

**DRUG REPURPOSING AND
INVESTIGATION OF NOVEL
COMBINATIONS FOR
GLIOBLASTOMA THERAPEUTICS
USING *IN VITRO* AND ZEBRAFISH *IN
VIVO* MODELS**

**A THESIS SUBMITTED TO
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MOLECULAR BIOLOGY AND GENETICS**

**By
Güneş Tok
January, 2024**

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By Güneş Tok

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We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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Abstract

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Glioblastoma is the most common and aggressive brain cancer type with the survival rate less than 2 years after diagnosis. Yet, potent drug treatments used in patients are limited and the field is in need of development of new potential drugs. In this study, repurposing of approved drugs alone or in combination and novel drugs are investigated in terms of inhibition of cell viability, glial fluorescent signals and their effects on behavior in zebrafish larval models. The main aim of this study was to test whether phenothiazines, trifluoperazine and a novel molecule **10**, could be repurposed for glioblastoma treatment with lower dosages and more potency when combined with Sorafenib, an approved drug, in glioblastoma cell lines and zebrafish larvae. Those drug combinations were not found as toxic in the dosages studied while acted on glia cells in zebrafish transgenic larval models. Last but not least, behavior and stress response of the wild type and heterozygous mutant *ache* larvae in comparison with homozygous siblings were tested upon drug administration to assess genotype by drug interactions. Combination treatments exhibited higher efficacies suggesting phenothiazines with sorafenib could have potential in glioblastoma treatment. Genotype specific effects of individual and

combination treatments on larval light-dark behavior, stress response and recovery exhibited potential for passage of blood brain barrier by the tested drugs. The established protocols for genotype and drug interactions could be applied to other kinases in combination with phenothiazines.

Keywords: Cancer, Glioblastoma, Drug, Drug Repurposing, Drug Combination, Zebrafish, Zebrafish behavior, Trifluoperazine, Sorafenib.



Özet

İLACIN YENİDEN KONUMLANDIRILMASI VE GLIOBLASTOMA TEDAVISINE YÖNELİK YENİ KOMBİNASYONLARIN İN VITRO VE ZEBRAFİSH İN VIVO MODELLER KULLANILARAK ARAŞTIRILMASI

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Glioblastoma, teşhisten sonra 2 yıldan az hayatta kalma oranıyla en yaygın ve agresif beyin kanseri türüdür. Ancak hastalarda kullanılan güçlü ilaç tedavileri sınırlıdır ve alanda yeni potansiyel ilaçların geliştirilmesine ihtiyaç vardır. Bu çalışmada, zebra balığı larva modellerinde onaylanmış ve yeni ilaçların yeniden konumlandırılması ve bu ilaçların kombinasyonlarının hücre canlılığı ve glial floresan sinyallerinin inhibisyonu ve davranış üzerindeki etkileri açısından araştırılmıştır. Bu çalışmanın temel amacı, fenotiazinlerden trifluoperazin ve molekül 10'un, glioblastoma hücre hatları ve zebra balığı larvalarında onaylanmış bir ilaç olan Sorafenib ile birleştirildiğinde daha düşük dozajlar ve daha fazla etki ile glioblastoma tedavisi için yeniden konumlandırılıp konumlandırılmayacağını test etmektir. Zebra balığı transgenik larva modellerinde glia hücreleri üzerinde etkili olduğu tespit edilirken, bu ilaç kombinasyonlarının incelenen dozajlarda toksik olmadığı görülmüştür. Son fakat bir o kadar da önemli olarak, vahşi tip ve *ache* heterozigot mutant larvalarının birbirleri ile karşılaştırıldığında ilaçlara verdikleri davranış ve stres tepkisi, genotipi ilaç etkileşimleri kapsamında araştırılmıştır. Kombinasyon tedavileri, sorafenibli fenotiyazinlerin glioblastoma tedavisinde potansiyele sahip olabileceğini düşündüren daha yüksek etkinlik sergiledikleri

görülmüştür. Bireysel ya da kombine olarak test edilen ilaçların kan beyin bariyerini geçme potansiyeli olduğu, larvaların aydınlık-karanlık davranışı, stres tepkisi ve iyileşmesi testlerinin genotip spesifik etkileri sayesinde bulgulanmıştır. Genotip ve ilaç etkileşimleri için oluşturulan protokoller, fenotiazinlerle kombinasyon halinde diğer kinazlar için de uygulanabilir.

Anahtar Kelimeler: Kanser, Glioblastoma, İlaç, İlaçların Yeniden Kullanılması, İlaç Kombinasyonu, Zebra balığı, Zebra balığı davranışı, Trifluoperazin, Sorafenib.





To All Women in Science...

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Table of Contents

Abstract.....	x
Özet.....	x
Acknowledgements.....	x
Table of Contents.....	x
List of Figures.....	xiv
List of Tables.....	xvi
Abbreviation.....	xvii
Chapter 1.....	1
1.Introduction.....	1
1.1 Cancer.....	1
1.1.1 Glioblastoma.....	2
1.1.1.1 Glioblastoma Diagnosis and Therapeutics.....	4
1.1.1.2 Drug Targeting.....	5
1.1.1.2.1 Candidate New Drugs In the Last Five Years.....	6
1.1.1.2.1.1 RTK Inhibitors Potentially Targeting GBM.....	6
1.1.1.2.1.2 MAPK Inhibitors Potentially Targeting GBM.....	8
1.1.1.2.1.3 GSK Inhibitors Potentially Targeting GBM.....	8
1.1.1.2.1.4 Other Inhibitors Potentially Targeting GBM.....	9
1.1.1.2.2 Trifluoperazine.....	11
1.1.1.2.3 Sorafenib.....	11
1.2 Acetylcholinesterase	12
1.3 Nicotinic Acetylcholine Receptors.....	14
1.4 Zebrafish as a Model Organism.....	14
1.4.1 Zebrafish and Cancer.....	15
1.4.1.1 Zebrafish and Glioblastoma.....	16
1.4.1.2 Zebrafish in Xenotransplantation Models.....	17
1.4.2 ache and p53 Genes in Zebrafish.....	19

1.4.2.1 TP53 Gene.....	19
1.4.2.2 Ache Gene.....	20
1.4.3 Zebrafish Behavior	20
1.4.3.1 Zebrafish Behavior in Light Dark Cycles.....	21
1.4.4 Zebrafish ache and WT Behavior in Diet and Alcohol Exposures.....	22
1.5 Aims & Rationale.....	24
Chapter 2.....	27
2. Materials & Methods.....	27
2.1. Materials.....	27
2.1.1 Chemicals, Reagents and Kits.....	27
2.1.2 Solution and Buffer Recipes.....	32
2.2 Methods.....	33
2.2.1 Cell Culture Methods.....	33
2.2.1.1 Cell Thawing.....	33
2.2.1.2 Cell Passaging.....	34
2.2.1.3 Cell Freezing.....	34
2.2.1.4 Cell Seeding.....	34
2.2.1.5 Drug Treatment.....	35
2.2.1.6 siP53 Transfection.....	36
2.2.1.7 MTT Cell Viability Assay.....	37
2.2.2 Gene Expression Analysis Methods.....	37
2.2.2.1. RNA Isolation.....	37
2.2.2.2 cDNA Synthesis.....	38
2.2.2.3 Quantitative Real-Time PCR.....	39
2.2.2.4 qPCR Analysis.....	40
2.2.3 Protein Assays & Analysis.....	40
2.2.3.1 Protein Isolation.....	40
2.2.3.2 Protein Quantification.....	41
2.2.3.3 Western Blot.....	42

2.2.3.4 Signal Quantification from Blots.....	43
2.2.4 In Vivo Experiments.....	44
2.2.4.1 Zebrafish Husbandry and Embryo Collection.....	44
2.2.4.2 Larval Drug Treatment.....	44
2.2.4.3 Behavior Assays.....	45
2.2.4.4 Brain Injection to Zebrafish Larvae.....	45
2.2.4.5 DNA Extraction from Zebrafish Larvae.....	46
2.2.4.6 ache Genotyping.....	46
2.2.4.7 Signal Quantification from Larvae.....	47
2.2.4.8 Larval DUAL Diet Introduction.....	47
Chapter 3.....	49
3. Results.....	49
3.1 Effect of VPA, NS-398, Topiramate and Trotabresib on Glioblastoma Cell Lines.....	49
3.2 Effect of TFP, SFB and Phenothiazine Derivative molecule ¹⁰ on Glioblastoma Cell Lines.....	52
3.3 Changes in Expression Profile After TFP & SFB Treatment in Glioblastoma Cell Lines.....	59
3.3.1 Alteration in mRNA Levels.....	59
3.3.2 Alteration in Protein Levels.....	64
3.4 Effect of Drugs on Zebrafish Larval Models.....	64
3.4.1 TFP & SFB Effect on Wild-Type Zebrafish.....	64
3.4.2 Drugs' Effect on Zebrafish Glia.....	69
3.4.3 Zebrafish Larval Behavior Investigation and Effect of Drugs on Larval Behavior.....	71
3.4.3.1 Zebrafish Larval Behavior Investigation on Wild-Type and Mutant Lines.....	71
3.4.4 Effect of Drugs on Wild-Type and Mutant Larval Behavior.....	75
3.4.4.1 Effect of Drugs on Wild-Type Larval Behavior.....	75
3.4.4.2 Effect of Drugs on ache Heterozygous Mutant Larval Behavior.....	81

3.4.5 Glioblastoma Xenograft Model and Behavior.....	83
3.4.6 DUAL Diet Effect on Larval Behavior	86
Chapter 4.....	88
4. Discussion & Conclusion.....	88
4.1 Investigation of Drugs in Different Glioma Cell Lines.....	88
4.2 Potential of Combinatorial Therapy for GBM.....	89
4.3 Understanding the Mechanism of TPF, SFB or TFP-SFB Combination.....	92
4.4 Investigation of Drugs in Zebrafish Models in vivo.....	94
4.5 Diving in Zebrafish Behavior.....	98
4.6 Future Perspectives.....	102
Chapter 5.....	104
5. References.....	104
Appendix.....	132

List of Figures

Figure 1.1: Workflow of the thesis	24
Figure 2.1: Drug dilutions example	36
Figure 3.1: VPA, NS-398, Topiramate and Trotabresib single treatment effect on U87-MG cell and A172 cell lines.....	49
Figure 3.2: VPA, NS-398, Topiramate combination with TMZ treatment effect on U87-MG cell and A172 cell lines.....	51
Figure 3.3:TFP, SFB & molecule10 single treatment effect on U87-MG cell and A172 cell lines.....	53
Figure 3.4: TFP & SFB combination treatment effect on U87-MG and A172 cell viability...	55
Figure 3.5: Microscopy image analysis of TFP & SFB single and combination treatment on A172 cell viability	56
Figure 3.6: SFB & TFP and siP53 treatment effect on A172 cell viability	57
Figure 3.7: Molecule10 & SFB combination treatment effect on U87-MG & A172 cell viability.....	59
Figure 3.8: TFP & SFB single and combination treatment effect on mRNA expression in U87-MG cell line.....	60
Figure 3.9: TFP & SFB single and combination treatment effect on Vimentin and Bax mRNA and protein expression in U87-MG cell line.....	63
Figure 3.10: Stereo microscopy images of wild-type zebrafish control groups.....	65
Figure 3.11: Stereo microscopy images of wild-type zebrafish TFP & SFB single treatment.	66
Figure 3.12: Stereo microscopy images of wild-type zebrafish TFP & SFB combination treatment.....	67
Figure 3.13: Stereo microscopy images zebrafish TFP, SFB & molecule10 single and combination treatment.....	68
Figure 3.14:EVOS scan of GFAP:GFP individual larvae with different treatments.....	69
Figure 3.15:Paired graphs individual signals.....	70
Figure 3.16: Locomotion changes in response to light/dark changes.....	72

Figure 3.17: Locomotion changes in p53 (-/-) genotyped larvae response to light/dark changes	73
Figure 3.18: Locomotion Differences in ache (+/+) and ache (+/-) larvae.....	74
Figure 3.19: Locomotion Differences in Wild-Type Larvae upon drug treatments.....	76
Figure 3.20: Locomotion Differences in Control and TFP Treatment in WT larvae.....	77
Figure 3.21: Regression Plots of Control and SFB Treatment in WT larvae.....	78
Figure 3.22: Regression Plots of Control and Combinatorial Treatment in WT larvae.....	79
Figure 3.23: Locomotion Differences in Control and molecule10 Treatment in WT larvae...	80
Figure 3.24: Locomotion Differences in ache (+/+) and ache (+/-) larvae with 10 μ M TFP...	82
Figure 3.25: Locomotion Differences in ache (+/+) and ache (+/-) larvae with Phenothiazine Derivative molecule10.....	83
Figure 3.26: U87-GFP xenograft images of 4 dpf and 5 dpf WT larvae in lateral and ventral views.....	84
Figure 3.27: Locomotion Differences of U87 xenograft and control larvae.....	85
Figure 3.28: Locomotion Differences in ache (+/+) and ache (+/-) larvae with DUAL diet...	86
Appendix Figure 1: Code for zantiks MWP device used for the behavior assays.....	133
Appendix Figure 2: Flow cytometer results of U87-GFP cells.....	134
Appendix Figure 3: Synthesis process and molecular shape of molecule10.....	135
Appendix Figure 4: Length of the larvae after drug treatment.....	136
Appendix Figure 5: Oil Red O Staining images of DUAL-diet group larvae.....	138

List of Tables

Table 2.1: Laboratory chemicals for general use.....	27
Table 2.2: Reagents for cell culture.....	28
Table 2.3: Laboratory equipment and purpose of use.....	29
Table 2.4: Primer list.....	30
Table 2.5: Antibody list.....	31
Table 2.6: Solution and buffer recipes.....	32
Table 2.7: RT-qPCR reaction protocol.....	39
Table 2.8: BSA standards dilution preparation.....	41
Appendix Table 1: Properties of molecule 10.....	135
Appendix Table 2: Sparos HFD Ingredients.....	137

Abbreviations

AB: Alexander's Disease

AchE: Acetylcholinesterase

ALKs: Activin Receptor-Like Kinases

APS :Ammonium Persulfate

BAX : BCL2 Associated X, Apoptosis Regulator (gene)

BBB: Blood-Brain Barrier

CCNE1: Cyclin E1

CD44: Cluster of Differentiation 44 (gene)

CD68: Cluster of Differentiation 68 (gene)

CDK1 - Cyclin-Dependent Kinase 1 (gene)

cDNA: Complementary DNA

CHEK1 - Checkpoint Kinase 1 (gene)

Chk: Checkpoint Kinase

CHRNA5 - Cholinergic Receptor Nicotinic Alpha 5 (gene)

CpG: Cytosine-phosphate-Guanine

CPP: Conditioned Place Preference

CT: Computed Tomography

ddH₂O: Double-Distilled Water

DDIT3 - DNA Damage Inducible Transcript 3 (gene)

DMEM: Dulbecco's Modified Eagle Medium

DMSO - Dimethyl Sulfoxide

DNA: Deoxyribonucleic Acid

DNA: Deoxyribonucleic Acid

dpf: Days post-fertilization

ECM: Extracellular Matrix

EDTA - Ethylenediaminetetraacetic Acid

EGF: Epidermal Growth Factor

EGFR: Epidermal Growth Factor Receptor

EGR2: Early Growth Response 2

EMT: Epithelial to Mesenchymal Transition

ERK: Extracellular Signal-Regulated Kinase

FBS: Fetal Bovine Serum

FDA: Food and Drug Administration

FGF: Fibroblast Growth Factor

GBM: Glioblastoma Multiforme

GFAP - Glial Fibrillary Acidic Protein

GFP - Green Fluorescent Protein

GSCs: Glioma Stem Cells

HCC: Hepatocellular Carcinoma

HCl: Hydrochloric Acid

HFD: High Fat Diet

IC50: Half-maximal Inhibitory Concentration

IDH: Isocitrate Dehydrogenase

IL1B - Interleukin 1 Beta (gene)

JAK: Janus Kinase

MAPK: Mitogen-Activated Protein Kinase

MDM2: Mouse Double Minute 2 Homolog

MGMT: O(6)-Methylguanine-DNA Methyltransferase

MIK67 - Marker of Proliferation Ki-67 (gene)

MRI: Magnetic Resonance Imaging

mRNA: Messenger Ribonucleic Acid

mTOR: Mammalian Target of Rapamycin

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

p53: Tumor Protein 53 (gene)

PCR: Polymerase Chain Reaction

PDX: Patient-Derived Xenotransplantation

PTEN: Phosphatase and Tensin Homolog

PVS: Perivitelline Space

qPCR - Quantitative Polymerase Chain Reaction

RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species

RTKs: Receptor Tyrosine Kinases

SEM: Standard Error of the Mean

SFB - Sorafenib

siP53: Small interfering RNA targeting P53

siRNA: Small Interfering RNA

SLC7A11 - Solute Carrier Family 7 Member 11 (gene)

SRSF7: Serine/arginine-rich splicing factor 7

TFP: Trifluoperazine

TGFβ: Transforming Growth Factor Beta

TGX: Trans-Blot Turbo Transfer System

TME: Tumor Microenvironment

TMZ: Temozolomide

TNFα: Tumor Necrosis Factor Alpha

TP53 - Tumor Protein p53 (gene)

VEGF: Vascular Endothelial Growth Factor

VEGFR: Vascular Endothelial Growth Factor Receptor

VIM - Vimentin (gene)

CHAPTER 1 : Introduction

1.1 Cancer

Cancer, initiated with the uncontrollable and unintended division of cells may lead to a large number of deaths worldwide with more than 10 million people diagnosed every year. (Siegel et al., 2016) (Mbeunkui & Johann, 2009). With the deteriorating living conditions in the world due to pollution, emergence of new pathogens, increased use of chemicals, these rising numbers of cancer incidents makes it necessary to better understand cancer biology to prevent and cure cancers. Causes listed but not limited to the above, lead to alterations in the genome, which ultimately change the behavior of the cell in terms of gene expression, and rates of cell growth and apoptosis (Hassanpour & Dehghani, 2017). Cancer is a highly heterogeneous disease that involves complex tissues including different cell types. Moreover, the presence of various types of cancers makes it difficult to diagnose and treat them. (Siegel et al., 2016).

Although mutations in the genome might have dangerous consequences, every alteration does not result in the formation of cancer. Cell's own defense mechanisms, e.g., cell cycle checkpoints, make it possible to detect the overgrowth and stop the cell division upon DNA damage or alteration (Murray, 1994). Cells that can escape cell cycle mechanisms can also be detected by the immune system of the body by T-cells. Humans and mice that are immune suppressed have been shown to be more prone to cancer (Corthay, 2014). Cells that manage to survive all these mechanisms are said to have completed some or all important hallmarks of the cancer (Hanahan & Weinberg, 2011)). Accordingly, the prognosis gets worse, now overpopulated cells reach immortality and eventually metastasize to a secondary site and induce angiogenesis (Hanahan & Weinberg, 2011).

Interactions of cells between each other are crucial for formation and progression of cancer as well as the interactions between the environment and cells. This crosstalk might include communication of fibroblasts, macrophages, epithelial cells and tumor cells as well as extracellular matrix (ECM) (Mbeunkui & Johann, 2009). As a result, these complex interactions, termed microenvironment, provide tumor cells an advantage of sending signals to the surrounding healthy tissue in a beneficial way for the tumor. Tumor microenvironment (TME) accelerates the formation and accelerates the progression of cancer. The communication is mostly provided by growth factors, chemokines, regulatory molecules, hormones, and enzyme secretion (Baghban et al., 2020). Thus, a better understanding of the signaling and communication between the tumor cells and TME is a crucial step towards treating cancers.

Another feature of the cancer cells is the plasticity indicating the cell's ability to shift stages from one phenotype to another (Shen & Clairambault, 2020). Cancer stem cells (CSCs) are considered as progenitors of cancer cells while recent studies have demonstrated a model where cancer cells can shift between non-CSC to CSC stage (Jang et al., 2015) (Pervan et al., 2022).). Cancer plasticity results in genetic and epigenetic alterations and modifications and thus, contributes to intra-tumor heterogeneity (da Silva-Diz et al., 2018)

1.1.1 Glioblastoma

Glioblastoma, one of the cancers of the central nervous system, is a type of brain cancer that occurs in astrocyte cells and is the most aggressive with one of the lowest survival rates (Adamson et al., 2009). Estimated life expectancy after diagnosis is less than 2 years (Tan et al., 2020). Also called glioblastoma multiforme (GBM) or glioblastoma pleomorphic, which means “able to reach different forms” (Omuro & DeAngelis, 2013), glioblastoma is very common among other brain tumors. Almost 50% of all brain cancer incidents are diagnosed as glioblastoma (Moliterno & Joseph, n.d.). Although not all risk factors are fully identified yet,

age is considered as the biggest risk factor for glioblastoma next to genetic tendencies (Adamson et al., 2009). Yet, ionizing radiation is also considered as an environmental risk factor for glioblastoma (Tsai et al., 2021) (Sharanek et al., 2020).

Glia cells, i.e., astrocytes, oligodendrocytes and microglia, are responsible for protecting and supporting neuronal cells. Glia has an additional role that is to provide an ionic balance, growth factor secretion, and even function as neural stem cells (He & Sun, 2007). The term glioma indicates the cancer occurs in glia cells. Glial cancers include astrocytoma, oligodendroglioma and glioblastoma, which is the stage 4 of all gliomas (Norden et al., 2010). The stages of glioma are vital to understand because they have implications for therapeutic approaches.

Isocitrate dehydrogenase (IDH-1) and isocitrate dehydrogenase 2 (IDH-2) play important roles in aerobic respiration, degrade isocitrate and give NADPH as a side product, which is essential for many cellular processes including fatty acid processing and immune response. IDH1 and IDH2 are found to be markers of glioblastoma and have been suggested as being drivers for early stages of gliomas such as pilocytic astrocytoma and oligodendroglioma (Han et al., 2020) (Nobusawa et al., 2009). In fact, astrocytoma and oligodendrogliomas are IDH mutants while glioblastomas are IDH wild type (Ohgaki & Kleihues, 2005). Therefore, patients with wild type IDH are more prone to have a poor diagnosis and low survival rate (L. J. Yang et al., 2014). Another biomarker of glioblastoma is the high expression of O (6)-methylguanine-DNA methyltransferase or MGMT, which is a protein that functions as a DNA repair enzyme upon DNA damage. MGMT enzyme's working principle involves transferring the alkyl group induced to the O6 positioned guanine (Wickström et al., 2015). Therefore, the low expression of MGMT results in failure of O6 guanine's alkyl group to be removed. Consequently, this damage creates mismatches during replication and triggers cell death (Weller et al., 2010) (P. Yang et al., 2015). Therefore, low MGMT levels give countenance to therapeutic approaches

inducing DNA damage in glioblastoma therapy. MGMT promoter's methylation pattern occurring in its CpG island is also essential. Upon methylation of the promoter, expression of the enzyme decreases. Herewith, patients with MGMT promoter methylation are less resistant to DNA damaging therapeutics (Bell et al., 2018). Additionally, glioblastomas are known to commonly have TP53 mutations, a tumor suppressor gene when not mutated. Another known susceptibility factor is the EGFR amplification, and PTEN mutations (Ohgaki et al., 2004) (Brennan et al., 2013). Accordingly, all these established and emerging markers contribute to the great complexity of glioblastoma.

However, factors that affect GBM formation and progression are not limited to mutated coding genes. Non-coding RNAs, microRNAs, crosstalk between cells and microenvironment, molecules and packages passing through Blood Brain Barrier (BBB) and tumor microenvironment support and contribute to the formation and progression of GBM (Valtorta et al., 2020; Crivii et al., 2022). All these factors mentioned above highlight the complexity of the disease where this complexity not only makes the disease challenging to detect but also poses significant difficulties in developing effective treatments.

1.1.1.1 Glioblastoma Diagnosis and Therapeutics

There are several diagnostic methods of the disease. Glioblastoma is often diagnosed by Magnetic Resonance Imaging (MRI) or Computerized Tomography (CT) scans after the doctor's consultation as well as biopsy samples taken from the brain for further histological examination of the tissue and after the diagnosis, the therapy generally includes the surgery of the tumor tissue (Weller et al., 2019) (Nuzzo et al., 2020). However, more frequently, the glioblastoma cells are able to grow inside and between normal brain cells, tumor tissue cannot be removed completely. Often radiation therapy and chemotherapy are applied together where the radiation therapy uses high energy beams focused on the tumor area in the brain (Medikonda

et al., 2021) On the other hand, the most common chemotherapy for glioblastoma includes Temozolomide (TMZ) followed by Lomustine, Carmustine, Irinotecan and Bevacizumab (Bai et al., 2011). Yet, due to drug resistance that is easily developed by glioblastoma cells in treatment and complexity of the tumor, the drugs in clinical use are not totally effective (Dymova et al., 2021) (Goldie, 2001). Although, scientific research field for drug discoveries is always developing, glioblastoma patients are still poorly prognosed with the percentage of survival after diagnosis remaining low as only less than 10% of patients receiving all the treatments live 5 years after diagnosis (Morales & Mousa, 2022) (Bai et al., 2011). Therefore, investigation of new and potential drugs and targeting mechanisms have been promoted for more than a decade (Haar et al., 2012) and there are many new developments in the last five years (Rajaratnam et al., 2020)

1.1.1.2 Drug Targeting

Due to all these reasons highlighting the complexity of GBM, the continuous development of new drug therapies is essential. The term drug discovery refers to new medications designed, tested and eventually applied to the clinic. The stages of drug discovery start with target identification and validation where the molecule or gene is suspected and tested for its responsibility in the formation of the disease. Informatics tools and transgenic or mutant model organisms are highly used for identifying a disease target (Chessell et al., 2005)(Y. Yang et al., 2009). Screening of synthesized drugs or drug libraries follows this step where the drugs or drug combinations with highest potential and best hits are chosen for testing. Then comes the preclinical research where research laboratories take part and test the hit drug or drugs by using primary cells, cell lines and model organisms (Michelini et al., 2010). Viability, reproduction and toxicology tests take the lead in this step as well as molecular identifications such as expression patterns, pharmacokinetics and behavior. Drugs or combinations of drugs that pass these steps are ready to be used in clinical research where human subjects are involved, often

known as phase studies (McFarthing et al., 2022) (Van Norman, 2019). After this step, authorities such as Food and Drug Administration (FDA) give approval to the therapy for further use (Hughes et al., 2011).

For drug targeting and drug-based therapeutics of glioblastoma, drugs that follow Food and Drug Administration (FDA) standards are commonly used. FDA-approved drugs are usually included into additional therapies mentioned above. Although TMZ is the leading FDA approved drug, there are several more, e.g., Lomustine, Carmustine, Carmustine wafer implants, and Bevacizumab, which results in DNA crosslinking as well as cell cycle arrest.(Hochberg et al., 1979; Westphal et al., 2003; Cohen et al., 2009; Fisher & Adamson, 2021). In the last five years, novel drugs or repurposed drugs have also become prominent in the quest to discover better glioblastoma treatments and in the section below I described each, specifically developed for glioblastoma, and others, e.g., sorafenib and trifluoperazine, that have been used for other purposes and had become the subject of repurposing studies. In fact drug repurposing, which means usage of a drug that is approved and in use for a certain disease, is applied and found effective for another disease, makes drug discovery process fast and more effective. Especially when treatment is needed in an urgent manner, drug repurposing is chosen, e.g., during COVID19 pandemic (Parvathaneni & Gupta, 2020) (Reay & Cairns, 2021).

1.1.1.2.1 Candidate New Drugs in the Last Five Years

1.1.1.2.1.1 RTK Inhibitors Potentially Targeting GBM

Receptor tyrosine kinases (RTKs) hold great responsibility in many cellular actions including cellular movement and migration, cell-cell communication, proliferation and increasing sensitivity to apoptosis (Paul & Mukhopadhyay, 2012) (Pottier et al., 2020). The mode of action of RTKs starts with the activation of the receptor with ligands such as EGF and continues with dimerization of the receptors (Maruyama, 2014). Activated RTKs start

phosphorylation cascades within the cell, activating many pathways. Since RTKs are involved in many intracellular survival activities, inhibitors of RTKs are used as cancer therapeutics and targeting (Masuda et al., 2023) (Mortensen et al., 2023) (Vinhas et al., 2017).

Several tyrosine kinase inhibitors are experimented in terms of gliomas as well. One of the recent investigated drugs, R406, a spleen tyrosine kinase inhibitor, was found to be a reversing agent of Warburg effect and activate apoptosis in glioma stem cells (GSCs) (Sun et al., 2019). Another recently highlighted inhibitor, Dasatinib, a Src family tyrosine kinase inhibitor (Milano et al., 2009) is studied in a case study where it was found to be associated with the mesenchymal subtype of glioblastoma (Alhalabi et al., 2022). Dasatinib is also tested with TMZ in GBM cell lines where their combination is found to enhance the therapeutic effect of TMZ (Sun et al., 2019). Another Src kinase inhibitor Bosutinib is recently found successfully triggering reactive oxygen species and subsequently apoptosis of IDH1 mutant glioma cells (Patrick et al., 2021). Continuing with RTK inhibitors, Canertinib, inhibits EGF receptors and found to be decreasing the viability of EGFRvIII expressing GBM cells by inducing apoptosis (Yeo et al., 2021). Canertinib is also mentioned as anti-angiogenic in glioma cells as along with other EFGR inhibitors (Li et al., 2022). Crizotinib targets RTKs such as ALK, MET and ROS1 (Cui et al., 2013) and recently tested in many case studies in the clinic for multiple cancers (Mansfield et al., 2022) including glioblastoma. In one particular study, crizotinib is found as efficiently inhibiting cell viability of MET-positive GSCs while prolonging the life of GSC mouse xenografts (Tasaki et al., 2016). Last but not least, Imatinib, a multiple tyrosine kinase inhibitor studied as a combination element with other cancer drugs, where p53 expression is elevated and proliferation is decreased in glioma cells (Lu et al., 2020). Although all drugs mentioned have not reached to a certain therapeutic conclusion, recent studies emphasize the great potential of RTK inhibitory molecules in glioma.

1.1.1.2.1.2 MAPK Inhibitors Potentially Targeting GBM

Mitogen-activated protein kinase (MAPK) plays an essential role in many cellular pathways including response to stress factors, proliferation, controlled cell death and defense against pathogens (Soares-Silva et al., 2016). Since MAPK pathways are involved greatly in apoptosis and pro-apoptotic cascades, MAPK inhibitors are under active research for cancer treatment (Yue & López, 2020). In mammalian cells, three MAPK pathways are present: ERK1/2, p38 and JNK cascades (Owens & Keyse, 2007). Especially the p38 and JNK pathways are activated upon environmental stress and result in production of immune response molecules such as interleukins and TNF α (Dong et al., 2002).

For all the reasons mentioned, MAPK inhibitors have been started to be frequently used in glioma drug research. One of the MAPK/ERK inhibitors, a molecule called PD0325901, was found to effectively inhibit glioblastoma cells as well as introduce a lower stiffness and viscosity. Besides, this drug is suggested to be related with fibronectin levels in the environment in the study (Shannon et al., 2017). Another study also successfully inhibited GBM cell survival using PD0325901 when combined with a peptide-drug conjugate (Hou et al., 2016). A p38 cascade inhibitor, SB203580 is also found to effectively decrease cell migration of GBM cells (Liu et al., 2014). In another study, SB203580, was used in order to verify the effect on p38 effect in GBM apoptosis (Q. Li et al., 2020). Although another p38 inhibitor called VX-702 is not yet studied in glioblastoma, it has been found to have significant therapeutic effects for breast cancer (Wang, Veeraraghavan, et al., 2021).

1.1.1.2.1.3 GSK Inhibitors Potentially Targeting GBM

Glycogen synthase kinase-3 is one of the kinases with most responsibility within the cell with two common isoforms GSK-3 α and β , actively involved in insulin and Wnt signaling (Patel & Woodgett, 2017). GSKs regulate glucose transfer to form glycogen and its inhibitors are

known to be used in many diseases such as depression-based mental disorders (Sonawane et al., 2021). Many GSK-3 inhibitors are studied in several cancer types including glioblastoma (Mathuram et al., 2016), gastrointestinal cancers (Mai et al., 2009), and pancreatic cancer (Santoro et al., 2020) in the preclinic studies. A GSK-3 inhibitor CHIR99021 combined with another drug forskolin was shown to successfully transform glioma into neurons without any tumorigenic phenotype or characteristics (Oh et al., 2017).

1.1.1.2.1.4 Other Inhibitors Potentially Targeting GBM

Many other kinase inhibitors have been tested for glioblastoma therapeutics in the last five years. AZD 7762, a checkpoint kinase (Chk) inhibitor and Ruxolitinib, a Janus Kinase (JAK) inhibitor are found to have a synergistic killing effect with TMZ when applied together (Chen et al., 2020) (Goker Bagca et al., 2023). Additionally, Necrostatin-1, a Receptor Interacting Protein (RIP) kinase inhibitor, is associated with ROS-induced necroptosis in different GBM cell lines (Huangfu et al., 2021). Another crucial kinase within the cell is mTOR. IN128, an mTOR inhibitor, was shown to potently arrest cell cycle control points of glioma cell lines (Heinzen et al., 2019).

Growth factors such as TGF β act as substrates to activin receptor-like kinases (ALKs) to start growth factor related cascades within the cells including transformation, migration and proliferation (Goumans et al., 2003). Previously, a TGF β inhibitor SB-431542 has been established as a molecule with both viability inhibitor and motility regulator of glioblastoma cells (Hjelmeland et al., 2004). A FGF inhibitor, PD-173974, has also recently been found as inhibiting the viability of different glioblastoma cell lines as well as decreasing the epithelial to mesenchymal transition (Bi et al., 2024).

Drugs used in preclinical and clinical studies for the treatment of glioblastoma are not limited to kinase inhibitors mentioned before. Novel molecules are frequently tested while approved drugs are tried to be repurposed for glioblastoma. A good example is valproic acid (VPA), an FDA approved drug for epilepsy and seizures (Pagliaro & Pagliaro, 2020). Many years after approval of the drug, its histone deacetylase (HDAC) inhibitory effect is discovered (Routy et al., 2012). Since HDACs hold a huge impact on changing chromatin structure and due to their direct effects on gene expression, they are in trials for immune diseases, viral infections and cancer (Duenas-Gonzalez et al., 2008). In the concept of glioblastoma, VPA has been tested and found to effectively increase cell autophagy (W. Han et al., 2021) and apoptosis when combined with TMZ (Z. Li et al., 2018). VPA is also used and found to have potential in enhancing life quality of patients in clinical studies (Kuo et al., 2020) (Su et al., 2020). Another approved drug is Topiramate, a carbonic anhydrase used for epilepsy and migraines. Similarly, topiramate has been repurposed for glioblastoma treatment in mouse model organism and successfully has increased the intracellular acidification and apoptosis (Marathe et al., 2016). Other examples of repurposing can be given as drugs produced as anti-inflammatory agents such as NS-398 and bromodomain inhibitor such as Trotabresib are recently applied to glioblastoma cell lines (Palumbo et al., 2020) and patients (Moreno et al., 2022).

Different from chemically produced molecules, natural compounds also have a place in cancer research, e.g., Emodin. Although only used in Chinese medicine (Sougiannis et al., 2021), this secondary plant product has been tested in various cancer types including glioblastoma after its discovery as a protein kinase inhibitor. Emodin showed evidence to enhance the effectiveness of beam therapy in glioblastoma cells (Kim et al., 2022) and trigger apoptosis and cell cycle arrest in glioblastoma cell lines (Arcella et al., 2018).

1.1.1.2.2 Trifluoperazine

Trifluoperazine (TFP), an antipsychotic, is an FDA-approved drug currently in use for schizophrenia patients and for mood disorder diseases such as anxiety and depression (Marques et al., 2004) (Garakani et al., 2022). The mode of action of TFP includes blocking the dopaminergic receptors by mimicking dopamine in the synaptic cleft, thus, it is referred as dopamine antagonist (Gene et al., 2021). Effects of TFP are also important in terms of muscle contraction because it works as a calmodulin inhibitor as well. Calmodulin regulates Ca^{2+} homeostasis which is very crucial in terms of initiation and continuation of many signaling pathways in the cell (Junho et al., 2020). TFP, as a calmodulin inhibitor, causes raised intracellular calcium levels within the cells. Since the effect of TFP includes both dopamine and Ca signaling, the consequences of TFP treatment in cancer cells are widely studied.

Although there is not much evidence that concludes the positive effect of TFP in zebrafish in terms of glioma, there are several studies where TFP was used *in vivo* in zebrafish, a highly promising vertebrate model for drug screens. In a recent study, researchers showed that TFP lowered the p53 amount and rescued the Diamond-Blackfan anemia phenotype in zebrafish (Taylor et al., 2020). In another study, TFP was found to be increasing the heart rate of zebrafish larvae and the change was dependent on the concentration of the drug. 5 μM TFP has raised the heart rate at 72 hours post fertilization (hpf) different from higher doses (Prill et al., 2023). In an early study, TFP has proven to be blocking cyclic nucleotide-gated current in adult zebrafish (Ma & Michel, 1998).

1.1.1.2.3 Sorafenib

Sorafenib (SFB), a kinase inhibitor, is an FDA-approved drug which is used for many cancer therapeutics including primary renal cell carcinoma, primary hepatocellular carcinoma and thyroid carcinoma resistant to irradiated iodine (Wilhelm et al., 2006). Sorafenib is a small

molecule that inhibits multiple kinases such as Raf and VEGFR that are playing important roles in cancer proliferation and angiogenesis (Rini, 2006). Expectedly, administration of SFB has been shown to diminish tumor growth and angiogenesis (Adnane et al., 2006). Thus, with the ability of affecting a variety of kinases, the value of SFB is advancing in cancer research. In an early study, SFB has been found successfully decreasing tumor burden in mouse xenograft models performed with renal carcinoma cells (Chang et al., 2007) while another study showed sorafenib is able to activate caspase dependent apoptosis in liver cancer cells (Rodríguez-Hernández et al., 2020) followed by many case studies where SFB is found as potential therapeutic agent in patients (Iyer et al., 2010) (Llovet et al., 2008). After FDA approval of SFB, there is a growing demand for repurposing this drug for different kinds of cancers. For instance, SFB was found effective in breast cancer xenograft models in terms of inhibiting p-Erk levels (Adnane et al., 2006), in colorectal cancer when applied with irradiation in terms of minimizing cell growth (Suen et al., 2010) and in glioblastoma (Siegelin et al., 2010). However, sorafenib has side effects that limit its use (Jeon et al., 2020) and hence lowering the concentration could be beneficial and therefore it has been combined with other molecules, e.g., doxorubicin (Richly et al., 2006) and bevacizumab (Azad et al., 2008).

SFB is also tested in zebrafish especially for hepatocellular carcinoma. Anti-proliferative and anti-migratory abilities of Sorafenib are observed in a zebrafish xenotransplantation test (Lin et al., 2019). In another experiment, SFB has demonstrated thyroid disruption in zebrafish (Wei et al., 2022).

1.2 Acetylcholinesterase

Acetylcholinesterase (AChE) is a carboxylesterase, produced by *ACHE* gene, working as a cholinergic enzyme that catalyzes the hydrolysis of acetylcholine, a neurotransmitter, into acetic acid and choline with high fidelity and specificity (Dvir et al., 2010). AChE is found as a

monomer in the cell and dimerizes when activated (Leung et al., 2018). Acetylcholine (ACh) is released to synaptic junctions as a neurotransmitter in order to initiate neuronal signaling and recognized by acetylcholine receptors. By degrading ACh, AChE prevents accumulation of ACh molecules in the synaptic junction and thus, prohibits ACh occupying the receptors. By this way, neuronal signaling is continued and skeletal muscle cells are triggered (Jolla, 2009).

Although *AChE* is mostly found in postsynaptic neuromuscular junctions, it has many other functions in different cell types. ACh is also a growth factor whose regulation affects immune responses such as inflammation, secretion of cytokine and cell differentiation. (Esterlis et al., 2013)(Intachai et al., 2018). *AChE* is found to be associated with many non-neuronal human diseases such as inflammatory bowel disease, heart failure, obstructive pulmonary disease (Richbart et al., 2021). Besides, *AChE* is an important enzyme in neuronal development; and is also associated with human cancers since neurotransmitters are not only synthesizing from neural cells but also cancer cells as well as immune cells (Jiang et al., 2020). ACh, released from non-neuronal cells, is recognized by its contribution to cell proliferation, cell-cell communication, migration, EMT changes and invasiveness of the cells (Wessler & Kirkpatrick, 2017) (Richbart et al., 2021) making modulation of *AChE* a great candidate for cancer research.

Alternative splicing of *ACHE* gene results in different spliced products. Differentially expressed isoforms in different cells are named as: AChE-S (synaptic), AChE-H (hydrophobic), AChE-R (read-through) (Nazim et al., 2017). AChE-S is found to be expressed in apoptotic cells while AChE-R mostly controls inflammatory response and coping with stress factors in the cell (Gilboa-Geffen et al., 2012) (Gnatek et al., 2012). In terms of cancer, AChE-S and AChE-R isoforms are seen in astrocytomas with changing AChE-S expression into AChE-R is associated with aggressiveness of the cancer (Perry et al., 2002). All *ACHE*-R, *ACHE*-S and *ACHE*-H isoforms are expressed in human squamous cell lung cancer, melanoma, colon carcinoma, neck cancer, renal cancer and head cancer (Zhang et al., 2017) (Castillo-González

et al., 2015) (Perry et al., 2002). Benign tumors are also found to be expressing AchE with AchE-R isoform (Grisaru et al., 2007).

Non-neuronal brain cancers like glioblastoma, astrocytoma are found to be expressing AchE with the highest enzymatic activity in all astrocytomas (Barbosa et al., 2001). However, when compared with the normal brain cells, AchE expression is lower in neuronal or non-neuronal cancers (Razon et al., 1984).

1.3 Nicotinic Acetylcholine Receptors

Nicotinic Acetylcholine Receptors (nAChRs) are integral membrane-bound polypeptides that sense and bind to a neurotransmitter called acetylcholine (Taly et al., 2009) as well as a drug called nicotine. nAChRs are expressed in many tissues in the central nervous system and muscles. Hence, they are extremely important for signal transmission, muscle contraction (Ho et al., 2020). Since they are expressed in many tissues and respond to nicotine, their role is highly studied in many cancers with increasing tobacco consumption. It has been shown that 14% of lung cancer incidents are associated with nAChR variations (Hung et al., 2008). Considering AChE is the enzyme that breaks down Ach, understanding the relation between nAChRs and AChE keeps its value for cancer research. However, the effects of increased Ach has not been thoroughly studied in the microenvironment in glioblastoma although there is a study showing its potential in increasing tumor size in liver cancer using xenografts in zebrafish (Avci et al., 2018).

1.4 Zebrafish as a Model Organism

Danio Rerio known as zebrafish is a freshwater fish living around South Asia which started to be used as model organisms in the 1970s for the ease of use when compared to mammals models (Streisinger et al., 1981) (VELDMAN & Lin, 2008). The advantages of this model organism can be listed as rapid reproduction, early development can be observed in

embryos, slightly shorter life cycle, susceptibility to genetic manipulation, physiological, genetic and systemic similarity to humans (Dooley & Zon, 2000). Starting from external fertilization, rapid cell division in the animal pole can be easily observed and allows manipulation such as reagent injection inside the cells. Later, gastrulation and organogenesis followed by hatching from the chorion occurs. Larvae that are now freely swimming pass to juvenile stage at 30 dpf and to adulthood after 90 dpf (Hosen et al., 2013). Transparency of the embryos and larvae makes observation of all developmental stages possible (Hörstgen-Schwark, 1993). Additionally, the similarity in both genetics and physiology between zebrafish and mammals, makes them great substitutes for rodent studies (Kalueff et al., 2014). Ability to use zebrafish embryos provides an enhanced approach to 3R (Replacement, Reduction, Refinement) since the usage of adult model organisms is decreasing.

With the first mutant zebrafish line identification, genes that involve notochord, spinal cord, blood and somites are also identified (Haffter et al., 1996). Now, an abundance of mutant lines is in use for many purposes. Another important use of zebrafish is in the neuroscience field where neurodegenerative diseases, neurotoxicology and behavior neuroscience are extensively studied (Wang et al., 2021) (D'Amora & Giordani, 2018). Drug repurposing area also allows the usage of zebrafish since drug administration is easy with delivery into the aqueous media of zebrafish. Besides, a large number of embryos provides a benefit for great scale of drug screening in many related disease and pathway analysis.

1.4.1 Zebrafish and Cancer

Zebrafish, a vertebrate, is able to generate tumors and embryos allowing visualization of the tumor through the development as well as delivery of small molecules and xenotransplantation. Since major drivers of human cancer can easily be mimicked in zebrafish models, cancer research area is currently in high focus in zebrafish. Transgenic zebrafish

models are established for various types of cancer. For example, for hepatocellular carcinoma (HCC), ribose-5-phosphate isomerase A expressing under *fab10a* promoter develops HCC tumors (Hason & Bartunek, 2019). Mutated KRAS expressing transgenic zebrafish under *ifabp* promoter is studied for intestine cancer (Q. Yang et al., 2019) while transgenic inactivated in *cdkn2a/b* gene in *tp53*^{-/-} genotyped fish shows peripheral nerve sheath tumor (Shim et al., 2017). The zebrafish model is also used for patient-specific small molecule treatment trials since injection of patient-derived tumor cells are possible. This model allows whole organism screening with potential small molecules (McCune, 1992) (Cagan et al., 2019). Either injected with patient-derived cells or cell lines, zebrafish forms a proper model for cancer invasion, extravasation, and metastasis. The transparency of the embryo allows tracking of the cell starting from the injection site to the metastasis route. Finally, angiogenesis, a very crucial step in cancer progression, is observable in zebrafish. Angiogenic factor released by injected cells promote angiogenesis and represent a tumor microenvironment. In an early study, formation of angiogenesis in the zebrafish model correlated with high or poor progression of the tumor (Nicoli & Presta, 2007).

1.4.1.1 Zebrafish and Glioblastoma

The establishment of the zebrafish model in cancer research, specifically glioblastoma, is widely studied (Almstedt et al., 2021). Importantly, brain divisions, neuronal cell differentiation, and neural signaling pathways are largely conserved in zebrafish and humans, making zebrafish an excellent model for brain and brain cancer research (Pensado-López et al., 2020). For mimicking glioblastoma, several transgenic models have been established. To illustrate *smoa1* and *akt* co-expressing fish formed brain tumors (Ju et al., 2009) and *Smoa1* expression and *Shh* activation developed a model for gliomagenesis (Ju et al., 2014), *kras*^{G12V}

mutant expression under neural promoters *gfap* and *krt5* resulted in tumor formation in cranial cavity (Ju et al., 2015). Zebrafish xenotransplantation models also let formation of GBM models. For example, normally non-invasive GBM cell line U87 was separated into CD133⁻ and CD133⁺ subpopulations and shown that CD133⁺ U87 cells were more invasive when injected into the yolk sac of the zebrafish larvae (X. J. Yang et al., 2013).

1.4.1.2 Zebrafish in Xenotransplantation Models

Xenograft models involve transplanting cells into a host animal to study the tumor's growth and response to therapies. Zebrafish are an ideal host animal for xenograft models due to their transparency, small size, and rapid development. Zebrafish xenograft models are also highly emerging due to ease of injection and manipulation of tumors into zebrafish embryo and larvae (Haldi et al., 2006) (Hamilton et al., 2016). Another advantage is the permeability of zebrafish embryos to the small molecules such as drugs and inhibitors. Hence, zebrafish xenotransplantation is also in use for patient specific drug investigation where cells are removed from the tumor of the patient and implanted in zebrafish. This makes it possible to screen many drugs specific to patients thanks to the high reproduction yield of zebrafish (Wu et al., 2017). Zebrafish xenograft models are also established in adult zebrafish in especially immune deficient zebrafish (Langenau et al., 2004). For those purposes, Casper line which has *roy* and *nacre* mutation is efficiently used due to transparency of the body. After injection, localization, characteristic, tumor formation, metastasis and angiogenesis can be screened in zebrafish larvae making these models crucial for cancer studies. For example, in one study, it has been found that a modification where microRNA-330-3p overexpression increases non-small cell lung cancer metastasis to the brain in zebrafish larvae (Fan et al., 2021) Injections are mostly performed into the perivitelline space (PVS), yolk sac, eye and brain while blastula animal pole injections can also be performed (Abercrombie & Ambrose, 1958). For xenograft model purposes, transgenic lines such as GFP expressing lines in their ventricles are highly in use

contributing to the metastatic characteristic of the tumor cell. With enhanced studies in zebrafish xenotransplantation, tools and platforms that collect the data in the literature about those studies has emerged. ZenofishDb, an R shiny application, provided the curated literature data about xenograft models in zebrafish. They have showed that, the most used cell line for xenotransplantation is breast cancer followed by melanoma and glioblastoma (Targen et al., 2020). Modifications to the cells before injection is also common where Targen et al. (2020) showed the leading modification is the overexpression with labelling vectors and silencing of different genes with siRNA treatment (Targen et al., 2020). Overall, all the data with developing zebrafish xenograft knowledge, understanding the tumor behavior rises in cancer research thanks to the zebrafish model.

Brain cancers are one of the most studied cancer types in zebrafish xenotransplantation studies since zebrafish provide a suitable vector for these purposes for several reasons. First, the neuronal system forms early in development (Schmidt et al., 2013). Starting with neuroectoderm determination, neuronal tube and neural plate formation follows (Papan & Campos-Ortega, 1999). After 3 days post fertilization (dpf), zebrafish brain and central nervous system development is completed (D'Amora & Giordani, 2018). Secondly, zebrafish brain development possesses high similarity between mammals. For instance, in a study, different dyes are injected in the blood circulation of the embryo and tracked for hours. At 3 dpf, dye molecules were not able to penetrate to the brain while other tissues such as muscles were well-stained (Zeng et al., 2017). This study indicated the formation of BBB in zebrafish larvae pointing out the similarity with mammals and advantage of early BBB development. Altogether, zebrafish are beneficial in brain cancer xenografts since it is a model for human-analog drug screening, tumor behavior and metastasis even in early development.

As mentioned, brain cancer microinjections such as glioma to the zebrafish is common and favorable. Usage of well-established cell lines for xenotransplantation provides conserved

and trusted information. Yet, they lack generating a personalized investigation and drug targeting. Thus, patient-derived xenotransplantation (PDX) to zebrafish larvae provides the possibility of a variety of personalized medicine. PDX might act differently upon the same drug or treatment in terms of cell viability or metastasis indicating the importance of personalized therapies (Fior et al., 2017). In a study, orthotopic injection of pediatric glioma cells into zebrafish forebrain ventricles has been performed and according to aggressiveness of the cell, different proliferative and metastatic behavior in the organism is observed (Larsson & Kettunen, 2022). Thus, in order to fully understand the cancer mechanism and target to inhibit, PDX holds great value.

1.4.2 *ache* and *p53* genes in Zebrafish

1.4.2.1 *tp53* gene

p53 so called *Guardian of the Genome* is a crucial tumor suppressor and protects the genome by shutting the cell cycle down upon stress of any kind by p21 cyclin-dependent kinase activation or p53-mediated apoptosis (Vousden & Lu, 2002). Mutations in p53 results in loss protection and defected DNA accumulation is seen that leads to tumorigenesis. For example, zebrafish p53 mutant line is shown to be developing malignant tumors in nerve sheaths (Berghmans et al., 2005). Besides, zebrafish p53 shows high similarity to human p53 with 48% identity in amino acid sequence of full length p53 protein (Storer & Zon, 2010). p53 starts to be expressed in high yields from 1 hpf (hour post fertilization) and expression continues in early development until 48 hpf (C. Thisse et al., 2000). Although development proceeds normally, zebrafish p53 knockdown embryos represent a reduced apoptosis pattern especially under DNA stress factors such as UV induction (Langheinrich et al., 2002) revealing p53 is an important apoptosis regulatory element not only in mammals but also in zebrafish. p53 downstream

interactions such as p53-mdm2 relation is also conserved in *D.rerio* (B. Thisse et al., 2000) (Langheinrich et al., 2002).

1.4.2.2 *ache* Gene

The *ache* gene in zebrafish encodes for the enzyme called acetylcholinesterase, that is involved in breaking down the neurotransmitter acetylcholine. This gene has been studied extensively in zebrafish as it plays an essential role at the synaptic transmission in development and function of the nervous system. Mutations in the *ache* gene have been associated to various developmental disorders and neurodegenerative diseases in zebrafish, making it an essential target for research in these fields. After identification of homozygous mutation *ache*^{sb55}/*ache*^{sb55} in zebrafish, it was shown that, non-functional *ache* gene results in complete paralysis of the larvae followed by immortality (Behra et al., 2002). Yet, *ache* is haplosufficient indicating a single allele of wild type *ache* is enough for rescuing paralysis phenotype of the larvae. Heterozygous organisms are able to live to maturity. Yet, there is evidence of *ache* heterozygous individuals have less AChE enzymatic activity (Behra et al., 2002).

1.4.3 Zebrafish Behavior

Zebrafish has emerged as an excellent model organism in behavior neuroscience field as well due to its high fertility rate and ease of pharmaceutical component absorption. Besides, zebrafish behavior assays are in use for brain disorders and mapping the potential damage markers. (Miller & Gerlai, 2007). One often studied pattern, conditioned place preference (CPP), indicating the choice of the animal of localizing in a specific place due to an award and a pattern learned before. For zebrafish, dopamine-related awards are used such as nicotine (Kily et al., 2008) and cocaine (Darland & Dowling, 2001) are commonly used. Also, in one study absence of one allele of *ache* resulted in lower CPP after reward of amphetamine (Ninkovic & Bally-Cuif, 2006).

Many behaviors of the adult zebrafish are also well-established. For instance, raised fin and mouth opening is associated with aggression while loss of movement is linked with ataxia or paralysis. Preference of fish of spending more time near an object is attraction and swimming away from one is called as avoidance, or often called chemotaxis. Another pattern is circling, indicating cycled swimming pattern and it is generally seen after drug application and results in seizure-like phenotype (Wolman & Granato, 2012).

1.4.3.1 Zebrafish Behavior in Light Dark Cycles

Light/dark sensation is also understood in zebrafish behavior and light stimulus is directly regulating the motor activity of the zebrafish (Burgess & Granato, 2007). Scototaxis is the dark preference (seen in adult zebrafish) while scotophobia is dark avoidance (seen in larval zebrafish) (Kalueff et al., 2013). Zebrafish has a natural circadian rhythm with 14 hours of light and 10 hours of dark where zebrafish is more active in light cycles due to lower risk of predation and higher change of feeding in the natural environment (Gahtan et al., 2005) (Neuhauss, 2003). Yet, changing light conditions unexpectedly results in differences in the behavior and response. In one study where fish are kept in constant light showed lower velocity but a higher response when a strong light introduced (Tuz-Sasik et al., 2022). Yet, it was also shown that sudden transition from light to dark illumination rather than circadian rhythm triggers the larval locomotion and velocity while transition from dark to light results sudden loss of activity (Ellis et al., 2012) (Burgess & Granato, 2007). The response against changing light stimuli starts at around 3 dpf and becomes highly activated at 5 dpf (Emran et al., 2008). An example can be given as the O-turn move that represents the angular turn responded to sudden dark transition (Wolman & Granato, 2012). There is evidence that dark to light transition makes larva more stressed and these tests are predominantly used to understand anxiety-like behavior (Lee et al., 2019). Both transition from dark to light and light to dark trigger stress responses which are seen as freeze/stop motion and hyperactivation, respectively (Könemann et al., 2022). In a

recent study, authors mentioned that the decrease in stress response after freeze/stop or hyperactivation movement is related with recovery from anxiety and neurotransmitter release (Mech et al., 2022).

Drugs' effects on zebrafish larvae behavior especially in the field of brain development and brain-related diseases could be studied and differential effects are seen in locomotion and stress responses (Könemann et al., 2022) (Mech et al., 2022). Especially with some of the drugs such as pentylenetetrazole, a GABA receptor antagonist, the response upon light changes is seen to be reversed (Siebel et al., 2015). Increased locomotion in the light after dark period is associated with epileptic behavior in mouse models as well (Shimada & Yamagata, 2018).

1.4.4 Zebrafish *ache* and WT Behavior in Diet and Alcohol Exposures

Recent studies have shown that liver steatosis along with diabetes, metabolic disease and hyperlipemia were associated with psychiatric disease patients in comparison with age and sex-matched controls significantly (H. Li et al., 2023). Moreover, the patients in this study were using different types of drugs some of which included phenothiazines alone or in combination with other drugs (10.9%) however this study did not find any significant differences between drugs with respect to liver steatosis or fibrosis. However, antipsychotics have already been associated with liver disease and steatosis, reviewed in Gangopadhyay et al. (2022). Another interesting study has shown that lipid transport was a significant link between the liver and brain with respect to antipsychotic drugs (Vik-Mo et al., 2009). Mental disorders are not the only brain related pathogenesis associated with liver steatosis. Glioblastoma (GBM), a deadly brain cancer, have also be studied in the context of metabolic syndrome and lipidogenesis. Interestingly, there is evidence that GBM tumors accumulate lipids, yet this does not create lipotoxicity (Guo, 2017). Moreover, higher lipid droplet (LD) formation was associated with lower survival in GBM patients. One of the links between liver and glioblastoma comes from

a drug that has been tested as a new therapeutic for GBM with significant effects in the liver (Altinoz et al., 2020). Caprylic (octanoic) acid, which induces ketogenesis has been found to reduce glycolysis in liver, was found to be effective in high grade gliomas including astrocytomas. Furthermore, there are reports for induction of hepatosteatosis by chemotherapies used in GBM treatment (Miller et al., 2012). Based on these it is important to test GBM therapies in the presence or absence of control and high fat diet (HFD) with or without treatment, or environmental agents, e.g., nicotine that induces steatosis further in mice and humans (Benedé-Ubieto et al., 2021). For this an *in vivo* paradigm is needed by which control and HFD can be combined with such anti-cancer and/or steatosis inducing agents. An example from mouse has shown that DUAL diet (HFD with alcohol) vs chow diet was successful in inducing a state of steatohepatitis (Benedé-Ubieto et al., 2021). Individual treatments with alcohol or high fat diet have been more effective in different genetic backgrounds in zebrafish, e.g., knockout of cirrhosis genes (Shihana et al., 2023). Another example of dual diet comes from zebrafish such that combination of alcohol with B[a]p in HFD results in more toxicity in liver than in standard diet (Bucher et al., 2018). A follow up study has shown that mitochondrial dysfunction as the culprit for transition from steatosis to steatohepatitis in zebrafish models of B[a]p and HFD in the presence or absence of ethanol (Imran et al., 2023). However, there is not a model of dual diet induced behavioral changes in zebrafish that is wildtype or *ache* mutant, which could be relevant to further development of steatohepatitis, and that can be later used in different models of human diseases, including GBM.

Previous studies in zebrafish locomotor behavior used HFD as a variable and showed that HFD reduces locomotion (Meguro et al., 2019). Hence, diet is an important modulator of nervous system activity and whose modulation can be detected by well-established locomotion assays. Moreover, genotypic background affects steatosis/fibrosis and/or locomotory behavior in response to diets as evidenced by multiple different studies in larval or adult zebrafish

(Shihana et al., 2023) (Picolo et al., 2021). Accordingly, locomotory behavior can be used as a proxy to link diet, especially HFD, to behavior, in combination with other environmental and pharmacological factors, and hence brain/nervous system activity.

1.5 Aims and Rationale

In this thesis, a certain workflow (Figure 1.1) is followed for the aims mentioned below.

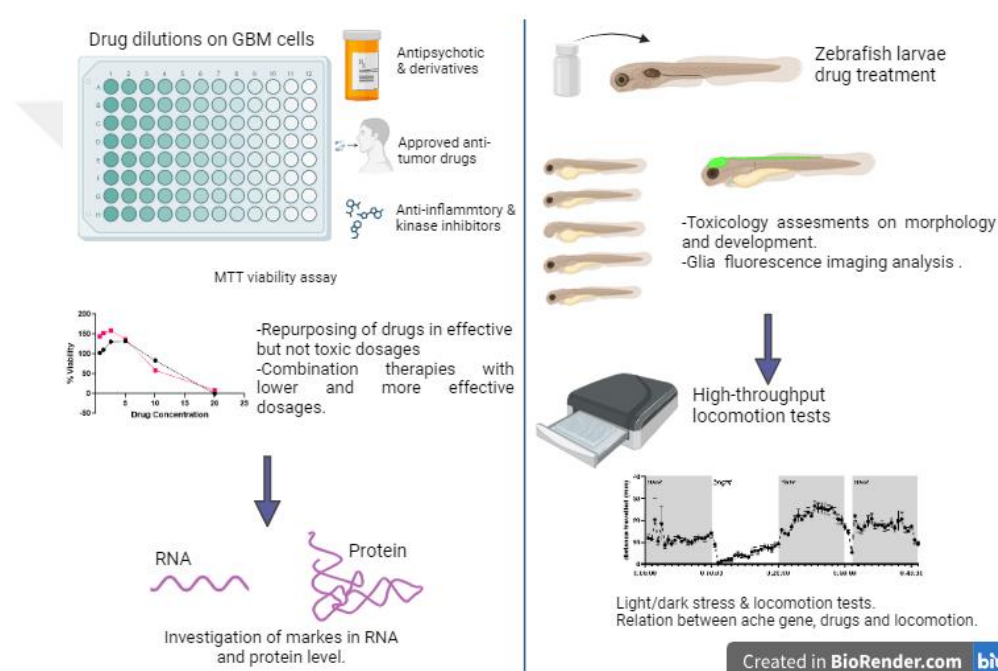


Figure 1.1: Workflow of the thesis.

An illustration of the workflow followed in the thesis study. This image is created with BioRender.com and not subject to copyright.

First and foremost, with the availability of the previously approved and novel synthesized drugs, usage of them in potential glioblastoma therapeutics has been aimed in this thesis. Furthermore, application of combination strategies was a secondary aim to provide a more effective influence in lowering the dosages *in vitro* and *in vivo*. The most used and mentioned in this thesis, are the drugs called trifluoperazine and sorafenib, which were targeted

before by our group in the context of liver cancer (Murat Yaman, PhD Thesis, Bilkent University) using in vitro and in zebrafish models. The rationale behind usage of lower dosages of SFB comes from the previous application of this drug in our lab (Murat Yaman, PhD Thesis; Büşra Korkmaz, MSc. Thesis at Bilkent University) in zebrafish larvae and optimized toxicology. The combination of TFP and SFB also showed great potential in the thesis mentioned above.

Previously, many phenothiazine derivatives were used and applied in cell lines and zebrafish larvae in our lab (Murat Yaman PhD Thesis & Büşra Korkmaz MSc. Thesis). A derivative, molecule **10** ($C_{26}H_{26}ClN_3O_2S \cdot 2.5HCl \cdot 0.6H_2O$) was found as an inhibitor of *ache* activity *in vivo*. This led to the hypothesis of using this drug to see the effect of *ache* mutation in zebrafish behavior and assess the genotype/drug relationship. The background of the genes that were to be investigated in this thesis comes from both literature reviews and previous application of those drugs in our group. Indeed, DDIT3, SRSF7 and SLC7A11 were found significantly altered in TFP and its combination with SFB in HCC RNA-Seq data (Murat Yaman PhD Thesis). CHRNA5 has also been well-studied in our lab and was associated with breast cancer and p53/p21 pathways previously (Burçin İrem Arıcı, MSc. Thesis). With the highlights of our group's extensive previous research, a unique approach is followed for repurposing those drugs in glioblastoma and zebrafish models.

The aims of the thesis are listed below.

- 1) Screening of cherry-picked FDA approved drugs in addition to novel synthesized drugs in different glioblastoma cell lines and obtain an IC₅₀ value.
- 2) Finding effective but not toxic lethal doses and repurposing these drugs that are previously in use for other therapeutics.

- 3) Approaching a more effective and potential drug treatment by combining two drugs together. By this way, lowering the concentration is intended. The selected drug combinations are TFP, molecule **10**, SFB and TFB+SFB and TFP + molecule **10** based on the above-mentioned reasons along with other recent drugs/kinase in the GBM field alone or in combination with TMZ.
- 4) Understanding the mechanism of the selected drugs and combinations in RNA and protein level by qPCR and Western Blot methods respectively.
- 5) Applying individual drugs and drug combination in a living model, i.e., zebrafish larvae to understand drug toxicology and assess morphological changes.
- 6) Understanding the changes in glial expression in the zebrafish model using GFAP: GFP reporter fish by optimizing a high-throughput fluorescence imaging method.
- 7) Understanding the toxicology of drugs by investigating zebrafish motility and locomotion, which could be useful for commenting on BBB possibilities as well as side effects of the drugs studied in this thesis.
- 8) Assessing the relationship between the *ache* gene and drugs via locomotion assays and genotyping in zebrafish homozygous wildtype and heterozygous *ache* mutant siblings.
- 9) Applying the DUAL diet, which includes high fat exposure together with EtOH in *ache* mutant (+/-) larvae and *ache* (+/+) siblings to establish a platform in which zebrafish larvae can be tested to find out the effect of DUAL diet on behavior and stress responses, as a proxy for future drug studies.

CHAPTER 2: Materials & Method

2.1. Materials

2.1.1 Chemicals, Reagents and Kits

Laboratory reagents and chemicals used in general purposes are listed in Table 2.1. Reagents are in use for buffer preparations, RNA and protein assays mentioned in method parts.

Table 2.1: Laboratory chemicals for general use

Product Name	Catalog Number	Company
NaCl	969.033.100	Isolab, Turkey
Dodecyl Sulfate Sodium Salt (SDS)	822050	EMD Millipore, USA
Ethanol	920.026.2500	Isolab, Germany
Methanol	947.046.2500	Isolab, Germany
2-Propanol	961.023.2500	Isolab, Germany
Trizma Base	T1503	Sigma-Aldrich, Germany
2-Mercaptoethanol	M3148	Sigma-Aldrich, Germany
Glycine	GLN001	Bioshop, Canada
Nonidet™ P40 (NP-40) Substitute	74385	Sigma-Aldrich, Germany
N,N,N',N'- Tetramethyl ethylendiamine (TEMED)	110732	EMD Millipore, USA
Ammonium Persulfate (APS)	420627	AppliChem, USA
4x Laemmli Sample Buffer	1610747	Bio-Rad, USA
Dodecyl Sulfate Sodium Salt (SDS)	822050	EMD Millipore, USA
Ponceu-S Solution	P-7170	Sigma-Aldrich, Germany
Tween-20	39796.51	Serva, Germany
Albumin Bovine Modified Cohn Fraction (BSA)	SE1194301	Serva, Germany
cOmplete, EDTA-Free Protease Inhibitor Cocktail	11873580001	Roche, Switzerland
PhosSTOP Easypack Phosphatase Inhibitor Coctail Tablets	4906845001	Roche, Switzerland

Pierce™ BCA Protein Assay Kit	23227	Thermo Fisher Scientific, USA
TGX Stain Free FastCast™ Acrylamide Kit 10%	1610183	Bio-Rad, USA
Amersham ECL Prime Western Blotting Detection Reagent	RPN2232	Cytiva, USA
Clarity Western ECL Substrate	170-5061	Bio-Rad, USA
PageRuler Prestained Protein Ladder	26616	Thermo Fisher Scientific, USA
PVDF Western Blotting Membranes	03010040001	Roche, Switzerland
Agarose	HS-8000	Prona, Spain
QIAzol Lysis Reagent	79306	Qiagen, Germany
Light Cycler 480 SYBR Green I Master	4887352001	Roche, Switzerland
OneScript® Plus cDNA Synthesis Kit	G236	ABM, USA
Ethidium Bromide (EtBr)	17898	Thermo Fisher Scientific, USA
Methyl Cellulose	M-0387	Sigma-Aldrich, Germany
Choloform	24216	Sigma-Aldrich, Germany

Reagents and kits used in cell culture are listed in Table 2.2. A172 and U87 cell lines are kindly gifted from Dr. Ayça Arslan Ergül and Dr. Tuğba Bağcı Önder respectively.

Table 2.2: Reagents for cell culture

Dulbecco's Modified Eagle Medium (DMEM) High Glucose,	BI01-052-1A	Sartorius, Germany
Penicillin-Streptomycin Solution	BI03-031-1B	Sartorius, Germany
Dulbecco's Modified Eagle Medium (DMEM) High Glucose, without phenol red	BI01-053-1A	Sartorius, Germany
Dulbecco's Phosphate Buffered Saline (D-PBS), 10X	BDL-002	Serena Europe, Australia
Trypsin-EDTA Solution, 10X	B.X0930-100	Sartorius, Germany
L-Glutamine Solution	B.X0550-100	Sartorius, Germany
Sodium Pyruvate Solution 100mM	BI03-042-1B	Sartorius, Germany
Fetal Bovine Serum (FBS)		Biological Industries, USA
Sterile-filtered. Dnase/Rnase/Protease free water	WAT333.1L	Bioshop Canada, Canada

Dimethyl Sulfoxide (DMSO)	A3672	AppliChem, Germany
MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)	M6494	Invitrogen, ThermoFisher
Trifluopeazine	T8516	Sigma-Aldrich, Germany
Sorafenib	S7397	Selleckchem
Hs_TP53_7 FlexiTube siRNA	SI02623747	Qiagen, Germany
All-Star Negative Control siRNA	SI03650318	Qiagen, Germany
HiPerfect Transfection Reagent	301705	Qiagen, Germany

Machines and equipment used in the experimentation that are mentioned in the materials part are listed in Table 2.3 according to their purpose of use.

Table 2.3: Laboratory equipment and purpose of use

Equipment Name	Company	Purpose
NanoDrop ND-1000	Thermo Fisher Scientific, USA	DNA & RNA quantification
PCR Thermal Cycler 2720	Applied Biosystems, USA	PCR & cDNA synthesis
LightCycler 96 Instrument	Roche, Switzerland	qPCR
Synergy HT Microplate Reader	BioTek, USA	BCA & MTT assay reads
The EVOS® FL Auto Imaging System	Thermo Fisher Scientific, USA	Imaging of cells and zebrafish
Themolyne Dri-Bath Heater	Themolyne, USA	DNA extraction & Protein sample preparation
FemtoJet Express	Eppendorf, Germany	Microinjection
MN 120 Microbiological Safety Cabinet	Nüve, Turkey	Cell culture
Nuaire Autoflow IR Direct Heat CO ₂ incubator	Nuaire, UK	Cell Culture
Universal 320 R Centrifuge	Hettich, Germany	Cell Culture
Nuve NB20 Water Bath	Nüve, Turkey	Cell Culture

1.5 mm Mini PROTEAN® 3 System Glass Plates	1653312	Western Blot
Thermo EC300XL2 Power Supply	EC300XL2	Western Blot
Trans-Blot Turbo Transfer System	690BR011989	Western Blot
Amersham™ Imager 600	GE Healthcare Life Sciences, USA	Western Blot
Thermolyne Roto Mix shaker	Thermolyne, USA	Western Blot

Primer sequences that are used for either qPCR expression analysis or genotyping are listed in Table 2.4 according to specific organism and purpose of use.

Table 2.4: Primer list

Gene name	Forward Sequence (5' to 3')	Reverse Sequence (5' to 3')	Organism	Purpose
Ache	AcheS: ACACGTGCCATATTGCAGAG AcheN: ACACGTGCCATATTGCAGAA	CTGCTCCAGGGAAGAACTTG	Danio rerio	Genotyping/qPCR
DDIT3	GTAAAGATGAGCGGG	TGGATCAGTCTGGAAAA	Homo sapiens	Expression analysis/ qPCR
SRSF7	GGTCTAGATCACATTCTCG	CCAGACCTAGATCTTCTG	Homo sapiens	Expression analysis/ qPCR
SLC7A11	TCTTTGTTGCCCTCTCC	GCTGGTAGAGGAGTGTGCTT	Homo sapiens	Expression analysis/ qPCR
CHECK1	TGGTCACAGGAGAGAAGGCA	CAGATAAACCACCCCTGCCA	Homo sapiens	Expression analysis/ qPCR
CD44	TGCCGCTTTGCAGGTGTATT	CCGATGCTCAGAGCTTTCTCC	Homo sapiens	Expression analysis/ qPCR

IL1B	TGGCAATGAGGATGACTTGTTTC	CTGTAGTGGTGGTCGGAGATT	Homo sapiens	Expression analysis/ qPCR
CCNE1	GCCAGCCTTGGGACAATAATG	CTTGACGTTGAGTTTGGGT	Homo sapiens	Expression analysis/ qPCR
Bax	GGGTTGTCGCCCTTTTCTAC	CTGGAGACAGGGACATCAGT	Homo sapiens	Expression analysis/ qPCR
CDK1	GGATGTGCTCAGGATTCC	CATGTACTGGAGGGATAG	Homo sapiens	Expression analysis/ qPCR
MIK67	GTGTCAAGAGGTGTGCAGAA	GCCTTACTTACAGAATTCAC	Homo sapiens	Expression analysis/ qPCR
N-Cadherin	ACAGTGGCCACCTACAAAGG	CCGAGATGGGGTTGATAATG	Homo sapiens	Expression analysis/ qPCR
CHRNA5	AGATGGAACCCTGATGACTATGGT	AAACGTCCATCTGCATTATCAAA	Homo sapiens	Expression analysis/ qPCR
VIMENTIN	CGTCACCTTCGTGAATACCA	CCAGAGGGAGTGAATCCAGA	Homo sapiens	Expression analysis/ qPCR
TPT1	GATCGCGGACGGGTTGT	TTCAGCGGAGGCATTTC	Homo sapiens	Expression analysis/ qPCR

Antibodies used in Western Blot experiments, catalog number, company and working condition is listed in Table 2.5.

Table 2.5: Antibody list.

Antibody	Catalog No	Company	Working Condition
Bax	D2E11	Cell Signaling Technologies	1:1000 diluted in 5% BSA/TBST
Vimentin	ab8069	abcam	1:1000 diluted in 5% BSA/TBST

2.1.2 Solution and Buffer Recipes

Preparation of buffers and solutions mentioned in the methods and results parts are listed in

Table 2.6. Used solutions and buffers are separated into related categories.

Table 2.6: Solution and Buffer Recipes

Name	Recipe
Cell Culture	
Complete DMEM	50 ml FBS, 10 ml 100 mM Sodium Pyruvate, 5 ml Penicillin/Streptomycin is added on top of 435 ml DMEM
1X PBS	5 ml 10X PBS is added on top of 45 ml sterile ddH ₂ O.
1x Trypsin-EDTA	5 ml 10X Trypsin-EDTA is added on top of 45 ml 1X PBS
MTT/DMEM Solution	For one 96-well plate, 5 mg of MTT is weighted and 1 ml 1X PBS is added on top. Dissolved well and filtered 0.45 µm filters into 10 ml phenol red-free complete DMEM.
SDS/HCl solution	For one 96-well plate, 1 gr SDS is weighted and dissolved in 10 ml 0.01M HCl.
Western Blot	
Protease Inhibitor (PI)	1 tablet of cOmplete PI is dissolved in 420 µl ddH ₂ O to make 25X PI
Phosphatase Inhibitor (PhosStop)	1 tablet of PhosStop is dissolved in 500 µl ddH ₂ O to make 20X PhosStop
RIPA Buffer	75 µl of 2M NaCl, 50 µl of 1M Tris-HCl (pH = 8.0), 10 µl 10% SDS, 10 µl NP-40 buffer, 40 µl of 25X PI, 40 µl of 20X PhosSTOP is mixed and filled up to 1 ml with ddH ₂ O.
SDS Loading Dye (4X)	50 µl of β-mercaptoethanol is added into 450 µl 4x Laemmli buffer.
10% APS	0.1 g Ammonium persulfate (APS) is dissolved in 1 ml of ddH ₂ O.
Running Buffer (10X)	144 g Glycine, 10.08 g of SDS and 30.3 g of Trizma Base is mixed and dissolved in 1 liter of ddH ₂ O.
Anode I Buffer	18.15 g of Trizma Base is added into 100 ml of Methanol and filled up to 500 ml with ddH ₂ O.
Anode II Buffer	1.5 g of Trizma Base is added into 100 ml of Methanol and filled up to 500 ml with ddH ₂ O.

Cathode Buffer	2.62 g of 6-aminocaproic acid is added into 100 ml of Methanol and filled up to 500 ml with ddH ₂ O.
Blocking Solution (5%)	2.5 g of BSA is added into 50 ml of 1x TBS-T.
Mild Stripping Buffer	1.5 g Glycine, 0.1 g SDS and 1 ml Tween20 is dissolved, pH is adjusted to 2.2 and volume is filled up to 50 ml with ddH ₂ O.
10X TBS	12 g Trizma Base, 44 g NaCl is mixed and dissolved. pH is adjusted to 7.6 and filled up to 500 ml with ddH ₂ O.
1X TBS	50 ml 10X TBS is added into 450 ml ddH ₂ O.
1X TBS-T	50 ml 10X TBS and 1 ml of Tween20 is added into 450 ml ddH ₂ O.
Zebrafish Experiments	
E3 medium (60X)	17.2 g NaCl, 2.9 g CaCl ₂ ·2H ₂ O, 4.9 g MgSO ₄ ·7H ₂ O, 0.76 g KCl is dissolved in 1 liter ddH ₂ O.
E3 medium (1x)	16.6 ml is taken from 10X stock and added into 1 liter ddH ₂ O 2-3 drops of methylene blue (0.01%) is added on top.
Tricane	400 mg tricane and 2 ml 1M Tris (pH: 9) is mixed. pH is adjusted to 7.5 and volume is filled up to 100 ml with ddH ₂ O.
DNA extraction buffer	2 ml 2.5M Tris, 1 ml 0.5M EDTA, 10 ml 1M NaCl, 2.5ml 10% SDS is mixed, and volume is filled up to 50 ml with ddH ₂ O.
Methylcellulose (3%)	6 g methylcellulose is added into 70 ml hot ddH ₂ O (80°C). 130 ml cold water (chilled in -20°C) is added on top and solution is chilled at 4°C.

2.2 Methods

2.2.1 Cell Culture Methods

Cell culture procedures were held in cell culture fume hoods and cells were incubated in the cell culture incubators shown in Table 2.3 .

2.2.1.1 Cell Thawing

Frozen cells that stored in cryovials as 1 ml at -196 °C liquid nitrogen tanks were thawed in 37°C water bath incubators quickly. When majority of the cells were melted, cryovials were placed in cell culture hoods and 8 ml pre-heated high glucose DMEM containing 10% FBS, 1% Penicillin/Streptomycin, 2% Na-P was mixed with the cells in a 15 ml tubes. Cells were centrifuged at 1500 rpm for 5 minutes. Supernatant was discarded using vacuum without

touching the cell pellet and 1 ml fresh media was put on top. Cells were suspended well and seeded into 25-cm flasks that contain 5 ml media. Cells were incubated at 37°C 5% CO₂ incubators until attached.

2.2.1.2 Cell Passaging

Cells that reached 70% confluency were washed with 6 ml pre-heated 1X PBS and 1 ml 1X Trypsin-EDTA was added for 25-cm flasks. Cells were incubated at 37°C for 2 minutes until detached. Trypsin was deactivated with 3 ml DMEM, and a part of the suspension was placed in a 15-ml tube. Amount of the cells to be passed was determined according to cell confluency. Centrifugation at 1500 rpm for 5 minutes was done and the supernatant was discarded. Cell pellet was dissolved in 1 ml fresh media and seeded in 8 ml DMEM containing 10-cm plates or 75-cm flasks. Rest of the cells were frozen.

2.2.1.3 Cell Freezing

Detached cells as described above were suspended in 10% DMSO containing DMEM. The total volume was determined according to the vial of cells to be frozen. Properly suspended cells were split as 1 ml per cryovial. Vials were gradually frozen in a decreasing manner as 4°C, -20°C, -80°C, -196°C.

2.2.1.4 Cell Seeding

Cells were washed with 6 ml pre-heated 1X PBS and 1 ml 1X Trypsin-EDTA was added. After cells were detached, 3 ml DMEM was applied for trypsin deactivation. After centrifugation at 1500 rpm for 5 minutes, supernatant was discarded, and 1 to 4 ml fresh media was added according to the amount of cell pellet. After cells were suspended in the media, 10 microliters were taken and loaded to the hemocytometer for cell seeding. By multiplying cell count with 10⁴ the total cell number in 1 ml media was found. Desired volume of media

containing cells was taken according to the number of cells that need to be seeded and mixed with the total media amount needed for the whole seed. Cells were equally distributed to the wells of multi-well culture plates. The number of media was fixed as 100 μ l, 1 ml, 2 ml for 96-well plate, 12-well plate, 6-well plate respectively.

2.2.1.5 Drug Treatment

Cells were seeded as 7000 cells/well and 10000 cells/well for U87-MG and A172 cell lines respectively and let attach for 24 hours. The next day drug treatment was performed. Starting with an unknown effect drug, a high concentration of drug (generally 20 μ M) was used as the initial dosage and serial dilutions were performed in 96-well plates with a multichannel pipette. Required amount of the stock was measured by a simple calculation of $M1 * V1 = M2 * V2$ where M1 was the drug stock concentration, V1 was the amount to be calculated, M2 was the starting concentration and V2 was the final volume needed. The calculated amount was taken and diluted into complete DMEM and content of DMSO was calculated as Volume taken from stock / DMEM volume. For the baseline, a DMSO/DMEM solution of the same percentage was prepared. 150 μ l of DMSO/DMEM was put on each well of the plate. For the first dilution, 150 μ l of starting concentration was taken and added on top of the first lane. A simplified explanatory scheme was seen in Figure 2.1. For the serial dilutions, 150 μ l from the first lane was measured with multichannel pipette and added on top of the second lane. By this way, the volume was doubled while concentration was divided into half. Serial dilutions were continued the same way by mixing up and down every time. The last lane contains no drugs and was used as a DMSO control further.

and spined down. Mix was incubated for 10 minutes at RT for the transfection complex to form. Complex was mixed with cell media and distributed as 100 µl/well.

2.2.1.7 MTT Cell Viability Assay

Cells were seeded as 7000 cells/ well and 10000 cells/ well for U87-MG and A172 cell lines respectively into 96-well plates as described above. After 24 hours, cells were checked for complete attachment. Drug treatments were applied, and cells were incubated at 37°C 5% CO₂ incubator for 72 hours without any media change. 5 mg of MTT powder was weighted and dissolved in 1 ml 1X PBS. MTT/PBS solution was filtered through 0.45 µm filters into 10 ml of phenol-red free high glucose DMEM for removal of any powder precipitate. MTT solution was protected from light until use and was not used after 7 days. At 72nd hour, media of the cells were removed carefully and 100 microliters of MTT/DMEM were given to each well by using a multi-channel pipette. To prepare SDS/HCL solution, 1-gram SDS was weighted into 10 ml of 0.01M HCl. After 4 hours of incubation at 37°C, prepared SDS/HCl solution was given to each as 110 microliters without discarding the MTT/media. After 18 hours of incubation at 37°C, absorbance of the wells was read at 570 nm by using a multiplate reader.

2.2.2 Gene Expression Analysis Methods

2.2.2.1. RNA Isolation

For RNA isolation 200,000 cells were seeded per well and cells were let attached for 24 hours. On the other day, the planned treatment was applied. After 72 hours, the old media was removed, and cells were washed with 1X PBS twice. 500 microliters of 1X Trypsin-EDTA was added on top of each well and incubated for 2 minutes. Detached cells were collected into respective tubes and snap frozen in liquid nitrogen. If pellet storage was required, tubes were stored at -80 fridges. If not, RNA isolation process takes place under fume hood starting with

600 microliters of Trizol that was added to cell pellets. 120 microliters of chloroform were placed on top and tubes were inverted 10-15 times. Tubes were let incubated at room temperature for 10 minutes. After, tubes were centrifuged at 13.000 rpm 20 minutes 4°C. During this time new tubes were prepared with 380 microliters of isopropanol. After centrifugation, three phased mix was obtained, and 120 microliters of the upper colorless part was taken for RNA and placed in tubes with isopropanol. Isopropanol was sterile and filtered with 0.45 micrometer filters. Tubes were inverted 10 times and incubated at room temperature for 10 minutes. Centrifugations with the same conditions were performed and after, the supernatant was discarded. 1 milliliter of 75% ethanol was added on top of the pellet that contained RNA. Ethanol must be also sterile and was filtered using 0.2 micrometer filters. Centrifugation with 13.000 rpm 8 minutes 4°C was performed and the supernatant was discarded. 1 milliliter of 100% ethanol was added and centrifuged as previously. Then, the supernatant was removed, and pellets were let air dry under the fume hood for around 20 minutes until no alcohol droplets were present. Pellet was dissolved in 20 microliters of sterile nuclease-free water. Concentration and quality were checked by using a Nanodrop machine. If storage of RNA was required, tubes were placed in -80°C fridges.

2.2.2.2 cDNA Synthesis

For cDNA synthesis OneScript® Plus cDNA Synthesis Kit (abm, USA) was used. For each sample, 4 µl 5X reaction buffer, 1 µl dNTP mix, 1 µl Oligo d(T) mix, 1 µl Reverse Transcriptase enzyme was required. Master mix of mentioned ingredients were prepared according to sample size. Required volume of RNA for obtaining 800 ng of RNA was taken and each reaction volume was filled up to 20 µl with sterile nuclease-free water in PCR tubes.

Reaction was run at 55°C for 15 minutes followed by inactivation step at 85°C for 5 minutes.

cDNA's were stored at -20°C.

2.2.2.3 Quantitative Real-Time PCR

LightCycler 480 SYBR Green I Master (Roche, Switzerland) was used for quantitative real-time PCR. For each well 5 µl 2X SYBR mix, 1 µl forward primer, 1 µl reverse primer, 1 µl sterile nuclease-free water was needed. For each primer, master mix was prepared according to the required well number. 8 µl of master mix was distributed to each well and 2 µl of DNA of interest was added into the wells of qPCR plate. Plate was centrifuged at 1000 rpm for 30 seconds and placed in LightCycler 96 Instrument. Reaction protocol was given in Table 2.7.

Table 2.7: RT-qPCR reaction protocol

Cycle Name	Temperature (degrees)	Duration (seconds)	Acquisition Mode	Ramp °C/s
Preincubation	95°C	300s	none	4.40 °C/s
3-step Amplification (45 cycle)	95 °C	10s	None	4.40 °C/s
	55-70 °C	20s	Single	2.2 °C/s
	72 °C	20s	none	4.40 °C/s
Melting	95 °C	5s	None	4.40 °C/s
	55 °C	60s	None	2.2 °C/s
	95 °C	1s	continuous	0.2 °C/s
Cooling	40 °C	30s	none	2.20 °C/s

2.2.2.4 qPCR Analysis

For qPCR relative expression analysis was done according to the $\Delta\Delta CT$ method (Livak & Schmittgen, 2001). First, the average of technical replicas was taken. Mean of the control group's $C(t)$ values were subtracted from every other group ($-\Delta C_T$) and $2^{-\Delta C_T}$ was taken. Same calculations were done to housekeeping genes as well. Normalization was done by dividing $2^{-\Delta C_T}$ (gene) to $2^{-\Delta C_T}$ (housekeeping). Finally, the obtained $2^{-\Delta\Delta CT}$ value was transformed to logarithmic base 2 and relative expression graphs were plotted.

2.2.3 Protein Assays & Analysis

2.2.3.1 Protein Isolation

For isolation of proteins, the culture plate well's media was discarded and 1 milliliter for 6-well plate and 500 microliters for 12-well plate of cold PBS was added and discarded. PBS of specified volumes were placed one more time and cells were scraped well with a sterile cell scraper. Collected cell suspension was centrifuged for 5 minutes at 1500 rpm. Rest of the protocol was performed outside of the cell culture hood and cell pellets were kept on ice. RIPA buffer was prepared as described in Table 2.6. PBS of the cells were discarded, and 60 microliters of RIPA buffer was added on top of pellets. Tubes were incubated on ice for 30 minutes with vortexed every 5 minutes for 10 seconds. After 30 minutes, tubes were centrifuged at 13,000 rpm 20 minutes 4°C . Supernatant was taken and placed in a new tube and labeled well. If storage needed proteins were kept in -80°C .

2.2.3.2 Protein Quantification

Investigation of protein concentrations was done via Pierce™ BCA Protein Assay Kit (Thermo, USA). Proteins were diluted 1:100 as 4 microliters of protein was mixed with 396 microliters of ddH₂O. BSA standards were prepared as described below.

Table 2.8: BSA standards dilution preparation.

Concentration (µg /ml)	100 µg / ml BSA stock (µl)	ddH ₂ O(µl)
0	0	400
10	40	360
20	80	320
30	120	280
40	160	240
50	200	200
70	280	120
100	400	0

BCA working solution was prepared by mixing 50 volumes of BCA reagent A and 1 volume of reagent B and vortexed shortly. Prepared BCA mix was distributed into 96-well plate as 100 µl per well. 100 µl BSA standard or protein dilution was put on top of the BCA mix as triplicates. Plate was covered with aluminum foil and incubated at 37°C for 30 minutes. After incubation, absorbance was read in Synergy HT Microplate Reader at 562 nm.

After reads were collected from the machine, average of triplicates was taken, and average blank absorbance read was subtracted from the sample absorbance reads. A standard curve with equation was plotted with known concentration on the x-axis and read values on the y-axis. Obtained protein sample's read was substituted for y in the equation and x, which was the concentration was drawn. Found concentration was multiplied by 100 to determine

undiluted protein concentration. For the sake of easy protein sample preparation for western blotting concentration was converted into $\mu\text{g}/\mu\text{l}$ by dividing concentration into 1000.

2.2.3.3 Western Blot

Desired amount of protein (generally 40 ng) was taken and mixed with 1:10 β -mercaptoethanol containing 4x Laemmli buffer. Laemmli buffer must be drawn to 1x working concentration and volume was filled up with RIPA buffer. WB samples were incubated at 95°C heater for 5 minutes followed by 5 minutes room temperature and 5 minutes ice incubation. Proteins were ready for loading into SDS-PAGE.

SDS gel was prepared by using 10% TGX Stain Free FastCast™ Acrylamide Kit (BioRad, USA). 1.5 mm Bio-Rad Mini PROTEAN® 3 System Glass Plates were set together. For separating gel 4 ml of solution A and B was mixed and 40 μl of freshly prepared 10% APS was added followed by 4 μl TEMED addition. Mix was vortexed and loaded between glasses. After the gel was hardened, stacking gel was prepared as 1.5 ml of A and 1.5 ml of B solution, 15 μl APS and 3 μl TEMED. Stacking gel solution was poured on top of the hardened separating gel and comb was placed without any bubbles formed in the gel. After 20 minutes, gels were completely hardened and placed into Bio-Rad Mini PROTEAN® TETRA System tanks filled with 1X running buffer. Protein samples were loaded as 6-15 μl for desired protein amount and 4 μl of protein ladder was loaded. Gel was run at 80V for stacking and 120V for separating gels with EC300XL2 power supply. After loading dye left the gel, run was stopped, and proteins were transferred to the PVDF membrane. Transfer was done by using Bio-Rad Trans Blot® Turbo Semi-Dry Transfer system. Sandwich system was formed by placing 4 Whatman papers soaked into the Anode I buffer, 2 into the Anode II buffer. PVDF membrane was activated with methanol for 1 minute on a shaker and placed on top of the Whatman papers. Gel was carefully removed from the SDS-PAGE glasses and put on top of the membrane. The sandwich was

completed by placing 6 Whatman papers soaked in Cathode buffer. Roller was used to remove any bubbles formed between the papers, membrane and gel. Excess buffers were removed from the system and lid was firmly closed. Transfer machine was run with the protocol of 1.3A 25V 55 minutes.

After transfer, the membrane was stained with Ponceu S dye and excess dye was washed off with distilled water. Pictures of the dyed membrane were taken, and the membrane was blocked with 5% BSA for 1 hour on a shaker with mild shaking. After blocking, membranes were washed with 1X TBS-T for 2 minutes two times. Meanwhile, antibodies were diluted in 5% BSA as described above in Table 2.5. Membranes were placed in antibodies overnight at 4°C or for 1 hour at room temperature. After primary incubation blots were washed with 1X TBS-T 3x10 minutes in high shaking. Then, secondary antibodies were prepared in 5% BSA. Incubation was done for 1 hour with mild shaking. After, blots were washed with 1X TBS-T 3x10 minutes. For visualization, Amersham ECL Prime WB Detection Reagent or Bio-Rad Clarity Western ECL substrate kit was used by mixing A and B solutions together as 1:1. ECL was put on membranes and incubated in the dark for 1 minute. Blots were visualized with Amersham Imager 600 chemiluminescence mode.

2.2.3.4 Signal Quantification from Blots

For the quantification of signal on the membranes, Image J application was used. First, image was converted to 8-bit and lanes were rotated to be horizontal fully. Under the analyze menu, gels were selected and the lane which was subjected to analysis was selected. From the same Gels menu, plot lanes were clicked. The graph's limits were defined by hand-drawing and label peaks selection was done under the same menu. All procedures were done to control protein as well. Quantification value of each sample's control protein was subtracted from the

protein of interest. Normalization was done by using biological control via subtracting biological control's average from all other samples. Results were graphed on log₂ base.

2.2.4 *In Vivo* Experiments

2.2.4.1 Zebrafish Husbandry and Embryo Collection

Adult zebrafish were kept in 27°C system water with a circadian rhythm of 14 hours of light/ 10 hours of dark. Feeding was performed everyday two times with dry flake in the mornings and dry flake combined with artemia in the afternoon. A day before embryo collection, one or two females were placed in a breeding set up with one male adult zebrafish of interested genotype. The breeding set-up includes a divider that separates male and female for the night. In the first hour of day circadian rhythm was started, the separator was removed, and fish were let interact. Embryos were ready to collect in the afternoon. Male and female were put back to their respective tanks and embryos were collected in a strainer. After embryos were washed with dH₂O in the strainer, they were placed in petri dishes as 100 embryo/ dish with E3 media (recipe can be found in Table 2.5). Embryos were incubated in 27°C incubators. The day after, dead or undeveloped embryos were discarded and E3 media was refreshed. Prior to experimentation, larval stages were examined under stereomicroscope and underdeveloped larvae were separated, not used for further assays.

2.2.4.2 Larval Drug Treatment

The drug treatments were started at either 2 dpf or 3 dpf. When drug treatment was started at 2 dpf, dechoriation was required. For this, chorion of the embryo was ripped with needles without harming or touching any part of the organism. For 3 dpf larvae, such a step was not needed since all fish leave the chorion naturally at the end of 2 dpf. For drug treatment, the required number of larvae was chosen randomly from all batches and placed in 96-well plates as one larvae per well. Drug dilutions were performed in E3 fish media as required for final

concentration. Old media of the fish were discarded before adding 100 μ l of drug dilution of interest. The drug media was discarded and renewed with the same concentration and volume every day until 5 dpf when imaging, behavior analysis or RNA extractions were done.

2.2.4.3 Behavior Assays

For behavior assays, Zantiks MWP Instrument (Zantiks Ltd., Cambridge, UK) was used. All experiments were performed between 13.00 – 17.00. Before the behavior test was run, room temperature was set to 27°C, drug media was changed with the same volume of fresh E3 fish water. Fish plate was let incubated in the incubator for 15 minutes and 96-well was placed in the instrument. A 10-minute dark acclimation period has been used. Furthermore, distance traveled data was collected in 10 minutes dark, 10 minutes bright, 10 minutes dark, 1 minute bright, 10 minutes dark periods with a time bin of 30 seconds (Appendix Figure 1). At the end of the locomotion test, 50 μ l of Tricaine was added on top of each well and fish were euthanized. All larvae individually were placed in different 1.5 ml tubes if genotyping was required. After the total distance travelled data were collected, the XY graph that contains distance travelled in each time period on the y in millimeters and the time in seconds was put on the x axis; and graphed. The transition from light to dark period was graphed separately and a regression analysis was done with slope and intercept comparisons. Also, the total distance travelled in each period was summed and plotted in log₂ base. Additionally, 10 was added to each period's total distance travelled data and a normalization was done by dividing the 2nd and 3rd dark period by baseline (1st dark period).

2.2.4.4 Brain Injection to Zebrafish Larvae

To make xenografts, U87-GFP cells were used. Cells were trypsinized, collected and counted. 150.000 cells were put into a new tube and centrifuged. Cell pellets were washed with 500 μ l of 1X PBS. Lastly the pellet was dissolved in 25 μ l PBS/phenol red mixture. 2 dpf

zebrafish larvae were dechorionated if necessary, anesthetized and lined into the 2% agarose mold plates. 5 µl cell mixture was taken and loaded into microinjection needles. Approximately 6 nl was pumped that corresponded to 30/50 cells per larvae. Injected larvae were placed in petri dishes containing fresh E3 media and incubated in a 32 degrees incubator.

2.2.4.5 DNA Extraction from Zebrafish Larvae

After a behavior assay, each euthanized larva was placed in 1.5 ml Eppendorf tubes separately and snap-frozen in liquid nitrogen. Larvae were thawed in ice and excess fish media was removed. 200 µl DNA extraction buffer (100 mM Tris pH:8.0, 10 mM EDTA pH:8.2, 200 mM NaCl, 0.5% SDS) was added with 1.5 µl Proteinase K (20 mg/ µl) (AppliChem, A4392) inside and incubated at 55°C for 18-20 hours. The day after, enzyme activity was stopped at 95°C for 20 minutes and tubes were centrifuged at 13.000 rpm for 20 minutes. Supernatant was taken and same amount of isopropanol was added for DNA precipitation. After centrifugation at 13.000 rpm 4°C for 20 minutes, the pellet was washed with 70% sterile ethanol. Centrifugation was done with same conditions and pellet was let air-dry before dissolving in nuclease-free water. Extracted zebrafish genomic DNA was subjected to genotyping.

2.2.4.6 *ache* Genotyping

In order to genotype the mixed population of *ache* (+/?) larvae, quantitative real-time PCR was done with allele specific primers of AcheN, AcheS and AcheR (Primer sequences were listed in Table 2.4). AcheN and AcheS represented the forward primers where S indicated wild-type allele while N indicated mutant one. AcheR was the reverse primer that was common for both forward primers. Each qPCR reaction was run with LightCycler 480 SYBR Green I Master (Roche, Switzerland) in LightCycler 96 Instrument. Resulting C(t) values between AcheS and AcheN mixes were compared as described previously (Avci et al., 2018).

2.2.4.7 Signal Quantification from Larvae

For investigation of GFAP: GFP signal from treated larvae, after anesthetization with Tricane, The EVOS® FL Auto Imaging System (USA) was used for fluorescence scanning. All larvae were placed on a microscopy slide and positioned with 3% methylcellulose. They were arranged and positioned laterally (on top of one eye, yolk faces down) on a slide and 4x scan mode was chosen with fluorescence GFP filter. Images were taken as scans. Larvae were placed in multi-well plates in the same order. After 24 hours of drug exposure, the larvae were imaged in the same way with the same order of larvae as well as the imaging was repeated after 48 hours of treatment (5 dpf). In order to quantify the GFP intensity, image J application (Schneider et al., 2012) was used. An area of individual fish with the signal present was hand-selected. The same area was copied and pasted into a background close to the larvae of interest. Then, from the measure tab, integrated density was measured. All larval data were normalized to its closest background by subtracting the background density from the GFP density. Each larva in each treatment group was normalized to its signal in the pretreatment day (3 dpf) by subtracting the log intensity density of the day of interest from log intensity density of pretreatment day. Graphs were performed using the GraphPad Prism application (Version 9.0.0).

2.2.4.8 Larval DUAL Diet Introduction

The larvae were placed in 6-well plates as 10-15 larvae/well starting from 5 dpf until 12 dpf. Each well was fed by a 2 mg/ml control larval diet (JBL NovoTom, Germany) or HFD (Sparos, Portugal) in 5 ml E3 fish water. The ingredients of Sparos HFD fish food is in the Appendix Table 2.1. The food was kept in wells for 30 minutes and food/media mix was removed. Wells were replaced with 12 ml fresh E3 fish water. The EtOH groups were introduced with 0.5% EtOH which was 60 µl of pure EtOH corresponding to 0.5% concentration. After 8 hours, media

or EtOH/media was removed, and the same feeding regime was applied to all wells for 30 minutes. Food was discarded and new E3 media was given. Fish were incubated without any treatment overnight until the next morning's feeding. At day 12, treatment was finished, and larvae were subjected to behavior experimentation.



CHAPTER 3: RESULTS

3.1 Effect of VPA, NS-398, Topiramate and Trotabresib on Glioblastoma Cell Lines

Based on the recent developments in the literature on candidate drugs used in glioblastoma treatment four drugs were tested: valproic acid (VPA); NS-398; Topiramate; and Trotabresib, alone or in combination with the well know glioblastoma drug Temolizomide (TMZ). Cell viability results of the two cell lines, U87 and A172, upon 72 hours of treatment is given in Figure 3.1 below.

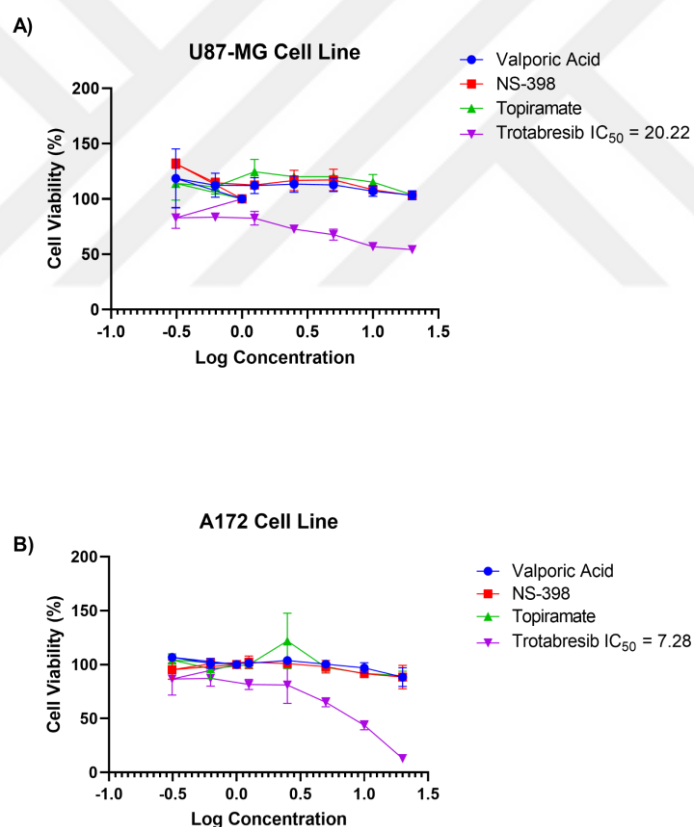


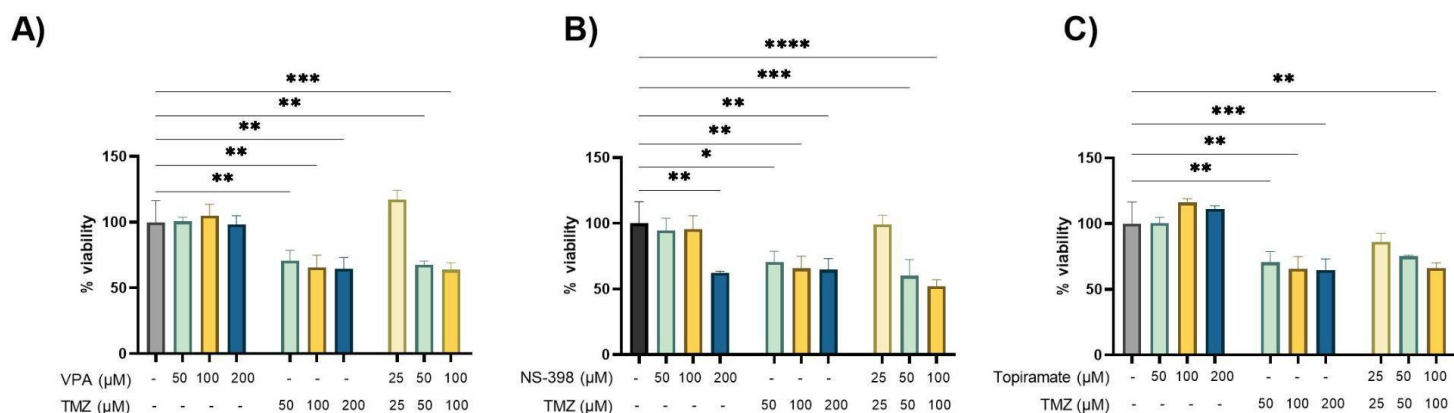
Figure 3.1: VPA, NS-398, Topiramate and Trotabresib single treatment effect on U87-MG cell and A172 cell lines.

Nonlinear fit of transformed data of A) U87-MG cell line and B) A172 cell line with log concentration values on the x-axis and % viability data on y-axis Scale bar represents mean $\pm$ SD.

As seen in Figure 3.1, Valproic acid, NS-398 and Topiramate did not decrease the cell viability even in 20 μM concentration with a stable slope observed in the cell viability. This was applicable to both U87-MG and A172 cell lines. Trotabresib on the other hand, gave a gradual decrease with the increasing concentrations. An IC_{50} value could be determined as 20.22 μM and 7.22 μM for U87-MG and A172, respectively.

Valproic acid, NS-398 and Topiramate which did not show a decrease, when combined with Temozolomide (TMZ), a known, approved and established drug for glioblastoma. The concentration of the drugs was also highly increased up to 50 μM minimum. Results in Figure 3.2 showed the drug's effect on both single and TMZ-combined exposures as well as TMZ single treatment. As seen, in the U87-MG cell line, the single treatments did not kill cells even in higher doses except an inhibition was seen in 200 μM NS-398. Although the combination with TMZ significantly decreased the viability, this was due to TMZ, where no change was occurring between TMZ single and combinatorial groups. The same pattern was seen for A172 cell line as well except NS-398. TMZ did not decrease the viability with these dosages. However, 100 μM NS-398 and 100 μM TMZ showed a significant decrease in cell viability when compared to control, the single treatments with 100 μM NS-398, or 100 μM TMZ.

U87-MG Cell Line



A172 Cell Line

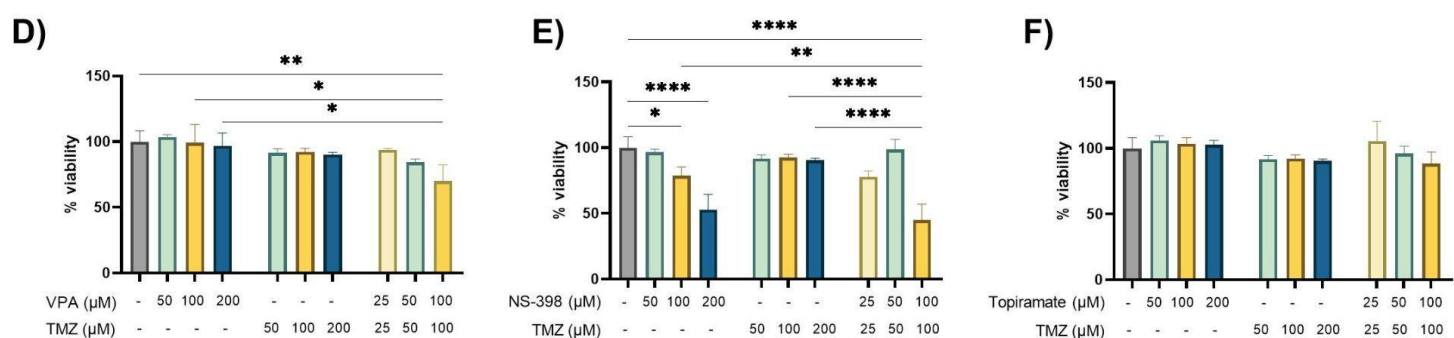


Figure 3.2: VPA, NS-398, Topiramate combination with TMZ treatment effect on U87-MG cell and A172 cell lines.

Column graph of % viability of U87-MG with A) VPA and TMZ B) NS-398 and TMZ C) Topiramate and TMZ and of A172 with D) VPA and TMZ E) NS-398 and TMZ F) Topiramate and TMZ.. Ordinary one-way Anova was performed, scale bar represents mean±SD, * indicates p value <0.05, ** indicates p value <0.005 , *** indicates p value <0.0005, **** indicates p value < 0.0001.

3.2 Effect of TFP, SFB and Novel Phenothiazine Derivative Molecule 10 on Glioblastoma Cell Lines

Previously Trifluoperazine, an FDA-approved phenothiazine and Sorafenib, an FDA-approved kinase inhibitor, was found effective in liver cancer cell viability and altering cell transcriptome in our lab (Büşra Korkmaz MSc. Thesis & Murat Yaman PhD. Thesis). Also, a phenothiazine derivative, we call molecule **10** was synthesized by Murat Kışla in Ankara University Faculty of Pharmacy (Appendix Figure 3). This molecule was also tested *in vitro* and *in vivo* previously in liver cancer cell lines and zebrafish; our lab's previous results showed that molecule **10** was less toxic than many of the phenothiazines tested *in vivo* in zebrafish but highly cytotoxic *in vitro* (Murat Yaman, PhD Thesis). Thus, here, these drugs individually and their combination were tested for glioblastoma cell viability in U87-MG and A172 cells.

It has been found that both TFP and SFB were effective in inhibiting U87-MG cell viability with an IC₅₀ value = 6.386 and 15.61 for SFB and TFP, respectively. Molecule **10** was not as successful as TFP in U87 cell line since even 20 µM concentration was not effective in reducing cell viability and metabolism of cells as measured by an MTT analysis while TFP was able to decrease cell viability down to 50% with 15 µM for the U87-MG cell line (Figure 3.3A). In Figure 3.3B, IC₅₀ values of the TFP, SFB and molecule **10** applied to A172 cell line were shown with 20.41, 10.51, 17.85 at micromolar concentration, respectively. Molecule **10** seemed to be inhibiting the A172 cell line more successfully than it did the U87-MG cell line.

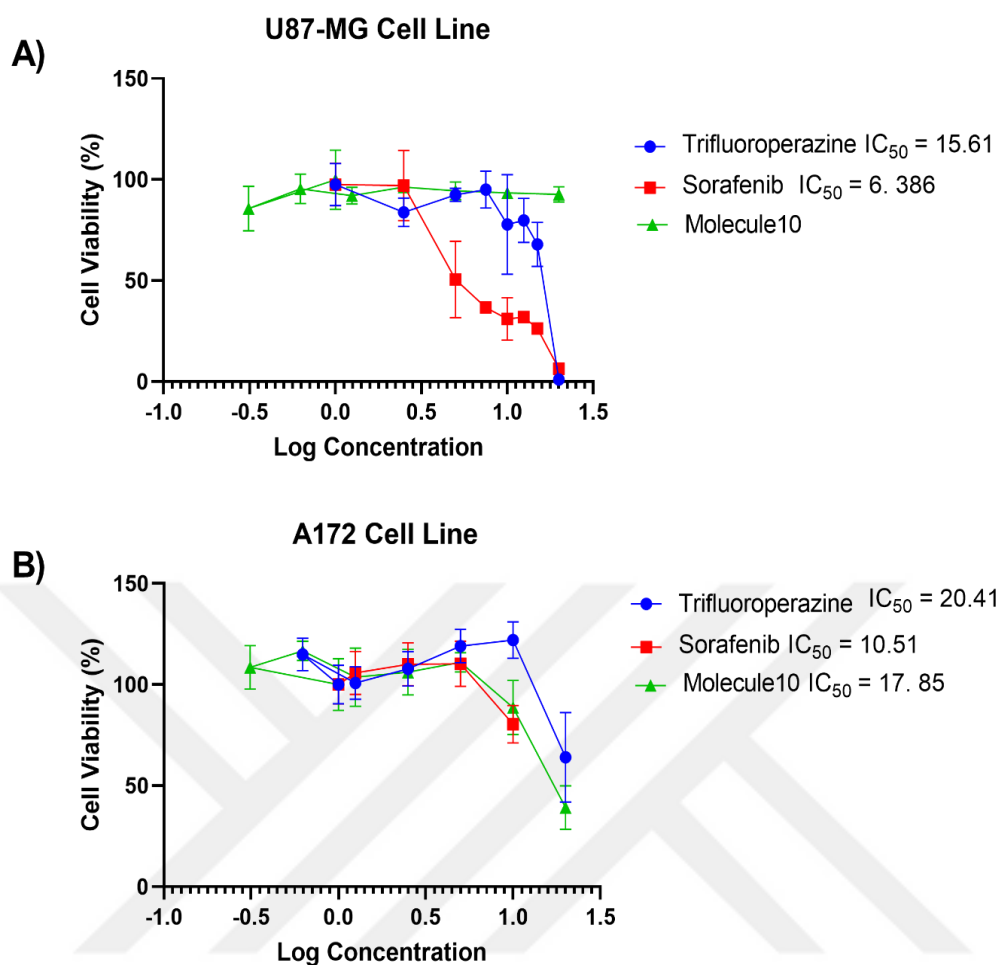


Figure 3.3: TFP, SFB & molecule10 single treatment effect on U87-MG cell and A172 cell lines.

Nonlin fit of transformed data of A) U87-MG cell line and B) A172 cell line with log concentration values on the x-axis and % viability data on y-axis. Scale bar represents mean $\pm$ SD.

Drug concentrations might be relatively high to test on model organisms and these have to be lowered and combination therapy is a solution. In addition, if an anticancer drug does not result in morphological deformity this would increase the effectivity of the drug. Therefore, the combination of those two drugs was tested in U87-MG and A172 cell lines *in vitro* first before going to *in vivo*. First, equal dosages of TFP and SFB drugs were combined and applied to the U87 cell line. Results shown in Figure 3.4A represented that although 10 μ M TFP + 10 μ M

SFB combinations had a major effect, this was lost in 5 μ M TFP + 5 μ M SFB. Also, when SFB was previously given to zebrafish larvae, it was seen that morphology and mortality was negatively affected at higher concentrations than 5 μ M. Therefore, TFP was a relatively less toxic drug, hence its concentration was kept higher while lowering the SFB dosage down to 2.5 μ M. Results in Figure 3.4B showed that combination of TFP and 2.5 μ M SFB decreased the cell viability significantly more than TFP single treatment could.

After determining the potential effect of TFP and SFB combination on the U87 glioblastoma cell line, the same treatment was applied to another GBM cell line, A172. Results shown in Figure 3.4C, indicated A172 cell line was more resistant to TFP or SFB than U87 cells. However, when 10 μ M TFP and SFB was combined, there was a significant decrease in viability when compared with the DMSO control and 10 μ M TFP single treatment. Also, 10 μ M combination decreased cell viability significantly while 10 μ M SFB was not significantly inhibitory (Figure 3.4C). In order to make sure the effect was due to the TFP and SFB combination and not SFB itself, the experiment was repeated with 10 μ M groups only. Clearly, the combination was more effectively killing A172 cells compared to DMSO control, TFP and SFB single treatments (Figure 3.4D). The synergy score is calculated for A172 cell line according to (Foucquier & Guedj, 2015), and found that TFP and SFB acts synergistically on this cell line with synergy score is lower than 1.

Cell Viability with Single and Combination of TFP & SFB

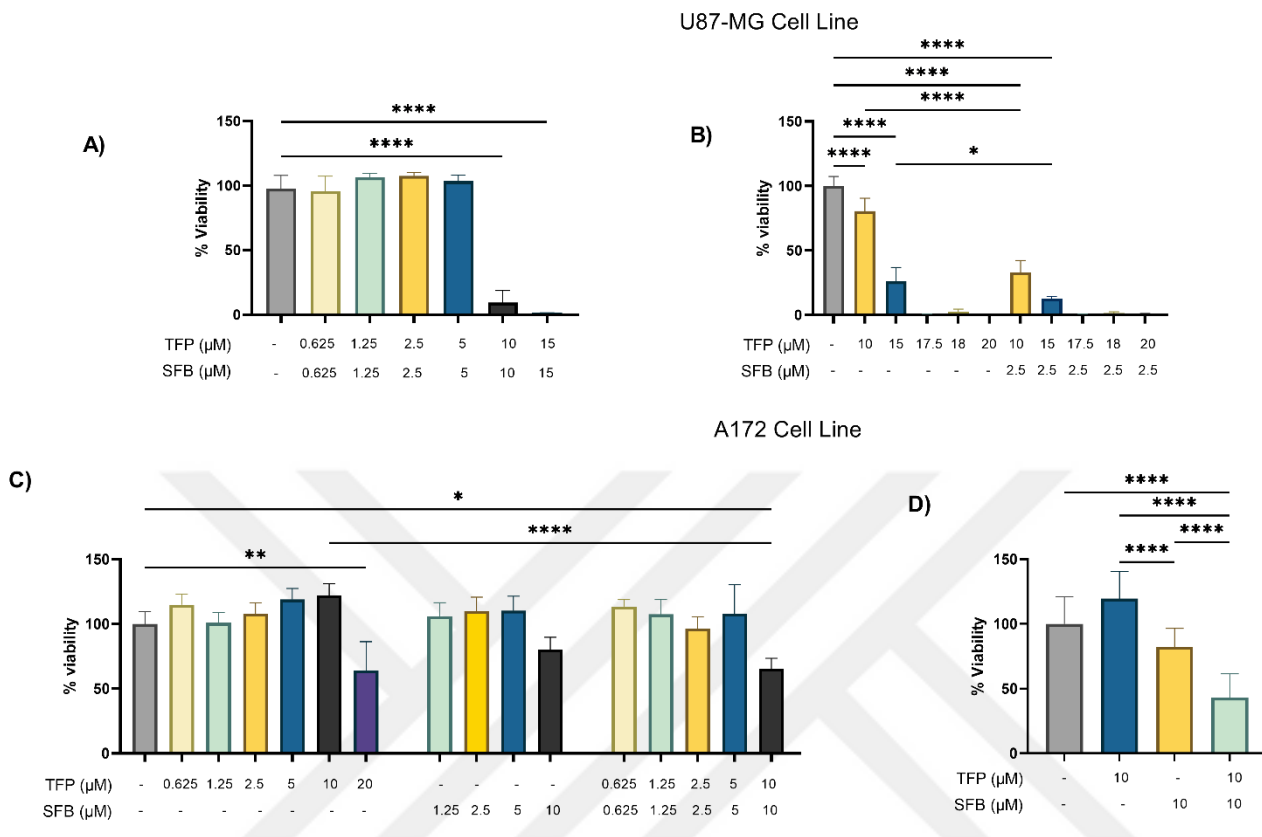


Figure 3.4: TFP & SFB combination treatment effect on U87-MG and A172 cell viability.

Column graph of % viability of U87-MG with A) equal dosages of TFP and SFB combined and B) high TFP and low SFB combination, and A172 C) single and equal dosages of TFP and SFB and D) 10 μM single and combinatorial treatment. Ordinary one-way Anova is performed, scale bar represents mean±SD, * indicates p value <0.05, ** indicates p value <0.005 **** indicates p value < 0.0001.

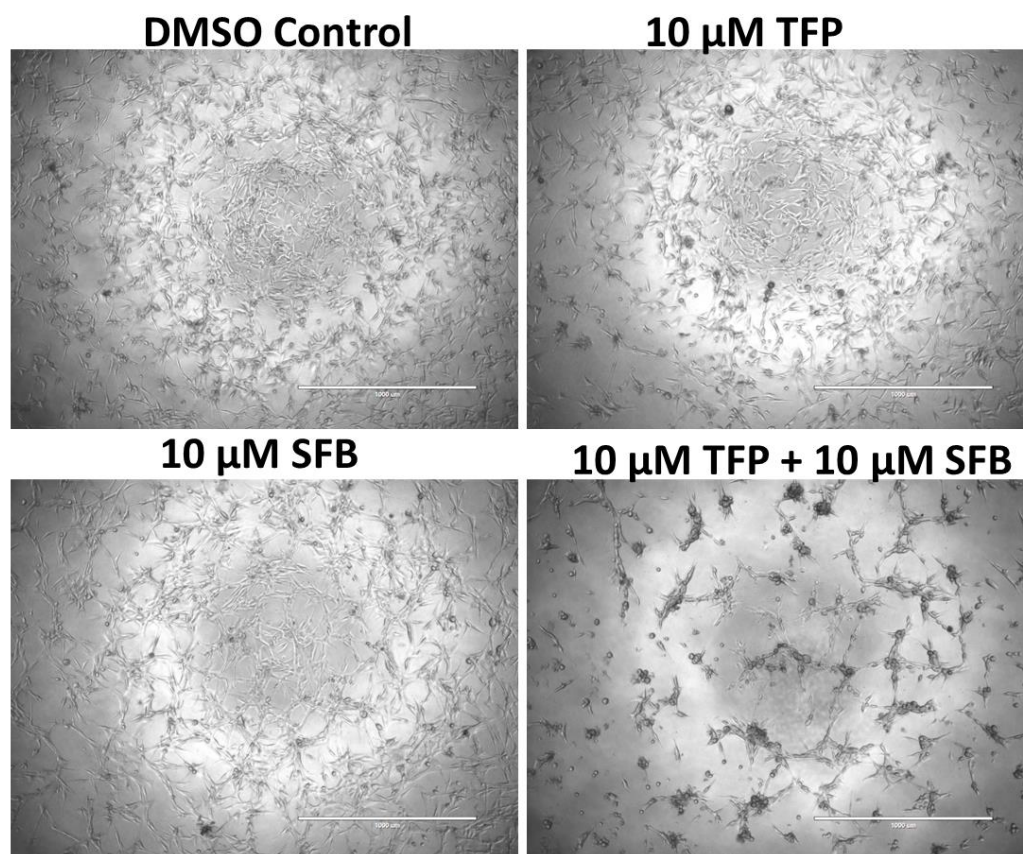


Figure 3.5: Microscopy image analysis of TFP & SFB single and combination treatment on A172 cell viability.

Microscopy images of A172 cell line after 72H of indicated drugs. Scale bar: 1000μm.

In order to determine the morphological changes in A172 cells after drug treatment, microscopy images were taken before the MTT assay. As seen in Figure 3.5, the number of living cells was reduced with combination therapy while there was not as clearer cell inhibition as with single treatments. Besides, the organization and attachment pattern of living cells to each other seemed to be reduced within combination therapy with shorter extensions of the cell. Therefore, both cell survival and morphology had more drastic alteration in TFP + SFB combination treatment.

Since A172 cell line was more resistant to TFP or SFB single treatment at the 10 μM dosage, breaking this resistance could be important and known mechanisms could be tried. Since GBM cells can lose P53 wildtype status we have hypothesized that TFP and/or SFB might

be acting on a p53-related manner and might induce resistance. In the literature there are cases where TP53 silencing or knockout increases apoptosis. Thus, both drugs were given to A172 cell line with an siP53 construct along with the controls of scrambled construct and the carrier HiPerfect only. HiPerfect was the transfection reagent that made the construct enter the cell membrane. In Figure 3.6A, a similar result with 10 μ M TFP with no inhibiting effect (Figure 3.4). This effect was not reversed with sip53 introduction as well as scrambled control. In Figure 3.6B the same application was done with SFB. Although, SFB and sip53 combination decreased cell viability significantly, this change was not unique to sip53 since the same decrease was seen in scrambled control as well. Overall, results showed that inhibition of cell viability was not dependent on TP53 silencing since similar effect was seen in scrambled control.

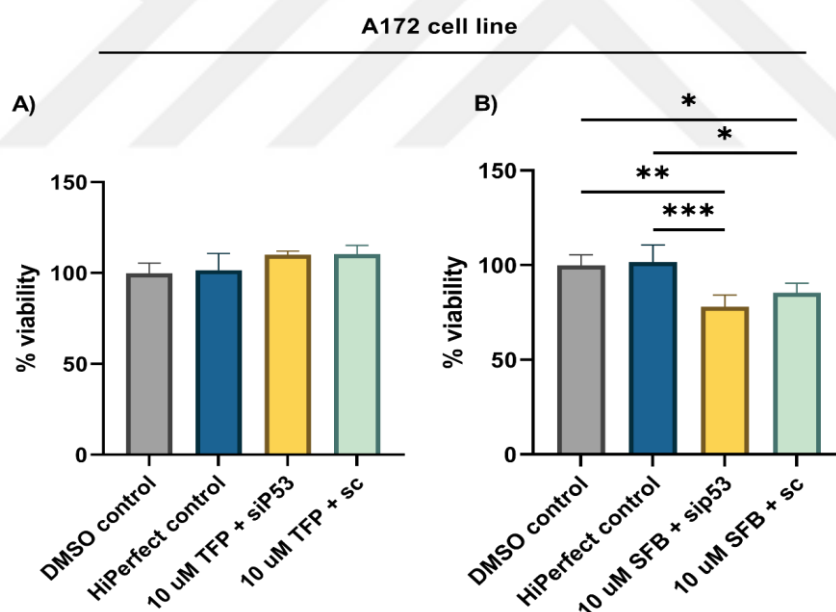


Figure 3.6: SFB & TFP and siP53 treatment effect on A172 cell viability.

A172 cell viability with A) TFP and B) SFB combined with sip53 and control graph with % viability on y-axis and treatment groups on x-axis. Ordinary one-way Anova is performed, scale bar represents mean $\pm$ SD, * indicates p value <0.05, ** indicates p value < 0.005, *** indicates p value < 0.0005.

Further, the effect of molecule **10**, which was a phenothiazine derivative synthesized in Ankara University by Murat Kışla, was tested on U87-MG and A172 glioblastoma cell line viability. Molecule **10** single treatment effect was shown in Figure 3.3B. Results showed that molecule **10** was not as successful as TFP in the U87 cell line since even 20 μ M concentration was not effective in killing cells while TFP was able to decrease cell viability down to 50% with 15 μ M. For A172 cell line, a very similar pattern was observed with TFP where 20 μ M is effective but this effect is lost after 10 μ M dosage.

In order to understand if a similar additive or synergistic effect of molecule **10** and SFB was present like observed in TFP and SFB, the combination of molecule **10** and SFB was applied to both cell lines. The aim was to create a more effective reduction of cell viability in the combination with lower concentration for glioblastoma therapeutics. As seen in Figure 3.7A, 10 μ M molecule **10** + 10 μ M SFB combination treatment was significantly decreasing cell viability compared to DMSO control while this effect was not seen with 10 μ M molecule **10** single treatment suggesting that molecule **10** resistance could be broken when combined with SFB. For A172 cell line it was seen that (Figure 3.7B), although 20 μ M molecule **10** and SFB single treatments were significantly decreasing cell viability, combination of them in 20 μ M concentration completely demolished viability. More importantly, 10 μ M molecule **10** and SFB combination decreased cell viability to a more workable percentage and this decrease was more significant than 10 μ M molecule **10** and SFB single treatments.

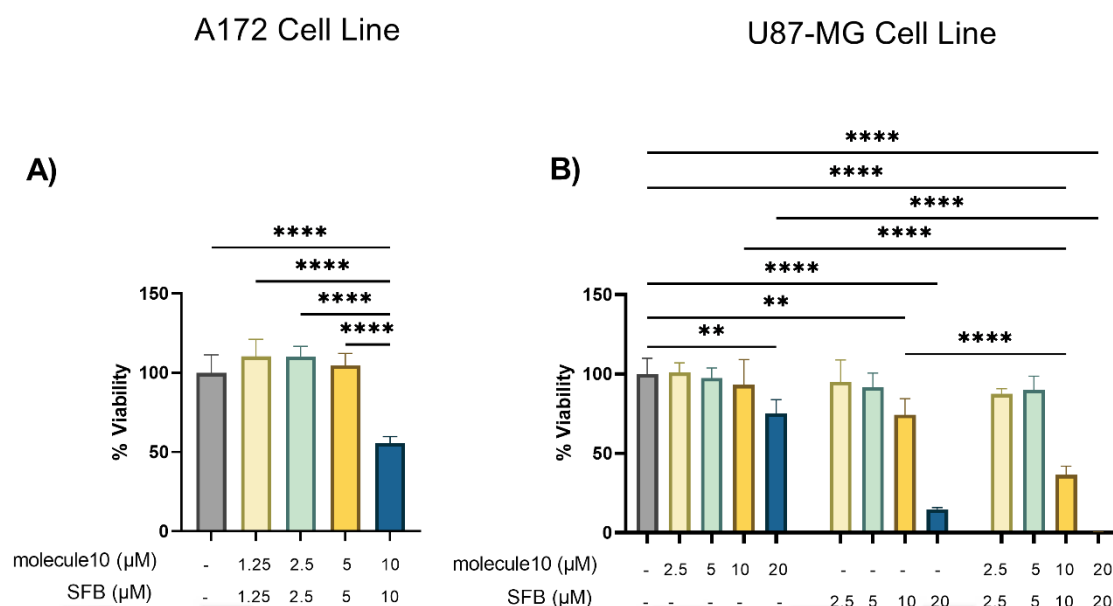


Figure 3.7: Molecule10 & SFB combination treatment effect on U87-MG & A172 cell viability.

A) Combination of molecule10 and SFB treatments in U87 cell line and B) A172 cell line with % viability on y-axis and treatment groups shown on x-axis. Ordinary one-way Anova is performed, scale bar represents mean $\pm$ SD, ** indicates p value < 0.005, **** indicates p value < 0.0001.

3.3 Changes in Expression Profile After TFP & SFB Treatment in Glioblastoma Cell Lines.

3.3.1 Alteration in mRNA Levels

After the viability and the resistance of the drugs on glioblastoma cell lines, the mRNA levels of specific genes are determined by RNA extraction followed by qPCR. Since 10 μ M + SFB was determined as more effective application in GBM cell inhibition than single treatments, both combination and drugs itself are applied to U87-MG cell line.

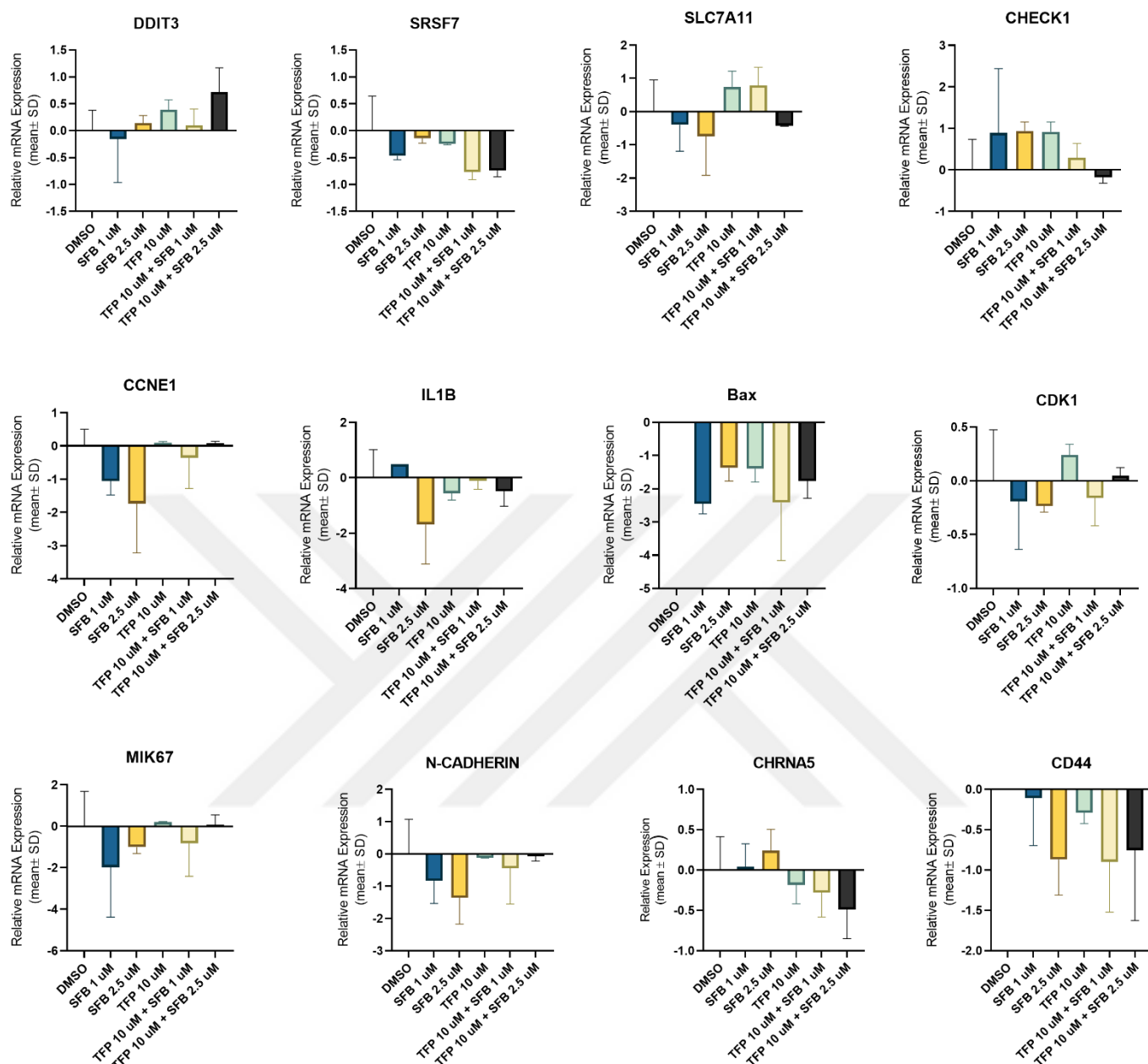


Figure 3.8: TFP & SFB single and combination treatment effect on mRNA expression in U87-MG cell line.

$\Delta\Delta C_T$ analysis of qPCR. Ordinary one-way Anova is performed, scale bar represents mean $\pm$ SD. Non-significant results are shown only.

DDIT3 is a gene that prevents the binding of the C/EBP family to DNA by preventing the formation of heterodimers, is mostly increased with endoplasmic reticulum stress and induces apoptosis (Xiao et al., 2021). This gene showed an increasing trend in the 10 μ M TFP

+ 2.5 uM SFB group. Although this increase was not statistically significant, the hypothesis that this dose combination may induce apoptosis by increasing DDIT3 to a certain extent. Overexpression of SRSF7 is known to improve the prognosis of some cancers (Fu & Wang, 2018). The almost 1-fold decrease in SRSF7 expression in TFP and SFB combination groups (as well as in low dose SFB) was significant. In the SLC7A11 gene, which is known to increase in gliomas (Polewski et al., 2017), although there was a slight increase in 10 uM TFP and 10 uM TFP + 1 uM SFB groups, this increase seems to be reversed with 10 uM TFP + 2.5 uM SFB. No significant difference was observed in CHEK1, CDK1 and MTK67 genes involved in cell division control and proliferation, respectively. In the IL1B gene, which is involved in inducing apoptosis after inflammation, a slight non-statistical decrease was observed only in the 2.5 uM SFB group. Therefore, these drugs cannot be said to induce inflammatory response and apoptosis. The CCNE1 gene, which is involved in the G1/S transition in cell division, shows a similar pattern to IL1B. Importantly, N-CADHERIN, one of the mesenchymal markers, is decreased especially in SFB groups, but this effect seems to be abolished by TFP. The CD44 gene, which plays a role in the Wnt signaling pathway and is involved in tumor metastasis, should be repeated with a better designed primer due to the high variation, although it is in a downward trend. Finally, CHRNA5, one of the cholinergic receptor subunits, has also been shown to be active in glioblastoma invasion (Gnatek et al., 2012). At the end of the experiment, a slight decrease was observed especially in our TFP and SFB combination groups.

Vimentin, another mesenchymal marker, shows no significant change in the single SFB groups, whereas it is statistically significantly decreased in the combination groups. Therefore, it can be interpreted that TFP and SFB combination therapy at the doses tested reduces mesenchymal behavior that plays an important role in cancer cell migration. In the literature, an increase in VIM in GBM has been shown to indicate a poor prognosis (Nowicki et al., 2019) and therefore the possibility that the combination of TFP and SFB prevents GBM invasion will

be tested in the future. Bax gene, which is also an apoptosis marker, decreased in all groups. It has been reported in the literature that an increase in BAX is anticancer in GBM and is associated with a poor prognosis (Daniele et al., 2018).



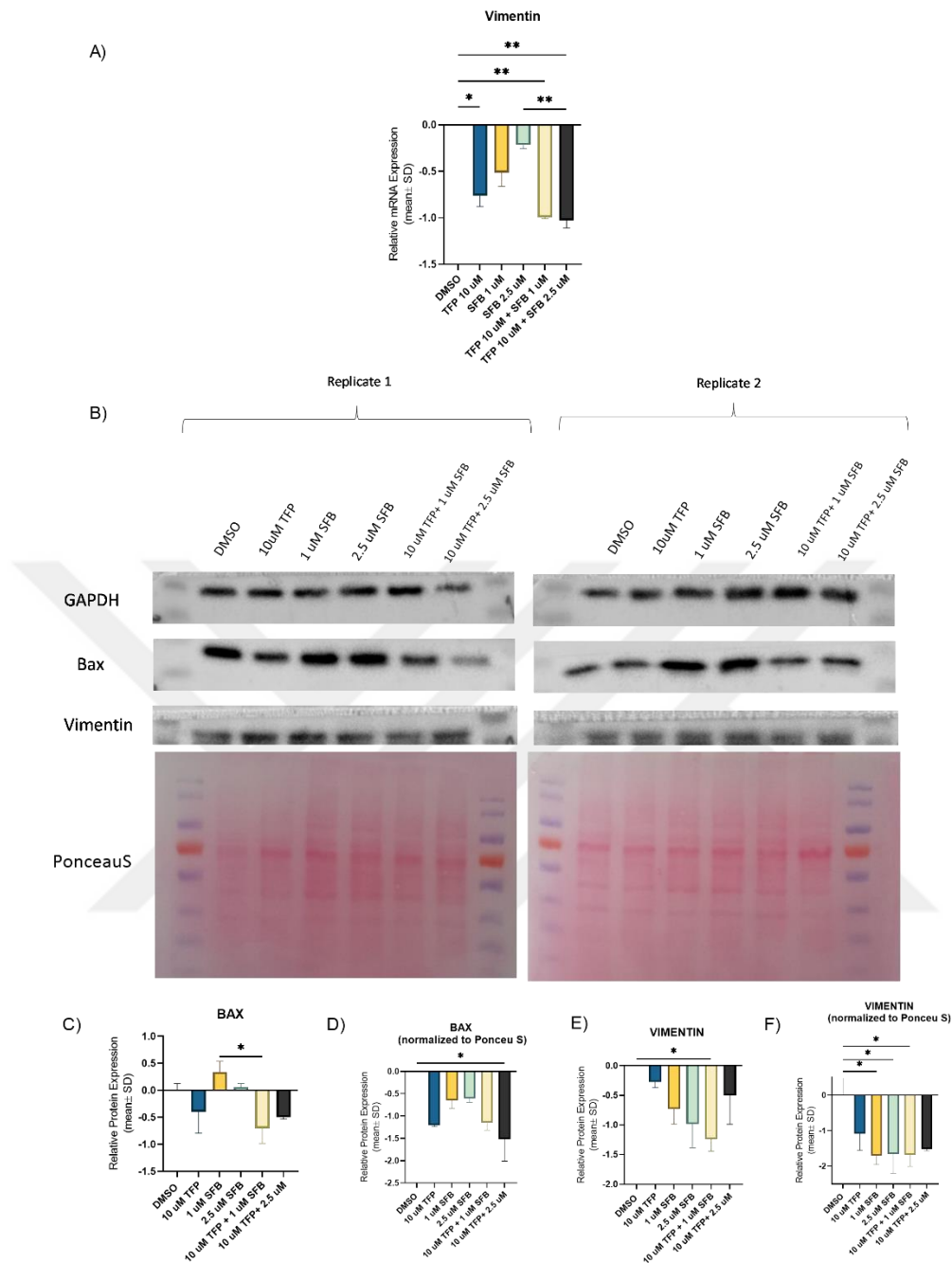


Figure 3.9: TFP & SFB single and combination treatment effect on Vimentin and Bax mRNA and protein expression in U87-MG cell line

A) Changes in mRNA expression in Vimentin. Western Blot results with B) gel image containing PonceauS, C) Bax and D) Vimentin normalization according to GAPDH band. E) Bax and F) Vimentin normalized to bands on Ponceau staining. Ordinary one-way Anova is performed, scale bar represents mean ± SD, * indicates p value < 0.05

3.3.2 Alteration in Protein Levels

Since Vimentin mRNA levels significantly decreased upon TFP treatment ($p < 0.05$) and more drastically reduced upon combinatorial application ($p < 0.005$) when compared to control, protein level of Vimentin is checked with Western Blot method (Figure 3.9B-F). Besides, Bax protein levels are also checked with the hypothesis that reduced Bax mRNA level is a compensation mechanism of increased Bax protein level.

In order to normalize protein signals on the blot, GAPDH control protein was used. Contrary to the hypothesis, Bax protein levels did not increase significantly. Vimentin showed a similar pattern as mRNA levels with decreasing vimentin protein especially in 10 μ M TFP + 1 μ M SFB. However, GAPDH protein, which is selected as an internal control, showed differences upon treatments. Therefore, PonceuS was applied to the blot after transfer and bands on the blot are used for normalization (Figure 3.9B-F). When considering two of the analyses, TFP, SFB and combination can be concluded as not acting on Bax-dependent apoptosis since the levels were decreased. Normalization with PonceuS resulted in decreased levels of Vimentin protein with SFB single treatments and a combination of 10 μ M TFP + 1 μ M SFB.

3.4 Effect of Drugs on Zebrafish Larval Models

3.4.1 TFP & SFB Effect on Wild-Type Zebrafish

After determining TFP and SFB combination is efficiently inhibiting glioblastoma cell viability, the effect of those drugs was also tested *in vivo* by using the model organism of zebrafish larvae younger than 5 dpf. Since SFB was previously established as highly toxic to zebrafish larvae in higher doses than 5 μ M, the combination of 10 μ M TFP + 1 μ M SFB and 10 μ M TFP + 2.5 μ M SFB. For understanding the effect of drug combination, single treatments were also applied to wild-type larvae. Drugs were administered to 3 dpf larvae, the stage when the zebrafish starts to develop blood-brain barrier (J. Xie et al., 2010) (Fleming et al., 2013). In

Figure 3.10, the control group images were shown where two control groups were present. E3 was the media zebrafish grows in and DMSO control includes the same DMSO percentage as in drug groups. As seen, morphologically and physiologically, both control groups presented healthy and expected phenotypes.

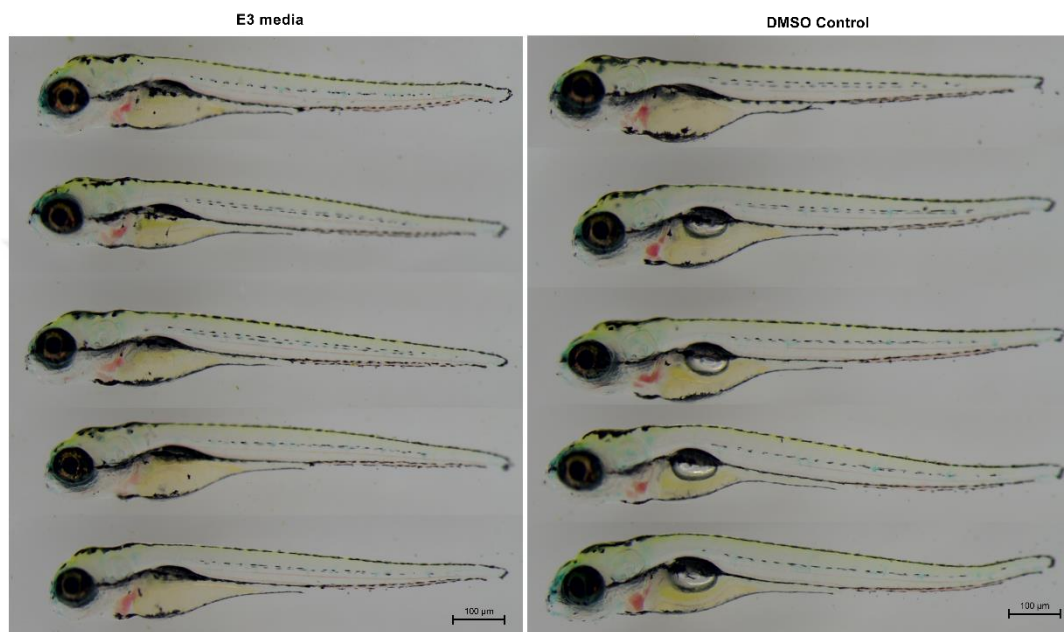


Figure 3.10: Stereo microscopy images of wild-type zebrafish control groups.

E3 media and DMSO control group 5 dpf larvae images taken under stereo microscope. Scale bar: 100 µm.

In order to understand the effects of TFP and SFB, single treatments were applied to 3 dpf larvae until 5 dpf. No significant deaths were observed although morphological differences were detected. As seen in Figure 3.10, with 10 µM TFP basic morphological markers of larvae such as body length, eye size, yolk sac size, swim bladder formation (Teixidó et al., 2019), melanin formation and accumulation (Curcio et al., 2021) was not affected. However, with the 1 µM SFB introduction yolk of the larvae started to become larger, and this effect was enhanced

with 2.5 μM SFB application. It was known that lipids start to enter the embryo's blood stream from yolk to feed and provide necessary compounds to larvae starting from 96 hpf (Anderson et al., 2011). Total utilization of yolk lipids begins at 120 dpf and continues up to around 200 hpf (Hölttä-Vuori et al., 2010). Therefore, SFB introduction, especially in 2.5 μM concentration may be related to lipid transfer from larvae to embryo correlating with the literature (Zoupa & Machera, 2017). The length comparison of single treatment groups was shown in Appendix Figure 3 and was not significantly different.

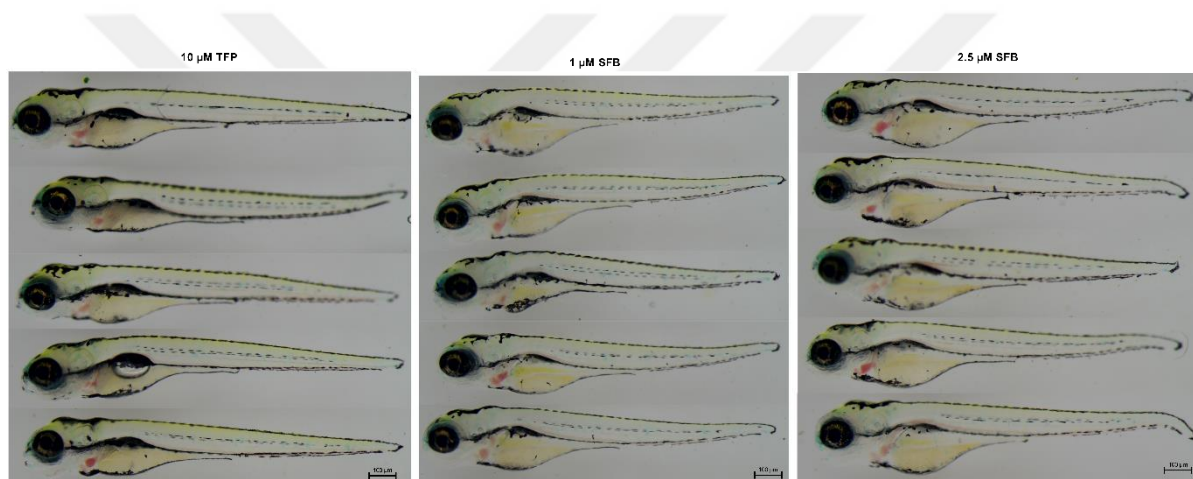


Figure 3.11: Stereo microscopy images of wild-type zebrafish TFP & SFB single treatment.

10 μM TFP, 1 μM and 2.5 μM SFB treated group 5 dpf larvae images taken under stereo microscope. Scale bar: 100 μm .

Next, the effect of those drugs when combined together was tested. Parallel to control and single treatments, drug combinations were applied starting from 3 dpf to 5 dpf. In Figure 3.12 stereo microscope images were seen where both drug combinations resulted in a major difference compared to control and single treatment groups. Although larvae were alive and in the presence of TFP, yolk enlargement seemed to be reversed, the morphology was

characterized as deformed with curved spine formation. Curvature in the spinal cord is often associated with scoliosis and well-studies in zebrafish (H. Xie et al., 2022)(Grimes et al., 2016). Importantly, curved spine is also associated with neuroinflammation (Van Gennip et al., 2018).



Figure 3.12: Stereo microscopy images of wild-type zebrafish TFP & SFB combination treatment.

10 μ M TFP +1 μ M and 10 μ M TFP + 2.5 μ M SFB treated group 5 dpf larvae images taken under stereo microscope. Scale bar: 100 μ m.

The toxicological assessments of TFP, SFB and combinations were performed using zebrafish larvae and tests were continued with measurement of glia signaling in zebrafish. By using another zebrafish strain (GFAP: GFP), one more toxicological identification was performed in EVOS FL Auto Imaging System (Thermo Fisher Scientific) scanning mode and with the addition 10 μ M molecule **10** this time. In Figure 3.13, results were shown where SFB

treatment resulted in an inflated yolk sac. With increased SFB concentration and addition of TFP, larvae showed curved spine in parallel to previous experiments with AB line. While control, TFP and molecule **10** treated larvae showed no morphological defects, 100% death was observed with 10 μ M TFP + 2.5 μ M SFB, suggesting different strains might have lower compensation for those combination concentrations.

Although molecule **10** itself was not toxic to larvae, combined with SFB, all larvae were observed as dead. As explained previously, molecule **10** molecule acts as Ache inhibitor while SFB is a known kinase inhibitor (Chang et al., 2007).

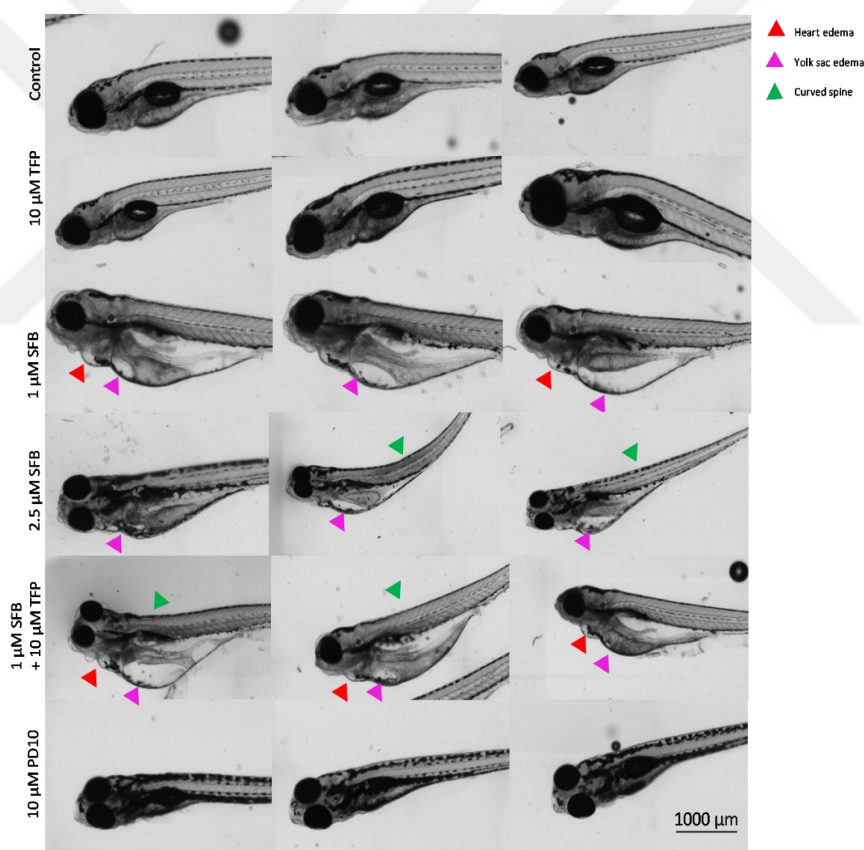


Figure 3.13: Stereo microscopy images zebrafish TFP, SFB & molecule10 single and combination treatment.

10 μ M TFP, 1 μ M, 2.5 μ M SFB, 10 μ M molecule10 treated group 5 dpf larvae images taken in EVOS scanning mode. Morphological abnormalities are indicated with red arrow as heart edema, purple as yolk sac edema and green as curved spine. Scale bar: 1000 μ m.

3.4.2 Drugs' Effects on Zebrafish Glia

Since glioblastoma is a cancer type occurring in glia cells, investigation of glia in zebrafish models has great value. For this purpose, a transgenic zebrafish line GFAP:GFP was used where GFP was expressed under GFAP (Glial fibrillary acidic protein) promoter, making this *in vivo* model a glia reporter. In Figure 3.14A, representative layout is seen after scanning fluorescence microscopy. The signal located on the GFAP promoter was quantified using Image J application. Each larva was scanned in 4X objective mode and signal is normalized to previous signal of the same larvae each day from 3 dpf to 5 dpf. 3 dpf larvae were imaged before any drug treatment was applied. Results were shown in Figure 3.14 below.

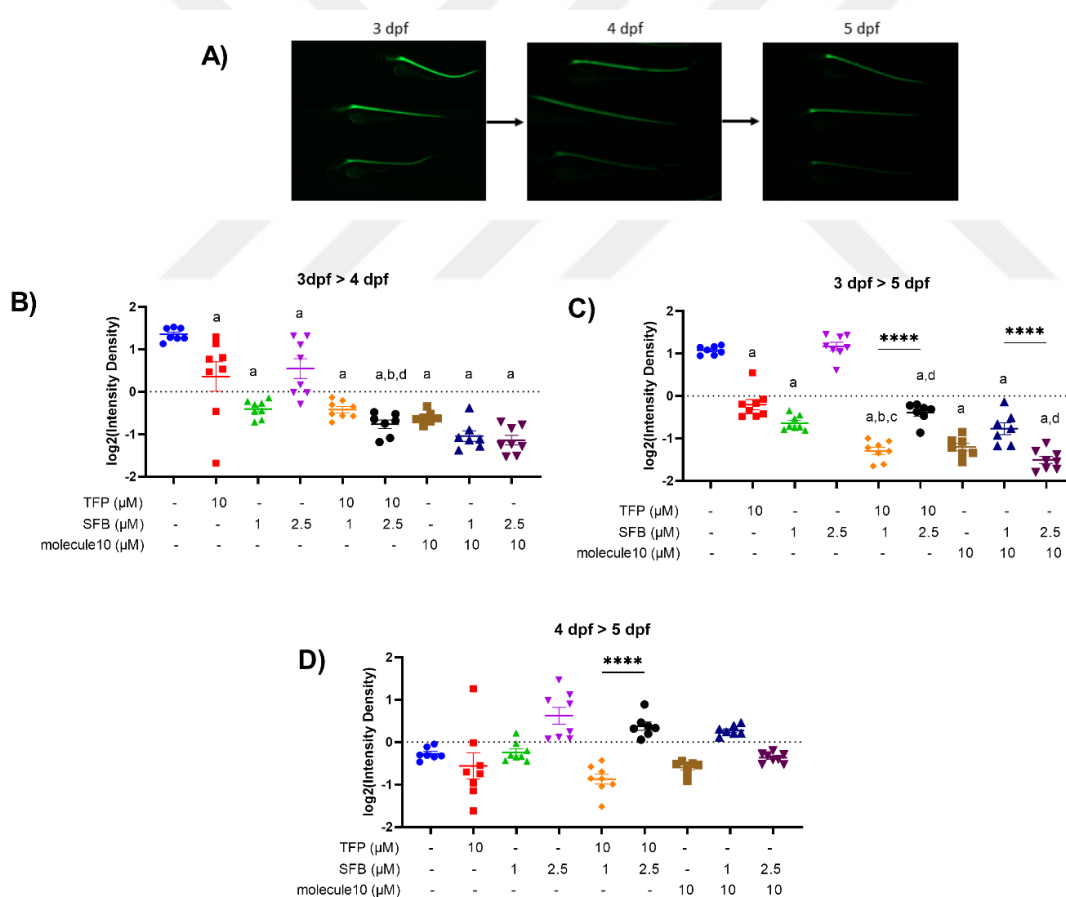


Figure 3.14:EVOS scan of GFAP:GFP individual larvae with different treatments.

A)3 dpf, 4 dpf and 5 dpf larvae images example taken in EVOS fluorescence scanning mode.. Normalized Intensity density of GFAP:GFP signal is shown as B) 4 dpf signal normalized to 3 dpf, C) 5 dpf signal normalized to 4 dpf and D) 5 dpf is normalized to 3 dpf. Letters on

graphs indicate groups significantly different. a is significantly different from DMSO control, b from 10 μ M TFP single treatment, c from 1 μ M SFB single treatment, d from 2.5 μ M SFB single treatment and e from 10 μ M molecule10 single treatment. Graph shows treatment groups on x-axis and normalized log(intensity density) on y-axis. N= 8 for all groups. Ordinary one-way Anova is performed, **** indicates p value <0.0001.

Quantifications of each individual larvae was performed to investigate drug's effect on first application (3 dpf > 4 dpf) (Figure 3.14B), the adjustment of the drugs (4 dpf > 5 dpf) (Figure 3.14C) and total effect of drugs (3 dpf > 5 dpf) (Figure 3.14D). In order to understand individual effects, each signal change from 3 dpf to 4 dpf and 4 dpf to 5 dpf in groups was also plotted.

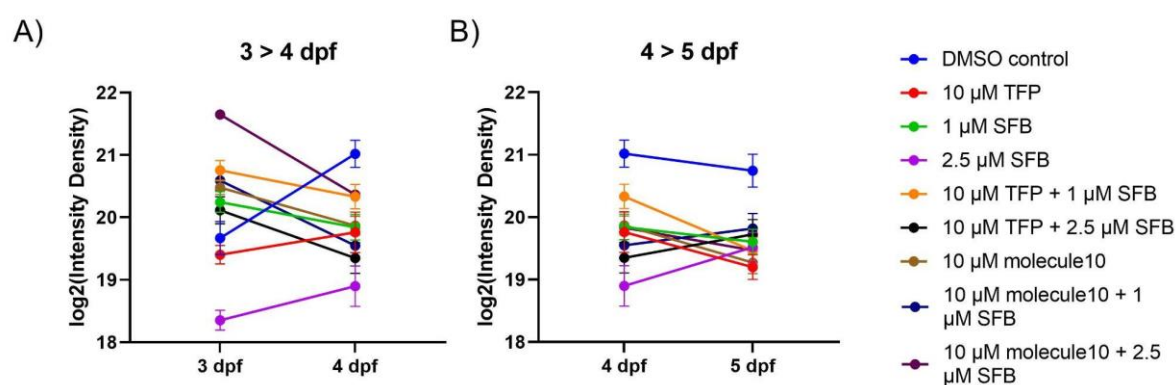


Figure 3.15: Paired graphs individual signals.

Change in signal in different groups from A) 3 dpf to 4 dpf and B) 4 dpf to 5 dpf.

Graph shows days (days post fertilization) on x-axis and normalized log(intensity density) on y-axis.

As expected, DMSO control groups larvae showed an increase from 3 dpf to 4 dpf since larvae were rapidly developing, and thus, glia cells expressing GFAP:GFP increased expectedly. It was also seen that this was relatively lower in 4 dpf to 5 dpf (Figure 3.15B). When 4 dpf signals were normalized to 3 dpf, DMSO control larvae increased their signal while all other groups significantly lowered GFP signal when compared with DMSO control (shown

as a) (Figure 3.14B). 10 μ M TFP + 1 μ M SFB groups decreased their signal significantly lower than 10 μ M TFP itself and 10 μ M TFP + 2.5 μ M SFB is significantly lower than 2.5 μ M SFB treatment. Signal lowering effect was not as significant in transition from 4 dpf to 5 dpf. Yet, 10 μ M TFP+ 1 μ M SFB was more successfully decreasing the signal intensity from 10 μ M TFP + 2.5 μ M SFB. As for total effect (3 dpf to 5 dpf), it was seen that except 2.5 μ M SFB group, all treatments lowered the signal compared with DMSO control. 10 μ M molecule10 + 2.5 μ M SFB is also significantly different than 10 μ M molecule10 single treatments. However, in this analysis each slide contained only one treatment or control group and did not include members from all groups or a control group the findings are preliminary and represent technical optimization efforts at this stage.

3.4.3 Zebrafish Larval Behavior Investigation and Effect of Drugs on Larval Behavior

3.4.3.1 Zebrafish Larval Behavior Investigation on Wild-Type and Mutant Lines

After addressing the questions of how drugs influenced on Zebrafish larvae morphology and glial expression, behavior analysis was next to be investigated. For this purpose, first, different mutants and wild type larvae behavior in different light/dark periods were optimized and studied. Below Figure included the results of changing behavior in terms of distance travelled in 30 seconds time bins of wild-type AB strain 5 dpf zebrafish larvae.

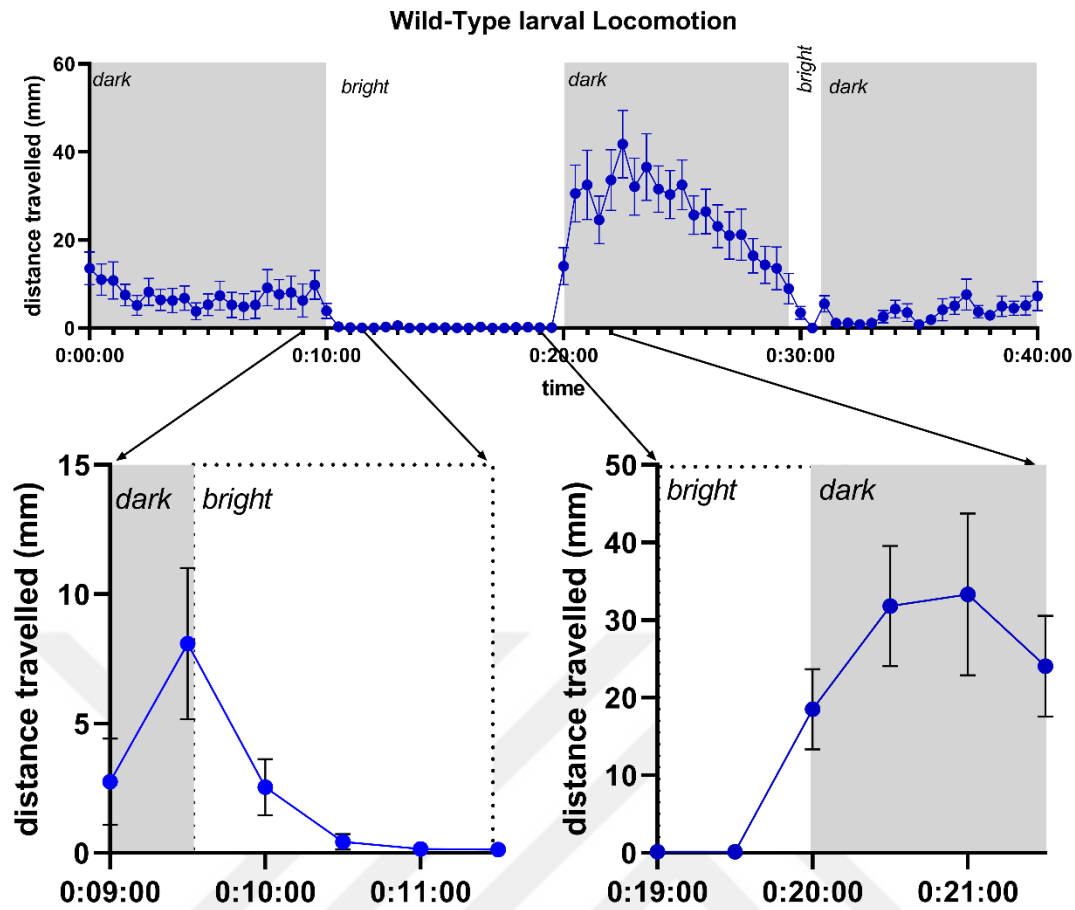


Figure 3.16: Locomotion changes in response to light/dark changes.

A) Locomotion of 5 dpf wild-type larvae in different light period. Enlarged graph of B) first dark to light and C) light to dark transition. Dark background shows dark cycle, bright background shows light cycle with elapsed time in the x-axis and distance travelled in millimeters in y-axis. Data shows mean $\pm$ SEM. All larvae are selected from the same breeding batch randomly (N= 11).

As seen in Figure 3.16, wild-type zebrafish larvae tend to change their locomotion and traveling in different light cycles. The first dark period is called the baseline period and is identified as their normal behavior without any stress response. When light is introduced as a stress response, the locomotion is completely abolished. Later when dark is introduced, the locomotion increases again with a decreasing slope. Following that, a very short (1 minute) light is given where the larva stops again. Lastly, the dark coming after that (last period) larvae starts to move again but with a lower locomotion.

The behavior assay was also applied to p53 (-/-) genotyped larvae to see if this pattern was specific to wild-type and if the p53 (-/-) genotype had an effect on behavior.

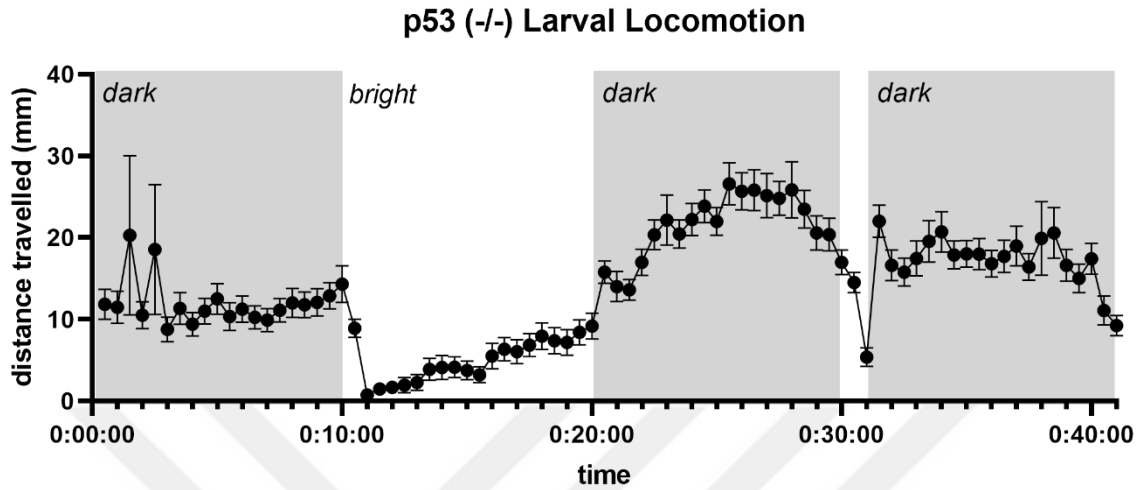


Figure 3.17: Locomotion changes in p53 (-/-) genotyped larvae response to light/dark changes

Locomotion of 5 dpf wild-type larvae in different light periods. Dark background shows dark cycle, bright background shows light cycle with elapsed time in the x-axis and distance travelled in millimeters in y-axis. Data shows mean $\pm$ SEM. All larvae are selected from same breeding batch randomly (N= 70).

As seen in Figure 3.17, p53(-/-) genotyped larvae show a similar pattern to wild-type larvae in terms of lowering the locomotion in light introduction while elevating it upon dark introduction. Differently, p53(-/-) larvae have increased locomotion slightly in the middle of the light period. Lastly, *Ache* (+/?) groups were also tested for larval locomotion and then each larva is genotyped after a behavior assay is performed (Figure 3.18).

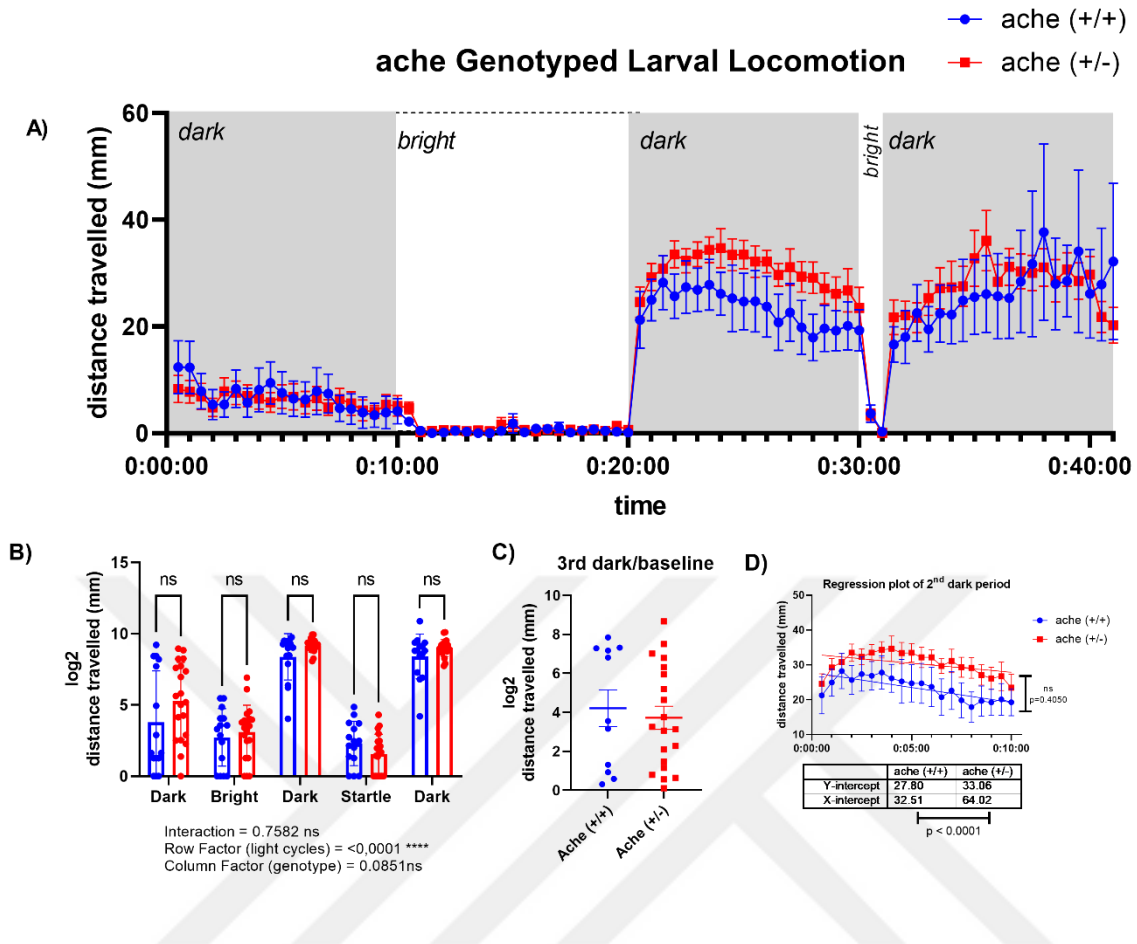


Figure 3.18: Locomotion Differences in *ache* (+/+) and *ache* (+/-) larvae.

A) Distance travelled graph of 5 dpf *ache* (+/?) larvae, blue indicates *ache* (+/+) and red *ache* (+/-) genotype. Dark background shows dark cycle, bright background shows light cycle. B) Total distance travelled graph in light periods differently. C) Normalized values of distance travelled in 3rd dark period to baseline period. D) Regression plot of distance travelled in 2nd dark period. Data shows mean±SEM for 1A and 1D and mean±SD for 1B and 1C. All larvae are selected from the same breeding batch randomly (N=11 for *ache* (+/+), N=20 for *ache* (+/-)).

Results showed that *ache* (+/+) and *ache* (+/-) genotyped larvae showed a similar pattern with no significant difference in the total distance traveled in any period (Figure 3.18B), normalized values (Figure 3.18C) and regression slope happening in 2nd dark period (Figure 3.18D).

3.4.4 Effect of Drugs on Wild-Type and Mutant Larval Behavior

3.4.4.1 Effect of Drugs on Wild-Type Larval Behavior

In order to investigate the effect of drugs used for cell culture and zebrafish toxicology assays on wild-type zebrafish behaviors, optimized concentrations were applied to 3 dpf zebrafish until 5 dpf.



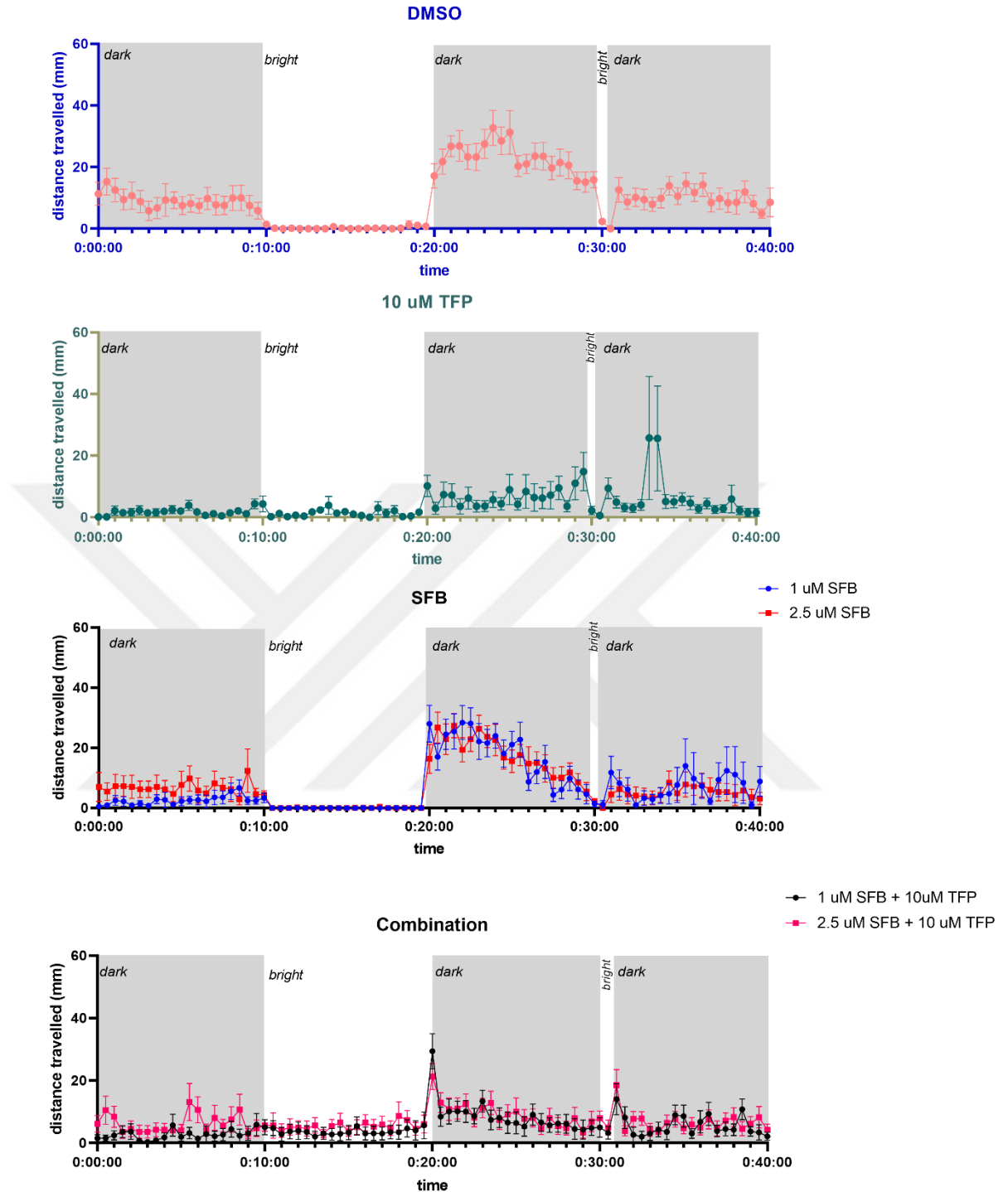


Figure 3.19: Locomotion Differences in Wild-Type Larvae upon drug treatments.

Distance traveled graph of 5 dpf wild-type larvae within different treatments. Dark background shows dark cycle, bright background shows light cycle. Data shows mean $\pm$ SEM. All larvae are selected from same breeding batch randomly (N=8 for DMSO, N=13 for 10 μ M TFP, N=14 for 1 μ M SFB, N=13 for 2.5 μ M SFB, N=14 for 10 μ M TFP + 1 μ M SFB, N=11 for 10 μ M TFP + 2.5 μ M SFB)

Results showed the similar pattern between DMSO control and SFB groups with increased locomotion in dark introduction and stop/freeze motion when light was on. Differently, TFP groups showed a different pattern where locomotion was lowered and no such hyperactivation in dark and stop response in light was observed. When TFP was combined with SFB, hyperactivation and freeze motions were observed in sudden changes in light. However, this effect seemed to be recovered fast where after one time-bin, locomotion lowered again. In order to better understand the effect of SFB and combination, each graph was analyzed separately.

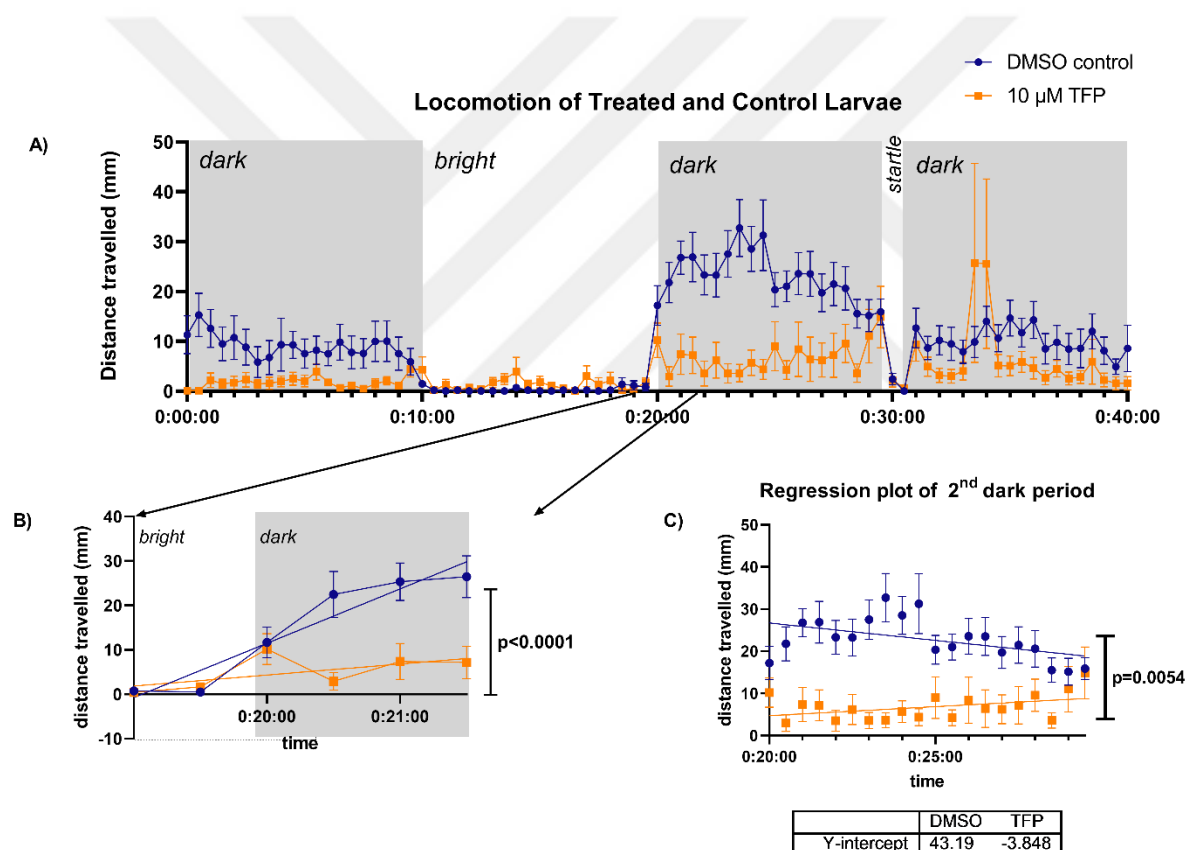


Figure 3.20: Locomotion Differences in Control and TFP Treatment in WT larvae

A) Distance traveled graph of 5 dpf *ache* AB (WT) larvae with indicated treatments. Blue indicates DMSO control while orange indicates 10 μ M TFP. Dark background shows dark cycle, bright background shows light cycle. B) Enlarged graph of first light to dark transition. C) Regression plot of distance traveled in 2nd dark period. Data shows mean $\pm$ SEM. Simple linear regression analysis is performed. All larvae are selected from same breeding batch randomly (N=8 for control N=13 for TFP)

First, DMSO and 10 μ M TFP groups were compared with each other, and new analysis methods were added. Although it was clear that TFP treated larvae tended to move less even in changing light cycles, it was also found that the slope of the transition from light to 2nd dark was significantly different from each other (Figure 3.20B) with a p value less than 0.0001. The regression plot which showed decreasing locomotion after hyperactivation occurs was also significantly different between groups ($p=0.0054$) as well as intercepts where slope met y-axis (Figure 3.20C).

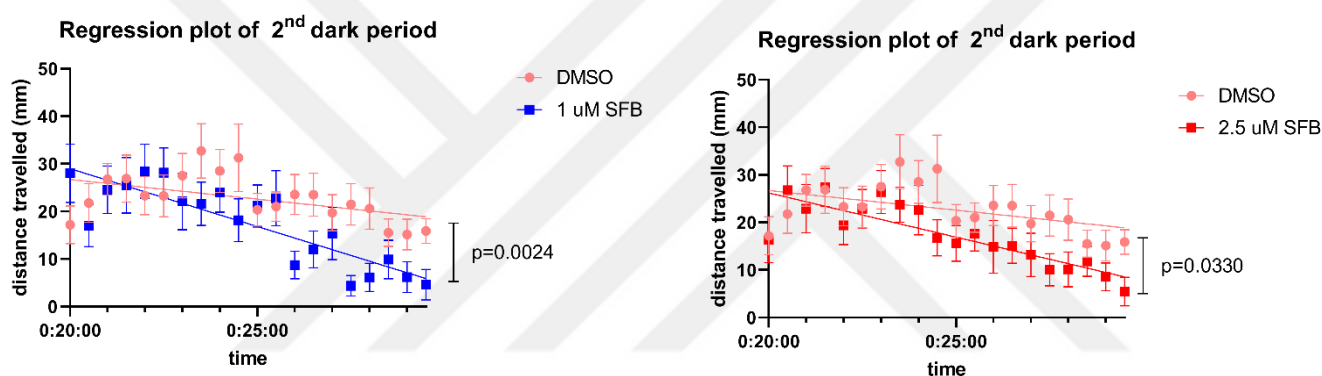


Figure 3.21: Regression Plots of Control and SFB Treatment in WT larvae.

Regression plot of distance traveled in 2nd dark period. Data shows mean $\pm$ SEM. Simple linear regression analysis is performed. All larvae are selected from same breeding batch randomly (N=8 for control, N= 14 for 1 μ M SFB, N= 13 for 2.5 μ M SFB)

As seen in Figure 3.21, both 1 μ M and 2.5 μ M SFB groups had a significantly more negative slope in 2nd dark period with p values of 0.0024 and 0.0330, respectively when compared to DMSO control. Therefore, for further analysis, drug combination groups were also tested for regression analysis.

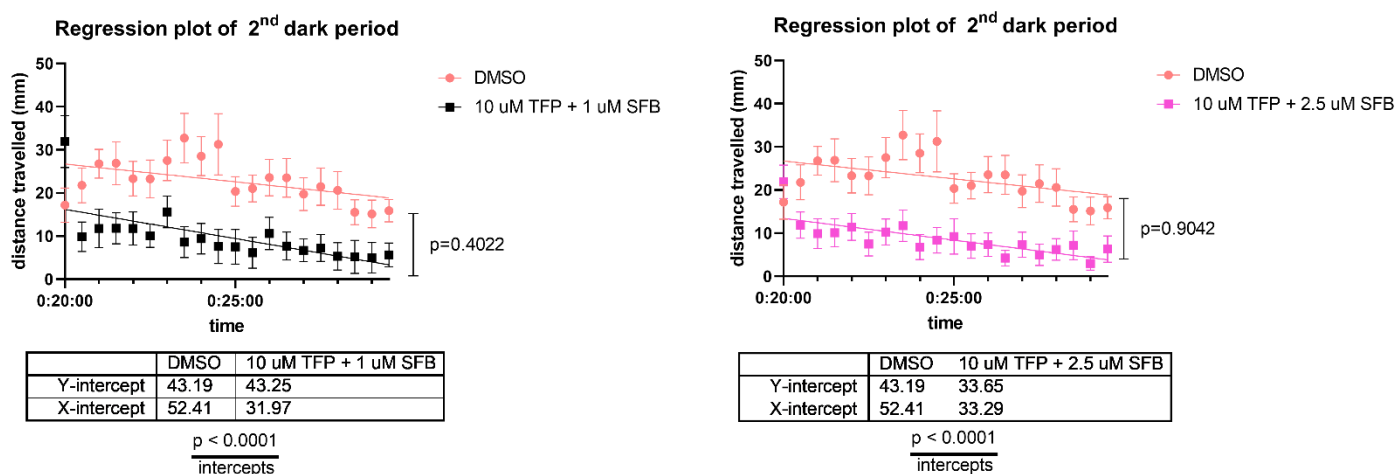


Figure 3.22: Regression Plots of Control and Combinatorial Treatment in WT larvae.

Regression plot of distance traveled in 2nd dark period. Data shows mean $\pm$ SEM. Simple linear regression analysis is performed. All larvae are selected from same breeding batch randomly (N=8 for control, N= 14 for 10 μ M TFP + 1 μ M SFB, N= 11 for 10 μ M TFP + 2.5 μ M SFB)

Interestingly, when the two drugs were combined, the regression slope of 2nd dark period was not significantly different from DMSO control with p values of 0.4022 and 0.9042, respectively (Figure 3.22). However, when intercepts were investigated, it was found that both combinatorial groups' intercepts (elevations) were significantly different from DMSO control with a p value less than 0.0001. In both graphs, DMSO groups have a higher elevation (higher locomotion) than drug combination groups.

Lastly, molecule **10**, which was previously identified as acetylcholinesterase inhibitor in our lab, and found effectively altering GBM cell viability, was tested on zebrafish larval locomotion and behavior. As seen in Figure 3.23A, molecule **10** treated groups moved less than DMSO (blue) in all periods. Interestingly, 10 μ M molecule **10** applied larvae showed a rather stable pattern except an unusual hyperactivation when light was introduced (Figure 3.23B-E). Total distance traveled in light and in the short startle period exhibited significantly more locomotion with p value <0.0001 (Figure 3.23C&E).

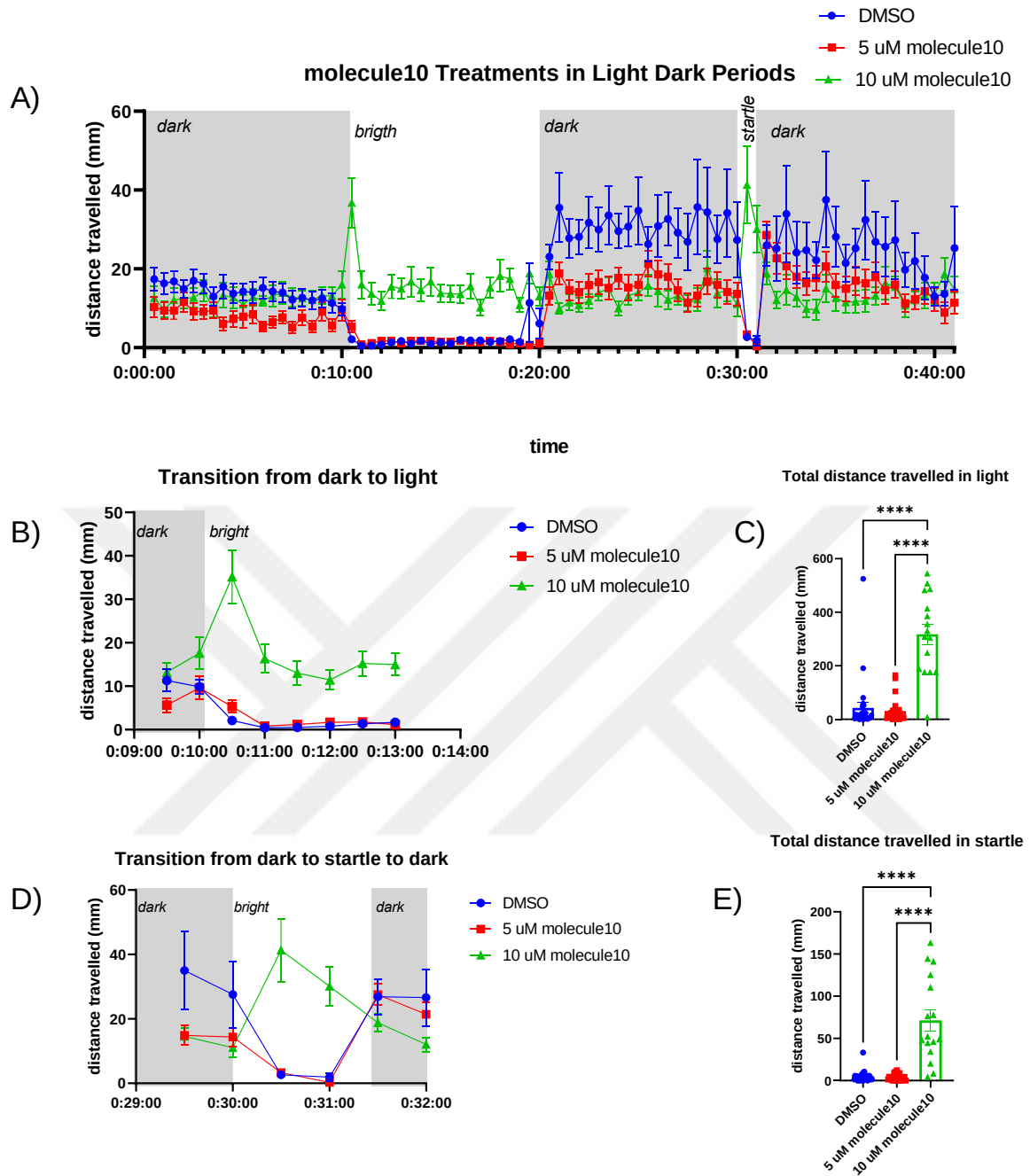


Figure 3.23: Locomotion Differences in Control and molecule10 Treatment in WT larvae

A) Distance traveled graph of 5 dpf *ache* AB (WT) larvae with indicated treatments. Blue indicates DMSO control while red indicates 5 μ M molecule10 and green 10 μ M molecule10. Dark background shows dark cycle, bright background shows light cycle. B) Enlarged graph of first dark to light transition. C) Total distance travelled graph in bright period. D) Enlarged graph of dark to startle to dark transition. Data shows mean $\pm$ SEM. **** indicates $p < 0.0001$. All larvae are selected from same breeding batch randomly (N=27 for control N=24 for 5 μ M molecule10, N=16 for 10 μ M molecule10)

3.4.4.2 Effect of Drugs on *ache* Heterozygous Mutant Larval Behavior

After addressing the question of how optimized drug concentrations acted on wild-type larval behavior, same tests were applied to *ache* mutants. Especially TFP and molecule **10** was hypothesized as they can be related with acetylcholinesterase activity and altering the larval locomotion differently when *ache* was mutated in one allele (*ache* (+/-)). In order to investigate this question, first TFP was given to 3 dpf *ache* (+/?) larvae until 5 dpf. After assay was completed in Zantiks, each larva was genotyped, and graphs were plotted accordingly. In Figure 3.24, it was also shown that *ache* (++) and (+/-) larvae had no significantly different behavior in the protocol used in this thesis. Figure 3.24 below showed 10 μ M TFP treated fish had no significant hyperactivation (Figure 3.24B) and no significant recovery differences upon regression analysis was performed (Figure 3.24C). Yet, the intercepts of regression plots were significantly different in the *ache* ++ and +/- larvae with a p value equaling to 0.0052.

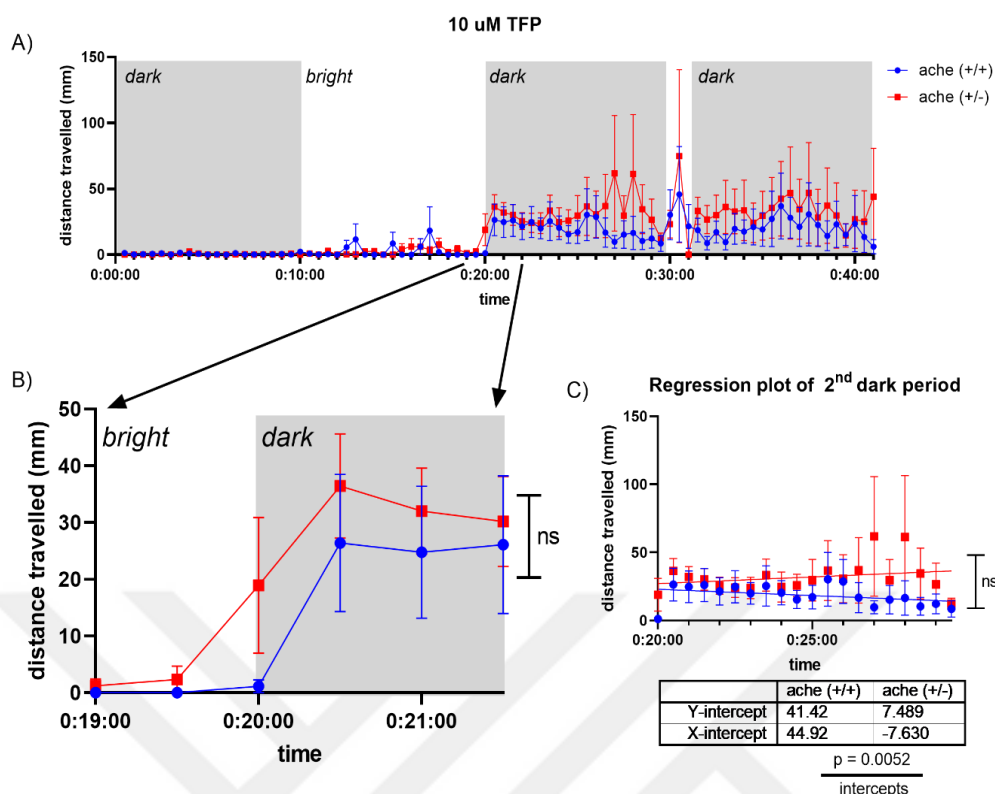


Figure 3.24: Locomotion Differences in *ache* (+/+) and *ache* (+/-) larvae with 10 μ M TFP

A) Distance traveled graph of 5 dpf *ache* (+/?) larvae, blue indicates *ache* (+/+) and red *ache* (+/-) genotype with respect to 10 μ M TFP. Dark background shows dark cycle, bright background shows light cycle. B) Enlarged graph of first light to dark transition. C) Regression plot of distance traveled in 2nd dark period. Data shows mean $\pm$ SEM. All larvae are selected from same breeding batch randomly (N=6 for *ache* (+/+) and N=6 for *ache* (+/-)).

Another drug mentioned, molecule **10**, an *ache* inhibitor was applied to the *ache* (+/?) pool same as previously. In Figure 3.25A and B both the control and molecule **10** treated groups were shown for pattern comparison. The statistical analysis was performed between the *ache* (+/+) and (+/-) larvae. The drastic difference was seen in the 3rd dark period with this drug. We had repeatedly seen that *ache* (+/+) recovery in 3rd dark was more drastic than (+/-) (Figure 3.25C). Besides, baseline period (first dark period) total distance traveled data was higher in (+/+) larvae upon 5 μ M molecule **10** treatment (Figure 3.25D).

Regression plots of 3rd dark period

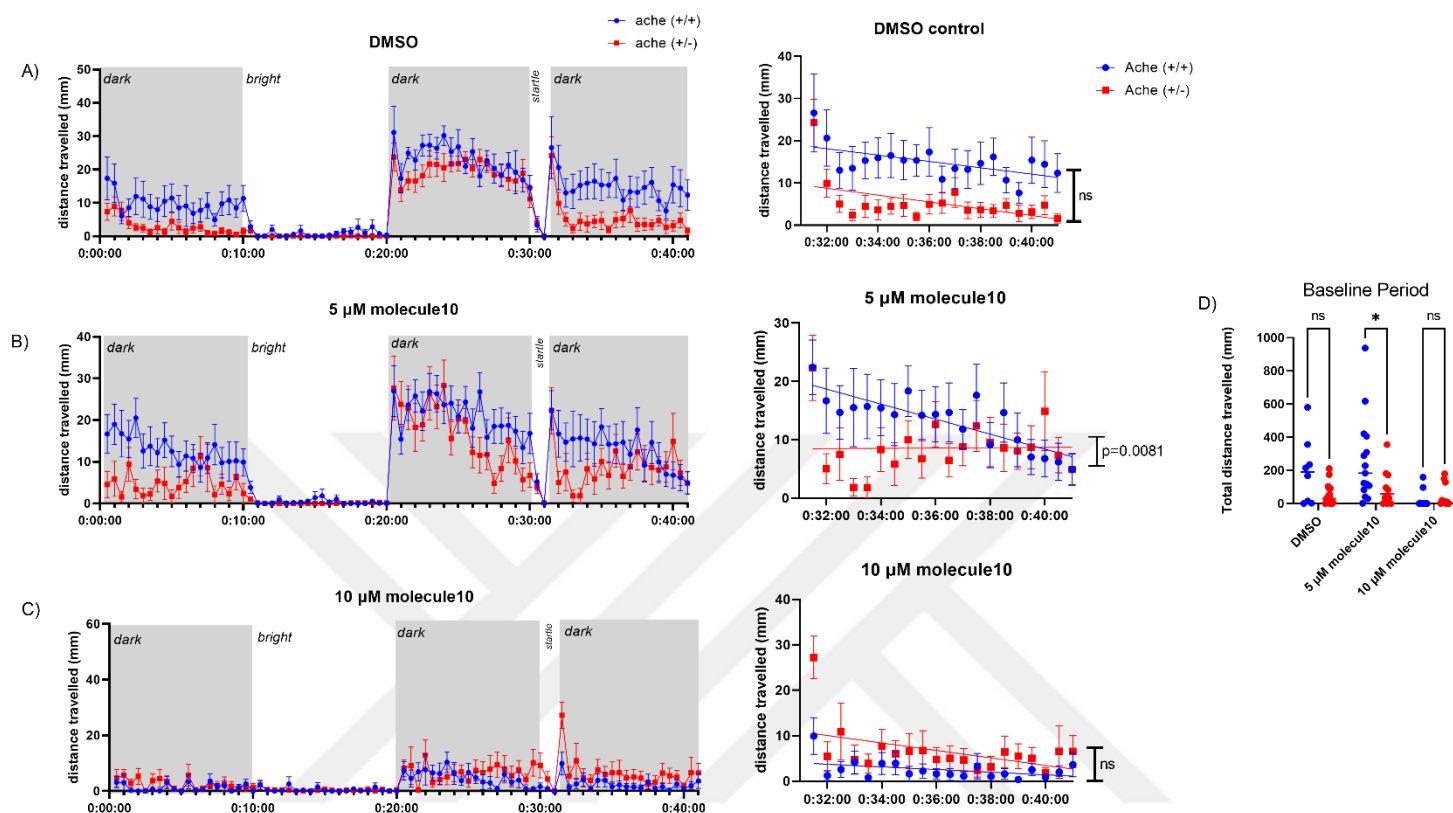


Figure 3.25: Locomotion Differences in *ache* (+/+) and *ache* (+/-) larvae with Phenothiazine Derivative molecule10

Distance traveled graph of 5 dpf *ache* (+/?) larvae, blue indicates *ache* (+/+) and red *ache* (+/-) genotype. Dark background shows dark cycle, bright background shows light cycle with respect to A) DMSO control B) 5 μM molecule10. C) Regression plot of distance traveled in 3rd dark period. D) Differences of total distance traveled in baseline period. Data shows mean mean±SEM for A and B and mean±SD for C. All larvae are selected from same breeding batch randomly (N=8 for control *ache* (+/+), N= 15 for control *ache* (+/-), N= 15 for *ache* (+/+) 5 μM molecule10, N=10 for *ache* (+/-) 5 μM molecule10, N= 10 for *ache* (+/+) and *ache* (+/-) 10 μM molecule10. * $p < 0.05$

3.4.5 Glioblastoma Xenograft Model and Behavior

In this part of the results section, U87-MG cells stable transfected with green fluorescent protein (GFP) were injected to the brain of 2 dpf zebrafish larvae. The images were taken under fluorescence microscope on day 4 and 5.

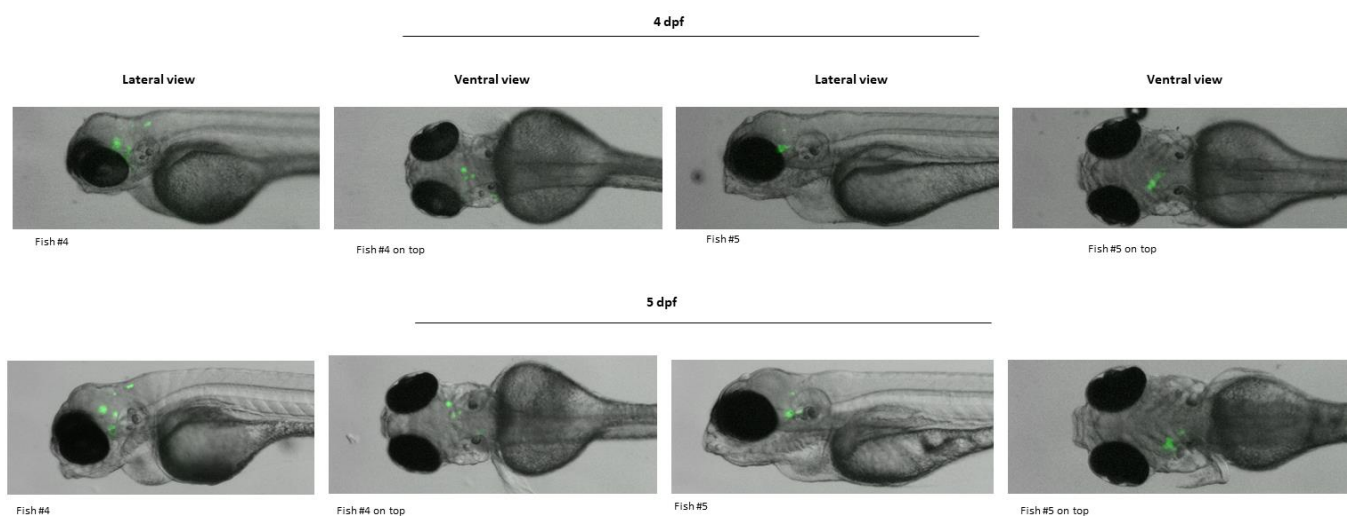


Figure 3.26: U87-GFP xenograft images of 4 dpf and 5 dpf WT larvae in lateral and ventral views.

After the injections were performed and the signal was visualized for 2 days, behavior assays were done on the xenografted and control larvae. Control larvae consisted of non-injected siblings of the xenografted larvae.

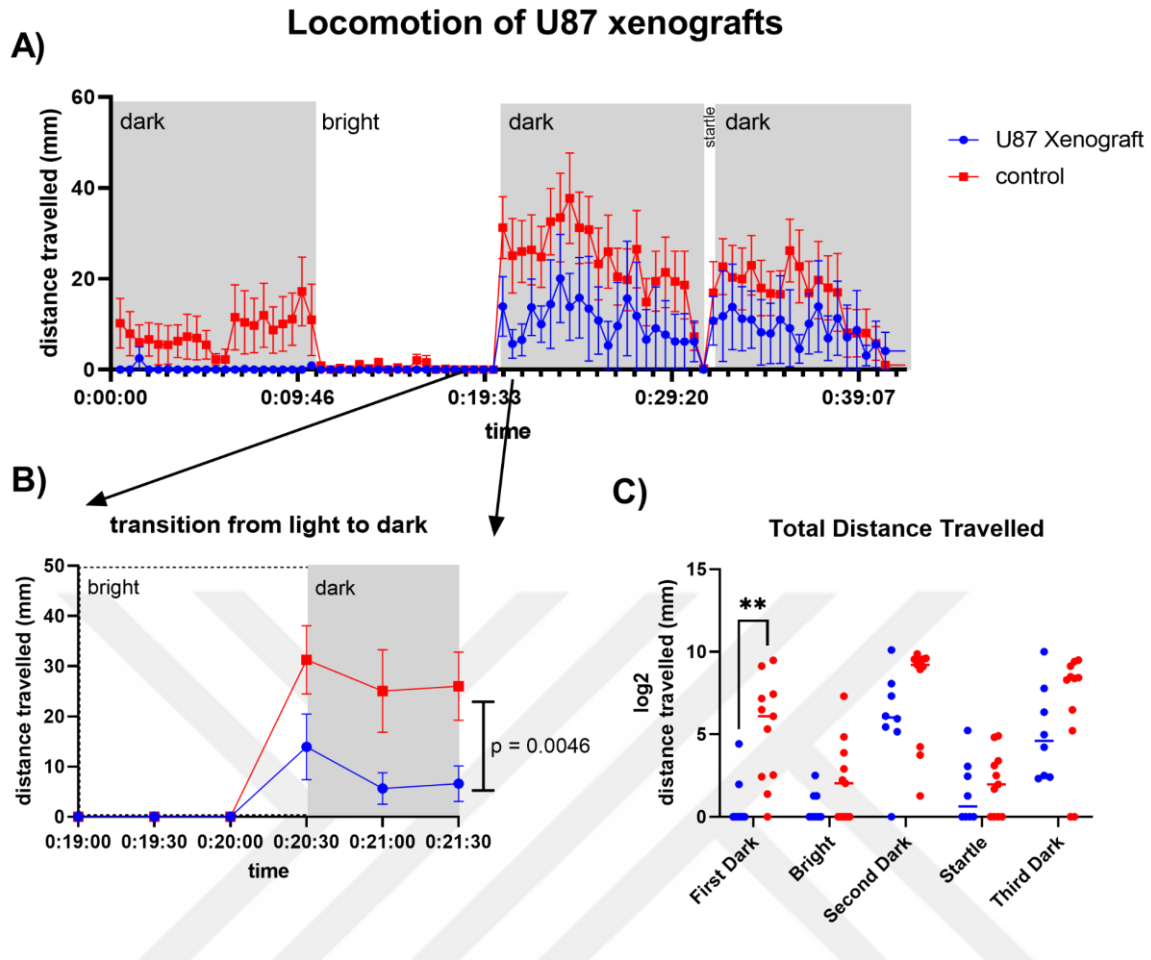


Figure 3.27: Locomotion Differences of U87 xenograft and control larvae.

A) Locomotion of 5 dpf U87-GFP xenograft and non-injected control larvae in different light period. B) Enlarged graph of first dark to light and transition. C) graph of log normalized total distance traveled in different periods. Dark background shows dark cycle, bright background shows light cycle with elapsed time in the x-axis and distance traveled in millimeters in y-axis. Data shows mean $\pm$ SEM. N= 8 for xenografted, N= 12 for control groups.

As seen in Figure 3.27, although sample size should be increased, glioblastoma xenograft model showed less locomotion especially in the baseline period (first dark period) (Figure 3.27 A,C). Additionally, xenografts showed significantly less hyperactivation in the transition from bright to dark (Figure 3.27B).

3.4.6 DUAL Diet Effect on Larval Behavior

Last but not the least, in this thesis study, the effect of DUAL diet (High Fat + EtOH) on larval behavior in *ache* (+/?) siblings was investigated. I wanted to see whether wildtype and mutant siblings exhibited differences in their locomotory activity in response to a dual diet applied from day 5 to day 12 with intermittent alcohol exposure (Figure 3.28). The efficiency of HFD is tested with whole-mount oil red o staining (Appendix Figure 4). A cohort of larvae from an *ache*-/+ x *ache* -/+ cross was used for identification of the locomotory differences between *ache* +/- and *ache* +/+ siblings (*ache* -/- fish were not used since they were paralyzed at 3dpf) when they were exposed to control diet, HFD diet, control diet with %0.5 alcohol and HFD with %0.5 alcohol. The findings in Figure 3.28 panel A, it was seen there were differences between feeding groups especially in dark periods. 2nd dark recovery regression graphs were plotted (Panel B) revealed the most effect was on the second dark period where hyperactivation occurs and recovery is expected.

As seen in Figure 3.28 although slopes between *ache* (+/+) and *ache* (+/-) siblings showed no significant difference, the intercepts of the regression lines were significantly different in control, EtOH and HFD groups. In these conditions, *ache* (+/+) larvae had a higher intercept which showed a more intense hyperactivation (stress response). However, this effect was removed when EtOH and HFD was applied together. In Panel C, when I plotted the graphs per genotype, HFD increased total distance travelled in *ache* (+/+) after normalization was done and no significance was seen in *ache* (+/-). However, more tests with higher sample sizes are needed to conclude whether stress behavior of *ache* siblings vary upon dual diet introduction.

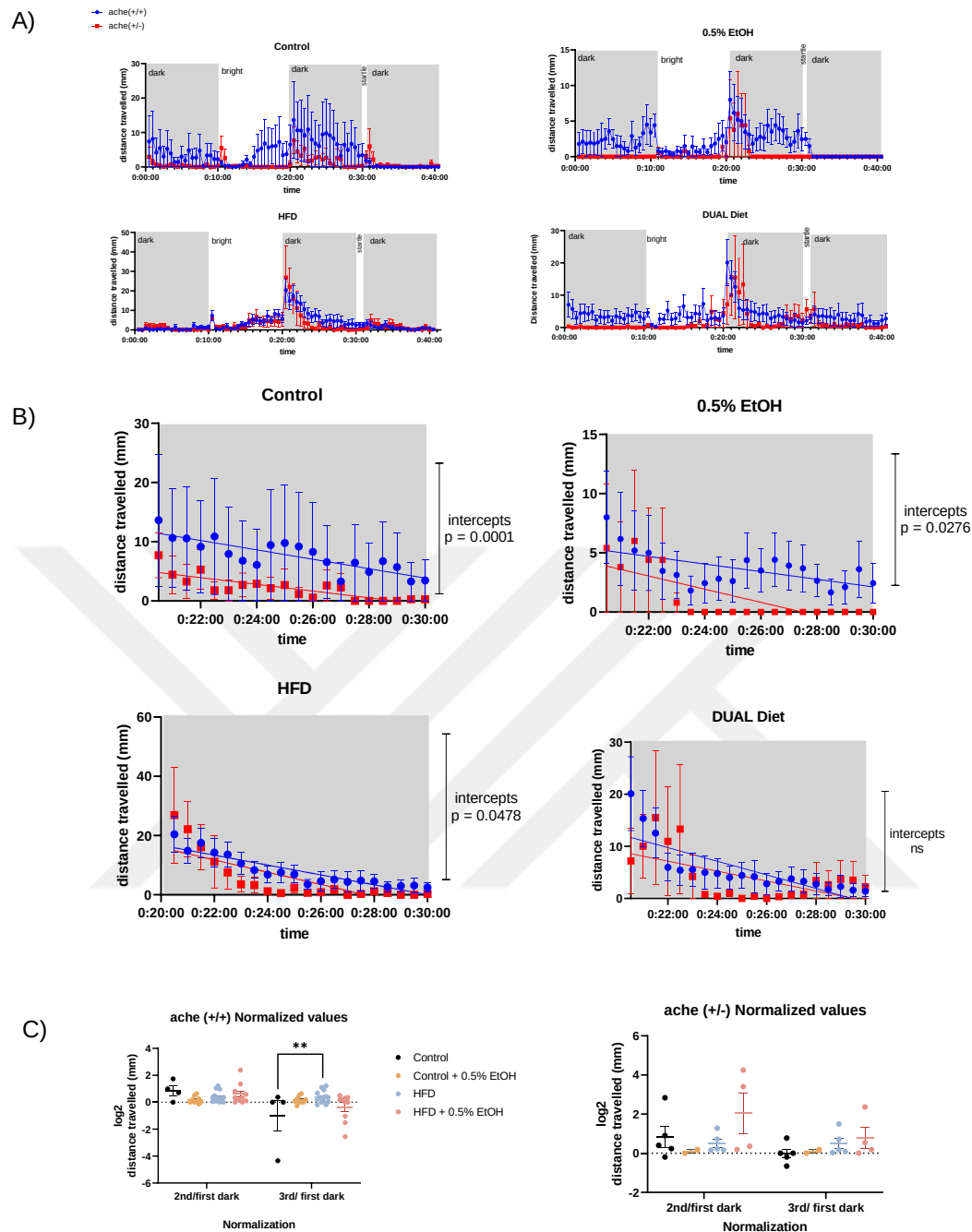


Figure 3.28: Locomotion Differences in *ache* (+/+) and *ache* (+/-) larvae with DUAL diet.

A) Distance traveled graph of larvae with indicated treatments. Blue indicates *ache* (+/+), red indicates *ache* (+/-). Dark background shows dark cycle, bright background shows light cycle. B) distance traveled in second dark period is shown. Graph shows elapsed time in the x-axis and distance traveled in millimeters in y-axis. C) Normalized graph of genotypes within itself. Data shows mean $\pm$ SEM.

CHAPTER 4: DISCUSSION & CONCLUSION

4.1 Investigation of Drugs in Different Glioma Cell Lines

In this study, several drugs, including Valproic acid (VPA), NS-398, Topiramate, Trotaresib, Trifluoperazine (TFP), Sorafenib (SFB) and a novel phenothiazine-derivative named molecule **10**, have been applied to glioblastoma cell lines U87-MG and A172. Although U87-MG cell line is an established GBM cell line in drug screening for novel therapeutics approaches (Akay et al., 2018) (Y. Fan et al., 2016), A172 is less abundantly used in drug screening research for glioblastoma. One of the strong points of this thesis has been the investigation of these drugs of interest on two different cell lines with a unique ability to generalize drug effects and cross-validation. In fact, it was found that different drugs showed different results where only one cell line could be more resistant or vulnerable to a certain drug. For instance, A172 showed more resistance to both TFP and SFB even in 10 μ M concentration where cell viability higher than 100% was observed which indicated that this drug could not inhibit the cell viability, but might be promoting its proliferation. As expected, to break down this resistance, higher concentrations of both drugs were combined together in A172 rather than U87-MG. This effect could be reversed with molecule **10** since A172 was more vulnerable to it than U87-MG. All this highlights the importance of variation in different cell lines as in between different GBM patients although they are derived from the same diseased tissue. Moreover, both U87-MG and A172 cell lines are wild-type for TP53 and similarly, no evidence exists about an ACHE null mutation in either cell line. It is important to test the effects of drug under different genetic backgrounds (Richter et al., 2017). Accordingly, we used siTP53 in A172 however we did not see an effect of loss of function in TP53 hence it is likely that the TFP and SFB tolerance is independent of TP53 status.

Drug repurposing exhibits a faster way to therapeutics rather than discovering a drug from scratch. Since commercially available drugs already passed the preclinical and clinical studies, registered, approved and used, their use in another concept is also possible in a shorter period of time. In this work, for TFP and SFB, qualify for repurposing since each has a specific domain that they are used for, psychosis and liver cancer, respectively (Zhang et al., 2017) (Lin et al., 2019). Molecule **10** on the other hand is a novel drug still in the development and preclinical state yet it has not been used previously for glioblastoma. In this aspect, the question of what makes a molecule a good drug needs to be answered. First size of the molecule could be important, and in the context of glioblastoma it is even more crucial since small molecules can pass through BBB. Yet, some other properties are also important such as molecular weight and hydrogen bond numbers (Pardridge, 2012). Based on that its molecular weight is 540.076 g/mol; it has a potent anticancer activity; and interestingly it is not highly toxic even at high concentrations in zebrafish larvae (Büşra Korkmaz MSc. Thesis). My findings with this molecule in GBM partially supported previous findings however GBM cells were more resistant to it although this could be lessened by SFB.

4.2 Potential of Combinatorial Therapy for GBM

VPA, NS-398 and Topiramate are novel and more recent drug candidates as indicated in the introduction yet when used individually MTT assays were not successful finding inhibitory effects even at relatively high concentrations. Therefore, they were combined with an established GBM drug, TMZ. However, the combination did not seem to be effective either, even in higher concentrations. Dosages like 200 μ M is relatively high for applying model organisms and eventually humans. In the literature, Valproic Acid (VPA) is mostly used in clinical trials with glioblastoma patients (Yuan et al., 2014) (Li et al., 2018) with beneficial potential usage of VPA in patients. In another study, VPA was tested on glioma cell lines including U87-MG where even 2.5 mM VPA single treatment was not inhibiting the cell

viability but combination of 2.5 mM VPA and 1 mM TMZ killed the cell significantly (Tsai et al., 2021). In an early study, A172 cell line, VPA was found effective after 10 mM concentration (Duenas-Gonzalez et al., 2008). Therefore, the dosages used in this thesis and resulting cell viability was expected and found in parallel with the literature. However, there was no study on the TMZ and VPA combination in the A172 cell line.

NS-398 was not studied with respect to glioblastoma cell viability, yet in another study, the working concentration ranged from 10 to 100 μ M (Palumbo et al., 2020). Therefore, in this thesis, the novel combination of NS-398 and TMZ was tested and I could conclude that they were not as not efficiently inhibiting cell viability in the used dosages which were relatively high. Similarly Topiramate was not studied in terms of cell viability in glioblastoma cell lines and in combination with TMZ. Here, this drug was also not found effective for reducing cell viability of glioblastoma cells. Trotabresib was not studied *in vitro* before, but used only in clinical trials for patients who received trotabresib before surgery (Moreno et al., 2022). Therefore, here it has been shown that Trotabresib could be suggested as inhibiting cell viability even in lower dosages alone itself; this could be a novel finding to pursue further. Trotabresib is under clinical trials for approval as a BET inhibitor for solid tumors and here it is suggested it can be repurposed for glioblastoma upon future preclinical studies as well. However, these drugs should be tested in zebrafish as well in the future. If the dosages used in this study are not toxic and lethal to the fish, then even higher dosages and combinations could be tested further.

As seen in Figure 3.4, combination of TFP with SFB changed the effect of TFP single treatment significantly more when TFP was combined with 2.5 μ M SFB. This SFB dosage was lower than the IC₅₀ value (Figure 3.3). This has indicated that TFP and SFB combinatory therapy could be potentially more effective on reducing the viability of U87-MG glioblastoma cells. By using a combinatorial approach, lower dosages can be used which is crucial for applying drug therapies into model organisms and humans (McCormack et al., 2011). In fact,

for A172 cell line, the synergy score calculations showed TFP and SFB acts synergistically on this cell line (Fouquier & Guedj, 2015).

TFP's inhibitory effect on glioblastoma cell lines has previously been demonstrated (Zhang et al., 2017) as well as that of the SFB nanoparticles on the U87-MG cell line (Dahis et al., 2023). Results and concentrations presented in this study are in parallel with the previous literature. As a novel finding, here it has been demonstrated that TFP and SFB combination could inhibit glioblastoma cell line viability more effectively than their individual treatments, suggesting a strong novel drug combination for glioblastoma therapeutics. In fact, combining two drugs together is a common approach in cancer therapeutics since it may break the chemoresistance if present, especially if the drugs are acting on different mechanism each with a lower toxicity level when on their own (Gilad et al., 2021). One of the reasons TFP and SFB has been combined together in this work was based on our lab's previous findings in liver cancer cell lines (Murat Yaman PhD. Thesis). Also, the actions of TFP and SFB on different pathways, dopamine and tyrosine kinase pathways relatively (Ghosh et al., 2003), may make them not compete with each other and cancel each other's effects out.

Combinatorial therapy in drug discovery or repurposing provides the ability of using lower drug concentrations which reduces the risk of toxicity and decreases the drug treatment duration for further usages (Mu et al., 2010). In the light of the abovementioned findings, hereby, a drug repurposing strategy has been tested and presented. Although, neither TFP or SFB is approved or marketed for glioblastoma, here we suggest the potential repurposing of these drugs in combination should be studied further using primary glioma cell lines.

4.3 Understanding the Mechanism of TPF, SFB or TFP-SFB Combination

It is known that increased levels of mutant p53 in some cancer types is associated with poor prognosis and drug resistance (Huang et al., 2014). On the other hand, many GBM tumors have tp53 mutations leading to loss of function (Gil-Perotin et al., 2006). In order to understand why a relatively more resistance occurs in A172 cell lines which has a wildtype tp53, and whether a null isogenic A172 will have increased resistance in the absence of TP53, sip53 was introduced where p53 protein could not be effectively translated into protein, mimicking loss of function. Inhibition difference between siP53 and siControl indicated that A172 cells were sensitive to the transfection rather than to the loss of TP53. However, this experiment is preliminary, future studies should include confirming that TP53 function was lost so that P21 was not induced. In addition, overexpression of a mutant TP53 in A172 cells could be a better option to make sure that the tested drugs were also effective in mutant GBM cell lines. In addition, TP53 might be activated by nutlin 3 and since both U87 and A172 cell lines express wild-type p53, increasing the levels of p53 might result in more regulation in the checkpoint of cell cycle and help overcome the resistance to drugs (Hientz et al., 2017) (Lee et al., 2023). Since one of the aims was to understand the effect of drugs in a genotype specific manner *in vivo* zebrafish studies were conducted on *ache* mutant fish. However, *in vitro* GBM models should also be tested using RNAi, CRISPR/Cas9 or overexpression models in the future.

Viability analyses provide a first glance into the effectiveness of drugs. However, it is important to understand the mechanisms of action by which these antiproliferative effects are conducted and looking at changes in expression levels has been one of the strategies taken by many studies in literature (Samarkina et al., 2023)(Manzoni et al., 2018). Importantly, a metastatic EMT gene, vimentin, VIM, showed significant decreases at both the mRNA and protein levels upon drug treatments but this seemed to be driven by TFP yet, in combination therapy, a lower p value was reached. The protein levels did not change as effectively as the

mRNA levels, possibly due to variability in gene expression control mechanisms (Perl et al., 2017). In order to reach a more conclusive finding, protein levels should be deeply investigated with other antibodies targeting different isoforms of VIM. Nevertheless, the changes in Vimentin was correlated with previous knowledge indicating VIM levels were associated with poor prognosis and blocking Vimentin lowered the migration of GBM cells (Zottel et al., 2023). Since Vimentin protein levels were not drastically altered in the western blot (only significant after quantification), this result should be repeated with different antibodies that target Vimentin.

Another important gene in cancer and ER stress is DDIT3, which was shown to be upregulated only in the combination treatment of TFP and SFB in Hep3B liver cancer cell line previously in our lab (Murat Yaman PhD Thesis). In U87 cells too the only significant increase was noted in 10 μ M TFP and 2.5 μ M SFB combination. DDIT3 gene is activated in DNA damage and triggers apoptosis (Copanaki et al., 2007) (Wang et al., 2019). Therefore, this can be an important finding about the mechanism of combinatorial treatment. In the same Hep3B TFP-SFB RNA-Seq and qPCR data from Murat Yaman (PhD Thesis), SRSF7 gene was shown to be downregulated upon combination of two drugs. Here a similar pattern was also shown in the U87-MG glioblastoma cell line.

The two drugs, TFP and SFB showed more effect in cell lines than they are by single treatments in terms of viability alteration, raising the question of what pathways SFB and TFP acted on. TFP is an inhibitor of dopamine receptor, both DR1 and DR2 (Moura & Vale, 2023), while SFB inhibits multiple tyrosine kinases. Dopamine signaling and tyrosine kinase signaling crosstalk over PI3K and PKC pathways (Perreault et al., 2014). Dopamine receptor activation results in a cascade where GSK-3 is phosphorylated. The phosphorylation status of GSK-3 is also regulated by activation of Akt which is a result of Tyrosine Kinase B activation. Therefore, TFP and SFB, might result in altered patterns of phosphorylation of the kinases in the Akt and

GSK-3 pathways (Ghosh et al., 2003). Future studies should test whether the biomarker proteins of these pathways are modulated differentially in the presence of TFP, SFB or their combination.

4.4 Investigation of Drugs in Zebrafish Models *in vivo*

Zebrafish is a valuable organism for drug screening, thus in our study, zebrafish larval models have been used for different morphological and behavioral assessments. TFP has previously been applied to zebrafish models for an anemia study (Macari et al., 2016) and a heart-rate related work (Prill et al., 2023) while SFB has also been applied in zebrafish before in parallel dosages used in the present study (Lin et al., 2019). Our results on morphological assessments, showed important highlights of the toxicology of these drugs. Although each drug was relatively less toxic in the AB fish, when combined they induced a severe change in the spinal cord formation of the larvae. As mentioned in the results part, there is evidence that spinal cord deformation can be an indicator of neuroinflammation (Van Gennip et al., 2018) and it is possible that combination treatment increases inflammation of the spinal cord. However, this needs to be tested further since such a curvature can also occur in cerebrospinal fluid flow anomalies (Sternberg et al., 2018)) or imbalanced notocord formation during development (Sun et al., 2020) in zebrafish.

Similar to what is done in cell culture toxicology assays, two different zebrafish lines were used in this study. It is also important to mention that different zebrafish lines have shown slightly different responses to TFP and SFB and combination of these drugs. These differences can be summarized as lower tolerance to relatively higher dosages as 2.5 μ M SFB combined with TFP and more drastic changes in heart and yolk sac edema. Some of the single treatments such as SFB administration resulted in slight changes such as increased yolk size but needs to be quantified further and additional measurements can also be taken such as inflation of swim

bladder since the larvae received TFP and SFB showed no inflated swim bladder. Although this can be a retardation in the development, swim bladder can be inflated until 6 dpf (Venuto et al., 2023). There was no change in the length of the larvae upon TFP and SFB single introductions (Appendix Figure 3) In the literature it is commonplace that different zebrafish strains respond differently due to their divergence in genetic background (Petzold et al., 2009)(Vignet et al., 2013)(Richter et al., 2017).

Molecule **10**, on the other hand, did not introduce any toxic effect on zebrafish. However, when combined with SFB, severe toxicity was seen where no fish survived upon administration of 10 μ M molecule **10** + 2.5 μ M SFB. Molecule **10** is a novel molecule and a PTZ-derivative (Appendix Figure 3). Previously, it has been found that molecule **10** is an inhibitor of Ache activity using zebrafish larvae (Murat Yaman PhD. Thesis). *ache* is a crucial gene for zebrafish neuronal development as well as neurotransmitter passage (Behra, 2002) (Verma et al., 2022). Its loss leads to paralysis and changes in locomotion. Therefore, it is likely that TFP, a dopamine receptor inhibitor, and also showing similarities to ache inhibitors (Murat Yaman, PhD Thesis) can behave differently in the presence of a mutant null ache allele, which halves the AChE enzyme activity (Behra et al., 2002) could provide opportunities to test genotype x drug effects. Therefore, this thesis represents an effective way to approach *ache* genotype x drug interactions. In the literature, drugs have been tested in zebrafish with varying genotypic backgrounds and findings indicated interesting leads, e.g., *ache*/+ and AB wild-type strain showing differences in conditioned place preference (Ninkovic & Bally-Cuif, 2006). This study now provided a pipeline for testing the effectivity of different drugs using *ache* wildtype and heterozygous mutants, as exemplified with TFP and SFB.

Sorafenib is an inhibitor of multiple serine/tyrosine kinases including VEGFR, RAF1 and FGFR (Gong et al., 2017). It can be discussed that, inhibiting ache activity as well as the effect of SFB of multiple kinase inhibition could be intolerable to zebrafish larvae. In an early

study, it was found that there exists a relation between tyrosine kinases and nicotinic acetylcholine receptors (nAChRs) (Wiesner & Fuhrer, 2006). Therefore, different strategies should also be improved to be able to conclude this combination for therapeutics such as lowering the concentration while combining a third drug or prolonging the exposure time.

Since the drugs and their more effective combinations could be repurposed for glioblastoma, it was crucial to check whether they affected healthy glia using biomarkers *in vivo*. For this purpose, GFAP: GFP zebrafish reporter strain which produces green fluorescent protein under the GFAP promoter was used in this screening study. Previously, several other studies have used GFAP:GFP in drug screening and have performed their screening using imaging evaluations in confocal microscopy (Candiani et al., 2020) and xenograft quantification as well as staining and RNA level investigation (Gao et al., 2018). However, it has been seen that GFP expression could be slightly different in each individual larvae possible due to differences in the introduction of transgene and expression of GFP, the larvae' exact developmental stage and the differences among larvae, that could be estimated by the standard length of the larvae. In the present study, a novel method has been introduced where imaging of each larvae individually for 3 days including one measurement taken before any drug was introduced (3 dpf), and another after 24 hours of drug introduction (at 4 dpf) and yet a final measurement at 48 hours of drug introduction (at 5 dpf). By normalizing all individual larvae to itself in 3 dpf, we saw how GFAP signal increased throughout the days. The most drastic change was seen from 3 dpf to 4 dpf transition; yet these results should be taken with caution since each scanned slide included only one group of larvae and could not be normalized within slide to a control, i.e., DMSO hence the findings might reflect signal differences among slides that were not due to biological changes but to microscopic parameters. However, the experiment is still important with respect to development of the technique. In between slides

the exposure was set to a point until any evidence of saturation in signal was observed since EVOS did not specify the intensity.

In literature, there was evidence that GFAP signal started localizing in the brain and spinal cord at 3 dpf in zebrafish (Bernardos & Raymond, 2006). Supporting this, GFAP: GFP signal was observed at 3 dpf in all groups but the signal change observed from 3 dpf to 4 dpf was significantly lower in many drugs when compared with DMSO control and 2.5 μ M SFB. Yet, in total transition graph (Figure 3.14) both 10 μ M TFP + 1 μ M SFB and 10 μ M TFP + 2.5 μ M SFB showed a significantly lower signal outcome when compared with the exposure to 10 μ M TFP alone. In this work, we have also shown the more drastic effect of SFB combination with molecule **10**, a novel PTZ derivative, on acting on glia. Therefore, combination of these two drugs, decreased the signal density, thus GFAP expression, suggesting their action on glia.

However, toxic effects on glia could not be a desired outcome. A more effective approach would be using zebrafish xenografted with glioblastoma cells stained with a red dye can be visualized at the same time in GFAP: GFP fish and a differential effect on glioblastoma and healthy glia can be tested in the future. However, acting on glia highlights that these drugs can pass through BBB. Nevertheless, this is the first attempt in this novel screening protocol and should be considered preliminary.

Variability of GFP signal has been observed even in 3 dpf, before any treatment. This could be possibly due to slight differences in the development and size of the larvae or systematic effects of using a separate slide for each treatment, as mentioned above. It would be better to use one or more larvae from each treatment and control group in each slide this way batch effects could be corrected. Moreover, scanning the fish every day until 5 dpf under GFP filter can result in photobleaching and at later days the detected signal may come from newly produced GFP. However, since this is going to happen in all treatment groups it may still be

negligible if the appropriate controls are present in each slide. For this, a control GFP-signal including molecule, bead or one or more control grouped larvae can also be included in the slides where imaging occurs.

4.5 Diving in Zebrafish Behavior

Zebrafish is a great model organism for behavior assays. When the drugs of interest have anti-cancer potential and/or a combinatorial effect, testing them for behavioral effects also is a must. Additionally, behavior assays can be considered as an index for crossing the BBB if any behavioral changes are observed (Yang et al., 2016). Moreover, testing a drug by genotype interaction could be performed using behavioral assays as a proxy for future assays. For those purposes, the *ache*^{+/?} zebrafish model has been utilized. The *ache* gene is essential this can be understood from non-viable embryos of *ache*^{-/-}. A single mutation occurring in *ache* enzyme's catalytic side makes larvae paralyzed and non-viable after 3 dpf. There is also striking relevance between acetylcholine receptors and glioblastoma (Thompson & Sontheimer, 2019) (Pucci et al., 2021). Since our cell lines showed no or very low expression of *ache*, it was crucial to use an *ache* zebrafish model to test the importance of Ach levels and/or dysfunction of *ache* in governing the behavioral effects of the selected drugs further. Besides, the novel phenothiazine derivative molecule **10** was found to be an AChE inhibitor from previous studies in our laboratory (Murat Yaman PhD Thesis). *ache* siblings from a heterozygous *ache*^{+/-} in cross has previously been used by our lab (Avci et al., 2018) and was shown to increase tumor size. This study adds to that and shows that *ache* heterozygous mutants also exhibit differential drug responsiveness, presenting a novel pipeline for future drug tests. In addition, since excess Ach might be equivalent to nicotine consumption this can further help understand interactions between drugs and genotypes in the context of dual diet in *ache* background.

Understanding the mechanism of action of a therapeutic compound, particularly in terms of behavior, stress response, and locomotion, holds significant importance, especially in the field of drug development for neurological diseases. Zebrafish behavioral changes can serve as an indicator that the applied compound has crossed the BBB. Moreover, there is evidence suggesting that many behavioral patterns observed in zebrafish closely resemble those found in human diseases. For instance, abnormal repetitive behaviors observed in human conditions such as autism spectrum disorders, obsessive-compulsive disorder, and Tourette's syndrome are also manifested in zebrafish (Zabegalov et al., 2019).

Both TFP and SFB are FDA-approved drugs; however, they have not been previously tested for inducing changes in zebrafish behavior. Importantly, for wild-type AB strain, here, TFP significantly reduced the stress response at light-dark transitions. This finding is correlated with the nature of TFP which is an antipsychotic and anti-anxiety drug (Zhang et al., 2017) (Moura & Vale, 2023). Another strong point of this study was to be able to show a very similar pattern in stress behavior and anxiety like response in humans and zebrafish upon TFP application ((Marques et al., 2004)). Yet, this drug's effect needs to be investigated in terms of behavior and stress response in higher mammals with more resemblance to human neuronal system and brain.

In here, SFB also showed differences in recovery from the stress response in terms of locomotion (Figure 3.21). Combination with TFP results also provide clues that TFP could be more dominantly acting on behavior since it changed the results into a similar pattern when combined with TFP as in the single TFP administration. Yet, TFP did not alter the results of homozygous wild-type *ache* and heterozygous mutant *ache* larvae. Thus, the effects mentioned may not be *ache* dependent. Further, a derivative of TFP, the novel molecule **10** was given to homozygous wild-type AB fish and in another set to heterozygous mutant *ache* larvae and their homozygous wildtype genotyped siblings. *ache* background siblings showed a rather different

behavioral pattern than AB strain wild-type larvae, which showed a striking behavior resembling epileptic seizure when transitioning to light period. This has highlighted the presence of the strain or batch effects on behavior in zebrafish. We also have realized, although the total distances travelled was not significantly different, *ache* (+/+) and (+/-) larvae showed different locomotion in different experimental set ups. This can be due to slight changes in the environmental and other background genetic factors that cannot be controlled (e.g., different broods) tested during experimentation. There is evidence that zebrafish larvae react to the environmental factors such as light and feeding (Kopp et al., 2018). Also, zebrafish with different genetic backgrounds have been shown altered behavior responses previously (Vignet et al., 2013) which can be the reason for observing differences in 3rd dark period between wild type larvae and *ache* (+/+) larvae although they both have the wild-type genes. As expected, *ache*^{+/-} fish recovered poorly after stress response was introduced when molecule **10** was applied (Figure 3.23). The absence of light induced hyperactivity in *ache* siblings warrants the repetition of this experiment in a dose dependent manner and in different strains to generalize the effects of Molecular **10**. Additionally, increased hyperactivity in the light is seen in epileptic seizure models where zebrafish behavior is reversed and hyperactivity is seen in dark to light rather than in light to dark transitions (Li et al., 2018). Therefore, observing a hyperactivated behavior the light period can be an index for inducing epileptic pathways. Besides, there is evidence that glioblastoma patients encounter epileptic seizures and repurposing anti-epileptic drugs is on topic for glioblastoma (Stella et al., 2023).

Although, this reverse effect seen after 10 μ M molecule **10** was not seen *ache* (+/?) larvae, a decrease in the overall distance travelled upon 10 μ M molecule **10** administration was apparant. This can be also associated with inhibition of *ache*, which might have an effect on visual perception, but the same pattern was seen in in homozygous wildtype siblings, so it is unlikley. Where we see the most effect on *ache* siblings however was with 5 μ M molecule **10**

administration which resulted in lower recovery after the stress response of *ache* (+/-) larvae as well as the decreased total distance travelled compared to *ache* (+/+) even in baseline period without any stress was introduced (Figure 3.25D). These tests should be repeated with additional doses and differing strains for confirmation of the strain specific effects of molecule **10**. However, although strain specific this thesis made it possible to test behavioral side effects of a novel compound and this protocol can be used with other drugs to see if they are epileptic when used alone or in combination with antiepileptics.

Another concept is to understand where the effect on behavior is originating from, i.e., the change in neuronal activity versus muscle contraction. For this, muscular markers should be investigated at the molecular level. For example, previous studies have used bringing of the muscle to see whether there is any degeneration (Mittal et al., 2010). Yet, some muscle changes might be expected to be seen in *ache* mutant larvae. The reason for that is the expression of *ache* occurs in neuromuscular junctions as well. Besides, TFP acts as a calmodulin inhibitor (Macari et al., 2016).

Rather than exposure to drugs, a diet that contains both high-fat and EtOH was also introduced to zebrafish larvae for 7 days followed by behavioral analysis. Since the larvae was older, the behavioral analysis has been done in bigger multi-well plates (24-well plate) to not to interfere with the swimming of these bigger and taller larvae. In the literature a significant association exists between increased lipidogenesis and glioblastoma (Gangopadhyay et al., 2022) (Altinoz et al., 2020). Previous studies have tested the effects of high fat diet on locomotion on zebrafish (Picolo et al., 2021) (Kopp et al., 2018). However, there was no study for DUAL diet in which effects of HFD alone or in combination with EtOH could be tested. This could be important as both a steatohepatitis model and its association with other diseases that have relationships with increased lipidogenesis, e.g., GBM. Moreover, a pipeline by which one can test the effect of DUAL diet in larval behavior in between different genetic

backgrounds, e.g., *ache* wt and heterozygous mutant siblings, could provide an effective strategy for genotype x environment interactions. Interestingly, *ache* (+/+) larvae showed a higher hyperactivation response while transitioning from light to dark and a more drastic recovery throughout the end of 10-min dark period (significantly higher intercept). This effect was removed by DUAL diet introduction where *ache* (+/+) and *ache* (+/-) larvae showed no differences. Yet, total sample size of the experiment was not very high due to presence of many groups where larvae were assigned blindly and genotyping that was done after the experiment. Therefore, more analysis and repeats are required. Yet, here DUAL diet was found as possibly acting on brain and/or cognition and locomotory behavior in zebrafish larvae and the effects of drugs in the presence of obesogenic diets could be tested using this experimental design.

4.6 Future Perspectives

- Drugs should be tested in other glioblastoma cell lines, patient derived cells as well as healthy glia or neuronal cells to conclude the inhibitory effects observed were specific to cancer cells.
- The effect on VIM should be deeply investigated with more advanced epithelial to mesenchymal transition (EMT) assays such as transwell migration and scratch assays.
- For deciphering the long-term effects of the drugs, exposure to the drugs could be extended to late larvae into juvenile. Alternatively, higher mammals can be subjected to drugs before repurposing for clinical use.
- The xenograft model needs more optimization to mimic human glioblastoma using more samples as well as incorporation of a mock-injection control group. Mock- injection control should be used to normalize the effect of injection hazards and stress occurring in zebrafish.

- Screening of the drugs mentioned in this thesis should be done in xenografts for future studies. In order to assess tumor burden within the zebrafish brain, high-throughput screening as well as molecular detection methods should be applied to xenografts.
- Different stress introductions might be helpful to further conclude the effect of drugs, especially for TFP and molecule **10** on *ache* wildtype and mutant siblings. These can be listed as temperature change, vibration, change in light wavelength, and sound inductions.
- DUAL diet effect on behavior and glioblastoma should be further investigated to test whether DUAL diet could be an inducer of behavior changes and/or have effects on glia or glioblastoma growth when xenotransplanted.

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Appendix I

```
SET(THERMOSTAT,28.5) #sets temp inside MWP

#defines parameters used later in the script in seconds
DEFINE ACCLIMATION 300 # 5 min 300 s
DEFINE BIN 30 # 1/2 min 30 s

DEFINE VIDEO_LENGTH 999999999

DEFINE X_LOGDATA_TRACKS 799 # development setting: log track lengths (total)
DEFINE X_DRAWTRACKS 30011 # development setting: enable track drawing

# Settings for writing labels to video
DEFINE X_OVERLAY_TEXT 30018
DEFINE X_OVERLAY_CLEAR 30019

DEFINE MAKEMAP 5682
DEFINE COLOURMAP 5683
DEFINE SUMSIZE 20

# tracking settings for larval zebrafish
SET(TARGET_SIZE,2) # Set target animal size, diameter in mm
SET(DETECTOR_THRESHOLD,6) # Higher threshold, less sensitive tracking

SET(AUTOREF_MODE,MOVEMENT)
SET(AUTOREF_TIMEOUT,300) # Change time to wait (in sec) for the autoref image by changing AUTOREF_TIMEOUT number. Increase for animals that are inactive. 300 sec

#Following 4 set commands for light stand. Turns it off before start of trial
SET(VOLTAGE4,0N)
SET(VOLTAGE_LEVEL,10000)

SET(GP03,0)
SET(GP04,0)

# Sets the data output counter to begin at 0. Used in the script below to label time bins in numerical order
SET(COUNTER1,COUNTER_ZERO)

# This is the whole experiment's outline/schedule. The actions listed here are always further explained below.
ACTION MAIN

    LOAD(ARENAS,"a96.bmp") # This .bmp file is required in assets directory for tracking
    LOAD(DETECTORS, "dz96.bmp")

    LOGCREATE("TEXT:RUNTIME|TEXT:TEMPERATURE|TEXT:CONDITION|TEXT:COUNTER|TEXT:COUNTER")

    LOGAPPEND("TEXT:A1|TEXT:A2|TEXT:A3|TEXT:A4|TEXT:A5|TEXT:A6")
    LOGAPPEND("TEXT:A7|TEXT:A8|TEXT:A9|TEXT:A10|TEXT:A11|TEXT:A12")
    LOGAPPEND("TEXT:B1|TEXT:B2|TEXT:B3|TEXT:B4|TEXT:B5|TEXT:B6")
    LOGAPPEND("TEXT:B7|TEXT:B8|TEXT:B9|TEXT:B10|TEXT:B11|TEXT:B12")
    LOGAPPEND("TEXT:C1|TEXT:C2|TEXT:C3|TEXT:C4|TEXT:C5|TEXT:C6")
    LOGAPPEND("TEXT:C7|TEXT:C8|TEXT:C9|TEXT:C10|TEXT:C11|TEXT:C12")
    LOGAPPEND("TEXT:D1|TEXT:D2|TEXT:D3|TEXT:D4|TEXT:D5|TEXT:D6")
    LOGAPPEND("TEXT:D7|TEXT:D8|TEXT:D9|TEXT:D10|TEXT:D11|TEXT:D12")
    LOGAPPEND("TEXT:E1|TEXT:E2|TEXT:E3|TEXT:E4|TEXT:E5|TEXT:E6")
    LOGAPPEND("TEXT:E7|TEXT:E8|TEXT:E9|TEXT:E10|TEXT:E11|TEXT:E12")
    LOGAPPEND("TEXT:F1|TEXT:F2|TEXT:F3|TEXT:F4|TEXT:F5|TEXT:F6")
    LOGAPPEND("TEXT:F7|TEXT:F8|TEXT:F9|TEXT:F10|TEXT:F11|TEXT:F12")
    LOGAPPEND("TEXT:G1|TEXT:G2|TEXT:G3|TEXT:G4|TEXT:G5|TEXT:G6")
    LOGAPPEND("TEXT:G7|TEXT:G8|TEXT:G9|TEXT:G10|TEXT:G11|TEXT:G12")
    LOGAPPEND("TEXT:H1|TEXT:H2|TEXT:H3|TEXT:H4|TEXT:H5|TEXT:H6")
    LOGAPPEND("TEXT:H7|TEXT:H8|TEXT:H9|TEXT:H10|TEXT:H11|TEXT:H12")

    LOGRUN()

    SETCOLOUR(15,0.5,0.5,0.8)
    SET(COLOURMAP,0)

    INVOKE(DARK,1) #do you want lights on for the acclimation if the first transition is dark??? #baseline dark olsun istiyorum. 0 zaman bunu invoke(dark,1) mÃ¼ yapacaÃŸÃızım?
    WAIT(ACCLIMATION) # System waits this amount of time before beginning the next command. (Which is defined above)
    AUTOREFERENCE() # This creates an autoreferenced image of the plate prior to invoking the trials. Needed for tracking.
    VIDEO(VIDEO_LENGTH,"LocomotionWithVibrationStartleMonitoring") # Record X seconds of video into file with given prefix. Change the name of your file by editing the name inside of the "".
    SET(X_DRAWTRACKS,1)

    INVOKE(DARK,1)
    LOAD(X_OVERLAY_TEXT,"1,2:DARK") # "DARK" is shown on the video screen (1:ROW and 2:COLUMN)
    INVOKE(WMDARK,20) # Runs the func 10 times for for 30 sec time bin each.
    LOAD(X_OVERLAY_CLEAR,"")

    INVOKE(BRIGHT,1)
    LOAD(X_OVERLAY_TEXT,"1,2:BRIGHT") # "LIGHT" is shown on the video screen (1:ROW and 2:COLUMN)
    INVOKE(WMBRIGHT,20)
    LOAD(X_OVERLAY_CLEAR,"")

    INVOKE(DARK,1)
    LOAD(X_OVERLAY_TEXT,"1,2:DARK") # "DARK" is shown on the video screen (1:ROW and 2:COLUMN)
    INVOKE(WMDARK,20) # Runs the func 10 times for for 30 sec time bin each.
    LOAD(X_OVERLAY_CLEAR,"")
```

```

|
    INVOKE(BRIGHT,1)
LOAD(X_OVERLAY_TEXT,"1,2: STARTLE BRIGHT")
    INVOKE(MMBRIGHT,2)
LOAD(X_OVERLAY_CLEAR,"")

    #INVOKE(STARTLE_LIGHT)

    INVOKE(DARK,1)
LOAD(X_OVERLAY_TEXT,"1,2:DARK")
    INVOKE(MMDARK,20)
LOAD(X_OVERLAY_CLEAR,"")

SET(MAKEMAP,SUMSIZE)

VIDEOSTOP

COMPLETE

ACTION MMDARK

    SET(COUNTER1,COUNTER_INC)                # Labels the data output in numerical order.
LOGDATA(DATA_SNAPSHOT,"begin") # DATA_SNAPSHOT starts data collection.
WAIT(BIN)                                   # Waits BIN amount of time before beginning the next command.
LOGDATA(DATA_SNAPSHOT,"end") # stops data snapshot.

#The following commands process the data between the two data snapshots and exports the parameters selected
LOGDATA(DATA_SELECT,"begin")
LOGDATA(DATA_DELTA,"end")

LOGCREATE("RUNTIME|TEMPERATURE1|TEXT:DARK|COUNTER1")
LOGAPPEND("COUNTER1|ARENA_DISTANCES:*")
LOGRUN() # Tells the unit to output the data from the previous LOGCREATE (and LOGAPPEND) command.

COMPLETE

ACTION MMBRIGHT

    SET(COUNTER1,COUNTER_INC)

    LOGDATA(DATA_SNAPSHOT,"begin")
WAIT(BIN)
LOGDATA(DATA_SNAPSHOT,"end")



    SET(COUNTER1,COUNTER_INC)

    LOGDATA(DATA_SNAPSHOT,"begin")

    SET(GP03,1)                #pulses the light stand at full brightness
    SET(GP04,1)
WAIT(0.5)                      # waits for 500ms
    SET(GP03,0)
    SET(GP04,0)

    #ZCOMMAND("G3#1,P2000,G3#0") #pulses the light stand at full brightness for 500ms (0.5sec)

    LOGDATA(DATA_SNAPSHOT,"end")

    LOGDATA(DATA_SELECT,"begin")
    LOGDATA(DATA_DELTA,"end")

    LOGCREATE("RUNTIME|TEMPERATURE1|TEXT:STARTLE_LIGHT|COUNTER1")
    LOGAPPEND("COUNTER1|ARENA_DISTANCES:*")
    LOGRUN()

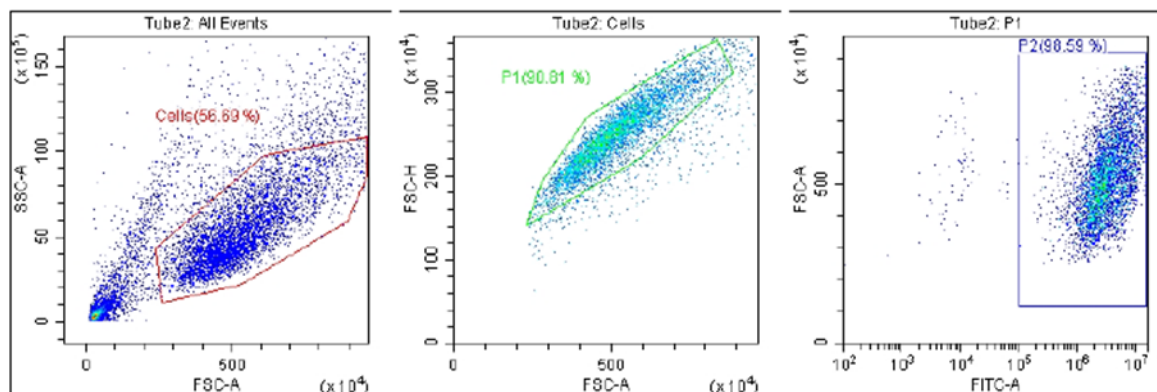
COMPLETE

```

Appendix Figure 1: Code for zantiks MWP device used for the behavior assays.

The script is written and stored in Zantiks device's own platform.

Appendix II



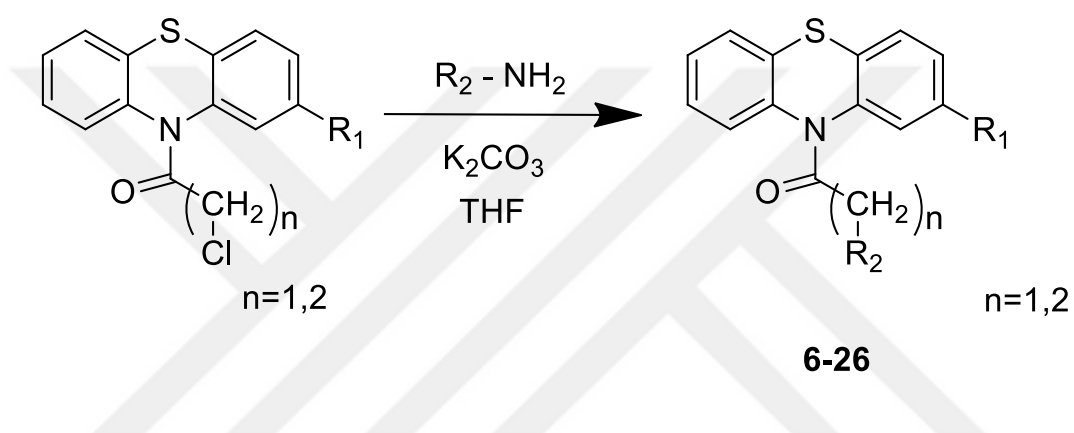
Appendix Figure 2: Flow cytometer results of U87-GFP cells

Cells that are used in xenograft experiments are shown. Last panel shows the cells that are GFP+ which is 98.56% of the population.

Appendix III

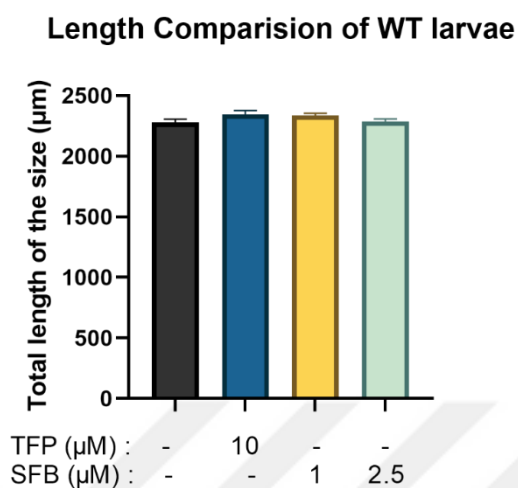
Appendix Table 1: Properties of molecule 10.

Molecule name	Molecular Weight (g/mol)	Molecular Volume ($\text{\AA}^3$)	QPlogp	%Oral absorption
10	540.076	1562.860	4.538	86.190



Appendix Figure 3: Synthesis process and molecular shape of molecule10.

Appendix III



Appendix Figure 4: Length of the larvae after drug treatment.

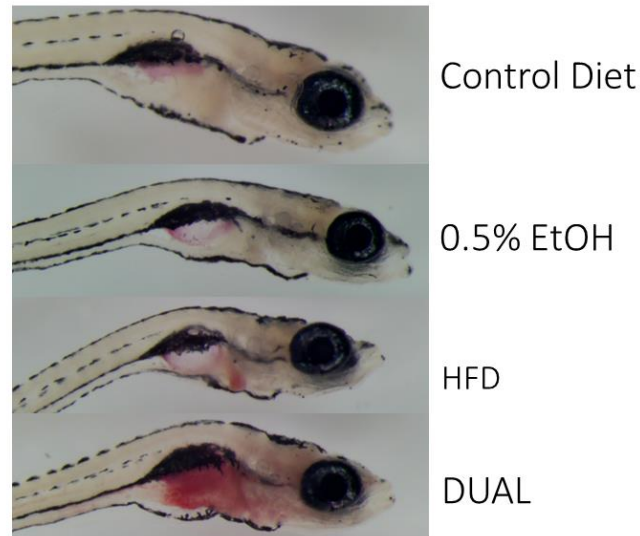
Larvae that receive single treatments of TFP and SFB is imaged, and length of the organism is scaled in ImageJ. No significant change occurs in the treatment groups.

Appendix IV

Appendix Table 2: Sparos HFD Ingredients.

Ingredient	Percentage
Fishmeal	30%
Squid Meal	33%
Fish Gelatin	5%
Wheat Gluten	5.5%
Palm Oil	12%
Soybean Oil	2.5%
Rapeseed Oil	2.5%
Vitamins And Minerals	2%
Vitamin E	0.1%
Antioxidant	0.4%
Monocalcium Phosphate	2%
Calcium Silicate	2.2%

Appendix V



Appendix Figure 5: Oil Red O Staining images of DUAL-diet group larvae.

Single images represent examples of the majority (at least 50%) of staining in each group. Whole mount oil red o protocol was done by 0.3% Oil Red O (Sigma-Aldrich, Germany) diluted in propylene glycol. Before dye is introduced, whole larvae were fixed in 4% PFA overnight followed by propylene glycol washes. Dye was incubated 1 hour and propylene glycol washes was applied again.