Anti-tumor Efficacy and Safety of the BAG3/HSP70 Inhibitor YM-1 in a Triple Negative Breast Cancer Model

David Rui-jing Guo

Abstract

Patients who treat with TNBC are usually with intense chemotherapy. Recently, BAG3 has been suggested as a potential therapeutic target for cancer. However, inhibiting BAG3 for therapy may result in unintended damage to cardiomyocytes. Thus, this research proposal will test whether or not the inhibition of BAG3 will significantly affect the heart while still having potent anti-tumor efficacy. Human TNBC cell line BT-549 cells will be inoculated into CB-17/SCID mice. When the tumor grows to 100 mm3, mice will be intraperitoneally injected with different doses of YM-1, an inhibitor of BAG3/HSP70. The clinically used TNBC chemotherapy drug doxorubicin will be used as the positive control. The tumor growth, expressed as the change in tumor volume and tumor growth inhibition percentage (TGI), will be measured. Troponin I level in the serum, ECG measurement, and Hematoxylin and Eosin staining will be used to assess heart damage. The results of our study will indicate whether the animals will tolerate the BAG3 inhibitor, YM-1, at its effective doses and whether inhibition of BAG3 may be a better option, particularly regarding unwanted effects on cardiomyopathy, compared to standard of care in TNBC. Future investigations are warranted.

Keywords: BAG3 inhibition, BAG3, YM-1, TNBC, doxorubicin, HSP:70

1. Introduction

TNBC, or triple-negative breast cancer, is a tough form of cancer to treat because of its lack of estrogen(ER) and progesterone receptors(PR) and its minute production of HER2 [1]. It is most effectively treated with chemotherapy, an aggressive and exhaustive form of treatment [2]. Thus, better and more effective therapeutic methods are highly demanded of TNBC.

Previous studies have identified Bcl-2-associated athanogene 3(BAG3) protein as a potential therapeutic target because it has shown tumor-promoting effects [3]. BAG3, a 575 amino acid-long co-chaperone protein, has many functions because of its multiple binding domains [4]. It can bind to heat shock protein 70(HSP:70) via its BAG domain and to other proteins via its proline-rich repeat, WW domain, and IPV motif [5]. Its interaction with HSP:70 is significant because BAG3's involvement can regulate proteostasis and major signaling pathways [6]. BAG3 has many means of aiding cancer growth. For example, BAG3 can enhance cancer cell proliferation through interactions with the FAK and AKT signaling pathways [3]. BAG3 can prevent apoptosis by binding to YAP/TAZ factor inhibitors so that YAP/TAZ factors can prevent apoptosis [5]. The overall result of BAG3 exposure is increased cancer cell proliferation and metastasis and decreased apoptosis [7]. Even though BAG3 is commonly expressed throughout the body, its expression is upregulated from stressful stimuli. As a result, it is found that much higher expression levels in cardiomyocytes, skeletal muscle, the central nervous system, and cancer cells [5]. In the heart, BAG3 lowers apoptosis, promotes autophagy, binds β-adrenergic receptor with L-type Ca2+ channel, and stabilizes the structure of the sarcomeres in cardiomyocytes [5]. Thus, the inhibition of BAG3 in the pursuit of decreasing TNBC cell proliferation might unintentionally kill heart cells. Fortunately, a study has hypothesized that the inhibition of BAG3 would not significantly affect the heart because of "inherent redundancy" in specific pathways that compensate for BAG3's functions [5].

Doxorubicin is a proven clinical chemotherapy drug that is the most frequently used treatment for TNBC [8]. However, it is very cardiotoxic, so its cardiotoxicity has limited its use [9]. The mechanism of action by which doxorubicin can affect the heart is through the enhancement of free radicle formation. This will increase oxidant stress in cardiomyocyte, which is already oxidant-sensitive, and leads to cardiomyocyte damage and thus instigate inflammation [10]. The reactive oxygen species produced by doxorubicin metabolism may cause cardiomyocyte cell death apoptosis [10]. Patients treated with doxorubicin can develop cardiomyopathy, acute ventricular dysfunction, arrhythmia, and congestive heart failure [9,11].

If the cardiac-related BAG3 pathways have inherent redundancies, then a BAG3 inhibitor should have less effect on the heart than doxorubicin. Thus, this discussion proposes a study that tests whether or not a BAG3

inhibitor would have less of an effect on the heart than doxorubicin while still killing as many TNBC cells. The inhibitor in question is YM-1, which prevents BAG3 from binding to Heat shock protein 70(HSP:70) by binding to HSP:70 before BAG3 can [12]. In this sense, YM-1 is technically an HSP70 inhibitor and a BAG3-HSP70 interaction inhibitor, not a BAG3 inhibitor. However, it is the closest thing to a BAG3 inhibitor that currently exists. The inhibition of BAG3 and HSP:70 interaction has decreased cancer cell proliferation and viability and is also hypothesized to prevent metastasis partially [3,13]. However, these interactions in the heart are crucial in that they stabilize the myofibril structure. Regulate the contractibility of the heart, and promote chaperone-assisted selective autophagy (which maintains cardiomyocytes and the lack thereof could cause cardiomyopathy) [5].

In this proposal, mice xenografted with TNBC will receive different doses of YM-1, and doxorubicin will be used as the positive control. The first phase of this proposal is to find a YM-1 dosage with the same tumor growth inhibition efficacy as doxorubicin. TNBC will be done by testing an extensive range of YM-1 dosages on mice and measuring the tumor growth rate associated with each dose of YM-1. The dose of YM-1 that has the most similar tumor growth inhibition to that of doxorubicin will be used to compare the side effects caused by doxorubicin and YM-1 on the heart. The level of Troponin I in the bloodstream, abnormalities in the ECG, and inflammation scoring of heart tissue samples will evaluate the two inhibitor's effect on the heart. All of these measures will be compared to mice treated with doxorubicin, an already proven clinical drug for TNBC, and mice treated with a sham injection of PBS. The research proposed does not include any in vitro experiments because previous studies have already proven YM-1 as an inhibitor of BAG3-HSP:70 interaction [12]. The inhibition of this protein interaction has resulted in the killing of TNBC cells [3].

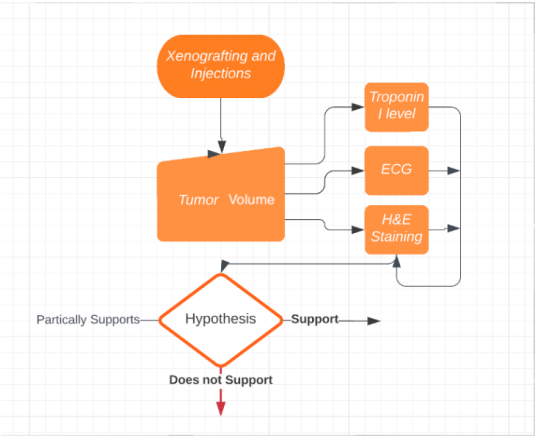

Figure 1: Methodology basic frame work

1.1. *Hypothesis*

Studies suggest that BAG3 pathways have "inherent redundancies" that compensate for BAG3's function [5]. It hypothesized that the inhibition of BAG3 will have fewer effects on the heart than doxorubicin while still killing as many TNBC cells.

1.2. *Materials:*

This experiment will use 90 adult CB-17/SCID mice from Charles River Laboratories and 1x106 BT-549 cells(for each mice) from ThermoFisher Scientific, YM-1 from Millipore Sigma, doxorubicin from Blink Health, PBS from Millipore Sigma, a caliper from WEN, 3 Mice Troponin I ELISA Kit from Abcam, an ECG machine from this study [14], and 3 Hematoxylin and Eosin stain kit from Vector Laboratories.

2. Methods

2.1. *Animal model*

CB-17/SCID female mice (4-6 weeks old) will be obtained from Charles River Laboratories. This type of mice is immunodeficient allowing BT-549, a human TNBC cell line, to be xenografted into the mice bodies without the risk of rejection. Mice will be allowed to have a period of adaptation in a sterile environment supplied with food and water. BT-549 cells will be harvested and inoculated into mice by bilateral injection into the inguinal mammary fat pad of mice. Afterwards, mice will be randomized into different treatment group and receive different dosages of YM-1 inhibitors once the tumor size reaches 100mm^3 (Table 1). Herein, 90 mice will be split into groups of 10(for nine groups), which are (1) negative control group receiving PBS injections; (2) positive control receiving doxorubicin injection at 5mg/kg; (3) group receiving YM-1 injection at 1mg/kg; (4) group receiving YM-1 injection 5mg/kg; (5) group receiving YM-1 injection at 10mg/kg; (6) group receiving YM-1 injection at 20mg/kg; (7) group receiving YM-1 injection at 40mg/kg; (8) group receiving YM-1 injection at 80mg/kg; and (9) group receiving YM-1 injection at 100mg/kg. For detailed information of each group refer to Table 1. A past study used a dosage of 25mg/kg in mice models and it effectively killed TNBC cells without causing any cardiac effects [15]. Thus, the 1-100mg/kg range is appropriate for the study. A previous study used 5mg/kg doxorubicin on mice models which was enough to show cardiac effects but not enough to kill them [16]. Thus, this dosage of doxorubicin is appropriate for this study. Figure 1 shows the whole studies' basic framework.

Table 1. Information on the injections of YM-1, doxorubicin, and PBS in mice

Group	Tumor cell Line	Drug and Dosages	Number of animals per group	Methods of injection	Frequency of injections	Total injections
1	BT-549	Phosphate Buffered Solution (Negative control)	10	*i.p.*	Every three days	5 times
2	BT-549	YM-1【1 mg/kg】	10	*i.p.*	Every three days	5 times
3	BT-549	YM-1【5mg/kg】	10	*i.p.*	Every three days	5 times
4	BT-549	YM-1【10mg/kg】	10	*i.p.*	Every three days	5 times
5	BT-549	YM-1【20 mg/kg】	10	*i.p.*	Every three days	5 times
6	BT-549	YM-1【40 mg/kg】	10	*i.p.*	Every three days	5 times
7	BT-549	YM-1【80 mg/kg】	10	*i.p.*	Every three days	5 times
8	BT-549	YM-1【100 mg/kg】	10	*i.p.*	Every three days	5 times
9	BT-549	Doxorubicin【15mg/kg】	10	*i.p.*	Every three days	5 times

For this study, the drug will be administered every three days for five injections (15 days). The tumor volume will be measured, and the blood sample and ECG will be taken for the individual mice every three days until the termination of this experiment. The experiment will be terminated three weeks after the first injection (18 days). The mice will be euthanized by exposure to an atmosphere of 100 percent CO2, immediately rendering them dead. The hearts of the mice will be collected for analysis.

2.2. *Tumor growth measurements*

To measure the tumor volume, the width and length of the tumor will be measured and recorded. The tumor volumes calculated by the formula:

$$V=W^2\times\frac{L}{2} \quad (1)$$

V=Volume(mm^3)
W=Width(mm)
L=Length(mm)

The tumor volume of each mice will be averaged within their group. Afterward, the graph with the X-axis as the number of days and the Y-axis as the average tumor volume in mm3 will be plotted. For each group, a line of best fit between their average tumor volume will be created, and the tumor inhibition rate will be calculated. The tumor growth rate for the mice treated with various dosages of YM-1 will be compared to that of the mice treated with the positive/negative control.

2.3. *Troponin I measurement*

Blood samples of mice will be collected one hour after drug administration and analyzed for Troponin I level, an indicator of myocyte damage. The collected blood samples will be processed through a Troponin I ELISA testing kit (Abcam) and the levels of Troponin I will be recorded. The Troponin I level of each mice will be averaged within their group.

2.4. *Electrocardiogram (ECG)*

An ECG (Iocare) will be conducted on each mice about one hour after drug administration. Previous research has developed methods for noninvasive ECG recordings for mice [14]. The protocol of the method will be followed s to take the mice's ECGs. The data from the ECGs will be collected and analyzed. Specifically, the ECGs will be scored based on a simplified ECG Scoring system developed in this study [17]. A lower score indicates normal heart function, while a higher score indicates abnormal heart function and a higher risk of cardiovascular (CV) mortality. Each mice's ECG will be scored and then averaged within its group.

2.5. *H&E Staining*

At necropsy, hearts will be collected. Five cross-sectional samples from evenly distributed areas of the heart will be analyzed using the Hematoxylin and Eosin stain kit from Vector Laboratories. Stained samples will be sent to a histopathology core, where a pathologist will score the inflammation levels of each tissue piece. Average the inflammation levels within each group.

3. Statistical analysis

Of the groups treated with dosages of YM-1, pick the group with the most similar tumor growth rate to those treated with doxorubicin. Then compare their average Troponin I level, average simplified ECG score, and average inflammation level to that of the group treated with doxorubicin. A T-test will be conducted between these two groups on each measure. If the difference

between the results of YM-1 and doxorubicin is not statistically significant, then their results are considered similar. For YM-1's measures to be considered lower or higher than doxorubicin, the difference must be statistically significant (P-value less than 0.05).

3.1. *Possible results:*

Please see Table 2 for more information

Table 2. Possible results

Measurement	Possible Result 1	Possible Result 2	Possible Result 3
Simplified ECG score	+	-	=
Measurement	Possible Result 4	Possible Result 5	Possible Result 6
Troponin I level	+	-	=
Measurement	Possible Result 7	Possible Result 8	Possible Result 9
Inflammation Grade	+	-	=

Note. "+" represents that YM-1 is safer compared to doxorubicin. "=" represent that YM-1 and doxorubicin are equally safe. "-" represents that YM-1 is more toxic than doxorubicin.

Table 3. Combinations of Possible Results

Measurements	Result 1	Result 2	Result 3	Result 4	Result 5	Result 6
Troponin I level	+	+	+	-	-	-
Simplified ECG score	+	+	-	+	-	+
Inflammation grade	+	-	+	+	+	-
Measurements	Result 7	Result 8	Result 9	Result 10	Result 11	Result 12
Troponin I level	+	-	+	+	=	=
Simplified ECG score	-	-	+	=	+	=
Inflammation grade	-	-	=	+	+	+
Measurements	Result 13	Result 14	Result 15	Result 16	Result 17	Result 18
Troponin I level	=	+	=	=	-	+
Simplified ECG score	+	=	=	-	+	=
Inflammation grade	=	=	=	+	=	-
Measurements	Result 19	Result 20	Result 21	Result 22	Result 23	Result 24
Troponin I level	-	+	=	-	=	-
Simplified ECG score	=	-	+	-	-	=
Inflammation grade	+	=	-	=	-	-
Measurements	Result 25	Result 26	Result 27			
Troponin I level	=	-	=			
Simplified ECG score	-	=	=			
Inflammation grade	=	=	-			

Note. "+" represents that YM-1 is safer compared to doxorubicin. "=" represent that YM-1 and doxorubicin are equally safe. "-" represents that YM-1 is more toxic than doxorubicin.

3.2. Abnormalities in the Cardiac Cycle.

Possible result 1: The average simplified ECG score of the mice that were treated with YM-1 was lower than that of mice treated with the positive control.
Possible result 2: The average simplified ECG score of the mice that were treated with YM-1 was higher than that of mice treated with the positive control.
Possible result 3: The average simplified ECG score of the mice that were treated with YM-1 is similar to that of the mice treated with the positive control.
Levels of Troponin I in The Blood
Possible result 4: The mice treated with YM-1 has a lower average Troponin I level compared to those that were treated with the positive control.
Possible result 5: The mice treated with YM-1 has a higher average Troponin I level compared to those that were treated with the positive control.
Possible result 6: The mice treated with YM-1 has the same average Troponin I level to those that were treated with the positive control.
H&E Staining:
Possible result 7: The average inflammation score of the heart tissues that have been exposed to YM-1 is lower than that of the positive control group.
Possible result 8: The average inflammation score of the heart tissues that have been exposed to YM-1 is higher than that of the positive control group.
Possible result 9: The average inflammation score of the heart tissues that have been exposed to YM-1 is similar to that of the positive control group.

4. Discussion

Triple-negative breast cancer (TNBC) is defined by the lack of expression of ER, PR, and HER2 [1]. This proposal aims to evaluate the potential of YM-1, a BAG3 and HSP70 inhibitor, as a possible therapeutic for treating TNBC.
An ECG displays the electrical activity and rhythm of an organism's cardiac cycle. It reflects the functioning of the heart. As shown in Table 2, possible result 1 indicates that YM-1 has a minor effect on cardiac function and will put patients at less risk of CV mortality than doxorubicin. Thus, YM-1 has less effect on the heart than doxorubicin while killing as many TNBC cells. This result will support the hypothesis. Possible result 2 indicates that YM-1 has a more significant effect on cardiac function and will put patients at more risk of CV mortality than doxorubicin. Thus, YM-1 has a larger effect on the heart than doxorubicin while killing the same amount of TNBC cells. This result will not support the hypothesis. Possible result 3 indicates that YM-1 has the same effect on cardiac function and has the same risk of CV mortality as doxorubicin. Thus, YM-1 and doxorubicin have the same effect on the heart while killing the same amount of TNBC cells. This result will not support the hypothesis.
When a cardiomyocyte dies, Troponin I, a protein that binds to actin, is released into the blood. The level of Troponin I positively correlates with cardiomyocyte cell damage. Possible result 4 indicates that YM-1 will result in less cardiomyocyte damage than doxorubicin. Thus, YM-1 has less effect on the heart than doxorubicin while killing as many TNBC cells. This result will support the hypothesis of this study. Possible result 5 indicates that YM-1 will result in more cardiomyocyte damage compared to doxorubicin. Thus, YM-1 has a more significant effect on the heart than doxorubicin while killing the same amount of TNBC cells. This result will go against the hypothesis of this study. Possible result 6 indicates that YM-1 will result in a similar amount of cardiomyocyte damage as doxorubicin. Thus, YM-1 and doxorubicin have the same effect on the heart while killing the same amount of TNBC cells. This result will not support the hypothesis of this study.
Inflammation is an immunological response that occurs when foreign substances have been recognized in the body and when cells are damaged. Possible result 7 indicates that YM-1 will result in less inflammation than doxorubicin. Thus, YM-1 has less effect on the heart than doxorubicin while killing as many TNBC cells. This result supports my hypothesis. Possible result 8 indicates that YM-1 will result in more inflammation than doxorubicin. Thus, YM-1 has a more significant effect on the heart than doxorubicin while killing the same amount of TNBC cells. This result does not support the hypothesis. Possible result 9 indicates that YM-1 and doxorubicin will result in the same amount of inflammation. Thus, YM-1 and doxorubicin have the same effect on the heart while killing the same amount of TNBC cells. This result does not support my hypothesis.
The possible results discussed above only evaluated each measurement (simplified ECG score, Troponin I level, and Inflammation grade) independently. However, now they will be evaluated and discussed collectively. As shown in Table 3, 27 possible combinations can be drawn from the possible results stated above. The result in 1 of the possible combinations is the only one supporting the hypothesis. All measures of YM-1 cardiac effect are lower than that of doxorubicin. Results 9, 10, and 11 are close to supporting the hypothesis; however, since one of YM-1's measures is not less than that of doxorubicin, these combinations only partially support the hypothesis. Results 2, 3, 4, 5, 6, 7, 13, 14, 16, 17, 18, 19, 20, and 21 of the possible combinations also partially support the

hypothesis because they all have at least one measure of YM-1 that is less than that of doxorubicin. Result from 8, 15, 22, 23, 24, 25, 26, and 27 do not support the hypothesis because none of their YM-1 measures are less than that of doxorubicin.

Based on the possible results described above, three statements can be drawn. YM-1's overall effect on the heart is less than doxorubicin; YM-1's overall effect on the heart is similar to doxorubicin; and YM-1's overall effect on the heart is greater than doxorubicin. If YM-1's overall effect on the heart turns out to be less than doxorubicin, YM-1 may be able to replace doxorubicin in certain clinical situations. However, YM-1's effect on the other systems in the body must also be investigated to confirm its safety. Suppose YM-1's overall effect on the heart is similar to that of doxorubicin. In that case, it still has potential clinical value because it may have less effect on the rest of the body than doxorubicin. Doxorubicin has many other adverse side effects which may prevent certain patients from being able to use it. For example, people with liver disease("Doxorubicin") are not recommended to use doxorubicin because of how the compound alters liver function. If YM-1 proves to have less of an effect on other systems in the body, YM-1 may be able to fill these niches left by doxorubicin. As long as YM-1 does not severely affect the heart, this inhibitor might be helpful for patients who cannot handle the other side effects of doxorubicin. Thus, YM-1 continues to hold potential therapeutic value, in all cases, YM-1's effects on the rest of the body should be explored.

However, suppose YM-1's overall effects on the heart are worse than that of doxorubicin. In that case, it is safe to assume YM-1 may be too toxic to use because doxorubicin is already very cardiotoxic. In the circumstance that YM-1 does severely affect the heart in any of the measurements, a pH-dependent BAG3 inhibitor may be explored. The tumor microenvironment (TME) is more acidic (6.4-7.0) than the rest of the body (7.4). Thus the pH-dependent BAG3 inhibitor can exclusively target cancer cells in TME. For example, a mask can bind to a YM-1s binding site at a pH of 7.4, but once the pH drops to 7.0, the mask will fall off.

5. Conclusion

This study will test whether or not YM-1 will affect the heart more than doxorubicin in an animal model. The results will indicate whether YM-1 would be safer for the heart than doxorubicin. Nevertheless, in a larger sense, this study partially reveals YM-1's therapeutic value in the current healthcare industry. For example, if this study results in YM-1 having minimal cardiac effects, YM-1 can be used for cancer patients with a history of cardiac problems. However, future studies should investigate YM-1's effects on the rest of the body, specifically areas like the brain or skeletal muscles where BAG3's function is prevalent. This study will reveal whether or not YM-1 can be employed in a clinical setting and in what cases it should use. If YM-1 is shown to be too toxic in the heart or any other part of the body, a pH-dependent form of YM-1 can be developed so that it only activates in acidic conditions like TME.

References

[1] "Treatment of Triple-Negative Breast Cancer: Treatment of TNBC." American Cancer Society, American Cancer Society, 12 Apr. 2022, https://www.cancer.org/cancer/breast-cancer/treatment/treatment-of-triple-negative.html.

[2] Isakoff, Steven J. "Triple-negative breast cancer: role of specific chemotherapy agents." Cancer journal (Sudbury, Mass.) vol. 16,1 (2010): 53-61. doi:10.1097/PPO.0b013e3181d24ff7

[3] Shields et al. BAG3 promotes tumour cell proliferation by regulating EGFR signal transduction pathways in triple negative breast cancer. Oncotarget. 2018;9(21):15673-15690

[4] Myers, Valerie D et al. "The Multifunctional Protein BAG3: A Novel Therapeutic Target in Cardiovascular Disease." JACC. Basic to translational science vol. 3,1 (2018): 122-131. doi:10.1016/j.jacbts.2017.09.009

[5] Kirk, Jonathan A., et al. "Therapeutic Targeting of BAG3: Considering Its Complexity in Cancer and Heart Disease." Journal of Clinical Investigation, vol. 131, no. 16, 16 Aug. 2021, https://doi.org/10.1172/jci149415. Accessed 1 July 2022.

[6] Meriin, Anatoli B et al. "Hsp70-Bag3 complex is a hub for proteotoxicity-induced signaling that controls protein aggregation." Proceedings of the National Academy of Sciences of the United States of America vol. 115,30 (2018): E7043-E7052. doi:10.1073/pnas.1803130115

[7] De Marco, Margot et al. "Role of BAG3 in cancer progression: A therapeutic opportunity." Seminars in cell & developmental biology vol. 78 (2018): 85-92. doi:10.1016/j.semcdb.2017.08.049

[8] Denard, Bray et al. "CREB3L1 as a potential biomarker predicting response of triple negative breast cancer to doxorubicin-based chemotherapy." BMC cancer vol. 18,1 813. 13 Aug. 2018, doi:10.1186/s12885-018-4724-8

[9] Chatterjee, Kanu et al. "Doxorubicin cardiomyopathy." Cardiology vol. 115,2 (2010): 155-62. doi:10.1159/000265166

[10] Volkova, Maria, and Raymond Russell 3rd. "Anthracycline cardiotoxicity: prevalence, pathogenesis and treatment." Current cardiology reviews vol. 7,4 (2011): 214-20. doi:10.2174/157340311799960645

[11] Mitry, Maria A, and John G Edwards. "Doxorubicin induced heart failure: Phenotype and molecular mechanisms." International journal of cardiology. Heart & vasculature vol. 10 (2016): 17-24. doi:10.1016/j.ijcha.2015.11.004

[12] Sherman, Michael Y, and Vladimir Gabai. “The role of Bag3 in cell signaling.” Journal of cellular biochemistry vol. 123,1 (2022): 43-53. doi:10.1002/jcb.30111

[13] Li, Xiaokai et al. “Validation of the Hsp70-Bag3 protein-protein interaction as a potential therapeutic target in cancer.” Molecular cancer therapeutics vol. 14,3 (2015): 642-8. doi:10.1158/1535-7163.MCT-14-0650

[14] Chu, Victor, et al. “Method for Non-Invasively Recording Electrocardiograms in Conscious Mice.” BMC Physiology, vol. 1, no. 1, 25 June 2001, pp. 1–6., https://doi.org/10.1186/1472-6793-1-6. Accessed 2 July 2022.

[15] Colvin, Teresa A et al. “Hsp70-Bag3 interactions regulate cancer-related signaling networks.” Cancer research vol. 74,17 (2014): 4731-40. doi:10.1158/0008-5472.CAN-14-0747

[16] Zhu, Wuqiang et al. “A mice model for juvenile doxorubicin-induced cardiac dysfunction.” Pediatric research vol. 64,5 (2008): 488-94. doi:10.1203/PDR.0b013e318184d732

[17] Tan, Swee Yaw, et al. “A Simplified Clinical Electrocardiogram Score for the Prediction of Cardiovascular Mortality.” Clinical Cardiology, vol. 32, no. 2, 12 Feb. 2009, pp. 82–86., https://doi.org/10.1002/clc.20288. Accessed 4 July 2022.