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Toxicity and metabolism of 3-bromopyruvate in *Caenorhabditis elegans*[*]

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Abstract: In this study, we aimed to evaluate the toxic effects, changes in life span, and expression of various metabolism-related genes in *Caenorhabditis elegans*, using RNA interference (RNAi) and mutant strains, after 3-bromopyruvate (3-BrPA) treatment. *C. elegans* was treated with various concentrations of 3-BrPA on nematode growth medium (NGM) plates, and their survival was monitored every 24 h. The expression of genes related to metabolism was measured by the real-time fluorescent quantitative polymerase chain reaction (qPCR). Nematode survival in the presence of 3-BrPA was also studied after silencing three hexokinase (HK) genes. The average life span of *C. elegans* cultured on NGM with 3-BrPA was shortened to 5.7 d compared with 7.7 d in the control group. *hxk-1*, *hxk-2*, and *hxk-3* were overexpressed after the treatment with 3-BrPA. After successfully interfering *hxk-1*, *hxk-2*, and *hxk-3*, the 50% lethal concentration (LC_{50}) of all mutant nematodes decreased with 3-BrPA treatment for 24 h compared with that of the control. All the *cyp35* genes tested were overexpressed, except *cyp-35B3*. The induction of *cyp-35A1* expression was most obvious. The LC_{50} values of the mutant strains *cyp-35A1*, *cyp-35A2*, *cyp-35A4*, *cyp-35B3*, and *cyp-35C1* were lower than that of the control. Thus, the toxicity of 3-BrPA is closely related to its effect on hexokinase metabolism in nematodes, and the *cyp-35* family plays a key role in the metabolism of 3-BrPA.

Key words: 3-Bromopyruvate; *Caenorhabditis elegans*; Hexokinase; Cytochrome P450
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1 Introduction

3-Bromopyruvate (3-BrPA) is an intermediate of the anthelmintic thiabendazole and is considered an inhibitor of glycolysis (Yadav et al., 2017b). It can combine with the active site of hexokinase (HK) II, the XH ($EXH+BrCH_2\text{-}CO\text{-}COOH \rightarrow EX\text{-}CH_2\text{-}CO\text{-}COOH+HBr$) base, and inhibit the activity of HK II during glycolysis (Kim et al., 2008). Under normal conditions, HK II is mainly expressed in the skeletal muscle, adipose and cardiac tissues, and it is not common in other tissues (Patra and Hay, 2013). HK II is highly expressed in tumor cells to enhance glycolysis (Chen GH et al., 2018; Dewaal et al., 2018). An anoxic microenvironment is easily formed in growing tumor tissues. Kim et al. (2007) demonstrated that

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increased mitochondrial stability is the main reason for hypoxia-induced overexpression of HK II. It has also been shown that the combination of HK II and the mitochondrial voltage-dependent anion channel protein inhibits membrane gap protein release and apoptosis and promotes tumor cell growth (Mycielska et al., 2012; Xu et al., 2017). 3-BrPA exhibits both tumoristatic and tumoricidal effects (el Sayed et al., 2017; Yadav et al., 2017a). It also possesses good antifungal properties (Dyląg et al., 2013).

Caenorhabditis elegans is a commonly studied model organism, especially in toxicology and pharmacology research, because approximately 83% of its protein sequences are homologous to those of humans (Lai et al., 2000; Qiao et al., 2014; Huang et al., 2017; García-Espiñeira et al., 2018). In this study, we aimed to evaluate the toxic effects of 3-BrPA and elucidate its metabolic pathway.

2 Materials and methods

2.1 Cultivation of *C. elegans*

C. elegans (N2 strain; *Caenorhabditis* Genetics Center, Saint Paul, MN, USA) was grown on a plate coated with *Escherichia coli* OP50 (*Caenorhabditis* Genetics Center) as nutrient at 20 °C. When the nematodes started to lay eggs, they were synchronized. The eggs hatched in the L4 stage.

2.2 Exposure of *C. elegans* to 3-BrPA

3-BrPA was added to the nematode growth medium (NGM) at 0, 0.7, 0.8, 0.9, and 1.0 mmol/L. Approximately 100 μL of *E. coli* OP50 suspension was plated on each plate and cultured overnight in an incubator at 37 °C. The nematodes (in L4 stage) were placed in each well containing the drug, and their survival was determined once daily. In another experiment, the nematodes (in L4 stage) were placed in Petri dishes and exposed to 0, 12.7, 25.4, and 50.8 mmol/L 3-BrPA, and the toxicity was observed. In an acute toxicity experiment, 3-BrPA was added to each well of a 96-well plate at 0, 2, 5, 7, 9, 11, 13, and 15 mmol/L in triplicates, and 15 L4 nematodes were placed in each well. After 24 h, the survival of nematodes was determined. Live adults were picked in a new NGM plate every day and counted until all the adults died; caution was exerted not to pick larvae, which could affect the experimental results. All experiments were repeated three times.

2.3 Construction of HK gene molecular phylogenetic tree

The FASTA sequence of HK genes was downloaded from the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov). The selected sequences included HK genes of *Homo sapiens*, *Mus musculus*, *Danio rerio*, *Xenopus laevis*, *Drosophila melanogaster*, *C. elegans*, and *Necator americanus*. An evolutionary tree was constructed using MEGA 5.05 software (https://www.megasoftware.net).

2.4 Total RNA extraction and reverse transcription of *C. elegans*

The synchronized nematodes (approximately 500) were homogenized in RNase-free water, centrifuged at 1788*g* for 2 min, and then the supernatant was discarded. After repeated freeze–thaw cycles, the pellet was centrifuged at 13545*g* for 5 min at 4 °C. Chloroform was added, and the solution was emulsified and centrifuged at 13545*g* for 15 min at 4 °C. Precooled isopropanol was added to the solution and mixed. Subsequently, the mixture was allowed to stand for 10 min and centrifuged at 13545*g* for 15 min at 4 °C. After aspirating the supernatant, precooled (4 °C) 75% ethanol was added and centrifuged at 13545*g* for 5 min at 4 °C. After discarding the supernatant and allowing the RNA extracts to dry naturally at 20 °C, the total RNA was extracted and reverse-transcribed using a reverse transcription kit (TaKaRa Bio Inc., Japan).

2.5 Determination of *hxk-1*, *hxk-2*, and *hxk-3* mRNA expression

The mRNA expression of nematode HK genes *hxk-1*, *hxk-2*, and *hxk-3* was determined using *act-1* as the internal control gene. The results of primer 5.0 design are shown in Table 1. According to the 50% lethal concentration (LC_{50}) of 3-BrPA in *C. elegans*, four concentration gradients—0, 1/10, 1/100, and 1/1000 of the lethal concentration—were set. The nematode RNA was extracted according to the procedure described earlier. The volume of the real-time fluorescent quantitative polymerase chain reaction (qPCR) system was set to 25 μL, with three sets of spacers per SYBR Premix Ex Tap II (TaKaRa Bio Inc., Japan).

2.6 Silencing of the target genes *hxk-1*, *hxk-2*, and *hxk-3*

2.6.1 Cloning the target genes

The primers for *hxk-1*, *hxk-2*, and *hxk-3* (upstream and downstream primer sequences for the *Xba*I and *Hin*dIII restriction sites; Table 2) were designed. The PCR products were subjected to polyacrylamide gel electrophoresis (120 V, 100 mA, 30 min). The target gene fragment was purified using the TaKaRa fragment purification kit (TaKaRa Bio Inc., Japan).

Single colonies of DH5α were inoculated in 5 mL of Luria-Bertani (LB) liquid medium and cultured overnight in a shaking incubator. The medium was aspirated and centrifuged at 300*g* for 10 min at 4 °C, and then the supernatant was discarded. Precooled $CaCl_2$-$MgCl_2$ (5 mL) was added, and the cells were resuspended and centrifuged at 1505*g* for 10 min at 4 °C. The cells were recovered and the above steps were repeated. Precooled $CaCl_2$ (0.5 mL) was added to 50 mL of the initial bacterial broth to prepare competent cells. Subsequently, 5 μL of ligation solution was added and incubated with the competent cells in ice-water mixture for 30 min. The tube was transferred to a water bath at 42 °C and allowed to stand for 1.5 min, and then transferred to the ice-water mixture and allowed to stand for 1.5 min. After the addition of 800 μL of start of cell to each tube, the tubes were allowed to stand at 37 °C for 1.5 min, and then transferred to a 37 °C incubator and incubated at 2351*g* for 46 min. The solution was then centrifuged at 2351*g* for 2 min. The supernatant was discarded, and 300 μL of the medium was removed and applied onto an LB solid plate supplemented with adenosine monophosphate (AMP) and cultured in a 37 °C incubator overnight. Five single colonies were placed in five tubes containing 5 mL of LB liquid medium supplemented with AMP and incubated overnight at 37 °C.

The cultured cells were transferred to a sterile Gene Era Biotech (GEB) microcentrifuge tube and centrifuged at 13545*g* for 2 min at room temperature. To the supernatant, 250 μL of Solution I and 250 μL of Solution II were added. After mixing, 350 μL of Solution III (precooled in a 4 °C refrigerator) was added and the solution was allowed to stand at room temperature for 2 min. After centrifugation at 13545*g* for 10 min at room temperature, the supernatant was collected and centrifuged at 13545*g* for 1 min at room temperature, and the supernatant was discarded. Subsequently, 500 μL of Buffer WA (washing buffer A) was added and centrifuged at 13545*g* for 30 s at room temperature, and then the supernatant was discarded. To the precipitate, 700 mg of Buffer WB (washing buffer B; in 56 mL of 100% ethanol) was added and centrifuged again at 13545*g* for 30 s at room temperature, and then the supernatant was discarded. The upper layer was transferred into a new sterilized Eppendorf tube and 30 μL of distilled water was added to the middle layer. After standing at room temperature for 1 min, the DNA was eluted by centrifugation at 13545*g* for 1 min at room temperature. The obtained plasmids were PCR-amplified. The electrophoresis-positive plasmid was sequenced for identification.

2.6.2 Expression of the target genes

According to the above steps, the plasmid in HT115-L4440 bacteria was isolated and double digested

Table 1 Primer sequences of *hxk-1*, *hxk-2*, and *hxk-3* for the qPCR

Gene	WormBase ID	Forward primer (5'→3')	Reverse primer (5'→3')
act-1	T04C12.6	CGCTTCTTCCTCTTCCCTC	TGATCGTATGCAGAAGGAAA
hxk-1	F14B4.2	GGAGAGTGTGCCCGAGTTGT	ATGCTCTCTGAAGATGGATCTGG
hxk-2	H25P06.1	GCTCTTTAATGGAATTGGCTCG	CGCAATCGTTTCGAGAGTCA
hxk-3	Y77E11A.1	CAAAGCAGTGATGAACGACACA	GACACAATTTGATCGGGAAGTCG

Table 2 Primer sequences of *hxk-1*, *hxk-2*, and *hxk-3* for the PCR

Gene	WormBase ID	Forward primer (5'→3')	Reverse primer (5'→3')
hxk-1	F14B4.2	GCTCTAGATTCACTTTCTCGTTTCCA	CCCAAGCTTCGAGTTTCCACCG
hxk-2	H25P06.1	CCTCTAGATCTCTCGACAAGCT	CCCAAGCTTCAGATCCTTCAGC
hxk-3	Y77E11A.1	GCTCTAGACAACTTCAAGAAATCAG	CCCAAGCTTGTGCATTCTG

(*Hin*dIII and *Xba*I). The digested solution was subjected to agarose gel electrophoresis to recover the target fragment. After cutting the gel and adding three volumes of Buffer GM (gel melting buffer), the thawed mixture was transferred into a collection tube and centrifuged at 13 545*g* for 1 min at room temperature. To the above collection tube, 700 μL of Buffer WB was added and filtered, and the filtrate was centrifuged at 13 545*g* for 30 s at room temperature. This procedure was repeated and the sample was centrifuged at 13 545*g* for 1 min at room temperature to collect the DNA fragments. The ligation solution was prepared from the DNA fragment using a reaction system of 25 μL (L4440 plasmid, approximately 0.03 pmol; target fragment, approximately 0.3 pmol; T4 DNA ligase, 1 μL; 5× T4 DNA ligase buffer, 2.5 μL; sterilized water, up to 25 μL) at 16 °C overnight. *E. coli* DH5α was prepared using the $CaCl_2$ method. The positive colony plasmid was extracted and the cloning results were analyzed.

2.6.3 Target gene silencing

HT115-positive bacteria were cultured in LB liquid (containing 100 μg/mL of ampicillin, 15 μg/mL of tetracycline, and 100 μmol/L of isopropyl-β-D-thiogalactopyranoside (IPTG)) at 37 °C for 2 h. The bacterial supernatant was centrifuged at 1581*g* for 10 min at room temperature. The bacterial suspension (100 μL) was applied on LB solid agar (ditto) and cultured at 37 °C overnight. Five L4 stage nematodes were placed in the LB plate and cultured at 20 °C until they laid eggs. After 24 h of culture, the adult worms were transferred onto a second plate for 24 h where they laid eggs, and then removed. After culturing in the LB plate for 24 h, a large number of interfering genes from the adult worms were obtained. The total RNA was extracted and reverse-transcribed, and then subjected to the qPCR to verify the interference efficiency.

2.6.4 Design of CYP35 family primers and determination of expression of *cyp35* genes

The primers for *cyp-35A1*, *cyp-35A2*, *cyp-35A4*, *cyp-35A5*, *cyp-35B3*, and *cyp-35C1* of the CYP35 family were designed (Table 3). The median concentrations, 1/10, 1/100, and 1/1000 of LC_{50} of 3-BrPA, were established to extract the total RNA from the nematodes, which was then reverse-transcribed. The qPCR system was set to 25 μL.

2.7 Action of 3-BrPA on RNAi or mutant nematode strains

The RNA interference (RNAi) *hxk-1*, *hxk-2*, and *hxk-3* gene strains and mutant nematode *cyp-35A1*, *cyp-35A2*, *cyp-35A4*, *cyp-35A5*, *cyp-35B3*, and *cyp-35C1* gene strains (from the *Caenorhabditis* Genetics Center) were treated with 3-BrPA and counted when they grew to the L4 stage (see Section 2.2). The LD_{50} was calculated using SPSS 19.0 software (SPSS Inc., Chicago, Illinois, USA) after exposing the nematodes to 3-BrPA for 24 h.

2.8 Data analysis

The median lethal concentration of 3-BrPA for nematodes was calculated using SPSS 19.0 software. The effects of 3-BrPA on the longevity of nematodes and the expression of metabolism-related genes were analyzed using GraphPad Prism 5 (GraphPad Software, Inc., California, USA). The difference was considered statistically significant when the *P* value was <0.05.

Table 3 Primer sequences of *cyp-35* for the qPCR

Gene	WormBase ID	Forward primer (5'→3')	Reverse primer (5'→3')
cyp-35A1	C03G6.14	ATTGTGTCCACGCTTGATCTGT	CACACGTTCAGAGATATGGGAG
cyp-35A2	C03G6.15	GCATCCATTCTTAACGTCAGCTTC	ATCTTCGGTAACCTTCTCCTTCG
cyp-35A4	C49G7.8	ACCAAATCAAGTCTGGGAGGTA	TCTTACTGACCGTGCTTCAACTC
cyp-35A5	K07C6.5	CATCTTCACCTTGTGGGTTGG	TTAGAAATATGGGCTTCGGGAAGG
cyp-35B3	K07C6.2	GTGATTATGAAACGTCGCAAGAAG	GCGGATGCTGTAAATGGAAAGAC
cyp-35C1	C06B3.3	AAAGTGACTAACGGAGGATCTCG	CTAGCAAGAGCCGAGCTGTATTT

3 Results

3.1 Effect of 3-BrPA on the life span of nematodes

The control group exhibited a normal life span. The activity of nematodes slowed down when treated with 3-BrPA at 2.7 mmol/L. When treated with 25.4 and 50.8 mmol/L 3-BrPA, the nematodes became rigid and many died (Fig. 1).

The average life span of *C. elegans* in the control group was 7.7 d, and all the nematodes died on Day 15 (P<0.05; Fig. 2). Compared with that of the control group, in the 0.7 mmol/L 3-BrPA group, the survival rate of the nematodes was only 30% and the average life span was 7.5 d (P<0.05; Fig. 2a). In the 0.8 mmol/L 3-BrPA group, the survival rate of the nematodes was 0 and the average life span was 6.3 d (P<0.05; Fig. 2b). In the 0.9 mmol/L 3-BrPA group, the survival rate of nematodes was 0 and the average life span was 6.1 d (P<0.05; Fig. 2c). In the 1.0 mmol/L 3-BrPA group, the average life span of nematodes was 5.7 d (P<0.05; Fig. 2d). Except for 0.7 mmol/L 3-BrPA group, the average age of other 3-BrPA groups was significantly lower than that of the control group (P<0.05; Fig. 2e).

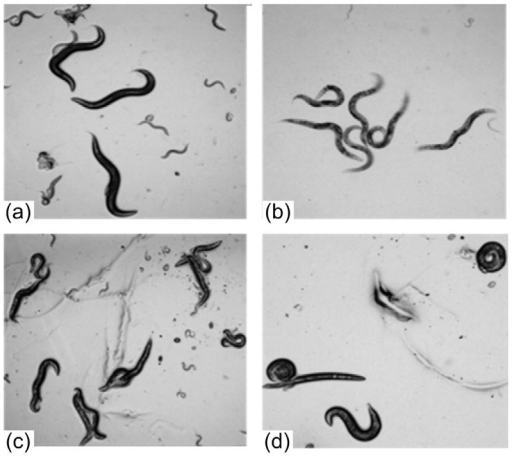

Fig. 1 Effect of 3-bromopyruvate (3-BrPA) on *C. elegans*
(a) Control; (b) 2.7 mmol/L 3-BrPA; (c) 25.4 mmol/L 3-BrPA; (d) 50.8 mmol/L 3-BrPA

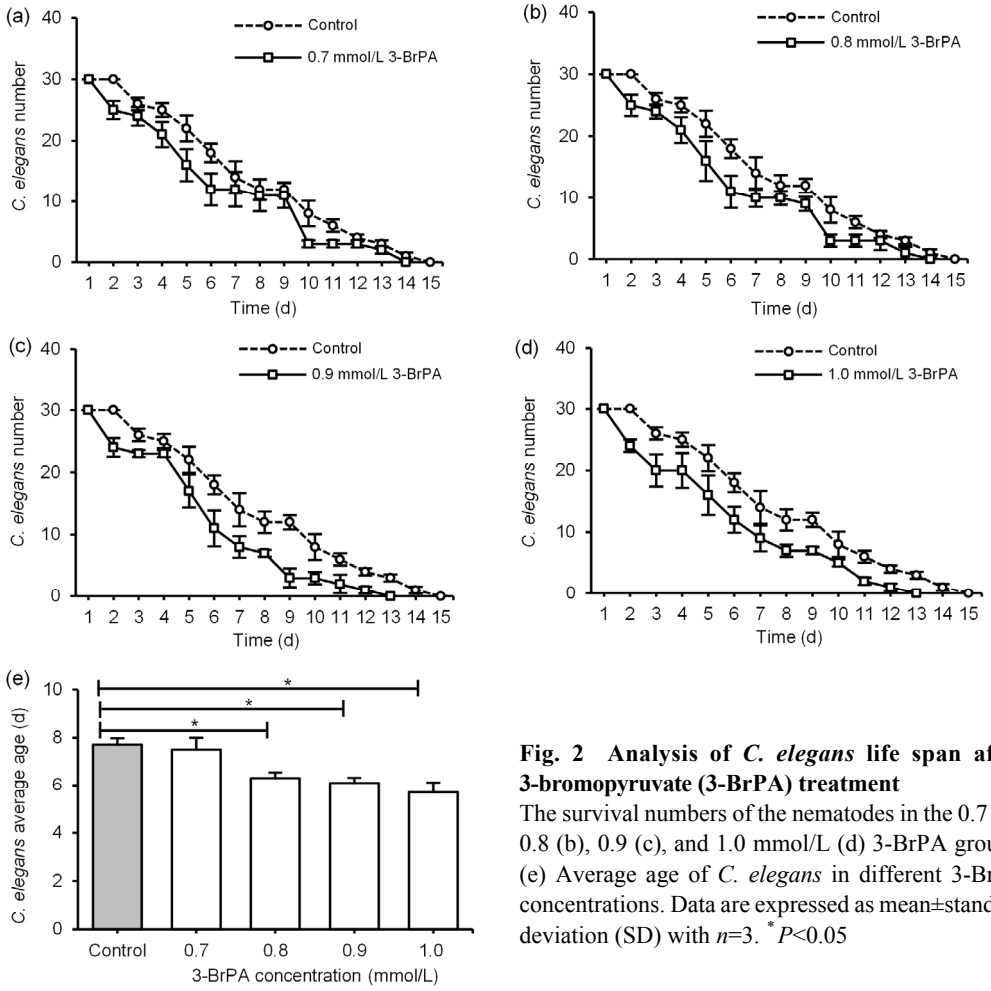

Fig. 2 Analysis of *C. elegans* life span after 3-bromopyruvate (3-BrPA) treatment
The survival numbers of the nematodes in the 0.7 (a), 0.8 (b), 0.9 (c), and 1.0 mmol/L (d) 3-BrPA groups. (e) Average age of *C. elegans* in different 3-BrPA concentrations. Data are expressed as mean±standard deviation (SD) with n=3. $^*P$<0.05

3.2 Evolutionary genetic comparison of the HK genes

The phylogenetic tree in Fig. 3 reveals that the *hxk-1* genes of *C. elegans* and *N. americanus* belonged to a branch with high genetic similarity; they belonged to the branch of *D. melanogaster* HK genes. The *hxk-2* and *hxk-3* genes of *C. elegans* presented higher approximate degree and lower genetic approximation to *hxk-1*, whereas those of humans and domestic mice have a higher degree of approximation to *HK II*. The *hxk-2* and *hxk-3* genes of *C. elegans* might be closely related to *HK II* in mammals, whereas *hxk-1* might be distant from *HK II* in mammals. The average evolutionary distance between the HK gene of *C. elegans* and that of other species was 0.6811 (Table 4).

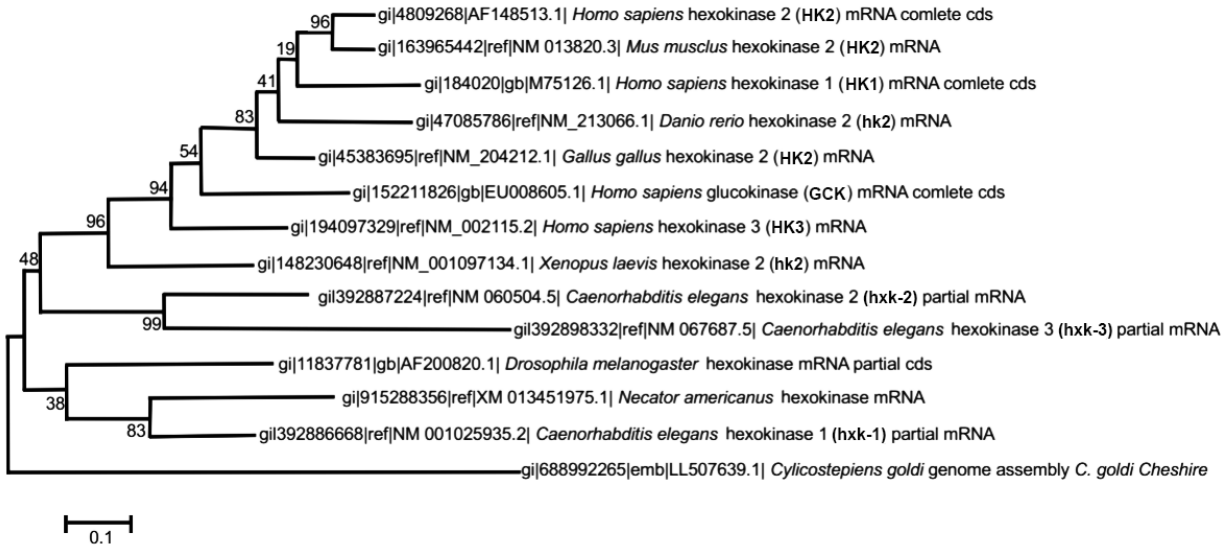

Fig. 3 Phylogenetic analysis of HK in *C. elegans*

Table 4 Pairwise comparison of the phylogenetic analysis of HK in *C. elegans*

No.	Genus	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	gi|915288356|ref|XM 013451975.1| *Necator americanus* hexokinase														
2	gi392886668|ref|NM 001025935.2| *Caenorhabditis elegans* hexokinase 1	0.472													
3	gi392887224|ref|NM 060504.5| *C. elegans* hexokinase 2	0.821	0.697												
4	gi392898332|ref|NM 067687.5| *C. elegans* hexokinase 3	1.056	0.850	0.767											
5	gi|688992265|emb|LL507639.1| *Cylicostepiens goldi* genome	1.127	1.087	1.351	1.454										
6	gi|4809268|gb|AF148513.1| *Homo sapiens* hexokinase 2	0.672	0.606	0.670	1.013	1.077									
7	gi|1840208|gb|M775126.1| *H. sapiens* hexokinase 1	0.660	0.545	0.669	0.964	1.098	0.254								
8	gi|194097329|ref|NM_002115.2| *H. sapiens* hexokinase 3	0.726	0.674	0.736	1.033	1.165	0.337	0.404							
9	gi|152211826|gb|EU008605.1| *H. sapiens* hexokinase	0.703	0.640	0.753	1.011	1.166	0.398	0.435	0.411						
10	gi|163965442|ref|NM_013820.3| *Mus musculus* hexokinase 2	0.648	0.607	0.663	1.022	1.067	0.107	0.256	0.358	0.384					
11	gi|47085786|ref|NM_213066.1| *Danio rerio* hexokinase 2	0.662	0.631	0.740	0.938	1.213	0.262	0.327	0.453	0.421	0.251				
12	gi|45383695|ref|NM_204212.1| *Gallus gallus* hexokinase 2	0.665	0.627	0.707	0.943	1.119	0.188	0.256	0.341	0.326	0.198	0.268			
13	gi|148230648|ref|NM_001097134.1| *Xenopus laevis* hexokinase 2	0.664	0.607	0.679	0.955	1.187	0.478	0.427	0.489	0.524	0.447	0.465	0.474		
14	gi|11837781|gb|AF2008201| *Drosophila melanogaster* hexokinase	0.700	0.586	0.738	1.085	1.231	0.619	0.634	0.635	0.581	0.618	0.671	0.636	0.667	

3.3 mRNA expression of the HK genes in *C. elegans* treated with 3-BrPA

The expression of the HK genes in the control group was set to 1 (Fig. 4); the mRNA expression of the objective gene with $\Delta C_T>1$ was considered upregulated and that with $\Delta C_T<1$ was considered downregulated. It can be seen from Fig. 4 that the mRNA expression of *hxk-1*, *hxk-2*, and *hxk-3* was upregulated. The mRNA expression of *hxk-1* and *hxk-3* was concentration-dependent, and the expression of *hxk-2* exhibited the highest change at 0.0127 mmol/L 3-BrPA.

3.4 Effect of 3-BrPA on the HK genes in RNAi nematodes

Fig. 5 shows the effects of silencing the *hxk-1*, *hxk-2*, and *hxk-3* genes, determined using the qPCR. The interference effect of *hxk-2* was better than those of *hxk-1* and *hxk-3* after silencing the *hxk*-1, *hxk-2*, and *hxk-3* genes ($P<0.01$).

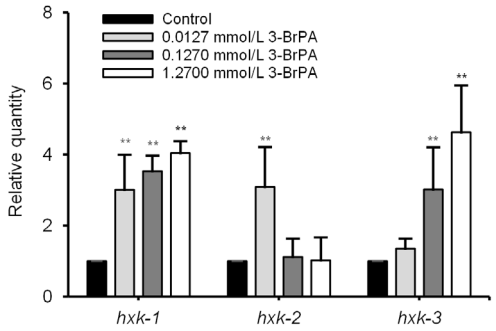

Fig. 4 Relative mRNA expression of *hxk-1*, *hxk-2*, and *hxk-3* after 3-bromopyruvate (3-BrPA) treatment
The expression of the HK genes in the control group was set to 1. Data are expressed as mean±standard deviation (SD), with n=3. $^{**}P<0.01$ vs. control

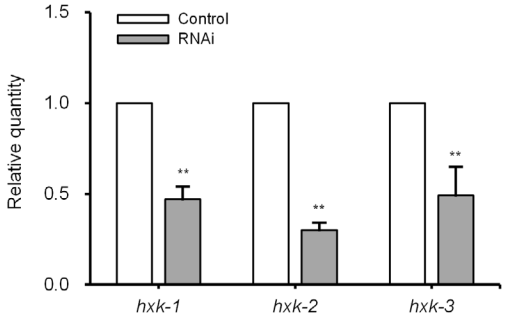

Fig. 5 Relative mRNA expression after *hxk-1*, *hxk-2*, and *hxk-3* gene interference
The expression of the HK genes in the control group was set to 1. Data are expressed as mean±standard deviation (SD), with n=3. $^{**}P<0.01$ vs. control

The LD_{50} was calculated using SPSS 19.0 software after exposing the *hxk* gene RNAi nematode to 3-BrPA for 24 h. Compared with that of the control group, the LC_{50} of nematodes decreased after silencing the *hxk-1*, *hxk-2*, and *hxk-3* genes ($P<0.05$; Table 5).

Table 5 3-Bromopyruvate (3-BrPA) LC_{50} of the HK gene RNAi nematodes

Gene	Strain	24 h LC_{50} (mmol/L)	95% CI (mmol/L)
hxk-1	N2	7.10**	6.37–7.98
hxk-2	N2	9.10**	8.29–9.99
hxk-3	N2	10.10*	9.74–10.87
Control	N2	12.70	4.58–26.38

* $P<0.05$, ** $P<0.01$ vs. control. LC_{50}: 50% lethal concentration; CI: confidence interval

3.5 Effect of 3-BrPA on the expression of the *cyp-35* gene in mutant strains

Fig. 6 shows the results of qPCR of the *cyp-35A1*, *cyp-35A2*, *cyp-35A4*, *cyp-35A5*, *cyp-35B3*, and *cyp-35C1* gene mutant strains; the mRNA expression was set as 1 in the control group. The expression of the target gene with $-\Delta\Delta C_T>1$ was considered upregulated and that with $-\Delta\Delta C_T<1$ was considered downregulated. The upregulation of *cyp-35A1* mRNA expression was the most obvious with 1.2700 mmol/L 3-BrPA, and it was concentration-dependent (Fig. 6a). When the concentration of 3-BrPA was increased from 0.0127 to 0.1270 mmol/L, the increase in the mRNA expression of *cyp-35A1*, *cyp-35A2*, and *cyp-35A4* was obvious; however, the increase in *cyp-35A5* mRNA expression was relatively moderate. Fig. 6b shows that the downregulation of *cyp-35B3* mRNA expression and upregulation of *cyp-35C1* mRNA expression were concentration-dependent.

3.6 LC_{50} of 3-BrPA on the *cyp-35* gene mutant strains

The LD_{50} was calculated using SPSS 19.0 software after exposing the *cyp-35* gene mutant strains to 3-BrPA for 24 h. Compared with that of the control group, the LC_{50} of the *cyp-35A1*, *cyp-35A2*, *cyp-35A4*, *cyp-35A5*, *cyp-35B3*, and *cyp-35C1* gene mutant strains decreased ($P<0.05$; Table 6).

Table 6 3-Bromopyruvate (3-BrPA) LC_{50} of the *cyp-35* gene mutant strains

Gene	Strain	Variation type	Variation genomic position	24 h LC_{50} (mol/L)	95% CI (mol/L)
cyp-35A1 (*ok1414*)	VC875	Allele: insertion/deletion	V:7359918..7361311	2.30**	0.15–4.75
cyp-35A2 (*gk317*)	VC710	Allele: deletion	V:7362185..7363429	4.88**	3.11–6.66
cyp-35A4 (*ok1393*)	RB1294	Allele	Unknown	9.69**	7.90–19.83
cyp-35A5 (*ok1985*)	KO7C6.2	Allele: deletion	V:3937831..3938637	5.26**	4.27–6.85
cyp-35B3 (*gk663902*)	K07C6.2	Allele: substitution	V:3945462..3945462	3.10**	1.27–4.30
cyp-35C1 (*ok2628*)	RB1993	Allele	Unknown	4.67**	3.22–5.68
Control	N2			12.70	4.58–26.38

** $P<0.01$, vs. control. LC_{50}: 50% lethal concentration; CI: confidence interval

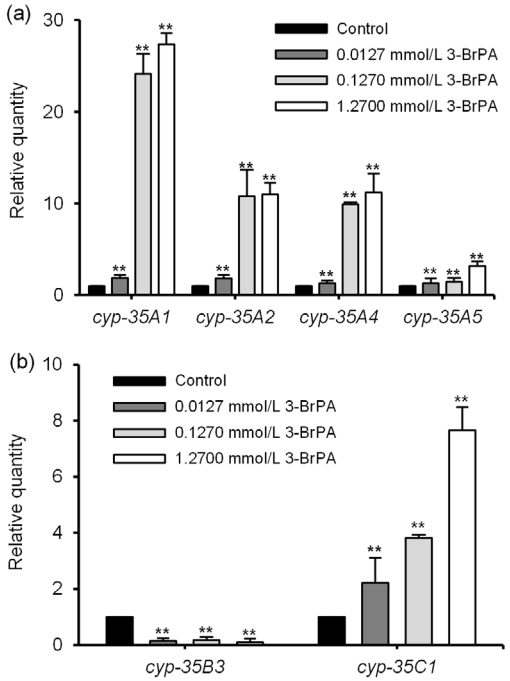

Fig. 6 Effect of 3-bromopyruvate (3-BrPA) on the mRNA expression of *cyp-35* genes

(a) *cyp-35A1*, *cyp-35A2*, *cyp-35A4*, and *cyp-35A5*; (b) *cyp-35B3* and *cyp-35C1*. The mRNA expression was set as 1 in the control group. Data are expressed as mean±standard deviation (SD), with n=3. ** $P<0.01$ vs. control

4 Discussion

HK is an enzyme that phosphorylates hexoses (six-carbon sugars), forming hexose phosphate. It is the first rate-limiting enzyme in glycolysis, which catalyzes almost all hexasaccharides (Cárdenas et al., 1998). It has been proved that 3-BrPA can bind to the active site of HK and inhibit its activity during glycolysis (Chen FZ et al., 2018). To the best of our knowledge, the present study is the first to use *C. elegans* to study 3-BrPA toxicity. The results showed that three different concentrations of 3-BrPA shortened the life span of nematodes. In the acute toxicity test for 24 h, the LD_{50} of 3-BrPA was 12.7 mmol/L, which indicated that 3-BrPA at concentrations higher than this can kill nematodes, and that at concentrations lower than this can effectively inhibit the growth and development of *C. elegans*.

HK was first identified in yeast, where it transferred an inorganic phosphate group from adenosine triphosphate (ATP) to the substrate (Slein et al., 1950). There are four important HK isozymes in mammals, which are designated as HK I, II, III, and IV (Wilson, 2003). In this study, through the comparison of genetic evolutionary tree, the genes *hxk-2* and *hxk-3* of *C. elegans* were found to be highly related to human *HK2*. As *hxk-1* did not belong to the same branch as *HK2* and *HK3* of *C. elegans*, and the genetic and evolutionary similarities were low, we speculated that HK encoded by *HK1* was different from that encoded by the *HK2* and *HK3* genes.

HK can catalyze almost all hexasaccharides and produce energy through glycolysis (Wilson, 2003). In normal tissues, its activity is limited by low energy demand and the level of its metabolite glucose-6-phosphate (de Meis et al., 1992). However, its activity is not restricted in tumor cells (Alcántar-Aguirre et al., 2013; Olsen et al., 2019). HK II is closely associated with carcinogenesis. Therefore, when tumor cells need more energy, the expression of HK II is high (Patra et al., 2013). In this study, we found that the expression of *hxk-1*, *hxk-2*, and *hxk-3* in *C. elegans* exposed to 3-BrPA was significantly higher than that in the control group, and the expression of *hxk-2* increased significantly. The results showed that 3-BrPA does not inhibit the expression of the HK genes, but

inhibits the activity of HK by interacting with the protein. With decrease in the HK level, the production of ATP decreased; however, the HK pathway feedback increased HK gene expression, resulting in a further increase in the HK level. These results suggest that 3-BrPA mainly inhibits the activity of HK I and HK III. Furthermore, the high expression of the HK genes might be due to the consumption of a large amount of ATP during drug metabolism.

The cytochromes P450 (CYP) are a heme-containing superfamily of proteins. They are the major enzymes involved in drug metabolism, accounting for approximately 75% of the total metabolism (Guengerich, 2008). Menzel et al. (2001) used 18 human CYP inducers to induce *cyp* gene expression in nematodes and found that naphthalene flavones and lansoprazole induced CYP35 expression, revealing that the CYP35 family is closely related to drug metabolism. Roh and Choi (2011) found that nematodes with the RNAi gene *cyp-35A2* exhibited a low metabolism of thiophosphate. In this study, 1.2700 mmol/L 3-BrPA upregulated the expression of *cyp-35A1*>*cyp-35A4*>*cyp-35A2*>*cyp-35C1*>*cyp-35A5* in the nematodes, and when these genes were mutated, the LD_{50} of 3-BrPA was lower than that of the control. The results elucidate the drug metabolism and detoxification effect of CYP.

5 Conclusions

This study showed that 3-BrPA at a certain concentration shortens the average life span of nematodes. The toxicity target of 3-BrPA is closely related to its effect on hexokinase metabolism of *C. elegans*. The *cyp*-35 family plays a key role in the metabolism of 3-BrPA in nematodes. These genes can act as a reference for further studies on the toxicity of 3-BrPA and metabolism of drugs.

Contributors

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Compliance with ethics guidelines

Qiao-ling GU, Yan ZHANG, Xi-mei FU, Zhao-lian LU, Yao YU, Gen CHEN, Rong MA, Wei KOU, and Yong-mei LAN declared that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题　目：3-溴丙酮酸对秀丽隐杆线虫的毒性和代谢的影响

目　的：通过 3-溴丙酮酸（3-BrPA）处理秀丽隐杆线虫（*Caenorhabditis elegans*），观察 3-BrPA 对线虫的毒性和生存周期的影响。通过秀丽隐杆线虫 RNA 干扰（RNAi）和突变株，分析 3-BrPA 对线虫糖酵解途径己糖激酶家族和药物代谢的关键酶细胞色素 P450（cytochrome P450，CYP）家族的影响。

创新点：首次报道了 3-BrPA 对秀丽隐杆线虫有毒性作用，己糖激酶是 3-BrPA 对线虫作用的重要靶点，而 CYP-35A 家族是线虫代谢 3-BrPA 的主要酶类。

方　法：用不同浓度的 3-BrPA 处理秀丽隐杆线虫，每 24 h 监测一次存活率；用实时荧光定量聚合酶链反应（qPCR）检测代谢相关基因的表达；通过 RNAi 沉默己糖激酶家族基因 *hxk-1*、*hxk-2* 和 *hxk-3*；计算 3-BrPA 处理 *hxk* 家族 RNAi 株和细胞色素 P450 *cyp-35* 家族突变株后的致死中浓度（LC_{50}）。

结　论：3-BrPA 对线虫有明显的毒性效应（图 1）；与对照组比较，3-BrPA 处理组的线虫平均寿命明显缩短（图 2）；3-BrPA 处理线虫后 *hxk-1*、*hxk-2* 和 *hxk-3* 的信使 RNA（mRNA）表达明显升高（$P<0.05$，图 5）；3-BrPA 处理 *hxk* RNAi 株后的 LC_{50} 均减小（$P<0.05$，表 5）；3-BrPA 处理 *cyp-35* 突变株后的 LC_{50} 也均减小（$P<0.05$，表 6）。综上所述，3-BrPA 的毒性与其对秀丽隐杆线虫己糖激酶代谢的影响密切相关；CYP-35 家族在线虫中对 3-BrPA 代谢中起着关键作用。

关键词：3-溴丙酮酸；秀丽隐杆线虫；己糖激酶；细胞色素 P450