

# BAG3's Role in Indicating the Recurrence of TNBC

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## Abstract

Triple-negative breast cancer (TNBC) cells tend to resist chemotherapy drugs, making it essential to find other therapies to treat this aggressive cancer. The inhibition of BAG3 with YM-1 is an option because BAG3 is overexpressed in over 50% of TNBC patients. BAG3 positively regulates the EGFR pathway and cell proliferation, which means inhibiting it could reduce TNBC tumor size and lessen the recurrence of TNBC. This paper looks at xenografting BT-549 cell lines in mice and treating them with docetaxel, PBS, and YM-1 at various concentrations. Changes in the tumor are tracked using the tumor volume and weight of the mice. The results of this work will provide a better understanding of how BAG3 inhibition impacts cell proliferation and whether it can be an indicator of TNBC recurrence. Studying YM-1 gives patients more options and the chance to find the best treatment.

**Keywords-xenograft,** triple-negative breast cancer (TNBC), BAG3, cancer recurrence, epidermal growth factor receptor (EGFR) pathway, cell proliferation

## 1. Introduction

Triple-negative breast cancer (TNBC) is a form of breast cancer that does not express estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [1]. TNBC is only present in 15-20% of breast cancer patients. However, it is one of the most challenging breast cancers to treat with its high invasiveness, a tendency to relapse, and poor prognosis [2,3].

The recurrence of cancer depends on the nature of cancer. Cancers that proliferate quickly, are more advanced, or spread out more in the body have a higher chance of returning after treatment [4]. Cancer can come back in cancer's original location, which is called local recurrence. When cancer recurs in the lymph nodes near the original region, that is called regional recurrence. The final type of recurrence is distant recurrence which refers to cancers that come back and are far from the start of cancer.

TNBC, compared to other types of breast cancer, has an increased likelihood of distant recurrence and death within 5 years of diagnosis [5]. The aggressiveness of TNBC and its lack of sensitivity towards endocrine and HER2 treatments causes TNBC to have limited treatment methods compared to other types of breast cancer [3]. The lack of treatment targets available results in surgery and chemotherapy as first-line therapy. Some types of treatment include neoadjuvant chemotherapy, adjuvant chemotherapy, radiation, or immunotherapy [3]. Neoadjuvant chemotherapy refers to giving chemotherapy to shrink tumors before performing surgery to remove the tumor. Adjuvant chemotherapy is performed if the tumor size is small enough for tumor resection surgery,

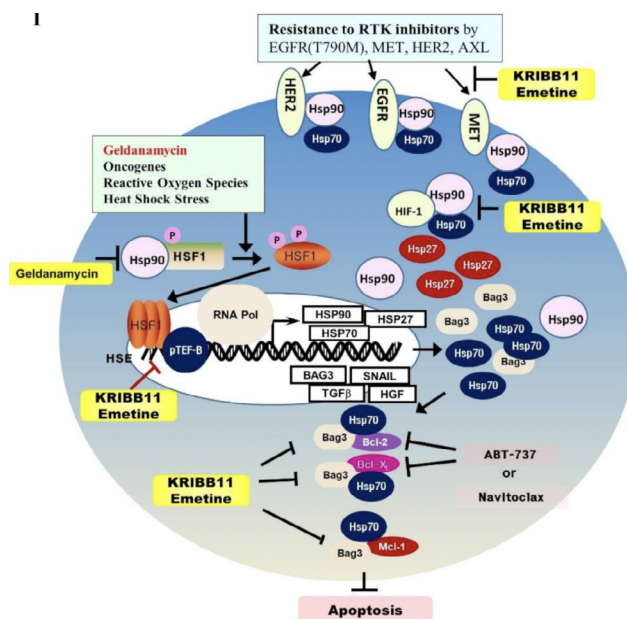
followed by chemotherapy to prevent cancer from recurring. A standard chemotherapy drug is Taxotere with the chemical compound name docetaxel. Taxotere treats cancers by preventing cell division through microtubule depolymerization. If a cell cannot form spindle fibers, it is unable to divide during mitosis, causing the cell to be stuck in the prometaphase [3].

The issue with relying on chemotherapy for treatment is that cancer cells can develop drug resistance causing cancer to be more challenging to treat [2]. There are two types of drug resistance: one is acquired resistance, and the other is de novo (intrinsic) resistance. Acquired resistance forms throughout the course of treatment, and intrinsic resistance implies that patients do not respond to conventional therapies [2]. TNBC's sensitivity towards chemotherapy can cause acquired resistance to occur. To improve the prognosis of TNBC, new therapeutic targets must be found. One such target is BCL-2-Associated Athanogene 3 (BAG3).

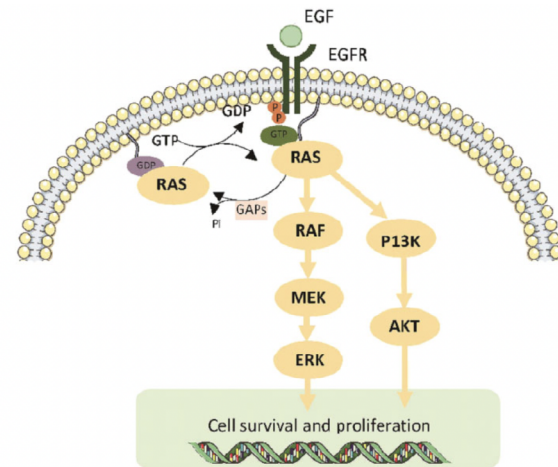
BAG3, an inhibitor of apoptosis in human cancer, is part of the Bcl-2-associated athanogene (BAG) protein family [2]. Another family is the BCL-2 family of co-chaperones with pro- and anti-apoptotic protein members [2, 6]. BAG3 stabilizes the anti-apoptotic members of the BCL-2 family, like Mcl-1, Bcl-2, and Bcl-xL, as shown in Figure 1, with BAG3 attached to the various anti-apoptotic member [2]. When the pro-survival proteins are stabilized, they increase in number, causing uncontrollable cell growth. Mcl-1, unlike the other anti-apoptotic proteins, is not affected by many novel anticancer agents like ABT-263 (Navitoclax). Therefore, the discovery that Mcl-1 is dependent on BAG3 is crucial in targeting cancer cell growth. In addition, BAG3 has been found to be present

in over half of TNBC patients at overexpressed levels [7]. This increased amount indicates that BAG3 could be a potential reason why TNBC is so much more challenging to treat than other breast cancers, making it a vital protein to study and inhibit.

BAG3 also plays a role in cell growth through the epidermal growth factor receptor (EGFR) signaling network [7]. The epidermal growth factor receptor pathway impacts tumor cell proliferation and cancer recurrence. Figure 2 shows that simply limiting EGFR is ineffective because mutations and other signaling networks downstream of the EGFR continue to affect cell proliferation. This is where BAG3 stands out because there is not only a correlation between it and the regulation of EGFR, but BAG3 also positively regulates the downstream signaling modules of the EGFR network. This includes signaling subnetworks Src, RAS, PI3K, and AKT. These subnetworks all play a role in cell survival and proliferation which means the inhibition of BAG3 will cause cancer growth to be reduced.



**Figure 1. The role of BAG3 and its interaction with other proteins [8]**



**Figure 2. Position of the various subnetworks of the EGFR signalling pathway and their activities [9].**

Frizzled 5 (FZD5) is another protein that impacts cancer cell proliferation. A study found that when high levels of FZD5 are present in TNBC patients, they have a shorter recurrence free survival and shorter overall survival (OS) [10]. Cancer stem cells (CSCs) also impact proliferation with their ability to proliferate unlimitedly. If they are not eliminated, proliferation will continue to occur, allowing the recurrence of tumors [11]. FZD5 and CSCs both impact cancer cell proliferation like BAG3, and there are studies indicating their connection to the recurrence of TNBC. If BAG3 is reduced, then most of the cancer cell proliferation will cease and recurrence of TNBC will stop which is akin to what happens when eliminating the CSCs.

If BAG3 is inhibited partially by the interaction inhibitor YM-1 in TNBC cell lines, the recurrence of TNBC will be reduced because BAG3 positively regulates the EGFR signaling network which controls cell proliferation [12]. The reduction of cell proliferation for TNBC and the association of high BAG3 levels with worse prognosis and survival in aggressive cancers make BAG3 a potential indicator for TNBC recurrence.

## 2. Hypothesis

Because BAG3 positively regulates cell proliferation in TNBC, it is hypothesized that the partial inhibition of BAG3 by YM1 in BT-549, a TNBC cell line, will shrink the size of tumors and the recurrence of TNBC will be reduced or stopped altogether.

## 3. Methods

### 3.1. Materials

This experiment uses BT-549, a known TNBC cell

obtained from the American Type Culture Collection (ATCC). Docetaxel (DOC) and YM-1 were purchased from Sigma-Aldrich.

Five-week-old female BALB/c nude mice are obtained from Charles River Laboratories. The mice are housed in sterile conditions with specific pathogen free conditions. They live in 12-hour light/dark cycles and are fed food and water ad libitum. All animal work is performed in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) guidelines.

### 3.2. Animal Experiment

Female Balb/c nude mice are randomly divided into 3 groups of 30: negative control, positive control, and YM-1 inhibitor treatments. The negative control receives sham phosphate buffered saline (PBS) injections. The positive control receives docetaxel, a common chemotherapy drug for triple-negative breast cancer. Docetaxel is diluted in .9% sodium chloride. The YM-1 inhibitor group is further broken down into 3 subgroups based on the concentration of the YM-1 injection. The three concentrations are low (1 mg/mL), medium (5 mg/mL), and high (10 mg/mL) concentrations of YM-1 dissolved in filter sterilized PBS. The mice in each YM-1 subgroup are randomly paired together to be parallel mice. This experiment is performed at least 5 times.

The BT-549 cell line (0.3 mL of  $9 \times 10^6$  cells/mouse) is

injected subcutaneously into the back of all the mice via xenografting. No treatments are given until the tumors reach an average volume of 60 mm<sup>3</sup> which is considered day 1. The volume of the tumors, measured by digital calipers, are estimated using the formula:  $V \text{ (mm}^3\text{)} = \text{length (mm)} \times \text{width (mm)} \times \text{height/2 (mm)}$ . On day 1 the negative control PBS (200 µL), positive control docetaxel (5 mg/kg), or one of the three YM-1 concentrations (500 µL) will be injected intraperitoneally. All treatments are given every 4 days for a total of 10 treatments. On the day treatments are given the volume of the tumor is measured with a digital caliper. The weights of the mice are also measured every 4 days.

After the treatments are over on day 44, one of the parallel mice from all the YM-1 injection pairs, a positive control mouse, and a negative control mouse are sacrificed. The tumor volume and mass are measured. The rest of the mice are observed for 80 days. The volume of the tumor on the back of the mouse and the mouse's weight is measured every 2 days. On day 120 the mice are sacrificed, and the final tumor mass and volumes are measured.

### 3.3. Statistical Analysis

The statistical significance of all numerical data collected from the animal experiment will be analyzed using the student's T-Test on GraphPad Prism® at  $p < 0.05$ .

## 4. Results

**Table 1. Possible Results of Tumor Size and Recurrence**

| Possible Observations   | Result 1 | Result 2  | Result 3  | Result 4  | Result 5  | Result 6 |
|-------------------------|----------|-----------|-----------|-----------|-----------|----------|
| Tumor Volume Decreases  | +        | +         | /         | /         | -         | -        |
| Tumor Mass Decreases    | +        | +         | /         | /         | -         | -        |
| No Recurrence of Cancer | +        | -         | +         | -         | +         | -        |
| Supports Hypothesis     | Yes      | Partially | Partially | Partially | Partially | No       |

Note: "+" represents positive result, "-" represents negative result, "/" represents no change

#### 4.1. Possible results of TNBC tumor size and recurrence

**1) Possible Result 1: The YM-1 inhibitor injection causes a decrease in tumor volume, tumor mass, and no new tumors form after the YM-1 injection.**

The YM-1 injections inhibit BAG3 causing the original tumor to decrease in mass and volume. By the end of the observation period, no new tumors are found after sacrificing the mice.

**2) Possible Result 2: The YM-1 inhibitor injection causes a decrease in tumor volume and tumor mass. However, new tumors form after the YM-1 injection.**

The YM-1 injections inhibit BAG3 causing the xenografted tumor to decrease in mass and volume. By the end of the observation period, new tumors are found after sacrificing the mice in comparison to the parallel mice.

**3) Possible Result 3: The YM-1 injection has no effect on the tumor mass and volume. There are no new tumors.**

The YM-1 injections cause no change in tumor mass or volume with the tumor growth being the same as the negative control. New tumors do not form in the mice at the end of the observation period.

**4) Possible Result 4: The YM-1 inhibitor injection does not change the tumor mass and volume but there are new tumors that form.**

The YM-1 injections cause no change in tumor mass or volume. There are new tumors that form in the mice other than the original xenografted tumor.

**5) Possible Result 5: The YM-1 inhibitor injection causes the tumor mass and volume to increase. No new tumors grow.**

The mass and volume of the original xenografted tumor increase after the YM-1 injection treatments. New tumors are not found in the mice after they are sacrificed.

**6) Possible Result 6: The YM-1 inhibitor injection causes the tumor mass and volume to increase. New tumors also form in the mice.**

The mass and volume of the original xenografted tumor increase after the YM-1 injection treatments. New tumors are found in the mice after they are sacrificed at 120 days.

**7) Possible Result 7: The effects of YM-1 are dose dependent**

Increasing the concentration of the YM-1 injection causes a greater decrease in tumor size and a lower chance of TNBC recurrence. The greater the concentration of YM-1 the more effective it is in inhibiting BAG3, reducing tumor size, and preventing TNBC recurrence.

**8) Possible Result 8: The effects of YM-1 are not dose dependent**

All concentrations of the YM-1 injections have the same effect on the tumor size and recurrence or new tumor growth of TNBC. A higher concentration is no more effective than a lower concentration of YM-1 inhibitor in inhibiting BAG3.

## 5. Discussion

Previous studies had found a connection between BAG3 and the proliferation of cancer cells in TNBC. To test whether BAG3 has a relationship with the recurrence of TNBC, TNBC cell lines are xenografted to mice, and the tumors that grow receive various treatments. There is a positive control, negative control, and 3 different concentrations of YM-1. Low, medium, and high concentrations of YM-1 are used to help determine which is most effective in preventing recurrence.

Possible result 1 fully supports the hypothesis. After the observation period, the final removed tumor compared to the tumor removed on day 40 is smaller, or there is no tumor left by the end of the experiment. The lack of

recurrence or new tumors means after sacrificing the mice no tumors other than the original xenografted tumor were found. The reduction in tumor size and lack of TNBC recurrence is similar to the results of the positive control docetaxel. The similarity in the outcomes of the positive control and experimental group match the hypothesis of this paper. It is also possible for YM-1 to have a more significant effect than docetaxel which still supports the hypothesis. YM-1 must cause similar or more significant changes in the mice compared to docetaxel because it indicates that inhibiting BAG3 can be a treatment just as effective as chemotherapy. To ensure the inhibition of BAG3 can be used as a treatment method, more studies need to be done looking at the side effects of silencing BAG3. Other BAG3 inhibitors can be used in future studies to test if they are as effective as the YM-1 inhibitor and how they affect mice or humans when inhibiting BAG3. The observation period of the mice can increase to see how long the mice remain recurrence-free.

Possible result 2 partially supports the hypothesis. The part of the result that matches this paper's hypothesis is the decrease in tumor size that is as much as the positive control and greater than the change that the negative control tumors receive. New tumors are found in the mice sacrificed at 120 days compared to those sacrificed right after the YM-1 treatment, which means recurrence has occurred. This causes possible result 2 to support the hypothesis only partially because it was believed that inhibiting BAG3 would stop recurrence and new tumor formation. A follow-up study can be designed to investigate the relation between changes in tumor size and recurrence of TNBC more thoroughly. Cell lines other than the BT-549 cell line can be used in future experiments to observe whether cell lines impact the experiment result.

Possible results 3 and 4 only partially support the hypothesis, but result 3 more fully supports the hypothesis. The two results both have the tumor size staying the same relative to the parallel mice and negative control. The tumors either stay the same size and don't change in size compared to the tumors removed right after receiving treatments or they grow like the tumors treated with the negative control. This means inhibiting BAG3 has a minimal effect on tumor size or no effect on tumor size if tumor growth is similar to that of the tumors that received PBS. A more extensive range of YM-1 concentrations can be used to investigate this further. Possible result 3 supports the hypothesis more than possible result 4 because result 3 does not have any new tumors appearing in the mice. The length of observation or treatment can be changed to see whether new tumors will form. Possible result 5 partially supports the hypothesis due

to no new tumors growing by the end of the observation period. Result 5 has the tumors increase in size, meaning the original tumors are bigger than the original tumors in the mice treated with the negative control. The final tumors removed will also be larger than the tumors removed on day 40 of the experiment. This result could indicate a possible issue with the BAG3 inhibitor. Further studies can examine increasing the dosage of YM-1 or the treatment length. The sensitivity of various TNBC cell lines to the YM-1 inhibitor could also be investigated.

Possible result 6 rejects the hypothesis because the original tumor increases in size, and new tumors form other than the xenografted tumor at the end of the observation period compared to the negative control and parallel mice. If the condition of the mice that received YM-1 is worse than those that received the sham saline injections, there could be issues with inhibiting BAG3 or using YM-1 as an inhibitor. Finding tumors in the second parallel mice that were not found when the first parallel mice were sacrificed at 40 days indicates further issues with the inhibition of BAG3. The potential reasons for the increased tumor size and tumor recurrence will have to be studied further. Other proteins that positively regulate cell proliferation can be inhibited and observed for similar side effects. The strength and speed tumors recur can be investigated to understand the recurrence process better. In addition, if new tumors form or there is some type of recurrence, it may be good to investigate whether TNBC cells can form a resistance to BAG3 inhibition. This would be comparable to the issues chemotherapy is causing for patients.

This study examines the effect YM-1, a BAG3 inhibitor, has on tumor size and cancer recurrence. Three different treatments with different concentrations of YM-1 are given to the mice to understand the inhibitor better. This leads to the study having two different results. One scenario is possible result 7, where the effect and strength of YM-1 depend on its concentration. The higher the concentration, the more potent and effective YM-1 is in inhibiting TNBC. The other result would be possible result 8, where no matter the concentration of YM-1, the changes in the mice are all the same. This means a higher concentration is no more effective in treating TNBC than a lower concentration.

## 6. Conclusion

This study explores the role BAG3 plays in predicting the recurrence of TNBC. The nature of the tumor growth after being subject to a BAG3 inhibitor indicates whether BAG3 impacts the recurrence of cancer cells or not. Having the tumors maintain the same size or even reduce

in size means BAG3 can be researched and developed into a new treatment for TNBC. Since BAG3 silencing is a novel therapy, more research needs to be conducted to accurately understand the role BAG3 plays in cancer and for potential side effects to be found. Due to our proposition only providing predicted results, further studies will need to be performed to determine the relationship between BAG3 and the recurrence of TNBC.

## Reference

- [1] Linder, B., et al., BAG3 is a negative regulator of ciliogenesis in glioblastoma and triple-negative breast cancer cells. *J Cell Biochem*, 2022. 123(1): p. 77-90.
- [2] Das, C.K., et al., BAG3 Overexpression and Cytoprotective Autophagy Mediate Apoptosis Resistance in Chemoresistant Breast Cancer Cells. *Neoplasia*, 2018. 20(3): p. 263-279.
- [3] Yin, L., et al., Triple-negative breast cancer molecular subtyping and treatment progress. *Breast Cancer Res*, 2020. 22(1): p. 61.
- [4] American Cancer Society. What is cancer recurrence? 2016, February 12; Available from: <https://www.cancer.org/treatment/survivorship-during-and-after-treatment/long-term-health-concerns/recurrence/what-is-cancer-recurrence.html#:~:text=If%20cancer%20is%20found%20after,somewhere%20else%20in%20the%20body>.
- [5] Dent, R., et al., Triple-negative breast cancer: clinical features and patterns of recurrence. *Clin Cancer Res*, 2007. 13(15 Pt 1): p. 4429-34.
- [6] Boiani, M., et al., The stress protein BAG3 stabilizes Mcl-1 protein and promotes survival of cancer cells and resistance to antagonist ABT-737. *J Biol Chem*, 2013. 288(10): p. 6980-90.
- [7] Shields, S., et al., BAG3 promotes tumour cell proliferation by regulating EGFR signal transduction pathways in triple-negative breast cancer. *Oncotarget*, 2018. 9(21): p. 15673-15690.
- [8] Lee, S., Jung, J., Lee, Y.-J., Kim, S.-K., Kim, J.-A., Kim, B.-K., Park, K. C., et al. (2021). Targeting HSF1 as a Therapeutic Strategy for Multiple Mechanisms of EGFR Inhibitor Resistance in EGFR Mutant Non-Small-Cell Lung Cancer. *Cancers*, 13(12), 2987. MDPI AG.
- [9] Li, Z.N., et al., BRAF and KRAS mutations in metastatic colorectal cancer: future perspectives for personalized therapy. *Gastroenterol Rep (Oxf)*, 2020. 8(3): p. 192-205.
- [10] Sun, Y., et al., FZD5 contributes to TNBC proliferation, DNA damage repair and stemness. *Cell Death Dis*, 2020. 11(12): p. 1060.
- [11] Duan, H., et al., Recent advances in drug delivery systems for targeting cancer stem cells. *Acta Pharm Sin B*, 2021. 11(1): p. 55-70.
- [12] Colvin, T.A., et al., Hsp70-Bag3 interactions regulate cancer-related signaling networks. *Cancer Res*, 2014. 74(17): p. 4731-40.