STING activation increases the efficiency of temozolomide in PTEN harbouring glioblastoma cells

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Abstract

Background/aim: Glioblastoma is one of the most aggressive tumour resistant to all applied therapy regiments and prone to relapse. Median survival rates are therefore only expressed as months. STING agonists are immunomodulatory molecules activating type I interferon expression; thus, can be used to regulate tumour microenvironment. Since, PTEN is a critical phosphatase in activating interferon regulating transcription factors and frequently mutated in glioblastoma cells, the aim of this study was to investigate STING activation in glioblastoma cell lines either harbouring the PTEN protein or not.

Materials and methods: T98G and U118MG glioblastoma cell lines were treated with the 2'3'-c-di-AM(PS)2(Rp,Rp) STING agonist together with or without the chemotherapeutic agent temozolomide. cGAS/STING pathway components were subsequently analysed by qRT-PCR, Western Blot, and ELISA methods.

Results: Our results showed that PTEN harbouring T98G cells responded well to STING activation along with increased temozolomide efficacy. In contrast, STING activation in U118MG cells did not affect temozolomide response. mRNA expression levels of *STING, IRF3, NF-KB* and *RELA* genes were significantly increased at the combine treatment groups in T98G cell line. Conversely, combined treatment with STING agonist and temozolomide did not affect mRNA expression levels of cGAS/STING pathway genes in U118MG cells.

Conclusion: Our data provides new evidence, that STING agonists can effectively be used to increase temozolomide response in the presence of PTEN protein. Therefore,

increased GBM therapy success rates can be achieved when using the PTEN expression status as a predictive biomarker before treating patients with a chemotherapeutic agent in combination with STING agonist.

Key words: cGAS/STING pathway, 2'3'-c-di-AM(PS)2 (Rp,Rp), STING agonist, PTEN, glioblastoma, temozolomide

1. Introduction

STING is discovered as an endoplasmic reticulum resident protein that facilitates innate immunity activated by viral infections [1]. Identification of cGAS as a cytosolic DNA sensor and cGAMP production as a second messenger provided more clarification for the cGAS/STING pathway in the host immune response [2]. STING can detect the genomic materials or cyclic dinucleotides (CDNs) derived from pathogens as well as self-DNA leaked from host nucleus or mitochondria. STING activation leads to type I interferon response immediately [3]. Besides the important roles in the innate immunity activation, STING-dependent cytosolic DNA sensing has also been related with immunogenicity and therapeutic sensitivity in cancer. STING activation was associated with cytotoxic T cell infiltration and improved PARP inhibitor response [4], enhanced radiation-mediated antitumor immunity [5] or immune checkpoint blockade therapy [6]. With the discovery of CDNs as cGAS/STING pathway agonists, several companies have started to develop activator compounds to benefit from the immunomodulatory functions of STING [7].

Glioblastoma (GBM) is the most frequent and aggressive malignant primary brain tumour in adults with a progression-free survival of 14 months and 5-year overall survival of 9.8% with the current standard-of-care involving surgery followed by radiotherapy and

temozolomide (TMZ), which is a DNA alkylating agent [8]. Although TMZ displays antitumor activity and limited toxicity, its survival benefit remains unsatisfactory and over 50% of the treated patients acquire resistance to TMZ in part due to the (re)expression of a gene called *O6-methylguanine-DNA methyltransferase* [9]. Recurrence of the tumour is an inevitable event in the GBM and most patients acquire it after 6-9 months of primary treatment [10]. Phosphatase and tensin homolog (PTEN) mutations found in 41% of GBM patients and has been linked to TMZ resistance [11-13]. The low expression of PTEN and the high expression of STING were associated with poor prognosis and shortened overall survival of patients diagnosed with tongue squamous cell carcinoma [14]. It was reported that human glioblastoma tumours express STING pathway components i.e. STING, TBK1, and IRF-3 [15]. STING activation triggered immune surveillance and hindered tumour development through vascular disruption in *in vivo* GBM models [16].

GBM is characterized with immunosuppressive microenvironment, therefore development of immunomodulatory compounds to activate immune response more important to increase success rates [17]. PTEN is one of the frequently altered tumour suppressor gene in cancers and associated with immunosuppressive tumour microenvironment [18]. During the antiviral innate immunity, PTEN controls the import of Interferon Regulated Factor 3 (IRF3) transcription factor into the nucleus to trigger interferon production [19]. Furthermore, PTEN and STING proteins are important for regulation of oxidative stress-induced liver inflammation and necroptosis in macrophage cells [20]. Therefore, we hypothesized that STING activation might generate different expression patterns and temozolomide response in cells either harbouring the PTEN protein or not.

2. Materials and methods

2.1. Cell culture

T98G and U118MG cell lines were obtained from American Type Culture Collection (ATCC). T98G cell line carries c.125T>G mutation in the 2nd exon of *PTEN* gene leading to increased mRNA and protein overexpression [21]. However, U118MG cell line carries a frame shift mutation, c.1026+1G>T, therefore lack of functional PTEN protein [22]. Both cell lines were grown in DMEM supplemented with 10% fetal bovine serum (FBS), L-glutamin and penicillin-streptomycin at 37°C under humidified atmosphere with 5% CO_2.

2.2. Cytotoxicity analysis

STING agonist (SA), 2'3'-c-di-AM(PS)2(Rp,Rp), was obtained from Invivogen (#tlrl-nacda2r-01) and dissolved in water at the 50 mg/ml concentration. Temozolomide was purchased from Sigma (#T2577) and dissolved at 50 mM concentration in DMSO. Cytotoxicity analysis were performed using xcelligance real time cell analyser system. Cells were plated at the density of $1x10^4$ T98G cells/well and $7.5x10^3$ U118MG cells/well. After 24h, 2 µg/ml STING agonist or IC_{50} dose of temozolomide were added to the wells [23]. Cell index was analysed for 72h and data was evaluated using instruments software.

2.3. qRT-PCR

All qRT-PCR primers were obtained from Oligomer Biotechnology, and sybr green enzyme were obtained from Biorad. mRNA expression levels of *STING (TMEM173), IRF3, NF-KB (P50),* and *RELA (P65)* genes were analysed using quantitative qRT-PCR. Glyceraldehyde 3-phosphate dehydrogenase *(GADPH)* expression was used as housekeeping gene for normalisation.

2.4. Western Blot

Primary antibodies and dilution concentrations used in the western blot analysis are as follows: Beta actin (Cell Signaling, 1/1000), STING (Cell Signaling, 1/1000), IRF3 (Cell Signaling, 1/1000) and NF-KB (Cell Signaling, 1/1000). Protein lysates were isolated using Complete Lysis-M Buffer (Roche Applied Science) and obtained protein amounts were assessed by the Bradford method. 30 µg of each protein extract was resolved in 8% SDS-PAGE gel and transferred to PVDF membranes. Western blot chromogenic detection kit (Invitrogen) was used to detection of proteins.

2.5. ELISA assay

Human interleukin (IL) 6 and IFNα (Elabscience) kits were used for ELISA analysis. Cells were treated with TMZ or STING agonist or both; after 48h treatment supernatants were collected and used for IL6 and IFNα analysis.

3. Results

IC_{50} concentration of temozolomide was 600 µM and 400 µM for the T98G and U118MG cells, respectively (Figure 1A and 1B). To investigate the effects of STING agonist on the TMZ response, we treated T98G and U118MG cell lines with 2 µg/ml SA, TMZ and both of them, and analysed proliferation for the next 72h. Combine treatment with 600 µM TMZ and 2 µg/ml SA showed more inhibitory effect on the T98G cells proliferation (Figure 1C and 1D). However, there was no significant difference between the combination therapy and temozolomide administration in U118MG cells (Figure 1E and 1F).

Downstream of STING signalling, IRF3 and nuclear factor kappa B subunit 1 (NF-KB, also known as P50) transfection factors work synergistically to activate type I interferons and cytokines [24, 25]. Therefore, we analysed both to investigate whether SA and TMZ upregulates IRF3 or NF-KB induced cytokine production. RELA (also known as P65) binds NF-KB to form the most abundant heterodimer form of NF-KB. Our results showed that *STING, IRF3, NF-KB* and *RELA* mRNA expression levels were significantly increased at the 24h combine treatment groups in T98G cell line (Figure 2A-2D). Western blot analysis also confirmed the elevated STING and NF-KB proteins after combined treatment (Figure 2E-F). When we analysed cell culture supernatants in terms of IFNα and IL-6 expression, did not observe significant change between treatment groups ($p=0.088$ and $p=0.363$; Figure 2G).

TMZ treatment significantly decreased *STING* mRNA expression in U118MG cells (Figure 3A). On the other hand, *IRF3, NF-KB* and *RELA* expressions did not significantly change in all treatment groups (Figure 3B-3D). Western blot analysis showed that U118MG cells express low levels of IRF3 and STING proteins (Figure 3E-F). U118MG cells also showed noticeably low IFNα and IL6 levels comparing to T98G cells, however ELISA assays did not show significant up- or downregulation between the treatment groups ($p=0.072$ and $p=0.085$; Figure 3G).

4. Discussion

In this study we aimed to compare the effects of STING agonist 2'3'-c-di-AM(PS)2 (Rp,Rp) on PTEN harbouring and PTEN deficient glioblastoma cell lines in terms of temozolomide response and cGAS/STING pathway. Several reports indicated that cGAS/STING signalling is frequently supressed in cancers [26, 27]. Colorectal cancer

patients with higher STING expression showed longer overall and recurrence-free survival therefore it was reported that higher STING expression may be an independent prognostic factor for overall survival [28]. STING activation was also revealed as a predictive biomarker in lung cancer to predict immunotherapy response [29].

Native and non-nucleotide agonists of STING are under development as potential agents to increase the efficacy of cancer therapy. [30]. For instance, local delivery of STING agonist with camptothecin provided tumour regression and increased animal survival [31]. IL-15 in combination with the STING agonist (ADU-S100) induced prostate cancer cell death by increasing natural killer cells [32]. Therapeutic efficacy of PARP inhibitors was associated with $CD8^+$ T-cell recruitment via STING pathway activation in triple-negative breast cancer (TNBC) [4]. Similarly, the efficacy of 5-Fluorouracil was associated with anti-tumour immunity triggered by cancer-cell-intrinsic STING activation [33].

ASA404, also known as DMXAA, showed strong effects on subcutaneous brain tumour model however did not exhibit an activity in orthotopic model [34]. Because the signalling strength is important for pro-apoptotic functions of STING, low penetration of ASA404 into the brain may responsible for insufficient effects in the intracranial tumours [35]. Boudreau et al. investigated the intratumoral administration of STING agonist (IACS-8779) to canine glioblastoma and reported well toleration up to 15 µg and higher doses were associated with radiographic responses [36]. Immunostimulatory mesoporous silica nanoparticles (immuno-MSN) carrying cyclic diguanylate monophosphate (cdGMP) and STING agonist were systemically delivered and facilitated circulating $CD8^+$ T cell activity and delayed tumour growth in mouse GBM model [37]. Combination therapy of anti-CD47 antibodies and STING agonists increased the macrophage

polarization to M1-phenotype, reduced tumour immunosuppression, and inhibited the orthotopic GBM growth [38]. These results from glioblastoma models indicate a potential use of STING agonists in enhancing the efficacy of immunotherapy and other treatments by shifting the tumour microenvironment towards to the immune active phenotype. In this study, we combined STING agonist with temozolomide, and compared the treatment response according to the PTEN genotype. Our results showed that PTEN expressing cells better responded to the combination treatment of STING agonist and temozolomide, whereas STING agonist did not change the temozolomide response of PTEN deficient cells.

PTEN is a dual phosphatase that have key functions in several cell regulatory mechanisms and tumour suppression. It was reported that PTEN controls the import of IRF3 transcription factor which responsible for IFN response, into the nucleus [19]. PTEN deficient cancers are associated with an immunosuppressive tumour microenvironment [18]. Molecular determinants of immunotherapeutic response in GBM were reported as specific molecular alterations, immune expression signatures, and immune infiltration that reflect the tumour's clonal evolution during treatment [39]. Different therapy strategies for GBM tried so far have failed to improve survival in randomized clinical trials and the standard of care has remained unchanged over the last decade [9]. Therefore, STING agonists have significant potential for the development of GBM therapy and hold promise for the invention of new treatment combinations in the near future.

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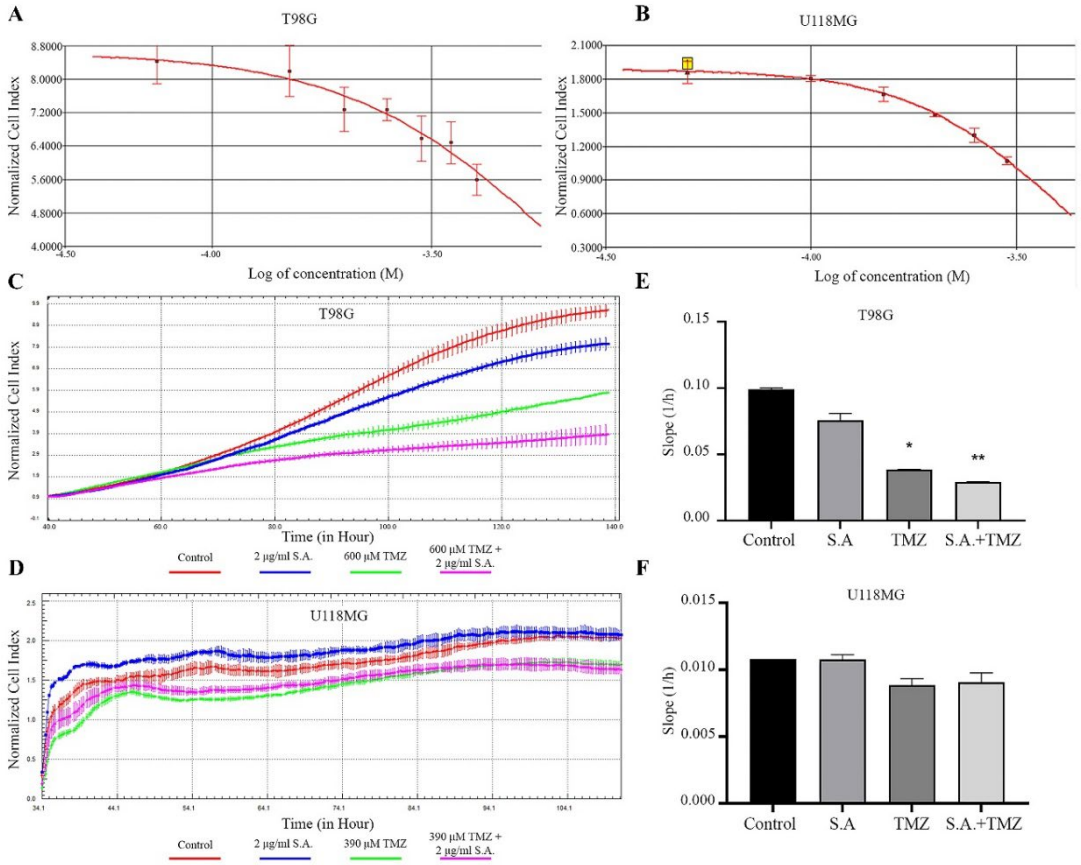
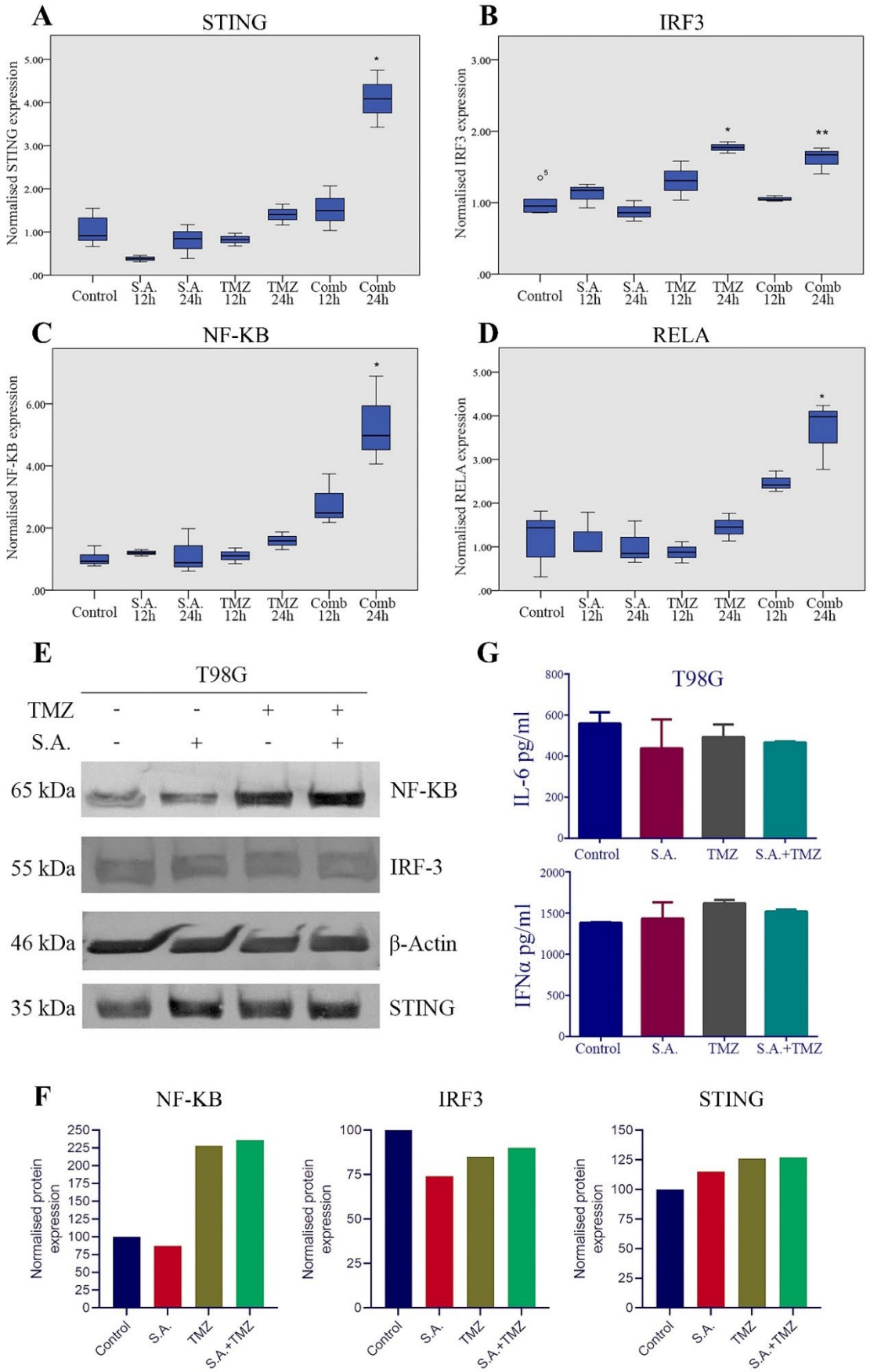
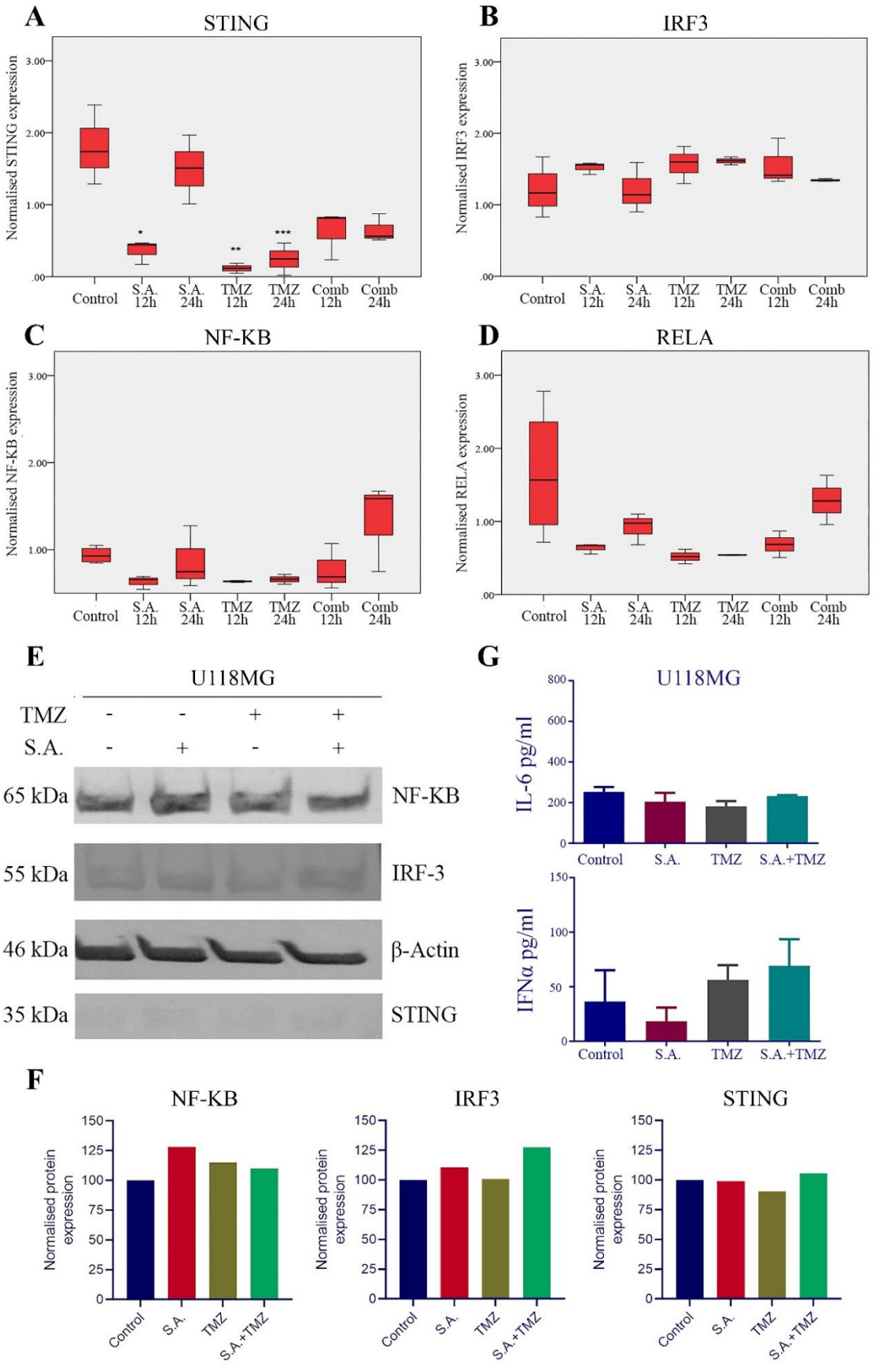


Figure 1: Effects of STING agonist (SA) on temozolomide (TMZ) response of T98G and U118MG cell lines. **A-B:** T98G and U118MG cells were treated with increasing concentrations of TMZ for 72h and IC_{50} levels were calculated using xcelligance software. **C-D:** Cells were treated with the 2 µg/ml SA, TMZ or both (SA + TMZ) and cell indexes analysed for 72 h. **E-F:** Slope values obtained from xcelligance software (*p=0.002; **p=0.004)


A
STING
Normalised STING expression
Control
S.A. 12h
S.A. 24h
TMZ 12h
TMZ 24h
Comb 12h
Comb 24h
B
IRF3
Normalised IRF3 expression
C
NF-KB
Normalised NF-KB expression
D
RELA
Normalised RELA expression
E
T98G
TMZ
S.A.
65 kDa
NF-KB
55 kDa
IRF-3
46 kDa
β-Actin
35 kDa
STING
G
T98G
IL-6 pg/ml
IFNα pg/ml
Control
S.A.
TMZ
S.A.+TMZ
F
NF-KB
IRF3
STING
Normalised protein expression

1

Figure 2: Effects of SA and TMZ treatment on cGAS/STING pathway in the T98G cell line. Normalized mRNA expressions of **A:** *STING* p=0.045 (Control vs Comb24h), p=0.049 (SA12h vs Comb24h), p=0.03 (SA24h vs Comb24h), **B:** *IRF3* *p=0.016 (SA24h vs TMZ24h) **p=0.012 (TMZ24h vs Comb24h), **C:** *NF-KB* p=0.001 (Control, SA12h, SA24h, TMZ12h and TMZ24h vs Comb24h), **D:** *RELA* p=0.001 (Control, SA12h, SA24h, TMZ12h and TMZ24h vs Comb24h); **E:** Western blot results of target proteins; **F:** Relative quantification graphs of western blot results; **G:** IFNα (p=0.088) and IL-6 (p=0.363) expression levels


A
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Normalised STING expression
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TMZ 24h
Comb 12h
Comb 24h
B
IRF3
Normalised IRF3 expression
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NF-KB
Normalised NF-KB expression
D
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Normalised RELA expression
E
U118MG
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S.A. - + - +
65 kDa
NF-KB
55 kDa
IRF-3
46 kDa
β-Actin
35 kDa
STING
G
U118MG
IL-6 pg/ml
800
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400
200
0
Control
S.A.
TMZ
S.A.+TMZ
IFNα pg/ml
150
100
50
F
NF-KB
IRF3
STING
Normalised protein expression
125
75
25

1

Figure 3: Effects of SA and TMZ treatment on cGAS/STING pathway in the U118MG cell line. Normalized mRNA expressions of **A:** *STING* *p=0.011 (Control vs SA12), **p=0.003 (Control vs TMZ), ***p=0.006 (Control vs TMZ24h), **B:** *IRF3*, **C:** *NF-KB*, **D:** *RELA*, **E:** Western blot results of target proteins; **F:** Relative quantification graphs of western blot results; **G:** IFNα (p=0.072) and IL-6 (p=0.085) expression levels