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Inhibitors of Mitogen-Activated Protein Kinase signaling transduction pathway are lethal to regenerating planarians

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Abstract
Regeneration requires the ability of stem cells to detect loss of tissue and respond using cell proliferation and differentiation to heal. These responses are regulated by signal transduction pathways. To explore these pathways, we investigated the role of the MAPK pathway in regenerating amputated planarians by inhibiting specific steps using the drugs U0126, EHT 1846, and imatinib. Each of these drugs not only inhibited regeneration, but also caused lethality to the planarian in a dose-dependent and time-dependent manner. Survival of non-regenerating planaria exhibited a reduced degree of sensitivity to the above inhibitors. Inhibitors of PKA and PKC kinases did not cause this lethality. The fragmentation of DNA in the treated planarians was consistent with those observed during apoptosis. These results suggest that loss of the MAPK pathway in regenerating planarians leads to apoptosis and fragmentation of planarians.

Keywords
Planarian, Regeneration, Signal transduction pathway, Mitogen-Activated Protein Kinase, Apoptosis, Cell proliferation

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Introduction

The regulation of cell proliferation and differentiation in adult tissues has important relevance to many fundamental biological processes including wound repair, regeneration, and cancer treatment. It has been suggested that the formation of tumors can be investigated as a pathological pathway of tissue regeneration. Both processes include the activation of stem cells to undergo cell proliferation either to replace missing tissues (regeneration) in a physiologically appropriate response to wounds or to pathologically create tumors (1,2). Thus, understanding the molecular regulation of regenerating tissues may offer important insights into the dysregulation of cell proliferation that occurs in adult tissues.

Planarians are a well characterized animal model of regeneration with a comprehensive scientific literature and ready accessibility for many different types of investigations (2, 3, 4). They show a remarkable ability to regenerate into new and complete organisms in response to a wide variety of amputations. This impressive regenerative capability is due to the large number of adult pluripotent stem cells called neoblasts that are scattered throughout their bodies. Upon wounding, for example, by amputation, neoblasts are activated to undergo immediate cell proliferation at the site of the wound. These cells form a specialized tissue that is called a blastema, which is required for eventual regeneration, generally 1 to 2 weeks after the amputation (as determined with head blastema) (5). After this proliferation, the neoblasts can differentiate into the specific cell type required to replace the missing tissue. Previous studies have shown that planarians possess exquisite sensitivity to the nature of the amputation and are able to fully regenerate regardless of the method of amputation. This phenomenon is known as “regeneration specificity,” although the specific cellular responses by which neoblasts specialize and migrate to repair wounds are incompletely characterized (2,4).

Regenerative properties of planarians may not rely on the tissue-specific expression of certain genes. Buchser, et al. suggest that peripheral nervous system neurons, which can show regenerative properties in the proper environment may express a unique set of genes with respect to neurons of the central nervous system, which have far more limited regenerative properties (6). However, recent results from Fan et al. show that secondary wounds to planarians can initiate regeneration at a distal site suggesting that genes that are not specific to a tissue may be sufficient to facilitate regeneration (7).

The epidermal growth factor (EGF) signaling system, specifically involving neuregulin-7 and EGF receptor-3, has been identified as an extracellular signal to initiate neoblast proliferation (8). Other studies suggest roles for the akt -TOR signaling pathway in regulating neoblast proliferation (9,10). It has also been established that mitogen-associated protein kinases (MAPKs or ERKs) are activated minutes after amputation and that inhibiting the activation of these kinases by knocking out upstream kinases results in the planarians failing to regenerate the wounded area (11). The action of the MAPK inhibitor U0126 on

the protein kinase ERK was shown to regulate neoblast formation and differentiation, perhaps regulating neoblast cell fate in the blastema (13). Tasaki et al. further showed that inhibition of ERK activity by an upstream inhibitor caused severe differentiation defects and resulted in multiple abnormal growths (12). In addition, it has been reported that inhibition of ERK blocks regeneration in amputated planarians (12). Thus, ERK has been established to play a crucial role in planarian regeneration (13).

We have investigated the role of the MAPK signaling pathway with neoblast proliferation during regeneration using planaria. Initially, we investigated the effects of U0126, an inhibitor of ERK kinase (also known as MEK), on the regenerating planarian. Another drug that we used was EHT 1846, an inhibitor of the Rac proteins that are involved in the MAPK pathway. Finally, we also used imatinib, which inhibits receptor tyrosine kinases, including ABL and PDGFR-beta (13) that activate the MAPK pathway. Downstream effects of this inhibition have been shown to include both inhibiting and enhancing the activity of the ras/MAPK signal transduction pathway in different cultured cell systems (14). The role of the ras/MAPK pathway as a tumor suppressor or pro-oncogenic signal is complex and appears to be dependent on cellular context (15).

Thus, in light of research on the diverse signaling pathways involved in planarian regeneration, we hypothesized that inhibition of the MAPK pathway would disrupt neoblast proliferation. Previous experiments reported diverse effects of various signaling pathway disruptors (11). Neither imatinib nor PZQ (praziquantel, which is believed to elevate intracellular calcium levels) showed any obvious effects on planarian regeneration while U0126 led to cyclopea and EHT 1864 was lethal (11). However, these studies used photo microscopy of a limited number of planarians. Our approach was similar in using the same inhibitors (except PZQ) but relied on quantitative analysis of multiple trials of each drug. We hypothesized that at some minimal dosage of each inhibitor, amputated planaria would not be able to regenerate if the MAPK pathway was disrupted.

Materials and Methods

Planarian

Brown planarian (*Dugesia tigrina*) purchased from Carolina Biological Supply were maintained at room temperature in spring water and not fed one week before the experiments were conducted.

Treatment with inhibitors

Planaria were placed in 3 ml spring water in twelve-well plates generally one or two planaria per well with each bisected, resulting in two or four blastema (two heads and two tails) per well. These blastema were then exposed to various concentrations of each inhibitor for various times at room temperature as indicated in the results section. Imatinib (https://pubchem.ncbi.nlm.nih.gov/compound/Imatinib), EHT 1864 (https://pubchem.ncbi.nlm.nih.gov/#query=%22EHT%201864%22), and U0126 (https://pubchem.ncbi.nlm.nih.gov/#query=U0126&input_type=text) were obtained from

Cayman Chemicals. The PKC/PKA inhibitor cocktail was obtained from Sigma-Aldrich. At the indicated times, the wells containing the planaria were photographed through a dissecting microscope using a cellphone camera or a video camera attached to a microscope (Moticam BTU8). Using the image criteria presented in Figure 1, living and dead planaria were counted for each well. Dead planaria were defined as planaria that exhibited fragmentation and failed to respond to physical stimuli. Each well was considered a single trial.

Figure 1. Change in planarian morphology at various times in the presence of U0126. Planarians in spring water were treated with U0126. Photomicrographs (using a video camera) were taken of planarians treated at the following doses (L to R) none, 16 μM, and 160 μM. Images representative of 6 biological replicates.

DNA extraction and analysis

Intact planaria or remaining tissues of dead planaria were collected by centrifugation on a tabletop centrifuge. The tissues were treated with a DNA extraction buffer (BioRad) containing 10 mM Tris, 2 mM MgCl, Triton X-100, pH 7.2. The tissue was gently triturated until it dissolved, and the solution was adjusted to contain 1 M sodium acetate. An equal volume of ice-cold ethanol was then added resulting in the visible precipitation of the DNA. The DNA was collected by centrifugation and the DNA pellet was gently resuspended in the Tris-EDTA buffer. Aliquots of these DNA solutions were then analyzed by agarose gel electrophoresis using 1% agarose and ethidium bromide staining. The gels were visualized by UV light and photographed using a cell phone camera.

Results

Planarians that were bisected and then treated with 16 μM U0126 exhibited an altered morphology within 24 hours of treatment (Figure 1, Left and Center). The worms flattened out and took on a round, disk-like shape. After 72 hours, the planarians had fragmented into small debris (Right). For the quantitative assays, we considered planaria appearing intact (Left) as survivors and planarians lysed into debris (Right) as dead.

The effects of the ERK kinase (MEK) inhibitor, U0126 exhibited a dose-and time-dependence (Figure 2). The effective micromolar molar range of the drug is comparable to its effects in previous experiments on cultured cells (17). To determine the specificity of this lethal effect of U0126, two other inhibitors of the MAPK

signaling pathway and cell proliferation were investigated. The Rac protein inhibitor, EHT 1864 also exhibited a dose- and time-dependent lethality to the planaria (Figure 3). The concentrations of EHT 1864 required to kill the regenerating planaria were in the micromolar range and similar to those required by U0126, consistent with other reported results (18). After four days, all of the planaria treated with 100 µM EHT 1864 were dead, while the 60% of planaria treated with 10 µM drug were dead. Imatinib, a tyrosine kinase inhibitor was also lethal to the planarians (Figure 4). The imatinib-treated planaria showed the same changes in morphology as depicted in Figure 1 for the U0126-treated planarians, transitioning with time from its intact shape to the disk-like shape eventually leading to a field of fragmented debris.

Not all inhibitors resulted in the death of planaria. Treatment of planaria with an inhibitor cocktail of both cAMP-dependent protein kinase and protein kinase C had no significant effect on the integrity of the planaria using high doses (Figure 5). The same results were seen with a one-hundred-fold range of the inhibitor cocktail (data not shown).

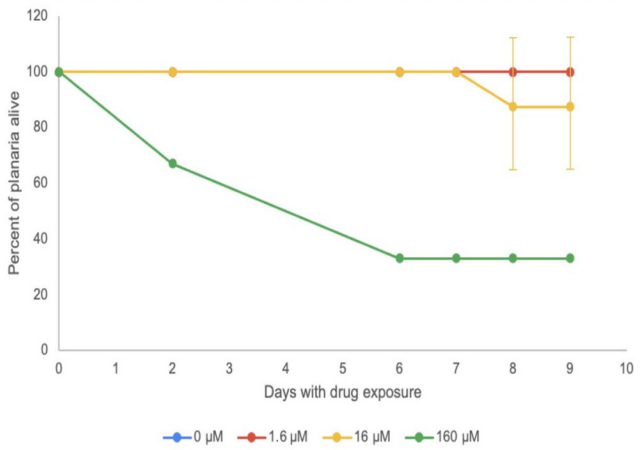

Figure 2. U0126 is lethal to planaria in a time- and dose-dependent manner. Bisected planaria were treated for various times with indicated concentrations of U0126. Planaria with intact morphology (as shown in Figure 1, control) were considered living while planaria showing morphology presented in Figure 1, (160 µM) were considered dead. Note that at 160 µM, standard deviations were zero. Each trial represents two planaria bisected into four total segments in a single well. n=4.

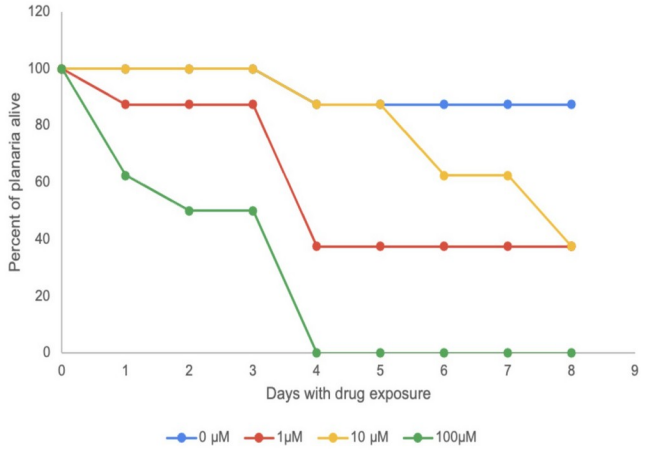

Figure 3. EHT1846 is lethal to planaria in a time- and dose-dependent manner. Bisected planaria were treated for various times with indicated concentrations of EHT1846. Planaria with intact morphology (as shown in Figure 1, left) were considered living while planaria showing morphology (presented in Figure 1 right) were considered dead. Each trial represents two planaria bisected into four total segments in a single well. Results are from four trials. $p<0.0001$ for control vs 100 µM. n=6.

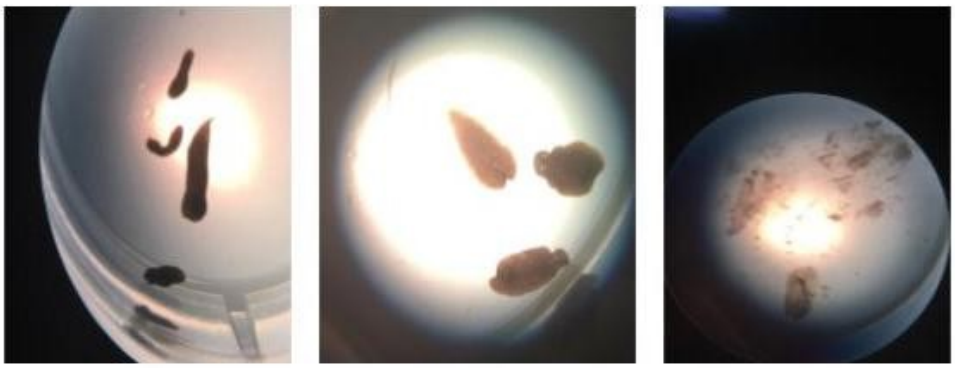

Figure 4. Change in planarian morphology at various times in the presence of imatinib. Planarians in spring water were treated with 100 µM imatinib. Photomicrographs (using a cellphone camera) were taken at the following times (L to R): time 0, after 24 hours, and after 72 hours.

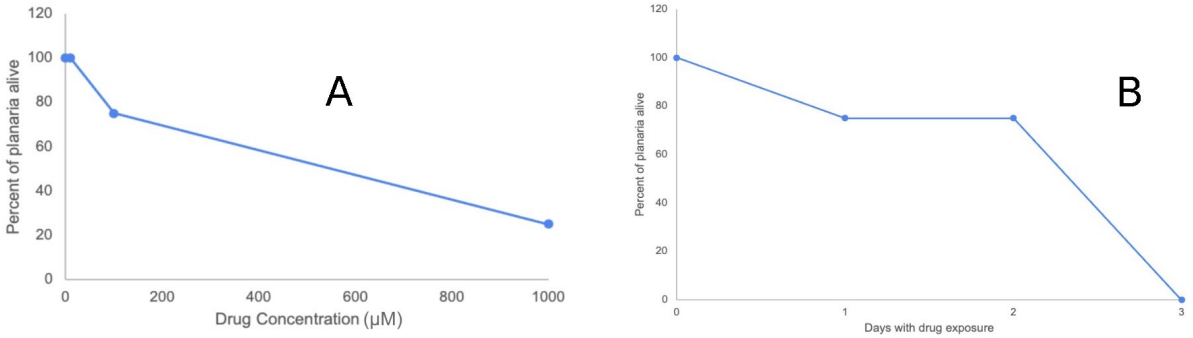

Figure 5. Imatinib lethality is time- and dose-dependent. **5*A*.** Bisected planarians were treated for 24 hours with indicated concentrations of imatinib. Planaria with intact morphology (as shown in Figure 1, Panel A) were considered living while planaria showing morphology presented in Figure 1, panel C were considered dead. Each trial represents two planaria bisected into four total segments in a single well. N=2. 5***B.*** Bisected planaria were treated with 10 mM imatinib for times indicated. Planaria with intact morphology (as shown in Figure 1, Panel A) were considered living while planaria showing morphology presented in Figure 1, panel C were considered dead. Each trial represents two planaria bisected into four total segments in a single well. n=2

In additional experiments, intact planaria that had not been bisected and therefore, not undergoing regeneration were treated with U0126 inhibitor. Non-regenerating planaria were significantly less sensitive to concentrations that were lethal to bisected planaria (Figure 7). The highest concentration of U0126 was eventually lethal, however it took fifteen days of treatment (significantly longer than for regenerating planaria).

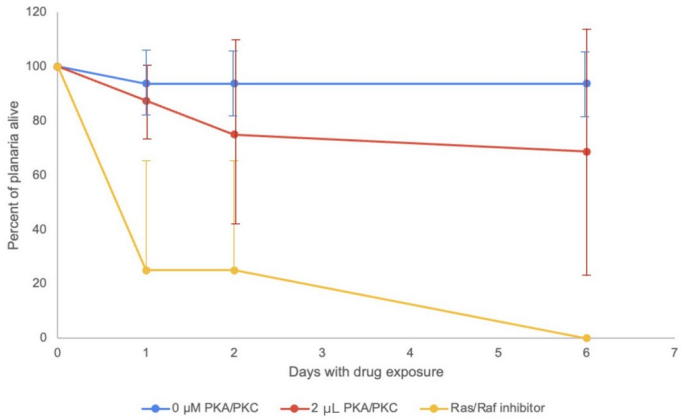

Figure 6. PKC/PKA inhibitor cocktail is not lethal to planaria. Bisected planarians were treated for various times with indicated concentrations of an inhibitor cocktail of both Protein kinase A and Protein kinase C. Planarians with intact morphology (as shown in Figure 1, left) were considered living while planarians showing morphology presented in Figure 1, right were considered dead. Each trial represents two planarians bisected into four total segments in a single well. n=4. Note that after 6 days, all the planarians were dead. Difference between MAPK-pathway inhibitor-treated and PKC/PKA inhibitor-treated survival after 6 days: p=0.0082. Difference between untreated and PKA/PKC inhibitor-treated survival after 6 days: p=0.54. Similar results were observed with treatment by 20 μL and 200 μL of the PKC/PKA inhibitor (data not shown).

After bisection, head and tail blastemas were equally sensitive to U0126 (Figure 7). Both fragments showed an identical time course of lethality to high concentrations of the inhibitor. DNA isolated from planaria treated by various concentrations of imatinib was displayed on agarose gels (Figure 9). Small fragments were evident in DNA samples isolated from planaria treated with 1 mM imatinib, which was a lethal dose. Similar DNA fragmentation was observed with treatment by EHT 1864. Furthermore, planaria treated with the PKA/PKC inhibitor cocktail, which is nonlethal, exhibited no such DNA fragmentation (data not shown).

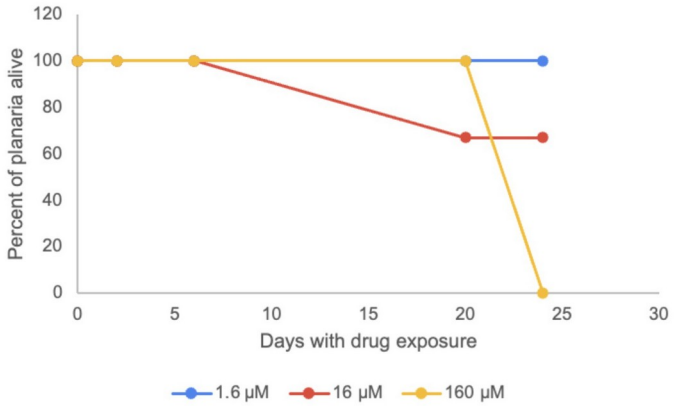

Figure 7. Intact planaria are significantly less sensitive to inhibitors. Planaria that were left intact (never bisected) were treated for various times with indicated concentrations of U0126. Planaria with intact morphology (as shown in Figure 1, Panel A) were considered living while planaria showing morphology presented in Figure 1, panel C were considered dead.

Discussion

Three different inhibitors of the MAPK signal transduction pathway were lethal to planarians. Treatment with U0126, an inhibitor of ERK1 and ERK2 (17), resulted in drastic changes of planarian morphology from its elongated arrowhead shape to a disk-like shape to eventual fragmentation (Figure 1). Similarly, EHT 1864 which inhibits the function of rac1 and its associated isoforms (18) led to the same morphological changes in a shorter time frame. Finally, imatinib, an inhibitor of receptor tyrosine kinases, particularly, abl, c-kit, and PDGF receptors (14), exhibited a similar lethality to the planarians (Figure 4).

On the other hand, a cocktail containing inhibitors of PKC and PKA did not induce planarian death at any concentration used (Figure 6), indicating some specificity to the MAPK signal transduction pathway that are involved in the observed lethality. Furthermore, non-regenerating planarians were significantly less sensitive to the effects of U0126 (Figure 7) suggesting that the formation of blastemas and activation of neoblasts increase vulnerability to MAPK-pathway inhibitors. Head and tail

sections of the amputated planarian were equally affected by the U0126 (Figure 8).

The lysed planarian tissues contained DNA that was fragmented into smaller sizes (Figure 9), consistent with an apoptotic mechanism of cell death in the inhibitor-treated planarians. Unfortunately, assays for caspase activity to confirm apoptosis were unsuccessful (data not shown).

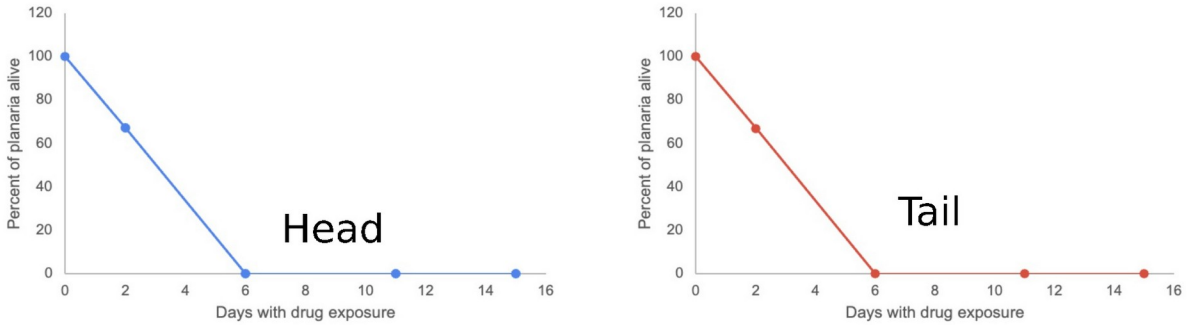

Figure 8. Head and tail blastema were equally affected by U0126. Planaria were bisected and the head and tail blastemas were placed into separated wells. The left figure shows head blastema and the right represents tail blastema. All fragments were treated for various times with 850 μM U0126. Planarians with intact morphology (as shown in Figure 1, left) were considered living while planarians showing morphology presented in Figure 1, right were considered dead. Each trial represents two planaria bisected into four total segments in a single well. Results are from a single trial.

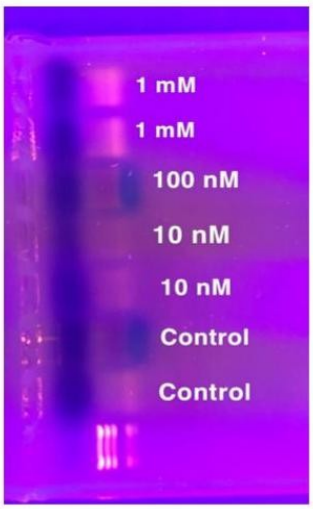

Figure 9. Treatment of planaria with imatinib resulted in DNA fragmentation. Planaria were treated with various concentrations of imatinib for 24 hours including control with no imatinib. DNA was extracted and separated by 1% agarose gel electrophoresis.

Our results were both similar and different from observations previously published (11). Like those results, we found that treatment with EHT 1864 resulted in fragmented planarians. However, our observations that both imatinib and U0126 were similarly lethal to regenerating planarians contrasted with the limited or nonexistent effects in the earlier study. Due to the limited experiments reported in the previously noted publication, it is difficult to reconcile these differences.

While many different signal transduction pathways are involved in the determination of positional polarity and regeneration of specific tissues (5, 7,8,9,11) in regenerating planarians, our results suggest that the Rac/MAPK pathway is important for their survival. The MAPK signaling pathways, including activation of ERK, in developing blastemas (19) have been found to be crucial for the early stages of regeneration (20). Tasaki et al noted that treatment of bisected planarians with U0126 caused disruptions of both head and tail regeneration that resulted in a reduction of neoblast number (12). In addition, the remaining neoblasts failed to differentiate into the appropriate cell type. They concluded that ERK signaling may be necessary for switching the neoblasts from undergoing cell proliferation to undergoing cell differentiation.

The effects of the other two inhibitors, imatinib (on receptor tyrosine kinases) and EHT 1864 (on rac) are consistent with disrupting the MAPK signaling pathway that includes ERK1/2 (19,20). Moraczewski et al have reported that PKC activity was an early event in the response to amputation in regenerating planarians (21). The role for ERK activity in planarian regeneration has been well established (current results, 7, 11, 12, 20, 28). The interaction between PKC activity and the ERK signaling pathway has been reported for many other cellular responses including: arrest of the cell cycle in epithelial cells (22), loss of cdx2 in carcinoma cell lines (23), trastuzumab resistance in HER2-positive breast cancer (24), calcium-stimulated CREB-dependent transcription in PC12 cells and hippocampal neurons (25), and reduced excitability in hippocampal neurons (26). These observations suggest that cross talk between PKC and ERK signaling regulate many biological processes. However, our observation that inhibition of PKC does not lead to the death of the planarians (Figure 6) while inhibition of ERK does (Figures 1, 3, and 4) suggest that the PKC and ERK pathways are independent of each other in the regenerating planarian. It may be that the PKC-dependent pathway as an early response may not be associated with the cellular decision to induce proliferation or undergo apoptosis but may instead be involved in some other aspect of wound repair.

However, if these inhibitors simply disrupt neoblast differentiation, planarian regeneration would be simply prevented resulting in inhibited growth of new tissue but leaving intact underdeveloped blastemas. In fact, we observed that the planarians underwent massive lethal fragmentation. The analysis of the DNA (Figure 9) suggested that apoptosis was involved. Apoptotic-mediated death of differentiated cells has been reported during uninhibited regeneration of planarians following amputation (27). Cell death was

observed both at the site of the amputation and subsequently scattered throughout the organism. It was proposed that the balance between cell proliferation and cell death during regeneration are used to remodel the newly produced tissue into the appropriate anatomy for the planarian (27). Our results showing massive fragmentation of planarians with treatment by MAPK signal pathway inhibitors and fragmentation of DNA suggest that, in the absence of neoblast proliferation or differentiation to maintain equilibrium of the cell population, neoblasts rapidly die due to the activation of apoptotic mechanisms. This possible response is speculative at this point and requires additional investigation.

Reactive oxygen species (ROS) may play a role in the wound repair process of planarians. Jaenen et al found that ROS and the ERK signaling pathway interact closely in the regeneration (28). Two of the inhibitors used in our investigation have been shown to affect ROS activity in opposite ways. Imatinib was shown to induce ROS-dependent apoptosis in melanoma cells (29), while U0126 reduced ROS in PC12 cells (30). This difference suggests that further investigations are necessary to determine the role of ROS in the action of imatinib, U0126, and EHT1864 on blocking planarian responses to amputation.

Fan et al recently discovered that, in response to amputation, planarians produce ERK signal waves that rapidly travel along the longitudinal body-wall muscles to the rest of the body of the wounded planarian (7). This signal propagation may help explain the planarians' robust regenerative abilities and why blocking ERK prevents this response. Fan et al. also discovered that creating a subsequent second wound at a distal site initiates wound repair at both the new and original sites (7). It would be interesting to investigate this response of the twice-wounded planarians using our inhibited planarian model by creating a second wound after treatment with the ERK inhibitors to determine if it could prevent the wholesale cell death that we have observed.

Conclusion

The drugs U0126, EHT 1846, and imatinib were used to investigate the role of the MAPK pathway in regenerating amputated planarians. Each of these drugs not only inhibited regeneration, but also caused lethality to the planarian in a dose-dependent and time-dependent manner. Survival of non-regenerating planaria exhibited a reduced degree of sensitivity to the above inhibitors. Inhibitors of PKA and PKC kinases did not cause this lethality. The fragmentation of DNA in the treated planarians was consistent with those observed during apoptosis. These results suggest that loss of the MAPK pathway in regenerating planarians leads to apoptosis and fragmentation in planarians.

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