UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

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Dissertação de Mestrado
Orientador: Élgion Lúcio da Silva Loreto

Porto Alegre
Dezembro 2021

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Dissertação submetida ao Programa de Pós-Graduação em
Genética e Biologia Molecular da
Universidade Federal do Rio Grande do Sul como requisito
parcial para a obtenção do grau de Mestre
em Genética e Biologia Molecular

Orientador: Élgion Lúcio da Silva Loreto

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Dezembro 2021

Este trabalho foi desenvolvido no Laboratório de Biologia Molecular e Sequenciamento (LabDros) da Universidade Federal de Santa Maria (UFSM), sendo financiado pela Coordenação de Aperfeiçoamento Pessoal de Nível Superior (CAPES) e pelo Programa de Pós-Graduação em Genética e Biologia Molecular (PPGBM) da Universidade Federal do Rio Grande do Sul (UFRGS).

Dedico esta dissertação à minha família, em especial ao José Carlos, à Ana Maria, ao Gabriel e à Maya, e a todos os amigos que me ajudaram e celebraram cada conquista comigo

A felicidade é apenas real quando compartilhada

RESUMO

A Cisplatina é amplamente usada em tratamentos para o câncer e é um dos melhores agentes citostáticos disponíveis para terapia antitumoral. *Drosophila melanogaster* tem uma das melhores anotações de genoma e de sequências de Elementos Transponíveis (TEs). O organismo-modelo é útil para analisar o modo de ação de diversos compostos *in vivo* e avaliar as consequências biológicas e comportamentais dos tratamentos. O objetivo do nosso estudo foi ampliar o conhecimento dos efeitos da Cisplatina em *Drosophila* através do sequenciamento de RNA (RNA-seq) juntamente com ensaios biológicos. O RNA-seq foi seguido por análises de expressão diferencial de genes (DEGs) e TEs (DETEs) e de vias e termos de ontologia. Os DETEs foram confirmados por qPCR. A Cisplatina foi avaliada a 50 e 100 µg/mL no meio de cultivo de *Drosophila* por 24 h. As análises de locomoção, sobrevivência, oviposição e desenvolvimento foram usadas como ensaios biológicos. A Cisplatina induz DEGs de forma dose-dependente e quatro TEs foram up-regulados. A maioria dos DEGs está ligada a dano de DNA e processos de detoxificação. A Cisplatina aumenta a atividade locomotora de *Drosophila* e interrompe o desenvolvimento. Genes e processos relacionados aos ensaios também foram identificados. Esse é o primeiro estudo a avaliar os efeitos da Cisplatina em moscas usando RNA-seq de nosso conhecimento. A alteração na expressão gênica foi limitada, preponderantemente, ao metabolismo de droga e de dano ao DNA, a droga aparenta não afetar amplamente a *Drosophila* na parte molecular. Ao contrário da hipótese de que o estresse altera dramaticamente a mobilização de TEs, apenas quatro foram up-regulados. O nosso estudo, juntamente com o conhecimento prévio, confirma a *Drosophila* como um organismo de relevância para o estudo com quimioterápicos.

Palavras-chave: RNA-seq, Cisplatina, *Drosophila*, Elementos Transponíveis.

ABSTRACT

Cisplatin is widely used in cancer treatment and is one of the best cytostatic agents available for antitumor therapy. Drosophila melanogaster has one of the best annotated genomes and one of the best characterized sets of transposable elements (TE) sequences. This model organism is useful for analysing the mode of action of several compounds in vivo and evaluating the behavioral consequences of treatments. The aim of our study was to increase the knowledge about the effects of Cisplatin in Drosophila by joining RNA sequencing (RNA-seq) and biological assays. RNA-seq was followed by analyses of differential expression of genes (DEGs) and TEs (DETEs), and of pathways and ontology terms. DETEs were confirmed by qPCR. Cisplatin was evaluated at 50 and 100 µg/mL in Drosophila culture medium for 24 h. The fly locomotor assay, survival analysis, oviposition and development were used as biological assays. Cisplatin induced DEGs in a dose-dependent fashion, and four TEs were up-regulated. Most DEGs are related to DNA damage and detoxification processes. Cisplatin increases Drosophila locomotor activity and interrupts development. Genes and processes related to the assays were also identified. To our knowledge, this is the first study to evaluate the effects of Cisplatin in flies using RNA-seq. Gene alteration was almost limited to drug metabolism and DNA damage, and the drug did not vastly affect Drosophila on the molecular level. Contrary to the hypothesis that stress dramatically alters TEs mobilization, only four TEs were up-regulated. Our study, together with previous knowledge, asserts Drosophila as a valuable organism in the study of chemotherapy drugs.

Keywords: RNA sequencing, Cisplatin, *Drosophila*, Transposable Elements

LISTA DE ABREVIATURAS E SIGLAS

CIN - Neuropatia Induzida por Quimioterapia

TEs - Elementos Transponíveis

LTRs - Longas Repetições Terminais

RNA-seq - sequenciamento de RNA

qPCR - PCR quantitativo

DEGs - Genes Diferencialmente Expressos

DETEs - TEs Diferencialmente Expressos

padj - Valor-P ajustado

Log2fc - Log 2 fold change

GO - Gene Ontology

ORFs - Quadro de Leitura Aberto

DSBs - Quebras de dupla fita

NHEJ - Junção de Extremidades não-Homólogas

GST - Glutationa-S-Transferase

CYP450 - Genes do Citocromo P450

Hsps - Proteínas de Choque Térmico

HSF - Fator de Transcrição de Choque Térmico

CPA - Ciclofosfamida

ARC - Proteína Associada ao Citoesqueleto Atividade-Regulada

SUMÁRIO

1 INTRODUÇÃO

1.1 - Quimioterápico Cisplatina

Quimioterápicos causam efeitos moleculares e biológicos que têm sido objeto de estudo aprofundado há muitos anos. A Cisplatina é usada no tratamento para o câncer há mais de três décadas e ainda é um dos melhores agentes citostáticos disponíveis para terapia antitumoral. A droga induz suas propriedades citotóxicas pela sua habilidade de fazer ligação cruzada entre as bases de purina no DNA e subsequente interferência com a transcrição e/ou mecanismos de replicação, assim, ela induz apoptose nas células cancerosas. Ademais, as evidências sugerem que os efeitos citotóxicos induzidos pela ligação da Cisplatina a alvos que não o DNA (em especial as proteínas) podem contribuir ao seu mecanismo de ação bioquímico[1]. A quimioterapia com Cisplatina também ativa múltiplas vias de transdução de sinal que podem levar a diversas respostas celulares, incluindo parada do ciclo celular, reparo do DNA e sobrevivência ou morte celular[2]. Os agentes quimioterápicos matam preferencialmente células cancerosas por causa das suas características únicas, como sua rápida proliferação. Assim, vários agentes acabam por causar dano a tecidos normais de alta proliferação. Porém, neurônios não proliferativos e células de suporte do sistema nervoso periférico também são suscetíveis ao dano. Com isso, é comum o desenvolvimento de neuropatia induzida por quimioterapia (CIN), e os pacientes tornam-se incapazes de completar o tratamento ou usar a dose adequada. Cerca de 30-40% dos pacientes tratados com Cisplatina experimentam sintomas e sinais sensoriais, frequentemente proeminentes em CIN[3]. Mesmo após a descontinuação da droga os sintomas podem progredir e a dor é considerada comum[3]. Outro limitante da quimioterapia com agentes de platina é o desenvolvimento de resistência pelos tumores. Os mecanismos que explicam a resistência à Cisplatina incluem a redução da acumulação do citostático dentro das células cancerosas por causa de barreiras ao longo da membrana celular, o rápido reparo de adutos de platina, a modulação de vias apoptóticas em várias células, a up-regulação de fatores de transcrição, a perda da p53 e outras funções proteicas e uma maior concentração de glutationa e metalotioninas em determinados tumores[4]. Portanto, a identificação de vias e proteínas relacionadas a CIN e a resistência de

quimioterápicos é essencial e o desenvolvimento de novas drogas que possam ser combinadas à Cisplatina e reduzir ou até reverter os sintomas é uma abordagem interessante para alcançar a melhor eficácia da quimioterapia. Por fim, organismos menos derivados, como *Drosophila*, nos proporcionam a identificação de vias e genes-alvo com menor redundância, os experimentos são considerados simples e não têm a necessidade de aprovação por conselhos de ética.

1.2 - *Drosophila melanogaster*

O genoma de *Drosophila* tem 60% de homologia ao de humanos e mais de 60% dos genes causadores de doenças têm um homólogo funcional nas moscas[5]. Além disso, famílias gênicas conservadas têm menos membros em *Drosophila*, o que facilita a análise funcional[5]. O organismo-modelo foi utilizado para importantes descobertas em diversas áreas. Em genética e epigenética, o descobrimento da herança genética cromossômica, a mutagenicidade dos raios-X, *linkage* e duplicação e deleção causadas por crossovers desiguais propulsionou a *Drosophila* como sistema de pesquisa principal[6,7,8,9,10,11]. No campo de desenvolvimento, a mosca-da-fruta conquistou espaço com o estudo de Poulson em *Notch* e de Lewis com os genes homeóticos[12,13]. Posteriormente, o estudo de Nüsslein-Volhard e Wieschaus, com análises genéticas de defeitos em embriões de *Drosophila*, levou a descoberta de numerosos participantes em quase todas as vias-chave de desenvolvimento, o que nos levou à compreensão do desenvolvimento humano e de doença, incluindo o câncer[14]. A área de neurobiologia e estudo de doenças neurológicas avançou com o entendimento das redes genéticas subjacentes à ritmicidade diurna encontradas e caracterizadas inicialmente na mosca[15]. Além disso, a *Drosophila melanogaster* é útil para analisar de forma rápida o modo de ação de diversos compostos *in vivo* e encontrar as vias e proteínas-alvo que a droga atinge. Já foi visto que, quando a Cisplatina é incluída no meio de cultura de *Drosophila*, isso resulta em nível de droga comparável àquele encontrado em roedores e humanos tratados com a mesma[16]. Ademais, adutos de platina-DNA e dano tecidual são qualitativamente similares àqueles observados em vertebrados[16]. Em *Drosophila*, também é possível quantificar a toxicidade do composto como a diminuição na fecundidade de moscas fêmeas após a ingestão da droga por via oral. A fecundidade é uma medida sensível ao efeito de quimioterápicos na fisiologia das moscas já que a oogênese (e a

espermatogênese) são as únicas células com rápida divisão em moscas adultas e drogas quimioterápicas são desenvolvidas justamente com o intuito de interromper a divisão celular[17].

1.3 - Elementos Transponíveis

Elementos Transponíveis (TEs) são sequências genéticas capazes de se mover nos genomas hospedeiros e aumentar seu número de cópias durante esse processo. Os TEs podem ser divididos em duas grandes classes baseadas em seus mecanismos de transposição e cada classe pode ser subdividida em subclasses baseadas em seus mecanismos de integração cromossômica. Elementos de Classe I, também chamados de retrotransposons, são mobilizados pelo mecanismo “copia-e-cola”, no qual um intermediário de RNA é transcrito reversamente em uma cópia de cDNA, a qual é integrada em outra localização do genoma[18]. Para retrotransposons de longas repetições terminais (LTR), a integração ocorre por meio de clivagem e uma reação de transferência de fita catalisada por uma integrase, semelhante a retrovírus[19]. Para retrotransposons não-LTR, o que inclui ambos elementos nucleares intercalados longos e curtos (LINEs e SINEs), a integração cromossômica é acoplada a transcrição reversa por um processo nomeado transcrição reversa *target-primed*[20]. Elementos de Classe II, também chamados de transposons de DNA, são mobilizados por um intermediário de DNA, diretamente por um mecanismo “corta-e-cola” ou, no caso dos *Helitrons*, um mecanismo replicativo “descasca-e-cola” envolvendo um intermediário de DNA circular[21,22,23]. Devido a sua mobilização e habilidade de se replicar, os TEs podem ser componentes ativos dos genomas[24]. A transposição dos TEs pode causar mutações que variam muito em termos de efeito biológico. A mobilização pode ser deletéria, como exemplificada pela disgenesia híbrida de *Drosophila*, em que uma transposição na linhagem germinativa pode levar à esterilidade, mas também pode criar variabilidade genética nova, útil ao hospedeiro como fonte de variabilidade para sua adaptabilidade[25,26]. Em relação ao estresse, tanto de origem oxidativa, radiativa ou quimioterápica, ele pode alterar a expressão dos TEs em células somáticas, como evidenciado em estudos de sequenciamento de transcriptoma em *Drosophila*[26,27,28]. Por isso, a transposição dos TEs em tecidos somáticos tem sido o foco de estudos recentes uma vez que também foi associada ao envelhecimento, ao câncer e a doenças

degenerativas[29,30,31]. A *D. melanogaster* tem uma das melhores anotações de genoma e sequências móveis. Além disso, ao menos 30% dos elementos da mosca estão inteiros e provavelmente ativos, portanto, a *Drosophila* é considerada o organismo modelo para estudo de TEs eucarióticos[24].

1.4 - Sequenciamento de RNA e Ensaios Biológicos

O sequenciamento de RNA (RNA-seq) permite a identificação da alteração de genes e TEs de modo abrangente. Análises *downstream* de vias e termos de ontologia também são informações importantes que nos apontam para ensaios biológicos de relevância. O nosso estudo avaliou a Cisplatina em duas concentrações diferentes no meio de cultivo de *Drosophila* por 24 h (50 e 100 µg/mL). Além disso, a expressão dos TEs também foi avaliada, quatro elementos foram encontrados up-regulados e dois deles foram confirmados por qPCR. A fim de estimar os efeitos biológicos da Cisplatina nas moscas, nós fizemos os ensaios de atividade locomotora, sobrevivência e oviposição e desenvolvimento. A Cisplatina a 100 µg/mL aumenta a atividade locomotora da *Drosophila* e interrompe o desenvolvimento. Genes e processos relacionados aos ensaios também foram identificados. O nosso estudo une análises moleculares de RNA-seq com ensaios de comportamento e desenvolvimento para ampliar o conhecimento dos efeitos da Cisplatina em *D. melanogaster*.

2 OBJETIVOS

2.1 - Objetivo Geral

Avaliar os efeitos moleculares e biológicos da Cisplatina em *Drosophila*.

2.2 - Objetivos Específicos

Com a abordagem molecular, identificar se a Cisplatina age de forma dose-dependente, se é capaz de mobilizar TEs e quais genes, processos e vias são alterados. Posteriormente, relacionar as análises moleculares com os ensaios biológicos de atividade locomotora, análise de sobrevivência e de oviposição e desenvolvimento.

3 ARTIGO PUBLICADO EM PERIÓDICO

Mombach, D. M., Gomes, T. M. F. F., Silva, M. M., Loreto, E. L. S. Molecular and biological effects of Cisplatin in Drosophila. Comparative Biochemistry and Physiology Part C 2021 https://doi.org/10.1016/j.cbpc.2021.109229

Comparative Biochemistry and Physiology, Part C 252 (2022) 109229

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Research article

Molecular and biological effects of Cisplatin in Drosophila

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ARTICLE INFO

Edited by Martin Grosell

Keywords:
Cancer
Differential expression
RNA sequencing
Transcriptome
Transposable elements

ABSTRACT

Cisplatin is widely used in cancer treatment and is one of the best cytostatic agents available for antitumor therapy. *Drosophila melanogaster* has one of the best annotated genomes and one of the best characterized sets of transposable elements (TE) sequences. This model organism is useful for analyzing the mode of action of several compounds *in vivo* and evaluating the behavioral consequences of treatments. The aim of our study was to increase the knowledge about the effects of Cisplatin in *Drosophila* by joining RNA-seq and biological assays. RNA-seq was followed by analyses of differential expression of genes (DEGs) and TEs (DETEs), and of pathways and ontology terms. DETEs were confirmed by qPCR. Cisplatin was evaluated at 50 and 100 μg/mL in *Drosophila* culture medium for 24 h. The fly locomotor assay, survival analysis, oviposition and development were used as biological assays. Cisplatin induced DEGs in a dose-dependent fashion, and four TEs were up-regulated. Most DEGs are related to DNA damage and detoxification processes. Cisplatin increases *Drosophila* locomotor activity and interrupts development. Genes and processes related to the assays were also identified. This is the first study to evaluate the effects of Cisplatin in flies using RNA-seq. Gene alteration was almost limited to drug metabolism and DNA damage, and the drug did not vastly affect *Drosophila* on the molecular level. Contrary to the hypothesis that stress dramatically alters TEs mobilization, only four TEs were up-regulated. Our study, together with previous knowledge, asserts *Drosophila* as a valuable organism in the study of chemotherapy drugs.

1. Introduction

Chemotherapy drugs induce biological and molecular effects that have been a matter of thorough study for many years. Cisplatin has been used in cancer treatments for more than three decades and is still one of the best cytostatic agents available in antitumor therapy. The drug induces its cytotoxic properties through its ability to crosslink with the purine bases on the DNA and subsequently interfere with normal transcription, and/or DNA replication mechanisms, thus inducing apoptosis in cancer cells. Moreover, evidence suggests that the cytotoxic effects induced by binding of Cisplatin to non-DNA targets (especially proteins) may contribute to its biochemical mechanism of action (Fuertes et al., 2003). Cisplatin chemotherapy also activates multiple signal transduction pathways, which can lead to several cellular responses, including cell cycle arrest, DNA repair, cell survival or cell death (Niedner et al., 2001).

The *Drosophila melanogaster* genome has 60% homology to that of humans, and more than 60% of human disease-causing genes have a functional homolog in flies (Wangler et al., 2015). Furthermore, conserved gene families have fewer members in *Drosophila*, which facilitates functional analyses (Wangler et al., 2015). This model organism is useful for rapid analysis of the mode of action of several active compounds *in vivo* and for elucidation of the target pathways and genes affected by the drug. When Cisplatin is included in *Drosophila* culture medium, it results in drug levels comparable to those found in rodents and humans treated with it (Podratz et al., 2011). Platinum-DNA adducts and tissue damage are also qualitatively similar to those observed in vertebrates (Podratz et al., 2011).

Transposable elements (TE) are genetic sequences that can mobilize within host genomes and increase their copy number in the process. Due to this mobilization and their capacity to replicate, TEs can be active components of genomes (Barrón et al., 2014). TE transposition can cause mutations that vary greatly in terms of biological effects. Mobilization can be deleterious, as seen in *Drosophila* hybrid dysgenesis, in which a germline transposition can lead to sterility, but it can also create new genetic variability, useful to the host, from an evolutionary perspective,

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https://doi.org/10.1016/j.cbpc.2021.109229
Received 3 July 2021; Received in revised form 20 October 2021; Accepted 27 October 2021
Available online 30 October 2021
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when submitted to stressful conditions (Kidwell, 1983; Capy et al., 2000) Regarding stress, whether its origin is oxidative stress, radiation or chemotherapy, it can alter TE expression in somatic cells, as seen by transcriptome sequencing studies in *Drosophila* (Capy et al., 2000; Stoffel et al., 2020; Oliveira et al., 2021). Thereby, TE transposition in somatic tissues has been the focus of recent studies as it has also been associated with aging, cancer and degenerative diseases (De Cecco et al., 2013; Sedivy et al., 2013; Jang et al., 2019). *D. melanogaster* has one of the best annotated genomes and one of the best characterized sets of TE sequences. In addition, at least 30% of the fly's elements are full length and probably active; therefore, *Drosophila* is considered the model organism for the study of eukaryotic TEs (Barrón et al., 2014).

RNA sequencing (RNA-seq) allows the detection of genes and TE alterations in a comprehensive fashion. Downstream analysis of pathways and ontology terms also provides valuable information that leads us to relevant biological assays. Our study evaluated Cisplatin in two different concentrations in *Drosophila* culture medium for 24 h (50 and 100 μg/mL). In addition, TE expression was addressed, and four elements were found to be up-regulated, two of them confirmed by quantitative polymerase chain reaction (qPCR). In order to estimate the biological effects of Cisplatin in flies, we carried out locomotor activity, survival and oviposition and development assays. Cisplatin at 100 μg/mL increased *Drosophila* locomotor activity and interrupted development. Genes and processes related to these assays were also identified. This study joins molecular analyses of RNA-seq with behavioral and developmental assays to improve knowledge of Cisplatin effects in *D. melanogaster*.

2. Methods

2.1. Fly maintenance and Cisplatin treatment

D. melanogaster Oregon R were raised in vials containing cornmeal, sucrose and yeast at 20 °C. Flies were treated with 0, 50 and 100 μg/mL Cisplatin (Blau Farmacêutica, SP, Brazil), diluted in the same medium in which they were raised, plus red food coloring. After a 7–8 h starvation period, 1–4 day-old flies were placed in vials containing medium and the appropriate drug concentration for 24 h. Since red food coloring was added to the medium, food intake was estimated by the flies' abdominal coloring. Flies visually screened for the same intensity of food coloring in the abdomen were used for the assays. The drug was not provided during the assays; the treatment consisted of only 24 h of Cisplatin exposure.

2.2. RNA isolation

Total RNA was obtained from 1 to 4 day-old adult female flies with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA integrity was analyzed with Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA) and quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and a QuBit 3.0 Fluorometer (Thermo Fisher Scientific). Each condition had two replicates, and 20 flies were used for each replicate. Only female flies were used for the RNA-seq analysis to reduce variability and to allow identification of genes related to the oviposition and development assay.

2.3. Library preparation and sequencing

For library preparation, mRNA was isolated with Dynabeads mRNA Purification kit (Invitrogen), according to the manufacturer's protocol. mRNA was fragmented with RNase III, with the Total Ion RNA-Seq v2 kit (Thermo Fisher Scientific). For adapters, barcode addition and cDNA synthesis, the kits Ion Adapter Mix v2 and Ion Xpress RNA-Seq barcode 01–16 were used. Amplified libraries were purified with nucleic acid binding and eluted in nuclease-free water. Emulsion PCR was performed with the One Touch system (Thermo Fisher Scientific). The Ion 540 sphere particles were prepared with the One Touch 2 Template v3 kit. Sequencing was performed in an Ion Torrent S5 sequencer (Thermo Fisher Scientific) using the Ion 540 Kit-OT2 and Ion 540 Chip. Data were collected with Torrent Suite v4.0 software.

2.4. Quantitative polymerase chain reaction

mRNA was isolated with the Dynabeads mRNA Purification kit (Invitrogen). Samples were quantified with a QuBit 3.0 Fluorometer (Thermo Fisher Scientific) and reversed transcribed using the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific), all according to the manufacturer's instructions. SYBR Green-based qPCR was then performed on a StepOnePlus (Applied Biosystems, Foster City, CA, USA). RP49 was used as the endogenous control, and all reactions were performed in quadruplicate (Saint-Leandre et al., 2017). Relative gene expression was calculated using the comparative cycle threshold ($2^{-\Delta\Delta CT}$) method. The primers used are described in Table 1.

2.5. Bioinformatics analysis of transcriptome sequence data

The library size, in millions of reads, for the control and treated conditions was congruent in the dataset: $\underline{x} = 27.6\ \sigma = \pm 8.0$. Cisplatin treatment was a single-end library, and each condition had two replicates. First, reads were trimmed with Trim Galore 0.5.0 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Five nucleotides were trimmed from the beginning of replicates and 40 from the end. Sequences with phred scores under 20 were dismissed. Since we sequenced in an Ion Torrent, and reads varied greatly in size, we made a custom script in Python to split reads above 100 bp in half, resulting in reads ranging from 50 to 100 bp. HISAT2 2.1.0 (Kim et al., 2019) was used for data alignment with *D. melanogaster* reference genome v6.32 obtained in FlyBase (https://flybase.org/). The output was processed by eXpress 1.5.1 (Roberts and Pachter, 2013) in order to calculate the estimated counts per gene using default parameters. TE table count was generated with TEcount, the first module of TEtools (Lerat et al., 2016). TEcount uses Bowtie2 for alignment and generates the total number of counts per TE insertion grouped by TE subfamily. The TE reference was FlyBase's v6.32 of all TEs for *D. melanogaster*. The parameter of the table count is unique mapped reads. Since the lengths of TE insertions in Oregon R strains are not known, the counts should not be normalized by length. Counts of TEs and genes per dosage were merged (Supplementary Table S1). Merged count tables were used to perform a principal component analysis (PCA) in order to compare similarities and dissimilarities between treatments and their replicates, using the *prcomp* function in the statistical programming environment R 3.4.0.

DEseq2 1.18.1 (Love et al., 2014) was used for differential expression analysis with default parameters. The results obtained were the levels of differentially expressed genes (DEGs) with Cisplatin treatment at both concentrations relative to the control. DEGs and differentially expressed TEs (DETEs) were considered significant with an adjusted p-value <0.05 (padj), which provides more accurate results in comparison with unadjusted p-values. A list with the DEGs/DETEs and their parameters is attached as a Supplementary Table S2.

Downstream analysis was performed using the DAVID v6.8 web

Table 1
Primers used in quantitative PCR analysis.

Target TE	Sequence (5′-3′)	Product length (bp)
flea-F	GTTTTACAACGGAGCATAGCAC	74
flea-R	AGGTGAAGTTGAAACAGTTGCT	
accord-F	GACGCAAACAACTACGCCAT	92
accord-R	AGCGAAGGATATGGGTCTGT	
RP49_66-F	CCAGTCGGATCGATATGCTAA	121
RP49_186-R	GTTCGATCCGTAACCGATGT	

server functional annotation tool (Huang et al., 2009). Gene Ontology (GO)(The Gene Ontology Consortium, 2019) and KEGG Pathways (Kanehisa et al., 2021) results were employed (Supplementary Table S3). Enriched GO terms were further refined using ReviGO (Supek et al., 2011) with a cut-off of $p < 0.05$ (Supplementary Table S3).

2.6. Transposable element analysis

Gene and TE coordinates in the chromosomes were crossed with the *GenomicRanges* (Lawrence et al., 2013) R package. TEs were searched inside the genes (Supplementary Table S4). However, it is worth mentioning that TE insertions are variable among *D. melanogaster* strains, and it is not possible to directly infer the Oregon R insertions found inside genes using the *iso-1* genome as a reference (Petrov et al., 2011). Rech et al. (2019) establishes fixed TEs insertions among sequenced *D. melanogaster* strains. Therefore, we used their findings to check if the insertions obtained are fixed (Rech et al., 2019).

Transcriptome assembly was performed with Trinity 2.9.1 (Grabherr et al., 2011) using the Galaxy repository (Afgan et al., 2018). Since we only found DETEs at 100 μg/mL, we performed an assembly for this concentration by merging its replicates. We then carried out blastn analysis with all the transcripts against the fasta file of TE consensus sequences from Sackton et al. (2009) with an E-value of 1e-50.

DETE transcripts with the longest length and highest bit scores were manually analyzed using the UGENE 37 (Okonechnikov et al., 2012) ORF prediction tool and Pfam 33.1 (El-Gebali et al., 2019) to verify the potential to produce either enzymes necessary for transposition or at least one enzyme that might have a biological impact. For representation, the alignment in Supplementary Fig. S1 was made with UGENE using the MUSCLE alignment tool with default parameters. The results of UGENE's ORF prediction, Pfam and blastn alignment are in Supplementary Table S5.

2.7. Code availability

The code for the bioinformatic analyses is available at https://github.com/DanielaMombach/CisplatinDrosophila.

2.8. Locomotor activity analysis

Registration and analysis of the locomotor activity of flies exposed to control and Cisplatin doses was performed using the DAM system (Drosophila Activity Monitor, TriKinetics). In this system, a single fly at a time is allocated to glass tubes (5 × 65 mm) containing agar medium and arranged horizontally on specific monitors supporting up to 32 tubes. The agar medium is used as a food source for this assay. The system was maintained in a BOD incubator with a 12:12 h light/dark cycle at 25 °C. The DAM system collects data such as the number of times an individual fly crosses the midline of its tube in 1 min. Flies were allocated and maintained in the DAM tubes for five days immediately after Cisplatin treatment. Since we obtained more data in the downstream analyses at 100 μg/mL Cisplatin, only this dose was used to measure the locomotor activity of flies. The first 24 h in DAM were considered as an acclimation period, and the data were not processed. Activity parameters were analyzed in the program ShinyR-DAM (Cichewicz and Hirsh, 2018), which converts the results from DAM to graphical data. A total of 32 individual male flies were used per group. Male flies are preferred in this assay because female flies lay eggs and carry the medium around, making it easier for them to stick to the tube wall. A Mann–Whitney *U* test was performed using GraphPad Prism 8.0 with flies' daily count table for the locomotor activity analysis. Other analyses, such as daytime *vs* nighttime activity, total sleep, daytime sleep, nighttime sleep and activity/sleep bout and length were performed; however, in none of them were control and treatment results significantly different.

2.9. Oviposition and development

A comparison of eggs laid, pupae formed and their eclosion to adult flies was made between flies treated at 0 and 100 μg/mL of the cytostatic drug. Cisplatin treatment was carried out as mentioned above. Flies were screened for red food coloring and placed in vials containing three females and two males for 17 h. Vials contained regular culture medium plus red food coloring to facilitate the counting of eggs. Differences between the number of pupae were analyzed with the chi-square test of GraphPad Prism 8.0. Pupae of both treatments ecloded to adults.

2.10. Survival analysis

Survival analysis was evaluated at 0 and 100 μg/mL. Cisplatin treatment was carried out as mentioned above. Flies were arranged in vials containing five females and five males and maintained with the same medium on which fly stocks were raised. A total of 10 vials were used for each treatment. Kaplan–Meier survival analysis followed by the Gehan–Breslow–Wilcoxon chi-square test were performed using GraphPad Prism 8.0.

3. Results

3.1. Transcriptome analyses

PCA of merged count tables shows agreement between control and treated replicates (Fig. 1). With padj <0.05, the following transcripts were differentially expressed. Of a total of 56 DEGs at 50 μg/mL, 25 were down-regulated and 31 up-regulated (Fig. 2a). There was no DETE subfamilies for the lowest dose. Of a total of 468 DEGs at 100 μg/mL, 238 were down-regulated and 230 up-regulated (Fig. 2b). There were four up-regulated TE subfamilies: *flea*, *mdg3*, *copia* and *accord* (Fig. 2c; Supplementary Table S2). *Flea* and *accord* mRNA expression levels were evaluated in the control and at 100 μg/mL treatment by qPCR. RNA-seq expression values are in Log 2-fold change (Log2fc); the control was set at 1 and the treatment at the Log2fc respective value plus 1 (Log2fc values and standard errors are in Supplementary Table S2). qPCR values were calculated using the comparative cycle threshold ($2^{-\Delta\Delta CT}$) method. Highly concordant results were observed between RNA-seq and qPCR, as shown in Fig. 3.

The 45 DEGs shared between treatments enabled us to perform a dose comparison in terms of Log2fc values (Fig. 4). Of these shared

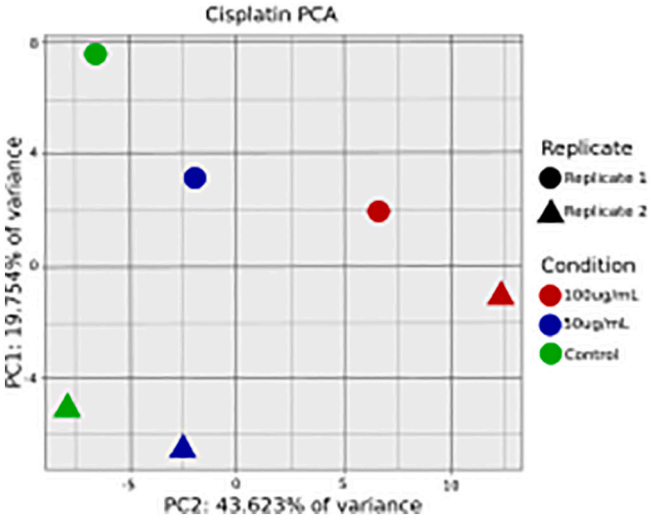

Fig. 1. Principal component analysis graph of the samples. The graph is colour-coded by sample and shape-coded by replicate. Proportion of the variance explained is 19.754% for PC1 and 43.623% for PC2.

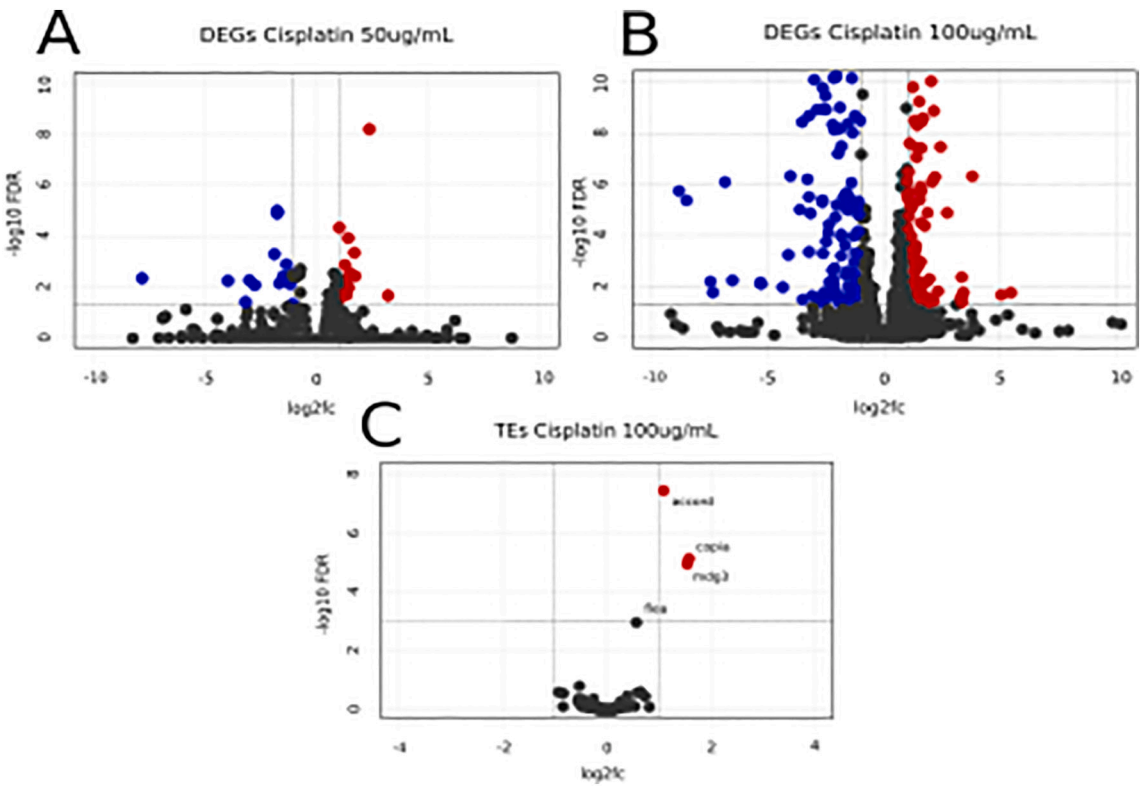

Fig. 2. Distribution of differentially expressed genes and TEs shown as a volcano plot. Each point represents a gene or TE. The blue dots represent the down-regulated differentially expressed genes, red dots represent the up-regulated differentially expressed genes and TEs, and black dots represent the genes or TEs that did not reached the parameters for the plot (Log2fc > |1| and padj <0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

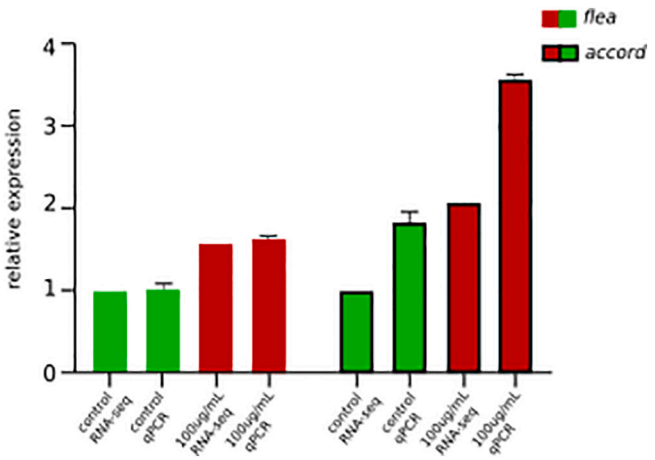

Fig. 3. The differentially expressed TEs, *flea* and *accord*, detected by RNA-seq confirmed by qPCR (bars indicate standard error).

DEGs, 38 worked in a dose-dependent fashion. The effects of the higher dose were enhanced, meaning they had higher/lower values of Log2fc than the lowest dose.

3.2. Downstream analysis

At 50 μg/mL Cisplatin, only one GO Process was identified: oxidation-reduction process. There was no significant KEGG pathway for the lowest dose. At 100 μg/mL Cisplatin, 43 GO terms and 9 KEGG pathways were identified. Most GO Processes were related to DNA damage and cell fate, but there were also interesting results, such as sleep and chorion-containing eggshell formation, and most pathways were involved in drug metabolism. The results of downstream analysis are in Supplementary Table S3. GO term's classification at 100 μg/mL Cisplatin and the number of DEGs for the specific term are shown in Fig. 5.

3.3. Transposable elements and gene regulation

We found three up-regulated genes with TE insertions from the also up-regulated TE subfamilies at 100 μg/mL Cisplatin. They were ionotropic receptor 40a and transglutaminase, both with a *copia* insertion, and tipE homolog 1 with a *flea* insertion (Supplementary Table S4). The four up-regulated TE transcripts, chosen by length and bit score, shared high identity with the TE consensus sequences from Sackton et al. (2009) (Supplementary Table S5). UGENE's ORF finder tool found ORFs of canonical sizes in all TE transcript sequences (Supplementary Fig. S1; Supplementary Table S5). Pfam identified a reverse transcriptase enzyme in the *flea* and *accord* transcript sequences. In the *accord* sequence, an integrase zinc binding domain was also identified, and in the *copia* sequence, a gag polypeptide (Supplementary Table S5).

3.4. Locomotor activity analysis

Locomotor activity analysis based on individual flies' daily count was significantly different between control flies and those treated with 100 μg/mL Cisplatin. Locomotor activity was higher in treated flies (788 counts; N = 124) than in controls (683 counts; N = 120) according to the Mann–Whitney *U* Test (approximate *p* value = 0.0276) (Fig. 6).

3.5. Oviposition and development

Control flies and those treated with 100 μg/mL Cisplatin laid a

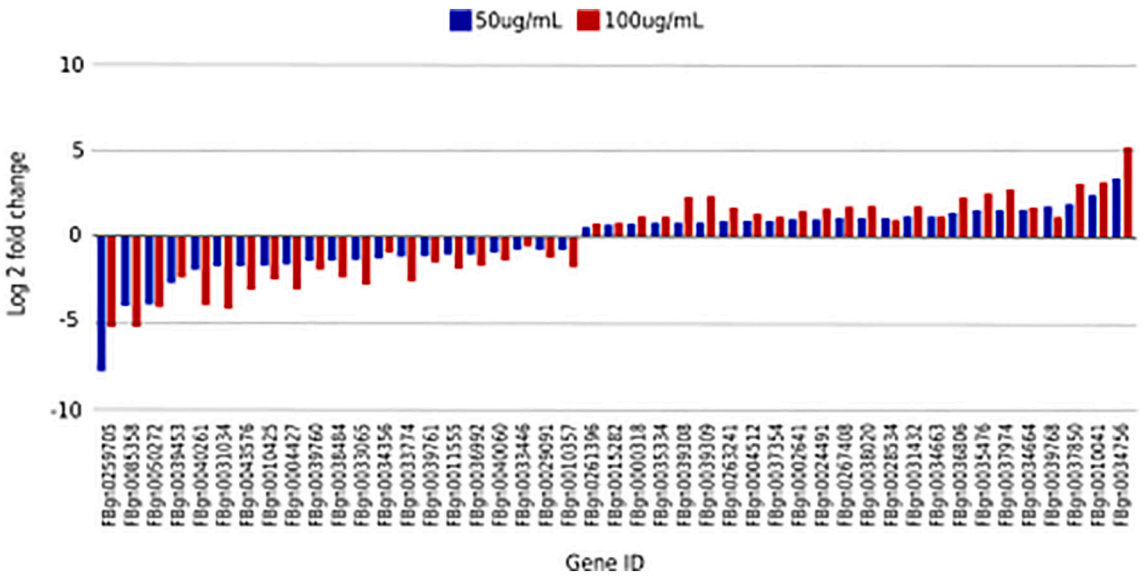

Fig. 4. Dose comparison of differentially expressed genes using Log 2 fold change. The colors represent the doses of Cisplatin, blue for 50 μg/mL and red for 100 μg/mL. The horizontal axis shows the DEGs FlyBase identification. The vertical axis displays the Log2fc for each gene according to the dose. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

similar number of eggs (112 and 110, respectively). However, there was a remarkable difference in pupa formation, with 92 eggs of control flies forming pupae, while only 11 treated flies did. The results were significantly different according to the chi-square test ($p < 0.0001$). We found that the eggs did not develop into larvae; therefore, there were no pupae.

3.6. Survival analysis

Kaplan–Meier survival analysis was performed between control and treated flies (100 μg/mL Cisplatin), and no significant difference was found according to the chi-square test.

4. Discussion

Both the volcano plot (Fig. 2) and the dose comparison graph based on Log2fc (Fig. 4) demonstrate that Cisplatin acts in a dose-dependent fashion in *Drosophila*. A number of 38 out of 45 genes shared between doses had higher/lower Log2fc at the highest concentration. With this, we can expect that, as the dose increases, the transcription of the same genes increases/decreases and new genes may appear dysregulated.

Cisplatin is a well-known double-strand break (DSB) inducer. Repair of DSBs can occur by either nonhomologous end joining (NHEJ) or homologous recombination. At 100 μg/mL Cisplatin, DSB repair *via* NHEJ as well as the cellular response to DNA damage stimulus (GO Processes) were enriched, and a gene of a DSB recognition protein, Ku80, was up-regulated. Moreover, both proteolysis and ubiquitination GO Processes and proteasome complex (GO Component) were enriched. The ubiquitin-proteasome system is an important regulator of cell growth and apoptosis. Isoe et al. (1992) reported that Cisplatin can inhibit the ubiquitin-ATP-dependent proteolysis and ubiquitination. The majority of genes related to the GO Processes are up-regulated. Finally, a caspase gene, *decay*, was found up-regulated at 100 μg/mL Cisplatin.

The enzyme glutathione-S-transferase (GST) catalyzes the formation of the platinum-glutathione conjugate, which is an important step in the inactivation and elimination of Cisplatin from the cell (Quintanilha et al., 2017). In this study, glutathione metabolic process (GO Process) was enriched at 100 μg/mL Cisplatin, and differentially expressed *GST* genes were found in both treatments. In addition, *Drosophila* treated at 100 μg/mL were enriched in two pathways related to P450. There were also differentially expressed *CYP450* genes in both doses of Cisplatin. In *Drosophila*, Cyp6g1 up-regulation mediates insecticide resistance (Chung et al., 2009). The upregulation is correlated with the presence of an *accord* inserted upstream of the Cyp6g1 transcription start site (Chung et al., 2009). However, in our study, Cyp6g1 was not differentially expressed. Most *P450* genes identified as dysregulated in our study are expressed in the larval midgut, Malpighian tubules and fat body, which is consistent with the detoxification role of CYP450 (Chung et al., 2009). Both *GST* and *CYP450* genes display the dose-dependency of Log2fc, meaning the dysregulation of these genes are expected to enhance with a higher dose of Cisplatin. These findings show *Drosophila*'s response to the drug and its attempt at detoxification.

The heat shock proteins (Hsps) are molecular chaperones capable of modulating the structure and folding of other proteins. Hsp expression is induced through the heat shock transcription factor pathway by stresses that cause protein denaturation (Tower, 2011). Our study found eight up-regulated *Hsps* genes at 100 μg/mL Cisplatin. The up-regulation of *Hsps* is in agreement with the heat shock-mediated polytene chromosome puffing and response to heat GO Processes. Up-regulation of *Hsps* genes are due to the cellular stress caused by Cisplatin. This also shows the cell response to the drug, since by mediating either protein refolding or degradation, the Hsps favor stress resistance (Tower, 2011).

The *Jonah* multigene family consists of approximately 20 genes organized in small clusters on different chromosomal sites (Carlson and Hogness, 1985). The functions of Jonah proteins are largely unclear, and this family has been identified as down-regulated in *Drosophila* aging transcriptome studies and up-regulated in an RNA-seq of *Drosophila* treated with Cyclophosphamide (CPA)(Curtis et al., 2007; Stoffel et al., 2020). The cause of the down-regulation of *Jonah* genes in these studies is also unclear. The present study found *Jonah* genes down-regulated, 10 in total at 100 μg/mL Cisplatin. *Jonah* genes are related to the following enriched GO terms: proteolysis (GO Process) and endopeptidase activity (GO Function).

The four up-regulated TEs belong to the LTR class of retrotransposons, a class containing direct long terminal repeats (LTRs) flanking the protein-coding sequence. TEs insertions found inside the up-regulated genes are not fixed among the sequenced *D. melanogaster* strains according to Rech et al. (2019). Nevertheless, there is a hypothesis that stress induces TEs mobility; however, recent studies show there is no general increase in TE transcription (Capy et al., 2000). Our study found only a few up-regulated TEs even though *Drosophila* has a

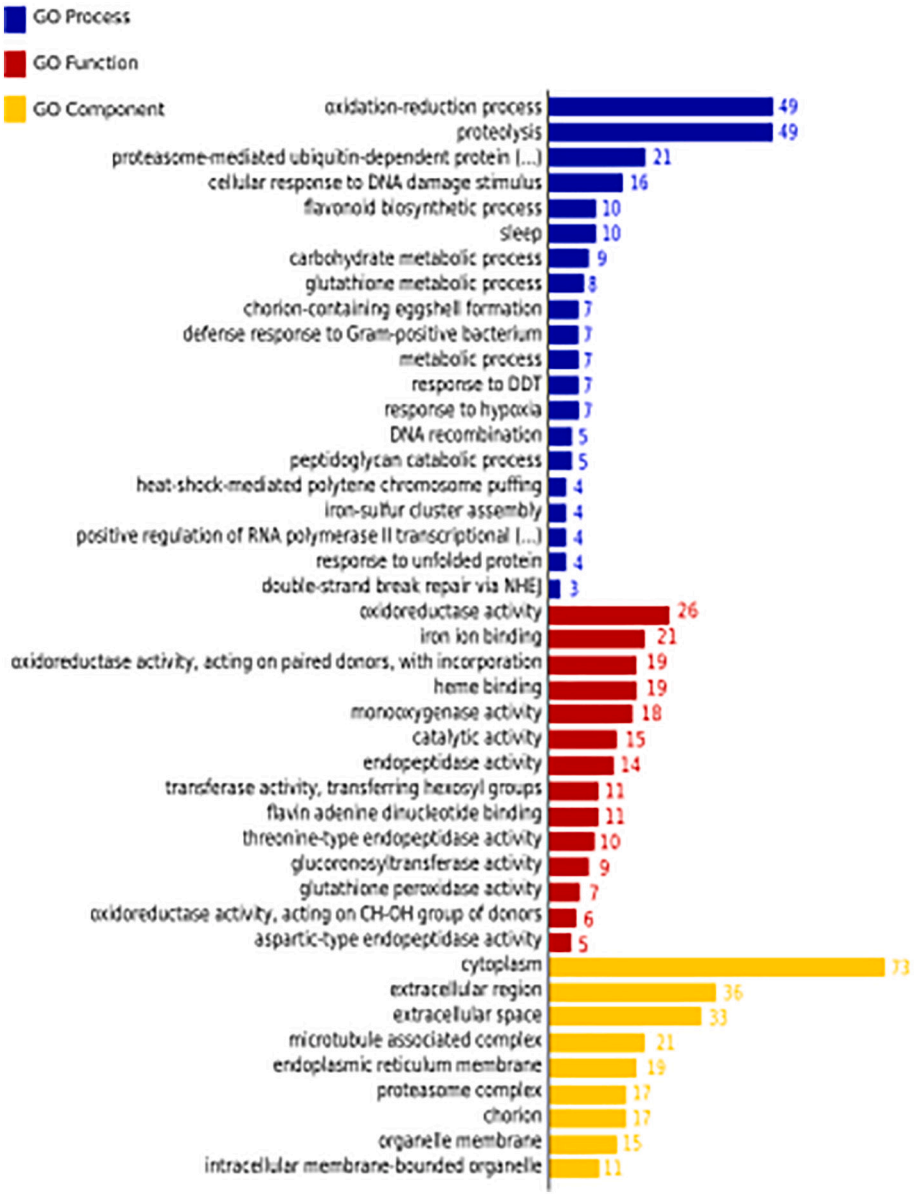

Fig. 5. GO classification for 100 μg/mL treatment. Annotation statistics of differentially expressed genes according to GO. The colors represent the three GO terms. The number beside each column is the number of DEGs for the specific class.

high percentage of active elements and a variety of TE families (Barrón et al., 2014). Stoffel et al. (2020) made a similar discovery when treating flies with CPA; they identified few members of the LTR class that were up-regulated. Oliveira et al. (2021) evaluated radiation and oxidative stress and also did not find a general increase in TE transcription. Furthermore, CPA treatment as well as radiation and oxidative stress exposure identified TE repression (Stoffel et al., 2020; Oliveira et al., 2021). Therefore, depending on the stress factor, TE activation appears to have a specificity regarding the class of elements. Other than that, it is interesting that LINEs, which account for the largest fraction of TEs in the human genome, are from the same class found up-regulated both in our study with Cisplatin and with CPA treatment (Stoffel et al., 2020). Perhaps it would be useful to evaluate whether platinum chemotherapy is also able to activate LINEs in human cells.

Activity-regulated cytoskeleton-associated protein (ARC) is a plasticity protein member of the immediate-early gene family and has a well-conserved protein sequence (Mattaliano et al., 2007). Mutational analysis of *Drosophila dArc1* indicated that its main role is in the behavioral responses to metabolic stress (Mattaliano et al., 2007). Our study shows that *Drosophila* treated at 100 μg/mL Cisplatin for 24 h increases its activity after treatment. Differential expression analysis also found *dArc1* and *dArc2* genes to be up-regulated. In addition, we found that flies treated at 100 μg/mL had *astray* down-regulated and were enriched in sleep (GO Process). Sonn et al. (2018) conducted a comprehensive study of genes involved in starvation-induced sleep loss in *Drosophila*. They found *astray* up-regulated in the starved group, a fly homolog of the rate-limiting phosphoserine phosphatase that catalyzes the last step in the biosynthesis of serine from carbohydrates (Sonn et al., 2018). Sonn et al. (2018) suggested that *astray* might differentially modulate sleep, depending on the neuronal loci and food availability. Our flies were not starved, which could be why *astray* was down-regulated, as was the case in their control group. Moreover, Cisplatin alters the carbohydrate metabolic process, which is possibly perceived as an internal nutrient deficit. If so, in response to the deficit, flies usually initiate food seeking behavior, and sleep is suppressed (Beckwith and French, 2019). This seeking behavior can also explain the increase in the locomotor activity of Cisplatin-treated flies. Other than that, even though control and treated flies showed no significant difference in sleep

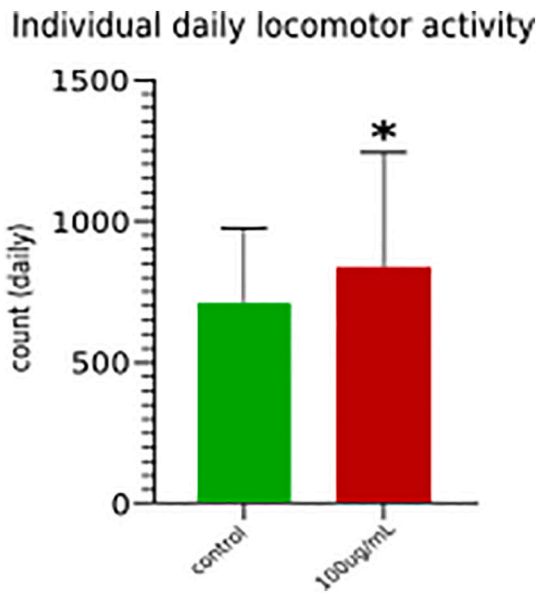

Fig. 6. Comparison of locomotor activity between control and flies treated at 100 μg/mL Cisplatin. Locomotor activity was measured in counts for four days. Statistical analysis was performed by GraphPad using Mann-Whitney *U* Test. *p < 0.05.

analysis, we observed an increase in activity overall. Therefore, it is likely that increased locomotor activity has a correlation with the up-regulated *dArc* genes and *astray* down-regulation as well as the alteration in carbohydrate metabolism.

It is possible to quantify a compound's toxicity by evaluating the female flies' fecundity after oral ingestion. Fecundity is a sensitive measure of the effect of chemotherapy drugs on the physiology of flies, since oogenesis are the only rapidly dividing cells in adult flies, and these types of drugs are designed to stop cell division (King et al., 2014). Our results with the oviposition and development assay show Cisplatin's severe toxic side effects. We also found a molecular base for the interruption of the larval stage. Two GO terms are altered, chorion-containing eggshell formation GO Process and chorion GO Component, and the seven genes related to the process are down-regulated. Recently, 11 new proteins have been identified as structural components of the eggshell (Fakhouri et al., 2006). Among these, seven are considered putative chorion components. Our study found that, besides the genes related to the GO Process, three genes of the putative chorion components were down-regulated. These gene alterations are most likely also related to the interruption of the larval stage.

In summary, we have identified some of the molecular and biological effects of Cisplatin in *Drosophila*. The drug acts in a dose-dependent manner, activating DNA damage and detoxification genes, processes and pathways. Four transposable elements of the LTR class of retrotransposons were up-regulated at the highest dose of Cisplatin, and two of them were confirmed by qPCR. We also found a molecular base to the increase in locomotor activity and interruption of the fly's development at 100 μg/mL Cisplatin. All findings were at a non-lethal dose, as seen by the survival analysis. RNA-seq followed by downstream analysis as a molecular approach leads us to relevant behavioral assays and can set a solid base to extend the findings in flies to vertebrates.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2021.109229.

Funding

This study was supported by research grants and fellowships from Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq (421255/2018-8) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors acknowledge partial support from CAPES and CNPq.

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4 DISCUSSÃO

5.1 - A Cisplatina funciona de forma dose-dependente

Os gráficos vulcão dos DEGs e o gráfico da comparação de dose baseada em Log2fc demonstram a Cisplatina funcionando de forma dose-dependente em *Drosophila*. Na comparação de dose, 45 genes são compartilhados pelas duas concentrações, sendo que a dose mais baixa tem 56 DEGs no total. Dos genes compartilhados, 38 mostram um Log2fc mais alto em módulo na concentração mais alta (100 µg/mL). Com isso, podemos esperar que ao aumentar a dose, os mesmos genes aumentem/diminuam a transcrição, como mostrado pelo Log2fc, e novos genes devem aparecer desregulados.

5.2 - A resposta da *Drosophila* à Cisplatina

A Cisplatina é um indutor de quebra-dupla de fitas do DNA (DSBs) muito conhecido. O reparo das DSBs pode ocorrer tanto por união de extremidades não-homólogas (NHEJ) ou recombinação homóloga. A 100 µg/mL de Cisplatina, o reparo de DSBs via NHEJ assim como resposta celular ao estímulo de dano de DNA (GO Processos) estavam enriquecidos e um gene de uma proteína de reconhecimento de DSBs foi encontrado up-regulado. Ademais, ambos GO Processos de proteólise e ubiquitinação e o GO Componente complexo proteassômico estavam enriquecidos. O sistema ubiquitina-proteassomo é um importante regulador de crescimento celular e apoptose. Isoe et al. (1992) mostrou que a Cisplatina pode inibir a proteólise ubiquitina-ATP-dependente e a ubiquitinação[32]. A maioria dos genes relacionados aos GO Processos estão up-regulados. Por fim, um gene de proteína do tipo caspase, *decay*, foi encontrado up-regulado. Esses achados sugerem que a Cisplatina está induzindo dano de DNA e apoptose celular, ainda que um ensaio de atividade de caspases não tenha mostrado diferença significativa entre controle e tratado (dados não mostrados).

A enzima Glutationa-S-Transferase (GST) catalisa a formação de um conjugado de platina-glutationa, o qual é um passo importante na inativação e na eliminação da Cisplatina da célula[33]. No presente estudo, o processo metabólico da glutationa (GO Process) estava enriquecido a 100 µg/mL de Cisplatina e genes de *GST* foram encontrados diferencialmente expressos em ambos tratamentos.

Ademais, a *Drosophila* tratada a 100 µg/mL teve duas vias relacionadas ao citocromo P450 enriquecidas. Também foram encontrados genes *CYP450* diferencialmente expressos em ambas doses de Cisplatina. Em *Drosophila melanogaster*, a up-regulação do gene *Cyp6g1* media a resistência aos inseticidas[34]. A up-regulação está correlacionada a presença de um elemento *accord* inserido upstream ao sítio de início de transcrição do *Cyp6g1*[34]. No entanto, no nosso estudo o gene *Cyp6g1* não foi encontrado diferencialmente expresso. A maioria dos genes P450 identificados como desregulados no nosso estudo são expressos no intestino da larva, túbulos de Malpighi e *fat body*, o que é consistente com o papel de desintoxicação de CYP450[35]. Ambos genes *GST* e *CYP450* apresentam a dose-dependência de Log2fc. Esses achados mostram a resposta da *Drosophila* à droga e a tentativa de detoxificação.

As proteínas de choque térmico (Hsps) são chaperonas moleculares capazes de modular a estrutura e o enovelamento de outras proteínas. A expressão das Hsps é induzida pela via do HSF (fator de transcrição de choque térmico) por estresses que causam desnaturação de proteínas[36]. Mediando o re-enovelamento ou a degradação das proteínas, as Hsps favorecem a resistência ao estresse. As Hsps são divididas em famílias: a família Hsp90/100, a família Hsp70, a família Hsp60, a família Hsp40, e as pequenas Hsps (sHsps). Elas também diferem em função de acordo com a família a qual pertencem. Dependendo da família, elas podem regular a atividade dos fatores de transcrição e componentes da via de sinalização e inibir divisão celular[36]. O nosso estudo encontrou genes de *Hsps* up-regulados a 100 µg/mL de Cisplatina pertencentes a todas as famílias, com exceção a Hsp40. A up-regulação das Hsps está em acordo com os GO Processos de *puff* de cromossomos politênicos mediado por choque térmico e resposta ao calor.

A família multigênica *Jonah* consiste de aproximadamente 20 genes organizados em pequenos *clusters* em diferentes sítios cromossômicos[37]. As funções proteicas de *Jonah* são amplamente desconhecidas e essa família tem sido identificada como down-regulada em estudos de envelhecimento em *Drosophila* e up-regulada em um estudo de RNA-seq de *Drosophila* tratada com Ciclofosfamida (CPA)[28,38]. O presente estudo encontrou genes de *Jonah* down-regulados, 10 no total a 100 µg/mL de Cisplatina. Os genes *Jonah* estão relacionados aos seguintes termos GO: proteólise (GO Processo) e atividade de endopeptidase (GO Função).

5.3 - Up-regulação de Elementos Transponíveis pela Cisplatina

Os quatro TEs up-regulados pertencem a classe LTR de retrotransposons, uma classe que contém LTRs flanqueando a sequência codificadora de proteína. As inserções de TEs encontradas dentro dos genes up-regulados não estão fixadas entre as linhagens sequenciadas de *D. melanogaster* de acordo com Rech et al. (2019)[39]. Mesmo assim, há uma hipótese que o estresse induz a mobilidade de TEs, no entanto, estudos recentes têm mostrado que não há um aumento geral na transcrição dos TEs[26]. O nosso estudo encontrou poucos TEs up-regulados ainda que *Drosophila* tenha uma alta porcentagem de elementos ativos e uma variedade de famílias de TEs[24]. Stoffel et al. (2020) obteve uma descoberta similar quando tratou as moscas com CPA, eles identificaram poucos membros da classe LTR up-regulados[28]. Oliveira et al. (2021) avaliaram os efeitos da radiação e do estresse oxidativo e também não encontraram um aumento geral na transcrição dos TEs[27]. Além do mais, o tratamento com CPA assim como radiação e exposição ao estresse oxidativo identificou repressão de TEs[27,28]. Portanto, dependendo do fator estressante, a ativação dos TEs aparenta ter uma especificidade em relação a classe de elementos. Além disso, é interessante que os LINEs, que contam com a maior fração de TEs do genoma humano, são da mesma classe encontrada up-regulada tanto no nosso estudo com Cisplatina como no tratamento com CPA[28]. Talvez, seja válido avaliar se a quimioterapia com platina também é capaz de ativar LINEs em células humanas.

5.4 - Aumento na Atividade Locomotora de *Drosophila*

A proteína associada ao citoesqueleto atividade-regulada (ARC) é uma proteína de plasticidade membro da família gênica *immediate-early* e tem uma sequência proteica muito conservada[40]. A análise mutacional da dArc1 de *Drosophila* indicou que seu papel principal é na resposta comportamental ao estresse metabólico[40]. O nosso estudo mostrou que a *Drosophila* tratada a 100 µg/mL de Cisplatina por 24 h aumenta sua atividade após o tratamento. A análise de expressão diferencial também encontrou os genes *dArc1* e *dArc2* up-regulados. Além disso, também achamos que as moscas tratadas a 100 µg/mL têm *astray* down-regulado e GO Processo sono enriquecido. Sonn et al. (2018) conduziram um amplo estudo de genes envolvidos em perda do sono por inanição em *Drosophila*[41].

Eles encontraram o *astray* up-regulado no grupo de inanição, um homólogo na mosca da fosfatase fosfoserina taxa-limitante que catalisa a última etapa na biossíntese da serina provinda de carboidratos[41]. Sonn et al. (2018) sugeriram que o *astray* deve modular o sono de forma diferente dependendo do loci neuronal e disponibilidade de alimento[41]. Já que as nossas moscas não sofreram inanição, pode ser por esse motivo que o *astray* está down-regulado, como foi o caso no grupo controle deles[41]. Além disso, a Cisplatina altera o processo metabólico do carboidrato, o que pode estar sendo reconhecido como um déficit interno de nutrientes. Se sim, em resposta ao déficit, as moscas, geralmente, iniciam um comportamento de busca por comida e o sono é suprimido[42]. Essas respostas também podem estar relacionadas ao aumento da atividade locomotora de moscas tratadas com Cisplatina. Ademais, mesmo que as moscas controle e as tratadas não tenham mostrado diferença na análise do sono, nós observamos um aumento de atividade geral. Portanto, é provável que a atividade locomotora aumentada tenha uma correlação tanto com os genes *dArc* up-regulados e o *astray* down-regulado como com a alteração no metabolismo de carboidratos.

5.5 - Interrupção do Desenvolvimento pela Cisplatina

A interrupção do desenvolvimento a 100 µg/mL de Cisplatina mostra o alto efeito tóxico do composto. A alteração é concordante com o GO Processo de formação da casca do ovo e o GO Componente córion. Os sete genes relacionados ao processo estão down-regulados. Recentemente, 11 novas proteínas foram identificadas como componentes estruturais da casca do ovo[43]. Entre esses, sete são considerados componentes putativos do córion. O nosso estudo encontrou, além dos genes relacionados ao GO Processo, três genes dos componentes putativos do córion down-regulados. As alterações dos genes estão muito provavelmente relacionadas à interrupção do estágio larval.

5.6 - Longevidade

Considerando o ensaio de longevidade, é interessante ressaltar que os efeitos da Cisplatina em *Drosophila* são altamente relevantes para a biologia e o comportamento da mosca mesmo em uma dose não-letal.

5 CONSIDERAÇÕES FINAIS

Com este estudo nós identificamos efeitos moleculares e biológicos da Cisplatina em *Drosophila*. A droga age de forma dose-dependente, ativa genes, processos e vias de dano de DNA e de detoxificação. Quatro elementos transponíveis da classe LTR de retrotransposons foram up-regulados na dose mais alta de Cisplatina e dois deles foram confirmados por qPCR. Nós também encontramos uma base molecular para o aumento da atividade locomotora e da interrupção do desenvolvimento a 100 µg/mL de Cisplatina. Todos os achados são em dose não letal, como visto pela análise de sobrevivência. O RNA-seq seguido de análises *downstream*, como abordagem molecular, nos leva a ensaios de comportamento relevantes e, em conjunto, as abordagens são capazes de formar uma base sólida para estender os achados em moscas a vertebrados.

A up-regulação de TEs pela Cisplatina agregada ao estudo prévio da up-regulação de TEs pela CPA, indicam que os citostáticos de platina alteram a expressão de TEs da classe LTR de retrotransposons em *Drosophila* e, por conseguinte, seria interessante avaliar se, em humanos, isso também é evidenciado. O nosso estudo, juntamente com estudos prévios, também muda a perspectiva de que o estresse aumenta a expressão de TEs de forma geral. Na verdade, o que é evidenciado não é um aumento geral na expressão, mas um aumento ou até mesmo uma diminuição na expressão de determinados TEs classe-específicos que dependem do tipo de estresse a que o organismo é submetido. Espera-se que, com o desenvolvimento de novas ferramentas, seja possível identificar as inserções das subfamílias de TEs diferencialmente expressos. Dessa forma, podemos identificar outras especificidades dos TEs que alteram sua expressão, como proximidade a genes, regiões de hetero ou eucromatina, integridade da inserção, entre outros. Outra abordagem possibilitada pelo estudo é de verificar os ovaríolos de fêmeas tratadas com Cisplatina, para identificar os danos que causam a interrupção do desenvolvimento da prole. Portanto, este estudo valida a *D. melanogaster* como organismo de estudo com quimioterápicos e fornece novas perspectivas para amplificar o conhecimento na área de câncer e desenvolvimento.

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7 MATERIAL SUPLEMENTAR

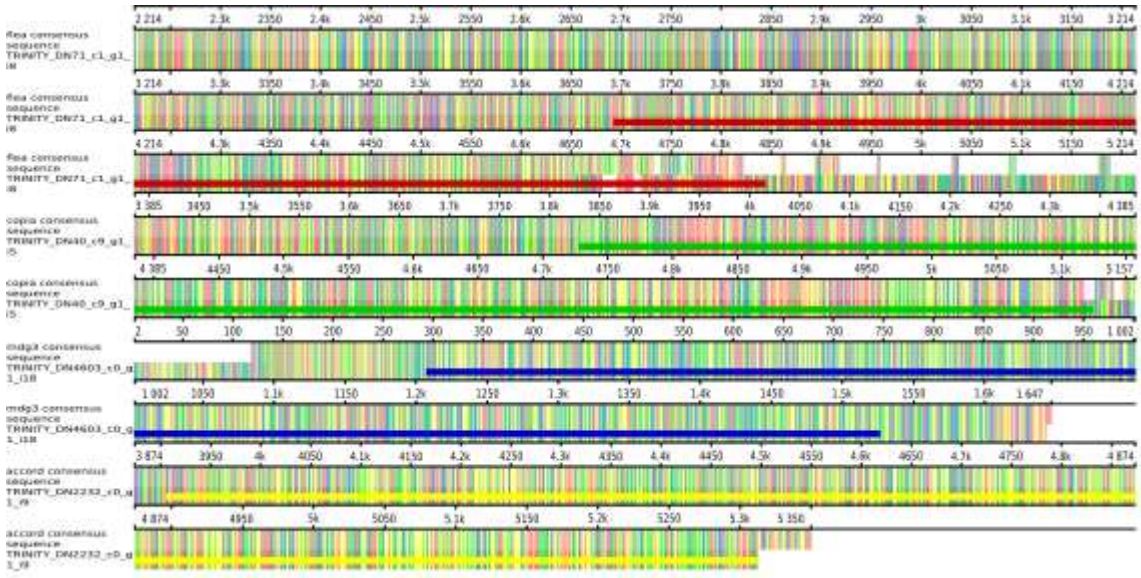

Figura Suplementar S1: Resultados do alinhamento para os 4 TEs up-regulados a 100 µg/mL de Cisplatina (*flea*, *copia*, *mdg3* e *accord*). Em vermelho, verde, azul e amarelo são as ORFs preditas pela ferramenta do UGENE. O alinhamento foi feito pela ferramenta MUSCLE através do UGENE.

Tabela Suplementar S1: Tabelas *count* agrupadas de genes e TEs das réplicas dos tratamentos (primeiros 20 genes e 18 TEs).

FlyBase ID	Control 1	Control 2	50ug/mL 1	50ug/mL 2	100ug/mL 1	100ug/mL 2
FBgn0000003	3467.587784	1969.737780	3906.520373	1814.176789	2166.095570	2781.392074
FBgn0000008	701.000000	465.000000	491.000000	517.000000	364.000000	436.000000
FBgn0000014	305.000000	267.000000	241.000000	211.000000	255.000000	369.000000
FBgn0000015	108.000000	99.000000	120.000000	73.000000	113.000000	107.000000
FBgn0000017	2263.000000	1796.000000	1928.000000	1837.000000	1485.000000	1751.000000
FBgn0000018	273.000000	116.000000	199.000000	144.000000	139.000000	195.000000
FBgn0000022	0.000000	4.000000	0.000000	1.000000	0.000000	0.000000
FBgn0000024	1416.000000	944.000000	773.000000	687.000000	592.000000	642.000000
FBgn0000028	37.000000	31.000000	32.000000	15.000000	14.000000	16.000000
FBgn0000032	1059.000000	522.000000	586.000000	630.000000	508.000000	566.000000
FBgn0000036	259.000000	214.000000	149.000000	159.000000	151.000000	123.000000
FBgn0000037	195.000000	169.000000	157.000000	128.000000	124.000000	168.000000
FBgn0000038	947.000000	677.000000	818.000000	360.000000	572.000000	725.000000
FBgn0000039	111.000000	88.000000	118.000000	83.000000	77.000000	108.000000
FBgn0000042	88191.288416	42136.589192	60030.000000	50646.000000	53190.000000	56565.000000
FBgn0000043	9957.000000	3309.000000	4785.000000	4486.000000	4156.000000	6062.000000
FBgn0000044	44572.000000	37802.410808	37078.320626	21278.000000	26226.000000	23280.034108
FBgn0000045	45680.000000	42955.000000	41614.000000	28406.000000	28989.000000	23301.000000
Cr1a	2359	1130	1406	1465	1735	1844
INE-1	4260	2245	2394	2188	2365	2702
BS	152	65	92	86	73	63
HMS-Beagle	1814	1247	1183	1158	1101	1404
1360	1478	646	943	785	803	1025
diver2	304	174	263	156	195	177
Tc1-2	94	40	57	49	48	58
G5A	147	59	128	80	66	85
3S18	1974	876	1257	983	1089	1602
jockey2	52	19	23	31	26	40
opus	787	380	323	274	282	347
297	3670	1802	1430	1505	1742	1073
Rt1a	104	89	181	91	98	118
blood	2115	1103	952	1224	1073	1067
Doc	4100	2388	2288	2742	3241	2711
HB	390	186	217	197	242	271
17.6	195	57	77	91	66	66
hobo	349	191	176	177	149	197

Tabela Suplementar S2: Genes e TEs diferencialmente expressos com parâmetros (Log 2 fold change e padj)(primeiros 15 up e down-regulados).

DEGs 50ug/mL

FlyBase ID	Log2fc	Padj
FBgn0259705	-7,787673372	0,004347259896
FBgn0085358	-3,999208571	0
FBgn0050272	-3,937406994	0,005215875206
FBgn0043791	-3,15285935	0,04337146929
FBgn0265084	-2,97665916	0,00509655656
FBgn0039453	-2,709221161	0,008126829661
FBgn0040261	-1,845358204	0,0004441930507
FBgn0031034	-1,740757354	0,00001166981167
FBgn0043576	-1,729956111	0,000009122762212
FBgn0010425	-1,649392351	0
FBgn0053199	-1,647111552	0,006858654859
FBgn0085447	-1,555300762	0,006858654859
FBgn0004427	-1,509001829	0,003450157655
FBgn0039760	-1,413183743	0,003450157655
FBgn0038484	-1,362455458	0,001275249593
FBgn0002641	0,8842651147	0,01715047743
FBgn0024491	0,9381640413	0,006228666571
FBgn0033294	0,9611245734	0,0002396239739
FBgn0002571	0,9721541618	0,003450157655
FBgn0267408	0,9921950002	0,000002996888223
FBgn0038020	1,017440935	0,00004122130574
FBgn0028534	1,021089855	0,03053863749
FBgn0031432	1,036639133	0,01031813801
FBgn0034663	1,08162512	0,02451754619
FBgn0036806	1,293865718	0,001275249593
FBgn0261361	1,355709353	0,01827242912
FBgn0267511	1,363532994	0,005215875206
FBgn0035476	1,445324348	0,0001081981188
FBgn0037974	1,447663882	0,009826964713
FBgn0034664	1,461488925	0,002742474029

DEGs 100ug/mL

FlyBase ID	Log2fc	Padj
FBgn0014903	-8,790440569	0,000001913508164
FBgn0266174	-8,486830467	0,000004490889941
FBgn0050274	-7,450992587	0,00713091559
FBgn0035886	-7,36065393	0,0179332575
FBgn0004426	-6,832579951	0,0000008173654415
FBgn0029170	-6,520944378	0,00621561743
FBgn0038028	-5,314510623	0,007744562291
FBgn0085358	-5,301197326	0
FBgn0259705	-5,258450598	0,008698169373
FBgn0004425	-4,327715182	0,01100671667
FBgn0031034	-4,200704673	0
FBgn0050272	-4,100895359	0,0006075523886
FBgn0013772	-4,01093927	0,0000004900523333
FBgn0040261	-4,002315512	0
FBgn0037996	-3,992833894	0
FBgn0051354	2,331988411	0,01561646346
FBgn0030189	2,440897129	0,00000003585948242
FBgn0035476	2,460217162	0
FBgn0037974	2,707571303	0
FBgn0033926	2,717142442	0,00001334533301
FBgn0037850	2,986445364	0
FBgn0010041	3,089044481	0
FBgn0038315	3,304507187	0,02950962113
FBgn0066365	3,3634745	0,004673306998
FBgn0259683	3,372467871	0,04461710517
FBgn0266168	3,400374182	0,02167826752
FBgn0010042	3,796610711	0,0000004804131458
FBgn0037099	5,085984996	0,02278764794
FBgn0034756	5,113214366	0
FBgn0037350	5,482466699	0,01807208898

Tabela Suplementar S3: Análise de enriquecimento do DAVID. Gene Ontology e KEGG Pathways para 50 e 100ug/mL de Cisplatina e o refinamento do ReviGO para 100ug/mL (primeiros cinco para cada classe).

DAVID

100ug/mL

Category	Term	Count	%	List Total	Pop Hits	Pop Total	Fold Enrichment	PValue	Benjamini
KEGG_PATHWAY	dme0305:Proteasome	20	4,3103448276	140	50	2737	7,82	0	0
KEGG_PATHWAY	dme00980:Metabolism of xenobiotics by cytochrome P450	17	3,6637931034	140	61	2737	5,4483606557	2,37E-08	9,73E-07
KEGG_PATHWAY	dme00982:Drug metabolism - cytochrome P450	16	3,4482758621	140	61	2737	5,1278688525	1,72E-07	4,71E-06
KEGG_PATHWAY	dme00040:Pentose and glucuronate interconversions	13	2,8017241379	140	43	2737	5,9104651163	7,25E-07	1,49E-05
KEGG_PATHWAY	dme00053:Ascorbate and aldarate metabolism	9	1,9396551724	140	30	2737	5,865	8,19E-05	0,0013428232

Category	Term	Count	%	List Total	Pop Hits	Pop Total	Fold Enrichment	PValue	Benjamini
GOTERM_MF_DIRECT	GO:0004175~endopeptidase activity	14	3,0172413793	348	42	9284	8,8927203065	2,1E-09	6,82E-07
GOTERM_MF_DIRECT	GO:0016705~oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	19	4,0948275862	348	91	9284	5,5701654667	5,1E-09	8,21E-07
GOTERM_MF_DIRECT	GO:0004497~monooxygenase activity	18	3,8793103448	348	88	9284	5,4568965517	2E-08	2,14E-06
GOTERM_MF_DIRECT	GO:0016491~oxidoreductase activity	26	5,6034482759	348	193	9284	3,5939491394	4,97E-08	3,99E-06
GOTERM_MF_DIRECT	GO:0004252~serine-type endopeptidase activity	31	6,6810344828	348	280	9284	2,9536535304	1,74E-07	1,12E-05

Category	Term	Count	%	List Total	Pop Hits	Pop Total	Fold Enrichment	PValue	Benjamini
GOTERM_CC_DIRECT	GO:0042600~chorion	17	3,6637931034	324	38	10026	13,8435672515	0	0
GOTERM_CC_DIRECT	GO:0000502~proteasome complex	17	3,6637931034	324	43	10026	12,2338501292	0	0
GOTERM_CC_DIRECT	GO:0005838~proteasome regulatory particle	11	2,3706896552	324	21	10026	16,208994709	3E-10	1,15E-08
GOTERM_CC_DIRECT	GO:0005839~proteasome core complex	9	1,9396551724	324	17	10026	16,3823529412	2,01E-08	6,43E-07
GOTERM_CC_DIRECT	GO:0031090~organelle membrane	15	3,2327586207	324	79	10026	5,8755274262	1,84E-07	4,7E-06

Category	Term	Count	%	List Total	Pop Hits	Pop Total	Fold Enrichment	PValue	Benjamini
GOTERM_BP_DIRECT	GO:0055114~oxidation-reduction process	49	10,5603448276	365	412	10996	3,5829498604	0	0
GOTERM_BP_DIRECT	GO:0006508~proteolysis	49	10,5603448276	365	475	10996	3,1077375631	0	9E-10
GOTERM_BP_DIRECT	GO:0043161~proteasome-mediated ubiquitin-dependent protein catabolic process	21	4,525862069	365	88	10996	7,1891656289	0	1,1E-09
GOTERM_BP_DIRECT	GO:0052696~flavonoid glucuronidation	10	2,1551724138	365	34	10996	8,8605962933	1,09E-06	0,000121
GOTERM_BP_DIRECT	GO:0009813~flavonoid biosynthetic process	10	2,1551724138	365	34	10996	8,8605962933	1,09E-06	0,000121

50ug/mL

Category	Term	Count	%	List Total	Pop Hits	Pop Total	Fold Enrichment	PValue	Benjamini

GOTERM_BP_DIRECT GO:0055114~oxidation-reduction process 8 14,28571428571 39 412 10996 5,47473238735 0,000454 0,03542392232

REVIGO
100ug/mL
Biological Process

term_ID	description	frequency	plot_X	plot_size		log10 p-value	uniqueness		dispensability	representative	eliminated		
GO:0008152	metabolic process	75387%	-3081	6986	-51945	981	0	8152	0				
GO:0043161	proteasome-mediated ubiquitin-dependent protein catabolic process		226%	-5029	4462	-3000000		588	0	43161	0		
GO:0006511	ubiquitin-dependent protein catabolic process		584%	null	4874	-20518	575	902	43161	1			
GO:0042176	regulation of protein catabolic process		112%	null	4158	-18237	621	708	43161	1			
GO:0030163	protein catabolic process		997%	null	5107	-38827	610	721	43161	1			

Celular Component

term_ID	description	frequency	plot_X	plot_size		log10 p-value	uniqueness		dispensability	representative	eliminated		
GO:0000502	proteasome complex	389%	4098	4583	-3000000	227	0	502	0				
GO:0031597	cytosolic proteasome complex	84%	null	3916	-26183	257	875	502	1				
GO:0008540	proteasome regulatory particle, base subcomplex		32%	null	3505	-55884	267	930	502	1			
GO:0008541	proteasome regulatory particle, lid subcomplex		19%	null	3264	-22314	281	898	502	1			
GO:0005839	proteasome core complex		183%	null	4255	-76968	249	933	502	1			

Molecular Function

term_ID	description	frequency	plot_X	plot_size		log10 p-value	uniqueness		dispensability	representative	eliminated		
GO:0003824	catalytic activity	65827%	276	6967	-36799	955	0	3824	0				
GO:0004175	endopeptidase activity	1918%	3653	5431	-86778	616	0	4175	0				
GO:0004252	serine-type endopeptidase activity		808%	null	5056	-67595	602	732	4175	1			
GO:0005506	iron ion binding	1548%	5271	5338	-62487	844	0	5506	0				
GO:0016758	transferase activity, transferring hexosyl groups		898%	-3747	5102	-56271	684	33	16758	0			

Tabela Suplementar S4: Resultados das coordenadas cruzadas de genes e inserções de TEs com Genomic Ranges.

first.X.seqnames	first.X.start	first.X.end	first.X.width	first.X.strand	first.X.name	first.name	second.X.seqnames	second.X.start	second.X.end	second.X.width	second.X.strand	second.X.name	second.name	gene strand	te strand
1	2L	8011405	8026898	15494	*	gene_id \"FBgn0031975\"; gene_symbol \"Tg\";	gene_id \"FBgn0031975\"; gene_symbol \"Tg\";	2L	8013669	8018814	5146	*	FBti0019136	plus (+)	plus (+)
2	3R	9826081	9868120	42040	*	gene_id \"FBgn0037766\"; gene_symbol \"Teh1\";	gene_id \"FBgn0037766\"; gene_symbol \"Teh1\";	3R	9845570	9850598	5029	*	FBti0019348	minus (-)	minus (-)
3	2L	22189526	22214376	24851	*	gene_id \"FBgn0259683\"; gene_symbol \"Ir40a\";	gene_id \"FBgn0259683\"; gene_symbol \"Ir40a\";	2L	22201408	22206551	5144	*	FBti0019250	minus (-)	minus (-)

Tabela Suplementar S5: Resultados da predição de ORF do UGENE, Pfam e alinhamento Blastn para os quatro TEs up-regulados com maior comprimento e *bit score*.

UGENE

Trinity ID	TE name	Length	ORF start	ORF end	ORF DNA length
TRINITY_DN71_c1_g1_i8	flea	3696	1558	2853	1296
			2867	3634	768
TRINITY_DN40_c9_g1_i5	copia	1772	445	1770	1326
TRINITY_DN4603_c0_g1_i18	mdg3	1643	414	1643	1230
TRINITY_DN2232_c0_g1_i9	accord	1493	7	1491	1485

Pfam

Trinity ID	TE name	Frame (sense)	Family	Description	Entry type	Clan	Bit score	E-value
TRINITY_DN71_c1_g1_i8	flea	1 (+)	zf-CCHC	Zinc knuckle	Domain	CL0511	17.6	0,003
		1 (+)	zf-CCHC	Zinc knuckle	Domain	CL0511	20.9	0.00026
		1 (+)	RVP	Retroviral aspartyl protease	Domain	CL0129	22.3	0.00012
		2 (+)	RVT_1	Reverse transcriptase	Domain	CL0027	103.4	1.2e-29
		3 (+)	eIF-1a	Translation initiation factor 1A / IF-1	Domain	CL0021	74.2	5.3e-21
TRINITY_DN40_c9_g1_i5	copia	1 (+)	Retrotran_gag_2	gag-polypeptide of LTR copia-type	Domain	CL0523	107.3	4.7e-31
		1 (+)	zf-CCHC	Zinc knuckle	Domain	CL0511	20.5	0.00035
TRINITY_DN4603_c0_g1_i18	mdg3	3 (+)	zf-CCHC	Zinc knuckle	Domain	CL0511	23.0	5.9e-05
TRINITY_DN2232_c0_g1_i9	accord	1 (+)	RT_RNaseH	RNase H-like domain found in reverse transcriptase	Domain	CL0219	126.5	4.7e-37
		1 (+)	RVT_1	Reverse transcriptase	Domain	CL0027	42.4	5.7e-11
		1 (+)	Integrase_H2C2	Integrase zinc binding domain	Domain	CL0361	39.9	3.3e-10

Blastn

Trinity ID	TE name	Subject ID	Align. start	Align. end
TRINITY_DN71_c1_g1_i8	flea	flea#LTR/Gypsy	73	2515
TRINITY_DN40_c9_g1_i5	copia	copia#LTR/Copia	1	1733
TRINITY_DN4603_c0_g1_i18	mdg3	mdg3#LTR/Gypsy	119	1643
TRINITY_DN2232_c0_g1_i9	accord	accord#LTR/Gypsy	1	1440

O material suplementar completo está disponível em: https://drive.google.com/drive/folders/1teK9k_4AwswRQ6Zh7XVpvrkcXa57QpmK?usp=sharing.