WHIMELANOMA CHEMOTHERAPY TREATMENT USING MODIFIED CELLULOSE-BASED INJECTABLE HYDROGEL COMBINED WITH TEMOZOLOMIDE

by

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ABSTRACT

MELANOMA CHEMOTHERAPY TREATMENT USING MODIFIED CELLULOSE-BASED INJECTABLE HYDROGEL COMBINED WITH TEMOZOLOMIDE

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A local delivery method of an injectable modified cellulose nanofiber hydrogel, DNCNF PAA 10%, loaded with the chemotherapy drug temozolomide, TMZ, was studied to investigate the most effective and safest therapy window on duke melanoma 6 (DM6) and human dermal fibroblast cells (HDF-α). Loaded hydrogel concentrations of 25μM-1000μM were administered inside PDMS microcurrent device for 72 hours, removed, cells stained, and quantified. For the long-term study, treatment was administered for 72 hours, treatment removed, and cells recovered for 72 hours. The data was quantified using a fluorescent live/dead cell assay and cells counted. There was a cell death dosage effect seen after the 250μM TMZ hydrogel treatment as the concentrations increased. The 500μM TMZ hydrogel had ~7-14% DM6 viability with ~50% HDF-α viability. Dosages higher than the 500μM TMZ hydrogel treatment and free 500μM TMZ had nonspecific killing of

both cell types. The long-term melanoma recovery study showed surviving melanoma have possible TMZ resistance. The most effective and safest therapy window was DNCNF PAA 10% 500μM-750μM TMZ. Currently, the treatment therapy window found is being studied further involving co-culturing of both cell types. Future studies recommended are TMZ resistance studies and in vivo animal studies.

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CHAPTER 1

INTRODUCTION

1.1 Background

Melanoma is the most lethal and mutagenic form of skin cancer with the highest mutation rate of any cancer (Davis et al., 2019). It is caused by UV radiation damaging melanocyte DNA and can have genetic origins. Despite affecting only 4% of people with skin cancer, melanoma is accountable for about 75% of all skin cancer deaths. When diagnosed correctly and treated early, patients have high survival rates with surgery alone, but once metastasis starts, the chances of survival drop dramatically (Davis et al., 2019). Other than surgery, few treatments have increased the survival or response rate for people affected by melanoma (Wolf, 2021). Overall, the high mutation rates of melanoma cause it to be the leading cause of skin cancer deaths.

Some ways to treat metastatic melanoma include radiation, chemotherapy, immunotherapy, and targeted therapy (Liu et al., 2016). Unfortunately, these treatments have low survival rates, are cytotoxic, and have high tumor recurrence, likely due to the mutagenic nature of melanoma that causes acquired resistance to many treatments (Liu et al., 2016). Chemotherapy remains the standard treatment of late-stage cancers and nonresectable tumors. (Gogas et al., 2019, Wolf, 2021). For melanoma, dacarbazine is the gold-standard chemotherapy treatment while temozolomide is a promising alternative (Wolf, 2021, Liu et al., 2016). However, intravenous methods of chemotherapy drugs kill non-specifically, causing severe systemic side-effects in patients (Liu et al., 2016, Wolf,

2021). Developing new localized therapies for melanoma patients is vital for increasing survival rates (Davis et al., 2019). Injectable hydrogels provide a compelling, innovative alternative to intravenous therapies. They have better drug solubility and bioavailability, high stability, sustained drug release, and can be locally delivered to minimize healthy tissue damage while increasing the anticancer effect to chemo-resistant melanoma (Capanema et al., 2018, Wolf, 2021, Tan et al., 2020).

1.1.1 Research Objective

To address these issues and improve the specific targeting of temozolomide (TMZ) on metastatic melanoma, a local delivery method of an injectable modified cellulose nanofiber hydrogel (DNCNF PAA 10%) with polyacrylic acid (PAA) 10% was studied to investigate the most effective and safest dosage therapy window. Our hypothesis was that the injectable hydrogel would stabilize and prolong the anticancer effect of TMZ for specific killing of melanoma cells. The hydrogel treatment was modified to give a positive charge and is antimicrobial, while the PAA 10% modification was used to create an acidic environment to stabilize the TMZ, providing sustained drug stability (Wolf, 2021). The cellulose nanofibers (CNF) also have a physical barrier property, a hydrophobic barrier, that can decrease cancer proliferation and maintains stability of the TMZ pro-drug form until released into a more neutral physiological pH (Pandey, 2021, Wolf 2021). The slow release of TMZ is expected to allow the healthy cells to recover and respond more favorably than a bulk delivery of free-form TMZ (Wolf, 2021).

1.2 Overview of Current Treatments

According to Davis et al. (2019), there are currently many other forms of approved melanoma treatments such as surgery, radiation, immunotherapy, targeted therapy, and

chemotherapy. Traditional treatments for resectable tumors involve surgical removal of the affected area and wide local excision to avoid regrowth commonly combined with radiation and chemotherapy (Davis et al., 2019). As Liu et al. (2016) mentions, current research of several chemotherapy drugs, including paclitaxel, cisplatin, doxorubicin, dacarbazine, and temozolomide, are used in treating later stages of melanoma. These drugs used in free form have been proven to kill cancer cells, but they are also cytotoxic to healthy cells (Liu et al., 2016). Wolf (2021) explains this is because free-form drugs degrade at a high rate and have off-site targeting, requiring higher dosages of the drugs to work as effectively as possible.

1.2.1 Chemotherapy

Traditional melanoma treatment involves surgical removal commonly combined with radiation or chemotherapy (Davis et al., 2019). Current research of several chemotherapy drugs is used in treating later stages of melanoma (Liu et al., 2016). Overall, these intravenous drugs are effective at killing cancer while also negatively affecting healthy tissue and if any cancer remains after treatment, they quickly acquire high drug resistance. Dacarbazine, the gold standard for melanoma chemotherapy, and TMZ, are the most common melanoma anticancer drugs used with response rates less than 20% from the acquired drug resistance of melanoma and toxic side effects (Trinh et al., 2009). Currently, all other drugs are compared to dacarbazine's effectiveness and side effects since it is FDA-approved and has the greatest survival rates among other drugs for melanoma (Wolf, 2021).

1.2.1.1 Temozolomide

The chemotherapy drug TMZ, an analog to dacarbazine, is used for the treatment of metastatic melanoma and glioblastomas (Wolf, 2021). TMZ is pH-sensitive, degrading at physiological pH (which reduces toxicity), often used orally with a more favorable side

effect profile when compared to dacarbazine, that works by depleting the DNA repair gene, MGMT, in tumors (Wolf, 2021, Trinh et al., 2009). However, TMZ is similar in response rate and survival rate to dacarbazine (Wolf, 2021) with common gastrointestinal side effects and myelosuppression (Trinh et al., 2009). Despite free-form TMZ's limited efficacy due to drug resistance and degradation, Fourniols et al. (2015) successfully created a polyethylene glycol dimethacrylate (PEG-DMA) injectable hydrogel that provided a sustained drug release and stabilized local delivery of TMZ administered to glioblastoma tumors in mice.

1.2.2 Hydrogels and Cellulose Nanofibers: An Alternative Delivery Method

There is a need for better drug delivery systems that buffer the cytotoxicity of free-form drugs and provide local delivery (Liu et al., 2016). According to Tan et al. (2020), injectable hydrogels have become popular in their usage for tumor treatments because of their modification ability, local delivery, and ability to stabilize drugs with less residual tumor cell metastasis.

Cellulose nanofibers have also risen in popularity for cancer treatments because of their mechanical strength, high specific surface area, reactive surface for modifications, biodegradability, and 90% drug entrapment efficiency (Pandey, 2021). CNF can be modified to work with different drugs and mimics the structure of cellular proteins which can restrict movement of cancer cells due to the physical barrier property. For example, Alizadeh et al. (2018) made an injectable CNF hydrogel with doxorubicin to combat melanoma, showing a greater anticancer effect than the free drug while confirming that CNF decreases cancerous growth.

CHAPTER 2

METHODS AND MATERIALS

2.1 Materials

Extra lab materials needed for these experiments were: polydimethylsiloxane (PDMS), PDMS curing agent, RPMI 1640 at 1X cell culture media supplemented with 10% FBS and penicillin-streptomycin (pen-strep) at 1X , image media, Duke Melanoma 6 cell line (DM6), Human Dermal Fibroblast-α cell line (HDF-α), temozolomide (TMZ), DNCNF PAA 10% hydrogel given by Dr. Seo's lab in the Republic of Korea, paraformaldehyde, 4′6-diamidino-2-phenylindole (DAPI), propidium iodide (PI), and Hoechst 33342 (Hoechst).

2.1.1 Cell Culture and Reagents

The DM6 and HDF-α cells were cultured in RPMI 1640 cell culture medium supplemented with FBS 10% and pen-strep during the experiments at 37℃ in a humidified incubator of 5% CO_2.

2.1.2 PDMS Device Manufacturing

The PDMS device used holds the DNCNF PAA 10% treatments in place so that the hydrogel remains in contact with the cells and contains channels that allow medium to flow throughout. The PDMS and curing agent were mixed and then vacuumed. Then, the mixture was poured into a pre-made silicon wafer mold that has microchannels, ~100 microns in height, and was cured by heat. The PDMS device was then cut to fit inside a

standard tissue-cultured 12 well plate with a 6mm hole punched in the middle of the microchannels. The devices were sterilized and washed. The dried devices were then placed inside the Novascan UV Ozone System to create charge for better adherence. Finally, PDMS devices were assembled in the 12 well plate channel down (Figure 2.1).

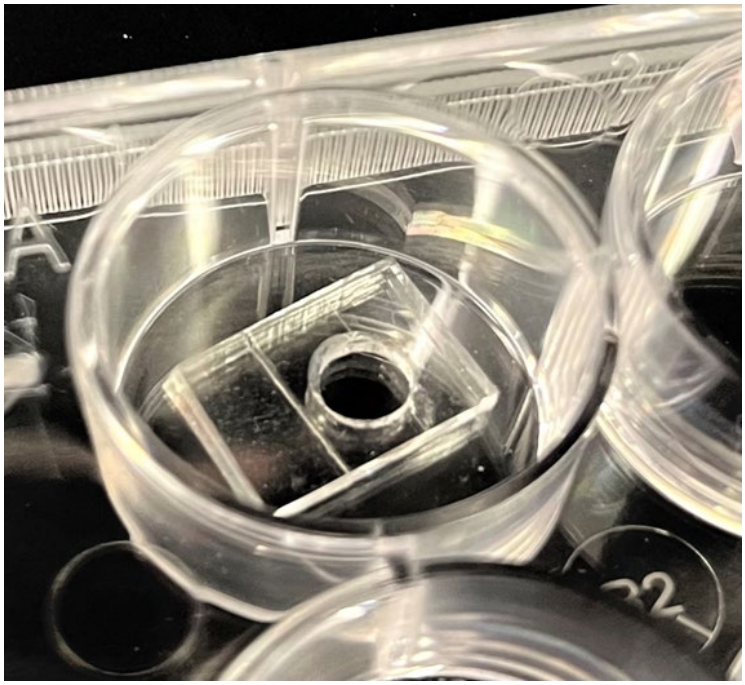

Figure 2.1: PDMS Device with microchannels face down inside 12 well plate

2.2 Cell Counting

The DNCNF PAA 10% hydrogel was prepared by removing the supernatant and replacing with HEPEs buffer. The stock solution of TMZ (10mM) in DMSO and DNCNF PAA 10% was mixed and diluted to get working TMZ concentrations of 25uM, 50µM, 100µM, 250µM, 500µM, 750µM, 1000µM respectively. The stock TMZ and 10% FBS cell culture medium was diluted to create the free-form TMZ working drug concentrations of 100µM and 500µM.

Prior to administration of treatment, the subcultured cells (5,000 cells/well) were seeded inside the PDMS device in each well with RPMI 1640 cell culture media FBS 10%.

The cells were placed in the incubator to attach for 24 hours. The plates were then imaged with brightfield microscopy at 2.5x magnification for confirmation of cell health and placed back in incubator. Day 0, 50µL of DNCNF PAA 10% TMZ working concentrations were introduced in the middle of the PDMS devices on top of the cells and placed in the incubator for 72 hours. The conditions were compared to several controls: free form 100µM and 500µM TMZ, DNCNF PAA 10% alone, and an undisturbed control cell culture.

Day 3, fresh image media was mixed with 2.5uL of Hoechst and 4uL of PI per 10mL of image media (PIH). One at a time, each plate was taken out of the incubator to carefully remove all the old media and various conditions from the PDMS device and well. Then, 1mL of the PIH was added to each well. After 30 minutes, PIH was replaced with fresh image media. Each well was immediately imaged using 10x magnification of 4x4 images (16 images per well) for stitching, while taking pictures of live cells (blue) stained with Hoechst using the DAPI filter first. Then, pictures of dead cells (red) stained with PI using PI filter second. Brightfield images at 10x magnification were taken of each well to observe the health of the cells in each condition and were repeated for each plate.

After imaging, ImageJ software was used to merge the DAPI images with the PI images. Then, the average, minimum, and maximum cell size was measured for each experiment using threshold to count/analyze the cells. The cell count was then put into Excel for tracking the cell counts of each image that were averaged per well condition and graphs generated for percentages of cell counts. Each well that had 16 images was then stitched by FIJI to show the overall image of the cells and the color adjusted to show blue and red.

2.3 Statistics

After looking at the stitched images for each experiment, images were not used if there was obvious human interference, such as pipette tip interference or hydrogel blockage. Once cell counting was finished, average cell size was used to quantify the cells in each image. A statistical outlier test using the interquartile range (IQR) method was then used to eliminate outliers. Graphs were generated with Excel from the finished data analysis. QQ plots were made using R to judge the normality of the data, and ANOVA analysis was performed with a p-value of $p<0.05$. Tukey-post hoc analysis in R was used to test the data set against statistical significance using $p<0.05$. From there, the graphs were normalized to DNCNF PAA 10% and the Control condition.

2.4 Long-term DM6 Cell Recovery Method

The beginning methods of the long-term cell recovery study were all the same as the cell counting section of the methods and materials, but after 72 hours, brightfield images were taken at 2.5x magnification and then allowed another 72 hours (without treatment) in the incubator with fresh cell culture media 10% FBS to study cell recovery. The well plate was then fixed with paraformaldehyde for 5min, stained with DAPI, and imaged at 2.5x magnification using brightfield and DAPI filter of their last state. Using ImageJ, the brightness of the images was adjusted.

CHAPTER 3

RESULTS

This section describes the results of the therapy window experiments for the DM6 and HDF-α monocultures as well as the implications from the long-term regrowth study.

3.1 DM6 Cell Viability of Low and High Dosage Ranges

This first study of the treatment after 72 hours shows the DM6 cell viability pictures (Figure 3.2) and normalized graph (Figure 3.1) of the low and high range TMZ concentrations compared to the undisturbed control, DNCNF PAA 10% alone, and free 100μM TMZ. Free 100μM TMZ was chosen based on Wolf (2021)'s research showing a plateau effect at the treatment DNCNF PAA 10% 100μM TMZ dosage and used as a comparison and control. The free 100μM TMZ had DM6 60% cell viability while the DNCNF PAA 10% alone had 39% cell viability compared to the control. The DNCNF PAA 10% 25μM, 50μM, and 100μM TMZ treatments had about the same cell viability around 40%, 35%, and 34% respectively, similar to DNCNF PAA 10% alone. The DNCNF PAA 10% 250μM and 500μM TMZ showed significantly lower viability than DNCNF PAA 10% alone with the former at 26% viability and the latter at 7% viability. The control, DNCNF PAA 10% alone, and free 100μM TMZ had the most variability due to clustering of the DM6 cells and/or DNCNF PAA 10% blockage. The DNCNF PAA 10% 500μM TMZ had the largest anticancer effect out of all the other dosages, which prompted another study to see the effects of a higher range of DNCNF PAA 10% TMZ dosages. Results could differ slightly from each study due to possible seeding differences and human error.

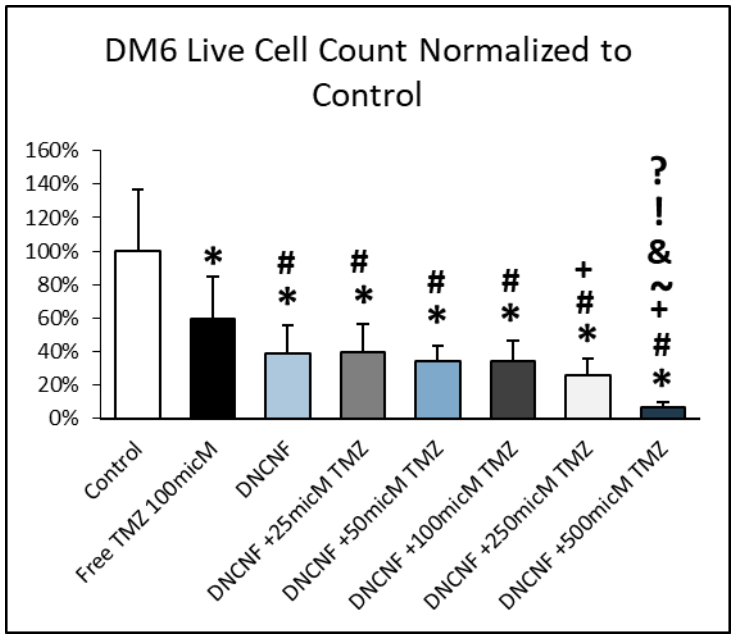

Figure 3.1: DM6 Cell Counting Viability Assay of low and high dosage ranges with TMZ and DNCNF PAA 10% after 72 hours of treatment graphical representation.

In Figure 3.1, it shows the graphical representation of cell viability percentage compared to each condition with cells counted from individual pictures by ImageJ and live cells averaged per image and normalized to Control. Significant difference was determined at a p-value of 0.05. The symbols to the right of the control indicate significant difference respectively. The symbol "*" indicates significant difference from Control; "#" indicates significant difference from Free 100μM TMZ; "+" indicates significant difference from DNCNF PAA 10%; "~" indicates significant difference from DNCNF PAA 10% 25μM TMZ; "&" indicates significant difference from DNCNF PAA 10% 50μM TMZ; "!" indicates significant difference from DNCNF PAA 10% 100μM TMZ; and "?" indicates significant difference from DNCNF PAA 10% 250μM TMZ.

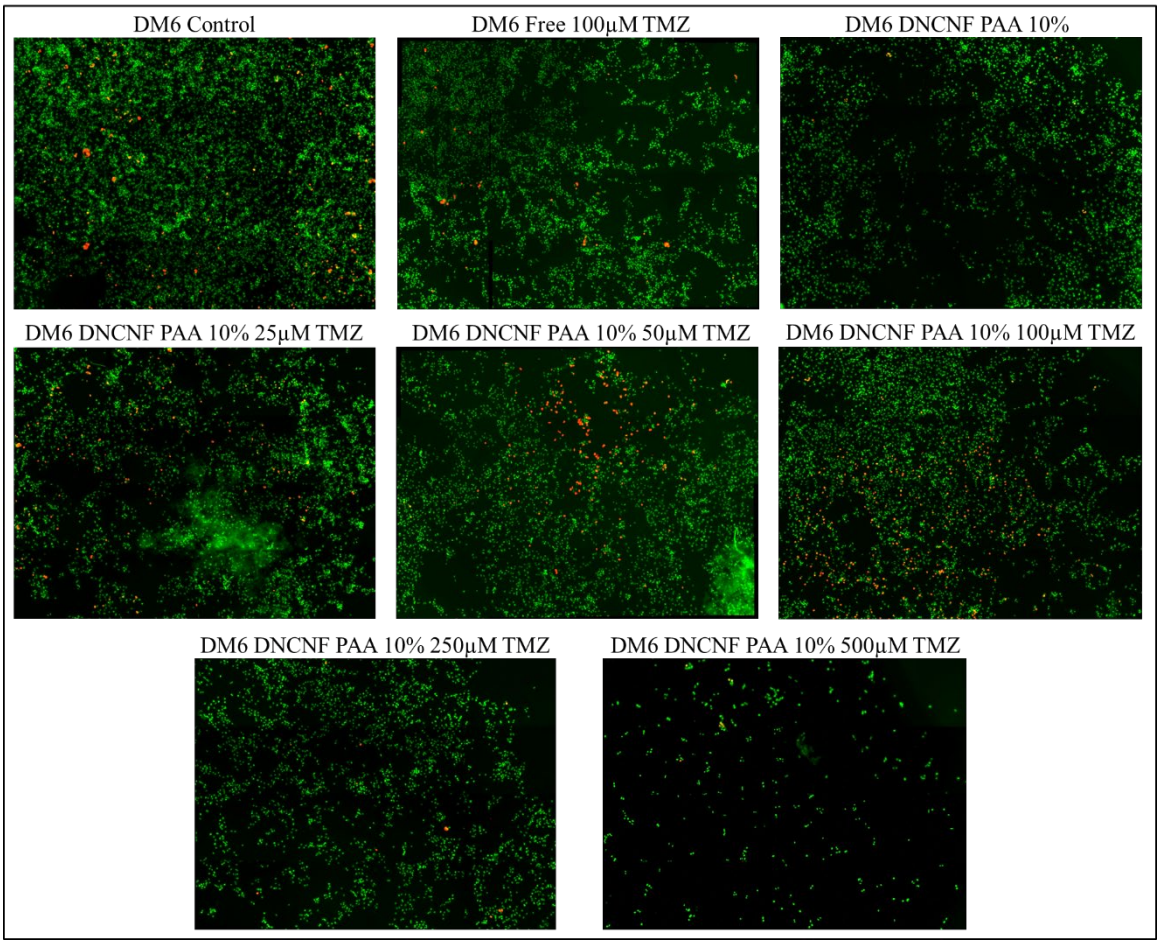

Figure 3.2: DM6 Cell Counting Viability Assay of low and high dosage ranges with TMZ and DNCNF PAA 10% after 72 hours of treatment image representation.

In Figure 3.2, it shows the stitched image representation of each well condition and controls, created by FIJI with 4 images by 4 images taken at 10x magnification of DM6 cells stained with PI (red, dead cells) and Hoechst (manipulated to be green, alive cells) for 30 minutes and replaced with fresh image media.

3.2 DM6 Cell Viability of Expanded Dosage Ranges

This second study of the treatment after 72 hours shows the DM6 cell viability pictures (Figure 3.4) and normalized graph (Figure 3.3) of the expanded high range TMZ concentrations compared to the undisturbed control, DNCNF PAA 10% alone, and free 500µM TMZ. Free 500µM TMZ was chosen since the first study of DM6 showed

significant cell death using the 500µM dosage in the hydrogel. The DNCNF PAA 10% alone had a cell viability around 53% compared to the control. The free 500µM TMZ had significantly lower viability than control and DNCNF PAA 10% alone with about 1% cell viability, but the DNCNF PAA 10% 500µM, 750µM, and 1000µM TMZ treatments were also all significantly lower viability than DNCNF PAA 10% but similar to free 500µM TMZ with significant cell death at about 14%, 3%, and 1% of cell viability respectively compared to the control. The cell death for the DNCNF PAA 10% 500µM TMZ condition in the second study is similar to the first DM6 study of low and high range dosages and within the variability range. This is also true for DNCNF PAA 10% alone. There is a clear dosage effect seen from DNCNF PAA 10% 500µM TMZ to 1000µM TMZ. The control and DNCNF PAA 10% had the most variability due to pipette disturbance of the DM6 cells and DNCNF PAA 10% blockage.

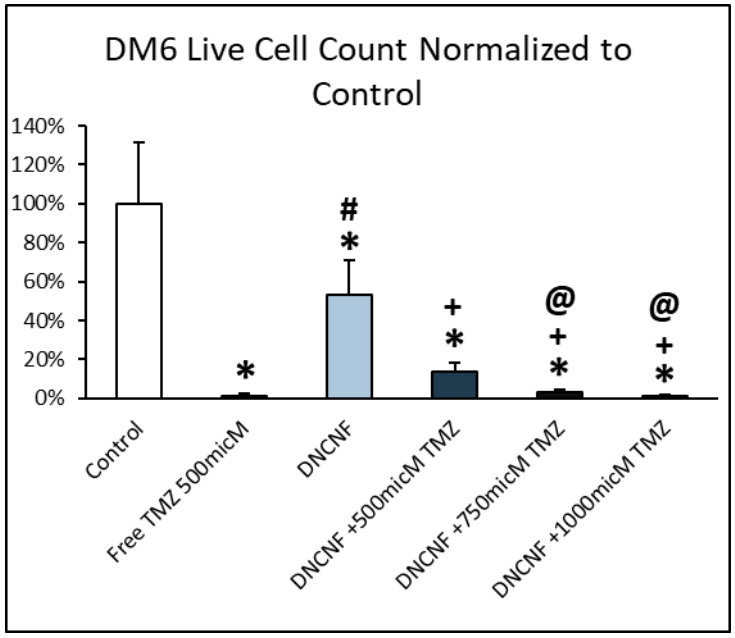

Figure 3.3: DM6 Cell Counting Viability Assay of expanded dosage ranges with TMZ and DNCNF PAA 10% after 72 hours of treatment graphical representation.

In Figure 3.3, it shows the graphical representation of cell viability percentage compared to each condition with cells counted from individual pictures by ImageJ and live cells averaged per image and normalized to Control. Significant difference was determined at a p-value of 0.05. The symbols to the right of the control indicate significant difference respectively. The symbol "*" indicates significant difference from Control; "#" indicates significant difference from Free 500μM TMZ; "+" indicates significant difference from DNCNF PAA 10%; "@" indicates significant difference from DNCNF PAA 10% 500μM TMZ.

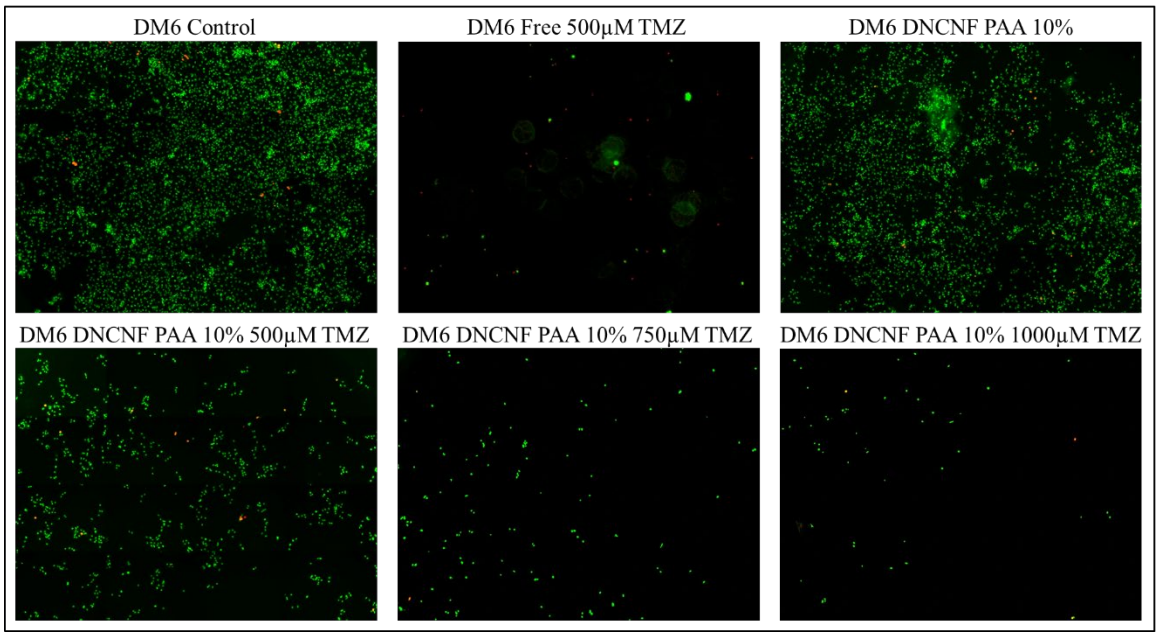

Figure 3.4: DM6 Cell Counting Viability Assay of expanded dosage ranges with TMZ and DNCNF PAA 10% after 72 hours of treatment image representation.

In Figure 3.4, it shows the stitched image representation of each well condition and controls, created by FIJI with 4 images by 4 images taken at 10x magnification of DM6 cells stained with PI (red, dead cells) and Hoechst (manipulated to be green, alive cells) for 30 minutes and replaced with fresh image media. Image 2 of DM6 Free 500μM TMZ is one image taken at 2.5x magnification using ImageJ for brightness/contrast manipulation (the cells may appear slightly larger than the other images).

3.3 HDF-α Cell Viability of Low and High Dosage Ranges

This first HDF-α study of the treatment after 72 hours shows the HDF-α cell viability pictures (Figure 3.6) and normalized graph (Figure 3.5) of the low and high range TMZ concentrations compared to the undisturbed control, DNCNF PAA 10% alone, and free 100μM TMZ. The free 100μM TMZ did not negatively affect the HDF-α. The DNCNF PAA 10% alone had cell viability of around 71% compared to the control. The DNCNF PAA 10% 25μM, 50μM, 100μM, 250μM, 500μM, and 1000μM TMZ treatments were all significantly similar to DNCNF PAA 10% and had cell viability of about 63%, 53%, 56%, 61%, 57%, and 61% respectively compared to the control. All the conditions have higher variability due to pipette disturbance of the DM6 cells and DNCNF PAA 10% blockage. The treatment was not completely taken out of most wells so there were less images used in cell counting which caused higher variabilities between this study and other studies.

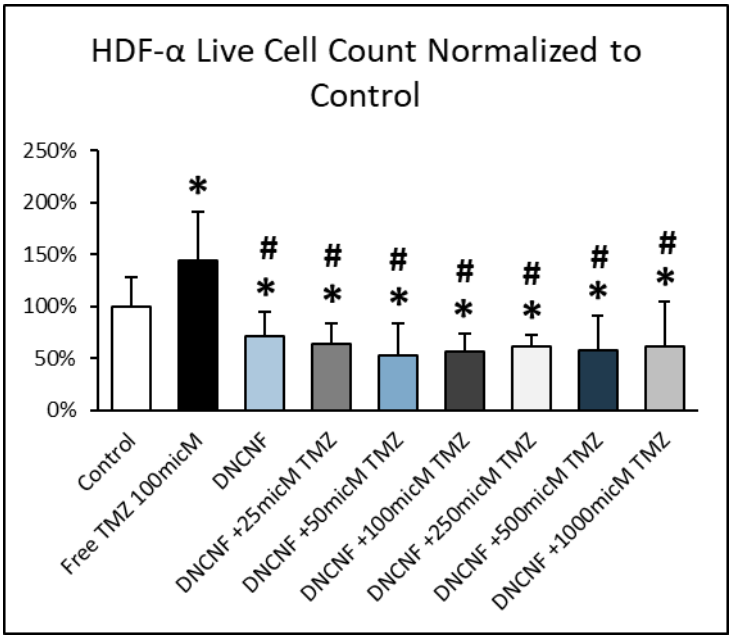

Figure 3.5: HDF-α Cell Counting Viability Assay of low and high dosage ranges with TMZ and DNCNF PAA 10% after 72 hours of treatment graphical representation.

In Figure 3.5, it shows the graphical representation of cell viability percentage compared to each condition with cells counted from individual pictures by ImageJ and live cells averaged per image and normalized to Control. Significant difference is at a p-value of 0.05. The symbols to the right of the control indicate significant difference respectively. The symbol "*" indicates significant difference from Control; "#" indicates significant difference from Free 100μM TMZ; and "+" indicates significant difference from DNCNF PAA 10%.

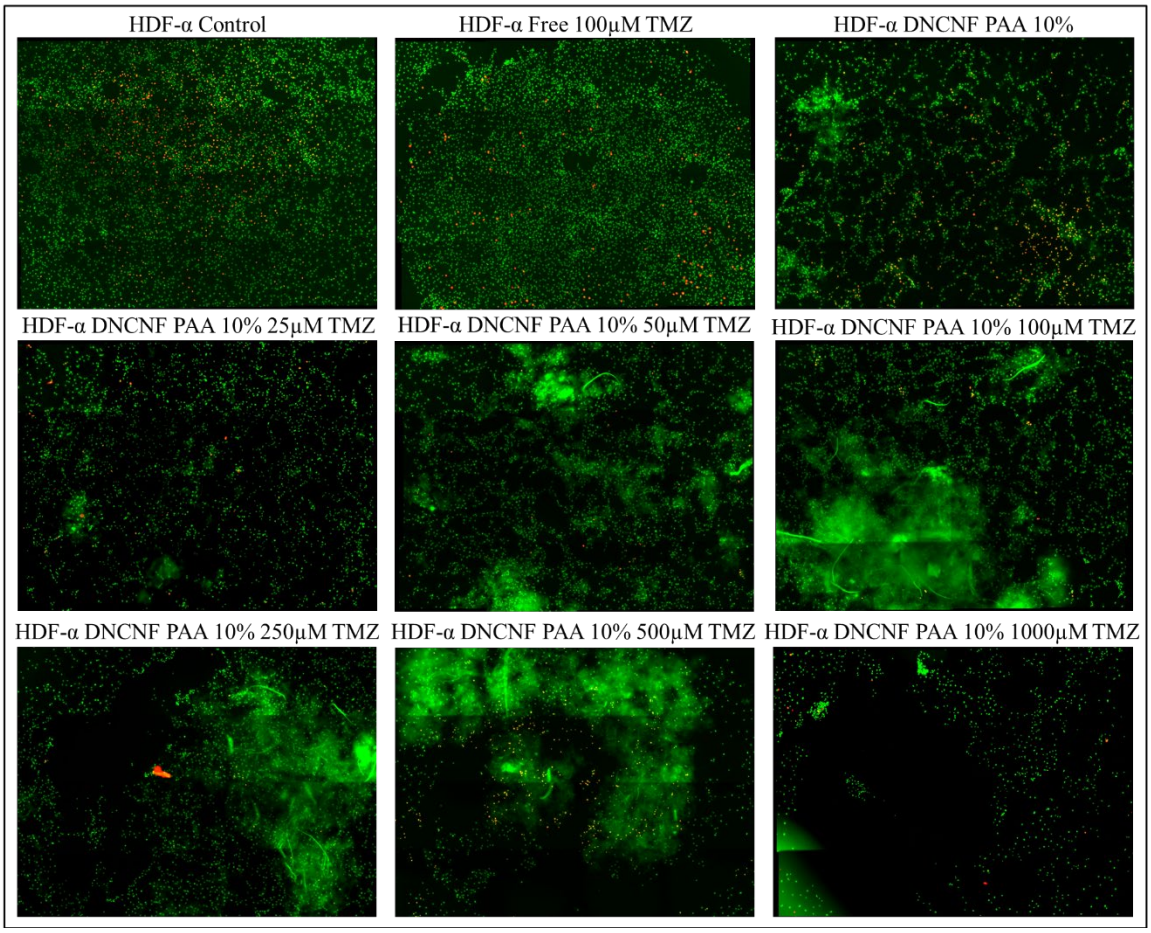

Figure 3.6: HDF-α Cell Counting Viability Assay of low and high dosage ranges with TMZ and DNCNF PAA 10% after 72 hours of treatment image representation.

In Figure 3.6, it shows the stitched image representation of each well condition and controls, created by FIJI with 4 images by 4 images taken at 10x magnification of HDF-α

cells stained with PI (red, dead cells) and Hoechst (manipulated to be green, alive cells) for 30 minutes and replaced with fresh image media.

3.4 HDF-α Cell Viability of Expanded Dosage Ranges

This second HDF-α study of the treatment after 72 hours shows the HDF-α cell viability pictures (Figure 3.8) and normalized graph (Figure 3.7) of the expanded high range TMZ concentrations compared to the undisturbed control, DNCNF PAA 10% alone, and free 500μM TMZ. Free 500μM TMZ was chosen since the first study of DM6 showed significant cell death using the 500μM dosage in the hydrogel and is compared to the HDF-α cell viability. The free 500μM TMZ is significantly different from control, DNCNF PAA 10% alone, and DNCNF PAA 10% 500μM TMZ with only 27% viability. The DNCNF PAA 10% alone exhibited a cell viability of about 43% compared to the control. The DNCNF PAA 10% 500μM, 750μM, and 1000μM TMZ treatments were all significantly similar to each other with cell viability of about 42%, 32%, and 30% respectively compared to the control. The cell viability for the DNCNF PAA 10% 500μM TMZ condition and DNCNF PAA 10% alone in the second study is similar to the first HDF-α study of low and high range dosages and within the variability range. All the conditions repeated from the first study are within variability range. There is a small dosage effect seen from 500μM TMZ to 1000μM TMZ with the most promising therapy window lying between DNCNF PAA 10% with 500μM and 750μM. Variability could be due to pipette disturbance of the DM6 cells and DNCNF PAA 10% blockage.

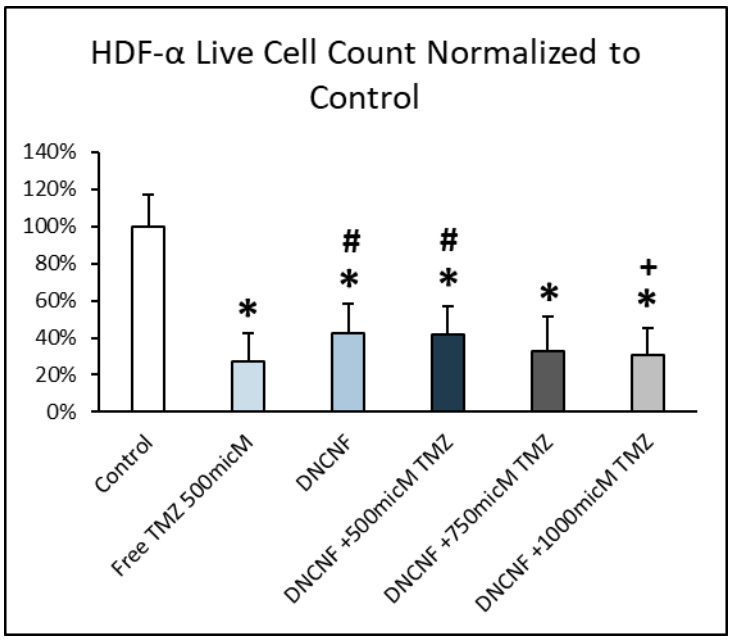

Figure 3.7: HDF-α Cell Counting Viability Assay of expanded dosage ranges with TMZ and DNCNF PAA 10% after 72 hours of treatment graphical representation.

In Figure 3.7, it shows the graphical representation of cell viability percentage compared to each condition with cells counted from individual pictures by ImageJ and live cells averaged per image and normalized to Control. Significant difference is at a p-value of 0.05. The symbols to the right of the control indicate significant difference respectively. The symbol "*" indicates significant difference from Control; "#" indicates significant difference from Free 500μM TMZ; and "+" indicates significant difference from DNCNF PAA 10%.

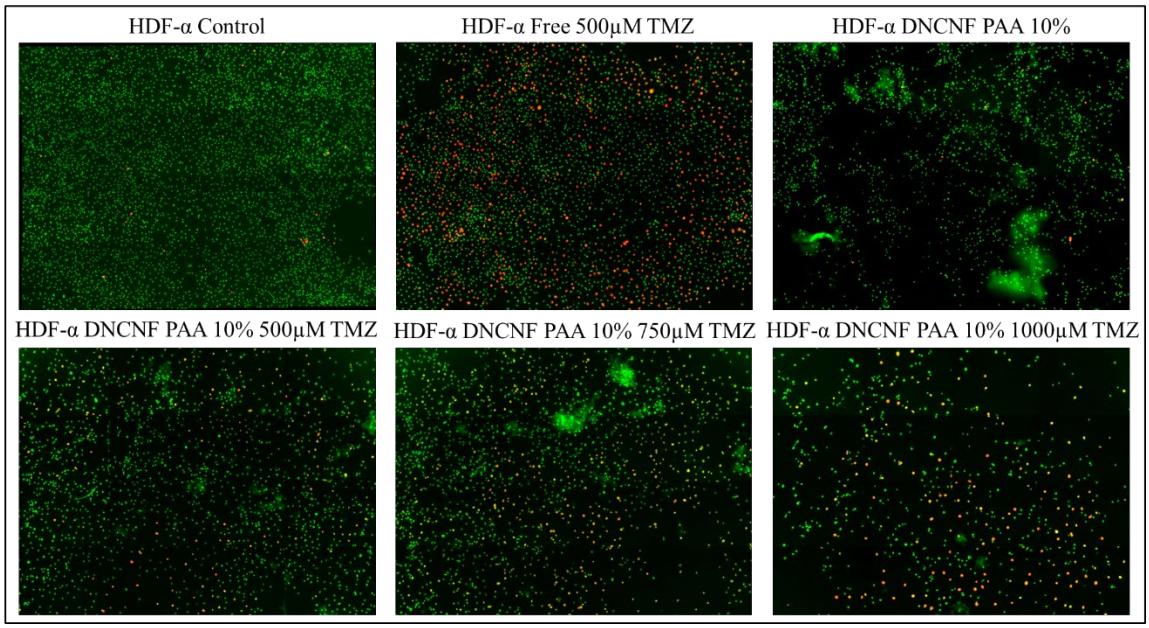

Figure 3.8: HDF-α Cell Counting Viability Assay of expanded dosage ranges with TMZ and DNCNF PAA 10% after 72 hours of treatment image representation.

In Figure 3.8, it shows the stitched image representation of each well condition and controls, created by FIJI with 4 images by 4 images taken at 10x magnification of HDF-α cells stained with PI (red, dead cells) and Hoechst (manipulated to be green, alive cells) for 30 minutes and replaced with fresh image media.

3.5 Long-term DM6 Cell Recovery Study

The long-term cell recovery study of DM6 cells with the expanded high dosage range of DNCNF PAA 10% 500μM, 750μM, and 1000μM TMZ was done to study the recovery of the cancer cells after the initial 72-hour treatments with an additional 72 hours without treatment. The procedure was the same until after Day 3. The brightfield images from Day 3 (Figure 3.9A) of conditions DNCNF PAA 10% alone, and 500μM-1000μM TMZ show a clear dosage anticancer response with greater concentrations. The brightfield microscopy images from Day 6 (Figure 3.9B middle column) of the same conditions with an additional 72 hours show the cells recover, with less cell recovery in increasing dosages. The Day 6 DNCNF PAA 10% alone DM6 cells cover most of the well showing clumps

and little free space. The Hoechst-stained cells in the DAPI filter confirm a great amount of regrowth compared to Day 3 (Figure 3.9B right column). The Day 3 DNCNF PAA 10% 500μM TMZ condition has more cell death than the DNCNF PAA 10% alone, and Day 6 brightfield microscopy also shows much less cell recovery than without TMZ. The fluorescent Day 6 images (Figure 3.9B right column) shows about 50% less cell recovery than the DNCNF PAA 10% alone. Both the DNCNF PAA 10% 750μM and 1000μM TMZ show the same descending trend of less cell regrowth the greater the TMZ concentration. However, the 1000μM TMZ condition has significantly less cell recovery than the other three conditions. This study shows that despite mass DM6 cell death from 72 hours of treatment with the expanded high dosage range, the surviving DM6 cells recover and have possibly acquired TMZ drug resistance. Given enough time, the cells could grow back to a metastatic state.

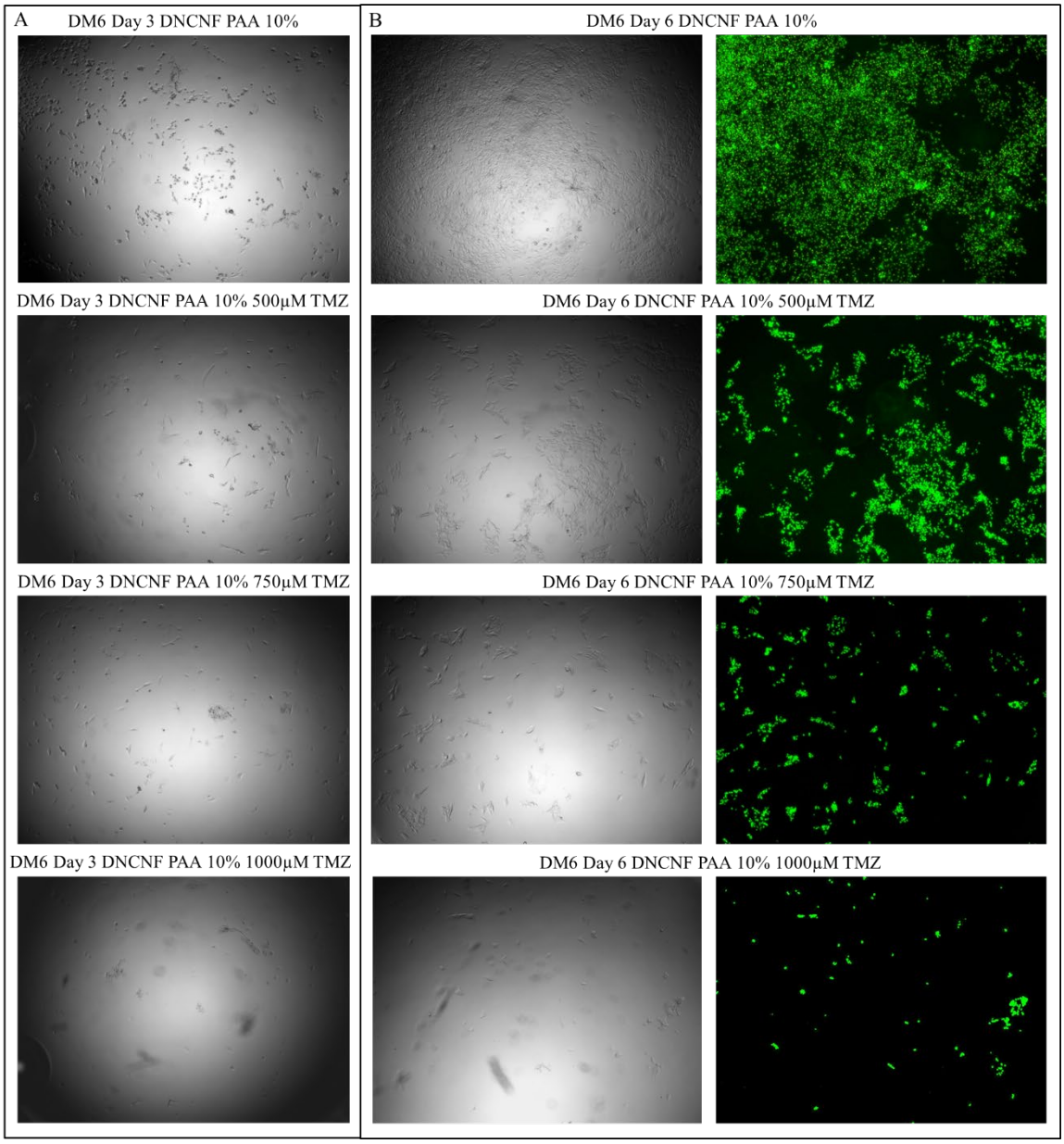

Figure 3.9: DM6 Long-term Cell Recovery Study with TMZ and DNCNF PAA 10% after 72 hours of treatment and an additional 72 hours without treatment.

In Figure 3.9A, the leftmost column is Day 3 brightfield images taken at 2.5x magnification of conditions after treatment of DM6 cells replaced with fresh image media. The left/middle column (Figure 3.9B) is an additional 72 hours, Day 6, brightfield images of DM6 cell conditions after Day 3 treatment, left to recover in 10% FBS media, taken at 2.5x magnification of conditions replaced with fresh image media compared to each

condition from Day 3. The right-most column is the same additional 72 hours, Day 6 of DM6 cell conditions after Day 3 treatment, left to recover in 10% FBS media. The right-most column images were taken at 2.5x magnification of DM6 cells stained with DAPI (manipulated to be green) in fresh image media after being fixed with paraformaldehyde and brightness/contrast manipulated by ImageJ compared to brightfield of Day 6 and to each condition from Day 3 to study cell recovery after treatment.

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1 Therapy Window

The hydrogel and TMZ treatment overall showed significant anticancer effects and coupled with high TMZ concentrations showed even greater cell death to DM6. The hydrogel treatment alone demonstrated a physical barrier effect, limiting cell proliferation. The hydrogel also seemed to stabilize the TMZ for better dosage effect. The free 100μM TMZ condition was not expected to have any effect on the cancer or healthy cells, and this was proved true. It is not known why the cells seemed to thrive in the free 100μM TMZ condition, but this could be due to a developed drug resistance of the cancer. The free 500μM TMZ was expected to have non-specific killing of cancer cells and the normal healthy cells, which was observed. The free 500μM TMZ, however, was not expected to kill to the degree it did. This could be due to the shock of a bulk drug delivery which could have ended up killing the cells before degrading, but the bulk drug delivery also had high cell death on the healthy cells. The DM6 low dosages of the TMZ loaded hydrogel had a plateau from 25-100μM TMZ with similar effects as the hydrogel alone. However, the low dosages were also similar to the expanded high range in cell viability of the healthy cells. The expanded high range dosages effectively killed the DM6 but also negatively affected the healthy cells. The 500μM TMZ loaded hydrogel had great cell death and was still statistically similar to the DNCNF PAA 10% alone. The 750μM TMZ loaded hydrogel also had great cell death but was possibly too harsh on the healthy cells. The 1000μM TMZ

loaded hydrogel was extremely harsh on the healthy cells despite great cell death on the DM6 cells which was similar to the free 500μM TMZ.

There was variability between the different studies and percentages of cell viability using free TMZ, DNCNF PAA 10% alone, and DNCNF PAA 10% 500μM TMZ, but the trends of the data remained the same between studies and was within the variability range allowing the comparison of experiments. The variability is likely due to the human seeding differences and possible pipette disturbance. Overall, the dosage range of 500-750μM TMZ loaded into the DNCNF PAA 10% is recommended for further testing. The 500-750μM TMZ loaded hydrogel range was effective for specific DM6 killing while having the best HDF-α cell viability compared to the other high range dosages and free 500μM TMZ.

4.2 Long-term DM6 Cell Recovery Study: Implications

The long-term DM6 cell recovery study demonstrated that even after treatment the DM6 cells can still recover, which calls into question how and why. The surviving melanoma cells have possible TMZ drug resistance. The hydrogel with 500-750μM TMZ still showed a significant decrease in cell growth while the 1000μM had little to no cell growth after treatment. The DNCNF PAA 10% 500-750μM TMZ treatment is recommended for treatment and should be researched further for different durations. The observed outcome shows that the DNCNF PAA 10% injectable hydrogel stabilized the TMZ for better dosage effects and allowed for specific killing of DM6 while keeping the HDF-α largely unharmed.

4.3 Conclusion

The therapy window found for DM6 and HDF-α is DNCNF PAA 10% 500-750μM TMZ. Based on the results, there is a need to research another in situ model to study the therapy window found by performing co-culture experiments of HDF-α and DM6. This would allow for observations of the dosages and interactions between the cancer and healthy cells for a closer human model. Another possible important study would be an injectable tumor model for melanoma treatment to observe the effects of underneath tumor treatment contact. To understand bulk delivery of drugs and degradation of TMZ in free form, a study could explore the free TMZ functionality and repeat the study but with different concentrations of TMZ. Lastly, the most important question to answer is how and why the cancer grew back after 72 hours of the TMZ loaded hydrogel treatment. This could be done by a series of long-term studies of DM6 and HDF-α using the therapy window. Finding an effective local treatment for melanoma is crucial for lowering the death rate.

4.3.1 Future Directions

The key takeaway from these experiments is that the local treatment had less severe side effects to healthy cells than the free drug in a high dose did, with specific killing and targeting of the melanoma cells. This opens the possibility of localized treatments for many kinds of cancers. Further studies using a co-culture method of HDF-α and DM6 should be done to see how the cells interact together with the treatment therapy window for a more refined and accurate demonstration of the cellular reaction. Another study that addresses TMZ resistance in combination with the loaded hydrogel treatment using DNA Repair inhibitors such as MGMT (DNA repair gene specific to melanoma) could be another option to combat surviving melanoma cells. Finally, for pre-clinical purposes, future *in vivo*

studies involving human melanoma xenografts on immunologically deficient nude mice would be a strong foundation to better understand the treatment on a living organism for relevance to possible clinical trials later.

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BIOGRAPHICAL INFORMATION

Kayla Meyers is a senior working towards her Honors Bachelor of Science in Biomedical Engineering degree. She is currently applying for graduate schools for biomedical sciences/engineering programs where she aspires to become a clinical researcher in a hospital to synergistically work alongside clinicians for the development of novel diagnostic and therapeutic interventions. Her interests lie in understanding the mechanisms behind diseases through cancer biology, cell and molecular biology, immunology, tissue engineering, and nanomedicine/drug delivery.

Her first research experience began as a junior, December 2021, in Dr. Young-Tae Kim's lab at the University of Texas at Arlington (UTA). She became a McNair Scholar and participated in the McNair Scholars Internship, summer 2022, where she performed independent research investigating the most effective therapy window of an injectable cellulose nanofiber hydrogel melanoma treatment loaded with temozolomide (TMZ).

Fall of 2022, she was awarded a position in the Undergraduate Research Opportunity Program (UROP) at UTA to continue her independent research on the melanoma treatment in via a co-culture system. Kayla was then awarded the Research Experience for Undergraduates (REU) grant at UTA with two peers. In Dr. Tang's tissue engineering lab at UTA. she is assisting a doctoral student on her research involving the adhesive for a damaged labrum. For senior design, Kayla and her group are designing a CT 3D post-processing software protocol and a post-processing toolkit for cleaning the printed models.