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The model beetle *Tribolium castaneum* can be used as an early warning system for transgenerational epigenetic side effects caused by pharmaceuticals

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ABSTRACT

Pharmaceuticals are not currently tested for transgenerational and epigenetic side effects. The use of vertebrates as preclinical research models is limited by their long generation times, low numbers of progeny and ethical concerns. In contrast, invertebrates such as insects breed rapidly, produce many offspring and are more ethically acceptable, allowing them to be used for high-throughput screening. Here, we established *Tribolium castaneum* as a model to screen for the effect of drugs on complex fitness parameters and the expression of epigenetic regulatory genes. We tested diets supplemented with the psychoactive drug valproic acid (VPA), which is a histone deacetylase inhibitor, or the antioxidant curcumin, which is a histone acetyltransferase inhibitor. We found that VPA delayed development, reduced longevity, and increased female body weight compared to a control diet. Fertility and fecundity declined and the expression of epigenetic regulatory genes was induced in the untreated F1 generation. In contrast, curcumin did not affect development or body weight, but it increased longevity, caused a significant reduction in fertility, and induced the expression of epigenetic regulatory genes mostly in the treated F0 generation. VPA and curcumin administered to vertebrate models have similar effects to those we observed in *T. castaneum*, confirming that this beetle is potentially useful as an alternative model to screen for the epigenetic effect of drugs. *T. castaneum* also provides a valuable early warning system for transgenerational epigenetic risk factors that are difficult to detect in mammals.

1. Introduction

Environmental effectors such as nutrients and pharmaceuticals may trigger unanticipated epigenetic effects. They have the ability to regulate gene expression, leading to heritable changes in DNA structure mediated by DNA methyltransferases, histone acetyltransferases (HATs), histone deacetylases (HDACs), and other histone-modifying enzymes (Lötsch et al., 2013). Research in the field of drug development is therefore turning to the analysis of potential epigenetic side effects, particularly those that may be transferable to the next generation (Csoka and Szyf, 2009; Skinner et al., 2010). Commonly-prescribed drugs are now known to affect the etiology of many diseases including diabetes and cancer (Mukherjee et al., 2015), and it is becoming clear that such effects could be passed to the next generation via epigenetic mechanisms (Whitelaw and Whitelaw, 2008). Preclinical studies usually require tests on at least two non-human species, typically mice and larger mammals. However, vertebrate models have many limitations, including their long generation times, low fecundity and the expense of housing large numbers of animals. The use of vertebrates for drug research also raises ethical concerns, and alternatives to reduce, refine and replace vertebrates (3Rs) are urgently required (Wilson-Sanders, 2011). Insects such as the model beetle *Tribolium castaneum* have short generation intervals, high fecundity and can be reared inexpensively in large numbers. They can be used to determine transgenerational effects and for high-throughput drug screening assays with few ethical limitations (Knorr et al., 2013; Mukherjee et al., 2015; Schmitt-Engel et al., 2015; Ulrich et al., 2015).

We investigated the use of *T. castaneum*, a global pest of stored food products that has recently emerged as a model organism for functional genomics and nutrition, to screen pharmaceutical products for epigenetic risk factors (Denell, 2008; Grünwald et al., 2013a). *T. castaneum* has been established as a screening platform for gene–food interactions (Grünwald et al., 2013b; Grünwald et al., 2014), and as a model for high-throughput studies involving RNA interference (Knorr et al., 2013), and

Abbreviations: DPH, diphenhydramine; HAT, histone acetyltransferase; HDAC, histone deacetylase; KAT, lysine acetyltransferase; MBD, methyl-CpG binding domain protein; Mt, methyltransferase; Nap1, nucleosome assembly protein 1; Sap, Sin3A-associated protein; Sin3A, SIN3 transcription regulator family member A; Sir, sirtuin; SIRT, sirtuin; VPA, valproic acid.

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DNA methylation has been recently confirmed in *T. castaneum* embryos (Feliciello et al., 2013).

To evaluate the potential epigenetic side effects of drugs using this model insect, we tested one known HDAC inhibitor and one known HAT inhibitor. We chose valproic acid (VPA), a commonly-prescribed psychoactive drug indicated for the treatment of epilepsy, migraine and bipolar disorder (Lagace et al., 2004; Karragiannis et al., 2006), which exerts epigenetic side effects by inhibiting histone deacetylation (Göttlicher et al., 2001; Phiel et al., 2001; Rosenberg, 2007). VPA is associated with common side effects such as weight gain (Dinesen et al., 1984; Morrell et al., 2003; Ness-Abramof and Apovian, 2005; El-Khatib et al., 2007; Verrotti et al., 2011) and teratogenic effects in early pregnancy (Lindhout and Schmidt, 1986; Oakeshott and Hunt, 1989; Phiel et al., 2001; Gurvich et al., 2004; Kimford and Loring, 2013). We also selected curcumin, a powerful antioxidant and inhibitor of histone acetylation best known as the yellow pigment in turmeric (*Curcuma longa*) (Balasubramanyam et al., 2004). Curcumin has been used for many centuries as a food additive and in Asian medicine to treat wounds, inflammation and tumors (Sharma et al., 2005; Shen et al., 2013). It is now used in the clinic to treat patients with pancreatic cancer (Aggarwal et al., 2003; Dhillon et al., 2008; Fu and Kurzrock, 2010). Diets rich in antioxidants are associated with longevity (Suckow and Suckow, 2006) and increases in lifespan have been reported in nematodes, fruit flies and mice presented with such diets (Kitani et al., 2004; Kitani et al., 2007; Shen et al., 2013; Soh et al., 2013; Chandrashekara, et al., 2014). However, curcumin reduces fertility when administered intravaginally, probably reflecting its concentration-dependent negative impact on sperm motility (Naz, 2011). Curcumin does not appear to be toxic in humans and other mammals even at high doses (Aggarwal et al., 2003; Sharma et al., 2005; Ganiger et al., 2007).

Here we established a pre-screening system to test the effect of VPA and curcumin on different complex fitness parameters, i.e. the duration of development, longevity, body weight and reproduction. We also analyzed the expression of genes encoding epigenetic regulators in the F0 and F1 generations to investigate the potential transgenerational effects of these drugs.

2. Materials and methods

2.1. Insect maintenance

Wild-type *T. castaneum* strain San Bernardino was reared on wholegrain flour (basal medium) supplemented with 5% yeast powder at 32 °C and 70% relative humidity in darkness (Knorr et al., 2009).

2.2. Drug supplements

The F0 generation was reared on basal medium containing 0.1 or 1% (*w*/*w*) VPA (2-propylpentanoic acid) or curcumin (diferuloylmethane), or 1% (*w*/*w*) diphenhydramine (DPH) as a control. All three substances were obtained from Sigma-Aldrich (Taufkirchen, Germany). They were ground with the basal flour diet to form a fine powder, which was sieved to ensure homogenous distribution. The F1 generation was fed on the non-supplemented basal diet. The oral dose of VPA in humans is up to 10 mg/kg body weight with a maximum dose of 60 mg/kg per day. The 0.1% and 1% VPA 116 supplements in the insect diets were therefore equivalent to doses of 0.1 and 1 mg/kg body weight 117 in humans.

2.3. Fitness parameters

The duration of development was investigated by transferring freshly laid eggs (F0 generation) onto supplemented diets. Neonates were transferred to 48-well plates and development was monitored daily until the adult stage. Longevity was measured for all treatments in a thermotolerance assay under heat stress at 42 °C as described elsewhere (Grünwald et al., 2013b). Synchronized adult beetles were transferred in groups of 25 to six-well plates with fresh basal or supplemented diets, and deaths were recorded every 14 days or daily for the thermotolerance assay. Weight differences were measured in the F0 generation in a pool of 10 adults per sex. Beetles were frozen at − 18 °C for 24 h and were then dried at 60 °C for 24 h to measure the dry weight.

To monitor reproduction, 10 adults per sex and treatment were allowed to mate for one day, 5–7 days after eclosion, before transfer to a fresh Petri dish where they laid eggs on supplemented white flour for 1 day. The eggs were separated from the flour by sieving through a 300-μm mesh and were placed on the basal diet medium. Egg production (fertility) and hatched larvae (fecundity) were counted daily until day 6.

2.4. RNA isolation, reverse transcription and quantitative real-time PCR

Quantitative real-time PCR was carried out as previously described (Knorr et al., 2015). Briefly, RNA was isolated from shock-frozen animals (final-instar larvae, n = 15, three replicates) using Direct-zol RNA MiniPrep (Zymo Research, Irvine, California, USA). The quantity and quality of the RNA were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). We prepared cDNA from 500 ng RNA using oligo(dT) primers and the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Gene-specific primers (Table 1) were designed with Oligo Explorer v1.1.2 (Gene Link Inc., Hawthorne, New York, USA) and purchased from Sigma-Aldrich. The ribosomal protein gene *RPS3* was used as an endogenous control (Toutges et al., 2010). PCR was carried out on a StepOnePlus system using the Power SYBR Green PCR Master Mix with 50 ng of cDNA per reaction (Applied Biosystems, Waltham, Massachusetts, USA). Gene expression was calculated using the $2^{-\Delta\Delta CT}$ method described by Pfaffl (2001).

2.5. Statistics

Statistical analysis was carried out using SigmaPlot v11.0 (Systat Software Inc., San Jose, California, USA). Statistical differences between groups (parametric data) were determined by one-way analysis of variance (ANOVA) with a significance threshold of $p < 0.05$ and a subsequent Holm–Sidak test. When the normality test failed, ANOVA on-Ranks with a subsequent Tukey's test was used instead. The longevity data were analyzed using Kaplan–Meier survival curves and compared using a log-rank test with a significance threshold of $p < 0.05$. For each experiment, comparisons to an untreated control group and DPH as an additional control were included at the same time points. All experiments were performed independently at least three times.

3. Results

3.1. Effects of VPA and curcumin on T. castaneum development and survival

The effects of VPA and curcumin on the development and survival of *T. castaneum* were tested by rearing the insects on a diet containing 0.1% or 1% of each drug and comparing them with controls fed on a non-supplemented diet. In the case of VPA, these doses represented 100-fold and 10-fold lower doses per kg body weight than the standard therapeutic dose in humans. The antihistamine DPH was used as an additional control because no epigenetic side effects have been reported for this compound. Accordingly we observed no differences in development or survival between beetles fed on the non-supplemented diet and the control diet supplemented with 1% DPH. The 0.1% VPA diet also showed no signs of toxicity and had no impact on development, but 1% VPA prolonged the larval stage by up to 3.62 ± 0.28 days (Table S1, $p < 0.001$) and increased lethality during the larval stage by 35.56 ± 3.85% compared to the control diets (Table S2, $p < 0.001$). Curcumin did not have any impact on larval development or survival.