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The effect of novel aromatic heterocycle substituted aminamidine derivatives on *Necator americanus*

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Background: The efficacy of current drugs against hookworms at a single dose is highly variable across regions, age groups and infection intensity. Extensive and repeated use of these drugs also leads to potential drug resistance. Therefore, novel drugs are required for sustained disease control.

Objectives: Novel aromatic heterocycle substituted aminamidine derivatives (AADs) were synthesized based on tribendimine (TBD), and their *in vivo* potency against *Necator americanus* was tested.

Methods: The efficacy of the AADs was tested in male hamsters. Oral and IV pharmacokinetic parameters were determined in male Sprague-Dawley rats. The proteomic profiles of *N. americanus* samples treated with AADs were compared using tandem mass tag-based quantitative proteomic analyses.

Results: Most AADs exhibited better anthelmintic activity than TBD at a single oral dose. Compound 3c exhibited improved solubility (>50×), and the curative dose was as low as 25 mg/kg. Similar to TBD, 3c was rapidly metabolized after oral administration and transformed into *p*-(1-dimethylamino ethylimino)aniline (dADT), an active metabolite against intestinal nematodes. dADT from 3c had better pharmacokinetic profiles than that from TBD and achieved an oral bioavailability of 99.5%. Compound 3c possessed rapid anthelmintic activity, clearing all worms within 24 h after an oral dose of 50 mg/kg. Quantitative proteomic analysis indicated that it might be related to ATP metabolism and cuticle protein synthesis.

Conclusions: Compound 3c is a novel and promising compound against *N. americanus in vivo*.

Introduction

Soil-transmitted helminthiases (STHs) are important neglected tropical diseases and are mainly caused by roundworms (*Ascaris lumbricoides*), hookworms (*Necator americanus* and *Ancylostoma duodenale*) and whipworms (*Trichuris trichiura*).[1] Hookworms infect over 400 million people and cause a disease burden of 4 million disability-adjusted life years.[2] Of the two major hookworm species that cause human infection, *N. americanus* accounts for the majority of all hookworm infections and is commonly distributed in southern China, Southeast Asia, the Americas and most of Africa.[2] Hookworms are parasitic in the small intestine of their hosts, causing iron deficiency anaemia, malnutrition in pregnant women, and an impairment of cognitive and/or physical development in children.[2,3]

The drugs most commonly used for the treatment of STHs are albendazole and mebendazole.[4] The regular treatment and preventive chemotherapy of high-risk groups is by means of mass drug administration, and single-dose anthelmintics are preferred for cost-effectiveness and convenience.[5] However, the therapeutic efficacy of albendazole and mebendazole varies among different worm species. Both are effective on *Ascaris* but not on *Trichuris* in a single dose.[6] For hookworms, a single dose of albendazole achieves higher efficacy than mebendazole,[6] but some references also report low efficacy of albendazole.[7,8]

Although hookworm infections can be adequately controlled through drug chemotherapy, for individuals inhabiting endemic areas, infections readily recur and may last a lifetime.[9] Meanwhile, large-scale and prolonged use of current drugs increases the risk of drug resistance.[10] On the other hand, after a long period of deworming treatment, *A. duodenale* is more sensitive, and *N. americanus* has become the dominant species among the two.[11] More selective drugs are needed to achieve better disease control.

Despite intensive research on new anthelmintics for STHs, only a few are clinically used. Amidantel is an aminophenylamidine compound approved in veterinary medicine in 1979.[12] It is efficient against *A. lumbricoides* and *A. duodenale*, but not against *N. americanus*.[13] Tribendimidine, a derivative of amidantel, was developed by the Chinese National Institute of Parasitic Diseases in the 1980s.[14] It exhibited high potency against *A. lumbricoides* and hookworms, especially for *N. americanus*, and was approved for human use by the China Food and Drug Administration in April 2004.[15]

In this study, a series of aromatic heterocycle substituted aminamidine derivatives (AADs) were synthesized based on the molecular structure of tribendimine. All synthesized compounds were evaluated for their efficacy against *N. americanus in vivo,* and the compounds with the best activity were further tested for solubility, cell toxicity, pharmacokinetic parameters and anthelmintic rate. Additionally, the proteomic profiles of *N. americanus* samples after AAD treatment were obtained using tandem mass tag-based (TMT-based) quantitative proteomic analysis to explore the putative mechanisms of anthelmintic action.

Materials and methods

Chemistry

AADs were synthesized according to the general procedures described in the Supplementary data (available at *JAC* Online). Tribendimine was provided by Shandong Xinhua Pharmaceutical Company, Ltd (Zibo, China). For *in vitro* studies, the compound was dissolved in DMSO to obtain a stock solution. For oral administration, the compound was suspended in 3% (v/v) Tween 80, 7% (v/v) ethanol and 90% (v/v) deionized water. For IV injection, the compound was dissolved in macrogol 400.

Reagents

Chemical reagents and solvents were purchased from Sigma-Aldrich (St Louis, USA) and Bidepharm (Shanghai, China). Biological reagents and solvents were purchased from Gibco (Grand Island, USA). FHs 74 Int cells and culture medium were provided by the Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China).

Animals

Male golden hamsters (aged 4–5 weeks) and male Sprague-Dawley (SD) rats (aged 6–7 weeks) were purchased from Shanghai Songlian Experimental Animal Farm (Shanghai, China) and Shanghai Jihui Laboratory Animal Care Co., Ltd (Shanghai, China), respectively. The animals were kept at the animal facility of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention (Chinese Center for Tropical Diseases Research) (Shanghai, China), and fed standard commercial pellets and water *ad libitum*. This study was compliant with the Chinese Laboratory Animal Administration Act (1988). The animal experiments were approved by the Animal Welfare and Ethics Committee of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention (Permit No: IPD-2019-6).

Animal infection

Our laboratory established an *N. americanus*-golden hamster model and has maintained the life cycle of *N. americanus* for decades. Egg-positive faeces were collected from infected hamsters at 40–50 days post-infection and hatched into third-stage infective larvae (NaL_3), as described previously.[16] For *in vivo* experiments, each hamster was infected with 0.2 mL of water containing 300 NaL_3 by subcutaneous injection.

Screening for the in vivo efficacy of AADs against N. americanus

According to the testing methods reported previously,[17] a total of 105 infected male hamsters were randomly divided into different groups (4–6 hamsters/group) on the 49th day post-infection. The animals in each group were administered one compound at a single oral dose. Tribendimine was used as a control. All faeces of each hamster were collected at 24 and 48 h, then the animals were sacrificed, and the hookworms from the small and large intestines were recovered and counted.

Solubility

The solubility was determined using the shake flask method. Excess amounts of test compound were added to the PBS (pH = 7.4) to allow saturation concentration to be reached. The samples were then incubated at 25°C at 1100 rpm in a thermostatic water bath shaker (Thermomixer Comfort, Eppendorf, Germany) for 24 h. All compounds were filtered and analysed by LC-MS/MS (Triple Quad 5500, SCIEX, USA).

Octanol-water partition coefficient (log P)

The log P value was determined using the shake flask method. The stock solution of test compound (10 mM) was partitioned between appropriately saturated 1-octanol and PBS (pH = 7.4). The phase mixtures were shaken in the shaker incubator (Thermomixer Comfort, Eppendorf, Germany) at 25°C at 1100 rpm for 1 h. After separation, the upper (1-octanol) and lower (buffer) phases were analysed by LC-MS/MS (Triple Quad 5500).

Cytotoxicity assay

FHs 74 Int cells and culture media were placed in 96-well plates (approximately 5000 cells per well) and incubated at 37°C in 95% air and 5% CO_2 for 24 h. The stock solution of test compounds (600 mM) was sequentially diluted by DMSO and added to the wells, and the final concentrations were 18.75–600 μM (0.1% v/v of DMSO in each well). Plates were incubated at 37°C in 95% air and 5% CO_2 for another 48 h. After the supernatants were removed, 90 μL of fresh medium and 10 μL of MTT solution were added, and the cell culture was further incubated for 4 h. The supernatants were removed, and 150 μL of DMSO was added to each well. The plates were swirled gently, and absorbance was measured using a multifunctional enzyme marker (Biotek Synergy H1, Agilent, USA) at 490 nm. The controls were subjected to the same procedures. The cell viability was calculated as follows:

$$\text{Cell viability (CV, \%)} = \frac{OD_{tested} - OD_{blank}}{OD_{control} - OD_{blank}} \times 100\% \quad (1)$$

Pharmacokinetic analysis

Male SD rats were randomly divided into two groups (3 rats/group). One group was orally administered with test compound at a dose of

Table 1. Efficacy of AADs in treating hamsters infected with NaL_3 for 49 days

	WRR (%) at different doses (mg/kg)		
Compound	50	25	12.5
3c	100[a]	100[b]	75.8 ± 9.2[a]
3d	100[a]	100[b]	44.9 ± 8.8[a]
3f	100[a]	90.5 ± 5.9[a]	NT
3i	100[a]	49.0 ± 18.2[b]	NT
3j	100[a]	100[b]	70.3 ± 9.2[a]
Tribendimine	89.4 ± 4.5[a]	NT	NT

The WRR was calculated as follows:

$$\text{WRR (\%)} = \left(\frac{\text{Worm Number}_{\text{large intestine+faeces}}}{\text{Worm Number}_{\text{small intestine+large intestine+faeces}}} \right) \times 100\%.$$

Values are presented as the means ± SEM. NT, not tested.
[a]$n = 5$/group.
[b]$n = 4$/group.

200 mg/kg, whereas the other group received an IV injection of the test compound at a dose of 20 mg/kg. Blood (0.2 mL) was collected at 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 24 and 48 h into centrifuge tubes containing sodium heparin. Plasma was isolated by centrifugation at 8000 g for 6 min at 4°C and analysed by LC-MS/MS (Triple Quad 5500).

Anthelmintic rate

After 35 days of infection, a total of 82 infected male hamsters were randomly divided into different groups (5–25 hamsters/group), test compound was administered at a single oral dose of 50 mg/kg and the control group was treated with placebo. Animals were sacrificed at 1, 2, 3, 6 and 24 h after treatment, and worms were collected from the small intestine. The anthelmintic rate was calculated as follows:

$$\text{Anthelmintic rate (AR, \%)} = \left(1 - \frac{\text{Worm Number}_{\text{treated}}}{\text{Worm Number}_{\text{control}}} \right) \times 100\% \quad (2)$$

Quantitative proteomic analysis

N. americanus treated with AADs at a single oral dose of 50 mg/kg for 1 h was collected from the small intestine and washed thrice with PBS. Worms (18–20 worms/sample, $n = 3$) were transferred into cryotubes and rapidly frozen in liquid nitrogen, then stored at −80°C. Placebo-treated worms were used as controls. Frozen worms were ground into a powder and subjected to TMT-based quantitative proteomic analysis as described previously.[18] In brief, the total protein of each sample was extracted and the concentration of protein was determined by bicinchoninic acid quantitative analysis. A 10 μg protein sample from each group was separated by 12% SDS-PAGE. According to the protein concentration, the amount of protein per sample was digested using trypsin-TPCK (*N*-α-tosyl-L-phenylalanine chloromethyl ketone) and labelled with the TMT Label Reagent Set (Thermo Scientific, USA) according to the manufacturer's instructions. The labelled samples were separated and analysed by LC-MS/MS (Q Exactive HF-X, Thermo Scientific, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad, USA). Pharmacokinetic parameters were calculated using Phoenix WinNolin® 7.0 (Pharsight, USA) with the non-compartmental model. Raw files of quantitative proteomics were analysed using Proteome Discover 2.4 software (Thermo Scientific, USA) and searched in the Uniprot-taxonomy_51031 database fasta.

Table 2. Solubilities and log P values of compounds 3c, 3j and tribendimine

Compound	Solubility[a] (μg/mL)	Log P (c)[b]	Log P[c]
3c	20.67 ± 1.75	2.00	1.74 ± 0.03
3j	21.67 ± 2.92	3.05	NT
Tribendimine	0.38 ± 0.06	5.13	NT

Values are presented as means ± SEM ($n = 3$). NT, not tested.
[a]Thermodynamic solubility in PBS (pH = 7.4) determined by the shake flask method and calculated as follows: [Sample] = $\frac{\text{AREA}_{\text{Sample}} \times \text{INJ VOL}_{\text{std}} \times \text{DF}_{\text{Sample}} \times [\text{STD}]}{\text{AREA}_{\text{std}} \times \text{INJ VOL}_{\text{Sample}}}$, where DF means the dilution factor.
[b]Octanol-water partition coefficient calculated by SwissADME based on the average of all five predictions.
[c]Octanol-water partition coefficient determined by the shake flask method and calculated as follows: Log P = $\text{Log}\left(\frac{\text{AREA}_{\text{Oct}} \times \text{INJ VOL}_{\text{Buf}} \times \text{DF}_{\text{Oct}}}{\text{AREA}_{\text{Buf}} \times \text{INJ VOL}_{\text{Oct}} \times \text{DF}_{\text{Buf}}} \right)$, where DF means the dilution factor.

Results

In vivo efficacy of the AADs against N. americanus at different dosages

At a 50 mg/kg dose, most compounds showed a better worm reductive rate (WRR) than tribendimine, whereas five compounds eliminated the worms completely (Table S1). Three compounds, 3c, 3d and 3j, showed 100% WRR at the 25 mg/kg dose, and the WRR of compound 3c (75.8 ± 9.2%) was higher than that of 3d (44.9 ± 8.8%) and 3j (70.3 ± 9.2%) when the dose was further reduced to 12.5 mg/kg (Table 1).

Physicochemical properties of compounds 3c and 3j

As shown in Table 2, compounds 3c and 3j had moderate solubilities (20.67 ± 1.75 and 21.67 ± 2.92 μg/mL) whereas tribendimine was practically insoluble (0.38 ± 0.06 μg/mL). The log P values of compounds 3c and 3j predicted by SwissADME (http://www.swissadme.ch) were 2.00 and 3.05 respectively, whereas that of tribendimine was 5.13. Notably, the tested log P value of 3c was 1.74 ± 0.03, consistent with the calculated result.

Cytotoxicity assay of compounds 3c and 3j on FHs 74 Int cells

The toxicities of compounds 3c and 3j were tested on the FHs 74 Int cells using the MTT assay (Figure 1). When the concentrations were less than 75 μM, compound 3c exhibited lower inhibition than 3j, and the result reversed when the concentration was above 150 μM. However, more than half of the cells treated with compound 3c still showed vitality, even at the highest test concentration of 600 μM (cell viability rate: 52.7 ± 2.1%).

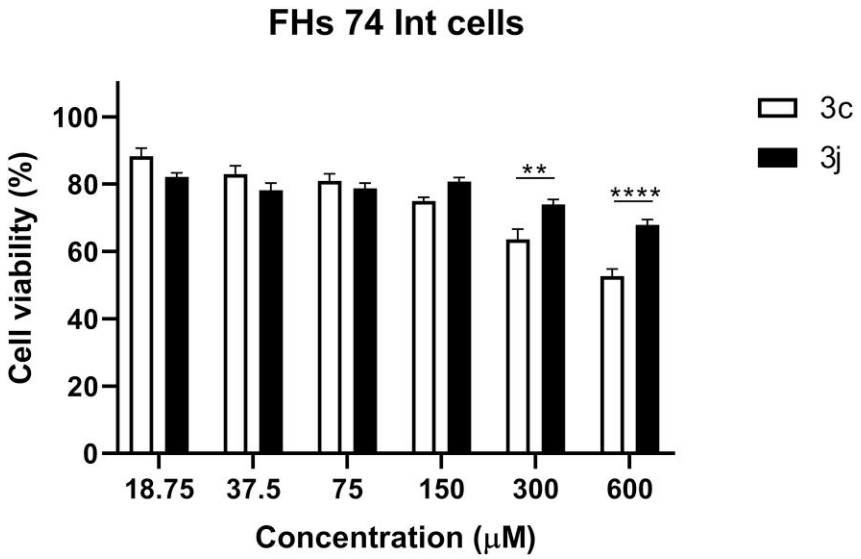

Figure 1. Cytotoxicity of compounds 3c and 3j against FHs 74 Int cells using the MTT assay. Values were compared by two-way analysis of variance with multiple comparison and are presented as the means ± SEM. $^{**}P < 0.01$, $^{****}P < 0.0001$.

Table 3. Pharmacokinetic parameters of compounds 3c and dADT *in vivo*

Compound	Dose (mg/kg)	Route	$t_{½}$ (h)	T_{max} (h)	C_{max} (ng/mL)	$AUC_{(0-t)}$ (h*ng/mL)	Apparent volume of distribution (mL/kg)	CL (mL/h/kg)	Oral bioavailability (%)
3c	200	Oral	7.79	0.083	397.3	304.7	—	—	12.0
dADT[a]			6.54	1.36	8621.1	35 138.9	—	—	99.5
3c	20	IV	1.53	0.083	400.5	254.2	1 66 193.2	78 702.6	—
dADT[b]			5.58	0.083	3299.1	3532.8	—	—	—

[a]dADT metabolized from 3c via the oral route.
[b]dADT metabolized from 3c via the IV route.

In vivo pharmacokinetic parameters of compound 3c

In vivo, compound 3c quickly broke down to *p*-(1-dimethylamino ethylimino)aniline (deacylated amidantel, dADT) after oral administration and subsequently transformed into other products (Figure S1). dADT is an effective metabolite against *N. americanus*.[19] The pharmacokinetic parameters of compounds 3c and dADT are presented in Table 3. At 200 mg/kg single oral dose, the plasma concentration of 3c attained the maximum (T_{max}) within 5 min, and the elimination half-life ($t_{½}$) was 7.79 h. Meanwhile, the peak plasma concentration (C_{max}) and the $AUC_{(0-t)}$ were 397.3 ng/mL and 304.7 h*ng/mL, respectively. The half-life of dADT was similar to that of 3c (6.54 h), but it had a longer T_{max} (1.36 h) than 3c. The C_{max} and $AUC_{(0-t)}$ of dADT (8621.1 ng/mL and 35138.9 h*ng/mL) were much higher than those of 3c. The oral bioavailability of 3c was 12.0%, whereas that of dADT was 99.5%.

In vivo anthelmintic rates of N. americanus after compound 3c treatment

The anthelmintic rates against *N. americanus* after treatment with 3c are summarized in Table 4. At 50 mg/kg single dose, nearly one-fifth and one-half of the worms were expelled from hamsters after treatment for 1 and 2 h, respectively. About 90% of worms were expelled after 3 h. The anthelmintic rate reached 98.3 ± 1.7% at 6 h, and all worms were excreted within 24 h.

Quantitative proteomic analysis of N. americanus treated by compound 3c

The proteomic profiles of *N. americanus* samples from the 3c- and placebo-treated groups were compared using TMT-based quantitative proteomic analysis. A total of 5660 protein groups were identified in the 38 574 peptides. Credible proteins were screened according to the criteria of score sequence Hypothesis Testing > 0 and unique peptide ≥1, and blank values were removed. The statistical results for credible proteins showed that the quantitative data were consistent (Figure S2). Furthermore, 437 differentially expressed proteins (DEPs, fold-change = 1.2 times and $P < 0.05$) were identified, 190 of which were up-regulated and 247 were down-regulated (Figure 2a).

Enrichment analyses, including Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) term

Table 4. Anthelmintic rates of *N. americanus* after treatment with compound 3c *in vivo*

Time (h)	Number of hamsters	Number of worms	Anthelmintic rate (%)
Control	25	19.2 ± 2.4	—
1	11	15.6 ± 2.3	18.9 ± 12.2
2	17	10.5 ± 1.8	45.0 ± 9.5
3	12	1.8 ± 0.8****	90.9 ± 4.1
6	12	0.3 ± 0.3****	98.3 ± 1.7
24	5	0***	100

Values were compared by one-way analysis of variance with multiple comparison and are presented as the means ± SEM.
***$P < 0.001$, ****$P < 0.0001$, compared with the number of worms in control group.

analyses, were performed to determine the functions of the DEPs. KEGG pathway analysis suggested that the significantly enriched pathways were mainly related to pyruvate, fatty acid, glycogen and amino acid metabolism, and protein synthesis (Figure 2b). The top 10 enriched GO terms for biological processes (BP), cellular components (CC) and molecular functions (MF) are shown in Figure 2(c). The main differences in BPs between normal and 3c-treated worms were translation and nucleosome assembly. CC annotation indicated that the significantly enriched DEPs located in the extracellular region, nucleus, ribosome, collagen trimer, endoplasmic reticulum membrane and cytoskeleton. According to MF analysis, the DEPs were primarily involved in chitin binding, lipid transporter activity, metallopeptidase activity, lipid binding and NAD binding.

Discussion

Tribendimine is an aminamidine derivative with a unique planar skeleton and symmetrical structure. Although it is a broad-spectrum anthelmintic agent, its efficacy is limited by its poor solubility, reducing the dissolution rate and absorption.[20] Thus, to improve the solubility and discover new compounds with higher anthelmintic activity, we designed and synthesized several classes of derivatives based on the molecular structure of tribendimine and found that compounds with aromatic heterocycles were better than tribendimine. The introduction of aromatic heterocycles probably contributed to the increased aqueous solubility of AADs, owing to the slightly polar and hydrogen bond-accepting properties of the heteroatom.[21]

In this study, 13 AADs were synthesized and evaluated for their efficacy against *N. americanus in vivo*. Screening from high to low doses revealed that the potency of most compounds was superior to that of tribendimine (Tables S1 and 1). The single dose of tribendimine to expel all worms was 150 mg/kg.[14] Five of the 13 compounds eliminated all worms at a single dose of 50 mg/kg, whereas the WRR of tribendimine was 89.4% at the same dose. Notably, compounds 3c and 3j exhibited excellent WRR at the dose of 25 mg/kg and it was >70% even at a dose of 12.5 mg/kg.

Compounds 3c, 3j and tribendimine were in accordance with the Lipinski rule of five except the log P value of tribendimine (Table S2).[22] The solubility and lipophilicity indicated that 3c and 3j exhibited better physicochemical parameters than tribendimine (Table 2). The solubilities of compounds 3c and 3j were about 20 μg/mL, 50 times more than that of tribendimine. The log P value of compound 3c was between 0 and 3, and that of 3j and tribendimine was over 3 and 5, respectively, indicating that 3c had higher permeability than 3j and tribendimine, which is beneficial for drug transport and pharmacodynamics.[23]

Compounds 3c and 3j showed low cytotoxicity (Figure 1); more than half of the cells were alive at the highest test concentration of 600 μM. Owing to the poor solubility of tribendimine, its cytotoxicity was not detected.

In view of its anthelmintic effect, physicochemical properties and cytotoxicity, compound 3c was chosen for further experiments. The major metabolite of 3c is dADT, which is also the main metabolite of tribendimine.[24,25] dADT plays an important role as a tribendimine in the treatment of intestinal nematodes.[19,26] dADT was also in accordance with the Lipinski rule of five and its log P value was 1.57 (Table S2). It is a soluble compound with a solubility of 1045.20 ± 28.33 μg/mL. Compound 3c rapidly metabolized to dADT after administration. Compared with the pharmacokinetic parameters of dADT metabolized from tribendimine at the same dose of 200 mg/kg,[27] dADT metabolized from 3c achieved better performance and very high bioavailability (Table 3). Compound 3c had a long half-life, but its oral bioavailability was low. Nevertheless, considering the bioavailability of its metabolite dADT, the total bioavailability was satisfactory. Tribendimine could not be administered via IV injection because of its poor solubility; therefore, the absolute oral bioavailability of the original drug and metabolite is unknown.

At a single dose of 50 mg/kg, compound 3c dewormed approximately half *N. americanus* in 2 h, most worms within 6 h, and all worms within 24 h (Table 4). The long half-life of compound 3c was favourable for maintaining a relatively high and long-lasting blood concentration, which contributed to its enhanced anthelmintic efficacy.

Early studies using the *Caenorhabditis elegans* model found that tribendimine-resistant mutants were also resistant to levamisole and could alter the same genes that mutated to levamisole resistance, indicating that the antiparasitic mechanism of tribendimine was similar to that of levamisole and that it was also a levamisole-type nicotinic acetylcholine receptor agonist.[28] But further research suggested that *Oesophagostomum dentatum* larvae with levamisole resistance remained sensitive to tribendimine.[29] Moreover, the efficiency of tribendimine and levamisole varies among different kinds of parasites. For instance, levamisole moderately inhibits *Clonorchis sinensis* but is not active on cestodes, whereas tribendimine is effective against them.[30,31] Therefore, tribendimine may work through different pathways.

Quantitative proteomic analysis of *N. americanus* treated with compound 3c revealed that AADs were involved in various physiological processes, and the most important pathways were related to ATP metabolism and cuticle proteins synthesis.

Phosphocreatine is an important source of energy for muscle contraction. Contraction of the worm body requires ATP expenditure, and the phosphate generated from phosphocreatine can

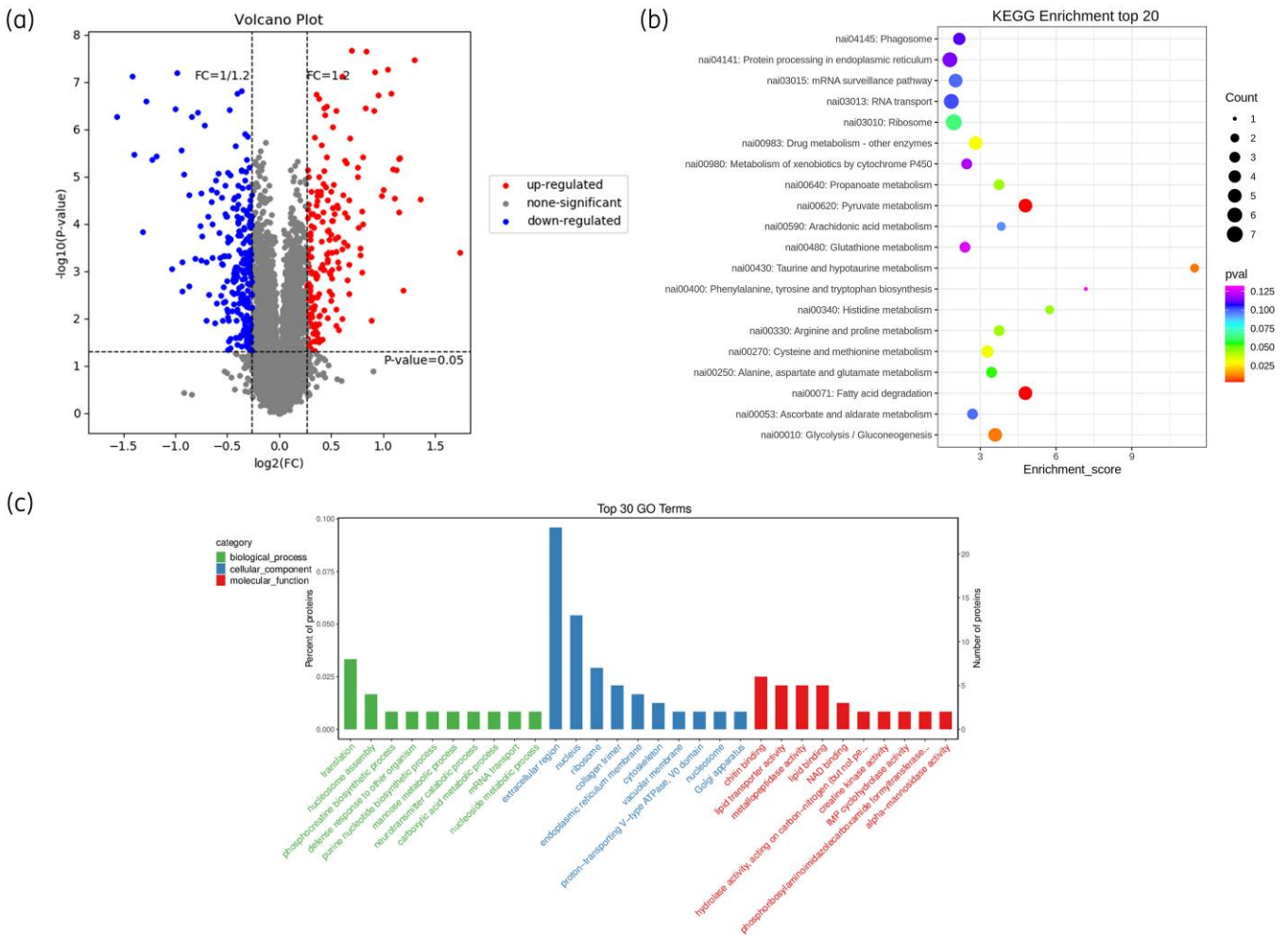

Figure 2. TMT-based quantitative proteomic analysis of *N. americanus* treated with compound 3c. (a) Volcano plot of DEPs between 3c-treated worms and controls. Up-regulated proteins are on the right, and down-regulated proteins are on the left. (b) The top 20 enriched KEGG pathways. The *x*-axis is the enrichment score, and the *y*-axis is the KEGG pathway. Colour represents the *P* value, and the size of the circle represents the number of involved DEPs. (c) The top 10 enriched GO terms in biological processes, cellular components and molecular functions, respectively. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

combine with ADP to form ATP. Moreover, the tricarboxylic acid cycle is a common pathway for energy supply via glucides, lipids and proteins. According to the KEGG and GO enrichment analyses, many DEPs were involved in phosphocreatine biosynthesis, glycolysis/gluconeogenesis, pyruvate metabolism, fatty acid degradation and amino acid metabolism (Figure 2). Additionally, purine nucleotide biosynthesis and nucleoside metabolic processes are related to ATP generation. The KEGG pathway and GO term results also indicated that the protein synthesis differed significantly between normal and 3c-treated worms due to the DEPs in translation, mRNA transport, ribosomes, endoplasmic reticulum membrane and Golgi apparatus (Figure 2). Moreover, the extracellular region and collagen trimer in the CC annotation of GO terms were also obvious differences (Figure 2). Collagen trimers locate in the extracellular matrix and are important components of nematode cuticles to protect the worm from the external environment.[32,33] Compound 3c might affect the synthesis of epidermal proteins such as collagen trimers. In addition, it is worth noting that the chitin binding in MF analysis of GO terms also showed significant differences (Figure 2). Chitin presents in the extracellular matrix. It is an essential component of the nematode eggshell and interacts with chitin-binding proteins to form an important protective barrier.[34,35] Compound 3c might inhibit the development of eggs by interfering with the synthesis of chitin-binding proteins; however, more research is needed to confirm that.

In conclusion, 3c is a novel compound that is potent against *N. americanus in vivo*. The solubility and absorption of 3c were significantly improved compared with those of tribendimine, and it was less toxic. Quantitative proteomic analysis suggested that the mechanism of action of compound 3c against parasites was probably related to ATP metabolism and cuticle protein synthesis. Our research indicates that compound 3c could be an anthelmintic candidate, and further studies are underway.

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Transparency declarations

None to declare.

Supplementary data

Figures S1 and S2 and Tables S1 and S2 are available as Supplementary data at *JAC* Online.

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