In vitro effect of low concentrations of oltipraz on the antioxidant defence of mouse hepatocytes and *Schistosoma mansoni* worms

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ABSTRACT

The direct effects of oltipraz (OPZ) on mouse hepatocytes and *Schistosoma mansoni* worms are studied *in vitro* at a concentration range of 5–25 μmol/L following one- and three-hour incubations. Oxidative stress is reflected by increases in malondialdehyde (MDA), representing the end products of lipid peroxidation, and depletion of glutathione (GSH), representing protective thiol groups. Activities of glutathione peroxidase isoenzymes, GST and GR as components of antioxidative defence are also determined. The opposite effects of low concentrations of OPZ on mammalian hepatocytes and *S. mansoni* worms were confirmed. In incubation with *S. mansoni*, addition of OPZ resulted in significantly increased production of MDA, together with depletion of GSH, both of which were time- and OPZ concentration-dependent. In incubation with mouse hepatocytes, however, there was little change in MDA concentrations, and a gradual increase in GSH levels, both of which were time- and concentration-dependent. Addition of OPZ to the incubation media also affected the activities of antioxidant enzymes. Although total GPx activity increased in both mammalian hepatocyte and *S. mansoni* experiments, the opposite was noted with the selenium-dependent isoenzyme. While there was a gradual increase in sGPx in hepatocytes, there was a time- and concentration-dependent inhibition in the worm isoenzyme. Contrasting results were also obtained with GR. While increased activity was obtained with the enzyme from mouse hepatocytes, the worm enzyme was inhibited, especially at the upper end of the OPZ concentration range and also following longer periods of incubation. The increase in GST activity followed the same qualitative pattern in both hepatocytes and schistosomes. Therefore, OPZ given in doses that maintain a serum concentration in the range 5–25 μmol/L induces biochemical changes in mouse hepatocytes that could be utilised for chemo-preventive purposes and prevention of oxidative damage. However, progressive oxidative damage to *S. mansoni* worms occurred despite some protective biochemical changes.

KEY WORDS: Antioxidants. Glutathione. Liver. Oltipraz. Schistosoma mansoni.

Introduction

The dithiolthione derivative oltipraz (OPZ) was introduced originally as an antischistosomal drug.[1] Although single oral doses achieved cure rates of more than 90% in field trials, the drug was withdrawn because of phototoxic side effects (photo-onycholysis).[2] Its schistosomicidal action was thought to be related to disruption of glutathione (GSH) metabolism; however, it was found to produce the opposite effect on GSH and its related enzymes in *Schistosoma mansoni* parasites and in their mammalian host.[3,4]

While schistosomicidal doses (20–30 mg/kg per day) cause depletion of GSH in the worms, much lower doses (1–3 mg/kg per day) increase GSH levels in mammalian cells and show chemopreventive action against cancer development. The latter effect has been noted in the lung, trachea, small intestine, colon, breast, skin, liver and urinary bladder in rodents.[5]

Reactive oxygen species (ROS) have been implicated in the development of many diseases including cancer. They have been suggested to play an important role in the initiation and promotion of multi-step carcinogenesis.[6] Thus, disruption of the antioxidant defence system has been implicated both in cancer development in mammals and in the susceptibility of *S. mansoni* to OPZ.

The release of ROS and other toxic molecules produced during the respiratory burst or nitric oxide release constitutes one mechanism that cells of the immune system may use to kill parasites.[7,8] The major immune effector mechanisms involve various cells of the immune system, stimulated by antibodies and cytokines.[9]

Therefore, the *in vitro* incubation of *S. mansoni* worms with OPZ would demonstrate the direct effect of the drug and exclude input from the host. In addition, the broad range of OPZ chemopreventive activity, coupled with its low toxicity in mammals when administered in low doses for prevention of cancer development, make it a promising agent in this field.[10] Thus, incubation of mammalian hepatocytes with OPZ would demonstrate its direct action without interference from hormonal, neurological, immunological or other factors.

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Table 1. Activity of LDH (U/mL) in the incubation media of mouse hepatocyes treated with OPZ[a].

	Oltipraz concentration (μmol/L)			
Control	5	10	20	25
One-hour incubation				
0.1388 ±0.0045	0.1420 ±0.0046)	0.1431 ±0.0048	0.1459 ±0.0034 (+5.1%)	0.1505* ±0.0022 (+8.5%)
Three-hour incubation				
0.1415 ±0.0049	0.1457 ±0.0034	0.1539* ±0.0035 (+8.8%)	0.1575* ±0.0034 (+11.4%)	0.1630* ±0.0039 (+15.3%)

[a] Data presented as the mean (±SD of four experiments.
* Significantly different from control value ($P<0.05$).
Number in parentheses represents percentage change from respective control value.

Table 2. Activity of LDH (U/mL) in the incubation media of *S. mansoni* worms treated with OPZ[a].

	Oltipraz concentration (μmol/L)			
0 (control)	5	10	20	25
One-hour incubation				
0.0283 ±0.0022	0.0341* ±0.0030 (+20.5%)	0.0451* ±0.0032 (+59.4%)	0.0502* ±0.0039 (+77.4%)	0.0536* ±0.0036 (+89.4%)
Three-hour incubation				
0.0306 ±0.0032	0.0468* ±0.0038 (+52.9%)	0.0585* ±0.0040 (+91.2%)	0.0641* ±0.0040 (+109.5%)	0.0685* ±0.0025 (123.9%)

[a] Data presented as the mean±SD of four experiments
* Significantly different from control value ($P<0.05$)
Number in parentheses represents percentage change from respective control value.

In view of the fact that differences between the doses used for schistosomicidal purposes (20–30 mg/kg)[3] and those used for chemopreventive purposes (1–3 mg/kg)[4] could be responsible for the reported differences in the effect of the drug on *S. mansoni* worms and hepatocytes, the present study aims to demonstrate the direct effect of the same drug concentrations on both experimental models and identify biochemical differences between parasite and host that could be used as drug targets. In addition, the effect on mammalian hepatocytes may provide an insight into the mechanism of the drug's chemopreventive action.

Material and methods

Experiments on *S. mansoni* were carried out on eight-week-old worms of a laboratory-bred local strain, recovered from the liver and mesenteric veins of infected mice by perfusion. Immediately after recovery the worms were washed (x4) with Eagle's minimal essential medium containing penicillin and streptomycin (MEM-PS) and suspended in the same medium at 37°C until exposed to OPZ.[11]

Groups of 10 pairs of worms were transferred to culture plate wells and incubated in 2.5 mL MEM-PS. Quantities of OPZ solution in dimethylsulphoxide (DMSO) were added to different wells to give final OPZ concentrations between 5 and 25 μmol/L. Control wells contained DMSO only.

In individual experiments, 50 pairs of worms were exposed to each concentration of OPZ. Incubations were carried out for 1 or 3 h at 37°C in a 5% CO_2 humid atmosphere. At the end of the incubation period, supernatants from wells containing the same concentration of OPZ were pooled to determine lactate dehydrogenase (LDH) activity.[12] Worms from the same wells were grouped, washed (x2) with sterile phosphate-buffered saline (PBS) and homogenised in 1.5 mL of the same solution. The homogenates were used to determine the other parameters tested. Each value was considered as a single result. The whole incubation experiment was repeated four times for proper statistical evaluation.

In the hepatocyte experiments, liver tissue was obtained from adult Swiss albino mice. Finely chopped pieces were treated with trypsin–EDTA solution and the separated hepatocytes were suspended in growth medium (RPMI 1640) supplemented with penicillin/streptomycin, 1% glutamine (200 mol/L), 1% non-essential amino acids and 10% fetal calf serum. The cells in suspension were counted and diluted to a final concentration of one million cells/mL.[13]

Samples (0.1 mL) of the cell suspension were seeded in sterile culture flasks containing 2.4 mL growth medium, and OPZ solution was added to give final concentrations ranging from 5 to 25 μmol/L. Four flasks were prepared for each concentration and incubated at 37°C in a 5% CO_2 humid atmosphere. Control flasks contained DMSO without OPZ. After incubation, duplicate flasks from each concentration were removed for immediate determination of supernatant LDH activity[12] to assess cellular injury. Cell viability was determined by trypan blue dye exclusion.[14] Hepatocytes were lysed by sonication (50 W for 20 sec at 4°C). Experiments were repeated four times.

In both *S. mansoni* worms and hepatocytes, GSH[15] and malondialdehyde (MDA)[16] levels were determined as

Table 3. Changes in the concentrations of free thiol groups (estimated as GSH) and lipid peroxidation products (estimated as MDA) in mouse hepatocytes incubated with oltipraz[a].

	Oltipraz concentration (μmol/L)				
	Control	5	10	20	25
GSH (nmol/mg protein)					
One-hour incubation time	0.319 ±0.018	0.362* ±0.013 (+13.5%)	0.383* ±0.016 (+20.1%)	0.402* ±0.013 (+26.0%)	0.417* ±0.098 (+30.7%)
Three-hour incubation time	0.321 ±0.013	0.375* ±0.008 (+16.8%)	0.408* ±0.014 (+27.1%)	0.424* ±0.016 (+32.1%)	0.430* ±0.016 (+34.0%)
MDA (nmol/mg protein)					
One-hour incubation time	6.37 ±0.35	6.09 ±0.20	6.15 ±0.20	6.41 ±0.15	6.49 ±0.16
Three-hour incubation time	6.57 ±0.32	6.51 ±0.32	6.56 ±0.23	6.64 ±0.33	6.71 ±0.24

[a] Data presented as the mean±SD of four experiments
* Significantly different from control value ($P<0.05$)
Number in parentheses represents percentage change from control value
GSH = glutathione
MDA = malondialdehyde.

Table 4. Changes in the concentrations of free thiol groups (estimated as GSH) and lipid peroxidation products (estimated as MDA) in *S. mansoni* worms incubated with oltipraz[a].

	Oltipraz concentration (μmol/L)				
	Control	5	10	20	25
GSH (nmol/mg protein)					
One-hour incubation time	0.421 ±0.012	0.350* ±0.031 (-16.9%)	0.328* ±0.030 (-22.1%)	0.320* ±0.029 (-24.0%)	0.297* ±0.028 (-29.5%)
Three-hour incubation time	0.462 ±0.040	0.362* ±0.035 (-21.6%)	0.331* ±0.033 (-28.4%)	0.304* ±0.018 (-34.2%)	0.290* ±0.027 (-37.2%)
MDA (nmol/mg protein)					
One-hour incubation time	4.38 ±0.28	4.81* ±0.23 (+9.8%)	5.01* ±0.25 (+14.4%)	5.48* ±0.31 (+25.1%)	5.52* ±0.33 (+26.0%)
Three-hour incubation time	3.37 ±0.30	4.00* ±0.11 (+18.7%)	4.19* ±0.19 (+24.3%)	4.61* ±0.27 (+36.8%)	4.73* ±0.17 (+40.4%)

[a] Data presented as the mean±SD of four experiments.
* Significantly different from control value ($P<0.05$).
Number in parentheses represents percentage change from control value.
GSH: glatathione.
MDA: malondialdehyde.

measures of the extent of oxidative stress. Also, the glutathione peroxidase (GPx) isoenzymes,[17] glutathione-S transferase (GST)[18,19] and glutathione reductase (GR),[20] which play an important role in the antioxidant defence system, were determined. Protein was assayed using the method described by Lowry *et al*.[21]

Results

Little change in LDH activity was seen in hepatocytes following incubation in OPZ for 1 h (Table 1). Following 3-h incubation, LDH reached its highest value in the presence of 25 μmol/L OPZ. However, incubating *S. mansoni* worms in media containing OPZ resulted in a significant increase in LDH activity, which was incubation time- and drug concentration-dependent (Table 2).

The effect of OPZ on the levels of the protective thiol groups (represented as GSH) and lipid peroxidation end-products (estimated as MDA) in hepatocytes is presented in Table 3. Although no change in MDA was seen, a gradual incubation time- and drug concentration-dependent increase in GSH was noted. A strong positive correlation was found between OPZ level and percentage increase in GSH

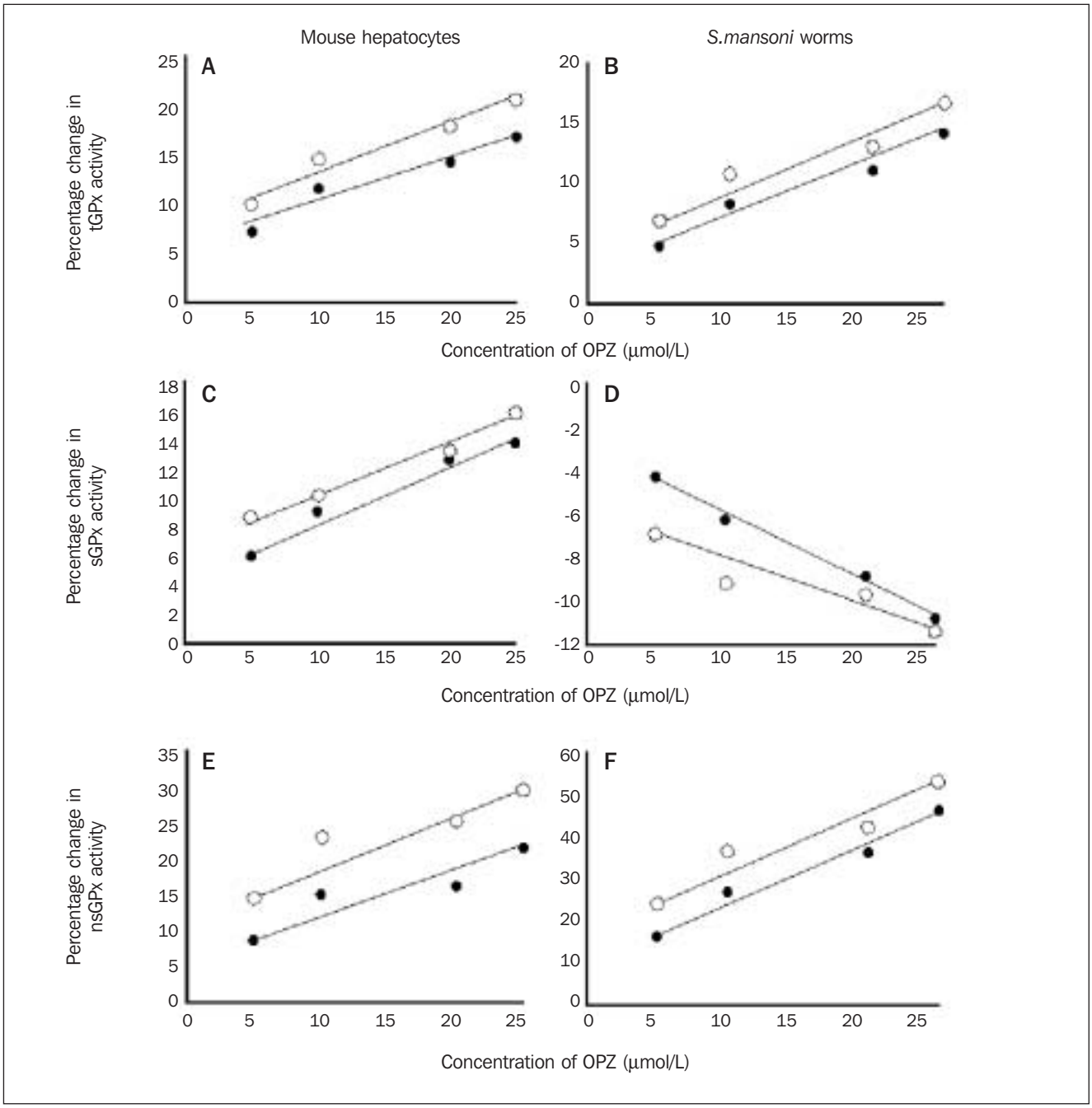

Fig. 1. Changes in the activities of total (tGPx), selenium dependent (sGPx) and non-selenium-dependent (nsGPx) glutathione peroxidase isoenzymes following 1 hour (●–●) and 3 hour (○–○) *in vitro* incubations with 5-25 µmol/L oltipraz.

for both the 1-hour (r=0.9331) and the 3-hour (r=0.9320) incubation times. However, incubation of *S. mansoni* with OPZ resulted in oxidative stress, indicated by depletion of GSH and accumulation of MDA (Table 4).

Incubation with OPZ also influenced the activity of the GSH-related enzymes tested. In mouse hepatocytes, both GR and GST showed increased incubation time- and drug concentration-dependent activity following incubation with OPZ (Table 5).

Incubation with OPZ produced mild inhibition of GR in *S. mansoni* worms (Table 6), and drug concentration appeared to be more important than incubation time in this case. The effect of OPZ on GST in *S. mansoni* worms was similar to that seen in the mouse hepatocytes

The response of GPx isoenzymes is illustrated in Figure 1.

Discussion

Opposite effects observed for OPZ in mammalian and schistosomal antioxidant defence systems may indicate differences in gene expression of antioxidant-related enzymes in response to the drug, raising the possibility that it can damage the parasite while preserving or even strengthening the integrity of host tissues. In the present study, LDH activity in the incubation medium was taken as

Table 5. Changes in the activities of glutathione reductase (GR) and glutathione S-transferase (GST) in mouse hepatocytes incubated with oltipraz[a].

	Oltipraz concentration (μmol/L)				
	Control	5	10	20	25
GR activity• (U/mg protein)					
One-hour incubation time	0.642 ±0.048	0.683 ±0.017	0.721* ±0.028 (+12.3%)	0.769* ±0.029 (+19.8%)	0.811* ±0.029 (+26.3%)
Three-hour incubation time	0.620 ±0.039	0.707 ±0.037 (+14.0%)	0.774* ±0.025 (+24.8%)	0.860* ±0.025 (+38.7%)	0.881* ±0.034 (+42.1%)
GST activity•• (U/mg protein)					
One-hour incubation time	0.0579 ±0.0046	0.0641* ±0.0018 (+10.7%)	0.0664* ±0.0017 (+14.7%)	0.0690* ±0.0026 (+19.2%)	0.0723* ±0.0025 (+24.9%)
Three-hour incubation time	0.0622 ±0.0037	0.0705* ±0.0023 (+13.3%)	0.0744* ±0.0022 (+19.6%)	0.0769* ±0.0025 (+23.6%)	0.0811* ±0.0031 (+30.4%)

[a] Data presented as the mean±SD of four experiments
* Significantly different from control value ($P<0.05$)
• One unit of GR activity will catalyse the oxidation of 1 μmol of NADPH/min.
•• One unit GST activity will catalyse the formation of 1 μmol S-conjugate/min under assay conditions.
Number in parentheses represents percentage increase over control value.

a measure of the effect of OPZ on hepatocytes and *S. mansoni* worms.

Incubation time- and drug concentration-dependent increases in LDH activity with the schistosomes was in direct contrast to the minimal changes seen with the mouse hepatocytes studied. This difference in cellular damage was the first indication of the cytotoxic action of OPZ on schistosomes and the lack of a damaging effect on mammalian hepatocytes.

It has been proposed that the effect of OPZ on the metabolic transformation of GSH may be central to its antiparasitic effect.[4] The increase in MDA observed is particularly significant because these toxic peroxidation products are usually neutralised rapidly by intracellular antioxidants. Involvement of GSH in the metabolism of peroxide and aldehyde products of lipid peroxidation[22] could be a contributing factor to the decrease in the GSH level observed in the present study.

Incubation of OPZ with mouse hepatocytes produced a significant increase in GSH without significant alteration in MDA. As GSH is a detoxifying factor for various carcinogens,[23] this effect of OPZ on mammalian cells may represent an important component of its chemopreventive action. Reduced glutathione has been designated a physiological nucleophile, which forms conjugates with reactive electrophiles including carcinogenic nitrosamines[24] and aflatoxins.[25]

In addition, this chemopreventive effect is mediated partly through inhibition of the enzymes of phase-1 xenobiotic biotransformation and induction of phase-2 enzymes, including GSTs.[26,27] Human deficiencies in GST activity have been associated with an increased risk of colorectal cancer.[28]

In the present study, GST activity in mouse hepatocytes increased gradually in both an incubation time- and drug concentration-dependent manner. These *in vitro* results parallel other *in vivo* observations of dose-dependent GST increased expression in mice.[26] This may apply only to certain concentration or dose ranges, however, as low rather than high doses of OPZ were reported to increase the reactivity of GST in colorectal mucosa.[29]

In order to become established in the vasculature of the host, schistosomes have evolved a number of immune evasive and protective mechanisms, including the development of antioxidant defence.[30,31] In the present study, the activity of GST in *S. mansoni* worms increased following incubation with low concentrations of OPZ. A similar increase has been reported in schistosomes exposed *in vitro* to 40 μmol/L OPZ for 18 h,[4] but is in contrast to other published data obtained following incubation with relatively high concentrations (0.5 mmol/L), which resulted in significant inhibition of GST.[32] The contrasting effects of low and high concentrations of OPZ on mouse hepatocytes and schistosoma worms point to a role for the drug's metabolites, especially as OPZ has been described as a prodrug.[33]

Glutathione peroxidase isoenzymes utilise GSH to catalyse the reduction of both lipid hydroperoxides and hydrogen peroxide as a cellular defence mechanism against oxidative stress.[34] In mouse hepatocytes, increases in activity of the different isoenzymes were evident following incubations with OPZ, which agreed with published *in vivo* results in rats.[35]

Owing to a lack of catalase activity in schistosomes, GPx may play a more important protective role. Although tGPx and nsGPx activities increased as a result of incubation with OPZ, the selenium-dependent isoenzyme responsible for reaction with hydrogen peroxide was inhibited. This is a significant observation because H_2O_2 causes more damage to the worms than does the superoxide radical.[36] Apparently, the increase in tGPx results from concurrent induction of GST isoenzymes, which lack selenium in their active site[37] and can reduce lipid peroxides but not H_2O_2.

Table 6. Changes in the activities of glutathione reductase (GR) and glutathione S-transferase (GST) in *S. monsoni* worms incubated with oltipraz[a].

	Concentration of oltipraz (μmol/L)				
	Control	5	10	20	25
GR activity• (U/mg protein)					
One-hour incubation time	0.381 ±0.024	0.365 ±0.007	0.353 ±0.008	0.333* ±0.010 (-12.6%)	0.326* ±0.007 (-14.4%)
Three-hour incubation time	0.373 ±0.028	0.341 ±0.008	0.327* ±0.012 (-12.3%)	0.319* ±0.010 (-14.5%)	0.307* ±0.009 (-17.7%)
GST activity•• (U/mg protein)					
One-hour incubation time	0.1248 ±0.0108	0.1372±0.0029	0.1399*±0.0033 (+12.1%)	0.1455*±0.0036 (+16.6%)	0.1519*±0.0044 (+21.7%)
Three-hour incubation time	0.1318 ±0.0065	0.1450* ±0.0033 (+10.0%)	0.1543*±0.0031 (+17.1%)	0.1611*±0.0045 (+22.2%)	0.1673*±0.0058 (+26.9%)

[a] Data presented as the mean±SD of 4 experiments.
* Significantly different from control value ($P<0.05$).
• One unit of GR activity will catalyse the oxidation of 1 μmol of NADPH/min.
•• One unit of GST activity will catalyse the formation of 1 μmol S-conjugate/min under the assay conditions.
Number in parentheses represents percentage increase over control value.

Another difference between mammalian and *S. mansoni* defences against oxidative stress is the response of GR to incubation with OPZ. These reductases play a key role in cellular defence against oxidative stress by preventing accumulation of oxidised glutathione and by maintainingthe redox state.[38] Thus, increased GR activity in mouse hepatocytes following incubation with OPZ speeds up the reduction of GSSG resulting from the high GPx activity. In contrast, the *S. mansoni* enzyme showed progressive inhibition with increasing concentrations of OPZ. In a previous study, *in vivo* treatment of infected mice with OPZ produced inhibition of *S. mansoni* GR, as did the *in vitro* incubation of worms with the disulphide metabolite.[39]

In summary, the present study showed that OPZ in a concentration range of 5 to 25 μmol/L affected *S. mansoni* worms in a concentration- and time-dependent manner but resulted in a much lower level of damage in mouse hepatocytes. In particular, the lowest concentration applied, (5 μmol/L) resulted in no damage to mouse hepatocytes. The similarity of this concentration to the reported serum levels obtained after the administration of chemopreventive doses of 1–3 mg/kg (i.e., 4 μmol/L)[40] shows that OPZ is safe at these levels.

In addition, differences in the effect of OPZ between *S. mansoni* worms and mouse hepatocytes also apply to their oxidative status. Oltipraz caused oxidative stress and decreased GSH concentration and GR activity in *S. mansoni* worms, but had the opposite effect in mouse hepatocytes. However, while it produced a concentration- and time-dependent increase in GST and GPX activity in both models, the effect was more pronounced in mouse hepatocytes. Increased GST activity could be partly responsible for the chemopreventive activity and thus be utilised to protect against drug-induced hepatotoxicity in mammalian hepatocytes. □

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