmach et al. 1996; Hanahan and Folkman 1996). Astrocytomas are strongly oxygen-dependent. When grade III astrocytomas progress to grade IV, they show features of hypoxic and necrotic palisades (Blouw, Song et al. 2003). The resulting hypoxia is promoting formation of new blood vessels that supply the tumor with necessary metabolites. The hypoxic response is triggered to a large extent by the hypoxia inducible factor-1 (HIF-1) (Semenza 2003). HIF-1 is overexpressed in human glioblastomas. Among the most prominent target genes of HIF-1 is the angiogenic factor, vascular endothelial growth factor (VEGF), which is expressed in the perinecrotic palisading cells (Folkman 1996). VEGF is also produced by tumor-associated cells like endothelial cells and macrophages, as well as by surrounding stromal cells and regulates endothelial cell proliferation and vascular permeability. The earliest stages are defined by vasodilatation and an increased vascular permeability of pre-existing capillaries or post-capillary venules in response to VEGF. The vascular basement membrane and the extracellular matrix are locally degraded to allow underlying endothelial cells to migrate into the perivascular space and multiply.

Astrocytic tumor cells do not require neovascularization. They acquire their nutrients by co-opting existing blood vessels without initiating angiogenesis, but by growing along blood vessels of the vascular-rich brain parenchyma (Brat and Van Meir 2004). However, when they progress into glioblastomas, they become hypoxic and necrotic, partially due to vessel regression and increased tumor cell proliferation. This initiates hypoxia-induced angiogenesis. As soon as these tumors are re-vascularized, they become extremely aggressive. Expression of tenascin-C, an extracellular matrix glycoprotein, has been found in the wall of the blood vessels and in the ECM of GBMs (Chiquet-Ehrismann, Mackie et al. 1986), suggesting a role for this protein in angiogenesis (Zagzag, Friedlander et al. 1995) (Annex 3).

## 2.4 Unchecked migratory and infiltration capacities

The ability of glioma cells to widely invade normal brain tissue is a key property of the malignant gliomas. Malignant lesions are usually considered to be metastatic; however, brain tumors differ from these by the fact that they rarely spread to sites outside of the CNS (Maher, Furnari et al. 2001; Merlo 2003). The progression is a diffuse, locally invasive growth into the normal brain coupled with an increase in tumor volume. Moreover, tumors may spread along blood vessels, white matter tracts and grey–white matter interfaces.

Invasion does not correlate with the grade, since low-grade astrocytomas already extensively invade throughout normal brain tissue. In the adult CNS, neurons, astrocytes and oligodendrocytes are fixed in position and do not migrate. A number of studies indicate that PTEN can regulate cell shape and movement (Merlo and Bettler 2004). In addition, the focal adhesion kinase (FAK) mediates signal transduction by integrins and by regulating cell adhesion and migration. Interestingly, FAK is overexpressed in primary GBMs (Jones, Machado et al. 2001). Overexpression of PTEN inhibits cell spreading and cell migration induced by integrins, whereas reduction of PTEN levels has the opposite effect (Maier, Jones et al. 1999). The inhibitory effects of PTEN can be blocked by the overexpression of FAK, which induces extensive reorganization of the actin cytoskeleton, formation of focal adhesions and directional migration (Jones, Machado et al. 2001). Thus, cells lacking functional PTEN would be expected to manifest increased migration and increased tendency to undergo metastasis. PTEN mutations are found in glioblastomas but rarely in low-grade astrocytic tumors (Knobbe, Merlo et al. 2002; Sansal and Sellers 2004).

## 2.5 Benefit of bad telomeres

Cells which derive from normal tissues are limited to a number of cell divisions named the “Hayflick limit” (Hayflick 1997). Telomeres are the protective ends of the chromosomes formed by repetitive nucleotide sequences and associated protein complexes that are shortened at each cell division. The telomerase lengthens telomeres by using an RNA template and is in adults only expressed in stem cells, and becomes reactivated in many cancers cells including malignant gliomas. Without telomerase, each chromosome loses a few telomere repeats every time it is duplicated. This makes the chromosomes unstable, and may lead to genomic instability. The integrity of the genome in normal cells is mediated by p53. A signaling circuit centered on p53 detects DNA damage and limits the emergence of cells with a mutant genome, either by arresting the cell cycle to allow DNA repair or by inducing cell death (Artandi, Chang et al. 2000). Since telomerase is reexpressed, cancer and immortalized cells have the ability to go indefinitely through cycles of chromosome replication followed by cell division (Hanahan 2000). Studies in glioblastomas have shown that the activity of telomerase may be an important marker of brain tumor malignancy (Nakatani, Yoshimi et al. 1997). Other studies done on brain tumor tissues show that telomerase reactivation is an essential event in the neuroepithelial cell immortalization in vitro (Hiraga, Ohnishi et al. 1998). Indeed, the detection rate of telomerase activity was examined in astrocytic tumors, where the detection rate was 20.0% (3 of 15) for grade II astrocytomas, 40.0% (6 of 15) for anaplastic astrocytomas, and 72.3% (34 of 47) for glioblastomas (Hiraga, Ohnishi et al. 1998). The concept of telomerase reexpression as a cause for genomic instability, however, is not uniformly accepted. It is likely that genomic instability is a multi-factorial process.

## 2.6. High aerobic glycolysis

**Glucose.** During aerobic glycolysis, glucose is taken up by specific transporters, GLUTs where it is converted first to glucose-6-phosphate by hexokinase and then to pyruvate, oxidized to  $\text{HCO}_3$  and reduced into lactic acid in the presence of oxygen. This process is called the “Warburg effect” (Warburg 1956). A common property of

invasive cancers is an altered glucose metabolism and increased aerobic glycolysis. This is only observed in cancer cells (Yamamoto, Seino et al. 1990; Smith 1999), whereas in most mammalian cells, glycolysis is inhibited in the presence of oxygen, which allows mitochondria to oxidize pyruvate to CO<sub>2</sub> and H<sub>2</sub>O (Gatenby and Gillies 2004).

Tumors are able to adapt their metabolism to survive under conditions of reduced oxygen availability by increasing glycolysis to maintain ATP production. But, if oxygen is not sufficiently supplied, under hypoxic conditions, cells become necrotic and a set of genes are induced by the hypoxia inducible transcription factors (HIF) that allow them to overcome hypoxic conditions by inducing angiogenesis. This is specifically the case for grade IV astrocytoma or GBM (Van Meir 1996; Kaur, Khwaja et al. 2005). In addition, under hypoxic conditions, some of the cells can acquire new mutations that allow them to grow and form new clones. The heterogeneity of these tumors parallels their aggressiveness.

An increased glucose uptake in cancer cells can be visualized through the clinical application of the imaging technique positron-emission tomography (PET) which uses the glucose analogue tracer fluorodeoxyglucose (FDG) (Novak, Molnar et al. 2005) (Annex 4). Imaging of thousands of oncology patients has demonstrated that most primary and metastatic human cancers show a significantly increased glucose uptake. The acceleration of glucose uptake and trapping occurs because of the upregulation of glucose transporters, notably GLUT3 (Boado, Black et al. 1994), but also GLUT1, and hexokinase II (Muzi, Freeman et al. 2001). These studies have correlated poor prognosis and increased tumor aggressiveness with increased glucose uptake, demonstrating the clinical importance of glucose metabolism in glioblastomas (Nelson, Wang et al. 1996) (Annex 5).

The high glycolytic activity of brain tumors leads to a considerable production of lactate. Lactate metabolism in normal brain tissue has been characterized by nuclear magnetic resonance (NMR) studies after infusion of <sup>13</sup>C-labeled lactate (Hassel and Brathe 2000). These studies have demonstrated that, depending on its blood concentration, lactate is able to cross the blood–brain barrier and penetrate the brain tissue, where it behaves as a preferred neuronal substrate.

**Lactate.** A particular type of glial cells, the astrocytes, plays a central role in regulating brain energy metabolism. Of particular interest was the observation that

astrocytes responded to a prominent index of neuronal activity, the excitatory neurotransmitter glutamate, by enhancing both glucose use and lactate production (Pellerin and Magistretti 1994). A transfer process between astrocytes and neurons was proposed, a concept that came to be known as the astrocyte-neuron lactate shuttle (Bittar, Charnay et al. 1996). The first component of the shuttle is constituted by the metabolic response of astrocytes to glutamate at excitatory synapses. An enhancement of aerobic glycolysis, defined as preferential glucose use and lactate production despite sufficient oxygen levels to support oxidative phosphorylation, occurs in astrocytes in response to neuronal activation at glutamatergic synapses. Lactate is released in the extracellular space where it adds to a preexisting extracellular pool. The second part of the shuttle concerns lactate use by neurons. It was clearly demonstrated recently that lactate is the preferential oxidative substrate for neurons but not for astrocytes, as compared with glucose (Bouzier-Sore, Voisin et al. 2003). Because the concentration of lactate in the extracellular space was found to be at least similar if not superior to glucose concentration under resting conditions (Abi-Saab, Maggs et al. 2002), it is likely that a substantial part of neuronal energy needs will be covered by lactate.

The concept that glucose is the major energy substrate provided by the circulation to the adult brain seems to hold true, even if under a number of circumstances, other substrates can contribute significantly to cover at least part of brain energy needs. Lactate and ketone bodies have been also demonstrated to provide a significant energy source for the brain during development but also in abnormal situations such as diabetes, prolonged starvation, or hypoglycemia (Abi-Saab, Maggs et al. 2002).

### 3. Targeted chemotherapy against GBMs

Therapeutic approaches for GBMs are multidisciplinary, involving surgical excision followed by radiotherapy and chemotherapy (Stupp, Mason et al. 2005). Despite prolonging survival, treatment of high-grade tumors essentially remains a compromise between control of tumor growth and maintenance of quality of life.

Modern imaging techniques have greatly improved surgical accuracy, but complications of surgical intervention are exacerbation of neurological deficit as well as the risk of infection, seizures, bleeding and cerebrospinal-fluid leakage. Even wide surgical resection inevitably leaves a large population of viable tumor cells (some  $10^8$ – $10^9$  cells) and is further limited by the need to conserve essential areas. Radiotherapy is well established in the treatment of brain tumors, extending life expectancy in the case of highly proliferative tumors by a factor of about two. Radiation is active against dividing cells which may form a relatively small proportion of the tumor mass. Late reactions like radiation-necrosis can occur after treatment. Radiotherapy principally affects the microvasculature, leading to the occlusion of small vessels and subsequent necrosis. Necrosis caused by radiation most commonly affects the white matter. Infants are more susceptible to its effects because of their incomplete state of myelination. In this perspective, chemotherapy is the first line of treatment following surgery for children and adults that do not respond well to radiotherapy. One traditional treatment for brain tumors involves non-specific cytotoxic drugs which act through damaging of DNA, like Taxol-like drugs (Dietzmann, Kanakis et al. 2003), or cisplatin, (Ashby and Shapiro 2001) since other targeted therapies are ineffective. However, these drugs have a marginal impact on patient survival (Rich and Bigner 2004). Nitrosoureas are moderately active against CNS tumors since they are soluble in lipids, a property that enables crossing the blood-brain barrier. Temozolomide permits slightly to prolong survival in selected GBM patients from 12 to 14 months when combined with radiotherapy (Stupp, Mason et al. 2005). The actions of such drugs are poorly understood. They can damage renal functions, affect bone marrow cells and cause peripheral nerve problems, besides being markedly immunosuppressive. Thus, the medical need for more effective cancer chemotherapies remains crucial.

### 3.1. 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, Furnari et al. 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. Holland and colleagues have shown that the expression of K-RAS and AKT (Holland, Celestino et al. 2000), PDGF-B (Dai, Celestino et al. 2001) or EGFR (Holland, Hively et al. 1998) can induce gliomas. Van Dyke (Xiao, Wu et al. 2002) has used tissue-specific expression of a RB inhibitor to generate brain tumors facilitated by disruption of PTEN expression. DePinho (Bachoo, Maher et al. 2002) found that activation of the EGFR pathway in a p16INK4A-null background induced astrocytic dedifferentiation and glioma formation.

A number of studies has demonstrated that expression of p21/WAF1/Cip1 causes a G1 cell cycle arrest protecting cells against chemotherapeutic agents (Fueyo, Gomez-Manzano et al. 1998; Burgess, Pavey et al. 2001; Schmidt and Fan 2001). p21/WAF1/Cip1 is a p53-inducible negative cell cycle regulator, and appears to mediate p53-dependent cell cycle arrest, but not apoptosis, via damaging DNA (Gorospe, Cirielli et al. 1997). Studies using cells from p21 knockout mice have also shown that p21-deficient cells are more sensitive to the induction of apoptosis by chemotherapeutic drugs and more defective in the repair of DNA damage than normal cells (Waldman, Lengauer et al. 1996). Interestingly, elevated p21 expression has

been shown in most gliomas suggesting a significant therapeutic window to be targeted (Arvanitis, Malliri et al. 1991; Jung, Bruner et al. 1995; Tsumanuma, Tanaka et al. 1997). Other studies claim the necessity of p21/WAF1/Cip1 for the induction of cell death in cancer cells, via HDAC inhibitors, as it is the case in the MCF-7 cell line (Chopin, Toillon et al. 2004). Mice lacking p21CIP1/WAF1 undergo normal development but are defective in G1 checkpoint control. (Deng, Zhang et al. 1995). Studies investigating the anti-tumor potential of p21 have shown that p21-null mice do not develop tumors, at least not at an early age (Jackson, Adnane et al. 2002). Molecular analysis of the p21 gene has been performed in diverse types of human tumors in which studies, 7 out of 102 (7%) human tumor samples were identified having point mutations within the coding region of the p21 gene; two of them showed gene rearrangements. The results suggest that the frequency of genetic alterations in the p21 gene is relatively low compared to several known tumor suppressor genes (Watanabe, Fukuchi et al. 1995) and may represent the background rate of mutations in genetically unstable cells.

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. These processes influence malignant cell transformation (Hanahan and Weinberg 2000). Methylation occurs only at position 5 of cytosines to guanosines in the normal mammalian genome. CpG dinucleotides have been progressively depleted from the eukaryotic genome during evolution (Herman, Merlo et al. 1995; Baylin and Herman 2000; Esteller 2002). Small CpG regions, so-called “ CpG islands”, are protected from methylation and are located within promoters of 40 to 50% of human genes, allowing

active transcription, whereas the remaining CpG have a high frequency of methylation. A growing number of tumor suppressor genes harbor dense methylation in normally unmethylated promoter CpG islands (Herman, Latif et al. 1994). Such changes contribute as much to tumor growth as do actual mutations, since methylation of CpG islands is associated with delayed replication of associated genes, chromatin condensation and inhibition of transcription initiation. The original indication that methylation status might be relevant in brain tumorigenesis came with the finding that the tumor suppressor gene *p16<sup>INK4</sup>*, encoding the cyclin-dependent kinase inhibitor, is methylated in its promoter in 25% of gliomas but not in normal brain (Merlo, Herman et al. 1995; Costello, Berger et al. 1996). Another example is the loss of DNA mismatch repair due to the methylation of the human mutL homologue 1 (*hMLH1*) gene promoter resulting in resistance of cancer cells to cisplatin *in vitro* and *in vivo* (Plumb, Steele et al. 2004). DNA methylation is linked to regulation control of gene expression. The functional implications of the association of methylation with transcriptionally repressive chromatin have been outlined (Hong, Bollen et al. 2003). Recent work has demonstrated that methylated CpG dinucleotides are recruiting histone deacetylases (HDACs) (Baylin and Herman 2000). HDACs are repressing transcription by deacetylating the amino groups on lysine residues in the amino-terminal tail domains of the histones. Knudson's hypothesis that "two hits" (Knudson 2001) are required for the full inactivation of a tumor suppressor gene has been shown to be fundamentally correct in almost all cases of human cancer (Jones and Laird 1999). The "two hits" by which tumor suppressors become disabled, are the intragenic mutations and the loss of heterozygosity (LOH) and homozygous deletion. The highest frequency of loss of heterozygosity (LOH) in glioblastoma cell lines is on chromosome 10q (Maier, Comparone et al. 1997; Maier, Zhang et al. 1998). PTEN on 10q23.3 was the first tumor suppressor gene identified (Li, Yen et al. 1997). Alternative reading frames on the *CDKN2A* locus on chromosome 9p21 encode two different cell cycle suppressors, called *p16<sup>INK4A</sup>* and *p14<sup>ARF</sup>*. Homozygous deletions of the *p16/CDKN2A* gene are found in 69% of glioblastoma cell lines (Ishii, Maier et al. 1999) and in 45% of human GBMs (Labuhn, Jones et al. 2001), and the *p14<sup>ARF</sup>* gene was always co-deleted with the *p16* gene. This high genetic instability developed during brain tumorigenesis leads to overgrowth of precursor cell populations with increasingly malignant tumor cell clones that develop higher resistance to selective

chemotherapy. 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 and van Lohuizen 2004) has clear implications on the therapeutic use of agents targeting the DNA methylation machinery and HDACs (Johnstone 2002; Noble and Dietrich 2004; Szyf, Pakneshan et al. 2004; Suzuki, Nagano et al. 2005).

**Blood-brain barrier (BBB): an obstacle to drug delivery.** Drug delivery is a major obstacle for successful treatment of malignant gliomas because of the BBB (Nagane, Huang et al. 1999). The BBB is a dynamic interface between the blood and the brain (Annex 6) (de Boer, van der Sandt et al. 2003), 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. This anatomical barrier is provided by tight junctions between endothelial cells and involves various transport systems at the BBB including P-glycoprotein (P-gp), a major ATP-dependent efflux pump consisting of the multidrug-resistance 1 gene (*mdr1*). The expression of P-gp was investigated in the brain where the protein might transport paclitaxel and other drugs away from central nervous system (CNS) tumors. It was found that P-gp is expressed at high levels in intact rat and human brain capillaries at the luminal surface of the endothelium, a location that could restrict permeation of drugs into the CNS (Fellner, Bauer et al. 2002). When human glioblastomas were transplanted into the brains of mice, the blood vessels developed within the tumors also expressed high levels of P-gp (Fellner, Bauer et al. 2002). Although frequently not intact in the center of malignant brain tumors, the presence of an intact BBB at the proliferating and especially invasive edge of a tumor beyond visible tumor margins has been proposed as a major factor for the failure of chemotherapy for brain tumors (Pardridge 2003). As delivery into the CNS can cause numerous side effects like leukoencephalopathy, most agents are specifically designed to have limited intracranial distribution. This is why potentially effective anti-cancer

drugs will be of limited use for the treatment of CNS tumors. 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, Langer et al. 2001).

### 3.2. Choice of drugs and combinatorial strategy

Genetics of human tumors and genetically engineered mouse models have improved the understanding of the molecular biology underlying glioma pathogenesis and have revealed abnormalities in a set of common cellular pathways and functions among the majority of these tumors that are now being targeted by novel agents in preclinical and clinical development (Merlo 2003). Such targeted agents offer the promise of improved tumor control with limited toxicity. Still, significant challenges in their development remain: the inability to predict tumor response and limitations of drug delivery into the tumor. Indeed, the lipid-mediated free diffusion of a drug through the BBB requires lipid solubility and a molecular mass less than about 400–500 Da. Most of the drugs that are selected as drug candidates by receptor-based high-throughput screening programs lack these dual characteristics and do not undergo transport across the BBB without the intervention of brain drug-targeting technology like liposomes or the infusion of agents into surgical resection cavities. For example, breast cancer patients treated with trastuzumab (Herceptin; Genentech/ Roche) have a significant rate of recurrence with brain metastases probably due to failure of antibody delivery into the brain.

In order to design a targeted for gliomas, high selectivity for tumour cells relative to normal cells, facilitated delivery to the brain parenchyma across the BBB and limited toxicity have to be taken into consideration. Several protein kinase inhibitors have been developed by pharmaceutical companies and are under preclinical and clinical evaluation. Measurement of biomarkers has become an essential element of targeted therapies by translating the *in vivo* efficacy of the drug to a tissue or cell culture model. The biomarker can be the target itself or an indirect marker. The target has to

be expressed in a high percentage of tumor cells but not in the surrounding normal tissues. It must be active in the tumors in which it is expressed and contribute to the tumor-phenotype.

One example for targeted therapies are drugs designed to specifically target tyrosine or serine/threonine kinase activity. The kinase regions bind ATP molecules and transfer phosphate groups to either the receptor or signaling transducers to initiate pathway activation. Pharmaceutical companies have screened large chemical libraries of ATP mimetics to develop selective inhibitors of kinases of interest. Recent enthusiasm has been generated by the remarkable success of some small-molecule protein kinase inhibitors (PKIs) in selectively inhibiting tumour growth. Imatinib mesylate (Glivec/ Gleevec/STI571/Novartis), which blocks the kinase activity of BCR-ABL, ABL, c-KIT and PDGF receptor (PDGFR), has been successful in the control of chronic myelogenous leukaemia (CML), and of gastro-intestinal stromal tumours (GIST) (Manley, Breitenstein et al. 2004), (Russell, Brady et al. 2003). Gefitinib (Iressa; AstraZeneca), an inhibitor of EGFR-TK activity, is the first targeted agent to be approved for the treatment of patients with advanced non-small-cell lung cancer (NSCLC) (Krishnan, Rao et al. 2003). Due to the small molecular size, PKIs might offer benefit in terms of delivery, but their polarity might limit intracranial delivery. Further drug design will permit increased lipophilicity or selective intracranial penetration.

Nevertheless, despite the relatively recent development of novel targeted therapies, several potential resistance mechanisms to these agents have become apparent. This is the case of chronic myelogenous leukaemia expressing BCR-ABL with resistance to Glivec (Bakalova, Ohba et al. 2003; von Bubnoff, Peschel et al. 2003; Weisberg and Griffin 2003), this resistance is associated with mutation of the ATP-binding site of ABL. Cell lines treated with inhibitors of growth-factor-receptor tyrosine kinases often regrow with increased expression of other mitogenic growth factors. Preclinical studies of GBMs resistant to inhibitors of the kinase activity of EGFR might have resistance due to activation of IGFR1/PI3K/AKT pathway (Jones, Goddard et al. 2004).

Because of the appearance of mechanisms of resistance, broad-spectrum kinase inhibitors or combination of inhibitors have been developed. Another possible

approach includes the simultaneous targeting of upstream (receptor) and downstream (intracellular) targets. Recent studies showed that the growth of GBMs might be synergistically inhibited with the combination of a tyrosin kinase C inhibitor (TKI) targeting EGFR and a mTOR inhibitor (Goudar, Shi et al. 2005). The complexity of these strategies might be increased by the need of a sequential application in order to avoid negative interactions. Some agents might require cycling cells for the induction of cell-cycle arrest. In addition to the combined use of inhibitors, targeted therapies are already under evaluation in combination with unspecific cytotoxic treatments like radiation or chemotherapy (taxol-like drugs, cisplatin, HDAC inhibitors an so on). Preclinical studies have shown additive and synergistic effects by these approaches. The mTOR inhibitor CCI-779 has shown to synergize with tamoxifen in tamoxifen-resistant breast carcinoma characterized by high AKT activity (deGraffenried, Friedrichs et al. 2004; Dutcher 2004) and synergizes with the inhibitor of EGFR's tyrosine-kinase (EGFR-TK) AEE788 (Novartis) in GBMs (Goudar, Shi et al. 2005). (Annex 7).

The goal of the thesis was to define, *in vitro*, an efficient chemotherapeutic treatment for human gliomas, since no real progress has been made in the domain of neurooncology with regard to drug development. In this work, human glioma cell lines were used as experimental tools for pre-clinical studies of various drugs that are already in clinical trials for the treatment of other cancers. Each cell line differs in its genetic background and is unique and representative of an individual patient. (Annex 8: glioma cell lines' table), (Ishii, Maier et al. 1999). We were focusing on four classes of drugs. The protein kinase inhibitors (biologicals) are specifically targeting tyrosine or serine/threonine kinases. These drugs are downregulating pathways that regulate cell growth, survival, invasion or angiogenesis. Among them, AEE788, Glivec and RAD001 are inhibiting EGFR/VEGFR, PDGFR and mTOR respectively. Cytotoxic drugs, including histone deacetylases (HDACs) inhibitors like trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), sodium butyrate (SB) and LAQ824 and microtubule dynamics blockers, like epothilones (e.g. EPO906A) or taxol, are less specific drugs. HDACs inhibitors target the epigenetic modifications in the promoter of a set of important genes regulating cell cycle, survival and proliferation, whereas taxol-like drugs are blocking mitosis by inhibiting

polymerization and depolymerization of microtubules. The use of glycolytic inhibitors, like 2-deoxy-D-glucose (2-DG) represents an innovative additional way to block signaling pathways, since tumor cells are highly dependent on ATP for regulating growth and survival. This choice was suggested by the results obtained by FDG-PET studies in humans with solid tumors that showed significantly increased glucose uptake, even more drastic in brain tumors (Annex 4). The non-steroidal anti-inflammatory drug (NSAID) Indomethacin is an inhibitor of the cyclo-oxygenase overexpressed in GBMs. The drugs we used in this study are described individually as follow:

### **AEE788.**

AEE788 inhibits both the epidermal growth factor (EGF) and the VEGF receptor tyrosine kinase family members. AEE788 is a small-molecule ATP mimetic that inhibits EGFR/VEGFR-associated kinase activity. EGFR is a transmembrane glycoprotein that mediates the response of cells to mitogenic polypeptides belonging to the EGF family as the epidermal growth factor (EGF). EGFR plays fundamental roles in development, cell proliferation and differentiation, and has been implicated in the deregulation of cell proliferation associated with human cancer, like breast cancer or GBM (Nathoo, Goldlust et al. 2004). The majority of GBM have aberrant EGFR activity through EGFR over-expression, amplification or mutation. The most common of these, the EGFRvIII, involves the loss of exons 2-7 that results in a loss of the extra-cellular ligand-binding domain and constitutive activation of the receptor. EGFR expression has been linked to decreased survival in patients with malignant gliomas. Most solid tumors establish their blood supply by forming vessels to allow unrestrained growth (Folkman 1996). Inhibition of VEGF-induced angiogenic signals will selectively target the tumor-associated vessels since VEGF-mediated endothelial cell proliferation in the normal vasculature is a rare event. In addition, EGFR inhibitors can inhibit production of VEGF by tumor cells; a more potent anti-tumor response may be achieved through the concomitant inhibition of both EGF and VEGF receptors.

Previous results indicate that there is potential for improved responses by combining EGFR inhibitors with other drugs. For example, the combination of gefitinib (Iressa,

ZD1839) with radiation therapy in non-small-cell lung cancer, head-and-neck cancers, and other solid tumors (Ochs 2004) or the synergistic interaction between gefitinib and paclitaxel against human gastric carcinoma cells. (Park, Lee et al. 2004). However, no efficient combinatorial effects have been reported on GBM so far. Currently, there are some ATP-competitive EGFR and VEGFR tyrosine kinase inhibitors in different stages of clinical development (Traxler, Allegrini et al. 2004). AEE788, as an anticancer agent, targets deregulated tumor cell proliferation as well as angiogenic and migratory capacities (Nathoo, Goldlust et al. 2004). AEE788 is currently in phase I clinical trials.

### **Glivec or Gleevec or STI571 or imatinib.**

The tyrosine kinase inhibitor, Glivec, is an example of a selective targeted oncologic therapy that induces improved survival in patients with chronic myelogenous leukemia (CML). Glivec is inhibiting specifically the Bcr-Abl kinase, a fusion protein with enhanced tyrosine kinase activity resulting from the Philadelphia chromosome (Ph) present in 95% of CML patients and formed by the translocation between chromosome 9 and 22, replacing the first exon of *c-abl* with sequences from the BCR gene. Glivec is an ATP-competitive inhibitor blocking the oncoprotein *c-Abl* and the Bcr-Abl fusion protein involved in the regulation of proliferation in CML. PDGFR and *c-Kit*, that play an important role in the proliferation of tumor cells, appeared to be two other main targets of Glivec, which block their autophosphorylation (Manley, Breitenstein et al. 2004). Gastrointestinal stromal tumors (GIST) are the most common mesenchymal neoplasms of gastrointestinal tract. The tumors occur in the whole gastrointestinal tract and express the cell surface transmembrane receptor KIT that has a tyrosine kinase activity and is a product of the *c-kit* protooncogene. Since PDGFR is involved brain tumors, Glivec appeared to be an interesting drug to be tested in human gliomas cell lines, as a single agent or combined with other drugs (Capdeville, Buchdunger et al. 2002).

In previous studies, Glivec has already been combined with other drugs for various cancer treatment. The microtubule-stabilizing agent Epothilone B (EPO906) combined with Glivec showed a positive interaction by exhibiting an anti-tumour effect using rat C6 glioma xenografted in nude mice (O'Reilly, Wartmann et al. 2004).

Glivec was tested in combination with radiotherapy (Russell, Brady et al. 2003) and in combination with the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) to overcome the resistance observed against Glivec during the treatment of a CML blast crisis (Nimmanapalli, Fuino et al. 2003; Nimmanapalli, Fuino et al. 2003; Yu, Rahmani et al. 2003). In another study, the mTOR inhibitor rapamycin enhanced Glivec-mediated killing of CML cell lines *in vitro* by overcoming Glivec resistance in cells with *Bcr-Abl* gene amplification (Ly, Arechiga et al. 2003).

### **RAD001.**

RAD001 is an analog of rapamycin, a natural product derived from the soil bacteria *Streptomyces hygrospilus*, approved for use in organ transplantation in 1999 due to its immunosuppressive property. Studies demonstrated antitumor activity, although initially the mechanism was unknown.

Rapamycin targets the serine/threonine protein kinase mTOR (mammalian target of rapamycin), a key regulator of cell cycle and growth. The drug does not directly inhibit mTOR, but binds to its immunophilin, FK binding protein (FKBP12). This complex then interacts with mTOR inhibiting its function and leading to reduced cell growth and proliferation. The downstream effects of rapamycin include inhibition of translational pathways, with loss of phosphorylation of the eukaryotic translation initiation factor 4EBP-1 (4E binding protein-1), and with inhibition of the 40S ribosomal protein kinase p70S6, blocking ribosomal biogenesis and mRNA translation. This effect results in a 15% to 20% reduction of overall protein levels and leads to cell cycle arrest. Other cellular functions that appear to be regulated by mTOR include actin organization, membrane trafficking, protein degradation, protein kinase C signaling, and tRNA synthesis (Schmelzle and Hall 2000). There are also regulatory effects on synthesis of essential cell cycle proteins, such as cyclin D1 and c-myc. Other data suggest that mTOR regulates protein synthesis when cellular ATP levels fluctuate (Dennis, Jaeschke et al. 2001).

Important upstream regulators may be altered in tumor cells as it is the case in glioblastoma. Both PI3K and Akt are thought to be upstream to mTOR, regulated by the tumor suppressor protein PTEN. (Inoki, Corradetti et al. 2005). An additional pathway influenced by mTOR involves the hypoxia-inducible factor (HIF). The

accumulation of the oxygen-sensitive transcription factors HIF-1 and HIF-2 leads to an increased stimulation of the expression of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and transforming growth factor (TGF- $\beta$ ). This effect is augmented by the activation of mTOR which increases HIF-1 activity (Brugarolas, Vazquez et al. 2003). In mice, rapamycin may inhibit production of the vascular endothelial growth factor VEGF that leads to a remarkable inhibition of tumor growth (Guba, von Breitenbuch et al. 2002).

In addition to its role in regulating cell proliferation, growth, differentiation, and survival, mTOR can control whether a cell undergoes programmed cell death type I (apoptosis) or type II (autophagy) (Castedo, Ferri et al. 2002). Rapamycin induces apoptosis in a cell type-specific fashion, yet in some cells in which rapamycin inhibits mTOR it stimulates autophagy rather than apoptosis (Shigemitsu, Tsujishita et al. 1999). It is still unclear whether cross-talk occurs between these two programmed cell death pathways and what determines why cells die from one type or the other.

It is known that half of glioblastomas have *PTEN* mutations, which could make them increasingly sensitive to mTOR inhibitors, and this is being investigated clinically (Sansal and Sellers 2004). Previous reports already showed that rapamycin and its derivatives RAD001 and CCI-779, inhibit glioblastoma tumor growth by specifically blocking the mTOR protein kinase, (Eshleman, Carlson et al. 2002), (Huang and Houghton 2003), (Sawyers 2003). Other studies showed synergism between rapamycin derivatives and other drugs, on growth inhibition or apoptosis induction. For example, rapamycin has been reported to enhance the efficacy of several cytotoxic agents in breast cancers: additive effects were observed in combination with doxorubicin and gemcitabine, whereas synergistic interactions were observed in combinations with paclitaxel, carboplatin, and vinorelbine (Mondesire, Jian et al. 2004). In the studies performed in a pediatric brain tumor model (Geoerger, Kerr et al. 2001), CCI-779 induced significant growth inhibition and additive effect when combined with cisplatin. More recently, the rapamycin derivative RAD001 was found to dramatically enhance cisplatin-induced apoptosis in wild-type p53, but not mutant p53 tumor cells (Beuvink, Boulay et al. 2005). In *In vivo* studies, RAD001 was shown to improve glioblastoma growth inhibition in combination with AEE788, in a mouse glioma xenograft model (Goudar, Shi et al. 2005). RAD001 and CCI-779 are in phase

I and II trials, respectively, as anti-cancer agents. But, used as single agents, only weak responses have been obtained when used for the treatment of GBM in some 5% of patients.

### **HDACs inhibitors: trichostatin A (TSA), sodium butyrate (NaB) and LAQ824**

The accessibility of DNA to proteins such as transcription factors and their co-factors is determined in part by a series of chromatin modifying enzymes. The DNA wrapped around nucleosomes can be further condensed into higher order chromatin, known as heterochromatin and euchromatin. Euchromatin is more transcriptionally active and contains most protein encoding genes. The nucleosome itself is made up of an octamer of histone proteins, two each of H2A, H2B, H3 and H4, with 145 bp of DNA wrapped around it. Modification of the highly charged lysine residues in the N-terminal histone tail is one mechanism whereby chromatin condensation is controlled. These lysine residues are subject to modification by acetylation and methylation, which are post-translational modifications that have a considerable impact on transcriptional activity (McLaughlin and La Thangue 2004), (Annex 9).

Histone deacetylases (HDAC) are evolutionarily conserved and expressed in organisms from archaeobacteria to man and, together with the conversely acting histone acetyl transferases (HATs), control the acetylation level of chromatin and subsequent transcriptional activity. HDACs remove the acetyl group from histones using a charge-relay mechanism consisting of two adjacent histidine residues, two aspartate residues and one tyrosine residue. Crucial for this charge-relay system is a  $Zn^{2+}$  ion (zinc finger), which binds deep in the pocket of the enzyme. The hydroxamic acid-based compound trichostatin A (TSA), fits into the HDAC active site, chelating the  $Zn^{2+}$  ion and inhibiting the enzyme at a low IC50 (nM).

The action of all HDACs inhibitors (HDIs) leads to a restricted alteration of gene expression profile since only 2 to 5% of genes, involved in proliferation, differentiation and apoptosis regulation, are re-expressed, as it is the case for the gene coding for the cyclin-dependent kinase inhibitor p21. This restricted gene re-expression may explain the apparent low toxicity seen in clinical trials to date. Subsequent hydroxamic acid based HDIs, including SAHA, PXD101 and LAQ824, are in clinical trials (Monneret 2005). Cancer cells overexpressing the membrane

ATPase P-glycoprotein (P-gp), that mediates multidrug resistance, do not exhibit resistance to LAQ824, which indicates that LAQ824 is not a substrate for P-gp.

An important finding in predicting the potential utility of HDIs in the clinics is their activity in cell-lines that are resistant to existing chemotherapeutics. For example, TSA sensitizes estrogen receptor  $\alpha$ -negative breast cancer cells to tamoxifen (Jang, Lim et al. 2004). Other interesting results were obtained in CML where Glivec-resistant Bcr/Abl cells are sensitized to Glivec upon co-treatment with SAHA (Yu, Rahmani et al. 2003).

### **EPO906: blocker of microtubule dynamics**

Drugs that target microtubules are among the most commonly prescribed anticancer therapies. EPO906 (epothilone B), is a potent member of a new class of microtubule-stabilizing cytotoxic agents known as epothilones. Like cytotoxics in the taxane family (paclitaxel or taxol), EPO906A exerts its anti-tumor activity effect by interfering with microtubule dynamics. EPO906 is able to induce polymerization of tubulin dimers into stable microtubules that blocks the cell cycle in G2/M and blocks mitosis (Goodin, Kane et al. 2004). Formation of microtubules involves polymerization of heterodimeric subunits of  $\alpha/\beta$ -tubulin which involves hydrolysis of guanosine 5'-triphosphate and is regulated by several microtubule-associated proteins. Intact microtubule function is required for formation and functioning of the mitotic spindle, and cells treated with agents that bind either tubulin subunits or polymerized microtubules exhibit alterations in spindle formation, as well as arrest in the G2/M phase of the cell cycle, which is associated with induction of apoptosis.

In contrast to paclitaxel, EPO906 is cytotoxic to multidrug-resistant cells overexpressing the P-glycoprotein (P-gp) efflux pump and to taxol-resistant cells. EPO906 is also able to pass the blood-brain-barrier in animals. Clinical studies have shown that DNA damaging agents like cisplatin or microtubule dynamic blockers like taxanes are cytotoxic chemotherapeutics, not well tolerated in patients due to their narrow therapeutic window of application. With respect to that, a combinatorial approach was developed in order to decrease the doses of these cytotoxic drugs. Recent results have already shown interesting combination of epothilones with other drugs. For example, O'Reilly *et al* have observed an enhanced antitumour activity in

vivo against experimental rat C6 glioma when EPO906 and Glivec were combined (O'Reilly, Wartmann et al. 2004). In another recent study, the glycolytic inhibitor 2-deoxy-D-glucose increased the efficacy of adriamycin and paclitaxel in human osteosarcoma and non-small cell lung cancers in vivo (Maschek, Savaraj et al. 2004).

### **The glycolytic inhibitor 2-deoxy-D-glucose (2-DG)**

The glucose analog 2-deoxy-D-glucose (2-DG) is an inhibitor of glycolysis in all eukaryotic cells. 2DG undergoes facilitated diffusion into cells via glucose transporters. Once intracellular, 2-DG is phosphorylated by hexokinase which is upregulated in cancer cells, yielding 2-deoxy-D-glucose-6-phosphate (2-DG-6-P). 2-DG-6-P is not a substrate for glucose-6-phosphate dehydrogenase or phosphohexoisomerase (Wick, Drury et al. 1957). Therefore, once formed, 2-DG-6-P is not further metabolized and will accumulate in the cell until de-phosphorylated by phosphatases. The accumulation of the anti metabolite leads to a drop in the cellular ATP/ADP ratio and an increase of AMP/ATP ratio. The increase of AMP activates the first cellular energy sensor, 5'AMP-activated protein kinase (AMPK) via the tumor suppressor LKB1 (Shaw, Bardeesy et al. 2004) and the tuberous sclerosis complex TSC1/2 (Inoki, Zhu et al. 2003) that is inhibiting mTOR which then adjusts the rate of ribosome biogenesis to the rate of intracellular ATP levels. By activating AMPK, mTOR may inhibit protein translation affecting the cell cycle and cell proliferation (Horman, Browne et al. 2002). Recently, a role of 2-DG in the modification of glycosylation of transcription factors and other essential proteins has been reported (Kang, Ju et al. 2003). This modification could alter the activity of these proteins, similarly to the phosphorylation status.

2-DG appears to selectively accumulate in cancer cells by metabolic trapping due to high intracellular levels of hexokinase or glucose transporters. This trapping is shown by the imaging technique positron emission tomography (PET) using the glucose analogue tracer <sup>18</sup>fluorodeoxyglucose (FDG) in patients with metastatic human cancers, which allows quantification of glucose uptake in the whole body compared to glucose uptake in the tumor (Wahl, Cody et al. 1991) (Annex 4). By using this method, previous studies have demonstrated that FDG accumulates in most of the cerebral gliomas and its accumulation correlates with the degree of malignancy (Di

Chiro, Brooks et al. 1984). Thus, tumor cells, and most glioblastoma cells, have a higher glucose uptake and use a huge part of their energy from the glycolytic pathway in order to maintain their high growth and proliferation rates (Gatenby and Gillies 2004). As tumor cells are highly dependent of ATP, a less conventional strategy would be to inhibit the “glycolytic” or the “energetic” pathway, which so far has generated little interest in terms of drug development (Aft, Zhang et al. 2002). Thus, the inhibition of the glucose transport and glycolysis appeared to be an interesting anti-tumor tool to alter tumor proliferation, migration and cell survival.

Previous studies have shown the effects of 2-DG on cancer cells. Mostly used as a tool to mimic glucose starvation, only a few reports have demonstrated that 2-DG, used as a single agent, is able to strongly reduce growth of tumor cells (Cay, Radnell et al. 1992). Mohanti *et al* reported the use of 2-DG in combination with radiotherapy for the treatment of human gliomas in poor countries (Mohanti, Rath et al. 1996). 2-DG might induces DNA repair deficiency after radiation leading to apoptosis (Dwarkanath, Zolzer et al. 2001).

### **The Non-Steroidal Anti-Inflammatory Drug (NSAID) Indomethacin (IND)**

Cyclooxygenase (COX) catalyzes the rate-limiting step in arachidonate metabolism, resulting in prostaglandin production. COX exists as two isoforms: COX-1 is constitutively expressed as a house keeping gene in several cell types of normal mammalian tissues, where it is involved in the maintenance of tissue homeostasis. In contrast, COX-2 is a highly inducible gene in response to various stimuli, like lipopolysaccharides and pro-inflammatory cytokines responsible for prostaglandin (PG) production. Cyclooxygenase is expressed in the CNS, where it plays a major role in inflammation, fever and pain, and is also involved in the sleep-awake cycle, body temperature control, cerebral blood flow and neuroendocrine function.

Normal astrocytes alone do not express COX-1, and COX-2 expression is minimal compared to astrocytoma cell lines. In contrast, COX-1, and especially COX-2, are expressed in all grades of astrocytomas, and high-grade tumors express higher levels of COX-2 than low-grade tumors, which is associated with resistance to apoptosis and induction of angiogenesis (Joki, Heese et al. 2000). In addition, recent studies in oncology have identified a role for COX-2 in cell proliferation, in cancer control and

apoptosis regulation (Zhou, Wong et al. 2001).

Cyclooxygenase inhibitors became of high interest for glioma treatment, since synergy with other forms of therapy was reported (New 2004). NSAIDs affect cyclooxygenase in several ways. The NSAID indomethacin is a temporary-dependent reversible inhibitor of cyclooxygenase. Aspirin, one of the oldest NSAIDs, irreversibly inactivates COX-1 and COX-2 by acetylating a serine moiety at the active site. Ibuprofen is a reversible competitive inhibitor. Salicylate alone does not inhibit COX-1 or COX-2 activity in vitro, but it is an effective inhibitor of prostaglandin at sites of inflammation. Previous studies showed that aspirin and other NSAIDs inhibit carcinogenesis in rats, and that both selective and nonselective NSAIDs are effective in inhibiting early stages of tumor development.

COMBINATORIAL STRATEGY FOR  
TREATMENT OF GBM:  
RESULTS OF PRECLINICAL *IN VITRO* TUMOR MODEL.  
AEE788, Glivec, RAD001, EPO906A,  
HDIs (TSA, SAHA, SB, LAQ824, 2-DG, IND)

Previous reports have shown that malignant GBMs are resistant to chemotherapeutic agents, due to the presence of the blood-brain barrier that is reducing drug diffusion, to a high level of genetic instability of tumor cells giving rise to heterogeneity and to mechanisms of resistance associated with tumor growth and the accumulation of mutations. Thus, the application of one single drug might not be sufficient to induce a therapeutic effect on survival or growth of these tumors. Drugs targeting various pathways, essential for tumor cell survival and maintenance, have been tested in this work. Among them, AEE788, Glivec and RAD001 inhibiting the protein kinases EGFR/VEGFR, PDGFR and mTOR respectively were used. The cyclo-oxygenase involved in the prostaglandin pathway has been inhibited by indomethacin. The HDIs LAQ824, NaB, TSA and EPO906A target HDACs and microtubules, respectively. Inhibition of the glycolytic pathway was performed by 2-DG. All these compounds have already been used as single agents in humans. However, we investigated if the exposure of human GBM cell lines to single or combined drugs could synergistically induce cell death at doses that would also be applicable to patients.

## 1. Effects of AEE788, Glivec, RAD001 (so called biologicals) on human glioma cell lines after single and combined treatment

First, using 10 human GBM cell lines (Annex 8) three “biologicals” have been tested in order to determine individual drug concentrations that are necessary to affect proliferation and cell survival. In a second set of experiments it was then analysed if the use of combinations of these three drugs, at clinical dose ranges, are capable to synergistically induce apoptosis.

### **AEE788 induces apoptosis only at unspecifically high concentrations in human GBM cells.**

The EGFR inhibitor AEE788 was tested on LN229, and its effect on cell survival and proliferation was tested. Cells were pre-incubated with AEE788 for 1 hour and then stimulated for 10 min with EGF. The Western Blot shows that 1 to 10  $\mu$ M AEE788

efficiently inhibits phosphorylation of EGFR after stimulation. 0.1  $\mu\text{M}$  AEE788 is able to partially reduce EGFR phosphorylation and 1  $\mu\text{M}$  AEE788 inhibits fully EGFR phosphorylation (Fig. 1A). AEE788 reduces proliferation by inducing an accumulation of cells in the G1 phase of the cell cycle, after 36 h treatment with 1  $\mu\text{M}$  on the drug (Fig. 1B). Increasing concentrations of AEE788 (0.1, 1, 10  $\mu\text{M}$ ) were applied to the cells for 4 days then cell survival was measured. Weak induction of apoptosis occurred at 1  $\mu\text{M}$  and did not exceed 40% apoptosis in 3 out of 10 cell lines tested (Hs683, LN18, U373). At 10  $\mu\text{M}$  the percentage of apoptosis reached 90% in LN215 and SF767 cell lines and between 50% and 70% in the other cell lines (Fig. 1C). However, 10  $\mu\text{M}$  is not usable for clinical trials, since serious side effects are expected to occur at such high doses. Therefore, 0.5  $\mu\text{M}$  AEE788 was used in following experiments.

### **Glivec weakly induces apoptosis in human GBM cell lines**

Increasing concentrations of Glivec were applied to the GBM cell lines for 4 days and survival was determined. Half of the cell lines were refractory to Glivec since no cell death was observed in 5 out of 10 cell lines (Fig. 2). Even unspecifically high concentration of 10  $\mu\text{M}$  Glivec was only able to induce apoptosis between 20 to 25% in half of the lines (Fig. 2).

### **RAD001 efficiently inhibits proliferation of GBM cell lines but does not induce apoptosis.**

A set of mutated PTEN or wild-type PTEN GBM cell lines were exposed to increasing doses of RAD001 from picomolar to micromolar ranges for 36 h. The 50% inhibitory concentration (IC<sub>50</sub>) was determined for each cell line (Fig. 3A). The results show that the IC<sub>50</sub> values of RAD001 are comprised between 2nM and 200 nM. The results showed also that the most sensitive cell lines are the PTEN mutated cell lines for which 2 to 10 nM RAD001 reduced proliferation to 50% (Fig. 3A), whereas 200 nM RAD001 are needed to reduce proliferation to the same extent in the PTEN wt cell lines (LN229) (Fig. 3A). LN229, SF767, LN401 and U373 cell lines were subjected to 20 nM RAD001 for 72 h and the cell cycle profile was determined by FACS after propidium iodide staining. The FACS analysis showed an increase of the number of all RAD001 treated cells in the G1 phase of the cell cycle when

compared to the untreated cells. In addition, the increase was more drastic for mutant PTEN than for the wild-type PTEN cells. RAD001 induced an increase of 5 to 10% of cells in G1 for LN229 and SF767, whereas an increase of 20% of cells was observed for LN401 and U373 (Fig. 3B) suggesting a higher sensitivity of the PTEN mutant cells to RAD001. This cytostatic effect is also demonstrated by the dephosphorylation of the cell cycle regulatory protein, the retinoblastoma protein (RB), upon RAD001 treatment in LN229 (Fig. 3C). The effect of 20 nM RAD001 in LN229 was lasting for 4 to 5 days as shown by the inhibition of the phosphorylation of the downstream biomarker, the small abundant ribosomal protein S6 (Fig. 3C). FACS analysis revealed that RAD001 did not induce apoptosis in none of the glioma cell lines tested, even after a 6 days exposure to 20, 200 or 2000 nM RAD001. In contrary, RAD001 even lowered the basal level of apoptosis in all 4 GBM cell lines tested (Fig. 3D).

These data confirm what was found in previous studies in other cancer cell lines concerning the highest sensitivity to RAD001 of the mutant PTEN compared to the wt PTEN cells. However, RAD001 is not able to induce apoptosis in GBM cells. Nevertheless, RAD001 was tested for the use as a sensitizer in combination with other drugs, in order to improve their effects on the inhibition of growth or on the induction of apoptosis in the human glioma cell lines. The effective concentration chosen for RAD001 was 20 nM for further combined experiments.

**Only 3/10 GBM cell lines weakly respond to a double or triple combination of “biologicals”.**

Various combinations were tested on 10 genetically well characterized GBM cell lines, the drugs were added simultaneously for 4 days and cell survival was determined by FACS. Three types of responses were obtained featuring different resistance of these cell lines to the drug combinations. The first type of response was found in 7 out of 10 cell lines. They did not respond to any of the drug combinations. The percentages of cell death observed in cells exposed to various drug combinations are similar to those observed in the untreated cells, except in presence of RAD001, where cell death appeared to be slightly reduced (Fig. 4A). The cell line LN18 represented the second type of response, with no response to the single agents but weak induction of cell death when AEE788 was combined with Glivec. Here again, the presence of RAD001 decreased the basal levels of apoptosis, even in the triple

combination (Fig. 4B). In the last type of response, LN215 was sensitive to the double and triple combinations. In the case of the triple combination, RAD001 increased apoptosis (Fig. 4C1). SF767 cell line was sensitive to AEE788, apoptosis was increased when AEE788 was combined to Glivec, and reduced in the presence of RAD001 in the triple combination (Fig. 4C2).

Colony formation assays were performed on two cell lines to test the effects of single and combined drugs on glioma cell proliferation and colony formation. The results showed a drastic reduction of proliferation and colony formation in the SF767 cell line, upon AEE788, RAD001 and upon the combined drugs. In contrary, the Hs683 cell line exhibited more resistance to the AEE788 and/or Glivec, but RAD001 could reduce its proliferation by half (Fig. 5).

**Conclusions:** Glioma cell lines exhibited a very high degree of resistance to the single agents AEE788, Glivec and RAD001, but also to double and triple combinations with respect to induction of cell death. Only 3 out of 10 cell lines were sensitive to some drug combinations, confirmed by a significant induction of apoptosis in LN18, SF767 and LN215 cells. However, the resistance or sensitivity of the cell lines to the drugs could not be correlated with the known genetic background. The results were not predictable and did not depend upon the p53 status (Fig. 6). Since these preliminary results obtained by the use of “biologicals” were disappointing, we expected to obtain more encouraging results when “cytotoxic” drugs like HDACs inhibitors (HDIs) or EPO906A were used as single drugs or in combinations.

## 2. Effects of the “cytotoxics” HDACs inhibitors (HDIs) and EPO906A on GBM cell survival

Cytotoxic drugs are not targeting specific kinases and act in a much more unspecific way. Transcription and the doubling of cells (mitosis) are the respective targets of HDIs and EPO906A. By inducing transcription of genes that have been epigenetically

silenced during tumorigenesis, the regulation of proliferation is restored and affects the behavior of the cancer cell that is finally directed to apoptosis. EPO906 is affecting the separation of chromosomes during mitosis that is controlled by microtubules. This generates abnormal mitosis that is directing the cells to apoptosis. By testing these drugs on our GBM cell lines, we intended to determine their potential anticancer effect.

### **The HDIs LAQ824 selectively induces apoptosis in cancer cells**

Seven glioma cell lines (LN229, LN401, SF767, U343, U373, LN71, Hs683) and the normal human embryonic kidney cell line HEK293 were exposed to increasing doses of the HDAC inhibitor LAQ824 (0, 10, 60, 200, 500 nM) for 72 h and cell death was measured by FACS analysis (Fig. 7A). LAQ824 induced apoptosis in all glioma cell lines at nanomolar drug concentrations and the induction of apoptosis (cytotoxicity) was dose-dependent. No cell death occurred in the normal HEK293 not even at high doses. The results supported the known property that HDIs like LAQ824 selectively targets cancer cells. In addition, several other human cancer cell lines originating from breast (MCF-7, HBL, SKBR-3), cervix (HeLa) and colon (Calu6), and the previous glioma cell lines were subjected to 60 nM LAQ824 for 72 h, in order to compare their sensitivity to the drug. In addition, the non-cancer cell line, HEK293, the human skin fibroblast cell line (HSF) and the rat aortic smooth muscle cell line (RASMC) were treated in the same way. The glioma cell lines were more resistant to LAQ824 than the other cancer cell lines, pointing to the obvious resistance of GBM towards treatment by such drugs in contrast to e.g. breast or colon tumors (Fig. 7B). Induction of cell death occurred neither in HEK293 nor in HSF nor in RASMC, even at higher LAQ824 doses or after a longer time of exposure (Fig. 7B).

### **HDIs reduce proliferation in cancer and non-cancer cells**

Cell proliferation was tested in all glioma cell lines at 30 nM HDI LAQ824, a concentration where no apoptosis was observed after 72 h exposure. HEK293 was also subjected to this treatment in order to know whether the drug affects at least its proliferation. Glioma cell lines and HEK293 were exposed for 48 h prior to BrdU staining followed by FACS analysis (Fig 7C). The results indicated that all cell lines,

GBM as well as normal HEK293, were responding to 30 nM LAQ824 by a significant reduction of cell proliferation (Fig 7C).

### **EPO906 is more cytotoxic for cancer cells than for normal cells**

In the same way, LN229, LN401, SF767, U343, U373, LN71, Hs683 GBM cell lines and HEK293 cells were exposed to increasing doses of EPO906A (0, 0.04, 0.2, 1, 5, 25 nM) for 72 h and cell death was measured (Fig. 7D). The data show that EPO906A induced apoptosis in some GBM cell lines in the sub-nanomolar range (1 nM) and that the cytotoxicity increased dramatically with higher doses. We only observed weak induction of cell death at high doses (25 nM) in HEK293 cell lines. These results show, similar to LAQ824, that EPO906A is selectively targeting cancer cells. The relative resistance of cancer cells to this drug was reported to be due to potential mutations in the  $\beta$ -tubulin, which would confer more resistance to EPO906A (Kowalski, Giannakakou et al. 1997) (Fig. 7D)

**Conclusions:** The results showed that the cytotoxic drugs are inducing apoptosis in the GBM cell lines at nanomolar concentrations, whereas they do not induce any cell death in non-cancer cells even at high doses. Nevertheless, efficient apoptosis is only triggered by high doses of HDIs (500 nM for LAQ824) in GBM cell lines, whereas other cancer cells exhibit already strong apoptosis at lower doses (60 nM for LAQ824). However, according to other studies performed in humans (Goodin, Kane et al. 2004), the disadvantage of using EPO906 *in vivo* is its significant toxicity. Therefore, HDIs were chosen for the combinatorial tests described in the following sections.

### 3. Indomethacin enhances HDACs inhibitors-induced apoptosis

The purpose for combining indomethacin with HDACs inhibitors (HDIs) was to sensitize cells by indomethacin in order to use lower doses of HDIs. Cyclooxygenase inhibitors become of high interest for glioma treatment, since GBMs express high levels of cyclo-oxygenase 2 (COX-2). Synergy between NSAIDs and other forms of

therapy was already reported (New 2004). In the following experiments, the effects of a combined treatment of glioma cell lines with the HDAC inhibitor LAQ824 and IND was investigated.

### **HDI and indomethacin synergistically induced apoptosis in human GBMs.**

Six glioma cell lines were exposed to both increasing doses of the HDI LAQ824 and 50  $\mu$ M indomethacin (IND) for 72h, and cell death was determined by FACS (Fig. 8A). The results revealed that IND, when used as single agent, did not induce apoptosis in any of the GBM cell lines tested within 72h. LAQ824 induced cell death only at higher concentration (180 nM). However, when both drugs were combined, apoptosis was synergistically induced at low LAQ824 doses (10, 30, 60 nM), but the induction of cell death was less strong in some cell lines. The synergism is p53-independent since all cell lines tested exhibited similar effects independently of their p53 status.

### **Indomethacin combined with HDI reduced p21 levels in Hs683 and induced apoptosis in other cancer cell lines like MCF-7 and Calu-6.**

In order to determine the mechanism of the synergism, phosphorylation levels of the proteins AKT, ERK and S6 and the levels of p21 were analysed 8 h after the exposure to 60 nM LAQ824, 50  $\mu$ M IND or the combination. Interestingly, western blot analysis revealed that the increase of p21 caused by LAQ824 was inhibited in the presence of IND, whereas the phosphorylation levels of the other proteins were not affected (Fig. 9A).

Other cancer cell lines were submitted to the same drug combination in order to test whether the synergism is also induced in non-glioma cell lines. The results indicate that a similar synergistic effect on the loss of survival is caused by the combination of both drugs. (Fig. 9B).

### **Indomethacin combined with HDI does not induce apoptosis in the non-cancer cell lines HEK293, HSF or RASMC.**

The human embryonic kidney cell line HEK293, the human skin fibroblast cell line HSF and the rat aortic smooth muscle cell line RASMC are non-cancer cell lines. They were used as controls to test the cytotoxicity of the combination. Increasing

amounts of LAQ824 (10, 30, 60, 90, 180 nM) were combined with 50  $\mu$ M IND for 72 h. No cell death was observed even when IND was combined with high concentrations of LAQ824 (180 nM) which, in contrary, induces strong apoptosis in glioma cell lines (Fig 10A,B,C).

**Conclusion:** According to the results, only tumor cell lines are responding to the combined inhibition of HDACs and COX-2 by a strong induction of apoptosis. In addition, the results concerning protein analysis in HS683 suggest that p21 could be involved in the synergistic induction of apoptosis by HDI and indomethacin.

#### 4. Effects of the glycolytic inhibitor 2-deoxy-D-glucose (2-DG) on human cancer cell lines

Since cancer cells have a high rate of glycolysis, we were interested in testing the glycolytic inhibitor 2-DG on our glioma cell lines, in order to determine whether these cancer cells are sensitive to energy depletion. The goal of the next experiments was to find out whether this compound could be used as a potential “sensitizer” to enhance induction of cell death when used in combination with other drugs.

##### **2-DG reduces strongly proliferation of glioma cell lines in a dose-dependent manner.**

To study the effect of the inhibition of glycolysis on the proliferation of tumor cells, 4 glioma cell lines, two PTEN wild-type (LN229, SF767) and two PTEN mutants cell lines (LN401, U373) were treated with increasing doses of 2-DG (1, 5, 25, 100 mM) for 48h in normal medium supplemented with 25 mM glucose. The proliferation rate was determined by BrdU incorporation for 1h before the end of the experiment. The data obtained showed already a notable reduction of cell proliferation at 1 mM 2-DG in the cell lines tested (Fig. 11A). When equimolar concentrations of glucose and 2-DG were present in the medium (25 mM), cell proliferation was dramatically reduced and no proliferation could be measured anymore upon 100 mM 2-DG (Fig. 11A).

Thus, 2-DG exhibits a strong cytostatic effect on glioma cell lines, in a dose-dependent manner.

### **2-DG induces apoptosis in cancer cell lines, but not in non-cancer cell lines.**

In order to test the sensitivity of cancer cell lines and non-cancer cell lines towards the inhibition of glycolysis, glioma cell lines (LN229, SF767, Hs683, U737, LN401, U343), other cancer cell lines (HeLa, MCF-7 and Calu-6) and non cancer cell lines (HEK293, HSF, RASMC) were exposed to increasing concentrations of 2-DG (1, 5, 25, 100 mM) for 72 h in medium supplemented with 25 mM glucose and cell death was measured by FACS analysis (Fig. 11B). Increased cell death was observed in a 2-DG dose-dependent manner in all cancer cell lines tested. Nevertheless, 100 mM 2-DG was inducing much stronger apoptosis in comparison to 25 or 5 mM, whereas 1 mM 2-DG was not able to induce cell death in any of the cancer cell lines tested. PTEN wt cell lines appeared to be more sensitive to 2-DG than PTEN mutant cell lines at 100 mM. Other cancer cell lines like HeLa, Calu-6 or MCF-7 appeared to be at least as sensitive to 2-DG as glioma cell lines (Fig. 11B). On the other hand, only high doses of 2-DG (25 and 100 mM) could induce weak apoptosis in the non-cancer cell lines HEK293, HSF or RASMC (Fig. 11B). HSF did not undergo any cell death following a drastic glycolytic block (100 mM). These results indicate that the inhibition of the use of glucose in the glycolytic pathway is affecting cancer cells by efficiently inducing cell death.

### **Glucose withdrawal induces strong cell death in all cancer cell lines**

Since 2-DG induced cell death in glioma cell lines, the previous cell lines and HeLa were incubated in glucose free medium in order to compare the sensitivity of cells to the absence of glucose with 2-DG treatment. Cell death was measured by FACS at the indicated time points (Fig 11C). The induction of cell death was more rapid and more effective following glucose starvation than after exposure to 2-DG, which was the case for all cell lines tested except for the SF767 cell line. This cell line appeared to be more sensitive towards 25 or 100 mM 2-DG than to glucose starvation. In addition, under glucose starvation, the most sensitive cell lines were the PTEN mutants, in contrast to their treatment with 2-DG. Cytochrome *c* release was used as a read-out for the induction of apoptosis via the mitochondrial pathway and its release was

measured after glucose starvation for 24 h. Cytochrome c release, especially in PTEN mutant cell lines, after 24 h incubation in glucose free medium, verifies the induction of apoptosis by the mitochondrial pathway (Fig 11C).

### **2-DG induces the dephosphorylation of the small ribosomal protein S6, but does not reduce the phosphorylation of AKT.**

In order to study the involvement of the glycolytic and the PI3K/AKT pathways in the 2-DG-induced cell death, their respective biomarkers S6 and AKT were studied. The total level and phosphorylation level of these two proteins were followed over 16 hours exposure to 100 mM 2-DG. The results showed that the total levels of the two proteins were maintained for at least 16h. As expected, the basal phosphorylation levels of AKT (Ser 473) in PTEN mutants were high in contrast to the PTEN wt cell lines (Fig 12A). Once treated with 100 mM 2-DG, all the cell lines exhibited a short increase in the phosphorylation of AKT that was more visible in the PTEN mutant cells, whereas S6 was rapidly and efficiently dephosphorylated in all the cell lines. No strong decrease in the levels of p-AKT was observed in the PTEN mutant cells during 16 h exposure to 100 mM 2-DG (Fig 12A).

### **2-DG reduces Rad51 levels and induces PARP cleavage.**

Rad51, a protein involved in DNA repair process, is cleaved by caspase-3 and subjected to degradation via the proteasome. PARP is also cleaved by caspase-3 and is a marker of apoptosis. In order to determine the effect of 2-DG on these two proteins, the four cell lines previously used were treated with 100 mM 2-DG for 48 h. The proteins were analysed at the indicated times by western blot. The results revealed that total protein levels of Rad51 were reduced after treatment for 24h and that PARP cleavage is appearing after 36 h of incubation with 2-DG, corresponding to the time where apoptosis is induced (Fig. 12B).

### **Effects of 2-DG on ATP levels and on the activation of AMPK**

ATP levels were examined upon 2-DG exposure or glucose withdrawal conditions, in order to determine whether the drop of ATP is initiating the induction of apoptosis and whether this drop is similar under the two conditions. U373 cell line was treated with increasing concentrations of 2-DG (1, 5, 25, 100 mM) or starved with glucose

free medium for 48h. The ATP levels were measured at 1, 8, 24 and 48 h after the beginning of the treatment. The results show that ATP levels are reduced upon 2-DG treatment as well as upon exposure to glucose-free medium. However, ATP levels are more dramatically decreased at the beginning of the exposure to 25 mM and especially to 100 mM 2-DG than after glucose starvation. Still, even 1 mM 2-DG is able to reduce glycolysis consequently lowering ATP levels in presence of 25 mM glucose into the medium (Fig. 13A). The results suggest that 2-DG is blocking glycolysis and reducing ATP levels by an active process, whereas the drop of ATP, as a consequence of glucose withdrawal, might happen via an inactive process following the decrease of intracellular stocks of ATP (Fig. 13A)

In addition, the data show that both, 2-DG and glucose-free medium, reduce ATP levels in a two-phase manner with time. A first fast drop occurs between 0 and 1 h after the start of the treatment followed by a small increase and a second drop of the ATP level after about 24 h. The same two-phase pattern can be observed with the activation/phosphorylation of AMPK that is regulated by the AMP/ATP ratio in the cell (Fig. 13B). Lower 2-DG did not induce apoptosis since the drop of ATP is less drastic and the stock of ATP was sufficient for cell survival.

### **Pyruvate is not able to rescue cell death induced after exposure to 25 mM 2-DG or glucose withdrawal treatment.**

Two moles of pyruvate result from of one mole of glucose during glycolysis. Pyruvate then enters the Krebs cycle and the respiratory chain that produces about 70% of the ATP in the cell. In order to investigate the involvement of ATP levels in the survival of cells in absence of glucose (glucose withdrawal) or in the situation of a restriction in glucose usage (2-DG), sodium pyruvate was added to the medium in presence of 2-DG or in the absence of glucose and cell death was measured after 72h. The results indicated that the supplementation of 4.5 g/L pyruvate to 2-DG-treated cells or glucose withdrawal only marginally affected the loss of cell survival (Fig. 13C), since no drastic reduction of cell death was observed in the presence of pyruvate.

**Conclusions:** At 25 mM, 2-DG induces cell death in all tested glioma cell lines. PTEN mutant cell lines exhibited a lower sensitivity to 2-DG than PTEN wt cell lines.

Compared to other cancer cell lines, glioma cell lines appeared to be less sensitive to 2-DG. Of note, cell death induced by the treatment of 2-DG is different from that induced by glucose withdrawal. Glucose withdrawal induced earlier and stronger apoptosis in glioma cell lines in contrast to 2-DG, except in the SF767 cell line. The addition of pyruvate did not bypass the induction of apoptosis in the absence of glucose or under the block of glucose usage or 2-DG treatment, except for SF767, where no cell death is measured after 72 h upon glucose-free medium treatment, in the presence of pyruvate. Taken together, it can be concluded that different mechanisms are involved in the induction of cell death by 2-DG or glucose withdrawal.

## 5. RAD001 delays 2-deoxy-D-glucose-induced apoptosis in human glioma cell lines

The PI3K/PTEN/AKT signaling pathway is one of the major pathway driving growth factor mediated cell survival and glycolysis. The bad prognosis of GBMs is linked to mutations or deletions in the tumor suppressor gene PTEN which negatively regulates the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) survival pathway. As it is the case with many malignant tumors, GBM are characterized by a high proliferation index, due to the upregulation of this PI3K/Akt pathway, correlated with an increased glucose metabolism through the glycolytic pathway. A common player in these two pathways is mTOR. Being downstream of the PI3K/Akt pathway, this protein kinase plays a critical role in translation and cell cycle regulation functioning as an energy sensor in the cell, since levels of cellular ATP are regulating the phosphorylation status of mTOR. The previous results show that the glycolytic inhibitor 2-DG is able to induce apoptosis in glioma cells but only at high doses which would not be applicable to patients. In addition, 2-DG is reducing phosphorylation of the small ribosomal protein S6, through the activation of AMPK (Fig. 11, 12, 13). As before, RAD001 blocks efficiently mTOR and reduces glioma cell growth by increasing the G1 cell population (Fig. 3). These results suggested that RAD001 could be a

“sensitizer” for the induction of cell death in glioma cells that are treated at the same time with low 2-DG concentrations.

**The combination of 2-DG and RAD001 does not reduce more efficiently the proliferation of glioma cell lines than the single drugs do.**

Four glioma cell lines were subjected either to 20 nM RAD001 or to 5 mM 2-DG or simultaneously to both drugs for 48 h and cell growth was determined by BrdU incorporation for 1 h. FACS analysis showed that 5 mM 2-DG more efficiently reduced proliferation of glioma cell lines than did 20 nM of RAD001 in PTEN wt cell lines (Fig. 14A). In contrast, RAD001 did not reduce cell growth in PTEN wt cell lines, but it strongly reduced proliferation of PTEN mutant cell lines. A slight additional inhibition of the proliferation of glioma cell lines was observed in PTEN mutant cell lines, when cells were exposed to the combination of 5 mM 2-DG and 20 nM RAD001 (Fig. 14A).

**2-DG more efficiently blocks colony formation of glioma cells than does RAD001**

A colony forming assay was performed of LN229 in order to test the capacities of the cells to form colonies upon 2-DG or RAD001 treatment. The data show that 20 nM RAD001 alone could reduce colony formation by 25% compared to the control, whereas 1 mM 2-DG reduced colony formation by 75%. When 20 nM RAD001 was combined with 1 mM 2-DG, the reduction was as strong as 90% (Fig. 14B). It can be concluded that the combination of RAD001 and 2-DG inhibits the formation of colonies in an additive way.

**2-DG blocks whereas RAD001 only reduces migration of glioma cells it.**

2-DG and RAD001 were tested for their ability to block migration. The migration capacity of LN229 glioma cell line was evaluated using the wound-healing assay. RAD001 was applied 24 h prior to the start of the migration test. The pictures show that the untreated cells migrated well to the middle of the wound (Fig. 14C). 2-DG-treated cells did not migrate, even at 5 mM 2-DG. In contrast, RAD001, even at 2000 nM, only weakly inhibited migration of LN229 (Fig. 14C).

### **RAD001 reduces 2-DG-induced apoptosis in glioma cell lines.**

It was investigated whether RAD001 was able to increase 2-DG-induced apoptosis in glioma cell lines. LN229, SF767, U373 and LN401 glioma cell lines were exposed to a combination of 20 nM RAD001 and 25 mM 2-DG for 72 h. No additive induction of apoptosis was observed in none of the glioma cell lines tested. RAD001 rather decreased apoptosis induced by 25 mM 2-DG (Fig. 15A) as demonstrated by Annexin-V staining and PARP cleavage (Fig. 15B,C) in LN401. To investigate further this antagonistic effect exhibited by RAD001, it was combined with lower and higher concentrations of 2-DG. RAD001 showed a strong pro-survival effect independently of the 2-DG concentrations, under the conditions where glycolysis or glucose usage is blocked (Fig. 15D).

### **2-DG is dominant over RAD001 concerning the inhibition of cell proliferation.**

In order to test the effect of the drug combination on cell proliferation and on the cell cycle profile, LN401 was exposed for 72 h to increasing doses of 2-DG (0.01, 0.1, 1, 5, 25, 50, 100 mM) in presence or absence of 20nM RAD001. The total number of cells was measured after 72 h. The histogram shows that RAD001 exerts a strong anti-proliferative effect in the absence of 2-DG or at concentrations where 2-DG has no effect on the proliferation of cells (0.01, 0.1 mM) (Fig. 16A). Indeed, up to 50% less cells were observed at these 2-DG concentrations in presence of RAD001 (Fig. 16A). At concentrations where 2-DG itself is reducing proliferation (1 mM-100 mM), the cytostatic effect of RAD001 is hidden by this one of 2-DG. The inhibition of proliferation is dominated by 2-DG.

### **2-DG and RAD001 induce opposite cell cycle arrest in LN401.**

In order to further analyse the effect of both drugs on the cell cycle profile, the cell line LN401 was exposed to 2-DG, RAD001 or both drugs for 36 h. The FACS analysis showed that RAD001 induced a strong accumulation of cells in G1, whereas 2-DG induced an accumulation of cells in G2-M of the cell cycle (Fig. 16B). When a combination of both drugs were applied, the cell cycle profile became similar to the one of the untreated cells (Fig. 16B), suggesting that both drugs were equally participating in the regulation of the cell cycle. The levels of cyclin A protein were

followed over 72 h after exposure to 2-DG or RAD001. The protein data showed that 2-DG decreased more efficiently cyclin A than RAD001 did (Fig. 16C).

#### **Effects of 2-DG and RAD001 on phosphorylation of AKT, ERK and S6.**

In order to investigate the pro-survival mechanism of RAD001 in combination with 2-DG, the cell lines LN229 and U373 were exposed for 8 h to 25 mM 2-DG, 20 nM RAD001 or to both drugs. Phosphorylation levels of AKT, ERK and S6 were determined using western analysis. The results revealed that the phosphorylation of S6 was abrogated by treatment with 2-DG and RAD001 in the PTEN wt LN229 cell line (Fig 17A), whereas it was only abrogated by RAD001 in the PTEN mutated U373 (Fig 17B). The phosphorylation levels of AKT in LN229 increased after treatment with RAD001 or 2-DG and even more with the combination of 2-DG and RAD001 (Fig 17A), whereas no striking change was observed in U373 after the exposure to the drugs. However, 2-DG decreased phosphorylation of ERK in both cell lines (Fig 17A,B). RAD001 alone had no effect, whereas when it was combined with 2-DG, the phosphorylation levels of ERK were the same as basal levels in LN229 (Fig 17A). In contrast, no effect was observed in U373 after 8 h exposure to the drugs (Fig 17B). Thus, the effects of RAD001 and 2-DG on the activity of these kinases are not clear, further experiments need to be performed.

#### **RAD001 delays apoptosis induced by glucose withdrawal in glioma cell lines.**

The question arose whether RAD001 is also able to reduce apoptosis induced by glucose withdrawal, as it was the case for 2-DG. In order to analyse that, 20 nM RAD001 was added to the cell culture of glioma cell lines using glucose-free medium. This treatment was applied to glioma cell lines during the indicated times. The data indicated that RAD001 was able to delay apoptosis induced by glucose withdrawal (Fig 18A), pointing again to the pro-survival effect exhibited by RAD001 in the absence of glucose.

#### **The combination of LY294002 and 2-DG synergistically induces apoptosis in LN401 glioma cell line.**

In order to determine the effect on cell survival caused by the simultaneous inhibition of the energy pathway and the PI3K/AKT/mTOR pathway on cell survival, the

inhibitor of PI3K, LY294002 (LY), was used in order to downregulate efficiently the PI3K/AKT/mTOR pathway. The effect of LY is determined by the block of the phosphorylation of AKT being under the control of PI3K. First, the phosphorylation of AKT was followed over 48 h in order to determine the efficiency of LY. Western analysis showed that LY blocked rapidly the phosphorylation of AKT, but the effect of the inhibitor is only lasting for 16 h (Fig. 19A). LY was used in combination with both drugs to determine whether this drug is able to potentiate the sensitivity of the cells towards RAD001 and 2-DG. The combination of 2-DG and RAD001 was applied to LN401 for 72 h in presence or in absence of LY and cell death was measured by FACS. LY was added every 16 h according to the inhibitory test (Fig 19A). The results show that the combination of 2-DG and LY have an additive effect on the induction of cell death (Fig 19B), whereas the addition of RAD001 in a triple combination, strongly reduced apoptosis induced by the combination of the two drugs (Fig 19B). Here again, RAD001 exhibited a pro-survival effect.

**Conclusions:** The results show that the combined treatment of RAD001 with 2-DG reduces apoptosis induced by 2-DG in human glioma cell lines acting like an antagonistic pro-survival agent. In contrast, the inhibition of the activation of AKT, consequently to the inhibition of PI3K by LY triggers additive cell death when combined with 2-DG.

6. 2-deoxy-D-glucose combined with HDAC inhibitors causes a synergistic induction of apoptosis in human glioma cell lines by inhibiting p21 upregulation

Burgess et al and Rahmani and colleagues showed that the simultaneous exposure of cells to agents that increase p21 expression (as HDIs) and agents that downregulate p21 levels (like caffeine or LY294002), induce apoptosis (Burgess, Ruefli et al. 2004), (Rahmani, Yu et al. 2003).

Another report (Kang, Ju et al. 2003) indicated that 2-DG dramatically decreases p21 protein levels. According to these facts, the combination of 2-DG and HDIs like TSA, NaB and LAQ824 was investigated using human glioma cell lines to determine whether 2-DG is able to sensitize these cells to HDI-induced apoptosis.

### **2-DG enhances trichostatin A- and sodium butyrate-induced apoptosis in HS683 and HeLa cancer cell lines.**

HDIs are potent inducers of cell death in immortalized cells and tumor cell lines. To investigate the effect of HDIs on loss of survival, we tested first to which extent trichostatin A (TSA) and sodium butyrate (SB) are capable to induce apoptosis in the GBM HS683 and the cervix carcinoma cell line HeLa and whether 2-DG is able to increase HDIs-induced cell death in a combined treatment. Increasing doses of TSA and NaB were applied together with 2-DG for 48 or 72h. Strong induction of cell death occurred in presence of 2-DG and TSA or NaB, whereas both HDIs alone did not induce apoptosis when used at low doses (Fig. 20A, B). Since p21 is re-expressed after HDIs treatment, p21 protein levels were analysed in both cell lines after 8h exposure to TSA or NaB. The results showed an increase of the p21 protein levels which was abrogated by co-treatment with 2-DG. Similar observations were made in previous studies where HDIs were combined with caffeine (Burgess, Ruefli et al. 2004), LY (Rahmani, Yu et al. 2003) or with 2-DG (Kang, Ju et al. 2003) (Fig 20C).

### **2-DG enhances HDIs-induced apoptosis in human glioma cell lines**

Also the new hydroxamic acid based HDAC inhibitor, LAQ824, was investigated on glioma cell lines. Increasing amounts of LAQ824 and/or 25 mM 2-DG were applied to six glioma cell lines for 72 h. Consistent with results obtained previously with TSA and NaB, LAQ824 induced apoptosis in a concentration-dependent manner in all glioma cell lines tested (Fig. 21). 2-DG alone had no strong effects on loss of survival, whereas the presence of 2-DG drastically enhanced cell death induced by LAQ824 in all cell lines tested. This synergism was found to be dependent on the concentration of LAQ824 since the effect was moderate at a LAQ824 concentration as low as 30 nM, whereas at higher concentrations the combination of 2-DG with LAQ824 exhibited the strongest synergism in that 6 times more cell death could be noticed at 60 nM LAQ824 in presence of 2-DG compared to use of the single drugs.

In addition, the synergism was induced in a p53-independent way since SF767 and U343MG glioma cell lines are mutated for the p53 gene and showed similar responses following a combined treatment. However, when the concentration of LAQ824 was increased up to 180 nM, the enhancing effect of 2-DG was lost because apoptosis was extensively induced by LAQ824 itself (Fig. 21).

**The synergism induced by the combination of both drugs is concentration-dependent.**

To test the contribution of 2-DG in this synergism, lower concentrations of 2-DG in combination with LAQ824 were tested in the cell line Hs683. The synergistic response is reduced concurrently to the concentrations of 2-DG, meaning that the synergism is also dependent of the concentration of 2-DG (Fig. 22A). In a time course experiment, the induction of apoptosis by a combination of 2-DG (25mM) and LAQ824 (60nM) was analysed by FACS. The single drugs showed almost no loss of survival after 72 h treatment whereas the drug combination induced an early cell death, observable already after 16 h (Fig. 22B). The detection of PARP cleavage, measured by the appearance of the 86 kDa product at 16 h confirmed the strong induction of apoptosis which occurred only when both drugs were used together. Only weak PARP cleavage was induced by 2-DG but not by LAQ824 after 36h. (Fig 22C). The data indicate that the two drugs are able to induce cell death by a synergistic mechanism.

**2-DG dominates LAQ824 when proliferation of Hs683 is inhibited.**

Given the amplified induction of cell death by combined treatment of 2-DG and LAQ824, the effect of both drugs on the proliferation of Hs683 was examined. LAQ824 affected Hs683 proliferation in a concentration-dependent manner inducing an approximate 50% inhibition at the cytotoxic concentration of 180 nM within 72 h of drug application, with cells accumulating in G1 phase of the cell cycle (Fig. 23A,B). Proliferation was reduced more efficiently in a concentration-dependent manner under 25 mM 2-DG treatment, whereas low or no cell death was detected at this concentration, and cells were accumulating in G2/M phase (Fig. 23B). The combined treatment of Hs683 with 25 mM 2-DG and 60 nM LAQ824 exhibited essentially the inhibitory effect of 2-DG, which appeared to be dominant over

LAQ824. This observation is confirmed by the cell cycle profile where cells, treated simultaneously with both drugs, are pushed in G2/M phase after 16 h treatment (Fig. 23B). Cyclin A levels are drastically reduced upon 2-DG compared to upon LAQ824 treatment (Fig. 23C).

**AKT and ERK are not responsible for the triggering of the synergistic induction of apoptosis upon the combination.**

Previous results demonstrated the major role of the kinases AKT and ERK into the regulation of the balance between survival and apoptosis, thus, the implication of the survival pathway proteins in driving cells into apoptosis was studied. We investigated whether AKT and/or ERK are involved in the synergistic induction of cell death in glioma cell lines. The phosphorylation of both proteins was analysed following treatment by 2-DG and/or LAQ824. 2-DG induced first an increase then a decrease of the phosphorylation of Ser473 of AKT (Fig. 24A). 2-DG did not induce dephosphorylation of ERK in Hs683. LAQ824 induced a weak decrease of the phosphorylation of AKT and an increase of ERK phosphorylation after 16 h. The levels of phosphorylation of Akt and ERK were both reduced 16h after the addition of both drugs around 16 h. At the same time PARP cleavage occurred (Fig. 24B). In the same way, the levels of Rad51 were followed over time upon the various treatment conditions. The Western-blot indicated that Rad51 decreased similarly to the kinases ERK and AKT upon the combined treatment of LAQ824 and 2-DG, consequently to the induction of apoptosis (Fig. 24C).

**ATP levels, AMPK and S6 phosphorylation are only affected by 2-DG, not by the HDIs.**

2-DG is blocking the glycolysis and the ATP production. A drop in ATP levels is leading to the activation of the energy sensing kinase AMPK, whose role is to block high energy consuming mechanisms by reducing, for example, the activation of S6K and the ribosomal S6 protein which blocks global translation. To determine whether the synergistic induction of apoptosis is requires the energy regulatoring pathway, ATP levels were first measured in Hs683 after treatment by 2-DG and/or LAQ824. Cells exposed to LAQ824 showed similar ATP levels as the untreated cells whereas 25 mM 2-DG induced a dramatic decrease in the levels of ATP. A similar drop of

ATP occurred when the drug combination was used. (Fig. 25A). Phosphorylation status and protein levels of AMPK and S6 were determined after exposing Hs683 to 2-DG and/or LAQ824 for 48 h. In presence of 2-DG, the glycolytic pathway did not produce enough ATP resulting in the increase of the AMP/ATP ratio, in the activation of AMPK (Fig. 25B) and in the inhibition of the phosphorylation of the downstream biomarker S6, whereas LAQ824 did not affect phosphorylation and total levels of these proteins. The data suggest that 2-DG inhibits by itself dramatically the energy regulating pathway and may considerably block translation by strongly decreasing phosphorylation of the ribosomal protein S6 (Fig. 25C).

### **2-DG inhibits the expression of p21 induced by LAQ824 treatment**

Previous studies have demonstrated that expression of the cdk inhibitor p21<sup>WAF1/Cip1</sup>, regulating the progression of the cells from G1 to S phase, causes G1 cell cycle arrest and protects cells against the cytotoxic actions of chemotherapeutic agents. In addition, many reports have shown that low doses of HDIs were upregulating p21 expression leading to an accumulation of cells in G1 phase of the cell cycle, whereas at high doses, HDIs were cytotoxic. According to these data, the role of p21 in the synergistic induction of apoptosis was investigated. First, p21 protein levels were followed over 24 h in Hs683 after the addition of 2-DG and/or LAQ824. LAQ824 increased the expression of p21 in Hs683 after a short time exposure to the drug, whereas the presence of 2-DG led to the total disappearance of p21 signal after 4h (Fig. 26A). The combination of 2-DG and LAQ824 was tested on glioma cell lines and similar effects on p21 levels were observed after 8h exposure to the drugs (Fig. 26B). Since the overexpression of p21 is responsible for preventing cells to undergo apoptosis, it was tested if p21 is still induced at higher concentrations of LAQ824. Increasing concentrations of LAQ824 (10, 60, 200, 500, 1000 nM) were applied to Hs683 and p21 protein levels were examined after 24 h exposure to the drug. In addition, 25 mM of 2-DG was added in order to know whether 2-DG is able to reduce p21 at high LAQ824 concentrations. Apoptosis was already detected after 24 h exposure to 500 nM and 1000 nM LAQ824 but the expression of p21 protein was still induced at high doses (500 nM) (Fig. 26C). The addition of 2-DG at high doses of LAQ824 efficiently reduced the levels of p21 protein (Fig. 26C). Therefore, the combination of 500 nM LAQ824 and 25 mM 2-DG was tested on Hs683 survival in a

time course, similar as in Fig. 22B. The results showed that a synergistic induction of apoptosis was also induced at the high dose of LAQ824 in presence of 25 mM 2-DG (Fig. 26D). Interestingly, apoptosis was not induced at an earlier time than 16h, similar to the lower dose of LAQ824 (60 nM) shown in figure 22B (Fig. 26D). In order to test whether p21 protein levels are also reduced at lower 2-DG concentrations, increasing doses of 2-DG (1, 5, 7.5, 25 mM) were combined with 60 nM LAQ824 in Hs683 and western-blot analysis was performed. The levels of p21 were significantly increased in presence of 60 nM LAQ824 after 8 h but reduced when increasing doses of 2-DG were added, even at doses of 2-DG as low as 1mM. Phosphorylation levels of S6 were also decreased in a 2-DG concentration-dependent manner, 1 mM 2-DG appeared to decrease phosphorylation of S6 and this decrease is sufficient to reduce p21 levels in presence of 60 nM LAQ824 (Fig. 26E). Taken together, the results suggested that, in parallel to the treatment of HDIs, the decrease of the levels of p21 protein after exposure to 2-DG might be involved in the triggering of apoptosis.

### **The synergism between 2-DG and HDIs requires the induction of p21 protein to induce apoptosis in Hs683.**

Sequential addition of 2-DG or LAQ824 was tested in order to determine the involvement of both drugs in the synergistic induction of apoptosis. First, 60 nM LAQ824 was tested in a time course and p21 protein levels of Hs683 were examined. The Western analysis showed that the expression of p21 was induced already 4 h after the addition of the drug to the cells (Fig 27A). The levels of p21 peak at around 16 h and then decrease to reach finally the undetectable basal levels after 48 h (Fig 27A). In the first sequential addition of the drugs (Fig 27B), 60 nM LAQ824 were added to Hs683 cell culture medium and 2-DG was added either simultaneously ( $t=0$ ) or after LAQ824 at the indicated times ( $t=16, 24, 36, 48$  h). Apoptosis was determined 72h after the addition of 2-DG. As control experiments for the use of single drugs, apoptosis was measured 72 h after the addition of 2-DG and 72h or 5 days after the exposure to LAQ824. The results show that the combination is exhibiting the best synergism when 2-DG was added when p21 protein expression induced by LAQ824 had reached high levels (Fig 27B). Cell death is decreasing proportionally to the levels of p21. No synergism is detected anymore when 2-DG is added 48h after the

addition of LAQ824 to the cells, that is when p21 expression was back to basal levels again.

In an analogue experiment first 25mM 2-DG was added to the cells followed by 60 nM LAQ824 at time=0 or after the indicated 16, 24, 36 and 48 h (Fig 27C). Apoptosis was determined 72 h after the addition of LAQ824. Again, as controls, apoptosis was measured for single drugs after 72 h in the case of LAQ824, and after 72 h and 5 days in the case of 2-DG. The results showed that once the cells had been exposed to 2-DG, strong cell death was induced after 72 h, independently of when LAQ824 was added (Fig 27C).

These results show that, in order to reach a strong synergism, 2-DG has to be combined with LAQ824 at time when the levels of p21 protein are high. In contrast, LAQ824 can be combined anytime with 25mM 2-DG, since the effect of 2-DG is not disappearing with time. The results suggest that high levels of p21 protein are crucial for triggering apoptosis, and that such high levels need to be induced by HDIs.

#### **ATP levels play a secondary role in the synergistic induction of apoptosis.**

In order to determine the involvement of energy levels in the synergistic induction of apoptosis by 2-DG and LAQ824, pyruvate and other glycolytic inhibitors or blockers of ATP utilization were tested in Hs683. First, in order to find out whether the drop of ATP levels caused by 2-DG is inducing cell death in presence of LAQ824, pyruvate, as another carbon source, was added at an equimolar concentration to glucose to allow the production of ATP by passing the block caused by 2-DG. The results indicated that pyruvate did not affect the induction of cell death caused by the combined drugs (Fig 28A). Also a combination of oligomycin and LAQ824 was tested. Oligomycin is known to block the ATP pump in the mitochondria, that is, in turn, blocking the use of ATP in the cell without affecting the metabolism of glucose. No cell death was observed in Hs683 after 72 h exposure to the single or to the combination of drugs (Fig. 28B). No strong change in the p21 protein levels occurred after the exposure to the combination, whereas the phosphorylation levels of S6K were dramatically reduced (Fig. 28B). The data suggested that the drop of ATP is of secondary importance for the synergistic induction of cell death by 2-DG and HDIs.

In an additional experiment, glucose-free medium was combined with LAQ824 in order to block the use of glucose in the cell, mimicking the effect of 2-DG. The

results show that glucose withdrawal induces apoptosis and that the presence of LAQ824 enhances the induction of apoptosis by 50% (Fig. 28C), suggesting that the block of glycosylation processes, active when glucose is metabolized, could be involved in the induction of apoptosis upon the synergism. Since both the addition of 2-DG or glucose withdrawal are affecting glycosylation processes, in contrast to oligomycin, the possibility arose that glycosylation could play a role in the synergistic induction of apoptosis and that ATP levels are only involved to a limited extent (Fig. 28A, B).

We wanted to investigate if the downregulation of p21 protein levels caused by the combination of 2-DG and LAQ824 was happening on the translational level. For this purpose, RAD001, known to efficiently bring down mTOR activity, was used in order to inhibit translation mimicking the effect of 2-DG on p21 levels. However, treatment of Hs683 with the combination of LAQ824 and RAD001 did not lead to the synergistic induction of apoptosis obtained before by the combination by LAQ824 and 2-DG although the phosphorylation of S6 was abrogated by RAD001. These results suggest that mTOR and S6 are not involved in the synergistic induction of apoptosis (Fig 28D).

### **2-DG and HDIs synergize in other tumor cell lines like HeLa, MCF-7, SKBR-3, HBL**

It was investigated whether the synergistic induction of cell death by the combined application of the two drugs is also occurring in other cancer cell lines. First, the combination was tested on the human cervix cancer cell line HeLa. HeLa cells were exposed to increasing doses of LAQ824 (10, 30, 60, 90, 180 nM), in presence or absence of 25 mM 2-DG. After 72 h treatment, loss of cell survival was measured by FACS. The results showed a strong synergistic induction of apoptosis (Fig. 29A). The Western analysis showed that p21 expression increased after treatment with HDI and was abrogated in presence of 2-DG as it was the case for the glioma cells. Also the phosphorylation of S6 was blocked as a consequence of treatment with 2-DG (Fig. 29B). To test the efficiency of the combination in other cancer cell lines, MCF7, SKBR-3 and HBL were exposed to increasing doses of LAQ824 and 5 mM or 25 mM 2-DG for 72 h and cell death was measured. These cell lines Also exhibited a synergistic induction of cell death by the two drugs (Fig. 29 C, D, E).

**No synergism is observed in the non-cancer HEK293 and RASMC cell lines, even at high HDIs concentrations, except in HSF cell line.**

In order to test the toxicity of the combination on non-cancer cells, the human embryonic kidney cell line HEK293 was tested. Interestingly, after 72 h, no cell death was induced upon the combination of LAQ824 and 2-DG, even when 2-DG was combined with higher concentrations of LAQ824 (Fig. 30A). Since no effect of the combination has been detected on the level of cell survival, the drugs have been tested on the proliferation of HEK293. The cells have been exposed for 72 h to increasing doses of the drugs and the number of cells was determined. The number of cells decreased when the doses of LAQ824 and 2-DG were increased (Fig. 30B). The results suggest that the drugs are efficiently inhibiting proliferation of HEK293, and that the absence of synergism in the induction of cell death was not due to the resistance of the cells to these drugs. Other non-cancer cell lines, like RASMC (rat aortic smooth muscle cells) and HSF (human skin fibroblast), were then also tested in the same way and analysed by FACS. No cell death was induced in RASMC by the combination (Fig 30C). However, the synergism could be observed in the HSF cell line (Fig 30D) but only at high LAQ824 doses.

**Conclusions:** The combination of low doses of the glycolytic inhibitor 2-DG and HDAC inhibitors is a promising way to efficiently induce apoptosis in glioma and other cancer cells whereas no or low toxicity is induced in non-cancer cells. p21 protein levels seem to play an important role in the mechanism by which apoptosis is triggered by the simultaneous inhibition of glycolytic pathway and HDAC. Thus, anti-metabolic intervention by 2-DG synergizes with HDIs by blocking re-expression of p21 and possibly other critical genes that are silenced.

## CONCLUSION AND DISCUSSION

Tumors of the central nervous system (CNS) occur at an incidence of approximately 1 per 10 000 people, representing 2% of all cancers. However, they account for some 23% of all pediatric malignancies, ranking second only after leukaemias. Furthermore, there has been a reported rise in the incidence of CNS tumors by at least one-third over the past 40 years in developed countries (Ohgaki, Dessen et al. 2004). Despite this trend which may be due in part to improved imaging, the primary causes of human CNS tumors remain hypothetical. Conventional therapies for brain tumors currently involve surgical resection together with adjuvant radiotherapy and chemotherapy. Despite a proven progress in slightly prolonging mean survival (Stupp, Mason et al. 2005), treatment of high-grade tumors remains a compromise between control of tumor growth and maintenance of quality of life.

On one hand, malignant gliomas accumulate many genetic and epigenetic alterations in genes involved in regulation of growth, apoptosis and repair. On the other hand, the clinical failure of potentially efficient therapeutics might not only be due to a lack of drug efficacy, but also to mechanisms of resistance, the heterogeneity in a single tumor and the non-liposolubility of the majority of the drugs which impairs or blocks the access through blood-brain barrier (BBB). Since chemotherapy and radiotherapy for primary brain tumors are non-specific and mostly ineffective, new strategies have been developed to overcome the problem of resistance.

### **The use of specific inhibitors of protein kinases (biologicals) to treat GBMs**

New classes of drugs were designed to specifically target protein kinases: the protein kinase inhibitors (PKIs). Some of these drugs also pass the BBB. The efficacy has already been demonstrated for AEE788 against human high-grade gliomas (Nathoo, Goldlust et al. 2004) or against tumor angiogenesis in mice (Traxler, Allegrini et al. 2004); for Glivec in chronic myelogenous leukemia (CML) (Capdeville, Buchdunger et al. 2002) or for RAD001 against tumor angiogenesis in an animal model (Guba, von Breitenbuch et al. 2002), (Chan 2004). The PK targeted by these drugs are frequently overexpressed in GBMs by constitutive activation or gene upregulation or as results of gain of function mutations in the region encoding the kinase domains. In addition, mutation in kinases working downstream in the pathway can occur. Regarding the efficiency of these drugs on other cancers and the upregulation of EGFR, VEGFR, PDGFR and mTOR in GBMs, we tested AEE788, Glivec and

RAD001 on 10 human GBM cell lines *in vitro*, in order to evaluate the potential of these drugs for this highly chemoresistant cancer. The results obtained were disappointing. Cell death was not observed at clinically meaningful dose ranges, even when the drugs were combined in double and triple combinations. Few cell lines responded to these combinations, but only at unspecific high drug doses. In addition, the results did not correlate with the known genetic background of the cell lines with respect to the tumor suppressor genes encoding p53, p16/p14 and PTEN. Thus, the response of the cell lines to the drugs cannot be predicted according to the genetic background.

The resistance we observed in these first results can be due the genetic instability which is a hallmark of cancer (Kinzler and Vogelstein 1997; Hanahan and Weinberg 2000). Indeed, as indicated in the name of the tumor, GBM (glioblastoma multiforme) is characterized by marked cytologic and histologic variation and displays extensive genetic and biological variability. These characteristics are the main obstacle that refrains the efficiency of biologicals in the treatment of GBMs (Hui, Lo et al. 2001). During the initial stages of oncogenesis, random alterations that are affecting oncogenes and tumor suppressor genes confer growth advantages allowing the selection of increasingly aggressive sublines. Thus, GBMs are formed by various clones of cells with distinct genetic background. In addition, the cross-talk between signaling pathways provide a possible role in chemo-resistance, too. The interconnections between distinct signaling cascades can circumvent the inhibition of specific protein kinases by alternative pathways. For example, treatment should not only consider the ability of RAD001 to inhibit mTOR, but also the inhibition of the molecular events downstream of mTOR signaling.

According to these results, other elements have to be taken into account in order to find an effective treatment for GBMs. Indeed, besides the alteration of the activity of many protein kinases, GBMs harbor epigenetic alterations (Reifenberger and Collins 2004) and have an abnormally high glycolytic rate (Gatenby and Gillies 2004) compared to normal cells.

### **Epigenetic alteration: use of histone deacetylase inhibitors**

Genomic alterations activating oncogenes and inactivating tumor suppressor genes result in unregulated tumor cell growth are the underlying basis of human tumors

(Lund and van Lohuizen 2004). The existing animal models of tumorigenesis, the selection of sets of genes contributing to this process and many cancer therapies are based on such genomic alterations. In addition, tumor suppressor genes can be epigenetically silenced by aberrant methylation and/or histone deacetylation of their promoter regions (Merlo, Herman et al. 1995; Baylin and Herman 2000; Hanahan and Weinberg 2000; Plumb, Steele et al. 2004). This mechanism of epigenetic regulation changes the accessibility of chromatin to the transcription machinery locally via modifications of the DNA and histones by modifying or rearranging nucleosomes (Mompalmer 2003). Epigenetic regulation and genetic alterations both collaborate in cancer development to act on cell growth and differentiation, cell cycle control, DNA repair, angiogenesis, migration, and evasion of host immuno-surveillance. In contrast to target the genetic causes of cancers, the possibility of reversing epigenetic changes provides new strategies for therapeutic intervention (Plumb, Steele et al. 2004).

Modulation of gene expression by acetylation and deacetylation of histones is allowing or restricting, respectively, the access of the transcriptional machinery on the gene promoter (Cress and Seto 2000). The anticancer potential of HDAC inhibitors (HDIs) is based on their ability to break several cellular processes that are dysregulated in neoplastic cells by restoring the transcription of tumor suppressor genes involved in cell cycle regulation, differentiation and proliferation (MacLachlan, Sang et al. 1995; Johnstone 2002).

Since cytotoxic compounds are not well tolerated in patients due to side effects, anti-cancer drug development has moved from conventional cytotoxic chemotherapeutics towards a more mechanism-based targeted approach. Nevertheless, the arrest of the proliferation of tumor cells delays growth of the tumor, but does not eradicate tumor cells. Regarding the importance of epigenetic alteration in cancer cells including GBM cells, in contrast to normal cells, we tested the effect of HDAC inhibitors on human glioma cell lines. From the large panel of HDIs currently available, we have chosen the two chemicals trichostatin A (TSA) and sodium butyrate (SB). In addition, we also tested LAQ824, a new cinnamic hydroxamic acid (Atadja, Gao et al. 2004), because this drug is already used in clinical trials for the treatment of leukaemia (Nimmanapalli, Fuino et al. 2003; Nimmanapalli, Fuino et al. 2003; Weisberg, Catley et al. 2004) and it can pass the BBB. In response to these HDIs, all glioma cell lines showed a significant induction of apoptosis in a short time range (72h) (at low

micromolar doses for LAQ824). However, low doses of these drugs only weakly triggered apoptosis, although a cytostatic effect on glioma cell lines was observed. Other cancer cell lines responded much better to these low doses of HDIs. These results support the well-known fact that malignant glioma cells are highly resistant. Interestingly, when HDIs were tested on the non-cancer cell lines HEK293, RASCM and HSF, no cell death could be induced, even at higher doses. These results indicate that HDIs are suitable for specifically targeting cancer cells, and suggests that the use of HDIs *in vivo* may only induce low toxicity in the surrounding tissues.

The mitochondrial cell death pathway is characterized by mitochondrial stress that can lead to apoptosome-mediated caspase-9 activation. Caspase-9 activates the effector caspases 3 and 7, which then trigger cell death by cleaving selected death substrates as PARP, and also process different caspases, thus leading to the generation of the pro-apoptotic amplification loop. An alternative extrinsic apoptotic pathway is triggered by the cell surface death receptors, which include caspase-8 and caspase-10 as upstream caspases. By cleaving BID, a Bcl-2 family member, these caspases can also induce permeabilization of the mitochondria and activation of the apoptosome (Igney and Krammer 2002). Histone deacetylase inhibitors have both pro-apoptotic and differentiating effects on tumor cell lines, but the mechanism of action underlying the effects of HDIs remains unclear. Some recent studies done in this purpose are contradictory and show some discrepancies. For example, the studies done by Komata and colleagues on glioma cell lines showed that HDI-induced apoptosis by sodium butyrate and trichostatin A (TSA) was caspase-dependent, involving caspase-8 but not caspase-9 (Komata, Kanzawa et al. 2005). This suggested that HDI-induced apoptosis was not mediated by the mitochondrial cell death pathway, also because no change in the mitochondrial membrane potential could be detected (Komata, Kanzawa et al. 2005). However, in another report, Henderson and colleagues showed that caspase-9 is critical for apoptosis induced by the HDI suberoylanilide hydroxamic acid (SAHA) and TSA and that efficient proteolytic activation of caspase-2, caspase-8, and caspase-7 strictly depends on caspase-9 (Henderson, Mizzau et al. 2003). They showed that SAHA and TSA induced translocation of BID into the mitochondria and cytochrome c release. They also claimed that the susceptibility to TSA- and SAHA-induced cell death is regulated by p53, whereas other studies demonstrated the opposite and identified the death receptor pathway

(Insinga, Monestiroli et al. 2005) as an alternative apoptotic pathway activated by HDIs.

### **Inhibition of glycolysis**

Energy production represents an interesting pathway to be targeted in GBMs. Indeed, tumor cells have abnormal mitochondrial function and essentially rely upon glycolysis to provide ATP for their metabolic requirements. This means that GBM cells have a low oxygen demand and can survive in an environment with low oxygen concentrations that would not be suitable for normal cells. Glycolysis is an evolutionary ancient metabolic pathway found in all cells that enables organisms to provide energy for biological processes, by the oxidation of glucose without the requirement of oxygen (Gatenby and Gillies 2004). Human GBMs are much more avid of glucose than normal cortex cells, since high glucose consumption was demonstrated by FDG-PET in these tumors (Annex 4) (Novak, Molnar et al. 2005).

The glycolytic inhibitor 2-deoxy-D-glucose (2-DG) efficiently inhibits glycolysis by blocking the access of glucose to the hexokinase (Kaplan, Navon et al. 1990). 2-DG itself is not metabolized but acts as a competitive inhibitor of glucose for hexokinase (Gallagher, Fowler et al. 1978). The anticancer effect of 2-DG has been demonstrated in combination with radiotherapy (Mohanti, Rath et al. 1996; Dwarkanath, Zolzer et al. 2001) for the treatment of human GBMs. 2-DG inhibits repair of radiation-based DNA damage by depleting energy levels leading to an enhancement of apoptosis. Used as a single agent, 2-DG reduces *in vivo* the size of liver tumors in rats (Cay, Radnell et al. 1992) and induces apoptosis *in vitro* in breast cancer cells by inducing cytochrome c release and PARP cleavage (Aft, Zhang et al. 2002).

We tested the effect of 2-DG on human GBM cell lines *in vitro*. The results showed that 2-DG was able to induce apoptosis in all GBM cell lines after 72 h of treatment, but only at high concentrations (equimolar with 25 mM glucose, or at 100 mM), whereas no apoptosis was induced at lower doses of 2-DG such as 1 mM or 5 mM after 72 h of exposure. Nevertheless, 2-DG was able to efficiently inhibit proliferation of all glioma cell lines at these lower doses that are suitable for clinical application. Interestingly, PTEN mutant cell lines exhibited a weaker sensitivity to 2-DG compared to the PTEN wild-type cell lines with regard to induction of cell death. Compared to other cancer cell lines, glioma cell lines exhibited lower cell death upon

2-DG treatment, whereas no cell death was induced in the non-cancer cell lines HEK293, HSF and RASMC, even at very high doses of 2-DG (100mM). Of note glucose withdrawal induced earlier and stronger apoptosis in glioma cell lines in contrast to 2-DG. Upon glucose withdrawal, cellular ATP is quickly used up. However, the addition of pyruvate (source of ATP) to the medium of the cells does not rescue significantly cell survival. In the case of an addition of 2-DG, glucose is still present, but the competitive inhibition of hexokinase activity by 2-DG might alter the normal use of glucose in the cell. This may explain why ATP depletion is faster following 2-DG addition than upon glucose withdrawal. The decrease of ATP is not correlated with cell death induction. Indeed, although ATP levels drop much more following 25 or 100 mM 2-DG than upon glucose withdrawal, cell death is induced earlier and more intensively by glucose starvation than upon 2-DG treatment. In consequence, we propose that 2-DG and glucose starvation induce cell death through different mechanisms, although both are inducing cytochrome c release and PARP cleavage.

Induction of cell death by energy depletion remains poorly understood. Since glucose is the major source for the process of glycosylation, the glycosylation of essential proteins like transcription factors might also be profoundly affected in the absence of glucose (Han and Kudlow 1997) or by blocking glucose usage by 2-DG treatment (Kang, Ju et al. 2003). This may explain why pyruvate cannot restore survival in cells treated with high doses of 2-DG. Some studies on c-Myc have suggested a role of this oncogene in the induction of cell death by energy depletion. For example, prolonged culture of pancreatic beta-cells in low-glucose concentrations increased c-myc expression and induces caspase-dependent apoptosis (Van de Castele, Kefas et al. 2003). Shim and colleagues observed that glucose deprivation or treatment with 2-DG caused arrest in the G0/G1 phase of the cell cycle of non-transformed cells. However, c-Myc-transformed fibroblasts, lymphoblastoid, or lung carcinoma cells underwent extensive apoptosis (Shim, Chun et al. 1998). These observations are providing insights into the coupling of glucose metabolism and apoptosis. In addition, c-Myc has been shown to be overexpressed in GBMs (Orian, Vasilopoulos et al. 1992). Thus, c-Myc could be the missing link between energy depletion and apoptosis in 2-DG-treated glioma cell lines.

Another interesting observation is that 2-DG is able to efficiently reduce the levels of

the repair protein Rad51. Clinically, the radiosensitivity of tumor cells after exposure to 2-DG in human patients (Dwarkanath, Zolzer et al. 2001) can be explained by the reduction in the levels of Rad51 protein (Russell, Brady et al. 2003). Indeed, radiotherapy induces various DNA lesions, including double strand breaks, in dividing cells. Repair of radiation-induced damages requires continuous supply of energy. In presence of 2-DG, the decrease of Rad51 protein levels may be the consequence of the inhibition of transcription and/or translation. Since Rad51, and probably other repair proteins play an essential role in the repair of radiation-induced damage and induction of cell death, cells that cannot recover from the damage will undergo cell death. Nevertheless, clinically applicable doses of 2-DG (1 and 5 mM) induced only a block of proliferation of GBM cell lines, but could not induce cell death.

Our initial observations indicated that GBM cells are resistant to “biologicals” which include specific targeting of essential kinases of pathways involved in the development and maintenance of the disease: mTOR, PDGFR, EGFR and VEGFR. Even upon the combination of RAD001, Glivec and AEE788, cell death was not increased *in vitro* in dose ranges suitable for clinical application. The glycolytic inhibitor 2-DG could induce apoptosis in glioma cell lines, but only at high doses (25 and 100mM), which could not be achieved in human patients. Serum concentration up to 7mM have been reported, corresponding to an administration of 200mg/kg body weight (Mohanti, Rath et al. 1996). Even the HDIs like TSA, SB, SAHA and LAQ824 could not trigger strong apoptosis in glioma cell lines at low nanomolar range. Nevertheless, an inhibitory effect on the proliferation of the GBM cells was observed at low doses for these drugs, which would be suitable for a clinical application. However, since the inhibition of proliferation of all cytostatic drugs is generally disappearing once the treatment is stopped, the only way to get rid of cancer cells is to induce cell death. From these disappointing results arose a new strategy where drugs from various classes (“biologicals”, “cytotoxics”, glycolytic inhibitors, NSAIDs) are combined with each other in order to trigger synergistically apoptosis or to induce additive tumor cell death.

### **Simultaneous inhibition of mTOR by RAD001 and glycolysis by 2-DG.**

The previous observations on RAD001 raise the possibility to use this drug as a “sensitizer” in order to enhance 2-DG-induced apoptosis at clinically applicable doses like 1 mM or 5 mM. We have proposed that RAD001 could be able to sensitize the AKT/mTOR signaling pathway to apoptosis induced after 2-DG treatment, since the PTEN-mutant GBM cells are more sensitive to RAD001. Previous studies have already shown potentiation in the inhibition of tumor growth or in the induction of apoptosis, when rapamycin derivatives are combined with cytotoxic agents (Mondesire, Jian et al. 2004), cisplatin (Georger, Kerr et al. 2001; Beuvink, Boulay et al. 2005) or AEE788 (Goudar, Shi et al. 2005). Since 2-DG is acting as a cytotoxic agent by inducing apoptosis at high doses and as a cytostatic agent at low doses, the combined application of low 2-DG and RAD001 was tested on GBM cells. The results show a negative effect of the combination in the induction of apoptosis in all human GBM cell lines. The level of apoptosis was delayed by about 30 to 50% depending on the cell line. This negative effect was reproducible when RAD001 was combined with glucose withdrawal, independently of the PTEN status.

The antagonistic effect in the induction of apoptosis is not understood yet, since neither ERK nor AKT appeared to be involved in this mechanism. RAD001 is mainly downregulating translation downstream of mTOR, whereas 2-DG is mainly affecting ATP production and protein glycosylation (Kang, Ju et al. 2003). It can be that upon blocking glycolysis, other mechanisms can be promoted as autophagy to provide energy to the cell. Autophagy is defined as the degradation of cellular proteins in response to nutrient deprivation or other types of stresses in order to allow ATP production (Klionsky and Emr 2000). mTOR has profound effects on the control of apoptosis and autophagy (Castedo, Ferri et al. 2002). Rapamycin has been found to induce autophagy rather than apoptosis in yeast and cultured mammalian cells, such as hepatocytes (Shigemitsu, Tsujishita et al. 1999). Rapamycin was found to induce autophagy in glioma cell lines whereas no induction of cell death could be observed (Takeuchi, Kondo et al. 2005). The delay in apoptosis observed upon the combination of 2-DG and RAD001 could be due to the induction of autophagy by RAD001 which would reduce 2-DG-induced cell death by providing more ATP. However, the stimulation of autophagy by mTOR inhibitors is not well defined.

mTOR plays a central role in the regulation of apoptosis. This has been demonstrated by the sensitization of tumor cells to cytotoxic agents by rapamycin (Beuvink, Boulay et al. 2005). Rapamycin has been reported to selectively kill mouse embryo fibroblasts deficient either in p53 or in p21 (Huang, Liu et al. 2001). The inhibition of apoptosis by mTOR can be due to the phosphorylation of BAD by S6K, a reaction that disrupts the binding of BAD to the mitochondrial death inhibitors Bcl-2 and Bcl-XL and thus inactivates BAD (Harada, Andersen et al. 2001). In contrast, the induction of apoptosis by mTOR was suggested by the combination of rapamycin with taxol, in which rapamycin could inhibit the taxol-induced cell death of human B-cell lines correlating with a dephosphorylation/deactivation of Bcl-2, whereas taxol induces phosphorylation of Bcl-2, activating apoptosis. In this report, it was found that mTOR was activated in cells treated with taxol although it was inhibited in cells pre-treated with rapamycin (Calastretti, Bevilacqua et al. 2001). Rapamycin inhibits the phosphorylation of p53 resulting in its transcriptional activation that induces pro-apoptotic proteins such as Bax and subsequent apoptosis (Castedo, Ferri et al. 2001). In order to answer the question by which RAD001 delays apoptosis induced by 2-DG, several additional experiments have to be done in order to determine whether some of these pro-apoptotic (Bax, BAD) or pro-survival proteins (Bcl-2, Bcl-xl) are deactivated or activated in the presence of RAD001. In contrast to RAD001 plus 2-DG, the combination of 2-DG and LY enhanced cell death in LN401 GBM cells. Inhibition of the PI3K/AKT pathway by LY sensitized the cells to 2-DG-induced apoptosis. A broader study has to be done in other glioma cell lines in order to define the extent and the limitation of this combination, for example, it would be interesting to know whether this combination depends on the PTEN status since PTEN-mutant cells exhibit a stronger activation of the PI3K/AKT pathway than wild-type cells.

### **Simultaneous inhibition of histone deacetylases and glycolysis**

Previous reports have already shown interesting effects on cancer treatment when HDACs inhibitors (HDIs) were combined with other drugs. For example, in acute promyelocytic leukemia with t(11;17)/*PLZF-RAR* translocation, a combination of all-*trans* retinoic acid with the HDI trichostatin A resulted in differentiation of all-*trans* retinoic acid-resistant acute promyelocytic leukemia cells (Ferrara, Fazi et al.

2001). An important finding in predicting the potential utility of HDIs in the clinic is their activity in cell lines that are resistant to existing chemotherapeutics. For example, Glivec-resistant Bcr/Abl human chronic myelogenous leukaemia (CML) cells are sensitized to Gleevec upon co-treatment with SAHA (Yu, Rahmani et al. 2003). In addition, CD34-positive progenitor cells from patients with Gleevec refractory CML respond to SAHA treatment and exhibit increased apoptosis and histone acetylation levels (Nimmanapalli, Fuino et al. 2003). In other studies, combinations of HDIs with other drugs such as the PI3K inhibitor LY294002 (Rahmani, Yu et al. 2003), the ATM inhibitor caffeine (Burgess, Ruefli et al. 2004) or DNA damaging agents (Kim, Blake et al. 2003) showed synergistic effect with respect to induction of cell death. We exploited new approaches for the treatment of GBMs, focusing on a combination of drugs that restores gene expression via inhibition of HDACs and glycolysis.

In order to enhance the efficiency of HDIs to induce apoptosis and according to the ease of these drugs to synergize with other drugs, we tested, *in vitro*, the combination of HDIs and the glycolytic inhibitor 2-DG on human GBM cells. The question was whether low concentrations of HDIs with reduced toxicity, would potentiate apoptosis when used in combination with 2-DG. It is already known that 2-DG as well as HDIs like SAHA, butyrates (NaB) or LAQ824 are well tolerated in patients and are able to pass the BBB. We found that 2-DG strongly increases HDI-induced apoptosis in all human GBM cells tested in a p53-independent manner, as Hs683, LN401, LN229 and U373 are mutated for *TP53* (Ishii, Maier et al. 1999). The cells exposed to this combination exhibited 95% cell death whereas only basal levels of apoptosis were detected after the exposure to the single drugs at concentrations with reduced toxicity. Of note is the finding that this synergism is specifically efficient in cancer cells whereas no cell death could be induced in non-cancer cells, even at high HDI doses (except in HSF).

Among 2% of genes that are targeted by HDIs, the up-regulated expression of the cyclin-dependent kinase inhibitor p21 is responsible for the anti-proliferative effect of the drug (Richon, Sandhoff et al. 2000; Blagosklonny, Robey et al. 2002). A number of studies have demonstrated that expression of p21 causes a G1 cell cycle arrest and protect cells against chemotherapeutic agents (Fueyo, Gomez-Manzano et al. 1998;

Burgess, Pavey et al. 2001; Schmidt and Fan 2001). Among cells that have HDI-induced p21 expression, only cells in late G1 and S phase proceed through an aberrant mitosis and rapid apoptosis, whereas cells in G1 phase accumulated p21 and arrest. We have shown that, at low doses, HDIs only induced weak cell death that did not increase with time, whereas at higher doses, HDIs could induce significant cell death. The expression of p21 was observed at low as well as at high concentrations and peaks around 8 to 16h and declines to almost control levels by 36h to 48h. After HDI treatment, the cells still undergo apoptosis later on which can be due to the decrease of the levels of p21 protein. If p21 is protecting cells against the induction of apoptosis, a decrease of p21 would allow the process of apoptosis. Our results showed that the HDIs TSA, NaB or LAQ824 all induced an increase in p21 protein levels after 8h treatment, and that this resulting p21 upregulation is abrogated in presence of the glycolytic inhibitor 2-DG. Sequential addition of HDI and 2-DG demonstrated that the expression of p21 needs to be induced in order to synergistically trigger apoptosis by this combination. Indeed, if 2-DG is added when p21 levels are low, no synergism was induced and no cell death observed. The effect of HDIs on p21 is transient whereas the inhibitory effect of 2-DG on glycolysis is lasting. We also observed that the decrease of the basal levels of p21 by 2-DG without HDIs treatment did not induce apoptosis. These results suggest that the inhibition of p21 upregulation upon HDIs by 2-DG is triggering apoptosis. However, many other factors might be involved in the synergism. Indeed, the exposure of cells to HDIs not only upregulates the expression of p21, but also concurrently activates other pathways which may be involved in the induction of apoptosis. On one hand, these pathways are inducing apoptosis, on the other hand, p21 upregulation is protecting cells against the activation of these pathways by blocking fundamental steps. The anti-apoptotic effect of p21 may be the result of a number of mechanisms. For example, p21 has been demonstrated to bind to procaspase-3, the inactive form of caspase-3, responsible for apoptosis triggering, and inhibits its proteolytic activation (Suzuki, Tsutomi et al. 1999). Procaspase-3/p21 complexes have been found in the mitochondria to resist Fas-mediated cell death in HepG2 cells (Suzuki, Tsutomi et al. 1999). In turn, caspase-3 contains p21-binding domain in the N-terminus, independent of the PCNA-binding site; p21 is also a substrate for caspase-3 cleavage, producing 14 and 7 kDa fragments (Gervais, Seth et al. 1998). Caspase-3 is cleaving other executioners

including PARP. The expression of p21 has also been reported to induce the expression of anti-apoptotic factors (Chang, Watanabe et al. 2000). HDIs are inducing the expression of pro-apoptotic factors like Bcl-2 (Facchetti, Previdi et al. 2004) which would trigger apoptosis via the mitochondrial pathway. An alternative to the 2-DG/HDI-mediated apoptosis would be the extrinsic pathway triggered via the death receptors, since HDIs have been reported to induce the expression of the ligand for death receptor, trail (Nebbioso, Clarke et al. 2005). However, whether this pathway can be activated in glioma cell lines remains unclear (Nebbioso, Clarke et al. 2005). Some additional experiments have to be done in order to find out how apoptosis is triggered by this synergism. For example, we could determine whether cytochrome c is released by this combination, this experiment will tell us whether cell death is mediated via the mitochondria pathway.

The main question remains, by which mechanisms 2-DG abrogates levels of p21. Either 2-DG affects the transcription of p21, or the translation of p21, or both. In our studies, we showed that RAD001 only weakly inhibited p21 upregulation induced by HDI and, in addition, no real synergism was observed when RAD001 and HDIs were combined, even when the concentration of RAD001 applied was ten times higher of its IC50 value to fully inhibit the mTOR pathway. The protein levels showed that RAD001 was not able to strongly reduce p21 levels. A drop of ATP is activating AMPK which in turn inhibits mTOR, S6 and 4EBP1 phosphorylation and finally ribosome biogenesis. The effect of 2-DG is comparable to the effect of RAD001. In our results, the addition of pyruvate in the combination of 2-DG and LAQ824, which allows production of ATP, does not affect the synergism. The combination of the inhibitor of the ATP-pump oligomycin with HDIs is not inducing cell death, whereas the combined treatment of HDIs and glucose withdrawal induced a comparable synergism comparable to this one induced by HDIs and 2-DG. These results suggest that the inhibition of translation could be responsible to a limited extent in the abrogation of p21 levels and that other mechanisms may also be involved in this synergism.

Previous studies support the hypothesis that 2-DG strongly reduced p21 levels at the transcriptional level. Indeed, the p21<sup>WAF1</sup> promoter is controlled by p53 and/or Sp1 transcription factors. Since we showed that p21 is still up-regulated upon HDIs treatment in p53 mutant cells, Sp1 appears to be the most important transcription

factor regulating p21 transcription. Kang et al (Kang, Ju et al. 2003) investigated how 2-DG and low glucose are affecting transcriptional activity of Sp1. They could show that poor ATP generation is involved only to a limited extent since gene transcription was only marginally affected by the inhibition of ATP synthesis. However, Sp1 is one of the nuclear proteins that are glycosylated at serine/threonine residues by N-acetylglucosamine (GlcNAc) through O-GlcNAcylation. Many key proteins within the cytoplasm and nucleus are now known to be glycosylated, including proteins of diverse functional groups: kinases, phosphatases, cytoskeletal proteins, chaperones, metabolic enzymes and transcription factors such as Sp1. Jackson et al. showed for the first time in 1988 that the human RNA polymerase II transcription factor Sp1 bears multiple O-linked N-acetylglucosamine monosaccharide residues and that these residues may play a role in the mechanism or regulation of transcriptional activation of RNA polymerase II (Jackson and Tjian 1988). The modification of proteins by O-linked- $\beta$ -N-acetylglucosamine (O-GlcNAc) is modulating cellular function in response to nutrition and stress. O-GlcNAc is acting in a manner analogous to protein phosphorylation (Schafer, Hamm-Kunzelmann et al. 1997). O-GlcNAc levels respond to mitogens, cellular signals, growth factors and modulate enzyme activity or regulation, protein-protein interaction, DNA binding, subcellular localization and the half life and proteolytic processing of proteins. Nevertheless, the way that O-GlcNAc is mediating its effects remains unclear. The addition and removal of O-GlcNAc is achieved by two enzymes: the O- $\beta$ -N-acetylglucosaminyltransferase (OGT) and the O-linked- $\beta$ -N-acetylglucosaminidase (O-GlcNAcase). Kang et al showed that 2-DG down-regulates Sp1 activity through hyper-GlcNAcylation, by affecting these enzymes, but the mechanism is not defined yet (Kang, Ju et al. 2003). In this regard, the finding that RAD001, an inhibitor of mTOR and by extension, S6, failed to enhance HDI lethality argues against a critical role for this pathway in the synergism and suggests the main intervention of the inhibition of Sp1 by hyperglycosylation and further on the inhibition of p21 transcription. Indeed, they show that 2-DG induced an early hyperglycosylation of Sp1, within 4 hours treatment upon 25mM 2-DG, responsible for the simultaneous decrease of p21 protein levels. This is correlated with our results showing that 2-DG reduces early p21 levels (also around four hours) in Hs683.

These findings indicate that other combinations of drugs can induce similar synergism regarding the implication of p21 proteins in the regulation of cell survival. First, important is that p21 is not mutated in GBMs (Tsumanuma, Tanaka et al. 1997). However, our results show p21 reexpression after treatment with HDIs. Second, many reports have demonstrated the pro-survival effect of p21 upon chemotherapeutic treatments. These facts suggest that any cytotoxic drug, that is increasing p21 levels, independently of p53 or not, can be combined with any agent that is strongly inhibiting p21 upregulation, as 2-DG. Several reports are supporting this hypothesis. Lane and colleagues (Beuvink, Boulay et al. 2005) have tested the combination composed by cisplatin and RAD001 on breast cancer cell lines *in vitro*. In this study, the cytotoxic agent cisplatin was upregulating p21 protein whereas RAD001 simultaneously induced a decrease of p21, synergistically induced apoptosis. In another study, sodium butyrate (SB) also synergized with the PI3K inhibitor LY294002 (Rahmani, Yu et al. 2003), inducing strong cell death consequently to p21 downregulation. Burgess and colleagues showed that the ATM inhibitor caffeine (Burgess, Ruefli et al. 2004) also abrogated p21 expression when combined with the HDI, suberic bishydroxamic acid (SBHA) leading to a synergistic induction of apoptosis. The mechanism by which the combination of 2-DG with HDIs is synergistically inducing apoptosis in glioma cell lines remains to be defined. Thus, we propose one possible model to explain the complexity of the mechanisms involved into the synergism (Fig. 31).

#### **The combination of HDIs with the NSAID Indomethacin (IND)**

In a similar way, since GBMs overexpress COX-2, the inhibition of HDACs was combined with indomethacin (IND), an inhibitor of cyclo-oxygenases. Our results showed an interesting synergism between the HDI LAQ824 and indomethacin on inducing apoptosis in human GBMs, whereas no effect was observed in the normal HEK293, RASMC or HSF cell lines. In addition, the results concerning protein analysis in HS683 upon the combination, suggests that p21 could, again, be involved in the synergistic induction of apoptosis. Nevertheless, additional experiments have to be done in order to understand the mechanisms underlying the effects observed on cell survival.

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, indomethacin and ibuprofen are the most widely used drugs for pain, arthritis (Schuna 1998), (Curtiss 2002), cardiovascular diseases (Goodnight 1996), the prevention of colon cancer (Sheng, Shao et al. 1997) and Alzheimer disease (Sloane 1998). However, NSAIDs produce gastroduodenal ulcers in about 25% of users (often with bleeding and/or perforations) and delay ulcer healing, presumably by blocking prostaglandin synthesis from cyclooxygenase COX-1 and COX-2. Other adverse effects have been noticed on the blood pressure and renal function. The hypothesis that the gastrointestinal side effects of NSAIDs result from inhibition of COX-1, but not COX-2, led to the development of NSAIDs that selectively inhibit only COX-2 (such as celecoxib and rofecoxib). Jones and colleagues showed that both COX-1 and COX-2 are important for angiogenesis and these findings challenge the premise that selective COX-2 inhibitors will not affect the gastrointestinal tract, ulcer and wound healing (Jones, Wang et al. 1999). Large-scale clinical outcome studies showed reductions in the range of 50% in clinical gastrointestinal events with COX-2 inhibitors compared with non-selective NSAIDs among patients not taking aspirin. Overall, the results indicate an advantage for COX inhibitors, but that their use is not devoid of serious gastrointestinal adverse events. (Laine, Connors et al. 2003).

### **Orthotopic glioma rat model**

*In vitro* assays allow to quickly determine the impact of a drug according to the genetic background. Considering that each cell line displays a specific combination of genetic defects, altogether they may represent the heterogeneous nature of GBMs, and the efficacy of the drug on a number of those could statistically mimic the potential effect of this drug in an *in vivo* model. Although xenograft studies are not fully predictive of the therapeutic efficacy of cancer therapies in clinical trials, xenograft studies may offer additional information over cell culture studies. These *in vitro* experiments are necessary since they allow to characterize the specific effects of this compound on its biomarker(s), on cell-cycle and cell survival.

Development of an orthotopic GBM rat models will further enable us to determine whether the synergism induced by the combinations of HDIs with 2-DG or with indomethacin, or a COX inhibitor, able to pass the BBB, can be reproduced *in vivo*.

These models will also give use some clues about their potential toxicity. These data are necessary prior to the introduction of the combinations into clinical studies.

### **Clinical relevance of the work**

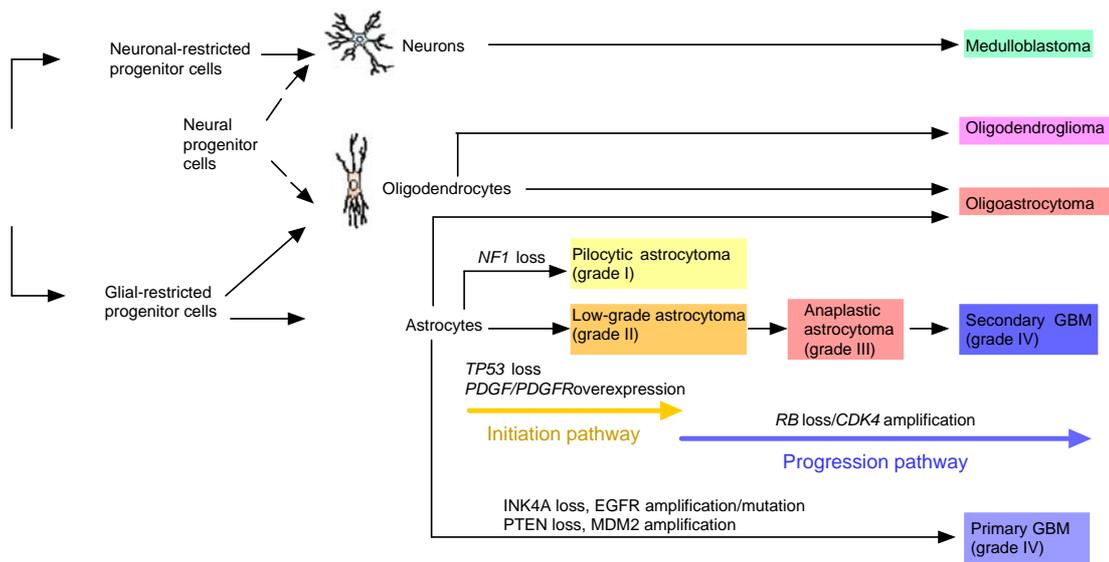
Our results show that the combination of HDAC inhibitors with 2-DG or with indomethacin are only inducing apoptosis in cancer cell lines whereas no cell death was observed in the non-cancer cell lines. Of importance is that the synergism is operating on the level of apoptosis and not only on the level of cell growth, which is more promising with respect to disease remission.

Clinically, 2-DG and indomethacin (and analogues) are well tolerated in humans and pass the blood-brain-barrier. HDIs may be more toxic in human application. However, since low doses of HDIs are sufficient to reach a strong synergism in presence of 2-DG, the combination of drugs is welcome in clinical trials for the treatment of GBMs. In addition, since we showed that other cancer cell lines exhibited similar synergism upon both combinations, we can conclude that they could be an attractive treatment for various solid cancers.

In this work we demonstrated that GBMs show high resistance to *in vitro* treatments that target individual pathways like the “biologicals” RAD001, AEE788 and Glivec but also to “cytotoxics” HDAC inhibitors (HDIs) and EPO906A at doses applicable in clinics. We have exploited new approaches in the treatment of GBMs by combining the anti-metabolite 2-DG with HDIs at doses of reduced toxicity, where synergistic effects were observed in 100% of the glioma and other cancer cell lines tested, but not in non-cancer cell lines. Thus, anti-metabolic intervention combined with HDIs might be a promising treatment for solid cancers. Our results and previous reports support the hypothesis that the simultaneous combination of agents that increase p21 expression (such as HDIs) with agents that abrogate p21 upregulation (such as 2-DG), might be good choice for the treatment of GBMs. The data obtained upon the treatment of 2-DG also suggest that future clinical trials designed for the treatment of GBMs must take into account the inhibition of the glycolytic pathway. Nevertheless, significant challenges remain for the development of drug combinations to treat GBMs, namely the inability to predict tumor response and genetic instability that may allow cells to escape from pro-apoptotic stimuli.

## ANNEXES

# Annex 1



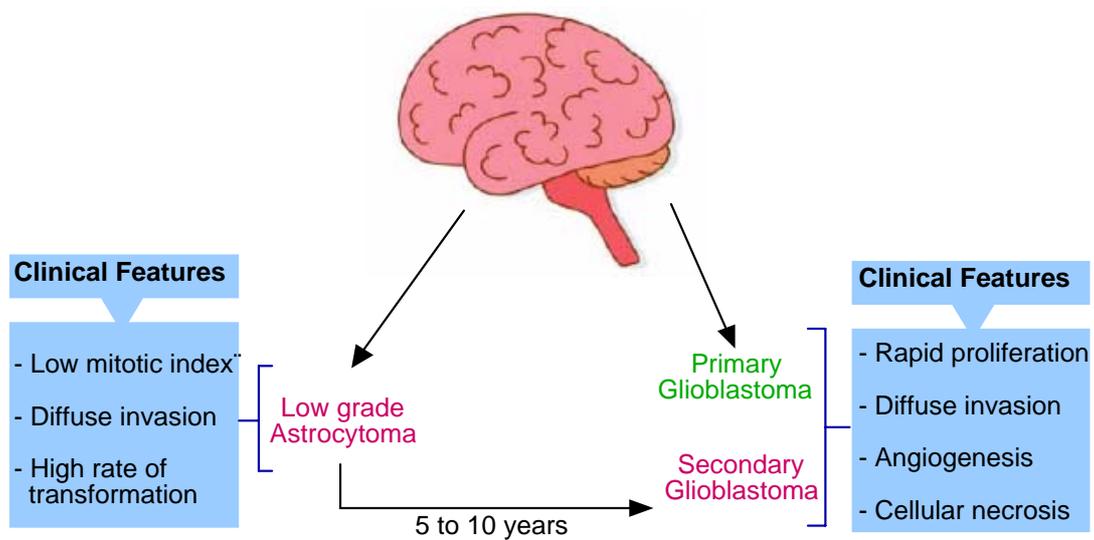
Zhu et al, Nature Reviews Cancer, Vol 2, Aug 2002- modified

## Precursors of CNS tumors

In the central nervous system (CNS), multipotent neural stem cells in the ventricular/subventricular zones of the embryonic neural tube give rise to three main cell types in the mature CNS: neurons, oligodendrocytes and astrocytes. It is suggested that that neural stem cells first differentiate into two distinct progenitor cells: neuronal restricted progenitor cells and glial restricted progenitor cells, which further differentiate into neurons and glia, respectively.

Several genetic pathways are involved in the initiation and in the progression of secondary GBMs. Loss of *p53* and activation of growth-factor-RTK-RAS pathway or loss of *NF1* are involved in the initiation of grade 1 and grade II astrocytoma. These can progress to grade IV GBM after disruption of *RB* pathway. In primary GBM, the same genetic pathways are dismantled, through different mechanisms. Amplification and/or mutation of *EGFR* is often detected and *PTEN* phosphatase activity is frequently disrupted.

## Annex 2

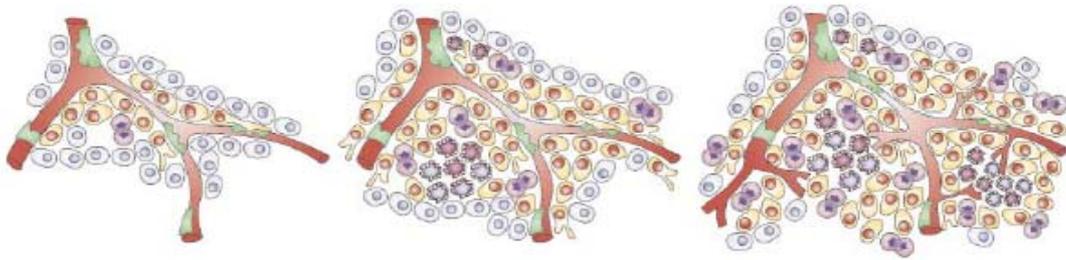


*Maher et al, Genes and Development, Vol 15, 2001*

### Two pathways to GBMs

GBM can develop over 5-10 years from a low-grade astrocytoma (secondary GBM), or can be the initial pathology at diagnosis (primary GBM). The clinical features of GBM are the same regardless of clinical routes.

## Annex 3



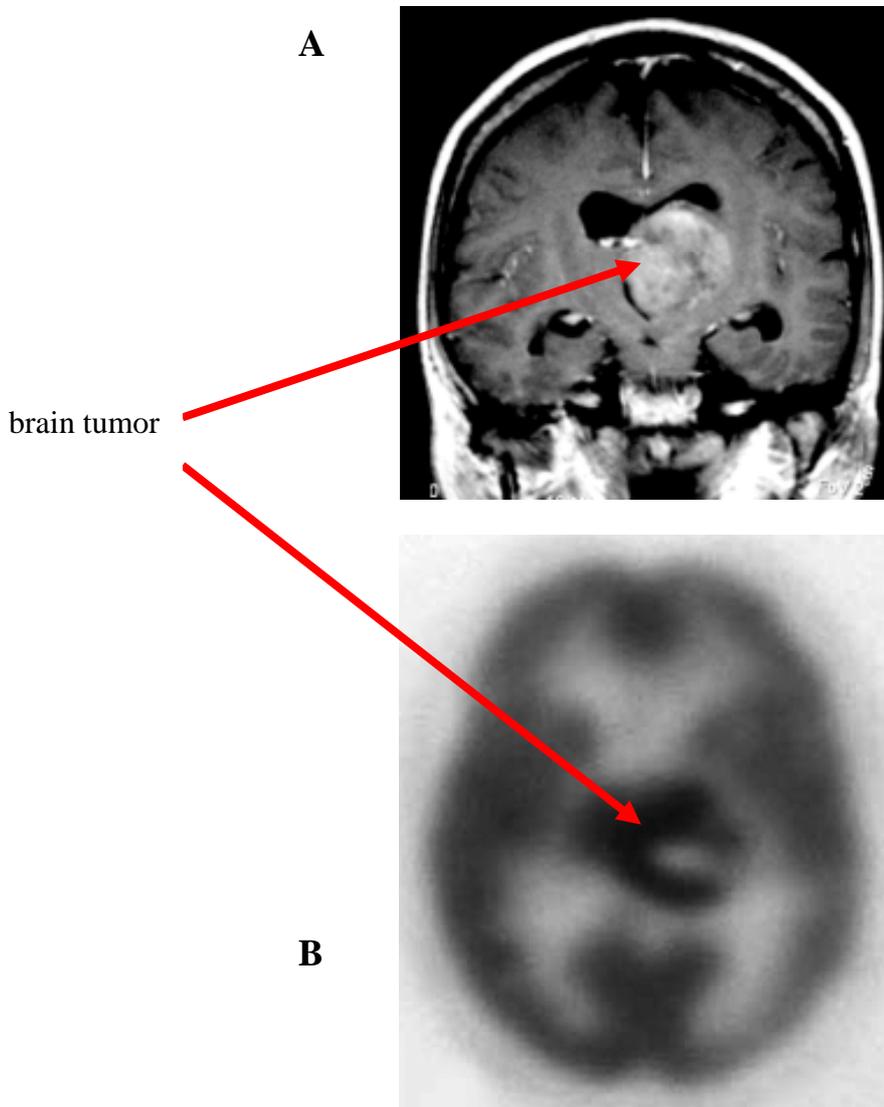
### **Blood vessel co-option precedes angiogenesis in astrocytoma progression.**

Astrocytomas first acquire their blood supply by co-opting existing normal brain blood vessels without the necessity to initiate angiogenesis.

**A.** They instead grow along blood vessels, without a tumour capsule, eliciting an invasive character.

**B.** When grade III astrocytomas progress into glioblastomas (GBM or grade IV astrocytoma), they become hypoxic and necrotic — partly due to vessel regression and increased tumour-cell proliferation. **C.** These conditions, in turn, induce formation of new blood vessels (angiogenic sprouting) that supply the tumour with the necessary metabolites. In fact, glioblastomas are partly defined by the appearance of proliferating endothelial cells and a high blood-vessel density that distinguishes grade IV tumours from the lower-grade astrocytoma

## Annex 4



*Neurosurgical University Clinic, Basel*

### **The glucose analog tracer $^{18}\text{F}$ fluoro-deoxy-glucose is used in humans for PET imaging**

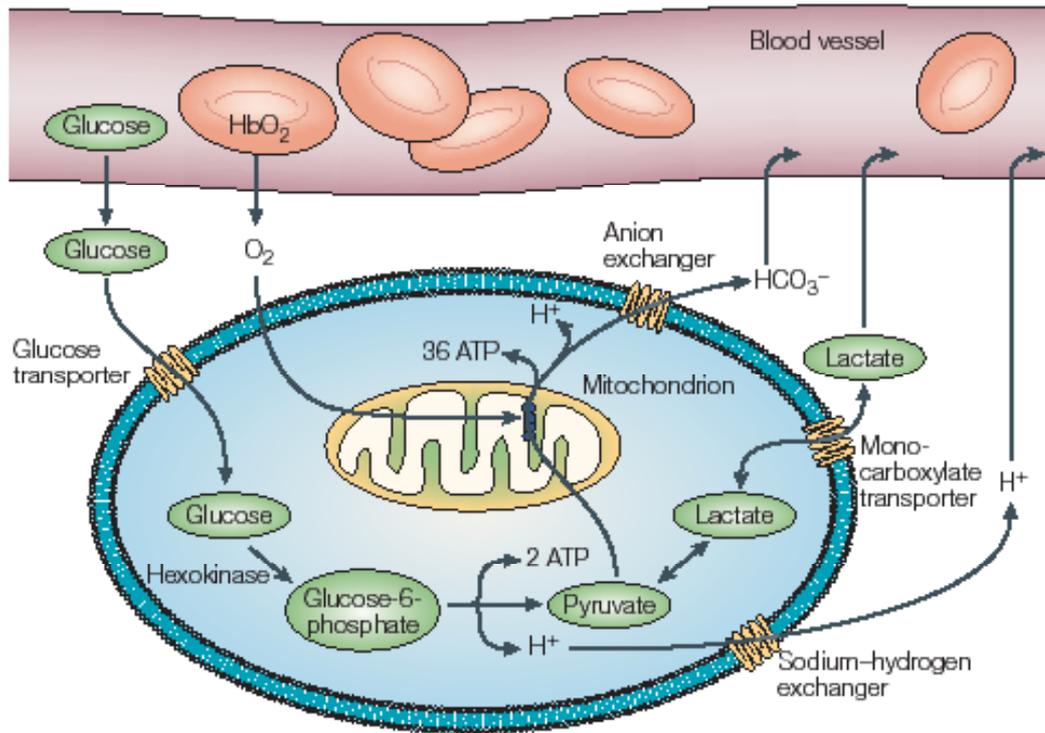
Malignant glial brain tumors display a high consumption of glucose, even more than the cells from the cortex. Malignant transformation is usually manifested by an increase of 18 fluorodeoxyglucose (FDG) uptake which is not metabolized.

(A): MRI: Magnetic resonance imaging: shows a large thalamic GBM on the left side. MRI is commonly used to determine the location and extent of cerebral GBMs.

(B): FDG-PET (positron emission tomography). Metabolic imaging using an analogue of glucose. Note the increased  $^{18}\text{F}$ FDG uptake in the tumor compared to normal cortex.

(Novak, Molnar et al. 2005)

## Annex 5



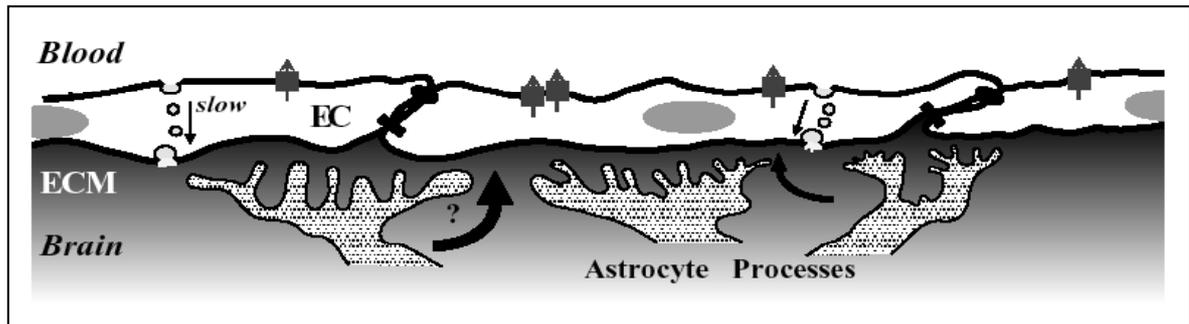
*Gatenby et al, Nature Review Cancer, Vol 4, Nov 2004*

### Glucose metabolism in mammalian cells.

Blood delivers glucose and oxygen (on haemoglobin) to tissues, where it reaches cells by diffusion. Glucose is taken up by specific transporters, where it is converted first to glucose-6-phosphate by hexokinase and then to pyruvate, generating 2 ATP per glucose. In the presence of oxygen, pyruvate is oxidized to HCO<sub>3</sub><sup>-</sup>, generating 36 additional ATP per glucose. In the absence of oxygen, pyruvate is reduced to lactate,

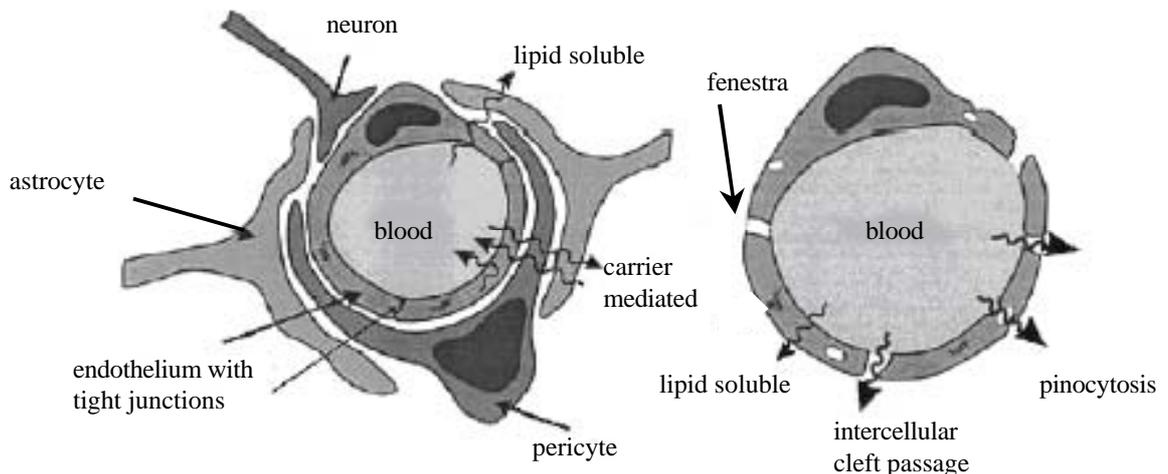
which is exported from the cell.

## Annex 6



- Tight junction
- Adherent junction
- ↑ P-Glycoprotein (P-gp)

*L. L. Rubin et al, Annu. Rev. Neurosci. 1999.*

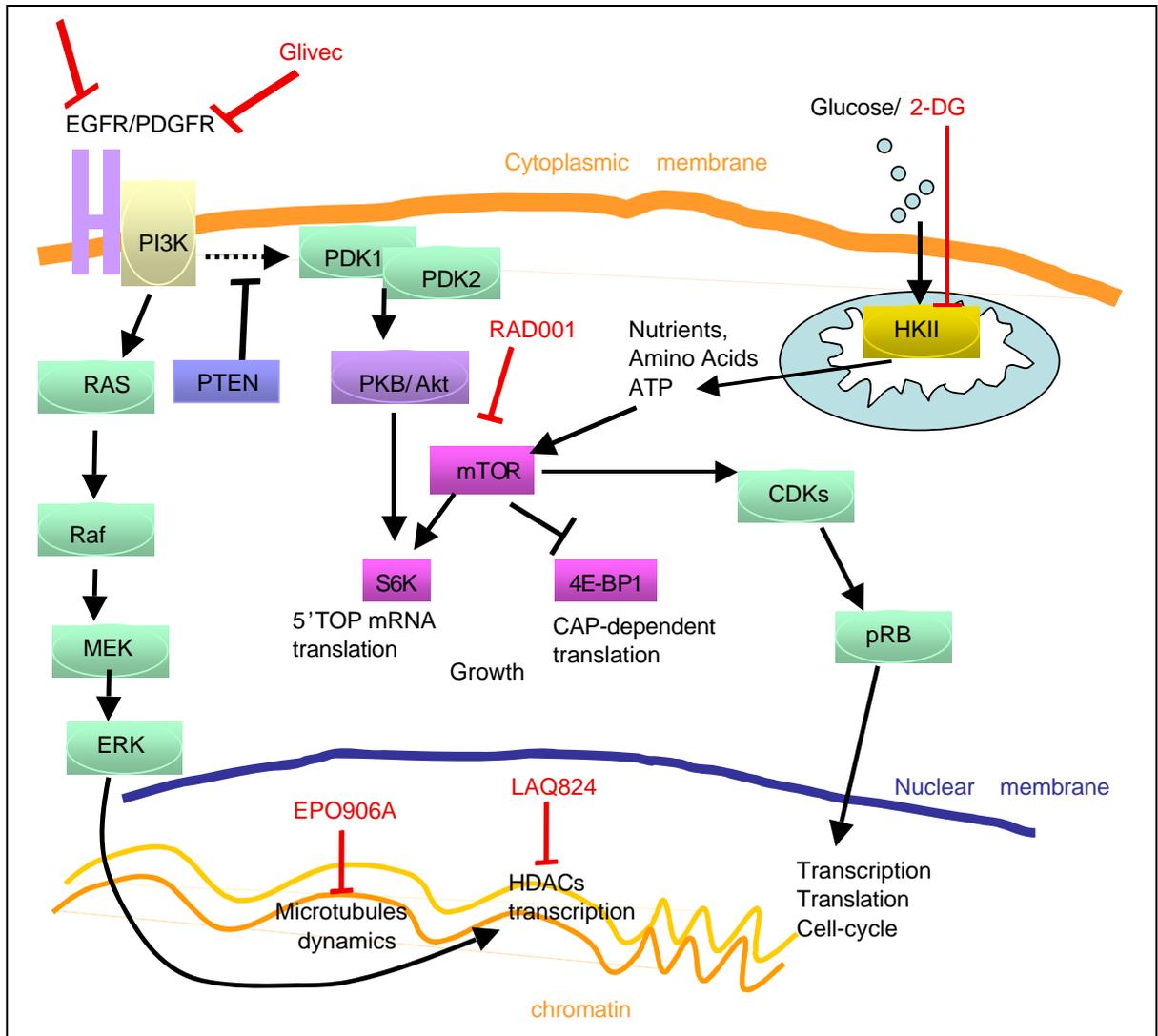


*Misra et al, J Pharm Pharmaceut Sci, Aug 2003*

### Structure of the Blood-Brain Barrier.

The BBB is an endothelial barrier present in capillaries that course through the brain. Brain capillary endothelial cells (ECs) are coupled by adherent and tight junctions limiting paracellular flux. P-glycoprotein is expressed in the apical membrane of the EC and actively ejects certain undesired substances from the central nervous system (CNS). ECs have very few endocytotic vesicles limiting transcellular flux. Transcytosis across brain ECs occurs slowly, minimizing transcellular movement into the CNS. The cell closest to brain capillary ECs is the astrocyte, whose endfeet cover the capillary's basal surface, although an extracellular matrix is interposed and may release molecules that influence their phenotype. Not shown are the transporters for essential nutrients, such as glucose and amino acids, and for macromolecules such as transferrin.

## Annex 7



### Targeting mitogenic pathways in gliomas.

Several growth-factor pathways are inappropriately activated in gliomas through overexpression, amplification or activating mutations, including EGFR and PDGFR that induce the activation of numerous downstream signal transduction pathways that regulate gene transcription, translation or cell cycle. In addition, tumor suppressor genes are frequently inactivated through deletion, mutation or promoter silencing.

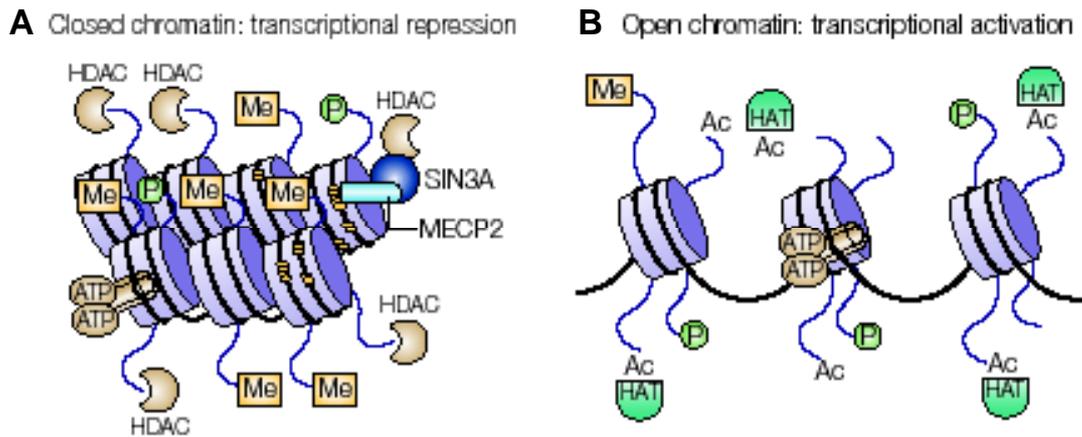
Several proteins in these cascades are the targets of therapies in development for malignant gliomas, like mTOR, PDGFR, EGFR, histone deacetylases (HDAC) and tubulin for microtubule dynamics. The glycolytic inhibitor 2-DG is targeting Hexokinase II (HKII). The drugs are indicated in red.

## Annex 8

	SF767	LN215	LN18	U373	U343	Hs683	LN319	LN229	LN401	LN71
<i>PTEN</i>	WT	mut	WT	mut	mut	WT	mut	WT	mut	mut
<i>p53</i>	WT	mut	WT	mut	WT	mut	mut	mut	mut	mut
<i>p16/p14</i>	WT	WT	del	WT	del	del	WT	del	del	del

WT: Wild-Type  
mut: mutant  
del: deleted

## Annex 9



*Ricky W. Johnstone, Nature Reviews Drug discovery, vol 1, Apr2002*

### **Chromatin structure regulates transcriptional activity.**

Nucleosomes consist of DNA (black line) wrapped around histone octamers (purple). Post-translational modification of histone tails by methylation (Me), phosphorylation (P) or acetylation (Ac) can alter the higher-order nucleosome structure. Nucleosome structure can be regulated by ATP-dependent chromatin remodellers (yellow cylinders), and the opposing actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Methyl binding proteins, such as the methyl-CpG-binding protein (MECP2), target methylated DNA (yellow) and recruit HDACs.

**A.** DNA methylation and histone deacetylation induce a closed-chromatin configuration and transcriptional repression.

**B.** Histone acetylation and demethylation of DNA relaxes chromatin, and allows transcriptional activation.

## MATERIALS AND METHODS

### **Cell lines and reagents:**

The mutation status of the human glioma cell lines Hs683, LN229, SF767, LN401, U373, U343, LN71, LN18, LN319, LN215 of *TP53*, *p16/p14* and *PTEN* genes has been previously established (Ishii, Maier et al. 1999). HeLa is a human cervix carcinoma cell line, MCF-7, HBL, SKBR-3 are human breast cancer cell lines, Calu6 is a colon cancer cell line. The human embryonic kidney cell line HEK293, the human skin fibroblast HSF and the rat aortic smooth muscle cell line RASMC are non-cancer cell lines and used as control cell lines for testing cytotoxicity. All the previous cell lines were cultured in Eagle medium containing 25mM glucose, glutamine, standard antibiotics and 10% FCS, except MCF-7, HBL, SKBR-3, cultured in RPMI medium with 25mM glucose, glutamine and standard antibiotics and 5% FCS. All cells were maintained at 37°C in 5% CO<sub>2</sub>. The pharmacological inhibitors Glivec, AEE788, RAD001, LAQ824 and EPO906A were kindly provided by Novartis-Pharma AG, Basel. The other drugs, namely 2-deoxy-D-glucose (2-DG), Trichostatin A (TSA), Sodium Butyrate (NaB), LY294002 (LY) and Indomethacin (IND) as well as Pyruvate, Oligomycin, Cycloheximide (CHX) and Sodium Azide (SA) were purchased from Sigma.

### **Western analysis and Antibodies:**

After drug treatment, cells were washed with 1xPBS, scrapped in cold SDS lysis buffer containing 2% sodium dodecyl sulfate (SDS), 50mM Tris pH 6.8, 0.1M DTT, boiled and frozen at -20°C. For the analysis of apoptotic and signal transduction markers, protein lysates were separated by size on denaturing SDS-polyacrylamide gels (SDS-PAGE) ranging from 8 to 13% depending on the molecular weight to be resolved and transferred to nitrocellulose membranes (Hybond, ECL, Amersham Biosciences). Membranes were probed with the following primary antibodies: Akt antibody (kindly provided by Dr Brian Hemmings, FMI Basel), Mouse anti-S6 (kindly provided by Dr George Thomas, FMI, Basel), phospho-RB (Ser 807-811), phospho-S6 (Ser-240/244), S6K and phospho-S6K (Ser-389), phospho-Akt (Ser-473), AMPK and phospho-AMPK- $\alpha$  (Thr-172), PARP antibodies provided by Cell Signaling (Beverly, MA). Total RB and cytochrome c antibodies were provided by BD pharmingen. p21, Cyclin A, ERK (p42 and p44) and phospho-ERK (Tyr 204 of p42 and p44) antibodies were obtained by Santa Cruz Biotechnology, (Santa Cruz,

California). Rad51 was purchased from Upstate Biotechnology (Inc, Lake Placid, NY). Decorated proteins were revealed using Horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins (obtained from New England Biolabs, Beverly, MA) visualized by chemiluminescence (ECL: Amersham Biosciences).

#### **Cell Viability Assay:**

DNA content and apoptosis were monitored by FACS analysis on a FACSCalibur (Becton Dickinson) and statistics were determined with Cell Quest software. All cell lines were seeded at a same density during 48h and treated with the drugs during 72h or the indicated time. The cells were trypsinized and fixed in 70% ice cold ethanol during 1h, stained with 50 $\mu$ g/ml propidium iodide for FACS analysis. The percentage of dead cells was determined by the content of cells in pro-G1. The percentage resulted from three independent experiments. The results show the average value and standard deviation resulting from three independent experiments.

#### **Proliferation determination by BrdU incorporation**

Cells were treated with various drugs in fresh medium during the indicated time. 10 $\mu$ M bromodeoxyuridine (BrdU) was added 30 minutes before the end of the treatment and cells were labelled with an anti-BrdU with DNase kit (Becton Dickinson, Pharmingen, San Diego, CA) according to the manufacturers' recommendations and 1 $\mu$ g/ml PI. Combined detection of BrdU incorporation and DNA content using PI was performed using FACS. Proliferating cells are BrdU positive and detected under FL-1 and FL-2 channels (FL = fluorescence). Results show the average value and standard deviation resulting from three independent experiments.

#### **Cell migration assay.**

LN229 cells were plated at 70% confluence in the normal DMEM in 6-well dishes. 48h after seeding, the monolayers were wounded by scoring with a sterile plastic 200 $\mu$ l micropipette tip in the middle of the wells, washed with 1X PBS and fed with normal DMEM or normal DMEM containing 2-DG or RAD001. After 36h, cells were fixed in 70% ice-cold ethanol during 1h at 4 $^{\circ}$ C and photographed using a high and

low-magnification phase-contrast microscope. The extent of migration into the wound area was evaluated qualitatively.

#### **Colony Forming Assay: CFA**

One thousand cells were plated in 6 cm dishes and grown for 72 hours in normal medium. 2-DG or RAD001 were then added for an additional 72h. Cells form colonies of various sizes. Cells were washed in 1X PBS and fixed with ice cold 70% ethanol for 1h. Plates were washed once more and fixed cells were stained with Giemsa for 5 to 20 minutes. Plates were washed with H<sub>2</sub>O until only cell colonies remain colored. The number of colonies was determined using a “colony counter”. Colony size is defined and colonies >50 µm diameter were counted. The histograms show the average value and standard deviation resulting from three independent experiments.

#### **Apoptosis assessment by Annexin-V staining**

LN401 were seeded and cultured for 48h in normal DMEM exposed to 25mM 2-DG and/or 20nM RAD001 during 72h. Dead and viable cells were pooled and stained with 1µg/ml PI and Annexin-V (Annexin V-FITC, BD Pharmingen, San Diego, CA). according to the instructions of the manufacturer. Cells were incubated at room temperature for 15 min and analyzed by flow cytometry. Annexin-V binds to those cells that present phosphatidylserine on the outer layer of the cell membrane, and propidium iodide stains the cellular DNA of those cells with a compromised cell membrane. This allows the discrimination between live cells (unstained with either fluorochrome), apoptotic cells (stained only with Annexin V) and necrotic cells (stained with both Annexin V and Pi). Dead cells are propidium iodide and/or Annexin-V positive.

#### **Measurement of cytochrome *c* release.**

LN229, SF767, Hs683, LN401, U373 and U343 cell lines were cultured in glucose free medium during the indicated times, floating and attached cells harvested by trypsinization were pooled and slowly centrifuged at less than 600 rpm. Cells were gently lysed for 2 minutes in an ice-cold buffer containing: 250 mM sucrose, 1 mM EDTA, 0.1% digitonin, 25 mM Tris, pH 6.8. Lysates were centrifuged for 2 minutes at 12,000g, supernatants and pellets were adjusted on same volumes and mixed with

6xSDS sample buffer before loading on a 15% SDS-PAGE gel. Polypeptides were transferred to nitrocellulose membranes (0.2  $\mu$ m; Schleicher & Schuell, Keene, NH), and cytochrome c was detected by immunoblotting with a monoclonal antibody purchased from Pharmingen, San Diego, CA.

**ATP measurement:**

Hs683 cells cultured in 6-well dishes were treated with 25mM 2DG and 60nM LAQ824. The ATP levels were measured using a luciferase-based assay kit (ATP Bioluminescence Assay Kit CLS II, Roche). Cells were lysed by a cell lysis buffer, incubated at room temperature for 5 min, before the measurement of luciferase activity. For each well, ATP levels were normalized by comparison to the amount of total protein by the Bio-Rad protein assay reagent. The measurement show the average value and standard deviation resulting from three independent experiments.

## ABBREVIATIONS

2-DG: 2-deoxy-D-glucose  
4EBP-1: 4E binding protein-1

AMP: adenosine mono-phosphate  
AMPK: 5' AMP-activated protein kinase  
ARF: p14  
ATP: adenosine tri-phosphate

BBB : blood-brain barrier  
BrdU: bromodeoxy-uridine

CDK: cyclin dependent kinase  
CHX: cycloheximide  
CNS: central nervous system  
CSF: cerebrospinal-fluid

EC<sub>50</sub>: 50% effective concentration  
EGF: epidermal growth factor  
EGFR: epidermal growth factor receptor  
ERK1/2: extracellular regulated-signal kinase 1 and 2

FAK: focal adhesion kinase  
FDG: fluorodeoxyglucose  
FKBP12: Immunophilin FK506 binding protein (FKBP12)

GBM: glioblastoma multiforme  
GIST: gastrointestinal stromal tumor  
GLUT: glucose transporter  
GlcNAc: N-acetylglucosamine

HDM2: human double minute 2  
HDAC: histone deacetylase  
HDIs: histone deacetylase inhibitors  
HIF-1: hypoxia inducible factor-1

IC<sub>50</sub> : concentration where 50% of apoptosis is reached  
IGFR-I: insulin-like growth factor receptor-1  
IGF1: insulin growth factor 1

PDGF: platelet-derived growth factor  
PDGFR: platelet-derived growth factor receptor

MDM2: mouse double minute 2  
mTOR: mammalian target of rapamycin  
NSCLC: non-small-cell lung cancer

O-GlcNAc: O-linked- -N-acetylglucosamine

PDGF: platelet-derived growth factor

PDGFR: platelet-derived growth factor receptor

PET: positron-emission tomography

Pgp: P-glycoprotein

PI3K: phosphatidil inositol (3,4,5) kinase

PI2P: phosphatidylinositol(3,4)-biphosphate

PI3P: phosphatidylinositol(3,4,5)-triphosphate

PKI: protein kinase inhibitor

PTEN: phosphatase tensin homolog

RB: retinoblastoma

RTK: receptor tyrosine kinase

RTKi: receptor tyrosine kinase inhibitor

S6: Small ribosomal protein 6

S6K: Small ribosomal protein 6 kinase

SA: Sodium Azide

SAHA: suberoylanilide hydroxamic acid

SBHA: suberic bishydroxamic acid

SCF: stem cell factor

SiRNA: small interfering RNAs

TGF $\beta$ : tumor necrosis factor

TSC1/2:Tuberous Sclerosis Complex

VEGF: vascular endothelial growth factor

VEGFR: vascular endothelial growth factor receptor

WHO: world health organization

## BIBLIOGRAPHY

- Abi-Saab, W. M., D. G. Maggs, et al. (2002). "Striking differences in glucose and lactate levels between brain extracellular fluid and plasma in conscious human subjects: effects of hyperglycemia and hypoglycemia." J Cereb Blood Flow Metab **22**(3): 271-9.
- Aft, R. L., F. W. Zhang, et al. (2002). "Evaluation of 2-deoxy-D-glucose as a chemotherapeutic agent: mechanism of cell death." Br J Cancer **87**(7): 805-12.
- Alt, K. W., C. Jeunesse, et al. (1997). "Evidence for stone age cranial surgery." Nature **387**(6631): 360.
- Artandi, S. E., S. Chang, et al. (2000). "Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice." Nature **406**(6796): 641-5.
- Arvanitis, D., A. Malliri, et al. (1991). "Ras p21 expression in brain tumors: elevated expression in malignant astrocytomas and glioblastomas multiforme." In Vivo **5**(4): 317-21.
- Ashby, L. S. and W. R. Shapiro (2001). "Intra-arterial cisplatin plus oral etoposide for the treatment of recurrent malignant glioma: a phase II study." J Neurooncol **51**(1): 67-86.
- Atadja, P., L. Gao, et al. (2004). "Selective growth inhibition of tumor cells by a novel histone deacetylase inhibitor, NVP-LAQ824." Cancer Res **64**(2): 689-95.
- Bachoo, R. M., E. A. Maher, et al. (2002). "Epidermal growth factor receptor and Ink4a/Arf: convergent mechanisms governing terminal differentiation and transformation along the neural stem cell to astrocyte axis." Cancer Cell **1**(3): 269-77.
- Bakalova, R., H. Ohba, et al. (2003). "Cross-talk between Bcr-Abl tyrosine kinase, protein kinase C and telomerase-a potential reason for resistance to Glivec in chronic myelogenous leukaemia." Biochem Pharmacol **66**(10): 1879-84.
- Barker, F. G., 2nd, M. L. Simmons, et al. (2001). "EGFR overexpression and radiation response in glioblastoma multiforme." Int J Radiat Oncol Biol Phys **51**(2): 410-8.
- Baylin, S. B. and J. G. Herman (2000). "DNA hypermethylation in tumorigenesis: epigenetics joins genetics." Trends Genet **16**(4): 168-74.
- Beuvink, I., A. Boulay, et al. (2005). "The mTOR inhibitor RAD001 sensitizes tumor cells to DNA-damaged induced apoptosis through inhibition of p21 translation." Cell **120**(6): 747-59.
- Bittar, P. G., Y. Charnay, et al. (1996). "Selective distribution of lactate dehydrogenase isoenzymes in neurons and astrocytes of human brain." J Cereb Blood Flow Metab **16**(6): 1079-89.
- Blagosklonny, M. V., R. Robey, et al. (2002). "Histone deacetylase inhibitors all induce p21 but differentially cause tubulin acetylation, mitotic arrest, and cytotoxicity." Mol Cancer Ther **1**(11): 937-41.
- Blouw, B., H. Song, et al. (2003). "The hypoxic response of tumors is dependent on their microenvironment." Cancer Cell **4**(2): 133-46.

- Boado, R. J., K. L. Black, et al. (1994). "Gene expression of GLUT3 and GLUT1 glucose transporters in human brain tumors." Brain Res Mol Brain Res **27**(1): 51-7.
- Bouck, N., V. Stellmach, et al. (1996). "How tumors become angiogenic." Adv Cancer Res **69**: 135-74.
- Bouzier-Sore, A. K., P. Voisin, et al. (2003). "Lactate is a preferential oxidative energy substrate over glucose for neurons in culture." J Cereb Blood Flow Metab **23**(11): 1298-306.
- Brat, D. J. and E. G. Van Meir (2004). "Vaso-occlusive and prothrombotic mechanisms associated with tumor hypoxia, necrosis, and accelerated growth in glioblastoma." Lab Invest **84**(4): 397-405.
- Brugarolas, J. B., F. Vazquez, et al. (2003). "TSC2 regulates VEGF through mTOR-dependent and -independent pathways." Cancer Cell **4**(2): 147-58.
- Burgess, A., A. Ruefli, et al. (2004). "Histone deacetylase inhibitors specifically kill nonproliferating tumour cells." Oncogene **23**(40): 6693-701.
- Burgess, A. J., S. Pavey, et al. (2001). "Up-regulation of p21(WAF1/CIP1) by histone deacetylase inhibitors reduces their cytotoxicity." Mol Pharmacol **60**(4): 828-37.
- Calastretti, A., A. Bevilacqua, et al. (2001). "Damaged microtubules can inactivate BCL-2 by means of the mTOR kinase." Oncogene **20**(43): 6172-80.
- Capdeville, R., E. Buchdunger, et al. (2002). "Glivec (STI571, imatinib), a rationally developed, targeted anticancer drug." Nat Rev Drug Discov **1**(7): 493-502.
- Castedo, M., K. F. Ferri, et al. (2001). "Human immunodeficiency virus 1 envelope glycoprotein complex-induced apoptosis involves mammalian target of rapamycin/FKBP12-rapamycin-associated protein-mediated p53 phosphorylation." J Exp Med **194**(8): 1097-110.
- Castedo, M., K. F. Ferri, et al. (2002). "Mammalian target of rapamycin (mTOR): pro- and anti-apoptotic." Cell Death Differ **9**(2): 99-100.
- Cavenee, W. K., H. J. Scrabble, et al. (1991). "Molecular genetics of human cancer predisposition and progression." Mutat Res **247**(2): 199-202.
- Cay, O., M. Radnell, et al. (1992). "Inhibitory effect of 2-deoxy-D-glucose on liver tumor growth in rats." Cancer Res **52**(20): 5794-6.
- Chan, S. (2004). "Targeting the mammalian target of rapamycin (mTOR): a new approach to treating cancer." Br J Cancer **91**(8): 1420-4.
- Chang, B. D., K. Watanabe, et al. (2000). "Effects of p21Waf1/Cip1/Sdi1 on cellular gene expression: implications for carcinogenesis, senescence, and age-related diseases." Proc Natl Acad Sci U S A **97**(8): 4291-6.
- Chiquet-Ehrismann, R., E. J. Mackie, et al. (1986). "Tenascin: an extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis." Cell **47**(1): 131-9.
- Chopin, V., R. A. Toillon, et al. (2004). "P21(WAF1/CIP1) is dispensable for G1 arrest, but indispensable for apoptosis induced by sodium butyrate in MCF-7 breast cancer cells." Oncogene **23**(1): 21-9.
- Costello, J. F., M. S. Berger, et al. (1996). "Silencing of p16/CDKN2 expression in human gliomas by methylation and chromatin condensation." Cancer Res **56**(10): 2405-10.
- Cress, W. D. and E. Seto (2000). "Histone deacetylases, transcriptional control, and cancer." J Cell Physiol **184**(1): 1-16.

- Curtiss, F. R. (2002). "Cost-effective use of Cox-2 drugs and NSAIDs." J Manag Care Pharm **8**(4): 295-6.
- Dai, C., J. C. Celestino, et al. (2001). "PDGF autocrine stimulation dedifferentiates cultured astrocytes and induces oligodendrogliomas and oligoastrocytomas from neural progenitors and astrocytes in vivo." Genes Dev **15**(15): 1913-25.
- de Boer, A. G., I. C. van der Sandt, et al. (2003). "The role of drug transporters at the blood-brain barrier." Annu Rev Pharmacol Toxicol **43**: 629-56.
- deGraffenried, L. A., W. E. Friedrichs, et al. (2004). "Inhibition of mTOR activity restores tamoxifen response in breast cancer cells with aberrant Akt Activity." Clin Cancer Res **10**(23): 8059-67.
- Deng, C., P. Zhang, et al. (1995). "Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control." Cell **82**(4): 675-84.
- Dennis, P. B., A. Jaeschke, et al. (2001). "Mammalian TOR: a homeostatic ATP sensor." Science **294**(5544): 1102-5.
- Di Chiro, G., R. A. Brooks, et al. (1984). "Issues in the in vivo measurement of glucose metabolism of human central nervous system tumors." Ann Neurol **15** Suppl: S138-46.
- Dietzmann, A., D. Kanakis, et al. (2003). "Nanomolar concentrations of epothilone D inhibit the proliferation of glioma cells and severely affect their tubulin cytoskeleton." J Neurooncol **65**(2): 99-106.
- Dutcher, J. P. (2004). "Mammalian target of rapamycin inhibition." Clin Cancer Res **10**(18 Pt 2): 6382S-7S.
- Dwarkanath, B. S., F. Zolzer, et al. (2001). "Heterogeneity in 2-deoxy-D-glucose-induced modifications in energetics and radiation responses of human tumor cell lines." Int J Radiat Oncol Biol Phys **50**(4): 1051-61.
- el-Deiry, W. S., T. Tokino, et al. (1993). "WAF1, a potential mediator of p53 tumor suppression." Cell **75**(4): 817-25.
- Eshleman, J. S., B. L. Carlson, et al. (2002). "Inhibition of the mammalian target of rapamycin sensitizes U87 xenografts to fractionated radiation therapy." Cancer Res **62**(24): 7291-7.
- Esteller, M. (2002). "CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future." Oncogene **21**(35): 5427-40.
- Facchetti, F., S. Previdi, et al. (2004). "Modulation of pro- and anti-apoptotic factors in human melanoma cells exposed to histone deacetylase inhibitors." Apoptosis **9**(5): 573-82.
- Fellner, S., B. Bauer, et al. (2002). "Transport of paclitaxel (Taxol) across the blood-brain barrier in vitro and in vivo." J Clin Invest **110**(9): 1309-18.
- Ferrara, F. F., F. Fazi, et al. (2001). "Histone deacetylase-targeted treatment restores retinoic acid signaling and differentiation in acute myeloid leukemia." Cancer Res **61**(1): 2-7.
- Fischer, P. M. and A. Gianella-Borradori (2003). "CDK inhibitors in clinical development for the treatment of cancer." Expert Opin Investig Drugs **12**(6): 955-70.
- Folkman, J. (1996). "Tumor angiogenesis and tissue factor." Nat Med **2**(2): 167-8.
- Fontana, A., S. Bodmer, et al. (1991). "Expression of TGF-beta 2 in human glioblastoma: a role in resistance to immune rejection?" Ciba Found Symp **157**: 232-8; discussion 238-41.

- Fueyo, J., C. Gomez-Manzano, et al. (1998). "Adenovirus-mediated p16 transfer to glioma cells induces G1 arrest and protects from paclitaxel and topotecan: implications for therapy." Int J Oncol **12**(3): 665-9.
- Gallagher, B. M., J. S. Fowler, et al. (1978). "Metabolic trapping as a principle of oradiopharmaceutical design: some factors responsible for the biodistribution of [<sup>18</sup>F] 2-deoxy-2-fluoro-D-glucose." J Nucl Med **19**(10): 1154-61.
- Gatenby, R. A. and R. J. Gillies (2004). "Why do cancers have high aerobic glycolysis?" Nat Rev Cancer **4**(11): 891-9.
- Georger, B., K. Kerr, et al. (2001). "Antitumor activity of the rapamycin analog CCI-779 in human primitive neuroectodermal tumor/medulloblastoma models as single agent and in combination chemotherapy." Cancer Res **61**(4): 1527-32.
- Gervais, J. L., P. Seth, et al. (1998). "Cleavage of CDK inhibitor p21(Cip1/Waf1) by caspases is an early event during DNA damage-induced apoptosis." J Biol Chem **273**(30): 19207-12.
- Goodin, S., M. P. Kane, et al. (2004). "Epothilones: mechanism of action and biologic activity." J Clin Oncol **22**(10): 2015-25.
- Goodnight, S. H. (1996). "Aspirin therapy for cardiovascular disease." Curr Opin Hematol **3**(5): 355-60.
- Gorospe, M., C. Cirielli, et al. (1997). "p21(Waf1/Cip1) protects against p53-mediated apoptosis of human melanoma cells." Oncogene **14**(8): 929-35.
- Goudar, R. K., Q. Shi, et al. (2005). "Combination therapy of inhibitors of epidermal growth factor receptor/vascular endothelial growth factor receptor 2 (AEE788) and the mammalian target of rapamycin (RAD001) offers improved glioblastoma tumor growth inhibition." Mol Cancer Ther **4**(1): 101-12.
- Guba, M., P. von Breitenbuch, et al. (2002). "Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor." Nat Med **8**(2): 128-35.
- Han, I. and J. E. Kudlow (1997). "Reduced O glycosylation of Sp1 is associated with increased proteasome susceptibility." Mol Cell Biol **17**(5): 2550-8.
- Hanahan, D. (2000). "Benefits of bad telomeres." Nature **406**(6796): 573-4.
- Hanahan, D. and J. Folkman (1996). "Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis." Cell **86**(3): 353-64.
- Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.
- Harada, H., J. S. Andersen, et al. (2001). "p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD." Proc Natl Acad Sci U S A **98**(17): 9666-70.
- Harper, J. W., G. R. Adami, et al. (1993). "The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases." Cell **75**(4): 805-16.
- Hassel, B. and A. Brathe (2000). "Cerebral metabolism of lactate in vivo: evidence for neuronal pyruvate carboxylation." J Cereb Blood Flow Metab **20**(2): 327-36.
- Hayflick, L. (1997). "Mortality and immortality at the cellular level. A review." Biochemistry (Mosc) **62**(11): 1180-90.
- Henderson, C., M. Mizzau, et al. (2003). "Role of caspases, Bid, and p53 in the apoptotic response triggered by histone deacetylase inhibitors trichostatin-A (TSA) and suberoylanilide hydroxamic acid (SAHA)." J Biol Chem **278**(14): 12579-89.

- Herman, J. G., F. Latif, et al. (1994). "Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma." Proc Natl Acad Sci U S A **91**(21): 9700-4.
- Herman, J. G., A. Merlo, et al. (1995). "Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers." Cancer Res **55**(20): 4525-30.
- Hiraga, S., T. Ohnishi, et al. (1998). "Telomerase activity and alterations in telomere length in human brain tumors." Cancer Res **58**(10): 2117-25.
- Holland, E. C., J. Celestino, et al. (2000). "Combined activation of Ras and Akt in neural progenitors induces glioblastoma formation in mice." Nat Genet **25**(1): 55-7.
- Holland, E. C., W. P. Hively, et al. (1998). "A constitutively active epidermal growth factor receptor cooperates with disruption of G1 cell-cycle arrest pathways to induce glioma-like lesions in mice." Genes Dev **12**(23): 3675-85.
- Hong, C., A. W. Bollen, et al. (2003). "The contribution of genetic and epigenetic mechanisms to gene silencing in oligodendrogliomas." Cancer Res **63**(22): 7600-5.
- Horman, S., G. Browne, et al. (2002). "Activation of AMP-activated protein kinase leads to the phosphorylation of elongation factor 2 and an inhibition of protein synthesis." Curr Biol **12**(16): 1419-23.
- Huang, S. and P. J. Houghton (2003). "Targeting mTOR signaling for cancer therapy." Curr Opin Pharmacol **3**(4): 371-7.
- Huang, S., L. N. Liu, et al. (2001). "p53/p21(CIP1) cooperate in enforcing rapamycin-induced G(1) arrest and determine the cellular response to rapamycin." Cancer Res **61**(8): 3373-81.
- Hui, A. B., K. W. Lo, et al. (2001). "Detection of multiple gene amplifications in glioblastoma multiforme using array-based comparative genomic hybridization." Lab Invest **81**(5): 717-23.
- Hunter, T. (2000). "Signaling--2000 and beyond." Cell **100**(1): 113-27.
- Hunter, T. and J. Pines (1994). "Cyclins and cancer. II: Cyclin D and CDK inhibitors come of age." Cell **79**(4): 573-82.
- Igney, F. H. and P. H. Krammer (2002). "Death and anti-death: tumour resistance to apoptosis." Nat Rev Cancer **2**(4): 277-88.
- Inoki, K., M. N. Corradetti, et al. (2005). "Dysregulation of the TSC-mTOR pathway in human disease." Nat Genet **37**(1): 19-24.
- Inoki, K., T. Zhu, et al. (2003). "TSC2 mediates cellular energy response to control cell growth and survival." Cell **115**(5): 577-90.
- Insinga, A., S. Monestiroli, et al. (2005). "Inhibitors of histone deacetylases induce tumor-selective apoptosis through activation of the death receptor pathway." Nat Med **11**(1): 71-6.
- Ishii, N., D. Maier, et al. (1999). "Frequent co-alterations of TP53, p16/CDKN2A, p14ARF, PTEN tumor suppressor genes in human glioma cell lines." Brain Pathol **9**(3): 469-79.
- Jackson, R. J., J. Adnane, et al. (2002). "Loss of the cell cycle inhibitors p21(Cip1) and p27(Kip1) enhances tumorigenesis in knockout mouse models." Oncogene **21**(55): 8486-97.
- Jackson, S. P. and R. Tjian (1988). "O-glycosylation of eukaryotic transcription factors: implications for mechanisms of transcriptional regulation." Cell **55**(1): 125-33.

- Jang, E. R., S. J. Lim, et al. (2004). "The histone deacetylase inhibitor trichostatin A sensitizes estrogen receptor alpha-negative breast cancer cells to tamoxifen." Oncogene **23**(9): 1724-36.
- Johnstone, R. W. (2002). "Histone-deacetylase inhibitors: novel drugs for the treatment of cancer." Nat Rev Drug Discov **1**(4): 287-99.
- Joki, T., O. Heese, et al. (2000). "Expression of cyclooxygenase 2 (COX-2) in human glioma and in vitro inhibition by a specific COX-2 inhibitor, NS-398." Cancer Res **60**(17): 4926-31.
- Jones, G., J. Machado, Jr., et al. (2001). "Loss of focal adhesion kinase (FAK) inhibits epidermal growth factor receptor-dependent migration and induces aggregation of nh(2)-terminal FAK in the nuclei of apoptotic glioblastoma cells." Cancer Res **61**(13): 4978-81.
- Jones, H. E., L. Goddard, et al. (2004). "Insulin-like growth factor-I receptor signalling and acquired resistance to gefitinib (ZD1839; Iressa) in human breast and prostate cancer cells." Endocr Relat Cancer **11**(4): 793-814.
- Jones, M. K., H. Wang, et al. (1999). "Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: insight into mechanisms and implications for cancer growth and ulcer healing." Nat Med **5**(12): 1418-23.
- Jones, P. A. and P. W. Laird (1999). "Cancer epigenetics comes of age." Nat Genet **21**(2): 163-7.
- Jung, J. M., J. M. Bruner, et al. (1995). "Increased levels of p21WAF1/Cip1 in human brain tumors." Oncogene **11**(10): 2021-8.
- Kang, H. T., J. W. Ju, et al. (2003). "Down-regulation of Sp1 activity through modulation of O-glycosylation by treatment with a low glucose mimetic, 2-deoxyglucose." J Biol Chem **278**(51): 51223-31.
- Kaplan, O., G. Navon, et al. (1990). "Effects of 2-deoxyglucose on drug-sensitive and drug-resistant human breast cancer cells: toxicity and magnetic resonance spectroscopy studies of metabolism." Cancer Res **50**(3): 544-51.
- Kastan, M. B. and J. Bartek (2004). "Cell-cycle checkpoints and cancer." Nature **432**(7015): 316-23.
- Kaur, B., F. W. Khwaja, et al. (2005). "Hypoxia and the hypoxia-inducible-factor pathway in glioma growth and angiogenesis." Neuro-oncol **7**(2): 134-53.
- Kim, M. S., M. Blake, et al. (2003). "Inhibition of histone deacetylase increases cytotoxicity to anticancer drugs targeting DNA." Cancer Res **63**(21): 7291-300.
- Kinzler, K. W. and B. Vogelstein (1996). "Lessons from hereditary colorectal cancer." Cell **87**(2): 159-70.
- Kinzler, K. W. and B. Vogelstein (1997). "Cancer-susceptibility genes. Gatekeepers and caretakers." Nature **386**(6627): 761, 763.
- Klionsky, D. J. and S. D. Emr (2000). "Autophagy as a regulated pathway of cellular degradation." Science **290**(5497): 1717-21.
- Knobbe, C. B., A. Merlo, et al. (2002). "Pten signaling in gliomas." Neuro-oncol **4**(3): 196-211.
- Knobbe, C. B., J. Reifenberger, et al. (2004). "Hypermethylation and transcriptional downregulation of the carboxyl-terminal modulator protein gene in glioblastomas." J Natl Cancer Inst **96**(6): 483-6.
- Knudson, A. G. (1996). "Hereditary cancer: two hits revisited." J Cancer Res Clin Oncol **122**(3): 135-40.

- Knudson, A. G. (2001). "Two genetic hits (more or less) to cancer." Nat Rev Cancer **1**(2): 157-62.
- Knudson, A. G., Jr., H. W. Hethcote, et al. (1975). "Mutation and childhood cancer: a probabilistic model for the incidence of retinoblastoma." Proc Natl Acad Sci U S A **72**(12): 5116-20.
- Komata, T., T. Kanzawa, et al. (2005). "Histone deacetylase inhibitors, N-butyric acid and trichostatin A, induce caspase-8- but not caspase-9-dependent apoptosis in human malignant glioma cells." Int J Oncol **26**(5): 1345-52.
- Kowalski, R. J., P. Giannakakou, et al. (1997). "Activities of the microtubule-stabilizing agents epothilones A and B with purified tubulin and in cells resistant to paclitaxel (Taxol(R))." J Biol Chem **272**(4): 2534-41.
- Krishnan, S., R. D. Rao, et al. (2003). "Combination of epidermal growth factor receptor targeted therapy with radiation therapy for malignant gliomas." Front Biosci **8**: e1-13.
- Labuhn, M., G. Jones, et al. (2001). "Quantitative real-time PCR does not show selective targeting of p14(ARF) but concomitant inactivation of both p16(INK4A) and p14(ARF) in 105 human primary gliomas." Oncogene **20**(9): 1103-9.
- Laine, L., L. G. Connors, et al. (2003). "Serious lower gastrointestinal clinical events with nonselective NSAID or coxib use." Gastroenterology **124**(2): 288-92.
- Lane, D. P. and P. M. Fischer (2004). "Turning the key on p53." Nature **427**(6977): 789-90.
- Lesniak, M. S., R. Langer, et al. (2001). "Drug delivery to tumors of the central nervous system." Curr Neurol Neurosci Rep **1**(3): 210-6.
- Levine, A. J., C. A. Finlay, et al. (2004). "P53 is a tumor suppressor gene." Cell **116**(2 Suppl): S67-9, 1 p following S69.
- Li, J., C. Yen, et al. (1997). "PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer." Science **275**(5308): 1943-7.
- Lokker, N. A., C. M. Sullivan, et al. (2002). "Platelet-derived growth factor (PDGF) autocrine signaling regulates survival and mitogenic pathways in glioblastoma cells: evidence that the novel PDGF-C and PDGF-D ligands may play a role in the development of brain tumors." Cancer Res **62**(13): 3729-35.
- Lund, A. H. and M. van Lohuizen (2004). "Epigenetics and cancer." Genes Dev **18**(19): 2315-35.
- Ly, C., A. F. Arechiga, et al. (2003). "Bcr-Abl kinase modulates the translation regulators ribosomal protein S6 and 4E-BP1 in chronic myelogenous leukemia cells via the mammalian target of rapamycin." Cancer Res **63**(18): 5716-22.
- MacLachlan, T. K., N. Sang, et al. (1995). "Cyclins, cyclin-dependent kinases and cdk inhibitors: implications in cell cycle control and cancer." Crit Rev Eukaryot Gene Expr **5**(2): 127-56.
- Maher, E. A., F. B. Furnari, et al. (2001). "Malignant glioma: genetics and biology of a grave matter." Genes Dev **15**(11): 1311-33.
- Maier, D., D. Comparone, et al. (1997). "New deletion in low-grade oligodendroglioma at the glioblastoma suppressor locus on chromosome 10q25-26." Oncogene **15**(8): 997-1000.
- Maier, D., G. Jones, et al. (1999). "The PTEN lipid phosphatase domain is not required to inhibit invasion of glioma cells." Cancer Res **59**(21): 5479-82.

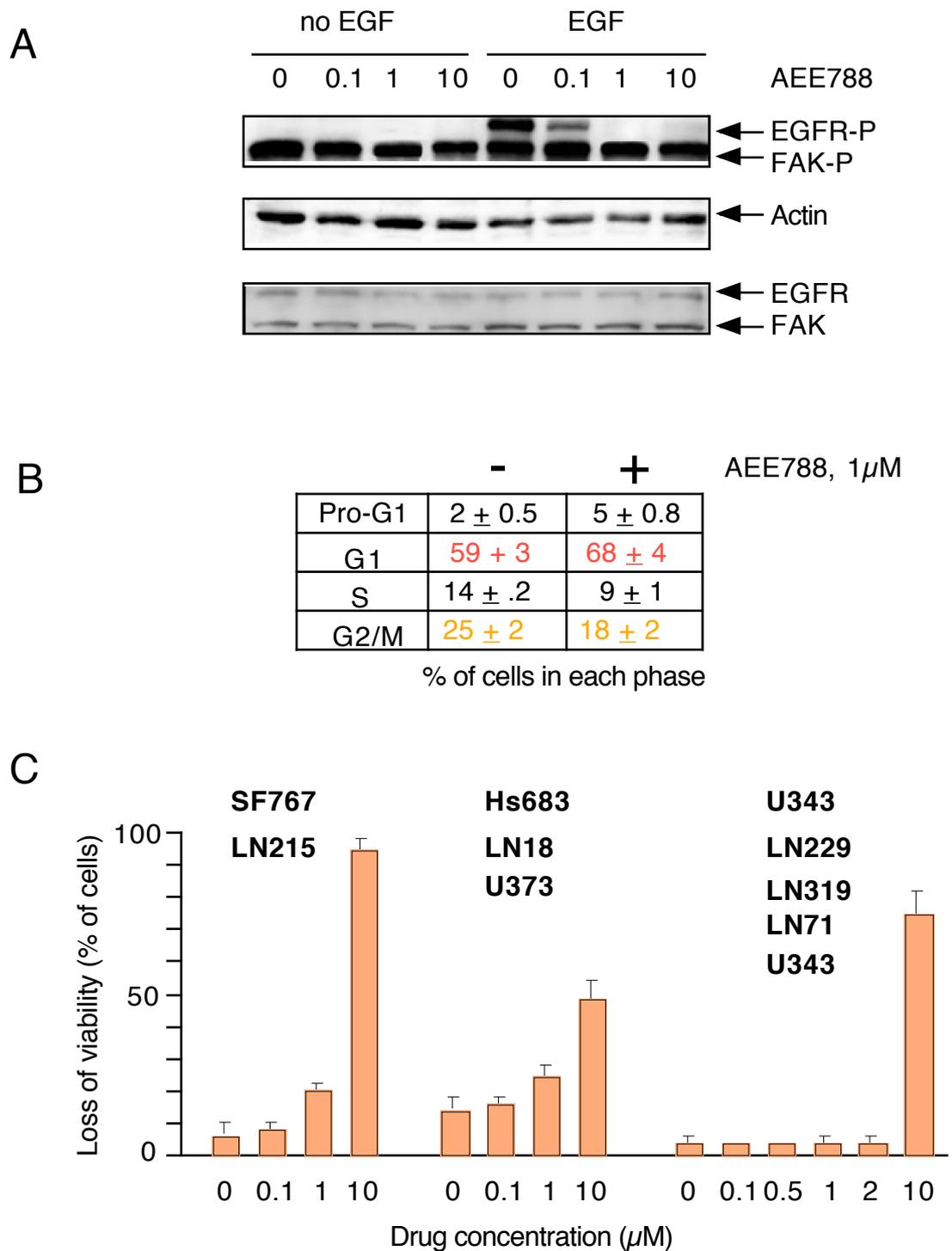
- Maier, D., Z. Zhang, et al. (1998). "Somatic deletion mapping on chromosome 10 and sequence analysis of PTEN/MMAC1 point to the 10q25-26 region as the primary target in low-grade and high-grade gliomas." Oncogene **16**(25): 3331-5.
- Maira, S. M., I. Galetic, et al. (2001). "Carboxyl-terminal modulator protein (CTMP), a negative regulator of PKB/Akt and v-Akt at the plasma membrane." Science **294**(5541): 374-80.
- Manley, P. W., W. Breitenstein, et al. (2004). "Urea derivatives of STI571 as inhibitors of Bcr-Abl and PDGFR kinases." Bioorg Med Chem Lett **14**(23): 5793-7.
- Maschek, G., N. Savaraj, et al. (2004). "2-deoxy-D-glucose increases the efficacy of adriamycin and paclitaxel in human osteosarcoma and non-small cell lung cancers in vivo." Cancer Res **64**(1): 31-4.
- McLaughlin, F. and N. B. La Thangue (2004). "Histone deacetylase inhibitors open new doors in cancer therapy." Biochem Pharmacol **68**(6): 1139-44.
- Merlo, A. (2003). "Genes and pathways driving glioblastomas in humans and murine disease models." Neurosurg Rev **26**(3): 145-58.
- Merlo, A. and B. Bettler (2004). "Glioblastomas on the move." Sci STKE **2004**(229): pe18.
- Merlo, A., J. G. Herman, et al. (1995). "5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers." Nat Med **1**(7): 686-92.
- Mohanti, B. K., G. K. Rath, et al. (1996). "Improving cancer radiotherapy with 2-deoxy-D-glucose: phase I/II clinical trials on human cerebral gliomas." Int J Radiat Oncol Biol Phys **35**(1): 103-11.
- Molinari, M. (2000). "Cell cycle checkpoints and their inactivation in human cancer." Cell Prolif **33**(5): 261-74.
- Momparler, R. L. (2003). "Cancer epigenetics." Oncogene **22**(42): 6479-83.
- Mondesire, W. H., W. Jian, et al. (2004). "Targeting mammalian target of rapamycin synergistically enhances chemotherapy-induced cytotoxicity in breast cancer cells." Clin Cancer Res **10**(20): 7031-42.
- Monneret, C. (2005). "Histone deacetylase inhibitors." Eur J Med Chem **40**(1): 1-13.
- Muzi, M., S. D. Freeman, et al. (2001). "Kinetic characterization of hexokinase isoenzymes from glioma cells: implications for FDG imaging of human brain tumors." Nucl Med Biol **28**(2): 107-16.
- Nagane, M., H. J. Huang, et al. (1999). "Causes of drug resistance and novel therapeutic opportunities for the treatment of glioblastoma." Drug Resist Updat **2**(1): 30-37.
- Nakatani, K., N. Yoshimi, et al. (1997). "The significant role of telomerase activity in human brain tumors." Cancer **80**(3): 471-6.
- Nathoo, N., S. Goldlust, et al. (2004). "Epidermal growth factor receptor antagonists: novel therapy for the treatment of high-grade gliomas." Neurosurgery **54**(6): 1480-8; discussion 1488-9.
- Nebbio, A., N. Clarke, et al. (2005). "Tumor-selective action of HDAC inhibitors involves TRAIL induction in acute myeloid leukemia cells." Nat Med **11**(1): 77-84.
- Nelson, C. A., J. Q. Wang, et al. (1996). "The interaction among glucose transport, hexokinase, and glucose-6-phosphatase with respect to 3H-2-deoxyglucose retention in murine tumor models." Nucl Med Biol **23**(4): 533-41.

- New, P. (2004). "Cyclooxygenase in the treatment of glioma: its complex role in signal transduction." Cancer Control **11**(3): 152-64.
- Nimmanapalli, R., L. Fuino, et al. (2003). "Histone deacetylase inhibitor LAQ824 both lowers expression and promotes proteasomal degradation of Bcr-Abl and induces apoptosis of imatinib mesylate-sensitive or -refractory chronic myelogenous leukemia-blast crisis cells." Cancer Res **63**(16): 5126-35.
- Nimmanapalli, R., L. Fuino, et al. (2003). "Cotreatment with the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) enhances imatinib-induced apoptosis of Bcr-Abl-positive human acute leukemia cells." Blood **101**(8): 3236-9.
- Noble, M. and J. Dietrich (2004). "The complex identity of brain tumors: emerging concerns regarding origin, diversity and plasticity." Trends Neurosci **27**(3): 148-54.
- Novak, L., P. Molnar, et al. (2005). "Does increased 18FDG uptake reflect malignant transformation of a low-grade glioma? A diagnostic dilemma." Neurol India **53**(1): 112-4.
- Nowell, P. C. (1976). "The clonal evolution of tumor cell populations." Science **194**(4260): 23-8.
- O'Reilly, T., M. Wartmann, et al. (2004). "Patupilone (epothilone B, EPO906) and imatinib (STI571, Glivec) in combination display enhanced antitumour activity in vivo against experimental rat C6 glioma." Cancer Chemother Pharmacol.
- Ochs, J. S. (2004). "Rationale and clinical basis for combining gefitinib (IRESSA, ZD1839) with radiation therapy for solid tumors." Int J Radiat Oncol Biol Phys **58**(3): 941-9.
- Ohgaki, H., P. Dessen, et al. (2004). "Genetic pathways to glioblastoma: a population-based study." Cancer Res **64**(19): 6892-9.
- Orian, J. M., K. Vasilopoulos, et al. (1992). "Overexpression of multiple oncogenes related to histological grade of astrocytic glioma." Br J Cancer **66**(1): 106-12.
- Pardridge, W. M. (2003). "Molecular biology of the blood-brain barrier." Methods Mol Med **89**: 385-99.
- Park, J. K., S. H. Lee, et al. (2004). "Synergistic interaction between gefitinib (Iressa, ZD1839) and paclitaxel against human gastric carcinoma cells." Anticancer Drugs **15**(8): 809-18.
- Pellerin, L. and P. J. Magistretti (1994). "Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization." Proc Natl Acad Sci U S A **91**(22): 10625-9.
- Plumb, J. A., N. Steele, et al. (2004). "Epigenetic approaches to cancer therapy." Biochem Soc Trans **32**(Pt 6): 1095-7.
- Rahmani, M., C. Yu, et al. (2003). "Inhibition of PI-3 kinase sensitizes human leukemic cells to histone deacetylase inhibitor-mediated apoptosis through p44/42 MAP kinase inactivation and abrogation of p21(CIP1/WAF1) induction rather than AKT inhibition." Oncogene **22**(40): 6231-42.
- Reifenberger, G. and V. P. Collins (2004). "Pathology and molecular genetics of astrocytic gliomas." J Mol Med **82**(10): 656-70.
- Rich, J. N. and D. D. Bigner (2004). "Development of novel targeted therapies in the treatment of malignant glioma." Nat Rev Drug Discov **3**(5): 430-46.

- Richon, V. M., T. W. Sandhoff, et al. (2000). "Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation." Proc Natl Acad Sci U S A **97**(18): 10014-9.
- Russell, J. S., K. Brady, et al. (2003). "Gleevec-mediated inhibition of Rad51 expression and enhancement of tumor cell radiosensitivity." Cancer Res **63**(21): 7377-83.
- Samuels, Y., Z. Wang, et al. (2004). "High frequency of mutations of the PIK3CA gene in human cancers." Science **304**(5670): 554.
- Sansal, I. and W. R. Sellers (2004). "The biology and clinical relevance of the PTEN tumor suppressor pathway." J Clin Oncol **22**(14): 2954-63.
- Sawyers, C. L. (2003). "Will mTOR inhibitors make it as cancer drugs?" Cancer Cell **4**(5): 343-8.
- Schafer, D., B. Hamm-Kunzelmann, et al. (1997). "Glucose regulates the promoter activity of aldolase A and pyruvate kinase M2 via dephosphorylation of Sp1." FEBS Lett **417**(3): 325-8.
- Schmelzle, T. and M. N. Hall (2000). "TOR, a central controller of cell growth." Cell **103**(2): 253-62.
- Schmidt, M. and Z. Fan (2001). "Protection against chemotherapy-induced cytotoxicity by cyclin-dependent kinase inhibitors (CKI) in CKI-responsive cells compared with CKI-unresponsive cells." Oncogene **20**(43): 6164-71.
- Schuna, A. A. (1998). "Update on treatment of rheumatoid arthritis." J Am Pharm Assoc (Wash) **38**(6): 728-35; quiz 735-7.
- Semenza, G. L. (2003). "Targeting HIF-1 for cancer therapy." Nat Rev Cancer **3**(10): 721-32.
- Shaw, R. J., N. Bardeesy, et al. (2004). "The LKB1 tumor suppressor negatively regulates mTOR signaling." Cancer Cell **6**(1): 91-9.
- Sheng, H., J. Shao, et al. (1997). "Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2." J Clin Invest **99**(9): 2254-9.
- Shigemitsu, K., Y. Tsujishita, et al. (1999). "Regulation of translational effectors by amino acid and mammalian target of rapamycin signaling pathways. Possible involvement of autophagy in cultured hepatoma cells." J Biol Chem **274**(2): 1058-65.
- Shim, H., Y. S. Chun, et al. (1998). "A unique glucose-dependent apoptotic pathway induced by c-Myc." Proc Natl Acad Sci U S A **95**(4): 1511-6.
- Shiraishi, S., K. Tada, et al. (2002). "Influence of p53 mutations on prognosis of patients with glioblastoma." Cancer **95**(2): 249-57.
- Sloane, P. D. (1998). "Advances in the treatment of Alzheimer's disease." Am Fam Physician **58**(7): 1577-86, 1589-90.
- Smith, T. A. (1999). "Facilitative glucose transporter expression in human cancer tissue." Br J Biomed Sci **56**(4): 285-92.
- Stupp, R., W. P. Mason, et al. (2005). "Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma." N Engl J Med **352**(10): 987-96.
- Suzuki, A., Y. Tsutomi, et al. (1999). "Caspase 3 inactivation to suppress Fas-mediated apoptosis: identification of binding domain with p21 and ILP and inactivation machinery by p21." Oncogene **18**(5): 1239-44.
- Suzuki, A., Y. Tsutomi, et al. (1999). "Mitochondrial regulation of cell death: mitochondria are essential for procaspase 3-p21 complex formation to resist Fas-mediated cell death." Mol Cell Biol **19**(5): 3842-7.

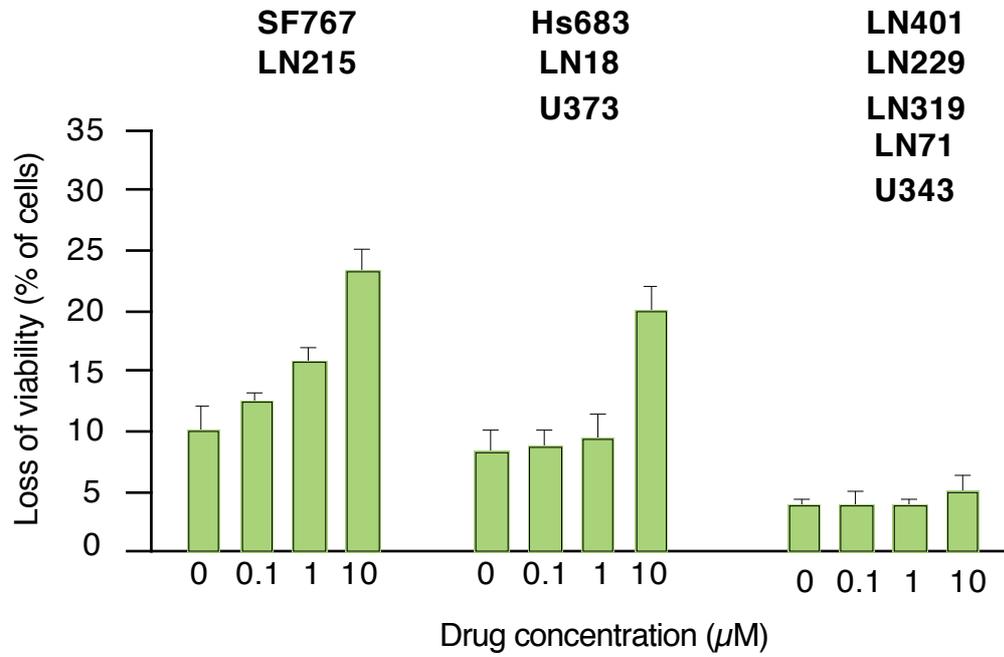
- Suzuki, T., Y. Nagano, et al. (2005). "Novel inhibitors of human histone deacetylases: design, synthesis, enzyme inhibition, and cancer cell growth inhibition of SAHA-based non-hydroxamates." *J Med Chem* **48**(4): 1019-32.
- Szyf, M., P. Pakneshan, et al. (2004). "DNA demethylation and cancer: therapeutic implications." *Cancer Lett* **211**(2): 133-43.
- Takeuchi, H., Y. Kondo, et al. (2005). "Synergistic augmentation of rapamycin-induced autophagy in malignant glioma cells by phosphatidylinositol 3-kinase/protein kinase B inhibitors." *Cancer Res* **65**(8): 3336-46.
- Traxler, P., P. R. Allegrini, et al. (2004). "AEE788: a dual family epidermal growth factor receptor/ErbB2 and vascular endothelial growth factor receptor tyrosine kinase inhibitor with antitumor and antiangiogenic activity." *Cancer Res* **64**(14): 4931-41.
- Tsumanuma, I., R. Tanaka, et al. (1997). "Infrequent mutation of Waf1/p21 gene, a CDK inhibitor gene, in brain tumors." *Neurol Med Chir (Tokyo)* **37**(2): 150-6; discussion 156-7.
- Van de Castele, M., B. A. Kefas, et al. (2003). "Prolonged culture in low glucose induces apoptosis of rat pancreatic beta-cells through induction of c-myc." *Biochem Biophys Res Commun* **312**(4): 937-44.
- Van Meir, E. (1996). "Hypoxia-mediated selection of cells with diminished apoptotic potential to solid tumours." *Neurosurgery* **39**(4): 878-9.
- Van Meir, E. G., K. Roemer, et al. (1995). "Single cell monitoring of growth arrest and morphological changes induced by transfer of wild-type p53 alleles to glioblastoma cells." *Proc Natl Acad Sci U S A* **92**(4): 1008-12.
- Vivanco, I. and C. L. Sawyers (2002). "The phosphatidylinositol 3-Kinase AKT pathway in human cancer." *Nat Rev Cancer* **2**(7): 489-501.
- Vogelstein, B., D. Lane, et al. (2000). "Surfing the p53 network." *Nature* **408**(6810): 307-10.
- von Bubnoff, N., C. Peschel, et al. (2003). "Resistance of Philadelphia-chromosome positive leukemia towards the kinase inhibitor imatinib (STI571, Glivec): a targeted oncoprotein strikes back." *Leukemia* **17**(5): 829-38.
- Wahl, R. L., R. Cody, et al. (1991). "Positron-emission tomographic scanning of primary and metastatic breast carcinoma with the radiolabeled glucose analogue 2-deoxy-2-[18F]fluoro-D-glucose." *N Engl J Med* **324**(3): 200.
- Waldman, T., C. Lengauer, et al. (1996). "Uncoupling of S phase and mitosis induced by anticancer agents in cells lacking p21." *Nature* **381**(6584): 713-6.
- Warburg, O. (1956). "On the origin of cancer cells." *Science* **123**(3191): 309-14.
- Watanabe, H., K. Fukuchi, et al. (1995). "Molecular analysis of the Cip1/Waf1 (p21) gene in diverse types of human tumors." *Biochim Biophys Acta* **1263**(3): 275-80.
- Wechsler-Reya, R. and M. P. Scott (2001). "The developmental biology of brain tumors." *Annu Rev Neurosci* **24**: 385-428.
- Weisberg, E., L. Catley, et al. (2004). "Histone deacetylase inhibitor NVP-LAQ824 has significant activity against myeloid leukemia cells in vitro and in vivo." *Leukemia* **18**(12): 1951-63.
- Weisberg, E. and J. D. Griffin (2003). "Resistance to imatinib (Glivec): update on clinical mechanisms." *Drug Resist Updat* **6**(5): 231-8.
- Wick, A. N., D. R. Drury, et al. (1957). "Localization of the primary metabolic block produced by 2-deoxyglucose." *J Biol Chem* **224**(2): 963-9.

- Xiao, A., H. Wu, et al. (2002). "Astrocyte inactivation of the pRb pathway predisposes mice to malignant astrocytoma development that is accelerated by PTEN mutation." Cancer Cell **1**(2): 157-68.
- Yamamoto, T., Y. Seino, et al. (1990). "Over-expression of facilitative glucose transporter genes in human cancer." Biochem Biophys Res Commun **170**(1): 223-30.
- Yu, C., M. Rahmani, et al. (2003). "Histone deacetylase inhibitors promote STI571-mediated apoptosis in STI571-sensitive and -resistant Bcr/Abl+ human myeloid leukemia cells." Cancer Res **63**(9): 2118-26.
- Zagzag, D., D. R. Friedlander, et al. (1995). "Tenascin expression in astrocytomas correlates with angiogenesis." Cancer Res **55**(4): 907-14.
- Zhou, X. M., B. C. Wong, et al. (2001). "Non-steroidal anti-inflammatory drugs induce apoptosis in gastric cancer cells through up-regulation of bax and bak." Carcinogenesis **22**(9): 1393-7.
- Zhu, Y. and L. F. Parada (2002). "The molecular and genetic basis of neurological tumours." Nat Rev Cancer **2**(8): 616-26.



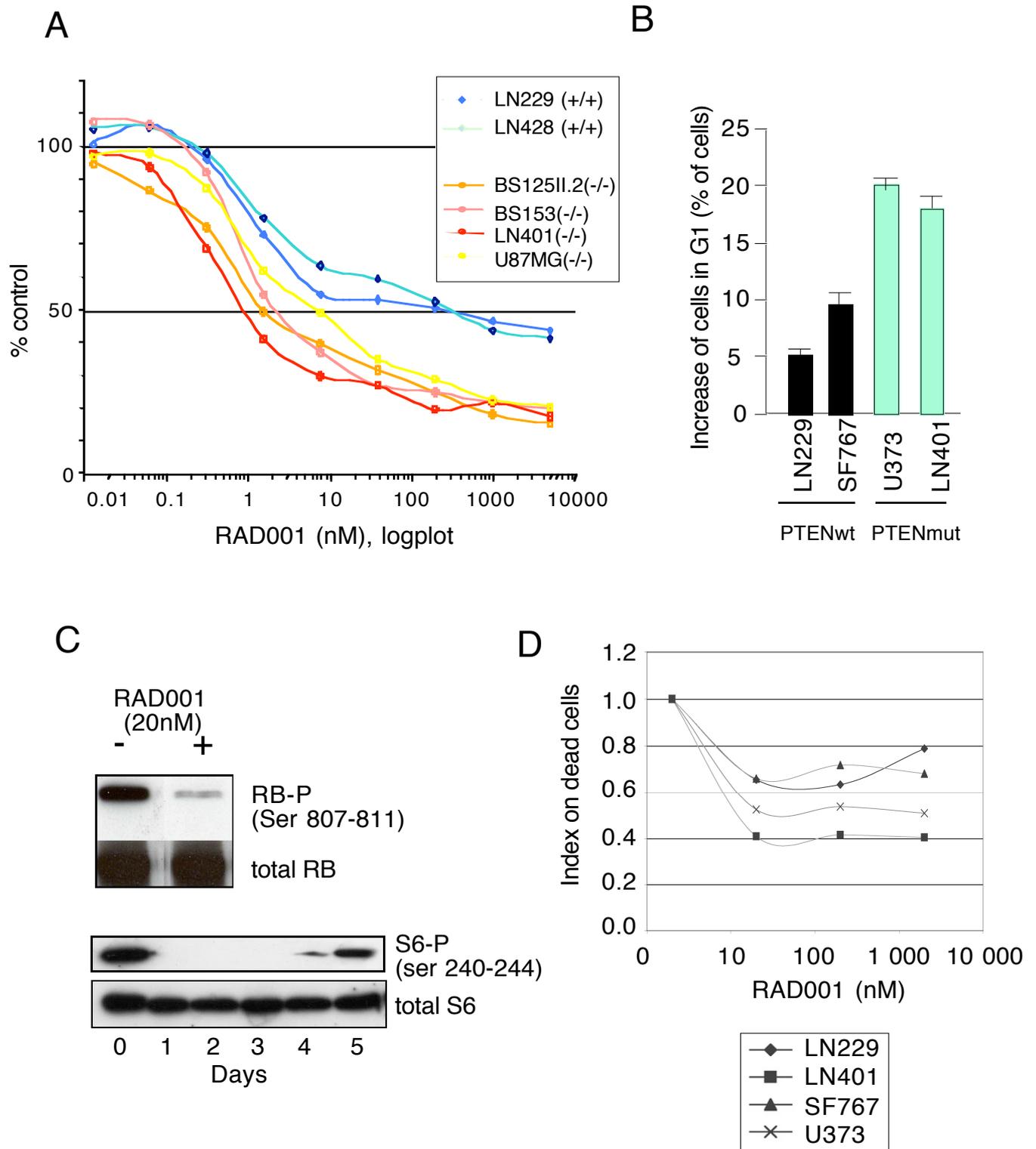
**Figure 1: AEE788 inhibits EGF-dependent activation of EGFR and induces apoptosis in human glioma cell lines**

(A) Western analysis using various antibodies described in Materials and Methods. Effect of AEE788 on EGFR and FAK phosphorylation in LN229 human glioma cell line. EGF was added for 10 minutes (B) FACS analysis: effect of AEE788 on cell cycle profile, after 36h incubation with 1 $\mu$ M AEE788, repartition of cells in each phase G1 or G2/M of the cell cycle. (C) AEE788 titration: effect of AEE788 on the survival of human glioma cell lines, after 4 days treatment. Cell lines were grouped according to their response to the drug.



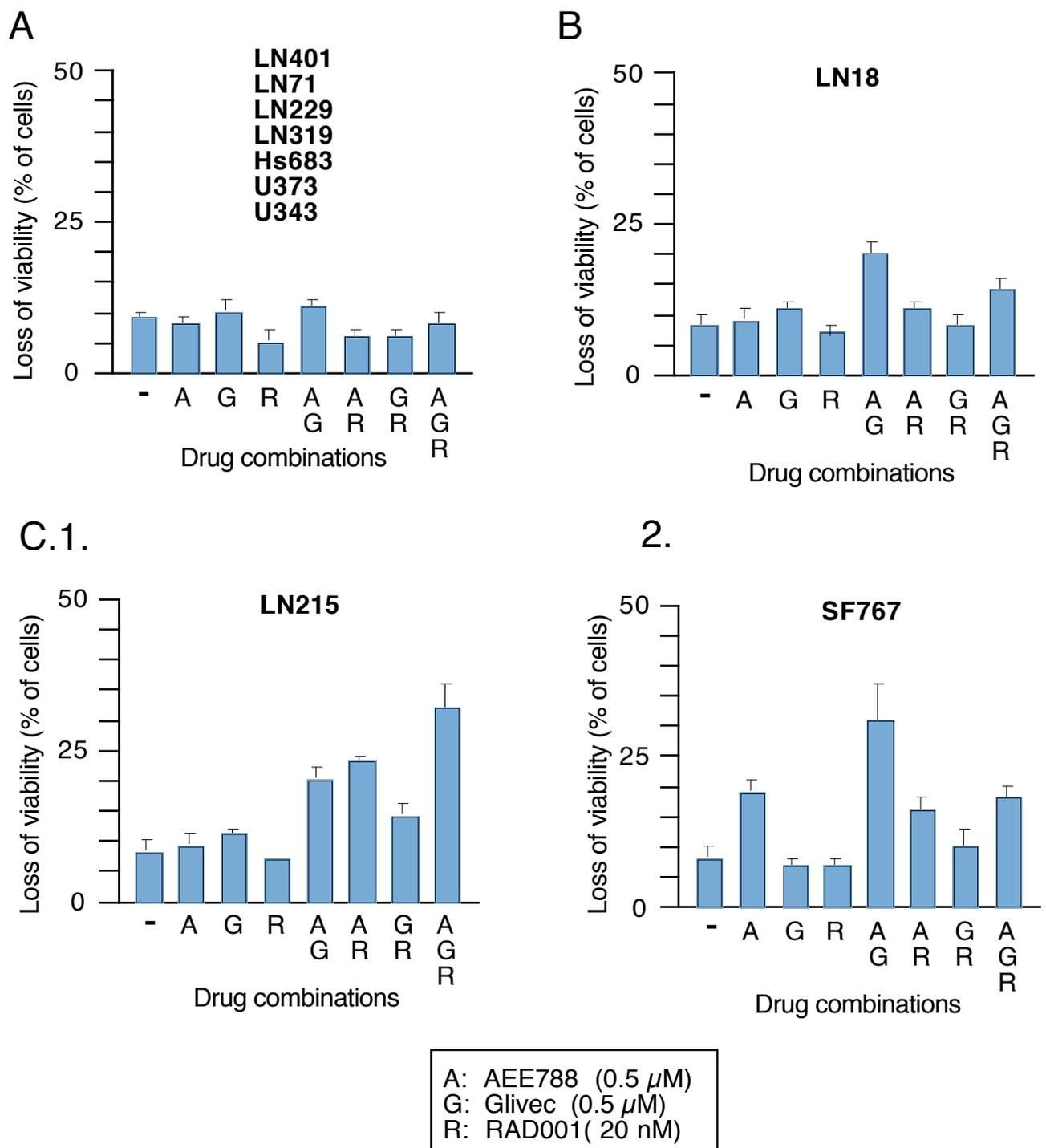
**Figure 2. Gleevec induces weakly apoptosis in human glioma cell lines.**

Glioma cell lines were treated for 4 days with increasing doses of glivec then cell survival was measured by FACS as described in Materials and Methods



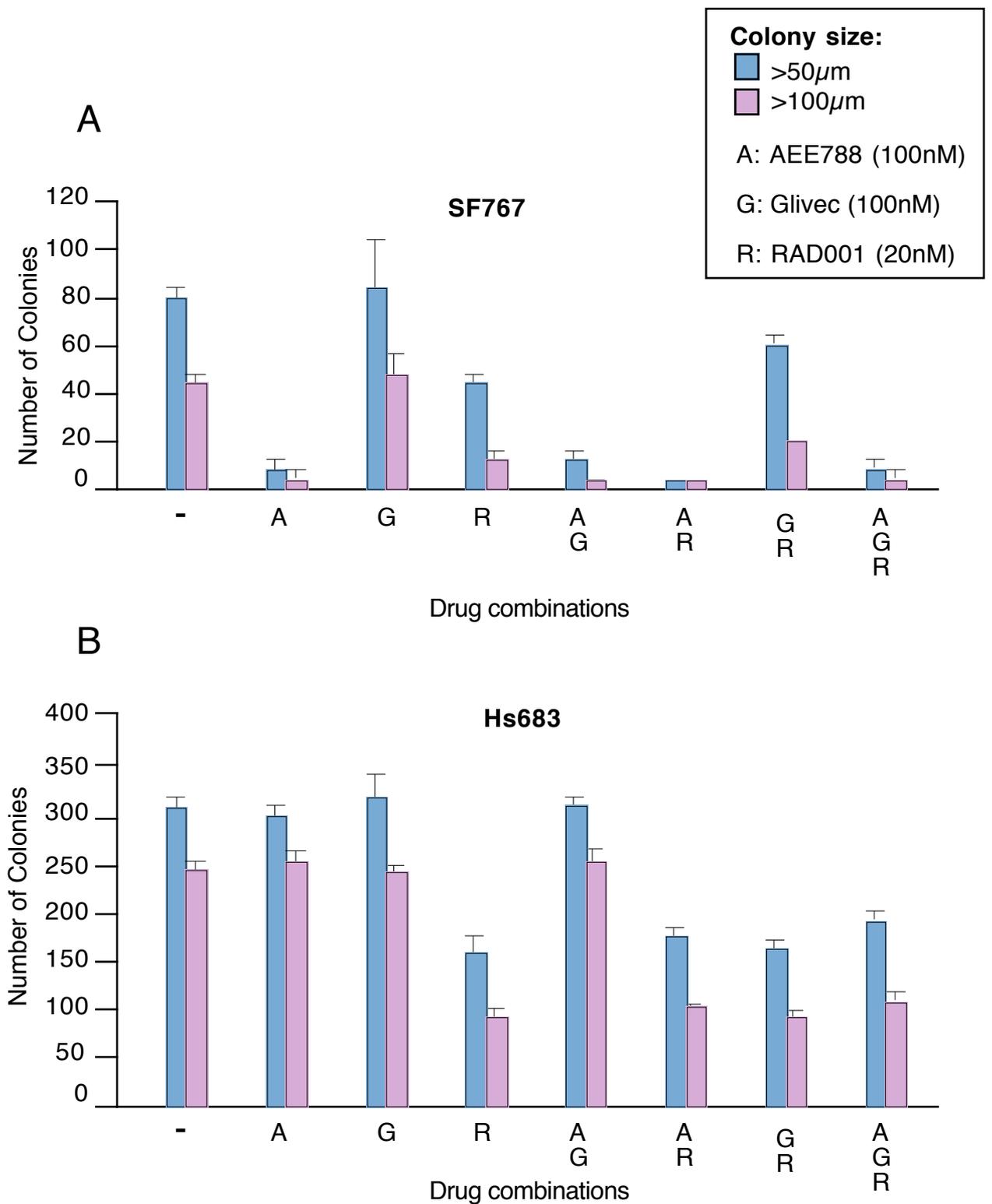
**Figure 3 : RAD001 has only a cytostatic effect on human glioma cell lines.**

(A) RAD001 IC50 titration: effect of RAD001 on the proliferation of PTENwt and PTEN mutant human glioma cell lines. (data from Heidi Lane, Novartis, Basel). (B) cytostatic effect: four glioma cell lines were treated with 20nM RAD001 for 72h, G1 accumulation of cells in the presence of the drug was compared with the % of cells in G1 under untreated condition. (C) LN229 cell lines were treated with RAD001 for 72h or 5days and western analysis was performed to determine phosphorylation and total protein levels of RB and S6 (D) Effect of increasing doses of RAD001 (20, 200, 2000) on survival of human glioma cell lines (LN401, LN229, U373, SF767) after 5 days exposure to the drug. Apoptosis was determined by FACS after PI staining. The initial basal levels of apoptosis were set to 1.



**Figure 4. Combinatorial effects of specific inhibitors: RAD001, AEE788 and Glivec on human glioma cell survival**

All drugs were added simultaneously for 4 days and cell survival was determined by FACS as described in Materials and Methods. **(A)** Response type 1: Resistance to apoptosis, RAD001 reduces basal levels of apoptosis. **(B)** Response type 2: Weak cell death induced by AEE788 and Glivec. **(C)** Response type 3: AEE788 and/or Glivec induce apoptosis. RAD001 enhances apoptosis induced by AEE788 in LN215 (1) or reduces the cytotoxic effect induced by AEE788 and Glivec in SF767 (2).



**Figure 5. Cytostatic effect by drug combinations of RAD001, AEE788 and Glivec on human glioma cell growth.**

SF767 (A) and Hs683 (B) cell lines were treated by the indicated drug combinations performing a colony formation assay (CFA) in which number of colonies were determined after 72h. Details of the CFA are described under Materials and Methods

Type 1										Type 2			Type 3	
	LN71	LN229	LN319	LN401	Hs683	U343	U373	LN18	SF767	LN215				
genetic background	PTEN mut	WT	mut	mut	WT	mut	mut	WT	WT	mut	WT	WT	WT	mut
	p53 mut	mut	mut	mut	mut	WT	mut	WT	WT	mut	WT	WT	WT	mut
	p16/p14 del	del	WT	del	del	del	WT	del	WT	WT	WT	WT	WT	WT
Single drug	AEE788 >1	10	>1	>1	>1	>1	>1	>0.5	0.1	>0.5				
	Glivec >1	10	10	10	>1	>1	>1	>1	>0.5	>0.5				
	RAD001 -	-	-	-	-	-	-	-	-	-				-
Combination	A + G -	-	-	-	-	-	-	+	++	++				++
	A + R -	-	-	-	-	-	-	-	++	++				++
	R + G -	-	-	-	-	-	-	-	-	-				+
	A+R+G -	-	-	-	-	-	-	-	++	++				++

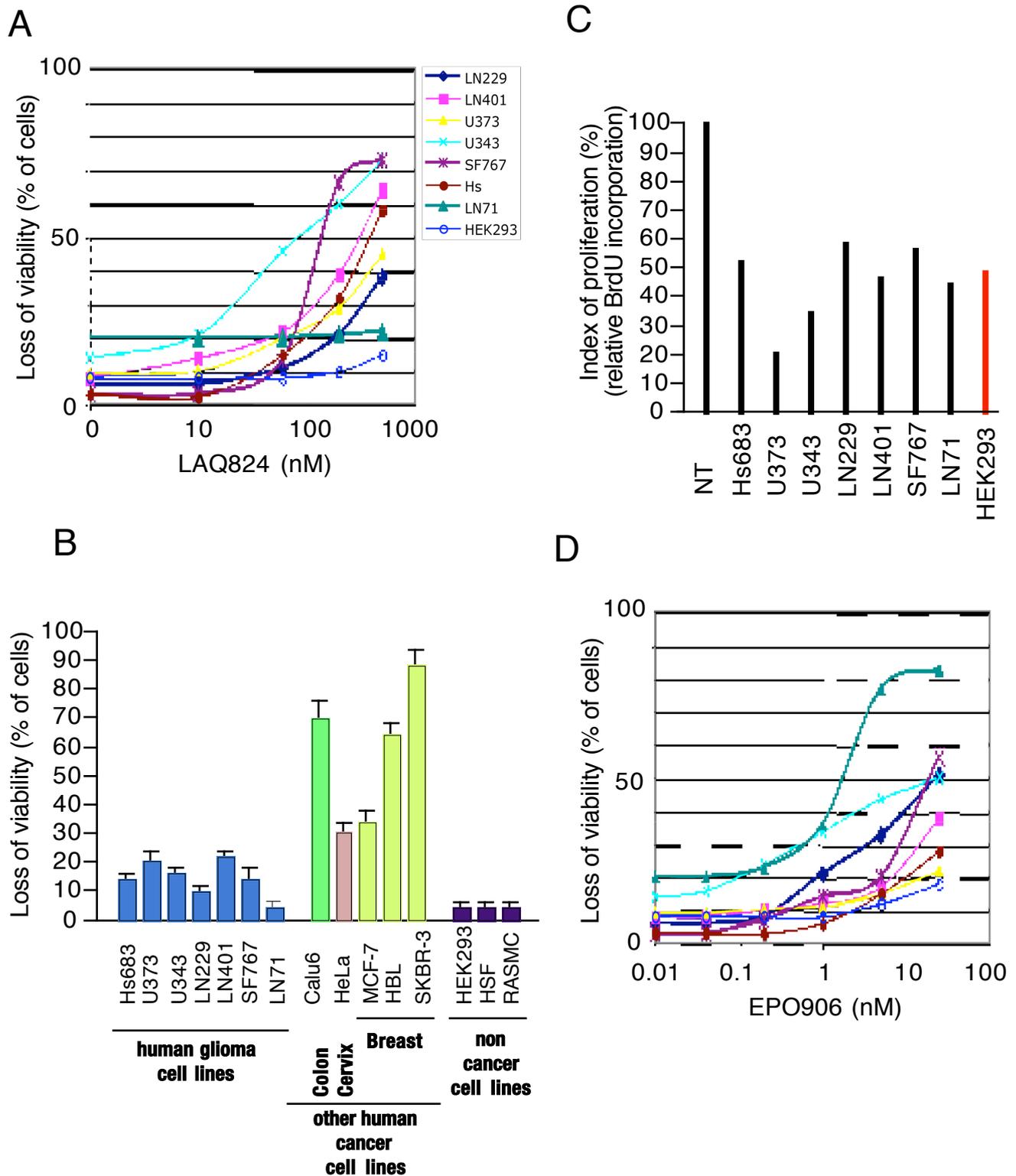
++++ High Apoptosis  
 ++ Moderate Apoptosis  
 + Weak Apoptosis  
 - No Apoptosis  
 -- Pro-survival

A: AEE788

G: Glivec

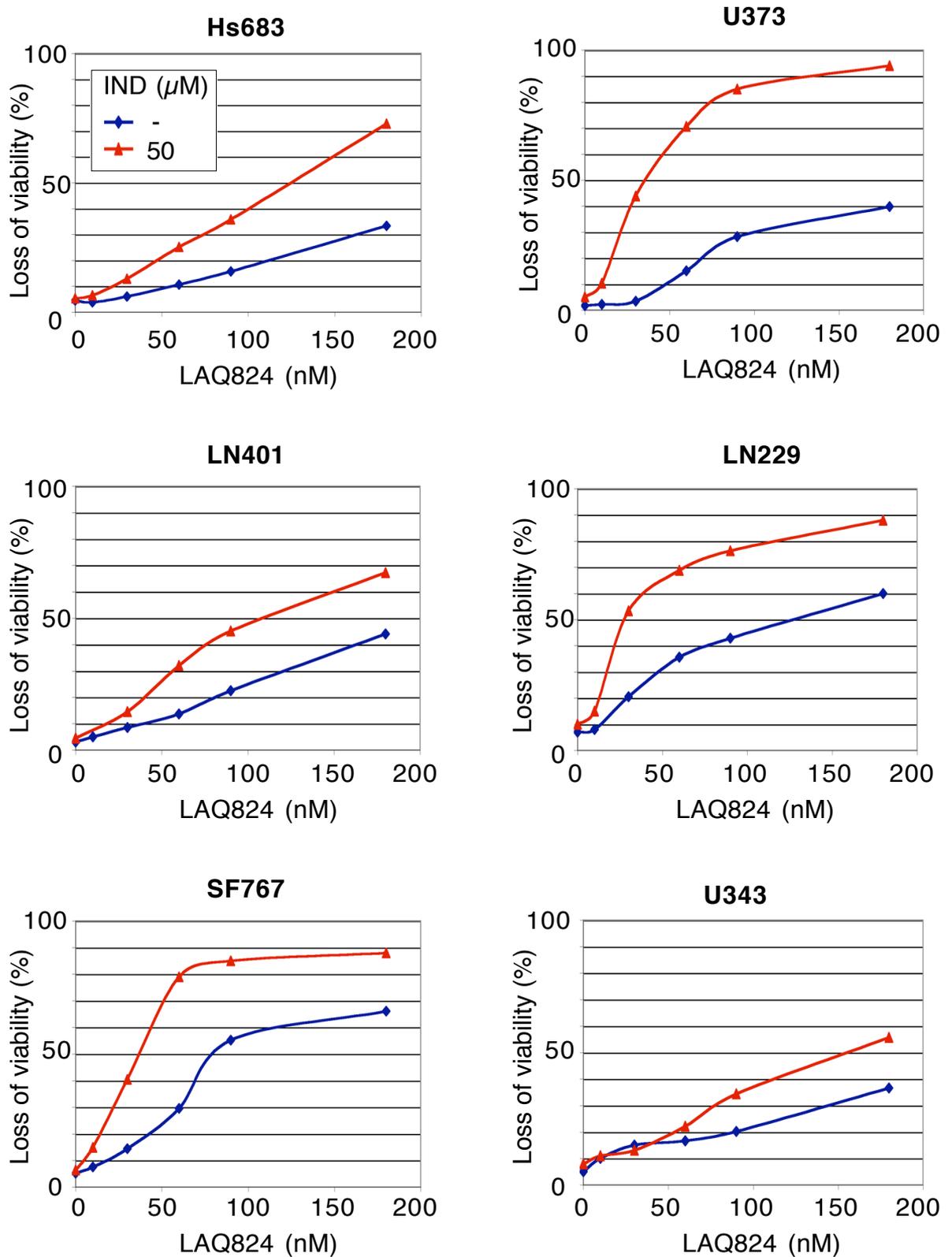
R: RAD001 20nM

Figure 6. Table summarizing the effects of AEE788, Glivec and RAD001 observed on the survival of 10 glioma cell lines upon single or combined application. No correlation between cell death and the genetic background was observed.



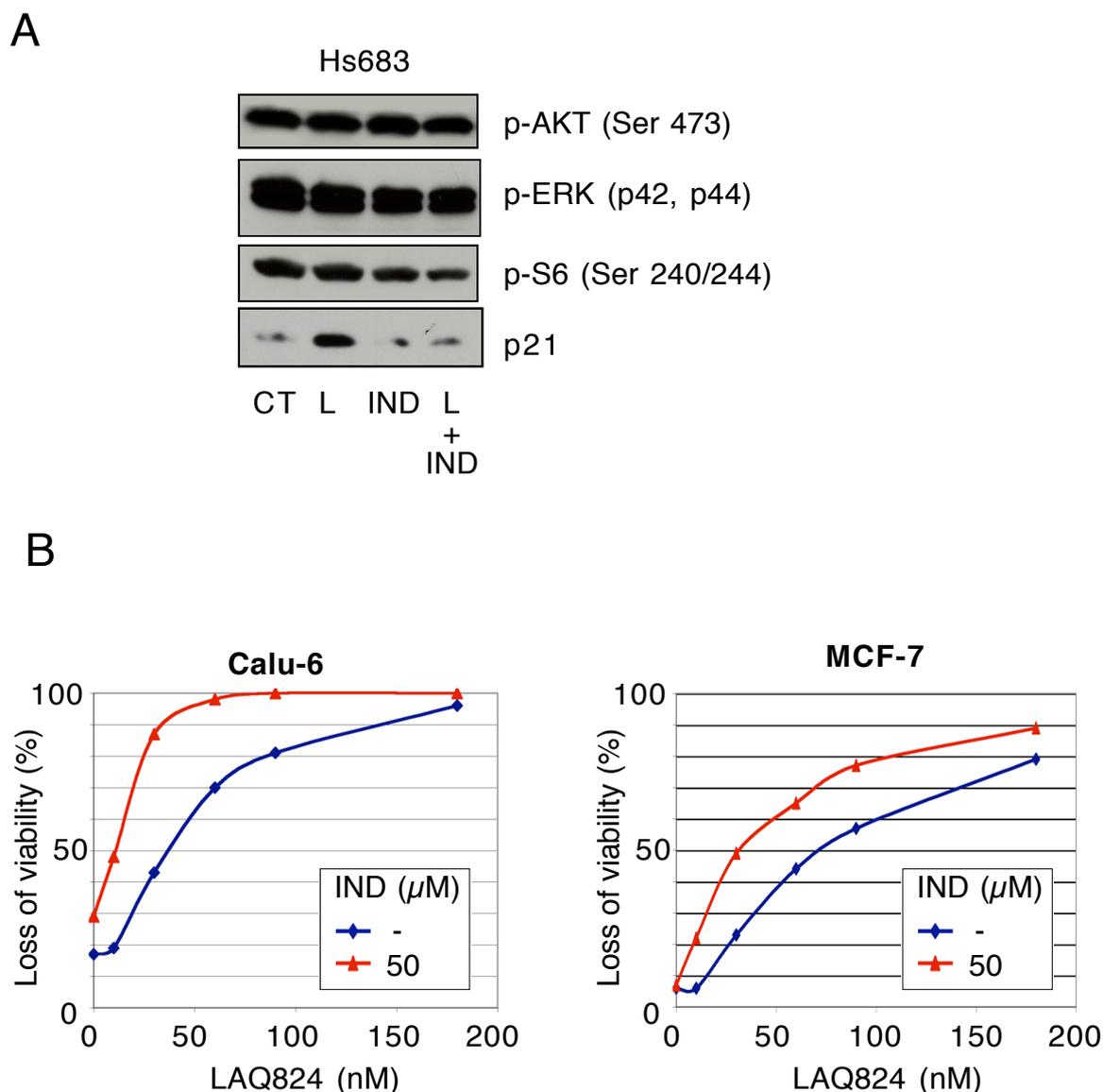
**Figure 7. Specific cytotoxicity of LAQ824 and EPO906A on cancer cell lines compared to non-cancer cell lines.**

(A) Titration of LAQ824: effect of LAQ824 on survival of human glioma and HEK293 cell lines. Cell survival was analysed by FACS as described in Materials and Methods (B) The various cell lines were treated with 60 nM LAQ824 for 72h and loss of viability was determined by FACS. (C) Various cell lines were treated with 30 nM LAQ824 for 48h and proliferation was determined by BrdU incorporation (NT=non treated) (D) Increasing doses of EPO906A were applied to human cell lines for 72h as in A and survival was determined by FACS.



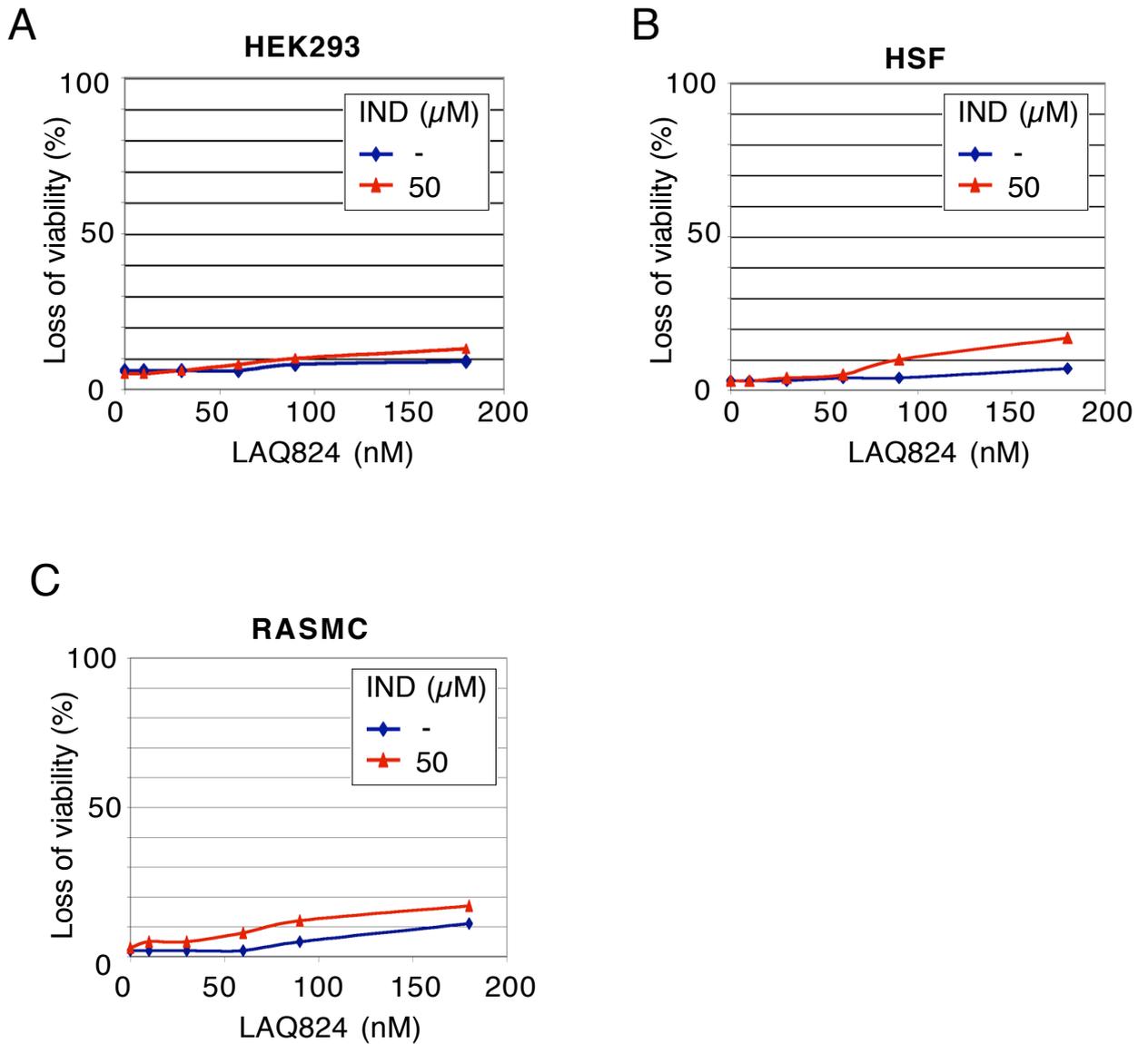
**Figure 8. Effect of the combinations of indomethacin (IND) and LAQ824 on the survival of six glioma cell lines.**

Hs683, LN401, LN229, U373, SF767 and U343 glioma cell lines were treated with increasing doses of LAQ824 (10, 30, 60, 90, 180nM), with or without 50μM IND during 72h and cell survival was determined by FACS as described in Materials and Methods



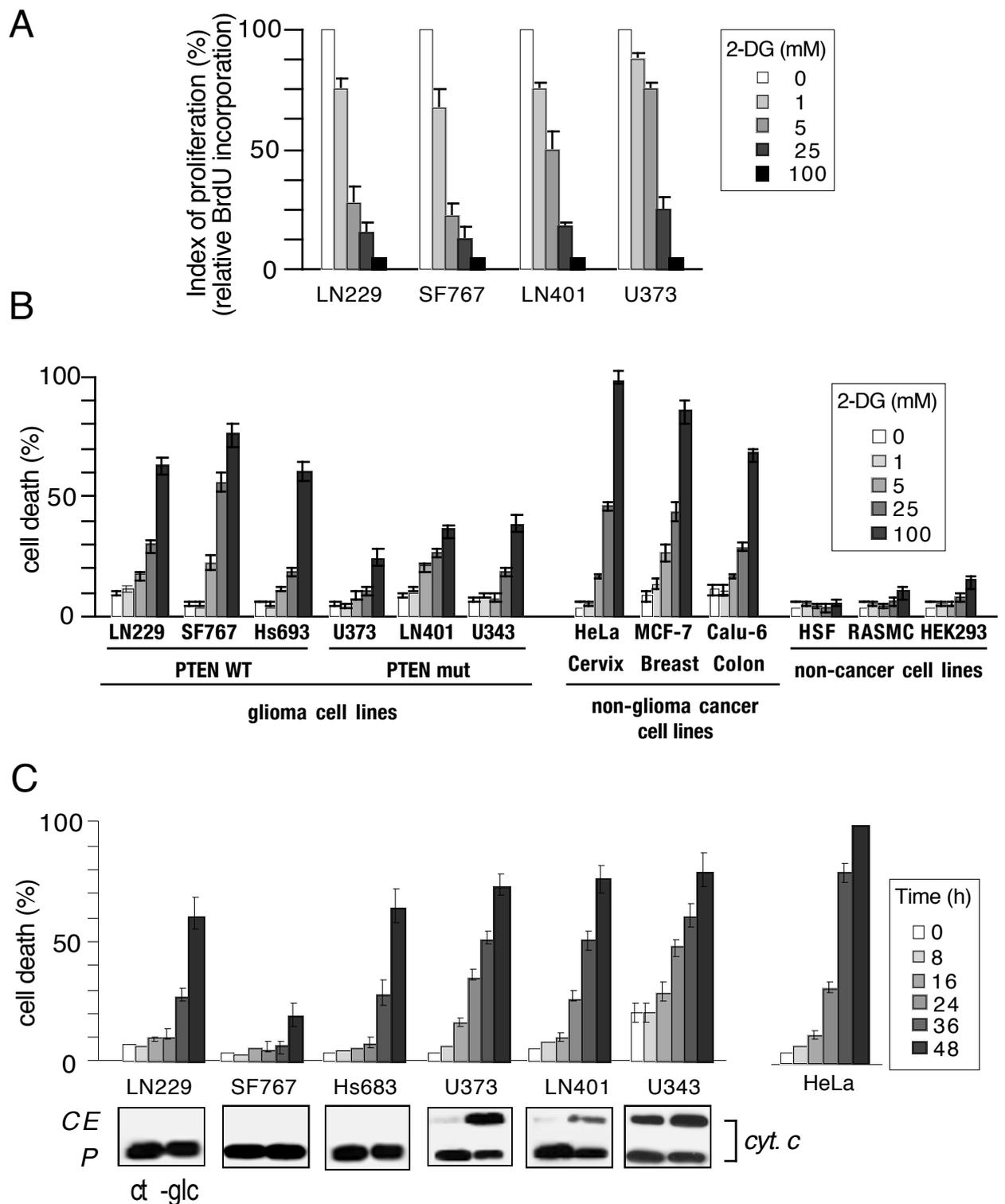
**Figure 9. Effect of the combinations of indomethacin (IND) and LAQ824 on protein levels and on the survival of other cancer cell lines (Calu-6, MCF-7) .**

(A) Hs683 was treated for 24h with 60nM LAQ824, 50μM indomethacin (IND) or a combination of both drugs. Cell extracts were done after 24h treatment and protein samples were loaded on a SDS-gel. Protein levels were determined by Western-Blot (B) The colon cancer cell line Calu-6 and the breast cancer cell line MCF-7 were cultured in triplicates in standard medium during 48h. Cells got fresh medium before to be treated with increasing doses of LAQ824 (10, 30, 60, 90, 180nM), and/or 50μM IND during 72h after which time floating and viable cells were pooled and cell death was measured using FACS analysis (pro-G1 phase) as described under „material and methods“



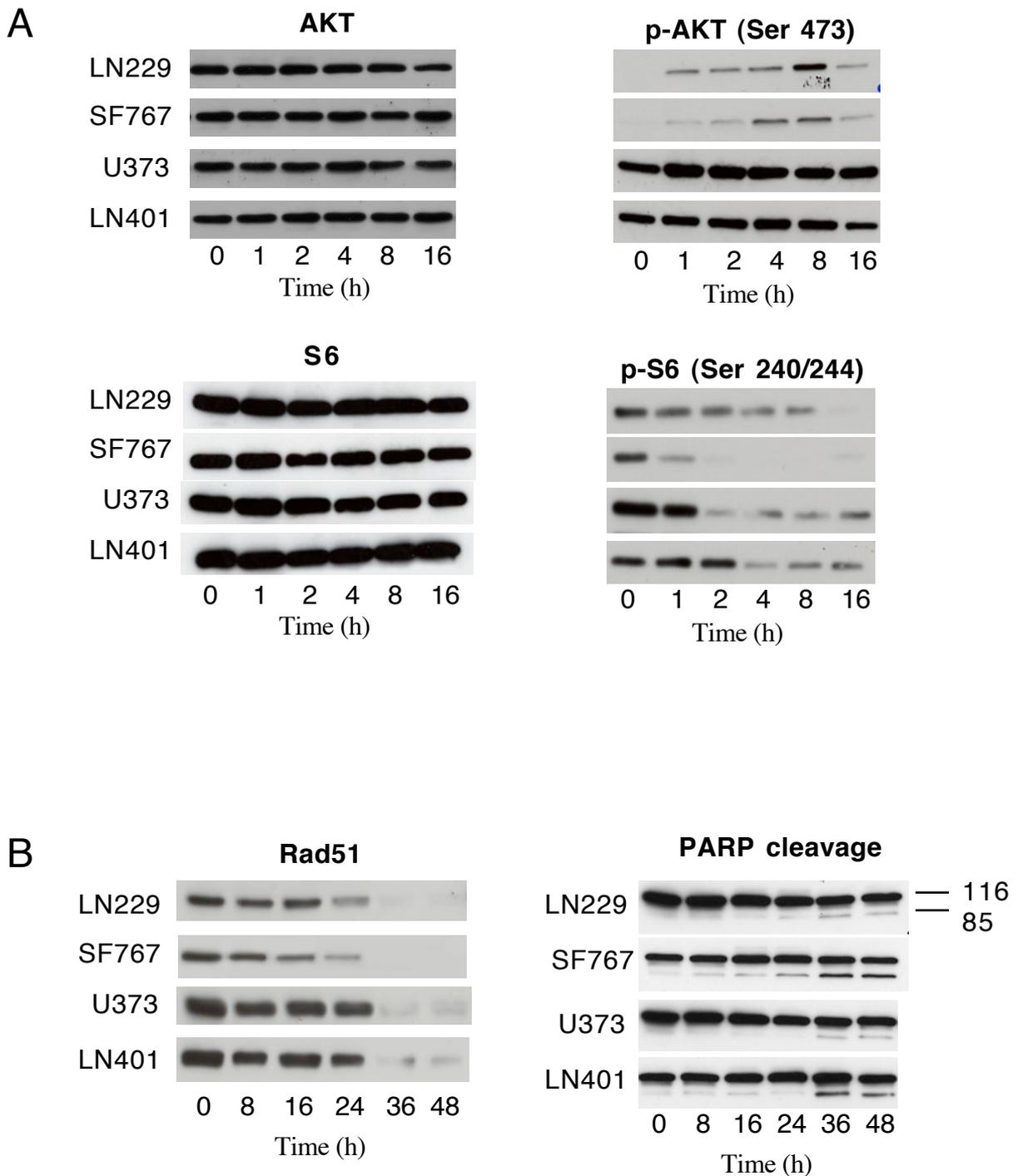
**Figure 10. Effect of the combinations of indomethacin (IND) and LAQ824 on the survival of non-cancer cell lines.**

HEK293, HSF and RASCM cell lines were cultured in triplicates in standard medium during 48h. Cells got fresh medium before to be treated with increasing doses of LAQ824 (10, 30, 60, 90, 180nM), and/or 50μM IND during 72h after which time floating and viable cells were pooled and cell death was measured using FACS analysis (pro-G1 phase) as described under „material and methods“ (A) Human embryonic kidney cell line (B) Human Skin Fibroblast cell line (C) Rat aortic smooth muscle cell line



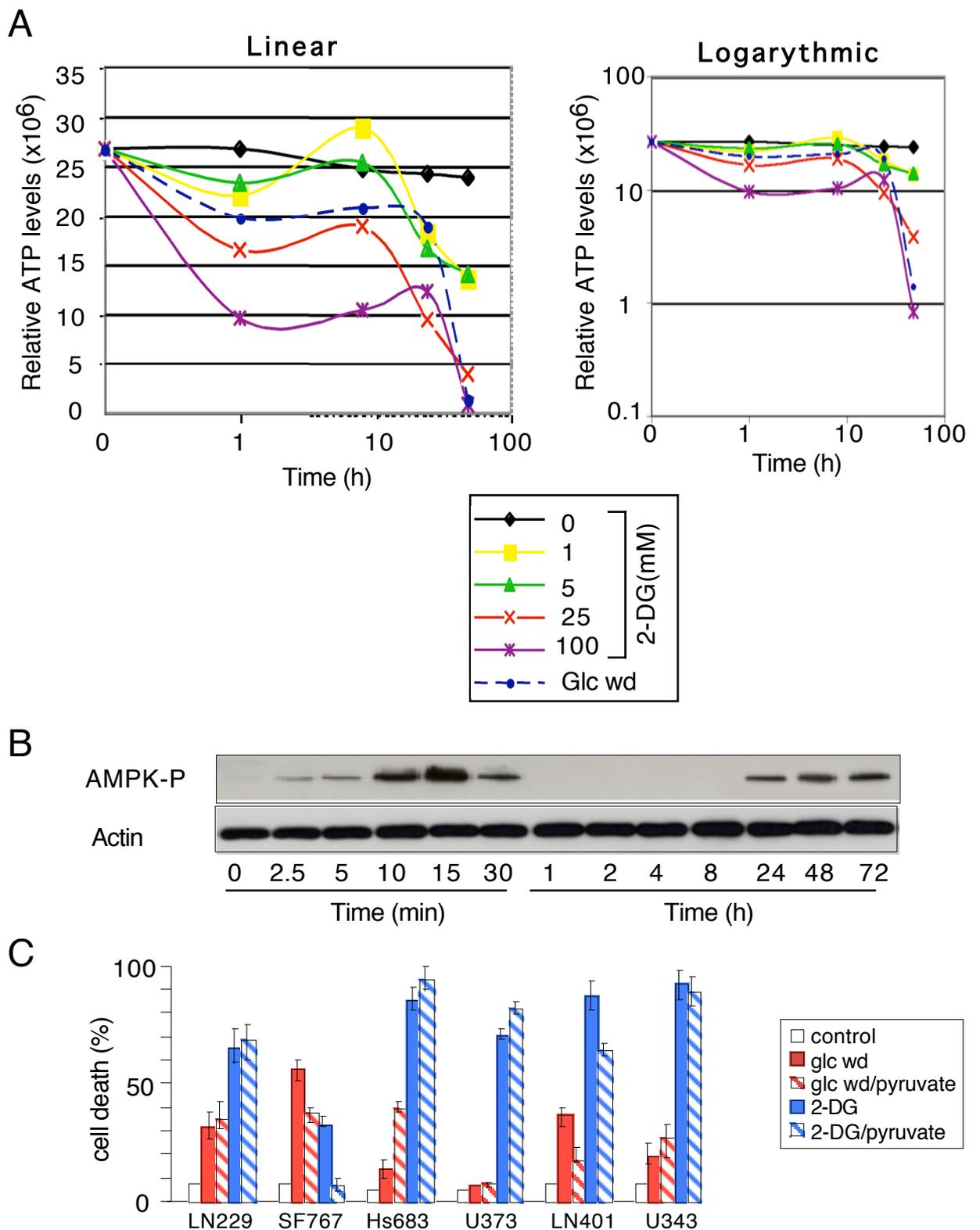
**Figure 11: 2-DG treatment and glucose withdrawal induce apoptosis in glioma cell lines**

(A) Various cell lines were treated with increasing doses of 2-DG for 48h and proliferation was determined after by BrdU staining and FACS analysis as described in Materials and Methods. BrdU incorporation in the non treated cells was set to 100%. (B) Various cell lines were treated with increasing doses of 2-DG for 72h and cell survival was determined by FACS (HSF: human skin fibroblast, RASMC: rat aortis smooth muscle cell, HEK: human embryonic kidney, HeLa: cervix, MCF-7: breast, Calu-6: colon). (C) Glioma cell lines and HeLa cell line were treated with glucose-free medium (-glc) and cell survival was determined after the indicated times. Cytochrome c release was measured after 24h under glucose starvation (CE: cytoplasmic extract, P: pellet) as described in Materials and Methods.



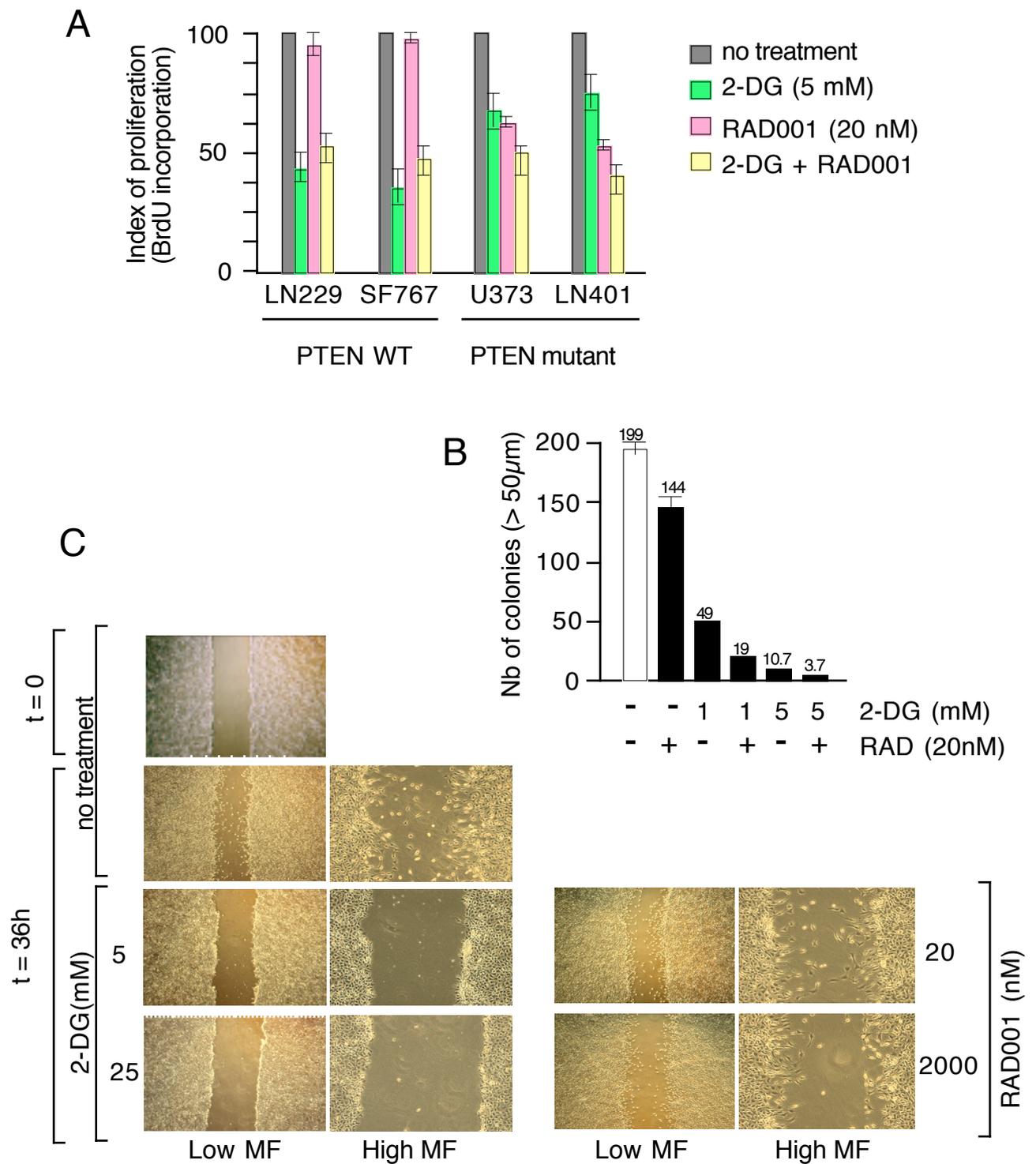
**Figure 12: Effect of 2-DG on glioma cell proliferation markers AKT and S6 and on the apoptosis markers Rad51 and PARP.**

(A) Phosphorylation and total protein levels of AKT and S6 were determined by Western analysis at the indicated times after the exposure of the glioma cell lines to 100mM 2-DG (B) Rad51 protein levels and PARP cleavage were determined by Western analysis at the indicated time after the exposure of the cells to 100mM 2-DG. The antibodies used are described in Materials and Methods.



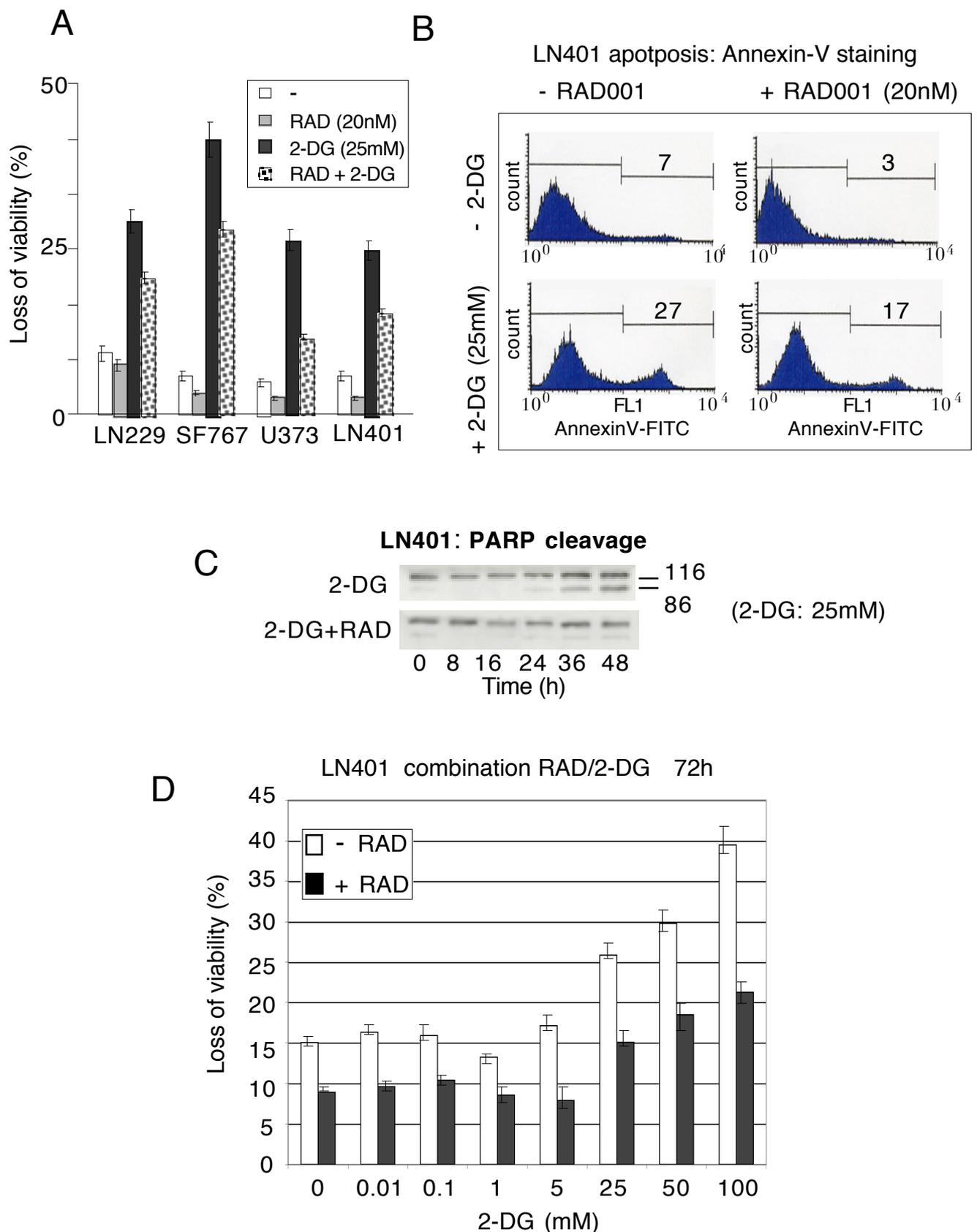
**Figure 13: 2-DG and glucose withdrawal reduce ATP levels in a two-phase manner . Pyruvate does not rescue cell survival upon 2-DG or glucose withdrawal.**

(A) U373 cell line was treated with increasing doses of 2-DG and glucose withdrawal (Glc wd) and ATP levels were measured 1h, 8h, 24h and 48h after the beginning of the treatment, as described in Materials and Methods. (B) U373 cell line was treated with 25mM 2-DG and the phosphorylation levels of AMPK was determined by Western analysis after the indicated times. (C) Glioma cell lines were exposed to the indicated treatments, and cell death was determined by FACS after 72h (4.5g/L pyruvate, 25mM 2-DG)



**Figure 14: 2-DG reduces more strongly proliferation, colony formation and migration of glioma cell lines than RAD001**

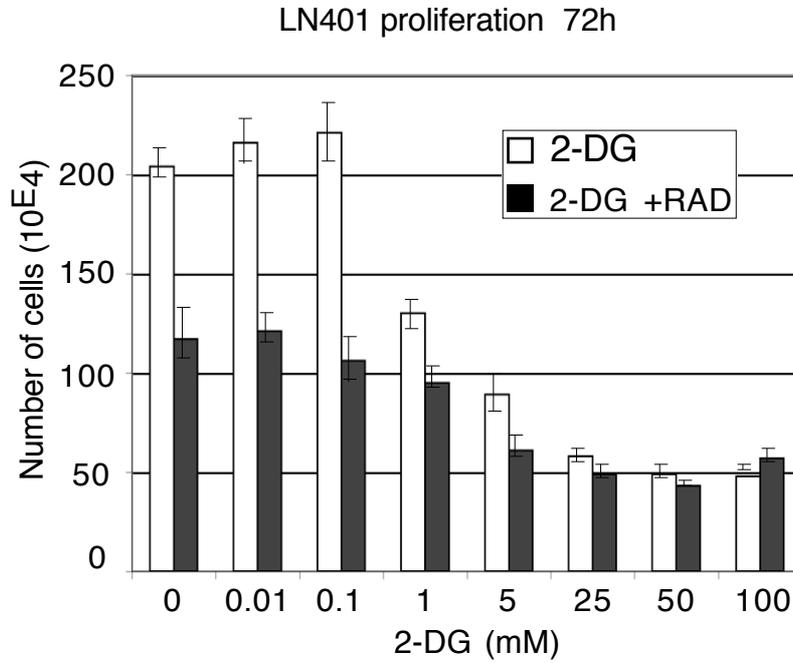
(A) BrdU staining: effect of 5mM 2-DG and/or 20nM RAD001 on the proliferation of four glioma cell lines, after 48h exposure. BrdU was added for 1h before the end of the treatment. BrdU incorporation in the non treated cells was set to 100%. (B) LN229 cell line was submitted to Colony Formation Assay for 72h in presence of 1 or 5mM 2-DG or 20nM RAD001 (RAD) or a combination of both drugs. The number of colonies > 50µm was determined by a colony counter instrument. (C) Migration assay: wound healing assay. LN229 was subjected to migration in presence of 2-DG or RAD001 at the indicated concentrations, for 72h. (RAD001 was added 24h before the wound was made). (MF: magnification)



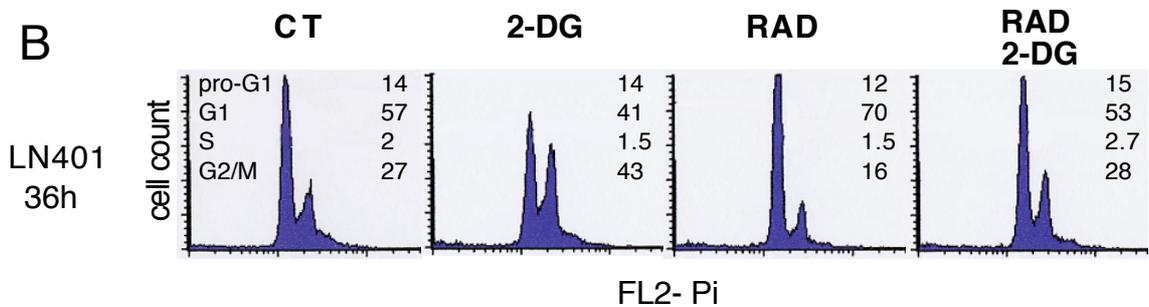
**Figure 15: RAD001 decreases 2-DG-induced apoptosis in glioma cell lines**

(A) LN229, SF767, U373 and LN401 cell lines are treated with the indicated drugs for 72h and cell survival was determined by FACS. (B) LN401 was exposed to the indicated drugs for 72h and apoptosis was determined by Annexin-V staining as described in Materials and Methods. (C) LN401 was exposed to the indicated drugs for the indicated times and PARP cleavage was analysed by Western. (D) LN401 was submitted to increasing doses of 2-DG or to the combination of 2-DG and RAD001 for 72h. Cell survival was determined by FACS. 20nM RAD001 was used for all treatments.

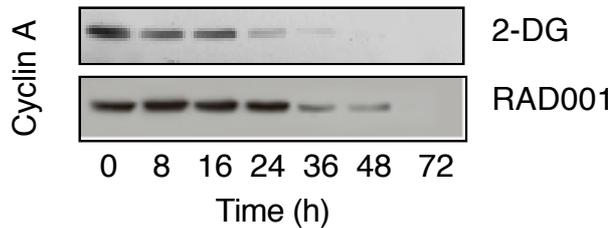
**A**



**B**

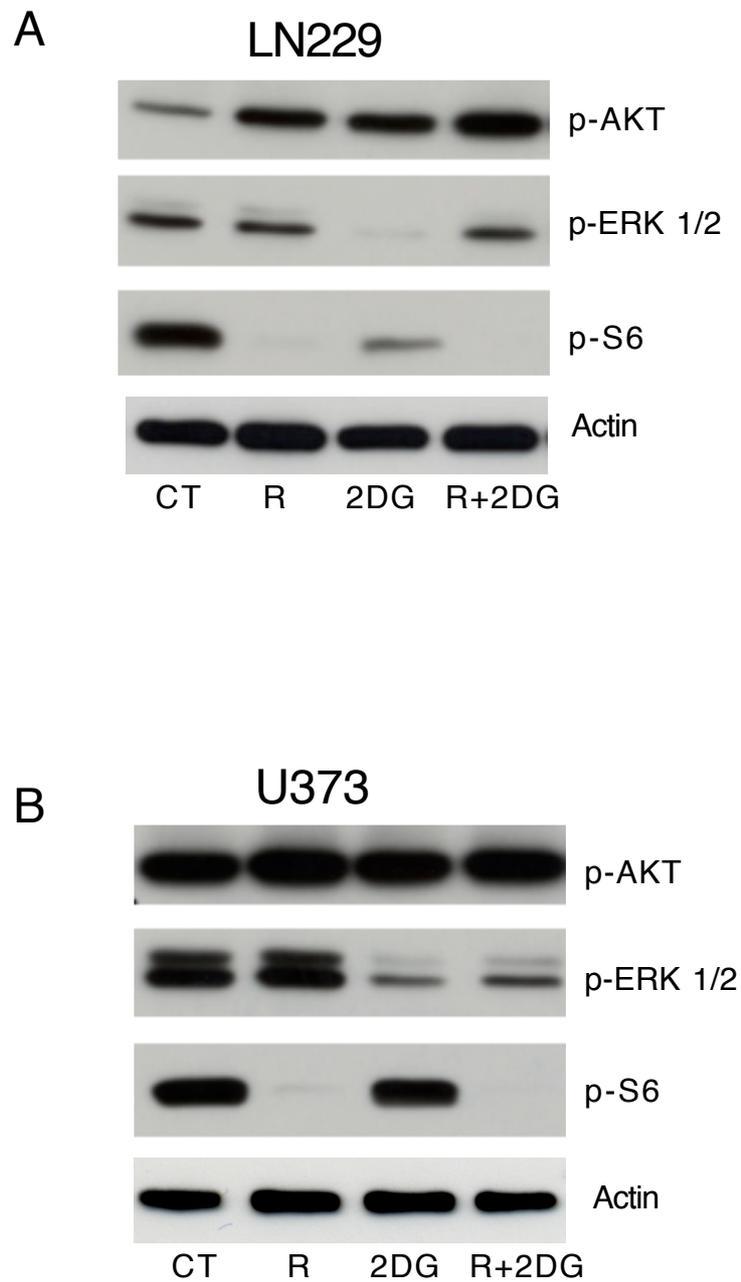


**C**



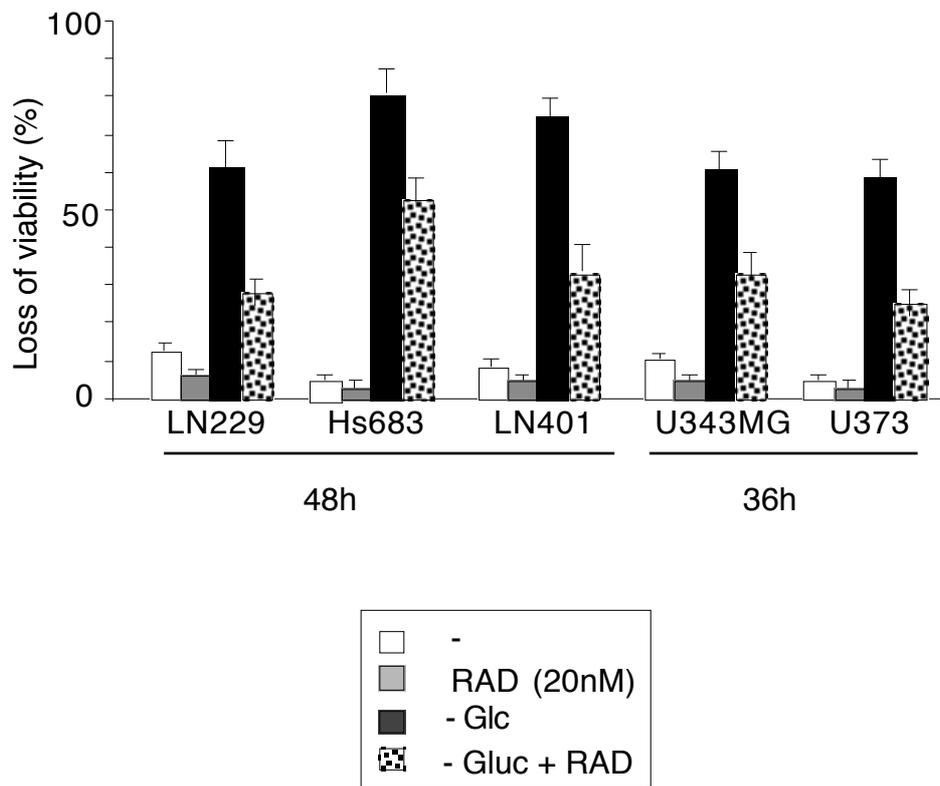
**Figure 16: 2-DG is dominant over RAD001 for inhibiting cell proliferation**

(A) LN401 were exposed to increasing doses of 2-DG in presence or not of 20nM RAD001 for 72h. At the end of the experiment, floating and attached cells were counted. (B) LN401 was exposed to 25mM 2-DG, 20nM RAD001 or both drugs for 36h and cell cycle profiles were determined by FACS after Pi staining. The percentages of cells in each phase are indicated on the histograms (C) Cyclin A levels were followed for 72h after 25mM 2-DG or 20nM RAD001 treatment by Western analysis. Equal amounts of protein were loaded. (RAD: RAD001)



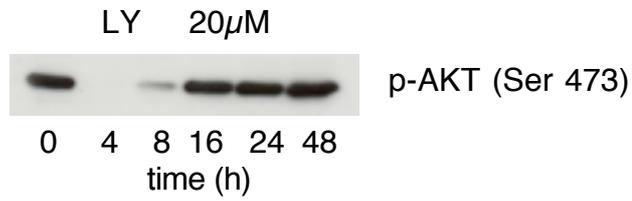
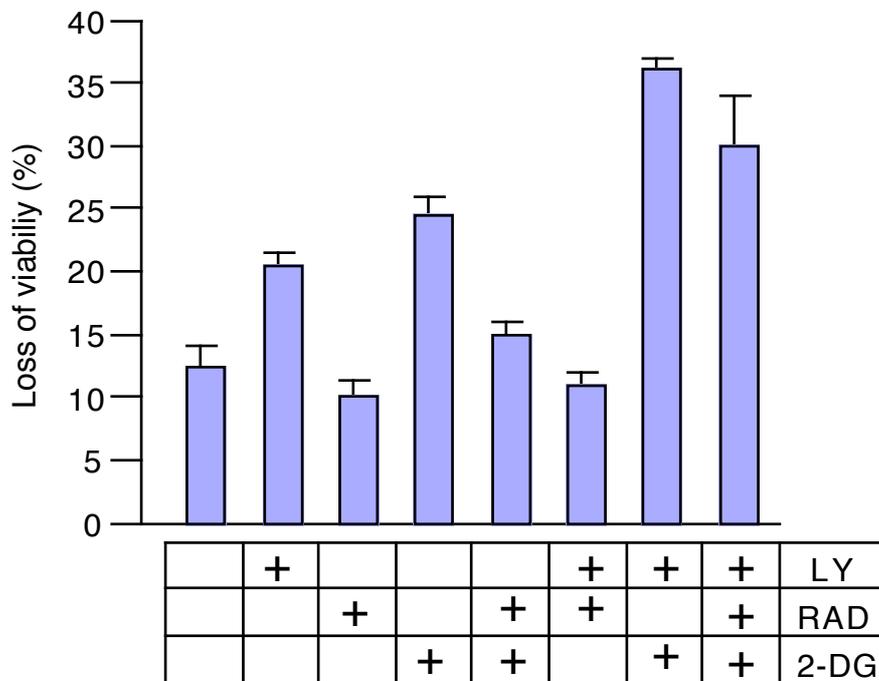
**Figure 17 : Effect of 2-DG and RAD001 on the phosphorylation of AKT, ERK and S6.**

(A) LN229 cells were treated for 8h with 20nM RAD001, 25mM 2-DG or the combination of both drugs and the phosphorylation of AKT (Ser 473), ERK(1/2) (p42/p44), and S6 (Ser 240/244) protein were determined by Western analysis as described in Materials and Methods. (B) U373 cell line was treated as in (A)



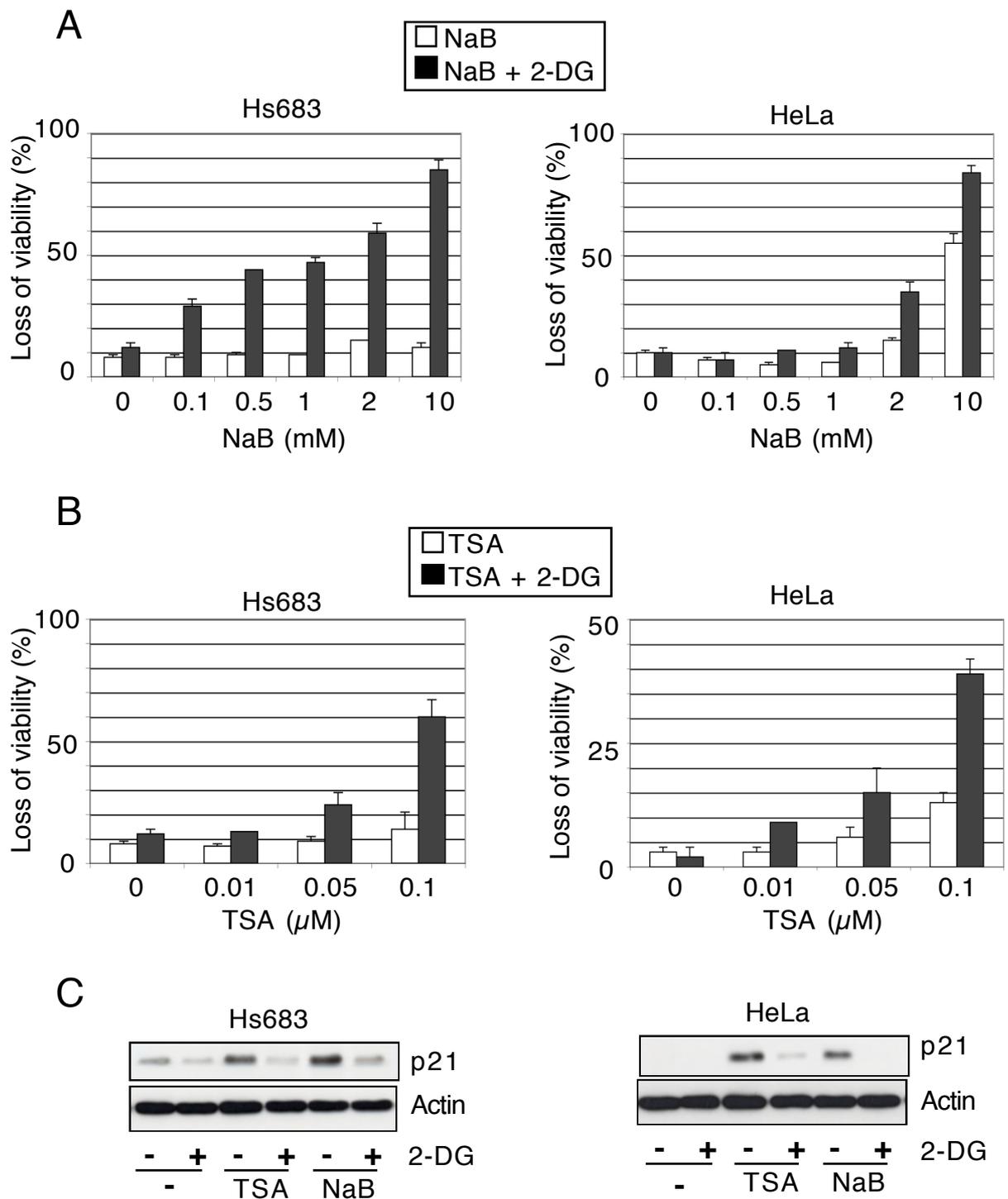
**Figure 18 : RAD001 delays apoptosis induced by glucose withdrawal**

Glioma cell lines were submitted to glucose starvation (- Glc), in presence or in absence of 20nM RAD001 (RAD) during the indicated times and cell death was measured by FACS as described in Material and Methods.

**A****B**

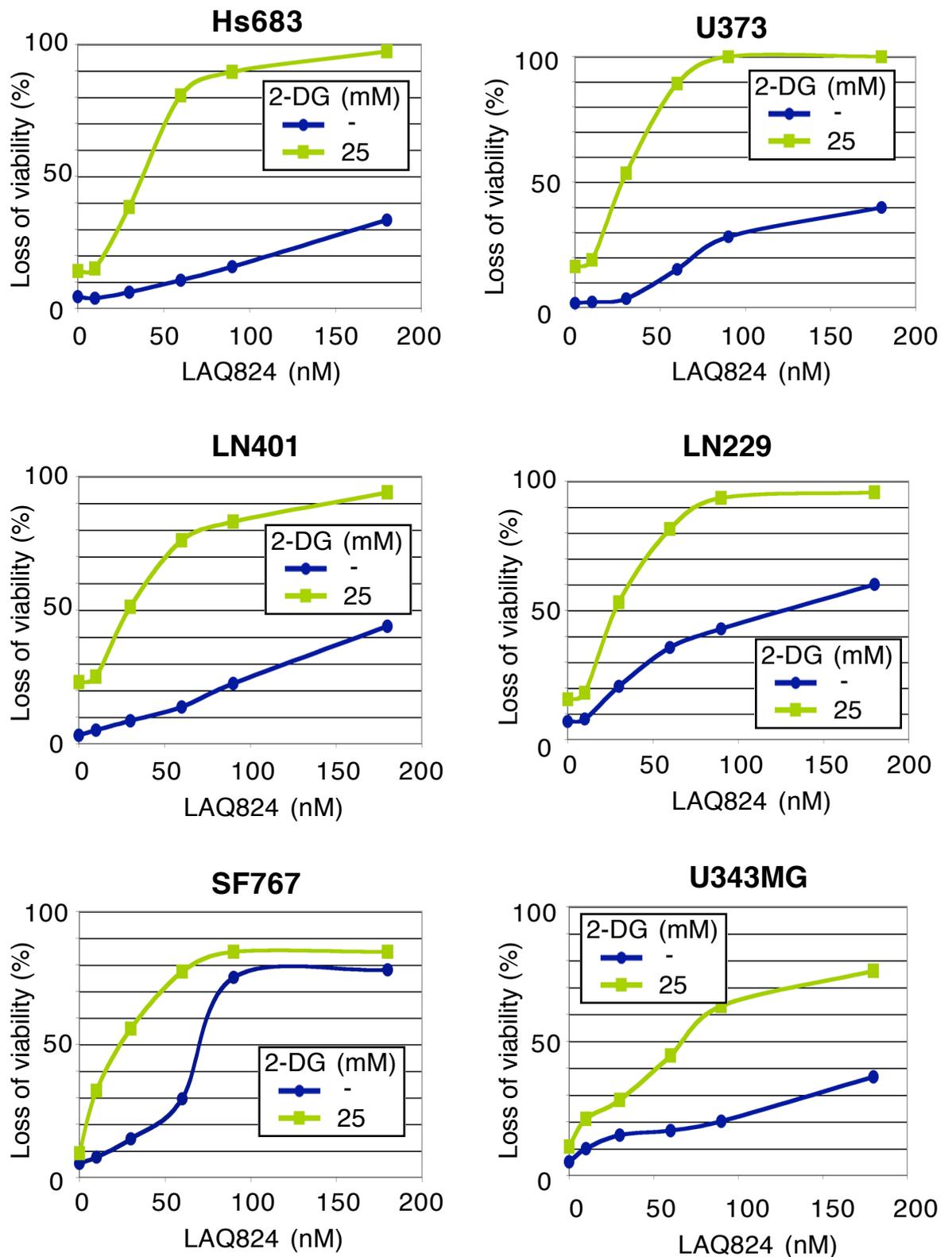
**Figure 19 : Effect of LY294002, RAD001 and 2-DG on the survival of LN401 cell line**

(A) LN401 was exposed to the PI3K inhibitor LY294002 (LY) and the phosphorylation levels of AKT were followed by Western analysis at the indicated times. (B) LN401 was treated with single or various drug combinations (25mM 2-DG, 20nM RAD001 and 20μM LY) for 72h and cell viability was determined by FACS. LY was added every 16h. Cell death is measured by FACS. (RAD: RAD001)



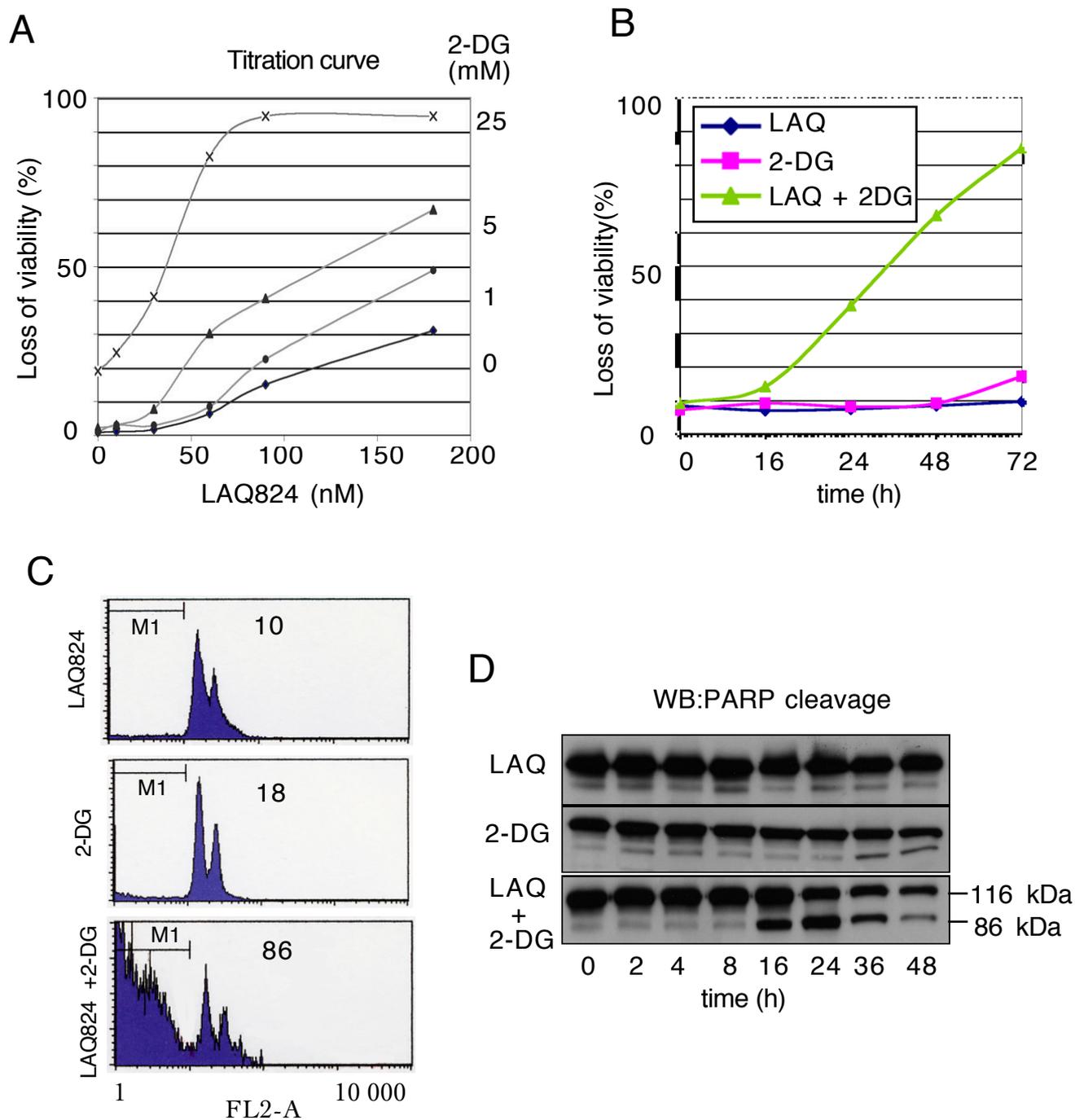
**Figure 20. Effect of the combinations of 2-deoxy-D-glucose (2-DG) and HDACs inhibitors (HDIs) on the survival of the glioma cell lines Hs683 and cervix carcinoma cell lines HeLa.**

(A) Hs683 and HeLa cells were treated with 25mM 2-DG and/or increasing doses of sodium butyrate (NaB) for 72h. Cell death was measured by FACS analysis after PI staining. (B) The same cell lines were treated with 25mM 2-DG and/or increasing doses of trichostatin A (TSA) for 48h. Cell death was measured as previously (C) Hs683 and HeLa cells were treated with 25mM 2-DG and/or TSA (100nM) or NaB (10mM) for 8h and p21 protein and actin levels were determined by Western analysis.



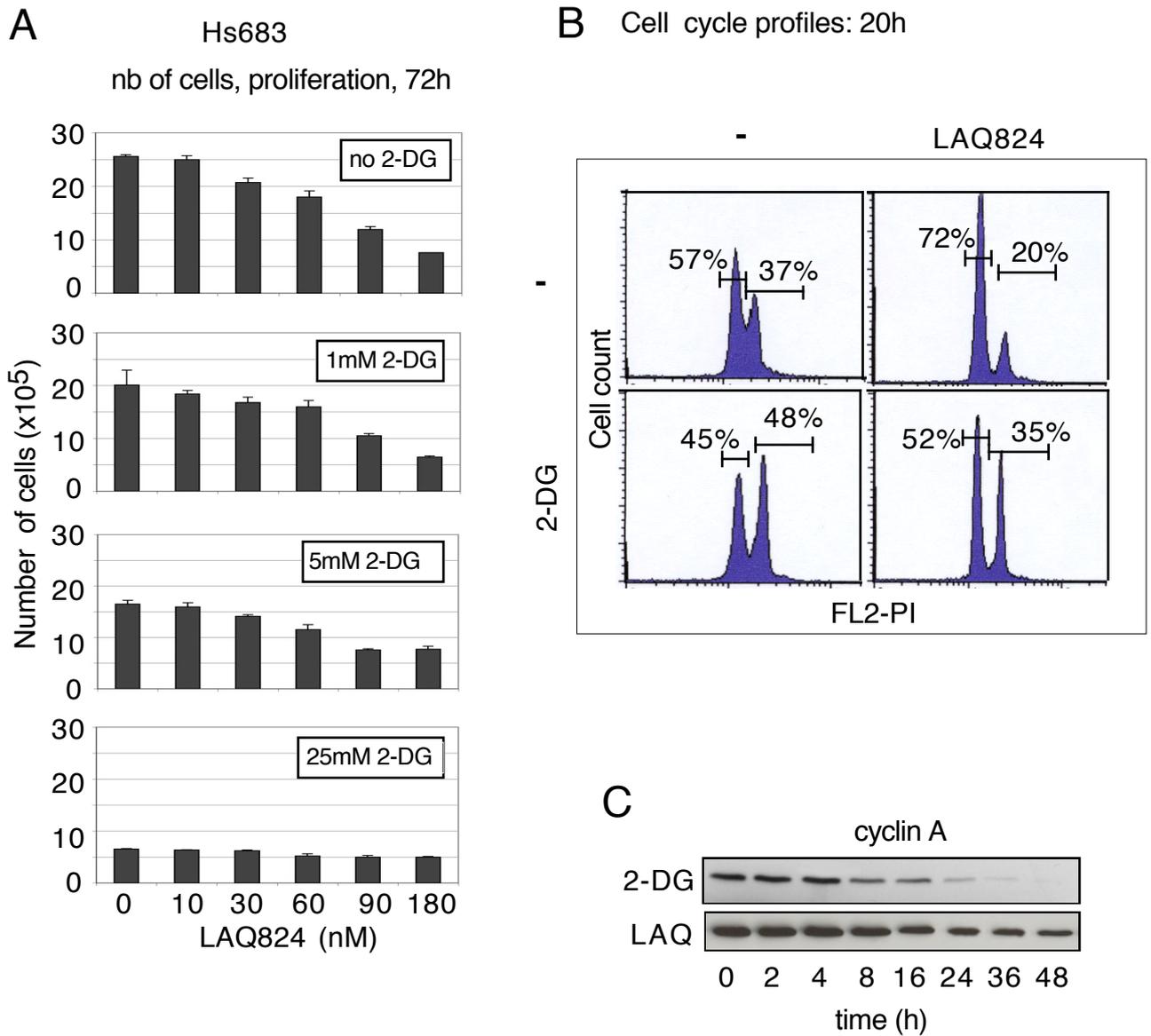
**Figure 21.** Effect of the combinations of 2-deoxy-D-glucose (2-DG) and LAQ824 on the survival of six glioma cell lines.

Hs683, LN401, LN229, U373, SF767 and U343 glioma cell lines were cultured in triplicates in standard medium during 48h. Cells got fresh medium before to be treated with increasing doses of LAQ824 (10, 30, 60, 90, 180nM), and/or 25mM 2-DG (only SF767 was treated with 5mM 2-DG) during 72h after which time floating and viable cells were pooled and cell death was measured using FACS analysis (pro-G1 phase) as described under “Experimental Procedures”.



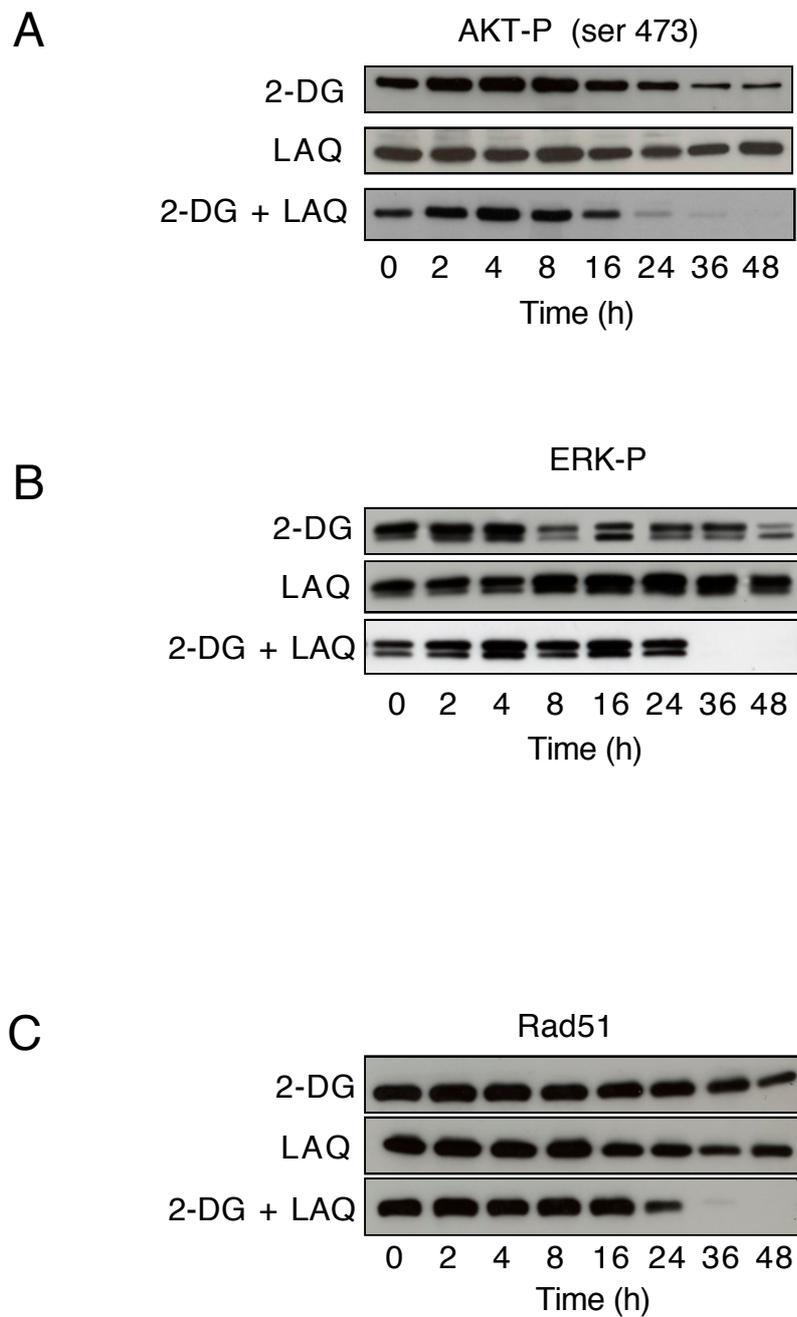
**Figure 22. The synergistic induction of apoptosis is 2-DG and LAQ824 dose-dependent and induces PARP cleavage.**

(A) Hs683 was treated with increasing concentrations of LAQ824 (10, 30, 60, 90, 180nM) and of 2-DG (1, 5, 25mM) for 72h cell death was measured by FACS. (B) Hs683 was treated with 25mM 2-DG and 60nM LAQ824 for 72h and cell survival was determined at the indicated time points by FACS.(C) HS683 was treated with 60nM LAQ824 and/or 25mM 2-DG for 72h and the cell cycle profile was established by FACS as described in Materials and Methods. M1 gives the percentage of cells in the pro-G1 phase. (D) Hs683 was exposed to 60nM LAQ824 and/or 25mM 2-DG, SDS samples were prepared at the indicated time points and submitted to SDS-Page. The cleavage of PARP was assessed by immunoblotting with anti-PARP. (PARP cleavage is defined by the appearance of the 86 kDa band.)



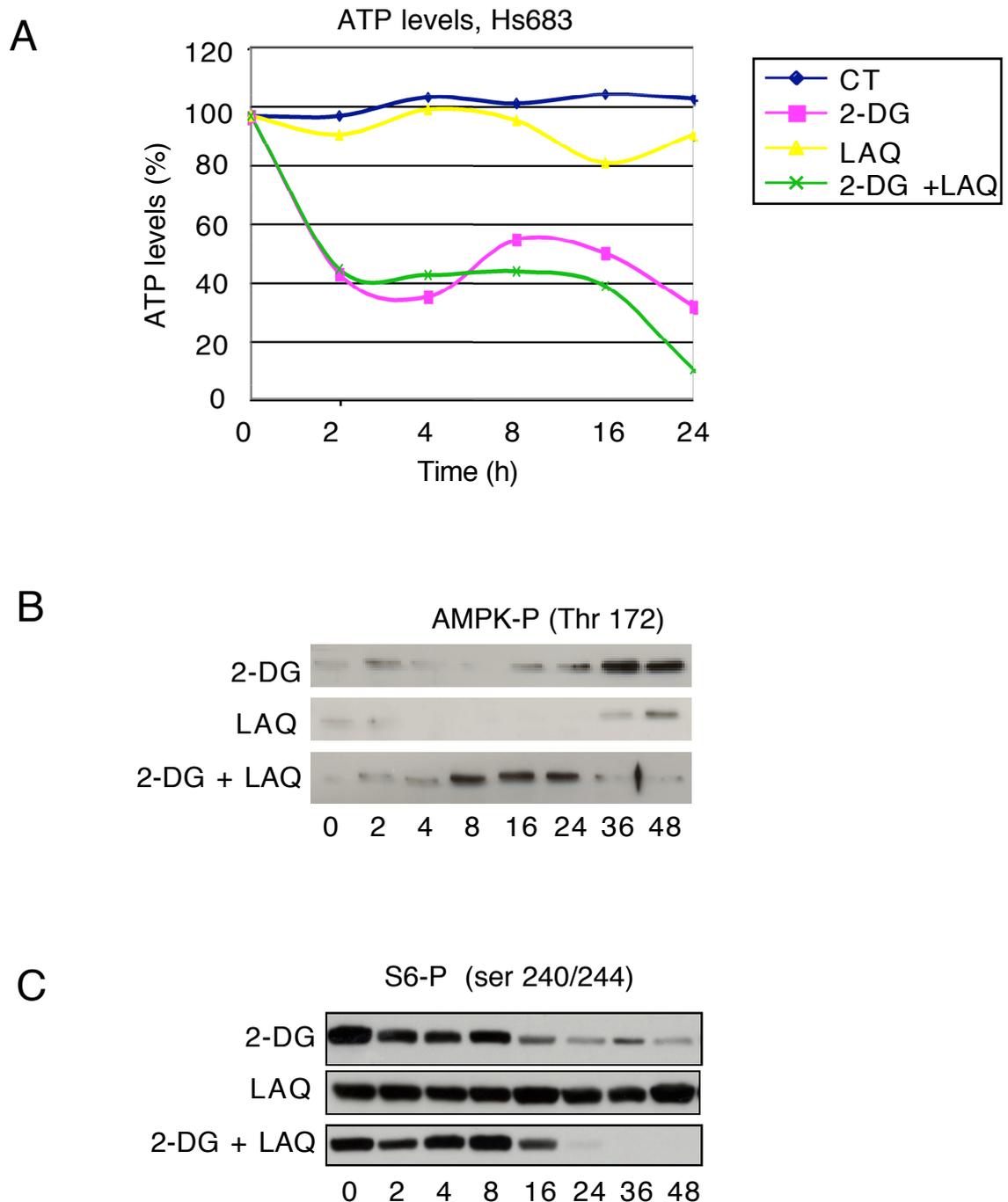
**Figure 23. Dominance of 2-DG over LAQ824 on the inhibition of Hs683 proliferation.**

(A) Hs683 was exposed to increasing doses of LAQ824 (10, 30, 60, 90, 180nM) and/or 2-DG (1, 5, 25mM), the total number of cells was determined after 72h treatment, by pooling attached and floating cells and counting total number of cells. (B) The cell cycle profile of Hs683 was determined by FACS after 16h treatment with 25mM 2-DG and/or 60nM LAQ824. Percentages of cells in G1, S and G2/M phases are represented on the histograms. (C) Hs683 was exposed to 25mM 2-DG or 60nM LAQ for the indicated time, equal amounts of protein were submitted to Western analysis to determine cyclin A protein levels.



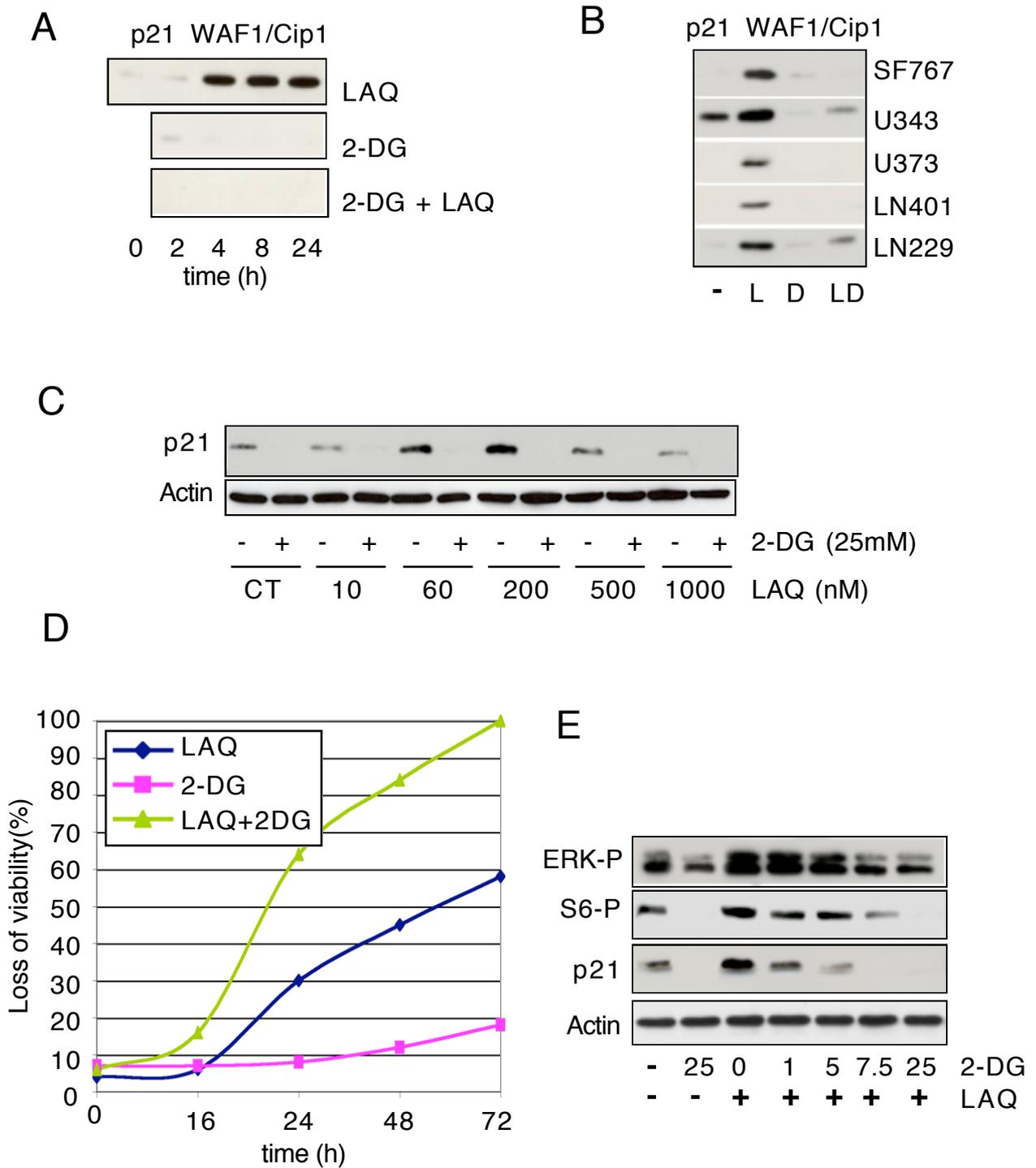
**Fig. 24. Effect of 2-DG, LAQ824 and the combination on AKT, ERK and Rad51 proteins .**

(A) Hs683 was exposed to 25mM 2-DG and/or 60nM LAQ824, cells were lysed at the indicated time and equal protein amounts were applied to Western analysis to determine the phosphorylated levels of Akt (B) Same protein samples as in (A) were applied to Western analysis to determine the phosphorylated levels of ERK protein and (C) for the total levels of Rad51 protein, as described in Materials and Methods.



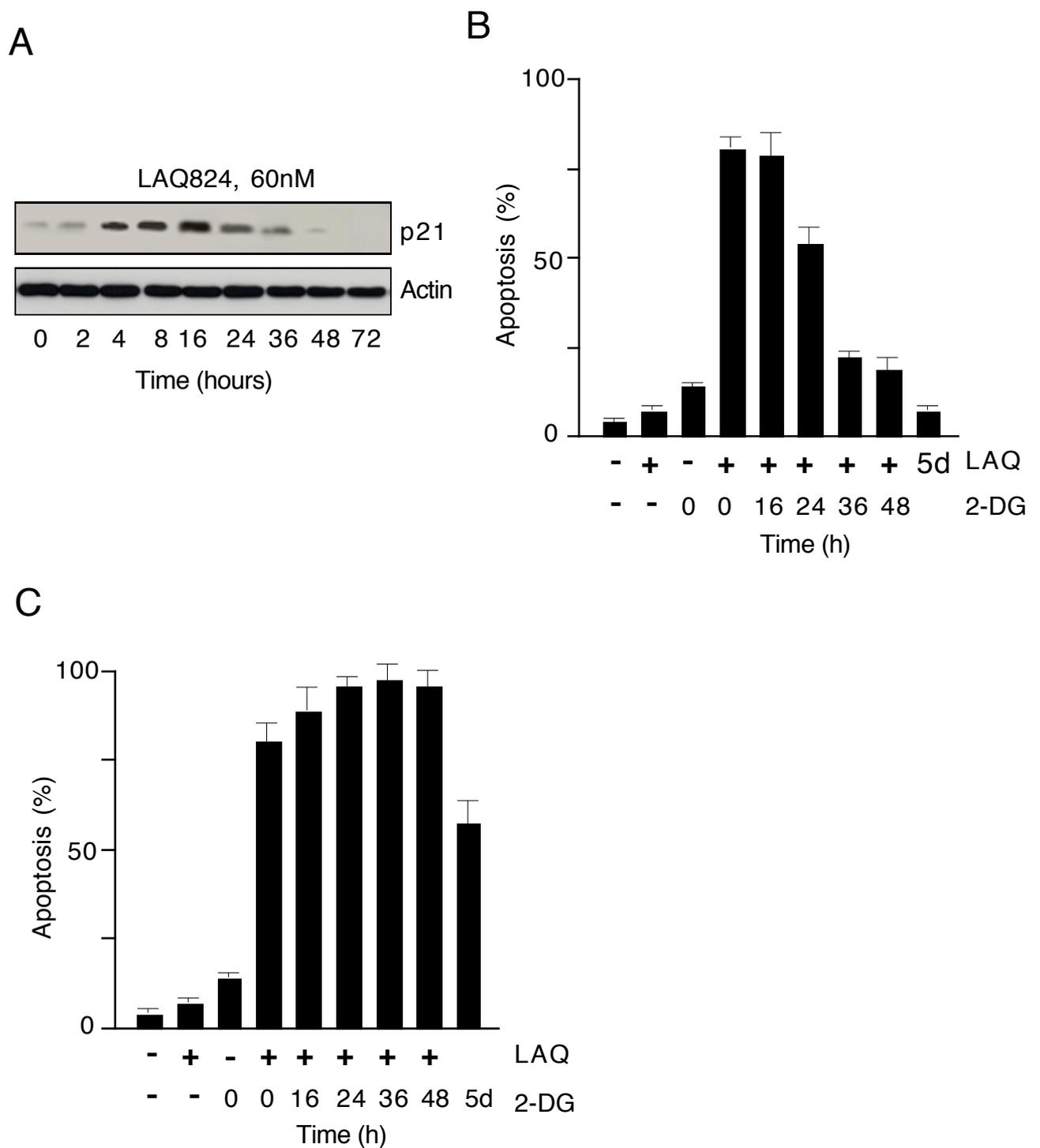
**Fig. 25. Effect of 2-DG and LAQ824 on ATP levels and on the proteins involved in the energy regulatory pathway.**

(A) Hs683 cells were incubated in the presence of 25mM 2-DG and/or 60nM LAQ824 (LAQ). At the indicated time points, ATP levels were determined as described upon Materials and Methods. ATP levels of the untreated cells at time  $t=0$  were set to 100%. (B) Hs683 was exposed to the same treatment as in (A) and protein samples were made at the indicated time points. Equal amounts of protein were applied to Western analysis to determine the phosphorylated form of AMPK (AMPK-P) and (C) S6 (S6-P).



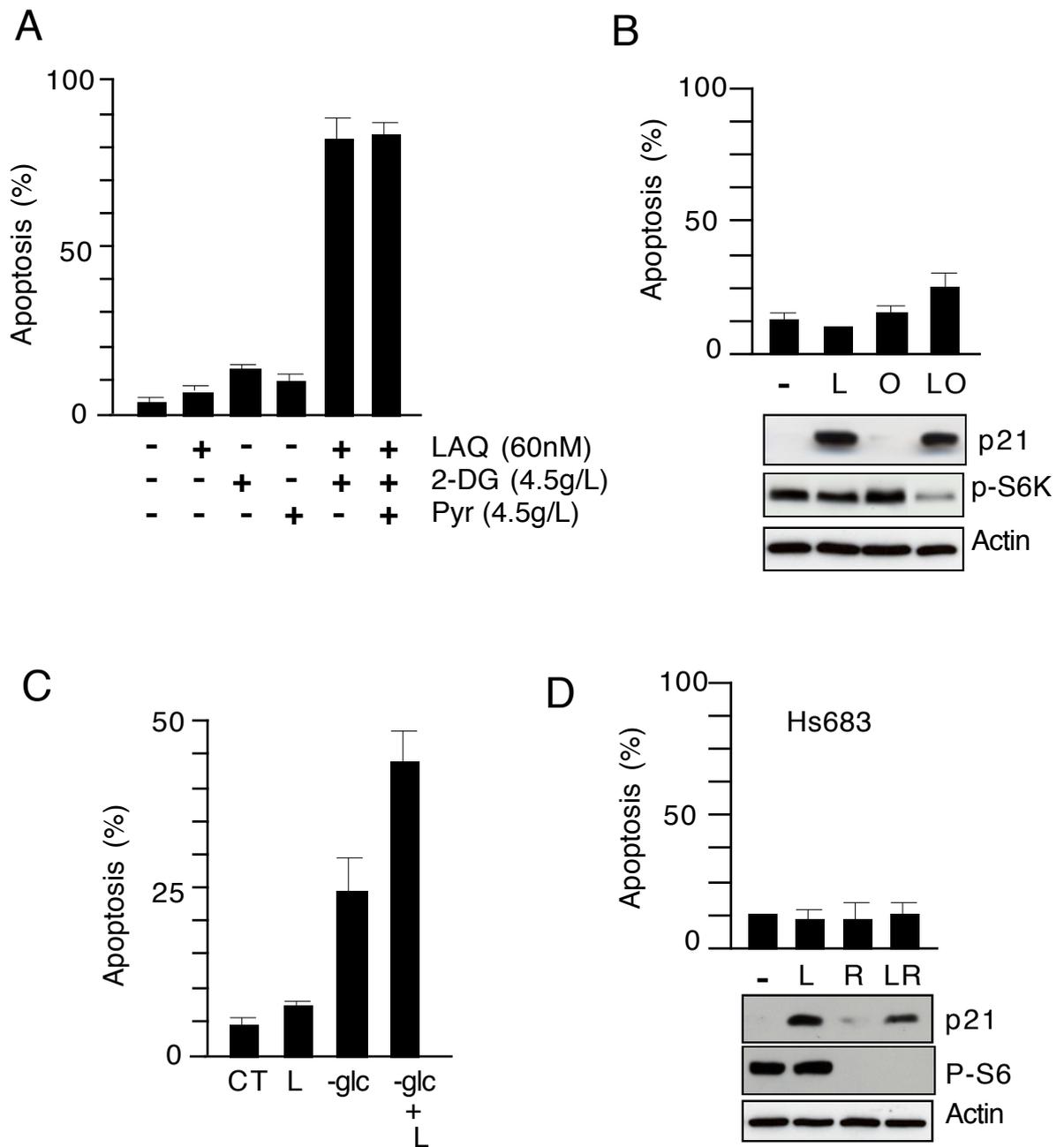
**Fig. 26. p21 plays a major role in the synergistic induction of apoptosis.**

(A) Hs683 cells were exposed to 25mM 2-DG and/or 60nM LAQ824 for 2, 4, 8 and 24h, and p21 protein levels were determined by Western analysis as described in Materials and Methods. (B) SF767, LN229, U343, U373 and LN401 were treated with 25mM 2-DG and/or 60nM LAQ824 for 8h and p21 protein cell content was determined by Western analysis as in (A). (C) Western analysis of p21 and actin proteins after 24h exposure of Hs683 to increasing doses of LAQ824, in combination or not with 25mM 2-DG. (D) Hs683 was treated with 500nM LAQ824, 25mM 2-DG or the combination of both drugs and cell death was measured by FACS analysis. (E) Hs683 was treated with increasing amounts of 2-DG (1, 5, 7.5, 25) for 8h in presence of not of 60nM LAQ824 and p21 protein levels were determined by Western analysis as in (A).



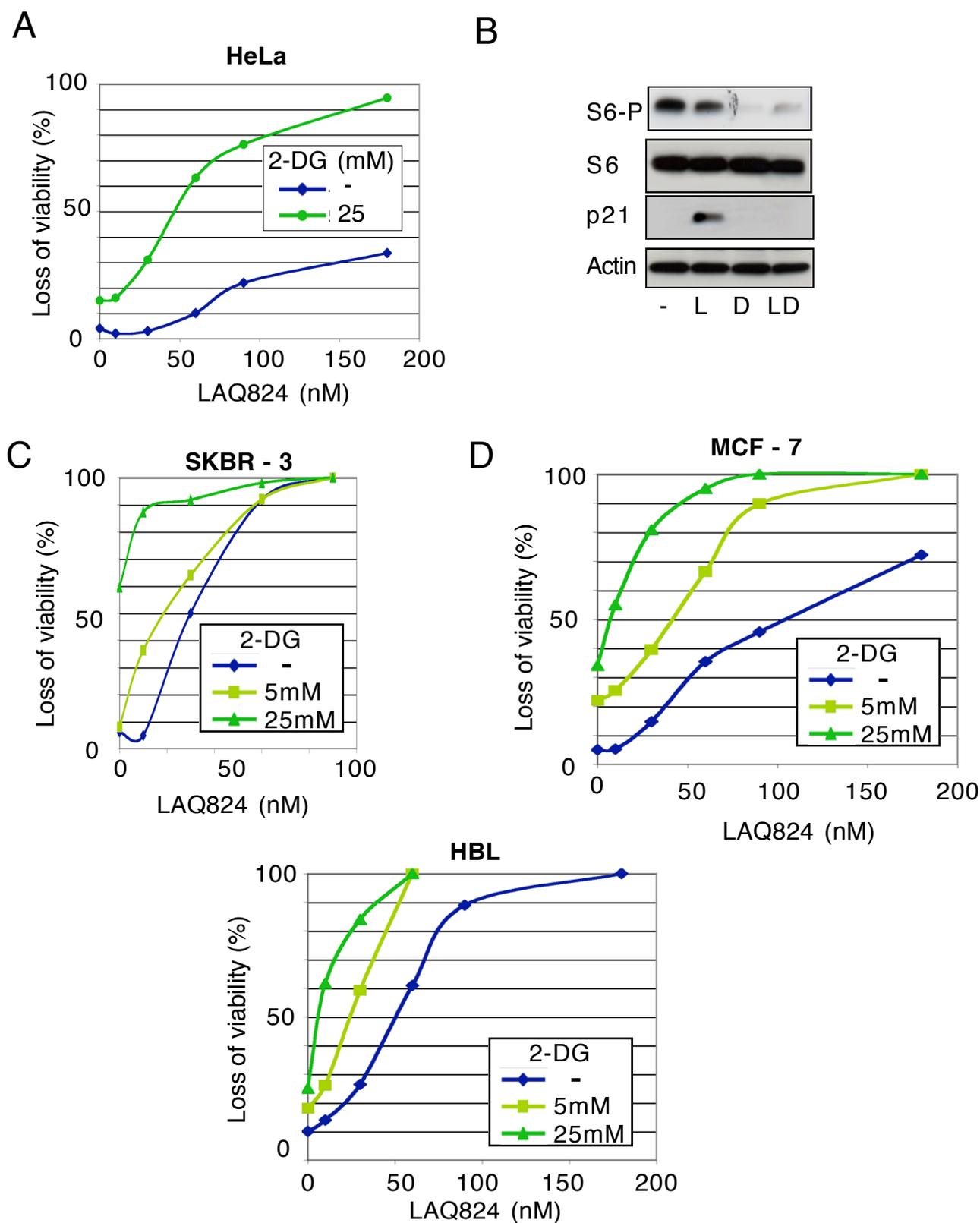
**Fig. 27. Effect of the sequential addition of LAQ824 or 2-DG on the cell survival of Hs683.**

(A) Hs683 was exposed to 60nM LAQ824 and p21 protein levels were determined at the indicated times by Western analysis. (B) Hs683 was treated with 60nM LAQ at time=0 and 25mM 2-DG was added simultaneously or after LAQ824 at the indicated times. For each sequential addition, cell death was measured after 72h treatment in the presence of both drugs, by FACS (C) The sequential addition was realized in the same way as in (B), but 2-DG was added first or before LAQ824 at the indicated times. Cell death was determined by FACS.



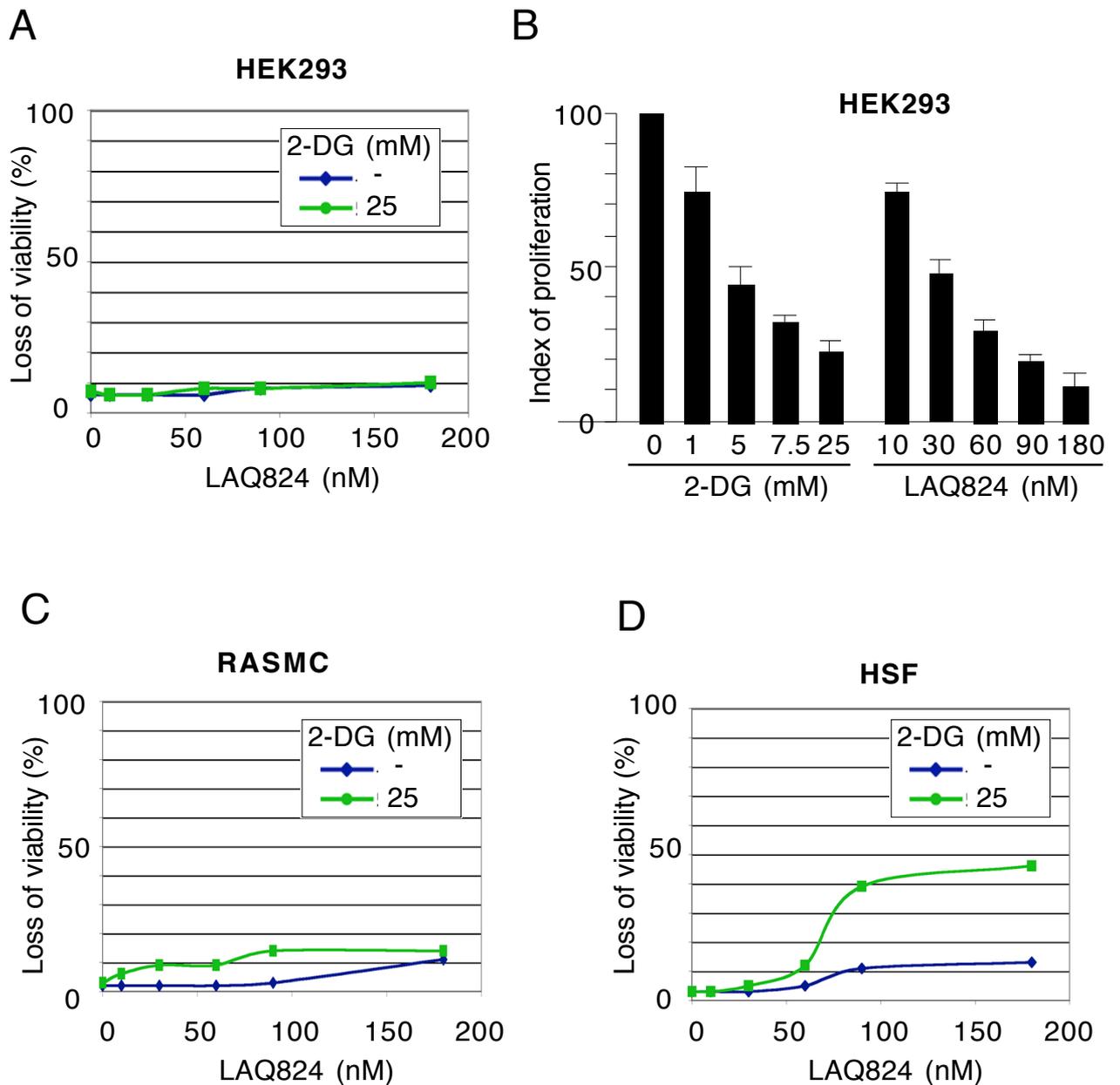
**Fig. 28. The synergistic induction of apoptosis in Hs683 cell line is not extensively linked with energy levels.**

(A) HS683 were treated with 4.5g/L (25mM) 2-DG and 60nM LAQ824 in presence or not of 4.5g/L pyruvate. Cell death was measure by FACS after 72h exposure to the compounds. (B) HS683 was exposed to 1 $\mu$ M oligomycin (O) in combination or not with 60nM LAQ824 for 72h and apoptosis was determined FACS. Analysis of p21 and actin proteins was done by Western after 8h exposure to the drugs. (C) Hs683 was incubated in glucose-free medium (-glc) in presence of not of 60nM LAQ824 (L) for 36h and cell death was determined by FACS. (D) Hs683 was treated with 200nM RAD001, 60 nM LAQ824 or the combination for 72h and cell survival was measured by FACS. The levels of p21, Actin and phosphorylation of S6 were determined by Western analysis after 24h exposure of Hs683 to 200nM RAD001, 60nM LAQ824 or both drugs.



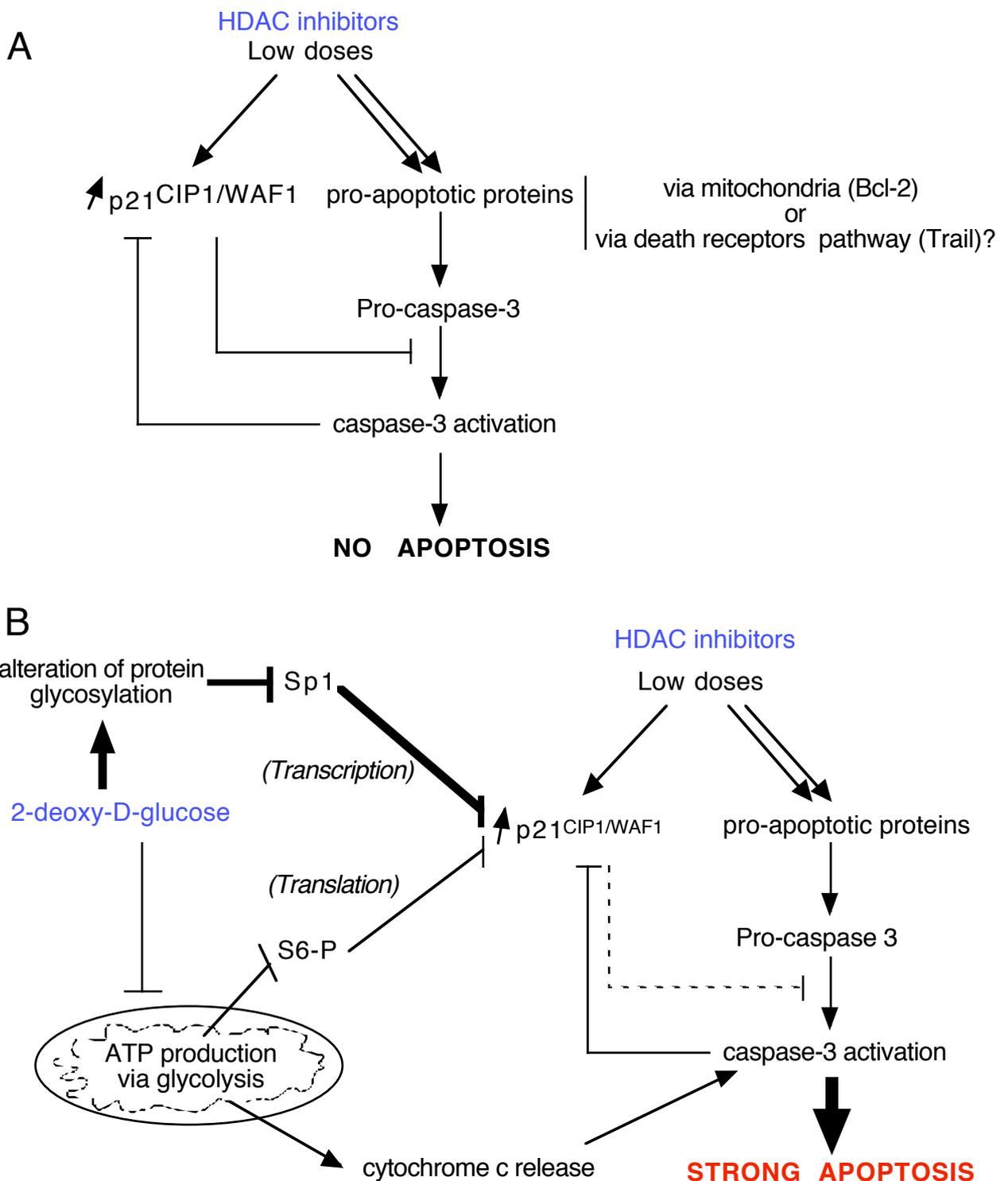
**Fig. 29. The combination of HDIs and 2-DG induces synergistically apoptosis in other cancer cell lines.**

(A) HeLa cell line was exposed to 25mM 2-DG and 60nM for 72h. Cell death was measured by FACS after Pi staining. (B) Phosphorylation and total levels of S6, p21 and Actin were determined by Western analysis after 12h exposure to 60nM LAQ, 25mM 2-DG or the combination of drugs. (C) Increasing doses of LAQ824 (10, 30, 60 and 90nM) and 5 or 25mM 2-DG were applied to SKBR-3 cell line and cell survival was determined by FACS after 72h (D) MCF-7 was treated as in (C), doses of LAQ824: 10, 30, 60, 90, 180nM (E) HBL was treated as in (D).



**Fig. 30. The combination of HDIs and 2-DG does not induce apoptosis in non-cancer cell line HEK293, RASMC, except in HSF.**

(A) HEK293 cell line was exposed to 25mM 2-DG and increasing amounts of LAQ824 (10, 30, 60, 90, 180nM) for 72h and cell death was measure by FACS after Pi staining. (B) HEK293 cells were exposed to increasing doses of 2-DG or LAQ824 for 72h and the total number of cells was determined. (C) The rat aortic smooth muscle cells (RASMC) were exposed to increasing doses of LAQ824 (10, 30, 60, 90, 180nM) and 25mM 2-DG for 72h and cell survival was determined by FACS (D) The human skin fibroblasts (HSF) was treated as in (C) and cell survival was determined by FACS.



**Figure 31: Model of the synergistic induction of apoptosis in cells by HDAC inhibitors and 2-DG**

(A) HDACs inhibitors (HDIs) are inducing reexpression of certain genes like p21, that regulate cell cycle progression through G1 phase and protect cells against apoptotic signals by blocking caspase-3 activation. In addition, HDIs allow reexpression of pro-apoptotic proteins that induce apoptosis via the mitochondria, like Bcl-2 or via the death receptors pathways, like Trail.

(B) In presence of the glycolytic inhibitor, the re-expression of p21 by HDACs inhibitors is abrogated via inhibition of transcription (by altering glycosylation of the transcription factor Sp1) that leads to a strong induction of apoptosis, via the mitochondrial or death receptor pathways. Inhibition of translation might also be involved in the decrease of p21 levels. In addition, 2-DG induces apoptosis via cytochrome c release.

**Dr. Viviane Egler**  
**Ph.D in Biochemistry (opt. neuro-oncology)**

Private Address: 86, rue des Lépiotes  
F- 01710 Thoiry (France)

Date of birth: March 03 1977

Nationality: French, E.U. citizenship

e-mail: [viviane.egler@sanofi-aventis.com](mailto:viviane.egler@sanofi-aventis.com)

Mobile : +33 (0) 6.77.82.29.14

**PROFESSIONAL EXPERIENCE**

Since July 2005

**Regulatory Affairs Officer at Sanofi-aventis, (Geneva, Switzerland)**

Responsible of regulatory affairs for oncology (Taxotere, Eloxatine...), diabetes (Lantus, Apidra...) and OTC portfolio, in an industrial merge and post-merge situation (Sanofi-Synthélabo with Aventis).  
Affiliate experience.

- Preparation of local documents and submission to Swiss HAs: new registration dossier as Acompla, variations dossiers for labelling, CMC), follow-up of existing registrations in order to maintain the product on the Swiss market.
- Analysis and constitution by critical way of registration dossiers in collaboration with Business Development and Marketing department.
- Collaboration with external consultants.
- Establish close interactions with local Health Authorities (Swissmedic).
- Advise marketing regarding promotional material and control of this material (Pharmacodex).
- Collaboration with Marketing, Corporate, Quality departments and production sites.
- Study of the CTD, clinical and post-marketing studies.
- Maintenance of labelling, leaflets and packaging material, publication by Documed.
- Updating of global and local data bases (MisAMM, DSOL, ...).

**EDUCATION**

Sept 2001-June 2005

**PhD in Biochemistry (opt. neurooncology)** in the department of research of the *University Hospital of Basel* (Switzerland), co-direction with *University of Strasbourg* (France).

Objective: rationally combine drugs for the treatment of human glioblastoma multiforme (GBM).  
Testing new treatments (new biological, cytotoxic and cytostatic drugs in preclinical development) in GBM cell lines:  
-Human glioma signaling pathways: impact of PI3K/Akt/mTOR axe (EGFR, VEGFR, PDGFR) and the energy pathway in cancer treatment, mechanisms of apoptosis. In collaboration with FMI, Basel.  
-*In vitro* drug screening. In collaboration with Novartis-Pharma, Basel.

2001 Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC, Illkirch, France)

Post graduate diploma in Molecular and Cellular Biology, directed by Prof Pierre Chambon, under the supervision of Dr Johan Auwerx and Dr Lluis Fajas.

Characterization of an important role of PPARgamma in cell cycle control and adipocyte differentiation through cross-talk with E2F and RB signaling pathways.

2000 Université Louis Pasteur (ULP), (Strasbourg, France)

Master's degree in Biochemistry

### TRAININGS

- 2009
- Autumn Introductory Course - TOPRA (Prague, Czech Republic)
  - e-CTD
- 2008
- Seminar on Biosimilars - SWISS BIOTECH ASSOCIATION (Bern, Switzerland)
- 2007
- Excel Level 2
  - German (Supercomm Geneva, Switzerland)
  - Colorectal cancer
- 2005
- Introduction in Regulatory Affairs, Pharmacovigilance – Swiss and european pharmaceutical regulatory system, *MARAS AG*, (Cham, Switzerland).
- 2002 - 2005
- Courses in cancer development and cancer treatments. (Biozentrum Basel, Switzerland)

### PUBLICATIONS

Egler V, Korur S, Faily M, Boulay JL, Imber R, Lino M M and Merlo A. Histone deacetylase inhibition and blockade of the glycolytic pathway synergistically induce cancer cell death. Clinical Cancer Research. 2008 May 15;14(10):3132-40.

Faily M, Korur S, Egler V, Boulay JL, Lino MM, Imber R, Merlo A.. Combination of sublethal concentrations of epidermal growth factor receptor inhibitor and microtubule stabilizer induces apoptosis of glioblastoma cells. Mol Cancer Ther. 2007 Feb;6(2):773-81.

Fajas L, Egler V, Reiter R, Miard S, Lefebvre AM, Auwerx J. PPARgamma controls cell proliferation and apoptosis in an RB-dependent manner. Oncogene. 2003 Jul 3;22(27):4186-93

Fajas L, Egler V, Reiter R, Hansen J, Kristiansen K, Debril MB, Miard S, Auwerx J. The retinoblastoma-histone deacetylase 3 complex inhibits PPARgamma and adipocyte differentiation. Dev Cell. 2002 Dec;3(6):903-10.

### COMMUNICATIONS

- 3rd Regional Oncology Symposium Landgut Castelen, Augst, Switzerland, May 2003. (oral)
- Forschungstag 2003, Departemente Chirurgie und Aneasthesie, Basel, August 2003. (oral)
- Neurex Symposium, Biozentrum, Basel, Switzerland, June 2003
- Keystone Symposium, Lake Tahoe (USA), February 2004 (Poster)

## ADDITIONAL QUALIFICATIONS

- IT skills:           Microsoft Word, Excel, Photoshop, Powerpoint and general knowledge of PC and Mac functions.  
                          Specific skills: MisAMM data base
  
- Languages:        French: mother tongue  
                          English: fluent  
                          German: level B2 (DFDB)
  
- Driving license since 1995