Determining potential anti-cancer properties of a leaf species with rainforest origins

DISCOVER BATH PROGRAMME

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Contents

Introduction

Cancer is a serious worldwide disease that can develop regardless of age, sex, or ethnicity and is the second leading cause of death in the US. (*American Cancer Society*, 2020) In addition to the person with cancer, the implications for those surrounding them can also be drastic. Due to the multiple distinct types, the fatality rate, and the commonality of the illness, it is no wonder that researchers are constantly working on more effective and efficient cancer treatment.

Exploration of tropical rainforests is one method of developing potential new treatments to combat cancer. There are an estimated 100 million plant and animal species on Earth, of which only 1.7 million have been identified thus far. Rainforests massively contribute to this, containing the most species diversity. In addition, they play a key role in climate regulation, indigenous communities, and drug discovery. (*Aljerf et al*, 2017) As a result, rainforests such as the Amazon are the primary natural drug resource on the planet, even dubbed a 'medicinal treasure chest'. (*Skirycz et al*, 2016)

Many currently used cancer drug therapies originated in tropical rainforests. One example being Taxol which is derived from the Pacific yew tree. Taxol is used to treat a variety of cancers, including breast, ovarian, lung and pancreatic cancer by stopping cell division, therefore reducing cancer cell growth. This highlights the significance that tropical rainforests can have within the medical industry today. (*Sanders et al*, 2014)

When a potential drug is found to possess anti-cancer properties, it must undergo clinical trials before being manufactured for medical use. Clinical trials involve several stages of testing to ensure the final product is effective and safe to use. Animal testing may occur prior to being tested on a small group of healthy people to determine safety. Phase two involves a larger group of people to also assess effectiveness and side effects. Hundreds or thousands of patients will then trial the drug for comparison to the existing treatments. If successful, the drug will be licensed and eventually be available for manufacture and prescription. A last trial can be used to assess wider use of the drug and any long-term complications following distribution. (*UK Clinical Research Collaboration*, 2014)

Only a minority of drugs will reach manufacture and distribution. On average 9 out of 10 potential compounds for drug therapies are rejected prior to even entering the first trial phase. This demonstrates that continual drug discovery is needed to advance the current cancer drug treatments available. (*Dowden & Munro*, 2019) Unfortunately, the incidence, mortality and survival rates for major types of tumour including lung and pancreatic, are not seeing significant improvement and there are calls for more effective treatments. (*Arnold et al,* 2019)

For the GFP protein to be useable in trials, it must go through several steps. The bacteria samples are spread onto petri plates using aseptic techniques and the colonies are screened under UV light after 48 hours. A sample of green (GFP) colony and a sample of a white (non-GFP) colony is collected and incubated or shaken for 24 hours at 32°C and then separated from the liquid cell culture in a centrifuge. Enzymes digest the cell wall, exposing the protein for extraction and purification, after which the protein can be tested.

Methodology

When a natural source is thought to have potential cancer treating properties, it must undergo a process before it is ready for use in any sort of clinical trials or testing. In this case, the GFP protein within leaves from a plant species located in a tropical rainforest is the desired sample. To isolate the protein, it is necessary to extract DNA segments, before multiplying with PCR and then producing bacterial colonies.

Cloning

To obtain the bacterial colonies necessary for producing the GFP protein, plasmid vectors containing the leaf DNA must be embedded. Then, using aseptic technique, the E. coli bacteria is spread onto petri plates and allowed to develop into colonies over a number of days. The typical generation time for E. coli in standard laboratory conditions is 15-20 minutes. This means that under standard conditions during the exponential growth phase of bacterial cell replication, when the cells are dividing regularly, it takes between 15 and 20 minutes for the cell population to double. (*Todar et al*, 2020) Leaving the colonies for an additional amount of time ensures they have all had time to progress through the stages of replication enough for a reasonable, reliable sample to be taken.

Screening

After 48 hours have passed, the colonies are screened using UV light. With exposure to UV light, the colonies containing the GFP gene will fluoresce green, enabling identification of which colonies contain the desired gene. Using sterile inoculation loops, a sample can be collected from a single green bacterial colony and a single white bacterial colony before being placed into two separate tubes labelled "+" and "-" for green and white respectively. The colony samples are then either shaken or incubated for 24 hours at 32°C or 48 hours at room temperature. Leaving the samples in ambient or warmer temperatures prevents denaturing and promotes cell growth.

Centrifuge

To separate the bacteria cells from the liquid cell culture, a centrifuge is required. As the centrifuge spins, gravity will separate the substances within the machine according to mass. This works due to the process of centrifugation, in which the less dense substances (liquid) will deviate away from the axis (upwards in this case), and the denser substances (bacterial cells) will move towards the axis (downwards in this case). (*Majekodunmi et al*, 2015) The bacterial cells will then collect at the base in the form of pellets, away from the liquid cell culture.

Release protein

Once the liquid has been removed, the protein can now be released from the living cell cultures using Lysozyme. This enzyme catalyses the hydrolysis of certain carbohydrate bonds in cell walls, essentially digesting them. Once the cells have been lysed, the proteins will be exposed for purification.

Purification

Finally, the GFP proteins are purified by passing through a hydrophobic interaction column. The proteins will stick to the hydrophobic beads as the remaining bacterial proteins flow past. Following removal of the salt, the proteins will eventually drip out of the column, ready for clinical testing.

Results

Varying dosages of the GFP treatment were administered to 11 of the rats, the other 11 received dosages of a placebo treatment. The number of tumours on the rats prior to and post administration can be seen in Table 1.

Table 1: Results of GFP administration on rat tumours

Rat number	Treatment administered to rat	Volume of treatment administered to rat (µl)	Number of tumours post treatment
1	GFP protein	1000	12
2	GFP protein	1700	6
3	GFP protein	1100	12
4	GFP protein	1600	7
5	GFP protein	1200	10
6	GFP protein	1900	5
7	GFP protein	1300	10
8	GFP protein	1800	8
9	GFP protein	1400	7
10	GFP protein	1500	6
11	GFP protein	2000	4
12	Placebo	1000	13
13	Placebo	1700	13
14	Placebo	1100	12
15	Placebo	1600	14
16	Placebo	1200	15
17	Placebo	1900	15
18	Placebo	1300	14
19	Placebo	1300	16
20	Placebo	1400	9
21	Placebo	1500	12
22	Placebo	2000	12

The volume of placebo adminstered against the volume of GFP treatment administered is shown in Figure 1. Figure 2 shows the number of tumours on each rat post treatment of either the placebo or GFP.

Figure 1: Relationship between volume of placebo administered and volume of GFP protein administered

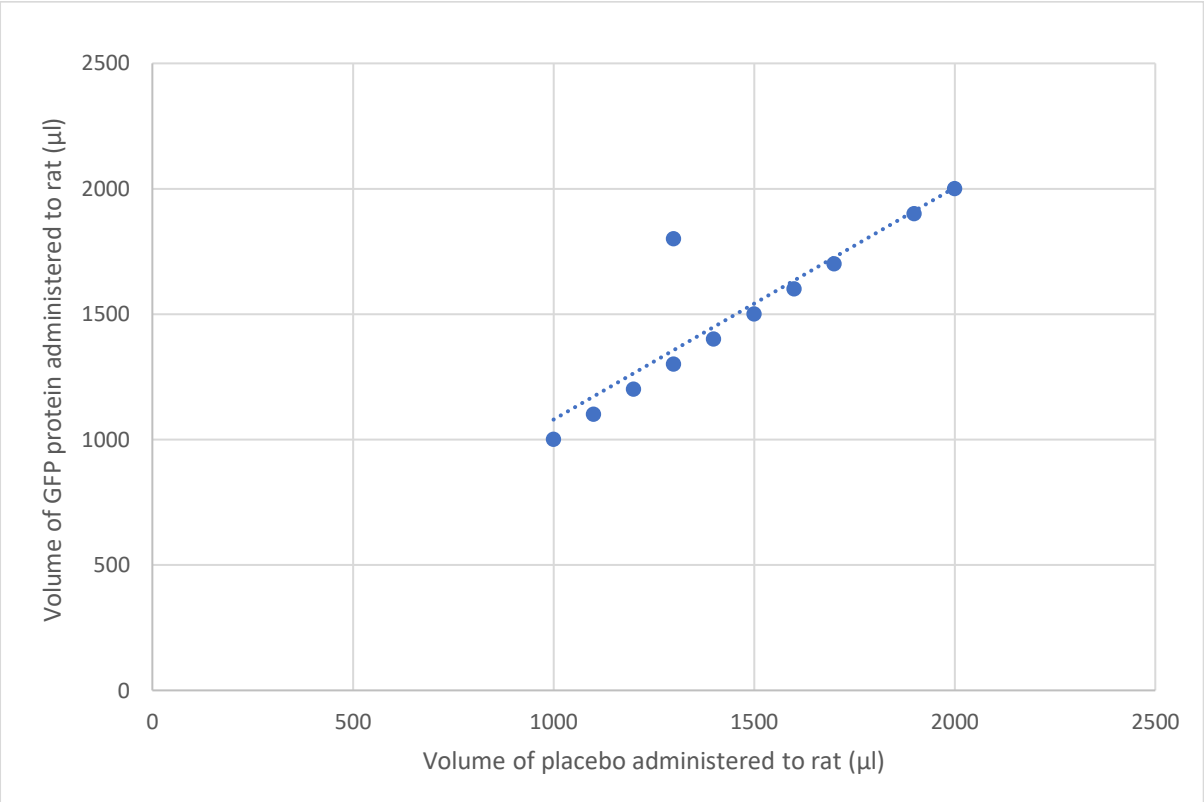
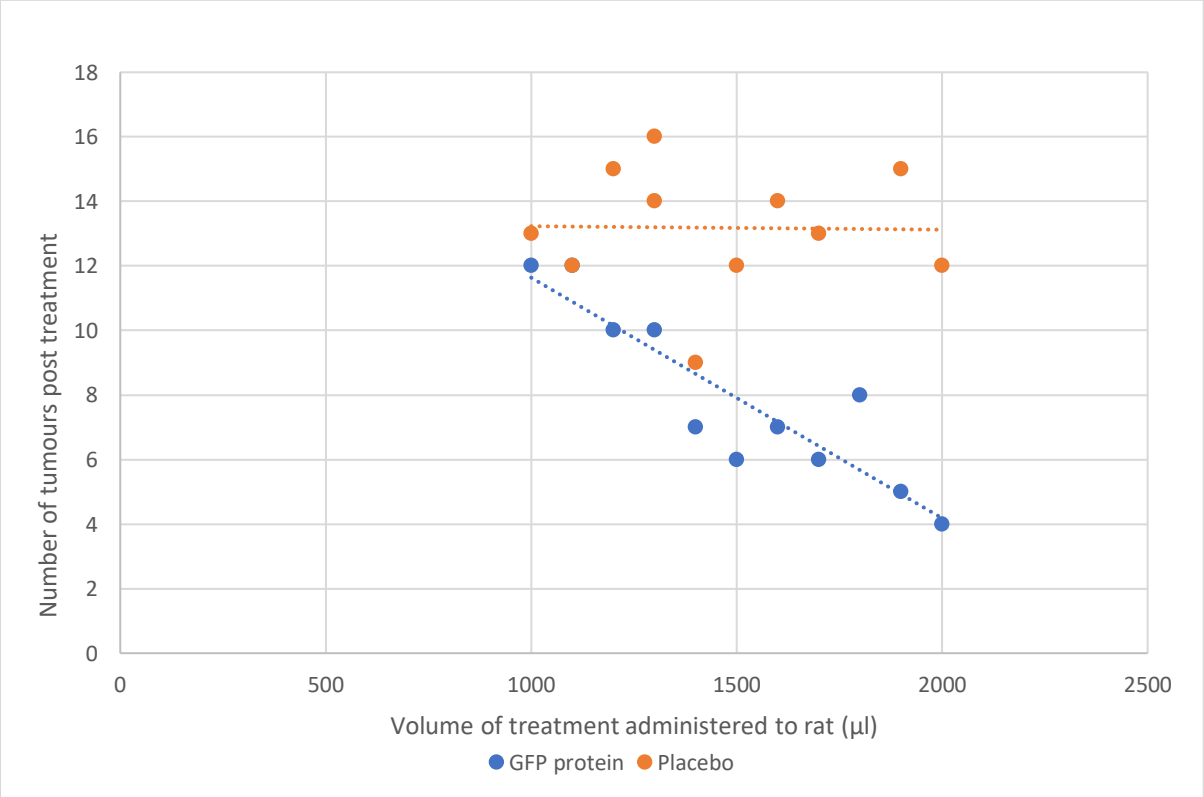


Figure 2: Effect of volume of treatment administered on number of tumours



Results Analysis

Figure 1 shows that generally the volume of placebo administered to each rat matched the volume of GFP treatment given to a different rat. The presence of an outlier can be seen, representing that rats 18 and 19 were given the same volume of placebo. This could have occurred for a number of reasons and does not affect the overall trend. Figure 2 displays the relationship between the volume of treatment administered and the number of tumours each rat exhibited post treatment. The data for the rats given a placebo suggests there is no correlation – as expected – compared to the data points for the rats given the GFP, which forms a clear downward line. Implying that as the dosage of the GFP treatment increased, the number of tumours decreased.

To evaluate the credibility and significance of this trend, a Spearman's Rank Correlation Coefficient can be calculated. To do so, the number of tumours present following the treatment and the volume of treatment must be ranked, enabling a difference between them to be found and eventually squared. This is represented in Table 2.

Table 2: Calculating components for Spearman's rank correlation coefficient from results

Rat number	Volume of GFP protein administered to rat (µl)	Volume ranking	Number of tumours post treatment	Number ranking	D	D^2
1	1000	1	12	10.5	-9.5	90.25
2	1700	8	6	3.5	4.5	20.25
3	1100	2	12	10.5	-8.5	72.25
4	1600	7	7	5.5	1.5	2.25
5	1200	3	10	8.5	-5.5	30.25
6	1900	10	5	2	8	64
7	1300	4	10	8.5	-4.5	20.25
8	1800	9	8	7	2	4
9	1400	5	7	5.5	-0.5	0.25
10	1500	6	6	3.5	2.5	6.25
11	2000	11	4	1	10	100

Calculating Spearman's Rank Correlation Coefficient:

$\Sigma D^2 = 90.25 + 20.25 + 72.25 + 2.25 + 30.25 + 64 + 20.25 + 4 + 0.25 + 6.25 + 100$

$= 410$

$r_s = 1 - 6\,\Sigma D^2 \div n(n^2 - 1)$

$= 1 - 6(410) \div 11(121 - 1)$

$= 1 - 2460 \div 1320$

$= -0.8636$ (4 s.f.)

As -0.8636 is a negative number that is relatively close to -1, it can be said that there is a fairly strong negative correlation between the volume of drug administered and the number of tumours that developed post treatment.

Calculating Probability:

n = 11

p = 0.05

Critical value = 0.618

0.8636 > 0.618

The r_s value is greater than the critical value, meaning we can reject the null hypothesis that there is no significant difference. There is a 95% probability that the negative correlation between the volume of treatment administered and the number of tumours that developed post treatment was not due to chance. We can confidently say that there is a strong negative correlation.

Discussion

This study explored whether the GFP protein developed from a species of leap discovered in a tropical rainforest has anti-cancer properties. The key findings suggest that administering this GFP protein significantly reduced the number of tumours present in rats, with a strong negative correlation (r_s = -0.86, p = < 0.05). This is significant because it means that the correlation is 95% certain. Hence, there is only a 5% possibility that the decrease in number of tumours paired with the increase of drug volume administered was due to chance. This allows the conclusion to be drawn with 95% certainty that the protein has cancer treatment properties. The presence of a statistical outlier (where a rat received a lower than expected volume of placebo) does not appear to affect the overall correlation.

The findings of this study are in line with the results of previous studies conducted into the effectiveness of potential anti-cancer treatments extracted from plants. A study conducted in 2013 focused on 'The Anti-Cancer Property of Proteins Extracted from Gynura procumbens' and found that the proteins extracted from the traditional South East Asian herb inhibited the growth of a breast cancer cell line. Due to this result and other proven benefits, they were able to conclude that the herb possesses anti-cancer properties although the exact mechanism is not clear. (*Hew et al,* 2013)

An article published in early 2017 systematically identified and collated data from 179 relevant studies focused on developing potential cancer treatments from plants and reviewed their findings. They evaluated the mechanisms by which extracts from the plants inhibited cancel cell replication and colony growth, concluding that the antioxidant compounds within the extracts could inhibit cell cycle and proliferation. However, as with other common cancer treatments, there may be side effects and harms associated with the use of these extracts. (*Kooti et al,* 2017)

As with any study of this nature, there are a few unavoidable limitations. To improve the degree of accuracy and determine the repeatability of the results recorded, multiple stages and repeats of testing would be encouraged, with as many patients as possible. Due to the fact that this potential drug is in its infancy of testing, one of the main problems arising is how effective or even successful the treatment will prove in humans. The nature and severity of any potential side effects exhibited in humans would also need further exploration.

Conclusion

The search for breakthroughs in cancer treatment and prevention is more important now than ever, with discoveries being made across the globe. Every available avenue needs to be explored, one being the life within tropical rainforests. The countless number of species residing in these habitats yields immeasurable potential when it comes to drug discovery. This study aimed to pursue one possible route, with the testing of a leaf species containing the Green Fluorescent Protein. The findings from this study suggested that, following administration of treatment containing the GFP, the number of tumours in rats decreased significantly. Though the findings of this particular study were successful, further research to bring about greater understanding of the mechanisms used with the protein would need to be done for more clarity and to allow effective use of the treatment. Additionally, as this study was conducted using a rat model, to determine the efficiency, safety and side effects of this treatment in humans, further trials should be done. Although this is a first step in drug discovery form the rainforest, it demonstrates the promise that such paths may hold.

References

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Pro Forma

Self-Reflection

The course was split into 8 weeks of material, each with a dedicated task to complete that would build up to form a hypothetical report of a study focused on drug testing and development. I followed the structure of the course, using the various resources provided to gain context and further my understanding of each task before completing and handing in for feedback. Following each piece of feedback and guidance, I adjusted my work accordingly until finally a full report was put together.

The current global circumstances – the COVID-19 pandemic – meant that the Discover Bath programme wasn't able to go ahead in the same way as originally planned. Instead of a weeklong residential stay in Bath with frequent trips to the university and team-based learning, the course proceeded as individual tasks set through Microsoft Teams. There were a few challenges when carrying out this programme, mostly due to the technical circumstances of getting to grips with navigating Microsoft Teams and communicating through its various channels. Though not a verified problem, it was a shame that we were unable to experience the programme in person at Bath as originally planned. Getting to know the university, working in the labs, and meeting new people were all things I was looking forward to.

Personally I found it challenging to balance the requirements of the work as well as the demands of A Level studies that were continuing online and progressing into the content of Year 13 curricula. Over the course of the project, I developed confidence in my own work with the knowledge it was reliable and done to a good standard. Additionally, I found that I furthered my written communication skills in explaining the situation and seeking guidance directly. I also am now confident using the desirable skills that will become extremely useful in further study, such as the Harvard referencing system, paraphrasing text, navigating academic publications, and presenting statistical analysis effectively. The specific topic chosen was one I found very interesting and thoroughly enjoyed learning more about the drug development industry and the science behind it. I now feel secure in my decision to pursue this field of study at university and possibly further, due to this experience and getting a taste of the world of academic biology and its significance.

Project Implementation Log

Student Name: Caitlin Shepherd

Student Email: caitlinlucyshepherd@gmail.com

The project you have decided on: Determining potential anti-cancer properties of a leaf species with rainforest origins

How was this project challenging for you:

The premise of undertaking an entire research project (aside from the actual clinical testing) completely independently seemed daunting. I'd never read a research paper or academic article prior to this programme, let alone reviewing and referencing one. Delving directly into the already established world of academic research was fairly intimidating.

Necessary equipment/software/other resources required and how you obtained them:

The key resources I utilised were internet access for gathering information in articles and publications, as well as Microsoft Teams for guidance, resources and feedback.

Initial research summary detailing what you have done so far in researching this project, including all relevant links and websites:

To gain some primitive context of the process, I researched the stages of clinical testing and how it fits into drug development. To further my understanding of this project specifically, I investigated protein extraction, centrifuge mechanics and bacterial colony growth patterns.

https://www.ukcrc.org/wp-content/uploads/2014/03/iCT_Booklet.pdf

http://article.sapub.org/10.5923.j.ajbe.20150502.03.html

http://textbookofbacteriology.net/growth_3.html

Ideas of what extra research you intend to do over the next few weeks

For each stage of the report I may need more understanding or knowledge to allow me to write about it and relate it to this specific study. This could include handling data, presenting it appropriately, and correct analysis and evaluation of results.

Planned work	**Deliverable**
Plan and write introduction - Introduce tropical rainforests and their relevance - Research examples of developing drugs from rainforests - Explain the process of development from testing to manufacture - Summarise methodology of extracting the required gene	Update implementation log Hand in introduction for feedback
Research stages of method, write explanation - Cloning bacteria - Screening colonies - Centrifuge - Extracting the protein - Purifying the protein	Update implementation log Hand in methodology for feedback
Present results clearly and appropriately. Create graphs to display data accurately and demonstrate any correlations.	Hand in results for feedback Update implementation log
Analyse results, present findings, and explain their significance. Calculate the degree of correlation and probability of the correlation occurring.	Update implementation log Hand in results explanation and analysis for feedback
Write discussion and conclusion - Summarise findings and significance of results - Compare with other studies, does it fit with their conclusions - Any limitations of the methodology and overall process - Evaluate if and how well the original question was answered	Hand in discussion for feedback Update implementation log
Collate the entire report together Finish pro forma, bibliography, header page, contents Complete whole report.	Hand in final report of the study.