HSP90 Inhibition as a Dual Therapeutic Strategy for Glioblastoma and Brain Tumour-Related Epilepsy

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Abstract

The aim of this research is to interrogate how inhibition of HSP90 activity in glioblastoma (GBM) might reduce seizures related to brain tumours, and/or affect tumour viability. This research has the potential to identify a novel treatment for the devastating diagnosis of the most severe type of brain tumour, GBM, and the seizures that often accompany it. Firstly, we established heat-shock proteins' gene expression patterns in publically available GBM samples. Secondly, cell viability assays were performed to measure the impact HSP inhibition on the tumour cells; both single agent and combined agent assays (HSP inhibitors +/- cannabidiol) were performed. Thirdly, preliminary local field recordings (LFP) were used to interrogate the potential anti-seizure properties of these drugs.

Key findings: HSP90B1 expression was associated with poorer survival in human GBM. HSP90 inhibition reduced CT2A glioma cell viability, although combination with CBD was predominantly additive rather than synergistic. Preliminary electrophysiological recordings further indicated that selective HSP90β inhibition with KUNB31 reduced the duration and magnitude of ictal-like activity.

Significance: Our findings provide preliminary evidence that HSP90, and particularly HSP90β, may represent a dual therapeutic target in GBM, with potential to influence both tumour viability and tumour-associated seizure activity. Further validation is required to establish efficacy and therapeutic relevance.

Keywords: Glioblastoma, heat shock protein, synergy

1. Introduction

1.1 Glioblastoma

Glioblastoma (GBM), is the most aggressive type of brain cancer (glioma) in adults. The WHO classifies it as a grade 4 diffuse astrocytic glioma: a malignancy that infiltrates surrounding brain tissue. Despite treatment, GBM remains incurable for most adults, with median survival of approximately 14–18 months and five-year survival around 5% or less. Standard treatment comprises maximal safe surgical resection followed by radiotherapy and temozolomide (TMZ). Although this reduces tumour burden and delays progression, infiltrating cells almost invariably remain and drive recurrence.

Some major barriers to treatment include infiltration and heterogeneity. GBM tumour cells tend to migrate beyond the tumour margin visible on MRI. Unlike liver or breast cancer, there is no safely resectable margin; as removing all infiltrative disease would cause unacceptable neurological injury. GBM cells are also highly heterogeneous and can transition among four major cellular states. The relative abundance of these states differs between tumours and reflects interactions between genotype, epigenome, and microenvironment. Because each state may depend on distinct signalling pathways, a drug targeting one pathway will become redundant if the cells 'switch' signalling pathways. This 'switching' between pathways is one contributor to treatment resistance. So, a key therapeutic challenge is to target several survival mechanisms simultaneously.

1.2 BTRE

Seizures are often the first presentation of a brain tumour. Brain tumour related epilepsy (BTRE) refers to epileptic seizures secondary to brain tumours and affect a substantial proportion of brain tumour patients, occurring in nearly 50% of high-grade glioma and up to 90% of low-grade glioma patients.[1] They profoundly affect quality of life, restricting driving, employment, and independence[2].

BTRE arises through interacting tumour and peritumoral mechanisms, including excess glutamate, reduced inhibitory GABA signalling, neuroinflammation, and immune-cell activation. Seizures are usually treated with standard antiseizure medications (ASMs), but BTRE mechanisms are not identical to those of non-tumour epilepsy. Drug interactions between ASMs and anticancer therapies can also compromise seizure management, oncological treatment, or both.

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[1] You et. al, 2021

[2] Vacher et. al, 2021

1.3 Heat-shock proteins: the common link

Heat shock proteins (HSPs) are present in every tissue and assist in baseline functions essential to keep us alive. They also take part in emergency responses when the body sustains a 'stressor', such as infection or inflammation (or cancer). Under these emergency states, signalling molecules will bind to DNA and cause 'upregulation' of HSP genes. HSPs then help damaged proteins to refold, or if damage is irreparable they direct proteins to be degraded. However this system can be used by cancer cells too. By protecting proteins and buffering cellular stress, HSPs support tumour cell survival, proliferation, and treatment resistance. HSP90 is particularly important because it stabilises multiple oncogenic signalling proteins and is therefore an attractive anticancer target

HSP90 inhibitors were first developed for non-GBM cancers. Yet despite decades of extensive research, only one HSP90 inhibitor has been approved for clinical use. Pimitespib is approved in Japan for advanced gastrointestinal stromal tumours (GIST). The majority of other trials failed or were terminated early on because of toxicity (ocular, hepatic, GI) and insufficient efficacy. The major challenge has been a process called the 'heat shock response' (HSR); when we use a drug to inhibit HSP90, the body responds by increasing levels of HSP90, HSP60, HSP70 and other chaperones, essentially undoing the work of the HSP inhibitor. This adaptive response limits the efficacy of HSP inhibitors in clinical practice and remains one of the major reasons why HSP90 inhibitors have not received FDA approval despite ~200 monotherapy/combination trials since 1999.

The strongest rationale for HSP90 inhibition in GBM has been as a chemo- and radiosensitizer[3]. However, non-selective inhibitors ("pan-HSP90") have limited efficacy due to compensatory resistance mechanisms discussed above. A novel approach could be targeting HSP90 *isoforms*. There are four isoforms that exist in humans: HSP90 alpha is inducible, beta is constitutive, and there are two isoforms selective to mitochondria and the endoplasmic reticulum. The toxicity issues mentioned earlier have been attributed to inhibition of the alpha isoform; so, targeting the beta isoform could reduce toxicity *and* avoid the HSR negative feedback loop (feedback less likely to be triggered if a smaller proportion of HSP90 are targeted). KUNB31 is a selective HSP90β inhibitor and demonstrates strong preclinical efficacy in other cancers[4]. It can also penetrate the CNS, meaning it is feasible to use in GBM.

HSP dysregulation has not only been linked to tumour proliferation, but also to drug-resistant epilepsy (outside the tumour setting)[5]. Yet, the role of HSPs in BTRE remains unexplored. Establishing this link between HSP and BTRE has the potential

[3] AUY922 phase I/II trial in molecular GBM with funding from NIH
[4] Khandelwal et al., 2018
[5] Hossain et al., 2021

to identify a novel therapeutic target capable of addressing both tumour progression and seizure burden.

1.4 CBD as an oncotherapy

CBD is the major non-intoxicating cannabinoid in Cannabis. Many preclinical models show CBD can reduce viability of cancer cells[6]. The effect has been observed across tumour types, including GBM[7]. Some emerging mechanisms by which CBD kills tumour include: increasing reactive oxygen species (ROS), disturbing the integrity of mitochondrial membrane, induction of ER stress and interfering with the cell cycle. In this way CBD shifts the balance in tumour cells towards death. Importantly, these effects are context dependent; in some settings CBD kills tumour cells, but in certain GBM models CBD reduces apoptotic signalling[8] and *protects* the tumour cell. Whether CBD kills tumour cells or protects them seems to depend on the baseline stress level of the cell. As such, the most promising approach is a combination strategy. Thus, a reasonable approach could be to treat with CBD- thus creating cellular stress- and simultaneously taking away a core defence by blocking HSPs with an inhibitor. This is the rationale behind the two-pronged strategy in this study; create stress *and* block the cell's defence system against the stress. Yet the broad effect spectrum effect makes CBD unreliable; its effects vary across tumour type, dosage, formulation, exposure time and experimental model[9], all of which creates barriers to clinical translation. But by treating cells with CBD *and* a heat shock protein, one could theoretically create the perfect conditions for CBD to inflict its cytotoxic effect.

[6] Twelves et al, 2021
[7] Odonkor et al 2026
[8] Picucci et al, 2026
[9] Heider et al, 2022

1.5 Aims and hypothesis:

The aim of this research is to investigate whether inhibiting heat shock proteins (HSPs) in brain tumour patients can reduce glioma-associated seizures and/or slow tumour progression. This work could identify a novel therapeutic approach for glioblastoma (GBM), the most aggressive form of brain tumour, and the seizures frequently accompanying it. We hypothesise that inhibition of HSP90-β via KUNB31 could reduce incidence of glioma related seizures while simultaneously killing tumour cells.

We therefore hypothesise that HSP90β selective inhibitors, designed to evade resistance mechanisms in other cancers, would have superior efficacy in GBM than currently tested HSP inhibitors.

Aim 1: Establish Expression patterns for various heat shock proteins and correlate them to survival outcomes in GBM

Aim 2: Determine the impact of HSP90 inhibition on electrophysiological activity and tumour cell viability in GBM

2. Results

2.1 Gene expression analysis via publicly available databases

Two online databases were used to investigate HSP expression patterns in human GBM. The first, the Ivy Glioblastoma Atlas Project (Ivy GAP), provides regionally resolved molecular data from human GBM tissue, enabling gene expression to be examined across distinct anatomical tumour regions, including the cellular tumour, infiltrating/leading edge, pseudopalisading regions and areas of microvascular proliferation. Representative regional expression patterns for HSPB3 and HSP90AA6P are shown in Figure 1.

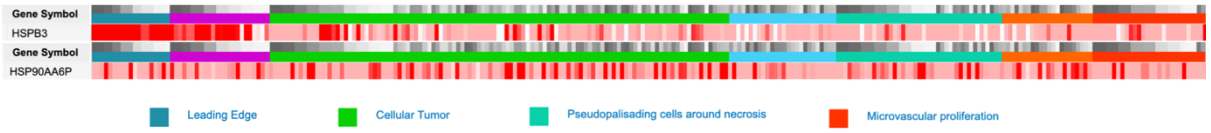


Figure 1 | Heatmaps show relative gene expression of HSPB3 and HSP90AA6P across anatomically defined GBM regions, including the leading edge, cellular tumour, pseudopalisading cells around necrosis and areas of microvascular proliferation. Columns represent individual tumour samples, with anatomical regions indicated by the annotation bars above each heatmap and relative gene expression represented by colour intensity. HSP90AA1 and HSP90-B1 were noted to have region specific expression patterns.

Publicly available transcriptomic data were next interrogated using GlioVis. Expression was examined in relation to overall survival, MGMT promoter methylation status, transcriptional subtype and patient sex. HSP90AA1 expression was not significantly associated with overall

survival (HR = 0.93, 95% CI 0.65–1.34; log-rank p = 0.7122; Wilcoxon p = 0.849; Figure 2). HSP90AA1 expression was additionally examined according to MGMT promoter methylation status, classical, mesenchymal and proneural transcriptional subtypes, and patient sex.

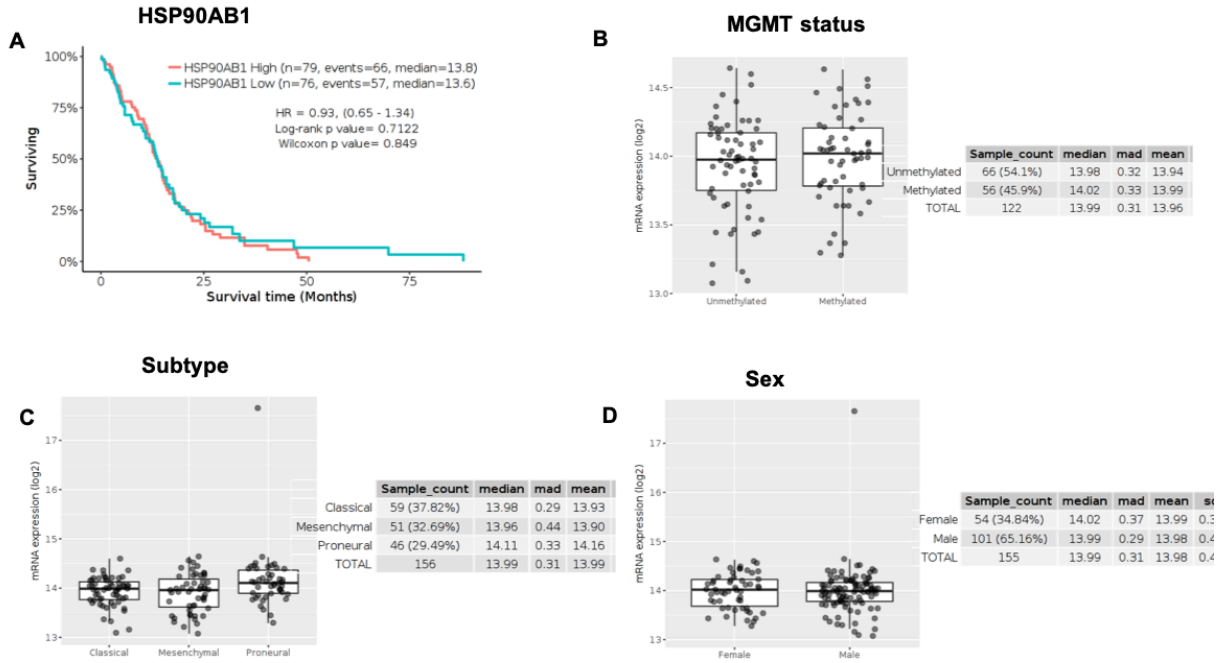


Figure 2 | HSP90AA1 expression and clinicopathological associations in human GBM. (A) Kaplan–Meier survival analysis comparing patients with high (n = 79) and low (n = 76) HSP90AA1 expression. (B) HSP90AA1 mRNA expression according to MGMT promoter methylation status, comparing unmethylated (n = 66) and methylated (n = 56) tumours. (C) HSP90AA1 mRNA expression across classical (n = 59), mesenchymal (n = 51) and proneural (n = 46) GBM transcriptional subtypes. (D) HSP90AA1 mRNA expression according to patient sex.

In contrast to HSP90AA1, HSP90B1 expression was significantly associated with overall survival, with high HSP90B1 expression associated with poorer survival (HR = 0.61, 95% CI 0.42–0.88; log-rank p = 0.0084; Figure 3). HSP90B1 expression was also examined in relation to MGMT promoter methylation status, transcriptional subtype and patient sex.

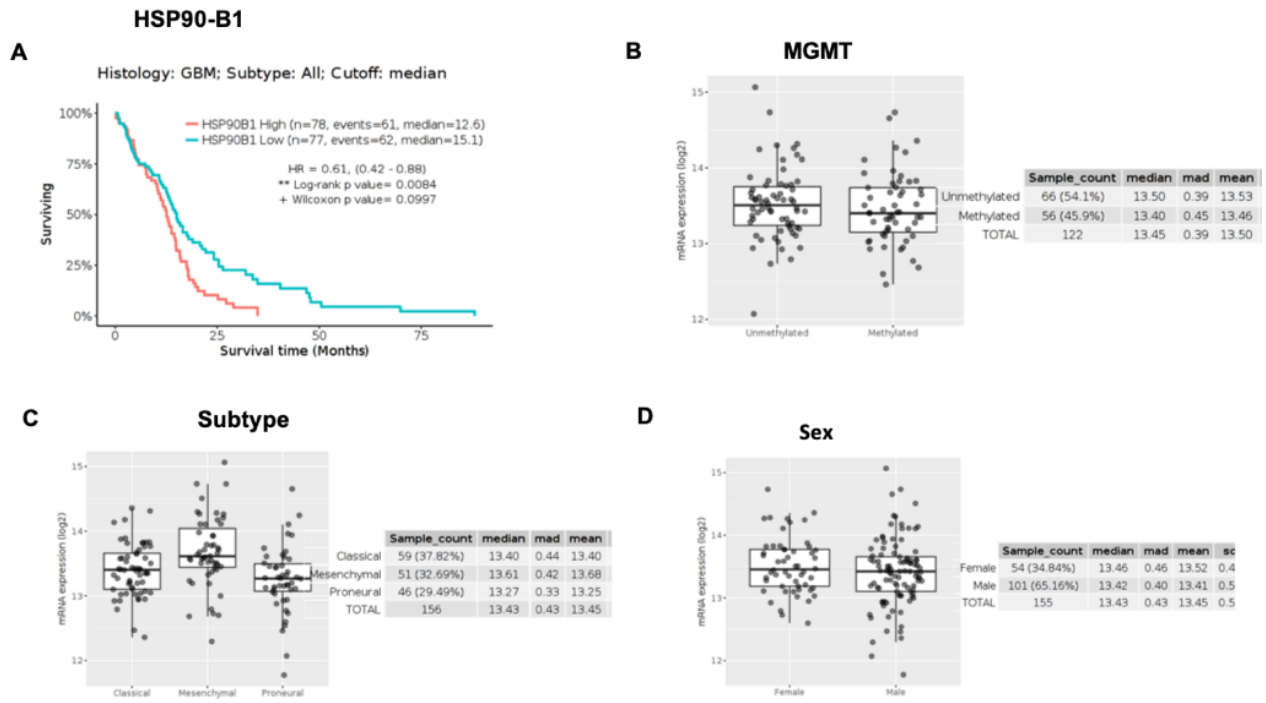


Figure 3 | HSP90B1 expression and clinicopathological associations in human glioblastoma. (A) Kaplan–Meier survival analysis comparing patients with high (n = 78) and low (n = 77) HSP90B1 expression, stratified using the median expression value. (B) HSP90B1 mRNA expression according to MGMT promoter methylation status, comparing unmethylated (n = 66) and methylated (n = 56) tumours. (C) HSP90B1 mRNA expression across classical (n = 59), mesenchymal (n = 51) and proneural (n = 46) GBM transcriptional subtypes. (D) HSP90B1 mRNA expression according to patient sex, comparing female (n = 54) and male (n = 101) patients.

2.2. HSP inhibitor effect on GBM cell viability

2.2.1 Methods

CT2A murine glioma cells were established for in vitro drug screening. Cells were maintained in DMEM supplemented with FBS. CT2A cells were seeded into 96-well plates at 5,000 cells/well and allowed to adhere for 24 h. Cells were subsequently treated with serial dilutions of onalespib (pan-HSP90 inhibitor), KUNB3 (HSP90β inhibitor), perampanel (non-competitive AMPA receptor antagonist), or cannabidiol (CBD). Cell viability was assessed at 24, 48 and 72 h following treatment using an MTT assay. Following incubation with MTT reagent, the resulting formazan product was quantified by absorbance using a microplate reader (Figure 4). Experiments were performed in biological triplicate. Data was analysed using GraphPad Prism.

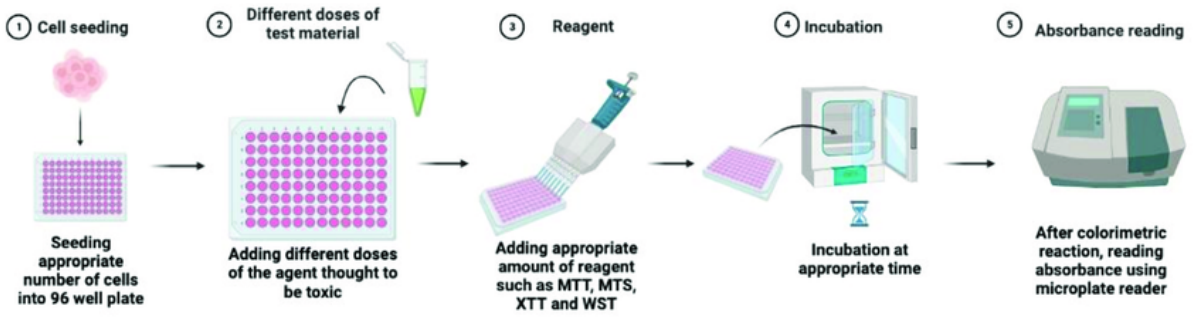
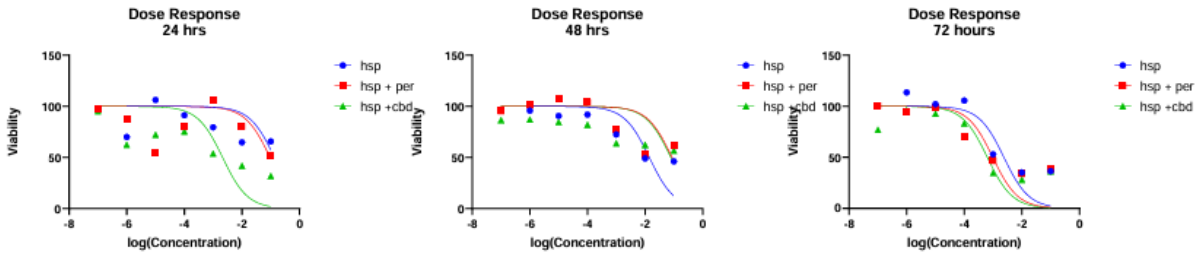


Figure 4 | Schematic representation of the MTT cell viability assay workflow. Cells were seeded into 96-well plates, allowed to adhere for 24 h and subsequently exposed to serial concentrations of the indicated compounds. Cell viability was assessed by MTT assay at 24, 48 and 72 h following treatment.

2.2.2 MTT assay results

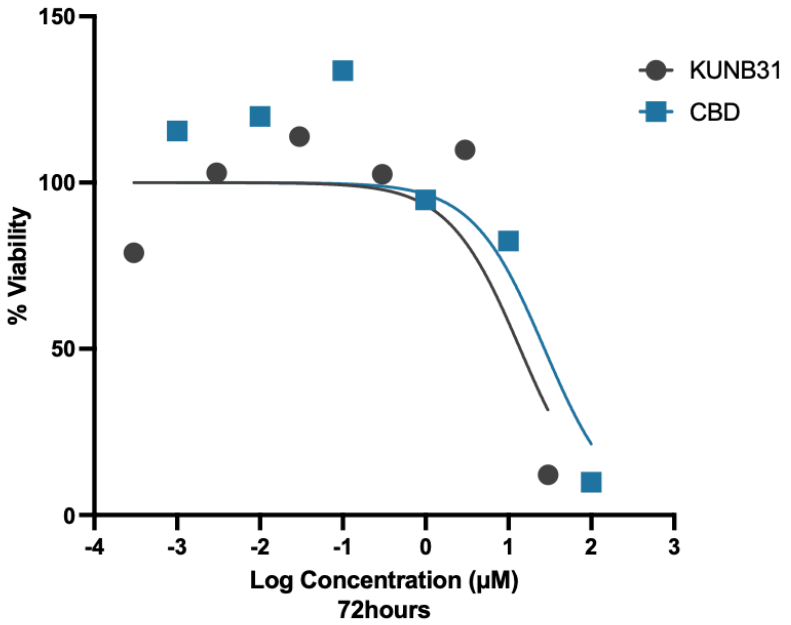
Cell viability was assessed over 72 h to identify concentration- and time-dependent responses (Figure 5). The effects of onalespib, alone and in combination with perampanel or CBD, varied across the treatment period. At 24 h, a significant effect was observed only for the onalespib + CBD combination (p = 0.0021), while onalespib alone and onalespib + perampanel did not reach statistical significance. At 48 h, onalespib alone produced a significant effect (p = 0.0142), whereas neither combination was significant. By 72 h, however, all three treatment conditions produced significant effects, with the strongest statistical evidence observed for the combination treatments (onalespib + perampanel, p = 0.00098; onalespib + CBD, p = 0.00063).



	Onalespib	Onalespib + perampenal	Onalespib + CBD
24hrs	0.1359	0.09522	0.002139
48hrs	0.01416	0.08760	0.07921
72hrs	0.002386	0.0009806	0.0006346

Figure 5 | Effect of single-agent and combination treatments on CT2A glioma cell viability. CT2A cells were treated with onalespib alone or in combination with perampanel or cannabidiol (CBD), and cell viability was assessed by MTT assay at 24, 48 and 72 h. Values shown represent the statistical significance (p-values) of the treatment effects at each time point.

The effects of KUNB31 (a HPS90 beta selective inhibitor), alone and in combination with CBD, also varied across the treatment period. Effects were observed at 24hrs and 72hrs. Significant effects were only observed at 72 hours, displayed in figure 6.

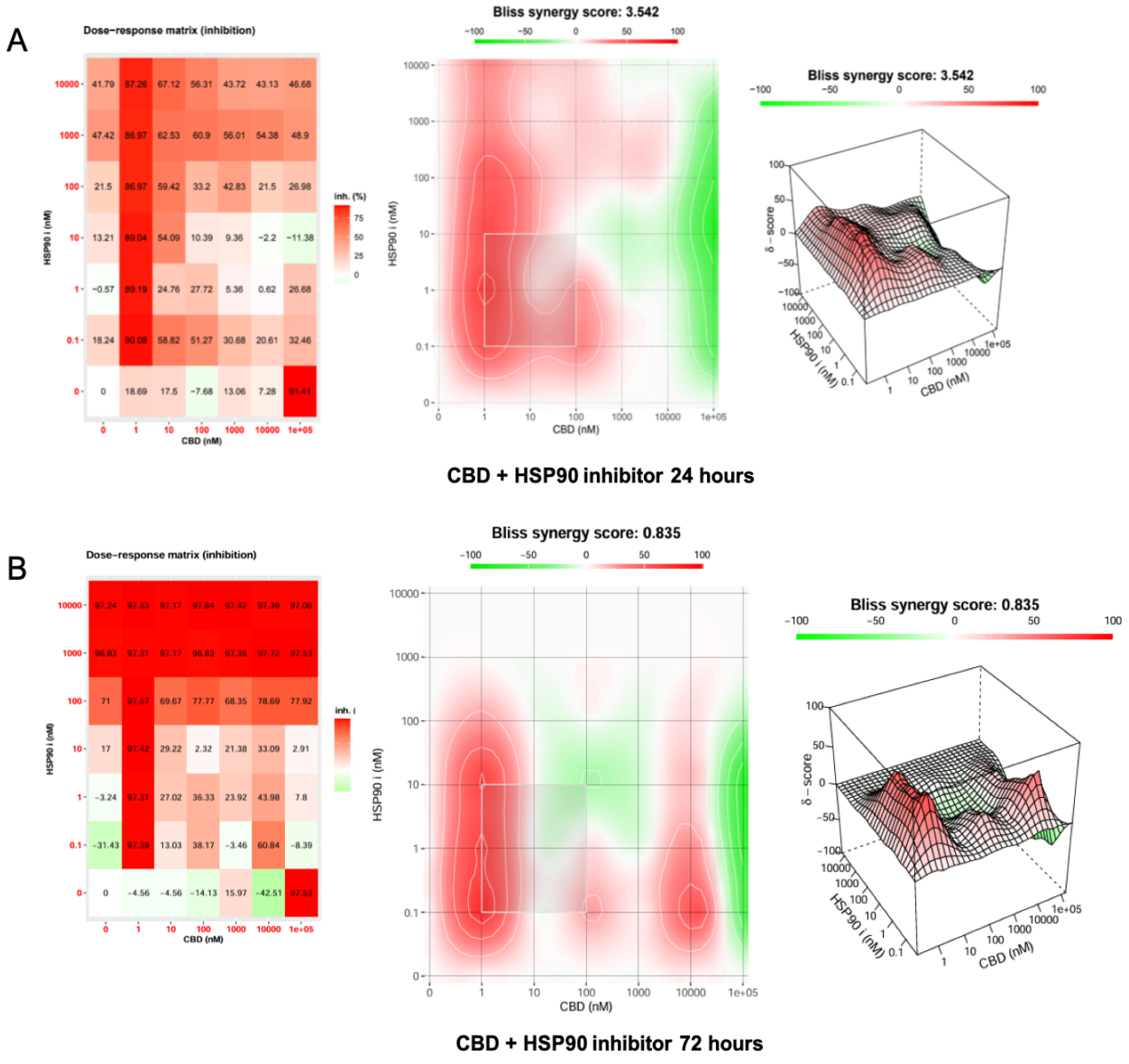


Agent	IC_{50}	95% CI
KUNB31	13.81	3.18–70.49
CBD	27.16	4.65–165.8

Figure 6 | Effect of KUNB31 and CBD treatments on CT2A glioma cell viability. Cell viability was assessed by MTT assay at 72 h. Values shown represent the IC50 values and 95% confidence intervals. The lower estimated IC50 of KUNB13 indicates greater apparent potency than CBD.

2.3 Bliss synergy analysis results

To next assess whether synergy is present or if an additive effect is being observed, bliss assays were performed[10]. Bliss analysis indicated that combined HSP90 inhibition with onalespib (broad spectrum) and CBD produced predominantly additive effects, with overall synergy scores of 3.542 at 24 h and 0.835 at 72 h. Although discrete concentration combinations showed regions of positive Bliss scores, these did not translate into substantial overall synergy. The lower score at 72 h suggests that the interaction became increasingly additive with prolonged exposure, despite high levels of inhibition being achieved at several drug concentrations (Figure 7).



[10] Ianevski et al, 2017

Figure 7 |Bliss synergy analysis of combined HSP90 inhibition and CBD treatment in CT2A cells. CT2A cells were treated with increasing concentrations of the HSP90 inhibitor and cannabidiol (CBD), and drug interactions were assessed using the Bliss independence model at 24 h (top) and 72 h (bottom). Dose–response matrices (left) show percentage inhibition across individual drug combinations. Bliss synergy maps (centre) and three-dimensional synergy plots (right) illustrate concentration-dependent drug interactions, with positive scores indicating synergy and negative scores indicating antagonism. Overall Bliss synergy scores were 3.542 at 24 h and 0.835 at 72 h.

Bliss assays were next performed for both Onalespib & CBD *and* KUNB31 & CBD. These plates were read at 24hr and 72hr timepoints across the week. The same synergy tool was used to analyse the results, which are as follows: (this time, a Bliss and Zip readout was performed for further analysis. Only Bliss is displayed as this was the most useful readout.)

Drug combination	Synergy score	Most synergistic area score	Method
CBD - KUNB	10.98	19.49	Bliss

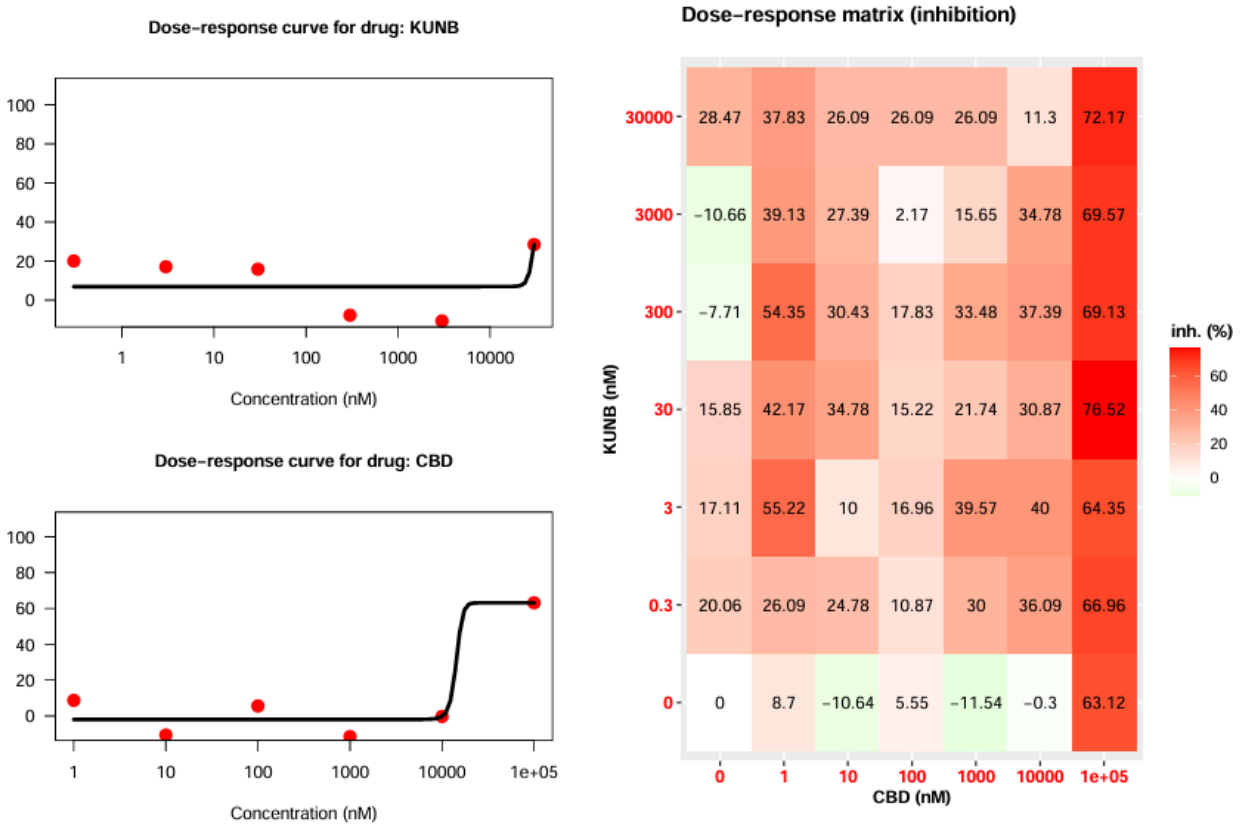


Figure 8 | KUNB31 and CBD bliss assay results after 24 hours.

Drug combinations:

Drug combination	Synergy score	Most synergistic area score	Method
CBD - KUNB	-9.95	6.72	Bliss

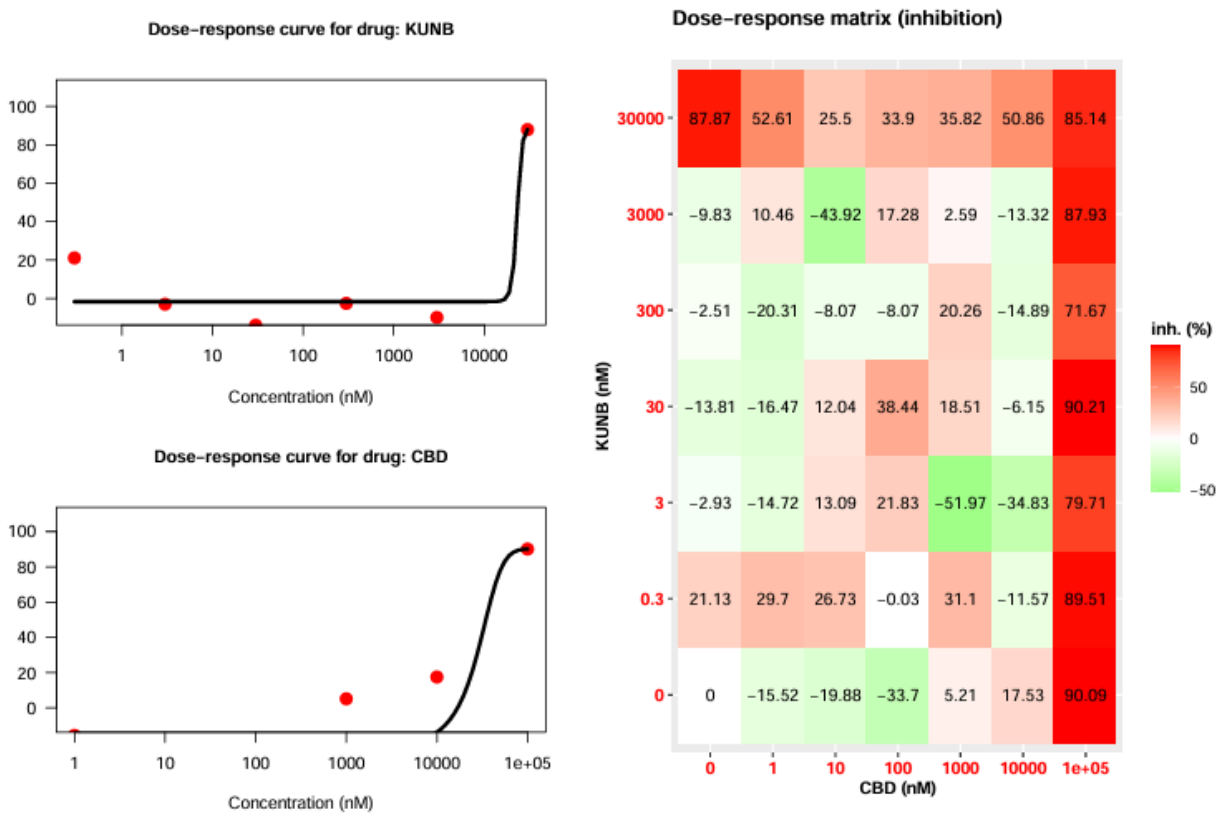


Figure 9 | KUNB31 and CBD bliss assay results after 72 hours

Drug combinations:

Drug combination	Synergy score	Most synergistic area score	Method
CBD - HSP90 i	-0.09	7.50	Bliss

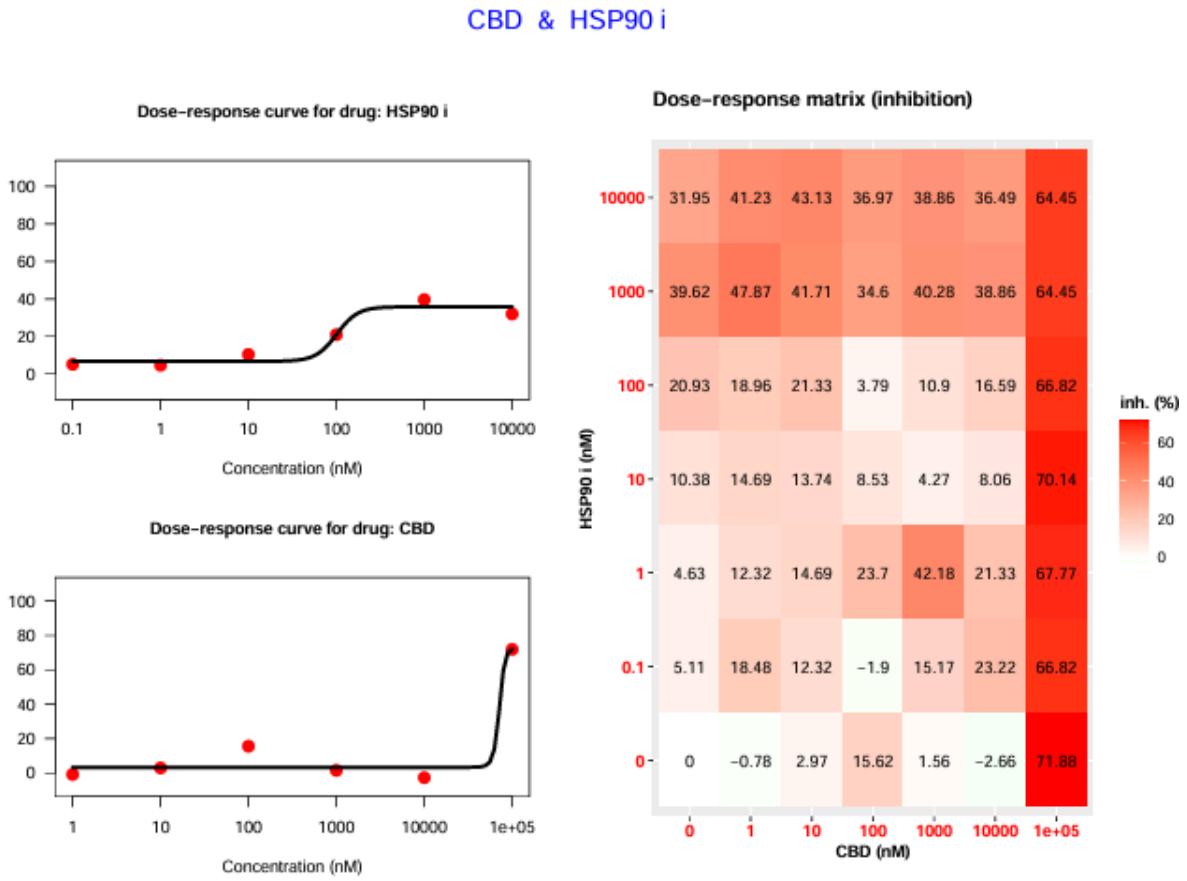


Figure 10 | Onalespib and CBD bliss assay results after 24 hours

Drug combinations:

Drug combination	Synergy score	Most synergistic area score	Method
CBD - HSP90 i	-7.86	10.20	Bliss

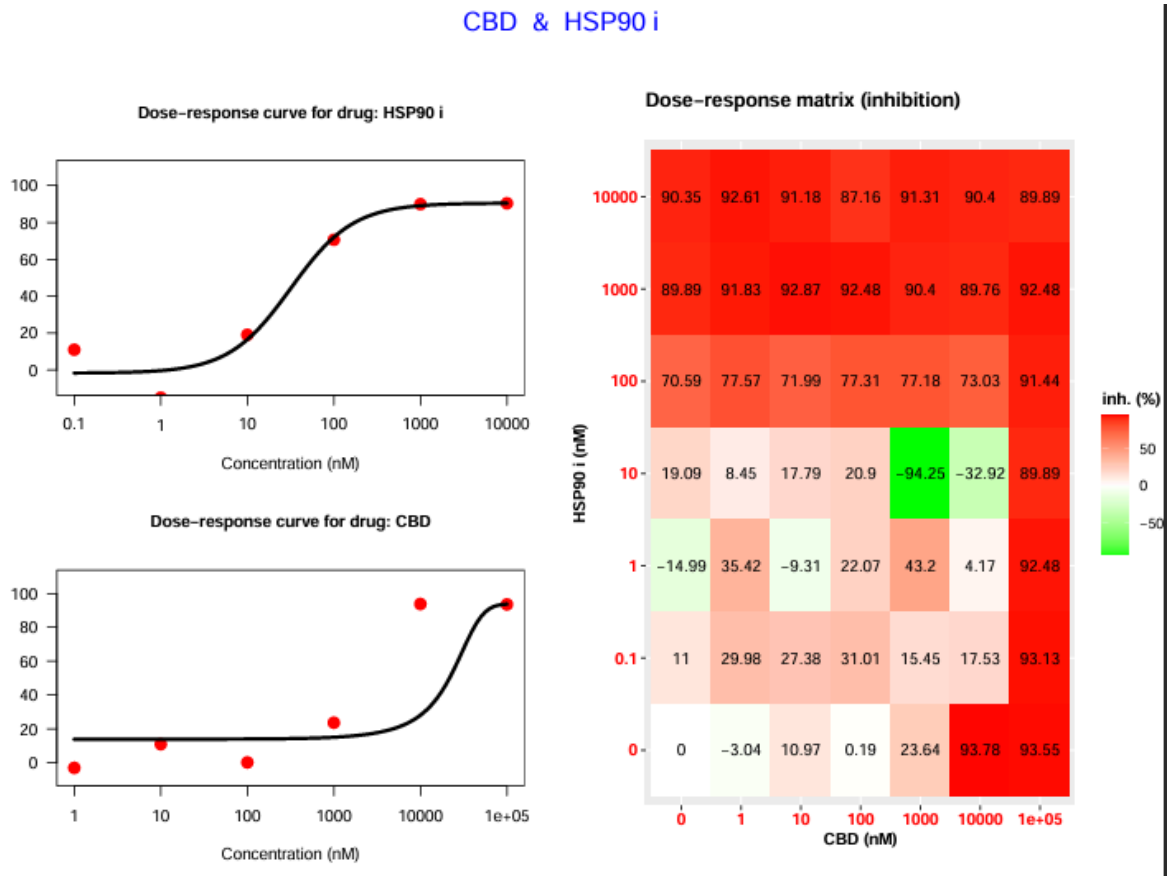


Figure 11 | Onalespib and CBD bliss assay results after 72 hours

2.4 Local field recordings

2.4.1 Methods

Mouse brains (provided by Dr Kate Connor) were sectioned horizontally at 400 µm and immersed in oxygenated artificial cerebrospinal fluid (aCSF; 126 mM NaCl, 3 mM KCl, 1.25 mM Na_2HPO_4, 24 mM $NaHCO_3$, 10 mM glucose, 1 mM $MgSO_4$ and 1 mM $CaCl_2$). Slices were allowed to stabilise for 1 h in a holding chamber at the interface between humidified carbogen (95% O_2/5% CO_2) and circulating aCSF before transfer to the recording chamber. For electrophysiological recordings, slices were continuously perfused with oxygenated aCSF and maintained at 30–34 °C. A glass microelectrode filled with aCSF was positioned within the medial entorhinal cortex (mEC). Local field potentials (LFPs) were recorded and analysed using Spike2 software (Cambridge Electronic Design Ltd.), with 50-Hz mains interference

removed using Humbug filtering. Spontaneous ictal-like activity was induced by perfusion with 4-aminopyridine (4-AP; 100 nM). Following stabilisation of ictal activity, slices were exposed to the compounds of interest. For KUNB31 experiments, 4 µL of 10 mM KUNB31 was added to 40 mL aCSF to achieve a final concentration of 1 µM. LFP activity was recorded throughout baseline, 4-AP exposure and drug treatment, followed by an approximately 40-min washout period.

2.4.2 Results

Preliminary LFP recordings indicated that KUNB31 altered several characteristics of 4-AP-induced ictal-like activity. KUNB31 treatment (1uM) was associated with a marked reduction in ictal event duration and AUC compared with the 4-AP baseline, while the number of detected events increased and the interval between events remained relatively unchanged. Following washout, event duration and AUC partially increased towards baseline values, suggesting some reversibility of the KUNB31-associated changes. These preliminary findings suggest that KUNB31 may reduce the duration and overall magnitude of ictal-like activity rather than suppressing event initiation. However, given the limited number of recordings, further biological replicates and statistical analysis are required before an anti-seizure effect can be concluded.

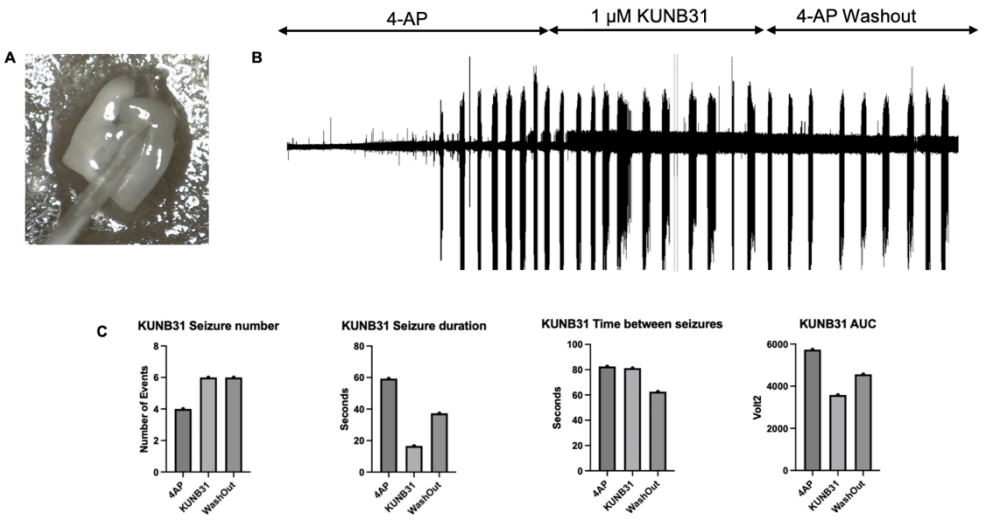


Figure 7 | Effect of KUNB31 on 4-AP-induced ictal-like activity in acute mouse brain slices. **(A)** Representative image showing placement of the recording electrode in the mEC. **(B)** Representative local field potential (LFP) recording illustrating 4-AP-induced spontaneous ictal-like activity. **(C)** Quantification of electrophysiological parameters during 4-AP baseline, treatment with KUNB31 (1 µM), and following washout, including number of ictal events, ictal event duration, inter-event interval and area under the curve (AUC). Data are presented descriptively from preliminary recordings.

3. Discussion

HSP90B1 expression was associated with poorer survival in human GBM, and inhibition of HSP90 in mouse cell glioma reduced glioma cell viability. The heightened toxicity from combination treatment with CBD was most likely due to an additive effect, not a synergistic one. This data offers preliminary support for our hypothesis that HSP90β selective inhibitors, designed to evade resistance mechanisms in other cancers, would have superior efficacy in GBM than currently tested HSP inhibitors.

A key limitation of the study is the electro-physiology data remains very preliminary. Future studies should interrogate this area further with more replicates using both onalespib and KUNB31. This would provide more evidence regarding the significance of HSP inhibition in preventing BTRE. E-phys experiments are, by nature, highly delicate and require careful monitoring of variables including temperature of the room and rigs. A period of more than six weeks is required to gather enough data in this form.

Another future direction could involve combination therapy with agents that disrupt epigenetic cell state programming (essentially 'locking' tumour cells in more vulnerable states), such as the HDAC inhibitor vorinostat. Seeing as vorinostat has been trialed on its own and in combination with chemo- and radiotherapy in GBM, there is potential for synergy with the HSP inhibitors investigated in this paper. Specifically, these state 'locking' agents could be used to prime GBM cells by making them more dependent on client proteins. This would heighten the toxicity of HSP inhibitors, as shown in some hematogenic cancers.[11]

In summary, our findings provide preliminary evidence that HSP90, and particularly HSP90β, may represent a dual therapeutic target in GBM, with potential to influence both tumour viability and tumour-associated seizure activity. Further validation is required to establish efficacy and therapeutic relevance.

[11] Rao et al, 2010