Combinatorial effect of maytansinol and radiation in Drosophila and human cancer cells

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SUMMARY
Combination therapy, in which two or more agents are applied, is more effective than single therapies for combating cancer. For this reason, combinations of chemotherapy with radiation are being explored in clinical trials, albeit with an empirical approach. We developed a screen to identify, from the onset, molecules that act in vivo in conjunction with radiation, using Drosophila as a model. Screens through two small molecule libraries from the NCI Developmental Therapeutics Program yielded microtubule poisons; this class of agents is known to enhance the effect of radiation in mammalian cancer models. Here we report an analysis of one microtubule depolymerizing agent, maytansinol isobutyrate (NSC292222; maytansinol), in Drosophila and in human cancer cells. We find that the effect of maytansinol is p53 dependent in Drosophila cells and human cancer cells, that maytansinol enhances the effect of radiation in both systems, and that the combinatorial effect of drug and radiation is additive. We also uncover a differential sensitivity to maytansinol between Drosophila cells and Drosophila larvae, which illustrates the value of studying cell behavior in the context of a whole organism. On the basis of these results, we propose that Drosophila might be a useful model for unbiased screens through new molecule libraries to find cancer drugs for combination therapy.

INTRODUCTION
No single mode of therapy presents a cure for cancer. Multi-modal therapy, in which more than one anti-cancer agent is applied in combination, has more promise and is being assessed in multiple clinical trials (www.clinicaltrials.gov). The precise efficacy and degree of tumor control exhibited by combination therapies, however, remains variable. Although the reasons for variability are unclear, discovery of additional novel drugs that synergize with an existing therapy, such as radiation, will allow multiple combinations to choose from, thereby increasing the likelihood of clinical success. How then can we identify drugs that work together with an existing agent to provide robust therapy against cancer? One solution would be to choose a suitable model and design screens that will allow co-discovery, from the onset, of anti-cancer agents that are effective in combination (as opposed to screening for an agent that has effect on its own and then testing whether it acts in combination with a second agent). Furthermore, a screening model that recapitulates the three-dimensional multicellular context of tumors might provide better predictive value than homogeneous single cell culture models.

We described previously a screen in Drosophila for chemical molecules that enhance the killing effect of ionizing radiation (IR) (Jaklevic et al., 2006). Briefly, third instar larvae were irradiated and cultured in food supplemented with different molecules. Survival to adulthood was quantified 10 days later. Molecules that produced a percent survival that was 2 standard deviations (s.d.) lower than the average survival for the cohort were considered potential hits and were further tested for reproducible effect. A pilot screen through a 1990-molecule library using Drosophila Chk1 (grapes) mutants yielded four molecules of interest (Jaklevic et al., 2006). Two of these are 20S-camptothecin and topotecan, a derivative of camptothecin. Camptothecin and its derivatives inhibit topoisomerase I. Topotecan is an FDA-approved anti-cancer agent that acts as a radiation sensitizer of mammalian tumors. These results serve as proof-of-concept that Drosophila can be a useful screening model for anti-cancer agents that can be used in combination therapy with a standard agent (i.e. radiation).

The pilot screen was performed on the Diversity Set library from the National Cancer Institute Developmental Therapeutics Program (NCI-DTP; http://dtp.nci.nih.gov/). Molecules in this library were chosen for structural diversity rather than biological activity, and the library contains both natural products and synthetic molecules. Of 1990 molecules in this library, approximately 2% are from natural products. Yet, all four hits in the pilot screen were of natural origin. This led us to explore the idea that natural products would be a good source of molecules that increase the effect of radiation. Therefore, we next screened through the Natural Products Set library from the NCI-DTP. This library consists of 235 molecules that were initially identified in extracts of plants or marine organisms. The active molecule in each extract was then identified, synthesized and supplied in a 96-well plate format at 10 mM concentration in DMSO by the DTP. The screen was done ‘blind,’ i.e. without knowledge of the identity of molecules in the whole library or in each well of the coded plate.

The screen through the Natural Product Set (235 molecules) using Drosophila Chk1 and p53 mutants identified 13 molecules...
A Drosophila screen for cancer drugs

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that reproducibly enhanced the effect of radiation (our unpublished data). These included three microtubule depolymerizing agents, a maytansine derivative (NSC292222), colchicine (NSC757) and vincristine (NSC67574). Colchicine was the drug we used in combination with radiation to design the Drosophila screen originally (Jaklevic et al., 2006). Its identification, therefore, was reassuring. Vincristine is an FDA-approved chemotherapy drug against several cancer types and is being tested in combination therapy with radiation in clinical trials (www.clinicaltrials.gov). It has also been used in experiments in Drosophila and was found to increase genetic instability in some assays (Clements et al., 1990; Tiburi et al., 2002). Maytansinol isobutyrate (NSC292222; hereafter referred to as maytansinol) was one of four hits in the pilot screen through the Diversity Set. Its repeat identification in the Natural Product screen attests to the robustness of the screening protocol. The effect of maytansinol and its derivatives in Drosophila has not been described before and forms the focus of this article.

Maytansine is an ansamycin antibiotic found in the Ethiopian shrub Maytenus (Kupchan et al., 1972). Maytansine inhibits microtubule assembly and induces microtubule disassembly in vitro, in cultured mammalian cells, and in embryos of sea urchins and clams (Ootsu et al., 1980; Remillard et al., 1975). Maytansin exhibits cytotoxicity against many tumor cell lines and inhibits tumor growth in vivo (e.g. Ootsu et al., 1980). Early clinical trials failed owing to toxicity at therapeutic levels. Currently there are multiple lines of effort to bypass toxicity by targeting cancer cells with maytansinoid-conjugated homing antibodies (e.g. Krop et al., 2010). Here we present a study of maytansinol in conjunction with IR in Drosophila larvae and human cell culture. The results support the idea that Drosophila larvae can be used to identify molecules that have efficacy in mammalian models of cancer. Furthermore, we report differences in the behavior of Drosophila cells and Drosophila larvae that illustrate the importance of studying cell behavior in a multicellular context in vivo.

RESULTS
We first confirmed that maytansinol depolymerizes microtubules in Drosophila. Wing imaginal discs were removed from third instar larvae were incubated in PBS containing either DMSO (–M) or DMSO with 10 μM maytansinol (+M) for 1 hour, fixed and stained with antibodies to phosphorylated Histone H3 (red in A and B; also shown in C,D,G-J) and β-Tubulin (blue in A and B; also shown in E,F). (A-F) Mitotic spindles (brackets) are visible in control but not drug-treated discs. Scale bar: 6.3 μm. (G,H) Various phases of mitosis are visible in controls but not in drug-treated discs. pm, prometaphase; m, metaphase; a, anaphase; p, prophase. Scale bar: 10 μm. (I,J) Wing imaginal discs from feeding-stage third instar larvae were incubated in PBS containing either DMSO (–M) or DMSO with 2 μM maytansinol (+M) for 2 hours, fixed and stained with antibodies to phosphorylated Histone H3. The number of mitotic cells increased after incubation in maytansinol. (K) Quantification of the data for the experiment shown in I and J. ‘none’ represents discs treated with solvent (DMSO for maytansinol and 95% ethanol for colchicine; n=13 from three different experiments). ‘C’ represents discs incubated in 50 μg/ml colchicine for 2 hours (n=5). ‘M’ represents discs incubated with 2 μM maytansinol for 2 hours (n=8 from two different experiments). The differences between drug-treated discs and controls are statistically significant (Student’s t-test).

Fig. 1. Maytansinol disrupts the mitotic spindle and prevents mitotic exit in Drosophila. (A-H) Wing imaginal discs from feeding-stage third instar larvae were incubated in PBS containing either DMSO (–M) or DMSO with 10 μM maytansinol (+M) for 1 hour, fixed and stained with antibodies to phosphorylated Histone H3 (red in A and B; also shown in C,D,G-J) and β-Tubulin (blue in A and B; also shown in E,F). (A-F) Mitotic spindles (brackets) are visible in control but not drug-treated discs. Scale bar: 6.3 μm. (G,H) Various phases of mitosis are visible in controls but not in drug-treated discs. pm, prometaphase; m, metaphase; a, anaphase; p, prophase. Scale bar: 10 μm. (I,J) Wing imaginal discs from feeding-stage third instar larvae were incubated in PBS containing either DMSO (–M) or DMSO with 2 μM maytansinol (+M) for 2 hours, fixed and stained with antibodies to phosphorylated Histone H3. The number of mitotic cells increased after incubation in maytansinol. (K) Quantification of the data for the experiment shown in I and J. ‘none’ represents discs treated with solvent (DMSO for maytansinol and 95% ethanol for colchicine; n=13 from three different experiments). ‘C’ represents discs incubated in 50 μg/ml colchicine for 2 hours (n=5). ‘M’ represents discs incubated with 2 μM maytansinol for 2 hours (n=8 from two different experiments). The differences between drug-treated discs and controls are statistically significant (Student’s t-test).
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Before declining in number. Red blood cells are the most resistant increase in number initially, owing to mobilization of reserves, a day after whole body radiation. Granulocytes, by contrast, are highly radiation sensitive and are significantly depleted within days before declining. All cell types can recover but the time course and the extent of recovery depends on the radiation dose. Macrophages, which are derived from the same progenitor stem cell type as granulocytes, are highly radiation sensitive.

Next we examined the combined effect of drug and radiation (Fig. 2B,C). The dose of X-rays that results in approximately 50% lethality for each genotype was used in these experiments. We found that the effect of drug and radiation was mostly additive. For example, for wild-type larvae, X-rays alone produced 55.8±7.2% survival and 0.5 nM of maytansinol alone produced 58.1±5.7% survival. The additive effect of drug and radiation was expected to extend beyond fractional survival of 1.0 for solvent controls (0 μM drug) simply because of normalization. WT, wild type; p53, p53 homozygous mutants. The data shown are averages from over 2500 pupae per genotype examined in four (wild type) or five (p53) different experiments. Error bars extend beyond fractional survival of 1.0 for solvent controls (0 μM drug) simply because of normalization. WT, wild type; p53, p53 homozygous mutants. The data shown are averages from over 2500 pupae per genotype examined in four (wild type) or five (p53) different experiments. Error bars extend beyond fractional survival of 1.0 for solvent controls (0 μM drug) simply because of normalization.

In humans, radiation exposure alters blood cell counts. The exact effect depends on cell type and radiation dose [pages 335-337 of Hall and Giaccia (Hall and Giaccia, 2006)]. In general, lymphocytes are highly radiation sensitive and are significantly depleted within a day after whole body radiation. Granulocytes, by contrast, increase in number initially, owing to mobilization of reserves, before declining in number. Red blood cells are the most resistant of all blood cell types and their counts remain unchanged for several days before declining. All cell types can recover but the time course and the extent of recovery depends on the radiation dose. Macrophages, which are derived from the same progenitor stem cell type as granulocytes, are highly radiation sensitive.

First, mitotic spindles were visible in control discs but not in drug-treated discs (Fig. 1A-F, brackets). Second, different phases of mitosis were visible in control discs but not in drug-treated discs. Instead, mitotic cells in drug-treated discs displayed a prometaphase configuration (Fig. 1G,H). This was expected because microtubule depolymerization would trigger the spindle checkpoint and prevent mitotic exit. Third, mitotic index increased after exposure to maytansinol (Fig. 1I-K). Again, this is consistent with the activation of the spindle checkpoint. We conclude that maytansinol depolymerizes microtubules in Drosophila, as do its derivatives in vertebrate cells, an echinoderm and a mollusk (Ootsu et al., 1980; Remillard et al., 1975).

Maytansinol was identified in a screen for molecules that decreased the survival of irradiated larvae. We investigated whether it also decreased the survival of un-irradiated larvae. Third instar larvae were either irradiated or left un-irradiated (control) and cultured in food containing maytansinol at different concentrations. Survival to adulthood was quantified 10 days later by counting the percentage of pupal cases that were left empty as adult flies eclosed. We found that treatment with maytansinol alone (i.e. without radiation) decreased the survival of wild-type and p53 mutant larvae, but to different levels. The effect was greater for p53 mutants than for wild type at 0.5 and 1 μM maytansinol (Fig. 2). At 2 μM maytansinol, the effect was still greater for p53 mutants than for wild type on average but, when compiled over a number of experiments, was found to not be significant.

We noted experiment-to-experiment variability in survival after drug treatment (for example, compare the numbers for wild type in panels A and B of Fig. 2). But two trends were reproducible over multiple experiments: (1) sensitivity to maytansinol was similar or greater for p53 mutants than wild type, and (2) the combined effect of drug and radiation was mostly additive.

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Although we do not know the basis for dose dependence, it was not significantly different on wild-type and p53 mutant cells. At intermediate doses, the effect was more severe for p53+/+ than for p53–/– cells at both low and high doses (Fig. 3). At intermediate doses, the effect was not significantly different on wild-type and p53 mutant cells. Although we do not know the basis for dose dependence, it is reproducible; the data shown depict the average of four independent experiments.

The effect of p53 status on maytansinol sensitivity in HCT116 cells is different from what was found in Drosophila larvae, in which p53 mutants were more sensitive to maytansinol than wild type at least at some concentrations. To address the basis for this difference, we investigated whether Drosophila p53 mutant cells show different sensitivity to killing by maytansinol than do wild-type cells. Third instar larvae were transferred to food containing maytansinol or DMSO carrier (control). Imaginal discs were extirpated 1 day after the transfer and stained with Acridine Orange (AO), a vital dye that is excluded from live cells. In Drosophila, AO is specific for apoptotic cells and does not stain necrotic cells (Abrams et al., 2003). We found that maytansinol induces apoptosis in imaginal discs of wild-type larvae but not p53 mutant larvae (Fig. 4). This parallels the finding in human HCT116 cells, in which maytansinol was more effective when p53 was present, at least at some doses (Fig. 3).

HCT116 cells also allowed us to investigate whether p53 status influenced the combinatorial effects of maytansinol with IR in human cells. The combined effects of two treatments were quantified in terms of Combination Index (CI; see Methods for the formula used) (Chou and Talalay, 1983). CI values of 1, >1 and <1 signify additive, antagonistic and synergistic effects, respectively, between two drugs. We found that adding 5 and 10 Gy of IR to drug treatment at concentrations shown in Fig. 3 produced CI values that are close to or above 1 for both p53+/+ and p53–/– isogenic HCT116 cells (Table 2), reflecting additive or antagonistic interaction between drug and radiation.

To investigate whether the combined effect of maytansinol and IR seen in HCT116 cells also applies to other cell lines, we

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Table 1. Expected and observed survival for Drosophila wild-type and p53 mutant larvae after exposure to a combination of maytansinol and IR

| Genotype      | Drug dose (µM) | Expected survival (%) | S.d. | Observed survival (%) | S.d. |
|---------------|----------------|-----------------------|------|-----------------------|------|
| Wild type     | 0.5            | 32.4                  | 5.3  | 34.5                  | 9.7  |
|               | 1              | 15.2                  | 9.1  | 12.4                  | 3.3  |
|               | 2              | 4.0                   | 1.4  | 2.3                   | 1.8  |
| p53 mutant    | 0.5            | 17.9                  | 8.2  | 18.1                  | 8.6  |
|               | 1              | 3.8                   | 3.0  | 4.7                   | 2.8  |
|               | 2              | 3.9                   | 1.4  | 0.8                   | 1.9  |

The values for expected and observed survival are shown for three different doses of drug. Expected numbers are computed for the scenario in which drug and radiation act in an additive manner.
Successful production of an adult fly from a larva requires proper growth and differentiation of diploid imaginal discs. Following irradiation and induced cell death, remaining cells undergo compensatory proliferation to re-generate imaginal discs (Jaklevic and Su, 2004). Chemical molecules that interfere with compensatory proliferation and regeneration are expected to reduce the fraction of irradiated larva that survives to reach adulthood (Jaklevic et al., 2006). Microtubule depolymerizing agents such as colchicine, vincristine and maytansine would disrupt mitotic progression and thus interfere with compensatory cell proliferation. We speculate that this is the basis for their ability to act in combination with radiation to kill Drosophila larvae. This would explain why two independent screens we have performed for chemical molecules that enhance the killing effect of radiation in Drosophila yielded three microtubule poisons from a total of 2225 molecules (Jaklevic et al., 2006) (and this article). We acknowledge, however, that we cannot rule out the possibility that it is some other activity of this drug, rather than microtubule depolymerization, that increases the radiation sensitivity.

Drosophila larvae and human cancer cells behave similarly in the following ways: (1) maytansinol is more effective at killing p53+/+ than p53−/− cells in Drosophila imaginal discs (Fig. 4) and is more effective on p53+/+ than p53−/− human HCT116 cancer cells at certain concentrations (Fig. 3). (2) In both Drosophila larvae and human cancer cells, maytansinol generally increased the effect of radiation. The extent of this increase varied, however, reflecting a mostly additive interaction between drug and radiation in Drosophila larvae, and additive-to-antagonistic interactions in various cancer cell lines.

In addition to these similarities, Drosophila offers another dimension of experimental analysis, namely the ability to compare cells and multicellular tissues and/or organisms. For example, Drosophila p53 mutant cells, like human p53 mutant HCT116 cells, were more resistant to maytansinol. In the context of a whole organism, the situation seems to be reversed; p53-deficient larvae are more sensitive to maytansinol than were p53 wild-type larvae. This provides a clear example of how cells and multicellular organs or organisms can behave differently and illustrates the value of studying drug effects in vivo in metazoan models.

IR induces apoptosis in imaginal discs of wild-type larvae but not p53 mutant larvae at 4–6 hours after exposure to radiation. At longer times after irradiation, however, p53-independent apoptosis occurs (Wichmann et al., 2006). Maytansinol induced apoptosis in imaginal discs of wild-type larvae but not p53 mutant larvae at 24 hours after exposure to drug. It remains possible that maytansinol also induces p53-independent apoptosis at longer times after drug exposure. Although the difference in apoptosis induction between wild type and p53 mutants is seen at both 1 and 2 μM drug, the

Table 2. CI values for the combination of maytansinol and IR in HCT116 cells

| Genotype | Irradiation (Gy) | nM maytansinol |
|----------|-----------------|----------------|
|          | 0.13            | 0.18           | 0.26 | 0.37 | 0.53 | 0.75 |
| p53+/+   | 5               | 1.934          | 0.78 | 0.912 | 1.373 | 1.446 | 1.479 |
|          | 10              | 1.481          | 1.432 | 1.045 | 1.359 | 1.761 | 2.044 |
| p53−/−   | 5               | 6.796          | 0.887 | 0.902 | 1.244 | 1.283 | 1.817 |
|          | 10              | 1.569          | 1.191 | 1.086 | 1.607 | 1.985 | 2.076 |

The data for 5 and 10 Gy of radiation are shown. CI values are not measured values but are computed from measured values. The numbers reflect the extent of antagonism or synergy between two treatments and can be very large (severe antagonism), very small (strong synergy) or close to 1 (additive effect). 100 R = 1 Gy.

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difference in eclosion is more pronounced and reproducible at 1 μM drug. We do not know the basis for this but note that, although apoptosis is assayed 24 hours after drug exposure, eclosion is assayed 10 days after drug exposure. It is possible that longer-term effects of the drug, such as p53-independent apoptosis, contribute to these findings.

There is precedent for differential sensitivity between cells and organisms. Drosophila p53-deficient cells were more resistant than cells with wild-type p53 to killing by IR. Under the same conditions, Drosophila p53 mutant larvae are more sensitive to IR than larvae with wild-type p53 (Wichmann et al., 2006). But what is the basis for this difference? In both Drosophila and in mice, cells undergoing apoptosis signal to neighboring survivors and induce the latter to proliferate (Li et al., 2010; Ryoo et al., 2004). This process is important for the generation of new cells to replace those lost to apoptosis, for wound healing and for regeneration. In Drosophila, dying cells signal to neighbors through the Wnt (wingless) and JNK pathways (Ryoo et al., 2004). In mice, this phenomenon requires caspases 3 and 7, and operates through prostaglandin E(2), a promoter of stem or progenitor cell proliferation and tissue regeneration (Li et al., 2010). On the basis of these previous findings, we speculate that, in the presence of p53, the most severely damaged cells die by a p53-dependent mechanism but are replaced by healthier survivors that have been induced to proliferate. This allows for regeneration of damaged tissue and survival of the organism. In the absence of p53, damaged cells accumulate but are not replaced. Indeed, irradiated Drosophila p53 mutants acquire elevated genetic instability in loss-of-heterozygosity assays compared with wild type (Sogame et al., 2003). The resulting genetic instability might be too high to be compatible with organism survival, even though cells survive. This could explain the differences we see between larval survival and cell survival in p53 mutants.

Drosophila p53 displays a subset of activities attributed to mammalian p53. Mammalian p53 acts in cell cycle checkpoint control as well as in the induction of apoptosis in response to genotoxin exposure. Drosophila p53 has a proapoptotic function but is not needed for cell cycle checkpoints, at least after IR exposure (Brodsky et al., 2000; Jin et al., 2000; Ollmann et al., 2000; Sogame et al., 2003). Instead, Drosophila p53 is needed for compensatory proliferation after experimental induction of cell death (Wells et al., 2006). Thus, p53 mutants might be doubly disadvantaged after exposure to maytansinol. Not only are proliferative signals from dying cells reduced (because cells fail to undergo p53-dependent apoptosis), but surviving cells additionally have a reduced ability to undergo compensatory proliferation.

We note that the range of drug concentrations used in Drosophila and human cells are different. The experiments in HCT116 cells measure cumulative survival over 6 or 7 days; this protocol is typically used to measure the effect of IR and combination therapies. The range of maytansinol used in Drosophila (1-10 μM) obliterates human cancer cells completely, regardless of genotype (A.E. and T.T.S., unpublished data). Experiments in Drosophila imaginal discs were set up to measure the effect of the drug on mitosis within a cell cycle, and apoptosis shortly after. We also wanted to use the same drug concentrations used on larvae in these experiments so that we could directly compare cellular responses and organismal responses in the same system. Low (<1 nM) concentrations of drug that are effective on human cells do not affect larval survival regardless of genotype. In short, we used drug concentrations that show some effect but still allow survival in each system so that we can then assay for the effect of mutations or radiation on top of the drug effect.

In conclusion, these results support the idea that Drosophila can be used to identify anti-cancer drugs. Furthermore, Drosophila offers an opportunity to compare the effect of small molecules on cells and on multicellular structures. We report here the results of a screen to identify small molecules that increase the effect of radiation. We are adapting the screen to identify small molecules that increase the effect of other standard therapies. Drosophila models of neurodegeneration are currently being used for drug identification or testing, both in academic laboratories and in industry (e.g. Bortvedt et al., 2010; Rana et al., 2010; Sarantseva et al., 2009) (www.vitruevan.com). We hope that Drosophila models of human cancers will likewise become useful for identifying new anti-cancer drugs in the future. Expression of the Drosophila RET receptor tyrosine kinase, which has been mutated to mimic human oncogenic mutants, causes defects in the adult eye. Importantly, these defects can be suppressed by a known chemical inhibitor of human RET (Vidal et al., 2005). Such results support the idea that small molecule inhibitors found in Drosophila models have the potential to apply to human cancers.

METHODS

Fly stocks

Wild-type flies were of the Sevelin stock. p53G4A-1-4 results from targeted deletion of the gene (Rong et al., 2002).

Irradiation

Feeding-stage third instar larvae were irradiated as previously described (Jaklevic et al., 2006). Briefly, 120-hour-old larvae were rinsed to remove food and passed through sizing sieves to obtain animals of uniform size. Larvae were placed in a Petri dish and irradiated using a TORREX X-ray generator, set at 115 kV and 5 mA (producing 2.4 Rads/second). Irradiated larvae were then cultured on cornmeal-agar media (Jaklevic et al., 2006) containing drug or DMSO carrier. Human cells in 96-well plates were irradiated with a RS2000 Biological Irradiator (Rad Source Technologies) delivering 1 Gy/minute.

AO staining

Larvae were dissected in PBS. Imaginal discs were incubated for 5 minutes in PBS + 0.5 mM AO (Sigma) at room temperature, washed once with PBS, mounted in PBS, and imaged immediately using a Leica DMR fluorescence compound microscope, a Sensicam CCD camera and Slidebook software (Intelligent Imaging). Images were compiled using Photoshop software.

Antibody staining

To detect phosphorylated Histone H3, larval imaginal discs were extirpated in PBS, fixed for 10 minutes in PBT (PBS with 0.2% Tween) containing 10% formaldehyde and washed three times with PBT. Samples were incubated with primary antibodies in blocking solution, which is PBT + 3% normal goat serum, for 2 hours at room temperature or overnight at 4°C. Primary antibodies were
rabbit polyclonal anti-phospho-Histone-H3 antibody (Upstate Biotechnology) diluted at 1:1000 and mouse monoclonal anti-β-Tubulin antibody (Developmental Hybridoma Bank) diluted at 1:100. Samples were then washed three times with PBT and incubated for 2-4 hours at room temperature with secondary antibody conjugated to rhodamine or fluorescein, diluted to 1:500 in blocking solution (Jackson ImmunoResearch). Samples were washed three times with PBT, stained with 10 μg/ml Hoechst 33258 in PBT for 2 minutes, and washed three times with PBT before mounting onto slides with Fluoromount G. Samples were imaged on a Leica DMR fluorescence microscope using a Sensicam CCD camera and Slidebook software (Intelligent Imaging). Images at different focal planes were combined using ImageJ (http://rsb.info.nih.gov/ij/), displayed using Photoshop software and the number of mitotic cells was counted manually.

**Cell lines**

The HNC and NSCLC cell lines were kindly provided by David Raben and Paul Bunn (University of Colorado Cancer Center, CO). These cell lines were maintained in RPMI media supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT) in a humidified incubator with 5% CO2. HCT116 cells were kindly provided by Joaquin Espinosa (University of Colorado, Boulder, CO) and grown in McCoy’s medium with 10% FBS.

**Cell growth assay**

The growth inhibitory effects of maytansinol with radiation were evaluated using a modified tetrazolium salt (MTT) assay (Carmichael et al., 1988). In the MTT assay, 1000-2000 viable cells were plated in 100 μl of growth medium in 96-well plates (Corning, Ithaca, NY). Following an overnight incubation, maytansinol was added at varying concentrations and the plates were irradiated on the same day (cotreatment) or 24 hours later (pre-treatment) and incubated for 6-7 days. The tetrazolium salt was added at a concentration of 0.4 mg/ml to each well following the 6- to 7-day treatment. The plates were incubated with the salt for 4 hours at 37°C. At 4 hours, the medium was aspirated off, leaving the dark blue formazan product at the bottom of the wells. The reduced MTT product was solubilized by adding 100 μl of 0.2 N HCl in 75% isopropanol, 23% MilliQ water to each well. Thorough mixing was done using a Titertek multichannel pipetman. The absorbency of each well was measured using an automated plate reader (Molecular Devices, Sunnyvale, CA). All experiments were performed in triplicate.

**Hemocyte counting**

Hemolymph was collected by opening two larvae lengthwise in 20 μl of PBS containing 10 μg/ml Hoechst 33342 (Molecular Probes), on a siliconized glass slide, using #5 forceps (Ted Pella). Larval carcasses were removed and hemolymph in PBS was incubated for 2 minutes in a humidified chamber to allow cell-permeable DNA dye to stain nuclei. 10 μl of stained cells in PBS was placed onto a hemocytometer and cells that were identified by UV illumination of Hoechst 33342 were counted. We used two larvae per sample to ensure that we had at least 10 μl of material left for counting after mixing and incubation; dissecting one larva in 10 μl of PBS resulted in less than 10 μl of recoverable material. ‘+IR’ was 4000 R for wild type and 3000 R for p53 for reasons explained in the Results.

**Results**

The authors report an in vivo system for the co-discovery of synergistic anti-cancer agents, using *Drosophila* as a model. They use a screen (US Patent No. 7,695,899) to identify molecules that act in vivo in conjunction with radiation. Following radiation exposure, organ precursors in *Drosophila* larvae undergo cell death followed by repopulation via proliferation; this process involves genes that are homologous to human genes. Thus, compounds that interfere with repopulation following radiation exposure in *Drosophila* larvae might be viable drug candidates for combination anti-cancer therapy. Screens of two molecule libraries from the NCI identify microtubule poisons (which are known to enhance the anti-cancer effect of radiation in mammalian cancer models) as candidates. More detailed analyses of one microtubule-depolymerizing agent, maytansinol, show that this compound enhances the effect of radiation both in *Drosophila* cells and human cancer cells, and that its effects are p53 dependent. Notably, the authors find that *Drosophila* cells and *Drosophila* larvae have differential sensitivities to maytansinol.

**Implications and future directions**

This study demonstrates the use of *Drosophila* as an in vivo model for the discovery of chemical compounds that enhance the effect of radiation, a standard therapy for cancer. The screen described could be adapted to identify chemical molecules that increase the effect of other standard therapies, such as taxol. The results also provide an example of how cells and multicellular organs or organisms can respond differently to such compounds, emphasizing the value of studying drug effects in vivo in a metazoan model.

We found that hemocytes are typically counted in one of two ways in published reports; total hemocytes per larva or hemocytes per unit volume of hemolymph. We used the former approach because of technical ease, but found that our numbers are in line with published counts from the latter approach. For example, we counted 725-5625 hemocytes per wild-type larva control (no IR, no drug, Sevelin strain). The amount of hemolymph that can be extracted from an individual *Drosophila* third instar larva ranges from 50 to 300 nl (Piyankarage et al., 2008). If we assume that our lower counts reflect a smaller volume of hemolymph, our counts translate to 725/50 nl or 14.5×10⁵ hemocytes per μl. At the high end, 5625/300 nl translate to 18.8×10³ hemocytes per μl. The published values are similar [e.g. 7.8×10³/μl for Canton S (Tokusumi et al., 2009) and ~5×10²/μl for a GAL4 control (Asha et al., 2003)]. The average hemocyte count per wild-type larva in our studies was ~2500. This is in line with an average of ~4000/animal measured for another wild-type strain, Oregon R (Sinенко et al., 2004).

**Combination index**

C1 values are calculated according to the formula: CI=(Dx)(Dx)/((Dx)1 + (Dx)2) (Chou and Talalay, 1983). (Dx)1 and (Dx)2 are the doses required to achieve a given effect level for each treatment, i.e. a
specified value of fraction affected, or $F_x$. (D1) and (D2) are the doses of each treatment in a given combination which gives the same $F_x$.

**Standard deviation**

S.d. for expected fraction survival for co-treatment of drug and radiation is computed according to the formula for the s.d. of a product of two normally distributed variables. For two normally distributed variables with means $m_1$ and $m_2$ and s.d. $s_1$ and $s_2$, respectively, the product will have mean $m_1 m_2$ and the s.d. $\sqrt{s_1^2 s_2^2}$ [page 140 of Menzel (Menzel, 1960)]. Fractional survival after genotoxin treatment shows a normal (Gaussian) distribution around a mean [figure 4 of Jaklevic et al. (Jaklevic et al., 2006) and our unpublished observations].

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**COMPETING INTERESTS**

T.T.S. is a co-founder, D.R. is a member of the scientific advisory board and B.F. is a consultant for Suvica. The University of Colorado has an issued patent for the Drosophila screen described here (US Patent #7,695,899).

**AUTHOR CONTRIBUTIONS**

T.T.S., B.F. and D.R. conceived and designed the experiments. A.E., M.G. and P.Y. performed the experiments. A.E., M.G., P.Y. and T.T.S. analyzed the data. D.R. contributed reagents and materials. M.G. and T.T.S. wrote the paper.

**SUPPLEMENTARY MATERIAL**

Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.006486/-/DC1

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