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The Evaluation of Mefloquine Drug Repurposing on Acute Myeloid Leukemia

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ABSTRACT

The aim of this study is to observe cell proliferation, cell viability, apoptosis, and autophagy on acute myeloid leukemia (AML) cell lines, NB4 and U937, with the drug repurposing of mefloquine (MQ). Methods such as the 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay and trypan blue staining have shown a decrease in live cells with high concentrations of mefloquine. Using their average perspective IC50 values of MQ concentration, Western blotting was applied by means of apoptosis and autophagy markers to determine if the induction of apoptosis and inhibition of autophagy was present in MQ-treated AML cells. The experiment will be continued with more cell lines, drugs, and other means of protocol in order to contribute to cancer therapy.

INTRODUCTION

BACKGROUND

Acute leukemia is a fast-growing blood cancer with an early onset in hematopoietic stem cells. DNA mutations block normal development in lymphoid and myeloid cells, leading to an accumulation of blast cells in the bone marrow. This can result in either acute lymphocytic leukemia (ALL) or acute myeloid leukemia (AML) as shown in Fig. 1. With 2-year survival rates of less than 20% in the elderly and 60% in children [2], AML became the main focus for this project.

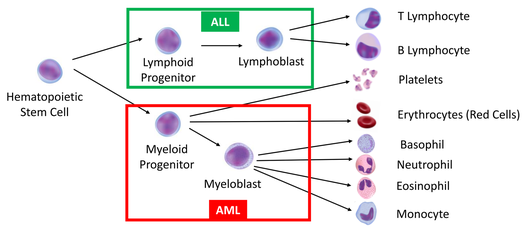


Fig. 1 - Hematopoiesis for Acute Leukemia

The growth maintenance of cancer cells depend partially on autophagy, a catabolic process that degrades and recycles unwanted proteins and organelles in the lysosome for cell survival. Based on previous studies, leukemic cells undergoing autophagy inhibition experience metabolic stress, triggering apoptosis [3].

The use of an antimalarial drug, mefloquine (MQ), has sparked interest in drug repurposing for leukemic cells due to its effective reduction of parasitemia from blood. Mefloquine's current mechanism of action for malaria includes damaging the membrane of free heme, interacting with phosphatidylinositol, and affecting volume-regulated anion channels (VRAC) to bring toxicity to parasites in blood [5]. A high-throughput screening panel of leukemic cell lines identified mefloquine as an anti-leukemic compound by lysosome disruption, releasing cathepsins into the cytosol and causing permeability in the lysosome membrane [2].

HYPOTHESIS

- The repurposing of mefloquine (MQ) as an anti-leukemic drug will result in decreased myeloid leukemia cell proliferation due to lysosome disruption, thereby inhibiting autophagy and inducing apoptosis.

METHODS

MTT Assay

1. Serial dilute 50 mM stock solution of MQ with DMSO. Seed NB4 and U937 with RPMI media (10% FBS, 1% PS) at 2 x 10^5 cells/mL and 1 x 10^5 cells/mL, respectively. Total volume per well: 100 µL.
2. Incubate plates for 48 hours. Add 10 µL of MTT reagent, incubate for 4 hours, then add 100 µL of detergent.
3. Store in the dark for 24 hours. Record absorbance at 595 nm and determine IC50.

Trypan Blue Staining

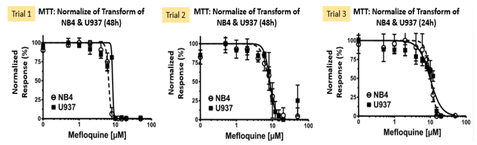
1. Perform similar pre-incubation steps as the MTT protocol
2. After 48 hours, perform a cell count with Trypan Blue to view cell viability at increasing MQ concentrations (include IC50s: 7 µM for NB4 and 8 µM for U937)

Western Blot

1. Prepare lysates of NB4 and U937 cells with MQ treatment for 0, 2, 4, and 24h. Run gel electrophoresis.
2. Perform electrotransfer of the proteins using polyvinylidene fluoride (PDVF) membrane
3. Wash membrane in Ponceau S staining solution. Block membrane in 10% milk (with TBST) to rid of background "noise."
4. Wash in 5% milk with the primary antibody (1:1000) overnight in 4ºC shaker. Wash membrane three times in TBST for 10 min.
5. Wash in 5% milk with secondary antibody (1:3000) for 1 hour. Wash membrane three times in TBST for 10 min.
6. Prepare ECL mix (1:2 dilution of solution A and B).
7. Visualize results in dark room for chemiluminescent detection.

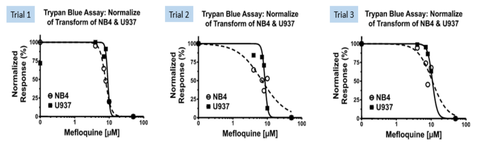
RESULTS

MTT Dose Response Results (48h)



Cell Line	MTT Mean IC50 (48h)
NB4	6.9 µM (~ 7 µM)
U937	8.2 µM (~ 8 µM)

Trypan Blue Results (48h)



Cell Line	Trypan Blue Mean IC50 (48h)
NB4	7 µM
U937	9.5 µM

Statistical Comparisons:

NB4: MTT vs. Trypan Blue IC50 Values (48h)			
Two-tailed p-value	**t-test value**	**Standard Deviation (SD)**	**Standard Error of Mean (SEM)**
P = 0.40	t = 1.1 (df = 2)	MTT: 1.4 Trypan Blue: 2.0	MTT: 0.82 Trypan Blue: 1.2

U937: MTT vs. Trypan Blue IC50 Values (48h)			
Two-tailed p-value	**t-test value**	**Standard Deviation (SD)**	**Standard Error of Mean (SEM)**
P = 0.33	t = 1.3 (df = 2)	MTT: 0.8 Trypan Blue: 1.2	MTT: 0.5 Trypan Blue: 0.7

Fig. 2 - Standard deviations (SD) and standard error of means (SEM) displayed in the table served as separate comparisons between the MTT and trypan blue assay trials from the graphs. The two-tailed p-value in paired t-tests were then computed to see the statistical significance between MTT and trypan blue; p-values were less than 0.05 for both NB4 and U937.

RESULTS (cont.)

Western Blot Results

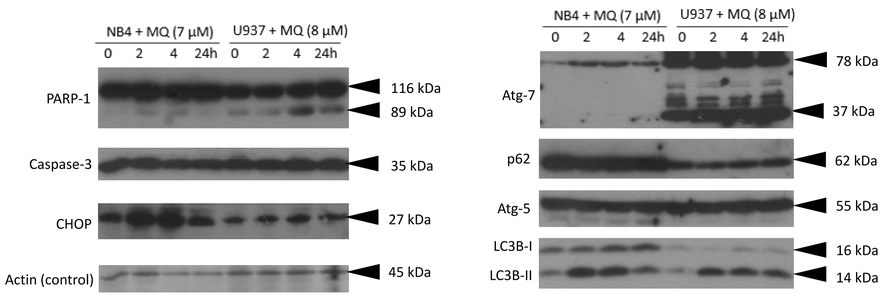


Fig. 3 - Apoptosis Markers: PARP-1, Caspase-3, CHOP; **Autophagy Markers**: Atg-7, Atg-5, p62, LC3B

DISCUSSION/CONCLUSIONS

We found that AML cells, NB4 and U937, had mean IC50 values respectively at 7µM and 8µM mefloquine (MQ) treatment through verification in MTT and trypan blue assays. The MTT assay uses tetrazolium dye reduction to measure metabolism as a marker for cell proliferation. Cells with increasing MQ concentration had the least metabolism by losing their ability to convert the MTT into formazan and revealing less color as quantified by absorbance. The trypan blue assay was used to evaluate anti-proliferative effects based on cell viability. The mean IC50 values between the MTT and trypan blue assays were statistically insignificant because $p > 0.05$ for both cell lines (Fig. 2). Having little difference in procedural methods, 7µM for NB4 and 8µM for U937 were used for future experiments. Western blotting was performed to detect protein expression using apoptosis and autophagy markers. These cells were treated at 0, 2, 4, and 24 hours of MQ treatment to investigate molecular processes before 48 hours.

Apoptosis Markers

The p53 tumor suppressor gene induces apoptosis by protease enzymes in the Caspase family. Cleaved Caspase-3 is needed in order to activate the proteolytic cleavage of PARP for the induction of apoptosis [4]. Since there was no Caspase-3 cleavage in the results, there was no induction of apoptosis for NB4 and U937 with MQ. PARP-1 repairs single-stranded DNA (ssDNA) breaks, helping to maintain cell viability. Unlike Caspase-3, there was an indication of PARP-1 cleavage, which allows for the likelihood of autophagy inhibition instead if cathepsins proteases are involved in autophagosomes [4]. Elevated levels of CHOP initiate apoptosis through ER stress. Since CHOP levels were inconsistent in the results, apoptosis may not have occurred. (Fig. 3)

Autophagy Markers

Atg-5 and Atg-7 are important molecules for the induction of autophagy through the formation of autophagosomes [1]. Although only Atg-5 was cleaved, it served as an indicator of apoptosis. A different pathway may be involved since there was no cleavage of Atg-7. Due to the inclusion of p62 in signal transduction pathways and degradation of ubiquitinated proteins, having no p62 cleavage in the cell lines implies that autophagy was not inhibited in this pathway [4]. LC3, a marker of autophagosomes, is the main protein in the autophagy pathway. A higher protein expression in LC3B-II means increased chances for autophagy [4], so based on the results, LC3 lipidation is not a contributor to autophagy inhibition. (Fig. 3)

CONCLUSIONS

- Although IC50 values were obtained for NB4 and U937 cells, there was little evidence that mefloquine (MQ) inhibited autophagy or induced apoptosis. A distinct DMSO control for Western blots, longer time points, IC90 trials, other cell lines, and different assays such as flow cytometry will have to be included to evaluate the drug repurposing of mefloquine. Additional anticancer drugs will also be tested. We hope that our results will eventually provide the rationale to continue with clinical trials.

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