Summary and Conclusion
Lymphatic filariasis and malaria are major public health and socioeconomic problems in the tropical and subtropical parts of the world, causing widespread destruction of mankind. Lymphatic filariasis is one of the leading causes of permanent and long-term disability in the world. The pathology associated with lymphatic filariasis results from a complex interplay of the pathogenic potential of the parasite, the immune response of the host, and external ('complicating') bacterial and fungal infections. At present no effective and safe chemotherapy is available against the adult filarial parasites, raising an urgent need for the development of macrofilaricidal drugs. Malaria, within its dominion affects almost every aspect of social and economic endeavor, including fertility, schooling and migration decisions. The emergence of parasite resistance to our limited armamentarium of antimalarial drugs contrives a formidable therapeutic challenge and necessitates the development of new drugs for the treatment of resistant cases of malaria.

Antioxidants are the cell's premier resource for protection against the diverse free radicals and other oxidative stressors to which it invariably becomes exposed. Glutathione (GSH) is the central constituent of the collective antioxidant defenses in a cell, and a highly potent antioxidant and antitoxin in its own right. In the face of endogenous and exogenous oxidative burden, the formidable reducing power of the GSH/GSSG couple is a profound physico-chemical asset for the cell. GSH participates in a plethora of biochemical processes, acting as a cofactor in the synthesis of proteins and deoxyribonucleotides, as a reducing agent in disulfide-sulfhydryl exchange reactions, an γ-glutamyl donor in amino acid transport and in protection against cellular damage due to radiation, ROS and toxic chemicals of both endogenous and xenobiotic origin.

Both filarial and malaria parasites appear to be more sensitive to oxidative stress than their mammalian hosts, and thus, the peculiarities and idiosyncrasies in these that can be exploited in drug design. The significance of GSH and related metabolism to filariae has been indicated by earlier findings that sustained severe reduction in endogenous GSH pools within adult filariae accelerate a drop in microfilarial output and eventually led to the death of the parasites. The finding that filariae in vitro are also able to salvage exogenous GSH, unlike most, if not all mammalian cell populations, points to a promising target for selective inhibition.
GSH is of major importance in filarial species as it has been proposed to constitute the antioxidant system responsible for the long term existence of filarial worms in mammalian host by protecting them from the ROS produced by the normal metabolism and the immune cells of the host. On the other hand, the role of GSH in important processes during the intra-erythrocytic life of the malaria parasite greatly increases the relevance of the *Plasmodium* enzymes involved in its biosynthesis and regeneration, as possible targets for the design of new effective drugs.

Glutamate cysteine ligase is the rate limiting enzyme responsible for *de novo* GSH biosynthesis, which catalyses the ligation of the L-glutamate and L-cysteine to form L-γ-glutamyl-L-cysteine. GSH oxidized by various metabolic processes to GSSG is regenerated by the enzyme glutathione reductase. Taking these facts into consideration, it was perceived that inhibition of these two enzymes in parasites presents potential targets for antifilarial and antimalarial drug development.
The present study demonstrates the presence of GCL and GR in adult filarial worms *S. cervi* and malaria parasites *P. yoelii nigeriensis*. Under the specified assay conditions the activities of cytosolic GCL and GR from *S. cervi* and *P. yoelii* were found to be fairly linear with respect to time (30 sec to 5 min) and amount of enzyme protein (17.3 to 276.3 μg and 14.6 to 468.3 μg for *S. cervi* and *P. yoelii* GCL, and 4.23 to 42.3 μg and 0.0053 to 0.063 μg for *S. cervi* and *P. yoelii* GR); the respective specific activities of GCL in cytosolic fractions were estimated to be around 0.577 and 0.0343 μmol/min, while those of GR were found to be around 0.0362 μmol/min and 0.780 μmol/min.

The catalytic activities of GCL from *S. cervi* and *P. yoelii* were found to be sensitive to pH of the assay buffer and concentrations of different components of the assay system, viz. PEP, Mg++, and NADH. The enzyme from both sources was found to be maximally active between pH 8.0 to 8.5. Like other biosynthetic pathways, GSH was found to exert a regulatory effect on its own synthesis, feedback-inhibiting GCL, the primary enzyme of its *de novo* biosynthesis. GSH analogs may thus be synthesized as GCL inhibitors and prospective drug candidates for antimalarial and antifilarial chemotherapy.

The substrate specificity and inhibitor profile of *S. cervi* and *P. yoelii* GCL appear to be different from each other and also from GCL from mammalian sources. The apparent Km of *S. cervi* and *P. yoelii* GCL with respect to ATP were found to be 1.66 mM and 1.25 mM, respectively. BSO, the known transition state inhibitor of GCL, caused enzyme inactivation; the Ki obtained for the *S. cervi* and *P. yoelii* enzymes, i.e. 7.0 mM and 4.0 mM, respectively, were lower than that found for the corresponding mouse liver enzyme (13 mM). Thus, the structure of BSO was utilized for the further development of GCL inhibitors. Cystamine showed appreciable inhibition on GCL from *S. cervi* and *P. yoelii*, the respective inhibition constants being 27 μM and 32 μM. The highly effective inhibition of the enzyme by cystamine observed is consistent with the possibility that cystamine may play a role in the physiological regulation of this enzyme. Heme, which is toxic to the malaria parasite, is formed when the intraerythrocytic malaria parasite ingests and digests its host cell cytosol that consists mainly of Hb, inside its food vacuole. In the present study, hemin was found to be a potent inhibitor of *P. yoelii* GCL, with Ki of 29 μM. The finding is
unique in that GCL in inhibited by hemin, which in turn is degraded by GSH, the final product of GCL reaction. Further studies into the structure and binding properties of hemin to GCL may be of critical importance for the development of therapeutic agents.

The results of the present study indicate that the catalytic activity of GR from both sources is sensitive to assay buffer concentration and to the presence of monovalent sodium salts, as reported for counterpart enzymes from other sources. The optimum pH was found centered around pH 7.0, similar to GRs obtained from human erythrocytes, porcine erythrocytes and calf liver. However, the *S. cervi* GR showed a broad pH optimum as compared to a sharp peak obtained for the *P. yoelii* GR. Kinetic experiments indicate that the affinity of NADPH to both the enzymes is higher than GSSG, in agreement with those reported for GR from other sources. The Km values obtained with respect to the substrates NADPH (20.8 µM and 10 µM for the *S. cervi* and *P. yoelii* GR, respectively) and GSSG (50 µM and 16 µM for the *S. cervi* and *P. yoelii* GR, respectively) were different from known values for calf liver, rat liver and human erythrocytes.

Inhibition profile shows that GR from both sources is inhibited by the product of its own reaction, NADP, in agreement with previous work on the counterpart enzyme from mammalian sources. However, the *S. cervi* enzyme had a lower Ki (48 µM) for NADP from the competition with NADPH as compared to that obtained for GR of human erythrocytes (70 µM), while the *P. yoelii* enzyme showed a higher Ki value (700 µM). The other product of the GR reaction i.e. GSH inhibited the *S. cervi* GR reaction with a Ki of 6.8 mM, while it showed no inhibition on the corresponding *P. yoelii* enzyme. The mammalian GR inhibitor menadione caused no inhibition on both filarial and malaria GRs, while it inhibited rat liver GR to the tune of 27 to 88% when tested in the concentration range from 0.5 to 4 µM. The inhibition of *S. cervi* and *P. yoelii* GR by MB gave lower Ki values (4 µM and 5.3 µM, respectively) as compared to human (16 µM) and *P. falciparum* (6.4 µM) GR, indicating they are more susceptible to inhibition by MB. Inhibition by MB on GR differs in that it is non-competitive with respect to GSSG in the case of GR from *S. cervi*, while it is competitive in the case of GR from *P. yoelii*. Inhibition patterns of *S. cervi* and *P. yoelii* GR by Carmustine have been found to be similar to that of the counterpart from
yeast; the Ki values from each source was calculated to be around 0.2 mM and 0.35 mM, respectively. Earlier studies on the inactivation of yeast GR by carmustine have shown that binding of an isocyanate derivative formed during carmustine degradation to the active site of the enzyme causes its irreversible inhibition. CDNB is an electrophilic compound alkylating SH groups used as a substrate in assays to determine GST, which is involved in elimination of CDNB in vivo. A known inhibitor of thioredoxin reductase, it is used for depleting intracellular GSH and also shows distinct immunomodulatory properties. It showed distinct inhibition properties on S. cervi and P. yoelii GR, and the inhibition constants calculated (Ki of 20 μM and 60.25 μM, respectively) in the present study on GR were comparable to those obtained for human thioredoxin reductase.

For the purification of GR, a combination of salt precipitation, ultrafiltration and affinity chromatography on 2', 5'-ADP Sepharose 4B was employed. The results obtained mean that this purification procedure is good enough to be used in other studies. This purification procedure has also an advantage of an experimental period as short as a day. The Km of the purified enzyme with respect to NADPH (19 μM) and GSSG (46 μM) showed little differences from that obtained with the crude enzyme. The Ki values obtained for product inhibition by NADP (30 μM) and GSH (5.9 mM), however, were slightly lower than those obtained with the crude enzyme.

In view of the key role of GCL in dictating GSH availability to filarial worms and malaria parasites, the differences obtained in the enzymes from the two sources, S. cervi and P. yoelii, with the host enzyme are consistent with GCL being a good but yet to be fully validated drug target. Further, due to its central role in cellular redox metabolism, inhibition of the GR of filarial worms and malaria parasites represents an important target in the design and synthesis of antifilarial/antimalarial agents.

Most of the currently available antifilarial drugs have been discovered empirically by screening of large number of compounds for efficacy against parasites in animal models. However, very few of these drugs have been rationally designed, the rational drug designing is usually based on biochemical and physiological differences between parasites and their host. The comparison of the parasitic GCL/GR and the isofunctional host enzymes detailed in the present investigation opens a new approach to studies on the redox metabolism of the parasites as a drug target for
chemotherapeutic intervention against malaria and filaria. With these basic differences in the enzymes from the two sources in mind, GR and GCL were assessed as possible targets for chemotherapeutic attack of these parasitic diseases. To this end, a total number of 731 compounds, synthesized by earlier reported methods, including cinnamate derivatives, coumarins and coumarin amides, glycoconjugates (glycosyl peptide glycoconjugates, glycosyl ureas and thioureas, DGDA, glycosylated amino acids, glycosylated amino esters, FMOC derivatives of glycosylated amino ester, glycosylated hydrantoin, glycosylated hydroxamic acid, glycosylated sulfone derivatives), imidazole derivatives, isoxazole and substituted isoxazole derivatives, morpholine derivative, nucleosides and C-nucleosides and S-containing nucleosides, organometallic rubidium complexes, piperazine derivatives, pyridine derivatives, pyrrolidine derivatives, substituted propane derivatives and thiazidine thiones were screened for their effect on isolated filarial and malarial GCL and GR.

A careful examination of biological activity of compounds synthesized revealed that two hundred and six compounds inhibited \( S. cervi \) GCL to the tune of 10 to 82% of which 2 DGDA, 6 glycoconjugates, 4 isoxazole derivatives, 1 nucleosides and C-nucleosides, 1 substituted propane derivative and 4 thiazidine thiones showed more than 50% inhibition on intact GCL, while three hundred and seventeen compounds inhibited \( S. cervi \) GR to the tune of 10 to 92% of which 1 DGDA, 24 glycoconjugates, 3 nucleosides and C-nucleosides, 1 pyridine derivative, 4 pyrrolidine derivatives, 1 substituted propane derivatives, 2 thiazidine thione compounds showed more than 50% inhibition on GR from \( S. cervi \). Noteworthy in this connection are 118 compounds that showed simultaneous inhibition on GCL and GR. The effect of the most promising ones have been looked on the counterparts from mammalian sources for the optimization of the leads and difference in the susceptibility towards enzyme activity inhibition were noted. Majority of the compounds showed no inhibitory activity on the mammalian enzymes except DGDA (compound 335) and glycosyl urea (compound 462), which showed 15.2 and 18% inhibition, respectively, on mammalian GCL, and glycosyl hydroxamate (compound 641), which showed 71.4% inhibition on mammalian GR.

The \textit{in vitro} antifilarial efficacies of the compounds were determined against human filarial worm \( B. malayi \) and were also looked for their antifilarial efficacy \textit{in}
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The criteria for assessment of *in vitro* efficacy were motility and MTT reduction tests. The motility of the lymphatic filarial worms *B. malayi* was completely lost with 1 coumarin, 7 DGDA, 19 glycoconjugates, 15 isoxazole and substituted isoxazole derivatives, 2 nucleosides and C-nucleosides, 1 piperazine derivative and 1 substituted propane derivatives. On the other hand, a total of 160 compounds were found active in MTT reduction assay, of which 1 cinnamate derivative, 6 DGDA, 23 glycoconjugates, 6 isoxazole and substituted isoxazole derivatives, 5 nucleosides and C-nucleosides, 2 pyrrolidine derivatives, 1 substituted propane derivative showed more than 50 percent reduction of MTT. The *in vivo* efficacy data of 18 compounds and the reference drug ivermectin against mf and adult worms revealed 8 compounds—Compounds 14, 18, 114, 175, 236, 237, 335 and 348 which showed microfilaricidal as well as macrofilaricidal action and sterilizing effect on reproductive potential of female worms.

The inhibition profile of the synthetic compounds against *P. yoelii* GCL and GR revealed a total number of two hundred and forty nine compounds inhibiting *P. yoelii* GCL to the tune of 10-89%, of which 1 glycoconjugate, 1 glycosyl peptide glycoconjugate, 3 glycosyl ureas, 5 isoxazole derivatives, 2 nucleosides and C-nucleosides and 1 pyrrolidine derivatives showed more than 50% inhibition on intact GCL, while two hundred and five compounds inhibited *P. yoelii* GR to the tune of 10-70% of which 1 glycoconjugate, 2 glycosyl ureas and thioureas, 1 glycosylated amino acid, 2 nucleosides and C-nucleosides and 2 thiazidine thione compounds showed more than 50% inhibition on GR from *P. yoelii*. Noteworthy in this connection are 41 compounds that showed simultaneous inhibition on *P. yoelii* GCL and GR.

Susceptibility to oxidative stress is a well-established feature of the malarial parasites. The tripeptide GSH is the crux of an important endogenous protective system against oxidative stress; inhibition of the GSH redox cycle has been shown to impair the development of the erythrocytic stages of *P. falciparum*. GSH of malarial parasites has thus been considered to be a drug target and therefore it is equally important to assess the modulation in its levels and that of the enzymes involved in GSH homeostasis, GCL and GR, of the affected host’s tissues. An attempt was thus made to measure the same in hepatic and splenic tissues of *P. yoelii* *nigeriensis* infected mice and in the same tissues after treatment with known antimalarial drugs.
Hepatic and splenic GSH levels, and GCL and GR activity profile during *P. yoelii* infection in Swiss mice show that infection by *P. yoelii nigeriensis* causes a decrease in GSH, GCL as well as GR as compared to the normal tissues. Treatment with known antimalarial drugs mefloquine, menadione and methylene blue caused a restoration in the levels of the GSH and the two enzymes. Therefore, it can be concluded from the present study that malaria infection can depress the hepatic and splenic GSH functions as compared to the normal tissues, and that these are normalized within a week after treatment with the antimalarials tested. Also, mefloquine and methylene blue were successful in curing the mice, causing a rapid decline in percent parasitaemia, while menadione caused a delay in maturation of infection, but could not cure the mice.

Results of the present investigations clearly demonstrate that there are some differences in the two enzymes i.e. GCL and GR in filarial worms and malaria parasites versus the host enzymes, that appear to be therapeutically promising, and may thus exploited for further development of antifilarial and antimalarial drugs.