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Effects of aryl hydrocarbon receptor agonists, antagonists and gemcitabine on gene expression in human pancreatic ductal adenocarcinoma cells

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„Effects of aryl hydrocarbon receptor agonists, antagonists and gemcitabine on gene expression in human pancreatic ductal adenocarcinoma cells“

The aryl hydrocarbon receptor (AHR) is a transcription factor that plays a crucial role in sensing environmental chemicals. Additionally, AHR has been found to be upregulated in several human cancers, including pancreatic ductal adenocarcinoma (PDAC).

The aim of this bachelor's thesis was to investigate the role of AHR in PDAC cell lines. We found that in PDAC cell lines, the AHR activity modulators do not have a major influence on PD-L1 and AHR levels. *COX-2* expression, however, is under AHR control. Interestingly, we observed a profound effect of gemcitabine (GEM) on gene expression that did not correspond to the protein levels, which remained unchanged.

Keywords: aryl hydrocarbon receptor, human pancreatic ductal adenocarcinoma, gemcitabine, PD-L1, COX-2.

CERCS code: B220 Genetics, cytogenetics; B200 Cytology, oncology, carcinology.

„Arüülsüsivesinike retseptori agonistide, antagonistide ja gemtsitabiini mõju inimese pankreasejuha adenokartsinoomi rakkude geeniekspressioonile“

AHR on transkriptsioonifaktor, mis osaleb mitmete keskkonnakemikaalide lagundamisel. On täheldatud, et AHR-i ekspressioon on üles reguleeritud mitmetes inimese kasvajates, sealhulgas pankreasejuha adenokartsinoomis (PDAC).

Käesoleva bakalaureusetöö eesmärk oli uurida AHR-i funktsiooni inimese PDAC rakkudes. Eksperimentide tulemusena selgus, et PDAC rakuliinides ei mõjuta AHR-i aktiivsuse modulaatorid PD-L1 ja AHR tasemeid oluliselt. Seevastu *COX-2* ekspressioon on AHR-i kontrolli all. GEM avaldas olulist mõju geeniekspressioonile, mis ei olnud korrelatsioonis valgu tasemetega, jäädes muutumatuks.

Märksõnad: arüülsüsivesinike retseptor, inimese pankreasejuha adenokartsinoom, gemtsitabiin, PD-L1, COX-2.

CERCS kood: B220 Geneetika, tsütogeneetika; B200 Tsütoloogia, onkoloogia, kantseroloogia.

TABLE OF CONTENTS

ABBREVIATIONS 5

INTRODUCTION 6

1. LITERATURE REVIEW 7

1.1 Aryl hydrocarbon receptor 7

1.1.1. Structure of the aryl hydrocarbon receptor 7

1.1.2. Aryl hydrocarbon receptor signalling pathway 8

1.1.3. Aryl hydrocarbon receptor ligands 9

1.2 The pancreas 10

1.2.1. Anatomy of the pancreas 10

1.2.2. Human pancreatic ductal adenocarcinoma 11

1.3. Aryl hydrocarbon receptor expression in tumours 12

1.3.1. Aryl hydrocarbon receptor and cyclooxygenase-2 13

1.3.2. Aryl hydrocarbon receptor and programmed death-ligand 1 14

1.4 Gemcitabine 15

1.4.1 Gemcitabine and aryl hydrocarbon receptor 16

2. PRACTICAL WORK 17

2.1. Aims 17

2.2. Material and methods 17

2.2.1. Cultivation of human pancreatic ductal adenocarcinoma cell lines 17

2.2.2. RNA isolation 17

2.2.3. Reverse transcription 18

2.2.4. Quantitative polymerase chain reaction (qPCR) 18

2.2.5. SDS-PAGE and western blot analysis 19

2.2.5.1 Membrane stripping 21

2.2.6. Statistical analysis of data 21

2.3 RESULTS AND DISCUSSION 22

2.3.1. Impact of AHR activity modulation alone or in combination with gemcitabine on AHR in PDAC cell lines. 22

2.3.2. Impact of AHR activity modulation alone or in combination with gemcitabine on COX-2 in PDAC cell lines. 24

2.3.3. Impact of AHR activity modulation alone or in combination with gemcitabine on PD-L1 in PDAC cell lines 26

SUMMARY 29
KOKKUVÕTE 30
REFERENCES 31
NON-EXCLUSIVE LICENCE 40

ABBREVIATIONS

AHR	aryl hydrocarbon receptor
AHRR	aryl hydrocarbon receptor repressor
ARNT	aryl hydrocarbon receptor nuclear translocator
bHLH	basic helix-loop-helix
COX-2	cyclooxygenase-2
Ct	cycle threshold
GEM	gemcitabine
HAH	halogenated aromatic hydrocarbon
KRAS	Kirsten rat sarcoma viral oncogene homolog
NF-κB	nuclear Factor kappa B
NSAIDs	non-steroidal anti-inflammatory drugs
PAH	polycyclic aromatic hydrocarbon
PAS	Per-ARNT-Sim (Period/ARNT/Single-Minded)
PBS	phosphate-buffered saline
PD-1	programmed cell death protein-1
PD-L1	programmed Death Ligand-1
PDAC	pancreatic ductal adenocarcinoma
qPCR	quantitative polymerase chain reaction
RIPA	radioimmunoprecipitation assay
RR	ribonucleotide reductase
SDS-PAGE	sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SMAD4	mothers against decapentaplegic homolog 4
TBP	TATA-box binding protein
TBST	Tris-buffer with Tween
TCDD	2,3,7,8-tetrachlorodibenzo-para-dioxin
XRE	xenobiotic response element

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer and has a high mortality rate. Late diagnosis and resistance to treatments like chemotherapy and radiation are the main causes of PDAC's high mortality rate. Thus, there is a need for new treatment options.

Aryl hydrocarbon receptor (AHR) is a transcription factor first discovered as a sensor for environmental chemicals such as 2,3,7,8-tetrachlorodibenzo-para-dioxin (TCDD) and polycyclic aromatic hydrocarbons (PAHs). Later studies clarified the role of AHR in multiple other processes, such as early development and cancer progression. Up-regulation of AHR has been shown in multiple different cancers, like breast and liver cancer, as well as in PDAC.

In cancer, high expression of AHR has been linked to increased cell invasion, migration, proliferation, and metastasis. On the other hand, AHR inactivation by certain ligands can lead to cancer cell growth inhibition and apoptosis. Although it is known that AHR is highly expressed in some PDAC cell lines and high-AHR cancer patients seem to have worse survival rates, making it a potential drug target, very little is known about the role of AHR in pancreatic cancer.

Gemcitabine, a chemotherapy drug, is a first-line treatment for PDAC patients. It gets metabolised in cancer cells and then targets DNA, inhibiting replication and transcription, leading to cell death. However, not all PDAC patients respond to the treatment the same way, and drug-resistant cell types often arise. Therefore, it is important to understand the molecular mechanisms affected by gemcitabine and figure out possible co-treatments to increase the efficacy of the drug.

The main aim of this study was to evaluate the impact of AHR activity modulation on select genes in human pancreatic cancer cell lines BxPC-3 and Su.86.86. We used AHR inhibitor BAY (currently in clinical trials for cancer treatment), AHR activator TCDD, and the chemotherapy drug gemcitabine. Specifically, we were investigating the short-term effect of AHR ligands, gemcitabine, and their combination on gene expression in PDAC.

1. LITERATURE REVIEW

1.1 Aryl hydrocarbon receptor

1.1.1. Structure of the aryl hydrocarbon receptor

The human ligand-dependent transcription factor aryl hydrocarbon receptor gene is located on the seventh chromosome. It consists of 11 exons, which produce an mRNA of 6243 base pairs (Ensembl). Notably, AHR consists of 848 amino acid residues and has a molecular weight of approximately 96 kilodaltons (Uniprot). Alongside AHR are nineteen proteins from the transcription factor family, including AHR nuclear translocator (ARNT) and AHR repressor (AHRR) (NCBI).

These proteins share three domains: the basic helix-loop-helix located within the N-terminal region, the PAS domain (Per-ARNT-Sim; Period/ARNT/Single-Minded), and the less conserved TAD (transactivation domain) positioned at the C-terminal side (Zhang et al., 2002). Most bHLH/PAS proteins form homodimers or heterodimers, being active in a dimeric state (Zhang & Hogenesch, 2017).

The bHLH region of AHR contains the nuclear localization signals (NLS) and export signals (NES) required for nuclear and cytoplasmic transport (Smith et al., 2009). The basic helix-loop-helix domain is also involved in how inactive AHR interacts with chaperones, how it forms dimers with ARNT, and how it binds to DNA. There are two PAS domains in the AHR protein. PAS-A is involved in dimerization, while PAS-B is involved in ligand binding and interactions with chaperones (Figure 1) (Hankinson, 1995). The C-terminal domain consists of three subdomains: a glutamate- and aspartate-rich region of acidic amino acid residues; a glutamine-rich region; and a region rich in serine, threonine, and proline (Okey, 2007). These domains are essential for the AHR to bind to its target genes and modulate gene expression in response to activating stimuli (Mimura & Fujii-Kuriyama, 2003).

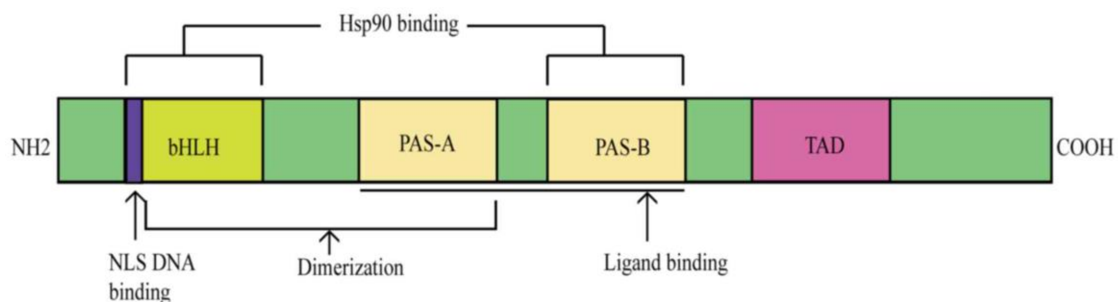

Figure 1. Structure of human AHR. (Zhu et al., 2019)

1.1.2. Aryl hydrocarbon receptor signalling pathway

The AHR signalling system controls numerous physiological processes, including cell proliferation, differentiation, and death. Within the cytoplasm, AHR is inactive and connected to a chaperone complex. This multiprotein complex includes a dimer of HSP90 (heat shock protein 90), a co-chaperone known as p23, AHR interacting protein (AIP), and the c-SRC (proto-oncogene tyrosine-protein kinase) (Bolton et al., 2019). Upon ligand binding, such as dioxins, polycyclic aromatic hydrocarbons (PAHs), and tryptophan metabolites, AHR translocates to the nucleus, where it binds to xenobiotic responsive elements (XRE) located in the regulatory region of its target genes and induces their expression. This complex activates the transcription of downstream target genes (Murray, Patterson, & Perdew, 2014).

AHR targets include cytochrome P450 enzyme genes, e.g. *CYP1A1*, *CYP1A2*, and *CYP1B1*, which are expressed due to the binding of TCDD; phase II detoxification enzymes, such as NAD(P)H, quinone oxidoreductase 1 (NQO1), and glutathione S-transferases (GSTs); as well as pro-inflammatory mediators, including interleukin-1β (IL-1β), interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α), and cyclooxygenase-2 (COX-2) (Lee et al., 2017). Following its dissociation from the XRE sequence, the aryl hydrocarbon receptor is translocated from the nucleus to the cytoplasm by the nuclear export signal. Upon reaching the cytoplasm, the AHR protein undergoes degradation by a proteasome (Figure 2) (Beischlag, Morales, Hollingshead, & Perdew, 2008). While NF-κB inhibitors do not function as AHR antagonists, they have the ability to block AHR-mediated signalling through the reduction of nuclear factor kappa-light-chain-enhancer activity in activated B cells, thereby indirectly blocking AHR-mediated signalling (Chen et al., 2019). Dysregulation of the AHR signalling pathway has been linked to several diseases, including cancer, immune dysfunction, and developmental disorders (Kerkvliet, 2009).

One of the target genes of AHR is *AHRR*. AHR-ARNT dimer binding to the *AHRR* promoter induces its expression. AHRR can compete with AHR by binding to ARNT, which results in the export of the monomeric AHR out of the nucleus and its degradation (Beischlag, Morales, Hollingshead, & Perdew, 2008). The transcriptionally inactive AHRR-ARNT dimer binds to XRE sequences, thereby silencing AHR-induced gene expression (Ohtake et al., 2007).

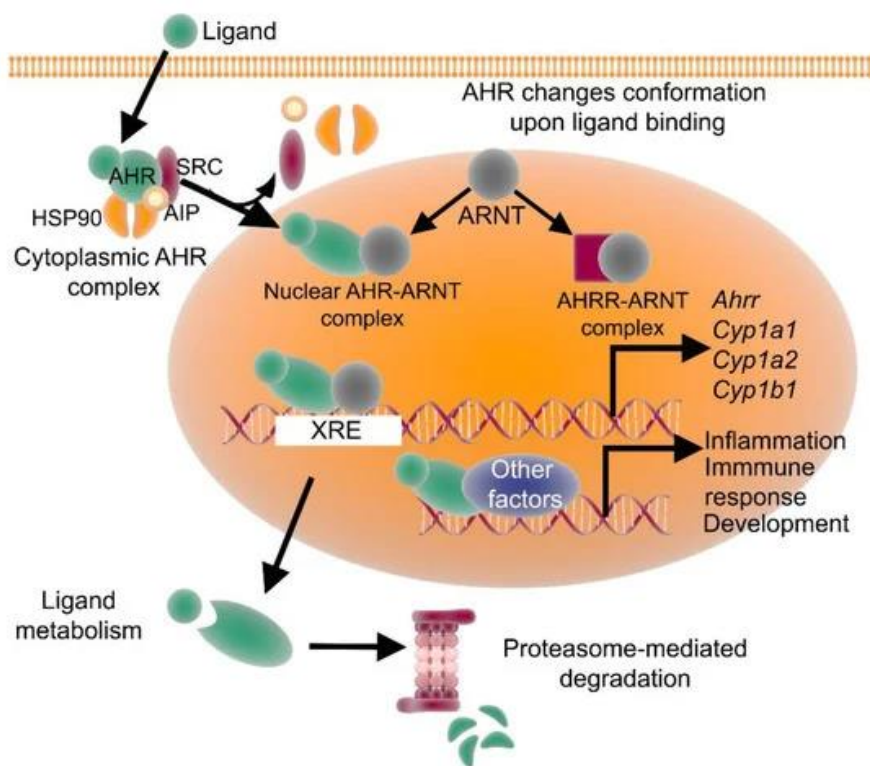

Figure 2. Aryl hydrocarbon receptor signalling pathway. (Choudhary & Malek, 2020)

1.1.3. Aryl hydrocarbon receptor ligands

AHR ligands, which belong to a heterogeneous class of compounds, have the ability to bind to and trigger activation of the aryl hydrocarbon receptor. The target genes expressed can depend on the ligand. The ligands show a variety of physiological effects on the organism, including the modulation of gene expression involved in metabolic processes, the immune response, and the development of cells (Denison & Nagy, 2003). Polycyclic Aromatic Hydrocarbons (PAHs) are among the frequently encountered classes of AHR ligands that occur due to the incomplete combustion of organic matter such as fossil fuels and tobacco. Benzo[a]pyrene, dibenzo[a,h]anthracene, and indeno[1,2,3-cd]pyrene are among the PAHs that function as AHR ligands (Cavallo et al., 2019). Halogenated Aromatic Hydrocarbons (HAHs) are a class of chemical compounds characterised by the presence of halogen atoms, such as chlorine or fluorine, and aromatic rings. Examples of HAHs that act as AHR ligands include 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and polychlorinated biphenyls (PCBs) (Teino et al., 2020). Some pharmaceutical drugs have been found to activate AHR. For example, the antimalarial drug chloroquine and its derivative, hydroxychloroquine, have been identified as AHR ligands. Similarly, certain selective serotonin reuptake inhibitors (SSRIs), used as

antidepressants, have been shown to interact with AHR (Giesy & Kannan, 2001). Various chemicals used in industrial and agricultural applications can act as AHR ligands. Examples include certain herbicides, such as 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and pesticides, such as the pesticide dichlorodiphenyltrichloroethane (DDT). These compounds can enter the environment through their use and may have implications for human and environmental health (Denison & Nagy, 2003). In addition, several naturally occurring chemicals, such as flavonoids, coumarins, and indoles, have been identified as ligands for the aryl hydrocarbon receptor. These include tryptophan, which can be metabolised into the AHR ligand kynurenine (KYN), resveratrol, and curcumin (La Merrill, Birnbaum, & Pompili, 2013).
Multiple aryl hydrocarbon receptor antagonists have been identified, such as GNF35 and CH223191, that are potent and selective AHR antagonists (Cummings et al., 2017). They bind to the AHR with high affinity and inhibit AHR-mediated signalling (Kozarich, Arlow, & Tropsha, 2010). BAY 2416964, an antagonist of the aryl hydrocarbon receptor, has been developed as a prospective medical treatment for the management of AHR-associated ailments such as cancer and inflammatory disorders. Preclinical studies conducted on animals have demonstrated that BAY 2416964 effectively restricts the AHR signalling pathway and decreases the progression of tumours associated with AHR (Han et al., 2020). The fact that BAY 2416964 has shown anti-inflammatory properties in vitro and in vivo suggests it could help treat immune-mediated diseases. BAY 2416964 shows beneficial pharmacological characteristics and significant therapeutic potential. However, further research is needed to fully understand the pharmacological properties and therapeutic potential of BAY 2416964 (Lin et al., 2022).

1.2 The pancreas

1.2.1. Anatomy of the pancreas

The pancreas is a glandular organ positioned in the abdominal region, posterior to the stomach (Figure 3). It has a length of approximately 15 centimetres and a flattened, elongated morphology. The pancreas is anatomically divided into discrete regions known as the "head," "body," and "tail" (Netter, 2014). The largest segment of the pancreas, the head, is situated in the right region of the abdominal cavity. The pancreatic duct acts as a conduit linking the pancreas to the duodenum, which is the first part of the small intestine (Standring, Borley, & Collins, 2016). The pancreatic body is the intermediary segment situated posterior to the stomach. The pancreas extends the abdominal region in a horizontal orientation, with its

narrowest segment located on the left side at the tail end. The spleen, an abdominal organ, is located left of the tail (Snell, 2012).

The pancreas consists of two distinct cellular populations: exocrine and endocrine cells. The former are responsible for the secretion of digestive enzymes, like amylase, lipase, and proteases, into the small intestine through the primary pancreatic duct. On the other hand, the endocrine cells release hormones such as insulin and glucagon in order to regulate the levels of glucose in the bloodstream (Alkaade & Vareedayah, 2017). The Islets of Langerhans are discrete cellular aggregates that represent the endocrine glands of the pancreas. The Langerhans islets comprise a variety of cell types, including but not limited to alpha, beta, delta, and pancreatic polypeptide cells (Kaur & Kaushal, 2019). Beta cells regulate blood glucose levels via insulin synthesis, while alpha cells increase blood glucose levels through glucagon synthesis (Junqueira, Carneiro, & Kelley, 2003).

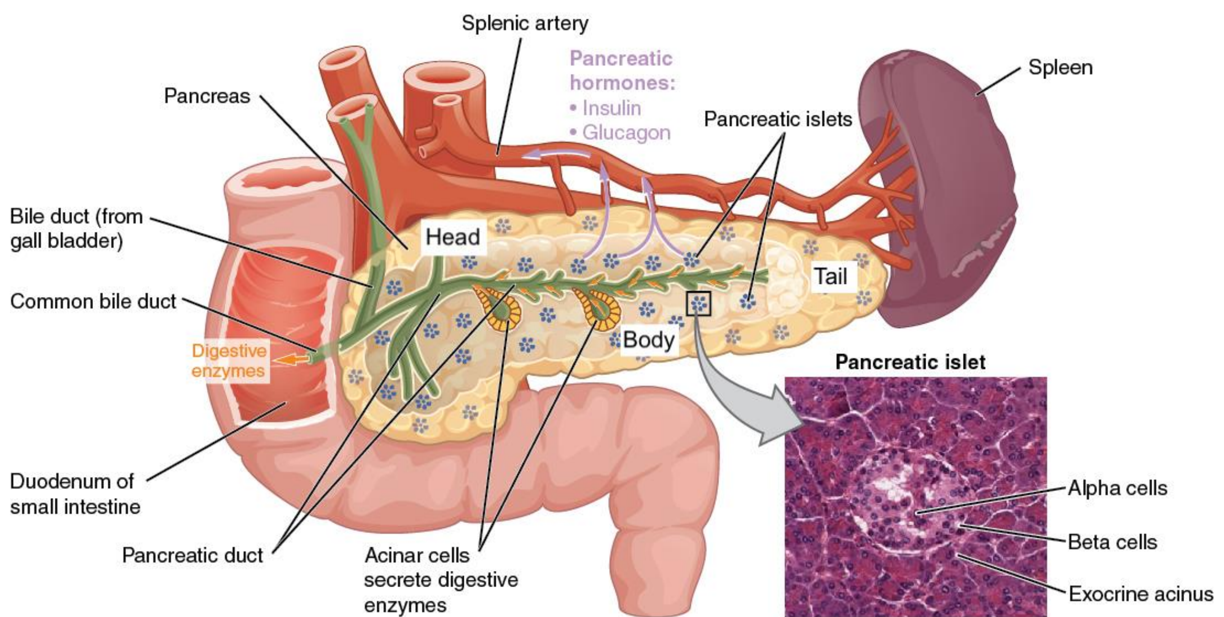

Figure 3. Anatomy of the pancreas Adapted from Pancreatic Parameters. Lecturio. Retrieved May 8, 2023, from https://www.lecturio.com/concepts/pancreatic-parameters/

1.2.2. Human pancreatic ductal adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) is a type of cancer that arises from the cells lining the ducts of the pancreas. It is the most common type of pancreatic cancer, accounting for about 90% of cases. PDAC is an aggressive and lethal cancer due to the absence of specific symptoms in the early stages of the disease. It is often diagnosed late and thus has a low 5-year survival rate (Kamisawa et al., 2016). PDAC typically arises from precursor lesions known as pancreatic

intraepithelial neoplasia that develop in the ducts of the pancreas. These precursors can develop into an aggressive, organ-spreading malignancy. Smoking, obesity, chronic pancreatitis, and family history are some risk factors associated with pancreatic cancer (Furukawa, Klöppel, & Adsay, 2012). PDAC is characterised by a variety of genetic mutations in several oncogenes and tumour suppressors. Among these, the most frequently observed mutations are in *KRAS* (Kirsten rat sarcoma viral oncogene homolog) and *HER2* (human epidermal growth factor receptor 2) oncogenes and the tumour suppressors p16, p53, and SMAD4 (mothers against decapentaplegic homolog 4). The *KRAS* oncogene mutation, occurring in 70-90% of PDAC cases, results in a constitutively active KRAS protein, which in turn activates several signalling pathways and transcription factors. This ultimately enhances cell proliferation, invasiveness, and transformation frequency (Bailey et al., 2016). The SMAD4 protein is a tumour suppressor frequently inactivated in PDAC tumours. Upon activation by TGF-β (transforming growth factor beta) ligands, SMAD4 inhibits cell growth and promotes apoptosis. However, inactive SMAD4 lacks any tumour-suppressive effect. Similarly, p53 is a tumour suppressor activated by strong genotoxic stress, leading to cell cycle arrest and apoptosis. In pancreatic cancer, p53 is mutated in 75% of cases (Wolfgang et al., 2013).

PDAC can cause a range of symptoms, including abdominal pain, weight loss, jaundice, and digestive problems. The diagnosis is typically made using imaging techniques such as computed tomography (CT) or magnetic resonance imaging (MRI) and confirmed by biopsy (Chun et al., 2015). Treatment for PDAC usually involves surgery, followed by chemotherapy and/or radiation therapy. Due to the aggressive nature of the disease, many patients are not eligible for surgery, and treatment options are limited. Early detection and treatment are being researched (Saif & Kim, 2014).

1.3. Aryl hydrocarbon receptor expression in tumours

The expression of aryl hydrocarbon receptor in tumours depends on the type and stage of cancer. In some instances, an increase in AHR expression has been linked to tumour progression as well as a negative prognosis; however, alternative studies show a diminishment or absence of AHR. (Fader et al., 2014; Hollingshead et al., 2008)

Upregulation of AHR expression can be evident in pancreatic ductal adenocarcinoma. Tumour cells exhibited an increased level of stemness, marked by the expression of pluripotency factors, leading to greater resistance to chemotherapy treatment. Similarly, AHR expression has also been found to be upregulated in breast cancer, colorectal cancer, and hepatocellular

carcinoma, where it has been linked to tumour progression and a poor prognosis (Vacher et al., 2018; Singh et al., 2017; Liu et al., 2013). The level of AHR expression can differ significantly depending on the type of cancer. Some cancers show limited or no expressions, such as non-small cell lung, where insufficient expressions are strongly linked to worse patient outcomes (Guan et al., 2021). A similar reduction in AHR levels has been observed in clear cell renal tumours, with proposals suggesting that upregulation of AHR can limit tumour growth (Tanaka et al., 2016).

1.3.1. Aryl hydrocarbon receptor and cyclooxygenase-2

One of the ways the immune system is regulated is through prostaglandins – hormone-like substances metabolised from arachidonic acid by cyclooxygenase enzymes. Cyclooxygenase-1 (COX-1) plays a role in maintaining normal physiological functions, such as protecting the gastrointestinal mucosa, regulating renal blood flow, and promoting platelet aggregation (Ferrara, 2004). Cyclooxygenase-2 (COX-2) is not normally expressed under basal conditions but is upregulated in response to inflammatory stimuli. It is primarily involved in the production of prostaglandins during inflammation and immune responses. To reduce inflammation or alleviate discomfort by preventing prostaglandin metabolism, COX-2 inhibitors are used (Lawrence et al., 2002).

NSAIDs (non-steroidal anti-inflammatory drugs) like aspirin and ibuprofen are another group of drugs that nonselectively inhibit both COX1 and COX-2 for pain relief but are known to often cause gastrointestinal side effects (Vane, 1971). In comparison, selective COX-2 inhibitors are preferred due to their lesser tendency towards side effects. Although effective at first, taking these drugs for extended periods has been associated with an increased risk of cardiovascular diseases like stroke or heart attack (Lawrence et al., 2002).

Chronic inflammation is a crucial factor in the development and progression of several types of cancer. Recent research shows a correlation between high levels of COX-2 and tumour development in various regions, such as colon cancer (Deng et al., 2020). According to recent preclinical research, COX-2 inhibitors demonstrate promise in reducing tumour development rates while enhancing cancer treatment effectiveness (Patrono & Baigent, 2019). One of the ways that COX-2 might affect tumour development and growth is by promoting angiogenesis through vascular endothelial growth factor (VEGF) (Ferrara, 2004). Angiogenesis is essential for tumour growth, as this process allows new blood vessels to form and supply cancer cells with nutrients and oxygen, making it a target for cancer treatments (Koki et al., 2002).

Furthermore, combining drugs like paclitaxel or 5-fluorouracil with COX-2 inhibitors can improve chemotherapy outcomes in select cancer types, such as breast or colon cancer. Additionally, studies have shown that human lung epithelial cells, as well as liver cells, exhibit elevated levels of COX-2 expression when exposed to AHR ligands (Vázquez-Gómez et al., 2022). Numerous hypotheses have been proposed regarding COX-2 relation to AHR. One theory suggests that AHR stimulation prompts human lung epithelial cells exposed to PAHs to produce more AHR and, thus, COX-2 by activating the transcription factor NF-kB (Vogel et al., 2020). Another study has found that activation of the MAPK (mitogen-activated protein kinase) pathway is responsible for triggering COX-2 production in the lung and gastrointestinal tract (Pizzorno & Bittoni, 2009).
The association between AHR and COX-2 has been shown in previous studies. However, much is still unknown regarding their collaborative role in tumorigenesis. Therefore, further research is needed to clarify their cooperation and the underlying molecular mechanisms in tumours.

1.3.2. Aryl hydrocarbon receptor and programmed death-ligand 1

PD-L1 (Programmed Death Ligand-1) is a membrane protein expressed in immune system cells such as NK and B cells as well as other cell types like endothelial cells. PD-L1 functions as a checkpoint within the immune system, regulating the immune response and blocking the immune system from targeting healthy cells (Herbst et al., 2014). PD-L1 interacts with a protein called PD-1 (Programmed Death-1) that is expressed on the surface of T cells. When PD-L1 binds to the PD-1 receptor, the T cell becomes inactive, preventing the immune system from attacking normal cells. However, cancer cells also exploit this mechanism. Cancer cells often express high levels of PD-L1, thereby suppressing T-cells, evading immune surveillance, and creating favourable conditions for tumour growth (Taube et al., 2014).
Inhibitors of PD-1 and PD-L1 are a class of anticancer drugs that inhibit the activity of the immune checkpoint proteins present on the surface of cancer cells. Although anti-PD-1 antibodies prevent PD-1 from interacting with both PD-L1 and PD-L2, the anti-PD-L1 antibodies atezolizumab, durvalumab, avelumab, and BMS-936559 selectively inhibit the PD-1/PD-L1 interaction. There is increasing evidence that PD-L1 inhibitors are providing a breakthrough in the treatment of multiple types of cancer, such as melanoma, non-small cell lung cancer, and bladder cancer, by aiding the immune system in targeting malignant cells (Keir et al., 2006). Recent studies have shown that activation of aryl hydrocarbon receptor signalling amplifies PD-L1 expression in tumour tissues, allowing malignant cells to evade

recognition from the host's immune system. Similarly to melanoma, lung cancer cells have been shown to exhibit higher PD-L1 levels upon AHR activation. The use of AHR inhibitors, in turn, has efficiently reduced PD-L1 levels and enhanced the efficacy of immune checkpoint inhibitors (Ganesan et al., 2018). Furthermore, AHR signalling can influence the expression of various immune checkpoint molecules. Notably, it has been demonstrated that activating AHR leads to increased levels of CTLA4 (cytotoxic T lymphocyte-associated protein 4), another important checkpoint molecule known to limit T-cell activity (Holmgaard et al., 2013).

1.4 Gemcitabine

Gemcitabine is a chemotherapy drug used to treat various types of cancer since its initial approval by the U.S. Food and Drug Administration (FDA) in 1996. (Burris III et al., 1997)
This pharmaceutical agent interferes with DNA synthesis, preventing malignant cell replication in patients with pancreatic, ovarian, lung, and breast cancer. Treatment is typically administered through intravenous infusion, with dosage and frequency determined by the type and stage of cancer. A combination of gemcitabine and other chemotherapy agents, such as paclitaxel, can increase its efficacy; however, side effects, such as hair loss or fatigue, may occur (von Hoff et al., 2011). Although infections and changes in blood cell count represent some adverse effects associated with using this drug, gemcitabine is an essential component in cancer treatment (Tempero et al., 2019).

Gemcitabine primarily functions by halting cellular processes like DNA synthesis or cell division, resulting in reduced growth and proliferation rates for tumours. Gemcitabine, in other words, dFdC (2',2'-difluorodeoxycytidine) is a deoxycytidine nucleoside analogue in which two fluorine atoms have been inserted into the deoxyribose ring. It is incorporated into the DNA of cancer cells during replication. This action leads to cell cycle arrest, causing the eventual death of these cells (von Hoff et al., 2011). Gemcitabine inhibits ribonucleotide reductase (RR), an enzyme used in producing dNTP molecules crucial in both replication and repairing the genetic material within cells. Inside the cell, gemcitabine is phosphorylated to gemcitabine monophosphate (dFdCMP) by deoxycytidine kinase (dCK), which is then converted to gemcitabine di- and triphosphate (dFdCDP and dFdCTP, respectively) (Tempero et al., 2019). Decreased levels of RR activity lower dNTP levels, thereby enhancing the incorporation of gemcitabine triphosphate instead. This process prevents polymerases from binding to the DNA. Damaged and unrepaired DNA eventually causes cell death through apoptosis (Stubbe & van der Donk, 2018).

Results from numerous clinical trials show that gemcitabine is highly effective, particularly when taken in conjunction with other chemotherapy medications like cisplatin. The National Cancer Institute's trial found that patients who received gemcitabine experienced better quality of life and longer survival rates than those who did not take it or were given a placebo. Additionally, the European Study Group for Pancreatic Cancer discovered that combining gemcitabine and cisplatin led to even more significant improvements (Tempero et al., 2019). Interestingly, a recent publication indicated that cisplatin could reduce AHR signalling by downregulating its protein levels (Sasaki-Kudoh et al., 2018).

1.4.1 Gemcitabine and aryl hydrocarbon receptor

The integration of gemcitabine into the DNA molecule and the suppression of DNA synthesis have both been shown to cause DNA damage, resulting in single-stranded and double-stranded breaks in the DNA (Mini et al., 2006). One of the systems involved in the repair of these DNA damages, which is critical for cell survival, is the nucleotide excision repair (NER) pathway. AHR signalling has been identified to influence NER pathway enzymes such as XPC (xeroderma pigmentosum group C protein) and XPD (xeroderma pigmentosum group D protein), which are involved in the identification and removal of DNA lesions (Esser et al., 2009).

According to the findings, gemcitabine boosted AHR expression in pancreatic cancer cells. Furthermore, activation of AHR was associated with the development of resistance to gemcitabine-induced cell death. Using a specific inhibitor or RNA interference to reduce AHR, on the other hand, improved the sensitivity of pancreatic cancer cells to gemcitabine treatment. These findings suggest that targeting AHR may have therapeutic effects in enhancing the efficacy of gemcitabine in the treatment of pancreatic cancer (Cheng et al.,2018). Recent research has revealed that activating the aryl hydrocarbon receptor with its endogenous ligand, 6-formylindolo[3,2-b]carbazole (FICZ), reduces the susceptibility of pancreatic ductal adenocarcinoma cells to gemcitabine. The study's findings suggest that AHR activation leads to pancreatic ductal adenocarcinoma resistance to gemcitabine. The study also implies that reducing AHR activity could be a potential strategy for increasing gemcitabine's therapeutic effectiveness in the treatment of PDAC (Huang et al., 2019).

More research is needed to thoroughly understand the interplay of gemcitabine and AHR signalling as well as its impact on DNA repair pathways in cancer cells, as these findings could have clinical significance for tumour growth inhibition and, thus, cancer patients.

2. PRACTICAL WORK

2.1. Aims

1. To describe the expression of *AHR*, *COX-2,* and *PD-L1* in PDAC cell lines following treatment with AHR activity modulators alone or in combination with GEM.
2. To analyse the protein levels of AHR, COX-2 and PD-L1 in PDAC cell lines after treatment with AHR activity modulators alone or in combination with GEM.

2.2. Material and methods

2.2.1. Cultivation of human pancreatic ductal adenocarcinoma cell lines

For this research, human pancreatic ductal adenocarcinoma (PDAC) cell lines SU.86.86 and BxPC-3 were obtained from collaborative partners at the Lithuanian University of Health Sciences. The cells were cultured in RPMI 1640 medium (Corning Inc.), supplemented with 10% foetal bovine serum (Capricorn Scientific) and 1% penicillin-streptomycin (Corning Inc.), in 100 mm dishes and 6-well plates (Greiner Bio-One), and maintained in a humidified incubator at 37 °C and 5% CO_2. The cell culture medium was changed every 2-3 days, and the cells were subcultured every 3-6 days using trypsin (Corning Inc.).

Cells were seeded on 6-well plates. After 24 hours, compounds or their combinations were added to the cells in 2 ml of cell culture media. Cells were lysed for RNA extraction or western blot 24 hours later.

Chemicals used: 10 nM 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) (Cambridge Isotope Laboratories), 10 µM BAY 2416964 (BAY) (MedChemExpress), gemcitabine (Acros Organics). Dilutions of the chemicals were made in dimethyl sulfoxide (Sigma-Aldrich). Gemcitabine (GEM) was used at concentrations of 36.96 nM and 26.67 nM for SU.86.86 and BxPC-3, respectively.

2.2.2. RNA isolation

For RNA isolation, the cells were first washed with phosphate-buffered saline (PBS, Corning Inc.), followed by the addition of FARB lysis solution (Favorgen Biotech Corporation) containing 1:100 beta-mercaptoethanol (Alpha Medthrift Scientific Co.). The cells were mechanically scraped off and transferred into microtubes for RNA extraction. The lysates were stored at -80 °C until further use. RNA purification was performed using the Tissue Total RNA

Purification Mini kit (Favorgen Biotech Corporation) in accordance with the manufacturer's instructions. The purified RNA was eluted in nuclease-free water to a final volume of 30 µl. The concentration and quality of RNA were assessed using a NanoDrop ND-100 spectrophotometer (Thermo Fisher Scientific).

2.2.3. Reverse transcription

For reverse transcription, the RevertAid RT kit (Thermo Fisher Scientific) was used. Prior to reverse transcription reaction, DNase treatment was conducted in a final volume of 10 µl containing 1000 ng RNA, 1x reaction buffer, 1 International Unit (IU) of DNase, and 10 IU of RiboLock, followed by incubation for 30 minutes at 37 °C. The DNase was then inactivated by adding 4.5 mM EDTA and incubating the samples at 65 °C for 10 minutes. Reverse transcription reaction was carried out in a final volume of 20 µl containing 1x reaction buffer, 20 IU RiboLock, 1 mM dNTPs, 5 µM Random Hexamer primer, and 200 IU revertase. The reverse transcription was conducted using a 2720 Thermal Cycler (Applied Biosystems) machine. The reverse transcription programme is given in Table 1.

Table 1. Reverse transcription programme

	Temperature (°C)	Time (min)
Activation	25	10
cDNA synthesis	42	60
Inactivation	70	10

2.2.4. Quantitative polymerase chain reaction (qPCR)

Gene expression analysis was performed using quantitative polymerase chain reaction (qPCR) on a 384-well plate (Bioplastics) with a LightCycler® 480 II machine (Roche). The reaction mix contained 500 nM forward and reverse primers, 5 µl SYBR™ Green/ROX qPCR Master Mix (Thermo Fisher Scientific), and 4 µl cDNA in a final volume of 10 µl. Negative controls were included with water instead of cDNA. The reactions were performed in triplicates, and the mean values were used for analysis. Cycle threshold (Ct) values were calculated using the LightCycler 480 II programme, and the results were normalised to the TATA-binding protein (TBP) housekeeping gene. The correctness of the reaction products was verified by melting

curve analysis. The Livak method (Livak and Scmittgen, 2001) was used to calculate relative gene expression results. The programme and primer sequences are detailed in Table 2 and Table 3, respectively.

Table 2. qPCR programme

	Temperature (°C)	**Time (min)**	**Cycles**
Primary denaturation	95	10	1
Denaturation	95	15 sec	40
Binding of primers and elongation	60	1	
Melting curve formation	45-95	7	1

Table 3. Oligonucleotides used in qPCR

Primer (F – forward / R – reverse)	Nucleotide sequence
*AHR*_mRNA_F	5'-ATTACAGGCTCTGAATGGCTTTG-3'
*AHR*_mRNA_R	5'-TGACATCAGACTGCTGAAACCCTAG-3'
*COX-2*_mRNA_F	5'-AGGGTTGCTGGTGGTAGGAA-3'
*COX-2*_mRNA_R	5'-GGTCAATGGAAGCCTGTGATACT-3'
*PD-L1*_mRNA_F	5'-TATGGTGGTGCCGACTACAA-3'
*PD-L1*_mRNA_R	5'-TGGCTCCCAGAATTACCAAG-3'
*TBP*_mRNA_F	5'-TGCACAGGAGCCAAGAGTGAA-3'
*TBP*_mRNA_R	5'-CACATCACAGCTCCCCACCA-3'

2.2.5. SDS-PAGE and western blot analysis

To lyse the cells for western blot, the radioimmunoprecipitation assay (RIPA) buffer was used, which included 50 mM Tris pH 7.4, 1% Triton X-100, 1 mM EDTA, 150 mM NaCl, and 0.1% SDS with 1x protease inhibitor (Roche). Cells were scraped from the dish in PBS, centrifuged for 5 minutes at 300 rcf, and resuspended in 50 μl of RIPA buffer. The cell lysis was performed

on ice for 1 hour with periodic vigorous vortex. Next, the lysate was centrifuged for 10 minutes at 4 °C and 18,000 rcf, and the supernatant was stored at -80 °C.
The protein concentration of the lysate was determined using a Pierce™ BCA protein measurement kit (Thermo Fisher Scientific) and a Multiskan Ascent apparatus (Thermo Fisher Scientific).
For SDS-polyacrylamide gel electrophoresis, 20 μl of the protein sample was mixed with 4 μl of 6x loading buffer and 100 mM dithiothreitol and heated for 5 minutes at 95 °C. Then, 25 μl of the sample was applied to a 10% SDS-polyacrylamide gel, and proteins were separated using a Mini-PROTEAN® Tetra Cell machine (Bio-Rad) for 45 minutes at 50 volts followed by 1 hour at 170 volts. After separation, the proteins were transferred onto a methanol-activated polyvinylidene difluoride (PVDF) membrane using a Trans-Blot® SD Semi-dry Transfer Cell machine (Bio-Rad). The transfer took place in 20 minutes at 15 volts. Non-specific signals were blocked in TBST buffer (Tris-buffer, Tween) containing 5% skimmed milk powder for 1 hour. The membrane was then incubated overnight with primary antibodies in blocking buffer, followed by 3 x 6 minutes and 1 x 9 minutes washes in TBST solution, and 1 hour of incubation with secondary antibodies. After repeated washes, the Immobilon Western Chemiluminescent HRP substrate mix (Merck Millipore) was applied to the membrane and incubated for 5 minutes. Finally, protein visualisation was carried out using a ChemiDoc XRS+ system (BioRad).

Primary antibodies used:

1. Rabbit polyclonal antibody against AHR, 28727-1-AP, 1:10 000 (Proteintech®)
2. Mouse polyclonal antibody against PTGS2 (COX-2), LS-C339544, 1:5000 (LS Bio)
3. Rabbit monoclonal antibody against PD-L1, EPR19759, 1:1000 (Abcam)
4. Mouse monoclonal antibody C4 against β-Actin, sc-47778, 1:10 000 (Santa Cruz Biotechnology)

Secondary antibodies used:

1. Goat antibody against mouse conjugated with horseradish peroxidase, sc-2005, 1:5000 (Santa Cruz Biotechnology)
2. Mouse antibody against rabbit conjugated with horseradish peroxidase, sc-2357, 1:5000 (Santa Cruz Biotechnology)

2.2.5.1 Membrane stripping

In order to remove antibodies from the western blot, ReBlot Plus 10X Mild Antibody Stripping Solution (MilliporeSigma™) was used. The solution was prepared by diluting it to a 1x concentration, after which the blot was incubated in the solution for 25 minutes. Next, the blots were washed in TBST for 5 minutes, followed by primary antibody incubation overnight, as described above.

2.2.6. Statistical analysis of data

In this study, the experimental procedures were carried out with meticulous attention to detail, ensuring the robustness and reliability of the results. All experiments were performed in triplicate to ensure reproducibility. To present the qPCR results, mean values were calculated, and the standard error of the mean (SEM) values were added as error bars. Statistical analysis was carried out using MS Excel. Student's t-test was used to assess the statistically significant difference between treatment groups. The predetermined level of statistical significance was set at $p < 0.05$. Bar graphs were drawn in MS Excel (version 16.0.13929.20206) for the visual representation of the results. For densitometry analysis, the ImageJ programme (version 1.8.0_172) was utilised. These methods and tools were chosen for their accuracy, precision, and established use in the field of research.

2.3 RESULTS AND DISCUSSION

2.3.1. Impact of AHR activity modulation alone or in combination with gemcitabine on AHR in PDAC cell lines.

One of the aims of this bachelor's thesis was to investigate the role of AHR in pancreatic ductal adenocarcinoma cell lines. Previous research has established that healthy pancreatic tissue typically exhibits a low level of AHR (Human Protein Atlas). Conversely, increased AHR expression has been observed in several PDAC cell lines, suggesting a potential association with pancreatic cancer development and progression (Jin et al., 2015; Koliopanos et al., 2002; Ogura et al., 2017).

For this thesis, cell lines SU.86.86 and BxPC-3 were chosen due to their relatively high AHR levels as determined previously in our lab (Konsa, 2021). To understand the role and function of AHR in these cell lines, AHR was activated and inactivated using agonists and antagonists, respectively. In addition, it was our goal to determine the impact of AHR activity modulator cotreatment with the PDAC chemotherapeutic gemcitabine on the expression of select genes.

First, we examined the effect of various chemical combinations on *AHR* expression. By RT-qPCR analysis, we found that in the SU.86.86 cell line, *AHR* mRNA levels are significantly reduced by 17% when treated with TCDD (Figure 4 A). Other treatments, however, did not influence *AHR* expression. Although there is not much published data about TCDD downregulating *AHR* expression, experiments performed previously in our lab have shown some minor downregulation of *AHR* in certain cell lines. In the BxPC-3 cell line, AHR activity modulators alone had no effect on its expression. GEM treatment alone and in combination with BAY and TCDD resulted in a significant 3.7-fold upregulation of *AHR* mRNA (Figure 4 B). This was somewhat unexpected, as the literature lacks sufficient data about different chemotherapeutic compounds regulating *AHR*. Considering the significance of AHR and its activity for cancer cells, this was alarming. On one hand, GEM kills cancer cells, but on the other, the upregulation of *AHR* could cause the remaining cancer cells to avoid cell death and hypothetically also contribute to their resistance to the treatment.

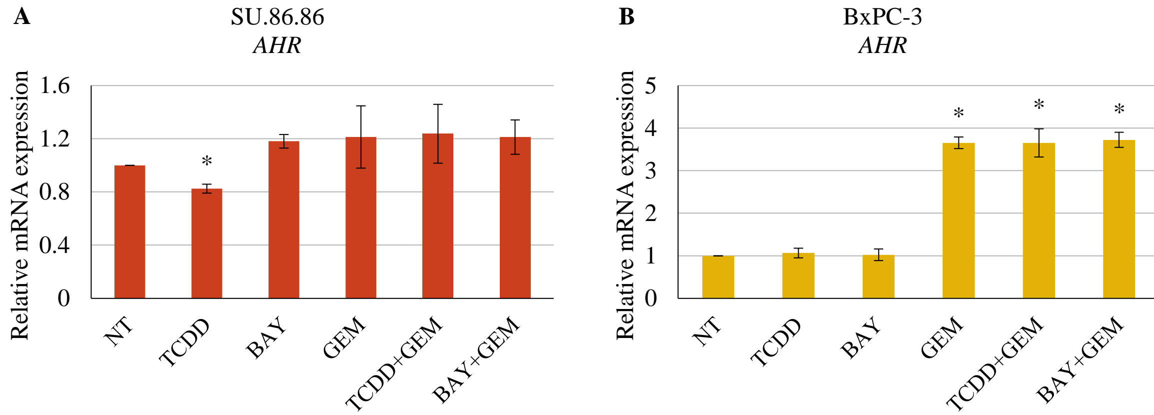

Figure 4. *AHR* mRNA expression in PDAC cell lines. SU.86.86 (A) and BxPC-3 (B) cell lines were treated with TCDD (10nM), BAY (10µM) and GEM (36.96 nM for SU.86.86 and 26.67 nM for BxPC-3), or their combinations. *AHR* mRNA levels were detected by RT-qPCR. Data are presented as fold change relative to non-treated (NT); average of n = 3 +/- SEM, * p < 0.05.

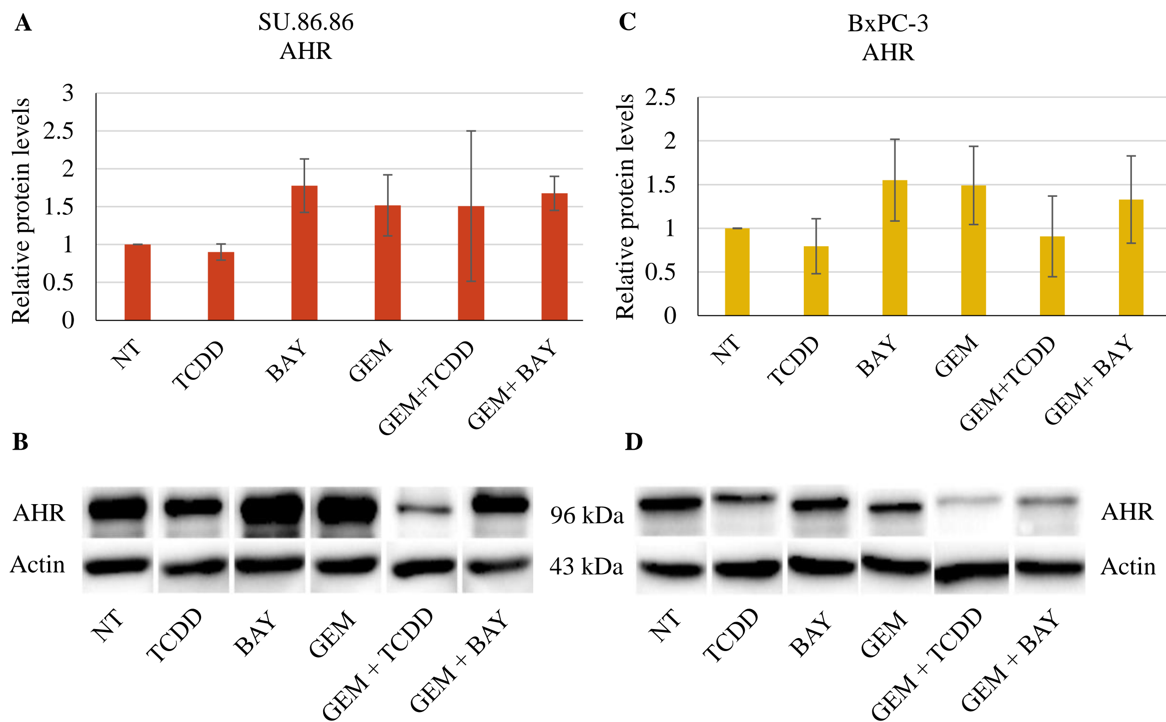

Figure 5. AHR protein levels in PDAC cell lines. SU.86.86 (A, B) and BxPC-3 (C, D) cell lines were treated with TCDD (10nM), BAY (10µM) and GEM (36.96 nM for SU.86.86 and 26.67 nM for BxPC-3), or their combinations. AHR protein levels were detected by western blot analysis. Densitometry analysis data is presented as fold change relative to non-treated (NT); average of n = 3 +/- SEM, * p < 0.05.

It is well established that changes in mRNA levels do not have to recapitulate in protein levels. There are several mechanisms that can influence the differential dynamics of mRNA and

protein. For example, microRNAs can potently regulate mRNA stability or translation (Bartel, 2009). Accordingly, there is data about miRNAs regulating AHR itself (Liu et al., 2018). Considering this, our next aim was to determine the protein levels of AHR following different treatments. Western blot analysis with an AHR-specific analysis revealed that no treatment significantly affected AHR protein levels in the SU.86.86 cell line (Figure 5 A, B). Similar results were observed in the BxPC-3 cell line (Figure 5 C, D). Thus, it appears that although GEM can increase *AHR* mRNA expression, protein levels remain unchanged. Although the underlying mechanisms remained unclear, it is reasonable to suggest that the translation of *AHR* transcripts may have been the rate-limiting step to avoid AHR protein increase.

2.3.2. Impact of AHR activity modulation alone or in combination with gemcitabine on COX-2 in PDAC cell lines.

Studies have shown that activation of AHR by its ligands, such as dioxins, can induce *COX-2* expression in various cell types (Pizzorno & Bittoni, 2009; Weiss et al., 1990). This suggests that AHR signalling can influence the inflammatory response mediated by COX-2.

By using RT-qPCR analysis, we found that TCDD upregulates *COX-2* expression 3-fold both in SU.86.86 and BxPC-3 (Figure 6 A, B), while BAY downregulates *COX-2* expression by 80% and by 43% in SU.86.86 and BxPC-3, respectively. TCDD-induced *COX-2* upregulation may involve multiple signalling pathways, such as NF-κB and MAPKs, which are involved in inflammatory responses. In addition, AHR may regulate *COX-2* expression by direct interaction with its promoter, as has been shown previously (Degner et al., 2009). And as AHR inhibitor BAY leads to a downregulation of *COX-2* mRNA expression levels, it seems likely that COX-2 is directly regulated by AHR in these cell lines.

Compared to non-treated cells, GEM treatment resulted in a 3.4-fold, although statistically insignificant, upregulation of *COX-2* in the SU.86.86 cell line (Figure 6 A). TCDD addition to the GEM treatment caused a 6.8-fold induction, whereas BAY addition rendered its expression to levels comparable with non-treated cells.

Treatment of BxPC-3 cells with GEM resulted in a 147-fold increase in *COX-2* expression (Figure 6 B). The addition of TCDD did not result in a further significant increase, indicating that *COX-2* expression may be at its maximum levels. Importantly, BAY significantly reduced GEM-induced *COX-2* expression by 67%. Nevertheless, it remained 48-fold higher than in the non-treated cells. This indicates that although AHR regulates *COX-2* expression, there are also other mechanisms that are capable of significantly modulating *COX-2* mRNA levels. Another

possibility is that AHR is not completely inactivated and still participates, to some extent, in the GEM-induced *COX-2* transcription. Furthermore, it is reasonable to suggest that this phenomenon is the result of GEM alone, as the COX-2 levels, treated with BAY, are significantly lower compared to treatment with GEM alone. It is thus within reason that AHR inhibition can reduce the effect of GEM.

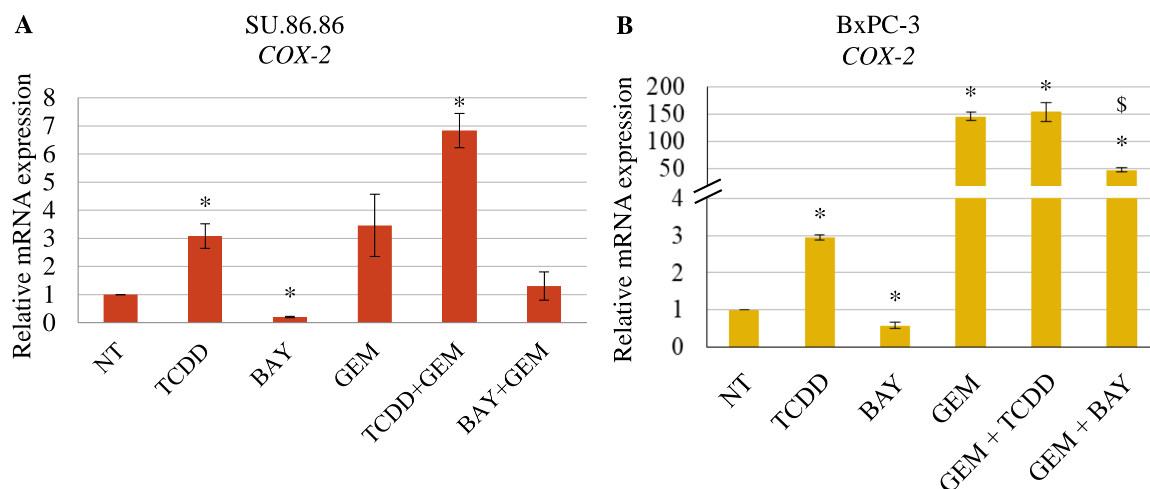

Figure 6. *COX-2* mRNA expression in PDAC cell lines. SU.86.86 (A) and BxPC-3 (B) cell lines were treated with TCDD (10nM), BAY (10µM) and GEM (36.96 nM for SU.86.86 and 26.67 nM for BxPC-3), or their combinations. *COX-2* mRNA levels were detected by RT-qPCR. Data are presented as fold change relative to non-treated (NT); average of n = 3 +/- SEM, * $p < 0.05$ vs NT, $^{\$}$ $p < 0,05$ vs GEM.

We next sought to determine whether the patterns seen by RT-qPCR recur at protein levels. Western blot analysis clearly indicated that TCDD increased COX-2 protein levels 1,7-fold in SU.86.86 cell line (Figure 7 A, B). BAY treatment, in turn, resulted in a robust 86% downregulation of COX-2. Although GEM treatment resulted in upregulation of *COX-2* mRNA, the protein levels remained unchanged regardless of the added compounds, although a trend of up and down regulation with TCDD and BAY, respectively, could be observed.

In the BxPC-3 cell line, TCDD increased COX-2 protein levels by 1,7-fold, whereas BAY reduced its amount by 36% (Figure 7 C, D). Again, GEM alone or in combination with TCDD did not significantly influence COX-2 levels. However, when BAY was added simultaneously with GEM, COX-2 amount was reduced by 36%, similar to treatment with BAY alone. These results indicate that although GEM is strongly influencing *COX-2* expression on mRNA level, protein levels are not affected. In addition, the data provided herein confirms that COX-2 is strongly and potentially directly regulated by AHR in these two PDAC cell lines.

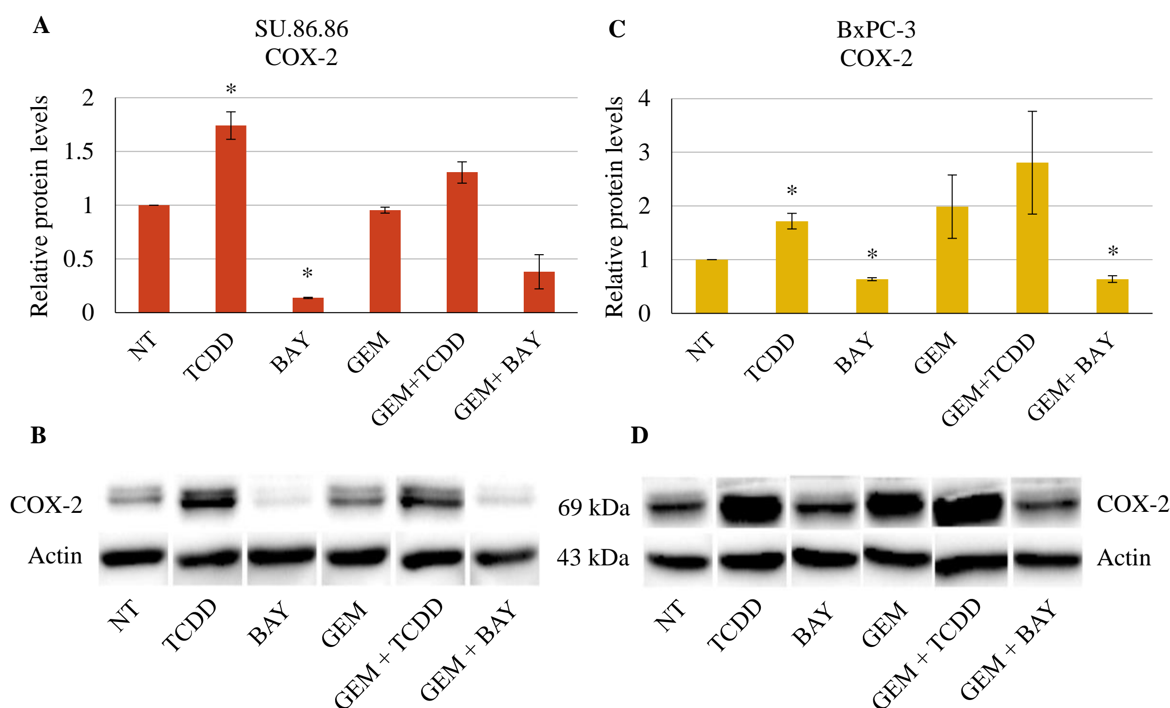

Figure 7. COX-2 protein levels in PDAC cell lines. SU.86.86 (A, B) and BxPC-3 (C, D) cell lines were treated with TCDD (10nM), BAY (10µM) and GEM (36.96 nM for SU.86.86 and 26.67 nM for BxPC-3), or their combinations. COX-2 protein levels were detected by western blot analysis. Densitometry analysis data are presented as fold change relative to non-treated (NT); average of n = 3 +/- SEM, * $p < 0.05$.

2.3.3. Impact of AHR activity modulation alone or in combination with gemcitabine on PD-L1 in PDAC cell lines.

There is evidence to suggest that PD-L1 expression can be influenced by AHR ligands. Exposure to certain environmental pollutant AHR ligands, such as dioxins, has been shown to increase PD-L1 expression in immune cells (Madej-Michniewicz et al., 2018). This implies that AHR activation by specific ligands may contribute to the regulation of PD-L1 expression. Tumours with high PD-L1 expression tend to have a poorer diagnosis, as they can effectively decrease the immune response and promote tumour growth and metastasis.

To investigate whether AHR might regulate PD-L1 in PDAC cell lines, we first analysed its mRNA expression in various treatment combinations by RT-qPCR. The results indicate that AHR activation or inhibition does not affect *PD-L1* expression in the SU.86.86 cell line (Figure 8 A). When cells were treated with GEM, there was, although statistically insignificant, a 7-fold increase in *PD-L1* transcripts. A similar trend was observed when TCDD or BAY were added in combination with GEM. In BxPC-3 cell line, AHR activity modulation again had no

effect on *PD-L1* expression (Figure 8 B). GEM alone or in combination with TCDD, in turn, increased *PD-L1* mRNA levels 55-fold, whereas with BAY 48-fold. This result seemed somewhat alarming, as increases in PD-L1 levels by the chemotherapeutic GEM would aid cancer cells to evade immune surveillance and thus reduce the treatment efficacy.

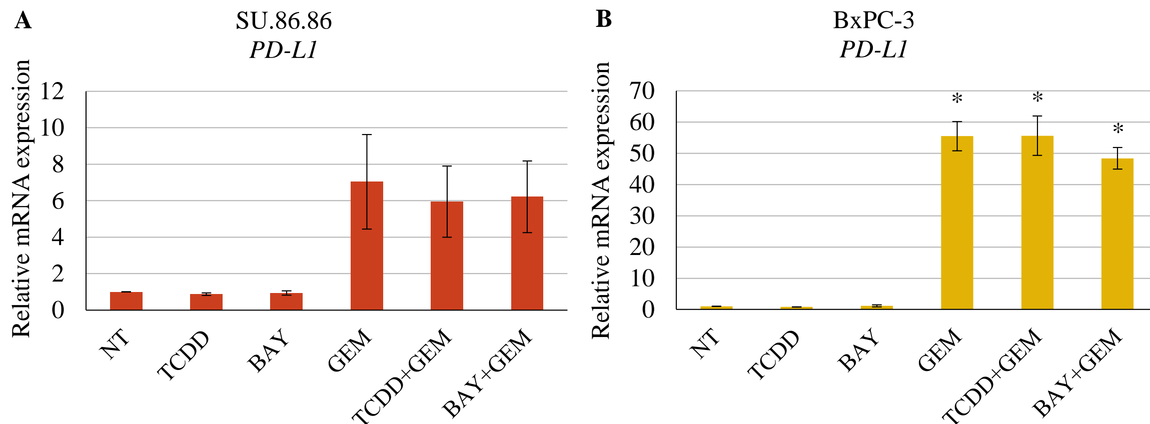

Figure 8. *PD-L1* mRNA expression in PDAC cell lines. SU.86.86 (A) and BxPC-3 (B) cell lines were treated with TCDD (10nM), BAY (10µM) and GEM (36.96 nM for SU.86.86 and 26.67 nM for BxPC-3), or their combinations. *PD-L1* mRNA levels were detected by RT-qPCR. Data are presented as fold change relative to non-treated (NT); average of n = 3 +/- SEM, * $p < 0.05$.

Next, we aimed to determine whether there are changes in PD-L1 protein levels. In general, we did not observe a significant change in PD-L1 protein, regardless of the treatments (Figure 9 A-D). Importantly, we noticed poor protein detection and high variation in our replicate experiments; thus, additional experiments would be necessary to draw any final conclusions. The poor quality of the western blot experiments with PD-L1-specific antibody can be attributed to the high N-linked glycosylation of PD-L1. It has been well established that the extracellular domain of PD-L1 is heavily glycosylated in cancer, and this greatly contributes to immunosuppression (Wang et al., 2020). Conversely, it has been reported that the removal of N-linked glycosylation enhances PD-L1 detection through anti-PD-L1 antibodies and increases the therapeutic efficacy of checkpoint inhibitor treatment (Lee et al., 2019).

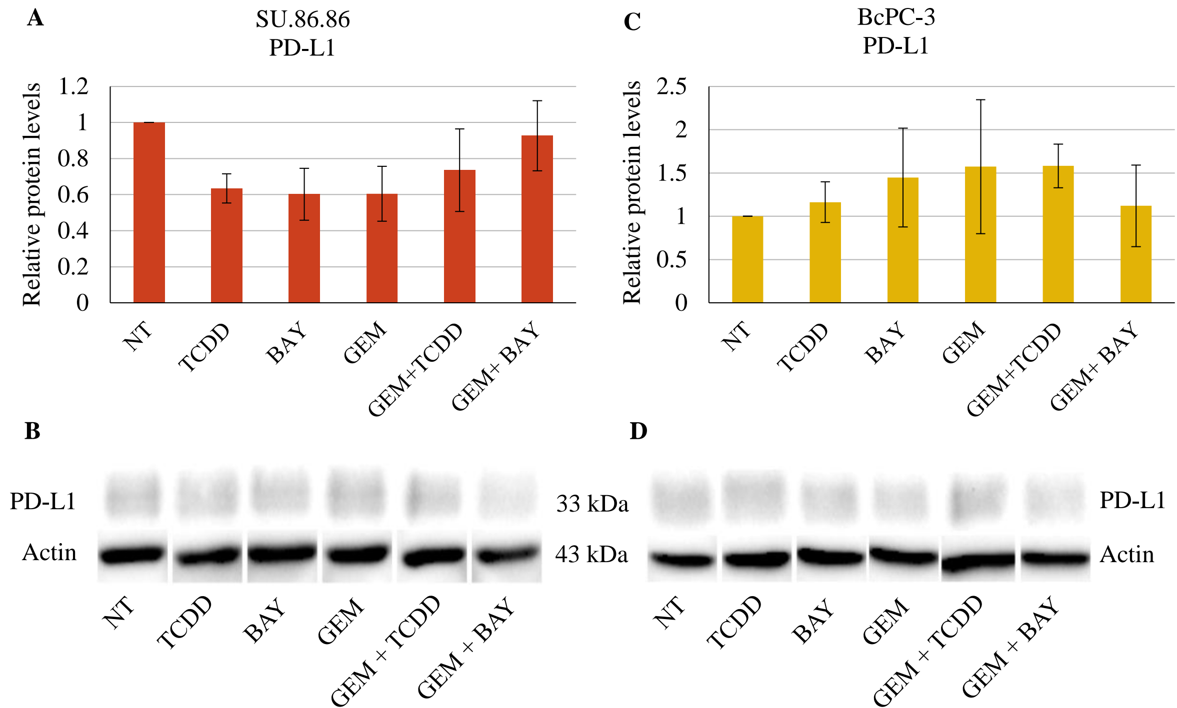

Figure 9. PD-L1 protein levels in PDAC cell lines. SU.86.86 (A, B) and BxPC-3 (C, D) cell lines were treated with TCDD (10nM), BAY (10µM) and GEM (36.96 nM for SU.86.86 and 26.67 nM for BxPC-3), or their combinations. PD-L1 protein levels were detected by western blot analysis. Densitometry analysis data are presented as fold change relative to non-treated (NT); average of n = 3 +/- SEM. * $p < 0.05$.

In conclusion, we have characterised the effects of AHR activity modulators alone or in combination with the chemotherapeutic gemcitabine on the expression of select genes. We found that in PDAC cell lines, the AHR activity modulators do not have a major influence on PD-L1 and AHR levels. COX-2 expression, however, is under AHR control. Whether this occurs via direct interaction of AHR with *COX-2* promoter remains to be determined in our ongoing chromatin immunoprecipitation experiments. Interestingly, we observed a profound effect of GEM on gene expression that did not correspond to protein levels, which remained unchanged. Importantly, the changes in gene expression were not uniform when comparing the cell lines and analysed genes. This may indicate that there is a specific mechanism that is responsible for the upregulation of certain genes, which should be clarified in future studies.

SUMMARY

Pancreatic ductal adenocarcinoma is the most prevalent form of pancreatic cancer and is associated with a high mortality rate. Late diagnosis and resistance to conventional therapies such as chemotherapy and radiation contribute to this high mortality rate, necessitating the ongoing search for novel treatment targets.

AHR is a transcription factor that is frequently upregulated in cancer cells and has been found to be expressed at higher levels in PDAC cells compared to non-cancerous pancreatic cells. While AHR is involved in various functions related to environmental chemical metabolism, its high expression in cancer has been linked to enhanced cell invasion, migration, proliferation, and metastasis. Conversely, AHR inactivation by specific ligands can induce cancer cell growth inhibition and apoptosis.

Gemcitabine disrupts DNA synthesis, causing single- and double-strand breaks. Repairing DNA lesions is vital for cell survival, and the AHR signalling pathway regulates this process. Research suggests that AHR can be a promising target for enhancing the effectiveness of gemcitabine in treating pancreatic cancer.

The aims of this study were to describe the expression of *AHR*, *COX-2*, and *PD-L1* mRNA and proteins in PDAC cell lines following treatment with AHR activity modulators alone or in combination with GEM. It was observed that mRNA levels widely differed between cell lines, with GEM treatments significantly upregulating BxPC-3 gene expression. Protein levels, however, did not significantly raise expression levels when treated with GEM.

We have characterised the effects of AHR activity modulators alone or in combination with the chemotherapeutic gemcitabine on the expression of specific genes. We discovered that the AHR activity modulators have little effect on PD-L1 and AHR levels in PDAC cell lines. COX-2 expression is, however, controlled by the AHR. Intriguingly, we observed a profound effect of GEM on gene expression that did not correspond to unchanged protein levels. Comparing cell lines and analysing genes revealed that the variations in gene expression were not uniform. This may suggest that there is a specific mechanism responsible for the upregulation of particular genes, which should be clarified in future research.

„Arüülsüsivesinike retseptori agonistide, antagonistide ja gemtsitabiini mõju inimese pankreasejuha adenokartsinoomi rakkude geeniekspressioonile“

Liisa Emilia Sorainen

KOKKUVÕTE

Pankreasejuha adenokartsinoom on levinuim kõhunäärmevähi vorm, moodustades ligikaudu 90% juhtudest. PDAC on agressiivne vähitüüp, mida iseloomustab spetsiifiliste sümptomite puudumine haiguse varases staadiumis. Kõhunäärmevähk diagnoositakse sageli hilja ja seetõttu on elulemus umbes 5 aastat.

Arüülsüsivesinike retseptor (AHR) on ligand-aktiveeritav transkriptsioonifaktor, mis omab olulist rolli erinevate keskkonnakemikaalide lagundamisel. Kemikaalide seondumisel AHR-iga käivitub signaalirada, mis mõjutab geenide ekspressiooni ning mitmeid füsioloogilisi protsesse. AHR-i hulga suurenemist on täheldatud mitmetes inimese kasvajates, sealhulgas pankreasejuha adenokartsinoomis.

Gemtsitabiin (GEM) on kemoterapeutiline ravim, mida kasutatakse mitmesuguste vähivormide, sealhulgas kopsu-, rinna-, pankrease- ja munasarjavähi raviks. Gemtsitabiin kuulub antimetaboliitide ravimiklassi ja toimib häirides DNA sünteesi, põhjustades üksikute ja kaheahelaliste katkete teket. DNA kahjustuste parandamine on oluline raku ellujäämiseks ning seda reguleerib AHR-i signaalirada.

Käesoleva bakalaureusetöö eesmärkideks oli kirjeldada *AHR*, *COX-2* ja *PD-L1* mRNA ekspressiooni ja valgutasemeid PDAC rakuliinides SU.86.86 ja BxPC-3 pärast töötlemist AHR-i aktiivsuse modulaatoritega üksi või kombinatsioonis GEM-iga.

Tulemustest selgus, et TCDD vähendas marginaalselt *AHR*-i ekspressiooni SU.86.86 rakuliinis. Seevastu kummaski rakuliinis rohkem AHR-i aktiivsuse modulaatorid *AHR*-i ega *PD-L1* ekspressiooni ei muutnud. Lisaks ilmnes, et *COX-2* ekspressioon on otseses seoses AHR-i aktiivsusega. SU.86.86 rakuliinis GEM uuritud geenide ekspressiooni oluliselt ei mõjutanud. See-eest BxPC-3 rakuliinis suurendas GEM oluliselt kõigi kolme geeni ekspressiooni. AHR-i ja PD-L1 valkude tasemetes kummaski rakuliinis olulisi muutusi ei täheldatud ei AHR-i aktiivsuse modulaatorite ega GEM poolt. Sarnaselt *COX-2* ekspressioonile täheldasime, et AHR-i agonistid suurendavad ning antagonistid vähendavad COX-2 taset mõlemas rakuliinis, viidates COX-2 otsesele regulatsioonile AHR-i poolt.

REFERENCES

Agency for Toxic Substances and Disease Registry (ATSDR). (1995). Toxicological profile for polycyclic aromatic hydrocarbons (PAHs) (Report No. TP-93/09). U.S. Department of Health and Human Services, Public Health Service.

Bailey, P., Chang, D. K., Nones, K., Johns, A. L., Patch, A. M., Gingras, M. C., Miller, D. K., Christ, A. N., Bruxner, T. J., Quinn, M. C., et al. (2016). Genomic analyses identify molecular subtypes of pancreatic cancer. Nature, 531(7592), 47-52.

Bartel, D.P. (2009). MicroRNAs: Target Recognition and Regulatory Functions. Cell, 136(2), 215-233.

Beischlag, T. V., Luis Morales, J., Hollingshead, B. D., & Perdew, G. H. (2008). The aryl hydrocarbon receptor complex and the control of gene expression. Critical Reviews™ in Eukaryotic Gene Expression, 18(3), 207-250.

Burris III, H. A., Moore, M. J., Andersen, J., Green, M. R., Rothenberg Jr, M. L., Modiano, M. R., et al. (1997). Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. Journal of Clinical Oncology, 15(6), 2403-2413.

Cavallo, D., Ursini, C. L., Cascio, C., & Iavicoli, S. (2019). The role of occupational exposure to polycyclic aromatic hydrocarbons and DNA damage-induced carcinogenicity: a review of the molecular mechanisms. Mutation Research/Reviews in Mutation Research, 779, 38-55.

Chen, Y. J., Lee, Y. J., Wu, C. C., Chen, G. Y., & Huang, S. H. (2019). The effect of NF-κB inhibitor on lipopolysaccharide-induced acute lung injury in mice. Mediators of inflammation, 2019.

Cheng, Y., Wang, K., Geng, L., Sun, J., Xu, W., Gong, S., ... & Xu, M. (2018). Role of aryl hydrocarbon receptor in cancer. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer, 1870(2), 280-289.

Choudhary, M., & Malek, G. (2020). The aryl hydrocarbon receptor: a mediator and potential therapeutic target for ocular and non-ocular neurodegenerative diseases. Journal of Clinical Medicine, 10(14), 3045.

Chun, Y. S., Pawlik, T. M., & Vauthey, J. N. (2015). 8th edition of the AJCC Cancer Staging Manual: pancreas and hepatobiliary cancers. Annals of Surgical Oncology, 22(2), 331-333.

Degner, S. C., Papoutsis, A. J., Selmin, O., & Romagnolo, D. F. (2009). Targeting of aryl hydrocarbon receptor-mediated activation of cyclooxygenase-2 expression by the indole-3-carbinol metabolite 3,3'-diindolylmethane in breast cancer cells. The Journal of nutrition, 139(1), 26–32.

Denison, M. S., & Nagy, S. R. (2003). Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annual review of pharmacology and toxicology, 43(1), 309-334.

Esser, C., Rannug, A., & Stockinger, B. (2009). The aryl hydrocarbon receptor in immunity. Trends in Immunology, 30(9), 447-454.

Fader KA, Nault R, Ammendola A, et al. The aryl hydrocarbon receptor modulates the function of human CD56^dim NK cells. Eur J Immunol. 2014;44(10):3068-3078.

Ferrara, N. (2004). Vascular endothelial growth factor: Basic science and clinical progress. Endocrine Reviews, 25(4), 581-611.

Furukawa, T., Klöppel, G., & Adsay, N. V. (2012). Classification of pancreatic cancer: pathology and molecular genetics of pancreatic ductal adenocarcinoma. Surgical oncology clinics of North America, 21(2), 215-225.

Ganesan, A. P., Johansson, M., Ruffell, B., Beltran, A., Lau, J., Jablons, D. M., ... & Coussens, L. M. (2018). Tumor-infiltrating regulatory T cells inhibit endogenous cytotoxic T cell responses to lung adenocarcinoma. The Journal of Immunology, 201(4), 1310-1319.

Giesy, J. P., & Kannan, K. (2001). Dioxin-like and non-dioxin-like toxic effects of polychlorinated biphenyls (PCBs): implications for risk assessment. Critical Reviews in Toxicology, 31(4-5), 353-368.

Guan, R., Wang, J. W., Li, Y. F., Cai, H., Li, X. Y., & Wan, J. Y. (2021). Aryl hydrocarbon receptor degradation induces lung cancer cell arrest at the G1 phase and decreases cyclin D1 expression. OncoTargets and therapy, 14, 2715-2725.

Han, S., Zhang, T., Yang, L., Cao, W., Zhang, R., Chen, H., ... & Yang, C. (2020). Discovery of BAY-2416964, an orally bioavailable pan-Aryl hydrocarbon receptor (AhR) antagonist. Journal of medicinal chemistry, 63(9), 4864-4878.

Hankinson, O. (1995). The aryl hydrocarbon receptor complex. Annual Review of Pharmacology and Toxicology, 35, 307-340.

Herbst, R. S., Soria, J. C., Kowanetz, M., Fine, G. D., Hamid, O., Gordon, M. S., ... & Sosman, J. A. (2014). Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature, 515(7528), 563-567.

Hollingshead, B. D., Beischlag, T. V., Dinatale, B. C., Ramadoss, P., & Perdew, G. H. (2008). Inflammatory signaling and aryl hydrocarbon receptor mediate synergistic induction of interleukin 6 in MCF-7 cells. Cancer research, 68(10), 3609-3617.

Holmgaard, R. B., Zamarin, D., Li, Y., Gasmi, B., Munn, D. H., Allison, J. P., & Merghoub, T. (2013). Tumor-expressed immune checkpoint CTLA-4 blockade induces T-cell–mediated regression of murine pancreatic tumors. Cancer research, 73(3), 2161-2170.

Huang, H., Huang, Q., Tang, Q., Zeng, Z., Li, S., & Wang, J. (2019). Aryl hydrocarbon receptor expression and its role in pancreatic cancer progression. Journal of Cancer, 10(19), 4535-4542. Human Embryonic Stem Cells and Early Differentiation. Int. J. Mol. Sci. 21, 9052.

Jin, U.-H., Kim, S.-B. and Safe, S. (2015). Omeprazole inhibits pancreatic cancer cell invasion through a nongenomic aryl hydrocarbon receptor pathway. Chem. Res. Toxicol. 28, 907–918.

Junqueira, L. C., Carneiro, J., & Kelley, R. O. (2003). Basic histology: text & atlas. McGraw-Hill Medical.

Kaelin Jr, W. G., & Ratcliffe, P. J. (2008). Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Molecular cell, 30(4), 393-402.

Kamisawa, T., Wood, L. D., Itoi, T., & Takaori, K. (2016). Pancreatic cancer. The Lancet, 388(10039), 73-85.

Kaur, H., & Kaushal, V. (2019). The endocrine pancreas and its role in metabolic homeostasis. Annals of pediatric endocrinology & metabolism, 24(4), 211-217.

Keir, M. E., Liang, S. C., Guleria, I., Latchman, Y. E., Qipo, A., Albacker, L. A., ... & Freeman, G. J. (2006). Tissue expression of PD-L1 mediates peripheral T cell tolerance. The Journal of experimental medicine, 203(4), 883-895.

Kerkvliet, N. I. (2009). AHR-mediated immunomodulation: the role of altered gene transcription. Biochemical pharmacology, 77(4), 746-760.

Koki, A. T., Leahy, K. M., & Masferrer, J. L. (2002). Potential utility of COX-2 inhibitors in chemoprevention and chemotherapy. Expert Opinion on Investigational Drugs, 11(8), 1215-1229.

Koliopanos, A., Kleeff, J., Xiao, Y., Safe, S., Zimmermann, A., Büchler, M. W., & Friess, H. (2002). Increased arylhydrocarbon receptor expression offers a potential therapeutic target for pancreatic cancer. Oncogene, 21(39), 6059–6070.

Konsa, P. (2021). Arüülsüsivesinike retseptori agonistide ja antagonistide mõju inimese pankreasejuha adenokartsinoomi rakkude geeniekspressioonile.

Kozarich, J. W., Arlow, D. H., & Tropsha, A. (2010). Development of the GNF-5 and GNF-2 inhibitors of the Hsp90 C-terminal domain. ACS medicinal chemistry letters, 1(6), 255-259.

La Merrill, M., Birnbaum, L. S., & Pompili, F. A. (2013). Denison protocol for assessing aryl hydrocarbon receptor activity in extracts of human and rodent tissues. In Toxicology mechanisms and analytical methods (pp. 273-294). Springer, New York, NY.

Lawrence, T., Willoughby, D. A., & Gilroy, D. W. (2002). Anti-inflammatory lipid mediators and insights into the resolution of inflammation. Nature Reviews Immunology, 2(10), 787-795.

Lee, H.-H., Wang, Y.-N., Xia, W., Chen, C.-H., Rau, K.-M., Ye, L., Wei, Y., Chou, C.-K., Wang, S.-C., Yan, M., Tu, C.-Y., Hsia, T.-C., Chiang, S.-F., Chao, K. S. C., Wistuba, I. I., Hsu, J. L., Hortobagyi, G. N., & Hung, M.-C. (2019). Removal of N-Linked Glycosylation Enhances PD-L1 Detection and Predicts Anti-PD-1/PD-L1 Therapeutic Efficacy. Cancer Cell, 36(2), 168-178.e4.

Lee, S. Y., Lee, S. H., Yang, H., Song, J. Y., & Kim, J. R. (2017). Naphtho[1,2-b]furan-4,5-dione (Gambogic Acid) Inhibits the Aryl Hydrocarbon Receptor and Aryl Hydrocarbon Receptor Nuclear Translocator-Mediated Signal Transduction Pathway. Biological and Pharmaceutical Bulletin, 40(3), 276-284.

Liu, C. C., Xia, M., Zhang, Y. J., Jin, P., Zhao, L., Zhang, J., Li, T., Zhou, X. M., Tu, Y. Y., Kong, F., Sun, C., Shi, L., & Zhao, M. Q. (2018). Micro124-mediated AHR expression regulates the inflammatory response of chronic rhinosinusitis (CRS) with nasal polyps. Biochemical and biophysical research communications, 500(2), 145–151.

Liu, Z., Wu, X., Zhang, F., Han, L., Bao, G., He, X., & Xu, Z. (2013). AhR expression is increased in hepatocellular carcinoma. Journal of molecular histology, 44(4), 455–461.

Ma, Q., & Pollenz, R. S. (2003). Aryl hydrocarbon receptor-mediated transcriptional regulation of detoxification and biotransformation enzymes. Expert opinion on drug metabolism & toxicology, 1(1), 9-21.

Madej-Michniewicz, A., Roszak, A., Siedlecki, P., & Domagała-Kulawik, J. (2018). The Role of PD-L1 in the Regulation of Antitumor Immune Responses in Lung Cancer and Its Implication as a Therapeutic Target. Cancer Immunology, Immunotherapy, 67(3), 459-470.

Mimura, J., & Fujii-Kuriyama, Y. (2003). Functional role of AhR in the expression of toxic effects by TCDD. Biochimica et Biophysica Acta (BBA)-General Subjects, 1619(3), 263-268.

Mini, E., Nobili, S., Caciagli, B., & Landini, I. (2006). Pharmacological aspects of the use of gemcitabine in lung cancer. Clinical Pharmacokinetics, 45(8), 795-816.

Murray, I. A., Patterson, A. D., & Perdew, G. H. (2014). Aryl hydrocarbon receptor ligands in cancer: friend and foe. Nature Reviews Cancer, 14(12), 801-814.

Netter, F. H. (2014). Atlas of human anatomy: including Student consult interactive ancillaries and guides. Elsevier Health Sciences.

Ogura, J., Miyauchi, S., Shimono, K., Yang, S., Gonchigar, S., Ganapathy, V. and Bhutia, Ohtake, F., Baba, A., Takada, I., Okada, M., Iwasaki, K., Miki, H., ... Kato, S. (2007). Dioxin receptor is a ligand-dependent E3 ubiquitin ligase. Nature, 446(7135), 562-566.

Okey, A. B. (2007). An aryl hydrocarbon receptor odyssey to the shores of toxicology: The Deichmann Lecture, International Congress of Toxicology-XI. Toxicological Sciences, 98(1), 5-38.

Patrono, C., & Baigent, C. (2019). Nonsteroidal anti-inflammatory drugs and the heart. Circulation, 139(18), 2142-2157.

Pizzorno, G., & Bittoni, A. (2009). 5-Fluorouracil in the management of colorectal cancer: review of literature. Clinical Medicine Insights: Oncology, 3, 13-27.

Saif, M. W., & Kim, R. (2014). Pancreatic cancer: advances in treatment, results and limitations. Future oncology, 10(6), 925-936.

Sasaki-Kudoh, E., Kudo, I., Kakizaki, Y., Hosaka, M., Ikeda, S.-I., Uemura, S., Grave, E., Togashi, S., Sugawara, T., Shimizu, H., & Itoh, H. (2018). Cisplatin inhibits AhR activation. American Journal of Molecular Biology, 8(1), 38-46.

Singh, N. P., Singh, U. P., Nagarkatti, M., & Nagarkatti, P. (2017). Role of aryl hydrocarbon receptor in breast cancer progression. PLoS One, 12(10), e0187541.

Smith, K. J., Murray, I. A., Tanos, R., Tellew, J., Boitano, A. E., Bisson, W. H., Kolluri, S. K., Cooke, M. P., Perdew, G. H., & Williams, D. E. (2009). Identification of a high-affinity ligand that exhibits complete aryl hydrocarbon receptor antagonism. Journal of Pharmacology and Experimental Therapeutics, 329(1), 64-73.

Snell, R. S. (2012). Clinical anatomy by regions. Lippincott Williams & Wilkins.

Standring, Borley, and Collins (2016) provided an anatomy reference for the head of the pancreas in Gray's Anatomy: The Anatomical Basis of Clinical Practice.

Stubbe, J., & van der Donk, W. A. (2018). Ribonucleotide reductases: radical enzymes with suicidal tendencies. Chemistry & biology, 25(11), 1147-1151.

Tanaka, Y., Sasaki, H., Moriyama, S., Shimura, T., & Miyano, S. (2016). A novel statistical method to estimate the tumor purity and ploidy from SNP microarrays. Cancer Research, 76(14 Suppl), abstract nr 2104.

Taube, J. M., Klein, A., Brahmer, J. R., Xu, H., Pan, X., Kim, J. H., ... & Anders, R. A. (2014). Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti–PD-1 therapy. Clinical cancer research, 20(19), 5064-5074.

Teino, I., Matvere, A., Pook, M., ... Maimets, T. (2020). Impact of AHR Ligand TCDD on

Tempero, M. A., Malafa, M. P., Behrman, S. W., Benson III, A. B., Casper, E. S., Chiorean, E. G., ... & Westin, J. R. (2019). Pancreatic adenocarcinoma, version 2.2019. Journal of the National Comprehensive Cancer Network, 17(2), 202-210.
therapy. Biochem. J. 474, 3391–3402.

Vacher, S., Castagnet, P., Chemlali, W., Lallemand, F., Meseure, D., Pocard, M., Bieche, I., & Perrot-Applanat, M. (2018). High AHR expression in breast tumors correlates with expression

of genes from several signaling pathways namely inflammation and endogenous tryptophan metabolism. PloS one, 13(1), e0190619.

Vane, J. R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nature: New Biology, 231(25), 232-235.

Vázquez-Gómez, G., Karasová, M., Tylichová, Z., Kabátková, M., Hampl, A., Matthews, J., Neča, J., Ciganek, M., Machala, M., & Vondráček, J. (2022). Aryl Hydrocarbon Receptor (AhR) Limits the Inflammatory Responses in Human Lung Adenocarcinoma A549 Cells via Interference with NF-κB Signaling. Cells, 11(4), 707.

Vogel, C. F. A., Van Winkle, L. S., Esser, C., & Haarmann-Stemmann, T. (2020). The aryl hydrocarbon receptor as a target of environmental stressors - Implications for pollution mediated stress and inflammatory responses. Redox biology, 34, 101530.

von Hoff, D. D., Ramanathan, R. K., Borad, M. J., Laheru, D. A., Smith, L. S., Wood, T. E., ... & Li, D. (2011). Gemcitabine plus nab-paclitaxel is an active regimen in patients with advanced pancreatic cancer: a phase I/II trial. Journal of Clinical Oncology, 29(34), 4548-4554.

Wang, YN., Lee, HH., Hsu, J.L. (2020). The impact of PD-L1 N-linked glycosylation on cancer therapy and clinical diagnosis. J Biomed Sci 27, 77.

Weiss, R. B., Donehower, R. C., Wiernik, P. H., Ohnuma, T., Gralla, R. J., Trump, D. L., ... & Von Hoff, D. D. (1990). Hypersensitivity reactions from taxol. Journal of Clinical Oncology, 8(7), 1263-1268.

Wolfgang, C. L., Herman, J. M., Laheru, D. A., Klein, A. P., Erdek, M. A., Fishman, E. K., Hsu, C. C., Le, D. T., Schulick, R. D., et al. (2013). Recent progress in pancreatic cancer. CA: A Cancer Journal for Clinicians, 63(5), 318-348.

Y.D. (2017). Carbidopa is an activator of aryl hydrocarbon receptor with potential for cancer
Zhang, J., & Hogenesch, J. B. (2017). An overview of the core circadian clock component, CLOCK: Structure, regulation, and function. In K. Hirota & P. Sassone-Corsi (Eds.), Circadian Clocks (pp. 79-97). Springer.

Zhang, J., & Liu, J. (2002). The PAS superfamily: Sensors of environmental and developmental signals. Annual Review of Pharmacology and Toxicology, 42, 519-561.

Zhu K, Meng Q, Zhang Z, Yi T, He Y, Zheng J and Lei W: Aryl hydrocarbon receptor pathway: Role, regulation and intervention in atherosclerosis therapy (Review). Mol Med Rep 20: 4763-4773, 2019

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