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RESEARCH ARTICLE

Vector-based RNA interference of cathepsin B1 in *Schistosoma mansoni*

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Abstract In helminth parasites, proteolytic enzymes have been implicated in facilitating host invasion, moulting, feeding, and evasion of the host immune response. These key functions render them potential targets for anti-parasite chemotherapy and immunotherapy. Schistosomes feed on host blood and the digested haemoglobin is their major source of amino acids. Haemoglobin digestion is essential for parasite development, growth, and reproduction. We recently reported the use of pseudotyped Moloney murine leukaemia virus to accomplish transformation of *Schistosoma mansoni*. Here, we report the design of a viral vector expressing a dsRNA hairpin to silence expression of the schistosome cathepsin B1 (*SmCB1*) gene. We observed 80% reduction in transcript level 72 h after virus exposure and complete silencing of enzyme activity in transduced worms. This is the first report using this technology in any helminth parasite. It will facilitate the evaluation of potential drug targets and biochemical pathways for novel interventions in schistosomes.

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Introduction

Schistosomiasis (bilharziosis) is one of the most severe parasitic diseases, threatening millions of people with chronic illness, disfigurement, or death, and is caused by blood flukes of the genus *Schistosoma*. The disease is endemic in 76 countries and over 280,000 deaths occur annually due to schistosomiasis mainly in rural areas of the developing world, which is testimony to its public health significance [1, 2]. The current therapy, chemotherapy with praziquantel, is faced with the problem of drug resistance, and vaccines are not available. Thus, the identification of new drug targets, development of effective vaccines, and diagnostic methods is a public health priority. The best prospect for achieving this aim resides in the analysis of the parasite's genome [3–6].

Schistosomes feed on the host blood and digestion of haemoglobin from erythrocytes provides the major source of nutrients and amino acids that are essential for the parasite's development, growth, and reproduction [7]. The erythrocytes are lysed by haemolysin(s) within the oesophagus of the parasites and the haemoglobin released is ultimately catabolized in the gut to dipeptides and/or free amino acids (Fig. 1). Broad-spectrum protease inhibitors have a profound anti-schistosomal and anti-pathology effect, demonstrating the essential role of this pathway in schistosome metabolism [8]. The importance of this crucial pathway was recently reinforced by RNAi silencing of cathepsin B (*SmCB1*) and cathepsin D

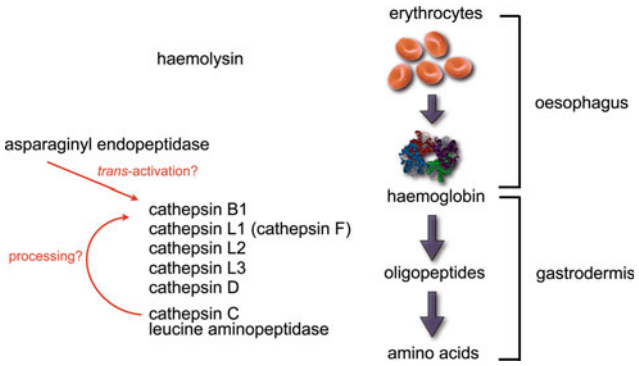

Fig. 1 Peptidases involved in the digestion of host haemoglobin in the gut of *S. mansoni*. Schistosomes utilize cysteine, aspartic and metallo-proteases to liberate peptides and amino acids from host haemoglobin. Haemolysin activity in the oesophagus lyses red blood cells and haemoglobin is released. It is speculated (*red arrows*) that an asparaginyl endopeptidase activates pro-cathepsin B1 and other pro-peptidases such as cathepsins L1 and L2, and cathepsin D in vivo. Cathepsin C may further process cathepsin B1 to its mature form

(*SmCD*), which resulted in a visible growth retardation phenotype in cultured schistosomes during early development in vitro [9, 10].

The potential of intestinal peptidases as targets for chemotherapy of schistosomiasis has driven the molecular study of these enzymes [11–14]. The entire pathway for haemoglobin degradation consists of at least eight proteolytic enzymes that break down haemoglobin [15–17]. Numerous questions remain regarding the exact mechanisms driving the digestive process in vivo; the structure of the signalling cascade; the contribution of the various components and degree of redundancy.

We have recently described the use of pseudotyped Moloney murine leukaemia virus to accomplish transformation of *S. mansoni* [18, 19]. Here, we report the design of a retroviral vector expressing a dsRNA hairpin loop, thus coupling a powerful delivery vehicle with a potent RNA interference (RNAi) mechanism to specifically silence expression of *SmCB1*. We have targeted *SmCB1* because RNAi knockdown of this protein delivers a visible phenotype [9] and it enables us to compare conventional RNAi with our vector-based delivery of a dsRNA hairpin. We observed striking suppression in transcript levels, accompanied by a complete ablation of the cognate enzyme activity in transduced worms. To our knowledge, this is the first report of this technology—retroviral vector-based RNAi—being successfully employed in any helminth parasite. These findings underscore the potential and power of vector-based RNAi to evaluate potential drug targets and biochemical pathways for novel interventions in schistosomes.

Materials and methods

Parasites

Biomphalaria glabrata snails, infected with the NMRI (Puerto Rican) strain of *S. mansoni,* were supplied by Dr. Fred Lewis (Biomedical Research Institute, Rockville, MD, USA). The schistosome lifecycle was maintained in our laboratory by passaging through BALB/c mice as previously described [20]. Adult schistosomes, perfused from mice 49 days after infection with cercariae, were cultured at 37°C and 5% CO_2 in DMEM (Gibco), supplemented with 10% foetal bovine serum, 100 U penicillin and streptomycin, and small quantities of washed mouse erythrocytes, 2 µl washed erythrocytes (50% packed red cells)/ml culture medium, before and after exposure to virus [19].

Design and construction of retroviral expression vectors

The retroviral expression vector was assembled in the pLNHX-SmAct-Luc plasmid [18] by substitution of the luciferase gene with a 250-nt cassette (GenBank accession number GQ497283) containing sequence in forward and reverse orientation from the transcription initiation site of the *SmCB1* (GenBank accession number M21309.1) joined by a stretch of six nucleotides comprising a *SacI* restriction site (Fig. 2a). The *SmCB1* sequence was amplified from genomic DNA (gDNA), isolated from mixed-sex adult schistosomes as described [18], with the following primers incorporating the recognition sequences for *Bgl*II, *Cla*I and *Sac*I restriction enzymes (underlined): CB_sense_fw: TTAGAAGCTTAGATCTATGCTCACATCTATTTTGTGTATTGCTTC; CB_sense_rv: TCTACTGTATTAGAGCATATAGTTACTTGTATCTCGAGGATT; CB_antisense_fw: TTAGGAGCTCAATGTTCATTGATATACGAGATTATGTCATCT; and CB_anti-sense_rv: CTTCGTTATGTGTTTTATCTACACTCGTACAAAAATAGCTAGATT. The amplified sequences were sub-cloned into the vector pLNHX-SmAct-Luc by *Bgl*II and *Cla*I replacing the luciferase gene.

Pseudo-typed retrovirus production and transduction of schistosomes

Production of VSVG-pseudotyped virions in packaging cells was accomplished as described [18]. In brief, GP2-293 packaging cells (modified HEK-293, Clontech) were co-transfected with either pVSVG and pLNHX-SmAct-Luc or pVSVG and pLNHX-SmAct-CB1^{RNAi} plasmids in the presence of lipofectamine 2000 transfection reagent (Invitrogen) as recommended by the manufacturer. Two days later, culture supernatants containing the virions

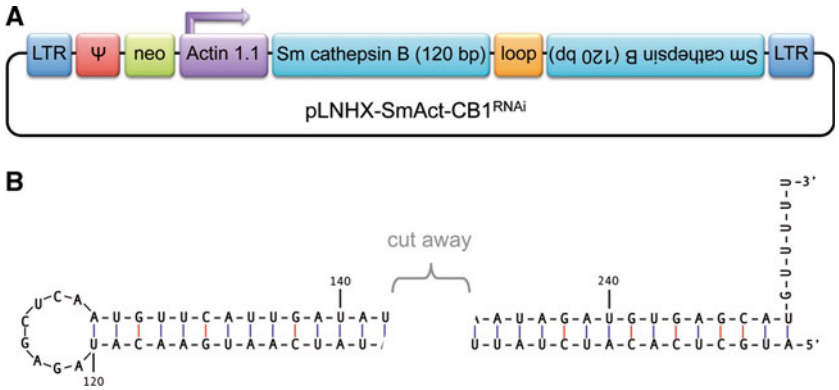

Fig. 2 Schematic representations of the retroviral vector and the dsRNA hairpin. **a** The pLNHX vector containing the MMLV long terminal repeats (*LTR*), the packaging signal (*Ψ*), and a neomycin selection marker (neo^r), modified to include the *S. mansoni* actin 1.1 gene promoter (Act 1.1) driving the transcription of inverted regions of the *S. mansoni* cathepsin B1 encoding sequence linked by a spacer (*loop*). **b** RNA hairpin structure of the transcribed sequence. For clarity, the central part of the molecule has been cut away. The structure was generated using mfold (http://mfold.burnet.edu.au/)

carrying either the luciferase gene (control virus) or the shCB1^{RNAi} cassette (for silencing SmCB1) were filtered through 0.45-µm-pore-size filters and treated with DNaseI (1,000 U/ml, NEB) for 1 h at room temperature to remove any contaminating DNA plasmid. Viral titre was determined using target NIH-3T3 mouse fibroblast cells in the presence of the antibiotic geneticin (G418, Gibco) for selection and crystal violet solution for determining infectivity in colony forming units (CFU). Three independent experiments were carried out where 50 mixed-sex adult *S. mansoni* worms were cultured in 3 ml of media containing 200 µl of virus with infectivity between 1×10^4 and 1×10^5 CFU/ml or in the range of one to several thousand virions per worm. Viral exposure was for 3 h only, in order to avoid toxicity of the cationic polymer polybrene (1.4 µg/ml culture medium [18], Sigma-Aldrich) used to increase the efficiency of infection. Worms were harvested at different time points after transduction, washed in PBS, and stored at −80°C for transcription and enzyme activity assays or cultured in vitro for analyses of long-term silencing effects.

Gene expression analyses

Total RNA was isolated from approximately 50 mixed-sex adult worms, either exposed to virus (24 or 72 h) or from non-exposed wild-type controls using TRIzol (Invitrogen) according to the manufacturer's instructions. Residual DNA in the samples was removed by treatment with RQ1 RNase-free DNase (Promega) at 37°C for 30 min. First strand cDNA was synthesised using random hexamers and AMV Reverse Transcriptase (Promega). The target-specific oligonucleotide pairs have been described elsewhere [21] with the modification of the CathepsinbR primer: TCGACCGCACCGAAAGCCCA where A was substituted to C at position 368 in accordance with the published cDNA sequence. Alpha tubulin was amplified as a reference gene with oligonucleotides as described [21]. The luciferase *trans*-gene and the cytochrome C oxidase 1 (*SmCox1*) were amplified by employing previously published oligonucleotides [18] while the Cathepsin D gene (U60995) was amplified to control for off target effect with the following oligonucleotides: CD_Fw: ATGCTGCTTCT AGTTCTTCTTCTAC and CD_Rv: AAACAATTTCT AGAGAAGTTTC. Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green PCR Master mix (Applied Biosystems) in a total volume of 20 µl according to the manufacturer's conditions. Three independent transduction experiments were performed and all samples were run in triplicate. The thermal profile regime followed included: (segment 1) 10:00 at 95°C for 1 cycle; (segment 2) 0:30 at 95°C, 1:00 at 58°C, 0:30 at 72°C for 40 amplification cycles, (segment 3) 1:00 at 95°C, 0:30 at 55°C, 0:30 at 95°C for 1 cycle using a Stratagene 3000 thermal cycler (Integrated Sciences). For relative quantification, REST-384 beta, described by Pffafl was employed [22, 23], using schistosome α-tubulin as the reference gene for each sample.

Enzyme activity assays

Activity assays were carried out as described previously [9, 21, 24] with minor modifications. Adult worms were homogenised at 4°C in 500 µl phosphate buffered saline. The total protein concentration in the soluble fraction was determined using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). A total of 1 µg of soluble protein from each group, wild-type and virus-treated (SmCB1^{RNAi}), was pre-incubated for 15 min at 37°C in 0.1 M phosphate buffered saline (pH 7) and 1 mM dithiothreitol prior to

addition of the cathepsin B-diagnostic substrate *Z*-Arg-Arg-AMC (Sigma-Aldrich) [9, 24] at 20 μM final concentration. Assays were carried out in triplicates in a total volume of 200 μl. In this assay, the fluorogenic substrate is cleaved by the protease and the resulting fluorescence is measured over 200 cycles with 15 flashes and a gain setting of five with a FLUOstar OPTIMA automated microplate based multi-detection reader (BMG Labtech) using excitation and emission wavelength filters of 360 and 460 nm, respectively. Inhibition of cysteine proteases was effected by incubation of the schistosome extracts with peptidyl-diazomethylketone *Z*-Phe-Ala-CHN_2 (Sigma-Aldrich), a potent inhibitor of Clan CA cysteine proteases including cathepsins B and L of *S. mansoni* [8, 25] for 15 min prior to the addition of the substrate. Relative cathepsin B activity was determined by comparing the relative fluorescence units (RFU) with those of the non-induced control or the negative control sample.

Statistical analyses

All data represent the mean of three independent transduction experiments (±SE). The analyses of qRT-PCR data are based on the pair-wise fixed reallocation randomisation test using the relative expression software tool (REST-384 beta) [22, 23]. Differences between experimental groups were assessed for statistical significance by Student's *t* test and *p* values of <0.05 were considered statistically significant. All changes were calculated relative to the unexposed wild-type control group. Linear regression analyses on the fluorescence measurements were performed using GraphPad Prism statistical software with results being expressed as a slope of the increase in fluorescent product with time (s). For clarity and simplicity of representation and interpretation of results, the slope was expressed as fold change in activity compared to the unexposed wild-type control sample. A Kaplan and Meier survival curve [26] was established and the 95% confidence interval for fractional survival at any particular time was calculated by both the log-rank (Mantel-Cox) test and the Gehan–Breslow–Wilcoxon test (GraphPad Prism 5, GraphPad Software, Inc, USA).

Results

We have previously demonstrated that replication-incompetent Moloney murine leukaemia virus (MMLV) virions pseudotyped with vesicular stomatitis virus glycoprotein (VSVG) were capable of transducing schistosomes and integrating into the schistosome genome [18, 19]. After exposure of schistosomes to virions, harvested from producer cell cultures, immunofluorescence studies indicated that the VSVG envelope on the pseudotyped virions interacted with the schistosome surface after which the retroviral capsid and RNA genome was released within the surface cells [18]. The ability of the retroviral particles to stably integrate into schistosome genome and express *trans*-genes (EGFP, luciferase, neomycin phosphotransferase) without causing any damage to the schistosomes, established the foundation for the experiments described below. Here, we have investigated whether the retroviral delivery system might be adapted as a tool for continuous generation of RNAi using dsRNA to achieve specific suppression of SmCB1 transcription and to investigate the long-term silencing.

RNAi mediated silencing of cathepsin B transcription

In order to demonstrate integration into the schistosome genome and confirm expression of the *trans*-gene, we have generated virions encoding luciferase from the firefly *Photinus pyralis* under the control of the endogenous *S. mansoni* actin promoter. Transcription of the *trans*-gene will only occur when the virus genome is integrated into the host genome. Approximately 50 mixed-sex adult worms were exposed to several thousand pLNHX-SmAct-Luc virions per worm for 3 h and harvested 24 and 72 h later. The equivalent number of non-exposed adult worms was also included in the experiment for comparison in the gene transcription analyses. Total RNA was extracted from either transduced or control worms and the presence of luciferase transcripts were investigated by qRT-PCR. Luciferase transcripts were detected at both 24- and 72-h samples after exposure to virions carrying the *trans*-gene in a dose-dependant manner, while no expression was detected in the control worms (Fig. 3).

Following the same experimental design, we then generated virions (pLNHX-SmAct-$CB1^{RNAi}$) that after integration into the genome lead to the transcription, under the control of the *S. mansoni* actin 1.1 promoter, of a double-stranded hairpin RNA. The dsRNA triggers the cell defence machinery [27–29] for degradation and generation of multiple short siRNAs targeting the *SmCB1* gene for silencing (Fig. 2b). The exposure to virus and gene-expression analyses were performed in parallel to the experiments described above. Transcripts encoding *SmCB1* and α-tubulin were measured in both transduced and non-exposed schistosomes. The results were normalised by the expression level of the α-tubulin gene (housekeeping control) and expressed as a fold difference compared to the non-exposed worms. Although only a moderate silencing efficiency of 50% was seen after 24 h (or 0.485 ± 0.126), strong 80% SmCB1-specific suppression of transcription was evident 72 h (or 0.239 ± 0.019; $p < 0.05$) after virus exposure (Fig. 4a). These rates are comparable to previous

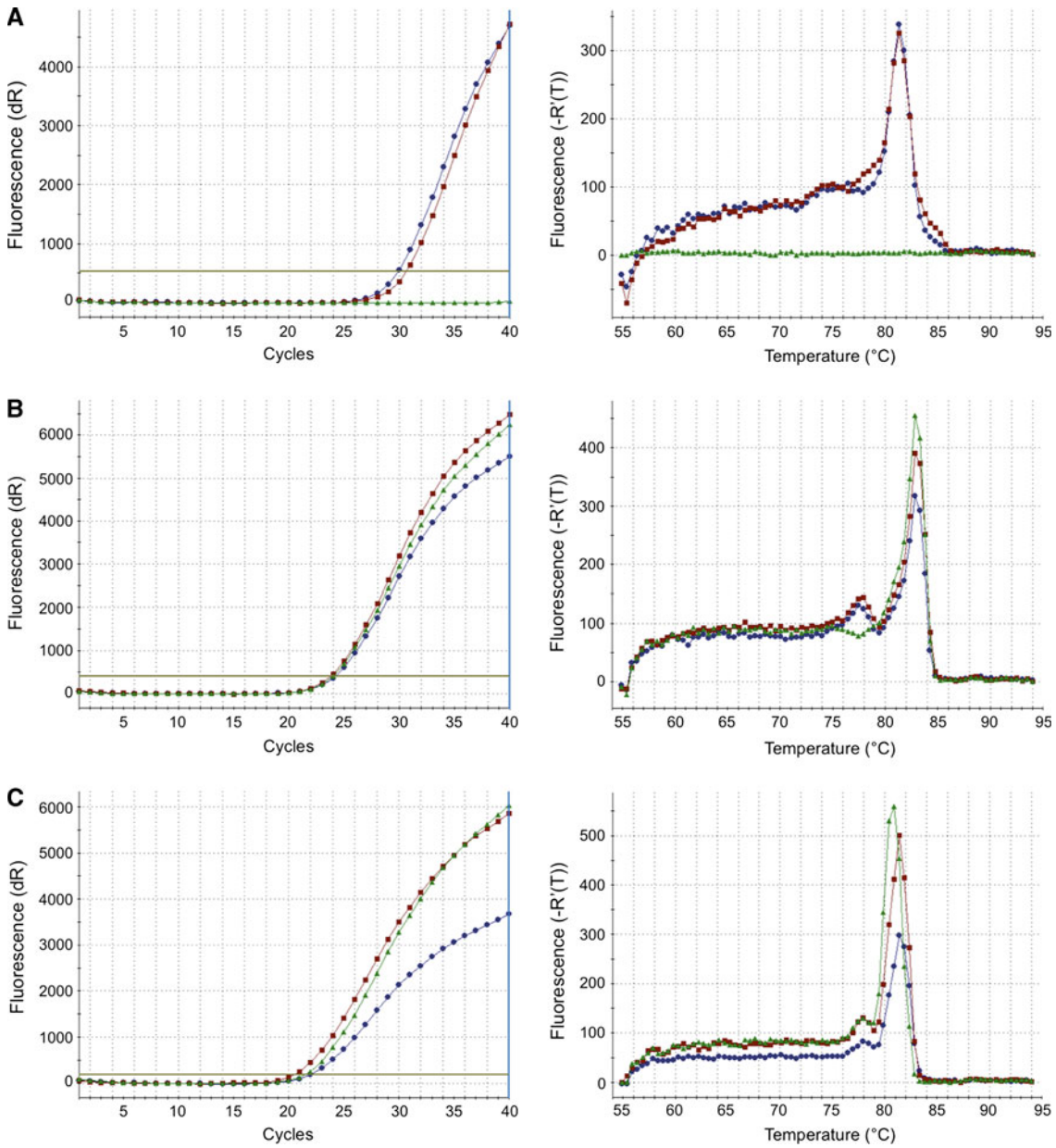

Fig. 3 Gene transcription analysis following transduction of *S. mansoni* with pLNHX-SmAct-Luc virions. Gene expression is demonstrated by the amplification of transcripts, and specificity of the reaction—by dissociation curves of the primers employed in a qRT-PCR. **a** Relative luciferase expression is detected at both 24 h (*blue symbols*) and 72 h (*red symbols*) after viral transduction. In contrast, no luciferase transcripts were present in the non-transduced parasites (*green symbols*). Transcription of the *S. mansoni* tubulin (**b**) and *SmCB1* genes (**c**) was apparent at all time points and also in the non-exposed parasites

results reported for RNAi-mediated silencing in schistosomes (reviewed in [6]) and on *SmCB1* expression in particular [9, 15, 21, 24].

Furthermore, we have measured the transcription of both the *SmCD* and *SmCox1* genes to control for off-target effect (Fig. 4b). The inhibitory effect mediated by exposure of worms to pLNHX-SmAct-CB1^{RNAi} virions was specific to *SmCB1* and expression of neither *SmCD* nor *SmCox1* transcript levels were affected.

Deficiency in enzyme activity in transduced schistosomes

To investigate whether the observed RNAi-mediated reduction in transcript levels was also reflected in the loss of protease activity or if residual SmCB1 activity could still be detected in worms transduced with the virus carrying SmAct-CB1^{RNAi}, we assessed SmCB1 activity in total soluble extracts of schistosomes by using the fluorogenic

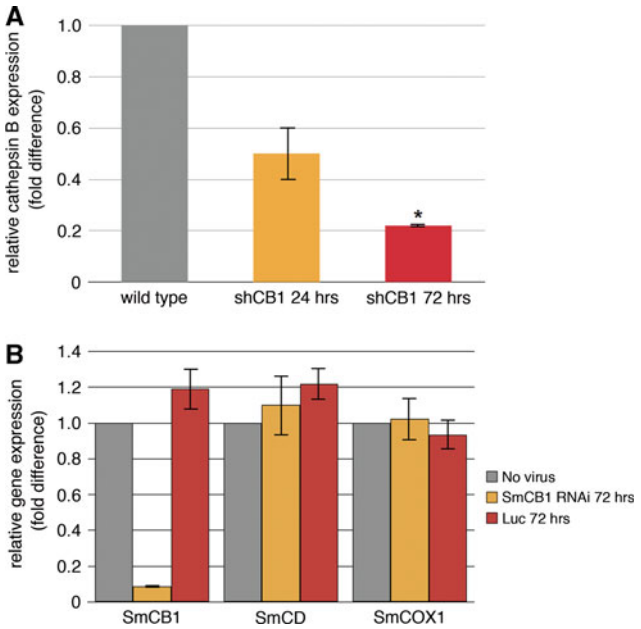

Fig. 4 Analyses of downstream molecular events following transduction of *S. mansoni* by virions carrying a dsRNA hairpin loop specific for cathepsin B1 (shCB1^{RNAi}). **a** Relative transcript levels of cathepsin B1. The results are normalised by the expression levels of the housekeeping gene α-tubulin and represented as a fold difference compared to the wild-type control sample (±SE). Results are averaged from three independent experiments. Significance at $p < 0.05$ is indicated by an *asterisk*. **b** To control for off-target silencing effect, the transcription level of two additional genes *SmCD* and *SmCox1* was also measured at 72 h post transduction with either pLNHX-SmAct-Luc (*red bars*) or pLNHX-SmAct-CB1^{RNAi} (*orange bars*) virions and compared to non-transduced worms (*grey bars*). While transcription of *SmCB1* was dramatically reduced after exposure to pLNHX-SmAct-CB1^{RNAi} virions, expression of both *SmCD* and *SmCox1* genes was not affected as a result of exposure to any of the viruses

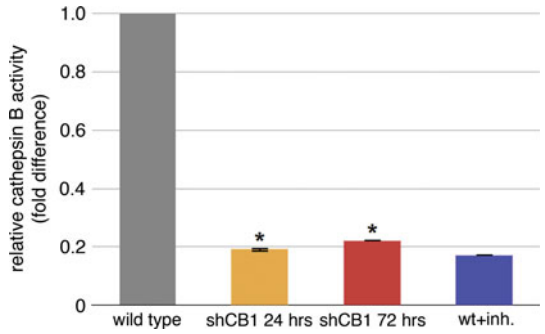

Fig. 5 Analysis of SmCB1 proteolytic activity. Fluorescence was measured in a fluorometer with excitation at 355 nm and emission at 460 nm in a 200-µl reaction mix of 1 µg total protein (1:200 dilution) and 20 µM substrate for 200 cycles, with 15 flashes at gain 5 and 37°C. In the control samples, 20 µM of inhibitor was pre-incubated with the extracts prior to addition of the substrate. Data were measured as relative cathepsin B activity in units/sec and expressed as a fold difference compared to the wild-type (not exposed to virions) control. Results are averaged from three independent experiments. Significance at $p < 0.0001$ is indicated by an *asterisk*

substrate, *Z*-Arg-Arg-AMC, which is relatively specific for cathepsin B under the conditions employed [9, 15, 21, 24, 25, 30–32]. In addition, the cathepsin B-specific protease inhibitor *Z*-Phe-Ala-CHN$_2$ was used to further enhance the specificity of the assay under the conditions applied and also as a positive control. Fluorescence emitted after cleavage of the substrate by the protease was detected in real time and the enzyme activity expressed as relative fluorescence units (RFU) per second, and then converted to fold difference compared to a wild-type control (Fig. 5). The observed suppression of cathepsin B transcription was accompanied by a profound reduction in enzyme activity at both time points monitored. Furthermore, the level of SmCB1 activity in the samples exposed to the virions was comparable to the activity in samples pre-incubated with the protease inhibitor, demonstrating the specificity and efficiency of silencing. Moreover, no difference in the level of enzyme activity was detected in the presence (or absence) of inhibitor in extracts of transduced worms (not shown).

Silencing effect on cultured worms in vitro

Retroviruses have the advantage of being able to stably integrate into the host genome, ensuring permanent and stable gene transfer for persistent expression of reporter genes and shRNA cassettes. This presents an ideal opportunity to investigate long-term silencing as opposed to the transient suppression of gene expression achieved in parasitic helminths so far (reviewed in [4]). Equal numbers of mixed-sex adult worms, either exposed to virus or unexposed, were cultured in DMEM media supplemented with washed erythrocytes in vitro at 37°C, 5% CO_2 and monitored daily for viability. The worms were considered dead when all evidence of motility, including gut peristalsis, had ceased. A significant 50% reduction in viability of worms exposed to pLNHX-SmAct-CB1^{RNAi} virions was observed within the first 10 days after exposure (Fig. 6). In parallel, there occurred an approximate 20% decrease in viability in schistosomes in the control groups (unexposed to virions or exposed to pLNHX-SmAct-Luc virions) over the entire period of the study, which was taken into consideration in the representation of the results. There was no significant difference between the survival curves of unexposed worms and worms exposed to pLNHX-SmAct-Luc virions. Each of the survival curves of the two control groups was significantly different to survival data obtained from worms transduced with pLNHX-SmAct-CB1^{RNAi} virions. The experiments were terminated with the death of all the worms exposed to pLNHX-SmAct-CB1^{RNAi} virions.

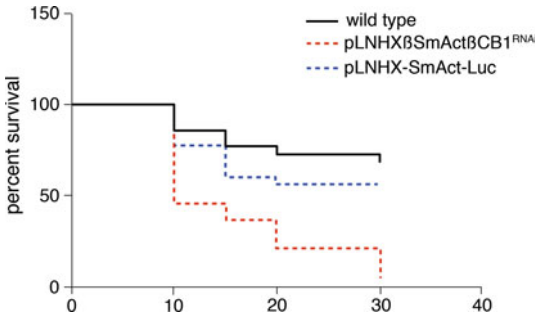

Fig. 6 Kaplan–Meier survival curve. A representative set of survival curves (out of three independent experiments) is shown. A 95% confidence interval for fractional survival at any particular time was calculated by both the log-rank (Mantel–Cox) test and the Gehan–Breslow–Wilcoxon test. The survival curves show significance when either the wild-type ($p < 0.0001$) or the pLNHX-SmAct-Luc control ($p = 0.0002$) is compared to pLNHX-SmAct-CB1^{RNAi} and no difference is observed when only the two controls are considered ($p = 0.07$) (GraphPad Prism 5) and [26]

Discussion

Retroviral vectors based on the Moloney murine leukemia virus (MMLV) have become established as standard tools for foreign gene transfer and expression in insect and mammalian cells, both in vitro and in vivo [33, 34]. For more than a decade, these defective, replication incompetent viruses have been shown to be safe when generated under laboratory conditions and incapable of self-propagation. Among the major advantages of the retroviral system is the ability of the virions to attach, uncoat, reverse-transcribe, and stably integrate into the genome to create transgenic organisms [35–38]. The high transformation efficiency demonstrated by VSVG pseudotyped viruses in mammalian cell culture (90–95%) [39], their ability to integrate in the host genome ensuring persistent expression of reporter genes and RNAi cassettes without otherwise harming the cell and the potential to tightly regulate the level of gene silencing by controlling the amount of virus (RNAi), renders them powerful tools not only for gene manipulation in schistosomes but also for functional genomics analyses requiring the continuous suppression of transcription. Since RNAi represents the only method currently available for manipulating gene expression in schistosomes, we have adapted the MMLV retroviral transduction system as a delivery tool to introduce short hairpin RNA molecules for long-term silencing of targeted gene expression and functional genomics studies in schistosomes. We recently reviewed the advances in mRNA silencing and transgene expression in the schistosomes [6] where we have summarized the methods for vector-mediated gene silencing by siRNAs in schistosomes. Different delivery approaches have been described for the introduction of siRNAs or shRNA expressed by different promoters. Zhao et al. [40] have used the mammalian Pol III promoter H1 to express shRNA targeting the Mago nashi gene for silencing and have introduced the expression vector by electroporation into schistosomula. The shRNA expressed from the mammalian Pol III promoter H1 specifically reduced the levels of Mago nashi mRNA and proteins in *S. japonicum* accompanied by pronounced phenotypic changes in the testicular lobes. Here we describe a fundamentally different approach for the generation of the expression cassette, in which the shRNA is driven from an endogenous schistosome promoter and a retroviral vector is used for the first time for the delivery and accomplishment of a long-lasting and specific gene silencing effect. We have taken advantage of the fact that schistosomes have the complete set of proteins required for gene silencing including Dicer and RNA-induced silencing complexes (RISCs) and that they can process long dsRNAs, similar to *C. elegans* and *Drosophila*, into functional 20 to 23-nt short-interfering RNAs (siRNAs), which in turn recognise and destroy homologous target mRNAs in an endonucleolytic manner [41–47]. To ensure effective silencing without screening through numerous siRNAs, we targeted the first 250 bp from the transcription start codon of the *SmCB1* gene and transcription of the shRNA molecule was driven constitutively by the endogenous SmAct1 promoter. Transcription results in the formation of a stem-loop structure (hairpin) molecule [48, 49], which is recognised by the cellular machinery as foreign [27–29] and targeted for degradation by generating nicks in the sequence and production of multiple siRNAs targeting the endogenous *SmCB1*. Two cathepsin B encoding genes, *SmCB1.1* and *SmCB1.2,* expressed predominantly in the gut and in the tegument, respectively, are known to be active in schistosomes [50]. We have targeted the expression of the *SmCB1.1* gene since it is localised in the gut where digestion of haemoglobin is accomplished [15]. The moderate effect observed at 24 h post-virus exposure might be due partly to the time required for the virus to be reversed transcribed into the provirus and/or integrate before transcription to occur and also the time needed to produce sufficient siRNAs to silence transcription of the target gene. The level was reduced dramatically 72 h post-virus exposure the *SmCB1* transcription. The suppression of 80% observed on *SmCB1* transcription is in agreement with previous observations in schistosomules [21].

Suppression of *SmCB1* transcription was accompanied by a profound reduction in protease activity at both time points, comparable to the effect of a relatively specific cathepsin B inhibitor, demonstrating the high specificity and efficiency of silencing achieved with this retroviral vector-based RNAi. Taken together, the results demonstrate the efficacy of the retroviral system in significantly

attenuating the level of *SmCB1* transcription, which was reflected at the protein level by the reduction in enzyme activity in crude extracts, and was accompanied by a lethal phenotype in vitro within 3 weeks post-viral transduction.

The phenotypic difference in the outcome of silencing *SmCB1* in schistosomules [9] compared to the lethal phenotype we have observed in adult worms might be due to the developmental and stage-specific differences. In schistosomules, the suppression of *SmCB1* transcription and protein activity results in a growth retardation phenotype. This might be explained by the fact that in schistosomules the gut is not yet fully developed and they are not entirely dependent on the digestion of haemoglobin to obtain nutrients as they are able to digest other serum proteins. In vivo, mature adult worms have ready access to abundant blood cells where they appear to rely on catabolism of haemoglobin released from ingested erythrocytes as the major source of nutrients [7, 15, 51]. Cathepsin B is also active against albumin and other plasma proteins [15]. Thus, silencing of *SmCB1* in this life cycle stage has a lethal outcome that may be due to starvation. Moreover, cathepsin B is a lysosomal enzyme (see [52]) and is likely present in the lysosomes of most or all schistosome cells, including those distant from the gut. A second cathepsin B certainly is known from the tegument of schistosomes [50], although the shRNA from our construct is only 48% identical to *SmCB2*. Accordingly, silencing of cathepsin B in these other cells may also have taken place, and contributed, in greater or lesser degree, to the demise of the worms. This deleterious phenotype was apparent within the first 10 days after viral exposure and only a few of the worms exposed to pLNHX-SmAct-CB1^{RNAi} virions survived for up to 3 weeks in culture. One explanation for the survival of a small number of worms might be their exposure to lower number of virions allowing sufficient protease synthesis to sustain their vitality. Additionally, it has been suggested that redundancy in activity of component proteases of the proteolytic cascade is likely, with at least partial overlap in the function of the proteases involved in digestion of the haemoglobin from ingested blood [15].

Our future work will target other members of the haemoglobin digestion pathway to further elucidate their individual function. Due to their potential as targets for chemotherapy of schistosomiasis, it has been long proposed that interference with the intestinal peptidases could provide an opportunity for the development of schistosomicidal agents [53]. In addition, gut peptidases are serodiagnostic for schistosomiasis [11, 12] and show potential as vaccine targets [13, 14]. The retroviral system employed here provides an opportunity to transduce worms with mixed populations of viruses, thereby targeting multiple proteases and ability to assess the combined double knock-down effect for further evaluation of protein interactions within the proteolytic signalling cascade. This powerful and functional experimental platform should allow us to study the pathway that leads to the degradation of haemoglobin in detail, and to identify and fully characterise the key enzymes involved in this process. Additionally, we anticipate gaining a better understanding of the interactions between these enzymes, their individual contribution to the proteolytic cascade, and the degree of redundancy present in this system. Severe (lethal) phenotypes that may arise from silencing the respective genes would also be a good indication of the suitability of the targeted enzymes for rational drug development. Furthermore, given that we now have a system for vector-based RNAi in schistosomes, in our future studies we also plan to undertake comparison of the vector-based versus conventional RNAi, targeting SmCB1 and other informative targets. Finally, on a wider scale, the technology described here, especially if the retroviral transgenes can be propagated through the germ line of the schistosome, has the potential for the evaluation of novel drug and vaccine targets, biochemical pathways for novel interventions, and genome-wide genetic analysis in schistosomes.

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References

1. Hotez PJ, Bethony JM, Oliveira SC, Brindley PJ, Loukas A (2008) Multivalent anthelminthic vaccine to prevent hookworm and schistosomiasis. Expert Rev Vaccines 7:745–752
2. Hotez PJ, Brindley PJ, Bethony JM, King CH, Pearce EJ, Jacobson J (2008) Helminth infections: the great neglected tropical diseases. J Clin Invest 118:1311–1321
3. Berriman M, Haas BJ, LoVerde PT, Wilson RA, Dillon GP, Cerqueira GC, Mashiyama ST, Al-Lazikani B, Andrade LF, Ashton PD, Aslett MA, Bartholomeu DC, Blandin G, Caffrey CR, Coghlan A, Coulson R, Day TA, Delcher A, DeMarco R, Djikeng A, Eyre T, Gamble JA, Ghedin E, Gu Y, Hertz-Fowler C, Hirai H, Hirai Y, Houston R, Ivens A, Johnston DA, Lacerda D, Macedo CD, McVeigh P, Ning Z, Oliveira G, Overington JP, Parkhill J, Pertea M, Pierce RJ, Protasio AV, Quail MA, Rajandream MA, Rogers J, Sajid M, Salzberg SL, Stanke M, Tivey AR, White O, Williams DL, Wortman J, Wu W, Zamanian M, Zerlotini A, Fraser-Liggett CM, Barrell BG, El-Sayed NM (2009) The genome of the blood fluke *Schistosoma mansoni*. Nature 460:352–358

4. Kalinna BH, Brindley PJ (2007) Manipulating the manipulators: advances in parasitic helminth transgenesis and RNAi. Trends Parasitol 23:197–204
5. Liu F, Zhou Y, Wang ZQ, Lu G, Zheng H, Brindley PJ, McManus DP, Blair D, Zhang QH, Zhong Y, Wang S, Han ZG, Chen Z (2009) The *Schistosoma japonicum* genome reveals features of host–parasite interplay. Nature 460:345–351
6. Tchoubrieva EB, Kalinna BH (2009) Advances in mRNA silencing and transgene expression: a gateway to functional genomics in schistosomes. Biotechnol Gen Eng Rev 26:273–292
7. Brindley PJ, Kalinna BH, Dalton JP, Day SR, Wong JY, Smythe ML, McManus DP (1997) Proteolytic degradation of host hemoglobin by schistosomes. Mol Biochem Parasitol 89:1–9
8. Abdulla MH, Lim KC, Sajid M, McKerrow JH, Caffrey CR (2007) *Schistosomiasis mansoni*: novel chemotherapy using a cysteine protease inhibitor. PLoS Med 4:e14
9. Correnti JM, Brindley PJ, Pearce EJ (2005) Long-term suppression of cathepsin B levels by RNA interference retards schistosome growth. Mol Biochem Parasitol 143:209–215
10. Morales ME, Rinaldi G, Gobert GN, Kines KJ, Tort JF, Brindley PJ (2008) RNA interference of *Schistosoma mansoni* cathepsin D, the apical enzyme of the hemoglobin proteolysis cascade. Mol Biochem Parasitol 157:160–168
11. Grogan J, Rotmans P, Ghoneim H, Deelder A, Yazdanbakhsh M, Klinkert MQ (1997) Recognition of *Schistosoma mansoni* cathepsins B and L by human IgG1 and IgG4 antibodies. Parasite Immunol 19:215–220
12. Ruppel A, Shi YE, Wei DX, Diesfeld HJ (1987) Sera of *Schistosoma japonicum*-infected patients cross-react with diagnostic 31/32 kD proteins of *S. mansoni*. Clin Exp Immunol 69:291–298
13. Chlichlia K, Bahgat M, Ruppel A, Schirrmacher V (2001) DNA vaccination with asparaginyl endopeptidase (Sm32) from the parasite *Schistosoma mansoni*: anti-fecundity effect induced in mice. Vaccine 20:439–447
14. Verity CK, McManus DP, Brindley PJ (2001) Vaccine efficacy of recombinant cathepsin D aspartic protease from *Schistosoma japonicum*. Parasite Immunol 23:153–162
15. Delcroix M, Sajid M, Caffrey CR, Lim KC, Dvorak J, Hsieh I, Bahgat M, Dissous C, McKerrow JH (2006) A multienzyme network functions in intestinal protein digestion by a platyhelminth parasite. J Biol Chem 281:39316–39329
16. Dvorak J, Mashiyama ST, Sajid M, Braschi S, Delcroix M, Schneider EL, McKerrow WH, Bahgat M, Hansell E, Babbitt PC, Craik CS, McKerrow JH, Caffrey CR (2009) SmCL3, a gastrodermal cysteine protease of the human blood fluke *Schistosoma mansoni*. PLoS Negl Trop Dis 3:e449
17. McCarthy E, Stack C, Donnelly SM, Doyle S, Mann VH, Brindley PJ, Stewart M, Day TA, Maule AG, Dalton JP (2004) Leucine aminopeptidase of the human blood flukes, *Schistosoma mansoni* and *Schistosoma japonicum*. Int J Parasitol 34:703–714
18. Kines KJ, Mann VH, Morales ME, Shelby BD, Kalinna BH, Gobert GN, Chirgwin SR, Brindley PJ (2006) Transduction of *Schistosoma mansoni* by vesicular stomatitis virus glycoprotein-pseudotyped Moloney murine leukemia retrovirus. Exp Parasitol 112:209–220
19. Kines KJ, Morales ME, Mann VH, Gobert GN, Brindley PJ (2008) Integration of reporter *trans*-genes into *Schistosoma mansoni* chromosomes mediated by pseudotyped murine leukemia virus. Faseb J 22:2936–2948
20. Heyers O, Walduck AK, Brindley PJ, Bleiss W, Lucius R, Dorbic T, Wittig B, Kalinna BH (2003) *Schistosoma mansoni* miracidia transformed by particle bombardment infect *Biomphalaria glabrata* snails and develop into transgenic sporocysts. Exp Parasitol 105:174–178
21. Krautz-Peterson G, Radwanska M, Ndegwa D, Shoemaker CB, Skelly PJ (2007) Optimizing gene suppression in schistosomes using RNA interference. Mol Biochem Parasitol 153:194–202
22. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45
23. Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30:e36
24. Skelly PJ, Da'dara A, Harn DA (2003) Suppression of cathepsin B expression in *Schistosoma mansoni* by RNA interference. Int J Parasitol 33:363–369
25. Dalton JP, Clough KA, Jones MK, Brindley PJ (1997) The cysteine proteinases of *Schistosoma mansoni* cercariae. Parasitology 114(Pt 2):105–112
26. Kaplan EL, Meier P (1958) Nonparametric estimation from incomplete observations. J Am Stat Assoc 53:457–481
27. Hammond SM, Bernstein E, Beach D, Hannon GJ (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature 404:293–296
28. Zamore PD, Tuschl T, Sharp PA, Bartel DP (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 101:25–33
29. Pham JW, Pellino JL, Lee YS, Carthew RW, Sontheimer EJ (2004) A Dicer-2-dependent 80s complex cleaves targeted mRNAs during RNAi in *Drosophila*. Cell 117:83–94
30. Caffrey CR, Ruppel A (1997) Cathepsin B-like activity predominates over cathepsin L-like activity in adult *Schistosoma mansoni* and *S. japonicum*. Parasitol Res 83:632–635
31. Lipps G, Fullkrug R, Beck E (1996) Cathepsin B of *Schistosoma mansoni* purification and activation of the recombinant proenzyme secreted by *Saccharomyces cerevisiae*. J Biol Chem 271:1717–1725
32. Wasilewski MM, Lim KC, Phillips J, McKerrow JH (1996) Cysteine protease inhibitors block schistosome hemoglobin degradation in vitro and decrease worm burden and egg production in vivo. Mol Biochem Parasitol 81:179–189
33. Anderson WF (1998) Human gene therapy. Nature 392:25–30
34. Blesch A (2004) Lentiviral and MLV based retroviral vectors for ex vivo and in vivo gene transfer. Methods 33:164–172
35. Burns JC, McNeill L, Shimizu C, Matsubara T, Yee JK, Friedmann T, Kurdi-Haidar B, Maliwat E, Holt CE (1996) Retrovirol gene transfer in *Xenopus* cell lines and embryos. In Vitro Cell Dev Biol Anim 32:78–84
36. Franco M, Rogers ME, Shimizu C, Shike H, Vogt RG, Burns JC (1998) Infection of lepidoptera with a pseudotyped retroviral vector. Insect Biochem Mol Biol 28:819–825
37. Lin S, Gaiano N, Culp P, Burns JC, Friedmann T, Yee JK, Hopkins N (1994) Integration and germ-line transmission of a pseudotyped retroviral vector in zebrafish. Science 265:666–669
38. Matsubara T, Beeman RW, Shike H, Besansky NJ, Mukabayire O, Higgs S, James AA, Burns JC (1996) Pantropic retroviral vectors integrate and express in cells of the malaria mosquito, *Anopheles gambiae*. Proc Natl Acad Sci USA 93:6181–6185
39. Burns JC, Friedmann T, Driever W, Burrascano M, Yee JK (1993) Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. Proc Natl Acad Sci USA 90:8033–8037
40. Zhao ZR, Lei L, Liu M, Zhu SC, Ren CP, Wang XN, Shen JJ (2008) *Schistosoma japonicum*: inhibition of Mago nashi gene expression by shRNA-mediated RNA interference. Exp Parasitol 119:379–384
41. Grishok A, Pasquinelli AE, Conte D, Li N, Parrish S, Ha I, Baillie DL, Fire A, Ruvkun G, Mello CC (2001) Genes and mechanisms related to RNA interference regulate expression of the small

temporal RNAs that control *C. elegans* developmental timing. Cell 106:23–34
42. Krautz-Peterson G, Skelly PJ (2008) *Schistosoma mansoni*: the dicer gene and its expression. Exp Parasitol 118:122–128
43. Grishok A, Mello CC (2002) RNAi (Nematodes: *Caenorhabditis elegans*). Adv Genet 46:339–360
44. Gomes MS, Cabral FJ, Jannotti-Passos LK, Carvalho O, Rodrigues V, Baba EH, Sa RG (2009) Preliminary analysis of miRNA pathway in *Schistosoma mansoni*. Parasitol Int 58:61–68
45. Bernstein E, Caudy AA, Hammond SM, Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409:363–366
46. Hamilton AJ, Baulcombe DC (1999) A species of small antisense RNA in post-transcriptional gene silencing in plants. Science 286:950–952
47. Krautz-Peterson G, Bhardwaj R, Faghiri Z, Tararam CA., Skelly PJ (2009) RNA interference in schistosomes: machinery and methodology. *Parasitology*, 1–11
48. Okamura K, Chung WJ, Lai EC (2008) The long and short of inverted repeat genes in animals: microRNAs, mirtrons and hairpin RNAs. Cell Cycle 7:2840–2845
49. McIntyre GJ, Fanning GC (2006) Design and cloning strategies for constructing shRNA expression vectors. BMC Biotechnol 6:1
50. Caffrey CR, Salter JP, Lucas KD, Khiem D, Hsieh I, Lim KC, Ruppel A, McKerrow JH, Sajid M (2002) SmCB2, a novel tegumental cathepsin B from adult *Schistosoma mansoni*. Mol Biochem Parasitol 121:49–61
51. Caffrey CR, McKerrow JH, Salter JP, Sajid M (2004) Blood 'n' guts: an update on schistosome digestive peptidases. Trends Parasitol 20:241–248
52. Turk B, Turk V, Turk D (1997) Structural and functional aspects of papain-like cysteine proteinases and their protein inhibitors. Biol Chem 378:141–150
53. Timms AR, Bueding E (1959) Studies of a proteolytic enzyme from *Schistosoma mansoni*. Br J Pharmacol Chemother 14:68–73