Diplomarbeit

Targeting epigenetic regulators in bladder cancer by the BET inhibitor JQ1

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Zürich, am 06.08.2018 *Jakob Schweighofer eh*

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Abkürzungen (Abbreviations)

UBC: urothelial bladder cancer

MIBC: muscle invasive bladder cancer

CIS: carcinoma in situ

UTUC: upper urinary tract carcinoma

CT: computer tomography

HG: high grade

LG: low grade

PUNLMP: papillary urothelial neoplasm of low malignant potential

WHO: world health organization

TURB: transurethral resection of the bladder

EAU: European association of urology

NMIBC: non-muscle invasive bladder cancer

BCG: Bacille Calmette – Guérin

MMC: mitomycin C

RC: radical cystectomy

TMT: trimodal therapy

LOH: loss of heterozygosity

DNA: Deoxyribonucleic acid

RNA: Ribonucleic acid

RNAP II: RNA polymerase II

FGFR: fibroblast growth factor receptor

BET: Bromo- and Extra-terminal

BRD 2-T: bromodomain containing protein

NMC: NUT midline carcinoma

P-TEFb: positive transcription elongation factor

EZH2: Enhancer of zeste homolog 2

FBS: Fetal Bovine Serum

PMSF: phenylmethylsulfonyl fluoride

EDTA: Ethylendiamintetraacetat

PBS: phosphate-buffered saline

TBS: tris-buffered saline

PVP: Polyvinylpyrrolidone

SDS: sodium dodecyl sulfate

A.d.: Aqua dest.

RT: room temperature

DMSO: dimethylsulfoxid

qPCR: quantitative polymerase chain reaction

IC50: half maximal inhibitory concentration

Rpm: revolutions per minute

CNS: central nervous system

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Zusammenfassung

Das Urothelkarzinom der Harnblase gehört zu den fünf am häufigsten diagnostizierten Tumoren in der USA, verursacht signifikante Krankheits- und Sterberaten und führt zu einer starken Reduktion der Lebensqualität. Therapeutische Ansätze hängen vom Fortschritt des Tumors ab, z.B. nicht Muskelinvasiv vs. Muskelinvasiv und bestehen sowohl aus chirurgischer Entfernung des Tumors als auch Immuntherapie und Chemotherapie.

Molekularbiologisch weist das Harnblasenkarzinom eine hohe Rate an somatischen Mutationen auf, unter anderem mehrere Mutationen in Gruppen von epigenetischen Regulatoren. Eine Studie hat gezeigt, dass Mutationen in diesen Regulatoren eine große Auswirkung auf die Regulation und Expression verschiedener Transkriptionsfaktoren und Pathways haben. Dies führt zu der Annahme, dass eine Hemmung dieser epigenetischen Regulationsmechanismen eine mögliche Therapiestrategie darstellen könnte.

Unser Hauptaugenmerk in dieser Studie liegt auf dem Protein BRD4. Hierbei handelt es sich um ein Protein welches in der Regulation von Onkogenen, zum Beispiel *c-Myc* beteiligt ist und zur Gruppe der Bromodomain and extra-Terminal Family gehört (BET). Diese Proteine sind wichtig in der Regulation der Genexpression, indem sie sich an Transkriptionsfaktoren binden.

Bei JQ1 handelt es sich um eine kleine molekulare Zusammensetzung welche die Fähigkeit besitzt, sich kompetitiv an spezielle Stellen (acetyl-lysine recognition) von BET Proteinen zu binden. Dieser Vorgang führt, unter anderem, zu einer Verhinderung von BRD4 Aktivität, welches sich direkt auf den Vorgang der Transkription auswirkt. In durchgeführten Studien konnte nach erfolgreicher Behandlung mit JQ1 eine antiproliferative Wirkung in mehreren verschiedenen Karzinomgruppen nachgewiesen werden. Im nicht-Muskelinvasiven Blasenkarzinom, führte JQ1 zur Hemmung von Proliferation und zu einem Stopp des Zellzyklus. Außerdem führte JQ1 dadurch zu einer progressiven Apoptose in BRD4 abhängigen Zellen.

In diesem Projekt versuchen wir die Wirkung von JQ1 an mehreren Zelllinen (Blasenkarzinom) zu analysieren um ein größeres Verständnis von epigenetischen und molekularbiologischen Vorgängen in UBC zu erhalten. Weiters wollen wir herausfinden auf welche Transkriptionsabläufe BRD4 eine Auswirkung hat und welche molekularen Abläufe hierfür notwendig sind.

Um die Effektivität von JQ1 herauszufinden erstellten wir IC50 Kurven mit allen verwendeten Zelllinien. Hierfür behandelten wir die Zellen mit zunehmenden Konzentrationen von JQ1, beginnend bei 0.01 µM bis zu 1000µM. Außerdem untersuchten wir mittels Western Blot die Protein Expression von BRD4 in den Zelllinien und analysierten die Genexpression verschiedener Gene vor und nach einer Behandlung mit JQ1.

Wir beobachteten, dass JQ1 eine mögliche Therapieoption für bestimme Arten von Blasenkarzinom darstellen könnte. Allerdings wäre es unter Umständen notwendig zuerst die Genexpressionen verschiedener Onkogene, besonders *c-Myc*, zu analysieren, um herauszufinden ob der Patient/die Patientin von einer möglichen JQ1 Therapie profitieren könnte.

Abstract

Urothelial bladder cancer is among the five most common cancers diagnosed in the United States and causes significant mortality and morbidity rates as well as a reduction in quality of live in affected patients. Therapeutic strategies to target the disease depend on the extent of local muscle invasion and comprise surgical resection, immunotherapy and chemotherapy. On the molecular level, UBC is characterized by high rates of somatic mutations, including several groups of epigenetic regulatory genes such as chromatin modifiers or nucleosome remodelers. Noteworthy, a recent study showed that mutations in these enzymes are likely to have profound impacts on the regulation and expression of various transcription factors and pathways involved in UBC, suggesting that inhibition of epigenetic regulatory mechanisms might repress tumor progression.

Our main focus will be BRD4. It is an epigenetic reader protein of acetylated histones that has recently been shown to be involved in the regulation of oncogenes such as *c-Myc* (REF) and belongs to the bromodomain and extra-terminal family. They play a vital part in the regulation of gene transcription by binding to acetylated histones and non-histone proteins and associating with transcription factors.

JQ1 is a small molecular compound that has the ability to bind competitively to acetyl-lysine recognition motifs found in BET proteins. Binding of JQ1 to these motifs leads to a displacement of BET bromodomains and prevents, for example, BRD4 activity, with a direct effect on transcription. JQ1 treatment has shown to have antiproliferative effects in several cancer types. In non-muscle invasive bladder cancer, JQ1 led to an inhibition of proliferation and G1 cell-cycle arrest and induced immediate and progressive apoptosis in BRD4 depending cells.

In this project, we aim to analyze the influence of JQ1 on a panel of UBC cell lines and characterize its effect on UBC and on epigenetic regulatory mechanisms pharmacologically and on a molecular level. We also will determine transcriptional targets of BRD4 and detect molecular pathways involved in BRD4 mediated transcription.

To determine the effect of JQ1 and to establishing IC50 doses on our UBC cell lines, we administered increasing concentrations of JQ1, staring form 0.01 µM to 1000µM.

We also investigated protein expression of BRD4 via Western blotting and looked at gene expression using qPCR.

We observed that JQ1 might be viable future treatment option in UBC. However it might be of importance to look at the expression pattern of a variety of oncogenes. Especially *Myc* genes are of special interest, to distinguish which cancer might be responsive to JQ1 and which one might rather profit from a combination treatment of JQ1 with other agents such as MMC or Romidepsin. This could lead to the development of an individualized and a more tailored treatment of cancer patients.

Introduction

Bladder Cancer

Epidemiology and Risk Factors

Urothelial carcinoma of the bladder is a major cause of morbidity and mortality worldwide, causing an estimated 150.000 deaths per year (1). It is the most common cancer of the urinary tract and ranks fifth among cancers in men in Western countries and sixth worldwide (2). Due to differences in reporting of low-grade tumors, there is a geographic variation. The incidence rates are highest in Europe, Northern America, Western Asia and Northern Africa, and lowest in Eastern, Middle and West Africa (2). Regarding mortality, among men, Turkey ranks first, with a mortality rate of 50% higher than the highest rates in Europe and 3 times as high as in the United States (2).

Risk factors comprise external and genetic causes. The most important external risk factor associated with bladder cancer is smoking. Tobacco smoke contains aromatic amines known to cause UBC, and duration as well as the amount of smoking are associated with an increased risk for urinary tract cancer (3, 4). It is estimated that approximately 50% of UBC is related to smoking (4).

The second main external risk factor is exposure to carcinogens, such as aromatic amines. In 20% of UBC cases, exposure to carcinogenic chemical substances such as paint, dye, metal and petroleum products can be found (4).

Besides smoking and exposure to carcinogens, dietary factors have also been associated with an increased risk in UBC development. Especially fluid intake plays a role in the development of UBC. Studies have shown that a high amount of ingested fluids may reduce the risk of developing UBC. It has been hypothesized that, this is due to an increased dilution of the urine, therefore reducing the concentration of carcinogens and by increasing the micturition frequency. It is, however important to differentiate between different types of fluids. For example, chlorination of drinking water leads to a higher risk for developing bladder cancer as chlorinated water counts as a relevant carcinogen (4). So far, no association between alcohol intake and an increased risk for UBC has been found (4).

Genetic predispositions are the second main contributor to the development of UBC. The risk of UBC development is two-fold higher in first-degree relatives of UBC patients (4). But so far, no specific genetic characteristic could be linked to the development of urothelial bladder cancer directly. It is also possible that smoking and dietary habits are

responsible for the higher risk in relatives, because these factors are often similar in families (5).

Signs and Symptoms

The main symptom for UBC is painless hematuria. In cases of advanced tumor stages with infiltration of the muscular layer of the bladder (muscle invasive bladder cancer or MIBC) or in cases of CIS (carcinoma in situ), a high grade intraepithelial neoplasm with a high tendency of progression, symptoms might also include dysuria, pelvic pain and, especially in MIBC, urinary tract obstruction (6, 7).

Diagnosis

The main examination for UBC is cystoscopy. Cystoscopy enables the surgeon to perform a full examination of the bladder itself, as well as performing a biopsy of the tissue for histological evaluation (6). Also, microscopic examination of cells from urine or bladder washings (cytology) has a high sensitivity in high – grade tumors and is a useful tool for diagnostic evaluation of cases of high–grade malignancy or carcinoma in situ. However, cytology has a low sensitivity for low-grade tumors. It is best used in combination with cystoscopy. A positive cytology has a high positive predictive value and can indicate a urothelial tumor anywhere in the urinary tract; a negative can however not exclude the presence of a malignancy (6, 8). Transabdominal ultrasound is a useful tool for detecting renal masses, hydronephrosis and intraluminal masses of the bladder. However, ultrasound usually only detects bladder masses >2 cm, and cannot be used to detect UTUC. It also cannot replace CT for the detection of urinary bladder cancer. CT with urography is the standard imaging modality to detect papillary tumors in the urinary tract and UBC of the upper tract is usually detected by filling defects or hydronephrosis (6).

Bladder Cancer Staging

Staging is based on the TNM system (6, 9). A table listing the current TNM system is shown below.

TX	Primary tumor can not be assessed
T0	No evidence of primary tumor
Ta	Non-invasive Tumor
Tis	Carcinoma in situ
T1	Invasion of subepithelial connective tissue
T2	Muscle invasion
	T2a: Invasion of the inner half of the muscle
	T2b: Invasion of the outer half of the muscle
T3	Invasion of perivesical tissue
	T3a: Microscopically
	T3b: Macroscopically
T4	Invasion of any of the following: prostate stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall
	T4a: Invasion of prostate stroma, seminal vesicles, uterus or vagina
	T4b: Invasion of pelvic or abdominal wall
NX	No assessment of regional lymph nodes
N0	No regional lymph node metastasis
N1	Metastasis in a single lymph node in the true pelvis
N2	Metastasis in multiple lymph nodes
N3	Metastasis in common iliac lymph nodes
M0	No distant metastasis
M1a	Metastasis in non-regional lymph nodes
M1b	Distant metastasis

1973 WHO grading system	
	Urothelial papilloma
	Grade 1: Well differentiated
	Grade 2: moderately differentiated
	Grade 3: Poorly differentiated
2004/2016 WHO grading system (papillary lesions)	
	Urothelial papilloma (completely benign lesion)
	PUNLMP
	LG papillary urothelial carcinoma
	HG papillary urothelial carcinoma
Flat lesions (2004 WHO grading system)	
	Urothelial proliferation of uncertain malignant potential (arcinia hyperplasia)
	Reactive atypia (flat lesion with atypia)
	Atypia of unknown significance

	Urothelial dysplasia
	Urothelial CIS (always HG)

CIS=Carcinoma in situ; HG= high grade, LG= Low grade, PUNLMP= papillary urothelial neoplasm of low malignant potential, WHO= World Health Organisation

Treatment

Depending on the stage of the tumor, there are different treatment options (6). The main choice of treatment for initial diagnosis of UBC is a complete surgical removal of the tumor. TURB itself is used for pathological staging, and if the tumor is small enough and has not yet invaded the muscle it is sufficient in removing the whole mass (10). For the procedure, a resectoscope is inserted through the urethra and the surgeon is able to resect the tumor by using an electrocautery sling. To improve the result of the procedure, the surgeon has the option of using chemotherapy in combination with TURB. The European Association of Urologists (EAU) Guidelines suggest that it is necessary to take adjuvant therapy into consideration to reduce the risk of recurrence. The most common used chemotherapeutic drugs are mitomycin C and epirubicin, which are installed immediately after the surgery (11). According to a 2016 study, a single immediate installation of chemotherapy immediately after resection reduces the risk of recurrence in NMIBC patients (12).

Adjuvant therapy for high-risk tumors

Bacille Calmette – Guérin is a bacterium mainly used as a vaccination for tuberculosis. In 1991 a study led to the conclusion that intravesical BCG therapy is an effective treatment for NMIBC (13). Since then a number of studies have proven the beneficial effect of intravesical BCG treatment when compared to TURB alone or TURB in combination with chemotherapy. In all of these studies, BCG showed the best results in preventing recurrences of NMIBC (14-18). BCG is indicated in cases of non-muscle invasive bladder cancer of stage Ta, T1 high grade and CIS (19).

The exact mechanism of the therapy is still unknown. It is hypothesized that intravesical installation of BCG leads to an immune reaction in the bladder and thereby induces an immune reaction against existing cancer cells. The treatment itself is divided into two sections, induction and maintenance. For induction, the BCG installations follows a schedule of six weeks followed by maintenance of the therapy (20). Even though the

beneficial effect of the maintenance therapy is proven, the optimal schedule has not yet been defined (21, 22).
In case of BCG-refractory tumors, according to the EAU-Guidelines, radical cystectomy is strongly recommended.

Treatment of MIBC

Radical cystectomy is the standard therapy for patients with muscle invasive bladder cancer. Usually, radical cystectomy is recommended in cases of T2-T4a MIBC (23). At the time of diagnosis, 10-15% of patients will show metastatic lesions (24). In this case, the therapy consists of a gemcitabine plus cisplatin-based chemotherapy (6, 25). In patients with NMIBC the EAU Guidelines suggest to perform an immediate radical cystectomy if the tumor belongs to one of the highest risk for fast progression (6).
Risk factors comprise high-grade/C3 tumors, carcinoma-in-situ or multiple and large recurring Ta G1- G2 tumors (26). Studies have shown that the 5-year survival rates for patients undergoing RC varies around 62-66% (27, 28).
In case of NMIBC after BCG therapy, the guidelines define the indications for RC as follows (6):

- Detection of MIBC during follow-up
- High-grade, NMIBC papillary tumor after three months
- CIS present at three and six months
- Despite initial success, high-grade recurrence after BCG therapy

To improve the survival rate of MIBC patients after radical cystectomy, neoadjuvant chemotherapy is used (29-33). Available options include chemotherapy with MVAC (combination of methotrexate, vinblastine, adriamycin and cisplatin), CMV (combination of cisplatin, methotrexate and vinblastine), CM (cisplatin and methotrexate), combinations of cisplatin/adriamycin or cisplatin/5-fluorouracil and CarboMV (carboplatin, methotrexate and vinblastine) (6). However, neoadjuvant chemotherapy should only be applied in patients eligible for chemotherapy with cisplatin. Other chemotherapy options have shown inferior outcomes in metastatic UBC and still need to be fully tested for neoadjuvant therapy (34-37). There are no guideline recommendations concerning pre-operative radiotherapy due to a lack of studies, looking into that treatment option (38). However, some retrospective analyses have shown a decrease in mortality and downstaging of tumors (clinical T1-3 down to T0) (39, 40). In cases where the patient may

not be fit enough for surgery, a combination therapy consisting of TURB followed by radiation and chemotherapy is given (6). This trimodal therapy (TMT), consists of complete TURB followed by cisplatin-based chemoradiotherapy (41). There are no studies that compare the outcomes of radical cystectomy to the outcomes of TMT, but trials performed for TMT showed similar outcomes (41).

Molecular Biology

In recent years more and more studies have shown the importance of molecular alterations in the development of bladder cancer. Currently two genetic pathways of tumor progression from normal urothelial cells into malignant cells are proposed (42, 43). The non-aggressive pathway involves mainly oncogenes with mutations in the FGFR3 and the PIK3CA genes. These mutations are predominantly present in tumors of low stage malignancies. Tumors baring these kinds of alterations usually didn't show progression to muscle invasive stages. The second pathway, the highly aggressive one predominantly shows mutations in tumor suppressors, such as TP53, PTEN, and an inactivation of the RB pathway.

A common denominator in both tumor pathways, the non-aggressive as well as the aggressive pathway, are alterations in chromosome 9. It is totally or just partially lost across all tumor stages and grades (44-46). The frequently deleted regions include 9p22–23 in the short arm, and 9q11–13, 9q12–13, 9q21–22, and 9q34 in the long arm of the chromosome. These regions comprise a number of tumor suppressor genes, namely BNC2, DAPK1, DBC1, CDKN2A/B, PTPRD, and TSC1, which are thought to be responsible for the initial transformation of urothelial cells (47). Thus, it has been suggested that a loss of heterozygosity of chromosome 9 is one of the earliest genetic alterations in UBC pathogenesis (48).

Depending on their gene expression profiles, NMIBC can be divided into three different groups (49). Group one, is defined by an up-regulation of early cell cycle genes (CCND1, IDN1 and RBL2). In group two NMIBC, mainly late cell cycle genes (CDC20, CDC25A, CDKs and PLK1) and cancer stem cell markers (ALDH1A1, ALDH1A2, PROM1, NES and THY1) have been shown to be expressed. Group three usually shows an increased expression of KRT (keratin gene family), which can, however also be found in the other two tumor groups.

In 2014 a study showed that 76% of all bladder tumors had at least one mutation in chromatin regulatory genes (50) suggesting that in more than three quarters of UBCs, alteration in chromatin regulatory genes play a major role in cancer development. In MIBC, chromatin remodeling genes are frequently mutated.
Genes with the highest mutation frequency in MIBC include mutations of TP53 (43%), KDM6A (28%), ARID1A (22%), PIK3CA (18%), MLL2 (17%), CREBBP (15%), RB1 (15%), STAG2 (13%), FGFR3 (13%), EP300 (13%), TSC1 (8%) and HRAS (8%).(50-52).

FGFR3 and RAS/MAPK pathway

As mentioned above, FGFR3 is commonly mutated in bladder cancer cases of low aggression. Four known members of Fibroblast Growth Factor receptors (FGFRs) family, *FGFR1-4* are known. They act as tyrosine kinases and consist of three immunoglobulin-like domains, a transmembrane domain, and a cytoplasmic domain with tyrosine kinase activity (53). *FGFR3* is a gene coding for the fibroblast growth factor receptor 3 protein. This protein has a regulatory role in the cell cycle. It affects proliferation, survival, migration, differentiation and metabolism (54, 55). Ligand binding induces receptor dimerization, which will lead to phosphorylation and receptor activation. Signaling downstream of *FGFR3* includes the PI3K/AKT, the RAS/MAPK, and the PKC pathways (70).
The most commonly expressed FGFR receptor in urothelial cells is *FGFR3*. By alternative splicing of the immunoglobulin-like domain III, IIIb or IIIc isoforms are produced. They show tissue-specific expressions, for instance, the IIIb isoform of FGFR3 is mainly expressed in epithelial cells (56). Approximately 60-65% of UBCs will show oncogenic point mutations in FGFR3. Point mutations in the *FGFR3* gene usually occur after chromosome 9 losses, a common denominator in urothelial cell cancer, and *TERT* promoter mutations (57). In case of mutations of the extracellular or transmembrane domains of *FGFR3*, spontaneous dimerization will occur, while mutations in the kinase domain will lead to constitutive receptor activation (58, 59). *FGFR3* gene mutations, may lead to uncontrolled cell proliferation by activating the RAS/MAPK pathway (60). This pathway that usually controls growth, proliferation and senescence by transmitting extracellular signals (61, 62).
As mentioned above, mutations in *FGFR3* are more frequently detected in non-aggressive tumors, particularly in those of lower stage and grade. Two large clinical studies have

shown that *FGFR3* oncogenic mutations are associated with a decreased risk in progression and a longer 5-year survival (63, 64).
In recent years inhibition of FGFR3 has been shown to be a viable treatment option in bladder cancer and are the topic of different studies (65).

Epigenetics

The first definition of the term Epigenetics goes back to the 1940s. At that time epigenetics was defined as "the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being" (66, 67). Nowadays, it is defined as "the study of changes in gene function hat are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence" (66, 68). This means that epigenetic modifications lead to heritable silencing of genes without having an effect on their coding sequence (69). Three modifications play a central role in this process, namely, DNA methylation, RNA-associated silencing and histone modifications (69). As changes in epigenetic processes may lead to gene alterations and malignant cell transformation, Hanahan & Weinberg defined epigenetic alterations as a hallmark of cancer (70).

Histones are proteins found in the nucleus of eukaryotic cells. They are part of the chromatin complex that consists of DNA, RNA and protein and are responsible for packing DNA into a more compact unit called nucleosome (71). The primary function of chromatin is to enable DNA replication, to control gene expression and to prevent DNA damage (72). An important component in this processes is the modification of histones (73). These posttranslational modifications modify the tails of the histones by methylation, acetylation and phosphorylation (73). Other posttranslational modification processes include ubiquination, deminiation or even histone tail clipping but acetylation and methylation are the most studied ones (51, 73). Changes in histone modifications may disrupt different cellular processes and lead to oncogenic transformation and cancer development (74).
In bladder cancer, genes that are responsible for histone modifications are frequently mutated. For example, *KDM6A* (lysine-specific demethylase 6A), which is responsible for removal of the *H3K27* trimethylation mark, is mutated in over 25% of all cases of bladder cancer (52). *ARID1A*, a gene which is a subunit of the SWI/SNF chromatin remodeling complex, shows alterations in 22% of bladder tumors (40). Another study found that the recurrence rate of bladder in cancer is increased if mutations in MLL, EP400 (part of the

NuA4 histone acetyltransferase complex) and PRDM2 (nuclear histone/protein methyltransferase) are present (75).

Another important epigenetic modification is the methylation of DNA. This describes the process in which a methyl group is added to the DNA.
In mammalian cells, methylation is catalyzed by methyltransferases such as DNMT1 (maintenance methyltransferase) and *DNMT3A* or *DNMT3B* (de novo methyltransferases). These three DNA methyltransferases catalyze the methylation of CpG sites, which are regions at which a cytosine nucleotide is followed by a guanine nucleotide. Genome-wide, DNA methylation can be found in about 70-80% of all CpG sites (76) and leads to stable silencing of genes, whereas promoter-located CpG island are usually kept free of methylation (77).
In bladder and other cancer cells, *DNMT1*, *DNMT3A* and *DNMT3B* are frequently overexpressed, which results in silencing of tumor suppressor genes (78).
Because of their difference in expression, DNA methylation has been proposed to be an useful biomarker in the diagnosis of UBC (79). Today it is being used in systems like Epicheck®. This technology uses a panel of DNA methylation biomarkers and has a high specificity for bladder cancer (88%). A study suggests that these kind of tests may be used in the follow-up of NMIBC cases and may reduce the need of repeated cystoscopies (80).

The third epigenetic modification is RNA silencing. Here, non-coding RNA negatively regulates gene expression via gene silencing (81). An important part in this process is fulfilled by miRNA. This is a group of small non-coding RNA molecules that usually consist of about 22 nucleotides. They are encoded by DNA and usually transcribed by RNA polymerase II (82). Their function is based on base-pairing of complementary sequences in mRNA, which subsequently leads to the silencing of the mRNA (83).
MicroRNAs play a role in post-transcriptional regulation of gene expression and also take part in RNA silencing (83). Depending on their part in gene expression, some miRNAs can be classified as oncogenes or tumor suppressors (84) with a role during cancer development (85).
In bladder cancer, certain microRNAs have been found to be upregulated (miR-224, miR-182, miR-183) or downregulated (miR-1, miR-101, miR-143, miR-145, miR29c, miR-127) (86).

In 76% of bladder cancer cases at least one chromatin regulatory gene shows some sort of mutation (50) and lead to dysregulation in the epigenetic landscape and to the development of cancer, which is why knowledge of epigenetic procedures is important.

BRD4 and JQ1

As mentioned above, histone modifications play an important role in the regulation of gene expression and histone-modifying enzymes, such as histone writers, histone readers and histone erasers, are responsible for adding different modifications to histone tails. In the last years, it has become increasingly clear that mutations or altered gene expression of these histone-modifying enzymes is frequently detected in cancer. Interestingly, bladder cancer has one of highest mutation rates in the genes coding for these enzymes among all cancers studied so far, thus it can be expected that de-regulation of epigenetic modifiers plays an essential part during bladder cancer development.

In this study, we focus on BRD4. It is an epigenetic reader protein of acetylated histones that has recently been shown to be involved in the regulation of oncogenes such as *c-Myc* and belongs to the bromodomain and extra-terminal family (87). The family itself consists of four proteins, BRD2, BRD3, BRD4 and BRDT (88). BRD2, BRD3 and BRD4 are present in many different cell types, while BRDT is especially present in testis (89). All of the four proteins have two tandem N-terminal bromodomains (BD1 and BD2), as well as an extra-terminal domain in common (90, 91). They play a vital part in the regulation of gene transcription by binding to acetylated histones and non-histone proteins and associating with transcription factors (92) (93).
For the transcription to function properly, a multiprotein complex called RNA polymerase II (*RNAP II*) is required (94). *RNAP II* is needed to catalyze transcription of the DNA to synthesize RNA (95). BRD4 is bound to the positive transcription elongation factor b, which plays a part in *RNAP II* – dependent transcription processes. This has led to the conclusion that BRD4 has the ability to regulate P-TEFb activity and by effecting its transcription (92, 96, 97).

BET proteins and cancer

Several studies have shown the important role of BET proteins in the development of different tumor types. The first tumor linked to the BET family was NUT midline carcinoma. NMC is a rare squamous cell epithelial cancer caused by chromosomal rearrangements involving the gene NUT (98). In approximately 75% of all NMC cases, the

coding sequence of NUT on chromosome 15q14 is fused to either BRD4 or BRD3 leading to chimeric genes that encode fusion proteins (BRD-NUT) (91, 98). The fusion protein then blocks cellular differentiation by interacting with chromatin and induces uncontrolled growth of tumor cells (98, 99). Regarding BRD4, a study showed that in primary and metastatic melanoma tissue, BRD4 is significantly upregulated and that its inhibition led to a decline in melanoma cell proliferation *in-vitro* and to a decline in tumor growth *in-vivo* (100). In contrary to this, BRD4 was shown to be frequently downregulated in human colon cancer cell lines and ectopic re-expression lead to a decline in *in-vivo* tumor growth, indicating that BRD4 function is tissue-dependent and its tumor-suppressive functions might be related to tissue-specific co-transcription factors present (101).
In bladder cancer, a study showed that BRD4 mRNA and protein levels are both significantly upregulated in comparison to normal tissue. Furthermore, a correspondence between the level of BRD4 expression and cancer grade as well as risk of lymph node metastasis was detected (87). Noteworthy, inhibition of BRD4 led also to a downregulation of the polycomb group gene EZH2, which is a histone writer protein overexpressed in UBC. EZH2 functions as a histone-lysine-N-methyltransferase by methylating lysine 27me2 on H3 and overexpression respectively deregulation of EZH2 is associated with bladder cancer (102-105).

JQ1

JQ1 is a small molecular compound that has the ability to bind competitively to acetyl-lysine recognition motifs found in BET proteins. Binding of JQ1 to these motifs leads to a displacement of BET bromodomains and prevents, for example, BRD4 activity, with a direct effect on transcription (106). JQ1 treatment has shown to have antiproliferative effects in several cancer types. In non-muscle invasive bladder cancer, JQ1 led to an inhibition of proliferation and G1 cell-cycle arrest and induced immediate and progressive apoptosis in BRD4 depending cells (106).
In leukemia cells, depending on expression of the *Myc* oncogene, JQ1 had a significant anti-proliferative effect by inhibiting *Myc* expression (91, 107-109). Recent studies have shown that *Myc* expression is directly regulated by BRD4, and silencing *Myc* expression by preventing BET protein binding to acetylated histones at *Myc* enhancer regions leads to a decline in cell proliferation (91, 110).
Finally, in UBC knockdown of BRD4 or treatment with JQ1 led to a decreased cell viability and JQ1 treatment also induced cell apoptosis (87).

In this project, we wanted to take a further look into the role of JQ1 and characterize its effect on UBC and on epigenetic regulatory mechanisms pharmacologically and on a molecular level. We want to check our cell lines for their dependency of BRD4 expression and if there are any underlying mutations leading to different dependencies of BRD4. Our goal is to illuminate the role of chromatin modifications in UBC and to identify bladder cancer patients that might potentially benefit from a treatment with JQ1.

Material and Methods

Solutions and reagents

Hunt buffer:

20mM Tris pH 8.0

100mM NaCl

1mM EDTA

0.5% NP-40 (10% stock solution)

1 pill protease inhibitor cocktail (Roche, Basel, Switzerland)

Phosphatase Inhibitor:

45 mM Na-Butyrat (0.45M stock),

0.1M PMSF (100mM stock),

1mM Na_3VO_4 (100mM stock, activated: pH 10 - NaOH added, then boiled 5-15 seconds until clear solution, cooled, HCl added - yellow color, cycle again until no yellow color),

10mM NaF (1M stock)

10xTBS (1liter) (0.5M)

60.5g Tris-Base

90g NaCl

With HCl to pH 7.5

(TBS-T: 0.1% Tween)

10xPBS (1liter)

Dissolve in 700mL A.d.:

28.83g Na_2HPO_4

5.244g $H_2NaO_4PH_2O$

90g NaCl

Dissolve: pH 7.2-7.6

Fill up with A.d. to 1000ml

Blocking Solution:

5g (1%) PVP

5g (1%) milk powder

500ul (0.1%) Tween

0.05g (0.01%) Na-Azid

Filled up with TBS to 500mL

Transfer Buffer, 1L:

200ml Methanol

3.03g Tris-Base

14.4 g Glycine

700ml H_2O

2x SDS loading buffer:

100mM Tris-HCl pH 6.8

200mM DTT

4% SDS

20% Glycerin

Bromphenolblue

Filled up with A.d. to 20ml

Ponceau Red:

0.1% Ponceau Red

5% acetic acid

Running buffer 10x, 1L

30g Tris-Base

144g Glycine

1% SDS

SDS loading dye with DTT 2X, 10ml:

20mM pH 6.8 Tris-HCl

2mM EDTA (stock, 0.5 M)

160mM DTT (stock, 1M)

20% Glycerol

2% SDS (stock, 10%)

Cell lines

We used four cell lines in these experiments. Three cell lines have shown JQ1 sensitivity (647V, 5637 and VMCUB1) and one cell lines has shown JQ1 resistance (SW780) in earlier experiments. Cells were grown, in RPMI1640 medium (GIBCO, Thermo Fisher Scientific, Waltham Massachusetts) with 10% Fetal Bovine Serum (GIBCO, Thermo Fisher Scientific, Waltham Massachusetts) and 1% Penicillin/Streptomycin (GIBCO, Thermo Fisher Scientific, Waltham Massachusetts) at 37°celsius, 5% CO_2 and a humidity of 95%. Cell lines were tested regularly for mycoplasma infection and if needed treated with BM cyclin (Roche, Basel, Switzerland) following the manufactures protocol.

Cell Culture

Cells were cultivated according to standard hygienic protocols. For splitting cells, we first removed and discarded the media. Then, the cells were gently washed with PBS to remove leftover medium that would inhibit the action of the dissociation reagent. To detach the cells from the surface trypsin (GIBCO, Thermo Fisher Scientific, Waltham Massachusetts. 0.5mL per 10cm2) was added and cells were incubated for about 5 minutes at 37°C. Detachment was observed under the microscope after 5 minutes. Then, detached cells were transferred to a 15mL tube and centrifuged at 200 x g for 10 minutes. The pellet was resuspended in RPMI media. For counting, a small sample was removed, and cells were counted using a hemocytometer (abcam, Cambridge, UK). Cells were diluted in media to the required level of density (usually 1:10 splitting) and 10mL media were added.

Freezing

After detaching the cells, they were resuspended in media and centrifuged for 5 minutes at 1500 x g. The supernatant was removed, and the cell pellet was resuspended in FCS/10% DMSO (Thermo Fisher Scientific, Waltham, Massachusetts). For freezing, the cell suspension was aliquoted, and a 1 ml aliquot was stored in cryogenic storage vials. The vials were then placed in isopropanol chamber (Mister Frosty, Thermo Fischer Scientific,

Waltham, Massachusetts) and frozen over night at -80°C before they were transferred to liquid nitrogen long-term storage.

Thawing

After taking the vials out of the freezer they were placed into a 37°C water bath. When the cells were nearly thawed, the vial was transferred to the laminar flow hood. The cells were transferred into a tube, suspended in 5mL media and centrifuged for 10 minutes at 200 x g. After centrifugation, the supernatant was carefully removed, the pellet was resuspended in media and transferred into culture flasks.

Molecular Biological Techniques

RNA Isolation, cDNA conversion and qPCR

After removing the medium, 1mL of trizol (Thermo Fisher Scientific, Waltham, Massachusetts) was added to the flask to induce cell lysis. Then the cells were incubated for 5 minutes at room temperature (RT) and 0.2mL chloroform per 1mL of trizol were added. After three more minutes of incubation at room temperature the cells were centrifuged for 30 minutes, 12.000rpm, at 4°C. To continue with RNA extraction, the upper liquid phase was transferred to a new tube. 0.5mL (per 1mL trizol) of 100% isopropanol were added and the solution incubated for 10 minutes at RT. The content was centrifuged for 10 minutes, 12.000rpm, at 4°C and the supernatant was removed. The remaining RNA pellet was subsequently washed with 75% ethanol, vortexed and centrifuged for 5 minutes, 7500rpm, at 4°C. Next, the ethanol was removed completely, and the pellet was left to dry at RT. After complete drying, the pellet was resuspended in RNA/DNAse free water (50µl) and incubated on a heat block for 15 minutes at 55° C to dissolve RNA secondary structures. To determine the RNA concentration, RNA samples were measured on a Nanodrop (NANODROP 2000, Thermo Scientific, Waltham, Massachusetts) (2µl per measure), frozen in liquid nitrogen and stored at -80° C until further use.

qPCR

The extracted RNA was then converted into cDNA using the TaqMan reverse transcription kit (Thermo Fisher, Waltham, Massachusetts) following the manufacturers protocol. For the conversion of 500ng of RNA into cDNA a mastermix containing the following ingredients was prepared:

- 10x TaqMan RT Buffer 5 µl

- 25mM $MgCl_2$ 11 µl
- dNTPs 10 µl
- OligoTs 2.5 µl
- RNase Inhibitor 1 µl
- Reverse Trancriptase 1.25 µl

For amplification of cDNA targets the following mastermix was prepared:

- SYBR Green master mix (Thermo Fisher) 7.5 µl
- ddH_2o 4.5 µl
- Primer 1 µl
- cDNA 2 µl

Primer sequences used were as follows:

MYC

Forward primer CATCCACGAAACTTTGCCCA

Reverse primer GTGAAGCTAACGTTGAGGGG

FGFR3 *(Homo sapiens fibroblast growth factor receptor 3)*

Forward primer TGCGTCGTGGAGAACAAGTTT

Reverse primer GCACGGTAACGTAGGGTGTG

GLI1 *(Homo sapiens GLI family zinc finger 1)*

Forward primer AGCGTGAGCCTGAATCTGTG

Reverse primer CAGCATGTACTGGGCTTTGAA

GLI2 *(Homo sapiens GLI1 family zinc finger 2)*

Forward primer CTGCCTCCGAGAAGCAAGAAG

Reverse primer GCATGGAATGGTGGCAAGAG

NOTCH1 *(Homo sapiens notch 1)*

Forward primer TGGACCAGATTGGGGAGTTC

Reverse primer GCACACTCGTCTGTGTTGAC

CCND2 *(Homo sapiens cyclin D2)*

Forward primer CTGTCTCTGATCCGCAAGCAT

Reverse primer GGTGGGTACATGGCAAACTTAAA

ERBB2 *(Homo sapiens v-erb-b2 erythroblastic leukemia viral oncogene homolog 2)*
Forward primer TGCAGGGAAACCTGGAACTC
Reverse primer ACAGGGGTGGTATTGTTCAGC

BCL6 *(Homo sapiens B-cell CLL/lymphoma 6)*
Forward primer GTTGTGGACACTTGCCGGAA
Reverse primer CTCTTCACGAGGAGGCTTGAT

SUFU *(Homo sapiens suppressor of fused homolog*)
Forward primer: GCCTGAGTGATCTCTATGGTGA
Reverse primer: TCTCTCTTCAGACGAAAGGTCAA

For qPCR, the QuantStudio 7 Flex (applied biosystems, Foster City, California) instrument with the following settings was used:

Settings:

- Number of PCR cycles: 40
- Hold Stage: 95°celsius for 10minutes
- PCR Stage:
    - 95°celsius for 15 seconds
    - 60°celsius for 30 seconds
    - 72°celsius for 30 seconds
- Hold Stage: 72°celsius for 10 minutes

Protein Extraction

For sample preparation the cells were harvested, washed and centrifuged. The cell pellet was dissolved in 100-200µl Hunt buffer and a phosphatase inhibitor was added. The tube was put into liquid nitrogen to shock-freeze the cells and subsequently thawed at 37°C. Afterwards cells were shock-frozen again and put on ice to thaw. After thawing, the contents were centrifuged for 10 minutes, 13.000rpm, at 4°C. To collect the proteins, the supernatant (containing the proteins) was carefully transferred to a new tube and protein concentration was measured with the help of Nanodrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts).

Western Blot

Reagent	8% Separating Gel (20ml)	5% Stacking Gel (8ml)
H_2O	9.3 ml	5.5 ml
30% acrylamide mix	5.3 ml	1.3 ml
Tris-Cl (1.5M, pH8.8)	5.0 ml	X
Tris-Cl (1.0M, pH6.6)	X	1.0 ml
SDS (10%)	0.2 ml	0.08 ml
10% Ammonium persulfate	0.2 ml	0.08 ml
TEMED	0.012	0.008 ml

The extracted proteins were run on SDS polyacrylamide gels for 2 hours at 120V and then transferred onto a nitrocellulose membrane for another 2 hours at 30V. The proteins on the membrane were stained with Ponceau Red Staining and then blocked for one hour in blocking solution.

For protein detection, the first antibody (anti-BRD4 antibody diluted 1:1000, abcam Cambridge UK) was diluted in blocking solution and the membrane was incubated with antibody-blocking-solution overnight at 4°celsius.

On the following day, the membrane was washed three times with 1 x TBS - T for five minutes and incubated in secondary antibody-solution, anti-rabbit (Thermo Fisher Scientific, Waltham, Massachusetts) at a dilution of 1:10 000 for 1h at room temperature. Before visualization, the membrane was washed 3 x with TBS-T and incubated for a few seconds in PierceECLPLUS Western blotting Substrate solution (Thermo Fisher Scientific, Waltham, Massachusetts) according to manufacturer's protocol. Visualization of proteins was carried out with plain films on a CURIX 60 (AGFA, Mortsel, Belgium).

IC_{50} Curves

The IC50 is defined as the half maximal inhibitory concentration and indicates the amount of substance (e.g. drug) needed to reduce a biological process by half.

For IC50 curves, approximately 3x10^3 cells/well were seeded out in 96 well plates (COSTAR) and kept overnight to let them attach to the ground. After overnight incubation, the media was removed and replaced with new media (100µl) containing different concentrations of inhibitor JQ1 (0.003 up to 3000µmol). The cells were then left to grow for up to 72 hours. After incubation, the plates were taken out of the incubator and brought to room temperature. 100µl of Cell Titer Reagent (CELLTITERGLO, Promega, Madison, Wisconsin) were added to each well in the ratio 1:1 and mixed with the contents for 2 minutes to induce lysis. Afterwards the plates were incubated for 10 minutes at room

temperature to stabilize the signal and luminescence was recorded with VICTOR Wallac1420 (PerkinElmer, Waltham, Massachusetts).

To create our IC50 curves we used the program aatbio.com/tools/ic50-calculator/

Results

IC50

To analyze the effect of JQ1 in bladder cancer cell lines 5637, 647V, SW780 and VMCUB1, IC50 curves with varying drug concentrations were created for each cell line. The IC50 is defined as the half maximal inhibitory concentration and indicates the amount of substance (e.g. drug) needed to reduce a biological process by half.

Results for cell line 5637

Cell line 5637 showed an IC50 value of 0.592 µM.

Concentration	Response 1	Response 2	Response 3
0.1	58360	50509	44803
0.3	48790	39816	33049
1	37505	30309	27517
3	23390	22950	20149
10	21079	21754	17484
30	9350	9890	5155
100	2289	2630	3264
300	1383	1394	1582
1000	2392	4758	6461

Table1.

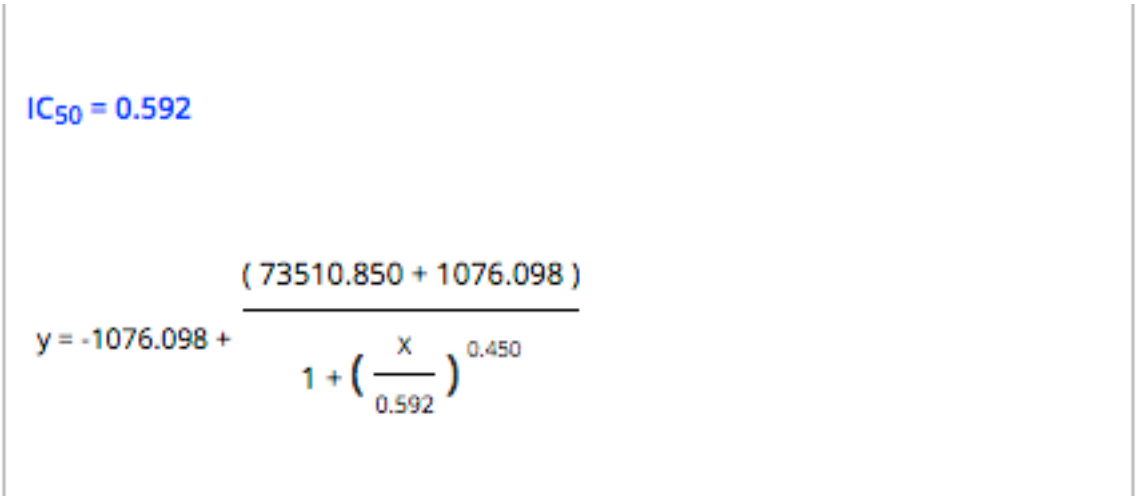

Figure1.

Cell counts and IC 50 calculation for Cell line 5637. Each cell count in comparison to its JQ1 concentration which we then converted into a dose – response curve (Figure 2).

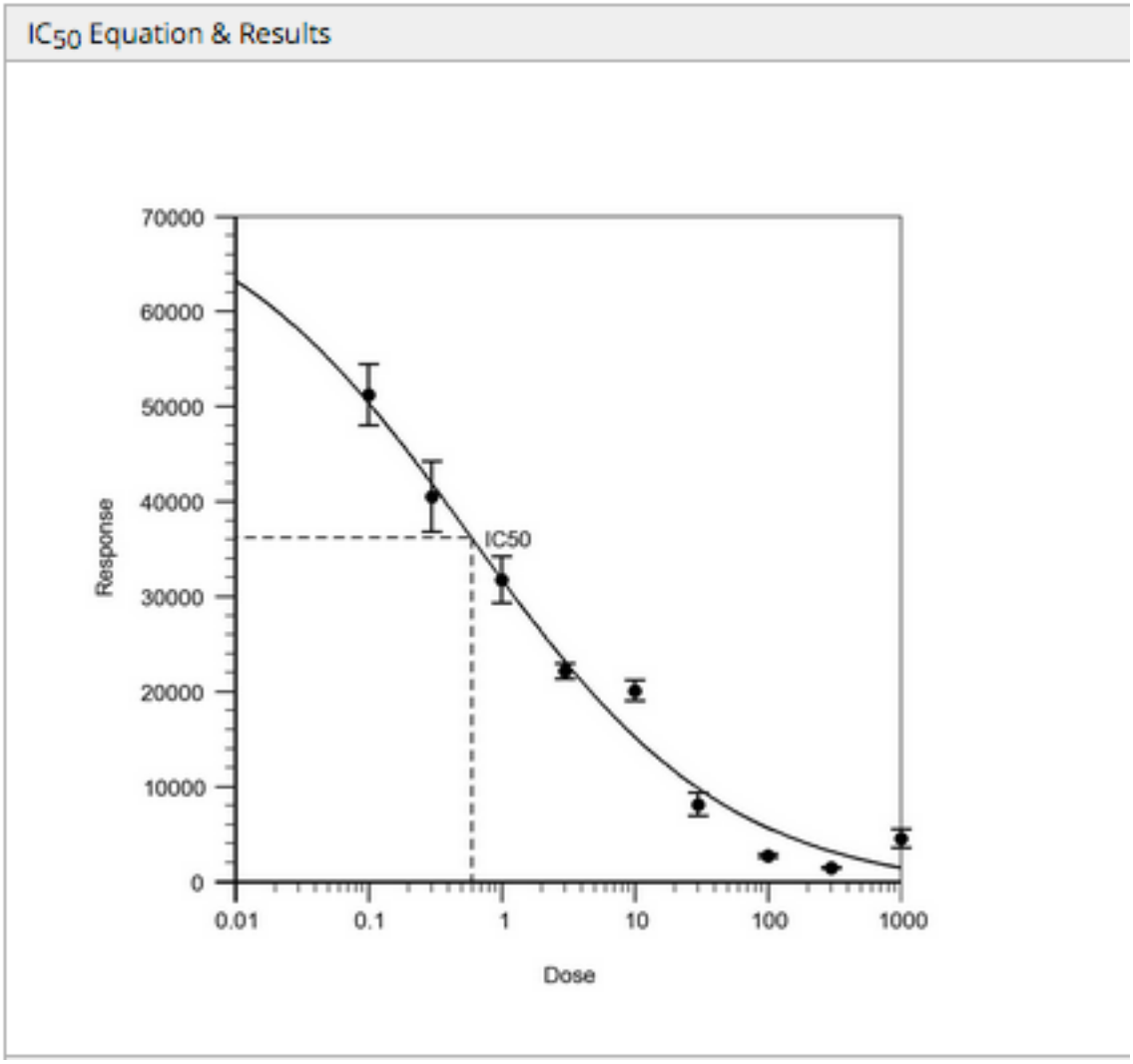

Figure 2. Dose-Response Curve for IC50 evaluation of cell line 5637.

Results for cell line 647V

Cell line 647V showed an IC50 value of 1.9 µM.

Concentration	Response 1	Response 2	Response 3
0.01	120919	119905	101913
0.03	127857	120990	118100
0.1	117181	104331	84450
0.3	113570	121986	105999
1	106930	75564	54498
3	52616	48386	35624
10	34636	22561	14285
30	11152	7572	7380
100	8093	6853	9763
300	1329	2157	2102

Table 2.

$IC_{50} = 1.972$

$$y = 4696.050 + \frac{(116690.700 - 4696.050)}{1 + \left(\frac{x}{1.972}\right)^{1.132}}$$

Figure 2.

Cell counts and IC 50 calculation for Cell line 647V. Each cell count in comparison to its JQ1 concentration which we then converted into a dose – response curve (Figure 3).

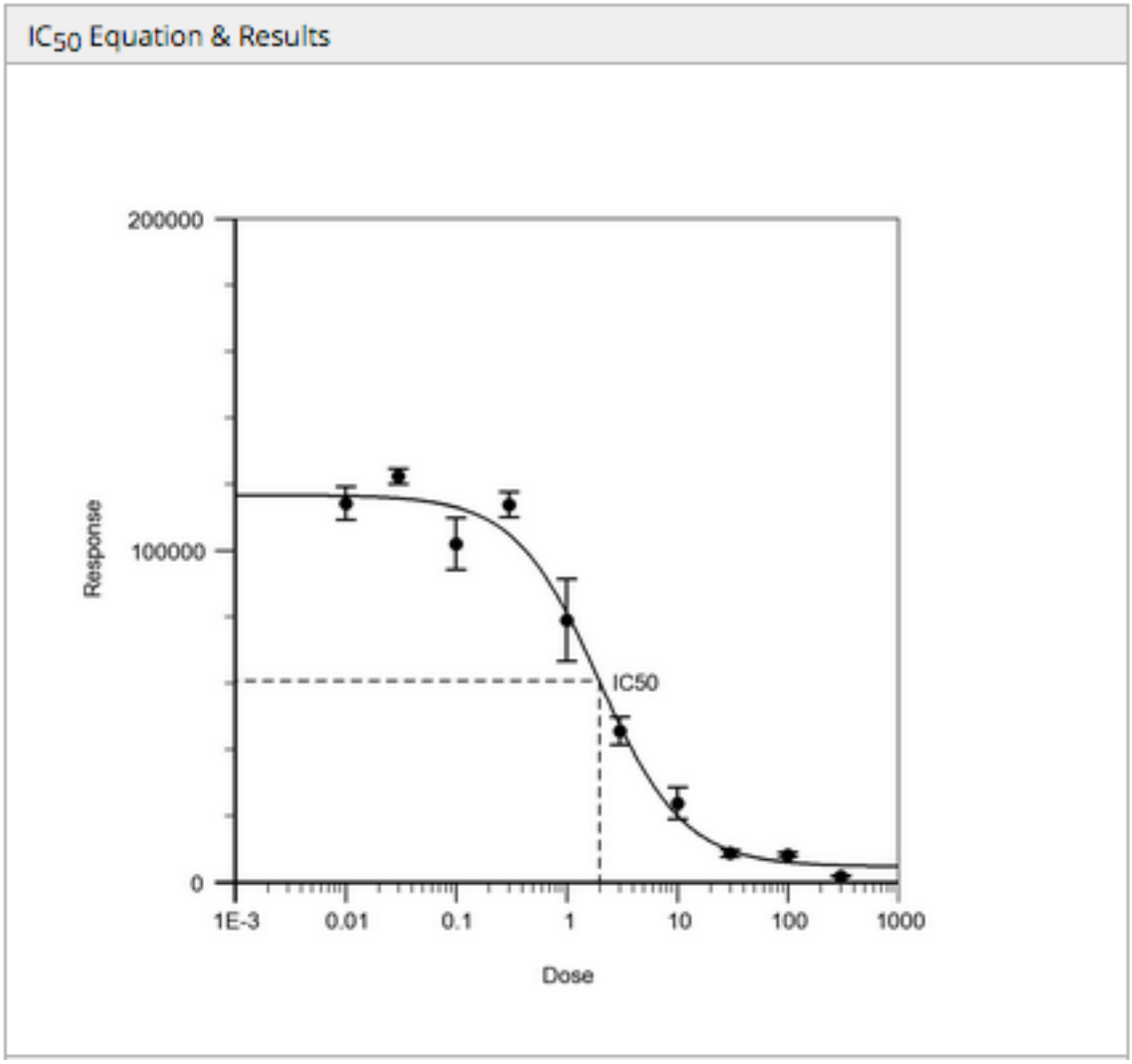

Figure 3. Dose-Response Curve for IC50 evaluation of cell line 647V.

Results for cell line Sw780

Cell line Sw780 showed an IC50 value of 41.75 µM.

Concentration	Response 1	Response 2	Response 3
0.03	20249	195226	189080
0.1	130607	118684	94088
0.3	178163	195226	189080
1	107147	98769	82263
3	134219	153719	141662
10	109707	109513	100766
30	85900	115237	112263
100	6960	9432	7159
300	5065	4723	4328
1000	9939	8501	9055

Table 3.

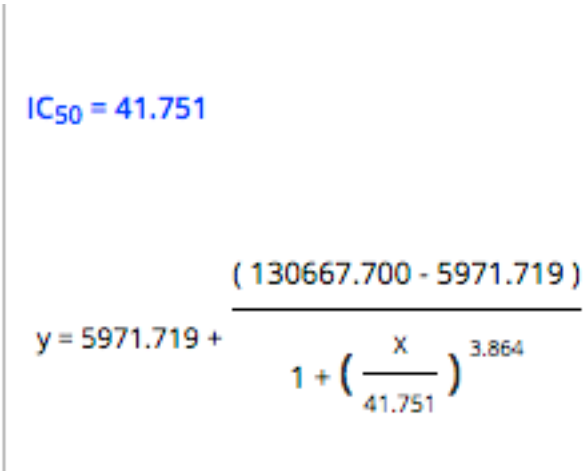

Figure 3.

Cell counts and IC 50 calculation for Cell line 647V. Each cell count in comparison to its JQ1 concentration which we then converted into a dose – response curve (Figure 4).

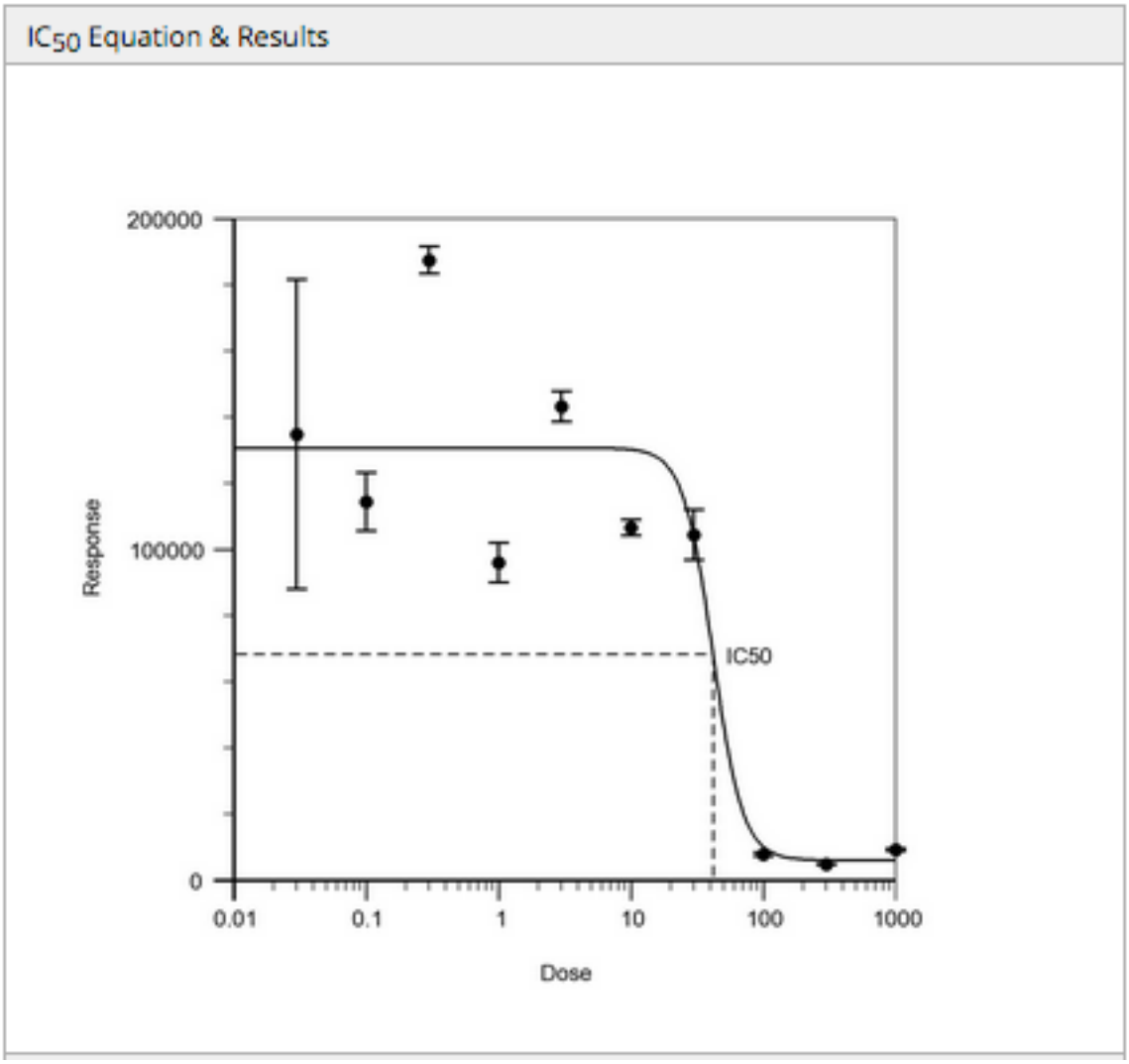

Figure 4. Dose-Response Curve for IC50 evaluation of cell line SW780.

Results for cell line Vmcub1

Cell line Vmcub1 showed an IC50 value of 12.9 µM.

Concentration	Response 1	Response 2	Response 3
3×10^{-3}	143382	92032	83459
0.03	158366	92745	83668
0.1	100551	75274	60537
0.3	145811	71016	63566
3	107789	55472	39648
30	82626	33909	26440
100	4159	4017	3300
300	3052	3407	1880
1000	6303	8010	5424

Table 4.

$IC_{50} = 12.886$

$$y = -12505.650 + \frac{(105816.900 + 12505.650)}{1 + \left(\frac{x}{12.886}\right)^{0.510}}$$

Figure 4.

Cell counts and IC 50 calculation for Cell line VMCUB1. Each cell count in comparison to its JQ1 concentration which we then converted into a dose – response curve (Figure 5).

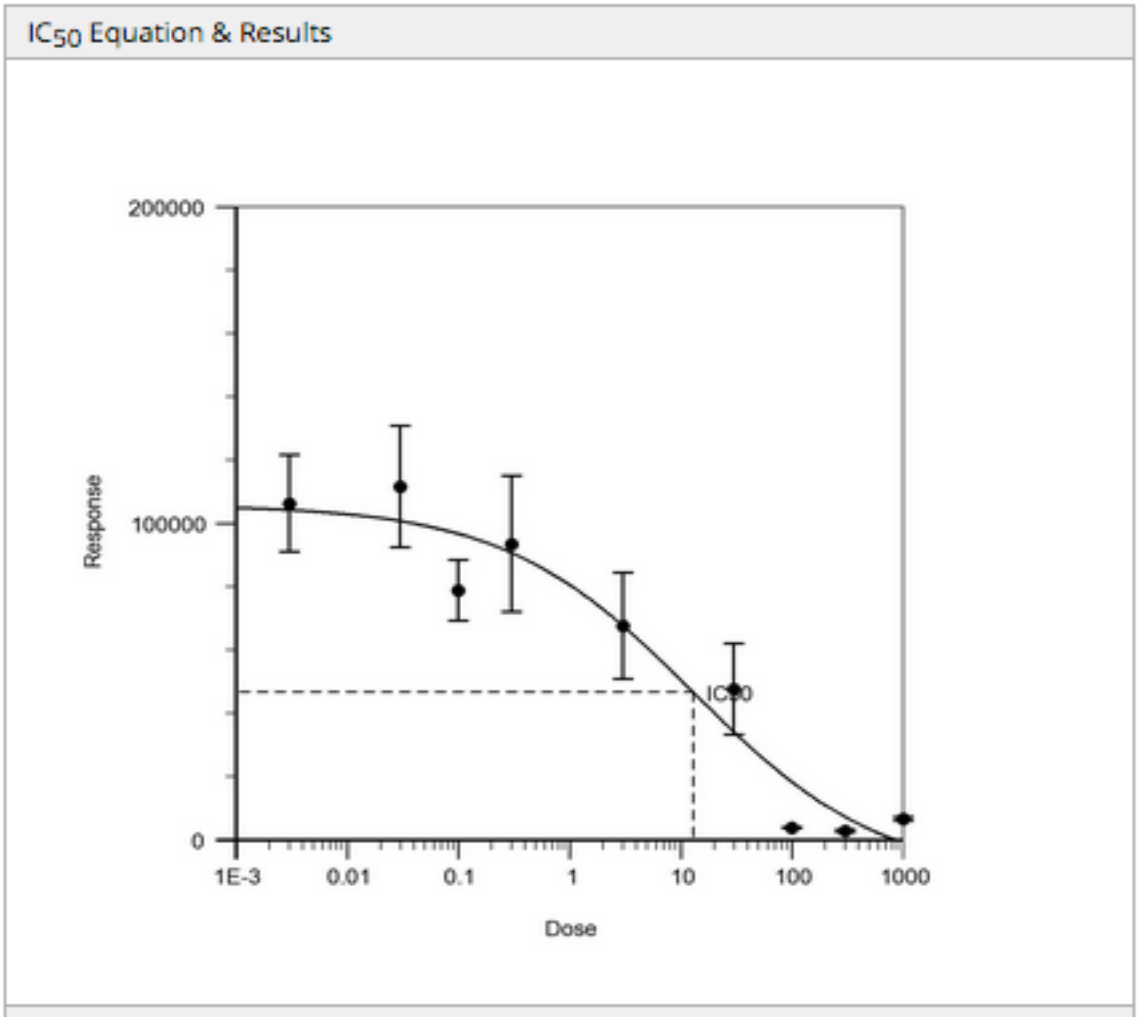

Figure 5. Dose-Response Curve for IC50 evaluation of cell line VMCUB1.

Gene expression

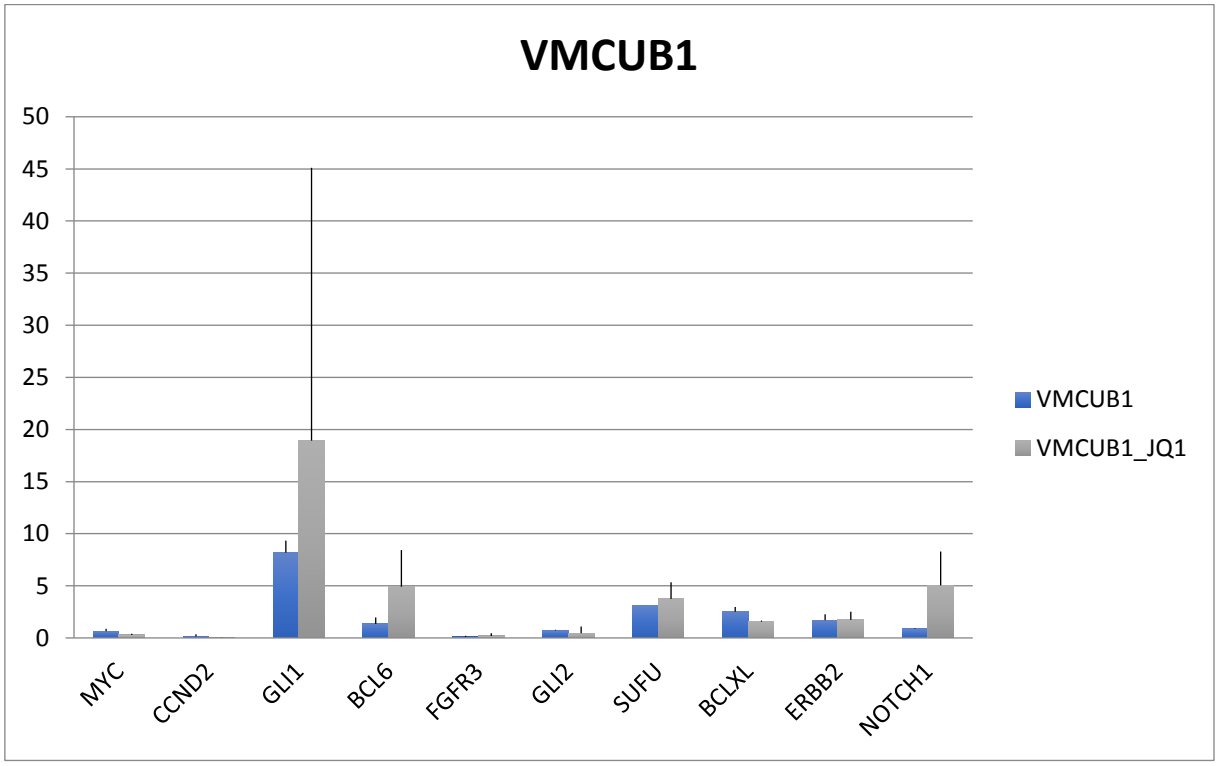

Figure 6.
Gene expression in cell line VMCUB1. Comparison between controlgroup (blue) and treated groups (grey).

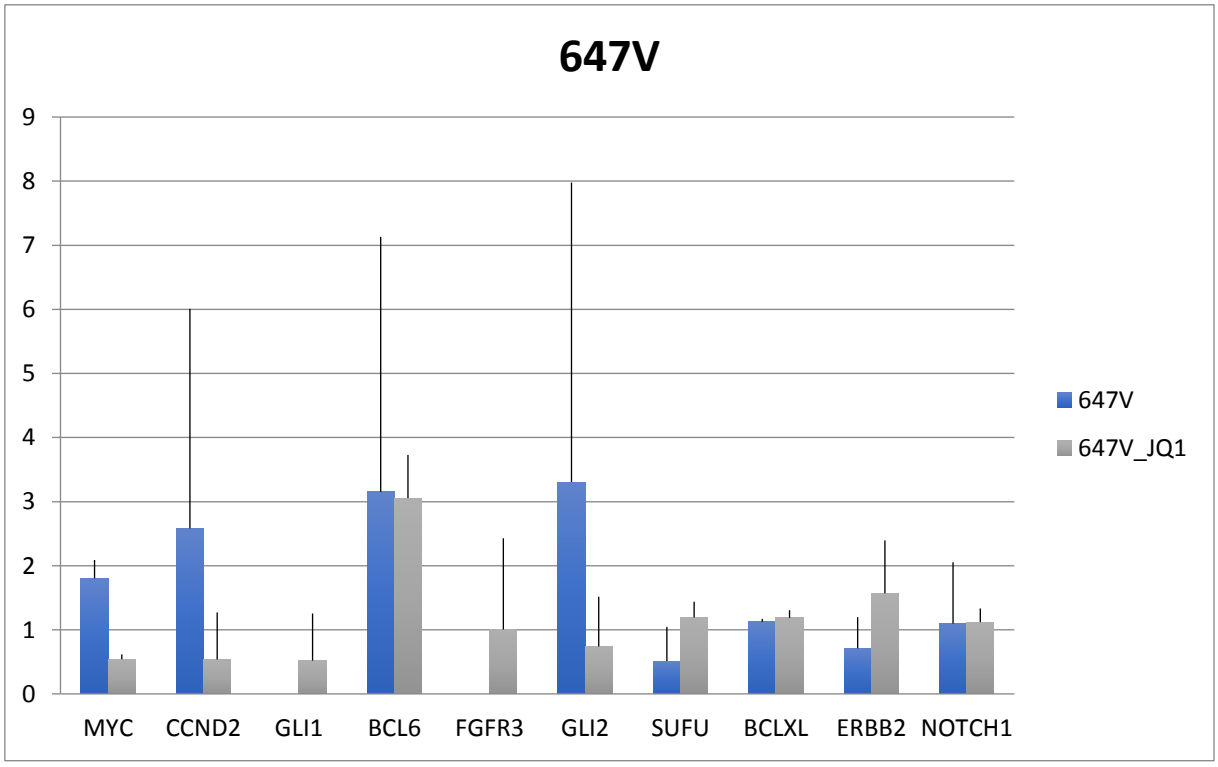

Figure 7.
Gene expression in cell line 647V. Comparison between control groups (blue) and treated groups (grey).

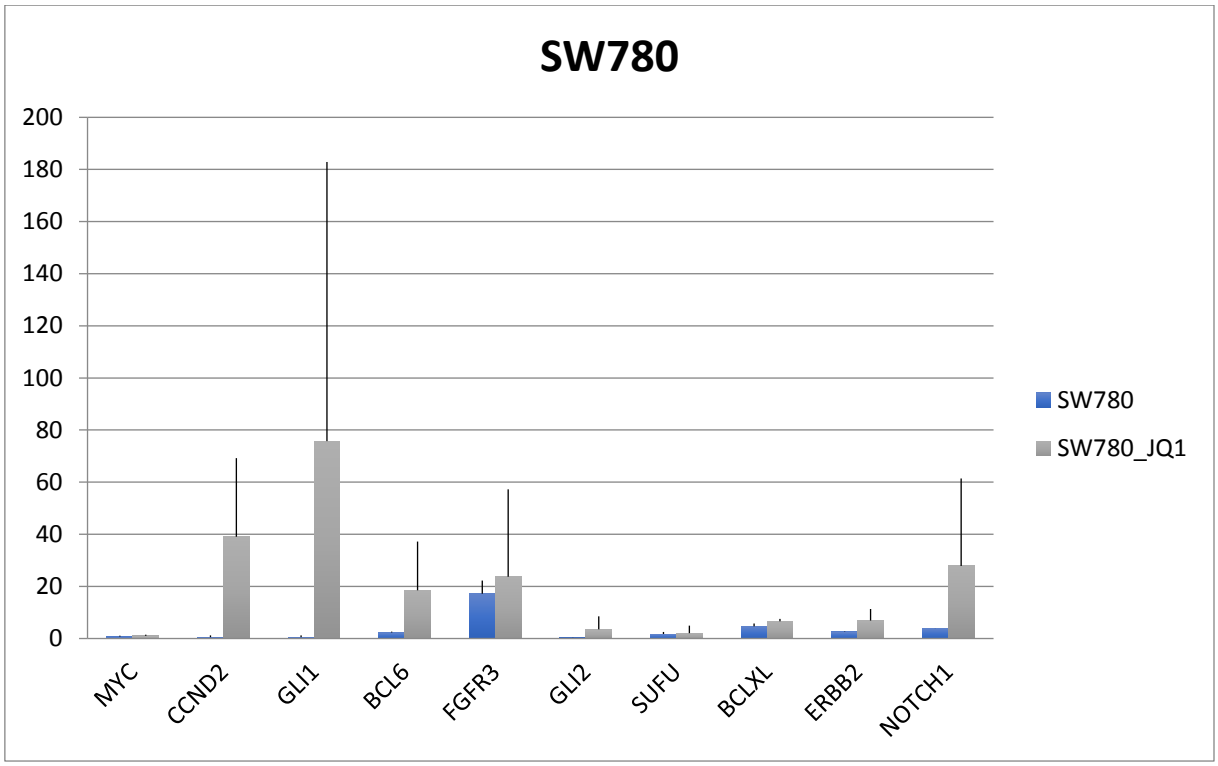

Figure 8.
Gene expression in cell line SW780. Comparison between control groups (blue) and treated groups (grey).

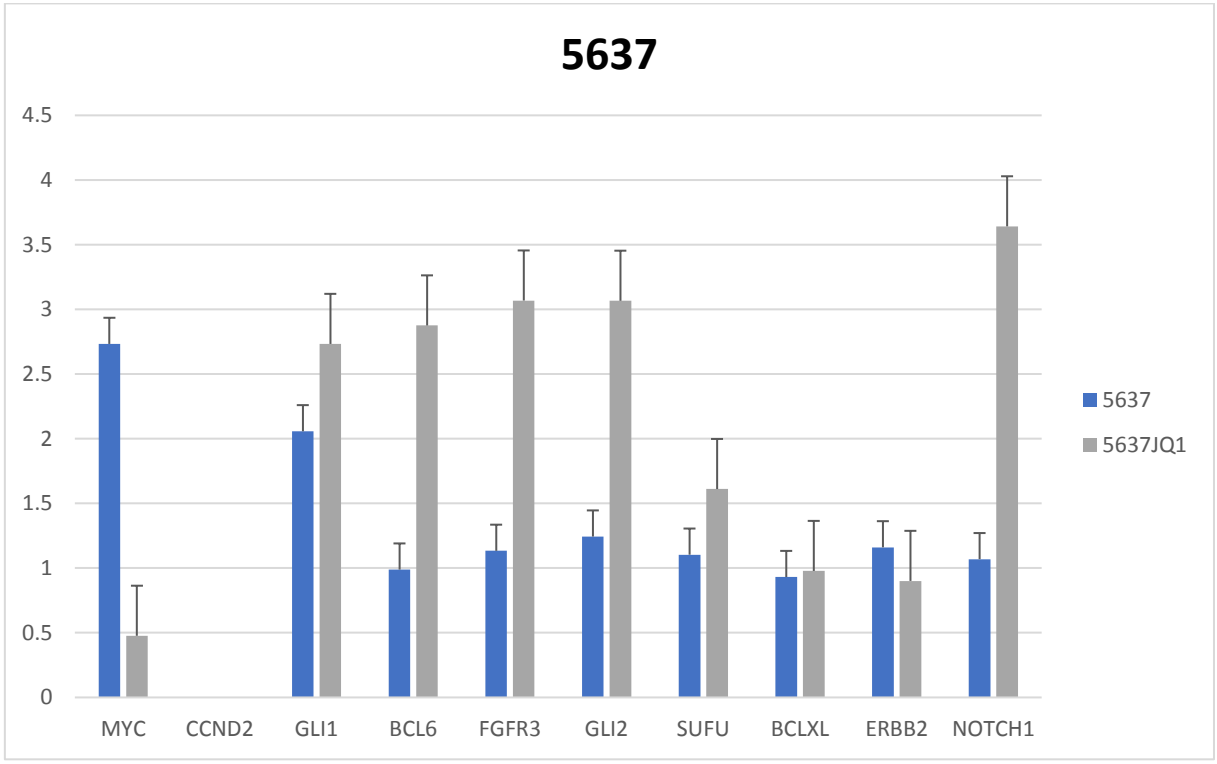

Figure 9.
Gene expression in cell line 5637. Comparison between control groups (blue) and treated groups (grey).

To study the gene expression in the cell lines used, we compared each cell line in untreated condition vs. itself after treatment with JQ1. Real time PCR analysis of RNA extracts of cell lines Vmcub1, 647V, Sw780 and 5367 revealed changes in the expression of genes as follows.
In Vmcub1 (Figure 6) major differences in gene expression were spotted for genes *Gli1* (1.3 times), *Bcl6* (2.6 times) and *Notch1* (4.8 times). We also detected changes in the expression patterns in genes *Sufu* (0.21 times) and *Bcl-xL* (-0.37 times). Genes *Erbb2*, *Fgfr3*, *Ccnd2* and *Myc* didn't show any real changes in their expression.

For the cell line 647V (Figure 7), difference in gene expression was spotted for genes *Myc* (-0.6 times), *Ccnd2* (-0.79 times), *Gli2* (-0.78 times), *Sufu* (+1.3 times) and *Erbb2* (+1.2 times).

In Sw780 (Figure 8), especially genes *Ccnd2* (+77 times), *Gli1* (+150 times), *Bcl6* (+7 times) and *Notch1* (+6 times), showed high overexpression after successful JQ1 treatment. *Fgfr3* (+0.4 times), *Erbb2* (+1.7 times) and *Bcl-xL* (+0.4 times) also showed upregulation, the other investigated genes did not show significant changes in their expression patterns.

Cell line 5637 (Figure 9) showed changes in expression in genes *Myc* (-77%), *Gli1* (+1.3 times), *Bcl6* (+1.9 times), *Fgfr3* (+1.7 times), *Gli2* (+1.5 times), *Sufu* (+1.5 times), *Erbb2* (-0.2 times) and *Notch1* (+2.4 times).
Figure 10 through figure 19 show the comparison of changes of gene expression for each gene itself. Cell lines are named 1 for line 5637, 2 for line Sw780, 3 for 647V and 4 for Vmcub1.

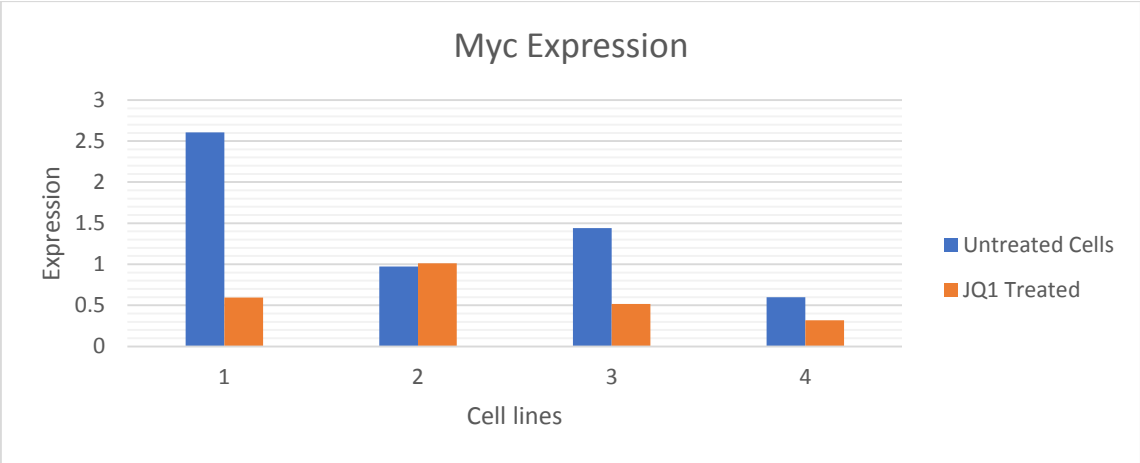

Figure 10.
Myc Expression compared between control group (blue) and treated group (orange). Myc downregulation in cell line 5637 (1), 647V (3) and Vmcub1 (4).

Figure 11.
Gli1 Expression compared between control group (blue) and treated group (orange). Gli1 upregulation in cell line Sw780 (2) and Vmcub1 (4).

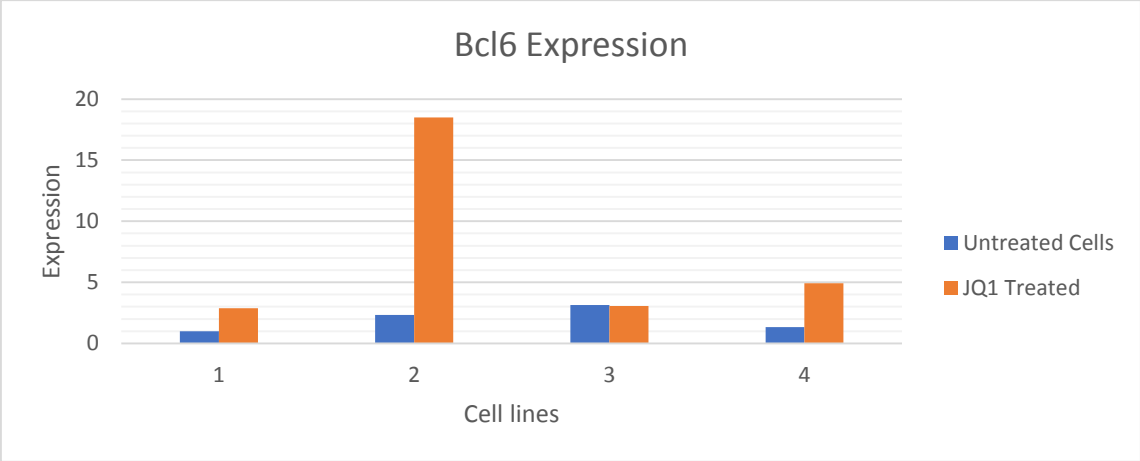

Figure 12.
Bcl6 Expression compared between control group (blue) and treated group (orange). Bcl6 upregulation in cell line 5637 (1), Sw780 (2) and Vmcub1 (4).

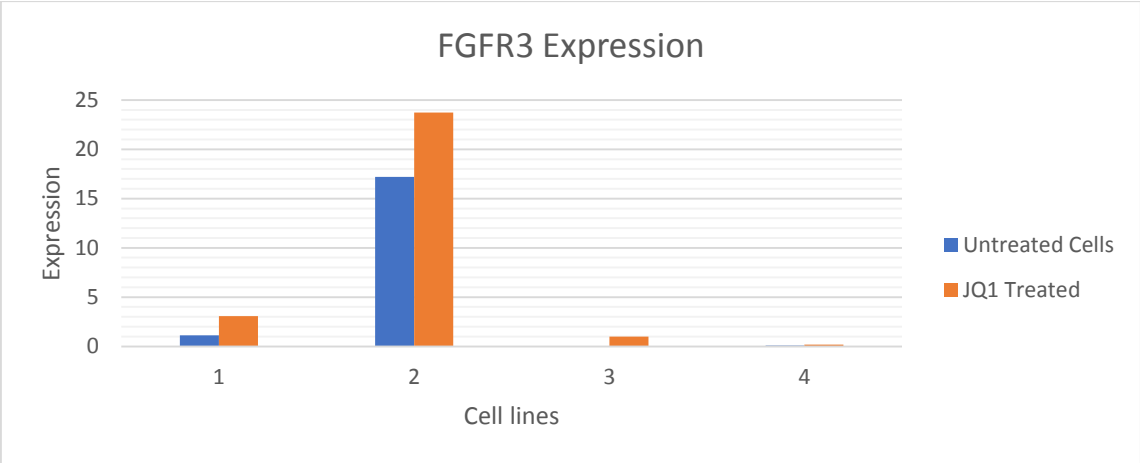

Figure 13.
FGFR3 Expression compared between control group (blue) and treated group (orange). FGFR3 upregulation in cell line 5637 (1), Sw780 (2) and 647V (3).

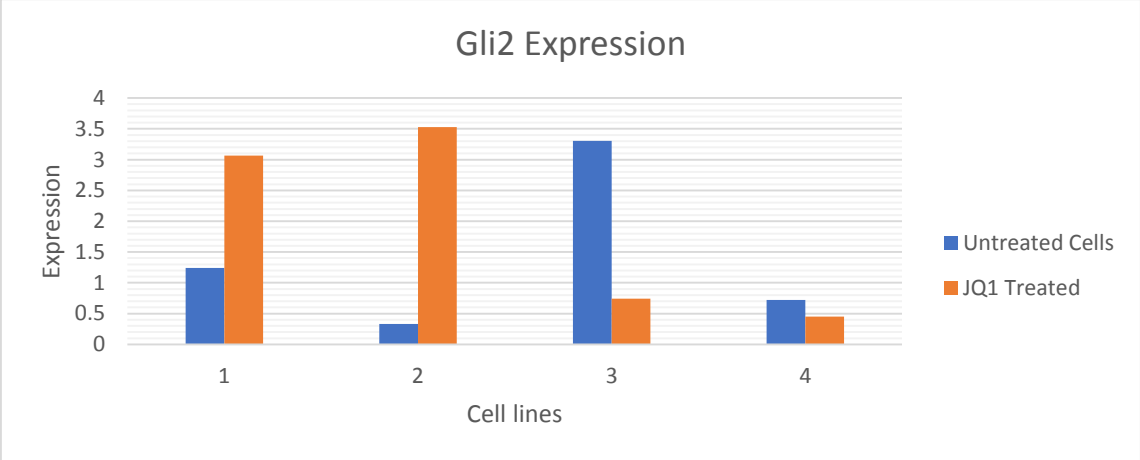

Figure 14.
Gli1 Expression compared between control group (blue) and treated group (orange). Gli1 upregulation in cell line 5637 (1), Sw780 (2) and downregulation in cell line 647V (3) and Vmcub1 (4).

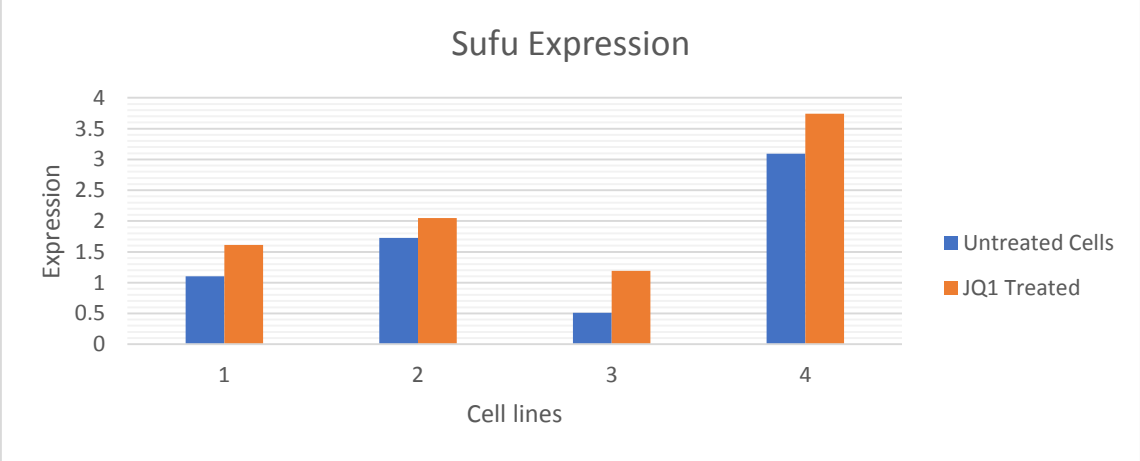

Figure 15.
Sufu Expression compared between control group (blue) and treated group (orange). Sufu upregulation observed in all cell lines.

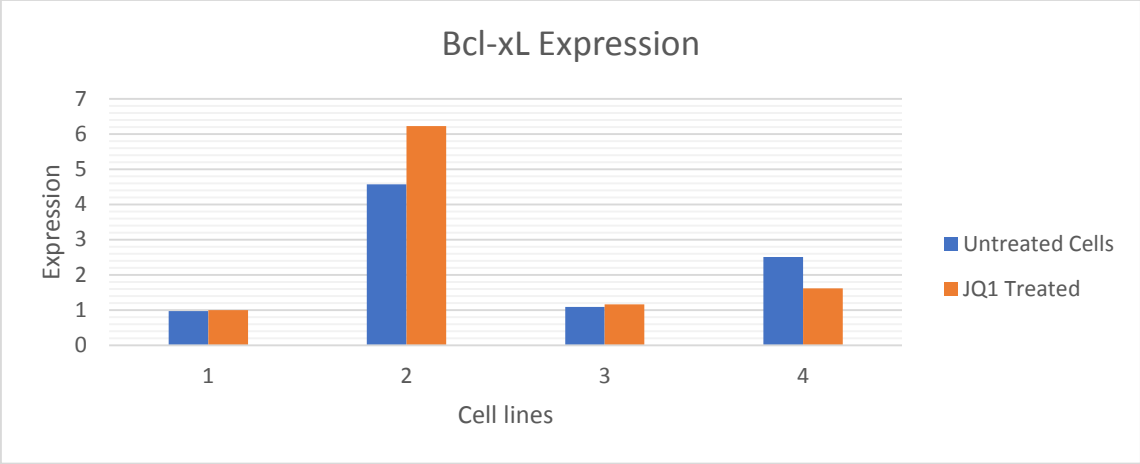

Figure 16.
Bcl-xL Expession compared between control group (blue) and treated group (orange). Bcl-xL upregulation in cell line Sw780 (2) and downregulation in Vmcub1 (4).

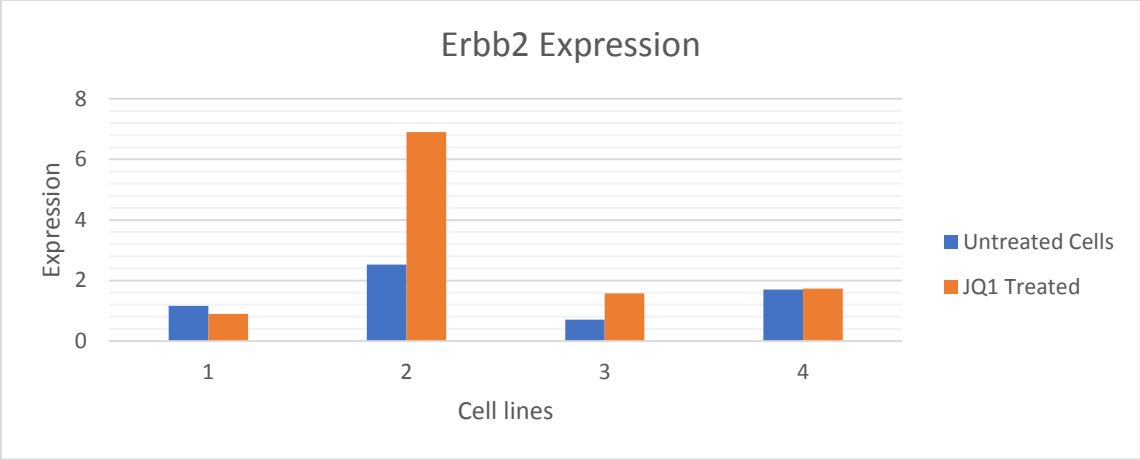

Figure 17.
Erbb2 Expession compared between control group (blue) and treated group (orange). Erbb2 downregulation in cell line 5637 (1) and upregulation in Sw780 (2) and 647V (3).

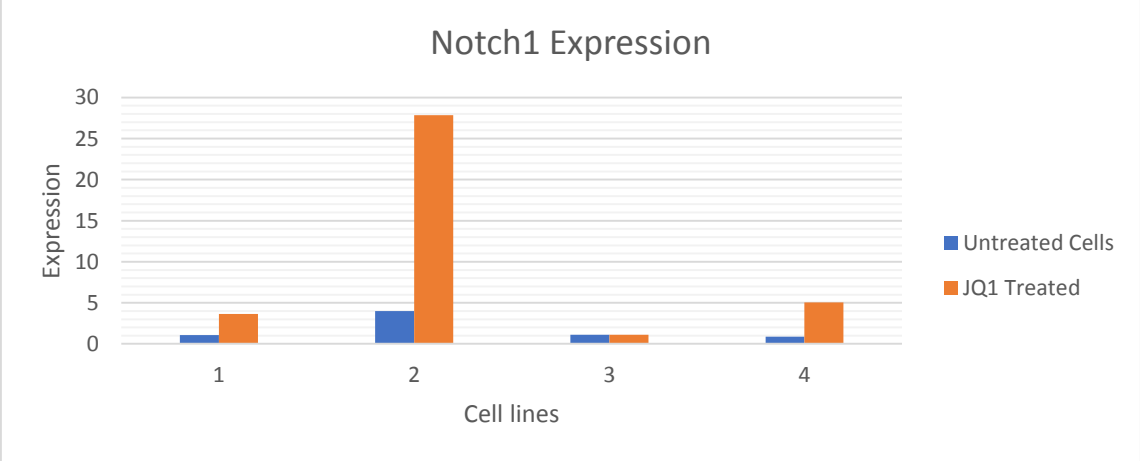

Figure 18.
Notch 1 Expession compared between control group (blue) and treated group (orange). Notch 1 upregulation in cell line 5637 (1), Sw780 (2) and Vmcub1 (4).

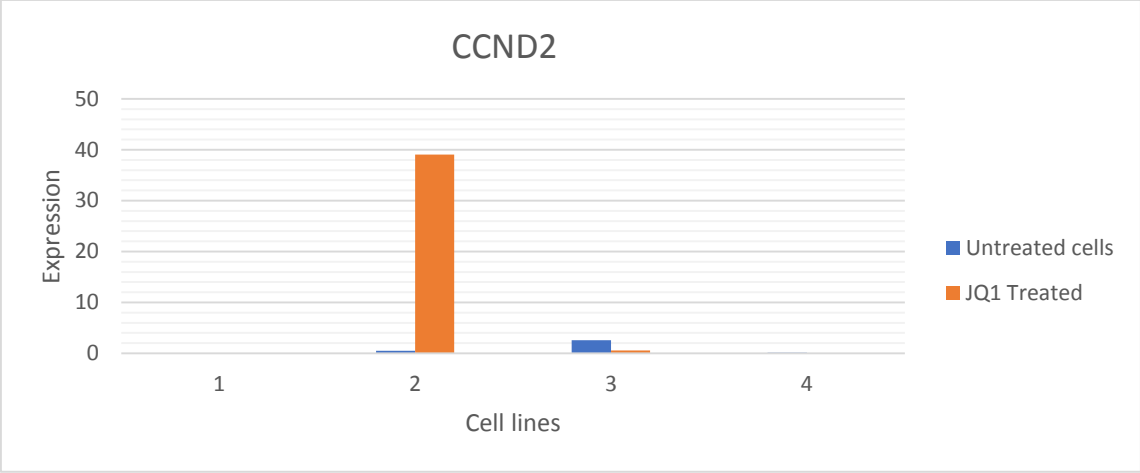

Figure 19.
Ccnd2 Expession compared between control group (blue) and treated group (orange). Ccnd2 upregulation in cell line Sw780 (2) and downregulation in cell line 647V (3).

Protein expression

Changes in the expression pattern of BRD4 protein level was measured by Western blotting. We used the same condition as in the qPCR. We compared four cell lines (5637, 647V, Sw780 and Vmcub1) in normal media vs treated cells with JQ1.

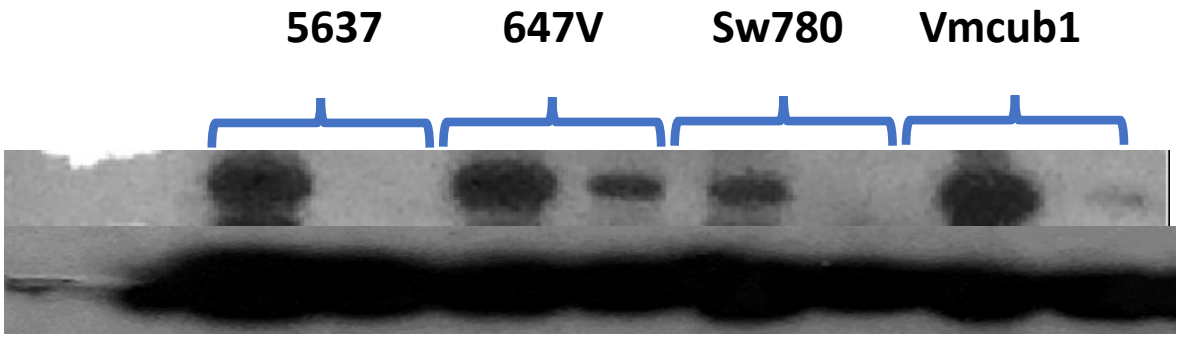

Figure 20.
Western Blot for protein expression of BRD4 in our cell lines. Comparison between control groups and treated groups. Below, Beta-Actin control.

Western Blot Analyses of JQ1 treated and non JQ1 treated Cell lines.

Cell 5637	Cell 5637 JQ!	Cell 647V	Cell 647V JQ1	Cell SW780	Cell SW780 JQ	Cell Vmcub1	Cell Vmcub1JQ
100	2	100	28	100	21	100	10

In the 5637 treated cells a reduction of 0.98 times of BRD4 production was seen. The 647V cells did show a reduction by 0.72 times. In the SW780 cell line the production of went down by 0.79 times and in the Vmcub1 cell line the production of BRD4 was reduced by 0.9 times (Figure 20, 22).

For Image analyses Image J 1.52a, NIH, USA was used.

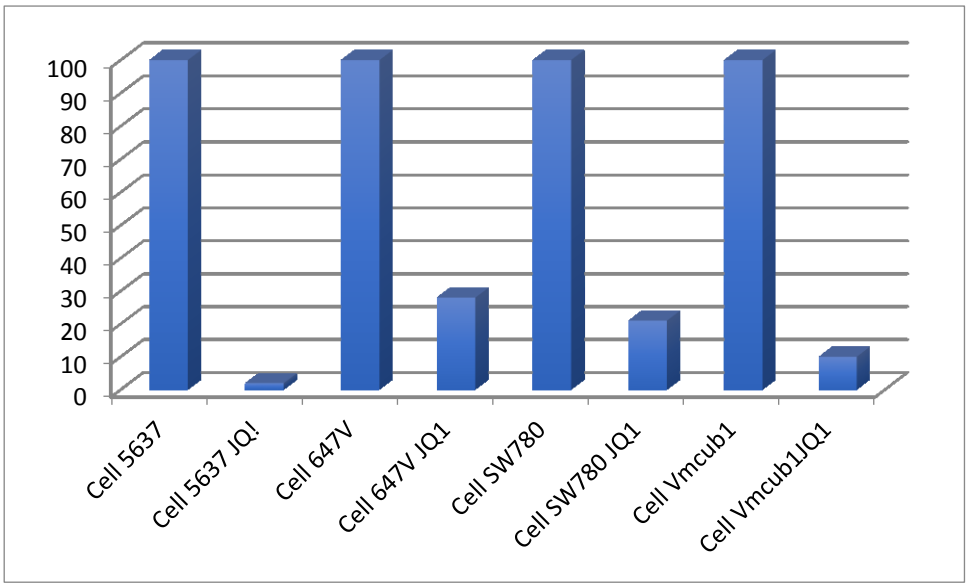

Figure 22. (Analysis of BRD4 protein expression.)

Discussion

Urothelial carcinoma of the bladder is a major cause of morbidity and mortality worldwide (1). TURB is still the main therapy of early stages of UBC, but the European Association of Urologists (EAU) Guidelines suggest adjuvant chemotherapy to be taken into consideration to reduce the risk of recurrence for low-risk UBC. The most commonly used chemotherapeutic drugs are mitomycin C and epirubicin, which are installed immediately after surgery (11). According to a 2016 study, a single immediate installation of chemotherapy immediately after resection reduces the risk of recurrence in NMIBC patients (12). However, despite the use of a large number of various chemo-therapeutics UBC- due to high recurrence rates- is the most expensive cancer in follow-ups, and novel treatment options are needed. In our thesis we identified JQ1 as a possible drug candidate in UBC. JQ1 is a thienotriazolodiazepine and a potent inhibitor of the BET family. It is currently being tested in clinical trials for a variety of cancers including NUT midline carcinoma. JQ1 acts mainly via BRD4 inhibition. BRD4 is a member of the bromodomain and extra terminal domain (BET) family of proteins that includes the ubiquitously expressed BRD2, BRD3, BRD4, and BRDT proteins.

Inhibition of BRD4 by JQ1 targets *c-Myc* expression and further down the pathway *Myc*-driven oncogenesis. Since, as described above, the efficacy of JQ1 is mainly attributed to its ability to suppress the expression of *c-Myc* in many malignancies, we looked at the expression levels of *Myc* mRNA in our UBC cell lines (647V, 5637, VMCUB1 and SW780). In untreated cells we were able to detect a significant mRNA expression for *Myc* in the cell lines 647V and 5637. In VMCUB1 and SW780 cell lines the levels of *Myc* mRNA expression was almost undetectable.

To determine the effect of JQ1 and to measure IC50 values of our UBC cell lines, we administered increasing concentrations of JQ1, starting form 0.01µM to 100µM. Two of the cell lines (647V and 5637) showed an IC50 value of approximately 1µM or less. This value has been also observed in other cell lines responsive to BRD4 inhibitors and is well in accordance to prior publications (111). In contrast to the two JQ1sensitive cell lines, in cell lines VMCUB1 and SW780, the IC50 values were at least 10 folds higher. The fact that these two cell lines, which seem to have a rather low *c-Myc* dependence, as suggested by their low *c-Myc* mRNA expression , need far larger amounts of JQ1 again fits well with the assumption that JQ1 acts mainly via BRD4 on *c-Myc* driven oncogenesis (112). Thus, since the epigenetic landscapes of cancers may vary drastically even in the same tissue as

seen in our bladder cancer cell lines, it might be necessary to determine the expression pattern for various oncogenes before planning treatment with JQ1 inhibitors.

We also observed an increased *Notch1* expression in our cell lines, especially in cell line Sw780. *NOTCH1* is part of the NOTCH pathway, which consists of four (*NOTCH1-4*) different transmembrane receptors. *Notch1* has been proven to play a role in bladder cancer as a tumor suppressor, as loss-of-function mutations of the *NOTCH* pathway accelerated bladder tumorigenesis (113). This might suggest a downregulation of *NOTCH1* in our untreated bladder cancer cell lines since successful treatment with JQ1 led to an increased *NOTCH1* activity. Because of this, JQ1 might lead to a slowdown of tumor progression through *NOTCH1* induced tumor suppression. Surprisingly, we found that in JQ1 resistant cell lines Sw780 (six times) and Vmcub1 (4.5 times), *NOTCH1* expression was significantly higher after treatment than in JQ1 sensitive cell lines, which will need further confirmation.
Another gene that showed the same result was *GLI1*. *GLI1*, as well as *GLI2* and *GLI3,* is part of the Kruppel family of zinc finger proteins. They are part of the Shh signaling pathway and thereby critical in the development of the CNS (114, 115). A study observed that *GLI1* expression is regulated through BRD4 (116). Because of this, we expected a change in *GLI1* expression after successful JQ1 inhibition. However, even though we observed some expression change, again it occurred mainly in cell lines Sw780 and Vmcub1. As already mentioned for *NOTCH1*, these results will need further confirmation, but maybe alternative pathways than the inhibition of *c-myc* mRNA expression might be responsible for the results seen in our study.

Recently, a study showed the improved outcome of combined therapy of mitomycin C and JQ1 over single therapy with just one of both substances (117). In the case of JQ1 resistant cell lines, a study has proven the positive effect of combining JQ1 with Romidepsin. The study treated ten cell lines with JQ1, but only observed apoptosis in eight lines. With the combination of JQ1 with Romidepsin they were able to induce apoptosis in all cell lines. They also observed that even though they were able to induce apoptosis in all bladder cancer cell lines, the combination of JQ1 and Romidepsin did not really affect benign cells and is therefore a promising therapy approach in urothelial carcinoma (118).
In conclusion, JQ1 might be a viable future treatment option in UBC. However, it might be of importance to look at the expression pattern of a variety of other oncogenes. Especially

Myc genes are of special interest, to distinguish which cancer might be responsive to JQ1 and which one might rather profit from a combination treatment of JQ1 with other agents such as MMC or Romidepsin. This could lead to the development of an individualized and a more tailored treatment of cancer patients.

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