Chapter 19
Mining Next-Generation Sequencing Data to Identify Anti-nociceptive Signaling Pathways

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Abstract Cisplatin is a platinum-based chemotherapy drug which causes damage to the peripheral nerves as a toxic side effect, resulting in cisplatin-induced peripheral neuropathy (CIPN). This diminishes patients' quality of life and possibly, their ability to work and live independently. A novel drug, CYM-5478, has been identified as a potential candidate which could ameliorate the pathology of peripheral neuropathy, although the mechanism in which it does this is not known in detail. Using RNA-Seq analysis, we identified genes in four pathways which have been shown to be significantly altered with cisplatin treatment—chemokine signaling pathway, cytokine-cytokine receptor interaction, NF-κB signaling pathway, and inflammatory medicated TRP channels. We then analyzed the genes identified in Rattus norvegicus treated with CYM-5478 to identify the pathways through which the drug exerts its protective effect. Through the characterization of genes using the data analysis method, t-test, we discovered multiple genes of interest, in particular, Gnb3 and Rac2. Finally, we validated observed pathway changes by evaluating homologous gene expression changes using an in vitro assay and performing RT-qPCR. Identified genes which were significantly altered in rats treated with cisplatin and with or without CYM-5478 did not show significant change in human astrocytes. However, we can better understand the mechanism through which CYM-5478 attenuates CIPN through the identification of these genes. This allows us to better understand the agonistic action of CYM-5478 on cisplatin toxicity, and the changes in their gene expression could be analyzed further in more vigorous future in vitro experiments involving human cells.

Keywords Cisplatin · CYM-5478 · Cisplatin-induced peripheral neuropathy (CIPN) · RNA-Seq · Anti-nociceptive · Volcano plots · Homologous gene expression · Immortalized normal human astrocyte (iNHA) cells

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19.1 Introduction

Cisplatin, a platinum-based chemotherapy drug, has been shown to be effective in targeting a wide variety of cancers such as ovarian, testicular, bladder, head, and neck cancer. However, approximately 92% of chemotherapy patients using the drug cisplatin develop neurotoxic symptoms—characterized by tingling, numbness, and mechanical and thermal hyperalgesia—which cause patient discomfort and compromise cancer treatment. Most are dose-limiting, especially cisplatin-induced peripheral neuropathy (CIPN), which is characterized by sharp, stabbing pains, or tingling in the hands or feet caused by damage to the peripheral nerves. Symptoms progressively become more severe with continued treatment. A major cause of persistent pain in cancer survivors, CIPN is a toxic side effect which is often tolerated as an unfortunate consequence of life-saving chemotherapy. S1P is a lipid-based signaling molecule involved in neuroinflammatory processes through interactions with its five cognate receptors $S1P_{1\text{-}5}$, and S1P-related signaling has been identified previously as a potential target for treatment of CIPN. A specific sphingosine 1-phosphate receptor 2 (S1P2) receptor agonist, known as CYM-5478, has been identified as a potential candidate which could ameliorate the pathology of peripheral neuropathy, although the mechanism in which it does this is not known in detail [1]. The purpose of this project is to identify the pathways involved in CIPN that are affected by the action of CYM-5478 such as inflammation, the production of reactive oxygen species, and apoptotic pathways through the characterization of genes using research methods such as RNA-Seq. This will allow us to understand the changes in expression of specific genes in the S1P2 signaling pathway due to cisplatin and CYM-5478 treatment. We sieved out information from big data generated by previous experiments, with a narrower focus on genes involved in pathways that are changed by the action of cisplatin and our drug candidate. Genes in four pathways previously recognized to be dysregulated in patients undergoing chemotherapy with cisplatin as drug used with CIPN as a side effect were identified. Furthermore, we have evaluated whether these pathways have been changed by CYM-5478. We then singled out 2 genes—Gnb3 and Rac2 that were significantly changed and whose change was consistent with activation of sphingosine 1-phosphate receptor 2 (S1P2) by CYM-5478. Providing a more detailed explanation about how these genes are related and how CYM-5478 affects the expression of these genes to attenuate the effects of cisplatin toxicity will enable the trial of more drugs such as CYM-5478 to attenuate the effects of chemotherapy drugs, as well as help to identify targets to ameliorate the symptoms of CIPN.

We hypothesized that

1. CYM-5478 is able to attenuate toxic changes in gene expressions due to the action of cisplatin, by upregulating specific genes downregulated by cisplatin.
2. CYM-5478 is able to attenuate toxic changes in gene expressions due to the action of cisplatin, by downregulating specific genes upregulated by cisplatin.
3. The alteration of gene expression is not because of secondary response mechanisms within the body due to changes in chemical environment caused by the

action of cisplatin, but due to a direct effect on gene expression (e.g. transcription factors) by the drug CYM-5478 as it causes changes in cell composition. Any upregulation of inflammatory genes is due to the infiltration of immune cells and is not the direct effect of CYM-5478.

19.2 Methodology

A. Quantitative data analysis

Data analysis was conducted using mRNA expression levels obtained from a previous in vivo study done regarding measuring the expression levels of CYM-5478. Three groups of Rattus norvegicus were present, with one being the vehicle and the other two having been treated with cisplatin only and cisplatin and CYM-5478. Comparisons of cisplatin vs vehicle, cisplatin vs cisplatin and CYM-5478, and vehicle versus cisplatin and CYM-5478 were made. With the use of GraphPad Prism software, mRNA expression levels were analyzed using unpaired t-test with Welch's correction to create volcano plots. These plots helped to identify and ascertain which genes were significantly upregulated and downregulated. The genes were then grouped according to the pathways that are involved in. Using data already procured from the same study, four main pathways that CYM-5478 had known to affect were identified—chemokine signaling pathway, cytokine-cytokine receptor interaction, NF-κB signaling pathway, inflammatory medicated TRP channels. Among these, two genes were chosen—Gnb3 and Rac2 due to the previous studies showing that CYM-5478 has been known to activate the S1P2 receptor and affect these genes. The remaining genes identified show significant upregulation and downregulation purely due to the infiltration of immune cells and not due to the effect of CYM-5478 and therefore were not taken into consideration. MCP-1, which is a gene that is involved in producing inflammatory chemokines and Bax, an apoptotic marker was also chosen as they have been previously identified to be contributors to the pathology of CIPN and these genes would be downregulated in CIPN-related pathways.

B. Cell Culture

Immortalized normal human astrocytes (iNHA) cells were prepared with Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids (Gibco, USA), 100 U/ml penicillin, 0.1 mg/ml streptomycin. They were incubated in 37 °C at 5% CO_2. 5×10^6 cells were cultured in 6-well plates overnight. The cells were subjected to four hour serum starvation before treatment began.

C. Treatment Conditions

iNHA cells were treated with the following conditions:

1. 10 μM CYM-5478,
2. vehicle control,

3. 20 μM cisplatin + 10 μM CYM-5478,
4. 20 μM cisplatin + vehicle control of CYM-5478 (10 μM).

D. RNA Extraction

1 ml of Trizol© Reagent was added, agitated using an orbital shaker for 5 min. 0.2 ml of chloroform was added per test tube. The cells were incubated at room temperature for two minutes and centrifuged at 12,000 × g for 15 min at 4 °C. The aqueous phase was removed and an equal amount of isopropanol was added and incubated for 10 min. It was then centrifuged at 12,000 × g for 10 min, and 75% ethanol was added to the resulting RNA pellet. The samples were centrifuged at 7500 × g for 10 min at 4 °C twice. The RNA pellets were then resuspended in 20 μl nuclease-free water, vortexed, and incubated in dry bath at 55 °C for 10–15 min. Yield and purity of RNA were quantified using Nanodrop.

E .Real-time Quantitative PCR (RT-qPCR)

Fast SYBR™ Green Master Mix (Thermo Scientific) was used to run RT-qPCR using the following primers: Bax, Rac2, Gnb3, and MCP-1. Beta-actin was used as a housekeeping gene. Applied Biosystems QuantStudio 6 Flex RT-qPCR machine was used to run a 3-step PCR (50 °C for 15 s, 95 °C for 20 s, 72 °C for 30 s) for 40 cycles.

19.3 Results

To identify the effects of CYM-5478 on gene expression, we generated volcano plots and identified genes that were significantly upregulated and downregulated (with significance achieved at p-value <0.05), which are highlighted in purple and are enlarged for clearer view (Figs. 19.1, 19.2, and 19.3). We primarily looked at four pathways—chemokine signaling pathway, cytokine-cytokine receptor interaction,

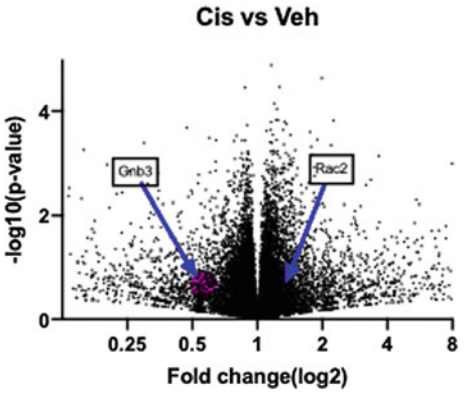

Fig. 19.1 Volcano plot depicting gene expression changes in in vivo analysis of rats treated with Cisplatin versus Vehicle

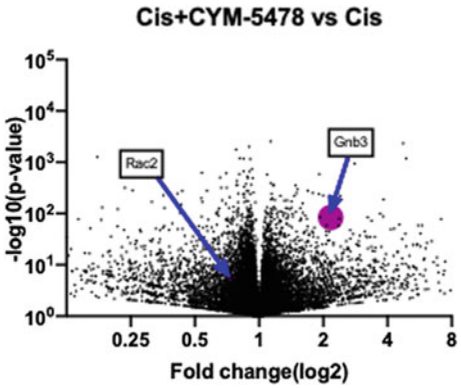

Fig. 19.2 Volcano plot depicting gene expression changes in in vivo analysis of rats treated with Cisplatin + CYM-5478 versus Cisplatin only

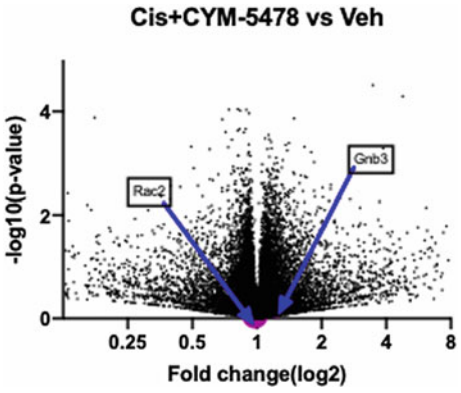

Fig. 19.3 Volcano plot depicting gene expression changes in in vivo analysis of rats treated with Cisplatin + CYM-5478 versus Vehicle

NF Kappa B signaling pathway, inflammatory medicated TRP channels that have been previously known to be primarily affected by CYM-5478 action. We sieved out genes based on the significance of regulation and whether they are involved in the 4 pathways that we had identified. These genes are displayed in Table 19.1, along with the p-values and q-values obtained from the volcano plots. The p-value refers to the probability of obtaining a change in gene expression at least as extreme as what is observed assuming that the null hypothesis (no change in gene expression observed) is correct. A p-value of less than 0.05 indicates a significant change in gene expression. The q-value is an adjusted p-value to control the false positive discovery rate.

In the RT-qPCR analysis, we measured the gene expression of 4 genes—*Bax, Rac2, MCP-1, and Gnb3. Rac2* and *Gnb3* were used from the array of genes that we identified from the volcano plot (Table 19.1). We used iNHA cell cultures exposed to 4 different types of treatments – vehicle, cisplatin only, CYM-5478 only and CYM-5478 and cisplatin and compared expression of the 4 genes across these treatment groups. *Bax* and *MCP-1* were used as means of comparison, as from the previous

Table 19.1 Table of p-values and q-values of genes of interest identified from the four pathways

Gene	CP versus V (p-value)	CP vs V (q-value)	CP versus CP + CYM-5478 (p-value)	CP versus CP + CYM-5478 (q-value)	Pathways involved
Dock2	0.0298	0.699	0.0491	0.838	Chemokine signaling, development HGF signaling, elevated platelet, and cytosolic Ca2+
Lyn	0.0382	0.764	0.0233	0.626	Chemokine signaling, NF-κB signaling, B cell receptor signaling, signaling by GPCR
Gnb3	0.0378	0.761	0.00585	0.305	Chemokine signaling, translation regulations by alpha-1 adrenergic receptors
Rac2	0.00665	0.704	0.018	0.559	Chemokine signaling, regulation of activated PAK-2p34, NGF pathway
Cntf	0.016	0.530	0.0016	0.144	Cytokine-cytokine receptor interactions, PEDF induced signaling, innate immune system
Camk2a	0.0488	0.840	0.0363	0.750	Inflammatory medicated TRP channels, translation regulations by alpha-1 adrenergic receptors
Trpm8	0.0000500	0.00994	0.0022	0.177	Inflammatory medicated TRP channels, CREB pathway, TRP channels

(continued)

Table 19.1 (continued)

Gene	CP versus V (p-value)	CP vs V (q-value)	CP versus CP + CYM-5478 (p-value)	CP versus CP + CYM-5478 (q-value)	Pathways involved
Asic1	0.007	0.339	0.0022	0.302	Inflammatory medicated TRP channels, transport of glucose and metal ions, ion channel transport
Asic3	0.00035	0.0472	0.0266	0.661	Inflammatory medicated TRP channels, transport of glucose and metal ions, ion channel transport
Syk	0.0465	0.823	0.0097	0.407	NF-κB signaling, B cell receptor signaling pathway, signaling by GPCR
Card11	0.00975	0.408	0.0142	0.498	NF-κB signaling, B cell receptor signaling pathway

literature, it has been established that they can be changed by the action of CYM-5478 action. As such, this gives us a means to check whether the expected increase of *Bax* and decrease of *MCP-1* is seen, thus allowing us to gauge the accuracy of our analysis.

The gene expression of the 4 genes was normalized to *β-actin*, and the results were plotted in the form of bar graphs (Figs. 19.4, 19.5, 19.6, and 19.7). However, the results of the analysis were inconclusive as the error bars charted in all 4 bar graphs overlapped across the 4 treatments, and hence, no definitive comparison could be made between the different treatments.

19.4 Discussion

For the sake of establishing the effect of CYM-5478 only, we used 2 comparisons only – cisplatin vs vehicle Fig. 19.1 and cisplatin vs cisplatin + CYM-5478 Fig. 19.2. The reason why these 2 comparisons were chosen is that they showed a clear contrast and change in gene expression of genes affected singularly by the introduction of the agonist into the treatment. We then further sieved out the genes that had contrasting regulations, as in upregulated in cisplatin vs vehicle and downregulated in cisplatin versus cisplatin + CYM-5478 and vice versa as we wanted to have a more focused and direct look on the effect of CYM-5478.

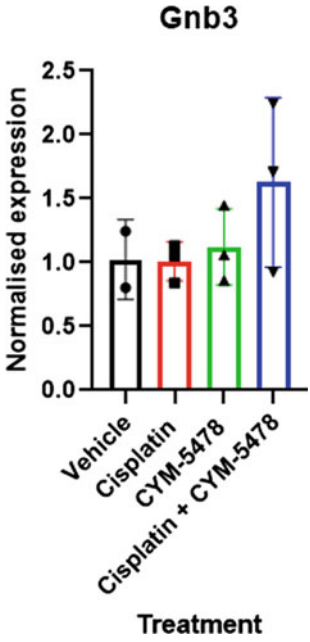

Fig. 19.4 RT-qPCR analysis of gene expression changes of *Gnb3* normalized to *β-actin* across the four treatments (n=3 per group)

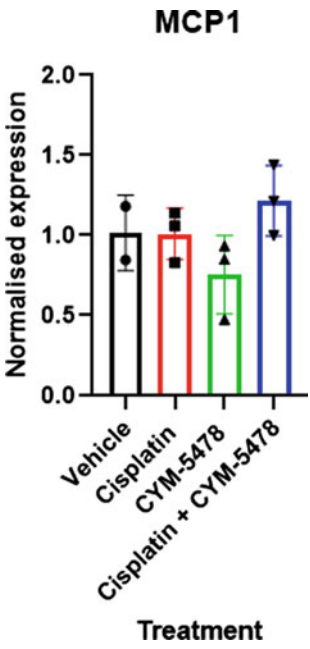

Fig. 19.5 RT-qPCR analysis of gene expression changes of *MCP-1* normalized to *β-actin* across the four treatments (n=3 per group)

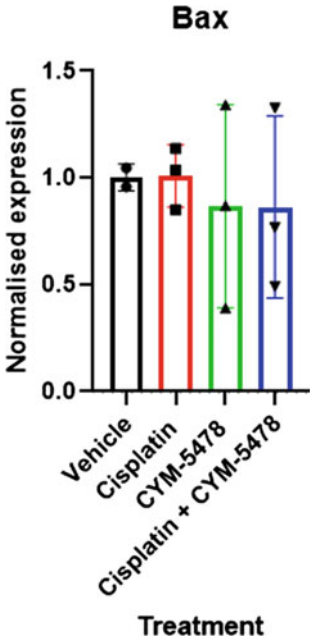

Fig. 19.6 RT-qPCR analysis of gene expression changes of *Bax* normalized to *β-actin* across the four treatments (n=3 per group)

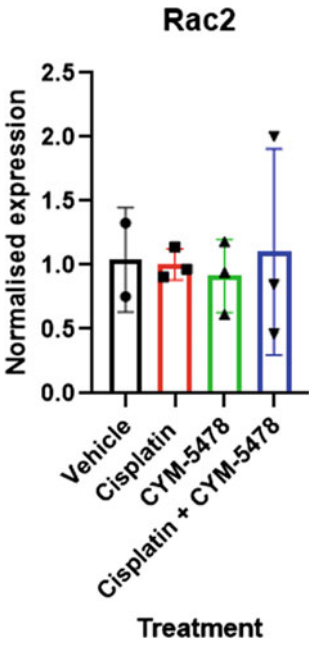

Fig. 19.7 RT-qPCR analysis of gene expression changes of *Rac2* normalized to *β-actin* across the four treatments (n=3 per group)

The panel of genes that we identified was *Dock2, Lyn, Gnb3, Rac2 (in chemokine signaling), Cntf (cytokine-cytokine receptor interaction), Camk2a, Trpm8, Asic1, Asic3* (inflammatory medicated TRP channels), *Syk*, *Card11,* and *Lyn* (NF Kappa B signaling pathway). Among these genes, with respect to possible directional gene changes evoked by CYM-5478 itself and secondary body responses such as immune responses triggered by cisplatin treatment, we singled out 2 genes—*Rac2* and *Gnb3*. *Rac2* is known to encode a member of the Ras superfamily of small guanosine triphosphate (GTP)-metabolizing proteins, which are involved in the generation of reactive oxygen species, consistent with a side effect commonly associated with cisplatin and it regulates processes, such as secretion, phagocytosis, and cell polarization. *Rac2* is also involved in the chemokine signaling pathway, which is associated with the action of CYM-5478. *Gnb3* codes for heterotrimeric guanine nucleotide-binding proteins (G proteins), which integrate signals between receptors and effector proteins and is involved in the chemokine signaling pathway.

After conducting RT-qPCR analysis with iNHA cells, we did not find any significant changes in gene expression across the different treatment groups. This could be due to the treatment time being too short for these genes to show any changes. This could also be due to the fact that the in vitro studies were conducted using human cells, unlike the in vivo study which involved rats, as such there might be significant differences in the gene expression pathways. Furthermore, only two genes were identified; therefore, this relatively small selection may not have included the target gene that we were looking for. The identification of these two genes may also be due to secondary body processes such as the possible mitochondrial damage caused by the action of cisplatin, which may have led to the marked upregulation and downregulation of the two genes identified.

19.5 Conclusion

We have identified genes whose expression was changed by the action of cisplatin, and then corrected by CYM-5478, in pathways which are known to be implicated in CIPN. No significant change in the gene expression of *Rac2* and *Gnb3* was observed. Hence, we identified genes that were changed through the cell-based assay, but they did not show the expected changes in gene expression in the in vitro conditions that we exposed them to.

19.6 Future Work

The optimization of treatment duration would allow for a more accurate picture of the effects of CYM-5478 in attenuating cisplatin toxicity and in ascertaining whether the changes in gene expression are still the same. This will allow us to better understand the mechanism of action of CYM-5478, which would be beneficial for the potential

co-administration of CYM-5478 to relieve the toxic side effects of cisplatin. This would allow patients to have a better quality of life and a better treatment chance. Repeating the experiment with different cell types such as oligodendrocytes and skin cells could also reveal changes in gene expression not apparent in astrocytes. Moreover, exploring different genes of interest expressed in other cell types to prevent other side effects such as low blood count and ototoxicity is possible to aid in narrowing the dose-limiting side effects of chemotherapy. We can also identify if CYM-5478 is able to attenuate similar toxic effects of other widely used cytotoxic drugs such as oxaliplatin and vincristine, via identifying genes from affected pathways and evaluating homologous gene expression changes in in vitro assays.

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