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Inhibition of Metastatic Cancer in Mice via Atopaxar: A Research Protocol

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Abstract

Introduction: When cancer becomes metastatic, tumour cells intravasate out of the primary tumour and spread to other organs, causing about 90% of cancer deaths. One way circulating tumour cells (CTCs) metastasize is by interacting with platelets, resulting in tumour cell-induced platelet aggregation (TCIPA) that shields CTCs from immune attack. Previous studies suggest that tumour cells promote metastasis and induce TCIPA by activating protease-activated receptor-1 (PAR-1) on platelets. Therefore, this study aims to investigate whether administering Atopaxar, a PAR-1 antagonist that has not yet been studied in cancer as other PAR-1 antagonists have, can limit metastasis in mouse models.

Methods: We will assess the effectiveness of Atopaxar and a placebo (or control) on adult C57BL mice inoculated with GFP-transfected Lewis lung carcinoma cells. Flow cytometry of blood samples taken 7, 14, and 21-days post-inoculation will be performed to quantify the number of GFP+ cells and activated CD8+ (cytotoxic) T cells in the samples.

Results: We expect that the Atopaxar treated mice will have reduced numbers of CTCs and higher numbers of cytotoxic T cells, suggesting that the inhibition of TCIPA via Atopaxar will correlate with reduced shielding of CTCs and metastasis rates.

Discussion: These results could provide novel insight into the use of PAR-1 antagonists in confining cancer to its primary site in patients and inhibiting CTCs' function as a seed for metastases.

Conclusion: Since CTCs will usually be present in the blood even after removal of a secondary tumour, limiting metastasis can significantly improve the prognosis and wellbeing of patients.

Keywords: cancer; metastasis; CTC; TCIPA; PAR-1; Atopaxar

Introduction

Cancer is the development of abnormal cells that divide uncontrollably and have the ability to infiltrate and destroy normal body tissue [1]. When a tumour becomes metastatic, cancer cells intravasate out of the primary tumour and spread to other organs, drastically decreasing the survival rate of patients and contributing to about 90% of cancer deaths [1,2]. Once cancer cells split away from the primary tumour and appear in the bloodstream, they are known as circulating tumour cells (CTCs). Interestingly, previous studies have shown that CTCs can interact and aggregate with platelets in order to be shielded from the immune system [3-5]. Specifically, it is suggested that CTCs activate protease-activated receptor-1 (PAR-1) on platelets to induce tumour cell-induced platelet aggregation (TCIPA). TCIPA has been shown to prevent the immune system from detecting the CTCs and activating immune cells for combat, ultimately enhancing the survival advantages of CTCs and their ability to spread to distant organs [3-5]. If the immune system cannot detect the CTCs and activate immune cells to combat them, the CTCs will have less stress on them as they spread across the body to different organs. Currently, there are many treatments and drugs being tested to try and limit cancer metastasis, including drugs that target the deactivation of PAR-1. A PAR-1 antagonist is a drug that can prevent the activation of the receptor PAR-1 on platelets [6]. As a result, PAR-1 antagonists are able to inhibit the natural functions of PAR-1, essentially "turning off" the receptor. However, PAR-1 is not often pursued as oncology drug targets, with the availability of mainly only two PAR-1 antagonists (Vorapaxar and Atopaxar) that have undergone clinical development but have not been extensively introduced into the field yet – especially Atopaxar [6]. Vorapaxar – a PAR-1 antagonist – has been shown to produce an increased risk of intracranial hemorrhage when being tested as a therapeutic target in a variety of metastatic cancers [7]. Thus, there would be great benefits to looking into other PAR-1 antagonists that can inhibit metastasis, but also have reduced side effects. This study aims to investigate whether administering Atopaxar, a PAR-1 antagonist that has not yet been studied in cancer, can limit metastasis in mouse

models. Atopaxar is a reversible, orally active PAR-1 antagonist discovered by scientists at Eisai, and is currently being investigated in thrombosis and cardiovascular diseases, such as coronary artery disease [8]. Given Atopaxar's nature as a PAR-1 antagonist, we hypothesize that Atopaxar treatment will inhibit TCIPA in cancerous mice, as opposed to control mice, allowing for reduced shielding of the CTCs from the immune system.

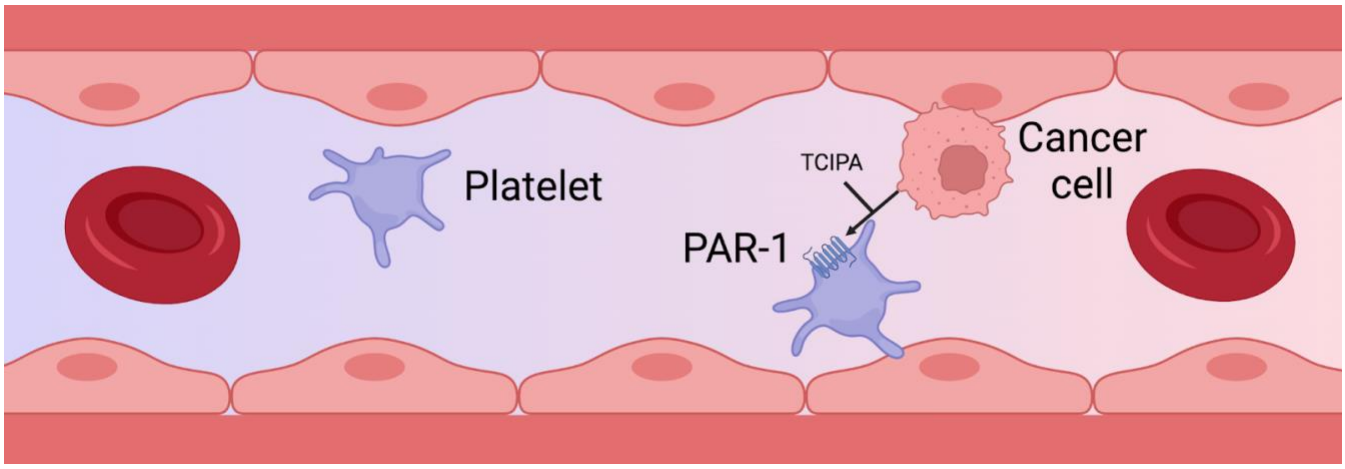

Figure 1. This diagram depicts the interaction between platelets and CTCs, through which CTCs induce TCIPA to bind to PAR-1 (created with BioRender.com).

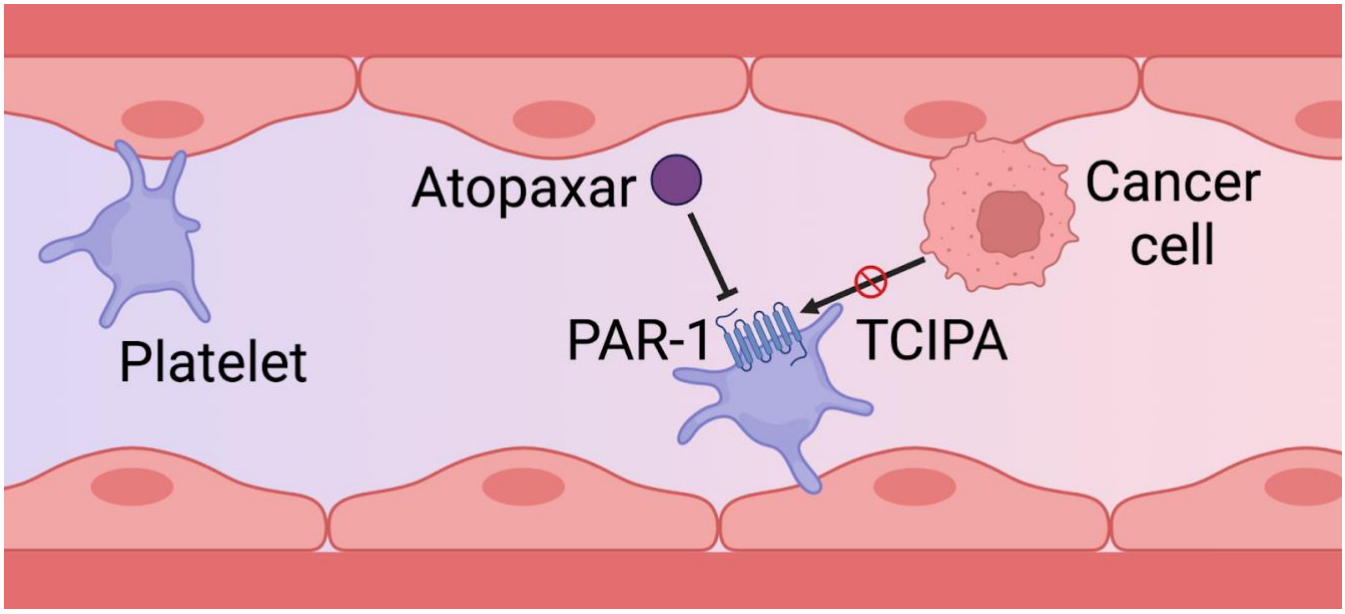

Figure 2. This diagram depicts the interactions between Atopaxar and PAR-1 to inhibit TCIPA and CTC binding to platelets (created with BioRender.com).

Methods

In this study, we will assess the effectiveness of Atopaxar and a placebo (or control) on adult C57BL mice inoculated with GFP-transfected Lewis lung carcinoma cells (LLC1). The reason for this is that C57BL mice are refractory to many tumours, but since LLC1 is highly tumourigenic, it will form metastatic tumours post-implantation into C57BL mice [9]. LLC1 is immunologically compatible with C57BL mice and can also be tracked *in vivo*, making it a great cell line for studying the mechanisms behind tumour growth and metastasis [9]. In addition, the tracking of LLC1 can be used to evaluate the effects of various drugs and treatments in mice, such as the implementation of Atopaxar in this study. Firstly, the LLC1 will be transfected with GFP so that the LLC1 can be quantified later on in the study. After,

by following the methods described by Yoo *et al*, the GFP+ LLC1 will be inoculated into the tail veins of the C57BL mice, allowing the LLC1 to metastasize to the lungs of the mice [9]. Right after inoculation, each mice group will be treated respectively: the Atopaxar group with Atopaxar and the control group with sugar. Syringes will be used for the Atopaxar and sugar injections, and the dosage will be variable. We will start with 100 µL for both the Atopaxar and placebo arms, and we will monitor the incidence of side effects in both groups. Should there be a high incidence of side effects, we will lower the dosage and future research will be conducted to determine the optimal dosage. In addition, we will monitor dose-dependent trends for efficacy and adjust accordingly. Following the treatments, flow cytometry of blood samples taken 7, 14, and 21 days post-inoculation will be performed. Flow cytometry allows for rapid analysis of single cells based on their physical properties and fluorescent-tagged markers. A variety of fluorescent reagents are utilized in flow cytometry, such as fluorescently conjugated antibodies, DNA binding dyes, viability dyes, ion indicator dyes, and fluorescent expression proteins [10]. In this study, flow cytometry will be used to quantify the number of GFP+ cells (or cancer cells) and activated CD8+ (cytotoxic) T cells in the blood samples of the two groups (one treated with Atopaxar and the other with sugar).

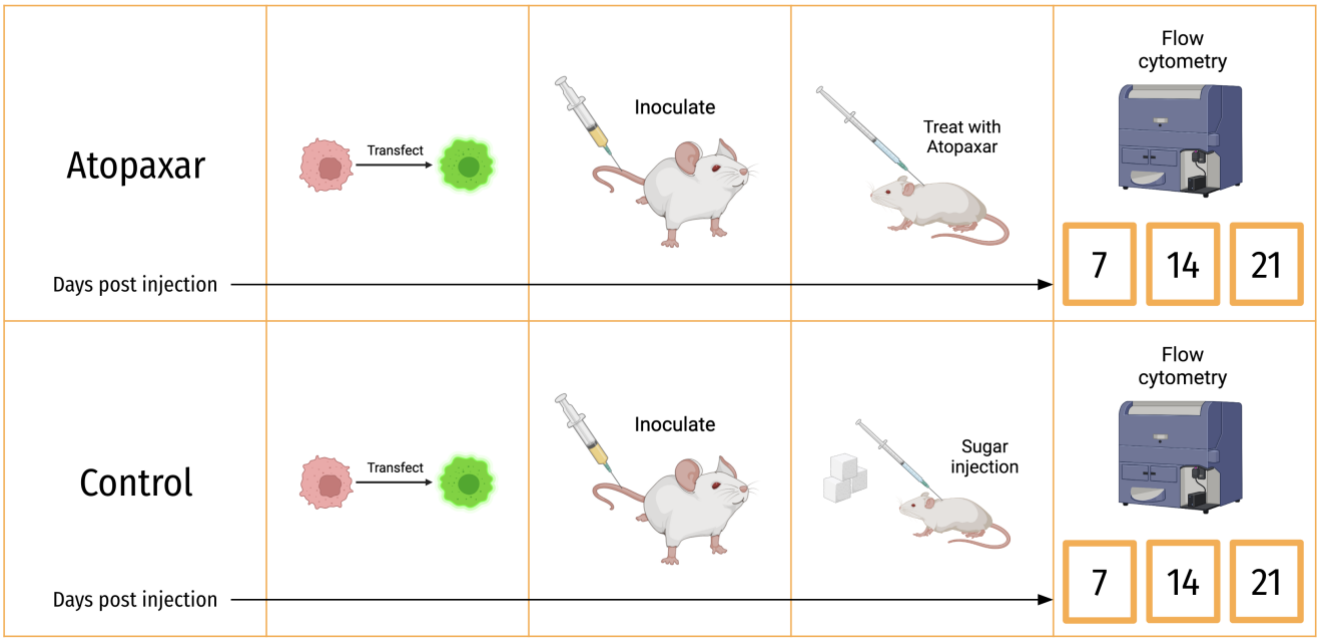

Figure 3. This diagram depicts the chronological methods and processes used to quantify the number of GFP+ cells and activated CD8+ T cells in the blood samples of the two groups (created with BioRender.com).

Results

This protocol is an outline of a prospective study; therefore, all results and analyses will remain hypothetical. After 21 days when the Atopaxar and placebo arms have been treated three times at 7-day intervals, we expect that the Atopaxar treated mice will have reduced numbers of CTCs (GFP+ cells) and higher numbers of CD8+ (cytotoxic) T cells. These results suggest that Atopaxar may be able to limit TCIPA, allowing the immune system to better detect and destroy the cancer cells, which leads to a decrease in metastasis rates. The anticipated results of this study should confirm that Atopaxar influences the shielding of CTCs and increases immune surveillance.

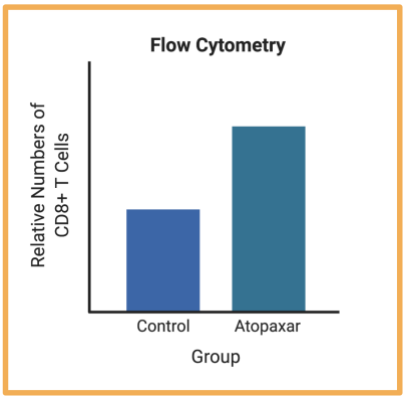

Figure 4. This graph visualizes the relative number of CD8+ T cells in both control and Atopaxar groups (created with BioRender.com).

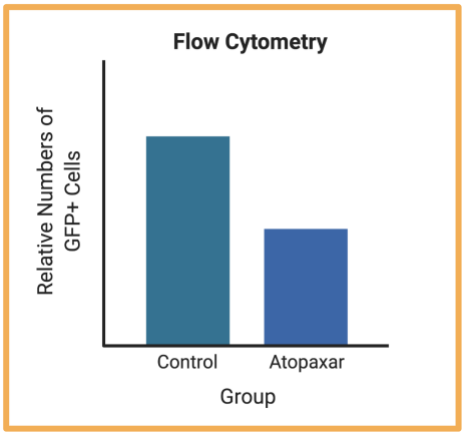

Figure 5. This graph visualizes the relative number of GFP+ cells in both control and Atopaxar groups (created with BioRender.com).

Discussion

We predict that these results would be due to Atopaxar acting as a PAR-1 antagonist, thereby inhibiting tumour cell interaction with platelets by preventing the activation of PAR-1. If the tumour cells cannot activate PAR-1 on platelets and induce TCIPA, then they will be more susceptible to immune detection and destruction as they will no longer be shielded.

As shown in Figure 4, the Atopaxar group results in a higher number of CD8+ (cytotoxic) T cells because the immune system is activated and can detect the CTCs. Furthermore, Figure 5 shows that the Atopaxar group results in a lower number of GFP+ cells because the CTCs have been eliminated by the CD8+ T cells since Atopaxar's inhibition of PAR-1 prevents the shielding of the CTCs.

The control group in Figure 4 shows that the number of CD8+ T cells is lower than that of the Atopaxar group. Since Atopaxar is not present to prevent the activation of PAR-1, CTCs are able to travel in the circulation undetected by the immune system; hence, there is a lower number of CD8+ T cells. Moreover, the control group in Figure 5 shows that there is a higher number of GFP+ cells compared to that of the Atopaxar group. Since the mice in the control group have not been treated with Atopaxar, the platelets' shielding of the CTCs persists and thus, the GFP+ cells (CTCs) are not attacked by the immune system and have a higher count.

As a result, these observations will correlate with reduced metastasis rates, indicating that Atopaxar has the potential to be a new form of treatment for cancer patients. In addition, these results present a correlation between the number of CD8+ T cells and GFP+ cells in each of the two mice groups. A lower number of CD8+ T cells correlates with a higher number of GFP+ cells (as shown in the control group), while a higher number of CD8+ T cells correlates with a lower number of GFP+ cells (as shown in the Atopaxar group).

Covic *et al* have shown that an adverse side effect of Vorapaxar is an increased risk of intracranial hemorrhage, while Atopaxar has no apparent complications. Both drugs could still cause increased bleeding, however, as they are anti-platelet agents [7]. Therefore, the next step in this study would be to monitor the effects that Atopaxar has on the mice under treatment. For example, a CT scan of the brain area in the Atopaxar-treated mice could be conducted to monitor if major side effects (such as intracranial hemorrhage) will arise.

Furthermore, another study has also concluded that Atopaxar can cause increased minor bleeding in patients. Wiviott *et al* have investigated Atopaxar as a treatment in patients with coronary artery disease. In this dose-ranging study, treatment with Atopaxar resulted in platelet inhibition, more minor bleeding, and numerically but not statistically fewer ischemic events [11].

Moreover, Goto *et al* have investigated the safety and efficacy of Atopaxar in addition to standard therapy in Japanese patients with acute coronary syndrome or high-risk coronary artery disease. Their study concluded that Atopaxar did not increase clinically significant bleeding, although there was a higher rate of any Thrombolysis in Myocardial Infarction (TIMI) bleeding – such as intracranial hemorrhage – with the highest two doses (100 mg and 200 mg of Atopaxar). Again, all doses tested achieved a significant level of platelet inhibition [12]. As such, our study expects similar results in the mice treated with Atopaxar: platelet inhibition to prevent the shielding of CTCs and minor bleeding side effects.

Since research into the use of Atopaxar for cancer treatment has not been conducted previously, further research will be needed to address a few current limitations in this study. These include testing the safety of Atopaxar in humans and the efficacy of Atopaxar compared to other standard treatments in cancer therapy. In addition, the optimal dosage for the highest efficacy of Atopaxar with a low incidence of severe side effects will need to be determined. Although further study is needed, PAR-1 antagonism may have the potential to provide a novel pathway for platelet inhibition to add on to the current standard therapies for cancer treatment. This study sets a foundation and outlines a possible treatment for cancer through the focus on one specific pathway by which cancer is able to metastasize successfully.

Conclusions

The results of this study provide a mechanistic rationale for the use of Atopaxar in hindering cancer metastasis, bringing novel insight into the use of PAR-1 antagonists in confining cancer to its primary site in patients and inhibiting CTCs' function as a seed for metastases. Since CTCs will usually be present in the blood even after removal of a secondary tumour, limiting metastasis can significantly improve the prognosis and wellbeing of patients. As a comparison between Atopaxar

and Vorapaxar, the former is shown to induce fewer, life-threatening side effects without any apparent complications [7]. Side effects caused by Vorapaxar, such as intracranial hemorrhage and severe bleeding, can lead to further complications that will only make treatment of the cancer more difficult [7]. In the future, further optimized experiments must be conducted to more accurately simulate cancer metastasis in humans and monitor possible side effects of the treatments. Experimentally confirming that Atopaxar will not have any adverse side effects will be a major step in implementing the drug as a future treatment option for metastatic cancer patients. Additionally, investigations of cancer's many different mechanisms for metastasis and the various inhibition methods that treatments can impose on metastatic cancer will allow for further development of more optimized drugs that can effectively prevent cancer from spreading out of its primary site. Thus, metastatic cancer is not impossible to prevent or hinder; rather, we believe that our research into specific cancer metastasis mechanisms can reveal new therapeutic approaches and drug targets that will allow us to successfully manage and treat metastatic cancer.

List of Abbreviations Used
CTC: circulating tumour cell
TCIPA: tumour-cell induced platelet aggregation
PAR-1: protease-activated receptor-1
GFP: green fluorescent protein
CD8: cluster of differentiation 8
LLC1: Lewis lung carcinoma cells

Conflicts of Interest
The author declares he has no conflicts of interest.

Ethics Approval and/or Participant Consent
This study did not require ethics approval or participant consent as it is a proposed research protocol. The research has not been conducted and no participants were involved.

Authors' Contributions
DS: made contributions to the topic idea, designed the methodology, interpreted the expected results, drafted and revised the manuscript critically, and gave final approval of the version to be published.

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