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LABORATORY INVESTIGATION

Profiling Hsp90 differential expression and the molecular effects of the Hsp90 inhibitor IPI-504 in high-grade glioma models

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Abstract Retaspimycin hydrochloride (IPI-504), an Hsp90 (heat shock protein 90) inhibitor, has shown activity in multiple preclinical cancer models, such as lung, breast and ovarian cancers. However, its biological effects in gliomas and normal brain derived cellular populations remain unknown. In this study, we profiled the expression pattern of *Hsp90α/β* mRNA in stable glioma cell lines, multiple glioma-derived primary cultures and human neural stem/progenitor cells. The effects of IPI-504 on cell proliferation, apoptosis, motility and expression of Hsp90 client proteins were evaluated in glioma cell lines. In vivo activity of IPI-504 was investigated in subcutaneous glioma xenografts. Our results showed *Hsp90α* and *Hsp90β* expression levels to be patient-specific, higher in high-grade glioma-derived primary cells than in low-grade glioma-derived primary cells, and strongly correlated with *CD133* expression and differentiation status of cells. Hsp90 inhibition by IPI-504 induced apoptosis, blocked migration and invasion, and significantly decreased epidermal growth factor receptor levels, mitogen-activated protein kinase and/or Akt activities, and secretion of vascular endothelial growth factor in glioma cell lines. In vivo study showed that IPI-504 could mildly attenuate tumor growth in immunocompromised mice. These findings suggest that targeting Hsp90 by IPI-504 has the potential to become an active therapeutic strategy in gliomas in a selective group of patients, but further research into combination therapies is still needed.

Keywords Hsp90 · IPI-504 · Glioma · Stem cells · Anti-tumor activity

Introduction

Heat shock proteins (HSPs) are a collection of molecules induced by a variety of stresses (such as hypoxia and starvation) and by certain pathological states including cancer [1, 2]. Hsp90 has two major isoforms in humans, Hsp90α and Hsp90β. While Hsp90β is thought to be constitutively expressed, Hsp90α expression is highly inducible by stressful stimuli [3]. As a molecular chaperone, Hsp90 can regulate the conformation and stability of many client proteins including signaling protein kinases [e.g. EGFR, MAPK cascades, Akt kinase], steroid hormone receptors and components of the telomerase complex [4].

Recently, targeting Hsp90 has been of great interest in cancer therapy, because abnormal levels of Hsp90 have been found in a number of malignancies [5, 6]. To date, there are over 20 drug candidates that target Hsp90 undergoing interventional trials in multiple cancer indications [7, 8]. Among them, 17-allylamino-17-demethoxygeldanamycin

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(17-AAG) is one of most advanced product candidates, acting through blocking the ATPase activity of Hsp90 by binding into its ATP-binding pocket [9]. 17-AAG has been proven to work in multiple glioma models, but its clinical development was limited by its relative low solubility [9]. In contrast, IPI-504 is the water soluble, hydroquinone hydrochloride salt of 17-AAG [10], uniquely designed to overcome the therapeutic limitations of earlier HSP90 inhibitors by allowing simple aqueous-based intravenous formulations for clinical administration [11]. In many cancer models IPI-504 has shown to have a much higher activity than 17-AAG. This can be explained by the fact that inside cells, 17-AAG is enzymatically reduced to the hydroquinone (free base of IPI-504) which is a 40- to 60-fold more potent inhibitor of HSP90 than 17-AAG [12]. IPI-504 is currently in clinical trials for patients with non-small cell lung cancer (http://www.ClinicalTrial.gov).

High-grade gliomas (HGGs) are the most common primary tumors in the central nervous system [13]. For the patients afflicted by the most aggressive and lethal HGG, glioblastoma multiforme (GBM), the prognosis remains poor, with a median survival of only 12–15 months [14]. Over-expression of Hsp90 has been found in GBM [15] and multiple Hsp90 client proteins, such as EGFR [16], are involved in gliomagenesis. To date, several Hsp90 inhibitors including 17-AAG have shown potential in preclinical GBM models [17]. However, the activity of IPI-504 in HGGs has not been evaluated.

Recent evidence suggested that the progression of HGGs is driven by a small group of glioma stem-like cells (GSCs), aka glioma tumor initiating cells [18, 19]. These GSCs contribute to tumorigenesis and the subsequent development of highly invasive phenotypes, as well as treatment resistance [20]. In this study, we measured and compared the expression pattern of *Hsp90α* and *Hsp90β* in stable glioma cell lines, glioma-derived primary cultures which are exhibiting GSC properties, and NSCs. Furthermore, the anti-tumor effect of IPI-504 as a single agent in HGGs was investigated both in vitro and in vivo.

Materials and methods

Reagents and drugs

All chemicals and reagents were obtained from Sigma (MO, USA) otherwise specified. IPI-504 was provided by Infinity Pharmaceuticals.

Cell lines and primary cultures

As described previously [21], high/low-grade glioma-derived primary cultures were isolated from surgical specimens (Table S1). Human NSCs (SC23, SC27, SC30) were derived by Dr. Philip Schwartz [22]. HuTuP01 cells were a gift from Dr. David Panchision [23]. The detailed culture conditions and verification of stem-cell like characteristics were previously published by our group [21, 24] and were included in Supplementary Methods. Early passage cells (both for the NSCs and GSCs) were defined as cultures that were collected and analyzed in the first four passages.

Quantitative RT-PCR (qRT-PCR) analysis

Total RNA was extracted using RNeasy Mini Kit (Qiagen, MD, USA), and cDNA was generated using the iScript^{TM} cDNA Synthesis Kit (Bio-rad, CA, USA). Quantitative PCR reactions (iQ^{TM} SYBR Green Supermix, Bio-rad) were conducted using a Bio-Rad CFX96 Real-time System, and the gene expression levels were normalized to those of *ACTB*. The primer sequences were shown in Supplementary Methods.

Antibodies for western blotting

Antibodies used were Hsp90 (ab1429, Abcam, MA, USA), EGFR (ab2430-1, Abcam), Phospho-Erk1/2 Pathway Sampler Kit (#9911, Cell Signaling, MA, USA), p44/42 MAPK (Erk1/2) (#9271, Cell Signaling), Phospho-AKT (Ser473) (#9271, Cell Signaling), pan-Akt (ab8805, Abcam) and β-actin (NB600-501, Novus, CO, USA).

Magnetic cell sorting

CD133+ cell selection was performed using CD133 MicroBead Kit (Miltenyi Biotec Inc., CA, USA) as indicated by manufacturer.

Cell proliferation assay

The viability of cells was determined with the Cell Proliferation Kit II (XTT) (Roche, IN, USA). The metabolic conversion of XTT to formazan by live cells was determined by measuring absorbance at 450 nm and expressed as percentage of control.

Flow cytometry

Cells were harvested and fixed in ice-cold 70 % ethanol, and then treated with RNase (10 μg/ml) and stained with propidium iodide (2.5 μg/ml) for 30 min at 37 ° C. Cell cycle analysis was performed on an FACScan flow cytometer (BD Biosciences, NJ, USA) and analyzed using the FlowJo software (Tree Star, Inc., OR, USA).

TUNEL assay

The DNA fragmentation in cells treated by IPI-504 was identified with the NeuroTACS™ II In Situ Apoptosis Detection Kit (Trevigen Inc., MD, USA) according to the manufacturer's instruction.

Invasion assay

Invasion assay was performed using BD BioCoat™ Matrigel™ Invasion Chamber with an 8 μm PET membrane (BD Biosciences). Cells were seeded in medium without serum, and medium containing 1.5 % FBS was used as chemo-attractant. After an incubation of 24 h at 37 °C, non-invasive cells were removed and invading cells were fixed with 100 % methanol. Cells were then stained in hematoxylin.

Immunocytochemistry

Cells were fixed with 10 % buffered formalin, incubated with 0.5 % Triton X-100 in PBS for 10 min, and then blocked with 3 % hydrogen peroxide. The avidin–biotin immunoperoxidase reaction (Vector Laboratories, CA, USA) was performed using an EGFR antibody (28-0005, Invitrogen, NY, USA). Cells were counterstained with hematoxylin.

VEGF ELISA

Cells were grown in serum-free medium for 1 day and then treated with 1 μM of IPI-504 in reduced serum medium (3 % FBS) for 24 h. The conditioned medium was collected and VEGF levels were analyzed by Human VEGF Immunoassay kit (R&D Systems, MN, USA).

Subcutaneous xenograft implantation and response to IPI-504 treatment

Female athymic mice (nu/nu genotype, Balb/c background, 6 to 8 weeks old) were used to implant D-54MG and U-251 xenografts maintained at the Preston Robert Tisch Brain Tumor Center (Duke University of Medical Center, USA) [25]. Groups of mice (9 or 10 mice in each group) were treated by IPI-504 or saline when the median tumor volumes were on average 250 mm^3. IPI-540 was injected intraperitoneally with the schedules as indicated in Table 1 for D-54MG xenografts (and the Table S2 for U-251). Tumors were measured twice weekly with hand-held calipers. The endpoint was defined as the number of days needed to reach a tumor volume five times greater than that measured at the start of the treatment. Growth delay, expressed as T–C, was defined as the difference in days between the median time required for tumors in treated (T) and control (C) animals. Tumor regression was defined as a decrease in tumor volume over two successive measurements.

Statistical analysis

Statistical analyses for the in vitro experiments were prepared using GraphPad Prism version 5.04 (GraphPad Software Inc., La Jolla, CA, USA). All values were presented as mean ± SE of the mean (SEM). Statistical significance was determined with unpaired t test. $p < 0.05$ was considered significant. For the animal experiments, statistical analysis was performed using the SAS statistical analysis program, the Wilcoxon rank order test for growth delay, and Fisher's exact test for tumor regression as previously described [26].

Results

The expression pattern of *Hsp90α/β* in stable glioma cell lines, low/high-grade glioma-derived GSCs and normal NSCs

We profiled *Hsp90α* and *Hsp90β* mRNA levels using four stable glioma cell lines (D-54, LN-229, U-251 and U-87), nineteen diverse histological GSCs derived from glioma patients (Table S1) and three NSCs (SC23, SC27 and SC30). Overall, compared to stable cell lines, low-grade glioma-derived GSCs and NSCs, the *Hsp90α/90β* expression was much higher in some of HGG-derived GSCs, such as DB33 (Grade III) and DB32/34 (GBM) (Fig. 1; Table S1). However, the *Hsp90α/90β* expression varied significantly between specimens within the same pathological diagnosis, raising the question of the role of *Hsp90α* and *Hsp90β* as prognostic indicator. As shown in the Table S1 (survival data), the GBM patients with the lowest *Hsp90α/β* levels (DB37 and DB50) had a better prognosis (13 and 14 month survival, respectively) compared to patients with the highest *Hsp90α/β* levels (DB32 and DB34; 9 month survival). The similar results could also be found in Grade III patients- the patient with the highest *Hsp90α* levels (DB33) and the poorest prognosis. Those data suggested a preliminary possible association between clinical prognosis and *Hsp90α/β* expression. Hsp90 over-expression is already reported to correlate with poor prognosis in breast cancer and gastric cancer patients but further adequate investigation in a larger number of glioma specimens is required to confirm our findings.

Hsp90α/β mRNA levels correlate with differentiation status and *CD133* mRNA levels in gliomas and NSCs

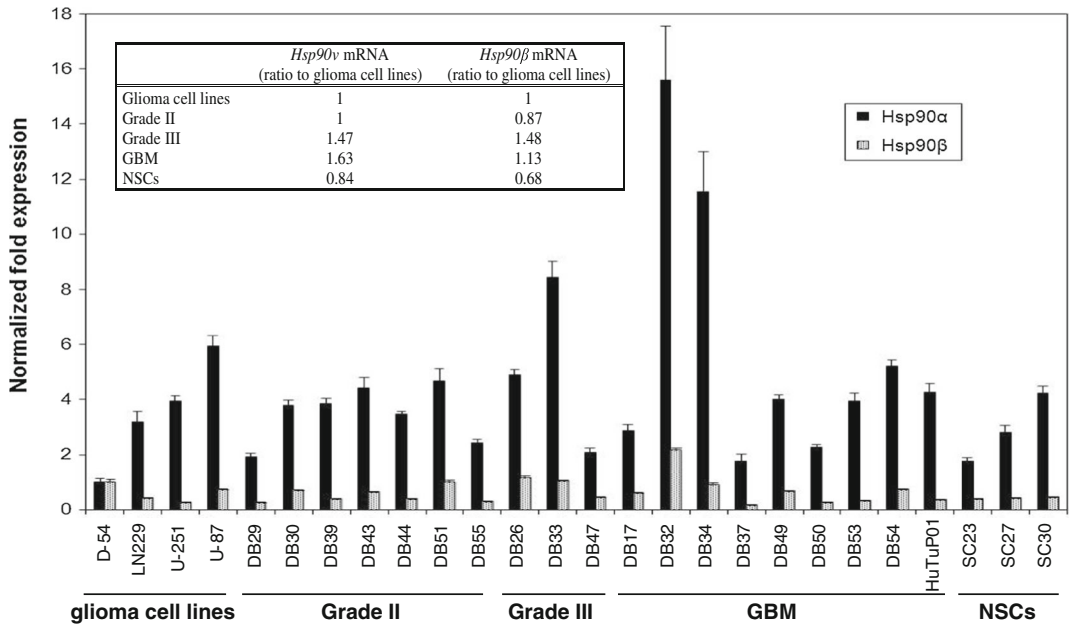
Hsp90 plays a role in embryonic cell differentiation [27, 28], and long-term in vitro expansion of stem/precursor

Table 1 The effect of IPI-504 treatment on the growth of subcutaneous human brain tumor D-54MG xenografts in mice

Experiment	Dose	Treatment	Duration	T–C (days)	*p* value	Regressions
1	100 mg/kg b.i.d.	Twice weekly	6 weeks	2.49	<0.062	3/10
2	100 mg/kg b.i.d.	5/2/5 schedule (5 days on, 2 days off, and then 5 days on)	6 weeks	3.45	<0.091	1/9

T–C The difference in days between the median time required for tumors in treated (T) and control (C) animals to reach a volume five times greater than that measured at the start of treatment

Tumor regression The decrease in tumor volume over two successive measurements



	Hsp90α mRNA (ratio to glioma cell lines)	*Hsp90β* mRNA (ratio to glioma cell lines)
Glioma cell lines	1	1
Grade II	1	0.87
Grade III	1.47	1.48
GBM	1.63	1.13
NSCs	0.84	0.68

Fig. 1 The expression of *Hsp90α/β* in stable HGG cell lines, low/high-grade glioma-derived glioma stem-like cells (GSCs) and neural stem/precursor cells (NSCs). *Hsp90α/β* mRNA expression was measured by qRT-PCR. The relative expression levels were normalized by *ACTB*, and the value of 1 was assigned for D-54. The insert table showed the average ratio of GSCs which belong to the same tumor grade and/or NSCs to glioma cell lines

cells may induce neuronal differentiation [29]. Our previous work [21] demonstrated that during continued in vitro expansion, DB32 and HuTuP01 GSCs underwent differentiation indicated by significantly decrease in the mRNA levels of *CD133*, a well-established stem cell marker in the brain. By comparing with early and late passages of DB32 and HuTuP01 GSCs, that expressed the highest and moderate levels of *Hsp90α/β* respectively, we found that *Hsp90α* and *Hsp90β* dramatically attenuated after continuous expansion (Fig. 2a and b), along with down-regulation of Hsp90 protein levels (Fig. 2b). These data suggested that *Hsp90α/β* levels are correlated with differentiation status and *CD133* expression in GSCs. To further identify the correlation between *CD133* and *Hsp90*, magnetic microbeads were used to separate CD133+ and CD133- population from HuTuP01 cells, and the expression of *Hsp90α/β* was then analyzed. As shown in Fig. 2c, both *Hsp90α* and *Hsp90β* levels were markedly higher in CD133 + cell population.

Our previous work demonstrated that when DB32 and HuTuP01 cells underwent differentiation by switching culture medium from serum-free stem cell medium (SCM) to serum-containing medium (differentiation medium), *CD133* revealed a dramatic decrease [21]. In this study, a significant drop of *Hsp90α/β* was also detected [Fig. 2d (left panel) and e]. Interestingly, when HuTuP01 was switched back to SCM (leading to a less differentiated state), both *Hsp90α* and *Hsp90β* increased again (Fig. 2d, right panel). Furthermore, when D-54MG cells were cultured in SCM which allowed cells to transit to an undifferentiated stem cell state, as indicated by *CD133* up-regulation and tumor sphere formation [21], a significant elevation on *Hsp90α/β* mRNA was found (Fig. 2f).

We also checked the expression pattern of *Hsp90α/β* in normal NSCs undergoing differentiation. When SC27 cells underwent continuous expansion, the NSC markers *CD133* and *nestin* dramatically decreased (Fig. 2g, left panel). In contrast with our results obtained from GSCs, SC27

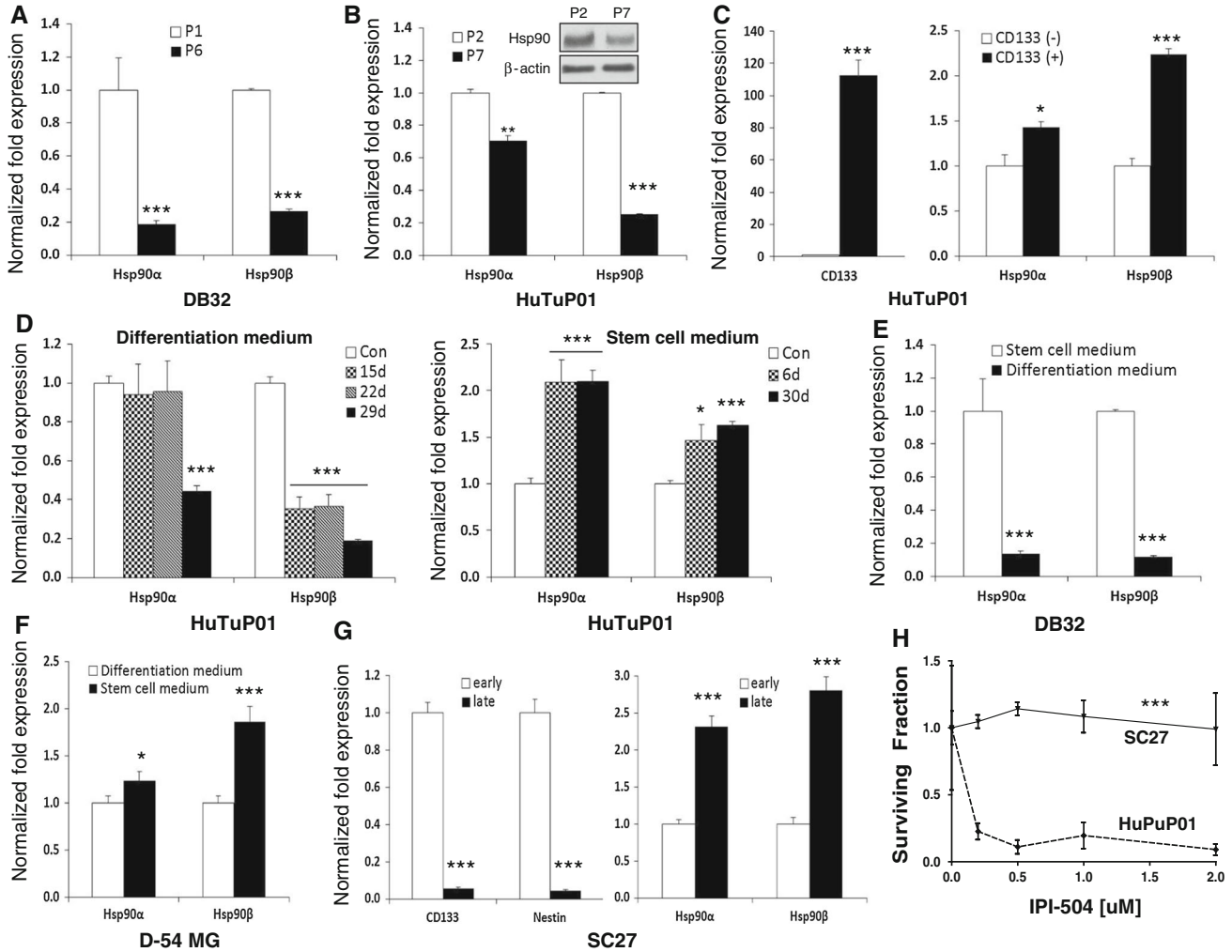


Fig. 2 *Hsp90α/β* mRNA levels correlate with differentiation status and *CD133* mRNA levels in HGG cells and NSCs. **a**, **b** RNA was extracted from early and late passages of DB32 (P1 and P6) and HuTuP01 (P2 and P7) cells, and qRT-PCR was then performed to measure the mRNA levels of *Hsp90α/β*. *ACTB* was an internal control. Western blotting was used to evaluate the expression of Hsp90 protein in **b**. **c** CD133+ and CD133- populations of HuTuP01 cells were separated and collected by magnetic bead cell sorting. **d** HuTuP01 cells were cultured with differentiation medium (*left*) and then switched back to stem cell medium (*right*). Cells were collected at the indicated time points. **e** DB32 cells were cultured with differentiation medium for 1 month. **f** D-54MG cells were cultured in stem cell medium for 1 month. **g** Early and late passages of SC27 cells were collected. RNA was extracted from cells collected in **c-g**, and qRT-PCR was then performed to measure the mRNA levels of *Hsp90α/β*, *CD133* and/or *nestin*. **h** Early passages of SC27 and HuTuP01 were treated with indicated concentrations of IPI-504 for 3 days. The effect of IPI-504 on cell proliferation was then measured. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

showed elevated expression of *Hsp90α/β* in late passage cells (Fig. 2g, right panel).

The question of potential neurotoxicity of new drugs is becoming more urgent, as more and more glioma patients are becoming long-term survivors. To further explore the clinical significance of these data, we exposed both NSC (SC27) and GSC (HuTuP01) cells to graded concentrations of IPI-504, a novel Hsp90 inhibitor. The SC27 cells which have low Hsp90 constitutive expression continued to proliferate at doses toxic to HuTuP01 cells (Fig. 2h), suggesting that IPI-504 might selectively target the GSCs while not eradicating the NSCs.

Taken together, our results suggest that the levels of *Hsp90α/β* correlate with differentiation status in both GSCs and NSCs, but displaying different expression patterns, which might protect NSCs from potential Hsp90 inhibition-related toxicity.

IPI-504 inhibits proliferation and induces apoptosis in glioma cells

We next focused on the effect of IPI-504 on glioma cells. The anti-proliferation effect of IPI-504 indicated by decreased S phase cells was accompanying with increased sub-G1

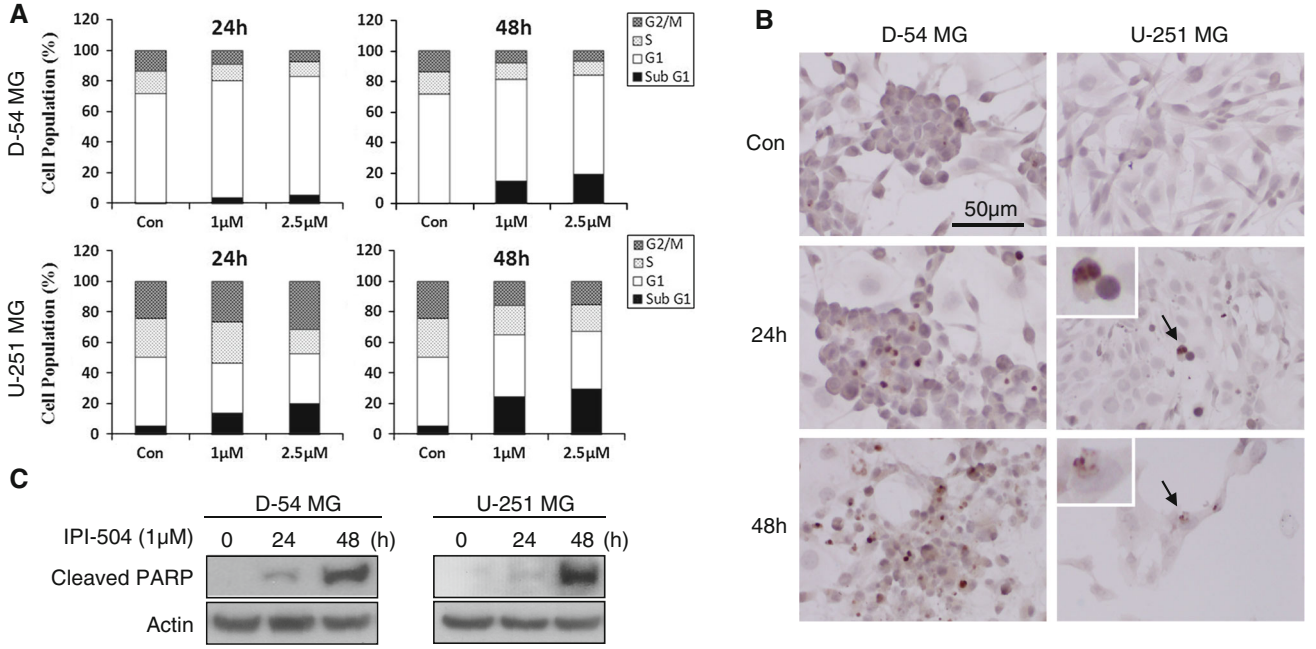


Fig. 3 IPI-504 inhibits proliferation and induces apoptosis in glioma cells. **a** Cells were exposed to 1 μM and/or 2.5 μM of IPI-504 for either 24 or 48 h, and cell cycle was analyzed by flow cytometry. **b** In situ apoptosis was detected by TUNEL in cells treated with 1 μM of IPI-504. The fragmented DNA of the apoptotic cells appeared as brown stained material. *Scale bar* 50 μm. **c** Cells were treated by 1 μM of IPI-504 for indicated timepoints. Western blot was used to detect cleaved PARP. Actin was used as the internal control

population of cells (Fig. 3a), and induction of fragmented DNA of the apoptotic cells (Fig. 3b) in a dose/time-dependent manner. Meanwhile, a significant up-regulation of cleaved PARP was detected in IPI-504 treated cells (Fig. 3c).

We next compared the toxicity of 17-AAG and IPI-504 on glioma cells. While IC50 was 0.045 μM and 0.011 μM (D-54MG and U-251MG, respectively) for IPI-504 treatment, IC50 was 0.143 μM and 5.87 μM in 17-AAG treated cells (Fig. S1).

GBM tumors are notoriously resistant to treatment, and new treatments are needed both for newly-diagnosed, chemotherapy naïve patients as well as for the patients that already developed resistance to the first-line chemotherapy treatment (temozolomide). Next, we used two glioma cell lines (D-54MG and U-251MG) and two temozolomide-resistant (TR) lines (D-54TR and U-251TR [25]), to investigate the anti-tumor effect of IPI-504 in vitro. As shown in Fig. S2a, both the parental and TR cells were sensitive to IPI-504, and their proliferation was dramatically decreased in a dose-dependent manner, while TR cells maintained their tolerance to temozolomide [25].

Hsp90 inhibition by IPI-504 suppresses migration and invasion of glioma cells, and displays dose- and time-dependent effects on Hsp90 client proteins

Since the invasive behavior of HGGs contributes to their poor prognosis [30], the role of IPI-504 on cell invasion was measured using the Matrigel invasion assay (Fig. 4a). In both D-54MG and U-251MG cells, the capability of invasion was robustly inhibited ($p < 0.001$). Wound closure assay (Fig. S2b) further confirmed these data. While the gap was only partially filled in IPI-504 treated cells, even after 24 h, control cells were able to migrate in and fill up the gap within 18 h.

Next, we investigated the underlying mechanism of IPI-504 function through Hsp90 client proteins [4], such as EGFR which is constitutively upregulated in GBM [16]. D-54MG and U-251MG cells were treated with IPI-504 and immunohistochemistry staining was then used to evaluate the expression of EGFR. As shown in Fig. 4b, IPI-504 decreased the expression of EGFR in both cell lines tested. Western blotting was then used to evaluate the expression levels of EGFR and some other Hsp90 client proteins. As shown in Fig. 4c, IPI-504 treatment down-regulated the expression of EGFR, phosphorylation of MAPKs and AKT, and total levels of AKT, indicating that the anti-tumor effect of IPI-504 was associated with Hsp90 client protein degradation.

VEGF, a potent angiogenic molecule, is highly expressed in glioblastoma [31], and the secretion of VEGF by tumor cells depends heavily on EGFR-mediated signaling [32]. Using VEGF ELISA, we examined the secretion of VEGF in IPI-504 treated cells. As shown in Fig. 4d, a significant reduction of VEGF was found in glioma cells upon IPI-504 treatment.

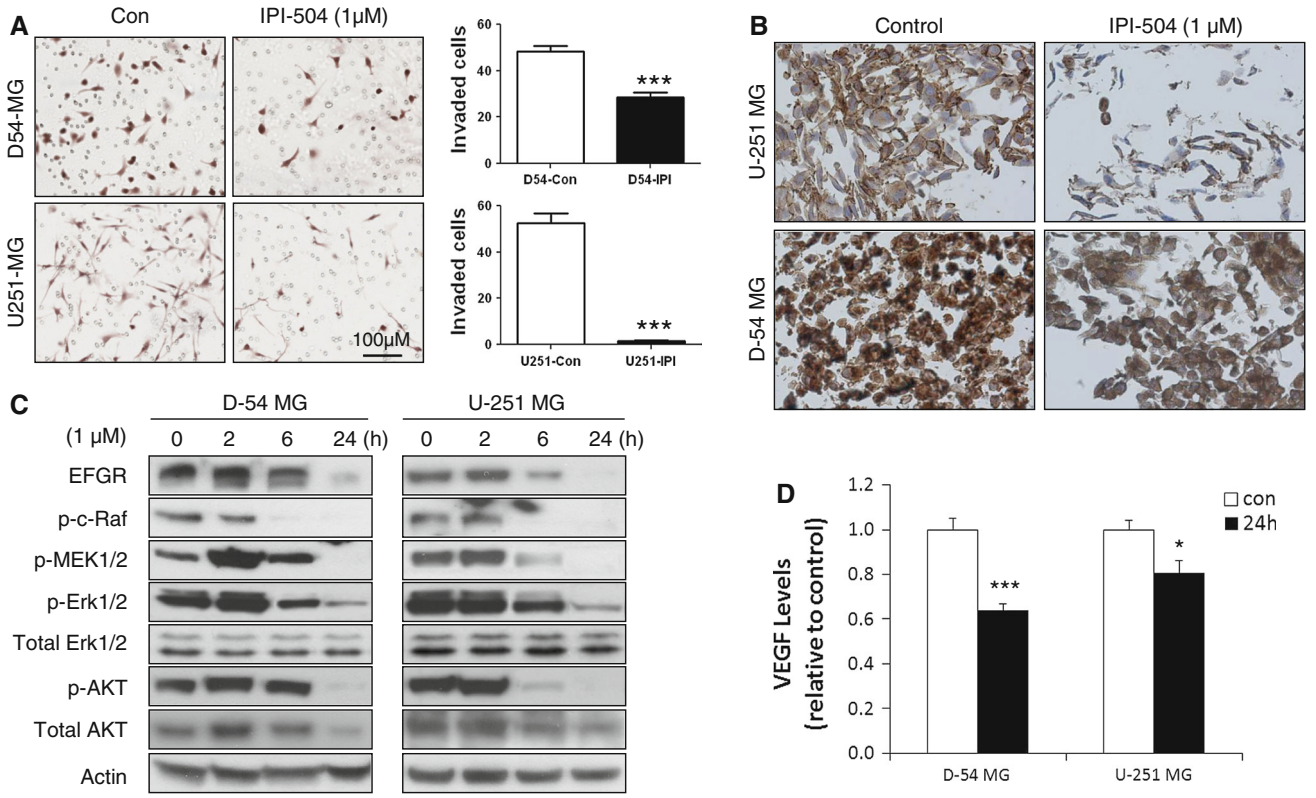


Fig. 4 Hsp90 inhibition by IPI-504 suppresses migration and invasion of glioma cells, and displays dose- and time-dependent effects on Hsp90 client proteins. **a** Cells were treated with 1 μM of IPI-504 for 24 h, and the invasion capability of cells was then analyzed using Matrigel Invasion chambers. The average of invaded cells for each counting grid was showed in right. **b** Cells were exposed to 1 μM of IPI-504 for 24 h. IHC staining was then used to detect the expression of EGFR. **c** Western blotting was used to analyze the expression of Hsp90 client proteins in cells treated with 1 μM of IPI-504 for the indicated durations. **d** Cells were grown in serum-free medium for 1 day and then treated with 1 μM of IPI-504 in reduced serum medium (3 % FBS) for 24 h. Conditioned medium was collected and VEGF levels were analyzed by ELISA. Values were normalized to un-treated control. $*p < 0.05$, $***p < 0.001$

IPI-504 has a very modest effect on in vivo tumor growth

To evaluate the anti-tumor activity of IPI-504 in vivo, D-54MG tumor xenografts were injected subcutaneously into the immunodeficient mice. As shown in Table, in two independent experiments, IPI-504 showed a trend to delay tumor growth with 2.49 and 3.45 days respectively when compared with saline-treated tumors ($p < 0.1$). In addition, 21 % of the mice (4 out of 19) used in this study displayed tumor regression, indicating an anti-tumor effect of IPI-504 in vivo. The same study was performed using U-251MG tumor xenografts. However, IPI-504 revealed a limited anti-tumor activity in U-251MG xenografts (Table S2). We next evaluated the direct effects of IPI-504 in tumor tissues harvested from the D-54MG xenograft model. Although the drug down-regulated the majority of Hsp90 client proteins in tissue culture, results varied from one tumor to another and no consistent effects were noted in xenograft tissues, except up-regulation of Hsp70 (Fig. S3). Similar results were previously reported by another group in a pancreatic cancer xenograft study [33].

Discussion

Our study profiles the expression pattern of *Hsp90α* and *Hsp90β* in human GSCs and NSCs (Fig. 1). It further demonstrates that *Hsp90α/β* mRNA levels correlate with differentiation status and *CD133* mRNA levels in human-derived GSCs (Fig. 2a-e). Those data are consistent with a previously published article, which Hsp90 showed remarkably high expression in undifferentiated human embryonal carcinoma cells and dramatic down-regulation during in vitro cellular differentiation [27]. Unlike the differentiation pattern seen in GSCs, the *Hsp90α/β* levels are up-regulated when NSCs are undergoing differentiation (Fig. 2g). Since the lineage connection between NSCs and GSCs is not established, the variance of *Hsp90α*/β expression pattern during differentiation in GSCs and NSCs requires further investigation.

Another difference between NSCs and GSCs is their divergent response to Hsp90 inhibition. Hsp90 inhibition by IPI-504 selectively targets GSCs while not affecting NSC survival (Fig. 2h), suggesting a favorable therapeutic

profile with limited neurotoxicity for Hsp90 inhibitors. The response of GSCs and NSCs to Hsp90 inhibitors has also been investigated recently by a few other laboratories, with similar results. For example, murine GSCs derived from normal NSCs are more susceptible to 17-AAG than wild-type NSCs [17]. In addition, a subset of GSCs having high-expressing Olig2 exhibits greater sensitivity to a novel Hsp90 inhibitor NVP-HSP990 [34]. Since more and more glioma patients are becoming long-term survivors, seeking new drugs with limited neurotoxicity is becoming more urgent.

The strong correlation between Hsp90 expression profile and tumor progression [7, 8] suggests that Hsp90 may be a potential biomarker. While Hsp90 over-expression is already reported to correlate with poor prognosis in breast cancer and gastric cancer patients [5, 6], our study implies that the Hsp90 over-expression may be a poor prognostic indicator for gliomas. The *Hsp90α/β* levels of GSCs seem to correlate with poor prognosis in high-grade glioma patients, as the GBM patients with the lowest *Hsp90α/β levels* have a better prognosis compared to patients with the highest *Hsp90α/β* levels (Table S1, survival data). However, with the limited patient number included in our study, a further and more extensive investigation is required.

It has been reported that 17-AAG is able to inhibit the intracranial tumor growth of both glioma cell lines and GSCs as a single agent [17, 35]. In our study, IPI-504 not only displays the anti-glioma activity in vitro by suppressing proliferation and inhibiting migration and invasion (Figs. 3 and 4), but shows a much stronger anti-proliferation effect than 17-AAG (Fig. S1). However, IPI-504 has a modest anti-tumor influence in vivo (Table S2), similar with a previous report that IPI-504 exerted a cytostatic effect on mantle cell lymphoma (MCL) cell lines, but only slightly inhibited tumor growth in vivo [36]. Although IPI-504 has limited activity as a single agent in our glioma model, it has a promising effect in other in vivo models as a combination therapy, including *Kras* mutant non-small cell lung cancer [37], and HER2-positive trastuzumab-resistant breast cancer [38]. The possibility of a combinational strategy of IPI-504 with other drugs in GBM is at the core of our future plans.

In summary, this study provides the first profile of the expression pattern of *Hsp90α*/β in GSCs and NSCs, and shows that human GSCs are particularly susceptible to Hsp90 inhibition. Our data suggests that *Hsp90α* and *Hsp90β* strongly relate to the differentiation status of glioma and/or neural precursor/stem cells. In our glioma models, inhibiting Hsp90 by IPI-504 does exert significant anti-tumor activity in vitro, but modestly alters tumor growth in vivo, implying a combinational strategy with others drugs may be an attractive therapeutic strategy for GBM patients.

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Conflict of interest No potential conflicts of interest were disclosed.

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