Diallyl Trisulfide as a Radiosensitizer for Glioblastoma Multiforme

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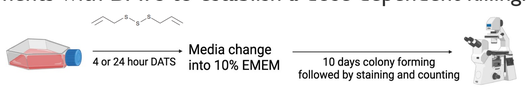
Introduction

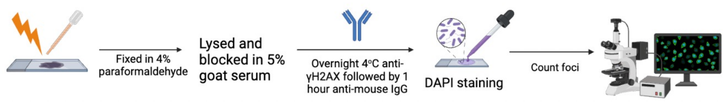
- Glioblastoma multiforme (GBM) is an extremely aggressive CNS cancer.
- Despite TMZ + radiation treatment, GBM results in death within 15-21 months of diagnosis. These poor statistics suggest the need for a novel therapy.
- The use of a GBM radio-sensitizing agent is a novel approach to minimizing radiation-mediated healthy tissue necrosis, while allowing use of lower radiation doses to achieve comparable GBM cytotoxic effects.
- Our previous research has shown that GBM cells treated with sodium sulfide (Na_2S), a hydrogen sulfide (H_2S) donor, along with photon or proton radiation, enhances cell killing while sparing normal human cerebral microvascular endothelial cells (Xiao, 2019).
- We are currently exploring Diallyl Trisulfide (DATS), another H_2S donor, as a radio-sensitizing agent.

Methods

1. Human T98G glioblastoma multiforme cells were cultured in EMEM medium supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2.
2. T98G clonogenic survival studies were performed after 4- and 24-hour treatments with DATS to establish a dose dependent killing.

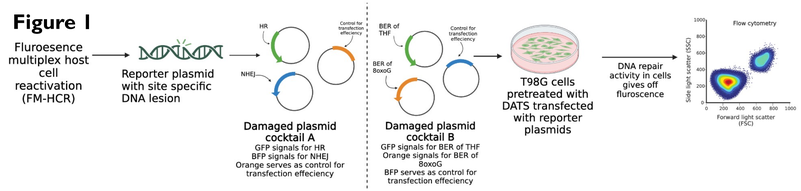


3. To determine if DATS increases DNA damage mediated cell death, T98G cells were treated ± DATS, radiation or DATS + radiation and then analyzed for γH2AX immunostaining, an indicator of DNA double stand breaks (DSB).



4. To elucidate the mechanism of increased γH2AX foci, a novel multiplex host cell reactivation (FM-HCR) reporter system (Piett, 2021) was used to assess repair activity of DSBs. T98G cells were pre-treated with DATS and transfected with reporter plasmids containing a site specific DSB, mimicking radiation-like DNA damage. Repair results in expression of a fluorescent reporter protein, which was quantitated using flow cytometry.

Figure 1



Results

T98G Clonogenic Survival Study

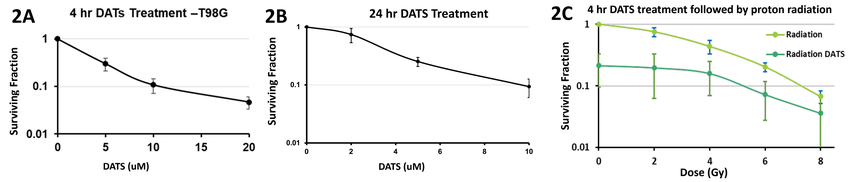
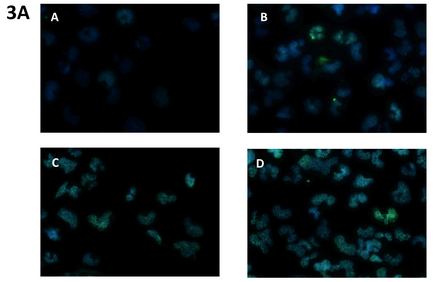


Figure 2- T98G cells were cultured in EMEM plus 10% FBS at 37°C and 5% CO_2 and allowed growth for 3 days. On day 4, cells were re-seeded in T25 flasks and treated with different doses of DATS for 4- (**2A**) or 24-hour (**2B**) time points. For radiation survival studies (**2C**), T98G cells were seeded at a standard density in T25 flasks and treated with 5uM DATS for 4 hours. Cells were then irradiated with photons and allowed 20 hours incubation for cell damage and cell death to occur. Following the treatments, cells were media changed back into normal 10% EMEM and allowed 10 days to form colonies. Colonies were stained with crystal violet and counted. >50 cells was considered a colony. Results show average of N=3 and standard deviation.

γH2AX Immunohistochemistry Staining



A- no treatment, **B-** 5uM DATS, **C-** 4 Gy radiation, **D-** 5uM DATS + 4 Gy radiation

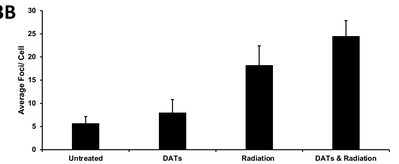
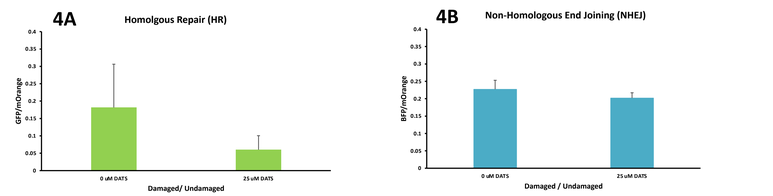


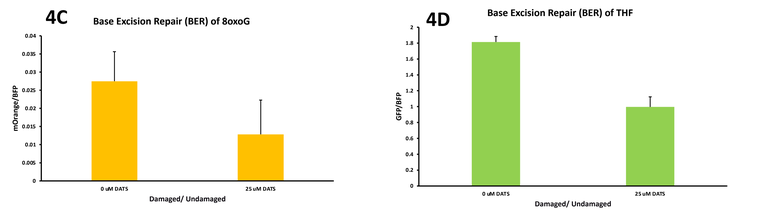
Figure 3- γH2AX foci were stained using immunohistochemistry. Briefly, T98G cells were cultured on glass coverslips and grown to confluency. Following treatment with DATs ± IR, cells were fixed in 4% paraformaldehyde. Cells were then lysed and blocked with 5% normal goat serum. Cells were incubated overnight at 4 °C in anti-γH2AX antibody, stained with anti-mouse IgG Alexa Fluor 488 secondary antibody for 1 hour, and finally DAPI stained. Images were taken using an Olympus BX43 microscope **(3A)**. Foci were counted using JQuantPlus and an average foci number per experiment calculated. An average from three experiments is shown +/- standard deviation **(3B)**.

DNA Damage Repair Assays

Figure 4- T98G (800,000 cells) were seeded in a T150 flasks and allowed 2 days to grow to 65-70% confluency. Cells were then pretreated with either DMF control or 25uM DATS for 4 hours. 1.2 million cells were used for each transfection. Cells pretreated with control DMF were transfected with either mock carrier DNA, single color controls (BFP, GFP, mOrange), or undamaged and damaged reporter plasmids. Cells pretreated with 25uM DATS were transfected with only undamaged or damaged reporter plasmids. Transfections were done using program O16 and the AMAXA nucleofector. Duplicate transfections for the damaged and undamaged reporter plasmids for each treatment were performed in each experiment. Experiments were performed for plasmid cocktail A and plasmid cocktail B. After the transfection, cells were transferred onto dishes and retreated with respective control DMF or 5uM DATS. Cells were allowed 20 hours for repair and reporter expression at 37°C and 5% CO_2, and then analyzed for fluorescence using flow cytometry. Results show average of N=3 and standard deviation.



Undamaged and damaged versions of reporter plasmids were developed. Damaged cocktail plasmid A (Figure 1) was engineered to contain a site-specific double strand break that can be repaired via HR or NHEJ pathways in vitro. % reporter expression for HR and NHEJ positively correlates with DSB repair. When the DSB is repaired, the coding region (coding for GFP or BFP) of the reporter is restored, fluorescence is emitted, and repair can be quantified using flow cytometry. ***GFP is produced with increased HR activity and BFP is produced with increased NHEJ activity.*** Expression was normalized to mOrange which served as a control for transfection efficiency. Damaged reporter plasmid fluorescence was calculated normalized to undamaged **(4A & 4B)**.



Similarly, undamaged and damaged versions of reporter plasmid B were developed. Damaged cocktail plasmid B (Figure 1) was engineered containing site specific DNA lesions (THF & 8oxoG) that can be repaired via the BER pathway in vitro. THF acts as an abasic lesion and 8oxoG is a common ROS mediated nucleotide mutation, both of which are repaired by the BER pathway. ***GFP expression positively correlates with increased repair of the THF abasic lesion***. On the contrary, mOrange expression inversely correlated with repair of the oxidized nucleotide. 8oxoG is located on the transcribed strand; when left unrepaired, A is incorporated and codes for mOrange fluorescence protein. ***mOrange expression correlates with decreased repair of 8oxoG lesion.*** Expression was normalized to BFP which served as a control for transfection efficiency. Damaged reporter plasmid fluorescence was calculated normalized to undamaged **(4C & 4D)**.

Key Takeaways

- At 4- and 24-hour treatment time points, T98G cell survival was similarly reduced with increasing DATS concentrations. 5uM DATS was able to achieve ~40% cell killing, similar to % killing achieved by 476uM Na_2S used in our previous studies.
- Clinically relevant dose of photon radiation is 2 Gy. Compared to no treatment in T98G cells, 2 Gy radiation alone decreased cell survival ~10%. 2 Gy radiation + 5uM DATS reduced cell survival ~70%.
- Compared to no treatment, 5uM DATS increased the number of γH2AX foci per cell by 1.5-fold, by 3-fold with 4 Gy radiation, and by 4-fold with combined DATS + radiation, an additive response.
- Results suggest that DATS does not significantly alter non-homologous end joining (NHEJ) activity but may decrease homologous repair (HR) activity.
- Results suggest that DATS may decrease base excision repair (BER) of THF lesions but increase BER of 8oxoG lesions.

Future Studies

- Treat T98G cells with 5uM and 25uM DATS for 4- and 24-hours time points each to collect for RNA and protein.
- Perform RT PCR on RNA collected from DATS treated T98G cells to identify changes in expression at the transcriptional level of genes involved in DSB repair and BER.
- Perform western blots on protein collected from DATS treated with T98G cells to analyze altered expression at the translational level of proteins involved in DSB repair such as RAD51, Ku70-80, DNA-PKcs and protein involved in BER such as OGG1 and APE 1.
- Perform protein activity assays to understand if DATS has effects on the on vs off state of proteins involved in DSB and BER of DNA.
- Repeat clonogenic survival studies in human cerebral endothelial cells (hCMEC/D3) to confirm normal brain tissue sparing with DATS treatment, as was seen in previous studies with Na_2S.

References

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2. Piett CG, Pecen TJ, Laverty DJ, Nagel ZD. Large-scale preparation of fluorescence multiplex host cell reactivation (FM-HCR) reporters. Nat Protoc. 2021 Sep;16(9):4265-4298. doi: 10.1038/s41596-021-00577-3. Epub 2021 Aug 6. PMID: 34363069; PMCID: PMC9272811

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