

ZEBRAFISH AS AN IN VIVO MODEL OF DRUG SCREENS FOR LIVER CANCER: THE ROLE OF PHENOTHIAZINES

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By
Büşra KORKMAZ

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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.

Özlen Konu Karakayalı (Advisor)

Gülçin Çakan Akdoğan (Co-advisor)

Onur Çizmecioğlu

Ceren Sucularlı

Approved for the Graduate School of Engineering and Science:

Ezhan Karaşan

Director of the Graduate School

ABSTRACT

ZEBRAFISH AS AN IN VIVO MODEL OF DRUG SCREENS FOR LIVER CANCER: THE ROLE OF PHENOTHIAZINES

Büşra KORKMAZ

MSc. in Molecular Biology and Genetics

Supervisor: Assoc. Prof. Özlen KONU KARAKAYALI

Co-supervisor: Dr. Gülçin ÇAKAN AKDOĞAN

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Hepatocellular carcinoma (HCC) is one of the most dangerous cancer types and sorafenib (SFB) is a commonly used drug against HCC, being the only FDA-approved medication until 2018. However, its limited efficacy and severe side effects emphasize the importance of developing alternative approaches. Regulation of cholesterol has been implicated in the progression of various cancers including HCC. Phenothiazines, which are in use as antipsychotic drugs and with effects on cholesterol biosynthesis, have drawn recent attention as anti-cancer drugs against different cancers. The elucidation of how phenothiazines exert their effects at cellular level might pave the way for proposing better strategies for HCC treatment. Zebrafish serves as a good cancer model for in vivo validations, since it enables efficient tumor formation and tracking. Its conserved genes and similar organ system to human make it even better of a model to reveal both molecular and morphological alterations caused by drugs. Based on previous cell viability studies from our lab, the combination of trifluoperazine (TFP) with SFB was found to exhibit synergism in Hep3B cells, while it was antagonistic in SkHep1 cells. Moreover, RNA-seq analysis on Hep3B cells has demonstrated that steroid biosynthesis and cholesterol metabolism was among the modulated pathways. Therefore, in the present thesis, the regulation of cholesterol levels in response to phenothiazine derivatives was investigated in HCC cell lines SkHep1 and Hep3B. The

combinatorial approach was examined on Huh7 cells in terms of the expression of cholesterol biosynthesis genes based on RT-qPCR. After showing the ability of phenothiazine derivatives and TFP-SFB combinations to modulate cellular cholesterol, zebrafish drug screens were performed to determine applicable derivative and combination doses. Assessment of safe doses for TFP, SFB and their combination directed us for xenograft studies, which resulted in enhanced Hep3B cell survival by the combination of low dose (6 μ M) TFP and 1 μ M of SFB. This finding was further validated based on Alu-based DNA quantification, a powerful method we have established. With the help of RNA-seq analysis results, orthologous zebrafish genes, involved in cholesterol biosynthesis were identified and used in cross-species testing. The impact of TFP on liver vasculature was evaluated using transgenic (Fli1a:EGFP) zebrafish and found insignificant. Overall, the findings presented in this thesis highlight that TFP alone or in combination was able to change cellular cholesterol levels and tumor growth.

Key words: Hepatocellular carcinoma, phenothiazines, sorafenib, drug repurposing, cholesterol, zebrafish, in vivo drug screening, xenograft

ÖZET

KARACİĞER KANSERİNDE İLAÇ TARAMALARI İÇİN İN VIVO MODEL OLARAK ZEBRA BALIĞININ İNCELENMESİ: FENOTİYAZİNLERİN ROLÜ

Büşra KORKMAZ

Moleküler Biyoloji ve Genetik Yüksek Lisans Programı

Tez Danışmanı: Özlen KONU KARAKAYALI

Tez Eş Danışmanı: Gülçin ÇAKAN AKDOĞAN

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Hepatoselüler karsinom (HCC) en tehlikeli kanser türlerinden biridir ve sorafenib (SFB) 2018 yılına kadar FDA onaylı tek ilaç olarak HCC'ye karşı yaygın olarak kullanılmıştır. Ancak bu ilacın sınırlı etkinliği ve ciddi yan etkileri, alternatif yaklaşımlar geliştirmenin önemini vurgulamaktadır. Kolesterolün düzenlenmesi, HCC dahil olmak üzere çeşitli kanserlerin ilerlemesinde rol oynamaktadır. Antipsikotik ilaç olarak kullanımda olan ve kolesterol biyosentezini etkileyen fenotiyazinler, farklı kanser türlerine karşı anti-kanser ilaçlar olarak son zamanlarda dikkat çekmiştir. Fenotiyazinlerin etkilerini hücresel düzeyde nasıl sergilediklerinin aydınlatılması, HCC tedavisi için daha iyi stratejiler önermenin yolunu açabilir. Zebra balığı verimli tümör oluşumu ve takibi sağladığından, in vivo doğrulamalar için iyi bir kanser modeli olarak hizmet etmektedir. Korunmuş genleri ve insana benzer organ sistemi, zebra balığını ilaçların neden olduğu hem moleküler hem de morfolojik değişiklikleri ortaya çıkartmak için daha iyi bir model yapmaktadır. Laboratuvarımızda daha önce gerçekleştirilen hücre canlılığı çalışmalarına dayanarak, trifluoperazinin (TFP) SFB ile kombinasyonunun Hep3B hücrelerinde sinerjizm sergilediği, SkHep1 hücrelerinde ise antagonistik olduğu bulgulanmıştır. Ayrıca, Hep3B hücreleri üzerinde gerçekleştirilen RNA-seq analizi, steroid biyosentezinin ve kolesterol metabolizmasının düzenlenen mekanizmalar

arasında olduğunu göstermiştir. Bu nedenle, sunulan tezde, fenotiyazin türevlerine yanıt olarak kolesterol seviyesinin düzenlenmesi HCC hücre hatları SkHep1 ve Hep3B’de araştırılmıştır. Kombinatorial yaklaşım, Huh7 hücrelerinde kolesterol biyosentez genlerinin ifadenmesi açısından RT-qPCR’a dayalı olarak incelenmiştir. Fenotiyazin türevlerinin ve TFP-SFB kombinasyonlarının hücresel kolesterolü modüle etme kabiliyeti gösterildikten sonra, uygunabilir türev ve kombinasyon dozlarını belirlemek için zebra balığı ilaç taramaları gerçekleştirilmiştir. TFP, SFB ve kombinasyon için güvenli dozların değerlendirilmesi bizi zenograft çalışmalarına yönlendirmiş, bu çalışmalar düşük doz (6 μ M) TFP ve 1 μ M SFB kombinasyonu ile Hep3B hücre sağkalımının artmasıyla sonuçlanmıştır. Bu bulgu, oluşturduğumuz güçlü bir yöntem olan Alu bazlı DNA kantifikasyonuna dayalı olarak doğrulanmıştır. RNA-seq analiz sonuçlarının yardımıyla, kolesterol biyosentezinde yer alan ortolog zebra balığı genleri tanımlanmış ve türler arası değerlendirmelerde kullanılmıştır. TFP’nin karaciğer damar sistemi üzerindeki etkisi transgenik (Fli1a:EGFP) zebra balığı kullanılarak değerlendirilmiş ve önemsiz bulunmuştur. Genel anlamda, bu tezde sunulan bulgular, tek başına veya kombinasyon halinde TFP’nin hücresel kolesterol seviyesini ve tümör büyümesini değiştirebildiğini vurgulamaktadır.

Anahtar kelimeler: Hepatoselüler karsinom, fenotiyazinler, sorafenib, ilaçların yeniden işlevlendirilmesi, kolesterol, zebra balığı, in vivo ilaç taraması, zenograft



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TABLE OF CONTENTS

ABSTRACT	iii
ÖZET.....	v
ACKNOWLEDGEMENTS	viii
TABLE OF CONTENTS	x
LIST OF FIGURES	xiii
LIST OF TABLES	xvi
ABBREVIATIONS.....	xvii
Chapter 1. INTRODUCTION	1
1.1 Cancer.....	1
1.2 Cholesterol Homeostasis	1
1.3 Cholesterol Biosynthesis	2
1.4 Cholesterol Uptake	4
1.4.1 LDLR-Mediated Endocytosis.....	4
1.4.2 NPC1L1-Mediated Cholesterol Uptake.....	4
1.5 Intracellular Cholesterol Transport.....	5
1.6 Cholesterol Efflux	5
1.7 The Role of Cholesterol Metabolism in Cancer	6
1.8 Liver Cancer/Hepatocellular Carcinoma (HCC).....	9
1.9 Clinical Strategies in HCC	9
1.9.1 Sorafenib	9
1.10 Alternatives for the Treatment of HCC: Drug Repurposing	10
1.11 Phenothiazines.....	10
1.11.1 TFP	12
1.12 Novel Phenothiazines	13
1.13 Regulation of Cholesterol Metabolism by Phenothiazines in Cancer	14
1.14 Zebrafish as a Model for Drug Screens	15
1.14.1 Zebrafish Xenograft Models.....	17
1.14.2 Cholesterol-Related Studies in Zebrafish	18
1.14.3 Phenothiazines in Cancer Studies using Zebrafish.....	20
1.14.4 Sorafenib in Zebrafish Studies	20
1.15 Aims and Rationale	21
Chapter 2. MATERIALS AND METHODS.....	23

2.1	Materials	23
2.1.1	Laboratory Reagents and Kits	23
2.1.2	Cell Culture Products	24
2.1.3	Primers.....	25
2.1.4	Antibodies	26
2.1.5	Solutions and Buffers	26
2.1.6	Laboratory Equipment.....	28
2.2	Methods	29
2.2.1	In Vitro Methods	29
2.2.2	Generation of Cholesterol Pathway Representations	33
2.2.3	In Vivo Methods.....	33
2.2.4	In Silico Analysis of Human and Zebrafish Conservation in terms of Cholesterol Biosynthesis Pathway Genes	38
Chapter 3. RESULTS		39
3.1	Gene Expression Analyses on Cholesterol Biosynthesis Pathway	39
3.1.1	The Effects of Phenothiazines on Cholesterol Biosynthesis Using SkHep1 and Hep3B Cells	39
3.1.2	The Effects of TFP-SFB Combination on Huh7 Cells	43
3.2	Investigation of the Change in Intracellular Cholesterol Levels	44
3.3	Cholesterol Pathway Representations Utilizing RNA-seq Analysis	47
3.4	Effects of Intermediary and Novel Derivatives on Cancer Pathway Protein Levels.....	56
3.4	In Vivo Experiments	58
3.4.1	Microscopic Examination on Casper Embryos	58
3.4.2	Xenograft Experiments on Casper Embryos	62
3.4.3	Microscopic Examination on Wild Type Embryos	63
3.4.3	Microscopic Examination on AB Larvae for Exposure of 12 μ M TFP, 2 μ M SFB, and Combination Therapy Starting from 1 dpi to 2 dpi	66
3.4.4	Xenograft Experiments on AB embryos by Transplantation and Drug Exposures Starting from 1 dpi to 3 dpi	67
3.4.6	Embryonic Toxicity of Novel Phenothiazine Derivatives	76
3.4.7	In Silico Evaluations to Understand the Conservation of Cholesterol Biosynthesis Genes Between Human and Zebrafish	79
3.4.8	In Vivo Experiments to Investigate Effect of Phenothiazines on Cholesterol Metabolism of Zebrafish	80
3.4.8.1	Effects of Selected Phenothiazines on Zebrafish Cholesterol Biosynthesis Gene Expression Levels.....	80
3.4.9	Change in Liver Vasculature Area of 7 dpf flil1a:EGFP Larvae After Exposure to 6 μ M of TFP for 48 hours	81

Chapter 4. CONCLUSIONS AND DISCUSSION	85
4.1 Expression of the genes involved in cholesterol biosynthesis increased in vitro and in vivo upon phenothiazine exposure	86
4.2 TFP 12 μ M combined with SFB increased intracellular cholesterol levels in Hep3B cells..	90
4.3 Cell cycle and apoptosis were regulated by novel and intermediary derivatives in Hep3B cells	92
4.4 Toxicity studies in zebrafish helped test and confirm the lead compounds	93
4.5 Xenograft studies implicated that drug dose is important	95
4.6 Microscopy was useful to identify toxic effects in a dose dependent manner	97
4.7 The conservation between human and zebrafish could be revealed within the scope of cholesterol biosynthesis genes.....	98
4.8 Future perspectives.....	100
REFERENCES.....	103
APPENDIX	114
Appendix A: Transplantation of HCC cells at Blastula Stage.....	114
Appendix B: RNA-seq Analysis for Differential Expression of Cholesterol-Related Genes in Hep3B cells.....	115
Appendix C: Effects of PD-3 and PD-9 on the Expression of <i>hmgcs1</i> and <i>lss</i> zebrafish genes.....	118
Appendix D: Nile Red Staining and Analysis of Zebrafish Liver Size after Exposure to TFP.....	119

LIST OF FIGURES

Figure 1. Cholesterol Biosynthesis Pathway.	3
Figure 2. Changes in the expression of cholesterol biosynthesis genes after treatment with commercial phenotiazines.	40
Figure 3. Changes in the expression of cholesterol biosynthesis genes after treatment with intermediary phenotiazines.	41
Figure 4. Changes in the expression of cholesterol biosynthesis genes after treatment with novel phenotiazines.	42
Figure 5. Gene expression profiles in Huh7 cells after treatments with TFP alone, SFB alone and TFP+SFB combined for 48 hours.	43
Figure 6. Cholesterol levels in Hep3B cells after exposure to TFP, SFB and their combination for 48 hours.	44
Figure 7. Representative images of 48 hour-treated Filipin III-stained Hep3B cells.	45
Figure 8. Normalization of cholesterol levels to cell viability results in Hep3B cells after exposure to TFP, SFB and their combination for 48 hours.	46
Figure 9. Color-coded representation of logFC values for cholesterol biosynthesis genes based on the comparison between TFP 12 μ M and DMSO control in 48 hour-treated Hep3B cells.	48
Figure 10. Color-coded representation of logFC values for cholesterol biosynthesis genes based on the comparison of A) TFP 12 μ M - SFB 1 μ M and B) TFP 12 μ M - SFB 2 μ M versus TFP 12 μ M in 48 hour-treated Hep3B cells.	49
Figure 11. Color-coded representation of logFC values for cholesterol biosynthesis genes based on the comparison between TFP 12 μ M - SFB 2 μ M and TFP 12 μ M - SFB 1 μ M in 48 hour-treated Hep3B cells.	50
Figure 12. Color-coded representation of logFC values for the genes involved in cholesterol metabolism based on the comparison between TFP 12 μ M and DMSO control in 48 hour-treated Hep3B cells.	52
Figure 13. Color-coded representation of logFC values for the genes involved in cholesterol metabolism based on the comparison between TFP 12 μ M - SFB 1 μ M combination and TFP 12 μ M in 48 hour-treated Hep3B cells.	53
Figure 14. Color-coded representation of LogFC values for the genes involved in cholesterol metabolism based on the comparison between TFP 12 μ M - SFB 2 μ M combination and TFP 12 μ M in 48 hour-treated Hep3B cells.	54
Figure 15. Color-coded representation of LogFC values for the genes involved in cholesterol metabolism based on the comparison between TFP 12 μ M - SFB 2 μ M and TFP 12 μ M - SFB 1 μ M combination groups in 48 hour-treated Hep3B cells.	55

Figure 16. Changes in protein levels after exposure of Hep3B cells with 10 μ M of novel and intermediary phenothiazines for 24 hours.	56
Figure 17. Quantification of T-CHEK1, CDK2, AKT and P38 levels as a result of 24-hour exposure with 10 μ M of novel and intermediary phenothiazines in Hep3B cells.	57
Figure 18. Quantification of p-CHEK1, p-P38 and DR5 levels as a result of 24-hour exposure with 10 μ M of novel and intermediary phenothiazines in Hep3B cells.	58
Figure 19. Representative images of 3 and 4 dpf casper larvae after 24 and 48 hours of exposure to TFP starting from 2 dpf.	59
Figure 20. Representative images of 3 and 4 dpf casper larvae after 24 and 48 hours of exposure to SFB starting from 2 dpf.	59
Figure 21. Representative images of 3 and 4 dpf casper larvae after 24 and 48 hours of exposure to TFP+SFB combination starting from 2 dpf.	60
Figure 22. The mortality of casper larvae as a result of treatment with A) TFP, B) SFB and C) TFP + SFB for 24 and 48 hours starting from 2 dpf.	61
Figure 23. The total length of 4 dpf casper larvae as a result of exposure to A) TFP, B) SFB and C) TFP + SFB for 48 hours starting from 2 dpf.	61
Figure 24. The xenograft experiment performed on casper embryos.	62
Figure 25. Fluorescence changes in Hep3B-injected embryos after being exposed to TFP, SFB or their combination.	62
Figure 26. Representative images of 4 and 5 dpf WT larvae after 48 and 72 hours of exposure to different concentrations of TFP starting from 2 dpf.	63
Figure 27. Representative images of 4 and 5 dpf WT larvae after 48 and 72 hours of exposure to different concentrations of SFB starting from 2 dpf.	64
Figure 28. Representative images of 4 and 5 dpf WT larvae after 48 and 72 hours of exposure to different concentrations of TFP + SFB combination starting from 2 dpf.	65
Figure 29. The mortality of WT larvae as a result of treatment with A) TFP, B) SFB and C) TFP + SFB for 48 and 72 hours starting from 2 dpf.	65
Figure 30. The total length of 5 dpf WT larvae as a result of exposure to A) TFP, B) SFB and C) TFP + SFB for 72 hours starting from 2 dpf.	66
Figure 31. Transplantation of 250-300 Hep3B cells and treatment of injected larvae for 24 hours.	67
Figure 32. Transplantation of 250-300 Hep3B cells and treatment of injected larvae for 48 hours.	68
Figure 33. Transplantation of 100-200 Hep3B cells and treatment of injected larvae for 48 hours.	69
Figure 34. Representative images of 4 and 5 dpf WT larvae after 48 and 72 hours of exposure to different concentrations of PD-9 starting from 2 dpf.	70
Figure 35. Representative images of 4 and 5 dpf WT larvae after 48 and 72 hours of exposure to different concentrations of PD-10 starting from 2 dpf.	71

Figure 36. Representative images of 4 and 5 dpf WT larvae after 48 and 72 hours of exposure to different concentrations of PD-11 starting from 2 dpf.	72
Figure 37. Representative images of 4 and 5 dpf WT larvae after 48 and 72 hours of exposure to PD-12 starting from 2 dpf.	73
Figure 38. The mortality of WT larvae as a result of treatment with A) PD-9, B) PD-10, C) PD-11 and D) PD-12 for 48 and 72 hours starting from 2 dpf.	74
Figure 39. The total length of 5 dpf WT larvae after being exposed to A) PD-9, B) PD-10, C) PD-11 and D) PD-12 for 72 hours starting from 2 dpf.	75
Figure 40. Representative images 5 dpf WT larvae after 48 and 72 hours of exposure to PD-3 and PD-5 starting from 2 dpf.	76
Figure 41. Representative images of 4 and 5 dpf WT larvae after 48 and 72 hours of exposure to PD-6 starting from 2 dpf.	77
Figure 42. The changes in WT larvae after being exposed to novel phenothiazines A) PD-3, B) PD-5 and C) PD-6 for 72 hours starting from 2 dpf.	78
Figure 43. The total length of A) 4 dpf WT larvae as a result of exposure to compound PD-3 for 48 hours and 5 dpf WT larvae after being exposed to compound B) PD-3, C) PD-5 and D) PD-6 for 72 hours starting from 2 dpf.	79
Figure 44. Zebrafish gene expression profiles for <i>hmgcs1</i> and <i>lss</i> after treatment with TFP, SFB and combination for 72 hours starting from 2 dpf.	81
Figure 45. Representative images of the liver of 7 dpf fli1a:EGFP zebrafish larvae after 48 hour-treatment with 6 μ M of TFP.	82
Figure 46. A) Liver vasculature area and B) vasculature intensity of TFP-treated fli1a:EGFP larvae at 7 dpf in comparison to DMSO control.	83
Figure 47. Representative images of the liver of 7 dpf fli1a:EGFP zebrafish larvae after 48 hour-treatment with 6 μ M of TFP as a result of the repetition of previous experiment.	83
Figure 48. A) Liver vasculature area and B) vasculature intensity of TFP-treated fli1a:EGFP larvae at 7 dpf in comparison to DMSO control as a result of the repetition of previous experiment.	84

LIST OF TABLES

Table 1. Different delivery methods used for drug screens on zebrafish.	16
Table 2. General laboratory products and kits used for the experiments.	23
Table 3. Products used in cell culture experiments.	25
Table 4. List of the primers used in RT-qPCR experiments.	25
Table 5. List of the antibodies used.	26
Table 6. The solutions/buffers used and their components.	27
Table 7. Equipment and the procedures in which they were used.	28
Table 8. Conditions for RT-qPCR.	32
Table 9. Conditions for Alu-based DNA Quantification.	36
Table 10. LogFC and adj. p-values of cholesterol-related genes that were significantly affected in Hep3B cells.	56
Table 11. Zebrafish orthologues/paralogs of human cholesterol biosynthesis genes and the similarity between them.	80

ABBREVIATIONS

ABCA1 : ATP-binding cassette transporter A1

ACAT2 : Acetyl-CoA acetyltransferase 2

AKT : Protein kinase B

ApoA1 : apolipoprotein A1

ApoB48 : apolipoprotein B-48

ApoB100 : Apolipoprotein B-100

ArBu : Arenobufagin

ATP : Adenosine triphosphate

Bax : Bcl-2 associated X protein

Bcl-2 : B-cell lymphoma 2

BSEP : Bile salt export pump

CDK2 : Cyclin-dependent kinase 2

CTCF : Corrected Total Cell Fluorescence

ddH₂O : Double-distilled H₂O

dpf : Days post fertilization

dpi : Days post injection

DR5 : Death Receptor 5

EMT : Epithelial-mesenchymal transition

EPA : Eicosapentaenoic acid

ER : Endoplasmic reticulum

ERC : Endocytic recycling compartment

FDA : Food and drug administration

FOXO1 : Forkhead box protein O1

GAPDH : Glyceraldehyde 3-phosphate dehydrogenase

GST : Glutathione S-transferase

HCC : Hepatocellular carcinoma

HDL : High-density lipoprotein

HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A

HMGCR : 3-hydroxy-3-methylglutaryl coenzyme A reductase

hpf : Hours post fertilization

IDL : Intermediate-density lipoprotein

INSIG-1 : Insulin-induced gene 1 protein

INSIG-2 : Insulin-induced gene 2 protein

IRR : Incidence rate ratio

JNK : c-Jun N-terminal kinase

LCAT : Lecithin-cholesterol acyltransferase

LDL : Low-density lipoprotein

LDLR : Low-density lipoprotein receptor

LINC00152: Long intergenic noncoding RNA 00152

LSS : Lanosterol synthase

LXRs : liver X receptors

MAPK : Mitogen-activated protein kinase

MCD : Methyl- β -cyclodextrin

MDR : Multiple drug resistance

mRNA : messenger RNA

MEKi : Mitogen-activated protein kinase kinase inhibitor

NAFLD : Non-alcoholic fatty liver disease

NASH : Non-alcoholic steatohepatitis

NO : Nitric oxide

NPC : Niemann-Pick

NPC1L1 : Niemann-Pick C1 like-1

P38 : P38 mitogen activated protein kinase

p-AKT : Phospo-AKT

p-CHEK1 : Phospo-Check point kinase 1

p-ERK : Phospo-Extracellular signal-regulated kinase

p-P38 : Phospo-p38

PCP : Prochlorperazine

PD-1 : Programmed death-1

PDAC : Pancreatic ductal adenocarcinoma

PDGFRs : Platelet-derived growth factor receptors

PD-L1 : Programmed death ligand-1

PDX : patient-derived-xenograft

PFA : Paraformaldehyde

P-gp : P-glycoprotein

PI3K : Phosphoinositide 3-kinase

PP2A : Protein phosphatase 2A

PPH : Perphenazine

PTZ : Phenothiazine

PVS : Perivitelline space

qPCR : Quantitative polymerase chain reaction

RCT : Reverse cholesterol transport

RETs : Ret proto-oncogenes

SCAP : SREBP-2 cleavage-activating protein

SCD : Stearoyl-coA desaturase

SFB : Sorafenib

SQLE : Squalene monooxygenase

SRB1 : Scavenger receptor class B type 1

SRC : Proto-oncogene tyrosine-protein kinase Src

SREs : sterol regulatory elements

SREBP-1 : Sterol regulatory element-binding protein-1

SREBP-2 : Sterol regulatory element-binding protein-2

StAR : Steroidogenic acute regulatory protein

T-ALL : T-cell acute lymphoblastic leukemia

T-CHEK1 : Total-Check point kinase 1

TFP : Trifluoperazine

TNBC : Triple-negative breast cancer

TRPM8 : Transient receptor potential melastatin 8 channel

VEGFRs : Vascular endothelial growth factor receptors

VLDL : Very-low density lipoprotein

Vps34 : Phosphatidylinositol 3-kinase VPS34

Chapter 1. INTRODUCTION

1.1 Cancer

Cancer is a disease which shows high heterogeneity and can originate from all types of cells. The presence of numerous cancer types makes this disease a highly serious problem worldwide. There are both genetic and environmental factors involved in the formation of cancer, but unregulated division and growth are the main features of cancer cells [1]. Fundamentally, these cells gain the ability to escape cell cycle controls based on genetic defects such as mutations. Various signaling pathways that regulate molecular processes including proliferation, apoptosis and angiogenesis become altered. Skipping the actions of growth suppressors, metastasis and being capable of unlimited replication can also be included in the common traits seen in cancer [2].

It is possible to classify cancers as benign or malignant according to their ability to spread in the body. Benign (noncancerous) tumors stay resident in the place where they originate, while malignant (cancerous) tumors expand throughout the neighboring organs by damaging the healthy cells [3]. The invasive characteristics of malignant tumors and the complex tumor microenvironment for each cancer type complicate the clinical options for treatment. Therefore, it is very important to elucidate mechanisms underlying cancer formation and progression.

1.2 Cholesterol Homeostasis

Cholesterol is one of the vital constituents of mammalian lipid cell membranes and alters membrane dynamics by modulating permeability and fluidity [4]. It takes part in the regulation of various signaling pathways by interacting with a number of membrane components such as lipids and proteins. Cholesterol also enables the production of different molecules including steroid hormones and bile acids, which are essential for certain biological events [5]. The key role of cholesterol in a broad array of molecular mechanisms brings along the necessity for its strict regulation. The insufficiency or accumulation of cholesterol has been associated with diseases such as atherosclerosis [6]. Therefore, it is crucial to control the level of cholesterol within the cells.

Cholesterol homeostasis involves important cellular events keeping the balance among uptake, synthesis, storage and export of cholesterol [6]. The first step to cholesterol

homeostasis is the cholesterol sensing, which takes place through either direct association with proteins that are sensitive to sterols or modulation of the cell membrane [4, 7]. The sensing and biosynthesis of cholesterol are highly connected [4]. Sterol-regulatory element-binding protein-2 (SREBP-2), a transcription factor, functions as the main mediator of the expression of cholesterol biosynthesis genes. SREBP-2 interacts with two ER membrane proteins, SREBP-2 cleavage-activating protein (SCAP) and Insulin-induced gene 1 protein (INSIG-1).

When the cholesterol level is high, sterol-sensing domain of SCAP becomes available to serve as a binding site for INSIG-1 through some conformational changes, generating INSIG-1-SCAP-SREBP-2 complex [7]. This complex stays in the ER membrane and hence the activation of the genes required for cholesterol biosynthesis is prevented.

In the case of cholesterol depletion in the cells, SCAP enables the translocation of SREBP-2 from ER to Golgi apparatus after dissociation of INSIG-1 [8]. With the cleavage of SREBP-2, the active domain of the protein becomes free to go into the nucleus, where it binds to sterol regulatory elements (SREs) [6]. By being located in the promoter region of genes responsible for cholesterol metabolism, SREs initiate the transcription.

The remaining mechanisms involved in cholesterol homeostasis are explained below in detail.

1.3 Cholesterol Biosynthesis

Cholesterol is synthesized through a multistep biosynthetic pathway, mevalonate pathway, in which more than 20 enzymes function [9]. The majority of these enzymes are found on endoplasmic reticulum (ER) membrane. Basically, it is possible to compartmentalize cholesterol biosynthesis in two main parts, one of which is the formation of squalene [10]. The second part involves the action of lanosterol synthase (LSS) to form lanosterol which is then converted to cholesterol as the final product after a series of 19 reactions. The process of constructing cholesterol starts with conversion of acetyl-CoA to HMG-CoA and is followed by the formation of mevalonic acid through functioning of HMG-CoA reductase (HMGCR), which is the rate-limiting enzyme. This role of HMGCR is often employed in the studies where cholesterol biosynthesis is desired to be interrupted. Besides, squalene monooxygenase (SQLE) is responsible for the first oxygenation reaction leading to the formation of the precursor for lanosterol. This enzymatic step has been presented as a productive enzymatic step to target as well [11].

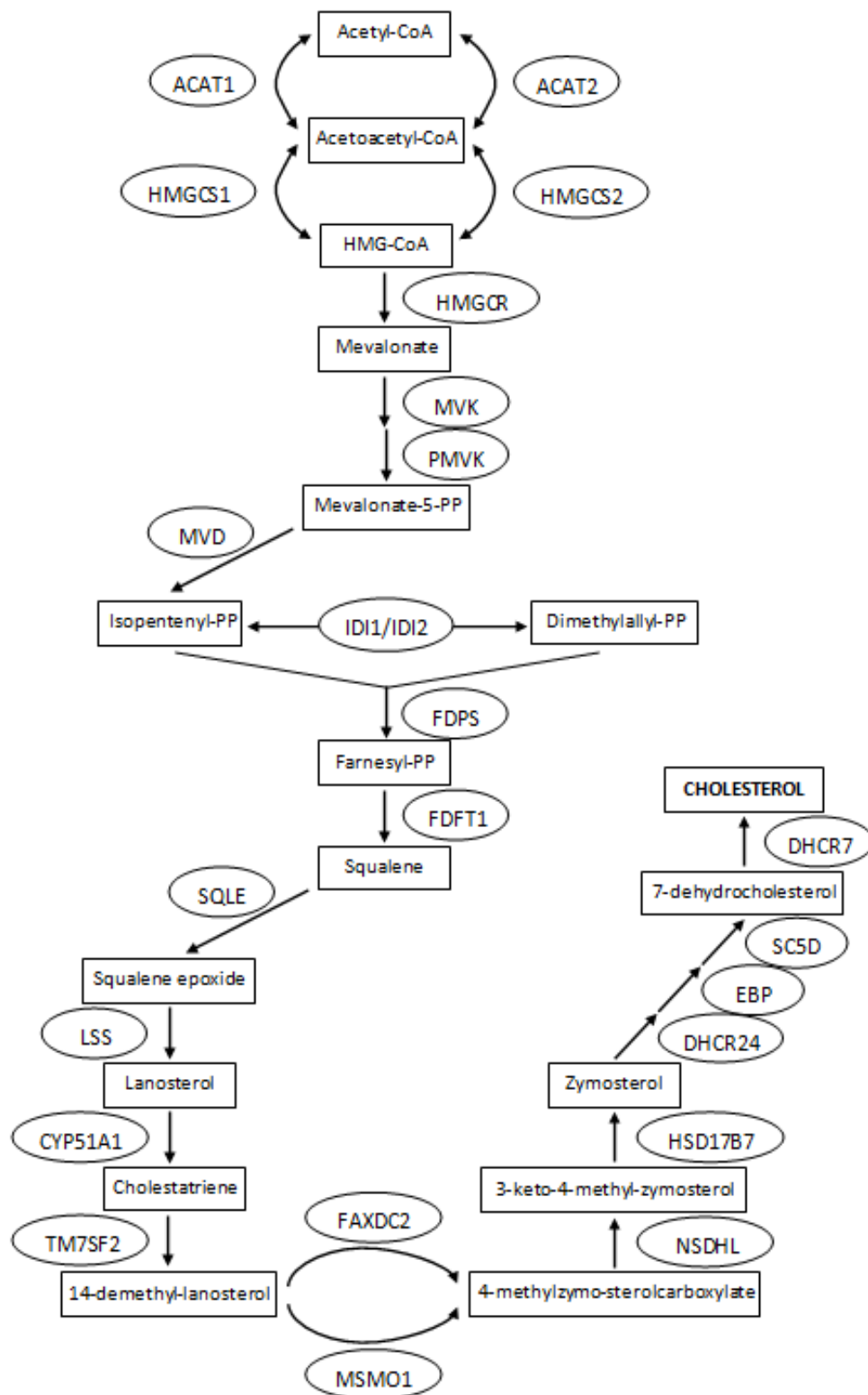


Figure 1. Cholesterol Biosynthesis Pathway. The chart is generated combining the information retrieved from “the medical biochemistry page” [12] and Kegg Pathway: Steroid Biosynthesis (hsa00100).

1.4 Cholesterol Uptake

Cholesterol biosynthesis is not the only source of cholesterol and there are two other mechanisms by which cholesterol levels are altered. They are mediated by low-density lipoprotein receptor (LDLR) and Niemann-Pick C1 like-1 (NPC1L1), respectively.

1.4.1 LDLR-Mediated Endocytosis

The uptake of cholesterol from blood circulation is achieved through low-density lipoprotein receptors (LDLRs) based on receptor-mediated endocytosis [6]. LDLRs are glycoproteins that are found on the cell surface to interact with lipoprotein particles. LDLs are the main carriers of cholesterol and their internalization occurs by means of clathrin-coated vesicles once they form a complex with LDLRs [13]. Then, the fusion of these vesicles with early endosomes takes place and LDLs become dissociated from LDLRs. While LDLRs localize to the cell surface again, cholesterol-containing early endosomes move forward to late endosomes which are incorporated into the Golgi apparatus forming lysosomes. After the degradation of cholesterol in the lysosomes, the free cholesterol becomes available to be transported to other membranes including plasma membrane and ER [14] or it can be stored [7].

1.4.2 NPC1L1-Mediated Cholesterol Uptake

Cholesterol can be exogenously obtained from dietary and biliary sources, via an uptake mechanism regulated by Niemann-Pick C1 like-1 (NPC1L1) protein. NPC1L1 is a transmembrane protein containing a sterol-binding pocket in the N-terminal domain and a conserved sterol-sensing domain [15]. It is present in the apical membrane of enterocytes and canalicular membrane of human hepatocytes.

The uptake from intestinal lumen into enterocytes is a function of clathrin-mediated endocytosis [6]. NPC1L1 primarily localizes to endocytic recycling compartment (ERC), but cholesterol depletion causes its translocation to the plasma membrane. When cholesterol levels become normal again, cholesterol binds to NPC1L1 and gets internalized via clathrin-coated vesicles [16]. This eventually leads to the enrichment of NPC1L1 in ERC, from where it may be sent back to plasma membrane depending on cholesterol availability [6]. Once in enterocytes, the absorbed cholesterol is esterified by ACAT2 in the ER membrane, while unesterified cholesterol travels back to the intestinal lumen by means of ABCG5/G8 transporters for excretion [9]. Then, chylomicrons incorporate the resulting cholesteryl ester together with apolipoprotein B-48 (apoB48) and are secreted to the circulation via lymphatic system after being processed in the Golgi apparatus [11]. Moreover, apolipoprotein A1

(apoA1)-containing HDL particles can also mediate the secretion of the absorbed cholesterol through ATP-binding cassette transporter A1 (ABCA1) [10].

After hepatocytes take up cholesterol-containing chylomicron remnants, the esterified cholesterol is packaged into VLDL particles through Apolipoprotein B-100 (apoB100) [9]. VLDL particles are released to the circulation where they are converted to IDL and LDL, respectively. The liver turns cholesterol into bile salts which then become secreted to the bile via bile salt export pump (BSEP) [11]. Biliary cholesterol can be retaken to hepatocytes via NPC1L1 [15].

Since NPC1L1 plays a central role in cholesterol homeostasis, the molecular changes modulated by it, should be highly regulated. This is achieved by liver X receptors (LXRs) LXR α and LXR β which are able to sense cholesterol and interact with LXR response elements located in the promoter of target genes [17]. These genes are responsible for NPC1L1 inhibition and also ABCG5/G8 activation which enables cholesterol excretion. Another function of LXR α and LXR β is to activate ABCA1, inducing cholesterol efflux [7].

1.5 Intracellular Cholesterol Transport

Intracellular cholesterol transport is the mechanism that enables efficient distribution of cholesterol in the cells. It is managed via two mechanisms, vesicular and non-vesicular. In vesicular transport, cholesterol travels in the membrane of vesicles among cellular entities via cytoskeleton elements [13]. However, most of intracellular cholesterol trafficking is based on non-vesicular mechanism [18]. The hydrophobic nature of cholesterol calls for mediators which are carrier proteins. Steroidogenic acute regulatory protein (StAR) is one such carrier protein, which has hydrophobic cavities enabling cholesterol binding [13]. An alternative to the action of diffusible carrier proteins is the release of cholesterol from one membrane to diffuse into another [14]. The cholesterol transfer between ER and plasma, also ER and Golgi apparatus can be given as examples, occurring through structural reorganization of their membranes to come into close proximity [19].

1.6 Cholesterol Efflux

One of the major requirements for cholesterol homeostasis is cholesterol efflux. In order to prevent excessive levels of cholesterol in the cells, a mechanism called reverse cholesterol transport (RCT) comes into play [14]. RCT involves the removal of cholesterol from

peripheral tissues and its trafficking to the liver to be secreted to bile, which in turn can be excreted to feces [11, 20].

The removal of cholesterol can take place via either an ATP-independent or apolipoprotein-mediated mechanism [14]. In the ATP-independent process, cholesterol mainly diffuses among lipid surfaces after being packaged into HDL particles [21]. In the apolipoprotein-mediated mechanism, ApoA1 serves as a modulator. The binding of ApoA1 to ABCA1 activates a process in which discoidal HDL is formed. Discoidal HDL comprises of ApoA1, phospholipids and cholesterol [7]. Later on, cholesterol inside the discoidal HDL is esterified via lecithin-cholesterol acyltransferase (LCAT). After this point, cholesteryl ester can follow different paths including selective uptake by the liver via SRB1 or transport to triglyceride-rich lipoproteins [22].

1.7 The Role of Cholesterol Metabolism in Cancer

Given these complex and multi-membered mechanisms of cholesterol homeostasis, it is not surprising that cholesterol has emerged as a molecule with significant impact on cancer. Since main nutrient sources such as glucose and also macromolecules that are needed for metabolic events become insufficient during extreme proliferation, cancer cells search for alternative sources to obtain energy. Cholesterol is one such example to these alternatives and serves to support biosynthesis of membrane components and aberrant growth in the cells [23]. Different studies have emphasized that mechanisms modulating cholesterol were involved in cancer progression. Increased cholesterol biosynthesis and overexpression of the transport proteins responsible for cholesterol efflux can be given as examples to them. The accumulation of cholesterol has been shown to cause cancer cells to skip several regulatory machineries, resulting in activation of oncogenic signaling pathways [24, 25], uncontrolled cell proliferation and attenuated cell death [26]. All these findings supported the idea of targeting cholesterol metabolism as an antitumor approach by either disrupting cholesterol biosynthesis or stimulating cholesterol efflux from cancer cells [26]. Statins, which are HMGCR-inhibitors, have been commonly used in research for this purpose and enabled to investigate the impact of lowering cholesterol in various cancer types [27]. Moreover, different methods used to measure intracellular cholesterol levels were also helpful to assess changes caused by various treatments and drugs including statins. Cell-based assays incorporating cholesterol-binding fluorescent dyes such as filipin [28, 29] or cholesterol extraction [30] from harvested cells provide examples of how to determine cholesterol abundance.

A patient-based study on pancreatic cancer revealed that lower risk of cancer-related death was observed in patients who have been using statins, especially in the case of nonmetastatic pancreatic cancer [31]. Similarly, statins showed protective effect on lung cancer patients when used more than 6 months [32]. LDLR was presented as an important regulatory molecule in progression of pancreatic ductal adenocarcinoma (PDAC) and inhibition of LDLR caused reduced growth of PK4A cells through a mechanism in which ERK1/2 pathway takes part [33]. In prostate cancer, the disruption of cholesterol-rich lipid rafts revealed decreased survival through the inhibition of AKT-mediated signaling [34]. The prevention of cholesteryl ester accumulation in a mouse model of prostate cancer led to less tumor invasion. Moreover, uncovering the accumulation of cholesteryl ester in prostate cancer tissues directed Yue et al. (2015) to elucidate the underlying mechanism [35]. The increase in the uptake of LDL was found to drive this process in PC-3 cells through the induction of PI3K/AKT/mTOR/SREBP pathway. Lowering the level of esterified cholesterol could revert the viability and metastasis of PC-3 cells, also decreased tumor growth in vivo [35]. The decreased expression of lipid biosynthesis genes through knockdown of SREBP-1 and SREBP-2 resulted in inhibited proliferation of colon cancer cells and less tumor growth in mouse xenograft model [36].

Besides, simvastatin treatment resulted in cell cycle arrest, induction of apoptosis and reduced metastasis in ovarian cancer cells [37]. These anticancer processes were proven to be related with ERK1/2 and AKT/mTOR pathways and also further validated in vivo after generating mouse models of ovarian cancer. Another study on ovarian cancer unraveled high expression of ACAT-1 leading to accumulation of esterified cholesterol in OC-314, SKOV-3 and IGROV-1 cells [38]. With the use of an ACAT inhibitor, the cellular processes that favor cancer progression such as proliferation and invasion could be reversed. When SQLE was overexpressed in HepG2 cell line, an in vitro model for HCC, the growth and migration were shown to be significantly enhanced [39]. Notarnicola et al. (2010) have shown that eicosapentaenoic acid (EPA) and lovastatin exerted an inhibitory effect on HMGCR expression, while they enhanced expression of LDLR in HepG2 cells both individually and in combination [40]. EPA and lovastatin together also impaired HepG2 cell proliferation and all these alterations were achieved at lower doses compared to single treatments. In the study of Athavale et al. (2018), providing HepG2 and Huh7 cells with exogenous LDL and also sorafenib showed protective effect on cancer cell survival compared to treatment with only sorafenib [41]. This regulation was found to be associated with RAF/MEK/ERK pathway.

In the study of Mohammad et al. (2014), the combination of tamoxifen with cholesterol depleting methyl- β -cyclodextrin (MCD) sensitized melanoma cells, as evident by lowered expression of Bcl-2 and p-AKT/-pERK [42]. When the mice that showed less tumor growth as a result of combination therapy were supplied with cholesterol, the antitumor effect was diminished. Moreover, MCD could exert the same sensitizing-effect in MCF7 and MDA-MB-231 cells when combined with either carboplatin or 5-fluorouracil, two DNA damaging medications [43]. In mouse xenograft models generated by using A549 lung cancer and MDA-MB-231 breast cancer cells, cepharanthine, an inhibitor of cholesterol trafficking, resulted in reduced tumor volume [44]. When it was provided in combination with cisplatin, the tumors became more susceptible and also angiogenesis was suppressed in comparison to the treatment with cisplatin alone. In the study of Liu et al. (2014), treatment of A549 cells with cholesterol oxidase, an enzyme replacing plasma membrane cholesterol with cholest-4-en-3-one, led to the regulation of signaling pathways including AKT, ERK1/2 and JNK/p38 [45]. This enzyme was also shown to induce apoptosis both in vitro and in vivo.

On the other hand, there are some conflicting studies in which low cholesterol levels have been implicated in the induction of cancer. Many patient studies have elucidated a link between targeted cholesterol biosynthesis and increased incidence of cancer [46]. Randomized controlled trials have demonstrated that lowered levels of LDL cholesterol by statins have a potential to enhance cancer risk [47, 48]. In the research by Lim et al. (2014), in vitro experiments unraveled that providing culture medium of gastric cancer cells with cholesterol resulted in enhanced autophagy and apoptosis [49]. Criscuolo et al. (2020) have highlighted that lovastatin treatment increased the resistance of chemoresistant ovarian cancer cells to cisplatin based on lowered cholesterol levels and reduction of cisplatin-derived apoptosis [50]. Prostate cancer aggressiveness was shown to be enhanced in simvastatin-fed *Pten* prostate-specific knockout mouse model, as evidenced by increased mass of prostate tissue [51]. Xenograft studies in which PC3 cells were transplanted into nude mice further confirmed this finding, since more tumor growth was observed after simvastatin treatment when compared to control group. However, this concept overall needs more investigation since it seems to depend on the cancer type [49].

1.8 Liver Cancer/Hepatocellular Carcinoma (HCC)

Liver cancer is the fourth most common cause of cancer-related deaths all around the world [52]. Hepatocellular carcinoma (HCC) corresponds to the majority of primary liver cancer [53]. There are different risk factors elevating the incidence of HCC, such as viral hepatitis infection, cirrhosis, non-alcoholic steatohepatitis (NASH), non-alcoholic fatty liver disease (NAFLD), alcohol consumption and smoking [53-55]. Among these, chronic hepatitis accounts for the highest risk for HCC development [55].

1.9 Clinical Strategies in HCC

Early diagnosis of HCC has been possible through improved liver imaging technologies and enables several treatment alternatives including surgical resection and transplantation [56]. Although systematic chemotherapy is applicable for patients of advanced HCC, it has been efficient especially in the case of small tumors and maintained liver function [52, 56]. There are different FDA-approved drugs that have been in use for HCC treatment such as sorafenib, the first systematic option approved in 2007 [57]. Recently, lenvatinib was validated as another first-line treatment for HCC. However, there is still a great need for new clinical approaches and effective medications against HCC.

1.9.1 Sorafenib

Before sorafenib was presented as the first systematic therapy with survival benefits in 2007, the treatment of advanced HCC remained a huge problem [53]. The members of important signaling pathways are targeted by the multikinase inhibitor sorafenib, such as VEGFRs, PDGFRs and RETs [58, 59]. Although this drug has been applied commonly in clinics, it has provided unsatisfactory results shown by low response rate to it [52], acquired resistance and serious side-effects including weight loss and fatigue [60]. The attempts to discover more efficient drugs have failed over ten years, leaving sorafenib as the only available option. Different clinical studies pointed out that lenvatinib, another multikinase blocking agent, showed non-inferiority and improved overall survival in comparison to sorafenib [52]. These findings then led to the approval of lenvatinib as an agent of first-line therapy for HCC in 2018 [57]. The developments in therapeutics against HCC stepped forward with combinatorial approaches. The use of atezolizumab and bevacizumab together was found to be highly effective for unresectable HCC, paving the way for a different level of first-line treatment [61]. The combination of these drugs also provided HCC patients a better life quality with more manageable side-effects. Moreover, second-line therapeutics regorafenib,

cabozantinib and ramucirumab which regulate VEGFRs have emerged as alternatives after treatment with sorafenib. These are also implicated in combination therapy with anti PD-1 and anti PD-L1 antibodies [57]. Studies have been ongoing for the discovery of impactful new drugs and combination strategies.

1.10 Alternatives for the Treatment of HCC: Drug

Repurposing

The challenges of novel drug discovery and insufficiency of already existing therapeutic options have created the need for different approaches against cancer. Drug repurposing can be defined as a strategy for the use of drugs available in the market with purposes other than the original [62]. Since it is safe, cost-effective and less time-consuming, attributing new targets to approved drugs has gained a lot of attention and drug repurposing research for cancer is accelerated recently [63]. In a study by Regan-Fendt et al. (2020), a transcriptomics-based approach was followed to determine novel drugs against sorafenib-resistant HCC [64]. As a result, dasatinib which is an anti-neoplastic drug was found to inhibit the growth of sorafenib-resistant Huh7 cells through the blockade of SRC kinases. Another study revealed that an FDA-approved glutathione S-transferase (GST) inhibitor acted synergistically with sorafenib to decrease viability of HepG2 and Huh7 cells [65]. Phenothiazines, a class of neuroleptic drugs, have also been strongly suggested as antitumor agents. Two phenothiazine derivatives, chlorpromazine and trifluoperazine, were shown to decrease HCC cell survival as evident by reduced cell growth; and moreover, tumor size was significantly suppressed in chlorpromazine- and trifluoperazine-treated in vivo liver tumor models as compared to control [66].

1.11 Phenothiazines

Phenothiazines are tricyclic, amphiphilic organic molecules that have been in use as antipsychotic drugs [67]. They have had a large place in pharmaceuticals for more than 60 years to treat psychiatric diseases such as schizophrenia and anxiety [68]. Phenothiazines and phenothiazine derivatives are known to affect a variety of molecular processes. The most striking and utilized action of these compounds is the inhibition of dopamine receptors in central nervous system (CNS), but they exhibit inhibitory effects on serotonin, histamine and acetylcholine receptors as well. Phenothiazines modulate protein interactions and receptor-ligand binding dynamics which are affected by substituents found on them [69, 70]. For example, the association of phenothiazines with calmodulin was found to be related to the

amino-containing group present in phenothiazine structure [71]. In addition, phenothiazines have been reported to show antimicrobial, antiviral and antiprion properties [67].

Phenothiazines are known to interact with lipid bilayers, which might be responsible for their biological actions [72]. The hydrophobic ring and hydrophilic moieties enable phenothiazines to form micellar structures that are affected by parameters such as temperature, pH and concentration [73]. These drugs aggregate in aqueous solution after the critical micellar concentration is achieved and surfactants are incorporated [74]. Based on the high affinity that they show for lipids, phenothiazines penetrate into cell membranes and alter lipid composition by interacting with membrane lipids [69]. At this point, the structural groups carried on them are of great importance, influencing the dynamics of the interaction [75]. Phenothiazines are also known to suppress the esterification of plasma membrane cholesterol like many other amphiphilic molecules [76], This is received as a false signal for cholesterol deficiency by the cells, accordingly resulting in enhanced HMG-CoA activity and cholesterol biosynthesis [77].

The anticancerous activity of phenothiazines has also gained great attention over the past years. Many studies have revealed that there has been a decreased incidence of cancers including lung and pancreas in schizophrenia patients [78]. This relationship between schizophrenia and cancer was suggested to be through the genetic factors and commonly regulated pathways [79]. The incidence of rectal cancer was lowered in Danish population in which antipsychotics were used with an adjusted incidence rate ratio (IRR) of 0.61 in women and 0.82 in men [80]. The women in that population also showed reduced risk of colon cancer (adjusted IRR: 0.78). These findings were therefore suggestive for possible role of such medications as anticancer agents.

It has been demonstrated that phenothiazines have a promising potential against different cancer types including melanoma, breast cancer, colorectal cancer and glioblastoma [28, 81-83]. Different biological mechanisms such as DNA-repair, proliferation, apoptosis and migration might be involved in such antitumor actions of phenothiazines [68]. Otreba et al. (2019) revealed that perphenazine (PPH) and prochlorperazine (PCP), two of the common phenothiazines, were capable of decreasing viability of human melanoma cell lines [84]. In another study, it has been shown that chlorpromazine led to enhanced sensitivity of breast cancer cells in combination with chemotherapeutic agents [83]. Trifluoperazine (TFP) resulted in decreased invasive and metastatic action in HCT116 colon cancer cells [82].

There have been many studies elucidating the anticancer impact of phenothiazines on HCC cell lines. Jiang et al. (2017) has determined that cell cycle arrest occurred in two HCC cell lines as a result of TFP exposure, also increased Bax/Bcl-2 ratio was another alteration indicating impaired survival caused by TFP [85]. Thioridazine was found to decrease the expression of PI3K and AKT in HepG2 cells at both mRNA and protein levels [86], pointing to lowered progression in HCC. It also resulted in inhibited epithelial-mesenchymal transition (EMT) in SNU449 and Huh7 cells by reducing migration [87]. These findings therefore support the anticancer activity of phenothiazines in HCC through different regulatory mechanisms.

This thesis aims at revealing the regulatory role of common phenothiazine derivatives phenothiazine (PTZ), PCP, PPH and especially TFP in HCC via different in vitro and in vivo approaches and with a specific focus on cholesterol biosynthesis pathway.

1.11.1 TFP

TFP is one of the commonly used phenothiazines which has been known to regulate dopaminergic signaling [88]. It also blocks calmodulin by impairing its interaction with calcium, which is utilized in cellular events including cell proliferation [89]. Multiple drug resistance (MDR) is another mechanism altered by TFP through the inhibition of P-glycoprotein (P-gp), a highly expressed protein in the case of resistance [90]. Wesolowska et al. (2005), showed that TFP reverted MDR in resistant human sarcoma cells via modulating P-gp levels [91]. Moreover, TFP was shown to effect cholesterol-related events by impairing the binding of cholesterol to SCAP [92], one of the most important proteins playing a role in the regulation of cholesterol production. The action of TFP was through the electronegative radical group $-CH_3$ and the side chain with amine group, which led to a change in the conformation of SCAP in a similar manner as cholesterol [93]. There are various molecular mechanisms through which TFP affects cancer development such as cell proliferation, apoptosis and cholesterol biosynthesis. Decreased expression of certain cyclin-dependent kinases, cyclin D1 and cyclin E by TFP resulted in cell cycle arrest in colorectal cancer cell lines [88]. TFP induced apoptosis through mitochondrial pathway in triple-negative breast cancer (TNBC) cell line MDA-MB-468 [94]. ATP production in pancreatic cancer cells was shown to decrease with increasing doses of TFP, being an indicative of the cell death [95]. In melanoma cells, TFP showed anticancer properties by impairing the action of lysosomes and accordingly leading to the blockade of autophagy flux [96]. TFP also decreased cholesterol transport to endoplasmic reticulum in CaCo-2 colorectal adenocarcinoma cells.

In addition to the cancer types mentioned above, different studies reported the possible anticancer mechanisms mediated by TFP in HCC. Gene expression-based profiling indicated TFP as a potential drug for HCC treatment [66]. Following in vivo studies in mice further validated efficacy of TFP in inhibition of tumor growth in the liver. In another study, the export of one of the tumor suppressors FOXO1 from the nucleus was prevented by TFP in HCC, maintaining antitumor effects both in vitro and in vivo [85].

Since there is an urgent need for alternative anticancer agents due to presence of side effects and resistance issues, development or repurposing of new therapeutic options is an important field in cancer research [97]. TFP showing a highly promising potency in the scope of anti-HCC activity therefore presents a great opportunity to investigate its role at both in vitro and in vivo levels.

1.12 Novel Phenothiazines

The diverse regulatory capability of phenothiazine derivatives arises from the structural alterations that can be introduced into their structure, resulting in different molecular activities [67]. Although phenothiazine derivatives that are available in the market have provided promising potential against different cancers, synthesis of new derivatives with defined structures would allow more precise elucidation of molecular interactions and resulting biological events. From this point of view, novel phenothiazine derivatives, which were used throughout this study, were generated (Murat Kislal, MS Thesis) and tested both in vitro and in vivo (Murat Yaman, PhD Thesis, 2020).

In the literature, there are many examples to studies involving newly generated phenothiazine derivatives. Bisi et al. (2008) synthesized phenothiazine derivatives with different moieties [98]; and MDR variant of human leukemia cells, HL60R, was shown to undergo cell cycle arrest at G1 phase and also apoptosis based on the action of phenothiazine derivatives 5a, 5f and 6a. Among all derivatives the authors synthesized, 5a, 5f, 5h and 7a showed synergy with doxorubicin in terms of survival of HL60R and CEM/VBL300 cells, another MDR variant of leukemia cells. However, only 5a, 5f and 5h were brought forward as efficient molecules against leukemia due to high cytotoxicity of 6a and 7a. Another study presented phenothiazine derivatives 4 and 13 as growth inhibitors of L-1210 leukemia cells and SW-948 colon cancer cells [99]. The proliferation of different types of cancer cells including MGC-803, EC-109, PC-3, MCF7 and HepG2 were demonstrated to be efficiently inhibited by

phenothiazine-1,2,3-triazole analogues [100]. Among them, 7d exerted more efficacy on all cancer cells tested in comparison to 5-fluorouracil.

All these information highlight the importance of synthesizing novel phenothiazines by modifying core phenothiazine structure with the addition of different substituents, which might be involved in biological functions against cancer.

1.13 Regulation of Cholesterol Metabolism by Phenothiazines in Cancer

Several studies in the literature have shown antitumor action of phenothiazines through the alteration of cholesterol metabolism. In one of the studies, the incubation of HepG2 cells with mevalonolactone following treatment for 12 hour-trifluoperazine resulted in inhibited cholesterol biosynthesis in a dose-dependent manner when compared to the control [101]. Another study has shown that the treatment of HepG2 cells with trifluoperazine and chlorpromazine after incubation with cholesterol for 24 hours caused high HDL uptake, while suppressing HDL degradation [102]. Nagy et al. (1989) showed that trifluoperazine prevented the internalization of plasma membrane cholesterol and functioning of ACAT in MA-10 cells which were obtained from Leydig cell tumor [103]. These three studies centered upon the calmodulin-antagonist property of phenothiazines. Providing R3230AC mammary carcinoma rat models with fluphenazine HCl for 21 days was on the other hand led to decreased cholesterol in the tumors [104]. An important research by Kuzu et al. (2017) elucidated that two phenothiazine derivatives, fluphenazine and perphenazine, were able decrease tumor growth via the inhibition of intracellular cholesterol trafficking in a xenograft model of melanoma [28]. Besides, the anticancer effect of these two drugs through the modulation of intracellular cholesterol transport was further explained by cellular mechanisms such as autophagy, endocytosis and apoptosis on UACC 903 cells. The introduction of lovastatin, one of the inhibitors of mevalonate pathway, could enhance the anticancer effect of thioridazine on glioblastoma cells, although it could not be recovered after supplying the cells with pathway intermediates [105]. In another study, three phenothiazine derivatives enabled reduced viability and induced apoptosis in doxorubicin-resistant colon cancer cells (LoVo/Dx) in combination with simvastatin, a type of statin, more efficiently when compared to single treatment [106]. These findings suggested a stronger anticancer effect by phenothiazine derivatives when cholesterol biosynthesis was impaired.

1.14 Zebrafish as a Model for Drug Screens

Zebrafish is a commonly used vertebrate model organism for human diseases based on high genetic conservation and organ similarity [107]. Zebrafish counterparts of 82% of the human genes have associations with diseases [108]. Zebrafish has become a very useful tool for toxicity assessments due to its ability to allow easy screening of drugs with large sample numbers (high fecundity) while different aspects of toxicity including developmental toxicity, cardiotoxicity and neurotoxicity can be investigated using zebrafish at any developmental stage [109].

The delivery of the drugs to zebrafish by means of the aqueous environment which enables chemical solubilization, is highly practical [110]. Small molecules introduced in the medium of zebrafish embryos can be taken up by skin and gills at early stages, while digestive system comes into play in zebrafish larvae which are able to swallow. Compound exposure can easily be performed using 24-, 96- or 384-well plates, which enable toxicology assessments in small quantity of treatment medium [111]. The presence of chorion, which possesses pores allowing oxygen and nutrient pass, at embryonic stages is an important parameter to consider, since pore size (0.5-0.7 μm in diameter) limits the molecules that can diffuse through [112, 113]. Oral administration has also been a choice of delivery method to zebrafish larvae and adults [114], due to its ability to enable precise control over the quantity and timing of the molecule delivered [115].

Since use of multiwell plates has been considered limited by not providing a dynamic environment for zebrafish to go through natural developmental processes, microfluidics has gained great attention in the field of zebrafish-based toxicology [116]. Microfluidic devices lead to efficient culturing and manipulation of zebrafish. For example, Yang et al. (2011) generated a microfluidic chip system in which zebrafish embryos were provided with drugs such as cisplatin and 5-fluorouracil via a concentration gradient generator [117]. The response of the embryos to these drugs could be easily assessed in the scope of developmental toxicity involving differences in pericardial impairment, heart rate, yolk shape, body length and pigmentation. Microfluidics seems to have eligibility enabling evaluation of the impact solely arising from the molecule tested.

For large molecules, proteins and hydrophobic compounds, injection can be utilized as an alternative method. The site of injection varies depending on the motive of the study, yolk sac, duct of cuvier and cardinal vein being commonly preferred sites [118].

The delivery of molecules can also be achieved via nanoparticles such as liposomes and polymer nanoparticles, either through direct addition into the medium or injection [114]. The transparent nature of zebrafish embryos and larvae serves for direct tracking of nanoparticle distribution and following biological outcomes [119]. However, there are several points that need special attention while performing nanoparticle-mediated delivery. Firstly, even if the nanoparticles are small enough to pass through the chorion, they might not be able to penetrate [120]. The uptake of these particles should be traced carefully to eliminate this possibility. Another consideration is due to the chemical composition of these nanoparticles. The structural components might get disrupted in the egg water, resulting in dispersion or aggregation [119].

Table 1. Different delivery methods used for drug screens on zebrafish.

Materials to be Delivered	Delivery Method	Suitable Developmental Stage	Notes
Drugs, Nanoparticles	Direct Immersion	Embryonic, Larval, Adult	Solubility and size of the material should be taken into account.
	Injection	Embryonic, Larval	Injection is usually applied when direct exposure is not possible, also enables direct release into blood circulation.
	Oral Administration	Larval, Adult	This method is performed by gavaging.
	Microfluidics	Embryonic, Larval	Microfluidic devices provide a dynamic environment for developmental processes.

Moreover, the changes occurring as a result of drug treatments can efficiently be tracked and monitored under microscope. The transparency of early stage zebrafish embryos further leads to detailed examination of morphological differences and organs [121]. Another great advantage of zebrafish is the comprehensive exemplification of vertebrate organs [122]. The zebrafish organ system mostly becomes fully functional by 96 hpf [123].

On the other hand, generation of xenograft models using zebrafish has been a great opportunity to test and develop new therapeutic options in cancer studies. These models have been enabling to see the response of different cancers to potential drugs in vivo [124], since there is a high histological similarity of tumors generated in zebrafish to human tumors [125].

Transplantation of cancer cells into zebrafish allows to generate pre-clinical models on which the efficiency of certain drugs can easily be determined.

1.14.1 Zebrafish Xenograft Models

Easy handling, manipulation and reproduction of zebrafish have been enabling the use of zebrafish as a host for xenotransplantation. Various cancer models that represent human cancers have been generated by implanting cancer cells into the yolk sac of zebrafish embryos [126]. Nonetheless, alternative injection sites such as perivitelline space (PVS) are also possible depending on the cell to be injected and the goal of the study [127]. Since the adaptive immune system of zebrafish is not fully developed at early stages, immunodeficiency is also not an issue in zebrafish xenograft studies, as it is in mice, providing cancer cells an unaltered environment in this sense [127, 128]. This approach paved the way for low-cost testing of therapeutic agents and elucidation of molecular mechanisms behind cancer progression [129]. Fluorescent dyes with which the cells can be labeled have made tracking the tumor formation, tumor size and metastasis very practical under fluorescence or confocal microscope.

Besides all these advantages, there are also some challenges of generating cancer models using zebrafish [127]. First of all, the incubation temperature that zebrafish can handle is less than 37°C which is the optimal temperature for human cancer cells to grow. This problem is overcome by incubating the xenotransplanted embryos at about 34°C, but the change in this parameter is very likely to affect molecular events [130]. Tumor microenvironment is another issue that needs to be taken into account, since there are some variations between zebrafish and humans in terms of non-cancerous cells and molecular cues [127]. Although immunosuppression is not necessary for the cancer models generated by implanting zebrafish at 2 dpf, the time period allowing for observation of the tumors becomes limited, usually up to 7 days [130]. Finally, the consistency and reproducibility of the xenografts is another challenge that has been faced [129].

There are numerous research indicating how zebrafish has been utilized to obtain efficient xenograft models to study cancer. In a model of prostate cancer which was generated using PC3-CTR cells, aggressive cell proliferation and increased expression of prostate cancer-related genes were specified based on quantification by qPCR [131]. JHH6 cells were used for the first time to obtain a zebrafish HCC model and exhibited less growth compared to control as a consequence of bortezomib, an anti-HCC drug, exposure [132]. Another xenograft study

on HCC employing HepG2 cells presented arenobufagin (ArBu) as a modulator of lipid homeostasis as a function of anticancer action [133]. In a melanoma xenograft model generated by using GFP-tagged cells, non-malignant Hermes 2B cells did not migrate while cutaneous melanoma cells C8161 invaded other regions than yolk sac of zebrafish including tail, eye and heart [134]. Later on, the angiotropic potential, a main feature involved in melanoma metastasis, of both cells could be observed based on live imaging. Moreover, combination therapy with trametinib and a novel Vps34, an autophagy-regulating protein, inhibitor was shown to cause less migration of MEKi-resistant melanoma cells from the injection site [135]. Another zebrafish xenograft model was obtained using K562 and NB-4 leukemia cells [136]. Treatment with imatinib mesylate on K562 cells and all-*trans* retinoic acid on NB-4 cells between 1 dpi and 3 dpi resulted in a reduction in cell growth. Besides, the screening of 10 anticancer agents on zebrafish that were transplanted with A549, SGC-7901 stomach cancer, HepG2, JF 305 pancreatic cancer and MCF-7 cells resulted in reduced cell proliferation, with different efficacy on each cell line [137]. Among these agents, furanodiene was found to be highly promising on JF 305 and MCF-7 cells. It also exhibited synergistic effect on MDA-MB-231 and BEL-7402 liver cancer cells together with 5-Fluorouracil, raising its efficiency. The novel quinoline derivative DFIQ, which was shown to exert anticancer potential in non-small-cell lung cancer cell lines, resulted in tumor growth inhibition in a zebrafish xenograft model. Finally, Shen et al. (2020) achieved to inhibit tumor-promoting actions of LINC00152, long intergenic noncoding RNA 00152, using a zebrafish xenograft model of lung cancer generated with LINC00152 siRNA-transfected SPCA1 and A549 cells [138]. In this model, suppressing LINC00152 was also shown to enhance the inhibitory effect of afatinib, an EGFR-blocking agent, on cancer cell growth. Based on the heterogenic nature of different cancers, there still remains unlightened molecular mechanisms and therapeutic agents targeting the members of those to be investigated. More studies on different zebrafish cancer models have the potential to accelerate cancer research through efficiency, feasibility and fast response. These in vivo models even offer the opportunity for personalized therapies, which might be highly striking in clinics.

1.14.2 Cholesterol-Related Studies in Zebrafish

Zebrafish has been preferred to study many cholesterol-associated research topics as an in vivo model. Besides modeling metabolic diseases such as dyslipidemia by performing a high cholesterol-diet on zebrafish [139, 140], there have also been gene expression analyses investigating the regulation of genes involved in cholesterol pathway and their downstream

effects. Statins have been tested on zebrafish in many studies, and decreased levels of cholesterol were found to be associated with myopathies. This might be based on cholesterol being one of the most important regulators of membrane dynamics and ion flow which is required for proper muscle function [141]. Hanai et al. (2007) have revealed that lovastatin-induced muscle damage in zebrafish embryos could be reverted by using morpholino against *atrogen-1* whose expression was shown to be increased by lovastatin. A similar phenotype could also be obtained by knocking down zebrafish *hmgcr* [142]. Another study similarly showed muscle abnormalities resulting from simvastatin, which could be mostly prevented with the administration of cholesterol to zebrafish embryos [143].

On the other hand, in one of the studies where the expression of individual members of cholesterol metabolism were examined, treatment of adult zebrafish with LXR ligand GW3965 led to increased expression of *lxr* and its targets *abcg1* and *cyp7a1* [144]. The regulatory role of LXR was enlightened by Pinto et al. (2016) in a way that the exposure of 4 dpf zebrafish larvae to LXR agonists for 48 hours led to an increase in the expression of cholesterol biosynthesis genes [145]. Weng et al. (2021) have revealed that one aspect of the toxicity caused by epoxiconazole, a fungicide, was related to regulation of cholesterol metabolism as evident by lowered expression of *hmgcra*, *hmgcrb*, *cyp51* and *cyp7a1* [146]. The effect of simvastatin on adult zebrafish could be demonstrated based on decreased cholesterol levels in liver after 90 days of exposure. At gene expression level, *hmgcra* was found to be significantly affected in both males and females with a simvastatin concentration of 40 and 200 ng/L [147]. Quintana et al. (2014) showed that cholesterol biosynthesis was associated with the action of *etv7*, a human oncogene involved in progression of leukemia, on the development of red blood cells in zebrafish [148]. Knockdown of *etv7* by using morpholino led to decreased *lss* expression and it could be rescued by providing zebrafish with exogenous cholesterol. Moreover, the administration of the cyanotoxin microcystin-LR to male zebrafish for 60 days resulted in enhanced total cholesterol levels, which was linked to upregulation of the genes such as *hmgcra* and *hmgcs1* [149].

As a result, zebrafish is an advantageous model organism to investigate mechanisms behind lipid-related diseases and also to understand the changes occurring in cholesterol biosynthesis and metabolism as a drug response. However, there is still a need for more comprehensive studies which elucidate the downstream effects of cholesterol regulation from various aspects including toxicity, morphology and liver functioning.

1.14.3 Phenothiazines in Cancer Studies using Zebrafish

Although there are zebrafish studies focusing on different properties of phenothiazine derivatives such as being a fluorescent probe for bioimaging [150, 151] and causing locomotion defects [152], the anticancer effect of them have not been extensively elucidated using zebrafish models. One study unraveled that colorectal cancer cells could go through apoptosis based on a p53- and JNK-dependent mechanism mediated by chlorpromazine [153]. The toxicity caused by chlorpromazine on zebrafish was also examined and it was not toxic or lethal up to 50 μ M concentration. Gao et al. (2019) demonstrated that nitric oxide (NO) donor-containing novel phenothiazine derivatives 20a-c and 21a-c had the ability to decrease survival of breast cancer cells more efficiently in comparison to trifluoperazine and thioridazine [153]. The effect of these novel derivatives were further assessed on zebrafish embryos and 20a and 21a came forward as promising compounds with the least toxicity at 250 μ M concentration throughout a 24 hour-treatment.

In the study of Gutierrez et al. (2014), a small molecule screening was performed on a zebrafish model for MYC-induced T-cell acute lymphoblastic leukemia (T-ALL); and perphenazine was found to cause apoptosis in MYC-overexpressing thymocytes [154]. The study of Morita et al. (2020) presented perphenazine as an activator of protein phosphatase 2A (PP2A) which usually suppresses tumor development [155]. Perphenazine and the novel phenothiazine analog iHAP1 that lacks dopaminergic receptor inhibitory action exerted anticancer effects by causing cell cycle arrest in T-ALL cells through the activation of PP2A. Five day-treatment with these compounds resulted in decreased survival of GFP-tagged T-ALL cells in transplanted zebrafish larvae, iHAP1 showing more efficacy [155]. Another study revealed the action of chlorpromazine as an inducer of apoptosis in human oral cancer cells by generating a zebrafish xenograft model [156]. Tumor growth could efficiently be reduced in zebrafish larvae after chlorpromazine treatment. These provide a great a basis to emphasize how zebrafish can be a great tool as a cancer model, however more studies are needed and trifluoperazine is not among the phenothiazines which have been commonly studied in zebrafish.

1.14.4 Sorafenib in Zebrafish Studies

Sorafenib has been included in many zebrafish studies investigating both toxicity and anticancer properties. In one of the studies, 4.5 μ mol/L of sorafenib was found to be cytotoxic to zebrafish up to 5 dpf, causing morphological defects such as heart edema, also deformed eye and somites [157]. Sorafenib was also tested on zebrafish which was used as a preclinical

model to assess cardiotoxicity [158]. It was shown to be cardiotoxic to zebrafish and impair cardiac function at certain doses based on a molecular mechanism involving ERK activation through α -adrenergic signaling. The inhibition of blood vessel growth was revealed to be another impact of sorafenib on zebrafish embryos [159]. As a concept, the molecular background of angiogenesis has commonly been elucidated by observing transgenic zebrafish (*fli-1:EGFP*) after sorafenib treatment [160, 161]. Many studies focused on the cardiotoxicity and anti-angiogenesis capability of sorafenib, elucidating that it caused shortness in ventricular dimensions [162], pericardial edema [163] and impaired blood vessel formation [164-166]. Besides, the research by Yao et al. (2018) indicated that sorafenib was able to reduce liver size in double transgenic zebrafish Tg(*Wnt&Kras*) and Tg(*Myc&Kras*), in vivo models of HCC, alone and in combination with MK8245, a SCD inhibitor [167]. By using two other zebrafish HCC models, sorafenib was presented as a regulator of cell-cycle, which was evident by lowered expression of *ccne1*, *cdk1* and *cdk2* [121]. It also resulted in inhibitory effect against proliferation and migration of EDN1 oncogene-induced 293T cells in xenograft model. This effect of sorafenib was further validated through a liver cancer patient-derived-xenograft (PDX) model in the same study. The study of Yamamoto et al. (2019) demonstrated that 1 μ M of sorafenib exposure from 1 dpi to 4 dpi could significantly reduce the fluorescence signal representing the tumor generated by injecting A375 melanoma cells into zebrafish embryos [168]. In another xenograft model of melanoma, the fluorescence intensity in the yolk sac of zebrafish embryos could be decreased after 0.5 μ M of sorafenib treatment for 48 hours [169]. In a zebrafish HCC model, the size of the tumors obtained after implanting Huh7 cells was reduced as a result of 1 μ M of sorafenib exposure for 72 h [170]. A very similar result was obtained with the exposure of 1 μ M of sorafenib to Hep3B-injected zebrafish embryos for 48 hours [171]. Sorafenib also led to tumor inhibition in a zebrafish xenograft model generated with using non-small-cell lung cancer cells [172]. Moreover, the combination of sorafenib with erlotinib reduced the tumor size and spread at 4 dpi in a zebrafish xenograft model of breast cancer generated using MCF7 cells [173]. These studies in which the metabolic role and toxicity of sorafenib were being investigated in zebrafish cancer models give hope to unravel the molecular mechanisms and clinical options of HCC. However, sorafenib has not been studied together with phenothiazines in zebrafish.

1.15 Aims and Rationale

Previous studies in our lab has shown that phenothiazines could be effective anticancer drugs for cancer including that of liver and breast (Murat Yaman, PhD Thesis; Zengin and Yaman et

al., 2020 [174]). One of these drug types is phenothiazines, widely used as antipsychotics and recently gained attention as anticancer agents. One study in the literature has shown that phenothiazines are also modulators of cholesterol metabolism in addition to being anticancer agents bringing the possibility for liver damage or modulation of their anticancer activity [28]. Cancer cell lines are powerful models for anticancer drug screens [175] as well as identification of lipid modulators [27]. However, in vivo models are important to assess the effects of microenvironment as well as screen drugs as the next step in preclinical studies.

In the present study, my first aim was to find out whether novel phenothiazines (developed by team of Prof. Dr. Zeynep Ates-Alagoz in Ankara University; Murat Kislal, MS Thesis; Murat Yaman, PhD Thesis), along with those known ones, modulate cholesterol metabolism at the mRNA level using two different cell lines and the in vivo zebrafish model, a promising vertebrate species with high fecundity and similar liver development and diseases to humans [107, 108]. Second, I wanted to test whether selected lead novel compounds have significant toxic effects on embryonic and larval development when given at 2 dpf and followed up until 5 dpf through microscopy and morphometric measurements. Third, I wanted to establish a xenograft model using a) blastula stage embryos and b) 2 dpf embryos using casper and WT zebrafish. My final aim was to test dose-dependent effects of trifluoperazine alone and together with a known kinase inhibitor sorafenib in zebrafish using microscopic and ALU-based methodology to determine if dose has any synergistic or antagonistic effects.

In brief, I tried to perform a comprehensive screening for known and novel phenothiazine derivatives, with the aim of elucidating the mechanism behind their anticancer potency both in vitro and in vivo. I have focused on the modulation of cholesterol biosynthesis upon derivative exposures utilizing HCC cell lines and zebrafish, which has served as a great organism for both toxicity assessments and generation of in vivo HCC model.

Chapter 2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Laboratory Reagents and Kits

General laboratory reagents and the kits used for experimental procedures were provided in **Table 2** with the information of their catalog number and company name. In addition to the commercial phenothiazines given, novel phenothiazine derivatives used in the experiments were synthesized by Ankara University in the scope of TUBITAK 1001 - 116Z388 project.

Table 2. General laboratory products and kits used for the experiments.

Product Name	Catalog Number	Company
NaCl	31434	Sigma-Aldrich Germany)
NaOH	6203	Sigma-Aldrich Germany)
HCl	7102	Sigma-Aldrich Germany)
SDS	71725	Sigma-Aldrich Germany)
EtOH	B2221	Sigma-Aldrich Germany)
MeOH	24229	Sigma-Aldrich Germany)
Chloroform	24216	Sigma-Aldrich Germany)
2-Propanol	24137	Sigma-Aldrich Germany)
HEPES	H-1016	Sigma-Aldrich Germany)
PFA	76240	Fluka (USA)
Triton X-100	T8787	Sigma-Aldrich (Germany)
Tween 20	39796.51	SERVA (Germany)
NP-40	A16940250	AppliChem (Germany)
Glycine	G8898	Sigma-Aldrich (Germany)
Glycerol	15524	Sigma-Aldrich Germany)
Dimethyl Sulfoxide (DMSO)	A1584	AppliChem (Germany)
Trizma Base	T1503	Sigma-Aldrich (Germany)
Aminocaproic acid	A2504	Sigma-Aldrich (Germany)
2-mercaptoethanol	M3148	Sigma-Aldrich (Germany)
Ponceau S Solution	P7170	Sigma-Aldrich (Germany)
TEMED	A11480100	AppliChem (Germany)
4X Laemmli Sample Buffer	161-0747	Bio-Rad (USA)
PageRuler Prestained Protein Ladder	26616	Thermo Scientific (USA)

TGX Stain-Free FastCast Acrylamide Kit, 10%	1610183	Bio-Rad (USA)
ECL Prime Western Blotting System	RPN2232	GE Healthcare (UK)
Ammonium Persulfate	420627	Carlo Erba Reagents (Italy)
QIAzol Lysis Reagent	79306	Qiagen (Germany)
Complete EDTA-free protease inhibitor cocktail	11873580001	Roche (Switzerland)
Phosphatase Inhibitor (PhosSTOP)	4906845001	Roche (Switzerland)
Nuclease Free Water	SH30538.01	HyClone (USA)
SDS	71725	Sigma-Aldrich (Germany)
Bovine Serum Albumin (BSA)	A7906	Sigma-Aldrich (Germany)
RNeasy Mini Kit	74104	Qiagen (Germany)
LightCycler 480 SYBR Green I Master	4887352001	Roche (Switzerland))
RevertAid First Strand cDNA Synthesis Kit	K1622	Thermo Scientific (USA)
BCA Protein Assay Kit	23227	Thermo Scientific (USA)
Cholesterol Cell-Based Detection Assay Kit	10009779	Cayman (USA)
MTT	M6494	Thermo Scientific (USA)
Phenothiazine	P14831-25g	Sigma-Aldrich (Germany)
Trifluoperazine	T8516-5g	Sigma-Aldrich (Germany)
Prochlorperazine	P9178-5g	Sigma-Aldrich (Germany)
Perphenazine	P6402-1g	Sigma-Aldrich (Germany)
Sorafenib	S7397	Selleckchem (USA)

2.1.2 Cell Culture Products

The reagents that were used in cell culture experiments, their catalog numbers and company information were given in **Table 3**. Additionally, HCC cell lines that have been used throughout the study were SkHep1, Hep3B and Huh7.

Table 3. Products used in cell culture experiments.

Product Name	Catalog Number	Company
DMEM with 1.0 g/L Glucose, without L-Glutamine-	BE12-707F	Lonza (Switzerland)
DMEM, low glucose, pyruvate, no glutamine, no phenol red	11880028	Thermo Scientific (USA)
Fetal Bovine Serum (FBS)	S181G-500	Biowest (France)
PBS (1X) without Ca and Mg	BE17-516F	Lonza (Switzerland)
L-Glutamine	BE17-605E	Lonza (Switzerland)
Penicillin/Streptomycin	DE17-602E	Lonza (Switzerland)
NEAA (100X)	BE13-114E	Lonza (Switzerland)
Sodium Pyruvate	BE13-115E	Lonza (Switzerland)
PBS	BE17-516F	Lonza (Switzerland)
Trypsin-Versene EDTA (1X)	BE17-161E	Lonza (Switzerland)

2.1.3 Primers

The primers that were used in RT-qPCR experiments to perform gene expression analyses were given in **Table 4** with their sequences.

Table 4. List of the primers used in RT-qPCR experiments.

Gene ID	Sequence	Species
HMGCR	F: 5'-TATCCGTTTCCAGTCCAGGT-3' R: 5'- GCTGGAATGACAGCTTCACA-3'	<i>Homo sapiens</i>
SQLE	F: 5'-CGTGAATATCCTTGCTCAGGC-3' R: 5'- AGACAATACAGAAAGCAGCCCA-3'	<i>Homo sapiens</i>
LSS	F: 5'-GGCGCTTAAGTATTTCCACAA-3' R: 5'-AACTCTAAGCCCTGCGTGAG -3'	<i>Homo sapiens</i>
TPT1	F: 5'-GATCGCGGACGGGTTGT-3' R: 5'-TTCAGCGGAGGCATTTCC-3'	<i>Homo sapiens</i>
AluYb8	F: 5'-CGAGGCGGGTGGATCATGAGGT R: 5'-TCTGTCGCCCAGGCCGGA-3'	<i>Homo sapiens</i>
ACHE	F: 5'- TCTCGAAACTACACGGCAGA-3 R: 5'- CGCAGGTCCAGACTAACGTA-3'	<i>Homo sapiens</i>
<i>actb2</i>	F: 5'-GCCTGACGGACAGGTCAT-3' R: 5'- ACCGCAAGATTCCATACCC-3'	<i>Danio rerio</i>
<i>hmgs1</i>	F: 5'-CCGTCCGCTTATTACACGAA-3' R: 5'- ACATGGACTCGCAAATCGC-3'	<i>Danio rerio</i>
<i>lss</i>	F: 5'-GATTCCTGACTATCTGTGGCTG-3' R: 5'- GTAAGGCACTCTGTAAATCTGGG-3'	<i>Danio rerio</i>

2.1.4 Antibodies

In **Table 5**, the antibodies used in western blot experiments were listed.

Table 5. List of the antibodies used.

Antibody Name	Catalog Number	Company
T-CHEK1	Sc-8408	Santa Cruz
P-CHEK1 (S345)	#2348P	Cell Signaling
CDK2**	C5223	Sigma-Aldrich
AKT*	#9272S	Cell Signaling
P38*	#9212	Cell Signaling
p-P38 (t180/y182)	#4511P	Cell Signaling
DR5	#8074P	Cell Signaling
GAPDH	Sc-47724	Santa Cruz

The antibodies that were marked were kindly provided by Rengül Çetin Atalay (*) and Özgür Şahin (**).

2.1.5 Solutions and Buffers

2.1.5.1 Zebrafish E3 Medium

After fertilization, zebrafish embryos were collected and kept in 1X E3 medium. In order to prepare 1 liter of 1X E3 medium, 60X stock E3 was diluted to 1X using Milli-Q water. 2-3 ml of methylene blue (0.01%) was added for antifungal purposes. The recipe for 60X stock E3 medium is as follows:

17.2 g of NaCl (Sigma-Aldrich, 13423), 0.76 g of KCl (Sigma-Aldrich, 12636), 2.9 g of CaCl₂.2H₂O (Carlo Erba, 327607) and 4.9 g of MgSO₄.7H₂O (Sigma-Aldrich, M2773-1kg) were weighed and mixed in 1 liter of Milli-Q water. After autoclaving, the stock was kept at +4°C.

2.1.5.2 Tricaine

Tricaine stock solution was prepared as 4 mg/ml (pH: 7.5). 400 mg of tricaine and 2 ml of 1 M Tris (pH: 9.0) were mixed and pH was adjusted to 7.5. The solution was topped up to 100 ml with distilled water and stored at +4°C.

2.1.5.3 4% PFA

In order to prepare 50 ml of 4% PFA, 40 ml of 1X PBS was heated in a water bath at 60°C and 2 g of PFA powder was added. 1 M NaOH was poured into the solution until it became clear and pH was adjusted to 6.8 – 7.0 using 1 M HCl. Finally, the solution was topped up to 50 ml using 1X PBS and filtered. It was kept at -20°C for long-term storage.

2.1.5.4 DNA Extraction Buffer

DNA extraction buffer was prepared using Tris (pH: 8.2), EDTA (pH: 8.2), NaCl and SDS. The final concentration of these components were 100 mM, 10 mM, 200 mM and 0.5%, respectively.

2.1.5.5 Western Blot Solutions

The solutions and buffers used to perform western blot experiments were provided in **Table 6** together with their component and recipe information.

Table 6. The solutions/buffers used and their components.

Solution/Buffer	Components
Running Buffer (10X)	10.08 g of SDS, 30.3 g of Trizma Base and 144 g of Glycine in 1 liter of ddH ₂ O
TBS (10X)	24 g of Trizma Base and 88 g of NaCl in 900 ml of ddH ₂ O After adjusting pH to 7.6, the volume was completed to 1 liter using ddH ₂ O.
TBS-T (0.2%) (10X)	100 ml of 10X TBS, 900 ml of ddH ₂ O and 2 ml of Tween-20
Anode I Buffer	18.15 g of Trizma Base and 100 ml of 100% MeOH in a final volume of 500 ml completed using ddH ₂ O
Anode II Buffer	1.5 g of Trizma Base and 100 ml of 100% MeOH in a final volume of 500 ml completed using ddH ₂ O
Cathode Buffer	2.62 g of aminocaproic acid and 100 ml of 100% MeOH in a final volume of 500 ml completed using ddH ₂ O
Blocking Solution (5%)	5 g of BSA or milk and 100 ml of TBS-T (2%)
Mild Stripping Buffer	1.5 g of Glycine, 0.1 g of SDS, 1 ml of Tween-20 After the volume was topped up to 80 ml using ddH ₂ O, pH was adjusted to 2.2 and the volume was completed to 100 ml with ddH ₂ O.
SDS Loading Buffer (4X)	100 µl of 2-mercaptoethanol and 900 µl of Laemmli Buffer (Biorad)

2.1.6 Laboratory Equipment

In **Table 7**, the equipment used to carry out the experiments were specified below with their company information.

Table 7. Equipment and the procedures in which they were used.

Equipment	Experimental Procedure	Company
NanoDrop ND-1000	DNA & RNA quantification	Thermo Scientific (USA)
PCR Thermal Cycler 2720	cDNA synthesis	Applied Biosystems (USA)
LightCycler 480 Instrument	RT-qPCR	Roche (Switzerland)
Synergy HT Microplate Reader	Protein quantification, MTT	Biotek (USA)
SpectraMax M5 microplate reader	Cholesterol Detection Assay	Molecular Devices (USA)
EVOS FL Auto Imaging System	Cholesterol Detection Assay	Life Technologies (USA)
PV820 pneumatic picopump	Xenograft	World Precision Instruments (USA)
FemtoJet Express	Xenograft	Eppendorf (Germany)
Narishige MN-151	Xenograft	Narishige (Japan)
Leica MZ10F	Imaging of zebrafish larvae	Leica (Germany)
SZX10 Stereomicroscope equipped with SC50 camera	Imaging (color) of zebrafish larvae	Olympus (Japan)
SZX16 Stereomicroscope equipped with XC50 camera	Imaging (fluorescent) of zebrafish larvae	Olympus (Japan)
Olympus IX71 Inverted Fluorescence Microscope	Imaging of zebrafish larvae	Olympus (Japan)
LSM 880 Confocal Laser Scanning Microscopy with Airyscan	Imaging of zebrafish larvae	Zeiss (Germany)
Amersham Imager 600	Western blot	GE Healthcare (USA)
Oasis CO ₂ Incubator	Cell Culture	Caron (USA)
MN 120 Microbiological Safety Cabinet	Cell Culture	Nüve (Turkey)
INCO 153 Incubator	Cell Culture	Memmert (Germany)
SAFE 2020 Biological Safety Cabinet	Cell Culture	Thermo Scientific (USA)

2.2 Methods

2.2.1 In Vitro Methods

2.2.1.1 Cell Culture

2.2.1.1.1 Cell Thawing

The frozen cells stored at -80°C were kept in water bath (37°C) for 2-3 minutes until most of them melted. Complete medium was added onto and mixed with the cells. The mixture containing the cells, freezing medium and complete medium was then centrifuged at 1500 rpm for 5 minutes. The supernatant was removed; and the cell pellet was dissolved in fresh complete medium. The cells were transferred into a T25 flask and kept in the incubator which was set to 37°C and 5% CO_2 . They were then passaged into a T75 flask when they were confluent enough (70-80%) to be passaged.

2.2.1.1.2 Cell Passaging

The medium on the cells was aspirated and cells were washed using PBS. 1X Trypsin was added onto the cells and the flask was kept in the incubator for 5 minutes to detach the cells. Then, fresh complete medium in at least twice the amount of trypsin was added and mixed with the detached cells. A desired amount was taken from the mixture to dilute the cells depending on the required confluency for experiments and transferred into a new T75 flask. Finally, about 10 milliliters of fresh complete medium was poured into the T75 flask and the flask was kept in the incubator until the next passage.

2.2.1.1.3 Cell Freezing

In order to have healthy and young cells in stock and ready for experiments, cells needed to be frozen in their early passages (passage number 2 or 3). After trypsinizing the cells and adding fresh medium as in cell passaging, cells were centrifuged at 1500 rpm for 5 minutes. Freezing medium (a mixture of 90% FBS and 10% DMSO) was added onto the cell pellet to dissolve it. The cells were distributed into cryovials which were then kept at -20°C for 1-2 hours. After the freezing medium containing the cells was completely frozen, cryovials were taken to -80°C . In order to store them for more than 2-3 weeks, they were placed into liquid nitrogen tanks.

2.2.1.1.4 Cholesterol Detection Assay

Cholesterol Cell-Based Detection Assay Kit (Cayman Chemical, Ann Arbor, MI) was used to determine intracellular cholesterol levels. For this procedure, 10.000 Hep3B cells were seeded into each well of a 96-well plate and treated with TFP, SFB or their combinations for 48 hours. In the process of treatment, the medium containing the drug in each well was renewed every 24 hours. After the exposure was completed, the medium was removed from the wells and cells were fixed using the fixative solution. Later on, they were washed 3 times and 100 µl of Filipin III solution was added into each well. At the end of 1 hour incubation at room temperature, the cells were washed 2 times and fluorescence was measured with an excitation of 355 nm and an emission of 460 nm using SpectraMax M5 microplate reader. Additionally, three images from different regions of each well were taken at 20X magnification using the DAPI filter (ex: 357/44, em: 447/60) of EVOS FL Auto Imaging System (Life Technologies). Filipin III absorbance values were normalized according to those of DMSO control. The resulting values were divided by the cell viability values -to control for cell number- obtained through MTT assay for the analysis performed in GraphPad Prism8.

2.2.1.1.5 MTT Cell Viability Assay

The effects of TFP, SFB and their combination on the viability of Hep3B cells were assessed based on MTT assay. Since it was performed simultaneously with Cholesterol Detection Assay, the number of Hep3 cells seeded in each well and the conditions for drug exposures were exactly the same. After the treatments ended, the medium in each well was removed and MTT solution was added. In order to prepare MTT solution, 5 mg/ml MTT was prepared in 1X PBS for 100 wells and filtered into 10 ml of phenol red-free DMEM. The resulting solution was distributed into each well as 110 µl and the cells were incubated for 4 hours at 37°C. Later on, 0.1 g/ml of SDS was freshly prepared in 0.01 M HCl and added onto each well as 100 µl. At the end of overnight incubation for ~16 hours at 37°C, the absorbance at 570 nm was measured using Synergy HT Microplate Reader (Biotek, USA). For the analysis, the average measurement for the blank was subtracted from that of each well and the obtained values were normalized to that of DMSO control. Moreover, the experiment was performed in triplicates for each treatment group with a fixed DMSO percentage (0.1%) for all.

2.2.1.1.6 Preparation of Cells for Injection

For the prepreparation of the cells to be injected, there were minor differences in the protocols followed at Bilkent and IBG. According to the protocol performed at Bilkent, Hep3B cells were detached from the cell culture plate using 1X trypsin and 3.000.000 cells were counted

and centrifuged at 1500 rpm for 3 minutes. After resuspension with 500 μ l of PBS, the centrifugation was performed at 1500 rpm for 1 minute. The cells were incubated with 1 μ l of 2 mg/ml DiI stock (V22886, Molecular Probes) in 49 μ l PBS for 5 minutes at 37°C and then 15 minutes at 4°C. They were then washed with 500 μ l of FBS. Two more wash steps were performed using 500 μ l of PBS. Lastly, the cells were resuspended in 5% phenol red-containing 50 μ l of PBS and were kept on ice.

In the protocol carried out at IBG, the number of cells counted was initially 500.000 or 1.000.000 according to the density of the cells. The centrifugation after washes were performed at 1500 rpm for 2 minutes and the incubation with DiI was for 20 minutes at 37°C instead. The resuspension was performed using 10% FBS containing PBS to a final density of 30.000 cells/ μ l. If more cells were counted initially, the amount of DiI stock used in DiI + PBS mixture and the final volume were adjusted accordingly.

2.2.1.2 Methods for Gene Expression Analyses

2.2.1.2.1 Treatment of the Cells

Huh7 cells were seeded into 6-well plates with each well having 180.000 cell density in complete media. 24 hours later, they were given different doses of TFP, SFB and their combination. The next day, the treatment procedure was repeated.

2.2.1.2.2 RNA extraction

After 48 hours of treatment, cell pellets were collected and frozen by putting them into liquid nitrogen. They were kept at -80°C until RNeasy Mini Kit (QIAGEN) was used to isolate total RNA based on the steps in manufacturer's instructions. The concentrations of RNA samples were obtained in ng/ μ l using NanoDrop.

2.2.1.2.3 cDNA Synthesis

The isolated RNAs were converted into cDNA by using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). A master mix containing 4 μ l of 5X reaction buffer, 2 μ l of 10 mM of dNTP mix, 1 μ l of Ribolock and 1 μ l of reverse transcriptase for each RNA sample was prepared and distributed as 8 μ l of mix. Then, a 12 μ l of mixture with 1000 ng of RNA template, 1 μ l of Oligo(dT) and nuclease-free water was added to obtain a total of 20 μ l reaction volume for each. 2720 thermal cycler (Applied Biosystems) was used to perform the cDNA synthesis reaction at the conditions 42°C for 60 minutes and 70°C for 5 minutes. The cDNAs were diluted in 1:10 ratio and both stock and diluted cDNAs were stored at -20°C.

2.2.1.2.4 Real time Quantitative PCR

In order to perform RT-qPCR, LightCycler 480 SYBR Green Master was used. A master mix with 1 µl of nuclease-free water, 1 µl of forward primer, 1 µl of reverse primer and 5 µl of SYBR green for each reaction was prepared and distributed in 8 µl volumes. Then, 2 µl of diluted cDNA was added resulting in a total of 10 µl of reaction volume. The 96-well plate containing the reaction mixtures was placed into LightCycler® 480 Instrument II which was set to the conditions specified in **Table 8**. For the calculation of logarithmically transformed relative expression ($-\Delta\Delta C_t$) levels, relative expression of each gene was normalized to the expression values of TPT1 which was used as the reference gene [176]. Statistical analyses were performed using GraphPad Prism (v. 6.05).

Table 8. Conditions for RT-qPCR.

Step	Temperature (°C)	Time	Cycles
Pre-incubation	95	5 min	1
Amplification	95	10 sec	45
	55-60	20 sec	
	72	20 sec	
Melting Curve	95	5 sec	1
	55	1 min	
	95	Acquisition per 5°C	
Cooling	40	30 sec	1

2.2.1.3 Western Blot

BCA Protein Assay Kit (Thermo Scientific, USA, 23227) was used to measure concentrations of isolated proteins. Proteins were boiled at 95°C for 5 minutes with 4X Laemmli Loading Dye (Biorad, USA, 161-0747). 10 µg of protein samples were run in 10% SDS-PAGE (161–0183, Bio-Rad Acrylamide Kit) gel. The transfer of proteins to PVDF membrane (3010040, Roche) was achieved by semi-dry transfer. Then, blocking was performed by keeping the membrane in blocking solution (5% BSA-Tris-buffered saline with Tween-20 (0.2%) (TBS-T)) for one hour at room temperature. After overnight incubation with primary antibodies (1:1000 dilution) at 4°C, the membrane was washed with TBS-T and incubated with anti-mouse (7076, Cell Signaling) or anti-rabbit (7474, Cell Signaling) secondary antibodies for an hour at room temperature. It was again washed with TBS-T and ECL Prime Western Blotting Detection Reagents (RPN2232, GE Healthcare) were used to visualize the proteins in Amersham Imager

600 (GE Healthcare). The analyses of the protein images were performed in ImageJ. Intensity curves were obtained and the area under those curves were determined as percentage. Each protein band was normalized to its own GAPDH, then also normalization to the control group (DMSO) was carried out. The values obtained from the analyses were taken to log₂ base and demonstrated in graphs generated in GraphPad Prism (v. 6.05).

2.2.2 Generation of Cholesterol Pathway Representations

In order to visualize fold change differences in cholesterol biosynthesis and metabolism pathways, RNA-seq analysis performed by Kubra Calisir, MS student, was utilized. The experiments for RNA-seq was performed by Murat Yaman (PhD Thesis, 2020) in a way that Hep3B cells were treated with DMSO (0.1%), 12 μ M of TFP, 1 μ M of SFB, 2 μ M of SFB, TFP 12 μ M - SFB 1 μ M combination and TFP 12 μ M - SFB 2 μ M combination for 48 hours. The raw RNA-seq data was processed using Biojupies web server [177] based on alignment to human reference genome and featuring (Kubra Calisir, MS student). Before TMM normalization was performed, the genes that had low expression (≤ 10 counts in 90% of the samples) were filtered out. Then, resulting data was analyzed using limma package in R to obtain differential expression among different treatment groups.

After the resulting gene list was scaled down to cholesterol-related genes by Kubra Calisir, Cytoscape (v3.8.2) software was used to generate a cholesterol biosynthesis pathway by editing Kegg Pathway: Steroid Biosynthesis (hsa00100), which was retrieved through KEGGscape app of Cytoscape. Comparisons between 12 μ M of TFP and DMSO, TFP 12 μ M - SFB 1 μ M and 12 μ M of TFP, TFP 12 μ M - SFB 2 μ M and 12 μ M of TFP, also TFP 12 μ M - SFB 2 μ M and TFP 12 μ M - SFB 1 μ M were performed by putting logFC values in a color scale from minimum to maximum. An additional parameter was provided by setting 0.05 as the threshold for adj. p-value, genes with adj. p-value lower than that as rectangles and with adj. p-value in the opposite direction as V-shapes.

A similar pathway representation for cholesterol metabolism was performed for by Kubra Calisir using 'pathview' package in R. This package allowed to represent Kegg Pathway: Cholesterol Metabolism (hsa04979) with a color scale for logFC.

2.2.3 In Vivo Methods

2.2.3.1 Drug Exposures on Zebrafish Embryos/Larvae

The commercial and novel phenothiazines were tested on wild-type zebrafish embryos starting from 2 dpf to 5 dpf. After dechoriation of the embryos at 2 dpf, they were

distributed into the wells of a 24-well plate in a way that each well contained 10 embryos. The treatment solutions were prepared simply by adding corresponding concentration of the drugs into 1X E3 medium. Two wells (a total of 20 embryos) were assigned for each treatment group tested. For the exposure, the excess E3 medium was removed from each well without harming the embryos and treatment solutions were added as 1 ml/well. Mortality and morphological differences such as heart edema, yolk size and body length in each embryo was observed under stereomicroscope (Olympus SZX10) and recorded every day. Normally developing as well as abnormal embryos were imaged daily using SC50 camera (Olympus, Japan) and this was followed by the renewal of the treatment solutions. At the end of 72 hours of treatment, the larvae were euthanized.

The toxicity of TFP, SFB and combination treatments was also investigated using casper zebrafish embryos from 2 dpf to 4 dpf. A similar experimental setup was applied for wild-type zebrafish embryos with the same treatment groups, however the time period was extended up to 5 dpf and the embryos were kept at 34°C starting with the first treatment given at 2 dpf instead of 28°C. This was because the xenograft conditions were tried to be mimicked to better understand the tolerable doses that could be applied after xenografting. All other drug exposures were carried out at 28°C, following the experimental steps explained above.

For the toxicology experiment which was conducted for RNA isolation, there were initially 20 embryos in each well and the larvae were snap-frozen in liquid nitrogen instead of being euthanized at 5 dpf. They were then kept at -80°C.

2.2.3.2 Xenografts

For the xenograft experiments which were conducted at IBG, the following protocol was applied/used. Dechoriation of 2 dpf zebrafish embryos was performed and they were injected with the cell suspension using an electronic microinjector (PV820 pneumatic picopump, World Precision Instruments) and a micromanipulator (Narishige MN-151). 250 - 300 cells were tried to be given into the yolk of each embryo. The embryos were kept at 33°C for the next 3 days. 6 hours after injection was completed, the embryos were checked using Olympus SZX16 (Olympus, Japan) fluorescent stereomicroscope which was connected to XC50 (Olympus, Japan) camera to determine efficiently injected ones. The embryos which had the targeted number of cells (250 - 300) were selected. The ones that contained less number of cells (100 - 200) were also kept separate to be treated and used for Alu-based DNA quantification later. At 1 dpi, representative bright field and fluorescent images of all embryos

selected were taken under fluorescent stereomicroscope using cellSens Entry software (Olympus, Japan). Then, the embryos injected with 250 - 300 cells were exposed to TFP, SFB or their combination in two different sets of treatment. For the first set, the larvae were provided with 12 μ M of TFP, 2 μ M of SFB and their combination for 24 hours. In the second set, the larvae were treated with 6 μ M of TFP, 1 μ M of SFB and their combination for 48 hours. The treatment solutions were renewed daily. Images were taken after drug exposures were completed using fluorescent stereomicroscope and the embryos were fixed using 4% PFA and kept at +4°C. Fixed embryos were mounted in 1% low melt agarose and imaged under inverted fluorescence microscope (Olympus, Japan). The changes in tumor size were determined based on the calculation of area with fluorescence using Fiji.

Statistical analyses were performed on GraphPad 8.0 platform in a way that each treatment group was compared to the control (DMSO) using One-way ANOVA (Dunnett's multiple comparison tests).

The xenografts at Bilkent were performed generally in the same way, with differences in the equipment used. The injections were performed using the microinjector Eppendorf FemtoJet express and the micromanipulator Nikon Narishige. While injecting, ~300 cells were aimed to be pumped into the yolk. Both the check and imaging of the embryos were carried out using Leica MZ10F stereo microscope (Leica, Germany) equipped with Olympus DP71 camera. At 1 dpi, 56 efficiently injected casper larvae were distributed into the wells of a 96-well plate. After bright field and fluorescent images of all embryos were taken using Dpcontroller (v2.3.1.292) (Olympus, Japan), The larvae were treated with DMSO control, 2.5 μ M of TFP, 0.25 μ M of SFB or their combination for 48 hours in a way that there were 14 larvae per group. At 3 dpi, the images of each larvae were captured again. The comparisons between 1 dpi and 3 dpi were performed using GraphPad after the analysis of fluorescence intensity change, which was calculated as CTCF [178] based on the following formula:

$$\text{CTCF} = \text{Integrated Density} - (\text{Area of Selected Cell} \times \text{Mean Fluorescence of Background Readings})$$

Apart from these, a differently designed xenograft experiment was performed on casper embryos at blastula stage. Two groups of Hep3B cells incubated in DiI, which were detached either by trypsin or scraping were injected to the cell portion of 3.5 hpf embryos. The fluorescence in the embryos were tracked every day and the larvae of both groups were imaged at 5 dpf as the final step of the experiment.

2.2.3.3 Alu-based DNA Quantification by qPCR

2.2.3.3.1 Zebrafish Genomic DNA Extraction

Each larvae was transferred into an eppendorf tube and excessive E3 medium was removed. 200 μ l of DNA extraction buffer was added onto each larvae and 3 μ l of proteinase K was also added. The tubes were incubated overnight at 55°C. The next day, the tubes were taken to a heat block set to 95°C for 20 minutes for inactivation of proteinase K. Centrifugation was performed at 13000 rpm for 20 minutes at room temperature after samples cooled down. The supernatant in each tube was transferred into a clean tube and 175 μ l of isopropanol was added. After mixing, the tubes were centrifuged at 13000 rpm for 20 minutes at +4°C and the supernatants were discarded. The wash step was performed with 500 μ l of 70% EtOH. The tubes were centrifuged at 13000 rpm for 20 minutes at +4°C again. After supernatants were discarded, the tubes were left to air dry. Finally, 30 μ l of ddH₂O was added for resuspension and DNA concentrations were measured using nanodrop.

2.2.3.3.2 Quantitative PCR

In order to determine the amount of Alu repeats, qPCR was performed using LightCycler 480 SYBR Green Master. A master mix with 1 μ l of nuclease-free water, 1 μ l of forward primer, 1 μ l of reverse primer and 5 μ l of SYBR green for each reaction was prepared and distributed in 8 μ l volumes. Then, 2 μ l of zebrafish-human DNA mixture was added resulting in a total of 10 μ l of reaction volume. The 96-well plate containing the reaction mixtures was placed into LightCycler® 480 Instrument II which was set to the conditions specified in **Table 9**. For the calculation of $-\Delta\Delta C_t$ levels, relative quantity of Alu was normalized to that of zebrafish ache which was used as the reference gene [179]. Statistical analyses were performed using GraphPad Prism (v. 8).

Table 9. Conditions for Alu-based DNA Quantification.

Step	Temperature (°C)	Time	Cycles
Pre-incubation	95	10 min	1
Amplification	95	15 sec	40
	62	5 sec	
	72	15 sec	
Melting Curve	95	30 sec	1
	60	1 min	
	95	Acquisition per 5°C	
Cooling	40	30 sec	1

2.2.3.4 Nile Red Staining and Imaging Vasculature of Fli1a:EGFP Zebrafish

Larvae

For the investigation of lipid accumulation, 5 dpf Fli1a:EGFP larvae were treated with DMSO and TFP for 48 hours with the renewal of treatment medium every 24 hours. At 7 dpf, they were stained with 5 ng/ml of Nile Red (in E3 medium) for 2 hours in the dark at 28°C. Later on, larvae were live-mounted in 1% low melt agarose with tricaine, since Nile Red staining on lipid droplets could only be observed on larvae when they were alive. They were then imaged using LSM 880 Confocal Laser Scanning Microscopy (Zeiss, Germany).

After live-imaging of larvae, the remaining larvae were fixed using 4% PFA. The vasculature was imaged again using confocal microscopy to evaluate liver in terms of size and structure. The vasculature area and GFP intensity in the liver were determined using Fiji. The comparison between DMSO control and TFP-treated group was performed by applying unpaired t-test on GraphPad 8.0 platform.

2.2.3.5 Zebrafish RNA Isolation using QIAzol

The larvae were taken out of the -80°C fridge and washed with 1 ml ice-cold PBS (+4). Throughout the whole experimental steps, larvae were kept on ice unless specified otherwise. After removing PBS, 600 µl of QIAzol lysis reagent was added for each group of 20 larvae. Then, they were homogenized using insulin syringes. 120 µl (1/5 volume of QIAzol) of chloroform was added and the eppendorf tubes containing the mixture were inverted 15 times. Following 10 mins of incubation at RT, the tubes were centrifuged at 13000 rpm for 20 mins at 4°C. The supernatant was carefully transferred into a clean eppendorf tube without touching the cloudy or colored phase. The same volume of isopropanol was added into each tube and the tubes were inverted 5 times. After incubation of the tubes at RT for 10 mins, centrifugation was performed at the previous conditions. The supernatant was discarded and the pellets were washed with 1 ml of 75% EtOH. Following centrifugation at 13000 rpm for 15 mins at 4°C and removal of the supernatant, another round of wash step was performed by adding 1 ml of 100% EtOH into each tube. When the supernatant in each tube was removed, the pellets were left to air-dry and dissolved in 20 µl of nuclease-free water. The concentration of RNA was measured using nanodrop.

2.2.4 In Silico Analysis of Human and Zebrafish Conservation in terms of Cholesterol Biosynthesis Pathway Genes

The comparison of human and zebrafish sequences for the members of cholesterol biosynthesis pathway was performed using Ensembl/BioMart web tool [180]. Firstly, ‘Human genes (GRCh38.p13)’ dataset under the selection of Ensembl Genes 104 was chosen and a list of gene IDs was provided for the genes involved in cholesterol biosynthesis. Homologue filters were applied by selecting ‘Orthologous Zebrafish Genes’. Later on, the attributes of interest were selected for ‘Homologues’ under the heading ‘Zebrafish Orthologues’. Finally, the resulting table was downloaded in XLS format.



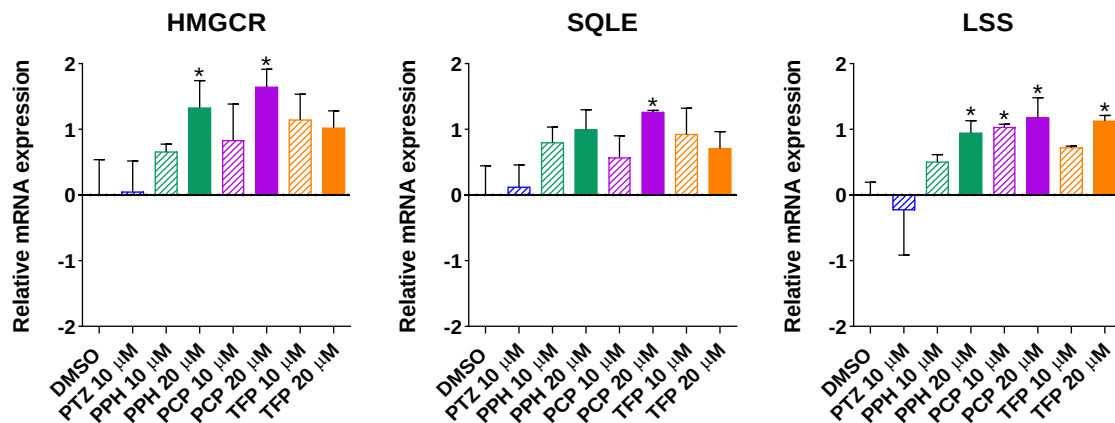
Chapter 3. RESULTS

3.1 Gene Expression Analyses on Cholesterol Biosynthesis Pathway

3.1.1 The Effects of Phenothiazines on Cholesterol Biosynthesis Using SkHep1 and Hep3B Cells

According to many studies in literature, regulation of cholesterol levels in cells is associated with different cancer types and cholesterol favors development of cancer cells as detailed in the introduction section. Therefore, checking the expression of genes involved in cholesterol biosynthesis and regulation pathways could be helpful to understand possible mechanisms driven by TFP or SFB in HCC.

A



B

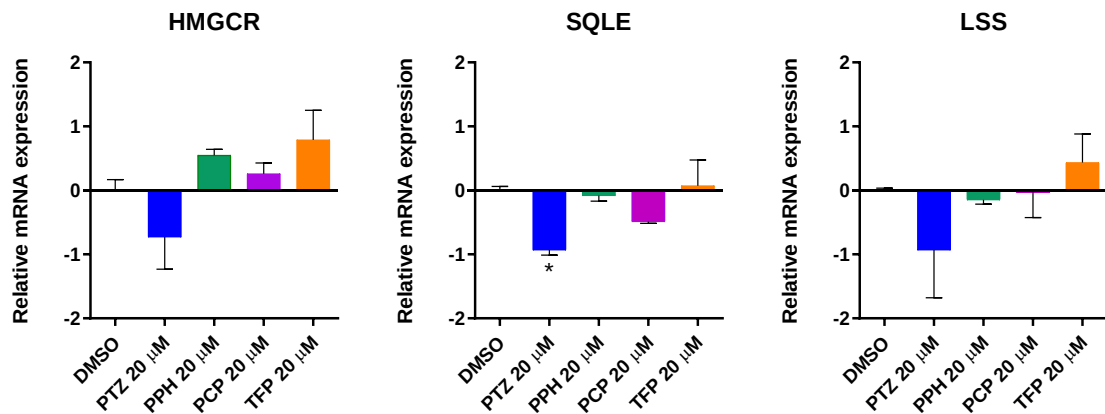
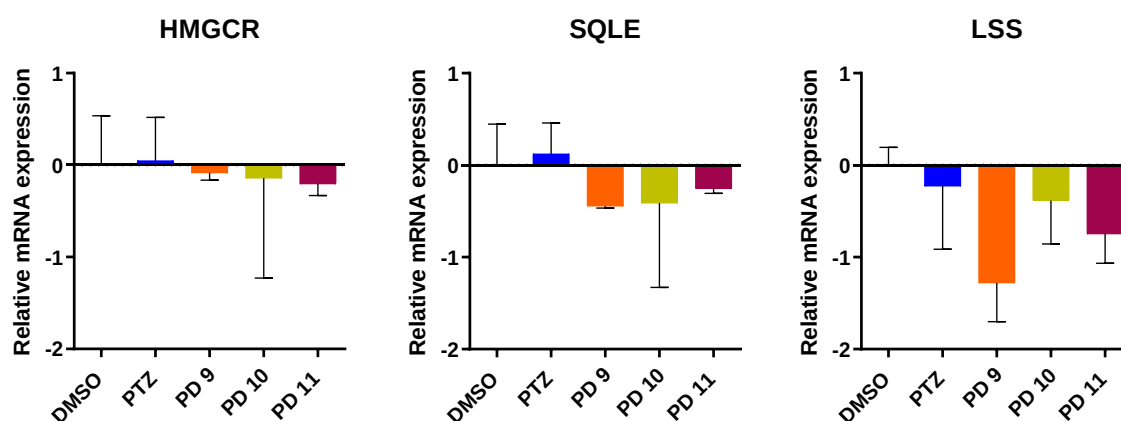


Figure 2. Changes in the expression of cholesterol biosynthesis genes after treatment with commercial phenothiazines. Cholesterol biosynthesis gene mRNA levels were quantified by qPCR in **A)** Hep3B and **B)** SkHep1 cells after 24 hour-treatment. The qPCR experiments for SkHep1 cell were performed by Rafeed Said Tiriyaki. While y-axis showed relative expression to TPT1 reference gene, treatment groups including DMSO control and PTZ, PPH, PCP and TFP at either 10 and/or 20 μ M concentration were given in x-axis. Expression values of HMGCR, SQLE and LSS genes were in log2 scale. One-way ANOVA (Dunnett's test) was the statistical analysis method used to compare each treatment group with DMSO control (*: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$). Dose-dependent changes were determined based on Two-Way ANOVA (Sidak's test) method (#: $p < 0.05$, ##: $p < 0.01$ and ###: $p < 0.001$).

In Hep3B cells, the expression of HMGCR, SQLE and LSS genes increased with many of the given treatments. While PCP at 20 μ M concentration was significantly effective for each gene, PPH at 20 μ M concentration increased the expression of HMGCR and LSS (**Figure 2A**). The most noticeable expression increase was seen in LSS gene which was affected by 20 μ M concentration of all commercial drugs and also 10 μ M of PCP significantly. PTZ did not affect expression of any of these three.

On the other hand, commercial phenothiazines even at 20 μ M concentration did not lead to any considerable change in SkHep1 cells in terms of cholesterol biosynthesis gene expression (**Figure 2B**). However, PTZ decreased the expression of all genes when compared to DMSO control and this effect was significant on SQLE gene only.

A



B

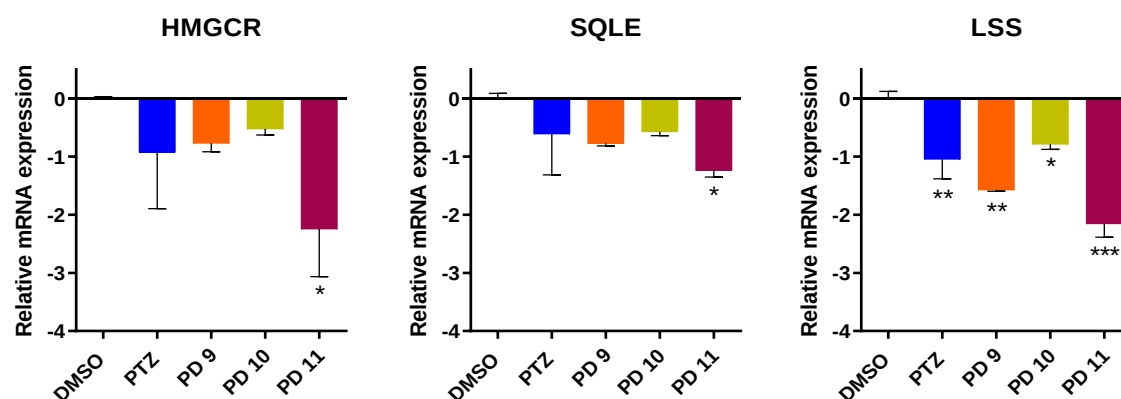
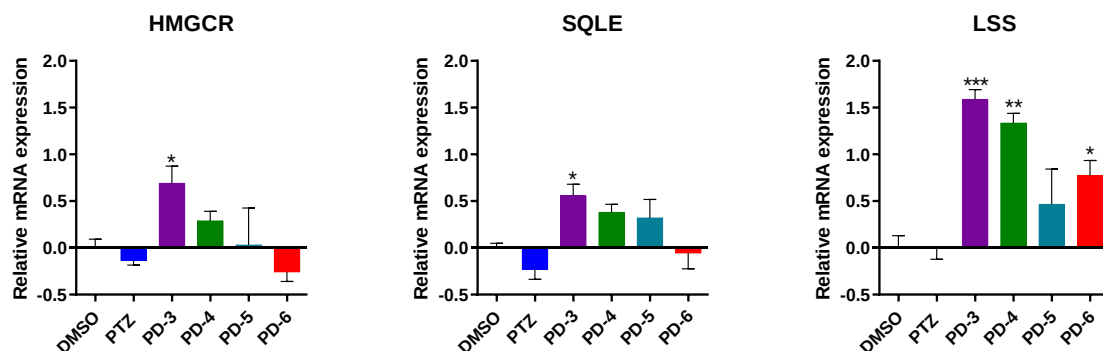


Figure 3. Changes in the expression of cholesterol biosynthesis genes after treatment with intermediary phenothiazines. Cholesterol biosynthesis gene mRNA levels were quantified by qPCR in **A**) Hep3B and **B**) SkHep1 cells after 24 hour-treatment. While y-axis showed relative expression to TPT1 reference gene, treatment groups including DMSO control and PTZ, PD-9, PD-10 and PD-11 at 10 μ M concentration were given in x-axis. Expression values of HMGCR, SQLE and LSS genes were in log2 scale. One-way ANOVA (Dunnett's test) was the statistical analysis method used to compare each treatment group with DMSO control (*: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$).

Contrary to known phenothiazines, it was seen that intermediary phenothiazines decreased the expression of HMGCR, SQLE and LSS genes in both HEP3B and SKHep1 cells (**Figure 3**). While the effect caused by PD-11 seemed to be important for all three genes in SkHep1, PD-9 and PD-10 were effective only on the expression of LSS gene.

A



B

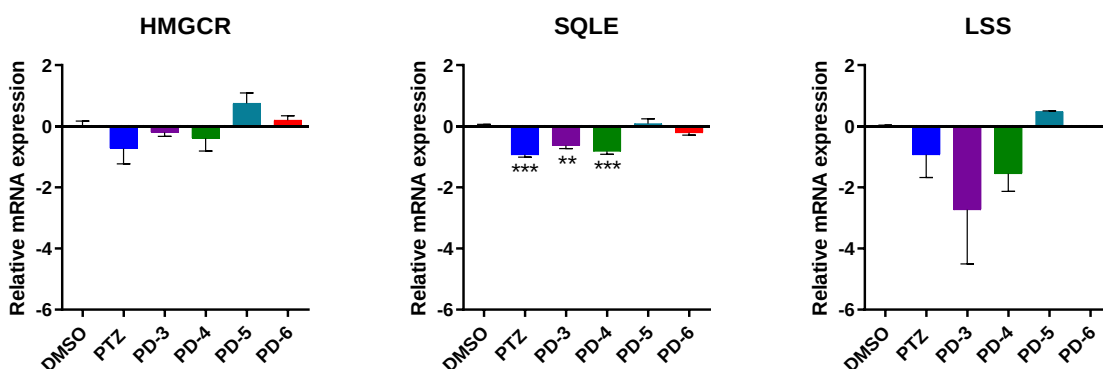


Figure 4. Changes in the expression of cholesterol biosynthesis genes after treatment with novel phenothiazines. Cholesterol biosynthesis gene mRNA levels were quantified by qPCR in **A)** Hep3B and **B)** SkHep1 cells after 24 hour-treatment. The qPCR experiments to investigate the effects of given novel phenothiazines were performed by Rafeed Said Tiryaki. While y-axis showed relative expression to TPT1 reference gene, treatment groups including DMSO control and PTZ, PD-3, PD-4, PD-5 and PD-6 at 20 μ M concentration were given in x-axis. Since commercial and novel phenothiazines belong to the same experimental setup, the same DMSO and PTZ groups were used for SkHep1 cells. Expression values of HMGCR, SQLE and LSS genes were in log2 scale. One-way ANOVA (Dunnett's test) was the statistical analysis method used to compare each treatment group with DMSO control (*: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$).

In general, novel phenothiazines caused an increase in the expression of HMGCR, SQLE and LSS genes in Hep3B cells (**Figure 4A**). While this change was significant for HMGCR and SQLE genes by PD-3, only, all novel derivatives except for PD-5 significantly enhanced the expression of LSS. In the case of SkHep1, a pattern for decreased gene expression was observed for all genes (**Figure 4B**). However, this effect was significant for SQLE expression since PD-3 and PD-4 effectively led to a significant decrease. Based on these results, PD-3

and PD-4 were distinguished among all novel derivatives as to affect cholesterol biosynthesis positively.

The investigation of gene expression changes caused by commercial, intermediary and novel phenothiazines showed that these drugs were able to regulate cholesterol biosynthesis pathway and the resulting effects varied depending on the drug and the cell line.

3.1.2 The Effects of TFP-SFB Combination on Huh7 Cells

In order to look into the changes occurring in cholesterol biosynthesis pathway with the combined treatment of TFP with SFB, the expressions of HMGCR, SQLE and LSS genes were studied after treating Huh7 cells with different concentrations of TFP only, SFB only or their combination for 48 hours. Here, the synergistic action of drugs, when combined, on Huh7 cells (Murat Yaman, PhD Thesis, 2020) was found promising and led us to determine TFP and SFB doses to be applied.

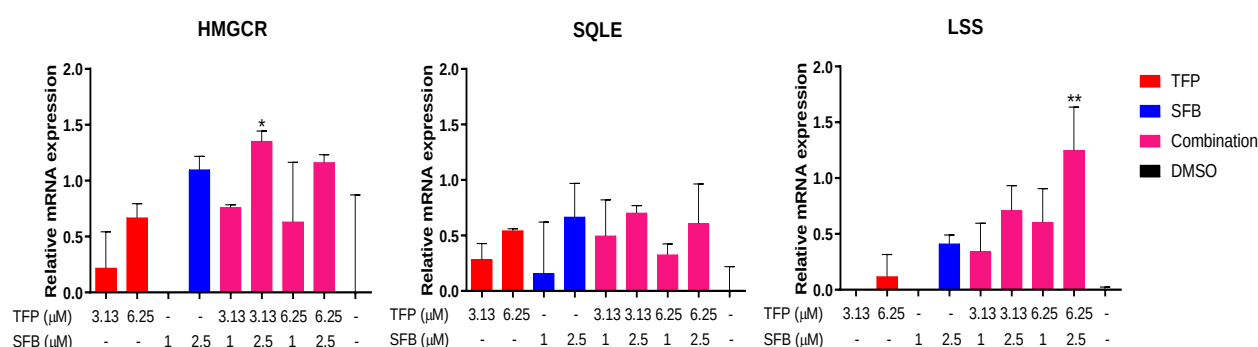


Figure 5. Gene expression profiles in Huh7 cells after treatments with TFP alone, SFB alone and TFP+SFB combined for 48 hours. While y-axis showed relative expression to TPT1 reference gene, treatment groups including DMSO control, TFP, SFB and their combinations were given in x-axis. Expression values of HMGCR, SQLE and LSS genes were given in log2 scale. One-way ANOVA (Dunnett's test) was the statistical analysis method used to compare each treatment group with DMSO control (*: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$).

In Huh7 cells, all treatment groups seemed to increase the expression of HMGCR, SQLE and LSS genes, but not always significantly (**Figure 5**). Treatments with TFP alone were not found effective for HMGCR gene, but the combinations at a given dose with increasing doses of SFB seemed to increase the expression of this gene; and TFP 3.13 μM - SFB 2.5 μM combination was significantly effective on HMGCR gene compared to DMSO control. Apart from this, none of the treatment groups caused a significant increase in SQLE gene. The increase caused by TFP 6.25 μM - SFB 2.5 μM combination was significant for LSS gene.

In general, the effects of higher concentrations of SFB and its combination with TFP at high doses were found to support the ability of phenothiazines to regulate cholesterol biosynthesis pathway. However, this conclusion was not applicable for the applications of single TFP doses.

3.2 Investigation of the Change in Intracellular Cholesterol Levels

Previous studies in our lab have demonstrated that single derivative treatments efficiently decreased Hep3B cell viability and TFP-SFB combinations acted synergistically at certain doses (Murat Yaman, PhD Thesis, 2020). These findings brought Hep3B cell line forward to be further evaluated. For a detailed examination of the effects of phenothiazines and TFP-SFB combinations on the regulation of cholesterol, intracellular cholesterol levels in Hep3B cells were determined after treating them with different concentrations of TFP, SFB and their combinations.

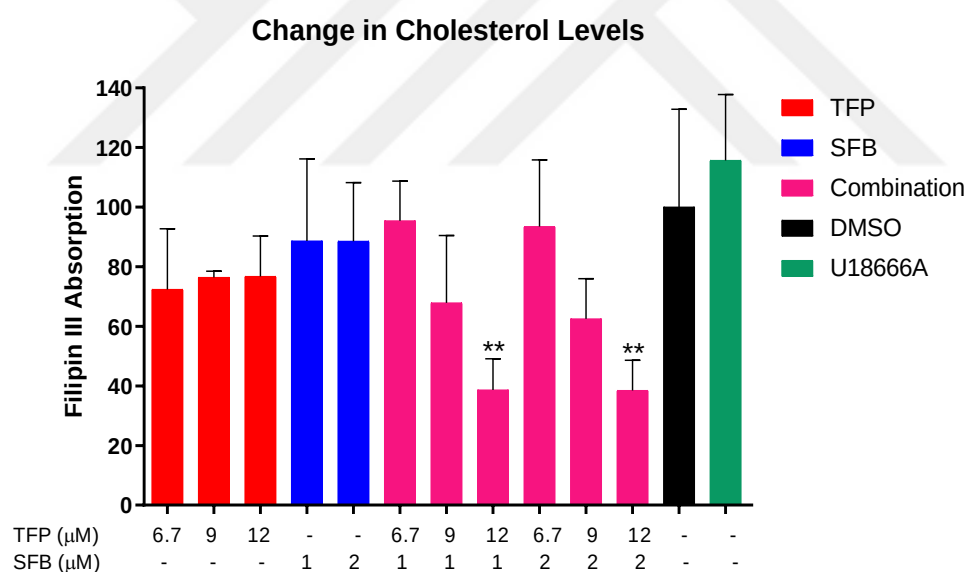


Figure 6. Cholesterol levels in Hep3B cells after exposure to TFP, SFB and their combination for 48 hours. While x-axis showed 0.1% DMSO control, the concentrations of the drugs and U18666A (1.25 μM) as the positive control, Filipin III absorption (ex: 355 nm, em: 460 nm) values were given in y-axis. One-way ANOVA (Dunnett's test) was used as the statistical method to compare each treatment group with DMSO control. (*: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$)

In **Figure 6**, changes in the absorption of Filipin III, a free cholesterol-binding fluorescent compound [181], arised from cholesterol binding and indicated higher cholesterol levels in Hep3B cells after treatment with TFP, SFB and their combinations. In comparison to DMSO control, all concentrations of TFP resulted in a decrease in cholesterol levels similarly. The

combination of 6.7 μM of TFP with both doses of SFB gave rise to more cholesterol as compared to either drug alone. However, combination groups with increasing concentrations of TFP gradually decreased cholesterol levels and this effect was significant for TFP 12 μM -SFB 1 μM and TFP 12 μM -SFB 2 μM exposures. Additionally, raising SFB concentration from 1 μM to 2 μM in the combination groups did not seem to make a difference.

As given in **Figure 7**, the representative images for Filipin III staining showing the change in cell numbers of these two groups were provided together with those of DMSO control and U18666A positive control. Cholesterol seemed to be present as droplets within Hep3B cells of positive control group although resolution was low (**Figure 7B**). The combination of TFP 12 μM with SFB 1 μM resulted in accumulated cholesterol. For TFP 12 μM SFB 2 μM , it was hard to draw a conclusion based on the image since the presence and accordingly fluorescence of the cells were much less comparatively.

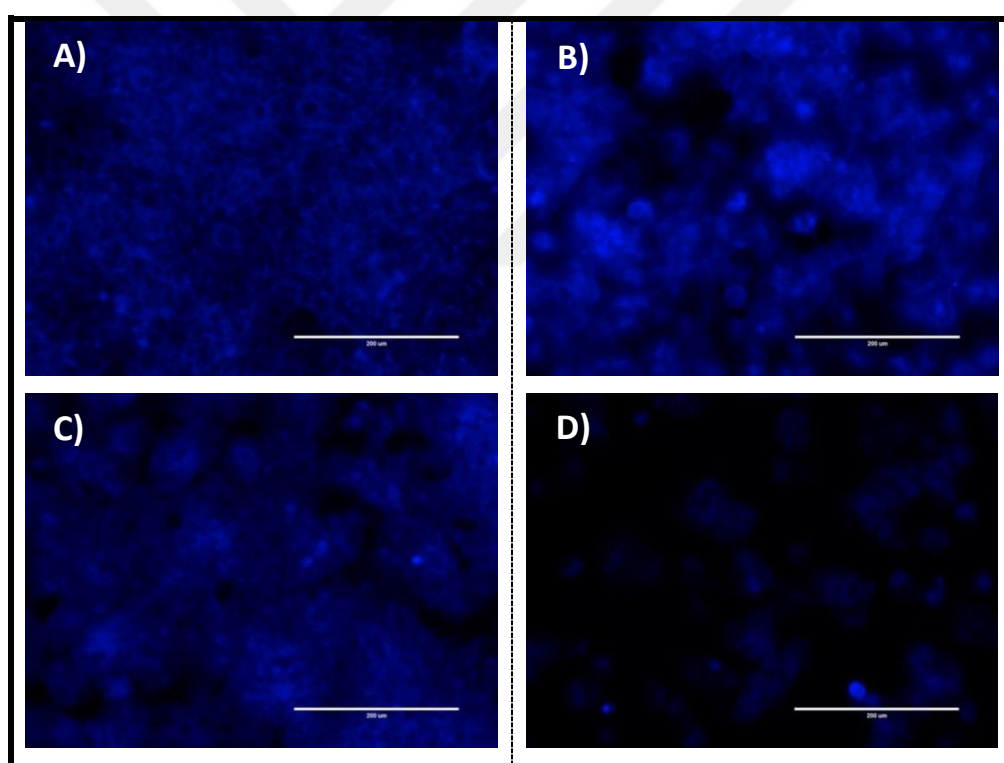


Figure 7. Representative images of 48 hour-treated Filipin III-stained Hep3B cells. A) DMSO control, **B)** U18666A positive control, **C)** TFP 12 μM -SFB 1 μM combination and **D)** TFP 12 μM -SFB 2 μM combination. The images were obtained with 20X objective. Scale bar: 200 μM .

Depending on these readings in **Figure 6**, it could have been concluded that high doses of TFP when combined with 1 μM or 2 μM of SFB were capable of lowering cholesterol levels in Hep3B cells. However, the analyses needed to be validated by normalizing cholesterol levels

to the cell number since **Figure 7** demonstrated that cell viability was significantly low in the combination treatment group.

In order to ensure that Filipin III absorbance was independent of the number of the cells for each treatment group, compound exposures were performed simultaneously for cholesterol detection and MTT cell viability assays. For normalization and further analysis, the values obtained for Filipin III absorption were divided into cell viability values.

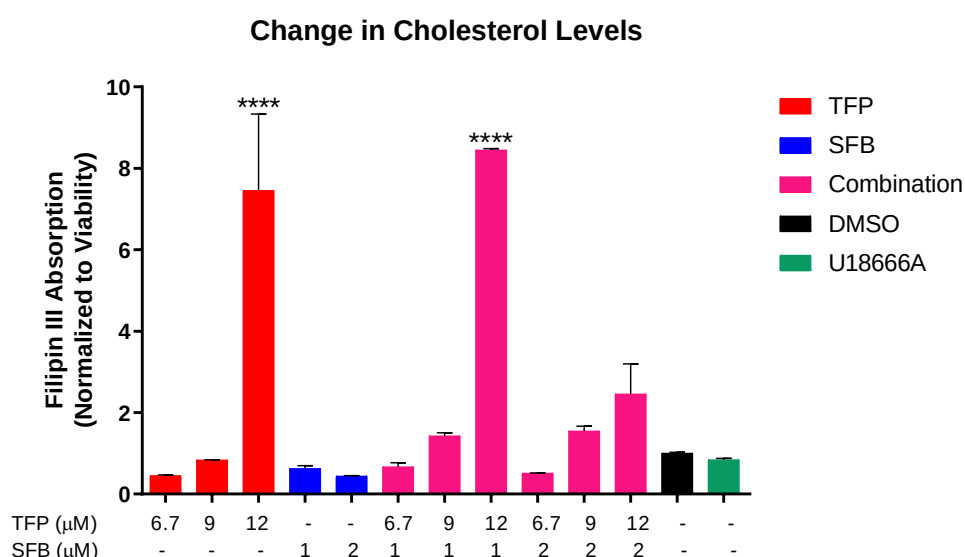


Figure 8. Normalization of cholesterol levels to cell viability results in Hep3B cells after exposure to TFP, SFB and their combination for 48 hours. While x-axis showed 0.1% DMSO control, the concentrations of the drugs and U18666A (1.25 μM) as the positive control, normalized Filipin III absorption (ex: 355 nm, em: 460 nm) values were given in y-axis. One-way ANOVA (Dunnett's test) was used as the statistical method to compare each treatment group with DMSO control. (*: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$)

The normalization of cholesterol levels to cell viability showed a trend that was the exact opposite of what was obtained with increasing concentrations of TFP, the highest dose and the combination of TFP 12 μM - SFB 1 μM being highly significant (**Figure 8**). Accordingly, the normalization demonstrated a significant gradual increase in cholesterol levels with increasing concentrations of TFP. Based on these results, inclusion of 1 μM of SFB to the combinations did not seem to cause a considerable difference in comparison to TFP only groups. Moreover, that much of an increase was not observed with TFP 12 μM - SFB 2 μM combination, indicating that 2 μM of SFB could act antagonistically to reduce the increase of cellular cholesterol caused by the high dose of TFP.

3.3 Cholesterol Pathway Representations Utilizing RNA-seq Analysis

To be able to determine the cholesterol-related genes that were most affected by 48 hours of TFP at a dose of 12 μ M and combination treatments in Hep3B cells, RNA-seq analysis performed by Kubra Calisir (please see methods) was used to generate pathway representations which enabled to visually compare log fold changes observed in expression levels.



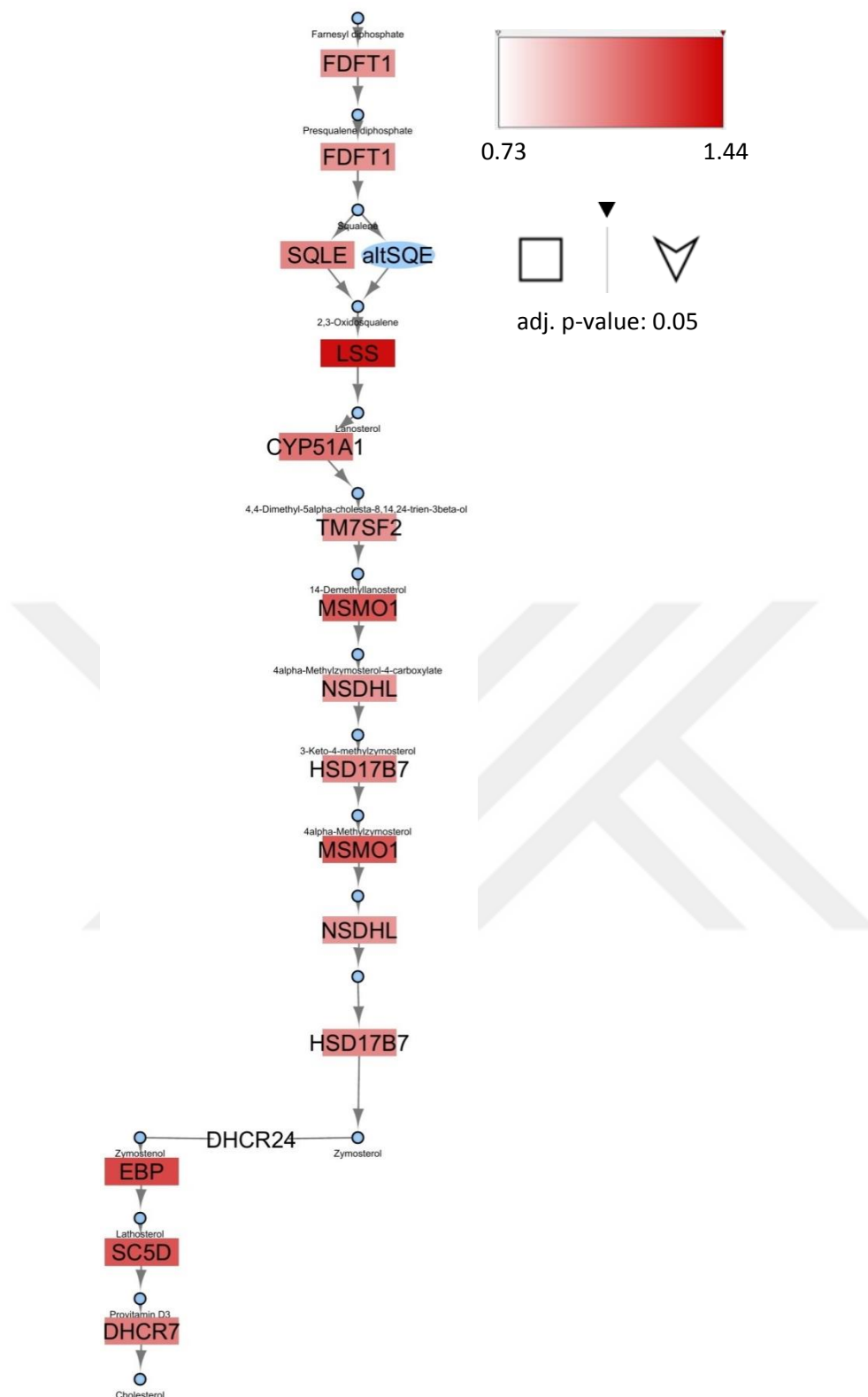


Figure 9. Color-coded representation of logFC values for cholesterol biosynthesis genes based on the comparison between TFP 12 μ M and DMSO control in 48 hour-treated Hep3B cells. The pathway was created via KEGGscape app in Cytoscape (v3.8.2), using Kegg Pathway: Steroid Biosynthesis (hsa00100) as a template. The color scale was generated starting from the minimum LogFC to maximum for the genes given. 0.05 was set as the threshold for adj. p-value, genes with lower than threshold values were shown as rectangular shapes and those with higher values were shown as v-shapes.

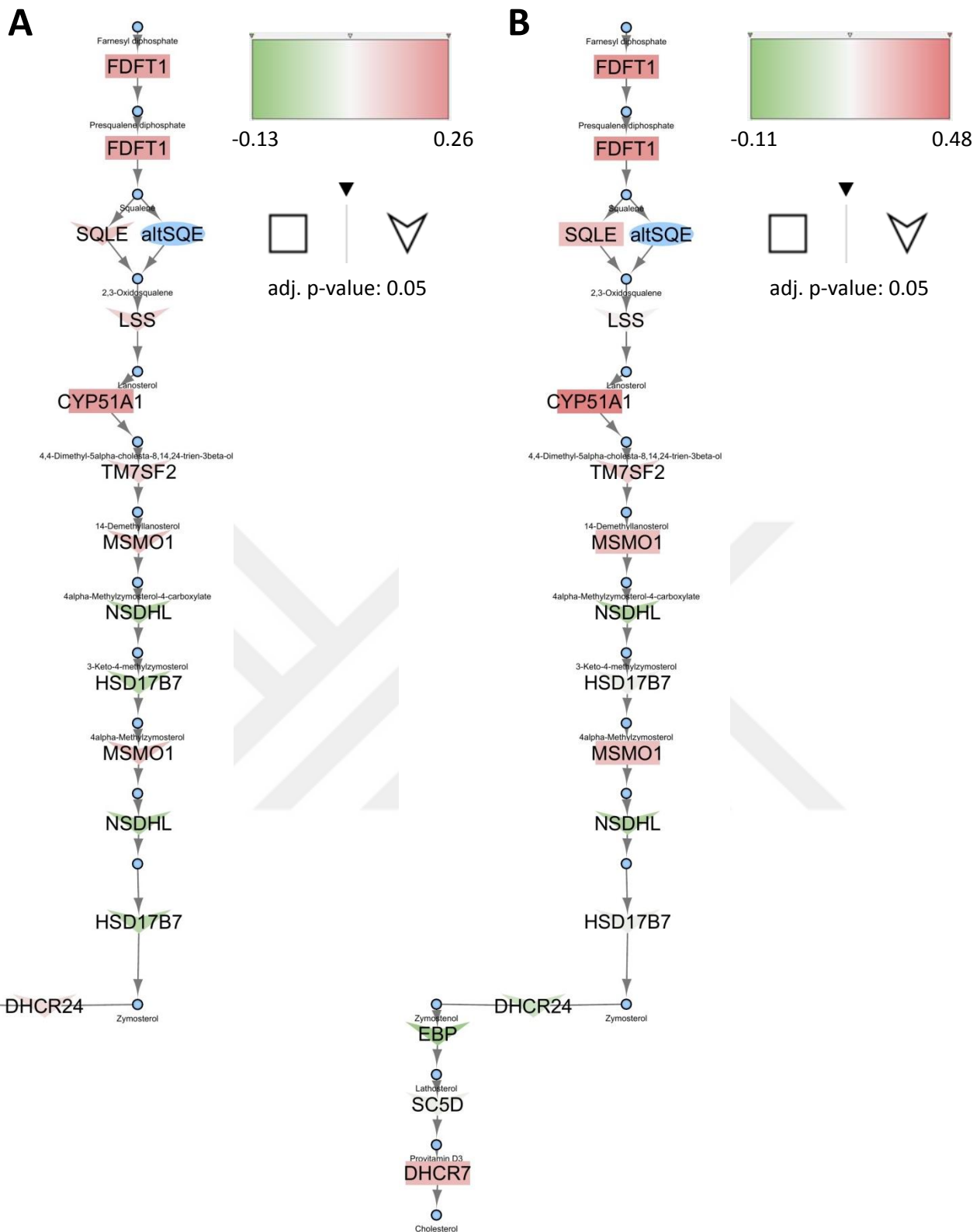


Figure 10. Color-coded representation of logFC values for cholesterol biosynthesis genes based on the comparison of A) TFP 12 μ M - SFB 1 μ M and B) TFP 12 μ M - SFB 2 μ M versus TFP 12 μ M in 48 hour-treated Hep3B cells. The pathway was created via KEGGscape app in Cytoscape (v3.8.2), using Kegg Pathway: Steroid Biosynthesis (hsa00100) as a template. The color scale was generated starting from the minimum LogFC to maximum for the genes given. 0.05 was set as the threshold for adj. p-value, genes with lower than threshold values were shown as rectangular shapes and those with higher values were shown as v-shapes.

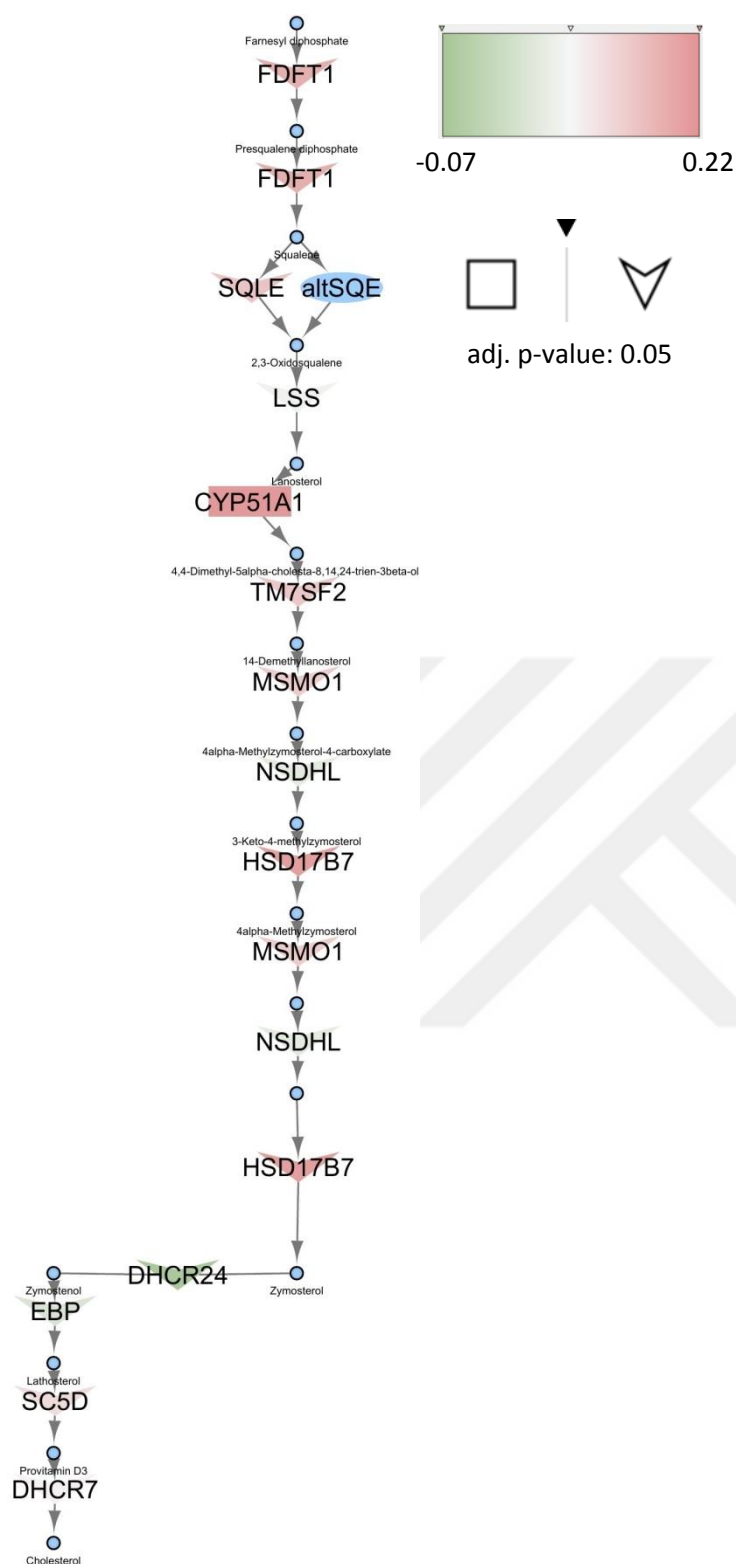


Figure 11. Color-coded representation of logFC values for cholesterol biosynthesis genes based on the comparison between TFP 12 μ M - SFB 2 μ M and TFP 12 μ M - SFB 1 μ M in 48 hour-treated Hep3B cells. The pathway was created via KEGGscape app in Cytoscape (v3.8.2), using Kegg Pathway: Steroid Biosynthesis (hsa00100) as a template. The color scale was generated starting from the minimum LogFC to maximum for the genes given. 0.05 was set as the threshold for adj. p-value, genes with lower than threshold values were shown as rectangular shapes and those with higher values were shown as v-shapes.

As seen in **Figure 9**, cholesterol biosynthesis genes were all upregulated by TFP 12 μ M in comparison to DMSO control, the LSS gene having the highest logFC (1.4391) (**Appendix B Table 1**). When combination groups were compared with TFP 12 μ M, it was seen that the addition of 1 μ M of SFB favored the upregulation of especially FDFT1, CYP51A1 and DHCR7 genes significantly (**Figure 10A**). Although not statistically important, it slightly reversed the effect coming from TFP 12 μ M for NSDHL, HSD17B7 and EBP genes. The combination of TFP 12 μ M with SFB 2 μ M caused minor changes when compared to TFP 12 μ M (**Figure 10B**). As for the comparison of the two combination groups, there seemed to be no significant difference except for CYP51A1 gene. Increasing SFB dose from 1 μ M to 2 did not result in a huge difference for the remaining members of the pathway (**Figure 11**).



Table 10. LogFC and adj. p-values of cholesterol-related genes that were significantly affected in Hep3B cells. The genes with an adj. p-value of 0.05 or lower for each comparison were filtered. (t1_control: TFP 12 μ M vs control, ts1_t1: TFP 12 μ M - SFB 1 μ M vs TFP 12 μ M, ts2_t1: TFP 12 μ M - SFB 2 μ M vs TFP 12 μ M, ts2_ts1: TFP 12 μ M - SFB 2 μ M vs TFP 12 μ M - SFB 1 μ M)

Gene	LogFC (t1_control)	adj. p-value (t1_control)	LogFC (ts1_t1)	adj. p-value (ts1_t1)	LogFC (ts2_t1)	adj. p-value (ts2_t1)	LogFC (ts2_ts1)	adj. p-value (ts2_ts1)
CYP51A1	1.14458	0.00000	0.25533	0.01050	0.47540	0.00023	0.22007	0.04829
FASN	0.80805	0.00000	0.62866	0.00006	0.39867	0.00102	-0.22999	0.04458
HMGCR	1.43171	0.00000	0.29781	0.00798	0.57625	0.00012	0.27844	0.02674
HMGCS1	1.86634	0.00000	0.21908	0.02212	0.53659	0.00009	0.31751	0.00688
SREBF2	0.54184	0.00004	0.32315	0.00240	0.61386	0.00003	0.29071	0.01016

In **Table 10**, the genes whose expression increased or decreased (logFC) that were significant for each and every comparison, were picked out to display. Based on higher logFC changes caused by it, TFP 12 μ M alone seemed to be an effective inducer of cholesterol biosynthesis, while combining TFP with the either dose of SFB was as impactful as single TFP treatment. In the appendix, I have included **Appendix B Table 1** for all of the a) cholesterol biosynthesis and b) cholesterol metabolism genes shown in the pathways above for a better understanding of which genes or clusters of genes were significantly modulated.

3.4 Effects of Intermediary and Novel Derivatives on Cancer

Pathway Protein Levels

In order to investigate anticancer characteristics of synthesized phenothiazine derivatives, protein levels of proliferation and apoptosis markers were studied (**Figure 16**).

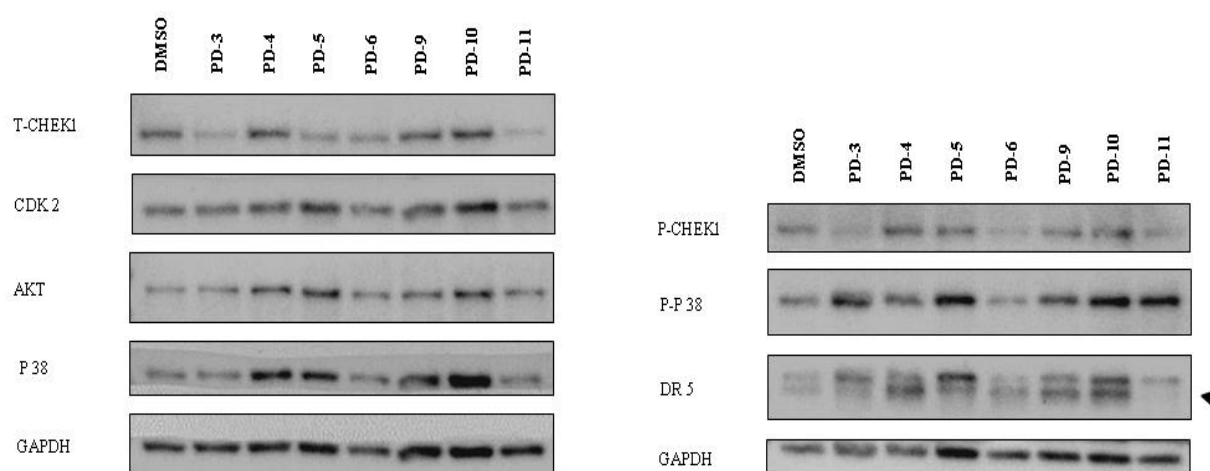


Figure 16. Changes in protein levels after exposure of Hep3B cells with 10 μ M of novel and intermediary phenothiazines for 24 hours. The bands belonging to T-CHEK1, CDK2, AKT, P38, p-CHEK1, p-P38, DR5 and GAPDH proteins were given.

24-hour treatment with 10 μ M of all derivatives was found to decrease total CHEK1 levels to some degree in Hep3B cells, while PD-11 having the most impact (**Figure 17**). This overall decrease in CHEK1 could be indicative of cell cycle regulation via CHEK1 by the derivatives yet for obtaining significance the experiment should be repeated. When it comes to phosphorylated form of CHEK1, it was observed that there was a decrease in general with the exception of PD-4 and PD-10 (**Figure 18**).

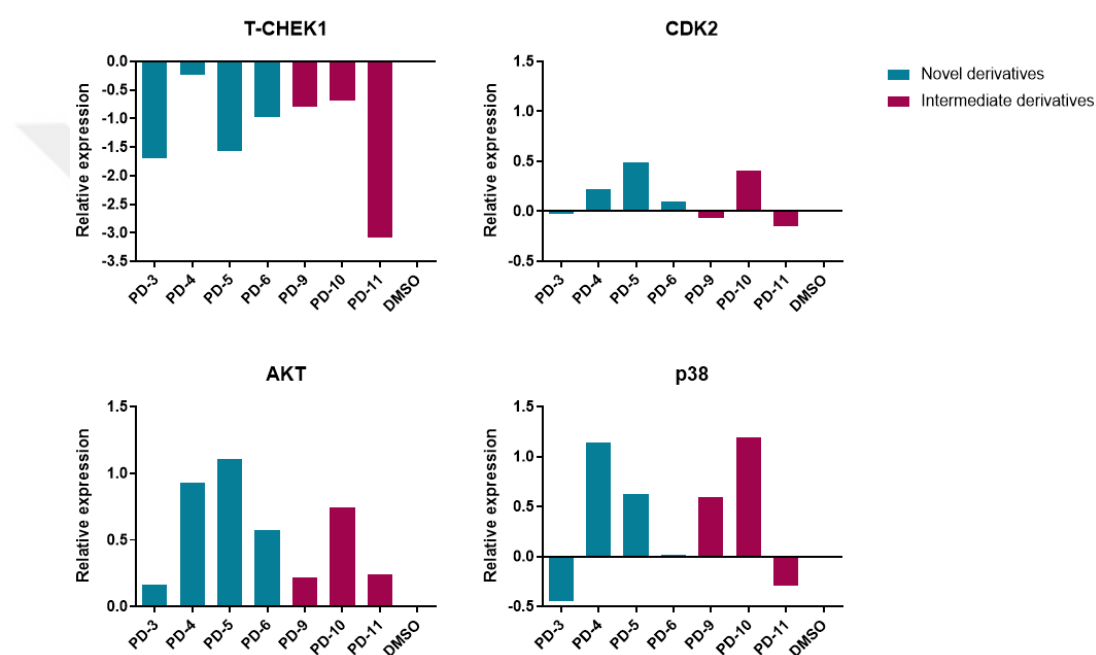


Figure 17. Quantification of T-CHEK1, CDK2, AKT and P38 levels as a result of 24-hour exposure with 10 μ M of novel and intermediary phenothiazines in Hep3B cells. Images were quantified based on the band intensity using ImageJ. The analysis was performed first by normalization to the reference gene GAPDH and then DMSO control. The values were provided at log2 scale.

The level of CDK2, another protein involved in cell cycle, was increased by PD-4, PD-5 and PD-10, however the change was relatively low (0.5). Moreover, AKT exhibited an increase with all derivatives. High levels of P38 were also obtained in Hep3B cells after treatment with PD-4, PD-5, PD-9 and PD-10 (**Figure 17**).

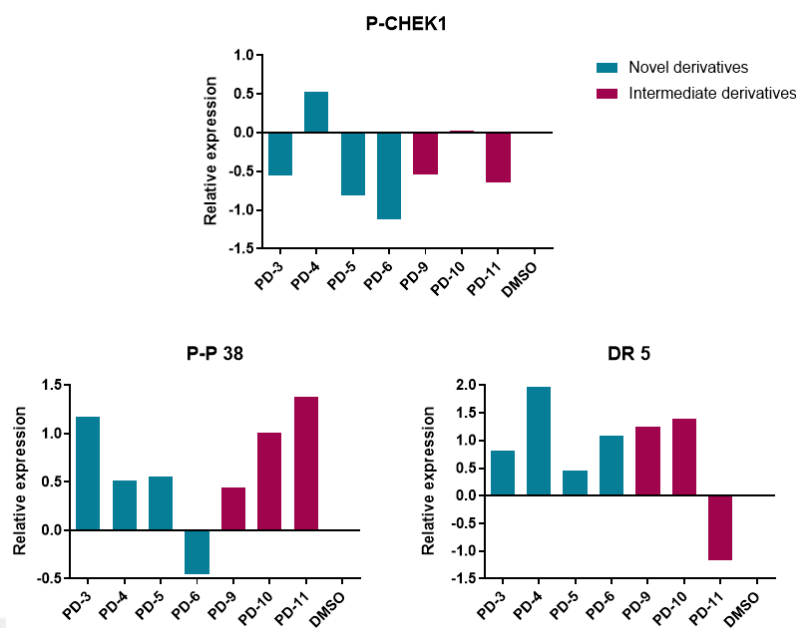


Figure 18. Quantification of p-CHEK1, p-P38 and DR5 levels as a result of 24-hour exposure with 10 μ M of novel and intermediary phenothiazines in Hep3B cells. Images were quantified based on the band intensity using ImageJ. The analysis was performed first by normalization to the reference gene GAPDH and then DMSO control. The values were provided at log2 scale.

The phosphorylated form of P38 also showed enhanced expression, except for PD-6. Finally, all derivatives tested, but not PD-11, led to increased DR5 levels (**Figure 18**). All these findings suggested that these derivatives might have the ability to modulate cell cycle, growth and apoptosis in Hep3B cells.

3.4 In Vivo Experiments

3.4.1 Microscopic Examination on Casper Embryos

In order to predict the safe range of drug concentrations that can be applied to zebrafish embryos, 2 dpf casper zebrafish embryos were exposed to gradually increasing concentrations of TFP, SFB and their combination.

Trifluoperazine

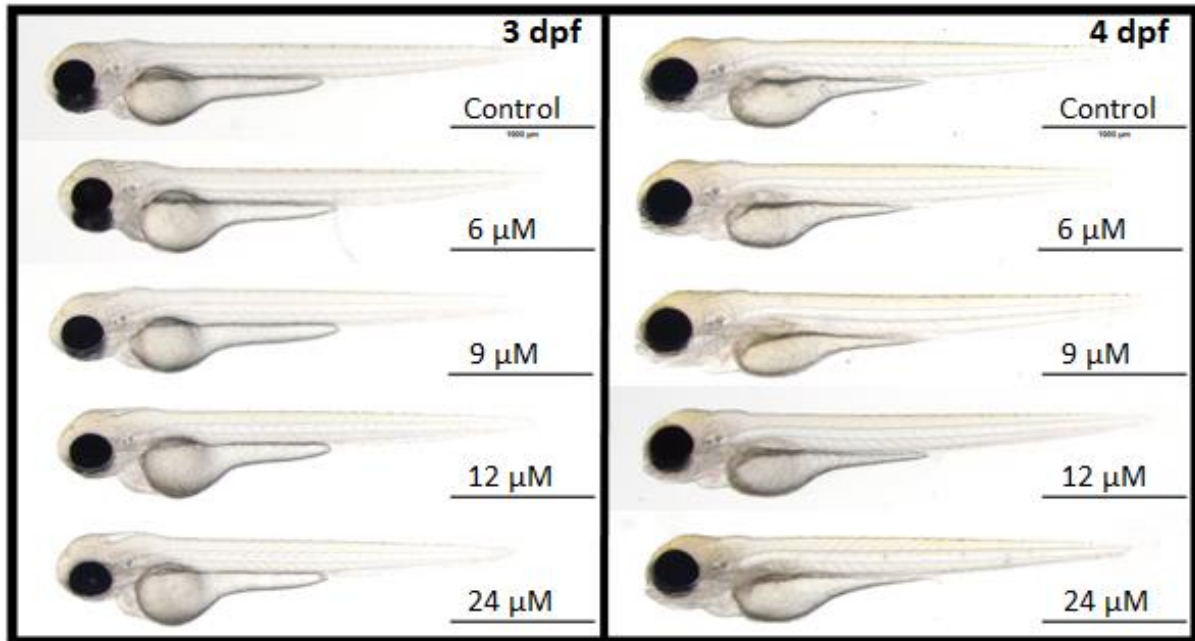


Figure 19. Representative images of 3 and 4 dpf casper larvae after 24 and 48 hours of exposure to TFP starting from 2 dpf. Scale bar: 1000 μ M.

In **Figure 19**, the morphologies of casper larvae after being exposed to TFP for a total of 48 hours were provided. Even at high concentrations of TFP no major changes in the appearance of the larvae were detected, apart from minor heart edema in some larvae.

Sorafenib

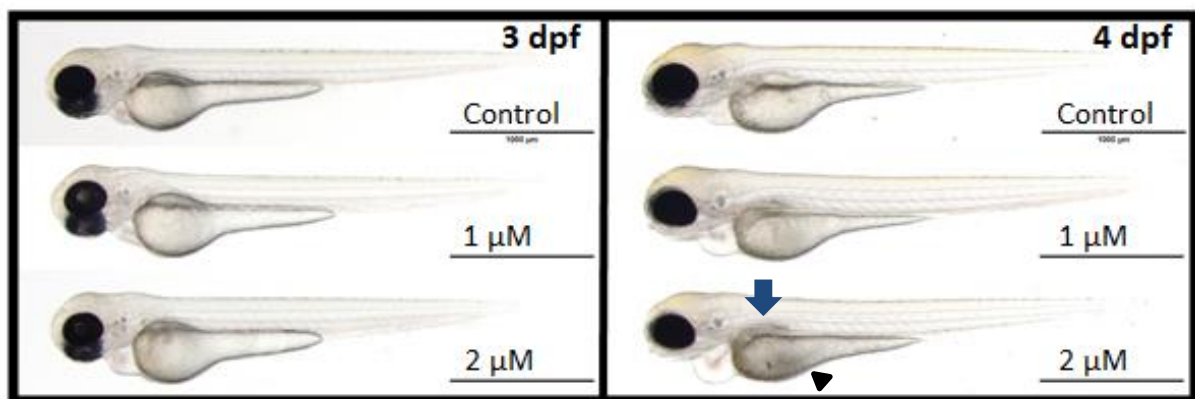


Figure 20. Representative images of 3 and 4 dpf casper larvae after 24 and 48 hours of exposure to SFB starting from 2 dpf. The black arrow indicates necrotic appearance in yolk and blue arrow indicates the developing gut region, also showing necrotic phenotype. Scale bar: 1000 μ M.

On the other hand, even 1 μM of SFB resulted in heart edema and this effect became highly enhanced at 4 dpf (**Figure 20**). A darkening was also observed in the yolk and the internal organs (**Figure 20**, blue arrow), which may indicate beginning of necrotic tissues [182] in the larvae treated with 2 μM of SFB for 48 hours, pointing out the toxic effect of SFB on zebrafish larvae.

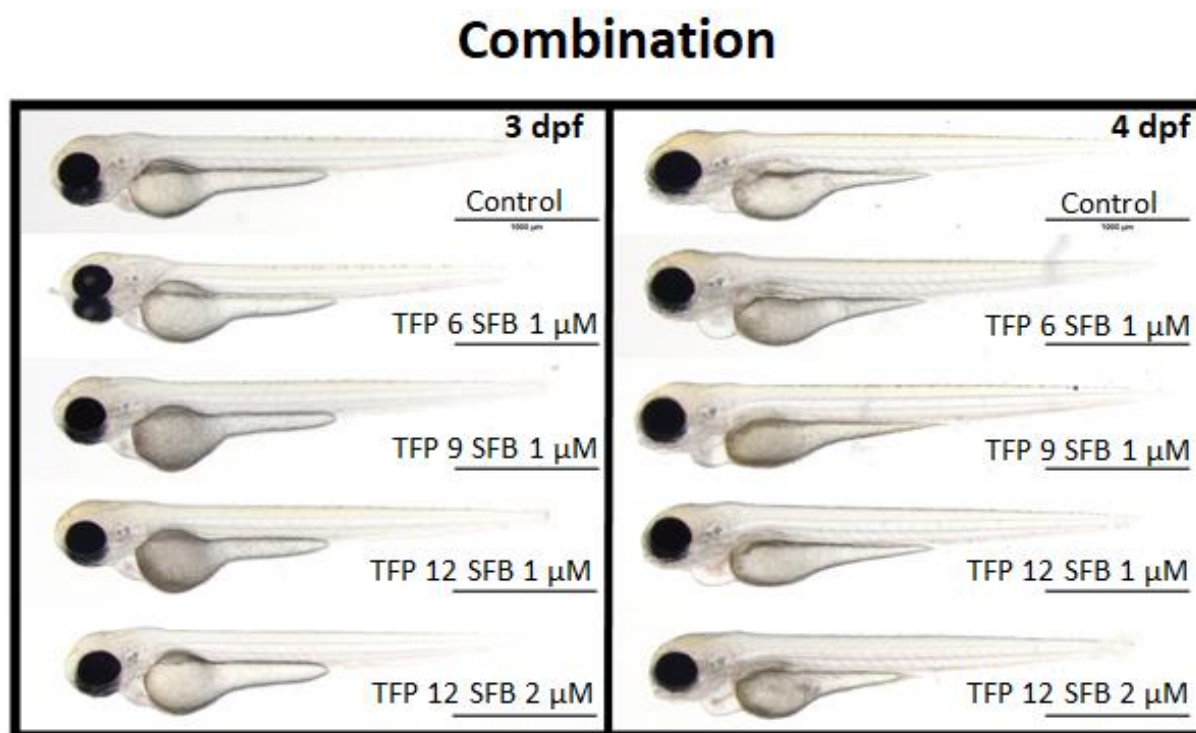


Figure 21. Representative images of 3 and 4 dpf casper larvae after 24 and 48 hours of exposure to TFP+SFB combination starting from 2 dpf. Scale bar: 1000 μM .

When the two drugs combined, the morphological abnormalities that were observed with either drug alone were apparent on the larvae at both 3 and 4 dpf (**Figure 21**). Most of the toxicity seemed to be coming from SFB indicating that treating injected embryos with high concentrations of SFB alone or in combination with TFP could be very harmful and prevent to reveal promising changes due toxic effects.

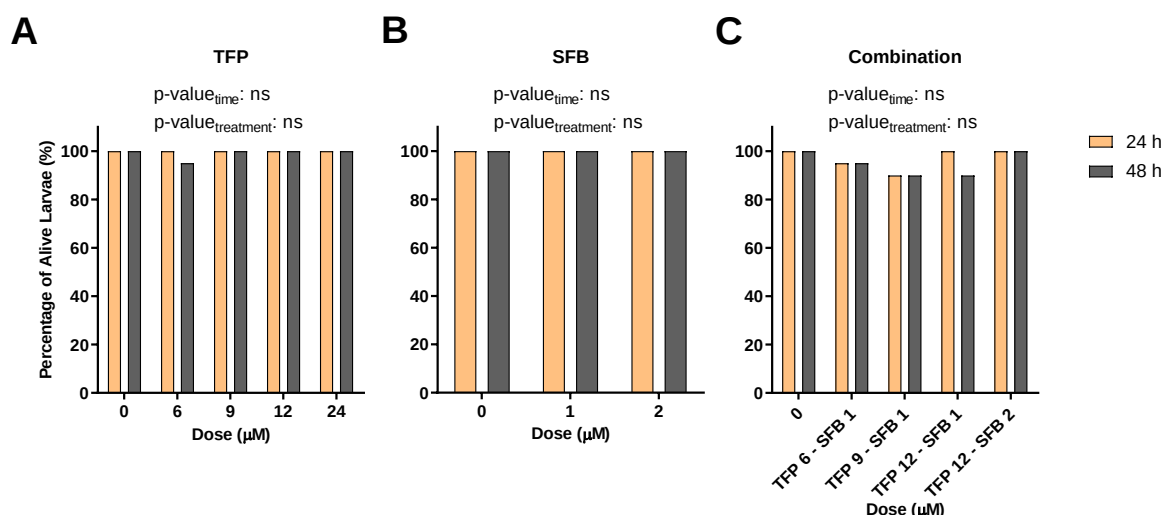


Figure 22. The mortality of casper larvae as a result of treatment with A) TFP, B) SFB and C) TFP + SFB for 24 and 48 hours starting from 2 dpf. Two-way ANOVA was applied as the statistical method (*: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$).

The mortality of the larvae after 24 and 48 hours of exposure to either drug alone or to their combination was not different when compared to DMSO control (**Figure 22**). Although SFB showed highly toxic effects, the doses applied did not cause lethality.

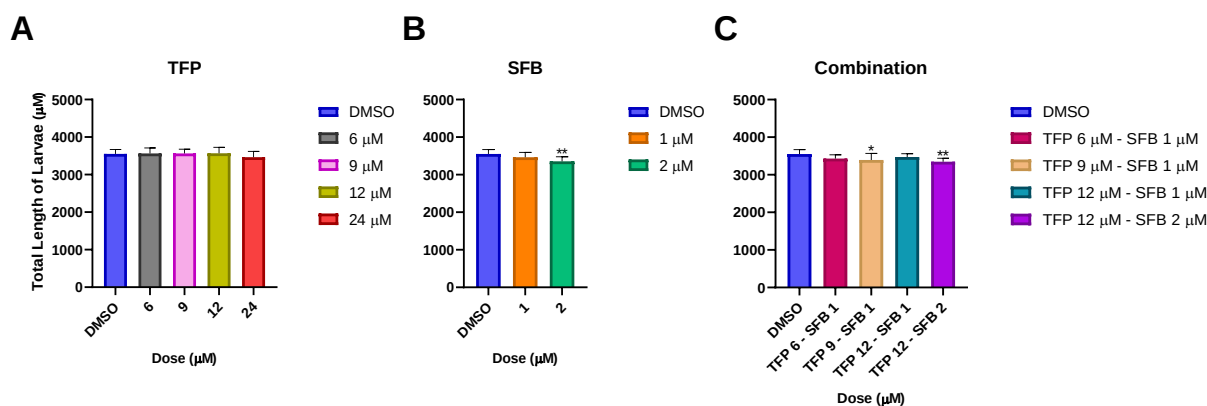


Figure 23. The total length of 4 dpf casper larvae as a result of exposure to A) TFP, B) SFB and C) TFP + SFB for 48 hours starting from 2 dpf. One-way ANOVA/Dunnett's test was applied as the statistical method for analysis (*: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$).

As another aspect of morphological effects of TFP, SFB and their combination, the total length differences of the larvae were compared (**Figure 23**). TFP did not cause any change in the total length of the larvae, although there was a significant but mild decrease caused by 2 μM of SFB compared to DMSO. As for combination, TFP 9 μM - SFB 1 μM decreased the size of the larvae after 48 hours of exposure significantly, while TFP 12 μM - SFB 2 μM even showed a more important impact.

3.4.2 Xenograft Experiments on Casper Embryos

3.4.2.1 Low dose TFP, SFB and TFP+SFB

For the xenograft experiment, casper embryos injected with Hep3B cells were exposed to 2.5 μM of TFP, 0.25 μM of SFB and the combination of the two drugs (**Figure 24**).

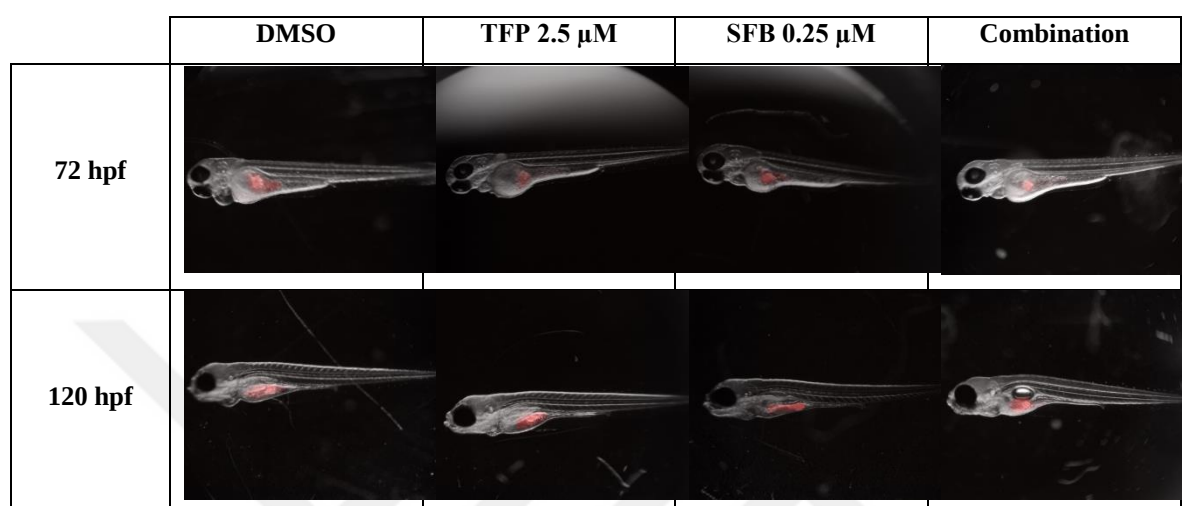


Figure 24. The xenograft experiment performed on casper embryos. 72 hpf and 120 hpf images of the casper fish after being injected with Hep3B cells are given. The embryos were exposed to 2.5 μM of TFP, 0.25 μM of SFB and their combination in 72 hpf. The images shown were obtained by merging brightfield and fluorescent images.

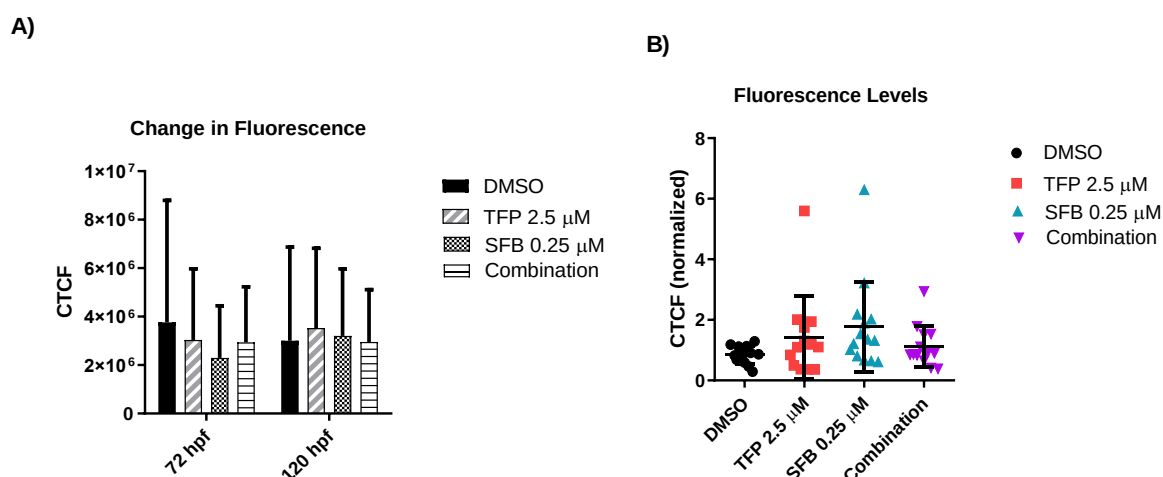


Figure 25. Fluorescence changes in Hep3B-injected embryos after being exposed to TFP, SFB or their combination. **A)** The fluorescence levels are given for each treatment group in 72 and 120 hpf. CTCF was used to measure the quantity of the cells with fluorescence. Two-way ANOVA (Sidak's test) was chosen as the statistical method. **B)** The fluorescence levels in 120 hpf were normalized to the levels obtained in 72 hpf. Statistical analysis was performed by applying one-way ANOVA (Dunnett's test).

When the CTCF values in 120 hpf were compared to the ones in 72 hpf, there was no significant difference between groups in terms of their mean CTCF (**Figure 25A**). Therefore, the changes caused by any of the treatment groups did not reduce the tumor size at these low doses of drugs alone or in combination. This conclusion was also supported as a result of the normalization of the fluorescence obtained in 120 hpf to in 72 hpf (**Figure 25B**). For these reasons, it can be said that TFP at 2.5 μM concentration and SFB at 0.25 μM concentration, either alone or in combination, were not effective enough to cause a distinct change in tumor volume as measured by fluorescence emitted from liver cells. This demonstrated that higher concentration should be tested.

3.4.3 Microscopic Examination on Wild Type Embryos

In addition to casper embryos, the nontoxic concentrations to be applied upon xenografting were also tested on WT zebrafish embryos. By keeping the embryos at 34°C and extending the exposure period from 2 dpf until 5 dpf, the conditions for xenograft were mimicked. Extension of the study to 5 dpf also led to identification of toxic effects beyond 4 dpf where no lethality was observed in casper embryos.

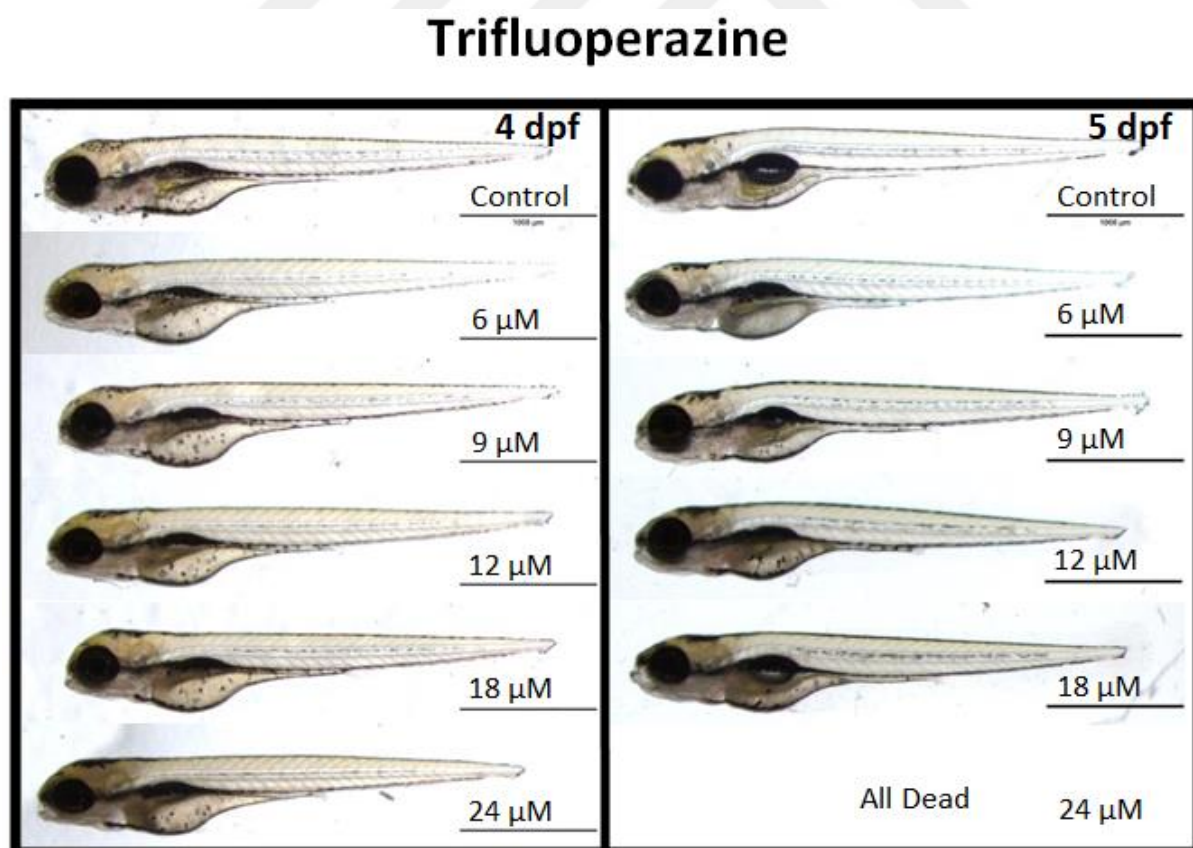


Figure 26. Representative images of 4 and 5 dpf WT larvae after 48 and 72 hours of exposure to different concentrations of TFP starting from 2 dpf. Scale bar: 1000 μM .

As seen in **Figure 26**, TFP caused mortality only at the highest dose after 72 hours of exposure. The yolk and the head of larvae darkened with increasing doses at both 4 and 5 dpf, pointing out potential necrosis and further confirming toxicity at high TFP concentrations.

Sorafenib

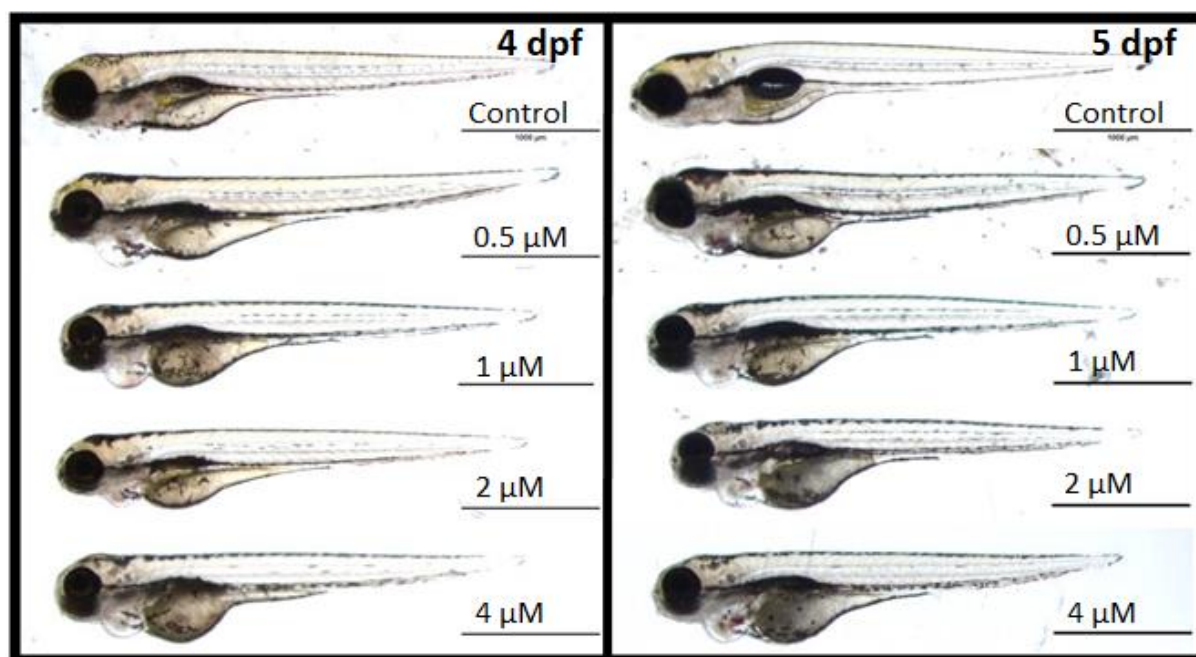


Figure 27. Representative images of 4 and 5 dpf WT larvae after 48 and 72 hours of exposure to different concentrations of SFB starting from 2 dpf. Scale bar: 1000 μ M.

On the other hand, SFB resulted in a severe heart edema even at low concentrations at both 4 dpf and 5 dpf (**Figure 27**). There was a significant decrease in the length of larvae with increasing concentrations (**Figure 29**). Also the head of SFB-treated larvae seemed to be smaller in comparison to control, demonstrating high toxicity of SFB in vivo.

Combination

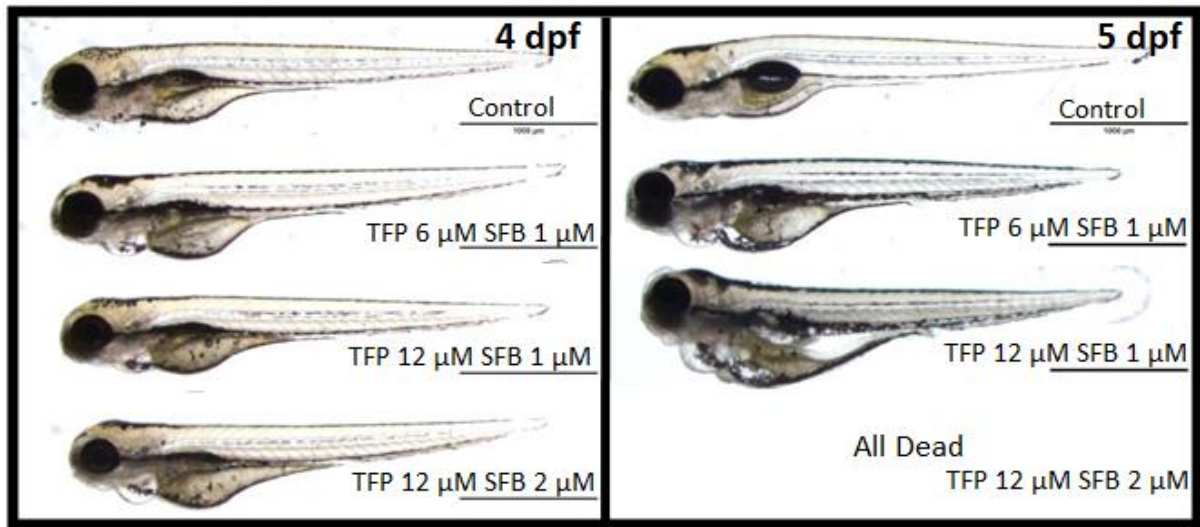


Figure 28. Representative images of 4 and 5 dpf WT larvae after 48 and 72 hours of exposure to different concentrations of TFP + SFB combination starting from 2 dpf. Scale bar: 1000 µM.

The toxic effect of TFP and SFB became enhanced when they were combined, especially at 5 dpf as given in **Figure 28**. The combination of 12 µM of TFP and 2 µM of SFB resulted in the death of all larvae treated at 5 dpf. These observations overall gave an idea about which doses can be applied to the embryos after transplantation of cancer cells.

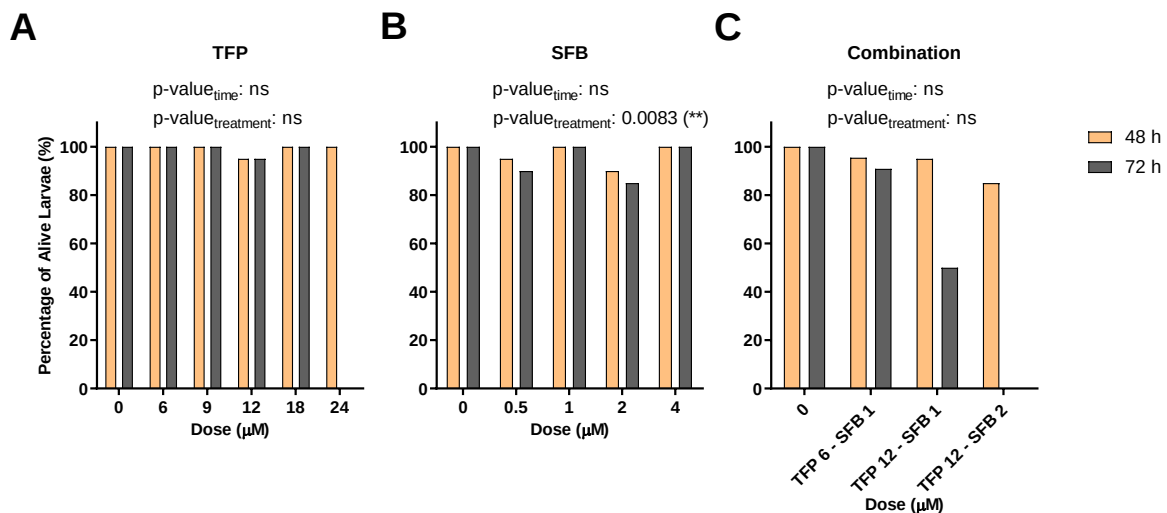


Figure 29. The mortality of WT larvae as a result of treatment with A) TFP, B) SFB and C) TFP + SFB for 48 and 72 hours starting from 2 dpf. Two-way ANOVA was applied as the statistical method (*: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$).

As seen in **Figure 29**, TFP did not lead to any lethal effects, while the differences in the dose of SFB had a significant impact on the percentage of alive larvae after both 48 and 72 hours

of treatment, demonstrating the increased lethality and defects when TFP was combined with SFB; and higher dose of SFB when together with TFP results in lethality.

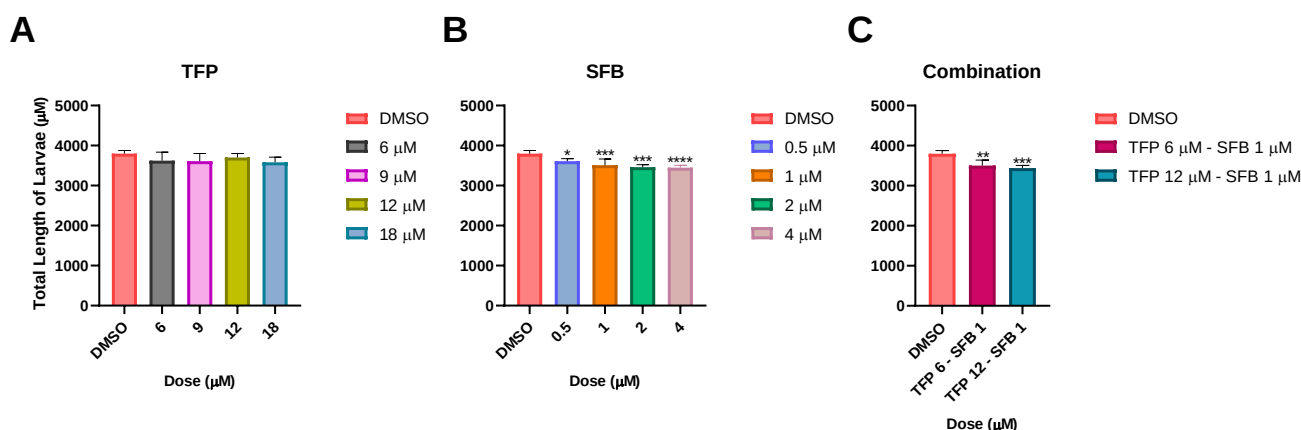


Figure 30. The total length of 5 dpf WT larvae as a result of exposure to A) TFP, B) SFB and C) TFP + SFB for 72 hours starting from 2 dpf. One-way ANOVA/Dunnett's test was applied as the statistical method for analysis (*: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$).

When the total length differences caused by the drugs were considered, it was seen that TFP did not show any effect similar to what was observed for casper embryos. However, all doses of SFB caused a size decrease in the larvae treated. These effects were significant, 4 µM of SFB being the most impactful. The combination of TFP and SFB also resulted in a significant shortness in total length by both combination doses given in **Figure 30**.

3.4.3 Microscopic Examination on AB Larvae for Exposure of 12 µM TFP, 2 µM SFB, and Combination Therapy Starting from 1 dpi to 2 dpi

After examination of the impact of the drugs on zebrafish larvae until 5 dpf, xenograft experiments were performed by transplantation of 250-300 Hep3B cells into the yolk of 2 dpf wild type embryos. Using these embryos, two different treatment setups were followed. In the first treatment setup, 1 dpi larvae were exposed to 12 µM of TFP, 2 µM of SFB and their combination for 24 hours. This was to test if the highest dose of combination treatment, which was effective in Hep3B cells in vitro, exhibited a reduction in tumor volume also in vivo. The 24 h exposure time was chosen to minimize the toxic effects of the combination treatment. Representative 1 dpi images and the images after 24 hours of treatment were given in **Figure 31A**.

images, providing 1 dpi images of the larvae and the images after exposing them with 6 μ M of TFP, 1 μ M of SFB and their combination for 48 hours. The selection of these doses dependent on lowering the toxic effect on the embryo although in vitro studies suggested higher levels of synergism at 12 μ M of TFP, 2 μ M of SFB (Murat Yaman PhD Thesis, 2020).

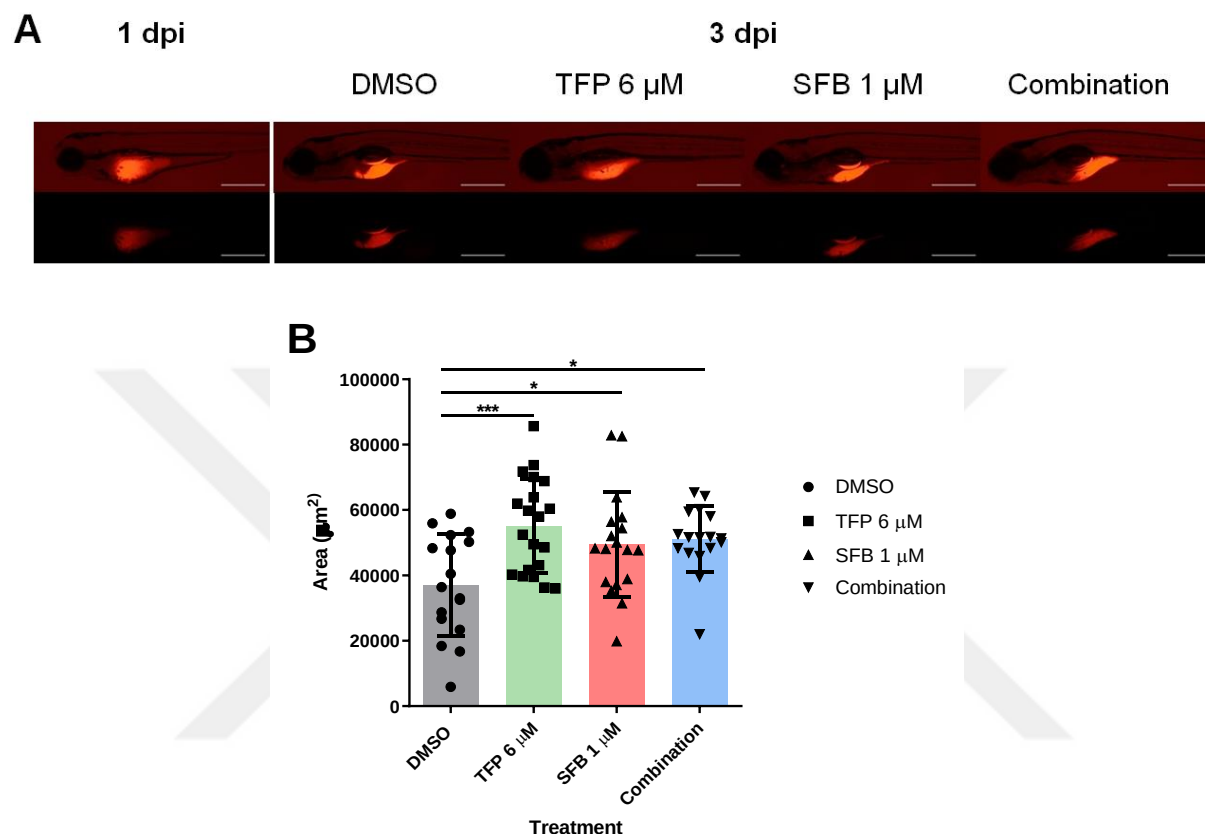


Figure 32. Transplantation of 250-300 Hep3B cells and treatment of injected larvae for 48 hours. **A)** Representative 1 dpi images of the larvae and 3 dpi images obtained as a result of treatment with 6 μ M of TFP, 1 μ M of SFB and their combination. All images were taken by using fluorescent stereo microscope before fixation of the larvae at 3 dpi. Scale bar: 500 μ m. **B)** The analysis of area of fluorescence was performed on the images of fixed 3 dpi larvae obtained under fluorescence microscope. One-way ANOVA (Dunnett's multiple comparisons test) was chosen as the statistical method to apply (*: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$).

The changes in fluorescence area (μm^2) with drug exposures were determined by comparing each treatment group with DMSO control. As seen in **Figure 32B**, each group significantly resulted in a bigger tumor area in comparison to DMSO which was evident by increased area of fluorescence. However, there was no difference between treatment groups in their effect sizes suggesting that at this dose of TFP, combination with SFB had no effect on tumor growth inhibition. All treatments led to increased survival of Hep3B cells in the zebrafish host.

3.4.4.2 Alu-based Quantification of Xenografts for 6 μ M TFP, 1 μ M SFB, and Combination Therapy

For Alu-based DNA quantification, embryos that were injected with 100-200 Hep3B cells were treated with 6 μ M of TFP, 1 μ M of SFB and their combination for 48 hours. The amount of Alu repeats in the zebrafish-human DNA mixtures obtained from these larvae was determined by qPCR as previously described [179].

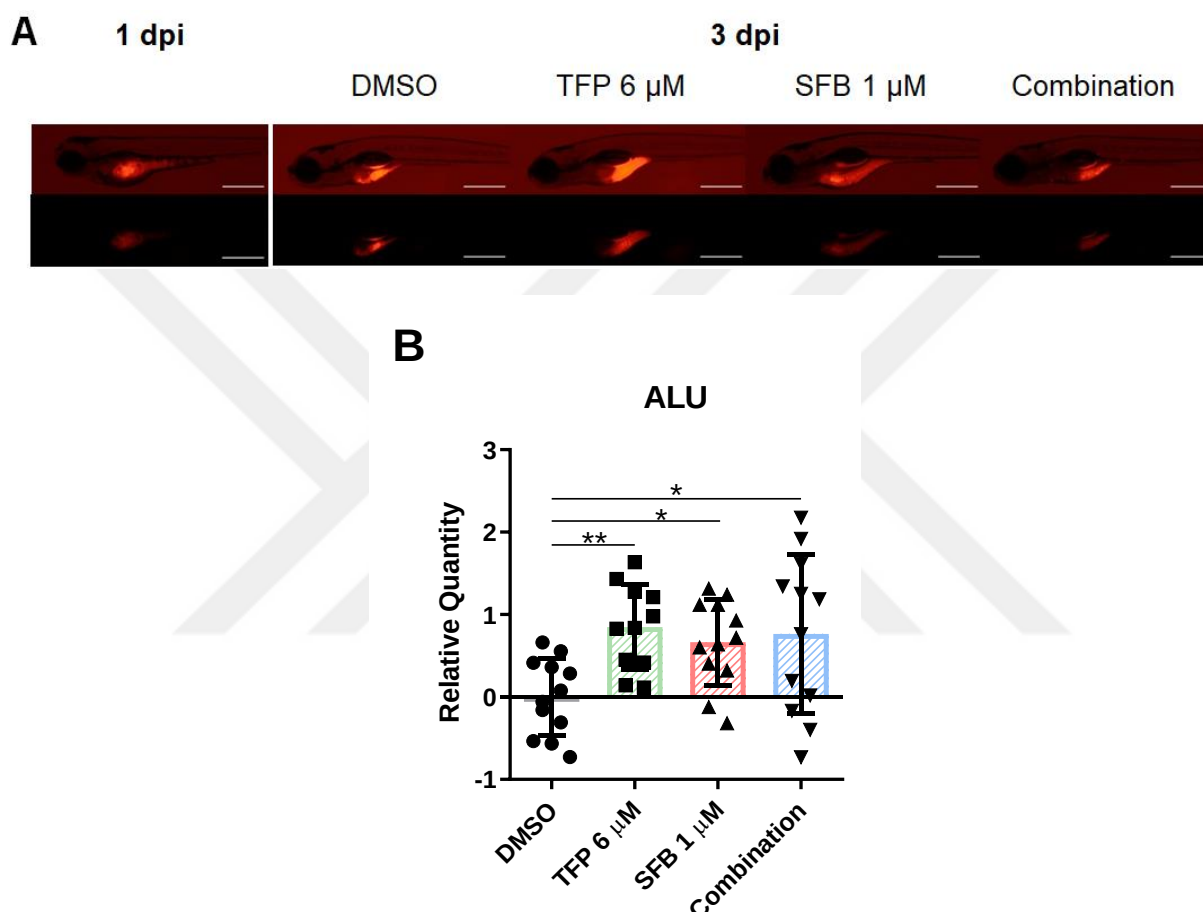


Figure 33. Transplantation of 100-200 Hep3B cells and treatment of injected larvae for 48 hours. **A)** Representative 1 dpi images of the larvae and 3 dpi images obtained as a result of treatment with 6 μ M of TFP, 1 μ M of SFB and their combination. All images were taken by using fluorescent stereo microscope before fixation of the larvae at 3 dpi. Scale bar: 500 μ m. **B)** Alu-based DNA quantification of the larvae was performed by calculating the amount of Alu for each treatment group relative to DMSO control. One-way ANOVA (Dunnett's multiple comparisons test) was chosen as the statistical method to apply (*: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$).

The quantity of Alu repeats given in **Figure 33B** demonstrated that the mean amount of human DNA in injected larvae that were exposed to drugs was significantly higher in comparison to DMSO. The number of human DNA that was obtained from 6 μ M of TFP group was also highly significant. These results substantially supported the previous

observations obtained by microscopy in which a highly similar pattern of increase was observed for tumor growth as a result of the same treatment groups. Although the ALU detection was based on lower number cell injections it was successful in detecting the human cell DNA in zebrafish host.

3.4.5 Embryonic Toxicity of Intermediary Phenothiazine Derivatives

Zebrafish embryos exposed to intermediary phenothiazines were investigated in detail to observe resulting toxicity and morphological changes.

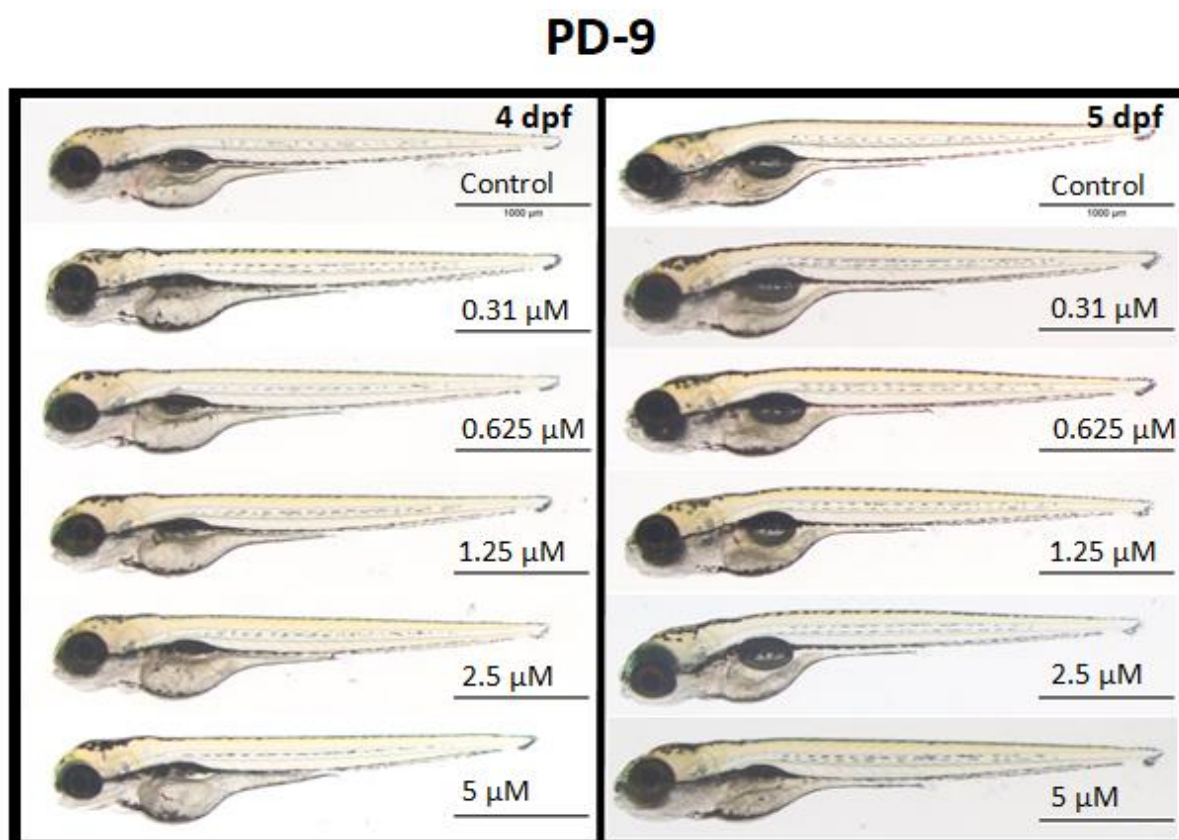


Figure 34. Representative images of 4 and 5 dpf WT larvae after 48 and 72 hours of exposure to different concentrations of PD-9 starting from 2 dpf. This toxicology experiment and image recordings were done by Emine Gelinci. Scale bar: 1000 μ M.

In **Figure 34**, the morphology of the larvae treated with PD-9 pointed out PD-9 as a safe derivative. There was not a distinct difference of PD-9-exposed larvae from control larvae at 4 and 5 dpf. However, 5 μ M of PD-9 seemed to impair the formation of swim bladder in larvae at 5 dpf.

PD-10

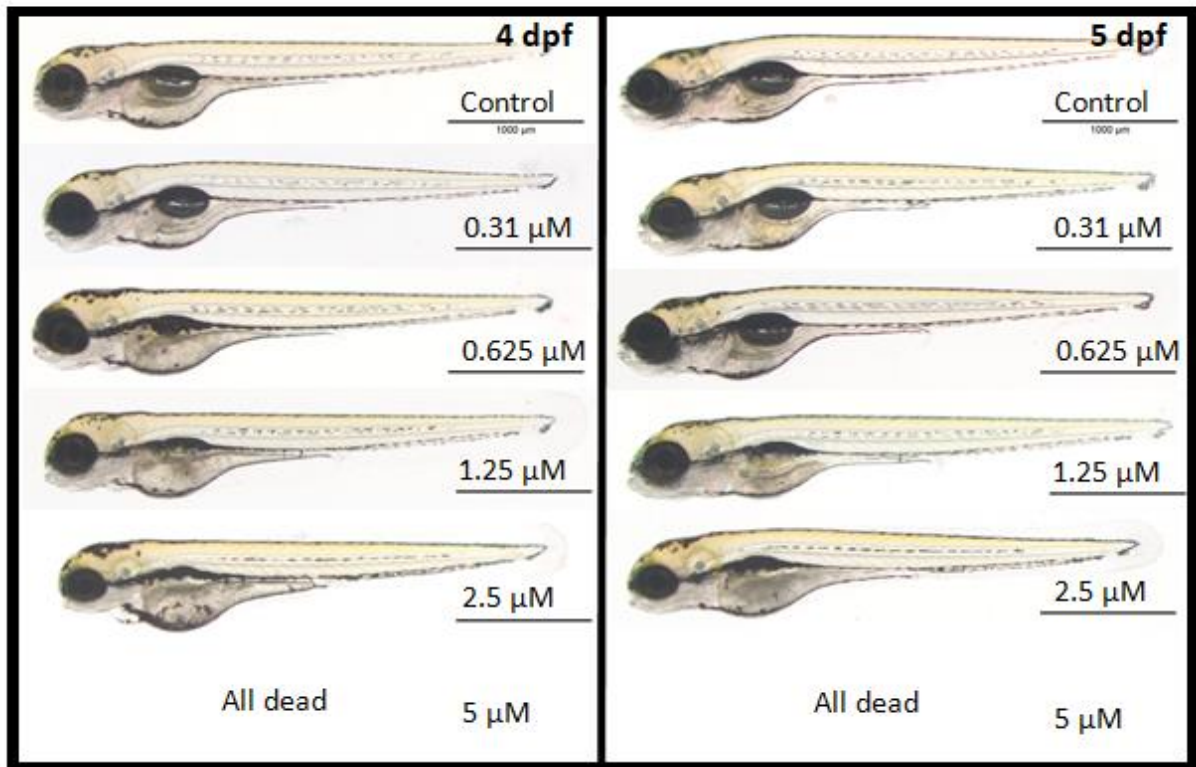


Figure 35. Representative images of 4 and 5 dpf WT larvae after 48 and 72 hours of exposure to different concentrations of PD-10 starting from 2 dpf. This toxicology experiment and image recordings were done by Emine Gelinci. Scale bar: 1000 μM .

When the impact of PD-10 was analyzed, it was clear that even 48 hours of treatment with 5 μM of PD-10 resulted in death of all embryos tested; therefore it was more toxic than PD-9. The toxicity resulting from this intermediary derivative was evident by the delay in swim bladder development starting from 1.25 μM concentration at both 4 dpf and 5 dpf embryos (**Figure 35**). Inside the yolk, a darkening, marker of necrosis, could be clearly seen at 5 dpf with 2.5 μM of PD-10.

PD-11

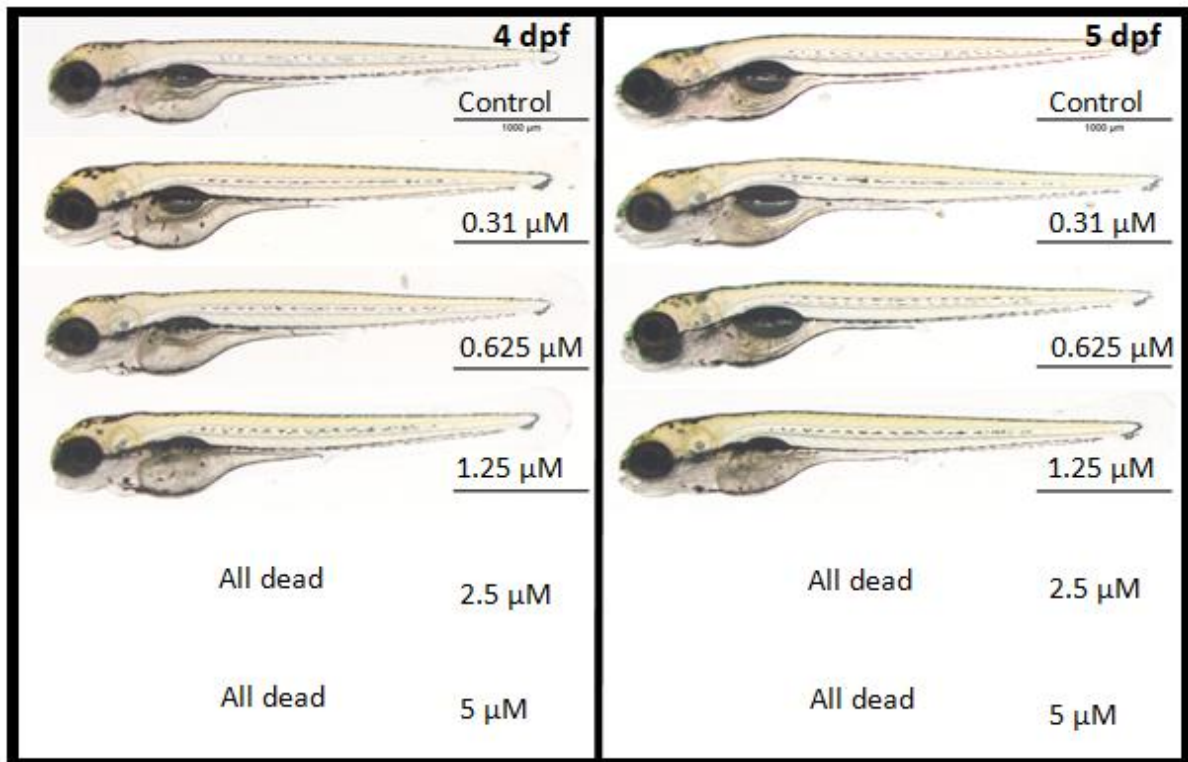


Figure 36. Representative images of 4 and 5 dpf WT larvae after 48 and 72 hours of exposure to different concentrations of PD-11 starting from 2 dpf. This toxicology experiment and image recordings were done by Emine Gelinci. Scale bar: 1000 μM .

PD-11 showed even more toxicity when compared to PD-10 (**Figure 36**). It caused 100% mortality at 2.5 and 5 μM , which highlighted its lethal effect even at low doses. Underdeveloped swim bladder was seen with 0.625 and 1.25 μM of PD-11 at 4 dpf. 72 hours of treatment with 1.25 μM dose affected the larvae in a way that swim bladder could not form completely and also there was a more apparent darkening in the yolk.

PD-12

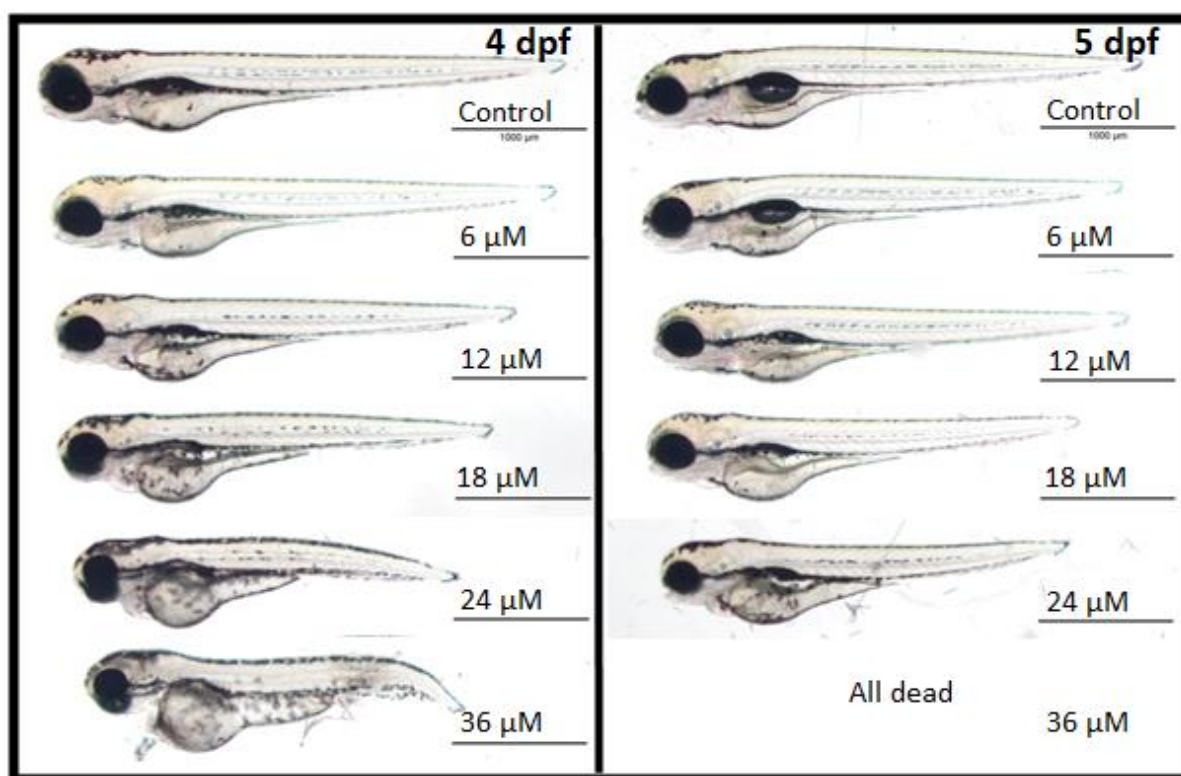


Figure 37. Representative images of 4 and 5 dpf WT larvae after 48 and 72 hours of exposure to PD-12 starting from 2 dpf. Scale bar: 1000 μ M.

PD-12 caused high toxicity after both 48 and 72 hours of exposure as seen in **Figure 37** and was less toxic than PD-9-11. The yolk was distorted significantly with increasing doses, although much higher than was used with the other phenothiazines when compared to DMSO control. The substantial and gradual decrease in body length could be observed starting from low doses at both 4 and 5 dpf. Additionally, the trunk of the larvae was bent especially after 18 μ M. These severe morphological abnormalities bring forward PD-12 as the least toxic at low doses among all intermediary derivatives tested. However, with increasing doses extensive morphological defects could be observed.

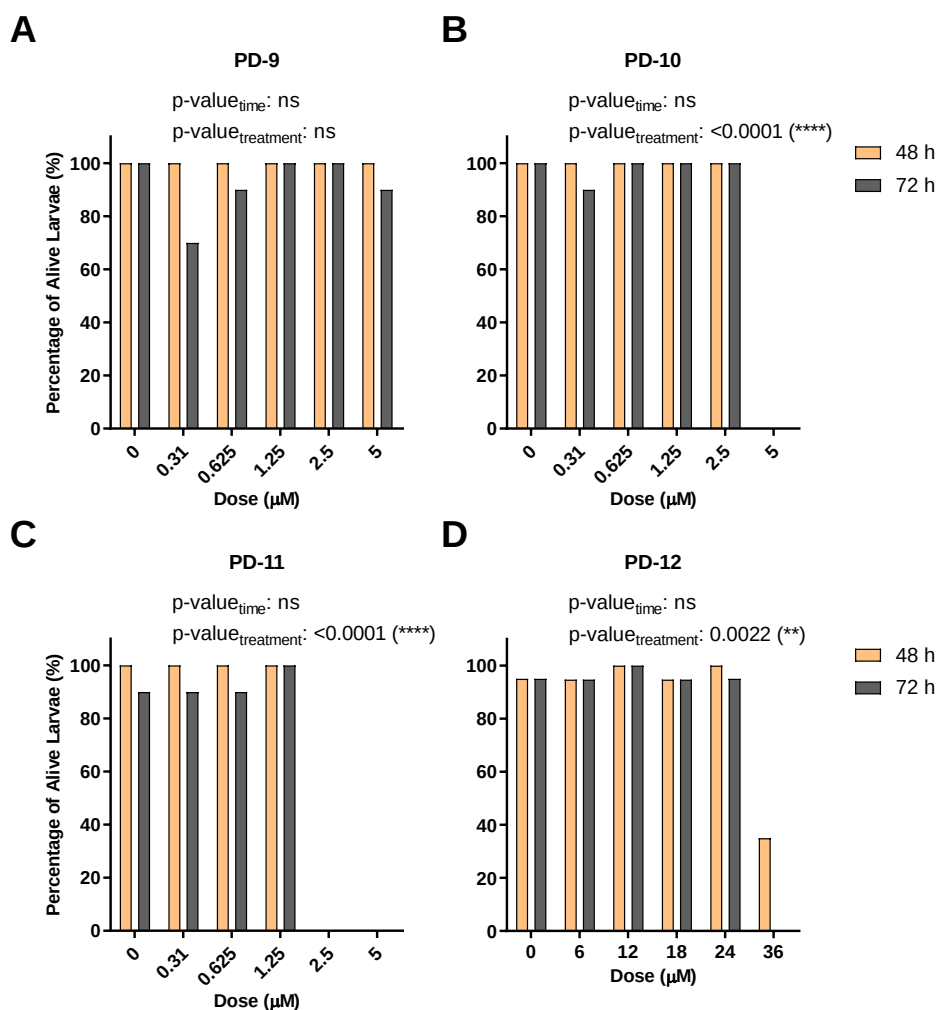


Figure 38. The mortality of WT larvae as a result of treatment with A) PD-9, B) PD-10, C) PD-11 and D) PD-12 for 48 and 72 hours starting from 2 dpf. Two-way ANOVA was applied as the statistical method (*: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$). The toxicology experiment to obtain these results was performed by Emine Gelinci, except for PD-12.

The lethal effect of PD-10 and PD-11 could also be confirmed through the analysis on the percentage of living larvae after 48 and 72 hours of exposure (**Figure 38**). Although PD-9 did not lead to a significant decrease in the number of living larvae, 5 μM of PD-10 killed all larvae after both 48 and 72 hours of treatment. PD-11, being the most toxic of all four, caused mortality of all larvae at 48 hours or after at 2.5 μM and 5 μM concentrations. Moreover, PD-12 decreased the percentage of surviving larvae at 36 μM ; and this further supported the toxic effects leading to developmental defects which can be caused by treatment for 48 and 72 hours at higher doses of PD-12.

Based on these observations, PD-9 came forward as a lead molecule among all intermediary derivatives tested because it was less toxic in vivo and had high effectiveness in vitro (Murat Yaman, PhD Thesis, 2020), even comparable to PD-11, which was more toxic.

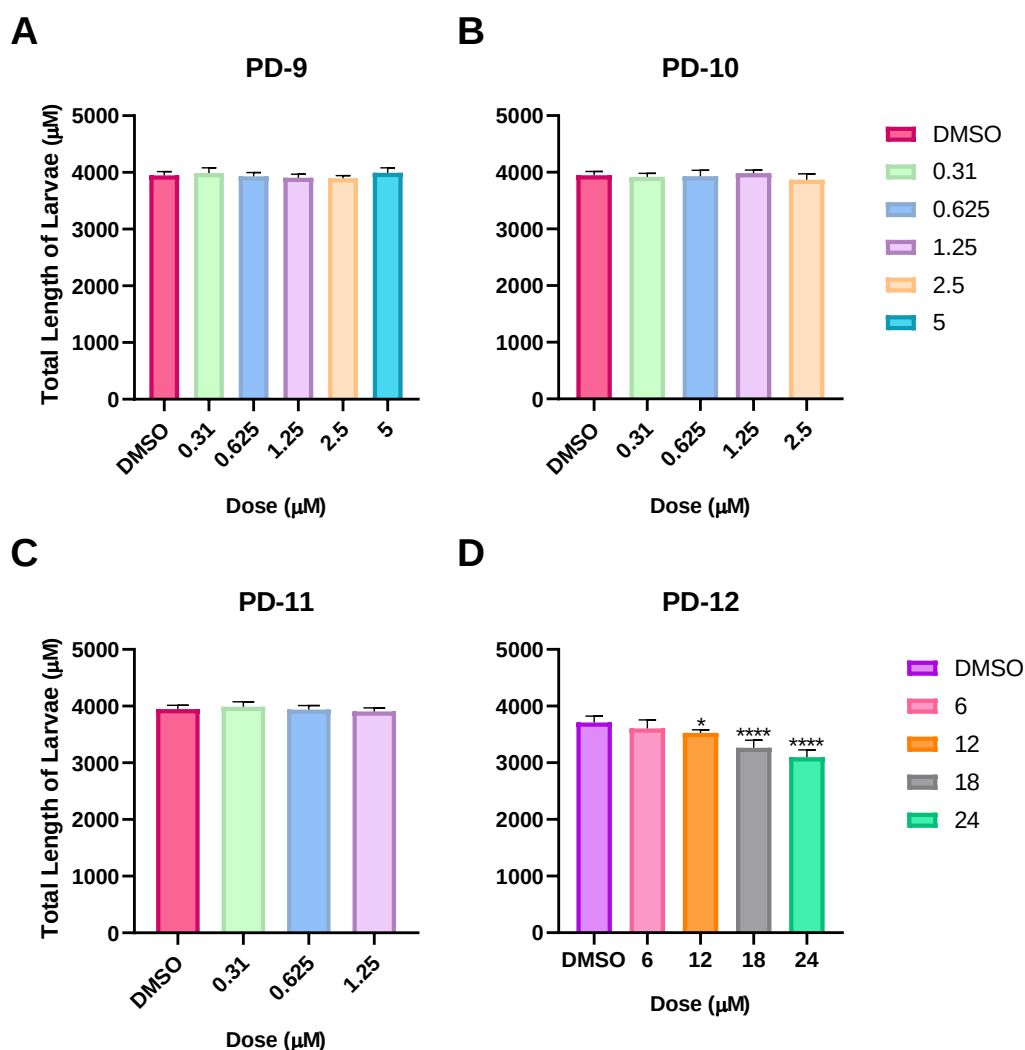


Figure 39. The total length of 5 dpf WT larvae after being exposed to A) PD-9, B) PD-10, C) PD-11 and D) PD-12 for 72 hours starting from 2 dpf. One-way ANOVA/Dunnett's test was applied as the statistical method for analysis (*: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$). The toxicology experiment to obtain these results was performed by Emine Gelinci, except for PD-12.

On the other hand, the measurement of total length for different doses of each intermediary derivative concluded that none of them, except for PD-12 (this was because higher doses were tested), caused a difference in total length of treated larvae compared to DMSO control at 5 dpf (**Figure 39**). PD-12 led to a gradual decrease in total length with increasing doses (**Figure 39D**). This effect was statistically important at 12, 18 and 24 μM , the last two doses being highly significant. Since all larvae were dead at the end of 72 hours of exposure to 36 μM of PD-12, the analysis on the total length could not be performed for this concentration.

3.4.6 Embryonic Toxicity of Novel Phenothiazine Derivatives

Zebrafish embryos at 2 dpf were exposed to novel phenothiazines for 72 hours to observe toxic effects caused by them.

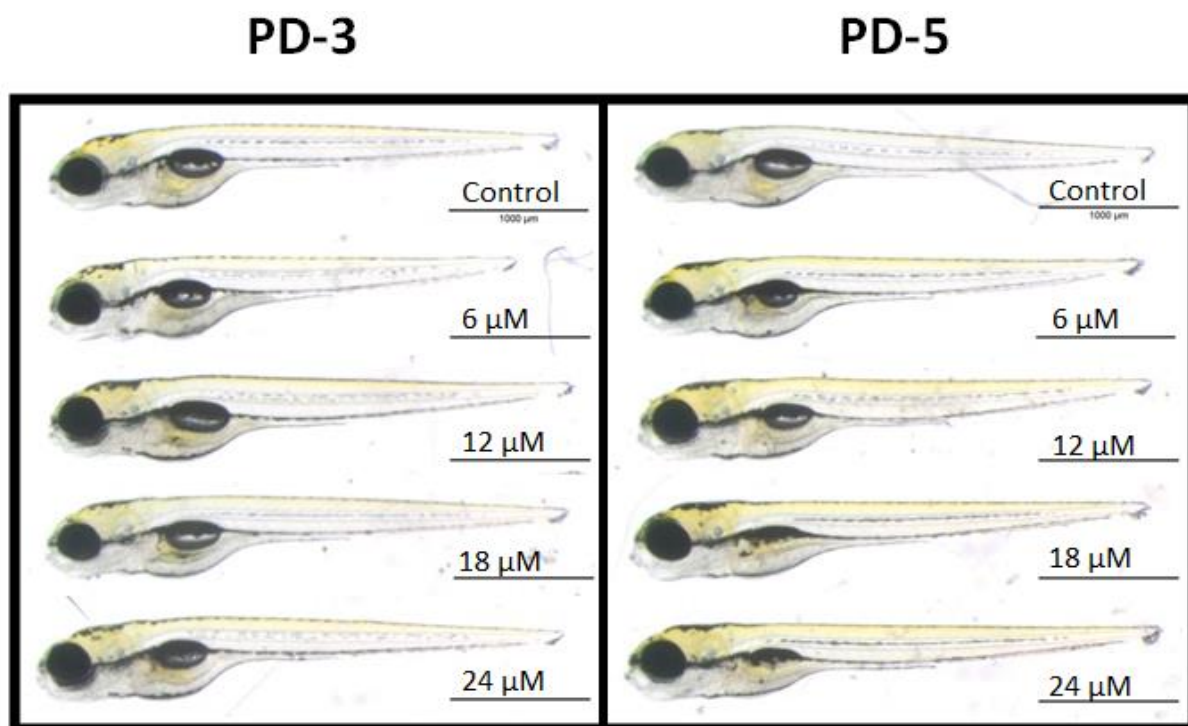


Figure 40. Representative images 5 dpf WT larvae after 48 and 72 hours of exposure to PD-3 and PD-5 starting from 2 dpf. Scale bar: 1000 μ M.

As seen in **Figure 40**; there was no major morphological deformation in the larvae treated with PD-3 when compared to DMSO control. However, the larvae treated with PD-5 showed problems in the development of swim bladder starting from 18 μ M, causing the larvae to have slimmer yolks.

PD-6

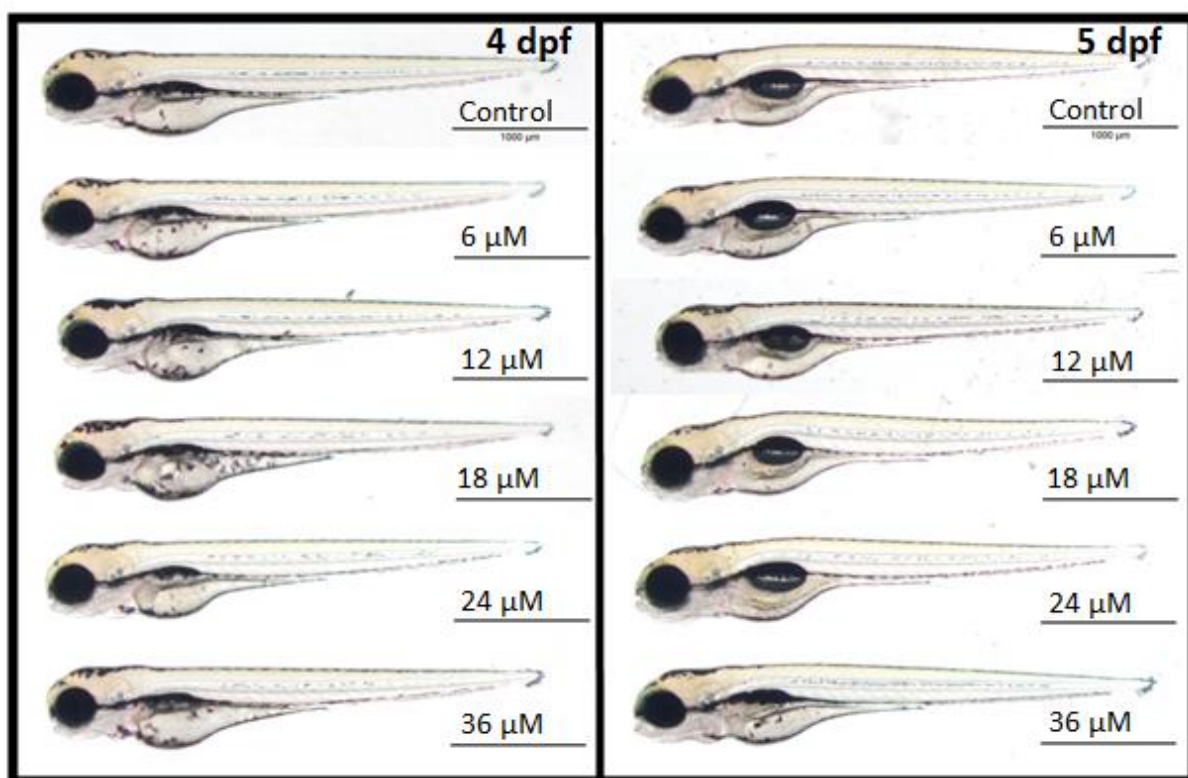


Figure 41. Representative images of 4 and 5 dpf WT larvae after 48 and 72 hours of exposure to PD-6 starting from 2 dpf. Scale bar: 1000 μ M.

When the outcome of PD-6 exposure was examined, it was seen that there was only a mild effect which was evident by the heart edema (**Figure 41**). At 5 dpf, the swim bladder of the larvae did not seem to inflate completely at the highest dose which was 36 μ M, supporting the mild toxicity of PD-6.

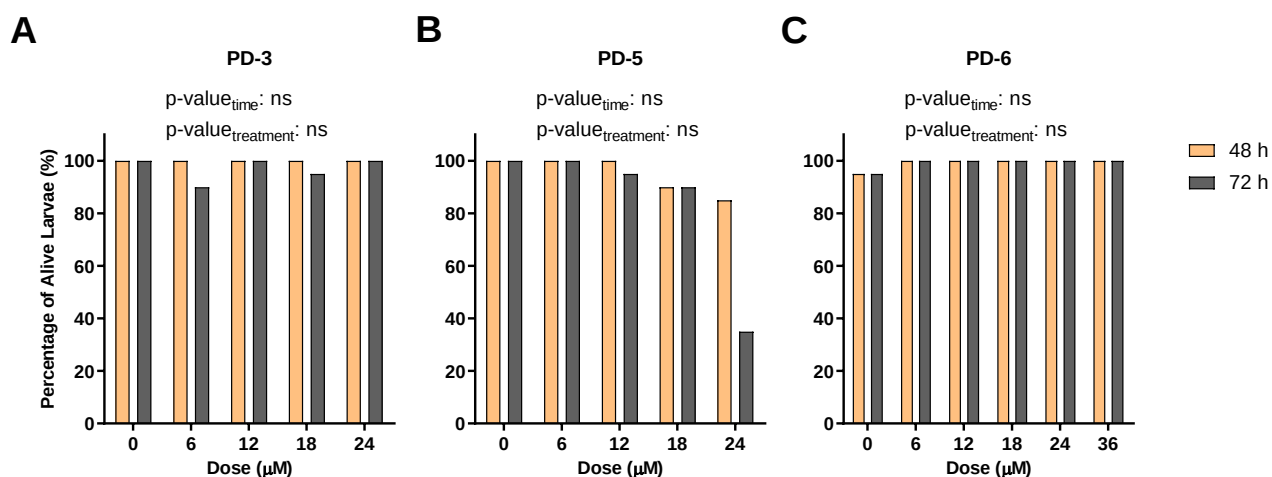


Figure 42. The changes in WT larvae after being exposed to novel phenothiazines A) PD-3, B) PD-5 and C) PD-6 for 72 hours starting from 2 dpf. The mortality of the larvae as a result of treatment with PD-3 and PD-5 for 48 and 72 hours. Two-way ANOVA was applied as the statistical method (*: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$).

The death of the larvae was also scored to see the mortality resulting from treatment with PD-3, PD-5 and PD-6 (**Figure 42**). Although not significant, 72 hours of exposure to PD-5 resulted in mortality at 24 μM to a large extent. However, compound PD-3 and PD-6 did not have a lethal effect at any doses.

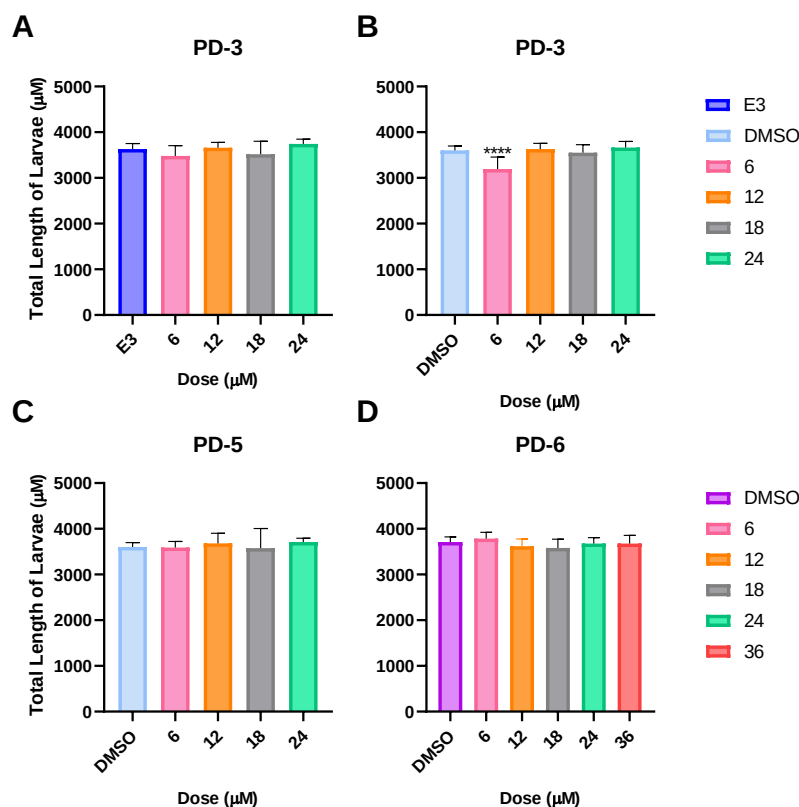


Figure 43. The total length of A) 4 dpf WT larvae as a result of exposure to compound PD-3 for 48 hours and 5 dpf WT larvae after being exposed to compound B) PD-3, C) PD-5 and D) PD-6 for 72 hours starting from 2 dpf. One-way ANOVA/Dunnett's test was applied as the statistical method for analysis (*: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$).

In general, the length of the larvae treated was not different from the larvae of DMSO control for any of the novel intermediates (**Figure 43**). However, 6 μM of PD-3 appeared as an outlier by causing a statistically significant decrease at 5 dpf and there was not such an effect for the same concentration of PD-3 at 4 dpf.

3.4.7 In Silico Evaluations to Understand the Conservation of Cholesterol Biosynthesis Genes Between Human and Zebrafish

In this study, the genetic similarity of zebrafish to human was a starting point for the use of zebrafish as a model of human HCC. Given the prominent role of cholesterol biosynthesis in physiology and cancers, human genes playing role in cholesterol biosynthesis were examined to see whether they had zebrafish orthologs and how similar they were to their zebrafish counterparts.

Table 11. Zebrafish orthologues/paralogs of human cholesterol biosynthesis genes and the similarity between them. The table was generated using Ensembl/BioMart web-based tool.

Gene name	Zebrafish gene name	Last common ancestor with zebrafish	%id. target Zebrafish gene identical to query gene	%id. query gene identical to target Zebrafish gene
CYP51A1	cyp51	Euteleostomi	74.8527	76.3527
DHCR24	dhcr24	Euteleostomi	79.4574	79.4574
DHCR7	dhcr7	Euteleostomi	72.6316	72.1757
EBP	ebp	Gnathostomata	57.8261	57.3276
FDFT1	fdft1	Euteleostomi	66.9065	66.7464
FDPS	fdps	Euteleostomi	52.9833	62.3596
HMGCR	hmgcra	Gnathostomata	77.8153	78.1674
	hmgcrb	Gnathostomata	67.2297	70.7346
HMGCS1	hmgcs1	Gnathostomata	78.2692	78.42
HSD17B7	hsd17b7	Euteleostomi	56.305	56.4706
LSS	lss	Vertebrata	74.7268	74.4218
MSMO1	msmo1	Vertebrata	78.4983	79.0378
MVD	mvda	Euteleostomi	63.5	63.5
MVK	mvk	Euteleostomi	56.8182	56.8182
NSDHL	nsdhl	Vertebrata	61.126	66.087
PMVK	pmvk	Euteleostomi	53.6458	49.2823
SC5D	sc5d	Euteleostomi	65.8863	65.6667
SQLE	sqlea	Gnathostomata	63.2404	65.1706
	sqleb	Gnathostomata	52.7875	61.4604
TM7SF2	tm7sf2	Euteleostomi	64.1148	63.658

According to **Table 11**, it was seen that there were zebrafish orthologues for all human members of cholesterol biosynthesis pathway. HMGCR and SQLE genes were found to have paralogous pairs in the form of a and b. Besides, the similarity of human genes to zebrafish genes and vice versa were generally very high, highlighting the sequence conservation of the given genes between the two species.

3.4.8 In Vivo Experiments to Investigate Effect of Phenothiazines on Cholesterol Metabolism of Zebrafish

3.4.8.1 Effects of Selected Phenothiazines on Zebrafish Cholesterol Biosynthesis Gene Expression Levels

Investigating the alteration of zebrafish cholesterol biosynthesis genes was also important to be able to complete previous findings on Hep3B cell line. Two genes, *hmgcs1* and *lss*, were selected to further study based on the RNA-seq analysis which was represented in **Section 3.3**. Although *hmgcs1* was not depicted in cholesterol biosynthesis pathways, logFC values

obtained in RNA-seq analysis revealed that both *hmgcs1* and *lss* were considerably affected by drug exposures (**Appendix B Table 1**).

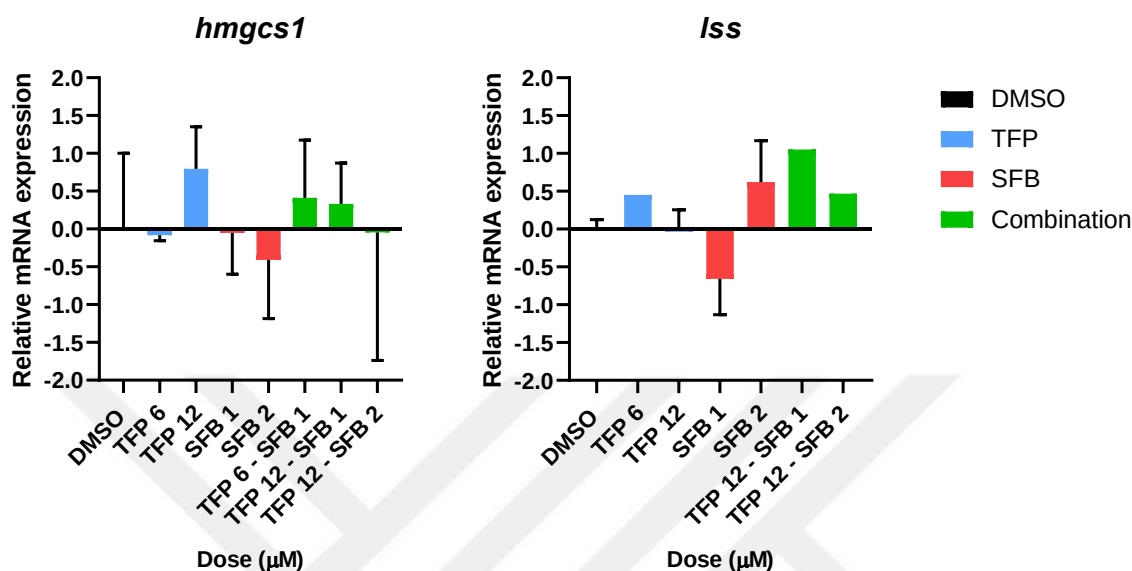


Figure 44. Zebrafish gene expression profiles for *hmgcs1* and *lss* after treatment with TFP, SFB and combination for 72 hours starting from 2 dpf. While y-axis shows relative expression to *actb2* reference gene, treatment groups are given in x-axis. Expression values of *hmgcs1* and *lss* genes are given in log2 scale. One-way ANOVA (Dunnett's test) is the statistical analysis method used to compare each treatment group with DMSO control (*: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$).

As seen in **Figure 44**, TFP alone or in combination showed the tendency to increase zebrafish *hmgcs1* and *lss* expression. Actually, there was mostly an increasing trend for all treatment groups except for SFB, which resulted in a decrease for both genes when applied at 1 μM . It also decreased the expression of *hmgcs1* at 2 μM , while the expression of *lss* was increased in response to the same treatment. Preliminary analyses performed with PD-3 and PD-9 suggested potential increases in expression of these genes (**Appendix C Figure 1**). Although the effect of none of the treatments was significant, elevated levels of *hmgcs1* with PD-3 drawn attention, which might support the potency of this derivative.

3.4.9 Change in Liver Vasculature Area of 7 dpf *fli1a:EGFP* Larvae After Exposure to 6 μM of TFP for 48 hours

In order to investigate if there was a change in liver size and vascularization in response to TFP, 5 dpf *fli1a:EGFP* zebrafish larvae, which had a fully developed liver [183], were

exposed to 6 μM of TFP for 48 hours and compared to DMSO control based on the images taken by using confocal microscopy at 7 dpf.

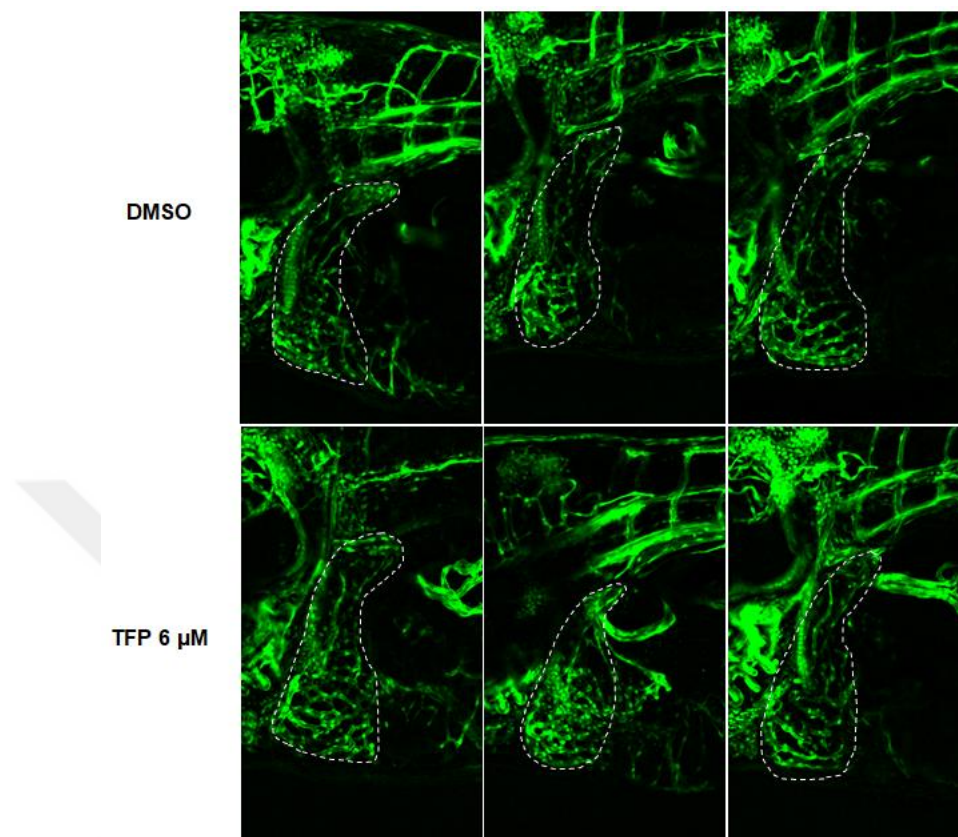


Figure 45. Representative images of the liver of 7 dpf *fli1a:EGFP* zebrafish larvae after 48 hour-treatment with 6 μM of TFP. The area with intense vasculature and branching vessels was where liver was located.

In **Figure 45**, three representative images for control and TFP-treated 7 dpf larvae were given, separately. Based on the GFP signal seen in the vasculature, the liver region could be easily distinguished as marked in the images.

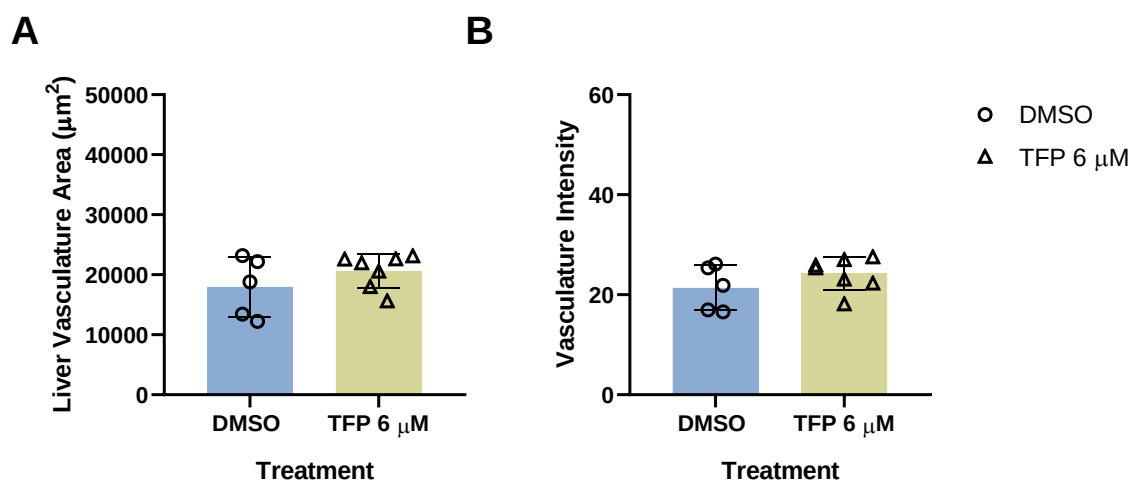


Figure 46. A) Liver vasculature area and B) vasculature intensity of TFP-treated *fli1a*:EGFP larvae at 7 dpf in comparison to DMSO control. The treatment was performed from 5 dpf to 7 dpf for 48 hours. Unpaired t-test was applied as the statistical analysis method.

By using the images including the ones given in **Figure 45**, the liver vasculature area and vasculature intensity in each larvae were determined. The analysis in which TFP-treated larvae were compared to control larvae revealed that liver area and GFP intensity of liver vasculature were not significantly affected by 6 μ M of TFP (**Figure 46**).

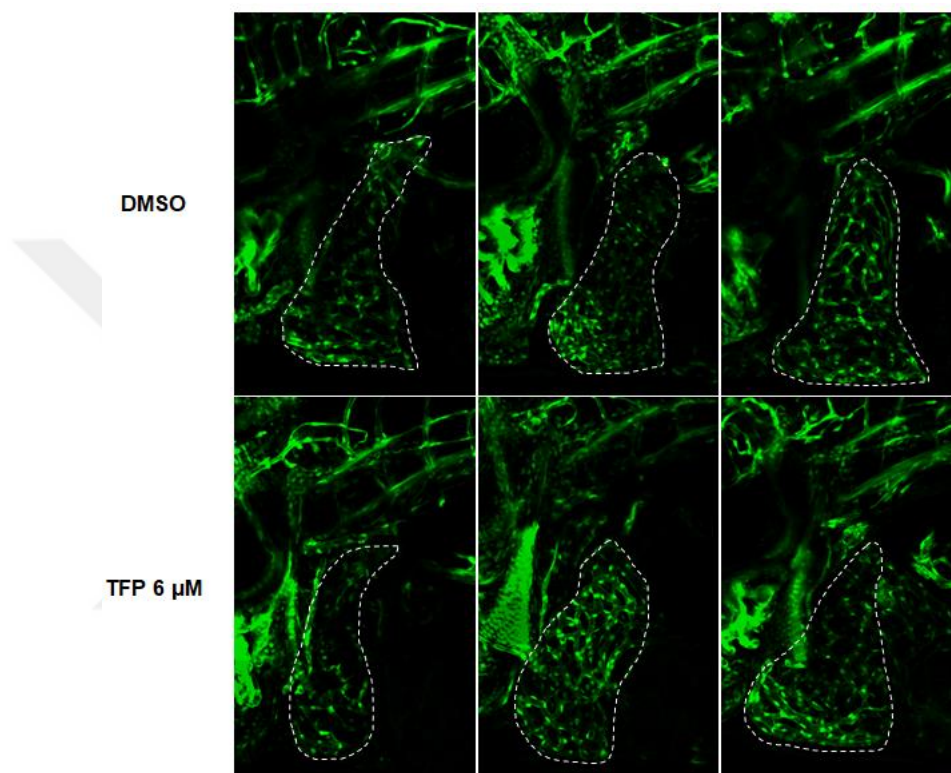


Figure 47. Representative images of the liver of 7 dpf *fli1a*:EGFP zebrafish larvae after 48 hour-treatment with 6 μ M of TFP as a result of the repetition of previous experiment. The area with intense vasculature and branching vessels was where liver was located.

The experiment was repeated by testing more number of larvae to minimize variation. **Figure 47** shows the images taken by using confocal microscopy for both DMSO and TFP group larvae.

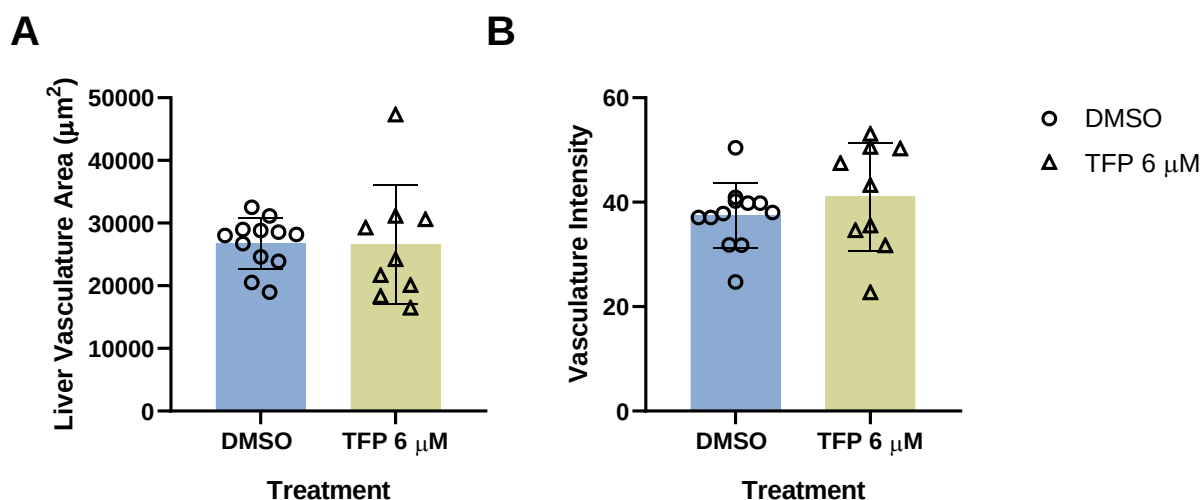


Figure 48. A) Liver vasculature area and B) vasculature intensity of TFP-treated *fli1a:EGFP* larvae at 7 dpf in comparison to DMSO control as a result of the repetition of previous experiment. The treatment was performed from 5 dpf to 7 dpf for 48 hours. Unpaired t-test was applied as the statistical analysis method.

Even if the sample size was increased, 6 μM of TFP again did not lead to a significant change in the liver area and GFP intensity of liver vasculature as a result of 48 hours of exposure (**Figure 48**). In addition, the images given in (**Appendix D Figure 2**) were obtained by using fluorescent stereomicroscope before switching to confocal microscopy for a better vision. Similarly, no change could be observed on GFP intensity with 6 μM of TFP exposure in comparison to DMSO (**Appendix D Figure 3**). Overall, these results suggested that the action of TFP at 6 μM concentration was not in the direction of impairing liver structure and vascularization in zebrafish larvae.

Apart from these, it was attempted to investigate whether exposure to TFP would affect lipid deposits in zebrafish larvae. After 48 hour-treatment of 5 dpf larvae with either 3 μM or 6 μM of TFP, Nile red staining was performed (**Appendix D Figure 1**). Since it was experienced before that fluorescence signal faded when the larvae were not alive, live-mounting was performed before imaging. However, this limited the sample size and Nile red staining could not be conclusive in terms of any changes caused by TFP on lipid deposits.

Chapter 4. CONCLUSIONS AND DISCUSSION

HCC is a type of cancer that results in high death rate every year and the treatment options available have not been sufficient for a full prevention. The side-effects and resistance observed after use of sorafenib, the first FDA-approved medication against HCC, urged scientists to search for new drugs and approaches. Although alternative first-line and second-line treatment options such as lenvatinib, regorafenib and cabozantinib have been developed [57, 184], new improved therapies are still in demand. Recently, drug repurposing and combinatorial therapies have served as great strategies for such improvements. Phenothiazines have recently emerged as anticancer molecules through in silico and in vitro studies in multiple cancers including liver cancer however there is a need for discovery and testing better acting and less toxic derivatives as well as gaining more knowledge on the drugs effectivity when used in combination with sorafenib in HCC. Zebrafish a valuable model in novel drug screening via high throughput embryonic/larval toxicity assays as well as effective xenograft models and high conservation of genes, serves well to screen such anticancer activity in vivo. However phenothiazines or sorafenib have not been elaborately studied in zebrafish models (please see introduction for details). At the same time, there is almost no zebrafish literature that compares human and zebrafish cholesterol pathway members in terms of their expression; and testing whether phenothiazines, known modulators of cholesterol biosynthesis [28], are modulated similarly in both liver cancer cell lines and zebrafish, with or without sorafenib. This thesis focuses on the above-mentioned research topics that have remained unexplored and shed light significantly, as discussed in detail below.

My work presented in this thesis has been built upon the hypothesis that exposure to known and/or novel phenothiazine derivatives might also modulate cholesterol biosynthesis in different liver cancer cell lines and in zebrafish larvae. Moreover, I wanted to test whether these derivatives were toxic to zebrafish early larval development, vessel formation, as well as lipid biosynthesis. Finally I inquired whether TFP alone or together with SFB can be used to treat liver tumors that occur when injected into the yolk of zebrafish embryos as xenografts using microscopic and novel Alu DNA based assays and test whether the effects were dose- and time-dependent. The idea of combining TFP with sorafenib to see if together they would increase the efficiency to treat liver cancer has emerged from the work of Murat Yaman (Murat Yaman, PhD Thesis, 2020; TUBITAK Z388). He has shown that TFP and SFB acted synergistically in Hep3B cells when combined at certain doses, while SkHep1 cells exhibited a relatively antagonistic profile, suggesting presence of cell type dependency.

On the other hand, RNA-seq analysis is a useful technology that helps enormously in gaining high throughput knowledge of the transcriptome in response to drug exposure [185, 186]. Previous studies on phenothiazines showed that several pathways, including cholesterol biosynthesis, have been affected by exposure to phenothiazines [28, 106]. In the present study, I followed up the findings from an RNA-seq study we performed in our lab (Kubra Calisir, MS student, and Murat Yaman, PhD (2020) analyzed the data) on significant increases in cholesterol biosynthesis and cholesterol metabolism gene expression in TFP when given at 12 μ M to Hep3B cells with or without SFB.

4.1 Expression of the genes involved in cholesterol biosynthesis increased in vitro and in vivo upon phenothiazine exposure

Cholesterol is the precursor of many substances that are able to provide cancer cells with energy and also an important component of plasma membrane, the main entity enabling cellular signaling [187]. Therefore, proper regulation of cholesterol levels inside the cells is highly crucial. The role of cholesterol-related mechanisms on the impact of phenothiazine derivatives was elucidated based on gene expression analysis in HCC cells. I have found that the expression of HMGCR, SQLE and LSS genes, which participate in cholesterol biosynthesis, showed different patterns for Hep3B and SkHep1 cells. The increasing trend with especially 20 μ M of PCP and PPH was indicative of the ability of phenothiazine derivatives to modulate cholesterol biosynthesis in Hep3B cells. When it comes to SkHep1 cells, PD-11 among intermediary derivatives resulted in an important decrease in all three genes. Novel derivatives PD-3 and PD-4 significantly decreased LSS gene expression. PD-3 also exerted significant effects in Hep3B cells on all genes, but the direction of these effects were opposite of what was observed with SkHep1 cells. These findings suggest that members of cholesterol biosynthesis pathway can be controlled by phenothiazine derivatives, although the changes occurring are cell line-specific. Moreover, LSS gene was found to be the most regulated among the genes tested, which might give a lead about the mechanism through which derivatives exert their effects.

Besides cell-line specificity, there were varying patterns of gene expression observed even within the same cell line as a result of the action of different derivatives. This could be related to the structural groups attached to the core phenothiazine ring of each derivative. Previously in our lab, possible targets of prominent commercial, intermediary and novel phenothiazines have been determined using SwissTargetPrediction web server (Murat Yaman, PhD Thesis, 2020). Among the targets provided, dopamine receptors (D2, D3 and D4) seem to be strong

partners for PD-3, PD-4, PD-5 and PD-6 to bind, while they were not the targets of PD-9, PD-10 and PD-11. It is known that lipid dynamics is important for dopamine receptors to be localized in the cell membrane [188]; and this could be an explanation for the relationship of dopaminergic signaling and cholesterol. In the literature, depleting cholesterol was found to impair the functioning of dopamine transporter in vitro [189, 190]. Another study has demonstrated that haloperidol, an antagonist of dopamine receptors, caused lowered cholesterol biosynthesis in neuroblastoma cells [191]. High levels of dopamine were shown to be associated with enhanced cholesterol production in astrocytes through a JNK3/SREBP-2-mediated mechanism [192]. Given this knowledge, cholesterol biosynthesis gene expression inducing activity of PD-3, PD-4, PD-5 and PD-6 could maybe be explained by their strong relationship with dopamine receptors.

With reference to synergistic action of combination therapies having TFP with doses less than 6.25 μ M, the expression of the three important cholesterol genes, HMGCR, SQLE and LSS genes were also analyzed in Huh7 cells after 48-hour single and combination treatments. Although TFP only or SFB only exposures did not lead to important changes, TFP 3.13 μ M - SFB 2.5 μ M and TFP 6.25 μ M - SFB 2.5 combinations increased the expression of HMGCR and LSS genes, respectively.

In the literature cholesterol pathway genes have been shown to be modulated by different drugs including statins [27], cisplatin [193], sorafenib [41] and phenothiazines [28, 101, 104]. The association between anticancer activity and cholesterol synthesis modulation or increasing/decreasing of anticancer activity via modulation of cholesterol levels is relatively indeterminate. Therefore, the direction of the modulation was not always towards lowering cholesterol levels, which might explain our results obtained for Hep3B cells. For example, Zheng et al. (2018) have obtained a panel for differential gene expression which showed upregulation of LDLR, HMGCR and FDFT1, all targets of SREBP-2, in cisplatin-resistant A2780 cancer cells comparatively to cisplatin-sensitive cells [193]. When they treated resistant A2780 cells with cisplatin, the treatment further induced the increased expression of these genes together with SREBP-2. Also, some studies have indicated cholesterol lowering did not always led to anticancer effects, as evident by increased mass of tumor in mouse model of prostate cancer after simvastatin treatment [49]. The nature of cancer cell type might be coming into play at this point. In our case, cell-type dependency has been observed with respect to alteration of cholesterol biosynthesis gene expression. This can be based on different characteristics of the three cell lines included in this study. SkHep1 was derived

from the liver tissue with adenocarcinoma and has been preferred to study hepatocellular carcinoma over the years. However, detailed characterization revealed that it may not even be considered as an HCC cell line given its endothelial origin [194]. Despite liver being the tissue of origin, SkHep1 cells do not entirely reflect the features of liver cells and show variation in mutational profile [195] and expression of genes including liver-specific genes [196]. SkHep1 now might be considered as a misclassified cell line and this is the reason why we did not move forward with it for our studies following gene expression evaluations. Hep3B is a well-differentiated, tumorigenic hepatoma cell line that enables studying characteristics of liver cancer cells [197-199]. It possesses an epithelial phenotype [200] and is distinguished from SkHep1 and Huh7 by its P53-null state [198] and presence of Hepatitis B virus integrants [201]. On the other hand, Huh7 is a hepatoma tissue-derived cell line with epithelial morphology [202]. It has been widely used in the studies involving Hepatitis C virus based on its high susceptibility and has been representing a good model for HCC [203]. Moreover, these HCC cell lines have been widely studied in research areas involving drug screening, being advantageous tools to understand cytotoxicity, drug metabolism and underlying molecular mechanisms [198, 204]. It is also possible that increases in cholesterol observed in the presence of phenothiazines might contribute to the side effects of singular or combinatorial treatments. Increased cholesterol biosynthesis might be short term or long term and may need to be stopped due to hazards that might be associated with it. In the literature, it has been shown that excessive amounts of free cholesterol exert toxic effects to cells [205]. Since cholesterol is able to regulate membrane integrity and membrane proteins that are crucial for cellular mechanisms [5], cholesterol indirectly might be impairing molecular events that are required for a healthy state of cells. Besides, oxidative stress caused by the formation of oxysterols following cholesterol oxidation might be another reason for the toxicity of enhanced cholesterol [206].

In addition to the evaluations on combinatorial approaches using Huh7 cell line, promising anticancer effect of Hep3B cells as evident by cytotoxicity and synergy profiles (Murat Yaman, PhD Thesis, 2020) enabled as to proceed with cholesterol pathway representations that were generated using logFC values acquired from RNA-seq analysis. These pathway representations helped pick the genes that we could observe important changes with. The main finding was that TFP 12 μ M was alone able to increase the expression of cholesterol biosynthesis genes. The combination with 1 μ M and 2 μ M of SFB both had similar effects when compared to TFP 12 μ M, enabling further increase in logFC of some genes playing role

in cholesterol biosynthesis pathway (**Figure 10, Appendix B Table 1**). Although there were minor decreases in logFC of the genes including NSDHL (-0.0819 for TFP 12 μ M - SFB 1 μ M and -0.04049 for TFP 12 μ M - SFB 2 μ M) and EBP (-0.1296 for TFP 12 μ M - SFB 1 μ M and -0.1084 for TFP 12 μ M - SFB 2 μ M) in comparison to single TFP treatment, the overall outcome was slight induction of cholesterol biosynthesis pathway which comprises of sequence of reactions that follow each other up. Based on all these findings, it is possible to conclude that TFP has a major ability to induce cholesterol biosynthesis alone; and SFB did not show a supportive effect with combination. Moreover, it was not possible to refer to a dose-dependency, since the changes between the two combination groups were not considerably different (**Figure 11, Appendix B Table 1**).

In the case of cholesterol metabolism, however, the effects of SFB in combination with TFP were more obvious. The enhanced logFC of LDLR, NPC1 and NPC2 was a sign of cholesterol uptake stimulation by TFP 12 μ M. The upregulation of PCSK9 might be associated with the increase in LDLR in response to LDLR-mediated endocytosis. Another regulation that attracted attention was lowered logFC of ABCA1, indicating decline in cholesterol efflux [207]. The combination of TFP 12 μ M with SFB 1 μ M made a slight contribution to the effects caused by single treatment in terms of cholesterol uptake, but cholesterol egress from peripheral tissues seemed to be negatively affected. The combination group with SFB 2 μ M exhibited similar changes as the previous combination, but the decrease in logFC of the genes related to cholesterol egress was more enhanced comparatively. Based on these findings, both cholesterol uptake and removal come forward as important mechanisms that are controlled by TFP alone or in combination.

Beyond these, SREBP-2-mediated cholesterol sensing, one of the most important events of cholesterol homeostasis [6], might be mediating differential expression of cholesterol-related genes in response to single or combination treatments with TFP. Since it involves a transcriptional regulation-based mechanism, checking expression of SREBP-2 and its downstream genes might be helpful to better understand differences in logFC values while comparing treatment groups. In the original RNA-seq data provided by Kubra Calisir, SREBP-2 was not among the 14540 genes that were listed due to the filtration performed before TMM normalization. However, the logFC values for the genes producing SCAP, INSIG1 and INSIG2 which are binding partners of SREBP-2 [208] were provided in **Appendix B Table 1**. SCAP and INSIG2 did not seem to be affected by any of the treatment groups compared. INSIG1 had a significantly increased logFC value with TFP 12 μ M

treatment in comparison to control. Although addition of neither doses of SFB resulted in a considerable difference, the increase caused by TFP 12 μ M alone could be explained by a mechanism which might possibly be related to significantly enhanced logFC of all cholesterol biosynthesis genes (**Figure 9**). Since INSIG1 has a regulatory role to keep cholesterol levels in balance [209], it might have acted to prevent further activation of cholesterol biosynthesis genes. In other words, the expression of INSIG1 might have been increased in Hep3B cells to keep SREBP-2 in the ER membrane, blocking its dissociation and following cleavage in Golgi apparatus. This way, the active domain of SREBP-2 might have not been released to initiate the transcription of cholesterol biosynthesis genes in the nucleus.

In this study, I have shown for the first time that TFP tends to increase zebrafish *hmgcs1* and *lss*, orthologs of human HMGCS1 and LSS in human. Literature research revealed that there was no comprehensive and comparative study of zebrafish and human cholesterol biosynthetic genes and mine provided the view that they were highly conserved. Given its crucial role in mediating initiation of cholesterol biosynthesis [210], the alteration of zebrafish *hmgcs1* was supportive of the findings obtained in vitro with Hep3B cells. Nonetheless, high error bars for *hmgcs1* gene and lack of results coming from some biological replicates for *lss* gene suggest for the improvement of experimental procedure for RNA isolation. The yield could be increased by increasing larvae number, enabling much consistent results. Moreover, looking at the orthologous cholesterol pathway genes provided testing of novel derivatives too. The increase in zebrafish *hmgcs1* with 24 μ M of PD-3 also stepped forward providing promising efficacy of this derivative (**Appendix C Figure 1**). Indeed, there are several examples in the literature that compares human and zebrafish genes with respect to their expression in response to perturbations [122, 211] and future studies comparing more genes could provide more insight into the degree of conservation of this pathway between zebrafish and humans.

4.2 TFP 12 μ M combined with SFB increased intracellular cholesterol levels in Hep3B cells

The investigation of cholesterol level alterations in Hep3B cells after TFP, SFB and combination applications highlighted that combination therapy as an effective strategy. Increasing doses of either TFP or SFB alone did not differ in terms of cholesterol level change intracellularly, but there was a decreasing trend in cholesterol with increasing concentration of TFP in the combination. More importantly, this effect was significant by the combination of TFP 12 μ M with both 1 and 2 μ M of SFB. Although these results point out the two

combination treatments as to modulate intracellular cholesterol levels effectively, the detection of cholesterol was based on its interaction with Filipin III inside the cells and less cell viability caused by these combinations might have been causing misleading interpretations. My results showed that without taking into account the cell viability and cell number, estimation of cholesterol levels would not be correct if there is significant cell death. Indeed, taken this into account resulted in comparable conclusions with the gene expression studies. Moreover, there was an exact opposite trend for combination of both SFB 1 and 2 μM with increasing concentrations of TFP when compared to previous results and the increased cholesterol level by TFP 12 μM - SFB 1 combination was highly significant. The gene expression results did not show striking differences between low and high dose SFB yet the intracellular cholesterol levels were higher at the lower dose combination. This discrepancy could be due to the fact that cholesterol levels depend on both synthesis, metabolism and efflux while gene expression increase is based on biosynthesis and may not reflect the negative and positive feedbacks between the biosynthetic-metabolic-transport signaling molecules. A metabolic modeling approach [212, 213] can help to test how cholesterol levels are affected given the expression level changes at the transcriptome. Moreover, transcriptomics changes may not be reflected at the protein levels in full [214], hence it would be important to check the protein expression levels of important rate-limiting enzymes and/or determine activity profiles of these enzymes. The findings on the cholesterol pathway modulation by phenothiazines might be modulating the lethal effects of the combination treatment however future research by inhibiting cholesterol biosynthesis along with exposure to phenothiazines are needed to decipher the mechanism at work and whether this would be the case.

Accordingly to see how cholesterol levels change within the cell based on solely intracellular mechanisms, it might be better to limit the availability of external cholesterol while growing the cells. If the cells were obtaining cholesterol by taking up from external sources rather than endogenously producing it, we would not be able to differentiate the cholesterol provided through regulation of intracellular cholesterol homeostasis. This could be achieved either by providing the cells with a medium containing delipidated FBS [215] or using charcoal-stripped FBS which would enable to eliminate steroids [216]. Thus, it would be possible to prevent the uptake of cholesterol from external sources.

At this point, it should also be mentioned that U18666A may not be the most optimum positive control. U18666A is a well-known cholesterol transport inhibitor [217] and with the

effect exerted by it, the localization of cholesterol is of importance rather than the level. However, the analysis that I performed was based more on absorbance, which enabled to measure cholesterol levels and could not give any information on where cholesterol was present in the cells. If we could have performed an analysis on high resolution images and see which cell compartments contained cholesterol, then using U16888A might have been a suitable option to see whether the derivatives tested were also resulting in intracellular accumulation of cholesterol due to impairment of cholesterol metabolism. Therefore, it might be better to include a group in which Hep3B cells were given a type of statin and compare the results with derivate-exposed cells in terms of cholesterol levels.

4.3 Cell cycle and apoptosis were regulated by novel and intermediary derivatives in Hep3B cells

The connection between phenothiazines and regulatory mechanisms that might be important in cancer can be studied through changes in protein level by Western Blotting. This was important because I had to select several leads to focus on zebrafish toxicity assays along with TFP and SFB, which I and others have shown that was effective in Hep3B. Multiple novel derivatives were tested to identify a potent one to further study. The results indicated that intermediary and novel derivatives showed distinctive regulatory impact on Hep3B cells based on several cycle cycle and apoptosis relevant protein amounts. The lack of biological replicates prevented to draw a conclusion on significantly effective derivatives, however there were important leads as for the molecular mechanisms that were likely to be modulated such as cell cycle and MAPK signaling, providing molecules for follow up. CHEK1 is known to be a regulatory protein for cell cycle arrest when there is DNA damage detected [218]. In literature, lowered CHEK1 action was shown to be involved in the process of apoptosis [219]. The drastic decrease of T-CHEK1 levels by PD-11 might be indicative of apoptosis, bringing this derivative forward as a potent anticancer agent. The increase in DR5 levels caused by most of the derivatives also highlighted apoptosis as a mechanism that was induced. DR5 is indeed a known target of phenothiazines [220]. Moreover, P38 MAPK is a protein that may act either in an apoptosis-favoring manner or support cell survival and growth according to the extracellular stimuli [221]. The increased levels of phosphorylated P38 by PD-3 and PD-11, while P38 levels were decreased by them, may point to the activation of MAPK signaling [222], which need to be further explored.

4.4 Toxicity studies in zebrafish helped test and confirm the lead compounds

After unraveling the ability of certain phenothiazine derivatives such as PD-3 and PD-11 and TFP-SFB combinations to alter mechanisms that affect cancer development, their impact was tested *in vivo* using zebrafish as a model organism. Microscopic examination of embryonic toxicities resulting from treatment with TFP, SFB and their combination has paved the way for the decision on which doses to apply to the embryos in xenograft studies.

One of the reasons to decide on using casper embryos was casper's transparency which allows to observe all morphological changes more efficiently after drug exposures. Also, they are very advantageous while tracking the engrafted tumors after xenograft, since pigments do not interfere with the fluorescence signal and the tumors can be localized precisely [129, 223]. As a result of toxicity screens on casper embryos, the heart edema caused by SFB was apparent especially after 48 hours of treatment. Pericardial edema was a phenotype that has been observed after SFB treatment in many studies [157, 163]. So, our results further supported this cardiotoxic effect of SFB in zebrafish. 2 μM of SFB also resulted in necrosis in the yolk, which was an indication of high toxicity [182]. On the other hand, TFP was tolerable for the larvae up to high doses, meaning that it was safe to be applied. Previously in zebrafish, TFP was not extensively studied. In one of the several studies in which TFP was included, TFP and two other phenothiazines were the only effective drugs that could rescue the phenotype of a zebrafish Parkinson's disease model in terms of movement [224]. Cornet et al. (2017) tested TFP HCl, the hydrochloride salt of TFP, on zebrafish from the point of mortality, cardiotoxicity, hepatotoxicity and motility [225]. The lethal concentration 50 (LC50) of TFP HCl was found as 31.62 μM for 96 hour-treatment starting from 0 hpf. This derivative was cardiotoxic to zebrafish larvae as a result of exposure from 96 hpf to 100 hpf. It also inhibited the locomotion significantly, while it did not affect liver area when applied from 96 hpf to 132 hpf. Given this limited number of studies, our findings were very helpful for a better understanding of the toxic effects of TFP. When it comes to the combination, the heart edema resulting from SFB application was observable for each combination group, however the overall body integrity of the larvae was not disrupted. But still, preserving the morphology of the zebrafish embryos as much as possible is highly important for xenograft studies. This is because, being a model organism, they serve as tools to allow efficient interpretation of the outcomes coming solely from tumors and any additional factor might have an unfavorable impact. Therefore, the toxicity screens were also performed on WT zebrafish embryos and it was checked whether they were more tolerable towards drug exposures.

SFB was found to show toxic effects in WT zebrafish embryos after 48 and 72 hours of exposure as shown in the literature. Heart edema and decrease in total body length were the most obvious among all. In the literature, there are different studies revealing that SFB results in cardiac dysfunction in zebrafish [158, 162]. Kawabata et al. (2015) showed that 1 μ M of SFB treatment starting from 3 dpf impaired ventricular dimensions and 3 μ M of SFB caused mortality at a high rate, leaving no alive larvae at 6 dpf [162]. What was observed after the screenings I performed supports the cardiotoxic effects caused by SFB.

Moreover, the highest dose of TFP, 24 μ M, was lethal to the larvae at 5 dpf. There were also necrotic tissues including liver and head of 4 dpf larvae at 24 μ M. The necrosis was more obvious for 5 dpf larvae. Although the darkening in the head and liver especially after 12 μ M may potentially point to necrosis, a better identification of necrosis might be performed by further investigation. Overall, TFP provided an advantage to obtain more efficacy when compared to SFB by not resulting in major abnormalities up to 12 μ M. When the two drugs were combined, the abnormality in the yolk and body was extreme at 5 dpf. Therefore, the applicable doses for xenograft experiments were chosen as low as possible, especially when the duration of the exposure was planned to be extended until 48 hours.

Herein, *in vivo* toxicity assessments were designed such that drug exposures were applied starting from the end of embryonic stage until 5 dpf of larval stage. This setup was helpful to make a statement on safety potential of the drugs tested, since larval observation provides an advantage in the sense that it enables to see all organs together. Additionally, drug screening using zebrafish holds great potential in clinics, which is beyond *in vitro* systems and provides leading information before moving to mice [226]. There are also studies in which certain molecules were identified on the basis of zebrafish screens without needing further confirmation by an additional model organism [226, 227]. As for the experimental setup, the fact that the formation of organs including liver was mostly complete by the end of specified time interval [183, 228] was also important for the evaluations we have made. However, zebrafish organ system continues to develop after 5 dpf [229], which might be influencing the internalization and resulting toxic effects of the drugs. Therefore, applying the drugs starting from 5 dpf instead of right after embryonic development might be an approach to better explain the metabolic outcomes of applying these drugs on zebrafish. If SFB and phenothiazine derivatives are poorly metabolized or show a direct toxic effect on functional organs, the toxicity profile would be entirely changed. Hence, exposure from 5 dpf to 7 dpf might enable us to obtain a much stronger evaluation for *in vivo* applicability of these drugs.

Apart from this, although adult zebrafish seems like an optimal tool for drug screens given its complete development, there are several disadvantages. The space taken up by adult zebrafish poses a problem in comparison to larvae [230]. Besides, full-pigmented body of adults prevents easy observation of the organs unlike transparency of early stage zebrafish [231]. These highlight the practicality of using larval zebrafish for elaborate screenings.

4.5 Xenograft studies implicated that drug dose is important

Xenograft studies using zebrafish have provided a different perspective on how TFP, SFB and the two drugs combined act upon tumor growth and size. The first xenograft experiment which was performed on casper embryos was an initiative to understand the importance of the dose applied to see changes caused by single or combination treatments (**Figure 24, Figure 25**). In the latter experiment, the analysis on tumor growth has revealed no change as a result of 24 hours of exposure to 12 μM of TFP, 2 μM of SFB and their combination when compared to DMSO control. Thus, we thought that this might point out the insufficiency of 24 hours for the drugs tested to regulate the mechanisms affecting Hep3B cell viability. Since more investigations could be required to fully understand the source of such an observation, the period of exposure was extended to 48 hour and the doses were decreased to half. This dose decrease was to compensate prolonged exposure which might have resulted in morphological deformations in the injected embryos. Hep3B cells survived more as a result of 6 μM of TFP exposure for 48 hours in comparison to DMSO group. It was also clear that TFP exerted the same effect when combined with 1 μM of SFB. When the studies that exist in the literature were checked, it was seen that anticancer agents were associated with decreased tumor growth in xenograft studies (please see introduction for details). However, in the cases where the efficacy of such agents could not be well-supported by in vivo xenograft studies, encapsulating them in nanoparticles served as a common and helpful strategy, eliminating high toxicity and insufficient efficiency. For example, in the study of Xie et al. (2018), growth of the tumors generated by engrafting MCF-7 cells to perivitelline space of zebrafish could be prevented much more efficiently with a novel doxorubicin-delivering nanoparticle in comparison to free doxorubicin [232]. Such a comparison for doxorubicin was performed in another study where tumors arising from the injection of HTC116 colon cancer cells into the yolk were inhibited significantly by nanocomposites loaded with doxorubicin compared to free doxorubicin [233]. Additionally, the nanocomposites exhibited much less cardiotoxicity comparatively, emphasizing the ability of such strategy to reduce unfavorable effects. Lipid nanocapsules delivering WS12, an agonist TRPM8, also served a purpose for the prevention

of TRPM-overexpressing prostatic cancer cells from metastasizing, enhancing anti-migratory effect of WS12 alone [234]. Although the anticancer potency of TFP and SFB could be shown in vitro, the toxicity caused by them may be prohibiting the ability to show their therapeutic role against Hep3B cells. Nonaocarriers may be a great solution to this problem, since they enable to direct the drugs precisely to the tumor site without having to expose zebrafish to direct toxic effects.

Besides, these findings were further confirmed based on the results of Alu-based DNA quantification, a quantitative method, modified from rodent models in our lab [179]. The impact of the treatment groups on human DNA amount after 48 hours of exposure was considerably parallel with changes in tumor growth. This finding verified the correlation between microscopic analysis which utilizes the fluorescence of Hep3B cells and Alu-based DNA quantification method. It also brought forward the efficiency and success of this method.

On the other hand, the microinjection that was performed to examine where Hep3B cells would localize after being injected into the cell portion of blastula stage embryos led to interesting results. Two methodologies were tried to be compared, one of which was trypsinizing the cells and the other was scraping the cells to prevent cell surface proteins from being affected [235]. At 3 dpi, the fluorescence was observable in only several embryos of the first group in the areas including yolk and eyes, while there was no fluorescence for the second methodology. At 5 dpi, the fluorescence was generally scattered through the body of the larvae which were injected with trypsinized Hep3B cells, being clearly visible in the gut area. For the larvae injected with scraped cells, the fluorescence could be observed in the gut area. There are research studies in which cancer cells were transplanted into zebrafish embryos at blastula stage. Pudenko et al. (2018) indicated that glioblastoma cells were localized in the nervous system of zebrafish 24 hours after injection, generating an efficient zebrafish glioblastoma model [236]. Injection of melanoma cells to blastula stage embryos resulted in cells being scattered in the body and not forming intact tumors [237]. It seems that the progress of transplanted cells vary based on the nature of cancer cell and our findings might lead future studies which will investigate the migratory action of HCC cells and the importance of the function of cell surface proteins.

From the point of methodology, xenografts which involve transplantation of cancer cells into the yolk sac are very challenging to manage and recapitulate [129]. It is very hard to provide

consistency in each embryo in terms of the number of cells injected. The exclusion of poorly injected embryos eventually results in small sample size, which may cause inconclusive results. In our experiments, we tried to use a minimum of twenty larvae per group and performed controls meticulously at every step. Even then we observed variation in the results. When these limitations are considered, although it is a practical method, xenografting might be regarded as a system which can lead to variations. However, it can be improved by creating an automated system for the number of cells injected or imaging. Actually, there is a study by Cabezas-Sainz et al. (2018) that was focused on analyzing the area and intensity coming from tumors in a standardized manner using a software called ZFtool [238]. If such strategies could be followed for pre-imaging processes as well, problems with reproducibility and time concerns could be eliminated.

Apart from the difficulty of methodology, the reason why our xenograft studies could not successfully reflect *in vitro* antitumor activity of single and combination treatments might be based on the experimental basis that we have followed. Since zebrafish xenograft models usually allow tracking cells more than 5 dpf [129], it might have been an improved strategy to follow how tumors generated by Hep3B cells were behaving for a longer period of time. However, considering the toxicity profiles of especially TFP-SFB combinations, prolonging exposure time was not possible. Another possibility might have been to start and end the exposure at a later time point. But still, finding the optimum conditions and generating an *in vivo* HCC model this way might have required an extensive effort and time. Therefore, finding alternative methods to obtain a zebrafish model of HCC rather than a xenograft model would be beneficial and more effective.

4.6 Microscopy was useful to identify toxic effects in a dose dependent manner

Apart from xenograft studies, microscopic examination of embryonic toxicities has revealed the morphological abnormalities caused by novel and intermediary phenothiazine derivatives, also enabled to point out the most suitable drugs for *in vivo* application. While PD-9, one of the intermediary derivatives, was not toxic to the zebrafish at the doses up to 5 μ M, PD-10 and PD-11 were found to show deadly effects. The dose just before the one which resulted in complete mortality for both molecules caused necrosis especially at 5 dpf. Moreover, PD-12 led to severe morphological defects at doses higher than 12 μ M and hence could not become prominent as an effective but non-deformative option. Among novel derivatives, only PD-3 and PD-6 could be lead candidates since they did not show any deleterious effect. Based on these observations, the drugs that are promisingly effective *in vivo* could be picked out.

By utilizing transgenic Fli1a:EGFP zebrafish larvae, the changes caused by TFP could be further evaluated in vivo. Fli1a:EGFP zebrafish line which has vascular system labeled due to GFP-expressing endothelial cells is very advantageous for especially angiogenesis studies and drug screens [239, 240]. There have been many studies that investigated how intersegmental vessels (ISVs) were morphologically changed after application of compounds to see if they exert anti-angiogenic activity [239, 240]. In our studies, GFP-labeled vasculature of this transgenic line enabled to locate liver in zebrafish body and hence the area and GFP intensity could be analyzed. However, methods that include better marking of the zebrafish liver tissue (e.g., transgenic strains with their liver tagged with dsRed [241, 242]) could be used. 6 μ M of TFP exposure which started from 5 dpf and continued for 48 hours did not seem to result in any significant changes on functional liver. When assessed from this aspect, the efficacy of TFP was not associated with liver size or vascularization. This could be due to experimental methodology as well as no effects on vascularization and maybe pursued further with higher doses, in which in vivo toxicity also seen. However exposure to TFP or SFB in late larvae for shorter periods could be tried for additional therapeutic options.

4.7 The conservation between human and zebrafish could be revealed within the scope of cholesterol biosynthesis genes

Zebrafish has been preferred as a model in many human disease studies due to numerous shared genes and high conservation [108]. Besides providing a great environment for tumor formation and drug screening, it could also be beneficial for the studies comparing genes across species [243]. Here, we thought that it would have been helpful to uncover the homology between human and zebrafish genes from the point of cholesterol biosynthesis. This way, we could successfully make a one-to-one gene comparison between the two species and determine sequence similarity.

The analysis performed on BioMart tool has shown that cholesterol biosynthesis pathway was conserved in zebrafish with all the genes having orthologues where more than 60% conservation of the genes HMGCR, SQLE and LSS was of great importance, since those were the genes being mainly focused on throughout this study.

HMGCR, the rate-limiting enzyme, had a sequence identity of 77.82% and 67.23% for zebrafish *hmgcra* and *hmgcrb* paralogues, respectively and turned out to be highly conserved in zebrafish. The last common ancestor of human with zebrafish for these paralogues is Gnathostomata, a superclass that is under Vertebrate classification and comprises of all jawed

vertebrates [244]. Investigation of HMGCR was important since the enzymatic reaction conducted by it has been the first to be targeted in cholesterol-related studies.

SQLE which has been suggested to mediate an important control point following HMGCR [245] was also present as a paralogous pair in zebrafish as *sqlea* and *sqleb*. They have shown 63.24% and 52.79% similarity to human SQLE, respectively. The last common ancestor with zebrafish was Gnathostomata as in HMGCR.

LSS is the enzyme responsible for the formation of lanosterol, the first sterol [246] and the precursor of cholesterol in the pathway [247]. Since it plays role in this key enzymatic reaction and is more close to the end-product, analyzing whether it had a zebrafish orthologue was important. Zebrafish *lss* gene was similar to LSS with a percentage of 74.73% and Vertebrate was the last common ancestor for the two species. Although Gnathostomata, the last common ancestor for HMGCR and SQLE, is one step closer to zebrafish in phylogeny [248] when compared to LSS, the percentage of *lss* gene identity to human orthologue enlightens its high conservation.

Herein, the following are the novel aspects that came out of the study given the lack or inadequacy of previous literature:

- 1) Phenothiazines are drugs that have therapeutic potential against HCC and are able to alter expression of genes involved in cholesterol biosynthesis both in vitro and in vivo, suggesting a lead for explaining how they exert their effects.
- 2) TFP and SFB combination might be a beneficial approach for anticancer purposes, however parameters including dose and time should be considered carefully. In this study, it could be effective by significantly increasing the expression of HMGCR and LSS genes in Huh7 cells, also by leading to enhanced expression of *hmgcs1* and *lss* in zebrafish.
- 3) Although tumor inhibition could not be observed as a result of TFP, SFB and combination applications, the consistency of microscopic analysis with Alu-based DNA quantification emphasized the usefulness of this methodology.
- 4) Zebrafish is a great model for in vivo drug screens and helped evaluate the toxicity of commercial, intermediary and novel phenothiazine derivatives elaborately. The derivatives that were promising in vitro showed varying toxic effects on zebrafish, enabling to scale down the ones that might be preferred clinically.

5) All human cholesterol biosynthesis genes have zebrafish orthologues and the sequences are highly conserved in zebrafish.

6) The impact of TFP on zebrafish larvae is not towards impairing liver vasculature when applied for 48 hours.

4.8 Future perspectives

I have provided the future perspectives for the sections I have discussed above and summarized what needs to be done to understand better my results or to follow them up:

The analysis of whether enhanced cholesterol levels are important for functioning of phenothiazines can be extended by exposing HCC cells or zebrafish embryos to exogenous cholesterol. Gene expression analyses can be elaborated by increasing the number of cholesterol-associated genes to be checked by RT-qPCR. Western blotting may contribute to this concept, enabling to see regulation of protein levels both *in vitro* and *in vivo*. Cholesterol-lowering drugs might also be utilized, even new combinatorial strategies with efficient anticancer activity and less *in vivo* toxicity can be developed.

For the detection of intracellular cholesterol levels, microscopic analysis instead of absorbance measurement might serve better for future studies to precisely determine where Filipin III-bound cholesterol is localized. Co-staining of the cells would also be helpful at this point to specify quantity and distribution of the cells. This way, the cellular structures that contain cholesterol and whether it accumulates due to impaired cholesterol trafficking can be better understood. It could also eliminate misinterpretation of absorbance results due to varying lethal effects of different derivatives and help reveal exactly through which mechanism takes part in altered cholesterol levels in the cells.

Zebrafish gene expression analysis should be improved. Problematic Ct values resulted in high differences between technical replicates and even lack of replicates for some groups. More number of larvae might be used per group to increase RNA concentration. Alternatively, RNA samples can be obtained from adult zebrafish instead of larvae, since organ-specific evaluations, especially for liver, might become possible.

Zebrafish drug screens may be evaluated more extensively to better understand how to determine safe doses for the derivatives and combinations, focusing on parameters such as measurement of pericardial edema and eye size. This way, different aspects of toxicity could

be discussed to extensively study the effects arising from the action of treatment options tested.

For xenograft studies, the sample size should be increased by injecting more number of embryos, providing a better evaluation. Moreover, performing xenograft experiments to see anticancer efficiency of 48 hour-treatment with TFP 12 μ M - SFB 2 μ M combination and also single drugs might be a good approach to see if extended duration of treatment would cause a difference in terms of anticancer efficacy. It might also help to make dose-dependent comparisons. Additionally, it might be required to test the anticancer efficiency of additional derivatives such as PD-3, which was found to be promising and not toxic to zebrafish up to high doses.

Further to these, zebrafish can be utilized from a different viewpoint to generate an HCC model, rather than transplanting Hep3B cells into zebrafish embryos. In the literature, a transgenic zebrafish (Tg(*fab10:EGFP-kras^{V12}*)) with expression of *kras^{V12}* oncogene under the promoter of *fabp10* which is a liver-specific gene was generated [249] and have been widely used in HCC studies. High expression of *kras^{V12}* in this model mediated efficient tumor progression. However, after some challenges such as early mortality appeared, new approaches that incorporated inducible systems were followed [250]. In one of the systems, mifepristone was used for such induction, enabling to keep tumor formation by *kras^{V12}* under control [251]. Another system enabled to generate a tetracycline-inducible (Tet-on) transgenic line in which liver tumors efficiently developed by means of doxycycline [252]. Therefore, employing such alternatives would be more beneficial to use a zebrafish HCC model for screening of SFB, phenothiazine derivatives and combination therapies. Lastly, since live-mounting was needed for microscopic examination of Nile red-stained zebrafish, other lipid-staining dyes such as oil red O or BODIPY [253] might help achieve more practical imaging and analysis.

Even though this thesis could shed light on important research topics, there are still many questions to be answered. If a follow-up study was designed to expand the findings obtained, there would be several approaches to pursue a better experimental strategy. First of all, cholesterol-based in vitro studies should be improved by going into details of molecular mechanisms. Preventing cells from taking up external cholesterol and also including statins as another set of drugs to test may be the first steps. As indicated above, a different zebrafish HCC model rather than xenograft models might be more helpful to obtain conclusive results,

since the anticancer activity of TFP, SFB and their combination could not be reflected well in the xenograft studies performed. The transgenic HCC model might provide a better environment for drug screening, eliminating the challenges of cancer cell transplantation and maybe enabling to confirm dose-dependent effects that were observed in vitro.



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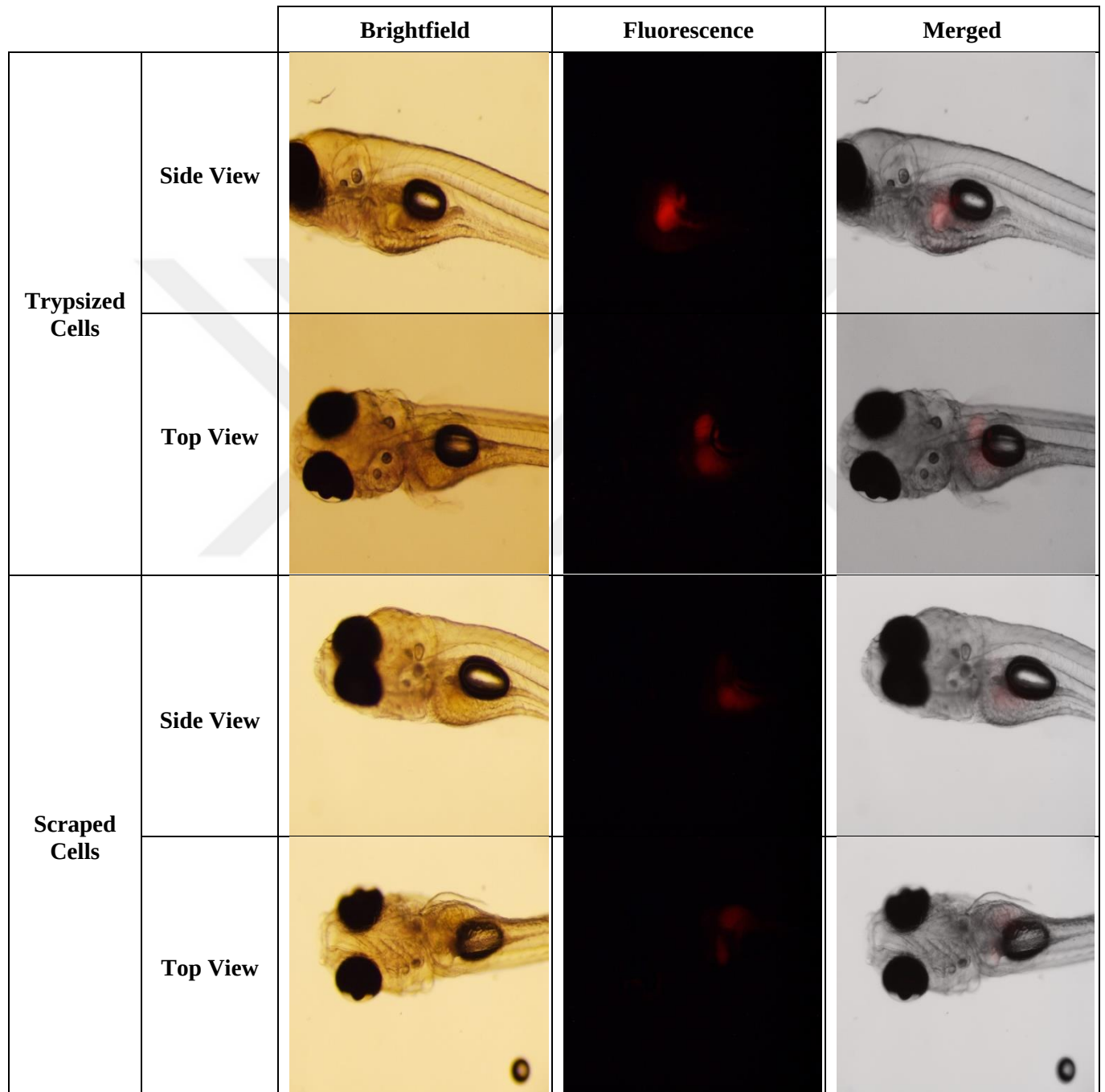
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APPENDIX

Appendix A: Transplantation of HCC cells at Blastula Stage



Appendix A Figure 1. Representative images of 5 dpi zebrafish larvae after injection with Hep3B cells at blastula stage (3.5 hpf). The cells were either trypsinized or scraped during preparation of the cells before injection.

Appendix B: RNA-seq Analysis for Differential Expression of Cholesterol-Related Genes in Hep3B cells

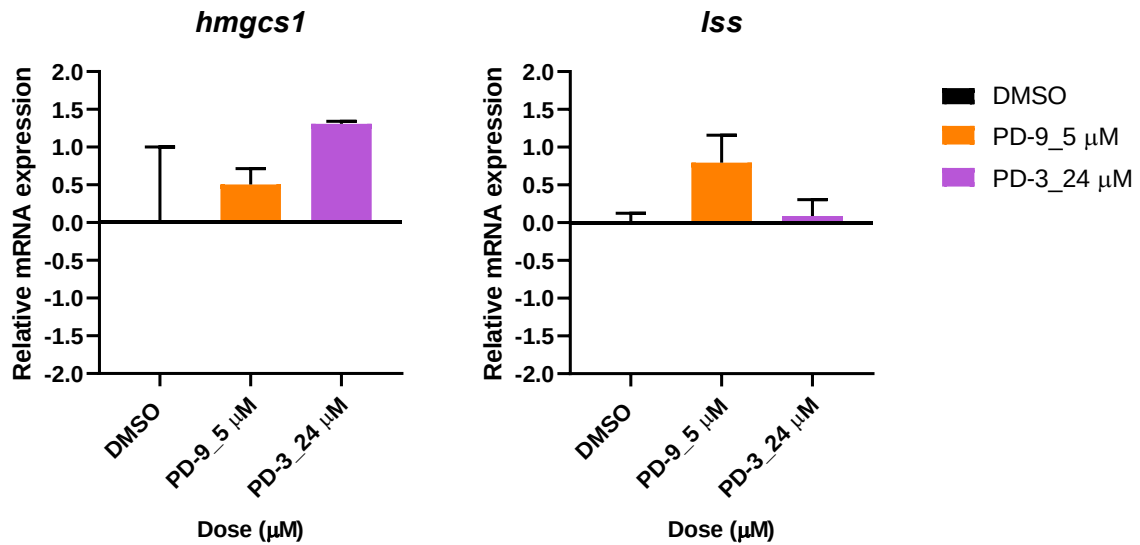
Appendix B Table 1. LogFC and adj. p-values of cholesterol biosynthesis and metabolism genes based on the RNA-seq analysis on Hep3B cells. (t1_control: TFP 12 μ M vs control, ts1_t1: TFP 12 μ M - SFB 1 μ M vs TFP 12 μ M, ts2_t1: TFP 12 μ M - SFB 2 μ M vs TFP 12 μ M, ts2_ts1: TFP 12 μ M - SFB 2 μ M vs TFP 12 μ M - SFB 1 μ M)

Gene	LogFC (t1_control)	adj. p-value (t1_control)	LogFC (ts1_t1)	adj. p-value (ts1_t1)	LogFC (ts2_t1)	adj. p-value (ts2_t1)	LogFC (ts2_ts1)	adj. p-value (ts2_ts1)
ABCA1	-1.17301	0.00001	-0.12711	0.52157	-0.64402	0.00672	-0.51691	0.04981
ABCG1	-2.15127	0.00269	1.97028	0.00565	1.32460	0.05083	-0.64567	0.25957
ACAA2	-0.24564	0.09579	-0.16597	0.29213	-0.12668	0.46345	0.03930	0.87838
ACACA	0.14706	0.24334	0.51812	0.00091	0.42395	0.00410	-0.09417	0.56442
ACACB	0.47197	0.20945	0.33856	0.34853	0.35544	0.34857	0.01688	0.97991
ACAT2	1.19208	0.00000	-0.19483	0.05067	-0.02295	0.85300	0.17188	0.15104
ACLY	0.71392	0.00000	0.21763	0.01386	0.22559	0.01564	0.00796	0.95441
ANGPTL3	-0.96760	0.00222	-0.04783	0.90516	-0.92663	0.02175	-0.87880	0.06307
ANGPTL4	-0.44179	0.40401	1.10510	0.03077	-1.12129	0.13980	-2.22639	0.01636
ANGPTL8	0.60715	0.00044	0.02598	0.88468	-0.47331	0.00449	-0.49930	0.00704
APOA1	0.58969	0.00011	-0.43410	0.00151	-0.46738	0.00127	-0.03328	0.85645
APOA2	0.12725	0.30885	0.15973	0.19561	-0.32483	0.01965	-0.48456	0.00418
APOA4	0.01901	0.98918	0.40906	0.71557	-1.21309	0.36187	-1.62215	0.30033
APOA5	0.37508	0.45058	-0.31833	0.55347	-0.51754	0.36662	-0.19921	0.82366
APOB	-0.22180	0.12650	-0.41480	0.00969	-0.80956	0.00019	-0.39476	0.03993
APOC1	0.10347	0.30223	0.10542	0.29402	-0.19432	0.07090	-0.29974	0.01800
APOC2	0.59265	0.09487	-0.46550	0.19986	-1.75074	0.00460	-1.28524	0.07344
APOC3	0.96260	0.00016	-0.47156	0.01692	-0.73531	0.00268	-0.26375	0.32385
APOE	0.32979	0.00076	0.22430	0.00962	0.13249	0.12092	-0.09182	0.35553
APOH	0.33025	0.09642	-1.27686	0.00011	-1.41069	0.00005	-0.13383	0.69130
ARV1	-0.18262	0.34190	-0.13513	0.52864	-0.25165	0.25386	-0.11653	0.70914
CD36	0.11590	0.44981	-0.62501	0.00099	-1.02881	0.00005	-0.40380	0.05163
CEL	0.27981	0.59813	-0.77224	0.16002	0.19487	0.73332	0.96711	0.14742
CES1	0.95368	0.03845	0.03780	0.94496	0.18039	0.70619	0.14259	0.82216
CFTR	0.02313	0.97891	-0.76719	0.30609	-1.50259	0.12120	-0.73541	0.60691
CNBP	-0.19259	0.04446	-0.25217	0.01428	-0.16530	0.11065	0.08687	0.50084
CYB5R3	0.09890	0.34377	-0.05008	0.67139	-0.21835	0.05232	-0.16827	0.18775
CYP24A1	-3.88706	0.05368	0.48171	0.87383	-1.30328	0.67268	-1.78499	0.63383
CYP27A1	0.70880	0.00020	-0.04742	0.78344	0.00232	0.99030	0.04974	0.82368
CYP2R1	0.08366	0.77530	0.45789	0.06480	0.65589	0.01418	0.19800	0.49959
CYP51A1	1.14458	0.00000	0.25533	0.01050	0.47540	0.00023	0.22007	0.04829
DHCR24	0.72556	0.00000	0.12238	0.16268	0.05562	0.57727	-0.06676	0.57231
DHCR7	1.10766	0.00000	0.25909	0.00515	0.34265	0.00113	0.08355	0.42192
EBP	1.27470	0.00000	-0.12963	0.22108	-0.10841	0.34333	0.02122	0.90380
ELOVL6	1.31388	0.00000	0.31910	0.00533	-0.00802	0.95569	-0.32712	0.01364
ERLIN1	-0.17241	0.17876	-0.08165	0.57250	-0.09500	0.52147	-0.01335	0.95304

ERLIN2	-0.12796	0.32120	0.10460	0.44047	0.24057	0.07888	0.13597	0.39715
FASN	0.80805	0.00000	0.62866	0.00006	0.39867	0.00102	-0.22999	0.04458
FDFT1	1.04764	0.00000	0.23834	0.02564	0.42689	0.00104	0.18854	0.12175
FDPS	1.08867	0.00000	0.06540	0.53366	0.04082	0.72397	-0.02458	0.87161
G6PD	0.08408	0.45544	0.13428	0.21427	0.17316	0.12940	0.03888	0.81231
GGPS1	-0.15182	0.25342	0.05529	0.72737	0.24102	0.09102	0.18573	0.25291
GPAM	0.08182	0.53542	-0.05904	0.68107	-0.10363	0.45198	-0.04459	0.81818
HMGCR	1.43171	0.00000	0.29781	0.00798	0.57625	0.00012	0.27844	0.02674
HMGCS1	1.86634	0.00000	0.21908	0.02212	0.53659	0.00009	0.31751	0.00688
HMGCS2	-1.56453	0.03110	0.08576	0.93846	-0.12168	0.91266	-0.20743	0.88061
HSD17B7	1.07962	0.00001	-0.07019	0.66021	0.14186	0.34146	0.21204	0.21158
IDI1	1.22651	0.00000	-0.06703	0.54243	-0.01822	0.88956	0.04881	0.74407
INSIG1	1.28161	0.00000	0.31279	0.01845	0.24739	0.06858	-0.06540	0.71844
INSIG2	0.34129	0.04391	0.26001	0.10948	0.12995	0.47664	-0.13005	0.55485
KPNB1	-0.12918	0.17510	-0.20811	0.03627	-0.05270	0.63795	0.15540	0.18970
LBR	-0.11174	0.27601	-0.28342	0.01091	-0.42843	0.00130	-0.14501	0.26721
LCAT	1.00325	0.01204	0.19869	0.58181	0.05277	0.90387	-0.14592	0.77569
LDLR	0.74523	0.00003	0.56036	0.00048	0.71723	0.00007	0.15687	0.26017
LDLRAP1	-0.15457	0.36976	0.23659	0.16465	0.01339	0.95587	-0.22320	0.30165
LIPA	0.81979	0.00004	0.29432	0.03131	0.36807	0.01350	0.07374	0.69222
LIPC	-0.00764	0.98315	0.63989	0.01581	0.53977	0.04628	-0.10012	0.77650
LIPG	0.12283	0.92903	0.10226	0.94207	-0.48884	0.71956	-0.59110	0.73401
LRP1	0.09436	0.50899	0.68155	0.00030	0.60773	0.00053	-0.07383	0.68559
LRPAP1	-0.12450	0.30252	0.01865	0.90681	-0.36306	0.01021	-0.38171	0.01636
LSS	1.43911	0.00000	0.14481	0.20987	0.20686	0.09121	0.06205	0.70615
MBTPS1	-0.03478	0.79855	0.00995	0.94943	0.02982	0.84084	0.01986	0.92098
MBTPS2	-0.01801	0.92734	-0.05464	0.76347	0.14033	0.38742	0.19498	0.29612
MSMO1	1.22383	0.00000	0.17987	0.09251	0.32377	0.00812	0.14390	0.26143
MVD	1.39415	0.00000	0.35396	0.00299	0.59346	0.00010	0.23950	0.05086
MVK	1.15030	0.00001	0.19155	0.20984	0.30497	0.06014	0.11341	0.57649
MYLIP	0.10309	0.84432	-0.36154	0.46153	-0.41787	0.43814	-0.05633	0.95037
NCEH1	-0.31182	0.06632	0.25115	0.14524	-0.02694	0.90760	-0.27809	0.20169
NFYA	-0.17849	0.12257	-0.20152	0.09062	-0.41804	0.00416	-0.21652	0.15505
NFYB	-0.15687	0.51081	-0.01732	0.95591	0.07405	0.79822	0.09137	0.80534
NFYC	-0.11149	0.43603	-0.12176	0.41049	0.15158	0.31358	0.27335	0.11242
NPC1	0.56643	0.00011	0.47004	0.00060	0.57425	0.00014	0.10421	0.41778
NPC1L1	0.64945	0.00020	0.24609	0.05825	0.63601	0.00026	0.38992	0.01271
NPC2	0.91819	0.00000	-0.04380	0.75688	-0.33398	0.01443	-0.29018	0.05278
NSDHL	1.03772	0.00000	-0.08192	0.51298	-0.04049	0.77555	0.04143	0.81904
PCSK9	1.52434	0.00000	0.47166	0.00031	0.52989	0.00011	0.05823	0.64440
PLPP6	0.21743	0.24274	0.05198	0.82132	0.23871	0.22082	0.18673	0.42565
PLTP	0.13735	0.66489	-0.38402	0.21457	-0.21365	0.53948	0.17037	0.72897
PMVK	0.14094	0.41308	0.15776	0.35311	0.26165	0.13384	0.10390	0.65273
POR	0.30156	0.00452	0.14141	0.13703	0.04652	0.67764	-0.09489	0.43402
PRKAA1	-0.08213	0.58289	-0.12332	0.40520	-0.01895	0.91976	0.10436	0.60032
PRKAA2	0.08560	0.63768	0.30350	0.06750	0.34646	0.05055	0.04295	0.86558

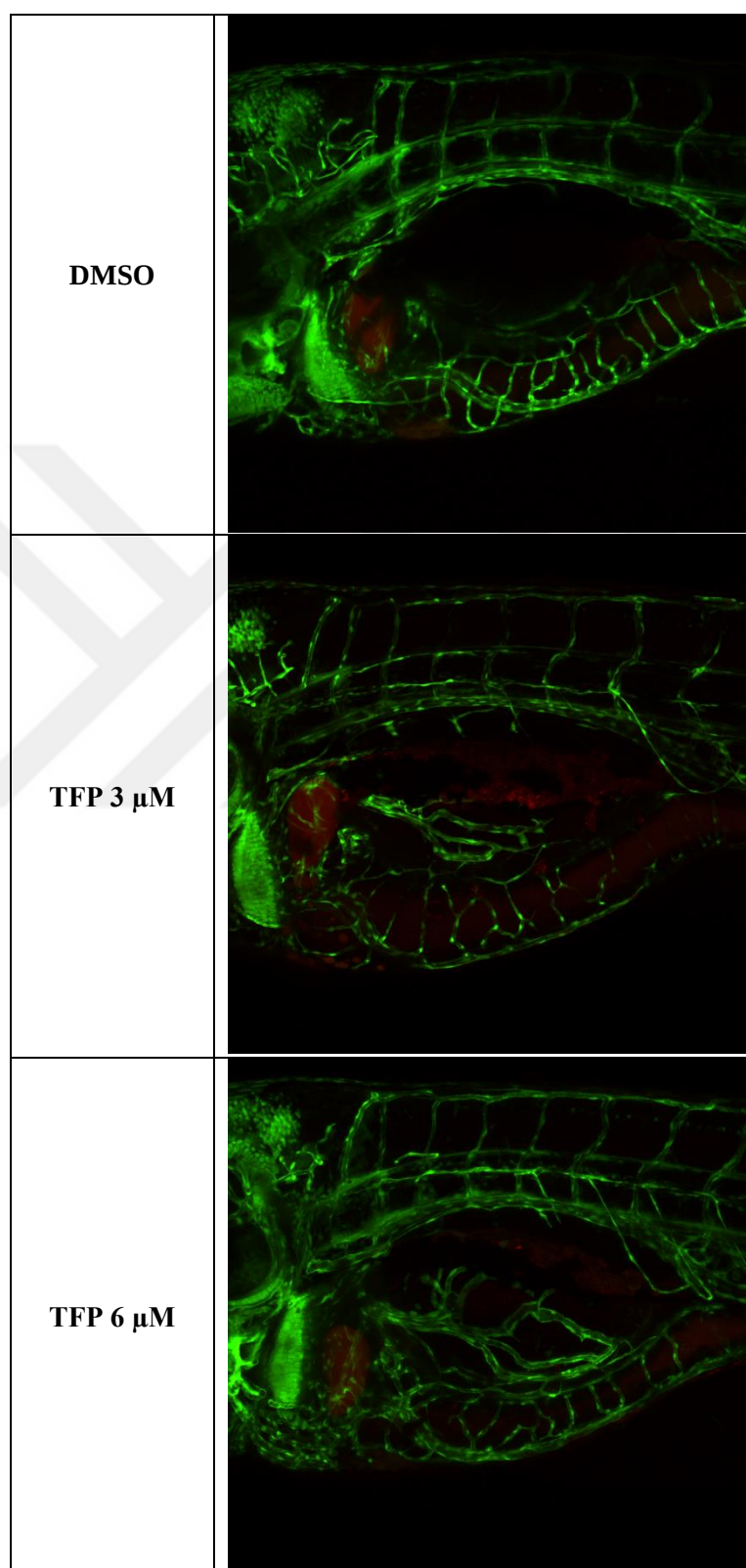
RAN	-0.33945	0.00177	-0.26002	0.01158	-0.29956	0.00762	-0.03954	0.79142
SC5D	1.23573	0.00000	0.02447	0.86961	0.14297	0.24307	0.11850	0.41459
SCAP	-0.01989	0.89135	0.12598	0.28391	0.31271	0.01530	0.18673	0.17640
SCARB1	0.31373	0.01014	-0.20847	0.06934	-0.59109	0.00032	-0.38262	0.01402
SCD	0.78051	0.00004	0.41424	0.00436	0.22559	0.10247	-0.18865	0.22530
SEC14L2	0.76971	0.04883	0.48392	0.15501	-0.30044	0.48636	-0.78436	0.09703
SOAT1	0.09001	0.51881	-0.01053	0.95230	-0.05349	0.73566	-0.04296	0.83588
SOAT2	0.49067	0.61515	-0.00874	0.99389	0.81219	0.33617	0.82093	0.39925
SOD1	-0.14652	0.22039	-0.37341	0.00621	-0.53203	0.00104	-0.15862	0.32622
SORT1	0.18799	0.05597	0.14666	0.13074	-0.07756	0.47617	-0.22422	0.06256
SP1	-0.09585	0.55650	-0.07841	0.66156	0.12444	0.46531	0.20285	0.29098
SQLE	1.09254	0.00000	0.16926	0.07648	0.32414	0.00410	0.15487	0.17388
SREBF1	-0.03963	0.75446	0.47550	0.00077	0.02548	0.85703	-0.45002	0.00298
SREBF2	0.54184	0.00004	0.32315	0.00240	0.61386	0.00003	0.29071	0.01016
STARD3	0.00149	0.99401	0.13362	0.30717	0.07844	0.59600	-0.05518	0.77872
TM7SF2	1.05064	0.00001	0.14480	0.33497	0.29427	0.06106	0.14947	0.41670
TSPO	0.30777	0.12252	0.09288	0.68251	-0.15978	0.48572	-0.25266	0.33504
VAPA	-0.08472	0.45553	-0.00773	0.95786	-0.00568	0.97101	0.00205	0.99370
VAPB	0.07585	0.61435	0.04262	0.80249	0.11154	0.46884	0.06892	0.73851
VDAC1	-0.14153	0.16423	-0.19298	0.06607	-0.35973	0.00434	-0.16675	0.20380
VDAC2	-0.01904	0.89277	-0.09370	0.43129	-0.03002	0.83212	0.06368	0.69951
VDAC3	-0.22121	0.06746	-0.47145	0.00195	-0.57343	0.00081	-0.10198	0.59017

Appendix C: Effects of PD-3 and PD-9 on the Expression of *hmgcs1* and *lss* zebrafish genes

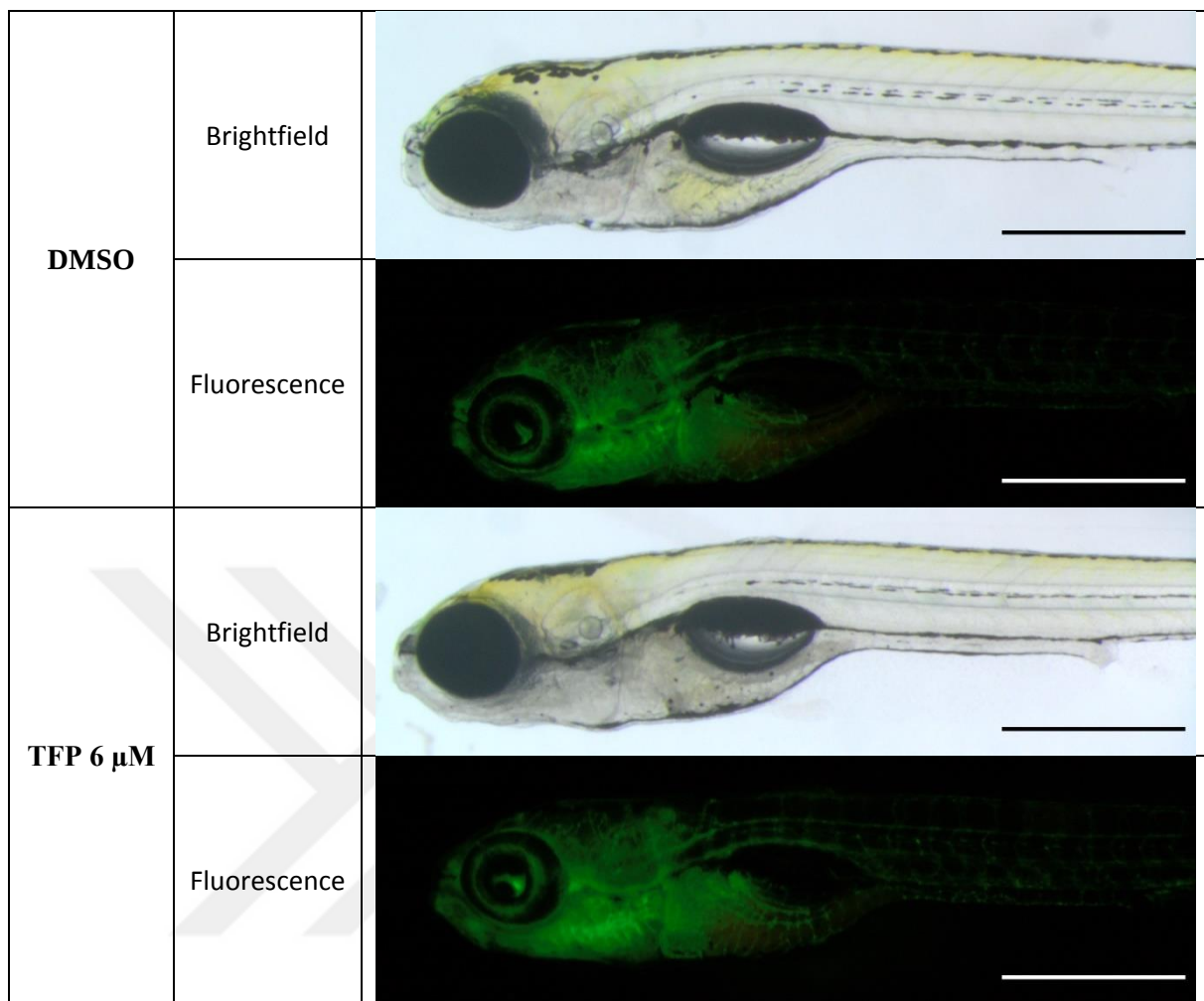


Appendix C Figure 1. Zebrafish gene expression profiles for *hmgcs1* and *lss* after treatment with PD-9 and PD-3 for 72 hours starting from 2 dpf. While y-axis shows relative expression to *actb2* reference gene, treatment groups are given in x-axis. Since PD-9 and PD-3 belong to the same experimental setup as TFP, SFB and combination, the same DMSO group was used. Expression values of *hmgcs1* and *lss* genes are given in log2 scale. One-way ANOVA (Dunnett's test) is the statistical analysis method used to compare each treatment group with DMSO control (*: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$).

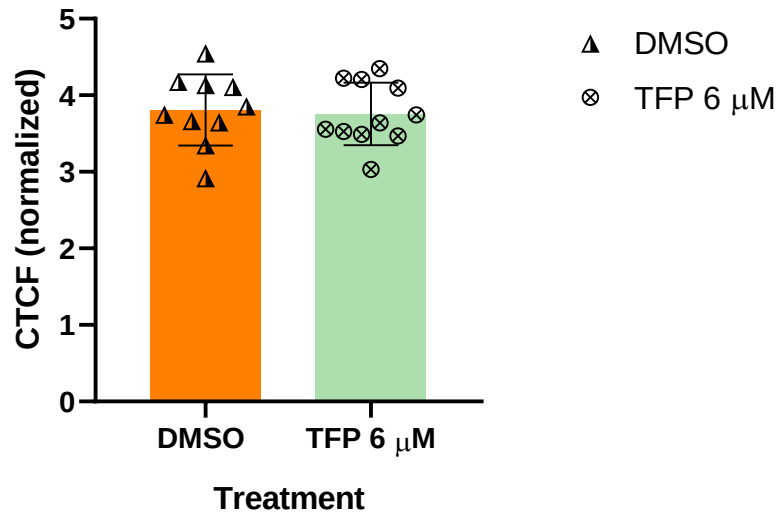
Appendix D: Nile Red Staining and Analysis of Zebrafish Liver Size after Exposure to TFP



Appendix D Figure 1. Nile red staining of 7 dpf *fli1a:EGFP* zebrafish larvae which were treated with 3 μ M and 6 μ M of TFP. The larvae were alive and immobilized in 1% agarose during imaging.



Appendix D Figure 2. Representative images of 7 dpf *fli1a:EGFP* zebrafish larvae treated with 6 μ M of TFP for 48 hours. All images were taken by using fluorescent stereomicroscope. Scale bar: 500 μ m.



Appendix D Figure 3. Fluorescence changes in 7 dpf *fli1a*:EGFP zebrafish larvae treated with 6 μ M of TFP for 48 hours in comparison to DMSO control. The treatment was performed from 5 dpf to 7 dpf for 48 hours. Normalized CTCF values were obtained by dividing GFP intensity values obtained from the yolk area involving liver and gut to that of the eye area. Unpaired t-test was applied as the statistical analysis method.