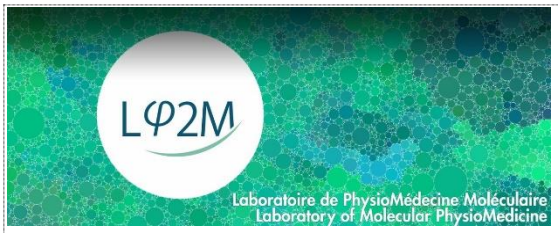

IISER PUNE

Cisplatin and Taxane side-effects: Kidney and Neuronal systems

A study into discerning the mechanism behind some common side-effects and resistance

Thesis submitted in partial fulfilment of the requirements of Five-year BS-MS Dual degree program at:

Indian institute of Science Education and Research, Pune

By

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Certificate

This is to certify that this dissertation entitled "*Cisplatin and Taxane side-effects: Kidney and Neuronal systems*" towards the partial fulfilment of the Five-year BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Suyash Naik at IISER Pune under the supervision of Pr. Laurent Counillon, Professor, LP2M, Uni. Of Nice-Sophia Antipolis, Nice during the academic year 2016-2017.

Pr Laurent Counillon

LP2M, Uni. Of Nice-Sophia Antipolis

Date: 28/03/2017

Declaration

I hereby declare that the matter embodied in the following report entitled "*Cisplatin and Taxane side-effects: Kidney and Neuronal systems.*" are the results of the work carried out by me at the Laboratoire de PhysioMédecine Moléculaire, University of Nice-Sophia Antipolis, under the supervision of Pr Laurent Counillon and the same has not been submitted elsewhere for any other degree.

Suyash Naik Date: 28/03/2017

20121075

Abstract

Cisplatin and taxanes are anticancer drugs commonly used to treat solid tumour type of cancers. The mechanisms of action of these drugs on the cells are vastly different, with cisplatin being a DNA intercalating agent and taxanes being microtubule polymerization inhibitors. However, clinical observations show similar debilitating side effects, which involve changes in sensory perception such as numbness, cold hypersensitivity, pain perception, and even renal toxicity in case of cisplatin. These side-effects could be explained by the changes in nociceptive and mechanosensory ion channels, which mediate these functions. Firstly we investigated the effect of these anticancer drugs on the mRNA levels of these channels and found significant modifications in cultured dorsal root ganglion and kidney cells. Using computational tools, we identified common transcription factors involved in these disregulations. Secondly, using electrophysiological techniques, we measured sodium and calcium currents to determined whether the changes in mRNAs were correlated with modification of functional expression. We also identified a possible mechanism involving oxidative stress in regulating the ion channels. Strikingly we had also observed the upregulation of CFTR mRNA and found it correlated with an increased in CFTR currents, suggesting a possible role in MDR.

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Also thanks to my new friends here who have let me be a social zombie as I always love to be. Travelling with them and getting to know them has been fun and I hope that we stay in touch always. Also my oldest friends Tanmoy, Mihir[2], Nishad and Roopak who have had my back even while I didn’t have my own. Thank you for keeping me grounded. Also to all my friends in IISER especially my roommate Suvi, for always being there for me. Also, people from the old lab in IISER, Manu, Mouli especially, who were the first in teaching me through the baby steps in experiments

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Last but probably the most, my deepest thanks to my parents, Ajay and Snehal, both of you have nudged me to always strive for better. Jivi, you are the best sister anyone could ask for.

Drifting on slipstreams to soar higher and onward.

1. **Introduction**:

The regulation of ion channels in a cell plays an important role in homoeostasis and ionic balance. Maintenance of a correct balance of all the ions is necessary for a variety of physiological process such as apoptosis, osmotic balance, etc. Anti-cancer drugs, which are very effective in kerbing cancerous cells, also pose a risk to the non-cancerous tissue causing a variety of dysfunctions in them (Neijit 1991). Most common among them are nausea, vomiting, hair loss, neurotoxicity, nephrotoxicity, ototoxicity and dysregulation of nociception. In this project, two types of drugs, which are regularly used for the treatment of solid tumour type cancers, namely taxanes and platinum-based drugs were used as a model. Though these drugs have vastly different modes of action described in some detail below the side effects seen in patients limiting treatment are strikingly similar. A body of clinical research has also shown the effectiveness of cisplatin and taxane combined therapy as an alternative first-line therapy, but the neurotoxic side effects limit its use (N Katsumata 2003, Davidson M. 1996). Many of these side effects are organ specific, but here we have focused on the effects that can explain the changes in nociception, touch perception and nephrotoxicity. Understanding the mechanism behind the side-effects of these drugs used in the treatment of solid tumours is necessary to alleviate these symptoms. Solid tumours show unique changes to their physiology such as increased angiogenesis, resistance to hypoxia, which may be important to devise a better mode of action for the drugs or to supplement the treatment with necessary anti-aggravating drugs (Giaccia 1998). Understanding the cause of these side effects could help the development of new drugs which while not compromising on the efficacy are not as adverse on non-cancerous cells. The unique physiology of solid tumours may be instrumental in the development of context-dependent activation of future drugs.

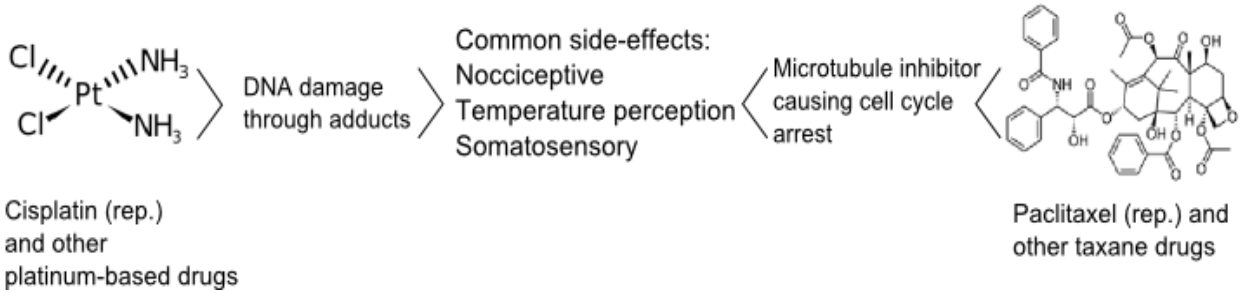

Figure 1: The chemical structures of cisplatin and paclitaxel a representative member of the taxane family of drugs. The different modes of actions of these two different types of drugs which lead to a variety of common nociceptive side effects.

Cisplatin is a very commonly used drug in the treatment of solid tumour cancers of the ovary, testes, and the head-neck. Cisplatin is transported through a variety of pathways including the copper transporter 2, P-type copper transporting ATPases ATP7 (A and B isoforms, the organic cation pathway (OCT) and multi-drug extrusion transporter (MATE 1). The different isoforms of OCT have an organ-specific expression pattern and have been shown to transport it at different efficiencies, hence leading to an organ specificity in its side-effects (Ciarimboli 2003).

Cisplatin's primary target in the cells is DNA, wherein it can form adducts and disrupt important cellular processes, DNA synthesis and cell cycle. It has also been shown that treatment with cisplatin directly inhibits protein functions, such as the non-competitive inhibition of NHE1 transporter and of mechanosensitive currents by VSORC and TREK-1 (Milosavljevic N 2010). The cytotoxic DNA adducts formed by cisplatin are due to the N7-sites nucleophilic interaction with purine bases forming DNA-protein or DNA-DNA intra and inter-strand crosslinks (Eastman 1987). Cisplatin-mediated adducts are recognised by a variety of damage sensing pathways in the cells, such as the non-homologous end-joining pathway. These changes stimulate both pro-apoptotic and pro-survival signals with the balance tipping in the favour based on the integration of the effect on downstream targets and damage done to the cells (Siddik 2003). Cisplatin-mediated damage also causes a transient S-phase arrest leading to an inhibition of cdc2-cyclin A or B leading to a G2/M arrest and G1 cdks (Jian 2001).

The MAPK pathway is an important pathway regulating the effects of cisplatin-mediated effects on the cells. This is of special importance as cisplatin-mediated nephrotoxicity as all three of the mediators of MAPK signalling viz. ERK, p38, JNK/SAPK pathways are activated in the kidney (Arany 2004). In the proximal cells of the kidney, the transport is mediated by the voltage-dependent bi-directional organic cation transporter OCT2. Cisplatin treatment could cause an osmotic imbalance in the kidney which explains the drop in renal blood flow and glomerular filtration rate (Shirali 2014). The damage to the kidney is dose dependent, and gradual worsening of the effects has also been observed even after the termination of the treatment (Yao 2007, Bokemeyer 1999). Its metabolism in the cell is intricately linked to the ROS pathway, as it is conjugated to glutathione and then broken down to give a reactive thiol through the activity of γ-glutmyl transpeptidase and cysteine S-conjugate β-layse (Townsend 2002, Townsend 2003). Some of the effects observed due to nephrotoxicity such as decreased mitochondrial function, altered ionic balance and altered solute transport are intricately linked to in the ion channels and transporters in the cells.

The other major side effects of cisplatin treatment are neurological in origin, especially the peripheral nervous system. Most commonly seen symptoms are numbness, paresthesia, vibration sensation threshold changes, nociception, proprioception and tinnitus arising from neuropathy (seen in almost 50% of the patients), neurotoxicity and ototoxicity (Nejit 1991). This effect is also dose dependent and has also been shown to worsen after cessation of treatment (Grundberg 1989). The effects are prominent in the peripheral nervous system than the central nervous system, due to the blood-brain barrier being able to filter out cisplatin (Thompson 1984). The active form of cisplatin has also been shown to inhibit tubulin microtubulin polymerization.

Taxanes, a class of diterpenoids, are microtubule polymerization inhibitors which prevent the polymerization of tubulin hence arresting the cell cycle and microtubule dynamics. They have also been shown to affect mitochondrial morphology and produce oxidative stress in the cell. This could be due to lack of force generation due to microtubules, and subsequent distended and enlarged mitochondria (Bennett 2006). The signature members of this family of drugs are paclitaxel (Taxol), and docetaxel

(Taxotere), known to cause axonal sensory neuropathy. These drugs share many common neuronal symptoms with platinum-based drugs such as paresthesia, dysesthesia, numbness and loss of sensation (Vahdat 2001). With the use of glutamine, an essential amino acid which acts a source of nitrogen and energy in proliferating cells, they showed that there was reduced severity in peripheral neuropathy.

Neurones being non-diving cells are not affected directly by the disruption of the cell cycle, but rather by other cellular pathways dependent on microtubule dynamics such as cellular transport. Neurones also contain a lot of mitochondria in the axons and are more susceptible to oxidative damage due to a higher amount of phospholipids and lower antioxidative capabilities(Hans J Tritschler 1997 Areti 2014). Taxanes induced peripheral neuropathy also is known to cause mitochondrial damage and hence oxidative stress (Areti 2014). The neuropathic axons show a prevalence of swollen and vacuolated mitochondria which leads to a loss of ATP production and respiration in the mitochondria (Bennett 2006). They proposed that the increase in the size of the mitochondria is a result of the increased efflux of calcium from the through the mitochondrial permeability transport pore (mPTP) opening. The increased intracellular calcium promotes hyperexcitability leading to an enhanced pain sensation leading to painful neuropathy. Though taxanes are not directly nephrotoxic in clinical trials previous work in the lab have shown some interesting changes in the levels of ion channels important for transport of Na^+, Ca^{2+} and mechanically gated cation currents similar to those observed in the cells from PNS origin. These changes being more subtle could lead to subtle changes in physiology which may not be as visible as other painful side effects.

In an effort to understand the changes in proprioception due to the side effects of anticancer drugs, cisplatin and taxanes, our lab tested the changes in gene expression levels of ion channels involved in proprioception. The major families involved in proprioception that were checked were K^+ channels, Na^+ channels, Ca^{2+} channels, transient receptor potential cation channels and mechanosensitive cation channels (check Appendix A for more details on the targets chosen and gene expression analysis

done).

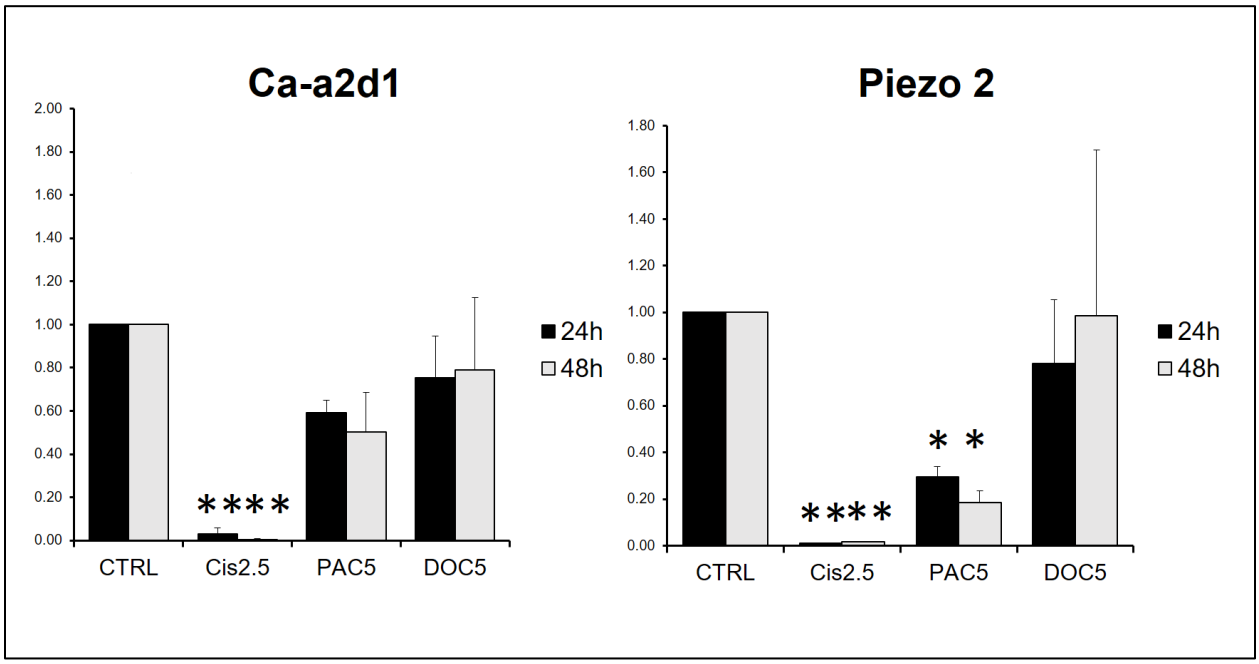

*Figure 2: Downregulation of gene expression of Cav alpha2-delta1 and piezo 2 on treatment with anticancer drugs cisplatin, paclitaxel and docetaxel in PWT2 cells measured using QPCR. Cisplatin used at 2.5 µg/ml, while paclitaxel and docetaxel were used at 5 µg/ml ,which corresponds to the concentrations used in chemotherapy. The treatment was done for both 24 hours (black) and 48 hours (grey). Significance calculated with the p-values from the t-test. * indicates a significant value for p<0.05, ** indicates a significant value for p<0.001.*

In PWT2 cells, we observed that calcium channel subunit alpha 2 d1 and piezo 2 mechanosensitive ion channels are downregulated after treatment with anti-cancer drugs. The effect was more severe for cisplatin-treated cells than taxane treated cells. Calcium homoeostasis as mentioned before is important to maintain proper perception in neurones. Similarly, in proximal convoluted cells cisplatin is known to cause toxicity, which may be due to the imbalance of currents in the cells. The Cav α2 δ1 subunit is involved in crosstalk with the extracellular partners as it is entirely extracellular and also interacts with a wide variety of calcium channels. The general effect on the calcium currents observed from the voltage-gated calcium channels is an increase in association with the delta subunit (Dolphin 2003). This is through both increased recruitment of the other subunits to the membrane and also by an increase in the peak current observed

through these channels. Piezo 2 is a mechanosensitive ion channel which can induce a cationic current when stimulated by an increase in the force applied to the channel. The mechanosensitive gating works in a physical deformation of the protein structure as observed from the structure of the other isoform in the piezo family, piezo 1 (Yang 2015). Interestingly this channel did not show any change in its expression on treatment with anticancer drugs such as cisplatin and taxanes.

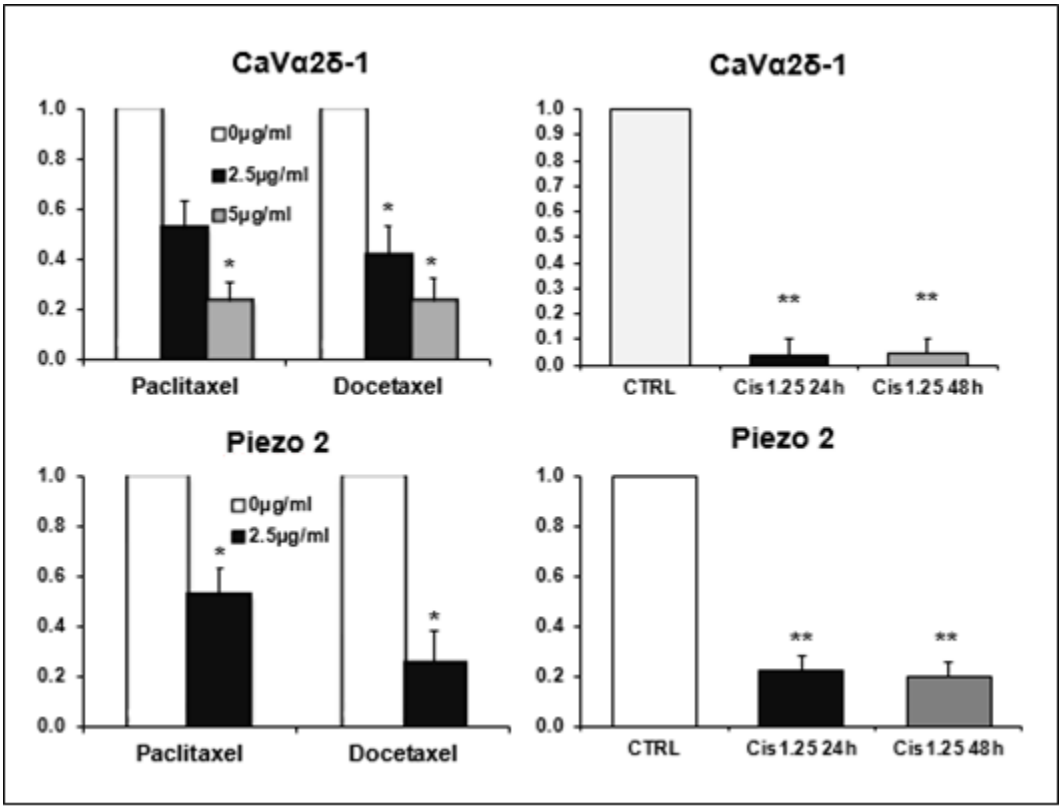

*Figure 3: Downregulation of gene expression of ion channels with QPCR in primary culture of dorsal root ganglion (DRG) cells. Similar to as observed in the PWT2 cells after treatment with cisplatin and taxanes showed a downregulation in Cav α2 δ1 subunit and piezo 2. For cisplatin 1.25 µg/ml was used and treated for both 24 hours (black) and 48 hours (grey). For taxanes, 2.5 µg/ml (black) and 5 µg/ml (grey) were used, and treatment was done for 24 hours and 48 hours (shown in the figure). * indicates a significant value for p<0.05, ** indicates a significant value for p<0.001.*

The major cell types that were tested were neuronal and proximal convoluted cells of the kidney. The neuronal cells were both of central nervous system origin as well as primary cultures of DRGs, from the peripheral nervous system, which as mentioned

before are the cells that are most affected by treatment of anticancer drugs. In DRG primary cultures, piezo2 showed a downregulation within 24 hours of treatment with cisplatin 1.25 µg/ml. The same could be observed for Cav α2 δ1 subunit gene expression with a downregulation observed in 1.25 µg/ml treatment of cisplatin. The effect of 1.25 µg/ml of cisplatin was enough to cause a downregulation with a higher concentration being more severe in its effects. This could be due to the difference in sensitivities of the two types of cells. Taxanes also showed a downregulation of the gene expression of these genes with the effect being more pronounced at 48 hours than 24 hours. Both 2.5 µg/ml and 5 µg/ml showed an effect in downregulation of the gene expression, showing a higher sensitivity to both cisplatin and taxanes.

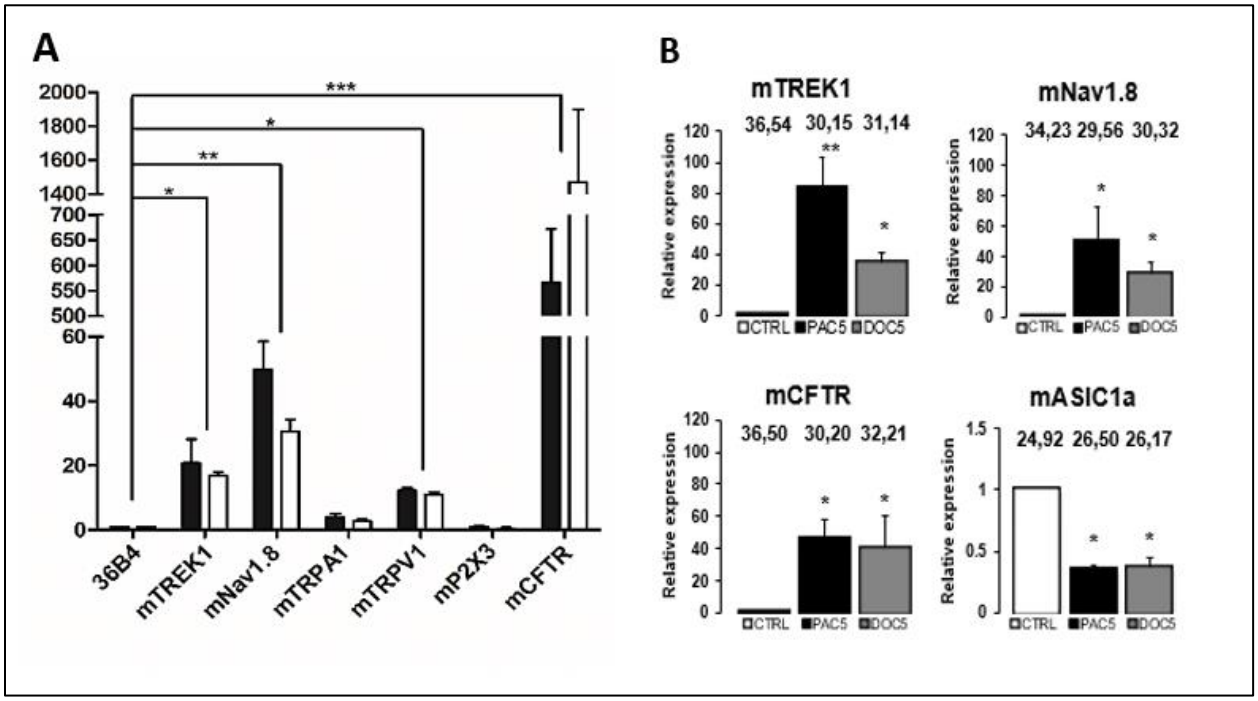

Figure 4: Changes in gene expression of nociceptive channels in HT22 cells observed using QPCR, hippocampal cells (n=3). Trek1, Nav1.8, Trpv1, P2x3 and Cftr are shown in the figure (the other targets (app. A) show no significant changes). A) treatment with cisplatin at 2.5 µg/ml (black) and 5 µg/ml (white). B) Effect of taxane treatment with paclitaxel (black) and docetaxel (grey) used at 5 µg/ml.

The changes seen in the PNS cells (DRG primary culture) and proximal convoluted tubule cells were not the same in the CNS cell line HT22. Instead, the

channels that were shown to be dysregulated in the CNS cell line, HT22, were sodium conducting channels Asic1a and Nav1.8, potassium conducting channels Trek1, chloride conducting channels CFTR in both taxane treatments (paclitaxel and docetaxel) and platinum-based drug treatments (cisplatin, oxaliplatin, carboplatin). CFTR though not involved in pain perception showed a changed expression. This was interesting as it may be involved in resistance to drugs. The difference in the behaviour of downregulation of gene expression between HT22, a CNS-derived cell line compared to that seen in DRG, a PNS-derived cell line and PWT2, a proximal convoluted tubule cell line showed an intrinsic change which could be due to the different regulation of responses to a xenobiotic stress such as anticancer drugs.

The genomic and physiological effects on neural and kidney-derived cells were used to understand the effect that they may have on the dysregulation of ion channels. We investigated the regulators of gene expression for the ion channels in an effort to understand a mechanism for the common side effects. In order to see if the changes in gene expression of the channels could be interpreted as a change in the electrical behaviours of the cells electrophysiological studies were done on some of the ion channels. The study showed that there might be a role of oxidative stress in the side effects of anticancer drugs. We also identified a link between the physiological effects of cisplatin to a pathway that may be involved in regulation of the ion channels. The similarity of changes in DRG cells and PWT2 cells could mean that the same mechanism can be a cause of some of the changes that lead to side effects after treatment with anticancer drugs.

2. **Materials and methods**:

2.1. Cell culture:

Mouse cell lines Ht22 and Pwt2 were cultured. Ht22 is an immortalised neuronal cell line from mouse hippocampus and has been used as a model for many neuro-degenerative diseases. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS, 1% Penicillin/streptomycin (PS) and 1% glutamine. This was used as a model for the central nervous system, but the blood brain barrier is not present. The blood-brain barrier proves as a shield against the entry of most drugs into the central nervous system as mentioned before.

Pwt2 is a primary cell line from the cortex of mouse kidneys. The cells are derived from the primary convoluted tubule from the segment 1-to 1.5-mm of the tissue immediately after the glomerulus under sterile conditions. The immortalization was done using infection of the 10-day old culture with pSV3 neo plasmid, and G418 selection. Pwt2 cells were maintained in Dulbecco's modified Eagle's medium/ Ham's F12 nutrient mixture with 15 mM $NaHCO_3$, 20 mM Hepes, 1% serum, 2 mM Glutamine, 5 mg/L insulin, 50 nM Dexamethasone, 10 µg/l epidermal growth factor, 5 mg/L transferrin, 30 nM sodium selenite, and 10 nM triiodothyronine (Barriere 2003). The cells were seeded in 10% collagen coated culture dishes so as to maintain physiological conditions. The cells were used as a model to understand nephrotoxicity that occurs with cisplatin.

DRG were dissected from mice and maintained in Dulbecco's modified Eagle's medium (day 1)/neurobasal medium with B27, NGF, glutamine and PS. For the dissections, the head, decapitated from recently sacrificed mice, at around the 3-4 vertebra, and the tail was discarded. The spinal cord was exposed from the ventral side, and the ganglions between the vertebral units were physically taken and kept in DMEM on the ice. The cells were then lysed and homogenised and plated in poly-l-lysine coated plates. They were allowed to settle for one hour after which more medium was added (2ml in 6 well plates/ 10 ml in 10mm dishes).

The cells were classically maintained at 37˚C in a water-saturated atmosphere of 5% CO2/ 95 % air. For the cisplatin treatment and taxane, treatments the drugs were obtained from Centre Antoine Lacassagne, an anticancer centre at the Hospital Pasture

which is involved in research in Head and neck and breast tumours. Cisplatin stock solution was made as 1 mg/ml stock solution in PBS, and a working concentration of 2.5 µg/ml was used. Taxanes were made as a stock solution of 1 mg/ml stock solution and used at 5 µg/ml. The treatment was done for 48 hours by direct dilution of the stock solutions in the cell culture medium and incubated at the same conditions as described above.

2.2. Real-time polymerase chain reaction and reverse transcription:

Total RNA was isolated by β-mercaptol based RNA extraction using the RNA extraction toolkit from Qiagen. QPCR was done to study the changes in m-RNA. The m-RNA extractions and reverse transcription were done by the MN Nucleospin kit. The primers were designed using the NCBI primer blast tool and verified using the BLAST algorithm. The primers were diluted to 100 pmol/µl in water and stored at -20˚C. 2 µl of 10 pmol/µl primer solution was used to make the primer mix containing SyBr green 50 µl and water 25 µl. For the QPCR 10 µl reaction was used with 2.5 µl c-DNA and 7.5 µl primer mix. The delta delta Ct values were calculated and used to estimate the expression levels.

Gene	**Left**	**Right**	**T_m**
Arnt2	aaacgcataccccagtcttg	cgccactctgtccactctc	59
Hes1	tgccagctgatataatggagaa	ccatgataggctttgatgacttt	59
Zfp423	gcgatcggtgaaagttgaag	tctccttccaggcctcct	60
Nhlh1	cccttggcagagtccttct	tgagctctgggaagcagttac	59
Mafb	tgaaagcccagtgttctgc	agggctaccggatgagaaac	60

Table 1: Primers used for studying transcription factors from the mouse genome.

The above primers were used to study the gene expression in both PWT2 and

DRG cells. The experiments were done with 2 technical replicates for each experiment and biologically replicated thrice. The primers listed below were made to study the regulation of MDR genes along with CFTR.

Gene	Left	Right	T_m
MDR1	tcgaagatgggcaaaaagag	agcgaaacatcccaaatacg	59
MDR3	ttgaactaggcagcatcagc	aacagtgtcaacaggccaatta	59
MRP1	cacgggcaagaatctggtat	tggttctcttctggccctataa	59
MRP2	tttcctggattacctccaacc	gccgagcagaagacaatca	60
MRP3	tccgaaactacgcaccagat	ctcatttgcattttgcaagg	60
MRP4	ggacccaagaacggatgag	tgagcaatggtgagcactgt	60
MRP5	ttatcctcttttatccagcaatga	ttctggacacggtcatctgt	59
MRP6	ctgctatggaggggctacaa	tctcccaagtgaccagaggt	60
MRP7	aacatcctgctggccaat	agaggtggtagcaggagcag	59
MXR	gccttggagtactttgcatca	aaatccgcagggttgttgta	60

Table 2: Primers for MDR genes

2.3. Computational analysis:

The expression of the ion channels in different organs of the body and the transcription factor binding sites were studied. Selected genes which showed a change in expression pattern after treatment with anticancer drugs were selected as targets. Target ids: Piezo2, P2rx2/3, Asic1a, Cacna1d2, Abcc7 (CFTR), Nav1.7. The Genevestigator tool by Nebion was used to study the expression in different regions of the body and cross-referenced to the Illumina body map data on the Gene cards database. Human genome U133 Plus 2.0 and mouse genome were studied. The transcription factor binding sites were also studied in both human and mouse genomes using the Swiss Regulon database. The predictions are made based on Bayesian probabilistic analysis with regards to the experimentally verified patterns of transcription factor binding and predictions of binding based on algorithms developed by Erik von Nimwegen et. al., database creators (Pachkov 2007). The locations of the promoters were taken based on the locations of the promoters annotated in the European promoter database or by browsing the genomes near the TSS

of the genes (Bucher 1998). The target transcription factors showing a probability of greater than 0.7 were curated, and common interesting targets were studied using Excel.

2.4. Electrophysiology:

Heka patch clamp EPC9 system was used to patch cells plated on collagen coated nuncolon 35-mm petri-dishes in whole cell configuration. Vitrex borosilicate micropipettes pulled for resistance between 5 MOhm to 10 MOhm, using a two-stage vertical puller, were used for the electrophysiology studies. The cells were plated on nuncolon plates at least one day prior to the experiment. The setup was done on an inverted microscope by Zeiss fitted with a water robot manipulator (WR 89, Narishige). The patch pipette was connected to the head stage of the amplifier using a silver (Ag/ AgCl by treatment with bleach) wire. The resistance of the pipette after obtained a seal was used to gauge the strength of the seal. The resistance of a seal had to be greater than 1 GOhm and with a leak current less than 75 pA at basal conditions for a patch to be used for whole-cell experiments.

For studying the chloride currents in HT22, the extracellular solution used contained (in mmol/L): 140 NMDGCl, 10 Hepes, 5 Glucose, 1 $CaCl_2$, pH7.4 and osmolarity maintained at 340 mOsm with glucose. The intrapipette solution used contained: 145 KCl, 10 HEPES, 1 MgATP, 1 EGTA ph7.4. The currents were observed in the Whole cell configuration to study the total current due to all ions channels in the cells. The cells were held at a resting membrane potential of -50 mV and stimulated with -100mV to 100mV currents in the time frame of 400ms. The activation of CFTR was done using Forskolin, which is a labdane diterpene used in activation in adenlycylase. The inhibitor CFTR-172 at 10 µg/ml was also used to inhibit the currents observed from CFTR. The addition of external components was done once a patch had been obtained with the help of a micropipette at very slow speeds so as not to disturb the patch. For highly viscous solutions the addition was done by addition of some excess bath solution so as to promote mixing throughout the solution.

For studying calcium currents in PWT2, the extracellular solution used contained (in mmol/L): 100 TEACl , 40 CaCl2, 10 Hepes, 5 Glucose, pH7.4 and osmolarity maintained at 340 mOsm with mannitol. The intrapipette solution used contained: 140 CsCl, 5 EGTA,

1 $MgCl_2$, 2 MgATP, 0.1 $CaCl_2$, 10 HEPES pH 7.2 and osmolarity maintained at 310 mOsm with CsOH. The cells were held at -70 mV and stimulated with a voltage from -60 mV to 60 mV. For studying calcium currents, generally, barium is substituted instead of calcium in the pipette solution. This is done as the peak current observed due to Ba^{2+} is more than that observed due to Ca^{2+}. But the behaviour is unchanged. We did not have access to $BaCl_2$. Hence calcium was used. If a current could be observed, it would only be magnified in the presence of barium instead of calcium.

For sodium currents in PWT2, the extracellular solution used contained (in mmol/L): 140 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, pH 7.4 and osmolarity maintained at 330 mOsm with mannitol. The intrapipette solution used contained: 130 CsCl, 1 EGTA, 10 NaCl and 10 HEPES with pH maintained at 7.4 using CsOH. The cells were held at -60 mV or -70 mV and stimulated with a voltage from -120 mV to 50 mV.

The Nernst potentials for the solutions were done using the Nernst equation. Check Appendix B for the Nernst potentials and Appendix C for representative currents for the channels studied here.

3. **Results**:

3.1. Regulation of downregulated ion channels

Various different families of ion channels play a part in nociception and touch reception. A QPCR based assay was done in the lab previously, to study the changes in these genes which included the Trek family, voltage-dependent calcium family, voltage-dependent sodium family, purigenic receptor family and the mechanosensitive ion channels. A change in these ion channels could potentially explain the changes in perception seen such as heightened pain perception even in the absence of pain and help to understand the regulation of perception changes after treatment with anti-cancer drugs. Of the genes tested there were a few targets which showed a significant change in gene expression values which belonged to a diverse family of ion channels. The major targets in Pwt2 cells included acid-sensing ion channel 1-a (asic1a), voltage-dependent calcium channel subunit α-1 delta-2 (cacna1d2), voltage-dependent sodium channel 1.7 ($Na_v1.7$), purinergic receptor P2X (P2RX) and piezo type mechanosensitive channel component 2 (piezo2).

In an effort to understand a common mechanism in the regulation of these ion channels, we tried to understand the regulation of the expression of the proteins through the transcription factors binding their respective promoters. This would be useful to understand the mechanism by which these genes can be regulated. Using the promoter database freely available at the Swiss regulon database transcription factor binding motifs were predicted. The common transcription factors regulating the genes were collected, and a few targets, which were shown to be involved in interesting pathways were studied further. Also, transcription factors regulating piezo type mechanosensitive channel component 1 (piezo1), which we saw to be not dysregulated by treatment with both the anti-cancer drugs, were neglected. The transcription factors selected were Aryl Hydrocarbon Receptor Nuclear Translocator 2 (arnt2), hairy and enhancer of split-1 (hes1), MAF BZIP Transcription Factor B (mafb) and zinc finger protein 423 (zfp423). Of all the transcription factors studied specificity protein 1 (sp1) was the only one that showed was common among all the targets. These targets are putative and only predict the possible binding sites of the transcription factors. To see if any of these may be involved

in response to the anti-cancer drugs and mediate the side effects we tried to see if the gene expression of these transcription factors changed after treatment.

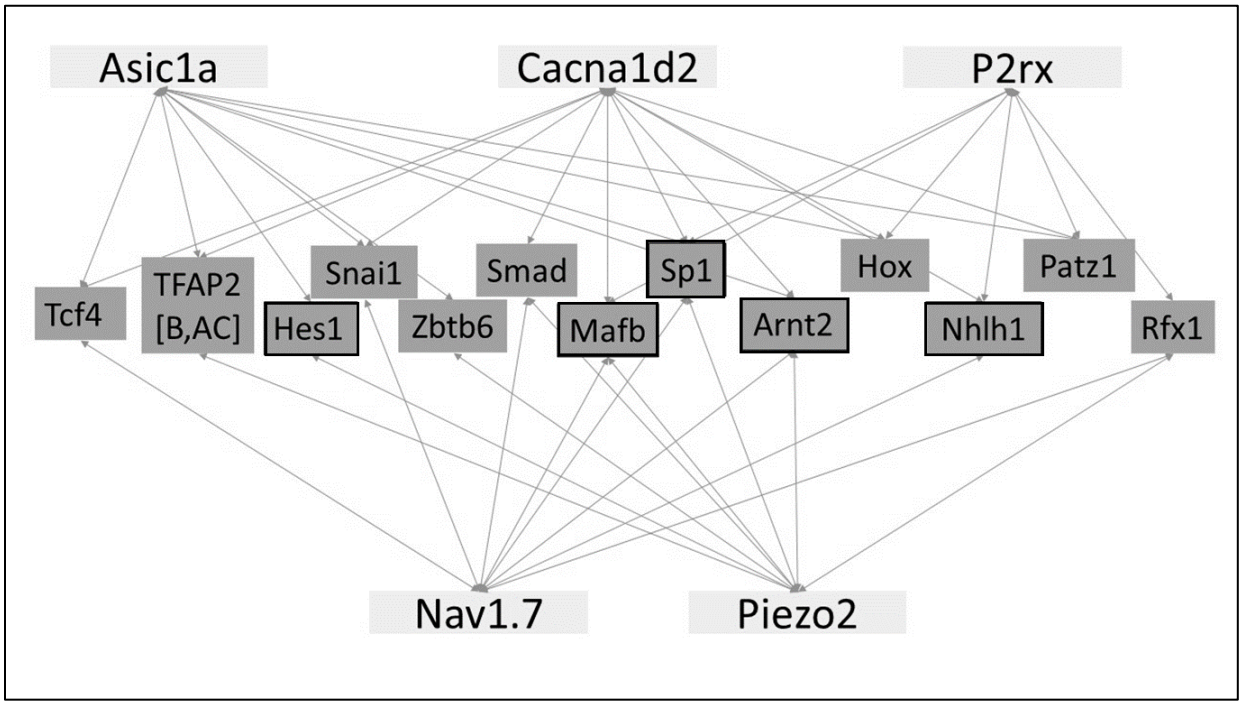

Figure 5: A map of the transcription factors commonly regulating the ion channels which were shown to be downregulated after treatment with anticancer drugs. The genes in darker boxes are the transcription factors while the grey boxes are the ion channels. The lines joining are regulations identified through swiss regulon. Important targets are marked with a box around them.

3.2. Changes in gene expression after treatment with taxanes and cisplatin

The cells were treated with cisplatin at 2.5 µg/ml and paclitaxel and docetaxel at 5 µg/ml for 48hrs before RNA was extracted. RNA was extracted and reverse transcribed into c-DNA and stored at -20˚C. Real-time polymerase chain reaction experiment was performed to see the change in the target transcription factors after treatment with anticancer drugs. We tested the changes occurring in both Pwt2 cells to understand the changes that occur during nephrotoxicity and primary DRG culture to explore the changes in peripheral neuropathy.

In PWT2 cells, we observed some significant changes in the expression of target transcription factors. Among the selected targets, zinc fingers protein 423 showed a

significant increase in expression on treatment with docetaxel at 5 µg/ml but not in the other treatments. Hes 1 showed no significant change in all of the treatments with a slight upregulation in cisplatin-treated cells while paclitaxel and docetaxel showed a slight downregulation. Mafb showed upregulation in both cisplatin and paclitaxel treated cells but not a significant upregulation in docetaxel treated cells. Arnt2 showed the most interesting significant changes in both cisplatin-based treatment and taxanes. Arnt2 was downregulated in all of the treatments. A more severe effect can be observed in cisplatin-treated while the paclitaxel and docetaxel were less severe but still significant.

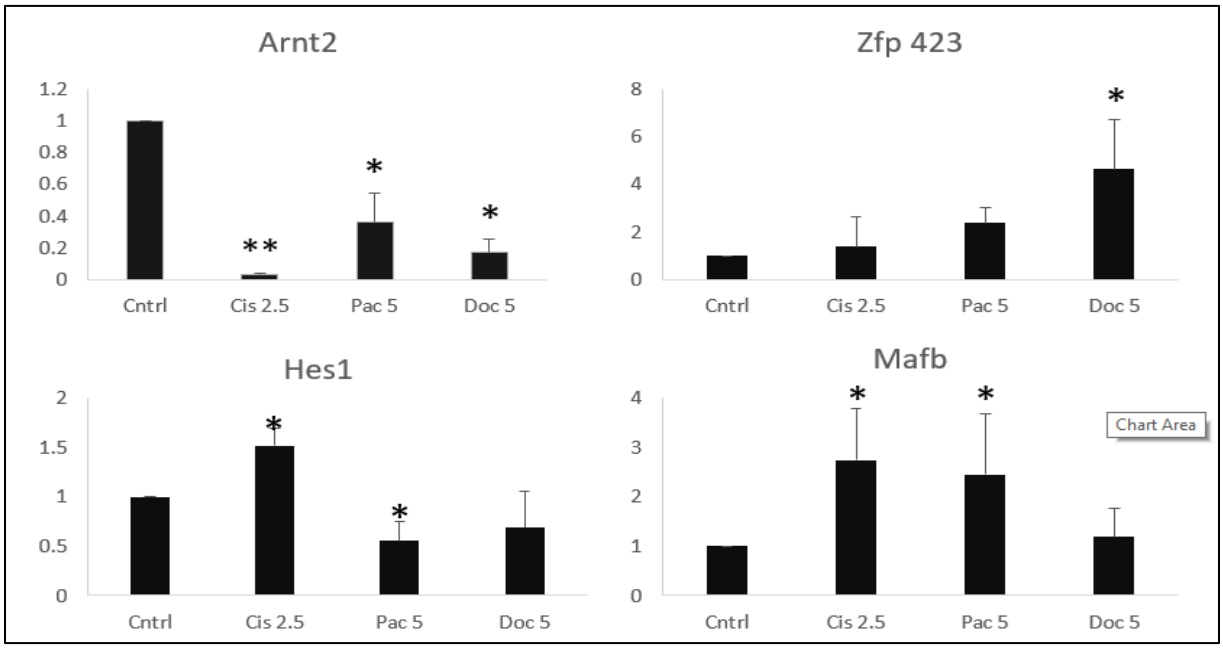

*Figure 6: The changes in expression of transcription factors in Pwt2 cells. The change on the y-axis is measured as a fold change of expression values obtained from QPCR experiments for 3 different biological replicates. The cells were treated with cisplatin at 2.5 µg/m/ paclitaxel and docetaxel at 5 µg/ml before the RNA was extracted. Nhlh1 (not shown) showed no measurable expression. The significance values were calculated using p-values from the student t-test. * indicates a significant value for p<0.05, ** indicates a significant value for p<0.001.*

If the changes observed in PWT2 are also repeated in DRG cells, a common mechanism could explain the changes observed, after treatment with anticancer drugs, with respect to the ion channels. Strikingly the changes in gene expression in ZFP 423, Arnt2 and Mafb were similar to that observed in Pwt2. Zinc finger proteins 423 was

upregulated in DRG cells with the highest upregulation observed in docetaxel treated cells. Hes1 showed a downregulation in cisplatin-treated cells unlike in PWT2 cells, and a similar change in paclitaxel and docetaxel-treated cells. Mafb showed a significant upregulation with an upregulation of over 20 fold observed in docetaxel treated cells.The change was less severe in cisplatin and paclitaxel treated cells than docetaxel treated cells with almost a similar change in both the treatments. Arnt2 again showed a similar change as observed in PWT2 with a downregulation in all of the treatments. The effect was most severe in cisplatin-treated cells and the effect being less severe in paclitaxel and docetaxel-treated cells.

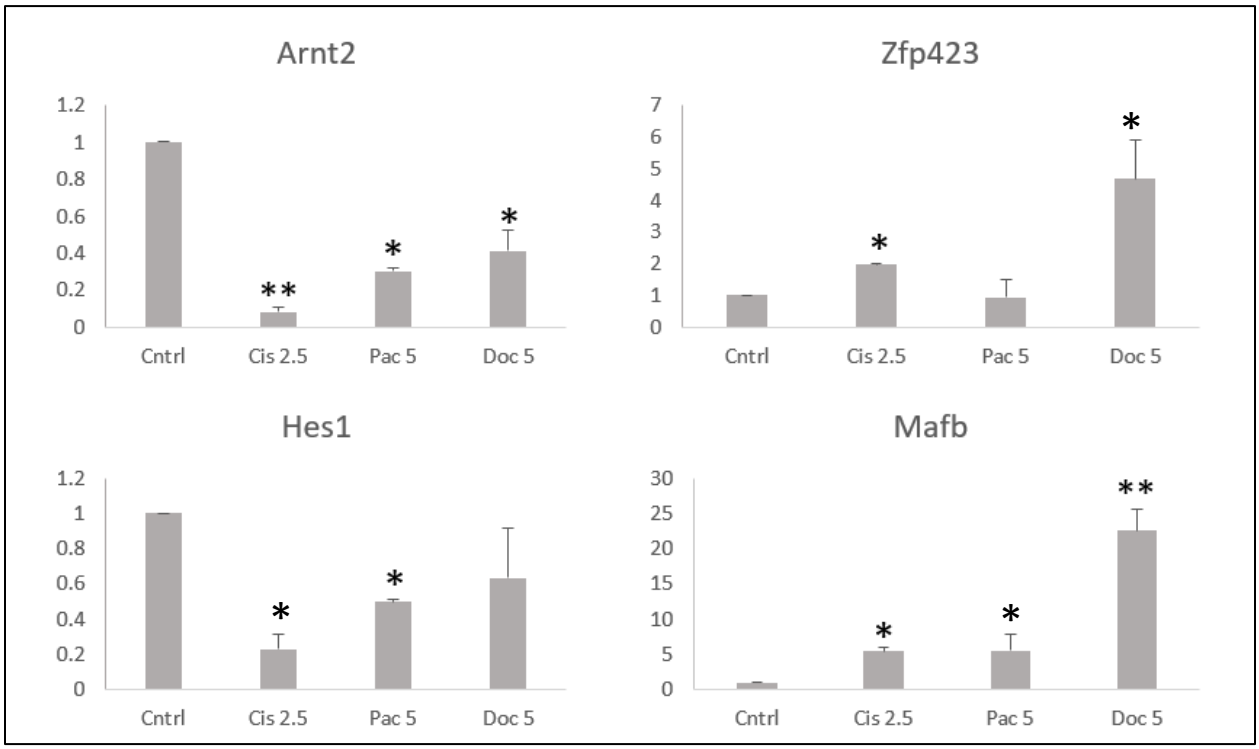

*Figure 7: The changes in expression of transcription factors in Drg cells as fold change of expression of 3 biological replicates in a QPCR experiment. The cells were treated with cisplatin at 2.5 µg/m/ paclitaxel and docetaxel at 5 µg/ml before the RNA was extracted. The significance values were calculated using p-values from the student t-test. * indicates a significant value for p<0.05, ** indicates a significant value for p<0.001.*

To understand if the regulation of these ion channels could occur due to the changes in any of the selected transcription factors, we tried to understand the regulation

of the transcription factors in time. This was done for both PWT2 and DRG cells for time points between 4, 8 and 16hrs.

In PWT2 cells, piezo 2 decreased at around 4 hours after treatment and stabilised at around 8 hours. Cav-alpha 2 delta 1, on the other hand, decreased more steadily reaching to a minimum value between 8 to 16 hours. Mafb did not show a significant change in expression with a slight rise at 4 hours and then a slight decrease. Hes1 showed no change until 8 hours but showed a slight increase after 8 hours to a little over two-fold expression. The time course of Arnt2 was similar to that of Cav-alpha 2 delta 1, with a decrease in 4 hours and a further decrease after 8 to 16 hours. This is interesting as it could be involved in the regulation of the ion channels.

In DRG cells, the changes observed were similar to that observed in PWT2 cells. Piezo2 showed a decrease at 4 hours until 16 hours to 0.4 fold change. Cav-alpha 2 delta 1 also showed a similar decrease going down to around 0.2 times control. Arnt2 showed a downregulation at 4 hours which coincided with the downregulation of the ion channels. This points toward its involvement in changes during those time points caused due to cisplatin. Mafb and hes1 did not show any significant changes during the selected time points.

From these experiments, Arnt2 proved to be an interesting target for regulation of the ion channels on treatment with anticancer treatment. Arnt family of proteins have been known to be involved in the regulation of hypoxia. We tried to see if oxidative stress played a role in this process, by downregulation the oxidative stress caused by the anticancer drugs.

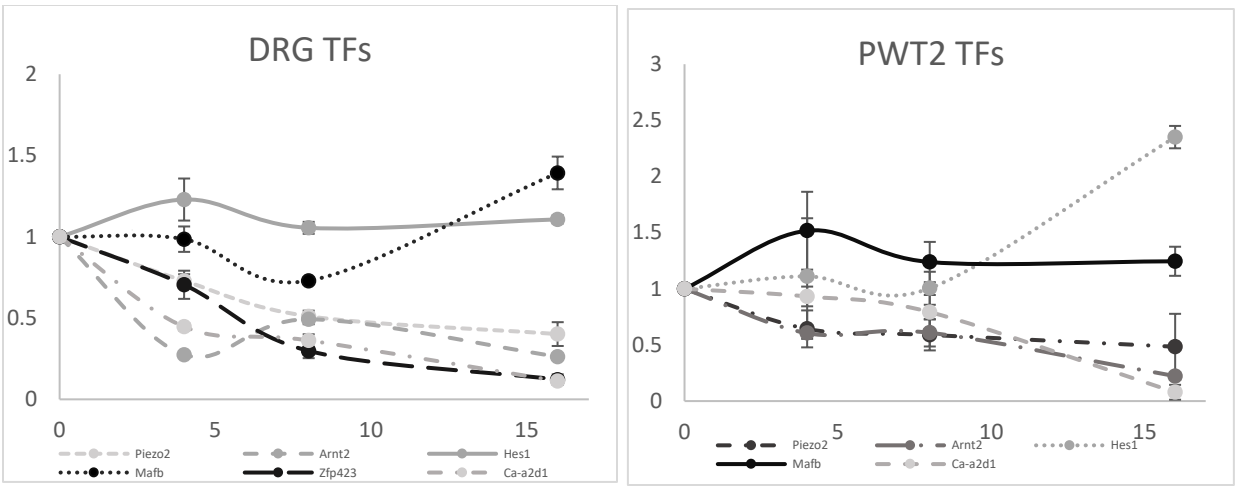

Figure 8: Time course QPCR assay was done on PWT2 and DRG cells after treatment with cisplatin 5 µg/ml to understand the regulation of the transcription factors and their targets. The time points chosen were 0 hours, 4 hours, 8 hours and 16 hours. RNA was extracted and frozen at -80˚C before reverse transcription was done together for 3 different biological replicates. The error bars show the standard deviation of the mean of the biological replicates. The fold change in the expression is represented in the y-axis and the changes between the fold change between time points are compared.

The treatment with 10 mM/ml of Nac as shown in S. L'hoste et. al. was done for PWt2 cells for 48 hours with and without cisplatin (L'hoste 2009). Nac treated cells alone showed no change in toxicity of the cells, but it did rescue the toxicity caused by cisplatin. We then tested to see if treatment with NAC affected the changes caused by anticancer drugs. Cisplatin treatment as observed before decreased the gene expression of Nav1.7, Ca-a2-d1, piezo 2 and Arnt2. Treatment with Nac rescued the expression of Nav1.7 to control values in cisplatin with 1.25 µg/ml and to half the control level in 2.5 µg/ml cisplatin-treated cells. Piezo 2 showed the same results with a rescue when treatment with Nac rescued the downregulation caused by cisplatin. Ca-a2-d1 showed a slight upregulation after treatment with Nac compared to control values. The gene expression of Arnt2 also increased after treatment with Nac was rescued to control values in both

cisplatin 1.25 µg/ml, and 2.5 µg/ml treated cells. This showed that *o*xidative stress was involved in the change in expression of the target ion channels and Arnt2.

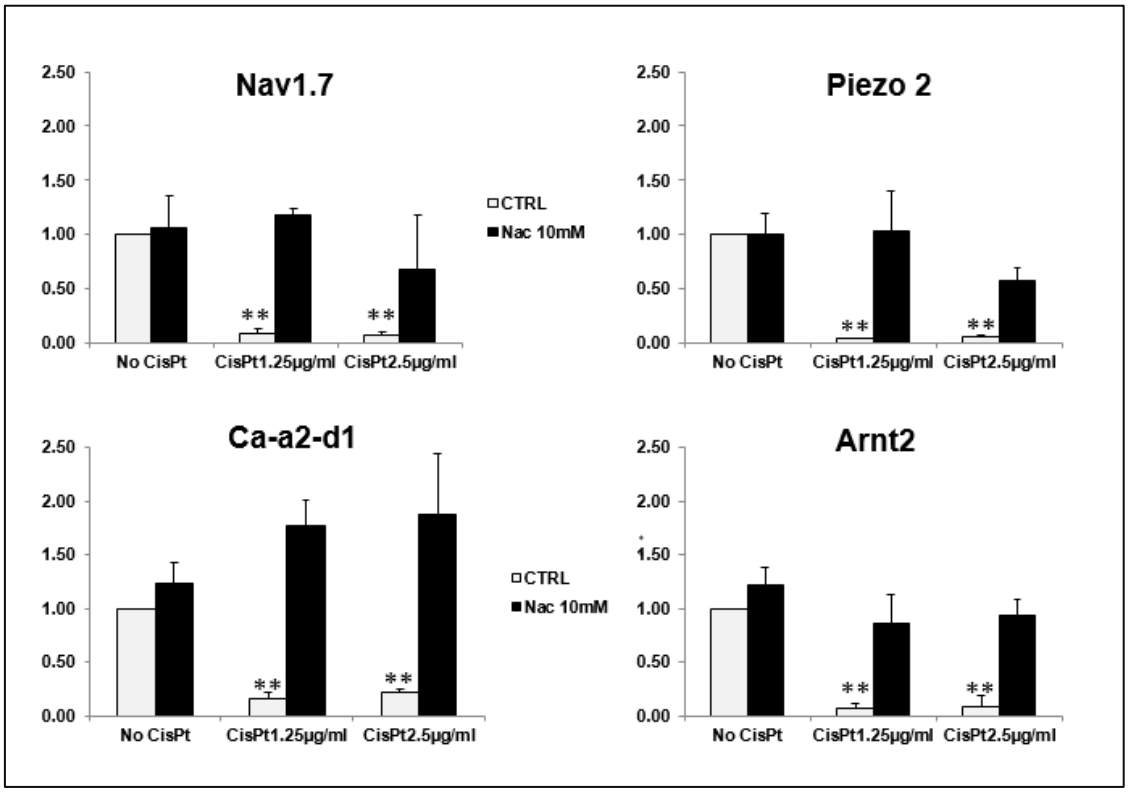

*Figure 9: QPCR mediated gene expression changes in ion channels and Arnt2 gene after treatment with N-acetylcysteine (Nac). Nac treatment was done with 10mM/ml in the cell culture as an antioxidant. The grey shaded columns represent the control samples with no treatment with Nac, and black shaded columns are Nac treated cells. The experiment was replicated on 3 different biological replicates. The significance values were calculated using p-values from the student t-test. ** indicates a significant value for p<0.001.*

3.3. Electrophysiological changes in sodium and calcium currents

The changes in gene expression levels could play a part in the changes in perception of touch and pain if the currents through these channels are changed. Electrophysiology is the perfect tool to understand the changes in currents observed. We tried to understand what happen to the calcium currents mediated by the calcium channel subunit Cacna1d2 and the sodium currents mediated by Nav1.7 downregulation.

From the real-time polymerase, chain reaction gave a medium level of expression hence we tried to measure the sodium currents. Sodium currents couldn't be observed in PWT2 cells. The only currents which were observed showed behaviours of that of leak currents. Hence the changes due to drugs on these currents could not be observed. The standard currents for Nav have been shown in the appendix for reference. Nav1.7 currents as shown are inward with a rapid activation and a slow late inactivation at higher voltage potentials with a step-wise activation from -120mV to 40 mV.

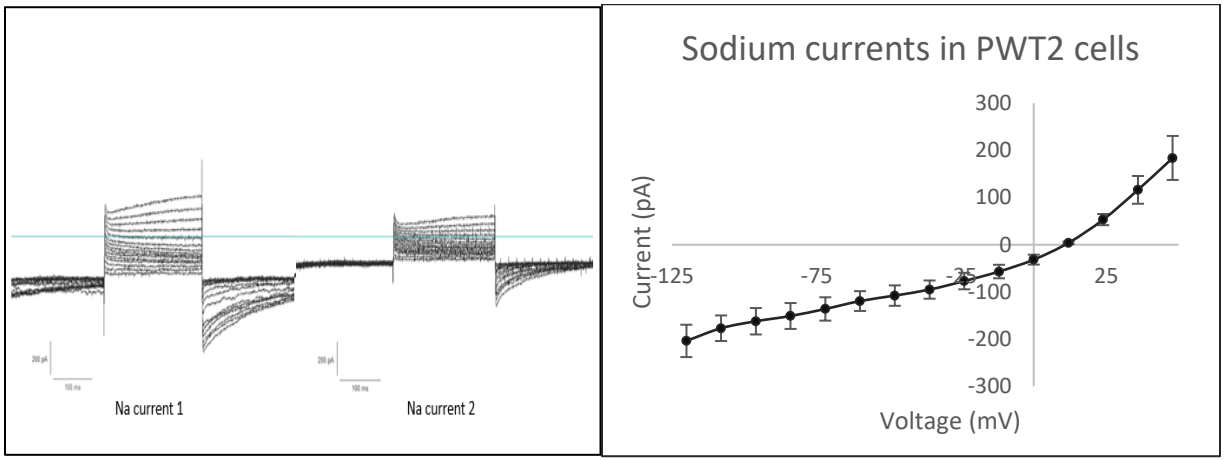

Figure 9: The sodium current electrophysiology in PWT2 cells. The sodium currents generally present as an inward current. Cells were placed in a bath solution containing only sodium and chloride as the moving ions with a lower concentration of sodium in the pipette to facilitate the movement of sodium. The cells were stimulated with a voltage gate from -120 mV to 50mV. A high potential outward current can be observed which gets activated at around 10 mV. As shown in the figure the average current is observed is about 400 pA.

Of the other ion channels downregulated piezo2 could not be measured as activation of mechanosensitive currents is tricky. Hence we tried to observe the calcium currents in PWT2 cells and changes in it after treatment with anticancer drugs. The subunit Cacna2d1 binds to many different types of calcium channels and acts as a regulatory subunit to regulate the current through these channels. Based on the downregulation of the subunit the amplitude as well as the control of the inactivation and activation of the current. The current amplitude did show a decrease in cisplatin-treated

cells vs. control cells. The current also showed a shift in reversal potential, the potential at which the current turns positive, or there is no net transfer of current between the two sides.

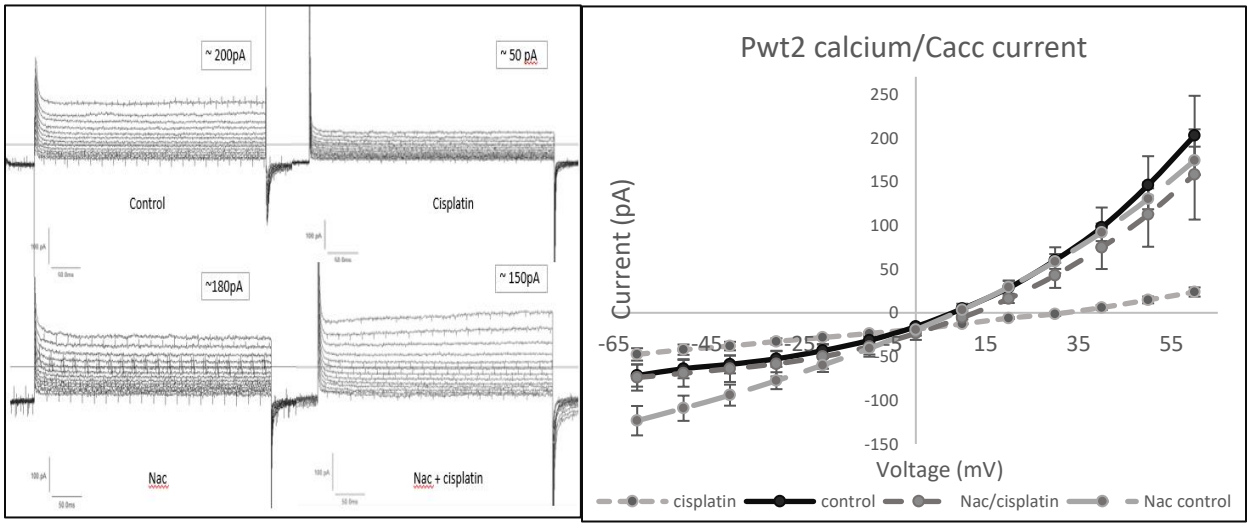

Figure 10: The calcium-activated chloride and calcium current in Pwt2 cells (n>5). Cisplatin treatment with 5 µg/ml was done on the cells for 24 hours. The cells were patched in solutions which only allowed the movement of calcium and chloride. As shown after treatment with cisplatin there is a loss of current observed to around 50 pA (grey dashed line in IV curve). This was also rescued after treatment with 10 mM/ml Nac to give an average current of around 150 pA. Nac alone did not show any change in current as the current amplitude of 200 pA observed in the Control was maintained. Nac and cisplatin treated current was not significantly different from the control current.

The expression levels of arnt2, as well as the subunit, increased when the oxidative stress in the cells was reduced by treatment with n-acetylcysteine (Nac). Hence it was interesting to understand the effect of oxidative stress on the level of protein expression and thereby the current experienced by the cells. Nac treated cells showed no significant change in the current observed in control cells. Hence Nac does not directly affect the currents induced due to calcium movement. The treatment with Nac and

cisplatin together did rescue the loss of current observed due to treatment with cisplatin alone.

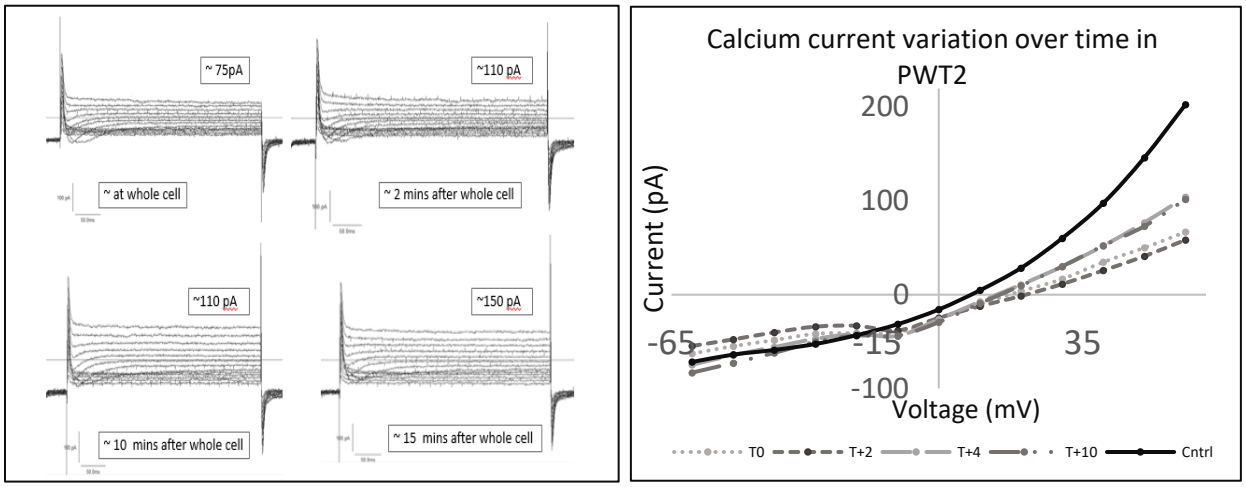

Figure 11: The transient inward current is observed in PWT2 cells (n=2). There were two distinct types of currents observed one outward and an inward current (see fig 12). The variation of the current with time for 15 mins is observed in the figures. Traces of the current (left) and the changing IC curves (right) are shown. The current is shown to increase in a time-scale studied with a peak current of 150 pA in the time-scale which could be observed. The inward current is characteristic of a calcium current.

Although the currents observed were not characteristic for calcium current but rather a calcium-activated chloride current, a rather interesting observation was seen. This showed that the calcium current could be observed for a very short time which led to an increase in the chloride current. As this current increased, the calcium current was inhibited. The current slowly increased over a time course of 10 mins with the calcium current was masked with the increase. Hence we tried to subtract the chloride current and see the behaviour of the remaining current.

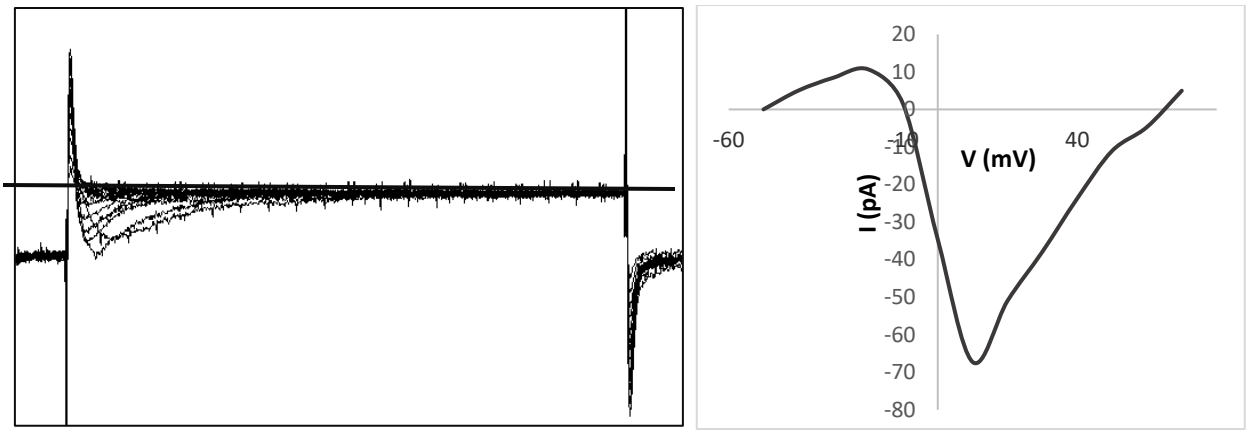

Figure 12: The current observed by subtracting the outward current from the total current. The current shows a behaviour same as that of calcium currents. The IV curve shows that a current of 70 pA inward current could be observed which peaks at around 10 mV after liquid junction potential corrections.

The chloride current, when subtracted from the total current, gave a calcium inward current with the amplitude of around 70 pA. It showed an inward activation which leads to a maximum current of 70 pA and a high potential deactivation at 60 mV.

Part II: CFTR in drug resistance studies

HT22 showed upregulation in some interesting ion channels, most of which was CFTR. The ion channel was not expressed in control cells as shown by QPCR and gene expression analysis. CFTR is a member of the ATP-binding cassette channel family of ion channels, many of which are involved in drug resistance. We tried to see if the regulation shared any similarities between the regulation of the MDR-related genes and CFTR .

3.4. Electrophysiology for CFTR in cisplatin-treated cells:

If the treatment of cisplatin, which led to an increase in the gene expression of CFTR, led to an increase in the protein expression it would lead to an increase in the

current observed by the channel. We observed the chloride current observed through the CFTR with the help of c-amp activator forskolin and CFTR inhibitor-172 (CFTR-172). In control cells, there was no CFTR current observed with or without the addition of forskolin. In cisplatin-treated cells, control cells showed a very weak current without forskolin but not significantly more than control cells. On addition of forskolin, an inwards current was elicited characteristic of a CFTR current which was around 100 pA in magnitude. This current was responsive to a specific CFTR inhibitor with a decrease observed in treatment with CFTR 172. CFTR 172 inhibited the current, and its effect was lost after washing off the inhibitor as characteristic for the drug. This showed that treatment with cisplatin did lead to an increase in the expression of CFTR protein and a functional increase in the current observed from CFTR. This led us to see if the same increase was also observed for other MDR family genes and if this was a response to resistance towards the drug.

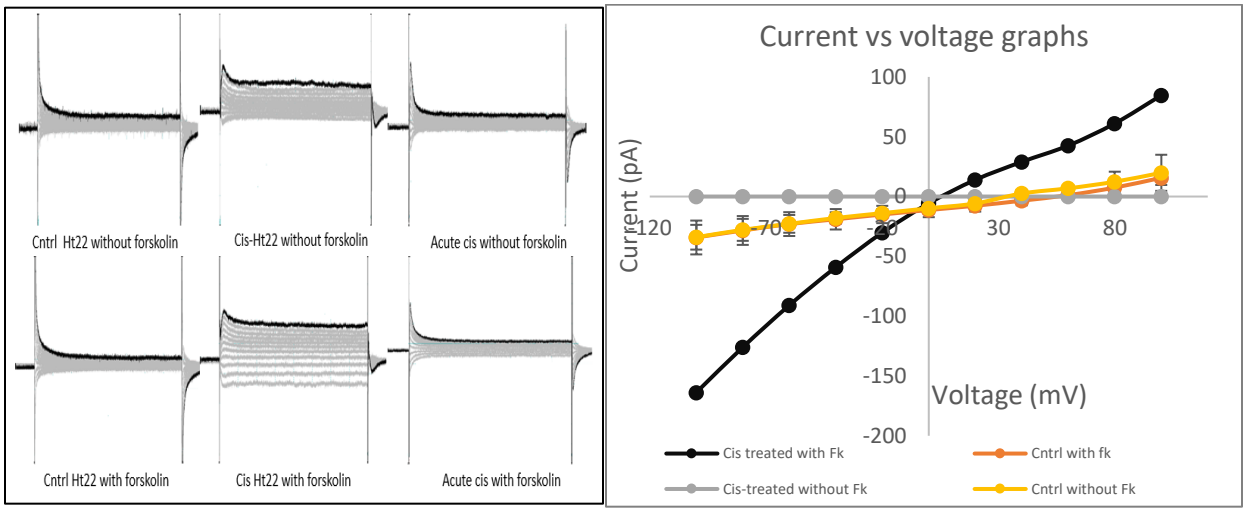

Figure 13: CFTR current is not observed in control HT22 before treatment with cisplatin or acute treatment with cisplatin. Cisplatin treatment showed an increase in the current but not significantly so. On treatment with forskolin 10 µM, there was an increase in current observed to an amplitude of around 250 pA current. The currents shown are the mean currents observed from at least 5 different cells.

3.5. Gene expression of MDR genes compared to the changes in CFTR

We checked the gene expression of CFTR and other MDR-related genes after anticancer drug treatment to see if they were similarly upregulated. We tested for MDR 1, MDR 3 from the multidrug resistance family, MRP 1, MRP 2, MRP 3, MRP 4, MRP 5, MRP 6, MRP 7 from the Multidrug resistance-associated protein and MXR a the latest member of the drug resistance family (Litmann 2001). We found no change in expression many of these genes including MDR 3, MRP 2, MRP 3, MRP4, MRP6 genes. MDR1 showed upregulation in cisplatin-treated cells but not to the same extent as that observed in CFTR. MRP 5 showed upregulation in cisplatin-treated cells, but no change was observed after taxane treatment. MRP7 was upregulated in all of the treatments with the effect in cisplatin being more than other treatments. MXR was upregulated in cisplatin-treated cells but not in the other treatments. Of the targeted MDR genes, MRP 7 showed an expression pattern similar to that of CFTR with a very low expression in control (~29 C_t) to an increased to higher expression in the treatments(~27 C_t). Hence the expression of the majority of the MDR genes did not increase similar to that of CFTR.

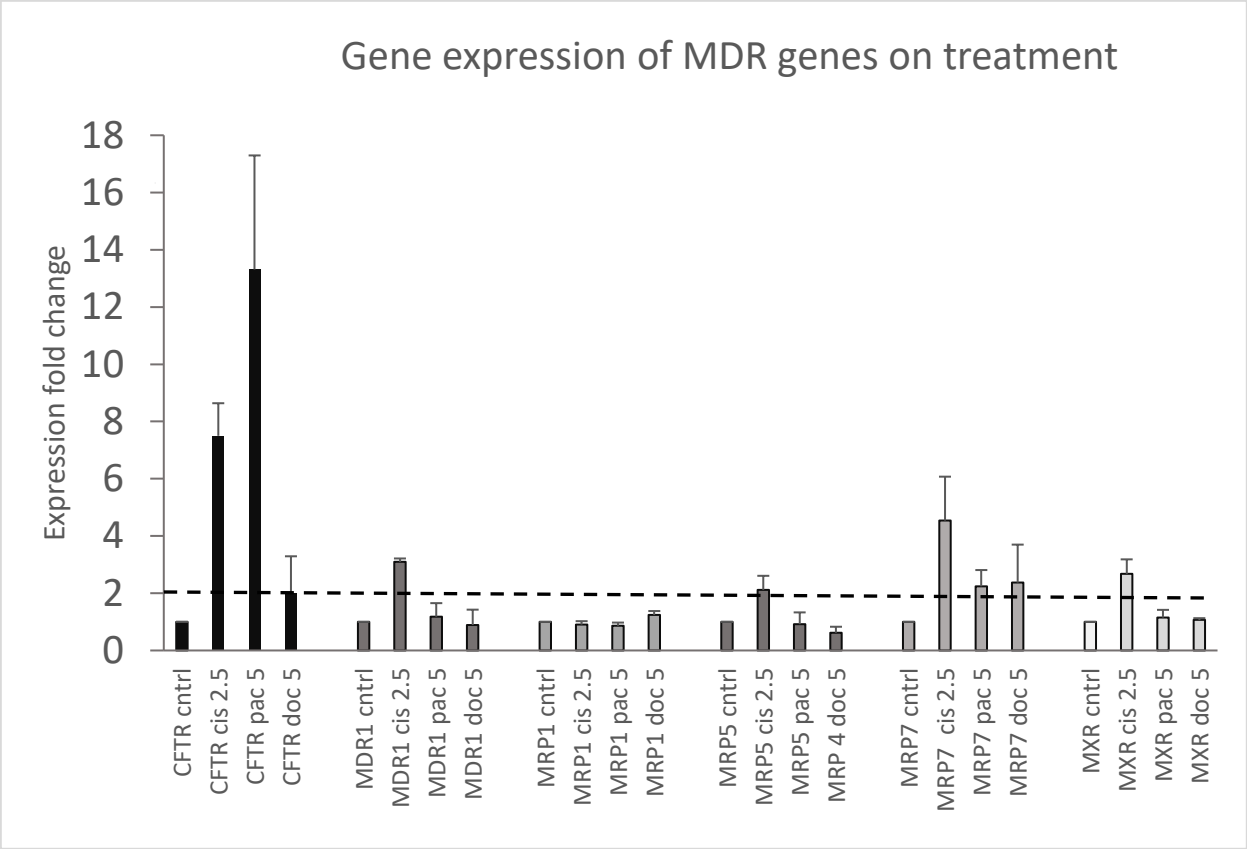

Figure 15: MDR genes compared to the expression of CFTR in Ht22 cells using QPCR (3 biological replicates). The cells were treated with cisplatin at 2.5 µg/ml, paclitaxel at 5 µg/ml and docetaxel at 5 µg/ml. Among all the targets observed only MDR1, MRP1, MRP5, MRP7, MXR showed a change in expression significantly compared to the control. The changes in gene expression of MRP7 are interesting when compared to that of CFTR.

4. Discussion

As discussed before, the painful neuropathie and nephrotoxicities are leading causes in limiting the use of the anti-cancer treatments based on cisplatin and taxanes. We tried to understand the role of ion channels involved in nociception and touch perception in the side effects observed due to these drugs. We found a similar change in expression in ion channels in both DRG and PWT2 cells and in some of the transcription factors regulating these ion channels. The regulation of the ion channels downregulated by anticancer treatments suggests having a common mechanism, which could be a probable reason why they behave similarly due to anticancer drug treatment.

Arnt2 one of the targets that we found in this study proved to be important in the regulation of the target ion channels. Arnt2, a bHLH/ PAS protein, is involved in the HIF pathway, along with a number of other important pathways such as VHL pathway (Hankinson 2009, Qin 2011). By the experiments done with n-acetylcysteine, we think that oxidative stress is involved in the side effects of anticancer drugs. Arnt2 has been observed earlier to be downregulation by oxidative stress in neuronal cells (Arrang 1999). Nac treatment rescued the effect on the ion channels as well as the downregulation of Arnt2.

Of the other targets, interesting observations were for Mafb, a bZip binding to Maf recognition elements (MAREs), especially in DRG cells. It has also been shown to be involved in renal development, but the change in its gene expression in PWT2 cells was not that significant (Moriguichi 2008). The mechanism for its involvement in the side-effects after anti-cancer drug treatment has not been studied yet. The transcription factor Nhlh1 has been implicated in neurogenesis and could be an important regulator in the neuronal side-effects observed (Murdoch 1999). But taken together in the gene expression of Arnt2 the most interesting observations have been observed hence its mechanism was studied in more detail.

Both cisplatin and taxanes have been shown to involved in causing oxidative stress (Areti 2014, Zheng 2011). Cisplatin directly affects the formation of reactive oxidative species through both mitochondrial damage and non-mitochondrial effects (Conklin 2004). Cisplatin causes mitochondrial damage through damage to the mitochondrial damage and damage to cristae structure in the mitochondria. Mitochondrial damage is also important in cisplatin-induced neuropathy. The oxidative stress which as proposed here may be involved in the downregulation of Arnt2 after treatment with cisplatin.

Taxanes, being microtubule inhibitors, also caused mitochondrial damage (Bennette 2006). Taxanes treatment to neurones showed an increased abundance of vacuolated and swollen mitochondria, which may be due to loss of a network around mitochondria and hence also problems in mitochondrial fusion and fission. This changed mitochondrial phenotype was associated with painful peripheral neuropathy but did not cause nerve degeneration.

This may be a common pathway linking damage by the two relatively unrelated types of anticancer drugs, platinum-based drugs and taxanes. Both affect mitochondria and cause damage to mitochondria, which in turn causes oxidative stress in the cells. The downregulation of HIF interactor Arnt2 is a possible mechanism which explains both the peripheral neuropathy as well as nephropathy in these cells. It has been shown that mitochondrial poisons magnify the effect of the taxanes and cisplatin-based drugs when administered together (Xiao 2012). When rotenone, oligomycin and auranofin were administered along with either oxaliplatin, a cisplatin-based drug or paclitaxel an increase in allodynia and hyperalgesia was seen (Xiao 2012). This leads us to propose that a possible mechanism for the common side effects of both cisplatin-based drugs and taxanes could be through mitochondrial damage induced oxidative stress. This stress then affects the signalling mediated by Arnt2 and possibly the HIF pathway. It would be interesting to understand the role of HIF pathway in the dysregulation of ion channels. Also if mitochondrial damage causes the downregulation of ion channels due to the treatment of anticancer drugs. This is further supported by the protection against side effects of cisplatin-induced nephrotoxicity seen with the use of anti-oxidative agent lycopene *in vivo* in rats (Atessahin 2005).

The downregulation of the ion channels can explain the side effects of anticancer drugs such as cisplatin and taxanes. Piezo 2 downregulation also causes a loss of mechanosensitive currents. In the nephron, loss of mechanosensitive currents could lead to a loss of osmotic regulation and possible loss of ions through the nephron. Touch perception is dependent on the regulation of mechanosensitive current by neurones. Hence downregulation of piezo 2 can cause a loss of sensation due to loss of mechanosensation by neurones. Calcium and sodium are involved in special pathways in the cell (such as apoptosis and action potentials respectively). A misregulation in the levels of these currents may be involved in disruption of these signalling mechanisms. A lower current in the kidney cells could also lead to loss of reabsorption of calcium and sodium which happens in these cells. These side effects hence could be mediated by the downregulation of ion channels through oxidative stress.

But nephrotoxicity is only seen in cisplatin-treated cells and not in taxane-treated cells. The oxidative stress caused by the drugs could be different in magnitude between cisplatin and taxane. It would be interesting to understand the regulation of oxidative stress and ion channels. The loss of expression in taxane treated cells may not be enough to cause toxicity in these cells. Neurones being more susceptible to oxidative stress than other cells in the body show damage by both cisplatin and taxanes.

HT22 being a derived from the central nervous system does not show the same regulation as kidney and DRG cells. This could be interesting in terms of the differential regulation which could be correlated with the central nervous system being shielded by the blood brain barrier. CFTR upregulation was interesting as it was not expressed in control cells but only upregulated with treatment with cisplatin or taxanes. But as shown in the results majority of the MDR genes didn't show a change in regulation. A possible reason for this could be due to the high level of expression of MDR genes in culture. This could be due to antibiotics added to the culture medium. A possible explanation for the upregulation could be that in low expressed ABC family genes showed an increase in expression after treatment with anticancer drugs. This was observed with both CFTR and MRP7. But the same cannot be said for other cell models as the observation when tested in other cell models did not hold true. It could be a cell type specific phenotype.

The role of CFTR in drug resistance is debatable, and some studies have shown its role in response to drugs (L'hoste 2009, Robinson 1996). Both CFTR and MDR proteins are members of the Abcc family of proteins, many of which are involved in drug removal using ATP hydrolysis in their ATP-binding cassette. CFTR transfectants have been shown to show a slight MDR phenotype, with also a depolarization in the plasma membrane (Robinson 1996).

To see if the regulation of the MDR genes occurs in the same way as CFTR we tried to understand the transcription factors binding to the promoters of the genes. We saw a few similarities between the genes and transcription factors regulating them. Among them interestingly SP1 was regulating CFTR, MRP1, MRP3, MRP4, MRP6 and Mrp7. Other common transcription factors were Tfdp1, Bptf4, Patz1, Spi1, Maz and Klf4.

As can be seen from the map, there is not much common regulation between CFTR and MDR genes.

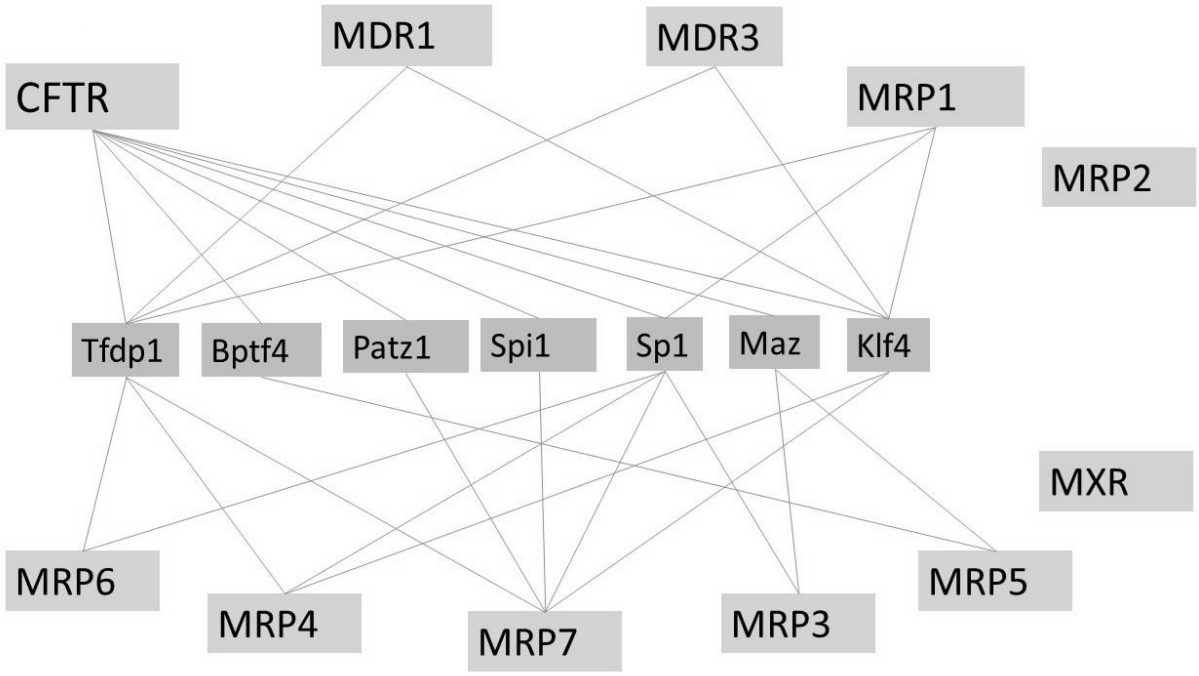

Figure 16: The transcription factors regulating the expression of MDR genes when compared to CFTR. As it can be seen, some common transcription factors were common between the MDR genes and CFTR. MXR and MRP2 show no common regulators with CFTR. Of the common targets observed, spi1 and klf4 are most interesting in understanding the changes that may be involved in upregulation of the interesting MDR proteins and CFTR.

It would be interesting to understand the effects of these anticancer drugs *in vivo* using mice as a model for the side effects. It would also be important to understand the role of the oxidative stress mechanism observed *in vitro* and if the treatment with Nac can be used in the treatment of side effects. The possible role of HIF in ion channel dysregulation after treatment with anti-cancer drugs has not been shown before this study. Also, it would be interesting to observe if cisplatin or taxane transport through CFTR is possible, as an explanation for the upregulation in terms of drug response.

In this study, we tried to understand a common mechanism, in which oxidative stress may play a role, involved in the side effects of anticancer drugs and also

investigated the regulation of CFTR, a gene which was upregulated after drug treatment, to see common mechanisms with MDR genes. In conclusion, we saw changes in chloride currents and calcium currents after treatment with anti-cancer drugs in neuronal and kidney cells respectively. This study points to oxidative stress which may be involved in nephropathy and neurotoxicity caused by cisplatin and taxanes. In kidney cell line studied, cisplatin and taxanes may mediate their effects through dysregulation of the HIF pathway through oxidative stress.Taken together, targeted upregulation of Arnt2 to compensate for the downregulation by oxidative stress, in non-cancerous cells may be useful to develop drugs to alleviate the side effects due to cisplatin and taxanes. CFTR currents could be seen after treatment with the anti-cancer drug, cisplatin but most MDR genes did not show an upregulation similar to CFTR.

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Appendix A:

Nociceptors	Role
TREK1	1. Neuroprotection 2. Pain sensation 3. Depression
TREK2	1. Polymodal pain/nociception 2. Cold and warm sensation 3. Cold allodynia associated with oxaliplatin treatment
K_V1.1/1.2/1.4	1. Peripheral neuropathic pain
ASIC1a/ASIC2a/ASIC3	1. Nociception during a tissue acidosis and inflammation
Nav1.7/1.8	1. Allodynia and inflammatory pain
P2X2-3/3	1. Allodynia and inflammatory pain
TRPV1/TRPM8/TRPA1	1. Thermosensitivity 2. Pain Sensation
Piezo 2	1. Touch and pain perception
CaVαδ-1/CaV1.2/1.3/CaV2.1-2.3	1. Touch sensitivity

Appendix B:

Nernst equation: $V_{EQ} = \frac{RT}{zF} \ln \frac{[X]_{out}}{[X]_{in}}$

Ion	Potential
$NMDG^+$	NA
Cl^-	-0.53 mV
Hepes	0 mV
Ca^{2+}	NA

Table 3: Chloride pipette and bath solutions

Ion	Potential
TEA^+	NA
Cl^-	6.05 mV
Ca^{2+}	NA
Hepes	0 mV
Mg^{2+}	NA
ATP	NA

Table 4: Calcium pipette and bath solutions

Liquid junction potential calculation: 10 mV

Ion	Potential
Cs^+	NA
Cl^-	-1.43 mV
Na^+	NA
Hepes	0 mV
Mg^{2+}	NA
Ca^{2+}	NA

Table 5: Sodium pipette and bath solutions

Appendix C:

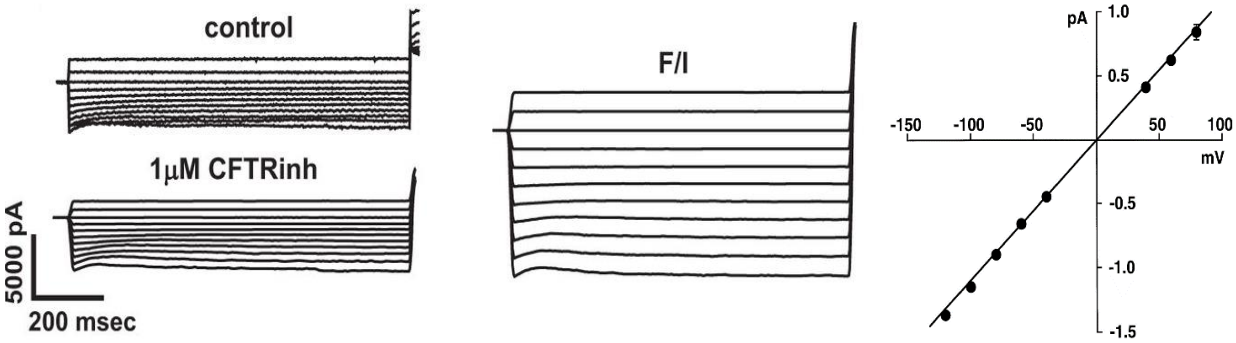

Figure 17: CFTR current standard currents and IV curves

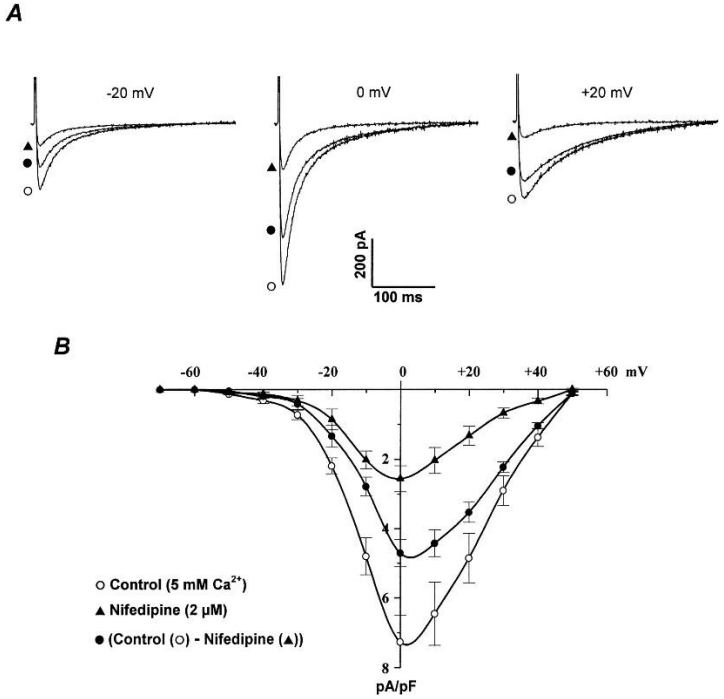

Figure 18: Standard calcium current and IV curves (adapted from Pignier C. 2000)

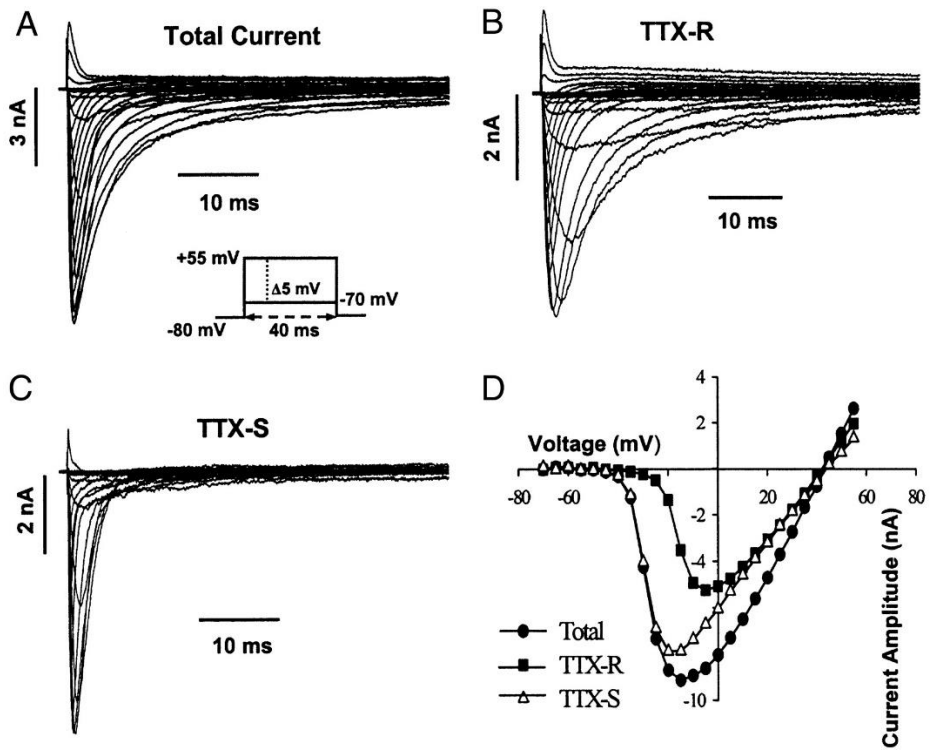

Figure 19: Sodium current and IV curves (adapted from Zhao J. 2007)