Combinatorial strategy using protein kinase inhibitors and a cytotoxic compound for highly resistant glioblastoma cells

-in vitro studies-

Inauguraldissertation

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Summary

Glioblastoma multiforme (GBM) is the most frequent and the most aggesssive malignant neoplasm of the human central nervous system (CNS), with a median survival of less than one year. These neoplasms are radio- and chemo-resistant, and are highly invasive, which renders their surgical resection inefficient. Because of the lack of efficiency of those conventional therapies, GBM patient prognosis has remained unchanged for the last forty years. Gene amplification and overexpression of the ErbB-related tyrosine-kinase receptor for epidermal growth factor (EGFR) are found in 40-50% of GBM, and correlate with progression of the disease and poor response to treatment. PKI-166 and AEE788 are small molecular weight protein kinase inhibitors (PKIs) of EGFR activity that can therefore potentially control progression of GBM.

In fact, PKIs against ErbB receptors did not induce apoptosis on GBM cells *in vitro*. We further assessed a pro-apoptotic effect of anti-ErbB PKIs in the presence of the microtubule stabilizer and cytotoxic drug patupilone applied at suboptimal doses. Combination of AEE788 with patupilone led to a synergistic induction of apoptosis in 50% of glioma cell lines. In the remaining resistant lines, the simultaneous use of PKIs against downstream EGF signaling mediators Phosphatidylinositol-3 kinase (PI3K) and mitogene-activated kinase/ERK kinase (MEK), Wortmannin and U0126, respectively, potentiated tumor cell apoptosis. The use of these particular drug combinations allows a novel approach for the treatment of glioblastoma patients.

Introduction

1.1 Cancer: general background

Cancer is a major cause of death in Western countries. For instance, in 2004, cancer was determined to be the cause of approximately 25% of deaths in Switzerland, while 15 000 new cases were diagnosed, according to the Federal Statistic Office (Quinto, 2004). Most cancers still lead to death, despite the progress in basic and clinical research, current treatment results only in a modest prolongation of life.

Environmental factors such as exposure to carcinogens (tobacco smoke) (Witschi *et al.*, 1995), food (Johnson, 2002) or UV light (Fisher & Kripke, 1977) play a critical role in the development of cancer. Carcinogenesis results from the accumulation of genetic alterations that target genes involved in the regulation of the cell-cycle, survival and genome integrity.

It is believed that a gain of oncogene function together with the loss of tumor-suppressor function cooperates to promote tumor development. Physiologically, many oncogenes accelerate cells during the G1/S phase of the cell cycle whereas tumor-suppressor genes act as blockers of cell growth and proliferation. Inactivation of tumor-suppressor genes requires alterations in both alleles as theorized by Knudson (2001). Alterations of oncogenes and tumor suppressor genes result from various genetic mechanisms, such as gene amplification, gain of function mutations, ectopic expression by translocation of oncogenes, and loss of heterozygosity (LOH), loss of function mutations (Pihan & Doxey, 2003) and also transcriptional silencing for tumor-suppressor genes (Merlo *et al.*, 1995; Herman & Baylin, 2003).

It is now widely accepted that the neoplastic process arises from a single cell and tumor progression results from the accumulation of acquired genetic alterations in tumor cells, allowing the selection of clones that have acquired growth advantage. This process leads to the progressive conversion of normal cells into cancer cells (Nowell, 1976). Mutations lead to self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, escape of apoptosis and sustained angiogenesis, all factors considered to be hallmarks of cancer cells (Hanahan & Weinberg, 2000). Upon an exogenous or an endogenous stress signal, a normal cell induces cell-cycle arrest and either repairs DNA damage or triggers the apoptotic machinery. In contrast, a cancer cell no longer induces

efficient cell cycle arrest and apoptosis, as a result of mutations accumulated in cell cycle, DNA repair and pro-apoptotic genes.

Mutations in cancer genes can be present in germline DNA, resulting in hereditary predispositions to cancer. Genes such as *NF-1, NF-2, PTEN, TSC1/2, MLH1/MSH2* (Turcot syndrome), *p53* (Li-Fraumeni syndrome) and *Rb* (He *et al.*, 1995) have been related to familial cancer syndromes (Fearon, 1997). On the other hand, cancer gene mutations can occur in a single somatic cell, resulting in the prevalent sporadic tumors. Among such genes involved in gliomagenesis are *Rb* (He *et al.*, 1995), *CDKN2A* which encodes p16/p14 (Labuhn *et al.*, 2001), *p27* (Alleyne *et al.*, 1999) and *HDM2* (Vogelstein & Kinzler, 2004).

1.2 Glioblastoma multiforme (GBM)

a. Main features

The most common neoplasms of the central nervous system (CNS) are of astrocytic origin. Like tumors of other cellular types, they show also resistance to chemo- and radio-therapy (Shapiro *et al.*, 1989). However, in contrast to other tumor types, they rarely metastasize via the cerebrospinal fluid (Giordana *et al.*, 1995), but show diffuse infiltration and rapid invasion of neighboring brain structures (Burger *et al.*, 1988).

The World Health Organization (WHO) classification into four grades (I-IV astrocytoma) is based on the degree of malignancy defined by histopathological criteria. Grade I is the benign pilocytic astrocytoma that rarely progresses to more advanced stages, whereas grades II to IV are malignant and infiltrate into the brain. Low-grade astrocytoma grade II patients have a survival time between 3 and 10 years. Grade III astrocytomas are anaplastic, with a mean survival of 2-5 years, and grade IV tumors also known as glioblastomas (GBM) are the most malignant forms, with a median of 10 to 12 months. The incidence rate per 100,000 population/year, adjusted to the World Standard Population, was 3.32 in males and 2.24 in females (Ohgaki *et al.*, 2004).

Progress in the field of molecular neuro-oncology revealed that the most relevant genes involved in gliomagenesis are those encoding growth factor receptors (e.g. *EGFR*), components of the cell cycle machinery (*Rb*, *Cdk4*, and the Cdk inhibitor $p16^{INK4a}$), and regulators of apoptosis (*p53*, *HDM2*, $p14^{ARF}$ and *PTEN*) (Maher *et al.*, 2001). These alterations have been shown to confer growth advantage not only to gliomas, but also to

tumors of various origins, leading to uncontrolled cell proliferation, high potential of invasion and drug resistance.

b. Molecular and genetic aspects of glioma development

GBMs are divided into two subgroups based on clinical features (Scherer, 1940). On one hand, primary GBMs arise as a *de novo* process, without a pre-existing lesion. On the other hand, the secondary GBMs regarded as grade IV astrocytomas, develop progressively from lower grade astrocytomas generally over a period of 5-10 years (Collins 1998 & Rasheed *et al.*, 1999). Remarkable insights into the origin and the behaviour of gliomas have emerged from *in vitro* cellular and *in vivo* animal models. It appears that GBMs can arise from adult neural progenitor cells and/or dedifferentiated astrocytes (Maher *et al.*, 2001). Astrocytes or oligodendrocytes may undergo dedifferentiation to multipotent progenitor cells as the result of an accumulation of genetic mutation(s) (Maher *et al.*, 2001; Bachoo *et al.*, 2002) that alter signal transduction pathways activated by receptor tyrosine kinases (RTKs) and cell cycle arrest regulation (Figure 1). Taken together, these observations have allowed novel cellular and molecular subclassifications that provide bases to improve diagnosis (Hermanson *et al.*, 1992; Leihues & Cavenee, 2000; Maher *et al.*, 2001; Holland, 2001).

Receptor tyrosine kinases

Components of mitogenic pathways, in normal and neoplastic cells, are activated by protein kinases located upstream in the signaling cascade. Among the growth factors and their receptors overexpressed in human malignant astrocytomas are the epidermal growth factor receptor (EGFR), the platelet-derived growth factor (PDGF) and its receptor, PDGFR. The *EGFR* gene is amplified in approximatively 50% of GBMs (Maher *et al.*, 2001), 40% of those express a truncated form of EGFR, that lacks a large portion of the extracellular ligand binding domain, resulting in a constitutive activation of the receptor (Ekstrand *et al.*, 1994). On the other hand, PDGF and PDGFR are expressed at high levels by malignant astrocytoma cell lines and tissue without gene amplification (Guha *et al.*, 1995), suggesting autocrine and/or paracrine stimulation.

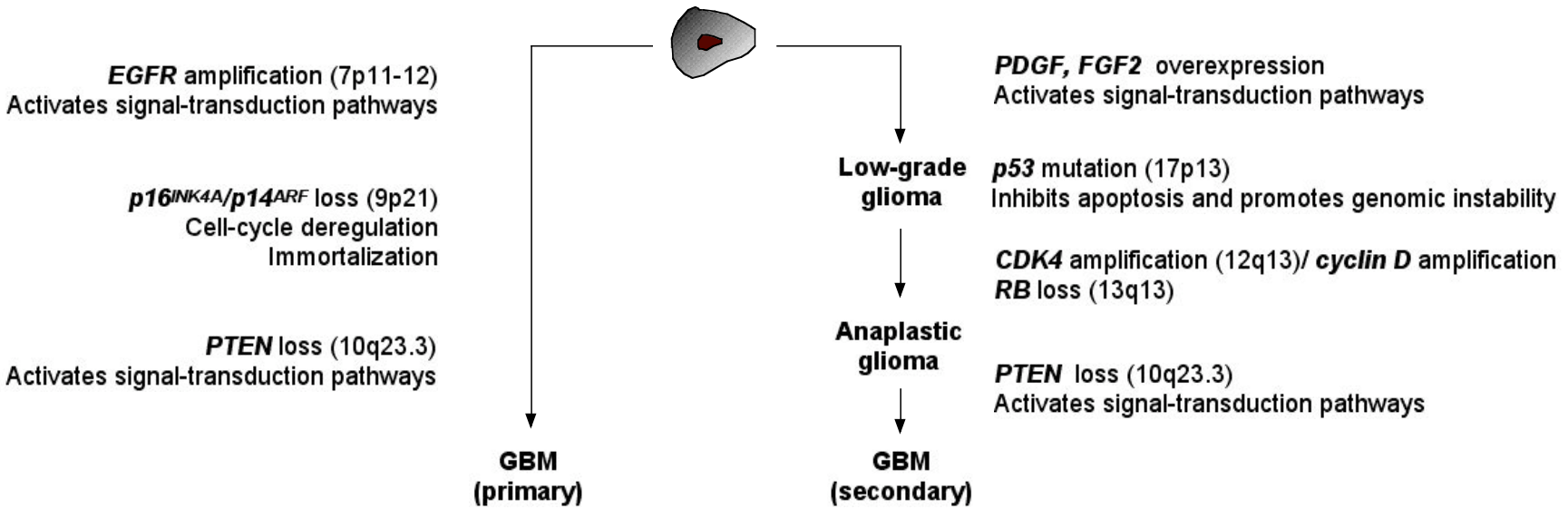


Figure 1: Pathways of gliomagenesis. Genetic analyses of glioblastoma show that primary and secondary glioblastoma occur through different genetic alterations. Secondary glioblastoma evolve through recurrence of lower grade astrocytoma. Acquiring mutations in *p53*, overexpression of *PDGFR*, *Rb* loss, *CDK4* amplification and *PTEN* loss. *EGFR* overexpression, *INK4A*/*ARF* deletion and *PTEN* mutations are typical features of primary glioblastoma that occurs *de novo*.

From these observations the idea has emerged to target growth factor pathways as a potential strategy of cancer therapy. The development of selective protein kinase inhibitors that can block or modulate diseases caused by abnormalities in these signaling pathways is widely considered as a promising approach for drug development. Two famous examples of tyrosine kinase activation have been used as targets for screening potentially inactivating low molecular weight compounds (Capedeville *et al.*, 2002). The BCR-ABL fusion protein, which results from the translocation of chromosomes 9 and 22 (Philadelphia chromosome) has been defined as the cause of chronic myeloid leukemia (CML) (Rowley, 1973). This allowed the identification of a new selective tyrosine kinase inhibitor, Gleevec. Interestingly, Gleevec has also been shown to block c-KIT constitutive activation in gastrointestinal stromal tumor (GIST) (Tuveson *et al.*, 2001). Following the same approach, mutations of EGFR in non-small cell lung cancer (NSCLC) are targeted by Gefinitib. These were the first examples of molecules that have resulted from the translation of basic cancer research into cancer therapeutics (Capedeville *et al.*, 2002; Linch *et al.*, 2004).

Cell-cycle deregulation

A hallmark of high-grade astrocytomas is their high mitotic activity. As a key checkpoint of mitosis, G1/S phase transition is regulated by the retinoblastoma tumor-suppressor (RB). Not surprisingly, the cyclin-dependent kinase inhibitor regulatory circuit RB– CDK4/6– $p16^{INK4a}$ is frequently disrupted in brain tumors. Homozygous deletion of $p16^{INK4a}$ and $p14^{ARF}$, both encoded by the same gene, *CDKN2A* are observed in 41% of GBMs (Labuhn *et al.*, 2001), while the combined amplification of CDK4 and loss of RB are identified in 14 to 33% of GBMs (He *et al.*, 1995; Fulci *et al.*, 2000). The remaining GBMs are due to mutations in other genes of this pathway (cyclin D1 and CDK6) (Costello *et al.*, 1997; Buschges *et al.*, 1999). Taken together, GBMs harbour mutations in the $p16^{INK4A}$/CDK4/RB pathway, whereas such mutations are rare in low-grade astrocytomas.

The tumor suppressor p53 regulates cell cycle progression and apoptosis (Fischer, 2001). Early loss of p53 in astrocytomas confers evasion of apoptosis. Healthy cells exposed to genotoxic stress either enter cell-cycle arrest or undergo apoptosis. Patients with the inherited Li-Fraumeni cancer syndrome carry a germ line mutation in the *TP53* gene and are predisposed to the development of various tumors, including astrocytomas (Malkin *et al.*, 1990; Srivastava *et al.*, 1990). Mutations in *TP53* are uniformly found in more than 60% of all grades of sporadic astrocytomas (Sidransky *et al.*, 1992).

Phosphatase and tensin homolog (PTEN) and Akt/PKB

LOH on chromosome 10 is the most frequent genetic alteration found in GBM. It was crucial to understand to which extent the loss of genetic material of this chromosome contributes to tumor development. Mutations in *PTEN* occur in approximatively 30% of primary GBMs, and only in 4% of secondary GBMs (Ishii *et al.*, 1999; Tohma *et al.*, 2000). The tumor-suppressor gene *PTEN* is located on chromosome 10q23.3. It negatively regulates PI3K by dephosphosphorylating phosphatidylinositol triphosphate (PIP_3) (Fig. 5). In PTEN mutant cells, PI3K hyperphosphorylates AKT (Maehama & Dixon, 1999) a modulator of survival, invasion and proliferation (Maier *et al.*, 1999; Ignatoski *et al.*, 2000). PTEN was shown to mediate growth inhibition (Furnati *et al.*, 1998) and to play a role in cell motility (Maier *et al.*, 1999). Finally, mutant PTEN status may be linked to an aggressive clinical course (Lin *et al.*, 1998; Rustia *et al.*, 2001).

Mismatch repair genes

The vast majority of glioblastomas are sporadic, with a few exceptions, e.g. the rare Turcot's syndrome predisposes to glioblastoma (Hamilton *et al.*, 1995). Turcot's syndrome is characterized by mutations in the DNA mismatch-repair genes mutS homologue 2 (hMSH2) or mutL Homologue 1 (hMLH1) operative during cellular replication (Jiricny & Nystrom-Lahti, 2000) while mutations at these loci are rarely observed in sporadic glioblastomas (Merlo, 2003). Survival of GBM patients with germline mutations in mutator genes is exceptionally high (3, 6 and 14 years as compared to about 8 months in sporadic cases) (Hamilton *et al.*, 1995), but not in all cases (Merlo *et al.*, 1996)

1.3 Glioma animal models

a. Introduction

Over the last 15 years, cellular, molecular and genetic comparison between glioma cell lines and normal brain tissue has provided essential insight into the biology of the disease. More recently, the microarray technology has allowed the identification of thousands of genes differentially expressed in glial tumors (Lal *et al.*, 1999). However, this approach only reveals an association between genetic expression changes and the neoplastic phenotype and from a mechanistic point of view, and does indicate genetic alterations that initiate

gliomagenesis. Such questions can only be solved by stably introducing the suspected genetic alterations into an *in vivo* experimental system and further testing for their ability to develop glial tumors, spontaneously, or in a given genetic background. For this reason, several genetic alterations hypothesised to initiate glioblastoma development have been stably introduced into the genomes of mice.

These animal models were especially adapted to these needs, they have several characteristics that make them valuable test systems, including defined and reproducible location of tumor formation, rate of tumor growth and life span. Animal models do not only provide information on the potential causes of astrocytoma formation, but they may also allow to provide novel targets for therapy and tumor-bearing animals for preclinical trials.

b. Models

Several transgenic mice that ectopically express oncogenes and that have been targeted for tumor suppressor genes (knock out) have been recently developed. A summary of mouse brain tumor models has been described in the Annex I. Interestingly, Holland and co-workers have shown that the co-expression of activated RAS and AKT in normal astrocytes or neural progenitors cells in mice induced GBM, whereas neither AKT nor RAS alone are sufficient to induce such neoplasm (Holland *et al.*, 2000). In addition, a recent study has shown that $p14^{ARF}$ loss combined with transgenic *Ras* and *Akt* in both astrocytes and glial progenitor cells, resulted in gliomagenesis. In this study, $p16^{INK4A}$ had a tumor suppressor function limited to glial progenitor cells (Uhrbom *et al.*, 2005). In contrast, others have shown that constitutive high Ras transgene expression in astrocytes can induce gliomas while a moderate expression level leads to low-grade atrocytoma development (Ding *et al.*, 2001).

On the other hand, in GBM, loss of function of *Ink4a/Arf* is frequently associated with EGFR activation (Holland *et al.*, 1998; Bachoo *et al.*, 2002). While Holland and co-workers (1998) proposed that tumors arise more efficiently from immature glial cells, another study demonstrated that both mature astrocytes and neural stem cells can serve as the cell-of-origin for gliomagenesis (Bachoo *et al.*, 2002). Their hypothesis that biological behaviour of GBM depends on disregulation of specific genetic background and not on the state of glial differentiation is supported by astrocytes dedifferentiation that undergo into a multipotent progenitor cell during tumorigenesis (Bachoo *et al.*, 2002).

Thus, genetic models have added significantly to the understanding of the cellular characteristics observed in malignant gliomas. This suggests potential ways for therapeutic

intervention in a broad range of tumors. This finding that different genetic alterations can generate malignant gliomas indicates that targeting proteins in crucial cellular pathways might be more successful than disrupting the function of a single protein.

However, transgenic and knock out mice models, in which only specific genes or combination thereof are targeted, do not take into account the genetic instability of tumor cells that generates the accumulation of additional genetic alterations occurring during tumorigenesis.

1.4 Molecular aspect of targeted therapies

a. Why targeting ErbB receptors?

The epidermal growth factor receptor (EGFR) family, also known as ErbB receptor family represents the subclass I of the RTK superfamily, and includes four members: EGFR/ErbB1/HER1, ErbB2/Neu/HER2, ErbB3/HER3, and ErbB4/HER4. They are normally expressed in a variety of tissues of epithelial, mesenchymal and neural origin, and play a fundamental role in cell development, proliferation, differentiation, migration and survival (Olayioye *et al.*, 2000; Holbro *et al.*, 2003). The *EGFR* gene, located on human chromosome 7p11-12, encodes a 170 kDa protein (EGFR/ErbB1/HER1) (Kondo *et al.*, 1983).

All members of the ErbB family have in common an amino-terminal extracellular ligand-binding, a single hydrophobic transmembrane and a carboxy-terminal cytoplasmic protein kinase domain. In the absence of ligand binding, EGFR is present as monomer and as dimer (Sako *et al.*, 2000). Ligand binding to ErbB receptors induces formation of different combinations of homo- and heterodimers, ErbB2 being its preferred partner (Graus-Porta *et al.*, 1997).

Signaling pathways activated by EGFR autophosphorylation

Dimerization leads to activation of the intrinsic kinase domain, which results in phosphorylation on specific tyrosine (Tyr) residues within the cytoplasmic tail of the receptor. These phosphorylated residues serve as docking sites for a variety of signaling molecules whose recruitment leads to the activation of intracellular pathways as described in Fig. 2a and b (Graus-Porta *et al.*, 1997; Yu *et al.*, 2002).

It has been shown that c-Src is involved in phosphorylation of the EGF receptor on Tyr845, which is associated with modulation of receptor function (Biscardi *et al.*, 1999). Phospho- Tyr 992 is a direct binding site for the phospholipase C-γ (PLC-γ) SH2 domain and results in activation of PLC-γ mediated downstream signaling (Emlet *et al.*, 1997). Phosphorylation of Tyr1045 creates a major docking site for Cbl-b that leads to assembly of ubiquitination machinery to the receptor, and enables receptor ubiquitination and degradation (Ettenberg *et al.*, 1999; Levkowitz *et al.*, 1999). Phospho-Tyr 1068 is a direct binding site for the Grb2/SH2 domain, whereas phospho-Tyr 1148 provides a docking site for Shc. Phospho-Tyr 1068 and phospho-Tyr 1173 are the major sites of autophosphorylation, which occurs as a result of EGF ligand binding (Batzer *et al.*, 1994; Rojas *et al.*, 1996; Ward *et al.*, 1996; Sakaguchi *et al.*, 1998).

All these sites are involved in activation of the Ras/MAP kinase signaling pathway (Rojas *et al.*, 1996; Zwick *et al.*, 1999). These phosphorylated residues in the EGFR and the activation of the corresponding signaling transduction pathways are summarized in Fig. 2a. In addition, ErbB2 was shown as the preferred heterodimerization partner within the ErbB family as it decreases ligand dissociation from the receptor heterodimer thus enhancing and prolonging the activation of the MAPK signaling pathway (Fig. 2b) (Graus-Porta *et al.*, 1995; Moriki *et al.*, 2001)

As described in chapter 1.2.b, genetic alterations that activate RTK signaling pathways frequently cooperate in the course of gliomagenesis. Moreover, aberrant EGFR and ErbB2 expressions have been associated with advanced stages in a number of tumor types (Annex II) and poor patient prognosis (Slamon et al., 1987; Allred *et al.*, 1992; Hynes & Stern, 1994; Salomon *et al.*, 1995; Nicholson *et al.*, 2001; Sjogren *et al.*, 2001). From these observations, strategies to develop drugs that target EGFR/ErbB2 have been designed (Annex III).

EGFR and glioblastoma

Amplification of the gene coding for *EGFR* has been observed in 40-50% of primary primary GBMs, but is rare in secondary GBMs (Lang *et al.*, 1994; Schlegel *et al.*, 1994). Glioblastoma with EGFR gene amplification typically show simultaneous loss of chromosome 10 (von Deimling *et al.*, 1992; Lang *et al.*, 1994) as well as deletion of the *INK4a*/*Arf* locus (Hegi *et al.*, 1997), while *TP53* is wild-type (Fulci *et al.*, 2000). EGFR

expression correlates with poor response to treatment, disease progression, and poor survival (Wong *et al.*, 1992; Salomon *et al.*, 1995; Nicholson *et al.*, 2001).

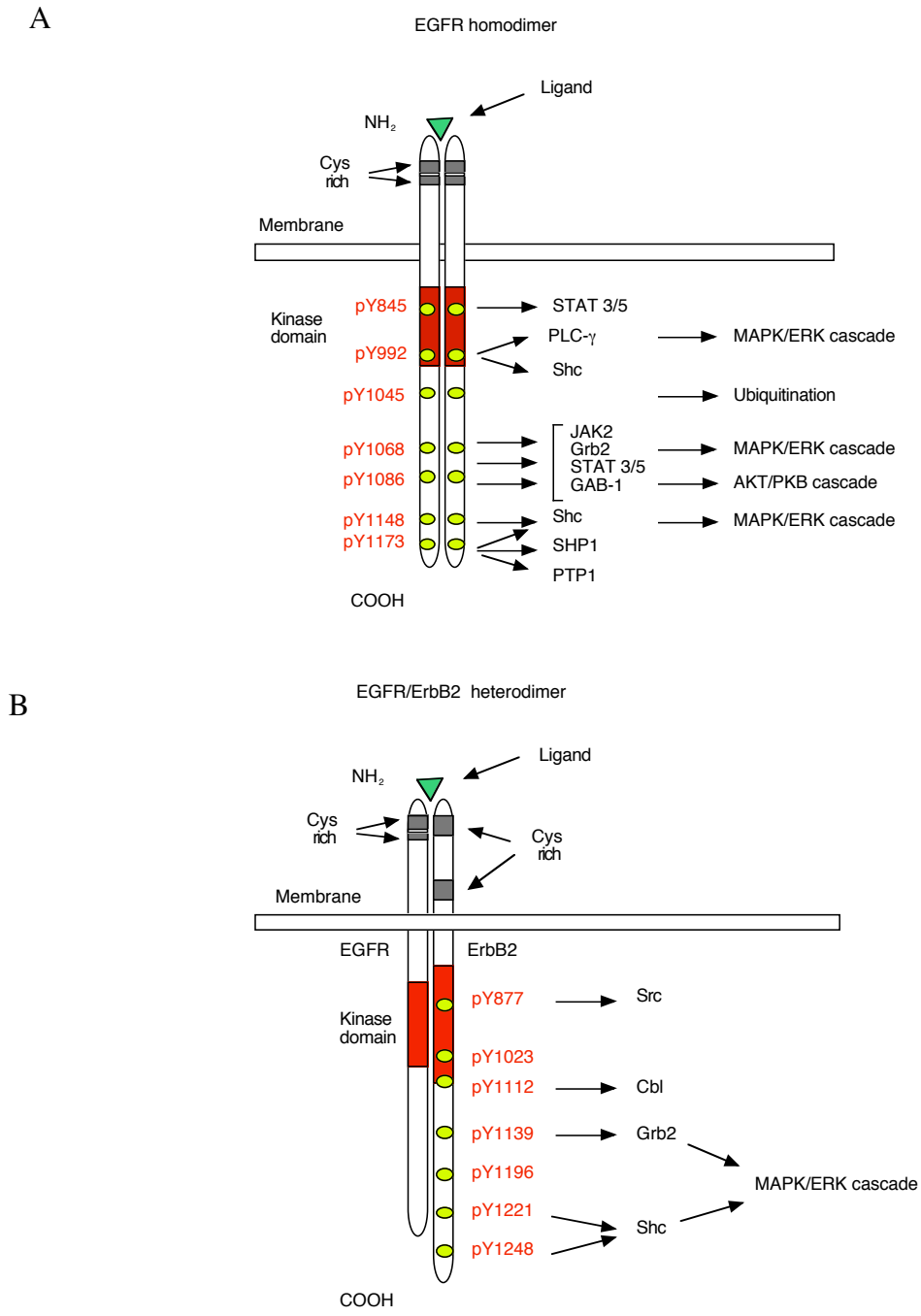


Figure 2: Autophosphorylation sites on the EGFR protein. We considered EGF alone as ligand (A) In absence of ErbB2 monomers, activation of the corresponding major signaling transduction pathways after EGFR homodimerization. (B) In presence of ErbB2 monomers, activation results in EGFR/ErbB2 heterodimers.

Mechanisms of activation and inhibition

In conclusion, aberrant activation of the mitogenic signals initiated by EGFR in GBMs, its mutant form and ErbB2 described in the literature (Fig. 2a and b) have been considered as particularly suitable targets to be inhibited by drug design for glioma therapy. Selective low molecular weight compounds have been developed to interact with the ATP binding site of the receptor as well as antibodies targeting either the extracellular ligand-binding region of EGFR or the intracellular tyrosine kinase region. Both approaches could result in interference with the signaling pathways that modulate mitogenic and other cancer-promoting responses such as cell motility, cell adhesion, invasion and angiogenesis.

b. PI3K/Akt (PKB) signaling pathway

EGFR can be activated by several ligands that lead to the activation of two disctinct pathways: the MAPK pathway downstream of Ras and the phospatidylinositol 3-kinase (PI3K) /Akt (PKB) pathway. The PI3K/Akt (PKB) pathway is a major survival pathway in epithelial cells, which also regulates other cellular processes such as proliferation, growth and cytoskeletal rearrangement (Vivanco & Sawyers, 2002). Several components of the PI3K/Akt (PKB) pathway are dysregulated in numerous types of human cancers. EGF stimulation results in the activation of PI3K, which generates phosphatidylinositol-trisphosphate (PIP_3) in the membrane, a second messenger activating downstream pathways including AKT (Datta *et al.*, 1999). AKT is a serine/threonine kinase with a wide range of substrates and is activated by phosphorylation at Thr308 and Ser473 (Andjelkovic *et al.*, 1997). Thr308 is phosphorylated by the 3-phosphoinoside-dependent protein kinase PDK1, whereas Ser473 was found to be phosphorylated by a DNA-dependent protein kinase (DNA-PK) (Feng *et al.*, 2004). Three main isoforms of AKT have been identified in mammalian cells: AKT1, 2 and 3, AKT1 may play a role in the pathogenesis of gastric adenocarcinoma (Staal, 1987), and AKT2 was found to be amplified in two ovarian carcinoma cell lines (Cheng *et al.*, 1992). In addition to AKT (Fig. 3), other signaling pathways are known to be regulated by PI3K activity and might be involved in PI3K-mediated tumorigenesis. Mutations in *PIK3CA* that encodes the p110 catalytic subunit of PI3K were also identified in 27% of glioblastomas (Samuels *et al.*, 2004).

Activation of the PI3K/Akt (PKB) pathway in brain tumors is due to excessive stimulation by growth factor receptors and by RAS. Loss of function of the tumor suppressor

gene PTEN also frequently contributes to upregulation of PI3K/Akt. In addition, active AKT was often detected in several human cancers *in vivo* (Holland *et al.*, 2000) and has been linked with poor clinical outcome (Lee *et al.*, 2001; Brognard *et al.*, 2001; Ermoian *et al.*, 2002; Perez-Tenorio & Stal, 2002). Moreover it has been correlated with resistance to chemotherapy and radiation (Brognard *et al.*, 2001; Clark *et al.*, 2002). Thus, these observations provide strong arguments for designing drugs that specifically target the PI3K/Akt (PKB) signaling pathway or the RTK upstream activators.

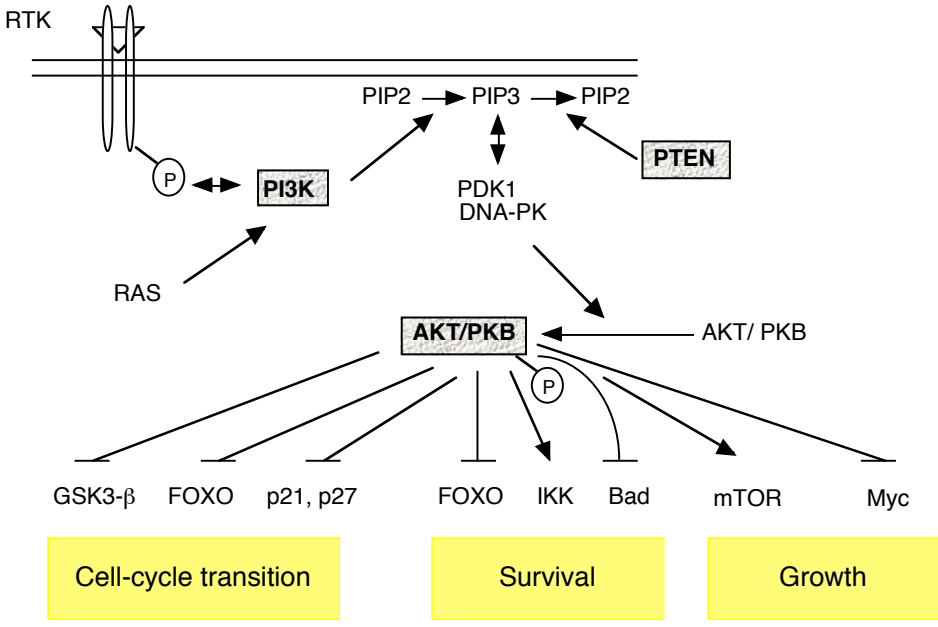


Figure 3: The PI3K/Akt (PKB) signaling pathway. Activated AKT mediates the activation and the inhibition of several targets such as mTOR, IKK, Bad, p21, p27, resulting in cell growth, survival (apoptosis inhibition) and proliferation through various mechanisms.

c. RAS/RAF/MEK/ERK

In the last decade, research has been focusing on the central importance of the Ras pathway. In tumorigenesis, it is one of the well-characterized signal-transduction pathways. This pathway is stimulated by PDGFR, EGFR and other RTKs (Downward, 2003). RAS is active when bound to guanosine triphosphate (GTP). Adapter proteins such as Grb2 bring the guanine nucleotide exchange factor (GEF) Sos to the receptor to form a stable complex, which is required for the activation of the membrane-bound RAS. RAS is inactive when the

GTP is hydrolysed to guanosine diphosphate (GDP). In GTP-bound form, RAS proteins have essential roles in controlling the activity of crucial signaling pathways, most notably the RAF pathway, which leads to the activation through phosphorylation of the mitogen-activated protein kinase (MAPK) also named ERK 1/2 (extracellular regulated-signal kinase 1 and 2). Substrates of ERK1/2 include cytosolic and nuclear proteins, reflecting the fact that they can be transported into the nucleus following activation (Fig. 4) (Downward, 2003).

Mutated and constitutively activated forms of Ras are found in around 50% of all human metastatic tumors (Chambers & Tuck, 1993). Although no RAS mutations have been detected in gliomas, high levels of Ras-GTP have been documented in high-grade astrocytomas (Guttmann *et al.*, 1996; Guha *et al.*, 1997). However, it was suggested that phosphorylated ERK (P-ERK) plays a role in the enhanced transformation and resistance to apoptosis seen in GBM (Wu *et al.*, 1999). Targeting RAS proteins and the downstream signaling pathway would therefore be valuable in treating tumors that have high activity of ERK.

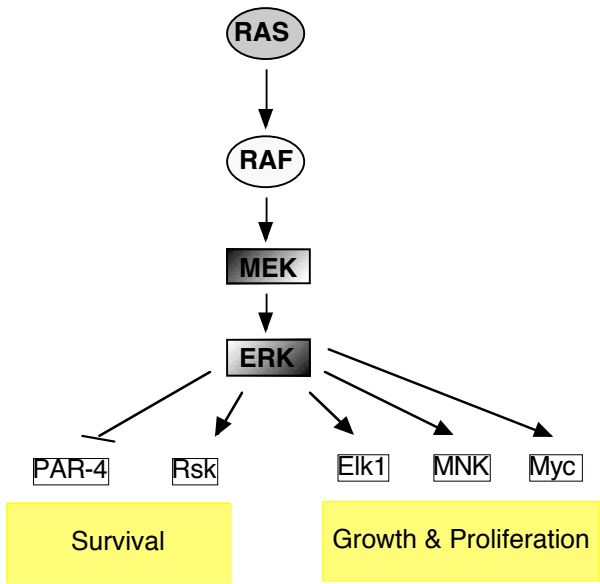


Figure 4: The RAS/RAF/MEK/ERK signaling pathway. The signaling pathway regulates survival, growth and cell-cycle progression through various mechanism such as RSK (p90 ribosomal protein S6 kinase), an inactivator of BAD, PAR-4, a transcriptional repressor of *Bcl2*, Elk1 and Myc, which are transcription factors involved in cell-cyle transition and in addition for myc, growth regulation. MNK (p38 MAPK –dependent kinase) can activate the translation initiation complex elF-4E to promote protein translation and cell growth.

1.5 Drug resistance

The recent success of Gleevec in chronic myeloid leukemia (CML) and in gastrointestinal stromal tumor (GIST), Herceptin (breast) and Iressa in non-small cell lung cancer (NSCLC), have opened new hopes in the treatment of cancer. Gleevec has established a paradigm for the treatment of tumors whose growth is acutely dependent on specific kinase targets. CML is driven by the constitutive ABL kinase activity of the fusion protein BCR-ABL resulting from the Philadelphia chromosomal translocation. Even if patients with late chronic-phase CML with which previous interferon therapy had failed (Kantarjian *et al.*, 2002), or patients with accelerated-phase CML (Talpaz *et al.*, 2002), Gleevec has been established as a safe and effective therapy in CML, except on the later stage of blast crisis (Sawyers *et al.*, 2002). The question is whether an analogy can be made between blast crisis and GBM, if both are considered as the most advanced stages of CML and gliomas, respectively. It has been shown that tumor cells in blast crisis have accumulated additional mutations that reactivate BCR-ABL, and therefore circumvent Gleevec treatment (Gorre *et al.*, 2001).

Single drug treatments are found to be insufficient, possibly because numerous genetic alterations have accumulated during gliomagenesis. A new strategy has to be reconsidered to counteract the redundant pathways. The first problem to be solved with GBM is to define the redundant pathways that may provide new potentially relevant targets for glioma therapy. In animal models, only few mediators of the growth factor/Ras, PI3K/PTEN/AKT, p53/ARF/HDM2 and p16/Rb/cyclinD/CDK4 pathways are targeted. In human gliomas, many additional genes and pathways are altered due to a high level of genetic instability leading to the accumulation of countless epigenetic and genetic alterations.

This is the reason why it is now conceivable that future therapies will select various combinations of drugs. There are obvious advantages to such approaches: (i) the drug specificity should lead to a greater therapeutic window and less toxicity; (ii) the use of combined therapies may limit acquired drug resistance; and (iii) many of the defective pathways in tumor cells also affect drug or radiation sensitivity. Combined therapies using conventional and designed biological agents may be especially effective.

1.6 Specific question

Molecular genetics has allowed fundamental advances in the knowledge of cancer biology (Hanahan, 2000). This knowledge has been exploited with success to designate cancer pathways, and use them as targets for low molecular weight compounds having potential anti-tumorigenic effects. For example, this strategy had been applied to identify the Gleevec molecule treating for chronic myeloid leukemia and gastro-intestinal tumor (Capdeville *et al.*, 2002), and Iressa for non-small cell lung cancer patients (Sordella *et al.*, 2004). However, no equivalent drug has been yet isolated for interfering with glioma progression.

With the aim to find drugs for the treatment of glioma that had been identified in the past in other cancer types using same signaling pathways. Further, we have evaluated drugs used in combination for a possible additive or synergistic effects. To achieve this goal, we applied the following approaches:

a) Protein kinase inhibition, single and combined drug approach

We investigated for the potential anti-tumor effect of protein kinase inhibitor (PKI) drugs. Because of the relevance of PKI such as Gleevec, PKI-166 and AEE788 as anti-tumor agents, we analyzed GBM cells for cell survival and induction of apoptosis by treatment of these compounds alone or in combination.

b) PKI and cytotoxic drug

Combination of the most promising PKI determined in the first part of the study, as a possible sensitizer with a cytotoxic compound in order to obtain additive or synergistic induction of apoptosis on glioma cell lines. The aim is to reduce toxicity of higher drug concentrations.

c) Targeting downstream key signaling components PI3K and MEK

Based on mouse models, altered components of distinct signaling pathways leading to gliomagenesis have been defined. Therefore, if two specific pathways have to be altered to develop a glioma in a mouse, we conclude that we have to specifically target them in order to at least block the progression or even better, kill the neoplasm. Several proteins have been shown to confer resistance to tumor cells and should, therefore, provide novel and defined

targets for cancer treatment. In this context, we investigated for pro-apoptotic effect by targeting specific downstream components of the EGFR pathway supposed to be involved in resistance mechanisms observed with the different drugs tested.

Results

1. Single drug treatment

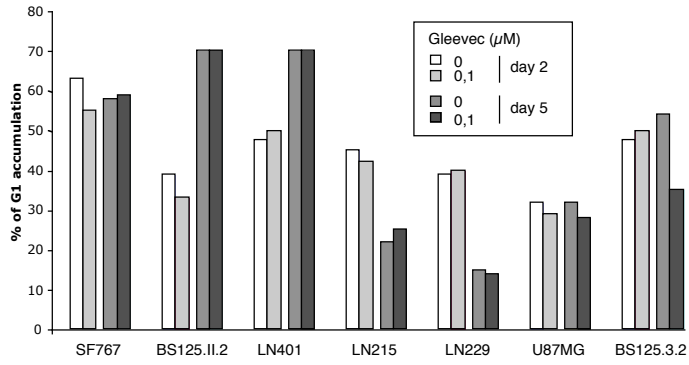
a. Gleevec

Gleevec® (Imatinib, Glivec or STI571) is registered in many countries for the treatment of CML in the accelerated phase or chronic phase as well as for the treatment of metastatic or recurrent GIST. Gleevec® is a protein-tyrosine kinase inhibitor which specifically targets the activated ABL oncoprotein, BCR-ABL (CML*),* PDGF receptor, C-KIT (Kilic *et al*., 2000).

PDGF is a ubiquitous growth factor with mitogenic properties in connective tissue and glial cells that also promotes angiogenesis. Under pathological conditions, the PDGF-dependent mitogenic pathway is involved in cancer and connective tissue disorders. PDGF plays a fundamental role in glial development (Maher *et al*., 2001), while PDGF receptors are expressed in most types of gliomas (Hermanson *et al*., 1992). Of note, PDGF receptor-A (PDGFR-A) amplification is only found in anaplastic oligodendrogliomas with glioblastomatous features (Smith *et al*., 2000). A number of experiments using antibodies neutralizing PDGFR and dominant-negative mutations of either PDGF or PDGFR suggest that PDGFR represents a potentially important therapeutic target in GBM treatment. These findings provided a rationale for testing Gleevec® in human gliomas in which these pathways may contribute to malignant growth.

The effect of on cell cycle profile of different glioma cell lines was determined (Fig. 5a). The drug did not affect the cell-cycle at a concentration of 0.1 μM except in BS125.3.2 cells, where a decrease of G1 phase accumulation was observed after 5 days. These observations led us to conclude that Gleevec does not affect cell cycle at this concentration. Since the anti-proliferative activity of Gleevec has been shown on cell lines derived from human bladder carcinomas, colon carcinomas and glioblastomas (Kilic *et al*., 2000), the drug was applied on glioma cell lines to confirm its effect on proliferation (Fig. 5b).

A



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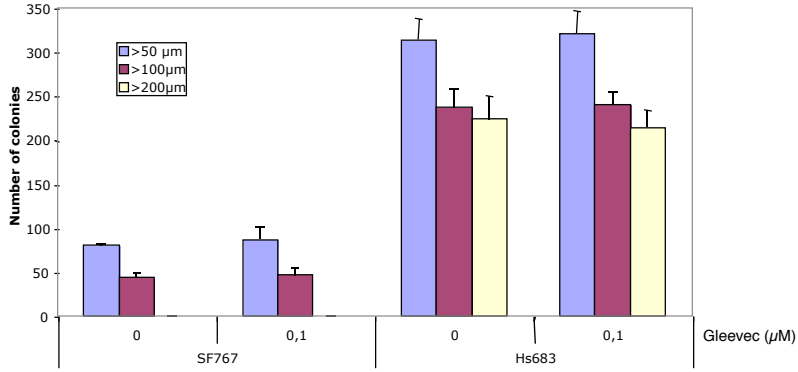

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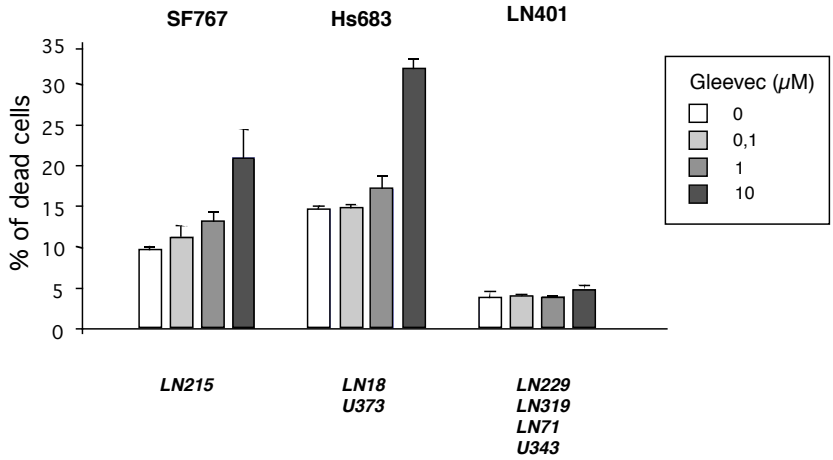


Figure 5: Cytostatic effect of physiological concentrations of Gleevec on glioma cells. (A) Cell cycle profile of glioma cell lines under Gleevec treatment. Cells were incubated with Gleevec during 2 or 5 days. G1 phase accumulation was determined by using flow cytometry. (B) Proliferation determined by Colony Forming Assay (CFA). SF767 and Hs683 cell lines were treated during 3 days with 0.1 μM of Gleevec. The size of the colonies was defined by using a colony counter that measures three cutoffs for colony size determination (> 10, >50, >200 μm). (C) Ability of Gleevec to induce apoptosis in glioma cell lines. Cells were treated with increasing concentrations of the drug (0; 0.1; 1 and 10 μM) during 4 days. Apoptosis was detected by using flow cytometry. Experiments were done in triplicate.

At a 0.1 μM concentration, no anti-proliferative activity was observed in the glioma cell lines tested. These results are in agreement with previous cell-cycle data described above. The cell lines were treated with increasing concentrations of Gleevec and induction of cell death was measured (Fig. 5c). No apoptosis was observed at a concentration of 0.1 μM. At a higher drug concentration (10 μM), apoptosis was detected but only in 5 out of 10 cell lines.

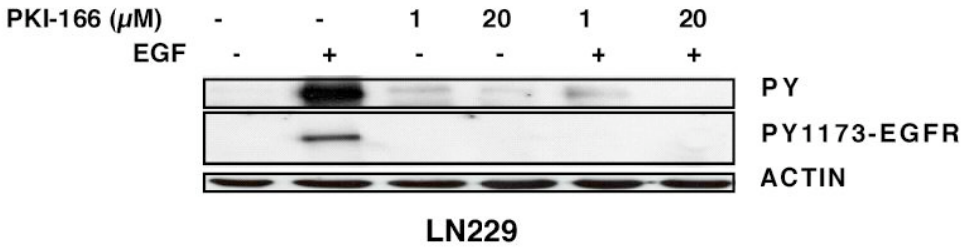
Taken together, these results show that Gleevec alone is inefficient in the induction of glioma cell death, except at high concentrations such as 10 μM, on part of analyzed cell lines only.

b. PKI-166

Pyrrolopyrimidines like PKI-166 were identified as lead structures by random screening, and were further optimized using a pharmacophore model of the ATP-binding site of EGFR (Traxler *et al.*, 1996). PKI-166 is a very potent dual inhibitor of EGFR and ErbB2 tyrosine kinase activities, having a reasonable degree of selectivity with respect to the inhibition of other tyrosine and Ser/Thr kinases (Caravatti *et al.*, 2001; Traxler *et al.*, 2001).

The antiproliferative activity and the *in vivo* antitumor activity of PKI-166 are closely related to the expression of the EGFR and/or ErbB2 in the human epidermoid carcinoma cells A-431 and in the lung carcinoma NCI-11596 (O'Reilly *et al.*, 2000; Traxler *et al.*, 2001). Based on these observations, PKI-166 is expected to be active against tumors associated with overexpressed or dysfunctional EGFR or ErbB2, thereby providing a rationale for testing PKI-166 in human glioma. The LN229 glioma cell line was treated with different concentrations of PKI-166 in the presence or absence of EGF in order to define the ability of this drug to block receptor activation. On one hand, the inhibitory effect of PKI-166 on all types of receptors of the ErbB family was evaluated by detection of phosphotyrosine activity level. On the other hand, specific inhibition of EGFR kinase activity was assayed with an antibody against EGFR phosphotyrosine-1173 (Fig. 6a). In the presence of 1 and 20 μM PKI-166 and in the absence of EGF, the anti-phosphotyrosine antibody revealed residual receptor activity. Addition of EGF did not activate the receptor whereas EGFR was fully inhibited with 20 μM PKI-166. The use of the EGFR phospho-tyrosine 1173 antibody confirmed that LN229 can be stimulated by EGF and that activation by EGFR is blocked with 1μM PKI-166.

A



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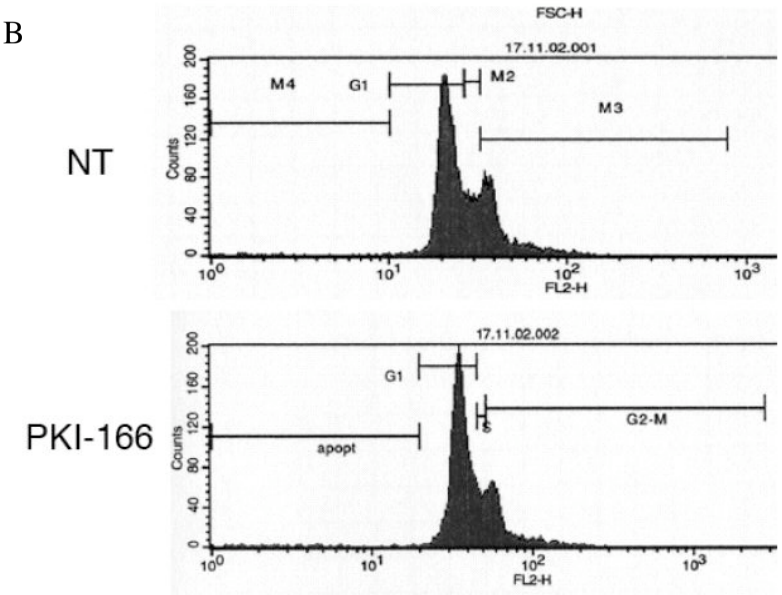

(%)	G1	S	G2/M
NT	59.39	14.03	27.34
PKI-166	68.73	9.63	21.87

C

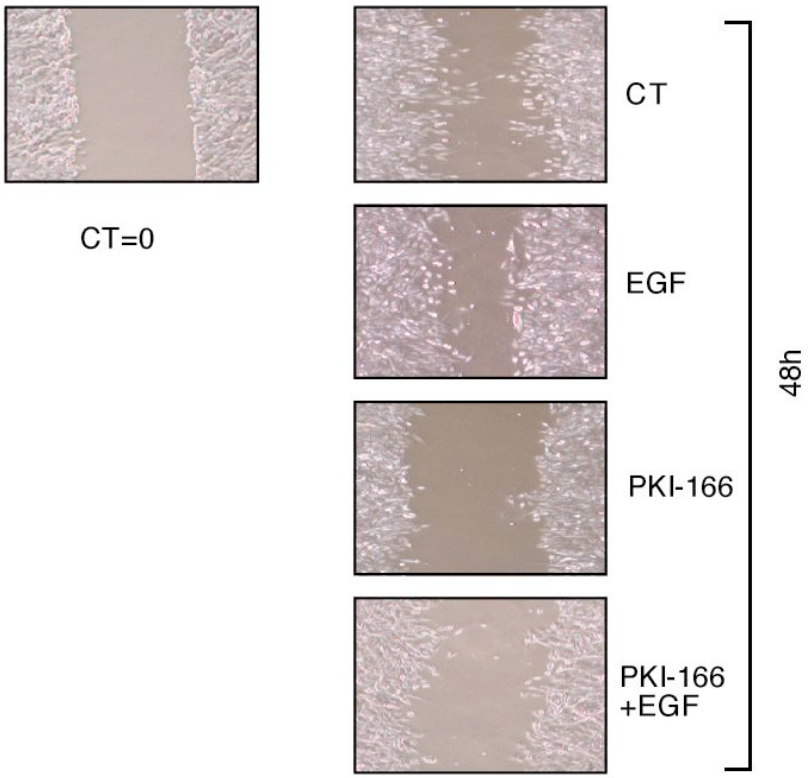

Figure 6: Anti-proliferative and anti-migratory capacity of PKI-166 on GBM cells. (A) Effect on biomarkers. LN229 cells were pre-incubated during 1 hour with increasing concentrations of PKI-166 (0; 1 or 20 μM) and stimulated in presence or absence of EGF (100 ng.ml^{-1}) during 10 minutes. Total levels of phosphorylation of the entire ErbB receptor family was detected by the anti-phosphostyrosine antibody (PY) whereas specific activation of EGFR by EGF was determined by measuring the activity of the PY-1173. (B) Effect of PKI-166 on proliferation. Cells were treated with PKI-166 (1 μM) for 48h. Cell cycle profiles were determined by flow cytometry. NT: no treatment. (C) Effect of PKI-166 on migration using the wound healing assay (WHA). Cells were grown at high confluence, then a strip was made (t=0). Then PKI-166 (1 μM) and/or EGF (100 ng.ml^{-1}) were added. In case of double combination, PKI-166 was added 1h before. The cells were allowed to migrate. After 48h, a picture was taken with an inverted microscope. CT: control

Since proliferation and invasion are fundamental features of GBM, they will both be used as readouts to assay effects of PKI-166. At a concentration of 1 μM, PKI-166 induces in LN229 cells a G1 accumulation reducing the number of cells in S phase (Fig. 6b), confirming the anti-proliferative effect of this compound. Regarding migration several conditions were tested such as PKI-166 (1 μM) and EGF alone (100 μg.ml^{-1}) or in combination (Fig. 6c). 1 μM PKI-166 inhibited EGF stimulated cell migration and reduced proliferation, leading to a complete stop of the migration of the cells. The simultaneous treatment with PKI-166 and EGF led to reduced migration. In fact, the cells treated with PKI-166 have a higher capacity of migration but lower when stimulated by EGF. The results demonstrated that PKI-166 was not able to fully block receptor activation. We finally tested the ability of PKI-166 to induce cell death. As shown in Fig. 7, a PKI-166 concentration as high as 5 μM was necessary to induce apoptosis whereas even after 6 days, no apoptosis happened at 1 μM.

Taken together, PKI-166 has been demonstrated to fully block EGFR activation, even in the presence of EGF, and to act as an anti-proliferative and anti-migratory compound. The combined treatment of EGF and PKI-166 resulted in an intermediate level of migration. This result could be explained either by a progressive degradation of the drug during the two days, or due to the drug characteristics, since PKI-166 is a reversible RTK inhibitor, meaning that it does not covalently bind to the receptor.

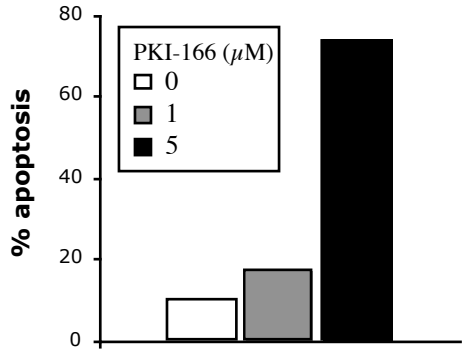

Figure 7: Weak pro-apoptotic effect of PKI-166 on GBM cells at 1 μM. LN229 glioma cells were incubated with increasing concentrations of the drug (0; 1 and 5 μM). After 6 days, levels of apoptosis were determined by flow cytometry.

Since apoptosis was only induced at a 5μM concentration, This drug would not be the appropriate drug for glioma. Another important issue is to know whether targeting EGFR/ErbB2 should be sufficient to induce apoptosis. As a consequence, a new compound has been tested: a dual inhibitor of the ErbB receptor family and VEGFR-2 (KDR), named AEE788.

c. AEE788

AEE788 conveys an anti-proliferative and anti-migratory effect onto the tumor cells by blocking signal transduction via EGFR and also exerts an anti-angiogenic effect on the endothelial cells by inhibiting KDR/VEGFR-2 (Traxler *et al.*, 2004). It is noteworthy that VEGF and its receptor, which play a crucial role during neovascularization are both expressed in glioblastomas (Heldin & Westermark, 1989; Plate & Risau, 1995). Inhibition of both VEGFR-2 and EGFR kinase activities render AEE788 highly attractive for the treatment of solid cancers including glioblastomas. Thus, one OG and six GBM cell lines in which the genetic status of the most critical cancer genes had been previously established (Ishii *et al.*, 1999), have been selected for further investigation.

First, the ability of AEE788 to block EGFR/ErbB receptor activation was determined in LN229 cell line with increasing concentrations. A partial reduction of EGFR activation was already observed at low concentration (0.1 μM), while higher concentrations up to 1 μM led to a complete blocking of EGFR activation (Fig. 8a). Addition of EGF activated EGFR phosphorylation, while pre-treatment with the drug blocked the activation in a dose-dependent manner.

Since EGFR and ErbB2-directed inhibitor effects were associated with increased expression of the inhibitor of cell cycle progression $p27^{kip1}$ (Peng *et al.*, 1996; Wu *et al.*, 1996; Cardiello *et al.*, 2001; Lane *et al.*, 2001), the effect of the drug on glioma cell proliferation was examined using flow cytometry (Fig. 8b) by measuring DNA synthesis in the presence of this drug. Cells were treated with increasing concentrations of AEE788. After 24 hours of treatment, all cell lines responded to the drug at a concentration as low as 2 μM except Hs683 which needed higher concentrations. A maximal reduction of 44% was found in SF767.

Taken together, these results indicate an anti-proliferative effect of AEE788 on the cell lines and further confirm a role for EGFR/ErbB2 receptor signaling in the potentiation of tumor cell proliferation.

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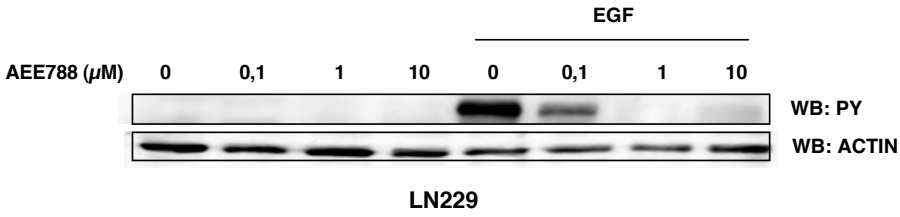

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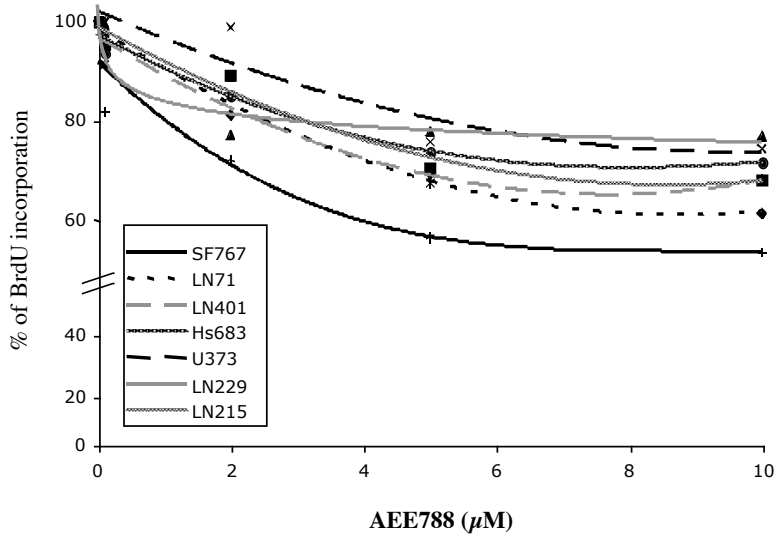

Figure 8: Effects of AEE788 on inhibition of EGFR activation and cell proliferation is concentration dependent. (A) AEE788 and ErbB receptor family activation. LN229 cells were pre-incubated for 1h with the drug and stimulated in presence or absence of EGF (100 ng.ml^{-1}) for 10 min. EGFR activation was evaluated by revealing phosphorylated EGFR on Western blot with anti-phosphotyrosine antibody. (B) AEE788 inhibits cell proliferation in all cell lines tested. Cells were incubated during 24 hours with increasing concentrations of AEE788 (0; 0.1; 1; 2; 5 and 10 μM), effect on proliferation was measured by BrdU incorporation.

The potential role of AEE788 as an anti-migratory compound was then analyzed. The ability of AEE788 to alter the migration capacity of glioma cell lines was tested by using the Boyden chamber technique. The cells were allowed to migrate after pre-treatment with AEE788 in presence or absence of EGF (Fig. 9a). The basal level of migration was reduced by AEE788 in six of the seven cell lines tested (LN71, LN215, U373, LN401, Hs683 and SF767). All cell lines responded to the drug and showed an inhibition of the motility after EGF stimulation when compared to EGF alone indicating a total or partial inhibition of EGF receptor activation. LN229, as an exception, AEE788 did not affect the basal level of migration even after EGF stimulation. In all the cell lines tested including LN229, the EGFR phosphotyrosine 1173 antibody confirmed that the drug was effective at 1 μM AEE788 and that activation of EGFR was blocked even when stimulated by EGF, then EGFR degradation process was stabilized (Fig. 9b).

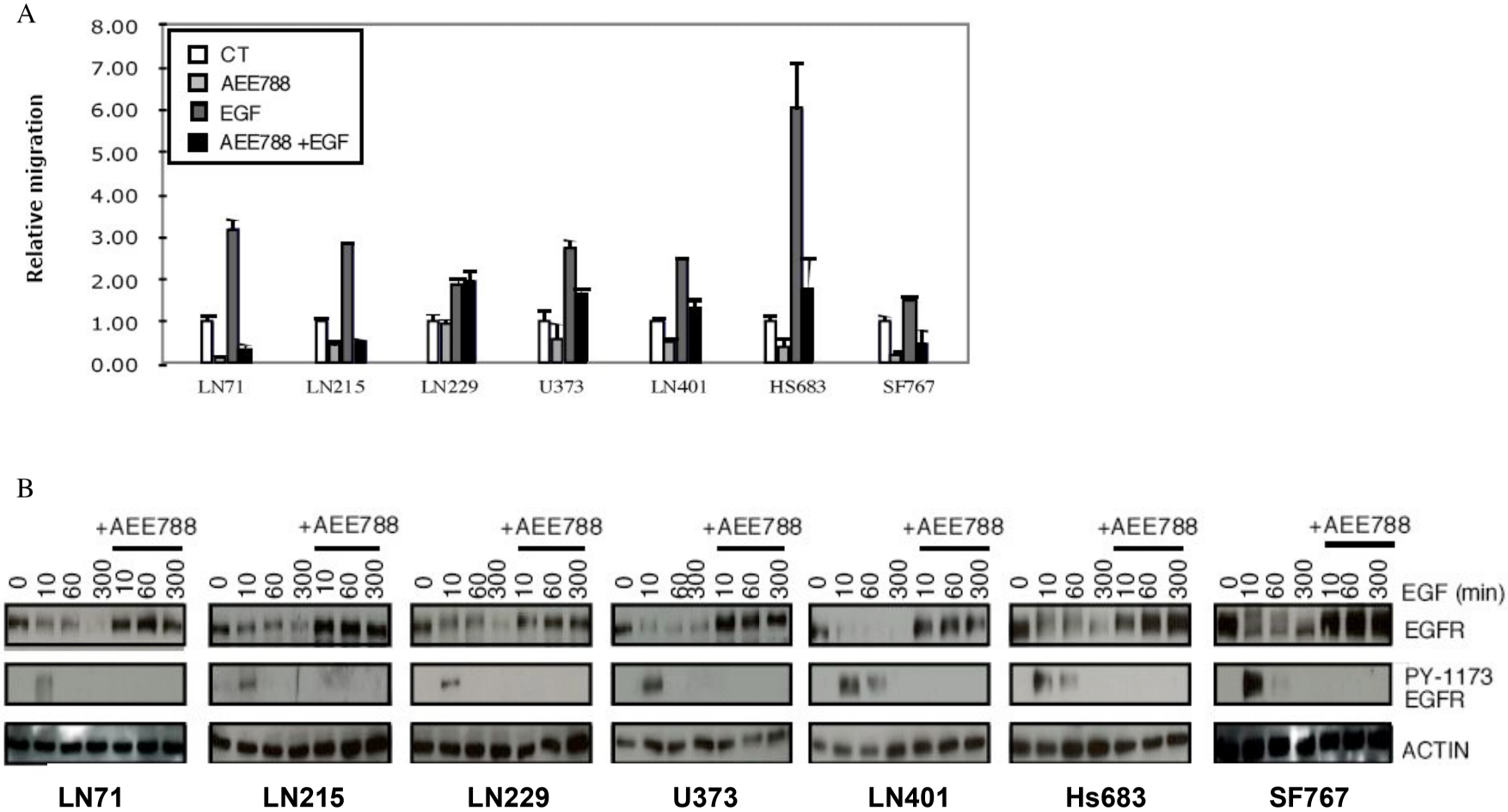

Figure 9: AEE788 acts as an anti-migratory compound and blocks EGF-dependent activation (A) Effect of AEE788 on migration. Cells were pre-incubated in Boyden chamber in presence or absence of 1 μM AEE788 for 30 min prior a 3 hr-migration. EGF concentration was 100 ng.ml^{-1}; CT: control. Results are expressed as fold increase of cell migration of treated case (AEE788 and/or EGF) as compared with untreated case (CT). (B) Cells were pre-incubated in presence or absence 1 μM AEE788 during 30 min and stimulated with 100 ng.ml^{-1} EGF for 10 min. EGFR, PY1173-

As shown above, 1 μM AEE788 inhibited EGFR activation, reduced proliferation and inhibited the basal level of migration, making this compound to a candidate for GBM treatment. Further, in order to determine cell viability in a dose-dependent manner, glioma cell lines were exposed to higher concentrations of AEE788 (Fig. 10). No apoptosis was induced except in SF767 and in LN215, in which only few dead cells were detected. 10 times higher drug concentrations were necessary to induce apoptosis. 10 μM of AEE788 treatment caused a very high level of apoptosis in five out of eight cell lines, (SF767, LN215, U373, LN229 and LN71) with a minimum percentage of apoptosis of 65% for the LN71. Although SF767 and LN215 have shown some cell death at a concentration as low as 1 μM, they exhibited in addition a dose-dependent response in the 0.1 to 10 μM range concentration, whereas, others cell lines such as LN401 or Hs683 showed very low or no cell death, even at 10 μM of drug treatment.

These results have shown that 1 μM AEE788 blocks proliferation and migration in addition to EGFR activation by its ligand, EGF as well as that the drug cannot induce apoptosis in the majority of the cell lines (5 out of 7).

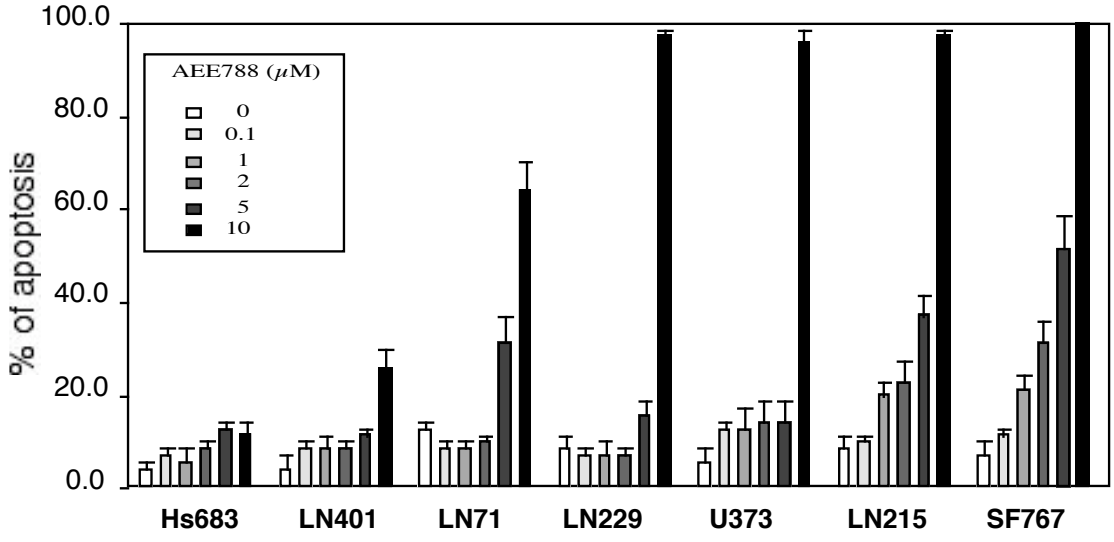

Figure 10: AEE788 does not induce cell death at physiological concentrations. Cells were incubated during four days with increasing concentrations of AEE788 (0; 0.1; 1; 2; 5 and 10 μM). Apoptosis was measured by flow cytometry.

d. Patupilone

Epothilones constitute a new class of microtubule-targeting compounds. One member of the epothilone family is patupilone, which has shown potent *in vitro* and *in vivo* antitumor activity (Altmann *et al.*, 2000; Goodin *et al.*, 2004). At nanomolar range, the drug exhibits taxol®-like properties and inhibits microtubule depolymerization, which potently inhibits the growth of human cancer cells in vitro. Since the function of the mitotic spindle is inhibited leading to mitotic arrest at the G2/M transition (Bollag *et al.*, 1995; Altmann *et al.*, 2000; Goodin *et al.*, 2004). The anti-proliferative (Chou *et al.*, 1998) and cytotoxic properties of the drug seem to be coupled to the disturbed mitotic spindle function (Blagosklonny & Fojo, 1999) (Fig. 11). Microtubules have already been used as targets for a multitude of antimitotic drugs that have been used successfully in the treatment of cancer (Jordan & Wilson, 2004).

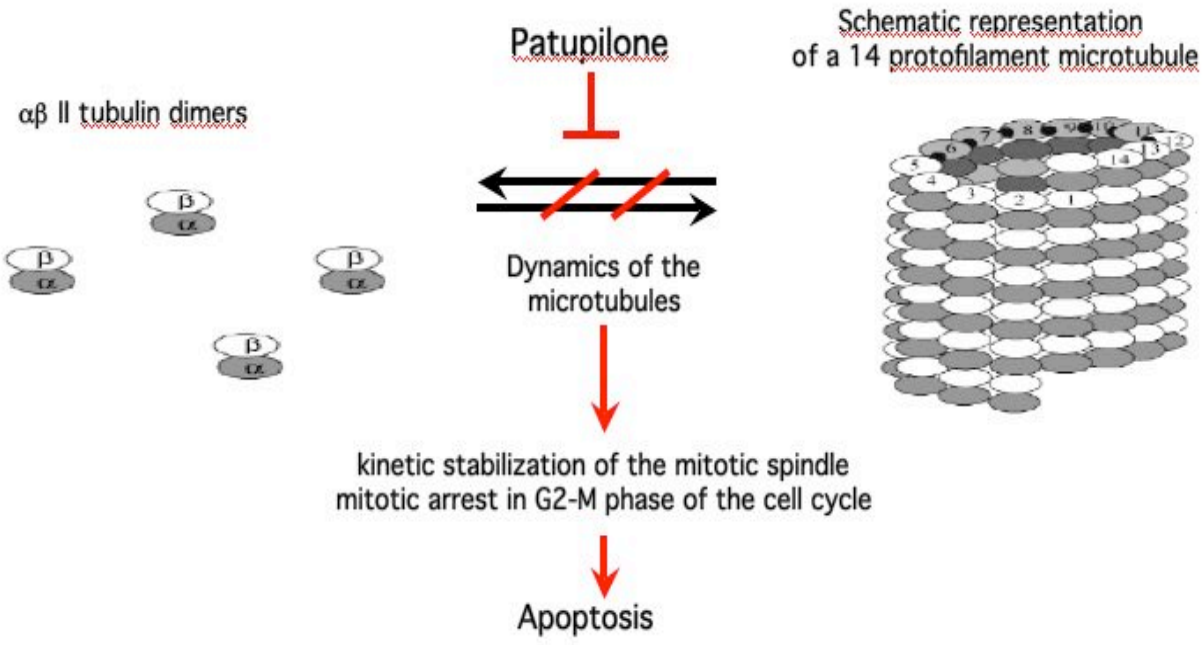

Figure 11: Mechanism of action of patupilone.

The effect of Patupilone was first analyzed on *in vitro* cell growth. Three glioma cell lines (LN71, LN229 and SF767), and one oligodendroglioma cell line (Hs683) carrying characterized mutations involved in glioma development were selected (Ishii *et al.*, 1999). The effect of the drug on cell cycle profile was determined on glioma cell lines. All cell lines were tested using nanomolar concentrations of patupilone and exhibited a G2/M phase accumulation at a concentration as low as 0.7 nM (Fig. 12). This accumulation increased at higher concentrations of the drug indicating a dose-dependence.

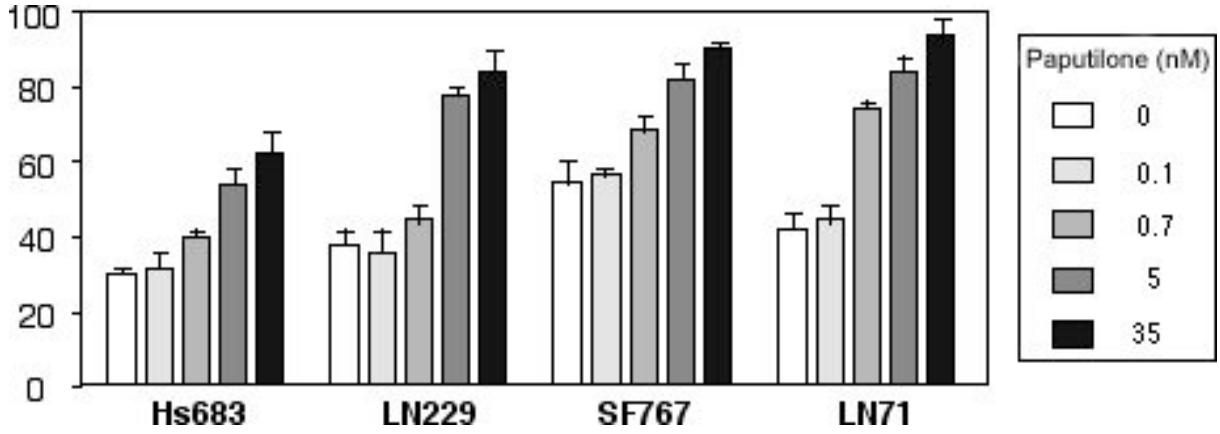

Figure 12: Patupilone induces G2/M accumulation at low nanomolar concentrations. Four cell lines were treated for 18 hours either with vehicule DMSO alone or with increasing concentrations of Patupilone. Percentage of cells in G2/M phase was measured by flow cytometry. Data represent the average ± SD of 3 independent determinations.

Changes of the microtubule network and of the shape of the nuclei were visualized with confocal laser microscopy. Prior patupilone treatment, the cells are large, showing variable shapes with filamentous staining, reflecting a high degree of organization of the microtubule system (Fig. 13a). After treatment with 5 nM patupilone much denser structures were formed with time, indicating an inhibition of depolymerization of the microtubule system already after 12hrs (c) of exposure to the drug. The microtubule network appeared damaged and shrunk and the nuclei became round after 12 hours (d). After 18h, the microtubules were still aggregated (e).

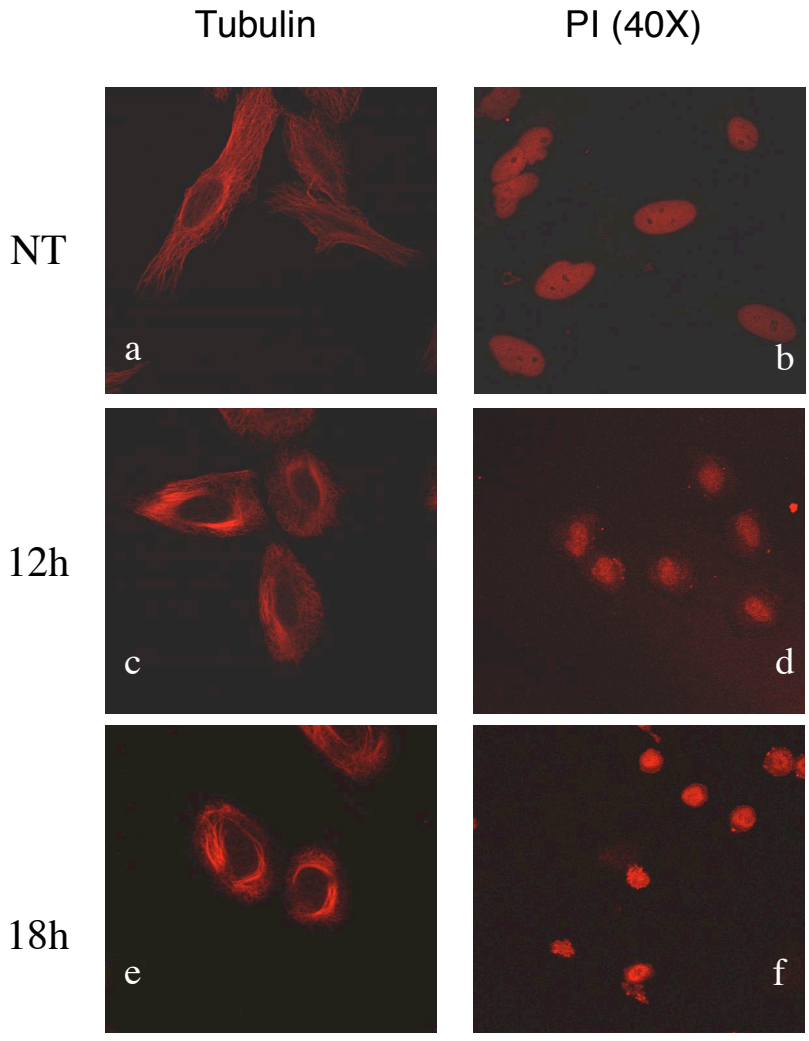

Figure 13: Patupilone inhibits microtubules depolymerization. The microtubules were detected by confocal laser microscopy with tubulin antibody. LN229 cells were treated with 5 nM paputilone for 18 hours. The pictures show the distribution of tubulin (a, c and e) and nucleus staining (b, d and f). Untreated control cells are shown in a and b. NT: no treatment

The ability of the drug to induce apoptosis in glioma cells was analyzed. Various human glioma cell lines were treated with patupilone using increasing concentrations in the nanomolar range (Fig. 14). The drug caused 100 % apoptosis in all cell lines tested at a 35 nM concentration. At lower concentrations, the cell lines responded differently to the drug. No induction of apoptosis was observed at 0.05 nM in any of the cell lines. The drug was able to induce apoptosis already at 0.1 nM in LN229 and at 0.7 nM in the case of LN71 and SF767,

whereas more than 0.7 nM was needed in Hs683. However, LN401 was much more resistant to the drug and apoptosis was observed only at concentrations of 5 nM or higher.
patupilone has demonstrated to cause cell death at a concentration as low as 0.1 nM in LN229 and 100% of apoptosis was reached at a concentration of 35 nM after four days.

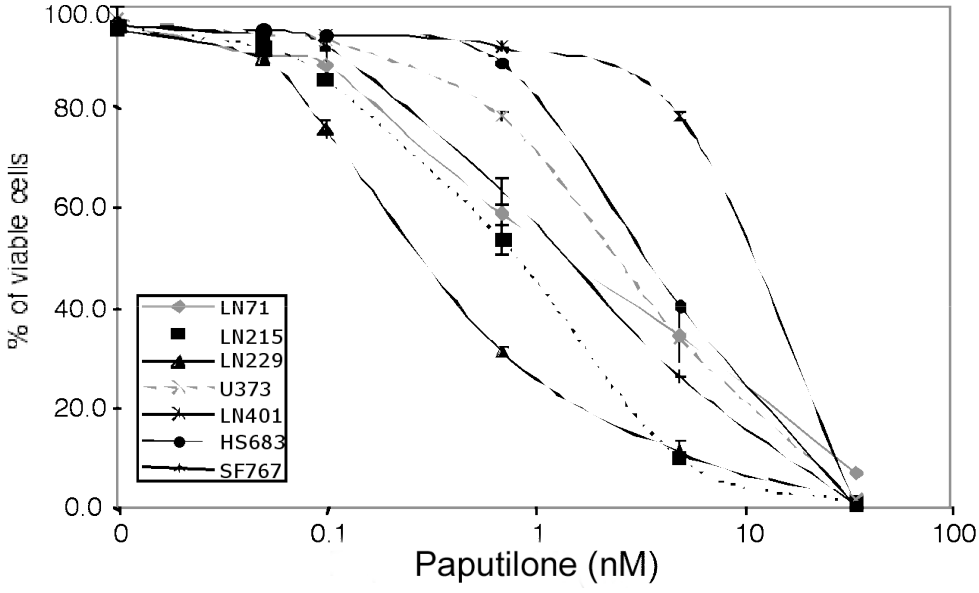

Figure 14: Patupilone is highly toxic for glioma cells. Increasing concentrations of paputilone (0.05; 0.1; 0.7; 5 and 35 nM) were applied to different cell lines during four days of teatment. The percentage of viable cells was measured after four days using flow cytometry. Data represent the average ± SD of 3 independent determinations.

e. Concluding remarks about single drug treatment

Glioma cell lines were found to be insensitive at the tested concentrations of PKIs. Although 10 μM or higher concentrations of Gleevec were necessary to induce apoptosis, moderate concentrations of PKI-166 have been demonstrated to inhibit receptor activation, migration and proliferation. However, the treatment was not sufficient to induce apoptosis. AEE788, which also inhibits angiogenesis, didn't either induce apoptosis. The question arises whether treatment with PKI alone is sufficient to induce apoptosis in glioma cells. While RTKi drugs such as Gefinitib or Gleevec have shown promising results on other tumor cell types, why is glioma still resistant to this kind of drugs? In contrast to PKIs, nanomolar concentrations of the cytotoxic compound patupilone were efficient in inducing apoptosis.

2. Combinatorial strategy

a. Combination of RAD001, Gleevec and AEE788

A major question is whether biologically active small drugs of the class of protein kinase inhibitors (PKIs) are sufficient to kill tumor cells (as proven by the paradigmatic Gleevec experience) or are combinations needed. Therefore, a basic question was addressed using an *in vitro* glioblastoma model. Combinations of Gleevec (0.5 μM), AEE788 (0.5 μM) and RAD001 (20 nM) have been tested for their capacity to induce apoptosis in glioblastoma cells highly resistant to cell death, either by biologicals alone as single or in combination.

RAD001 is a derivate of the natural compound rapamycin that induces G1 growth arrest in yeast, *Drosophila*, and mammalian cells. Genetic and biochemical studies have established that the target of rapamycin is the protein kinase mTOR. In vivo experiments were done in nude mice xenografts. CCI-779, another analogue of rapamycin - when given for five consecutive days - inhibited glioblastoma tumor growth for up to two weeks after drug withdrawal, whereas normal immune function was restored as early as one day following withdrawal, indicating that an intermittent dosage of CCI-779 could be effective (Gibbons *et al.*, 1999). Thus, RAD001 may be a promising therapeutic agent for the treatment of cancers in which a dysregulation of the PI3K pathway is involved.

Each compound targets critical pathways in gliomagenesis (Fig. 15). We looked for additive and/or synergistic effects in order to limit toxicity and lack of specificity of these drugs, concentrations were selected according to the results obtained by the use of single drugs that means 20 nM for RAD001 and 0.5 μM for AEE788 and Gleevec.

We intended to correlate the induction of apoptosis by the drugs with the genetic status of the PTEN/p53/$p16^{INK}$/$p14^{ARF}$ genes. To do so, various cell lines were selected based on their respective genetic backgrounds for these genes (Annex IV). Therefore, a larger panel of genetically well-defined tumor lines has been tested for their proliferative, invasive and pro-apoptotic properties upon exposure to these drugs as single agents and in various combinations. Cells were incubated with single drugs and in combinations. A phenomenological classification of cell lines according to similar patterns of the response to the drugs has been established (Fig.16).

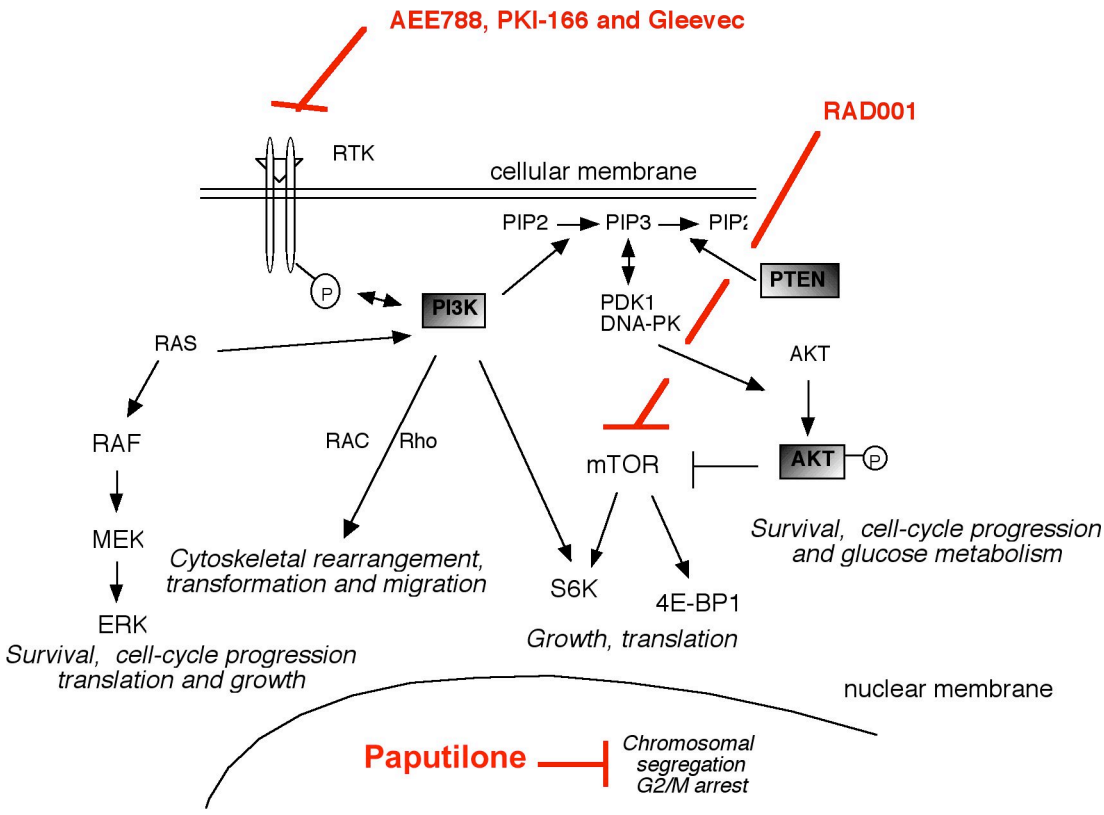

Fig. 15: Major signaling pathways in gliomas. The PKIs Gleevec, AEE788, PKI-166, RAD001 and the cytotoxic drug patupilone are shown together with their molecular targets.

The cell lines that turned out to be resistant to all kind of treatments, represented the first group (LN401, LN71, LN229 and LN319) (Fig. 16a). The second group (LN18, LN215 and SF767) featured a specific sensitivity to AEE788, to which combination with Gleevec resulted in an additive induction of apoptosis. While RAD001 had no effect on LN18 cell death, RAD001 enhanced the cytotoxic effect induced by AEE788 in LN215, and RAD001 reduced the cytotoxic effect caused by AEE788 in SF767. The last group of cell lines, Hs683, U373 and U343, showed a cytostatic response to each one of the drugs. The basal level of apoptosis is reduced in all the combinations tested (Fig. 16c).

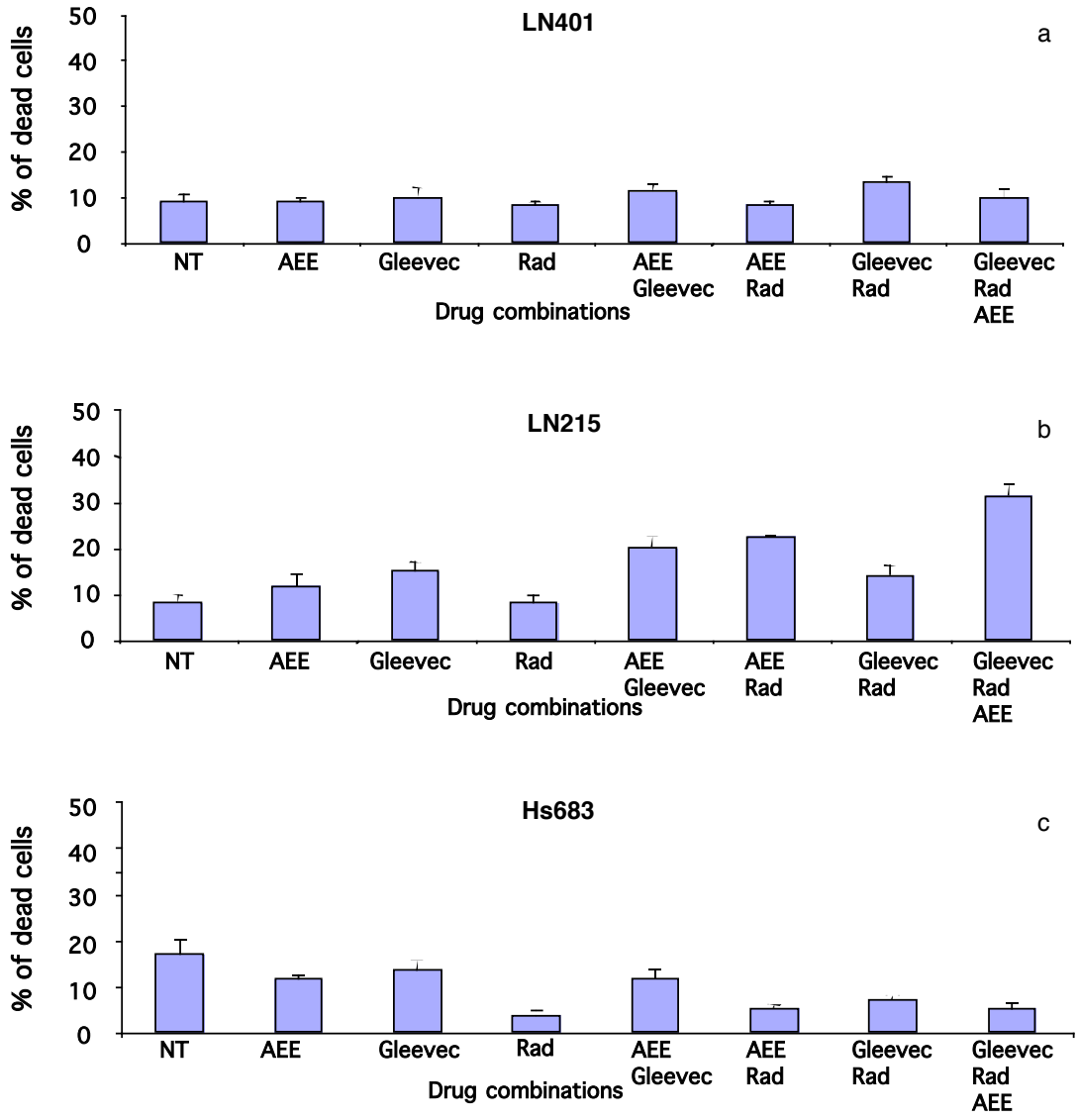

Figure 16: Induction of apoptosis by the various PKIs alone or in combination. Cell lines were incubated with the drug(s) during 4 days. Percentage of dead cells was measured by flow cytometry. Data represent the average and standard deviation of 3 independent experiments. NT: no treatment, AEE: AEE788 (0.5 μM), Rad: RAD001 (20 nM) and Gleevec (0.5 μM).

All these results obtained by combinations of the three biologicals are summarized in Figure 17. In addition to the apoptosis data, the genetic status of the PTEN/p53/p16^{INK}/p14^{ARF} genes in GBM was added in order to correlate them with the apoptosis results. In conclusion, neither single drugs nor double and triple combinations caused substantial levels of apoptosis. Only in SF767 and LN215 (second group), combination of drugs led to 30% of apoptosis at best.

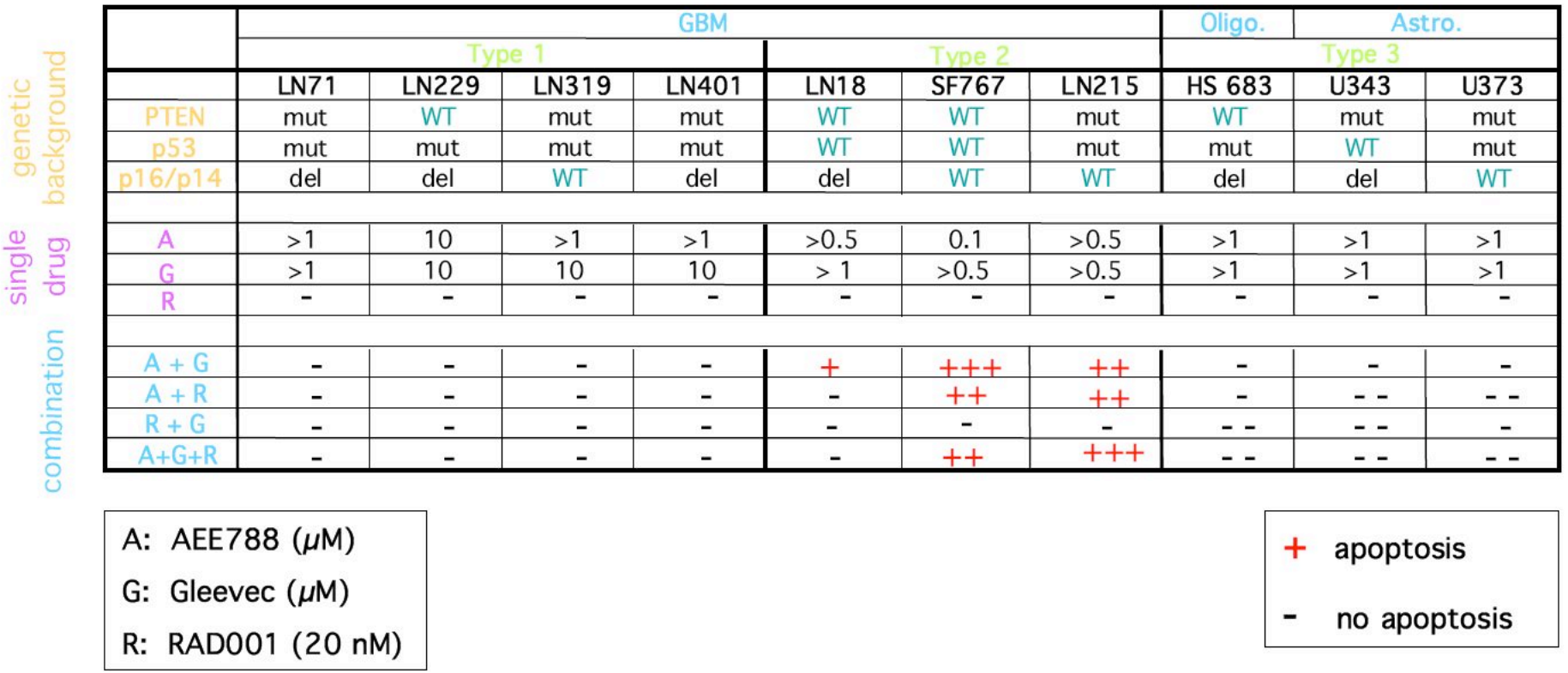

Figure 17: Induction of apoptosis with single and combined drug applications. Genetic background of PTEN, p53 and p16/p14 are shown. For single drug, the concentrations necessary to induce apoptosis obtained from the experiments are mentioned. Induction of apoptosis in the combination is described by: (-) no apoptosis, and the number of (+) correlated with higher percentage of apoptosis. mut: mutated, del: homozygous deletion, WT: wild-type.

b. AEE788 and Patupilone

All drugs alone or in combination tested so far gave either no substantial induction of apoptosis or exhibited cytotoxicity when used at concentrations inducing apoptosis (patupilone). We then investigated whether a combination of the most promising biological, AEE788, and subcytotoxic concentrations of patupilone could lead to a synergistic induction of cell death.

As shown in Fig. 8a, treatment of the cell lines with AEE788 alone at 1 μM concentration led to a complete block of EGFR activation. Addition of EGF was able to activate EGFR phosphorylation, whereas pretreatment with the drug blocked the activation in a dose dependent manner. However, this concentration did not induce apoptosis (Fig. 10). A combinational treatment has been performed using 1 μM AEE788 with increasing concentrations of patupilone (Fig. 18a). Both drugs caused apoptosis in a synergistic manner in LN71, SF767 and LN401. In contrast, only an additive effect was observed in U373, LN215, and only a slight effect in Hs683 and LN229. In addition, the determination of the concentration necessary to induce 50% of apoptosis ("AC_{50}" modified IC_{50} for apoptosis instead of proliferation) of the AEE788/ Patupilone combination revealed that AEE788 acted as a sensitizer in all the cell lines (Fig. 18b). AEE788 alone gave "AC_{50}" values in the micromolar range (LN71: 5.5, LN215: 5.1, SF767: 4.9 and LN229: 5.5) while "AC_{50}" of Hs683, U373 and LN401 could not be determined since the drug, even when combined, did not reach 50% of apoptosis at these defined subcytotoxic concentrations. patupilone induced apoptosis at a concentration in the nanomolar range. In combination, AEE788 decreased the "AC_{50}" values in all cell lines at a lower induction of apoptosis in cell lines such as LN229 and Hs683 than in the other cell lines where an additive or synergistic induction of apoptosis was observed.

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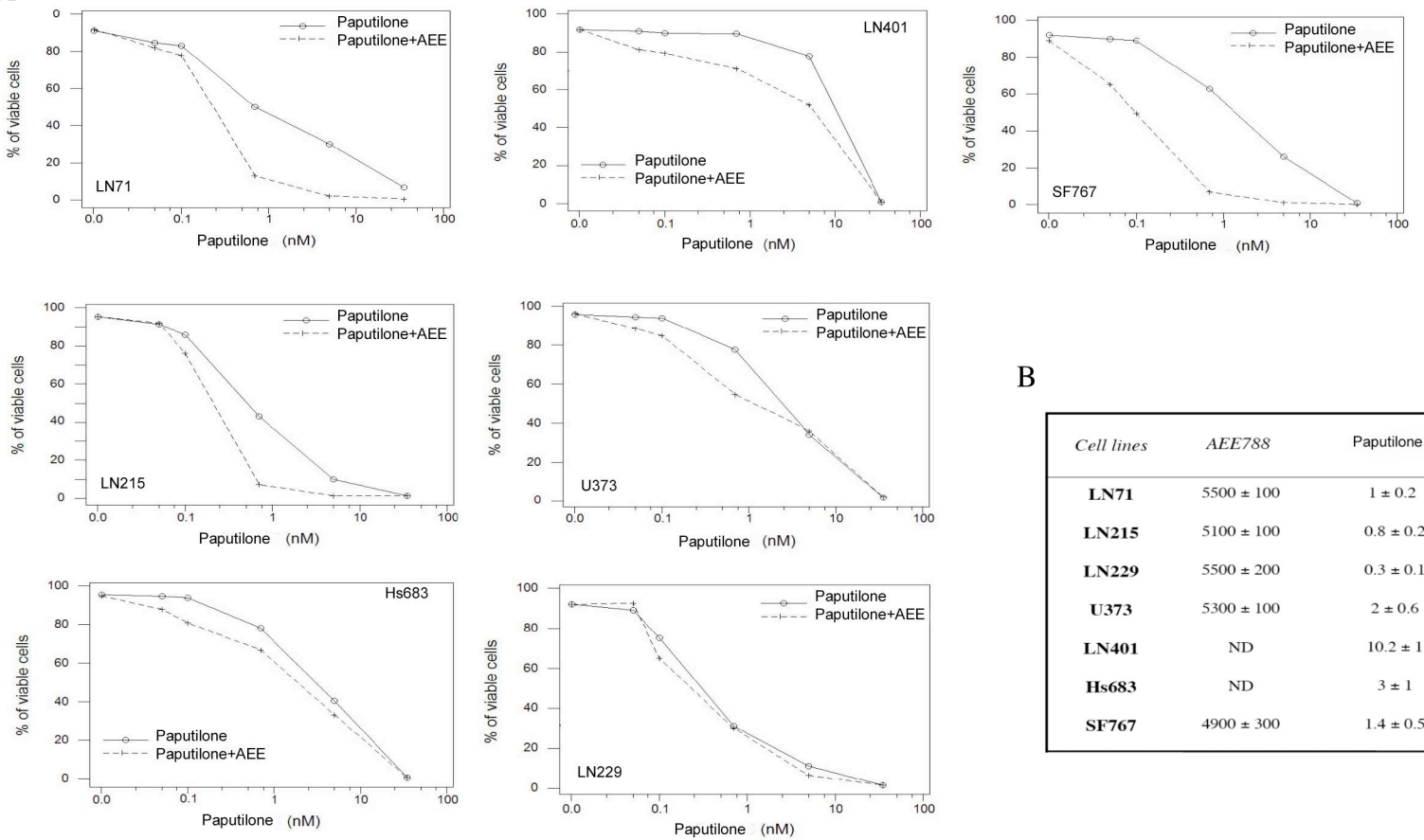

B

Cell lines	*AEE788*	Paputilone	*AEE788+*
LN71	5500 ± 100	1 ± 0.2	0.5 ± 0.3
LN215	5100 ± 100	0.8 ± 0.2	0.2 ± 0.1
LN229	5500 ± 200	0.3 ± 0.1	0.25 ± 0.1
U373	5300 ± 100	2 ± 0.6	1 ± 0.8
LN401	ND	10.2 ± 1	5 ± 0.5
Hs683	ND	3 ± 1	2 ± 0.5
SF767	4900 ± 300	1.4 ± 0.5	0.1 ± 0.1

Figure 18: Synergistic induction of apoptosis by the combined use of AEE788 and Paputilone. (A) Cells were treated for four days either with PATUPILONE alone or in combination with 1 μM AEE788. Cell survival was measured by flow cytometry. Data represent the average ± SD of 3 independent experiments. (a) Synergistic induction of apoptosis effects observed at low nanomolar concentrations of Paputilone and AEE788 (1 μM). In some cell lines (SF767, LN401 and LN71), synergistic effects are observed between 0.1 and 0.7 nM. Low or no additive effect on apoptosis by AEE788 in the LN229, Hs683, LN215 and U373. (B) "AC_{50}" (nM) values for induction of apoptosis in glioma cell lines of AEE788, PATUPILONE alone or combined. Results are expressed as average and standard deviation of 3 independent experiments. ND: not determined.

The synergistic induction of apoptosis occurred in LN71, LN401 and SF767 cell lines at concentrations between 0.05 and 0.1 nM, that is in a range where both drugs were not effective when they were used alone. The lowering of the dosage of paputilone has the advantage that also its toxicity is reduced. This result indicated that the specific combination of these two drugs led to a strong potentiation of their ability to induce apoptosis in some of the glioma cell lines.

c. Conclusion about drug combinations

Taken together, the data obtained from single as well as combined drugs demonstrated that the glioma cell lines exhibited a wide spectrum of resistance. On one hand, the triple combination (Gleevec, AEE788 and RAD001) was not able to induce a high level of apoptosis, on the other hand the combination between paputilone and AEE788 revealed that a synergistic effect was obtained at low nanomolar range by double treatment, but only in 50% of the cell lines treated.

3. Resistance of glioma cells towards drug treatment

a. Introductory questions

Why were the PKIs alone or in combination drugs rarely efficient in the induction of apoptosis? What are the reasons for the low sensitivity? How can the low sensitivity be overcome? In this context, we investigated a possible correlation between induction of apoptosis and the genetic status of EGFR, PTEN, p53 and $p16^{INK4A}$/$p14^{ARF}$ and/or basal expression levels of specific proteins like e.g. ErbB receptors and others.

b. Comparison of the genetic status of EGFR-PTEN-p53-p16/p14 of the cell lines and their sensitivity to the drugs

Of note, although some cell lines showed similar genetic status regarding glioma genes, they responded differently to the various treatments, for example, Hs683 and LN229, which are both wild type for PTEN. Similarly, LN215 and U373 both harbour deletions at the p16/p14 locus; while LN401 and LN71, which both have the same genetic status for PTEN, p53 and p16/p14 genes (Annex IV) and LN71 and SF767 exhibited similar EGFR RNA expression (Fig. 19a). There was no obvious correlation between the status of these four genes and the drug resistance of the respective cell lines.

c. Comparison of various protein levels of the cell lines and their sensitivity to the drugs

With respect to their importance in gliomagenesis, levels of PTEN or ErbB receptors were measured. Phosphorylated AKT and ERK, components of the PI3K/Akt and Ras/Raf/MEK/ERK signaling cascades, shown to play a critical role in gliomagenesis (Maher *et al.*, 2001), have also been tested.

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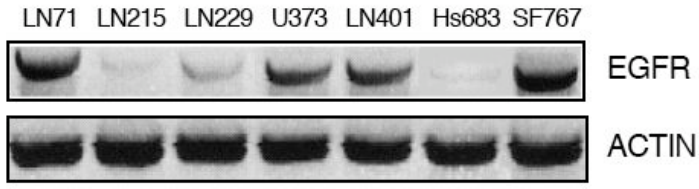

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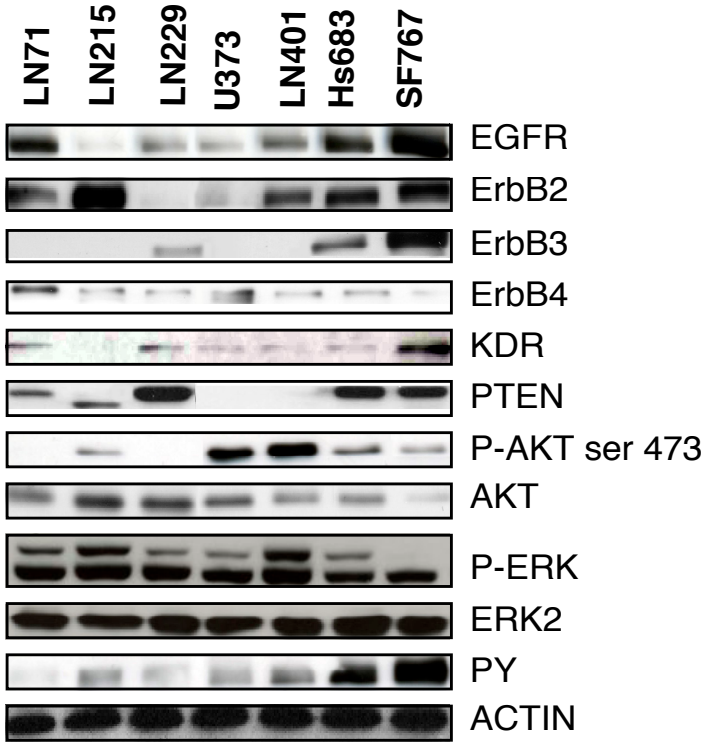

Figure 19: Basal expression of specific proteins of cell lines. (A) EGFR/ErbB1 gene expression using RT-PCR is also shown. (B) Expression levels of proteins potentially involved in gliomagenesis determined by Western blot analysis.

Interestingly, although LN71 and SF767 exhibited similar EGFR protein levels, the induction of apoptosis appeared to be different. The efficacy of EGFR inhibitors on proliferation, migration and receptor activation does not correlate with the degree of overexpression of EGFR or ErbB2 (Fig. 10 and 19b). Then, expression of other biomarkers of the drug was also analyzed, including VEGFR-2 (KDR), ErbB3, ErbB4 which showed a high level of variation of proteins levels. Basal ERK1/2 activities were also found to be high in all the cell lines, excepted in SF767, where no ERK1 (p44) activity was detected. Finally, several cell lines tested overexpressed phosphorylated AKT (P-AKT) mainly due to the PTEN deletion, and all displayed high level of phosphorylated ERK.

d. De-activation of AKT and/or ERK as readout for treatment of GBM cells

Simultaneous constitutive activation of *Akt* and *Ras* has been shown to induce gliomagenesis in transgenic mice (Holland *et al.*, 2000). Similarly, activation of both *Akt* and *Ras* combined with *Ink4a/Arf* knock out in mice also develop gliomas (Uhrbom *et al.*, 2005) (Annex I). Based on the results of these *in vivo* experiments, we intended to use AKT and ERK as readout for the induction of glioma cell death.

Two cell lines (U373 and SF767) exhibiting different levels of apoptosis at the concentrations used were selected (Fig. 20). Cells were treated with either 1 or 10 μM of AEE788 to investigate induction of apoptosis and ERK/AKT activities at 1, 8 and 24 hours.
In U373 treated with 1 μM AEE788, no change in the phosphorylation status of AKT and ERK was observed, despite blocking of the EGF receptor activation, and no apoptosis was detected. In contrast, SF767 treated with 1 μM AEE788 had inhibition of AKT and ERK activities as early as one hour after treatment and showed apoptosis. At 10 μM concentration, in both cell lines AKT and ERK activities were partially (U373) or completely (SF767) inhibited after twenty-four hours, in parallel to the induction of apoptosis (Fig. 20). Thus, SF767 showed the highest sensitivity to AEE788 with respect to inhibition of both ERK and AKT activities and apoptosis levels obtained in these conditions (Fig. 20). From these results, the hypothesis evolved that drug resistance in U373 was due to the high level of phosphorylation of ERK and/or AKT. In order to test this hypothesis, the PI3K inhibitor wortmannin and the MEK inhibitor U0126 were used (Fig. 21a) to completely abrogate AKT or ERK activation.

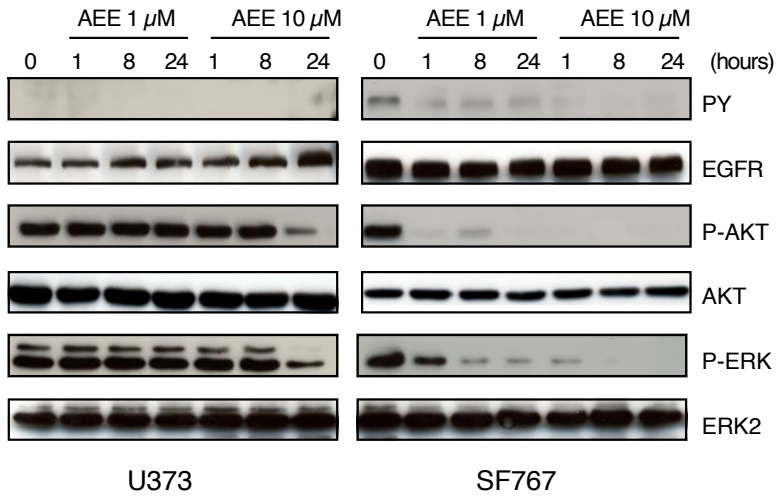

Figure 20: Inactivation of ERK and AKT activities by AEE788. Cells were incubated with 1 or 10 μM of AEE788. To determine the potential correlation between induction of apoptosis and protein inhibitions, drug effects were observed during 24 hours. Level of proteins and respective activities were determined using the following antibodies. PY: anti-phosphotyrosine, P-AKT: Ser 473 of AKT and P-ERK: ERK1/2 activities.

In order to investigate the pathways involved in the glioma tumor lines, cell lines were treated with each compound individually and in combination (Fig. 21b). Drugs alone, except U0126, did not induce apoptosis. The MEK inhibitor (U0126) induced apoptosis in 45% of LN215. Interestingly, an increased induction of apoptosis was observed for several drug combinations: AEE788 plus wortmannin led to 42% apoptosis in SF767, AEE788 and U0126 to 41% in LN71 and to 54% in LN215. In fact, the combination of U0126 and wortmannin appeared to be most efficient to trigger cell death. The frequency of apoptotic cell population resulting from the individual drug treatments provided a baseline for evaluating whether drug combinations may act in an additive or in a synergistic manner. Thus, by killing 33% of LN401, 41% of Hs683 and 73% of LN215, we could establish that the combination of U0126 and wortmannin acted in an additive way, while U0126 and wortmannin synergized to induce apoptosis in 58% of LN71 and in 75% LN229.

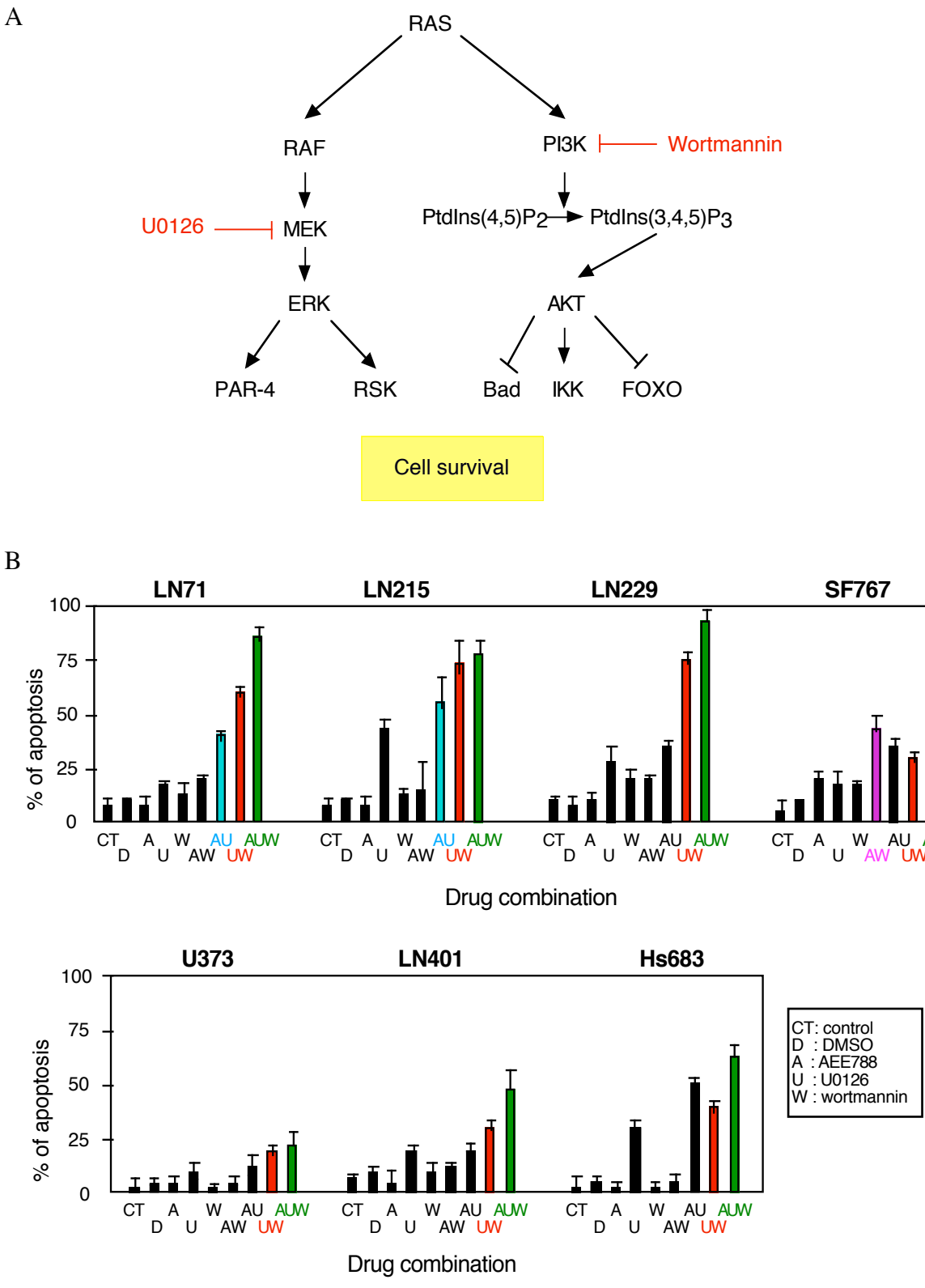

Figure 21: Synergistic induction of apoptosis by wortmannin and U0126. (A) Signaling pathways targeted by U0126 and wortmannin. (B) Induction of apoptosis of U0126, wortmannin and AEE788 alone or in combination. Combination of AEE788 in presence or absence of the MEK inhibitor and/or the PI3K inhibitor can induce a synergistic induction of apoptosis. U0126 and wortmannin were added every day whereas AEE788 only once at day 0. Apoptosis was measured after 4 days of treatment and experiments were made in triplicates. Percentage of apoptosis was determined by flow cytometer.

In response to a given combination, cell lines showed quite constant levels of apoptosis. Indeed, combination of U0126 and wortmannin was efficient in 6 out of 7 cell lines and the triple combination of AEE788, wortmannin and U0126 caused more than 50% of apoptosis in 6 out of 7 cell lines. These results supported the hypothesis that the AKT- and ERK- related pathways may serve as potential targets in glioblastoma treatment. However, some cell lines such as U373 and LN401 were resistant to combined treatment. The nature of the resistance was further investigated.

Abrogation of ERK and AKT activities was determined upon single and combinatorial treatments. After one hour, wortmannin and U0126 inhibited the phosphorylation of AKT and ERK in all the cell lines tested (Fig. 22). Nevertheless, as shown in parallel FACS analysis 4 days after drug treatment, these cell lines showed diverse apoptotic reponses despite the fact that the combination between both inhibitors completely abrogated both ERK and AKT activities (Fig. 22).

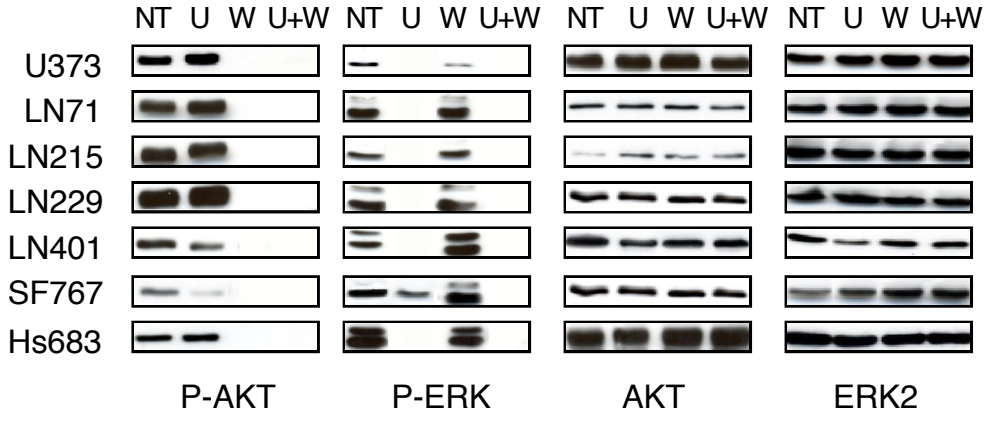

Figure 22: Wortmannin and U0126 inhibit their respective biomarkers. After one hour, both compounds inhibit ERK and AKT activities at the concentrations selected. CT: control, A: AEE788 (1 μM), U: U0126 (20 μM), W: wortmannin (1 μM). After treatment, SDS was added and western analysis was performed.

e. Simultaneous inhibition of both AKT and ERK activities is needed to induce cell death

To test the hypothesis that inhibition of both AKT and ERK activations are needed to induce glioma cell death, the phosphorylation status of both proteins was determined after 72 hours drug treatment, in parallel to the apoptosis assay performed after 96h, as described above (Fig. 18 and 22).

When compounds, AEE788 and Paputilone, were combined, only LN71 and SF767 showed an inhibition of phosphorylation of both ERK1/2 and AKT after 24 and 72 hours whereas LN229 and Hs683 still exhibited strong phosphorylation of both kinases (Fig. 23a). When compared to the apoptosis data shown above (Fig. 18a), these results indicate a possible correlation between the simultaneous inhibition of both pathways and induction of apoptosis.

In order to test this hypothesis in a new context, the activities of AKT and ERK were specifically inhibited by wortmannin and U0126 (Fig. 23b). The cells were treated with single or with combined drugs and the phosphorylation status was defined after 72 hours. In response to U0126 and wortmannin, ERK and AKT were no longer phosphorylated in 3 of the 6 cell lines (LN215, LN229 and SF767). This simultaneous inhibition correlated with the additive or the synergistic induction of apoptosis observed previously (Fig. 21). These results have to be compared with those presented in Fig. 21. SF767 exhibited a full decrease of P-AKT under the same conditions, while LN229 and LN215 showed a less pronounced effect. In contrast, cell lines with high levels of P-AKT (i.e PTEN $^{-/-}$ cells such as U373 and LN401) or sustained ERK activity such as U373, LN401 and Hs683 demonstrated to be more resistant and exhibited sustained signal of P-ERK and P-AKT. Thus, these results are in agreement with the additive or the synergistic induction of apoptosis of these drugs observed in the same cell lines (Fig. 21).

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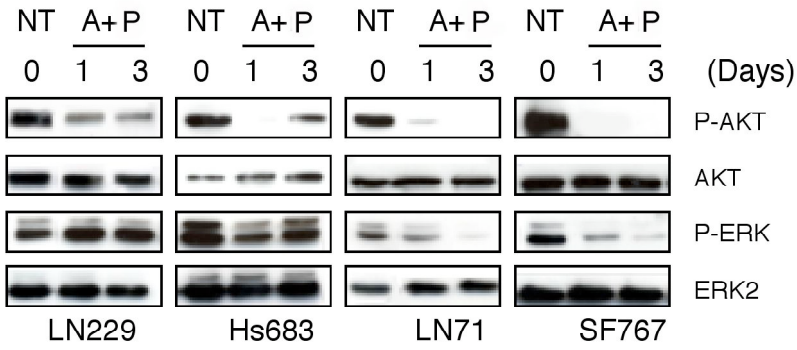

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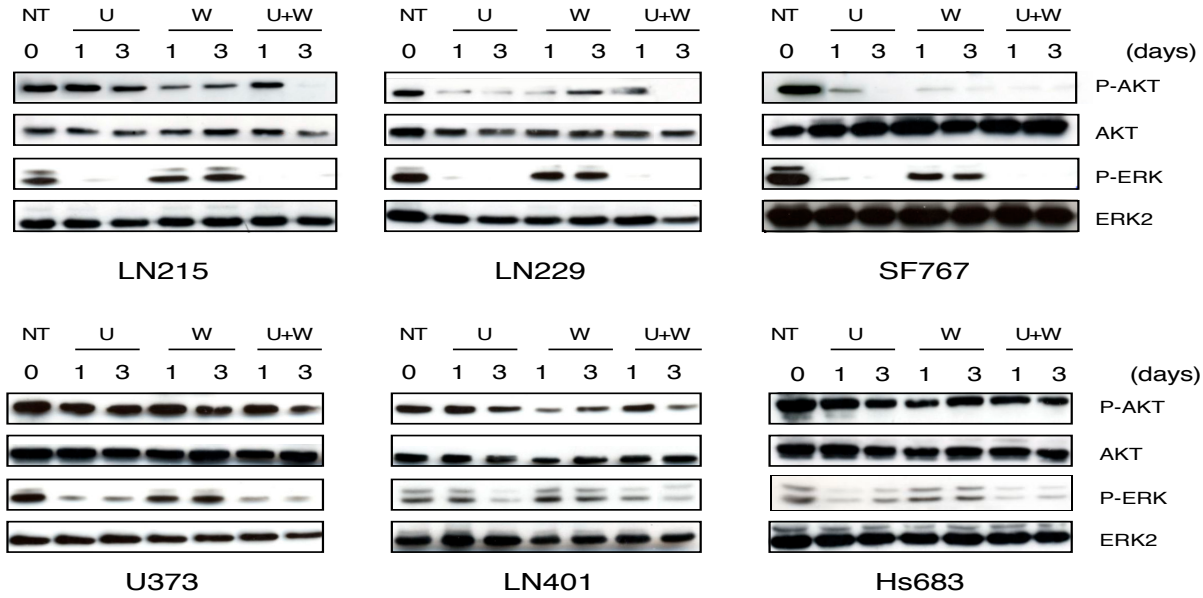

Figure 23: Combination of AEE788 and paputilone or U0126 and wortmannin can result in the inhibition of both phosphorylated AKT and ERK. (A) Cells were incubated during three days with Paputilone (0.7 nM) with or without AEE788 (1 μM). SDS was added at the indicated time and a western analysis was performed. (B) Cell lines were treated either with U0126 (20 μM) or wortmannin (1 μM) or in combination. After three days of treatment, SDS was added and western analysis was performed. NT: no treatment. A: AEE788, U: U0126, P: Patupilone, W: wortmannin.

f. Restoration of ERK activity abrogated the pro-apoptotic effect of U0126

In contrast to wortmannin, U0126 alone was sufficient to induce apoptosis (Fig. 21). In order to test the hypothesis that sustained activity of ERK should increase cell survival, the

phorbol ester TPA was used. This compound is activating PKC and ERK1/2 thereby modifying mitogenic signaling pathways (Crews *et al.*, 1992; Lang *et al.*, 1994).

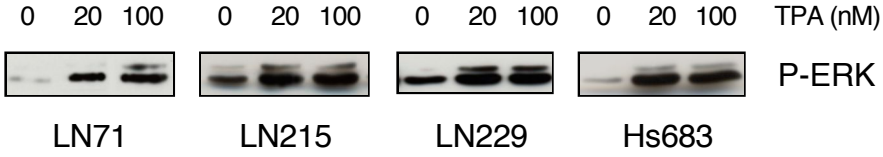

Figure 24: Activation of ERK in glioma cell lines by TPA. ERK activation in cell extracts was determined by western analysis. Cells were treated either with 20 or 100 nM of TPA during 30 min.

When exposed to 20 nM TPA, LN71, LN215, LN229 and Hs683 cells showed strong increase of phosphorylated ERK levels (Fig. 24).

In order to test a possible correlation between decreased phosphorylation of ERK and apoptosis, we selected the LN71 cell line because of its strong induction of apoptosis by the use of the combination of AEE788 and paputilone. LN71 was treated with AEE788 and Patupilone in presence or absence of TPA (20 and 100 nM) as described above (Fig. 25a). As expected, the combination of AEE788 and Paputilone induced apoptosis in a synergistic manner (Fig 18a), whereas the addition of TPA protected the cells from apoptosis. Higher activities of ERK1/2 were detected upon TPA treatment compared to the drug combination alone (Fig. 25b). Using the same protocol, cell lines were treated with the U0126 and wortmannin for 4 days in presence or absence of TPA (20 or 100 nM) (Fig. 25c). As described for AEE788 and patupilone, addition of 20 nM TPA reduced the fraction of cells undergoing apoptosis in response to U0126 and wortmannin. This correlated with the increased levels of ERK1/2 activation whereas in contrast to ERK, inhibition of phosphorylated AKT did not correlate with induction of apoptosis (Fig. 25d).

Although activition of ERK1/2 seemed to have a dominant effect over that of AKT (Fig. 25d) on apoptosis, cell lines such as LN215 retained high phosporylation levels of both proteins, even when the apoptosis level was reduced (Fig. 25c and d). It remained therefore unclear whether complete inhibition of AKT activity is required for the induction of apoptosis. Comparison of basal P-AKT levels before and after treatment revealed that partial or full reduction of P-AKT levels were associated with cell death (LN71, LN229 and Hs683)

(Fig. 25) but this was not the case with U373 and LN401 (Fig. 23). In contrast, in LN215 apoptosis occurred at higher level of AKT phosphorylation than in the control.

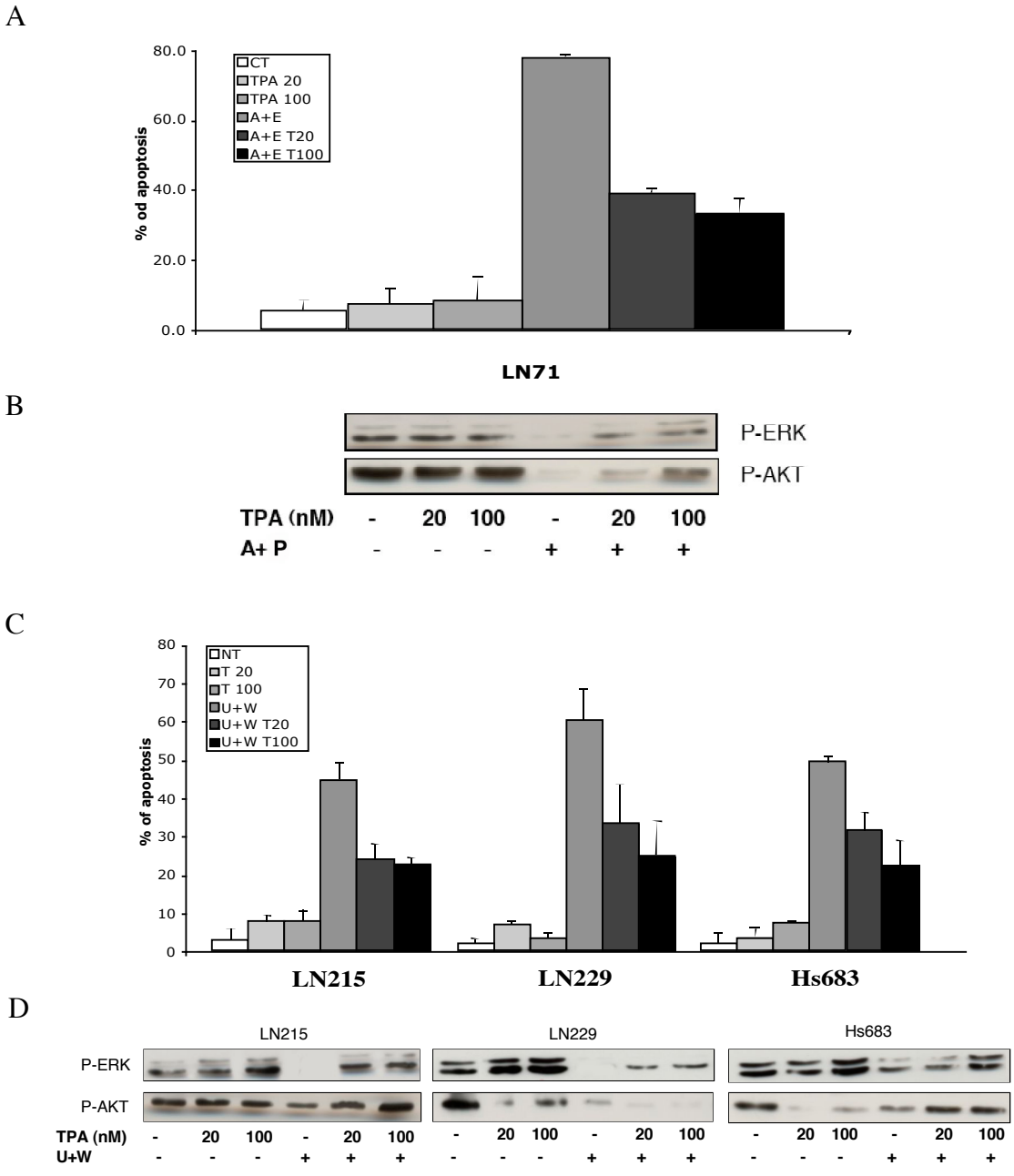

Figure 25: Addition of TPA caused a decrease in the induction of apoptosis by the drugs. (A) AEE788 and patupilone combination in presence or absence of TPA. In contrast to AEE788 and Paputilone, TPA was added every day. Cells were treated during 4 days prior to FACs analysis. (B) Correlation between protein activities and apoptosis of the AEE788 and paputilone combination. Cells were treated during 3 days prior to western analysis. (C) As described in (A) but with the combination of U0126 and wortmannin. (D) As described in (B) but with the U0126 and wortmannin combination. U: U0126 (20 μM), W: wortmannin (1 μM), A: AEE788 (1 μM) and P: paputilone (0.7 nM). Cells were treated during 3 days. After incubation, cell extracts were prepared by the addition of SDS sample buffer.

In order to further confirm the possible correlation of induction of apoptosis and low levels of AKT and ERK activities, induction of apoptosis was investigated with cell lines resistant to the AEE788/paputilone combination, for this purpose, LN229 and Hs683 cells were selected (Fig. 18a). Addition of either wortmannin (PI3K inhibitor) or U0126 (MEK inhibitor) should reveal which of these two protein kinase activities are critical for the induction of apoptosis (Fig. 26a). Addition of the two inhibitors caused induction of apoptosis. In LN229, the level of apoptosis induced by the inhibitors are only additive whereas in Hs683, an additive induction of apoptosis was observed with U0126 or synergy with wortmannin. Interestingly, the various cell lines responded in completely different ways to the two inhibitors. In contrast to LN229, Hs683 exhibited a higher sensitivity to the PI3K inhibitor than to the MEK inhibitor.

Whereas the triple combination of AEE788, U0126 and wortmannin induced 95 and 70% of apoptosis in LN229 and Hs683 (Fig. 22), respectively the addition of either U0126 or wortmannin to the AEE788 plus Patupilone only led to 30 to 40% of apoptosis in LN229 and Hs683. Western analysis of extracts from cells treated by these combinations was performed at the same time. While inhibition of both protein activities led to induction of high levels of apoptosis (compare Fig. 18a /23a and Fig. 21b/23b), a partial reduction of signaling only led to apoptosis in a fraction of the cell population ranging from 30 to 40% (Fig. 26a and b).

These results indicated that targeting either one of the kinases only partially sensitizes the cells to drug treatment (Fig. 26a). Only when both kinase activities are inhibited simultaneously high levels of apoptosis are induced. Taken together, the results suggest that the strong induction of apoptosis, by the two drugs is due to the inhibition of both protein kinases, ERK1/2 and AKT. Cell lines that were more resistant to the drug combination exhibited remaining activities of one or both kinases.

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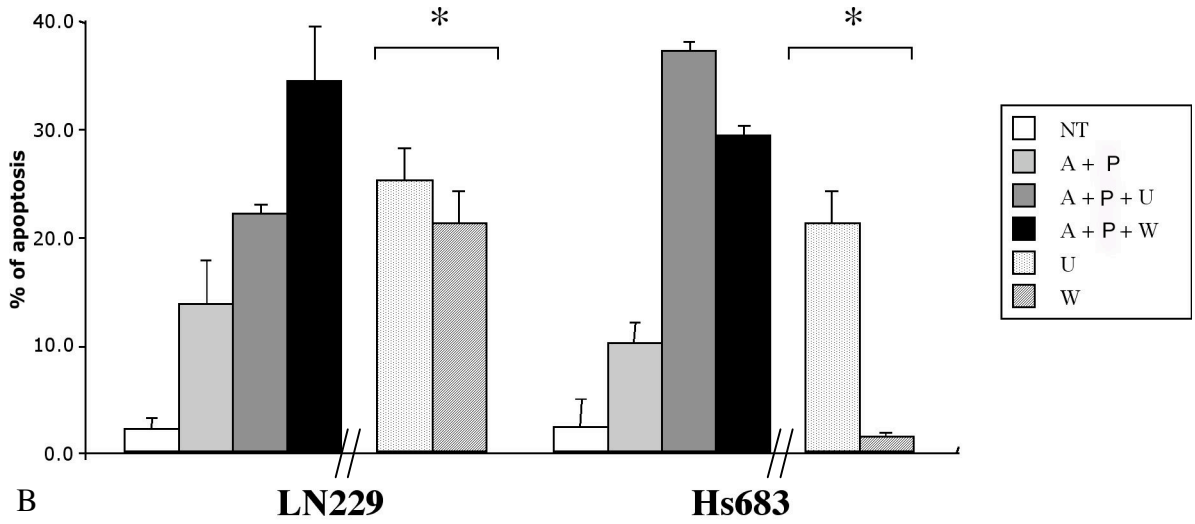

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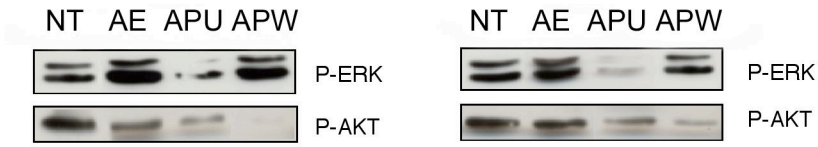

Figure 26: Induction of apoptosis by targeting either ERK or AKT activity. (A) LN229 or Hs683 were treated with AEE788 (1 μM) and Patupilone (0.7 nM) in combination either with U0126 (20 μM) or wortmannin (1 μM) during 4 days. (B) Western analysis was performed in order to determine the relative activities of ERK1/2 and AKT after 3 days of treatment. U: U0126, A: AEE788, W: wortmannin, P: patupilone. (*) Results from a previous experiment (Fig. 21).

Discussion

Although surgical resection, radiotherapy and chemotherapy can significantly improve the prognosis of patients with breast, lung or prostate cancers, these therapies remain of limited efficacy in the treatment of GBM. Progress made in molecular neuro-oncology has established that the PDGF and EGF pathways via PI3K/Akt and Ras/Raf/MEK/ERK signaling cascades play a critical role in gliomagenesis (Maher *et al.*, 2001). Accumulation of genetic alterations in these pathways confers a growth advantage to transformed cells leading to uncontrolled cell proliferation, high potential of invasion and drug resistance. Thus, identification of the altered signaling mediators allows the designation of potential molecular targets for isolating novel anti-tumor compounds. The epidermal growth factor receptor (EGFR) had been designated early as a major target for anti-tumoral drug screening for glioma, breast and NSCL cancer therapy (Annex III). The purpose of this work was to study drugs targeting EGFR and other signaling mediators for the treatment of gliomas and to define factors that allow induction of apoptosis in response to these anti-tumoral drugs in GBM cells in an *in vitro* cellular system.

Targeted strategies: the problem of cross-talk

The process of cellular signal transduction has been viewed for a long time as a linear cascade of successive events of activation and/or inhibition. Interconnections between distinct signaling cascades, designated *cross-talk*, provide a possible explanation on how inhibition of specific proteins can be circumvented through alternative pathways, as depicted in Fig. 27.

Treatment should not only consider the capability of AEE788 to block EGFR activation, but also the inhibition of molecular events that occur downstream of EGFR signaling. For example, many GBM cells carry PTEN mutations activating AKT/PKB. In this scenario, inhibition of RTK activation cannot be sufficient if downstream signaling components remain activated, thereby providing an explanation for the resistance of cell line U373 in the combination of AEE788 and patupilone at the concentrations tested. For this reason, drugs designed to target components located downstream of EGF signaling, such as MEK, would be better strategic choices for specifically acting on this pathway. This may be superior to targeting EGFR itself.

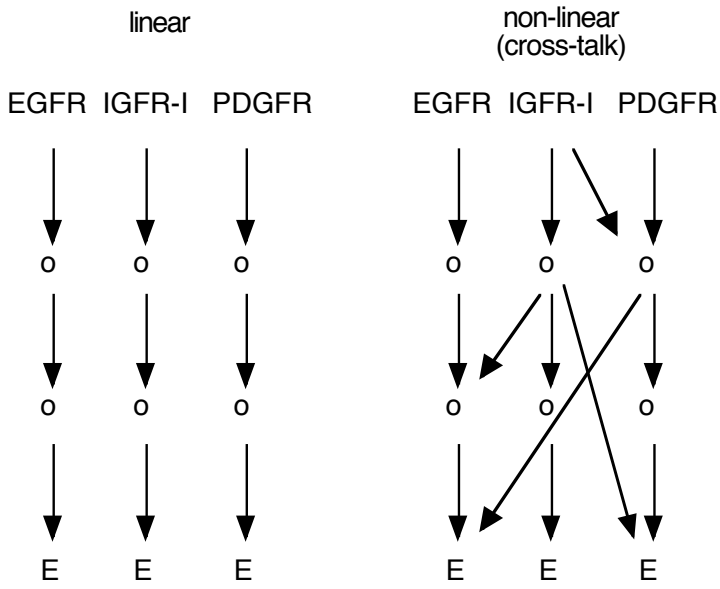

Figure 27: Linear and non-linear signaling pathways. EGFR, IGFR-I and PDGFR represent the upstream component whereas downstream components (o) lead to the activation or inhibition of the effectors (E).

A ubiquitous mechanism of resistance of tumor cells to intracellular drugs is related to the alterations of drug transport and efflux by the P-glycoprotein (P-gp) family of membrane transporters: P-gp, being the product of the multidrug-resistance gene *mdr1* (Schinkel et al., 1994). On the other hand, the ABC (ATP-binding cassette) transporter P-glycoprotein (Pgp) has been demonstrated as a key element of the blood-brain barrier (BBB) that can actively transport a wide variety of lipophilic drugs out of the brain capillary endothelial cells that form the BBB. In addition to Pgp, other ABC efflux transporters such as members of the multidrug resistance protein (MRP) family and breast cancer resistance protein (BCRP) seem to contribute to BBB function. Consequences of ABC efflux transporters in the BBB include minimizing or avoiding neurotoxic adverse effects of drugs that otherwise would penetrate into the brain. However, ABC efflux transporters may also limit the central distribution of drugs that are beneficial to treat CNS diseases (Loscher& Potschka, 2005).

In this context, the tumor resistance seen in EGFR targeted therapies can be attributed to the following reasons (Fig. 28):

(a) The simultaneous activation of tyrosine kinase receptors such as insulin-like growth factor receptor I (IGFR-I) or PDGFR. In glioma cell lines, inhibition of AKT/PKB activation is counteracted by sustained PI3K activation by IGFR-I (Chakravarti *et al.*, 2002).

(b) The existence of specific EGFR mutations. In contrast to NSCLC, where tumors with mutations at the EGFR kinase domain showed increased responsiveness to gefinitib (Sordella *et al.*, 2004), no EGFR mutations were observed in glioma lines (Barber *et al.*, 2005) providing an argument on the resistance of gliomas to anti-EGFR drugs.

(c) The constitutive activation of downstream mediators. Deletion of PTEN, which is highly prevalent in gliomas, upregulates AKT and counteracts ErbB1/2 inhibition by preventing apoptosis.

Gleevec and blast crisis: analogy with GBM treatment

The designation of a target for anti-tumoral drug screening must be based on its role in tumor induction. A famous example is provided by Gleevec, a compound used for chronic myeloid leukemia (CML) treatment. CML results from the Philadelphia chromosome translocation. This translocation generates a chimaeric BCR-ABL protein fusion that renders the tyrosine kinase of the proto-oncogene ABL constitutively active (Sawyers, 1999), and subsequently causes the malignant transformation. For this reason, it turned out to be a target of choice for drug screening (Daley *et al.,* and Lugo *et al.,* 1990). Gleevec was the first selective protein kinase inhibitor developed for targeted cancer therapies and treatment as a single agent has demonstrated remarkable clinical efficacy in CML, except at the blast crisis stage (Drucker, 2004) where mechanisms of resistance to Gleevec occur. Either BCR–ABL is reactivated through mutation(s) in the ABL kinase domain (Gorre *et al.*, 2001), or secondary tyrosine kinases of alternative signaling pathways become activated (Hingorani *et al.*, 2003).

Thus, these genetic modifications render Gleevec alone insufficient to counteract the tumorigenic effect produced by other signaling pathways. In contrast, EGFR alone, although overexpressed in gliomas, cannot induce glioma formation in mouse models (Bachoo *et al.*, 2002). Retrospectively, it is not surprising that targeting of EGFR by AEE788 alone does not induce cytotoxicity in glioma cells.

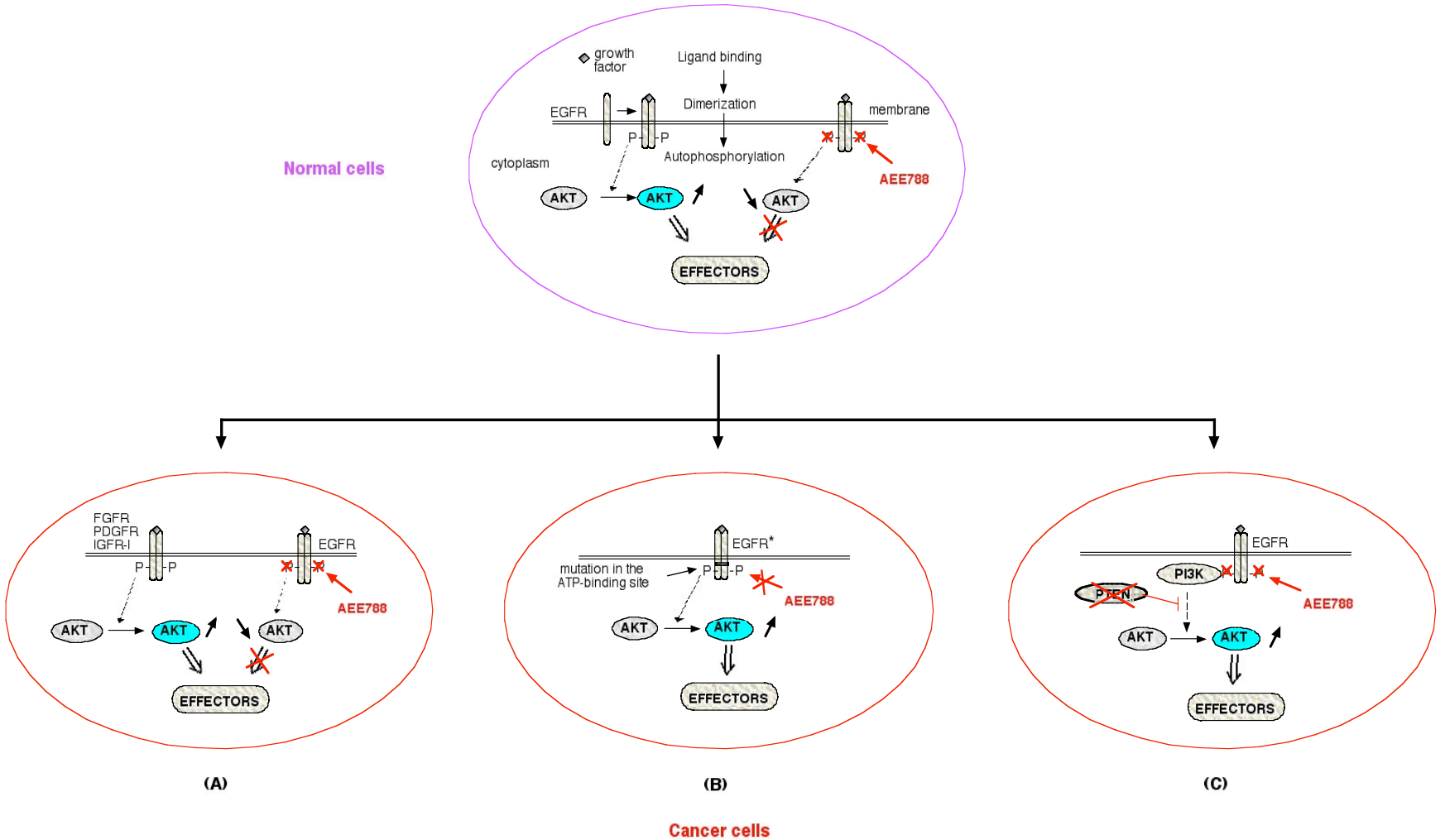

Figure 28: Different mechanisms of drug resistance in EGFR targeted strategy.. (a), (b) and (c) refers to the text. In normal cells, AEE788 blocks EGFR activation. Inhibition of AKT is not counteract by constitutive other signaling pathways (a) AEE788 blocks EGFR activation but other RTKs such as PDGFR counteract this inhibition by activation of AKT which results in activation of downstream signaling components. (b) Putative mutations in the ATP binding site of EGFR represented by (*). EGFR* is mutated and AEE788 cannot bind to the ATP binding site and block receptor activation. (c) AEE788 blocks EGFR but PTEN mutations leads to constitutive active AKT in GBM

Mouse brain models used: definition of new targets

Analyses of transgenic mouse models have established that gliomagenesis can be initiated from a specific combination of genetic alterations of AKT and RAS. Therefore, one can expect that molecular therapies targeting both pathways might be more successful than those disrupting the function of only a single pathway.

One other example is EGFR. EGFR gene amplification has been associated with poor prognosis for GBM patients (Nicholson *et al.*, 2001). New small molecular weight compounds such as PKI-166 or AEE788 have been generated to target EGFR. In this study, we used the two drugs to target EGFR in GBM cells. As it was defined in EGFR transgenic and Ink4a/Arf KO mice (Holland *et al.*, 1998) and in orthotopically transplanted brain tumor models (Bachoo *et al.*, 2002), activated EGFR cooperates with inactivation of Ink4a/Arf to induce glioma-like lesions. In fact, inactivation of both $p16^{INK4a}$ and $p19^{Arf}$ genes in cooperation with activated EGFR is of importance for the development of astrocytic tumors.

If two pathways have to be altered to develop a glioma in a mouse, we conclude that at least two pathways need to be targeted for treatment in humans, too. Among the seven glioma cell lines with defined genetic background for glioma genes (Ishii *et al.*, 1999) we had analyzed, six displayed expression of activated EGFR and four were *Ink4a/Arf* $^{-/-}$. We therefore considered these four latter cell lines to be suitable for testing EGFR as a drug target and the other ones as negative controls. Consistent with our hypothesis, SF767 and LN215 lines, both wild-type for *Ink4a/Arf*, were sensitive to AEE788. Conversely, GBM lines with activated EGFR together with a homozygous deletion at the *Ink4a/Arf* locus did not respond at all to AEE788.

Targeted combinations against EGFR, PDGFR and mTOR do not induce apoptosis

We have shown that AEE788 treatment induces little to no apoptosis in the cell lines U373 and LN401, in a similar manner as other PKI e.g. Gleevec is targeting overexpressed PDGFR in glioma (Hermanson *et al.*, 1992). Single targeting of RTKs did not show induction of apoptosis in GBM lines, according to the hypothesis on the necessity to hit two distinct glioma pathways. Consequently, we combined the anti-EGFR drug AEE788 and the anti-PDGFR drug Gleevec for glioma treatment. However, this combination did not lead to apoptosis. Retrospectively, this is consistent with the redundancy between both RTKs signaling pathways, and with the fact that overexpression of both EGFR and PDFDR may not

be sufficient to induce gliomagenesis.

Next, we performed the triple combination consisting of targeting the putative growth factor pathways (EGFR and PDGFR), together with the downstream component of the PI3K/Akt pathway –mTOR- that regulates cell growth (Inoki *et al.*, 2005). It turned out that application of AEE788, Gleevec and RAD001 resulted in a very modest induction of apoptosis in 3 out of 10 GBM cell lines. This is consistent with our hypothesis that EGFR, PDGFR and mTOR may be not critical to maintain the neoplastic phenotype in these GBM cells.

On the other hand, the results obtained by treating GBM cell lines with AEE788 have confirmed EGFR as a promoter of glioma cell proliferation and migration. In addition, AEE788 also targets the pro-angiogenic receptor KDR (VEGFR-2) with a high specificity (IC_{50}=77 nM) (Traxler *et al.*, 2004), although KDR inhibiton has not been established *in vivo* mouse xenogragft model. Interestingly, other *in vivo* data have shown AEE788 to increase survival of animals with intracranial tumor at concentration below the maximally tolerated dose (Goudar *et al.*, 2005) and confirmed a potential role of AEE788 as anti-tumor agent.

Combination of PKIs with a cytotoxic compound leads to a synergistic induction of apoptosis

Altogether, these results obtained with AEE788, Gleevec and RAD001 alone or combined did not induce tumor cell death *in vitro*. We intended to associate AEE788 with the highly cytotoxic compound Paputoline, in order to sensitize glioma cells to apoptosis.

Patupilone is a microtubule-targeting compound. Microtubules (MTs) are key components of the cytoskeleton, They are not only crucial for the development and the maintenance of cell shape, in the transport of vesicles, mitochondria and other components throughout cells, in cell signalling, but essential in cell division and mitosis. Moreover, a body of evidence suggests that aneuploidy is often caused by a particular type of genetic instability, called chromosomal instability, which may reflect defects in mitotic segregation in cancer cells (Rajagopalan & Lengauer, 2004). For these reasons, MTs have been designated as critical targets for anticancer drugs (Jordan & Wilson, 2004).

Patupilone exhibits *in vitro* cytotoxic properties on tumor cell lines of various lineages, including multi-drug resistance (MDR) cell lines (Chou *et al.*, 1998). Patupilone treatment revealed potent *in vivo* antitumor activity (Altmann *et al.*, 2000). However, mice exhibited loss of body weight, followed by death (Chou *et al.*, 1998) and clinical trials revealed very strong side effects (Diarrhea, fatigue, nausea and vomiting) (Goodin *et al.*,

2004). From the results obtained in the glioma cell lines tested, patupilone appeared to be efficient as low as at a nanomolar range. When exposed to patupilone at a concentration of 35 nM concentration, no glioma cell line tested showed resistance to apoptosis which render this compound much more efficient than PKI in the induction of apoptosis. The question is whether even lower concentrations of the drug, leading to a reduction of the side effects, would exhibit the same ability to induce apoptosis when used in combination with AEE788. Indeed, AEE788 has been shown to act as an anti-proliferative, anti-migratory and anti-angiogenic compound (Traxler *et al.*, 2004). Interestingly, we detected a synergistic induction of apoptosis in glioma cell lines when we combined AEE788 and Patupilone using concentrations that do not induce cell death when the two drugs are applied alone. This synergy occurred in LN71 and SF767 and slightly in LN229, U373 and Hs683 at picomolar range concentrations of patupilone (between 0.1 and 0.7 nM), whereas an additive effect was observed in LN401 and LN215. Evaluation of the concentration where 50% of apoptosis was reached (AC_{50}) demonstrated the strong potentiation of AEE788 (1 μM) to act as sensitizer in these GBM cell lines (Fig. 27).

Targeting downstream components of the EGFR signaling pathways induces glioma cell death

According to results shown in Fig. 18, the combination between AEE788 and Patupilone leads to non-additive, additive or synergistic effects. What are the reasons why synergistic induction of apoptosis was not observed in all lines, while low effect was found in other cell lines. MEK kinase 1 (MEKK1) has been reported to transduce c-Jun NH2-terminal kinase activation in response to changes in the microtubule cytoskeleton of mouse embryonic stem cells thereby protecting the cells from apoptosis (Yujiri *et al.*, 1998). c-Jun NH2-terminal kinase activation upon drug treatment may explain the induction of this synergy. However, when we treated the glioma cells with the taxol-like microtubule toxin patupilone, the JNK pathway was not affected in none of the glioma cell lines, regardless of their respective sensitivities towards the drug with respect to induction of apoptosis (data not shown). Besides the fact that our analysis has been performed with different and transformed cell types, we have measured JNK activation at much later time points namely at 24 and 72 hours. It appears that MEKK1-JNK pathway was not involved in the sensitivity to the drug.

Next, we analyzed whether constitutive activation of downstream mediators may lead to drug resistance, counteracting the effect of the EGFR inhibitor by maintaining activated

downstream regulators involved in survival or proliferation which could explain the inefficacy of targeted EGFR therapies in some cell lines. Downstream cascades of EGFR are the Ras/Raf/MEK/ERK and PI3K/Akt signaling pathways.

With regard to glioma animal models, transgenic mice developed gliomas with activated Ras and Akt (Holland *et al.*, 2000) or associated with the deletion of *Ink4a/Arf* locus (Uhrbom *et al.*, 2002), whereas single activated Ras or Akt failed to induce tumor formation. Altogether, these data argue for an involvement of AKT and ERK in the synergistic induction of anti-apoptotic signaling. This argumentation was confirmed in our AEE788-PATUPILONE combination: inhibition of their activities could explain the synergistic induction of apoptosis observed in the AEE788 and patupilone combination. We have demonstrated that a very strong downregulation of phosphorylation of both AKT and ERK1/2 occurs in the cells that show this synergy upon co-treatment with AEE788 and patupilone. This result suggests that induction of apoptosis requires the combined/simultaneous down regulation of both downstream signaling pathways of EGFR.

Inactivating AKT/PKB and ERK: a new strategy for glioblastoma

Efficacy of novel drug therapies should not only take into consideration the ability to block EGFR activation, but should also be tested for their potential to inactivate downstream signaling mediators. This idea is further supported by *in vitro* experiments, where downregulation of ERK and AKT signals correlates with induction of apoptosis by activation of the pro-apoptotic protein Bad (Ellert-Miklaszewska *et al.*, 2004).

We have shown that inhibition of AKT and ERK was not necessarily dependent upon EGFR activity. In addition, mouse models have shown that activation of both pathways is needed for tumor induction (Holland *et al.*, 1998 & Bachoo *et al.*, 2002). One exception is the transgenic mouse model reported by Ding *et al.*, (2001) in which activated Ras gene alone can promote growth of malignant human astrocytomas and exhibit genetic instability. Nevertheless, whether high levels of Ras-GTP are responsible for this genetic instability or whether this is simply due to other genetic alterations remains to be clarified.

In this context, we aimed to specifically inactivate the two main effectors in the Ras/Raf/MEK/ERK and PI3K/Akt signaling pathways, namely ERK and AKT by targeting MEK and PI3K with inhibitors. Consistently, the use of protein kinase inhibitors known to downregulate AKT and ERK activity confirmed our hypothesis about the need to target these two distinct signaling pathways.

The combination of these inhibitors was efficient in 6 out of 7 cell lines tested. Our experiments demonstrate that biologically based therapies, which target two components within complex signaling cascades, may effectively cooperate in the preclinical treatment of brain cancer.

Basically, we have demonstrated that dual inhibition of PI3K by wortmannin and ERK by the MEK inhibitor (U0126) results in a high induction of apoptosis in glioma cell lines. In addition to inhibition of PI3K, wortmannin inhibits myosin light chain kinase, phospholipases C, D andA_2, and DNA-dependent protein kinase (West *et al.*, 2002). Lack of specificity of wortmannin may be taken into consideration since the PI3K related kinases (PIKKs) comprise a family of proteins that play central roles in the control of cell growth, gene expression, genome surveillance and repair in eukaryotic cells. Mammalian cells express six PIKK family members, five of which-ATM, ATR, mTOR, DNA-PK, and hSMG-1-function as protein serine-threosine kinases (Abraham, 2004). U0126 inhibits 25 other protein kinases (Cohen, 1999), interleukin-2 synthesis and T cell proliferation (DeSilva *et al.*, 1999). In addition to their lack of specificity, both are only soluble in organic solvents, which limits their clinical application. Then, other members of these signaling pathways have to be defined (e.g. RAF in the RAS/MEK/ERK pathway). A compound such as BAY 43-9006 has recently entered phase III clinical trial (Strumberg & Seeber, 2005) and could be used, whereas drugs targeting the PI3K/Akt pathway are in development.

Outlook

Genomic instability is a hallmark of cancer and leads to the expansion of a cell population resistant to apoptosis (Hanahan & Weinberg, 2000). Since disabling apoptosis by itself might favour genetic instability, it becomes plausible that both mechanisms could cooperate to increase the oncogenic and metastatic potential of transformed cells. During the initial stages of oncogenesis, an accumulation of random alterations in the unstable genome will be selected for growth advantage that affects a restricted set of oncogenes (for example, oncogenes that encode anti-apoptotic genes) and tumor-suppressor genes.

Finally, genetic instability renders each tumor clonally heterogeneous and different from another one. In this study, cell lines showed different responses to various inhibitors. These results confirmed the interconnection between the signaling pathways and the singularity of each individual glioma cell line. In other words, each glioma cell line (or human primary tumor) needs to be specifically typed for its respective RTK expression pattern and

mutations in the EGFR, PDGFR and IGFR-I genes as of activities of downstream components (AKT and ERK). This genetic profiling of primary tumors would allow to provide an individualized (customized) therapy that may improve patient outcome.

Another feature of GBM is a striking and dramatic induction of angiogenesis. VEGF signaling plays a crucial role in the process of neovascularisation. Interestingly, VEGF and its receptor are both expressed in glioblastomas (Heldin & Westermark, 1989; Plate & Risau, 1995). Besides the proliferative and migratory effect mediated by EGFR and ErbB2, these tyrosine kinase receptors might also mediate the expression of pro-angiogenic molecules. Blocking EGFR and/or ErbB2 has been demonstrated to decrease pro-angiogenic molecules of tumor cells to inhibit tumor-associated angiogenesis. Then, inhibition of VEGF-induced angiogenic signals will target the essential role of angiogenesis in GBM development. AEE788 blocks EGFR and KDR, and also reduces secretion of VEGF via inhibition of EGFR. These combined effects render this compound highly attractive for the treatment of solid cancer such as GBM.

Endothelial cells in the tumor vasculature are genetically more stable than tumor cells, and thus less likely to develop resistance. Anti-angiogenic therapy is directed specifically against endothelial cells in the newly-formed tumor microvasculature, so that the passage of drugs through the blood-brain-barrier (BBB) is not required and the therapy does not appear to induce acquired drug resistance. This approach allows escaping from the lack of effective methods of drug delivery, due to the presence of the BBB, which prevents the effective therapy of brain tumors. Then, the anti-angiogenic strategy (e. g. AEE788) to target both EGFR and KDR in combination with future inhibitors of AKT and ERK could be one potential therapy of glioblastoma. Impairing neovascularization of the tumor would result in starvation of the tumor. Since the potential anti-angiogenic ability of AEE788 cannot be tested *in vitro*, an orthotopic xenograft model is needed to address this question.

In vitro assays allow to determine easily and quickly the impact of a drug according to the genetic background. Considering that each cell line displays a specific combination of genetic defects, they represent the heterogeneous nature of gliomas, and the efficacy of the drug on a number of those could statistically mimic the potential effect of this drug on an *in vivo* model. Although xenograft studies are not fully predictive of the therapeutic efficacy of cancer therapies in clinical trials, orthotopic xenograft studies may offer additional information over cell culture studies. Thus, preliminary *in vitro* experiments are required for the determination of the spectrum of actions of given compounds on cell survival, on cell-

cycle and on biomarkers. Development of such models enables to study simultaneously all the parameters previously described.

Conclusion

We found that GBM cells show resistance to treatment with drugs that target individual signaling pathways. As a single drug, AEE788 has provided new hope since its anti-migratory, cytostatic effect and anti-angiogenic properties have been shown *in vitro* and *in vivo* experiments. We have opened new approaches in the treatment of GBM, by combining AEE788 with the cytotoxic compound paputilone at doses of reduced toxicity, where synergistic effects were observed in 50% of the cell lines. Induction of apoptosis in these conditions was associated with downregulation of P-AKT and P-ERK. On the other hand, our results strongly suggest that such cells might successfully be brought to apoptosis by an additional treatment with compounds e.g. wortmannin or U0126 leading to the simultaneous downregulation of the PI3K/Akt and of the Ras/Raf/MEK/ERK signaling pathways. In the two strategies proposed, the readout of a successful therapy correlates with downregulation of proteins ERK and AKT. In the context of GBM treatment, we propose a combined therapy based on the simultaneous targeting of the Ras/Raf/MEK/ERK pathway with a protein kinase inhibitor and of the PI3K/Akt (PKB) signaling pathway with a phosphatidylinositol 3-kinase inhibitor.

Materials and methods

Cell culture

All cell lines were grown at 37°C in a 5% CO_2 humidified atmosphere. Culture medium contained 10% fetal bovine serum (FBS, No S1810, Labforce, Basel, Switzerland) and Dulbecco's Modified Eagle Medium (DMEM, No 10938-25, Invitrogen Corporation, UK) supplemented with glutamax (No 35050-038, Invitrogen Corporation, UK) and antibiotics solution (No 15240-062, Invitrogen Corporation, UK). Cell detachment was performed with 1 ml trypsin-EDTA 1X for 5 minutes at 37°C.

Pharmacological inhibitors

AEE788, patupilone, Gleevec and PKI-166 were provided by Novartis Pharma AG, Basel, Switzerland. They were prepared as a 10 mM stock solution dissolved in DMSO, aliquots were stored a –20°C. Wortmannin (Sigma) was prepared as a 1 mM stock solution in DMSO and stocked at 4°C. U0126 (Promega) as a 10 mM stock solution in DMSO, aliquoted and stocked at –20°C. TPA (LC laboratories) as a 2 mM stock solution in DMSO, aliquoted and stocked at –20°C. As remarks, TPA, U0126 and wortmannin were added every day during the experiment at their respective concentrations.

Cell cycle profile and apoptosis

Growing cells were treated during 24h, briefly washed with PBS and trypsinized. Cell suspension, was centrifuged at 800 rpm for 5 min, pelleted cells were fixed in ice cold 70% ethanol and kept at 4°C for 30 min. Cells were resuspended in a 1X PBS solution containing 50 ng/ml propidium iodide (PI) and 50 μg/ml of RNAse. Percentages of cells were determined by flow cytometer (FACs) in G1 and G2/M for cell cycle and in sub-G1 for apoptosis, respectively.

Proliferation

BrdU incorporation analysis

Cells were plated to grow during 24 hours in DMEM/10% FCS. Then, cells were incubated with AEE788 alone for 24h. BrdU was added to 10μM final concentration and cells incubated at 37°C during 1 hour prior harvesting cells, cells were prepared according to the

manufacturer's instructions (Becton Dickinson, Franklin Lakes, NJ) and BrdU incorporation was analyzed by FACs analysis.

Colony Forming Assay (CFA)

Effect of the drug on cell proliferation was evaluated by measurement of the diameter of the resulting colony. 10^3 cells were plated in a 6 cm dish. After 24h, the drug was added and cells grown during 72h. Cells were washed twice with PBS, fixed during 10 min with MetOH/Ace. Acid (3:1)(v/v), and finally stained during 20 min with giemsa, washed several times with H_2O. The colony counter takes the 3 parameters which defin the colony size (> 10, >50, >200 μm).

Migration

Boyden chamber

Migration assays were performed by precoating Boyden chamber well inserts with a 8.0 mm pore size (Becton Dickinson, Franklin Lakes, NJ) with 5 mg/ml collagen (Roche Diagnostics, Switzerland) for 2 h at room temperature and washed with PBS. Cells were pre-incubated in presence or absence of AEE788 (1 μM). After resuspension at a concentration of $1x10^6$ cells/ml in 10% FCS/DMEM, 100 μl were added to the upper chamber of the well insert, whereas 500 μl of DMEM 10% FCS containing also the appropriate conditions (EGF 100 $ng.ml^{-1}$, Sigma) is added to the lower chamber. Cells were allowed to migrate for 3 hours, after which time they were fixed in methanol and stained using Hematoxylin (Fluka). Cells remaining in the upper chamber were removed by wiping, and remaining cells adhering to the underside of the membrane were counted. Each condition was done in triplicate.

Wound Healing Assay (WHA)

Cells were plated in 6-well plates at 70% confluence in DMEM with 10% serum. At 24 h after seeding, the cell monolayers were wounded by scraping with a 200-μl plastic micropipette tip, washed, and then refed with complete DMEM. After 48 h, cells were fixed with 4% paraformaldehyde and photographed with an inverted microscope.

Immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde, permeabilized in phosphate-buffered saline (PBS) containing 0.5% Triton X-100, and blocked with FCS. Cells were incubated with anti-

tubulin antibody for 1 h at room temperature, washed, and incubated with Cy3 (Molecular Probes, Eugene, OR). After final washes and mounting, nuclei were visualized by incubating the cells for 5 min in 0.1 mg/ml propidium iodide (Sigma, St. Louis, MO), cells were examined using a laser scanning deconvolution microscope with a 60X or 40X oil immersion objective.

Nucleic acids analysis

RNA isolation

10 cm plates from each cell line were grown to a 90% confluence (2 per cell line). Medium was sucked off, and cells washed with 1x PBS, resuspended into 1,5 mL Trizol (Invitrogen Corporation, UK) and incubated for 3min at room temperature (RT), cell lysates were homogenized the cells and after 5 min the cells were transferred into 3 microfuge tubes and left at RT for another 3-5 min. 200 μl choloroform was added and tubes were vortexed for 30 sec, and left at RT for 5 min. After centrifugation at 14000 rpm for 15 min at 4°C. To avoid genomic DNA and proteins contaminations, the upper (aqueous) phase was carefully transferred to fresh tubes. RNA was precipitated by addition of 500 μl isopropanol. RNA precipitate was pelleted down at 14000 rpm for 10 min at 4°C. Supernatant was removed and the RNA was washed with 1 mL ethanol and pelleted down at 12000 rpm for 5 min at 4°C, air-dried on ice and resuspended in 100 μl DEPC-treated H_2O. 5 μl of RNA solution was added to 495 dH_2O. Optical density was measured at 260 and 280 nm. The RNA in the remaining 95 μl was precipited by addition of 9,5 μl 4M LiCl, and 380 μl 100% EtOH.
The concentration of RNA was calculated based on the OD_{260nm}, 1 OD unit is equivalent to 40 μg/mL

cDNA synthesis

The volume corresponding to 5µg of RNA was centrifuged at 14000 rpm and 4°C for 20 min. Supernatant was carefully withdrawn. Pellets were air-dried on ice and resuspended into 9 µl of DEPC-treated H_20 + 1 µl of oligo dT (random hexamer). RNA samples were denatured at 65°C for 5 min. cDNA synthesis master mix was added.

cDNA Synthesis Master Mix :

5X buffer	**4µl**	**34.0**
(0.1M) DTT	**1**	**8.5**
(10mM) dNTPs	**2**	**17.0**
dH_20	**1**	**8.5**
RNAse out	**1**	**8.5**
Thermoscript-RT	**1**	**8.5**
	10µl/tube	**8.5 reactions**

10µl of cDNA Master Mix is made for each tube

The cDNA synthesis took place at 50 °C for 1 hour. Thermoscript-RT was heat-inactivated at 85 °C for 5 min and the RNA template was degraded by 1 µl RNAse H at 37 °C for 20 min. Finally, sample volume was adjusted to 50 µl by addition of DEPC-treated H_20.

PCR Master Mix

dH_20	**18.5µl**	**222.0µl**
10x	**2.5**	**30.0**
dNTPs	**0.5**	**6.0**
Actin F	**0.4**	**4.8**
Actin R	**0.4**	**4.8**
Taq	**0.2**	**2.4**
cDNA	**2.5**	**---**
	25 µl/tube	**For 12 tubes**

Program used for amplification:

95°C	2 min	
95°C	30 sec	40 cycles
58	1 min	
72	1 min	
72	5 min	

12.5µl of each sample was mixed with 2,5 μl of gel loading buffer (GLB) and run on a 1.0% agarose gel to check for amplification.

EGFR Primer PCR Master Mix

dH_20	**18.3µl**	**73.2µl**
10x	**2.5**	**10.0**
dNTPs	**0.5**	**2.0**
Primer sens	**0.5**	**2.0**
Primer antisens	**0.5**	**2.0**
Taq	**0.2**	**0.8**
cDNA	**2.5**	**-**
	25 µl/tube	**4 tubes**

The above samples were amplified in the thermo-cycler with following cycles:

95°C	2 min	
94°C	30 sec	35 cycles
60°C	1 min	
72°C	3 min 30	
72°C	5 min	

The PCR product (12,5 μl) was loaded on a 1% agarose gel.

Primers (5'-3')

EGFRs: gcg **atg** cga ccc tcc gga cg (initiation codon)

EGFRas: gg**t ca**t gct cca ata aat tc (stop codon)

Protein analysis

Protein extraction

Cell were washed with PBS, scraped and resuspended into 1X SDS Sample Buffer (62,5 mM Tris/Hcl, 2% SDS, 10% Glycerol, 50 mM DTT). Cell lysates were boiled at 95°C during 5 minutes and aliquots were made and stored at –20°C.

Western analysis

Protein were separated by size on a SDS-PAGE gel (8 to 14%), and transferred on a nitrocellulose membrane (Hybond-C, Amersham Life Sience) for 3 hours at 0.8 A at 4°C. Membrane was blocked with 5% non fat dry milk in TBS-Tween (10 mM Tris, 150 mM NaCl, 0,05% Tween 20) and 1% BSA. Primary antibodies were diluted (next section) either in TBS-Tween or in 5% non fat dry milk, 1% BSA in TBS-Tween overnight at 4°C. Three additional washes with TBS-Tween were performed before incubation with the appropriate secondary antibody. Then, after three washes in TBS-Tween, signal detection by chemiluminescence (ECL detection kit, Amersham) was performed.

List of antibodies

Name	Host	1st dilution	2nd dilution	Reference	Company
4G10	M	2000	20000	05-321	U
ACTIN	R	2000	20000	A5060	S
AKT	R	500	5000		*
P-AKT	M	5000	10000	05-669	U
ERK2	R	1000	10000	sc-154	SC
P-ERK	M	500	10000	sc-7383	SC
EGFR	G	100	10000	sc-03	SC
EGFR-PY 992	R	500	5000	9922	C
EGFR-PY 1173	G	100	5000	sc-12351	SC
ErbB2	R	500	20000	06-562	U
ErbB2-PY 877	R	250	5000	9922	C
ErbB2-PY 1248	R	250	5000	9922	C
ErbB3	R	100	10000	sc-285	SC
ErbB4	R	100	10000	sc-283	SC
PTEN	R	1000	10000	07-016	U
KDR	M	200	10000	07-158	U
PARP	R	1000	10000	9542	C
p21	M	1000	5000	sc-6246	SC

anti-mouse				31432	P
anti-rabbit				31462	P
anti-goat				31402	P

CONFOCAL

Tubulin	M	100		sc-6251	SC
Cy3	anti-M		500	715-165-150	JIR

M: mouse, R: rabbit, G: Goat, U: Upstate Biotechnology, Lake NY, SC: Santa Cruz, S: Sigma, St. Louis, MO, C: Cell Signaling, JIR: Jackson Immunoresearch Laboratories , P: Pierce

* gift from Bryan Hemmings (FMI).

Abbreviations

AC_{50} (nM): concentration where 50% of apoptosis is reached
CDK4: cyclin-dependent kinase 4
CML: chronic myeloid leukemia
CNS: central nervous system
DNA PK: DNA dependent ptotein kinase
EC_{50}: 50% effective concentration
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
ERK1/2: extracellular regulated-signal kinase 1 and 2
GBM: glioblastoma multiforme
GIST: gastrointestinal stromal tumor
GSK3β: glycogen snthase kinase-3β
HDM2: human double minute 2
IGFR-I: insulin-like growth factor receptor-1
MAPK: mitogen activated protein kinase
MEK: mitogen-activated kinase/ERK kinase
MEKK1: mitogen-activated protein kinase kinase 1
MNK: p38 MAPK –dependent kinase
NSCLC: non small cell lung cancer
NF1: neurofibromin
NF-κB: nuclear factor of κB
PDGF: platelet-derived growth factor
PDGFR: platelet-derived growth factor receptor
PDK1: 3-phosphoinoside-dependent protein kinase
PI3K: phosphatidylinositol 3-kinase
PLA_2 :phospholipase A_2
PKI: protein kinase inhibitor
PTEN: phospatase tensin homolog
RB: retinoblastoma susceptibility protein
RSK: p90 ribosomal protein S6 kinase
RTK: receptor tyrosine kinase
RTKi: receptor tyrosine kinase inhibitor
SCF: stem cell factor
VEGF: vascular endothelial growth factor
VEGFR: vascular endothelial growth factor receptor
WHO: world health organization

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Annex I: Mouse models of glioma and their construction. Signal transduction pathways are activated by expression of the indicated genes. Cell cycle arrest pathways are experimentally disrupted by the indicated genetic alterations. GFAP: Glial Fibrillary Acidic Protein, NSC: Neural Stem Cells.

Signal transduction abnormalities	Cell cycle arrest disruption	Cell of origin	Reference
Kras and Akt		Nestin expressing progenitor	Holland *et al.*, 2000
EGFR	Ink4a/Arf-/-	Nestin expressing progenitor	Holland *et al.*, 1998
EGFR	Ink4a/Arf-/- Ink4a/Arf+/-	NSC and Astrocyte	Bachoo *et al.*, 2002
NF1 deletion	p53 deletion	Astrocytoma	Reilly *et al.*, 2000
Ras	MDM2,CDK4 overxexpression p53 deletion	GFAP expressing astrocyte	Ding *et al.*, 2002
Hras	p53, RB deletion and telomere maintenance	Astrocyte	Rich *et al.*, 2001
Kras and Akt	Ink4a/Arf-/-	GFAP expressing astrocyte and Nestin expressing progenitor	Uhrbom *et al.*, 2002
Kras and Akt	Arf -/-	GFAP expressing astrocyte	Uhrbom *et al.*, 2005
Kras and Akt	Ink4a -/- OR Arf-/-	Nestin expressing progenitor	

Annex II: Expression of ErbB receptors and their ligands in cancer. (TGF-α, transforming growth factor-α; NRG1, neuregulin-1; HB-EGF, heparin-binding epidermal growth factor).

Molecule	Nature dysregulation	Type of cancer	Notes	References
Ligands				
TGF-α	Overexpression	Prostate	Expressed by stroma in early, androgen-dependent prostate cancer and by tumours in advanced, androgen-independent cancer	Scher *et al.* , 1995
	Overexpression	Pancreatic	Correlates with tumour size and decreased patient survival; may be due to overexpression of Ki-Ras, which also drives expression of HB-EGF and NRG1	Yamanaka *et al.* , 1993
	Overexpression	Lung, ovary, colon	Correlates with poor prognosis when co-expressed with ErbB1	
NRG1	Overexpression	Mammary adeno-carcinomas	Necessary, but not sufficient for tumorigenesis in animal models	Krane & Leder, 1996
Receptors				
ErbB1	Overexpression	Head and neck, breast, bladder prostate, kidney, NSCLC	Significant indicator for recurrence in operable breast tumours; associated with shorter disease-free and overall survival in advanced breast cancer;may serve as a prognostic marker for bladder, prostate, and non-small-cell lung cancers	Gorgoulis *et al.* , 1992 Irish & Bernstein, 1993
	Overexpression	Glioma	Amplification occurs in 40% of gliomas; overexpression correlates with higher grade and reduced survival	Wong *et al.* , 1992
	Mutation	Glioma, lung, ovary, breast	Deletion of part of the extracellular domain yields a constitutively active receptor	Moscatello *et al.,* 1995
ErbB2	Overexpression	Breast, lung pancreas, colon oesophagus, endometrium, cervix	Overexpressed owing to gene amplification in 15–30% of invasive invasive ductal breast cancers Overexpression correlates with tumour size, spread of the tumour to lymph nodes, high grade, high percentage of S-phase cells, aneuploidy and lack of steroid hormone receptors	Ross *et al.* , 1998
ErbB3	Expression	Breast, colon gastric, breast cancer and other carcinomas	Co-expression of ErbB2 with ErbB1 or ErbB3 improves predicting power	Lyne *et al.* , 1997 Xia *et al.* , 1999
	Overexpression	Oral squamous cell cancer	Overexpression correlates with lymph node involvement and patient survival	Schintani *et al.* , 1995
ErbB4	Reduced expression	Breast, prostate	Correlates with a differentiated phenotype	Kew *et al.* , 2000
	Expression	Childhood medulloblastoma	Co-expression with ErbB2 has a prognostic value	Gliberston *et al.* , 1997

Adapted from Yarden Y. & Sliwkowski M. X. Nat. Rev. Mol. Cell Biol. 2: 127-37 (2001)

Annex III: Some examples of drugs targeting EGFR/ErbB2

Compound	Type	Company	Target	Status
Cetuximab (C225)	Chim. mAb	Imclone Systems	EGFR	Phase III
EMD 72000	Hum. mAb	Merck	EGFR	Phase I/II
Thera CIM	Hum. mAb	YM Biosciences	EGFR	Phase I/II
ABX-EGF	Hum. mAb	Abgenix	EGFR	Phase I
Iressa (ZD1839, Gefinitib)	TKI	AstraZeneca	EGFR	Phase II
Tarceva (OSI-774)	TKI	Roche/Genentech/OSI	EGFR	Phase III
PKI-166	TKI	Novartis	EGFR/ErbB2	Phase I
CI-1033	TKI	Pfizer	EGFR/ErbB2	Phase II
GW2016	TKI	GlaxoSmith Kine	EGFR/ErbB2	Phase I
EKB-569	TKI	Wyeth	EGFR	Phase I
AEE788	TKI	Novartis	EGFR/ErbB2	Phase I

Adapted from Holbro *et al*., 2003

Annex IV:

a. List of glioma cell lines and their respective genetic status for PTEN-p53-p16/p14

Name:	**PTEN**	**p53**	**p16/14**
LN-71	**mut**	**mut**	**del**
LN-215	**mut**	**mut**	**wt**
LN-229	**wt**	**mut**	**del**
LN-401	**mut**	**mut**	**del**
LN-405	**mut**	**mut**	**wt**
U87MG	**wt**	**wt**	**del**
SF767	**wt**	**wt**	**wt**
U343MG	**mut**	**wt**	**del**
BS-125.3.2	**mut**	**del**	**+/-**
BS-125II.2	**mut**	**del**	**+/-**

TP53, PTEN, p16 status in glioma cell lines. mut: mutated, del: homozygous deletion, wt: wild-type, +/-: one allele deleted. (Ishii *et al.,* 1999)

b. Other cell lines tested

Name:	**PTEN**	**p53**	**p16/p14**
U373	**mut**	**mut**	**wt**
Hs683	**wt**	**mut**	**del**

Combination of sublethal concentrations of epidermal growth factor receptor inhibitor and microtubule stabilizer induces apoptosis of glioblastoma cells

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Abstract

The oncogenic epidermal growth factor receptor (EGFR) pathway triggers downstream phosphatidylinositol 3-kinase (PI3K)/RAS-mediated signaling cascades. In transgenic mice, glioblastoma cannot develop on single but only on simultaneous activation of the EGFR signaling mediators RAS and AKT. However, complete blockade of EGFR activation does not result in apoptosis in human glioblastoma cells, suggesting additional cross-talk between downstream pathways. Based on these observations, we investigated combination therapies using protein kinase inhibitors against EGFR, platelet-derived growth factor receptor, and mammalian target of rapamycin, assessing glioblastoma cell survival. Clinically relevant doses of AEE788, Gleevec (imatinib), and RAD001 (everolimus), alone or in combinations, did not induce glioblastoma cell apoptosis. In contrast, simultaneous inactivation of the EGFR downstream targets mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase and PI3K by U0126 and wortmannin triggered rapid tumor cell death. Blocking EGFR with AEE788 in combination with sublethal concentrations of the microtubule stabilizer patupilone also induced apoptosis and reduced cell proliferation in glioblastoma cells, accompanied by reduced AKT and ERK activity. These data underline the critical role of the PI3K/AKT and the RAS/RAF/mitogen-activated protein/ERK kinase/ERK signaling cascades in the cell-intrinsic survival program of sensitive glioblastoma cell lines. We conclude that drug combinations, which down-regulate both ERK and protein kinase B/AKT activity, may prove effective in overcoming cell resistance in a subgroup of glioblastoma. [Mol Cancer Ther 2007; 6(2):773–81]

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Introduction

Glioblastoma multiforme is the most frequent malignant neoplasm of the human central nervous system. Surgery can only control the highly proliferative component of the disease, whereas widespread tumor cell infiltration into normal brain areas resists radiotherapy and chemotherapy (1, 2). Amplification and overexpression of the gene encoding the epidermal growth factor (EGF) receptor (EGFR) are detected in ~50% of glioblastomas and are mainly associated with disease progression (3, 4). EGFR is a member of the ErbB family of receptor tyrosine kinases (5). Small molecular weight compounds with EGFR protein kinase inhibitory (PKI) activity, such as PKI-166 or AEE788, have a cytostatic effect *in vitro* on tumor cells that overexpress EGFR (6, 7). In addition, treatment of non–small cell lung cancer with the EGFR PKI imatinib (gefitinib) resulted in tumor growth control in 10% of patients (8). In fact, tumors that responded to gefitinib specifically carried specific mutations in the EGFR tyrosine kinase domain (8–10). Although responses to gefitinib have also been observed in a limited number of glioblastoma cases (11), a specific molecular profile is associated with response that differs from the lung signature (12–15). To date, several small molecular weight inhibitors, such as Gleevec or erlotinib/gefinitib, applied as monotherapies for the treatment of gliomas, only resulted in limited effectiveness (16). This has supported the hypothesis that combination of drugs would be a more appropriate treatment for glioma.

EGF mediates signaling via phosphatidylinositol 3-kinase (PI3K)/AKT and RAS/mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK pathways (17–19), which are redundantly stimulated by other activated growth factor receptors (e.g., the insulin-like growth factor receptor-I; ref. 20). In at least 50% of glioblastoma, PI3K is activated either by loss of function of the tumor suppressor PTEN (21) or by gain-of-function mutations in the *PIK3CA* gene, which encodes the p110 catalytic subunit of PI3K (22). Interestingly, activated protein kinase B (PKB)/AKT cooperated with RAS in the induction of malignant gliomas in a murine brain tumor model (23). Furthermore, activation of PKB/AKT has been detected in several types of human cancers and found to be associated with poor clinical outcome (24–26) and resistance to chemotherapy and radiotherapy (24, 27, 28).

Although no RAS mutations have been detected in glioblastoma, high levels of RAS-GTP have been documented in high-grade astrocytomas (29, 30). It has been suggested that ERK plays a critical role in cellular transformation and resistance to apoptosis (31). Evidence for a cooperation between AKT and RAS has been proven by a transgenic mouse model in which both oncogenes are ectopically expressed in normal astrocytes, giving rise to glioblastoma, whereas neither AKT nor RAS alone was sufficient for tumor induction (23).

Several examples of combined therapies have recently undergone advanced clinical trials in cancer treatment. For example, lapatinib with tamoxifen has been tested together in breast cancer (32), and in xenografted tumors (32), PKI AEE788 has been associated with the rapamycin-derivative RAD001 for the treatment of glioblastoma (33–35). Successful drug combinations allow the use of lower doses, possibly reducing toxicity and limiting the degree of acquired drug resistance. Given the limited efficacy of PKI to induce cell death in rapidly proliferating tumor cells, these inhibitory drugs may depend on the coadministration of a cytotoxic drug for the induction of cell death rather than on the coadministration of a second cytostatic compound. The principle of cytotoxicity of patupilone (epothilone B, EPO906) relies on the inhibition of microtubule depolymerization, which is lethal for all dividing cells. The antitumor activity of patupilone has been proven *in vitro* and *in vivo* in lung, breast, colon, and prostate cancers (36). Patupilone also shows clinical activity in a range of solid tumors (37) and is now in phase III clinical development.

In the present study, we examined the induction of cell death on glioblastoma lines *in vitro* by targeting EGFR/ErbB2 by the receptor tyrosine kinase inhibitor AEE788 alone or in combination with the cytotoxic compound patupilone. We further evaluated the activation status of the EGFR downstream signaling mediators PKB/AKT and ERK following combined treatment with patupilone and AEE788. Our study points to a critical role of both PI3K and ERK in glioblastoma cell survival.

Materials and Methods

Cell Culture

All cell lines were grown at 37°C in a 5% CO_2 humidified atmosphere. DMEM culture medium was supplemented with 10% FCS and standard antibiotics. Cells were detached with 1 mL trypsin-EDTA (1×) for 5 min at 37°C. The genetic status of all cell lines at *p53, p16, p14ARF*, and *PTEN* tumor suppressor genes has been previously reported (38, 39).

Pharmacologic Inhibitors

AEE788, RAD001, and patupilone (provided by Novartis Pharma AG, Basel, Switzerland) and U0126 (Promega, Madison, WI) were dissolved in DMSO as 10 mmol/L stocks and stored as aliquots at −20°C. Wortmannin (Sigma, St. Louis, MO) was prepared as a 1 mmol/L stock solution in DMSO and stored at 4°C, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA; LC Laboratories, Woburn, MA) was prepared as a 2 mmol/L stock solution in DMSO, aliquoted, and stored at −20°C. Treatment of the cells with TPA, U0126, or wortmannin consisted of daily additions without replacing the medium.

Measurement of Cell Proliferation and Cell Cycle Profile

In experiments shown in Fig. 1B, cells were grown for 24 h in DMEM supplemented with 10% FCS and for an additional 24 h in the presence of the drug. Bromodeoxyuridine (BrdUrd) was added 1 h before cell harvesting to a 10 μmol/L final concentration. Fluorescence-activated cell sorting analysis was done according to the manufacturer's instructions (Becton Dickinson, Franklin Lakes, NJ). The cell proliferation assays presented in Figs. 2C and 3B were done with the Biotrak ELISA System version 2 (Amersham Biosciences Corp., Piscataway, NJ) according to the manufacturer's instructions. Cells (5×10^3) were seeded in 96-well plates and grown for 24 h, and drugs were applied for an additional 24 h. BrdUrd incorporation was allowed during the last 2 h of treatment. In experiments shown in Fig. 1D, cells were grown for 24 h in DMEM supplemented with 10% FCS and for an additional 24 h in the presence of the drug. Cell DNA content and apoptosis were analyzed on a FACSCalibur. Cells were trypsinized and fixed in 70% ice-cold ethanol for 1 h and stained with 50 μg/mL propidium iodide for fluorescence-activated cell sorting analysis. The percentages of cells in the cell cycle phases are reported (results from three independent experiments).

Measurement of Apoptosis

Expanding cells were subjected to drug treatment for 24 h, briefly washed with PBS, and trypsinized. Cell suspension was pelleted at 800 rpm for 5 min, fixed in ice-cold 70% ethanol, and kept at 4°C for 30 min. Cells were resuspended in a 1× PBS solution containing 50 ng/mL propidium iodide and 50 μg/mL RNase. Percentage of cells in sub-G_1 for apoptosis was determined by flow cytometry.

Protein Extraction and Western Blot Analysis

Cells were washed with PBS, resuspended in 1× SDS sample buffer (62.5 mmol/L Tris-HCl, 2% SDS, 10% glycerol, 50 mmol/L DTT), and boiled at 95°C during 5 min; aliquots were stored at −20°C. Proteins (30 μg) were separated by size on SDS-PAGE gels (10%) and transferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences). Western blot analysis was done using antibodies against the following proteins: phosphorylated AKT (Upstate Biotechnology, Lake Placid, NY), ERK1/2, phosphorylated ERK, phosphorylated EGFR (Tyr^{1173}), and EGFR (Santa Cruz Biotechnology, Santa Cruz, CA) and actin (Sigma). The anti-AKT antibody was a gift from Dr. Brian Hemmings (Friedrich Miescher Institute, Basel, Switzerland). Western blots were developed with enhanced chemiluminescence reagents (Pierce, Rockford, IL).

Results

Specific Targeting of the EGF Signaling Pathway by AEE788

Nearly 50% of primary glioblastomas exhibit amplification of the *EGFR* gene (3, 4), which is also associated with

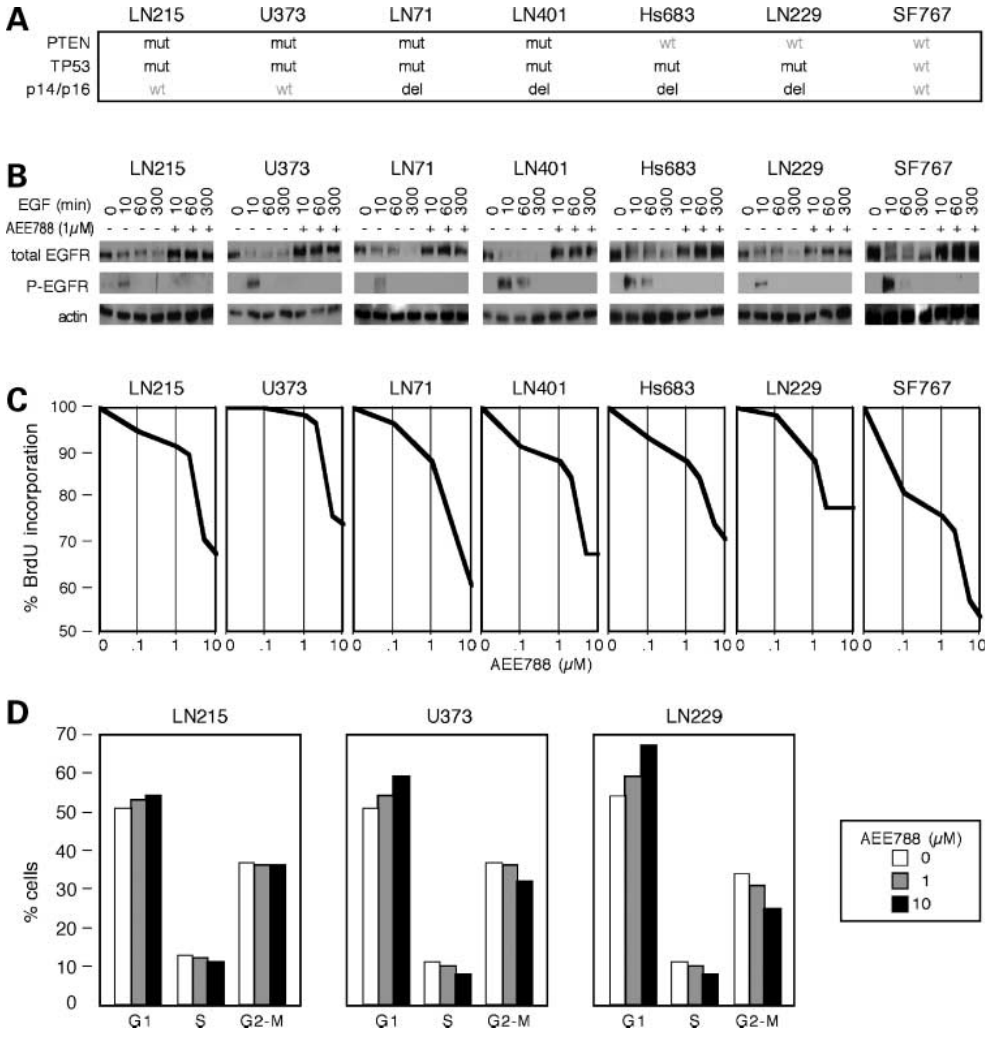

Figure 1. Treatment of glioblastoma cell lines with EGFR inhibitor. **A,** genotyping of used glioma cell lines by Ishii et al. (39). **B,** cells were preincubated in the presence or absence of 1 μmol/L of the EGFR inhibitor AEE788 during 30 min and stimulated with 100 ng/mL EGF for 10, 60, and 300 min. EGFR, phosphorylated EGFR (Tyr^{1173}), and actin were detected by Western blot analysis of cell lysates. **C,** cells were incubated during 4 d with increasing concentrations of AEE788 (0, 1, 2, 5, and 10 μmol/L). Proliferation was measured by BrdUrd and flow cytometry. Experiments were done in triplicate. *Points,* average of three independent determinations; *bars,* SD. **D,** cell cycle phase analysis on glioblastoma cell lines on 1 and 10 μmol/L of AEE788 treatment.

poor prognosis. For this reason, the inhibitor of EGFR and of ErbB receptor protein kinase activities AEE788 has been considered to be a potent drug to induce cell death in glioblastoma cell lines. AEE788 was tested for its ability to induce cell growth arrest and apoptosis in various cell lines derived from glioblastoma, for which the genetic status of established cancer genes had been previously defined (Fig. 1A; refs. 38, 39). For a preliminary determination of the minimal drug concentration needed to fully prevent EGFR activation by EGF, increasing concentrations of AEE788 were applied to the LN229 glioblastoma cell line, and EGFR/ErbB receptor activation status was monitored by the presence of EGFR phosphorylated at Tyr^{1173}. To completely block receptor phosphorylation, 1 μmol/L was needed (data not shown). Further time course experiment done with 1 μmol/L AEE788 showed a strong inhibition of the transient phosphorylation of Tyr^{1173} as well as of the degradation of the receptor protein in all seven glioblastoma cell lines tested (Fig. 1B).

Increasing concentrations of AEE788 were applied to the glioblastoma cell lines, and proliferation was assayed by integration of BrdUrd after 1 day. At a 1 μmol/L concentration, AEE788 gives up to 20% of proliferation reduction compared with the control cell population (Fig. 1C). Cell cycle analysis after 24 h of 1 and 10 μmol/L of AEE788 treatment shows an increase of cell population in the G_1 phase (Fig. 1D).

Cell viability was assayed by flow cytometry after 24, 48, and 96 h. Very low or no apoptosis was induced at drug concentrations <2 μmol/L as late as 96 h (Fig. 2A). High levels of apoptosis were observed at unphysiologically high concentration (10 μmol/L) in five of the seven cell lines (SF767, LN215, U373, LN229, and LN71). Under the same condition, LN401 and Hs683 did not show significant levels of apoptosis. Because AEE788 was not capable of inducing strong apoptosis in glioblastoma cells when used as a single drug, we tested whether a specific double or a triple combination of compounds would significantly increase the induction rate of apoptosis. For this purpose, we combined suboptimal doses of AEE788 (0.5 μmol/L) with two additional biological drugs: Gleevec (imatinib, 0.5 μmol/L) and RAD001 (everolimus, 20 nmol/L). In gliomas, Gleevec is being explored as a PKI of platelet-derived growth factor receptor (34), which is activated in

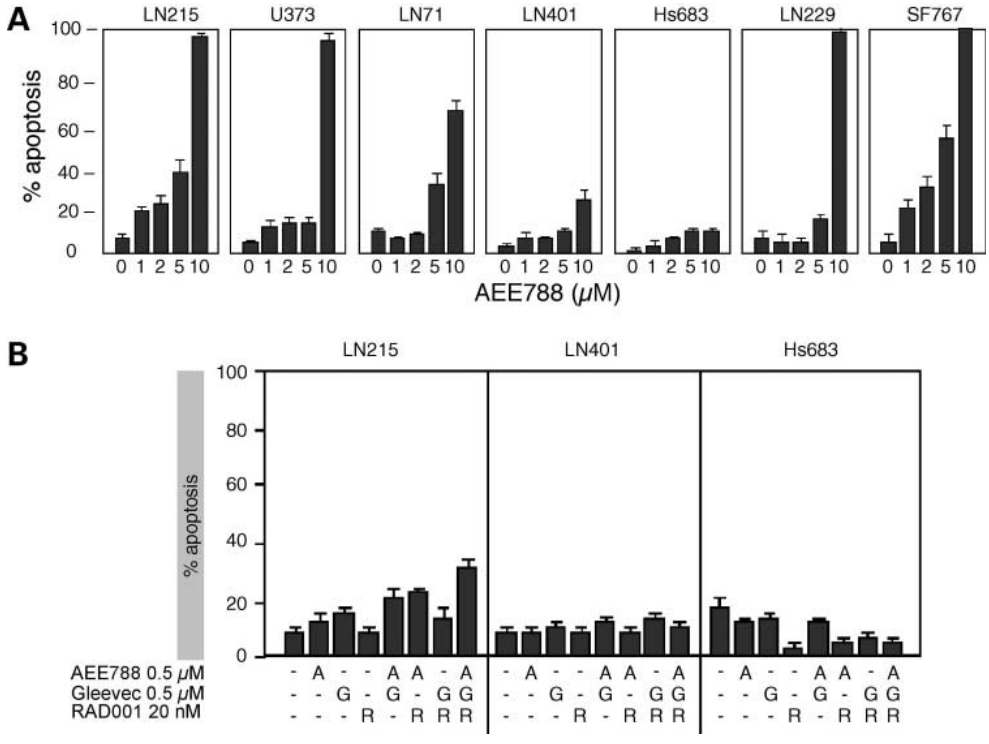

Figure 2. Survival of glioblastoma cell lines on treatment with EGFR inhibitor and in combination with Gleevec and RAD001. **A,** cells were incubated during 4 d with increasing concentrations of AEE788 (0, 1, 2, 5, and 10 μmol/L). Apoptosis was measured by flow cytometry. Experiments were done in triplicate. *Columns,* average of three independent determinations; *bars,* SD. **B,** combinatorial study of biological drugs AEE788 (A), Gleevec (G), and RAD001 (R) on glioma cell survival.

primary tumors (40, 41). Platelet-derived growth factor receptor phosphorylation levels correlated with sensitivity to Gleevec in glioma primary cultures (42). RAD001, a derivative of rapamycin, inhibits mammalian target of rapamycin, leading to inactivation of ribosomal S6K1 and inhibition of cap-dependent translation (43).

The application of the combined drugs to 10 glioblastoma cell lines for 4 days revealed that there was a combination-specific and a cell line–specific response with regard to induction of cell death (Fig. 2B). The cell lines could be divided into three categories. One group (SF767, LN18, and LN215; Fig. 2B, *left*) showed a modest additive induction of apoptosis by all of the three drugs, reaching a maximum of 35% of cell death with the triple combination. A second group of glioblastoma cell lines (LN401, LN71, LN229, and LN319; Fig. 2B, *middle*) was basically insensitive to all drug combinations, showing the same rate of apoptosis as untreated cells. The third group (Hs683, U373, and U343; Fig. 2B, *right*) exhibited a relatively high level of basal apoptosis (15–20%), which was, however, decreased by the three drugs applied together. Especially, RAD001 exhibited an antiapoptotic effect, alone or in combination. No differences in apoptosis rate were evidenced between PTEN-mutant (LN215, LN401, LN71, and U373) and wild-type (Hs683, SF767, and LN229) cell lines on RAD001 application. Although, in theory, RAD001 was expected to overcome the constitutive AKT activation resulting from loss of PTEN activity.

Taken together, the results indicated that neither single nor combined application of the three biological drugs, at physiologic concentration, was able to strongly induce cell death in glioblastoma cell lines.

Induction of Apoptosis in Glioblastoma Cell Lines by Patupilone

Because neither the single nor the combined application of the three biological drugs led to a consistent induction of apoptosis, we tested whether the combination of AEE788 with a cytotoxic drug could result in a more cooperative induction of apoptosis. Patupilone is a member of the group of epothilones, which represent a new class of low molecular weight compounds that target microtubules by inhibiting their depolymerization and therefore impairs cell division (44). Patupilone prevents chromosome alignment at metaphase and drives cells to undergo apoptosis (45). It has been shown to exhibit antitumor activity *in vitro* and *in vivo* (36, 37). As a result of inhibition of microtubule depolymerization, after addition of patupilone, dramatic changes in cell morphology occurred within 18 h (Fig. 3A, *right*). To investigate its ability to induce apoptosis, glioblastoma cells were treated with increasing concentrations of patupilone in the picomolar and nanomolar concentration range for 4 days (Fig. 3A, *left*). Although 100% apoptosis was induced in all cell lines when the highest drug concentration (35 nmol/L) was applied, levels of apoptosis induced by 0.7 nmol/L patupilone strongly varied between the cell lines, from Hs683 and LN401, the most resistant, to LN229, the most sensitive one.

Combined Treatment with AEE788 and Patupilone Strongly Induced Apoptosis and Reduced Proliferation of Glioblastoma Cells

AEE788 (1 μmol/L) was applied together with increasing concentrations of patupilone on cell lines LN71, LN229, Hs683, and SF767, and cell survival was determined after 4 days (Fig. 3B). The combined drugs induced apoptosis in

a cooperative manner in LN71 and SF767 but not in Hs683 and LN229 cell lines. In LN71 and SF767 lines, the patupilone concentration required to induce apoptosis in 50% of cells (AC_{50}) revealed that AEE788 acted as a sensitizer, reducing the patupilone concentration from a nanomolar to a picomolar concentration range. Thus, addition of AEE788 shifted down the amount of patupilone required to induce apoptosis in 50% of cells, up to 2 orders of magnitude in SF767 cells. It is noteworthy that the synergistic effect of patupilone on those glioma cell lines seemed independent of their respective sensitivities to AEE788. Consistent with the absence of synergy of both compounds on LN229 cells, also proliferation was not affected either by AEE788 or patupilone or by the combination of both drugs. In contrast, SF767 cells, on which the AEE788/patupilone synergized the best, had a significant reduction of proliferation up to 80% ($P < 0.001$) after each individual or combined drug application (Fig. 3C).

Simultaneous Inhibition of Both ERK1/2 and AKT Kinase Activities Parallels the Induction of Apoptosis

Both signaling pathways, PI3K/AKT and RAS/MEK/ERK, are under the control of EGF-directed activation of ErbB receptors. To investigate the activation of these two pathways, phosphorylation status of the kinases AKT and ERK was chosen as readouts. AEE788 (1 μmol/L) and patupilone (0.7 nmol/L), alone and in combination, were applied for 24 and 72 h to the four glioblastoma cell lines that differed remarkably in their sensitivities toward those drugs. In contrast to LN229 and Hs683, LN71 and SF767

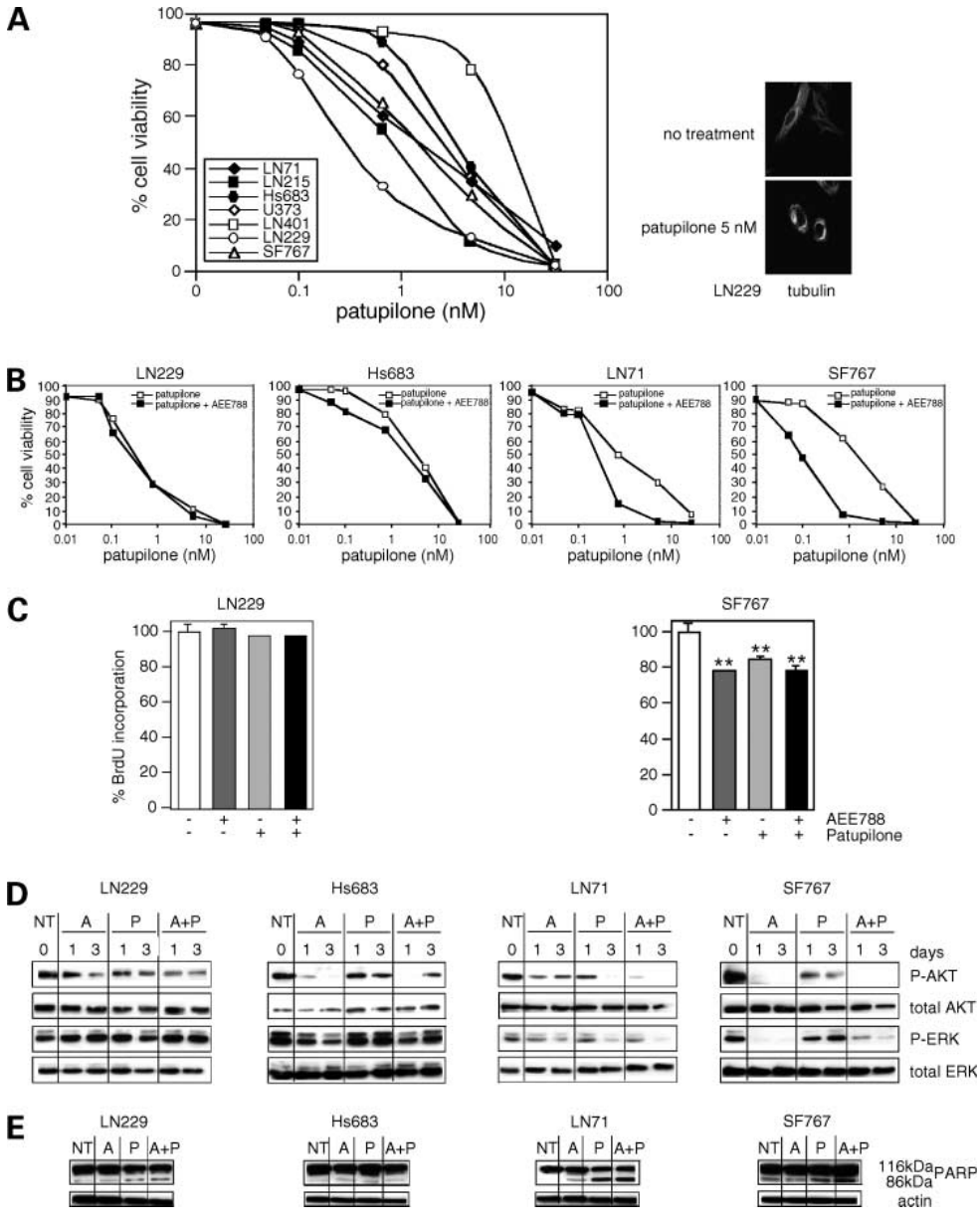

Figure 3. Combined treatment of glioblastoma cells stabilizing microtubules and blocking EGFR. **A,** increasing concentrations of patupilone (0.05, 0.1, 0.7, 5, and 35 nmol/L) were applied to seven cell lines for 4 d and the percentage of viable cells was determined using flow cytometry. *Points,* average of three independent determinations; *bars,* SD. Confocal microscopy of LN229 cells after 18 h of patupilone treatment. **B,** cells were treated for 4 d either with increasing concentrations of patupilone alone or in combination with 1 μmol/L AEE788. Cell survival was measured by flow cytometry. *Points,* average of three independent experiments; *bars,* SD. **C,** cells were treated with 1 μmol/L AEE788 and/or 0.7 nmol/L patupilone and assayed for proliferation with BrdUrd by ELISA. **D,** *top,* cells were incubated during 24 h and 3 d with patupilone (0.7 nmol/L), AEE788 (1 μmol/L), or both. Western blot analysis was done on cell lysates. *NT,* no treatment; *A,* AEE788; *P,* patupilone. *Bottom,* poly(ADP-ribose) polymerase (*PARP*) cleavage after 24 h.

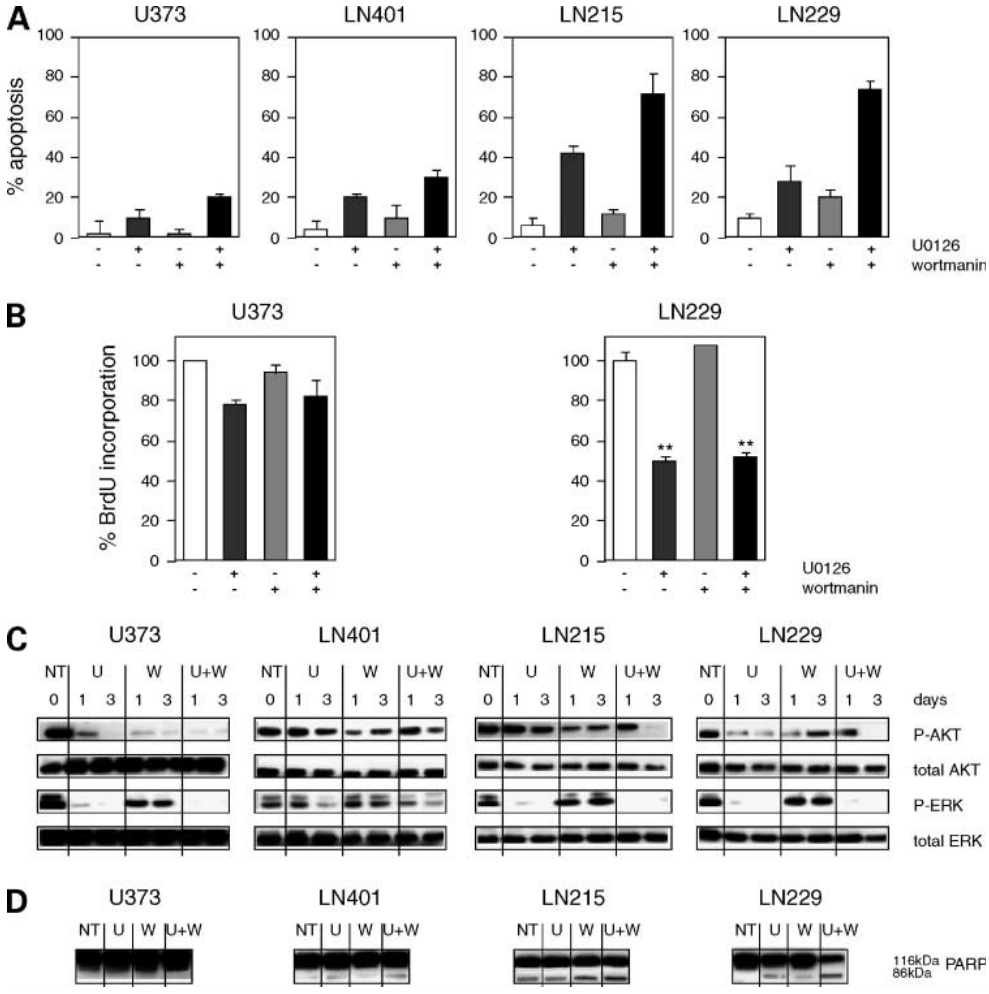

Figure 4. Combined treatment of glioblastoma cells blocking PI3K and MEK. **A,** cell lines were treated with the MEK inhibitor U0126 (20 μmol/L) and/or the PI3K inhibitor wortmannin (1 μmol/L) for 3 d, whereby the addition of both drugs was repeated every day. Percentage of apoptotic cells was measured using flow cytometry. *Columns,* mean of three independent experiments; *bars,* SD. **B,** cells were treated with 20 μmol/L U0126 and/or 1 μmol/L wortmannin and assayed for proliferation with BrdUrd by ELISA. **C,** Western blot analysis was done to determine the relative phosphorylation levels of ERK1/2 and AKT after 3 d of treatment. *U,* U0126; *W,* wortmannin. **D,** poly(ADP-ribose) polymerase (PARP) cleavage after 24 h.

showed full down-regulation of phosphorylation of both protein kinases after 3 days of combined treatment (Fig. 3D), accompanied by widespread apoptosis between 85% and 95% of cells. In contrast, LN229 and Hs683 still retained strong phosphorylation of the two protein kinases and showed much lower levels of apoptosis. These results suggested a link between the simultaneous inhibition of the two EGF-dependent signaling pathways and the induction of apoptosis triggered by the combination of patupilone and AEE788. To support this hypothesis, we analyzed the cleavage status of poly(ADP-ribose) polymerase, which triggers caspase-dependent apoptosis (46, 47). The cleaved form of poly(ADP-ribose) polymerase was indeed detected in glioblastoma cells undergoing apoptosis (Fig. 3E).

Simultaneous Inhibition of AKT and MEK Cooperatively Induces Apoptosis in a Subgroup of Glioblastoma Cell Lines

To test whether sensitivity of LN71 and SF767 cells to the AAE788-patupilone combination is due to low phosphorylation levels of AKT and/or ERK, inhibitors of PI3K (wortmannin) and MEK (U0126) were used to abrogate activation of AKT and ERK pathways. The potential to induce cell death was tested by applying single or combined inhibitors to four glioblastoma cell lines and measuring the proportion of apoptotic cells (Fig. 4A). When used as a single compound, U0126 was able to induce a significant level of apoptosis in LN215 and LN229 cells but to a much lesser extent in U373 and LN401 cells, whereas wortmannin alone had no or very little effect. However, strong induction of apoptosis was caused by the combination of the two inhibitors. On U0126 application, proliferation was significantly decreased up to 50% in LN229 cell line ($P < 0.001$), whereas U373 cells showed a 20% reduction. Again, wortmannin alone had no effect on reduction of proliferation on both LN229 and U373 cells, and no synergistic effect was present after drug combination (Fig. 4B).

U373 and LN401 generally exhibited much less sensitivity toward the two inhibitors. To examine the long-term effect of the inhibitors on phosphorylation of AKT and ERK, the two most sensitive and the two much less sensitive cell lines were treated for 24 h as well as for 3 days by applying fresh doses of the compounds daily. The activation status of AKT and ERK was determined as a readout for drug efficiency (Fig. 4C). In response to the combination of U0126 and wortmannin, a decrease of

phosphorylated ERK and phosphorylated AKT occurring in all glioblastoma lines confirmed the activity of the two drugs used. Unexpectedly, on treatment with the MEK inhibitor, U0126, U373, and LN229 cell lines show a decrease of phosphorylated AKT levels, suggesting a cross-talk between the RAS/MEK/ERK and PI3K/AKT pathways.

In the sensitive cell lines LN215 and LN229, simultaneous inactivation of protein kinases AKT and MEK was associated with poly(ADP-ribose) polymerase cleavage and apoptosis (Fig. 4D). However, in the resistant cell lines U373 and LN401, AKT and MEK inactivation was not sufficient to trigger apoptosis. There was no correlation between glioblastoma cell line sensitivity or resistance to apoptosis and a given genotype (Fig. 1A).

Phorbol Ester TPA Restores Activation of MEK and Inhibits Induction of Apoptosis

Phorbol ester TPA activates protein kinase C and ERK1/2, thereby modifying mitogenic signaling pathways (44, 48, 49). When exposed to 20 or 100 nmol/L of TPA, LN215 and LN229 cells showed a strong increase of phosphorylated ERK levels (Fig. 5A). Treatment of the cell lines with combined U0126 and wortmannin for 4 days in the presence of TPA (20 or 100 nmol/L) led to a substantial increase of activated ERK1/2, whereas phosphorylation was completely down-regulated without TPA (Fig. 5A). In parallel, the extent of the induction of apoptosis was determined after 4 days under the same conditions, revealing that the addition of TPA to both glioblastoma cell lines substantially decreased the induction of apoptosis caused by the two inhibitors (Fig. 5B). Taken together, the results further supported the model of a correlation between down-regulation of the two signaling pathways and induction of apoptosis.

Discussion

We report that the combination of the inhibitor of EGFR kinase AEE788 and of the microtubule depolymerization inhibitor patupilone synergistically induced death of glioblastoma cells. Of high interest is that this synergy occurred at drug concentrations that were not effective to induce cell death when each drug was applied alone. AEE788 alone at 1 μmol/L only induced low levels of apoptosis in glioblastoma cells *in vitro*. Its main antitumor activity consists of inhibition of cell growth and motility (50). Consistently, survival of animals bearing intracranial tumors had been extended by giving AEE788 at concentration below the maximally tolerated dose (34) and confirmed a role of AEE788 as an antitumor agent. However, lasting responses cannot be obtained with a cytostatic effect but require induction of tumor cell death, which may only be achieved by drug combinations. Therefore, we designed a strategy to trigger glioblastoma cell apoptosis by combining the PKI AEE788 with patupilone, a compound with strong cytotoxic potential against various cancer types and also against multidrug resistance cancer cell lines (51). Glioblastoma cell death could be triggered in all tumor cell lines *in vitro* by 35 nmol/L patupilone, a dose that can already be toxic *in vivo*. Interestingly, synergistic induction of apoptosis was observed in glioblastoma cells when using 1 μmol/L AEE788 in combination with only 0.1 nmol/L patupilone. Of interest, the genetic backgrounds of the cell lines that responded the best to the combination therapy with AEE788 and patupilone were different with regard to the three main glioma pathways: TP53, PTEN, and p14/p16 (Fig. 1A). Whereas SF767 cells are wild-type at all three loci, LN71 cells are either mutated or null (39), suggesting that such a combination could be applied to a wide spectrum of gliomas. When analyzing the activation status of EGFR, PKB/AKT, and ERK as readouts for critical pathways in the glioblastoma signaling network, we found that synergistic induction of apoptosis by combining patupilone and AEE788 was paralleled by inactivation of PKB/AKT and ERK. Conversely, the two critical cell signaling mediators remained active in glioblastoma cells that did not show this drug synergism. Although AEE788, patupilone, and U0126, applied individually, reduced glioblastoma cell growth, AEE788/patupilone and U0126/wortmannin combinations

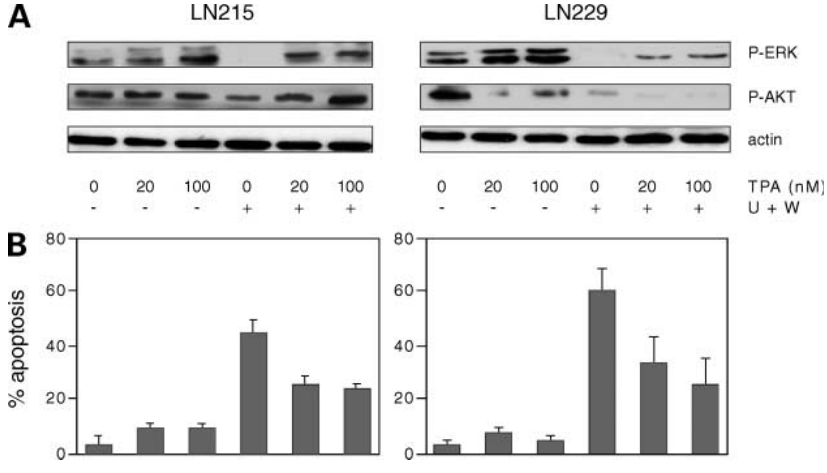

Figure 5. Phorbol ester TPA antagonizes induction of apoptosis and maintains AKT and ERK1/2 activities. **A,** cells were treated with 20 and 100 nmol/L of TPA in the presence or absence of wortmannin (W) and U0126 (U) for 3 d, whereby the additions were renewed every day. Cell extracts were prepared by the addition of SDS sample buffer, and Western blot analysis was done. U0126, 20 μmol/L; wortmannin, 1 μmol/L; TPA, 20 or 100 nmol/L. **B,** cells were treated in the same way for 4 d and the percentage of dead cells was determined by flow cytometry.

did not affect cell growth in a synergistic manner, suggesting that these processes are driven by distinct pathways. The importance of the two signaling pathways PI3K/PKB and RAS/RAF/MEK/ERK for glioblastoma signaling is further supported by the finding that PI3K is activated in the majority of human glioblastoma either by loss of PTEN function (38, 39) or by activating mutations of PI3KCA (22), whereas growth factor stimulation causes activation of RAS in glioblastoma (52). Further evidence stems from a murine transgenic glioblastoma model, in which simultaneous activation of RAS and PKB gives rise to malignant gliomas (53, 54). Glioma animal models have further shown that the combination of ectopic expression of activated RAS and AKT in the glial lineage (23) led to spontaneous induction of murine gliomas, whereas an activated allele of either RAS or AKT alone failed to induce tumor formation. In analogy to this animal model and the effects on signaling induced by the described synergism, we hypothesized that direct inhibition of signaling mediators downstream of EGFR within the PI3K/PKB and RAS/RAF/MEK/ERK pathways will overcome glioblastoma cell resistance. Therefore, we specifically blocked activity of both MEK and PI3K, which induced strong apoptosis in a subgroup of glioblastoma cell lines, indicating a critical role of these two pathways in glioblastoma signaling. Combining the MEK inhibitor U0126 and the PI3K inhibitor wortmannin led to synergistic induction of apoptosis in LN215 and LN229 cell lines. Interestingly, although phosphorylation of ERK1/2 and PKB/AKT was decreased in all cell lines, it was not associated with apoptosis in resistant lines, suggesting additional defects in the proapoptotic machinery. Activation of protein kinase C and ERK1/2 by TPA counteracted U0126- and wortmannin-dependent apoptosis, confirming the specificity of drug effect.

An unresolved question is at which cellular level the intervention has to take place. Our data argue that combined blocking of signaling mediators downstream of growth factor receptors, interfering with the signaling cross-talk, may be more effective than inhibition of a single cell surface receptor (e.g., EGFR; ref. 13). This view is supported by other findings in glioblastoma cell lines, where inhibition of PKB/AKT could be counteracted by a stimulation of insulin-like growth factor receptor-I, resulting in sustained activation of PI3K (19, 20).

In conclusion, our results indicate that the induction of apoptosis in glioblastoma cell lines requires drug combination, which down-regulate distinct pathways. Blocking EGFR activation alone did not induce apoptosis unless complemented with the microtubule stabilizer patupilone. If PKIs were targeted without the help of cytotoxic drugs, combined inhibition of MEK and PI3K was found to be the most efficient combination to induce glioblastoma cell death. Drug effects were independent of mutation statuses at the major glioblastoma pathways (Fig. 1A). Additional studies are needed to develop other target for treatment of resistant glioblastoma cells to directly down-regulate key members of the critical pathways.

Acknowledgments

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