PREVENTION OF SPONTANEOUS TUMORS BY PHARMACOLOGICAL INHIBITION OF THE EPIDERMAL GROWTH FACTOR RECEPTOR

An Undergraduate Research Scholars Thesis

by

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Approved by Research Advisor: Dr. David W. Threadgill

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Major: Biomedical Science
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ABSTRACT

Prevention of Spontaneous Tumors by Pharmacological Inhibition of the Epidermal Growth Factor Receptor

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The epidermal growth factor receptor (EGFR) is a ligand-activated tyrosine kinase receptor that is often overexpressed in cancers and plays a significant role in cell proliferation and inhibition of apoptosis. Mouse model studies have demonstrated the ability of EGFR inhibition to hinder spontaneous colorectal cancer occurrence by 50-80% when EGFR inhibition is induced before the establishment of tumors.1 The success rates reported in human clinical trials differ from mouse model studies, with a 15% success rate among colorectal cancer patients receiving anti-EGFR treatment.2 Timing of treatment is the major difference between animal/mouse trials and human therapeutic treatment of end-stage cancers. In our study, it was hypothesized that earlier preventative doses of the AG1478 chemotherapy inhibitor would reduce the frequency of spontaneous tumor occurrence in a preventative, rather than therapeutic, manner in a murine population. This current study provides additional specificity and histopathological analysis of the spontaneous tumor occurrence in a large murine sample of four genetically distinct wild-type strain mice to better simulate a variable human population. Such specificity includes, but is not limited to, the number, type and severity of the tumors that did
occur and whether the concentration of the drug is correlated to a reduction in tumor occurrence.

Such information will broaden the discussion of EGFR’s role in tumor treatment.
ACKNOWLEDGEMENTS

This project is the culmination of numerous undergraduate research experiences that were made available to me over the past two years in the Threadgill Lab by Dr. David Threadgill and my team leader, PhD candidate Selene Howe. I owe both of you my thanks for your continued support and encouragement. Thanks are also due to Dr. Rachel Lynch for making the Threadgill Lab such a welcoming environment for undergraduate students, Dr. Amie Perry for her histopathology work analyzing the tumor tissue in this study, and the past and present Team Baymax members, including Ashtyn Zapletal and Justin Dam, and other members of the Threadgill lab.
NOMENCLATURE

AG1478  EGFR kinase inhibitor chemotherapy used in this murine experiment
A/J     One of four strains of mice studied in this project
Apc\textsuperscript{Min/+}  Heterozygous for the hyperactive oncogenic allele leading to colorectal cancer
BALB/cJ One of four strains of mice studied in this project
C57BL/6J One of four strains of mice studied in this project
EGF     Epidermal growth factor
EGFR    Epidermal growth factor receptor (protein)
EGFR-TKI Epidermal growth factor receptor tyrosine kinase inhibitor
\textit{Egf}_{wa2/wa2}  Homozygous for the hypomorphic allele of EGFR
FVB/NJ  One of four strains of mice studied in this project
\textit{IC}_{50}  Half maximal inhibitory concentration measures a substance’s effectiveness to inhibit a specific biological or biochemical process
Mitotic index Ratio of the number of cells undergoing mitosis to the total number of cells
\mu m    Micrometer (1x10\textsuperscript{-6} meters)
Murine  Mouse model (study)
CHAPTER 1
INTRODUCTION

The ability of EGFR inhibition to hinder spontaneous tumor occurrence has been demonstrated by multiple studies which have expanded the body of knowledge about the efficacy of such treatment.\textsuperscript{1,3} Previous research shows a strong, positive correlation between EGFR over activity and the occurrence of several types of cancers prevalent in humans, including colorectal cancer,\textsuperscript{4} pulmonary adenocarcinoma,\textsuperscript{5} head and neck cancer,\textsuperscript{6} soft tissue sarcoma,\textsuperscript{7} prostate cancer,\textsuperscript{8} glioblastoma,\textsuperscript{9} and breast cancer.\textsuperscript{10}

However, in human subjects, EGFR inhibition is generally used only in terminally ill cancer patients, due to known, and possible unknown, long-term consequences of EGFR inhibition. As a result, EGFR inhibition as a preventative and therapeutic chemotherapy have often been studied through murine genetic model experiments. Mouse trials have shown that EGFR inhibition can hinder spontaneous tumor occurrence by 50-80\% when EGFR is inhibited before the establishment of tumors.\textsuperscript{1} By contrast, later human therapeutic treatment has shown a 15\% success rate among colorectal cancer patients receiving anti-EGFR treatment for end-stage cancers.\textsuperscript{2} In our study, the mice receive preventative doses of the chemotherapy inhibitor AG1478 at two months of age.

Mice with hypomorphic \textit{Egfr}\textsuperscript{wa2/wa2}, or hyperactive oncogenic \textit{Apc}\textsuperscript{Min/+} alleles, are were previously the subjects in genetic models to study anti-tumor capabilities of EGFR inhibition and have shown significant results. The hypomorphic \textit{Egfr}\textsuperscript{wa2/wa2} allele, for example, showed a 90\% reduction in murine colorectal cancer,\textsuperscript{3} while an 80\% reduction in murine polyps was seen in an \textit{Apc}\textsuperscript{Min/+} model.\textsuperscript{1} The preventative opportunities of AG1478 chemotherapy are the subject of this
study, using four genetically-distinct strains of wild-type mice which receive AG1478 drug administration (0mg control, 5mg, 50mg, or 144mg). Wild-type A/J, BALB/cJ, C57BL/6J, FVB/NJ mice are used in this study because expected frequencies of spontaneous tumor occurrence allowed for the determination of the efficacy of AG1478.

**Objectives**

As there are numerous gaps in the knowledge base concerning EGFR inhibition, this study uses histopathological analysis of four genetically-distinct wild-type strains of mice predisposed to cancer to study tumor development and growth by assessing the extent to which, if any, factors such as sex, genetics, and dosage of medication impact tumor development and growth. We hypothesize that the positive correlation between EGFR inhibition and the occurrence of cancer will result in the treatment group of mice having a lower occurrence of cancer, whether such drug administration is at the therapeutic or subtherapeutic dosage level, when compared to the control group of mice that do not receive the drug AG1478 chemotherapy.
CHAPTER II

METHODS

This study utilized 640 total mice, evenly divided among four genetically-distinct strains predisposed to certain cancers, as shown in Table 1. Table 2 shows the expected spontaneous tumor occurrence of each genetically distinct mouse strain.

Table 1. Number of mice in each cohort by genetic strain, sex and dosage level.

<table>
<thead>
<tr>
<th>640 total mice</th>
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<tbody>
<tr>
<td>160 per genetically-distinct strain A/J, BALB/cJ, C57BL/6J, FVB/NJ</td>
</tr>
<tr>
<td>80 per sex per strain (male or female) of each of A/J, BALB/cJ, C57BL/6J, FVB/NJ</td>
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<tr>
<td>20 per dosage level per sex per strain (dosages 0 mg/kg control, 5 mg/kg subtherapeutic, 50 mg/kg subtherapeutic, &amp; 144 mg/kg therapeutic)</td>
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</table>

Table 2. Expected spontaneous tumor occurrence of each genetically distinct mouse strain [from the Jackson Lab MGI (mouse genome informatics) website] (http://www.informatics.jax.org/phenotypes.shtml).

<table>
<thead>
<tr>
<th>Expected Spontaneous Tumor Occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A/J</strong></td>
</tr>
<tr>
<td>● Mammary gland (28% of females)</td>
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<tr>
<td>● Lung (90% by 18 months of age)</td>
</tr>
<tr>
<td>● Muscle/soft tissue (79% after 20 months of age)</td>
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</table>
Wild-type A/J, BALB/cJ, C57BL/6J, and FVB/NJ mice were used in this study because the known frequencies of spontaneous tumor occurrence could be used to determine the efficacy of AG1478. The spontaneous tumor predispositions of the four strains of mice to various cancers are shown in Table 2 above. Chi-squared and Pearson's tests were used to determine the statistical significance and efficacy of AG1478 with respect to pulmonary adenomas and soft tissue sarcomas at varying doses in the A/J strain, as shown in Figures 6 and Table 4. Such information is not available at this time for other tissue types with respect to the A/J strain, and any tissue types with respect to the BALB/cJ, C57BL/6J, and FVB/NJ strains.

**Administration of Medication**

In addition to an untreated control group, other groups of mice received alternative doses of the AG1478 drug, which blocks ATP from binding at the kinase domain of the receptor. Such doses were incorporated into the mouse chow. AG1478 is very specific to EGFR (IC₅₀ = 3 nM), and we therefore hypothesized that a subtherapeutic dosage might yield significant results due to the minimal amount of drug necessary to inhibit EGFR. Each group of 20 mice, distinguished by sex for each genetically-distinct strain, received drug administration (0mg control, 5mg, 50mg, or 144mg) starting at two months of age and ending at 18 months of age. Actual medication dosage for an individual mouse might have varied from the group average, as a result of the mice being fed from a common, abundant food supply per cage housing 4-5 mice and individualized altered feeding behavior due to possible factors such as the taste and neurologic effects of the drug. The calorimetry chamber machine measured the average amount of food eaten, categorized by the four strains of mice.

The mice were fed Research Diets, D12079B formula, [high fat (21% gm% and 41 kcal%), high carbohydrate (50% gm% and 43 kcal%) and low protein diet (20% gm% and 17&...
kcal%), referred to as a ‘Western’ diet, to better simulate a common diet of the American population.

**Euthanasia and Necropsy**

The majority of mice were euthanized by carbon dioxide inhalation after receiving 16 months of AG1478, some mice were found deceased and their tissue was discarded, and other mice were euthanized by carbon dioxide inhalation and necropsied early due to aggressive tumors that visibly inhibited their quality of life. Tissue was harvested post-necropsy. Preliminary analysis of this tissue provided the initial assessment of cancer and hyperplasia occurrence, with measurement of weight, size and tissue coloration to assess potential abnormalities and visual examination.

**Tissue Handling**

At necropsy, all tissue was organized and placed in histology cassettes. The cassettes were placed into containers of formalin where they were fixed for 48 hours before being transported to the Texas A&M Department of Veterinary Pathobiology Histology Laboratory for processing. In this lab, the tissue was processed, including the absorption and homogenous infusion of paraffin wax into the tissue in order to allow for easily hydrated tissue that could be evenly sectioned into 5µm thick segments, without tearing. The lung, lymph node, and spleen tissue were then individually placed in a metal mold, and liquid paraffin wax was poured over the tissue to orient it in an optimal position for sectioning. Spleen and kidney tissue required an additional step of cutting the organ in half with a scalpel, in order to produce a full view of the inside of the organ without wasting tissue due to excess sectioning. The embedded tissue was cooled, trimmed, sectioned via microtome to a thickness of 5 µm, placed on the surface of a hot water bath at 42.0 degrees Celsius, placed onto a glass slide, and dried overnight.
Slide Preparation

The slides underwent a staining process which first involved the chemical removal of excess paraffin wax by xylene, acid wash (1% HCl and 99% ethanol), and various concentrations of ethanol (ranging from 70% to 100%). The remaining tissue was stained first with hematoxylin, which stains the nucleus blue, and next with eosin, which stains the cytoplasm red, and then viewed under a compound microscope.

Histopathological Analysis

Slide production sequence was determined by two criteria, with the first being those mouse line strains which are most predisposed to developing adenomas and are thus most likely to be affected by EGFR inhibition, such as BALB/cJ. The second criteria for earlier slide production was any tissue that was visually noted as appearing abnormal in a way that indicated it was potentially cancerous.

This initial assessment determined the sequence in which such stained tissue was submitted to further examination under microscope by Dr. Amie Perry, a veterinary anatomic pathologist (DACVP 2015) who is pursuing a PhD in veterinary pathobiology at Texas A&M - College Station, Texas. Dr. Perry identified abnormalities in the type of tumor, aggressiveness of growth (using a Mitotic index methodology), and presumed direction of growth of any cancerous tissue.
CHAPTER III

RESULTS

Histological examination of tissue, including lungs and various soft tissue masses, was performed to quantify and categorize tumor incidences. Tumor tissue was evaluated both grossly by visual inspection, and histopathologically using H&E staining and a compound microscope, to draw conclusions on the severity, tissue sensitivity, timing, sex preference, and strain preference of the chemotherapy at different dosages. Figures 1 - 5 below show examples of such abnormal tissue. Results showed that subject sex and medication dosage impacted tumor development and growth in the subject wild mice, as detailed in Tables 3 and 4, as well as Figure 6.

Figure 1. Lung of A/J mouse (50 mg/kg dose). Pulmonary adenoma, 1mm diameter (20X magnification) 50 mg/kg dose.
Figure 2. Hindlimb of A/J mouse (50 mg/kg dose). Soft tissue sarcoma invading muscle (100X magnification) 50 mg/kg dose. This slide shows the migration of bone marrow cells invading the hindlimb muscle. This reveals the severity of this sarcoma and the likelihood of metastasis for similar cancers in this mouse.

Figure 3. Hindlimb of A/J mouse (0 mg/kg). Soft tissue sarcoma with high Mitotic index. Red circles indicate mitotic cellular divisions (400X magnification) 0 mg/kg dose. This slide shows a high number of cells (seven) in the “mitosis” phase of the cell cycle. This Mitotic index indicates that these cells are proliferating more continuously than is typical and thus are likely cancerous.
Figure 4. Evidence of hyperplasia (abnormally large growth of white pulp) in splenic tissue of a C57BL/6J mouse at 13 months (5X magnification).

Figure 5. Evidence of white pulp hypoplasia in splenic tissue of 13-month old C57BL/6J mouse at 5X magnification. (Note, to better visualize this evidence, the brightness and contrast were adjusted slightly, on both the microscope and computer image, after the image was captured.)
Table 3. Number of A/J male and female mice that survived until the end of the 16-month study (mice were 18 months of age).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gender</th>
<th>Control pulmonary adenoma occurrence</th>
<th>5 mg adenoma occurrence</th>
<th>50 mg adenoma occurrence</th>
<th>144 mg adenoma occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/J</td>
<td>Female</td>
<td>15</td>
<td>12</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>A/J</td>
<td>Male</td>
<td>14</td>
<td>16</td>
<td>17</td>
<td>16</td>
</tr>
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</table>

Figure 6. The frequency of A/J pulmonary adenomas of two cohorts (A/J male and A/J female) necropsied at 18 months of age. The significance bar (p=0.0443 with 95% certainty) indicates that the 144 mg/kg female A/J cohort had a statistically significant reduction in the frequency of pulmonary adenomas, but the male A/J cohort did not.
Table 4. The frequency percentages of A/J soft tissue sarcomas of two cohorts (A/J male and A/J female). The data includes A/J mice necropsied at 18 months of age. Using Chi-squared and Pearson’s tests, it was determined that neither the therapeutic nor subtherapeutic doses of AG1478 to the A/J population resulted in a statistically significant reduction of the frequency of soft tissue sarcomas. An inverse relationship between AG1478 drug concentration and spontaneous soft tissue sarcomas was more prevalent in the female A/J cohort, however, a statistically significant reduction was not observed in either cohort.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sex</th>
<th>Tumor Type</th>
<th>Control Frequency</th>
<th>5 mg Frequency</th>
<th>50 mg Frequency</th>
<th>144 mg Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/J</td>
<td>Female</td>
<td>Adenoma</td>
<td>26.3%</td>
<td>22.2%</td>
<td>26.3%</td>
<td>17.6%</td>
</tr>
<tr>
<td>A/J</td>
<td>Male</td>
<td>Adenoma</td>
<td>18.8%</td>
<td>11.1%</td>
<td>5.9%</td>
<td>21.1%</td>
</tr>
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</table>
CHAPTER IV

CONCLUSION

Preliminary results support the hypothesis that subject sex and medication dosage will impact tumor development and growth arising from EGFR overexpression in wild mice, under certain circumstances. Specifically, outcomes for the female A/J mice, but not for the male A/J mice, show a significant reduction in adenomas, but only for the highest medication dose group ($p=0.0443$ with 95% certainty). As histopathological analysis is not complete at this time and has only been done on spleen and lung tissue from approximately 200 (BALB/cJ + A/J) of the 640 mice, our conclusions may change as we analyze additional tissue samples.

It was hypothesized that long term exposure of preventative subtherapeutic doses of either 5mg or 50mg of AG1478 would reduce the frequency of spontaneous tumor occurrence in a preventative, rather than therapeutic, manner. However, Chi-squared and Pearson's tests were used to determine that such subtherapeutic doses did not show statistical significance and efficacy of AG1478 with respect to reduction of pulmonary adenomas and soft tissue sarcomas in the A/J strain. At this time, we are unable to draw a conclusion as to whether the subtherapeutic doses were able to significantly reduce the occurrence of tumor formation in the remainder of the wild-type murine population.

As histopathological analysis is not complete on the BALB/cJ, C57BL/6J and FVB/NJ mice, no conclusions on such strains have been drawn to date from this study.

One reason why the A/J female cohort might be more responsive to EGFR inhibition than was the male cohort is hormonal (estrogen, progesterone) differences between murine males and females, which is also observed in human populations/studies. These hormones lead to cellular
proliferation through a mechanism known as “EGFR cross talking.” The pulmonary adenomas observed in the A/J population depend on EGFR to elicit uncontrolled cellular proliferation, since the inhibition of EGFR in this population resulted in statistically significant reduction. There are many possible reasons for the lack of statistically significant reduction in the frequency of soft tissue sarcomas in the A/J population. One possible reason is that the soft tissue sarcomas may have been independent of the EGFR activation process. Therefore, these soft tissue adenomas would depend on alternate pathways (such as insulin-like growth factor or MAPK proliferation cascades) rather than the EGFR-TK proliferation cascade. Another possible reason is that these soft tissue sarcomas bypassed the EGFR protein/ligand activation yet found other mechanisms of activating the EGFR proliferation cascade downstream of the receptor. These hypotheses could be answered using qPCR analysis at a later date.
REFERENCES


