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Validating the Efficacy of shRNA vs. siRNA In Silencing *Hsp90α* In Gliomas

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Abstract

The stress dependent tumour cells found to harbour Hsp90 and its inducible component Hsp90α in activated superchaperone complexes are highly sensitive to pharmacological Hsp90 inhibitors compared to normal cells where Hsp90 is present in an uncomplexed state. Downregulating Hsp90α can be achieved using chemical inhibitors and RNAi such as siRNA's or shRNA's. This study aimed at assessing the efficacy of siRNA (targeting Hsp90α exon 5) and shRNA (targeting Hsp90α exon 4 and 5 border) in three glioma cell lines (1321N1, GOS-3 and U87-MG). Hsp90α expression at mRNA and protein levels was monitored using qRT-PCR and immunofluorescence, respectively. The downstream effect of silencing Hsp90α was determined by measuring the Akt/PKB kinase activity level. While siRNA treatment decreased *Hsp90α* mRNA copy numbers by ~35%, shRNA decreased it by ~63% (three glioma cell lines). Furthermore, *Hsp90α* inhibition by siRNA resulted in downregulating Akt/PKB kinase by ~29%, whereas shRNA downregulated it to ~3% (three glioma cell lines). Considering the vital role of Akt kinase in cell signalling, anti-apoptotic and drug resistant pathways in tumours, the treatment induced sensitivity of Akt to degradation results in a novel therapeutic strategy emphasising a greater potential of shRNA as opposed to siRNA in silencing Hsp90α and subsequently Akt in glioma cells.

Keywords: Glioma; Hsp90α; siRNA; shRNA; Akt/PKB Kinase

Introduction

The over expression of heat shock protein 90 (Hsp90) has been a common factor in several cancers [1]. Hsp90 has a number of client proteins involved in apoptosis, cell survival and growth pathways [2]. Several of these client proteins are cancer associated proteins and thus, inhibiting Hsp90 or the inducible component Hsp90α could result in their disruption by influencing oncogenic signal transduction pathways. Inhibiting Hsp90α can be achieved using natural inhibitors (Radicicol), synthetically processed inhibitors (Geldanamycin and its analogues) [3], and RNA interference (RNAi) using small interfering RNA (siRNA) or a vector based short hairpin RNA (shRNA) [4,5].

The RNA interference potential in gene therapy [6-8] has been confirmed by preclinical studies carried out in the treatment of cancers. *In vivo* studies involving the targeting of critical components for tumour cell growth [9-11] metastasis [12,13], angiogenesis [14] and chemoresistance [15,16] have shown favourable usage of RNAi in the treatment of tumours. The silencing attained by siRNA is effective, however, there are disadvantages which can be overcome by the use of shRNA. Although siRNA's are stable and their delivery to the cytoplasm is more easily attained as opposed to the shRNA's delivery into the nucleus, the shRNA hairpin is a more efficient substrate for the dicer with improved RISC loading [17,18]. Furthermore, shRNA plasmids are amplified by transcription. siRNA's, on the other hand, are not amplified intracellularly and are more susceptible to metabolism [19].

siRNA's have high degradation rates resulting in less than 1% siRNA remaining within the cell after 48 hours post administration and subsequently the loading efficiency is 10 times lower than that of shRNA's [17,20,21]. Interestingly, shRNA's are more durable and are continuously synthesized in the host cells have been shown to be 250 fold more effective than siRNA in a luciferase expression system [19].

Identical siRNA and shRNA strands have been identified to target the firefly luciferase gene in HeLa cells, resulting in shRNA showing greater inhibition of the gene compared with siRNA [18]. Furthermore, the internal ribosome entry site of the hepatitis C virus (HCV) has been shown to be targeted by both shRNA and siRNA inhibiting the site driven gene expression in cultured cells, resulting in shRNA being more potent than the corresponding siRNA [21]. shRNA's prove to be effective *in vivo* [22,23] with their oligonucleotides at 5' being less immunogenic than the 5' ends of the siRNA oligonucleotides and thus, shRNAs are less likely to induce an inflammatory response as opposed to siRNAs [17,24].

Although various studies have been carried out to evaluate the silencing efficacy of shRNA versus siRNA, none to date have been carried out on Hsp90 in tumours. Studies from our laboratory have shown the presence of the inducible Hsp90α protein in glioma cell lines and tissue and its absence in normal cells and tissues [25]. The downregulation of Hsp90α in glioma cells by siRNA resulted in enhanced sensitivity to chemotherapeutic agents [26]. The investigation aimed at silencing the therapeutic target Hsp90α in three different grades of glioma cell lines using siRNA and shRNA. Hsp90α silencing efficiency was determined by measuring gene expression at the mRNA and protein levels. In addition, the level of AKT/PKB kinase, which is directly regulated by Hsp90 and plays a major role in anti-apoptotic pathway empowering the cancercells the property of "immortality" [27] was monitored.

Materials and Methods

Cell lines

The human brain tumour cell lines 1321N1 (astrocytoma) (ECCAC,

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UK), GOS-3 (mixed astro-oligodendroglioma) (DMSZ, Germany) and U87-MG (glioblastoma astrocytoma) (ECCAC, UK) were routinely cultured in the laboratory. 1321N1 and GOS-3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, UK). The U87-MG cells were cultured in Eagle's minimum essential medium (Sigma, UK).

siRNA transfection

Pre-designed Hsp90α siRNA duplex composing of 21-nt was designed by Ambion, UK. Based on our previous findings, siRNA oligonucleotide used in this study was: siRNA2 (NM_005348; targeted exon 5 at nucleotides 1387-1408 bp) (sense) 5'CGUGAUAAAGAAGUAAGCGtt3' (antisense) 5'CGCUUACUUCUUUAUCACGtt3' [26]. A negative control was used. The annealed siRNA was resuspended at a final concentration of 20 μM and was delivered into the cells using siPORT™ siRNA Electroporation Kit according to the manufacturer's protocol [26]. The cells were incubated in a 5% CO_2 incubator for 48 hours before being used to further analysis.

shRNA transfection

The purified and sequence verified pGFP-V-RS expression plasmids with Hsp90α specific shRNA cassettes were purchased from Origene, USA. The sequence of the shRNA verified to silence Hsp90α (data not shown) were: shRNA2 29-nt (NM_005348; targeted bordering exon 4 and 5 at nucleotides 2137-2166 bp) (sense) 5'CTCTCAAGGACTACTGCACCAGA3' (antisense) 5'GAGAGTTCCTGATGACGTGGTCTTACTTC3'. The shRNA plasmids were diluted with 50 μL of dH_2O to obtain a final concentration of 100 ng/μL, from which 1 μg of the shRNA expression plasmid DNA was used to transfect cells according to the manufacturer's protocol. The cells were then incubated in a 5% CO_2 incubator for 48 hours before being used for further analysis.

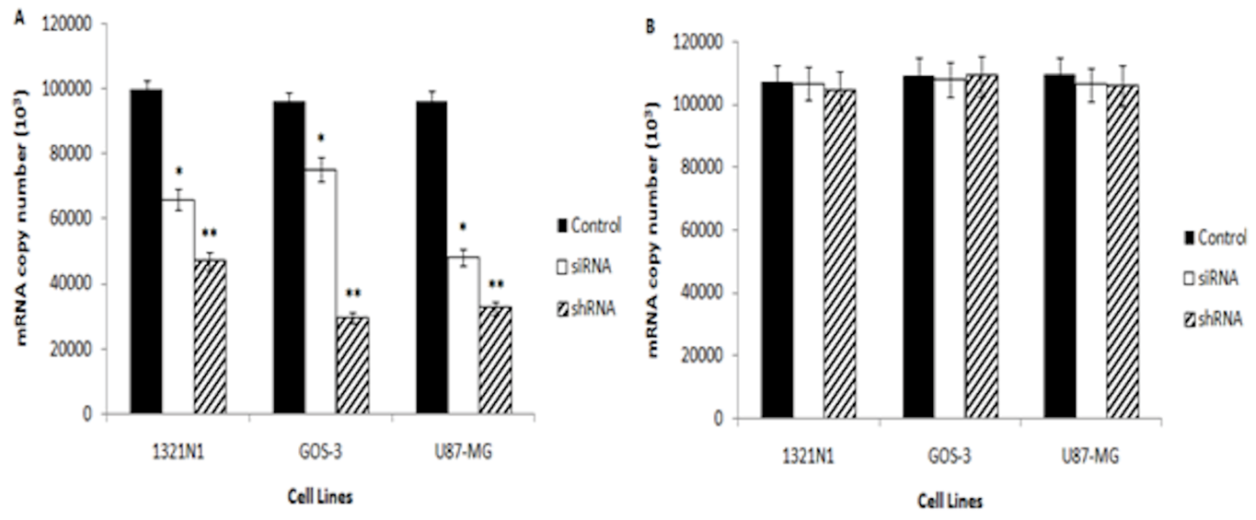
mRNA isolation, reverse transcription and qRT-PCR

Averaging 1 ρg/cell of mRNA was extracted from cell lines using mRNA Isolation Kit (Roche, UK) [28]. 100 ng of the isolated mRNA was then transcribed to cDNA using a First Strand cDNA Synthesis Kit (Roche, UK). Using the cDNA as a template for PCR, qRT-PCR (quantitative real time PCR) was used to evaluate the expression of Hsp90α and *GAPDH* as a control. The primer sequence (TIB MOLBIOL, Germany) and length of amplicons were: Hsp90α (189 bp) sense: 5'tctggaagatccccagacac, antisense: 5'agtcatccctcagccagaga, and *GAPDH* (238 bp) sense: 5'gagtcaacggatttggtcgt, antisense: 5'ttgattttggagggatctcg. The qRT-PCR was performed using Fast Start

Cell Lines	*Hsp90α* mRNA/100 ng cells extract (1 x 10^6 cells)	% cells expressing *Hsp90α*	% Akt/PKB kinase activity
Control			
1321N1	99772	78	100
GOS-3	95945	67	100
U87-MG	96292	81	100
siRNA			
1321N1	65973	51	14.97
GOS-3	75123	52	45.6
U87-MG	48204	41	27.29
shRNA			
1321N1	46856	37	0.58
GOS-3	29623	21	7.87
U87-MG	32482	28	0.32

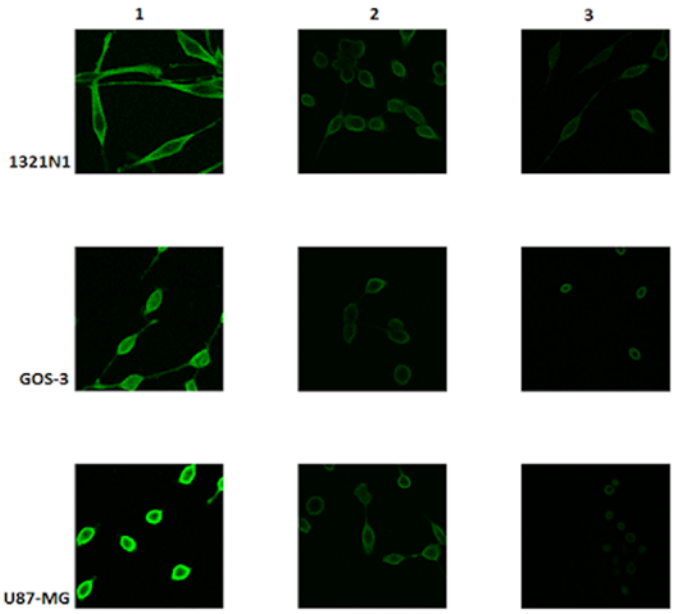
Data values are mean ± standard deviation, n=3

Table 1: A comparison between siRNA vs shRNA in three glioma cell lines.



Data values are mean ± standard deviation, n=3, *p < 0.05 and **p < 0.001 are considered to be statistically significant.

Figure 1: Copy numbers of gene expression level of (A) Hsp90α and (B) *GAPDH* in 1321N1, GOS-3 and U87-MG cell lines in control, siRNA and shRNA treated samples.



These immunofluorescence results are typical of 3 different experiments.

Figure 2: Hsp90α protein levels assessed using immunofluorescence in 1) Untreated 2) Treatment with siRNA 3) Treatment with shRNA. Hsp90α antigen is detected with FITC conjugated secondary antibody.

DNA master^{PLUS} SYBR Green 1 (Roche, UK) in a LightCycler real time PCR detection system (Roche Diagnostics, Germany) [28]. The level of fluorescence reflecting the cycle number at the detection threshold (crossing point) was used to monitor quantitative amplification. All the work was carried out in triplicate and a negative control (no DNA) was included.

Immunofluorescence

The cells were initiated on culture slides in 6 well plates and post 48 hours achieved more than 60% adherence. Once this was attained, the cells were fixed with 4% paraformaldehyde and washed thrice with warm PBS before being permeabilised with 0.3% TritonX-100 (Sigma,UK) for 7 minutes at room temperature. The cells were then removed and washed thrice with PBS and then incubated in a blocking solution before the addition of the monoclonal antibody against primary Hsp90α (1:50) (Cambridge Bioscience, UK). Post washing, the primary antibody was detected with a secondary antibody [goat anti-rat IgG FITC (1:128, Cambridge Bioscience, UK)]. Following washing with PBS, the cells were fixed with 1.5μg/ml VECTASHIELD® mounting medium (Vectashield, UK) and then mounted on slides for further analysis. The cells were visualized using Axiovert 200M LSM 510 laser scanning confocal microscope (Carl Zeiss Ltd, UK). For each slide, a total of 250 cells were counted in at least 5 random microscopic fields [26].

Akt/PKB kinase activity assay

Akt/PKB kinase activity was assayed using a solid phase enzyme linked immune-absorbent assay (ELISA) kit, (Assay Deign, UK) that detects the Akt/PKB activity in the solution phase. Proteins were extracted from the cells and 1 μg protein from each sample was used to perform the Akt/PKB kinase activity assay (manufacturer's protocol).

Results and Discussion

Silencing Hsp90α could contribute towards future therapeutic options in the treatment of glioma [25]. The RNAi machinery can substitute chemical inhibitors in downregulating Hsp90α which in turn, could destabilize various cancerous client proteins. The RNAi mechanism involves the use of either siRNA or shRNA. In spite of having similar functional outcomes, siRNA and shRNA are two intrinsically different molecules that have different molecular mechanism pathways [6].

In order to evaluate the differential silencing of Hsp90α achieved in glioma cell lines using siRNA and shRNA based techniques, the level of Hsp90α was monitored at the mRNA, protein and enzyme levels. The mRNA level of Hsp90α and *GAPDH* (control) were measured using qRT-PCR in 1321N1, GOS-3 and U87-MG cell lines. The percentage difference of mRNA copy numbers between shRNA compared to siRNA silencing was 1.8 fold (**$p < 0.001$) (Figure 1 and Table 1). Thus, at the genetic level, shRNA targeted against Hsp90α performs better silencing capability than siRNA targeted against Hsp90α in glioma cells.

The protein level of Hsp90α was measured by immunofluorescence. Figure 2 represents a sample of the stained cells indicating positive staining for Hsp90α. The Hsp90α antigen was detected with FITC conjugated secondary antibodies in the cytoplasm. The quantification of the immunofluorescent detection of the Hsp90α protein expression for each sample was determined by counting 250 Hsp90α positive cells (Table 1). A distinct correlation between the mRNA copy numbers and the protein level of Hsp90α in the samples used was achieved. In order to determine the downstream effect of silencing Hsp90α, the level of Akt/PKB kinase activity was measured. A commercially available Akt/PKB kinase activity assay kit (Assay Design, UK) was used to measure the activity of Akt/PKB kinase in the glioma cell lines. Irrespective of the cell line used, the Akt/PKB kinase activity was reduced significantly (**$p < 0.001$) when shRNA targeted against Hsp90α was used as compared to siRNA targeted against Hsp90α (Table 1).

shRNA achieved better results than siRNA in targeting Hsp90α at both the genomic and proteomic levels, and subsequently reduced the Hsp90α mRNA, protein and activity levels in glioma cell lines. In addition to the reduced efficacy achieved by siRNA, there are limitations attached to its use. siRNA needs to overcome several cell internalization barriers such as the extra cellular space, cell membranes, endosomes, RNAses and other nucleases in the cytoplasm, whereas, shRNA is based in a plasmid vector and is easily transfected into the cells.

Furthermore, shRNA transcribes into the genome and is used for stable transfections, whereas, siRNA has a transient transfection that remains in the cytosol and has a shorter effect than shRNA [38]. Moreover, the expression of shRNA could be controlled by inducible promoters thereby resulting in constitutive silencing of the gene(s) of interest and could possibly prove toxic to the cells [39]. Previous studies have shown that factors such as high GC content of the siRNA [29-32], the inaccessibility of the target region [33-35] and the unfavourable strand symmetry of the siRNA [36,37], could lead towards poor siRNA efficacy. The results from this study demonstrate that siRNA decreases Hsp90α mRNA in all three glioma cell lines by ~35 %, whereas, shRNA decreases Hsp90α mRNA in the same three glioma cell lines by ~63%. Furthermore, the post Hsp90α inhibition by siRNA of the Akt/PKB kinase levels downregulated to ~29 %, whereas, Hsp90α inhibition by shRNA resulted in the reduction of Akt/PKB kinase levels to ~3 % in all three glioma cell lines. Interestingly, both shRNA and siRNA target exon 5, however, the shRNA used in this study was 29-mer while the siRNA was 21-mer rendering it a more potent inhibitor [17,18].

Previous studies have reported that the 29-mer shRNA suppressed genes approximately two fold greater than the corresponding 21-mer siRNA [18]. The shRNA constructs were computer designed against multiple splice variants at Hsp90α gene locus for its optimal suppression. Four designed and sequence verified shRNA vectors were provided by Origene (USA), one of which was to achieve >90% Hsp90α expression inhibition. The vectors were previously validated for transient transfection and for the ability to inhibit the targeted gene i.e. Hsp90α. The four shRNA constructs bind to exon 2 and 3 border, exon 4 and 5 border, exon 11 and exon 12, respectively. It was noted that shRNA construct 2 which binds to the exon 4 and 5 border suppresses Hsp90α expression more effectively than the other three constructs in glioma cell lines (data not shown) and hence, was used in this study. Similarly, previously validated siRNA [26] that binds to exon 5 was also used in this study for comparative analysis.

Based on the literature and the results obtained from this study it could be inferred that shRNA works as a more effective silencer of Hsp90α mRNA and protein in 1321N1, GOS-3 and U87-MG glioma cell lines.

Conclusion

The inhibition of Hsp90α in glioma could be used as a future therapeutic approach to conventional chemotherapies. In this study, shRNA proved to be more effective than siRNA in silencing Hsp90α in glioma cell lines at both the genetic and proteomic levels. shRNA targeted against Hsp90α provides a better reduction profile of the Akt/PKB kinase activity. Given the role of Akt/PKB kinase, this in turn could possibly mean that the glioma cells used in this study are no longer immortal and could thereby undergo apoptosis which is of therapeutic importance. Thus, silencing of Hsp90α using shRNA targeted against it could be used as a future therapeutic option in glioma treatment.

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