Validation of potential CIP2A inhibitors based on computer modeling in triple-negative breast cancer

Master's Thesis

University of Turku

Department of Biotechnology

Biochemistry

June/2021

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LEHTINEN ALEKSI: Validation of potential CIP2A inhibitors based on computer modeling in triple-negative breast cancer
Master's Thesis, 50 pages
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6/2021
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Cancer is a wide range of diseases that are caused by unchecked multiplication of the cells due to underlying mutations. Rapidly dividing cells may achieve metastasis, which is the stage where cancer cells are able to spread through blood stream to other organs. Breast cancer is the major cause of cancer related death in women. Triple-negative breast cancer (TNBC) accounts for about 10-15% of all breast cancers. There are no targeted therapies for this subtype as they lack three molecular markers that drugs are based on, leading to poor prognosis. Inhibition of the tumor suppressor PP2A and the overexpression of its inhibitor CIP2A is observed in many cancer types, including TNBC. CIP2A consist of two monomers forming a dimer. Based on computer modeling four compounds were predicted to be novel inhibitors of CIP2A. These four molecules were predicted to prevent dimerization of CIP2A leading to its degradation.

These compounds were tested by cell proliferation assays in five different TNBC cell lines. IC50 was calculated for each of the compounds across the five cell lines. Only one compound was able to potently kill 50% of the cells in 24 hours. The effect of this compound was also observed using colony growth experiment. CIP2A inhibition was evaluated by western blotting, which showed that CIP2A protein levels were decreased in response to CIP1 compared to the control cells. However, using dimerization assay with recombinant proteins, it was seen that CIP1 was not able to prevent CIP2A monomers to form the dimer as suggested by the modeling. RNA experiment using qPCR showed that the CIP2A RNA level also decreased in response to CIP1. With all the data from the experiments, it can be concluded that these compounds though promising in the beginning but after detailed evaluation cannot be used as direct CIP2A protein inhibitor and because of off-target effects research with these compounds should not be prioritized.

Key words

TNBC, PP2A, CIP2A, cell viability, colony growth, western blot, dimerization assay

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1 Literature overview

1.1 Cancer

Cancer is the second leading cause of mortality worldwide making this disease a serious problem. No one can predict for sure if someone will get cancer, there is only estimations that some behaviors may be a risk factor to get cancer. The problem with cancer is that it is a group of genetic diseases at the tissue level. This means that notable changes (mutations) in the genome of the cell makes them to grow uncontrollably. In normal situation, a body is able to find and repair these mutations, which occur in the cell. But sometimes some mutations are able to "slip" through the repair machinery and these can ultimately lead to cancer. The efficiency of repair mechanisms also goes down with old age, hence we see more people affected by cancer in older ages. The challenge currently is being able to detect the cancer in early stage. Wide range of cancers makes it harder to be specifically diagnosed and treated with the most efficient way possible. (Fisher et al., 2013; Meacham & Morrison, 2013.)

Among men, most frequent cancer types are prostate, lung, colorectal, and bladder cancers respectively. Among women, most frequent ones are breast, lung, colorectal, uterine corpus and thyroid respectively. Statistically, prostate and breast cancers are the major portion of all occurring cancers among men and women. (Siegel et al., 2016.) When it comes to children, the most frequent cancer types are blood, brain and lymph nodes, respectively.

Cancer develops when multiple mutations in vital genes, that does not diminish the vitality of the cell, occur and the body cannot repair these, leading to change in cellular functions. There are certain chemical compounds called carcinogens that are known to have a crucial role in the formation of gene mutations and thereby cancer. For example, smoking has a direct correlation in the likelihood of getting lung cancer. (Aizawa et al., 2016.) Also, nucleus and cytoplasm can be directly or indirectly influenced by exposure to environmental chemical compounds, leading to gene mutations and genetic disorders (Antwi et al., 2015; Poon et al., 2014). Viral and bacterial infections and radiation might also expose one to cancer, and these account for 7 % of all cancer cases (Parkin, 2006).

In normal cell there is a balance between proteins that cause cell division etc, and their counter parts. Cancer can disrupt this cellular balance, resulting in the abnormally functioning vital genes. This dysfunction is able to disturb cellular functions for example, cell cycle, leading to abnormal proliferation. (Parkin, 2006; Seto et al., 2010.) Carcinogens can cause mutations in genes (oncogenes) that are responsible for growth and cellular division under normal condition causing them to multiply without any kind of control. This type of mutation is the most crucial for the survival of the cancer cells. (Shtivelman et al., 1985.) Other cancer dysfunction is when mutations in genes (tumor

suppressor) are not able to prevent the uncontrolled cell division. There are repair proteins, which can remove the main DNA lesions induced by ultraviolet light, bypass the DNA damage and remove uracil from DNA. By these properties, DNA can be repaired successfully when the repair proteins function correctly. (Wood et al., 2001.)

Genetic changes can occur via chromosomal translocation, point mutation, deletion, amplification, or insertion activation. These lead to oncogene generation and to genetic disorders. One example is chronic myeloid leukemia (CML), which often occurs in the elder population (median of 65 years). The genetic abnormality, that is the cause of the disease, happens in the long arms of chromosome 9 and 22. In these chromosomes, there is change of genetic material, which leads to the production of fusion protein BCR-ABL, when Bcr gene is partly fused to Abl oncogene. It will lead to the production of a biomarker ph1, which is found in 95 % of patients and is it used to diagnose chronic myeloid leukemia. Because of the Abl gene, BCR-ABL protein also has a kinase activity, being able to disrupt the normal functions of the cell. (Heinrich et al., 2002; Joensuu & Dimitrijevic, 2001; Thomas et al., 2007.)

TP53 is one of the most studied tumor suppressors. Loss of function mutation in the TP53 gene leads to formation of misfunctioning p53 protein. p53 has a crucial role in cellular functions being called the guardian of the genome. It has roles in DNA metabolism angiogenesis, differentiation, cell division, senescence and cell death. This mutated protein is found in approximately 60 % of all cancer cases. Most of the TP53 mutations has been found in the DNA-binding position. The main role of p53 is to participate in DNA repair and induce cell cycle arrest for the response to cellular stress. Cell cycle is fragile mechanism that needs to be regulated and checkpoints are ensuring proper timing in the cell cycle for example, when DNA damage occurs, cell cycle is not allowed to continue to the mitosis by the G2/M checkpoint. The most important function in the cellular arrest is the p53 ability to maintain the cell in the G1 and G2 phase of the cell cycle via CDK1-P2 and CDC2. When p53binds to DNA damage it leads to CDC2 inhibition by stimulation of WAF1 gene. (Bukholm & Nesland, 2000; Roninson, 2002.) Holding cells in these checkpoints provides time for the DNA repair mechanisms to repair DNA damage or if the damage cannot be repaired, cell will go to apoptosis in normal conditions. (Chae et al., 2011; Taylor & Stark, 2001). During the first decade of when p53 was found, it was thought to be an oncoprotein. Nowadays, it is known to be a tumor suppressor. p53 anti-cancer properties are active during three routes of stimulation, which are DNA repairing proteins, induction of apoptosis and arresting of cell cycle in G1/G2 phase. (Muller & Vousden, 2014.)

Another genetic change is hypomethylation. Methylation has many functions in the normal cells. It regulates gene expression, retro-element silencing, centromere stability and chromosome segregation

in mitosis, X-chromosome inactivation and monoallelic silencing of imprinted genes. Hypomethylation induces chromosomal instability, increased movement and deletion of genes. Hypomethylations are mostly found in the highly and moderately repeated sequences. (Kanai & Hirohashi, 2007.) One hypomethylation that is absent in many cancer types like breast, lung, and bladder cancer, is L1 from the LINE family. Ectopic expression is one of the cancer promoting effect, which occurs via hypomethylation. One example of this kind of hypomethylation is with MASPIN, which is a tumor suppressor gene. MASPIN dysfunction has been found in breast and prostate cancer. Hypomethylation has been found in many other cancers as well. Other examples of hypomethylation include MAGE and DPP6 in melanoma, SNGG in breast and ovarian cancer, S100P in pancreatic cancer. (Futscher et al., 2004.)

Hypermethylation is more specific than hypomethylation. Unlike hypomethylation, hypermethylation only targets specific CpG region. Hypermethylation of promoter induces transcription inactivation, which influences many genes. Because of this specific targeting, hypermethylation is found to affect tumor suppressor genes via inhibition, whereas hypomethylation affects to aberrant activation of oncogenes. There are lot of evidence that hypermethylation silences many genes, that are involved in DNA repair (BRCA1), cell cycle (p16, WRN, P161NK4b, P161NK4a), response to vitamin (CRBP1, RARB2), cell adhesion and apoptosis (TMS1, DAPK1 and WIF-1). These can be used as biomarkers for diagnosis and prognosis because aberrant DNA methylation can be tracked in body fluids. Also many studies are focused to the CpG regions of the promoters. It is worth of noticing, that from 45 % to 65 % cases, hypermethylation has been found to occur in some point of the cancer. (Doi et al., 2009; M. Li et al., 2009.) There are many possibilities of how hypermethylation occurs, one being DNA methyltransferase (DNMT). It has been associated to have high level of expression in tumors. Interestingly, miRNA regulates DNMT expression and it has been found that MIR-29 family reduces the expression of DNMT3a, DNMT3b and DMNT1. (Miremadi et al., 2007).

Histone acetylation is controlled by two opposite functions by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histone acetylation is widely studied posttranslational histone modification. HDACs are able to undo chromatin acetylation alternating transcription of tumor suppressor genes and oncogenes, and misfunction of HDACs have been associated in many cancers. HDACs are grouped into four classes, where sirtuins family is the main family, containing from seven sirtuins. This family regulates many cellular processes (stress response, metabolism survival). The increased SIRT1 expression and activity varies and high expression is associated with poor prognosis and advanced disease. Sirtuin cooperate with other substances for example, SIRT1 and DNMT1 affects to the DNA methylation together. HDAC have many regulators, one being microRNA. One

example of these microRNAs is miR449a, which regulates survival and cell growth via suppression of HDAC1 expression in prostate cancer. In addition to alteration of HDAC expression in many cancers such as leukemia, colon and lung cancer, disturbance in histone acetylation occurs trough deletion in HAT and genes related to it as well as trough ectopic mutations. These situations might be the main driver of cancer formation. (Noonan et al., 2009.) Many cancers have been observed to have lost H4k16 acetylation, H3K4, H4k20 and H3K27 methylation which are epigenetic modifications of the DNA packaging protein Histone H4 (Kondo et al., 2007). Also histone methylation can be problematic. Distribution of histone methylation is controlled by histone methyltransferases and histone demethylases. Examples of these are found in renal carcinoma and in leukemia. One histone methyltransferase (SETD2) and one histone demethylase (UTX) has been found to occur in a renal carcinoma. Another example is with some leukemia cases where MLL oncoprotein has been found to cause misfunctioning patterns in H3K4 and H3K29 methylation. These patterns are able to change the expression of the target genes of MLL. (P. Wang et al., 2009.)

There are many other chromatin modeling alterations that can be tumorigenic. One is SWI/SNF chromatin-remodelling complex (CRC), which is needed in creation of several thousand biochemically distinct complexes and it functions as a tumor suppressor. Alternations in the function of this complex has been found to have a crucial role in 25 % of all cancers. These crucial mutations in the subunits are mostly focused on the ARID family and on the ATPase subunits BRG1 and BRM. BRG1 is crucial for DNArepair, differentiation, and organ development. BRG1 role in tumors is controversial, showing overexpression and suppression of BRG1 depending on tumor type. It is also able to interact with promoting and inhibiting genes. BRG1 and BRM affects TP53 and BRG1 has been found to destabilize p53 and SWI/SNF complex. This destabilizing effect is beneficial for cancer formation because of the alternative reactions with p53, BRCA1, MLL, RB and MYC. By the effects of alterations, inhibiting SWI/SNF complex should disrupt cell growth. (Mittal & Roberts, 2020; Mulero-Navarro & Esteller, 2008.)

1.2 Kinases and Phosphatases

Kinases and phosphatases are known to play a crucial role in many cellular functions. These cellular components are known to have an effect in cells life cycle, for example growth and development, proliferation, cell division as well as maintaining survival or activating programmed cell death known as apoptosis. Cell must be able to activate or inactivate functional proteins to regulate its physiological functions. This shifting from one state to another is often done via dephosphorylation (inactivation) and phosphorylation (activation), but inactivation and activation can also function vice versa. The transaction is regulated by kinases and phosphatases. (Mumby & Walter, 1993.) One of the most and

widest studied signaling mechanism in eukaryotic cells is phosphorylation via protein kinases. This mechanism is used in regulating almost every property of a protein. Importance of kinases can be easily noticed because of one-third of all intracellular proteins may be phosphorylated. Understanding the complex network of signaling that is based on phosphorylation is crucial for therapeutic approaches. Kinases are divided into two groups, which are protein tyrosine kinases and protein serine threonine kinases. (Johnson & Hunter, 2005.) Like the kinases, the phosphatases are also further divided into protein tyrosine phosphatases (PTPs) and protein serine/threonine phosphatases (PSP). PTPs ever further divided to four classes: Class 1-3 Cysteine-Based PTPs and fourth class Asp-Based PTPs. (Alonso et al., 2004.) Phosphatases are normally found to exist in an active monomeric unit or in an active holoenzyme complex consisting from up to three subunits. These subunits are known as catalytic, regulatory and scaffold subunits. There is a larger number of different kinases when compared to phosphatases, but still lower number of phosphatases are able to control higher number of kinases. Phosphatases capability is explained by different subunit forms and combinations controlling specific kinases. Kinases are thought to control the amplitude of a signaling response and phosphatases control the rate and duration of the response. (Tonks, 2006.)

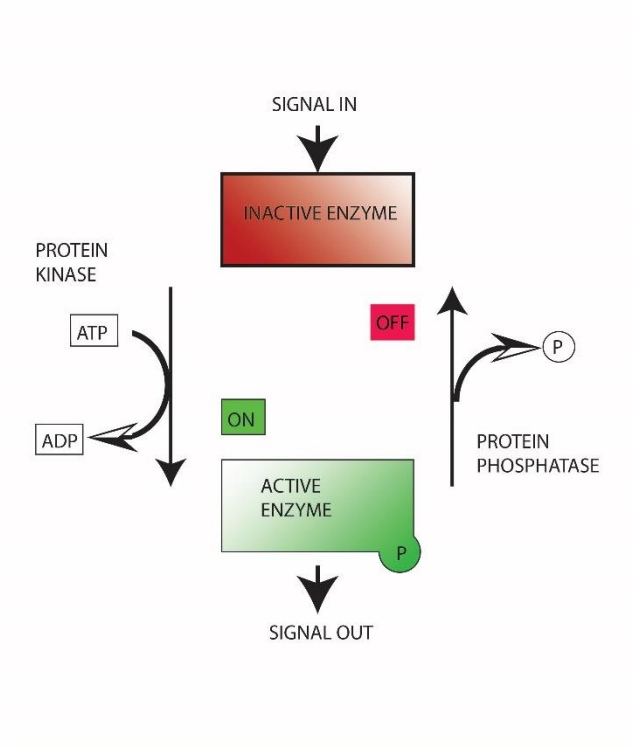

Figure 1: Protein is activated and inactivated by protein kinases and protein phosphatases. Figure was drawn by Adobe Illustrator CS6.

1.3 Protein phosphatases

Kinases are mainly affecting on the hydroxyl-group of the target protein, which mostly consists of serine (86,4%), threonine (11,8%) and tyrosine (1,8%) amino acid residues (Olsen et al., 2006). Protein phosphatases are targeting the phosphate group catalyzing removal of phosphate in the

presence of a water molecule leading to conformation changes, thus change of function (Barford, 1996), Just like PTPs, PSPs are also further divided into three sub-groups, which are: phosphoprotein phosphatases (PPPs), metal dependent protein phosphatases (PPMs) and more recently found aspartate-based phosphatases that utilize an aspartate-based catalytic mechanism. PPPs and PPMs dephosphorylate phosphoserine and phosphothreonine. PPPs are even further divided into sub-groups: PP1, PP2A, calcium-activated PP2B, PP4, PP5, PP6 and PP7. PPM and PPP subgroups are sequential unrelated, implicating of being evolved from two unique ancestral genes, but still having related catalytic centre structure. PTPs are highly diverse in domain structure and substrate preference. Some family members of PTPs are found to be able to dephosphorylating complex carbohydrates, mRNA and phosphoinositides. (Barford, 1996; Moorhead et al., 2009.) PTPs can be thought to be a counterbalance for protein tyrosine kinases. One example of this balance is regulation of AKT signaling and other is MAP kinase pathway, which is mainly regulated by MAP kinase phosphatases. PTPs are functioning by targeting post-translationally modified tyrosine residue via removing the phosphate group. (Tonks, 2006.)

1.4 Protein Phosphatases 2A (PP2A)

Previously shortly mentioned PPPs sub-group, protein phosphatase 2A (PP2A), has a crucial role in many cellular functions. It affects the cellular metabolism by regulating enzyme activity, lipid metabolism and catecholamine synthesis. (TUNG et al., 1985.) PP2A also regulates biological processes (cell cycle), DNA replication, transcription and translation, signal transduction, cell proliferation, cytoskeleton dynamics and cell mobility, apoptosis as well as cell transformation. PP2A functions under a strong autoregulatory mechanism, which ensures constant level of PP2A synthesis. If PP2A mRNA is highly elevated, there is still no corresponding increase in PP2A protein levels. (Baharians & Schönthal, 1998; Glenn & Eckhart, 1993.) PP2A is ubiquitously expressed and contributes to 0,3-1 % of the total cellular protein (Ruediger et al., 1991). It catalyzes most of the soluble phosphatase's activity at the phospho serine and phospho threonine residues. PP2A is found in a dimeric form ($PP2A_D$) (TUNG et al., 1985) and in a trimeric form ($PP2A_T$) (Mayer-Jaekel & Hemmings, 1994). The difference between these two forms is that the dimeric form is the core enzyme, which is not active, and the trimeric form is an active holoenzyme. The dimeric form has two subunits, the catalytic subunit (PP2A-C) and scaffold subunit (PP2A-A). The trimeric form of PP2A is like the dimeric form with the addition of regulatory subunit (PP2A-B) (Kamibayashi et al., 1994). The B-subunits are determining the specificity of the substrate and the subcellular localization of the trimeric PP2A complex.

1.4.1 Catalytic subunit (C-subunit)

The catalytic subunit of PP2A (PP2Ac) is expressed in all the tissues, with highest levels found in brain and heart. The size of this subunit is 36 KDa and its translation is regulated to maintain constant levels in the cells. (Baharians & Schönthal, 1998). $PP2A_c$ has two isoforms Cα and Cβ, which share 97 % similarity in the primary sequence. The expression of Cα is approximately 10-fold more than Cβ. These two are encoded by two different genes consisting of 7 exons and 6 introns. From these exons, the exon 2 to 6 are involved in catalysis and substrate binding, whereas exons 1 and 7 help in regulation. The promoter for Cα is approximately 7-10- fold stronger in comparison with the promotor of Cβ, which might explain the several fold higher expression of Cα protein. Cα is localized to the plasma membrane whereas Cβ is found in both cytoplasm and nucleus. (Hemmings et al., 1990.) PP2Ac is uniquely conserved and binds to the scaffolding and regulatory subunit (Xu et al., 2006).

1.4.2 Scaffold subunit (A-subunit)

Scaffolding subunit (PP2Aa or PR65) has two variants, Aα and Aβ, which were identified to have 86 % sequence similarity and were found to be ubiquitously expressed. PR65 size is 65 kDa (Hemmings et al., 1990). From all possible PP2A combinations, about 90 % have Aα scaffold subunit, which is highly abundant in all normal tissues, but Aβ is found to be expressed 10-fold less than Aα even in the cells with highest Aβ expression, which would indicate of its role being a minor one (Zhou et al., 2003). Scaffolding subunit has on dual function depending on if PP2A is in a dimeric or in a heterotrimeric form. When in heterotrimeric form, A-subunit functions as a structural assembly base to bring B-subunits together with C-subunit. On the other hand, the A-subunit plays an important role in regulating the substrate specificity of the catalytic subunit in the absence of the B-subunit, when in dimeric form. A-subunit has an important property of being able to bind to the many forms of B-subunit and to two different forms of C-subunits. This is remarkable because when comparing B-subunits with each other, they are unrelated or weakly related, but still can bind to different or overlapping sites of A-subunit. It is no wonder that large number of holoenzymes exists. Aβ isoform mutation is found in many human cancers, which are defective in the binding of B- and C-subunits. This would indicate that Aβ-variant has a special role in growth control, whereas Aα has more like housekeeping functions. Aβ was also found to be unable to bind with B55-subunit (Zhou et al., 2003). Scaffolding subunit contains 15 tandem repeats of a conserved 39-residue sequence known as HEAT (huntingtin elongation-A subunit-TOR) motif, which folds into a horseshoe shape, and thereby hold the catalytic and regulatory subunits together on the same side. These repeats are formed as extended, L-shape molecule. (Xu et al., 2006.)

1.4.3 Regulatory subunit (B-subunit)

PP2A has four different B-subunit families in human genome, which are denoted as B (B55), B´(B56), B´´(B72) and B´´´(STRN), which determine substrate specificity and cellular location as well as spatio-temporal functions of PP2A. Each of these families have multiple isoforms, and these isoforms are theoretically allowing 60 different PP2A holoenzyme assemblies to exist in human cells. The numbers indicate the molecular weight in kDa. PP2A has a diverse structure and it is encoded by 15 different genes in the human genome. B55 is thought to be the most important regulator of the PP2A holoenzyme and it is thought to act as a targeting modulator providing spatio-temporal specificity. (Zolnierowicz et al., 1994.) A certain isoform of B55 functions as tumor suppressor and the best characterized oncogenic target for B55 is AKT. Different B55 variants bind to similar recognition motifs of PP2A Aα, but their protein sequences are not similar. B55 has four different isoforms, which are α, β, γ and δ and these are expressed in a tissue-specific manner. Studies have shown that, the levels of B55γ increase and B55β decrease sharply after birth and these are highly expressed in brain. B55α and B55β are mainly cytosolic and have a distinct localization patterns within neurons. B55γ is enriched in the cytoskeletal fraction. B55δ and B55α have a widespread tissue distribution. B55 has a role in cytoskeletal forces, movements and in nuclear translocation in mammals. B55 fibroblast knockouts cannot dephosphorylate vimentin, resulting in alteration in the interphase dynamics, differentiation and migration. (Turowski et al., 1999.)

B56, has five isoforms, which are α, β, γ, δ and ε. These isoforms have one feature in common, which is being able to be phosphorylated and they are mostly found in α helical shape. These different isoforms are directly bound to the core enzyme and enhance the reaction. The most noticeable difference between these isoforms is found in N- and C-terminals regions, but otherwise they have 80 % identical sequences in their central region. By looking at the differences in N and C terminal parts, the variable expression in different tissues can be explained. Different isoforms of B56 and their intracellular localization varies from each other. B56α, B56β and PR56ε are expressed in the cytoplasm, B56γ in the nucleus and B56δ in both of these. (Janssens & Goris, 2001.)

The third include from two isoforms that are PR72 and PR130. The only difference between these two is in N-terminal region. This might be caused of alternative splicing. These are also expressed in different tissues, where PR72 is exclusively expressed in the heart and skeletal muscle, whereas PR130 is found to be expressed in almost all tissues but still having the highest expression in the heart and muscle. Other isoforms included in this family are PR48, G5PR and PR59 (Voorhoeve et al., 1999.)

Last family of B-subunits is the striatin (STRN) family consisting from STRN, STRN3 and STRN4. This family was poorly understood and studied, until just recently. It was found out that one STRN, STRN3, functions as upstream activator of YAP via interaction of MST1/2 dephosphorylation and subsequent inhibition of the tumor suppressor Hippo pathway. Overexpression of STRN3 has been found in human gastric tumors. (Tang et al., 2020.)

Each regulatory subunit of PP2A has different functions. Each PP2A trimer may have specific physiological activity depending on the cell type, as well as the tissue type. Tissue and cell type also impose other constraints, such as what part of the cell they are located at, as well as the selective protein interactions between different B subtypes and their target protein. These different functions partly explain why only some of the B subunits suppress tumors, due to the fact that certain B subunits act as antagonists of oncogenic signaling pathways. (Meeusen & Janssens, 2018; Sablina et al., 2010; Westermarck & Hahn, 2008.) Certain B subunits have been studied much more than others, such as B56α, b56γ. The best known of these functions are the ability of B56α to regulate MYC phosphorylation (Myant et al., 2015), as well as the negative ability of B56α or B56γ to regulate AKT kinase phosphorylation (Meeusen & Janssens, 2018; Sablina et al., 2010). The importance of these functions in PP2A-mediated tumor suppression was investigated by transforming several human cells. In these cells, PP2A was inhibited, but the effects could be restored by activating MAPK, c-MYC, Wnt and / or P13K signaling. (Laine et al., 2013; Sablina et al., 2010; Tan et al., 2010.)

1.5 PP2A in breast cancer

One of the most common cancer types among female population is breast cancer, which is the most common cancer-related cause of death among women. Annually, there are approximately over one million cases worldwide (Polyak, 2007). This year, it is estimated that 281,550 women in the United States alone, will be diagnosed with invasive breast cancer and 49,290 women with non-invasive breast cancer. Also, it is estimated that 2,650 men in the United States will be diagnosed with invasive breast cancer. Estimations on the part of deaths are 44,130 from which 43,600 are women and 530 are men (*Breast Cancer: Statistics | Cancer.Net*, n.d.).

Transformation of human cells to become oncogenic requires dysfunction of oncogenes and tumor suppressors (Zhao et al., 2004). One of these tumor suppressor is protein phosphatase 2A (PP2A), that prevents the transformation of human breast epithelial cells (Rangarajan et al., 2004). PP2A's role as tumor suppressor was shown when the somatic mutations occurring in PP2A Aβ were found in 13 % of human breast cancers and from these cases, PP2A failed to suppress the oncogenic activity of RalA GTPase (Sablina et al., 2007), which has been implicated to the regulation of exocytosis, transcription, secretion, cell transformation and tumor progression (Feinstein, 2005). Another

mechanism of suppressing tumor is PP2A's ability to mediate dephosphorylation of serine 62 on c-Myc, resulting ubiquitination and proteolytic degradation of c-Myc, inhibiting malignant cell growth and cellular transformation (Junttila & Westermarck, 2008). Dysfunction of this mechanism can be seen in malignant cells isolated from hematologic cancers, where c-Myc is more stable (Malempati et al., 2006). Interestingly, c-Myc overexpression is found in 40-45 % of human breast cancers, but the amplification of the c-Myc gene is only present in 20-25 % of human breast cancers (Chrzan et al., 2001; Naidu et al., 2002). These observations are suggesting that stabilization of c-Myc protein might contribute to its oncogenic activity in breast cancer.

1.5.1 Reactivation of PP2A tumour suppressor function

The B55 and B56 family PP2A complexes are known to function astumor supressors. Unlike B55 targeting AKT, B56 is targeting MYC. This might explain why these two families are the best studied isoforms and last two has just recently been in the interest of research. Recruitment of these two subunits to the PP2A dimer is sensitive to carboxy methylation of Leu ^{309} in the C-terminal tail of catalytic subunit. This methylation is driven by leucine carboxyl methyltransferase 1 (LCMT1) (Fowle et al., 2019). This methylation can only be reversed by oncogenic protein phosphatase methylesterase 1 (PME1) (Puustinen et al., 2009). Also, cancerous inhibitor of PP2A (CIP2A) (Puustinen et al., 2009), SET (M. Li et al., 1996), and cyclic adenosine monophosphate-regulated phosphoprotein 19 (ARPP19) (Gharbi-Ayachi et al., 2010) has been identified to inhibit PP2A function. Because the overexpression of these inhibitors, cancer can occur without mutation in PP2A genes through PP2A inhibition. It is also important to understand that PP2A gene is rarely mutated in human cancer (Kauko et al., 2020; Kauko & Westermarck, 2018), highlighting the importance of mechanism of function in PP2A inhibitors. Low mutation rate can be explained by multifunctional properties of PP2A. PP2A inhibition never inhibits all PP2A functions because its multifunctional properties of regulating normal cellular homeostasis is needed even in cancer cells. In other words, selective inhibition of one property of PP2A does not compromise organismal well-being. Understanding of mutations in PP2A inhibitor proteins opens new approaches in therapeutic therapies, for example activating PP2A's tumor suppressor activity to the normal level by inhibiting overexpressed inhibitor protein (Westermarck & Hahn, 2008a; Westermarck & Neel, 2020).

Currently, it is known that CIP2A and SET are selectively inhibiting PP2A complexes by interacting with PP2A/B56 complexes (Kauko & Westermarck, 2018). Other specific inhibitions based on specific PP2A complexes are also found, for example ARPP19 is interacting with PP2A/B55 complex. First, PP2A was only thought to function as tumor suppressor. This is not the case in all situations. Two PP2A B-subunits, STRN3 and STRN4, has been found to function as oncogenic B-subunits,

being tumorigenic when dysfunctioning, promoting dephosphorylation of tumor suppressive Hippo kinases MST1/2 and MAP4K4. This leads to oncogenic yes-associated protein 1 (YAP1) hyperactivation (Kim et al., 2020; Kurppa & Westermarck, 2020; Tang et al., 2020). The balance between tumor-suppressor and oncogenic PP2A-complexes is important to determine which role PP2A is playing in different cancers (Kurppa & Westermarck, 2020) and to find out new possibilities in the field of therapeutic approaches. One example is a peptide capable of inhibiting YAP1, which was developed and found to be effective in the way of disrupting PP2A-STRN3 interactions. This molecule is known as STRN3-derived Hippo-activating peptide (SHAP). SHAP was shown to have antitumor activity in gastric cancer (Tang et al., 2020) . Questions remain. When PP2A-STRN3 interactions is disrupted, theoretically there is open complex for another B-subunit to form PP2A trimer. It should be studied more what functions are regulating which B-subunit is binding to the dimeric form? Then other questions rise, how inhibiting of one subunit changes the balance if it is replaced with some other subunit?

Activation of PP2A´s tumor suppressor activity via natural inhibitor mechanisms has been one approach for over a one and half decade. It has faced many obstacles and one is pharmacological reactivation being problematic because of possible side effects (Junttila & Westermarck, 2008; Neviani et al., 2005; Westermarck & Hahn, 2008b). Some promising results has been found in knockout mice that are lacking CIP2A. It was seen that there were reduced tumorigenesis without any major side effects, which is suggesting minimal side effects with PP2A-B56 activation (Laine et al., 2013; Ventelä et al., 2012; Jiao Wang et al., 2017). These promising results were further studied by two independent research groups that developed a series of small molecules being safe and effective in cancer animal models (Morita et al., 2020;Leonard et al., 2020; Sangodkar et al., 2017). These studies used derivatives of phenothiazine (PPZ) that was earlier found to be reactivating PP2A (Gutierrez et al., 2014). Downside with these series were found to have side effects in the doses required for tumor suppressor activity.

Two promising compounds were recently studied, DT-061(Leonard et al., 2020) and iHAP1(Morita et al., 2020). These function in a way that increases the affinity of the methylation-sensitive B55 and B56 subunits to the PP2A dimer containing PP2A-C methylated at L309 (Westermarck & Neel, 2020).

1.6 Cancerous inhibitor of PP2A (CIP2A)

CIP2A can do what small T-antigen does to promote transformation of immortalized HEK-TERV cells, which implicates CIP2A's role as an oncogenic PP2A inhibitor (Junttila et al., 2007). There is also evidence which indicates that CIP2A is essential for transformation of JNK2 mouse embryo fibroblasts, that are defective for RAS-driven transformation (Mathiasen et al., 2012). Published

research from several groups have established that CIP2A directly promotes malignant transformation and CIP2A inhibition in fully malignant cancer cells leads to decreasing cell viability and anchorage-independent growth (Böckelman et al., 2011; Chen et al., 2010; Côme et al., 2009; Dong et al., 2011; Junttila et al., 2007; Khanna et al., 2009; Lucas et al., 2011; Niemelä et al., 2012; J. Wang et al., 2011). CIP2A is also known to promote resistance to therapy-induced apoptosis, senescence induction and cell self-renewal (Laine et al., 2013; Tseng et al., 2012; Ventelä et al., 2012). CIP2A has a crucial role in regulating cell cycle and mitosis by directly targeting PLK1. Several studies indicate that depletion of CIP2A by small interfering RNAs, inhibits the growth of xenografted tumors of various cell types (Côme et al., 2009; Khanna et al., 2013; Xue et al., 2013).

Current understanding about proteins interacting with CIP2A is not complete. In order to understand why CIP2A is overexpressed in breast cancer instead of retaining its normal level, it would be useful to know the protein interactions with CIP2A. Also in order to target CIP2A therapeutically, understanding the crystal structure is very critical. Jiao Wang et al (Jiao Wang et al., 2017) found out that homodimerization occurs in the region between amino acids 388 and 559 by using the SID analysis. They also did structure folded ability analysis, which proposed that this novel homodimerization domain comprises of well-folded domain that is followed by a flexible linker and a predicted coiled-coil domain that is probably disordered. Another study they did was gel filtration analysis. There it was found out that C-terminal fragment tends to aggregate by itself. CIP2A homodimerization was further studied by them and confirmed that CIP2A forms a dimer comprising of two identical monomers. (Jiao Wang et al., 2017.)

Jiao Wang et al crystalized CIP2A(1-560) fragment and determined its structure using the selenium-methionine single-wavelength anomalous scattering method at 3.0 Å. They found out that there are two CIP2A(1-560) molecules in the crystal lattice, related by a non-crystallographic twofold axis, conforming CIP2A homodimerization. In this published crystal structure, two CIP2A (1-560) monomers interact with each other at the C-terminal end in a structure that resembles an oppositely twisted double hook. CIP2A(1-560) monomer is an all-helical protein, mostly formed from armadillo and armadillo-like repeats. This monomer can be divided to three parts: tip (1-185 amino acids), stem (188-505), and C-dimerization subdomains. Tip domain consist from five shortened armadillo repeats. Stem domain comes after twist-forming loop. It consists of armadillo repeats 6-11. Stem domains residue 507-559 is responsible for CIP2A dimerization by its three helicases. Closest structural similarity of stem domain is found with HEAT-repeat domain of Wapl. (Jiao Wang et al., 2017.)

The dimerization subdomain is formed by the last three helicases of CIP2A(1-560). The last two C-terminal ends of the CIP2A homodimer are close to each other, both pointing to the top side of twisted

double hook. The most important interaction residues to be noted are V525, L529, L533, L546 and L550 amino acids. Jiao Wang et al did single-point mutations to the spots that were predicted to interfere with the homodimerization of CIP2A. From those mutations, L533E was disrupting dimerization by 70% which was the highest among all mutants tested. L553E was found to directly have an interaction in the dimerization whereas other mutation, R552D, only disrupted dimerization by 50%. This can be explained by R552 not being directly in interaction with CIP2A dimerization. Mutational analysis proved CIP2A homodimerization being mediated by three helix subdomain, (507-559) forming a planar interaction surface. (Jiao Wang et al., 2017.)

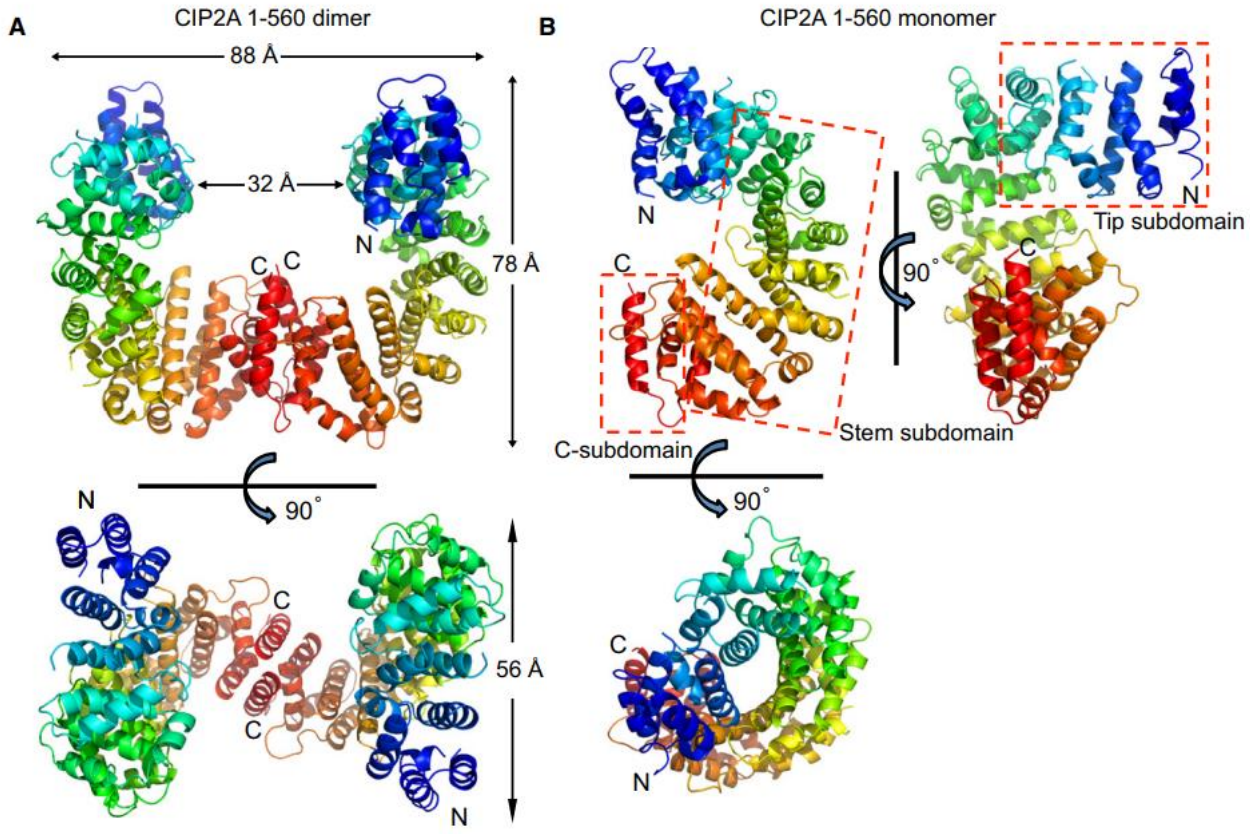

Figure 2: 3D crystal structure of CIP2A(1-560) protein in dimer (A) and in monomer (B) (Jiao Wang et al., 2017)

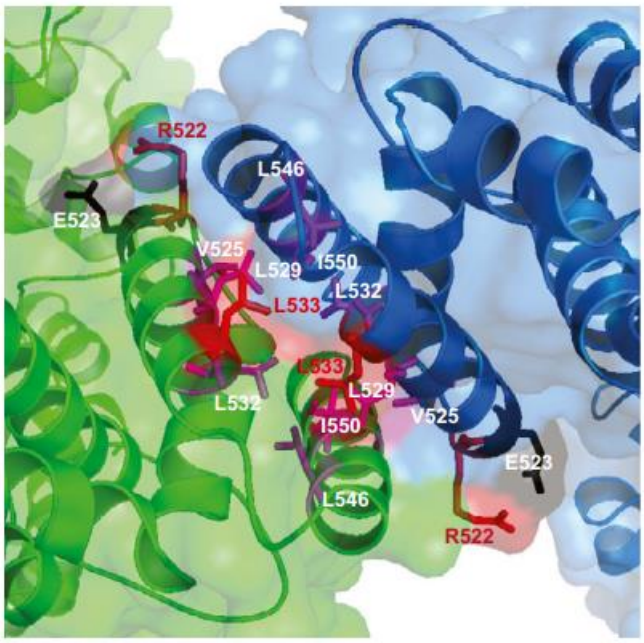

Figure 3: Key amino acids interactions in CIP2A dimerization. (Jiao Wang et al., 2017)

Previously, there were no sufficient evidence of CIP2A directly interacting with PP2A, inhibiting its normal function. Jiao Wang et al were able to identify one of the PP2A subunit (B56γ) being in direct contact with full-length of CIP2A using Y2H assay. They also reported that CIP2A is only directly in contact with B56γ and not with A and C subunits. They repeated these results with GST pulldown and MST experiments. Another important tumor suppressor B-subunit (B56α) was also found to directly interact with CIP2A. (Jiao Wang et al., 2017.)

Jiao Wang et al hypothesized from their previous results that because of CIP2A dimerization mutants inhibited binding only by 50%, there most likely are other binding region to B56 than CIP2A(1-560). They proved that by using mutants created via site-directed mutagenesis, CIP2A binds to PP2A via co-operation of N-terminal region of CIP2A and CIP2A dimerization. These results are giving reasonable reason to think of new approach in the therapeutic treatments. By being able to target CIP2A binding to B56, it could be potential mechanism on structure-based strategy for therapeutic inhibition of CIP2A protein stability and activity. Jiao Wang et al show that the binding of CIP2A to PP2A/B56 stabilizes this protein. With a single-point mutation in the CIP2A dimerization interface, they were able to detect degradation of CIP2A protein in cancer. In other words, if CIP2A dimerization can be inhibited, CIP2A will be degraded being unable to inhibit PP2A normal functions. (Jiao Wang et al., 2017.) My practical part of thesis will focus on this hypothesis.

1.6.1 CIP2A in cancer

The overexpression of CIP2A protein has been found to be a common phenomenon in many tumor types for example, ovarian, lung, gastric and in breast cancers (Soofiyani et al., 2017). The overexpression is found from 39 % to 90 % of the patient samples. This indicates that CIP2A overexpression is one of the most popular molecular alteration among human cancers. The expression of CIP2A is one of the prognostic biomarkers in cancers, for example gastric, bladder, ovarian, tongue, hepatocellular, colon and non-small cell lung carcinoma. (Soofiyani et al., 2017.) This overexpression has been correlated with tumor grade in many cancers (Soofiyani et al., 2017). It can also be seen that CIP2A negative tumors respond significantly better to cancer therapies (Böckelman et al., 2011; Laine et al., 2013), highlighting CIP2A's importance for cancer cells. CIP2A overexpression is driven by many oncogenic signaling pathways, like MYC (Khanna et al., 2009), activation of EGFR-MEK-ETS1 pathway (Khanna et al., 2011), inactivation of p53, or the overexpression of E2F1 (Laine et al., 2013) and ATF-2 (Mathiasen et al., 2012). From these examples, only MYC-responsive region that binds to the CIP2A promoter has not been found. Recently CHK1 has been identified to stimulate CIP2A transcription in cancer cells, where CHK1 is phosphorylated on serine 345 by another DNA-damage kinase (Khanna et al., 2013). Often, cancer is caused by problems and deregulation in DNA repair mechanisms being unable to repair the damaged DNA, which is found in most cancer cells. This could be the explanation for the widespread overexpression of CIP2A in many cancers.

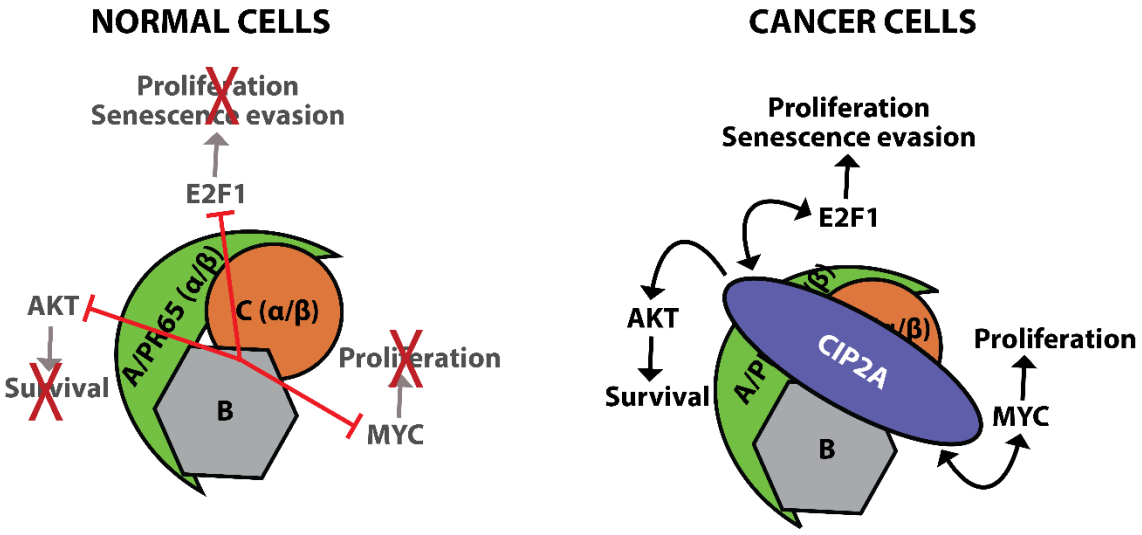

Figure 4: Schematic presentation of PP2A and CIP2A.

MYC is a well-studied oncogene, and its presence is found in multiple malignant cellular processes and it is regulated in many ways. It is worth noticing, that MYC mRNA and gene amplification fail to explain the high frequency of MYC protein overexpression in most of the human cancers. This might be caused by MYC posttranslational regulation, which leads to high MYC levels in human tumors. MYC is regulated by several kinases, ERK, JNK2, CK2 and Cdk1 that phosphorylates MYC

on serine 62, which is the target of PP2A activity. (Khanna et al., 2011; Yeh et al., 2004.) When PP2A is inhibited, MYC phosphorylation on serine 62 increases, which leads to enhanced MYC stability and enhanced transcriptional and oncogenic activities (Alonso et al., 2004; Yeh et al., 2004; Zolnierowicz et al., 1994). This stability is found in many human cancer types, especially in breast cancer.

Another CIP2A related protein is found to be overexpressed in breast cancer. It is CIP2A-E2F1 feedback loop, where E2F1 is oncogenic transcription factor. This transcription factor binds to CIP2A promoter in 375 base pairs upstream of the transcription starting site. CIP2A is found to be inhibiting PP2A's ability to phosphorylate E2F1 on serine 364, which would inactivate E2F1. When p53 activity was decreased, it led to increased expression of CIP2A by E2F1. This forced expression of CIP2A entirely prevented inhibition of E2F1 by p53 and senescence induction. These results are indicating that CIP2A-E2F1 positive feedback loop has a crucial role in modulating the p53 response in cancer cells. This might be a potential target mechanism for pro-senescence therapies, mainly in cancers where p53 mutation has been found. (Laine et al., 2013.)

1.7 Triple-negative Breast cancer (TNBC)

Triple negative breast cancer (TNBC) lacks on expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (Ryu et al., 2011)(Ryu et al., 2011). This cancer type is diagnosed in 10-15 % of all cancer types among Latin, African and African American women, it being considerably one of the most found cancer type among women populations (Lund et al., 2009). TNBCs are also known to have aggressive behavior, early relapse and metastatic spread to the lung, liver, and central nervous system. This indicates poor prognosis and survival for patients diagnosed with TNBC. When patients are diagnosed with TNBC, they usually have a positive axillary lymph node, larger primary tumor size, pushing borders and poorer Nottingham prognostic index, calculated using pathological criteria and used to determine prognosis following surgery for breast cancer. (Rebecca Dent et al., 2007.) These findings indicate poor prognosis.

TNBCs tumor heterogeneity has been highlighted to be the reason for different clinical outcomes, with different response rates when using traditional treatments or new targeted therapies. This leads to unpredictable probability of patient surviving TNBC. (Burstein et al., 2015; Lehmann et al., 2011; Prat et al., 2010; Sørlie et al., 2001). Genomic expression profile (GEP) assays have been used for molecular characterization of TNBC subgroups defining their molecular fingerprints, but like Lehmann et al. (Lehmann et al., 2011) and Burstein et al. (Burstein et al., 2015) showed, interactions between proposed subclassification groups may occur. Best subclassification was proposed and TNBCs were divided into 7 molecular subtypes: immunomodulatory (IM), mesenchymal (M),

mesenchymal stem-like (MSL), luminal androgen receptor (LAR), unstable (UNS) subtype and basal-like subtypes (BL1 and BL2). Later Lehmann performed subclassification only to four groups (BL1, BL2, M and LAR) (Lehmann et al., 2016) . With cluster analysis of GEP and immunohistochemistry (IHC), this subclassification is used as prognostic and predictive tool. This way, specifications for discovering new drugs and design of clinical trials with better patient selection for personalized treatment leading to improved response and survival outcomes can be done.

1.7.1 Current treatments for TNBC

In practice, cancer is rarely treated by a single treatment. Instead, it is usually a combination of different treatments. Surgery is one of the most effective treatments for the patient with cancer, but it is often difficult and always has a risk of failure. To minimize the risks, patient is often treated before and/or after with radiation therapy, chemotherapy or by using both. The goal is to minimize the risk of surgery by reducing the size and number of cancer cells and to control the progression of the cancer before the actual treatment. After the surgery the goal is to support the treatment by preventing the growth and survival of the remaining cancer cells in the body. These pre- and after treatments refer to the adjuvant and neoadjuvant treatments, respectively. It is worth of noticing that radiation therapy and chemotherapy has risks on their own, so it needs to be validated what treatment or combinations of treatments would most likely have the best outcome for the patient. (Shien & Iwata, 2020.)

In general, treatment of TNBC patients is initiated with anthracycline-based chemotherapy, followed by treatment with a taxane (Cardoso et al., 2019). According to clinical data, TNBC cancers would be susceptible to platinum salts, which in turn would support the use of platinum-based chemotherapies in advanced TNBC patients. It has been noticed that platinum-based neoadjuvant therapies could be combined with a more positive pathological response, but there is no information on the long-term benefits because there is not enough and reliable data on the long-term benefits. (Poggio et al., 2018.) Some recent long-term data have been obtained relating to platinum therapy, without anthracycline (Pandy et al., 2019; Yu et al., 2020; Zhang et al., 2021). The problem with this data, however, is that the use of carboplatin in a neoadjuvant environment is still controversial and widely discussed (Balic et al., 2019; Poggio et al., 2018; Torrisi et al., 2019). This is due to inconsistencies in the available data and differences in experimental setups.

Nowadays, TNBC therapies are mostly based on the quality of BRCA mutations and its associated specific features as well as PDL1 expression (Caparica et al., 2019). Finding appropriate treatment for such TNBC patients begins with testing the response with PARP1 inhibitors or platinum-based chemotherapy in the case of germline BRCA mutation. In the case of TNBC, which is due to overexpression of PD-L1, the starting point for the treatment of the cancer should be atezolizumab

and nabpaclitaxel. Chemotherapy is advised when the TNBC is identified as wild-type BRCA without PD-L1 expression, because of the outcome would be the most beneficial one. (Caparica et al., 2019.)

In most cases, sequentical single type of medication is the most optimal starting point. This is because the combination of treatments should be reserved for patients who have a great deal of physical difficulties, such as pain and life affecting symptoms. Combination therapies should also be reserved for patients whose cancer is progressing rapidly or whose organs are failing (visceral crisis). The primary treatment options are taxanes and anthracyclines, which slowed down the progression of the cancer. There are many other treatments available, but the use of these should be discussed with the patient. When deciding proper treatment, patient opinion is taken into account, as well as the medical history of the patient and safety profile of the treatment in planning phase. However, a combination of treatments should always be considered at each stage of the disease if its use would be more beneficial than individual treatments. (Cardoso et al., 2018.)

The use of once approved therapies with other therapies is widely studied. One large study called The CREATE-X study statistically showed that the use of adjuvant capecitabine after neoadjuvant chemotherapy (NAC) improved patients´ chances of recovering completely from cancer (DFS) or increased their chances of surviving another five years (5 OS), and the positive result was even higher for TNBC patients with 21 % DFS. It needs to be highlighted, that the study only included patients with HER2-negative breast cancer and whose cancer was found to be residually invasive in pathological test, meaning that the cancer cells have survived after chemotherapy and these are spreading cancer cells. (Masuda et al., 2017;van Mackelenbergh et al., 2020).

Thanks to the results of CREATE-X study, two major organizations, Society for Medical Oncology (ESMO) ja American Society of Clinical Oncology (ASCO), have align that the use of the adjuvant capecitabine should be considered as normal follow-up treatment in situations where residually invasive cells are detectable after chemotherapy (Cardoso et al., 2019; Denduluri et al., 2018). The results of this study are also supported by two other studies. According to these studies, the adjuvant capecitabine improved DFS values and was found to be a safe treatment for the patient (J. Li et al., 2020; X. Wang et al., 2020).

There are other alternatives treatments that are being investigated for use in TNBC cases when surviving cancer cells are detected after chemotherapy. An example of such a treatment is Platinum-based chemotherapy or capecitamine for patients with a surviving cancer cell size of more or equal to 1 cm after chemotherapy. Alternatively, cisplatin plus gemcitabine post-neoadjuvant is indicated for the treatment of patients with no evidence of surviving cancer cells. The purpose of the use of

Cisplatin plus gemcitabine is to eliminate microscopic undetectable cancer cells that are likely to exist (Agostinetto et al., 2021.)

It is estimated that in 11 % of all TNBC cases, patients have a pathogenic variant of the germline in BRCA1 or BRCA2 (gBRCA) regardless of age or family history of cancer. Based on that assessment, TNBC patients should be tested for those variants. (Couch et al., 2015.) Proteins expressed by these genes are crucial for the function of homologous repair of double-stranded DNA breaks (HRR) (Pothuri, 2013). When BRCA is mutated in cells, the only mechanism which the cell is able to repair DNA damage is complementary DNA repair processes, using poly ADP-ribose polymerase (PARP) proteins. Because this repair mechanism is the only working repair process in the cell, preventing this will cause the cancer cells to die because the cell will not be able to survive as multiplying amount of DNA mistakes accumulate. Two compounds have been approved as PARP inhibitors, which are Olaparib and talazoparib (Jennifer K. Litton et al., 2018; Robson et al., 2017). The OlympiaD (Olaparib) and EMBRACA (talazoparib) studies showed that these PARP inhibitors significantly inhibited the progression of the cancer compared to the chemotherapy decided by the investigator (Zimmer et al., 2018;J. K. Litton et al., 2020). It should be noticed that these studies did not show a significant improvement in survival, but the studies were not optimized for this point of interest. These two inhibitors are used as a monotherapy treatment option for advanced gBCRA-mutated HER2-negative breast cancer. Unfortunately, PARP inhibitors appear to be weaker in the treatment of metastatic breast cancer patients than in the treatment of metastatic gBRCA breast cancer patients, which on the other hand was to be expected.

The potentiality of PARP inhibitors in therapies is constantly being explored and its use as combination therapy with chemotherapy, targeted agents, radiotherapy or immunotherapy in patients with or without gBRCAm (Zimmer et al., 2018). Brocade 3 was a study evaluating effects of carboplatin and paclitaxel with (test group) or without (control) veliparib in advanced HER2-negative breast cancer patients with gBRCA. According to that study, the use of veliparib significantly increased the value of progression-free survival (FPS). On average the development of cancer was observed as early as one year in the control group, but only after 2-3 years in the treatment group. PARP inhibitors are being studied in use of before or after chemotherapy as a treatment for BRCA wild-type breast cancer patients. (Diéras et al., 2020.) Up to 10 % of gBRCA-WT TNBC have pathogenic mutations leading to homologous recombination deficiency (HRD), leading to a BRCA-like phenotype despite the gBRCA-WT status (Sharma et al., 2020). In such cases, the addition of PARP inhibitors to cisplatin should be investigated. Another alternative approach to the treatment of breast cancer is combining of PARP inhibitors with immunotherapy treatments. This combination is

based on the increase in DNA damage caused by PARP inhibitors, which on the other hand, stimulates the antitumor immune response, leading to the formation of neoantigens. This improves the response to immune checkpoint inhibitors. The amount of neoantigens present in cancer has been found to be dependent on the mutation load. On the other hand, as the amount of neoantigens increases, so does the ICI response. These observations are the basis for combination therapy and immunotherapy with PARP inhibitors. (Hellmann et al., 2018; Mouw et al., 2017.)

The I-SPY 2 study showed that the addition of olaparibine and durvalumab with paclitaxel to patients with high-risk stage II/III, HER2-negative breast cancer, significantly reduced pCR compared with patients receiving chemotherapy alone. The problem with the usefulness of the results is once again the lack of reliable data on the long-term effects of the treatment. Also, the likelihood of the cancer becoming resistant to the treatment or treatment behavior in combinations with other treatments in unknown. One of the special features of TNBC is its genomic stability compared to other breast cancers. This difference can be exploited when using ICI (Pusztai et al., 2020.)

TNBC has a useful property which is greater genomic stability than with other breast cancer subtypes. This particular difference is more optimal for ICI treatments. (Budczies et al., 2015; Denkert et al., 2017.) TNBC´s stromal tumor-invading lymphocytes (sTIL) have been shown to have a strong prognostic value as well as a predictive value for the early response to NAC. Certain sTILs (CD4+, CD8+ and FOXP3+) predicted a positive long-term prognosis of treatment benefits. A number of TNBC patients were randomized to two Phase III trials. This experiment showed that TILs can themselves be used as indicators when assessing the chances of a full recovery from cancer, the likelihood of cancer recurrence, and the likelihood of still surviving after a certain period of time. (Gao et al., 2020; Loi et al., 2017.) Still, there are problems with using immunotherapy. Response rate is lower by the start of the treatment and the more advanced the cancer, the lower the response. (Adams, Loi, et al., 2019; Adams, Schmid, et al., 2019; Dirix et al., 2018; Emens et al., 2019.)

Because of problems with immunotherapy, combination therapies are showing more favorable outcomes and have shown more activity in metastatic TNBC. The combination of the anti-PD-LI antibody atezolizumab and nab-paclitaxel has been found to be significantly more effective than nab-paclitaxel alone when treated with untreated PD-LI-positive advanced TNBC patients. This method of treatment is nowadays the usual treatment (Schmid et al., 2018.) There is a problem to have nab-paclitaxel available worldwide, so in Impassion-131 study, they tried to identify if paclitaxel could be used instead of nab-paclitaxel. Results was disappointing because of use of paclitaxel did not have desired outcome.

Another drug that has been studied and used is pembrolizumabia. One study (Phase III KEYNOTE 355) questions the treatment where this drug alone acts as acting agent (Cortes et al., 2020). This drug has been approved by the U.S. Food and Drug Administration (FDA) and has been used for a some time by now based on some trials (Cheng et al., 2020; Lemery et al., 2017), although KEYNOTE 355 states that pembrolizumabia is not more effective than other alternatives options.

In general, immunotherapy has a high potentiality to be the answer for the TNBC patients that express PD-L1, especially when used as combination therapy with chemotherapy. Currently, it is investigated whether immunotherapy could be utilized in TNBC cases other than PD-L1 positive ones alone, as well as in utilization in the early stages of the disease. The usage of immunotherapy and its benefits should be studied more. By more data, specific biomarkers could be wound and used for more reliable prognosis. At this point, there are no reliable individual biomarkers to be used in immunotherapy. (Agostinetto et al., 2021.)

1.7.2 New target therapies in TNBC treatment

Targeted therapies in the treatment of cancer are beneficial because these kinds of therapies mainly affect only cancer cells, with little effect on healthy cells. Examples of such a methods are antibody-drug conjugates (ADC). The problem with chemotherapy is the side effect to the healthy cells, which reduces life expectancy the more chemotherapy you have to go through. The benefits of chemotherapy in TNBC patients are also small because of high likelihood of recurrence and treatment is often ineffective. Of course, chemotherapy is only used when the benefits outcomes the disadvantages, but still, there is urgent need for more effective treatments. However, this is not the case when using an ADC. This treatment targets cancer cells without damaging healthy ones. One example of an ADC is Sacituzumab-govitecan-hziy, which is an antibody-drug conjugate that is a combination of SN38 and Trop-2 antigen. SN38 is a topoisomerase 1 inhibitor fused to the humanized antitrophoblast cell surface antigen 2 antibody hRs7IgG1K. These bind to the cleaved protein. Trop2 (on the surface of many epithelial cells) has been found to be present in TNBC cases. It functions as transmembrane calcium signal transducer and is overexpressed inducing cell growth. The principle of the drug is that the Trop 2 antibody delivers the drug to the cancer cells by releasing SN-38 into both microenvironmentally, causing drug-bounded cancer cell death, and intracellularly causing surrounding cancer cells death. No significant side effects were observed with the drug. The studies showed that the medicine had a lasting effect compared with other treatments. (Bardia et al., 2019.) This is a very potential option in the treatment of TNBC patients, and based on Phase I/II studies, the DFA approved the drug for use in pretreated TNBC patients (Agostinetto et al., 2021).

In addition to Sacituzumab-govitecan-hziy, the ladiratuzumab inhibitor is also under investigation. It is an anti-LIV-1 antibody combined with monomethyl auristan (MMAE) through a protease-cleavable linker (Han et al., 2020). LIV-1 is overexpressed in metastatic TNBCs. The principle of action of the compound is similar to that of Sacituzumab-govitecan-hziyn. One phase I/II study investigated the interaction of ladiratuzumab-vedotin and pembrolizumab in patients with first-line TNBC. This combination shows promising clinical activity (Han et al., 2020).

A third drug with the same principle is Trastuzumab deruxteca, which consists of anti-HER2, a cleavable tetrapeptide-based linker, and a cytotoxic topoisomerase I inhibitor. The study found out that the drug had a lasting antitumor effect in the treatment of treated HER2-positive metastatic breast cancer patients. However, the problem is the observed side effect of the drug such as nausea and myelosuppression as well as interstitial lung disease. (Modi et al., 2020.)

ADC is not the only new target therapies in TNBC therapies. One of the interesting ones is P13K/AKT/mTOR pathway as an alternative in the treatment of TNBC. In TNBC, these pathways have been found to be overactive due to dysfunction of key factors such as P13K mutated catalytic subunit PIK3CA or in mutated AKT1 or loss of PTEN function. (Cossu-Rocca et al., 2015.) The key effect of AKT is proliferation and survival, so its overactivity benefits cancer cells. Logically, inhibition of that protein should prevent cell survival. There is and AKT inhibitor (capivasertib) which effectivity was evaluated in randomized Phase II study (Schmid et al., 2020). In that study, capivasertib was added in combination with first-line paclitaxel treatment to TNBC patients, resulting in significantly higher PFS and OS values than placebo-treated patients. The difference between these two groups was even highlighted in patients with PIK3CA/AKT1/PTEN-altered tumors. The LOTUS study supported the potentiality of ATK inhibitors as treatment for TNBC patients because of similarity in study set up, but instead of capivasertib as ATK inhibitor, ipasertib was used. Paclitaxel remained (R. Dent et al., 2020). Inspired by these results, a Phase III study (IPATunity130) was conducted to investigate the effect of ipatasertib in advanced TNBC patients in combination with paclitaxel and paclitaxel alone. However, no significant benefit was observed as a result of the study and it was concluded as negative result (*Ipatasertib Plus Paclitaxel Falters in Advanced Triple-Negative Breast Cancer Phase III Trial - The ASCO Post*, n.d.). The phase II study, FAILANE, also proved out to be disappointing because ipatasertib was not able to significantly increase pCR values clinically or statistically (Oliveira et al., 2019). Phase III CAPItello-290 trial is ongoing study investigating capivasertib. If this trial ends up being negative, it might be the death sentence for at least ATK inhibitors are singe agents (*January 25, 2021 - EPOV Yuan Yuan (1) - The ASCO Post*, n.d.). It may be that AKT is not as important driver for cancer as it was thought to be, which might

be the reason of disappointing results. ATK inhibitors have also been found to cause a variety of side effects, which is why ATK inhibitors are being studied for use in combination with other inhibitors. For example, mTOR inhibitors, dual inhibitors and combination with immunotherapy are being studied (Agostinetto et al., 2021).

There are many disappointments in breast cancer treatment research, but one approach has been successful. This is one of the major advances in the field of cancer therapies in recent years, which is successful development and adoption of cyclin-dependent kinase (CDK) 4 and 6 inhibitors for metastatic HR + and HER2 metastatic breast cancers (Spring et al., 2020). Examples of such inhibitors are palbociclib, ribocyclib and abemaciclib. These are showing significantly increased PFS values when used in combination with endocrine therapy. Ribociclib and abemaciclib have observed to have even better OS value (Spring et al., 2020). More research is still needed to determine the resistance to these inhibitors and possible side effects. One feature of TNBC is the loss of the tumor suppressor retinoblastoma (Rb). This tumor suppressor is used as a biomarker for the sensitivity of CDK4/6 inhibitors in vitro. Rb loss is more common in the case of basal-like TNBC, which explains the lower activity of CDK4/6 inhibitors compared to luminal models (Herschkowitz et al., 2008). Interestingly, palbociclib appears to be able to reduce the adverse effects of paclitaxel on RB-positive TNBC cell lines. However, CDK therapies require identification of the TNBC type in order for these inhibitors to be used with or without chemotherapy.

Androgen receptor positivity has been observed in approximately 24 % of all TNBC cases, and this property has been found to predict a lower risk of cancer recurrence (C. Wang et al., 2016). AR positivity is indeed one of the TNBC subclasses, but sometimes it is overlapping with the LAR subclass (Pascual & Turner, 2019). Two AR inhibitors, bicalutamide and enzalutamide, the latter of which is approved for use in certain cancers were studied. These two were tested in Phase II trial, which showed a weak effect in AR-positive TNBC patients (Gucalp et al., 2013). AR-expression has also been found to be associated with Rb expression, which in turn would indicate the benefits of the interaction between AR inhibitors and CDK4/6 inhibitors (Patel et al., 2020). One study investigated the combined effect of bicalutamide and palbociclib in AR-positive TNBC patients and suggested that the combination would be safe and effective (Gucalp et al., 2020).

1.7.3 Other immunotherapeutic approaches

As mentioned before, immunotherapies are characterized by limited efficacy. This is due in part to the presence of various immunomodulatory factors in the microenvironment of cancer cells, such as purine nucleoside adenosine. It is a metabolite produced by cancer cells, that can weaken immune response. By this function, purine nucleoside adenosine is able to improve cancer growth, metastasis

and avoidance of the immune response. (Arab & Hadjati, 2019.) Therefore, inhibition of adenosine production in cancer cells in combination with immunotherapies is one potential option in the treatment of cancer. CD73 is responsible for adenosine production, and overexpression of that protein is associated with TNBC as well as poor prognosis. Inhibition of the adenosine receptor (A2R) has also been found to inhibit the growth and migration of breast cancers (Buisseret et al., 2018). Based on these observations, there has been increased interest in components that target purigenic signaling pathway and are in clinical trials. 195TiP-SYNERGY is phase I/II study investigating the effect of oleclumab (an anti-CD73 antibody) in combination with durvalumab and chemotherapy in previously untreated, locally recurrent, inactive or metastatic TNBC patients (Maurer et al., 2019).

Another approach in cancer treatments are innate immune activators. The efficacy of the combination of ICI and imprime-PGG as a treatment was evaluated in the IMPRIME 1 study, according to which imprime-PGG in combination with pembrolizukab would be useful as a first-line treatment for metastatic TNBC patients. Imprime-PGG has been found to activate the innate immune response against cancer cells by improving antigen representation and T cell activation. This would be due to the pathogenic molecular structure of imprime PGG. (O'Day et al., 2019.)

The growth of blood vessels (angiogenesis) is essential for organ growth and repair. This is even more crucial for tumor cells these cells need more oxygen and nutrition. Tumor cells secure this by growing blood vessels. These blood vessels formed are structurally chaotic, grow in all directions and form ending vessels and the blood pressure in those blood vessels is high. By cutting off the cancer cells nutrition and oxygen should theoretically reduce the cancer cell growth, which is being attempted by angiogenesis inhibition drugs. However, before using these drugs, neovessel permeability and tumor interstitial pressure needs to be reduced. (Aguiar & Moraes, 2019.) These drugs have been seen to improve patient's response to chemotherapy by mediating interactions with various chemotherapy agents. Antiantogenic agent combinations in combinations of different therapies may be used in the future in TNBC patients, but there is not yet sufficient evidence of efficacy, and in some cases these compounds seems to worsen the situation (Pusztai et al., 2020).

The last treatment I am considering is histone deacetylase inhibitors (HDACi) Histone deacetylase induces genes that in turn increase cell growth and proliferation. Overexpression of this protein has been observed in malignancies. However, histone deacetylase inhibitors alone show a poor response, so their use in combination is being investigated (Garmpis et al., 2017). ENCORE 602 studied the effect of entinostat (a class 1 selective histone deacetylase inhibitor) in combination with atezolizumab in advanced TNBC patients but did not show better results compared to placebo. This combination was also found to be more toxic(O'Shaughnessy et al., 2020). The use of these inhibitors

in TNBC therapies needs to be further studied as potential combinations have been found, but the amount of data is insufficient.

There are other potential therapies that exists for targeted treatment of breast cancer and TNBC but many attempts have failed. Many new approaches are being explored, but the problem is too little evidence against disadvantages and being too new approach, leading to situation when even promising treatments can be total failures because there is a lack of long-term data.

2. Materials and Methods

2.1 Cell culturing and compounds

All cell lines used were cultured using a humidified incubator at 37°C and 5% CO_2. MDA-MB-231, MDA-MB-436 and MDA-MB-468 cell lines were cultured in DMEM (D6171-500ML, Sigma Life Science). HCC38 and HCC1937 were cultured in RPM1-1640 media (R5886-500ML, Sigma Life Science). All mediums were supplemented with 10% (V/V) fetal bovine serum (FBS), 0.5% (V/V) penicillin/ streptomycin (10,000 U/10 mg per mL, Sigma) and 2mM L-Glutamine (Biowest). MDA-MB-231 also was supplemented with 1 % of MEM Non-essential Amino Acid Solution (Sigma Life Science, M7145). All the cell lines were tested negative for *Mycoplasma* during the study. The compounds used are from Maybridge Screening library:AW00907 as CIP1, MBX515163 as CIP2, MBX523401 as CIP3 and MBX523408 as CIP4 were dissolved in DMSO

2.2 Protein isolation

In 6 x well plates, medium was collected, centrifuged at 1000 g for 4 min. Plates were trypzinised until cells detached and collected. Then cells were lysed with RIPA buffer (50 mM TRIS HCl, pH 7.5, 150 mM NaCl, 1 % NP-40, 0.5 % Sodiumdeoxycholate, 0.1 % SDS, supplemented with protease and phosphatase inhibitors (Roche) and incubated for 10 min on ice. Samples were sonicated (UCD-200 Sonicator) 5 min 50 % on/off cycle (medium) and centrifuged at 13200 x g for 15 min.

2.3 Western blot using TNBC cell lines

After lysis and sonication, protein quantification was done by using Pierce™ BCA Protein Assay kit (Thermo scientific, 23227) and absorbance measured at 570 nm. Samples were made for 12 µg per well, using 6x SDS. Gel used in gel run was 4-20 % Mini-PROTEAN TGX Gel (BIO-RAD,). Gel was run in Mini Protean Tetra Cell (BIO-RAD, Serial No 552BR) and power source was PowerPac Basic (BIO-RAD). Gel run was done using 150 V for 50 min. Gel transfer to membrane was done by using Trans-Blot Turbo Transfer Pack (BIO-RAD, #1704158) in Trans-Blot Turbo Transfer System (BIO-RAD) using 1.25 A, 25 V, 7 min. After membrane transfer, primary antibodies were TBS supplemented with 0.1% Tween20 and 0,5 % milk and were incubated overnight at 4°C. Next, secondary antibody was supplemented in TBS like above and incubated 1 H at RT. Then, membranes were scanned using Odyssey fluorescent scanner. Page Ruler Prestained Protein Ladder (26619 Thermo Scientific) was used as a protein size reference. Membranes were visualized by Odyssey CLx (Serial No. 1005, LI-COR) and bands were quantified using ImageJ.

Following antibodies were used: Primary antibodies were 1:1250000 GAPDH (mM Ab (6C5) from Hytest and 1:500 anti-CIP2A (mM Ab(2G10-3B5)) from Santa Cruz Biotechnology. Secondary antibody was IRDye 800CW Donkey anti-Mouse (926-32212).

2.4 Cell viability

Cells (3,000 per well) were seeded into 96-well plates overnight for attachment and then treated with CIP1, CIP2, CIP3 or CIP4 using concentrations 0, 1, 2, 5, 10, 20, 30, 40, 50 and 75 µM. After 24 h, medium was changed and cell proliferation reagent WST-1 (Sigma 11644807001) was added 10 µl per well for 2 hour incubation in the cell culturing conditions. Plate was measured at 450 nm using plate reader (Thermo Multiskan Ascent).

2.5 mRNA using qPCR

MDA-MB-468 were seeded (150,000 per well) into 6-well plates overnight for attachment and then treated with CIP1 using concentration of 12 µM. Cells were lysed, mRNA collected and extracted according to manufacturer's kit protocol (NucleoSpin® RNA MACHEREY-NAGEL, 740955.250). Then, mRNA was converted to cDNA using SuperScript™ II Reverse Transcriptase kit (ThermoFisher Scientific, C.n 18064200). Next 96-well qPCR plate was prepared and qPCR run was done following: hold stage with 50.0 ̊C for 2 min and 95.0 ̊C for 2 min, PCR stage (40 cycles) with 95.0 ̊C for 15 min and 60.0 ̊C for 1 min, melt curve stage with 95.0 for 15 s and 60.0 ̊C for 1 min and back to 95.0 ̊C for 15 s and finally on holding stage of 4.0 ̊C. qPCR was done by using QuantStudio ®12 K Flex Software v1.2.3.

2.6 Colony growth

8 K/well HCC38 cells were seeded to 12 well plate for overnight to attach. Second day CIP1 drug was added to wells. After 24 h, medium was changed and again changed on the sixth day. When control wells were full, crystal violet staining was done by removing medium and adding 1 ml of ice cold methanol per well and incubated 15 min. Next methanol was removed and 0,2 % crystal violet was added for 10 min. Wells were washed with PBS twice and scanned using EPSON PERFECTION V33 Colony area was calculated using Image J ColonyArea plugin (Guzmán et al., 2014)

2.7 Dimerization assay

2.7.1 Protein production

CIP2A(1-560) and CIP2A-V5-His are cloned in pGEX vector. Proteins are regularly expressed in BL21 E. coli cells and in LB medium using 4 l scale. After inoculation of 1l of LB medium with 35 ml of the corresponding night culture, the cells are grown at 37°C (180-200rpm) until optical

density 600 nm (A600) reaches 0.6-0.9. Protein expression is induced by 0.2 mM IPTG (isopropyl-β-D-1-thiogalactopyranoside) for 4 h at 23°C. Bacterial pellets were collected by centrifugation at 6,000 g at 4°C and stored at -20°C. For protein extraction, the pellets were suspended in lysis buffer (200 mM Tris pH 8, 500 mM NaCl, 2 mM DTT, 0.5% Triton X-100 and 1 x Roche Protease Inhibitor Mini Tablets, EDTA-Free, 12575015). Lysozyme (0.2 mg/mL, Calbiochem®, 4403-1GM), DNase (0.02 mg/mL), and 10 mM $MgCl_2$ were added to the suspension and incubated for 30 min at 4°C with gentle shaking. After incubation, the suspension was centrifuged at 10,000 g at 4°C for 30 min and the supernatant was collected to fresh 50 ml Falcon tube. 1 ml of Glutathione Sepharose 4B (GE Healthcare, 27-0846-01) slurry (1:1, washed with the lysis buffer according to the manufacturer protocol) was added to the cell lysate and incubated for 3h at 4°C for GST and GST-CIP2A proteins and overnight at 4°C for GST-CIP2A-V5-His protein. After incubation, the beads were pelleted by centrifugation at 500 g for 5 min at 4°C.

2.7.2 GST and GST-CIP2A(1-560) protein purification

Pelleted beads were washed with 25 ml washing buffer (lysis buffer without lysosome and DNase) in 1 ml polypropylene columns and eluted with 5x 500 µl of elution buffer (100 mM Tris pH 8, 200 mM NaCl, 5 mM DTT, 0.1% Tx-100 and 20 mM Glutathione). Protein samples were dialysed using Slide-A-Lyzer ® Dialysis Casette 10.000 MWCO (Thermo scientific) using dialysis buffer 1 (20 mM Tris pH 8, 100 mM NaCl, 2 mM DTT, 0.05% Tx-100) and stored in a buffer 2 containing 20 mM Tris pH 8, 150 mM NaCl, 2 mM DTT, 0.05% Tx-100 and 10% glycerol. Purity of the proteins were checked by SDS-PAGE after staining the gels with Coomassie dye.

2.7.3 CIP2A(1-560)-V5 His protein purification

After overnight incubation, the beads washing is done like above. The beads were resuspended in 1 ml of dialysis buffer 1. 100 units of Thrombin (17-0756-01) was added to the beads and incubated overnight at 4°C with gentle shaking. After overnight incubation, the samples were centrifuged at 3000 g for 1 min 4°C. Next, the supernatant was passed through a polypropylene column and collected in an Eppendorf tube. Then the beads were suspended in 0.5 mL of dialysis buffer 1 and passed through the same column and collected separately. Fractions were analysed by SDS-PAGE. Next, the protein was dialysed to the storage buffer.

2.7.4 Assay

CIP1 were prepared in DMSO and final concentration in reaction was 20 µM and protein amount was10 pmol in reaction buffer (50 mM Tris HCI pH 7.5, 150 mM NaCL, 2 mM DTT, 0.2 % Igepal (SIGMA, I8896) 10 % glycerol) to the final reaction volume of 150 µl.

GST or GST-CIP2A bait proteins were preincubated with or without 20 uM of CIP1 for 30 min at 37°C (controls have reaction buffer instead of CIP1). After incubation, CIP2A-V5-his prey protein was added and the reaction was incubated for 1 H at 37 °C. Next, 5 µl from each tube were taken as input samples and 5 µl of 2xSDS buffer were added and the samples were heated for 10 min at 95°C.

20 µl of Glutathione Sepharose 4B beads (5 µl of beads and 15 µl of reaction buffer) were added to the reaction tubes and incubated for another 1 H at RT with gentle rotation. Then, the beads were washed with 250 µl of reaction buffer and incubated 10 min at 4°C, and centrifuged at 500 g for 5 min. This wash step was repeated 2 additional times. After washes, bound complexes were eluted off the beads by adding 30 µl 2 x SDS-Page buffer and incubated 10 min at 95 °C in thermoblock, then centrifuged at 3,000 for 1 min. Supernatants were collected to new Eppendorf tubes and gel was loaded with 10 µl of sample. Gel run was done in 100 V.

Input samples and eluted samples are resolved on 4-20 % SDS-PAGE, transferred on PVDF membrane and analysed by western blotting. Antibodies used were anti-V5 (mM Ab, Thermo Fisher Scientific E10/V4RR, used at 1:5,000), and anti-GST (polyclonal rabbit (pR Ab), Thermo Fisher Scientific CAB4169, used at 1:5,000). Antibodies were diluted in TBS supplemented with 0.1% Tween20 and 5% milk and were incubated overnight at 4°C. Secondary antibodies used were: polyclonal goat anti-mouse immunoglobulin-HRP (P0447) and polyclonal swine anti-rabbit (P0399), both from Dako and used at 1:5,000 dilution for 1 hour at RT.

To visualize antibody-antigen complexes, Pierce ECL Western Blotting Substrate (32106, Thermo Scientific) was used and incubated with the membranes for 1 min. The following films were used: Fuji Medical X-Ray Film (47410 19284, Fuji Film) and UltraCruz Autoradiography Film (sc-201697, Santa Cruz Biotechnology). Page Ruler Prestained Protein Ladder (26619 Thermo Scientific) was used as a protein size reference. Images were quantified using Image J. Data was plotted with GraphPad Prism6.1 showing mean + S.E.M.

3 Results

3.1 Aim of the study

My master's thesis aim is to identify if four compounds, could be used as direct CIP2A protein inhibitors as predicted based on computer modeling (Bhowmick et al., 2020). CIP2A is a homodimer consisting of two identical monomers. These compounds are predicted to prevent CIP2A dimerization leading to cellular death of CIP2A-dependent TNBC cell lines. These compounds are proposed to affect dimerization by binding to specific residues in the CIP2A homodimer interface binding site. When the dimer cannot form, CIP2A will be degraded leading to PP2A normal cellular activity and TNBC cell death. CIP2A inhibition would also prove the concept of it being possible target in drug discovery and not kept as "undruggable". We have an urgent need for new cancer therapies because existing drugs and treatments are not able to affect some cancers like triple-negative breast cancers. Especially, basal like TNBC lacks receptors that nowadays drugs are based on and other treatments has been found to be insufficient. This leads in the loss of hope when patient is diagnosed with basal like TNBC. By TNBC being one of the leading causes of mortality among female population, it has a negative impact socially and economically. This has to chance and new approaches have to be studied. This justifies the importance of my study.

3.2 Computer modeling for potential CIP2A inhibitors

"Maybridge Screening Collection" compound library, consisting of approximately 62 000 small molecules, was used to find out potential CIP2A inhibitor candidates for possible therapeutic cancer applications. Small compounds having a strong binding affinity for CIP2A homodimer interface region, were screened out by setting XP-glide threshold score to -8.00 Kcal/mol manually to reduce chemical space and to select best-top scored compounds. With these thresholds only 17 compounds were left. With IFD, seven best IFD score poses, using threshold of less or equal to -2000.00 Kcal/mol, were chosen based on combination of Glide Score functions and Prime energy values for subsequent molecular modeling analyses. This score threshold was chosen to include both receptor and ligand flexibility into account. The binding interaction stability and understanding the structural and conformational changes, these seven compounds were run with long range atomistic MD stimulations studies, which diminished possible compounds to four: MBX515163, MBX523401, MBX523408 and AW00907. For clarifying, proposed compounds are named AW00907 as CIP1, MBX515163 as CIP2, MBX523401 as CIP3 and MBX523408 as CIP4 in my thesis.

3.3 Cell viability assay

Computer modeling only predicted that If these compounds are able to interact with CIP2A, these will compete with dimerization leading to CIP2A degradation. There were no guarantee of

compounds being able to have any kind of effect in vitro, which was the first study that had to be done. Five different cell lines were chosen to be tested with these compounds based on Laine et al (Laine et al., 2020) . The chosen cell lines and their CIP2A dependency according to Laine et al (Laine et al., 2020) are: HCC1937, MDA-MB-231, MDA-MB-468, MDA-MB-436, and HCC38. They found out that CIP2A dependency varies from lowest HCC1937-> to highest HCC38 in order. Cell lines were cultured normally, and cell viability assay was done. Cell viability assay has become a one of the most used technology in life sciences and from wide variety of assays I used cell proliferation reagent WST-1. Here, cell viability assay was based on WST-1 cleavage to a soluble formazan by a complex cellular mechanism mostly occurring at the cell surface. This bio reduction is largely dependent on the glycolytic production of NAD(P)H in viable cells. Amount of formazan dye formed is directly correlated to the number of metabolically active cells in the culture. This technique has many advances in comparison with other cell viability assays for example there is no need for washing or harvesting of the cells and reading can be done in the same microplate where treatment was done. WST-1 reagent was used because of its advances in contrast with MTT that is cleaved to water-insoluble formazan crystal, which has to be solubilized before being able to get the data. In comparison with MTT, WST-1 has a wider linear range and shows accelerated color development. Results of the cell viability assay are shown in figure (Figure 5) and table (Table 1) below. The IC50 were calculated for every cell line using PrismGraph 8, which corresponds to the CIP1 concentration where 50 % of the cells are dead after 24 h in comparison with the control (untreated).

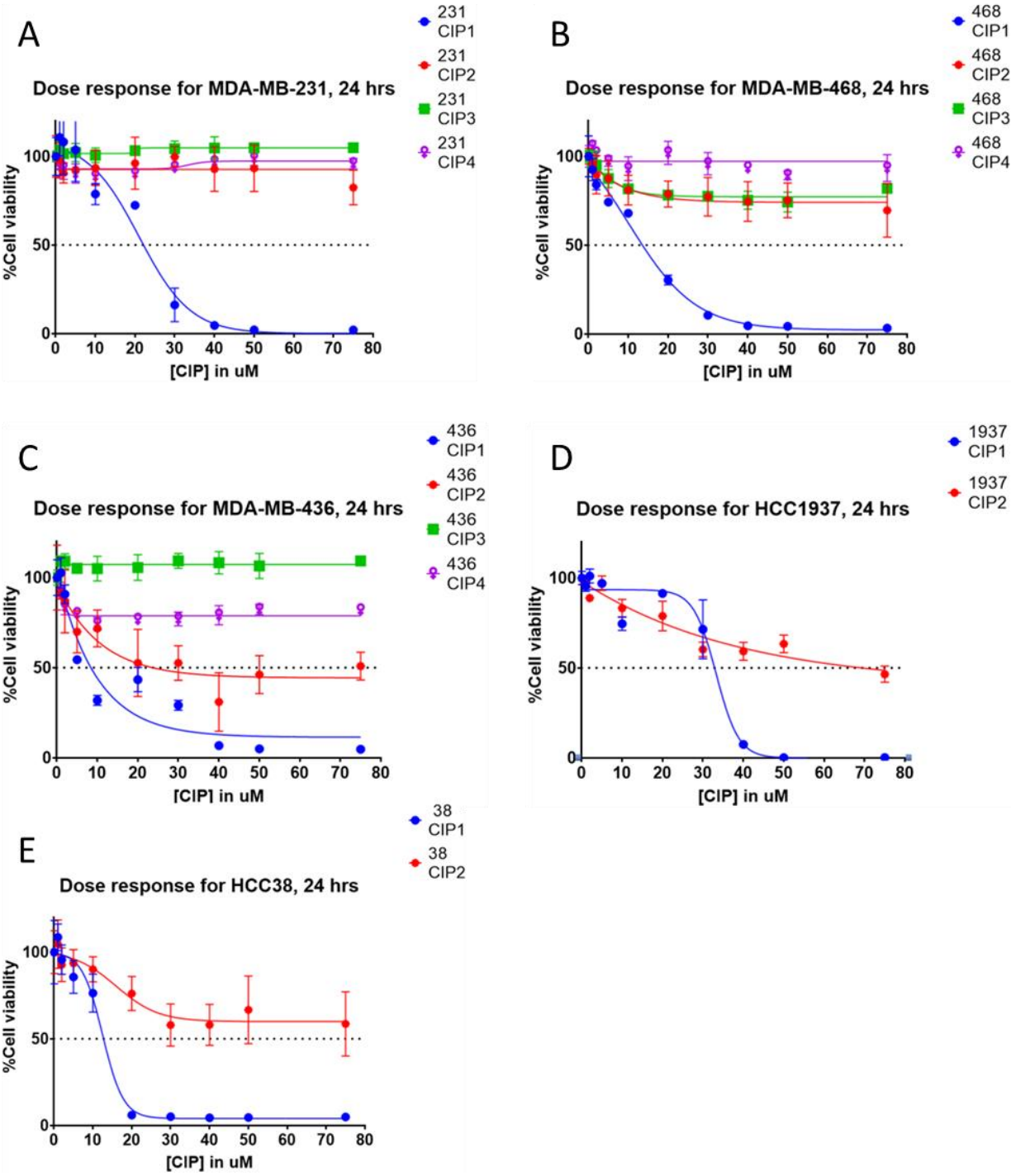

Figure 5: MDA-MB-231 (A), MDA-MB-468 (B), MDA-MB-436 (C) cell lines were treated with compounds CIP1, CIP2, CIP3 and CIP4. HCC38 (D) and HCC1937 (E) cell lines were treated with compounds CIP1 and CIP2. Cells were treated with indicated drug concnetrations for 24 hours. Compound treatment was done in 24 h time point. y-axisindicates percentage of cell viability and x-axis indicates concentration of compounds used in µM. Graph was drawn by using PrismGraph

Table 1: Calculated IC50 for MDA-MB-231, MDA-MB-468, MDA-MB-436, HCC38 and HCC1937 cell lines treated with CIP1. Calculations were done by using PrismGraph

Cell line	MDA-MB-436	HCC38	MDA-MB-468	MDA-MB-231	HCC1937
IC50 CIP1 µM	7,11	11,94	14,68	21,05	32,96

3.4 Colony growth

Next, HCC38 cell line was decided to be tested using colony growth experiment. Advances of this method is that the result can be seen by eye and also it can be quantified easily. Colony growth was done by using HCC38 cell line with four concentrations of CIP1. Results are shown in the colony growth figure (Figure 6) and as quantified data (Figure 7).

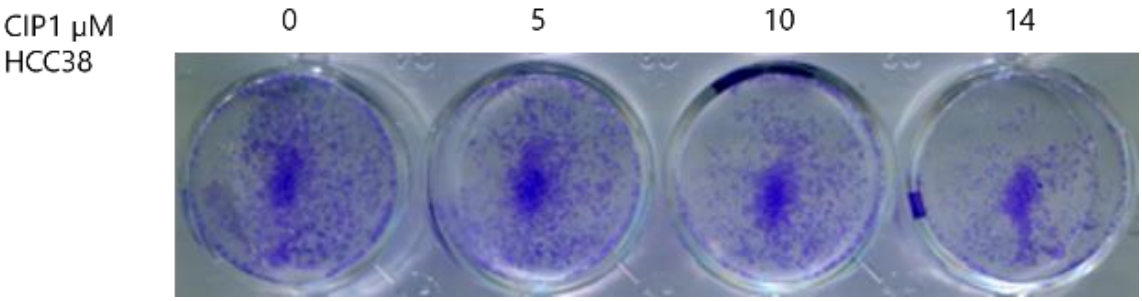

Figure 6: HCC38 cell line was grown without (control) CIP1 or in presence of 5, 10, and 14 µM of CIP1. When the control cells were 70% confluent, the colonies were stained with crystal violet.

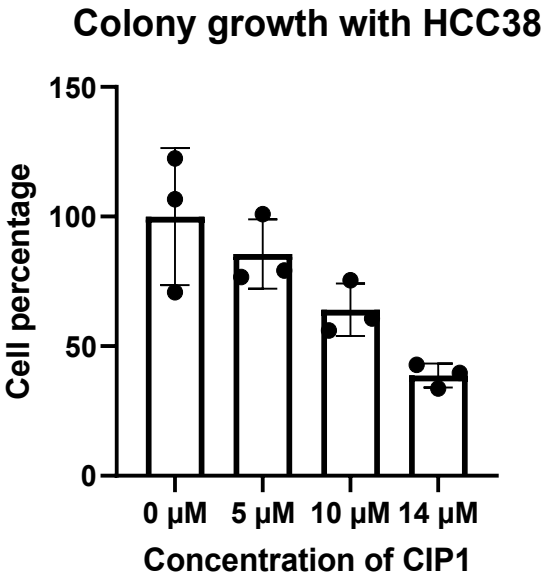

Figure 7: Quantified data from HCC38 colony growth plate (figure 5). Colony areas were quantified using ImageJ ColonyArea pluginGraph (Guzmán et al., 2014) was done by using PrismGraph 8.

3.5 Western-blot using TNBC cell lines

Protein expression was studied using western blot. It was easily implicated, because, if CIP2A dimerization is inhibited, CIP2A will be degraded. In western, this should be seen as decreased band in comparison with control that was not treated. CIP2A antibodies used in this experiment were

routinely used in the research group and found out to be working well. Experiment was done by finding suitable CIP1 dose that does not kill too many cells and has an effect on CIP2A level in Western blot comparing with the control. Concentrations used to find suitable CIP1 dose was found by using concentrations between no effect to all cells dead so there are no western results with highest concentrations used. This is because of there were not enough of cells to be collected in the culture plate for protein extraction. CIP3 and CIP4 were also studied with western blot using 40 µM of compound. Commonly when researching early stage drug discovery, this concentration is way too high and often can be seen as toxic effects on the organ. It can be said that by this concentration CIP2A expression should be decreased significantly if more research with CIP3 and CIP4 would be justified to be done.

Western blots were first done with cell lines HCC38, MDA-MB-436, MDA-MB-231 and MDA-MB-468. Whit these four westerns, cell lines were treated with four different concentrations of CIP1 and a control (untreated). This was done to identify if CIP1 is able to decrease protein expression level in these cell lines. Results are shown in the figures (Figure 8, Figure 9, Figure 10) below.

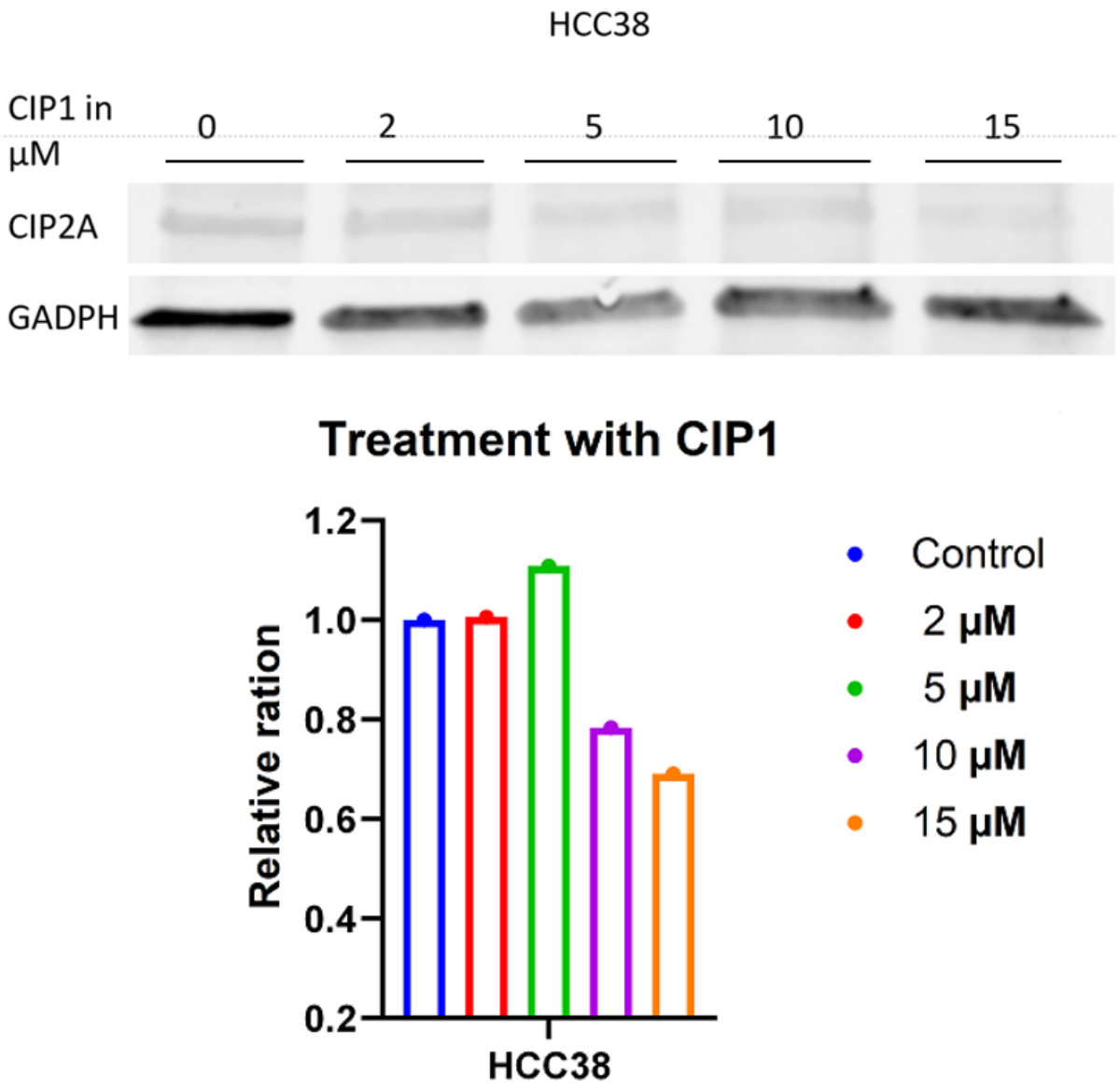

Figure 8: **Western blot data using HCC38 cell line. CIP1 (0, 2, 5, 10, 15 μM in 24 h) effect to CIP2A protein level was quantified from western blot using ImageJ and PrismGraph 8.**

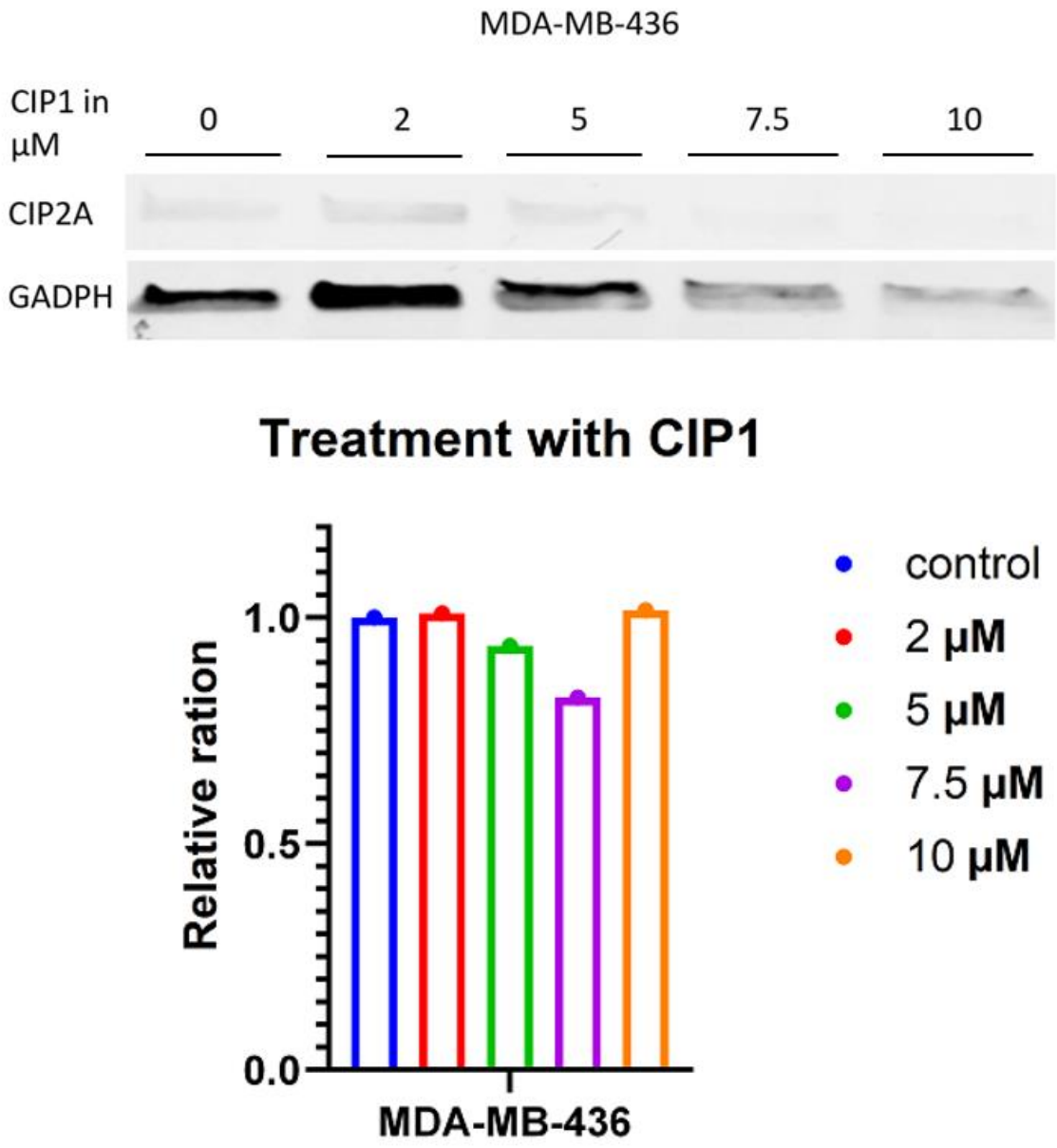

Figure 9: Western blot data using MDA-MB-436 cell line. CIP1 (0, 2, 5, 7.5, 10 µM in 24 h) effect to CIP2A protein level was quantified from western blot using ImageJ and PrismGraph 8.

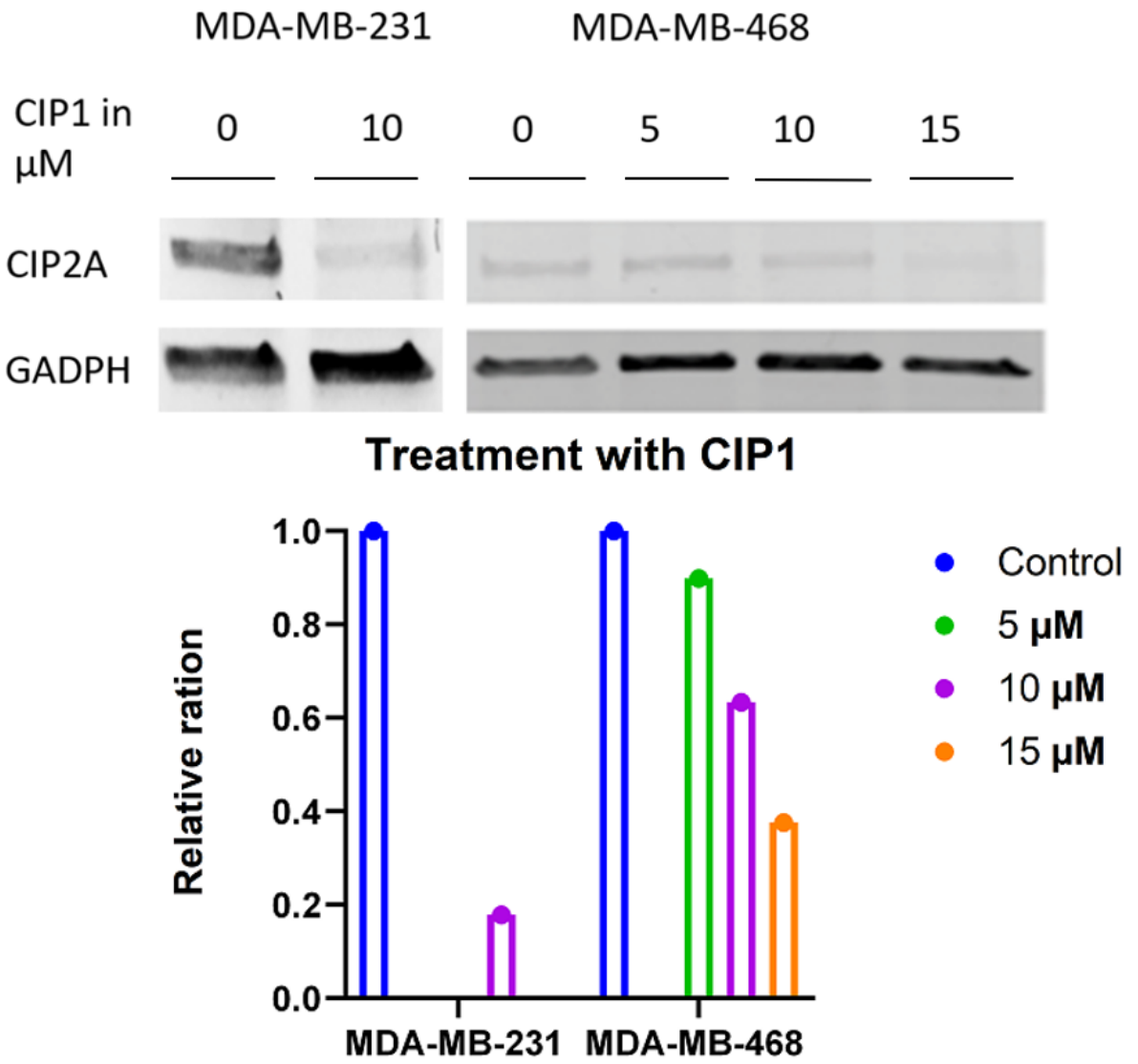

Figure 10: Western blot data using MDA-MB-231 and MDA-MB-468 cell lines. CIP1 (0, 10 µM in 24 h with MDA-MB-231 and 0, 5, 10, 15 µM in 24 h with MDA-MB-231) effect to CIP2A protein level was quantified from western blot using ImageJ and PrismGraph 8.

Then, usable CIP1 concentrations showing decrease in CIP2A protein expression level were chosen and run in a same gel (Figure 11).

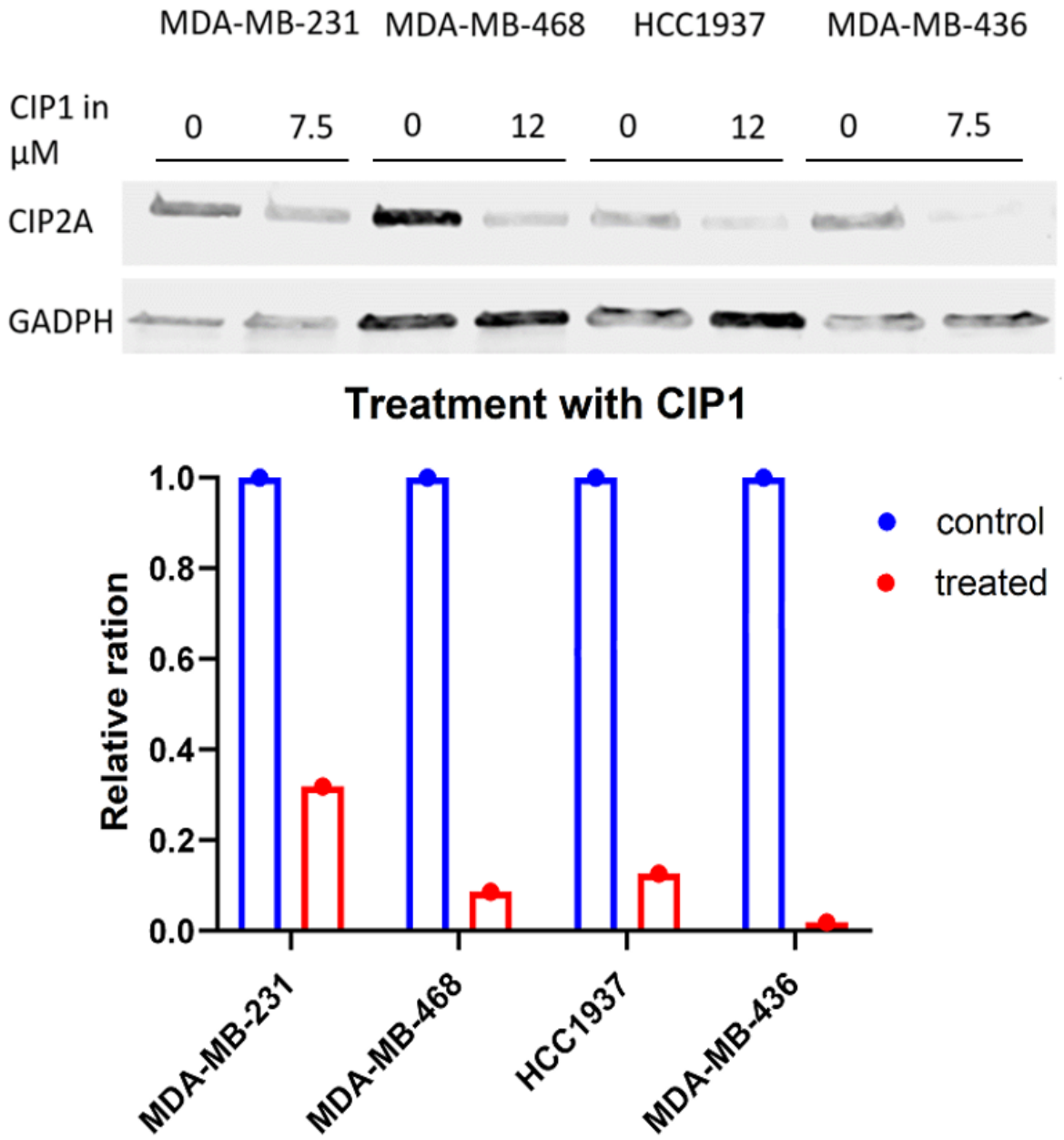

Figure 11: Western blot data using MDA-MB-231, MDA-MB-468, HCC1937 and MDA-MB-436 cell lines with one effective concentrations of CIP1 (24 h) with each cell line. CIP1 effect to CIP2A protein level was quantified from western blot using ImageJ and PrismGraph 8.

Next, CIP3 (Figure 12) and CIP4 (Figure 13) were studied by using concentration of 40 µM just to see if these two compounds have considerably effect in CIP2A expression level. Cell lines used were MDA-MB-231, MDA-MB-436, MDA-MB-468 and HCC1937. High concentration of drugs have often unwanted side effects, which can cause toxicity to the organ. By using concentration of this high at this level of study, decrease of CIP2A protein expression level should be decreased considerably if more study is done.

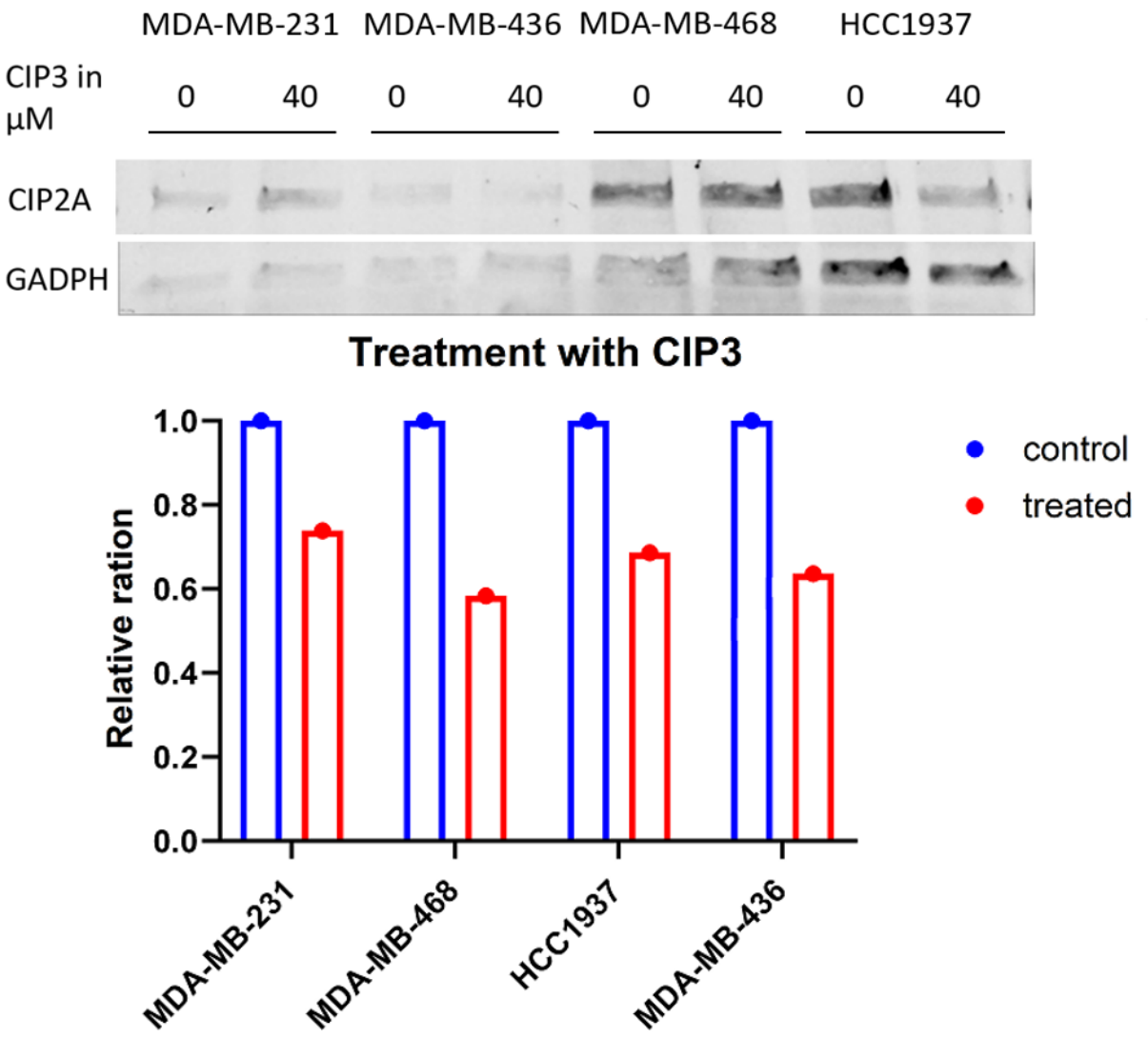

Figure 12: Western blot data using MDA-MB-231, MDA-MB-436, MDA-MB-468 and HCC1937 cell lines with CIP3 (40 µM) 24 h treatment. CIP3 effect to CIP2A protein level was quantified from western blot using ImageJ and PrismGraph 8.

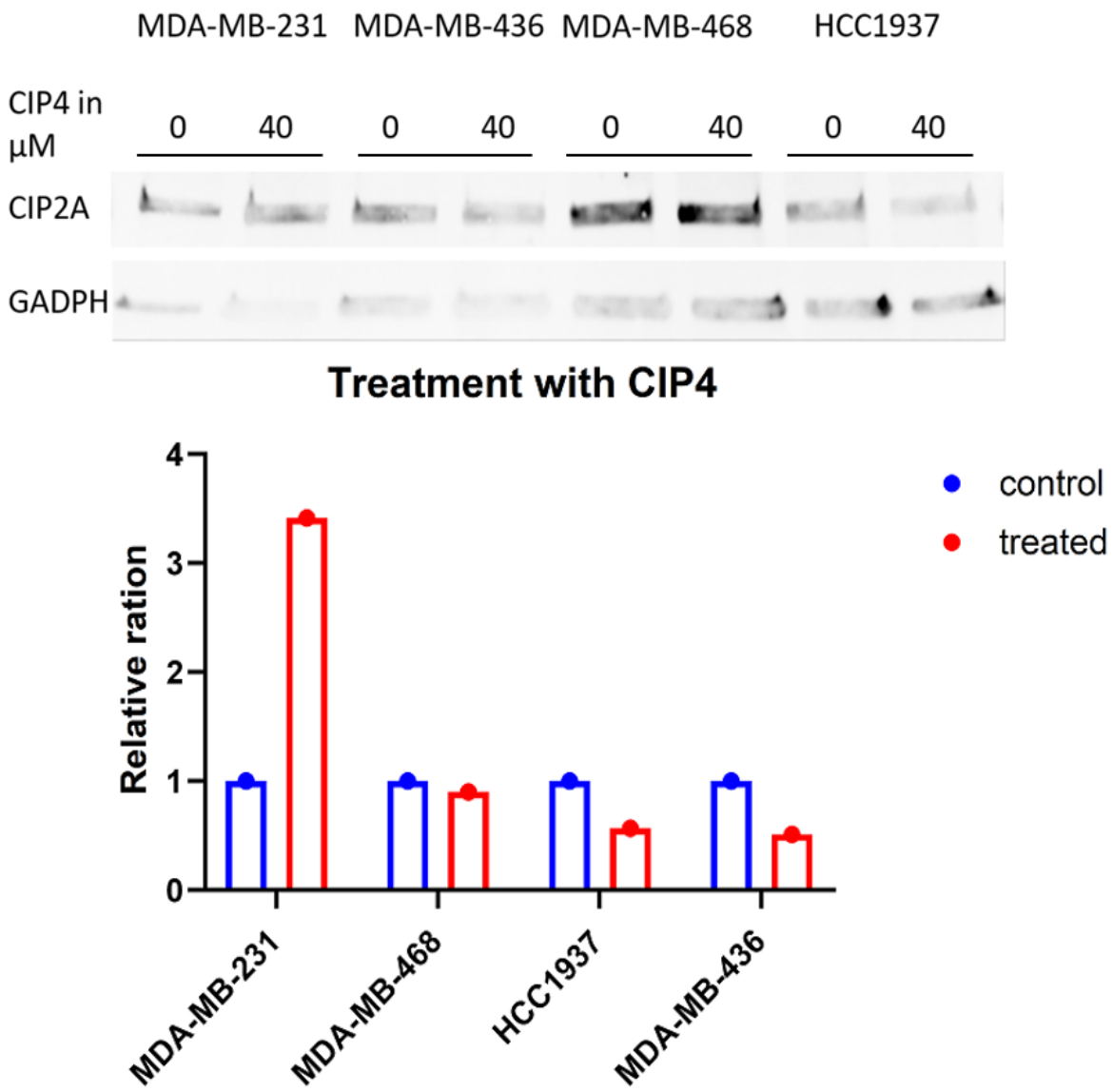

Figure 13: Western blot data using MDA-MB-231, MDA-MB-436, MDA-MB-468 and HCC1937 cell lines with CIP4 (40 µM) treatment. CIP4 effect to CIP2A protein level was quantified from western blot using ImageJ and PrismGraph 8.

To simplify all the westerns, final western blot (Figure 14) was done by using cell lines MDA-MB-231. MDA-MB-436, MDA-MB-468 and HCC38 with CIP1, CIP3 and CIP4.

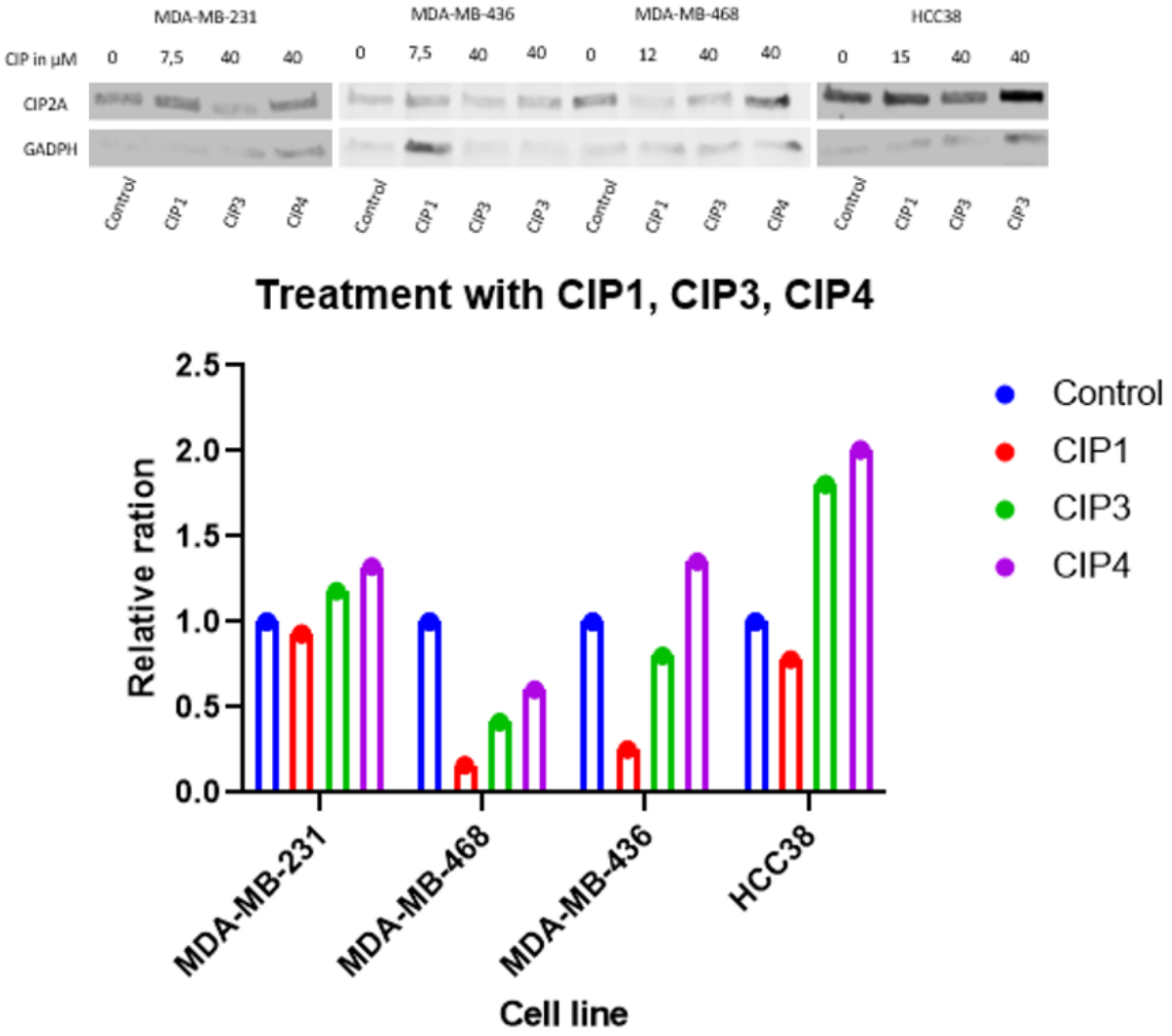

Figure 14: Western blot data using MDA-MB-231, MDA-MB-468, MDA-MB-436 and HCC38 cell lines with CIP1, CIP3 and CIP4 treatment. CIP effect to CIP2A protein level was quantified from western blot using ImageJ and PrismGraph 8.

3.6 Dimerization assay using recombinant proteins

In this experiment three different proteins had to be produced in E.coli and purified (Figure 15). One protein was GST, other was CIP2A with GST-tag and last one was GST-CIP2A-V5. The GST tag was used for protein purification with GSH beads. The GST tag was removed from GST-CIP2A-V5 variant by proteolytic cleavage with thrombin. The GST control and GST-CIP2A were used in dimerization assay with CIP2A-V5. Purified proteins are shown in figure below (Figure 15).

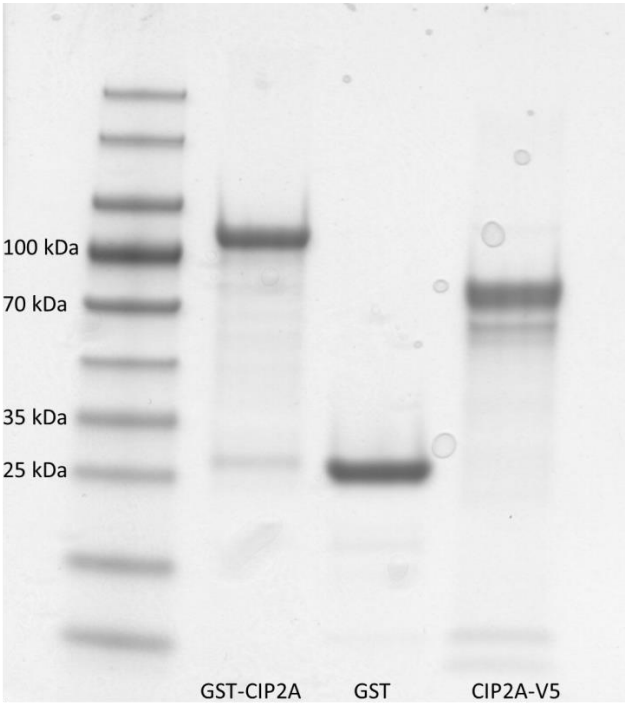

Figure 15: Purified, GST-CIP2A, GST and CIP2A-V5proteins. Protein ladder used was PageRulerTM Plus Prestained Protein Ladder #26619 (Thermofisher).

The protein complexes were allowed to form during 1h incubation, and then the complexes were pull-down with GSH beads which binds GST tag. The GSH beads were then extensively washed with reaction buffer to remove all unspecific binding and the proteins were eluted from beads with 2xSDS-PAGE buffer. Protein compositions of the complexes were visualized by Western blotting with anti-V5 and anti-GST antibodies. The influence of CIP1 on CIP2A dimerization was studies by incubation of GST or GST-CIP2A with CIP1 prior to incubation with CIP2A-V5. If CIP1 would be preventing CIP2A dimerization, it would be seen as an absence or lower abundance of the band corresponding to CIP2A-V5 in GST-CIP2A pull-down.

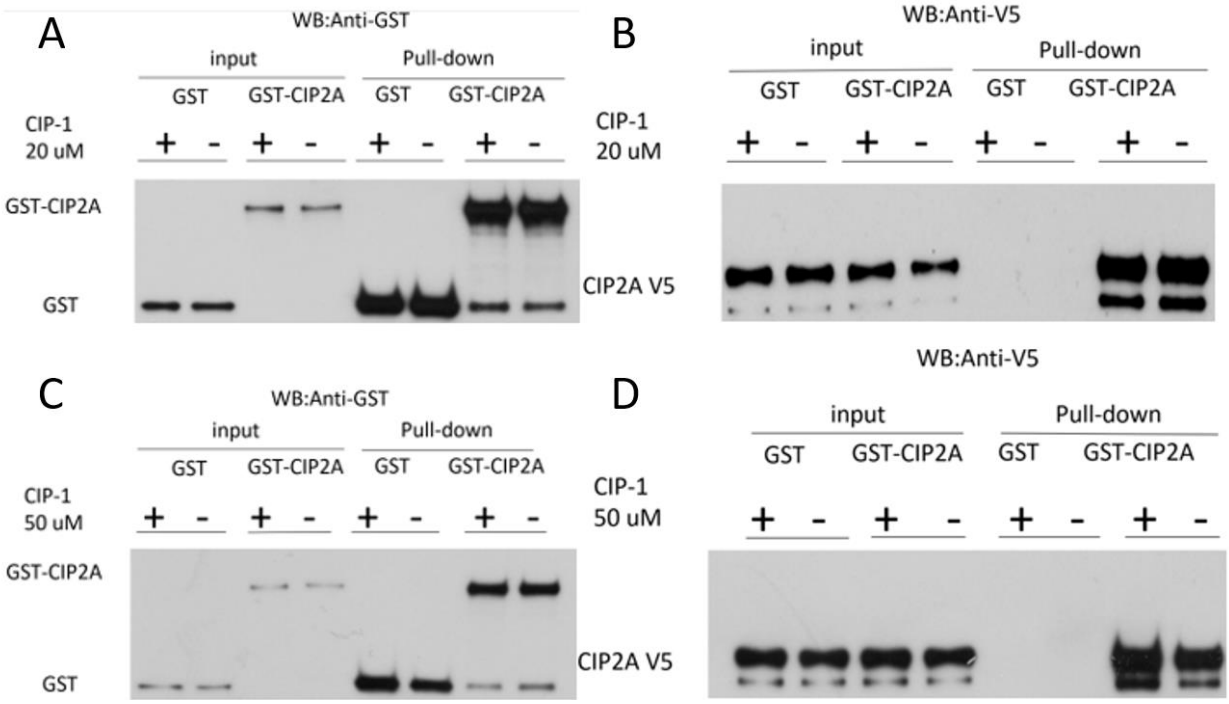

Figure 16: Dimerization assay using CIP2A(1-560) fragment analyzed by GST pulldown. Incubation was done using 20 µM (A and C) or 50 µM (B and D) of CIP1. Equal molar amounts of GST and GST-CIP2A(1-560) were incubated with CIP2A(1-560)-V5 fragment for 1h at 37° C.

Input samples were taken before incubation with beads (Figure 16). It can be seen that when membrane was labeled with Anti-V5 antibody, there is no or little difference in input sample bands. This showed that the equal amount of CIP2A-V5 is present in all samples prior to the pull-down. The absence of CIP2A-V5 band in the pull-down with GST control (Figure 16) after washing of beads demonstrate the absence of unspecific binding of CIP2A-V5 to GST alone. In contrast CIP2A-V5 band is visible in the pull-down sample with GST-CIP2A showing the strong dimerization of CIP2A (Figure 16).

The effect of CIP1 on the dimerization was studied by preincubation of GST and GST-CIP2A with CIP1, and the rest of the experiment was done as described above. Three concentrations of CIP1 were tested: 20 µM (Figure 16 A and B), 50 µM (Figure 16 C and D) and 100 µM (Figure 17).

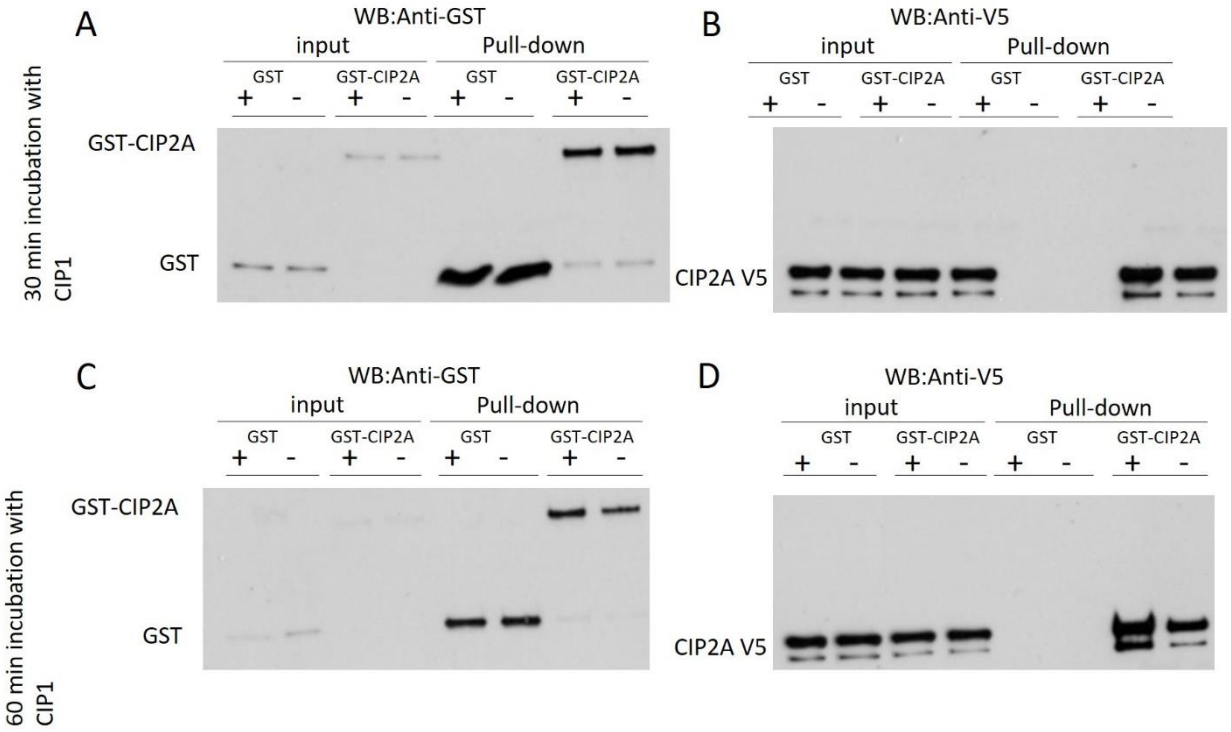

Figure 17: Dimerization assay using CIP2A (1-560) fragment analyzed by GST pulldown. Incubation was done by using 100 µM of CIP1. Equal molar amounts of GST and GST-CIP2A(1-560) were incubated with CIP2A(1-560)-V5 fragment for 30 min (A and B) or 60 min (C and

3.7 Timepoint with mRNA using qPCR and protein with western blot

Next, effect on mRNA level was reasonable approach to study unknown function of inhibition of CIP2A by CIP1. This was done by extracting and purifying mRNA from MDA-MB-468 cell lines with different treatment durations of CIP1 (timepoints). mRNA was transformed to cDNA and qPCR was used to see what effect does CIP1 have to mRNA level of CIP2A. From the same MDA-MB-468 wells treated with different CIP1 treatment durations, also protein samples were made for western blot. Results are shown below (Figure 18) and experiment was repeated one additional time with same results.

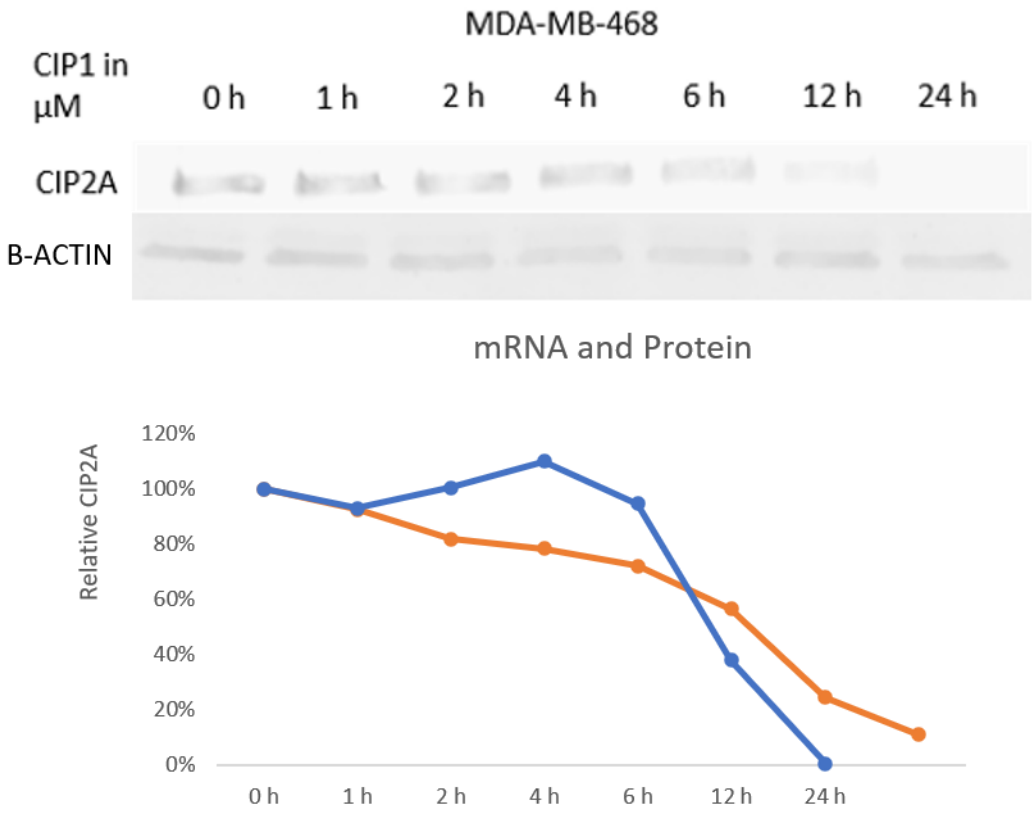

Figure 18: Western blot membrane from timepoint treatment with CIP1 using MDA-MB-468 cell line. mRNA and protein level chance in seven different CIP1 treatment timepoints, using MDA-MB-468 cell line. Protein level quantification was done from western blot A using ImageJ.

4. Conclusions and discussion

Based on the results from cell viability assay (Figure 5 and Table 1), only CIP1 was able to kill the cells effectively and IC50 was able to be calculated. The results are in accordance with the study of Laine et al (Laine et al., 2020), where CIP2A dependency of the used cell lines was shown, IC50 should be highest for HCC1937 (32,96 µM) and MDA-MB-231 (21,05 µM) cell lines and lowest for MDA-MB-436 (7,112 µM), MDA-MB-468 (14,68 µM) as well as for HCC38 (11,94 µM) cell lines and it would mean that CIP1 effects somehow to CIP2A protein level. This assumption is justified because when CIP2A dependent cell lines are treated with compound that should decrease CIP2A protein expression level in the cell line meaning that higher dependency cell lines should have lower IC50 values. The order of the lowest CIP2A dependency differs from Laine et al, but this is most likely because of quality difference in cell culturing, leading to unhealthy cells being more affected by the CIP1 or because of CIP1 effectivity to get inside the cell, and finally affecting to CIP2A protein expression, might differ with each cell line. This indeed was promising results because it was not known for sure if these compounds would even be able to get inside the cell or have any kind of effect to the cells. Still, more experiments had to be done to identify if CIP1 actually affects to CIP2A protein expression by preventing CIP2A dimerization. Also, CIP2 was able to kill the cells, but because of small amount of compound was available and effect was not significant, experiments with CIP2 were not continued.

The cell viability assay results and the colony growth experiment supports each other. Effect of the CIP1 can clearly be seen by eye (Figure 6) and from the quantified data (Figure 7). By increasing the dose of CIP1, the percentage of dead cells increases almost linearly. Also, the IC50 value looks like being between dose concentrations of 10 µM and 14 µM, which is in accordance with the cell viability assays value of 11,94 µM.

Like mentioned before, without CIP2A dimerization, monomers will be degraded. This should be seen in the western blots by stronger bands in treated samples versus control samples, which would support the hypothesis of CIP1 being able to prevent dimerization. Protein expression level was seen to be decreased by higher the concentration of CIP1 in comparison with control (untreated) by using western blot. This was done with cell lines HCC38 (Figure 8), MDA-MB-436 (Figure 9), MDA-MB-231 and MDA-MB-468 (Figure 10). Cell lines were treated with four different concentrations of CIP1 and a control (untreated). With MDA-MB-231, all cells were dead past concentration of 10 µM (control, 10 µM, 15 µM, 20 µM, 25 µM). With HCC38 cell line, by increasing the dose of CIP1, CIP2A protein expression level shows a decrease after concentration of 5 µM increased by the higher the concentration of CIP1. Similar phenomenon can be seen with other cell lines (Figure 9, Figure

10). With cell line MDA-MB-436, there is a deviation with the concentration of 10 µM. This is most likely caused by mistake in sample preparation which can be seen as a faint GADPH band meaning amounts of protein in the samples are not the same. When quantifying bands, difference with the GADPH bands lowers the quality of the quantification. Still, it can be ignored because these four different cell lines shows same kind of phenomenon, CIP1 is able to decrease CIP2A protein expression level increased by higher the concentration.

It needs to be noticed that IC50 values does not correlate with the level of CIP2A protein expression. Like mentioned before, these cell lines dependency for CIP2A differs, meaning that some cell lines only die after CIP2A protein level decreases significantly and for some cell lines, CIP1 effect can be seen with low concentrations in cell viability assay and in western blot, protein level decrease is low.

Next, previous western results were repeated in the same gel with four cell lines using one specific concentration of CIP1 for each cell line (Figure 11). CIP2A protein expression level can be clearly seen to decrease but the way of function still needed to be studied. Despite the cell viability results from the behave of CIP3 and CIP4, also these compounds were studied via western blot. Potential drugs often have side effects even with low concentrations. By using concentrations of 40 µM with these two compounds, the effect of CIP2A protein decrease should be significant. This is not the case with CIP3 (Figure 12) and CIP4 (Figure 13). There is a deviation in the MDA-MB-231, treated with CIP4, sample (Figure 13) but it is most likely problem in the sample preparation. Like previously, when loading control of GADPH in the treated sample and in the control sample differs from each other, it decreases the accuracy of the quantification. This abnormally can be ignored because other cell lines are showing reasonable results.

To conclude western results, final western was done to simplify results. Cell lines MDA-MB-231, MDA-MB-436, MDA-MB-468 and HCC38 were treated with CIP1, CIP3 and CIP4 (Figure 14). The decrease in protein expression level can be seen to be high with low CIP1 concentrations. CIP3 and CIP4 also have an effect with some of the cell lines but the concentration of the compound is high and decrease is lower than with the usage of CIP1. Based on cell viability assay and western blots, I conclude that CIP3 and CIP4 cannot be used as direct CIP2A protein inhibitors and more study on the behave of these to compounds are not needed. CIP2 should be studied similar way, but like mentioned lack of compound, I did not do this.

CIP1 research was continued with the dimerization assay. Three concentrations of CIP1 were tested: 20 µM (Figure 16 A and B), 50 µM (Figure 16 C and D) and 100 µM (Figure 17). In all cases there were no significant effect of CIP1 on CIP2A dimerization, when comparing Pull-down GST-CIP2A treated and GST-CIP2A control samples. Based on these three identical results from dimerization

assay and from previous experiments by now it is clear that CIP1 is able to inhibit CIP2A protein expression but by via some other mechanism than affecting directly CIP2A dimerization. This pretty much was killer experiment for the beneficial usage of CIP1. In fact, it looks like CIP1 stabilizes dimerization in this experiment because of Pull-down treated samples shows a stronger band. Importantly, circumstances of the dimerization assay differ from experiments done with TNBC cell lines, meaning that this experiment does not show any kind of upstream effects. This is because of assay only includes from three components, not from entire cell. Even thought, western blot supported hypothesis, dimerization assay proved that CIP1 is not able to prevent dimerization but yet, CIP2A protein expression level can be seen to decrease in western blot experiments. Unfortunately there is still question if CIP3 and CIP4 would have prevented CIP2A dimerization but because of high concentrations needed in western blot and lack of IC50 values in cell viability assay, these two compounds are very unlikely to be used in the future as CIP2A inhibitors. Next approach was mRNA experiment via qPCR by using MDA-MB-468 cell line.

By looking at results from timepoint experiment (Figure 18), it is clear that mRNA level of CIP2A is decreased almost linearly with time. This experiment was repeated one additional time showing similar results. mRNA results were expected based on dimerization assay. It can be seen that CIP2A mRNA level decreases in correlation with time passed. This explains the problem with western blots CIP2A protein level decrease, when using CIP1 and dimerization assays results where CIP1 was not able to prevent CIP2A dimerization.

From the four proposed compounds, only CIP1 was able to kill the triple negative breast cancer cell lines effectively in the cell viability assay and decrease CIP2A protein expression in the cell lines based on western blot experiments. Though promising start, CIP1 was not able to prevent CIP2A dimerization, which was seen in dimerization assay. Interestingly CIP1 was affecting by mRNA level, that was seen in mRNA experiment using qPCR. Pathways where CIP1 might affect are many and it is difficult to start finding what is the point of affection in signaling pathway leading to CIP2A inhibition. One possibility would be ERK pathway, which has been shown to be crucial for CIP2A mRNA expression (Khanna et al., 2011). Because of lack of successful drug in triple- negative breast cancer therapies, this would still be useful to study, if CIP1 would only target specific signaling pathway. But like said, possible off-target effects must be researched, which might take time. Even though the result was not what was hypothesized, these results prove something else. The idea of concept of CIP2A being a novel target in cancer therapies. Here I proofed that CIP2A can be inhibited leading to TNBC cell lines dying, so identifying other compounds by computer modeling or some other methods might be beneficial approaches.

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