Molecular characterization and expression of *Onchocerca volvulus* glutathione reductase

Sylke MÜLLER*‡, Tim-Wolf GILBERGER*, Alan H. FAIRLAMB† and Rolf D. WALTER*

*Bernhard Nocht Institute of Tropical Medicine, Department of Biochemistry, D-20359 Hamburg, Germany, and †University of Dundee, Medical Sciences Institute, Dundee DD1 4HN, Scotland, U.K.

Glutathione metabolism represents a potential target for anti-parasite drug design. The central role of glutathione reductase (GR) in maintenance of the thiol redox state and in anti-oxidative defence has to be evaluated in more detail in order to establish the essential function of this enzyme for the survival of the filarial parasite *Onchocerca volvulus*. The *O. volvulus* GR (*OvGR*) gene was cloned and sequenced. The gene is composed of 13 exons and 12 introns and spans 4065 bp. The first intron is located within the 5'-untranslated region of the gene, 16 nucleotides upstream of the translation initiation codon. Southern-blot analysis and structural characterization of the genomic sequence indicate that *OvGR* is encoded by a single-copy gene. Isolation of various cDNA clones revealed a polymorphism of polyadenylation initiation with no consensus polyadenylation sites in any of the cDNAs analysed. The entire cDNA is 1977 bp long

and carries the nematode-specific spliced leader sequence SL1 at its 5' end, 236 nucleotides upstream of the first in-frame methionine. The cDNA codes for a polypeptide of 462 amino acids with 53.5% sequence identity with human GR (*HsGR*). A total of 18 out of 19 residues contributing to glutathione binding are identical in *OvGR* and *HsGR*. However, one of the arginine residues (Arg-224 in *HsGR*) involved in discrimination between NADPH and NADH in all known GRs is substituted by tryptophan (Trp-207 in *OvGR*). The coding region of *OvGR* was expressed in *Escherichia coli* as a histidine-fusion protein, and it was established that the parasite protein still favours the binding of NADPH (K_m 10.9 μ M) over NADH (K_m 108 μ M). The histidine-fusion protein has a subunit size of 54 kDa and is active as a homodimer of 110 kDa.

INTRODUCTION

Onchocerca volvulus is the causative agent of onchocerciasis, a disease that affects about 20 million people in Africa, the Arabian Peninsula and Central/South America. One of the major pathological consequences of the infection is ocular damage (river blindness) caused by the millions of larvae (microfilariae) released by the adult females (macrofilariae) during their 10–14 year life span in subcutaneous nodules [1]. Although annual doses of ivermectin are effective in killing the microfilariae, there is a pressing need for the development of better and safer drugs that are effective against adult worms.

GSH is the most abundant low-molecular-mass thiol within almost all eukaryotes [2]. Apart from its function as an antioxidant, it is responsible for the maintenance of intracellular thiol redox status and thus for the function of many biological processes within the cell [3,4]. For most of its functions glutathione must be in the reduced form. The flavoprotein glutathione reductase (GR) catalyses the reduction of GSSG to GSH using NADPH as reducing cofactor [5,6].

It is well known that organic arsenicals have macrofilaricidal effects, and some evidence suggests glutathione metabolism of filarial worms might be the drug target [7–9]. In support of this hypothesis, we have demonstrated that filarial GR is more susceptible towards inhibition by the trivalent organic arsenical melarsen oxide than the human isoenzyme is [10]. These data suggest structural differences exist between host and parasite enzyme that might be exploited for the design of specific inhibitors for subsequent development as anti-filarial drugs. Here we report

on the molecular characterization and expression of *O. volvulus* GR (*OvGR*) and compare the amino acid sequence obtained with the structural features of human GR (*HsGR*).

MATERIALS AND METHODS

Parasites

Adults of *O. volvulus* were isolated from nodules that had been surgically removed from Liberian patients as previously described [11]. Parasites were released from nodules by treatment with collagenase (*Clostridium histolyticum*; Boehringer Mannheim) in RPMI 1640 medium, followed by snap freezing and storage in liquid nitrogen.

Preparation of genomic DNA and total RNA from *O. volvulus*

Frozen adult *O. volvulus* female worms were thawed then homogenized in an all-glass homogenizer in guanidinium thiocyanate, and extracts were layered on a CsCl step gradient. After centrifugation for 18 h at 27000 g (TST 41.14 rotor; Kontron), RNA and DNA were prepared as previously described by Chirgwin et al. [12].

Amplification of *OvGR* by PCR

In order to amplify a portion of the *OvGR* cDNA, degenerate oligonucleotides based upon conserved regions of GRs of other organisms (residues 26–32 and 466–473 in *HsGR*) were designed and used for a PCR with *O. volvulus* cDNA (prepared from a *O.*

Abbreviations used: GR, glutathione reductase; *OvGR*, *Onchocerca volvulus* GR; *HsGR*, *Homo sapiens* GR; 'CeGR', putative *Caenorhabditis elegans* GR; UTR, untranslated region; IPTG, isopropyl β -thiogalactoside; SL1, spliced leader sequence; AdoMetDC, S-adenosylmethionine decarboxylase; r, recombinant.

‡ To whom correspondence should be addressed.

The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers Y11830 and X90528.

tttatattacccaaglltggaacgaagtcctccatggtgccttagttacattactttgtttcc 64
 acgtatttttttgcattgacgatgaatgcgcgtcctcattatcggtgtgcctacacatttc 128
 cctactgattgttcttcgacgggtttgcgaagaattgcacacaaaagtcacatacaatatt 192
 ccgatttgcgaataattccctacgatcgacgaatagacgtattatgacacatgggtgacgat 256
 M S S G D T M
 GAAACGTACGATTATCTGGTATCGGTGGTCTCGGAGGTATTCGAGTGGCCCGAGCT 320
 K T Y D Y L V I G G G S G G I A S A R R A 27
 OVRGSP1
 GCTGTATCAAGTATCTGCGGATTAATGAAGGAAGACATCTCGGTGTACATCGTAAATA 384
 A E F K V S V G L I E G R H L G T C V N 48
 OVRGSP3
 AAGGATCGCTACCGAAGAGATTATGTATTAATGTTCGCCGATCGAAGCAATCATCGATCA 448
 K G C V P F K K I M Y N C S R H A E A I I D H 71
 TTCGATTACGGTTTGTATGTAACATTAATGGATTCAATTGAAAAATAAGGATAGTCGC 512
 S D Y G F D V T L N G F N W K K I K D S R 92
 GATGCATATGTCAGGCGTTTAAATGGAATATATGAATCCAATCTATGCAATCAAGTCGACT 576
 D A Y V R R L N G I Y E S N L C K S Q V D 113
 TCGATTGAGGAAAAGCGTCATTACCAAGATGGTGTATTGACGTTAACGGCAAGAAATATT 640
 L I R G K A S F T K D G V I D V N G K K Y F 135
 CGGAAACACATATTATAGCTGTGCGGAGGATATCCAAAGCGGCTAACGTGCGGGAGCAGAG 704
 G K H I L I A V G G Y P K R P N V P G A E 156
 TATGGTATCAATAGTGTGGCTTCTTCATCTTGATGTTTACCAACGACGCTAGTAGTGG 768
 Y G I N S D G F F H L D V L P K R T V V V 177
 GAGGTGTTACATAGCGATCGAATATCATGATTTGTCGGCATTAGGATCTGATGTTCAATT 832
 G G G Y I A I E I T L S M L S A L G S D V H L 199
 GTTGATACGAAACCAAGGTTTGTGGAATTTGATCAGACAGTGTCTGAATCTTAAACGGA 896
 L I R K P R V L W N F D H T V S E S L T E 220
 TCCATTGATAGAGGCCAACGAACTTTATAAAAAACGGAAGTGAATCAGTGGTAAAGAAAC 960
 S I D R G P T K L Y K N T E V K S V V K K 241
 CTAATGATTACTTACCGTTAACCAACAAATGATACCATGATGAAGTAATGTTTAACTCTG 1024
 P N G L T T V N T T N D T I D E V N C L I W 263
 GGCATTGGCGCTCTTCTGAACAAAGTGAATTAATCTAAATATGTTGGTGGGAAACGTGAT 1088
 A V G R L P E T S D L N L N Y V G G K T D 284
 GAAATCGGCATGTGATTGTGGACGAATATCAGAACACATCGACTAAAAATATTATGCGATG 1152
 E I G H V I V D E Y Q N T S T K N I Y A V 305
 GAGATTGCTGTGAAGAACATTTAGACAGCTTGGGATTGCTGCTGACGATGCTGCGCCA 1216
 G D C C G K A L L T P V A I A A G R C L A Q 327
 GAGATTGTTCAATAACGAAATAAATCTGCTGATTACGAAACATCCCGACTGTTGTGTTT 1280
 R L F N N E I N N R L D Y E N I P T V V F 348
 AGCCATCCACATTAGGAACCGTTGGGCTTACAGAGCGGGAAGCTATCAACAATATGAAAAA 1344
 S H P P L G T V G L T T E A E A I K Q Y G K 369
 ACAATCTTCAATTTATAAAACGAATTAATTCCTTGTATCATGCTGTACTCAACACAAAGA 1408
 N N L T I Y K T K F N S L Y H A V T Q H K E 391
 ACCAAGCATAATGAAATTAATCTGTGCTGGTAAATGAACAGGTGTGCGTTTGCACATGCTT 1472
 P T I M K L I C A G K N E Q V V G L H M L 412
 GGTGAGGATGTGACGAATGCTGCAAGGTTTGTGCTTGCATCAAGTGGGTGGCAGCAAGA 1536
 G E G C D E M L Q G F A V A I K M G A T K 433
 AGGATTTCGATAATACAGTTCGATTATCAACAGGTGCGGAAGAACTAGTTACAATCGGTAA 1600
 K D F D N T V A I H P T G A E E L V T M R N 455
 TGGTACAAACCCGAAACAGCATAAagcatgaattctctgatgatttcgttaacacagaagttt 1664
 G T K F E T A * 462
 aaatgaatgcacagactcgtc~~caaaaac~~cctatgaattgtttgtgttatcgga~~aaaaac~~catcttgc 1728
 ttttacgaattcctgtc~~ca~~taagatttcatcatttttattgaattttgtgcacaaacagttgt 1792
 aaacatttttaga~~tt~~ttattgttttagtgtaattatcaatttatttagaccggaatttcaatga 1856
 tttccctagaagctaaaagcatcatgaattcagaaaattctgtttattttgaattttag~~aa~~ga 1920
~~aa~~catctttttcaaaattttttg~~taataa~~taacttgcactaaaaa~~aaaaaaaaaaaa~~ 1976

Figure 1 Nucleotide sequence and deduced amino acid sequence of *OvGR*

The spliced leader sequence (SL1) is shown in italics and double underlines. Oligonucleotides used for 5'-rapid amplification of cDNA ends are underlined, and their names *OvGRGSP1*, *OvGRGSP2* and *OvGRGSP3* are given. The potential polyadenylation sites are double underlined. The asterisk indicates the stop codon.

volvulus lambda unizap library) as template. The degeneracy of the oligonucleotides was reduced by introducing inosine at some positions instead of all four nucleotides. Using the sense primer 5'-AT(A/T/C)GG(A/T/G/C)G(G/C)(A/T/G)GGI(A/T)-(G/C)IGGIGG-3' and the antisense primer 5'-AA(C/T)TC-(C/T)TC(A/T/G/C)GCIGCI(G/C)(A/T)IGG(A/G)TG-(A/T/G)AT-3' and 100 ng of *O. volvulus* cDNA, PCR was performed as follows: 30 cycles at 94 °C for 2 min, 50 °C for 2 min and 72 °C for 2 min [13]. To obtain the expected 1.3 kb PCR fragment, a second round of PCR was performed using an aliquot of the first reaction product as a template. The 1.3 kb PCR fragment was analysed by agarose-gel electrophoresis, isolated (GeneClean; Bio 101) and subsequently subcloned into the pCRII™ vector (Invitrogen) for sequence analysis. The nucleotide sequence was determined using the Sanger dideoxy-chain-termination reaction on double-stranded DNA [14], using ³⁵S-labelled dATP and Sequenase (Amersham-Buchler).

Genomic Southern-blot analysis

O. volvulus genomic DNA was digested with *EcoRI*, *EcoRV* and *DraI* (Boehringer Mannheim) (10 µg of DNA with each enzyme), and 10 µg of human genomic DNA was digested with *DraI*. The

restricted DNA was separated on an 0.8%-agarose gel, transferred on to a positively charged nylon membrane (Boehringer Mannheim) as previously described by Sambrook et al. [14] and subsequently probed overnight with the radiolabelled 1.3 kb *OvGR* cDNA PCR fragment (Random Primed DNA Labelling Kit; Boehringer Mannheim). The membrane was washed with 2 × SSC containing 0.1% SDS for 1 h at 65 °C and 0.2 × SSC containing 0.1% SDS for 1 h at 65 °C and autoradiographed (1 × SSC contains 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0).

Screening of cDNA and genomic DNA libraries

The 1.3 kb *OvGR* cDNA PCR product was used to screen a lambda unizap cDNA library of adult *O. volvulus* (K. J. Henkle-Dührsen, Bernhard Nocht Institute, Hamburg, Germany) for a full-length cDNA-clone of *OvGR* containing the 5' and 3' ends of the mRNA. The probe was radiolabelled (Random Primed DNA Labelling Kit; Boehringer Mannheim), and 80 000 plaques were screened [14,15]. After the first screen, four positives were purified and excised *in vivo* (Stratagene), and the nucleotide sequences of both strands of the longest cDNA clone (*OvGR5a3*, 1693 bp) were determined using sequence-specific primers. In a second round of screening a 5' portion (nt 266–521; see Figure 1) of the clone *OvGR5a3* was amplified using sequence-specific primers. The PCR product was used as a radiolabelled probe in order to identify a cDNA clone containing the entire 5' portion of the *OvGR* mRNA. Eight positives were isolated, and after *in vivo* excision their nucleotide sequences were determined as described above [14]. None of the clones contained the entire 5' region of the *OvGR* mRNA.

The cDNA clone was also used to screen a lambda fixII genomic DNA library of adult *O. volvulus* (H. von Besser, Bernhard Nocht Institute, Hamburg, Germany) in order to obtain information about the genomic organization of the *O. volvulus* GR gene. Some 80 000 phages were screened, and 10 positives were isolated and plaque purified [14,15]. Genomic phage DNA of these clones was isolated as previously described [14], and the DNA was restricted with *EcoRI*, *EcoRV* and *XbaI*, blotted on to a nylon membrane and probed with radiolabelled probes. One probe contained one of the *OvGR* cDNA clones. To identify the restriction fragment that encompassed the 5'-flanking region of the *OvGR* gene, a 655 bp 5' fragment (see Figure 4, middle panel) of one of the genomic clones, obtained by PCR using sequence-specific sense and antisense oligonucleotides (sense primer: nt –41-5'-GCGATATTGTGGAGATAATC-3'; antisense primer: nt 614-5'-GTCACAAAATTTTCGAGG-3'), was radiolabelled and used to hybridize the phage DNA Southern blot. The genomic phage DNA fragments hybridizing to the probes were isolated and subcloned into pBSSK+, restricted with the respective enzyme and their nucleotide sequences determined [14].

Rapid amplification of cDNA ends

To obtain the 5' end of the mRNA and the sequence of the N-terminal part of *OvGR*, the rapid amplification of cDNA ends or 'anchored PCR' was performed according to the manufacturer's instructions (Gibco BRL). First-strand cDNA was synthesized using total *O. volvulus* RNA and *OvGRGSP3* (nt 418–436; Figure 1) as antisense oligonucleotide. The cDNA was purified and tailed with a homopolymeric dC tail. Portions of the tailed cDNA were used to perform various PCRs using the nested

antisense primers *OvGRGSP2* (nt 351–370; Figure 1) or *OvGRGSP1* (nt 321–341; Figure 1) and the anchor primer. The 450 bp PCR fragment obtained was cloned into pCRIITM, and the nucleotide sequence was determined [14].

Construction of expression plasmids

In order to synthesize the *OvGR* coding region for expression in *E. coli*, the sense oligonucleotide *OvGREx4* encoding the first 17 amino acids and the antisense oligonucleotide *OvGREx2* encoding the last 7 amino acids were used to perform a PCR using the *OvGR5a3* cDNA clone as template. The sense primer contained a *XhoI* restriction site and the antisense primer an *ApaI* restriction site to simplify directed, in-frame cloning into pJC40 [16]. To facilitate subcloning into the expression vector, the PCR fragments were first subcloned into pCRIITM, using T/A cloning, followed by preparative digestion with *XhoI* and *ApaI* and isolation of the restricted *OvGR* coding region before they were ligated into the expression plasmids digested with these restriction enzymes. Cloning of the expression fragment into pCRIITM and subsequent restriction with *XhoI* and *ApaI* increased subcloning efficiency into the expression vector. The constructs were transformed into *E. coli* BL21 (DE3) and used for the expression of the recombinant *OvGR*.

Purification of recombinant *OvGR* and GR-assay conditions

Transformed bacterial cells were grown in Luria–Bertani medium containing 50 µg/ml ampicillin overnight. This stock culture was diluted 1:100 in the same medium, and the bacteria were grown to A_{600} 0.5 before induction with 1 mM isopropyl β-thiogalactoside (IPTG). After 4 h at 37 °C, bacterial cells were harvested and recombinant *OvGR* was isolated by resuspension of bacterial cells in 20 mM Tris/HCl buffer, pH 7.9, containing 0.5 M NaCl and 5 mM imidazole, followed by sonication and centrifugation at 100 000 g (1 h, 4 °C; TFT 55.38 rotor, Centrikon T-1065; Kontron). The supernatant was subsequently used to isolate soluble recombinant *OvGR* histidine-fusion protein by chelating Sepharose loaded with Ni²⁺. The procedure was performed according to the manufacturer's instructions (Novagen). GR activity of the recombinant protein was determined as previously described [10].

RESULTS

OvGR cDNA sequence

The 1.3 kb PCR *OvGR* cDNA fragment was used as a probe to screen a cDNA library of *O. volvulus* adult worms, and the longest clone obtained (*OvGR5a3*) was 1693 bp, containing the entire 3'-non-coding region of the *OvGR* mRNA. However, to obtain the N-terminal part as well as the 5'-non-coding part of the *OvGR* mRNA a rapid amplification of cDNA ends was necessary. The entire cDNA sequence (1976 bp) and the deduced amino acid sequence (462 amino acids) of *OvGR* are shown in Figure 1. Upstream (236 nucleotides) of the first in-frame methionine, a nematode-specific spliced leader sequence (SL1) [17,18] was identified.

Different populations of cDNA clones were isolated from the cDNA library, not only differing at the 5' end but also showing a polymorphism at their 3' ends. Since no consensus polyadenylation signal in any of the cDNAs could be found, the sequences were analysed for potential polyadenylation sites (Figure 1). Such potential sites were found within 6–30 bp

Table 1 Polyadenylation polymorphism of *OvGR* mRNA

Bases identical with the consensus polyadenylation sequence (AATAAA) are underlined.

Potential polyadenylation site	Position in <i>OvGR</i> cDNA clone (bp)	Length of <i>OvGR</i> cDNA clone (bp)
AAAAAC	1455	1500
ATTAAG	1479	1500
AATTA	1515	1543
AAGAAA	1651	1684
TATAAA	1676	1693

<i>OvGR</i>	-----MSSGDTMKTYDYLVIGGGSGGIASARRAAEFKVSGLI-	38
<i>CeGR</i>	MLLSTFKRHLPIRRLFSNNKFDL****A****LSCSK****DLGAN*A**D	50
<i>HsGR</i>	ACRQEPQPGPPPAAGAVAS*****L*****LGARAAV-	49
<i>OvGR</i>	-----EGRHLGGTCVMKGCVPKIMYNCSRHAEEIIDSXDYGFVDTL	80
<i>CeGR</i>	AVEPTPHGHSWGI****A**V**I**ALNSKFQLKH*DKYGWNGIDQEKI	100
<i>HsGR</i>	-----*SHK*****V*****V*W*TAV*S*FMB**A****PSCE	91
<i>OvGR</i>	NGFNWKKIKDSRDAYVRLNGIYESNLCKSQV-----DLIRKASFTK	123
<i>CeGR</i>	KHDWNVLS*NVN*-R*KAN*W**RVQ*NQKINYFNAYAEFVDDKIVIT	149
<i>HsGR</i>	GK**RV**EK*****S**A**QN**T**HI-----EI**H*A**S	134
<i>OvGR</i>	DGVIDVN--GKKYFGKHILIAVGGYPKRPN---VPGAEGYINSDDGFFHLD	168
<i>CeGR</i>	GTDKNKTKNFLSAP--NVV*ST*LR**Y*---I****L**T**DL*T*A	194
<i>HsGR</i>	*PKPTIEVS***TAP*****T**M*ST*HESQI***SL**T****Q*E	184
<i>OvGR</i>	VLPKRTVVVGGGYIAIELSSMLSALGSDVHLLIRKPRVLWNFDHTVSESL	218
<i>CeGR</i>	SV*GK*LI****V*L*CAG**VR**KRS*KILA**FRA*CNGV*****IV	243
<i>HsGR</i>	E**G*S*I**A****V*MAGI*****KTS*M**HDK**RS**SMI*TNC	234
<i>OvGR</i>	TESIDRGPTKLYKNTEVKS*VVKPNGL-----LTVN--TTNDTIDEVNC	260
<i>CeGR</i>	M*HLKTTGV*VKEHV**ER--EAVGSKKKVFTGN--GGVEEY**VI----	287
<i>HsGR</i>	**ELENAGVEVL*FSQ**E*K*TLS**EVSMTAVPGRPLVMTM*PD*D*	284
<i>OvGR</i>	LIWAVGRLPETS*DLNLYVGGKTD*EI--GHVIVDEYQNTSTKNIYAVGDCC	309
<i>CeGR</i>	---*A**V*NLKS**DNA**VR**KRS*KILA**FRA*CNGV*****IV	335
<i>HsGR</i>	*L*I**V*N*K**S**KL*IQ**DK--I*I**F**MV*G*****V*	333
<i>OvGR</i>	--GKALLTPVAIAAGRCIAQRLFNNEINNRLDYENIPTVVFVSHPLGTVGL	358
<i>CeGR</i>	QDRQE***L**QS*KL**D***S*SKQI--VRFDGVA*T**TPLE*S****	384
<i>HsGR</i>	-----*****K**H**EYKEDSK**N*****I*****	382
<i>OvGR</i>	TEAERMKQYGKNNLTIIYKTKFNSL-YHAVTQHQEPTIMKLICAGKNE-Q-	405
<i>CeGR</i>	**E*AIQKH*EDSIEVFHSH*TPFE*VVPQNDGFCYKAVCTRD*S*K	434
<i>HsGR</i>	**D*AIHK**IE*VKT*S*S*TPM-----*KR*TKCV**MV**N**E*-K	429
<i>OvGR</i>	VVGLHMLGEGCDEMLQGFVAIKMGATKKDFDNTVAIHPTGAELVTMRN	455
<i>CeGR</i>	IL***FV*PNAA*VI**Y***FRV*ISMS*LQ**I***CSS**F*KLH-	483
<i>HsGR</i>	***I**Q*L*****V*****A*****SS*****L*	478
<i>OvGR</i>	GTKPETA	462
<i>CeGR</i>	I**RSGQDPRTQGCGG	499

Figure 2 Alignment of the deduced amino acid sequences of *OvGR*, '*CeGR*' and *HsGR*

Residues involved in ligand and substrate binding as well as dimer formation, based upon X-ray studies on *HsGR*, are in bold letters. The asterisks indicate identical amino acids in comparison with the *O. volvulus* sequence. Dashes indicate gaps that were introduced to improve fit. The alignment was done using the multiple alignment mode of the software package DNASIS (Hitachi).

upstream of the poly(A)⁺ tails of the different cDNA clones and differ in one or two nucleotides from the consensus AATAAA (Table 1).

Table 2 Residues involved in binding to the substrate glutathione disulphide

Glutathione group	Residue in <i>HsGR</i>	Residue in ' <i>CeGR</i> '	Residue in <i>OvGR</i>
Glu I	T-339	T-342	T-314
	I-343	I-346	I-318
	R-347	K-350*	R-322
	H-467'	H-472'	H-442'
	T-476'	K-481'	T-451'
Cys I	S-30	S-30	S-19
	V-59	A-68*	V-48
	V-64	I-73*	V-53
	Y-114	Y-122	Y-103
	H-467'	H-472'	H-442'
Gly I	A-34	S-34*	A-21
	R-37	K-37*	R-24
	Y-114	Y-122	Y-103
Glu II	M-406'	F-408**	L-381**
	H-467'	H-472'	H-442'
	P-468'	P-473'	P-443'
	T-469'	C-474**	T-444'
	E-472'	E-477'	E-447'
	E-473'	E-478'	E-448'
Cys II	L-110	N-118*	L-99
	Y-114	Y-122	Y-103
	H-467'	H-472'	H-442'
Gly II	I-113	I-121	I-102
	Y-114	Y-122	Y-103
	N-117	Q-125*	N-106

* Residues that are replaced within '*CeGR*' or *OvGR* with respect to *HsGR*.

Deduced amino acid sequence in comparison with other GR sequences

The deduced amino acid sequence of *OvGR* reveals a high similarity to the human GR (*HsGR*) sequence (53.5% identity), whereas the identity with the putative GR of *Caenorhabditis elegans* ('*CeGR*'; see below) is only 30% [19]. The sequences are aligned in Figure 2, and it is obvious that the *OvGR* encodes the characteristic four-domain structure of GRs [20]. The catalytic-site residues of *HsGR* (Cys-58, Cys-63, Lys-67, Tyr-197, Glu-202, His-467' and Glu-472') have identical counterparts in the filarial GR. As summarized by Aboagye-Kwarteng et al. [21], 19 residues are implicated in GSSG binding to *HsGR* (Table 2). Out of these 19 amino acids, 18 are conserved in *OvGR*. One difference identified involves a conservative substitution of Met-406 in *HsGR* with Leu-381 in *OvGR*. In contrast, '*CeGR*' shows nine differences in these residues, which can also be considered as homologous replacements (Table 2).

The dinucleotide binding motifs for FAD and NADPH as well as the specific FAD-binding motif are well conserved (Figure 2) [22]. However, only one of the side chains that favour the binding of NADPH rather than NADH to the enzyme (Arg-201 in *OvGR*; Arg-218 in *HsGR*) is conserved, whereas the second arginine (Arg-224 in *HsGR*) is replaced by Trp-207 in the *OvGR* genomic and the cDNA sequences (Figures 1 and 3).

The C-terminal part of the protein is highly conserved; within the intersubunit contact area (residues 440–476 in *HsGR*; residues 415–451 in *OvGR*) 87% of the amino acids of *OvGR* are identical with those of the human enzyme.

Genomic structure of *OvGR*

Several genomic *OvGR* phage clones were isolated and partially characterized by PCR, restriction-enzyme cleavage, Southern transfer and subsequent hybridization with the radiolabelled

1.3 kb *OvGR* cDNA PCR fragment or 5'-specific radiolabelled probes (see Figure 4) to identify genomic restriction fragments corresponding to the *OvGR* gene. Three *EcoRI* fragments of 2600 bp, 1330 bp and 774 bp were subcloned into pBSSK⁺, and their nucleotide sequences were determined. Further, a 1285 bp *XbaI* fragment and an approx. 5500 bp *EcoRV* fragment, which specifically hybridized to a genomic *OvGR* 5' probe, were subcloned and their nucleotide sequences determined. The cloning strategy is shown in Figure 4. In order to analyse the complexity of the *OvGR* gene, a genomic Southern blot was performed using the 1.3 kb cDNA PCR product, which hybridized specifically to *O. volvulus* genomic DNA while no signal was obtained with the host DNA (Figure 4). The genomic Southern-blot analysis revealed the presence of at least one *EcoRI* restriction site within the *OvGR* gene. The *EcoRI* fragments seen on the blot represent the internal fragments of the *OvGR* gene (2.6 kb and 1.33 kb). The 3' and 5' portions of the gene do not hybridize to the probe, which only encompasses part of the entire genomic *OvGR* sequence. The sequence analysis of the *OvGR* genomic clone 4.1 revealed the presence of three *EcoRI* restriction sites within the *OvGR* gene (Figure 3). There exists one *EcoRV* site in the genomic DNA sequence of *OvGR*, but there is only one 3.1 kb *EcoRV* band to be seen on the blot probed with the *OvGR* cDNA. This restriction pattern is probably due to the size of the probe used and the fact that the *OvGR* gene has only three small exons interrupted by two large introns, not hybridizing to the cDNA probe, within the 5' region, whereas in the 3' region of the gene the intergenic areas are smaller and almost the entire coding sequence can hybridize to the cDNA probe.

Nucleotide sequence analysis shows that the *OvGR* gene is composed of 13 exons and 12 introns. The intron–exon boundaries show conserved 5'-gt-ag-3' intron splicing junctions. A comparison of the 5'-untranslated region (UTR) of the *OvGR* cDNA with the corresponding region in the gene revealed that the 5' end of the primary mRNA transcript is interrupted by a 155 bp intron (Figure 3). This intron is located 16 nucleotides upstream of the translation initiation codon, and it is removed from the mature mRNA, as it is no longer present in the cDNA sequence of *OvGR*. The size of the entire *OvGR* gene including 5'- and 3'-UTRs is 4065 bp. Further, the sequences of the 5'- and 3'-flanking regions, 677 bp and 459 bp respectively, of the *OvGR* gene were determined (Figure 3).

Expression and partial characterization of recombinant *OvGR*

Using the expression vectors pJC40 it was possible to produce active, recombinant (r) *OvGR* (Figure 5). More than 70% of the protein was located in insoluble inclusion bodies. Only the soluble fraction of *OvGR*, obtained after centrifugation of sonified resuspended bacteria at 100 000 g, was used in the purification procedure, yielding 400–600 µg of soluble, purified r(His)₁₀*OvGR* per litre of bacterial culture. The recombinant histidine-fusion protein produced in pJC40 has a specific activity of 78 ± 3 (S.E.M.) units·mg⁻¹ of protein. The subunit molecular mass of the histidine-fusion protein was determined to be 54 kDa using SDS/PAGE. The theoretical subunit molecular mass deduced from the amino acid sequence without the His tag is 50.5 kDa. The recombinant histidine-fusion protein is active as a dimer of 110 kDa, as shown by gel filtration on a calibrated Sephadex S-200 FPLC column. The *K_m* values for GSSG and NADPH were determined to be 130.7 ± 13.0 µM (*n* = 6) and 10.9 ± 2.4 µM (*n* = 4) respectively. The recombinant protein shows a strong preference for NADPH (*K_m* 10.9 ± 2.4 µM) over NADH (*K_m* 108.0 ± 17.0 µM) (*n* = 4) as reducing cofactor.

acatgcagtgagcgttagaagaataagggaataatgattctcttctatctagttacttag
 atattggccttggtttatctccaattatattgagatcatagctggcaactaattcagtc
 cagtaaatatctcccaatagggaataatattgcttcccatccatcggaagaaagt
 gttcaaacacaaagctcaactcaactcaactcaattggtggaattggtttgatgaacca
 catacttctgcttcaattagggtggtggcaaaaacatagattgctctctgtaaa
 gtttgaattactgatcgcaattctgcttttgcattctaatgctgtttcttagcttaa
 atcgcttatatctggcgtggcaatagctgataatcgatgcacattatgctagcgaa
 aatgcaagagcaaaagcaaaaacatgcccacacatgaggaatccgattctctaat
 atattcagggcagttatctgggtttaaagcagaagatcccaacccagataacgacatcat
 acatggttctctccagagttctaatctggaacactgctccgagaataacgagtgatct
 ctgagagctgcccggccgagctcaattcaacccctcaataaaggagatcgactcgatcgt
 ttgtaaatatttaagggtgattttatgtgacattttcgatattgtggagataat
 \$ trans-splicing site
 cttgttaatttactcattatagGAACGAAGTCTCCATGGTGCCTTAGTTACATTACTT
 TTGTTCCACGATTTTTTTCGATTGATCGATGAATCGCGCTCCCTCATTTATCGGTGT
 CGCTACGATTTCCTTACTGTTTGTCTTCGACCGGTTTCGCAGCAATGCCACACAAA
 GTCCCATACATCAATTTCCGATTGCGAAATTTCCCTAACATAGCgaagaactgttga
 aaatctgctgatagataactaagcgaagtgaacttccatgacacactctgctaaactta
 ttattgttctgaaatttcaacttgcactgtgttttttttttttttttttttttttttttt
 tcagtgcttcaaaaagttttcagcagCAATGACAGCTATTATGAGCAGTGGTATACGAT
 M S S G D T H
 GAAAACGTCAGATTATCTGGTGATCGGTGGTGGCTGAGGATTTGCGAGTGCCCGCC
 K T Y D Y L V I G G S G G I A S R
 GAGCTCTGTAATTCAAAGTATCTGGGATTAATGGAAGGAAGACATCTCGGTGTGACA
 R A A E F K V S V G L I E G R H L G T
 TGGCTtagtttttaactatttccagaactcggaatttttccatatttagttatttagaag
 C
 aagaaataagtttaaaacttttccctcgaaattttttgtgacaattttcagGTAAATA
 V N
 AAGATGCGTACCGAAGAAGATTATGTATAATTGTTCCCGCATGCAGAAGCAATCATC
 K G C V P K K I M Y N C S R H A E A I
 GATCATCTCCGATTACGGTTTGTATGTACATTGAAATGGATTCAATTGGAAGtagattaa
 D H S D Y G F D V T L N G F N W K
 ttttccattctcttctgttccatagttataaaaaagtttttgcgtccgaagaaacaa
 aatctgtcttaatttaagaataatgggcaaatcgatttatcatcatatttgggaac
 cgcaacatttaagattgcaatgaatcaatttgaaggaatttcatataaaatggataaca
 catgattatctattgttttgcactcagtaaatgatttaatacaaatgcatgattgaa
 atttggggaaagcagattgctgattgctgtgattgctgatttttaatttttgatcct
 ctactacgacttaacccaataagaagtttcaaaataaagactgattcaaaaagtacttt
 agattatttttccagttgtttaaagtatttttgaatttctgtttcttagagactgtata
 gattattcttttaccgatttttctgttttttctgctaatcaacttttttttttagtaaaac
 acattagtgatttccatttagAAAAATAAGGATAGTCGCGATGCATATGTCAGGCGTTT
 K I K D S R D A Y V R R L
 AAATGGAATATATGAATCCAACCTATTATGCAAGtagttttaaacttttcaacttttttt
 N G I Y E S N L C K
 cccatcgaaatgaacacttttttgcatacaatttaataaagaacaaatttgcgaataaaaa
 cctgcatcatccggatttttctcttcaataatgcatcttctgctgtgacattgtttcgt
 ttttctctcagtgaaacacttaattttgtgatttttttttttttttttttttttttttt
 ctgtttatgaatcattttttctgtgttttaaagagtcacgcaaaaagctcatttggta
 tatttatacaacttttaacaatagcatgaataaaaaactggatgttacacataaaggtt
 ttattatgcataaaaaattacagaacagttccacaataaattgtataaactttcaacaacaa
 gaataaagctgagctgagttgtgtgctcaggtttccatcagacagatattgattatgttt
 ttttgcacaactgtcaaaacttaacttaactatgctgcagtaatttcaattattgtttttg
 ttttaattgttcttatttttagTCACAACTCGACTTGGATTCGAGGAAAGCGTCATTTA
 S Q V D L I R G K A S F
 CCAAGATGGTGTATTGACGTTTACCGCAAGAAATATTTCGAAACACATATTGATA
 T K D G V I D V N K G K Y F G K H I L I
 GCTGTGCGAGGtagtagtagttaaatttttttctgaaattataaactttgatgaa
 A V G G
 aattattctgatgagaagtagttgcttgatttcttattgtcagcattatagATATCCAAA
 Y P K
 GCGGCCTAACGTGCCGGGTGCAGAGTATGGTATCAATAGTATGGCTTCTTCCATCTTG
 R P N V P G A E Y G I N S D G F F H L
 ATGTTTACCAGtagtgatttttgcattttttctccattttctgtggaaggaccaag
 D V L P K
 acttattttttgttactattttatcatcataaacttttggctctatgaaattttt
 attctagACGCACGGTAGTAGTGGGAGTGGTTACATAGCGATCGAATATCATCGATG
 R T V V V G G G Y I A I E L S S M
 TTGTCGCGATTAGGATCTGATGTTCAATTTGTGATACGAAACCAAGGTTTGTGGAA
 L S A L G S D V H T L L I R K P R V L W N
 TTTTGTACACACAGTGTCTGAATCTTAAAGCAATCTGATAGAGGCCAACGAAC
 F D H T V S E S L T T E S I D R G P T K
 TTTATAAAATACGGAAGtagtaatttttattacattctgacatatttcaataaatatg
 L Y K N T E
 ctattataatattttgttttaaaattattctgacatttttgacaaaagtttag
 ataattccatgatattgctgataattttacaagttatctcaacttccgacagataaact
 aatagtgactctaatatgatcatcagctaaaaaacagtttgagaataagcgaatgaa
 cggtttttttgatgtagacgattccagatttttcttcaattcttttgggttcaataatga
 aatcaaatagctaaactgattaccaaatttaagaaggccttctgttatctgtaagtgaac
 aatttttaacttcaattcaatttaataattagcatttttctgtttaaagttatgctttt
 tcaagttttgaaagattttgtcataataatcagtggtgcaattcatgaaactattattt
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 V K S V V K K P N G L L T V
 AACACACAAATGATACCATTTGATGAAGTAATTTGTTAATCTGGGAGTTGGCGTCT
 N T T N D T I D E V N C L I W A V G R L
 TCTTGAACCAAGTATTAAATCTAAATTTATGTgtttgtaatttttatttaactgaa
 P E T S D L N L N Y V
 acttccgttgcattctatctaaaaatagaatttcccaaaaaataagcaagttttgtagGGT
 G
 GTGAAACTGATGAAATCGGCCATGTGATTGTGGACGAATATCAGAACACATCGACTAA
 V K T D E I Q V D E Y Q N T S T
 AAATATTATTGTCAGTAGGAGATTGCTGTGGAAAGCAATTATTGACACCGgtttttgt
 N I Y A V G D D C C G K A L L T P
 tgaatcagtttttaacttagtgagcattgttttaatttttcaataactgtaataagcat
 tccagtttttaaaaattttctgttataatacccaaaaattacagctcattagtttgaana
 tttcgaagattatatacagcatttttggaaaaataataatttgaanaataaact
 cttttgtttttgttctgataaatttataatatttaataatttttaagtgaattcagTTG
 V
 CGATTGCTGTCGACGATGTCTCGCCAGAGATTGTTCAATAACGAATTAACAAATCGT
 A I A A G R C L A Q R L F N N E I N N R
 CTGGATTACGAAACATCCCGATGTTGTGTTAGCATTCCACCATAGGAACCGTGG
 L D Y E N I P T V V F S H P L G T V G

GCTTACAGAGGGtaggaattgtctttaaattcttattcttcaattttaaacaacaaagctg
 L T E
 ccatagcgaattttctgtagcttttctgtcttacttttttcccaacaaatttttat
 tagttttgtatagttttgatttttagCGGAAGCTATCAAAATATGGAAGAAACATCTT
 A E A I K Q Y G K N N L
 ACAATTTATAAAACGAAATTTAATCTCTGTATCTGTACTCAACACAAAGAACCT
 T I Y K T K F N S L Y H A V T Q H K E P
 AACGATAATGAAATTAATCTGTCTGGTAAAGAGGtagaattgtaaacgtatctt
 T I M K L I C A G K N E Q
 cggagtgagagcgggtgcttctagaaagttttattcttattttcaaatatttactttca
 agtttcgaaatgaaatttcaaaaactcatgtaaaactattttagGTGTCGGTTTG
 V V G L
 CACATGCTTGGTGGAGGATGTGACGAATGCTGCAAGGTTTGTGTTGCCATCAAGt
 H M L G E G C D E M L Q G F A V A I K
 gagttataattcgaattgctgtagctgcgaagtagtaataaattcttatttttt
 ggaataataaattttctgcaattccacattgtgaagaataaattgagattttaaag
 tagttttttttaagATGGGTGCGACGAAGAGGATTTCGATATACAGTTGCTATTCA
 M G A T K K D F D D T V A I H
 TCCAACAGGTGCGGAAGAACTAGTTACAACTGATGACAAAACCCGAACAGCAT
 P T G A E E L V T M R N G T K P E T A
 AAGcatgaatttctgatttctgttaacacagaaggaactttaaataagtcacagactc
 *
 gtcaaaaacotgaattgtttgtgttctggaaaaacatttctgtttttcgaattcc
 tgcatttaagatttcaacttttactgaatttctgtgcacaaacaaagttgtaaacattt
 ttagaataaattgttttagtgaattatcatgaaattatttagtaccggaattcaatgattt
 ccctagaagctaaaaagcacaatgaaatcagaataactctgtttattttgattgataa
 gaacattttttcaaaattttgtataaaacttctgataaatttttctagctctctc
 ctgagttatagaaaaagctcttttataaaactgataacgacagctgacacaagacg
 tgacacacagctgataaacaacagatttttcaagaatttttcaagaatcatcaaaat
 gattatttggcagcttttattgttttttattctgtgacgaaaaaagaatttaggaat
 aatctgagaaggttaactgctacgatttctgcaatttattgataaactctggcatt
 tttcagctttatttaggatttctagtagaattttgcaactcgcggaacacatatata
 ggactactcaatttcaacagctgataaggtgctgcaatgaggtgtttttatttatacaca
 aaaccatttttaaaatttttaaaaaaaattttaaactcattctctgaaaaagga
 tcaattatttaaaagcattctctgagacacaaatagaagatttaacagcattcttata
 aaagacaagttcatattgttgaaaaactcagctattttgttacttgcattttaaagtaa
 agatttgggtttaaataagaatt

Figure 3 Genomic sequence of *OvGR*

Exonic and intergenic sequences are shown in bold upper-case letters and lower-case letters respectively. The *trans*-splicing site and the translation initiation codon (ATG) are underlined. Nucleotide position 1 is assigned to the *trans*-splicing site (\$); negative numbers refer to the 5'-flanking sequence. The amino acid change Gly → Val compared with the cDNA sequence is double underlined. The asterisk indicates the stop codon.

DISCUSSION

GR cDNA was isolated from the human parasite *O. volvulus*. The cDNA nucleotide sequence has a 236 bp 5'-UTR and carries a nematode-specific SL1 at its 5' end [18,19,23–27]. The 5'-UTR of the *OvGR* mRNA is unusually long when compared with other *O. volvulus* cDNAs, such as Cu/Zn superoxide dismutases, Mn superoxide dismutase and *O. volvulus* glutathione S-transferase 1, where the length of the 5'-UTR is between 0 and 76 bp [25–27]. Similarly the 5'-UTR of *O. volvulus* S-adenosyl-methionine decarboxylase (AdoMetDC) mRNA [27] was also found to be very long, possibly indicating a specific function of this region. It is well established that the AdoMetDC is a highly regulated gene in eukaryotes and that the formation of secondary structures within the 5'-UTR is involved in mRNA stability and translational control [28,29].

Within the 3'-UTRs of several cDNA clones, no consensus polyadenylation site could be identified. The 3'-UTRs of these cDNA clones are identical up to the site of polyadenylation, and each clone has a potential polyadenylation signal upstream of the poly(A)⁺ tail (Figure 1). None of the cDNA clones analysed contained an A-rich stretch within the 3'-UTR that could have been responsible for wrong priming of the oligo(dT) primer during cDNA library construction. These results suggest that due to alternative polyadenylation several *OrGR* mRNAs might be synthesized.

An *O. volvulus* genomic Southern blot, using the 1.3 kb cDNA PCR product as a probe, resulted in a restriction pattern consistent with that of the isolated *OrGR* gene (Figure 4). Unlike in *Nicotiana tabacum*, where more than one copy of GR per haploid genome was identified [30], the results shown here

Figure 4 Genomic Southern-blot analysis, cloning strategy and genomic structure of *OvGR*

Upper panel: genomic DNA of *O. volvulus* was digested with *EcoRI*, *EcoRV* and *DraI*, and human genomic DNA was digested with *DraI*. The DNA fragments were subsequently separated by 0.8%-agarose electrophoresis, blotted on to a nylon membrane and probed with the 1.3 kb cDNA PCR product. M, 1 kb ladder (kb). Middle panel: genomic phage DNA was digested with *EcoRI*, *EcoRV* and *XbaI*, separated by 0.8%-agarose electrophoresis, blotted on to nylon membrane and probed with either the 1.3 kb cDNA PCR product or a 655 bp genomic DNA probe. Hybridizing bands were subcloned into pBSSK⁺, and their nucleotide sequence was determined. RI, *EcoRI*; RV, *EcoRV*. Lower panel: the genomic structure of *OvGR* was determined by sequence analysis of the genomic phage DNA. The locations of *EcoRI*, *EcoRV* and *XbaI* restriction-enzyme sites in the gene are shown. SL1, spliced leader acceptor site; ATG, translation initiation codon; TAA, stop codon; Poly(A)⁺-tail, site of polyadenylation, determined in the longest cDNA clone *OvGR5A3*. Boxes represent intergenic sequences (hatched box is in 5'-UTR).

suggest that the *OvGR* is encoded by a single-copy gene. The *OvGR* gene spans 4065 bp and comprises 13 exons and 12 introns. The first intron of *OvGR* is located in the 5'-UTR, 16 nucleotides upstream of the ATG (Figure 3). Thus the *OvGR* cDNA has a 236 bp 5'-UTR that undergoes both *cis*- and *trans*-splicing processes. Similar results were obtained for the *O. volvulus* AdoMetDC [27]. The processing of the first intron might have potential regulatory functions.

Figure 5 SDS/PAGE analysis of recombinantly expressed *OvGR*

Lanes 1–3 represent soluble proteins extracted from *E. coli* BL21 (see the Materials and methods section) before and after IPTG induction. Proteins were separated by SDS/PAGE on a 10%-polyacrylamide separation gel and stained with Coomassie Blue. Lane 1, *E. coli* BL21 lysate containing pJC40*OvGR* before IPTG induction. Lane 2, *E. coli* BL21 lysate containing pJC40*OvGR* after IPTG induction. Lane 3, purified *rOvGR* after passing through Ni²⁺-chelating Sepharose (Novagen). M, high-molecular-mass markers (Bio-Rad).

The deduced amino acid sequence of *OvGR* cDNA encodes a polypeptide of 462 amino acids. The amino acid sequence has 53 % identity with *HsGR*. This is much higher than the similarity to the putative GR of the free living nematode *C. elegans*, with only 30 % sequence identity [19,31,32]. As shown in Table 2, 8 out of 19 residues involved in GSSG binding are different in 'CeGR' compared with the *HsGR*, whereas the *OvGR* deduced amino acid sequence only shows one conservative replacement within these residues. These results suggest that the 'CeGR' sequence might encode another flavoprotein oxidoreductase rather than GR. Recently, the human and *Plasmodium falciparum* thioredoxin reductase have been cloned, and the amino acid sequences of both proteins show a high degree of homology to the 'CeGR' deduced amino acid sequence [33,34]. It remains for investigation whether the 'CeGR' belongs to this new family of large thioredoxin reductases, as suggested by Müller et al. [34].

The residues around the catalytic active cysteines (Cys-46 and Cys-51 in *OvGR*) are almost identical with those in the human GR. However, one substitution was identified: Val-61 in *HsGR* is changed into Lys-50 at the equivalent position in *OvGR*. This change might have an impact on the enzyme activity or the sensitivity of the protein towards inhibitors. Another marked difference from all other GRs is the substitution of Arg-224, one of the residues responsible for NADPH binding [22], into a Trp-207 in *OvGR*.

Recombinant *OvGR* was expressed in the *E. coli* system. The expression level was between 400 and 600 µg of protein per litre of bacterial culture. The protein was expressed as a histidine-fusion protein and subsequently was purified using chelating Sepharose. The specific activity of 78 units · mg⁻¹ of protein and the k_{cat} of 4200 ± 180 (S.E.M.) min⁻¹ appear to be lower for the parasite GR when compared with the *HsGR*, with a k_{cat} of 12600 min⁻¹ [35], indicating a lower efficiency of catalysis for the parasite enzyme. The purified native protein of *Setaria digitata* and the *Ascaris suum* GR have specific activities of 24 and 32 units · mg⁻¹ of protein respectively, also lower than *HsGR*, with 240 units · mg⁻¹ of protein [10,36–38].

Compared with *HsGR*, the affinity of r(His)₁₀*OvGR* to the substrate GSSG is slightly lower: 130 µM compared with 65 µM. Both proteins show a strong preference for NADPH (K_m

10.9 μM) over NADH (K_m 108 μM for OvGR and K_m 500 μM for HsGR). These results indicate that the substitution of Arg-224 in HsGR by Trp-207 in OvGR still favours NADPH binding over NADH binding to the enzyme. It remains to be investigated by structural analysis and mutagenesis studies whether Trp-207 is really involved in ligand binding, or whether another positively charged residue is responsible for the discrimination between NADPH and NADH binding to the parasite protein. We are currently investigating the mechanism of action of several potential GR inhibitors, such as tervalent organic arsenicals and isoalloxazine derivatives, which were reported to inhibit effectively human and *P. falciparum* GR [39], in order to test the validity of the filarial glutathione metabolism as a potential target for chemotherapy.

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