EFFECTS OF THE QUERCETIN DERIVATIVE CHNQ, A POTENT ALDO-KETO REDUCTASE INHIBITOR, ON AKR1B1 SILENCED HCT-116 COLORECTAL CANCER CELLS

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ABSTRACT

EFFECTS OF THE QUERCETIN DERIVATIVE CHNQ, A POTENT ALDO-KETO REDUCTASE INHIBITOR, ON AKR1B1 SILENCED HCT-116 COLORECTAL CANCER CELLS

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Aldo-keto reductases (AKRs) are NAD(P)H dependent oxidoreductases that are known to be involved in the biosynthesis, metabolism and detoxification of a number of substrates including glucose. These enzymes are therefore implicated in the development of diabetic complications. Additionally, this family of enzymes, particularly AKR1B1, has been shown to be involved in pathology of inflammation-associated diseases such as atherosclerosis, asthma, uveitis, sepsis, arthritis, periodontitis and cancer, including colorectal cancer (CRC).

To better understand the role of AKR1B1 in CRC, we selected the cell line HCT-116 that robustly expresses AKR1B1. Next, we either pharmaceutically inhibited AKR1B1 using 3,7-dihydroxy-2-[4-(2-chloro-1,4-naphthoquinone-3-yloxy)-3-hydroxyphenyl]-5-hydroxychromen-4-one (CHNQ), a novel quercetin derivative and AKR1B1 inhibitor, or stably silenced the gene using shRNA.

Contrary to our expectations, the cellular effects of CHNQ appeared to be unrelated to its inhibitory effects on AKR1B1; rather it had a significant pro-oxidant effect both *in vitro* and *in vivo* by virtue of its 1,4 napthoquinone moiety. Confirming this, we

observed an upregulation of the ERK pathway, cell cycle arrest at G2/M phase, induction of apoptosis and decreased cell migration in HCT-116 cells. These effects were similar both in cells with a stable knockdown of AKR1B1 and control cells. On the other hand, we observed that silencing of AKR1B1 itself in HCT-116 cells resulted in decreased proliferation, delayed cell cycle progression, downregulation of the NF-κB pathway as well as impaired cell motility and migration. Taken together, this study provides new data that AKR1B1 may have oncogenic properties in CRC.

Keywords: aldo-keto reductase, AKR1B1, colorectal cancer, ROS, NF-κB

ÖZ

ALDO KETO REDÜKTAZ İNHİBİTÖRÜ OLAN KUERSETİN TÜREVİ CHNQ MADDESİNİN AKR1B1 SUSTURULMUŞ HCT-116 KOLOREKTAL KANSERİ HÜCRELERİNDE ETKİLERİNİN ARAŞTIRILMASI

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Aldo-keto redüktazlar (AKR), glikoz da dahil olmak üzere birtakım substratların biyosentezinde, metabolizmasında ve detoksifikasyonunda önemli bir rol oynayan NAD(P)H bağımlı oksidoredüktazlardır. Bu sebeple, bu enzimler diyabetik komplikasyonların oluşmasında önemli bir yere sahiplerdir. Buna ek olarak, bu enzim ailesi, özellikle AKR1B1, aterosklerosis, astım, üveit, sepsis, artrit, periyodontit ve kolon kanseri de dahil olmak üzere bir çok kanser tipi üzerinde inflamasyona bağlı patolojilerde yer almaktadır.

Kolon kanserinde AKR1B1 enziminin rolünü daha iyi anlamak için, bu enzimi aşırı derecede ifadeleyen HCT-116 kolon kanseri hücre hattı model olarak seçilmiştir. Sonrasında, AKR1B1 farmasötik yollarla özgün bir kuersetin türevi ve AKR1B1 inhibitörü 3,7-dihydroxy- 2-[4- (2-chloro- 1,4- naphthoquinone-3- yloxy)-3- hydroxyphenyl]-5- hydroxychromen-4- one (CHNQ) ile inhibe edilmiş ya da shRNA kullanılarak genetik olarak susturulmuştur. Beklentilerin aksine, CHNQ'nun hücresel etkileri AKR1B1 enzimini inhibe etmekten ziyade, 1,4, naphtoquinone grubundan dolayı önemli bir pro-oksidant olma yönündedir. Bunu doğrular nitelikte, HCT-116 hücrelerinde, ERK yolağının aşırı regülasyonu, hücre döngüsünde G2/M tutuklanması,

apoptozun tetiklenmesi ve hücre göçünün azalması gözlemlenmiştir. Bu etkilerin, hem AKR1B1 susturulmuş hücrelerde hem de kontrol hücrelerde benzer olduğu görülmüştür. Bunun yanında, AKR1B1'in HCT-116 hücrelerinde susturulması kendi başına oksidatif stresi düşürerek hücre proliferasyonunu azaltmış, hücre döngüsünde gecikmeye sebep olmuş, NF-κB yolağının regülasyonunu düşürerek inflamatuar tepkileri azaltmış, ve düzgün işlemeyen hücre hareketliliği ve göçüne sebep olmuştur. Sonuç olarak, bu çalışma, AKR1B1'in kolon kanserinde hücre çoğalmasını, hücre döngüsünü, hareketliliğini ve NF-κB aktivasyonunu tetikleyerek onkojenik özelliklerini vurgulamaktadır.

Anahtar kelimeler: aldo-keto redüktaz, AKR1B1, kolorektal kanser, ROT, NF-κB

To my family

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LIST OF ABBREVIATIONS

CRC	colorectal cancer
AKR	aldo-keto reductase
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
CHNQ	3,7-Dihydroxy-2-[4-(2-chloro-1,4-naphthoquinone-3-yloxy)-3-hydroxyphenyl]-5 hydroxychromen-4-one
IC_{50}	half maximal inhibitory concentration
MAPK	mitogen-activated protein kinase
ERK	extracellular signal-regulated kinase
G1	Gap 1 phase of cell cycle
S	Synthesis phase of cell cycle
G2	Gap 2 phase of cell cycle
M	mitosis
CDK	cyclin-dependent kinase
pRb	retinoblastoma protein
NF-κB	nuclear factor – kappa B
shRNA	short hairpin RNA
DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
PBS	phosphate buffer saline
TBS	tris buffer saline
TBS-T	tris buffer saline- Tween 20
PVDF	polyvinylidene fluoride
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
BrdU	bromodeoxy uridine
DHE	dihydro ethidium
NBT	nitrotetrazolium blue
ROS	reactive oxygen species
NAC	N-acetyl cysteine
YPD	yeast-peptone-dextrose

CHAPTER 1

INTRODUCTION

1.1.Cancer

Cancer is a disease of uncontrolled cell division. It can have severe health consequences and it is one of the major causes of death all over the world. While most cancers are sporadic and may be caused by environmental factors such as tobacco usage, unhealthy lifestyle or infectious organisms, it can also be inherited; both environmental and genetic elements may be involved in the development of this disease (American Cancer Society, 2016).

According to the latest report of World Health Organization (WHO) in 2012, each year 8.2 million people die from cancer, which is equivalent to 13% of all deaths worldwide. On the other hand, in Turkey, cancer is the second leading cause of death with a 20% death rate (Turkish Statistical Institute, 2015).

During the development of cancer, a cancer cell acquires several characteristics to form a tumor. These characteristics mainly involve maintaining continuous proliferative signals to have self-sufficiency in growth signals and insensitivity to anti-growth signals, limitless replicative potential, avoiding and resisting apoptosis, initiating metastasis and tissue invasion, and activating angiogenesis (Hanahan & Weinberg, 2000). Moreover, over the past decade, new characteristics such as alterations in cellular metabolism and escape from immune responses have also emerged (Hanahan et al., 2011).

1.2.Colorectal Cancer

Colorectal cancer (CRC) is defined as the cancer of colon and rectum. Regardless of gender, it is the third most frequent cancer type in the world (American Cancer Society, 2016). Development of CRC can be highly associated with inherited factors, as well as environmental factors like eating habits and poor lifestyle. Consumption of a high-fat diet, red meat, insufficient intake of fiber-containing foods are linked to the development of CRC (Pericleous, Mandair, & Caplin, 2013; Song, Garrett, & Chan, 2015).

The development of CRC with the adenoma-carcinoma sequence model has been well defined in the literature and begins with hyper-proliferation of the colon mucosal cells. This leads to the formation of small, benign clumps of cells that are called adenomatous polyps. Then, these adenomatous polyps transform into larger pre-cancerous polyps with severe dysplasia that still hold their benign features. From polyps to adenocarcinoma, the cells of the colon start to acquire more malignant characteristics. During these processes, two types of genomic instabilities are observed: microsatellite instability (MIN) that is caused by mismatch repair defects at the nucleotide level and chromosomal instability (CIN) that is caused by gain or loss of chromosomes or their parts during cell division (Cunningham et al., 2010). This established description of colon carcinogenesis is regulated and determined by several different molecular pathways, some of which include the activation of proto-oncogenes such as Ras and inactivation of tumor suppressor proteins such as APC and p53 and (Michor, Iwasa, Lengauer, & Nowak, 2005).

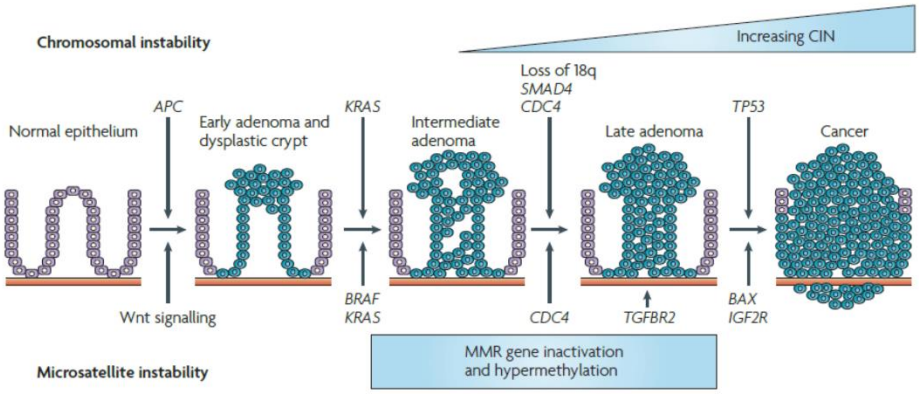

Figure 1.1. Representation for the model of colon carcinogenesis; retrieved from (Walther et al., 2009).

Although there is no certain way to prevent the development of CRC, there are ways to decrease the risk. Moreover, if detected at an early stage through sophisticated endoscopic and colonoscopic techniques, the disease can be cured through advancements in surgical techniques. However, death rates from CRC are still high all over the world and much remains to be identified in the signaling mechanisms that underlie a complex disease such as cancer. This may then lead to novel ways of prevention of the disease either through physiological or pharmacological means.

1.3.Aldo-Keto Reductases (AKRs)

The superfamily of Aldo-Keto Reductases (AKRs) are nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) - dependent oxidoreductases that can catalyze the reduction of many substrates such as retinals, steroid hormones, bile acid precursors, glucose, lipid peroxidation products, prostaglandins etc. and exogenous substrates like xenobiotics. The superfamily consists of 15 families (AKR1-AKR15) and more than 190 members. These enzymes are 34-37 kDa monomeric proteins and generally found in the cytosol. Although there is high functional diversity among AKRs, all of them contain a conserved $(\beta/\alpha)_8$ barrel structure and a pyridine nucleotide binding site in common with high sequence identity, which makes them structurally

very similar to each other (Mindnich & Penning, 2009). The common $(\beta/\alpha)_8$ barrel motif designates a crucial functionality to AKRs. It is involved in the oligomerization of tertiary and quaternary structures, and binding of cofactors and metals, so that they can generate an active site geometry (Wierenga, 2001).

In the nomenclature of AKR proteins, the first number represents the family (e.g. AKR1), then a letter represents the subfamily (e.g. AKR1B) and finally a second number represents the unique protein (e.g. AKR1B1). Human aldo-keto reductases fall into 3 main families: AKR1, AKR6 and AKR7. Within these three families, 15 AKR proteins have been described. Systematic and functional names of each member of human AKRs can be found in ***Table 1.1***.

Table 1.1. List of human AKR members.

Systematic Name	Functional Name
AKR1A1	Aldehyde reductases
AKR1B1	Aldose reductases
AKR1B10	Small intestine-like aldose reductase
AKR1B15	Aldose reductase
AKR1C1	3α(20α)-hydroxysteroid dehydrogenase
AKR1C2	Type 3 3α-hydroxysteroid dehydrogenase
AKR1C3	Type 2 3α-hydroxysteroid dehydrogenase
AKR1C4	Type 1 3α-hydroxysteroid dehydrogenase
AKR1D1	Steroid 5β-reductase
AKR1E2	1,5-Anhydro-D-fructose reductase
AKR6A3	Potassium voltage gated channel, β-subunit-1
AKR6A5	Potassium voltage gated channel, β-subunit-2
AKR6A9	Potassium voltage gated channel, β-subunit-2
AKR7A2	Aflatoxin aldehyde reductase 1
AKR7A3	Aflatoxin aldehyde reductase 2

Although all human AKRs share highly common features with each other, they all have different substrates and therefore, different functions. They catalyze a wide range of reactions and some of these reactions can overlap with each other.

1.3.1. AKRs and Disease

AKR1B subfamily of AKRs is known as aldose reductase, which is involved in the reduction of aldehydes to alcohol and many other substrates. More specifically, AKR1B1 is involved at the beginning of polyol pathway, where it converts glucose into sorbitol using NAD(P)H as the cofactor. Then, the polyol pathway continues with sorbitol dehydrogenase (SDH) by the conversion of sorbitol to fructose by through an NAD-linked oxidation process.

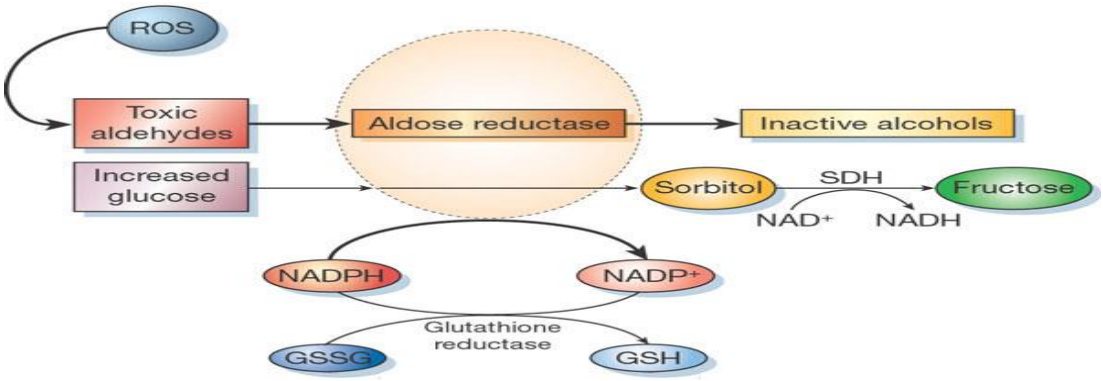

Figure 1.2. Aldose reductase and its role in polyol pathway; retrieved from (Brownlee, 2001).

These enzymes are best characterized in the implications of general diabetic pathophysiology (Saraswathy et al., 2014). Under normal glycemic conditions, polyol pathway uses ~3% of total glucose flux, however, under hyperglycemic conditions polyol pathway affects more than 20% of total glucose metabolism. This increased usage of polyol pathway induced by hyperglycemia leads to primary and secondary diabetic complications like hyperglycemic tissue injury (S. K. Srivastava, Ramana, & Bhatnagar, 2005). AKRs are involved not only in diabetic complications, but also in

the reduction of aldehydes derived from lipid peroxidation and their corresponding glutathione conjugates (Ramana, 2011). Aldehydes, such as 4-hydroxy-trans-2-nonenal (HNE) an its glutathione conjugate (GS-HNE), are reduced by AKRs to their corresponding alcohols 1,4-dihydroxy-nonene (DHN) and glutathionyl-1,4-dihydroxynonene (GS-DHN). These reduced compounds serve as inflammatory signals, which mediate reactive oxygen species (ROS) related signaling, leading to an inflammatory response (Srivastava et al., 2011). Furthermore, elevated expression of AKRs have been linked to many chronic inflammatory diseases including sepsis, asthma, arthritis, uveitis and many cancers such as breast, livers, lung and colon (Ramana, 2011; Tammali, Srivastava, & Ramana, 2011).

In cancer, the expression of AKRs, specifically AKR1B1, is highly variable in different cancer types and tissue origin. Based on an Oncomine gene expression database study, AKR1B1 expression was observed to be significantly increased in cancer when compared to the corresponding normal tissue in bladder, cervical, brain, esophageal, kidney, leukemia, lymphoma and melanomas with 1.2-5 fold increase in expression (Laffin & Petrash, 2012). In another study, it was shown that lung, breast, prostate, cervix, ovarian and colon cancers display increased expression of AKR1B1. Moreover, not only expression but also the activity of AKRs were shown to be higher in these cancer types (Saraswat et al., 2006).

The role of AKR1B1, its aberrant expression and methylation has been reported in CRC previously (Cebola et al., 2015; Laffin & Petrash, 2012; Moon et al., 2014). Based on these studies, AKR1B1 was found to be a potential screening marker for CRC in a DNA methylation profiling study (Wei et al., 2016). Moreover, previous studies indicate that inflammation is one of the major actors of carcinogenesis of colon (Terzić et al., 2010). The role of AKRs is shown to be regulating carcinogenic signaling pathways by inducing growth factors and cytokines, and this provides AKRs a mitogenic and an inflammatory role for colon carcinogenesis (Tammali, Ramana, Singhal, Awasthi, & Srivastava, 2006; Tammali, Ramana, & Srivastava, 2007).

1.3.2. AKR Inhibitors

A number of AKR inhibitors were developed in order to inhibit the generation of polyol pathway products such as sorbitol that is implicated in diabetic complications or to reduce oxidative stress. Based on their chemical structures, these inhibitors fall into 3 major groups: succinimide class (e.g ranirestat), acetic acid derivatives (e.g. tolrestat, zopolrestat) and spiro hydantoins (e.g. sorbinil, fidarestat) (Liu, Wen, & Cao, 2009). These drugs were developed as soon as the role of AKRs and polyol pathway were discovered to be involved in diabetic complications. However, many of them had excessive toxicity and low specificity, which hindered clinical applications (Alexiou, Pegklidou, Chatzopoulou, Nicolaou, & Demopoulos, 2009).

Recently, the development of phytochemical therapeutics has been paid considerable attention. Flavonoids are an important group of phytochemicals that are widely available in the human diet and are known to be involved in inhibitory mechanisms of diabetes-related complications such as oxidative stress and the polyol pathway (Stefek, 2011). Quercetin, a flavonol, is a widely consumed flavonoid in the human diet and its AKR inhibitory action is well-defined (Stefek & Karasu, 2011). However, quercetin has limited application in clinics because of its low bioavailability and poor water solubility. Recently a series of semi-synthetic derivatives of quercetin were synthesized and characterized as bi-functional agents combining ARI activity with antioxidant action (Veverka et al., 2013). Among the derivatives studied, the chloronaphthoquinone derivative of quercetin (CHNQ) appeared to be most promising compound with very high ARI and antioxidant activities.

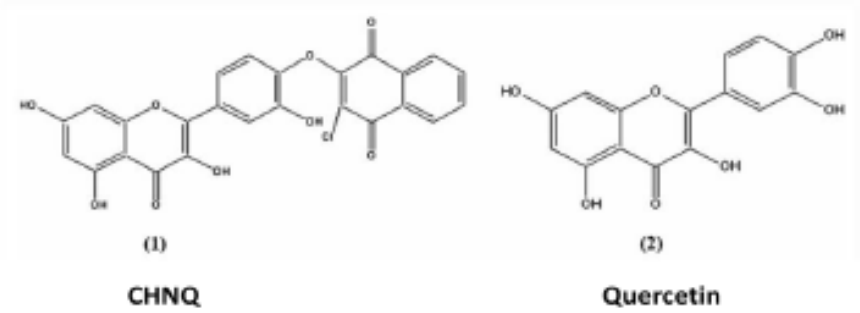

Figure 1.3. CHNQ and Quercetin.

1.4. Cell Proliferation and the Mitogen Activated Protein Kinase (MAPK) Pathway

One of the most critical pathways in the regulation of differentiation, apoptosis and proliferation of cells is Mitogen Activated Protein Kinase (MAPK) signaling cascade (English et al., 2002). The cascade basically obtains signals from cell surface receptors and transmits them to transcription factors and gene expression (McCubrey et al., 2007). There are 3 subfamilies of MAPKs: **i.** the extracellular-signal-regulated kinases (ERK), **ii.** c-Jun N-terminal or stress-activated protein kinases (JNK or SAPK) and **iii.** MAPK.

Among these subfamilies, ERK1/2 (i.e. Ras/Raf/MEK/ERK) pathway is a key regulator of cell proliferation (Chang et al., 2003). ERK1/2 signaling cascade is directly linked to the Receptor Tyrosine Kinases (RTKs) activation through stimulation by growth factors. This activation recruits the adaptor protein Grb2 (growth factor receptor bound proteins) together with the guanine nucleotide exchange factor SOS (son of sevenless). SOS activates Ras, a proto-oncogene, provides the recruitment and activation of Raf by phosphorylating it (Fang & Richardson, 2005). With this, MEK1/2 is also phosphorylated, which subsequently phosphorylates ERK1/2 (Mebratu & Tesfaigzi, 2009). When, ERK1/2 is activated, it is translocated to the nucleus, and then phosphorylates and activates several transcription factors

including AP-1, c-Myc and NF-κB, which links this pathway directly to cell proliferation, cell cycle and inflammation (Chang et al., 2003).

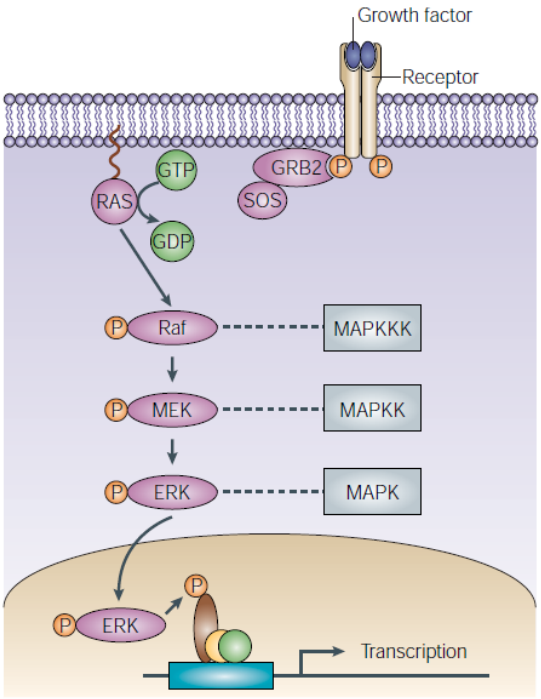

Figure 1.4. Summary of the ERK/MAPK cascade; retrieved from (Kim & Bar-Sagi, 2004).

1.5.Cell Cycle and Cancer

Cell cycle of a eukaryotic organism contains a series of events in the formation of two daughter cells. It consists of 4 well-defined phases, which are G1, S (DNA synthesis phase), G2 and M (mitosis phase). Genetic material of the cell is duplicated in the S phase and is then segregated into the two daughter cells after mitosis in the M phase., S and M phases are separated by two gap phases G1 and G2. Both of these gap phases are responsible for cell growth. In the G1 phase, the size of the cell increases, and many metabolic activities of the cell are slowed down. The checkpoint at the end of this phase controls whether the cell is ready for DNA synthesis or not. Likewise, in the

G2 phase, the cell continues growing and the G2 phase checkpoint controls whether the cell is ready for entering M phase and divide (Suryadinata, Sadowski, & Sarcevic, 2010). Progression through these four phases in a cyclical manner is mainly regulated by Cyclin proteins and their Cyclin-Dependent Kinases (CDKs). The protein levels of Cyclins can oscillate up and down in a cell cycle and thereby binding to and activating their corresponding CDK partners in a cyclical manner. For each phase of cell cycle, a different Cyclin-CDK complex is required, and when the phase ends, the responsible Cyclin-CDK complexes are rapidly degraded, allowing other cyclin-CDKs to take part (Hochegger, Takeda, & Hunt, 2008).

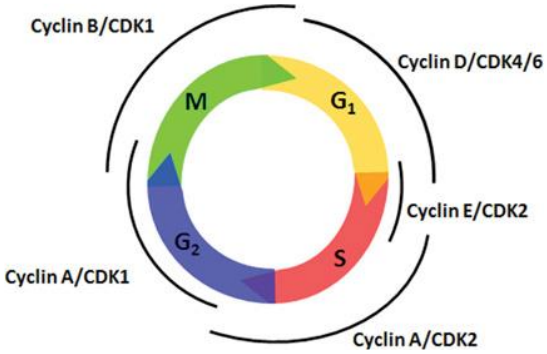

Figure 1.5. A model for Cyclin-CDK regulation of mammalian cell cycle; retrieved from(Suryadinata et al., 2010).

Beginning from the early G1 phase, Cyclin D associates with CDK4/6 to relieve the inhibition of cycle progression caused by a tumor suppressor protein, Retinoblastoma protein (pRb). When pRb is bound to E2F transcription factor, which is responsible for the transcription of S phase specific genes, it recruits chromatin remodeling complexes such as histone deacetylases (HDACs) and methyl transferases (HMTs), which gives "off" signals for transcription. Cyclin D-CDK4/6 complex phosphorylates pRb so that it can no longer stay bound to E2F (Thurlings & de Bruin, 2016). This release of E2F allows the formation and recruitment of the S phase specific Cyclin E-CDK2 complex, which is important in G1/S transition of the cell cycle. Cyclin E-CDK2 complex then hyper-phosphorylates pRb. This forms a positive feedback loop

to overcome the Restriction Point "R", the point where the cell is committed to complete the cell cycle (Bertoli, Skotheim, & de Bruin, 2013). After this point, cell continues its cycle with phase specific Cyclin-CDK complexes as shown in ***Figure 1.5*** and completes cell division.

Many tumor-related cell cycle defects are observed in cancer. For instance, due to genetic alterations or epigenetic changes in CDKs or their upstream regulators, the activity of a cell cycle changes in cancer cells to enhance proliferation (Malumbres & Barbacid, 2009). Other targets for deregulation of cell cycle in cancer include the cyclin proteins. In many cancers, Cyclin E is overexpressed in exchange for giving advantage to the cancer cells to pass through the restriction point (Hwang & Clurman, 2005). Moreover, mutations in the Ras protein, a proto-oncogene that initiates the ERK signaling pathway, can cause deregulations on cell cycle. A transcriptional target of ERK signaling, c-myc, directly promotes proliferation by upregulating Cyclin D (Chambard, Lefloch, Pouysségur, & Lenormand, 2007). Thus, it is an important key point to find out how the cell cycle is regulated in cancer.

1.6. Cancer and Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are molecules or ions that are highly reactive and derived from molecular oxygen. They are produced by activity of mitochondrial electron transport system in aerobic metabolism or by activity of oxidation/reduction enzymes. When the physiological conditions are at normal levels, reactive oxygen species are detected by antioxidant defense system and kept under control in order to maintain oxygen homeostasis (Pan, Hong, Ren, Submissions, & Com, 2009). In cancer cells, ROS-sensitive pathways that participate in cell proliferation and growth, glucose metabolism and inflammation are continuously upregulated driving these cells further to carcinogenesis. ROS targets many pathways including the MAPK/ERK1/2 pathway and NF-κB pathway. For instance, in ERK1/2 pathway, Ras can be activated by ROS activity through an oxidative modification at Cys118 residue, leading to an inhibition

in GDP/GTP exchange and thus hyperactivation of the pathway. Moreover, ROS is capable of inducing NF- κB activation and translocation into the nucleus through various ways (Morgan & Liu, 2011). Other targets of ROS include upregulation of mRNA levels of G1/S transition responsible cyclins such as Cyclin E (Liou & Storz, 2010). Another target of ROS includes the regulators of cell motility and metastasis, where ROS increases migration and cell adhesion related molecules in the cancer microenvironment (Avery et al., 2011).

1.7.NF-κB Pathway and Cancer

The connection between inflammation and cancer is well-defined and a lot effort has been made to understand this link from genetic and pharmacological perspectives. Most tumors display an inflammatory milieu with migrated immune cells and secreted cytokines and chemokines (Coussens & Werb, 2002). Colorectal cancer (CRC), in particular, is one of the cancers that is highly related with chronic inflammation (Terzić et al., 2010).

Nuclear factor κB (NF- κB) is one of the major activators of inflammation that regulates the transcription of pro-inflammatory chemokines, cytokines, growth factors and adhesion molecules, etc. (Lawrence, 2009). The family consists of five different transcription factors including RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52) and each of these subunits work as homo- or heterodimers (Baeuerle & Baltimore, 1996). In the canonical pathway, RelA (p65) - p50 heterodimer is responsible for transcriptional activity. Under normal conditions, these protein dimers exist inactive in the cytoplasm bound to IκB (inhibitor of κB) protein, which hides the nuclear translocation domains of NF-κB dimers. Phosphorylation and degradation of IκB by IκB kinases (IKKs) releases NF-κB, thus, it translocates into the nucleus and regulates the transcription of NF-κB target genes (Naugler & Karin, 2008).

1.7.1. NF-κB in Regulation of Proliferation and Cell Cycle

NF-κB activity includes but is not limited to regulation of transcription of pro-inflammatory cytokines. It can be targeted by Reactive Oxygen Species (ROS). High NF-κB activity is implicated in chronic inflammatory conditions as a result of high generation of ROS (Morgan & Liu, 2011). Moreover, it is also a key component of proliferation and cell cycle regulation. The relationship between these two important cell processes were first discovered in 1991 in mouse fibroblasts (Baldwin, Azizkhan, Jensen, Beg, & Coodly, 1991), in which NF-κB was bound to DNA in a cell cycle dependent manner at the beginning of G1 phase. After this finding, many studies showed the probable involvement of NF-κB in proliferation and cell cycle progression in various cell types and models. Out of many cell cycle related proteins, Cyclin D was shown to be one of the best characterized target of NF-κB. The promoter region of Cyclin D gene contains 3 binding sites for NF-κB and all NF-κB subunits are capable of binding these sites to regulate its expression (Barré & Perkins, 2007). Other target genes involved in the regulation of proliferation and cell cycle include Cyclin E, CDK2 and c- myc (Jourdan et al., 2007).

1.8.Aims of the Study

AKR1B1 is an aldo-keto reductase that takes part in the polyol pathway of glucose metabolism, by reducing glucose into a membrane impermeable compound sorbitol. Overexpression or over-activity of this enzyme leads to excessive sorbitol accumulation in cytosol and this creates oxidative stress, which may lead to complications in diabetes and inflammatory diseases including cancer. We hypothesized AKR1B1 expression and activity may be involved in colorectal carcinogenesis.

For this we selected HCT-116 cells that express high amounts of AKR1B1 as a model. The enzyme was inhibited in two different ways:

1. Pharmacologically, with the use of CHNQ, a novel semi-synthetic inhibitor of AKR1B1
2. Stably knocking down AKR1B1 in HCT-116 cells with shRNA.

By using this dual approach, we have identified novel effects of AKR1B1 on cellular proliferation, cell cycle, apoptosis, generation of ROS, inflammation and cellular motility.

CHAPTER 2

MATERIALS AND METHODS

2.1.Reagents and Chemicals

3,7-Dihydroxy-2-[4-(2-chloro-1,4-naphthoquinone-3-yloxy)-3-hydroxyphenyl]-5 hydroxychromen-4-one (CHNQ) was synthesized by BEL/NOVAMANN (Bratislava, Slovakia) and provided by Dr. Milan Stefek's Group from Slovak Academy of Sciences, Bratislava, Slovakia.

Colorimetric BrdU proliferation kit was obtained from Roche (Mannheim, Germany), FITC Annexin V Apoptosis Detection Kit was obtained from BD Pharmingen (USA), Nitro-blue Tetrazolium (NBT) and Propidium Iodide were purchased from Sigma-Aldrich (Taufkirchen, Germany). Antibodies against ERK1/2, p-ERK1/2, p65, p50, α-tubulin, Topo-II, β-actin and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); p-pRb was purchased from Cell Signaling (Danvers, MA); Cyclin E was purchased from Merck Millipore (Massachusetts, ABD).

2.2.Cell Culture and Stable Transfection

The colorectal cancer cell line HCT-116 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and was cultured under ATCC specified conditions in RPMI-1640 medium, that is, supplemented with 1% Penicillin-Streptomycin, 2mM L-glutamine and 10 % fetal bovine serum (FBS). Cells were grown in a humidified incubator containing with 5% CO_2 at 37 ^{o}C. Cells were stored in complete growth medium as described above supplemented with 5% (v/v) DMSO and kept inside liquid nitrogen vapor. All consumables used for cell culture were purchased from Biochrom AG, Germany.

AKR1B1 knockdown in HCT-116 cells was performed with the SureSilencing shRNA plasmid kit (Qiagen, Germany). shRNA vectors were transfected into 70-80% confluent cells in OptiMEM (Thermo Fischer Scientific, USA) by using X-tremegene HP in 1:2 (1 µg vector: 2 µl of X-tremegene HP) ratio. The cells were selected for stable expression in the presence of 500µg/ml of the antibiotic G418 (Roche, Switzerland) for 3-4 weeks and 2 monoclones having stably incorporated the shRNA plasmid for AKR1B1 and 1 polyclone of their corresponding empty vector were obtained. The stably transfected monoclones were maintained in 225 µg/ml of G418. The G418 concentrations was based on a kill curve generated in our lab (Tuncay Cagatay, Cimen, Savas, & Banerjee, 2013). Related kill curve can be seen on **APPENDIX C.** Stably transfected cells were stored in complete growth medium with 225 µg/ml G418 supplemented with 5% DMSO (v/v) and kept under liquid nitrogen vapor. All experiments with stably transfected cells were done before 8th passage.

2.3.Treatments

CHNQ was prepared as 10mM stocks in DMSO, and kept at -20^{o}C as 50 µl aliquots. Cells were treated with around 10-20 µM of CHNQ in serum-free medium for 24 hours. This concentration was based on IC_{50} values generated from our lab (Enayat, Şeyma Ceyhan, Taşkoparan, Stefek, & Banerjee, 2016). For all treatments, DMSO was used as vehicle control.

2.4.Total Protein Isolation

For the isolation of total proteins, after cells were collected, they were washed twice with cell culture grade PBS at 300 x *g* for 5 min. Then, Mammalian Protein Extraction Reagent (M-PER) (Thermo Fisher Scientific, USA) was added on the cell pellets, with the addition of phosphatase and protease inhibitors (Roche, Germany).

2.5.Cytoplasmic-Nuclear Protein Isolation

In order to isolate of nuclear and cytoplasmic proteins, ~10^7 cells in a cell culture plate were washed once with 1-2 mL of cell culture grade PBS, collected by scraping and transferred to eppendorf tubes. Following centrifugation of cells at 300 x *g* for 5 min, the supernatant was removed and 1mL of cell culture grade PBS containing 1X phosphatase inhibitor was added. Samples were centrifuged once again at 300 x *g* for 5 min and the supernatants were removed. The pellets were resuspended in 300 µl of hypotonic buffer (100mM HEPES, 40mM NaF, 100µM Na_2MoO_4 and 1 mM EDTA, pH:7.5), transferred to pre-chilled eppendorf tubes and incubated on ice for 15 min. Then, 75 µl from NP-40 (10 %) was added and mixed. Then, the samples were spun down at high speed for 30 s in order to obtain whole cytoplasmic fraction in the supernatant. Supernatants were transferred into new pre-chilled eppendorf tubes while the pellets were resuspended in 80 µl of nuclear extraction buffer (20 mM HEPES, 0.2 mM EDTA, 3 mM $MgCl_2$, 840 mM NaCl, 20 % glycerol, pH:7.9) and vortexed for 15 s. After this step, samples were placed on ice and shaken at high speed for 15 min, followed by 30 s vortexing and 15 min of shaking again. Finally, samples were centrifuged at 14000 x *g* for 10 min at 4^{o}C and supernatants were transferred into new eppendorf tubes as the nuclear fraction.

2.6.Protein Quantification

Isolated proteins were measured by Bradford Assay with the Coomassie Protein Assay Reagent (Thermo Fisher Scientific, USA) and amounts were determined relative to a standard curve generated with bovine serum albumin.

2.7.Western Blotting

30-50 μg of proteins from each sample were loaded and run on 10% SDS-polyacrylamide gel together with a PageRuler Prestained protein ladder (10-250 kDa) and electrophoresis was carried out at 100 V for 1-2 h. After electrophoresis, wet transfer was performed from the gel to a Polyvinylidene fluoride (PVDF) membrane at 115 V for 75 min at 4^{o}C. Blocking of the membranes was done for 1 h at room temperature either with 5% skimmed milk in TBS-T for non-phosphorylated proteins or 5% bovine serum albumin (BSA) in TBS-T for phosphorylated proteins. Then, the membranes were probed with determined primary antibodies overnight at 4^{o}C, washed extensively, followed by probing with its secondary antibody for 1 h at room temperature. Visualization of the bands was performed by using Clarity ECL Substrate (BioRad, USA) and imaged on a Chemi-Doc MP (BioRad, USA). The membranes were also blotted with β-actin or GAPDH antibody as loading control to ensure equal protein loading. Antibody dilutions and other details were given in ***Table 2.1***.

Table 2.1. List of antibodies used in this study.

Description	Origin	Media	Dilution
B-actin	mouse	TBS-T	1/4000
GAPDH	rabbit	TBS-T	1/1000
α-tubulin	mouse	TBS-T	1/500
Topoisomerase II β	rabbit	TBS-T	1:500
p65	mouse	TBS-T	1/500
p50	rabbit	TBS-T	1/500
ERK1/2	rabbit	TBS-T	1/500
phospho-ERK1/2	rabbit	TBS-T	1/500
Cyclin E	mouse	TBS-T	1/1000
p-pRb	rabbit	TBS-T	1/1000
anti-rabbit	goat	TBS-T	1/2000
anti-mouse	rabbit	TBS-T	1/2000

Before re-probing the membranes with different antibodies, a mild stripping was carried out with mild stripping buffer twice for 10 min at 60ºC, followed by extensive washing in TBS-T, blocking and probing steps.

2.8.BrdU Incorporation Assay

Effects of CHNQ and AKR1B1 knockdown on cell proliferation was determined with a Cell Proliferation Elisa, BrdU (Colorimetric) kit (Roche, Switzerland) according to the manufacturer's instructions. Basically in this kit, BrdU, an analogue of thymidine, gets incorporated into the DNA during S (synthesis-DNA replication) phase of cell cycle. In this way, newly synthesized DNA and newly divided cells are detected colorimetrically at 370 nm in a micro-plate reader (Thermo Fisher Scientific, USA).

To determine the effects of CHNQ, ~10^4 HCT-116 cells were plated per well in a 96-well plate and allowed to attach for 24 h. Then, the cells were treated for 24h with different concentrations of CHNQ ranging between 0-50 µM. To determine the effects of AKR1B1 knockdown, 10^4 cells were seeded per well in a 96-well plate and allowed to attach for 24 h. Then, the cells were incubated with serum-free medium overnight, during which the cells were arrested at the G1 phase of their cell cycle (Iyer et al., 2007). The cells were released from this arrest with the addition of complete medium at the 0, 24 and finally 48 h.

At the end of 24 h of treatment with CHNQ or the different times after release of the cells from G1/S arrest, the medium was removed and a 90 µl fresh complete medium together with 10 µl BrdU labeling solution was added to cells and incubated for at least 2 h at 37ºC, which enabled BrdU to incorporate into DNA. At the end of BrdU incorporation, the labeling medium was disposed. The cells were fixed with 200 µl/well Fix/Denat solution for 30 min at room temperature. Then, the fixing solution was removed and 100 µl/well of anti-BrdU-POD was added and incubated for 90 min in the dark at room temperature in order to obtain BrdU immunocomplexes. The anti-

BrdU-POD solution was removed and the cells were washed 3 times by adding 200 µl/well of 1X washing buffer. Finally, 100 µl/well of substrate solution (tetrametyhlbenzidine) was added and measurements were taken in a microplate reader at 370 nm within 5-15 minutes.

2.9.Cell Cycle Assays

To determine the effects of CHNQ on cell cycle, cells were treated either with vehicle or two concentrations of CHNQ (10 and 20 µM) for 24 h in serum free medium. The cells were collected and fixed with the drop-wise addition of 70% ice-cold ethanol and kept at -20ºC for at least 2 h. Fixed cells were then centrifuged at 300 x *g* for 5 min, re-suspended in 500 µl of PBS (10^6 cells /0.5 mL), centrifuged once again at 300 x *g* for 5 min, and the supernatant was discarded. Pellets were resuspended in 200µl of staining solution which was prepared in cell culture grade PBS containing 0.1% Triton X-100, 2mg/mL RNase A (DNase free) and 20 µg/mL Propidium Iodide (Sigma-Aldrich, USA) and incubated in the dark at room temperature for 30 min. Depending on the DNA content, the percentage of cells at different stages of cell cycle was determined by using the Accuri C6 Flow Cytometer (BD Biosciences, USA) at the FL-3 channel and compared with vehicle controls.

For determination of the effects of AKR1B1 knockdown on cell cycle progression, two monoclones (Clone 1 and Clone 2) with stable knockdown of AKR1B1 and the scrambled control cells were synchronized as described above. Then, at different time points (0, 6, 12, 24, 30 hours) the cells were fixed, stained and the cell cycle distribution was analyzed as described above.

2.10. Luciferase Assays

In order to assess the effects of CHNQ and AKR1B1 knockdown on HCT-116 cells on the activity of NF-κB pathway, NF-κB luciferase assays were carried out. NF-κB transcriptional activity was analyzed using pNF-κB Luc Pathdetect reporter plasmid (Agilent Genomics, USA), which contains 5 copies of the binding sites for NF-κB, and pRL-TK Renilla plasmid (Promega, USA) was used as an internal control. $5x10^4$ HCT-116 scrambled (NC) and AKR1B1 knockdown (Clone 1 and 2) cells/well were seeded in a 48 well-plate. After the cells were attached, they were first treated with 20 μM CHNQ for 24 h and after removal of treatment medium, the cells were transfected at 1:250 ratio of Firefly: Renilla vectors for 24 h. For the analysis, Dual-Glo Luciferase Assay Kit (Promega, USA) was used by following manufacturer's guidelines. Briefly, cells were washed once with use of cell culture grade PBS and lysed in 50 μl/well Passive Lysis Buffer for 20 min on a shaker at room temperature. Then from each sample, 20 μl lysate was transferred into a white, opaque 96-well plate and luminescence from Firefly and Renilla luciferase was measured after addition of 25 μl of Luciferase Assay Reagent (LARII) and Stop & Glo reagent respectively in a luminometer (Turner Biosystems, USA).

2.11. Annexin V Apoptosis Assay

Effect of CHNQ and AKR1B1 knockdown on apoptosis and cell death was evaluated by FITC Annexin V Apoptosis Detection Kit (BD Biosciences, USA) according to manufacturer's instructions.

Briefly, ~$5x10^5$ HCT-116 scrambled (NC) and AKR1B1 knockdown (Clone 1 and Clone 2) cells were treated either with vehicle or 20 μM CHNQ for 24 hours. Cells were treated with 50 μM etoposide (Selleckchem, USA) for 24 h as a positive control for apoptosis. At the end of the treatment, the cells were trypsinized, collected, washed twice with ice-cold cell culture grade PBS and resuspended in 1X Binding Buffer (0.1

M HEPES/NaOH (pH 7.4), 1.4 M NaCl, 25 mM CaCl2) at a concentration of 10^6 cells/mL. 100µl of the resuspended cell suspension (~10^5 cells) was then transferred to a new eppendorf tube. Then, 10 µl of Propidium Iodide solution from a 50 µg/mL stock and 5 µl of Annexin V was added to the cell suspension. After a gentle vortex, the cells were incubated in the dark at room temperature for 15 min. Finally, 400 µl of the 1X binding buffer was added to the cells and analysis was carried out with an Accuri C6 Flow Cytometer (BD Biosciences, USA) at the FL-3 channel for propidium iodide and FL-1 channel for Annexin V-FITC dye within 1 hour. Proper controls were also included as unstained, only propidium iodide stained and only Annexin V-FITC stained samples. The results were expressed as percentages of late apoptotic/dead cells, early apoptotic cells and live cells.

2.12. NBT (Nitro-blue tetrazolium) Assay

Nitro-blue tetrazolium (NBT) is a reagent that can absorb superoxide anions in the cells. By this way, this assay determines the superoxide levels inside the cells. To determine relationship between ROS and AKR1B1, 1 x 10^4 HCT-116 scrambled (NC) and AKR1B1 knockdown (Clone 1 and Clone 2) cells were seeded on 96-well plates. 24 h after seeding, cells were incubated with NBT solution (2 mg/mL for each well) for 5 h at 37^{o}C. Then, cells were fixed by use of 100% methanol for 5 min and air dried at room temperature. The fixed cells were then mixed thoroughly by pipetting with 120 µl KOH and 140 µl DMSO to dissolve the blue formazan crystals of NBT by superoxide radicals. The absorbances was read colorimetrically at 620 nm in a micro-plate reader.

2.13. In vitro Scratch Wound Healing Assay

To determine the effect of CHNQ and AKR1B1 knockdown on cell motility, ~5x10^5 cells were seeded on 12-well plates. When cells were attached and reached 80-90% confluency, the cells were treated with 10 µM CHNQ for 24 hours. This time, a lower

concentration was chosen in order to avoid excess cell death caused by CHNQ. At the end of the treatment, a scratch wound was opened using a sterile 100 µl-pipette tip. Cell debris was removed by washing the cells twice with cell culture grade PBS. Then, the cells were incubated in complete medium and at 0, 24 and 48 h time points, the cells were visualized with the Juli Smart Fluorescent Cell Imager at 10X magnification. The width of the wounds at each time point was measured with a real-sized ruler.

2.14. Transwell Migration Assay

In order to observe the effects of CHNQ and AKR1B1 knockdown on cellular migration properties, a Transwell migration assay was carried out. Each Transwell insert contains an upper and a lower chamber system, which are separated by a 0.8 µm polycarbonate porous structure, which enables cells to move from upper compartment to the lower compartment.

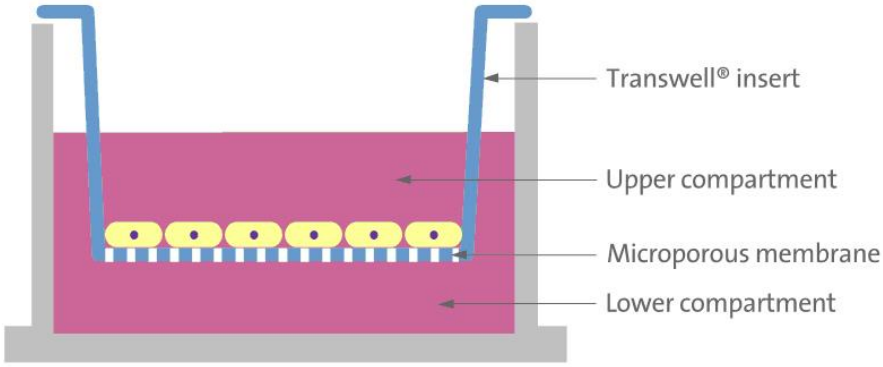

Figure 2.1. Schematic representation of Transwells.

First of all, HCT-116 scrambled (NC) and AKR1B1 knockdown (Clone 1 and Clone 2) cells were treated with 10 µM CHNQ for 24 h, harvested by trypsinization, washed twice and resuspended in 1% FBS containing medium. Then, the cells were counted with a hemocytometer and ~$5x10^4$ in a 100 µl of cell suspension were seeded on

Transwell chambers having membranes with 8 μm pores (ThinCert™ Cell Culture Inserts, Greiner Bio-One, Germany). At the same time lower chamber was also filled by complete medium (10% FBS). The cells were allowed for migration for 48 h. The Transwells were removed out and non-migrated cells were cleared with sterile cotton swabs; this step was repeated at least twice. Afterwards, the Transwells were fixed in 100% methanol for 10 min, followed by staining with Giemsa solution (Merck Millipore, USA) for 2-3 min at room temperature, and washed with sterile distilled water extensively in order to remove all excess dye. Finally, the Transwells were left to air dry inside a fume hood. When the membrane filters were completely dried, the membranes were removed with a bistoury and placed on a sterile, glass slide with a drop of immersion oil. The total number of cells was counted at 20X magnification under an inverted light microscope (Leica, Germany).

2.15. Yeast Culture

S. cerevisiae cells (S288c parental strain- RDKY3615 Wild type) were taken from Dr Kyungjae Myung (National Human Genome Research Institute, MD, USA) (Myung, Smith, & Kolodner, n.d.) grown in 2% glucose containing YPD media at 30^{o}C. Before starting with experiments, Yeast cells were inoculated in YPD medium and incubated overnight with shaking at 200 rpm at 30 ^{o} C. The next day, the cell suspension was transferred into 45 mL of YPD in sterile glass flask and incubated at 30^{o}C with shaking until the OD_{600} reached 0.6-0.8.

Yeast cells were chosen because they are easy to work with and good eukaryotic models for *in vivo* studies since each yeast cell is considered as one individual.

2.16. Yeast Spotting Assay

Before beginning, cells were propagated as described in **Section 2.15.** Then, the cells were divided into 5 tubes in aliquots of 5 mL and treated overnight with either vehicle (DMSO) or 3 increasing concentrations of CHNQ (100, 500 and 750 μM). These

concentrations were decided on the basis of preliminary toxicity experiments carried out with CHNQ concentrations between 10 and 1000 μM. At the end of treatment, each sample was serially diluted as 10^0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}, spotted onto YPD agar plates and incubated 24-36 hours at 30 ^{o}C to observe the ability of colony formation.

2.17. Dihydroethidium (DHE) Staining for Detection of Reactive Oxygen Species (ROS)

It was previously shown that CHNQ has antioxidant activities *in vivo* (Milackova, Kovacikova, Veverka, Gallovic, & Stefek, 2013). To see whether this is also valid for yeast, a Dihydroethidium (DHE) staining was carried out. DHE is an indicator of superoxide. It exhibits blue fluorescence in the cytoplasm until it is oxidized. When it is oxidized, it is converted to ethidium, which then intercalates with DNA and selectively gives red fluorescence.

For this purpose, first of all, yeast cells were propagated as described in **Section 2.15.** Then, the culture was divided into 5 tubes in equal volumes (5mL culture/tube) and treated with either vehicle control, 500 μM CHNQ, 20 mM N-acetyl cysteine (NAC), 500 μM CHNQ together with 20 mM NAC and 5 μg/mL Rapamycin for 3 h for 24 h. Rapamycin was used as a positive control, NAC was used as an antioxidant that can reduce ROS formation. At the end of the treatments, OD_{600} was measured for each sample and equalized to 0.6 with the addition of YPD. The cells were centrifuged at 200 x *g* for 5 min. The supernatant was discarded and 1 mL of 5 μg/mL DHE was added to the cell pellet. The samples were incubated 30 min at 30^{o}C. Cells were analyzed by using Accuri C6 Flow Cytometer (BD Biosciences, USA) at FL-3 channel due to red fluorescent exhibited by oxidized DHE.

2.18. Statistical Analysis

In all experiments, 2 or 3 independent biological replicates were carried out, each containing at least 3 technical replicates. GraphPad Prism 6.1 (GraphPad Software Inc., USA) software was used for data analysis and graphs. One-way or Two-way ANOVA was employed to determine significance. $p < 0.05$ was taken to be statistically significant.

CHAPTER 3

RESULTS

3.1.shRNA mediated silencing of AKR1B1 in HCT-116 cells

AKR1B1 is known to be robustly expressed in HCT-116 cells (Ebert et al., 2011). Therefore, it was stably silenced in HCT-116 cells by shRNA and two silenced monoclones were selected along with a scrambled shRNA control. In these two monoclones (Clone 1 and Clone 2), the loss of expression of AKR1B1 with respect to the scramble control (NC) was confirmed by western blotting) (***Figure 3.1***).

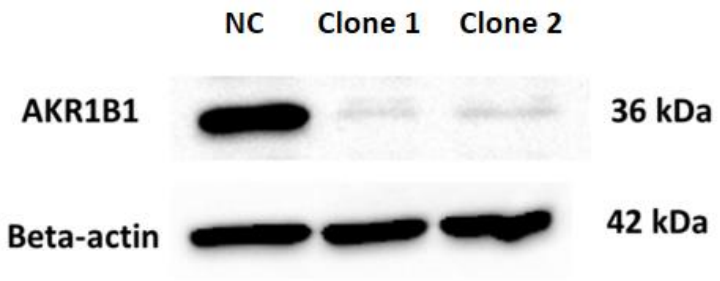

Figure 3.1. Protein levels of AKR1B1 in stably transfected HCT-116 scrambled cells and silenced monoclones.

NC represents cells stably transfected with the scrambled shRNA, clone 1 and clone 2 represent two selected monoclones. From each sample, 50 µg protein was loaded to a 10% SDS-PAGE. Beta-actin was used as protein loading control.

3.2. Effects of CHNQ on ROS production in yeast cells

Naphtoquinones like CHNQ are known to induce ROS formation and control redox signaling (Verma, 2006). To validate whether CHNQ treatment resulted in ROS formation *in vivo*, yeast cells were incubated with 500 µM of CHNQ for 24 h and then stained with Dihydroethidium (DHE). Robust ROS formation was seen upon treatment with CHNQ. The induction by CHNQ was even seems to be stronger than rapamycin (the positive control). Treating the cells together with CHNQ and an antioxidant NAC resulted in reversal in ROS production, while no induction of ROS was observed in treatment with only NAC (***Figure 3.2***).

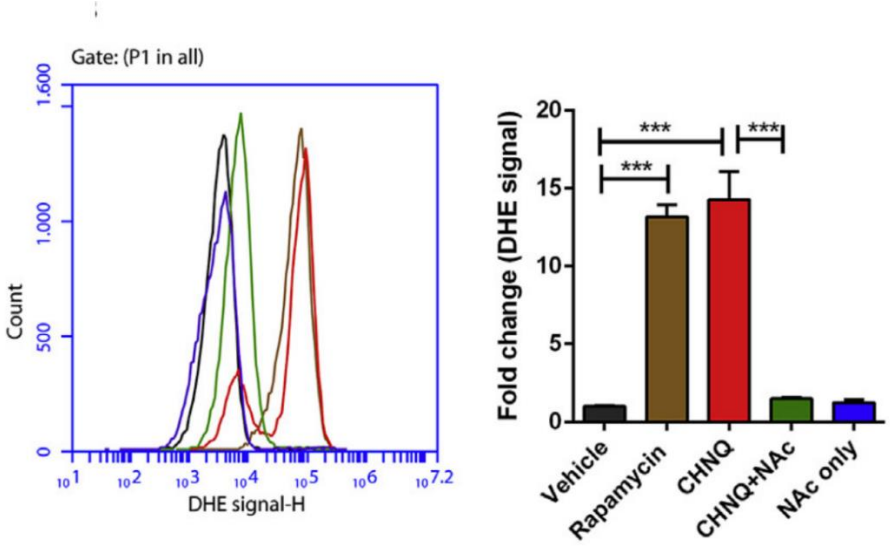

Figure 3.2. Production of oxidative stress in S.cerevisiae treated with CHNQ.**.**

A high induction of oxidative stress was observed in cells treated with CHNQ (red line) when compared with rapamycin (the positive control; brown line). A successful reversal in ROS production was seen in cells treated with CHNQ and NAC together (green line). ROS formation was not observed with NAC alone (blue line). Statistical significance was determined by one-way ANOVA with post hoc Tukey's multiple comparison test (*** $p < 0.001$).

3.3.Investigating the effects of CHNQ on AKR1B1 silenced HCT-116 cells

CHNQ is a napthoquinone derivative that has previously been known to inhibit the activity of purified rat lens AKRs (Milackova et al., 2015). Therefore, to determine whether treatment with CHNQ resulted in alterations in cellular characteristics and whether those changes were dependent on the expression of AKR1B1, AKR1B1 silenced HCT-116 cells were investigated from several different perspectives including proliferation, cell cycle, apoptosis and cell migration.

3.3.1. Effects of CHNQ on cell viability and proliferation of AKR1B1 silenced cells

The BrdU incorporation assay performed to understand the effects of CHNQ on cellular proliferation. AKR1B1 silenced and control cells were treated with 0-50 μM CHNQ for 24 h and then BrdU incorporation assay was conducted as described in Section **2.8** and the IC_{50} value (the concentration that is able to kill 50% of the cells) was determined. We have previously shown that treatment of HCT-116 wild-type cells with CHNQ resulted in an IC_{50} value of 10.3±2.6 (Enayat et al., 2016). The IC_{50} values of CHNQ in AKR1B1 silenced HCT-116 monoclones clone 1 and clone 2 were 11.05±2.35 μM and 12.4±2.6 μM, respectively and 9.5±1.95 μM in the scrambled control (NC) (***Table 3.1***).

Table 3.1. IC50 values of CHNQ on AKR1B1 silenced and control cells.

	IC50
Wild type HCT-116	10.3±2.6
Scrambled HCT-116 (NC)	9.5±1.95
AKR1B1 silenced clone 1	11.05±2.35
AKR1B1 silenced clone 2	12.4±2.6

HCT-116 wild-type, scrambled and AKR1B1 silenced cells were treated with different concentrations of CHNQ ranging from 0-50 μM for 24 h in serum free medium. Then by the use of BrdU incorporation assay, growth inhibition was determined. The results were represented as the mean ± SD of 3 independent biological replicates, each of them containing 3 technical replicates.

3.3.2. Changes in MAPK pathway upon treatment with CHNQ on AKR1B1 silenced cells

CHNQ treatment resulted in the inhibition of proliferation in both wild type, scrambled and AKR1B1 silenced HCT-116 cells (***Table 3.1***). In order to find out the molecular basis of CHNQ's antiproliferative effects, we first assayed a well-known mitogenic signaling pathway, the Mitogen Activated Protein Kinase (MAPK) pathway. MAPK pathway plays an important role in cell proliferation, cell growth and cell survival (Seger & Krebs, 1995). Treatment with pre-optimized concentrations of CHNQ (a concentration close to IC_{50}: 10 μM and a concentration that is above IC_{50}: 20 μM) for 24 h led to enhanced ERK1/2 phosphorylation, the active form of the protein, when compared with untreated control samples in both scrambled NC cells and AKR1B1 silenced monoclones clone 1 and clone 2.

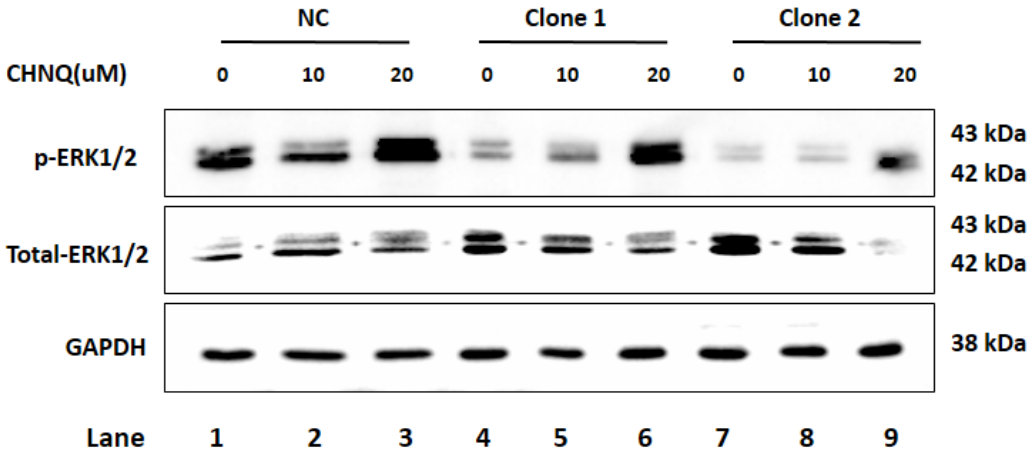

Figure 3.3. CHNQ leads to upregulation of ERK/MAPK pathway in both HCT-116 scrambled (NC) and AKR1B1 silenced monoclones Clone 1 and Clone 2.

50 µg protein from each sample was run on 10% SDS-PAGE. GAPDH was used as protein loading control. p-ERK1/2 and total-ERK1/2 antibodies were diluted with 1/500 ratios in 5% BSA or 5% skimmed milk in TBS-T, respectively; GAPDH antibodies was diluted with 1/1000 dilution in 5% skimmed milk in TBS-T.

The Western blot results gave two important leads. First, CHNQ treatment for 24 h enhanced the ERK1/2 phosphorylation in NC, Clone 1 and 2 cells; *i.e.* irrespective of whether AKR1B1 was silenced or not. Second, a reduction in phosphorylated ERK1/2 was observed in untreated AKR1B1 silenced Clone 1 and 2 cells when compared to untreated NC cells (compare lane 1 with lanes 4 and 7). These data indicate that AKR1B1 silencing was likely to have profound effects on cellular characteristics.

3.3.3. Effects of CHNQ on cell cycle progression

In order to determine if the growth inhibitory effect of CHNQ was as a consequence of an arrest in cell cycle progression, scrambled NC and AKR1B1 silenced Clone 1 and Clone 2 cells were treated with CHNQ at 10 μM and 20 μM concentrations for 24 h. Sorbinil (20 μM) is a known AKR1B1 inhibitor (Ramana, Tammali, & Srivastava, 2010) and was used here as a control. DNA content of the cells was measured after PI staining and flow cytometry analysis.

Treatment with CHNQ led to a significant arrest at the G2/M phase of cell cycle in all cells, however, the number of cells in G1 phase was reduced significantly as well (***Figure 3.4***). These effects were attributed to AKR independent effects of CHNQ; the cell cycle distributions were similarly altered in NC, Clone 1 and Clone 2 upon CHNQ treatment.

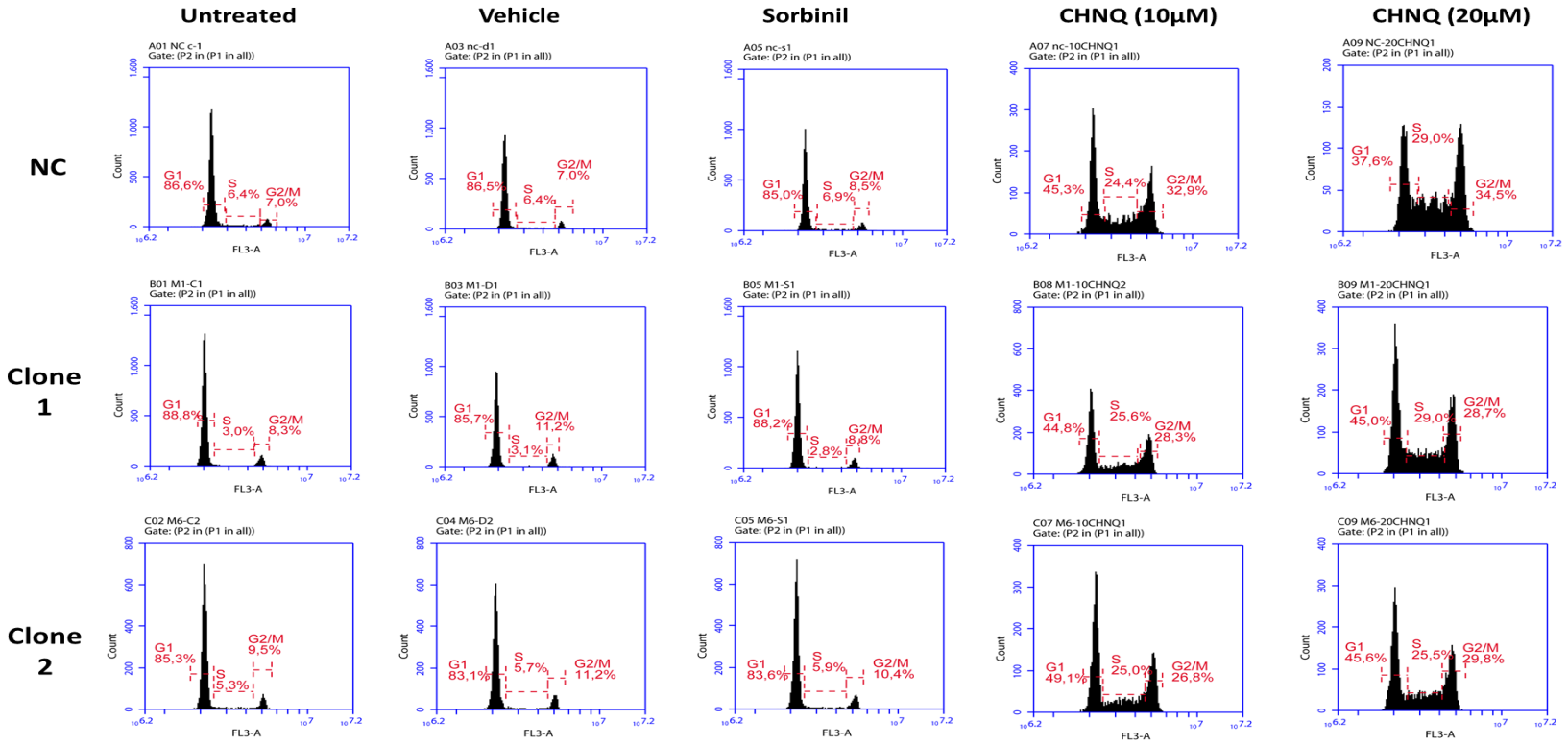

Figure 3.4. G2/M arrest in cell cycle in HCT-116 scrambled (NC) and AKR1B1 silenced Clone 1 and Clone 2 cells treated with CHNQ.

Cell cycle and its distribution analyzed by PI staining and flow cytometry of HCT-116 scrambled (NC) and AKR1B1 silenced clone 1 and 2 cells treated with CHNQ..Data was analyzed by BD Accuri C6 flow cytometer software. Each graph is a representative figure for each treatment and cell type.

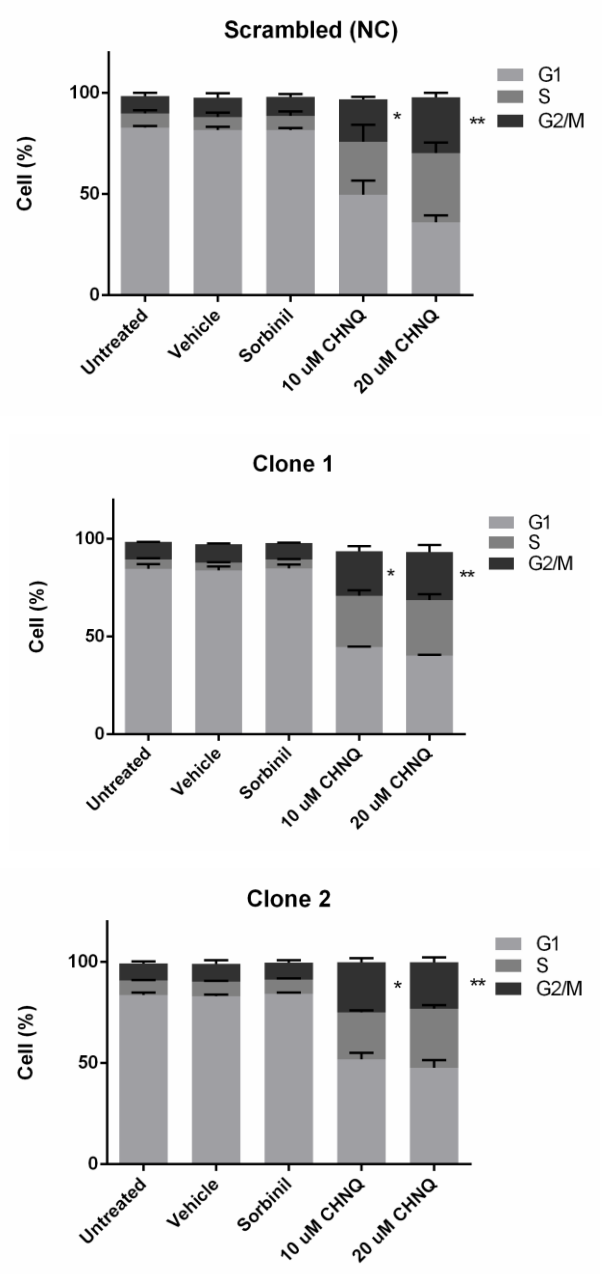

Figure 3.5. Cell cycle distribution of scrambled (NC) and AKR1B1 silenced cells HCT-116 upon treatment with CHNQ.

Cells treated with 10 and 20 μM CHNQ in serum free medium for 24 h were stained with PI and analyzed by BD Accuri C6 flow cytometry. Sorbinil (20μM) was used as a known AKR1B1 inhibitor. Significances were obtained at * $p<0.05$, ** $p<0.01$ by one-way ANOVA with post hoc Tukey's multiple comparison test.

3.3.4. Effects of CHNQ on apoptosis

We have previously shown that incubation of HCT-116 cells with CHNQ led to the activation of apoptosis after 24 h of treatment (Enayat et al., 2016). To observe if CHNQ also leads to an initiation of apoptosis on AKR1B1 silenced HCT-116 cells, Annexin V staining was carried out. ***Figure 3.6 and Figure 3.7*** show that CHNQ significantly induced apoptosis in these cells compared to the corresponding untreated cells. Treatment with 50 µM etoposide a known inducer of apoptosis (Kang, Lee, Yoo, & Nho, 2010) for 24 h was used as positive control.

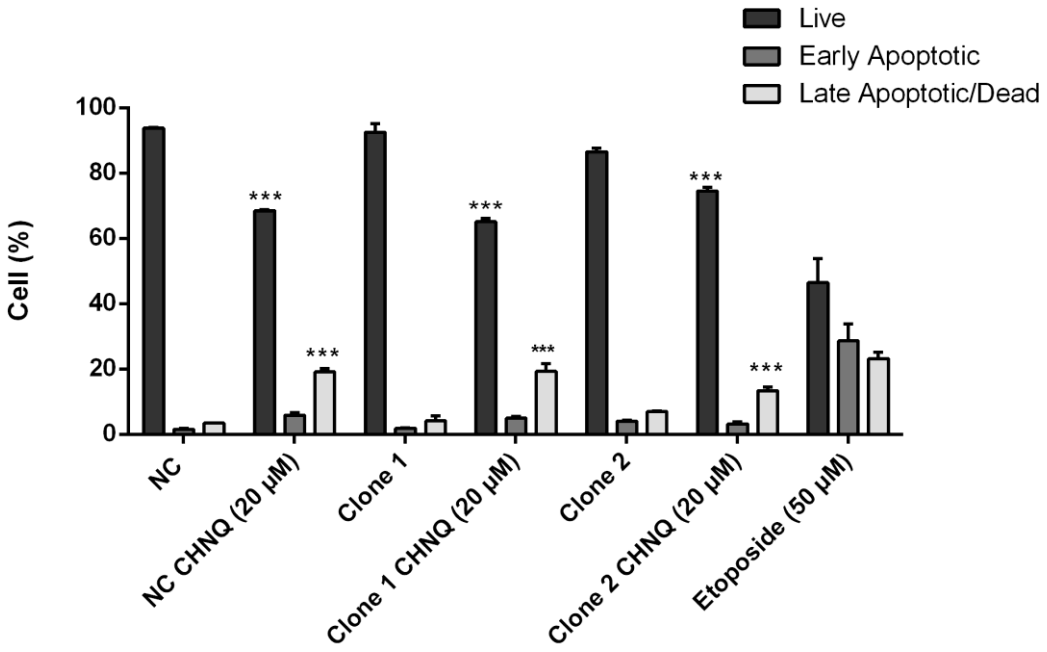

Figure 3.6. Annexin V staining for detection of apoptosis in scrambled (NC) and AKR1B1 silenced Clone 1 and Clone 2 cells upon treatment with CHNQ for 24h.

Etoposide (50 µM) was used as positive control. Significant differences were determined by two-way ANOVA with post hoc Tukey's multiple comparison test (***$p<0.001$).

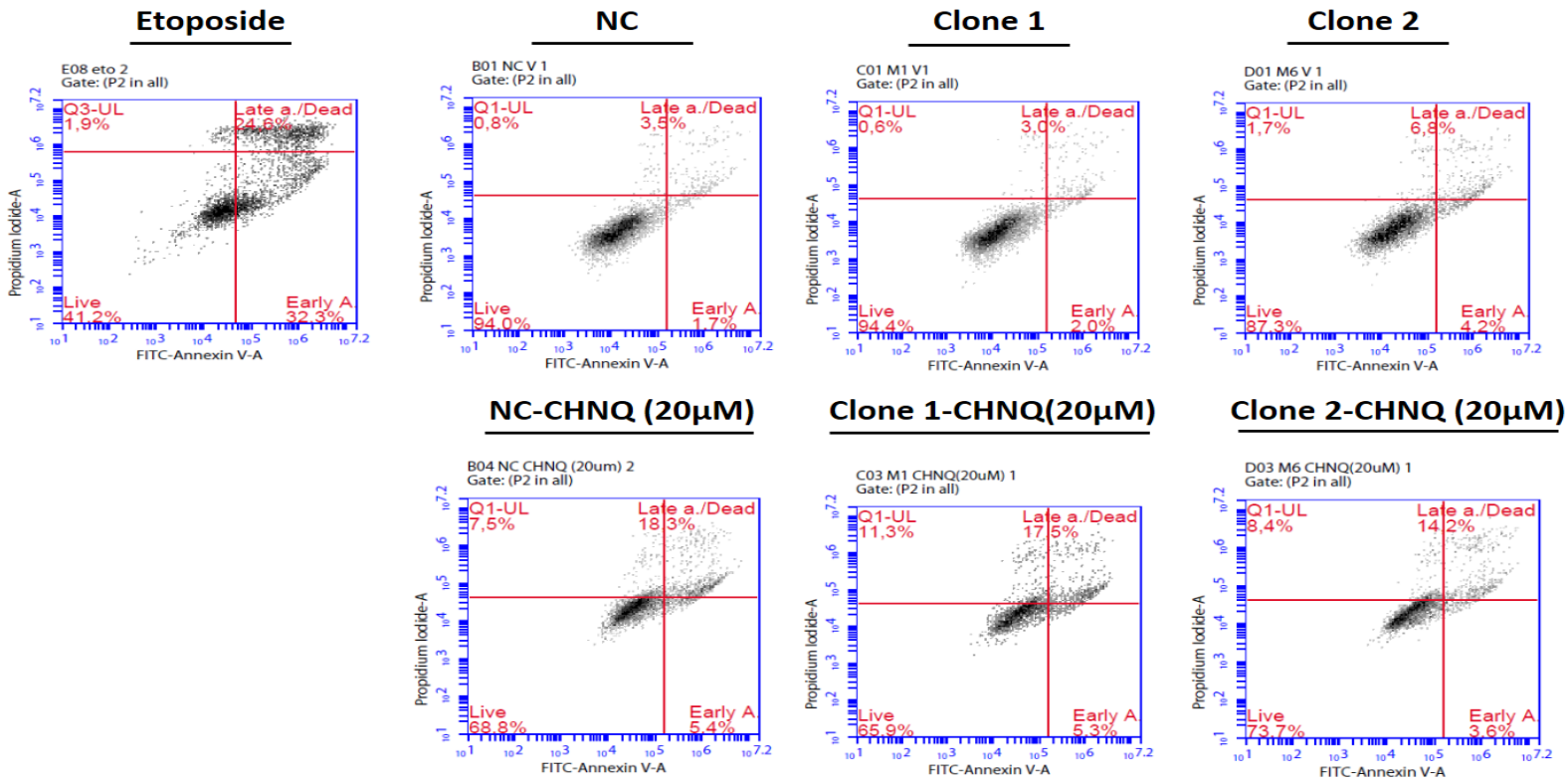

Figure 3.7. CHNQ leads to apoptosis in scrambled (NC) and AKR1B1 silenced Clone 1 and 2 HCT-116 cells.

Cells treated with CHNQ were stained with FITC-Annexin V and PI, and analyzed by flow cytometry. Etoposide (50 µM) was used as a positive control. Representative graphs show the distribution of cells. Lower left quadrants show live and viable cells; lower right quadrants show early apoptotic cells and upper right quadrants show late apoptotic and dead cells.

3.3.5. Effects of CHNQ on cell migration

Metastatic spread, represented by cellular motility, is an essential hallmark of cancer (Hanahan et al., 2011). In order to evaluate the effect of CHNQ on the motility of AKR1B1 silenced HCT-116 cells, an *in vitro* Transwell migration assay was performed. The migration assay was performed for 48 h, following a 24 h of CHNQ treatment. A significant decrease was seen in migration of CHNQ treated HCT-116 scrambled (NC) and AKR1B1 silenced monoclone (Clone 2) compared to their vehicle (DMSO) treated controls. Moreover, a significant decrease was also observed in migration of vehicle treated AKR1B1 silenced HCT-116 cells (Clone 2) when compared to vehicle treated scrambled HCT-116 (NC) cells (***Figure 3.8***).

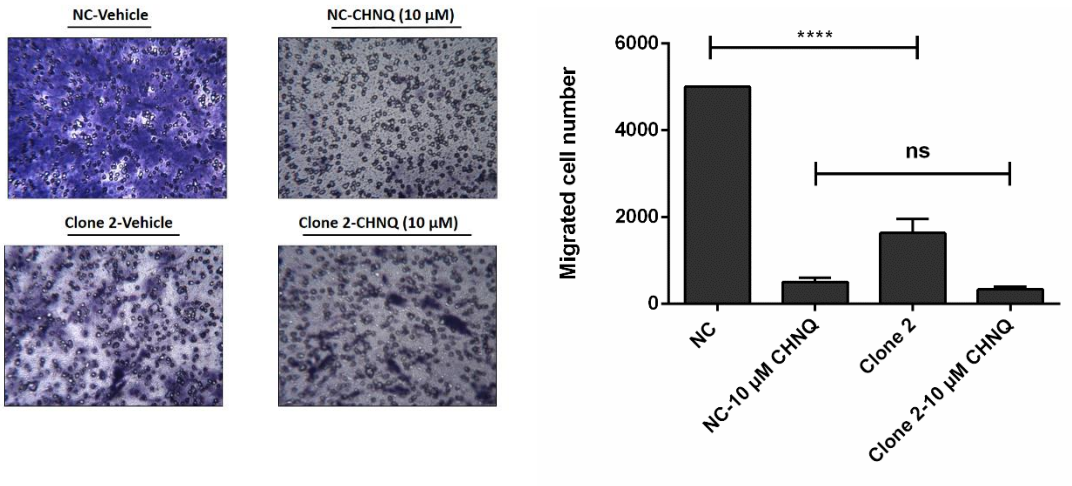

Figure 3.8. CHNQ inhibits cell migration in HCT-116 scrambled (NC) and AKR1B1 silenced Clone 2 cells.

Left panel shows representative images from Transwell migration assay (20X magnification) with less migration in CHNQ treated HCT-116 scrambled (NC) and AKR1B1 silenced Clone 2 compared to their corresponding vehicle treated controls. **Right** panel shows quantitative analysis of cell migration through the Transwell. Statistical significance was determined by one-way ANOVA with post-hoc Tukey's multiple comparison test (****$p<0.0001$).

3.4.Investigating the effects of AKR1B1 silencing on HCT-116 cells

The data presented above showed that CHNQ showed profound effects on various cellular characteristics, however, the effects were similar irrespective of whether AKR1B1 was silenced or not. On the other hand, when the vehicle treated AKR1B1 silenced cells were compared with vehicle treated scrambled control cells, a number of changes in cellular characteristics were observed. These data indicate that loss of AKR1B1 expression itself had tumor suppressive properties (Neli Hevir, Jasna Šinkovec & A, 2013) which we examined further.

3.4.1. Effects of AKR1B1 silencing on cell proliferation

According to the data given in ***Figure 3.3***, HCT-116 AKR1B1 stably silenced monoclones Clone 1 and Clone 2 showed reduced phosphorylation of ERK1/2, which may indicate a consequent decrease in cell proliferation. A BrdU incorporation assay was carried out using HCT-116 scrambled (NC) and AKR1B1 silenced monoclones (Clone 1 and Clone 2), as described in ***Section 2.8***. 24 h after plating, HCT-116 scrambled (NC) and AKR1B1 silenced monoclones (Clone 1 and Clone 2) were synchronized at G1/S phase of the cell cycle by overnight serum starvation. Then, serum-containing medium was given to the cells thereby releasing the cells from the arrest and the BrdU assay was carried out 0h, 24h and 48h later. A significant reduction in proliferation in AKR1B1 silenced HCT-116 cells (Clone 1 and 2) was observed when compared with HCT-116 scrambled (NC) cells (***Figure 3.9***).

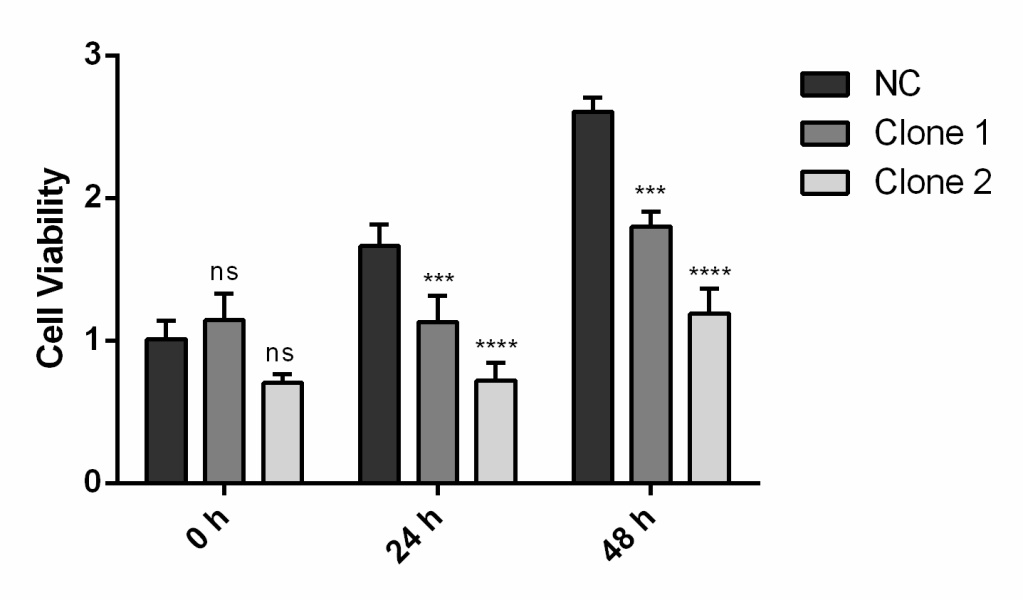

Figure 3.9. BrdU incorporation assay (colorimetric) showing decrease in cellular proliferation in AKR1B1 silenced HCT-116 cells Clone 1 and Clone 2 compared to HCT-116 scrambled (NC) cells.

By overnight serum starvation, HCT-116 cells were synchronized and then released by incubating with serum containing medium for 24 and 48h. All data are represented as mean ± SD of 3 independent biological replicates. Statistical significance was assesed by one-way ANOVA with post-hoc Tukey's multiple comparison test (***$p<0.001$).

3.4.2. Effects of AKR1B1 silencing on cell motility

An *in vitro* scratch wound healing assay was performed in order to determine the role of AKR1B1 in the motility of HCT-116 cells. The results showed that while HCT-116 scrambled cells (NC) were able to close to wounded area within 48 h, AKR1B1 silenced HCT-116 cells (Clone 1 and Clone 2) were not capable of closing the wounded area within that period (***Figure 3.10***).

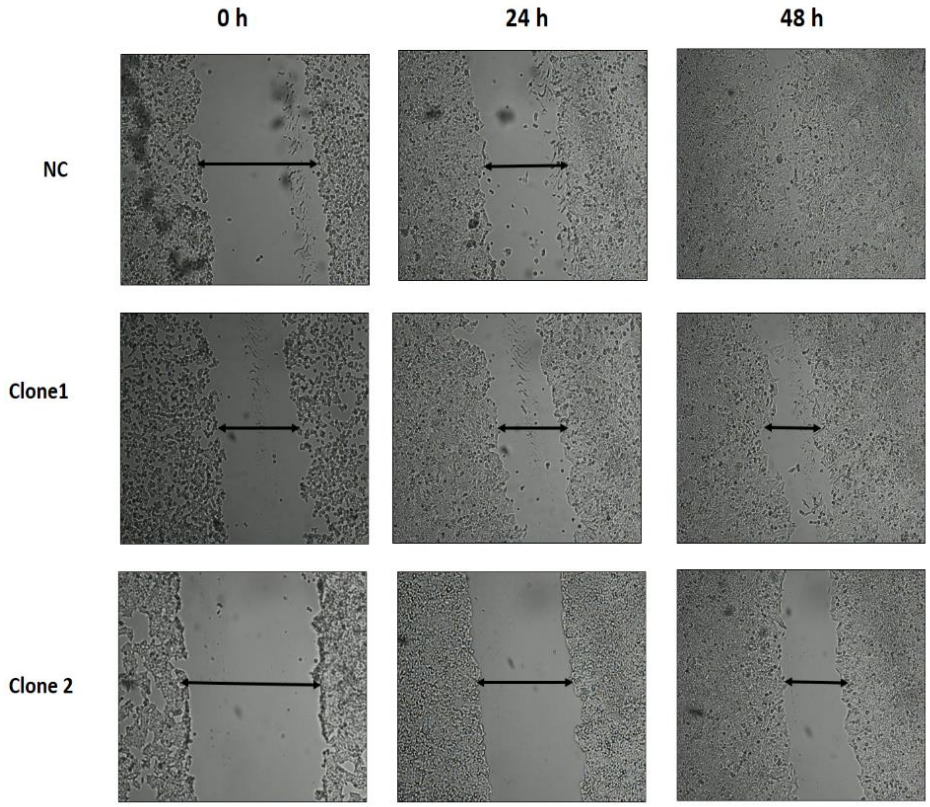

Figure 3.10. Wound closure representative figures of HCT-116 scrambled (NC) and AKR1B1 silenced cells (Clone 1 and 2) within 48 hours.

HCT-116 scrambled (NC) and AKR1B1 silenced Clone 1 and Clone 2 cells were grown to 100% confluency, and wounded with a 100 µl pipette tip. The areas of wound closures were visualized under microscope over 48h. At each time point cells were washed once and then photographed.

The quantitative evaluation of wound closure in HCT-116 scrambled (NC) and AKR1B1 silenced (Clone 1 and 2) cells are given in ***Figure 3.11***. Although AKR1B1 silenced (Clone 1 and 2) cells still had the ability to close the wounded area, these cells were less motile than the scrambled (NC) cells. Since the wound healing assay was carried out in serum containing medium, the assay can also be a measure of cellular proliferation. The delayed would closure in the AKR1B1 silenced cells therefore corroborate with reduced cell proliferation.

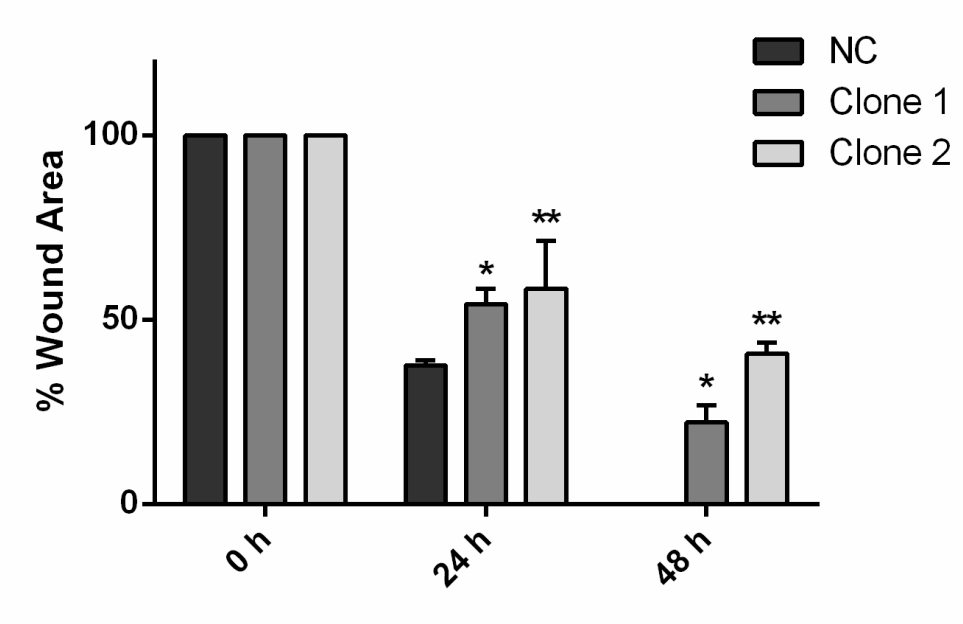

Figure 3.11. Quantification of cell motility in the in vitro scratch assay in scrambled (NC) and AKR1B1 silenced (clone 1 and clone 2) HCT-116 cells.

Bars represent the % wounded area. Significances were determined by one-way ANOVA. * $p<0.05$ and ** $p< 0.01$. 48h scrambled bar cannot be seen, since the wound was completely closed.

3.4.3. Effects of AKR1B1 silencing on production of reactive oxygen species

Recently, it was shown that AKR1B1 plays a key role on the development of oxidative stress mediated inflammation (Maccari & Ottanà, 2014). We have shown in ***Figure 3.3*** that, phosphorylation of ERK1/2 levels decreases in AKR1B1 silenced HCT-116 (Clone 1 and Clone 2) when compared to scrambled HCT-116 (NC) cells (please see lanes 1, 4 and 7) which could be a possible indicator of decrease oxidative stress. To further confirm this, cellular oxidative stress was assessed by NBT assay. We observed a significant decrease in AKR1B1 silenced HCT-116 (Clone 1 and 2) when compared with scrambled HCT-116 (NC) cells (***Figure 3.12***.).

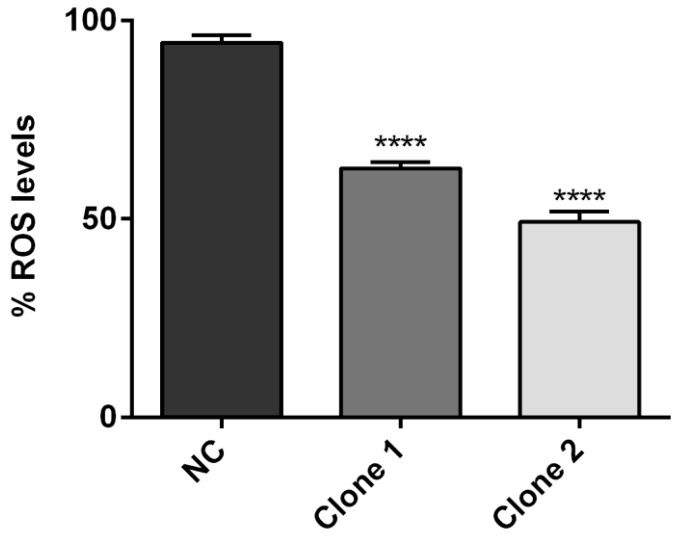

Figure 3.12. Reactive oxygen species (ROS) levels of HCT-116 scrambled (NC) and AKR1B1 silenced Clone 1 and 2 cells.

NC, Clone 1 and Clone 2 cells were treated with NBT solution for 5 h, fixed and dried. Formed formazan crystals were dissolved in alkaline DMSO. The blue color obtained from dissolution was measured at 620 nm and represented as percentages. Statistical significance was determined by one-way ANOVA with post hoc Tukey's test (****$p<0.0001$). (Unpublished data obtained by Dr. Sinem Tunçer).

3.4.4. Effects of AKR1B1 silencing on NF-κB pathway

Chronic inflammation is strongly involved in the development of colorectal cancer (Coussens & Werb, 2002). The activation of the master inflammatory transcription factor Nuclear Factor Kappa B (NF-κB) is also known to contribute to malignant transformation in colorectal cancer (Ullman & Itzkowitz, 2011).

AKR1B1 has an important role in regulation of inflammation (Srivastava et al., 2011). In order to observe the effects of AKR1B1 silencing in HCT-116 cells on the NF-κB pathway, the nuclear translocation of the NF-κB subunits p65 and p50 was assayed in AKR1B1 silenced (Clone 1 and 2) and scrambled (NC) HCT-116 cells by western

blotting. Reduced nuclear localization of p65 was seen in the AKR1B1 silenced HCT-116 cells when compared to the scrambled (NC) cells (***Figure 3.13***); this was especially obvious in the Clone 2.

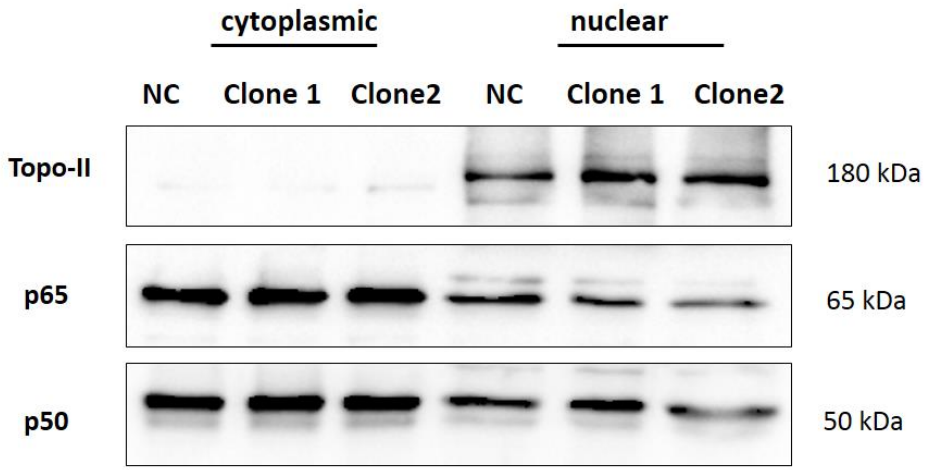

Figure 3.13. Representative western blot analysis of nuclear and cytoplasmic proteins obtained from AKR1B1 silenced (Clone 1 and 2) and scrambled (NC) HCT-116 cells, showing nuclear levels of p65 and p50.

50 µg of protein from each sample was loaded on 10 % SDS-PAGE. All antibodies were diluted with 1/500 ratios in 5% skimmed milk in TBS-T. Reduced translocation and localization of p65 to the nucleus was observed in AKR1B1 silenced cells indicating an inhibition of NF-κB.

Reduced nuclear translocation of p65 is generally associated with an inhibition of the transcriptional activity of NF-κB. To determine the NF-κB transcriptional activity, a commercial luciferase plasmid containing 5 copies of the NF-κB responsive elements in the upstream region of firefly luciferase gene was used. Following transfection and treatment with 20µM CHNQ, firefly luminescence levels normalized to Renilla luciferase levels were expressed as fold changes with respect to untreated HCT-116 scrambled cells (NC).

A significant reduction in NF-κB activation in AKR1B1 silenced (Clone 1 and 2) HCT-116 cells was observed when compared to scrambled (NC) cells, which supports the data shown in ***Figure 3.13***.

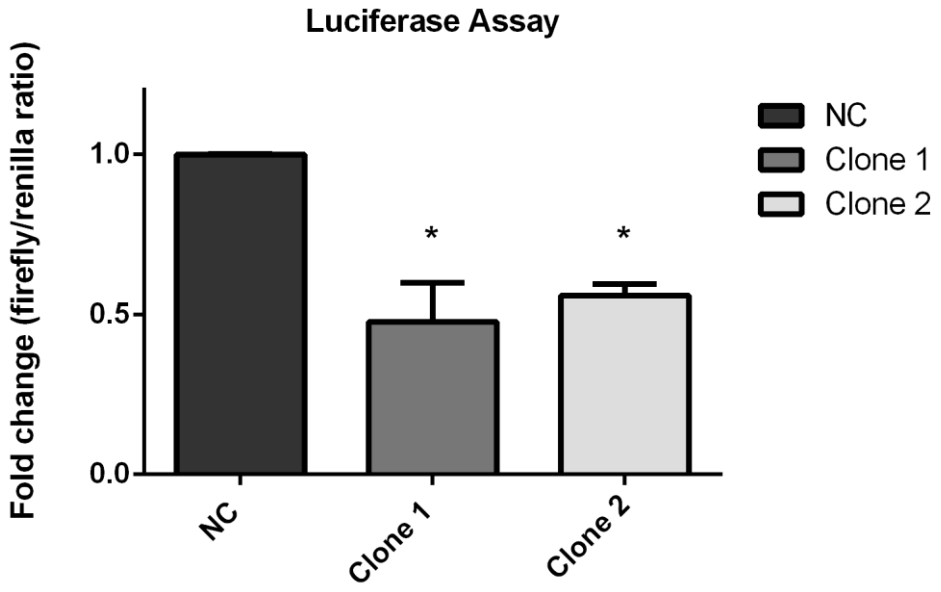

Figure 3.14. Luciferase assay utilizing NF-κB Pathdetect plasmid in untreated and 20 μM CHNQ treated HCT-116 scrambled (NC) and AKR1B1 silenced Clone 1 and Clone 2 cells.

NF-κB Pathdetect Firefly luciferase plasmid was transfected with pRL-TK Renilla plasmid in 1:250 ratio with X-tremegene transfection reagent for 24 h and assay was performed with Dual-Glo Luciferase Assay System. Statistical significance was determined by two-way ANOVA with post hoc Tukey's test (*$p<0.05$).

3.4.5. Effects of AKR1B1 silencing on cell cycle progression

We hypothesized that the reduced proliferation, oxidative stress and inflammation of AKR1B1 silenced HCT-116 cells (Clone 1 and Clone 2) observed may result in a defect or delay in cell cycle progression. Therefore, a time course cell cycle analysis was performed. For this, 24 h after plating, HCT-116 scrambled (NC) and AKR1B1

silenced (Clone 1 and Clone 2) cells were synchronized at G1/S phase by overnight serum starvation. Then, serum containing medium was given to the cells and assay was carried out at 0, 6, 12, 24 and 30 h time points.

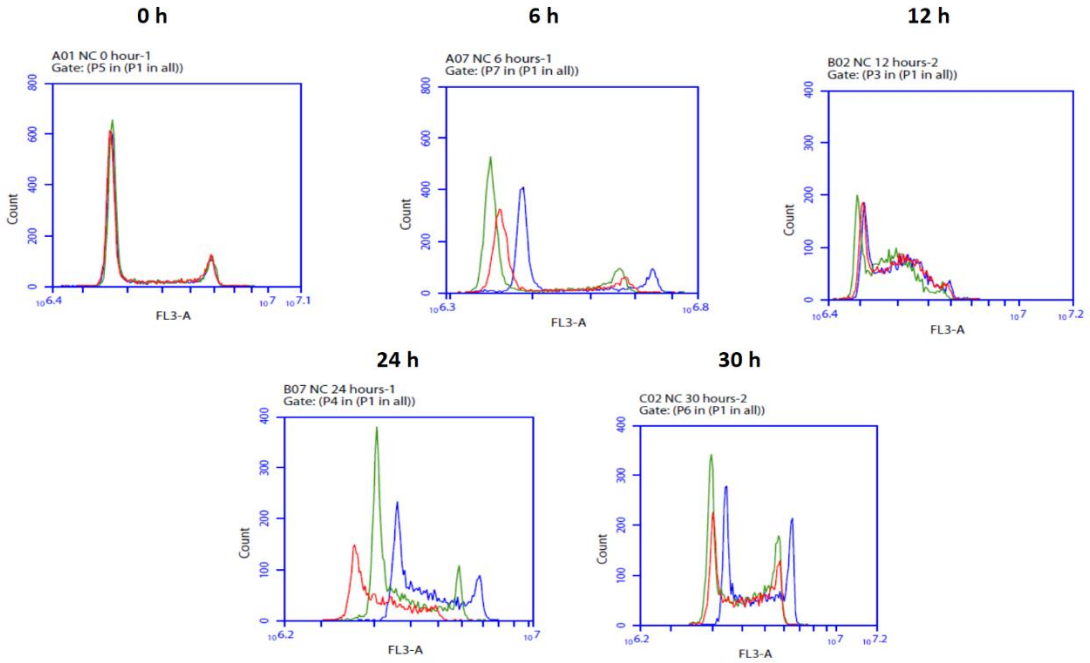

Figure 3.15. Time course cell cycle analysis by propidium iodide staining and flow cytometric analysis of HCT-116 scrambled (NC) and AKR1B1 silenced Clone 1 and Clone 2 cells at 0, 6, 12, 24 and 30h time points.

Both HCT-116 scrambled (NC) and AKR1B1 silenced (Clone 1 and 2) cells were synchronized by overnight serum starvation. Then, cells were released up to 30 h with serum containing medium, stained with PI and analyzed by BD Accuri C6 flow cytometer. Blue lines represent NC cells; green lines represent Clone 1 cells; red lines represent Clone 2 cells.

As seen in ***Figure 3.15***, at 0 h time point, scrambled (NC) and AKR1B1 silenced (Clone 1 and 2) cells were synchronized. However, beginning from the 6 h time point, an obvious delay in the cell cycle progression of Clone 1 (green) and Clone 2 (red) cells were observed when compared to NC (blue) cells.

To further confirm the delay in cell cycle, the expression of cell cycle related proteins such as Cyclin E, P-pRb, Cyclin B1 was assayed at two selected time points, 0 h and 30 h. These time points were selected since 0 h is the indicator of cell synchronization and 30 h is the time point that cells has already passed one cycle of division and started a new one. During cell cycle, Cyclin E predominantly takes role in G1 phase, where it binds to Cyclin Dependent Kinase-2 (CDK-2) and control the transition from G1 phase to S phase. At this point, the Cyclin E/CDK-2 complex phosphorylates the retinoblastoma protein (pRb) and triggers the progression from G1 to S phase. When this transition is achieved, Cyclin E is degraded rapidly in order for activation of phase-specific cyclins. Cyclin B, on the other hand, is the G2/M phase cyclin where it controls the initiation of mitosis together with its partner CDK1 (Suryadinata et al., 2010).

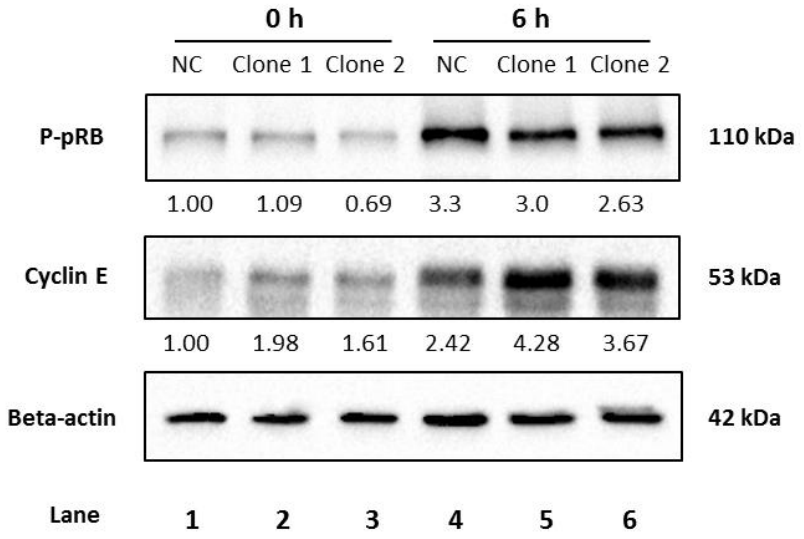

Figure 3.16. Western blot analysis of the cell cycle related proteins pRb, Cyclin B1 and Cyclin E in HCT-116 scrambled (NC) and AKR1B1 silenced (Clone 1 and 2) cells at 0 h and 6 h after cell synchronization by overnight serum starvation.

Beta actin was used as calibrator protein loading control. P-pRb and Cyclin E antibodies were diluted in 1/1000 ratio in 5% BSA and 5% skimmed milk, respectively. Beta-actin antibody was diluted in 1/4000 ratio in 5% skimmed milk in TBS-T. Numerical values under each blot shows the densitometric analysis of each sample normalized with respect to Beta actin.

Decreased levels of phosphorylated pRb at Serine 807/811 residues, mediated by Cyclin D- CDK4/6 complex, was observed in AKR1B1 silenced Clone 1 and 2 cells at 6 h time point, which supports the delay in cell cycle seen in these cells beginning at G1/S transition of cell cycle. Following this, at 6 h time point after synchronization, an accumulation of Cyclin E was observed in AKR1B1 silenced HCT-116 cells (Clone 1 and Clone 2, please see lanes 5 and 6) compared to scrambled HCT-116 cells (NC, lane 4). Thus, silencing of AKR1B1 mediates a signaling mechanism that leads to a delay in cell cycle progression.

CHAPTER 4

DISCUSSION

The AKR family of enzymes have unequivocally been shown to be very important in the development of diabetes and its complications (Alexiou et al., 2009). Latest studies have indicated that the enzyme is also implicated in inflammatory diseases. However, not much is known about the role played by AKR1B1 in colorectal cancer (CRC). Inhibition of AKR1B1 with known inhibitors such as sorbinil and zopolrestat was shown to inhibit growth factor driven activation of proliferation, cell cycle progression and generation of ROS in CRC cell lines (Ramana et al., 2010). Here, we have aimed to understand the importance of AKR1B1 in CRC through both pharmacological inhibition and RNAi approaches.

We have first aimed to investigate the effects CHNQ, a semi synthetic quercetin derivative of quercetin, which under *in vivo* conditions, inhibited AKR1B1 with a lower IC_{50} value than quercetin (Milackova et al., 2015). Moreover, the selectivity of CHNQ towards AKR1B1 in relation to other AKRs was reported to be higher than quercetin (Milackova et al., 2015). Quercetin is a flavonoid that is found in many natural products such as fruits, vegetables and grains. Besides having effects on AKR inhibition (Barski et al., 2008), it also has strong anti-cancer properties; however, its low water solubility and low bioavailability makes it difficult to be used in clinical applications (Cai, Fang, Dou, Yu, & Zhai, 2013). Therefore, we examined whether CHNQ showed any anti-cancer properties in CRC.

HCT-116 cells robustly express AKR1B1 (Ebert et al., 2011). When these cells were stably knocked down for AKR1B1 and treated with CHNQ, they showed reduced cell

proliferation and viability when compared to the control cells. However, the IC_{50} values (***Table 3.1***) were highly comparable irrespective of whether AKR1B1 was silenced or not indicating that the inhibitory effect of CHNQ on cell proliferation was independent of its inhibitory effects on AKR1B1 in HCT-116 cells. We examined then whether CHNQ showed any effects on cellular viability *in vivo*. For this, we used baker's yeast (*S. cerevisiae)* that provided us with an easy to use model. Yeast cells treated with 500µM CHNQ showed high cytotoxicity.

We have recently reported that HCT-116 wild type cells treated with CHNQ generated high amounts of ROS and showed ROS related activation of the MAPK pathway and inhibition of proliferation (Enayat et al., 2016). This was also replicated *in vivo,* where treatment of *S. cerevisiae* with CHNQ induced oxidative stress very strongly (almost 15 folds) when compared to the vehicle treated control (***Figure 3.2***). Furthermore, co-treatment of CHNQ with the antioxidant N-acetyl cysteine (NAC) resulted in a reversal of the oxidative stress. This is expected since CHNQ is a naphthoquinone derivative; naphtoquinones are known inducers of oxidative stress (Klotz, Hou, & Jacob, 2014). Corroborating these findings, we observed enhanced phosphorylation of ERK1/2 protein in the AKR1B1 silenced or control cells treated with CHNQ by western blotting (***Figure 3.3***). However, we again observed no selective effect of CHNQ on AKR1B1 silenced HCT-116 cells, since the level of induction of MAPK was similar for both control and AKR1B1 silenced cells. On the contrary, a decreased phosphorylation of ERK1/2 protein was observed in untreated AKR1B1 silenced cells in comparison to untreated control cells.

We next wanted to determine whether the reduction in proliferation and induction of ERK1/2 levels with CHNQ treatment, affected cell cycle progression of AKR1B1 silenced or control HCT-116 cells. We observed that CHNQ treatment showed a significant G2/M arrest in all cell types, irrespective of whether AKR1B1 was silenced (***Figure 3.4***). The G2/M arrest was most likely related to the increased levels of ROS caused by CHNQ treatment, since it is known that high levels of ROS can induce cell

cycle arrest by creating defects in cell cycle checkpoints (Verbon, Post, & Boonstra, 2012). However, consistent with our other data, the effects of CHNQ did not show any selectivity for AKR1B1 expression.

To determine whether the reduced proliferation in CHNQ treated cells was related to enhanced induction of apoptosis, HCT-116 AKR1B1 silenced or control cells were treated with 20 μM CHNQ for 24 h and Annexin V-PI staining was carried out as described before in ***Section 2.11***. Etoposide was used as positive control, since it is a known Topoisomerase II inhibitor, causes G2/M arrest in cell cycle and induces apoptosis (Kang et al., 2010). We observed that CHNQ treatment caused an increase in early apoptotic and late apoptotic/dead cell populations in all cells compared to the vehicle (***Figure 3.7***) irrespective of whether AKR1B1 was silenced or not. Interestingly, we observed no differences in apoptosis induction between the control and AKR1B1 silenced cells.

It was previously shown both *in vivo* and *in vitro* that pharmaceutical inhibition of AKRs could inhibit metastasis in CRC (Tammali, Reddy, Saxena, et al., 2011). In order to assess the effects of CHNQ on cell migration, an *in vitro* Transwell migration assay was performed. A relatively low dose of CHNQ was used in this experiment to ensure that migration was not affected by cell viability. A significant decrease in migration was observed in CHNQ treated control HCT-116 and AKR1B1 silenced cells when compared to their corresponding vehicle treated controls. However, again, this decrease did not reflect any relationship with AKR1B1, since the inhibition in migration was comparable in all the cells examined (***Figure 3.8***). On the other hand, when vehicle treated HCT-116 scrambled control cells were compared with vehicle treated HCT-116 AKR1B1 silenced cells, less migration of AKR1B1 silenced cells was observed, which supports that loss of AKR1B1 resulted in reduced cell migration. Moreover, MAPK signaling pathway is known to be a major pathway that is involved in epithelial to mesenchymal transition (EMT) together with other signaling pathways. Activated ERK1/2 is able to facilitate EMT by inducing the increased expression of

EMT-specific regulators and transcription factors of cell motility, migration and invasion (Lamouille, Xu, & Derynck, 2014). Therefore, reduced cell migration with AKR1B1 knockdown is also supported by the reduced activation of ERK1/2 observed in AKR1B1 silenced cells.

We have consistently observed that AKR1B1 silenced cells showed cellular properties that were considerably different from the control (scrambled shRNA) cells. This led us to a new hypothesis that AKR1B1 may have oncogenic properties in CRC. There are limited number of studies in the literature that have shown that pharmaceutical inhibition of AKR1B1 led to inhibition of inflammatory signaling, proliferation, metastasis and angiogenesis in colon cancer (Ramana et al., 2010; Tammali, Reddy, Srivastava, & Ramana, 2011; Tammali, Reddy, Saxena, et al., 2011). To examine our hypothesis, we examined the proliferation, cell cycle distribution, cell motility and inflammatory signaling in the AKR1B1 silenced cells.

Deregulation in cell proliferation and cell cycle in normal epithelial cells are known to be one of the major cause of cellular transformation (Evan & Vousden, 2001). Based on our incidental observation that AKR1B1 silenced HCT-116 cells grew more slowly than their corresponding control cells, we examined the proliferation using the BrdU assay. In this assay, BrdU (instead of Thymidine) is incorporated during S phase of cell cycle, which can be measured. The choice of this assay for cellular proliferation and not the MTT assay has been made since the MTT assay determines metabolic activity of the cells via NAD(P)H-dependent cellular oxidoreductases enzymes (which also includes the AKR enzymes) and may therefore result in artifacts for the current study. In a time course experiment with synchronized cells, the proliferation rates of the AKR1B1 silenced cells were significantly decreased after 24 and 48h of growth in comparable to the control cells (***Figure 3.9***). These data corroborate with the decrease in cell viability reported with growth-factor induced CRC cell lines treated with AKR inhibitors (Ramana et al., 2010).

Cancer cells treated with fidarestat, a potent AKR inhibitor used in treatment of diabetic neuropathy, has previously been shown to delay the entry of cancer cells from G1 to the S phase (Ramana et al., 2010). When we examined the cell cycle distribution of synchronized cells in a time dependent manner, AKR1B1 silenced cells showed a considerably delayed progression through the cells cycle starting from as early as 6 h (***Figure 3.15***). For instance, when the control HCT-116 cells were starting to enter their S phase at the 6 h time point, the AKR1B1 silenced cells were observed to still be progressing in their G1 phase. Similarly, at 30 h time point, while the control cells were proceeding towards the end of S phase of a new cycle, the AKR1B1 silenced cells were at the G1 phase. A considerably higher amount of Cyclin E was observed in the AKR1B1 silenced cells at 6 h compared to the control cells (***Figure 3.16***). Although high levels of Cyclin E should be accompanied by hyper-phosphorylation of pRB, we did not observe this in our western images. This is most likely because the antibody recognizes mono-phosphorylated pRb exclusively at Ser807/811 residues and not its hyper-phosphorylation. Thus, at 6 h, the AKR1B1 cells were in their G1/S phase, therefore, Cyclin E was highly active; however, the control cells most likely had already completed and passed that phase, thus the Cyclin E levels were lower. Overall, these results claim that AKR1B1 inhibition may have a regulatory role in cell proliferation and serves a delay in cell cycle progression.

Deregulations in cell cycle and its progression and proliferation may go hand in hand and simultaneously with metastatic properties (Ouhtit et al., 2013). Therefore, we examined whether silencing of AKR1B1 in HCT-116 cells showed any functional changes that could affect cellular motility of the cells. We have shown in ***Figure 3.8*** that AKR1B1 silenced HCT-116 cells showed decreased migration in a Transwell migration assay compared to the control cells. Confirming this, an in vitro scratch assay indicated that the AKR1B1 silenced cells were significantly less motile than the control cells (***Figure 3.10***). This is expected since pharmacological inhibition of AKR1B1 inhibition is known to inhibit growth factor-induced migration and invasion

of colon cancer cells by downregulating adhesion molecules ICAM, VCAM and E-cadherin (Tammali, Reddy, Saxena, et al., 2011).

Cell cycle progression, motility and proliferation are among the major hallmarks of cancer (Hanahan et al., 2011). Such fundamental characteristics of cells are regulated through nodal signaling mechanisms of which the NF-κB pathway is among the most important ones. Moreover, NF-κB and AP-1 are redox sensitive transcription factors that can be activated by oxidative stress. Under physiological conditions, ROS levels in cells exist in a balance between ROS production and their detoxification by antioxidant systems (Srivastava et al., 2011). Disruption of this balance results in oxidative stress, either resulted by enhanced generation of ROS or failed antioxidant defenses. We have observed a reduction in ROS levels in the AKR1B1 silenced HCT-116 cells (***Figure 3.12***). Moreover, since AKR1B1 activity has previously been reported to modulate inflammatory signaling (Yadav, Ramana, & Srivastava, 2011), activation both AP-1 and NF-κB in AKR1B1 silenced cells were examined. We did not observe any activation of AP-1 in a luciferase assay (data not shown). However, both nuclear translocation and transcriptional activity of NF-κB were significantly reduced in AKR1B1 silenced cells compared to control cells. Overall, our data suggest that one of the principal cellular alterations induced through the downregulation of AKR1B1 is the reduction in ROS formation. We propose that this reduction lead to a decrease in NF-κB activation and subsequently reduction in cell proliferation, motility and cell cycle progression.

CHAPTER 5

CONCLUSION

Aldo-keto reductases (AKRs) are enzymes that play important roles in the polyol pathway of glucose metabolism and are strongly implicated in diabetic complications. It is also reported in the literature that AKR1B1, a member of AKR family, is involved in development of inflammation-associated such as colorectal cancer (CRC) (Ramana et al., 2010).

To understand the underlying mechanisms of AKR1B1 in CRC, we have selected the HCT-116 colorectal cancer cell line as our model. Treatment with CHNQ (quercetin derivative that is also an AKR1B1 specific inhibitor) reduced cell viability and proliferation, caused cell cycle arrest, induced apoptosis and reduced cell migration irrespective of the expression of AKR1B1. However, these effects could be attributed to the high ROS generation capacity of CHNQ (observed also *in vivo* in yeast) through its 1,2 napthoquinone moiety rather than a specific inhibitory effect on AKR1B1.

When we examined the effects of RNAi of AKR1B1 in HCT-116 cells, we detected a major decrease in the generation of ROS. Oxidative stress affects several cellular pathways, and the decrease in ROS levels in the AKR1B1 silenced cells is likely to result in major changes in cell behavior. Supporting this, we observed concomitant decreases in proliferation and cell motility, decrease in NF-κB subunit p65 nuclear localization and downregulation of NF-κB activity as well as delay in cell cycle progression (***Figure 5.1***).

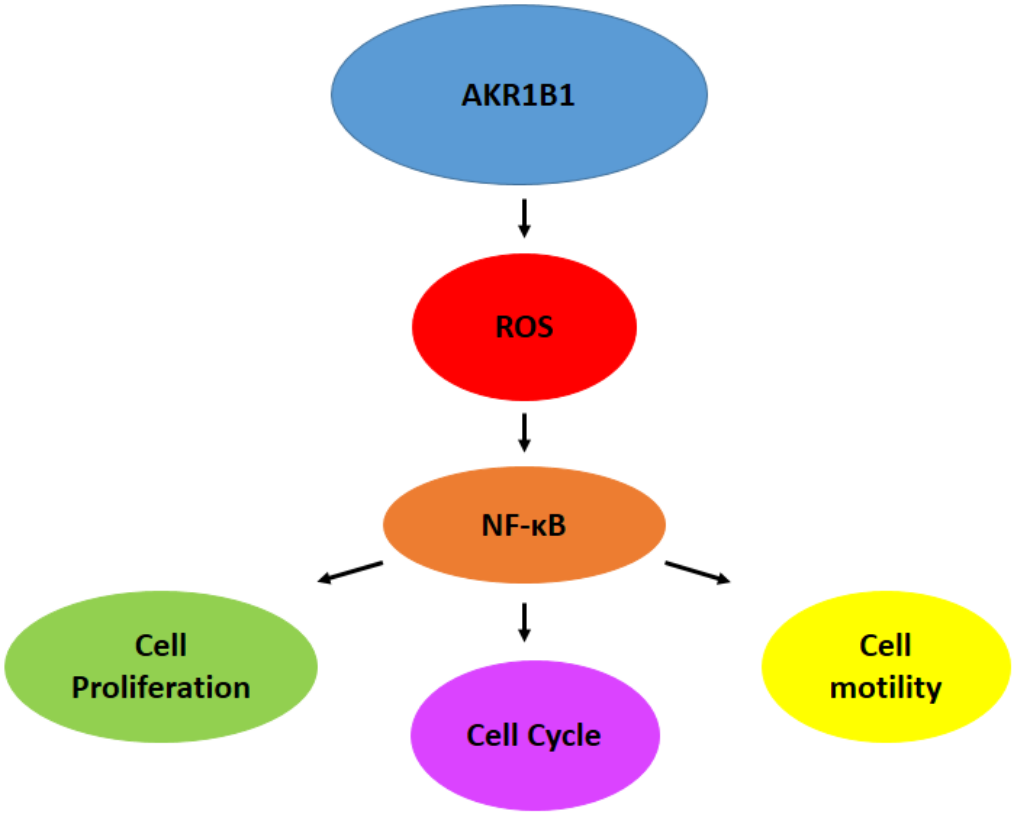

Figure 5.1.Overall mechanism for AKR1B1 where its inhibition leads to a decrease in NF-κB activation and nuclear translocation, cell proliferation, cell motility and delay in cell cycle through reduced ROS.

Our data support previous reports on the cellular functions of AKR1B1 where the enzyme was inhibited by different AKR1B1 inhibitory drugs. This study is the first to examine the role of AKR1B1 after shRNA mediated silencing in CRC cells. We believe that these results highlight the mechanisms behind the oncogenic function of AKR1B1 and can help to develop novel strategies to regulate AKR1B1 expression in colorectal cancer.

REFERENCES

Alexiou, P., Pegklidou, K., Chatzopoulou, M., Nicolaou, I., & Demopoulos, V. J. (2009). Aldose reductase enzyme and its implication to major health problems of the 21(st) century. *Current Medicinal Chemistry*, *16*(6), 734–52. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19199934

American Cancer Society. (2016). *Cancer Facts & Figures 2016*. *Cancer Facts & Figures 2016*.

Avery, S. V, Avery, S. V., Limon-Pacheco, J., Gonsebatt, M. E., Roberts, R. A., Laskin, D. L., … Shang, F. (2011). Molecular targets of oxidative stress. *The Biochemical Journal*, *434*(2), 201–10. http://doi.org/10.1042/BJ20101695

Baeuerle, P. A., & Baltimore, D. (1996). Nf-κB: Ten years after. In *Cell* (Vol. 87, pp. 13–20). http://doi.org/10.1016/S0092-8674(00)81318-5

Baldwin, A. S., Azizkhan, J. C., Jensen, D. E., Beg, A. A., & Coodly, L. R. (1991). Induction of NF-kappa B DNA-binding activity during the G0-to-G1 transition in mouse fibroblasts. *Molecular and Cellular Biology*, *11*(10), 4943–51. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1922027

Barré, B., & Perkins, N. D. (2007). A cell cycle regulatory network controlling NF-kappaB subunit activity and function. *The EMBO Journal*, *26*(23), 4841–4855. http://doi.org/10.1038/sj.emboj.7601899

Barski, O. A., Tipparaju, S. M., Bhatnagar, A., Endo, S., Matsunaga, T., Mamiya, H., … Bhatnagar, A. (2008). The aldo-keto reductase superfamily and its role in drug metabolism and detoxification. *Drug Metabolism Reviews*, *487*(4), 1–9. http://doi.org/10.1016/j.abb.2009.05.009

Bertoli, C., Skotheim, J. M., & de Bruin, R. A. M. (2013). Control of cell cycle transcription during G1 and S phases. *Nature Reviews. Molecular Cell Biology*, *14*(8), 518–28. http://doi.org/10.1038/nrm3629

Cai, X., Fang, Z., Dou, J., Yu, A., & Zhai, G. (2013). Bioavailability of quercetin:

problems and promises. *Current Medicinal Chemistry*, *20*(20), 2572–82. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/23514412

Cebola, I., Custodio, J., Muñoz, M., Díez-Villanueva, A., Paré, L., Prieto, P., … Peinado, M. A. (2015). Epigenetics override pro-inflammatory PTGS transcriptomic signature towards selective hyperactivation of PGE2 in colorectal cancer. *Clinical Epigenetics*, *7*, 74. http://doi.org/10.1186/s13148-015-0110-4

Chambard, J.-C., Lefloch, R., Pouysségur, J., & Lenormand, P. (2007). ERK implication in cell cycle regulation. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1773*(8), 1299–1310. http://doi.org/10.1016/j.bbamcr.2006.11.010

Chang, F., Steelman, L. S., Lee, J. T., Shelton, J. G., Navolanic, P. M., Blalock, W. L., … McCubrey, J. A. (2003). Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention. *Leukemia*, *17*(7), 1263–1293. http://doi.org/10.1038/sj.leu.2402945

Coussens, L. M., & Werb, Z. (2002). Inflammation and cancer. *Nature*, *420*(6917), 860–867. http://doi.org/10.1038/nature01322

Cunningham, D., Atkin, W., Lenz, H.-J., Lynch, H. T., Minsky, B., Nordlinger, B., & Starling, N. (2010). Colorectal cancer. *Lancet*, *375*(9719), 1030–47. http://doi.org/10.1016/S0140-6736(10)60353-4

Ebert, B., Kisiela, M., Ws??l, V., Maser, E., Wsól, V., & Maser, E. (2011). Proteasome inhibitors MG-132 and bortezomib induce AKR1C1, AKR1C3, AKR1B1, and AKR1B10 in human colon cancer cell lines SW-480 and HT-29. In *Chemico-Biological Interactions* (Vol. 191, pp. 239–249). http://doi.org/10.1016/j.cbi.2010.12.026

Enayat, S., Şeyma Ceyhan, M., Taşkoparan, B., Stefek, M., & Banerjee, S. (2016). CHNQ, a novel 2-Chloro-1,4-naphthoquinone derivative of quercetin, induces oxidative stress and autophagy both in vitro and in vivo. *Archives of Biochemistry and Biophysics*, *596*, 84–98. http://doi.org/10.1016/j.abb.2016.03.004

English, J. M., Cobb, M. H., Pearson, G., al., et, Pearson, G., al., et, … al., et. (2002).

Pharmacological inhibitors of MAPK pathways. *Trends in Pharmacological Sciences*, *23*(1), 40–45. http://doi.org/10.1016/S0165-6147(00)01865-4

Evan, G. I., & Vousden, K. H. (2001). Proliferation, cell cycle and apoptosis in cancer. *Nature*, *411*(6835), 342–348. http://doi.org/10.1038/35077213

Fang, J. Y., & Richardson, B. C. (2005, May). The MAPK signalling pathways and colorectal cancer. *Lancet Oncology*. Elsevier. http://doi.org/10.1016/S1470-2045(05)70168-6

Hanahan, D., & Weinberg, R. A. (2000). The Hallmarks of Cancer. *Cell*, *100*(1), 57–70. http://doi.org/10.1016/S0092-8674(00)81683-9

Hanahan, D., Weinberg, R. A. R. A. R. A. A., Adams, J. M., Cory, S., Aguirre-Ghiso, J. A., Ahmed, Z., … Christofori, G. (2011). Hallmarks of cancer: The next generation. *Cell*, *144*(5), 646–674. http://doi.org/10.1016/j.cell.2011.02.013

Hochegger, H., Takeda, S., & Hunt, T. (2008). Cyclin-dependent kinases and cell-cycle transitions: does one fit all? *Nature Reviews. Molecular Cell Biology*, *9*(11), 910–916. http://doi.org/10.1038/nrm2510

Hwang, H. C., & Clurman, B. E. (2005). Cyclin E in normal and neoplastic cell cycles. *Oncogene*, *24*(17), 2776–2786. http://doi.org/10.1038/sj.onc.1208613

Iyer, N., Xian, J., Chin, S.-F., Bannister, A., Daigo, Y., Aparicio, S., … Caldas, C. (2007). p300 is required for orderly G1/S transition in human cancer cells. *Oncogene*, *26*, 21–29. http://doi.org/10.1038/sj.onc.1209771

Jourdan, M., Moreaux, J., Vos, J. De, Hose, D., Mahtouk, K., Abouladze, M., … Klein, B. (2007). Targeting NF-kappaB pathway with an IKK2 inhibitor induces inhibition of multiple myeloma cell growth. *British Journal of Haematology*, *138*(2), 160–168. http://doi.org/10.1111/j.1365-2141.2007.06629.x

Kang, K., Lee, S. B., Yoo, J.-H., & Nho, C. W. (2010). Flow cytometric fluorescence pulse width analysis of etoposide-induced nuclear enlargement in HCT116 cells. *Biotechnology Letters*, *32*(8), 1045–52. http://doi.org/10.1007/s10529-010-0277-x

Kim, H. J., & Bar-Sagi, D. (2004). Modulation of signalling by Sprouty: a developing story. *Nature Reviews Molecular Cell Biology*, *5*(6), 441–450.

http://doi.org/10.1038/nrm1400

Klotz, L. O., Hou, X., & Jacob, C. (2014). 1,4-naphthoquinones: From oxidative damage to cellular and inter-cellular signaling. *Molecules*, *19*(9), 14902–14918. http://doi.org/10.3390/molecules190914902

Laffin, B., & Petrash, J. M. (2012). Expression of the aldo-ketoreductases AKR1B1 and AKR1B10 in human cancers. *Frontiers in Pharmacology*, *3 JUN*, 104. http://doi.org/10.3389/fphar.2012.00104

Lamouille, S., Xu, J., & Derynck, R. (2014). Molecular mechanisms of epithelial–mesenchymal transition. *Nature Reviews Molecular Cell Biology*, *15*(3), 178–196. http://doi.org/10.1038/nrm3758

Lawrence, T. (2009). The Nuclear Factor NF-kB Pathway in Inflammation. *Cold Spring Harbor Perspectives in Biology*, *1*(6), 1–10. http://doi.org/10.1101/cshperspect.a001651

Liou, G.-Y., & Storz, P. (2010). Reactive oxygen species in cancer. *Free Radic Res.*, *44*(5). http://doi.org/10.3109/10715761003667554

Liu, J., Wen, G., & Cao, D. (2009). Aldo-keto reductase family 1 member B1 inhibitors: old drugs with new perspectives. *Recent Patents on Anti-Cancer Drug Discovery*, *4*(3), 246–253. http://doi.org/10.2174/157489209789206931

Maccari, R., & Ottanà, R. (2014). Targeting aldose reductase for the treatment of diabetes complications and inflammatory diseases: New insights and future directions. *Journal of Medicinal Chemistry*, *58*(5), 2047–2067. http://doi.org/10.1021/jm500907a

Malumbres, M., & Barbacid, M. (2009). Cell cycle, CDKs and cancer: a changing paradigm. *Nature Reviews. Cancer*, *9*(3), 153–166. http://doi.org/10.1038/nrc2602

McCubrey, J. A., Steelman, L. S., Chappell, W. H., Abrams, S. L., Wong, E. W. T., Chang, F., … Franklin, R. A. (2007). Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochimica et Biophysica Acta*, *1773*(8), 1263–84. http://doi.org/10.1016/j.bbamcr.2006.10.001

Mebratu, Y., & Tesfaigzi, Y. (2009). How ERK1/2 activation controls cell

proliferation and cell death: Is subcellular localization the answer? *Cell Cycle (Georgetown, Tex.)*, *8*(8), 1168–75. http://doi.org/10.4161/cc.8.8.8147

Michor, F., Iwasa, Y., Lengauer, C., & Nowak, M. A. Dynamics of colorectal cancer, 15 Seminars in Cancer Biology 484–493 (2005). http://doi.org/10.1016/j.semcancer.2005.06.005

Milackova, I., Kovacikova, L., Veverka, M., Gallovic, J., & Stefek, M. (2013). Screening for antiradical efficiency of 21 semi-synthetic derivatives of quercetin in a DPPH assay. *Interdisciplinary Toxicology*, *6*(1), 13–17. http://doi.org/10.2478/intox-2013-0003

Milackova, I., Prnova, M. S., Majekova, M., Sotnikova, R., Stasko, M., Kovacikova, L., … Stefek, M. (2015). 2-Chloro-1,4-naphthoquinone derivative of quercetin as an inhibitor of aldose reductase and anti-inflammatory agent. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *30*(1), 107–113. http://doi.org/10.3109/14756366.2014.892935

Mindnich, R. D., & Penning, T. M. (2009). Aldo-keto reductase (AKR) superfamily: genomics and annotation. *Human Genomics*, *3*(4), 362–70. http://doi.org/10.1186/1479-7364-3-4-362

Moon, J. W., Lee, S. K., Lee, J. O., Kim, N., Lee, Y. W., Kim, S. J., … Park, S.-H. (2014). Identification of novel hypermethylated genes and demethylating effect of vincristine in colorectal cancer. *Journal of Experimental & Clinical Cancer Research : CR*, *33*, 4. http://doi.org/10.1186/1756-9966-33-4

Morgan, M. J., & Liu, Z. (2011). Crosstalk of reactive oxygen species and NF-κB signaling. *Cell Research*, *21*(1), 103–15. http://doi.org/10.1038/cr.2010.178

Myung, K., Smith, S., & Kolodner, R. D. (n.d.). Mitotic checkpoint function in the formation of gross chromosomal rearrangements in Saccharomyces cerevisiae.

Naugler, W. E., & Karin, M. (2008). NF-kappaB and cancer-identifying targets and mechanisms. *Current Opinion in Genetics & Development*, *18*(1), 19–26. http://doi.org/10.1016/j.gde.2008.01.020

Neli Hevir, Jasna Šinkovec, T. L. R. ˇner a, & A. (2013). Decreased levels of AKR1B1 and AKR1B10 in cancerous endometrium compared to adjacent non-cancerous

tissue. In *Chemico-Biological Interactions* (Vol. 202, pp. 226–233). http://doi.org/10.1016/j.cbi.2012.11.001

Ouhtit, A., Gaur, R. L., Abdraboh, M., Ireland, S. K., Rao, P. N., Raj, S. G., … Raj, M. H. G. (2013). Simultaneous Inhibition of Cell-Cycle, Proliferation, Survival, Metastatic Pathways and Induction of Apoptosis in Breast Cancer Cells by a Phytochemical Super-Cocktail: Genes That Underpin Its Mode of Action. *Journal of Cancer*, *4*(4), 703–715. http://doi.org/10.7150/jca.7235

Pan, J.-S., Hong, M.-Z., Ren, J.-L., Submissions, O., & Com, W. W. (2009). Reactive oxygen species: A double-edged sword in oncogenesis. *World J Gastroenterol World Journal of Gastroenterology ISSN World J Gastroenterol*, *15*(1514), 1702–1707. http://doi.org/10.3748/wjg.15.1702

Pericleous, M., Mandair, D., & Caplin, M. E. (2013). Diet and supplements and their impact on colorectal cancer. *Journal of Gastrointestinal Oncology*, *4*(4), 409–23. http://doi.org/10.3978/j.issn.2078-6891.2013.003

Ramana, K. V. (2011). Aldose reductase: new insights for an old enzyme. *Biomolecular Concepts*, *2*, 103–114. http://doi.org/10.1515/BMC.2011.002.ALDOSE

Ramana, K. V, Tammali, R., & Srivastava, S. K. (2010). Inhibition of aldose reductase prevents growth factor-induced G1-S phase transition through the AKT/phosphoinositide 3-kinase/E2F-1 pathway in human colon cancer cells. *Molecular Cancer Therapeutics*, *9*(4), 813–824. http://doi.org/10.1158/1535-7163.MCT-09-0795

Saraswat, M., Mrudula, T., Kumar, P. U., Suneetha, A., Rao Rao, T. S., Srinivasulu, M., & Reddy, B. (2006). Overexpression of aldose reductase in human cancer tissues. *Medical Science Monitor : International Medical Journal of Experimental and Clinical Research*, *12*(12), CR525–529. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17136009

Saraswathy, R., Anand, S., Kunnumpurath, S. K., Kurian, R. J., Kaye, A. D., & Vadivelu, N. (2014). Chromosomal Aberrations and Exon 1 Mutation in the AKR1B1 Gene in Patients with Diabetic Neuropathy. *The Ochsner Journal*,

14(3), 339–342.

Seger, R., & Krebs, E. G. (1995). The MAPK signaling cascade. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *9*(9), 726–35. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7601337

Song, M., Garrett, W. S., & Chan, A. T. (2015). Nutrients, foods, and colorectal cancer prevention. *Gastroenterology*, *148*(6), 1244–1260. http://doi.org/10.1053/j.gastro.2014.12.035

Srivastava, S. K., Ramana, K. V, & Bhatnagar, A. (2005). Role of aldose reductase and oxidative damage in diabetes and the consequent potential for therapeutic options. *Endocrine Reviews*, *26*(3), 380–92. http://doi.org/10.1210/er.2004-0028

Srivastava, S. K. S., Yadav, U. C. S., Reddy, A. B. M., Saxena, A., Tammali, R., Shoeb, M., … Ramana, K. V. (2011). Aldose reductase inhibition suppresses oxidative stress-induced inflammatory disorders. In *Chemico-Biological Interactions* (Vol. 191, pp. 330–338). NIH Public Access. http://doi.org/10.1016/j.cbi.2011.02.023

Stefek, M. (2011). Natural flavonoids as potential multifunctional agents in prevention of diabetic cataract. *Interdisciplinary Toxicology*, *4*(2), 69–77. http://doi.org/10.2478/v10102-011-0013-y

Stefek, M., & Karasu, C. (2011). Eye lens in aging and diabetes: effect of quercetin. *Rejuvenation Research*, *14*(5), 525–34. http://doi.org/10.1089/rej.2011.1170

Suryadinata, R., Sadowski, M., & Sarcevic, B. (2010). Control of cell cycle progression by phosphorylation of cyclin-dependent kinase (CDK) substrates. *Bioscience Reports*, *30*(4), 243–255. http://doi.org/10.1042/BSR20090171

Tammali, R., Ramana, K. V., Singhal, S. S., Awasthi, S., & Srivastava, S. K. (2006). Aldose reductase regulates growth factor-induced cyclooxygenase-2 expression and prostaglandin E2 production in human colon cancer cells. *Cancer Research*, *66*(19), 9705–9713. http://doi.org/10.1158/0008-5472.CAN-06-2105

Tammali, R., Ramana, K. V, & Srivastava, S. K. (2007). Aldose reductase regulates TNF-alpha-induced PGE2 production in human colon cancer cells. *Cancer*

Letters, *252*(2), 299–306. http://doi.org/10.1016/j.canlet.2007.01.001

Tammali, R., Reddy, A. B. M., Saxena, A., Rychahou, P. G., Evers, B. M., Qiu, S., … Srivastava, S. K. (2011). Inhibition of aldose reductase prevents colon cancer metastasis. *Carcinogenesis*, *32*(8), 1259–67. http://doi.org/10.1093/carcin/bgr102

Tammali, R., Reddy, A. B. M., Srivastava, S. K., & Ramana, K. V. (2011). Inhibition of aldose reductase prevents angiogenesis in vitro and in vivo. *Angiogenesis*, *14*(2), 209–221. http://doi.org/10.1007/s10456-011-9206-4

Tammali, R., Srivastava, S. K., & Ramana, K. V. (2011). Targeting aldose reductase for the treatment of cancer. *Current Cancer Drug Targets*, *11*(5), 560–71. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3142792&tool=pmcentrez&rendertype=abstract

Terzić, J., Grivennikov, S., Karin, E., Karin, M., Tenesa, A., Dunlop, M. G., … Assche, G. Van. (2010). Inflammation and Colon Cancer. *Gastroenterology*, *138*(6), 2101–2114.e5. http://doi.org/10.1053/j.gastro.2010.01.058

Thurlings, I., & de Bruin, A. (2016). E2F Transcription Factors Control the Roller Coaster Ride of Cell Cycle Gene Expression. *Methods in Molecular Biology (Clifton, N.J.)*, *1342*, 71–88. http://doi.org/10.1007/978-1-4939-2957-3_4

Tuncay Cagatay, S., Cimen, I., Savas, B., & Banerjee, S. (2013). MTA-1 expression is associated with metastasis and epithelial to mesenchymal transition in colorectal cancer cells. *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, *34*(2), 1189–204. http://doi.org/10.1007/s13277-013-0662-x

Tunçay Çağatay, S. (2014). *CHARACTERIZATION AND PROMOTER IDENTIFICATION OF METASTASIS ASSOCIATED PROTEIN 1 IN HUMAN COLORECTAL CARCINOMA CELL LINES*. Middle East Technical University.

Ullman, T. A., & Itzkowitz, S. H. (2011). Intestinal Inflammation and Cancer. *Gastroenterology*, *140*(6), 1807–1816.e1. http://doi.org/10.1053/j.gastro.2011.01.057

Verbon, E. H., Post, J. A., & Boonstra, J. (2012). The influence of reactive oxygen species on cell cycle progression in mammalian cells. *Gene*, *511*(1), 1–6. http://doi.org/10.1016/j.gene.2012.08.038

Verma, R. (2006). Anti-Cancer Activities of 1,4-Naphthoquinones: A QSAR Study. *Anti-Cancer Agents in Medicinal Chemistry*, *6*(5), 489–499. http://doi.org/10.2174/187152006778226512

Veverka, M., Gallovič, J., Švajdlenka, E., Veverková, E., Prónayová, N., Miláčková, I., & Štefek, M. (2013). Novel quercetin derivatives: synthesis and screening for anti-oxidant activity and aldose reductase inhibition. *Chemical Papers*, *67*(1), 76–83. http://doi.org/10.2478/s11696-012-0240-5

Walther, A., Johnstone, E., Swanton, C., Midgley, R., Tomlinson, I., & Kerr, D. (2009). Genetic prognostic and predictive markers in colorectal cancer. *Nature Reviews. Cancer*, *9*(7), 489–99. http://doi.org/10.1038/nrc2645

Wei, J., Li, G., Dang, S., Zhou, Y., Zeng, K., & Liu, M. (2016). Discovery and Validation of Hypermethylated Markers for Colorectal Cancer. *Disease Markers*, *2016*, 2192853. http://doi.org/10.1155/2016/2192853

Wierenga, R. . K. (2001). The TIM-barrel fold: A versatile framework for efficient enzymes. *FEBS Letters*, *492*(3), 193–198. http://doi.org/10.1016/S0014-5793(01)02236-0

Yadav, U. C. S., Ramana, K. V., & Srivastava, S. K. (2011). Aldose reductase inhibition suppresses airway inflammation. In *Chemico-Biological Interactions* (Vol. 191, pp. 339–345). http://doi.org/10.1016/j.cbi.2011.02.014

APPENDICES

APPENDIX A

MAPS OF VECTORS USED IN THIS STUDY

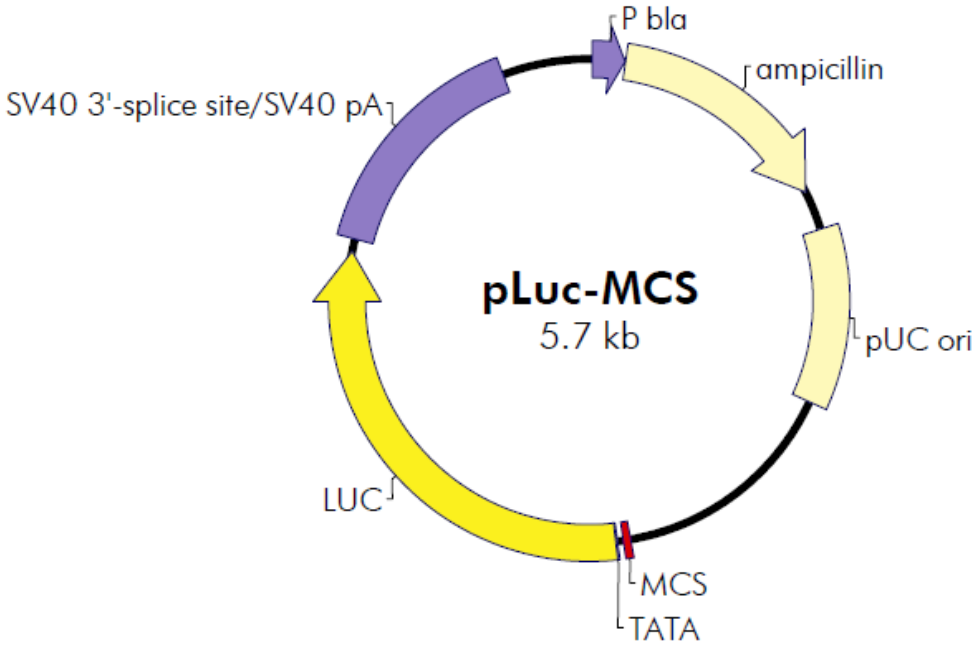

Figure A. 1. The map for Pathdetect Cis-Reporter plasmid (Agilent Technologies). The NF-κB Pathdetect plasmid used in this study contains 5 tandem repeats of NF-κB response element ((TGGGGACCTTTCCGC) cloned at Multiple Cloning Site (MCS) of this vector.

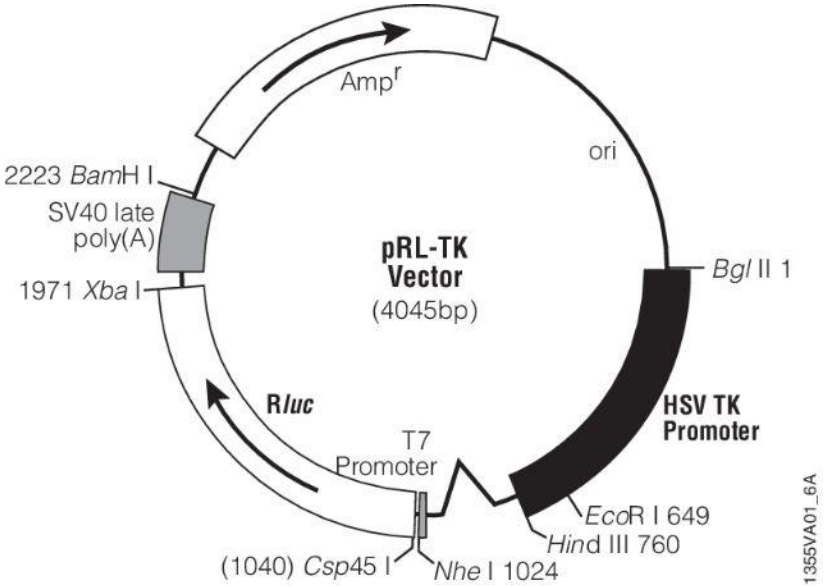

Figure A. 2. The map for pRL-TK Renilla Luciferase Vector.

APPENDIX B

RECIPES OF BUFFERS USED FOR WESTERN BLOT EXPERIMENTS

6X Sample Loading Dye

12% SDS

30% β-mercaptoethanol

30% Glycerol

0.012% Bromophenol Blue

0.375 M Tris-HCL pH 6.8

10% Separating Gel Mix

5.4 mL dH_2O

3.8 mL 10% SDS + 1.5 M Tris-HCl pH 8.8

5.6 mL 30 % Acrylamide+Bisacrylamide solution

150 µl 10 % Ammonium persulfate (APS)

20 µl TEMED

4% Stacking Gel Mix

4.7 mL dH_2O

2 mL 10% SDS + 1.5 M Tris-HCl pH 6.8

1.2 mL 30 % Acrylamide+Bisacrylamide solution

50 µl 10 % Ammonium persulfate (APS)

10 µl TEMED

SDS-PAGE Running Buffer

25 mM Tris

190 mM Glycine

0.1% SDS

TBS-T

50 mM Tris-HCl pH 7.4 } Autoclaved

150 mM NaCl }

Before use, 0.1% Tween-20 added.

10X Blotting Buffer

0.25 M Trizma Base

1.92 M Glycine

pH adjusted to 8.3 in 1 mL dH_2O

Transfer Buffer (1L)

200 mL methanol

100 mL 10X Blotting Buffer

700 mL dH_2O

Mild Stripping Buffer

15 g Glycine

1 g SDS

10 mL Tween-20

pH adjusted to 2.2 in 1 L dH_2O.

APPENDIX C

KILL CURVE OF G418 IN HCT-116 CELL LINE

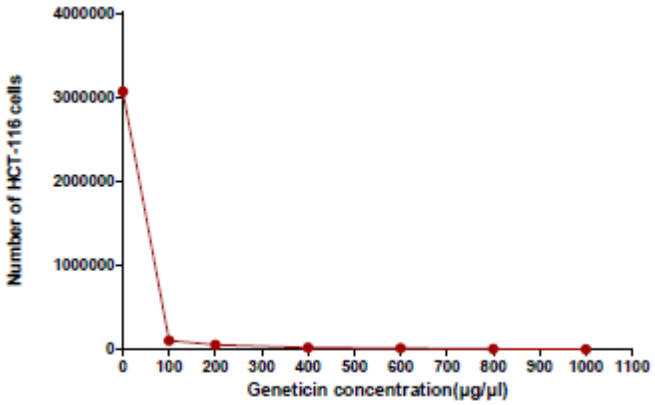

Figure A. 3. G418 kill curve in HCT-116 cells. Obtained from (Tunçay Çağatay, 2014).

APPENDIX D

EFFECTS OF CHNQ ON CELL VIABILITY OF YEAST CELLS

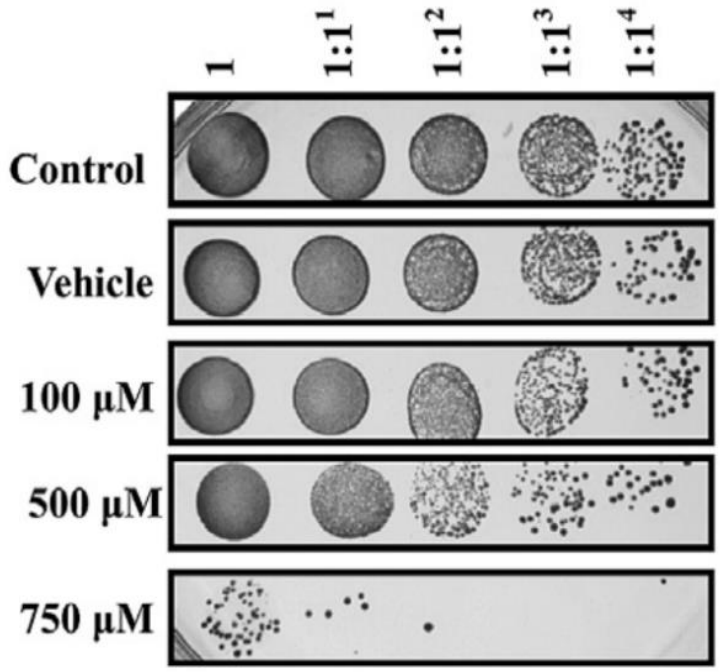

Figure A. 4. A representative figure showing cell viability of yeast cells upon treatment with 0-750 µM CHNQ for 24 h. Following CHNQ treatment, cells were serially diluted and then spotted on YPD-agar plate.

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