THE ROLE OF AKR1B10 IN OXIDATIVE STRESS RESPONSE IN HEPATOCELLULAR CARCINOMA

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ABSTRACT

THE ROLE OF AKR1B10 IN OXIDATIVE STRESS RESPONSE IN HEPATOCELLULAR CARCINOMA

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Hepatocellular carcinoma (HCC) is projected to account for 4.7 percent of all new cancer cases and 8.2 percent of all cancer-related deaths globally. NADPH-dependent aldoketoreductases (AKRs) can reduce carbonyl substrates like glucose, sugar aldehydes, keto-steroids, keto-prostaglandins, retinals, quinones, and lipid peroxidation by-products. A member of this family of enzymes, AKR1B10, is thought to be a robust early marker for HCC as well as a predictor of poor prognosis. Oxidative stress in cells may result from an imbalance between the synthesis and buildup of reactive oxygen species (ROS). Mitigation of oxidative stress is largely dependent on the activation of enzymes that can reduce ROS using electrons from the cofactor NADPH. NADPH can be consumed by reductive reactions such as fatty acid synthesis, which mainly occurs in the liver. The pentose phosphate pathway (PPP) is one of the primary sources of NADPH in the cell. We have hypothesized that AKR1B10 may have a cross talk with the PPP and alter the antioxidant response or fatty acid metabolism. To address this, I treated HCC cell lines expressing AKR1B10 with the pro-oxidant Manumycin A or with 6-AN, a PPP inhibitor. We observed that HuH-7 cells showed a robust antioxidant response and survival when treated with Manumycin A, whereas SNU-423 cells were highly vulnerable to the

drug. A higher antioxidant response was observed in stressed SNU423 cells expressing AKR1B10, however, this response was not sufficient to restore survival. Unlike SNU423 cells, Huh-7 cells showed high survival when treated with to 6-AN, indicating that these cells were not reliant on NADPH from the PPP for their survival. 6-AN treatment, however, increased the phosphorylation of ACC as well as AMPK in HuH7 cells, especially in cells that ectopically expressed AKR1B10, suggesting the induction of fatty acid oxidation. The same cells were also highly vulnerable to $CoCl_2$ indicating high reliance of oxygen for survival. Taken together, our results suggest that AKR1B10 expression in HCC can not only increase an antioxidant response, but also increase fatty acid oxidation for energy generation, providing a mechanistic basis for poor prognosis observed with high expression of AKR1B10 in HCC.

Key words: Aldo-keto reductases, ROS, NADPH, pentose phosphate pathway

ÖZ

HEPATOSELLÜLER KARSİNOMDA OKSİDATİF STRES YANITINDA AKR1B10 ROLÜ

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Hepatoselüler karsinomun (HCC), dünya çapında tüm yeni kanser vakalarının yüzde 4,7'sini ve kansere bağlı tüm ölümlerin yüzde 8,2'sini oluşturduğu tahmin edilmektedir. Nikotinamid adenin dinükleotid fosfatlara (NADPH) bağlı aldoketoredüktazlar (AKR'ler), glikoz, şeker aldehitler, ketosteroidler, ketoprostaglandinler, retinaller, kinonlar ve lipid peroksidasyon yan ürünleri gibi karbonil substratlarını azaltabilir. Bu enzim ailesinin bir üyesi olan AKR1B10'un, HCC için güçlü bir erken belirteç olduğu kadar, kötü prognozun bir öngörücüsü olduğu düşünülmektedir. Hücrelerdeki oksidatif stres, reaktif oksijen türlerinin (ROS) sentezi ve birikmesi arasındaki dengesizlikten kaynaklanabilir. Oksidatif stresin hafifletilmesi, büyük ölçüde, kofaktör NADPH'den elektronları kullanarak ROS'u azaltabilen enzimlerin aktivasyonuna bağlıdır. NADPH, esas olarak karaciğerde meydana gelen yağ asidi sentezi gibi indirgeyici reaksiyonlarla tüketilebilir. Pentoz fosfat yolu (PPP), hücredeki birincil NADPH kaynaklarından biridir. AKR1B10'un PPP ile çapraz konuşma yapabileceğini ve antioksidan tepkisini veya yağ asidi metabolizmasını değiştirebileceğini varsaydık. Bunu ele almak için, AKR1B10'u eksprese eden HCC hücreleri pro-oksidan Manumycin A

veya bir PPP inhibitörü olan 6-AN uygulandı. HuH-7 hücrelerinin Manumycin A ile tedavi edildiğinde güçlü bir antioksidan tepki ve hayatta kalma gösterdiğini, oysa SNU-423 hücrelerinin ilaca karşı oldukça savunmasız olduğunu gözlemledik. AKR1B10'u eksprese eden SNU423 hücrelerinde daha yüksek bir antioksidan tepki gözlendi, ancak bu tepki hayatta kalmak için yeterli değildi. SNU423 hücrelerinin aksine, Huh-7 hücreleri, 6-AN ile tedavi edildiğinde ölmediler; bu durum, bu hücrelerin hayatta kalmaları için PPP'den NADPH'ye bağımlı olmadığını göstermektedir. Ancak 6-AN tedavisi, HuH7 hücrelerinde, özellikle AKR1B10'u ektopik olarak eksprese eden hücrelerde, ACC'nin yanı sıra AMPK'nin fosforilasyonunu arttırdı, bu da yağ asidi oksidasyonunun indüklendiğini düşündürdü. Aynı hücreler ayrıca kobalt klorüre ($CoCl_2$) karşı oldukça savunmasızdı ve bu da hayatta kalmak için oksijene bağlı olduklarını göstermektedir. Birlikte ele alındığında, sonuçlarımız HCC'de AKR1B10 ekspresyonunun sadece bir antioksidan tepkisini arttırmadığını, aynı zamanda enerji üretimi için yağ asidi oksidasyonunu arttırdığını ve HCC'de yüksek AKR1B10 ekspresyonu ile gözlenen kötü prognoz için mekanik bir temel sağladığını göstermektedir.

Anahtar Kelimeler: Aldo-keto redüktaz, ROS, NADPH, pentoz fosforilasyonu yolağı

This thesis is dedicated to my family & love.

For their endless love and support...

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ABBREVIATIONS

AKR Aldo-keto reductases

NADPH Nicotinamide adenine dinucleotide phosphate, reduced form

$NADP^+$ Nicotinamide adenine dinucleotide phophate, oxidized form

PAGE polyacrylamide gel electrophoresis

PBS Phosphate- buffered saline

ROS Reactive Oxygen Species

PPP Pentose Phosphate Pathway

HCC Hepatocellular Carcinoma

IDH Isocitrate Dehydrogenase

ME Malic Enzyme

FAO Fatty Acid Oxidation

CHAPTER 1

INTRODUCTION

1.1 Hepatocellular Carcinoma

Liver cancer is the sixth most common cancer type in prevalence and the fourth in mortality worldwide. 90% of the liver cancer cases are hepatocellular carcinoma (HCC) which makes it the most common form (Fitzmaurice et al., 2017). Even though cirrhosis, alcohol consumption, hepatitis B and C infections are the most prominent causes for HCC, predominance of HCC in individuals with a western diet suggest that metabolic syndrome and diabetes may also enhance the risk of developing HCC (Estes, Razavi, Loomba, Younossi, & Sanyal, 2018).

The development of HCC occurs in several steps. The first step is chronic liver disease after chronic inflammation, which results in increased hepatocyte regeneration. Following hepatocyte formation, telomerase reactivation and increased genomic instability are seen, which can eventually result in malignant transformation (Farazi & DePinho, 2006).

HCC has one of the poorest prognosis rates at the later stages of the disease. The mortality rate can be decreased by 37% if the patients can access screening early (Lederle & Pocha, 2012). HCC has many molecular classifications that can be used to identify the stage and treatment options, however molecular biomarkers are limited. Increased α-fetoprotein in the serum is the only known and widely used biomarker and it shows poor prognosis at all stages of HCC (Galle et al., 2019). Robust biomarkers that can be evaluated from liquid biopsies are preferred to increase screening. In a recent study, it was shown that AKR1B10 can be a good

biomarker for early HCC; AKR1B10 can be secreted into the serum and its levels in the serum was 18 times higher in HCC patients compared to the control group (Ye et al., 2019b). Similar results were also shown in another study with 78 HCC patients (Han et al., 2018). Moreover, serum levels of AFP and AKR1B10 together may predict the incidence of HCC better than AFP alone (Zhu et al., 2019). It is, however, unknown why the expression of AKR1B10 is increased early during the transformation of hepatocytes and mechanistically why AKR1B10 expression predicts worse prognosis.

1.2 AKR1B10

The aldo-keto reductase superfamily of enzymes are cytosolic NADPH-dependent monomeric proteins (Borski et al., 2008). These enzymes utilize reducing electrons from NADPH and act as catalysts in redox reactions including biosynthesis, detoxification, reduction of some carbonyl compounds such as aromatic aldehydes, ketones, steroids, xenobiotics, and products of lipid peroxidation (Penning, 2015).

AKR1B10 is a member of the AKR1B subfamily along with AKR1B1 and AKR1B15 (Salabei, Li, Petrash, Bhatnagar, & Barski, 2011). Unlike its partners, AKR1B10 is predominantly expressed in the small intestine and adrenal gland with low expression also detected in the liver and colon (Hyndman & Flynn, 1998). Several studies have found that the expression of AKR1B10 increases dramatically in the early stages of HCC and then decreases as the disease progresses to metastatic stages. AKR1B10 expression in HCC has been associated with Hepatitis-B virus (HBV) infection. The expression of AKR1B10 was reported to be higher in moderately differentiated HCC tumors compared to well-differentiated and poorly differentiated tumors. (Kanno et al., 2019). Knockdown of AKR1B10 in HCC cell lines resulted in increased cell death with apoptosis, sensitivity to doxorubicin and smaller colony formation (Tsuzura et al., 2014). Oxidative stress was discovered to upregulate AKR1B10 in hepatocellular carcinoma (HCC) cells via the

transcriptional activation of NRF2, the master regulator of oxidative stress response (Endo, Matsunaga, & Nishinaka, 2021a). In normal conditions NRF2 is located in the cytosol; when activated via oxidative stress it translocates to nucleus and acts as a transcription factor for antioxidant genes such as hemeoxygenase-1 (HMOX1), thioredoxin reductase-1 (TXNRD1), and glutathione peroxidase 2 (GPX2), among others (Raghunath, Sundarraj, Arfuso, Sethi, & Perumal, 2018).

As an enzyme, AKR1B10 exhibits more restricted substrate specificity than general human aldo-keto reductases. Farnesyl, geranylgeranyl, retinal and carbonyls are among the compounds that are known to serve as suitable substrates for AKR1B10 (Chung et al., 2012). Reduction of farnesyl and geranylgeranyl enable them to post translationally modify proteins via prenylation, which in turn can change the localization of the protein to the membrane, enable protein-protein interactions and affect signal transduction pathways. AKR1B10 is also known to efficiently reduce retinal to retinol, which prevents the conversion of retinal to retinoic acid, the latter known to serve as an anti-neoplastic metabolite. Retinol formed from retinal can be reconverted to retinal, which in turn can be irreversibly oxidized to retinoic acid, a signaling molecule that enhances cell differentiation. Increased expression of AKR1B10 in cancer cells may increase the conversion of retinal to retinol along with a reduction in the levels of retinoic acid thereby leading to a shift from differentiation to proliferation. AKR1B10 can also catalyze the conversion of highly reactive aldehydes and ketones into hydroxyl groups. Carbonyl compounds can often induce apoptosis; therefore, AKR1B10 expression in cancer cells may protect them from carbonyl compounds particularly those found in chemotherapy drugs.

AKR1B10 was also shown to enhance lipid synthesis in a breast cancer cell line RAO-3. A physical interaction of AKR1B10 with Acetyl CoA Carboxylase (ACC) was reported, leading to enhanced degradation of the latter (Ma et al., 2008). Silencing of AKR1B10 in human colon carcinoma cells (HCT-8) and lung carcinoma cells (NCI-H460) cells was shown to reduce the cellular lipid content, particularly of phospholipids, by more than 50% (C. Wang et al., 2009). Therefore,

in addition to its role as a reductase, we have hypothesized that AKR1B10 may affect survival in HCC by modulating lipid synthesis, mitochondrial function, and oxidative status.

1.3 Nicotinamide Adenine Dinucleotide Phosphate (NADPH)

NADPH acts as a co-enzyme and supplies electrons for anabolic reactions and redox homeostasis in all living organisms. NADPH fuels activities of some enzymes such as nicotinamide adenine dinucleotide phosphate-oxidase, superoxide dismutase, catalase, and glutathione peroxidase (Ying, 2008). NADPH predominantly works in antioxidant mechanisms against cellular redox stress by supplying reducing agents to antioxidants like glutathione and thioredoxin. In addition, NADPH also acts as an electron donor for several anabolic reactions such as synthesis of fatty acids, steroids, and nucleotides (Xiao, Wang, Handy, & Loscalzo, 2018). Due to the high energy need of tumor cells, their NADPH/NADP ratios are generally higher compared to non-transformed cells. In neoplastic cells, reactive oxygen species (ROS) levels are important in signaling, but too much ROS may increase DNA damage and induce cell death via different pathways. Therefore, tumor cells use NADPH not just to maintain their rapid growth via activation of anabolic pathways, but also increase the capacity of their redox defense systems (Moreno-Sánchez et al., 2018).

1.3.1 Synthesis of NADPH

NADP is produced from NAD+ with mitochondrial and cytosolic NAD Kinases (NADK) using ATP. NADKs are mostly expressed in the liver, heart, kidney and muscle (Zhang, 2013). Silencing of cytoplasmic NADK was shown to reduce the NADPH levels in HEK293 cells, whereas overexpression rescued the NADPH pool (Pollak, Niere, & Ziegler, 2007). The NADPH pool is regulated by several mechanisms and enzymes including the oxidative pentose phosphate pathway (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase),

folate metabolism (methylenetetrahydrofolate dehydrogenase and aldehyde dehydrogenase), cytosolic and mitochondrial malic enzymes (ME1 & ME2), cytosolic and mitochondrial isocitrate dehydrogenase (IDH1 &IDH2), the nicotinamide nucleotide transhydrogenase (NNT) and glutamate dehydrogenase (GDH). However, the biggest contributors of cellular NADPH pool are the PPP, ME1, IDH1 and folate metabolism in cytosol and mitochondria of cancer cells (Koju, Qin, & Sheng, 2022).

1.3.1.1 Pentose Phosphate Pathway

The Pentose Phosphate Pathway (PPP) is divided into two parts: oxidative and non-oxidative. In the oxidative part of the PPP, NADPH is generated via two enzymes, G6PD and 6PGD, during the metabolism of glucose-6- phosphate (Patra & Hay, 2014). Many cancer cells are known to be addicted to glucose; therefore, in different malignancies, increased the input of glucose into the oxidative branch of the PPP greatly increases NADPH synthesis (Yi et al., 2012). G6PD expression is associated with a poor prognosis in a variety of cancer patients, and it plays an important role in both carcinogenesis and chemoresistance. Inhibition of G6PD activity was shown to diminish NADPH levels (Hecker, Leopold, Gupte, Recchia, & Stanley, 2013).

1.3.1.2 Isocitrate Dehydrogenase

Isocitrate dehydrogenases (IDH) function via the oxidative decarboxylation of isocitrate to α- ketoglutarate using NADP as the electron acceptor (M. Gagné, Boulay, Topisirovic, Huot, & Mallette, 2017). There are three isomers for IDH enzymes including IDH1, IDH2 and IDH3. IDH3 uses NAD+ as its cofactor, whereas IDH1 and IDH2 use NADP as a coenzyme. The NADPH produced from this reaction can increase the cell's antioxidant capacity. IDH1 works in cytosol, while IDH2 is in the mitochondria (Al-Khallaf, 2017). Increased activity of IDH1

could be a typical response to reducing oxidative stress and higher synthesis of macromolecules, resistance, and growth (Calvert et al., 2017). Moreover, silencing of IDH1 results in decreased NADPH levels and increased oxidative stress, sensitivity to chemotherapy and radiotherapy (Tan et al., 2012). Similar to IDH1, ectopically expressed IDH2 can reduce oxidative stress in cancer cells and increase cell survival (Bergaggio & Piva, 2019). However, some studies have pointed out that in metastatic HCC, IDH2 expression is low. Lacking IDH2, these cells increase their invasive capacity due to high levels of metalloproteases (Tian et al., 2015). Notwithstanding, IDH1 and IDH2 also consumes NADPH while generating isocitrate from α-ketoglutarate. The produced isocitrate then translocates into the mitochondria to reduce mitochondrial ROS (Mullen et al., 2011). In addition, mutations of these two genes causes the proteins to catalyze an oncometabolite called 2- hydroxyglutarate (2-HG) from α-ketoglutarate using NADPH for the reaction. The 2- HG generated promotes tumorigenesis and suppresses the immune environment making it more favorable for the growth and proliferation of cancer cells (Du & Hu, 2021).

$$\text{Isocitrate} + NADP^+ \leftrightarrow \text{2 - oxoglutarate} + NADPH + H^+ + CO_2$$

1.3.1.3 Malic Enzyme

Another major contributor of cellular NADPH is ME that is also located both in the cytosol (ME1) and mitochondria (ME2). Malic enzymes regulate the decarboxylation of L- malate to pyruvate and CO_2, while using NADP as the electron acceptor and generating NADPH (Sanz et al., 1997).

$$\text{L-Malate} + NADP^+ \leftrightarrow \text{Pyruvate} + CO_2 + NADPH$$

According to a quantitative flow analysis, ME's direct contribution to NADPH generation is expected to be equal to the contribution of the PPP (Ciccarese &

Ciminale, 2017). NADPH generated from the enzymatic action of ME is used in long-chain fatty acid synthesis and it may also provide electrons for detoxification activity in the liver (Fernandes et al., 2018).

1.3.1.4 Nicotinamide Nucleotide Transhydrogenase (NNT)

Nicotinamide nucleotide transhydrogenase (NNT) is an integral membrane protein located in the inner membrane of the mitochondria in eukaryotes. The enzyme catalyzes the transfer of a hydrogen anion between NADH and NADP+ and therefore contributes towards the cellular NADPH pool (Murphy, 2015).

$$NADH + NADP^{+} \leftrightarrow NAD^{+} + NADPH$$

NNT also appears to be a significant enzyme for antioxidant defense mechanisms, since it can produce NADPH from NADH directly (Nickel et al., 2015). A mutation in NTT causes cortisol deficiency in humans which suggests that NNT- produced NADPH can be used in steroid synthesis in addition to antioxidant defense mechanisms (Meimaridou et al., 2012).

1.3.1.5 Glutamate Dehydrogenase

Glutamate dehydrogenase (GDH) catalyzes reversible reaction of glutamate to alpha-ketoglutarate. In this reaction NADP+ is reduced to NADPH (Smith, Li, Stanley, & Smith, 2019).

$$NADP^{+} + \text{glutamate} \leftrightarrow \ NADPH + \alpha\text{- ketoglutarate} + NH_4^{+}$$

GDH is a highly conserved enzyme that is also found in life forms such as yeast and bacteria and has several isoenzymes that can utilize either NAD+ or NADP+. However, mammalian GDH can use both NAD+ and NADP+ in anabolic and catabolic reactions (Plaitakis, Kalef-Ezra, Kotzamani, Zaganas, & Spanaki, 2017). In cancer cells, the GDH pathway plays a significant role, since it can produce nitrogen which is used in amino acid synthesis and supply an additional carbon source for TCA cycle (Deberardinis & Cheng, 2009). Moreover, it provides NADPH for redox metabolism. In humans, it is shown that glutamate dehydrogenases (hGDH1 and hGDH2) are localized in the brain, steroid- producing organs and testis, but not in the liver (Spanaki, Zaganas, Kleopa, & Plaitakis, 2010).

1.3.1.6 Folate Metabolism

This folate- mediated pathway produces one carbon unit for nucleic acids, methionine generation, homocysteine methylation and mitochondrial protein translation while providing NADPH as a side product (Lewis et al., 2014). There are two major enzymes that link this pathway to NADPH including methylene tetrahydrofolate dehydrogenases (cytosolic MTHFD1 and mitochondrial MTHFD2) and 10- formyl-tetrahydrofolate dehydrogenases (cytosolic ALDH1L1 and mitochondrial ALDH1L2). MTHFD enzymes catalyze 5,10-methylene-THF and form 10-formyl-THF, first. Then, the enzyme ALDH1L enzyme oxidizes 10- formyl -THF into carbon dioxide and THF, generating NADPH (Ducker et al., 2016).

Cytosolic ALDH1L1 predominantly controls folate pools, whereas mitochondrial ALDH1L2 generates NADPH for oxidative stress. In one study, it was shown that inhibition of ALDH1L2 decreased NADPH and glutathione levels and lessened the metastatic burden (Noguchi et al., 2018). In human cancers, the expression of both MTHFD1 and MTHFD2 are increased and is associated with poor prognosis (Sheraj,

Guray, & Banerjee, 2021). Using a quantitative flux analysis, knockdown of MTHFD1L gene was shown to diminish MTHFD1 or MTHFD2 levels, which in turn decreased NADPH and GSH levels and induced growth inhibition in HCC (Piskounova et al., 2015).

1.3.2 Consumers of NADPH

Both NADH and NADPH acts as cofactors. They are recognized by different enzymes, and both have different functions. NADH acts primarily in catabolic processes, while NADPH functions in antioxidant metabolism and reductive biosynthesis (Xiao et al., 2018).

1.3.2.1 Oxidative Stress and Antioxidant Mechanism

Reactive oxygen species (ROS) produced as derivatives of healthy cell metabolism which have oxidizing abilities. ROS act as secondary messengers in many signaling pathways and in low amounts, they are crucial for cellular metabolism, but in high doses they can create excessive oxidative stress for the cell and become deadly. Cells therefore have various mechanisms to reduce ROS levels to the extent that they cannot affect cell survival. The enzymes and metabolites that play a role in the mitigation of ROS include superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and thioredoxins (TXR). NADPH contributes to the maintenance of glutathione levels in cells (GSH) by donating its electrons for glutathione reductase activity, as well as thioredoxin activity (Arfin et al., 2021).

Cancer cells are metabolically more active compared to healthy cells; therefore, their energy demand is higher. Active metabolism brings ROS with it; therefore, cancer

cells mostly need a robust antioxidant mechanism to survive. Elevated ROS levels are deadly for cancer cells as much as for healthy cells; however, it was shown that to some extent ROS can contribute towards several hallmarks of cancer cells including angiogenesis, invasiveness, and metastatic ability (Hanahan & Weinberg, 2011).

In both cancer cells and healthy cells, redox homeostasis is controlled by the transcription factor nuclear factor like-2 (Nrf2) encoded by the *NFE2L2* gene. Under normal conditions, Nrf2 remains in the cytosol via protein-protein interaction with Kelch-like-ECH-associated protein 1 (Keap1), which ubiquitinates Nrf2 for degradation (Baird & Yamamoto, 2020). This ubiquitination is crucial to maintain low levels of Nrf2 in the cytosol. When there is increased oxidative stress in cell, Keap1 undergoes a conformational change and the interaction between Nrf2 and Keap1 is lost (Suzuki et al., 2019). Nrf2 then translocates to the nucleus where it binds to antioxidant response elements (ARE) of multiple antioxidant genes including aldo-keto reductases (AKR), aldehyde dehydrogenase (ALDH), superoxide dismutase (SOD), enzymes of the glutathione system [glutathione reductase (GR), glutathione peroxidase (GPX)], thioredoxin system enzymes (thioredoxin-1, thioredoxin reductase-1), heme metabolism (heme oxygenase-1) and NADPH generation (malic enzyme, isocitrate dehydrogenase, glucose- 6 phosphate dehydrogenase) (Tonelli, Chio, & Tuveson, 2018). Many of these enzymes utilize NADPH as a cofactor for their enzymatic functions (Xiao et al., 2018). Therefore, the antioxidant mechanism is one of main consumers of NADPH. Glutathione contributes maximally towards cellular antioxidant pathways. Glutathione disulfide (GSSG) is reduced to glutathione (GSH) with glutathione reductases using NADPH (Ju, Lin, Tian, Xie, & Xu, 2020). Glutathione is a substrate for GSH peroxidases, which can reduce hydrogen peroxide (H_2O_2) to water (H_2O) and alcohol. The enzyme thioredoxin reductase also uses NADPH to reduce thioredoxin and detoxify H_2O_2 (Cebula, Schmidt, & Arnér, 2015).

1.3.2.2 Fatty Acid Synthesis

Fatty acid synthesis (FAS) is a reductive process in which NADPH is the main source of electrons. FAS is needed for signaling, membrane biosynthesis and energy storage (Röhrig & Schulze, 2016). The rate limiting step in fatty acid synthesis is the production of malonyl- CoA from Acetyl- CoA with the enzymatic activity of Acetyl- CoA Carboxylase (ACC). Once produced, malonyl-CoA can inhibit fatty acid oxidation and produce acetyl-CoA via malonyl CoA decarboxylase (Chien, Dean, Saha, Flatt, & Ruderman, 2000). Then, both malonyl-CoA and acetyl-CoA bind to a large protein complex called Fatty Acid Synthase (FAS) and produce Butyryl-CoA in which malonyl-CoA acts as a carbon donor. This cycle of carbon donation continues until palmitate is produced. During this pathway, there are several reduction steps for which NADPH is needed (Maier, Leibundgut, & Ban, 2008).

The activation of ACC is mediated by insulin, citrate, carbohydrate response element binding protein (ChREBP) while its inhibition is mediated by glucagon, palmitoyl-CoA, and AMPK (Kim, 1983). AMPK is a nutrient sensor that is usually activated when ATP/AMP ratio in the cell decreases below a critical level. AMPK can also be activated by Ca^{2+} mediated signaling via CAMKK2. Active AMPK can phosphorylate ACC at serine 79 to inhibit its function in fatty acid synthesis. In this case, the cell undergoes β-oxidation of fatty acids (Mihaylova & Shaw, 2011).

Fatty acid oxidation (FAO) is the hydrolysis of fatty acids into acetyl-CoA and eventually to ketones. FAO mostly occurs in the liver; the acetyl-CoA generated can either enter the TCA cycle to provide NADH and $FAHD_2$ for the electron transport chain or can be used to generate ketone bodies for the brain and muscle cells (M. Wang et al., 2016). The chain length of fatty acids determines where the oxidation will take place. Short to long chain fatty acids are oxidized in the mitochondria, whereas very long chain fatty acids can only be oxidized in peroxisomes

(Demarquoy & Borgne, 2015). Unlike fatty acid synthesis, fatty acid oxidation uses NADH as its fuel and is not reliant on NADPH at all (Sangineto et al., 2020).

1.4 Research hypothesis and aims of the thesis

The primary motivation for this research came from *in silico* observations (Sheraj, 2021) in our lab using data from The Cancer Genome Atlas (TCGA) that HCC patients [Liver Hepatocellular Carcinoma (LIHC) patients] with high expression of AKR1B10 had significantly lower survival compared to patients with lower expression of AKR1B10. When the LIHC patients were stratified by the median expression of AKR1B10, the hazard ratio (HR) for patients expressing high levels of AKR1B10 was 1.55 ($p= 0.015$). Thus, a patient with a higher than median expression of AKR1B10 was approximately 1.55 times more likely to die than a patient with lower AKR1B10 expression. This highlighted the importance of AKR1B10 in HCC. Additionally, the same study showed that the expression of the NADPH consuming enzyme AKR1B10 was very strongly and significantly correlated with the expression of all the NADPH generating enzymes.

Based on these observations, my thesis had the following aims:

1. To evaluate the crosstalk between the NADPH consuming enzyme AKR1B10 and the NAPDH generating PPP enzymes. We hypothesized that ectopic expression of AKR1B10 can lead to an antioxidant response in cells via the NADPH generated from the PPP, leading to the mitigation of oxidative stress and enhanced cell survival. To evaluate this, I treated cells with Manumycin A, a pro-oxidant drug and 6-AN, a PPP inhibitor and evaluated cell survival and the expression of antioxidant genes.

2. Since NADPH can also be consumed anabolic reactions such as fatty acid synthesis, which mainly occurs in liver, we also hypothesized that AKR1B10 expression may alter fatty acid metabolism. I tested this hypothesis by evaluating the activation of proteins crucial for FAS and FAO in AKR1B10 expressing cells.

Although AKR1B10 has already been reported to be an antioxidant response gene, a cross talk with the PPP has not been reported to date, which is considered as a novelty of the hypothesis. Additionally, very few studies have addressed a role of AKR1B10 in fatty acid metabolism; none of which were in liver cancer.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Line Characteristics

Table 2.1 Characteristics of SNU-423 & HuH-7

SNU-423	HuH-7
Damaging KEAP1 Mutation	Silent KEAP1 Mutation
Poorly Differentiated, Mesenchymal- like	Well- Differentiated, epithelial -like
Male- Korea- 40 y/o	Male- Japanese - 57 y/o
Truncating mutation on TP53	Missense mutation on TP53
DMEM High Glucose	DMEM Low Glucose
2 mM L-glutamine (In Medium)	4 mM L-glutamine (In Medium)

https://lccl.zucmanlab.com/hcc/cellLines/SNU423

https://lccl.zucmanlab.com/hcc/cellLines/Huh7

2.2 Cell Culture

The hepatocellular carcinoma cell lines SNU423 and Huh7 were a kind gift from Dr. Rengül Atalay (Middle East Technical University). SNU423 cells were grown in DMEM Medium supplemented with 10% FBS, 2 mM L-glutamine, and 1% Penicillin- Streptomycin solution. Huh7 cells were grown in DMEM Low Glucose Medium supplemented with 10% FBS, 4 mM L-glutamine, and 1% Penicillin-Streptomycin solution. Both cell lines were grown in a humidified incubator with 5% CO2 at 37.C. All cell culture consumables were purchased from Biological Industries (Israel).

2.3 AKR1B10 Cloning and Generation of Lentiviruses

For cloning, the AKR1B10 cDNA was amplified with NEB Phusion Taq polymerase from pcDNA3.1- cloned with the AKR1B10 gene (available in Banerjee Lab). Primers used in the PCR amplification were designed with flanking *EcoRI* and *XbaI* restriction sites. pLenti-puro vector (Addgene plasmid #39481) was restriction digested with EcoRI-HF and XbaI. Ligation of the vector and PCR-amplified AKR1B10 fragment was carried out using T4 DNA Ligase at 16ºC overnight (Thermo Fisher). For transformation, the *E. coli Stbl3* strain was used. The selection antibiotic was ampicillin for pLenti-puro. Colonies were confirmed via PCR with AKR1B10 primers. This cloning was conducted by a graduate student, Esin Gülce Seza, in Banerjee lab. PCR confirmed isolated plasmids were sent to sequencing for further confirmation (BMLabosis)

For generation of the viruses, HEK293FT cells were used. Packaging plasmid psPax2 (Addgene plasmid #12260), envelope plasmid p-CMV-VSV-G (Addgene plasmid #8454) and cloned transfer plasmid pLenti-puro including the AKR1B10 cDNA transfected into HEK293FT cells with transfection reagent PEI ((Polyethyleneimine, Sigma-Aldrich) with a ratio of 1:3 [DNA in (µg) to PEI (1 mg/ml) in µl]. Transfection carried out in 10-cm dishes. 24 hours after seeding cells in a 10 cm dish, transfection was carried out with three plasmids at a ratio of 2.5:2.5:5 (packaging- envelope- transfer) in OPTIMEM. 16 hours after the transfection, medium is renewed with DMEM complete medium. 48 hours later, first set of viruses were collected and medium was renewed. After 24 hours, 72hours viruses were collected. Media containing viruses were filtered through 0.22 µM filters, snap-frozen in liquid nitrogen and stored at -80ºC. These viruses were generated by İsmail Güderer, a graduate student in Banerjee Lab.

Table 2.2 Primer Sequences for AKR1B10 Cloning & Lentivirus

Primer	Sequence
AKR1B10 Cloning Forward	GCCGGGAATTCGCCACCATGGCCACGTTTGTGGAG
AKR1B10 Cloning Reverse	GTTCTAGAACCTCAATATTCTGCATTGAAGGG
AKR1B10 Forward	CAGAATGAACATGAAGTGGGG
AKR1B10 Reverse	GCTTTTCCACCGATGGC

2.4 Transduction of HuH-7

For transduction experiments of Huh7 cells, 50,000 cells/well was seeded in a 12-well plate. After 24 hours, two dilutions of viruses were used including a 1:1 ratio (1 volume virus – 1 volume medium), or undiluted virus (full virus) with the addition of 10 µg/mL Polybrene (Merck Millipore) in antibiotic-free media. At the same time, Huh7 cells were also reverse- transduced with 50,000 cells/well using 1:1 ratio in antibiotic-free medium with polybrene. After 72 hours of incubation, the medium was renewed with the medium of Huh7 cells supplemented with 3 µg/ml puromycin (Invivogen) as the selection antibiotic. When all untransduced (wild type) cells were dead, the selection was ended, and the remaining cells were grown with a maintenance dose of puromycin. (1.5 µg/ml for Huh7). The cells were confirmed for overexpression of AKR1B10 by PCR and western blot, expanded and stored in liquid nitrogen.

2.5 Treatments

The drugs were used in this thesis included manumycin A, 6- aminonicotinamide (6-AN), N-acetylcysteine, and cobalt chloride ($CoCl_2$). The solvent for manumycin A, 6-aminonicotinamide was DMSO. Pure water was used to dissolve cobalt chloride and N-acetylcysteine. All drugs except $CoCl_2$ were at -20ºC, $CoCl_2$ was kept at 4 ºC. Manumycin A, and 6-aminonicotinamide were prepared as a 10 mM stock and diluted as necessary.

2.6 Proliferation Assay (MTT)

To determine cell viability, an MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was carried out according to manufacturer's instructions (Thermo Fisher, USA). 5,000 cells/well were used per well for a 96-well plate. 0.05 g MTT was dissolved in 1 mL PBS and further diluted in cell culture medium. At the start of the experiment, the cell culture medium (containing drugs or vehicle) was aspirated and 100 µL of MTT-Medium mixture was added onto the cells. After 4 hours incubation, 100 µL 1%SDS- 0.01M HCl was added to the MTT added wells and incubated for 18 hours at 37 ºC. The absorbance was measured at 570 nm, using Multiskan-GO spectrophotometer (Thermo Fisher, USA).

2.7 Colony Formation Assay

Colony formation assay was done to test the viability of AKR1B10 overexpressing SNU423 and Huh7 cell lines treated with cytotoxic agents. 500 cells/wells were seeded in a 6-well plate. After allowing them to attach for 24 hours, several drugs and their combinations (including manumycin A, erastin, cobalt chloride, 6-AN and manumycin + 6-AN) were tested. Cells were incubated at 37ºC and treated every 48 hours for 7 days. After colonies grew large enough to be seen with the naked eye (a colony should consist at least 50 cells), the medium was aspirated, and the colonies

were washed twice with PBS. Then, they were fixed with 700 µL 4% paraformaldehyde (PFA) solution (Sigma Aldrich, USA) for 15-20 minutes at room temperature. PFA was aspirated and the wells were washed with PBS again. 700 µL 0.5% crystal violet (Sigma Aldrich, USA) solution was added onto the wells and incubated for 20 minutes at room temperature on a shaker. After the second incubation was completed, the wells were washed with tap water for 4-5 times until all remaining staining solution was removed. The lid of the plate was left open overnight to air-dry the plates. The colonies were imaged using white tray of the ChemiDoc Imaging system and Image Lab software (BioRad, USA) and counted manually.

2.8 Protein Isolation and Quantification

For total protein isolation, M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, USA) 1X protease inhibitor and 1X phosphatase inhibitor (Roche, Germany) were used in a mixture. After aspirating medium, cell culture samples were washed with PBS once. Protein isolation mixture was applied directly onto samples and scraped with a scraper. The protein mixture was collected into a 1.5 mL Eppendorf tube and incubated on ice for 30 minutes with vortexing in every 10 minutes. After incubation, samples were centrifuged at 14000 x g for 10 minutes. Without touching the pellets, supernatants containing protein were collected into new 1.5 mL Eppendorf tubes.

Protein amount was measured with Coomassie Protein Assay (Thermo Fisher, USA). A standard curve was generated for quantification. Samples were diluted in Coomassie Blue with a 1:5 ratio and seeded into 96-well plate. Proteins were measured at 596 nM with MultiSkan GO Microplate Spectrophotometer (Thermo Scientific) in plate segment. After measurement, proteins were mixed with 6X-Protein Loading Dye and boiled at 95ºC for 6 minutes. Then, proteins were stored at -20 ºC.

2.9 Western Blot

Proteins were loaded onto 10% SDS-PAGE gels. Gels were run at 50V until proteins were all stacked, then voltage was increased to 100V. After electrophoresis, proteins on the gel were wet transferred to polyvinylidene fluoride (PVDF) membranes with cassette system and run for 100 minutes at 115V. 5% Skim Milk (AppliChem) prepared in 0.1% TBS-T was used to block membranes for 1 hour at room temperature. Primary antibodies were incubated overnight at +4 ºC. Once incubation was over, membranes were washed with TBS-T for 10 minutes, three times. Then, secondary antibodies were incubated for 1 hour at room temperature or overnight at +4 ºC. Once secondary antibody incubation finished, membranes were washed again with TBS-T for 10 minutes three times. Bands on the membranes were imaged by using Clarity ECL Substrate (Bio-Rad) with ChemiDoc MP Imaging System (Bio-Rad). Band intensities were measured with Image Lab 6.0.1 software. GAPDH and α- tubulin was used as housekeeping which were used to normalize protein data. Antibodies used in the thesis are shown in Table 2.3.

Table 2.3 Antibodies used in this study.

Antibody	Size (kDa)	Origin	Brand	Cat#
AKR1B10	35	Rabbit	Thermo Fisher	PA5-23017
p-ACC	265	Mouse	Santa Cruz	Sc- 271965
Gpx2	26	Mouse	Santa Cruz	Sc- 133160
t-AMPK	63	Mouse	Santa Cruz	Sc- 74461
p-AMPK	62	Rabbit	Cell Signaling	2535
P62	62	Mouse	Santa Cruz	Sc- 28359
TKT	68	Rabbit	Cell Signaling	64414
G6PD	58	Rabbit	Cell Signaling	Sc- 25778
α-tubulin	52	Mouse	ProteinTech	HRP-66031
GAPDH	37	Mouse	Santa Cruz	Sc-47724

2.10 RNA Isolation and cDNA Synthesis

After treatment of the cells with manumycin A or 6-AN, the wells were washed with PBS twice and cells were collected via scraper. RNA isolation was conducted with NucleoSpin RNA Kit (Macherey Nagel, Germany) or Ribozol^{TM} RNA Extraction Reagent (VWR, USA) according to the manufacturer's guidelines. RNA samples were kept at -80 ºC. For cDNA synthesis 1 μg RNA was used with the Revertaid First Strand cDNA Synthesis Kit (Thermo Scientific). cDNAs were kept at -20 ºC.

2.11 RT- PCR and qRT- PCR

Reverse Transcriptase- Polymerase Chain Reaction was conducted in a total of 20 µL mix including 4 µL 5X FirePOL Master Mix (Solis Biodyne, Estonia), 1 µL 10 µM forward and reverse primers, 1 µL cDNA and pure water. The reaction mixes were put in a thermal cycler (Applied Biosystems, USA). Reaction conditions are shown in Table 3. The PCR products were loaded onto agarose gel (with different percentages) with 6X loading dyes and run at 200V. The gel was visualized under UV light using Quantum ST4 Imaging System (Vilber Lourmat, France). To determine the expression of our genes of interest, we performed qRT-PCR with total volume of 10 µL including 8 µL reaction mix (Table 2.4) and 2 µL cDNA (1:10 dilution). For each primer pair (Table 2.5) used in this thesis a standard curve was generated. β-actin was used as housekeeping gene. The qRT-PCRs were carried out in Rotor-Gene Q 6000 System (Qiagen, Germany). Threshold cycle (Ct) values which were given based on the standard curves were used to analyze the qRT-PCR with Pfaffl Method (Pfaffl,2001).

Table 2.4 RT-qPCR Conditions for FIREPOL Master Mix

Initial Denaturation	95 ºC	3 minutes	
Denaturation	95 ºC	30 seconds	
Annealing	55 ºC (for AKR1B10)	30 seconds	35 cycles
Elongation	72 ºC	30 seconds	
Final Elongation	72 ºC	7 minutes	
Hold	4 ºC	∞	

Table 2.5 Reaction Mix of Gene of Interest and βactin (1X)

Mixture	GOI	βactin
Forward Primer	1 µL	0.5 µL
Reverse Primer	1 µL	0.5 µL
GoTaq qPCR Master Mix (Promega, USA)	5 µL	5 µL
cDNA	2 µL	2 µL
Molecular Biology Grade Water	1 µL	2 µL

Table 2.6 List of Primer Pairs used in this Study

Gene	Primer Sequence	TM (ºC)
AKR1B10 Forward	CAGAATGAACATGAAGTGGGG	55
AKR1B10 Reverse	GCTTTTCCACCGATGGC	55
P62 Forward	ATGAGGACGGGGACTTGGTT	57
P62 Reverse	TTGCAGCCATCGCAGATCA	57
TXNRD1 Forward	AGTAGTAGCTCAGTCCACCA	55
TXNRD1 Reverse	GGCACATTGGTCTGTTCTTCA	55
HMOX1 Forward	CGGGCCAGCAACAAAGTG	58
HMOX1 Reverse	AGTGTAAGGACCCATCGGAGAA	58
G6PD Forward	TGACCTGGCCAAGAAGAAGA	56
G6PD Reverse	CAAAGAAGTCCTCCAGCTTG	56
TALDO Forward	GTCATCAACCTGGGAAGGAA	60
TALDO Reverse	CAACAAATGGGGAGATGAGG	60
TKT Forward	GAAGATCAGCTCCGACTTGG	60
TKT Reverse	GTCGAAGTATTTGCCGGTGT	60
ME1 Forward	AACTCTGACTTTGACAGGTATCTT	60
ME1 Reverse	TGCCCTCGATCGTGGATAGT	60
βactin Forward	CAGCCATGTACGTTGCTATCCAGG	-
βactin Reverse	AGGTCCAGACGCAGGATGGCATG	-

2.12 Statistical Analysis

All experiments were conducted with at least 2 independent biological replicates with 3 technical replicates each. Graph Pad Prism 6 (Graph Pad Software Inc., USA) was used for the analysis. Two-way Anova or Student's t-test was performed for determining the significance. P-value < 0.5 was taken.

CHAPTER 3

RESULTS

3.1 Oxidative Stress and AKR1B10

One of the transcription factors that can regulate the expression of AKR1B10 is NRF2, the master regulator of cellular antioxidant response (Endo, Matsunaga, & Nishinaka, 2021). AKR1B10 activity may enhance ROS levels in the cell via several pathways including using NADPH as an electron donor for its own enzymatic functions thereby consuming cellular NADPH or by metabolizing PAH trans-dihydrodiols to redox-active quinones (Banerjee, 2021). By increasing cellular ROS, AKR1B10 can also stimulate NRF2, leading to the expression of NRF2 regulated antioxidant genes (Endo et al., 2021). Based on this potential crosslink between antioxidant homeostasis and AKR1B10, we first sought to examine the expression of oxidative-stress related genes with AKR1B10^{HIGH} and AKR1B10^{LOW} tumors in hepatocellular carcinoma (HCC) using publicly available transcriptome data from The Cancer Genome Atlas (TCGA). We observed that oxidative stress related genes were overall upregulated in AKR1B10^{HIGH} HCC tumors but AKR1B10^{LOW} tumors showed no specific pattern of expression of these genes (Figure 3.1, heat map generated by Ilir Sheraj, a post-doc in our lab). Therefore, we decided to overexpress AKR1B10 in HCC lines to evaluate the effects of AKR1B10 expression in oxidative-stress response.

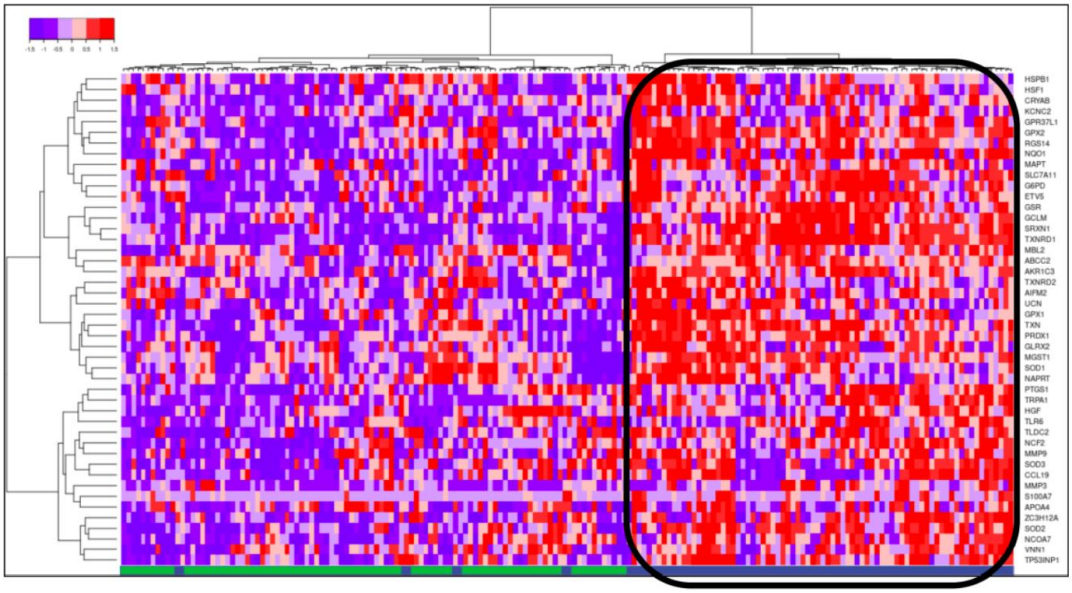

Figure 3.1. Heatmap showing antioxidant genes upregulated in AKR1B10^{HIGH} LIHC Tumors.

The bottom bar shows sample classification with blue representing AKR1B10^{HIGH} and green AKR1B10^{LOW} samples. The AKR1B10^{HIGH} samples with high expression of antioxidant genes were found in the outlined cluster. The heatmap was constructed using the heatmap.2 function from the gplot package in R with Euclidean distance and Ward.d2 linkage. This data and figure were generated by Ilir Sheraj.

3.2 Evaluation of oxidative stress response in HCC cell lines ectopically expressing AKR1B10

HuH-7 and SNU-423 cell lines were chosen for the ectopic expression of AKR1B10 as neither cell line expresses any AKR1B10 within the limits of qPCR. Both cell lines have a mutation in the KEAP1 gene that can lead to the constitutive activation of NRF2. The KEAP1 protein normally binds to NRF2 in the cytosol and induces its degradation by ubiquitination. Upon oxidative stress, KEAP1 is degraded, leading to the nuclear translocation of NRF2 and the activation of an antioxidant response

(Bellezza, Giambanco, Minelli, & Donato, 2018). While the HuH-7 cell line has a silent mutation on KEAP1, keeping the protein functionally active, SNU-423 cell line has a damaging mutation. Therefore, the SNU-423 cells are likely to have a deregulated antioxidant response whereas HuH-7 cell line are likely to have a more regulated response. To test the response of these cells to oxidative stress, we first treated both wild type cell lines with different doses of the pro-oxidant drug manumycin A and evaluated their viability. We found that HuH-7 cells had a stronger antioxidant response and survived better compared to SNU-423 cells (Figure 3.2).

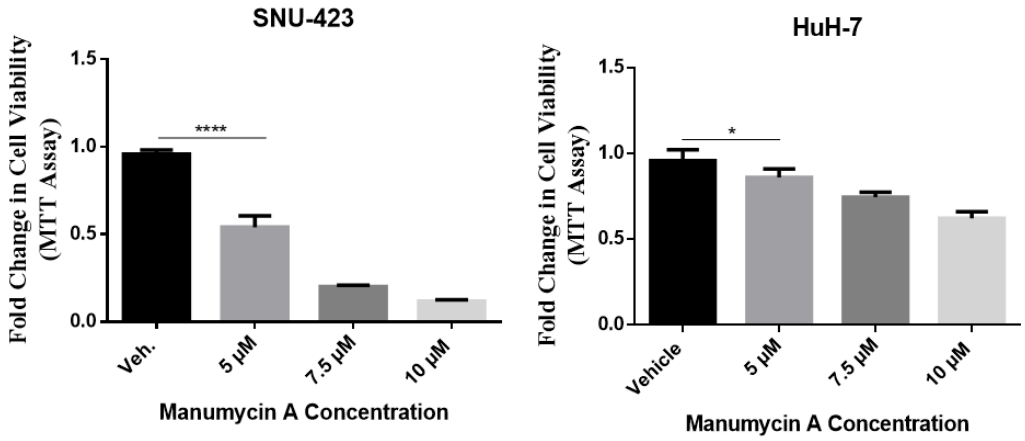

Figure 3.2. Determination of viability of SNU-423 and HuH-7 cell lines treated with different concentrations of the pro-oxidant drug Manumycin A.

*5,000 SNU-423 or HuH-7 cells were plated in 96 well plates and treated with 5µM, 7.5µM, 10µM Manumycin A for 24h followed by an MTT assay. Statistical analyses were carried out using ANOVA followed by Dunnett's multiple comparison test. ****p< 0.0001. Data are mean of two independent replicates.*

We next evaluated the mechanism by which the Manumycin A treated SNU423 cells lost their viability. For this, we treated the cells with Manumycin A, along with the apoptosis inhibitor QVD-OPH and the oxidative stress inhibitor N-acetyl cysteine (NAC). Manumycin A- induced cell death was not reversed with QVD- OPH but could be reversed with NAC. Manumycin A is known to target sulfhydryl homeostasis in cells; therefore, a thiol-based antioxidant such as NAC could successfully block cell death in manumycin A treated cells (Singha, Pandeswara, Venkatachalam, & Saikumar, 2013). Manumycin A killed the SNU-423 cells with ROS-induced cell death, but not apoptosis (Figure 3.3).

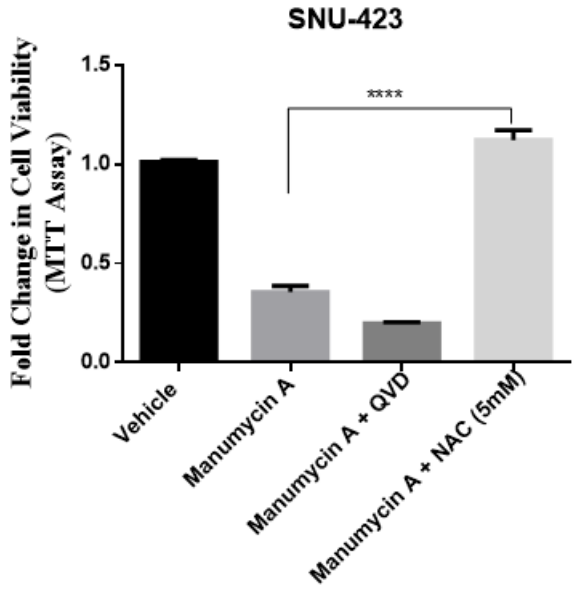

Figure 3.3. Cell viability of SNU-423 cells treated with Manumycin A, Manumycin A + QVD-OPH and Manumycin A + NAC.

*5000 SNU-423 cells were plated in 96 well plates and treated with 4µM Manumycin A, 4µM Manumycin A + QVD-OPH, and 4µM Manumycin A + 5mM n-acetylcysteine (NAC). Statistical analyses were carried out using ANOVA followed by Tukey's multiple comparison test. ****p< 0.0001. The mean of two independent experiments is shown here.*

3.3 Overexpression of AKR1B10 gene in SNU423 and Huh-7 Cell Lines

Having established the overall response of SNU423 and HuH7 cells to oxidative stress, we next generated stable cell lines with the ectopic expression of AKR1B10. Lentiviruses carrying AKR1B10 cDNA sequences were generated in HEK293FT cells previously by İsmail Güderer (Güderer, MSc, 2021). The lentiviral particles were transduced to HuH-7 and SNU-423 cells. The selection was continued until the wild-type non-transduced cells were death. Then, the cells were expanded and stored under a maintenance dose of puromycin (1.5 µg/ml for HuH-7 and 1 µg/ml for SNU-

423). The overexpression of AKR1B10 in HuH-7 and SNU-423 cells were confirmed via western blot (Figure 3.4). Overexpression of AKR1B10 in SNU423 cells was carried out by a graduate student, Esin Seza Gülce, in our laboratory while the overexpression of AKR1B10 in HuH7 cells was carried out by me.

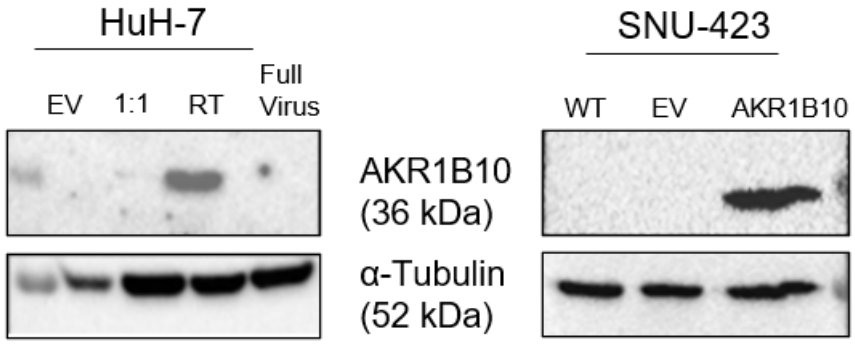

Figure 3.4. Confirmation of AKR1B10 expression in stably infected HuH7 and SNU423 cells with western blot.

AKR1B10 expression was evaluated in 1:1 ratio [Virus: Medium] empty vector (EV) transduced, 1:1 ratio AKR1B10 transduced, AKR1B10 Reversed- transduced (RT) and undiluted AKR1B10 Virus (Full) transduced total cell lysates of Huh7 cells. 20µg protein was loaded. α-tubulin was used as the loading control. AKR1B10 overexpression in SNU-423 cells was carried out by Esin Gülce Seza.

Based on our *in-silico* data that AKR1B10 expressing tumors showed a stronger antioxidant response, I evaluated the survival of AKR1B10 expressing SNU-423 cells treated with manuymcin A. For this, a colony formation assay (clonogenic assay) was carried out that can measure a cells capacity to divide and produce progeny. This technique is extensively used to determine cytotoxic effects of chemicals and anti-cancer drugs. Manumycin A (4 µM) treated SNU423 cells expressing both AKR1B10 and EV (Control) showed complete cell death that could be reversed with NAC (Figure 3.5).

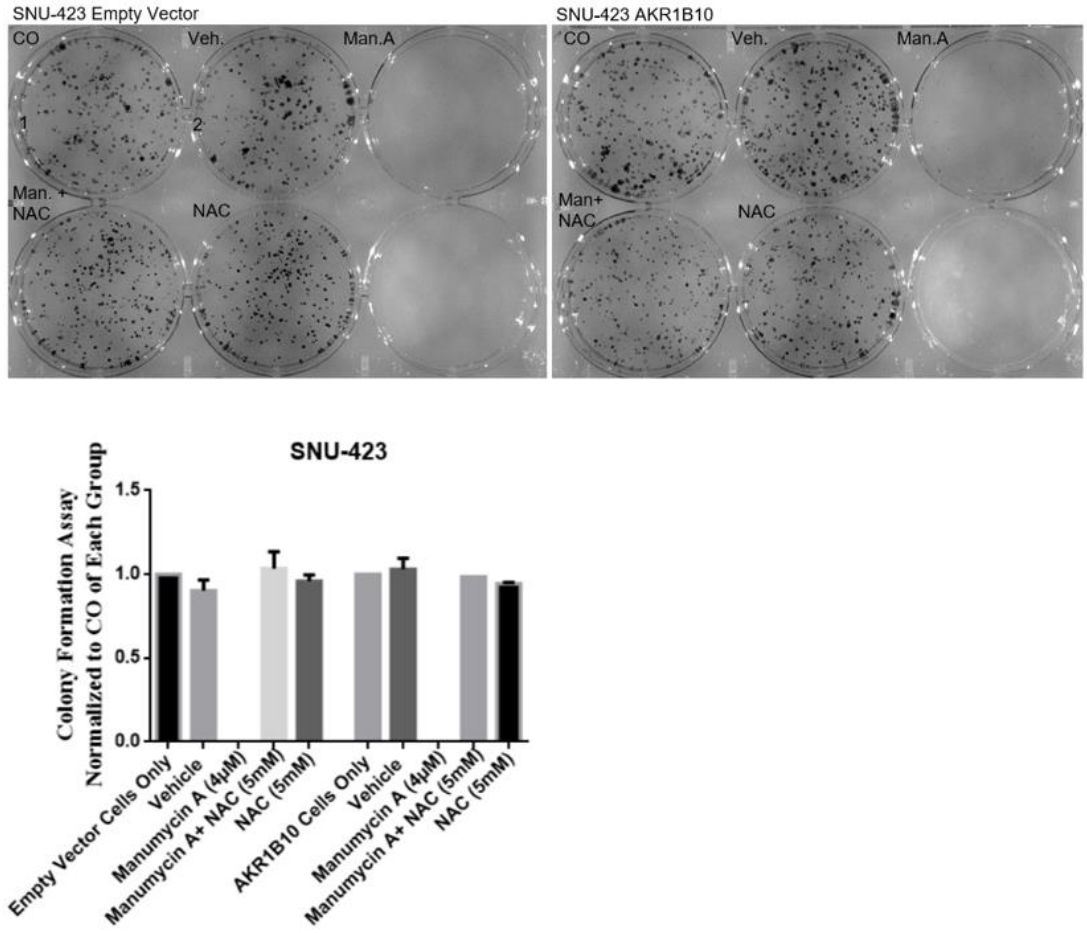

Figure 3.5. Colony Formation in Huh7 cells stably expressing AKR1B10 or empty vector (EV) and treated with Manumycin A (4μM), NAC, Manumycin A + NAC or vehicle.

500 SNU-423 cells expressing AKR1B10, or control cells were plated in 6-well plate. The cells were treated with the respective drugs for 8 days and the medium was renewed every 48 hours. After 8 days, the cells were fixed with 4% PFA and stained with 0.5% crystal violet solution. The upper row (left to right): Well #1 corresponds to untreated cells, well #2 vehicle (DMSO), well #3 Manumycin A (4μM), well #4 Manumycin A + NAC (5 mM), well #5 NAC (5mM) treated cells. Data from the average of 2 independent biological replicates are shown.

Since extensive cell death was seen with 4μM Manumycin A, I performed the same experiment with 1μM Manumycin A (Figure 6). This time some colonies were observed with the Manumycin A treated cells; however, the number of colonies was similar between AKR1B10 and control cells.

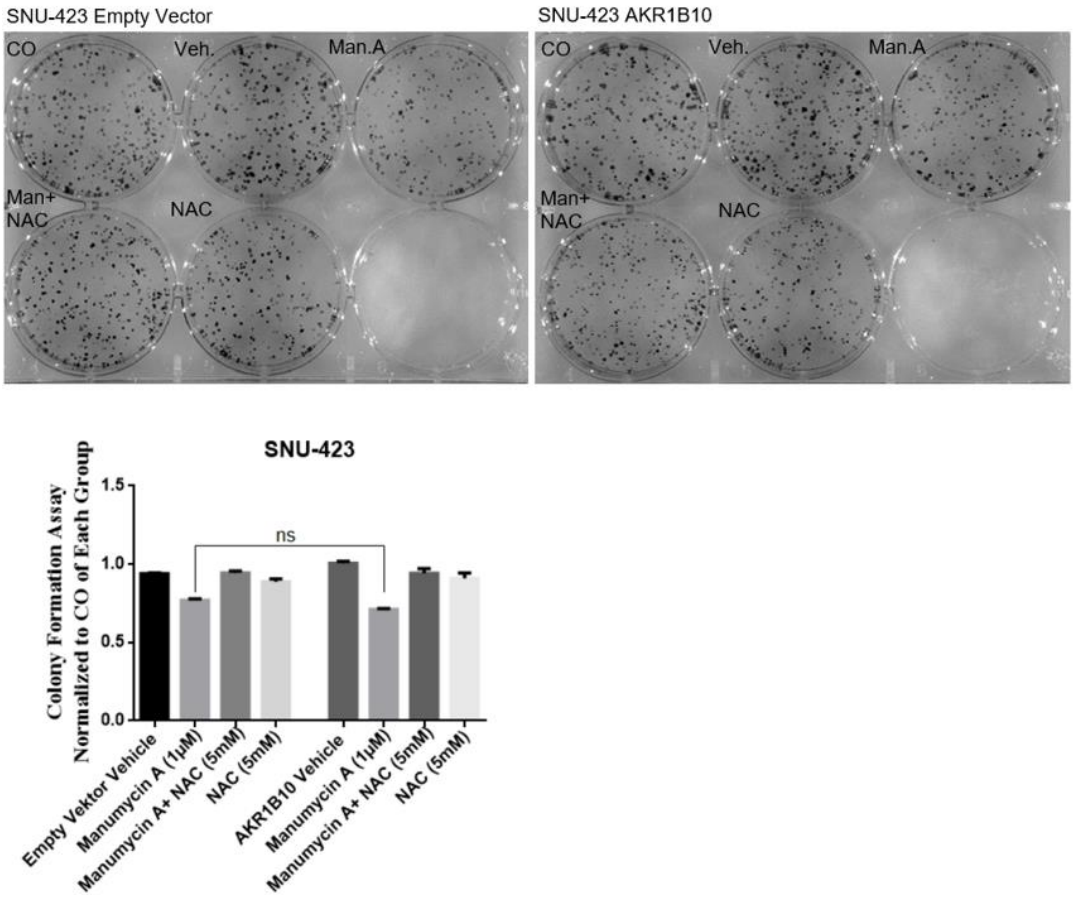

Figure 3.6. Colony Formation of SNU-423 cells stably expressing AKR1B10 or empty vector (EV) and treated with Manumycin A (1μM), NAC, Manumycin A + NAC, or vehicle.

500 SNU-423 cells expressing AKR1B10, or control cells were plated in 6-well plate. The cells were treated with the respective drugs for 8 days and the medium was renewed every 48 hours. After 8 days, the cells were fixed with 4% PFA and stained with 0.5% crystal violet solution. The upper row (left to right): Well #1 corresponds to untreated cells, well #2 vehicle (DMSO), well #3 Manumycin A (1μM), well #4 Manumycin A + NAC (5 mM), well #5 NAC (5mM) treated cells. Data from the average of 2 independent biological replicates are shown. Statistical analyses were carried out by 2-way ANOVA followed by Tukey's multiple comparison test.

3.4 Effects of AKR1B10 overexpression in SNU423 on oxidative stress response

Our data so far indicated that SNU423 cells expressing AKR1B10 did not lead to better survival when grown under oxidative stress. To further substantiate this data, I evaluated the expression of certain key antioxidant response genes. NRF2 regulates many genes in several branches including glutathione pathway, thioredoxin pathway, NADPH production and iron metabolism (Tonelli et al., 2018).

I first evaluated the mRNA expression of TXNRD1 (thioredoxin pathway) and HMOX1 (iron metabolism) in manumycin A treated SNU-423 cells expressing AKR1B10 or EV. The expression of both genes is transcriptionally regulated by NRF2 (Tonelli et al., 2018). A significant decrease in the expression of both HMOX1 and TXNRD1 was observed in untreated (vehicle) AKR1B10 expressing cells compared to the untreated (vehicle) empty vector control cells. However, when treated with Manumycin A, both genes were expressed more in the AKR1B10 expressing cells ($p<0.0001$ for TXNRD1 and trend towards increase in HMOX1) (Figure 3.7) supporting the idea of increased antioxidant response in AKR1B10 expressing cells grown under oxidative stress.

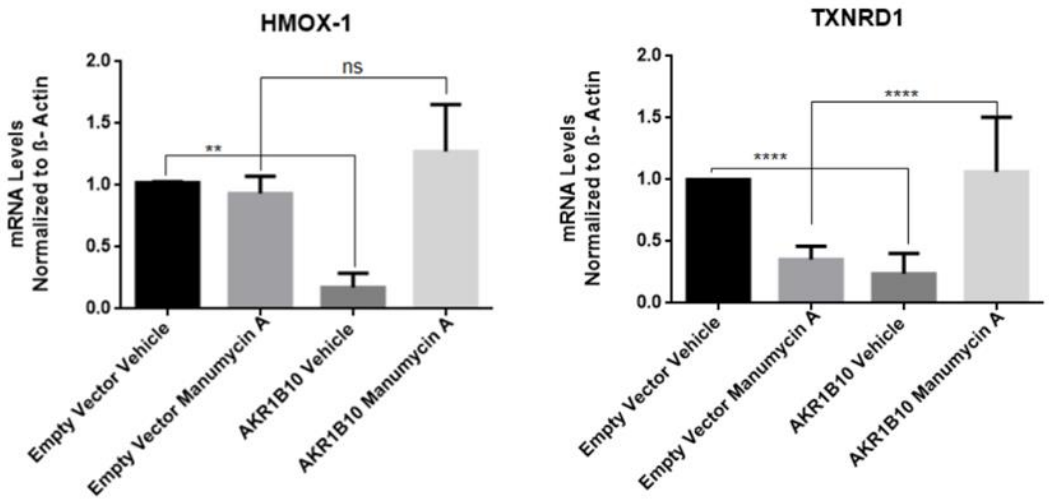

Figure 3.7. Expression of the antioxidant pathway genes HMOX1 and TXNRD1 in stably AKR1B10 expressing SNU-423 cells or control (EV) cells.

*SNU-423 cells expressing AKR1B10, or control were treated with 4 Manumycin A for 48 hours and collected for RNA isolation and RT-qPCR. Data from the average of 2 independent biological replicates are shown. Statistical analyses were carried out using ANOVA followed by Tukey's multiple comparison test. (****p< 0.0001, **p<0.05) (ns: not significant)*

Next, I evaluated the protein expression of antioxidant genes including glutathione peroxidase 2 (GPx2), a protein involved in the glutathione pathway and p62, a cargo protein that is known to facilitate the degradation of KEAP1 during an oxidative stress response. Again, AKR1B10 expressing cells under oxidative stress (Manumycin A treated) showed an increase in GPx2 protein compared to the empty vector control cells (Figure 3.8). A decrease in the protein levels of p62 was seen in the stressed AKR1B10 expressing cells compared to the control cells, which could be due to the enhanced degradation of p62 along with Keap1.

Among the many sources of cellular NADPH, the pentose phosphate pathway (PPP) is a primary source in cancer (Patra & Hay, 2014). The expression of G6PD, an enzyme of the oxidative branch of the PPP that generates NADPH and TKT, an

enzyme of the non-oxidative branch was evaluated. Although no change in the expression of G6PD was observed in the stressed (Manumycin A treated) AKR1B10 expressing cells, a decrease in TKT was observed. We speculate that this decrease in TKT levels may channel the pathway towards purine metabolism rather than feeding back into glycolysis; however, more experiments are needed to substantiate this speculation.

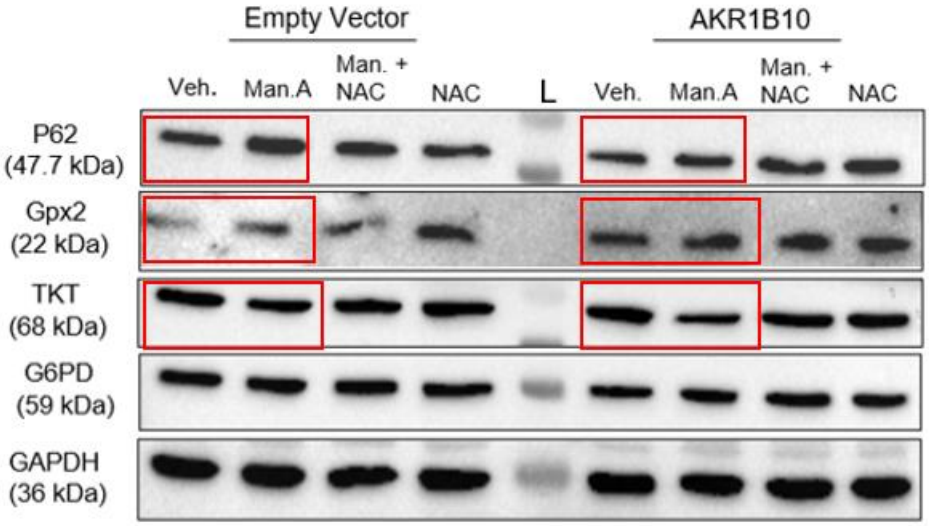

Figure 3.8. Evaluation of the expression of Gpx2, p62, TKT and G6PD in SNU-423 cells stably expressing AKR1B10.

SNU-423 cells were treated with 4 μM Manumycin A, NAC (5mM) or a combination of Manumycin A and NAC for 48 hours and the cells were collected for protein isolation. 20 μg proteins were loaded on 10% SDS-PAGE gel. GAPDH was used as a loading control. Representative blot from 2 independent biological replicates is shown.

Overall, the data suggest that when faced with oxidative stress, AKR1B10 expression can enhance an antioxidant response. Since we did not observe an increase in the survival of AKR1B10 expressing SNU423 cells treated with

Manumycin A compared to control cells, it appears that this antioxidant response is not robust enough to enable cell survival.

3.5 Effects of ectopic expression of AKR1B10 on oxidative stress response in HuH-7 cells

We observed that HuH-7 cells treated with Manumycin A survived better than SNU423 cells, most likely due to a stronger antioxidant response (Figure 3.2). This strong antioxidant response could have resulted from a properly functioning NRF2-Keap1 system, or robust cellular levels of NADPH, or a well-functioning glutathione antioxidant system. To test this hypothesis, we evaluated the expression of the PPP enzymes (as the source of NADPH) in AKR1B10 overexpressing HuH-7 cells and control cells (Figure 3.9).

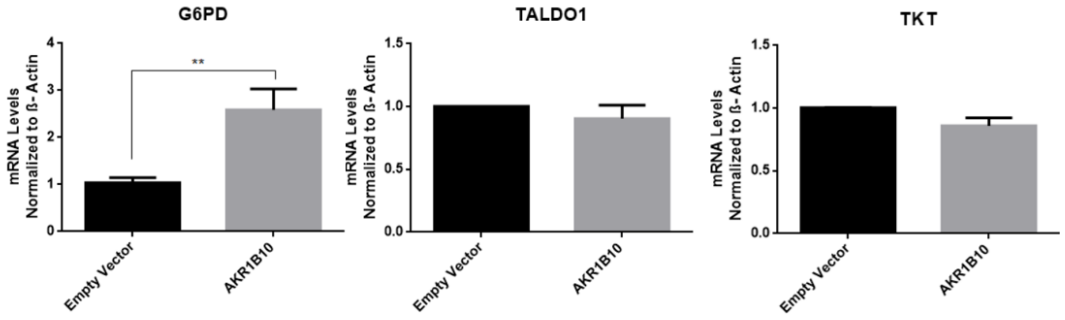

Figure 3.9. Expression of enzymes of the oxidative and non-oxidative branches of the PPP in HuH7 cells stably expressing AKR1B10 compared to EV (Empty Vector) at the transcript level.

*Pellet was collected from HuH-7 untreated empty vector and AKR1B10 expressing cells and RNA was isolated with MN RNA Isolation Kit. Results are presented as fold change with respect to the control (empty vector transfected) cells. Data from the average of 2 independent biological replicates are shown. Statistical analyses were carried out by t-test. *p<0.05*

An approximately 3-fold increase in the mRNA levels of G6PD was observed in AKR1B10 expressing HuH7 cells, whereas the expression of TKT and TALDO did not change. These data suggest that the oxidative branch of the PPP may be more active in AKR1B10 expressing HuH-7 cells. G6PD is also an NRF2 target (Hayes and Ashford, 2012) therefore overexpression of AKR1B10 may also increase the mRNA expression of G6PD in an NRF2 dependent manner. To better evaluate the NFR2 mediated transcriptional regulation of antioxidant genes in AKR1B10 expressing HuH7 cells the mRNA expression of several antioxidant genes was determined (Figure 3.10). Although a trend towards an increase or decrease of expression of these genes was observed, AKR1B10 expression did not lead to a significant change in the expression of any of the antioxidant genes tested.

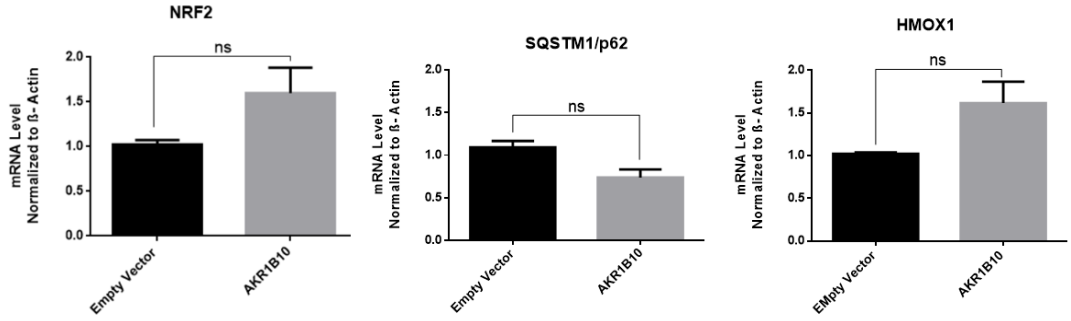

Figure 3.10. Expression of NRF2, SQSTM1 (p62) and HMOX1 in HuH7 cells stably expressing AKR1B10 compared to EV (Empty Vector) at the transcript level.

*RNA was collected from HuH-7 untreated empty vector and AKR1B10 expressing cells and isolated with MN RNA Isolation Kit. Results are presented as fold change with respect to the control (empty vector transfected) cells. Data from the average of 2 independent biological replicates are shown. Statistical analyses were carried out by t-test. *p<0.05*

The data presented above suggest that the increase in G6PD mRNA expression may be related to other functions of the increased NADPH pool in AKR1B10 expressing HuH-7 cells. To better evaluate this, the PPP was inhibited with 6-aminonicotinamide (6-AN) which blocks both G6PD and 6PGD. A colony formation assay was first carried out to determine whether HuH7 cells deprived of NADPH from the PPP showed any difference in survival when AKR1B10 was expressed. To our surprise, we observed that HuH7 cells were not reliant on the PPP since robust proliferation was seen in both 6-AN (2μM and 5μM) treated and untreated cells (Figure 3.11). At these concentrations almost a complete loss of survival in SNU423 cells was observed (data not shown; generated by Esin Gülce Seza). These data suggest that other mechanisms that can compensate for G6PD inhibition are likely to be functional in HuH-7.

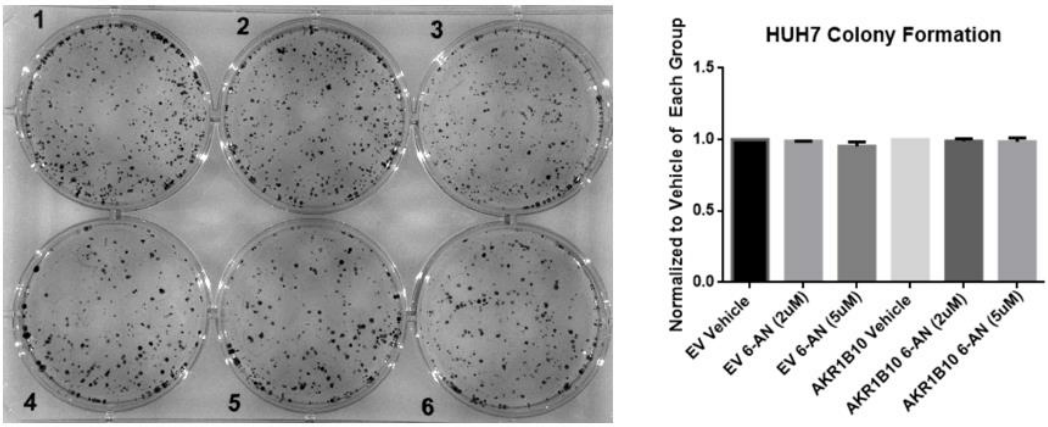

Figure 3.11. Colony Formation of Huh7 cells stably expressing AKR1B10 or empty vector (EV) and treated with 6-AN or vehicle.

500 AKR1B10 expression or EV transfected HuH-7 cells were plated in a 6-well plate. The cells were treated for 8 days with 6-AN or vehicle, and the medium was renewed every 48 hours. After 8 days, cells were fixed with 4% PFA and stained

Figure 3.11. (continued) Colony Formation of Huh7 cells stably expressing AKR1B10 or empty vector (EV) and treated with 6-AN or vehicle.

with 0.5% crystal violet solution. The upper row: Well #1 corresponds to vehicle treated EV cells, well #2 EV cells treated with 2µM 6-AN and well #3 corresponds to EV cells treated with 5µM 6-AN. Bottom row: Well #4 corresponds to vehicle (DMSO) treated AKR1B10 expressing cells, well #2 AKR1B10 expressing cells treated with 2µM 6-AN and well #3 corresponds to AKR1B10 expressing cells treated with 5µM 6-AN. Data from the average of 2 independent biological replicates are shown.

In addition to the oxidative branch of the PPP, several other enzymes can generate NADPH including ME1 and IDH1. To test whether any of these other enzymes were activated and therefore contributed to the NADPH pool when the PPP was inhibited, we treated AKR1B10 expressing HuH-7 cells with 10 µM 6-AN for 24 hours and evaluated the mRNA expression of ME-1 and G6PD. (Figure 3.12). We observed a decrease in the mRNA expression of G6PD with 6-AN treatment regardless of the expression of AKR1B10. However, an increase in the expression of ME-1 was seen in the 6-AN treated cells suggesting that alternative pathways can compensate for the loss of NADPH when the PPP was inhibited with 6-AN both in AKR1B10 expressing and empty vector control cells.

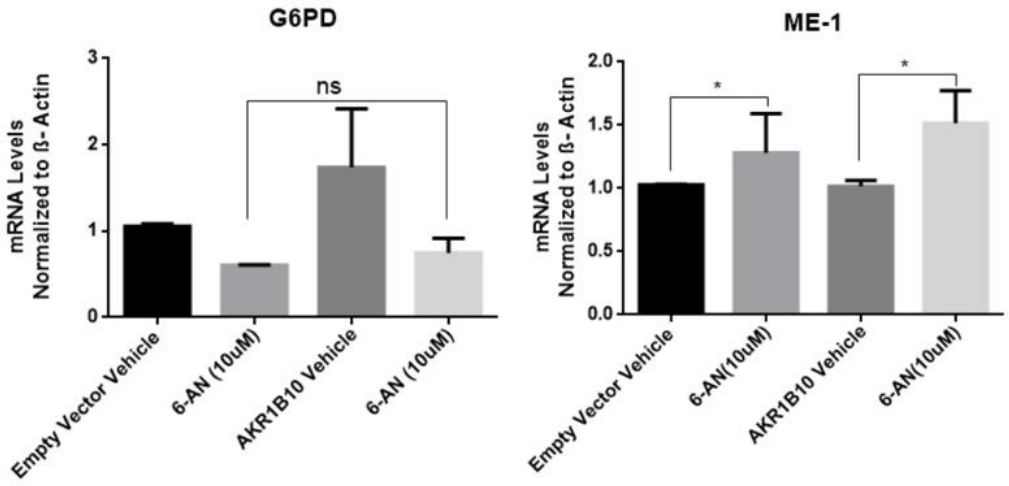

Figure 3.12. Expression of G6PD and ME1 in HuH7 cells stably expressing AKR1B10 compared to EV (Empty Vector) at the transcript level.

*HuH-7 cells expressing AKR1B10, or the empty vector (control) were treated with 6-AN (10 μM) for 24 hours and the cells were collected for mRNA and RT-qPCR. 6-AN treatment led to an increase in ME1 expression. Results are presented as fold change with respect to the control (empty vector transfected) cells. Data from the average of 2 independent biological replicates are shown for ME-1. Statistical analyses were carried out by two-way ANOVA followed by Tukey's comparison test for ME-1. *p<0.05*

3.6 Effect of ectopic expression of AKR1B10 on fatty acid metabolism in HuH-7 cells

The NADPH generated in cells can be used, in addition to mitigation of oxidative stress, in anabolic pathways such as fatty acid synthesis. Since we did not observe any change in the survival of AKR1B10 expressing HuH-7 cells treated with 5 and 10 μM of 6-AN and no significant change in the expression of NRF2 targets, we next evaluated a potential role of NADPH on anabolic pathways. For this, AKR1B10

expressing HuH-7 cells or control empty vector cells were treated with 5 and 10 μM 6-AN and the cells were collected for protein isolation (Figure 13). NADPH is extensively used for fatty acid synthesis and a key regulatory protein that contributes to the decision between fatty acid synthesis and oxidation in a cell is Acetyl CoA Carboxylase (ACC). When cellular NADPH and ATP levels are low, ACC undergoes an inhibitory phosphorylation on serine 79 (S-79) via AMPK and mediates β-oxidation of fatty acids to restore ATP levels (Mihaylova & Shaw, 2011). We observed an increase in the phosphorylation of ACC on serine 79 when the control cells were treated with increasing concentrations of 6-AN. This was expected, since 6-AN treatment inhibits one of the major NADPH generating pathways (PPP) in the cell and which should preclude any fatty acid synthesis. Interestingly, the phosphorylation of ACC was higher in AKR1B10 expressing HuH7 cells, which increased even further when the cells were treated with 6-AN (Figure 3.13). These increases were accompanied by a concomitant increase in p-AMPK on threonine 172 (T-172), suggesting that signaling via AMPK was responsible for the phosphorylation of ACC and increase in fatty acid oxidation. Therefore, it is likely that HuH-7 cells rely on fatty acid oxidation for energy generation, which is exacerbated with AKR1B10 overexpression.

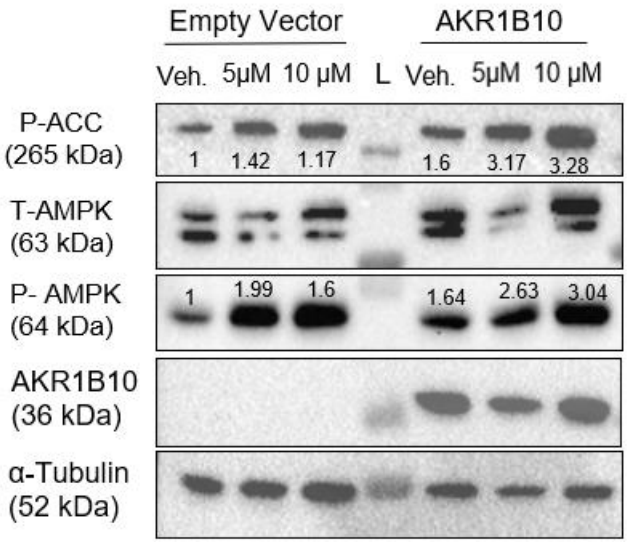

Figure 3.13. Evaluation of p-ACC (S79), p-AMPK (T172), and total AMPK proteins in HuH-7 cells stably expressing AKR1B10.

L represents the protein ladder. Lanes 1-6 correspond to: Empty Vector (EV) vehicle treated cells (Veh.), EV cells treated with 5μM 6-AN, EV cells treated with 10μM 6-AN, AKR1B10 expressing vehicle treated cells with vehicle (Veh.), AKR1B10 cells treated with 5μM 6-AN, AKR1B10 cells treated with10μM 6-AN. 20μg protein was loaded and α-tubulin was used as the loading control.

For AKR1B10 expressing HuH-7 cells to be reliant on β-oxidation of fatty acids, the presence of oxygen is crucial. To evaluate this, I treated HuH7 cells expressing AKR1B10 or the control cells with cobalt chloride ($CoCl_2$), a chemical that can stabilize HIF1α and therefore mimic a state of hypoxia (Muñoz-Sánchez & Chánez-Cárdenas, 2019). The treated cells were plated for a colony formation assay where the treatment with $CoCl_2$ was continued for the duration of the assay. We observed that HuH7 cells were highly vulnerable to 50μM $CoCl_2$ treatment and 100% of the cells regardless of whether AKR1B10 was expressed or not, were killed (Figure

3.14). With 5μM $CoCl_2$, nearly all cells survived, indicating that at this concentration the hypoxia mimicking role of $CoCl_2$ was not functional.

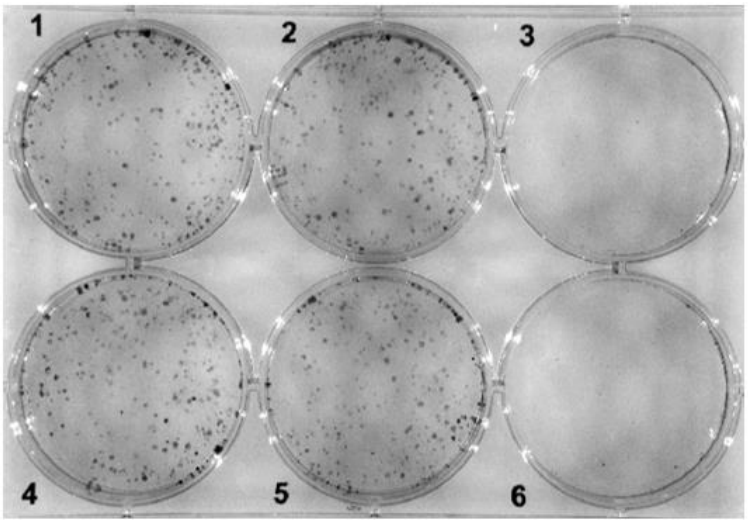

Figure 3.14. Colony Formation of HuH-7 cells stably expressing AKR1B10 or empty vector (EV) and treated with $CoCl_2$.

500 HuH-7 cells expressing AKR1B10, or the empty vector control were plated in 6-well plate. Cells were treated with 5 or 50 μM CoCl2 for 8 days the medium was renewed every 48 hours. After 8 days, cells were fixed with 4% PFA and stained with 0.5% crystal violet solution. The upper row corresponds to Empty Vector transfected cells and the lower row corresponds to AKR1B10 expressing cells: Well #1 corresponds to Empty Vector vehicle (DMSO) treated cells, well #2 5 μM Cobalt Chloride, well #3 50 μM Cobalt Chloride, well #4 AKR1B10 vehicle (DMSO) treated cells, well #5 5 μM Cobalt Chloride and well #6 50 μM Cobalt Chloride treated cells.

CHAPTER 4

DISCUSSION

Tumor cells are characterized with high energy demand for which catabolic pathways are active; rapid cell division means that generation of biomass is important for which anabolic pathways are active. This aberrant metabolic rewiring can eventually cause increased ROS levels (Hayes, Dinkova-Kostova, & Tew, 2020). High ROS levels can increase Nrf2 activity, therefore cancer cells possess a hyperactive antioxidant environment that facilitates anabolic pathways and increases tumor growth. Thus, high Nrf2 activity, so called NRF2 addiction, can protect cancer cells from their own oxidative stress and is also associated with chemoresistance. Moreover, mutations in KEAP1 are common in tumors such as lung cancer, whereby NRF2 is no longer sequestered in the cytosol and remains constitutively active (Emanuele et al., 2021).

AKR1B10 is a NADPH-dependent enzyme that catalyzes reduction reactions using various aldehydes and ketones as substrates (Penning, 2015). Most AKRs, including AKR1B10, are transcriptionally regulated by NRF2 (Macleod et al., 2016). While consuming NADPH for enzymatic activities, AKR1B10 can also produce redox-active quinones. In response, AKR1B10 can activate Nrf2 and maintain a high antioxidant response (Banerjee, 2021). Additionally, by reducing cytotoxic carbonyl, AKR1B10 can help maintain a redox balance. Since AKR1B10 is primarily expressed in the gastrointestinal tract, it can reduce the toxic carbonyls produced by the gut microbiota and protect cells from further damage (Balendiran, Martin, El-Hawari, & Maser, 2009). Supporting this, using *in silico* analyses of publicly available transcriptome data, qPCR evaluation of cDNA samples from colorectal cancer (CRC) patients and a panel of CRC cell lines, we have previously shown that AKR1B10 was strongly downregulated in CRC compared to non-transformed cells (Taskoparan et al., 2017).

Contrary to the tumor suppressive features of AKR1B10 in CRC, the enzyme was shown to be highly expressed in hepatocellular, non-small cell lung, breast, uterine and pancreatic cancers (Torres-Mena et al., 2018). Data from our lab suggest that AKR1B10 was upregulated very early during the transformation process of HCC, from the stage of cirrhosis (Sheraj I, unpublished data). A strong case has been made over the years for the use of AKR1B10 as a marker for poor prognosis. However, in a recent study, it was shown that AKR1B10 may be upregulated in liver tumorigenesis not as an indicator of cancer, but to act as a protector (Ye et al., 2019).

The motivation from my thesis emerged from *in silico* data generated in our lab showing that most antioxidant response genes were upregulated in high AKR1B10 expressing ($AKR1B10^{HIGH}$) HCC. To substantiate this finding in wet lab experiments, we have generated HuH7 and SNU423 HCC cells lines ectopically expressing AKR1B10 via stable lentivirus infection. Neither cell line expresses any AKR1B10 endogenously. SNU423 cells have a damaging mutation in *KEAP1* (https://depmap.org/portal/) that should lead to the hyper-activation of Nrf2 and a strong antioxidant response. Nonetheless, we observed that SNU423 cells were highly susceptible to the pro-oxidant drug Manumycin A; cell death could be rescued with NAC. SNU423 cells were also found to be highly reliant on NADPH from the pentose phosphate pathway (PPP) since we observed that treatment of these cells with 6-Aminonicotinamide (6-AN), a PPP inhibitor, caused extensive cell death. A recent study that evaluated metabolic dependencies in cancers such as lung adenocarcinoma (LUAD) with NRF2 activation, revealed that these cells were highly dependent on the availability of G6PD (Ding et al., 2021). Loss of G6PD led to cell death that could not be rescued with antioxidants or nucleosides, suggesting that cell death was not caused by NADPH depletion dependent oxidative stress or loss of ribonucleotides from the non-oxidative branch of the PPP. Rather, the loss of G6PD activated enzymes such as ME1 and IDH1 to replenish cellular NADPH levels, leading to a depletion in the intermediates of the TCA cycle. SNU423 cells also have a KEAP1 mutation, were highly dependent on the PPP for survival, and did not show any reversal of cell death when treated with 6-AN plus the antioxidant

NAC (data not shown), suggesting that a similar mechanism may be in place in these cells as well. Of note, HuH7 cells that have a functional KEAP1 protein, were not as susceptible to 6-AN and showed nearly 100% survival at concentrations that resulted in complete death of SNU423 cells (Figure 3.11).

Increased expression of AKR1B10 was shown to be associated with mitigation of oxidative stress in HCC (Liu et al., 2019). However, in the current study, induction of oxidative stress with Manumycin A, a pro-oxidant drug, did not lead to improved survival in AKR1B10 expressing SNU-423 cells, compared to empty vector controls (Figure 3.5). Data from our lab indicates that untreated AKR1B10 expressing SNU423 cells had higher ROS levels [detected by dihydroethidium (DHE) specific for superoxide and hydrogen peroxide] compared to the empty vector transfected cells (Korkmaz, N, unpublished data). Additionally, we observed a decrease both in TXNRD1 and HMOX1 in AKR1B10 expressing SNU-423 cells (Figure 3.7), further corroborating the increased ROS levels observed. When AKR1B10 expressing cells were treated with Manumycin A, a significant increase in the mRNA expression of TXNRD1 (Figure 3.7) and increase in the protein expression of GPX2 (Figure 3.8) was seen, suggesting that when faced with oxidative stress, AKR1B10 expression in SNU423 cells may ameliorate the ROS levels by increasing the expression of antioxidant genes, particularly those related to GSH replenishment. However, considering that AKR1B10 expression was not sufficient to reverse cell death from treatment with Manumycin A, it is likely that the antioxidant function of AKR1B10 was not sufficient for survival.

Despite the well-established crosstalk between AKRs and oxidative stress, the response of AKR1B10 expressing SNU423 and HuH7 HCC cells to treatment with Manumycin A or 6-AN suggested that AKR1B10 was not a major player in cellular response to these specific cellular insults. It is possible that the role of AKR1B10 in an antioxidant response is more relevant to other types of stress, or another member of the AKR family may be more functional with these types of stresses. AKR enzymes are thought to be stress-related genes, since the regulation is stimulated by oxidative, osmotic and electrophilic stress (Penning, 2017). There are fifteen AKRs

that have a role in the metabolism of lipid, sugar, chemical carcinogens, and cancer chemotherapeutic agents. In a recent study, eight AKR genes including AKR1B10, AKR1C1-4, AKR1D1, AKR7A2-3) were showed significantly dysregulated in HCC (Dai et al., 2021). Aflatoxin, which induces HCC, was shown to be reduced by AKR7A3 to dialcohols in humans (Chow et al., 2016). AKR1A1, AKR1C1-4, AKR1B10 were shown to metabolize tobacco carcinogens polycyclic aromatic hydrocarbons (PAH) and 4-(methylnitrosoamino)-1-(3- pyridyl)-1-butanone (NNK) (Quinn, Harvey, & Penning, 2008). Moreover, it was shown that AKR1C1-3 enzymes played important roles in the detoxification of PAH transhydrodiols in lung adenocarcinoma cells (Park et al., 2008). In a recent study, it was shown that AKR1C3 expression in HCC can regulates metastasis via activation of NF-κB and STAT3 (Zhou et al., 2021). It was shown that exposure to Nrf2 activators including ROS may increase the AKR1C1 mRNA levels by 3 to 10 folds in both HepG2 and the CRC cell line HT29 (Penning, 2016). Finally, knockdown of Nrf2 resulted in increased mRNA and protein levels in AKR7A3 in HepG2 cells. Moreover, sensitivity against acetaminophen-induced toxicity was increased in these cells (Ahmed et al., 2011). In another study, it was shown that acetaminophen treatment depleted GSH and reduced the viability of L-02 (non-transformed) liver cells; moreover, acetaminophen-induced cell death could be reversed with NAC (Liang et al., 2010). Taken together, these data suggest that other AKRs, especially AKR1C1-3 and AKR7A3, may be more relevant in cellular survival against stress.

Lipids have high energy storage and can provide ATP for the biosynthetic reactions. Fatty acids are utilized for the synthesis of structural lipids such as phospholipids and triglycerides, for signal transduction via phosphatidyl inositol and for post-translational modifications such as prenylation or geranylgeranylation of different proteins (Koundouros & Poulogiannis, 2020). Fatty acids can be taken up from the environment or synthesized from excess glucose or glutamine via fatty acid synthesis (de novo synthesis) (Nakagawa et al., 2018). The synthesized lipids can either be stored as lipid droplets (thereby diminishing toxicity due to excess glucose or lipids) or used up when the cell is under metabolic stress or in high energy demand via fatty

acid oxidation or β-oxidation. Fatty acid oxidation generates NADH and $FADH_2$ via the TCA to be used in ATP production (M. Wang et al., 2016). To maintain high rates of proliferation, cancer cells can reprogram their metabolism according to their requirement of higher energy output or synthesis of macromolecules. This increased proliferation requires more membrane biosynthesis and lipids, hence aberrant fatty acid metabolism is often seen in cancer cells. Saturated and monounsaturated lipids, which are produced during de novo synthesis, are important for these cells (Koundouros & Poulogiannis, 2020). In a study, it was shown that cancer cells with more saturated phospholipids in their membrane were resistant to chemotherapeutic agent induced oxidative stress, inhibition of fatty acid synthesis via the use of an ACC inhibitor increased cytotoxicity (Rysman et al., 2010).

AKR1B10 was shown to bind to the ACC protein and prevent its degradation and increase fatty acid synthesis in RAO-3 breast cancer cell line (Ma et al., 2008). Moreover, silencing of AKR1B10 in HCT-8 cells and NCI-H460 cells resulted in enhanced depletion of cellular lipid species, increase in lipid peroxidation, damage to mitochondria, and enhanced oxidative stress due to uneliminated carbonyl species. The research group indicated that AKR1B10 mediated stabilization of ACC protein resulted in enhanced lipid synthesis, and elimination of carbonyl groups resulting in better cell survival (C. Wang et al., 2009). However, in a more recent study, several breast cancer cells lines with high AKR1B10 expression were found to reduce the FA synthesis significantly. Moreover, the authors reported that AKR1B10 expression was associated with high fatty acid oxidation under elevated oxidative stress (van Weverwijk et al., 2019).

In HCC, both FA synthesis and oxidation have been observed and even though a role of fatty acid synthesis is recognized in HCC, the role of oxidation in carcinogenesis is currently unclear. A reduction in β-oxidation has been shown in many studies; however, some tumor types show different phenotypes upon fatty acid oxidation (Berndt et al., 2019). Moreover, the expression of FA oxidation related genes can vary widely among patients (Björnson et al., 2015). These differences may emanate from the high heterogeneity of liver cancer.

In β-catenin- activated HCC (with a mutation in the CTNNB1 gene), increased fatty acid oxidation together with increased PPARα and CPT2 were observed in both human and mice tumors (Nakagawa et al., 2018). CTNNB1 mutation has been reported to be found in 19.5% of human HCC tumors (Ando, Shibahara, Hayashi, & Fukayama, 2015). Chemical or genetic inhibition of fatty acid oxidation was shown to diminish tumor development in β-catenin activated HCCs (Senni et al., 2019), although neither SNU423 nor HuH7 cells are known to harbor any mutations in the CTNNB1 gene (https://depmap.org/portal/).

In a recent study, three different subclusters of HCCs were identified and designated as iHCC1-2-3. The iHCC1 subcluster tumors had the most elevated β-oxidation rate, while iHCC2 tumors were characterized by elevated expression of the β-catenin target genes such as glutamine synthase and only 75% of the tumors were prone to FAO. On the other hand, the iHCC3 subcluster was associated with higher fatty acid synthesis (Bidkhori et al., 2018).

Although the expression and function of AKR1B10 has been widely studied in HCC (Distefano & Davis, 2019), the crosstalk of FAO with AKR1B10 in HCC has not been reported to the best of my knowledge. Most of the attention has been devoted to the role of AKR1B10 in detoxification reactions, particularly of chemotherapy drugs and a role in oxidative stress response (Endo et al., 2021b). In the current study, we observed an antioxidant effect of AKR1B10 in SNU423 cells, particularly when the cells were either under oxidative stress or with a deficient PPP; however, ectopic expression of AKR1B10 in these cells was not sufficient to prevent cell death.

HuH7 cells, on the other hand, showed no change in susceptibility to oxidative stress or to reduced levels of NADPH irrespective of whether AKR1B10 was expressed. HuH7 cells, however, showed an increase in the inhibitory phosphorylation of ACC especially in AKR1B10 expressing HuH-7 cells, suggesting that FAO in these cells may be exacerbated when the cells were treated with 6-AN. Moreover, we also observed an increase in phosphorylation of AMPK, suggesting that p-AMPK can stimulate the phosphorylation of ACC and enhance FAO. Moreover, when these

cells were treated with $CoCl_2$ (a hypoxia mimic) we saw extensive cell death at 50 μM again regardless of the AKR1B10 expression, suggesting that these cells may rely more on FAO for energy.

A recent study divided HCCs into different subgroups as CL1 hepatoblast-like, CL2 mixed epithelial-mesenchymal and CL3 mesenchymal-like via whole-exome RNA and miRNA sequencing. HuH-7 cells were classified as CL1 while SNU-423 cells were classified as CL3 (Caruso et al., 2019). A more recent study with similar methods showed that HuH-7 cells have a more hepatoblast-like phenotype. Moreover, the same study reported that HuH-7 cells have increased β-oxidation, cholesterol biosynthesis, sulfur-amino acid metabolism, along with decreased glycolysis and fructose metabolism (Sun, Tang, Ye, & Ding, 2021). These two recent studies support my finding that AKR1B10 may facilitate FAO in cells that are reliant on this mode of energy generation.

CHAPTER 5

CONCLUSION AND FUTURE STUDIES

My thesis aimed to identify whether the expression of AKR1B10 in HCC cells affected pathways generating NADPH and any potential biological effect of such a crosstalk. My initial hypothesis was to identify a role for NADPH availability for an antioxidant effect in AKR1B10 expressing cells. SNU423 cells did rely on NADPH for survival and were sensitive to pro-oxidant stress. Ectopic expression of AKR1B10 in these cells led to an antioxidant response when under oxidative stress, however, such an antioxidant response was not sufficient to reverse any cell death.

HuH-7 cells showed high survival under both pro-oxidant stress (manumycin A) and inhibition of PPP (6-AN) irrespective of whether AKR1B10 was expressed or not suggesting that these cells had a strong endogenous antioxidant response and could efficiently compensate for the loss of NADPH via PPP from other metabolic sources. However, we observed an increase in phosphorylation of ACC and AMPK with 6-AN treatment which was exacerbated when AKR1B10 was ectopically expressed in these cells. HuH7 cells were also seen to be reliant on oxygen for their survival as treatment with $CoCl_2$ (hypoxia mimic) lead to extensive cell death. This is one of the first reports on the contribution of AKR1B10 in enhancing FAO in HCC cell lines. Data currently available in the lab is insufficient to address the source of fatty acids that could be oxidized in these cells; further studies are being designed to address this.

Taken together, my thesis work indicates a role of AKR1B10 in an antioxidant response, as reported from other studies previously. However, AKR1B10 may also have a previously under-appreciated role in fatty acid metabolism. Fatty acid metabolism can significantly affect cellular pools of cofactors such as NADPH and

NADH. Therefore, our hypothesis on a role of AKR1B10 in determining NADPH availability may be relevant to a regulation of fatty acid metabolism and an antioxidant response.

Several experiments can be conducted in the future to better substantiate the findings of this thesis:

- We have hypothesized changes in NADPH levels in cells expressing AKR1B10. The NADPH/NADH ratio needs to be determined in both SNU-423 and HuH-7 cells expressing AKR1B10.

- If FAO is the predominant means of energy generation in AKR1B10 expressing cells, then the role of FAO needs to be evaluated in more detail. For this, the oxygen consumption rate (OCR) of the cells can be evaluated. Additionally, inhibition of FAO with etomoxir (2[6hexyl] oxirane-2-carboxylate, an irreversible inhibitor of carnitine palmitoyltransferase-1) can be evaluated. HuH7 cells with high FAO should have a high OCR and should be highly susceptible to death when treated with etomoxir.

- The substrate for FAO needs to be identified. Cells can either use fatty acids from the environment or can generate their own from sources such a glucose or glutamine (de novo FAS). We need to establish which of these two mechanisms provide the fatty acids for oxidation.

- We have observed high p-AMPK in AKR1B10 expressing cells cultured in complete medium. We will need to establish how the AMPK is activated in these cells and whether the inhibition of AMPK will affect FAO.

- Finally, if possible, we need to determine exactly how AKR1B10 mediates fatty acid oxidation. Previous studies have identified a physical interaction of AKR1B10 with ACC in a breast cancer cell line. However, in the current study we did not observe any changes in total ACC levels, suggesting that such an interaction may not be occurring in HCC. The mechanism for enhanced FAO was not addressed in the study by van Weverwijk et al., (2019). For this, metabolomics studies can be

designed, which can provide data on how carbons from nutrients such as glucose or glutamine are channeled through the metabolic pathways when AKR1B10 is expressed.

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APPENDICES

A. COMPOSITIONS OF THE BUFFERS USED IN THIS STUDY

6X SDS-PAGE SAMPLE LOADING DYE	
SDS	12%
B-mercaptoethanol	30%
Glycerol	30%
Bromophenol Blue	0.012%
Tris-HCL, pH=6.8	0.375%

4% SDS-PAGE STACKING GEL MIXTURE (1.5 mm Glass)	
dH_2O	3.1 mL
0.5 m Tris-HCl pH= 6.8	1.25 mL
Acrylamide/Bisacrylamide Solution	650 µL
10% Ammonium Persulfate (APS)	50 µL
TEMED	5 µL

10% SEPARATING GEL MIXTURE (1.5 mm Glass)	
dH_2O	4.1 mL
1.5 M Tris-HCl pH= 8.8	2.5 mL
Acrylamide/Bisacrylamide Solution	3.33 mL
10% Ammonium Persulfate (APS)	100 µL
TEMED	10 µL

MILD STRIPPING BUFFER pH= 2.2	
Glycine	15 g
SDS	1 g
Tween-20	10 mL
dH_2O	1 L

10X TRANSFER BUFFER pH=8.3	
Tris	25 mM
Glycine	192 mM
dH_2O	1 L

SDS-PAGE RUNNING BUFFER	
Tris	25 mM
Glycine	190 mM
SDS	0.1%

1X TRANSFER BUFFER	
dH_2O	700 mL
Methanol	200 mL
10X Transfer Buffer	100 mL