PLANARIAN REGENERATION IN RESPONSE TO DRUG DISRUPTION OF THE WNT AND MAPK PATHWAYS

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ABSTRACT
The regenerative ability of planarians depends largely on its complex signaling pathways. The Wnt pathway regulates the anterior/posterior (A/P) polarity formation after regeneration, while the MAPK pathway plays a role in anterior regeneration. This experiment uses various drugs to disrupt the aforementioned pathways. Imatinib targets the receptor tyrosine kinases (RTKs), a common type of surface receptors that play a role in the Wnt pathway. PZQ is expected to affect the Wnt noncanonical calcium pathway. EHT 1864 inhibits Rac1, a GTPase involved in the noncanonical PCP pathway. Finally, U0126 disrupts the MAPK pathway and blastemic cell differentiation. After drug treatment, abnormal planarian regeneration is expected. The drug assays demonstrated that while both Imatinib and PZQ have no effect on planarian regeneration, EHT 1864 under high concentration has a potent effect on the viability of planarians during regeneration. Furthermore, U0126 caused cyclopia, a condition in which organisms only develop one eye instead of the normal number, in planarians under high concentrations. These observations suggest that the RTKs play a limited role in planarian regeneration, Rac1 plays a greater role than just A/P determination during regeneration, and that U0126 affects eye and head regeneration. Our assays with PZQ also show that different species of planarians might have different noncanonical calcium pathways.

KEY WORDS
Regeneration; Planarian; Drugs; Wnt; MAPK

INTRODUCTION
Planarians have been long known for their regenerative abilities; even a fragment as tiny as 1/279th of the planarian body can fully regenerate into a complete planarian¹. A large number of pluripotent, highly undifferentiated cells called neoblasts are distributed throughout the planarian body; neoblasts are able to differentiate into all planarian cell types and upon injury, differentiate and seem to migrate in response to wound signals². Central to planarian regeneration are the Wnt pathway and the MAPK pathway that determine the
polarity of planarian regeneration after receiving injury. The Wnt pathway can be categorized further into three pathways: the canonical pathway, the noncanonical calcium pathway, and the noncanonical PCP pathway.

The canonical pathway is marked by the presence of the protein β-catenin. The binding of the Wnt ligand to the Frizzled receptor activates the Dsh protein, and through a series of cascades promotes cytoplasmic β-catenin accumulation. β-catenin binds to the transcription factor TCF and acts as a cotranscription factor. Studies on the planarian species S. mediterranea have found that the canonical pathway is active in the posterior end, while it is suppressed by sFRP, a protein expressed in the anterior end. Furthermore, RNAi of the Smed-βcatenin-1 gene, which codes for β-catenin, resulted in posterior head regeneration. It appears that the canonical pathway determines posterior tail formation.

The role of the noncanonical pathways in regeneration is not thoroughly studied. The noncanonical calcium pathway regulates intracellular calcium ion concentrations. It couples Dsh with a G-protein to stimulate production of either PLC or PDE, whose actions ultimately increase intracellular calcium ion levels. In the noncanonical PCP pathway, Rac1, a GTPase, is activated. Studies have shown that Rac1 is involved in actin polymerization, and may play a role in cell structure and early embryogenesis.

Our experiment involved four drugs: Imatinib, PZQ, EHT 1864, and U0126. Imatinib, a common anticancer drug, has been found recently to inhibit RTKs. RTKs phosphorylate the tyrosine components of β-catenin, which in turn stabilizes it, promotes its accumulation and binding to TCF and consequent gene transcription. We hypothesized that the downregulation of the canonical pathway due to Imatinib would cause RTK inhibition, and consequently, posterior head formation during regeneration.

The anthelmintic drug PZQ promotes the release of intracellular calcium ions. Current theory suggests that PZQ targets membrane calcium channels, and through some unknown mechanism, causes an influx of calcium ions into the cell. PZQ affects the Wnt noncanonical calcium pathway; its actions lead to an increased inhibition of gene transcription downstream of the pathway. Previous experiments have shown that PZQ treatment caused planarian posterior head formation. Therefore, we hypothesized that PZQ would cause posterior head growth.

EHT 1864 inhibits the GTPase Rac1 by inhibiting its guanine nucleotide association. This affects various components downstream and causes a number of unknown events. We hypothesized that the inhibition of the Rac1 by EHT 1864 would disrupt the normal functioning of planarian stem cells.

U0126 inhibits MEK1. MEK1 protein functions in the MAPK pathway to activate ERK, which induces blastema cell (planarian stem cell) differentiation. Thus, MEK1 is essential for planarian development. The inhibition of MEK1 by U0126 limits ERK activity and thus limits blastema cell differentiation. Since ERK is primarily involved with blastema cells on the anterior end, we expected abnormal head formation on the planarians treated with U0126.

A diverse set of pathways is involved in planarian A/P polarity formation. The canonical pathway is mainly involved in posterior regulation. Its activation in the posterior end of the planarian promotes transcription of certain genes that facilitate the posterior end to develop into a tail. The noncanonical pathways’ functions are unclear for the most part, but nonetheless they seem to complement A/P polarity formation due to their simultaneous activation with the canonical pathway. Finally, the MAPK pathway has the important role of regulating anterior regeneration. The main objective of the study, as presented previously, is to disrupt these pathways using drugs and to study whether A/P polarity formation is affected.

**MATERIALS AND METHOD**

**Drug Concentrations:**

For the drugs PZQ, EHT 1864, and Imatinib, we prepared five concentrations for each drug: 100 µM, 50 µM, 25 µM, 12.5 µM, and 6.25 µM in spring water supplied by Carolina®. These dilution concentrations were estimations based off of research papers on these drugs and the IC50 of the drugs on non-planarian cells. For U0126, the five dilution concentrations were 10 µM, 5 µM, 2.5 µM, 1.25 µM, and 0.625 µM. U0126 dilutions are 10 times more diluted than the other three drugs because its IC50 is very low compared to the rest. Each drug was given one 6-well plate to house the five dilutions. A control well with only spring water was set up.
within the Imatinib plate. We had an inadequate supply of brown planarians after we had finished transferring the planarians, so we instead decided to use black planarians for the U0126 dilutions.

**Planarian Drug Treatment:**

The planarians used were the brown and black planarians of the species Dugesia tigrina, from Carolina® that had been starved for 24 hours. The planarians were cut on iced plates by removing their heads approximately half way between the anterior end and the anterior end of the pharynx, and their tails at half way between the posterior end and the posterior end of the pharynx. The remaining fragments were washed in spring water and two trunk fragments were pipetted into each well.

The worms were observed and photographed every 24 hours for 8 days using an optical microscope with a camera attached and connected to a computer with appropriate software for image acquisition.

After 48 hours of drug treatment, the worms on each plate were transferred into a new plate with wells containing spring water only.

**RESULTS**

**EHT 1864 disintegrated planarians under high concentration and stunted planarian regeneration**

EHT 1864 was fatal to the planarians at concentrations of 50µM and 100µM. After 18 hours of EHT 1864 treatment at 50 and 100 µM concentrations, the planarians disintegrated (Figure 1A), leaving behind body remnants (For this reason, we did not plate these planarian in the spring water after the 48 hours). In the EHT 25 µM, the worms never fully regenerated (Figure 1B) even after eight days, and seemed to be unable to move. Both worms in the plate squirmed and twitched in place, and failed to respond when prodded with forceps. One of the worms developed a growth on the back after 3 days (Figure 1C); the growth was round and raised on the back. In addition to the growth, the worm seemed to have trouble moving and flipping back over. By the next day, however, the growth disappeared and in its place was a small white dot. There was also a bit of mucus on the end of the worm (Figure 1D). The worms in the 12.5µM well (Figure 1E) exhibited a greater degree of regeneration, with visible eyespots. However, the regeneration appeared to be incomplete when compared to the control (Figure 1F), with the tail regeneration stunted and the anterior head still in the process of regeneration. In the 6.25µM, we found one worm (Figure 1G) that had fully regenerated within a day of being cut.

**Imatinib produced no abnormal changes**

All of the worms in all wells of the Imatinib plate exhibited normal anterior and posterior regeneration. After 72 hours, eyespots were visible in all planarians in the Imatinib plate except for those in the 100 µM well (Figure 2A). After 96 hours (4 days), eyespots were clearly visible on the planarians in all wells including the 100 µM (Figure 2B). By the end of the experiment, after 192 hours (8 days), it was clear that both the head and

![Figure 1 EHT 1864 Treated Planarians](image)

A. EHT 1864 50µM. The worm has fully disintegrated and no longer shows the same body outline of typical planarians. Scale bar indicates 2.5mm. B. EHT 1864 25µM, 8 days. The worms have shown little signs of regenerations, and are unable to move out of place. Scale bar indicates 2.5mm. C. EHT 1864 25µM, 3 days. The worm has a strange growth on the back of its body, and is having trouble twisting over. Scale bar indicates 2.5mm. D. EHT 1864 25µM, 4 Days. The growth is no longer present. Instead, the worm exhibits a white spot (indicated by arrow), and has some mucus coming off of the worm. (Not Shown) Scale bar indicates 1 mm. E. EHT 1864 12.5µM, 5 days. The worms have shown signs of eye development (blue arrow), but have not developed a fully formed head or tail. F. Control worm, 5 days. Near complete regeneration of head and tail. Pronounced head shape has developed. Scale bar indicates 1 mm. G. EHT 1864 6.25µM, 1 day. Fully regenerated worm after just one day. Complete with head and tail development. (Tail not shown) Scale bar indicates 1 mm.
The tail of all Imatinib treated planarians had regenerated (Figure 2C, 2D). There were no abnormal tail growth or abnormal regeneration seen in the planarians.

**PZQ produced no abnormal changes**

PZQ treated planarians showed normal anterior and posterior regeneration. After 96 hours (4 days), all planarians in all PZQ wells except those in the 100 µM well formed visible eyes on the anterior blastema, indicating head regeneration (Figure 3A). After 120 hours (5 days), the planarians in the 100 µM well formed visible eyes. After 192 hours (8 days), all planarians exhibited normal head and tail growth (Figure 3B, 3C, 3D).

**Figure 2 Imatinib Treated Planarians**

A. Imatinib 50µM, 3 days. The planarians show clear eye development three days after being exposed to the drug (eyes indicated by arrows). The development of the eyes coincided with the development of the eyes within the control plate, indicating that Imatinib does not inhibit the regeneration and development of the head at concentrations as high as 50µM. Scale indicates 0.75 mm. B. Imatinib 100µM 4 days. Even at the highest concentration, the planarian is showing signs of eye development indicating minimal effect of the drug on regeneration. Scale indicates 0.75 mm. C. Imatinib 100 µM, head, 8 days. Scale indicates 0.75 mm. D. Imatinib 100µM, tail, 8 days. After 8 days, the planarian has a fully regenerated head and tail, showing that the worm is able to fully regenerate with no consequence after being exposed to the Imatinib. Scale indicates 0.75 mm.

**Figure 3 PZQ Treated Planarians**

A. PZQ 50µM Day 4. Clear development of eyes on the anterior, indicating that there was minimal impact of the drug on regeneration. Development of eyes was consistent with the timeframe of the control worms. B, C, D. Development of the head and tails after 8 days in PZQ 6.25µM. B is the head, C is the tail, and D is the entire body. There are no observable defects or abnormalities. Scale indicates 1.5 mm for all except D. On D, Scale indicates 3 mm.

**Figure 4 U0126 Treated Planarians**

A. U0126 5µM 3 days. The worm had developed an eye, but did not show a sign of a second eye (a condition known as cyclopia). B. U0126 10µM 4 days. The worm had developed the same cyclopia condition as the 5µM concentrations. C. The worms have expanded eyes, but still seem to be part of a single eye, fused together. D. U0126 10 µM 6 days. A planarian had an inconspicuous eye on the left, the other overly large. Scales all indicate 1mm.

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noticed that a planarian in 5 µM well had only one eye on its anterior blastema (Figure 4A). The next day, at 96 hours, we noticed only one visible eye forming on the planarian in the 10 µM (Figure 4B). As for the planarians in the 5 µM well, we observed that the previously identified single eyes became larger and wider, almost slit-like (Figure 4C). At 120 hours (5 days), the single eyed planarians in 10 µM and 5 µM were still spotted with no signs of a second visible. At 144 hours (6 days), however, we observed a very faint and inconspicuous second eye on all worms in the 10µM and 5µM worms (Figure 4D). It appeared that in the 10 µM well, the two planarians had eyes of different sizes, one of which has a significantly larger eye on the left, the other a significantly larger eye on the right; in the 5 µM well, the two planarians had eyes of roughly equal size, but are joined together so they appeared to be only one single eye slit. All the planarians in the other concentrations (2.5 µM, 1.25 µM, 0.625 µM) exhibited normal eye development and regeneration. Normal eyes in those low concentration wells were visible starting after 72 hours.

**DISCUSSION**

EHT 1864 disintegrated the planarians at concentrations of 100 µM and 50 µM, consistent with the idea that the high concentrations inhibited much of the Rac1 within the planarians, thus disrupted actin polymerization and cytoskeletal integrity (also a possible explanation of the growth observed). The planarians in the 25µM well also exhibited stunted regeneration and movement. This may be due to EHT 1864 binding to Rac1 outside of the Wnt pathway as well. Rac1 is a multipurpose protein responsible for cell growth and motility within the body in humans. Planarian Rac1 may have similar roles. This would explain the planarians’ failure to regenerate, because the stem cells within the planarians would be unable to move towards the wound and proliferate.

Imatinib did not cause abnormal regeneration. Since it inhibits RTKs, and there are no changes in regeneration, we suggest that RTKs play a non-essential role in the Wnt pathway and the other pathways associated with regeneration. The downregulation of the canonical pathway due to Imatinib inhibition of RTKs would cause posterior head growth, if the RTKs were integral to the pathway. However, the possibility remains that the drug concentrations were not sufficient to cause inhibition, or the treatment time was not sufficient.

Carefully following the procedures of Chan and Marchant’s (2011) study on PZQ’s effects on planarians, we expected both anterior and posterior head formation during regeneration, which did not occur. The previous study used *D. japonica* as their test subjects, whereas we used *D. tigrina*. The paper mentioned that PZQ has different levels of penetrance on different species of planarians. Thus, the lack of posterior head formation might be due to the low level of PZQ penetrance on *D. tigrina*, or that the *D. tigrina* noncanonical calcium pathway does not play as large a role as that of *D. japonica*. We propose that the two species may have different noncanonical calcium pathways, for the same drug yielded different results.

U0126 treatment effected unusual eye formation. At 10µM and 5µM, a cyclops condition was first observed, and then an abnormally large eye next to a minuscule eye. In contrast, the eyes of planarians in the control group developed at the same rate and have roughly the same size each time they were photographed (representative of the normal mode of planarian eye development). U0126’s inhibition of the MEK1 at high concentrations resulted in low ERK levels, downregulation of the MAPK pathway, and abnormal cell differentiation into eye cells. This is supported by the observation that at concentrations of 0.625, 1.25, and 2.5 µM, the planarians regenerated normal eyes earlier than those in 5 and 10 µM. The general trend is that the higher the U0126 concentration, the more abnormal the eye development. Since the MAPK pathway seems to be directly involved in eye development, we speculate that it may regulate a gene that participates in eye development.

Though the experiment was successful, we did make some errors. For instance, in the 6.25µM EHT 1864 plate, we found a worm (Figure 5G) that had fully regenerated within a day. We decided to replicate a new 6.25µM EHT 1864 well the next day. The other worms in the original 6.25µM and the new 6.25µM wells did not show complete regeneration, confirming that there was a human error in worm cutting. Furthermore, our experimental procedure had some limitations, primarily because we had to cut the planarians under microscopes and approximate the cutting locations; different cutting locations might result in different rates of regeneration.

**FUTURE DIRECTIONS**

Due to time constraints, we were unable to repeat our drug assays. To further confirm the validity of our results, more tests need to be conducted.

Furthermore, more research need to be conducted on the drugs. The major limitation of the research is that it relies heavily on pure observational analysis and is lacking in...
quantitative measurements. Future experiments can complement observations with procedures such as protein expression staining to quantify the extent of protein expression during development. In addition, in order to determine whether the disruption of the Wnt pathway is solely responsible for the results from EHT 1864, a second experiment using a drug, or RNAi, that is specific for the noncanonical PCP pathway needs to be performed. More research is needed to determine the cause behind the different responses of \textit{D. tigrina} and \textit{D. japonica} to PZQ. Furthermore, the concentration of Imatinib should be increased to clarify whether RTKs serve an important role in planarian regeneration, to eliminate the possibility of having inadequate concentration. Finally, in order to elucidate the functions of the MAPK pathway in regards to eye development, RNAi should be used on further downstream components.

**ABBREVIATIONS**

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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>A/P</td>
<td>Anterior/Posterior</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
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<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
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<td>PZQ</td>
<td>Praziquantel</td>
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<td>Dsh</td>
<td>Dishevelled</td>
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<tr>
<td>TCF</td>
<td>Transcription Factor</td>
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<tr>
<td>sFRP</td>
<td>Secreted Frizzle Related Protein</td>
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<td>PCP</td>
<td>Planar cell polarity</td>
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<td>RNAi</td>
<td>RNA interference</td>
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<td>ERK (a.k.a. MAPK)</td>
<td>Extracellular-signal-regulated kinases</td>
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<td>MEK1 (a.k.a MAP2K1)</td>
<td>Dual specificity mitogen-activated protein kinase kinase 1</td>
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**REFERENCES**


