Research Articles

J Vect Borne Dis 44, September 2007, pp. 164–180

Prolonged somatostatin therapy may cause down-regulation of SSTR-like GPCRs on *Schistosoma mansoni*

Shyama Chatterjee[a], Jeff Op De Beeck[a], Archana V. Rao[b], Dattatraya V. Desai[b], Gunther Vrolix[a], Frank Rylant[a], Thomas Panis[a], Amit Bernat[a], Jonathan Van Bergen[a], Dieter Peeters[a] & Eric Van Marck[a]

[a]*Pathology Laboratory, Faculty of Medicine, Antwerp University, Universiteitsplein-1, Belgium;* [b]*Department of Biotechnology, B.V. Bhoomaraddi College of Engineering & Technology, Hubli, India*

Abstract

Background & objectives: Chemotherapy with praziquantel remains the only control measure to *Schistosoma mansoni* infections to date. The neuropeptide hormone somatostatin gives relief from gastrointestinal disturbances, liverpathology, and reduces egg production in *S. mansoni* infected mice, suggesting an interaction of somatostatin with the parasite rather than with the host alone. Using antibodies directed to epitopes of the seven somatostatin transmembrane receptors (SSTRs), the presence of SSTRs (or proteins that contain these epitopes) was shown on both worm- and egg-stages of *S. mansoni*. The present study was undertaken to investigate whether SSTRs on *S. mansoni* displayed homo/heterodimerisation properties as well as agonist induced down-regulation.

Results: Somatostatin therapy was effective after two days of treatment with no further reduction in pathology after five days of therapy. Immunohistochemistry performed on parasite sections showed reactivity of the anti-SSTR antibodies to the tegument and internal parts of adult *S. mansoni* worms. SDS-PAGE-Western blotting identified protein bands of 70–100 and 200–250 kDa molecular weight. Upon carboxymethylation of the sulfhydryl groups of proteins in the worm lysate, a reduction in density of the protein band at 200–250 kDa and an increase in density of the protein band at 70–100 kDa were noted. This suggested that a substantial amount of the proteins detected on the blot are present as a homo/heterodimer. A protein microarray was used to investigate whether somatostatin therapy induced receptor down- or up-regulation on the adult worm of *S. mansoni*. Slides spotted with primary anti-SSTR antibody were exposed to lysates of worms collected from infected C3H mice that received none, two days or five days somatostatin treatment, followed by a secondary anti-SSTR antibody coupled to a fluorophore. Comparison of the different samples in terms of parasite dilution till when the fluorescence was detectable, and the fluorescence intensity, proved that the proteins detected in the parasite worm have been down-regulated after five days of somatostatin treatment.

Conclusion: SSTR-like GPCRs are being expressed by adult *S. mansoni* worms and extended somatostatin treatment may cause down-regulation of these receptors, thus reducing the therapeutic capacities of this neuropeptide. However, the presence of SSTRs on *S. mansoni* has not yet been proven on a genetic basis. Cross-reactivity of anti-SSTR antibodies with other G-protein coupled receptors (GPCR) thus cannot yet be excluded.

Key words GPCR – immunohistochemistry – *Schistosoma mansoni* – somatostatin – SSTR

Introduction

Hepatic fibrosis can lead to fatal conditions in *Schistosoma mansoni* infected patients that amounts to 200 million mostly in Africa and South America. Our previous studies have indicated a direct association between severe hepatic fibrosis and low levels of circulating somatostatin levels in *S. mansoni* infected patients in the Richard Toll region of Northern Senegal[1]. Somatostatin is a neuropeptide produced by neuroendocrine, inflammatory and immune cells that inhibits various cellular functions including secretions, motility and proliferation. Based on our results, new therapeutic possibilities can be devised[2]. Our initial experiments explored the therapeutic potential of somatostatin in an experimental model of hepatic fibrosis associated with *S. mansoni* infections. Somatostatin therapy in *S. mansoni* infected outbred Swiss mice caused a decrease in hepatic fibrosis levels as compared to untreated animals (represented by liver hydroxyproline values) after two days of treatment. In parallel a decrease in the number of *S. mansoni* eggs in the liver of the treated animals was observed as compared to untreated animals. Our data suggested that somatostatin might have therapeutic potential in *S. mansoni* mediated liver pathology[3].

Within the human body, the multiple actions of this inhibitory peptide are mediated by specific membrane-bound receptors [the seven somatostatin transmembrane receptors (SSTR1-SSTR5)] belonging to the G-protein-coupled receptor family[4]. Our recent experiments have localised SSTR2, SSTR3 and SSTR5 on the surface of the egg and worm stages of the parasite in host liver and ileum using immunohistochemistry, dot blot and western blotting techniques (Fig. 1)[5]. The presence of these SSTRs on the parasite was probed using antibodies to human, mouse and rat SSTRs. Soluble egg antigen (SEA) secreted by the egg stage parasite in the liver triggers inflammation and fibrosis. We believe that the administration of exogenous somatostatin to *S. mansoni* infected mice may (besides other pathways) inhibit SEA production via interaction with these SSTRs on the parasite surface.

High concentrations of ligand *in vivo* can, however lead to receptor inactivation. Internalisation upon ligand exposure is a major characteristic of the G-protein-coupled receptor family and some somatostatin receptor subtypes such as SSTR2a internalise particularly rapidly and efficiently[6]. In the case of somatostatin, ligand binding is known to induce endocytosis of ligand-receptor complexes that are degraded in lysosomes. Prolonged ligand binding can alter the conformation of the receptor so that it can no longer bind the ligand. Alternately, it can bind the ligand without activating a membrane-bound enzyme or ion channel. These possibilities may cause down-regulation of the specific signals.

In this study, our aim was to investigate if somatostatin therapy over two to five days could modulate liver collagen content in inbred mice strains infected with

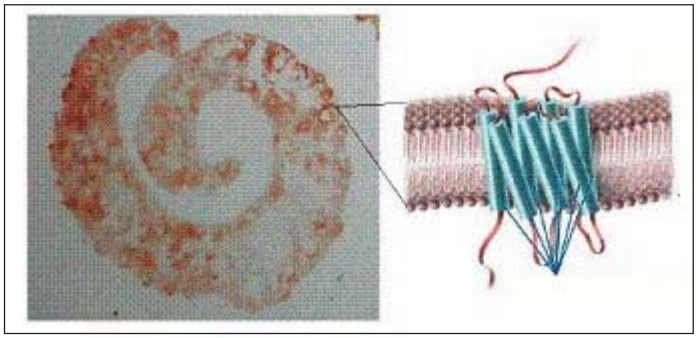

Fig. 1: Immunohistochemical localisation of SSTR-like GPCRs in *S. mansoni* worm stage[5].

S. mansoni. In parallel we wished to delineate whether prolonged somatostatin therapy would up/down-regulate SSTR expression on the parasite stages. The low pathology mice strain C57BL6 shows light pathology upon *S. mansoni* infection due to a balanced T1/T2 immune reaction. In contrast the high pathology mice strain C3H shows serious pathology upon *S. mansoni* infection due to strong T1 and T2 responses. Therapy with somatostatin *in vivo* could cause down-regulation of the somatostatin receptors. Worm stage parasites from *S. mansoni* infected mice that were treated with somatostatin till five days were assayed for the presence and concentration of somatostatin receptors using the protein micro-array method. Results were compared to that obtained from infected and untreated mice.

Material & Methods

Schistosoma mansoni infection: The maintenance of the *S. mansoni* life-cycle and the transcutaneous infection of mice with *S. mansoni* have been previously described[7]. Six week old male inbred (C57BL6, C3H/He) and outbred (Swiss) mice (Janvier, BioServ, Schaijk, NL) were maintained in animalarium with food and water *ad libitum*, in compliance with the guidelines of the University's Ethical Committee. To infect with *S. mansoni,* mice were anaesthetised with Nembutal® (60 mg/kg) and their abdomen was shaved. A metal ring was placed on the abdomen and then filled with treated water containing infectious cercariae of a Puerto Rican strain of *S. mansoni*. The cercariae were allowed to penetrate during 20 min after which the water was removed and checked for remaining cercariae.

Experimental setup: Groups of C57BL6 and C3H mice were infected with 60 *S. mansoni* cercariae each as mentioned above. Age-matched mice were maintained as uninfected control animals. For each mouse strain, groups of 10 mice were maintained till eight weeks following infection (acute stage of infection), while another group was maintained till 16 wk of infection (chronic stage). At such times animals of various groups were treated with somatostatin (Somatostatin-ucb®, UCB Pharma, Brussels) administered in two regimens — a two-day treatment or a five-day treatment. Separate groups of uninfected and infected mice were injected with 30 µg of somatostatin intraperitoneally (in the abdomen) or intravenously (in the caudal vein in the tail). The two-day treatment consisted of six doses of 30 µg somatostatin each, administered over 48 h. The five-day treatment entailed 15 doses of 30 µg somatostatin to each mouse, in total. One week after the last somatostatin administration, mice were killed, the weight of the animal and liver were noted, and plasma was extracted and stored. Untreated animals of the acute and chronic stages were also sacrificed at the respective times together with their treated counterparts. Research protocols involving rodents received ethical clearance by the University of Antwerp Ethical Committee.

Parasite recovery after somatostatin treatment: All infected animals of various groups were controlled for the presence of parasite worms and eggs at the time of sacrifice. The livers were cut out and snap frozen in liquid nitrogen. For cryosectioning, liver fragments were embedded in Tissue-Tek OCT compound, 4 µm thick transverse sections were cut on a cryostat, mounted on slides coated with 0.1% ploy-L-lysine and stored at –20°C until use. To study parasite egg count, hepatocyte status, granuloma size and histology, series of sections were stained with Haematoxylin-Eosin stain. In parallel, two frozen liver fragments from different parts of the liver were collected, each with a weight of at least 150 mg. Each fragment was dissolved in 1 ml of 4% KOH for 18 h at 37°C and total volume was determined. *S. mansoni* eggs were counted in three samples of 0.1 ml under a compound microscope. The number of eggs obtained was extrapolated to the total number of eggs per liver. GraphPad Prism® was used for statistical calculations.

Hydroxyproline determination: The collagen concen-

tration in infected host liver was determined by assessing hydroxyproline content. Herein is described the protocol of technique B for the biochemical assessment of fibrosis used by Bergman and Loxley[8]. Just as was done by Cheever *et al*[9], we neutralised our samples for the colour reaction.

Hydrolysis of liver: For the measurement of the hydroxyproline content in the liver, about 200 mg of liver was treated with 5 ml of 6N HCl for 18 h at 110°C. This acidic hydrolysis breaks down the collagen to individual amino acids. Remaining undissolved matter was removed by adding 40 mg Dowex/ Norit in 5 ml of distilled water. After centrifugation for 15 min at 2000 rpm, the supernatant was filtered with the aid of 0.22 µm millipore filters (Millipore S.A., Molsheim, France).

Neutralisation: About 2 ml of hydrolysate was pipetted out to which one drop (40 µl) of 1% phenolphthalein was added. When the solution became colourless, 10N NaOH was added drop wise till the colour changed to purple red. Return titration was done with 5 µl drops of a 3N HCl solution, till all red colour was lost. The total volume was next restored to 4 ml with distilled water and the solution was kept stable at 4°C.

Colour reaction: Starting from this step we used a series of standard hydroxyproline concentrations made from 0–25–50–75–100 µmol/l (200 µl/test tube). From the test sample about 200 µl was placed in a separate test tube. After vortexing 200 µl test sample/ 200 µl standard mixed together with 400 µl of isopropanol, 200 µl of solution A (chloramine T/citrate-acetate buffer) was added that provided an optimal binding between the colour and tissue. This reaction needed at least 4 min to work after which 2.5 ml of solution B was added and the contents mixed well. The tubes were covered with aluminium foil and incubated for 25 min in a warm water bath maintained at 60°C. To stop the reaction the test tubes were cooled in cold water for 3 min.

Measurement: Within 30 min, the absorbance for each sample was measured in an Ultrospec 3000 UV/Visible Spectrophotometer at a wavelength of 558 nm.

Measurement of somatostatin levels in plasma: To collect plasma, animals were anaesthetised with Nembutal® (60 mg/kg), the thoracic cavity of the animal was cut open and blood collected from the right ventricle of the heart into chilled syringes containing EDTA (1 mg/ml) and Aprotinin (500 KIU/ml blood). The collected blood was centrifuged at 3000 rpm for 15 min at 0°C. The plasma was immediately frozen at –80°C. Untreated naïve mice were also bled to ascertain background levels of somatostatin. The measurement of somatostatin concentrations in the mice plasma was carried out in the laboratory of gastrointestinal hormones, at Gasthuisberg, K.U. Leuven, by means of a radioimmunoassay (RIA). The RIA was performed by incubating the samples with 1.7 pM 3-[^{125}I] iodotyrosyl[10] somatostatin-14 (specific activity 2000 Ci/mmol, Amersham Pharmacia Biotech, Buckinghamshire, UK) and a rabbit antibody against human Somatostatin [1-14] in a 50 mM sodium phosphate buffer (pH 7.4, 0.25% EDTA, 0.5% charcoal-BSA, 500 U/ml Trasylol) for at least two days at 4°C. At the end of the incubation period the somatostatin bound to the antibody was separated from the free somatostatin by adding 500 µl dextran-charcoal followed by centrifugation for 15 min at 3000 rpm. Both fractions were counted in a gamma counter and the results were read from a standard curve (0–250 pg/ml) included in the RIA. The minimal detectable dose was 2.5 pg/ml.

Retrieval of SSTR sequences: The sequences of human, mouse and rat SSTR2, 3 and 5 were retrieved from Swiss-Prot[10–12] using the sequence retrieval system 5 (SRS) (Table 1a–c).

Search for similar sequences in S. mansoni: Computational studies did not indicate the presence of somatostatin receptor on the parasite, possibly due to their

Table 1. Representation of human, mouse and rat SSTRs

Protein name	Accession number(s)	Sequence length (Amino acid residues)
(a) *Human SSTRs*		
Somatostatin receptor type 2 (SS2R) (SRIF-1)	P30874 Q96GE0 Q96TF2 Q9BWH1	369
Somatostatin receptor type 3 (SS3R) (SSR-28)	P32745	418
Somatostatin receptor type 5 (SS5R)	P35346 P34988 Q9UJI5	364
(b) *Mouse SSTRs*		
Somatostatin receptor type 2 (SS2R) (SRIF-1)	P30875 P30934 Q91Y73	369
Somatostatin receptor type 3 (SS3R) (SSR-28)	P30935	428
Somatostatin receptor type 5 (SS5R)	O08858 O08998	362
(c) *Rat SSTRs*		
Somatostatin receptor type 2 (SS2R) (SRIF-1)	P30680	369
Somatostatin receptor type 3 (SS3R) (SSR-28)	P30936	428
Somatostatin receptor type 5 (SS5R)	P30938	363

absence from the databases. The *S. mansoni* genome database maintained at the European Bioinformatics Institute (EBI) was searched for the presence of sequence(s) of SSTR2, 3 and 5 using tBLASTn[13,14] using default parameters (Table 2). Results of tBLASTn indicated some similarity of the SSTR sequences with Histamine-responsive GPCR and RHO-GPCR of *S. mansoni* and the absence of sequence of SSTRs in *S. mansoni* in the database. Albeit some sequence similarity with the *S. mansoni* putative neuropeptide receptor was seen, it is insignificant as it is putative.

Sequence-structure comparison with S. mansoni and GPCR sequences—Search for protein family sequence patterns: The PROSITE[15,16] database was searched using ScanProsite[17]. Maximum number of matching sequence was asked for in order to exclude patterns with high probability of occurrence. The human, mouse and rat SSTR2, 3 and 5 sequences as well as the translated amino acid sequences of *S. mansoni* RHO-GPCR and *S. mansoni* Histamine-responsive GPCR sequences were scanned against PROSITE. All the SSTR sequences invariably showed the presence of unique pattern characteristic of the GPCR family. The pattern (Pattern ID: PS00237) is as given below:

Pattern: [GSTALIVMFYWC] – [GSTANCPDE] – {EDPKRH} – x – {PQ} – [LIVMNQGA] – {RK} – {RK} – [LIVMFT] – [GSTANC] – [LIVMFYWSTAC] – [DENH] – R – [FYWCSH] – {PE} – x – [LIVM]

Pattern description: The first position in the pattern is occupied by any one amino acid residue among G, S, T, A, L, I, V, M, F, Y, W & C; the next position has any one amino acid residue among G, S, T, A, N, C, P, D & E; the next position has any amino acid residue other than E, D, P ,K, R & H; the next position has any amino acid residue; the next position has any amino acid other than P & Q; the next position has any one of the following amino acids: L, I, V, M, N, Q, G & A; the next two positions can have any amino acid other than R & K; the next position has any one amino acid residue among L, I, V, N, F & T; the next position has any one amino acid residue among G, S, T, A, N & C; the next position has any one amino acid residue among L, I, V, M, F, Y, W, S, T, A & C; the next position has any one amino acid residue among D, E, N & H; the next position has R; the next position may have any one amino acid residue among F, Y, W, C, S & H; the next position has any amino acid other than P & E; the next position can have any amino acid residue; the next position has any one amino acid residue among L, I, V & M.

Table 2. Result of tBLASTn search to identify SSTR-like sequences in *Schistosoma mansoni*

Alignment	Database ID	Source	Length	Score	%Identity	%Positives	E()
1	EMBL:AY354457	*Schistosoma mansoni* putative neuropeptide receptor mRNA, complete cds	2000	288	27	51	1.3e-24
2	EMBL:AF031196	*Schistosoma mansoni* histamine-responsive G-protein coupled receptor (GPCR) mRNA, complete cds	2002	214	27	50	6.8e-22
3	EMBL:AF155134	*Schistosoma mansoni* RHO G-protein coupled receptor (RHO) mRNA, complete cds	1857	153	27	46	6.5e-08
4	EMBL:CD161155	ML1-0070T-M239-H02-U.G ML1-0070 *Schistosoma mansoni* cDNA clone ML1-0070T-M239-H02.G, mRNA sequence	630	140	27	50	2.5e-07
5	EMBL:BH207973	Sm1-53O10.TF Sm1 *Schistosoma mansoni* genomic clone Sm1-53O10, DNA sequence	509	109	33	50	0.00077
6	EMBL:CD096414	ME1-0008T-L087-D11-U.B ME1-0008 *Schistosoma mansoni* cDNA clone ME1-0008T-L087-D11.B, mRNA sequence	607	111	29	44	0.00095
7	EMBL:CD096475	ME1-0008T-L088-F05-U.B ME1-0008 *Schistosoma mansoni* cDNA clone ME1-0008T-L088-F05.B, mRNA sequence	551	90	31	42	0.24

Antibodies used to screen for SSTRs on parasite: Antibodies used to screen for SSTRs on *S. mansoni* worm and egg stages, via immunohistochemistry, Dot blot and Western blot techniques, were commercially obtained (Biognost Benelux, Heule, Belgium), based on their reactivity to human and mouse SSTRs. These chosen sequential epitopes of human and mouse SSTRs (against which the antibodies were raised and screened on parasite sections) were: SSTR2A (RSDSKQDKSRLNETTC), SSTR3 (TAGDKASTLSHL) and SSTR5 (KSGRPQTTLPTRSC).

Parasite lysate preparation: In order to prepare antigen samples to be run on SDS-PAGE, parasite lysates were prepared from the worm stages of *S. mansoni*. About 30 worms obtained by perfusion with citrate saline (0.85% sodium chloride; 1.5% sodium citrate) from the portal circulation of infected and/or treated mice were washed several times in PBS buffer, homogenised to crush the worms and then sonicated in the presence of glass beads (60 A, pulses of 10 sec repeated six times with a resting period of 30 sec in between when the samples were cooled on ice). Supernatants were removed and centrifuged at 13,000 rpm for 5 min. The resulting supernatant was collected and frozen at –20°C.

Using a BCA Protein Assay Reagent Kit (Pierce), the quantity of protein was determined in the worm lysates. Total protein content was adjusted to 1.5 mg/ml.

SDS-PAGE and Western blotting with parasite lysates (Decarboxylation protocol): The parasite lysates were separated on the basis of protein molecular weights by one-dimensional SDS-PAGE. The samples were loaded onto Criterion™ XT precast gels, and a current of 200 V was applied for 50 min. The protein bands were transferred to 0.45 µm nitrocellulose membranes using western blot apparatus driven by a current of 50 V for 2 h. All necessary reagents and materials for these techniques were obtained from BIORAD. The blots were stained using a primary antibody directed against cytoplasmic epitopes of human SSTRs, a secondary antibody coupled to Horse Radish Peroxidase, and the respective colour reaction was obtained using appropriate substrate medium. A part of the prepared parasite worm lysate was carboxymethylated using the iodoacetamide method. Firstly, a chaotropic agent like guanidium chloride was added to unfold the proteins. The disulphide bridges were reduced using B-mercaptoethanol, iodoacetamide was allowed to carboxymethylate the free sulfhydryl groups. Finally, dialysis in 0.1% TFA was performed to separate guanidium chloride from the proteins.

Protein profiling on antibody microarrays to study somatostatin receptor expression: Protein microarray is a powerful screening tool in high-throughput proteomics with applications in receptor-epitope binding studies. For our purposes the protein microarray method detailed the following steps (Fig. 2).

Printing antibodies on glass slides: Antibodies were spotted on amino-reactive glass slides. Antibodies were spotted in multiple numbers per standard glass slide (25.3 × 75.5 mm), spot diam of about 150 µm and spot spacing about 300 µm from centre-to-centre. The anti-SSTR2 and anti-SSTR5 were spotted at four different concentrations each (1/10, 1/100, 1/1000 and 1/10000 dilutions). For each concentration, the spotting was done in triplicate. With the negative control (spotting buffer) 27 spots per grid were present in total (adding both antibodies). On each slide several grids (up to 16) were spotted allowing multipe experiments to be performed on the same slide. Spotted slides were stored under regular storage conditions of 4°C to remain functional for at least one month.

Multiple quantification of SSTRs on capturing chips (Multiple ELISA): To determine the concentration of SSTRs in the parasite lysates under conditions of somatostatin therapy or without therapy, multiple ELISA on chips was proposed (Fig. 3). The strategy used here was that multiple sandwich ELISA could be implemented using as a second antibody, an antibody against the alternate somatostatin receptor (second epitope) of the target parasite lysate. On antibody

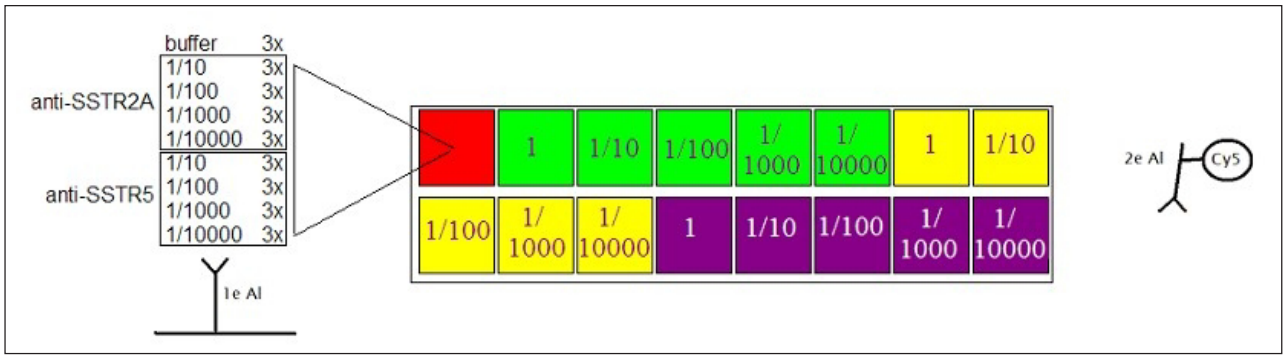

Fig. 2: Scheme of the protein microarray: left—spotting of the primary antibodies on the aminoreactive slides, each antibody in four different dilutions; middle—exposure to different dilutions of the samples, Red = Control, Green = Untreated, Yellow = 2 days treated, and Purple = 5 days treated; right—exposure of the whole slide to a secondary antibody coupled to the fluorophore Cy5; 1e AI—Primary antibody; 2e AI—Secondary antibody

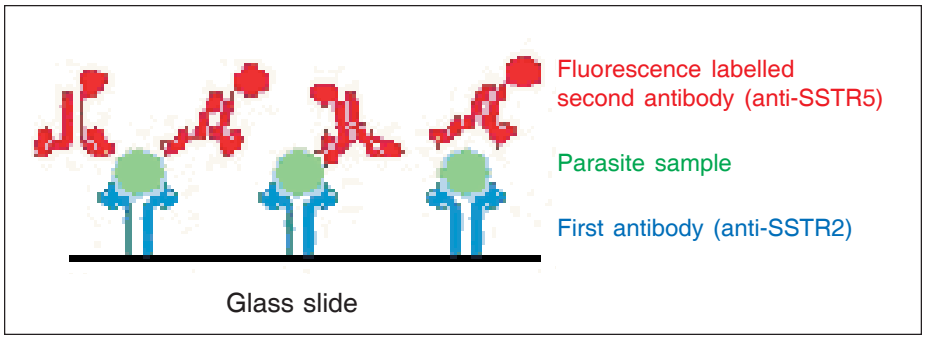

Fig. 3: Diagrammatic schema of the Sandwich ELISA

chips (Eurogentec, Belgium), the analysis of differential protein expression involved spotting of one antibody on glass slide; assay (the binding of the SSTR receptor to the antibody array was performed upon incubation of the spotted slide with 50 µl of buffer or parasite lysate (obtained from untreated/ 2 days somatostatin therapy received/5 days somatostatin therapy received mice); incubation with the second antibody that was labelled with a fluorophore (Cy5).

Statistical analysis: Statistical analysis of the fluorescence intensities of different parasite samples used in the protein micro-array was carried out using the statistical package SPSS. Non-parametric tests like Kruskal Wallis and Mann Whitney U-test were performed to show differences in SSTR receptor concentrations on parasite samples obtained from somatostatin untreated and treated groups of mice.

Results

The evolution of inherent somatostatin levels in low and high pathology inbred mice: In outbred Swiss mice, *S. mansoni* infection caused endogenous somatostatin levels to increase at the acute stage of infection as compared to uninfected mice (119 ± 11.99 pg/ml) ($p = 0.01$). At chronic stages somatostatin levels were reduced (Table 3). This trend was repeated in groups of C3H (high pathology) mice, with a decrease in endogenous somatostatin levels from acute to chronic stage, in contrast to the low pathology C57BL6 mice where the reverse was noticed.

Table 3. Comparison of circulating somatostatin levels in mice strains infected with *S. mansoni*

Mouse strain	Circulating somatostatin levels at acute infection (pg/ml)	Circulating somatostatin levels at chronic infection (pg/ml)
Outbred Swiss mice	297 ± 37.24	206 ± 13.3
Inbred C3H mice	151 ± 20.35	50 ± 21.8
Inbred C57BL6 mice	174 ± 48	311 ± 15

Modulation of fibrosis and parasite count after somatostatin treatment in the low pathology C57BL6 mice strain: Somatostatin administration showed little toxicity, probably due to its short half-life. Total liver and spleen weights of *S. mansoni* infected animals increased over time, with no changes observed due to somatostatin therapy. Total body weights decreased after infection but were not affected by somatostatin therapy.

Following were the results (mean ± SEM) obtained after somatostatin therapy in *S. mansoni* infected low pathology mice: Infection with *S. mansoni* caused an increased hydroxyproline levels (2.31±1.17µmol at Week 8; 5.41±2.14 µmol at Week16) as compared to uninfected animals at similar age time (0.97 ± 0.12 µmol; 0.78 ± 0.17 µmol respectively) (Fig. 4). This significant increase in collagen content ($p < 0.0001$) marks the fibrosis observed at these time points. Treatment with somatostatin, however, did not result

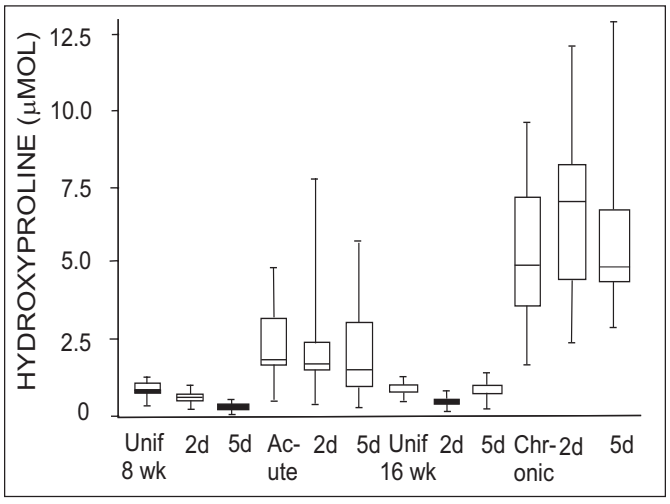

Fig. 4: Variations in hydroxyproline levels in C57BL6 mice after somatostatin treatment. Box and whiskers plot depicting the 5th, 25th, 50th, 75th and 95th percentiles represent hydroxyproline in uninfected C57BL6 animals, variations after 2 or 5 days of somatostatin treatment, at acute stage of infection, variations after 2/5 days of treatment; and at chronic stages of infection with variations after 2/5 days of treatment

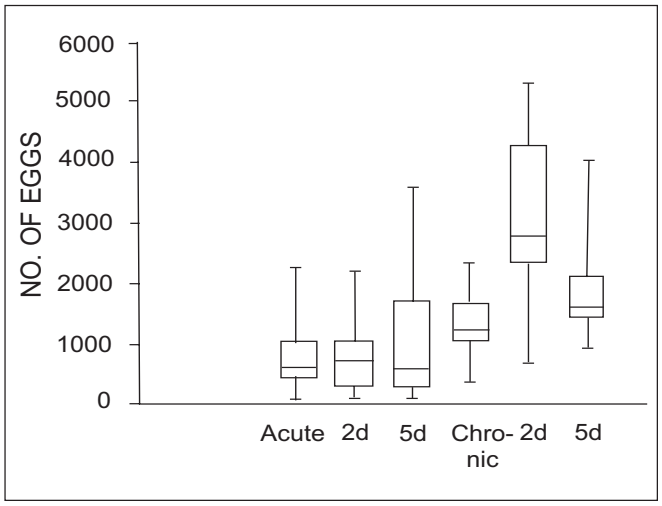

Fig. 5: Parasite egg counts in infected C57BL6 mice after somatostatin treatment. Figure depicts the egg numbers after *S. mansoni* infection and/or somatostatin treatment. Values are represented as a box and whiskers plot as described in Fig. 4

in any significant change in hydroxyproline levels at Week 8 (2.28 ± 1.53 µmol after two days of somatostatin treatment; 2.05 ± 1.35 µmol after five days of somatostatin treatment), or even at Week 16 (6.57 ± 1.13 µmol after two days treatment; 5.72 ± 2.17 µmol after five days treatment) (p = 0.06; 0.68, respectively). Circulating somatostatin levels in infected animals were not significantly affected by somatostatin treatment.

Somatostatin treatment over two days did not cause the total egg count per infected liver (8324 ± 6013) to be significantly reduced as compared to the egg counts in untreated mice at the acute stages of infection (7816 ± 5091), or even at the chronic stages of infection (31680 ± 12870 after treatment; 13640 ± 5580 without treatment) (Fig. 5). Similarly, no significant reduction in parasite egg counts was observed after somatostatin treatment over five days in the total egg count in acute infected animals (10600 ± 9250) and chronic infected animals (18520 ± 7180) as compared to untreated animals.

Modulation of fibrosis and parasite count after somatostatin treatment in the high pathology C3H mice strain: Following were the results obtained after 2 and 5 days of therapy in *S. mansoni* infected high pathology mice: Infection with *S. mansoni* caused an increased hydroxyproline levels (3.41 ± 0.21µmol at Week 8; 4.22 ± 1.47 µmol at Week16) as compared to age matched, uninfected animals (0.52 ± 0.27 µmol; 0.44 ± 0.10 µmol, respectively) (Fig. 6). This

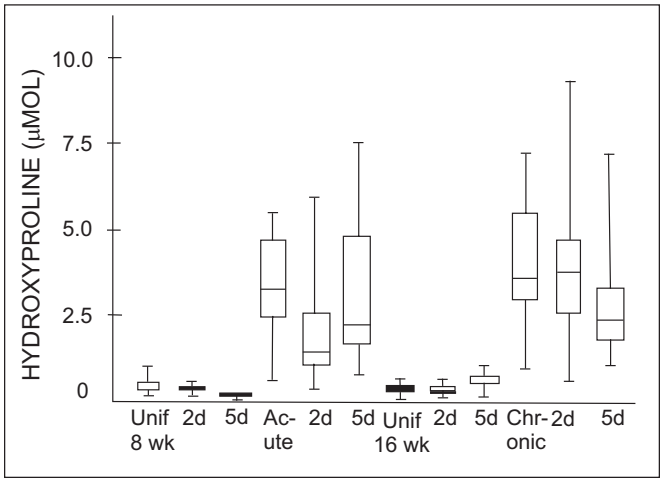

Fig. 6: Variations in hydroxyproline levels in C3H mice after somatostatin treatment. Values are represented as a box and whiskers plot as described in Fig. 4

significant increase in collagen content ($p < 0.0001$) marks the fibrosis observed at these time points. Somatostatin treatment resulted in a significant decrease in hydroxyproline levels at Week 8 (2.03 ± 0.16µmol) and at Week16 (2.88 ± 0.18 µmol) ($p < 0.0001$), when compared to the respective values in untreated animals. Circulating somatostatin levels in infected animals were not significantly affected by somatostatin treatment. Two days of somatostatin treatment caused the total egg count per infected liver (9363 ± 1404) to be significantly reduced as compared to the egg counts in untreated mice at the acute stage of infection (15450 ± 1630) ($p = 0.007$) (Fig. 7).

Sequence comparison of human, mouse and rat SSTRs with S. mansoni: The presence of somatostatin receptors (SSTR) on the parasite was probed by immunohistochemistry using anti-SSTR antibodies. Computational studies did not indicate the presence of somatostatin receptors on the parasite which might have been so due to their absence in the databases. The presence of GPCR (histamine-responsive GPCR and RHO-GPCR) has been described on the parasite surface. As the SSTRs belong to the GPCR family, the binding of anti-SSTR antibody to the parasite surface as well as the reduction in pathology may well be a case of genuine interaction.

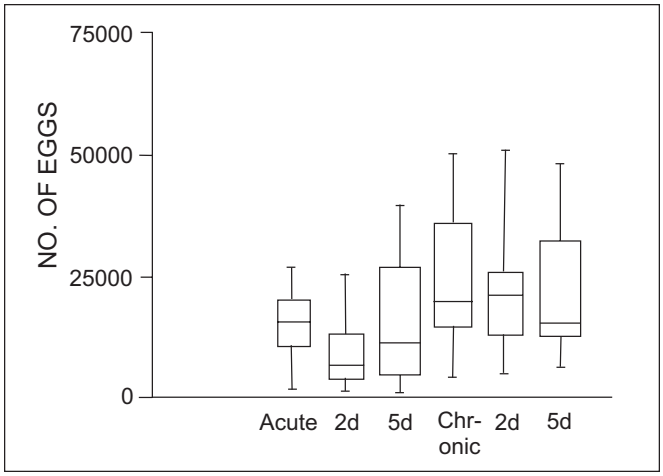

Fig. 7: Variations in parasite egg count in infected C3H mice after somatostatin treatment. Values are represented as a box and whiskers plot as described in Fig. 4

The Protein Data Bank (PDB)[18], was queried for "*Schistosoma* proteins" which showed the presence of nine entries of structures of proteins and their complexes of *S. mansoni* (22 structure entries of all *Schistosoma* proteins and their complexes) as on the date of communication, but none of them belong to the GPCR family, further confirmed by mapping GPCR family sequence pattern on the known protein structures. The sequential epitopes of human and mouse SSTRs (against which the antibodies were raised and used for immunohistochemistry on parasite sections)—SSTR2A (RSDSKQDKSRLNETTC) showed identical stretch of five amino acid residues, SSTR3 (TAGDKASTLSHL) showed identical stretch of ten amino acid residues while sequential epitopes of human and mouse SSTR5 (KSGRPQTTLPTRSC) showed three and four consecutive identical amino acid residues with a non-identical residue in between on comparison with the GPCR family sequence pattern. Thus any reactivity (cross-reactivity if any) of anti-SSTR antibodies would have been with the member(s) of GPCR family rather than with other *Schistosoma* proteins.

Protein content in parasite lysates: The protein content in the worm lysates was determined using the Pierce kit incorporating BSA protein standards and absorption at 562 nm. Respective protein contents were determined ($y = 0.5672\mathbf{x}$). The different samples were worm lysates collected from mice that at Week 8 of infection received none (AC), 2 days (A2) or 5 days (A5) somatostatin treatment, and worm lysates collected from mice that at Week 16 of infection received none (CC), 2 days (C2) or 5 days (C5) somatostatin treatment.

SDS-PAGE and Western blots (Decarboxylation): When the blots were analysed, two distinct protein bands were found; one between 70 and100 kDa and the other between 200 and 250 kDa (Fig. 8 a and b). When the carboxymethylated lysates (W3) were compared with the normal samples (W1/W2), a reduction in density of the top band was observed.

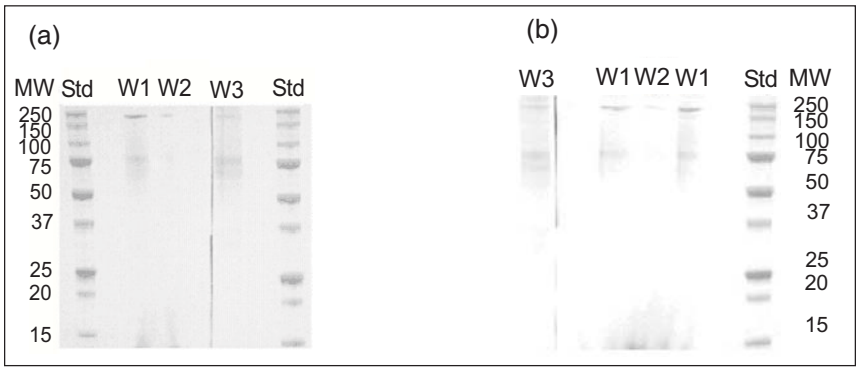

Fig. 8: (a) Western blots of worm lysates W2, W3 compared to carboxymethylated sample W3. Blots were treated with anti-SSTR2A; and (b) Similar blots treated with anti-SSTR5 (W1: Untreated worm sample/ W2: 1/10 diluted untreated worm sample/W3: Carboxymethylated worm sample)

Whereas, the lower band gained in density, suggesting that a part of the identified proteins between 200 and 250 kDa was present as hetero- or homo-dimers. The remaining proteins at 200–250 kDa could have modifications like glycosylation or palmitoylation.

Protein micro-array: Antibodies spotted onto micro-array slides captured specific receptors on the parasite lysates, whereas the detection antibody coupled to the fluor Cy5 bound this complex thus providing a signal that was measured (Fig. 9 a and b). The detection of bound parasite receptor protein is based on the generation of fluorescence. Quantification of the bound detection antibody provided a measure of receptor abun-

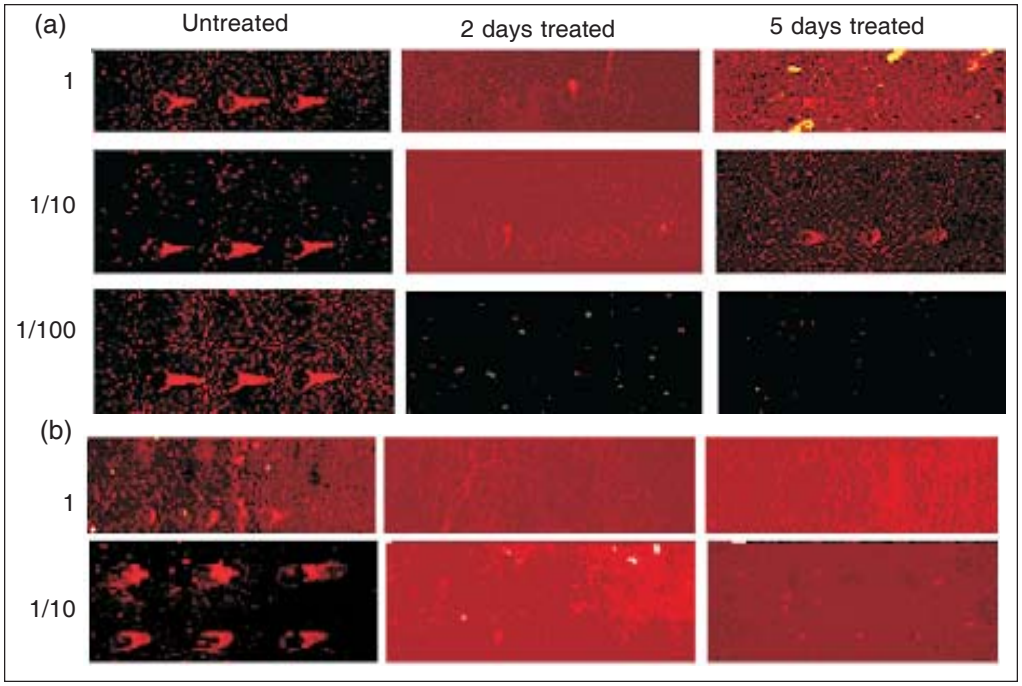

Fig. 9: (a) SSTR expression on worm lysates obtained from C3H mice at acute stage of infection; and (b) same at chronic stage of infection. Comparison is made between spots of untreated worm samples and that after 2/5 days somatostatin treatment

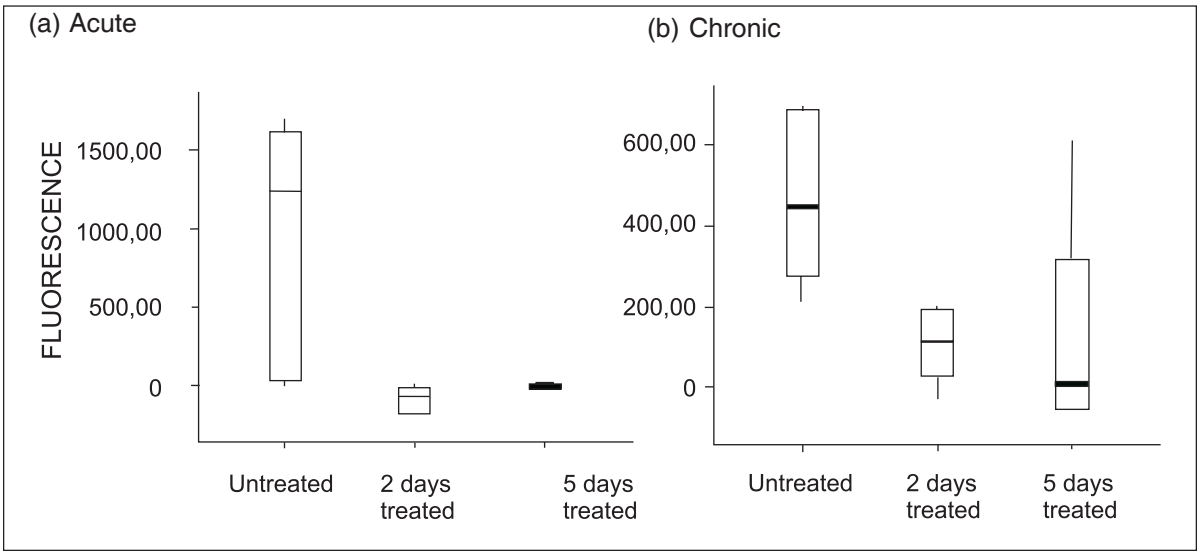

Fig. 10: Statistical analysis showing fluorescence expressed on worm lysates from C3H mice at acute (a) or chronic (b) stages of infection, after 2/5 days of somatostatin treatment

dance. Finally, analysis was done to determine the differential receptor expression. Sandwich assays are more sensitive than the direct labelling method because background is reduced through the specific detection of two antibodies instead of one.

When the pictures of the slides were compared, we found that fluorescence was detectable at a greater dilution for the lysates from untreated mice as compared to those collected from two and five days treated mice. We could only show this for the slides treated with lysates from C3HeN mice, the data from slides treated with lysates from C57BL/6J mice could not be used for interpretation.

We compared the fluorescence intensities of the dilutions where there was still fluorescence detectable for the three samples. For both slides these are the undiluted samples.

Statistical analysis: Kruskal Wallis and Mann Whitney U-tests (SPSS) revealed that worm samples obtained from untreated mice generated significantly higher levels of fluorescence as compared to that extracted from treated animals (Fig.10 a and b). The p-values were obtained via SPSS, and depicted that fluorescence intensity did not vary significantly between worm extracts of 2 and 5 days treated mice (Table 4).

Table 4. SPSS derived statistical differences in fluorescence intensity between untreated, 2 days and 5 days of somatostatin treatment

Variables	Acute (Fig. 10a)	Chronic (Fig. 10b)	Statistically significant difference
Untreated vs 2 days vs 5 days treated	0.006	0.025	Yes
Untreated vs 2 days treated	0.006	0.004	Yes
Untreated vs 5 days treated	0.02	0.078	Unsure
2 days vs 5 days treated	0.109	0.631	No

Discussion

The neuropeptide somatostatin is one of the major regulatory peptides in the central nervous system and the digestive tract. Somatostatin receptors (SSTRs) are present in somatostatin target tissues, such as brain, pituitary, pancreas and gastrointestinal tract. The SSTR2A receptor protein is found in the human GI lymphatic and nervous components, SSTR3 mRNA is identified in intestinal smooth muscle cells pointing to its role in regulating gut motility, SSTR5 has been reported to play a role in cell proliferation[19].

Somatostatin receptors are also expressed in pathological states, particularly in neuroendocrine tumors of the GI tract. Ninety percent of the carcinoids and a majority of islet-cell carcinomas, including their metastases usually have a high density of somatostatin receptors. Since somatostatin receptors in gastroenteropancreatic tumors are functional, their identification can be used to assess the therapeutic efficacy of somatostatin or its analogues to inhibit excessive hormone release in the patients. Somatostatin and its major, clinically employed analogues are now widely used for the treatment of a variety of diseases including neuroendocrine tumor disease, portal hypertension and gastrointestinal motility disorders[20].

A considerable amount of ongoing research is involved with the manner in which Schistosomes interact with their environment, more specifically the nature of signals received from the host environment that could influence the development and maturation of this parasite. Delineation of such mechanisms and the identification of responsible pathways activated as a result would lead to new chemotherapeutic and immunoprophylactic therapies to eliminate *Schistosoma* infections. Neuropeptides secreted by the host and/or the parasite may play an important causative role in this respect. We have indicated in previous reports the use of somatostatin therapy to alleviate *Schistosoma* caused pathology[3,21]. Considering that our previous work has shown a direct association between *S. mansoni* induced fibrosis and low endogenous somatostatin levels in human subjects from N. Senegal, it is proposed that exogenously administered somatostatin would further modulate fibrosis via interaction with specific G-protein coupled receptors (GPCRs)[1].

These receptors (SSTRs), presence of whom have been confirmed in humans, rats and mice, have now been shown by us also on the miracidia, worm tegument and internal structures of *S. mansoni* via immunohistochemistry. Moreover, when worm lysates were run on SDS-PAGE and protein bands blotted onto nitrocellulose membranes, immunostaining with enzyme-coupled SSTR antibodies identified specific bands. The screening antibodies were selected due to their reactivity to the cytoplasmic sequences of SSTR2A and SSTR5- RSDSKQDKSRLNETTC and KSGRPQTTLPTRSC respectively, epitopes present in human, rat and mouse SSTRs.

A scrutiny of the *S. mansoni* genome provided no single known gene coding for SSTRs, as a consequence at this moment the exact amino acid sequence of this parasite protein is unknown[22]. As our screening antibodies are not confirmed to be raised to *S. mansoni* SSTRs, we could not certify with certainity that the proteins identified on worm and egg parasite sections and the blots are indeed SSTRs. Cross-reactivity of the screening antibodies with other *S. mansoni* proteins was indeed a possibility. Upon sequence comparison of human, mouse and rat SSTR2A and SSTR5 amino acid sequences (Swissprot database) with proteins coded for by the *S. mansoni* genome, tBLASTn showed unique sequence similarity with *S. mansoni* RHO-GPCR and *S. mansoni* histamine-responsive GPCR. The prosite database confirmed unique, identical, amino acid sequence pattern similarity, characteristic of the GPCRs. The different epitopes, amongst which also reside the sequences to which our screening antibodies were raised (Chemicon®), showed a sequence

similarity of 5–10% with the GPCR family sequence pattern, evident in both *S. mansoni* RHO-GPCRs and histamine-responsive GPCRs.

None of the 28 different *Schistosoma* proteins with known 3D structure in the Protein Data Bank, shared structural similarity with the GPCR family. Comparison of the typical GPCR family sequence pattern with the sequences of these 28 proteins confirmed this. This observation confirmed with surety that the protein sequences identified by our screening antibodies were indeed members of the GPCR family, thus any possible cross-reactivity of such antibodies would only be restricted to them, and not to any other protein coded by the *S. mansoni* genome.

To date the presence of *S. mansoni* RHO-GPCR and the *S. mansoni* histamine-responsive GPCR on the parasite has been delineated only at the genetic level. The EST sequence of *S. mansoni* RHO-GPCR was first described[23] while analysing *S. mansoni* cercarial gene expression profiles. Amino acid sequence coded for displayed high sequence similarity with Rhodopsin and was thus thought to bear photosensitive properties[24]. *S. mansoni* stages that express this protein might use such light sensitive receptors to identify their path, but are such receptors required by adult worms residing in the blood vessels of their vertebrate host? In the absence of any functional activity of *S. mansoni* RHO-GPCR on adult worms, we doubt that our experiments have shown any cross-reactive expression of this protein.

Regarding the expression profile of the *S. mansoni* histamine-responsive GPCR[25] in the different parasite stages, there is even less known. At a functional level the purpose of the SSTRs on the parasite was indicated in our previous reports[3,5]. Somatostatin therapy may cause a reduction in the total egg count in the infected liver, via SSTRs present on the parasite worms. Future experiments will involve coincubation of worms with somatostatin concentrations *in vitro*, and assay for secretory levels of worm antigens. Similar studies as have been described by Kahama *et al*[26], can be set up to assay for SEA levels.

Western blots screened with anti-SSTR antibodies generated to the cytoplasmic epitopes of this neuropeptide recognised protein bands of 200–250 kDa and a diffuse band of 70–100 kDa. Literature has shown SSTR expression on western blots with a molecular weight of 80–100 kDa, when expressed in HEK 293 cells[27]. These authors also claim that such receptors may form hetero- or homodimers due to non-covalent interactions, causing protein bands higher than 200 kDa on the blots. Carboxymethylation, leading to the break down of the dimerisation products resulted in a lesser dense band at 200–250 kDa, and a higher density band at 70–100 kDa in our experiments. These data prove the eventual presence of oligomeric SSTR like GPCRs in adult *S. mansoni* worms. The persistence of light protein band at 200–250 kDa in our results may be caused by unknown SSTR post-translational modifications. Different consensus sites exist for glycosylation, also on most SSTRs, palmitoylation sites are present[28,29].

The strength of a cellular response to any stimulus depends upon the presence of receptors available on the cell surface, which is regulated by genetic or epigenetic mechanisms. SSTRs in cellular membranes, sensitive to hormonal and physiological changes, are cell-type specific and development-dependent[30]. A separate important mechanism of GPCR regulation is the homologue regulation via agonists. Cell stimulation with high agonist concentrations could ultimately lead to a reduced cellular response. This process of desensitisation occurs via the interactions of the agonist bound receptors and the subsequent signal pathways[31]. At a functional level, further interactions between the receptor and G-proteins fail, whereas the agonist-receptor complexes are internalised in the cellular compartments where they are protected from the G-protein. Down-regulation is not the only option, upregulation of the receptors also occurs after long-term presence of the agonists[32]. The

regulation of SSTR like GPCRs is a topic that deserves much attention since stable somatostatin analogues are being used in various therapeutic fields. In such fields of study receptor desensitisation is hardly welcome[33]. Various studies have denoted that SSTR density on different cell and tissue types were reduced after agonist stimulation due to the internalisation of the receptors[34]. In many cases an association was suggested between phosphorylation and internalisation or desensitisation process. The formation of homo- or heterodimers could also influence affinity for the ligand, the signal transduction, internalisation and up-regulation of the SSTRs. In the present study we also noted an association between somatostatin therapy and down-regulation of the SSTR2A and SSTR5 like GPCRs on the surface of the adult *S. mansoni* worms, from C3H/HeN mice.

The protein microarray method, based on the principle that a large number of microspots could be studied at the same time point, promised a high level of sensitivity in result depiction. Screening antibodies at four different dilutions, were spotted on to grids in triplicate conditions, and subsequently incubated with the test worm lysates, followed by the corresponding secondary antibody coupled to a fluorophore. Quenching in the microspots was observed occasionally when the concentrations of the spotted antibodies were too high. In such cases unfortunately fluorescent signals could not be detected. As a consequence results in terms of fluorescence intensity was best obtained using the six most diluted spotting samples of the screening antibodies.

In the worm lysates obtained from the C3H/HeN mice, Mann Whitney U-tests revealed differences in signal intensity between grids with CC (chronic infected untreated C3H), C2 (chronic infected, 2 days somatostatin treatment) and C5 (chronic infected, 5 days somatostatin treatment) worm sample incubation, and this was observed both when the spotted antibodies were directed to SSTR2A- or SSTR5 like GPCRs. Significant differences were observed in fluorescence intensity between CC samples and C2 or C5, but none was observed between C2 and C5. Moreover, comparison of the separate grids informed us that the CC samples could be detected till greater dilutions of both screening antibody and antigen protein concentrations with respect to C2 and C5.

We conclude from these data that the SSTR-like GPCRs on the *S. mansoni* worm stages could be subjected to somatostatin (agonist) induced down-regulation. This indicates that somatostatin therapy directed to adult worms may be ineffective when administered over an extended period of time. Our results using inbred mice strains depicted that based on the events that somatostatin therapy has an effect on egg count and liver fibrosis, this therapeutic effect was noted after two days of treatment. No further reduced effect was noted after five days of somatostatin therapy.

However, from a broader point of view this does not signify that somatostatin therapy would have no further effect on *S. mansoni* caused disease pathology, given the knowledge that alternate pathways and foci of action of somatostatin receptor-ligand interactions exist in the diseased host.

Acknowledgement

We acknowledge the collaboration of Prof. T. Peeters and Prof. I. Depoortere, KULeuven, in the measurement of somatostatin levels at all experimental time points. Dr. Marie-Claire Beckers, Eurogentec (Belgium) helped us in the spotting and development of the protein microarray protocol. Dr. Tom Gheskiere (Biognost, Benelux) kindly provided us the amino acid sequence to which our screening antibodies to SSTRs 2, 3 and 5 were generated. Financial support for this study was obtained from the Interuniversity Poles of Attraction Programme of the Services of the Prime Minister, Federal Agency for Scientific, Technical and Cultural Affairs (Belgium).

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Corresponding author: Dr. Shyama Chatterjee, Pathology Laboratory, Faculty of Medicine, Antwerp University, Universiteitsplein-1, Antwerp, B-2610 Belgium.
E-mail: Shyama.Chatterjee@ua.ac.be

Received: 18 January 2007 *Accepted in revised form:* 16 March 2007