# An *in vivo* large-scale chemical screening platform using *Drosophila* for anti-cancer drug discovery

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#### SUMMARY

Anti-cancer drug development involves enormous expenditure and risk. For rapid and economical identification of novel, bioavailable anti-tumour chemicals, the use of appropriate *in vivo* tumour models suitable for large-scale screening is key. Using a *Drosophila* Ras-driven tumour model, we demonstrate that tumour overgrowth can be curtailed by feeding larvae with chemicals that have the *in vivo* pharmacokinetics essential for drug development and known efficacy against human tumour cells. We then develop an *in vivo* 96-well plate chemical screening platform to carry out large-scale chemical screening with the tumour model. In a proof-of-principle pilot screen of 2000 compounds, we identify the glutamine analogue, acivicin, a chemical with known activity against human tumour cells, as a potent and specific inhibitor of *Drosophila* tumour formation. RNAi-mediated knockdown of candidate acivicin target genes implicates an enzyme involved in pyrimidine biosynthesis, CTP synthase, as a possible crucial target of acivicin-mediated inhibition. Thus, the pilot screen has revealed that *Drosophila* tumours are glutamine-dependent, which is an emerging feature of many human cancers, and has validated the platform as a powerful and economical tool for *in vivo* chemical screening. The platform can also be adapted for use with other disease models, thus offering widespread applications in drug development.

#### INTRODUCTION

The identification of bioavailable, tumour-specific anti-cancer compounds for clinical use by the conventional pharmaceutical pipeline of *in vitro* screening has been highly inefficient. The most direct manner of identifying such compounds would be to use in vivo screening using whole-animal tumour models. For such screens to be effective, very large lead-like chemical libraries need to be assayed and, at present, the screening of mouse models in this way is unfeasible due to cost and time constraints. However, the vinegar fly Drosophila melanogaster is ideal for large-scale screening. Indeed, recent studies have highlighted the strong conservation in signalling pathways from flies to humans that enables the cross-reactivity of 'human' drugs in flies, and the capacity of drugs to be efficacious in fly larvae and adults when ingested in their food (e.g. Kang et al., 2002; Agrawal et al., 2005; McBride et al., 2005; Vidal et al., 2005; Stilwell et al., 2006; Aritakula and Ramasamy, 2008; Edwards et al., 2011). A great deal this pioneering work has been carried out using of

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neurodegeneration models in the adult fly (reviewed in Pandey and Nichols, 2011); however, with Drosophila now being increasingly recognised as an excellent organism for modelling tumour formation (reviewed in Brumby and Richardson, 2005), there are considerable opportunities for their use in anti-cancer drug development and discovery (reviewed in Gladstone and Su, 2011). Key to the success of this approach is the use of relevant Drosophila models for human cancer. Recently, a Drosophila model of multiple endocrine neoplasia type 2, driven by overexpression of Ret kinase (dRet), was used to powerfully demonstrate how in vivo screening of polypharmacological kinase inhibitors could be combined with genetic analysis to fine-tune compounds for increased chemical efficacy and reduced toxicity (Dar et al., 2012). This type of focussed chemical screening has significant potential and its principles can be readily adapted to other disease models. However, for large-scale chemical screening using diverse chemical compound libraries, screening platforms and assays also need to be developed to enable rapid identification of novel lead-like compounds suitable for followup development.

We have developed *Drosophila* epithelial cancer models that are driven by the ectopic expression of orthologues of activated human oncogenes, Ras ( $Ras^{V12}$ ) or Notch ( $N^{intra}$ , the intracellular domain of Notch). When overexpression of either of these oncogenes is combined with loss of the epithelial cell polarity regulator, *scribbled* (*scrib*), in mitotic clones within the eyeantennal disc, massive tumours develop throughout the larval stage of development. These tumours recapitulate many of the hallmarks of human cancers, including increased cell proliferation, survival, a failure to differentiate and increased invasion and metastasis (Brumby and Richardson, 2003; Pagliarini and Xu, 2003). Localised inflammation also drives tumour development (Pastor-Pareja et al., 2008; Cordero et al., 2010), thus further paralleling the development of human cancers. The models are

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## **TRANSLATIONAL IMPACT**

#### **Clinical issue**

The development of new anti-cancer therapeutics is very costly and has a low success rate. Conventional drug-development involves the identification of a suitable candidate gene or protein to target, followed by in vitro screening for chemical inhibitors of the candidate and, finally, chemical modifications to increase in vivo bioavailability and efficacy. It is often only during the final stages of drug development that issues of inefficacy or toxicity are fully revealed, leading to a high attrition rate amongst candidate drugs. An alternative approach to drug development is to carry out unbiased chemical screening in vivo using appropriate cancer models. This approach has the advantage of being able to quickly select compounds with good drug-like potential and high specificity for tumour cells, and without adverse host toxicity. Time and financial restraints mean this approach is currently unfeasible using mouse models, but the high conservation of signalling pathways between Drosophila and mammals, as well as the speed and economy of Drosophila in vivo screens, make the fly a powerful tool to exploit for drug development.

#### Results

Using a *Drosophila* epithelial Ras-driven cancer model, the authors demonstrate that tumour development can be abrogated by feeding tumourbearing larvae with chemicals of known efficacy against human tumour cells and with pharmacokinetics essential for drug development. They design a 96well plate *in vivo* screening platform for large-scale chemical screening and, in a proof-of-principle pilot screen of 2000 compounds, identify the glutamine analogue acivicin (a chemical with activity against human tumour cells) as a potent and specific inhibitor of *Drosophila* tumour formation. They then use RNAi-mediated knockdown of candidate genes to show that CTP synthase, an enzyme involved in pyrimidine biosynthesis, might be an essential target of acivicin-mediated inhibition in *Drosophila*.

## Implications and future directions

The validation of this large-scale *in vivo Drosophila* chemical screening platform highlights the significant but largely untapped potential of an important system for anti-cancer drug development. The platform could be adapted to screen for compounds that target other types of tumour, or even for other diseases that can be modelled in *Drosophila* larvae. Sophisticated *Drosophila* genetics tools can complement this chemical screening approach to aid in the elucidation of chemical targets and in understanding the mechanism of drug efficacy. Such unbiased and integrated approaches should help to identify important new drug targets, and will hopefully improve the outlook for drug development.

ideally suited to large-scale chemical screening because tumour development within the larvae can be monitored in situ by GFP expression. Clones of mutant tissue are induced in the 1st instar stage of larval development (day 1-2), and by the 3rd instar stage (day 5) these have formed large GFP-positive tumours. Larvae normally initiate pupal development at day 5-6; however, the continual proliferation of the tumour cells blocks pupariation in ~80% of the tumour-bearing larvae, resulting in an extended larval stage of development during which the GFP-positive tumour cells massively overgrow and invade surrounding tissues. The extent of GFP signal acts as a reproducible parameter to gauge antitumour chemical efficacy. In this report we develop a large-scale chemical screening platform that scores this parameter for drug discovery. The results of a pilot screen identify the glutamine analogue and known anti-tumorigenic compound acivicin as a potent inhibitor of Drosophila tumour formation. Genetic analysis suggests that CTP synthase could be the crucial target of acivicinmediated inhibition. Thus, the screen both validates the potential of the screening platform for drug discovery and has revealed a glutamine-dependency of the *Drosophila* tumours that is shared by some human cancers and is an area of increasing therapeutic interest.

## RESULTS

## *Drosophila* tumours are responsive to compounds with good bioavailability and known anti-cancer activity

To initially establish the suitability of the *Drosophila* cancer models for chemical intervention, we used the Ras-driven tumour model to screen a panel of inhibitors targeting mitogen-activated protein kinase kinase (MEK), a key downstream target of Ras signalling through the MAP kinase pathway. Five MEK inhibitors were analysed, all of which were capable of reducing ERK phosphorylation, a downstream target of MEK signalling, in vitro using Drosophila S2 cells (Fig. 1A,B). However, of the five MEK inhibitors, only exposure of the tumour-bearing larvae to PD0325901 (50 µM final concentration in the food) was efficacious in reducing GFP-positive tumour burden (Fig. 1C,D), and also in restoring pupariation to the tumour-bearing larvae (supplementary material Fig. S1). Significantly, PD0325901 was derived from the same chemical series as CI-1040, and both exhibit similar affinities for their target kinase. However, CI-1040 was not efficacious against the Drosophila tumour model and has poor aqueous solubility, cell permeability, and is rapidly metabolized compared with PD0325901 (Barrett et al., 2008). Therefore, the data are consistent with the *in vivo* screening, preferentially identifying compounds with both favourable physiochemical properties and the metabolic stability necessary for good drug bioavailability.

To establish whether the tumour model could also be used to isolate compounds with efficacy against human tumours, we then used the Ras-driven tumour model to conduct a boutique screen using a small panel of anti-neoplastic compounds, including drugs that are routinely used in the clinic (supplementary material Table S1). Significantly, exposure of the tumour-bearing larvae to 50µM idarubicin, an anthracycline drug that intercalates into DNA, resulted in a significantly reduced GFP-positive tumour burden, compared with those treated with DMSO alone. Also, like PD0325901, idarubicin increased the frequency of pupariation (supplementary material Fig. S2). Idarubicin is clinically used to treat acute myeloid leukaemia, thus demonstrating the utility of the tumour model to isolate clinically relevant compounds. Furthermore, idarubicin can be orally administered to patients, having been developed from daunorubicin for increased bioavailability (Goebel, 1993). Because daunorubicin was ineffective at restraining the Drosophila tumours at concentrations an order of magnitude greater than that of idarubicin (supplementary material Table S1), these data again highlight the potential of the screening approach to identify compounds with good bioavailability.

## Screening platform and proof-of-principle pilot screen

Having established that the cancer model was amenable to chemical screening, we developed parameters to grow larvae in a 96-well plate format for large-scale screening (Fig. 2A). Seven larvae were grown per well, which is a significant enough number to gauge a reliable response without overcrowding. After growth of the larvae



**Fig. 1. PD0325901 inhibits tumour development** *in vivo.* (A) Western blot of S2 cell lysates, prepared 30 minutes after treatment of cells with carrier alone (0.1% DMSO) or various MEK inhibitors (final concentration, 10  $\mu$ M), and probed with antibodies to phosphorylated ERK (p-ERK), total ERK (t-ERK) and a-tubulin (a-Tub). (B) Quantification of phosphorylated ERK levels compared with total ERK levels from the western blot, normalised to DMSO alone. (C) Representative examples of 9-day-old larvae, bearing GFP-expressing (white) *Ras<sup>V12</sup> scrib*<sup>1</sup> tumours, after 5 days of exposure to carrier alone (0.5% DMSO) or MEK inhibitor (final concentration, 50  $\mu$ M). (D) Quantification of GFP-positive tumour burden per larva after MEK inhibitor exposure, as measured by the number of white pixels (from binarised images of GFP fluorescing larvae) within a defined area from the anterior end of each larva (one way ANOVA, \**P*<0.005; *n*=6; error bars indicate s.e.m.).

in the chemical-containing food for five days, a 30% sucrose solution was added to each well which, with agitation, enabled larvae and pupae to float to the surface where they could be imaged under both white and fluorescent light (Fig. 2B). The number of larvae and pupae per well were recorded digitally and, after binarising the fluorescent images of every plate, the number of white pixels in each well was divided by the number of individual entities (larvae + pupae) to give a numerical representation (pixel count) of the amount of GFP-labelled tumour tissue per larva or pupa in each well. We used this assay to determine the efficacy of chemicals in abrogating tumour growth.

To demonstrate the utility of the screening platform, we undertook a proof-of-principle pilot screen with the MicroSource Discovery Systems Spectrum Collection. This library contains ~2000 compounds, many of which have known biological targets. The library was stored at a concentration of 10 mM in DMSO and diluted to a final concentration of 50  $\mu$ M/well in the food. Significantly, both PD0325901 and idarubicin exhibited efficacy at this concentration, and the resulting final DMSO concentration of 0.5% is not lethal to *Drosophila* larvae (supplementary material Fig. S3). Compounds were screened in duplicate to facilitate verification of hits, with a hit only being recognised if the average pixel count from the combined duplicate wells was two standard deviations lower than the average pixel count across the entire screen.

Across the 2000 compounds screened, we observed an average of 16.56±0.24% (s.e.m.) pupariation, which remained largely consistent throughout the screen (supplementary material Fig. S4), and the average pixel count per larva or per pupa in each well was 352.68±3.20 (Fig. 2C). Forty-six compounds were recorded as giving pupariation frequencies higher than 50%; however, only ten hits were identified with pixel counts that were two standard deviations lower than the average pixel count. Examination of white light images indicated that eight of the ten compounds were larval-lethal; however, two compounds reduced GFP pixel count without adversely effecting larval viability. One of these two compounds failed to be validated upon retesting; however, compound 2O1E3 (Spectrum ID 01502002) gave a highly reproducible reduction in GFP pixel count, although pupariation frequencies were not noticeably increased (Fig. 3A). Closer examination of the dissected tumours confirmed the reduction in tumour overgrowth compared with DMSO-exposed control larvae (Fig. 3A).

## Pharmacogenetic analysis reveals glutamine utilisation and CTP nucleotide synthesis as crucial tumorigenic targets

Compound 2Q1E3 is a glutamine analogue known as acivicin (AT-125 or U-42126), which has been extensively investigated for its anti-tumorigenic activity (Earhart and Neil, 1985). It irreversibly inhibits glutamine-dependent amidotransferases, a class of enzymes that catalyse the transfer of the amido group of glutamine (thus generating glutamate) to a substrate and are involved in a variety of processes, including nucleotide and amino acid biosynthesis (Earhart and Neil, 1985). Sourcing an independent supply of acivicin confirmed its strikingly potent anti-tumour efficacy (supplementary material Fig. S5). Significantly, identical exposure of wild-type flies to acivicin showed that it was non-toxic and produced no developmental defects (data not shown), thus demonstrating its tumour specificity. The capacity of acivicin to restrain Ras-driven tumour overgrowth in Drosophila was not larval-stage-specific because it was also an effective therapeutic when fed to adult flies that had been injected with Ras-driven tumour cells (Fig. 3B). Furthermore, it was also an effective therapeutic when used against a Notch-driven (N<sup>intra</sup> instead of  $Ras^{V12}$ ) tumour model (supplementary material Fig. S6). By contrast, the MEK inhibitor PD0325901 lacked efficacy against the Notch tumour model, thus indicating that although different Drosophila tumour types can exhibit unique profiles of drug susceptibility, acivicin exhibits broad anti-tumour activity.

To determine which glutamine-dependent amidotransferase could be the key anti-tumorigenic target of acivicin, we made use of fly genetics and commercially available RNAi overexpression lines directed against nine of eleven identified glutamine-dependent amidotransferases in the fly genome. Using a variant of the Ras<sup>V12</sup>-driven tumour model in which it was possible to carry out F1 screening (Willecke et al., 2011), and which was also responsive to acivicin-mediated inhibition (data not shown), we overexpressed individual RNAi lines in the tumour and assayed for tumour development by quantifying GFP fluorescence. The strongest reduction in tumour growth was elicited by overexpression of  $CTPsyn^{RNAi}$  (CG6854) and CG9674<sup>RNAi</sup> (Fig. 3C). CG9674 encodes a glutamate synthase with no mammalian orthologue; however, CTPsyn expresses CTP synthase, an enzyme responsible for generating CTP from UTP and glutamine (Fig. 3F), and,



**Fig. 2. The** *Drosophila* **chemical screening platform and summarised screen results.** (A) Schematic of the chemical screening protocol. The chemical library, stored as 96-well plates, was diluted with water and used to reconstitute powdered instant *Drosophila* media in 96-well deep-well plates (final compound concentration, 50 μM, DMSO <0.5%). Seven 4-day-old *Ras<sup>V12</sup> scrib<sup>1</sup>* tumour-bearing larvae were then added to each well. The plate was sealed with fine mesh, held firmly in place by a perspex lid (containing a hole over every well, to allow air access to each well). After 5 days of incubation at 25°C, sucrose solution was added to each well, resulting in all larvae and pupae floating to the surface, before imaging. The number of larvae and pupae in each well were then scored from a white light image. A binarised GFP image was used to calculate the white pixel count per larva in each well. These data were stored in a database, from which positive hits were identified on the basis of a decrease in pixel count. (B) Example screening data showing white light, GFP and binary images captured for an entire plate. Note the location of compound 2Q1E3 in the third column, fifth row (circled). (C) Histogram summary of the complete screen data, showing the average pixel count per larva for each compound (*n*=2 replicates for each compound), and potential hits on the basis of a decreased pixel count.

significantly, mammalian CTPsyn is known to be a key target of acivicin-mediated inhibition (Jayaram et al., 1975). The expression of *CTPsyn*<sup>*RNAi*</sup> in *Drosophila* strongly reduces *CTPsyn* protein levels (Chen et al., 2011), and expressing it within the original Ras-driven tumour model confirmed its capacity to dramatically restrain tumour overgrowth (Fig. 3D), although pupariation was not noticeably increased (data not shown). Interestingly, knockdown of *CTPsyn* in otherwise wild-type clones of tissue did not impede clonal growth as compared with wild-type clones, thus indicating

that CTPsyn was not crucially rate-limiting for the growth or proliferation of normal cells, but was specifically required for tumour cells (supplementary material Fig. S7). CTPsyn as a drugable anti-tumorigenic target was further validated through exposure of the tumour-bearing larvae to another known CTP synthase inhibitor, the uridine analogue 3-deazauridine (McPartland et al., 1974), which also elicited significant reductions in tumour overgrowth (Fig. 3E). This analysis confirms the importance of CTPsyn in *Drosophila* tumour development and



Fig. 3. Acivicin inhibits tumour development, and CTP synthase could be a key anti-tumorigenic target. (A) Nine-day-old larvae, bearing GFP-labelled Ras<sup>V12</sup> scrib<sup>1</sup> tumours, after 5 days exposure to carrier alone (0.5% DMSO) or 50 µM acivicin. A representative eye-antennal imaginal disc pair, dissected from these larvae, is shown below. (B) Representative examples of  $w^{1118}$  flies two days after receiving transplantation into their abdominal cavity of GFPlabelled Ras<sup>V12</sup> scrib<sup>1</sup> tumour tissue from 9-day-old larvae (day 0 of treatment regime), and 4 days later after treatment with either carrier alone (DMSO) or 50 uM acivicin (day 4). On the right is shown quantification of fold increase in tumour area in adult flies after 4 days of treatment (t-test, P=0.0007; n=10). (C) Quantification of GFP-positive tumour overgrowth in a Ras<sup>V12</sup> dlg<sup>RNA</sup> tumour model (Willecke et al., 2011) upon knockdown of candidate glutamine-dependent amidotransferases (n=6 pupae). The overexpression of dap (Drosophila p21 homologue) was used as a positive control. Knockdown of CTPsyn and CG9674 (a glutamate synthase with no mammalian orthologue) exerted a significant reduction in tumour overgrowth. Representative images of the tumour-bearing pupae are shown on the right. (D,E) Quantification of pixel counts at day 9, upon knockdown of CTPsyn in GFP-labelled Ras<sup>V12</sup> scrib<sup>1</sup> tumour-bearing larvae (D), and after 5 days exposure of Ras<sup>V12</sup> scrib<sup>1</sup> tumourbearing larvae to 50 µM acivicin or 500 µM 3-deazauridine (E); one way ANOVA, \*P<0.0001; n=8 wells (D) and n=3 wells (E). (F) Above: Drosophila glutamine-dependent amidotransferases involved in nucleotide biosynthesis potentially targeted by acivicin, with CTPsyn highlighted as a potentially key biologically relevant target. Below: simplified pathway of glutamine utilisation for energy production in tumours that is inhibited by AOA. Error bars in B-E indicate s.e.m.

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suggests that the anti-tumorigenic effects of acivicin in *Drosophila* might be mediated through CTPsyn inhibition.

Finally, the isolation of acivicin and identification of a glutaminedependent step in pyrimidine synthesis as rate limiting for tumour development (Fig. 3F), suggested that Drosophila tumours might exhibit additional levels of glutamine dependency, as has been shown for some human cancers (DeBerardinis and Cheng, 2010), Indeed, the tumour cells are also likely to be dependent upon glutamine as a carbon source for energy production because tumour overgrowth was significantly abrogated (supplementary material Fig. S8) by exposure of the tumour-bearing larvae to amino-oxyacetic acid (AOA), a transaminase inhibitor and potentially promising anticancer therapeutic that prevents the entry of glutamine-derived glutamate into the TCA cycle (Moreadith and Lehninger, 1984) (Fig. 3F). The Drosophila tumours therefore depend upon multiple glutamine-dependent metabolic processes, which is a feature shared by some human cancers and is an area of increasing therapeutic interest (Wise and Thompson, 2010).

## DISCUSSION

In this report, we have described development of a novel in vivo chemical screening platform and demonstrated its capacity to identify anti-cancer compounds with pharmacokinetics favourable for in vivo drug efficacy, thereby validating it as an effective and economical tool for large-scale chemical screening. Our criterion for identifying chemical hits was based upon the assay of GFP fluorescence within the tumour-bearing larvae. By using a stringent cut-off for hit selection, two of the 2000 compounds screened were flagged for re-screening, although only one of these, acivicin, was subsequently validated. Abrogation of tumour overgrowth can also be associated with a restoration of pupariation, and 46 of the 2000 compounds were identified with pupariation frequencies above 50% in both duplicates. However, none of these compounds were additionally identified as having significantly reduced the GFPpositive tumour burden, even though these two properties were correlated in our original analysis of PD0325901 and idarubicin efficacy. Indeed, acivicin was striking for its capacity to reduce tumour burden, even though pupariation frequencies were unaffected. Normally, pupariation is tightly coordinated with imaginal disc growth (Colombani et al., 2012; Garelli et al., 2012), and if normal growth is not restored to the tumour-bearing discs, the signals that drive pupariation will not be produced at appropriate levels. Possibly, acivicin treatment of Ras-driven tumours only slows down tumour overgrowth and stronger inhibition might be required to restore the normal developmental program of pupariation to the larvae. Interestingly, acivicin also reduced the GFP-positive tumour burden in Notch-driven tumourbearing larvae, although in this case, it was correlated with a restoration of pupariation. Thus, the pupariation response can be highly variable and, overall, fluorescence serves as a more reliable measure of tumour development than puparium formation. We also note that 13 of the 2000 compounds proved to be lethal to the larvae in both duplicate wells. Because toxicity might mask an antitumorigenic activity, the re-screening of these compounds at lower concentrations will be important to reassess their anti-tumorigenic potential.

A key aspect of our screening approach was the growing of larvae in 96-well plates to facilitate analyses at a medium to high throughput level. Although this practice has not yet been widely documented, a prior study adopted a similar approach to identify chemicals capable of increasing puparium formation and adult viability to larvae carrying a mutation in the fragile X mental retardation 1 (FMR1) gene (Chang et al., 2008). This screen utilised an automated large particle flow cytometer (COPAS $^{\text{TM}}$  Select) to automatically sort and distribute embryos of the right genotype into wells containing food mixed with chemicals, and efficacy was established through counting the number of adults and pupae after 10-15 days. Our approach has been similar, although there were three differences: first, we manually distributed larvae of the right genotype into the 96-well plates. Second, we used a commercial dry food that can be reconstituted with water and chemical, as opposed to the adding and mixing of chemicals into food that has been melted. Third, we used a relatively rapid method of scoring that relied upon floating the larvae and pupae to the surface of the wells where they could be batch-imaged. With this scoring technique, and using acivicin as a positive control, we calculated a Z-prime factor (a statistical measure of an assay's suitability for screening purposes) of 0.67 (supplementary material Fig. S9), which is well within the range of 0.5-1.0 (the ideal assay) that is recommended for high-throughput screening (Zhang et al., 1999). Thus, for our approach, the single-most limiting factor in screening potential is the time taken to manually sort and distribute Drosophila larvae into 96-well plate format, although the use of an automated larvae sorter has the potential to overcome this bottleneck. High-throughput screening of large lead-like chemical libraries then becomes feasible and, with it, the potential to isolate compounds with even greater potency than acivicin. Indeed, although our relatively small screen failed to identify a compound with the capacity to rescue Drosophila tumour-bearing larvae to adulthood, such efficacy is possible because we have previously shown that genetically blocking tumour proliferation through overexpressing the p21 homologue (Cyclin/Cdk inhibitor), Dacapo, within the tumour was able to rescue larval lethality and resulted in the eclosion of adult flies (Brumby and Richardson, 2003).

The isolation of acivicin, a chemical with known antitumorigenic activity, in the pilot screen validated the screening platform. However, a number of other compounds with known antitumorigenic activity were not identified. There could be many reasons for this, including poor stability of the compound in food over 5 days, poor cellular uptake, rapid chemical metabolism, the irrelevance of the target for the particular tumours being modelled in the fly, failure of the chemical to effectively inhibit the homologous Drosophila target or failure to screen the chemical at an efficacious concentration. Some of these issues can be addressed through, for instance, the use of fly strains with compromised xenobiotic detoxification machinery to avoid premature clearance of the chemical (Shah et al., 2012); however, it is clear that only a small proportion of anti-cancer compounds with clinical potential will be picked up in such a screening strategy. Although these limitations in identifying anti-tumorigenic hits are significant, they might also constitute an advantage in using this assay for drug development because only chemicals with highly favourable biophysical properties are likely to be identified for subsequent follow-up. Our identification of PD0325901 and idarubicin, compounds known for their bioavailability, supports this hypothesis and is consistent with the necessity of chemical hits to retain

efficacy over a number of days in fly food and following larval

Drosophila methodologies. Not only is it possible to rapidly and economically investigate the efficacy of a chemical series of modified compounds, but also genetic analysis can be used to facilitate chemical target identification, probe modes of chemical efficacy, as well as expose additional targets for chemical intervention, thus creating a powerful and integrated in vivo chemical genetics approach to drug discovery and development. Acivicin, for instance, is known to inhibit a broad range of glutamine-dependent amidotransferases; however, other glutamine analogues also capable of inhibiting glutamine-dependent amidotransferases, such as DON and azaserine, were ineffective against the Drosophila tumour model (data not shown). This probably reflects a combination of both differing pharmacokinetics for the three glutamine mimetics, as well as differing activities towards specific glutamine-dependent amidotransferases. To identify potential key targets of acivicin-mediated inhibition, we used RNAi knockdown of nine candidate glutamine-dependent amidotransferases to determine whether they would phenocopy the effects of acivicin on tumour overgrowth. Although a failure to impede tumour development in this assay cannot be used to infer that a particular candidate enzyme is not a crucial in vivo target of acivicin, this analysis demonstrated that both CG9674 and CTPsyn were rate limiting for tumour growth. Together with the knowledge that, firstly, acivicin is a potent inhibitor of CTP synthase in mammals (Denton et al., 1982) and, secondly, that exposure of the tumour-bearing larvae to an additional inhibitor of CTP synthase (3-deazauridine) also rescued tumour formation, leads us to favour the hypothesis that CTPsyn is a key target of acivicin in Drosophila. Confirmation of this, however, will require overexpressing CTPsyn in tumours to see if it can counteract the efficacy of acivicin, and biochemical verification of the capacity of acivicin to inhibit CTPsyn in Drosophila. Although human clinical trials with acivicin were abandoned many years ago, due in part to adverse side effects (Ahluwalia et al., 1990), specific inhibitors of CTP synthase, as opposed to broad inhibition of multiple glutamine-dependent amidotransferases, could therefore represent a potentially more promising avenue for further investigation. Indeed, many cancers are known to exhibit increased CTP synthase levels (Williams et al., 1978; Kizaki et al., 1980; Ellims et al., 1983) and, overall, there is considerable interest in developing new strategies for targeting metabolic activities, such as glutamine dependency, that are unique to tumour cells. Furthermore, the capacity of AOA to abrogate tumour formation in the Drosophila larvae suggests that the tumours are also sensitive to inhibition of other glutaminedependent pathways. Our results, therefore, highlight the use of a chemical genetics approach in Drosophila for identifying additional therapeutic avenues for targeting Ras-driven tumours.

Interestingly, although a Notch-driven tumour model was also susceptible to acivicin-mediated restraint, the MEK inhibitor PD0325901 lacked efficacy against this tumour model. This indicated that although acivicin exhibits broad anti-tumorigenic

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activity, different tumour types also have unique properties that can be exploited with the screening platform for the development of specific therapeutics. Indeed, although our work focussed upon a Ras-driven Drosophila tumour model because of the high significance of Ras signalling in tumorigenesis, the increasingly recognised importance of Notch signalling in human cancers makes the Notch-driven model also particularly appealing for the development of Notch-specific therapeutics. Furthermore, the repertoire of cancer models suitable for use with the screening platform can be custom designed through combining various oncogenic and tumour-suppressor mutations, and by using alternative drivers to induce tumours in different tissues. At a broader level, the 96-well plate in vivo screening platform can also be applied to any other Drosophila larval disease model with a readout amenable to high-throughput analysis, thus making it a unique tool for in vivo drug discovery.

## MATERIALS AND METHODS **Chemicals and compound libraries**

The following chemicals were used: acivicin (United Bioresearch Products, EI-113-0010); α-amanitin (Calbiochem #129741); actinomycin D (Calbiochem #114666); amino-oxyacetic acid (AOA) (Sigma C13408); blebbistatin (Calbiochem #203390); CI-1040 (Selleck Chemicals S1020); cisplatin (Calbiochem #232120); colchicine (Calbiochem #234115); dacarbazine (Sigma D2390); daunorubicin (Calbiochem #251800); 3-deazauridine (Sigma D6011); doxorubicin (Calbiochem #324380); idarubicin (Sigma I1656); LY294002 (Calbiochem #440202); MEK inhibitor 1 (Calbiochem #444937); ML-7 hydrochloride (Calbiochem #475880); nocodazole (Calbiochem #487928); paclitaxel (Calbiochem #580556); PD0325901 (Sapphire Bioscience #13034); PD98059 (Calbiochem #513000); roscovitine (Calbiochem #557360); SAHA (a gift from Ricky Johnstone, Peter MacCallum Cancer Center, Melbourne, Australia); U0126 (Calbiochem #662005); Y-27632 (Calbiochem #688000). All compounds were dissolved in DMSO and added to the Drosophila food such that the final DMSO concentration did not exceed 0.5%. DMSO at 0.5% was used as a carrier control. The MicroSource Spectrum Collection of chemicals was purchased from MicroSource Discovery Systems (Gaylordsville, CT) and housed and aliquoted at the WEHI High Throughput Chemical Screening Facility.

## Western blot analysis of MEK inhibitor efficacy

Drosophila melanogaster S2 cells were cultured in Schneider's media with 10% fetal calf serum. MEK inhibitors (final concentration 10 µM) were added to cells 30 minutes prior to harvesting. Whole cell lysates were prepared using an SDS lysis buffer. The antibodies used for protein detection on western blots were; anti-phosphorylated-ERK (Sigma M8159), anti-ERK (Cell Signaling #4695S) and anti-α-tubulin (Sigma T5168).

## Drosophila stocks and tumour models

The tumour models used in this study have been described previously (Brumby and Richardson, 2003; Willecke et al., 2011). For all experiments except those in Fig. 3C, the tumours were developed from clones of tissue in the eye-antennal disc that were generated from eyeless (ey)-driven expression of FLPase from the early 1st instar stage onwards, and were mutant for the cell polarity

regulator scribbled (scrib) and expressed activated alleles of either Ras ( $Ras85D^{V12}$ ) or Notch ( $N^{intra}$ , the intracellular domain of Notch). Normally, scrib mutant clones die; however, in combination with oncogenic Ras or Notch signalling, cell death is prevented and tumour overgrowth ensues. The tumour-bearing larvae were generated from crossing either UAS-Ras85D<sup>V12</sup>; FRT82B,scrib<sup>1</sup>/ *TM6B* male flies or *UAS-N<sup>intra</sup>*; *FRT82B*,*scrib*<sup>1</sup>/*TM6B* male flies to ey-FLP,UAS-GFP; ; tub-GAL4,FRT82B,tub-GAL80/TM6B females. The crosses were allowed to lay eggs on grape juice agar plates and the embryos incubated at 25°C for 4 days. Tumour-bearing larvae were selected at the late 2nd to early 3rd instar stage on the basis of the absence of the dominant Tubby phenotype carried on the TM6B balancer chromosome. For the experiments in Fig 3C, knockdown of discs large (dlg) was combined with the expression of Ras<sup>V12</sup> in the eye-antennal disc by outcrossing the following stock: ey-FLP; UAS-Ras85D<sup>V12</sup>, UAS-dlg<sup>RNAi#41134</sup>/CyO, tub-GAL80; act>CD2>GAL4,UAS-GFP. For all experiments that did not involve the use of tumour-bearing larvae,  $w^{1118}$  larvae were used. All Drosophila stocks and crosses were maintained on standard fly media. All RNAi overexpression lines were obtained through the Vienna Drosophila RNAi Center.

## **Chemical screening platform**

To prepare the screening plate, instant Drosophila media (Southern Biological) was ground to a powder-like consistency and  $\sim 2 \text{ mg}$ was added to each well of a 1.3-ml 96-well deep-well plate (NUNC 260252). To transfer ~2 mg of instant Drosophila media into the screening plate, a 130×85×6 mm perspex sheet containing 96 holes (5 mm diameter, in an 8×12 hole arrangement, with 9 mm spacing between each hole) was sealed at the bottom to create a device with wells, which were then filled with the dry media; the perspex was then inverted over the screening plate. The aliquoted chemicals (1.2 µl of a 10 mM stock from the MicroSource Spectrum Collection) were sourced in a 96-well plate format and 240 µl yeast solution (3% w/v baker's yeast, inactivated by 10 minutes at maximum power in a microwave) was added to each chemical. This mixture was then transferred into the screening plate, where it reconstituted the instant Drosophila media (resulting in a final compound concentration of  $\sim$ 50  $\mu$ M for the MicroSource library, DMSO concentration was <0.5%). Seven tumour-bearing larvae were transferred to each well, and the screening plate containing the larvae was sealed using a 125×80 mm, 0.4 mm wire mesh, held in place by a 130×85×3 mm perspex sheet containing 96 holes (to allow air access to each well). After incubation for 5 days at 25°C, the perspex lid and mesh were removed and a 30% sucrose solution was added to each well. With gentle agitation, all larvae and pupae within each well floated to the surface, and further sucrose solution was added to generate a slightly convex meniscus such that the larvae and pupae floated towards the centre of the well. The plate was viewed under a dissecting microscope and a digital camera used to capture images of the screening plate using white light and GFP fluorescence. The number of larvae and pupae in each well were recorded and the GFP images analysed using MetaMorph (Molecular Devices) to manually set a pixel intensity threshold that included the GFP-labelled tumours, but not background fluorescence. Images were then binarised such that GFP-labelled tumours were represented by white pixels, with everything else represented by black pixels. The binary image was subdivided into 96 equally sized boxes (one box per well) and then the number of white pixels per box was counted and divided by the number of larvae or pupae in that well to generate what we termed the pixel count. All compounds were screened in duplicate. The data for each compound, number of larvae, pupae and pixel count as well as the white light, GFP and binary images for both replicates were then stored in a Microsoft Access database. Potential hits were identified on the basis of a decrease in pixel count, with a compound being considered a potential hit if the average pixel count from the combined duplicate wells was two standard deviations lower than the average pixel count across the entire screen (a few compounds exhibited strong auto-fluorescence, and these were removed from consideration in generating the standard deviation). Because lethal compounds also led to a considerable reduction in pixel count, potential hits were then referenced to the white light images to determine larvae health. If the compound was lethal, it was no longer considered as a potential hit.

## **Tumour implantation**

Nine-day-old tumour-bearing larvae (reared at 25°C) were dissected in PBS and the GFP-labelled tumour tissue was isolated. This tissue was sectioned into small segments of equal size, and single fragments were injected into the abdomen of 4-day-old virgin w<sup>1118</sup> females, as previously described (Ursprung, 1967). The injected flies were allowed to recover for 2 days at 25°C (in our hands, we observed between 10-20% mortality of injected flies). The ventral surface of the injected flies were then imaged using a fluorescent dissecting microscope to identify GFP-labelled tumour size and location (day 0). The adult flies were then paired on the basis of similar size and location of the tumour, and the members of each pair were randomly assigned to either a control or a treated group. Single flies were added to 5 ml Falcon tubes containing approximately 2 mg of powdered instant Drosophila media reconstituted with 240 µl of 3% w/v yeast solution containing 1.2 µl of either 10 mM acivicin (treated group) or DMSO (control group). After incubation for 4 days at 25°C, the ventral surface of each fly was again imaged (day 4), and the total area of GFP-labelled tumour tissue for each image was determined in Photoshop by tracing the outline of the GFP-labelled tumour and then calculating the number of pixels within that area.

## RNAi overexpression in the *dlg*<sup>RNAi</sup> Ras<sup>V12</sup> tumour model

To screen the knockdown of different glutamine-dependent amidotransferases in *Drosophila* eye disc tumours, male flies carrying the RNAi overexpression transgene (or  $w^{1118}$  males as a negative control) were crossed to female flies of the following genotype: *ey-FLP*; *UAS-Ras85D<sup>V12</sup>,UAS-dlg<sup>RNAi#41134</sup>/CyO,tub-GAL80*; *act>CD2>GAL4,UAS-GFP*. Progeny larvae expressing the RNAi within the tumour cells were staged and analysed for GFP expression 10 days after egg laying. The following RNAi lines were used: *bur* (*CG9242*), RNAi#24153; *CTPsyn* (*CG6854*), RNAi#12759; *r* (CG18572), RNAi#33375; *ade2* (*CG9127*), RNAi#47972; *Gfat1* (CG12449), RNAi#24540; *Prat* (*CG2867*), RNAi#20926; *Prat2* (*CG10078*), RNAi#48823; *CG9674*, RNAi#24089, *CG9940*, RNAi#40756. RNAi lines against two other identified glutaminedependent amidotransferases (*Gfat2/CG1345* and *CG33486*) were not available for screening at the time.

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#### **COMPETING INTERESTS**

The authors declare that they do not have any competing or financial interests.

#### AUTHOR CONTRIBUTIONS

L.F.W. designed experiments, contributed ideas to the development of the project, prepared the figures, contributed to the writing of the paper and carried out most of the experiments. T.S. carried out the western blot analysis for Fig. 1. S.A.M. assisted with the screening of candidate drugs. J.P.P. and I.P.S. contributed ideas and facilitated access to the chemical library used in the study. L.F.W. and A.M.B. wrote the paper, and H.E.R. and P.O.H. contributed editorial guidance. H.E.R., P.O.H. and A.M.B. designed and supervised the project.

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#### SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.009985/-/DC1

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