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Original Article

Effects of (−)-6,6′-dinitrohinokinin on adult worms of *Schistosoma mansoni*: a proteomic analyses

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ABSTRACT

Schistosomiasis, a chronic disease that affects million people worldwide, is caused by trematode flukes of the genus *Schistosoma*. The lack of an anti-schistosomiasis vaccine and massive monotherapy with praziquantel reinforces the need for search and development of new therapeutic drugs. Recently, we demonstrated that the essential oil of *Piper cubeba L., Piperaceae*, and their derivative dibenzylbutyrolactolic (−)-6,6′-dinitrohinokinin, presents *in vitro* and *in vivo* activities against *Schistosoma mansoni*. Here, we identified changes in the protein expression after exposure to dibenzylbutyrolactolic (−)-6,6′-dinitrohinokinin. We applied two-dimensional gel electrophoresis (2-DE) to *S. mansoni* soluble protein extracts and observed at least 38 spots to be affected by dibenzylbutyrolactolic (−)-6,6′-dinitrohinokinin. We further identified 25 differentially expressed proteins by mass spectrometry. Enrichment for biological processes and predictive analyses of protein-protein interactions suggest that dibenzylbutyrolactolic (−)-6,6′-dinitrohinokinin targets proteins involved mainly in metabolic processes, especially carbohydrate metabolism. In summary, this study provides an interesting approach to understand the anti-parasitic activity of semi-synthetic (−)-6,6′-dinitrohinokinin a derivative compound from lignan and for the development of new therapy strategies.

Introduction

Schistosomiasis is caused by a parasitic infection with trematode flukes of the genus *Schistosoma* and affects more than 200 million people worldwide (Rollinson et al., 2013). Currently, an antischistosomal vaccine is not available, whereas Praziquantel (PZQ) is the drug of choice for the treatment of patients (Caffrey, 2015). Although PZQ have been effective in the treatment of the disease, the possibility of drug resistance caused by repeated and massive monotherapy reinforces the need to develop new safe and effective drugs for the prevention and treatment against schistosomiasis (Cioli et al., 2014; Colley et al., 2014).

Piper cubeba L., Piperaceae, known as the tailed pepper, has been used in traditional medicine for the treatment of dysentery, syphilis, abdominal pain, diarrhea, enteritis and asthma (Usia et al., 2005; Gutierrez et al., 2013). We demonstrated previously that extracts and isolated compounds from *Piper* species present anti-parasitic activity (Saraiva et al., 2007, 2010; Esperandim et al., 2013). Furthermore, we showed that the essential oil of *P. cubeba* and their derivative dibenzylbutyrolactolic (−)-6,6′-dinitrohinokinin (**1**, DNH), presents *in vitro* and *in vivo* activities against *Schistosoma mansoni* (Magalhães et al., 2012; Pereira et al., 2015).

High-throughput strategies including genomics, transcriptomics, proteomics and metabolomics have become important approaches to understand schistosome biology and pathogenesis, but also constitute important tools to the exploration of novel drugs, vaccines or diagnosis (De Marco and Verjovski-Almeida, 2009; Chuan et al., 2010). In view of these facts, we identified

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changes in the protein expression induced by exposure to DNH. We applied a strategy based on two-dimensional gel electrophoresis (2-DE) followed by mass spectrometry, which provided insights into key proteins, molecular functions and biological processes targeted by DNH.

Materials and methods

Obtainment of DNH

Prior to the DNH synthesis, (−)-cubebin (**2**) was isolated from *Piper cubeba L., Piperaceae*, seeds and (−)-hinokinin (**3**) was obtained by oxidation of (−)-cubebin (Da Silva et al., 2005). (−)-Hinokinin was then submitted to a nitration reaction in the presence of fuming HNO_3. The reaction was processed under magnetic stirring at 0 °C for 2 h. The obtained DNH (**1**) was crystallized as yellowish powder from MeOH. The purity of the synthesized DNH was estimated to be higher than 95% by both HPLC and ^1H and ^{13}C NMR analyses, as well as by its melting point (Setchell et al., 1981; Costa, 2000).

Yield 97.6%; $[\alpha]_D^{25}$ −29.07° (*c* 0.008, $CHCl_3$, *ee* > 99%); mp 191–193 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 7.5 (s, 1H); δ 7.48 (s, 1H); δ 6.8 (s, 1H); δ 6.6 (s, 1H); δ 6.1 (m, 2H); δ 4.35 (dd, 1H, *J* = 7.1 Hz and *J* = 9.1 Hz); δ 4.0 (dd, 1H, *J* = 7.3 Hz and *J* = 9.1 Hz); δ 3.26 (d, 2H, *J* = 7.1 Hz); δ 3.2 (dd, 1H, *J* = 6.3 Hz and *J* = 13.6 Hz); δ 3.0 (dd, 1H, *J* = 7.8 Hz and *J* = 13.6 Hz); δ 2,8 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): 178.0, 152.3, 152.2, 147.6, 143.1, 142.9, 130.9, 130.7, 112.5, 111.2, 106.6, 106.2, 103.6, 103.5, 71.4, 45.7, 41.7, 37.2, 34.21.

Parasite strain

The LE (Luiz Evangelista) strain of *S. mansoni* was maintained by passage through *Biomphalaria glabrata* snails and BALB/c mice. After 49 ± 2 days, *S. mansoni* adult worms were recovered under aseptic conditions from mice previously infected with 200 cercariae by perfusion of their livers and mesenteric veins (Smithers and Terry, 1965). Experiments were approved by Animal Research Ethics Committee from Ribeirão Preto Medical School – University of São Paulo, under the protocol 021/2009.

S. mansoni *soluble protein extraction and two-dimensional polyacrylamide gel electrophoresis (2-DE)*

Parasites exposed to 25 μM DNH or RPMI 1640 medium (0.1% DMSO) were transferred to 0.5 ml of homogenization buffer [25 mM Tris–HCl, pH 7.5, 1 mM phenylmethanesulfonyl fluoride and 1 mM dithiothreitol] and worms were lysed by sonication (5 × 10 s, 21 kHz at 7 μm amplitude) at 10 s interval, on ice. Cellular debris was removed from the lysate by centrifugation for 1 h at 15,000 × *g*, 4 °C. The supernatant containing soluble proteins was collected and quantified using the Coomassie Protein Assay Kit (Pierce Biotechnology).

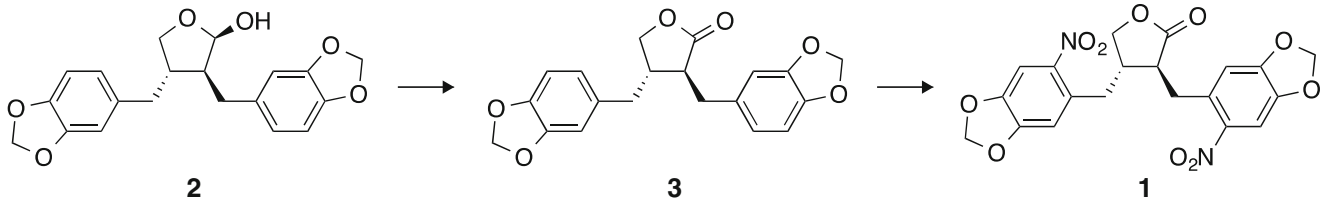
Soluble proteins (200 μg) were dissolved in an isoelectric focusing buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% (v/v) carrier ampholytes (IPG buffer 3–10 l, GE Healthcare), 40 mM dithiotreitol (DTT) and 0.002% (v/v) bromophenol blue] and rehydrated into linear 13-cm Immobiline dry strips pH 3–10 (GE Healthcare) for 16 h at 20 °C. Isoelectric focusing was carried out on Ettan IPGphor (GE Healthcare), by applying a current of 50 mA/strip, following the steps: 1 h at 500 V (step), 1 h at 1000 V (gradient), 2.5 h at 8000 V (gradient) and 0.5 h at 8000 V (step), totaling 16 kVh. The focused strips were equilibrated for 15 min in 15 ml equilibration buffer [50 mM Tris–HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS], supplemented with 1% (w/v) DTT and subsequently for 15 min in 15 ml equilibration buffer with 2.5% (w/v) iodoacetamide. Proteins were separated in 12.5% (w/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the Ruby 600 apparatus (GE Healthcare) and SDS electrophoresis buffer (25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS). Each gel was performed in triplicate.

Protein visualization and image analysis

2-DE gels were fixed for 1 h with 3% (v/v) phosphoric acid and 50% (v/v) ethanol, followed by staining with Coomassie Brilliant Blue G250 (GE Healthcare) during 24 h. 2-DE protein profiles were obtained after gel distaining in MilliQ water. Gels were digitized at 300 dpi and 16 bits depth resolution with an ImageScanner (GE Healthcare) operated by the LabScan software (GE Healthcare). The software ImageMaster 2D Platinum 6.0 (GE Healthcare) was used to merge the image of each experimental replicate onto a master 2-DE gel image, using the six well-defined landmark as reference to spot detection. Differential protein expression was analyzed by comparison of spot intensity in gels containing proteins from *S. mansoni* exposed to DNH and unexposed controls. Spots that presented at least 1.5-fold change in intensity given by Student's *t* test ($p < 0.05$) were manually excised and analyzed by mass spectrometry.

In gel *digestion and identification of proteins by liquid chromatography-tandem mass spectrometry (LC-MS/MS)*

Spots obtained from 2-DE gels were digested with grade-modified trypsin (Promega), as described by Shevchenko et al. (1996). After digestion, peptides were extracted with 50% (v/v) acetonitrile plus 5% (v/v) formic acid solution. The resulting peptides were dried again and re-suspended in 12 μl of 0.1% (v/v) formic acid. An aliquot of 4.5 μl of the digested protein was then separated on a C18 RP-UPLC column (100 mm × 100 μm I.D. × 1.7 μm particle size, nanoAcquity UPLC, Waters) using a linear gradient of 15–90% (v/v) acetonitrile in 0.1% (v/v) formic acid for 10 min at 0.6 μl/min. The HPLC system was coupled to a Q-Tof Ultima API mass spectrometer (MicroMass/Waters) fitted with a nano-electrospray source. The parameters were 35 V cone voltage, nano-electrospray voltage was 3.5 kV, and 100 °C source temperature. The equipment was operated in the MS positive ion mode, with continuous data acquisition from a mass-to-charge ratio (*m*/*z*) 100–2000 Da, at a scan rate of 1 s and an interscan delay of 0.1 s, which was followed by MS/MS of the three most intense ions. To optimize peptide coverage, a mass/charge exclusion list was maintained so that the same peptide was not selected for MS/MS within a period of 40 s. The collision energy used for peptide fragmentation was varied according to the mass and charge of the ion.



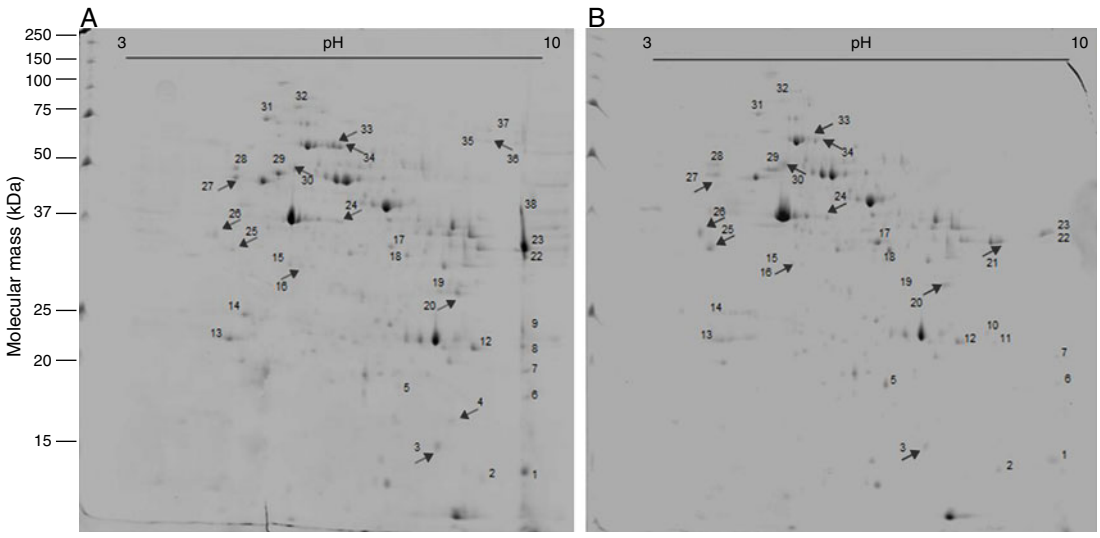


Fig. 1. Effect of (−)-6,6′-dinitrohinokinin on *Schistosoma mansoni* adult protein profile. Comparative bidimensional gels analyses of *S. mansoni* soluble proteins before (A) and after exposure to 25 μM DNH (B). Numbers represent 38 spots whose intensity was significantly affected ($p < 0.05$) by DNH.

The data obtained were processed using Mascot Distiller v.2.3.2.0 (Matrix Science Ltd.) and proteins were identified by correlation of tandem mass spectra against public available *S. mansoni* (27,729 sequences and 12,093,356 residues) deposited into NCBI database. Search parameters for MASCOT were as follows: *S. mansoni* taxonomic restriction; trypsin cleavage, one missed cleavage; carbamidomethyl–cys as fixed modification (monoisotopic mass 57.0215 Da), methionine oxidation as variable modification (monoisotopic mass 15.9949) carbamidomethyl (C); variable modification, oxidation (M); peptide tolerance, 0.1 Da; MS/MS fragment ion tolerance. Results were regarded as significant with an allowed likelihood for a random hit of $p < 0.05$, according to the MASCOT.

Bioinformatic analysis

Identified proteins were analyzed for Gene Ontology (GO) enrichment and protein class using the bioinformatics tools PANTHER (Mi et al., 2013) and UniProt (Consortium, 2014). The networks of interaction for the identified proteins were performed using STRING database v 9.05, employing prediction methods as neighborhood, gene fusion, co-occurrence, co-expression, experiments, database and textmining sources (Jensen et al., 2009). We performed analysis in both Cluster of Orthologous Groups/Eukaryotic Orthologous Groups (COG/KOG) (with a high confidence of 0.700) and PROTEIN-modes (with a medium confidence of 0.400). The identity of the putative proteins were considered significant if they presented at least 75% of homology with sequences from *Schistosoma japonicum*.

Results

Differential protein analyses revealed approximately 38 spots (*i.e.*, proteins) that were significantly affected by 25 μM DNH (Fig. 1A and B). Most of the separated proteins have molecular weights greater than 25 kDa and are distributed among the isoelectric points from 4 to 10. Basic proteins (*i.e.*, proteins that present an isoelectric point from 8 to 10) were directly affected by exposure to DNH, which resulted in their absence or lower expression. Although, we observed that spots numbered as 4, 8, 9, 19, 35, 36, 37 and 38 are present in protein profile of *S. mansoni* unexposed to DNH, these spots were not identified after drug exposure (Fig. 1A and B). After exposure to DNH, the spots 2, 5, 10, 11, 17, 18 and 21 were present or had at least a 2-fold increase in expression, while nineteen proteins had at least a 2-fold reduction or no expression (Fig. 1A and B).

To gain further knowledge on changes of protein expression by *S. mansoni* after exposure to DNH, we performed LC-MS/MS. For each spectrum, a search for protein sequences from *S. mansoni* was performed with MASCOT at NCBI nr protein database (27,729 sequences and 12,093,356 residues). We further identified 25 differentially expressed proteins by mass spectrometry, however, twenty four identified proteins had MASCOT scores higher than 28 ($p < 0.05$) and only one protein spot (spot n° 19) had MASCOT score below 28 ($p > 0.05$) (Table 1). Of interest, the tegumental antigen Sm 20.8 and phosphoglycerate mutase (spots 10 and 11, respectively) were expressed following exposure to DNH, whereas the peroxiredoxin, gelsolin and a major egg antigen (spots numbered as 5, 17 and 18, respectively) were the proteins with the highest expression after exposure to DNH (Fig. 1A and B, and Table 1). Notably, exposure to DNH decreased the expression of the peptidyl-prolyl *cis-trans* isomerase, glutathione-*S*-transferase (GST), 14-3-3 protein, 14-3-3 epsilon, calreticulin and paramyosin (spots numbered as 1, 12, 13, 14, 28 and 32, respectively) (Fig. 1A and B, and Table 1). MASCOT did not retrieve significant matches to spots 3, 4, 16, 20, 21, 24, 25, 26, 27, 30, 33, 34 and 36, therefore we were unable to identify their protein content (Fig. 1).

To evaluate which biological processes might be affected by exposure to DNH, we performed a predictive functional analysis with public available tools as PANTHER classification system and UniProt. *S. mansoni* adult proteins were mainly enriched in Gene Ontology (GO) biological processes such as: metabolic process (45.5%), cellular process (22.7%), localization (13.6%), biological regulation (9.1%) and cellular component organization or biogenesis (9.1%) (Fig. 2A and Table 2). Furthermore, enrichment by GO molecular function resulted in 52.9% of proteins enriched in catalytic activity, followed by proteins related to binding (17.6%), enzyme regulator activity (11.8%), structural molecule activity (11.8%) and antioxidant activity (5.9%) (Fig. 2B and Table 2). In addition, proteins enriched in Panther protein class resulted in 20% of oxidoreductases, 13.3% of isomerases, 13.3% enzyme modulators, 13.3% of cytoskeletal proteins, chaperones, 6.7% of calcium-binding proteins, 6.7% of kinases, 6.7% of lyases and 6.7% of transferases (Fig. 2C).

Table 1
Schistosoma mansoni adult proteins identified by LC-MS/MS.

Spot n°	Protein name	Accession number[a]	MW (kDa)/pI	MW (kDa) experimental	Coverage %[c]	Mascot Score[d]	Expression after exposure DNH[e]
1	Peptidyl-prolyl *cis-trans* isomerase	Smp_040130	18/8.26	12	46	390	Down
2	Nucleoside diphosphate kinase	Smp_092750	17/7.74	10	79	453	Up
5	Peroxiredoxin, Prx1	Smp_059480	21/6.10	18	36	231	Up
6	Calponin-related	Smp_086330	21/8.68	17	55	453	Down
7	Putative adenylate kinase	Smp_071390	22/8.52	19	45	331	Down
8	Triosephosphate isomerase	Smp_003990	28/4.63	22	54	481	Down
9	Putative carbonyl reductase	Smp_033540	31/7.64	24	2	37	Down
10	Putative tegumental antigen Sm20.8	Smp_086530	21/6.90	23	56	196	Up
11	Phosphoglycerate mutase	Smp_096760	28/7.71	22	50	234	Up
12	Glutathione S-transferase	Smp_054160	24/6.56	22	16	132	Down
13	14–3-3 protein, putative	Smp_009760	28/4.74	23	46	553	Down
14	14–3-3 epsilon	Smp_034840	28/4.85	25	52	354	Down
15	Camp-dependent protein kinase type II-alpha regulatory subunit	Smp_079010	43/5.12	32	33	286	Down
17	Putative gelsolin	Smp_008660	42/5.57	34	46	433	Up
18	Putative major egg antigen	Smp_049300	39/6.23	33	18	287	Up
19	Putative four and A half lim domain	Smp_048560	32/7.61	26	14	20	Down
22	Malate dehydrogenase	Smp_047370	37/8.70	34	31	746	Down
23	Glyceraldehyde-3-phosphate dehydrogenase	Smp_056970	37/8.16	36	32	305	Down
28	Calreticulin autoantigen homolog, putative	Smp_030370	45/4.69	49	39	231	Down
29	Putative alpha tubulin	Smp_090120	51/4.97	49	53	1416	Down
31	Putative heat shock protein	Smp_072330	81/4.92	75	25	623	Down
32	Putative paramyosin	Smp_021920	90/5.3	98	14	142	Down
35	Taurocyamine kinase	Smp_194770	77/7.87	72	11	91	Down
37	Aconitate hydratase	Smp_063090	66/8.45	74	18	88	Down
38	Putative uncharacterized protein	Smp_042170	40/7.63	38	20	167	Down

[a] *S. mansoni* Genedb database identifier.
[b] Predicted molecular weight (MW) and isoelectric point (pI) recovered in the NCBI protein database and Protparam tool.
[c] Coverage %: The Sequence Coverage is the percentage of the database protein sequence covered by matching peptides.
[d] Score: Relative for the probability that the observed match between the experimental data and the database sequence is a random event.
[e] Spots that changed at least 1.5-fold in intensity (down or up) after exposure to DNH compared to unexposed control.

Next, to understand whether the identified proteins interact with each other and therefore contribute to common biological processes, we performed predictive protein-protein interaction analyses using String. Analysis of differentially expressed proteins of *S. mansoni* assigned to clusters of orthologous groups (COG) or eukaryotic orthologous groups (KOG) shows that all proteins, except for glutathione-*S*-transferase (GST) (KOG1695), might interact with one or more nodes in the predicted network (Fig. 3A). As expected, the network depicts an upper cluster of proteins assigned with COGs, while in a second cluster, the majority of nodes were assigned with KOG. Further analysis resulted in a more specific network of potential targets induced or repressed by exposure to DNH, which possess significant identity with proteins of *S. japonicum* (Fig. 3B). Of note, five proteins enriched in this network were reduced after exposure to DNH, while only nucleoside diphosphate kinase and phosphoglycerate mutase presented a significant increase in intensity when compared to unexposed controls (Fig. 1 and Table 1).

Discussion

Here, we show that DNH modulates the profile of a protein set from *S. mansoni* adult worms. Several of those proteins, affected by exposure to DNH, participate of important metabolic processes and have already been exploited as potential vaccine candidates (Pearce, 2003; Kohama et al., 2010; Fonseca et al., 2012; Beaumier et al., 2013) or for immunodiagnosis (El Aswad et al., 2011; Qian et al., 2012; Yu et al., 2014). For example, reduced expression of paramyosin, a major structural component that has been associated as potent mucosal immunogen against *Schistosoma* ssp. (Lanar et al., 1986; Jiz et al., 2009; Kohama et al., 2010), might reflect similar activity with the essential oil of *P. cubeba* and DNH by affecting the motility of adult worms (Magalhães et al., 2012; Pereira et al., 2015).

Analysis of protein–protein interactions operated on COG/KOG mode resulted in two major clusters of interactions, whereas proteins assigned to COG were mainly enriched in metabolic processes, such as adenosine triphosphate (ATP) generation and carbohydrate metabolism (Fig. 3A and Table 2). For example, *S. mansoni* nucleoside diphosphate kinase (COG0105) and adenylate kinase (COG0563) catalyze reactions that generate ATP (Senft and Crabtree, 1983). Our results demonstrated that adenylate kinase was decreased after exposure to DNH and suggest that the compound might affect the activity of directly and thus impair the levels of ATP. In contrast, the increased expression of nucleoside diphosphate kinase could be a compensatory response in view of the low adenylate kinase activity. Furthermore, predicted interactions of proteins mainly enriched in carbohydrate metabolism, such as phosphoglycerate mutase (COG0588), triosephosphate isomerase (COG0149), malate dehydrogenase (COG0039), glyceraldehyde-3-phosphate dehydrogenase (COG00570), aconitate hydratase (COG1048) indicate that DNH targets pathways crucial to development and survival of *S. mansoni*, such as glycolysis and gluconeogenesis (Zhou et al., 2008). This is further corroborated due the level of agreement in protein expression, since only phosphoglycerate mutase increased after exposure to DNH (Table 1). Indeed,

Table 2
Enrichment analysis by Gene Ontology and PANTHER terms.

IDs[a]	Protein (COG/KOG IDs)[b]	GO molecular function	GO biological process	PANTHER protein class
Smp_040130	Peptidyl-prolyl cis-trans isomerase (COG0652)	Isomerase activity	Protein folding, Intracellular protein transport, Nuclear transport	Isomerase
Smp_092750	Nucleoside diphosphate kinase (COG0105)	ATP binding	CTP biosynthetic process, UTP biosynthetic process	–
Smp_059480	Peroxiredoxin, Prx1 (COG0450)	Oxidoreductase activity, Peroxidase activity	Metabolic process	Peroxidase
Smp_086330	Calponin-related (COG5199)	–	–	–
Smp_071390	Putative adenylate kinase (COG0563)	Nucleotide kinase activity	Purine nucleobase metabolic process, Pyrimidine nucleobase metabolic process	Nucleotide kinase
Smp_003990	Triosephosphate isomerase (COG0149)	Isomerase activity	Glycolysis	Isomerase
Smp_033540	Putative carbonyl reductase (COG1028)	Oxidoreductase activity	Steroid metabolic process	Dehydrogenase reductase
Smp_086530	Putative tegumental protein Sm 20.8	Catalytic activity, Structural constituent of cytoskeleton, Protein binding, Enzyme inhibitor activity	Nitric oxide biosynthetic process, Nucleobase-containing compound metabolic process, Cell cycle, RNA localization, Intracellular protein transport, Vesicle-mediated transport, Regulation of catalytic activity, Cellular component organization	Enzyme modulator, Microtubule family, Cytoskeletal protein
Smp_096760	Phosphoglycerate mutase (COG0588)	Bisphosphoglycerate 2-phosphatase activity, Bisphosphoglycerate mutase activity, Phosphoglycerate mutase activity	Glycolytic process	–
Smp_054160	Glutathione-*S*-transferase (KOG1695)	Glutathione transferase activity	Transferase activity	–
Smp_009760	14-3-3 protein, putative (COG5040)	Protein binding, ATP binding, Protein domain specific binding	Cell cycle	Chaperone
Smp_034840	14-3-3 epsilon (KOG0841)	Protein binding, ATP binding, Protein domain specific binding	Cell cycle	Chaperone
Smp_079010	Camp-dependent protein kinase type II-alpha regulatory subunit, putative (KOG0616)	Kinase activity, Protein binding, Kinase regulator activity	Protein phosphorylation Cellular process Regulation of catalytic activity	Kinase modulator
Smp_008660	Putative gelsolin (KOG0443)	Structural constituent of cytoskeleton, Cytoskeletal protein binding	Cellular process, Cellular component organization or biogenesis	Cytoskeletal protein
Smp_049300	Putative major egg antigen (KOG3591)	–	–	–
Smp_048560	Putative four and A half lim domains (KOG1704)	Zinc ion binding, Metal ion binding	–	–
Smp_047370	Malate dehydrogenase (COG0039)	Oxidoreductase activity	Generation of precursor metabolites and energy, Carbohydrate metabolic process, Tricarboxylic acid cycle	Dehydrogenase
Smp_056970	Glyceraldehyde-3-phosphate dehydrogenase (COG00570)	Glyceraldehyde-3-phosphate dehydrogenase (NAD+) (phosphorylating) activity, NAD and NADP binding	Glucose metabolic process Glycolysis	Oxidoreductase Dehydrogenase
Smp_030370	Calreticulin autoantigen homolog, putative (KOG0674)	Calcium ion binding	Protein folding	Calcium-binding protein
Smp_090120	Putative alpha tubulin (COG5023)	Structural constituent of cytoskeleton	Cellular component movement, Mitosis, Intracellular protein transport, Cellular component organization	Tubulin
Smp_072330	Putative heat shock protein (COG0071)	ATP binding	Protein folding	Chaperone
Smp_021920	Putative paramyosin (KOG0161)	Motor activity, Structural constituent of cytoskeleton, Protein binding, Enzyme regulator activity	Metabolic process, Cytokinesis, Cellular component movement, Mitosis, Cell communication, Muscle contraction, Sensory perception of sound, Sensory perception, Mesoderm development, Anatomical structure morphogenesis, Intracellular protein transport, Vesicle-mediated transport, Regulation of catalytic activity, Cellular component organization	G-protein modulator, Actin binding motor protein, Cell junction protein

Table 2 (*Continued*)

IDs[a]	Protein (COG/KOG IDs)[b]	GO molecular function	GO biological process	PANTHER protein class
Smp_194770	Taurocyamine kinase (KOG3581)	ATP binding, Metal ion binding, Oxidoreductase activity, Taurocyamine kinase activity	Catalyzes	–
Smp_063090	Aconitate hydratase (COG1048)	Hydrolyase activity	Generation of precursor metabolites and energy, Carbohydrate metabolic process, Tricarboxylic acid cycle, Cellular amino acid biosynthetic process	Dehydratase, Hydratase
Smp_042170	Putative uncharacterized protein (KOG1557)	–	–	–

[a] *S. mansoni* Genedb database identifier.

[b] COG, cluster of orthologous groups; KOG, eukaryotic orthologous groups; GO, Gene Ontology terms; PANTHER classification resultant from enrichment in UNIPROT and PATHER tools.

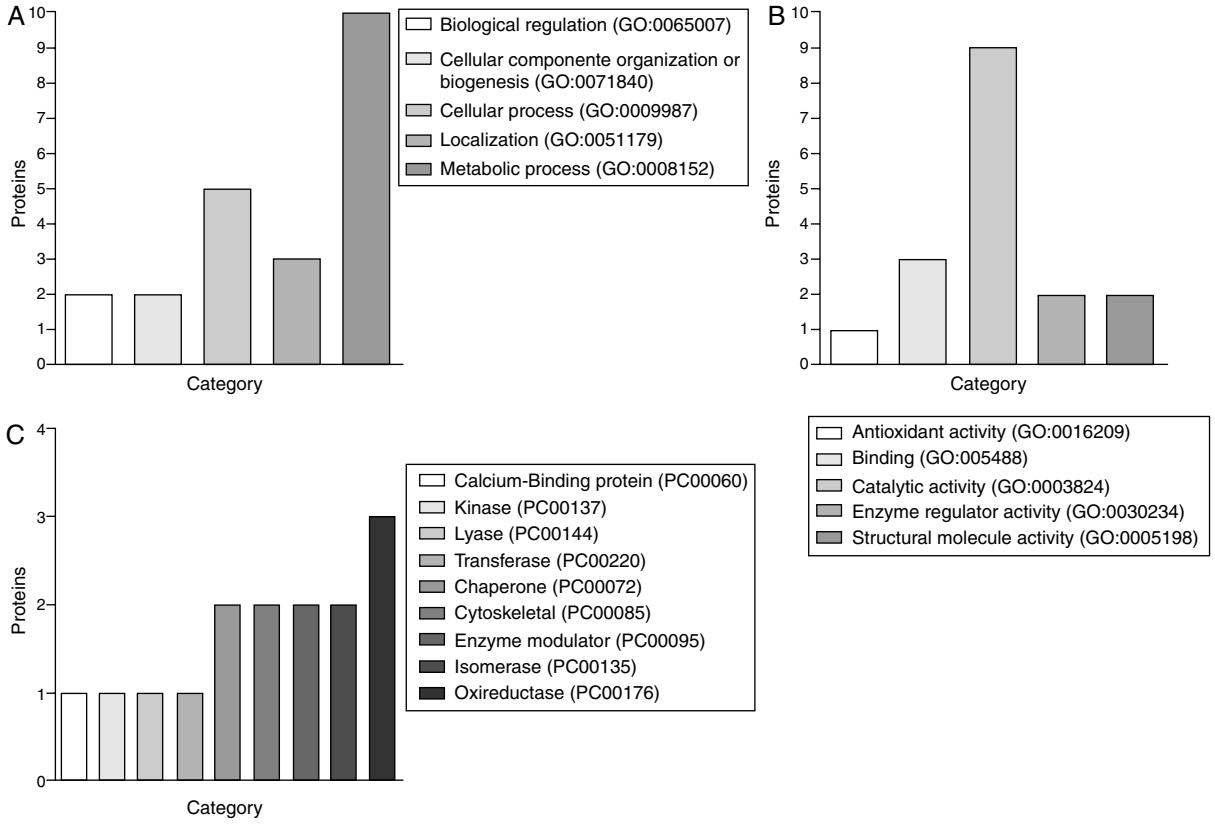


Fig. 2. Classification of *Schistosoma mansoni* adult proteins according to biological processes and molecular function. Enrichment of GO biological processes (A), GO molecular function (B) and PANTHER protein class (C) retrieved by PANTHER classification system.

parasitic helminthes depend heavily on carbohydrate metabolism to supply their energy demand, whereas one quarter to one third of their energy derives from aerobic respiration (Coles, 1973; Barrett, 2009).

Evaluation of putative interactions on STRING protein mode resulted in a distinct and smaller network (Fig. 3B). Paramyosin, GST, triosephosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase and their reduced expression after exposure to DNH indicates that those proteins participate of common biological processes. Indeed, all of them were enriched in metabolic processes, while triosephosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase were specifically associated to glycolysis (Fig. 3B and Table 2). Previous study demonstrated that *S. mansoni* GST has a low level of homology with GST found in mammalians, which reinforces its potential as a target for development of new drugs and vaccines (Taylor et al., 1988). In addition, several anthelmintics can also bind to glutathione transferases, especially those containing a phenolic ring (Barrett, 2009).

Previous study revealed that peroxiredoxin is significantly more abundant after exposure to PZQ (Aragon et al., 2009), thus as expected, we observed an increase in expression of this enzyme after exposure to DNH. Overall, our data suggest that schistosomes exposed to DNH may increase the production of peroxiredoxins as a defense mechanism against the stress and oxidative process, which might be a common process between parasitic worms.

Acidic proteins of the 14-3-3 family are widely expressed by *S. mansoni*. These proteins self-assemble spontaneously and form phosphoserine–threonine-binding dimers that can interact with over 70 different proteins (Tzivion and Avruch, 2002). Studies have shown that these proteins could be involved in the regulation of PKC functions during the worm development (Wiest et al., 1992; Siles-Lucas and Gottstein, 2003). In addition, it has been

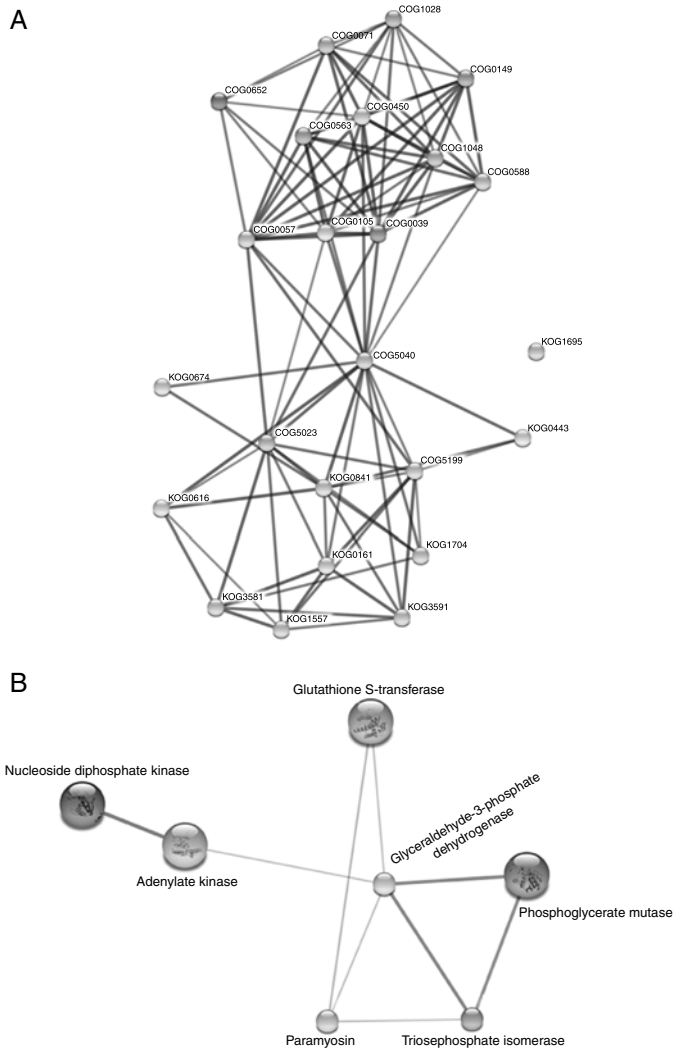


Fig. 3. Network of interactions from *Schistosoma mansoni* proteins affected by exposure to DNH. Identified *S. mansoni* proteins were matched to *S. japonicum* on STRING database v9.05. (A) Network of interactions on Cluster of orthologous groups/Eukaryotic orthologous groups (COG/KOG) mode. (B) Network of interactions on protein mode; nucleoside diphosphate kinase, adenylate kinase, glutathione *S*-transferase, glyceraldehyde-3-phosphate dehydrogenase, paramyosin, triosephosphate isomerase, phosphoglycerate mutase.

found that 14-3-3 epsilon interacts with the intra-cytoplasmic phosphorylated *S. mansoni* receptor kinase-1, but also binds to and modulate the activity of human trans-forming growth factor β receptor-1 (TβRI) (McGonigle et al., 2001; Siles-Lucas and Gottstein, 2003).

Tegumental antigens mediate several signaling and transport processes, such as the regulation of calcium levels and control of muscle contraction (Mohamed et al., 1998). Therefore, these proteins represent potential targets to new therapy compounds and vaccines (Fonseca et al., 2012). DNH may also act on parasite structure, since we observed a high expression of the tegumental antigen Sm20.8 in response to DNH. This hypothesis is further corroborated by the increased expression of gelsolin that has been associated with inhibition of cell apoptosis by blocking mitochondrial membrane loss and preventing the release of cytochrome C (Koya et al., 2000). Indeed, tegumental antigen Sm20.8, gelsolin, calponin, alpha tubulin and paramyosin are proteins particularly important for the organization of the syncytial tegument of schistosomes and have a significant role in host-parasite interactions (Jones et al., 2004).

Overall, our study adds new knowledge into the understanding of anti-schistosomicidal activity of semi-synthetic (−)-6,6′-dinitrohinokinin a derivative compound from lignan and raises new perspectives for future therapeutic approaches.

Author contributions

TCL, MLAS, RSL and JKB contributed by synthesis of DNH and drafting the paper. LGM, RGP, DPG and ERM contributed by proteome analysis and drafting the paper. LGM, LGG and GRG contributed by analyzing the data. APY, VR, AASF and WRC supervised the proteome analysis and critically read the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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