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Full Length Research Paper

Amino acyl tRNA synthetase inhibitors is therapeutics for gliomas at sub nanomolar concentrations

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Glioblastoma is the most malignant and fatal of all primary brain tumors. Each year in the United States, about 22,000 cases of glioma are diagnosed and about 70% of these patients die. The current treatment modality options available for glioma patients are limited to temozolomide, radiation, and surgery. The poor outcome with these treatment options has necessitated the search for a better chemotherapeutic strategy to improve patient outcomes. In a previous study, a genome-scale clustered regularly interspaced short palindromic repeats (CRISPR) screen in glioblastoma was performed under human type II topoisomerase (TOP2) poison selection and found that various aminoacyl tRNA synthetases (AARS) were the most enriched in our screen. This high expression of AARS was validated by western blot and the gliomas were treated with sub-nanomolar doses of AARS inhibitors. Our results showed that AARS inhibitor treatment effectively killed 75 to 90% of the tumors within 72 h and that this killing is independent of DNA damage repair machinery. Taken together, the findings suggest that application of AARS inhibitors might be curative for glioma, but more experimental *in-vivo* tests will be needed to validate this.

Key words: Borrelidin, mupirocin.

INTRODUCTION

According to surveillance epidemiology end result programme (SEER) (Ostrom et al., 2018), about 23,820 cases of brain tumor are diagnosed annually in the US. 17,760 of the affected patients die, which is approximately 70% of the cases, making glioblastoma a lethal tumor, and among brain tumors, the most malignant. The current modality for treating these patients includes radiation and chemotherapy and tumor treating field and survival is 14 months, at best (Mehta et al., 2018).

Aminoacyl tRNA synthetase is a very important enzyme which ensures fidelity in the transmission of genetic information. They play a key role in the loading of amino acids unto the charged tRNA, which then transfers it to ribosomes for decoding of the information (Walter, 2017; Carter and Wolfenden, 2015). Aminoacyl tRNA synthetases (AARS) is conserved across the phyla of life. Comparative evolutionary studies have shown that AARS is conserved from fungi to yeast to apicomplexa and humans. Evidence has shown that non-ribosomal peptides of fungi show conservation to AARS from eukaryotes (Luque et al.,

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2008). AARS are broadly classified into two groups: Classes I and II, based on the position of their active site. Class I AARS show Rossman's fold and form active sites at the interface between parallel β strands, while class II AARS, have their active sites on the anti-parallel β strands (Cavarelli and Moras, 1993). Orthogonal AARS and tRNA pairs have enabled the expansion of the genetic code (Normanly et al., 1990; Wang et al., 2001; Hancock et al., 2010; Anderson and Schultz, 2003; Sakamoto et al., 2002; Zhang et al., 2004) via the introduction of unnatural amino acids into the genome.

AARS has been shown to be highly over-expressed in tumors such as glioblastoma (Awah et al., 2019; Kim et al., 2012), prostate cancer (Vellaichamy et al., 2009), and other tumors. Furthermore, various small molecules have been shown to specifically inhibit different types of amino acyl tRNA synthetases. Both natural and synthetic AARS inhibitors have been described (Fang et al., 2015). These inhibitors are broadly classified into: (1) mimetics binding to ATP pockets or amino acids, (2) mimetics binding to tRNA-binding sites, (3) mimetics binding to tRNA editing sites, and (4) all substrate-binding blocking drugs by geometry fitting.

Mupirocin and borrelidin are potent inhibitors of amino acyl tRNA synthetase. They belong to categories 1 and 4, respectively and have been used in treating different diseases. Mupirocin is a topical skin ointment which inhibits isoleucine tRNA synthetase at IC50 of 2.5 - 32 nM (Fang et al., 2015). Borrelidin is a threonine tRNA synthetase inhibitor (IC50 = 0.8 - 7 nM) (Fang et al., 2015), and is very effective in treating malaria at the very low concentration of 0.93 ng/ml. It is effective in the treatment of both drug-sensitive and drug-resistant malaria (Sugwara et al., 2013). In addition, some studies have shown that borrelidin possesses angiogenic, antiviral and antifungal activities. Since borrelidin is effective against many diseases, efforts are being made to use it to treat cancers such as metastatic breast cancer (Jeong et al., 2018), oral cancer (Sidhu et al., 2015), and acute lymphoblastic leukaemia (Habibi et al., 2012).

To repurpose a well-known DNA damaging agent, etoposide for glioblastoma, we performed a genome-scale CRISPR knockout screen in a glioma cell line under etoposide selection (Awah et al. 2019). It was found that the most enriched themes were genes in protein translation (Awah et al., 2019), which belonged to amino acyl tRNA synthetase and ribosomal protein subunits. In addition, using the genome-scale CRISPR, we also found that glioblastoma expresses high levels of AARS and inhibition of AARS with borrelidin and mupirocin specifically kills the glioma cell lines *in-vitro* and at a very low concentration.

Therefore, the aim of this study was to investigate *in-vitro* if the inhibition of AARS is a potential therapy for glioblastoma.

METHODOLOGY

Genome scale CRISPR screen knockout

Briefly, as previously described (Awah et al., 2019), we obtained Brunello SgRNA library from Addgene (73179-LV), this library contains 70,000 sgRNA. 60,000 of which target the 20,000 genes at 3-4 guides per gene and 10,000 non-targeting controls. The guides were transduced into lentivirus. Using lentiviral particles, we spinfected 70,000 SgRNA Brunello library into 0.5 billion of SNB19 cells and selected them with puromycin at 0.6 µg/ml for 96 h, to achieve an MOI of 21%. About 140 million cells were harvested to ascertain the initial representation of the library after puromycin selection. The cells were then expanded to 200 million cells; 100 million of each were treated with etoposide (5 µM) and DMSO, respectively for 14 days. At the end of 14 days, the cells were harvested and the genomic DNA from the DMSO and the etoposide treated groups were harvested using Zymo Research gDNA kit and the SgRNA were amplified by a unique set of barcodes, then using NextSeq, the SgRNA abundance was determined. The data was analyzed as described (Awah et al., 2019) using CRISPRAnalyzer (Winter et al., 2017). We used 8 statistical methods from the CRISPRAnalyzer to call a guide a hit using a very stringent $p<0.001$ and an FDR of 0.01.

Western blot

To determine the protein expression level of AARS genes in various GBM, we extracted the proteins using M-PER (Thermoscientific: 78501) and a cocktail of phosphatase and protease inhibitors. The cells were lysed using a water bath ultrasonicator for 4 min. Subsequently, we spun down the cells and collected the supernatant. We measured the concentration and denatured the proteins in laemmli SDS buffer at 100°C for 14 min and directly loaded them unto 4 to 20% Tris-glycine gels (Novex), before separation at 180 V for 2 h. The gels were transferred unto a PVDF membrane by semi-dry blotting for 1 h. We blocked the membrane in 5% non-fat milk TBST for 30 min and incubated with primary antibodies AlaRS (Santa Cruz) (1:500) and IleRS, ThreRS (Santa Cruz) (1:500) and/or ACTB (Cell Signalling) (1:1500) in 5% BSA, respectively, with overnight shaking at 4°C. Primary antibodies were removed and we added the secondary polyclonal HRP (1:20,000) in TBST and incubated, with shaking for 2 h at room temperature. The membrane was washed 6X in TBST and developed with ECL (Cat No: 1705061), then band-imaged on a Biorad Chemi-doc imaging system.

Cell viability assay

GBM PDX and glioma cells were seeded at 4,000 cells per well in a 96 well plate and treated with 0.5 to 30 nM Borrelidin or DMSO. The plate was incubated for 72 h, after which we added cell titre glo (Cat No: G7572) and then incubated the cells while shaking for 5 min. We read the viability by measuring the luminescence. We normalized the intensity against DMSO treated cells of each cell line or PDX or the edited cell and then determined the survival.

Kaplan Meier survival analysis

To ascertain if high levels of AARS expression conferred survival

benefit or not to different cancers, we used the KM plotter online software platform (Nagy et al., 2018) to make a determination if patients whose tumors expressed high levels of AARS survived better or not. The algorithm from KM plotter calculates the KM survival based on the gene expression profiles based on RNA seq or tiled microarray probe and calculates the FDR for the p values of the survival.

GBM patient-derived xenografts

GBM patient-derived xenografts (MES83, GBM43, GBM6, GBM12) were originally from Dr Ichiro Nakano (University of Alabama, USA), they were a kind gift to me from Dr Peng Zhang (Northwestern University, USA). The cells were all grown in 1% fetal bovine serum in DMEM media with antibiotics, while SNB19, KS1, and GB1 (commercially obtained from ATCC) were all grown in 10% fetal bovine serum in MEM with antibiotics. All cells were grown to 80% confluency before they were harvested with trypsin for further analysis.

RESULTS

AARS is expressed in glioblastoma and its high expression is associated with poor survival outcome

Since AARS has been shown to be expressed in other tumors, we explored if it is also highly expressed in glioblastoma. First, we interrogated our CRISPR screen and found that many aminoacyl tRNA synthetases were enriched in glioblastoma (Figure 1A and B and Table 1). Chief amongst these were CARS (p = 1E-6), AARS (p = 2E-5), LARS (p = 0.00071), GARS (p = 0.001), TARS (p = 0.05), VARS (p = 0.006), MARS (p = 0.008), and HARS (p = 0.01). To verify the high expression of aminoacyl tRNA synthetase observed in the CRISPR screen, we performed a western blot analysis on three glioblastoma patient-derived xenografts (MES83, GBM6, GBM43) and on a glioma cell line SNB19. We found that alanyl tRNA synthetase was markedly enriched in some of the cell lines (Figure 1B). To ascertain, if the high expression of AARS confers any prognostic values to patients' survival, we used the KM-plotter (Nagy et al., 2018), an online survival algorithm that calculates the survival based on the mRNA expression obtained by RNA seq and microarray probe expression to test 13 different cancers, breast carcinoma, lung squamous cell carcinoma (Figure 1C), bladder carcinoma, cervical squamous cell carcinoma, testicular germ cell tumor, head and neck squamous cell carcinoma, kidney renal clear cell carcinoma, and liver carcinoma (Supplementary Figure 1A and B). We found that high expression of AARS in these 13 different cancers confers an unfavorable survival prognosis from the tumors. This indicates that tumors over-express AARS in order to usurp translational fidelity and outgrow normal tissue. In addition, inhibition of AARS might represent a therapeutic target for gliomas.

Borrelidin and mupirocin specifically inhibit classes I and II of AARS in gliomas at nanomolar concentration

As AARS has been established to be over-expressed in many tumors, we explored if the inhibition of AARS might be lethal for the glioblastoma PDX and glioma cell lines. We acquired two glioblastoma patient-derived xenografts (PDX-MES83, GBM6) and two glioma cell lines (Figure 2A) and performed a viability assay by treating with Borrelidin for 72 h. We found that at nanomolar concentrations (7 - 18 nM), borrelidin kills 99% of the glioma (Figure 2A). Next, to ascertain if treating gliomas with AARS inhibitors specifically targets aminoacyl tRNA synthetase (AlaRS, isoleucine and threonine tRNA synthetase), we treated cell lines: three patient-derived xenografts (GBM6, GBM12, GBM43) and three glioma cell lines (SNB19, GB1, KS1), with borrelidin for 36 h and performed a western blot with antibodies against alanyl, isoleucine and threonine tRNA synthetase and GAPDH as loading control. it was found that borrelidin and mupirocin (Figure 2B and C) inhibit AARS expression.

Inhibition of AARS does not impact DNA damage repair nor anti-apoptotic machinery

To determine if inhibition of AARS impacted other pathways such as DNA damage repair machinery or the anti-apoptotic pathway, we performed a western blot against FANCB, a DNA damage repair protein that has been validated previously (Awah et al., 2019), among others, to be involved in response to chemotherapy. We found that gliomas treated with inhibitors of AARS do not show a reduction nor an increase in the expression level of FANCB (Figure 2D). Likewise, we explored if the anti-apoptotic machinery were impaired upon AARS inhibition of glioma cells and found no changes in the expression of BCL2L2 (Figure 2D), which have been shown to be triggered in the presence of chemotherapeutics. Taken together, these findings suggest that AARS inhibition is very specific and targets the unique pathway of amino acid loading unto tRNAs for ribosomal translation.

DISCUSSION

A cure for glioblastoma remains elusive. The current therapies are limited to temozolomide, radiation and electric field treatment. So far, these regimens have remained inefficient, which has called for reevaluation into the strategies for curing this disease. The revelation of AARS as a gene involved in carcinogenesis has opened new possibilities for targeting gliomas and many other cancers. Evidence showing the expression of

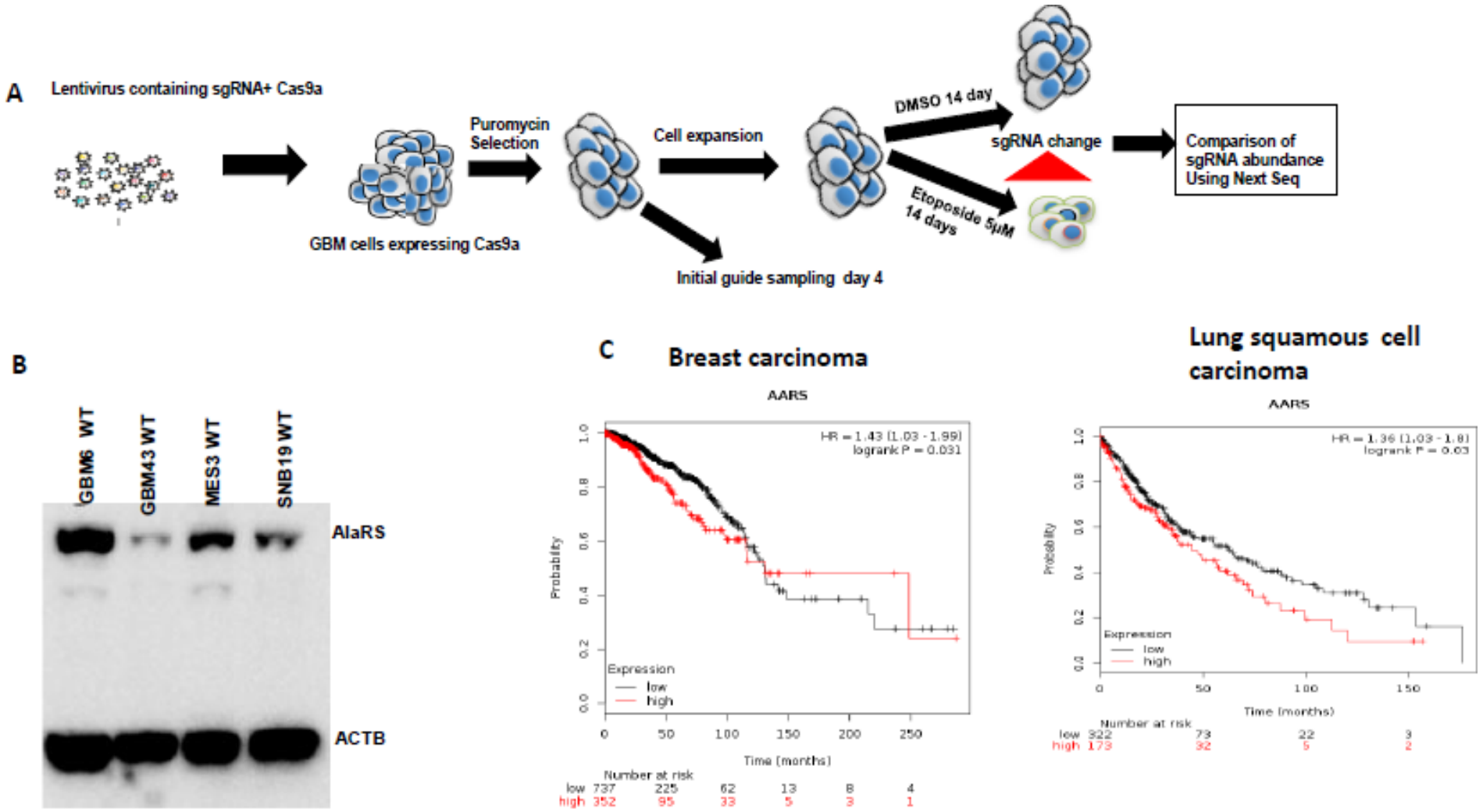

Figure 1. Genome wide CRISPR identifies Aminoacyl tRNA synthetase in glioblastoma. A. Schematic depiction of CRISPR screen in glioblastoma, the numbers ''x'' represents the coverage of sgRNA library in that experimental condition after sequencing. Briefly, as previously described (Awah et al., 2019), we spinfected 70,000 SgRNA Brunello library into 0.5 billion of SNB19 cells and selected them with puromycin at 0.6 µg/ml for 96 h. We achieved an MOI of 21%. About 140 million cells were harvested to ascertain the initial representation of the library after puromycin selection. The cells were then expanded to 200 million cells; 100 million of each were then treated with etoposide (5 µM) and DMSO, respectively, for 14 days. At the end of 14 days, the cells were harvested and the SgRNA were amplified by a unique set of barcodes and using NextSeq, the SgRNA abundance was determined. The data was analyzed as described in (Awah et al., 2019) using CRISPRAnalyzer (Winter et al., 2017). B. Western blot shows the expression of AlaRS in different glioma patient derived xenografts (GBM6, 43, MES83 and SNB19). C. Kaplan Meier survival curve shows high expression of AlaRS is a poor prognostic marker for breast cancer and lung squamous cell carcinoma.

Table 1. Table shows different AARS that were enriched and the p values showing enrichment of the guide.

Etoposide vs DMSO	P value
CARS	1.05E-06
AARS	2.51E-05
LARS	0.00071
GARS	0.001855
TARS	0.005013
VARS	0.006298
MARS	0.008141
HARS	0.010263

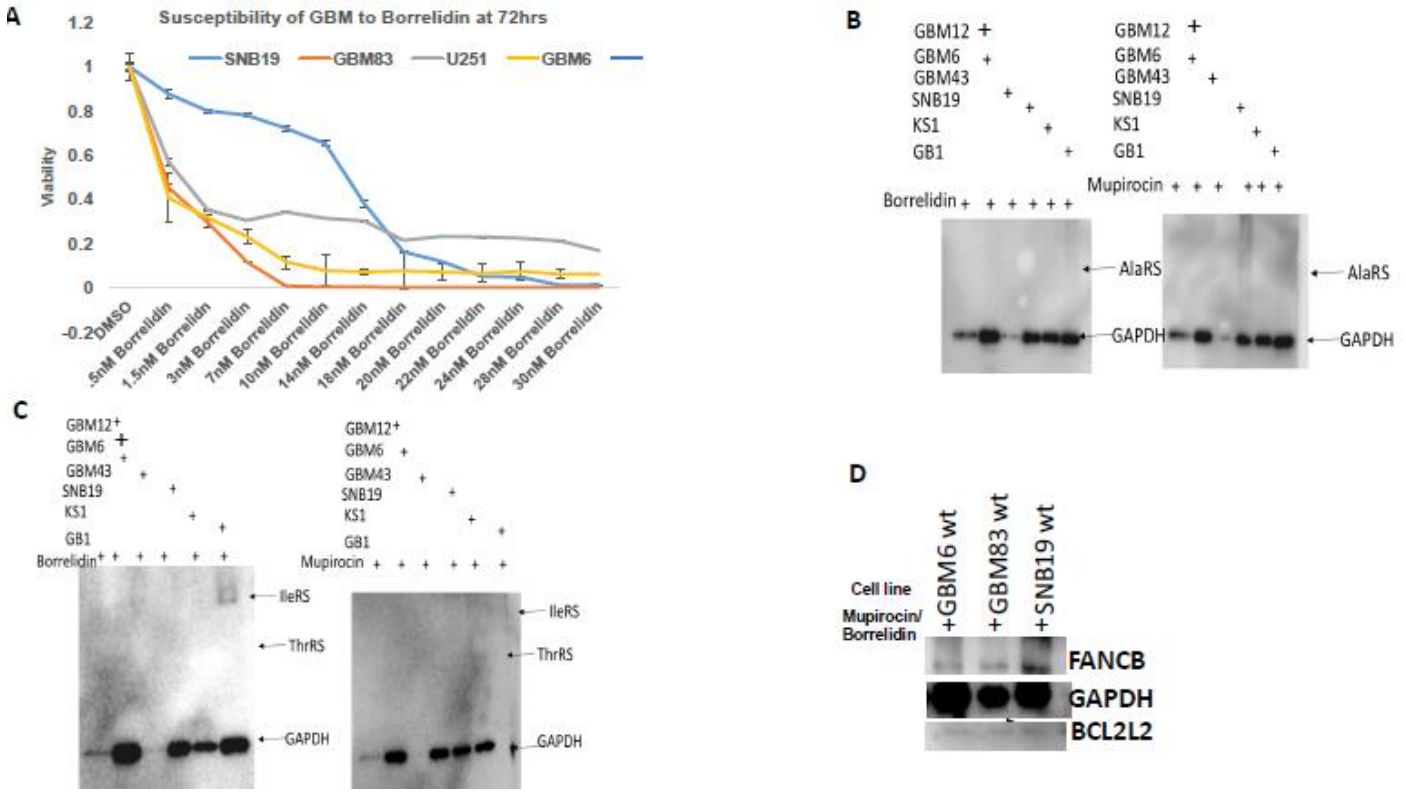

Figure 2. Borrelidin and mupirocin inhibits class II AARS and I and does not impair FANCB and BCL2L2. A. Viability assay shows dose dependent killing of glioma by AARS inhibitor borrelidin for 72 h normalized against DMSO. B. Western blot shows the inhibition of AlaRS by both Borrelidin and Mupirocin across different glioma. C. Western blot shows inhibition of IleRS and ThrRS by Borrelidin and Mupirocin across different glioma. D. Western blot shows expression of FANCB and BCL2L2 on gliomas treated with Mupirocin or Borrelidin.

AARS in glioblastoma is beginning to emerge. Kim et al., (2012), demonstrated through gene expression analysis that AARS were expressed in glioblastoma. We leveraged an unbiased genome-scale CRISPR knockout (Awah et al., 2019) and could show that AARS are indeed, highly expressed in some glioma cell lines and in some glioblastoma patients derived xenografts, with validation by western blot. The results presented here show that AARS plays a key role in glioma malignancy.

AARS have been characterized and broadly classified into classes I and II based on the position of their ATP binding, editing, amino acid, and tRNA A76 active

sites (Carter and Wolfenden, 2015; Fang et al., 2015). Various inhibitors are also classified based on their ability to inhibit each specific sites. Borrelidin and mupirocin are specific inhibitors of AARS. Studies have demonstrated their effectiveness in treating malaria and other cancers (Sugwara et al., 2013; Jeong et al., 2018; Sidhu et al., 2015; Habibi et al., 2012). We repurposed the AARS inhibitor into treating glioma. Using borrelidin, we demonstrated *in-vitro*, that AARS inhibitors are indeed specific and can target AARS without impact on DNA damage and on anti-apoptotic machinery; our observation under the microscope shows cells blebbing and bursting from the nuclei when treated with borrelidin. A key concern for the use of AARS inhibitors is toxicity, it has been demonstrated that this can be alleviated using niacin and tryptophan supplement treatment (Cooperman et al., 1951). AARS inhibition is lethal to cancers (Sugwara et al., 2013; Jeong et al., 2018; Sidhu et al., 2015; Habibi et al., 2012), when compared against cancer resistance to most chemotherapeutics. The AARS inhibitors kill glioma cell lines and patient derived xenografts at sub-nanomolar concentration compared to etoposide, which we demonstrated kills gliomas at micromolar concentration (Awah et al., 2019). Cancers and indeed the human genome, have not been exposed to AARS inhibitors in general, except for mupirocin, which is a skin ointment. Thus, the feasibility of AARS inhibitors being very effective is very plausible.

The fundamental function of AARS is to load amino acids unto tRNA for translation of the genetic code into proteins via the ribosome (Walter, 2017). The over-expression of AARS across cancers points to the fact that cancers usurp translation fidelity to gain evolutionary advantage over normal cells. AARS has extensively been used for genetic code expansion (Normanly et al., 1990; Wang et al., 2001; Hancock et al., 2010). The overexpression of AARS in cancers offers the unique opportunity to use the genetic code expansion technique to encode unnatural amino acids, drugs, and diagnostic compounds into glioma, for the purpose of treating them. This approach can be broadly applied to many cancers that over express AARS, presenting a general platform to address untreatable, undruggable and even chemo resistant cancers. Taken together, our *in-vitro* findings present evidence that AARS inhibitors can be applied to treat gliomas. However, this finding requires further validation *in-vivo* in an animal model to prove the effectiveness of this drug, how much of it can be tolerated and its toxicity in the animal model.

Conclusion

It was concluded that AARS is over-expressed in gliomas and that the inhibition of AARS might be a potential therapy for gliomas. This finding will require further *in-vivo* investigation.

LIMITATION

This study represents preliminary findings and would require validation in mouse models to confirm the therapeutic benefits of AARS inhibitors in a glioma model.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

CRISPR, Clustered regularly interspaced short palindromic repeats; **AARS,** aminoacyl tRNA synthetase; **Mupirocin AlaRS,** alanine tRNA synthetase; **TheRS,** threonine tRNA synthetase; **IleRS,** isoleucine tRNA synthetase; **GAPDH,** glyceradehyde 3-phosphate dehydrogenase; **SEER,** surveillance epidemiology end result programme; **Glioma cell line,** SNB19. KS1, GB1; **Glioblastoma PDX,** MES83, GBM6, GBM43, GBM12.

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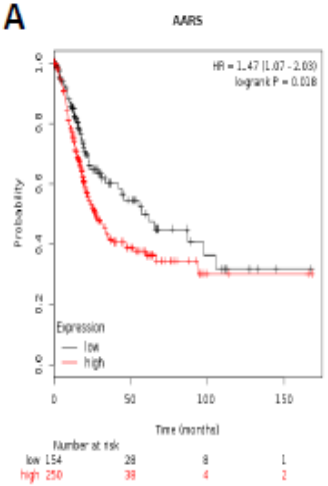
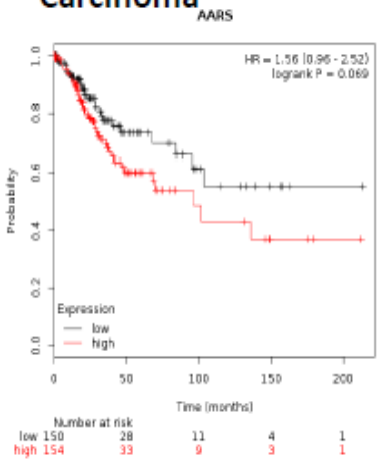
Supplementary Fig 1. High expression of Alanyl tRNA synthetase is a poor prognostic factor for many cancers.

A

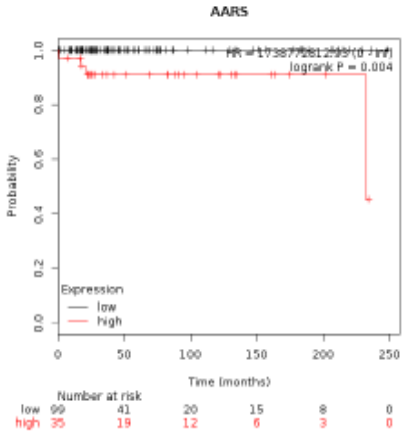
Bladder carcinoma



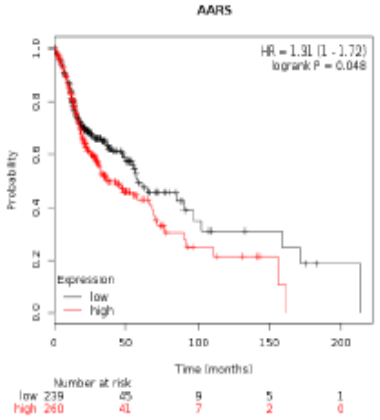
Cervical Squamous cell Carcinoma



Testicular Germ cell tumor

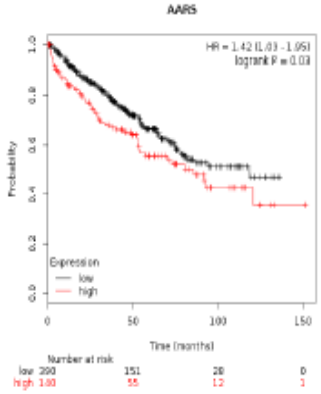


Head and neck Squamous cell Carcinoma

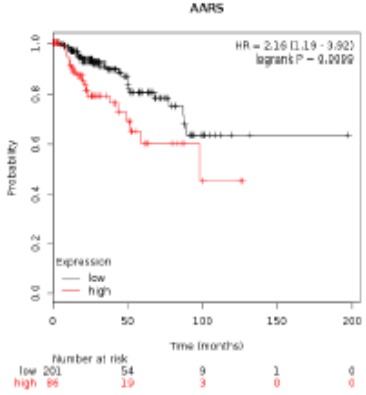


B

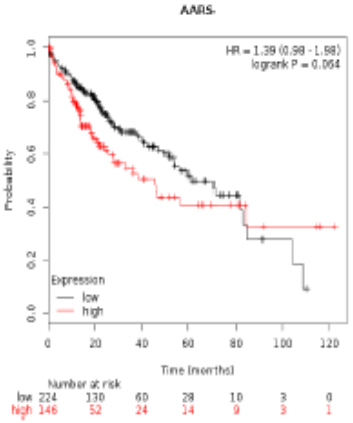
Kidney Renal Clear carcinoma



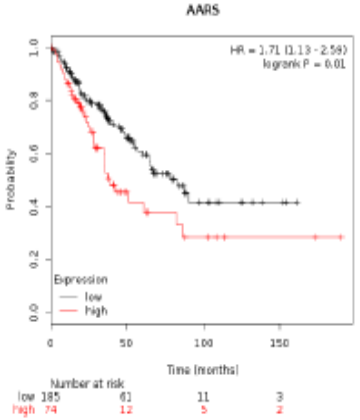
Kidney Renal papillary cell carcinoma



Liver carcinoma



Sarcoma



Uterine carcinoma