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Determining effective treatment regimens for breast cancer using combined immunotherapy and chemotherapy in vivo

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STEM

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ABSTRACT

Breast cancer has the highest incidence rate of all cancers globally in women, and those of African descent, especially West African females, face higher rates of triple-negative breast cancer (TNBC), a more aggressive form of breast cancer. Immunotherapy for breast cancer is a relatively new treatment option, and research is ongoing to identify the best combination treatments for increasing survival of those diagnosed with TNBC. Eganelisib (IPI-549: a PI3K-gamma inhibitor that works to shift M2 macrophages to M1 to augment T cell function) with other combinatory treatments has shown promising results in reducing tumor growth and increasing survival in mice. We have been conducting experiments to determine the most effective treatment regimen that will reduce growth of 4T1 mammary cancers, a murine TNBC model in syngeneic BalbC female mice. Combinations of eganelisib, cyclophosphamide, and anti-PD-1 or anti-PD-L1 have been tested to determine how immunotherapy and chemotherapy can induce a strong immune response, resulting in better responses to chemotherapy. Our current data indicates that a treatment regimen combining eganelisib, cyclophosphamide, and anti-PD-1 was most effective at suppressing tumor growth, compared to other treatments that only included one or two of these treatments. Mean tumor sizes of mice treated with a combination of eganelisib and other treatments were 187±70 mm^3, 232±71 mm^3, and 227±59 mm^3 at the end of the experiments, compared to control data of 576±137 mm^3, 414±31 mm^3, and 591±200 mm^3, respectively. These results could lead to further research on effective immunotherapy treatment combinations for TNBC.

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KEYWORDS

Breast Cancer • Chemotherapy • Immunotherapy • TNBC • Eganelisib • Cyclophosphamide • Anti-PD-L1

Introduction

A) Significance

Breast cancer has the highest incidence rates among all cancer types and is the most commonly diagnosed cancer globally (Arnold et al., 2022). Similarly, mortality rates for breast cancer are one of the highest for women in the United States, after lung cancer, although they have been slowly decreasing as a result of screening and better treatment options. In addition to more classical treatments, such as surgery and radiation, chemotherapy and immunotherapy have major impact on patient outcomes, especially for triple negative breast cancers (TNBC, lacking estrogen and progesterone receptors and not overexpressing HER-2). Immunotherapy for breast cancer is a relatively new treatment option, and research is still ongoing to identify combinations of treatment regimens that would prove to be the most effective in increasing survival.

To focus on this issue, we are testing different regimens of combined immunotherapy treatments, such as anti-PD-1 / anti-PD-L1 (blocks T cell suppression and stimulates an immune response against the tumor cells) and eganelisib (IPI-549: PI3K-gamma inhibitor that works to shift M2 macrophages to M1 in order to further stimulate T cells), and chemotherapy treatments, such as cyclophosphamide (decreases T-regulatory cells in tumor microenvironment) and guadecitabine / decitabine (targeting myeloid-derived suppressor cells, MDSC). We have performed preliminary in vivo experiments with syngeneic BalbC female mice, which were injected with 4T1 cancer cells in the mammary fat pad, and produced results where a combined approach of immunotherapy and chemotherapy was effective in inhibiting tumor growth. We hope to identify a treatment regimen that will produce the best results in terms of completely curing mice of 4T1 tumors, a murine TNBC model.

B) Study Aims

Aim 1: To determine whether inhibiting PI3K-gamma using eganelisib increases efficacy of checkpoint blockade combined with chemotherapy. Experiments with eganelisib and other combined treatments will be conducted to identify which dosages and sequences of the treatments produce the best results for curing the mice of cancer. We will identify how best to produce a strong immune response from the mice to elicit a better response to chemotherapy. We will also determine whether anti-PD-1 or anti-PD-L1, in combination with eganelisib and chemotherapy, is a better option.

Aim 2: To perform correlative studies to determine how these treatments affect macrophage subsets and to study the immunologic response after treatment. With this aim, we hope to conduct further studies such as flow cytometry and multiplex immunofluorescence staining of tumors to determine how macrophages and T cells are affected by the various combination treatments outlined in Aims 1 & 2. Metastases to the lungs after resection may also be studied, using bioluminescent tumor cells (4T1-luciferase) to gain a more thorough picture of the effects of treatment on outcomes and to determine that the effects we are seeing are caused by T cell responses to the tumors.

With this proposed study, Aim 1 will allow us to formulate an effective treatment regimen, combining immunotherapy with chemotherapy to result in maximal inhibition of tumor growth. We will further investigate the effects of treatments such as eganelisib and anti-PD-1 or anti-PD-L1 on the tumor microenvironment, following the regimen established after studies into the first aim outlined in this paper. The second aim will help to gather more evidence for the studies

that we will be performing. At the end of this study, we hope to have a better understanding of how these treatment options affect the growth of tumors in mice and to identify what timing or sequence of the various treatments will produce the most effective anti-tumor effects.

C) Background

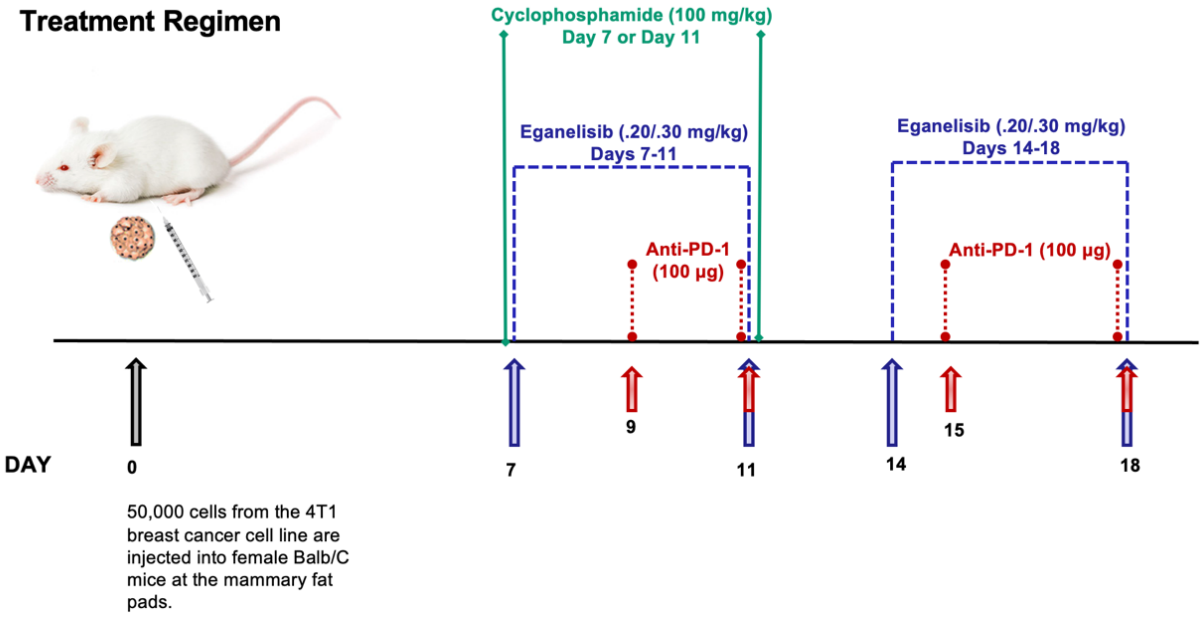
Of the types of breast cancer, triple-negative breast cancer is a more aggressive form of breast cancer, and as immunotherapeutic treatments are being studied, the use of immune checkpoint inhibitors shows promise as an effective treatment when administered in combination with chemotherapy (Emens et al., 2021). The use of tumor-infiltrating lymphocytes for pinpointing tumors of TNBC and HER2+ breast cancer types that would benefit from immune checkpoint inhibitors, anti-PD-1 or anti-PD-L1, has also been in the process of development (Loi et al., 2021). The usage of this biomarker could improve prognosis rates by being able to target certain tumors that would show the best positive results when treated with PD-1 or PD-L1 inhibition. Early-stage TNBC provides an immune microenvironment that could be more responsive to the PD-1 or PD-L1 checkpoint inhibitors, and studies showing neoadjuvant immunotherapy has a greater impact on TNBC models compared to adjuvant immunotherapy (Emens et al., 2021).

Another treatment option that is being explored in our current studies is a PI3Kγ inhibitor, for which we are using eganelisib or IPI-549 to test its efficacy for reducing tumor growth when given in conjunction with chemotherapy and immune checkpoint blockade. In studies with myeloid cells, a correlation between immune checkpoint blockade resistance and increase of immune-suppressive myeloid cells/macrophages warranted a need for an agent that would enhance the tumor immune microenvironment and overcome resistance to immune checkpoint blockade (De Henau et al., 2016). Inhibiting PI3Kγ has been shown to block the transition of M1 macrophages to immunosuppressive M2 macrophages and can augment immune responses to cancer when combined with ICB. Thus, the use of eganelisib could greatly benefit the immune microenvironment and reduce tumor volume, especially when combined with immunotherapy and chemotherapy. As PI3Kγ signaling plays a role in suppressing immune response and leading to tumor growth, a PI3Kγ inhibitor, such as eganelisib, can show major improvements in establishing immune responses by decreasing M2 macrophages and increasing the activity of $CD8^+$ T cells and increasing cytotoxicity in the immune microenvironment (Kaneda et al., 2016). Results from multiple studies show the importance of the PI3Kγ signaling pathway in macrophages, and our aim is to determine how we can translate the success of those studies for using a PI3Kγ inhibitor to increase the efficacy of immune checkpoint blockade and reduce tumor growth and metastases of breast cancer in a mouse preclinical model.

Methods

Aim: To determine the sequence and dosages of a treatment regimen with both immunotherapy and chemotherapy to inhibit tumor growth of mice treated with 4T1 breast cancer cells and to conduct correlative studies to establish the effects of treatment on macrophage subsets and immunologic responses

Our primary studies currently involve in vivo experiments to focus on the aim outlined above. For each study, the 4T1 breast cancer cell line is used, and in the week prior to the administration of the cancer cells into the mice, the 4T1 cells are thawed and split over the next few days to prepare for



injection. Syngeneic BalbC female mice are used for all of the studies, and on day 0 of the study, approximately 50,000 cells are injected into each mouse through mammary fat pad injections. After the administration of the cancer cells, the mice are randomized and sorted into different treatment and control groups. The mice do not start receiving treatment until day 7 of the study, providing a week for the cancer cells to grow, in order to realistically represent how cases are presented in patients in the clinical setting. For the following two weeks, the identified treatments are administered until day 18 or day 20.

For the current studies being conducted, these following treatments are being used: eganelisib, guadecitabine, cyclophosphamide, anti-PD-1, and anti-PD-L1. Eganelisib is administered through oral gavage, and the treatment is combined with corn oil to ease the process of giving the treatment to the mice. Guadecitabine, cyclophosphamide, anti-PD-1, and anti-PD-L1 are all administered through intraperitoneal injections in the quadrant opposite that of the one where the cancer cells were injected. For eganelisib, the dosage has been 0.2 mg/kg in our initial experiment, but in our later experiments, it was increased to 0.3 mg/kg. Egeanelisib is given daily; in some experiments, the weekends were skipped. Either guadecitabine or cyclophosphamide was combined with the other treatments, and guadecitabine was given at a dosage of 50 µg and cyclophosphamide at 2 mg. Guadecitabine has always been administered days 7-11 in our studies, but different timings for cyclophosphamide were tested (either day 7 or day 11) to determine the most effective sequence. Anti-PD-1 and anti-PD-L1 were administered to the mice at 100 mg every three to four days until the end of the treatment regimen.

Mouse tumors were measured every three to four days to document the growth of the tumor volume which was calculated based on the measurements of the length and width of the tumor that were taken. These measurements were subsequently added onto tumor growth curves, and Kaplan-Meier survival curves were also created to study the effects of the treatment on survival of the mice. If the mice showed indications of being sick or having very deep or large tumors, they were euthanized through a CO_2 euthanasia chamber.

Results and Discussion

All the experiments conducted showed that a combined approach of eganelisib with anti-PD-1 and/or cyclophosphamide was effective in decreasing tumor growth. When eganelisib was given at a dose of 0.2 mg/kg, along with anti-PD-1, the final average tumor volume was 187±70 mm^3, compared to 576±137 mm^3, the final average tumor volume of the control group (Figure 1). Similarly, when the same dosage of eganelisib was combined with anti-PD-1 and cyclophosphamide in a different experiment, the final average tumor volume of the experimental group that received all three treatments was 232±71 mm^3, compared to the control group's tumor volume of 414±31 mm^3 (Figure 2). Increasing the dosage of eganelisib from 0.2 mg/kg to 0.3 mg/kg and administering cyclophosphamide at day 11 instead of day 7 seemed to have an effect on tumor growth - the tumor volume of the experimental group that received this increased dosage of eganelisib and different treatment sequencing of cyclophosphamide with the addition of anti-PD-1 had an average tumor volume of 227±59 mm^3, while the untreated control group had a mean tumor volume of 591±200 mm^3 (Figure 3). However, tumors for the group treated with three drugs including eganelisib was not significantly different from the group treated with anti-PD-1 and CYP alone (Figure 3). The survival in the experimental group showed more promise compared to the control group mice but again was not different from anti-PD-1 + CYP without eganelisib (Figure 4). The latest experiment conducted with eganelisib, cyclophosphamide, and anti-PD-1 showed results of the untreated control group reaching a mean tumor volume of 412±87 mm^3 by day 21 of the experiment, and the experimental groups receiving treatments for all three medications resulted in a mean tumor volume of 409±42 mm^3 on day 28 (Figure 7). While the mice survival curve favored groups of mice receiving the treatments, the results revealed that eganelisib was not making as much of an impact as cyclophosphamide. This latest experiment, where the different combinations of treatments with eganelisib, did not seem to have any positive effects in reducing the tumor volume of the mice.

These results show that the sequence of the treatment regimen may play a role on the impact on tumor volume, as well as overall survival. Studying when cyclophosphamide is administered can improve results for decreasing tumor growth by strengthening the immune microenvironment of the tumor. It may be that increasing the immune response prior to chemotherapy may increase the efficacy of the latter. Alternatively, cyclophosphamide, which has been found by some to selectively deplete immunosuppressive T-reg cells may increase the efficacy of the immune therapies. Future experiments with different sequences of the same treatments can further reveal the impact of timing for establishing effective treatment regimens that will decrease or even stop tumor growth and increase survival of mice with breast cancer. Different methods of administering eganelisib (in particular, solubilizing the drug exactly as outlined previously rather than as recommended by our supplier) are also being explored to attempt to replicate the results that were found in previous studies. Replication of studies following the methods outlined in the current studies of eganelisib will be conducted, as well as possibly exploring the administration of eganelisib through IP injections. Continued studies will hopefully point in the right direction of implementing eganelisib in treatment regimens for breast cancer with in vivo studies.

Our initial experiments with combinations of guadecitabine,

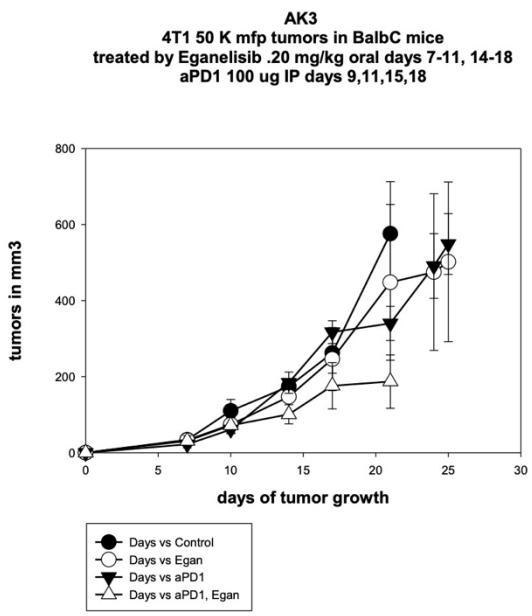


Fig 1: Introductory experimental results of tumor growth of mice treated with varying combinations of Eganelisib (IPI-549) and anti-PD-1

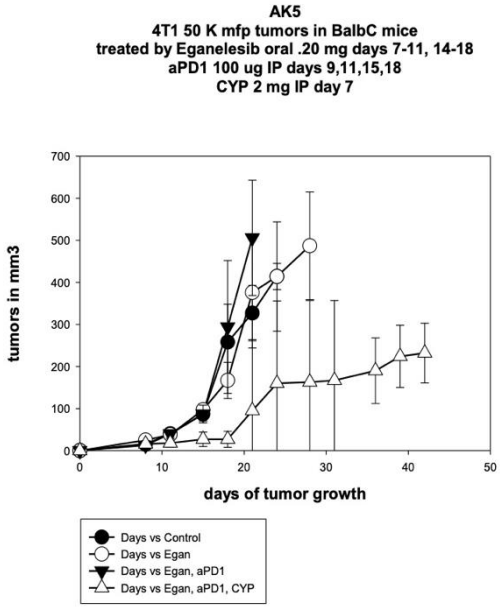


Fig 2: Experimental results of tumor growth of mice treated with varying combinations of eganelisib, cyclophosphamide, and anti-PD-1

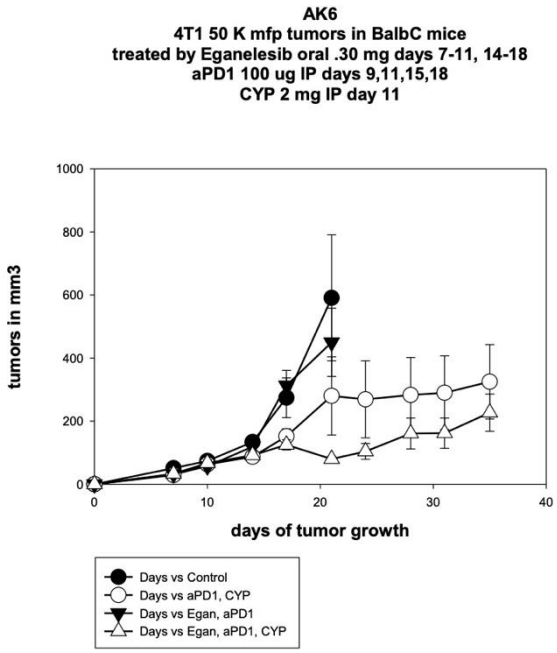


Fig 3: Experimental results of tumor growth of mice treated with varying combinations of eganelisib, cyclophosphamide, and anti-PD-1

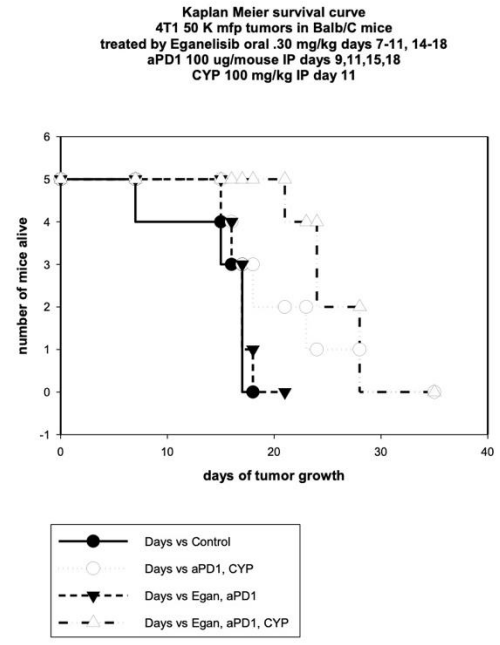


Fig 4: Kaplan Meier survive cure of AK6 experiment with experimental groups treated with combinations of eganelisib, cyclophosphamide, and anti-PD-1

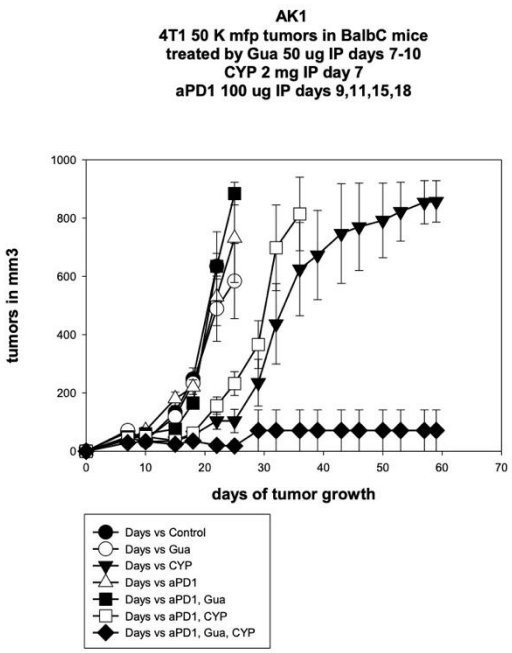


Fig 9: Experimental results of tumor growth of mice treated with varying combinations of guadecitabine, cyclophosphamide, and anti-PD-1

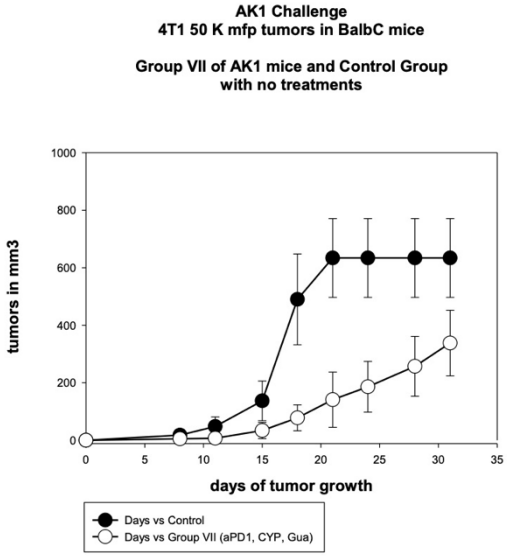


Fig 10: Experimental results of tumor growth of AK1 mice of the group that received treatments of guadecitabine, cyclophosphamide, and anti-PD-1 after reintroduction of tumor cells

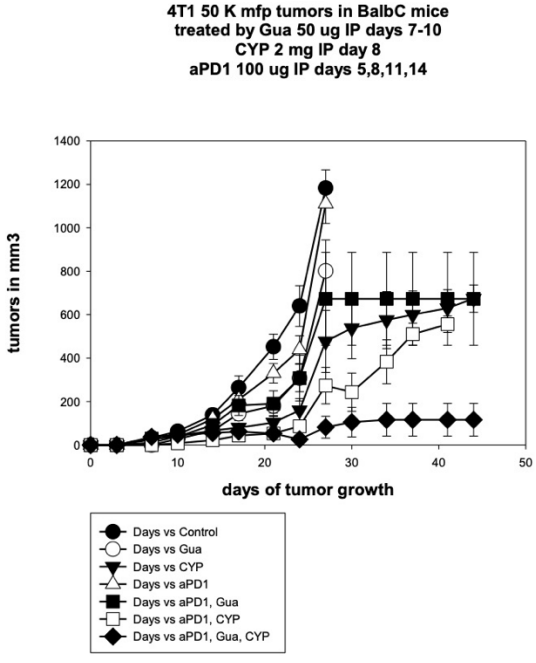


Fig 11: Experimental results of tumor growth of mice treated with varying combinations of guadecitabine, cyclophosphamide, and anti-PD-1

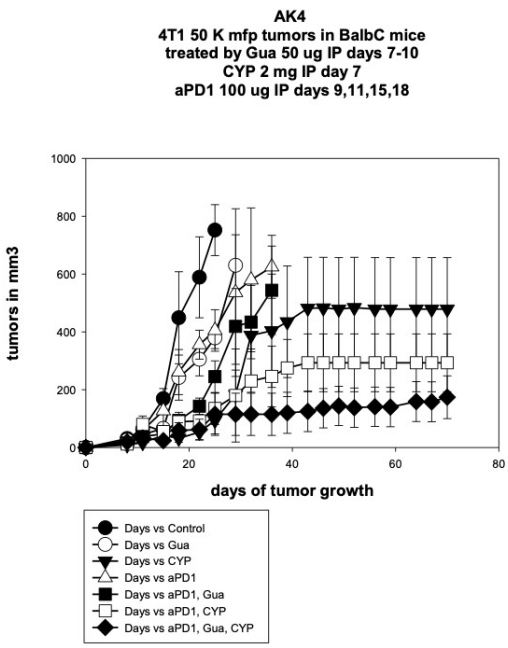


Fig 12: Experimental results of tumor growth of mice treated with varying combinations of guadecitabine, cyclophosphamide, and anti-PD-1

cyclophosphamide, and anti-PD-1 showed promising results with mice being cured and becoming resistant. One experiment involving the treatment of guadecitabine 50 µg/mouse IP on days 7-10, cyclophosphamide 2 mg/mouse IP on day 7, and anti-PD-1 100 µg on days 9, 11, 15, and 18 resulted in this treatment group reaching a final average tumor volume of 71±71 mm^3, while the untreated control group was 635±44 mm^3 (Figure 9). The mice who were given the treatments survived longer than those who were not provided any, with four of the five mice in the group being cured. A repeat of the same experimental procedure had similar results, and in that experiment, the treatment group that had received all three treatments reached a final average tumor volume of 174±74 mm^3, while the untreated control group was 752±88 mm^3 (Figure 12). We hope to use these promising results, as we work to implement eganelisib in the treatment regimens.

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