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Brugia malayi: Depletion of Glutathione by Buthionine Sulfoximine

خفض كمية الجلوتاثيون في طفيل البروجيا مالايا بواسطة مركب الباثيونين سالفوكسامين

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Abstract

Glutathione is an intracellular reducing agent. It is synthesized by a two step reaction catalyzed by γ-glutamylcysteine synthetase (γGCS) and glutathione synthetase. γGCS, which is the rate limiting enzyme in the synthesis of glutathione, is inhibited specifically by buthionine sulfoximine (BSO). The enzyme was partially purified from the parasitic nematode *Brugia malayi*, the causative agents of lymphatic filaraiasis. BSO inhibits the enzyme activity in an irreversible manner. The *Brugia* enzyme was found to be 24-fold more sensitive to BSO than its mammalian counterparts. The inhibitor was also found effective in depleting the glutathione contents of the parasites with low concentration. The results may conclude that γGCS is a potential target for the development of drugs against filariasis.

Running head: BSO inhibits glutathione synthesis in *Brugia malayi.*

ملخص

الجلوتاثيون هو مركب خلوي يحافظ على درجة اختزال الخلية. يصنع هذا المركب على مرحلتين متتاليتين بواسطة انزيمات جاما جلوتاميل سيستيت سينثاتيز (γGCS) و جلوتاثيون سينثاتيز. ومن المعروف أن الانزيم يثبط بواسطة مركب يسمى الباثيونين سالفوكسامين (BSO). تمت دراسة تاثير المثبط على الانزيم (γGCS) المستخرج والمنقى جزيئيا من طفيل البروجيا مالايا. وكذلك جزئيا تمت دراسة تأثير المثبط (BSO) على كمية الجلوتاثيون في الطفيليات عند زراعتها بوجود المثبط في بيئة مناسبة. وقد اثبتت الدراسة أن (BSO) يثبط الانزيم بكميات قليلة مقارنة مع تاثيره على خلايا الثديات. اضافة لذلك وجد أن المثبط (BSO) له تأثير فعال في خفض كمية الجلوتاثيون في الطفيل بكميات قليلة. هذه الدراسة لها دلالة على (γGCS) يمكن أن يكون هدفا محتملا لتطوير أدوية لعلاج الديدان اللمفاوية.

Introduction

Glutathione (GSH) is the most abundant intracellular thiol and acts as a major cellular antioxidant. It is widely distributed in most living cells and has many functions, such as the maintenance of a reducing environment and defence against cytotoxic compounds of endogenous and exogenous origin by the actions of GSH-transferase and GSH-peroxidases (Meister, 1991). All aerobic living organisms are exposed to reactive oxygen species, which confer modifications of nucleic acids, thiol containing proteins and membrane lipids (Meister, 1991). A part from enzymatic anti-oxidants such as superoxide dismutase and catalase, the tripeptide is part of the GSH redox cycle. GSH is considered an important endogenous protective system against oxidative stress, in which peroxides are detoxified by GSH-peroxidase (Flohe, 1985). During the reaction, GSH is oxidized to glutathione disulfide (GSSG), which is regenerated by the NADPH-dependent GSH-reductase (Schirmer *et al*., 1989).

GSH is synthesized intracellularly from its constituent amino acids by two consecutive enzymatic reactions. Glutamate and cysteine are ligated by the action of γ-glutamylcysteine synthetase (γGCS) forming glutamylcyateine dipeptide, the rate limiting step of GSH *de novo* synthesis. The following addition of glycine to the glutamylcyateine dipeptide is catalyzed by GSH-synthetase.

$$\text{L-glutamate + L-cysteine + ATP} \xrightarrow{\gamma\text{GCS}} \text{L- }\gamma\text{-glutamyl-L-cysteine + ADP + } P_i$$

Filarial parasites such as *Brugia malayi* and *Wuchereria bancrofti*, the causative agents of lymphatic filaraiasis and *Onchocerca volvulus* causing Onchocerciasis (river blindness) are widely distributed throughout the tropical regions of the world.

Lymphatic filariasis is a serious disease which currently affects 120 million people worldwide. Adults of filarial parasites inhabit the lymphatic vessels and produce the microfilaria into blood. Those microfilaria are taken up by insects when they feed on human blood. Humans are infected when larvae are injected into their skin by these arthropods when they feed on human blood.

Existing chemotherapy against these parasites is limiting since only the microfilaria are killed and there is no effect on adult parasites, which survive in their human host for up to 15 years (WHO, 1995). It has been suggested that one of the drug targets is the GSH redoxcycle, and inhibition of GSH-reductase by melarsenoxide was also shown for the proteins isolated from *Setaria digitata* and *O. gutturosa* (Muller *et al*., 1995). GSH depletion, which had been shown to sensitize cells to radiation (Renschler, 2004), cisplatin (Anderson *et al*., 1990) and compounds that produce oxidative cytolysis, was suggested to be a useful procedure in the treatment of certain tumours and parasitic diseases (Luersen *et al*., 2000; Reschler, 2004; D'Alessio *et al*., 2004; Lopez and Luderer, 2004). One goal to achieve this is through inhibition of GSH synthesis by DL-buthionine-SR-sulfoximine (BSO) (Meister, 1991; Luersen *et al*., 2000a; Lopez and Luderer, 2004). BSO is a specific transition-state inhibitor of γGCS and was used successfully to deplete GSH in mammalian tissues and tumor cell lines as well as in the protozoan parasites *Trypanosoma brucei*, *Leishmania donovani* and *Plasmodium falciparum* (Meister, 1991; Arrick *et al.*, 1981; Bailey, 1998; Weldrick *et al*. 1999; Luersen *et al*., 2000).

Methodology

Given the proposal of using BSO as a chemical lead for the development of drug against parasitic diseases, (Arrick *et al*., 1981, Meister, 1991, Luersen *et al*., 2000), BSO was tested whether it could inhibit the γGCS from *B. malayi* and whether it could lead to a lethal effect on the parasites. The enzyme (γGCS) was partially purified and the inhibition constant of BSO was determined as follows: adults *B. malayi* parasites were recovered from the peritoneal cavity of Mongolian jirds (*Meriones unguiculatus*) infected more than 3 months earlier and obtained from TRS Labs, Athens, Greece. The parasites were washed three times in RPMI medium and homogenized in 5 vol. of 50 mM Tris-HCl (pH 7.5) containing 1 mM glutamate, 5 mM $MgCl_2$ and 0.1 mM phenyl methyl sulfonyl fluoride (Buffer A) at 1400 rpm with a glass/glass homogenizer. The homogenate was centrifuged at 100,000 g for 1 h. The 100,000 g supernatant was applied to a DEAE DE52-cellulose column (1.5 cm x 6 cm) previously equilibrated with buffer A. After washing

with starting buffer the enzyme was eluted with 0.2 M NaCl in buffer A. The DEAE-cellulose eluant was used for the determination of the K_m- and K_i-values for glutamate and BSO, respectively. The assay for the enzyme activity was performed according to Hussein and Walter (1995). The standard incubation mixture of 100 µl contained 0.1 M Tris-HCl buffer (pH 8.0/20 mM $MgCl_2$/2 mM EDTA/1 mM L-[U-^{14}]glutamate (25 nCi)/10 mM aminobutyrate/10 mM ATP/5 mM creatine phosphokinase (1 U) and the enzyme preparations. Protein was determined according to Bradford (1976) using bovine serum albumin as standard.

Following incubation at 37˚C for 15 min, the reaction was terminated by 10 µl $HCLO_4$ (2 M) and heating for 3 min at 95˚C. After cooling on ice, the samples were centrifuged at 1000 g for 3 min. The product of the enzyme reaction γ-glutamylaminobutyrate was determined by reverse-phase HPLC or thin layer chromatography (TLC).

Living parasites were next treated with low concentration of BSO (20 µM) to test whether BSO at low concentration could deplete the GSH content of the organisms. *Brugia malayi* adults were washed gently and maintained for 24 h in phenol red-free RPMI 1640 containing 2 mM glutamine, 1% glucose, 100 U of penicillin, and 100 mg of streptomycin at 37°C and 5% CO_2 before laboratory experimental procedures were done. Two adult female parasites were transferred to 2 ml RPMI medium supplemented with 0, 20, 100, 200 and 500 µM BSO. The adult females were incubated at 37°C and 5% CO_2. At the end of the incubation period, the parasites were removed and washed three times with cold phosphate-buffered saline (pH 7.3) and homogenized in three vol. of 5% sulfosalicylic acid at 1400 rpm with a glass/glass homogenizer. The homogenate was centrifuged at 10,000 g for 5 min and the supernatant was used for GSH and protein determination. GSH content was determined by HPLC as described by Hussein and Walter (1996). Protein content was determined according to Bradford (1976).

The viability of the parasites was assayed as follows (see table 1). Uptake and reduction of the tetrazolium salt 3-[4,5-diethylthiazol-

2-yl]-2,5-diphenyltetrazolium bromide (MTT) was utilized as a measure of parasite viability (Comley *et al*., 1989; Mosmann, 1983). Two size-matched adult females were incubated in 0.5 mg MTT ml in

phenol red-free RPMI 1640 at 37°C for 1 h. Formazan crystals formed within the parasites were solubilized by transferring parasites to 200 ml of dimethyl sulfoxide and incubating at room temperature for 1 h with shaking. Parasites were removed, and the absorbance of the supernatant was determined at 510 nm with dimethyl sulfoxide as a blank. Background levels were established by performing the assay on parasites killed by heating to 65°C for 10 min. All assays were carried out in triplicate.

Results and Discussion

The specific activities of the γGCS in the parasite homogenate and in DEAE-cellulose eluant were determined to be 88 and 250 nmole/min/mg protein, respectively. The K_m-value for glutamate was found to be 1.34 mM and thus similar to those reported for the enzymes from rat kidney and *Ascaris suum* (Chang and Chang, 1994; Hussein and Walter, 1995). BSO showed a time-dependent irreversible inhibitory effect on the enzyme activity with a K_i-value of 0.30 μM (Fig.1). A similar effect of the inhibitor was reported for the rat kidney enzyme (Griffith and Meister, 1979; Meister, 1988; Hussein and Walter, 1995). However, the *Brugia* enzyme was found to be 24-fold more sensitive to BSO than its mammalian counterparts, since the K_i-value for BSO of the rat kidney γGCS was found to be 7.19 μM (Hussein and Walter, 1995).

Depletion of the GSH levels of adult *B. malayi*, which was found to be dose dependent, was rapid (Table 1). Treatment of the parasites with different concentrations of 20, 100, 200 and 500 μM BSO lowered the GSH content by 70%, 47%, 35% and 23%, respectively. A similar effect of BSO has been reported for other organisms (Grifith *et al*., 1979; Arrick *et al*., 1981; Hussein and Walter, 1995, Luersen et *al*., 2000a,b). Surprisingly, a marked decrease of GSH levels by an extremely low concentration of BSO was not comparable with a decline in the viability of the treated parasites (Table 1). The later result may indicate that other thiols might spare the function of GSH.

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Table 1 *In vitro* inhibition of glutathione synthesis of *Brugia malayi* by DL-buthionine-SR-sulfoximine (BSO)

BSO (μM)	Glutathione content (nmole/mg protein)	Inhibition (%)	Reduction of MTT (ABS at 510 nm)	Viability (%)
0*	10.12 ± 0.9	100	1.7 ± 0.21	100
20	3.12 ± 0.1	31	1.2 ± 0.10	70
100	1.10 ± 0.0	11	0.8 ± 0.13	47
200	0.85 ± 0.10	8	0.6 ± 0.11	35
500	0.40 ± 0.09	4	0.4 ± 0.10	23

The results, based upon duplicates, represent mean determinations ± SD from two adult worms. The parasites were treated *in vitro* with various concentrations of BSO at 37°C and 5% CO_2 for 24 h.

* Control is parasites treated with 0.0 μM BSO concentration

Fig. 1 Time- and concentration-dependent inhibition of γ-glutamylcysteine synthetase from *Brugia malayi* by DL-buthionine-SR-sulfoximine (BSO). Aliquots of 200 μl of the enzyme preparations were pre-incubated in 0.1 M Tris.HCl (pH 8.0)/10 mM $MgCl_2$/10 mM ATP at 37˚C for 0-15 min with 0.025 (○), 0.05 (●), 0.10 (Δ) and 0.25 (▲) μM BSO. Aliquots of 10 μl were assayed for the enzyme activity as described in the text. In the insert figure, the times of half inactivation, ($t_{1/2}$) are plotted against the reciprocal of the inhibitor concentrations. Untreated parasites (0.0 μM BSO) parasites had 100% activities. The values represent the mean of duplicate determinations.