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Oral Presentation

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Expression of TNFAIP8 and ATP7A in cisplatin-adapted HeLa cell line

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Cisplatin is one of the most-commonly used platinum-based treatments for chemotherapy in various types of cancer. However, the high incidence of chemo-resistance has become the main limitation. TNFAIP8 (an anti-apoptotic protein) and ATP7A (a copper-efflux transporter protein) have been associated with the development of cisplatin resistance. Both proteins are highly expressed in cancer cells that are resistant to platinum drugs such as cisplatin. The objective of this study is to generate a cisplatin-adapted Hela cell line and determine the expression level of TNFAIP8 and ATP7A. The IC50 of cisplatin on parental HeLa cells was 1,75 μM as determined by MTT assay. Cisplatin-adapted cells were induced by continuously exposing the HeLa cell line to 1 and 2 μM cisplatin until stable cell growth was observed. The growth curve analysis showed that in the normal complete media, the cisplatin-adapted Hela cell line grows a bit slower as compared to the growth of the parental cell line. Cisplatin-adapted HeLa cell line showed higher IC50 compared to the parental Hela cell line by 2.4-fold as measured by trypan blue dye exclusion assay. Quantitative RT-PCR was used to measure the level of TNFAIP8 and ATP7A expression against GAPDH as the control. TNFAIP8 and ATP7A expression increased when the parental cells were treated with an incremental amount of cisplatin. The basal expression of TNFAIP8 and ATP7A is higher in cisplatin-adapted cells compare to those in the parental cell line. The expression level of TNFAIP8 and ATP7A was higher in the cisplatin-adapted cells as compared to the parental cells that were treated briefly with 1 μM and 2 μM of cisplatin. The results of this preliminary study corroborated the previous reports in the literature about the role of TNFAIP8 and ATP7A in the cancer resistance to cisplatin and suggest that the two proteins might be drug targets. Generation of cisplatin-resistance HeLa cell lines with higher IC50 can be used to screen for new cytotoxic drugs that might reverse resistance into sensitive cell lines, as well as investigating the mechanism and pathways involved in resistance and its reversal.

Keywords: HeLa cell, cisplatin, drug resistance, TNFAIP8, ATP7A

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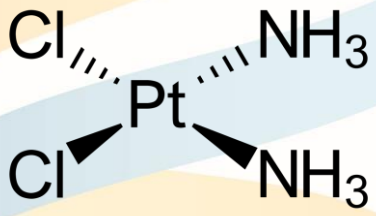
Introduction

- Cisplatin is one of the most common anticancer drugs that are used to treat patient against various cancers.
- Cisplatin often results in successful treatment, however high incidence of chemoresistance has become the main limitation of cisplatin as anticancer drug.
- New anticancer drug that will kill both cisplatin-sensitive & cisplatin-resistance cancer cells will be needed in the future.
- *In vitro* assay to do drug screening experiment is very important.
- To make this *in vitro* assay possible, the availability of cisplatin-resistant cancer cell lines are crucial.

Objective

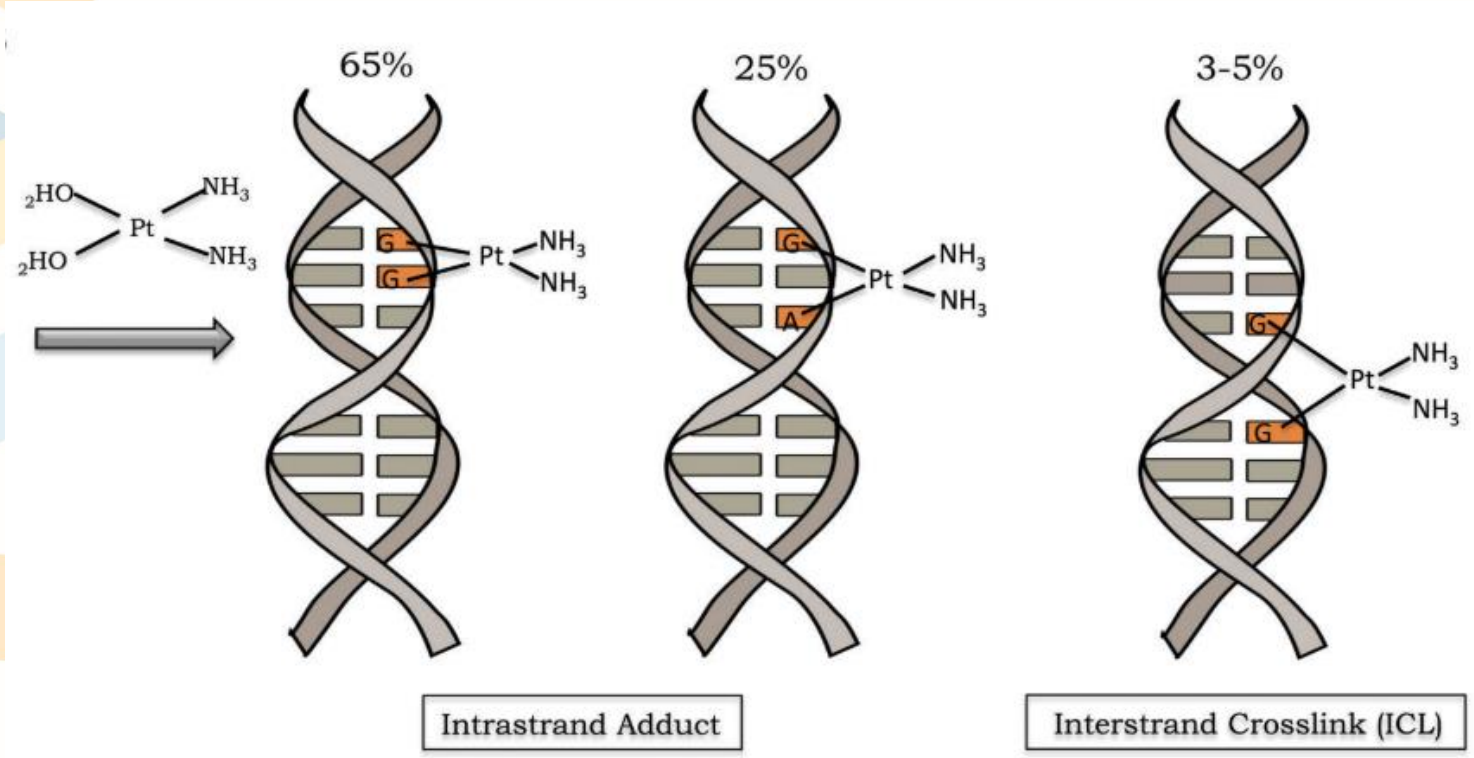
To generate cisplatin-resistant Hela cells that later can be used for the screening of new cytotoxic drugs, and for basic research to investigate the mechanisms and pathways involved in resistance and its reversal.

Cisplatin Mechanism of Action



<u>Cisplatin induce DNA damage in cancer cells by activation of irreversible apoptotic program.</u>

Cisplatin interact with the purine bases in the DNA to form DNA-DNA inter-strand and intra-strand crosslink.



Mechanism of Resistance

The molecular mechanisms underlying cisplatin resistance in cancer cells are complex and usually associated with these following features:

- Reduction in the intracellular accumulation of the platinum compounds
- Inactivation of apoptosis

Reduced Accumulation of Cisplatin

Decrease Influx

- Defective transporter or channels or binding protein
- Functional/structural changes in organelles/membrane potential.

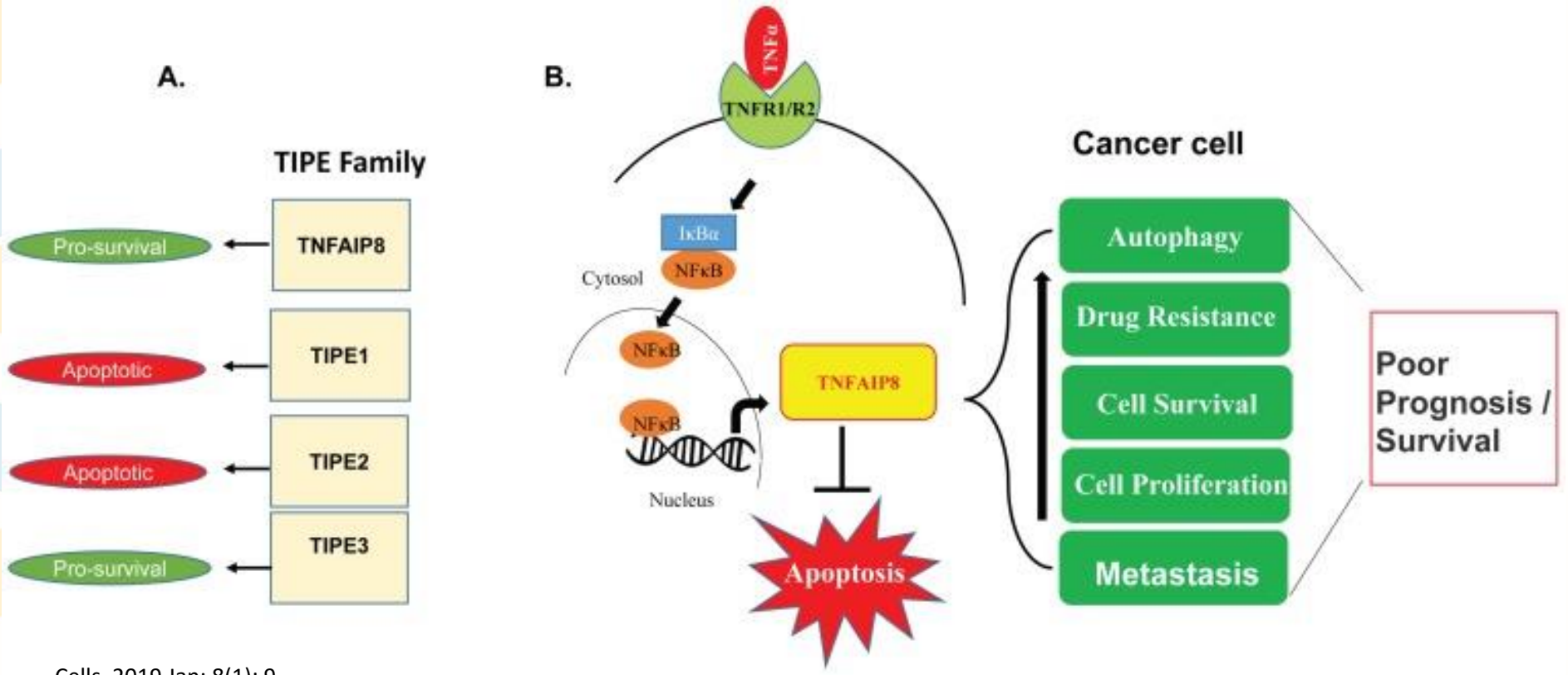
Increase Efflux

- Increased export, secretion, or exocytosis of the platinum compound.

Copper transport protein mediates the efflux of platinum drugs

- The association between ATP7A/7B & platinum drug resistance (Dolgova et al., 2013).
- The expression level of ATP7A found to be higher in platinum-resistant cells than in platinum-sensitive cells during *in vitro* studies.
- Patients with lower level of ATP7A expression reported to response better to chemotherapeutic response thus better survival.
- High expression of ATP7B can increase the resistance in cells towards platinum drugs (Mangala et al., 2009; Xu et al., 2008).

Tumor Necrosis Factor α-induced Protein 8 (TNFAIP8)



Cells. 2019 Jan; 8(1): 9.

- TNFAIP8 is anti-apoptotic and pro-oncogenic signaling molecule (Goldsmith et al., 2017)
- TNFAIP8 regulates Bcl-2 expression and suppressing cellular apoptosis (Yang et al., 2009).
- The expression level of TNFAIP8 was high in cervical cancer tissues compared with the adjacent normal tissues. Depletion of TNFAIP8 increased cisplatin sensitivity and promoted cisplatin-induced cellular apoptosis (Wu et al., 2019).

Higher TNFAIP8 expression in cisplatin-resistant **NSCLC A549** as compared to the WT, and administration of shRNA reverse the resistancy (Xing et al., 2018)

Investigate the level of TNFAIP8 expression in cisplatin-resistant **HeLa cells**

Can we reverse the cisplatin resistance in HeLa cells with high TNFAIP8 expresion by downregulate it using antisense oligonucleotide?

Adapting HeLa cells to Cisplatin

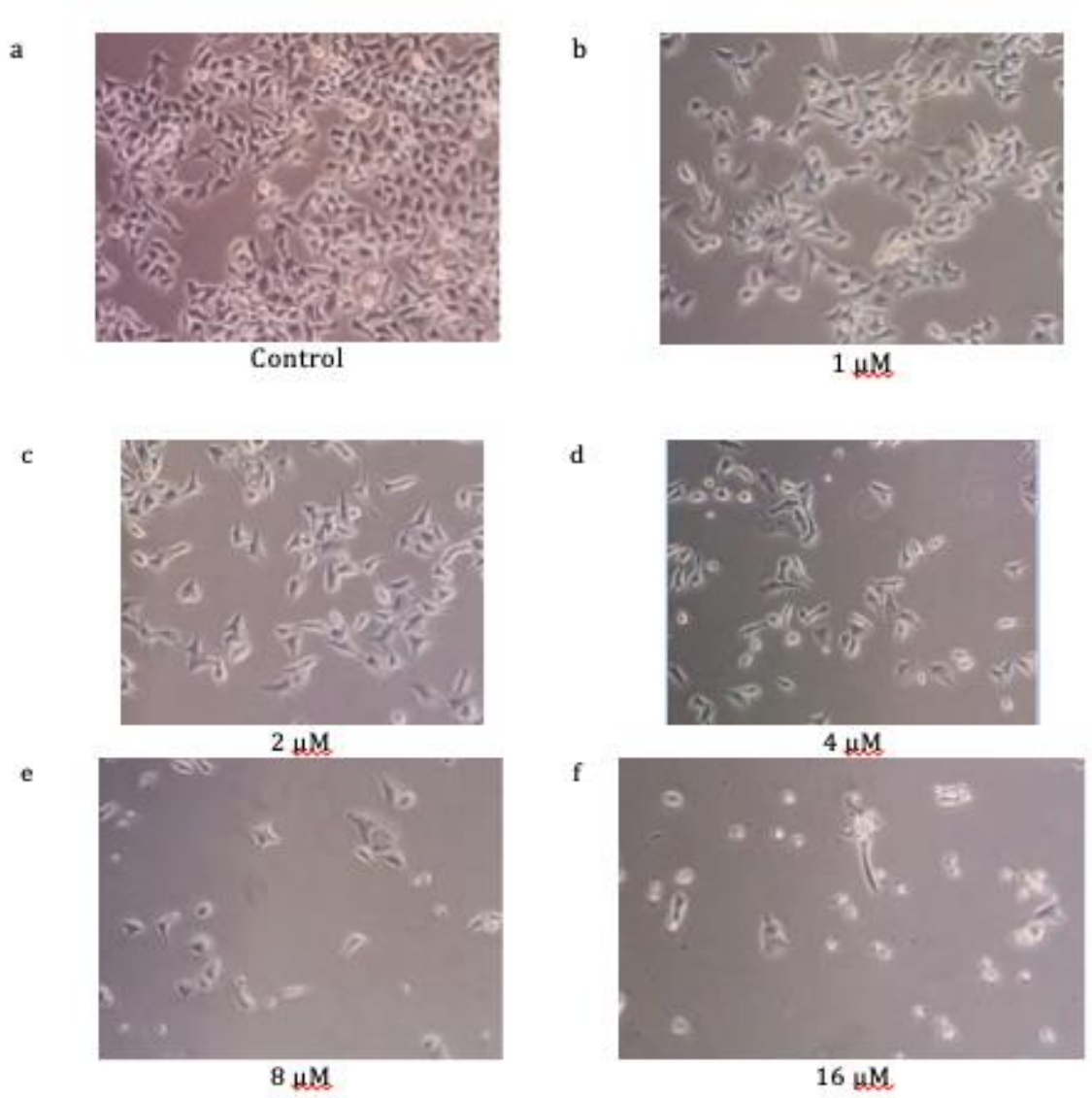
Hela cells were grown in normal media until it is nearly confluent.

Media changed into starving media

Hela cells were subjected to cisplatin treatment for 48 hours.

After 48 hrs, media was changed to normal media and wait until it was at least 60% confluent to be again treated with cisplatin.

Effects of cisplatin on cell morphology



Control cell: angular shape, patchy monolayer.

Treated cells: no morphology changes that can be observed, the density of cells in different concentrations can be observed; higher concentration of cisplatin will cause decrease in cell density.

Sensitivity to cisplatin - MTT assay

Hela cells were cultured in DMEM, 10% FBS and 1% penstrep.

Cells were suspended in 96-well plate and were left overnight to adhere to the vessels.

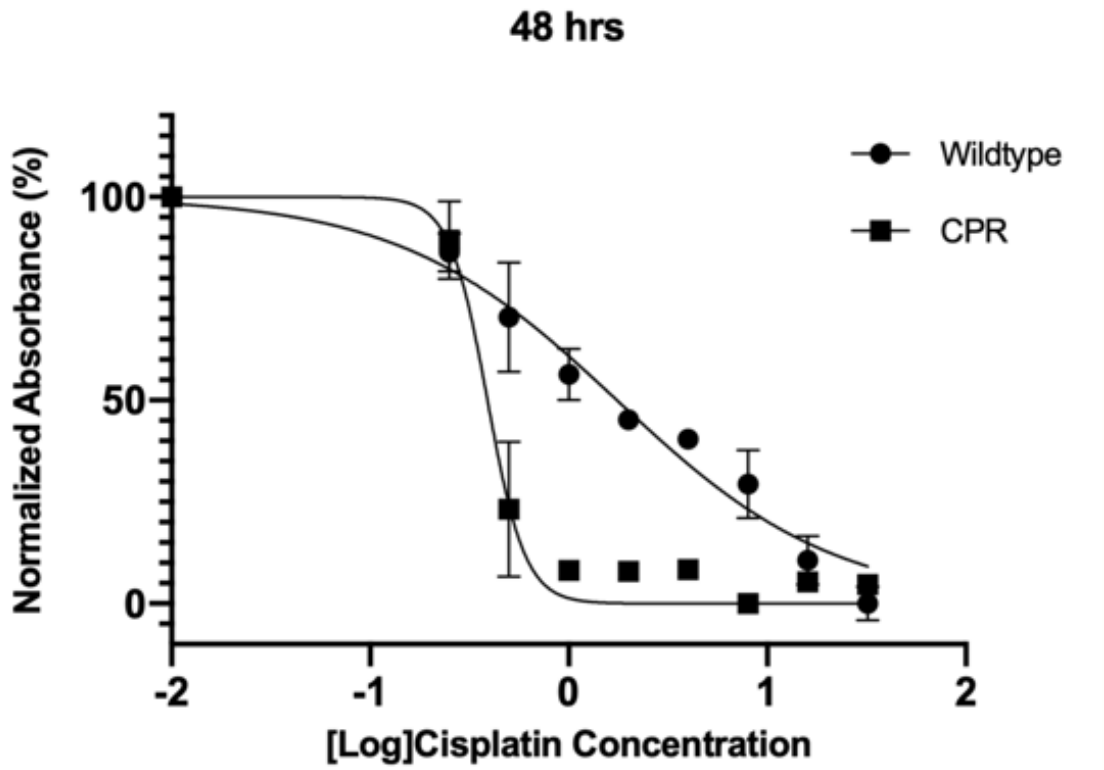
After 24 hours, the media were changed to starving media (0.1% FBS) & incubated for 4 hrs.

Cisplatin were given in various concentration including: 32 µM, 16 µM, 8 µM, 4 µM, 2 µM, 1 µM, 0.5 µM, & 0.25 µM.

After given cisplatin, cells were incubated for 48 hrs.

After 48 hrs incubation, MTT reagent was added & incubated for 4 hrs and then the absorbance of the formazan product was measured at 570 nm with a microplate reader.

Sensitivity to cisplatin (MTT assay)



WT HeLa IC50 1,749 uM
CPR HeLa IC50 0,389 uM

The resistance ratio for cisplatin-resistance Hela cell line shown to be lower than wildtype Hela cell line.

Trypan Blue Dye Exclusion Assay for Cell Viability

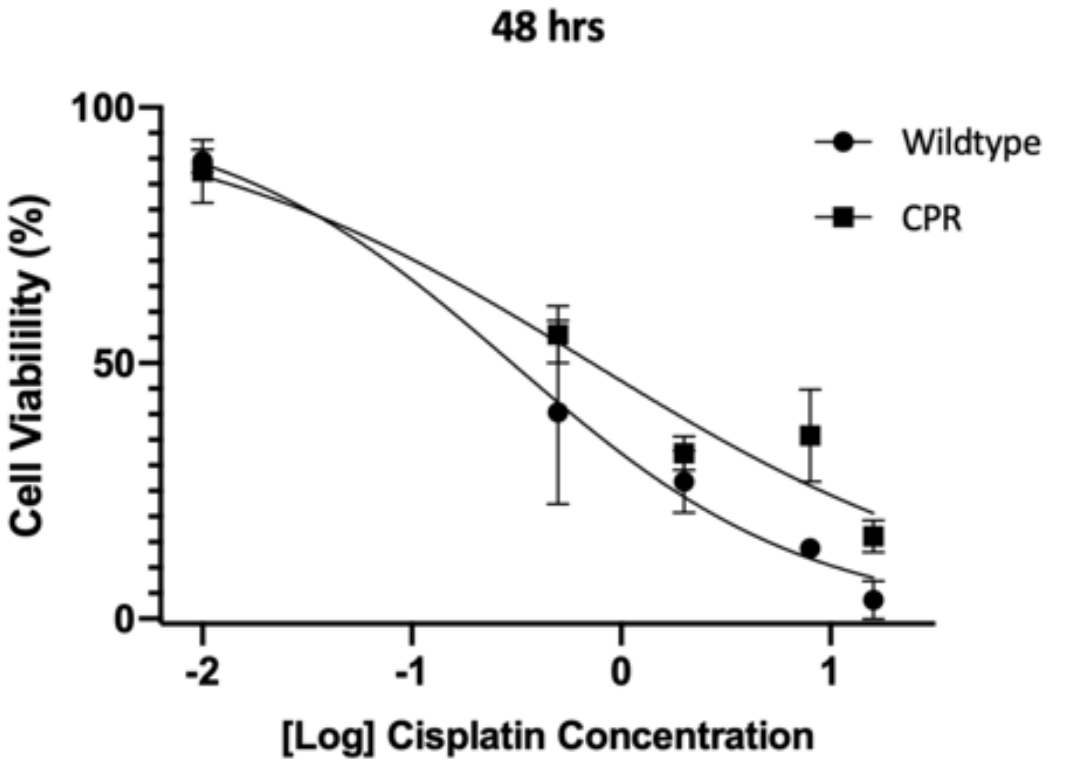
All cell lines underwent treatment as describe in MTT assay method with various concentrations of cisplatin 0.5 µM, 2 µM, 8 µM, and 16 µM..

Old media and trypsinized cells were combined in 1.5 ml Eppendorf tube.

10 µl of cell suspension were mixed with trypan blue dye with ratio of 1:1 in a new 1.5 ml Eppendorf tube.

The cell suspension were counted for viable and non-viable cells

Trypan blue exclusion dye assay for cell viability



WT HeLa IC50 0,3012 uM
CPR HeLa IC50 0,7260 uM

The resistance ratio for cisplatin-resistance Hela cell line shown to be higher by 2.4 fold compared to wildtype Hela cell line.

According to MTT assay, IC50 value of cisplatin-resistant Hela cell line is observed to be lower compared to the wildtype Hela cell line (resistance ratio of 0.22 : 1)

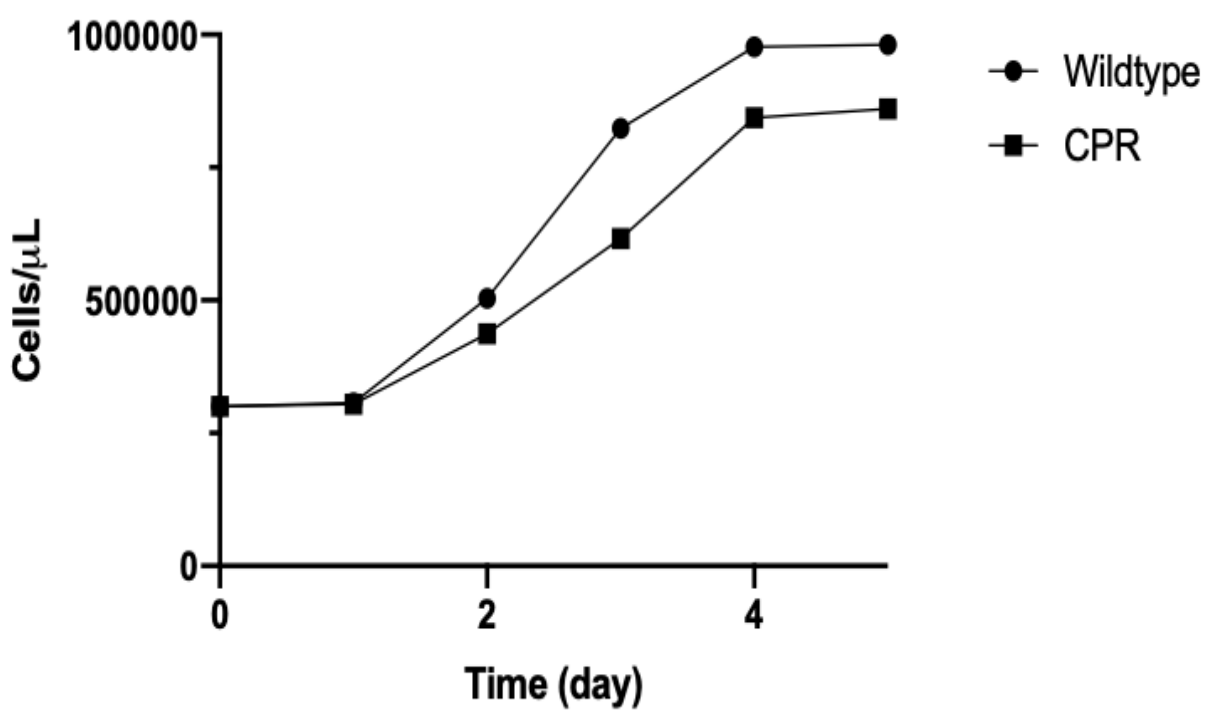
MTT assay measures the metabolic activity of cells

According to trypan blue exclusion assay, IC50 value of cisplatin-resistant Hela cell line is higher compared to the wildtype Hela cell line (resistance ratio of 2.4 : 1)

Trypan blue exclusion assay examined live or dead cells by blue dye that is taken by the cells

Low metabolic activity might have been associated with the chemoresistance properties of the cell

Growth Curve Analysis



Cell line	Population Doubling Time (hrs)
Wildtype Hela Cell	42 (1.75 day)
Cisplatin-resistant Hela	48 (2 days)

- Wildtype & cisplatin-resistant Hela cell line present a growth curve consisting lag, log, and stationary phase in 5 days of observation.
- The doubling time for cisplatin-resistant Hela cell line is a few hours longer than wildtype.
- Slower growth rate was shown in cisplatin-resistant Hela cell line compared to the parental wildtype Hela cell line.
- Slower growth rate is a component of drug resistance (Wosikowski et al., 2000)
- Barr et al., (2013) found similar observation with non-small-cell lung carcinoma (NSCLC) treated with cisplatin, significant growth delay along with delay recovery period.

Quantitative Reverse Transcriptase Polymerase Chain Reaction

Target gene:

- ATP7A
  (forward primer 5'-GCCTGCGTACGTGGATTTAT-3' and reverse primer 5'-TCAATGGTCCAAACACAGGA-3')
- TNFAIP8
  (forward primer 5'-TCCATCGCCACCACCTTA-3' and reverse primer 5'-CTCTGCCTCCTTCTTGTTTT-3'

Reference genes:

- GAPDH
- Beta actin

Set 1:

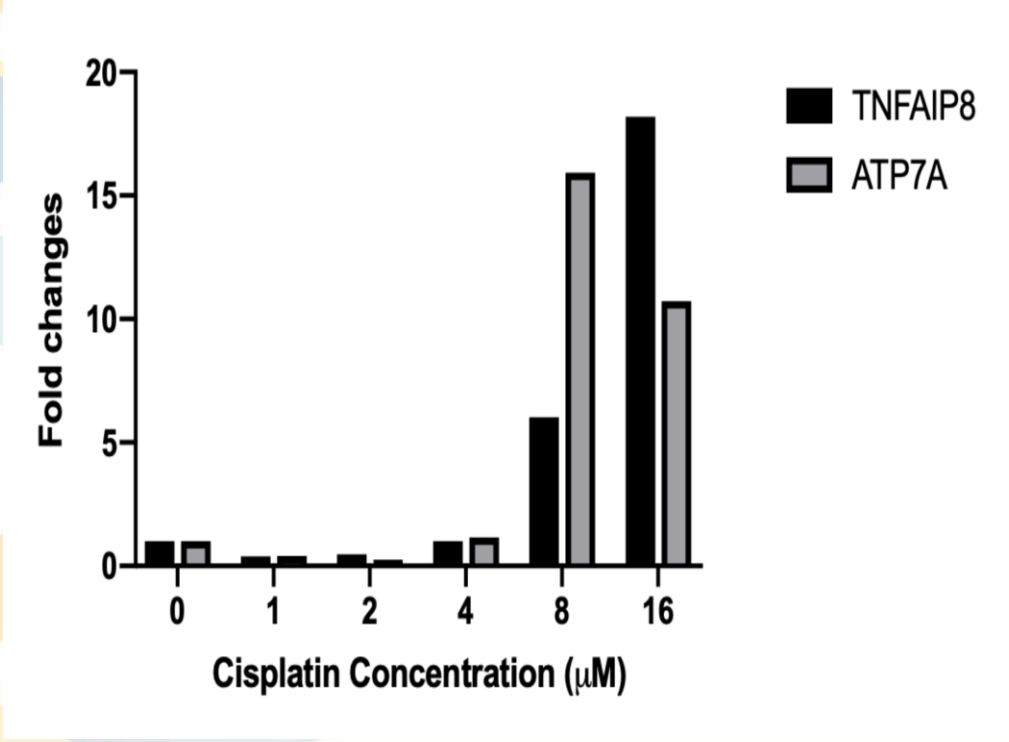
Wildtype Hela cell line was treated with cisplatin with concentration of 1 µM, 2 µM, 4 µM, 8 µM, and 16 µM prior to qRT-PCR analyses.

Set 2:

Hela cell line already adapted to cisplatin 1 µM and 2 µM of cisplatin.

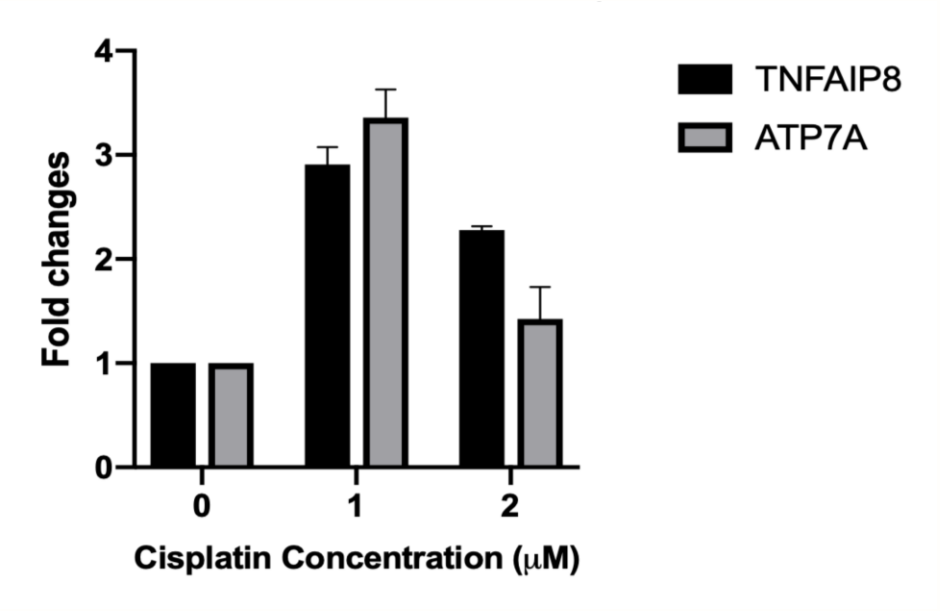
Expression of TNFAIP8 and ATP7A

Set 1



Cisplatin treatment increased the TNFAIP8 mRNA and ATP7A mRNA levels

Set 2



TNFAIP8 mRNA and ATP7A mRNA level was significantly increased in the cisplatin-adapted HeLa (1 uM and 2 uM), compared to that in the WT HeLa.

Conclusion

- The IC50 results showed that cells have not reached higher resistance yet. Other studies reported that they need at least 6 months to generate resistant cells.
- TNFAIP8 & ATP7A expression in cells adapted to cisplatin showed to have increase in fold changes compared to control cells.
- The expression of TNFAIP8 in resistance cells can be investigated further to elucidate the molecular mechanism of drug resistance in HeLa cells and the use of oligonucleotides to downregulate TNFAIP8 and reverse the resistance.
- TNFAIP8 might be the therapeutic target for reversing cisplatin resistance in cancer patients with high TNFAIP8 expression.

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Thank you