Summary
Filarial nematodes, like all aerobic organisms, require enzymatic and non-enzymatic antioxidant systems to cope with reactive oxygen species (ROS) generated during cellular metabolism. Additionally, they have to protect themselves against ROS produced by the host. It has been postulated that the antioxidant enzymes and molecules are essential for the parasites to defend themselves against ROS generated by macrophages, neutrophils, and eosinophils of the host, in addition to their normal functions in aerobic organisms. These antioxidant systems may be particularly important for long-lived filarial parasites that are involved in chronic infections.

The tripeptide GSH (L-γ-glutamyl-L-cysteinyl-glycine), the most abundant nonprotein sulphydryl present in cells, plays a central role in the protection of cells against oxidative and electrophilic stress. GSH can either act as substrate to various enzymes like glutathione reductase, glutathione peroxidases, and glutathione-S-transferases in the GSH redox cycle or is able to directly inactivate ROS such as \( \text{O}_2^- \) and \( \text{OH}^- \). GSH and related enzymes also constitute an antioxidant system in filarial parasites. GSH metabolism thus can be harnessed as a drug target. This metabolism needs to be investigated in detail, particularly in the adult filarial worms, in order to discover a reliable macrofilaricidal agent, in the absence of which filariasis keeps on relapsing. In the present studies, GSH has been estimated to be around 0.025 and 0.048 mg/g wet weight, respectively, in the extracts/homogenates of filarial worms’ *B. malayi* and *S. cervi* by the highly sensitive HPLC method, against the total thiol content of around 0.23 and 0.36 mg/g wet weight, respectively.

Gamma glutamyl cycle is the metabolic pathway responsible for the biosynthesis, degradation, and regeneration of GSH (Fig. 35). In the present studies, attempts have been made for the assessment of its enzymes in adult *S. cervi* filarial worms. Glutamate cysteine ligase (GCL) and glutathione synthetase (GS) are the enzymes responsible for the *de novo* biosynthesis of GSH and have been identified as cytosolic enzymes in the filarial worms. Their cytosolic activity was determined to be around 0.12 and 0.02 μmol/min/mg protein, respectively. The GSH breakdown initiator, γ-glutamyl transpeptidase (γ-GT) was identified in both cytosolic and microsomal fractions of filarial worm homogenate. Particulate and triton-X-100 solubilised microsomal γ-GT activity was determined to be around 0.052 and 0.025 μmol/min/mg protein respectively whereas the cytosolic activity was determined to be
around 0.050 μmol/min/mg protein. γ-Glutamyl cyclotransferase (γ-GCT) and

Cysteine

Glycine

Cys-gly

γ-Glutamyl amino acid

Amino Acid

GSH

γ-Glutamyl cysteine

ATP

5-Oxo-proline

Amino Acid

γ-Glutamyl amino acid

Fig. 35 Diagramatic Representation of γ-Glutamyl Cycle. (1) Glutamate cysteine ligase; (2) Glutathione synthetase; (3) γ-Glutamyltranspeptidase; (4) Cysteinylglycine dipeptidase; (5) γ-Glutamyl cyclotransferase; (6) 5-oxoprolinase

5-oxo-prolinase, on the other hand, were not detected under the stated experimental conditions. Cysteinylglycine dipeptidase activities need to be investigated for the complete elucidation of γ-glutamyl cycle enzymes in filarial worms.

Attempts have been made for the elucidation of kinetic and inhibition properties of S. cervi GS. All the three substrates of GS viz. L-γ-glutamyl-L-cysteine, glycine and ATP were analyzed for their Michaelis Menton constant Km. For glycine and ATP, it was calculated to be around 0.41 mM and 0.95 mM, respectively. As far as the third substrate L-γ-glutamyl-L-cysteine is concerned, a non linear Lineweaver Burk plot was obtained. Km was calculated to be around 33.3 μM at lower concentrations (<0.1 mM) and 400 μM at higher (>1 mM) concentrations, respectively. The enzyme thus proved to be allosteric similar to its mammalian counterpart. The effect of various amino acids viz. L-Met, L-Gln, L-Asn, L-Ala, L-Cys, L-Lys, L-Arg, L-His, L-Leu, DL-Ile, DL-Val, DL-B-Phe, DL-Ser, DL-Thr, L-Pro, L-Hyp, L-Glu, L-Asp, L-Tyr, DL-Trp and L-cystine at 1 mM concentration was studied on GS. Almost all amino acids caused decrease in the enzyme activity. They might be averting the substrates to attach to the active site of GS by acting as substrate analogs. Further studies need to be done in this regard to pave the way to an
effective enzyme inhibitor. Maximum decrease (20%) in GS activity was observed in the case of L-Lys. Moreover, N-ethylmaleimide inhibited GS in concentration dependent fashion when tested in the concentration range of 2.5-20 mM, inhibiting GS to the tune of 9.5 to 99.9% respectively, which confirms that sulfhydryl groups are necessary for GS activity.

Efforts have also been made for the elucidation of kinetic and inhibition properties of S. cervi γ-GT. Km for the substrate L-γ-glutamyl-p-nitroanilide was determined to be around 0.13 mM and 0.21 mM respectively for triton solubilised microsomal and cytosolic γ-GT. The effect of all the amino acids which were tested against GS, was also studied against γ-GT. Except L-cystine, no other amino acid showed any remarkable effect at 1 mM concentration. A concentration dependent increase in enzyme activity was observed in the case of both solubilised microsomal and cytosolic γ-GT in the concentration range of 0.25-2 mM L-cystine. In contrast, both GSH and GSSG caused concentration dependent decrease in γ-GT activity, when their effect was studied over the concentration range of 0.25-5 mM. Decrease in γ-GT activity with GSH and GSSG was because both are natural γ-glutamyl group donors and thus prevented the artificial substrate L-γ-glutamyl-p-nitroanilide to give its γ-glutamyl group to the enzyme in the assay system. Ki of GSH for solubilised microsomal and cytosolic γ-GT was determined to be 3.5 mM and 11.0 mM respectively. Microsomal γ-GT thus has approximately three times more affinity for GSH, as compared to cytosolic γ-GT. In contrast, Ki of GSSG for solubilised microsomal and cytosolic γ-GT was approximately same i.e 0.85 and 0.9 mM respectively. Serine-borate, the known mammalian γ-GT inhibitor was studied over a wide concentration range of 0.25-5 mM against γ-GT. For solubilised microsomal and cytosolic γ-GT, the Ki for serine-borate was calculated to be around 1.58 and 1.13 mM, respectively. Likewise, for acivicin, the known mammalian irreversible inhibitor, Ki was found to be around 0.88 and 0.28 mM for solubilised microsomal and cytosolic γ-GT, respectively.

Buthionine sulfoximine (BSO), dimethylmaleate (DMM), phorone, carmustine and menadione are the known mammalian GSH depletors. BSO is a specific and competitive inhibitor of the rate-limiting GSH biosynthesizing enzyme, GCL. Dimethylmaleate and phorone (2, 6-Dimethyl-2,5-heptadien-4-one) are α, β-
unsaturated compounds which forms conjugate with GSH. These are weak electrophiles that react with GSH in the presence of endogenous glutathione S-transferase. Carmustine and menadione, on contrary are oxidizing agents. However, their effect on filarial parasites is mostly unidentified. With the aim to get leads for the synthesis of new classes of antifilarial drugs, the effect of these compounds on GSH content of bovine filariids *S. cervi* was studied over a wide concentration range in the present studies. GSH depletion was apparent in *S. cervi* worms when incubated in Hanks balanced salt solution for a time period of 6 h at 37°C in the presence of above said compounds, thus they need to be harnessed as chemotherapeutic agents against filariasis. All these standard mammalian GSH depletors depleted GSH in *S. cervi* in a concentration dependent manner. Varying concentrations of BSO viz. 10, 50, 250, 500 and 1000 μM depleted GSH to the tune of 11, 27, 36, 46 and 59%. Furthermore, 10, 50 and 250 μM DMM depleted GSH around 22, 38 and 95% respectively, whereas same concentrations of phorone depleted 50, 79 and 93% of GSH respectively. Similar concentrations of carmustine and menadione depleted GSH to the tune of 19; 35; 69 and 44.4; 77.2; 96% respectively.

Three classes of compounds viz. diglycosylated diaminoalkanes (DGDA), glycosyl amino esters and coumarin derivatives were evaluated for their antifilarial efficacy in the present studies. The criterion behind taking the first two classes was that these include amino acid analogs grafted on to the sugar backbone and it is presumed that they may serve as substrate analogs for GSH biosynthesis and hamper its *de novo* synthesis. Additionally, sugar moiety offers better stability, less toxicity, better pharmacokinetic parameters and facilitates the transport of drugs. Coumarins and derivatives are also of great interest as they possess a diverse array of pharmacological and biochemical properties, some of which may be of potential pharmaceutical interest. Moreover, because of low toxicity and comparative cheapness, it appears judicious to evaluate their properties and relevance further.

A prerequisite for the development of new antifilarial compounds would be the availability of excellent *in vitro* and *in vivo* drug-screening systems. In the present studies, *in vitro* and *ex vivo* preliminary screening against filarial GSH metabolism has been conducted, followed by *in vivo* antifilarial testing, in order to overcome the drawbacks of *in vitro* system, which has the major disadvantage of not being able to
assess accurately the synergistic effect of host immunological and other responses in parasite killing, nor the effects of drug metabolites and toxicity.

GCL, GS and γ-GT are three important enzymes of the γ-glutamyl cycle. GCL and GS are involved in the de novo GSH biosynthesis whereas γ-GT is involved in the salvage of extracellular GSH. Particular emphasis has been granted to these enzymes for the preliminary screening of synthetic compounds. Prospective compounds which inhibited the activity of either of these filarial enzymes, in vitro, were of relevance and screened for their effect on GSH content in adult filarial worms by incubation studies. In such studies filarial worms S. cervi were incubated in the presence of potential test compounds for 6 h at 250 μM concentration. Most of such compounds severely affected the GSH homeostasis and were evaluated by Motility and MTT reduction in vitro assays against the human lymphatic filarial worms, B. malayi. The in vivo microfilaricidal, macrofilaricidal and sterilization effect was determined in B. malayi- M. coucha model.

Two lead molecules i.e. one DGDA analog and another coumarin derivative, have been identified for further exploration to develop two new classes of antifilarial drugs. Compound number 12, a DGDA, showed the remarkable antifilarial effects and can be further tested as lead compound. It caused 37% reduction in microfilariae (mf). This compound also caused 40% mortality in adult parasites and 39% sterilization of female B. malayi. Among coumarins derivatives, the compound number 20 showed significant in vivo antifilarial efficacy to be promoted for drug development. It caused 82% reduction of mf, 60% mortality in adult worms and 41% sterilization of living female filarial worms.