Proceedings of the 4th International Conference on Biological Engineering and Medical Science
DOI: 10.54254/2753-8818/40/20241228

A novel approach to breast cancer – Assessing the promise of RF16662 on MCF-7 breast cancer cells

Yancheng Lu

School of Life Sciences, University of Liverpool, Liverpool, L69 3BX, United Kingdom

luyanchengzs@gmail.com

Abstract. Breast cancer, a prevalent global health issue, often poses challenges due to resistance apoptosis. This study explores the potential of RF16662, a derivative of naphthyridine, as a novel compound for breast cancer treatment. Naphthyridine compounds, known for inducing necroptosis in cancer cells, offer a unique avenue for addressing apoptosis-resistant cancers. RF16662's efficacy was evaluated through three experiments on MCF-7 breast cancer cells. The MTT cell viability assay demonstrated a strong negative correlation between RF16662 concentration and cell viability, indicating its effectiveness in inhibiting MCF-7 cellular activity. DNA fragmentation analysis revealed substantial DNA fragmentation in cells treated with RF16662, suggesting necroptosis induction. MLKL immunofluorescence confirmed the significant difference in relative MLKL expression between untreated and treated cells, further supporting the induction of necroptosis. This study provides robust evidence of RF16662's efficacy against MCF-7 breast cancer cells. However, variations in MLKL immunofluorescence data suggest the need for repeated experiments to minimize the standard deviation. The results highlight RF16662's potential as an anti-breast cancer drug, offering a promising avenue for future research and potential therapeutic development. While its efficacy against other cancer types remains unknown, RF16662 emerges as a noteworthy candidate in the ongoing quest for innovative breast cancer treatments.

Keywords: Necroptosis, Naphthyridine, Breast Cancer

1. Introduction

Breast cancer is one of the most common types of cancer in the world. According to the WHO, in 2020 alone, 2.3 million women received diagnosis of breast cancer, and the disease claimed the lives of 685,000 individuals worldwide [1]. Despite these shocking statistics, there is even more despairing discovery – some breast cancer cells are resistant to apoptosis (programmed cell death), rendering effective efficiencies such as Tamoxifen no longer potent [2, 3]. Against this ominous backdrop, the exploration of novel compounds has become imperative, bringing the focus to compounds like RF16662 as potential drug to treat breast cancer.

RF16662 is a derivative of naphthyridine which is a class of heterocyclic compounds characterized by a distinctive nitrogen arrangement within a naphthalene ring [4]. Such compounds often possess the property of causing necroptosis, which has different pathways to apoptosis, in cancer cells [5]. Necroptosis is another form of programmed cell death that shares characteristics with both apoptosis

DOI: 10.54254/2753-8818/40/20241228

and necrosis (unregulated cell death) and it is a regulated process that occurs under specific conditions and involves a controlled mechanism for cells to die [6].

In this essay, RF16662's potential in breast cancer treatment is assessed via three different experiments on MCF-7 cells, which were obtained from breast cancer tissues. The first test was MTT cell viability assay which tested how concentration can affect cell viability. The second test was DNA fragmentation analysis which confirmed if RF16662 triggered necroptosis instead of other forms of cell death. The third test was MLKL (Mixed Lineage Kinase Domain-Like Protein) immunofluorescence, which was done to test the relative expression level of the gene that produces MLKL protein. This experiment is relevant because MLKL is responsible for necroptosis. Test for expression level may further support the result from DNA fragmentation analysis by comparing quantified data between the test subjects and control group.

2. Methods

2.1. MTT Cell Viability Assay

This tested how concentration can affect cell viability.

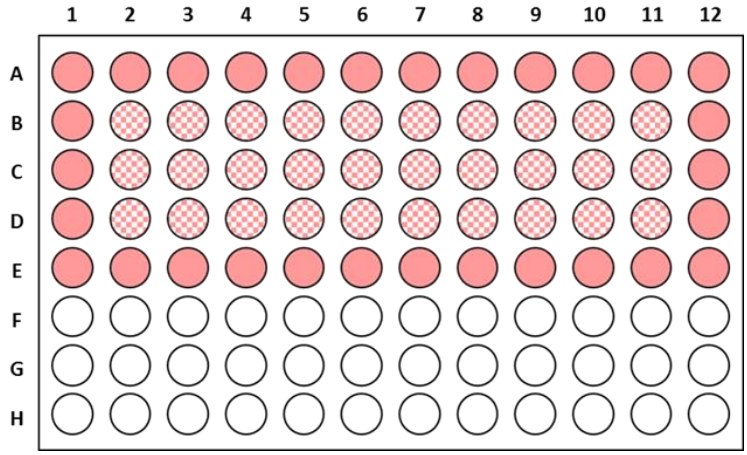

Figure 1. The arrangements of the wells

A 96 well plate was loaded with MCF-7 cells with 10000 cells per well as figure 1. Only cell culture media was added to solid wells, and the cell suspension was added to the checked wells. A series of 2-fold dilutions of 4mM stock RF16662 solution was performed by adding sterile PBS 8 times.

Sterile PBS was added to well 2B-2D; RF16662 solution of each concentration during the serial dilution was added to the test wells (rows B, C, D, columns 2-11). The plate was incubated at 37 ºC for 1 hour. Ten μL of MTT labeling reagent was added to the test wells. The plate was incubated at 37 ºC for another 3 hours. One hundred μL of 10% SDS was added to the test wells. Then the plate was incubated overnight at 37 ºC. The optic density of the test wells under 550 nm light was recorded.

2.2. DNA Fragmentation Analysis

A solution of 1% (w/v) agarose power dissolved in 1× TAE buffer is made. Ten μL of SYBR safe is added to 100 mL of the agarose solution. The solution was heated to 100 ºC for 2 minutes. 50 mL of the solution was poured into the casting tray. It was allowed to set for 30 minutes. In this way, the gel was made.

Two μL of gel loading buffer (#B7025) was added to each of three DNA samples which were cells incubated in PBS for 6 hours, cells incubated in IC_{50} of RF16662 for 6 hours and cells incubated in IC_{75} of RF16662 for 6 hours. The mixture of buffer and DNA samples is then vortexed.

DOI: 10.54254/2753-8818/40/20241228

One kb ladder (#N3232 New England Biolabs) and three DNA samples were loaded from left to right in order. The voltage of 100 V was applied to run the gel electrophoresis for 90 minutes. The result was photographed under UV transilluminator.

2.3. MLKL Immunofluorescence

MCF-7 cells were firstly seeded in chamber slides in the concentration of 4×10^4 cells per well and then incubated for 24 hours at 37°C for 24 hours. After incubation, growth media (Dulbecco's Modified Eagle Medium with 10% v/v fetal calf serum) was removed and the cells were exposed to IC_{50} of RF16662 diluted in growth media for 6 hours at 37°C. Media containing the IC_{50} drug were removed by washing in PBS for 5 minutes and repeating the wash 2 more times. To stain the slides, protein blocking buffer was added and allowed to set for 1 hour (1% w/v BSA) at room temperature. Anti-MLKL was diluted in blocking buffer 1:100, added and allowed to set for 1 hour at room temperature. The slides were washed in PBS for 5 minutes and repeat the washing for another 2 times. Finally, prolong gold was mounted in.

3. Results

3.1. MTT Cell Viability Assay

Note that the RF16662 solution is added to the 50 μL of MCF-7 cell solution, the final concentration of the drug is the half of the concentration of the drug before being added to cell solution.

Figure 2 presents the raw data. Because the raw data is obtained via different groups of researchers, it must be normalised to bring reasonable comparisons of cellular responses at different concentrations. To normalize the data, we need to plot % cell viability against RF16662 concentration. % Cell viability is the percentage of cells survived (in proportion to optic density) in a certain concentration of RF16662 in relation to those that survived at a concentration of RF16662 is 0.

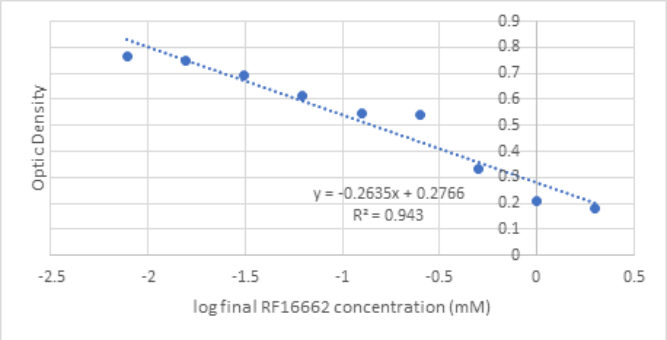

Figure 2. The relationship between RF16662 (on a log scale) and optic density

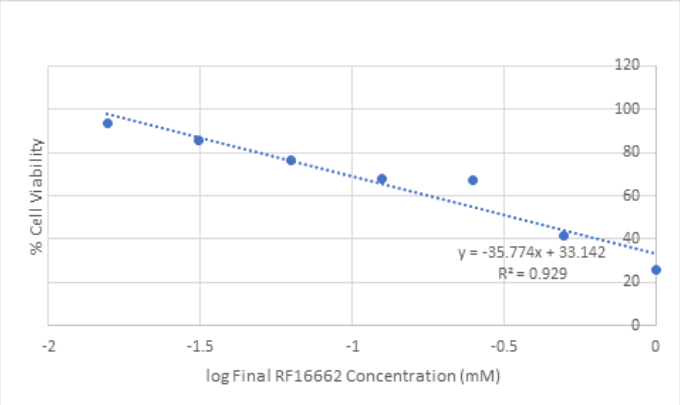

Figure 3. The relationship between RF16662 concentration (on a log scale) and % cell viability.

DOI: 10.54254/2753-8818/40/20241228

IC_{50} is the concentration of RF16662 where % cell viability is 50.
So, when y=50, x=-0.471
Because RF16662 concentration is on a log scale, -0.170 needs to be anti-logged.
$10^{-0.471}$=0.338
Therefore, IC_{50} is 0.338 mM.

3.2. DNA Fragmentation Analysis

There are smears shown in Lane 3 and Lane 4 in figure 4, and the start of the smear is lower than the end of the cluster in lane 2. This suggests that after being incubated in IC_{50} and IC_{75} of RF16662, DNA of MCF-7 cells was fragmented, which indicates necroptosis of cells.

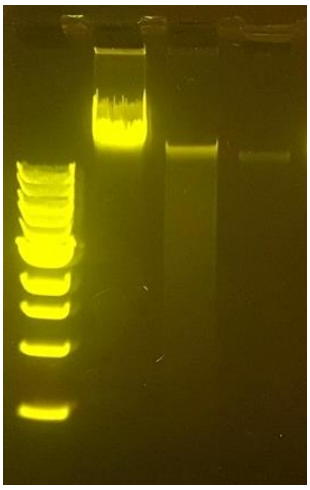

Figure 4. Gel electrophoresis results the lanes from left to right are 1kb ladder, control untreated cells, cells treated with RF16662 at IC_{50}, cells treated with RF16662 at IC_{75}.

3.3. MLKL Immunofluorescence

To test the significance of the difference between the relative MLKL expression between, cells treated with RF16662 and untreated cells, Shapiro-Wilk tests are carried out for both data sets first to test if they are normally distributed. Data from untreated cells are tested first.

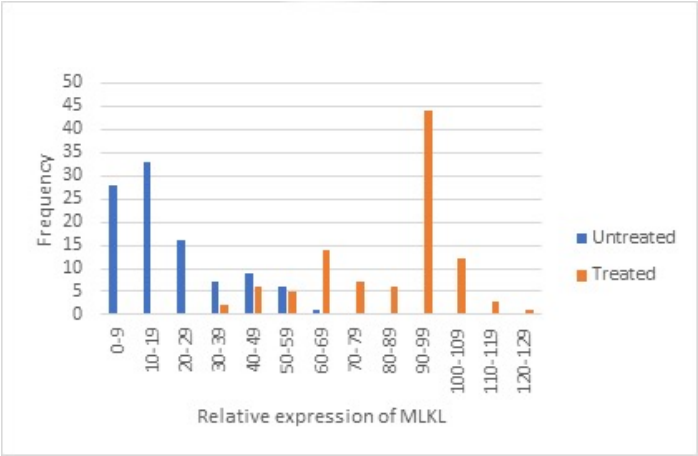

Figure 5. The bin size (frequency) of data falls into different ranges.

H_0 - Relative MLKL expression of untreated cells is normally distributed.
p-value = 0.00125
p-value < 0.05, H_0 is rejected, data from untreated cells is not normally distributed.

DOI: 10.54254/2753-8818/40/20241228

Data from treated cells are then tested.

H_0 - Relative MLKL expression of treated cells is normally distributed.

p-value = 0.000160

p-value < 0.05, H0 is rejected, relative MLKL expression of treated cells is not normally distributed.

Since both sets of data are not normally distributed, Mann-Whitney U test is used to test the significance of difference.

H_0 – The difference between relative MLKL expression of untreated cells and relative MLKL expression of treated cells is not significant.

p-value < 2.2×10^{-16} (The exact p-value was too small to be given by R studio, thus, only a range is given here).

p-value < 0.05, H_0 is rejected, the difference between relative MLKL expression of untreated cells and relative MLKL expression of treated cells is significant. Cells treated with RF16662 have higher levels of MLKL than the untreated cells.

4. Discussion

In figure 3, the correlation coefficient is -0.964 and the R^2 value is 0.929, which indicates very strong negative correlation between RF16662 and % cell viability. This means RF166662 can effectively inhibit cellular activity of MCF-7. In addition, the sample size is 98, which makes the results more convincing.

Figure 4 shows a cluster of bands larger than 1000 kb in lane 2 (ordered from left to right) and smear in Lane 3 and Lane 4. And the start of the smear is lower than the end of the cluster in lane 2. This suggests that DNA of MCF-7 cells was fragmented after being incubated in IC_{50} and IC_{75} of RF16662 and fragmentation suggests the cells underwent necroptosis. The smear in lane 4 is fainter than the one in lane 3 because in higher concentration of RF16662, more cells underwent necroptosis and DNAs were more fragmentised.

In figure 5, the sample size is 100 for each group, which makes the results reliable. The standard deviation for frequency of relative expression of MLKL of untreated cells is 11.3, for treated cells, it is 27.1 which is not ideal. However, the result from Mann-Whitney U test still proves the significance of the difference of MLKL expression between two sets of data.

5. Conclusion

In conclusion, the results are in general reliable, but MLKL immunofluorescent test may be repeated more often to minimize standard deviation. These experiments prove the effectiveness of RF16662 against MCF-7 cancer cell. Yet, its effectiveness against other types of cancer cells is unknown. RF16662 may be used to test against other types of cancer. RF16662 may play an important role in anti-breast cancer drug in the future.

References

[1] World Health Organization (2023). Breast cancer. [online] www.who.int. Available at: https://www.who.int/news-room/fact-sheets/detail/breast-cancer. (Accessed 04/Dec/2023)

[2] Mohammad R., Muqbil I., Lowe L., Yedjou C., Hsu H., Lin L. et al. (2015). Broad targeting of resistance to apoptosis in cancer, Seminars in Cancer Biology, 35(0), 78–103

[3] Mandlekar S., Kong A. (2001). Mechanisms of tamoxifen-induced apoptosis, Apoptosis, 6(6), 469-477

[4] Chabowska G., Barg E.,Wójcicka A. (2021). Biological Activity of Naturally Derived Naphthyridines. Molecules, 26(14), 4324

[5] Kong D., Lv J., Yan S., Chang K., Wang G. (2018). A Novel Naphthyridine Derivative, 3u, Induces Necroptosis at Low Concentrations and Apoptosis at High Concentrations in Human Melanoma A375 Cell. International Journal of Molecular Sciences, 19(10), 2975

[6] Yan J., Wan P., Choksi S., Liu Z. (2022). Necroptosis and Tumor Progression. Cell Press: Trends in Cancer, 8(1), 21–27