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Paper Title: The Potential of Chloroquine Phosphate to Reduce Chemotherapy Drug Resistance in Acute Myeloid Leukemia Cells

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Abstract

Uncontrolled proliferation of hematopoietic cells could result in acute myeloid leukemia (AML). However, drug resistance leads to the reduction of the effectiveness of traditional chemotherapy, which is responsible for a five-year survival rate less than 30%. This study obtained primary AML cells from 33 Chinese patients and divided them into low or high drug resistant groups. Potential mechanisms of drug resistance in AML were analyzed using high throughput sequencing technology. Interestingly, autophagy-related genes were often elevated in the drug-resistant AML cells. Thus, chloroquine phosphate, as a safer and widely used analog of chloroquine, was selected for its well-known capability of autophagy inhibition. To verify its effectiveness in the AML cells, *in vitro* experiments were conducted, in which two AML cell lines were selected to explore the effects of chloroquine phosphate combined with chemotherapeutic medicines such as cytarabine (Ara-C), idarubicin, and homoharringtonine. The results indicated that the IC_{50} of the conventional chemotherapy drugs were reduced at the presence of 20 μM chloroquine phosphate. Our study suggested that chloroquine phosphate could increase the AML cell sensitivity to chemotherapy of the AML through the inhibition of autophagy pathway and would be informative for developing new molecular targets for AML treatment.

Key words: acute myeloid leukemia; chemotherapy drug resistant; autophagy; chloroquine phosphate

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1 Background

1.1 Drug-resistance in acute myeloid leukemia (AML)

AML is a heterogenous malignancy of myeloid progenitor cells. During the past decades, understanding of AML molecular and cellular biology dramatically advances the clinical treatment of AML[1]. However, despite numerous of research projects and clinical trials, existing therapy, and the recurrence rate after complete remission is also high. Overall, current treatment leads to varying results by patient population, along with many secondary effects. More than 45% of patients would experience relapse and the 5-year survival rate is less than 30%[2]. Drug resistance is considered as the leading cause of treatment failure and several research are interested in elucidating the mechanisms of resistance to the drug[3]. Many cellular and molecular reprogramming such as metabolism, apoptosis, and autophagy[4] have been found after the exposure of AML cells to chemotherapy drugs. Consequently, several inhibitors targeting on these drug-resistant mechanisms were reported[3]. Still, more resistance mechanisms are expected to be explored, and despite numerous of research projects and clinical trials, existing therapy the effectively application of these mechanisms into improve clinical outcome requires further investigation.

1.2 Chloroquine analogues and autophagy

Since the synthesis of chloroquine in 1934, chloroquine and its analogues have

been the most commonly used drugs to prevent and treat malaria because of their reliable efficacy, low toxicity, and affordability[5]. Also, several clinical trials in progress have shown favorable effects, suggesting that they might be effective for cancer therapy[6]. Although the precise mechanism remains unclear, the anticancer effects of chloroquine and its analogues may be partially due to the inhibitory action on autophagy. Autophagy is an intracellular regulated mechanism, in which unnecessary or dis-functional macro-molecules are sequestered by membrane-wrapped vesicles. During autophagy, components inside cells are degraded and recycled, so that cells can survive under stressful conditions. In cancer biology, autophagy contributes to tumor development and proliferation. It plays dual roles in both tumor promotion and suppression[7]. Once undergoing chemotherapy, autophagy as a protective mechanism supports cancer cells survive. Meanwhile, the upregulation of autophagy is often accompanied with the increase in resistance of cancer cells to a number of anticancer drugs[8]. For example, the most commonly used medicines for AML treatment, a combination of anthracyclines with cytarabine (Ara-C), induce cytoprotective autophagy in AML cells but not in normal leukocytes [9]. It is worth noticing that most researches using primary AML cells were conducted based on the Western population. Meanwhile, blockade of autophagy by chloroquine and hydroxychloroquine, markedly increases cytarabine cytotoxic effects in AML cells[10]. However, not until recently, the potential side effects and risks of chloroquine and hydroxychloroquine were identified and limited their further development[11]. On the other hand, chloroquine phosphate has

acceptable safety profile[12]. Yet the effect of chloroquine phosphate on the drug resistance of AML cells has never been determined.

1.3 Research Goals

This study aimed to evaluate the contribution of autophagy in the drug-resistance of primary AML cells that were isolated from the Chinese population. In addition, the role of chloroquine phosphate in enhancing sensitivity of AML cells to the chemotherapy drugs would be determined.

2 Materials and Methods

2.1 Gene Expression Test

Total RNA from the AML patients' primary cells were isolated, from which complementary DNA (cDNA) was prepared. Then, the gene expression profiles of these cells were obtained through high throughput sequencing. Filtering out the fitting sequence pieces, we compared the sequences of the sample with the existing profile of human hg19 genomic. Thus, the gene expression of the sample and the standardized values of the gene expression were obtained.

2.2 Gene Pathway Identification

In accordance with the half maximal inhibitory concentration (IC_{50}) of distinct medicines, we evaluated the sensitivity of AML cells to the selected chemotherapeutic agents. By Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway data base and Gene Set Enrichment Analysis (GSEA), we then recognize the function of the pathway that led to the distinction.

2.3 Inhibitive Medicine Determination

After discovering the resistant gene pathway, we were to determine a medicine that could restrain the expression of the autophagy pathway by using Connectivity Map (clue.io). We input the abnormally expressed gene sets (autophagy pathway)

of the resistant group on the website and compared them with the database to identify the effective inhibitor of resistant related pathways.

2.4 Evaluation of the IC_{50} value of different drugs

Using MTS Cell Proliferation Assay Kit (CTB169, Promega biotechnology, Beijing, China) to detect cell proliferation. In 96-well plate, eight thousand MV-4-11 cells or twenty thousand THP-1 cells were cultured with 100μl of complete media. The cells were treated with indicated drugs at various concentration. Seventy-two hours post-drug treatment, 20 μl of MTS agents were added for three hours. The absorbance data was recorded and calibrated at 490nm and 690nm by using a Spectra max Molecular Devices microplate reader. The assay was repeated four times under the same conditions. the following equation was used to calculate the inhibition rates of cell proliferation:

Inhibition ratio = $(OD_{DMSO}-OD_{Compd})/(OD_{DMSO}-OD_{blank})\times 100\%$.

2.5 Western blot assay

Total protein of primary human cells and AML cell lines was detected by a BCA Protein Assay Kit (P0012, Beyotime Biotechnology, Shanghai, China). Anti-LC3B (1:1000, 3868S, CST, USA), Anti-actin primary antibody (1:3000, ab8224, Abcam UK).

2.6 Primary AML cells can be divided into drug-resistant and sensitive groups.

To evaluate drug-resistance of Chinese primary AML cells against various chemotherapeutic medicines, the study obtained primary cancer cells from 33 Chinese AML patients. The cells were treated with ten medicines that could cover three main types of chemotherapeutics. For example, Ara-C is a DNA synthesis inhibitor; homoharringtonine, an alkaloid, prevents the initial elongation step of protein synthesis; and idarubicin, an anthracycline drug, suppresses topoisomerase. The IC_{50} of each medicine that roughly indicated the level of drug-resistance was determined as stated in the Materials and Methods. As shown in Figure 1, red color represented higher IC_{50} with drug resistance, while blue color represented lower IC_{50} with drug sensitivity.

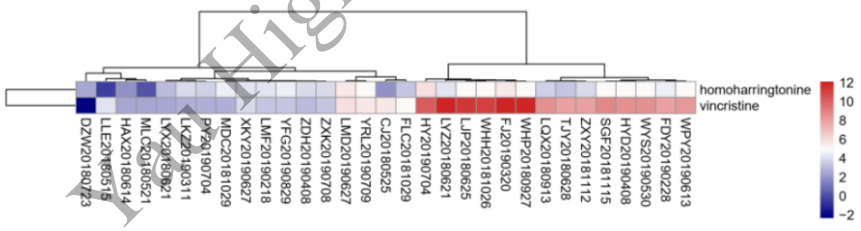


Figure 1 (a)

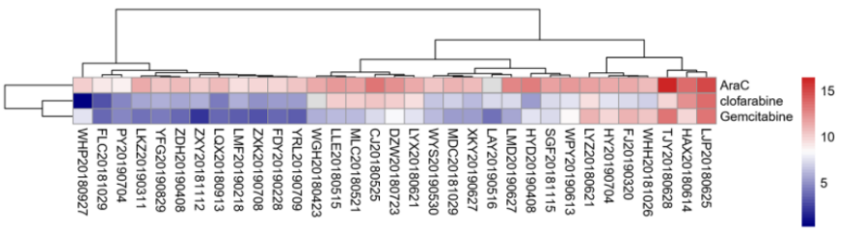


Figure 1 (b)

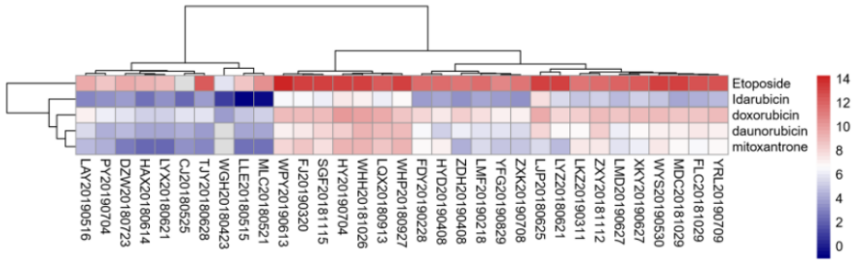


Figure 1 (c)

Figure 1. Sensitivity of AML Primary Cells to 10 Medicines. (a) The thermal pattern of alkaloid medicines. **(b)** The thermal pattern of DNA synthesis Inhibitor. **(c)** The thermal pattern of Topoisomerase-IIsuppressors. horizontal axis: medical record number; vertical axis: chemotherapeutic medicines;

It was evident that the majority of primary AML cells possessed significant resistance to the DNA synthesis inhibitors, such as Ara-C. Protein synthesis inhibitors and topoisomerase suppressors were used for further analysis. As shown in Figure 2, the human samples were divided into the sensitive group and the resistant group through hierarchical cluster.

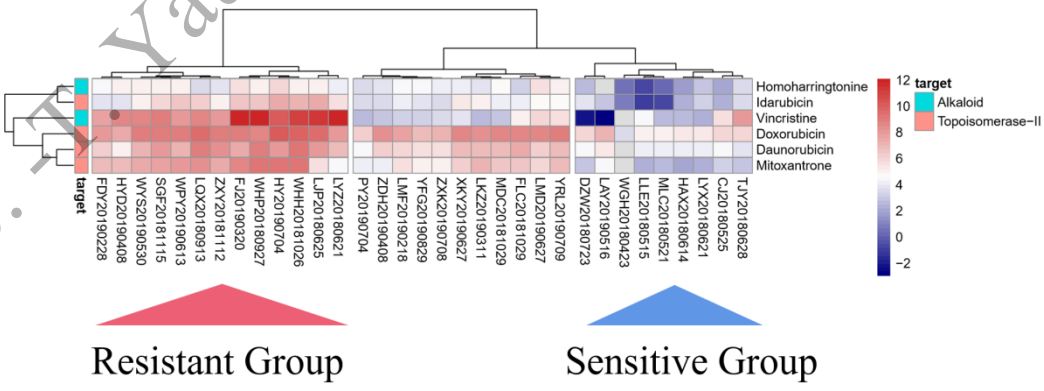


Figure 2. Sensitivity of AML primary cells to protein synthesis inhibitors and topoisomerase suppressors. horizontal axis: medical record number; vertical axis: chemotherapeutic medicines;

2.7 Autophagy pathway was activated in the resistant group.

Next, the integrity of total RNA samples was shown in Figure 3a. RNA-seq analysis were performed and the results was further analyzed by the GSEA software. As shown by the bubble chart statistics, among all the pathways that were activated in the indicated group, the autophagy pathway was up-regulated most significantly. These results suggested that autophagy pathway might contribute to AML cancer cell survival when challenged by cytotoxic drugs.

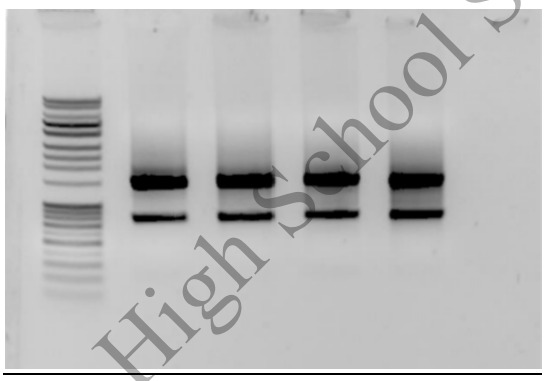


Figure 3(a)

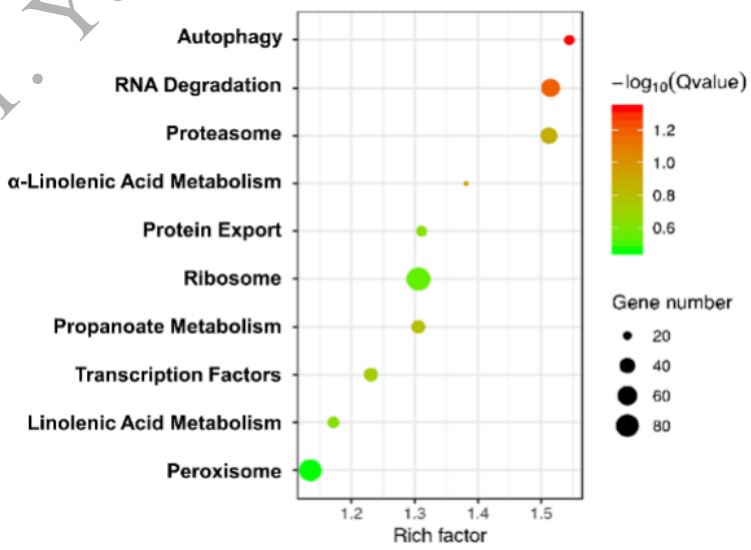


Figure 3(b)

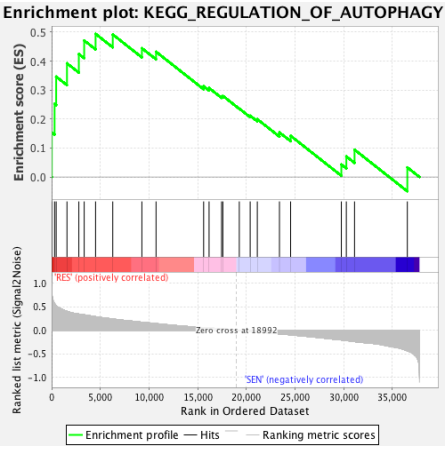


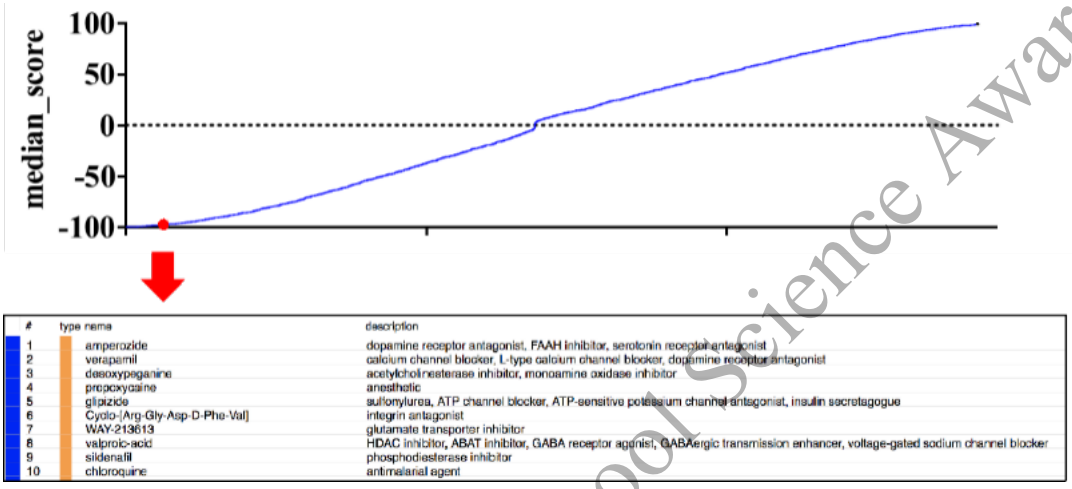
Figure 3(c)

Figure 3. Transcriptional characteristics of drug-resistant AML primary cells. **(a)** RNA agarose electrophoresis of the AML primary cells. **(b)** The bubble chart of the top 10 significant activated gene pathways in the drug-resistant group. The color represented the degree of significance, the size of the dot represented the size of gene set, the ordinate was the GSEA enrichment score, and the abscissa was gene pathway. **(c)** The thermal pattern indicated that the autophagy pathway was highly expressed in the resistant group (red), and lowly expressed in the sensitive group (blue).

2.8 Restraining Effect of Chloroquine on the Resistance of AML Cells

Furthermore, we used Connectivity Map to identify the potential medicines that may inhibit the autophagy pathway (Figure 4). Among these drugs, Chloroquine is most related to autophagy (there are more than 1,500 scientific articles describing the relationship between chloroquine and autophagy), and

chloroquine analogs were under investigation in many clinical trials for a variety of cancer types. Recently, it was reported that chloroquine and hydroxychloroquine had side effects, such as cardiotoxicity. Thus, we chose another safer and widely used analog, chloroquine phosphate, to conduct the following *in vitro* experiments.



#	type name	description
1	amperozide	dopamine receptor antagonist, FAAH inhibitor, serotonin receptor antagonist
2	verapamil	calcium channel blocker, L-type calcium channel blocker, dopamine receptor antagonist
3	desoxypeganine	acetylcholinesterase inhibitor, monoamine oxidase inhibitor
4	prepoxycaine	anesthetic
5	glipizide	sulfonylurea, ATP channel blocker, ATP-sensitive potassium channel antagonist, insulin secretagogue
6	Cyclo-[Arg-Gly-Asp-D-Phe-Val]	integrin antagonist
7	WAY-213613	glutamate transporter inhibitor
8	valproic-acid	HDAC inhibitor, ABAT inhibitor, GABA receptor agonist, GABAergic transmission enhancer, voltage-gated sodium channel blocker
9	sildenafil	phosphodiesterase inhibitor
10	chloroquine	antimalarial agent

Figure 4. The Fitting Score of Chloroquine on Restraining the Resistance Genes

2.9 The Chloroquine phosphate could induce the accumulation of LC3B in AML cell lines

To determine the activation of autophagy pathway under various conditions, Western blot analysis was performed tree times against LC3B, a well-known autophagy marker that will be increased under inhibition of autophagy. The results indicated that Ara-C, the chemotherapeutic drug, slightly reduced LC3B in the MV-4-11 (a kind of AML cell line), while chloroquine phosphate could inhibit autophagosomes to make the accumulation of LC3B[13]. After co-administrated with 10μM chloroquine phosphate, LC3B protein was enhanced significantly compared with treated with Ara-C alone, further indicating the ability of chloroquine

phosphate to inhibit autophagy in the AML cells.

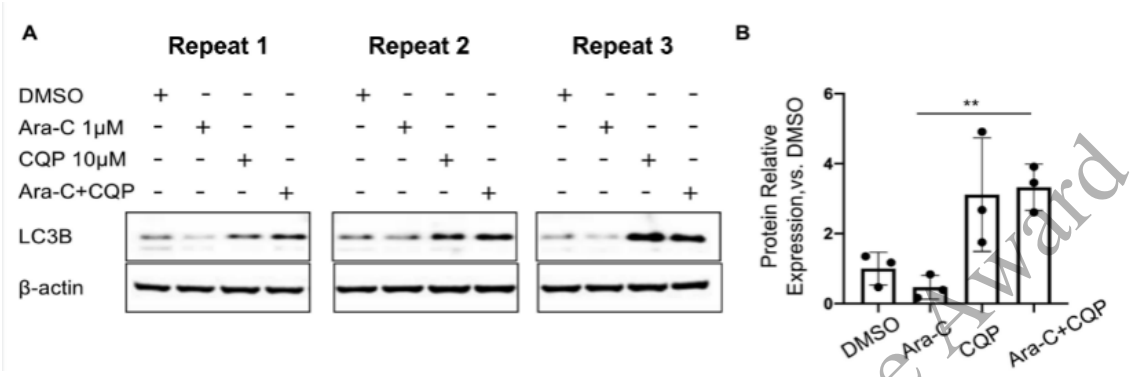


Figure 5. Western blot of chloroquine phosphate to the expression of LC3B protein in MV411 cell line. (a) The tendency of LC3B protein expression post-drug treatment was similar in three parallel experiments. **(b)** The results of figure 5a were quantified by Image J software (**indicates $P < 0.01$）.

2.10 The effect of chloroquine phosphate was verified through *in vitro* experiments

In two kinds of AML cell lines (MV-4-11 and THP-1), chloroquine phosphate alone did not significantly change the growth of cancer cells under experimental conditions (Figure 6). Yet combined with common chemotherapeutic agents, chloroquine phosphate raised the sensitivity of cancer cells to chemotherapeutic medicines. Therefore, it could increase curative effects, resulting in an IC_{50} with over 80% reduction (Figure 7).

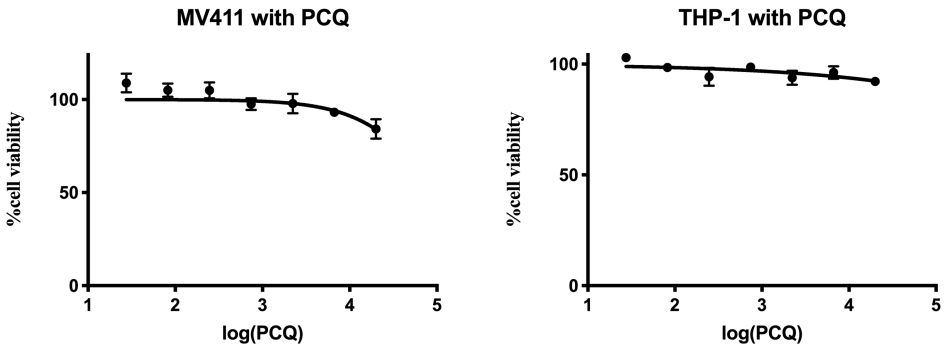


Figure 6. The effect of chloroquine phosphate alone on the AML cell growth

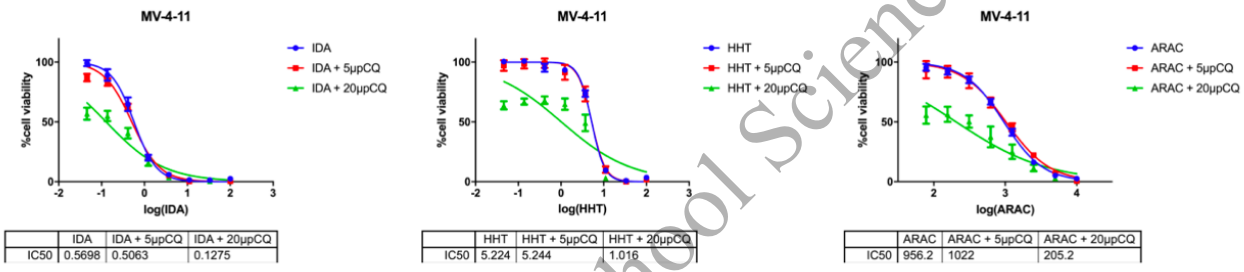
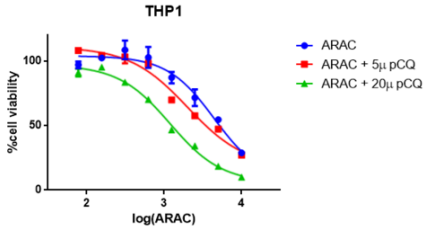


Figure 7. Influence of Chloroquine Phosphate Combined with Idarubicin (Left), Homoharringtonine (Middle), and Ara-C (Right) on MV-4-11

As Ara-C is basic for standard induction therapy, we also testified its effects on THP-1 cell line. High concentration of chloroquine phosphate also did not influence the growth of cancer cells. However, when it was combined with Ara-C, the survival rate of THP-1 decreased significantly, and its IC_{50} declined as much as 75% (Figure 8).



	ARAC	ARAC + 5μ pCQ	ARAC + 20μ pCQ
IC50	4424	2001	1198

Figure 8. Influence of Chloroquine Phosphate Combined with Ara-C on TPH-1

3 Discussions

Autophagy is an intracellular mechanism that degrades unwanted proteins, organelles, and invading pathogens. Since methods for investigating autophagic activity are complicated and consequently, the results are sometimes misinterpreted[14]. Except LC3B used in our research, there are many other existing autophagosome indicators, such as ULK1, WIPI1, ATG5, which are required to be determined in the future. Furthermore, it is necessary to have direct observation of autophagy-related structures in the treated cells, which requires transmission electron microscopy. This is beyond the capability of a high school student. Hopefully, I will continue this research during undergraduate study.

Chloroquine and its analogues have been used for the treatment of various diseases. The inside molecular and cellular mechanisms include interfering with lysosome and autophagy, disrupting membrane stability, and inhibiting immune activation[15]. Accumulating evidences demonstrate that chloroquine sensitizes cancer cells to radiation and other chemotherapy drugs. However, the precise mechanism is unclear. The antineoplastic effects of chloroquine stem from the modulation of pathways other than autophagy, such as inducing apoptosis, activating cell cycle arrest, etc[16]. Whether these pathways are affected upon treatment of AML cells with chloroquine phosphate is of interest. In addition to its own mechanism, the side effect of chloroquine combined other drugs is also an important clinic relevant consideration. For example, it was reported that co-medication of tamoxifen with chloroquine was accompanied by an

increased risk of eye toxicity[17]. Thus, the combination use of chloroquine phosphate with chemotherapy drugs requires further investigation.

In this study, we demonstrated that autophagy pathway is activated in the primary AML cells with drug-resistance from the Chinese population. Meanwhile, chloroquine phosphate is capable of rescuing the drug-resistance, probably through inhibition of the autophagy pathway. Our research was preliminary because the effect of chloroquine phosphate was only determined on two AML cell lines. Although THP-1 and MV-4-11 are two typical cell lines that have properties of self-renewal and pluripotency similar to those of the myeloid leukemia cell, primary AML cells obtained from patients are required to evaluate the effect of chloroquine phosphate. In addition to the *in vitro* experiments, animal models offer a powerful approach to evaluate the potential role of various kinds of drugs[18]. The available animal models include zebrafish, drosophila, and mammalian rodent systems, such as rats and mice. Furthermore, to investigate patient-derived, primary AML cells *in vivo*, immune deficient mice have been explored[19]. Although it is not desirable for a high school student to perform experiments with immune deficient mice, I wish to explore the related experiments in the future.

4 Acknowledgements

In 2017, the 2nd Academic Conference on Hematological Tumor Biology and Combined Therapy was held in the Ruijin Hospital, Shanghai. I was one of the volunteers there, in charging of information desk. It was my pleasure to listen to the speech of Professor Xiaobin Zhang. I was told that even though many research projects and clinical trials regarding blood cancer had been already carried out, existing therapy remains ineffective and the recurrence rate is still high. Therefore, with the enthusiasm of life science, I wanted to do some changes and dove into the research of blood cancer.

My science teacher in the Shanghai Pinghe school, Miss. Yinghe Lv, not only helped me search basic knowledge of AML, but also introduced me to Dr. Jia Li. It was my honor to be one of Dr. Li's students and stayed in his laboratory during summer 2019, and many weekends afterwards. He directed me through the whole journey of scientific research, and sowed a seed of science in my heart. I started to know the scientific spirit and pursue the truth of science.

I would like to thank Mr. Hanlin Wang and Miss. Ning Song, both of whom were Dr. Li's graduate students. During the past two years, they led my way through this magical world of microorganisms, and guided me in the field of bioinformatics and biological experiments. When I failed an experiment, they would analyze the reason with me and always encouraged me to continue and repeat. No matter how late I performed the experiments, they would be in the laboratory with me. Without them, my experiments would not be accomplished in time. They helped me consolidate my

knowledge and better understand the theory behind the practice.

Lastly, I would like to thank my parents and my brother. They support me to pursue what I am interested in and accompany me along my growth. It is from the vegetative state of my parents that illuminated the sparkle in my heart and led me into Biology and Medicine.

5 Reference

1. Estey EH. Treatment of acute myeloid leukemia. *Haematologica.* 2009;94(1):10-16.
2. Medeiros BC, Satram-Hoang S, Hurst D, Hoang KQ, Momin F, Reyes C. Big data analysis of treatment patterns and outcomes among elderly acute myeloid leukemia patients in the United States. *Ann Hematol.* 2015;94(7):1127-1138.
3. Zhang J, Gu Y, Chen B. Mechanisms of drug resistance in acute myeloid leukemia. *Onco Targets Ther.* 2019;12:1937-1945.
4. Wilde L, Ramanathan S, Kasner M. B-cell lymphoma-2 inhibition and resistance in acute myeloid leukemia. *World J Clin Oncol.* 2020;11(8):528-540.
5. Boudhar A, Ng XW, Loh CY, et al. Overcoming chloroquine resistance in malaria: Design, synthesis and structure-activity relationships of novel chemoreversal agents. *Eur J Med Chem.* 2016;119:231-249.
6. Kimura T, Takabatake Y, Takahashi A, Isaka Y. Chloroquine in cancer therapy: a double-edged sword of autophagy. *Cancer Res.* 2013;73(1):3-7.
7. Yun CW, Lee SH. The Roles of Autophagy in Cancer. *Int J Mol Sci.* 2018;19(11).
8. Xiao X, Wang W, Li Y, et al. HSP90AA1-mediated autophagy promotes drug resistance in osteosarcoma. *J Exp Clin Cancer Res.* 2018;37(1):201.
9. Auberger P, Puissant A. Autophagy, a key mechanism of oncogenesis and resistance in leukemia. *Blood.* 2017;129(5):547-552.
10. Kim Y, Eom JI, Jeung HK, et al. Induction of cytosine arabinoside-resistant human myeloid leukemia cell death through autophagy regulation by hydroxychloroquine. *Biomed Pharmacother.* 2015;73:87-96.
11. Chen Y, Shen T, Zhong L, et al. Research Progress of Chloroquine and Hydroxychloroquine on the COVID-19 and Their Potential Risks in Clinic Use. *Front Pharmacol.* 2020;11:1167.
12. Cui C, Zhang M, Yao X, et al. Dose selection of chloroquine phosphate for treatment of COVID-19 based on a physiologically based pharmacokinetic model. *Acta Pharm Sin B.* 2020;10(7):1216-1227.
13. Mauthe M, Orhon I, Rocchi C, et al. Chloroquine inhibits autophagic flux by decreasing autophagosome-lysosome fusion. *Autophagy.* 2018;14(8):1435-1455.
14. Yoshii SR, Mizushima N. Monitoring and Measuring Autophagy. *Int J Mol Sci.* 2017;18(9).
15. Schrezenmeier E, Dorner T. Mechanisms of action of hydroxychloroquine and chloroquine: implications for rheumatology. *Nat Rev Rheumatol.* 2020;16(3):155-166.
16. Zhang Y, Liao Z, Zhang LJ, Xiao HT. The utility of chloroquine in cancer therapy. *Curr Med Res Opin.* 2015;31(5):1009-1013.
17. Toimela T, Tahti H, Salminen L. Retinal pigment epithelium cell culture as a model for evaluation of the toxicity of tamoxifen and chloroquine. *Ophthalmic Res.* 1995;27 Suppl 1:150-153.
18. Almosailleakh M, Schwaller J. Murine Models of Acute Myeloid Leukaemia. *Int J Mol Sci.* 2019;20(2).
19. Skayneh H, Jishi B, Hleihel R, et al. A Critical Review of Animal Models Used in Acute Myeloid Leukemia Pathophysiology. *Genes (Basel).* 2019;10(8).