A pharmacological screen for compounds that rescue the developmental lethality of a Drosophila ATM mutant

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Abstract

Ataxia-telangiectasia (A-T) is a neurodegenerative disease caused by mutation of the A-T mutated (ATM) gene. ATM encodes a protein kinase that is activated by DNA damage and phosphorylates many proteins, including those involved in DNA repair, cell cycle control, and apoptosis. Characteristic biological and molecular functions of ATM observed in mammals are conserved in Drosophila melanogaster. As an example, conditional loss-of-function ATM alleles in flies cause progressive neurodegeneration through activation of the innate immune response. However, unlike in mammals, null alleles of ATM in flies cause lethality during development. With the goals of understanding biological and molecular roles of ATM in a whole animal and identifying candidate therapeutics for A-T, we performed a screen of 2400 compounds, including FDA-approved drugs, natural products, and bioactive compounds, for modifiers of the developmental lethality caused by a temperature-sensitive ATM allele (ATM8) that has reduced kinase activity at non-permissive temperatures. Ten compounds reproducibly suppressed the developmental lethality of ATM8 flies, including Ronnel, which is an organophosphate. Ronnel and other suppressor compounds are known to cause mitochondrial dysfunction or to inhibit the enzyme acetylcholinesterase, which controls the levels of the neurotransmitter acetylcholine, suggesting that detrimental consequences of reduced ATM kinase activity can be rescued by inhibiting the function of mitochondria or increasing acetylcholine levels. We carried out further studies of Ronnel because, unlike the other compounds that suppressed the developmental lethality of ATM8 flies, Ronnel was toxic to the development of heterozygous ATM8 flies. Ronnel did not affect the innate immune response of ATM8 flies, and it further increased the already high levels of DNA damage in brains of ATM8 flies, but its effects were not harmful to the lifespan of rescued ATM8 flies. These results provide new leads for understanding the biological and molecular roles of ATM and for the treatment of A-T.
Introduction

Ataxia-telangiectasia (A-T) is an autosomal recessive disorder characterized by progressive cerebellar atrophy, immunodeficiency, and cancer predisposition [1–3]. The cause of A-T is mutation of the A-T Mutated (ATM) gene, which encodes a phosphatidylinositol-3-kinase (PI3K)-related protein kinase involved in the recognition and repair of DNA damage. Loss of ATM function leads to a variety of cellular and molecular abnormalities, including accumulation of DNA damage, oxidative stress, insulin resistance, mitochondrial dysfunction, cell cycle dysregulation, and neurodegeneration. Currently, no specific treatment is available for A-T, but studies of ATM-deficient cells and animals are making progress toward this goal. High-throughput screens have identified compounds that induce translational read-through of nonsense mutations in ATM [4]). In addition, large-scale proteomic, metabolomic, and transcriptomic studies have identified potential cellular therapeutic targets [5]. Lastly, directed approaches have identified possible therapeutics for specific physiological manifestations of A-T; antioxidants reduce oxidative stress [6], glutamine supplementation blocks neuronal cell cycle reentry and improves the DNA damage response [7], and increasing intracellular NAD+ promotes the elimination of dysfunctional mitochondria by mitophagy, a selective form of autophagy [8]. Nevertheless, an opportunity that is yet to be explored because of issues of cost and feasibility is an unbiased screen for compounds that improve ATM mutant phenotypes in a whole animal.

Screens of libraries of compounds in Drosophila melanogaster have identified candidate therapeutics for diverse human diseases, including Fragile X syndrome [9], thyroid cancer [10], and Alexander disease [11]. Medium-throughput screens of compounds are possible in flies because flies have a short lifecycle, are maintained in small vials, and feed on inexpensive food, which collectively make it economical and feasible to screen large numbers of animals. Flies also have a short lifespan, which allows age-related phenotypes to be analyzed in a reasonable period of time. Lastly, compounds can be easily administered to flies in food and are only needed in small quantities.

ATM mutant flies exhibit phenotypes similar to those in A-T, including DNA damage, sensitivity to ionizing radiation, and progressive neurodegeneration [12–17]. A key difference between flies and mammals is that ATM is essential in flies. The essential nature of ATM in flies may be due to the fact that flies lack the catalytic subunit of DNA-dependent protein kinase (DNA-PK(cs)), which like ATM is a PI3K-related protein kinase. In support of this hypothesis, mice deficient for both ATM and DNA-PK(cs) are embryonic lethal [18]. Because ATM is essential for viability in flies, a temperature-sensitive ATM allele (ATM8) has been tremendously useful for investigating the mechanistic basis of adult phenotypes such as neurodegeneration [16, 17]. ATM8 flies contain a missense mutation that changes the final amino acid of the ATM protein from leucine to phenylalanine [15]. When raised at 25˚C, ATM8 flies die during development, often as pupae, but lowering the temperature to 18˚C largely prevents the developmental lethality. By assaying phosphorylation of the ATM substrate histone H2Av in response to ionizing radiation-induced DNA damage, we determined that ATM8 kinase activity is inhibited at 25˚C but not at 18˚C [16]. Furthermore, ATM8 flies that are raised at 18˚C and shifted to 25˚C as adults undergo progressive neurodegeneration that is caused by hyperactivation of the innate immune response in glial cells [17]. The innate immune response in flies and humans not only functions to combat pathogens but also influences the process of neuroprotection [19, 20]. In flies, the Toll and Imd (Immune deficiency) innate immune response pathways activate distinct NF-κB transcription factors to control the transcription of genes that encode antimicrobial peptides (AMPs) [21]. In ATM8 flies, the expression of AMP genes is substantially upregulated [16, 17]. Inactivation of the Imd pathway in ATM8 flies by
mutation of the NF-κB transcription factor Relish not only reduces AMP gene expression but also blocks neuron death and increases lifespan [17]. Chronic inflammation in A-T and links between the innate immune response and neurodegeneration in disorders such as Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, and Amyotrophic lateral sclerosis indicate that ATM flies are a pertinent model of neurodegeneration in A-T as well as other neurodegenerative diseases [19, 20, 22, 23].

To identify potential therapeutics for A-T and other neurodegenerative diseases, we screened a library of 2400 compounds for modifiers of the developmental lethality of ATM flies. We identified 10 compounds that reproducibly suppressed the developmental lethality of ATM flies, and we investigated the physiological and molecular effects of one of these compounds Ronnel because it paradoxically and uniquely suppressed the developmental lethality of homozygous ATM flies but enhanced the developmental lethality of heterozygous ATM flies.

**Results and discussion**

**Identification of a phenotype that is sensitive to the function of ATM**

Dominant modifier screens in flies have been extremely successful in identifying genes involved in specific biological processes, including genes involved in ATM-mediated neuroprotection [14]. The key feature of dominant modifier screens is a sensitized, intermediate phenotype that permits heterozygous mutations in genes that function in relevant biological processes to dominantly suppress or enhance the phenotype. Based on this paradigm, we set out to identify a sensitized, intermediate ATM-dependent phenotype where compounds that affect the function of ATM or its downstream targets would suppress or enhance the phenotype. It was previously documented that in ATM flies, ATM kinase activity and development to adulthood is temperature-sensitive [12, 15, 16]. Indeed, we found that when heterozygous ATM/TM3 flies (TM3 is a balancer chromosome that contains a wild-type ATM allele) were self-crossed at 25˚C, no ATM F1 progeny eclosed, indicating that ATM kinase activity is below the threshold needed for viability (Table 1). In contrast, at 18˚C, 24% of the F1 progeny were ATM, which approaches the Mendelian ratio of 33% expected for fully viable ATM flies. Moreover, an intermediate temperature of 21˚C produced an intermediate ratio of ATM F1 progeny, 16%. These data suggest that 21˚C is a sensitized condition where compounds that affect ATM kinase activity or signaling through ATM pathways might alter the level of ATM progeny up to as high as 33% or down to as low as 0%.

**A primary screen of 2400 compounds identifies many modifiers of ATM lethality**

To identify compounds that affect the function of ATM, we performed a blinded screen of 2400 compounds from the Spectrum Collection (MicroSource Discovery System) for those that altered the percent eclosion of ATM flies at 21˚C. The library included FDA approved drugs, natural products, and bioactive components. Details of the screening protocol are

| Temperature | ATM total | Total | % ATM |
|-------------|-----------|-------|-------|
| 18˚C        | 31        | 127   | 24    |
| 21˚C        | 19        | 120   | 16    |
| 25˚C        | 0         | 71    | 0     |

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described in the Materials and methods and illustrated in Fig 1. In brief, for each compound, 200 μl of 0.2 mM compound in 2% dimethyl sulfoxide (DMSO) was evenly applied to the surface of newly prepared molasses food in vials. This concentration was selected based on other screens in flies that used 0.05 or 0.1 mM compounds that were homogenously mixed in food [10, 11, 24]. 50–60 young ATM<sup>y</sup>/TM3 flies were added to the vials, and the flies were cultured at 25˚C for 5–6 days to allow egg-laying, at which time the parental flies were removed and the vials were transferred to

**Fig 1. Schematic diagram of the primary and secondary screen protocols.** Details are provided in the Materials and methods section as well as the Results and discussion section.

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21 °C for about 12 days, enough time for eggs to have developed to adults. This protocol allowed compounds to be consumed \textit{ad libitum} by parental flies as well as F1 larvae. \textit{ATM}^8 and \textit{ATM}^8/\textit{TM3} F1 adults were counted and the percent \textit{ATM}^8 flies was determined. In the primary screen, we only tested each of the 2400 compounds once, but, in a secondary screen, we retested the top 100 compounds two more times to identify compounds that had a reproducible effect.

Table 2 provides an overview of the findings, and S1 Table provides data for each compound. 147 compounds completely blocked the production of \textit{ATM}^8 progeny. Of these, 23 compounds, including several insecticides, also blocked the production of \textit{ATM}^8/\textit{TM3} flies either by killing the parental flies and preventing egg-laying or by inhibiting the development of progeny. The other 124 compounds that preferentially blocked production of \textit{ATM}^8 progeny may provide insights into ATM function; however, there were no obvious commonalities among these compounds. 892 compounds had little or no effect on the development of \textit{ATM}^8 relative to \textit{ATM}^8/\textit{TM3} flies leading to 10–20\% \textit{ATM}^8 progeny, which was not substantially different than 14\% \textit{ATM}^8 progeny for the control (i.e., 2\% DMSO) (Table 3). 819 compounds were detrimental to the development of \textit{ATM}^8 relative to \textit{ATM}^8/\textit{TM3} flies, reducing \textit{ATM}^8 progeny to 1–10\%. In contrast, the remaining 542 compounds were beneficial to the development of \textit{ATM}^8 relative to \textit{ATM}^8/\textit{TM3} flies, increasing \textit{ATM}^8 progeny to >20\%. In summary, the primary screen served the desired purpose of broadly categorizing the collection of 2400 compounds into those that were detrimental, neutral, or beneficial to the development of \textit{ATM}^8 flies.

A secondary screen of 100 compounds identifies reproducible suppressors of \textit{ATM}^8 lethality

We performed a secondary screen of the 100 compounds that produced the highest percent \textit{ATM}^8 progeny in the primary screen (S2 Table). Each compound was tested twice under the same conditions as the primary screen. However, for unknown reasons, controls in the secondary screen had 1\% rather than 14\% \textit{ATM}^8 progeny observed in the primary screen (Table 3). Despite these more stringent conditions, 18 compounds substantially increased the percent \textit{ATM}^8 progeny in one of the two trials. Moreover, 10 compounds, listed in Table 3 and hereafter referred to as the top 10 compounds, substantially increased the percent \textit{ATM}^8 progeny in both trials. Thus, in three independent biological trials, the top 10 compounds reproducibly suppressed the developmental lethality due to reduced ATM kinase activity.

Three of the top 10 compounds, Ronnel (also known as fenchlorphos), estragole (also known as p-allylanisole), and stigmasterol, are inhibitors of the enzyme acetylcholinesterase. 

| % \textit{ATM}^8 | Number of compounds |
|-----------------|---------------------|
| 0               | 147                 |
| 1–10            | 819                 |
| 10–20           | 892                 |
| 20–30           | 425                 |
| 30–40           | 99                  |
| 40–50           | 16                  |
| 50–60           | 0                   |
| 60–70           | 1                   |
| 70–80           | 0                   |
| 80–90           | 0                   |
| 90–100          | 1                   |

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(AChE), which catalyzes breakdown of the neurotransmitter acetylcholine (ACh) to choline [25–27]. Inhibition of AChE leads to accumulation of ACh at synapses and stimulation of nerves and muscles in nervous systems. AChE inhibitors have been used in the treatment of Alzheimer’s and Parkinson’s disease patients who have reduced levels of ACh in the brain [28, 29]. Thus, we retested AChE inhibitors (i.e., donepezil hydrochloride, tacrine hydrochloride, dichlorvos, galantamine, and rivastigmine tartrate) from the primary screen that were not included in the secondary screen. In the primary screen, donepezil hydrochloride, tacrine hydrochloride, and dichlorvos weakly suppressed the developmental lethality of **ATM**8 flies, but neither these nor the other AChE inhibitors affected **ATM**8 lethality in the retest (S3 Table). Similarly, four of the AChE inhibitors failed to suppress **ATM**8 lethality at other concentrations, ranging from 0.002 to 2 mM or when used in combination (S4 Table). Nevertheless, direct evaluation of AChE activity and ACh levels are needed to definitively determine the extent to which inhibition of AChE leads to suppression of **ATM**8 lethality.

Ronnell may also act through other mechanisms, as it is a member of the organophosphate class of compounds that not only inhibit AChE but also have non-cholinergic effects, including disruption of mitochondrial oxidative phosphorylation and mitochondrial membrane potential [30]. Interestingly, six of the other top 10 compounds cause mitochondrial dysfunction through a variety of mechanisms; hypoxanthine inhibits oxidative phosphorylation [31], quassin and usnic acid cause mitochondrial depolarization [32, 33], sodium thioglycolate (also known as 2-mercaptoacetate) and avocatin B inhibit mitochondrial fatty acid oxidation [34, 35], and tyramine inhibits mitochondrial respiration [36]. Thus, based on the finding that elimination of defective mitochondria by mitophagy in ATM-deficient neurons and animals has beneficial consequences [8], the identified compounds may trigger mitophagy of partially dysfunctional mitochondria in **ATM**8 cells by further reducing their function and thereby improve the mitochondrial network’s integrity and functionality.

### The top 10 compounds are allele-specific suppressors of **ATM** lethality

To investigate the mechanisms by which the top 10 compounds suppress the developmental lethality of **ATM**8 flies, we examined their effect on other recessive lethal **ATM** alleles, **ATM**3 and **ATM**4, that were identified in the same chemical mutagenesis screen that identified **ATM**8 flies.
ATM³ contains a nonsense mutation that truncates the ATM protein at residue 600 out of 2429, and ATM⁴ contains a missense mutation that changes leucine 284 to histidine. By genetic criteria, ATM³ and ATM⁴ are both null alleles. In three independent tests of each compound at 0.2 mM, we found that none of the top 10 compounds rescued the developmental lethality of even a single ATM³ or ATM⁴ fly. These data argue that some level of ATM activity is required for suppression of developmental lethality by the top 10 compounds.

Ronnel is a dose-dependent suppressor of ATM⁸ and enhancer of ATM⁸/TM3 lethality

Among the top 10 compounds, Ronnel (also known as fenchlorphos) stood out because >90% of the F1 progeny from self-crosses of ATM⁸/TM3 flies were ATM⁸, which is considerably higher than the maximum expected 33% if both ATM⁸ and ATM⁸/TM3 flies are fully viable (Table 3). These data indicate that Ronnel is both beneficial to the development of ATM⁸ flies and toxic to the development of ATM⁸/TM3 flies. In support of this conclusion, the number of ATM⁸ flies that eclosed per vial with Ronnel was similar to the number for the other top 10 compounds, but the total number of eggs from which the flies developed was substantially lower because Ronnel was lethal to the parental flies (Table 3). In other words, since a similar number of ATM⁸ flies eclosed from fewer eggs, it means that ATM⁸ flies that usually would have died during development were able to survive, indicating rescue by Ronnel rather than tolerance to Ronnel. Furthermore, the effect of 0.2 mM Ronnel during fly development is dependent on the level of ATM activity; Ronnel was unable to rescue the lethality of flies with no ATM activity (i.e., ATM³ and ATM⁴ at 25°C), it rescued the lethality of flies with low ATM activity (i.e., ATM⁸ flies at 21°C), and it increased the lethality of flies with higher ATM activity (i.e., ATM⁸/TM3 flies at 21°C and ATM³/TM3, ATM⁴/TM6B, and wild-type flies at 25°C).

To further characterize Ronnel, we used the screening protocol to examine the effect of different concentrations of Ronnel on the developmental lethality of ATM⁸ flies. As observed in the primary and secondary screens, 0.2 mM Ronnel produced >90% ATM⁸ progeny (Table 4). A 10-fold lower concentration of Ronnel (0.02 mM) reduced ATM⁸ progeny to 59% and even lower concentrations (0.002–0.00002 mM) reduced ATM⁸ progeny to 14–19%, which was similar to the 20% ATM⁸ progeny observed in DMSO controls. Fewer total progeny were produced at high concentrations of Ronnel because it killed most of the parental flies within 48 hrs thereby reducing the number of eggs laid. Moreover, the percent ATM⁸ flies at 0.02 mM Ronnel was lower than at 0.2 mM Ronnel because of increased viability of ATM⁸/TM3 progeny rather than reduced viability of ATM⁸ progeny. These data indicate that molecular events targeted by Ronnel that suppress ATM⁸ lethality and enhance ATM⁸/TM3 lethality are sensitive to different concentrations of Ronnel.

Ronnel does not adversely affect the lifespan of ATM⁸ flies

To investigate whether treatment with Ronnel during the development of ATM⁸ flies has long-lasting effects during adulthood, we determined the lifespan of ATM⁸ and ATM⁸/TM3 flies at

| Ronnel (mM) | ATM⁸ | Total | % ATM⁸ |
|------------|------|-------|--------|
| 0.2        | 19   | 20    | 95     |
| 0.02       | 19   | 32    | 59     |
| 0.002      | 24   | 170   | 14     |
| 0.0002     | 27   | 183   | 15     |
| 0.00002    | 34   | 180   | 19     |
| % DMSO Control (2–0.0002) | 181 | 893 | 20 |

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25˚C after being raised at 21˚C on molasses food, molasses food with 0.2% DMSO (i.e., DMSO), or molasses food with 0.02 mM Ronnel in 0.2% DMSO (i.e., Ronnel). We used 0.02 mM rather than 0.2 mM Ronnel for this and subsequent analyses of Ronnel because it was less toxic to parental ATM8/TM3 flies and ATM8/TM3 progeny, which led to more ATM8/TM3 flies to analyze (Table 4). This study showed that Ronnel had no effect on the lifespan of either ATM8 or ATM8/TM3 flies (Fig 2). Kaplan-Meier analysis revealed that the mean lifespan of ATM8 and ATM8/TM3 flies was not significantly different when raised with or without Ronnel (S5 Table). Furthermore, with and without Ronnel, ATM8 flies had similar mean lifespans that were significantly shorter than those of ATM8/TM3 flies (P<0.01). Thus, the mechanism by which Ronnel affects the developmental lethality of ATM8 and ATM8/TM3 flies does not cause lasting damage or repair that affects the lifespan of adult flies.

**Ronnel does not affect the innate immune response of ATM8 flies**

The innate immune response is hyperactivated in glial cells of ATM8 flies, and genetic manipulations that reduce the innate immune response prevent neurodegeneration and increase the lifespan of ATM8 flies [16, 17]. Thus, Ronnel-mediated suppression of the developmental lethality of ATM8 flies might involve effects on the innate immune response. To test this hypothesis, we monitored activation of the innate immune response by quantifying mRNA
levels of AMP genes *Diptericin B* (*DiptB*), *Metchnikowin* (*Mtk*), and *Attacin C* (*AttC*). At 24 hrs after development of *ATM*<sup>8</sup> flies on molasses food containing 0.2% DMSO (i.e., DMSO) or 0.02 mM Ronnel in 0.2% DMSO (i.e., Ronnel), *Mtk* and *AttC* expression was equivalent between DMSO- and Ronnel-treated flies and *DiptB* expression was about 2-fold lower in Ronnel-treated flies (Fig 3A). Similarly, the expression of all three AMP genes was not affected in *ATM*<sup>8</sup> flies fed Ronnel as adults, that is, raised on molasses food at 18˚C and transferred to molasses food containing DMSO or Ronnel for 24 hrs (Fig 3B). Longer treatments of *ATM*<sup>8</sup>
adult flies with 0.02 mM Ronnel were not possible because it killed too many flies within 48 hrs. These data indicate that the innate immune response is unlikely to play a role in Ronnel-mediated suppression of the developmental lethality of ATM8 flies or the lethality of adult ATM8 flies.

DNA damage accumulates in the brain of ATM8 flies

The primary function ascribed to ATM is in the recognition and repair of DNA double-strand breaks [1–3]. Abnormally high levels of DNA damage have been reported in tissue culture cells and organisms, including flies, that contain ATM mutations, and DNA damage may be responsible for triggering neurodegeneration and other phenotypes in A-T patients [12–17]. To test the possibility that DNA damage in the brain contributes to the developmental lethality of ATM8 flies, we measured DNA damage using the Comet assay, which involves lysing cells in low-melt agarose and electrophoresing the released DNA [37]. Fragments of damaged DNA migrate faster than intact chromosomes during gel electrophoresis and when visualized with a fluorescent dye resemble a comet (Fig 4A). The amount of DNA in the comet tail relative to the total amount of DNA (i.e., percent tail DNA) is a measure of the amount of DNA damage. We found that cells from dissociated brains of 0–3 day old ATM8 flies had a significantly higher percent tail DNA than w1118 flies (a standard laboratory strain), whereas ATM8/TM3 flies had the same percent tail DNA as w1118 flies (Fig 4B and 4C). Therefore, ATM activity is required in the brain to prevent the accumulation of DNA damage, and the level of ATM activity determines the extent of DNA damage.

To confirm that the Comet assay quantitatively detects DNA damage in the brain, we used it to analyze cells from dissociated brains of 3 day old flies 1 hr after exposure to 50 Gy of ionizing radiation (IR), which induces DNA double-strand breaks. In all of the flies examined, w1118, ATM8/TM3, and ATM8, IR significantly increased the percent tail DNA relative to unirradiated flies (Fig 4B and 4C). Thus, the Comet assay is able to detect a wide range DNA damage levels in brain cells.

Ronnel exacerbates DNA damage in ATM8 flies

To investigate whether Ronnel suppresses the developmental lethality of ATM8 flies by affecting the level of DNA damage, we used the Comet assay to determine the level of DNA damage in 0–3 day old ATM8 and ATM8/TM3 flies that were raised at 21˚C on molasses food, molasses food with 0.2% DMSO (i.e., DMSO), or molasses food with 0.02 mM Ronnel in 0.2% DMSO (i.e., Ronnel). As observed in Fig 4, ATM8 flies had higher percent tail DNA than ATM8/TM3 flies under all of the culturing conditions (Fig 5). Moreover, relative to molasses food and DMSO, Ronnel increased the percent tail DNA in both ATM8 and ATM8/TM3 flies, indicating that Ronnel promotes DNA damage. This is consistent with studies showing that organophosphate compounds induce DNA damage in multiple cell types [38, 39]. Since Ronnel had the same effect on DNA damage in ATM8 and ATM8/TM3 flies but had opposite effects on the development of these flies, the effects of Ronnel on DNA damage and development are unlikely to be mechanistically related.

Conclusions

Our pharmacological screen identified 10 compounds that suppressed the developmental lethality caused by reduced ATM kinase activity in flies (Table 3). Studies of Ronnel argue against a suppression mechanism that affects the level of the innate immune response or DNA damage (Figs 3–5). Subsets of the 10 compounds are known to inhibit the enzyme acetylcholinesterase (AChE) or the function of mitochondria, and Ronnel inhibits both AChE and
Fig 4. DNA damage accumulates in the brain of ATM flies. (A, top row) Microscopy images of four examples of individual cells subjected to the Comet assay. The cells are arranged left to right from low to high levels of DNA damage. Dotted lines indicate the area of electrophoresed DNA. (A, bottom row) Output from the CometScore Pro Software analysis of the cells shown in the top row. (B) The Comet assay was used to determine the percent tail DNA for brain cells of flies of the indicated genotype and either not exposed to ionizing radiation (IR) (−) or exposed to 50 Gy of IR (+). All data points were graphed using a box and whisker plot in Prism 7.0 (Graphpad) statistical software. Boxes indicate the middle 50% of the data points, lines in the middle of the boxes indicate the median, +s in the boxes indicate the mean, and the maximum and minimum whiskers indicate 95% of the data points (minimum whisker begins at 2.5% and maximum whisker ends at 97.5%). >200 comets were analyzed for each condition. (C) P-values for data presented in panel B were based on an unpaired equal-variance two-tail t-test.

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mitochondria [27, 30]. These data implicate acetylcholine (ACh) in the suppression mechanism. Inhibition of AChE increases ACh levels by preventing its breakdown, and inhibition of mitochondrial function may increase ACh levels by triggering mitophagy of dysfunctional mitochondria, thereby improving the overall functionality of mitochondria, which produce acetyl-CoA, a substrate for ACh synthesis. In support of this hypothesis, AChE inhibitors have shown benefits in Alzheimer’s and Parkinson’s disease patients [28, 29], and reduced

Fig 5. Ronnel increases the accumulation of DNA damage in ATM^{	ext{f}} flies. (A) The Comet assay was used to determine the percent tail DNA for brain cells of flies of the indicated genotype and raised on molasses food (M), molasses food with 0.2% DMSO (D), or molasses food with 0.02 mM Ronnel in 0.2% DMSO (R). All data points were graphed using a box and whisker plot in Prism 7.0 (Graphpad) statistical software. Boxes indicate the middle 50% of the data points, lines in the middle of the boxes indicate the median, +s in the boxes indicate the mean, and the maximum and minimum whiskers indicate 95% of the data points (minimum whisker begins at 2.5% and maximum whisker ends at 97.5%). >200 comets were analyzed for each condition. (B) P-values for data presented in panel A were based on an unpaired equal-variance two-tail t-test.

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mitophagy contributes to mitochondrial dysfunction in many neurodegenerative diseases, including A-T [8, 40, 41]. However, the mechanism is likely to be complex, since some AChE inhibitors did not suppress the developmental lethality of ATM mutant flies (S3 and S4 Tables). Nevertheless, the pharmacological screen has uncovered specific avenues to explore for the treatment of A-T.

Materials and methods

Drosophila strains

Fly stocks were maintained on standard cornmeal-molasses food at 25˚C, unless otherwise stated. ATM<sup>8</sup>/TM3,Sb, ATM<sup>3</sup>/TM3,Sb, and ATM<sup>4</sup>/TM6B,Hu flies (referred to as ATM<sup>8</sup>/TM3, ATM<sup>3</sup>/TM3, and ATM<sup>4</sup>/TM6B, respectively) were obtained from the Bloomington Drosophila Stock Center. To generate ATM<sup>8</sup> flies, ATM<sup>8</sup>/TM3 flies were raised at 18˚C or 21˚C. Heterozygous ATM<sup>8</sup>/TM3 flies were maintained at 25˚C.

Pharmacological screens

2400 compounds from the Spectrum Collection (MicroSource Discovery Systems) were obtained in a 96-microplate format, 30 plates of 80 compounds (columns 1 and 12 empty), and were stored at -80˚C. Each plate was thawed at room temperature in light protective packaging for 24 hrs before use. After use, all plates were flushed with nitrogen gas, immediately capped, and stored at -80˚C to prevent degradation. All compounds were provided in 100% DMSO at a concentration of 10 mM. 6 μl of each compound was diluted in 300 μl of deionized, distilled H<sub>2</sub>O (ddH<sub>2</sub>O) to a final concentration of 0.2 mM in 2% DMSO. 200 μl of 0.2 mM compound in 2% DMSO or control 2% DMSO was evenly distributed over the top of freshly made molasses food [42] in vials before the food completely solidified. Compounds were allowed to absorb into the molasses food overnight at room temperature. As described in Fig 1, one plate was tested at a time. Compounds were blindly tested through identification by plate number, row, and column position. For each plate screened, four vials of control 2% DMSO were screened. 0–7 day old ATM<sup>8</sup>/TM3 flies raised at 25˚C were allowed to lay eggs on molasses food with compound for 5–6 days at 25˚C. ATM<sup>8</sup>/TM3 parents were removed from the vials, the vials were incubated at 21˚C for ~12 days, and the percent ATM<sup>8</sup> progeny was determined. For the primary screen, each compound was tested once. For the secondary screen, the 100 compounds with the highest percent ATM<sup>8</sup> from the primary screen were retested in duplicate.

Lifespan assay

ATM<sup>8</sup> and ATM<sup>8</sup>/TM3 flies were raised on molasses food, molasses food with 200 μl of 0.2% DMSO, or molasses food with 200 μl 0.02 mM Ronnel in 0.2% DMSO at 21˚C, collected at 0–5 days old, transferred to fresh molasses vials at approximately 20 flies per vial, and aged at 25˚C. The day of collection was designated day 1. Surviving flies were counted daily until all flies had died. Flies were transferred to new vials approximately every 3 days. Lifespan assays for the different genotypes and compounds were performed at the same time. >186 flies were examined for each assay condition. The number of flies examined is provided in the legend for Fig 2. Kaplan-Meier survival analysis, standard t-tests, and chi square tests were performed using Oasis online application for survival analysis (sbi.postech.ac.kr/oasis/) and Rstudio statistical software.
RT-qPCR analysis

To determine the effect of Ronnel on AMP gene expression when fed during development, 
*ATM*8 flies were raised on molasses food with 0.2% DMSO or molasses food with 0.02 mM Ronnel in 0.2% DMSO at 21˚C, collected at 0–7 days post-eclosion, transferred to molasses food vials without compound or DMSO at approximately 50 flies per vial, and incubated at 25˚C for 24 hrs. Total RNA was isolated from 50 whole flies for each experimental condition, *ATM*8 DMSO (n = 13) and *ATM*8 Ronnel development (n = 14). To determine the effect of Ronnel on AMP gene expression when fed to adult flies, *ATM*8 flies were raised on molasses food at 18˚C, collected at 0–7 days old, transferred to fresh molasses food with 2% DMSO or molasses food with 0.02 mM Ronnel in 0.2% DMSO at approximately 50 flies per vial, and incubated at 25˚C for 24 hrs. Total RNA was isolated from 50 whole flies per experimental condition, *ATM*8 DMSO (n = 15) and *ATM*8 Ronnel (n = 12). RNA was isolated using the RNeasy Plus Mini Kit (Qiagen). cDNA was generated by reverse transcription (RT) with the iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR (qPCR) was carried out as described by Katzenberger *et al.* (2006) [42]. Primer sequences for DiptB, Mtk, AttC, and Rpl32 are described in Katzenberger *et al.* (2016) [42].

Comet assay

The Comet Assay Kit (Trevigen, Catalog #4250-050K) was used with the following modifications, fly brains were dissected in cold phosphate-buffered saline (PBS) and placed in 2 ml Eppendorf tubes at a concentration of 1 brain/10 μl of PBS. A minimum of 6 brains was dissected per sample. Samples were maintained on ice until they all were dissected. Samples were homogenized with plastic pestles for Eppendorf tubes, 10 μl of brain homogenate was added to 75 μl of LMA-garose that had been boiled and cooled to 40˚C in a heat block. 50 μl of LMAgarose/homogenate mix was immediately added to a CometSlide that had been warmed at 37˚C. A 200 μl pipette tip was used to spread the sample evenly across the sample area. Slides were placed flat, at 4˚C for 30 min in the dark to cool and harden. Slides were immersed at room temperature in Lysis Solution and incubated at 4˚C overnight in the dark. Slides were removed from the Lysis Solution and washed by immersion twice for 10 min in 50 ml 4˚C tris-borate-EDTA (TBE). Slides were then placed in an electrophoresis unit side by side lengthwise with the white slide label at the top. The electrophoresis box was filled with 4˚C TBE to just covering the sample on the slide and electrophoresed at 23V for 10 min. Excess TBE was drained from the slides, slides were washed twice for 5 min in ddH2O and then in 70% ethanol once for 5 min, and dried overnight at room temperature in the dark. DNA was stained by placing 100 μl of SYBR Gold solution (30 ml tris-EDTA (TE) buffer and 1 μl SYBR Gold (Molecular Probes, S11494)) on each circle of dried agarose for 30 min at room temperature. Excess SYBR Gold solution was drained from the slides and the slides were quickly rinsed in 50 ml ddH2O and dried for 20–25 min at 37˚C. Comets were imaged using a Zeiss LSM 510 confocal microscope with an Imager.M1 module using a 20X/0.8 NA Plan-APOCHROMAT objective. Fluorescence was excited using an Argon laser at 488 nm. Scan mode was set to frame, frame size was 2048x2048, scan speed was 4 sec, and bit depth was 16 bit. For each sample, >200 comets were imaged and analyzed using CometScore Pro Software (TriTek Corporation). One-way ANOVA and Tukey's post hoc test was performed using RStudio statistical software to compare percent tail DNA between pairs of samples.

Ionizing radiation

Three day old flies were placed in empty vials at approximately 20 flies per vial. Vials were exposed to 50 Gy (5,000 rad) of gamma rays using a Cesium-137 irradiator (J. L. Sheppard Mark I unit). Brains were dissected and processed for the Comet assay 1 hr after irradiation.
Supporting information

S1 Table. Effects of 2400 compounds from the Spectrum Collection (MicroSource Discovery System) on percent eclosion of ATM\(^8\) flies at 21˚C.
(XLSX)

S2 Table. A secondary screen of the 100 compounds that produced the highest percent ATM\(^8\) eclosion in the primary screen.
(XLSX)

S3 Table. A secondary screen of AChE inhibitors from the Spectrum Collection.
(XLSX)

S4 Table. Effects of AChE inhibitors at different concentrations and in combination.
(XLSX)

S5 Table. Kaplan-Meier analysis of the mean lifespan of ATM\(^8\) and ATM\(^8\)/TM3 flies raised with or without Ronnel.
(XLSX)

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References

1. Rothblum-Ovitt C, Wright J, Lefton-Greif MA, McGrath-Morrow SA, Crawford TO, Lederman HM. Ataxia telangiectasia: a review. Orphanet J Rare Dis 2016; 11: 159. https://doi.org/10.1186/s13023-016-0543-7 PMID: 27884168

2. Choy KR, Watters DJ. Neurodegeneration in ataxia-telangiectasia; Multiple roles of ATM kinase in cellular homeostasis. Dev Dyn 2018; 247: 33–46. https://doi.org/10.1002/dvdy.24522 PMID: 28543935
3. Guleria A, Chandna S. ATM kinase: Much more than a DNA damage responsive protein. DNA Repair 2016; 39: 1–20. https://doi.org/10.1016/j.dnarep.2015.12.009 PMID: 26777338

4. Du L, Damoiseaux R, Nahas S, Gao K, Hu H, Pollard JM, et al. Nonaminoglycoside compounds induce readthrough of nonsense mutations. J Exp Med 2009; 206: 2285–2297. https://doi.org/10.1084/jem.20081940 PMID: 19770270

5. Jung M, Timofeeva O, Cheema AK, Varghese R, Ressom H, Dritschilo A. Human fibroblasts for large-scale “omics” investigations of ATM gene function. Adv Exp Med Biol 2011; 720: 181–190. https://doi.org/10.1007/978-1-4614-0254-1_15 PMID: 21901628

6. Du L, Damoiseaux R, Nahas S, Gao K, Hu H, Pollard JM, et al. Nonaminoglycoside compounds induce readthrough of nonsense mutations. J Exp Med 2009; 206: 2285–2297. https://doi.org/10.1084/jem.20081940 PMID: 19770270

7. PLOS ONE | https://doi.org/10.1371/journal.pone.0190821 January 16, 2018 16 / 17

8. Vicente L, Duque M, Silveira R, Sanches A, Carvalho M, et al. ATM kinase inhibition in glioma cells activates the innate immune response and causes neurodegeneration. Proc Natl Acad Sci USA 2012; 109: E656–E664. https://doi.org/10.1073/pnas.1110470109 PMID: 22355133

9. Petersen AJ, Rimkus SA, Wassarman DA. ATM kinase inhibition in glioma cells activates the innate immune response and causes neurodegeneration. Proc Natl Acad Sci USA 2012; 109: E656–E664. https://doi.org/10.1073/pnas.1110470109 PMID: 22355133

10. Petersen AJ, Katzenberger RJ, Wassarman DA. The innate immune transcription factor Relish is necessary for neurodegeneration in a Drosophila model of Ataxia-telangiectasia. Genetics 2013; 194: 133–142. https://doi.org/10.1534/genetics.113.150854 PMID: 23502677

11. Amor S, Puentes F, Baker D, van der Valk P. Inflammation in neurodegenerative disease. Immunology 2010; 129: 154–159. https://doi.org/10.1111/j.1365-2567.2009.03225.x PMID: 20651356

12. Lemaitre B, Hoffmann J. The host defense of Drosophila melanogaster. Annu Rev Immunol 2007; 25: 697–743. https://doi.org/10.1146/annurev.immunol.25.022106.141615 PMID: 17201680

13. McGrath-Morrow SA, Collaco JM, Detrick B, Lederman HM. Serum interleukin-6 levels and pulmonary function in ataxia-telangiectasia. J Pediatr 2016; 171: 256–261. https://doi.org/10.1016/j.jpeds.2016.01.002 PMID: 26851119

14. McGrath-Morrow SA, Collaco JM, Crawford TO, Carson KA, Leighton-Greif MA, Zeitlin P, et al. Elevated serum IL-8 levels in ataxia telangiectasia. J Pediatr 2010; 156: 682–684. https://doi.org/10.1016/j.jpeds.2009.12.007 PMID: 20171651
24. Willoughby LF, Scholsser T, Manning SA, Parisot JP, Street IP, Richardson HE, et al. An in vivo large-scale chemical screening platform using Drosophila for anti-cancer drug discovery. Dis Model Mech 2013; 6: 521–529. https://doi.org/10.1242/dmm.009985 PMID: 22996645

25. Farag MA, Ezzat SM, Salama MM, Tadros MG, Serya RA. Anti-acetylcholinesterase activity of essential oils and their major constituents from four Ocimum species. Z Naturforsch C 2016; 71: 393–402.

26. Gade S, Rajamanikyam M, Vadiapudi V, Nukala KM, Aluvala R, Ciddigari C, et al. Acetylcholinesterase inhibitory activity of stigmasterol & hexacosanol is responsible for larvicidal and repellent properties of Chromolaena odorata. Biochem Biophys Acta 2017; 1861: 541–550. https://doi.org/10.1016/j.bbagen.2016.11.044 PMID: 27916676

27. Čolović MB, Kristić DZ, Laxarević TD, Bondžić AM, Vasić VM. Acetylcholinesterase inhibitors: Pharmacology and toxicology. Curr Neuropharm 2013; 11: 315–335.

28. Galimberti D, Scarpini E. Old and new acetylcholinesterase inhibitors for Alzheimer’s disease. Curr Alzheimer Res 2016; 13: 1100–1111.

29. Pagano G, Rengo G, Pasqualetti G, Femminella GD, Monzani F, Ferrara N, et al. Cholinesterase inhibitors for Parkinson’s disease: a systematic review and meta-analysis. J Neurol Neurosurg Psychiatry 2015; 86: 767–773.

30. Karami-Mohajeri S, Abdollahi M. Mitochondrial dysfunction and organophosphorus compounds. Toxicol Appl Pharmacol 2013; 270: 39–44.

31. Biasibetti-Brendler H, Schmitz F, Pierozan P, Zanotto BS, Prezzi CA, de Andrade RB, et al. Hypoxanthine induces neuroenergetic impairment and cell death in striatum of young adult Wistar rats. Mol Neurobiol 2017 https://doi.org/10.1007/s12035-017-0634-z PMID: 28593435

32. Rosati A, Quaranta E, Ammirante M, Turco MC, Leone A, De Feo V. Quassinoids can induce mitochondrial membrane depolarization and caspase activation in human cells. Cell Death Diff 2004; 11: S216–S218.

33. Bessadottir M, Egilsson M, Einarsdottir E, Magnusdottir IH, Ogmundsdottir MH, Omsdal S, et al. Proton-shuttling lichen compound usnic acid affects mitochondria and lysosomal function in cancer cells. PLoS One 2012; 7: e51296. https://doi.org/10.1371/journal.pone.0051296 PMID: 23227259

34. Mansouri A, Koss MD, Brandt K, Geary N, Langhans W, Leonhardt M. Dissociation of mercaptoacetate’s effects on feeding and fat metabolism by dietary medium- and long-chain triacylglycerols in rats. Nutrition 2008; 24: 360–365. https://doi.org/10.1016/j.nut.2007.12.008 PMID: 18234475

35. Lee EA, Angkla L, Rota SG, Hanlon T, Mitchell A, Hurren R, et al. Targeting mitochondria with Avocatin B induces selective leukemia cell death. Cancer Res. 2015; 75: 2478–2488. https://doi.org/10.1158/0008-5472.CAN-14-2676 PMID: 26077472

36. Gluck MR, Zeevalk GD. Inhibition of brain mitochondrial respiration by dopamine and its metabolites: implications for Parkinson’s disease and catecholamine-associated diseases. J Neurochem 2004; 91: 788–795. https://doi.org/10.1111/j.1471-4159.2004.02747.x PMID: 15525332

37. Liao W, McNutt MA, Zhu W-G. The comet assay: A sensitive method for detecting DNA damage in individual cells. Methods 2009; 48: 46–53. https://doi.org/10.1016/j.meth.2009.02.016 PMID: 19269328

38. Ojha A, Yaduvanshi SK, Pant SC, Lomash V, Srivastava N. Evaluation of DNA damage and cytotoxicity induced by three commonly used pesticides individually and in mixture, in rat tissues. Environ Toxicol 2013; 28: 543–552. https://doi.org/10.1002/tox.20748 PMID: 21786386

39. Sunkaria A, Wani WY, Sharma DR, Gill KD. Dichlorvos-induced cell cycle arrest and DNA damage repair activation in primary rat microglial cells. J Neurosci Res 2013; 91: 444–452. https://doi.org/10.1002/jnr.23173 PMID: 23280485

40. Fivenson EM, Lautrup S, Sun N, Scheibye-Knudsen M, Stevnsner T, Nilsen H, et al. Mitophagy in neurodegeneration and aging. Neurochem Int 2017 https://doi.org/10.1016/j.neuint.2017.02.007 PMID: 28235551

41. Whitworth AJ, Pallanck LJ. PINK/Parkin mitophagy and neurodegeneration-what do we really know in vivo? Curr Opin Genet Dev 2017; 44: 47–53. https://doi.org/10.1016/j.gde.2017.01.016 PMID: 28213158

42. Katzenberger RJ, Ganetzky B, Wassarman DA. Age and diet affect genetically separable secondary injuries that cause acute mortality following traumatic brain injury in Drosophila. G3 2016; 6: 4151–4166. https://doi.org/10.1534/g3.116.036194 PMID: 27754853