**Inhibition of glutathione production by L-S,R-buthionine sulfoximine
activates hepatic ascorbate synthesis--a unique anti-oxidative stress
mechanism in mice**

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Abstract

Glutathione (GSH) and ascorbate, the cytoplasmic antioxidants, can regenerate and replace each other in scavenging reactive oxygen species reaction. Mice, but not guinea pigs, produce ascorbate endogenously. L-Buthionine-S,R-sulfoximine (L-S,R-BSO) substantially inhibited GSH production at a greater degree and caused a higher toxicity to guinea pigs than mice, implying that mice may have an additional protective mechanism against oxidative stress injury. Indeed, administration of L-S,R-BSO to mice inhibited tissue GSH production while increasing ascorbate levels. L-S,R-BSO also increased tissue ascorbate levels in mice fed a ascorbate and dehydroascorbate-free diet suggesting activation of ascorbate synthesis, which was further confirmed by increased urinary ascorbate excretion. Other reagents inhibiting GSH production also increased tissue ascorbate levels. The results of Northern blot and promoter assay showed that L-S,R-BSO increased mRNA expression and promoter activity of mouse liver L-gulono- γ -lactone oxidase, the critical enzyme for ascorbate synthesis. Taken together, our study demonstrates that inhibition of GSH production activates ascorbate synthesis to protect mice against oxidative stress injury, the mechanism which is not present in guinea pigs or humans.

Key words: GSH; ascorbate; oxidative stress; mouse; guinea pig; L-gulono- γ -lactone oxidase

Abbreviations: GSH: glutathione; L-S,R-BSO: L-buthionine-S,R-sulfoximine;
GCL: glutamyl-cysteine ligase; ROS: reactive oxygen species; L-Gulo: L-gulono-
 γ -lactone oxidase

Introduction

Reactive oxygen species (ROS) include hydrogen peroxide (H_2O_2), hydroxyl free radical ($\cdot\text{OH}$) and other free radicals. Several cellular compartments, such as mitochondria, peroxisomes and cytoplasm, can produce ROS by enzymatic or/and non-enzymatic reactions in normal and drug metabolism[1]. ROS at normal levels can serve as the second messengers in many redox-dependent cell signal pathways, thereby playing an important role in different biological processes[2]. However, the excess ROS can result in oxidative stress in which ROS modifies many classes of molecules and cause cell injury or death. Oxidative stress has been implicated in pathophysiological status of many diseases including aging, cancer and cardiovascular diseases[3].

Cells have both enzymes (e.g. superoxide dismutase, GSH-dependent peroxidases) and small-molecular-weight reductants [e.g. glutathione (glutamate-cysteine-glycine, GSH), ascorbate, carotene and ubiquinone] to protect against the adverse effects of ROS[4]. GSH is a tripeptide thiol, and mammalian cellular GSH content can be up to millimolar concentrations. In addition to being a co-factor for various enzymatic reactions, GSH mainly functions as an antioxidant to scavenger cellular ROS[5]. Ascorbate has been demonstrated to have multiple biological functions[6]. For example, it is a cofactor for synthesis of collagen and norepinephrine. Similar to GSH, ascorbate can play an important role in anti-oxidative stress by reducing ROS, lipid peroxides and other free radicals, and converting its self into dehydroascorbate.

GSH can be produced by many species, and glutamate-cysteine ligase (GCL) functions as the rate-limiting enzyme for GSH production[7]. L-Buthionine-

S,R-sulfoximine (L-S,R-BSO) is a potent GCL inhibitor, and substantially inhibits GSH production[8]. In contrast, ascorbate production is in a species-dependent manner. Mutation of L-gulonolactone oxidase (Gulo), the enzyme for the last step of ascorbate synthesis, occurs to guinea pigs and humans[9]. Therefore, they can't produce ascorbate endogenously and solely depend on dietary ascorbate plus dehydroascorbate supplement.

Because of the hydrophilic properties, interactions between GSH and ascorbate, such as regeneration or/and replacement each other in scavenging ROS reactions, can be determined both *in vitro* and *in vivo*[10]. For instance, administration of L-S,R-BSO to newborn rats, which have not fully developed ascorbate synthesis, inhibited GSH production, caused oxidative stress injury in multiple tissues, and induced cataracts, which can be prevented by simultaneous administration of ascorbate[11]. Reciprocally, the onset of scurvy in guinea pigs fed ascorbate plus dehydroascorbate-free diet can be delayed by administration of GSH ester[12]. Compared to mice, guinea pigs can't produce ascorbate. Thus, we hypothesized that guinea pigs may have reduced capacity of protecting against oxidative stress than mice. In addition, inhibition of GSH production may activate ascorbate synthesis in mice, which can provide additional protective mechanism.

Material and methods

Materials

L-S,R-BSO, L-buthionine-S-sulfoximine (L-S-BSO) and L-buthionine-R-sulfoximine (L-R-BSO) were prepared as described[13]. BNL CL.2 cells, a mouse hepatic cell line, were purchased from ATCC (Rockville, MD) and cultured in complete DMEM medium containing 10% FCS, 50 $\mu\text{g/ml}$ of penicillin/streptomycin.

In vivo study

The protocols for animal study were approved by the Ethics Committee from Nankai University and conform to the Guide for the Care and Use of Laboratory Animals published by NIH.

Swiss-Webster mice (male, ~20 g) and guinea pigs (male, ~200 g) were purchased from the Animal Center of Nanjing University (Nanjing, China). Normal chow (ascorbate plus dehydroascorbate: 46 $\mu\text{g/g}$) and ascorbate plus dehydroascorbate-free diet (ascorbate plus dehydroascorbate: ≤ 1.5 $\mu\text{g/g}$) were purchased from U.S. Biochemical, Corp. (Cleveland, OH).

After treatment, animals were anesthetized and euthanized in a CO_2 chamber. A piece of tissue was removed, weighed, and homogenized in 5 volumes (g/ml) of an ice-cold solution containing 5% metaphosphoric acid and 0.6% sulfosalicylic acid. After centrifugation for 10 min with a Microfuge at 13,000 rpm at 4°C , the supernatant was collected and stored at -20°C until the assay.

Urine samples from groups of 5 mice in metabolic chamber were collected into 5 ml of the same solution above, and the metabolic chamber walls was rinsed with another 5 ml of the solution at the 12 h interval. The mixture was centrifuged to remove precipitates, and the supernatant was also stored at -20°C.

Determination of GSH, ascorbate and dehydroascorbate

Total GSH [GSH plus GSH disulfide (GSSG)] was determined using an enzymatic method[14]. Tissue and urine ascorbate plus dehydroascorbate were determined fluorometrically after fractionation by ion-pair HPLC and post-column derivation with 4,5-dimethyl-*o*-phenylenediamine; ascorbate was oxidized post-column by cupric sulfate prior to reaction with the phenylenediamine[15].

Determination of L-Gulo mRNA expression and promoter activity

A piece of mouse liver (~50 mg) was used to prepare total RNA, and liver L-Gulo mRNA expression was determined by Northern blot as described[16]. The probe for L-Gulo mRNA was generated by RT-PCR with mouse liver total RNA and the following primers: forward, 5'-TGCCACAGTTCCACCTTC-3'; backward, 5'-GGTTGCTGCTCTCCTTCT-3'.

Mouse L-Gulo promoter (pGL4-Gulo) was constructed by PCR with mouse genomic DNA and the following primers: forward, 5'-CCGCTCGAGTCAGGTTTCGTTACGGATG-3'; backward, 5'-CCGATATCCCCAAGCCTTCCACTTTCAT-3'. To analyze L-Gulo promoter activity, BNL CL.2 cells (~90% confluence) were transfected with DNA for pGL4-Gulo and *Renilla* (for internal normalization) using Lipofectamine™ 2000

(Invitrogen, Carlsbad, CA). After 24 h transfection plus treatment, cells were lysed and cellular lysate was determined the activity of firefly and *Renilla* luciferases using the Dual-Luciferase[®] Reporter Assay System (Promega, Madison, WI) as described[16].

Data analysis

All samples were assayed for GSH, ascorbate, dehydroascorbate in duplicate ($n \geq 3$) except urine samples. Data were presented as mean $\pm$ standard errors, and analyzed by Student's *t* test using Prism (GraphPad Software) with significant difference at $P < 0.05$.

Results

Inhibition of GSH production is associated with increased ascorbate and dehydroascorbate levels in mouse tissues

We initially injected guinea pigs with L-S,R-BSO subcutaneously (s.c.) at different doses, and determined that L-S,R-BSO reduced bodyweight at low doses while inducing a high mortality at high doses (Table 1). For instance, L-S,R-BSO at 2.5 and 1.25 mmol/kg/t.i.d (thrice injection per day) caused no and 60% guinea pigs survived after 5 days treatment, respectively.

To determine if the high toxicity of L-S,R-BSO to guinea pigs is related to inhibition of GSH production, we collected liver and kidney samples from the survived animals at the end of treatment (5 days), and found that tissue GSH levels were substantially reduced (Table 1). For instance, L-S,R-BSO at 1.25 and 0.625 mmol/kg/t.i.d. reduced liver GSH levels to 1% and 9%, and kidney GSH levels to 2% and 7% of controls. Even at the lowest dose used (0.325 mmol/kg/t.i.d.), L-S,R-BSO reduced liver and kidney GSH levels to 15% and 13% of controls (Table 1).

In contrast, all mice survived well with the same L-S,R-BSO treatment. No dead mouse was found by treatment of L-S,R-BSO at 2.5 mmol/kg/t.i.d, even for 2 weeks. Compared to controls, the bodyweight of L-S,R-BSO-treated mice increased similarly to controls indicating that mice well tolerated to L-S,R-BSO treatment. However, L-S,R-BSO (2.5 mmol/kg/t.i.d) still reduced mouse tissue GSH levels but at much lower degrees than that in guinea pigs. Figure 1A shows that after 2 days treatment, GSH levels were reduced to 32% and 15% of controls in liver and kidney, but maintained stable thereafter. Although GSH

levels in other tissues (lung, pancreas and brain) were progressively reduced by L-S,R-BSO treatment, >20% of controls was determined at all the time points of treatment. Therefore, L-S,R-BSO inhibited tissue GSH production very differently between guinea pigs and mice.

Reduced GSH levels may affect tissue ascorbate and dehydroascorbate levels because they can replace or/and regenerate each other in scavenging ROS reactions. In guinea pigs, tissue ascorbate plus dehydroascorbate and ascorbate levels were reduced by L-S,R-BSO treatment with a greater effect on ascorbate alone (Table 1), which also suggests that the redox status of ascorbate (percentage of ascorbate in ascorbate plus dehydroascorbate) in guinea pigs was changed by inhibition of tissue GSH production. For instance, ~100% of ascorbate plus dehydroascorbate is ascorbate in liver and kidney of control guinea pigs. However, the percentages of ascorbate in ascorbate plus dehydroascorbate were reduced to 52% and 61% in liver and kidney, respectively, by L-S,R-BSO treatment at 1.75 mmol/kg/t.i.d. (Table 1). Taken together, the substantial reduction of tissue GSH and ascorbate levels in guinea pigs indicates that the animals have a severe oxidative stress by L-S,R-BSO treatment.

We also determined mouse tissue ascorbate and ascorbate plus dehydroascorbate levels in response to L-S,R-BSO treatment (Figures 1B, 1C). Surprisingly, we did not see reduced ascorbate and ascorbate plus dehydroascorbate levels. In contrast, mouse tissue ascorbate and ascorbate plus dehydroascorbate levels were increased at most time points of L-S,R-BSO treatment. For instance, liver and kidney ascorbate plus dehydroascorbat levels

were increased by 46% on day 7 and 37% on day 5, respectively. Meanwhile, ascorbate plus dehydroascorbate levels in brain and lung were also increased by >20% on day 5. In addition, the percentages of ascorbate in ascorbate plus dehydroascorbate were not affected in all tissues indicating that the redox status of ascorbate in mice is well maintained although GSH production was inhibited.

L-S-BSO, but not L-R-BSO, is the active form inhibiting GCL and GSH production[13]. To further link the elevation of mouse tissue ascorbate levels to reduction of GSH levels, we injected mice with L-S-BSO and L-R-BSO separately, and determined tissue GSH, ascorbate and ascorbate plus dehydroascorbate levels 4 h after a single dose (2.0 mmol/kg) injection. Figure 2A shows that L-S-BSO reduced GSH levels in liver, kidney and pancreas to 19-27% of controls, while it slightly reduced GSH levels in lung and brain which might be due to the slower GSH turnover in those tissues. In contrast, L-R-BSO had little effect on tissue GSH levels, and the minor reduction of GSH levels found in liver and kidney is attributed to a little L-S-BSO contained in L-R-BSO[13].

Similar to L-S,R-BSO treatment, L-S-BSO increased ascorbate and ascorbate plus dehydroascorbate levels in mouse liver and lung (Figures 2B, 2C). In the liver, ascorbate plus dehydroascorbate or ascorbate levels were increased by 20% or 22%; in the lung, they were increased by 37% and 61%, respectively, by L-S-BSO treatment. However, little change in either ascorbate or ascorbate plus dehydroascorbate was determined in mouse tissues by L-R-BSO treatment. The different effects of L-S-BSO and L-R-BSO on ascorbate and ascorbate plus dehydroascorbate levels suggest that increases of tissue ascorbate and ascorbate plus dehydroascorbate levels correlate to reduction of

GSH levels. L-S-BSO had no effect on ascorbate and ascorbate plus dehydroascorbate levels in other tissues which might be related to the rate of ascorbate transport from the liver to other tissues by a single L-S-BSO injection.

Other reagents, such as aminopyrine, can also reduce mouse tissue GSH levels[17]. When mice were fed normal chow containing 0.2% aminopyrine (w/w) for 8 days, tissue GSH levels were reduced (left panel, Figure 2D) although no difference of food intake and bodyweight gain was observed. Associated with reduced GSH levels, tissue ascorbate and ascorbate plus dehydroascorbate levels were increased (middle and right panels, Figure 2D). For instance, aminopyrine decreased GSH levels by 34% while increasing ascorbate and ascorbate plus dehydroascorbate levels by ~50% in mouse liver.

Selenium is essential for GSH-dependent peroxidases which catalyze reactions reducing cellular levels of hydrogen peroxide and lipid peroxides using GSH as a substrate[18]. Therefore, sodium selenite can decrease tissue GSH levels by activating GSH-dependent peroxidases. Correspondingly, mouse tissue ascorbate and ascorbate plus dehydroascorbate levels were increased (Figure 2E).

Inhibition of GSH production activates ascorbate synthesis

Increased mouse tissue ascorbate levels associated with inhibition of GSH production might be contributed by increased dietary ascorbate plus dehydroascorbate intake, reduced ascorbate or dehydroascorbate excretion, and activated ascorbate synthesis.

Compared to control mice, we determined that L-S,R-BSO treatment did not cause difference in food intake implying that ascorbate plus dehydroascorbate supplement is not involved in increased tissue ascorbate levels. We further confirmed it by completing the following experiment. Mice were pre-fed a ascorbate plus dehydroascorbate-free diet for 3 weeks, then injected L-S,R-BSO (2.5 mmol/kg/t.i.d.) for 4 days while they were continued on the ascorbate plus dehydroascorbate-free diet feeding. Compared to normal chow, ascorbate plus dehydroascorbate-free diet feeding had little effect on mouse tissue GSH, ascorbate and ascorbate plus dehydroascorbate C levels (Figures 3A-3C vs. Figure 1). Interestingly, treatment of mice on ascorbate plus dehydroascorbate-free diet with L-S,R-BSO resulted in more reduction of tissue GSH levels than mice fed normal chow. For example, 4 days L-S,R-BSO treatment decreased liver GSH levels of mice on ascorbate plus dehydroascorbate-free diet to 14% of control (Figure 3A) while 5 days L-S,R-BSO treatment decreased liver GSH levels to 37% of control in mice fed normal chow (Figure 1A). Thus, the dietary ascorbate plus dehydroascorbate supplement may further enhance GSH regeneration.

Inhibition of GSH production by L-S,R-BSO also increased tissue ascorbate and ascorbate plus dehydroascorbate in mice fed ascorbate plus dehydroascorbate-free diet (Figures 3B, 3C). For instance, after 4 days treatment, liver ascorbate plus dehydroascorbate and ascorbate levels were increased by 61% and 56%, respectively. Compared to mice fed normal chow, L-S,R-BSO increased liver ascorbate plus dehydroascorbate and ascorbate levels with greater effect in mice fed ascorbate plus dehydroascorbate-free diet. For

example, ascorbate plus dehydroascorbate levels were increased by 61% and 34%, while liver ascorbate levels were increased by 56% and 31% by L-S,R-BSO in mice fed ascorbate plus dehydroascorbate-free diet and normal chow, respectively. Therefore, we clearly see that increased ascorbate or ascorbate plus dehydroascorbate levels in mouse tissues by L-S,R-BSO treatment is not contributed by dietary ascorbate plus dehydroascorbate intake.

To determine the effect of L-S,R-BSO treatment on ascorbate excretion, we collected urine samples from mice receiving L-S,R-BSO treatment (2.5 mmol/kg/t.i.d.) at intervals of day (10 AM-10 PM) and night (10 PM-10 AM). In general, more excreted ascorbate and dehydroascorbate in nights than days can be determined, which might be related to that mice are nightly activating animals. More importantly, Figures 3D and 3E demonstrate that L-S,R-BSO treatment substantially increased excretion of ascorbate and dehydroascorbate, particularly in nights. For instance, 4 and 3-fold increase of excreted ascorbate and dehydroascorbate were observed, respectively, in the second night of treatment.

The increased excretion of dehydroascorbate suggests that more ascorbate replaces/regenerates GSH to reduce ROS, while the generated dehydroascorbate in the anti-oxidative reactions cannot be reduced back to ascorbate when tissue GSH production is inhibited.

Taken together, Figure 3 demonstrates that neither dietary ascorbate plus dehydroascorbate intake nor ascorbate excretion makes contribution to increased mouse tissue ascorbate levels when GSH production is inhibited, which suggests that endogenous ascorbate synthesis must be activated. To obtain the direct evidence, we initially determined that L-S,R-BSO activated

mRNA expression of mouse liver L-Gulo (Figure 4A), the critical enzyme for ascorbate synthesis in mice. We then constructed an L-Gulo promoter and transfected it into BNL CL.2 cells. The transfected cells were treated with L-S,R-BSO followed by determination of promoter activity. Figure 4B shows that L-S,R-BSO increased L-Gulo promoter activity in a concentration-dependent manner which further confirms activation of ascorbate synthesis at the transcriptional level.

Discussion

Both GSH and ascorbate have multiple biological functions to prevent some diseases. Deficiency of GSH production can cause several symptoms, such as hemolytic anemia, 5-oxoprolinuria, metabolic acidosis and neurological symptoms, while lack of ascorbate induces scurvy in humans[19,20]. In addition, as cytosolic antioxidants, they can regenerate or replace each other in scavenging ROS reactions. Cellular GSH production is mainly determined by GCL activity, the rate-limiting enzyme for GSH synthesis, which can be feedback regulated by cellular GSH levels. Compared to mice and many other animals, guinea pigs and humans cannot produce ascorbate indicating that mice and guinea pigs may have different anti-oxidative stress mechanisms, and interfering these systems can result in different consequences. Indeed, in our study, we found that inhibition of GSH production by L-S,R-BSO had little effect on mice but caused severe toxicity to guinea pigs. More importantly, we found that inhibition of GSH production activated ascorbate synthesis in mice which provides an additional protective mechanism in anti-oxidative stress injury. Thus, even in the absence of ascorbate plus dehydroascorbate supplement, mice still tolerated to L-S,R-BSO treatment well indicating the importance of activation of ascorbate synthesis in response to GSH inhibition.

In this study, we provided several lines of evidence demonstrating activation of ascorbate synthesis by inhibition of GSH synthesis in mice. L-S,R-BSO reduced tissue GSH to lower levels in guinea pigs than that in mice, which we interpret that activation of ascorbate synthesis in mice enhances GSH regeneration or ascorbate reduces ROS at a larger degree to spare GSH.

Inhibition of GSH production also shifted redox status of ascorbate in guinea pigs since reduced levels of ascorbate or ascorbate plus dehydroascorbate and percentages of ascorbate in ascorbate plus dehydroascorbate were determined. However, activation of ascorbate synthesis in mice increased tissue ascorbate and ascorbate plus dehydroascorbate levels, and maintained redox status of ascorbate unaffected. Dehydroascorbate, formed in anti-oxidant reactions of ascorbate, can be either catabolized to oxalate or regenerated to ascorbate by GSH-dependent reactions[21,22]. In addition, excess ascorbate or dehydroascorbate can be excreted into urine[23]. Inhibition of GSH production enhanced urinary excretion of ascorbate or plus dehydroascorbate in mice, which suggests that ascorbate synthesis has been induced enough to meet the requirement for clearance of ROS *in vivo*. Finally, activation of L-Gulo expression at the transcriptional level in mouse liver was determined.

The last step of ascorbate synthesis is completed in mouse liver by action of L-Gulo[24], the enzyme which is highly mutated and non-functional in guinea pigs and humans. Lack of ascorbate or plus dehydroascorbate supplement results in severe problems including scurvy in humans. Similarly, ascorbate plus dehydroascorbate insufficiency to mice with genetic deletion of L-Gulo expression can bring severe defects in multiple tissues including heart, liver, brain and lung[24,25,26,27]. Therefore, our study demonstrates that activation of L-Gulo expression by inhibition of GSH production can provide additional protection to other biological systems besides reduction of ROS. And the loss of such protective mechanism due to L-Gulo mutation in species, such as guinea

pigs and humans, leads to reduction of the anti-oxidative stress capacity, and suggests the importance of enough ascorbate plus dehydroascorbate intake.

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Footnotes

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Figure Legends

Figure 1. Administration of L-S,R-BSO reduces mouse tissue GSH levels while increasing ascorbate and ascorbate plus dehydroascorbate levels

Mice (6/group) were s.c. injected with L-S,R-BSO solution (2.5 mmol/kg, t.i.d.) or saline [control (C), 7 days injection] for indicated times. Animals were sacrificed 2 h after the last injection. Tissue samples were collected for determination of GSH (A), ascorbate (Asc) plus dehydroascorbate (DeHAsc) (B) and ascorbate (C). ¶, *, #: $p < 0.05$ vs. corresponding controls.

Figure 2. L-S-BSO, aminopyrine and sodium selenite, but not L-R-BSO, reduce mouse tissue GSH levels while increasing ascorbate and ascorbate plus dehydroascorbate levels

A-C: mice (5/group) were s.c. injected with 2 mmol/kg L-S-BSO or L-R-BSO or saline. Animals were sacrificed 4 h after injection and tissue samples were collected for determination of GSH (A), ascorbate plus dehydroascorbate (B) and ascorbate (C). *, ¶: $p < 0.05$ vs. control and L-R-BSO group, respectively; D, E: mice (5/group) were fed normal chow (control) or chow containing 0.2% (w/w) aminopyrine (D); or s.c. injected with 20 $\mu\text{mol/kg}$ sodium selenite solution (E). Animals were sacrificed after 8 days feeding (D) or 24 h injection (E), followed by collection of tissue samples, and determination of GSH, ascorbate and ascorbate plus dehydroascorbate levels. *, #: $p < 0.05$ vs. corresponding controls.

Figure 3. L-S,R-BSO regulates tissue GSH, ascorbate and ascorbate plus dehydroascorbate levels in mice fed ascorbate plus dehydroascorbate-free diet, and urinary excretion of ascorbate and dehydroascorbate in mice fed normal chow

A-C: after pre-fed a ascorbate plus dehydroascorbate-free diet for 3 weeks, mice (5/group) were s.c. injected with L-S,R-BSO (2.5 mmol/kg t.i.d.) or saline, and continued ascorbate plus dehydroascorbate-free diet feeding for 4 days. Mouse tissues were then collected for determination of GSH (A), ascorbate plus dehydroascorbate (B) and ascorbate (C) levels. ¶, *, #: $p < 0.05$ vs. corresponding controls; D-E: mice in each group (n=5) were placed in metabolic chambers with free access to food and water, and s.c. injected with L-S,R-BSO (2.5 mmol/kg t.i.d.) or saline. Urine samples were collected at the end of each interval of days (D, 8 AM-8 PM) or nights (N, 8 PM-8 AM) followed by determination of ascorbate (D) and dehydroascorbate (E).

Figure 4. L-S,R-BSO increases mouse liver L-Gulo mRNA expression and L-Gulo promoter activity

A: after 5 days treatment with L-S,R-BSO (2.5 mmol/kg t.i.d) or saline, total RNA was extracted from the liver of two mice in each group and used to determine L-Gulo mRNA expression by Northern blot; B: BNL CL.2 cells in 24-well plate (~90% confluence) were transfected with mouse L-Gulo promoter DNA (pGL4-Gulo, 0.4 µg/well) and *Renilla* luciferase DNA (for internal control, 0.02

μg/well) for 6h. Cells were then treated with L-S,R-BSO for 18 h followed by determination of activity of firefly or *Renilla* luciferase. *: p<0.05 vs. control (n=4).

Table 1. Effect of L-S,R-BSO on lethality, bodyweight gain and tissue GSH, ascorbate and ascorbate plus dehydroascorbate levels in guinea pigs

Dose ^a	Animals survived	Bodyweight gain ^b	Tissue GSH (μmol/g) ^e		Tissue ascorbate plus dehydroascorbate (μmol/g) ^e		Tissue ascorbate (μmol/g) ^e	
			Liver	Kidney	Liver	Kidney	Liver	Kidney
Control	10/10	+18%	10.23±1.75	3.34±0.47	0.90±0.11	0.53±0.07	0.89±0.10	0.52±0.04
0.325	10/10	+13%	1.55±0.28 [§]	0.43±0.05 [§]	0.87±0.12	0.51±0.05	0.84±0.10	0.46±0.03
0.625	10/10	-8%	0.93±0.09 [§]	0.22±0.03 [§]	0.85±0.09	0.50±0.05	0.75±0.09 [§]	0.40±0.04 [§]
1.25	6/10 ^c	-10%	0.12±0.09 [§]	0.07±0.06 [§]	0.83±0.08	0.48±0.05	0.58±0.07 [§]	0.34±0.04 [§]
1.75	4/10 ^c	-12%	0.06±0.01 [§]	0.04±0.02 [§]	0.82±0.05 [§]	0.46±0.04 [§]	0.43±0.04 [§]	0.28±0.04 [§]
2.5	0/10 ^d	-12%	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

a: guinea pigs were s.c. injected with L-S,R-BSO at the indicated doses (mmol/kg) or saline (Control) thrice a day (8 AM, 4 PM and midnight) for 5 days or till the animals died; b: after 3 days treatment; c: after 5 days treatment; d: after 3-4 days treatment; e: tissues were collected from the survived animals after 5 days treatment. §: P<0.05 vs. Control (n≥4). N.D: not determined.

Figure 1

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Figure 3

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