EFFECT OF ANTISENSE-SGTPS ON THE GLUCOSE UPTAKE OF THE BLOOD FLUKE *SCHISTOSOMA MANSONI*: OBSERVATIONS IN ADULT WORMS AND SCHISTOSOMULA

Waranya Wongwit[1], Ezequiel Rivera[2] and Liang-Feng Tao[2]

[1]Department of Social and Environmental Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; [2]Department of Biological Sciences, University of Massachusetts Lowell, Lowell, Massachusetts, USA

Abstract. AS-ODNs, complementary to *Schistosoma mansoni* glucose transporter proteins (SGTP1 and SGTP4), were chosen as potential therapeutic agents for schistosomiasis. AS-SGTP1 oligos lowered the glucose uptake of adult worms both *in vitro* and *ex vivo*. The most effective AS-ODN was that of 21 nucleotides complementary to the SGTP1 nucleotide sequence, including the initiation region of mRNA translation. This oligo was found to decrease glucose uptake *in vitro* by as much as 50% and at a concentration of 4.0 mg/ml, it killed all male worms within 24 hours. A significant decrease, up to 34%, in glucose uptake was also noted when 100 mg/kg x2 (with a 2 hours interval) of AS-ODN was administered *ex vivo*. Two out of six anti-SGTP4 oligos also decreased the glucose uptake of adult worms *in vitro* by 25-44%. Added to the culture of schistosomula, two AS-SGTP4 oligos were found to decrease glucose uptake by 20-43%.

INTRODUCTION

Schistosomiasis is a debilitating tropical parasitic disease caused by the trematode *Schistosoma* spp. It afflicts in excess of 200 million people worldwide (WHO, 1996; Carter Center, 2002). Despite the unavailability of either prophylactic drugs or effective vaccines for clinical uses, the parasite has shown the potential to develop some resistance to praziquantel, the only effective drug at the time (Gryseels, 1992; Geerts and Gryseels, 2001; Doenhoff *et al*, 2002; Ismail *et al*, 2002).

The uptake and utilization of glucose by schistosomes represents a major dependence on the host. Adult schistosomes live in the vertebrate bloodstream and have been reported to consume their dry weight in glucose every five hours (Bueding, 1950), especially when male and female parasites have paired and females produce eggs (Coles, 1984). Observations on the uptake of glucose by adult worms suggest the mechanism of facilitated diffusion (Isseroff *et al*, 1972; Uglem and Read, 1975; Cornford *et al*, 1988). A more recent study by Camacho and Agnew (1995), employing a simple flow culture apparatus, showed that the uptake of glucose is entirely carrier mediated. Therefore, glucose transporter proteins localized within the parasite tegument might be potential targets for the disruption of glucose uptake or even of the disease.

With the gene sequence encoding schistosome glucose transporter proteins revealed by Skelly *et al* (1994), we chose AS-SGTP1 and AS-AGTP4, had them synthesized (Hybridon, Worcester, MA) and added them to the culture of adult worms and schistosomula. The intention was that these oligos would inhibit the synthesis of glucose transporter proteins and, therefore, decrease glucose uptake. In investigating this effect, *in vitro* uptake of radiolabelled glucose was observed and the *ex vivo* glucose uptake by adult worms was determined.

MATERIALS AND METHODS

AS-SGTPs

Phosphorothioate oligonucleotides with 21-

Correspondence: Dr Waranya Wongwit, Department of Social and Environmental Medicine, Faculty of Tropical Medicine, Mahidol University, 420/6 Rajvithi Road, Bangkok 10400, Thailand.
Tel: +66 (0) 2354 9100-19 ext 1562-3

24 mer and non-schistosomal gene sequence specific, GEM91, of 25 mer (5′-CTC-TCG-CAC-CCA-TCT-CTC-TCC-TTC-T-3′) were synthesized, purified and analyzed by Hybridon, Inc, Worcester, MA, employing the method reported by Padmapriya *et al* (1994). Gene sequences for schistosome glucose transporter proteins were chosen according to Skelly *et al* (1994). The SGTP1 comprised five different sequences, whereas SGTP4 comprised eight. Their sequences were as follows.

SGTP1: HYB0085 5′-AGC-TAC-TCC-CAT-CTT-CTA-GCC-3′, HYB0086 5′-ATC-TAA-TTC-TGG-AGT-ATT-TGG-3′, HYB0087 5′-TTG-ATT-TTT-TGC-AAC-TTC-ATT-3′, HYB0088 5′-TTG-TGT-AAA-TTT-AAA-AAC-TGG-3′, HYB0089 5′-AAG-TAA-TGA-CTC-AGA-AGC-TGG-3′, SGTP4: Oligo171 5′-AGC-TAC-TCC-CAT-CTT-CTA-GCG-3′, Oligo172 5′-ATC-TAA-TTC-TGG-AGT-ATT-TGG-3′, Oligo173 5′-TGG-TTG-ATT-TTT-TGC-AAC-TTC-AAT-3′, Oligo174 5′-TTG-TGT-AAA-TTT-AAA-AAC-TGG-3′, Oligo175 5′-AGG-ATA-AAG-TAA-TGA-CTC-AGA-AGC-3′, Oligo176 5′-AGC-AAT-TAA-AAC-TGG-CAT-ACG-3′, Oligo177 5′-ACC-AGG-AAT-TGG-ACC-TAA-ACC-3′, Oligo178 5′-AGC-ATT-CTT-TGT-TTC-AAT-CAT-3′.

Adult worms

S. mansoni worms were harvested from out-bred CD1 female mice previously infected with 100 cercariae shed by infective *Biomphalaria glabrata*. Perfusion of the worms from hepatic portal vasculature, following the method of Smithers and Terry (1965), was performed 7-8 weeks after the mice were infected.

Schistosomula

Mechanically transformed schistosomula were prepared according to the method of Colley and Wikel (1974) with minor modification by Kojima *et al* (1985).

In vitro culture

Harvested worms were washed twice in a sterile serum-free medium prior to culture. Five males and five females were co-cultured in each well of a 48-well culture plate containing 0.4 ml of pre-warmed (37ºC) culture medium (RPMI 1640 with 5% FBS, 25 mM HEPES, 2.2 mg/ml $NaHCO_3$, 100 IU/ml of penicillin and 100 μg/ml of streptomycin). After 30 minutes of mild agitation at 37ºC in flowing 5% CO_2 and 95% air, AS-ODNs were added to the culture. The oligo concentration was 4.0 mg/ml and the same amount of water was added to each well of the control group. The worms were let stand in the culture medium/AS-ODNs for four hours before radiolabelled deoxy-D-glucose was added to the culture. The radioactivity of ^{3}H-glucose in each well was 1.0 μCi/ml.

In the case of schistosomula, after the organisms were allowed to transform for a period of three hours, the schistosomula suspension was centrifuged for three minutes at 1,500 rpm. The supernatant was removed and replaced by a fresh culture medium (the same as that used in the worm culture). Estimation of the number of schistosomula was carried out by taking at least eight aliquots of 10 μl of the suspension, counting the organisms seen under the compound microscope, and calculating the number of schistosomula in one ml of suspension. The amount of the schistosomula suspension to be cultured in each well was calculated on the basis of maintaining a concentration of 4.0 mg/ml for AS-ODNs and 1.0 μCi/ml radioactivity in each well.

Ex vivo experiment of glucose uptake by adult worms

AS-SGPT1s (HYB0085-8) were intravenously given to infected mice on day 59 post-infection. The mice were sacrificed for worm recovery 1-1.5 hours later. Recovered worms were washed and cultured according to protocol.

Determination of the uptake of radiolabelled deoxy-D-glucose

Two time points were assigned for the determination of glucose uptake, one hour and four hours after the addition of radiolabelled glucose. At the end of the incubation period, worms were washed three times with cold (4ºC) PBS, 10 minutes each. They were then transferred to scintillation counting vials and solubilized in 0.5 ml solvable tissue and gel solubilizer (New England Nuclear Research Products, Boston, MA) at 55ºC for a minimum of two hours, or until the solution appeared clear. The amount of 5 ml of Scintiverse (Fisher Scientific, Fairlawn, NJ) was added to each vial, and the vials were

left at room temperature for 30 minutes before the radioactivity was measured by the Beckman 1801 liquid scintillation system.

In the case of schistosomula, at the end of the incubation period, the culture was transferred to microfuge tubes and centrifuged for 0.5 minute in a microcentrifuge (Fisher Scientific, Model 235B). The supernatant was removed and the pellet was suspended in cold PBS. The washing procedure was repeated three times, five minutes each. At the last wash, after centrifugation and removal of PBS, the pellet was resuspended in 0.5 ml of solvable tissue and gel solubilizer and immediately transferred to the scintillation counting vial. The solubilization condition and radioactivity determination were the same as for the worms.

RESULTS

Effect of AS-SGTP1 on the glucose uptake of adult worms

When five AS-SGTP1 oligos were added to the culture of adult worms, they were found to decrease the uptake of glucose (Table 1). A maximum of almost 50% reduction was observed in the wells HYB0085 and HYB0088. However, an antisense oligonucleotide to human immunodeficiency virus type 1 (gene expression modulation, GEM91) introduced here as a non-specific oligo, enhanced the glucose uptake of the worms. In a separate experiment, when the same dose of AS-SGPT1 (4.0 mg/ml) was added to the culture of adult worms and their effect on the viability of the worms was studied, HYB0085 killed all the male worms within 24 hours (Table 2). No death of female worms was observed in any AS-SGTP1 except that one out of five females was found dead at 72 hours. It was noted that GEM91 had no effect on the viability of the adult worms.

Ex vivo study of the glucose uptake of adult worms exposed to AS-SGPT1

A reduction of as much as 33.8% was observed in worms recovered from the infected mice treated with HYB0085 (Table 3). This reduction was seen 30 minutes after the worms were cultured in the culture medium supplemented with the radiolabelled glucose and it was also noted that the reduction rate decreased with time in each AS-SGTP tested.

In vitro effect of AS-SGTP4 on the glucose uptake of adult worms

Of the eight AS-SGTP4 oligos added to

Table 1
Effect of AS-SGTP1 on the glucose uptake of adult worms *in vitro.*

Oligos	Reduction of ^{3}H-glucose uptake (%)	
	1-hour observation	4-hour observation
HYB0085	49.9[a]	23.8
HYB0086	0	16.7
HYB0087	40.6[a]	41.8[a]
HYB0088	51.0[a]	11.5
HYB0089	11.1	22.6
GEM91	Enhanced uptake was observed	

ANOVA, [a]$p < 0.05$

Table 2
Effect of AS-SGTP1s on the viability of *S. mansoni* worms *in vitro.*

Oligos	Oligo/medium changes	No. of worms (M/F)	No. of dead worms at time point						
			0 hour	4 hours	8 hours	12 hours	24 hours	48 hours	72 hours
HYB0085	3 every 4 hours	5/5	0/0	0/0	0/0	2/0	5/0	5/0	5/1
HYB0087	4 every 4 hours	5/5	0/0	0/0	0/0	1/0	3/0	4/0	4/0
HYB0088	4 every 4 hours	5/5	0/0	0/0	0/0	1/0	1/0	3/0	3/0
HYB0089	4 every 4 hours	5/5	0/0	0/0	0/0	0/0	2/0	4/0	5/0
GEM91	4 every 4 hours	5/5	0/0	0/0	0/0	0/0	0/0	0/0	0/0
H_2O control	4 every 4 hours	5/5	0/0	0/0	0/0	0/0	0/0	0/0	0/0

Table 3
Effect of AS-SGTP1 on the glucose uptake of adult worms *ex vivo.*

Oligos	Reduction of ^{3}H-glucose uptake (%)			
	0.5 hour	1 hour	2 hours	4 hours
HYB0085	33.8[a]	20.1	17.0	4.6
HYB0086	3.0	0	0	0
HYB0088	2.1	2.2	0	0
HYB0089	14.4	11.1	0.49	0

ANOVA, [a]$p < 0.05$

the culture of adult worms, only oligos 171 and 178 caused a reduction in ^{3}H-glucose uptake. Oligo 171 exerted its effect only at 1 hour (one hour after the addition of ^{3}H-glucose) whereas oligo 178 had a prolonged effect, up to 4 hours (Table 4). The reduction rate, again, decreased with time, as observed in the other experiments (Tables 1 and 3). Comparing these two oligos, oligo 178 had more effect than oligo 171.

Effect of AS-SGTPs on the glucose uptake of schistosomula

One of AS-SGTP1, HYB0085, was chosen for the study of its effect on the glucose uptake of schistosomula. There was no reduction in the uptake observed for up to four hours. However, when oligos 171 and 178 were administered, it was found that both decreased the uptake of glucose (Table 5). In the latter experiment when GEM91 and DHBV (another non-specific oligo) were employed as oligo controls, they both lowered the uptake of glucose, as well. The difference in reduction observed in schistosomula and adult worms was that the reduction rate in the schistosomula increased with time, *ie*, the reduction observed at four hours after the addition of ^{3}H-glucose was higher than that after 1-hour observation.

Table 4
Effect of AS-SGTP4 on the glucose uptake of adult worms *in vitro.*

Oligos	Concentration mg/ml	Reduction of ^{3}H-glucose uptake (%)	
		1-hour observation	4-hour observation
Oligo 171	4.0	25.0[a]	0
	2.0	14.1	0
Oligo 178	4.0	43.9[a]	23.4
	2.0	18.4	1.5

ANOVA, [a]$p < 0.05$

Table 5
Effect of AS-SGTP4 on the glucose uptake of schistosomula.

Oligos	Reduction of ^{3}H-glucose uptake (%)	
	1-hour observation	4-hour observation
HYB0085	0	0
Oligo 171	39.0	37.0
Oligo 178	20.6	43.3
GEM91	14.2	21.3
DHBV	12.6	16.3

ANOVA

DISCUSSION

A report of the uptake of oligodeoxynucleotide by *S. mansoni* (Tao *et al*, 1995) resulted in the development of effective antisense oligonucleotides for the treatment and prevention of schistosomiasis. The glucose transporter protein is one of the potential targets for antisense inhibition of RNA processing.

By separating males and females before culturing them together, both male and female worms had a good chance to absorb nutrients as well as AS-ODNs of their own. There was no doubt that AS-ODNs were taken up by the worms and with the overall culture of eight hours, at least 45% of the oligonucleotide should remain sequestered within the cells of the worms (Tao *et al*, 1995). A reduction, as a result of

AS-SGTP1 oligos, of as much as 50% was observed at 1-hour and it was noted that the reduction was lower at 4-hour observation (Table 1). This pattern of reduction was confirmed by the effect of the AS-SGTP4 oligos (Table 4) when oligos 171 and 178 showed decreases in glucose uptake. This might due to the release of oligonucleotide as the efflux after the uptake of AS-ODN reached its plateau. The decrease in glucose uptake should arise from the effect of the oligo itself, not because of the worms, since worms cultured in the presence of oligos can live up to 12 hours, which is when the male worms were first found dead in the well HYB0085 (Table 2).

Comparing the results of *in vitro* and *ex vivo* experiments, the reduction of glucose uptake by worms *ex vivo* was much lower than *in vitro*. The highest reduction (33.8%) was observed in the well of HYB0085, within 30 minutes (Table 3), and the reduction decreased rapidly with time. This might be because utilization and degradation of the oligos occurred in the vertebrate host before the worms were recovered.

The effect of AS-SGTPs on the glucose uptake of schistosomula was studied with the assumption that schistosomula have the same sequence of glucose transporter protein as adult worms, and with the intention to inhibit the glucose uptake of the organism at its younger stage. Table 5 shows the decrease in glucose uptake as the effect of oligos 171 and 178. The reduction was assumed to be the effect of oligos since no deaths of schistosomula were found when cultured in the presence of oligos during the first eight hours (data not shown). What should be noted here are GEM91 and DHBV, non-specific oligos, also exerted an effect by lowering glucose uptake. However, the effect of non-specific oligo controls was mentioned before at a meeting sponsored by Nature Medicine, September 21-22, 1995 in New Orleans, USA. It raised the question of whether antisense compounds could be used as therapies for cancer, AIDS and other diseases (Gura, 1995).

The pattern of reduction observed in schistosomula was the reverse of that observed in adult worms, *ie*, more reduction was obtained at 4-hour observation for schistosomulae. This might be due to the fact that the oligos were synthesized for the worms and the uptake of oligos by schistosomula had never been studied before. A time course study of the uptake, stability of oligos in the schistosomula, efflux, as well as toxicity of oligo against this early stage of schistosome might solve the mystery of the pattern of reduction. It is probably worth determining the glucose uptake of schistosomulae in the presence of other AS-SGTP4 oligos, which, previously, did not show any effect on adult worms.

With the suggestion that glucose transporter proteins are localized within the parasite tegument, observation of any damage to the tegument of adult worms and schistosomula is encouraged.

From the above experiments, we concluded that both AS-SGTP1 and AS-SGTP4 could lower the glucose uptake of adult schistosome worms. *In vitro* study revealed that AS-SGTP1 reduced glucose uptake up to 50% while AS-SGTP4 lowered the uptake by 14-44% depending on the dose of oligos administered and the time the investigation was made. Comparing the reduction of glucose uptake by different assays, *ie*, *in vitro* versus *ex vivo*, it was found that the reduction revealed by *ex vivo* study was much lower that than by *in vitro*. It is possible that natural utilization and degradation of the oligos occurred in the mice before the worms were recovered.

With the assumption that schistosomula have the same sequence of glucose transporter protein as adult worms, and with the intention to inhibit glucose uptake at a younger stage, AS-SGTPs were added to the schistosomula culture and glucose uptake was determined. HYB0085 seemed to have no impact on schistosomula, while AS-SGTP4s and two other non-specific oligos did (Table 5).

The pattern of reduction observed in the schistosomulae was different from that in adult worms. In schistosomulae, the longer the organisms stayed in oligo, the more reduction in glucose uptake was observed. The authors

speculated on the higher specificity of the sequence of oligos, which were synthesized for the worms, not for the schistosomula.

With the intention to destroy schistosomes at the earliest stage possible, we suggest the revelation of baseline information, such as uptake, stability, efflux and toxicity of oligos in schistosomula, and investigation of any damage of the tegument of both adult worms and schistosomulae, where glucose transporter proteins are localized.

ACKNOWLEDGEMENTS

The authors would like to thank Hybridon, Inc, Worcester, MA for the AS-ODNs and the University of Massachusetts Lowell Research Foundation for the use of facilities. The study was partially funded by the UML Research Foundation.

REFERENCES

Bueding E. Carbohydrate metabolism in *Schistosoma mansoni. J Gen Physiol* 1950; 33: 473-95.

Camacho M, Agnew A. Glucose uptake rates by *Schistosoma mansoni*, *S. haematobium*, and *S. bovis* adults using a flow in vitro culture system. J *Parasitol* 1995; 81: 637-40.

Carter Center. Schistosomiasis Control Program. 2002. [Accessed 2003 October 15]. Available from: URL: http://www.cartercenter.org/healthprograms/showdoc.asp?programID=4&submenu=healthprograms

Coles GC. Recent advances in schistosome biochemistry. *Parasitology* 1984; 89: 603-37.

Colley DG, Wikel SK. *Schistosoma mansoni*: simplified method for the production of schistosomulae. *Exp Parasitol* 1974; 35: 44-51.

Cornford EM, Fitzpatrick AM, Quirk TL, Diep CP, Landaw EM. Tegumental glucose permeability in male and female *Schistosoma mansoni*. *J Parasitol* 1988; 74: 116-28.

Doenhoff MJ, Kusel JR, Coles GC, Cioli D. Resistance of *Schistosoma mansoni* to praziquantel: is there a problem? *Trans R Soc Trop Med Hyg* 2002; 96: 465-9.

Geerts S, Gryseels B. Anthelminthic resistance in human helminthes: a review. *Trop Med Int Health* 2001; 6: 915-21.

Gryseels B. Praziquantel shows unexpected failure in recent schistosomiasis outbreak. *TDR News* 1992; 41: 1.

Gura T. Antisense has growing pains. *Science* 1995; 270: 575-7.

Ismail MM, Farghaly AM, Dyab AK, Afify HA, el-Shafei MA. Resistance to praziquantel, effect of drug pressure and stability test. *J Egypt Soc Parasitol* 2002; 32: 589-600.

Isseroff H, Bonta CY, Levy MG. Monosaccharide absorption by *Schistosoma mansoni*. 1. Kinetic characteristics. *Comp Biochem Physiol* 1972; 43A: 849-58.

Kojima S, Yamamoto N, Kanazawa T, Ovary Z. Monoclonal IgE-dependent eosinophil cytotoxicity to heptenated schistosomula of *Schistosoma japonicum*: enhancement of the cytotoxicity and expression of Fc receptors for IgE by *Nippostrongylus brasiliensis* infection. *J Immunol* 1985; 134: 2719-22.

Padmapriya AA, Tang J, Agrawal S. Large-scale synthesis, purification and analysis of oligonucleotide phosphorothioates. *Antisense Res Dev* 1994; 4: 185-99.

Skelly PJ, Kim JW, Cunningham J, Shoemaker CB. Cloning, characterization, and functional expression of cDNAs encoding glucose transporter proteins from human parasite *Schistosoma mansoni*. *J Biol Chem* 1994; 269: 4247-53.

Smithers SR, Terry RJ. The infection of laboratory hosts with cercariae of *Schistosoma mansoni* and the recovery of adult worms. *Parasitology* 1965; 55: 695-700.

Tao LF, Marx KA, Wongwit W, Jiang Z, Agrawal S, Coleman RM. Uptake, intracellular distribution, and stability of oligodeoxynucleotide phosphorothioate by *Schistosoma mansoni*. *Antisense Res Dev* 1995; 5: 123-9.

Uglem GL, Read CP. Sugar transport and metabolism in *Schistosoma mansoni*. *J Parasitol* 1975; 61: 390-7.

WHO. Fact sheet: Schistosomiasis. 1996. [Accessed 2003 October 15] Available from: URL: http://www.who.int/inf/inf-fs/en/fact115.html