Trends in Pharmacological Sciences

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Review

Cancer Drug Development Using *Drosophila* as an *in vivo* Tool: From Bedside to Bench and Back

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The fruit fly *Drosophila melanogaster* has been used for modeling cancer and as an *in vivo* tool for the validation and/or development of cancer therapeutics. The impetus for the use of *Drosophila* in cancer research stems from the high conservation of its signaling pathways, lower genetic redundancy, short life cycle, genetic amenability, and ease of maintenance. Several cell signaling pathways in *Drosophila* have been used for cancer drug development. The efficacy of combination therapy and uptake/bioavailability of drugs have also been studied. *Drosophila* has been validated using several FDA-approved drugs, suggesting a potential application of this model in drug repurposing. The model is emerging as a powerful tool for high-throughput screening and should significantly reduce the cost and time associated with drug development. In this review we discuss the applications of *Drosophila* in cancer drug development. The advantages and limitations of the model are discussed.

Trends

Cancer drug development is a lengthy process and is associated with high cost and high failure rates.

Drosophila is an alternative *in vivo* tool for high-throughput screening of cancer therapeutics.

The common cell signaling pathways studied for cancer drug development in *Drosophila* include JAK–STAT, RET receptor tyrosine kinase, Hedgehog, EGFR, APC–Wnt, and Notch.

The common anticancer agents studied in *Drosophila* include methotrexate, aminopterin, acivicin, gefitinib, erlotinib, indomethacin, imipramine, artemisinin, curcumin, and triptolide.

Drosophila in Cancer Research: A Brief Overview

The investment in cancer drug development has not resulted in proportionate quantities of novel drugs. Only one of every 5000 to 10000 prospective anticancer agents achieves US FDA approval and only 5% of cancer drugs from Phase I clinical trials are ultimately approved for clinical use [1]. The failure rates are normally due to limitations of existing preclinical models. Unfortunately, the problem of untranslatability to humans is associated with all preclinical model systems. Although *in vitro* cell lines and xenograft mouse models have been extensively used, they do not effectively mimic and predict human conditions [2]. During recent years, patient-derived xenograft (PDX) models have been used as a reliable platform for drug development [3]. However, the requirement for the stroma and vasculature of the immunocompromised mice to support tumor growth limits the PDX model. The large-scale use of mice for high-throughput screening is not feasible because of the associated cost and ethical issues raised by animal rights organizations. Furthermore, it is relatively difficult to create genetic manipulations in mouse models for preclinical studies. Therefore, there is a need for suitable model organisms that can be easily manipulated for preclinical studies and high-throughput screening of cancer drugs. Moreover, using diverse model organisms can reduce the rate of false positives and identify novel hits at early stages of drug development.

The 'Cinderella of Genetics', *Drosophila melanogaster*, is one such fly model that has contributed significantly in modeling cancer and the identification of cancer therapeutics in recent years.

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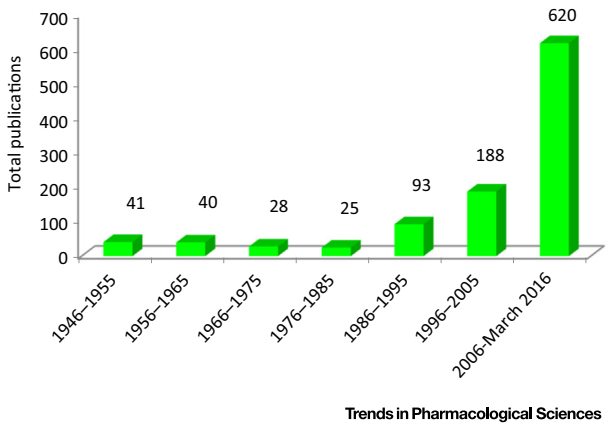


Figure 1. The Number of Publications on *Drosophila* Cancer Research Over the Years. Source: http://www.ncbi.nlm.nih.gov/sites/entrez.

Evidence suggests that as many as 75% of human disease genes are conserved in *Drosophila* [4]. Furthermore, 68% of human cancer genes have homologs in *Drosophila* [5]. To our knowledge, 2016 is the centennial year of *Drosophila* cancer research. The utility of the model in cancer research was first documented in 1916, when Bridges reported 'black granules' in *Drosophila* larvae that were caused by the 'lethal 7 factor' and were lethal to the organism. Later, the granules were characterized as 'melanotic tumors' [6]. The field of *Drosophila* cancer research has increased tremendously over the years (Figure 1). On 30 March 2016, the keywords "*Drosophila*, cancer" entered into the National Institutes of Health PubMed database (http://www.ncbi.nlm.nih.gov/sites/entrez) produced more than 1000 articles, two-thirds of which appeared during the past decade. The model can exhibit classic hallmarks of cancer such as evasion of apoptosis, sustained proliferation, metastasis, prolonged survival, and genomic instability [7–9]. The fly model has been used to unravel the mechanism/signaling molecules of several human cancer types including those of brain, thyroid, colorectal, prostate, ovarian, and skin (Table 1).

Although *Drosophila* has contributed significantly in several fields of cancer biology, the focus of this review is to discuss the applications of this model in cancer drug discovery. The utility of the model in combination therapy and drug uptake/bioavailability is also discussed.

Table 1. A List of Human Cancer Types Studied in *Drosophila*

Cancer Type	Pathway Involved	Refs
Brain cancer	PDGF, EGFR, PI3K, Notch	[106–109]
Medullary thyroid carcinoma	RET, Ras, EGFR, ERK	[30,32,34,110]
Tuberous sclerosis	TSC-1, TSC-2	[111,112]
Colorectal cancer	Wnt, EGFR, Ras	[40,113,114]
Prostate cancer	MRGBP, CNPY2, MEP1A	[115]
Ovarian cancer	YAP/Hippo	[116]
Skin cancer	Hh/Ptc	[117]

MEP1A, meprin 1 alpha; MRGBP, MRG-binding protein; PDGF, platelet-derived growth factor; TSC, tuberous sclerosis complex; YAP, yes-associated protein.

Drosophila in Cancer Drug Discovery

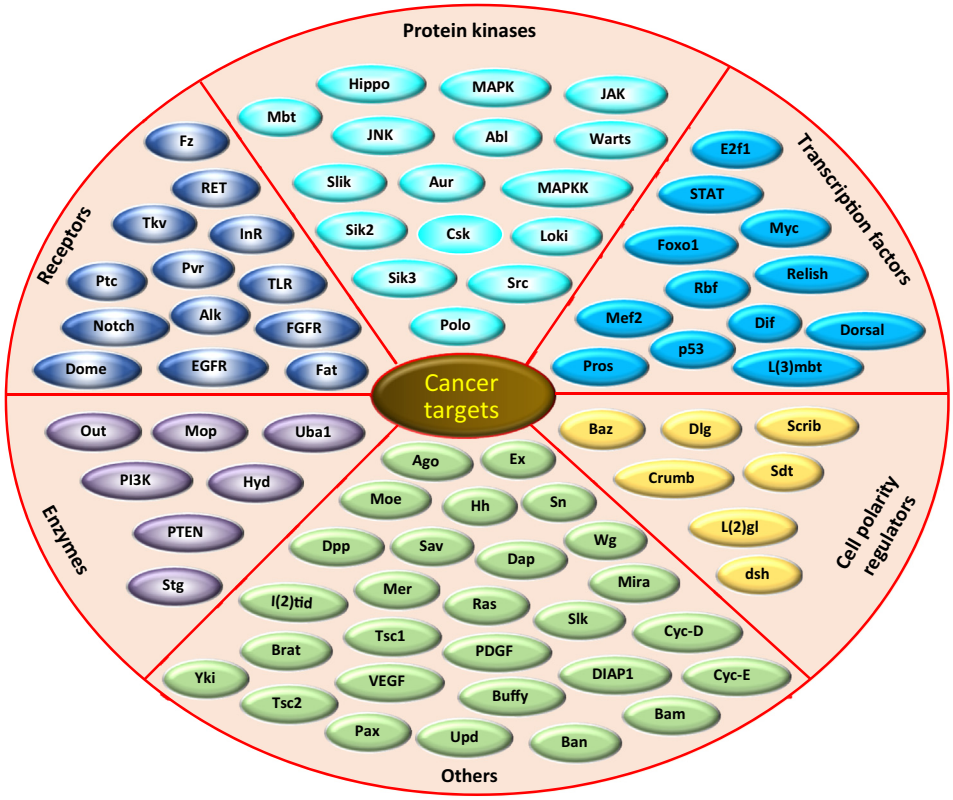
The fact that *Drosophila* contains functional homologs for several human cancer-related genes has provided a rationale for using this model in cancer drug discovery. The model has made seminal contributions to the discovery of several oncogenes and tumor suppressor genes. For example, Notch, Hedgehog (Hh), and Salvador–Warts–Hippo (SWH) were first identified in the fly [10]. Similarly, the Janus-activated kinase (JAK)–signal transducer and activator of transcription (STAT) pathway was observed to cause overgrowth in fly hemocytes before the discovery of its role in human leukemia [11]. Toll-like receptor (TLR), which is involved in mediation of the inflammatory response through activation of the proinflammatory transcription factor nuclear factor kappa B (NF-κB) [12], was originally discovered in *Drosophila* [13]. NF-κB, which is a master regulator of tumor development [14], has three homologs in *Drosophila* (Dorsal, Dif, Relish) [13]. Moreover, *Drosophila* has contributed significantly to the deciphering of the sequential events of the epidermal growth factor receptor (EGFR)–RAS–RAF–MEK–extracellular signal-regulated kinase (ERK) signaling pathway [15,16]. The model has also contributed significantly to our understanding of the mechanism of metastasis, which is a major cause of mortality in cancer patients [17,18]. The cancer-related genes identified in this model can be grouped into enzymes, receptors, protein kinases, transcription factors, cell polarity regulators, and others (Figure 2).

Although numerous cancer targets have been identified in this model, only a few have been used for drug development (Table 2). Several cancer drugs have been studied using this model as an *in vivo* tool (Table 3). The model has also been used to identify new drugs that are subsequently validated in mammalian system. These drugs are structurally diverse (Figure 3) and some are approved for use in cancer patients (Table 4). The most commonly used models for cancer drug screening include cultured cells, organs (eye, wing), whole larvae, and whole fly. To induce cancer in the model, overexpression, suppression, or mutation of specific genes/pathways has been employed and subsequently used to examine the efficacy of drugs.

Perhaps one of the most appealing applications of this model is in high-throughput screening of cancer drugs. High-throughput screening is an extremely powerful assay that allows quick screening of drugs *in vivo* against a predetermined target at large scale in a cost-effective manner. In brief, cells, embryos, larvae, or adult *Drosophila* with cancer-causing

Table 2. Cancer Targets and Pathways Studied in *Drosophila* for the Validation/Development of Cancer Drugs

Cancer Target/Pathway	Cancer Type	Model Used	Refs
JAK/STAT	Multiple	Cells	[21,26–28]
RET receptor tyrosine kinase	MTC	Adult fly and larvae	[31–33,35]
APC/Wnt	Colorectal	Cells and adult fly	[40,42]
Notch	Leukemia	Stem cells	[47]
Ras	Not defined	Larval eye antennal disc	[22]
EGFR	Not defined	Eye and wing imaginal disc	[56]
l(2)gl	Brain	Larvae	[60]
ALK	Neuroblastoma	Adult eye	[70,71]
Fascin	Glioma	Neurons	[76]
TFIIH	Not defined	Larval wing disc	[84]
Topo II	Not defined	Adult eye cells	[87]
Hh	Not defined	Tissues	[89]



Trends in Pharmacological Sciences

Figure 2. Common Cancer Targets Identified in *Drosophila*. These targets can be grouped into enzymes, receptors, protein kinases, transcription factors, cell polarity regulators, and others. Abl, abelson murine leukemia; Ago, archipelago; Alk, anaplastic lymphoma kinase; Aur, aurora-A; Bam, bag of marbles; Ban, bantam; Baz, bazooka; Brat, brain tumor; Csk, C-terminal Src kinase; CycD, cyclin D; CycE, cyclin E; Dap, dacapo; Diap1, death-associated inhibitor of apoptosis1; Dif, dorsal-related immunity factor; Dlg, disc large; Dome, domeless; Dpp, decapentaplegic; Dsh, disheveled; EGFR, epidermal growth factor receptor; Ex, expanded; FGFR, fibroblast growth factor receptor; Foxo1, forkhead box protein o1; Fz, frizzled; Hh, hedgehog; Hyd, hyperplastic disc; InR, insulin-like receptor; JAK, Janus-activated kinase; JNK, c-Jun N-terminal kinase; l(2)gl, lethal(2)giant larvae; l(2)tid, lethal(2)tumorous imaginal disc; l(3)mbt, lethal(3)malignant brain tumor; Lkb1, liver kinase b1; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; Mbt, mushroom bodies tiny; Mef2, myocyte enhancer factor2; Mer, merlin; Mira, miranda; Moe, moesin; Mop, myopic; Myc, myelocytomatosis; Out, ovarian tumor; Pax, paxillin; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; Pros, prospero; Ptc, patched; PTEN, phosphatase and tensin homolog deleted on chromosome 10; Pvr, PDGF and VEGF receptor; Ras, rat sarcoma; Rbf, retinoblastoma family; RET, rearranged during transfection; Sav, Salvador; Scrib, scribble; Sdt, stardust; Sik2, salt-inducible kinase2; Sik3, salt-inducible kinase3; Slik, sterile 20-like kinase; Slk, schluckless; Sn, singed; Src, sarcoma; STAT, signal transducer and activator of transcription; Stg, string; Tkv, thickveins; TLR, toll-like receptor; Tsc1, tuberous sclerosis complex1; Tsc2, tuberous sclerosis complex2; Uba1, ubiquitin-activating enzyme1; Upd, unpaired; VEGF, vascular endothelial growth factor; Wg, wingless; Yki, yorkie.

genes tagged with a luciferase or GFP reporter can be cultured with food containing drugs. After a specific period of time, the effect of the drug on the cancer phenotype can be quantified by various means (Figure 4). The most commonly used end points are: (i) viability of the organism; (ii) eclosion of the pupa/adult; (iii) luciferase activity; (iv) GFP expression; and (v) other biochemical assays [19–22]. Whole-organism-based drug screening permits assessment of drug absorption, distribution, metabolic stability, and toxicity and reduces the possibilities of false positives. Furthermore, the use of whole organisms allows drug screening in a multicellular context and can reproduce the complexity of the disease *in vivo*.

Table 3. A List of Anticancer Drugs Developed/Validated in *Drosophila*

AUH-6-96	Inhibited JAK–STAT signaling in *Drosophila* Schneider cells that were engineered to express a transcriptional reporter for STAT92E [26]
BOT-4-one	Inhibited tyrosine phosphorylation of STAT92E and exhibited anticancer activities in *Drosophila* Schneider cells [27]
Methotrexate, aminopterin	Inhibited the JAK–STAT pathway in *Drosophila* [28]
FDA-approved drugs	*Drosophila* stem cell tumors were sensitive to a wide range of drugs (gemcitabine, methotrexate, thiotepa, topotecan, rapamycin); paradoxically, a subset of drugs (actinomycin, bortezomib, paclitaxel, vincristine, vinblastine, mitomycin, daunorubicin) that inhibited growth of cancer stem cells also induced hyperproliferation of wild-type stem cells driven by the JAK–STAT pathway [21]
ZD6474	Oral dose of the drug suppressed the defects associated with wild-type and oncogenic forms of dRET [31,32]; exhibited efficacy in patients with advanced MTC [33]
MS0019266	Exhibited anticancer activities in *Drosophila* expressing oncogenic MEN2B and increased the viability of the *Drosophila* larvae and the number of organisms reaching the pupal and adult stages [35]
Indomethacin	Enhanced hAPC-induced eye defects in the fly [40]
Oxazoles, thiazoles, thiazolidinedione	Exhibited potent inhibitory effects on β-catenin-responsive transcription in *Drosophila* [42]
DAPT, Cpd E, thapsigargin, cyclopiazonic acid	Inhibited the differentiation of stem cells into enterocytes [47]
Acivicin	Inhibited tumor formation in *Drosophila* by inhibiting CTP synthase [22]
Gefitinib and erlotinib	Suppressed eye and wing phenotypes induced by EGFR in *Drosophila*; inhibited dp-ERK1/2 in the eye and wing imaginal discs of wild-type larvae [56]
Artemisinin, curcumin	Exhibited anticancer activities against brain cancer through generation of reactive oxygen species [60]
TAE684, crizotinib	Ameliorated the rough-eye phenotype caused by overexpression of $hALK^{F1174L}$ and $hALK^{R1275Q}$ in *Drosophila*; the effect of crizotinib was less than that of TAE684 [70,71]
Imipramine	Inhibited fascin pathway in *Drosophila*; known mediator of tumor invasion and metastasis [76]
Triptolide	Induced apoptosis in third-instar larval wing discs of *Drosophila* by inhibiting the ATPase activity of the XPB subunit of TFIIH [84]
F14512	Exhibited antiproliferative properties in *Drosophila* cells; stabilized ternary Topo II–DNA-cleavable complexes at unique sites located in moderately repeated sequences [87]
AY9944	Inhibited Hh-induced internalization of the transmembrane protein Ptc as well as the expression of the Hh target gene en; depleted cholesterol from the plasma membrane and its intracellular accumulation in *Drosophila* tissues [89]
Bouvardin	Enhanced the effects of radiation in *Drosophila* larvae through inhibition of the elongation step of protein synthesis [92]
Trametinib and fluvastatin	Improved tracheal development and reduced over-proliferation and whole-animal toxicity in a Ras–PTEN *Drosophila* lung cancer model [94].

In the following sections, we discuss the common targets that have been used for cancer drug screening in *Drosophila*. The common cancer drugs that have been studied to date are also discussed.

STAT

STAT is a family of transcription factors that regulate several aspects of cell growth, survival, and differentiation and are activated by JAK. Because dysregulation of STAT signaling is linked with

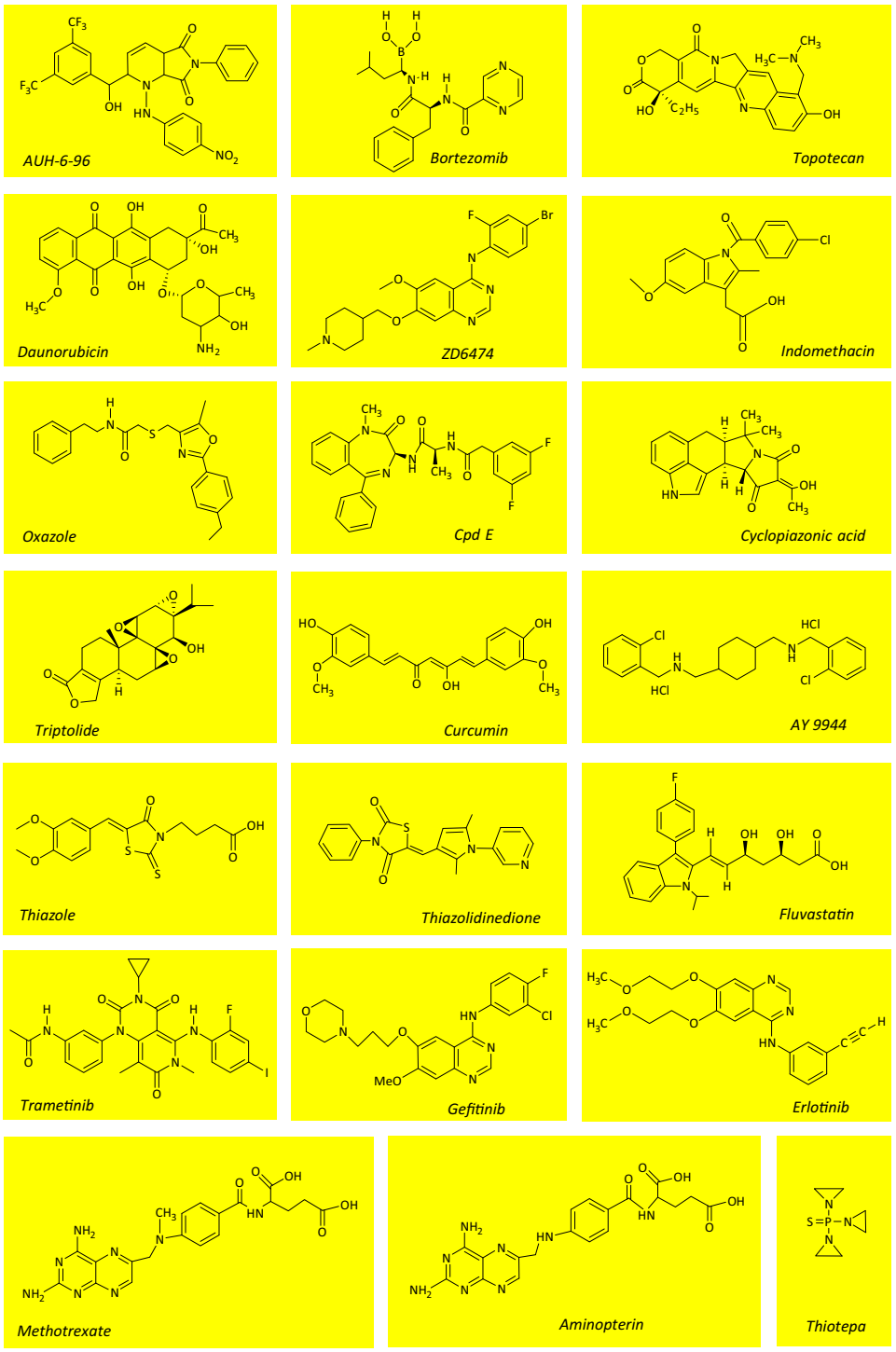


Figure 3. Chemical Structure of Anticancer Drugs Developed/Validated Using *Drosophila*. These drugs are structurally diverse.

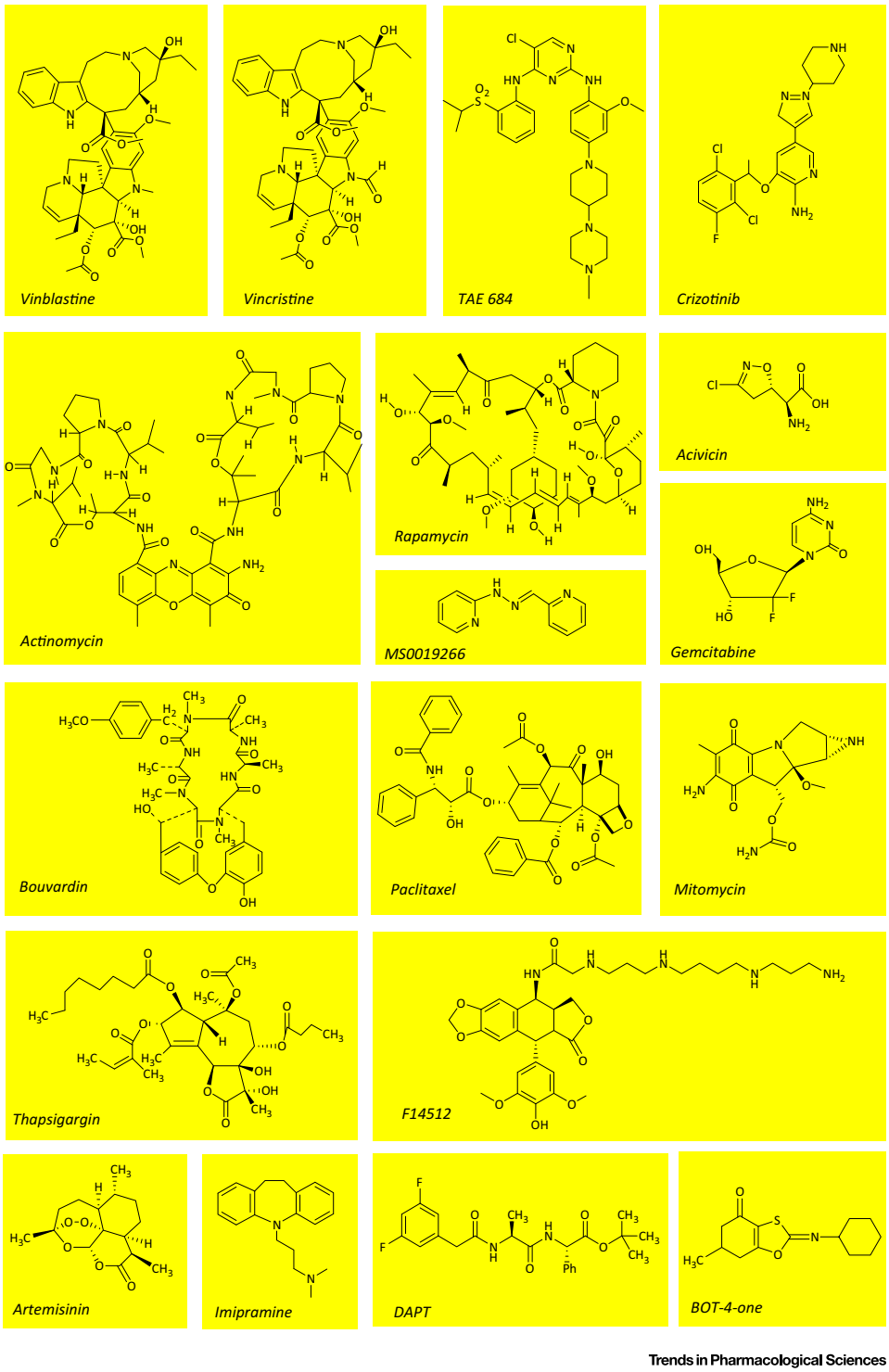


Figure 3. (Continued).

Table 4. A List of Clinically Relevant Anticancer Drugs Validated/Developed in *Drosophila*

Drug	Human Cancer Type	Current Status	Refs
Methotrexate	Acute lymphoblastic leukemia	FDA approved	[21,28]
Gemcitabine	Breast, lung, ovarian, pancreatic	FDA approved	[21]
Topotecan	Cervical, lung, ovarian	FDA approved	[21]
ZD6474/vandetanib	MTC	FDA approved	[33]
Gefitinib, erlotinib	Lung cancer	FDA approved	[56,118]
Curcumin	Multiple	Under clinical trial	[61]
Crizotinib	Neuroblastoma	FDA approved	[70]
F14512	Acute myeloid leukemia	Under clinical trial	[88]

several aspects of tumor development [23], small molecules that disrupt the function of STAT, such as sunitinib and dasatinib, are now being developed for cancer therapy [24]. In mammals, four JAK and seven STAT genes have been identified, while the JAK–STAT pathway in *Drosophila* comprises only three highly related activating ligands of the Unpaired (upd) family, one receptor [Domeless (Dome)], one JAK [hopscotch (hop)], and one STAT (STAT92E) [25].

In a recent study, *Drosophila* Schneider cells were engineered to express a transcriptional reporter for STAT92E [26]. The engineered cells were then used in a high-throughput screen of a library of novel polysubstituted imidopiperidines that resulted in the identification of 2-[(3,5-bis-trifluoro-methyl-phenyl)-hydroxy-methyl]-1-(4-nitrophenylamino)-6-phenyl-1,2,4a,7a-tetrahydro-pyrrolo [3,4-b]-pyridine-5,7-dione (AUH-6-96) as a potent inhibitor of JAK–STAT signaling. Interestingly, AUH-6-96 affected the growth and survival only of human cancer cells with aberrant JAK–STAT signaling. AUH-6-96 also inhibited the growth of Hodgkin lymphoma L540 cells and induced

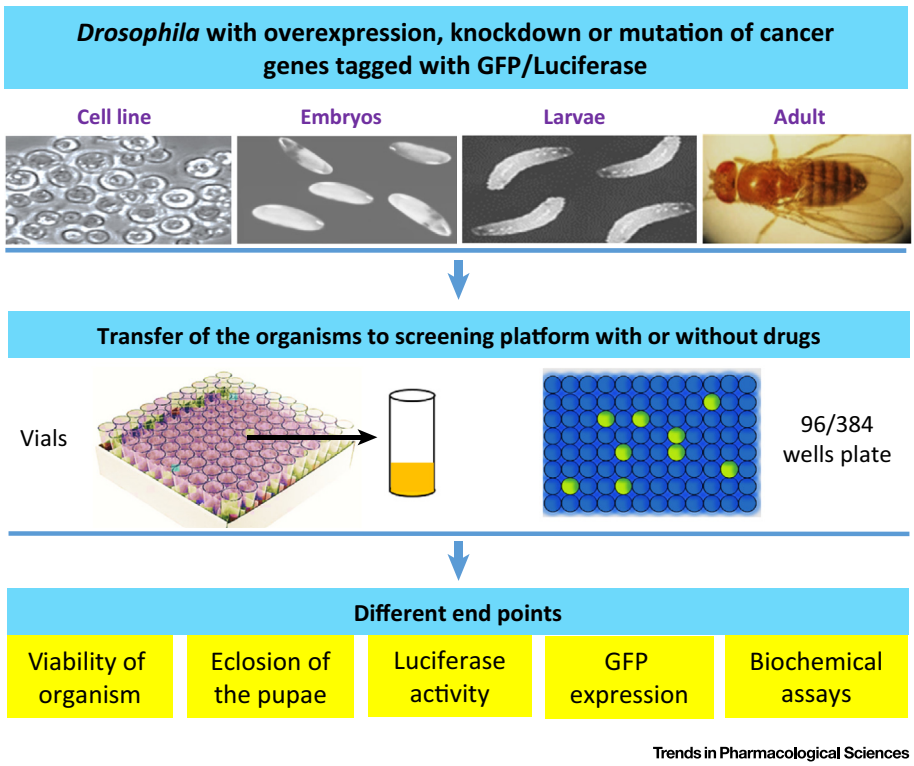


Figure 4. Basic Steps for High-Throughput Screening of Cancer Drugs in *Drosophila*.

apoptosis by downregulating the expression of STAT3-regulated cell survival genes. In another study, 2-cyclohexylimino-6-methyl-6,7-dihydro-5H-benzo[1,3]oxathiol-4-one (BOT-4-one), a derivative of benzoxathiol, was found to possess anticancer activity in both *Drosophila* Schneider and human cancer cells [27]. BOT-4-one suppressed upd-induced tyrosine phosphorylation and transcriptional activity of STAT92E in *Drosophila* cells. While upd was found to induce STAT92E transcriptional activity by more than 21-fold, BOT-4-one suppressed the activity in a dose-dependent manner. The inhibition of upd-induced tyrosine phosphorylation of STAT92E by BOT-4-one could contribute to its anticancer activity.

The *Drosophila* JAK–STAT pathway has also been used as a therapeutic target for screening FDA-approved drugs. For example, a luciferase-based transcriptional assay was used to screen 2000 small molecules [28] that produced methotrexate and aminopterin as strong inhibitors of the JAK–STAT pathway. Furthermore, a HEL cell line with constitutive activation of the JAK–STAT pathway was used to validate the relevance of *Drosophila* observations to human myeloproliferative neoplasms. Methotrexate caused significant suppression of JAK–STAT activation in HEL cells at clinically relevant concentrations, thus confirming the translation of *Drosophila* observations to humans.

The above studies suggest *Drosophila* JAK–STAT as a novel target for anticancer drug screening. However, a *Drosophila* screen also revealed that some chemotherapeutic agents can induce hyperproliferation of cells through modulation of this pathway [21]. In an attempt to identify inhibitors of stem-cell-derived tumors in adult *Drosophila*, the authors screened FDA-approved chemotherapy drugs for effects on 'stemness'. The stem cell tumors were found to be sensitive to a wide range of drugs including gemcitabine, methotrexate, thiotepa, topotecan, and rapamycin. Paradoxically, a subset of drugs (actinomycin, bortezomib, paclitaxel, vincristine, vinblastine, mitomycin, daunorubicin) that inhibited the growth of cancer stem cells also induced hyperproliferation in wild-type cells via the JAK–STAT pathway. Because hyperproliferation is one of the hallmarks of cancer cells, chemotherapeutic agents were suspected to refuel the growth of the tumors. These observations further corroborate the side effects of the current cancer chemotherapeutics in inducing inflammatory pathways in cancer patients [29].

Rearranged During Transfection (RET) Receptor Tyrosine Kinase (RTK)

The *RET* proto-oncogene encodes a RTK that is a key regulator of development and vulnerable to mutations. An increase in RET activity can lead to several cancer syndromes, including multiple endocrine neoplasia type 2A and 2B (MEN2A and MEN2B) and familial medullary thyroid carcinoma (FMTC). *Drosophila* models for MEN2A and MEN2B have been generated [30]. Specifically, three classes of transgenic flies that misexpressed *Drosophila* RET (dRET) were generated: wild type (mimicking FMTC), MEN2A-like, and MEN2B-like. Each dRET isoform was directed to the developing eye to create a cancer phenotype in adult *Drosophila*. Oral administration of the kinase inhibitor ZD6474 [vandetanib (Caprelsa)] was found effective in suppressing the defects associated with wild-type and oncogenic forms of dRET [31] at doses well below the observed toxic doses. This was the first direct evidence demonstrating the efficacy of ZD6474 against RET-related defects in a whole organism. Furthermore, the drug did not suppress *Drosophila* EGFR or downstream components of the RET–rat sarcoma (Ras) pathway [32]. The conclusion of this study was that targeting the oncogenic forms of RET by ZD6474 may be a useful strategy for the treatment of RET-dependent carcinomas.

Based on these *Drosophila in vivo* studies, a Phase II clinical trial was conducted to assess the efficacy of vandetanib in patients with advanced MTC [33]. Vandetanib (300 mg) was administered orally to 30 patients with unresectable locally advanced or metastatic hereditary MTC. The primary assessment was objective tumor response using the Response Evaluation Criteria in Solid Tumors (RECIST). Six of 30 patients (20%) experienced a confirmed partial response

(10.2 months) and 16 of 30 patients (53%) experienced stable disease. Furthermore, serum calcitonin levels showed a 50% or greater decrease from baseline that was maintained for at least 4 weeks in 24 patients, and 16 patients showed a similar reduction in serum carcinoembryonic antigen levels. The most common adverse events reported in these patients were diarrhea, rash, fatigue, and nausea. Overall, vandetanib demonstrated durable objective partial responses and disease control with manageable adverse effects. Based on these observations, vandetanib was approved by the FDA in April 2011 for MTC patients [33]. The approval of this drug validated the potential utility of flies as a powerful tool for anticancer drug development. However, toxicity and development of drug resistance were major issues with vandetanib use. Therefore, the drug has been structurally modified to develop molecules with improved efficacy and minimal toxicity, such as AD57, AD58, AD80, and AD81 [19,34].

The MEN2B model of *Drosophila* has also been used to examine the anticancer activities of a small molecule, 2-pyridinecarbaldehyde 2-pyridinylhydrazone (MS0019266) [35]. Administration of this small molecule was found to increase the viability of *Drosophila* larvae. Furthermore, the number of organisms reaching the pupal and adult stages was also significantly increased and the molecule was found to inhibit ribonucleotide reductase in human prostate cancer cells.

Adenomatous Polyposis Coli (APC)–Wint (Wnt) Signaling

APC is a large protein that is encoded by the *APC* gene in humans [36,37]. *APC* is a tumor suppressor that acts by downregulating and inactivating β-catenin, a transducer of Wnt signaling [38]. Mutations in human *APC* are associated with the development of both familial and spontaneous colorectal cancer [39]. Targeted expression of either full-length human APC (hAPC) or its β-catenin-binding domain negatively regulates the functions of armadillo (Arm) (*Drosophila* β-catenin) and causes eye defects during fly development [40]. Whether transgenic *Drosophila* expressing hAPC can be used as a tool for anticancer drug screening was examined in one study [40]. Of the four drugs tested, indomethacin was found to enhance hAPC-induced eye defects in the fly. Although the precise mechanism of the action of indomethacin remains elusive, the drug is known to reduce β-catenin levels and/or activity in mammals [41]. The study suggested that the action of indomethacin is to antagonize Wnt signaling in both mammals and *Drosophila* and that transgenic *Drosophila* with eye-directed expression of hAPC could be a valuable tool for anticancer drug screening.

In another study, chemical inhibitors of β-catenin-responsive transcription (iCRTs) [42] were examined in *Drosophila*. *Drosophila* clone 8 (Cl8) cells derived from wing imaginal discs [43] were engineered to express a reporter in which luciferase was under the control of a Wingless (Wg)-responsive promoter, dTF12. A double-stranded RNA (dsRNA) was used to knock down Axin, which led to the stabilization of β-catenin and constitutive expression of dTF12-luciferase reporter activity that was used for high-throughput screening. The screening produced oxazoles, thiazoles, and thiazolidinedione as potent classes of iCRT. The specificity of the compounds was further tested using reporters for the Hh and JAK–STAT pathways in *Drosophila*. The specificity and relevance of the iCRTs were confirmed using reporters for human Wnt and Notch in HEK293 cells. Overall, the study provided evidence for the successful use of a *Drosophila* model to identify drug candidates against Wnt-associated human cancers.

Notch

Notch is a highly conserved cell signaling pathway present in most multicellular organisms [44]. Originally discovered as an important component of the developmental pathway in *Drosophila*, accumulating evidence supports a pro-oncogenic function of Notch signaling in multiple tumor types [45]. Therefore, Notch inhibitory agents such as γ-secretase inhibitors (GSIs) are being investigated as cancer therapeutics. Loss-of-function mutations in the only *Drosophila*

sarco/endoplasmic reticulum calcium ATPase (SERCA) homolog (Ca-P60A) have been shown to produce Notch loss-of-function phenotypes [46].

A recent study evaluated a *Drosophila* intestinal stem cell model in which Notch inhibition perturbs differentiation [47]. When Notch was inhibited by feeding flies with two different GSIs [DAPT or compound E (Cpd E)], stem cell daughters failed to differentiate into enterocytes (ECs) and instead gave rise mostly to additional stem cells, as well as some enteroendocrine (ee) daughters. Cyclopiazonic acid and thapsigargin treatment also expanded the stem cell and ee cell populations, thus phenocopying the effects of the GSIs. Furthermore, knockdown of Ca-P60A produced effects on stem cell and ee cell pools similar to those induced by GSIs, thapsigargin, or cyclopiazonic acid. Overall, the study suggested that *Drosophila* can be used as a tool for *in vivo* drug screening of Notch inhibitors. These observations were subsequently validated in human cell lines and xenograft mouse models [47].

An epithelial *Drosophila* cancer model has been developed in which ectopic expression of orthologs of the activated human oncogene Ras (Ras^{V12}) or Notch (N^{intra}) (the intracellular domain of Notch) drives tumor formation. When overexpression of either of these oncogenes is combined with loss of the epithelial cell polarity regulator *scribble* (*scrib*), massive tumors develop within the eye antennal disc throughout the larval stages of *Drosophila* development. These tumors have been reported to recapitulate many of the hallmarks of human cancers, including increased cell proliferation and survival, failure to differentiate, increased invasion, and metastasis [17,48]. Using the same model, Willoughby *et al.* screened a library of 2000 compounds and found that acivicin, a glutamine analog with known activity against human tumor cells, inhibits tumor formation in *Drosophila* [22]. Furthermore, an RNAi-mediated knockdown approach revealed CTP synthase as a possible target of acivicin-mediated inhibition of tumor formation.

EGFR

EGFR is a transmembrane glycoprotein comprising an extracellular ligand-binding domain and an intracellular TK domain. Activation of the EGFR pathway requires autophosphorylation at its TK domain. Because dysregulation of EGFR accounts for nearly 80% of all lung cancers [49], the TK domain of EGFR is an important target for therapeutic development. Gefitinib and erlotinib are two important TK inhibitors (TKIs) of EGFR, with reported efficacy in cancer patients [50,51]. Both are reversible inhibitors that compete with ATP for binding to the catalytic site of the enzyme [52].

Considerable similarity between the TK domains of EGFR in humans and *Drosophila* has been reported [53]. EGFR signaling is essential for morphogenesis of the eye [54] and wing development [55] in *Drosophila*. Because a large number of transgenics and mutants for EGFR have been reported in *Drosophila*, this alternative animal model provides an ideal tool to identify TKIs. Using enhancer–suppressor assays and *in silico* analysis, the model has been employed to examine the probable mechanism by which gefitinib and erlotinib block EGFR signaling [56]. Gefitinib and erlotinib were found to suppress eye phenotypes induced by EGFR in *Drosophila* and gefitinib also suppressed wing phenotypes induced by EGFR. Both of these inhibitors inhibited diphosphorylated forms of ERK1/2 (dp-ERK1/2) in the eye and wing imaginal discs of wild-type larvae. These results suggest that gefitinib and erlotinib are potent inhibitors of EGFR signaling in *Drosophila*. Both drugs were approved by the FDA in 2003–2004, well before their validation in *Drosophila* [57], which further supports the utility of the model in cancer drug screening.

lethal(2)giant larvae [l(2)gl]

Human giant larvae (Hugl-1/Llg1/Lgl1) is a human gene that encodes a protein (LLGL), reduced expression of which leads to colorectal cancer [58]. The *Drosophila* homolog of Llg1 is *l(2)gl*, a tumor suppressor whose deletion leads to brain tumors at the larval stage. A brain tumor model

with mutations in the *l(2)gl* gene was used to examine the efficacy of artemisinin and curcumin in *Drosophila* larvae [59,60]. Both agents had antitumor activities, individually and in combination. The antitumor activities of these agents were mediated through the generation of reactive oxygen species. Furthermore, the median lifespan and locomotory response of the organisms were improved by both agents. Curcumin is a polyphenol derived from golden spice, or turmeric. The antitumor activity of curcumin is well established by both preclinical and clinical studies [61]. Although artemisinin is an antimalarial drug, some of its derivatives possess antitumor properties [62]. Overall, these studies provided evidence that *Drosophila* can be used as an *in vivo* tool to screen anticancer drugs against brain cancer. The study also suggests that an antimalarial drug may be repurposed for anticancer activity.

Anaplastic Lymphoma Kinase (ALK)

ALK is a member of the insulin receptor superfamily of RTKs [63]. It acts like an oncogene by forming a fusion gene with nucleophosmin. Although aberrant ALK activity plays a role in the progression and maintenance of various solid and hemopoietic tumors [64], only a few ALK inhibitors have been approved for cancer patients. Crizotinib (Xalkori®) is one such FDA-approved drug that has been reported to possess clinical efficacy in both non-small-cell lung cancer and inflammatory myofibroblastic tumors [65,66]. The drug has also been reported to possess anticancer activities in neuroblastoma patients harboring ALK mutations [67,68].

The two most common mutations in the *ALK* gene ($hALK^{F1174L}$ and $hALK^{R1275Q}$) have been reported in neuroblastoma patients [69]. In one study, these mutant genes were ectopically expressed in the *Drosophila* eye using pGMR-Gal4, which directs protein expression in the developing photoreceptors of the eye [70]. Expression of the wild-type hALK did not result in any obvious phenotype in adult flies [71]. However, expression of $hALK^{F1174L}$ and $hALK^{R1275Q}$ resulted in a rough-eye phenotype [70]. Although both mutants displayed a robust phenotype, a more severe phenotype was observed with ALK^{F1174L}. Furthermore, treatment with a small-molecule ALK inhibitor, TAE684, improved the rough-eye phenotype of both mutants, whereas crizotinib had little effect on either phenotype. These differential responses of both mutants to the inhibitors *in vivo* were in good agreement with the *in vitro* cell culture experiments. Overall, these studies suggest that *Drosophila* can be used as an alternative animal model for cancer drug development against neuroblastoma.

Fascin

Fascin is a highly conserved actin-bundling protein and an essential regulator of development and physiology [72]. While fascin is a key mediator of tumor invasion and metastasis [73], its deficiency leads to developmental brain disorders [74]. *Drosophila* has a single fascin-coding gene [75], named *singed*. To identify the modulators of the fascin pathway, a cell-based bidirectional drug screening assay was developed in *Drosophila* for the identification of agents with antimetastasis or cognitive-enhancing activities [76]. Fascin-deficient mutant *Drosophila* neurons, whose neurite arbors manifest the 'filigree' phenotype, were used for the study. Employing a drug-repurposing approach, authors screened a library of 1040 compounds containing structurally diverse FDA-approved drugs. The screen yielded 34 blockers and 48 enhancers of the fascin pathway, with potential antimetastasis or cognitive-enhancing activity, respectively. Imipramine, a tricyclic antidepressant, was identified as one of the most potent blockers of the fascin pathway. A previous study has also demonstrated the anti-invasive activities of this antidepressant [77]. The authors of this study proposed that bidirectional screening is an efficient and multipurpose strategy for drug discovery.

Transcription Factor IIH (TFIIH)

TFIIH is a multisubunit complex that participates in transcription, nucleotide excision repair, and control of the cell cycle [78]. Mutations in the xeroderma pigmentosum group B protein (XPB),

XPD, and p8 subunits of TFIIH are associated with various human diseases, including cancer [79]. The Dmp52 subunit of TFIIH in *Drosophila* has been shown to directly interact with the fly homolog of p53 (Dp53). Previous studies have demonstrated that p53 requires the presence of intact TFIIH to induce apoptosis [80,81].

Triptolide is a diterpene triepoxide derived from *Tripterygium wilfordii*, a plant used in traditional Chinese medicine. It has the potential to specifically inhibit the ATPase activity of the XPB subunit of TFIIH [82] and to induce apoptosis in cancer cells [83]. In one study, the efficacy of triptolide was examined in third-instar larval wing discs of *Drosophila* that were deficient in Dp53 [84]. Triptolide was found to induce apoptosis in the larval wing discs in a dose- and time-dependent manner. The rate of apoptosis in triptolide-treated wing discs was similar to that observed in discs expressing the dsRNA against Dmp52. When wing discs expressing the dominant-negative form of Dp53 were incubated with triptolide, an increase in apoptosis was observed. A similar observation was observed with double depletion of Dmp52 and Dp53 in the wing compartment. Inhibition of the ATPase activity of the XPB subunit of TFIIH by triptolide in cells deficient in functional Dmp53 was found to generate the same phenotype as when the Dmp52 and Dp53 subunits of TFIIH were simultaneously depleted. Furthermore, the observed increase in apoptosis generated by the combined action of triptolide and Dp53 depletion occurred in a JNK-dependent manner. These observations support the idea that *Drosophila* can also be used as a tool to screen agents derived from 'Mother Nature'.

Topoisomerase II (Topo II)

Topo II is an essential enzyme for DNA replication, transcription, and chromosome segregation [85]. While the functions of Topo II are to ensure genomic integrity, agents with an ability to modulate Topo II activities such as podophyllotoxins, acridines, and anthracyclines have been extensively used in anticancer therapies [86].

In one study, *Drosophila* mutants were used to delineate the mechanism of action of F14512 [87], which is a known Topo II inhibitor containing a spermine moiety [88]. F14512 exhibited antiproliferative properties in *Drosophila* cells. It also stabilized ternary Topo II–DNA cleavable complexes at unique sites located in moderately repeated sequences, suggesting that the drug specifically targets a select subset of genomic sequences. When developing mutant larvae were fed with F14512, flies with one eye replaced by a first thoracic segment were recovered. Similarly, other F14512-induced gain- and loss-of-function phenotypes corresponded to precise genetic dysfunctions. These observations in the developing organisms can be reconciled with known genetic anomalies and constitute a remarkable instance of specific alterations of gene expression by the ingestion of a drug. The authors of this study concluded that *Drosophila* can be used to elucidate the fundamental mechanisms of action of candidate drugs of therapeutic interest in humans. F14512 is currently in under Phase I/II clinical trial for patients with acute myeloid leukemia [88].

Multiple Pathway-Based Drug Screening

In most of the studies discussed above, only one signaling pathway was used for anticancer drug screening in *Drosophila*. However, one study used multiple pathways to examine the degree of conservation of activity/efficacy of known drugs between *Drosophila* and humans [89]. More specifically, the group tested 27 small molecules with known targets in mammalian systems for their *in vivo* activity in *Drosophila* [89]. The pathway-specific developmental phenotypes were generated by ectopic expression of components of the Hh, insulin–phosphoinositide 3-kinase (PI3K), EGFR–MAPK, JNK, Wnt, cell cycle, and apoptosis pathways in a temporally controlled manner. The activities of several molecules were confirmed directly on target tissues using pathway-specific target gene expression as read outs. The activities of 20 of 27 compounds were found to be highly conserved between *Drosophila* and mammals.

Furthermore, one agent, AY9944, inhibited Hh-induced internalization of the transmembrane protein Patched (Ptc) as well as expression of the Hh target gene engrailed (en). From epistasis analyses, AY9944 was found upstream of protein kinase A (PKA) and Ptc, two negative regulators of Hh signaling. AY9944 was also found to deplete cholesterol from the plasma membrane and its intracellular accumulation in *Drosophila* tissues. Interestingly, the cholesterol moiety on the Hh protein was necessary for the inhibitory effect of AY9944 on Hh signaling.

Drosophila in Combination Therapy

Because cancer is caused by dysregulation of multiple genes, the current paradigm of cancer therapy is either to combine multiple monotargeted drugs or to design a molecule that can target multiple pathways. Combination therapy minimizes the chances of drug resistance and toxicity. One such approach uses agents that can enhance the effects of radiation for cancer therapy.

A tool has been developed in *Drosophila* (US Patent No 7 695 899) for the identification of small molecules that can enhance the effects of radiation. The tool takes advantage of similarities between mammalian tumors and the primordia of *Drosophila* larvae; both are capable of regeneration through 'accelerated repopulation' [90]. The tool has been successfully validated in published proof-of-concept studies [20,91]. Using the same system, two molecule libraries from the National Cancer Institute Developmental Therapeutics Program (NCI-DTP) were screened [92], resulting in the identification of three molecules that can enhance the effect of radiation in *Drosophila* larvae. One of these inhibitors, bouvardin, also enhanced the effect of radiation in human cancer cells and in tumor xenografts. Mechanistically, bouvardin inhibited the elongation step of protein synthesis. Bouvardin was also identified independently in a screen for selective inhibitors of engineered breast cancer stem cells [93]. Overall, these results suggest that *Drosophila* can be used to identify radiosensitizers.

In another recent study, a Ras-phosphatase and tensin homolog deleted on chromosome 10 (PTEN) lung cancer model was developed in the *Drosophila* tracheal system [94]. The model was associated with overproliferation of tracheal tissue, formation of tumor-like growths, and animal lethality. Screening of over 1000 FDA-approved drugs in the same model produced trametinib and fluvastatin, which showed therapeutic efficacy. Both of these agents improved tracheal development and reduced over-proliferation and whole-animal toxicity. The oncogenic phenotypes and lethality were further suppressed by the combination of the two agents. Similar observations were made in human lung cancer cell lines [94].

Drosophila in Drug Uptake and Bioavailability

Bioavailability refers to the extent to which a drug is absorbed or becomes available at the site of physiological activity after administration. Only limited studies have examined the bioavailability of cancer therapeutics in *Drosophila*.

The polyamine transporter (PAT) is frequently upregulated in many tumor types and is crucial for importing exogenous polyamines [95]. The imaginal disc epithelium of *Drosophila* has been successfully used to screen PAT-selective molecules [96]. More specifically, a library of polyamine–anthracene conjugates was found to possess similar PAT selectivity and toxicity profiles in mammalian cell culture and *Drosophila* imaginal discs. Furthermore, polyamine uptake in *Drosophila* S2 cells was found to be sensitive to pH in another independent study [97]. That *Drosophila* could be used for the screening of compounds with known bioavailability was reported in a recent study [22]. More specifically, the group found that the bioavailable tumor-specific MEK inhibitor PD0325901 was highly efficacious in reducing tumor burden in a Ras-driven *Drosophila* model. Further, the low-bioavailability parent compound CI-1040 was less effective. However, the model could not identify several compounds with known anticancer activities, which could be due to their limited bioavailability in *Drosophila*. Collectively, these

results demonstrate the potential of the model to discriminate drug candidates with greater bioavailability and clinical efficacy. However, more thorough studies are required focused solely on bioavailability and its relation to the efficacy of known drugs.

Concluding Remarks

Cancer drug development is associated with high failure rates, high cost, and a lengthy design and testing process that necessitates alternative approaches for drug discovery. Reduced genetic redundancy, greater conservation of signaling pathways, small size, low cost of maintenance, and ease of generation of mutant flies have enabled *Drosophila* to become a powerful tool for high-throughput screening of cancer drugs in a physiologically relevant environment. One area where *Drosophila* can contribute significantly in the future is drug repurposing. This approach is based on the fact that many different human diseases share common molecular pathways and targets in the cell and thus a single drug may be useful for more than one human disease. As a tool *Drosophila* can be used to examine the efficacy of non-cancer drugs for cancer activities (bedside to bench). Because these drugs have well-defined preclinical data, fewer tests will be required before they can be translated into advanced clinical trials for anticancer activities (bench to bedside). This will greatly reduce the cost and time associated with cancer drug development. While employing *Drosophila* for drug repurposing, we however recommend extra care in selecting only those non-cancer drugs that have well-defined molecular targets and pharmacokinetic/pharmacodynamic data. Some researchers have validated *Drosophila* in high-throughput screening of FDA-approved cancer drugs, as discussed in this review. Future studies should be focused more on repurposing non-cancer FDA-approved drugs for anticancer activities. The model has also been used to examine the efficacy of multitargeted agents and combination therapy [19,60]. Although *Drosophila* holds promise for cancer drug discovery, there are limitations that raise several questions and deserve attention (see Outstanding Questions).

First, the anatomy and physiology of *Drosophila* are significantly different from those of humans and thus can produce only a partial picture of human symptoms. The possibility that potential drug candidates can produce pseudonegativity and/or pseudopositivity in *Drosophila*-based screening platforms cannot be excluded. Second, a drug that has demonstrated efficacy in *Drosophila* cannot be tested directly in cancer patients; the drug must first be validated in mammals. So the question is: why begin with *Drosophila* in the first place? Screening in *Drosophila* may help to bypass several steps of preclinical testing and may determine the suitability of a drug at a very early stage before it is tested in costly rodent assays and in clinical trials. Furthermore, the reduced genetic redundancy of the organism will help to delineate the molecular mechanism of drug action. Third, the drug doses, formulations, and routes of administration in *Drosophila* are potentially different from those in humans. Observations on these parameters in *Drosophila* will require extrapolation and may hamper the translation of drugs into the clinic. If the extrapolated drug doses are not readily achievable in humans, further modifications of the original structure might be needed to achieve pharmacokinetic and pharmacodynamic profiles for human use. Finally, it would be interesting to examine the efficacy of those drugs in *Drosophila* that have not been successful in humans. It is also important to examine and compare the efficacy of drug candidates in mice implanted with tumors from human and *Drosophila* in a simultaneous but independent manner. The suitability of the model for cancer drug development will be further strengthened if these drugs produce similar effects in two groups of xenografts.

With the advent of the clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) 9-based genome editing tool, researchers can now alter the genomes of a large variety of organisms, including *Drosophila*, with unprecedented ease, specificity, efficiency, and low cost [98–100]. The technology has the potential to permanently correct genetic

Outstanding Questions

What is the reason for the high failure rate of the current cancer drug development process?

Why have only a few cancer targets been used for cancer drug screening in *Drosophila*?

Can cancer drugs with efficacy in *Drosophila* be tested directly in humans (bench to bedside)?

Are the pharmacokinetic/pharmacodynamic properties of drugs similar in *Drosophila* and humans?

Can the drugs produce similar effects in two groups of mice, one group implanted with tumors from humans and the other with tumors from *Drosophila*?

mutations *in vivo* [101] and thus provides an opportunity for better understanding and therapeutic targeting of cancer. The technology has been used to identify genes required for the development of drug resistance in cancer [102,103]. The tool has also been used to uncover the mechanism of action, resistance [104], and efficacy of drug combinations [105] in human cancer cells. However, the tool has yet to be employed in *Drosophila* cancer research. By employing this technology, it is our hope that the contribution of *Drosophila* to the identification of novel cancer targets and development of cancer therapeutics will be further enhanced.

In conclusion, *Drosophila* has emerged as a promising tool for cancer drug screening. The model has already produced a drug for the treatment of MEN2 thyroid cancer. The model should significantly reduce the cost and time associated with the cancer drug development process. However, while numerous tumor models have been generated in *Drosophila* only a few have been utilized for cancer drug discovery. Pharmacokinetic and pharmacodynamic studies of the drugs are even less common in this model. Therefore, more studies are required before the model can be recommended as a powerful *in vivo* tool for cancer drug screening and/or development.

Acknowledgments

A.K.Y. is highly thankful to the Indian Council of Medical Research for providing a Senior Research Fellowship. S.S. greatly acknowledges support by the Science Engineering Research Board-funded project SB/EMEQ-389/2015 (P-07/598) and the Department of Biotechnology-funded project BT/PR6712/MED/30/873/2012 (P07/564), Government of India. S.C.G. thanks the University Grant Commission and Banaras Hindu University (BHU), India for financial support; office and laboratory space is provided by BHU's Interdisciplinary School of Life Sciences. He is a recent recipient of an Early Career Research Award from the Science and Engineering Research Board, India (ECR/2016/000034).

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