MINERALOCORTICOID AND GLUCOCORTICOID RECEPTORS AS NOVEL TARGETS IN BREAST AND LIVER CANCER THERAPIES

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Mineralocorticoid and Glucocorticoid Receptors as Novel Targets in Breast and Liver Cancer Therapies

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December 2020

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

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ABSTRACT

MINERALOCORTICOID AND GLUCOCORTICOID RECEPTORS AS NOVEL TARGETS IN BREAST AND LIVER CANCER THERAPIES

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MSc in Neuroscience

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Cell signaling is a complex phenomenon and is maintained through intertwined signal transmissions within and in-between the cells. Anti-cancer therapies are often challenged by this fact due to crosstalk-associated activation of alternative survival routes. Hence, development of new treatment strategies and identification of novel prognostic markers depends on in-depth knowledge on cell signaling routes altered in cancer and possible crosstalk paths. Herein, mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) signaling, two closely related members of steroid receptor hormone family, and their possible crosstalk were studied across breast and liver cancer cell lines. In breast cancer cell lines, estrogen responsive and MR expressing T47D was used in order to study possible crosstalk among Estrogen receptor (ER) and MR. MR-GR ligand aldosterone (ALDO) and ER ligand estrogen (E2) administered to breast cancer cells alone and in combination and, MR, ER and GR and their downstream signaling members were studied employing qRT-PCR and Western blot assays. Furthermore, ALDO, E2, ALDO-E2 hormone administrations were also used for cell viability assessments. Our results implied possible interactions of ALDO-E2 signaling at the level of cell viability, and at mRNA levels of progesterone receptor. In liver cancer cell lines, MR and GR was investigated as targets of a novel treatment. Liver cancer subtype hepatocellular carcinoma (HCC) has high mortality rate with limited treatment options. Multi-kinase inhibitor Sorafenib (SFB) with mild effectivity is most known systemic therapy against HCC. To potentiate the

effectiveness of SFB and overcome to the crosstalk associated limitations, combinatorial drug treatment approach targeting multiple signaling modalities has been adopted in literature. Previously in our lab, SFB was combined with repurposed anti-psychotic drug TFP as a novel combinatorial treatment against hepatocellular carcinoma (HCC) and liver cancer cell lines. Cellular viability was synergistically reduced by SFB-TFP in HCC cell line Hep3B, while antagonistic effects on viability in SkHep1 was apparent. Herein, two liver cancer cell lines Hep3B and SkHep1, were used in comparison to unravel mechanism of action of SFB-TFP combination at the protein level. Apoptosis, cell cycle, PI3K/AKT/mTOR and MAPK pathways were investigated in addition to MR and GR. Our results revealed several markers indicating success of drug combinations and targeted pathways at protein level which needs to be pursued further.

Key words: Steroid hormone receptors, Breast cancer, Drug repurposing, Drug combination, Phenothiazines, Sorafenib, hepatocellular carcinoma, Pathway crosstalk

ÖZET

MEME VE KARACİĞER KANSERLERİNDE ÖZGÜN HEDEFLER OLARAK MİNERALOKORTİKOİD VE GLUKOKORTİKOİD RESEPTÖRLERİNİN İNCELENMESİ

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Hücresel sinyal yolakları hücre içi ve hücreler arası sinyallerle yönetilen kompleks yapılardır. Kanser tedavileri çoğunlukla bu karmaşık ve iletişim halindeki yolaklar tarafından alternatif yaşam yolaklarının aktifleşmesi sebebiyle etkilerini kaybeder. Bu sebeple sinyal yolakları arasındaki ilişkileri deşifre etmek yeni tedaviler geliştirmek ve işaretçiler bulmak açısından önemlidir. Bu tez kapsamında yakın ilişkili olan iki adet steroid hormon reseptörü mineralocortikoid (MR) ve glucocortikoid (GR) reseptörleri meme kanseri ve karaciğer kanseri hücrelerinde incelenmiştir. Meme kanseri kapsamında östrojene duyarlı ve MR ifade eden T47D hücresi Östrojen reseptörü (ER) ve MR reseptörleri arasındaki olası iletişimi anlamak açısından kullanılmıştır. Bu amaçla hücreler ER ligandı östrojen ve/veya MR-GR ligandı aldosteron uygulamasına tabi tutulmuş ve MR, ER ve GR tarafından etkilenen genler mRNA ve protein seviyesinde incelenmiştir. Ayrıca hücre canlılığına olan etkilerde incelemelere dahil edilmiştir. Bulgularımız östrojen ve aldosteron sinyal yolaklarının iletişiminin progesteron reseptörü seviyesinde gerçekleşebildiğini ve ek olarak hücre canlılığı üzerinde de etkili olduğunu göstermiştir. Karaciğer kanseri hücrelerinde bu reseptörler özgün ilaç tedavisi hedefi olarak incelenmiştir. Karaciğer kanseri alt türü olan hepatocellüler karsinom (HCC) yüksek ölüm oranı ve kısıtlı tedavi olanakları nedeniyle kritiktir. HCC'ye karşı bilinen ilaçlardan birisi

kısıtlı bir etkiye sahip olan Sorafenib (SFB) dir. SFB'nin etkisini arttırabilmek ve yolak iletişimlerinden kaynaklı kısıtlamaları aşmak amacıyla birden fazla yolağı hedef alan ilaç kombinasyonları literatürde denenmektedir. Laboratuvarımızda daha önce özgün olarak SFB ilacı yeniden işlevlendirilmiş TFP ilacıyla beraber karaciğer kanseri hücre hatlarına uygulanmıştır. Hücresel canlılık sinerjik olarak Hep3B hücre hattında düşerken Skhep1 hücre hattında tam tersi antagonisttik bir etki görülmüştür. Bu çalışma kapsamında SFB-TFP kombinasyonunun etki mekanizması bu iki hücre hattında karşılaştırılmalı olarak protein seviyesinde incelenmiştir. Bu kapsamda apoptoz, hücre döngüsü, PI3K/AKT/mTOR ve MAPK sinyal yolaklarına ek olarak GR ve MR özgün ilaç hedefleri olarak incelenmiştir. Çalışmalar kapsamında kombine ilaç etkisi mekanizmasına dair ipuçları yakalanmıştır.

Anahtar kelimeler: Steroid hormon receptörleri, Meme kanseri, İlaçlarınyeni amaçlarla kullanılması, İlaç kombinasyonları, Fenotiyazin türevleri, Sorafenib, Hepatosellüler karsinom, Sinyal yolaklarının iletişimleri

For everybody still trying,

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CHAPTER 1

INTRODUCTION

Cancer is a heterogeneous disease with diverse drivers. By escaping the cellular control mechanisms on growth and division, cells can divide in unregulated manner. Hence, they can form benign or malignant tumors which can spread to tissues, organs throughout the body. Uncontrolled cancer cell behavior can be caused by accumulation of multiple underlying abnormalities such as mutations and replication errors. Continues proliferative signals, evasion from growth suppressors, resistance to cell death, invasion and metastasis, angiogenesis and replicative immortality are some of common features of cancers that has been reviewed in literature and new ones emerges as the knowledge on the field accumulates (Hanahan & Weinberg, 2011). Each cancer may have different formation and behavior pattern that should be analyzed within itself. Most cancers arise from the epithelial tissues that covers the organs and body. Carcinomas originated from the epithelial tissues can be classified as adenocarcinomas and squamous cell carcinomas (Weinberg, 2013); and breast and liver cancers are mostly adenocarcinomas. Breast cancers are more common in female population while mostly diagnosed liver cancer type, the hepatocellular carcinoma in men (HCC) with high mortality rates. Hence, studies focusing on these two cancers contribute to scientific knowledge that may lead better insight on diseases and treatment options for patients.

1.1 Breast cancer

Breast cancer is one of the most widely diagnosed cancer type among the females in the world. There is a small population with genetic predisposition that increase the risk of developing the disease, however most breast tumors arise sporadically. According to 2018's WHO data, 15% cancer related death of women is caused by breast cancer. Screening and early diagnosis are important for successful treatment against the disease. Although there are several known and developing treatment strategies are being studied around the world, there are still need for more effective options of treatments to eradicate disease or increase the life quality of patients.

Since cancer has a clinically and genetically heterogeneous structure, breast cancers have been classified into certain subtypes for differentiation of prognosis and treatment modalities (Harbeck et al., 2019). Criteria for these classifications are constantly changing as scientific world gains more detailed insight about the disease. There are several ways of classifications using histological, functional, and molecular profiles as basis (Malhotra et al., 2010). Intrinsic/molecular classification provides better insight on disease progression, prognosis, and new targeted therapeutic strategies (Dai et al., 2017). Hormone receptors, growth factors and proliferation markers are keys for the classifications among other markers added as the knowledge accumulates. As shown in **Figure1.1**, expression of 50 genes (PAM50) evaluated for intrinsic/molecular classification. Currently, surrogate intrinsic subtypes which are classified based on ER, PGR and HER2 status are used in clinical studies to differentiate hormone receptor positive and triple negative cases (Harbeck et al., 2019). However, there is a need to address the importance of other nuclear receptors such as Mineralocorticoid Receptor (MR) and its paralog Glucocorticoid Receptor (GR) in diagnosis and treatment of breast cancer.

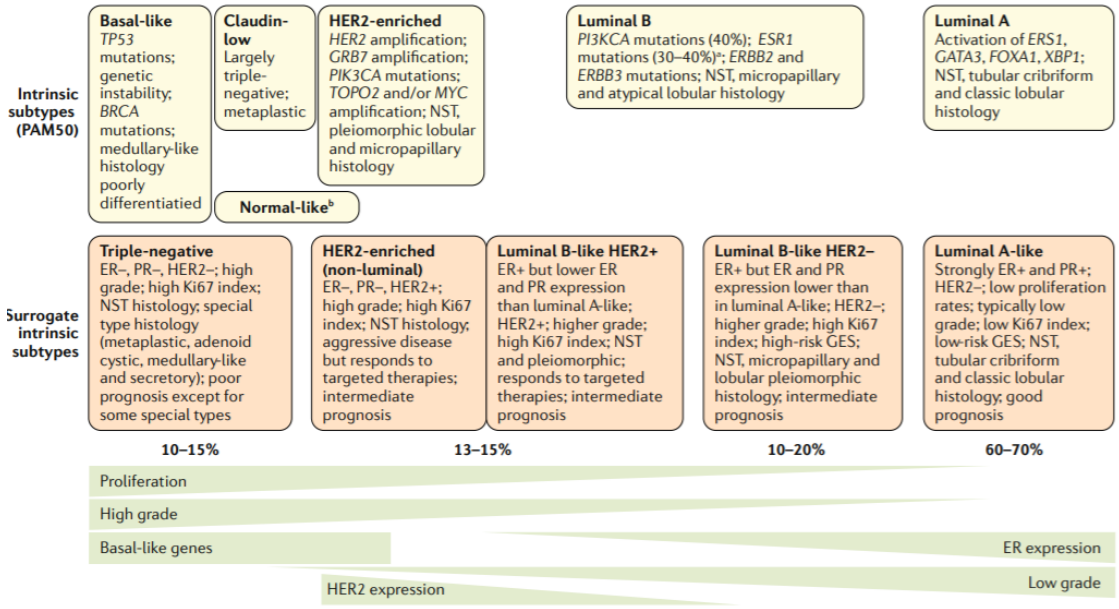


Figure1. 1 Molecular classification of breast cancers (*adapted from Harbeck et al., 2019*).

In this thesis, invasive ductal carcinoma cell line T47D (ER+, PR+, HER-) that has been classified as a part of Luminal A subtype which is highly common among diagnosed women together with Luminal B, was used as model (S. Yu et al., 2017).

1.2 Liver Cancer/Hepatocellular carcinoma

Liver cancer was ranked as the fourth main cause of the cancer related deaths worldwide by World Health Organization (*World Health Organization*, 2018). 5 years of survival chance for the liver cancer patients are the second worst after the pancreatic cancer with a rate of 18%. Moreover, among primary liver cancers hepatocellular carcinoma is the mostly encountered aggressive, solid liver cancer type (80%), and it is found to be more common in men than women. Existing liver dysfunctions, such as Hepatitis C and B viruses (HBC, HCV) and extreme alcohol consumption, have correlation with most of the diagnosed hepatocellular carcinoma cases (C. Y. Liu et al., 2015). HCC which is the sixth most common cancer with high mortality rate has known to be developed by alterations leading to dysregulation of below mentioned pathways with or without underlying reasons such as cirrhosis (**Figure1.2).**

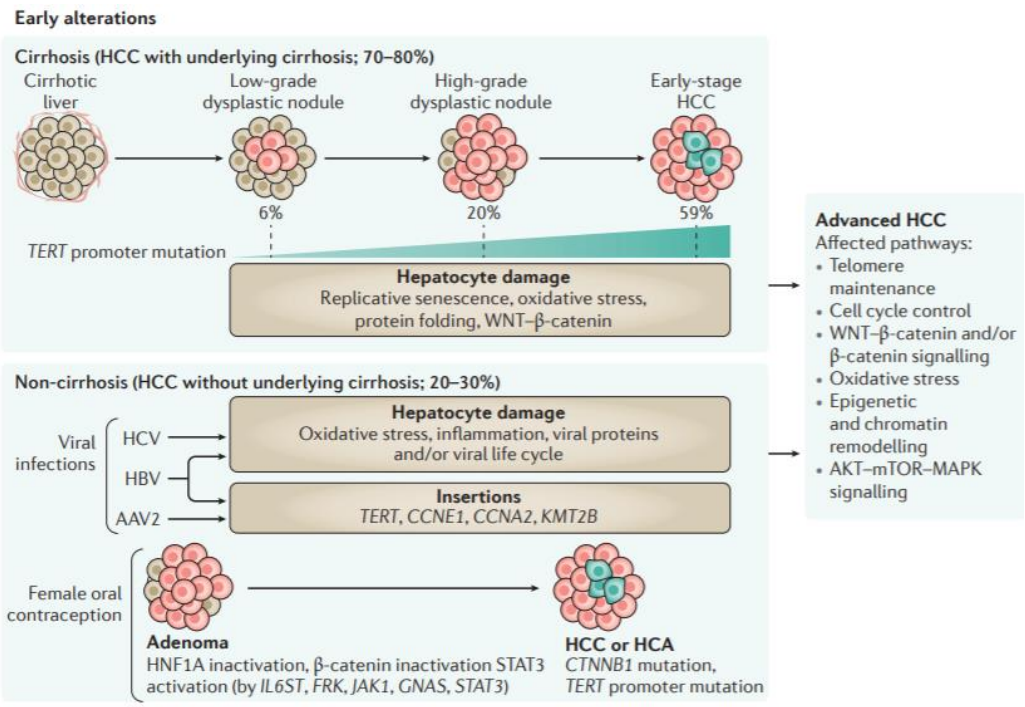


Figure1. 2 Liver carcinogenesis (*adapted from Llovet et al., 2016*)**.**

Accumulated changes such as mutations and chromosomal alterations in DNA of somatic cells correlate with the most of HCCs. Especially, mutations in the TERT promoter is the most frequent aberration followed by mutations in in cell cycle, WNT pathway and chromatin remodeling related genes (Hartke et al., 2017). Developing effective therapies is a challenge due to low number of somatic mutations and lack of distinctive mutations to estimate the

efficiency of therapeutics which underlies the seriousness of disease. If detected in early stages, there are highly invasive surgical treatment options. However, most of the time HCC is diagnosed at the advanced stages of the disease that has almost no treatment options. A multi-kinase inhibitor Sorafenib (SFB) was the only FDA approved first line treatment against HCC for long years. Recently, FDA approved new multi-kinase inhibitors and antibodies and combinatorial treatments to have better results than SFB (*FDA Approves Atezolizumab plus Bevacizumab for Unresectable Hepatocellular Carcinoma*, 2020; *Regorafenib*, 2017). Heterogeneity of the disease and acquired drug resistance cause restriction in therapies to have satisfactory benefits for patients. Hence, treatment strategies with better outcomes are needed for this detrimental disease such as combination of multi-drug modalities.

In this thesis, SkHep1 and Hep3B cell lines were used to model liver cancer and hepatocellular carcinoma. SkHep1 cell line which first time isolated from adenocarcinoma of liver and considered as HCC in 1975. However, origin of SkHep1 has been discovered as endothelial. Even though, this cell line is commonly used in cancer research; it may not be categorized as hepatocellular carcinoma. It differs from other HCCs at mRNA and protein levels while having endothelial features. In a more recent article, it has been suggested as model for liver sinusoidal endothelial cells rather than HCC (Y. Tai et al., 2018).

In 1980, Hep3B cell line was isolated from hepatocellular carcinoma of an HCC developed child and categorized as hepatoma derived cell line (Knowles et al., 1980; Yin et al., 2008). This p53 null cell line has been used as HCC model over the years. However, this fully differentiated, (Yuzugullu et al., 2009) epithelial hepatoma cell line has not been considered as a good model for HCC by a part of scientific community.

However, both SkHep1 and Hep3B are widely studied cell lines with respect to SFB alone or combination treatments thus make good models for subjects of my thesis. In addition, they represent fully differentiated and poorly differentiated models of liver cancers.

1.3 Signaling pathways altered in cancers

In cancer, genetic or epigenetic alterations leads the dysregulation of the critical pathways regulating normal physiology of the cells. Cell growth and division, death, and movement related pathways are the above-mentioned altered ones in general as the hallmarks of cancer. T-P53, cell cycle, MYC, PI3K/AKT/mTOR, RTK/RAS/MAPK pathways can be accepted as several of most altered pathway (Sanchez-Vega et al., 2018; Sever & Brugge, 2015) .These pathways are also common for breast cancer and hepatocellular carcinoma along with growth factor signalling (Guille et al., 2013; Moeini et al., 2012). Steroid hormone receptor (SHRs) family members as transcription factors can also be considered as the part of altered mechanisms in cancer as reviewed in Ahmad &Kumar 2011, especially in hormone driven cancers such as breast cancer (Ahmad & Kumar, 2011).

Complicated interactions of the above-mentioned pathways are crucial to have in-depth knowledge to understand pathophysiology of cancers and evaluating and discovering new treatment options. Interaction of major signalling pathways evolved to secure the homeostasis of cells however, cancer can take advantage of this to activate secondary routes of survival against treatments. Suppression of one cancer related pathway can activate many others through pathway interactions and feedback mechanisms (Prahallad & Bernards, 2016). Existence of crosstalk and redundancy of dysregulated pathways in cancers are known in breast and liver cancer literature. Interaction of pathways signifies the need for combinatorial treatments targeting multiple pathways acting together at once. In HCC tumorigenesis complex interaction of AKT/mTOR and RAS/MAPK pathways were previously evinced (Chunmei Wang et al., 2013). Since the crosstalk of AKT and ERK signalling is apparent in several cancers, co-inhibition draw attention against cancers (Cao et al., 2019). MAPK pathways (ERK, JNK, p38 MAPKs) can crosstalk within each other and both AKT/mTOR and MAPK pathways are regulators of cell growth, proliferation, and apoptosis. Moreover, Wnt/β-catenin, Notch and Hedgehog signalling pathways (among others) and their crosstalk with each other and other pathways contributing hepatocellular carcinoma should be considered in HCC studies (Giakoustidis et al., 2015). Steroid hormone receptor (SHR) crosstalk within each other and other pathways (including kinases) especially in hormone driven cancers, such as breast cancer, are also critical for endocrine therapy efficiency as mentioned above. Interaction of SHR family members and with growth factors, and MAPK superfamily was reviewed in literature for breast

cancer, too (Truong & Lange, 2018). Hence, interactions of pathways must be investigated for estimation of prognosis and therapy developments.

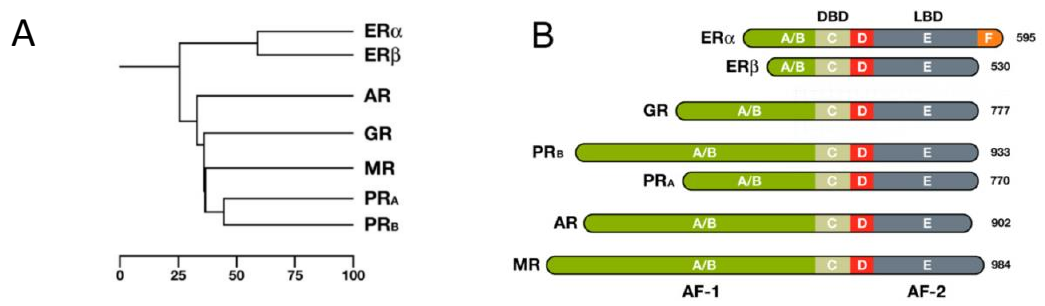
In the scope of this thesis, I have explained several cross-talking signaling pathways involved development and progression of breast and liver cancer/HCC which are also targeted by drug modules that was explained in this thesis.

1.3.1 Steroid Hormone Receptor family

One of the most known and abundant family of transcription factors is Nuclear Receptor (NR) Family of Transcription factors that can be activated by several, relevant ligands including steroids. This ligand activated transcription factor family can control the essential disease related pathways in cancers and regulate cellular homeostasis, development, and metabolism (P. Huang et al., 2009). Since NRs are critical for pathological or physiological functions, understanding the complicated relations in between the members might provide better insight for future drug targeting purposes or prognostic marker studies (Sever & Glass, 2013).

NR superfamily can be examined under three subfamilies: steroid hormone receptors, thyroid retinoid, and orphan receptors. In this thesis, focus is the “Steroid Hormone Receptor Family” activated by hydrophobic, lipid-soluble steroids.

Steroid Hormone Receptor Family (SHR) is one of the NR families that draw most of the attention. Members are listed as Estrogen (ER), Androgen (AR), Glucocorticoid (GR), Mineralocorticoid (MR) and Progesterone (PGR) receptors as in **Figure1.3**. They have evolutionary connections and share structural modules as in other NRs (Doan et al., 2017; Vladic, 2012).



C

Name	Nomenclature	Symbol	Entrez ID	Ligands	Expression in BC vs normal
Steroid hormone receptors					
Estrogen receptor	NR3A1	ESR1	2099	Estogens	Increased
	NR3A2	ESR2	2100	Estradiols	Decreased
3-Ketosteroid receptor	NR3C1	GR	2908	Cortisols (hydrocortisone)	Increased
	NR3C2	MR	4306	Aldosterone	Decreased
	NR3C3	PGR	5241	Progesterone	Expressed in BC
	NR3C4	AR	367	Testosterone, dihydrotestosterone	Increased

Figure1. 3 Steroid Hormone Receptors A) phylogenetic tree, B) shared structural modalities of family members, C) family members, relevant ligands and expression in breast cancer *(adapted from Szmuilowicz, et al 2006*).

SHRs share common structural modalities as showed in **Figure1.3** such as highly variable amino terminal with trans activation reagents (A/B, AF1). They all have central domain for DNA binding with Zinc finger domains (C). D domain is for translocation to nucleus. E, LBD domains are for the ligand binding in the conserved carboxy terminal which has role in heterodimeric interaction of the members. F is the carboxy terminal tail with a no detailly known function (Mangelsdorf et al., 1995).

These members can be found as monomers, homodimers, and heterodimers. They are associated with heat shock proteins before activated, in cytoplasm. When activated with appropriate ligand, conformational alterations followed by nuclear translocation and dimerization. Together with the relevant co-regulators they can induce the transcriptional alterations of several genes by binding hormone response elements (HRE) in DNA (W. Liu et al., 1995; Stepanova et al., 2006).

SHRs intricate interactions have been under attention and revealed for some of the members in literature. Especially in hormone driven cancers as in breast cancer their interactions are critical and under study to gain better understanding on cancer development, treatment, and prognosis (Ko & Balk, 2005; Truong & Lange, 2018).

As shown in **Figure1.3 (C)**, SHRs are known to be dysregulated in breast cancers. Moreover, inside of cells are filled with various signals including hormones hence, action of SHRs cannot be considered solely by single receptor-ligand interaction. Closely related SHRs can crosstalk in cancers to antagonize or potentiate each other in terms of transcriptional or non-genomic functions. In addition to crosstalk between family members, other critical pathway such as growth factor receptors and kinases also can be crosstalk partners in breast cancers (D'Uva & Lauriola, 2016; Need et al., 2012; Skandalis et al., 2014; Tonsing-Carter et al., 2019; Truong & Lange, 2018). These interactions may cause resistance against anti-SHRs focused treatments as in crosstalk of estrogen receptor/growth factor signaling which can be overcame by mechanistic understanding on it (Osborne et al., 2005). Hence, establishing the mechanism of crosstalk in SHRs and their possible outcomes can pave the way for more convenient therapy strategies against hormone driven cancers such as breast cancer. In terms of crosstalk in between SHRs in breast cancers, most of the interactions have been discovered except for MR's which needs further attention.

Even though SHRs mainly focused on hormone driven cancers, they are known to be expressed in cancers that are not driven by hormone which is worth to study. Since the HCC has more dominant occurrence among the male population than female, relationship of hormones to HCC has become an interesting subject. SHR members, especially ER and AR has been found to be key modulators in HCC at several levels of development and progression of disease (Truong & Lange, 2018).

Therefore, cancer caused alterations, cross-talks among family members and drug induced expressional changes of SHRs are relevant to both breast and hepatocellular carcinomas. In this thesis, several members of SHRs, namely MR, GR, ER and PGR, are explained in detail in terms of functions, targets, and crosstalk to underly the importance of the aims of this project for breast cancer and HCC literature.

1.3.1.1 Mineralocorticoid receptor and aldosterone in cancer

Mineralocorticoid Receptor (MR) encoded by NR3C1gene is a member of SHRs that has more recently been focused on in literature since it was under shadow of closely related GR. Aldosterone is a mineralocorticoid that is synthesized from zona glomerulosa and regulated by

renin-angiotensin system (RAS). MR and its primary ligand, aldosterone found to act on epithelial tissues such as distal nephrons to balance sodium-water (fluid homoeostasis) in kidneys via transcriptional regulations. Physiological functions that MR signaling involves can be listed as blood pressure, intracellular pH, cardiac action potential etc.(Ong & Young, 2017). In addition to its activity in epithelial cells, MR activity is present in neurons, cardiac cells, adipocytes and macrophages (Marzolla et al., 2014,McCann et al., 2019). Disease related alterations in MR-ALDO activity have also been reported in hypertension (hyperaldosteronism), cardiac failure, some nervous system disorders such as depression which underlies need for extended research on MR for therapeutic action (Barrett et al., 2013; Jia et al., 2017). MR signaling involves in oxidative stress, inflammation, endothelial dysfunction among other critical functions (Bigas et al., 2018; Nagase et al., 2012). Artificial MR antagonists have been used as medicine against heart failure, chronic kidney disease etc. such as spironolactone, eplerenone, finerenone, and mexrenone (Y. Nagata et al., 2019).

MR mRNA undergoes to alternative splicing event (Alnemri et al., 1991). MR has multiple isoforms and protein variants which might be tissue specific with differential functions (Maria Christina Zennaro et al., 1997). Moreover, MR is subject to post transcriptional modifications including phosphorylation (Bloem et al., 1995; M. C. Zennaro et al., 1995). Rapid phosphorylation of MR linked to ion transport, and phosphorylation in ligand binding domain can cause impaired transcriptional activity (Tallec & Lombès, 2005).

As mentioned before, SHRs are closely related and have similar activation processes. Ligands other than aldosterone can interact with MR such as glucocorticoids (cortisol/corticosterone), and progesterone (mostly antagonize) with same affinity (Fuller et al., 2019; Myles & Funder, 1996; Ong & Young, 2017a). Hence, there should be a selectivity mechanism that can lead differential activation of MR and GR in different functional contexts in crowded environment. 11β -hydroxysteroid dehydrogenase (11HSD2) is the enzyme regulates cell/tissue specific regulation of receptor activation by converting cortisol to cortisone allowing ALDO-MR interaction in epithelial cells. Besides, ALDO-MR interaction is more stable than other interactions which supports specific regulation.

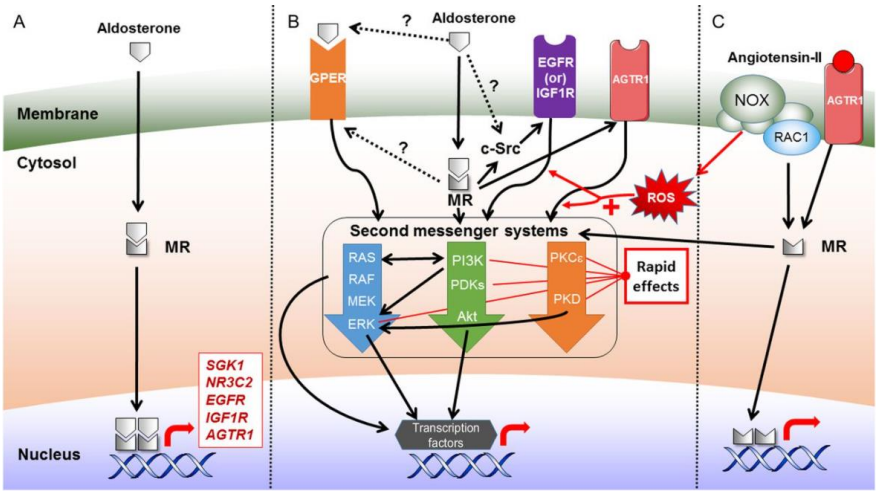


Figure1. 4 Aldosterone activated MR action *(adapted from Ong & Young, 2017*)

MR-ALDO actions classified under three categories as shown in **Figure1.4** (Gekle et al., 1998; Ong & Young, 2017). Transcriptional activity of MR-ALDO action is relatively time-consuming process takes couple of hours due to activation-translocation-transcription-translation cascade. Regulation of several cellular processes including signaling, stress, inflammation, structure, migration, and many others trough alterations of MR, SGK1, ENaC, IGFR, EGFR, AGTR1 and some of the genes is possible by this genomic action (Viengchareun et al., 2007). Fast responded, non-genomic actions can be triggered within minutes by usage of secondary messengers. Effectors of this rapid activation are MAPK, PI3K and PKC/D signaling pathways that have critical roles in cellular trafficking, survival, migration etc.(Grossmann et al., 2005; Nagoshi et al., 2012).As shown in **Figure 1.4** aldosterone-MR signaling is highly intertwined with other signaling pathways in terms of crosstalk and interactions. It can interact with or transactivate other hormone activated receptors in rapid action such as GPER, EGFR, IGFR etc. (Griol-Charhbili et al., 2011; Rigiracciolo et al., 2015).

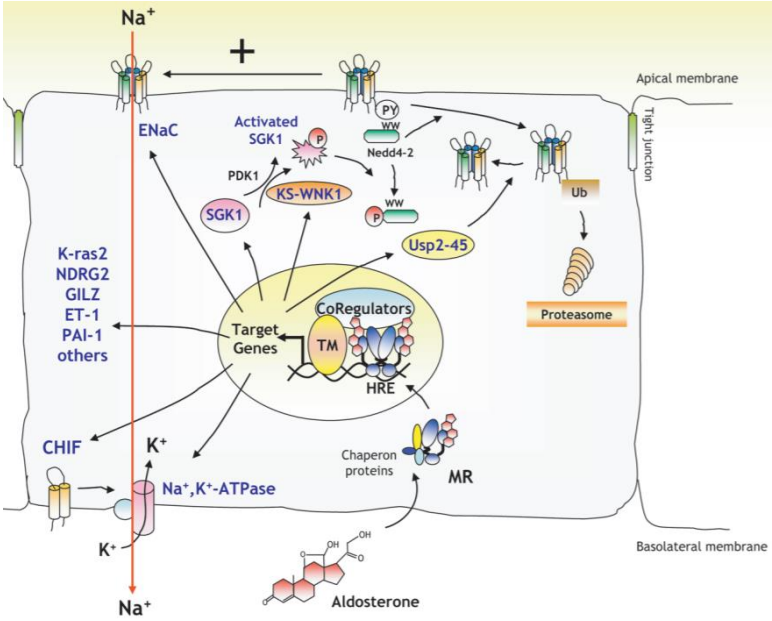


Figure1. 5. MR and ALDO action in polarized epithelial kidney*(adapted from Viengchareun et al., 2007*)

In **Figure 1.5** transcriptional activity of the MR-ALDO signaling in epithelial cells were exemplified. In epithelial cells upon activation by ALDO, cytoplasmic MR dissociates from chaperons (such as Hsp90) and conformational changes reveal nuclear translocation signal. Nuclear translocated and dimerized active MR acts on transcription of target genes. Hormone response element (HRE) in DNA of target genes is triggered together with co-activators. Among various transcriptional targets, MR itself, Na^+ channel ENaC and serum and glucocorticoid-regulated kinase- 1 (SGK1) are the ones that take part on ion homeostasis in renal epithelial cells and investigated in this thesis. In addition to the direct transcriptional activation on ENaC and SGK1, SGK1 activation by phosphorylation can lead ubiquitin protein ligase NEDD4-2 phosphorylation which loosens up its interaction and prevents proteasomal degradation of apical epithelial sodium channel ENaC (Debonneville et al., 2001; Valinsky et al., 2018). It is also shown than SGK1 can enhance αENaC subunit transcription (D. Pearce & Kleyman, 2007).

MR and aldosterone mediated signaling in cancer is a more recent area in literature compared to other well-known family members such as ER, PGR, AR signaling. Lung, breast, liver, colon, kidney, pancreas, and prostate are examples of cancers investigated in literature in terms of MR and its pro or anti-cancer functions. In non-small cell lung cancer, its expression has been shown to be correlated with the good prognosis, hence its role predicted as protective against lung

tumor progression (Jeong et al., 2010; Konu & Targen, 2019). Tumor suppressive function of MR which has reverse interaction with VEGFR-2 also identified in colorectal cancer patients with better survival with high expression (Di Fabio et al., 2007). In pancreatic ductal adenocarcinoma its tumor suppressiveness has been suggested while in prostate cancer crosstalk with AR is draw the attention on MR (Shiota et al., 2018; S. Yang et al., 2016).In renal cell neoplasm MR and 11β-HSD2 expression has been proposed to be used in subtype specification (Yakirevich et al., 2008). Moreover, another study shows involvement of ALDO to progression of renal cancer by upregulation of specific KRas isoform and yet another study indicates that persistent ALDO exposure can cause carcinogenesis via ERK1/2 and STAT3 related signals in kidney (King et al., 2014) . MR also tested in a targeted drug delivery system in cancer cells in terms of apoptosis induction and promising results were obtained for used cell lines (P. Sharma et al., 2016). In the scope of this thesis, MR in breast and liver cancer is detailed in paragraph below.

In HCC cell lines proliferation, cell cycle progression and apoptosis has been found to be altered by MR-ALDO activation (Nie et al., 2015). This phenomenon explained by MR's transcriptional action on miR-338-3p/pyruvate kinase and suppression of Warburg effect which accelerates proliferation in cancers. Further *in vivo* study in mouse model also has supported MR-ALDO action in HCC which draws attention to MR-ALDO action as anti-cancer target and prognostic marker (Nie et al., 2015). Moreover, in liver fibrosis, non-genomic MR-ALDO action in hepatic satellite cells was discovered (Münzberg et al., 2015).

SHRs member MR is probably the less known and studied them all, especially for breast cancer. Even though there are studies on this receptor in other cancer types such as lung cancer and hepatocellular carcinoma as mentioned above, their role in breast cancer is relatively less studied (Konu and Targen, 2019).

For long time now MR expression in mammary tissue has been known and more recently it was found that GR expression is absent during some mammary gland development stages can substituted by MR in mouse models (Jääskeläinen et al., 2019; Kingsley-Kallesen et al., 2002; Raynaud & Rolland, 1981). However, its function on tumorigenesis and tumor progression has received more recent attention in breast carcinomas. In a study on ductal differentiation of breast carcinomas, MR was identified as related to this differentiation (Sasano et al., 1997). In another study focusing breast cancer patients revelated that there is a distinction between TNBC and non-TNBC patients in terms of prognostic value of SHRs highlighting MR. Moreover,

cytoplasmic MR expression was correlated with worse relapse free survival in HER$^-$ patients and exist in opposite trend TNBC (Jääskeläinen et al., 2019).

SHR family with structurally and functionally intertwined signaling members have raised the question of MR's crosstalk with other members in breast cancer. Previously expression of MR has been established for several breast cancer cell lines by Bircan Coban (MSc Thesis, 2016). Furthermore, separate ALDO and E2 exposures in several breast cancer cell lines were investigated in terms of possible crosstalk at the levels of previously determined ALDO-MR signaling targets and effectors which are mentioned below (MSc Thesis, 2016). Promising leads on Bircan Coban's results were the basis of the work in this thesis.

1.3.1.1.1 Serum and glucocorticoid-regulated kinases

Evolutionary conserved AGC family's (protein kinase A, protein kinase G, and protein kinase C) serine/threonine subfamily member "serum and glucocorticoid-regulated kinases" has three members SGK1, SGK2, and SGK3 (L. R. Pearce et al., 2010). They have been known to be part of mechanisms regulating ion channel activity, survival, proliferation, migration, cell cycle etc. (Hong et al., 2008).

Even though SGK1's primary function has thought to be on ion transport across membranes, its other critical functions have been revealed in time involving cancer and immunology. It even has been targeted in major depressive disorder to develop better antidepressant therapies due to its functions controlled by glucocorticoids in brain (Dattilo et al., 2020). It is discovered to be another effector of PI3K signaling, which is critical in cancers in addition to AKT (they have sequence homology). Hence, it has been marked as a possible therapeutic target in cancers (Di Cristofano, 2017; Orlacchio et al., 2017). SGK1 can be induced by mineralocorticoids, stress, growth factors and cytokines. Moreover, short-term transcription of SGK-1 mRNA takes place with the half-life of 20 minutes (Waldegger et al., 1997). As an effector of PI3K signaling, its neoplastic transformation ability is evaluated in cancers. Its role has been known for colon, prostate, myeloma, and ovarian cancers (Lang et al., 2010; Z. Zhang et al., 2020). In non-small cell lung carcinoma, expression of SGK1 is correlated with worse prognosis(Z. Tang et al., 2018). Moreover, downregulation of SGK1 by shRNA results in decreased cancer cell existence which shows itself in dysregulated cell cycle markers in multiple myeloma cells

(Fagerli et al., 2011). Besides its known functions, there are still other targets awaiting to be discovered that can contribute to SGK1 function in cancers (Di Cristofano, 2017). In liver regeneration SGK1 acts together with ERK2 and increase its activity (Won et al., 2009). From a study adopting human microarray data profiling in normal and HCC tissues, SGK1 also was shown to be over expressed in HCC (Chung et al., 2002). For the scope of this thesis, SGK1's role in breast cancer detailed in following paragraph.

PI3K signaling pathway activation is critical for breast cancers due to pathway's anti-apoptotic functions. Importance of activation of PI3K/ SGK1 levels in stressed cells is known in breast cancer survival (Sahoo et al., 2005). In a breast cancer patient study, SGK1 is found to be overexpressed in 48% of the cases with high correlation with p-AKT-1 overexpression (Sahoo et al., 2005). SGK1 expression was also correlated to resistance against AKT inhibitors in another study (Sommer et al., 2013). Metastasis study in breast cancer has revealed that SGK1 can promote metastasis together with PDGF and their double silencing cause stronger anti-cancer effect (L. Yang et al., 2020). Bone metastasis through breast cancer has also been related to SGK1 expression in a mouse model study (Z. Zhang et al., 2020). In MCF7 cells, it has been shown that membrane Androgen Receptor (AR) inhibition causes pro-apoptotic alteration which can be boost by SGK1 inhibition (G. Liu et al., 2015). Its role in cancers has been established in literature within the scope of PI3K action however, how SGK1 is regulated by MR-Aldosterone signaling in breast cancer models need to be studied for more detailed, mechanistic understanding.

1.3.1.1.2 Neuronally expressed developmentally downregulated 4L/NEDD4-2

NEDD4 (Neuronally expressed developmentally downregulated 4) is a family of E3 ubiquitin ligases with 9 members that has been investigated in scope of cancer development and progression apart from Na^+ homeostasis (Ye, 2012). As mentioned earlier, ALDO-MR signaling controls the Na^+ balance through ENaC and other cotransporters in renal epithelial cells which is critical for blood pressure regulation and ion homeostasis. ENaC levels were regulated by ubiquitination through a member of NEDD4 family NEDD4-2 (ubiquitin mediated endocytosis and proteasomal degradation) by ALDO induced SGK1 phosphorylation to prevent ENaC-NEDD4-2 interaction causing degradation (Arroyo et al., 2011). In addition to this well-known function, NEDD4L has a vast number of ubiquitination targets that may explain its

involvement in many other signaling pathways including MAPK (JNK), PI3K- AKT (PIK3CA), TGFβ (Hallows et al., 2010; Imamura et al., 2013; Z. Wang et al., 2016).

Role of the NEDD4L in the several cancers has been known in literature for a while. In colorectal cancer patients, it has been suggested as a tumor suppressor by its repressive effect on WNT signaling (Tanksley et al., 2013). Prognostic value of NEDD4L which is negatively correlated with HIF1α also has been acknowledged in gastric cancer patients since reduced expression of NEDD4L has been correlated with invasion and metastasis (C. Gao et al., 2012; X. Jiang et al., 2019). Similar downregulation in tumors are also apparent in malignant glioma patients (S. He et al., 2012). However, cancer specific action might be the case for NEDD4L since its over expression has been associated with gallbladder cancer invasion (Takeuchi et al., 2011). HCC has also been investigated in literature in terms of NEDD4L levels which indicated a reduction in tumors when compared to normal tissue. Mechanistic understanding of how NEDD4L acts as a tumor suppressor in HCC is explained through its action on MAPK/ ERK related apoptosis induction (F. Zhao et al., 2018). In breast cancer, NEDD4L expression which is repressed by miR-106b-25 clusters is also linked to better relapse free survival (Guarnieri et al., 2018). Another study focusing on androgen regulated NEDD4L levels revealed NEDD4-2 isoforms are highly expressed in mammary glands and differentially exist in breast cancer cell lines (H. Qi et al., 2003). Even though some other members of this family have been found to be functioning in breast cancer (Zou et al., 2015), studies explaining proper function of NEDD4L and its regulation by MR-ALDO signaling is missing in the literature.

1.3.1.1.3 Epithelial sodium channel ENaC/SCNN1A-B-G

Epithelial sodium channel (ENaC) which has been mentioned to regulated by ALDO-MR to balance blood pressure, sodium absorption, and extracellular fluid volume, and is composed of three homologous subunits: α, β, γ ENaCs (1:1:1 heterotrimer). This Degenerin/ENaC mechanosensitive protein family member has a role in lung airway surface liquid balance, urinary bladder, sweat glands etc. besides of above-mentioned functions in kidney and colon (Boscardin et al., 2016). This channel located in apical membrane has activity regulated by cleavage in α, γ ENaCs by proteases beside NEDD4-2 induced ubiquitination and others (Hallows et al., 2010; Kleyman et al., 2006; Rossier & Jackson Stutts, 2009). ENaC activity can also be regulated by ROS and PI3K signaling in distal nephron cells (Q. S. Wang et al.,

2018) and TGFβ action through SMAD4 (Chang et al., 2008). Their interaction with p38 MAPK and ERK MAPK is also known in literature (Niisato et al., 2007). ENaCs role in several pathologies has been known for a while including cancers (Mehta, 2008; Mirshahi et al., 1998; Xu et al., 2016).

ENaC/DEG family members are known for their roles in cancer development and progression including ENaCs in terms of invasion, migration, and proliferation (C. Liu et al., 2016). αENaC has been determined as a therapeutic target in in small cell lung cancer/neuroendocrine tumor (M. He et al., 2018). Same subunit has also related to proliferation of liver cancer cell line HepG2 (Bondarava et al., 2009).This isoform's increment with ALDO or other cancer related abnormalities can cause migration (Jagirdar et al., 2016). Knockdown of α and, γ ENaCs causes reduction in glioma migration that makes these channels anticancer targets (Kapoor et al., 2009, 2011).

ENaC expression has been established both in human and mouse mammary gland and cancer cells. Steroid hormone dependent induction of ENaC also is investigated in cancer cell lines T47D and MCF7 together with noncancerous breast cells for dexamethasone, progesterone, and prolactin (Boyd & Náray-Fejes-Tóth, 2007). Dexamethasone increased the expression of subunits except α in both cancer cells while the same trend is only observed in T47D with progesterone. ERK signaling activation by ENaC abundance in membrane has been shown by another study in breast cancer cells (Soundararajan et al., 2009). Moreover, γ ENaCs has been identified having role in inflammatory injury with probable pro-carcinogenic effect caused by excessive salt in breast cancers (Amara et al., 2016). Hence, there is a limited number of studies on ENaCs in breast cancers at all or related to MR-ALDO-ENaC relation.

1.3.1.2 Glucocorticoid receptor and glucocorticoids in cancer

Glucocorticoid Receptor (GR/NR3C1) has 5 isoforms with different localizations and functions; and two of them, α and β, have been focused on more in literature (I. Wu et al., 2013; J. Zhou & Cidlowski, 2005). GR is activated by glucocorticoids (corticosteroids and dexamethasone) and act on transcriptional activity as other SHR (through glucocorticoid response elements) (Jantzen et al., 1987). Widely expressed, dominant α form is the responsible one for known action of the receptor such as immune response-inflammation, proliferation

(Pufall, 2015). PI3K and GR interaction has been found to be part of innate immune response (Arancibia et al., 2011).However, β isoforms found to be repress MR activity hence regulate ALDO sensitivity of cells and GRα independent from glucocorticoids (Bamberger et al., 1997). In breast cancer research, α isoform is the focus but there is evidence suggesting β isoform's involvement, as well (Vilasco et al., 2011).

GRs and glucocorticoids have been investigated in several cancers in literature such as lung, prostate, breast, endometrial, bladder cancer, and pancreas cancer(Hu & Chen, 2017; McBeth et al., 2016; Tangen et al., 2017; Taylor et al., 2016). Tumor progression related function of GR in association with AR has been assessed in prostate cancer (S. Narayanan et al., 2016). GR expression has been found to be contributing to apoptosis in lymphoid malignancies through BCL family members (Schmidt et al., 2004; West et al., 2018). Glucocorticoid receptor activation causing cell cycle arrest in cancer via FOXO1 was previously shown (Prekovic et al., 2019), glucocorticoids are in use against liver failure for a long time (Xue & Meng, 2019). Non-alcoholic fatty liver disease with a risk of HCC development can be improved by targeting glucocorticoids and GR action (Woods et al., 2015). In hepatoma cell line HepG2 glucocorticoids and GR has been associated with tumor growth (Psarra et al., 2005). Moreover, in TNBC cell line MDA-MB-231, GRα agonized by Pseudopterosin causes the anti-tumor effect (Sperlich & Teusch, 2018). In a breast cancer cohort ER positiveness and negatives have been found to be differentially related to GR expression levels. In positive patients high GR expression correlates with the better prognosis when compared to negative in which GR expression is a negative prognostic marker(D. Pan et al., 2011). Another study suggested that co-expression of ER and GR cause less aggressive breast cancer outcome (West et al., 2016) . GR draw attention as a target in a study in TNBC, in which antagonizing GR increased apoptosis induction in resistant cells (Skor et al., 2013). Hence, consequences of GR expression have context dependent action in breast cancer which depends on its interaction with ER. In addition to effects on tumor growth and progression, GR has also been shown to modulate metastasis and can induce EMT trough GR- insulin receptor substrate 1- ERK2 signaling in breast cancer (W. Shi et al., 2019). ER-GR crosstalk has also been established in endometrial cancer which results in more aggressive tumor progression (Vahrenkamp et al., 2018). Crosstalk of the SHR members and their compensation for each other has been shown for AR-GR, and ER-GR crosstalk. MR-GR interaction has been shown in skin and a rat mammary cell line (Pooley et al., 2020; Sevilla & Pérez, 2018). Moreover, above mentioned MR target genes and downstream members can also be altered by GR activities. Glucocorticoids have found to

be regulating SGK1 level in lung and renal epithelial cells, and in rat brain (Hinds et al., 2017; Itani et al., 2002). In human distal airway epithelial cell line glucocorticoid dependent induction of αENaC has been shown (McTavish et al., 2009). Hence, SGK1-NEDD2-2-ENaC action can also be controlled by Glucocorticoid/GR actions.

1.3.1.3 Estrogen receptors and estrogen in cancer

Primary female sex hormone estrogen (estrone, estradiol-dominant in productive years-, estriol, and estretrol) is synthesized primarily in ovaries and regulate reproduction, menstruation, bone density, brain functions, inflammation besides from development of female sexual organs as part of its broad spectrum of functions (Hua et al., 2018; Novella et al., 2012). Even though it has striking functions in females, estrogen is also crucial for male reproductivity, spermatogenesis (Carreau et al., 2011). In this thesis, 17β-estradiol (E2) has been used as estrogen. Estrogens show their function mostly through the Estrogen Receptors (ER) which act like the other members of the SHRs as mentioned above. ERα (ESR1), ERβ (ESR2), and more recently discovered trans-membrane G protein-coupled estrogen receptor (GPER-rapid non-genomic response) are mainly investigated E2 receptors (Prossnitz & Arterburn, 2015). Activated receptors can bind Estrogen Receptor Elements (EREs) of DNA to induce transcriptional alterations on relevant genes as direct activity (Klinge, 2001). However, there are indirectly regulated genes which do not have ERE element in their promoters. This type of activations regulated by protein interactions of transcription factors such as AP-1 and Sp-1 (Björnström & Sjöberg, 2005). Regulation of cyclinD1, IGFI and NF-kb etc. is dependent to in-direct activity of ER (Umayahara et al., 1994). α and β receptors might have opposite transcriptional effects on genes as in cyclin D1 which is repressed by ERβ while ERα cause production (M. M. Liu et al., 2002). When both receptors are expressed, they can act on the same targets in opposite directions (Madeira et al., 2013; Nilsson & Gustafsson, 2011). Non-genomic action of GPER1 acts on secondary messengers to regulate signaling pathways such as MAPK and PI3K/AKT (Prossnitz & Barton, 2014). Ligand independent actions of ER through phosphorylation have also been known (Vrtačnik et al., 2014; Yuanzhong Wang et al., 2014)

In lung, colon, endometrial, ovary, prostate and breast cancers, increasing levels of estrogen have been related to tumor development or progression and related angiogenesis (Liang &

Shang, 2013). In sexually dimorphic HCC, ERα expression has been found as protective against tumorigenesis in mouse models while AR acts in opposite direction. Moreover, there are studies suggesting ER downregulation in HCC (Chaturantabut et al., 2019; Ohnishi et al., 1986; Y. Zhao & Li, 2015). In HepG2, an HCC cell line, E2 has been shown as anti-proliferative and pro-apoptotic. Moreover, in Hep3B cell line, ESR2 (ERβ) gene expression evaluated to be against HCC in a ligand dependent manner (Y. S. Liu et al., 2015). In another study in colon cancer, possible protective act of estrogen was associated with ERβ (Stevanato Filho et al., 2018).

ER is the most investigated SHR in the family in terms of breast cancer research both as a diagnostic marker and therapeutic target since expression of ER is prominent most of the cases (Stevanato Filho et al., 2018). E2/ER signaling leads to proliferation and cell cycle progression and may suppress apoptosis by regulating its direct or indirect target gene expression (T. T. Y. Wang & Phang, 1995). However, high doses of estrogen may have opposite, pro-apoptotic results (R. X. D. Song & Santen, 2003). E2/ER signaling can crosstalk with vast number of pathways critical for cellular metabolism and survival in cancer including other members of SHRs family. For example, E2/ER can crosstalk with growth hormone/factors such as EGFR/IGF-IR signaling pathways, and others such as PGR, GR, MR and NF-kB (Katzenellenbogen, 2000; Smith, 1998; Tonsing-Carter et al., 2019; Vahrenkamp et al., 2018). In a study applying E2 to ovariectomized female rats showed that, E2 treatment can reduce both MR and GR mRNA levels in hippocampus (Burgess & Handa, 1993). In vascular context, MR and ERs have opposite functions. Excessive activation of the MR by ALDO can cause oxidative stress (ROS production) in vascular smooth muscle cells which is shown to be suppressed by active ERs preventing the NAPDH deficiency in cells (Muehlfelder et al., 2012) In breast cancer, GPER has also been shown to support ALDO's action on ERK/EFGR pathway which contributes to proliferation in breast cancer cell line SKBR3 (Caroccia et al., 2019; Rigiracciolo et al., 2015). ALDO's adverse cardiovascular effects has been mostly seen in post-menopausal woman hence it was hypothesized that E2 levels might have protective role against ALDO's effect (Barrett Mueller et al., 2014). When they have examined ALDO-activated MR and E2-activated ERα in HEK293, the ER has exhibited negative effect on MR transcription activation. This inhibition was validated with the observation of E2's inhibition of ALDO induced ICAM-1 gene activation. On the contrary there was no evidence suggesting MR' suppressive or potentiating effect on ER. In this study, they suggested that upon activation, ER and MR interact in nucleus and ER control the MR's transcriptional activity (Barrett Mueller et al., 2014). The

powerful interaction has been proven in this study underlies the crosstalk between SHRs which propose the importance of revealing MR/ALDO-ER/E2 interaction in breast cancer.

E2 treatment to ovariectomized rat results in restored SGK1 levels in myocardium. However, this was not observed in E2 treated rat cardiomyocytes suggesting E2 regulates SGK1 levels by an indirect mechanism (Cong et al., 2015). In the case of endometriosis, E2 caused increase in SGK1 expression which leads anti-apoptotic results has been shown to be controlled by ERβ (Monsivais et al., 2016). ER and PI3K signaling pathway can crosstalk in breast cancer and, recently SGK1 has been shown to have role in this phenomenon in terms of ER transcriptional regulation. Moreover E2/ER can control the SGK1 activation when PI3Kα is inhibited (Castel & Toska, 2019). Rapamycin resistance in breast cancer cell lines has been shown to be related to SGK1 (Ser442) levels in ER positive cells hence, silencing of SGK1 in ER negative MDA-MB-231 increased the effects of drug (Hall et al., 2012). ENaC levels with E2 treatment is variable in different tissues and, contradictory studies exist in literature. Increase in γENaC activity has been shown in kidney cortical collecting duct cells via non-genomic action of E2. Another study revealed increased levels of αENaC in kidneys of E2 treated ovariectomized rats (Gambling et al., 2004). An *in vivo* study performed in rats revealed that E2 has regulatory function on NEDD4-2 in kidneys, moreover, causes reduction in ENaC expression in mouse collecting duct cell line through ubiquitination (X. Zhang et al., 2019).

1.3.1.4 Progesterone receptors and progestin in cancer

Progesterone receptor (PGR) is the last member of SHRs that has been covered in the context of this thesis. This well-known receptor known to be expressed primarily in female reproductive systems. Upon activation proliferation, development and differentiation of the relevant tissues can be controlled by its actions (Grimm et al., 2016). Ligand progesterone can be produced during of menstrual cycle, and its expression have also been established in adrenal glands and placenta during the pregnancy (development and lactation) (Trabert et al., 2019). There are vast numbers of literature supporting on progestin treatments increasing risk of breast cancer development while it may show opposite protective effects on other cancer types such as ovary and lung (Banks et al., 2003; Cenciarini & Proietti, 2019; Hunter et al., 2010; Knutson & Lange, 2014). There is little or almost no expression in HCC according to study using HCC patient

data (Nagasue et al., 1991). Interestingly, there are specific cases that progestins can interact with AR and GR and less commonly MR and ER as reviewed in literature (Moore et al., 2012).

There are two common PGR isoforms: isoform B with full length and isoform A with truncated amino terminal domain with overlapping and distinct actions. PGR is expressed in ERα expressing cell lines and considered as marker for proper ERα activation (Horwitz et al., 1978). B isoform has more activity upon hormonal activation and A isoform has been associated with repressive function on progestin actions (Giangrande & Mcdonnell, 1999; Richer et al., 2002). In breast cancer 1:1 ratio of these receptors can be altered in the favor of isoform A which is caused by high A isoform levels or loss of B isoform. Moreover, PRB is related to bad response to chemotherapy (Cork et al., 2008; Hopp et al., 2004). PGRs can be subject of post-translational modifications including phosphorylation as reviewed in literature as possible effectors on activity of receptors (Abdel-Hafiz & Horwitz, 2014). As in other family members PGRs can act directly or indirectly on transcription and there are also non-genomic functions (Cenciarini & Proietti, 2019).

ER and PGR interaction has been known for a while. Progestin induced PGR and ERα crosstalk via kinase activation is known in literature (Diep et al., 2016). ER-PRA crosstalk through miRNAs has been investigated to reveal progesterone's role as invasion and migration inducer against E2's effect on luminal breast cancers (McFall et al., 2018; Mohammed et al., 2015). Another interesting study on MR gender specific expression in female mice endothelial cells showed increased MR activation through active PGR (Faulkner & Belin de Chantemèle, 2019). Progesterone itself known to have high affinity for the MR which competes with ALDO (Baker & Katsu, 2020). In neurons, *in vivo* and *in vitro*, progesterone treatment increases the activation of MR (Castrén et al., 1995). A-isoform of human PGR has been identified as MR repressor without any mechanistic knowledge on interaction but with a suggestion on competition for common TFs (McDonnell et al., 1994). Relevant to context of this thesis, MR, GR and PGR crosstalk has been suggested in breast cancer focusing on focal adhesion and growth in literature. This study proposes that treatment of relevant ligands in PGR expressing cell lines mimics progesterone induced alterations in cell lines such as induction of focal adhesion and inhibition of cellular growth (J. C. L. Leo et al., 2004). PGR's crosstalk to other signaling mechanisms are known. ERK MAPK- PGR interaction in breast cancer has been explained since dysregulation of ERK signal suppressed PGR induced gene expression (Treviño et al., 2013). Moreover, in endometrial cancers resistant to progestin induced autophagy can crosstalk at the level of PI3K/AKT/mTOR-PGR as escape mechanism (H. Liu et al., 2017).

Regardless of PR expression progestins shown to upregulate the SGK1 levels in breast cancer cell lines (Godbole et al., 2018). In another study, progesterone's suppressive effect on ENaC opening probability has been associated with NEDD4L actions in *Xenopus* oocyte model (Michlig et al., 2005).

1.3.2 Apoptosis signaling pathway

Genetically controlled cell death mechanisms are the essential part of development and homeostasis. There are several cell death mechanisms including necroptosis, ferroptosis, apoptosis which are reviewed in cancer literature (Karsch-Bluman et al., 2019). As mentioned before, one of the main hallmarks of cancer has been determined as the escape from the apoptosis (A. Sharma et al., 2019). In here, we are focusing on programmed cell death, apoptosis. Other than being a widely dysregulated pathway in cancers, alterations in apoptosis found to be related to resistance to anti-cancer therapies as in HCC (Lohitesh et al., 2018; Pommier et al., 2004). Hence, it is often targeted in anti-cancer research.

Apoptosis has two pathways: intrinsic (mitochondrial) and extrinsic (death receptor mediated) apoptosis as shown in **Figure1. 6**. Cysteinyl, aspartate-specific proteases, caspases are identified as executioners: caspase 3, 6, 7 (and initiators caspase 2,8,9,10) of the apoptosis which are the merging point for both pathways. Auto-proteolytic cleavage of initiators with help of adapters lead to cleavage of executors leading cleavage of cellular components to induce morphological and chemical changes such as condensation of chromatins, plasma membrane asymmetry, cell shrinkage and budding of membrane in a controlled manner (A. Sharma et al., 2019).

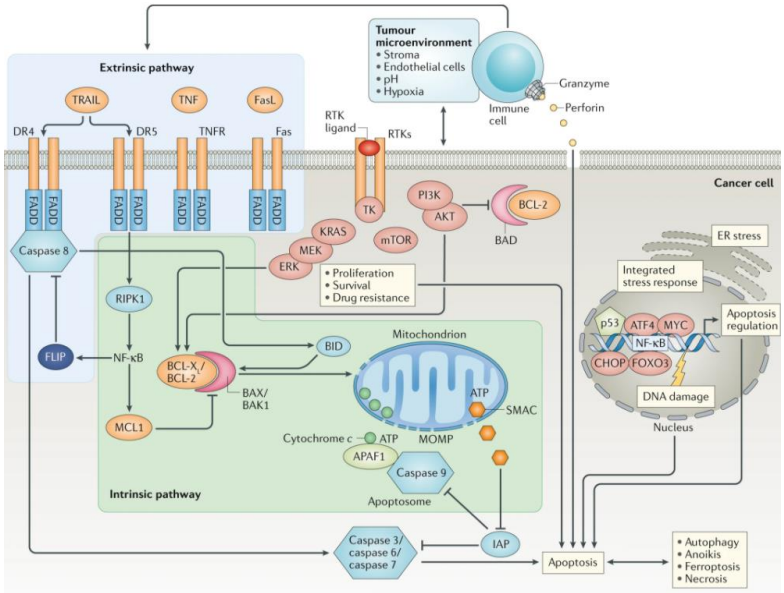


Figure1. 6 Apoptosis pathways (*adapted from Carneiro & El-Deiry, 2020*).

Extrinsic pathway is activated by extracellular signals depending on death receptors which are part of tumor necrosis factor receptor (TNFR) family. These extrinsic receptors activation, TNFR1-TNFα, FAS-FasL, DR4-TRAIL, DR5-TRAIL, lead to conformational changes revealing death domains of the receptors. FADD/TRADD adaptors and initiators caspase 8 and 10 form a complex with receptors which is called death-inducing signaling complex (DISC) which shifts the balance for pro-apoptotic proteins and eventually merge to executor caspases for apoptosis (Carneiro & El-Deiry, 2020).

CASP8 has been shown to contribute to hepatocarcinogenesis in early steps and associated with less aggressive cancer when its expression is lower (Boege et al., 2017; Stupack, 2013). Reduced CASP8 levels has been reported in several cancers such as in neuroblastoma, previously (Tummers & Green, 2017). Cascade of cellular events that activates this caspase starts with the activated death receptors such as FAS and DR5. DR5 receptor has two isoforms. Moreover, TRAIL dependent DR5 action has been associated to pro-survival of tumours which is related to MAPK pathway (Shlyakhtina et al., 2017). Another study supports this idea by showing the involvement of p-ERK1/2 in blocked CASP8 induced apoptosis through cell cycle specific phosphorylation (Mandal et al., 2014). DR5-caspase8 cascade has also been shown for endoplasmic reticulum (ER) stress related apoptosis induction (Lam et al., 2018). HCC and surrounding tissues highly express DR5, and TRAIL induced activation of DR5 might be

effective but development of resistance is inevitable through decoy receptors competing for ligand (X. P. Chen et al., 2003; W. Jiang et al., 2019).

Intrinsic apoptotic pathway depending on mitochondria is triggered by internal signals such as DNA damage, hypoxia, increased Ca^{+2} concentrations as the consequence of cellular stress (S. Nagata, 2018). The B-cell lymphoma 2 (Bcl-2) family proteins are widely known and investigated for intrinsic apoptotic pathway as apoptotic switch since they are critical for balancing the pro and anti-apoptotic actions/interaction on mitochondrial membranes (Frenzel et al., 2009). Anti-apoptotic proteins can be listed as Bcl-2, Bcl-xL, Bcl-W, Mcl-1, A1, and Bcl-B while pro-apoptotic ones are Bax, Bak, Bok, Bid, Bim, Bad, PUMA, NOXa, and BIK (Pistritto et al., 2016; R. Singh et al., 2019). Pro-apoptotic members suppress the anti-apoptotic ones leading to permeability of outer membrane of mitochondria (MOMP). Hence, factors belong to intermembrane leak to cytosol such as cytochrome-c and together with other activators it forms apoptosome to activate initiator caspases (Würstle & Rehm, 2014). This pathway also merges with executor caspases (R. Singh et al., 2019).

In intrinsic apoptosis morphological changes of mitochondria involves the active caspase 9 while caspase 3 is needed for proper apoptosis induction and caspase 7 (casp3/7 are mostly known as redundant in terms of function) leads to detachment of cells going through apoptosis (Brentnall et al., 2013; P. Li et al., 2017). Even though intrinsic and extrinsic pathways have been distinctly specified, they interact at certain levels. In some conditions extrinsic activation can induce intrinsic pathway through caspase 8 activated Bid (Tummers & Green, 2017). Activation of caspase 8 and caspase 9 has been shown as common phenomena in both apoptotic pathways in human leukemic cell lines as example(McComb et al., 2019).

BCL-2 overexpression has been observed in several cancers (Frenzel et al., 2009). For example, the ratio of BCL-2 and pro-apoptotic BAX has been indicator of the apoptosis in several cancers (Perlman et al., 1999). BCL-xL/BAX ratio dysregulation has also been investigated in the context of apoptosis in colon cancer (Baillat et al., 2005). In this thesis, BCL-xL (*BCL2L1*) has been investigated upon drug treatments in addition to BCL-2 and BAX. Bcl-x gene expresses two isoforms with alternative splicing: antiapoptotic Bcl-xL (long), proapoptotic Bcl-xS (short) (Stevens & Oltean, 2019). Being an anti-apoptotic protein, Bcl-xL promotes the cell survival and proliferation. Moreover, high expression of BCL-xL can also be associated with drug resistance in cancer (R. Liu et al., 1999). It can also be part of non-apoptotic cell death by binding Beclin1 which is a tumour suppressor (Michels et al., 2013). There are also several

cellular functions it can be involved in other than cell death such as migration and invasion (Choi et al., 2016).

GR associated apoptosis induction in neurons was found to be dependent on MR action which act on opposite proliferation direction (Crochemore et al., 2005). Moreover, there are several studies showing the glucocorticoid induced apoptosis in various cellular models (H. Li et al., 2012; S. L. Planey & Litwack, 2000; Sonia L. Planey et al., 2002).

1.3.3 DNA damage and cell cycle pathways

Aberrations in cell cycle progression in cancer resulting in uncontrolled cell proliferation due to several failed control mechanisms inducing genetic instability (Funk, 2006). In mitotic cells, the transition from one phase to another is controlled by specific proteins such as cyclin dependent kinases (CDKs-serine/threonine). CDKs in complex with cyclins which have oscillating levels during the cell cycle, are switches of cellular progressions leading mitosis (Otto & Sicinski, 2017).

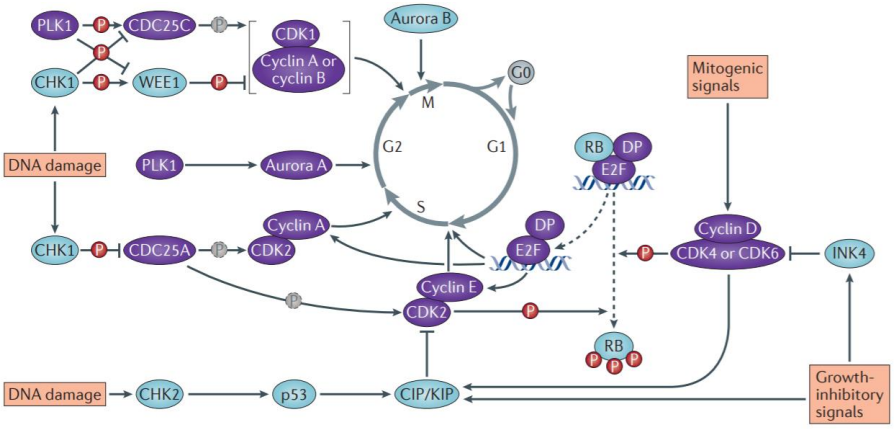


Figure1. 7 Cell cycle and DNA damage checkpoints (*adapted from Otto & Sicinski, 2017*)

Purple color indicates positive regulators of cell cycle progression and blue color indicates negative regulators of cell cycle progression.

Progression of cell cycle is controlled by these complexes act as switches in between cell cycles phases as shown **in Figure1. 7**. Upon approach to mitogenic signals and passing the irreversible restriction point, the cycle starts with G_1phase. In G_1, homologous CDK4 and 6 are activated and coupled with cyclin D (1-3). Active complex phosphorylates several targets including

tumor repressor Retinoblastoma protein (RB). This complex also has ability to sequester and lead to ubiquitin mediated proteolysis of p21 and p27 CDK inhibitors that work on CDK2. Later in G_1 due to CDK4/6-cyclinD function active CDK2/cyclin E complex is formed for G_1/S transition. Involvement of PI3K/AKT/mTOR signaling reviewed in literature for their effect on cycle by controlling G_1 to S phase transition via cyclin D1 (Vadlakonda et al., 2013). In addition to ERK, p38 MAPK has similar progressive effect in this transition, p38 also interacts with G_2/M upon stress induction (MacCorkle & Tan, 2005; Thornton & Rincon, 2009). In the entry of S phase, cyclin E degrades, and the CDK2/cyclin A complex is then formed (De Boer et al., 2008). CDK2 kinase activity can be enhanced in several cancers: for example, in HCC, CDK2 activity is quite higher than healthy liver tissue (S. Shen et al., 2019). In G_2 phase to M transition, CDK1/cyclin A2/B complexes are essential for progression (Gong & Ferrell, 2010). Upon mitotic entry CDK1/cyclin B complex is formed. CDK1 activity ends with completion of mitosis via Anaphase promoting complex/ cyclosome (APC/C)(Van Zon et al., 2010). Overall, examination of cell cycle is critical for cell cycle progression after treatment and CDK2 is a relevant marker for this purpose.

MR has been associated with cell cycle blockage in HCC cell lines (Nie et al., 2015). Moreover, DNA damage induction by MR was shown in rat kidneys (Schupp et al., 2011). DNA damage was also related to glucocorticoids in breast cancer besides of GR function in cell cycle arrest (Rogatsky et al., 1997).

Control on proper cell cycle is critical for healthy proliferation. There are several checkpoints ensures this including DNA damage and spindle checkpoint (Barnum & O'Connell, 2014).

Upon DNA damage, checkpoints which are positioned in G1/S, G2/M transitions and during S and M phases ceased the cell cycle to give time for DNA repair (DNA damage response) (Weinberg, 2013). If the damage is too severe to repair, induction of apoptosis is the solution to prevent proliferation of errored cells. Recognition of DNA damage in cells can be performed by several kinases such as ATM (ataxia telangiectasia mutated protein kinase) and ATR (ataxia telangiectasia and Rad3-related) (Funk, 2006). ATM (double strand break) and ATR (single strand break-replication fork stress) protein kinases can activate CHK1 upon relevant DNA damage, and ATM can also activate CHK2 by phosphorylation which activates p53 (Otto & Sicinski, 2017). p53 dependent G_1 arrest induced by increased transcriptional activity of p21 inhibitor results in ceased activation of CDK2 by cyclin E (Barnum & O'Connell, 2014). Activation of CHK1 by phosphorylation can induce S phase and G_2 phase arrest by halting Cdc25 family phosphatases (WEE1). CHK1 dependent cell cycle checkpoint and cell cycle

arrest at G_2 phase is critical since it can work regardless of p53 status (Gottifredi et al., 2001). CHK1 status in cancer cell has controversy in terms of its function (Otto & Sicinski, 2017; Vermeulen et al., 2003; Y. Zhang & Hunter, 2014). Recent evidence supports the CHK1 related progression in cancers; moreover, over-expression of CHK1 proposed to be anti-cancer target in TNBC and HCC (Verlinden et al., 2007; Xie et al., 2014). Hence, examination of CHK1 levels in liver cancer cell lines such as p53 null Hep3B is quite informative on cell cycle status.

Damaged genetic material induces genomic instability leading the tumorigenesis. In the case of DNA damage, γH2AX histone protein can be considered as a marker when it is phosphorylated as a part of DNA damage response (DDR). DDR has been proposed as anti-cancer target together with PI3K inhibition in ovarian cancers (T. T. Huang et al., 2020). Moreover, MAPK pathway is also has role in DDR via interaction with CHK1 (Rezatabar et al., 2019). After introduction of double stranded break (DSB) by ionizing radiation or genotoxic anti-cancer drugs, MRN complex containing three proteins (MRE11-RAD50-MBS1) recognize the break and DDR proteins gather around the damaged foci(Jackson & Bartek, 2009). As a part of this mechanism, checkpoint kinase ATM directed to the site which can activate checkpoint mediators CHEK1 and CHEK2 are recruited the damage site, and this can activate the γH2AX by phosphorylation at ser 139 (Mah et al., 2010). p-γH2AX foci acts as docking site for repair proteins and concentrate them on it by maintaining them in correct site for repair. Even though ATM is the major mediator for this marker, ATR and DNA-PK proteins also can induce this phosphorylation (M. Ando et al., 2014). In HCC, γH2AX has been evaluated in patient samples and concluded as possible biomarker for HCC risk due to its high expression in preneoplastic lesions (Matsuda et al., 2013). It has also been related to HCC angiogenesis (H. Xiao et al., 2015). Basically, above mentioned proteins can be effective to predict anti-cancer drug responses.

1.3.4 PI3K/AKT/mTOR signaling pathway

mTOR (mammalian target of rapamycin) signaling (**Figure1. 8)** which can be described as master regulator of proper cell physiology by regulating cell growth, proliferation, aging, survival, mRNA and protein synthesis, cellular metabolism, cytoskeleton, invasion, and metastasis. Several members of the pathway become highly valuable as oncogenes and tumor suppressors which are dysregulated in cancers (Porta et al., 2014).

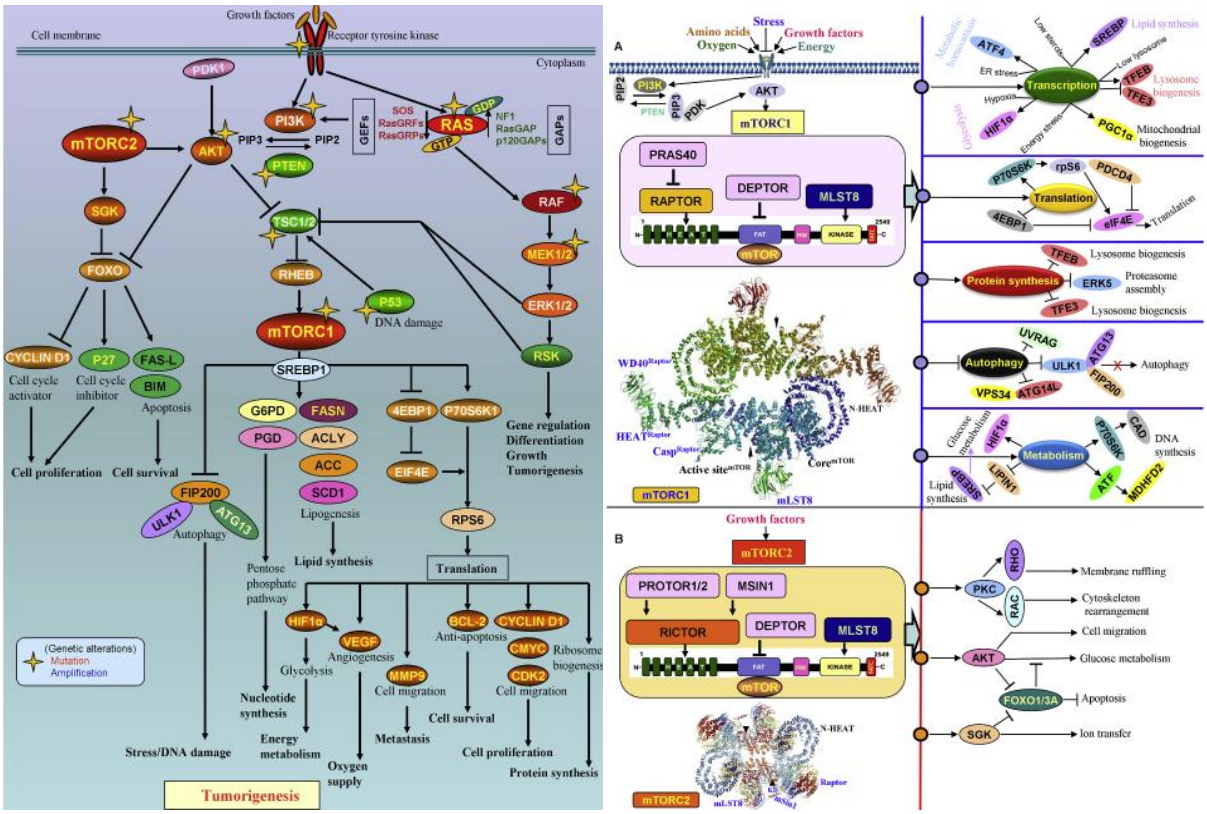


Figure1. 8 mTOR signaling pathway *(adapted from Murugan, 2019).*

mTOR can form two cross-talking complexes with their regulators: mTORC1 and mTORC2. These two proteins complex have their own distinct activation routes and physiological functions even though they can crosstalk. mTORC1 can be regulated positively or negatively by mainly PI3K pathway and AMPK, RAS/MAPK pathways. Activated mTORC1 regulates S6K1 (ribosomal S6 kinases), 4EBP1 (repressor of mRNA translation, suppressed by phosphorylation) and others to induce control over cell growth, proliferation, and survival. TSC1/TSC2 complex inhibits the activity of this complex. Receptor Tyrosine Kinase (RTK)-PI3K produces second messenger PIP3 which leads SGK1, PDK1/2 and AKT to membrane. Membrane engaged AKT gets phosphorylated by PDK1/2 at Thr308. Active AKT phosphorylates TSC2 and activated mTORC1 by ser2448 phosphorylation. On the other hand, mTORC2 (Rictor as scaffold) complex is less known than mTORC1. It can be activated by growth factors and phosphorylate AKT at ser473, SGK and PKC isoforms. These proteins lead regulation of apoptosis, motility, cytoskeletal arrangement by mTORC2.

Since this pathway's up and downstream regulators and effectors are critical for cancer, dysregulation of the members of the pathways have been established in several cancers (Murugan, 2019). For example, in almost 50% of HCC patients, upregulated mTOR pathway which is associated with the bad prognosis draw attention as therapy target (Villanueva et al., 2008; L. Zhou et al., 2010). Activation of this pathway in HCC cause less differentiated tumors and fast recurrence. Phosphorylated P70s6K's biomarker capacity has been detected before

because of its indication of more aggressive tumors in HCC (Baba et al., 2009). Although mutation in the pathway members are not common in HCC, low frequency mutation in mTOR has been detected (Matter et al., 2014). AKT and mTOR phosphorylated forms with loss of PTEN in HCC was determined to be overexpressed and induce invasion and metastasis (J. S. Chen et al., 2009). PTEN dysregulation is a known phenomenon for HCC and in hepatoma cell lines its was targeted/downregulated by epigenetic mechanisms (L. Wang et al., 2007). Moreover, in literature, PI3K/AKT/mTOR pathway together with several others are associated to hepatocarcinogenesis hence, targets for therapy against HCC (Whittaker et al., 2010). mTOR expression is often upregulated in HCC cases which attract attention as therapeutic target due to related adverse prognosis. Moreover, there are clinical and pre-clinical trials targeting mTOR in HCC (Matter et al., 2014).

In a cancer irrelevant context, MR-mTOR-p70s6 kinase interaction was identified in rat kidney Mineralocorticoid receptor-dependent proximal tubule injury is mediated by a redox-sensitive mTOR/S6K1 pathway (Whaley-Connell et al., 2012). Renal fibrosis claimed to be induced via aldosterone related AKT/mTOR signaling (X. Yuan et al., 2019). A recent study revealed ALDO regulated mTOR activity which was not conclusive on MR activation performed in cardiac and renal tissues of male mice (Brooks et al., 2019). Through gene signature analysis in T-cell acute lymphoblastic leukemia (T-ALL), glucocorticoid resistance was found to be induced by PI3K downstream signaling which was reversed by inhibitors of this pathway (Silveira et al., 2015). In T-ALL, direct phosphorylation of GR by AKT was linked to glucocorticoid resistance and inhibition of GR related apoptosis through impaired nuclear translocation of GR (Piovan et al., 2013). In prostate cancer, targeting PI3K pathway was discovered to sensitize cells to AR therapy through GR inhibition (Adelaiye-Ogala et al., 2020). Moreover. autophagy in osteoclast induced glucocorticoids claimed to crosstalk with this pathway (Fu et al., 2020).

To sum up, in the context of HCC treatment evaluation members of these pathway are relevant.

1.3.5 MAPK signaling pathway

The mitogen-activated protein kinases (MAPKs) which are widely expressed in eukaryotic cells have role in several critical cellular functions by transferring extracellular signals with a cascade of kinase activation to reflect physiological responses. Relaying, amplifying, and integrating various signals to regulate development, proliferation, differentiation, and survival etc. by this signaling pathway has been shown to be critically deregulated in several cancers (Braicu et al., 2019). Extracellular signals such as growth factors, cytokines, stress transduced to intracellular components via several members of MAPK family: extracellular signal-regulated kinase (ERK) 1/2, Jun N-terminal kinase (JNK) 1/2/3, p38 a/b/c/d, ERK5, ERK 3/4, ERK 7/8and Nemo-like kinases (NLKs) (**Figure1. 9)** (Krishna & Narang, 2008).

In this thesis, mainly MAPK family members ERK1/2 and p38 MAPK were covered. **Figure1. 9** shows the ERK signaling pathway in detail. Effector kinase of the pathway, ERK two isoforms ERK1 and ERK2 which are 88% similar in sequence. Even though they have similar regulatory functions, their induvial and distinct function are also mentioned in several studies underlying isoform specificity. ERK1/2 has been effective in cell cycle regulation especially in G1 to S phase transition, and cause G2/M arrest in its absence (Dumesic et al., 2009). ERK's regulatory effect on cell survival acts through suppression of apoptosis. It protects cells from death receptor initiated apoptotic pathway which is clarified by FasR and TRAIL-R sensitivity upon blockage of ERK. This relation has been linked to caspase machinery (caspase 8 underlined) rather than alterations in gene expression (Tran et al., 2001). ERK1/2 can induce the expression of BCL-xl without affecting BAX activation in an indirect way in human pancreatic cancer cells which can also be regulated by PI3K/PKB pathway (Boucher et al., 2000). Based on its activity on survival and proliferation, dysregulation of these pathway members in several cancers is not an unexpected phenomenon. Persistent activation of the ERK has been shown in several cell lines and primary tumors. In HCC tumorigenesis, activation of ERK by HBV and HCV proteins leads to tumor formations. As in normal conditions, in HCC MAPK pathway acts together with PI3K/AKT/mTOR pathway in complex and diverse (positive or negative effects) signaling levels. AKT and RAS act more robustly on hepatocarcinogenesis causing progression of cell cycles in HBV infected tissue (Chin et al., 2007). Besides of p-ERK levels, activating downstream targets to promote HCC progression, other members of the pathway have been shown to be altered in HCC (upregulated expression of Ras/Raf/MEK/ERK kinase in HCC cells and tissue). Even though both isoforms are needed

for proliferation of the HCC cells, studies targeting ERK1 and ERK2 by RNA interference suggesting ERK2's role rather than ERK1 in reduction in tumor proliferation in liver cancer cell lines and in vitro in rodent models (Bessard et al., 2008). In IL-2-dependent Kit 225 T lymphocytes, MAPK dependent activation of CDK2 which is an important regulator of G1 to S phase transition and S phase (Blanchard et al., 2000). In HCC, activation of MEK/ERK signaling has been associated with proliferation, survival, metastasis, and desensitization to TGFβ induced apoptosis (Caja et al., 2009).

Figure1. 9 shows the p38 MAPK signaling pathway. Stress response kinase p38 together with other family member JNK has been discovered as signaling pathways dysregulated in several cancers. Interestingly, they can antagonize the tumor proliferation, but cancer can manipulate these signals to induce survival and invasion (Hui, Bakiri, Stepniak, et al., 2007; Ventura et al., 2007). Hence, they have cancer and stage specific opposite effects. A study in HCC patients highlights the importance of p38 since its phosphorylated levels are associated with tumor size which is antagonized by p-JNK and, worse prognosis (S. N. Wang et al., 2012). Another study claims that resistance to apoptosis leading to proliferation of HCCs can be induced by decreased p38 and MKK6 (p38 upstream MAPK) levels (Spaziani et al., 2006). In HCC cell lines MKK3 (p38 upstream MAPK) expression has been shown to interfere cell cycle to cause arrest at G1 through p38 activation. p38α has been linked to HBV- and HCV related hepatitis. Hepatitis C virus (HCV) proteins can manipulate the cell cycle via p38α mediated regulation of cyclin-CDK complexes (Spaziani et al., 2006).There are other studies showing its proliferation inhibitory function in tumorigenesis by antagonizing JNK pathway in liver (Hui, Bakiri, Mairhorfer, et al., 2007). In HCC cell lines, Cathepsin C upregulation associated with proliferation and metastasis. A recent paper has linked Cathepsin C to TNF-α/p38, hence concluding their interaction caused to proliferation in HCC cell lines (G. P. Zhang et al., 2020).

Family members of the MAPK signaling shares upstream regulators and downstream effector hence, they interact at several steps of signaling. ERK and p38 crosstalk in human primary cell culture hepatocytes discovered by p38 inhibitor which leads to activation of ERK pathway (Henklova et al., 2008). Active/sustained JNK can suppress ERK activation via mitogens (Y. H. Shen et al., 2003). These family members can be targeted as promising anti-HCC proteins due to their involvement in HCC initiation and progression.

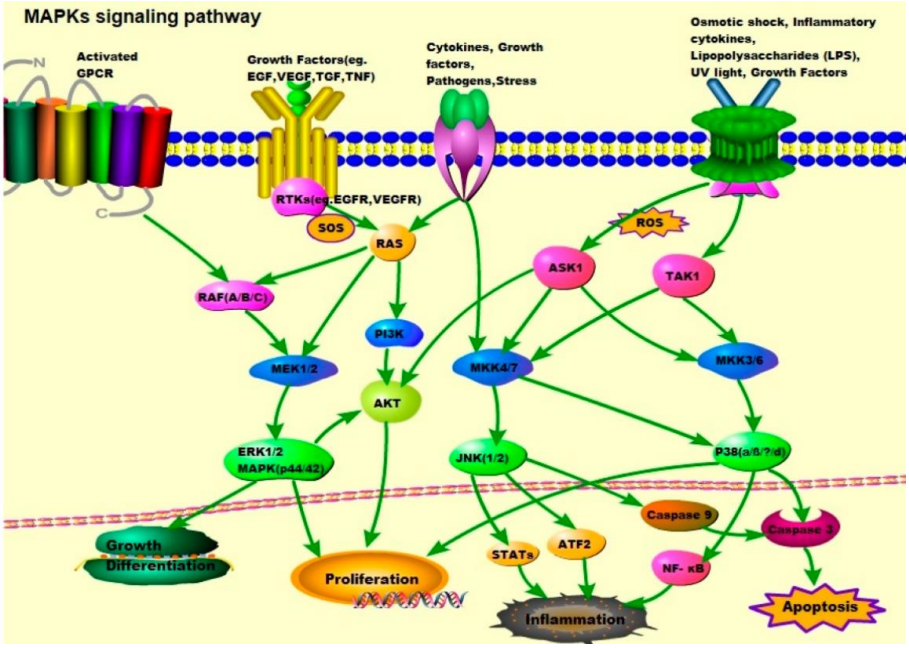


Figure1. 9 Signaling of main MAPKs ERK1/2, p38 and JNK (a*dapted from C. Chen et al., 2019*)

Transduction of extracellular signal to nucleus starts with Receptor Tyrosine Kinases (RTKs) or G-protein coupled receptors (GPCRs). This surface receptors induces MAPK signaling cascade, which is composed of MAPKKK, MAPKK, MAPK kinases activating each other in an orderly form by phosphorylation. These can be de-phosphorylated by dual-specificity phosphatases (DUSPs). Active MAPK phosphorylates serine and threonine residues of several targets to induce stimuli related gene expression regulating responses including proliferation, apoptosis, migration, inflammation etc. MAPK pathway members are heavily crosstalk with other pathways including TGFβ/Smad, Wnt/β-catenin etc. to regulate cellular functions. PI3K/AKT/mTOR pathway is one of the crosstalk partners. Active ERK is translocated to nucleus to activate several TFs including AP-1 family TFs, cJun and cFos to induce cell cycle progression, proliferation etc. Another MAPK family member p38 MAPK generally activated by stress and cytokine signals to induce responses such as inflammation and cell death. p38 has 4 isoforms: α, β, γ, δ p38, α (most studied) and β isoforms being the most widely expressed ones.

GR's ligand independent transcriptional response to stress has been associated with phosphorylation at Ser134 residue was found to be induced by p38 kinase in rat hepatoma cell lines (Galliher-Beckley et al., 2011). Their interaction in bronchial epithelial cells for inflammation regulation was also investigated (Lea et al., 2020). MAPK-GR intensive crosstalk in terms of inflammation is extensively studied in the literature (Kassel et al., 2001; Miller et al., 2007; Petta et al., 2016) In pediatric leukemia, targeting MAPK resulted in reversal of glucocorticoid resistance which suggest combinatorial treatment of glucocorticoids with MAPK inhibitors.GR identified as post translational modification target of p38 MAPK in terms of stress sensing which can be ligand independent and underlies GR as target for therapy in

TNBC (Parker et al., 2018; Perez Kerkvliet et al., 2020). ALDO exposure (via MR activation) has been associated to ERK1/2/STAT3 pathways induce DNA damage and proliferation which might lead to kidney tumorigenesis (Queisser et al., 2017). ALDO might not act on MR to regulate ERK signaling in cortical collecting duct cell line (Rossol-Haseroth et al., 2004). In literature SHRs has been reviewed for their ability to sense MAPK pathway through phosphorylation of PGR and GR (Dwyer et al., 2020). In myocytes, MR and p38 was identified as crosstalk via ALDO activation (Y. S. Lee et al., 2004). MR-MAPK interaction also has literature for inflammation (C. J. Zhu et al., 2012). Hence, MR and GR has crosstalk with MAPK pathway at various levels.

1.4 Treatment strategies in cancers

There are several cancer treatment strategies from traditional ones to new targeted approaches depending on the type and stage of the cancer. Invasive surgeries, hormone therapies, radio and chemotherapies are most applied and known traditional treatment modalities. However, recently more targeted, less disruptive strategies are addressed such as immunotherapies and taking advantage of nanotechnological advances (Pucci et al., 2019; Yildizhan et al., 2018). Often, combination of these treatment modalities is applied to patients to treat these multifactorial diseases. Cytotoxic chemotherapy agents, small molecule inhibitors and monoclonal antibodies together with more recent immunotherapy are systemic therapy approaches against cancers. Although, there is a vast literature on anti-cancer drugs, new approaches and drugs are required for stronger treatment models (Pucci et al., 2019). Targeting proteins and pathways dysregulated specifically in cancers is the one of the most adopted treatment approaches. These targets were listed by **Kumar et al., 2017** as: 1. kinases such as RTKs and CDKs to develop kinase inhibitors 2. Tubulins and microtubules to destabilize them, 3. Tumor vasculature, 4. Cancer stem cells by targeting Notch, Hedgehog, Wnt and NF-kB pathways (Kumar et al., 2017). Still, development of drug resistance and side effects on healthy cells limits the success of drugs. Hence, detailed physiology and pathology of cancer cells needed to be known for designing better drug modalities. Strategies such as multi-target agents and combining multiple drugs to prevent/reverse the drug resistance are the main focuses in anti-cancer drug research (Kumar et al., 2017). In the context of this thesis, drug treatment options for breast cancer and HCC will be covered.

1.4.1 Breast cancer

Breast cancer treatment strategies are varies depending on tumor status. Subtype of the tumor, metastasis levels etc. are critical criteria to decide suitable treatment modality. Local invasive surgeries and radiation, and systemic pre/post-operative (neo/adjuvant) applications are available (Waks & Winer, 2019). Only the endocrine therapy approaches targeting SHR family member ER is covered in this thesis.

1.4.1.1 Targeting steroid hormone receptor family

Hormone regulations in breast cancer has been critical to subtype the disease for prognostic evaluations and determining the proper treatment strategies. Downregulation of estrogen receptor (ER) signaling by using endocrine agents have been the main strategy for hormone receptor positive tumor treatments. This downregulation have been achieved by antagonists can selectively modulate (SERMs) or degrade (SERDs) the ER (Patel & Bihani, 2018). Most known SERMs are used in HR positive cancers is Tamoxifen. Estrogen production by androgen conversion can be targeted by aromatase inhibitors leading to reduced hormone levels in circulation can only be applied after menopause (Waks & Winer, 2019). Even though there are several other drugs available, limitations of these strategies are undeniable including side-effects and acquired resistance. Weak estrogenic effect of Tamoxifen causing unintended proliferation might be considered as an example (Wong & Chen, 2012). Cross talking signaling pathways responsible for the resistance most of the time (Knowlden et al., 2003; M. Sun et al., 2001). Hence, new strategies should be considered with these cross-talks in mind. In this sense, positive or negative crosstalk of ER with other proteins/pathways become important. Hence, clarifying the ER interaction with the other members of SHRs will be a valid point for breast cancer research.

1.4.2 Hepatocellular carcinoma

Since HCC is a high mortality rate disease with limited treatment options, new options against HCC are essential part of liver cancer research. When diagnosed in early stages invasive surgical resections and liver transplantations have success to improve the short-term survival rate. Moreover, stage of disease and underlying abnormalities are critical for treatment and recurrence of the diseases. However, there are few, recently emerged systemic treatment options for severe HCC cases. Hence, there was a great need of systemic therapies and first effective drug against HCC was discovered, Sorafenib (SFB).

1.4.2.1 Sorafenib

Discovery of Sorafenib (Nexavar), a multi-kinase inhibitor, was a game changer in the HCC treatment. It was the first systemic drug that was beneficial for survival of HCC patients (Abou-Alfa et al., 2006). However, these improvements were not extended for long term, only achieved couple of months increase in life expectancy. Studies on better options were failed for long time which made Sorafenib the only available drug against advanced HCC (Lurje et al., 2019). Recently, more treatment options are approved by FDA as single or combinatorial drugs in first or second line of treatments. Another multi-kinase inhibitor Lenvatinib was approved for unresectable HCC cases as first line of treatment (Kudo et al., 2018). Nivolumab, a checkpoint inhibitor also found to be promising for clinical applications (El-Khoueiry et al., 2017). As second line of treatment after SFB, another multi-kinase inhibitor Regorafenib is also promising for survival of HCC patients (Bruix et al., 2017; Personeni et al., 2018). Combination of monoclonal antibodies Atezolizumab and Bevacizumab in un-operable HCC patients was discovered to be more successful compared to SFB (Finn et al., 2020). Effects of new drugs have been evaluated by comparing their effectiveness to SFB's. Hence, SFB still a key drug for HCC treatment.

Multi-kinase inhibitor SFB can target critical pathways including MAPK (RAF), VEGFR, PDGFR, and STAT3 among others acts on proliferation, apoptosis, and angiogenesis etc. (L. Liu et al., 2006; Rosmorduc & Desbois-Mouthon, 2011; Wilhelm et al., 2004). Since HCC is a heterogenous disease, altering multiple genes, with diverse cell populations has diverse

responses to drugs. Moreover, as in most cancer treatments development of resistance against SFB is an issue (W. Tang et al., 2020). Modest benefits of the Sorafenib were aimed to be improved by combinatorial drug treatment strategy. Targeting HCC with multiple molecules can enhance the efficiency of treatment by preventing phenomena such as drug resistance. Resistance mechanisms against SFB in HCC further reviewed by Tang et al., 2020 under headlines of epigenetics, transporters, cell death manipulation, tumor microenvironment (W. Tang et al., 2020). Possible combination partners of Sorafenib inducing cytotoxic drugs or targets of signaling pathways have been reviewed by Gao JJ et al., as agents against angiogenesis, histone deacetylase (HDAC), MEK/ERK pathway, EGF/EGFR signaling, mTOR signaling and HGF/c-Met pathway (J. J. Gao et al., 2015).

Acquired SFB resistance via constant exposure can be induced by AKT activation through mTOR feedback and induce protective autophagy which was reversed synergistically by combination of SFB and AKT inhibitor GDC0068 in HCC (Zhai et al., 2014). In mouse xenografted with SFB insensitive cells, SFB and Rapamycin combination found to be effective against cancer growth via reduction of SFB induced mTOR signaling and cyclin expression (Huynh et al., 2009). In another study, SFB and a dual PI3K/mTOR inhibitor PI-103 synergistically act against HCC cell line HUH7. HUH7 cell line treated with EGF (epithelial growth factor) and these drugs alone and combination showed that SFB induced p-AKT and PI-103 reduced it while activating MEK but, combination reduced both AKT and MEK activation (Gedaly et al., 2010). EGFR (epithelial growth factor receptor) is identified as Sorafenib resistance marker in HCC (Ezzoukhry et al., 2012). Since expression of HDAC has been associated to HCC aggressiveness, it was targeted as combination partner of SFB (Rikimaru et al., 2007). HDAC inhibitor MPT0E028 and SFB synergistically induce apoptosis in HCC cell lines by activating apoptosis and partial ERK inhibition (C. H. Chen et al., 2014). Even though SFB targets the MAPK signaling, long term exposure causes a feedback increasing the phosphorylation of ERK and MEK eventually reducing effectiveness of SFB. However, combinational treatment of SFB with MEK inhibitors trametinib/selumetinib reversed the situation synergistically in terms of viability and act on cyclin D1 and c-MYC levels (Hou et al., 2019).

Interestingly, Sorafenib has been examined as combination partner in breast cancer treatments in literature. SFB has been shown to desensitize cells to Tamoxifen by downregulating ER levels (Pedersen et al., 2014). Phase I/II clinical studies was followed with SFB and combined

with and Aromatase Inhibitor for resistant patients and showed modest clinical benefit (Isaacs et al., 2011).

Even though there is a need for more effective drugs than SFB, it is still a promising candidate for treatment of HCC and other cancers when combined with appropriate second effectors.

1.4.3 New approaches in cancer treatment: Drug repurposing/repositioning

Increasing number of cancer patients and limitations in treatment options arise a need for more successful anti-cancer drugs. This urgency direct scientists to repurposing of clinically used or preclinical promising drugs which means revisiting known unrelated drugs for their anti-cancer effects as new candidates against cancers. This strategy might be considered as safe and cost effective and discover unknown functions of drugs in cancer. Sleire et al., 2017 reviewed and categorize these as cancer preventive and therapeutic drugs (Sleire et al., 2017). They can be used as single treatment options or in combination therapies. Spironolactone is a Mineralocorticoid Receptor antagonist which is a widely used diuretic drug repurposed and combined with other drugs against cancers, effectively (Sanomachi et al., 2019). Computational approaches have been adopted to identify candidate drugs for SFB resistance in HCC cell lines by transcriptomic analysis (Gouri et al., 2019; Regan-Fendt et al., 2020; L. Yu et al., 2020). Anti-psychotic Phenothiazines (PTZs) are one of the drug families attract attention in drug repurposing due to their anti-cancer effects.

1.4.3.1 Phenothiazines

Phenothiazine is a long-known anti-psychotic drug for treatment of patients with Schizophrenia and similar anxiety symptoms since 1940s (Koch et al., 2014). Core structure of PTZ has been used to develop more efficient drugs with variety of biological acts constructing the drug family called Phenothiazines/ Phenothiazine derivatives (PTZs) as reviewed in literature (Gopi & Dhanaraju, 2019). PTZs known for their anti-dopaminergic activities in central nervous system besides of antibacterial, antifungal, anti-inflammatory, immunosuppressive, multidrug resistance reversal functions by targeting various biological mechanisms (Y. Gao et al., 2019;

Varga et al., 2017; Wesołowska et al., 2009). Besides of their act on dopaminergic receptors, they also have cholinesterase inhibitory activity (Jończyk et al., 2020). Schizophrenic patients found to be less prone to develop certain cancers which draw attention to drug administrations(Hodgson et al., 2010; Ji et al., 2013). Hence, this correlation paved the way for drug repurposing of this derivatives.

In addition to several known, available PTZs, newly synthesized derivatives are under investigation for their anti-cancer features in several cancer types. Targeting SIRT1 (histone deacetylase) in colorectal cancer, cancer stem cells in breast cancer and drug resistance in colon cancer, autophagy and apoptosis induction are several beneficial features of PTZs (Gutierrez et al., 2014; W. Y. Lee et al., 2015; Omoruyi et al., 2020; Środa-Pomianek et al., 2018). Anti-cancer effect of generic Perphenazine (PPH), Prochlorperazine (PCP) against melanoma cell lines was also observed in literature (Otręba Michałand Pajor & Warncke, 2019).

PTZs have been studied against HCC, too. In hepatoma tissue culture PTZs effect on mitochondria permeabilization was detected as reason for cytotoxic effects of these drugs (de Faria et al., 2015) Their possible apoptosis induction effect has been shown *in vivo* in melanoma cancer xenografts with Thioridazine (Gil-Ad et al., 2006) PTZ derivatives has been evaluated as anti-cancer drugs in HepG2, an HCC cell line, and cause induction of caspase dependent apoptosis through check-point activation and interfering mitochondrial function (Yuanxi Wang et al., 2019) Thioridazine induce cell cycle arrest, suppress EMT to prevent migration, suppress stemness, reduce viability in HCC cell lines (Lu et al., 2015) They have also been used in drug combinations in colon cancer and melanoma to potentiate drug efficiency of other relevant drugs (Hait et al., 1988; M. S. Lee et al., 2007; Środa-Pomianek et al., 2019). Hence, PTZs are promising anti-cancer candidates alone and can be effective combination partners.

In the scope of this thesis, among the derivatives being studied in our lab (Phenothiazine (PTZ), Perphenazine (PPH), Prochlorperazine (PCP) Trifluoperazine (TFP) derivative was further investigated and explained in cancer research, below.

Trifluoperazine (TFP) is a derivative of PTZ with calcium calmodulin inhibitor besides of being dopaminergic receptor antagonist (Feldkamp et al., 2010). TFP is a modulator of multidrug resistance (MDR) which was examined in breast cancer patients together with Doxorubicin in Phase II trials even though clinical trials did not established its effectiveness (Budd et al., 1993). TFP modulates MDR by regulating P-glycoprotein (P-gp) function which is overexpressed in resistant cancers (S. Y. Shin et al., 2006). In glioma cell lines TFP reversed the downregulated

expression of early growth response gene-1 (Egr-1) and exhibits anti-proliferative effects (S. Y. Shin et al., 2004). Moreover, in leukemic cells it showed selective cytotoxic and anti-proliferative features which was related to mitochondrial defects leading reduced ATP levels (Zhelev et al., 2004). Impairment of double strand break repair mechanism and helping to sensitize cell to DNA damaging agents by TFP was discovered in non-small cell lung carcinoma. Follow up study showed involvement of reactive oxygen species (ROS) production, caspase activation and γH2AX regulation upon TFP combinations, synergistically (Polischouk et al., 2007; Zong et al., 2011). To identify drugs acting similar to AXL knockdown (receptor tyrosine kinase which regulates tumor dissemination in TNBCs) revealed PTZ derivates as promising candidates through gene signature-based profiling. Derivatives including TFP acted on PI3K/AKT/mTOR and MAPK pathways to induce apoptosis and reduce cancer viability (Goyette et al., 2019). In oral cancer cell lines TFP modulates the protein levels of AKT, p38 and ERK and caspase dependent apoptosis in a dose dependent manner (C. H. Wu et al., 2016). This derivative has been associated with signaling of steroid hormone receptor family members. Glucocorticoid Receptor-TFP interaction is known and its effects on secretion of glucocorticoid and mineralocorticoid was studied (Ning & Sánchez, 1995).

TFP was identified as promising anti-HCC drug in a high throughput study searching for candidate drugs by gene expression profiling. TFP's effect on HCC was established in vivo by this study (M. H. Chen et al., 2011). Moreover, in TFP treated HCC cell lines, FOXO1 a tumor suppressor re-localized to nucleus and increment in expression was observed which leads G1/S arrest (J. Jiang et al., 2017).

TFP's above mentioned features such as inducing sensitivity to treatments, targeting critical pathways and being effective in several HCC cell lines supports the idea of combinatorial treatment of SFB and TFP in HCC or other cancers. Since both TFP and SFB targets critical pathways dysregulated in cancers, and I focused on those pathways detailed above.

1.5 Aims

Steroid Hormone Receptor family members mineralocorticoid and glucocorticoid receptors (MR and GR) have been studied in several cancers as prognostic markers and drug targets. In the present study, these hormone-activated transcription factors were studied in breast and liver cancer cell lines to reveal their crosstalk in response to hormone activation and a novel drug combination. Understanding these crosstalks can pave the way for novel avenues to pursue further to decipher cancer biology and drug responses. Aims and rationale of the studies in breast and liver cancer was given in two parts, below.

Signaling pathways are rarely act alone. The combinatorial action or crosstalk of ligand activated steroid hormone receptors (SHRs) can act on each other to antagonize or synergize their responses. SHR members MR and Estrogen Receptor (ER) crosstalk is a missing in breast cancer which could be a crucial addition to literature for further prognostic and therapeutic studies. Previous preliminary studies (Bircan Coban MSc Thesis, 2016) in our lab supported the existence of this crosstalk. In the first part of this thesis, I aimed to investigate the potential crosstalk of MR and ER signaling upon hormone dependent activation by Estrogen (E2) and Aldosterone (ALDO) at mRNA and protein level together with cellular viability assessment in T47D cell line (MR and ER positive). GR was also studied in the concept of crosstalk along with the plethora of MR downstream targets of epithelial salt transport in response to exposure to ALDO, E2, and ALDO-E2 combination. Hence, I intended to discover the hints of MR-ER-GR crosstalk with combinatorial hormone exposure in breast cancer.

Since there is a need for advanced treatment strategies against liver cancer/hepatocellular carcinoma, repurposing of drugs and drug combinations are widely examined in literature. Previously in our lab, FDA approved multi-kinase inhibitor Sorafenib and repurposed Trifluoperazine as a novel drug combination was found to be synergistic in terms of reduced cellular viability in HCC cell line, Hep3B. Moreover, antagonistic viability profile with combination was observed in liver cancer cell line, SkHep1 (Murat Yaman PhD Thesis, 2020). In the second part of my thesis, I focused on mechanism of action of SFB and TFP combination inducing sensitivity in Hep3B cell line in comparison to in-sensitive SkHep1. I tried to answer whether pathways altered in HCC and targetable by SFB and/or TFP alone affected by combination synergistically, explaining the observed viability reduction in Hep3B in comparison with SkHep1. With this purpose, protein markers of apoptosis, cell cycle, DNA-

damage/repair, PI3K/AKT and MAPK signaling pathways were examined with effective doses of TFP and SFB alone and in combination. Moreover, MR and GR and their downstream effector SGK1 was investigated in an effort to identify a crosstalk between multi-kinase inhibitors and GR/MR signaling. Overall, expression and crosstalk of MR and GR upon steroid and anti-cancer treatments in breast and liver cancers were the main focuses of this thesis to gain deeper insight on the crosstalk between nuclear receptors as well as novel drug combinations of kinase inhibitors.

CHAPTER 2

MATERIALS

T47D breast cancer cell line (ER +, PR+) was used for Nuclear Receptor crosstalk project. Liver cancer cell lines SkHep1 and Hep3B was used for phenothiazine repurposing and combination experiments.

2.2 General chemicals, reagents, and kits

Chemicals, reagents, and kits that were used routinely during this study is listed in **Table2.1** below.

Table 2. 1 List of chemicals, reagents, and kits

Catalog No.	Reagent/Chemical/Kit	Company
A7905	Bovine Serum Albumin (BSA)	Sigma Aldrich, USA
3010040	Immobilon-P PVDF Membrane	Roche (Switzerland)
17008891	iScript cDNA Synthesis Kit	Bio-Rad (USA)
K1622	RevertAid First Strand cDNA Synthesis Kit	Fermentas (Canada)
23227	Pierce BCA Protein Assay Kit	Thermo Scientific, USA
RPN 2232	Pierce ECL Western Blotting Substrate	GE Healthcare (UK)
79306	QIAzol lysis reagent	Qiagen, Germany
74104	RNeasy Mini Kit	Qiagen (Germany)
SH30538.01	Nuclease free water	HyClone (USA)

4729692001	Light Cycler 480 Multiwell plates-96, White	Roche (Switzerland)
4887352001	LightCycler SYBR Green I Master	Roche (Switzerland)
11873580001	Complete EDTA-free protease inhibitor cocktail tablets	Roche (Switzerland)
4906845001	PhosSTOP easy pack phosphatase inhibitor cocktail tablets	Roche (Switzerland)
161-0747	4X Laemmli Sample Buffer	Bio-Rad (USA)
P-7170	Ponceau S solution	Sigma Aldrich, USA
26616	PageRuler Prestained Protein Ladder	Thermo Scientific (USA
1610183	TGX Stain-Free™ FastCast™ Acrylamide Kit, 10%	Bio-Rad (USA)
M6494	Vybrant MTT Cell Proliferation Assay Kit	Invitrogen
2-mercaptoethanol	2-mercaptoethanol	Sigma-Aldrich (Germany)
24137	2-Propanol	Sigma-Aldrich (Germany)
A1584	Dimethyl sulfoxide (DMSO)	Applichem (Germany)
B2221	Ethanol	Sigma-Aldrich (Germany)
G8898	Glycine	Sigma-Aldrich (Germany)
15524	Glycerol	Sigma-Aldrich (Germany)
A16940250	NP-40	Applichem (Germany)
7102	Hydrochloric acid	Sigma-Aldrich (Germany)
6203	NaOH	Sigma-Aldrich (Germany)
71725	SDS	Sigma-Aldrich (Germany)
A11480100	TEMED	Applichem (Germany)
T1503	Tris (Trizma Base)	Sigma-Aldrich (Germany)
T8787	Triton X-100	Sigma-Aldrich (Germany)
822184	Tween-20	Merck (USA)

A3678	Ammonium persulfate	Sigma-Aldrich (Germany)
24229	Methanol	Sigma-Aldrich (Germany)
24216	Chloroform	Sigma-Aldrich (Germany)
31434	Sodium chloride	Sigma-Aldrich (Germany)
A2504	6-aminocaproic acid	Sigma-Aldrich (Germany)
H-1016	HEPES	Sigma-Aldrich (Germany)
E2758	17ß-Estradiol	Sigma-Aldrich
A9477	Aldosterone	Sigma-Aldrich
P14831-25g	Phenothiazine	Sigma-Aldrich (Germany)
P6402-1g	Perphenazine	Sigma-Aldrich (Germany)
P9178-5g	Prochlorperazine	Sigma-Aldrich (Germany)
T8516-5g	Trifluoperazine	Sigma-Aldrich (Germany)
S7397	Sorafenib	Selleckchem(USA)

2.3 Primers

Primers used in this study listed in **Table2.2,** below**.**

Table 2. 2 List of primers.

Gene	Primer sequence
TPT1	5’-GATCGCGGACGGGTTGT-3’ 5’-TTCAGCGGAGGCATTTCC-3’
DDIT3	5’-GTTAAAGATGAGCGGGTGGC-3’ 5’-TGGATCAGTCTGGAAAAGCACA-3’
SRSF7	5’-GGTCTAGATCACATTCTCG-3’ 5’-CCAGACCTAGATCTTCTG-3’
NOC3L	5’-ACCCAAAGGAAAAGCGACCA-3’ 5’-CGCATGAACAGGCTCACTAGA-3’
NIP7	5’-GGTGTACTATGTGAGTGAGAAGA-3’ 5’-GCACCAGGCTTTATCCAAAC-3’
RRM2	5’-TCCGGTTCTTTTGCGTCGAT-3’ 5’-TCCGATGGTTTGTGTACCAGG-3’
BAX	5’-GGGTTGTCGCCCTTTTCTAC-3’

	5’-CTGGAGACAGGGACATCAGT-3’
FAS	5’-AATAAACTGCACCCGGACCC-3’ 5’-AGAAGACAAAGCCACCCCAA-3’
BCL2	5’-TGAACTGGGGGAGGATTGTG-3’ 5’-CGTACAGTTCCACAAAGGCA-3’
MR	5’-AGTGGAAGGGCAACACAACT-3’ 5’-CTGCTCCTCGTGAATCCCTTT-3’
GR	5’-CTGGGGTGGAGATCATATAGACA-3’ 5’-CATAAGATACCTGAAGCCTGTGT-3’
ZBTB16	5’-AGACGTACCTCTACCTGTGCTATGTGT-3’ 5’-TGTCTAGTCCTTCCTTCATCTCACT -3’
PGR	5’-GGAGGAGGAGGGAGGTGTCA-3’ 5’-CTGCTTGGAAGACTCAGGGA-3’
SGK1	5’-AACCCTTCTCCTCCACCAAG-3’ 5’-TTCCAAAACTGCCCTTTCCG-3’
α-ENaC	5’-GGTTTCTCACACCAAGGCAG-3’ 5’-AGCCCTTACCCATCTTGCTT-3’
B-ENaC	5’-TCCTACCCTCGTCCCTACCT-3’ 5’-CCAGGAAGGAGAAAACCACA-3’
Ɣ-ENaC	AAGGTGACACAGTGAGGAGG-3’ GGAAGGGTCAGCTCTGTCTT-3’
NEDD4-2	5’-CATCAGCGAAGACTTGGAGC-3’ 5’-GCTGAACTGTTCCCCATTGG-3’
PFS/TTF1	5’-CCATGGAGAACAAGGTGATCTGC-3’ 5’-TTAGGATAGAAGCACCAGGGGAC-3’
ANLN	TAAAGCAGGTGATTGTTCGG GTTCTTCATCAACACAGCAG
CCNE2	5’- GTAGCTGGTCTGGCGAGGTTT -3’ 5’- GGGCTGCTGCTTAGCTTGTAA -3’
CCND1	5’- CTGCGAAGTGGAAACCATCC -3’ 5’- GCACTTCTGTTCCTCGCAGA-3’

2.4 Antibodies

Antibodies used in study listed in **Table2.3**, below.

Table 2. 3 List of antibodies

Antibody	Catalog No.	Company
CASPASE 9*	# 9502	Cell Signaling
DR5	#8074P	Cell Signaling
BCL-xL*	sc-8392	Santa Cruz
BCL2	#2870	Cell Signaling
BAX	#5023P	Cell Signaling
T-CHK1	sc-8408	Santa Cruz
p-CHK1(S345)	#2348P	Cell Signaling
c-PARP(Asp214)	#5625	Cell Signaling
P70s6K	#9202	Cell Signaling
p-p70s6K (Thr389)	#9234S	Cell Signaling
mTOR	#2972	Cell Signaling
FAS (4C3)	#8023S	Cell Signaling
c-CASPASE 8	#9496S	Cell Signaling
p38 MAPK**	#9212	Cell Signaling
p-p38 MAPK (t180/y182)	#4511P	Cell Signaling
ERK 1/2	9107S	Cell Signaling
p-ERK 1/2	9106S	Cell Signaling
CDK2***	C5223	Sigma
AKT**	#9272S	Cell Signaling
p-AKT (Ser473) **	# 9271L	Cell Signaling
p-MTOR (Ser2448) **	# 2971S	Cell Signaling
p-γH2AX	sc-517348	Santa Cruz
GAPDH	sc-47724	Santa Cruz
β-actin	Ab1801	Abcam
Anti-mouse	# 7076P2	Cell Signaling
Anti-rabbit	#7074S	Cell Signaling
MR	rMR1-18 1D5	DSHB/ University of Iowa

ESR1	#2512	Cell Signaling
GR	#12041S	Cell Signaling
PGR	# MS-298-P0	Thermo Fisher Scientific (USA)
SGK-1	S5188	Sigma
NEDD4-2****	300-376; A27 (AP)	Cocalico/Dan
α-ENaC****	JL- #1132	JL
β-ENaC****	JL- #135	JL
γ-ENaC****	JL- #552	JL

Marked antibodies are kind gifts from (*) Işık Yuluğ (**), Rengül Çetin Atalay, (***) Özgür Şahin, (****) Olivier Staub (University of Lausanne, JL:Johannes Loffing from Institute of Anatomy, University of Zurich).

2.5 General equipment and materials

Equipment that was used in this study are listed in **Table2.4**, below.

Table 2. 4 Equipment are listed.

Equipment	**Company**
PCR Thermal Cycler 2720	Applied Biosystems (USA)
NanoDrop ND-1000	Thermo Scientific (USA)
LightCycler 480 Instrument	Roche (Switzerland)
Amersham (TM) Imager 600	GE Healthcare Life Sciences (USA)
Synergy HT Microplate Reader	BioTek (USA)
Eclipse TS100 Inverted light microscope	Nikon (Japan)

2.6 Common buffers and solutions

Buffers and solutions were used in this study are listed below in **Table2.5**.

Table 2. 5 Buffers and solutions are listed.

Buffer/ Solution	Components
4X SDS Loading Dye	100uL 2-mercaptoethanol, 900 µl BioRad Laemmli Buffer
RIPA buffer	75 µl 2M NaCl, 50 µl 1M Tris-HCl, 10 µl 10% SDS, 40 µl 25x proteinase inhibitor, 40 µl 20X PhoStop,775 µl ddH2O (for 1mL)
Protein Lysis Buffer	25 ml from 1M HEPES, 15 ml from NaCl, 2.5 ml from 200 mM pH:8.0 EGTA, 57.4 ml from 87% Glycerol, 50 ml from 10% Triton x100, volume up to 500 ml with 27 ddH2O. Stored at 4 °C.
10x Running buffer	30.3g Trisbase, 144.1g glycine, 100 ml 10% SDS, ddH2O up to 1L
Anode I buffer	18.15g Trisbase, 100ml 100%MetoH, ddH2O up to 500mL
Anode II buffer	1.5g Trisbase,100ml 100%MetoH, ddH2O up to 500mL
Cathode buffer	2.62g aminocaproic acid, 100ml 100%ethanol, ddH2O up to 500mL
Blocking solution (5%)	5g BSA or milk, 100 mL TBS-T (2%)
10% APS	0,5g APS, 5mL ddH2O
10X TBS	24 g Tris, 88g NaCl dissolve in 900 ml ddH2O. pH adjusted to 7.6, volume bring up to 1000ml.
1X TBS-T (Tween 0.2%)	100ml from 10X TBS, 900 ml ddH2O, 2ml Tween-20.
10X Running Buffer	10.08g SDS, 30.3g Trismabase, 144g Glycine, volume up to 1000ml with ddH2O.
Mild Stripping Buffer	1.5g Glycine, 0.1g SDS, 1ml Tween-20 volume up to 50 ml; adjust pH 2.2, complete volume to 100mL
MTT solution	5 mg MTT, 1ml Sterile 1X PBS
10% SDS-PAGE gel (1.5 mm)	Stacker: 1.5 ml Solution A, 1.5 ml Solution B, 15µl APS, 3µl TEMED Resolver: 4 ml Solution A, 4 ml Solution B, 40µl APS, 4µl TEMED

2.7 Cell culture products

Materials and solutions were used in cell culture studies are listed below, **Table2.6**.

Table 2. 6 List of cell culture solutions.

Product	Catalog No.	Company
DMEM w/1.0 g/L glucose w/o L-Glut	BE12-707F	Lonza, Switzerland
RPMI 1640, Low Glucose	BE12-702F	Lonza, Switzerland
RPMI w/o phenol red	BE12-918F	Lonza, Switzerland
PBS-1X w/o Ca, Mg	BE17-516F	Lonza, Switzerland
FBS	S181G-500	Biowest
Charcoal stripped FBS	S18F-100	Biowest
Trypsin	BE-17-161E	Lonza, Switzerland
L-Glutamine	BE17-605E	Lonza, Switzerland
Sodium pyruvate	BE13-115E	Lonza, Switzerland
Penicillin/Streptomycin	DE17-602E	Lonza, Switzerland
NEAA 100X	BE13-114E	Lonza, Switzerland
Trypsin-Versene (EDTA) Mix(1X)	BE17-161E	Lonza, Switzerland

CHAPTER 3

METHODS

3.1 General cell culture maintenance

In this study, a breast cancer cell line T47D was used and maintained in 10% FBS complete RPMI media. Liver cancer cell lines Hep3B and SkHep1 was maintained in 10% FBS complete DMEM media. Both complete DMEM and RPMI were supplemented with 10% FBS, 2%NaPyr, 2% L-glut, 1%Pen-strep, 1%Neaa.

Stock cell lines were stored in liquid nitrogen tanks or -80 ˚C freezers and thawed in 37˚C water bath before usage. Then, cells were collected from cryovials with DMEM or RPMI depending on the cell type and centrifuged for 5 minutes at 1500 rpm. Freezing media was discarded, and cells were seeded in T25 flasks. Seeded cells were left to grow in incubators providing 5% CO_2 at 37˚C. Cells were transferred to T75 flasks and passaged when they reached to 80-90 % confluency. For passaging, media was discarded, and cells were washed with 1X PBS, and trypsinized with 1X Trypsin for 5 minutes. This mix was diluted with fresh DMEM or RPMI and depending on cells' doubling time the required amount was transferred to new T75 flask.

New stocks of cell lines were created at the early passages of the cells by freezing them in freezing media. Freezing media was prepared as 10% DMSO and 90% sterile, filtered FBS. Pelleted cells were resuspended with this media and distributed to cryo-vials. Cryo-vials were kept in -20 ˚C for an hour then transferred to -80˚C for overnight. Stocks were stored in liquid nitrogen tanks.

To start experiments, required numbers of the cells were seeded to relevant multi-welled plates. For seeding, cells were collected-pelleted, re-suspended and mixed with trypan blue (1:1) and 10µl of the mixture was loaded into hemocytometer to count cell number under light microscopy. Total amount of cells was calculated and required volume of cells were taken from suspension completed with fresh media to seed each well.

3.2 Hormone treatments

Aldosterone and/or Estrogen treatments were performed on T47D cells that were seeded in either 6 well plates or 100 mm petri dishes for mRNA and protein collection, respectively. One day after the seeding with complete media, the phenol red free media with charcoal free FBS (%5) was added into the cells which were PBS washed upon disposal of phenol red containing media. After incubation for 24 hours with phenol red free media, hormones were introduced to cells in the same but fresh media. Both E2 and ALDO were given as 10nM in the treatment volumes. Since both ALDO and E2 were dissolved in EtOH, treatment groups always had EtOH control groups. Stock of ALDO was 5mM, and E2 was 2mM (all stored at -20˚C refrigerator). Required concentration of the treatments was obtained using a dilution series of the working solutions. All groups had equal concentrations of EOH. All experimental groups had two biological replicas.

3.3 Drug treatments

Depending on the experiment's duration and the duplication time of the cells, specified number of Hep3B and SkHep1 cells were seeded into multi welled plates or 100mm dishes. 24 hr. after seeding, drugs were introduced to cells. Since both, the Phenothiazine derivatives and Sorafenib were dissolved in DMSO as 100µM stocks at -20˚C refrigerator, DMSO was the control of the experiments. All the experimental dilutions of the drugs alone or combination treatments contained same fixed amount of DMSO which was fixed as 0.01%. For 48 hours of treatments, media changed with freshly prepared drug contained media in each 24 hr.

3.4 MTT assay for evaluation of cell proliferation

For the MTT assay, the hormone treatment protocol described in section **3.2** was directly used or altered based on the optimization trials. Duration of the phenol red free media incubation was the altered step of the protocol which used as 1 or 3 days (specified in Results). Rest of the protocol was the same.

Depending on the treatment duration and the duplication time of the cell line, 2000-5000-10000 cells (indicated in figures) were seeded into 96 well plates a day before the experiments. At the end of the treatments (at least 3-5 replicates for each group) cell were given the MTT solution. The solution was prepared by dissolving 5mg of MTT in 1 ml 1X PBS. After mixing, the

solution was filtered using 0.22 µm pore size filters. For each well with fresh 100 ul phenol red free media, 10 µl of MTT mixture was added which are prepared as master mix for all used wells. After an incubation period of 4 hours at 37˚C incubator, 100 µl of fresh SDS-HCl (2g SDS, 0.01M HCL) mixture was added to each well. Followed by 12-16 hours incubation (4-18 hr. recommended), the absorbance of the wells was measured at 570nm by using Microplate Spectrophotometer (Thermo Scientific, USA). Blank and control normalized results were analyzed in GraphPad Prism6 by using One-Way ANOVA Tukey Multiple Comparison method and Three-Way ANOVA Multiple comparison method.

3.5 RNA isolation with QIAzol

At the end of the experiments, cells were pelleted and stored at -80 ˚C until RNA isolation. RNA isolation with the QIAzol is one the isolation method that was used in this thesis. All steps were performed on ice and under the chemical hood. 600-1000µl QIAzol was added into each pellet and mixed thoroughly. Chloroform was added in a 1:5 ratio to QIAzol and mixed again. After tubes were incubated at RT for 10 minutes, mixtures were centrifuged at 13200 rpm for 15 minutes at 4˚C. Three phases were observed and the aqueous upper phase was collected without touching the middle, cloudy phase. Next, isopropanol was added to the tubes 1:1 ratio to lysate and samples were inverted for 15 times. After 10 minutes incubation at RT another round of centrifugation was followed for 15 minutes at 4˚C. Supernatants were discarded and white pellets were washed with 75% EtOH. Samples were centrifuged at 8000 rpm for 8 minutes at 4˚C. Pellets also washed with 100% EtOH and left for air drying under the hood. After drying, pellets were dissolved in 20-30µl RNase/DNase free water and RNA concentration and RNA quality of samples were measured by using Nanodrop spectrophotometer. Stocks were stored at -80˚C.

3.6 RNA isolation with RNeasy mini kit

RNeasy mini kit was used to isolate RNA by following the manufacturer's protocol (Qiagen, Germany). RNA was elucidated from columns with 20-30µl RNase/DNase free water and RNA concentration and RNA quality of samples were measured by using Nanodrop spectrophotometer. Stocks were stored at -80˚C.

3.7 cDNA synthesis

For the cDNA synthesis, 1µg of RNA was taken from each sample for the reaction. Two kits were used one of them is RevertAid first strand cDNA (Fermentas, Canada) synthesis kit (liver cancer cell lines) and the other one is iScript cDNA synthesis kit (Bio-Rad) (breast cancer cell line). In both cases, manufacturer's protocols were followed. cDNA samples were diluted 1:10 with nuclease free water before Real-time PCR protocol.

With RevertAid kit protocol, 4µl 5X reaction buffer, 10nM dNTP,1µl RiboLock and 1µl Reverse Transcriptase were mixed with 1µl Oligonucleotide and 1000ng RNA sample; and for one reaction a total volume of 20µl was obtained by adding nuclease free water. Reaction was run in thermal cycler (Applied Biosystems, USA) with following conditions; 42˚C 60 min, 70˚C 5 min, 4˚C α.

In iScript protocol, 1000ng RNA sample was added into each reaction together with 4µl reaction buffer and 1µl Reverse Transcriptase (RT). Reaction volume was completed to 20µl by adding nuclease free water. Reaction conditions are as following: 5 min 25˚C priming, 20 min 46˚C RT, 1 min 95˚C RT inactivation.

3.8 Real-time PCR and analysis of relative expressions

10µl reaction volume composed of 10 µM-1µl forward, 10µM-1µl reverse primers, 5µl 2X SYBR Green I mix, 1µl nuclease free water and 2µl of cDNA samples was run in Light Cycler 480 Instrument (Roche, Switzerland). Reaction conditions (**Table3.1**) of samples were the same except the annealing temperature which was determined based on the primer sequences (**Table2.2**). For internal control normalization, reference gene TPT1 (Pilbrow et al., 2008) was used for each sample. Each group (with two biological replicates) had two technical replicates to have statistical significance.

At the end of the reaction, each sample gave Tm (melting temperature) for primers and CT (cycle threshold) values of genes which were used to determine the presence of a single amplicon to evaluate the alterations in gene expressions, respectively. To analyze the gene expression CT values of genes that were normalized to TPT1 values and experiment controls. One way-ANOVA Dunnett or Tukey Multiple comparison test were performed on the log2 transformed 2ˆ(–ΔΔCT) (**Table3.2,** Rao et al., 2013) of groups in GraphPad Prism6.

Table3. 1 RT-qPCR steps and conditions for mRNA quantification.

	Temp. (ºC)	Time (mm:ss)	Cycle
Pre-incubation	95	5:00	1
Amplification	95 58-60 72	0:10 0:20 0:20	45-50
Melting curve	95 55 95	0:05 1:0 Acquisition per 5ºC	1
Cooling	40	0:30	1

Table3. 2 Formula of ΔΔCT calculation for RT-qPCR results

	Reference Sample	Target Sample
Reference gene	A	B
Target gene	C	D

$\Delta\Delta CT = \Delta CT$ (a target sample) $-\Delta CT$ (a reference sample) $= (CT_D - CT_B) - (CT_C - CT_A)$.

3.9 Total protein extraction with RIPA buffer and protein lysis buffer

To collect the proteins from treated liver cancer cell lines freshly prepared RIPA buffer was used. RIPA buffer mixture was added with Complete EDTA-free Protease inhibitor cocktail and PhosSTOP Phosphatase inhibitor cocktail tablets to have complete lysis buffer. For 100 mm dishes 250 µl of RIPA buffer was added to PBS washed cells and cells were scrapped. Cell and RIPA mixtures were collected in 1.5 ml tubes and incubated on ice for 30 minutes. During the incubation on ice, tubes were vortexed every 5 minutes. Tubes were centrifuges at 13.200 rpm and the liquid portion was collected into new tubes without touching the cloudy component. Protein solutions were stored at -80˚C.

Even though ‘protein lysis buffer’ contained different ingredients than RIPA buffer, the experimental steps to follow for extraction was the same. 1 tablet of Complete EDTA-free Protease inhibitor cocktail was dissolved in 20 ml of pre-lysis buffer and half of it stored for future usage while rest was used after 1 tablet of PhosSTOP Phosphatase inhibitor cocktail was dissolved in it. This protocol was followed for T47D cell line.

3.10 Quantification of proteins with BCA assay

Protein concentrations were measured with BCA Protein Assay Reagent Kit (Thermo Scientific, USA). Kit's protocol was followed, and samples were loaded as duplicates or triplicates. 25 µl of standards was loaded while 2µl of the samples was used to keep enough samples. Standard curve was drawn from the standards' absorbance to obtain y=ax+b (y=absorbance, x= unknown concentration) equation from the graph. Unknown protein concentrations were calculated by comparison to standard curve. Calculations were performed by taking the dilution factor into account.

Depending on the required protein concentration (15-30µg), proteins were mixed with 4X Laemmli sample buffer (Bio-Rad, USA, 161-0747) loading dye and volume completed using RIPA buffer/protein lysis buffer. These mixtures were incubated at 95˚C heat block for 5 minutes to denature proteins. Stocks were kept in -80˚C. Denatured proteins either used immediately or stored at -20˚C for next day to use.

3.11 SDS-PAGE, Western Blot

SDS-PAGE (10 %, Bio-Rad, USA) gel was casted according to the manufacturer's protocol. Required volumes of samples was loaded into the wells along with the 3-5 µl PageRuler Prestained Protein Ladder (Thermo Scientific, USA). Gel was run at 80V until proteins left the stacker gel, then voltage switched to 120 V for resolver gel. Proteins were blotted to PVDF membranes (3010040, Roche) by using semi-dry transfer protocol (30 minutes, 25V, 1A). Blotting success was assessed with Ponceau staining after each transfer (5 min at RT), and the membrane was washed off with distilled water. Blocking step performed either with 5% milk or 5% BSA in TBS-T for an hour at RT. Membranes were cut according to expected band sizes for antibodies and incubated overnight at 4˚C in primary antibodies. Membranes were washed 10 minutes for three times with TBS-T before the secondary antibody incubation for an hour (or up to 4hr for phosphorylated proteins) at RT. Membranes were washed 10 minutes for three times with TBS-T and visualized with ECL Plus Western Blotting Detection System (RPN 2232, GE Healthcare) by using Amersham Imager 600 (GE Healthcare Life Sciences, USA).

3.12 Mild stripping protocol

Mild stripping protocol was used to re-probe the existing blots with new antibodies. In order to do that, striping buffer was prepared. Membranes were washed with mild stripping buffer 10 minutes for 2 times at RT. TBS wash was performed twice for 10 minutes. Finally, 5 minutes TBS-T wash was performed twice. Membranes were blocked at RT for an hour as mentioned earlier before the primary antibody incubation.

3.13 Western Blot and quantification of protein bands

For the quantification of the bands ImageJ program ((Schneider et al., 2012)was used. All images were converted to 16-bit and all the relevant bands were selected for obtaining band densities. Density plots were drawn for all bands at once, and band density boarders were determined, manually. Area and percentage measurements for each plot were pasted into an Excel file. Taking log2 of the percentage intensities and normalizing them to GAPDH and mean DMSO (drug treatment experiment) or ETOH (hormone treatment experiments) values were the method for the quantifications when n=2. All values on graphs were represented in log2 format. Graphs were drawn in Graph-pad Prism6. One-way ANOVA Dunnett and Tukey multiple comparison tests, and Two-way ANOVA Multiple comparison test (row: SFB and column: TFP effect) were performed as stated in figure legends.

3.14 Heatmap generation

Differentially expressed genes identified from *RNA-seq* data of TFP and SFB single and combinatorial treatment in Hep3B cell line was used to generate heatmaps. Gene lists in log2 forms were uploaded to online tool Heatmapper (Babicki et al., 2016). Average linkage was used as clustering method and distance was based on Pearson correlation in all heatmaps. These heatmaps used to visualize differential responses of interested genes against the given treatments.

3.15 PCA analysis

Principal Component Analysis (PCA) was performed by Ayşe Gökçe Keskus in R environment and visualized by “ggfortify” package of R (Y. Tang et al., 2016).

CHAPTER 4

RESULTS

4.1 ALDO mediated mineralocorticoid receptor and estrogen mediated estrogen receptor signaling pathways' possible crosstalk in breast cancer cell lines.

Herein, I mainly focused on ALDO mediated MR signaling and its possible interaction with E2 mediated ER signaling in mechanistic and functional aspects using T47D breast cancer cell line.

It is important to note that, the fundamentals of this study were initially established by Bircan Çoban (MSc Thesis, 2016) where ALDO and E2 hormones were applied solely and well-established ER and MR downstream target genes were studied both at the mRNA and protein levels. Yet, a more comprehensive approach, i.e., combinatorial hormone therapy application, remained to be pursued.

Herein, I aimed to investigate the role of combinatorial ALDO and E2 hormone therapy in an endogenously MR and ER expressing T47D cell line by identifying alterations in expression of MR and ER and downstream members both at the mRNA and protein levels. Additionally, effect of ALDO, E2 and ALDO-E2 combinatorial therapy on cell viability was further pursued for studying the possible role of MR-ER crosstalk on breast cancer cell proliferative capacity.

Hence, it was the best fit to study possible interactions, so-called crosstalk, among MR-ALDO and ER-E2 signaling pathways when both hormones were applied in combination.

4.1.1 Establishment of ALDO mediated MR signaling in T47D cell line

ALDO and E2 treatment doses were initially determined by literature mining and based on their expression levels in plasma/blood. Physiological concentration of ALDO is 0.5-1nM and E2 is 0.1-10nM (Celojevic et al., 2011; Hermidorff et al., 2017). For the purpose of this study, an

intermediate dose of ALDO (10nM) and E2 (10nM) were applied for ALDO, E2 and combinatorial ALDO-E2 applications on T47D cell line.

Initially, effectiveness of the hormone treatments was established by studying well-established hormone mediated MR and ER targets. For MR, ZBTB16 gene was selected as the positive control as it was previously shown to be induced by ALDO in kidney (Billan et al., 2015). Therefore, ZBTB16 positive control target gene was shown to be significantly upregulated upon 24-hour, 10nM ALDO exposure (**Figure 4.1.1)**. E2 treatment was also shown to upregulate ZBTB16 expression significantly (ALDO vs E2 p value 0.0687). Furthermore, combination of hormones increased the level of gene expression, as ALDO did, but it was significantly more than E2, alone (**Figure 4.1.1).** Increases obtained by ALDO treatments indicated the successful activation of signaling pathways by this hormone and helped understand that ALDO and E2 was not synergistic nor antagonistic with respect to a common target of MR and GR.

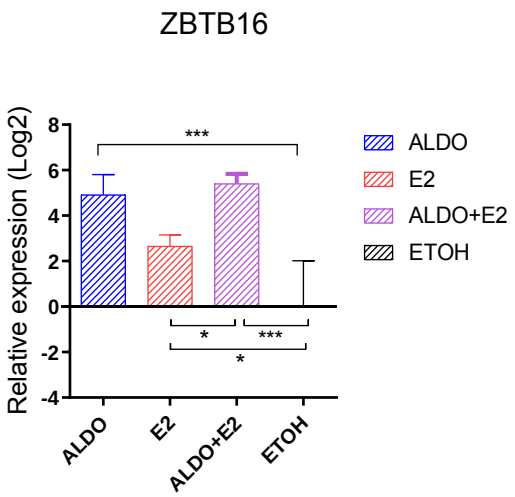


Figure 4.1 1ALDO treatment upregulates ZBTB16 gene expression.

T47D cell line upon 10 nM ALDO and/or E2 treatment for 24 hr. One-Way ANOVA Tukey Multiple comparison test was applied (* $p \leq 0.05$, ** ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$, # $p < 0.1$).

4.1.2 Establishment of E2 mediated ER signaling in T47D cell line

E2 and ER signaling have been comprehensively studied in breast cancer biology and has been long known to induce the expression of Progesterone Receptor (PGR) and TFF1/PS2 (Trefoil factor 1) primary target genes and they have been widely accepted as the two of the primary

downstream effectors of ER (Bourdeau et al., 2008). Hence, initial evaluation on E2 mediated ER signaling pathway was performed by testing changes observed in expression levels of these genes upon ALDO, E2, and ALDO-E2 treatments.

As shown in **Figure 4.1.2,** E2 significantly increased the PGR expression levels in each of the E2 exposure groups, namely E2 and E2-ALDO. Yet only ALDO exposed group resulted in significant reduction in PGR levels in T47D cell line. TFF1/PS2 exhibited significant upregulation upon E2 and E2-ALDO exposure yet did not show the same reduction trend with ALDO alone group as observed in PGR mRNA. On the contrary TFF1 levels exhibited an upregulation trend (instead of downregulation as in PGR) approaching significance (p-value: 0.0673) upon ALDO exposure.

Overall, E2 mediated ER signaling activation was confirmed and ALDO was shown to be significantly downregulating PGR expression upon 24-hour exposure in T47D cell line.

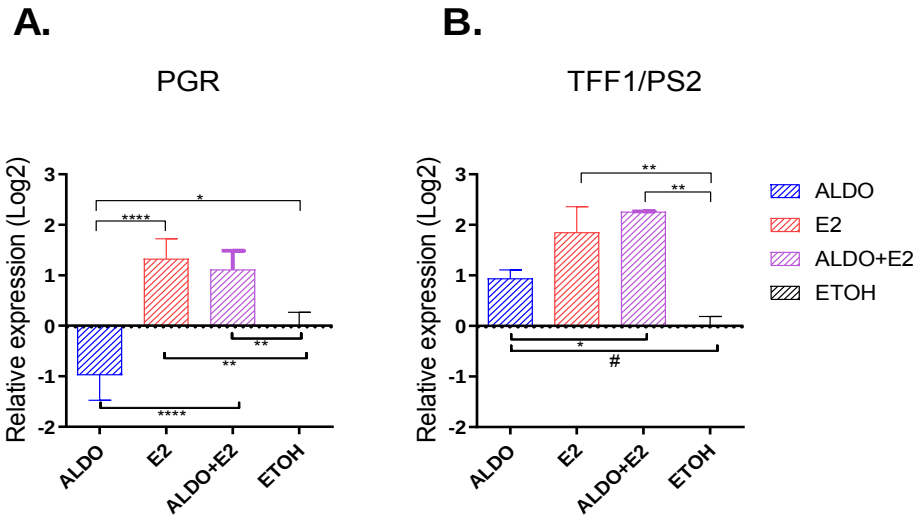


Figure 4.1 2 E2 treatment upregulates PGR and TFF1/PS2 gene expressions.

A) PGR and B) TFF1/PS2 expression upon 24-hour exposure to ALDO and/or E2 in T47D cell line. Statistical analyses were performed by applying One-Way ANOVA Tukey Multiple comparison test (* $p \leq 0.05$, ** ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$, # $p < 0.1$).

Moreover, there was no sign for synergism. However, opposite changes of ER targets PGR and TFF1 genes upon ALDO treatment can be marked as an interesting discussion point to explain possible interaction.

4.1.3 Effects of ALDO and/or E2 on members of MR and ER signaling at the mRNA level

Herein, ALDO, E2 and combinatorial ALDO-E2 exposed T47D cells were used for testing MR, ER and their well-established downstream effector genes at the mRNA level. Within this context, ALDO's compatible receptors, MR (primary) and GR, and estrogen responsive ERα was tested at the doses expected to activate only MR and ER. Furthermore, MR signaling members NEDD4-2, α-,β-,γ- ENaC, both MR, GR, ER effector SGK1 was also studied at the mRNA level (**Figure 4.1.3**). MR, GR and ERα did not exhibit any significant alteration regardless of given treatment groups for 24-hour treatment (**Figure 4.1.3**). Only MR expression with ALDO treatment was approaching significance in terms of reduction (p-value: 0.0608). Increasing sample size may be needed since fold changes were relatively small and variations were high. However, this was an interesting finding for a hormone activated self-inducible transcription factor such as MR. Changes in these receptors' expressions might be related to doses and time interval of treatment groups, hence should be investigated at more advanced levels.

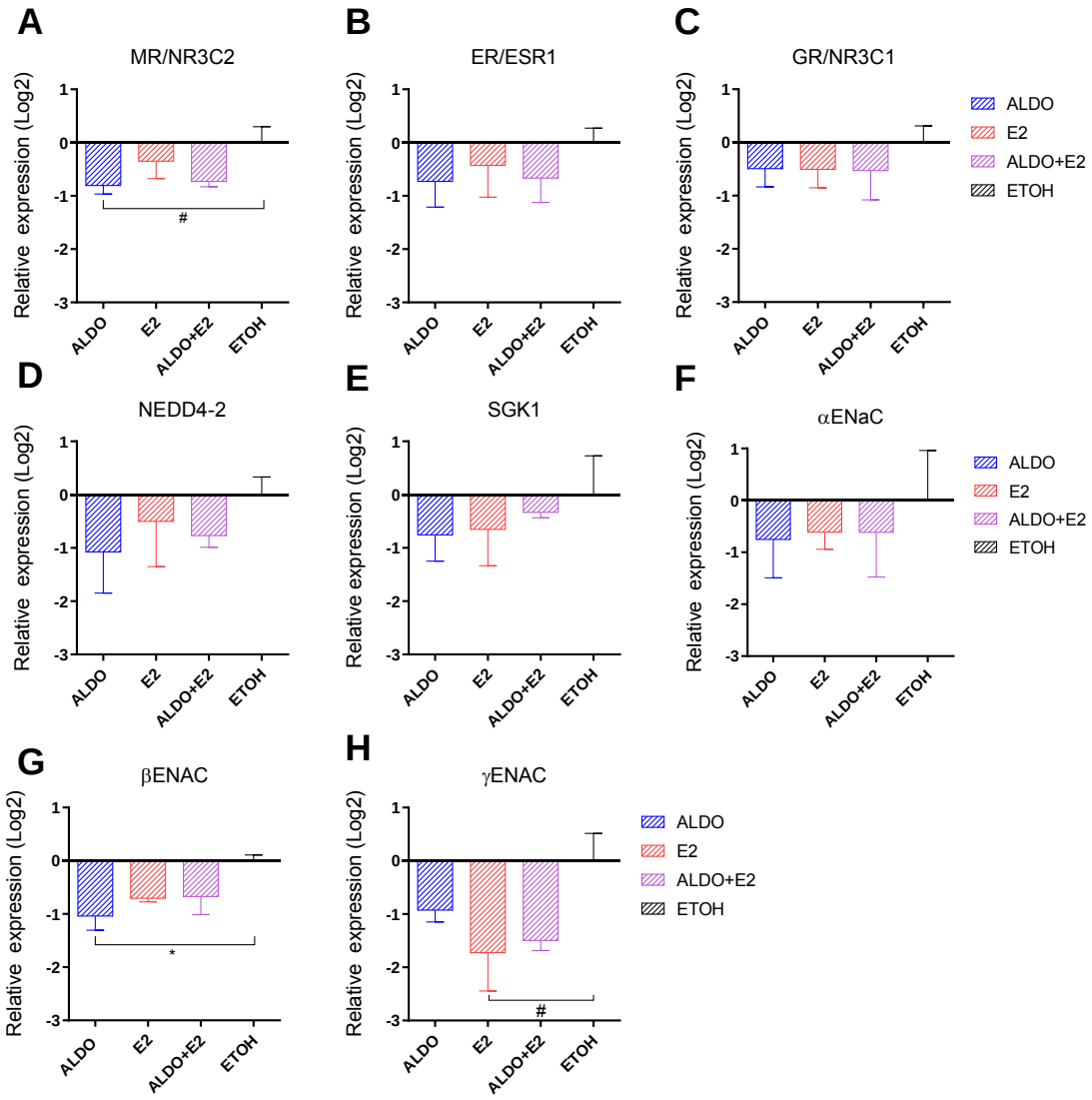


Figure 4.1 3 Gene expression of downstream signaling members of MR-ALDO and/or ER-E2 signaling.

A) MR, B) ESR1, C) GR, D) NEDD4-2, E) SGK1, F) αENaC, G) βENaC, H) γENaC upon 10 nM ALDO and/or E2 treatment in T47D cell line for 24 hr. One-Way ANOVA Tukey Multiple comparison test was performed (* $p \leq 0.05$, ** ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$, # $p < 0.1$).

There are several genes known to be modulated downstream of both ALDO and/or E2 signaling pathways. Some of them have been tested previously such as NEDD4-2, SGK1, α, β, γ ENaCs upon exposure to ALDO or E2, alone (Bircan ÇOBAN, MSc Thesis, 2016). Understanding how these members of the pathways were affected upon combinatorial treatments, however, was required to gain insight on possible interactions of MR and ESR1 pathways. Chosen genes in **Figure 4.1.3** showed the same yet mostly insignificant trends with E2 and ALDO treatment groups. Moreover, the combination treatment of E2 and ALDO resulted in no significant

changes in expression suggesting E2 and ALDO do not attenuate or potentiate the effects of each other for selected targets with roles in epithelial salt transport.

Decreasing trend upon single or combinatorial treatments can be observed in NEDD4-2, SGK1, α, β and γENaCs. However, only ALDO's effect on β-ENaC became significant moreover, E2 almost significantly reduced γENaC levels which might indicate differential regulation of subunits. Even though reduction trend was apparent in all cases, insignificancies might be caused by variations implicated in error bars. Moreover, ALDO is known to induce expression of these genes; hence these negative effects were not expected.

When these results are compared to previous results (Bircan Çoban, MSc Thesis, 2016), 100nM E2 treatment in T47D resulted in same trend as in here for ESR1, PGR, SGK1, NEDD4-2, ENaCs, and MR levels. Reduction in MR and MR signaling members by E2 activated ER signaling is underlined. Moreover, ALDO signaling caused PGR reduction and its rescue by E2 is also supports interaction possibility.

4.1.4 Effects of ALDO and/or E2 on members of MR and ER signaling at the protein level

I investigated singular E2 and ALDO with respect to the effect of combination treatment to test the presence of a possible crosstalk of MR and ESR1 signaling pathways in breast cancer at protein level. As a first step, changes in MR, ER and GR protein levels upon treatments were evaluated as shown in **Figure 4.1.4**.

Initially, MR protein levels were tested in this experimental set-up (**Figure 4.1.4**.). Herein, no significant changes were detected in MR protein levels upon ALDO, E2, ALDO-E2 treatments when both sets were used for statistical analyses. However, E2 groups had mild increasing trend but this was inconclusive due to variations especially in ALDO group.

Next, ER protein levels were tested in this experimental set-up. ER protein levels were reduced with E2 treatment in E2-treated groups, namely E2 and E2-ALDO. E2 induced suppression of ESR1 is known in literature (Ellison-Zelski et al., 2009). Hence, ALDO did not cause differential response to ER expression when applied in combination with E2 treatment (**Figure 4.1.4**.). These findings at the protein levels were compatible with mRNA levels, yet at different degrees (**Figure4.1.3.)**. Reduction trend in ESR1 levels at mRNA with ALDO treatment shows itself as minimal, exhibiting insignificant reduction. Besides of these findings, another member

of steroid receptor family, GR, was checked since it can also be activated by ALDO. However, due to variations and mild alterations of GR protein regulation, it was not possible to conclude GR protein alterations as significant findings.

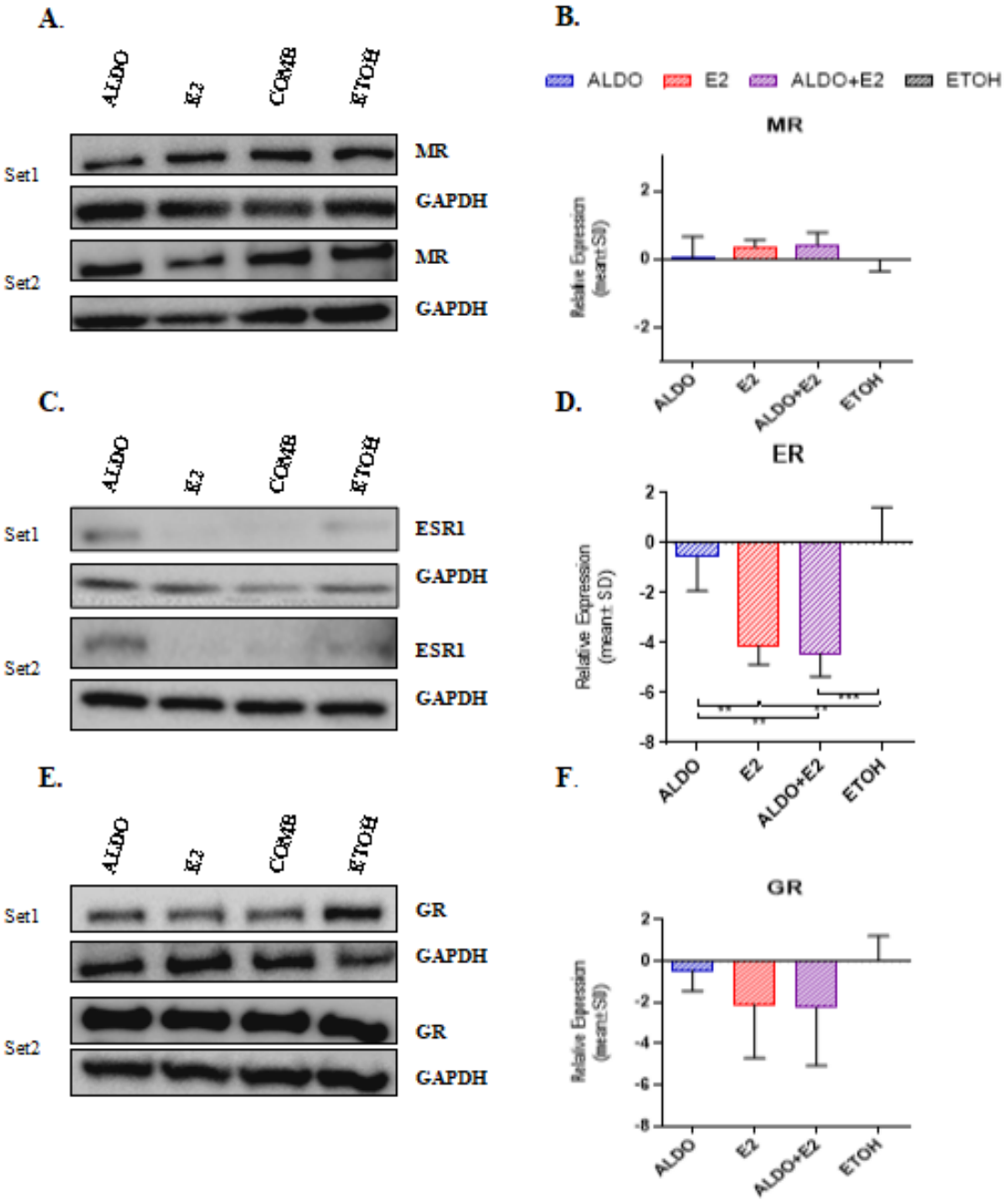


Figure 4.1 4 MR, ESR1 and GR protein levels upon ALDO and/or E2 exposure.

T47D cell line upon 10 nM ALDO and/or E2 treatment for 24 hr. (n=2). Protein images and quantification graphs of A, B) MR, C, D) ESR1, E, F) GR were represented. One-way ANOVA-Tukey Multiple Comparison test was applied (* $p \leq 0.05$, ** ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$, # $p < 0.1$).

In addition to MR, ERα and GR proteins, downstream effectors were also tested at the protein levels. Again, NEDD4-2, SGK1, αENaC, βENaC, and γENaC proteins were tested for ALDO, E2, ALDO-E2 exposed T47D cell line with two replicates (**Appendix A Figure1**).

As shown in **Figure4.1.5** protein levels were not significantly altered in most of them. Minimal increase in SGK1 levels in ALDO and E2 groups unlike mRNA levels was interesting since trend seems to be reversed in combination. NEDD4-2 trends were also insignificant but opposite to mRNA levels. αENaC reduced in E2 given group significantly. This significance was lost in combination. βENaC insignificantly increased in E2 given groups at protein levels. Unfortunately, second replicate of γENaC was not available to have strong comments however, reduction trend was clear for E2 group. MR – NEDD4-2 – βENaC and αENaC – γENaC shown similar trends, and these two groups acted towards different directions. To be able to have strong conclusions on possible crosstalk, these results were not powerful enough.

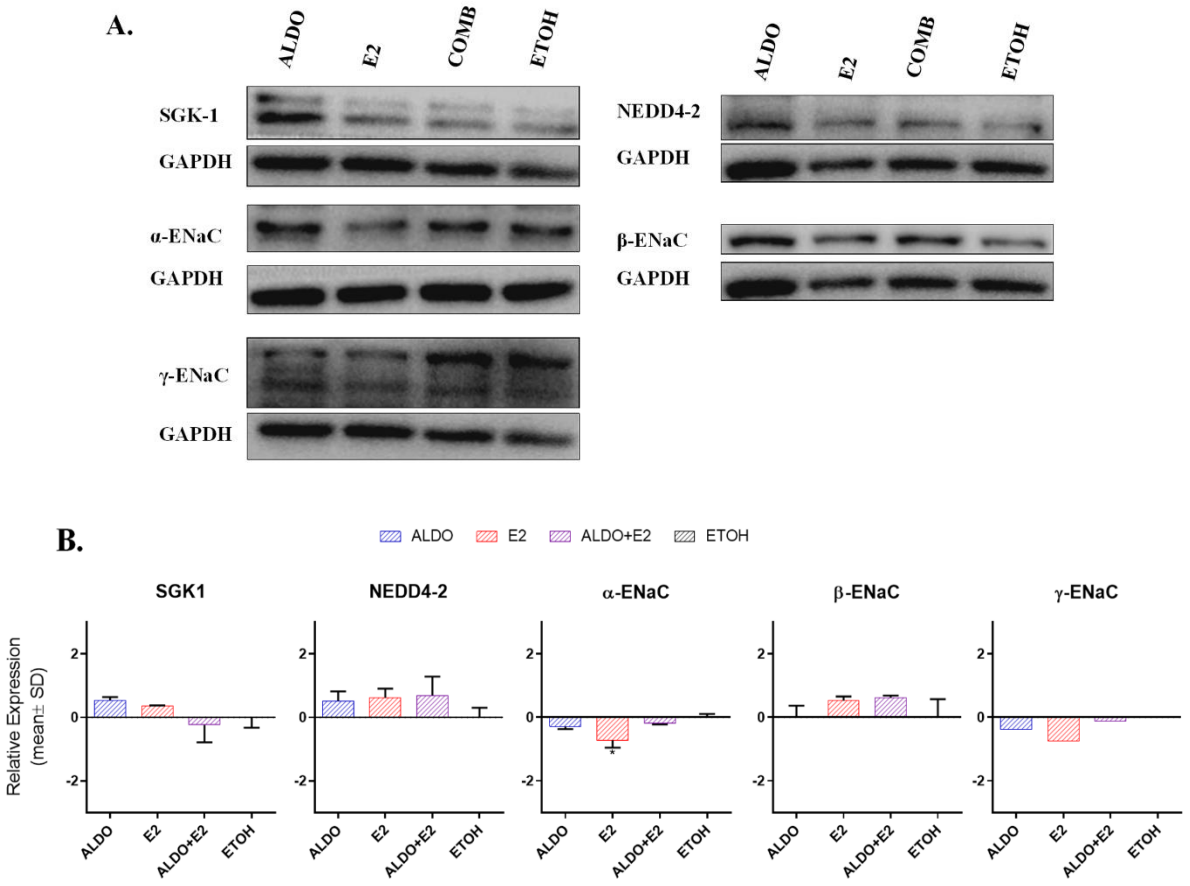


Figure 4.1 5 Protein levels of downstream signaling members of MR-ALDO and/or ER-E2 signaling.
T47D cell line upon 10 nM ALDO and/or E2 treatment for 24 hr. One-Way ANOVA-Tukey Multiple comparison test was applied. (* $p \leq 0.05$, ** ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$, # $p < 0.1$).

I also tried to validate mRNA results of PGR expression at protein levels. PGR has two isoforms with overlapping but differential functions. I was able to detect two of these isoforms in one replicate however, in second replicate second isoform was not clear enough to be used (**Appendix A Figure 1**). When the common isoform, presumably PGR-A, in these two replicates were quantified, mRNA results were supported. ALDO reduced this PGR isoform while E2 increased. In combination, again, E2 masked the ALDO reduction. Hence, possible E2-ALDO-PGR interaction could be further tested to drive more conclusive results.

4.1.5 Effects of ALDO and/or E2 on cell proliferation and viability

Functional effects of the single (ALDO, E2) and combinatorial (ALDO-E2) hormone treatment was investigated in T47D cell lines by using MTT cell viability test. Significant differences that were observed could give clues about the interaction of E2-ER and ALDO-MR signaling pathways on cell proliferation and viability.

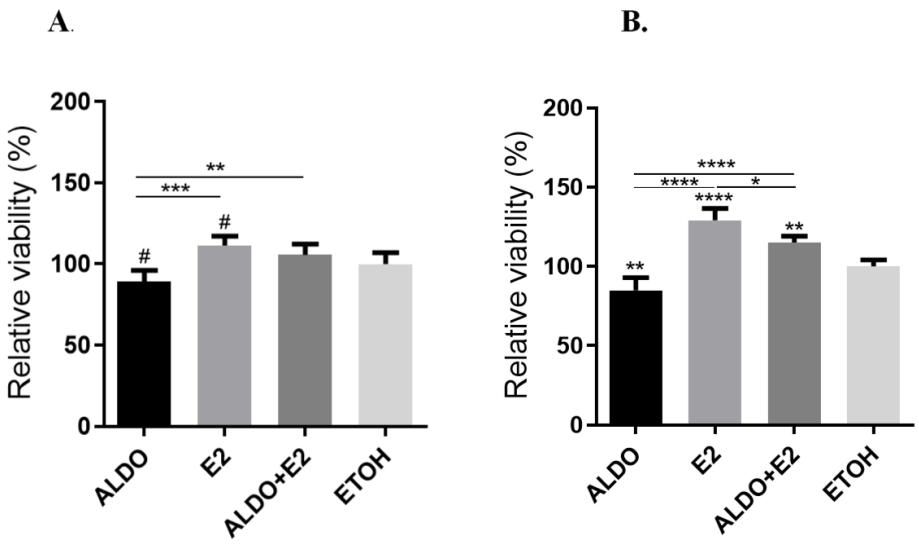


Figure 4.1 6 Cellular viability alterations upon 24 /48 hr ALDO and/or E2 exposure. A)10000 cells for 24hr, B) 5000 cells for 48hr treated with 10nM ALDO and/or 10nM E2 in T47D cell line. One-way ANOVA Tukey multiple comparison test was applied (* $p \leq 0.05$, ** ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$, # $p < 0.1$).

Herein, two different optimization methods were pursued. In the first trial 24hr hormone deprivation given cells were treated with hormones in two setups. For the first set-up 10.000 T47D cells were seeded and exposed to 10nM ALDO and/or E2 treatment for 24 hours. For the second set-up 5.000 T47D cells were seeded and exposed to 10nM ALDO and/or E2 treatment for 48 hours. Cell proliferation and viability indices were then calculated for the above-mentioned treatments.

For the first set up, **Figure 4.1.6 A.,** 24hour ALDO (p-values: 0.0761) exposure resulted in decreased cell viability whereas E2 exposure (p-value 0.0602) resulted in increased cell viability (these results are significant for One-way ANOVA-Dunnett test). In the second experimental set up, 48-hour ALDO exposure again resulted in decreased cell viability and again E2 exposure resulted in increased cell viability. Decreasing cellular viability was observed in both time points with ALDO. Moreover, reduction was more distinguishable at 48hr, suggesting time dependency. Success of these treatments were apparent due to increased proliferation in E2 groups. In combinations, ALDO was not able to exert its full reduction effect compared to control. Moreover, proliferation in combination group become significant at 48 hr. However, in 48hr treatment combination was not able to induce proliferation as E2 suggesting ALDO related regression. Hence, ALDO-E2 mediated possible MR and ER crosstalk was supported.

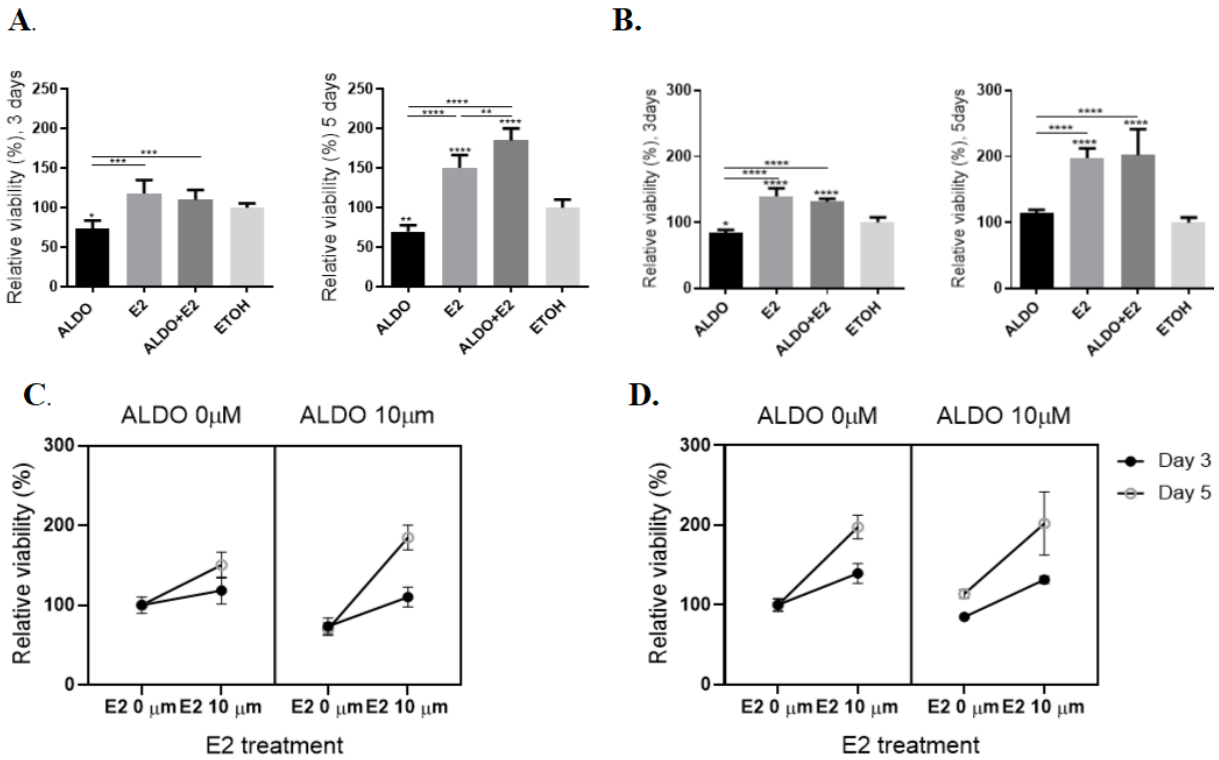


Figure 4.1 7 Cellular viability alterations upon 3/5 days of ALDO and/or E2 exposure.

3 and 5 days of 10nM ALDO and/or 10nM E2 treatment in A, C) 2000, B, D) 5000 T47D cell tested A, B) by One-way ANOVA Tukey multiple comparison test, and C, D) Three-way ANOVA test was applied (* $p \leq 0.05$, ** ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$, # $p < 0.1$)

I also tried to optimize hormone deprivation interval to 72 hrs. I used two set-ups by seeding 2000 and 5000 cells and tested hormone treatments in a time dependent manner (**Figure 4.1.7**). In the experimental set up wherein 2000 cells were seeded, ALDO caused reduction was prominent while E2 increased the proliferation in both time points (for 3 days of E2 treatment p-value is 0.0717 for One-way ANOVA Dunnett multiple comparison test). In combinations, E2 masked ALDO's effect, and at day 5 this increment even more than E2 alone group. I tested the time dependency, ALDO and E2 variables for viability by Three-way ANOVA to reveal their interactions (**Appendix A Table 1 and Table 2**). Time and E2 separately and together interacts with ALDO in this case. In the experimental set up wherein 5000 cells were seeded, time dependent loss of ALDO's reduction effect was a clear point. Responses at day 3 was similar in both cell densities however, ALDO's effect was less striking in higher density. Three-way ANOVA showed loss of interactions in 5000 cell seeded set-up. These results indicate almost consistent ALDO induced reduction in viability and supports ALDO-E2 interaction. However, variation of results based on hormone deprivation interval, treatment duration and cell density reveal complexity of these treatments affected by multiple parameters and requirement of a more robust methodology.

4.1.6 Effects of ALDO and/or E2 on cell cycle markers

Upon findings indicating differential and significant alterations in cellular proliferation and viability, I checked the levels of cell cycle markers shown below.

Cyclins CCND1 (cyclin D1) and CCNE2 (cyclin E2) was investigated because of their roles in cell cycle progression (**Figure 4.1.8**). Even though there were no statistically significant changes, reduction trend by ALDO treatment was observed in both. E2 containing groups were inconclusive due to variations. However, ALDO could not show its negative trend in combination groups as ALDO alone groups as in 24hr cell viability results. Cell cycle progression marker ANLN had the same minor reduction with ALDO which was opposite of E2 and combination group underlying the differential induction by these hormones. After cell

viability results, these alterations remind possible cell cycle arrest caused by ALDO treatment. Since the alterations in these genes are minimal, to be able have more valid conclusions experiments can be repeated with optimized conditions.

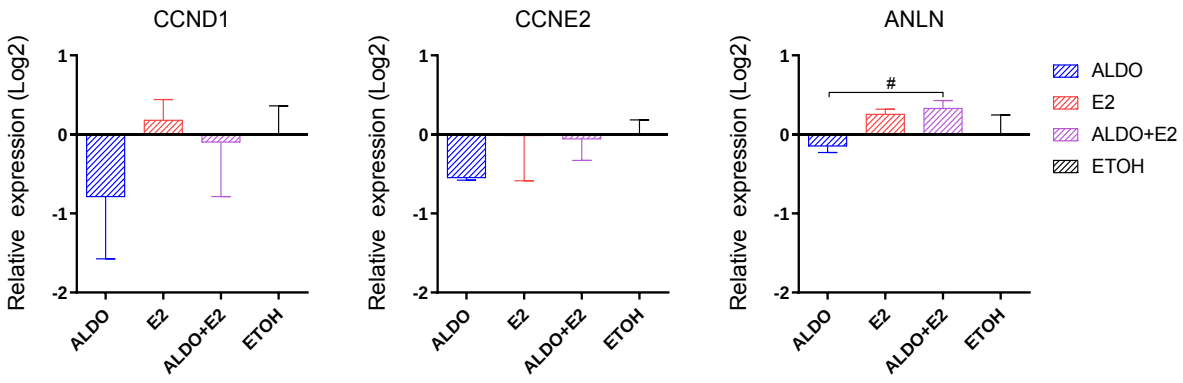


Figure 4.1 8. Gene expression of cell cycle markers upon ALDO and/or E2 treatment. T47D cell line upon 10 nM ALDO and/or E2 treatment for 24 hr. One-Way ANOVA Tukey Multiple comparison test was applied (* $p \leq 0.05$, ** ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$, # $p < 0.1$).

4.2. Sorafenib and Trifluoperazine combinatorial treatment on liver cancer/HCC cells and association with MR-GR signaling.

HCC has limited treatment options with bad prognosis and, Sorafenib is one of the few FDA approved drug choices as the first line of treatment. Recent studies showed that HCC patients can benefit from combinatorial treatments potentiating the drug responses. Phenothiazine derivates, drug repositioning candidates, were tested alone to find promising combination partners to SFB against HCC, previously. For that, commercial derivatives PTZ, PPH, PCP and TFP were tested in two liver cancer cell lines, Hep3B and SkHep1, at doses determined accordingly to IC_{50} values obtained by Murat Yaman (PhD thesis, 2020). Several marker genes were tested upon treatments to find indications supporting effectiveness of drugs. Our preliminary single treatment experiments in both cell lines at mRNA **(Appendix B Figure 1,2)** and protein **(Appendix B Figure.3**) levels highlighted two promising candidates for further experiments; PCP and TPF. Based on the Murat Yaman' (PhD thesis, 2020) work, TFP was determined as combinatorial partner of SFB in the scope of this thesis.

Since the reduced effectiveness and resistance to drugs are common problems for cancer treatments, combinatorial drug treatment strategies stood out to overcome these limitations. Especially, drugs like Sorafenib (SFB), with almost no alternative, is main part of this strategies to increase their effect against liver cancers and HCC. Herein, combinatorial treatment approach was adopted to enhance SFB's anticancer effects by combining it with one of the promising commercial PTZ derivatives, TFP. Combination investigations were carried on Hep3B cell line with TFP due to its promising and synergistic results and compared to SkHep1 due to opposite antagonistic results that came from Combination Index (CI) values of drug combinations (Murat Yaman, PhD thesis, 2020). Basically, several doses of TFP and SFB was combined and Hep3B cell line was exposed to this treatment for 48 hr. to evaluate cellular viability. Results were suggesting reduced cellular viability in combination groups which was found to be synergistic at certain doses by Murat Yaman (PhD thesis, 2020). These relevant doses were chosen for deeper investigations on mechanistic understanding on synergy at mRNA and protein levels. Moreover, SkHep1 cell line exposed similar combination dose scale showed antagonism in terms of viability with increased proliferation. Hence, this cell line was also studied to differentiate the mechanisms affected by drug combinations to induce sensitivity. Hence, functional results on viability were tried to be linked to a mechanistic understanding on

sensitivity to drug combinations in Hep3B cell line in comparison with SkHep1 in the scope of this thesis.

Whether the combination had synergetic effects on main cellular pathways several markers were investigated at protein level for Hep3B and compared to SkHep1 to understand the mechanisms driving these diverse directions of events in two different cell lines of liver cancers. Apoptosis and cell cycle makers together with PI3K/AKT/mTOR and MAPK signaling markers were evaluated. Moreover, whether this novel treatment targets MR and/or GR in differential manner as novel targets was investigated.

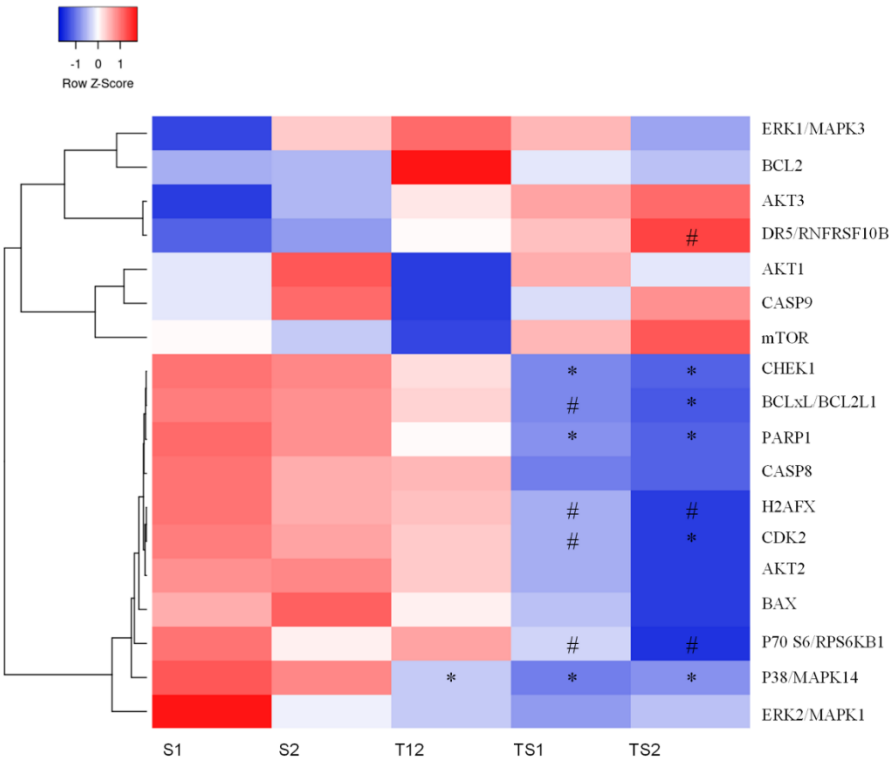


Figure 4.2. 1 Heatmap of differentially expressed signaling pathway marker genes upon exposure to SFB-TFP combination in Hep3B RNA-seq data.

Differentially expressed genes list was generated by Murat Yaman (PhD thesis, 2020) for RNA-seq results of 48 hr. S1: SFB 1 µM, S2: SFB 2 µM, T12: TFP 12 µM, TS1: TFP 12+ SFB 1 µM, TS2: TFP 12+ SFB 2 µM treatments in Hep3B cell line. Log2FC and p-values are represented as heatmap (* $p \leq 0.05$, ** ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$, # $p < 0.1$).

Previously, an RNA-seq experiment was performed in Hep3B cell line to identify differentially and synergistically altered pathways by drug combinations (TFP 12 µM, SFB 1 µM, SFB 2 µM- doses determined from synergism graphs/Combination Index). I used differentially expressed genes list generated by Murat Yaman (PhD Thesis, 2020) to evaluate and visualize the alterations in markers of several pathways critically altered in HCC and targeted by SFB

and/or TFP. Log fold change of gene expressions and their p values are represented in **Appendix C Table 1**. To visualize the data, heatmap was generated as shown in **Figure 4.2.1**. Alterations in expression of these genes were easily readable. Especially, genes accumulated together starting from CHK1 to RPS6KB1 had a notable division suggesting reduction in expression with drug combinations which was not the case for single treatments. This cluster was especially interesting to decipher possible interactions. Cell cycle, apoptosis and mTOR pathway markers in this cluster direct us to validate these results at protein levels. High concentration drug combination caused approaching significant increment in DR5 which was also noted. Moreover, p38 and ERK2 MAPK genes clustered together, and suggested the TFP caused reductions in these genes. Especially, p38 was significantly altered. Hence, MAPK pathway draw our attention. I aimed to validate these results at protein levels.

4.2.1. Effects on the cell cycle and DNA damage pathways

Hep3B and SkHep1 were treated with specified drugs to gain insight on mechanistic alterations caused their differential pattern (synergism and antagonism) on cell survival. Three TFP and two SFB doses were determined based on synergism map of Hep3B for combinations, and effective doses were also tested in SkHep1 cell line (Murat Yaman, PhD Thesis, 2020). Cell cycle and DNA damage markers were investigated for combinatorial treatment approach as shown in **Figure 4.2.2** below.

Check-point protein CHEK1 was investigated at both total and phosphorylated forms and phosphorylated form normalized to total to evaluate increment in activation. In both cell lines, single treatments of TFP had reduction trend, which was significant in SkHep1 compared to control, DMSO. TFP 12 μM combined with the SFB doses became significant in both cell lines. In Hep3B cell line this was more striking since single treatments were not significant. p-CHEK1 levels in SkHep1 became significant or approaching significant with combinations which was not the case for Hep3B. Even though variations interfered with the proper conclusion, active p-CHEK1 levels increased in single TFP and combination with increasing TFP doses in Hep3B. Again, in SkHep1, presumably, TFP induced increase was clear. Since these analyses performed with One-way-ANOVA Dunnett multiple comparison test, simply comparing each group to DMSO, two variables TFP and SFB doses were evaluated by Two-way-ANOVA-Tukey multiple comparison test to have an extensive view.

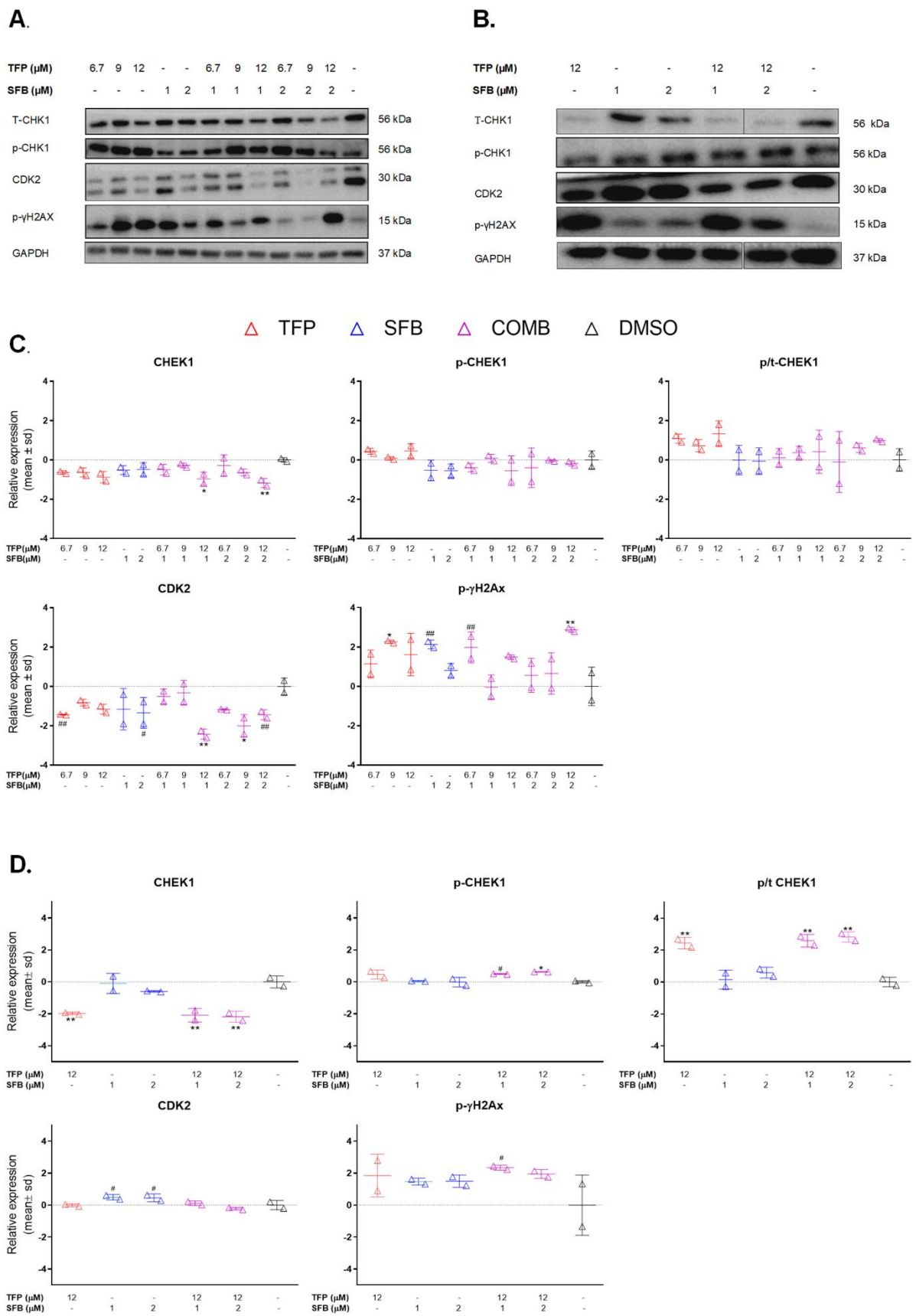

A.
TFP (μM) 6.7 9 12 - - 6.7 9 12 6.7 9 12 -
SFB (μM) - - - 1 2 1 1 1 2 2 2 -
T-CHK1 56 kDa
p-CHK1 56 kDa
CDK2 30 kDa
p-γH2AX 15 kDa
GAPDH 37 kDa
B.
TFP (μM) 12 - - 12 12 -
SFB (μM) - 1 2 1 2 -
T-CHK1 56 kDa
p-CHK1 56 kDa
CDK2 30 kDa
p-γH2AX 15 kDa
GAPDH 37 kDa
TFP SFB COMB DMSO
C.
CHEK1
p-CHEK1
p/t-CHEK1
CDK2
p-γH2Ax
Relative expression (mean ± sd)
TFP(μM) 6.7 9 12 - - 6.7 9 12 6.7 9 12 -
SFB(μM) - - - 1 2 1 1 1 2 2 2 -
D.
CHEK1
p-CHEK1
p/t CHEK1
CDK2
p-γH2Ax
Relative expression (mean± sd)
TFP (μM) 12 - - 12 12 -
SFB (μM) - 1 2 1 2 -

Figure 4.2. 2 Investigation of cell cycle and DNA damage pathway members at protein levels in Hep3B and SkHep1 cell line upon treatment of TFP and/or SFB in indicated doses.

48 hours of TFP and/or SFB treatments were given in indicated doses in A, C) Hep3B, B, D) SkHep1. All proteins were normalized to internal control GAPDH and experimental control DMSO. Results were represented in log2 scale, One-way ANOVA Dunnett's test was applied (n=2).

Interaction between drugs might be the indicators of synergism/antagonism, and this advanced test helped us to comment on possible synergism. Two-way ANOVA results of Hep3B is shown in **Appendix C Figure 5**. CHEK1 levels did not indicate any interactions of drug doses, and TFP doses found as the source of reduction alone. However, Sorafenib doses were found as main factor for p-CHEK1 alteration. p/t CHEK1 ratio was unconclusive. Hence, in terms of CHEK1 levels in Hep3B, there was no evidence for synergistic alterations. In SkHep1 (**Appendix C Figure 6**), all alterations in CHEK1 forms caused by column factor, TFP dose. As a result, one can conclude that both Hep3B and SkHep1 cell lines exhibited similar alterations against drugs for CHEK1 levels at different extent. Hence, this alone may not be able to explain the differences observed with respect to opposing effects in cell proliferation and drug resistance based on synergy tests conclusions.

These two cell lines were most distinguished with respect to the changes observed in cyclin dependent kinase, CDK2 levels. Almost all treatment groups caused a reduction in Hep3B cell line which were just trends with TFP or SFB alone and significant in combination groups especially at TFP 12 μM-SFB1 μM suggesting synergism between TFP and SFB. However, CDK2 levels in SkHep1 cell line have almost no change besides of slight increase in SFB alone groups. In Hep3B cell line, CDK2 levels were found to be altered by interaction of the drugs (**Appendix C Figure 5**). Especially TFP 12-SFB1μM combination shined out as possibly synergistic treatment for CDK2. However, in SkHep1, there was no interaction and TFP dose were the main effector. Altogether, differential alterations in cell cycle marker, CDK2 might determine the responsiveness of a cell line to the above-mentioned treatments which can explain their distinct behavior. Differential behavior of CDK2 should be discussed together with similar response to another cell cycle protein CHEK1 to have wider perspective.

DNA damage marker phopho-γH2AX had general increasing trend in all groups of both cell lines in which high combination doses or single treatments become significant or approaching significant. In Hep3B, TFP 12-SFB 2μM combinatorial dose was strikingly upregulated this

marker. Interestingly, TFP9, SFB1 and TFP 6.7-SFB1μM groups were also increased the expression in higher levels than most of the combination groups. Two-way ANOVA revealed interaction of these drugs in Hep3B while in SkHep1 there was no significant difference. TFP 12- SFB 2μM treatment might be synergistic in Hep3B cell line, however due to high variation it cannot be concluded. Moreover, reduction TFP 9-SFB1μM combination might indicate and antagonistic effect. Drugs alone and in combination can induce DNA damage, hence, may not contribute to sorting the divergence alone.

To sum up, CHEK1 in cell cycle levels and DNA damage response might be recognized as TFP induced effects. Differential CDK2 protein levels might help to understand sensitivity of Hep3B to combinations unlike Skhep1.

4.2.2 Effects on the intrinsic and extrinsic apoptosis

Determining that Hep3B but not SkHep1 cell exhibited reduced cell proliferation in combination, (Murat Yaman, PhD thesis, 2020) apoptosis attracted the attention as the second cellular mechanism to investigate. Chosen markers of intrinsic and extrinsic pathways were investigated at the protein level as shown in **Figure4.2.3** below.

In terms of extrinsic apoptosis pathway, DR5 was selected to be tested since Phenothiazines were shown to modulate death receptors (K. Yuan et al., 2015). Both cell lines showed significant increases in DR5 (mature form) protein levels with different doses of TFP alone as well as TFP-SFB combinations indicating TFP's singular effect on this protein since SFB does not cause any alterations. To test this statistically, I have employed a Two-Way ANOVA Multiple comparison test (**Appendix C Figure 5, Appendix C Figure 6**) and demonstrated that TFP was responsible for the effect observed in combination treatments.

On the other hand, intrinsic pathway members CASP9, BAX and BCL2 was not influenced mostly by the tested drugs and combinations. Moreover, Two-way ANOVA results did not indicate any interaction except Casp9 in SkHep1 cell line which shows drugs interact to change each other's effect on protein, but probably not in a synergistic or antagonistic manner (**Appendix C Figure 5, Appendix C Figure 6**).

Another intrinsic apoptosis pathway member, anti-apoptotic BCL-xL protein levels were found to have differential response to drug combinations in these cell lines. Even Sorafenib single

treatments were in opposite trends. Reduction of BCl-xL levels in increasing doses of drugs became significant in TFP 9 SFB 2 and TFP 12 SFB 2 μM groups in Hep3B which suggest synergism. However, drug combinations failed to alter BCL-xL levels in SkHep1 cells. To test the synergism (**Appendix C Figure 5**), Two-way-ANOVA Multiple comparison tests was performed. As expected, interaction of drugs was significant in Hep3B underlying effectiveness of TFP 12 SFB 2 μM group, specifically. In SkHep1 cell line, same analysis revealed significant interaction which underlies differential behavior in combination but does not support synergistic effect. Differential regulation of BCL-xL stands out as a candidate might involve the mechanism causing differential cellular responses (reduced BCl-xL levels activates apoptosis in HCC (Takehara et al., 2001). However, minimal alterations in other intrinsic apoptosis markers should not be ignored to understand these results. Crosstalk of pathways, regulations by other mechanisms should be considered.

Caspase8 is a caspase involved in extrinsic apoptosis signaling. Cleaved products of CASP8 showed the active extrinsic apoptosis in Hep3B cells at TFP 12 SFB1 and TFP 12 SFB2 μM combination treatments (**Figure4.2.3**). In SkHep1, these proteins have similar pattern with treatments but not as drastic as in Hep3B cell line. These results indicate existence of apoptosis which is required to be confirmed by several other markers and assays to support this idea. Even though proteins were in the same direction in both cell lines, levels of activation could be a clue for difference. To demonstrate the probable apoptosis was a result of combination therapy I performed a Two-Way ANOVA test and both cell lines failed to have drug interaction. Result showed that TFP and SFB acts on the protein at the same direction. However, when simple row (SFB) and simple column (TFP) tests were applied, TFP 12 SFB2 μM combination supports to idea of synergism.

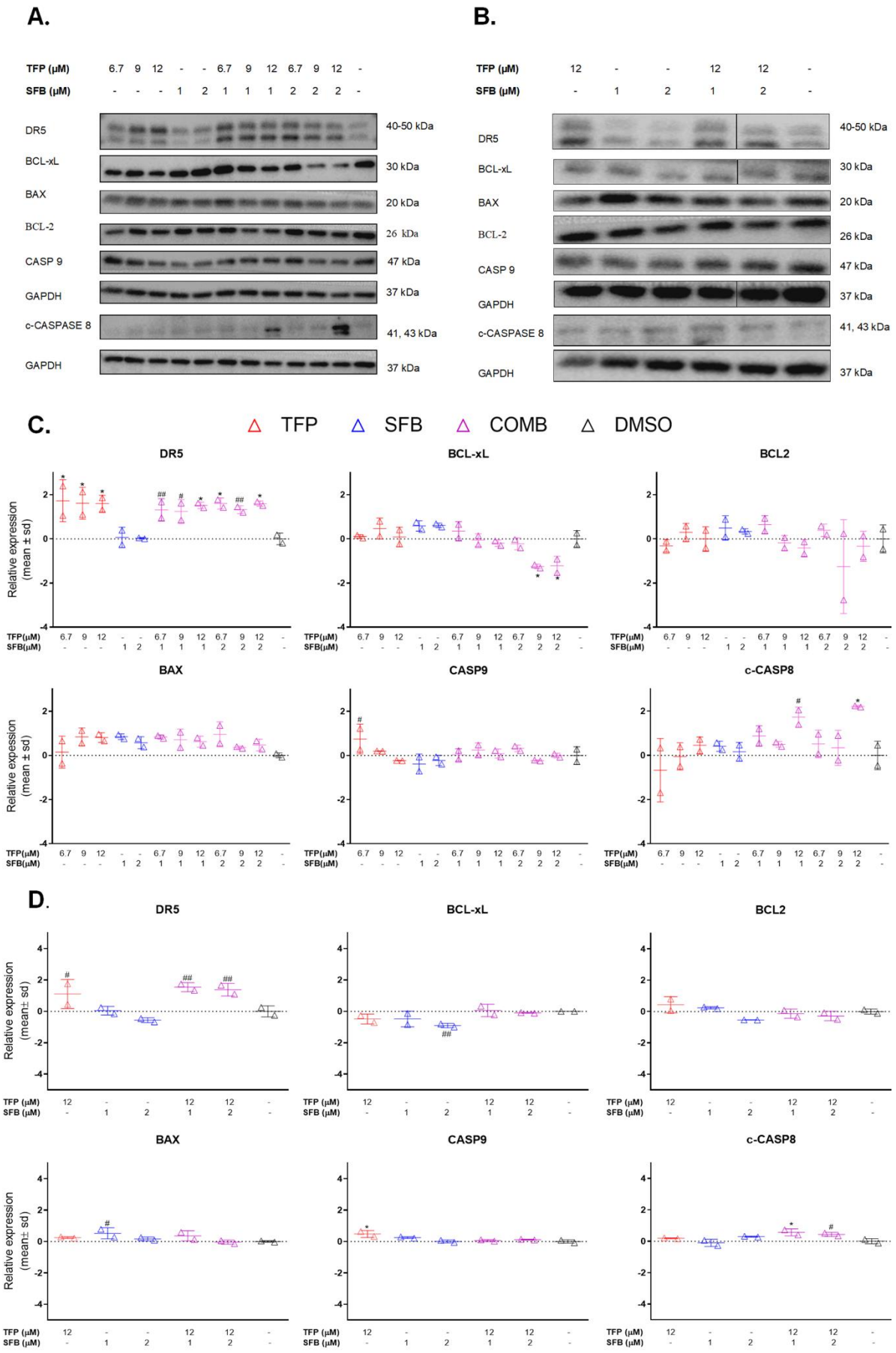

A.
TFP (μM) 6.7 9 12 - - 6.7 9 12 6.7 9 12 -
SFB (μM) - - - 1 2 1 1 1 2 2 2 -
DR5
BCL-xL
BAX
BCL-2
CASP 9
GAPDH
c-CASPASE 8
GAPDH
40-50 kDa
30 kDa
20 kDa
26 kDa
47 kDa
37 kDa
41, 43 kDa
37 kDa
B.
TFP (μM) 12 - - 12 12 -
SFB (μM) - 1 2 1 2 -
DR5
BCL-xL
BAX
BCL-2
CASP 9
GAPDH
c-CASPASE 8
GAPDH
40-50 kDa
30 kDa
20 kDa
26 kDa
47 kDa
37 kDa
41, 43 kDa
37 kDa
C.
TFP
SFB
COMB
DMSO
DR5
BCL-xL
BCL2
BAX
CASP9
c-CASP8
Relative expression (mean ± sd)
TFP(μM) 6.7 9 12 - - 6.7 9 12 6.7 9 12 -
SFB(μM) - - - 1 2 1 1 1 2 2 2 -
D.
DR5
BCL-xL
BCL2
BAX
CASP9
c-CASP8
Relative expression (mean± sd)
TFP (μM) 12 - - 12 12 -
SFB (μM) - 1 2 1 2 -

Figure 4.2. 3 Investigation of apoptotic pathway members at protein levels in Hep3B and SkHep1 cell line upon treatment of TFP and/or SFB in indicated doses.

48 hours of TFP and/or SFB treatments were given in indicated doses in A, C) Hep3B, B, D) SkHep1. All proteins were normalized to internal control GAPDH and experimental control DMSO. Results were represented in log2 scale, One-way ANOVA Dunnett's test was applied (n=2). (* $p \leq 0.05$, ** ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$, ## $p < 0.1$, # $p < 0.2$)

Induction of extrinsic apoptosis in both cell lines supported by TFP induced DR5 levels and active CASP8 protein products. However, most striking, and distinct difference was in BCL-xL. Understanding how this protein is part of drug combination induced responses regardless of similar alterations in other apoptosis markers should be the primary future focus.

4.2.3. Effects on cellular growth and proliferation by kinase driven pathways: AKT/mTOR and MAPK pathways

In addition to the above-mentioned pathways, other critical cellular pathways known to be affected by SFB and/or PTZs were examined further. These pathways known for their regulation on cell cycle and apoptosis. Hence, these results might be related to previous observations. Similarly, Hep3B's synergism with drug combination was evaluated together with the differential pattern of SkHep1 at the protein level.

PI3K/AKT pathway member AKT can be indicator of mTOR pathway activation. Total-AKT levels were similar and insignificant in terms of alterations in both cell lines (**Figure 4.2.4**). A striking difference was the double band formation in SFB and DMSO groups in SkHep1 unlike Hep3B cell line. These lower bands might be another isoform, modified form of the AKT or a non-specific occurrence. Existence of double band formation and TFP's effects on AKT should be investigated in detail to explain this interesting phenomenon.

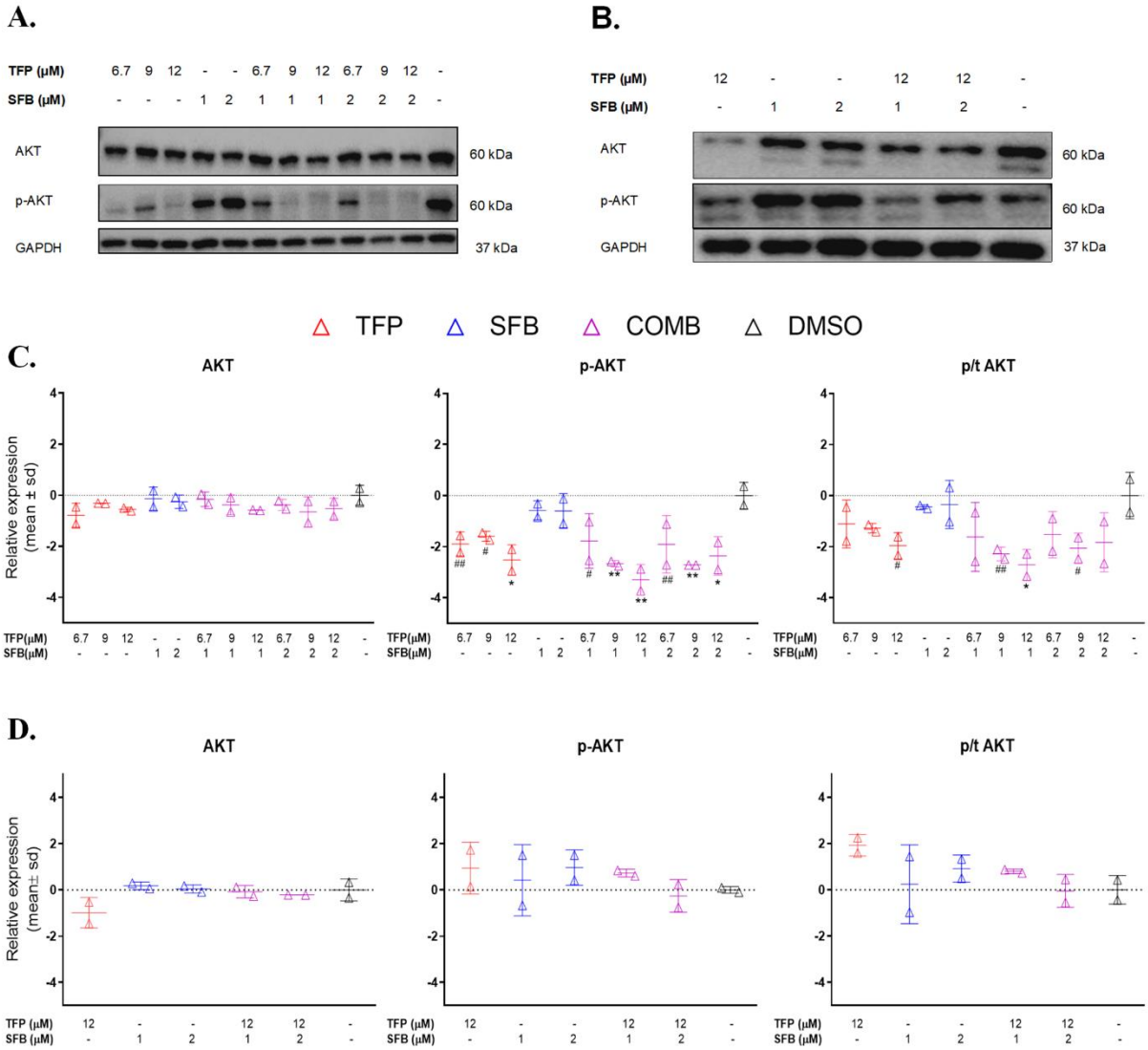


Figure 4.2. 4 Investigation of AKT protein levels in Hep3B and SkHep1 cell line upon treatment of TFP and/or SFB in indicated doses.

48 hours of TFP and/or SFB treatments were given in indicated doses in **A, C)** Hep3B, **B, D)** SkHep1. All proteins were normalized to internal control GAPDH and experimental control DMSO. Results were represented in log2 scale, One-way ANOVA Dunnett's test was applied (n=2) (* $p \leq 0.05$, ** ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$, ## $p < 0.1$, # $p < 0.2$).

In Hep3B cells, p-AKT (Ser/473) which is phosphorylated by mTORC2, decreased drastically with TFP only and combination groups. I want to clarify whether further decrease in combinatorial doses in Hep3B is an indication of synergism. However, there was no sign for synergism suggesting TFP induced reduction in all cases **(Appendix C Figure 5, Appendix C Figure 6**). In SkHep1, p-AKT levels were not significantly influenced by drug treatments. Even when these protein levels were normalized to total form, reduction in Hep3B was apparent unlike SkHep1 (mild trend). Hence, activation of p-AKT (Ser-473) varies among cell lines which might be relevant to differential responses. Next, I wanted to investigate alterations in

m-TOR activation and mTORC1 induced protein p70 s6 kinase to connect how AKT/mTOR pathway altered by combinations in a cell line specific manner (**Figure 4.2.5**).

mTOR and p-mTOR (Ser2448) levels which is activated PI3K/AKT pathway was investigated. In both proteins and both cell lines, there was no notable alterations, statistically. Active forms of mTOR with combination showed mild alterations but in opposite direction in both cell lines. Moreover, subtle alterations in phosphorylated form found to be related to TFP alone (**Appendix C Figure 5,6**). Interestingly, mTOR downstream kinase p70s6K was found to be affected by TFP and combinations in a cell line specific manner. In Hep3B, reduction in total and phosphorylated (Thr389) forms of this kinase were notable. Moreover, this trend in phosphorylated form and active kinase give clues on synergism since increased doses of combinations decreased the levels more, in the opposite direction of single treatments. Existence of drug interaction strengthens the synergism claim for this kinase in TFP 12 SFB1 and TFP 12 SFB2 µM groups (**Appendix C Figure 5**). Hence, active p70s6 kinase levels were found to be synergistic for relevant combinatorial treatment doses. Since the mTOR levels were almost constant (there is a subtle reduction trend with combinations), mechanisms of action of combinations might be independent from upstream signaling, and involvement/crosstalk of other pathways should be investigated. In SkHep1, total p70s6 kinase levels were similar to Hep3B. However, most striking differential behavior of cell lines against drugs was encountered at the levels of active p70s6 kinase. Unlike in Hep3B cells, phosphorylated form of this kinase and its activation levels were increased with TFP and combination doses. These alterations were found to be TFP dependent (**Appendix C Figure 6**). Even though active mTOR levels were having increasing trend with TFP groups unlike Hep3B, this subtle difference may not be able to explain the difference. Altogether, active p70s6 kinase has synergistic reduction actions in Hep3B unlike SkHep1 with opposite effect which should contribute to explanation of sensitivity and resistance of cell lines to drug combinations. Hence, mTOR downstream signaling and their crosstalk became important for deeper examinations.

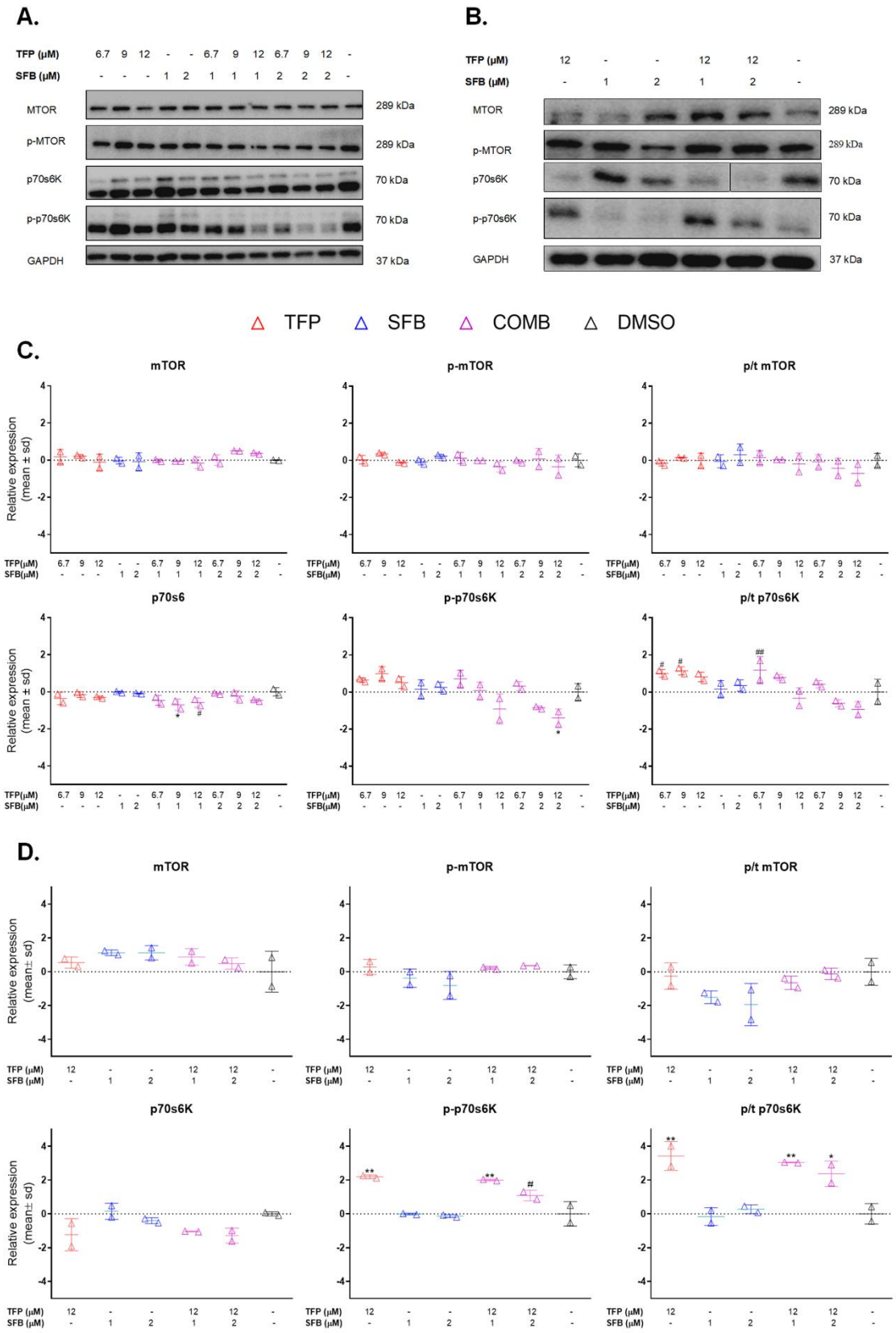


Figure 4.2. 5 Investigation of mTOR signaling members at protein levels in Hep3B and SkHep1 cell line upon treatment of TFP and/or SFB in indicated doses.

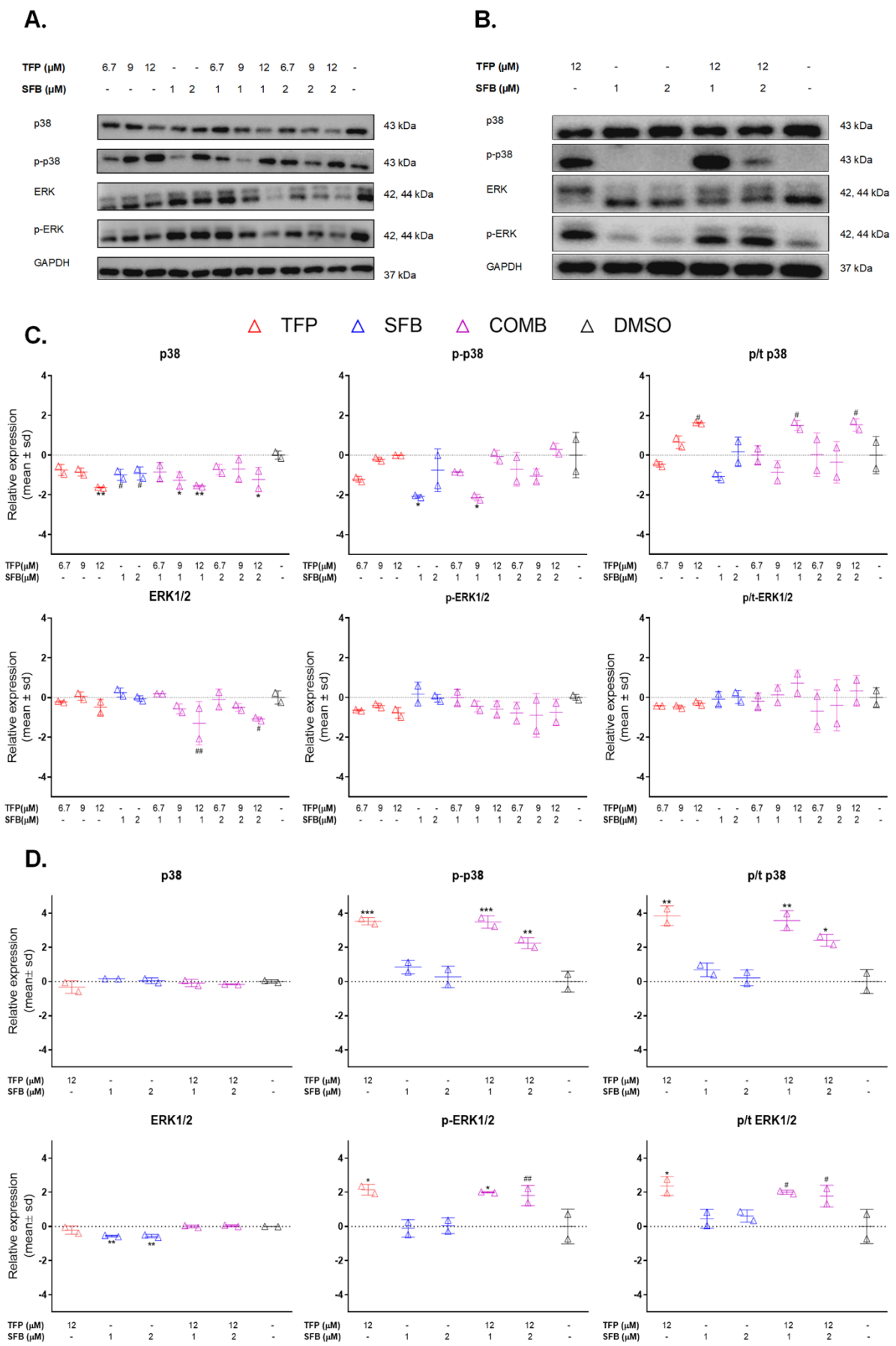
48 hours of TFP and/or SFB treatments were given in indicated doses in **A, C)** Hep3B, **B, D)** SkHep1. All proteins were normalized to internal control GAPDH and experimental control DMSO. Results were represented in log2 scale, One-way ANOVA Dunnett's test was applied (n=2) (* $p \leq 0.05$, ** ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$, ## $p < 0.1$, # $p < 0.2$).

MAPK pathway can control cell survival and proliferation alone; however, crosstalk of this pathway with other critical ones such as PI3K/AKT/mTOR is known, too. Hence, I wanted to investigate mitogen activated ERK1/2 and stress activated p38 effector kinase levels.

p38 MAPK levels were reduced in a TFP and/or SFB groups in Hep3B while this was not notable in SkHep1 cell line. In both cell lines, Thr180/Tyr182 phosphorylated p38 levels had the same increment trend with TFP doses and combinations but with different start points which should be considered to evaluate differential responses. Same trend was clearer in total form normalized active form levels in both cell lines. Dual role of p38 should be considered to have an opinion on these results. Moreover, synergism investigation was failed to point out any promising candidate except for approaching significant interaction levels in p-p38 in Hep3B cells suggesting drugs' effect on each other.

Effector kinases ERK1 and ERK2 with sequence similarity and overlapping functions were investigated together in **Figure 4.2.6**. I have evaluated both kinases together in Hep3B cell line since they were acting together. Since the relevant isoform in HCC is the ERK2, I separately evaluated ERK2 and performed statistical analysis which revelated same results as shown in appendix (**Appendix C Figure 4**). Total ERK 1/2 levels almost significantly reduced in TFP12-SFB 1 or SFB2 μM combination groups which suggests synergism due to un-effected single treatments. Similar reduction trend was also observed in Thr202/Tyr204 phosphorylated forms however, variations prevented any strong comment on synergism. Ratio of phosphor-total active forms shows increasing trend of protein levels with increasing TFP doses in combinations. However, variations make it impossible to deduce a significant result. When synergism investigated more advanced level (**Appendix C Figure 4, 5, Appendix C Figure 6**), there were no interactions to support such synergy.

In SkHep1, drugs act on total ERK1 and ERK2 diversely. In **Figure 4.2.6.,** both kinases evaluated together as in Hep3B. I also quantified and analyzed ERK1 and ERK 2 bands separately (**Appendix C Figure 3A**) and found that both kinases altered at the same levels in terms of phosphorylated forms. However, total forms of ERK1 and ERK2 differs. In ERK1 TFP increases the protein levels while in ERK2 cause reductions which significant for TFP 12 μM dose. Ratio of active p-ERK1 and p-ERK2 has the same trend of increment with TFP and combinations. These increments are significant for ERK2. When synergism was investigated at more advanced level (**Appendix C Figure 3B**) interaction factors became significant with total ERK2 and approached to significance with active ERK2 ratios which suggests interaction of drugs at these levels but there was no evidence supporting synergy. When cell lines were compared, differential ERK1 and ERK2 regulations, opposite p-ERK regulations and magnitude of active ERK levels by treatments draw attention. Interestingly, each cell line within itself showed similar ERK and p38 kinase pattern. Moreover, to be able to conclude on synergism and differential effect of combinations on MAPK pathway, stronger results will be needed.


A.
TFP (μM) 6.7 9 12 - - 6.7 9 12 6.7 9 12 -
SFB (μM) - - - 1 2 1 1 1 2 2 2 -
p38
p-p38
ERK
p-ERK
GAPDH
43 kDa
43 kDa
42, 44 kDa
42, 44 kDa
37 kDa
B.
TFP (μM) 12 - - 12 12 -
SFB (μM) - 1 2 1 2 -
p38
p-p38
ERK
p-ERK
GAPDH
43 kDa
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C.
TFP
SFB
COMB
DMSO
p38
p-p38
p/t p38
ERK1/2
p-ERK1/2
p/t-ERK1/2
Relative expression (mean ± sd)
TFP(μM) 6.7 9 12 - - 6.7 9 12 6.7 9 12 -
SFB(μM) - - - 1 2 1 1 1 2 2 2 -
D.
p38
p-p38
p/t p38
ERK1/2
p-ERK1/2
p/t ERK1/2
Relative expression (mean± sd)
TFP (μM) 12 - - 12 12 -
SFB (μM) - 1 2 1 2 -

Figure 4.2. 6 Investigation of MAPK signaling pathway members at Protein levels in Hep3B and SkHep1 cell line upon treatment of TFP and/or SFB in indicated doses.

48 hours of TFP and/or SFB treatments were given in indicated doses in **A, C)** Hep3B, **B, D)** SkHep1. All proteins were normalized to internal control GAPDH and experimental control DMSO. Results were represented in log2 scale, One-way ANOVA Dunnett's test was applied (n=2) (* $p \leq 0.05$, ** ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$, ## $p < 0.1$, # $p < 0.2$).

4.2.4 Effects of SFB and/or TFP on nuclear receptor family members.

As discussed at the beginning of this thesis, I wanted to elucidate whether MR and/or GR take part in conducting the observed synergistic anticancer and drug resistance effects with respect to TFP-SFB combination in liver cancer cells. Since ALDO activated MR related reduction in HCC proliferation is known, drug combinations' effect on MR was an interesting point to further investigate. In literature TFP, calcium calmodulin inhibitor, claimed to interfere with glucocorticoid action through competition for steroid binding sites. Moreover, data showing TFP's involvement to ALDO signaling supported our objective to combine two projects at the levels of MR and GR. With this goal, I identified several Steroid Hormone Receptor family members and their downstream effectors and examined their alterations in combination RNA-seq data in Hep3B cell line (Murat Yaman, PhD thesis,2020). I visualized these genes in **Figure4.2.7.** In **Appendix D Table 1**, their Log2FC and p values are available.

Heatmap showing the visualized RNA-seq results helped us to determine genes clustered together (**Figure 4.3.1**). NR3C1 (GR) gene clustered with related genes STAT3, SGK1, FKBP. NR3C2(MR) is clustered with NEDD4L, IGFR and EGFR. From these genes, I chose GR and SGK1 which had significant or approaching significant results with combinations, and MR showing opposite trend in combination.

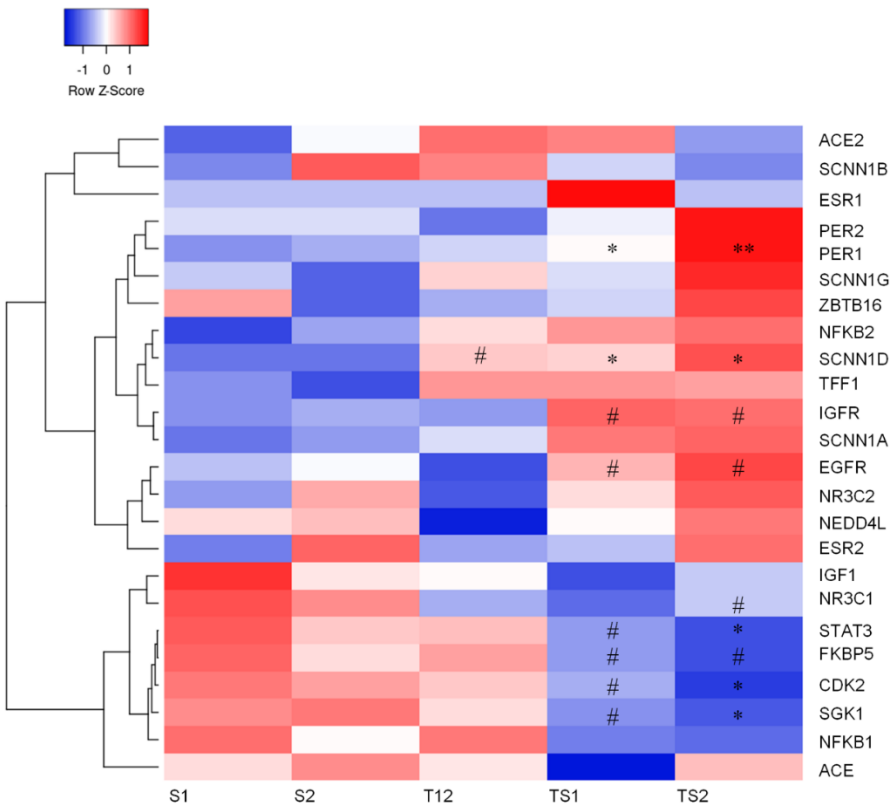


Figure 4.2. 7 Heatmap of RNA-seq data on Steroid Hormone Receptor family members and downstream effectors of their signaling pathways.
Differentially expressed genes list was generated by Murat Yaman (based on RNA-seq analysis used in his PhD thesis, 2020) for RNA-seq results of 48 hr. S1: SFB 1 µM, S2: SFB 2 µM, T12: TFP 12 µM, TS1: TFP 12+ SFB 1 µM, TS2: TFP 12+ SFB 2 µM treatments in Hep3B cell line. Log2FC and p-values were represented as heatmap (* $p \leq 0.05$, ** ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$, # $p < 0.1$).

To validate chosen gene's alterations were found in RNA-seq, the expression levels were analyzed both at mRNA and protein levels.

MR, GR and SGK1 was validated by RT-qPCR method and results were given in **Figure 4.2.8**. Combination groups of GR and SGK1 were compatible with the RNA-seq results which manifested themselves as reduction in expression. MR levels were not significantly altered by combinations to conclude anything however, mild increasing trend in RNA-seq was not observed by RT-qPCR.

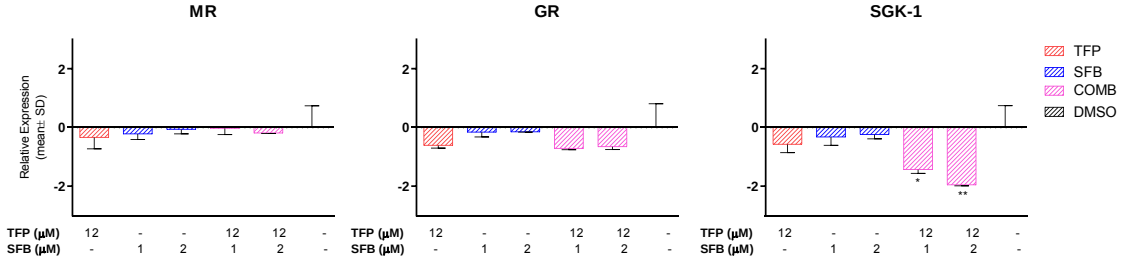


Figure 4.2. 8 Investigation of Nuclear Receptor Family members and downstream effectors at mRNA levels in Hep3B cell line.

48 hours of TFP and/or SFB treatments were given in indicated doses. Results were represented in log2 scale, One-way ANOVA Dunnett's test was applied (* $p \leq 0.05$, ** ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$).

Protein levels of the chosen genes were controlled in SkHep1 cells together with Hep3B to detect any possible variations in between these cell lines (**Figure 4.2.9**). GR levels in both cell lines reduced with combinations which is quite coherent with the mRNA levels for Hep3B. However, high SFB and TFP combinations downregulated more in Hep3B. These potentially synergistical alterations in GR levels will be a very intriguing subject for detailed analysis. I performed Two-way ANOVA test to evaluate interaction of drugs especially potentially promising GR levels in Hep3B (**Appendix D Figure 2A and 2B**). Interestingly, GR levels were not significant for interaction in both cell lines. However, with simple row and column tests in Two-way-ANOVA shows synergistic pattern in TFP9- SFB2 µM combination. Even more unexpected result was obtained in SkHep1 cell lines SGK1 result which showed interaction of drugs which suggest differential behavior of drug together without any clue on synergism. SFB caused reduction in both cell lines was apparent while TFP combination reduced the levels of reduction trend in SGK1 levels. This gene can be affected by multiple mechanisms besides from SHRs such as AKT signaling. Hence, SGK1 levels should be evaluated in a much wider concept. Lastly, levels of MR were tried to be obtained. Reduction trend in TFP and combination groups are easily seen with only one replicate. I was not able to detect MR levels in SkHep1 cells which might be related to low expression. However, based on these validations, SHRs member can be affected by these drug combinations which can widen our perspective to understand mechanism of action of these drugs.

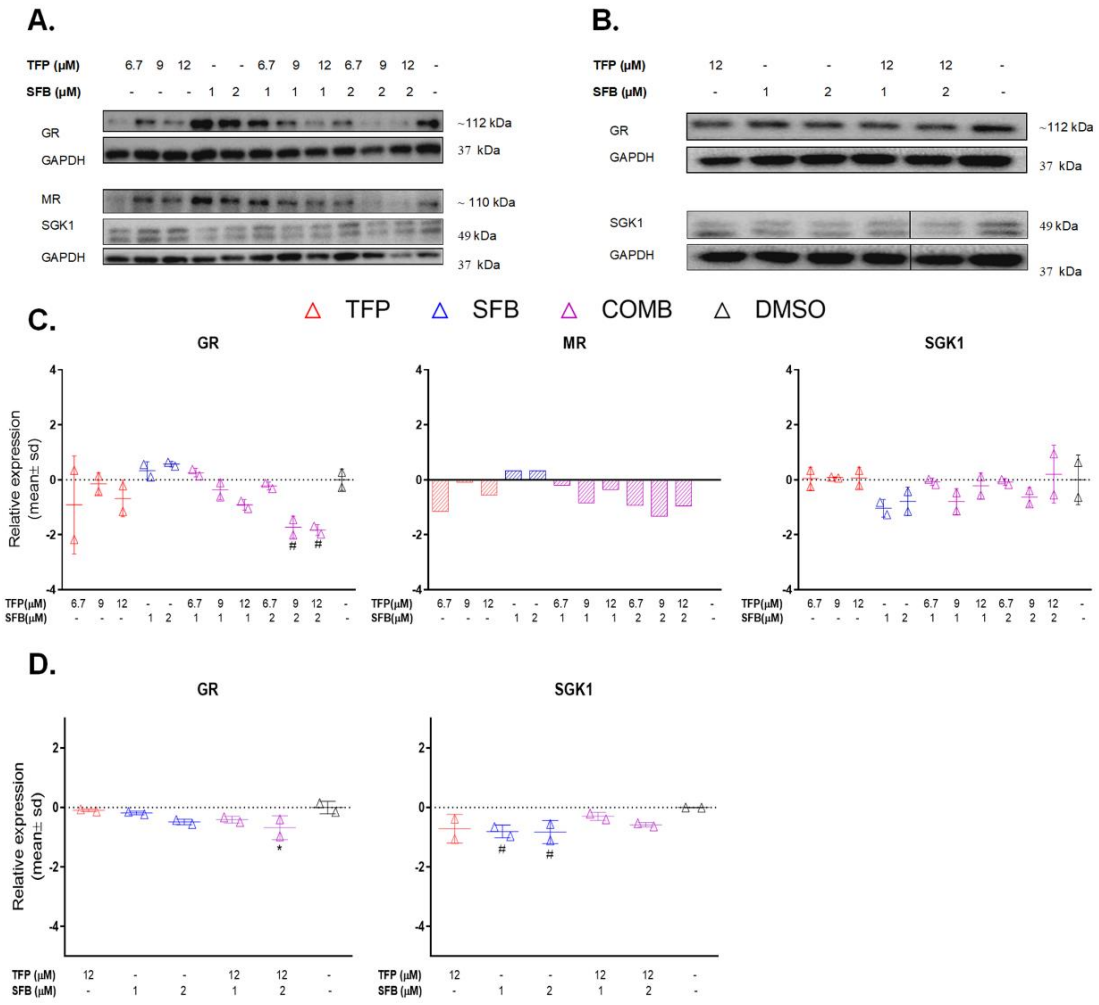


Figure 4.2. 9 Investigation of MR, GR and SGK1 at protein levels in Hep3B and SkHep1 cell line upon treatment of TFP and/or SFB in indicated doses.

48 hours of TFP and/or SFB treatments were given in indicated doses to **A, C)** Hep3B, **B, D)** SkHep1. All proteins were normalized to internal control GAPDH and experimental control DMSO. Results were represented in log2 scale, One-way ANOVA Dunnett's test was applied (n=2) (* $p \leq 0.05$, ** ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$, ## $p < 0.1$, # $p < 0.2$).

4.2.5. Principal component analysis (PCA) of protein markers

To be able to evaluate the distinct patterns of treatment groups within cell lines and cell line specific actions of proteins, Principal Component Analysis (PCA) was performed with common doses of cell lines. Clustering proteins and cell lines in an understandable visual representation might help to understand distinct behaviors.

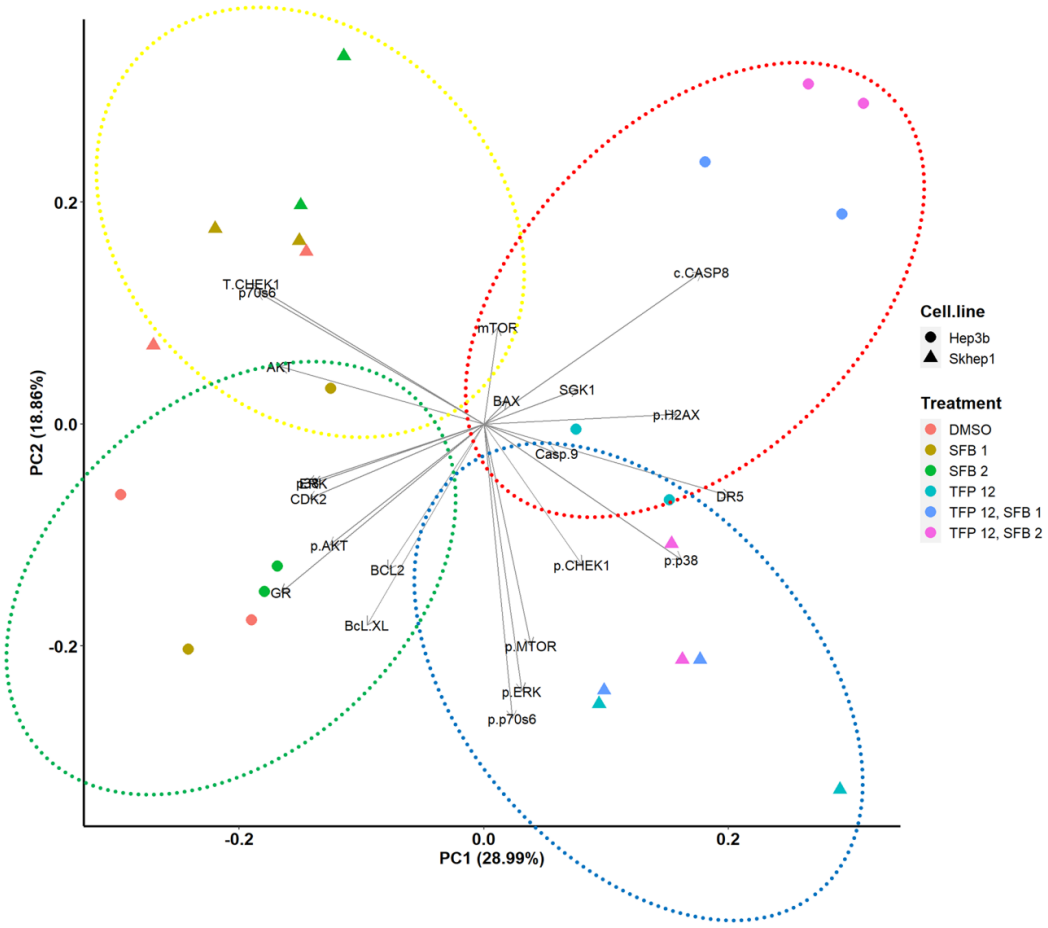


Figure 4.2. 10 Principal Component Analysis of common drug doses and cell lines for protein levels.

48 hours of TFP and/or SFB treatments were given to Hep3B and SkHep1 in indicated doses. Protein markers of cellular pathways were analyzed with PCA analysis to investigate cell specific alteration.

Principal component 1 (PC1) and PC2 explained 28.99% and 18.86% of variance, respectively (PC3 was around 1%). As shown in **Figure4.2.10.** I observed 4 groups that are clustered together. Groups are: 1) red group: Hep3B TFP alone and combination groups, 2) blue: group SkHep1 TFP alone and combination groups, 3) green group: Hep3B DMSO and SFB doses, 4)

yellow group: SkHep1 DMSO and SFB doses. Differential clustering of these groups is meaningful to represent differential behaviors of cell lines and treatment groups. Red and green groups clustered in opposite direction suggesting effectiveness of TFP and combinational treatments in Hep3B. Within Red group, combinations clustered together and away from TFP alone groups which might indicate the strength of the combinatorial treatments with increasing SFB doses. Moreover, c-CASP8 seems to be affected by combinations more than TFP alone in Hep3B. Similar divisions were not seen in Blue group which might suggest similar effects of combinations and TFP alone groups in SkHep1. However, opposite directions of Yellow and Blue groups may advice TFP's distinct effect on protein levels opposing the control and SFB alone groups.

One may suggest based on this analysis that alterations in some proteins were more prominent in Hep3B cell line upon treatment which can be listed as ERK, BCL-xL, BCL2, GR, p-AKT, CDK2, and p38 in negative direction (decreased levels) and c-CASP8, SGK1 and p-γH2AX (moderately) in a positive direction (increased levels). Especially CASP8 and GR's opposite but distinct regulation by combination groups of Hep3B cell line should be further investigated in terms of sensitivity to treatment. Proteins relatively more effected in SkHep1 can be listed as T-CHEK1, p70s6K being in negative direction, and p-p38, p-CHEK1, p-ERK, p-p70s6 (moderately) being in positive direction. Especially, accumulation of phosphorylated ERK, p70 s6K and mTOR in SkHep1's blue group might explain the differential responses. Hence, these diverse impacts on proteins will be helpful to understand mechanistic effects of drug combinations together with cell line specific act on viability.

CHAPTER 5

CONCLUSIONS AND DISSCUSSIONS

Cancer is a highly heterogenous disease with numerous, diverse underlying pathologies. Hence, understanding pathophysiology, mechanisms driving tumorigenesis and its progression is critical for discovery of drugs targeting precise mechanisms. With this in mind, I aimed to attribute Mineralocorticoid and Glucocorticoid Receptors and their interactions and/or responses as novel effectors of hormones (Aldosterone and Estrogen) and anticancer drugs (Sorafenib, Trifluoperazine) in breast and liver cancer cells, respectively. To be able to explain my work in an understandable manner, I will separately conclude and discuss the work in breast and liver cancers and extend my discussion into the intersection of these two projects.

ALDO-E2 crosstalk in breast cancer cell line T47D

Especially in cancer, crosstalk of the pathways can be a burden due to activation of alternative and/or redundant survival pathways reducing treatment efficiency. Hence, for targeting a certain gene or pathway, there is a need for detailed study to reveal such crosstalk. In hormone driven cancer cells, expression of steroid hormone receptor (SHR) family regulates critical functions. SHRs including ER, AR, PGR, GR and MR has ability to crosstalk within the family and with other critical cellular pathways. Hence, SHRs do not act alone but instead interact with each other's functions. ER crosstalk with AR, GR, and PGR has been known in breast literature while the extent to which MR crosstalk with ER is lacking.

First part of my study was based on Bircan Coban's (MSc Thesis, 2016) preliminary data on exposure of breast cancer cell lines to ALDO and E2, individually, with the aim of deciphering probable crosstalk of pathways at the mRNA and protein levels. My follow-up study included combinatorial hormone exposure in ER and MR positive T47D cell line with the same objective. SHRs are heavily cross-talking TFs family which have been investigated in cancer literature. For example, ligand activated GR can suppress proliferation by occupying ER enhancers hence regulates genes including CDK2 and CCND1 in ER positive breast cancer cell

lines. TFF1/PS2 is also identified as one of the genes regulated by this interaction (Tonsing-Carter et al., 2019; F. Yang et al., 2017). However, MR is less studied with respect to ER signaling in cancer literature.

MR-ER crosstalk has been referred to in one study where E2-ER suppressed the ALDO mediated MR transcriptional activity in HEK-293 cell line (Barrett Mueller et al., 2014). However, this interaction was not reciprocal; and ALDO did not affect the luciferase activity of E2-ER. In addition, this repression was not at the level of HRE, but MR and ER were found in the same protein complex. The authors confirmed their results by evaluating the activity and expression of an ALDO induced gene in combinatorial hormone treatments (Barrett Mueller et al., 2014).

Estrogen induced upregulation of ZBTB16/ PLZF (Zinc finger and BTB domain containing-16) gene was the first interesting finding of this thesis. ZBTB16 was proposed as possible biomarker since it was found as tumor suppressor expressed less in breast cancers (J. He et al., 2020). ALDO responsive TF ZBTB16 was previously fused with ESR1 to repress E2 induced gene expression in MCF7 including PGR and TFF1 (Buluwela et al., 2005). This gene was also shown to be responsive to progesterone-PGR signaling in human endometrial stromal cell (Kommagani et al., 2016). In the study presented in this thesis one of the most striking finding was ALDO induced significant reduction in PGR levels. This was reversed by ALDO-E2 exposure suggesting the involvement of ER suppression on ALDO driven MR activity in T47D cells. However, since another ER primary target, TFF1, was not repressed by ALDO alone, and even upregulated, ALDO's inhibitory effect on PGR mRNA expression in relation to ESR1-E2 primary activity needs to be investigated further. Estrogen responsive TFF1 (Trefoil factor 1) is small secretory protein present in several cancers including breast. This protein with anti-apoptotic and invasive features was shown to involve in resistance to doxorubicin in MCF7 cell line (Pelden et al., 2013). Moreover, ALDO exposed MCF7 cell line did not induce the TFF1 protein levels in the medium. It is important to understand why PGR and TFF1 oppositely affected by ALDO in T47D if both are primary targets of ESR-E2 binding. Furthermore, ZBTB16-PGR-TFF1 axis should be further investigated to reveal crosstalk of pathways and its consequences.

ER and MR can control and can be controlled by similar mechanisms. For example, ALDO can activate IGF-1R, and IGF-1 can stimulate TFF1 expression in an ER mediated manner in different cell models (Baron et al., 2007; Holzman et al., 2007). Another mechanism of ALDO action in breast cancer acts via GPER regulation. When ER and PGR negative SKBR3 cell line

exposed to ALDO, ALDO induces GPER activation together with MR and both contribute to proliferation and migration induced by Na^+/H exchanger-1 (Rigiracciolo et al., 2015). Hence, ALDO and MR can induce diverse functions depending on the cellular context within cancer cell lines as in different cancer types. Moreover, complex intersection of SHRs complicates the generalization of the results. Yet, ER and PGR status might be effective on MR function in breast cancer. On a further note, high expression of MR in TNBC cytoplasm has been detected and MR expression associated with elevated overall survival, however in non-TNBC phenotypes high MR levels have been related to worse relapse free survival which supports the idea of tumor status also is related with functions of MR (Jääskeläinen et al., 2019).

The decrease in PGR upon ALDO treatment was in line with ALDO's anti-proliferative effects in this study. In literature, progesterone was found to reduce proliferation in a PGR dependent manner since, PGR silencing revered the inhibitory effect (C. C. Chen et al., 2011). Even though there is a need for further validation, I was able to observe a significant reduction of cell viability with ALDO treatment. In accord with this result, ALDO induced MR signaling has been shown to reduce proliferation in HCC cell lines (Nie et al., 2015). Indeed, induction of apoptosis and cell cycle arrest at G1 was suggested as an explanation and miRNA mediated regulation of pyruvate kinase enzyme was found to reduce the proliferative Warburg effect (Nie et al., 2015). Progesterone induced Warburg effect was observed in HEK293 cell lines which was related to degradation of progesterone receptor membrane component 1 (Sabbir, 2019). Hence, the promising results obtained in T47D proliferation by ALDO and modulation of PGR in breast cancer could be studied with respect to inhibition of Warburg effect. MR crosstalk related with Warburg effect in MCF7 cell line has been shown such that MR and retinoic acid signaling are reactivated (lost in tumorigenesis) and this crosstalk suppresses proliferation regardless of ER activation status (Doan et al., 2020).

ALDO induced reduction in cell viability might be related to cell cycle arrest, apoptosis, senescence, or other mechanisms. Even though the reductions in mRNA expressions of CCND1, CCNE2 and ANLN were minor upon 24-hr of exposure, increasing the duration of exposure might explain that the reduced viability was due to inhibition of cell cycle or G1 arrest at the mRNA level. Moreover, CCND1 is known as one of the primary targets of ERα and CCNE2 is a secondary target which depends on CCND1 activity in breast cancer cell lines (Caldon et al., 2009; Cicatiello et al., 2004; W. Zhou et al., 2015). These genes have also been identified as progestin responsive in breast cancer (Cicatiello et al., 2004; Reyes et al., 2014). This is important because MR appear to be at the cross-roads of ER and PGR and further studies

using progestins in connection with ALDO may shed more light into this crosstalk. Moreover, ALDO induced reduction in PGR might be related to MR-ER-PG or MR-PGR alone which needs to be validated via RNAi approaches in the presence or absence of progestins. Interaction of MR and other pathways including ER signaling seem to be a promising avenue to further pursue based on the findings presented herein and supported by literature.

PGR-ER crosstalk is long known in literature, E2 induced ER upregulates PGR levels as downstream effector. There are few studies explaining progesterone and ALDO-MR interaction. Increased levels of progesterone reduce the ALDO's binding to MR by competing for receptor (Fuller et al., 2019). Increased progesterone levels suppress ALDO-MR interaction, but in turn activates ALDO production in menstrual cycle (Szmuilowicz et al., 2006). Adversely, E2 induced ERβ and GPER was shown to reduce the production of aldosterone in adrenocortical cells (Caroccia et al., 2014). Moreover , PR-A isoform competes for common TFs with MR and reduces MR's transcriptional activity (McDonnell et al., 1994). Growth factors such as IGF-1 are repressors of PGR levels through PI3K/AKT/mTOR activity resulting in more cancerous ER+/PGR- cells; however, increased levels of cell cycle markers coincide with reduced PGR levels (Cui et al., 2003). Hence, effects of prolonged exposure and connecting PGR reductions to possible crosstalk would be critical to assess.

In literature, ALDO an DEX's separate crosstalk with PGR in breast cancer was associated with reduction in growth. Progestin like growth inhibition (via G1/S arrest) and focal adhesion reduction was induced by ALDO and DEX separately which leads to questioning of this crosstalk. DEX and progesterone act to increase p21 and decrease p-ERK1/2 expression to exert their effects. The authors confirmed the crosstalk via silencing and blocking PR receptor and found that E2 shows no such affect in this model suggesting specific crosstalk of ALDO, DEX and PGR. However, mechanistic explanation of this crosstalk was not detailed for MR (J. C. Leo et al., 2004). This ALDO induced reduction in proliferation in breast cancer cell lines via PGR correlates with the reduced proliferation observed in the present thesis study and the trends regarding reduction of cell cycle related proteins. Since CCND1 and CCNE2 regulates G1 and G1/S transitions in cell cycle, reduction of these may correlate with G1/S phase arrest (Rezaei et al., 2012). Moreover, reduced proliferative marker ANLN has also association with cell cycle arrest in G2/M transition of T47D (Magnusson et al., 2016). Inhibition of PGR-cyclin complexes suggested as a target for endocrine resistance in breast cancer, reduction of CCND1 upon ALDO may act through this interaction (Dressing et al., 2014). Interestingly, PGR activity levels in breast cancer cell lines are shown to be regulated depending to cell cycle phase. PGR

levels found to be reduced in G1 and G2/M transitions and reaches to a maximum in the S phase (R. Narayanan et al., 2005). Hence, reduced PGR levels might correlate with aberrations in cell cycle which remains to be validated. RNA-sequencing of overexpression (OV) models of MR negative ER positive MCF7 and ZR75-1 cells was performed with 100 nM ALDO treatment in our lab by Seniye Targen, PhD *(unpublished data)*. In MCF7 cell line, MR OV vs OV+ALDO showed minor reduction in PGR levels (log2FC= -0.365, p value= 0.793603). Moreover, control (empty vector) vs ALDO+ control group was also reduced the PGR (log2FC= -1.227, p value= 0.669901). In ZR75-1 cell line with minimal MR and GR expression, ALDO treatment reduced the PGR levels in MR OV group (log2FC=-0.468, p-value= 0.445006). However, similar reduction in empty vector-ALDO groups were prominent (log2FC=-0.67, p value=0.249894). Even though validation is needed with greater sample size and other methods, these findings suggest that MR expression alone may not be responsible for reduced PGR levels upon ALDO exposure.

As mentioned in introduction, many of the chosen ALDO-MR target genes exert their effects within minutes or couple of hours (Verrey et al., 2003). This is indicative of feedback loops on these genes with 24 hr. exposure. ALDO induces an MR size shift due to rapid ERK caused phosphorylation of receptor leading to ubiquitin degradation in renal epithelial cells indicating the presence of a negative loop controlling MR levels (Faresse et al., 2012). Hence, reduction in MR levels that was observed in T47D cells herein might be explained by similar negative feedback loop. E2 induced reduction of ESR1 has been associated to ERα action in literature, too (Ellison-Zelski et al., 2009). Increased ZBTB16 gene (a target of MR and GR signaling via ALDO) has been shown to reduce ENaC expression via a negative feedback loop in renal cortical collecting duct cell lines. Moreover, long time exposure of ALDO in mouse cortical and medullary collecting duct cells was found to express less NEDD4-2 as in our mRNA levels (Loffing-Cueni et al., 2006). These results from literature support the observed reduction of MR, ER, SGK1, and ENaC genes and minor alterations in proteins in this thesis via a potential feedback mechanism.

Even though ENaC activation has been associated with several cancer related dysregulation such as migration, E2 induces ubiquitination of αENaC in rat kidneys (C. Liu et al., 2016; X. Zhang et al., 2019). However, there are many more studies suggesting E2 has an activating role in ENaCs (Chang et al., 2007; Gambling et al., 2004; D. Qi et al., 2014; G. Z. Yang et al., 2011). SGK1 can crosstalk with a wide range of signaling pathways making it an anti-cancer target and is inducible by E2 in short treatment intervals (Monsivais et al., 2016; R. Zhu et al., 2020).

SGK is known to be activated by ALDO within 30 minutes in kidney cells (Verrey et al., 2003). ALDO also induces ENaC subunits differentially depending on the cell type (Frindt & Palmer, 2012). Due to above mentioned literature, 24-hr hormone exposure might induce several feedbacks to regulate expression levels of these genes involved in ALDO-MR signaling. In addition to the time dependency, hormone doses can also be critical criteria together with ER/PGR status of cells.

Overall, study presented in this thesis can be considered as a preliminary study on promising MR-ER hormone activated crosstalk which has potential to contribute breast cancer literature with deeper studies.

Understanding the cellular and molecular effects of combinatorial treatment with Sorafenib and Trifluoperazine in liver cancer and novel association with MR-GR signaling

Liver cancer subtype Hepatocellular carcinoma is a detrimental disease with limited treatment options. Sorafenib-a multi-kinase inhibitor and still is the most known, used first line anti-HCC drug; however, SFB has mild benefits and can be applicable to only subgroups of patients and eventually these patients develop acquired resistance. Combining FDA approved Sorafenib with other anti-cancer drugs is a widely used strategy against HCC. Our lab previously repurposed a generic Phenothiazine derivative TFP as a novel combination partner of SFB (Murat Yaman PhD thesis, 2020). Functional assessment in Hep3B cell line showed synergistic reduction in cellular viability by several doses of combination of TFP-SFB. Another liver cancer cell line SkHep1 was antagonistic in terms of cellular viability with given combinations which provides natural comparative platform to detect the mechanistic differences of these two cell lines. In present study mechanisms of sensitivity and insensitivity to this novel combination was studied regarding cancer related pathways, such as, cell cycle, apoptosis, and kinase activities. Moreover, MR and GR which are known to be altered in HCC was investigated as possible novel targets of TFP-SFB combination.

In the present thesis I have investigated protein markers of several pathways which are critical for HCC and liver cancer progression and SFB and/or TFP might act on, and hence, to discover

the behavior of known (e.g., DR5) and novel (i.e., MR and GR) targets which could help understand why the combinatorial treatment could be better than singular in liver cancer.

Apoptosis, cell cycle and DNA damage pathways are generally altered in cancers as hallmarks. Hence, to evaluate the success of drug combinations protein markers of these pathways was investigated in Hep3B and SkHep1 cell lines. Check-point inhibitors have been studied in the cancer literature due to their control on cell cycle and apoptosis; indeed checkpoint inhibitor treatment with cytotoxic drugs is a way of increased therapy efficiency (K. Ando et al., 2019; Luo et al., 2001; Rogers et al., 2020). Among these, PTZ, Thioridazine, was shown to disrupt ATR/CHK1 in response to DNA damage and induce apoptosis (V. Singh et al., 2019). In p53 null cell lines (such as Hep3B), apoptosis can be induced upon DNA damage via CHK1 inhibition and caspase 2 related apoptosis mechanism which is independent from classical apoptotic pathways (Meuth, 2010; S. Shin et al., 2005; Sidi et al., 2008). Since the loss of p53 contributes to anti-apoptotic cancer phenotype, CHK1 controlled alternative apoptotic pathway would be an anti-cancer target to sensitize cells to DNA damage induced apoptosis. Moreover, CHK1 reduction potentiates drug induced DNA damage and this was associated with caspase 8 cleavage. This relation was validated in literature via si-CHK1 plus DNA damage inducing agent given cells which show increased caspase 8 cleavage, p-γH2AX activation, and cleavage of PARP (Z. Xiao et al., 2005). PTZ derivative, A4 induced ROS production and DNA damage was shown in literature (C. H. Wu et al., 2016) . In the present thesis, I observed in TFP12 - SFB1/2 μM combination groups in Hep3B, a reduction in total-CHEK1 levels correlates with increased p- γH2AX, increased c-CASP8, decrease in CDK2 levels which are compatible with CHK1 inhibited alternative apoptosis induction context. Moreover, previously, TFP has been found to repress double stranded DNA repair in lung cancer cell lines in combination with the DNA damaging agent bleomycin (Polischouk et al., 2007). These studies support the induction in DNA damage marker p-γH2AX. Moreover, c-CASP8 levels supports extrinsic apoptosis induction (Kruidering & Evan, 2000). Minimal alterations or insensitivity to combinations in classical apoptosis markers are also supportive of CHK1 dependent potentiation of apoptosis in Hep3B cell line. In a study on prostate cancer cell lines, a novel PTZ derivative has bypassed the DDR through not activating CHK1 arrest hence induce apoptosis which may not be the case observed with SFB+TFP in Hep3B cells (V. Singh et al., 2020). This mechanism can be challenged by other observations in this study. In SkHep1 which was resistant to combination, a similar reduction in T-CHEK1 levels by combinations was observable as in Hep3B. Even though CHK1 levels were in distinguishable, in the SkHep1 cell line, CDK2 was not reduced

as in Hep3B via combinatorial treatment. ATM-ATR-p53-CDK2 mechanism might differentially regulate CDK2 levels upon CHK1 inhibition. Moreover, activity of CDK2 levels in S phase is associated with sensitivity against CHK1 inhibitors which might explain our results (Sakurikar et al., 2016). However, this CDK2-CHEK1 hypothesis should be validated further. Downregulation of CDK2 protein as anti-cancer agent has been shown in several studies in literature (Feng et al., 2018; B. Wang et al., 2017; Xia et al., 2019). In a study examining novel drugs on Hep3B and invasive SkHep1 cell lines, cell cycle arrest via reduced CDK2 was associated with differential mechanisms. These reductions were associated with G0/G1 arrest and apoptosis together with reduced p-AKT and p-JNK levels. In Hep3B CDK2 reduction was explained by p53 independent arrest via p21 and p27, and in SkHep1 by WNT/β-catenin pathway (Yadunandam et al., 2015). Hence, differential pattern of CDK2 activation can be related to pathways targeted by drug combinations. β-catenin reduction by TFP has been shown to be a must for TFP's anti-metastatic behavior together with reduced p-AKT in a study comparing HT1080 and NIH/3T3 cell lines. When TFP has failed to reduce p-β-catenin in cells, anti-metastatic phenotype has also been lost (Pulkoski-Gross et al., 2015). Hence, targeting crosstalk of β-catenin pathway by the combinatorial SFB+TFP treatment in Hep3B and SKHep1 and remains to be assessed in future studies.

Another important finding of our study was the reduction of anti-apoptotic BCL-xL with SFB2/TFP9-12μM doses in Hep3B, showing different pattern than above mentioned markers. Crosstalk of signaling pathways might explain this reduction which will be mentioned in the following paragraphs. Altogether, we might say that existence of apoptosis in DNA damage-CHEK1-CDK2-cCASP8 axis is probable in Hep3B cell line which diverges in SkHep1 at the level of CDK2 and BCL-xL.

In hepatocellular carcinoma, BCL-xL levels correlates with worse prognosis; and moreover, combination of regorafenib with BCL-xL inhibitors increases the therapeutic efficiency in HCC cell lines (Cucarull et al., 2020; Michels et al., 2013). Moreover, BCL-xL was suggested as an anti-mitotic target in cancers since it can control the switch between cell cycle arrest and induction of apoptosis (Bah et al., 2014). Autophagy is yet another mechanism with a critical role in HCC. For example, tumor suppressor Beclin1 can be inhibited by BCL-xL, which leads to a possibility of the role of autophagy in the actions of SFB+TFP combinatorial treatment, remaining to be addressed in future studies (Nikoletopoulou et al., 2013; Y. H. Shi et al., 2009). SFB alone can induce autophagy both in Hep3B and SkHep1 cell lines in an Mcl-1-STAT3 dependent manner, and independently of BCL-xL (W. T. Tai et al., 2013). Activation of

autophagy can be suppressive or promoting in SFB given HCC, and the differential regulation would be an interesting point to explain diverse cellular response (T. Sun et al., 2017). TFP induced reduced viability in pancreatic carcinoma cell lines also determined to be via induction of apoptosis and necrosis. This dual activation could be related to mitochondrial stress inducing ROS (aberrant energy metabolism) and ER (Ca+2) stress which activates unfolded protein response (UPR) (C. Huang et al., 2019). TFP related apoptosis protection of rat pheochromocytoma upon H_2O_2 has been shown to be due to regulation of mitochondrial membrane potential in a Ca^{+2}-calmodulin dependent manner (reduced ROS generation) (S. Liu et al., 2011). Moreover, low doses of TPF are also protective against apoptosis in glioma cells via control on Ca+2 levels (Wen et al., 2018). Hence, TFP associated apoptosis protection is also relevant to the findings of this study. Crosstalk of cellular death mechanisms are needed to be investigated in this context with SFB or TFP alone or in combination, as done and explained below.

Another striking common phenomenon as in the case of total CHEK1 levels in between Hep3B and SkHep1 was the upregulation of DR5 in a TFP dependent manner. In a study on TRA-8 resistant (DR5 agonist) TNBC breast cancer, calmodulin antagonist TFP sensitized cells through DR5 (direct calmodulin binding) activation and reduction in phosphorylated AKT and ERK levels as observed with combinatorial treatment of DR5 agonist and TFP (Fancy et al., 2018). Even though DR5 levels was not potentiated by TFP-SFB combinations in the present thesis, TFP induced DR5 activation may contribute to c-CASP8 activity observed in Hep3B and SkHep1 cells which might lead to apoptosis. In TRAIL resistant cancer cell lines mainly DR5 and related complex members were associated with both apoptotic and survival stimulus (Shlyakhtina et al., 2017). Hence, activation of DR5 and c-CASP8 levels in both cells can be explained by TFP action alone. In another study, TFP has induced BCL-xL suppression in combination with cisplatin sensitizing the resistant cells and inducing apoptosis in bladder urothelial carcinoma. Accordingly, TFP dose and combination dependent induction of p-H2AX and CHOP upregulation has been noted in resistant cells aside from G0/G1 arrest (Kuo et al., 2019). Induction of CHOP suggests ER stress in relationship with TFP which might be investigated further in the context of this thesis as well. In ER stress induced apoptosis, not only DR5 and c-CASP8, but BIM and PUMA inductions determine the outcome. Knowing that BIM expression can be directly induced by FOXO1 (which is suppressed by AKT activity and retained in nucleus by TFP), suggests the potential TFP-SFB-FOXO1-BIM axis involvement in sensitivity of Hep3B cells to SFB+TFP combinatorial treatment (H. M. Song et al., 2015).

Opposite direction of BIM and BCL-xL would be explanatory for this hypothesis and should be tested. Interestingly, a study on glioma cell lines showed that DR5 upregulation and caspase 8 activity is dependent on drug induced ER stress and p38 activity on CHOP which sensitizes cell to TRAIL induced apoptosis (Byun et al., 2018). This study also supports of the TFP induced DR5 levels in both cell lines.

Above mentioned results support synergistic viability reduction in Hep3B upon exposure to SFB and TFP together. Identifying the mechanisms that explain improved efficiency of SFB is crucial. Since literature suggest the efficient combination partners could be MAPK, mTOR, c-MET, HDAC, angiogenesis and EFGR inhibitors, we have chosen to investigate two of these: mTOR and MAPK pathways (kinases).

In oral cancer cell lines, TFP was shown to reduce AKT and mTOR and increase p38 together with caspase 8 and 9 cleavages in connection with its multi-targeting capacity. However, dose dependent TFP exposure did not show effectivity for down-regulation of p-ERK levels which seems to be increased (C. H. Wu et al., 2016). TFP induced p-AKT reduction is also valid in mesangial cells (B. Wang et al., 2018). Furthermore, when wild type vs SFB resistant HUH7 HCC cells have been compared, differential response of AKT is found as the driver of resistance through mTOR feedback. Protective autophagy has been adopted by the resistant cells and this could be reversed by AKT inhibition.(Zhai et al., 2014). In another study resistant HUH7 cells treated with a PI3K inhibitor could successfully reverse the resistance phenotype (H. Zhang et al., 2018). There are various studies showing increased SFB sensitivity and reversal of SFB resistance with AKT inhibition including in HCC cell lines and animal models (Jilkova et al., 2018; Zhai et al., 2014). In the findings presented in this thesis the observed reduction in p-AKT levels upon TFP alone and as well as in combination groups in Hep3B cells only might be explained as a TFP induced phenomenon which might differentiate the cell lines as in the case between Hep3B and SKHep1 but may not be attributed to the synergy observed in Hep3B (since this was observed also in TFP alone). PI3K signaling mediated CHK1 activation was previously observed and inhibition of CHK1 induced apoptosis in those models (Kurosu et al., 2013). Accordingly, a simultaneous reduction of both p-AKT and CHK1 in Hep3B cells may be able to explain the success of the SFB+TFP therapy in Hep3B cells.

Between the two cells studied herein, total mTOR protein levels did not reveal a striking difference yet, mTORC1 regulated p-p70s6 kinase levels were reduced with the increasing combination doses in Hep3B only. This reduction was one of the most promising candidates for explaining a synergistic regulation between SFB and TFP at the highest combination doses.

Inhibition of p70s6K which controls translation of several pro-survival genes can be a candidate to explain synergistic growth reduction (Bahrami-B et al., 2014). Reduction in p70s6 kinase in cisplatin induced DNA damage was associated with caspase3 causing cleavage of p70s6 which might support the potential of apoptosis in Hep3B cell line (Dhar et al., 2009). Indeed, TFP dependent upregulation of p-p70s6K was prominent in SkHep1 as opposed to Hep3B. In literature, SFB resistant SkHep1 model cells showed increased p-p70s6 kinase level similar to our results (M. Li et al., 2016). Moreover, p-ERK levels were also increased in SkHep1 in TFP given groups. ERK induction was previously observed in gastrointestinal cancer cells and is related to the induction of autophagy with TFP; however, increased p-p70s6K levels was not observed (Yen et al., 2019). One may speculate about the existence of p-ERK induced feeding loop on p-p70s6 kinase in SkHep1 based on their similar incremental pattern to the exposed drugs. Even though AMPK (AMP-activated protein kinase) was not included in this thesis it is another kinase involved in energy metabolism. Under stress, AMPK can activate AKT inhibitor TSC2 and suppresses downstream kinases to protect cells (Inoki et al., 2003). This kinase can also be regulated by internal Ca^{+2} levels. When activated, AMPK can control apoptosis, survival, and autophagy. Even though its involvement in apoptosis is controversial and dependent on the cell line, it can induce apoptosis via reducing mTORC1 activity which downregulates anti-apoptotic proteins as in MCL-1 to activate mitochondrial apoptosis. AMPK can also induce protective autophagy in the same conditions (Villanueva-Paz et al., 2016). AMPK related crosstalk of apoptosis and autophagy is known for colon cancer, too. It is possible that AMPK can be responsible for Hep3B apoptosis via reduction in p70s6K which coincides with reduced BCL-xL levels-intrinsic apoptosis induction and requires further attention.

FOXO 1 is a Fork-head box protein O protein which is known to be tumor suppressor (Hornsveld et al., 2018). AKT phosphorylates FOXO1 to inhibits its function via degradation, however PI3K inhibitors adversely increase ERK phosphorylation derived resistance. In a study on prostate cancer cells, cytoplasmic FOXO1, which is phosphorylated by AKT, reduces the ERK phosphorylation/resistance which means that FOXO1 can have a cytoplasmic tumor suppressor role. FOXO-derived phosphor-mimicking peptides have been suggested as solutions to FOXO1 induced ERK activation in used model (C. Pan et al., 2017).. In HCC cell lines, FOXO1 nuclear localization is associated with TFP action which induces apoptosis (J. Jiang et al., 2017). In literature, SFB exposure unexpectedly increases p-ERK levels in Hep3B which makes it more insensitive to the drug while SkHep1 shows sensitivity to SFB which is related

to BIM activity. BIM degradation can be induced by ERK and AKT and reduced by JNK signaling and consequently controls BCL-2 proteins (Y. Chen et al., 2017). As mentioned before, FOXO1 and AMPK can increase BIM expression to induce intrinsic apoptosis. Even though BAX, BCL2 and CASP9 was not informative in the present thesis' findings explaining the synergism between SFB and TFP, BCL-xL reduction in Hep3B might support the induction of apoptosis along with FOXO1. Since CDK2 can phosphorylate FOXO1 to induce proteasomal degradation, reduced CDK2 levels in Hep3B combinations groups may support FOXO1 related sensitivity (H. Huang et al., 2006). FOXO (not specified as FOXO1) and BCL-xL was also shown to be related since FOXO activity can reduce BCL-xL levels by intermediate BCL-6 action (Ho et al., 2008). Moreover, FOXO1 expression shown to be minimal in SkHep1 cell line which might support the insensitivity of SKHep1 to SFB+TFP together if FOXO1 is playing a role, which remains to be assessed (Dong et al., 2017).

Modulations in MAPK pathway which can crosstalk with AKT/mTOR could also be critical for SFB sensitization. In a study carried out on HCC cell lines for SFB sensitization, the level of p-ERK has been determined as a sensitivity marker. In particular, reducing the p-ERK2 levels with MEK/ERK inhibitors successfully induces SFB sensitivity (Cun Wang et al., 2018). Hence, the reduced p-ERK in Hep3B and increased levels in SkHep1 in the present thesis can help explain the opposite sensitivity observed in response to combinatorial treatment.

On the other hand, p38 levels acted in the same direction in both cell lines in a TFP dependent manner herein. General reduction in total p38 protein levels in Hep3B should be further analyzed to understand its contribution to results. In literature, in HCC, induction of p-38 levels generally correlates with SFB resistance (Witt-Kehati et al., 2018). However, p38 has dual roles which might have meanings in different context. For example, reduced p-38 levels are also correlated with HCC cell growth (Iyoda et al., 2003). Everolimus-SFB combination has bene shown to induce this phosphorylation to activate AMPK-p38 related apoptosis (Pignochino et al., 2015). Moreover, p-p38 in HCC patients has been associated with poor survival which is antagonized by p-JNK. Exposure to TFP alone in Ca922 cell lines induces p-38 phosphorylation in a dose dependent manner (C. H. Wu et al., 2016). Hence, there might be a balance between p38-JNK which needs to be investigated further. Studying DNA-damage-AKT-AMPK-p38-autophagy and apoptosis axes might be explored deeply to understand the mechanisms of drug combinatorial action of SFB and TFP. Moreover, doses of combinations, analyzed phosphorylated sites of proteins, uninvestigated crosstalk of pathways should be considered to evaluate existing results and planning the future experimentations.

One of the most interesting and novel aim of the second chapter of this thesis was examination of MR and GR modulation in response to SFB, TFP or in combination in liver cancer cells. Whether MR and GR are targets for combinatorial therapy in HCC to potentiate drug efficiency still remains relatively unexplored. Since HCC is diagnosed more commonly in men and breast cancers in women, differential sex hormone levels which are under SHRs makes investigation of this family valuable (Sukocheva, 2018). Reduced expression of MR in HCC patients through chromosomal aberrations or acetylated chromosomes has been established and proposed to be a negative prognostic marker. Downregulation of MR has been shown in liver cancer patients which underlies its pathogenic act in liver cancer. As mentioned before ALDO-activated MR reduces HCC viability. In another study, cirrhosis of liver cancer has been reduced via MR-antagonist eplerenone (Schreier et al., 2018). Moreover, rapid-genomic action of MR signaling crosstalk with the above mentioned PI3K and MAPK pathways is known as mentioned in introduction. In the present study GR was downregulated significantly while MR still needs to be validated. There is no literature on the effects of TFP or SFB on MR and but there are some for GR. MR antagonist spironolactone induces PI3K/AKT/mTOR signaling which cause autophagy has been shown in diabetic rat podocytes (D. Li et al., 2016) and can be a way to manipulate MR signaling in association with GR.

On the other hand, calcium-calmodulin antagonists, such as TFP and chlorpromazine, was shown to have direct interactions with GR interfering with GR's transcriptional actions in a Ca^{+2} dependent manner (Basta-Kaim et al., 2002). In neurons, glucocorticoids were shown to reduce Ca^{+2} levels to protect cells from Ca^{+2} overload and cell death. Moreover, in SFB given cells, the signaling pathway members downstream of GR were found to be dysregulated (Suwanjang et al., 2013).GR dependent induction of proliferation and metastasis in colon cancer draws further attention to diverse GR activities (W. Shi et al., 2019). Moreover, GR related ERK2 activity and induction of EMT are also relevant which are shown in breast cancer. Hence, reduction in GR protein in high combination of SFB+TFP that happened in Hep3B cells can be antiproliferative as suggested by the above-mentioned literature. Moreover, GR has been found to control BCL-xL levels when activated by its ligand (Gross et al., 2011). In Hep3B, GR and BCL-xL exhibited similar reduction patterns which were not observed in SkHep1. Moreover, mTOR-FOXO-GR-DEX crosstalk has also been also shown in skeletal muscle (Shimizu et al., 2011). However, more detailed investigations are required to decipher this possible crosstalk. SGK-1 which is inducible by both MR and GR signaling and target of mTORC1 provides another lead to pursue. In literature, TFP and Tamoxifen treated nerve

sheath tumors reduces SGK1 to prevent tamoxifen resistance (Brosius et al., 2014). However, SGK1 which is under control of above-mentioned pathways did not lead to significant changes in protein levels upon treatment in either of the cell lines. Moreover, one may speculate about the MR-GR ratio being a sensitivity indicator for this therapy in HCC after completed experimental setups. MR and GR acting on opposite directions in HCC and failed observation of MR bands in SkHep1 probably due to low expression might support this idea. Moreover, other MR-GR downstream target might be included to understand their regulation in an extended level.

Altogether, in Hep3B cell line the sensitivity of cellular viability to novel TFP-SFB combinatorial approach has supported at protein level via connection with multiple cancer related pathways and a novel change in GR levels, that needs to be further studied. However, to decipher the full mechanism of action requires more detailed investigations and several suggestions have been made in the discussion above. Sorting out the cellular death mechanism leading to reduced cell viability in a cell line/type specific manner and detecting protein targets of synergy would be beneficial to decipher combinatorial action and understand resistance of SkHep1. These findings are not only important in liver cancer but can be extended to other cancers where MR, GR are modulators and SFB+TFP can be an effective strategy as an anti-cancer treatment.

CHAPTER 6

FUTURE PERSPECTIVES

Herein, I have listed the future experimental approaches should be followed to clarify preliminary or inconclusive results of this study in two parts.

ALDO-E2 crosstalk in breast cancer cell line T47D

Our findings were novel and interesting yet requires further experimental approaches to be adopted for having stronger conclusions on possible mechanism of interaction. Hence, there is a requirement for more advanced experimental setups including dose (range of crosstalk) and time dependence together with a vast range of signaling markers to provide a better perspective on the mechanistic view. PGR and MR specific targets, and markers of cell cycle, apoptosis and other related pathways can be included.

I was T47D in this study which is a MR and ER positive breast cancer cell line. To be able to conclude MR and ESR1 as the responsible receptors in ALDO-E2 crosstalk, RNAi studies targeting these receptors alone and in combination, with/without hormone exposures can be included into experimental setup. Besides of RNAi, blocking receptors with small molecules to investigate rescue of crosstalk specific phenotypes might be beneficial. MR signaling antagonist spironolactone and drospirenone can be candidates. More detailed studies can be carried on cell lines without endogenous ER and/or MR expression to establish overexpression models to validate interactions. In this part, involvement of ALDO inducible GR signaling should be considered. Hence, involvement of cell lines that has no endogenous GR expression or RNAi silenced GR should be added to the experimental sets to sort out the ALDO-GR activation to prove ALDO induced MR involvement. Moreover, ER isoforms can be investigated due to their diverse functions in these treatment groups.

Furthermore, RNA-seq of ALDO and/or E2 treated groups in appropriate cellular model to identify gene clusters acting the same or diverse upon treatments can be a high throughput

approach to identify their distinctive inductions and interactions. To be able to sort out the possible mechanism of crosstalk we should investigate how these receptors or ligands interacts at cellular level. Co-localization studies can be performed via Immunofluorescence with antibodies targeting MR, ER, and their targets (possible mediators). Ligand induced nuclear translocation of proteins and their overlaps in nucleus would be a clue on their nuclear interactions. However, whether they form a protein complex in nucleus, regulate each other through secondary protein or directly target HRE elements of each other would be another question. For this purpose, Co-Immunoprecipitation of proteins can be evaluated upon hormone treatments in a nuclear and cytoplasmic levels to reveal protein-protein interactions. For the HRE regulation, Chromatin Immunoprecipitation Sequencing (ChIP-Seq) approach can be adopted to evaluate protein-DNA interaction upon treatments. Moreover, hormone regulated differential activity of receptors can be evaluated with Luciferase activity assays.

ALDO's effect on cell viability which seems to be reversed by E2 suggesting crosstalk of these signals, should be investigated further. Additional cell viability assessments involving optimization of hormone deprivation, increasing, or decreasing ALDO and E2 concentration in a time and cell concentration dependent manner might underly the effects on viability. Moreover, understanding the mechanism of this reduction, cell cycle and other related pathways should be investigated. In this context, cell cycle progression via PI- staining and evaluation of apoptosis via Annexin-PI or protein markers might be useful.

Since ALDO mediated metastasis is known in literature of renal cancer via GPER, metastasis might be relevant in this concept. Moreover, MR induced activities are known in brain. Hence, EMT markers might be evaluated to related breast cancer treatments which would be guided by ENaCs (has been related to metastasis in literature) in above mentioned models. Identification such mechanisms would be a valuable contribution to literature.

In more advanced future perspective, upon validation of ALDO induced reduction in cell viability, effectiveness can be tested *in vivo* by zebrafish xenograft models.

Reversed ALDO-induced PGR reduction by E2 signaling was a novel finding in this thesis. Whether ER is required for ALDO's reduction on PGR or ALDO-induced MR directly acts on PGR should be investigated at mechanistic and functional levels. With this purpose, above mentioned RNAi approaches can be adopted. Since PGR has two isoforms isoform specific action upon ALDO should be investigated. Whether silencing of PGR affects ALDO and E2 induced phenomena with hormone induction would be another question to ask. Over-expression

of PGR, may be in an isoform specific manner, should be investigated in the context of MR-ALDO signaling to reveal whether PGR expression rescues ALDO induced functional changes.

Understanding the cellular and molecular effects of combinatorial treatment with Sorafenib and Trifluoperazine in liver cancer and novel association with MR-GR signaling

The findings in the present thesis have indicated the possibility of apoptosis induction by combinatorial treatments in a cell-line specific manner, which should be validated with additional protein markers. Moreover, autophagy or other cell death mechanism should not be ignored. Annexin-PI staining, caspase 3/7 assays with pan-caspase inhibitors would be beneficial in terms of this validation. Moreover, energy metabolism dysfunction via AMPK and mitochondrial membrane dysfunction should be investigated through TMRE-mitochondrial membrane potential kit. Since the pathways that have been investigated have the ability to crosstalk with many others additional protein markers such as JAK-STAT, β-catenin, JNK, FOXO, MCL-1, BIM, and autophagy markers might be more informative in deciphering the mechanism. PKC signaling pathway can also be investigated in this combination due to its crosstalk with examined pathways and relativeness to calcium levels which can be targeted by TFP.

Since we have Hep3B cell line RNA-seq results which can be used as a reference to us for future validations, and a similar approach can be adopted for SkHep1 cell line via mRNA isolation, RNA-seq and/or qRT-PCR. Heterogeneity of HCC/liver cancer is a concern for drug developing hence addition of more cell lines to these panels would be beneficial to extend the view of this thesis. Since the cell lines used in this thesis differ in terms of p53 levels, importance of p53 should be validated. Moreover, the treatment has reduced p-AKT levels in the sensitive cells which might be beneficial to observe this activity in PTEN mutant cell lines. Additional cell lines of other cancers would be important to study to validate success of combination. In that sense, online tools can be used to predict treatment efficiency.

We have investigated the cell lines at mRNA and protein levels in addition to cell viability. More detailed functional analysis can be added to reveal effectiveness of this new treatment approach such as colony formation, soft agar, sphere formation, wound healing, real-time cell

analysis to assess morphology etc. would be beneficial to evaluate migration, stemness and proliferation upon treatments. Especially, EMT markers would be beneficial which can be induced by combinatorial targets.

Since we are proposing this treatment to enhance SFB induced effects in HCC cell lines, establishing SFB resistant cell lines and evaluate combination efficiency would be informative to display possible TFP induced sensitization.

In the cross-section of SHRs and SFB-TFP treatment, promising reduction in GR has drawn attention. Hence, GR might be affected by this treatment to relay anti-cancer effects with cross talking critical cellular pathways namely MR. Although a reduction in GR was seen, MR was not significantly altered, and this will may lead to higher MR activity and should be pursued further. These implicate whether GR rescue (may be MR too depending on completed experimental sets) can reverse the drug induced apoptotic phenotype in the absence or over expression of MR (hormones can be included to setup).

REFERENCES:

Abdel-Hafiz, H. A., & Horwitz, K. B. (2014). Post-translational modifications of the progesterone receptors. In *Journal of Steroid Biochemistry and Molecular Biology*. https://doi.org/10.1016/j.jsbmb.2013.12.008

Abou-Alfa, G. K., Schwartz, L., Ricci, S., Amadori, D., Santoro, A., Figer, A., De Greve, J., Douillard, J. Y., Lathia, C., Schwartz, B., Taylor, I., Moscovici, M., & Saltz, L. B. (2006). Phase II study of sorafenib in patients with advanced hepatocellular carcinoma. *Journal of Clinical Oncology*. https://doi.org/10.1200/JCO.2005.01.3441

Adelaiye-Ogala, R., Gryder, B. E., Nguyen, Y. T. M., Alilin, A. N., Grayson, A. R., Bajwa, W., Jansson, K. H., Beshiri, M. L., Agarwal, S., Rodriguez-Nieves, J. A., Capaldo, B., Kelly, K., & VanderWeele, D. J. (2020). Targeting the PI3K/AKT Pathway Overcomes Enzalutamide Resistance by Inhibiting Induction of the Glucocorticoid Receptor. *Molecular Cancer Therapeutics*. https://doi.org/10.1158/1535-7163.MCT-19-0936

Ahmad, N., & Kumar, R. (2011). Steroid hormone receptors in cancer development: A target for cancer therapeutics. In *Cancer Letters*. https://doi.org/10.1016/j.canlet.2010.09.008

Alnemri, E. S., Maksymowych, A. B., Robertson, N. M., & Litwack, G. (1991). Overexpression and characterization of the human mineralocorticoid receptor. *Journal of Biological Chemistry*.

Amara, S., Ivy, M. T., Myles, E. L., & Tiriveedhi, V. (2016). Sodium channel γENaC mediates IL-17 synergized high salt induced inflammatory stress in breast cancer cells. *Cellular Immunology*. https://doi.org/10.1016/j.cellimm.2015.12.007

Ando, K., Nakamura, Y., Nagase, H., Nakagawara, A., Koshinaga, T., Wada, S., & Makishima, M. (2019). Co-inhibition of the dna damage response and chk1 enhances apoptosis of neuroblastoma cells. *International Journal of Molecular Sciences*. https://doi.org/10.3390/ijms20153700

Ando, M., Yoshikawa, K., Iwase, Y., & Ishiura, S. (2014). Usefulness of monitoring γ-H2AX and cell cycle arrest in HepG2 cells for estimating genotoxicity using a high-content analysis system. *Journal of Biomolecular Screening*. https://doi.org/10.1177/1087057114541147

Arancibia, S., Benítez, D., Núñez, L. E., Jewell, C. M., Langjahr, P., Candia, E., Zapata-Torres, G., Cidlowski, J. A., González, M. J., & Hermoso, M. A. (2011). Phosphatidylinositol 3-kinase interacts with the glucocorticoid receptor upon TLR2 activation. *Journal of Cellular and Molecular Medicine*. https://doi.org/10.1111/j.1582-4934.2009.00958.x

Arroyo, J. P., Lagnaz, D., Ronzaud, C., Vázquez, N., Ko, B. S., Moddes, L., Ruffieux-Daidié, D., Hausel, P., Koesters, R., Yang, B., Stokes, J. B., Hoover, R. S., Gamba, G., & Staub, O. (2011). Nedd4-2 modulates renal Na+-Cl- cotransporter via the aldosterone-SGK1-Nedd4-2 pathway. *Journal of the American Society of Nephrology*. https://doi.org/10.1681/ASN.2011020132

Baba, H. A., Wohlschlaeger, J., Cicinnati, V. R., Hilgard, P., Lang, H., Sotiropoulos, G. C., Takeda, A., Beckebaum, S., & Schmitz, K. J. (2009). Phosphorylation of p70S6 kinase predicts overall survival in patients with clear margin-resected hepatocellular carcinoma. *Liver International*. https://doi.org/10.1111/j.1478-3231.2008.01798.x

Babicki, S., Arndt, D., Marcu, A., Liang, Y., Grant, J. R., Maciejewski, A., & Wishart, D. S. (2016). Heatmapper: web-enabled heat mapping for all. *Nucleic Acids Research*. https://doi.org/10.1093/nar/gkw419

Bah, N., Maillet, L., Ryan, J., Dubreil, S., Gautier, F., Letai, A., Juin, P., & Barillé-Nion, S. (2014). Bcl-xL controls a switch between cell death modes during mitotic arrest. *Cell Death and Disease*. https://doi.org/10.1038/cddis.2014.251

Bahrami-B, F., Ataie-Kachoie, P., Pourgholami, M. H., & Morris, D. L. (2014). P70 Ribosomal protein S6 kinase (Rps6kb1): An update. *Journal of Clinical Pathology*. https://doi.org/10.1136/jclinpath-2014-202560

Baillat, G., Garrouste, F., Remacle-Bonnet, M., Marvaldi, J., & Pommier, G. (2005). Bcl-xL/Bax ratio is altered by IFNγ in TNFα- but not in TRAIL-induced apoptosis in colon cancer cell line. *Biochimica et Biophysica Acta - Molecular Cell Research*. https://doi.org/10.1016/j.bbamcr.2004.12.005

Baker, M. E., & Katsu, Y. (2020). Progesterone: An enigmatic ligand for the mineralocorticoid receptor. In *Biochemical Pharmacology*. https://doi.org/10.1016/j.bcp.2020.113976

Bamberger, C. M., Bamberger, A. M., Wald, M., Chrousos, G. P., & Schulte, H. M. (1997). Inhibition of mineralocorticoid activity by the β isoform of the human glucocorticoid receptor. *Journal of Steroid Biochemistry and Molecular Biology*.

https://doi.org/10.1016/S0960-0760(96)00167-7

Banks, E., Beral, V., Bull, D., Reeves, G., Austoker, J., English, R., Patnick, J., Peto, R., Vessey, M., Wallis, M., Abbott, S., Bailey, E., Baker, K., Balkwill, A., Barnes, I., Black, J., Brown, A., Cameron, B., Canfell, K., … Timadjer, A. (2003). Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet*. https://doi.org/10.1016/S0140-6736(03)14065-2

Barnum, K. J., & O'Connell, M. J. (2014). Cell cycle regulation by checkpoints. In *Methods in Molecular Biology*. https://doi.org/10.1007/978-1-4939-888-2_2

Baron, S., Escande, A., Albérola, G., Bystricky, K., Balaguer, P., & Richard-Foy, H. (2007). Estrogen receptor α and the activating protein-1 complex cooperate during insulin-like growth factor-I-induced transcriptional activation of the pS2/TFF1 gene. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M610079200

Barrett Mueller, K., Lu, Q., Mohammad, N. N., Luu, V., McCurley, A., Williams, G. H., Adler, G. K., Karas, R. H., & Jaffe, I. Z. (2014). Estrogen receptor inhibits mineralocorticoid receptor transcriptional regulatory function. *Endocrinology*. https://doi.org/10.1210/en.2014-1270

Barrett, K. V., Mccurley, A. T., & Jaffe, I. Z. (2013). Direct contribution of vascular mineralocorticoid receptors to blood pressure regulation. In *Clinical and Experimental Pharmacology and Physiology*. https://doi.org/10.1111/1440-1681.12125

Basta-Kaim, A., Budziszewska, B., Jaworska-Feil, L., Tetich, M., Leśkiewicz, M., Kubera, M., & Lasoń, W. (2002). Chlorpromazine inhibits the glucocorticoid receptor-mediated gene transcription in a calcium-dependent manner. *Neuropharmacology*. https://doi.org/10.1016/S0028-3908(02)00149-1

Bessard, A., Frémin, C., Ezan, F., Fautrel, A., Gailhouste, L., & Baffet, G. (2008). RNAi-mediated ERK2 knockdown inhibits growth of tumor cells in vitro and in vivo. *Oncogene*. https://doi.org/10.1038/onc.2008.163

Bigas, J., Sevilla, L. M., Carceller, E., Boix, J., & Pérez, P. (2018). Epidermal glucocorticoid and mineralocorticoid receptors act cooperatively to regulate epidermal development and counteract skin inflammation article. *Cell Death and Disease*. https://doi.org/10.1038/s41419-018-0673-z

Billan, F. Le, Khan, J. A., Lamribet, K., Viengchareun, S., Bouligand, J., Fagart, J., & Lombès, M. (2015). Cistrome of the aldosterone-activated mineralocorticoid receptor in human renal cells. *FASEB Journal*. https://doi.org/10.1096/fj.15-274266

Björnström, L., & Sjöberg, M. (2005). Mechanisms of estrogen receptor signaling: Convergence of genomic and nongenomic actions on target genes. In *Molecular Endocrinology*. https://doi.org/10.1210/me.2004-0486

Blanchard, D. A., Mouhamad, S., Auffredou, M. T., Pesty, A., Bertoglio, J., Leca, G., & Vazquez, A. (2000). Cdk2 associates with MAP Kinase in vivo and its nuclear translocation is dependent on MAP Kinase activation in IL-2-dependent Kit 225 T lymphocytes. *Oncogene*. https://doi.org/10.1038/sj.onc.1203761

Bloem, L. J., Guo, C., & Pratt, J. H. (1995). Identification of a splice variant of the rat and human mineralocorticoid receptor genes. *Journal of Steroid Biochemistry and Molecular Biology*. https://doi.org/10.1016/0960-0760(95)00162-S

Boege, Y., Malehmir, M., Healy, M. E., Bettermann, K., Lorentzen, A., Vucur, M., Ahuja, A. K., Böhm, F., Mertens, J. C., Shimizu, Y., Frick, L., Remouchamps, C., Mutreja, K., Kähne, T., Sundaravinayagam, D., Wolf, M. J., Rehrauer, H., Koppe, C., Speicher, T., … Weber, A. (2017). A Dual Role of Caspase-8 in Triggering and Sensing Proliferation-Associated DNA Damage, a Key Determinant of Liver Cancer Development. *Cancer Cell*. https://doi.org/10.1016/j.ccell.2017.08.010

Bondarava, M., Li, T., Endl, E., & Wehner, F. (2009). α-ENaC is a functional element of the hypertonicity-induced cation channel in HepG2 cells and it mediates proliferation. *Pflugers Archiv European Journal of Physiology*. https://doi.org/10.1007/s00424-009-0649-z

Boscardin, E., Alijevic, O., Hummler, E., Frateschi, S., & Kellenberger, S. (2016). The function and regulation of acid-sensing ion channels (ASICs) and the epithelial Na+channel (ENaC): IUPHAR Review 19. In *British Journal of Pharmacology*. https://doi.org/10.1111/bph.13533

Boucher, M. J., Morisset, J., Vachon, P. H., Reed, J. C., Lain, J., & Rivard, N. (2000). MEK/ERK signaling pathway regulates the expression of Bcl-2, Bcl-X(L), and Mcl-1 and promotes survival of human pancreatic cancer cells. *Journal of Cellular Biochemistry*. https://doi.org/10.1002/1097-4644(20001201)79:3<355::AID-JCB20>3.0.CO;2-0

Bourdeau, V., Deschênes, J., Laperrière, D., Aid, M., White, J. H., & Mader, S. (2008). Mechanisms of primary and secondary estrogen target gene regulation in breast cancer cells. *Nucleic Acids Research*. https://doi.org/10.1093/nar/gkm945

Boyd, C., & Náray-Fejes-Tóth, A. (2007). Steroid-mediated regulation of the epithelial sodium channel subunits in mammary epithelial cells. *Endocrinology*. https://doi.org/10.1210/en.2006-1741

Braicu, C., Buse, M., Busuioc, C., Drula, R., Gulei, D., Raduly, L., Rusu, A., Irimie, A., Atanasov, A. G., Slaby, O., Ionescu, C., & Berindan-Neagoe, I. (2019). A comprehensive review on MAPK: A promising therapeutic target in cancer. In *Cancers*. https://doi.org/10.3390/cancers11101618

Brentnall, M., Rodriguez-Menocal, L., De Guevara, R. L., Cepero, E., & Boise, L. H. (2013). Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. *BMC Cell Biology*. https://doi.org/10.1186/1471-2121-14-32

Brooks, D. L., Garza, A. E., Katayama, I. A., Romero, J. R., Adler, G. K., Pojoga, L. H., & Williams, G. H. (2019). Aldosterone Modulates the Mechanistic Target of Rapamycin Signaling in Male Mice. *Endocrinology*. https://doi.org/10.1210/en.2018-00989

Brosius, S. N., Turk, A. N., Byer, S. J., Longo, J. F., Kappes, J. C., Roth, K. A., & Carroll, S. L. (2014). Combinatorial therapy with tamoxifen and trifluoperazine effectively inhibits malignant peripheral nerve sheath tumor growth by targeting complementary signaling cascades. *Journal of Neuropathology and Experimental Neurology*. https://doi.org/10.1097/NEN.0000000000000126

Bruix, J., Qin, S., Merle, P., Granito, A., Huang, Y. H., Bodoky, G., Pracht, M., Yokosuka, O., Rosmorduc, O., Breder, V., Gerolami, R., Masi, G., Ross, P. J., Song, T., Bronowicki, J. P., Ollivier-Hourmand, I., Kudo, M., Cheng, A. L., Llovet, J. M., … Han, G. (2017). Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, phase 3 trial. *The Lancet*. https://doi.org/10.1016/S0140-6736(16)32453-9

Budd, G. T., Bukowski, R. M., Lichtin, A., Bauer, L., Van Kirk, P., & Ganapathi, R. (1993). Phase II trial of doxorubicin and trifluoperazine in metastatic breast cancer. *Investigational New Drugs*. https://doi.org/10.1007/BF00873916

Buluwela, L., Pike, J., Mazhar, D., Kamalati, T., Hart, S. M., Al-Jehani, R., Yahaya, H., Patel,

N., Sarwarl, N., Heathcote, D. A., Schwickerath, O., Phoenix, F., Hill, R., Aboagye, E., Shousha, S., Waxman, J., Lemoine, N. R., Zelent, A., Coombes, R. C., & Ali, S. (2005). Inhibiting estrogen responses in breast cancer cells using a fusion protein encoding estrogen receptor-α and the transcriptional repressor PLZF. *Gene Therapy*. https://doi.org/10.1038/sj.gt.3302421

Burgess, L. H., & Handa, R. J. (1993). Estrogen-induced alterations in the regulation of mineralocorticoid and glucocorticoid receptor messenger RNA expression in the female rat anterior pituitary gland and brain. *Molecular and Cellular Neuroscience*. https://doi.org/10.1006/mcne.1993.1023

Byun, H. S., Zhou, W., Park, I. W., Kang, K., Lee, S. R., Piao, X., Park, J. B., Kwon, T. K., Na, M. K., & Hur, G. M. (2018). C-27-carboxylated oleanane triterpenoids up-regulate TRAIL DISC assembly via p38 MAPK and CHOP-mediated DR5 expression in human glioblastoma cells. *Biochemical Pharmacology*. https://doi.org/10.1016/j.bcp.2018.10.019

Caja, L., Sancho, P., Bertran, E., Iglesias-Serret, D., Gil, J., & Fabregat, I. (2009). Overactivation of the MEK/ERK pathway in liver tumor cells confers resistance to TGF-β-induced cell death through impairing up-regulation of the NADPH oxidase NOX4. *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-09-1482

Caldon, C. E., Sergio, C. M., Schütte, J., Boersma, M. N., Sutherland, R. L., Carroll, J. S., & Musgrove, E. A. (2009). Estrogen Regulation of Cyclin E2 Requires Cyclin D1 but Not c-Myc. *Molecular and Cellular Biology*. https://doi.org/10.1128/mcb.00269-09

Cao, Z., Liao, Q., Su, M., Huang, K., Jin, J., & Cao, D. (2019). AKT and ERK dual inhibitors: The way forward? In *Cancer Letters*. https://doi.org/10.1016/j.canlet.2019.05.025

Carneiro, B. A., & El-Deiry, W. S. (2020). Targeting apoptosis in cancer therapy. In *Nature Reviews Clinical Oncology*. https://doi.org/10.1038/s41571-020-0341-y

Caroccia, B., Seccia, T. M., Campos, A. G., Gioco, F., Kuppusamy, M., Ceolotto, G., Guerzoni, E., Simonato, F., Mareso, S., Lenzini, L., Fassina, A., & Rossi, G. P. (2014). GPER-1 and estrogen receptor-β ligands modulate aldosterone synthesis. *Endocrinology*. https://doi.org/10.1210/en.2014-1416

Caroccia, B., Seccia, T. M., Piazza, M., Prisco, S., Zanin, S., Iacobone, M., Lenzini, L., Pallafacchina, G., Domening, O., Poglitsch, M., Rizzuto, R., & Rossi, G. P. (2019).

Aldosterone Stimulates Its Biosynthesis Via a Novel GPER-Mediated Mechanism. *Journal of Clinical Endocrinology and Metabolism*. https://doi.org/10.1210/jc.2019-00043

Carreau, S., Bouraima-Lelong, H., & Delalande, C. (2011). Estrogens - New players in spermatogenesis. *Reproductive Biology*. https://doi.org/10.1016/S1642-431X(12)60065-5

Castel, P., & Toska, E. (2019). Chromatin regulation at the intersection of estrogen receptor and PI3K pathways in breast cancer. *Molecular and Cellular Oncology*. https://doi.org/10.1080/23723556.2019.1625620

Castrén, M., Patchev, V. K., Almeida, O. F. X., Holsboer, F., Trapp, T., & Castrén, E. (1995). Regulation of rat mineralocorticoid receptor expression in neurons by progesterone. *Endocrinology*. https://doi.org/10.1210/endo.136.9.7649087

Celojevic, D., Petersen, A., Karlsson, J. O., Behndig, A., & Zetterberg, M. (2011). Effects of 17β-estradiol on proliferation, cell viability and intracellular redox status in native human lens epithelial cells. *Molecular Vision*.

Cenciarini, M. E., & Proietti, C. J. (2019). Molecular mechanisms underlying progesterone receptor action in breast cancer: Insights into cell proliferation and stem cell regulation. *Steroids*. https://doi.org/10.1016/j.steroids.2019.108503

Chang, C. T., Hung, C. C., Chen, Y. C., Yen, T. H., Wu, M. S., Yang, C. W., Phillips, A., & Tian, Y. C. (2008). Transforming growth factor-β1 decreases epithelial sodium channel functionality in renal collecting duct cells via a Smad4-dependent pathway. *Nephrology Dialysis Transplantation*. https://doi.org/10.1093/ndt/gfm786

Chang, C. T., Sun, C. Y., Pong, C. Y., Chen, Y. C., Lin, G. P., Chang, T. C., & Wu, M. S. (2007). Interaction of estrogen and progesterone in the regulation of sodium channels in collecting tubular cells. *Chang Gung Medical Journal*.

Chaturantabut, S., Shwartz, A., Evason, K. J., Cox, A. G., Labella, K., Schepers, A. G., Yang, S., Acuña, M., Houvras, Y., Mancio-Silva, L., Romano, S., Gorelick, D. A., Cohen, D. E., Zon, L. I., Bhatia, S. N., North, T. E., & Goessling, W. (2019). Estrogen Activation of G-Protein–Coupled Estrogen Receptor 1 Regulates Phosphoinositide 3-Kinase and mTOR Signaling to Promote Liver Growth in Zebrafish and Proliferation of Human Hepatocytes. *Gastroenterology*. https://doi.org/10.1053/j.gastro.2019.01.010

Chen, C. C., Hardy, D. B., & Mendelson, C. R. (2011). Progesterone receptor inhibits proliferation of human breast cancer cells via induction of MAPK phosphatase 1 (MKP-1/DUSP1). *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M111.295865

Chen, C. H., Chen, M. C., Wang, J. C., Tsai, A. C., Chen, C. S., Liou, J. P., Pan, S. L., & Teng, C. M. (2014). Synergistic interaction between the HDAC inhibitor, MPT0E028, and sorafenib in liver cancer cells in vitro and in vivo. *Clinical Cancer Research*. https://doi.org/10.1158/1078-0432.CCR-12-3909

Chen, C., Nelson, L. J., Ávila, M. A., & Cubero, F. J. (2019). Mitogen-Activated Protein Kinases (MAPKs) and Cholangiocarcinoma: The Missing Link. In *Cells*. https://doi.org/10.3390/cells8101172

Chen, J. S., Wang, Q., Fu, X. H., Huang, X. H., Chen, X. L., Cao, L. Q., Chen, L. Z., Tan, H. X., Li, W., Bi, J., & Zhang, L. J. (2009). Involvement of PI3K/PTEN/AKT/mTOR pathway in invasion and metastasis in hepatocellular carcinoma: Association with MMP-9. *Hepatology Research*. https://doi.org/10.1111/j.1872-034X.2008.00449.x

Chen, M. H., Yang, W. L. R., Lin, K. T., Liu, C. H., Liu, Y. W., Huang, K. W., Chang, P. M. H., Lai, J. M., Hsu, C. N., Chao, K. M., Kao, C. Y., & Huang, C. Y. F. (2011). Gene expression-based chemical genomics identifies potential therapeutic drugs in hepatocellular carcinoma. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0027186

Chen, X. P., He, S. Q., Wang, H. P., Zhao, Y. Z., & Zhang, W. G. (2003). Expression of TNF-related apoptosis-inducing Ligand receptors and antitumor tumor effects of TNF-related apoptosis-inducing Ligand in human hepatocellular carcinoma. *World Journal of Gastroenterology*. https://doi.org/10.3748/wjg.v9.i11.2433

Chen, Y., Liu, Y. C., Sung, Y. C., Ramjiawan, R. R., Lin, T. T., Chang, C. C., Jeng, K. S., Chang, C. F., Liu, C. H., Gao, D. Y., Hsu, F. F., Duyverman, A. M., Kitahara, S., Huang, P., Dima, S., Popescu, I., Flaherty, K. T., Zhu, A. X., Bardeesy, N., … Duda, D. G. (2017). Overcoming sorafenib evasion in hepatocellular carcinoma using CXCR4-targeted nanoparticles to co-deliver MEK-inhibitors. *Scientific Reports*. https://doi.org/10.1038/srep44123

Chin, R., Earnest-Silveira, L., Koeberlein, B., Franz, S., Zentgraf, H., Dong, X., Gowans, E., Bock, C. T., & Torresi, J. (2007). Modulation of MAPK pathways and cell cycle by replicating hepatitis B virus: Factors contributing to hepatocarcinogenesis. *Journal of*

Hepatology. https://doi.org/10.1016/j.jhep.2007.03.025

Choi, S., Chen, Z., Tang, L. H., Fang, Y., Shin, S. J., Panarelli, N. C., Chen, Y. T., Li, Y., Jiang, X., & Du, Y. C. N. (2016). Bcl-xL promotes metastasis independent of its anti-apoptotic activity. *Nature Communications*. https://doi.org/10.1038/ncomms10384

Chung, E. J., Sung, Y. K., Farooq, M., Kim, Y., Im, S., Tak, W. Y., Hwang, Y. J., Kim, Y. Il, Han, H. S., Kim, J. C., & Kim, M. K. (2002). Gene expression profile analysis in human hepatocellular carcinoma by cDNA microarray. *Molecules and Cells*.

Cicatiello, L., Addeo, R., Sasso, A., Altucci, L., Petrizzi, V. B., Borgo, R., Cancemi, M., Caporali, S., Caristi, S., Scafoglio, C., Teti, D., Bresciani, F., Perillo, B., & Weisz, A. (2004). Estrogens and Progesterone Promote Persistent CCND1 Gene Activation during G1 by Inducing Transcriptional Derepression via c-Jun/c-Fos/Estrogen Receptor (Progesterone Receptor) Complex Assembly to a Distal Regulatory Element and Recruitment of Cyclin D1 t. *Molecular and Cellular Biology*. https://doi.org/10.1128/mcb.24.16.7260-7274.2004

Cong, B., Du, J., Zhu, X., Lu, J., & Ni, X. (2015). Estrogen enhancement of SGK1 expression induced by urocortin contributes to its cardioprotection against ischemia/reperfusion insult. *International Journal of Cardiology*. https://doi.org/10.1016/j.ijcard.2014.10.113

Cork, D. M. W., Lennard, T. W. J., & Tyson-Capper, A. J. (2008). Alternative splicing and the progesterone receptor in breast cancer. In *Breast Cancer Research*. https://doi.org/10.1186/bcr2097

Crochemore, C., Lu, J., Wu, Y., Liposits, Z., Sousa, N., Holsboer, F., & Almeida, O. F. X. (2005). Direct targeting of hippocampal neurons for apoptosis by glucocorticoids is reversible by mineralocorticoid receptor activation. *Molecular Psychiatry*. https://doi.org/10.1038/sj.mp.4001679

Cucarull, B., Tutusaus, A., Subías, M., Stefanovic, M., Hernáez-Alsina, T., Boix, L., Reig, M., de Frutos, P. G., Marí, M., Colell, A., Bruix, J., & Morales, A. (2020). Regorafenib alteration of the BCL-xL/MCL-1 ratio provides a therapeutic opportunity for BH3-mimetics in hepatocellular carcinoma models. *Cancers*. https://doi.org/10.3390/cancers12020332

Cui, X., Zhang, P., Deng, W., Oesterreich, S., Lu., Y., Mills, G. B., & Lee, A. V. (2003). Insulin-like growth factor-I inhibits progesterone receptor expression in breast cancer cells via the

phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway: Progesterone receptor as a potential indicator of growth factor activity in brea. *Molecular Endocrinology*. https://doi.org/10.1210/me.2002-0318

D'Uva, G., & Lauriola, M. (2016). Towards the emerging crosstalk: ERBB family and steroid hormones. In *Seminars in Cell and Developmental Biology*. https://doi.org/10.1016/j.semcdb.2015.11.004

Dai, X., Cheng, H., Bai, Z., & Li, J. (2017). Breast cancer cell line classification and Its relevance with breast tumor subtyping. In *Journal of Cancer*. https://doi.org/10.7150/jca.18457

Dattilo, V., Amato, R., Perrotti, N., & Gennarelli, M. (2020). The Emerging Role of SGK1 (Serum- and Glucocorticoid-Regulated Kinase 1) in Major Depressive Disorder: Hypothesis and Mechanisms. In *Frontiers in Genetics*. https://doi.org/10.3389/fgene.2020.00826

De Boer, L., Oakes, V., Beamish, H., Giles, N., Stevens, F., Somodevilla-Torres, M., DeSouza, C., & Gabrielli, B. (2008). Cyclin A/cdk2 coordinates centrosomal and nuclear mitotic events. *Oncogene*. https://doi.org/10.1038/onc.2008.74

de Faria, P. A., Bettanin, F., Cunha, R. L. O. R., Paredes-Gamero, E. J., Homem-de-Mello, P., Nantes, I. L., & Rodrigues, T. (2015). Cytotoxicity of phenothiazine derivatives associated with mitochondrial dysfunction: A structure-activity investigation. *Toxicology*. https://doi.org/10.1016/j.tox.2015.02.004

Debonneville, C., Flores, S. Y., Kamynina, E., Plant, P. J., Tauxe, C., Thomas, M. A., Münster, C., Chraïbi, A., Pratt, J. H., Horisberger, J. D., Pearce, D., Loffing, J., & Staub, O. (2001). Phosphorylation of Nedd4-2 by Sgk1 regulates epithelial Na+ channel cell surface expression. *EMBO Journal*. https://doi.org/10.1093/emboj/20.24.7052

Dhar, R., Persaud, S. D., Mireles, J. R., & Basu, A. (2009). Proteolytic cleavage of p70 ribosomal S6 kinase by caspase-3 during DNA damage-induced apoptosis. *Biochemistry*. https://doi.org/10.1021/bi801840s

Di Cristofano, A. (2017). SGK1: The Dark Side of PI3K Signaling. In *Current Topics in Developmental Biology*. https://doi.org/10.1016/bs.ctdb.2016.11.006

Di Fabio, F., Alvarado, C., Majdan, A., Gologan, A., Voda, L., Mitmaker, E., Beitel, L. K.,

Gordon, P. H., & Trifiro, M. (2007). Underexpression of mineralocorticoid receptor in colorectal carcinomas and association with VEGFR-2 overexpression. *Journal of Gastrointestinal Surgery*. https://doi.org/10.1007/s11605-007-0234-8

Diep, C. H., Ahrendt, H., & Lange, C. A. (2016). Progesterone induces progesterone receptor gene (PGR) expression via rapid activation of protein kinase pathways required for cooperative estrogen receptor alpha (ER) and progesterone receptor (PR) genomic action at ER/PR target genes. *Steroids*. https://doi.org/10.1016/j.steroids.2016.09.004

Doan, T. B., Cheung, V., Clyne, C. D., Hilton, H. N., Eriksson, N., Young, M. J., Funder, J. W., Muscat, G. E. O., Fuller, P. J., Clarke, C. L., & Graham, J. D. (2020). A tumour suppressive relationship between mineralocorticoid and retinoic acid receptors activates a transcriptional program consistent with a reverse Warburg effect in breast cancer. *Breast Cancer Research*. https://doi.org/10.1186/s13058-020-01355-x

Doan, T. B., Graham, J. D., & Clarke, C. L. (2017). Emerging functional roles of nuclear receptors in breast cancer. In *Journal of Molecular Endocrinology*. https://doi.org/10.1530/JME-16-0082

Dong, T., Zhang, Y., Chen, Y., Liu, P., An, T., Zhang, J., Yang, H., Zhu, W., & Yang, X. (2017). FOXO1 inhibits the invasion and metastasis of hepatocellular carcinoma by reversing ZEB2-induced epithelial-mesenchymal transition. *Oncotarget*. https://doi.org/10.18632/oncotarget.13786

Dressing, G. E., Knutson, T. P., Schiewer, M. J., Daniel, A. R., Hagan, C. R., Diep, C. H., Knudsen, K. E., & Lange, C. A. (2014). Progesterone receptor-cyclin D1 complexes induce cell cycle-dependent transcriptional programs in breast cancer cells. *Molecular Endocrinology*. https://doi.org/10.1210/me.2013-1196

Dumesic, P. A., Scholl, F. A., Barragan, D. I., & Khavari, P. A. (2009). Erk1/2 MAP kinases are required for epidermal G2/M progression. *Journal of Cell Biology*. https://doi.org/10.1083/jcb.200804038

Dwyer, A. R., Truong, T. H., Ostrander, J. H., & Lange, C. A. (2020). Steroid receptors as MAPK signaling sensors in breast cancer: Let the fates decide. In *Journal of Molecular Endocrinology*. https://doi.org/10.1530/JME-19-0274

El-Khoueiry, A. B., Sangro, B., Yau, T., Crocenzi, T. S., Kudo, M., Hsu, C., Kim, T. Y., Choo, S. P., Trojan, J., Welling, T. H., Meyer, T., Kang, Y. K., Yeo, W., Chopra, A., Anderson,

J., dela Cruz, C., Lang, L., Neely, J., Tang, H., … Melero, I. (2017). Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. *The Lancet*. https://doi.org/10.1016/S0140-6736(17)31046-2

Ellison-Zelski, S. J., Solodin, N. M., & Alarid, E. T. (2009). Repression of ESR1 through Actions of Estrogen Receptor Alpha and Sin3A at the Proximal Promoter. *Molecular and Cellular Biology*. https://doi.org/10.1128/mcb.00383-09

Ezzoukhry, Z., Louandre, C., Trécherel, E., Godin, C., Chauffert, B., Dupont, S., Diouf, M., Barbare, J. C., Mazière, J. C., & Galmiche, A. (2012). EGFR activation is a potential determinant of primary resistance of hepatocellular carcinoma cells to sorafenib. *International Journal of Cancer*. https://doi.org/10.1002/ijc.27604

Fagerli, U. M., Ullrich, K., Stühmer, T., Holien, T., Köchert, K., Holt, R. U., Bruland, O., Chatterjee, M., Nogai, H., Lenz, G., Shaughnessy, J. D., Mathas, S., Sundan, A., Bargou, R. C., Dörken, B., Børset, M., & Janz, M. (2011). Serum/glucocorticoid-regulated kinase 1 (SGK1) is a prominent target gene of the transcriptional response to cytokines in multiple myeloma and supports the growth of myeloma cells. *Oncogene*. https://doi.org/10.1038/onc.2011.79

Fancy, R. M., Kim, H., Napier, T., Buchsbaum, D. J., Zinn, K. R., & Song, Y. (2018). Calmodulin antagonist enhances DR5-mediated apoptotic signaling in TRA-8 resistant triple negative breast cancer cells. *Journal of Cellular Biochemistry*. https://doi.org/10.1002/jcb.26848

Faresse, N., Vitagliano, J. J., & Staub, O. (2012). Differential ubiquitylation of the mineralocorticoid receptor is regulated by phosphorylation. *FASEB Journal*. https://doi.org/10.1096/fj.12-209924

Faulkner, J. L., & Belin de Chantemèle, E. J. (2019). Mineralocorticoid Receptor and Endothelial Dysfunction in Hypertension. In *Current Hypertension Reports*. https://doi.org/10.1007/s11906-019-0981-4

FDA approves atezolizumab plus bevacizumab for unresectable hepatocellular carcinoma. (2020). https://www.fda.gov/drugs/drug-approvals-and-databases/fda-approves-atezolizumab-plus-bevacizumab-unresectable-hepatocellular-carcinoma

Feldkamp, M. D., O'Donnell, S. E., Yu, L., & Shea, M. A. (2010). Allosteric effects of the

antipsychotic drug trifluoperazine on the energetics of calcium binding by calmodulin. *Proteins: Structure, Function and Bioinformatics*. https://doi.org/10.1002/prot.22739

Feng, Z., Xia, Y., Gao, T., Xu, F., Lei, Q., Peng, C., Yang, Y., Xue, Q., Hu, X., Wang, Q., Wang, R., Ran, Z., Zeng, Z., Yang, N., Xie, Z., & Yu, L. (2018). The antipsychotic agent trifluoperazine hydrochloride suppresses triple-negative breast cancer tumor growth and brain metastasis by inducing G0/G1 arrest and apoptosis. *Cell Death and Disease*. https://doi.org/10.1038/s41419-018-1046-3

Finn, R. S., Qin, S., Ikeda, M., Galle, P. R., Ducreux, M., Kim, T.-Y., Kudo, M., Breder, V., Merle, P., Kaseb, A. O., Li, D., Verret, W., Xu, D.-Z., Hernandez, S., Liu, J., Huang, C., Mulla, S., Wang, Y., Lim, H. Y., … Cheng, A.-L. (2020). Atezolizumab plus Bevacizumab in Unresectable Hepatocellular Carcinoma. *New England Journal of Medicine*. https://doi.org/10.1056/nejmoa1915745

Frenzel, A., Grespi, F., Chmelewskij, W., & Villunger, A. (2009). Bcl2 family proteins in carcinogenesis and the treatment of cancer. In *Apoptosis*. https://doi.org/10.1007/s10495-008-0300-z

Frindt, G., & Palmer, L. G. (2012). Regulation of epithelial Na + channels by adrenal steroids: Mineralocorticoid and glucocorticoid effects. *American Journal of Physiology - Renal Physiology*. https://doi.org/10.1152/ajprenal.00480.2011

Fu, L., Wu, W., Sun, X., & Zhang, P. (2020). Glucocorticoids Enhanced Osteoclast Autophagy Through the PI3K/Akt/mTOR Signaling Pathway. *Calcified Tissue International*. https://doi.org/10.1007/s00223-020-00687-2

Fuller, P. J., Yao, Y. Z., Jin, R., He, S., Martín-Fernández, B., Young, M. J., & Smith, B. J. (2019). Molecular evolution of the switch for progesterone and spironolactone from mineralocorticoid receptor agonist to antagonist. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.1903172116

Funk, J. O. (2006). Cell Cycle Checkpoint Genes and Cancer. In *Encyclopedia of Life Sciences*. https://doi.org/10.1038/npg.els.0006046

Galliher-Beckley, A. J., Williams, J. G., & Cidlowski, J. A. (2011). Ligand-Independent Phosphorylation of the Glucocorticoid Receptor Integrates Cellular Stress Pathways with Nuclear Receptor Signaling. *Molecular and Cellular Biology*. https://doi.org/10.1128/mcb.05866-11

Gambling, L., Dunford, S., Wilson, C. A., McArdle, H. J., & Baines, D. L. (2004). Estrogen and progesterone regulate α, β, and γENaC subunit mRNA levels in female rat kidney. *Kidney International*. https://doi.org/10.1111/j.1523-1755.2004.00593.x

Gao, C., Pang, L., Ren, C., & Ma, T. (2012). Decreased expression of Nedd4L correlates with poor prognosis in gastric cancer patient. *Medical Oncology*. https://doi.org/10.1007/s12032-011-0061-3

Gao, J. J., Shi, Z. Y., Xia, J. F., Inagaki, Y., & Tang, W. (2015). Sorafenib-based combined molecule targeting in treatment of hepatocellular carcinoma. *World Journal of Gastroenterology*. https://doi.org/10.3748/wjg.v21.i42.12059

Gao, Y., Sun, T. Y., Bai, W. F., & Bai, C. G. (2019). Design, synthesis and evaluation of novel phenothiazine derivatives as inhibitors of breast cancer stem cells. In *European Journal of Medicinal Chemistry*. https://doi.org/10.1016/j.ejmech.2019.111692

Gedaly, R., Angulo, P., Hundley, J., Daily, M. F., Chen, C., Koch, A., & Mark Evers, B. (2010). PI-103 and sorafenib inhibit hepatocellular carcinoma cell proliferation by blocking Ras/Raf/MAPK and PI3K/AKT/mTOR pathways. *Anticancer Research*.

Gekle, M., Silbernagl, S., & Wünsch, S. (1998). Non-genomic action of the mineralocorticoid aldosterone on cytosolic sodium in cultured kidney cells. *Journal of Physiology*. https://doi.org/10.1111/j.1469-7793.1998.255bi.x

Giakoustidis, A., Giakoustidis, D., Mudan, S., Sklavos, A., & Williams, R. (2015). Molecular signalling in hepatocellular carcinoma: Role of and crosstalk among Wnt/β-catenin, Sonic Hedgehog, Notch and Dickkopf-1. In *Canadian Journal of Gastroenterology and Hepatology*. https://doi.org/10.1155/2015/172356

Giangrande, P. H., & Mcdonnell, D. P. (1999). The A and B isoforms of the human progesterone receptor: Two functionally different transcription factors encoded by a single gene. *Recent Progress in Hormone Research*.

Gil-Ad, I., Shtaif, B., Levkovitz, Y., Nordenberg, J., Taler, M., Korov, I., & Weizman, A. (2006). Phenothiazines induce apoptosis in a B16 mouse melanoma cell line and attenuate in vivo melanoma tumor growth. *Oncology Reports*. https://doi.org/10.3892/or.15.1.107

Godbole, M., Togar, T., Patel, K., Dharavath, B., Yadav, N., Janjuha, S., Gardi, N., Tiwary, K., Terwadkar, P., Desai, S., Prasad, R., Dhamne, H., Karve, K., Salunkhe, S., Kawle, D.,

Chandrani, P., Dutt, S., Gupta, S., Badwe, R. A., & Dutt, A. (2018). Up-regulation of the kinase gene SGK1 by progesterone activates the AP-1–NDRG1 axis in both PR-positive and -negative breast cancer cells. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.RA118.002894

Gong, D., & Ferrell, J. E. (2010). The roles of cyclin A2, B1, and B2 in early and late mitotic events. *Molecular Biology of the Cell*. https://doi.org/10.1091/mbc.E10-05-0393

Gopi, C., & Dhanaraju, M. D. (2019). Recent Progress in Synthesis, Structure and Biological Activities of Phenothiazine Derivatives. *Review Journal of Chemistry*. https://doi.org/10.1134/s2079978019020018

Gottifredi, V., Karni-Schmidt, O., Shieh, S.-Y., & Prives, C. (2001). p53 Down-Regulates CHK1 through p21 and the Retinoblastoma Protein. *Molecular and Cellular Biology*. https://doi.org/10.1128/mcb.21.4.1066-1076.2001

Gouri, N., Hema Sree, G. N. S., Saraswathy Ganesan, R., Subeesh, K. V., & Jayaraman, A. (2019). *Drug Repurposing for Hepatocellular Carcinoma via Virtual Screening Integrated with Bioinformatics Analysis* . https://doi.org/10.1039/9781839160783-00073

Goyette, M. A., Cusseddu, R., Elkholi, I., Abu-Thuraia, A., El-Hachem, N., Haibe-Kains, B., Gratton, J. P., & Côté, J. F. (2019). AXL knockdown gene signature reveals a drug repurposing opportunity for a class of antipsychotics to reduce growth and metastasis of triple-negative breast cancer. *Oncotarget*. https://doi.org/10.18632/oncotarget.26725

Grimm, S. L., Hartig, S. M., & Edwards, D. P. (2016). Progesterone Receptor Signaling Mechanisms. In *Journal of Molecular Biology*. https://doi.org/10.1016/j.jmb.2016.06.020

Griol-Charhbili, V., Fassot, C., Messaoudi, S., Perret, C., Agrapart, V., & Jaisser, F. (2011). Epidermal growth factor receptor mediates the vascular dysfunction but not the remodeling induced by aldosterone/salt. *Hypertension*. https://doi.org/10.1161/HYPERTENSIONAHA.110.153619

Gross, K. L., Oakley, R. H., Scoltock, A. B., Jewell, C. M., & Cidlowski, J. A. (2011). Glucocorticoid receptor α isoform-selective regulation of antiapoptotic genes in osteosarcoma cells: A new mechanism for glucocorticoid resistance. *Molecular Endocrinology*. https://doi.org/10.1210/me.2010-0051

Grossmann, C., Benesic, A., Krug, A. W., Freudinger, R., Mildenberger, S., Gassner, B., &

Gekle, M. (2005). Human mineralocorticoid receptor expression renders cells responsive for nongenotropic aldosterone actions. *Molecular Endocrinology*. https://doi.org/10.1210/me.2004-0469

Guarnieri, A. L., Towers, C. G., Drasin, D. J., Oliphant, M. U. J., Andrysik, Z., Hotz, T. J., Vartuli, R. L., Linklater, E. S., Pandey, A., Khanal, S., Espinosa, J. M., & Ford, H. L. (2018). The miR-106b-25 cluster mediates breast tumor initiation through activation of NOTCH1 via direct repression of NEDD4L. *Oncogene*. https://doi.org/10.1038/s41388-018-0239-7

Guille, A., Chaffanet, M., & Birnbaum, D. (2013). Signaling pathway switch in breast cancer. *Cancer Cell International*. https://doi.org/10.1186/1475-2867-13-66

Gutierrez, A., Pan, L., Groen, R. W. J., Baleydier, F., Kentsis, A., Marineau, J., Grebliunaite, R., Kozakewich, E., Reed, C., Pflumio, F., Poglio, S., Uzan, B., Clemons, P., VerPlank, L., An, F., Burbank, J., Norton, S., Tolliday, N., Steen, H., … Aster, J. C. (2014). Phenothiazines induce PP2A-mediated apoptosis in T cell acute lymphoblastic leukemia. *Journal of Clinical Investigation*. https://doi.org/10.1172/JCI65093

Hait, W. N., Lazo, J. S., Chen, D. L., Gallichio, V. S., & Filderman, A. E. (1988). Antitumor and toxic effects of combination chemotherapy with bleomycin and a phenothiazine anticalmodulin agent. *Journal of the National Cancer Institute*. https://doi.org/10.1093/jnci/80.4.246

Hall, B. A., Kim, T. Y., Skor, M. N., & Conzen, S. D. (2012). Serum and glucocorticoid-regulated kinase 1 (SGK1) activation in breast cancer: Requirement for mTORC1 activity associates with ER-alpha expression. *Breast Cancer Research and Treatment*. https://doi.org/10.1007/s10549-012-2161-y

Hallows, K. R., Bhalla, V., Oyster, N. M., Wijngaarden, M. A., Lee, J. K., Li, H., Chandran, S., Xia, X., Huang, Z., Chalkley, R. J., Burlingame, A. L., & Pearce, D. (2010). Phosphopeptide screen uncovers novel phosphorylation sites of Nedd4-2 that potentiate its inhibition of the epithelial Na+ channel. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M109.084731

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. In *Cell*. https://doi.org/10.1016/j.cell.2011.02.013

Harbeck, N., Penault-Llorca, F., Cortes, J., Gnant, M., Houssami, N., Poortmans, P., Ruddy,

K., Tsang, J., & Cardoso, F. (2019). Breast cancer. *Nature Reviews Disease Primers*. https://doi.org/10.1038/s41572-019-0111-2

Hartke, J., Johnson, M., & Ghabril, M. (2017). The diagnosis and treatment of hepatocellular carcinoma. *Seminars in Diagnostic Pathology*. https://doi.org/10.1053/j.semdp.2016.12.011

He, J., Wu, M., Xiong, L., Gong, Y., Yu, R., Peng, W., Li, L., Li, L., Tian, S., Wang, Y., Tao, Q., & Xiang, T. (2020). BTB/POZ zinc finger protein ZBTB16 inhibits breast cancer proliferation and metastasis through upregulating ZBTB28 and antagonizing BCL6/ZBTB27. *Clinical Epigenetics*. https://doi.org/10.1186/s13148-020-00867-9

He, M., Liu, S., Gallolu Kankanamalage, S., Borromeo, M. D., Girard, L., Gazdar, A. F., Minna, J. D., Johnson, J. E., & Cobb, M. H. (2018). The Epithelial Sodium Channel (αENaC) Is a Downstream Therapeutic Target of ASCL1 in Pulmonary Neuroendocrine Tumors. *Translational Oncology*. https://doi.org/10.1016/j.tranon.2018.01.004

He, S., Deng, J., Li, G., Wang, B., Cao, Y., & Tu, Y. (2012). Down-regulation of Nedd4L is associated with the aggressive progression and worse prognosis of malignant glioma. *Japanese Journal of Clinical Oncology*. https://doi.org/10.1093/jjco/hyr195

Henklova, P., Vrzal, R., Papouskova, B., Bednar, P., Jancova, P., Anzenbacherova, E., Ulrichova, J., Maurel, P., Pavek, P., & Dvorak, Z. (2008). SB203580, a pharmacological inhibitor of p38 MAP kinase transduction pathway activates ERK and JNK MAP kinases in primary cultures of human hepatocytes. *European Journal of Pharmacology*. https://doi.org/10.1016/j.ejphar.2008.07.007

Hermidorff, M. M., de Assis, L. V. M., & Isoldi, M. C. (2017). Genomic and rapid effects of aldosterone: what we know and do not know thus far. *Heart Failure Reviews*. https://doi.org/10.1007/s10741-016-9591-2

Hinds, L. R., Chun, L. E., Woodruff, E. R., Christensen, J. A., Hartsock, M. J., & Spencer, R. L. (2017). Dynamic glucocorticoid-dependent regulation of Sgk1 expression in oligodendrocytes of adult male rat brain by acute stress and time of day. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0175075

Ho, K. K., Myatt, S. S., & Lam, E. W. F. (2008). Many forks in the path: Cycling with FoxO. In *Oncogene*. https://doi.org/10.1038/onc.2008.23

Hodgson, R., Wildgust, H. J., & Bushe, C. J. (2010). Cancer and schizophrenia: is there a paradox? In *Journal of psychopharmacology (Oxford, England)*. https://doi.org/10.1177/1359786810385489

Holzman, J. L., Liu, L., Duke, B. J., Kemendy, A. E., & Eaton, D. C. (2007). Transactivation of the IGF-1R by aldosterone. *American Journal of Physiology - Renal Physiology*. https://doi.org/10.1152/ajprenal.00214.2006

Hong, F., Larrea, M. D., Doughty, C., Kwiatkowski, D. J., Squillace, R., & Slingerland, J. M. (2008). mTOR-Raptor Binds and Activates SGK1 to Regulate p27 Phosphorylation. *Molecular Cell*. https://doi.org/10.1016/j.molcel.2008.04.027

Hopp, T. A., Weiss, H. L., Hilsenbeck, S. G., Cui, Y., Allred, D. C., Horwitz, K. B., & Fuqua, S. A. W. (2004). Breast Cancer Patients with Progesterone Receptor PR-A-Rich Tumors Have Poorer Disease-Free Survival Rates. *Clinical Cancer Research*. https://doi.org/10.1158/1078-0432.CCR-03-0141

Hornsveld, M., Dansen, T. B., Derksen, P. W., & Burgering, B. M. T. (2018). Re-evaluating the role of FOXOs in cancer. In *Seminars in Cancer Biology*. https://doi.org/10.1016/j.semcancer.2017.11.017

Horwitz, K. B., Koseki, Y., & McGuire, W. L. (1978). Estrogen Control of Progesterone Receptor in Human Breast Cancer: Role of Estradiol and Antiestrogen. *Endocrinology*. https://doi.org/10.1210/endo-103-5-1742

Hou, W., Xia, H., Zhou, S., Fan, Z., Xu, H., Gong, Q., Nie, Y., Tang, Q., & Bi, F. (2019). The MEK inhibitors enhance the efficacy of sorafenib against hepatocellular carcinoma cells through reducing p-ERK rebound. *Translational Cancer Research*. https://doi.org/10.21037/tcr.2019.07.11

Hu, J., & Chen, Q. (2017). The role of glucocorticoid receptor in prostate cancer progression: from bench to bedside. In *International Urology and Nephrology*. https://doi.org/10.1007/s11255-016-1476-8

Hua, H., Zhang, H., Kong, Q., & Jiang, Y. (2018). Mechanisms for estrogen receptor expression in human cancer. *Experimental Hematology & Oncology*. https://doi.org/10.1186/s40164-018-0116-7

Huang, C., Lan, W., Fraunhoffer, N., Meilerman, A., Iovanna, J., & Santofimia-Castaño, P.

(2019). Dissecting the anticancer mechanism of trifluoperazine on pancreatic ductal adenocarcinoma. *Cancers*. https://doi.org/10.3390/cancers11121869

Huang, H., Regan, K. M., Lou, Z., Chen, J., & Tindall, D. J. (2006). CDK2-dependent phosphorylation of FOXO1 as an apoptotic response to DNA damage. *Science*. https://doi.org/10.1126/science.1130512

Huang, P., Chandra, V., & Rastinejad, F. (2009). Structural overview of the nuclear receptor superfamily: Insights into physiology and therapeutics. In *Annual Review of Physiology*. https://doi.org/10.1146/annurev-physiol-021909-135917

Huang, T. T., Lampert, E. J., Coots, C., & Lee, J. M. (2020). Targeting the PI3K pathway and DNA damage response as a therapeutic strategy in ovarian cancer. In *Cancer Treatment Reviews*. https://doi.org/10.1016/j.ctrv.2020.102021

Hui, L., Bakiri, L., Mairhorfer, A., Schweifer, N., Haslinger, C., Kenner, L., Komnenovic, V., Scheuch, H., Beug, H., & Wagner, E. F. (2007). p38α suppresses normal and cancer cell proliferation by antagonizing the JNK-c-Jun pathway. *Nature Genetics*. https://doi.org/10.1038/ng2033

Hui, L., Bakiri, L., Stepniak, E., & Wagner, E. F. (2007). p38α: A suppressor of cell proliferation and tumorigenesis. In *Cell Cycle*. https://doi.org/10.4161/cc.6.20.4774

Hunter, D. J., Colditz, G. A., Hankinson, S. E., Malspeis, S., Spiegelman, D., Chen, W., Stampfer, M. J., & Willett, W. C. (2010). Oral contraceptive use and breast cancer: A prospective study of young women. *Cancer Epidemiology Biomarkers and Prevention*. https://doi.org/10.1158/1055-9965.EPI-10-0747

Huynh, H., Ngo, V. C., Koong, H. N., Poon, D., Choo, S. P., Thng, C. H., Chow, P., Ong, H. S., Chung, A., & Soo, K. C. (2009). Sorafenib and rapamycin induce growth suppression in mouse models of hepatocellular carcinoma. *Journal of Cellular and Molecular Medicine*. https://doi.org/10.1111/j.1582-4934.2009.00692.x

Imamura, T., Oshima, Y., & Hikita, A. (2013). Regulation of TGF-β family signalling by ubiquitination and deubiquitination. *Journal of Biochemistry*. https://doi.org/10.1093/jb/mvt097

Inoki, K., Zhu, T., & Guan, K. L. (2003). TSC2 Mediates Cellular Energy Response to Control Cell Growth and Survival. *Cell*. https://doi.org/10.1016/S0092-8674(03)00929-2

Isaacs, C., Herbolsheimer, P., Liu, M. C., Wilkinson, M., Ottaviano, Y., Chung, G. G., Warren, R., Eng-Wong, J., Cohen, P., Smith, K. L., Creswell, K., Novielli, A., & Slack, R. (2011). Phase I/II study of sorafenib with anastrozole in patients with hormone receptor positive aromatase inhibitor resistant metastatic breast cancer. *Breast Cancer Research and Treatment*. https://doi.org/10.1007/s10549-010-1226-z

Itani, O. A., Liu, K. Z., Cornish, K. L., Campbell, J. R., & Thomas, C. P. (2002). Glucocorticoids stimulate human sgk1 gene expression by activation of a GRE in its 5′-flanking region. *American Journal of Physiology - Endocrinology and Metabolism*. https://doi.org/10.1152/ajpendo.00021.2002

Iyoda, K., Sasaki, Y., Horimoto, M., Toyama, T., Yakushijin, T., Sakakibara, M., Takehara, T., Fujimoto, J., Hori, M., Wands, J. R., & Hayashi, N. (2003). Involvement of the p38 mitogen-activated protein kinase cascade in hepatocellular carcinoma. *Cancer*. https://doi.org/10.1002/cncr.11425

Jääskeläinen, A., Jukkola, A., Haapasaari, K. M., Auvinen, P., Soini, Y., & Karihtala, P. (2019). Cytoplasmic Mineralocorticoid Receptor Expression Predicts Dismal Local Relapse-free Survival in Non-triple-negative Breast Cancer. *Anticancer Research*. https://doi.org/10.21873/anticanres.13792

Jackson, S. P., & Bartek, J. (2009). The DNA-damage response in human biology and disease. In *Nature*. https://doi.org/10.1038/nature08467

Jagirdar, R., Molyvdas, P. A., Gourgoulianis, K., Hatzoglou, C., & Zarogiannis, S. (2016). *The role of amiloride sensitive epithelial sodium channel (ENaC) in cell migration of mesothelioma cells is dependent on the cell type and the extracellular matrix*. https://doi.org/10.1183/13993003.congress-2016.pa5072

Jantzen, H. M., Strähle, U., Gloss, B., Stewart, F., Schmid, W., Boshart, M., Miksicek, R., & Schütz, G. (1987). Cooperativity of glucocorticoid response elements located far upstream of the tyrosine aminotransferase gene. *Cell*. https://doi.org/10.1016/0092-8674(87)90752-5

Jeong, Y., Xie, Y., Xiao, G., Behrens, C., Girard, L., Wistuba, I. I., Minna, J. D., & Mangelsdorf, D. J. (2010). Nuclear receptor expression defines a set of prognostic biomarkers for lung cancer. *PLoS Medicine*. https://doi.org/10.1371/journal.pmed.1000378

Ji, J., Sundquist, K., Ning, Y., Kendler, K. S., Sundquist, J., & Chen, X. (2013). Incidence of cancer in patients with schizophrenia and their first-degree relatives: A population-based study in Sweden. *Schizophrenia Bulletin*. https://doi.org/10.1093/schbul/sbs065

Jia, G., Jia, Y., & Sowers, J. R. (2017). Role of mineralocorticoid receptor activation in cardiac diastolic dysfunction. In *Biochimica et Biophysica Acta - Molecular Basis of Disease*. https://doi.org/10.1016/j.bbadis.2016.10.025

Jiang, J., Huang, Z., Chen, X., Luo, R., Cai, H., Wang, H., Zhang, H., Sun, T., & Zhang, Y. (2017). Trifluoperazine Activates FOXO1-Related Signals to Inhibit Tumor Growth in Hepatocellular Carcinoma. *DNA and Cell Biology*. https://doi.org/10.1089/dna.2017.3790

Jiang, W., Wu, D. B., Fu, S. Y., Chen, E. Q., Tang, H., & Zhou, T. Y. (2019). Insight into the role of TRAIL in liver diseases. In *Biomedicine and Pharmacotherapy*. https://doi.org/10.1016/j.biopha.2018.12.004

Jiang, X., Zhang, S., Yin, Z., Sheng, Y., Yan, Q., Sun, R., Lu, M., Zhang, Z., & Li, Y. (2019). The correlation between NEDD4L and HIF-1α levels as a gastric cancer prognostic marker. *International Journal of Medical Sciences*. https://doi.org/10.7150/ijms.34646

Jilkova, Z. M., Kuyucu, A. Z., Kurma, K., Ahmad Pour, S. T., Roth, G. S., Abbadessa, G., Yu, Y., Schwartz, B., Sturm, N., Marche, P. N., Hainaut, P., & Decaens, T. (2018). Combination of AKT inhibitor ARQ 092 and sorafenib potentiates inhibition of tumor progression in cirrhotic rat model of hepatocellular carcinoma. *Oncotarget*. https://doi.org/10.18632/oncotarget.24298

Jończyk, J., Godyń, J., Stawarska, E., Morak-Młodawska, B., Jeleń, M., Pluta, K., & Malawska, B. (2020). Dual Action of Dipyridothiazine and Quinobenzothiazine Derivatives-Anticancer and Cholinesterase-Inhibiting Activity. *Molecules (Basel, Switzerland)*. https://doi.org/10.3390/molecules25112604

Kapoor, N., Bartoszewski, R., Qadri, Y. J., Bebok, Z., Bublen, J. K., Fuller, C. M., & Benos, D. J. (2009). Knockdown of ASIC1 and epithelial sodium channel subunits inhibits glioblastoma whole cell current and cell migration. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M109.037390

Kapoor, N., Lee, W., Clark, E., Bartoszewski, R., McNicholas, C. M., Latham, C. B., Bebok, Z., Parpura, V., Fuller, C. M., Palmer, C. A., & Benos, D. J. (2011). Interaction of ASIC1 and ENaC subunits in human glioma cells and rat astrocytes. *American Journal of*

Physiology - Cell Physiology. https://doi.org/10.1152/ajpcell.00199.2010

Kassel, O., Sancono, A., Krätzschmar, J., Kreft, B., Stassen, M., & Cato, A. C. B. (2001). Glucocorticoids inhibit MAP kinase via increased expression and decreased degradation of MKP-1. *EMBO Journal*. https://doi.org/10.1093/emboj/20.24.7108

Katzenellenbogen, B. S. (2000). Mechanisms of action and cross-talk between estrogen receptor and progesterone receptor pathways. In *Journal of the Society for Gynecologic Investigation*. https://doi.org/10.1177/1071557600007001s10

King, S., Bray, S., Galbraith, S., Christie, L., & Fleming, S. (2014). Evidence for aldosterone-dependent growth of renal cell carcinoma. *International Journal of Experimental Pathology*. https://doi.org/10.1111/iep.12074

Kingsley-Kallesen, M., Mukhopadhyay, S. S., Wyszomierski, S. L., Schanler, S., Schütz, G., & Rosen, J. M. (2002). The mineralocorticoid receptor may compensate for the loss of the glucocorticoid receptor at specific stages of mammary gland development. *Molecular Endocrinology*. https://doi.org/10.1210/me.2002-0103

Kleyman, T. R., Myerburg, M. M., & Hughey, R. P. (2006). Regulation of ENaCs by proteases: An increasingly complex story. In *Kidney International*. https://doi.org/10.1038/sj.ki.5001860

Klinge, C. M. (2001). Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Research*. https://doi.org/10.1093/nar/29.14.2905

Knowlden, J. M., Hutcheson, I. R., Jones, H. E., Madden, T., Gee, J. M. W., Harper, M. E., Barrow, D., Wakeling, A. E., & Nicholson, R. I. (2003). Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells. *Endocrinology*. https://doi.org/10.1210/en.2002-220620

Knowles, B. B., Howe, C. C., & Aden, D. P. (1980). Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science*. https://doi.org/10.1126/science.6248960

Knutson, T. P., & Lange, C. A. (2014). Tracking progesterone receptor-mediated actions in breast cancer. In *Pharmacology and Therapeutics*. https://doi.org/10.1016/j.pharmthera.2013.11.010

Ko, Y., & Balk, S. (2005). Targeting Steroid Hormone Receptor Pathways in the Treatment of Hormone Dependent Cancers. *Current Pharmaceutical Biotechnology*. https://doi.org/10.2174/1389201043376616

Koch, K., Mansi, K., Haynes, E., Adams, C. E., Sampson, S., & Furtado, V. A. (2014). Trifluoperazine versus placebo for schizophrenia. In *Cochrane Database of Systematic Reviews*. https://doi.org/10.1002/14651858.CD010226.pub2

Kommagani, R., Szwarc, M. M., Vasquez, Y. M., Peavey, M. C., Mazur, E. C., Gibbons, W. E., Lanz, R. B., DeMayo, F. J., & Lydon, J. P. (2016). The Promyelocytic Leukemia Zinc Finger Transcription Factor Is Critical for Human Endometrial Stromal Cell Decidualization. *PLoS Genetics*. https://doi.org/10.1371/journal.pgen.1005937

Konu, O., & Targen, S. (2019). Investigating the Role of Mineralocorticoid Receptor Signaling in Cancer Biology in the Genomic Era. In *Aldosterone-Mineralocorticoid Receptor - Cell Biology to Translational Medicine*. https://doi.org/10.5772/intechopen.87233

Krishna, M., & Narang, H. (2008). The complexity of mitogen-activated protein kinases (MAPKs) made simple. In *Cellular and Molecular Life Sciences*. https://doi.org/10.1007/s00018-008-8170-7

Kruidering, M., & Evan, G. (2000). Caspase-8 in Apoptosis: The Beginning of "The End"? *IUBMB Life*. https://doi.org/10.1080/713803693

Kudo, M., Finn, R. S., Qin, S., Han, K. H., Ikeda, K., Piscaglia, F., Baron, A., Park, J. W., Han, G., Jassem, J., Blanc, J. F., Vogel, A., Komov, D., Evans, T. R. J., Lopez, C., Dutcus, C., Guo, M., Saito, K., Kraljevic, S., … Cheng, A. L. (2018). Lenvatinib versus sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. *The Lancet*. https://doi.org/10.1016/S0140-6736(18)30207-1

Kumar, B., Singh, S., Skvortsova, I., & Kumar, V. (2017). Promising Targets in Anti-cancer Drug Development: Recent Updates. *Current Medicinal Chemistry*. https://doi.org/10.2174/0929867324666170331123648

Kuo, K. L., Liu, S. H., Lin, W. C., Hsu, F. S., Chow, P. M., Chang, Y. W., Yang, S. P., Shi, C. S., Hsu, C. H., Liao, S. M., Chang, H. C., & Huang, K. H. (2019). Trifluoperazine, an antipsychotic drug, effectively reduces drug resistance in cisplatin-resistant urothelial carcinoma cells via suppressing bcl-xl: An in vitro and in vivo study. *International Journal of Molecular Sciences*. https://doi.org/10.3390/ijms20133218

Kurosu, T., Nagao, T., Wu, N., Oshikawa, G., & Miura, O. (2013). Inhibition of the PI3K/Akt/GSK3 pathway downstream of BCR/ABL, Jak2-V617F, or FLT3-ITD downregulates DNA damage-induced Chk1 activation as well as G2/M arrest and prominently enhances induction of apoptosis. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0079478

Lam, M., Lawrence, D. A., Ashkenazi, A., & Walter, P. (2018). Confirming a critical role for death receptor 5 and caspase-8 in apoptosis induction by endoplasmic reticulum stress. In *Cell Death and Differentiation*. https://doi.org/10.1038/s41418-018-0155-y

Lang, F., Perrotti, N., & Stournaras, C. (2010). Colorectal carcinoma cells-Regulation of survival and growth by SGK1. In *International Journal of Biochemistry and Cell Biology*. https://doi.org/10.1016/j.biocel.2010.05.016

Lea, S., Li, J., Plumb, J., Gaffey, K., Mason, S., Gaskell, R., Harbron, C., & Singh, D. (2020). P38 MAPK and glucocorticoid receptor crosstalk in bronchial epithelial cells. *Journal of Molecular Medicine*. https://doi.org/10.1007/s00109-020-01873-3

Lee, M. S., Johansen, L., Zhang, Y., Wilson, A., Keegan, M., Avery, W., Elliott, P., Borisy, A. A., & Keith, C. T. (2007). The novel combination of chlorpromazine and pentamidine exerts synergistic antiproliferative effects through dual mitotic action. *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-07-2235

Lee, W. Y., Lee, W. T., Cheng, C. H., Chen, K. C., Chou, C. M., Chung, C. H., Sun, M. S., Cheng, H. W., Ho, M. N., & Lin, C. W. (2015). Repositioning antipsychotic chlorpromazine for treating colorectal cancer by inhibiting sirtuin 1. *Oncotarget*. https://doi.org/10.18632/oncotarget.4768

Lee, Y. S., Kim, J. A., Kim, K. L., Jang, H. S., Kim, J. M., Lee, J. Y., Shin, I. S., Lee, J. S., Suh, W., Choi, J. H., Jeon, E. S., Byun, J., & Kim, D. K. (2004). Aldosterone upregulates connective tissue growth factor gene expression via p38 MAPK pathway and mineralocorticoid receptor in ventricular myocytes. *Journal of Korean Medical Science*. https://doi.org/10.3346/jkms.2004.19.6.805

Leo, J. C., Guo, C., Woon, C. T., Aw, S. E., & Lin, V. C. (2004). Glucocorticoid and mineralocorticoid cross-talk with progesterone receptor to induce focal adhesion and growth inhibition in breast cancer cells. *Endocrinology*, *145*(3), 1314–1321.

Leo, J. C. L., Guo, C., Woon, C. T., Aw, S. E., & Lin, V. C. L. (2004). Glucocorticoid and

Mineralocorticoid Cross-Talk with Progesterone Receptor to Induce Focal Adhesion and Growth Inhibition in Breast Cancer Cells. *Endocrinology*. https://doi.org/10.1210/en.2003-0732

Li, D., Lu, Z., Xu, Z., Ji, J., Zheng, Z., Lin, S., & Yan, T. (2016). Spironolactone promotes autophagy via inhibiting PI3K/AKT/mTOR signalling pathway and reduce adhesive capacity damage in podocytes under mechanical stress. *Bioscience Reports*. https://doi.org/10.1042/BSR20160086

Li, H., Qian, W., Weng, X., Wu, Z., Li, H., Zhuang, Q., Feng, B., & Bian, Y. (2012). Glucocorticoid receptor and sequential P53 activation by Dexamethasone mediates apoptosis and cell cycle arrest of Osteoblastic MC3T3-E1 cells. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0037030

Li, M., Wang, W., Dan, Y., Tong, Z., Chen, W., Qin, L., Liu, K., Li, W., Mo, P., & Yu, C. (2016). Downregulation of amplified in breast cancer 1 contributes to the anti-tumor effects of sorafenib on human hepatocellular carcinoma. *Oncotarget*. https://doi.org/10.18632/oncotarget.8812

Li, P., Zhou, L., Zhao, T., Liu, X., Zhang, P., Liu, Y., Zheng, X., & Li, Q. (2017). Caspase-9: Structure, mechanisms and clinical application. In *Oncotarget*. https://doi.org/10.18632/oncotarget.15098

Liang, J., & Shang, Y. (2013). Estrogen and cancer. In *Annual Review of Physiology*. https://doi.org/10.1146/annurev-physiol-030212-183708

Liu, C. Y., Chen, K. F., & Chen, P. J. (2015). Treatment of liver cancer. *Cold Spring Harbor Perspectives in Medicine*. https://doi.org/10.1101/cshperspect.a021535

Liu, C., Zhu, L. L., Xu, S. G., Ji, H. L., & Li, X. M. (2016). ENaC/DEG in tumor development and progression. In *Journal of Cancer*. https://doi.org/10.7150/jca.15693

Liu, G., Honisch, S., Liu, G., Schmidt, S., Pantelakos, S., Alkahtani, S., Toulany, M., Lang, F., & Stournaras, C. (2015). Inhibition of SGK1 enhances mAR-induced apoptosis in MCF-7 breast cancer cells. *Cancer Biology & Therapy*. https://doi.org/10.4161/15384047.2014.986982

Liu, H., Zhang, L., Zhang, X., & Cui, Z. (2017). PI3k/AKT/mTOR pathway promotes progestin resistance in endometrial cancer cells by inhibition of autophagy. *OncoTargets and*

Therapy. https://doi.org/10.2147/OTT.S95267

Liu, L., Cao, Y., Chen, C., Zhang, X., McNabola, A., Wilkie, D., Wilhelm, S., Lynch, M., & Carter, C. (2006). Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-06-1377

Liu, M. M., Albanese, C., Anderson, C. M., Hilty, K., Webb, P., Uht, R. M., Price, R. H., Pestell, R. G., & Kushn, P. J. (2002). Opposing action of estrogen receptors α and β on cyclin D1 gene expression. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M201829200

Liu, R., Page, C., Beidler, D. R., Wicha, M. S., & Núñez, G. (1999). Overexpression of Bcl-X(L) promotes chemotherapy resistance of mammary tumors in a syngeneic mouse model. *American Journal of Pathology*. https://doi.org/10.1016/S0002-9440(10)65505-8

Liu, S., Han, Y., Zhang, T., & Yang, Z. (2011). Protective effect of trifluoperazine on hydrogen peroxide-induced apoptosis in PC12 cells. *Brain Research Bulletin*. https://doi.org/10.1016/j.brainresbull.2010.12.008

Liu, W., Wang, J., Sauter, N. K., & Pearce, D. (1995). Steroid receptor heterodimerization demonstrated in vitro and in vivo. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.92.26.12480

Liu, Y. S., Tsai, Y. L., Yeh, Y. L., Chung, L. C., Wen, S. Y., Kuo, C. H., Lin, Y. M., Vijaya Padma, V., Bharath Kumar, V., & Huang, C. Y. (2015). Cell cycle regulation in the estrogen receptor beta (ESR2)-overexpressing Hep3B hepatocellular carcinoma cell line. *Chinese Journal of Physiology*. https://doi.org/10.4077/CJP.2015.BAC239

Llovet, J. M., Zucman-Rossi, J., Pikarsky, E., Sangro, B., Schwartz, M., Sherman, M., & Gores, G. (2016). Hepatocellular carcinoma. *Nature Reviews Disease Primers*. https://doi.org/10.1038/nrdp.2016.18

Loffing-Cueni, D., Flores, S. Y., Sauter, D., Daidié, D., Siegrist, N., Meneton, P., Staub, O., & Loffing, J. (2006). Dietary sodium intake regulates the ubiquitin-protein ligase Nedd4-2 in the renal collecting system. *Journal of the American Society of Nephrology*. https://doi.org/10.1681/ASN.2005060659

Lohitesh, K., Chowdhury, R., & Mukherjee, S. (2018). Resistance a major hindrance to

chemotherapy in hepatocellular carcinoma: An insight. In *Cancer Cell International*. https://doi.org/10.1186/s12935-018-0538-7

Lu, M., Li, J., Luo, Z., Zhang, S., Xue, S., Wang, K., Shi, Y., Zhang, C., Chen, H., & Li, Z. (2015). Roles of dopamine receptors and their antagonist thioridazine in hepatoma metastasis. *OncoTargets and Therapy*. https://doi.org/10.2147/OTT.S77373

Luo, Y., Rockow-Magnone, S. K., Kroeger, P. E., Frost, L., Chen, Z., Han, E. K. H., Ng, S. C., Simmer, R. L., & Giranda, V. L. (2001). Blocking CHK1 expression induces apoptosis and abrogates the G2 checkpoint mechanism. *Neoplasia*. https://doi.org/10.1038/sj.neo.7900175

Lurje, I., Czigany, Z., Bednarsch, J., Roderburg, C., Isfort, P., Neumann, U. P., & Lurje, G. (2019). Treatment strategies for hepatocellular carcinoma—A multidisciplinary approach. In *International Journal of Molecular Sciences*. https://doi.org/10.3390/ijms20061465

MacCorkle, R. A., & Tan, T. H. (2005). Mitogen-activated protein kinases in cell-cycle control. In *Cell Biochemistry and Biophysics*. https://doi.org/10.1385/CBB:43:3:451

Madeira, M., Mattar, A., Logullo, Â. F., Soares, F. A., & Gebrim, L. H. (2013). Estrogen receptor alpha/beta ratio and estrogen receptor beta as predictors of endocrine therapy responsiveness-a randomized neoadjuvant trial comparison between anastrozole and tamoxifen for the treatment of postmenopausal breast cancer. *BMC Cancer*. https://doi.org/10.1186/1471-2407-13-425

Magnusson, K., Gremel, G., Rydén, L., Pontén, V., Uhlén, M., Dimberg, A., Jirström, K., & Pontén, F. (2016). ANLN is a prognostic biomarker independent of Ki-67 and essential for cell cycle progression in primary breast cancer. *BMC Cancer*. https://doi.org/10.1186/s12885-016-2923-8

Mah, L. J., El-Osta, A., & Karagiannis, T. C. (2010). γh2AX: A sensitive molecular marker of DNA damage and repair. In *Leukemia*. https://doi.org/10.1038/leu.2010.6

Malhotra, G. K., Zhao, X., Band, H., & Band, V. (2010). Histological, molecular and functional subtypes of breast cancers. In *Cancer Biology and Therapy*. https://doi.org/10.4161/cbt.10.10.13879

Mandal, R., Raab, M., Matthess, Y., Becker, S., Knecht, R., & Strebhardt, K. (2014). PERK 1/2 inhibit Caspase-8 induced apoptosis in cancer cells by phosphorylating it in a cell cycle

specific manner. *Molecular Oncology*. https://doi.org/10.1016/j.molonc.2013.11.003

Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., & Evans, R. M. (1995). The nuclear receptor superfamily: The second decade. *Cell*. https://doi.org/10.1016/0092-8674(95)90199-X

Marzolla, V., Armani, A., Feraco, A., De Martino, M. U., Fabbri, A., Rosano, G., & Caprio, M. (2014). Mineralocorticoid receptor in adipocytes and macrophages: A promising target to fight metabolic syndrome. In *Steroids*. https://doi.org/10.1016/j.steroids.2014.05.001

Matsuda, Y., Wakai, T., Kubota, M., Osawa, M., Takamura, M., Yamagiwa, S., Aoyagi, Y., Sanpei, A., & Fujimaki, S. (2013). DNA damage sensor γ-H2AX is increased in preneoplastic lesions of hepatocellular carcinoma. *The Scientific World Journal*. https://doi.org/10.1155/2013/597095

Matter, M. S., Decaens, T., Andersen, J. B., & Thorgeirsson, S. S. (2014). Targeting the mTOR pathway in hepatocellular carcinoma: Current state and future trends. In *Journal of Hepatology*. https://doi.org/10.1016/j.jhep.2013.11.031

McBeth, L., Nwaneri, A. C., Grabnar, M., Demeter, J., Nestor-Kalinoski, A., & Hinds, T. D. (2016). Glucocorticoid receptor beta increases migration of human bladder cancer cells. *Oncotarget*. https://doi.org/10.18632/oncotarget.8430

McCann, K. E., Lustberg, D. J., Shaughnessy, E. K., Carstens, K. E., Farris, S., Alexander, G. M., Radzicki, D., Zhao, M., & Dudek, S. M. (2019). Novel role for mineralocorticoid receptors in control of a neuronal phenotype. *Molecular Psychiatry*. https://doi.org/10.1038/s41380-019-0598-7

McComb, S., Chan, P. K., Guinot, A., Hartmannsdottir, H., Jenni, S., Dobay, M. P., Bourquin, J. P., & Bornhauser, B. C. (2019). Efficient apoptosis requires feedback amplification of upstream apoptotic signals by effector caspase-3 or -7. *Science Advances*. https://doi.org/10.1126/sciadv.aau9433

McDonnell, D. P., Shahbaz, M. M., Vegeto, E., & Goldman, M. E. (1994). The human progesterone receptor A-form functions as a transcriptional modulator of mineralocorticoid receptor transcriptional acitivity. *Journal of Steroid Biochemistry and Molecular Biology*. https://doi.org/10.1016/0960-0760(94)90190-2

McFall, T., McKnight, B., Rosati, R., Kim, S., Huang, Y., Viola-Villegas, N., & Ratnam, M.

(2018). Progesterone receptor A promotes invasiveness and metastasis of luminal breast cancer by suppressing regulation of critical microRNAs by estrogen. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M117.812438

McTavish, N., Getty, J., Burchell, A., & Wilson, S. M. (2009). Glucocorticoids can activate the α-ENaC gene promoter independently of SGK1. *Biochemical Journal*. https://doi.org/10.1042/BJ20090366

Mehta, A. (2008). Cystic fibrosis as a bowel cancer syndrome and the potential role of CK2. *Molecular and Cellular Biochemistry*. https://doi.org/10.1007/s11010-008-9815-4

Meuth, M. (2010). Chk1 suppressed cell death. In *Cell Division*. https://doi.org/10.1186/1747-1028-5-21

Michels, J., Kepp, O., Senovilla, L., Lissa, D., Castedo, M., Kroemer, G., & Galluzzi, L. (2013). Functions of BCL-XL at the interface between cell death and metabolism. In *International Journal of Cell Biology*. https://doi.org/10.1155/2013/705294

Michlig, S., Harris, M., Loffing, J., Rossier, B. C., & Firsov, D. (2005). Progesterone down-regulates the open probability of the amiloride-sensitive epithelial sodium channel via a Nedd4-2-dependent mechanism. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M506308200

Miller, A. L., Garza, A. S., Johnson, B. H., & Thompson, E. B. (2007). Pathway interactions between MAPKs, mTOR, PKA, and the glucocorticoid receptor in lymphoid cells. *Cancer Cell International*. https://doi.org/10.1186/1475-2867-7-3

Mirshahi, M., Mirshahi, S., Golestaneh, N., Nicolas, C., Mishal, Z., & Agarwal, M. K. (1998). Mineralocorticoid hormone receptor and the epithelial sodium channel in a human leukemic cell line. *Endocrine Research*. https://doi.org/10.3109/07435809809032633

Moeini, A., Cornellà, H., & Villanueva, A. (2012). Emerging Signaling Pathways in Hepatocellular Carcinoma Introduction: Mainstream Pathway De-regulation in HCC. *Liver Cancer*.

Mohammed, H., Russell, I. A., Stark, R., Rueda, O. M., Hickey, T. E., Tarulli, G. A., Serandour, A. A. A., Birrell, S. N., Bruna, A., Saadi, A., Menon, S., Hadfield, J., Pugh, M., Raj, G. V., Brown, G. D., D'Santos, C., Robinson, J. L. L., Silva, G., Launchbury, R., … Carroll, J. S. (2015). Progesterone receptor modulates ERα action in breast cancer. *Nature*.

https://doi.org/10.1038/nature14583

Monsivais, D., Dyson, M. T., Yin, P., Navarro, A., Coon, J. S., Pavone, M. E., & Bulun, S. E. (2016). Estrogen receptor β regulates endometriotic cell survival through serum and glucocorticoid-regulated kinase activation. *Fertility and Sterility*. https://doi.org/10.1016/j.fertnstert.2016.01.012

Moore, N. L., Hickey, T. E., Butler, L. M., & Tilley, W. D. (2012). Multiple nuclear receptor signaling pathways mediate the actions of synthetic progestins in target cells. In *Molecular and Cellular Endocrinology*. https://doi.org/10.1016/j.mce.2011.09.019

Muehlfelder, M., Arias-Loza, P. A., Fritzemeier, K. H., & Pelzer, T. (2012). Both estrogen receptor subtypes, ERα and ERβ, prevent aldosterone-induced oxidative stress in VSMC via increased NADPH bioavailability. *Biochemical and Biophysical Research Communications*. https://doi.org/10.1016/j.bbrc.2012.06.053

Münzberg, C., Hellerbrand, C., Seufferlein, T., Gekle, M., & Dollinger, M. (2015). Aldosterone stimulates the non-genomic signaling pathway of the mineralocorticoid receptor in hepatic stellate cells. *Zeitschrift Für Gastroenterologie*. https://doi.org/10.1055/s-0035-1559131

Murugan, A. K. (2019). mTOR: Role in cancer, metastasis and drug resistance. In *Seminars in Cancer Biology*. https://doi.org/10.1016/j.semcancer.2019.07.003

Myles, K., & Funder, J. W. (1996). Progesterone binding to mineralocorticoid receptors: In vitro and in vivo studies. *American Journal of Physiology - Endocrinology and Metabolism*. https://doi.org/10.1152/ajpendo.1996.270.4.e601

Nagase, M., Ayuzawa, N., Kawarazaki, W., Ishizawa, K., Ueda, K., Yoshida, S., & Fujita, T. (2012). Oxidative stress causes mineralocorticoid receptor activation in rat cardiomyocytes: Role of small GTPase Rac1. *Hypertension*. https://doi.org/10.1161/HYPERTENSIONAHA.111.185520

Nagasue, N., Kohno, H., Yamanoi, A., Kimoto, T., Chang, Y. - C, & Nakamura, T. (1991). Progesterone receptor in hepatocellular carcinoma. Correlation with androgen and estrogen receptors. *Cancer*. https://doi.org/10.1002/1097-0142(19910515)67:10<2501::AID-CNCR2820671019>3.0.CO;2-N

Nagata, S. (2018). Apoptosis and Clearance of Apoptotic Cells. *Annual Review of Immunology*. https://doi.org/10.1146/annurev-immunol-042617-053010

Nagata, Y., Goto, T., & Miyamoto, H. (2019). The Role of Mineralocorticoid Receptor Signaling in Genitourinary Cancers. *Nuclear Receptor Research*. https://doi.org/10.32527/2019/101410

Nagoshi, T., Date, T., Fujisaki, M., Yoshino, T., Sekiyama, H., Ogawa, K., Kayama, Y., Minai, K., Komukai, K., Ogawa, T., & Yoshimura, M. (2012). Biphasic action of aldosterone on akt signaling in cardiomyocytes. *Hormone and Metabolic Research*. https://doi.org/10.1055/s-0032-1316343

Narayanan, R., Edwards, D. P., & Weigel, N. L. (2005). Human Progesterone Receptor Displays Cell Cycle-Dependent Changes in Transcriptional Activity. *Molecular and Cellular Biology*. https://doi.org/10.1128/mcb.25.8.2885-2898.2005

Narayanan, S., Srinivas, S., & Feldman, D. (2016). Androgen-glucocorticoid interactions in the era of novel prostate cancer therapy. In *Nature Reviews Urology*. https://doi.org/10.1038/nrurol.2015.254

Need, E. F., Selth, L. A., Harris, T. J., Birrell, S. N., Tilley, W. D., & Buchanan, G. (2012). Research resource: Interplay between the genomic and transcriptional networks of androgen receptor and estrogen receptor α in luminal breast cancer cells. *Molecular Endocrinology*. https://doi.org/10.1210/me.2011-1314

Nie, H., Li, J., Yang, X. M., Cao, Q. Z., Feng, M. X., Xue, F., Wei, L., Qin, W., Gu, J., Xia, Q., & Zhang, Z. G. (2015). Mineralocorticoid receptor suppresses cancer progression and the Warburg effect by modulating the miR-338-3p-PKLR axis in hepatocellular carcinoma. *Hepatology*. https://doi.org/10.1002/hep.27940

Niisato, N., Taruno, A., & Marunaka, Y. (2007). Involvement of p38 MAPK in hypotonic stress-induced stimulation of β- and γ-ENaC expression in renal epithelium. *Biochemical and Biophysical Research Communications*. https://doi.org/10.1016/j.bbrc.2007.04.192

Nikoletopoulou, V., Markaki, M., Palikaras, K., & Tavernarakis, N. (2013). Crosstalk between apoptosis, necrosis and autophagy. In *Biochimica et Biophysica Acta - Molecular Cell Research*. https://doi.org/10.1016/j.bbamcr.2013.06.001

Nilsson, S., & Gustafsson, J. Å. (2011). Estrogen receptors: Therapies targeted to receptor subtypes. *Clinical Pharmacology and Therapeutics*. https://doi.org/10.1038/clpt.2010.226

Ning, Y. M., & Sánchez, E. R. (1995). Evidence for a functional interaction between calmodulin

and the glucocorticoid receptor. *Biochemical and Biophysical Research Communications*. https://doi.org/10.1006/bbrc.1995.1303

Novella, S., Heras, M., Hermenegildo, C., & Dantas, A. P. (2012). Effects of estrogen on vascular inflammation: A matter of timing. *Arteriosclerosis, Thrombosis, and Vascular Biology*. https://doi.org/10.1161/ATVBAHA.112.250308

Ohnishi, S., Murakami, T., Moriyama, T., Mitamura, K., & Imawari, M. (1986). Androgen and estrogen receptors in hepatocellular carcinoma and in the surrounding noncancerous liver tissue. *Hepatology*. https://doi.org/10.1002/hep.1840060320

Omoruyi, S. I., Ekpo, O. E., Semenya, D. M., Jardine, A., & Prince, S. (2020). Exploitation of a novel phenothiazine derivative for its anti-cancer activities in malignant glioblastoma. *Apoptosis*. https://doi.org/10.1007/s10495-020-01594-5

Ong, G. S. Y., & Young, M. J. (2017a). Mineralocorticoid regulation of cell function: The role of rapid signalling and gene transcription pathways. In *Journal of Molecular Endocrinology*. https://doi.org/10.1530/JME-15-0318

Ong, G. S. Y., & Young, M. J. (2017b). Mineralocorticoid regulation of cell function: The role of rapid signalling and gene transcription pathways. In *Journal of Molecular Endocrinology*. https://doi.org/10.1530/JME-15-0318

Orlacchio, A., Ranieri, M., Brave, M., Arciuch, V. A., Forde, T., De Martino, D., Anderson, K. E., Hawkins, P., & Di Cristofano, A. (2017). SGK1 is a critical component of an AKT-independent pathway essential for PI3K-mediated tumor development and maintenance. *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-17-2105

Osborne, C. K., Shou, J., Massarweh, S., Schiff, R., Come, S., Santen, R., & Johnston, S. (2005). Crosstalk between estrogen receptor and growth factor receptor pathways as a cause for endocrine therapy resistance in breast cancer. *Clinical Cancer Research*.

Otręba Michałand Pajor, M., & Warncke, J. D. (2019). Antimelanoma activity of perphenazine and prochlorperazine in human COLO829 and C32 cell lines. *Naunyn-Schmiedeberg's Archives of Pharmacology*. https://doi.org/10.1007/s00210-019-01668-5

Otto, T., & Sicinski, P. (2017). Cell cycle proteins as promising targets in cancer therapy. In *Nature Reviews Cancer*. https://doi.org/10.1038/nrc.2016.138

Pan, C., Jin, X., Zhao, Y., Pan, Y., Yang, J., Karnes, R. J., Zhang, J., Wang, L., & Huang, H.

(2017). AKT - phosphorylated FOXO 1 suppresses ERK activation and chemoresistance by disrupting IQGAP 1- MAPK interaction . *The EMBO Journal*. https://doi.org/10.15252/embj.201695534

Pan, D., Kocherginsky, M., & Conzen, S. D. (2011). Activation of the glucocorticoid receptor is associated with poor prognosis in estrogen receptor-negative breast cancer. *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-11-0362

Parker, B. M., Wertz, S. L., Pollard, C. M., Desimine, V. L., Maning, J., McCrink, K. A., & Lymperopoulos, A. (2018). Novel insights into the crosstalk between mineralocorticoid receptor and G protein-coupled receptors in heart adverse remodeling and disease. In *International Journal of Molecular Sciences*. https://doi.org/10.3390/ijms19123764

Patel, H. K., & Bihani, T. (2018). Selective estrogen receptor modulators (SERMs) and selective estrogen receptor degraders (SERDs) in cancer treatment. In *Pharmacology and Therapeutics*. https://doi.org/10.1016/j.pharmthera.2017.12.012

Pearce, D., & Kleyman, T. R. (2007). Salt, sodium channels, and SGK1. In *Journal of Clinical Investigation*. https://doi.org/10.1172/JCI31538

Pearce, L. R., Komander, D., & Alessi, D. R. (2010). The nuts and bolts of AGC protein kinases. In *Nature Reviews Molecular Cell Biology*. https://doi.org/10.1038/nrm2822

Pedersen, A. M., Thrane, S., Lykesfeldt, A. E., & Yde, C. W. (2014). Sorafenib and nilotinib resensitize tamoxifen resistant breast cancer cells to tamoxifen treatment via estrogen receptor $α$. *International Journal of Oncology*. https://doi.org/10.3892/ijo.2014.2619

Pelden, S., Insawang, T., Thuwajit, C., & Thuwajit, P. (2013). The trefoil factor 1 (TFF1) protein involved in doxorubicin-induced apoptosis resistance is upregulated by estrogen in breast cancer cells. *Oncology Reports*. https://doi.org/10.3892/or.2013.2593

Perez Kerkvliet, C., Dwyer, A. R., Diep, C. H., Oakley, R. H., Liddle, C., Cidlowski, J. A., & Lange, C. A. (2020). Glucocorticoid receptors are required effectors of TGFβ1-induced p38 MAPK signaling to advanced cancer phenotypes in triple-negative breast cancer. *Breast Cancer Research*. https://doi.org/10.1186/s13058-020-01277-8

Perlman, H., Zhang, X., Chen, M. W., Walsh, K., & Buttyan, R. (1999). An elevated bax/bcl-2 ratio corresponds with the onset of prostate epithelial cell apoptosis. *Cell Death and Differentiation*. https://doi.org/10.1038/sj.cdd.4400453

Personeni, N., Pressiani, T., Santoro, A., & Rimassa, L. (2018). Regorafenib in hepatocellular carcinoma: Latest evidence and clinical implications. In *Drugs in Context*. https://doi.org/10.7573/dic.212533

Petta, I., Dejager, L., Ballegeer, M., Lievens, S., Tavernier, J., De Bosscher, K., & Libert, C. (2016). The Interactome of the Glucocorticoid Receptor and Its Influence on the Actions of Glucocorticoids in Combatting Inflammatory and Infectious Diseases. *Microbiology and Molecular Biology Reviews*. https://doi.org/10.1128/mmbr.00064-15

Pignochino, Y., Dell'Aglio, C., Inghilleri, S., Zorzetto, M., Basiricò, M., Capozzi, F., Canta, M., Piloni, D., Cemmi, F., Sangiolo, D., Gammaitoni, L., Soster, M., Marchiò, S., Pozzi, E., Morbini, P., Luisetti, M., Aglietta, M., Grignani, G., & Stella, G. M. (2015). The combination of sorafenib and everolimus shows antitumor activity in preclinical models of malignant pleural mesothelioma. *BMC Cancer*. https://doi.org/10.1186/s12885-015-1363-1

Pilbrow, A. P., Ellmers, L. J., Black, M. A., Moravec, C. S., Sweet, W. E., Troughton, R. W., Richards, A. M., Frampton, C. M., & Cameron, V. A. (2008). Genomic selection of reference genes for real-time PCR in human myocardium. *BMC Medical Genomics*. https://doi.org/10.1186/1755-8794-1-64

Piovan, E., Yu, J., Tosello, V., Herranz, D., Ambesi-Impiombato, A., DaSilva, A. C., Sanchez-Martin, M., Perez-Garcia, A., Rigo, I., Castillo, M., Indraccolo, S., Cross, J. R., deStanchina, E., Paietta, E., Racevskis, J., Rowe, J. M., Tallman, M. S., Basso, G., Meijerink, J. P., … Ferrando, A. A. (2013). Direct Reversal of Glucocorticoid Resistance by AKT Inhibition in Acute Lymphoblastic Leukemia. *Cancer Cell*. https://doi.org/10.1016/j.ccr.2013.10.022

Pistritto, G., Trisciuoglio, D., Ceci, C., Alessia Garufi, & D'Orazi, G. (2016). Apoptosis as anticancer mechanism: Function and dysfunction of its modulators and targeted therapeutic strategies. In *Aging*. https://doi.org/10.18632/aging.100934

Planey, S. L., & Litwack, G. (2000). Glucocorticoid-induced apoptosis in lymphocytes. In *Biochemical and Biophysical Research Communications*. https://doi.org/10.1006/bbrc.2000.3922

Planey, Sonia L., Derfoul, A., Steplewski, A., Robertson, N. M., & Litwack, G. (2002). Inhibition of glucocorticoid-induced apoptosis in 697 pre-B lymphocytes by the

mineralocorticoid receptor N-terminal domain. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M205085200

Polischouk, A. G., Holgersson, Å., Zong, D., Stenerlöw, B., Karlsson, H. L., Möller, L., Viktorsson, K., & Lewensohn, R. (2007). The antipsychotic drug trifluoperazine inhibits DNA repair and sensitizes non-small cell lung carcinoma cells to DNA double-strand break-induced cell death. *Molecular Cancer Therapeutics*. https://doi.org/10.1158/1535-7163.MCT-06-0402

Pommier, Y., Sordet, O., Antony, S., Hayward, R. L., & Kohn, K. W. (2004). Apoptosis defects and chemotherapy resistance: Molecular interaction maps and networks. In *Oncogene*. https://doi.org/10.1038/sj.onc.1207515

Pooley, J. R., Rivers, C. A., Kilcooley, M. T., Paul, S. N., Cavga, A. D., Kershaw, Y. M., Muratcioglu, S., Gursoy, A., Keskin, O., & Lightman, S. L. (2020). Beyond the heterodimer model for mineralocorticoid and glucocorticoid receptor interactions in nuclei and at DNA. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0227520

Porta, C., Paglino, C., & Mosca, A. (2014). Targeting PI3K/Akt/mTOR signaling in cancer. In *Frontiers in Oncology*. https://doi.org/10.3389/fonc.2014.00064

Prahallad, A., & Bernards, R. (2016). Opportunities and challenges provided by crosstalk between signalling pathways in cancer. In *Oncogene*. https://doi.org/10.1038/onc.2015.151

Prekovic, S., Schuurman, K., Manjón, A. G., Buijs, M., Peralta, I. M., Wellenstein, M. D., Yavuz, S., Barrera, A., Monkhorst, K., Huber, A., Morris, B., Lieftink, C., Silva, J., Győrffy, B., Hoekman, L., van den Broek, B., Teunissen, H., Reddy, T., Faller, W., … Zwart, W. (2019). Glucocorticoids regulate cancer cell dormancy. In *bioRxiv*. https://doi.org/10.1101/750406

Prossnitz, E. R., & Arterburn, J. B. (2015). International union of basic and clinical pharmacology. XCVII. G protein–coupled estrogen receptor and its pharmacologic modulators. *Pharmacological Reviews*. https://doi.org/10.1124/pr.114.009712

Prossnitz, E. R., & Barton, M. (2014). Estrogen biology: New insights into GPER function and clinical opportunities. In *Molecular and Cellular Endocrinology*. https://doi.org/10.1016/j.mce.2014.02.002

Psarra, A. M. G., Solakidi, S., Trougakos, I. P., Margaritis, L. H., Spyrou, G., & Sekeris, C. E. (2005). Glucocorticoid receptor isoforms in human hepatocarcinoma HepG2 and SaOS-2 osteosarcoma cells: Presence of glucocorticoid receptor alpha in mitochondria and of glucocorticoid receptor beta in nucleoli. *International Journal of Biochemistry and Cell Biology*. https://doi.org/10.1016/j.biocel.2005.06.015

Pucci, C., Martinelli, C., & Ciofani, G. (2019). Innovative approaches for cancer treatment: Current perspectives and new challenges. In *ecancermedicalscience*. https://doi.org/10.3332/ecancer.2019.961

Pufall, M. A. (2015). Glucocorticoids and cancer. *Advances in Experimental Medicine and Biology*. https://doi.org/10.1007/978-1-4939-2895-8_14

Pulkoski-Gross, A., Li, J., Zheng, C., Li, Y., Ouyang, N., Rigas, B., Zucker, S., & Cao, J. (2015). Repurposing the antipsychotic trifluoperazine as an antimetastasis agent. *Molecular Pharmacology*. https://doi.org/10.1124/mol.114.096941

Qi, D., He, J., Wang, D., Deng, W., Zhao, Y., Ye, Y., & Feng, L. (2014). 17β-estradiol suppresses lipopolysaccharide-induced acute lung injury through PI3K/Akt/SGK1 mediated up-regulation of epithelial sodium channel (ENaC) in vivo and in vitro. *Respiratory Research*. https://doi.org/10.1186/s12931-014-0159-1

Qi, H., Grenier, J., Fournier, A., & Labrie, C. (2003). Androgens differentially regulate the expression of NEDD4L transcripts in LNCaP human prostate cancer cells. *Molecular and Cellular Endocrinology*. https://doi.org/10.1016/j.mce.2003.08.009

Queisser, N., Schupp, N., Schwarz, E., Hartmann, C., Mackenzie, G. G., & Oteiza, P. I. (2017). Aldosterone activates the oncogenic signals ERK1/2 and STAT3 via redox-regulated mechanisms. *Molecular Carcinogenesis*. https://doi.org/10.1002/mc.22643

Rao, X., Huang, X., Zhou, Z., & Lin, X. (2013). An improvement of the 2ˆ(-delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. *Biostatistics, Bioinformatics and Biomathematics*.

Raynaud, J. P., & Rolland, P. H. (1981). Macromolecular binding of dexamethasone as evidence for the presence of mineralocorticoid receptor in human breast cancer. *Cancer Research*.

Regan-Fendt, K., Li, D., Reyes, R., Yu, L., Wani, N. A., Hu, P., Jacob, S. T., Ghoshal, K.,

Payne, P. R. O., & Motiwala, T. (2020). Transcriptomics-based drug repurposing approach identifies novel drugs against sorafenib-resistant hepatocellular carcinoma. *Cancers*. https://doi.org/10.3390/cancers12102730

Regorafenib. (2017). https://www.fda.gov/drugs/resources-information-approved-drugs/regorafenib

Reyes, D., Ballaré, C., Castellano, G., Soronellas, D., Bagó, J. R., Blanco, J., & Beato, M. (2014). Activation of mitogen-and stress-activated kinase 1 is required for proliferation of breast cancer cells in response to estrogens or progestins. *Oncogene*. https://doi.org/10.1038/onc.2013.95

Rezaei, P. F., Fouladdel, S., Ghaffari, S. M., Amin, G., & Azizi, E. (2012). Induction of G1 cell cycle arrest and cyclin D1 down-regulation in response to pericarp extract of Baneh in human breast cancer T47D cells. *DARU, Journal of Pharmaceutical Sciences*. https://doi.org/10.1186/2008-2231-20-101

Rezatabar, S., Karimian, A., Rameshknia, V., Parsian, H., Majidinia, M., Kopi, T. A., Bishayee, A., Sadeghinia, A., Yousefi, M., Monirialamdari, M., & Yousefi, B. (2019). RAS/MAPK signaling functions in oxidative stress, DNA damage response and cancer progression. In *Journal of Cellular Physiology*. https://doi.org/10.1002/jcp.28334

Richer, J. K., Jacobsen, B. M., Manning, N. G., Abel, M. G., Wolf, D. M., & Horwitz, K. B. (2002). Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M110090200

Rigiracciolo, D. C., Scarpelli, A., Lappano, R., Pisano, A., Santolla, M. F., Avino, S., de Marco, P., Bussolati, B., Maggiolini, M., & de Francesco, E. M. (2015). GPER is involved in the stimulatory effects of aldosterone in breast cancer cells and breast tumor-derived endothelial cells. *Oncotarget*. https://doi.org/10.18632/oncotarget.6475

Rikimaru, T., Taketomi, A., Yamashita, Y. I., Shirabe, K., Hamatsu, T., Shimada, M., & Maehara, Y. (2007). Clinical significance of histone deacetylase 1 expression in patients with hepatocellular carcinoma. *Oncology*. https://doi.org/10.1159/000111106

Rogatsky, I., Trowbridge, J. M., & Garabedian, M. J. (1997). Glucocorticoid receptor-mediated cell cycle arrest is achieved through distinct cell-specific transcriptional regulatory mechanisms. *Molecular and Cellular Biology*. https://doi.org/10.1128/mcb.17.6.3181

Rogers, R. F., Walton, M. I., Cherry, D. L., Collins, I., Clarke, P. A., Garrett, M. D., & Workman, P. (2020). CHK1 inhibition is synthetically lethal with loss of B-family DNA polymerase function in human lung and colorectal cancer cells. *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-19-1372

Rosmorduc, O., & Desbois-Mouthon, C. (2011). Targeting STAT3 in hepatocellular carcinoma: Sorafenib again... In *Journal of Hepatology*. https://doi.org/10.1016/j.jhep.2011.06.005

Rossier, B. C., & Jackson Stutts, M. (2009). Activation of the epithelial sodium channel (ENaC) by serine proteases. In *Annual Review of Physiology*. https://doi.org/10.1146/annurev.physiol.010908.163108

Rossol-Haseroth, K., Zhou, Q., Braun, S., Boldyreff, B., Falkenstein, E., Wehling, M., & Lösel, R. M. (2004). Mineralocorticoid receptor antagonists do not block rapid ERK activation by aldosterone. *Biochemical and Biophysical Research Communications*. https://doi.org/10.1016/j.bbrc.2004.04.026

Sabbir, M. G. (2019). Progesterone induced Warburg effect in HEK293 cells is associated with post-translational modifications and proteasomal degradation of progesterone receptor membrane component 1. *Journal of Steroid Biochemistry and Molecular Biology*. https://doi.org/10.1016/j.jsbmb.2019.105376

Sahoo, S., Brickley, D. R., Kocherginsky, M., & Conzen, S. D. (2005). Coordinate expression of the PI3-kinase downstream effectors serum and glucocorticoid-induced kinase (SGK-1) and Akt-1 in human breast cancer. *European Journal of Cancer*. https://doi.org/10.1016/j.ejca.2005.07.018

Sakurikar, N., Thompson, R., Montano, R., & Eastman, A. (2016). A subset of cancer cell lines is acutely sensitive to the Chk1 inhibitor MK-8776 as monotherapy due to CDK2 activation in S phase. *Oncotarget*. https://doi.org/10.18632/oncotarget.6364

Sanchez-Vega, F., Mina, M., Armenia, J., Chatila, W. K., Luna, A., La, K. C., Dimitriadoy, S., Liu, D. L., Kantheti, H. S., Saghafinia, S., Chakravarty, D., Daian, F., Gao, Q., Bailey, M. H., Liang, W. W., Foltz, S. M., Shmulevich, I., Ding, L., Heins, Z., … Schultz, N. (2018). Oncogenic Signaling Pathways in The Cancer Genome Atlas. *Cell*. https://doi.org/10.1016/j.cell.2018.03.035

Sanomachi, T., Suzuki, S., Togashi, K., Sugai, A., Seino, S., Okada, M., Yoshioka, T.,

Kitanaka, C., & Yamamoto, M. (2019). Spironolactone, a classic potassium-sparing diuretic, reduces survivin expression and chemosensitizes cancer cells to non-DNA-damaging anticancer drugs. *Cancers*. https://doi.org/10.3390/cancers11101550

Sasano, H., Frost, A. R., Saitohi, R., Matsunaga, G., Nagura, H., Krozowski, Z. S., & Silverberg, S. G. (1997). Localization of mineralocorticoid receptor and 11 beta-hydroxysteroid dehydrogenase type II in human breast and its disorders. *Anticancer Research*.

Schmidt, S., Rainer, J., Ploner, C., Presul, E., Riml, S., & Kofler, R. (2004). Glucocorticoid-induced apoptosis and glucocorticoid resistance: Molecular mechanisms and clinical relevance. In *Cell Death and Differentiation*. https://doi.org/10.1038/sj.cdd.4401456

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. In *Nature Methods*. https://doi.org/10.1038/nmeth.2089

Schreier, B., Wolf, A., Hammer, S., Pohl, S., Mildenberger, S., Rabe, S., Gekle, M., & Zipprich, A. (2018). The selective mineralocorticoid receptor antagonist eplerenone prevents decompensation of the liver in cirrhosis. *British Journal of Pharmacology*. https://doi.org/10.1111/bph.14341

Schupp, N., Kolkhof, P., Queisser, N., Gärtner, S., Schmid, U., Kretschmer, A., Hartmann, E., Oli, R. G., Schäfer, S., & Stopper, H. (2011). Mineralocorticoid receptor- mediated DNA damage in kidneys of DOCA- salt hypertensive rats. *The FASEB Journal*. https://doi.org/10.1096/fj.10-173286

Sever, R., & Brugge, J. S. (2015). Signal transduction in cancer. *Cold Spring Harbor Perspectives in Medicine*. https://doi.org/10.1101/cshperspect.a006098

Sever, R., & Glass, C. K. (2013). Signaling by nuclear receptors. *Cold Spring Harbor Perspectives in Biology*. https://doi.org/10.1101/cshperspect.a016709

Sevilla, L. M., & Pérez, P. (2018). Roles of the glucocorticoid and mineralocorticoid receptors in skin pathophysiology. In *International Journal of Molecular Sciences*. https://doi.org/10.3390/ijms19071906

Sharma, A., Boise, L. H., & Shanmugam, M. (2019). Cancer metabolism and the evasion of apoptotic cell death. In *Cancers*. https://doi.org/10.3390/cancers11081144

Sharma, P., Banerjee, R., & Narayan, K. P. (2016). Mineralocorticoid receptor mediated

liposomal delivery system for targeted induction of apoptosis in cancer cells. *Biochimica et Biophysica Acta - Biomembranes*. https://doi.org/10.1016/j.bbamem.2015.11.023

Shen, S., Dean, D. C., Yu, Z., & Duan, Z. (2019). Role of cyclin-dependent kinases (CDKs) in hepatocellular carcinoma: Therapeutic potential of targeting the CDK signaling pathway. *Hepatology Research*. https://doi.org/10.1111/hepr.13353

Shen, Y. H., Godlewski, J., Zhu, J., Sathyanarayana, P., Leaner, V., Birrer, M. J., Rana, A., & Tzivion, G. (2003). Cross-talk between JNK/SAPK and ERK/MAPK pathways: Sustained activation of JNK blocks ERK activation by mitogenic factors. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M303264200

Shi, W., Wang, D., Yuan, X., Liu, Y., Guo, X., Li, J., & Song, J. (2019). Glucocorticoid receptor-IRS-1 axis controls EMT and the metastasis of breast cancers. *Journal of Molecular Cell Biology*. https://doi.org/10.1093/jmcb/mjz001

Shi, Y. H., Ding, Z. Bin, Zhou, J., Qiu, S. J., & Fan, J. (2009). Prognostic significance of Beclin 1-dependent apoptotic activity in hepatocellular carcinoma. *Autophagy*. https://doi.org/10.4161/auto.5.3.7658

Shimizu, N., Yoshikawa, N., Ito, N., Maruyama, T., Suzuki, Y., Takeda, S. I., Nakae, J., Tagata, Y., Nishitani, S., Takehana, K., Sano, M., Fukuda, K., Suematsu, M., Morimoto, C., & Tanaka, H. (2011). Crosstalk between glucocorticoid receptor and nutritional sensor mTOR in skeletal muscle. *Cell Metabolism*. https://doi.org/10.1016/j.cmet.2011.01.001

Shin, S., Lee, Y., Kim, W., Ko, H., Choi, H., & Kim, K. (2005). Caspase-2 primes cancer cells for TRAIL-mediated apoptosis by processing procaspase-8. *EMBO Journal*. https://doi.org/10.1038/sj.emboj.7600827

Shin, S. Y., Choi, B. H., Kim, J. R., Kim, J. H., & Lee, Y. H. (2006). Suppression of P-glycoprotein expression by antipsychotics trifluoperazine in adriamycin-resistant L1210 mouse leukemia cells. *European Journal of Pharmaceutical Sciences*. https://doi.org/10.1016/j.ejps.2006.03.002

Shin, S. Y., Kim, C. G., Hong, D. D., Kim, J. H., & Lee, Y. H. (2004). Implication of Egr-1 in trifluoperazine-induced growth inhibition in human U87MG glioma cells. *Experimental and Molecular Medicine*. https://doi.org/10.1038/emm.2004.50

Shiota, M., Fujimoto, N., Higashijima, K., Imada, K., Kashiwagi, E., Takeuchi, A., Inokuchi,

J., Tatsugami, K., Kajioka, S., Uchiumi, T., & Eto, M. (2018). Mineralocorticoid receptor signaling affects therapeutic effect of enzalutamide. *Prostate*. https://doi.org/10.1002/pros.23661

Shlyakhtina, Y., Pavet, V., & Gronemeyer, H. (2017). Dual role of DR5 in death and survival signaling leads to TRAIL resistance in cancer cells. *Cell Death & Disease*. https://doi.org/10.1038/cddis.2017.423

Sidi, S., Sanda, T., Kennedy, R. D., Hagen, A. T., Jette, C. A., Hoffmans, R., Pascual, J., Imamura, S., Kishi, S., Amatruda, J. F., Kanki, J. P., Green, D. R., D'Andrea, A. A., & Look, A. T. (2008). Chk1 Suppresses a Caspase-2 Apoptotic Response to DNA Damage that Bypasses p53, Bcl-2, and Caspase-3. *Cell*. https://doi.org/10.1016/j.cell.2008.03.037

Silveira, A. B., Laranjeira, A. B. A., Rodrigues, G. O. L., Leal, P. C., Cardoso, B. A., Barata, J. T., Yunes, R. A., Zanchin, N. I. T., Brandalise, S. R., & Yunes, J. A. (2015). PI3K inhibition synergizes with glucocorticoids but antagonizes with methotrexate in T-cell acute lymphoblastic leukemia. *Oncotarget*. https://doi.org/10.18632/oncotarget.3524

Singh, R., Letai, A., & Sarosiek, K. (2019). Regulation of apoptosis in health and disease: the balancing act of BCL-2 family proteins. In *Nature Reviews Molecular Cell Biology*. https://doi.org/10.1038/s41580-018-0089-8

Singh, V., Bhoir, S., Chikhale, R. V., Hussain, J., Dwyer, D., Bryce, R. A., Kirubakaran, S., & De Benedetti, A. (2020). Generation of Phenothiazine with Potent Anti-TLK1 Activity for Prostate Cancer Therapy. *IScience*. https://doi.org/10.1016/j.isci.2020.101474

Singh, V., Jaiswal, P. K., Ghosh, I., Koul, H. K., Yu, X., & De Benedetti, A. (2019). Targeting the TLK1/NEK1 DDR axis with Thioridazine suppresses outgrowth of androgen independent prostate tumors. *International Journal of Cancer*. https://doi.org/10.1002/ijc.32200

Skandalis, S. S., Afratis, N., Smirlaki, G., Nikitovic, D., Theocharis, A. D., Tzanakakis, G. N., & Karamanos, N. K. (2014). Cross-talk between estradiol receptor and EGFR/IGF-IR signaling pathways in estrogen-responsive breast cancers: Focus on the role and impact of proteoglycans. *Matrix Biology*. https://doi.org/10.1016/j.matbio.2013.09.002

Skor, M. N., Wonder, E. L., Kocherginsky, M., Goyal, A., Hall, B. A., Cai, Y., & Conzen, S. D. (2013). Glucocorticoid receptor antagonism as a novel therapy for triple-negative breast cancer. *Clinical Cancer Research*. https://doi.org/10.1158/1078-0432.CCR-12-3826

Sleire, L., Førde-Tislevoll, H. E., Netland, I. A., Leiss, L., Skeie, B. S., & Enger, P. Ø. (2017). Drug repurposing in cancer. In *Pharmacological Research*. https://doi.org/10.1016/j.phrs.2017.07.013

Smith, C. L. (1998). Cross-talk between peptide growth factor and estrogen receptor signaling pathways. *Biology of Reproduction*. https://doi.org/10.1095/biolreprod58.3.627

Sommer, E. M., Dry, H., Cross, D., Guichard, S., Davies, B. R., & Alessi, D. R. (2013). Elevated SGK1 predicts resistance of breast cancer cells to Akt inhibitors. *Biochemical Journal*. https://doi.org/10.1042/BJ20130342

Song, H. M., Song, J. L., Li, D. F., Hua, K. Y., Zhao, B. K., & Fang, L. (2015). Inhibition of FOXO1 by small interfering RNA enhances proliferation and inhibits apoptosis of papillary thyroid carcinoma cells via Akt/FOXO1/Bim pathway. *OncoTargets and Therapy*. https://doi.org/10.2147/OTT.S95395

Song, R. X. D., & Santen, R. J. (2003). Apoptotic action of estrogen. In *Apoptosis*. https://doi.org/10.1023/A:1021649019025

Soundararajan, R., Melters, D., Shih, I. C., Wang, J., & Pearce, D. (2009). Epithelial sodium channel regulated by differential composition of a signaling complex. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.0809892106

Spaziani, A., Alisi, A., Sanna, D., & Balsano, C. (2006). Role of p38 MAPK and RNA-dependent protein kinase (PKR) in hepatitis C virus core-dependent nuclear delocalization of cyclin B1. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M512536200

Sperlich, J., & Teusch, N. (2018). Pseudopterosin inhibits proliferation and 3D invasion in triple-negative breast cancer by agonizing glucocorticoid receptor alpha. *Molecules*. https://doi.org/10.3390/molecules23081992

Środa-Pomianek, K., Michalak, K., Palko-Łabuz, A., Uryga, A., Świątek, P., & Majkowski Michałand Wesołowska, O. (2019). The combined use of phenothiazines and statins strongly affects doxorubicin-resistance, apoptosis, and Cox-2 activity in colon cancer cells. *International Journal of Molecular Sciences*. https://doi.org/10.3390/ijms20040955

Środa-Pomianek, K., Michalak, K., Świątek, P., Poła, A., Palko-Łabuz, A., & Wesołowska, O. (2018). Increased lipid peroxidation, apoptosis and selective cytotoxicity in colon cancer

cell line LoVo and its doxorubicin-resistant subline LoVo/Dx in the presence of newly synthesized phenothiazine derivatives. *Biomedicine and Pharmacotherapy*. https://doi.org/10.1016/j.biopha.2018.06.170

Stepanova, M., Lin, F., & Lin, V. C. L. (2006). In silico modelling of hormone response elements. *BMC Bioinformatics*. https://doi.org/10.1186/1471-2105-7-S4-S27

Stevanato Filho, P. R., Aguiar Júnior, S., Begnami, M. D., Ferreira, F. O., Nakagawa, W. T., Spencer, R. M. S. B., Bezerra, T. S., Boggiss, P. E., & Lopes, A. (2018). Estrogen Receptor β as a Prognostic Marker of Tumor Progression in Colorectal Cancer with Familial Adenomatous Polyposis and Sporadic Polyps. *Pathology and Oncology Research*. https://doi.org/10.1007/s12253-017-0268-5

Stevens, M., & Oltean, S. (2019). Modulation of the Apoptosis Gene Bcl-x Function Through Alternative Splicing. In *Frontiers in Genetics*. https://doi.org/10.3389/fgene.2019.00804

Stupack, D. G. (2013). Caspase-8 as a therapeutic target in cancer. *Cancer Letters*. https://doi.org/10.1016/j.canlet.2010.07.022

Sukocheva, O. A. (2018). Estrogen, estrogen receptors, and hepatocellular carcinoma: Are we there yet? In *World Journal of Gastroenterology*. https://doi.org/10.3748/wjg.v24.i1.1

Sun, M., Paciga, J. E., Feldman, R. I., Yuan, Z. Q., Coppola, D., You Yong Lu, Shelley, S. A., Nicosia, S. V., & Cheng, J. Q. (2001). Phosphatidylinositol-3-OH kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor α (ERα) via interaction between ERα and PI3K. *Cancer Research*.

Sun, T., Liu, H., & Ming, L. (2017). Multiple Roles of Autophagy in the Sorafenib Resistance of Hepatocellular Carcinoma. *Cellular Physiology and Biochemistry*. https://doi.org/10.1159/000485285

Suwanjang, W., Holmström, K. M., Chetsawang, B., & Abramov, A. Y. (2013). Glucocorticoids reduce intracellular calcium concentration and protects neurons against glutamate toxicity. *Cell Calcium*. https://doi.org/10.1016/j.ceca.2012.12.006

Szmuilowicz, E. D., Adler, G. K., Williams, J. S., Green, D. E., Yao, T. M., Hopkins, P. N., & Seely, E. W. (2006). Relationship between aldosterone and progesterone in the human menstrual cycle. *Journal of Clinical Endocrinology and Metabolism*. https://doi.org/10.1210/jc.2006-1154

Tai, W. T., Shiau, C. W., Chen, H. L., Liu, C. Y., Lin, C. S., Cheng, A. L., Chen, P. J., & Chen, K. F. (2013). Mcl-1-dependent activation of Beclin 1 mediates autophagic cell death induced by sorafenib and SC-59 in hepatocellular carcinoma cells. *Cell Death and Disease*. https://doi.org/10.1038/cddis.2013.18

Tai, Y., Gao, J.-H., Zhao, C., Tong, H., Zheng, S.-P., Huang, Z.-Y., Liu, R., Tang, C.-W., & Li, J. (2018). SK-Hep1: not hepatocellular carcinoma cells but a cell model for liver sinusoidal endothelial cells. *International Journal of Clinical and Experimental Pathology*.

Takehara, T., Liu, X., Fujimoto, J., Friedman, S. L., & Takahashi, H. (2001). Expression and role of Bcl-xL in human hepatocellular carcinomas. *Hepatology*. https://doi.org/10.1053/jhep.2001.25387

Takeuchi, T., Adachi, Y., Nagayama, T., & Furihata, M. (2011). Nedd4L modulates the transcription of metalloproteinase-1 and -13 genes to increase the invasive activity of gallbladder cancer. *International Journal of Experimental Pathology*. https://doi.org/10.1111/j.1365-2613.2010.00740.x

Tallec, L. P. Le, & Lombès, M. (2005). The mineralocorticoid receptor: A journey exploring its diversity and specificity of action. In *Molecular Endocrinology*. https://doi.org/10.1210/me.2005-0089

Tang, W., Chen, Z., Zhang, W., Cheng, Y., Zhang, B., Wu, F., Wang, Q., Wang, S., Rong, D., Reiter, F. P., De Toni, E. N., & Wang, X. (2020). The mechanisms of sorafenib resistance in hepatocellular carcinoma: theoretical basis and therapeutic aspects. In *Signal Transduction and Targeted Therapy*. https://doi.org/10.1038/s41392-020-0187-x

Tang, Y., Horikoshi, M., & Li, W. (2016). Ggfortify: Unified interface to visualize statistical results of popular r packages. *R Journal*. https://doi.org/10.32614/rj-2016-060

Tang, Z., Shen, Q., Xie, H., Zhou, Z., Shi, G., Zhang, C., Mohammed, A., Wu, Y., Ni, S., & Zhou, X. (2018). Serum and glucocorticoid-regulated kinase 1 (SGK1) is a predictor of poor prognosis in non-small cell lung cancer, and its dynamic pattern following treatment with SGK1 inhibitor and γ-ray irradiation was elucidated. *Oncology Reports*. https://doi.org/10.3892/or.2018.6181

Tangen, I. L., Veneris, J. T., Halle, M. K., Werner, H. M., Trovik, J., Akslen, L. A., Salvesen, H. B., Conzen, S. D., Fleming, G. F., & Krakstad, C. (2017). Expression of glucocorticoid receptor is associated with aggressive primary endometrial cancer and increases from

primary to metastatic lesions. *Gynecologic Oncology*. https://doi.org/10.1016/j.ygyno.2017.09.013

Tanksley, J. P., Chen, X., & Coffey, R. J. (2013). NEDD4L is downregulated in colorectal cancer and inhibits canonical WNT signaling. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0081514

Taylor, K. M., Ray, D. W., & Sommer, P. (2016). Glucocorticoid receptors in lung cancer: New perspectives. In *Journal of Endocrinology*. https://doi.org/10.1530/JOE-15-0496

Thornton, T. M., & Rincon, M. (2009). Non-classical p38 map kinase functions: Cell cycle checkpoints and survival. In *International Journal of Biological Sciences*. https://doi.org/10.7150/ijbs.5.44

Tonsing-Carter, E., Hernandez, K. M., Kim, C. R., Harkless, R. V., Oh, A., Bowie, K. R., West-Szymanski, D. C., Betancourt-Ponce, M. A., Green, B. D., Lastra, R. R., Fleming, G. F., Chandarlapaty, S., & Conzen, S. D. (2019). Glucocorticoid receptor modulation decreases ER-positive breast cancer cell proliferation and suppresses wild-type and mutant ER chromatin association. *Breast Cancer Research : BCR*. https://doi.org/10.1186/s13058-019-1164-6

Trabert, B., Sherman, M. E., Kannan, N., & Stanczyk, F. Z. (2019). Progesterone and breast cancer. In *Endocrine Reviews*. https://doi.org/10.1210/endrev/bnz001

Tran, S. E. F., Holmström, T. H., Ahonen, M., Kähäri, V. M., & Eriksson, J. E. (2001). MAPK/ERK Overrides the Apoptotic Signaling from Fas, TNF, and TRAIL Receptors. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M010384200

Treviño, L. S., Bingman, W. E., Edwards, D. P., & Nl, W. (2013). The requirement for p42/p44 MAPK activity in progesterone receptor-mediated gene regulation is target gene-specific. *Steroids*. https://doi.org/10.1016/j.steroids.2012.12.014

Truong, T. H., & Lange, C. A. (2018). Deciphering Steroid Receptor Crosstalk in Hormone-Driven Cancers. *Endocrinology*. https://doi.org/10.1210/en.2018-00831

Tummers, B., & Green, D. R. (2017). Caspase-8: regulating life and death. In *Immunological Reviews*. https://doi.org/10.1111/imr.12541

Umayahara, Y., Kawamori, R., Watada, H., Imano, E., Iwama, N., Morishima, T., Yamasaki, Y., Kajimoto, Y., & Kamada, T. (1994). Estrogen regulation of the insulin-like growth

factor I gene transcription involves an AP-1 enhancer. *Journal of Biological Chemistry*.

Vadlakonda, L., Pasupuleti, M., & Pallu, R. (2013). Role of PI3K-AKT-mTOR and Wnt signaling pathways in transition of G1-S phase of cell cycle in cancer cells. *Frontiers in Oncology*. https://doi.org/10.3389/fonc.2013.00085

Vahrenkamp, J. M., Yang, C. H., Rodriguez, A. C., Almomen, A., Berrett, K. C., Trujillo, A. N., Guillen, K. P., Welm, B. E., Jarboe, E. A., Janat-Amsbury, M. M., & Gertz, J. (2018). Clinical and Genomic Crosstalk between Glucocorticoid Receptor and Estrogen Receptor α In Endometrial Cancer. *Cell Reports*. https://doi.org/10.1016/j.celrep.2018.02.076

Valinsky, W. C., Touyz, R. M., & Shrier, A. (2018). Aldosterone, SGK1, and ion channels in the kidney. In *Clinical Science*. https://doi.org/10.1042/CS20171525

Van Zon, W., Ogink, J., Ter Riet, B., Medema, R. H., Te Riele, H., & Wolthuis, R. M. F. (2010). The APC/C recruits cyclin B1-Cdk1-Cks in prometaphase before D box recognition to control mitotic exit. *Journal of Cell Biology*. https://doi.org/10.1083/jcb.200912084

Varga, B., Csonka, Á., Csonka, A., Molnár, J., Amaral, L., & Spengler, G. (2017). Possible biological and clinical applications of phenothiazines. In *Anticancer Research*. https://doi.org/10.21873/anticanres.12045

Ventura, J. J., Tenbaum, S., Perdiguero, E., Huth, M., Guerra, C., Barbacid, M., Pasparakis, M., & Nebreda, A. R. (2007). p38α MAP kinase is essential in lung stem and progenitor cell proliferation and differentiation. *Nature Genetics*. https://doi.org/10.1038/ng2037

Verlinden, L., Bempt, I. Vanden, Eelen, G., Drijkoningen, M., Verlinden, I., Marchal, K., De Wolf-Peeters, C., Christiaens, M. R., Michiels, L., Bouillon, R., & Verstuyf, A. (2007). The E2F-regulated Gene Chk1 is highly expressed in triple-negative estrogen receptor-/progesterone receptor-/HER-2- breast carcinomas. *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-06-3545

Vermeulen, K., Van Bockstaele, D. R., & Berneman, Z. N. (2003). The cell cycle: A review of regulation, deregulation and therapeutic targets in cancer. In *Cell Proliferation*. https://doi.org/10.1046/j.1365-2184.2003.00266.x

Verrey, F., Loffing, J., Zecevic, M., Heitzmann, D., & Staub, O. (2003). SGK1: Aldosterone-induced relay of Na + transport regulation in distal kidney nephron cells. In *Cellular Physiology and Biochemistry*. https://doi.org/10.1159/000070246

Viengchareun, S., Le Menuet, D., Martinerie, L., Munier, M., Pascual-Le Tallec, L., & Lombès, M. (2007). The mineralocorticoid receptor: insights into its molecular and (patho)physiological biology. In *Nuclear receptor signaling*. https://doi.org/10.1621/nrs.05012

Vilasco, M., Communal, L., Mourra, N., Courtin, A., Forgez, P., & Gompel, A. (2011). Glucocorticoid receptor and breast cancer. In *Breast Cancer Research and Treatment*. https://doi.org/10.1007/s10549-011-1689-6

Villanueva-Paz, M., Cotán, D., Garrido-Maraver, J., Oropesa-Ávila, M., de la Mata, M., Delgado-Pavón, A., de Lavera, I., Alcocer-Gómez, E., Álvarez-Córdoba, M., & Sánchez-Alcázar, J. A. (2016). AMPK Regulation of Cell Growth, Apoptosis, Autophagy, and Bioenergetics. In *EXS*. https://doi.org/10.1007/978-3-319-43589-3_3

Villanueva, A., Chiang, D. Y., Newell, P., Peix, J., Thung, S., Alsinet, C., Tovar, V., Roayaie, S., Minguez, B., Sole, M., Battiston, C., van Laarhoven, S., Fiel, M. I., Di Feo, A., Hoshida, Y., Yea, S., Toffanin, S., Ramos, A., Martignetti, J. A., … Llovet, J. M. (2008). Pivotal Role of mTOR Signaling in Hepatocellular Carcinoma. *Gastroenterology*. https://doi.org/10.1053/j.gastro.2008.08.008

Vladic, Y. (2012). Progesterone in Human Pregnancy and Parturition. In *Sex Hormones*. https://doi.org/10.5772/27055

Vrtačnik, P., Ostanek, B., Mencej-Bedrač, S., & Marc, J. (2014). The many faces of estrogen signaling. In *Biochemia Medica*. https://doi.org/10.11613/BM.2014.035

Waks, A. G., & Winer, E. P. (2019). Breast Cancer Treatment: A Review. In *JAMA - Journal of the American Medical Association*. https://doi.org/10.1001/jama.2018.19323

Waldegger, S., Barth, P., Raber, G., & Lang, F. (1997). Cloning and characterization of a putative human serine/threonine protein kinase transcriptionally modified during anisotonic and isotonic alterations of cell volume. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.94.9.4440

Wang, B., Luo, Y., Zhou, X., & Li, R. (2018). Trifluoperazine induces apoptosis through the upregulation of Bax/Bcl-2 and downregulated phosphorylation of AKT in mesangial cells and improves renal function in lupus nephritis mice. *International Journal of Molecular Medicine*. https://doi.org/10.3892/ijmm.2018.3562

Wang, B., Zhou, X., Wang, Y., & Li, R. (2017). Trifluoperazine inhibits mesangial cell proliferation by arresting cell cycle-dependent mechanisms. *Medical Science Monitor*. https://doi.org/10.12659/MSM.902522

Wang, Chunmei, Cigliano, A., Delogu, S., Armbruster, J., Dombrowski, F., Evert, M., Chen, X., & Calvisi, D. F. (2013). Functional crosstalk between AKT/mTOR and Ras/MAPK pathways in hepatocarcinogenesis: Implications for the treatment of human liver cancer. *Cell Cycle*. https://doi.org/10.4161/cc.25099

Wang, Cun, Jin, H., Gao, D., Lieftink, C., Evers, B., Jin, G., Xue, Z., Wang, L., Beijersbergen, R. L., Qin, W., & Bernards, R. (2018). Phospho-ERK is a biomarker of response to a synthetic lethal drug combination of sorafenib and MEK inhibition in liver cancer. *Journal of Hepatology*. https://doi.org/10.1016/j.jhep.2018.07.004

Wang, L., Wang, W. L., Zhang, Y., Guo, S. P., Zhang, J., & Li, Q. L. (2007). Epigenetic and genetic alterations of PTEN in hepatocellular carcinoma. *Hepatology Research*. https://doi.org/10.1111/j.1872-034X.2007.00042.x

Wang, Q. S., Liang, C., Niu, N., Yang, X., Chen, X., Song, B. L., Yu, C. J., Wu, M. M., Zhang, Z. R., & Ma, H. P. (2018). Palmitate stimulates the epithelial sodium channel by elevating intracellular calcium, reactive oxygen species, and phosphoinositide 3-kinase activity. *Oxidative Medicine and Cellular Longevity*. https://doi.org/10.1155/2018/7560610

Wang, S. N., Lee, K. T., Tsai, C. J., Chen, Y. J., & Yeh, Y. T. (2012). Phosphorylated p38 and JNK MAPK proteins in hepatocellular carcinoma. *European Journal of Clinical Investigation*. https://doi.org/10.1111/eci.12003

Wang, T. T. Y., & Phang, J. M. (1995). Effects of Estrogen on Apoptotic Pathways in Human Breast Cancer Cell Line MCF-7. *Cancer Research*.

Wang, Yuanxi, Gao, F., Ooi, K. K., Tai, Q., Zhang, J., Zhu, Y., Kang, J., Zhong, F., Cai, L., Fang, F., Ang, K. P., & Gao, Y. (2019). 10h-3,6-diazaphenothiazine-induced, caspases-mediated mitochondrial-dependent apoptosis on hepg2 hepatocellular carcinoma cells and suppressed cancer cells invasion via inhibiting mitochondrial thioredoxin reductase enzymatic activities. *Journal of Environmental Pathology, Toxicology and Oncology*. https://doi.org/10.1615/JEnvironPatholToxicolOncol.2019030482

Wang, Yuanzhong, Zhou, D., & Chen, S. (2014). SGK3 Is an androgen-inducible kinase promoting prostate cancer cell proliferation through activation of p70 S6 kinase and up-

regulation of cyclin D1. *Molecular Endocrinology*. https://doi.org/10.1210/me.2013-1339

Wang, Z., Dang, T., Liu, T., Chen, S., Li, L., Huang, S., & Fang, M. (2016). NEDD4L protein catalyzes ubiquitination of PIK3CA protein and regulates PI3K-AKT signaling. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M116.726083

Weinberg, R. A. (2013). The Biology and Genetics of Cells and Organisms. In *The Biology of Cancer*.

Wen, Y., Zhang, Y., Li, J., Luo, F., Huang, Z., & Liu, K. (2018). Low concentration trifluoperazine promotes proliferation and reduces calcium-dependent apoptosis in glioma cells. *Scientific Reports*. https://doi.org/10.1038/s41598-018-19413-y

Wesołowska, O., Molnar, J., Ocsovszki, I., & Michalak, K. (2009). Differential effect of phenothiazines on MRP1 and P-glycoprotein activity. *In Vivo*.

West, D. C., Kocherginsky, M., Tonsing-Carter, E. Y., Dolcen, D. N., Hosfield, D. J., Lastra, R. R., Sinnwell, J. P., Thompson, K. J., Bowie, K. R., Harkless, R. V., Skor, M. N., Pierce, C. F., Styke, S. C., Kim, C. R., De Wet, L., Greene, G. L., Boughey, J. C., Goetz, M. P., Kalari, K. R., … Conzen, S. D. (2018). Discovery of a glucocorticoid receptor (gr) activity signature using selective gr antagonis in er-negative breast cancer. *Clinical Cancer Research*. https://doi.org/10.1158/1078-0432.CCR-17-2793

West, D. C., Pan, D., Tonsing-Carter, E. Y., Hernandez, K. M., Pierce, C. F., Styke, S. C., Bowie, K. R., Garcia, T. I., Kocherginsky, M., & Conzen, S. D. (2016). GR and ER coactivation alters the expression of differentiation genes and associates with improved ER+ breast cancer outcome. *Molecular Cancer Research*. https://doi.org/10.1158/1541-7786.MCR-15-0433

Whaley-Connell, A. T., Habibi, J., Nistala, R., Demarco, V. G., Pulakat, L., Hayden, M. R., Joginpally, T., Ferrario, C. M., Parrish, A. R., & Sowers, J. R. (2012). Mineralocorticoid receptor-dependent proximal tubule injury is mediated by a redox-sensitive mTOR/S6K1 pathway. *American Journal of Nephrology*. https://doi.org/10.1159/000335079

Whittaker, S., Marais, R., & Zhu, A. X. (2010). The role of signaling pathways in the development and treatment of hepatocellular carcinoma. *Oncogene*. https://doi.org/10.1038/onc.2010.236

Wilhelm, S. M., Carter, C., Tang, L. Y., Wilkie, D., McNabola, A., Rong, H., Chen, C., Zhang,

X., Vincent, P., McHugh, M., Cao, Y., Shujath, J., Gawlak, S., Eveleigh, D., Rowley, B., Liu, L., Adnane, L., Lynch, M., Auclair, D., … Trail, P. A. (2004). BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-04-1443

Witt-Kehati, D., Fridkin, A., Alaluf, M. B., Zemel, R., & Shlomai, A. (2018). Inhibition of pMAPK14 Overcomes Resistance to Sorafenib in Hepatoma Cells with Hepatitis B Virus. *Translational Oncology*. https://doi.org/10.1016/j.tranon.2018.02.015

Won, M., Park, K. A., Byun, H. S., Kim, Y. R., Choi, B. L., Hong, J. H., Park, J., Seok, J. H., Lee, Y. H., Cho, C. H., Song, I. S., Kim, Y. K., Shen, H. M., & Hur, G. M. (2009). Protein kinase SGK1 enhances MEK/ERK complex formation through the phosphorylation of ERK2: Implication for the positive regulatory role of SGK1 on the ERK function during liver regeneration. *Journal of Hepatology*. https://doi.org/10.1016/j.jhep.2009.02.027

Wong, C., & Chen, S. (2012). The development, application and limitations of breast cancer cell lines to study tamoxifen and aromatase inhibitor resistance. In *Journal of Steroid Biochemistry and Molecular Biology*. https://doi.org/10.1016/j.jsbmb.2011.12.005

Woods, C. P., Hazlehurst, J. M., & Tomlinson, J. W. (2015). Glucocorticoids and non-alcoholic fatty liver disease. In *Journal of Steroid Biochemistry and Molecular Biology*. https://doi.org/10.1016/j.jsbmb.2015.07.020

World Health Organization. (2018). https://www.who.int/news-room/fact-sheets/detail/cancer

Wu, C. H., Bai, L. Y., Tsai, M. H., Chu, P. C., Chiu, C. F., Chen, M. Y., Chiu, S. J., Chiang, J. H., & Weng, J. R. (2016). Pharmacological exploitation of the phenothiazine antipsychotics to develop novel antitumor agents-A drug repurposing strategy. *Scientific Reports*. https://doi.org/10.1038/srep27540

Wu, I., Shin, S. C., Cao, Y., Bender, I. K., Jafari, N., Feng, G., Lin, S., Cidlowski, J. A., Schleimer, R. P., & Lu, N. Z. (2013). Selective glucocorticoid receptor translational isoforms reveal glucocorticoid-induced apoptotic transcriptomes. *Cell Death and Disease*. https://doi.org/10.1038/cddis.2012.193

Würstle, M. L., & Rehm, M. (2014). A systems biology analysis of apoptosome formation and apoptosis execution supports allosteric procaspase-9 activation. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M114.590034

Xia, Y., Jia, C., Xue, Q., Jiang, J., Xie, Y., Wang, R., Ran, Z., Xu, F., Zhang, Y., & Ye, T. (2019). Antipsychotic drug trifluoperazine suppresses colorectal cancer by inducing G0/G1 arrest and apoptosis. *Frontiers in Pharmacology*. https://doi.org/10.3389/fphar.2019.01029

Xiao, H., Tong, R., Ding, C., Lv, Z., Du, C., Peng, C., Cheng, S., Xie, H., Zhou, L., Wu, J., & Zheng, S. (2015). γ-H2AX promotes hepatocellular carcinoma angiogenesis via EGFR/HIF-1α/VEGF pathways under hypoxic condition. *Oncotarget*. https://doi.org/10.18632/oncotarget.2942

Xiao, Z., Xue, J., Sowin, T. J., Rosenberg, S. H., & Zhang, H. (2005). A novel mechanism of checkpoint abrogation conferred by Chk1 downregulation. *Oncogene*. https://doi.org/10.1038/sj.onc.1208309

Xie, Y., Wei, R. R., Huang, G. L., Zhang, M. Y., Yuan, Y. F., & Wang, H. Y. (2014). Checkpoint kinase 1 is negatively regulated by miR-497 in hepatocellular carcinoma. *Medical Oncology*. https://doi.org/10.1007/s12032-014-0844-4

Xu, S., Liu, C., Ma, Y., Ji, H. L., & Li, X. (2016). Potential roles of amiloride-sensitive sodium channels in cancer development. In *BioMed Research International*. https://doi.org/10.1155/2016/2190216

Xue, R., & Meng, Q. (2019). The management of glucocorticoid therapy in liver failure. In *Frontiers in Immunology*. https://doi.org/10.3389/fimmu.2019.02490

Yadunandam, A. K., Yoon, J. S., Jeong, Y. T., Kim, W. Y., Lee, S. Y., & Kim, G. Do. (2015). Differential effects of tetrahydropyridinol derivatives on β-catenin signaling and invasion in human hepatocellular and breast carcinoma cells. *International Journal of Molecular Medicine*. https://doi.org/10.3892/ijmm.2015.2240

Yakirevich, E., Morris, D. J., Tavares, R., Meitner, P. A., Lechpammer, M., Noble, L., De Rodriguez, A. F., Gomez-Sanchez, C. E., Wang, L. J., Sabo, E., Delellis, R. A., & Resnick, M. B. (2008). Mineralocorticoid receptor and 11β-hydroxysteroid dehydrogenase type II expression in renal cell neoplasms: A tissue microarray and quantitative RT-PCR study. *American Journal of Surgical Pathology*. https://doi.org/10.1097/PAS.0b013e31815f2362

Yang, F., Ma, Q., Liu, Z., Li, W., Tan, Y., Jin, C., Ma, W., Hu, Y., Shen, J., Ohgi, K. A., Telese, F., Liu, W., & Rosenfeld, M. G. (2017). Glucocorticoid Receptor:MegaTrans Switching Mediates the Repression of an ERα-Regulated Transcriptional Program. *Molecular Cell*.

https://doi.org/10.1016/j.molcel.2017.03.019

Yang, G. Z., Nie, H. G., Lu, L., Chen, J., Lu, X. Y., Ji, H. L., & Li, Q. N. (2011). Estrogen regulates the expression and activity of epithelial sodium channel in mouse osteoblasts. *Cellular and Molecular Biology*. https://doi.org/10.1170/170

Yang, L., Li, N., Xue, Z., Liu, L. R., Li, J., Huang, X., Xie, X., Zou, Y., Tang, H., & Xie, X. (2020). Synergistic therapeutic effect of combined PDGFR and SGK1 inhibition in metastasis-initiating cells of breast cancer. *Cell Death and Differentiation*. https://doi.org/10.1038/s41418-019-0485-4

Yang, S., He, P., Wang, J., Schetter, A., Tang, W., Funamizu, N., Yanaga, K., Uwagawa, T., Satoskar, A. R., Gaedcke, J., Bernhardt, M., Ghadimi, B. M., Gaida, M. M., Bergmann, F., Werner, J., Ried, T., Hanna, N., Alexander, H. R., & Hussain, S. P. (2016). A novel MIF signaling pathway drives the malignant character of pancreatic cancer by targeting NR3C2. *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-15-2841

Ye, X. (2012). NEDD4: A Promising Target for Cancer Therapy Xiantao. *Changes*.

Yen, C. C., Chen, L. T., Li, C. F., Chen, S. C., Chua, W. Y., Lin, Y. C., Yen, C. H., Chen, Y. C., Yang, M. H., Chao, Y., & Fletcher, J. A. (2019). Identification of phenothiazine as an ETV1-targeting agent in gastrointestinal stromal tumors using the Connectivity Map. *International Journal of Oncology*. https://doi.org/10.3892/ijo.2019.4829

Yildizhan, H., Barkan, N. P., Turan, S. K., Demiralp, Ö., Demiralp, F. D. Ö., Uslu, B., & Ōzkan, S. A. (2018). Treatment strategies in cancer from past to present. In *Drug Targeting and Stimuli Sensitive Drug Delivery Systems*. https://doi.org/10.1016/B978-0-12-813689-8.00001-X

Yin, C., Lin, Y., Zhang, X., Chen, Y. X., Zeng, X., Yue, H. Y., Hou, J. L., Deng, X., Zhang, J. P., Han, Z. G., & Xie, W. F. (2008). Differentiation therapy of Hepatocellular carcinoma in mice with recombinant adenovirus carryinghepatocyte nuclear factor-4α gene. *Hepatology*. https://doi.org/10.1002/hep.22510

Yu, L., Xu, F., & Gao, L. (2020). Predict New Therapeutic Drugs for Hepatocellular Carcinoma Based on Gene Mutation and Expression. *Frontiers in Bioengineering and Biotechnology*. https://doi.org/10.3389/fbioe.2020.00008

Yu, S., Kim, T., Yoo, K. H., & Kang, K. (2017). The T47D cell line is an ideal experimental

model to elucidate the progesterone-specific effects of a luminal A subtype of breast cancer. *Biochemical and Biophysical Research Communications*. https://doi.org/10.1016/j.bbrc.2017.03.114

Yuan, K., Yong, S., Xu, F., Zhou, T., McDonald, J. M., & Chen, Y. (2015). Calmodulin antagonists promote TRA-8 therapy of resistant pancreatic cancer. *Oncotarget*. https://doi.org/10.18632/oncotarget.4490

Yuan, X., Wang, X., Li, Y., Li, X., Zhang, S., & Hao, L. (2019). Aldosterone promotes renal interstitial fibrosis via the AIF‐1/AKT/mTOR signaling pathway. *Molecular Medicine Reports*. https://doi.org/10.3892/mmr.2019.10680

Yuzugullu, H., Benhaj, K., Ozturk, N., Senturk, S., Celik, E., Toylu, A., Tasdemir, N., Yilmaz, M., Erdal, E., Akcali, K. C., Atabey, N., & Ozturk, M. (2009). Canonical Wnt signaling is antagonized by noncanonical Wnt5a in hepatocellular carcinoma cells. *Molecular Cancer*. https://doi.org/10.1186/1476-4598-8-90

Zennaro, M. C., Keightley, M. C., Kotelevtsev, Y., Conway, G. S., Soubrier, F., & Fuller, P. J. (1995). Human mineralocorticoid receptor genomic structure and identification of expressed isoforms. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.270.36.21016

Zennaro, Maria Christina, Farman, N., Bonvalet, J. P., & Lombès, M. (1997). Tissue-specific expression of α and β messenger ribonucleic acid isoforms of the human mineralocorticoid receptor in normal and pathological states. *Journal of Clinical Endocrinology and Metabolism*. https://doi.org/10.1210/jcem.82.5.3933

Zhai, B., Hu, F., Jiang, X., Xu, J., Zhao, D., Liu, B., Pan, S., Dong, X., Tan, G., Wei, Z., Qiao, H., Jiang, H., & Sun, X. (2014). Inhibition of akt reverses the acquired resistance to sorafenib by switching protective autophagy to autophagic cell death in hepatocellular carcinoma. *Molecular Cancer Therapeutics*. https://doi.org/10.1158/1535-7163.MCT-13-1043

Zhang, G. P., Yue, X., & Li, S. Q. (2020). Cathepsin C interacts with TNF-α/p38 MAPK signaling pathway to promote proliferation and metastasis in hepatocellular carcinoma. *Cancer Research and Treatment*. https://doi.org/10.4143/crt.2019.145

Zhang, H., Wang, Q., Liu, J., & Cao, H. (2018). Inhibition of the PI3K/Akt signaling pathway reverses sorafenib-derived chemo-resistance in hepatocellular carcinoma. *Oncology*

Letters. https://doi.org/10.3892/ol.2018.8536

Zhang, X., Ge, Y., Bukhari, A. A. S., Zhu, Q., Shen, Y., Li, M., Sun, H., Su, D., & Liang, X. (2019). Estrogen negatively regulates the renal epithelial sodium channel (ENaC) by promoting Derlin-1 expression and AMPK activation. *Experimental and Molecular Medicine*. https://doi.org/10.1038/s12276-019-0253-z

Zhang, Y., & Hunter, T. (2014). Roles of Chk1 in cell biology and cancer therapy. In *International Journal of Cancer*. https://doi.org/10.1002/ijc.28226

Zhang, Z., Xu, Q., Song, C., Mi, B., Zhang, H., Kang, H., Liu, H., Sun, Y., Wang, J., Lei, Z., Guan, H., & Li, F. (2020). Serum- And Glucocorticoid-inducible Kinase 1 is Essential for Osteoclastogenesis and Promotes Breast Cancer Bone Metastasis. *Molecular Cancer Therapeutics*. https://doi.org/10.1158/1535-7163.MCT-18-0783

Zhao, F., Gong, X., Liu, A., Lv, X., Hu, B., & Zhang, H. (2018). Downregulation of Nedd4L predicts poor prognosis, promotes tumor growth and inhibits MAPK/ERK signal pathway in hepatocellular carcinoma. *Biochemical and Biophysical Research Communications*. https://doi.org/10.1016/j.bbrc.2017.11.139

Zhao, Y., & Li, Z. (2015). Interplay of estrogen receptors and FOXA factors in the liver cancer. In *Molecular and Cellular Endocrinology*. https://doi.org/10.1016/j.mce.2015.01.043

Zhelev, Z., Ohba, H., Bakalova, R., Hadjimitova, V., Ishikawa, M., Shinohara, Y., & Baba, Y. (2004). Phenothiazines suppress proliferation and induce apoptosis in cultured leukemic cells without any influence on the viability of normal lymphocytes: Phenothiazines and leukemia. *Cancer Chemotherapy and Pharmacology*. https://doi.org/10.1007/s00280-003-0738-1

Zhou, J., & Cidlowski, J. A. (2005). The human glucocorticoid receptor: One gene, multiple proteins and diverse responses. *Steroids*. https://doi.org/10.1016/j.steroids.2005.02.006

Zhou, L., Huang, Y., Li, J., & Wang, Z. (2010). The mTOR pathway is associated with the poor prognosis of human hepatocellular carcinoma. *Medical Oncology*. https://doi.org/10.1007/s12032-009-9201-4

Zhou, W., Wang, Z., Shen, N., Pi, W., Jiang, W., Huang, J., Hu, Y., Li, X., & Sun, L. (2015). Knockdown of ANLN by lentivirus inhibits cell growth and migration in human breast cancer. *Molecular and Cellular Biochemistry*. https://doi.org/10.1007/s11010-014-2200-6

Zhu, C. J., Wang, Q. Q., Zhou, J. L., Liu, H. Z., Hua, F., Yang, H. Z., & Hu, Z. W. (2012). The mineralocorticoid receptor-p38MAPK-NFB or ERK-Sp1 signal pathways mediate aldosterone-stimulated inflammatory and profibrotic responses in rat vascular smooth muscle cells. *Acta Pharmacologica Sinica*. https://doi.org/10.1038/aps.2012.36

Zhu, R., Yang, G., Cao, Z., Shen, K., Zheng, L., Xiao, J., You, L., & Zhang, T. (2020). The prospect of serum and glucocorticoid-inducible kinase 1 (SGK1) in cancer therapy: a rising star. In *Therapeutic Advances in Medical Oncology*. https://doi.org/10.1177/1758835920940946

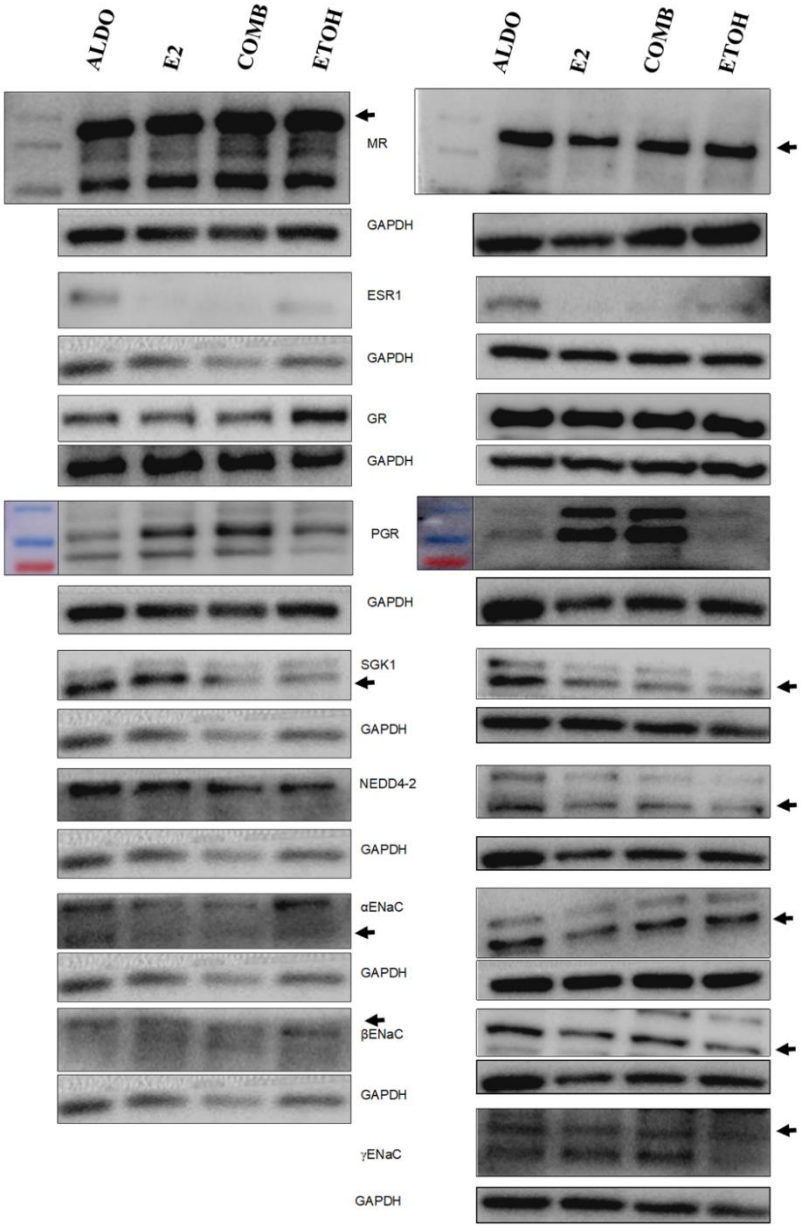
Zong, D., Hååg, P., Yakymovych, I., Lewensohn, R., & Viktorsson, K. (2011). Chemosensitization by phenothiazines in human lung cancer cells: Impaired resolution of γh2AX and increased oxidative stress elicit apoptosis associated with lysosomal expansion and intense vacuolation. *Cell Death and Disease*. https://doi.org/10.1038/cddis.2011.62

Zou, X., Levy-Cohen, G., & Blank, M. (2015). Molecular functions of NEDD4 E3 ubiquitin ligases in cancer. In *Biochimica et Biophysica Acta - Reviews on Cancer*. https://doi.org/10.1016/j.bbcan.2015.06.005

APPENDIX

APPENDIX A: ALDO/MR and E2/ER signaling pathways and their possible crosstalk in breast cancer.

In the main text, Set2 of the protein experiments were used for representation. Below in Figure, Set1 and Set2 were given together.



Appendix A Figure 1. **ALDO/MR and/or E2/ER downstream signaling members' protein levels.** Western blot images of proteins obtained from 24 hr., 10nM ALDO and/or E2 exposed T47D.

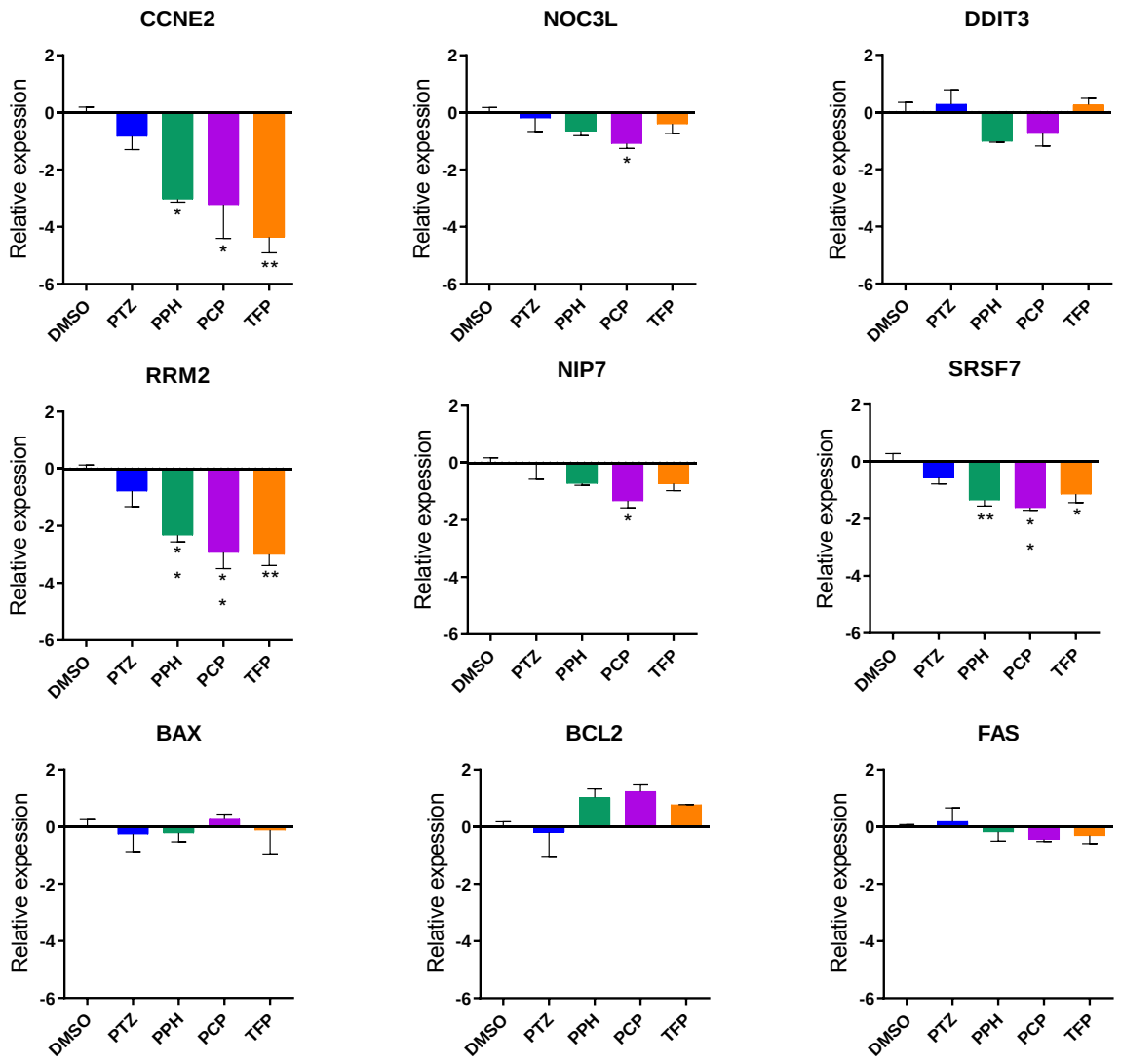
Appendix A Table 1. Three-way ANOVA interaction table of T47D cellular viability levels upon combinatorial hormone treatment in a time dependent manner (2000 cells).

Source of Variation	% of total variation	P value	P value summary	Significant?
E2	53,40	<0,0001	****	Yes
(ALDO 0 vs ALDO 10 µM)	1,008	0,0645	ns	No
(Day 3 vs Day 5)	11,74	<0,0001	****	Yes
E2 x (ALDO 0 vs ALDO 10 µM)	7,590	<0,0001	****	Yes
E2 x (Day 3 vs Day 5)	13,41	<0,0001	****	Yes
(ALDO 0 vs ALDO 10 µM) x (Day 3 vs Day 5)	1,689	0,0187	*	Yes
E2 x (ALDO 0 vs ALDO 10 µM) x (Day 3 vs Day 5)	2,356	0,0063	**	Yes

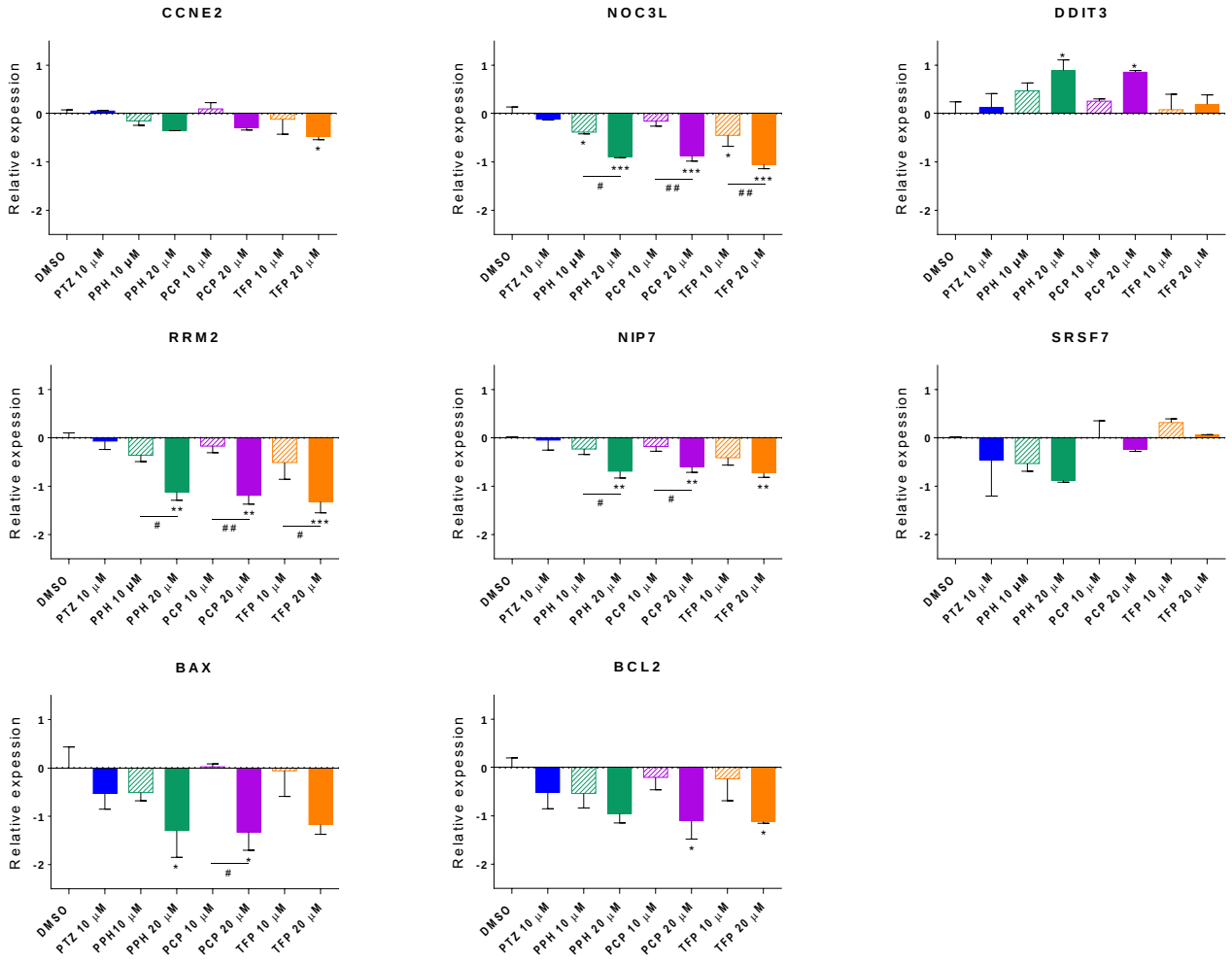
Appendix A Table 2. Three-way ANOVA interaction table of T47D cellular viability levels upon combinatorial hormone treatment in a time dependent manner (5000 cells).

Source of Variation	% of total variation	P value	P value summary	Significant?
E2	59,54	<0,0001	****	Yes
(ALDO 0 vs ALDO 10 µM)	0,01901	0,8150	ns	No
(Day 3 vs Day 5)	19,95	<0,0001	****	Yes
E2 x (ALDO 0 vs ALDO 10 µM)	0,003589	0,9190	ns	No
E2 x (Day 3 vs Day 5)	7,962	<0,0001	****	Yes
(ALDO 0 vs ALDO 10 µM) x (Day 3 vs Day 5)	1,378	0,0530	ns	No
E2 x (ALDO 0 vs ALDO 10 µM) x (Day 3 vs Day 5)	0,2219	0,4262	ns	No

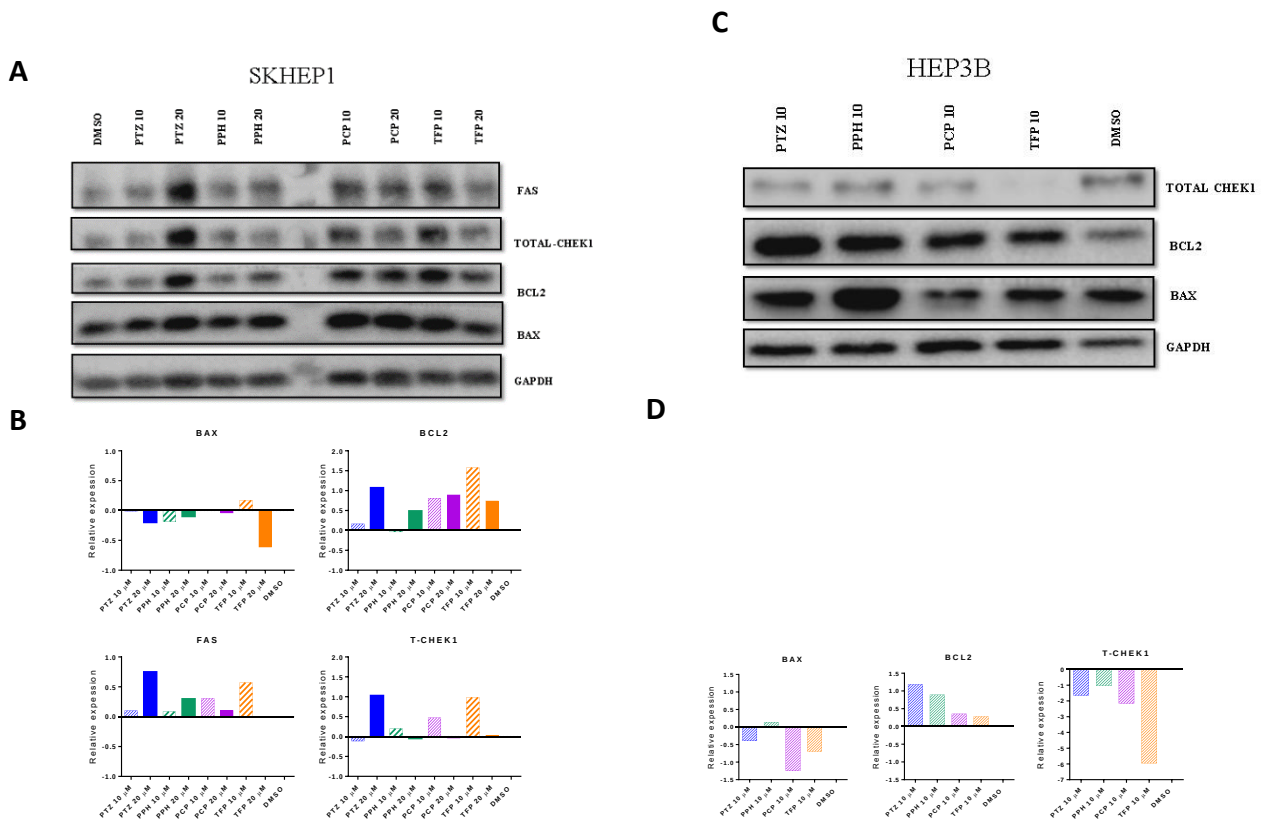
APPENDIX B: Single, commercial phenothiazine derivative treatments in SkHep1 and Hep3B to evaluate expression of cell cycle, nucleic acid processing and apoptosis markers.



Appendix B Figure 1. Expression levels of apoptosis, cell cycle, nucleic acid processing markers upon treatment of Phenothiazine derivatives in SkHep1 cell line. 20µM of commercial PTZs were applied for 24 hours. One-Way ANOVA Dunnett and Tukey Multiple comparison test was applied (n=2) (* p≤ 0.05, **≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001, ## p<0.1, #<0.2).



Appendix B Figure 2. Expression levels of apoptosis, cell cycle, DNA damage, nucleic acid processing markers upon treatment of Phenothiazine derivatives in Hep3B cell line. 20 µM of commercial PTZs were applied for 24 hours. One-Way ANOVA Dunnett and Tukey Multiple comparison test was applied (n=2) (* $p \leq 0.05$, ** ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$, ## $p<0.1$, #<0.2)).



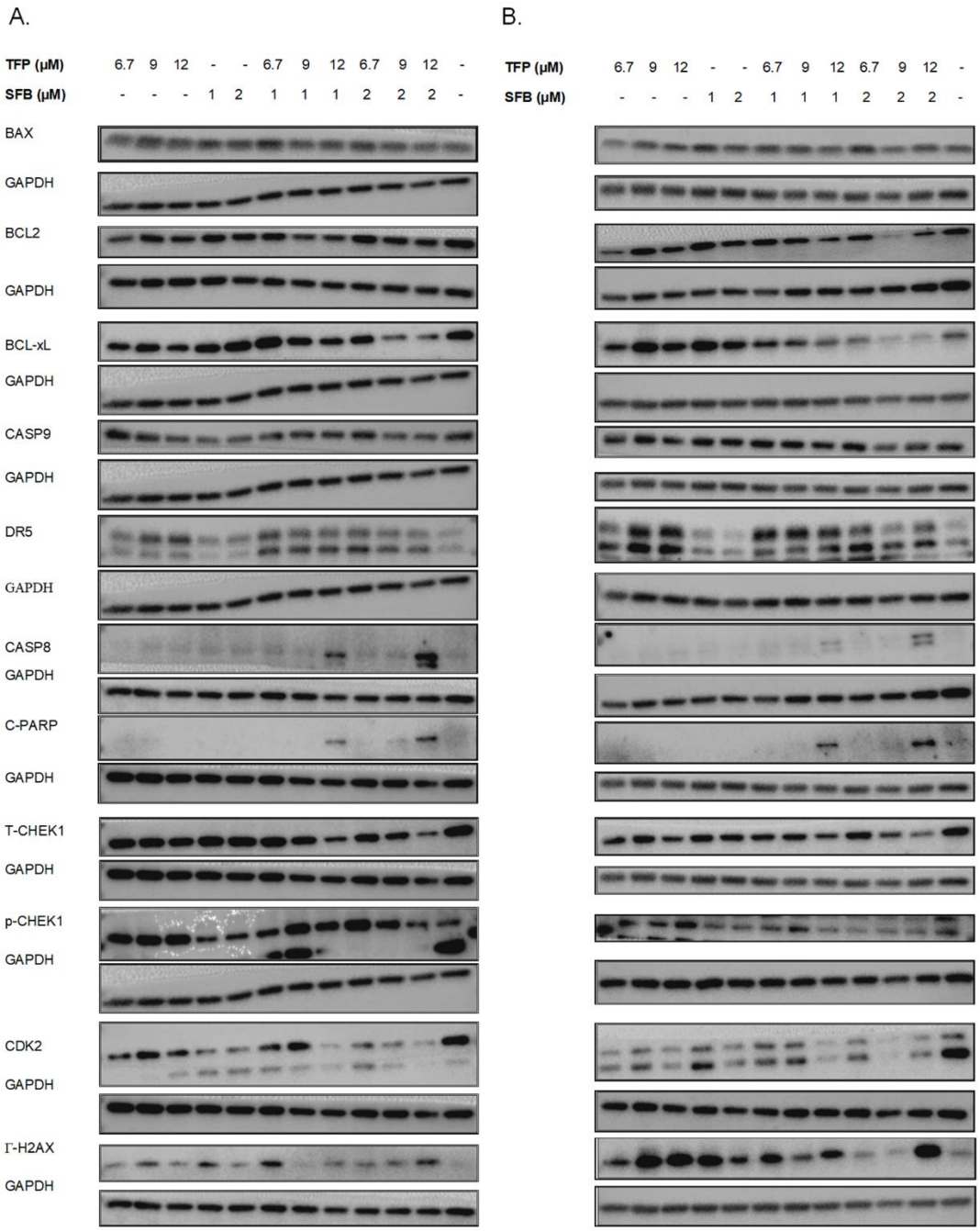
Appendix B Figure 3. Investigation of apoptosis at protein levels in SkHep1 and Hep3B cell lines upon treatment of commercial derivatives. 10 and 20µM of PTZ treatments were applied for 24 hours. A) SkHep1, b) quantification of bands in SkHep1, c) Hep3B, d) quantification of bands in Hep3B. Band quantifications were performed in ImageJ program. All proteins were normalized to internal control GAPDH and experimental control DMSO. Results were represented in log2 scale in graphs drawn in Graphpad Prism.

APPENDIX C: TFP and SFB combinatorial treatment in Hep3B and SKHep1 cell lines at protein level.

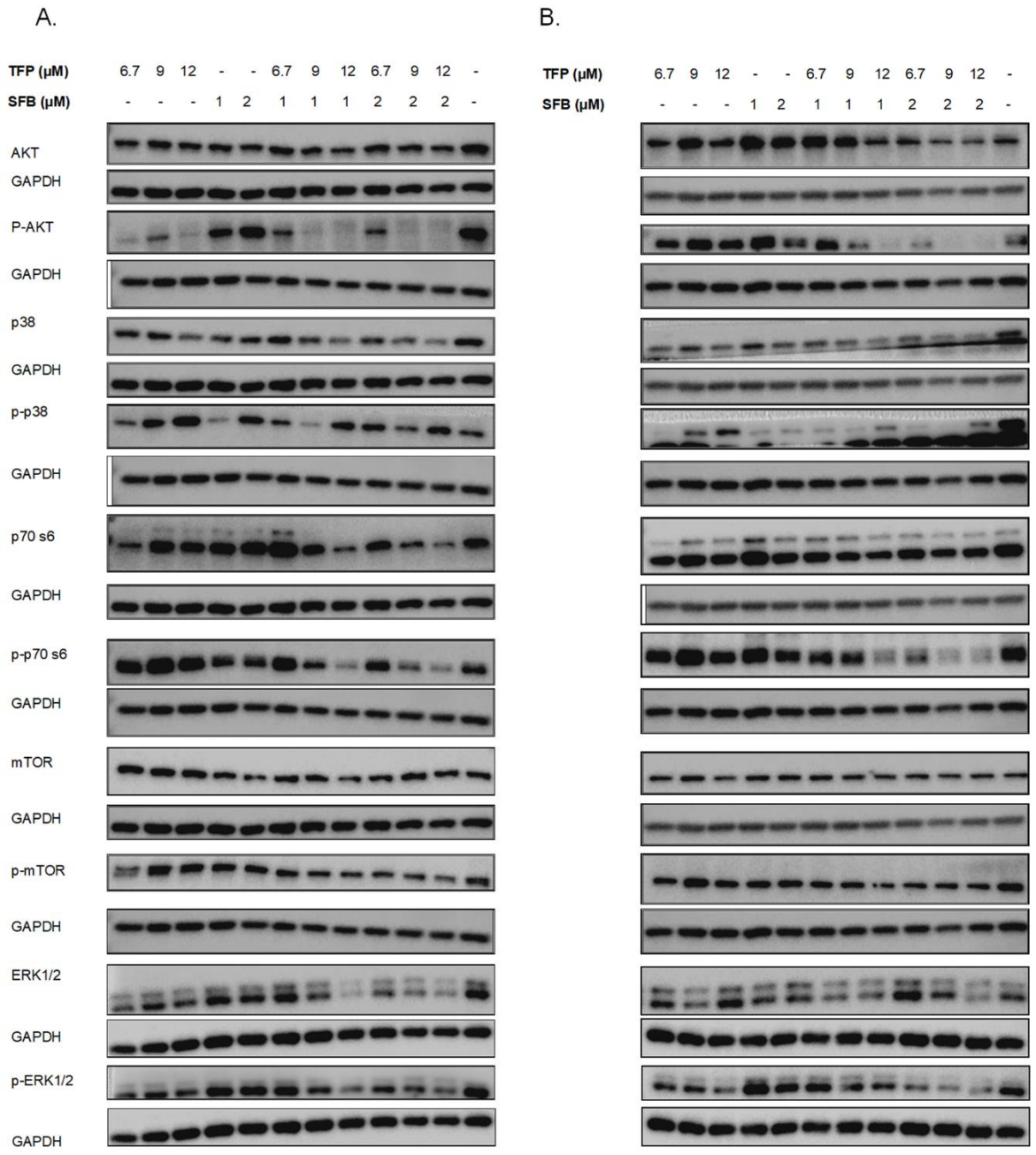
Appendix C Table 1. Log2FC and p values of chosen genes of pathways involved in HCC in Hep3B drug combination RNA-seq data.

Gene	logFC_s1	adj.P.Val_s1	logFC_s2	adj.P.Val_s2	logFC_t1	adj.P.Val_t1	logFC_ts1	adj.P.Val_ts1	logFC_ts2	adj.P.Val_ts2
TNFRSF10B	-0.03169	1	0.021765	1	0.12401707	0.510203	0.1844519	0.153707	0.310098	0.096411
BCL2L1	-0.01307	1	-0.03116	0.729992	-0.07050559	0.31751468	-0.1802957	0.060616	-0.21475	0.046943
BAX	-0.01007	1	0.007035	1	-0.0259784	1	-0.04116533	0.469692	-0.07131	0.285487
BCL2	-0.0441	1	-0.04122	1	0.01898054	1	-0.03168576	0.883008	-0.03948	0.951995
CASP9	-0.01615	1	0.060636	0.904398	-0.09345819	0.59804698	-0.01850672	1	0.044094	0.883277
PARP1	-0.0251	1	-0.05437	0.357217	-0.14505969	0.1137013	-0.24609496	0.043371	-0.2889	0.022157
CASP8	-0.01578	1	-0.03488	1	-0.03832406	1	-0.11063447	0.357505	-0.12095	0.391512
CHEK1	0.003123	1	-0.00593	1	-0.0603167	0.21812654	-0.15073169	0.047578	-0.17438	0.030744
CDK2	0.015805	1	-0.02441	0.958411	-0.06497185	0.39141997	-0.18832173	0.062912	-0.30378	0.035886
H2AFX	-0.0037	1	-0.10449	0.545369	-0.1455939	0.36370342	-0.40972243	0.061197	-0.61131	0.037253
AKT1	0.024251	1	0.046018	0.657338	0.00541680	1	0.03649119	0.436074	0.024033	0.815941
AKT2	0.009626	1	0.013283	1	-0.00587494	1	-0.04274559	0.432439	-0.07255	0.269184
AKT3	-0.09788	1	0.040858	1	0.14851189	0.62679479	0.22114362	0.203838	0.291266	0.174948
MTOR	-0.00295	1	-0.0221	1	-0.06329656	0.65285852	0.01878226	1	0.047188	0.623022
RPS6KB1	-0.04301	0.87494	-0.09249	0.275627	-0.06297017	0.33819803	-0.11689235	0.077753	-0.18553	0.05169
MAPK14	0.002905	1	-0.01468	0.304511	-0.07919369	0.02524805	-0.10691158	0.028299	-0.10108	0.010732
MAPK1	0.010816	1	-0.03148	0.951984	-0.03873587	0.78456706	-0.04721308	0.368436	-0.04038	0.542517
MAPK3	0.018631	1	0.045558	0.94711	0.05633231	0.76793045	0.04702378	0.56939	0.0286	1

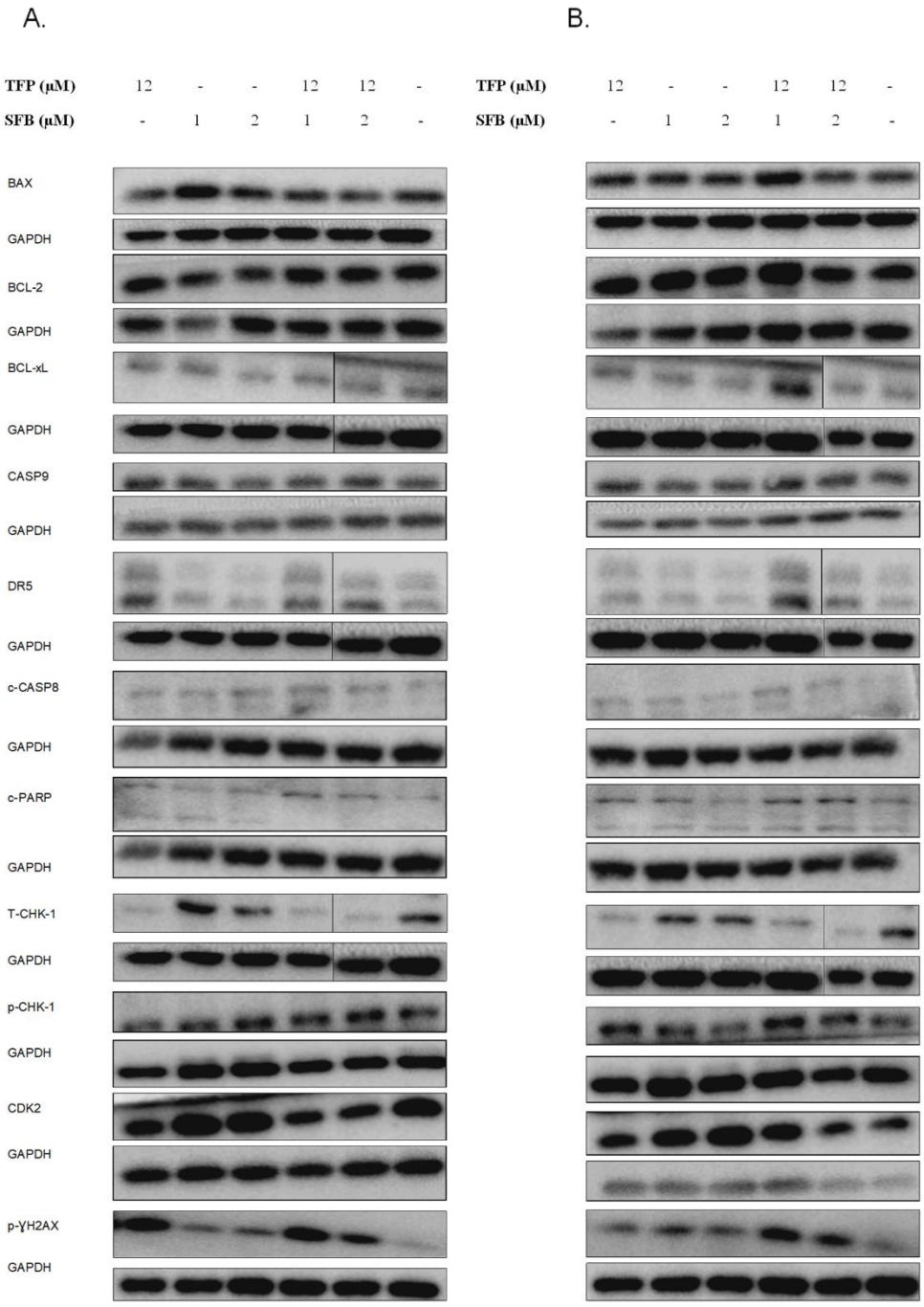
Quantified two replicates of Western Blot images of combinatorial drug treatment in liver cancer cell lines are given in the Figures below.



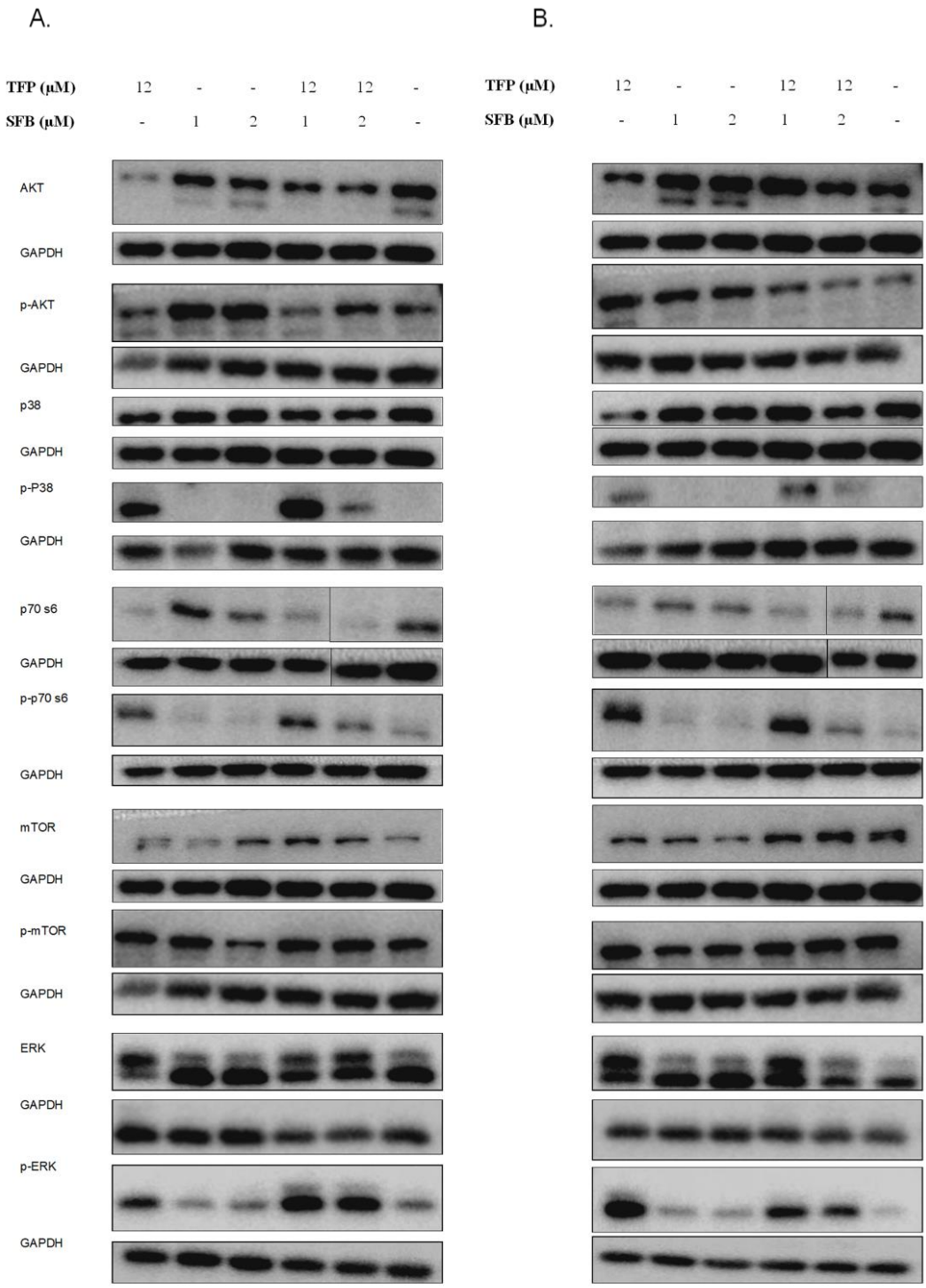
Appendix C Figure 1. Investigation apoptosis, cell cycle, DNA damage, mTOR, AKT and MAPK pathway members at protein levels in Hep3B cell line upon treatment of TFP and/or SFB in indicated doses. 48 hours of TFP and/or SFB treatments were given in indicated doses (n=2, A is replicate 1, B is replicate 2).



Appendix C Figure 1 cont. Investigation apoptosis, cell cycle, DNA damage, mTOR, AKT and MAPK pathway members at protein levels in Hep3B cell line upon treatment of TFP and/or SFB in indicated doses. 48 hours of TFP and/or SFB treatments were given in indicated doses (n=2, A is replicate 1, B is replicate 2).

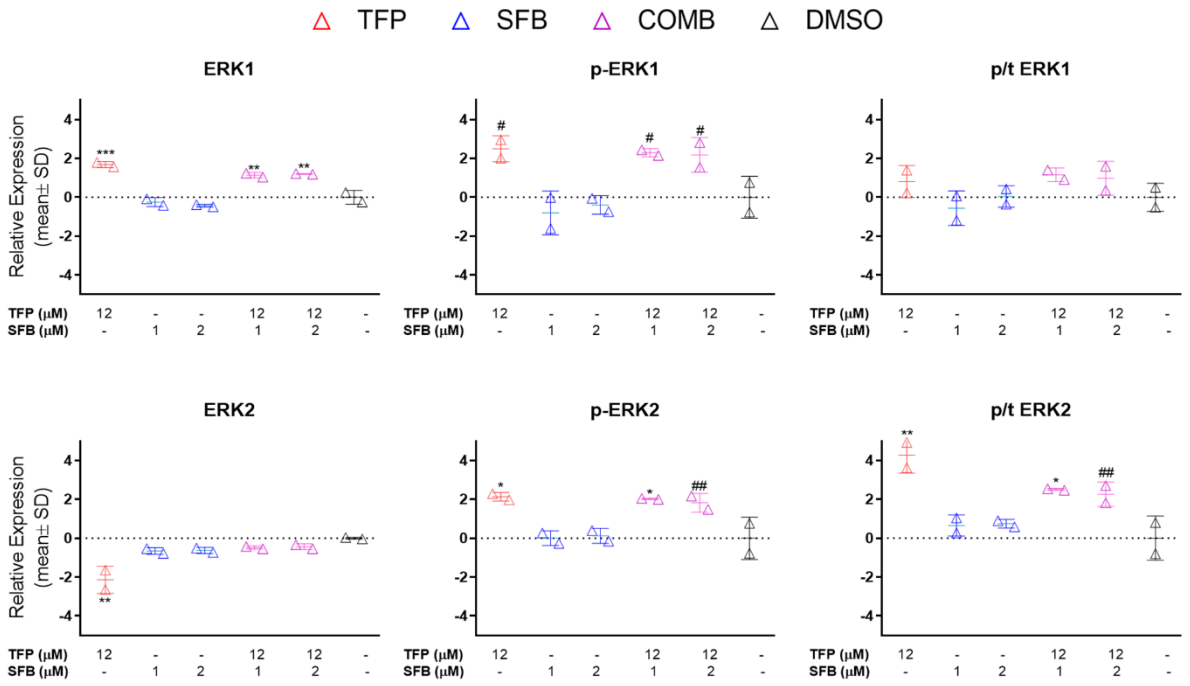


Appendix C Figure 2. Investigation apoptosis, cell cycle, DNA damage, mTOR, AKT and MAPK pathway members at protein levels in SkHep1 cell line upon treatment of TFP and/or SFB in indicated doses. 48 hours of TFP and/or SFB treatments were given in indicated doses (n=2, A is replicate 1, B is replicate 2).

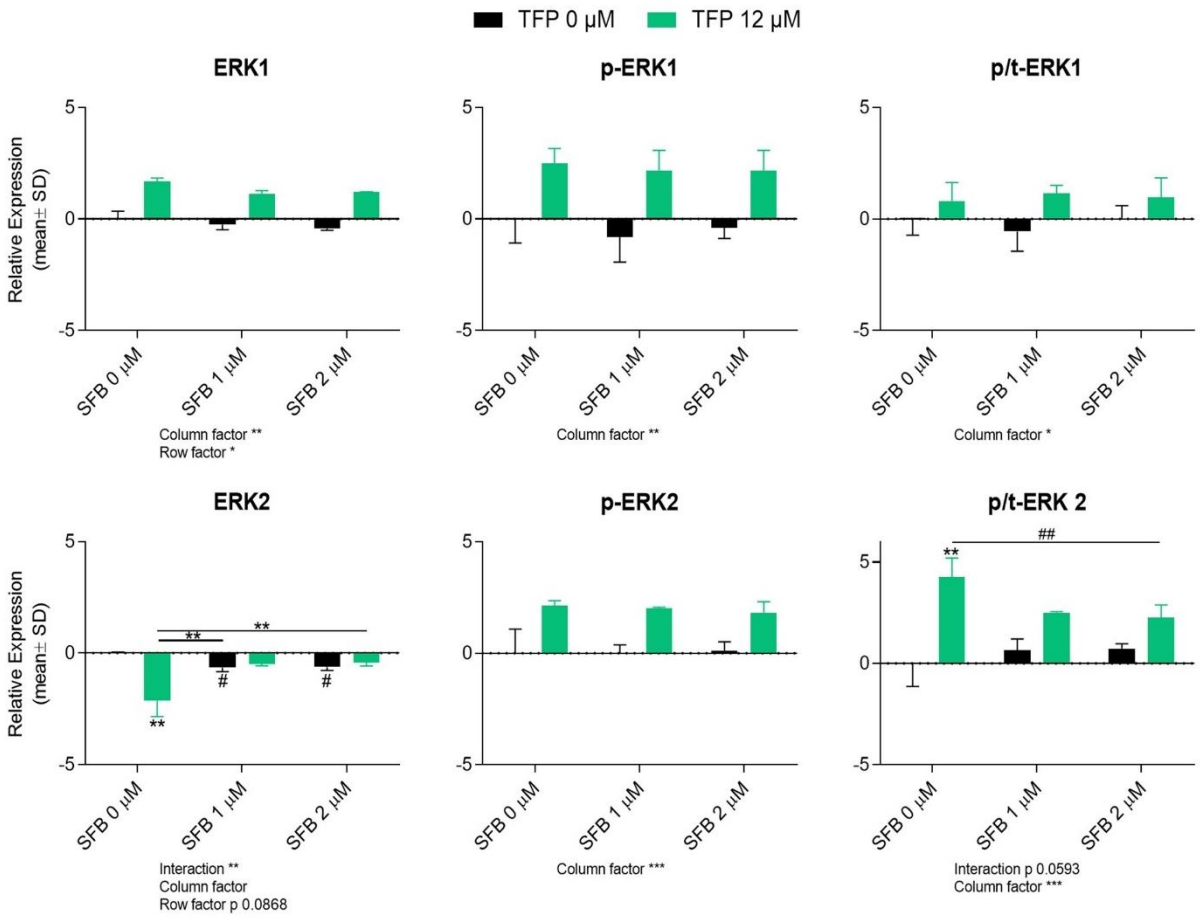


Appendix C Figure 2 cont. Investigation apoptosis, cell cycle, DNA damage, mTOR, AKT and MAPK pathway members at protein levels in SkHep1 cell line upon treatment of TFP and/or SFB in indicated doses. 48 hours of TFP and/or SFB treatments were given in indicated doses (n=2, A is replicate 1, B is replicate 2).

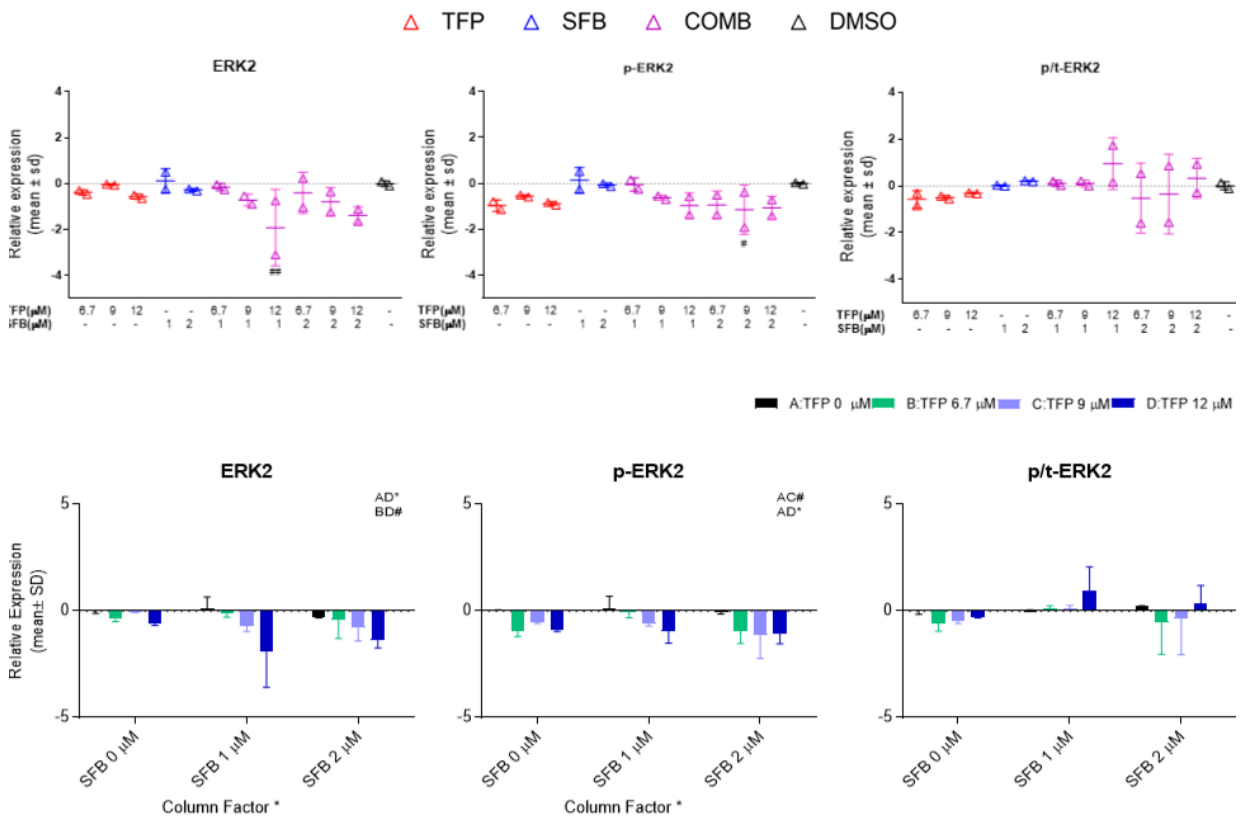
A.


TFP
SFB
COMB
DMSO
ERK1
p-ERK1
p/t ERK1
ERK2
p-ERK2
p/t ERK2
Relative Expression (mean± SD)
TFP (µM)
SFB (µM)

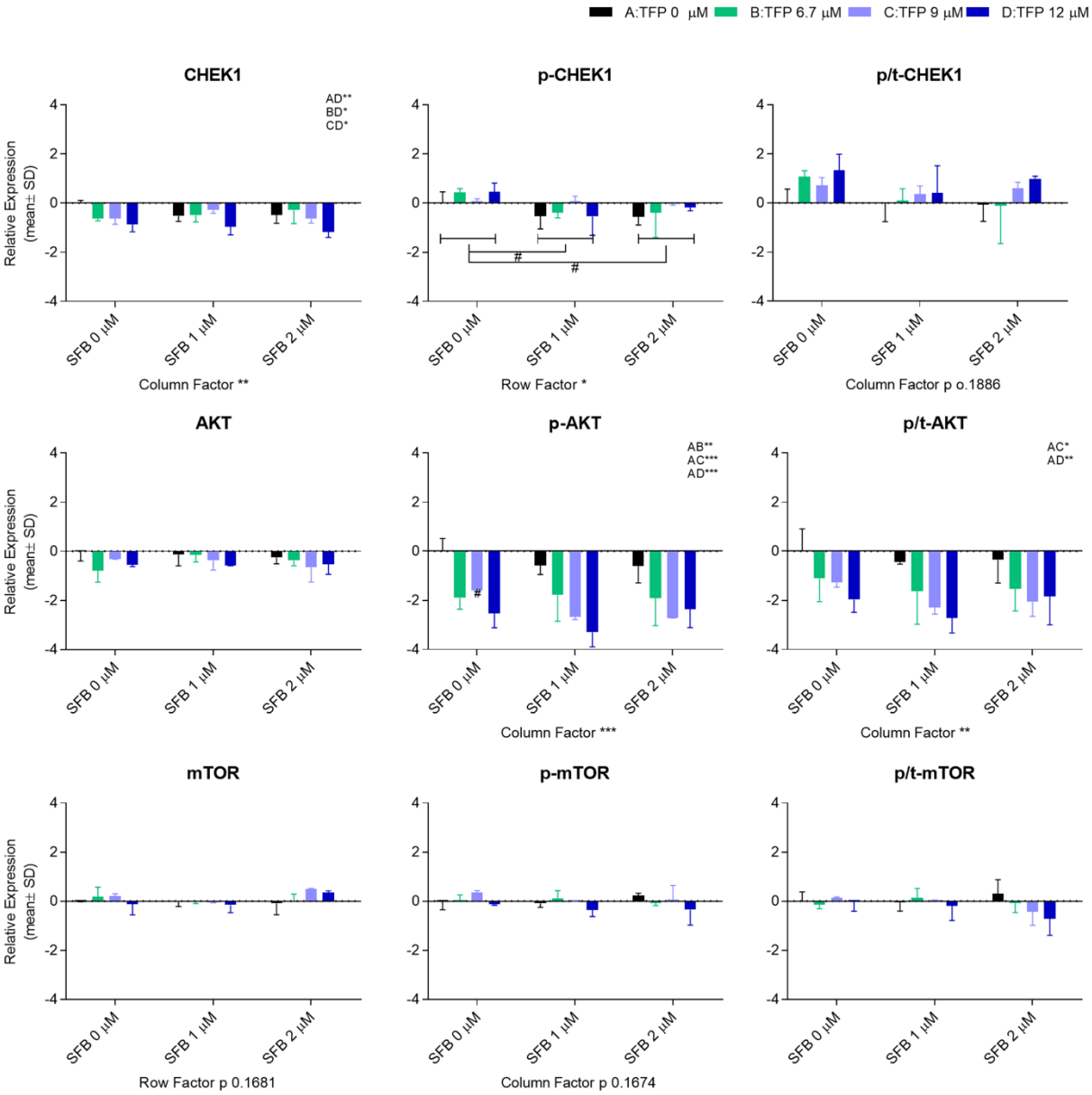
B.


TFP 0 µM
TFP 12 µM
ERK1
p-ERK1
p/t-ERK1
ERK2
p-ERK2
p/t-ERK 2
Relative Expression (mean± SD)
SFB 0 µM
SFB 1 µM
SFB 2 µM
Column factor **
Row factor *
Column factor **
Column factor *
Interaction **
Column factor
Row factor p 0.0868
Column factor ***
Interaction p 0.0593
Column factor ***

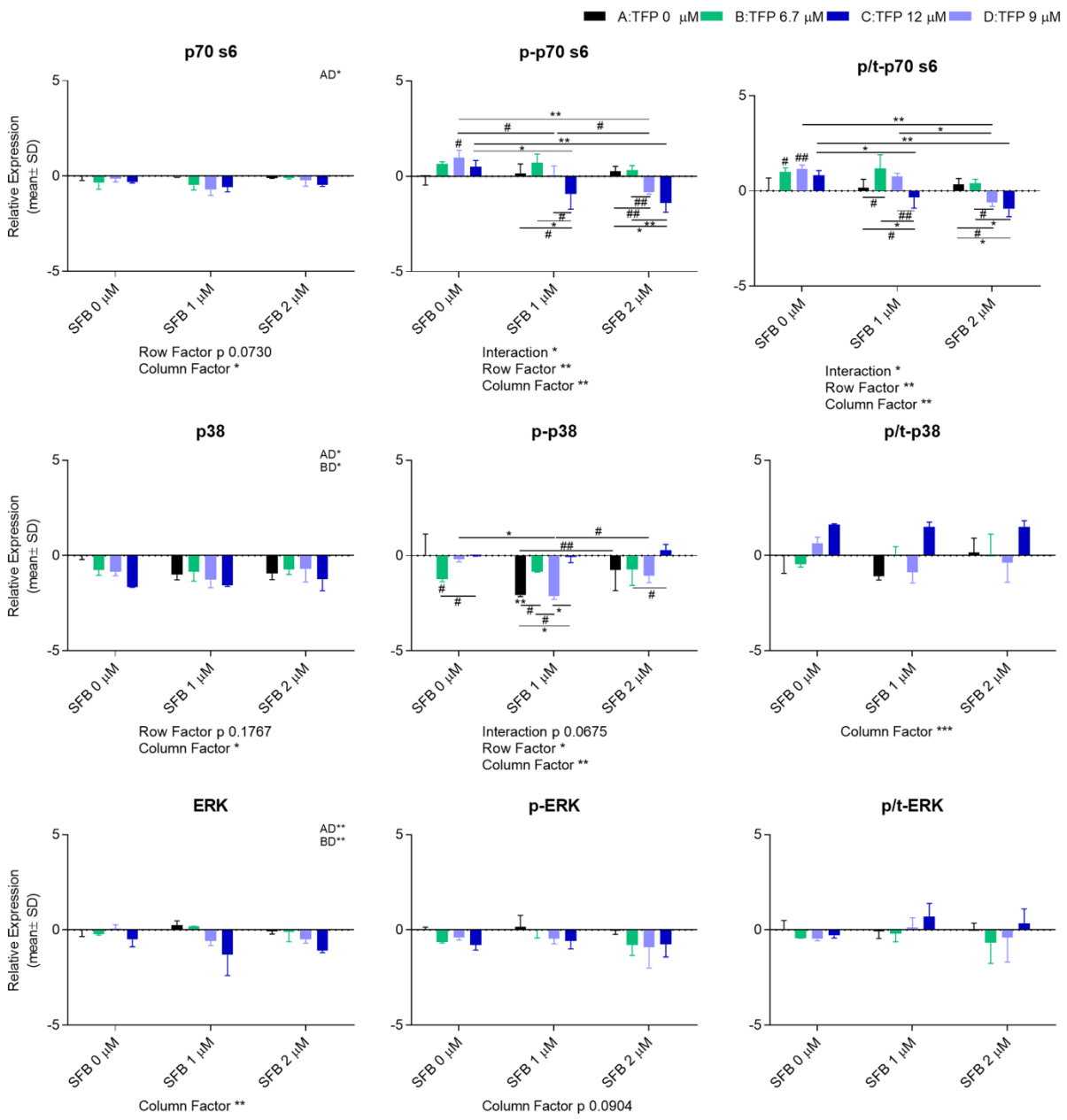
Appendix C Figure 3. Investigation of ERK 1 and ERK 2 levels in SkHep1 cell line upon treatment of TFP and/or SFB in indicated doses. 48 hours of TFP and/or SFB treatments were given in indicated doses (n=2,). A) One-way-ANOVA Dunnett Multiple comparison test. B) Two-way-ANOVA Tukey Multiple comparison test.



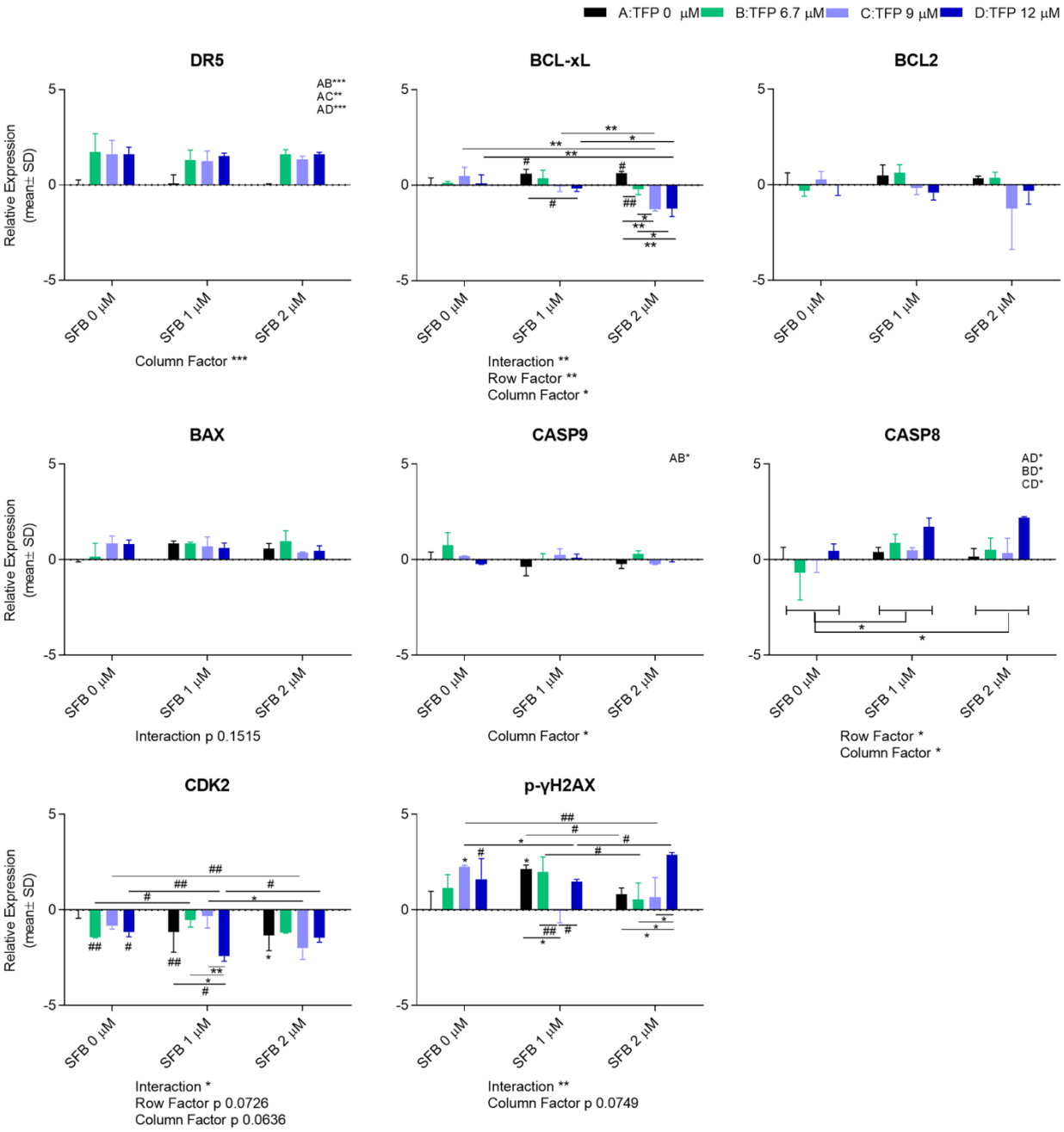
Appendix C Figure 4. Investigation of ERK 2 levels in Hep3B cell line upon treatment of TFP and/or SFB in indicated doses. 48 hours of TFP and/or SFB treatments were given in indicated doses (n=2). A) One-way-ANOVA Dunnett Multiple comparison test. B) Two-way-ANOVA Tukey Multiple comparison test.



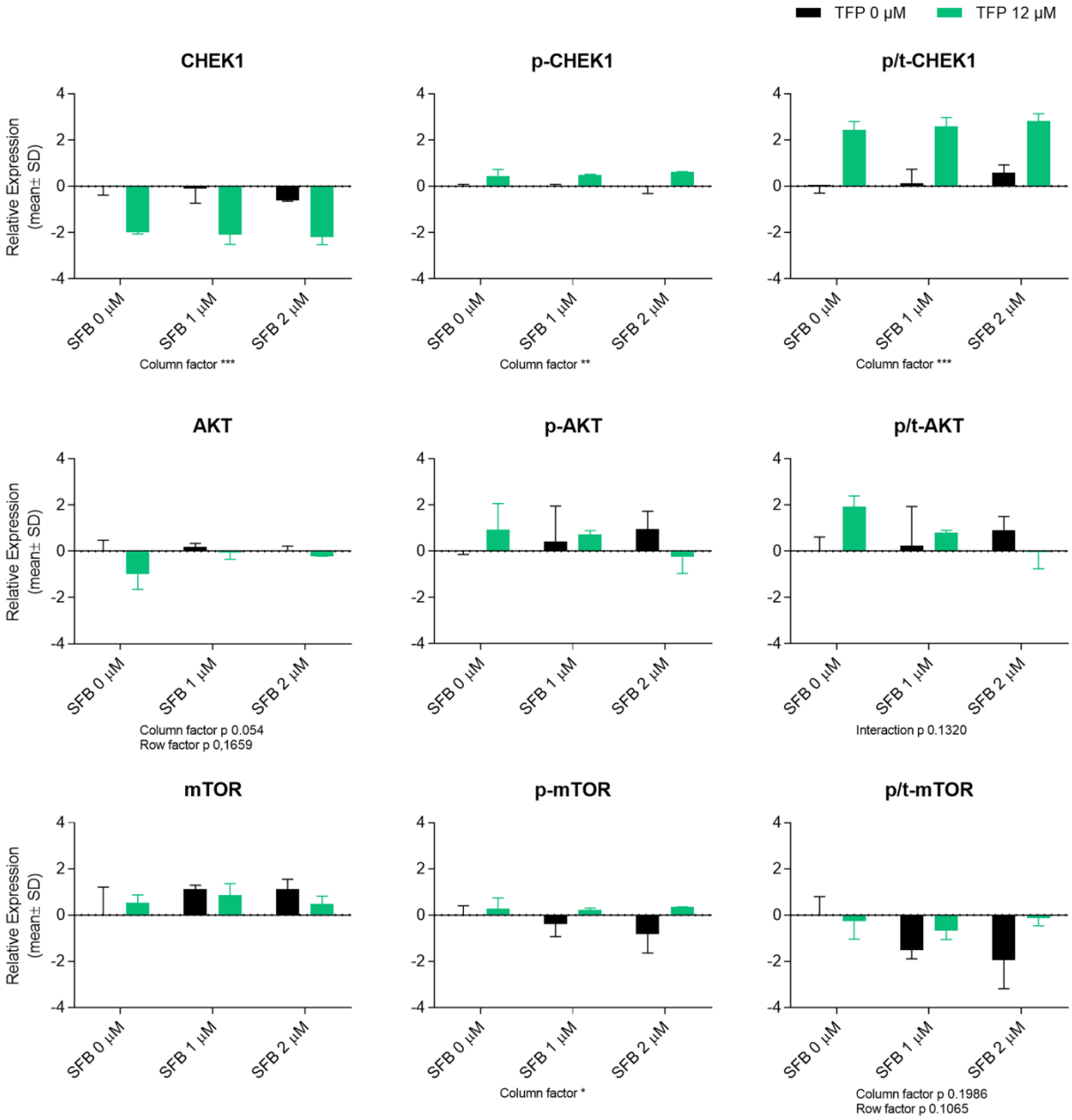
Appendix C Figure 5. Evaluation of possible synergy of drug combination in terms of protein markers in Hep3B cell line. Two-Way ANOVA-Tukey Multiple comparison test (n=2) (* $p \leq 0.05$, ** ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$, ## $p < 0.1$, # $p < 0.2$).



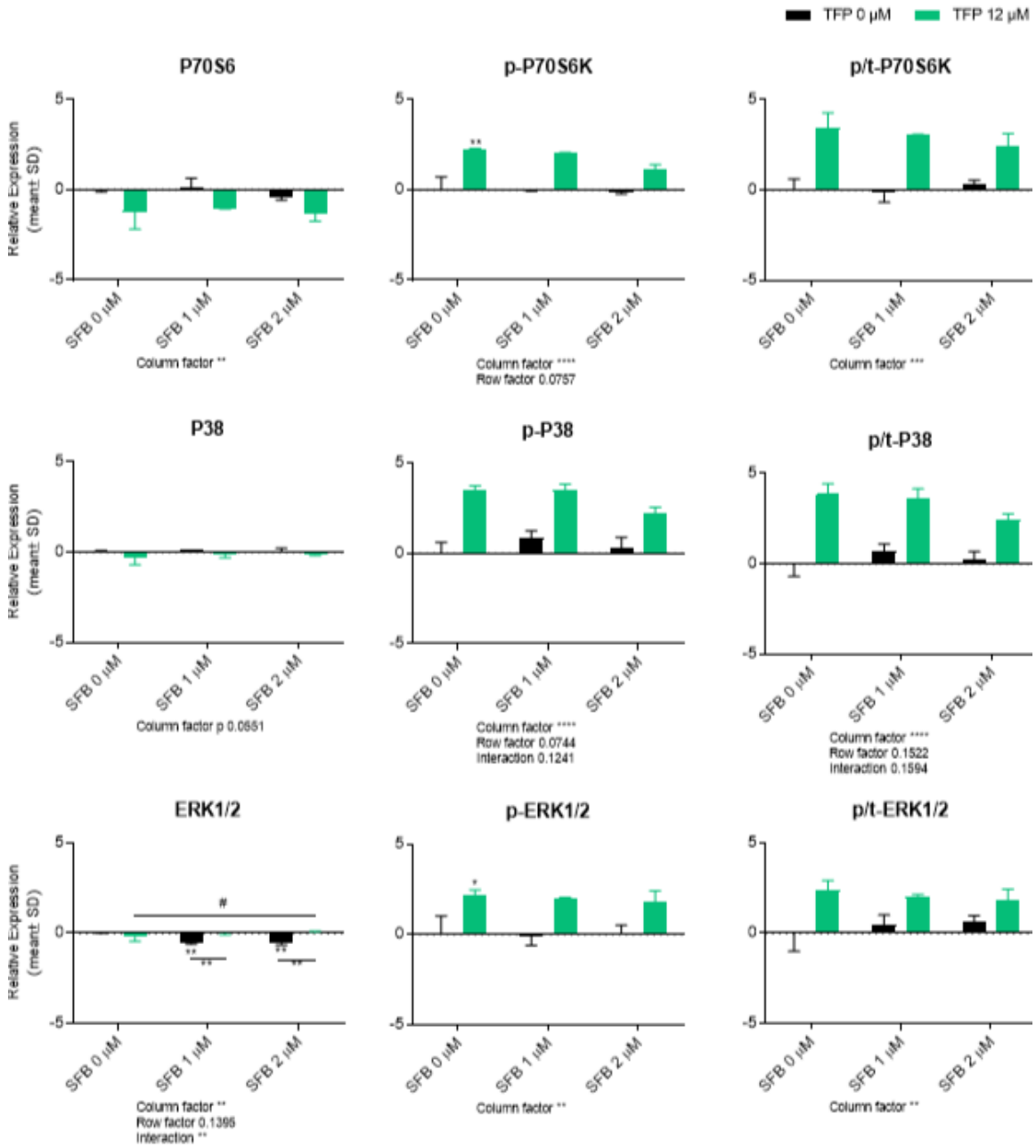
Appendix C Figure 5 cont. Evaluation of possible synergy of drug combination in terms of protein markers in Hep3B cell line. Two-Way ANOVA-Tukey Multiple comparison test (n=2) (* $p \leq 0.05$, ** ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$, ## $p < 0.1$, # $p < 0.2$).



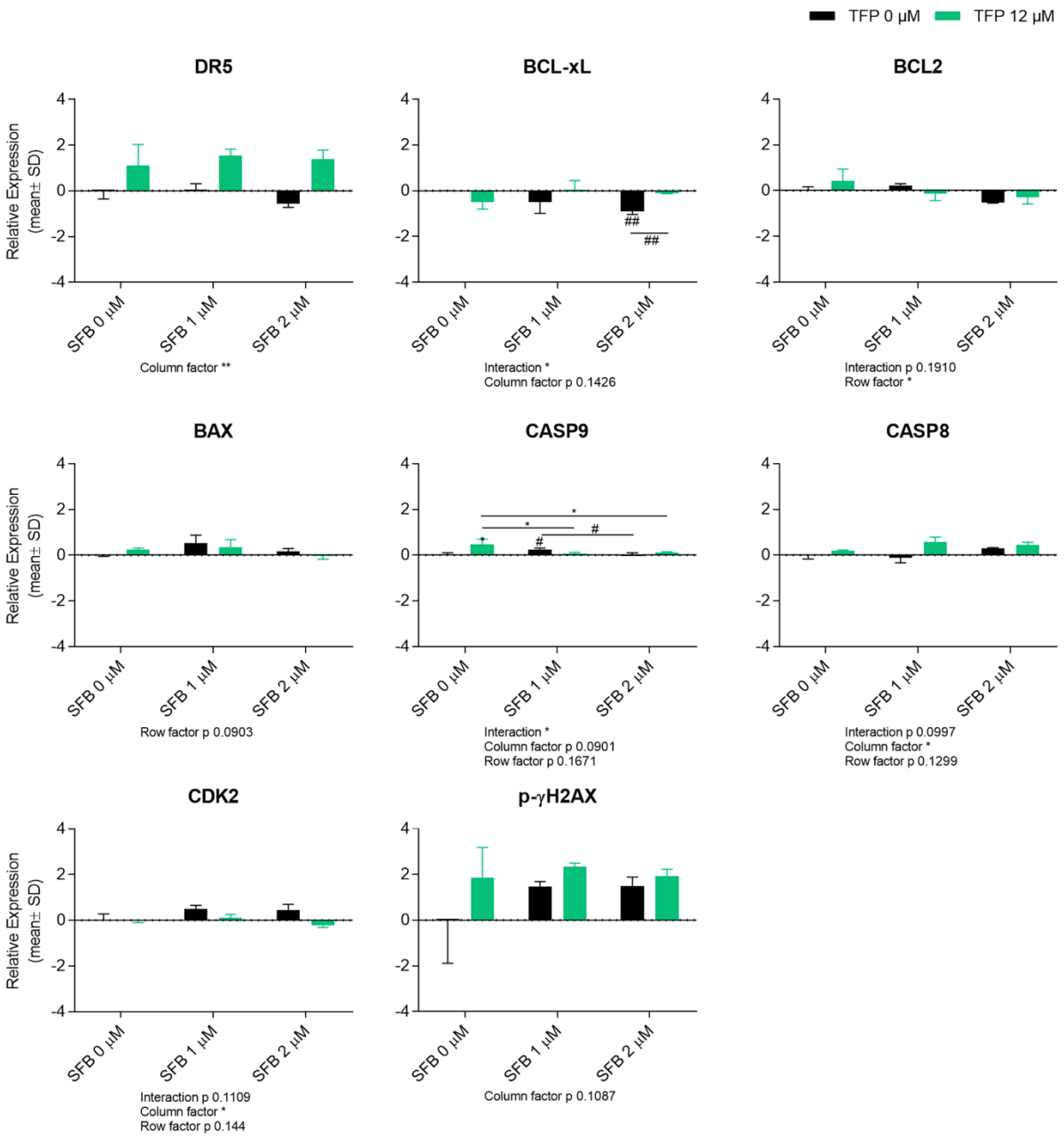
Appendix C Figure 5 cont. Evaluation of possible synergy of drug combination in terms of protein markers in Hep3B cell line. Two-Way ANOVA-Tukey Multiple comparison test (n=2) (* p≤ 0.05, **≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001, ## p< 0.1, # p< 0.2).



Appendix C Figure 6. Evaluation of possible synergy of drug combination in terms of protein markers in SkHep1 cell line. Two-Way ANOVA-Tukey Multiple comparison test (n=2) (* $p \leq 0.05$, ** ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$, ## $p < 0.1$, # $p < 0.2$).



Appendix C Figure 6 cont. Evaluation of possible synergy of drug combination in terms of protein markers in SkHep1 cell line. Two-Way ANOVA-Tukey Multiple comparison test (n=2) (* $p \leq 0.05$, ** ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$, ## $p < 0.1$, # $p < 0.2$).



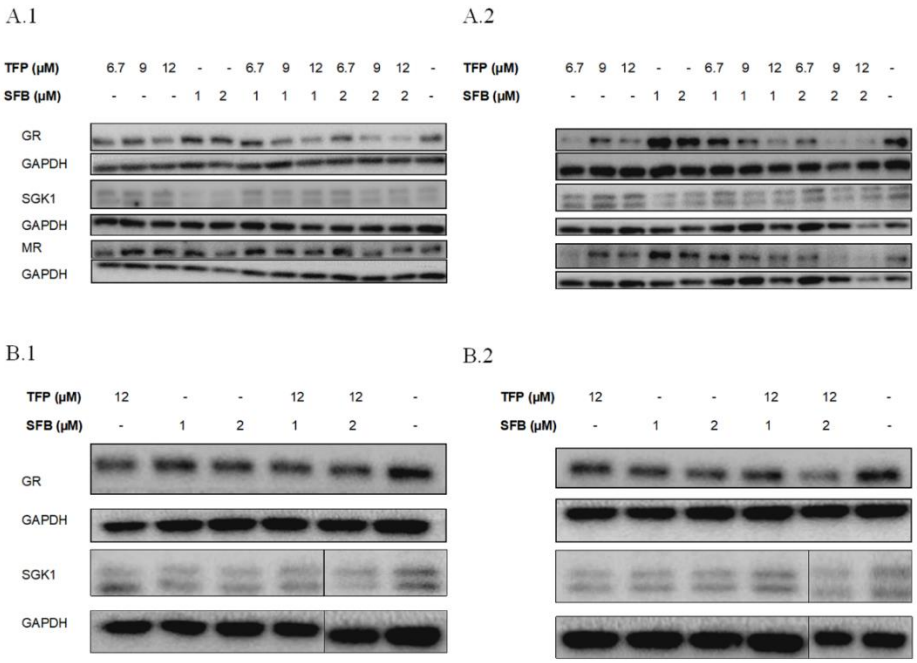
Appendix C Figure 6 cont. Evaluation of possible synergy of drug combination in terms of protein markers in SkHep1 cell line. Two-Way ANOVA-Tukey Multiple comparison test (n=2) (* $p \leq 0.05$, ** ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$, ## $p < 0.1$, # $p < 0.2$).

APPENDIX D: Nuclear Receptor Family in liver cancer cell lines upon combinatorial TFP-SFB treatment

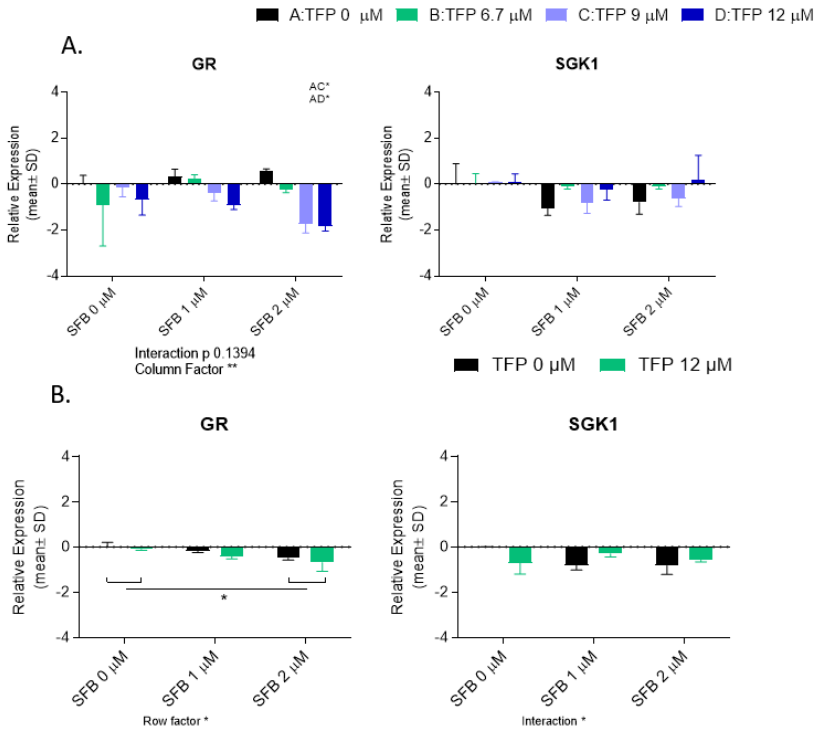
Nuclear Receptor Family members were evaluated upon combinatorial treatment in Hep3B SkHep1 cell lines at protein level. Below, two replicates evaluated for statistical analysis are given.

Appendix D Table 1. RNA-seq differential gene expressions of chosen SHRs and their downstream effectors of their signaling pathways (s1: SFB 1µM, s2: SFB 2µM, t1: TFP 12µM, ts1: TFP 12µM-SFB 1µM, ts2: TFP 12µM-SFB 2µM).

Gene	logFC_s1	adj.P.Val_s1	logFC_s2	adj.P.Val_s2	logFC_t1	adj.P.Val_t1	logFC_ts1	adj.P.Val_ts1	logFC_ts2	adj.P.Val_ts2
NR3C2	0.040399	1	0.16857	0.513334506	-0.00868	1	0.135307	0.298277549	0.220052	0.196595202
NR3C1	0.043457	0.791907178	0.007265	1	-0.10637	0.187844826	-0.14992	0.061173007	-0.08771	0.101747977
PGR	0	1	0	1	0	1	0	1	0	1
ESR1	-0.01797	1	-0.01797	1	-0.01797	1	0.025547	1	-0.01797	1
ESR2	-0.01045	1	0.108982	1	0.00484	1	0.01797	1	0.10602	0.850696927
TFF1	-0.05281	1	-0.07021	0.931426943	-0.00468	1	-0.00456	1	-0.00726	1
SGK1	-0.04708	1	-0.01343	1	-0.16656	0.290320132	-0.40094	0.058974754	-0.48694	0.044005494
NEDD4L	0.064977	1	0.080737	0.662260452	-0.06378	0.752907765	0.051275	0.577558501	0.113481	0.259291768
SCNN1A	-0.01526	1	0.028381	1	0.107772	0.661850473	0.291586	0.10702942	0.305992	0.117092424
SCNN1B	0	1	0.039652	0.566065538	0.034001	0.5941104	0.011347	0.883007739	0	1
SCNN1D	0.105237	0.374471355	0.109256	0.232454708	0.287808	0.05474636	0.280475	0.044226844	0.398792	0.018758627
SCNN1G	0.009386	1	-0.00802	1	0.025986	1	0.014116	1	0.054937	0.528497094
IGF1	-0.01536	1	-0.07127	1	-0.07631	1	-0.13458	0.411159566	-0.09398	0.736656797
IGF1R	-0.01382	1	0.012244	1	-0.0042	1	0.176603	0.057386551	0.168524	0.054088303
ACE	0.029937	1	0.03487	1	0.029075	1	0.013359	1	0.031411	1
ACE2	-0.0102	1	0.013862	1	0.036143	1	0.032986	1	-0.00137	1
ZBTB16	0.068758	1	-0.01448	1	0.011401	1	0.023191	1	0.09692	0.830966712
PER1	-0.02592	0.732702581	0.017059	0.619658298	0.054611	0.191805169	0.119241	0.04748522	0.378491	0.005678643
PER2	-0.04615	1	-0.0471	1	-0.12379	0.446951438	-0.03175	0.883007739	0.136638	0.230061303
FKBP5	-0.06262	1	-0.13224	0.382238136	-0.10099	0.433814832	-0.21713	0.083913036	-0.26724	0.076494977
NFKB1	-0.04113	1	-0.09883	0.444135677	-0.04464	0.802096366	-0.15927	0.100879191	-0.16795	0.109304999
NFKB2	-0.01796	1	0.04646	1	0.134728	0.757914399	0.179673	0.309256289	0.207116	0.317411928
CDK2	0.015805	1	-0.02441	0.958410804	-0.06497	0.391419973	-0.18832	0.062912473	-0.30378	0.035885858
STAT3	-0.03165	0.555843579	-0.05487	0.209116486	-0.05292	0.177566591	-0.08828	0.053088796	-0.10507	0.036942833
EGFR	0.046707	0.904106441	0.082208	0.352010006	-0.0224	0.960562626	0.129846	0.080303553	0.196476	0.05721563



Appendix D Figure 1. Investigation of Nuclear Receptor Family members and downstream effectors at Protein. A.) Hep3B and B.) SkHep11 cell lines**.** 48 hours of TFP and/or SFB treatments were given in indicated doses (n=2).



Appendix D Figure 2. Evaluation of possible synergy of drug combination in terms of GR and SGK1. A) Hep3B, B) SkHep1 cell lines**.** Two-Way ANOVA-Tukey Multiple comparison test (n=2) (* $p \leq 0.05$, **≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$, ## $p < 0.1$, # $p < 0.2$).

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