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Comparative *in vivo* Lead Mobilization of *meso*- and *rac*-2,3-Dimercaptosuccinic Acids in Albino Wistar Rats

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Abstract: Comparison of the *racemic* and *meso* forms of 2,3-dimercaptosuccinic acid (DMSA) in lead mobilization from lead-loaded albino Wistar rats demonstrates that the *racemic* form is significantly more effective in reducing femur lead levels. After four oral doses at 0.5 mmol/kg, femur lead levels were reduced to 87% of control values by *meso*-DMSA and to 50% of control levels by *rac*-DMSA. Similarly, when the dose was increased to 1.0 mmol/kg, femur lead levels were reduced to 69% of control levels by *meso*-DMSA and to 45% of control levels by *rac*-DMSA. A similar pattern was found for renal lead levels. Brain lead concentrations were significantly lower in treated groups than in control groups, but no differences were found between *rac*- and *meso*-DMSA. *Rac*-DMSA is more soluble than *meso*-DMSA in acetonitrile, ethyl acetate, and ethyl ether. The partition coefficient of *rac*-DMSA in the *n*-octanol/water system was found to be about 2.8. These results indicate that *rac*-DMSA deserves further attention as a possible substitute for *meso*-DMSA.

The importance of chronic lead intoxication as a public health problem of some urgency (Bresnitz *et al.* 1992; Chao & Kikano 1993; Fett *et al.* 1992; Gittleman *et al.* 1994) has been given added emphasis by a recent study (Needleman *et al.* 1996) demonstrating a correlation between bone lead levels and risk of antisocial behaviuor in children. Lead exposures may result in neurological problems as well as chronic renal damage in children (Nowack & Ritz 1992). The usual treatment of chronic lead intoxication requires repeated courses of chelation to achieve reduced serum lead levels because after reduction following a single course of chelation therapy, there is a release of lead from the bone which can bring serum lead levels back to their pretreatment levels (Graziano 1986 & 1993; Graziano *et al.* 1985). The problem of lead intoxication is made more intractable because the majority of absorbed metal remains in the bone, where its biological half-life is estimated to be about 20 years (Weeden 1992). The possible exploitation of new agents which are more effective in reducing both bone and renal lead levels is thus of some interest. A number of agents have been used singly or in combination with other agents for the treatment of lead intoxication in animal models and humans (Glotzer & Bauchner 1992; Nowack & Ritz 1992). These include ethylenediaminetetraacetic acid (EDTA), sodium 2,3-dimercaptopropanesulfonate (DMPS), 2,3-dimercaptopropan-1-ol (BAL), and *meso*-2,3-dimercaptosuccinic acid (*meso*-DMSA) (Aaseth *et al.* 1995; Chisolm 1992; Glotzer & Bauchner 1992; Graziano *et al.* 1992; Liebelt *et al.* 1994; Linz *et al.* 1992; Mortensen 1994; O'Connor 1992; Tandon *et al.* 1994). At the present time *meso*-2,3-dimercaptosuccinic acid (*meso*-DMSA), which is administered orally, is considered to be one of the most effective chelating agents for the treatment of chronic lead intoxication (Aaseth *et al.* 1995; Aposhian & Aposhian 1990; Chisolm 1992; Graziano *et al.* 1992; Liebelt *et al.* 1994; Mortensen 1994). However, a recent study has clearly demonstrated that the stability constants for the lead complexes of *rac*-2,3-dimercaptosuccinic acid(*rac*-DMSA) are significantly greater than those of *meso*-DMSA (Fang & Fernando 1995). Previous studies comparing these two forms of DMSA have demonstrated the superiority of *rac*-DMSA to *meso*-DMSA as an antidote for experimental mercury intoxication in an animal model (Okonishnikova & Nirenburg 1970) and that the stability constant of the mercury complex with the *rac*-DMSA is greater than that of the mercury complex with *meso*-DMSA (Egorova *et al.* 1972). While there have been several recent studies on the chemistry of the *in vitro* interaction of lead with DMSA (Aposhian 1983; Aposhian & Aposhian 1990; Harris *et al.* 1991), no previous study has compared the *in vivo* behaviour of *meso*-DMSA and *rac*-DMSA in an experimental animal model of lead intoxication. The present study was undertaken to determine if *rac*-DMSA possessed any advantage over *meso*-DMSA *in vivo* in mobilizing lead from lead-loaded albino Wistar rats.

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Materials and Methods

Acetylene dicarboxylic acid (**1**) and thiolacetic acid were purchased from Aldrich Chemical Company (Milwaukee, WI, U.S.A.). Lead acetate was obtained from Kemika (Zagreb, Croatia). All solvents were degassed before use. Melting points were determined on a Thomas-Hoover stirred liquid apparatus. ^{1}H NMR spectra were

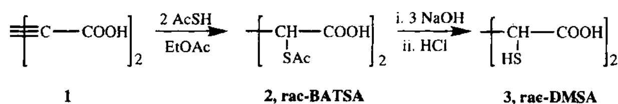

Fig. 1. Scheme showing the preparation of *rac*-2,3-dimercaptosuccinic acid (rac-DMSA).

recorded on 300-MHz FT NMR spectrometer using deuterated solvents as standards. Elemental analyses (C, H, S) were obtained from Atlantic Micro Lab (Norcross, GA, U.S.A.). The *rac*-DMSA was prepared in two steps (fig. 1) following reported methods (Fang & Fernando 1995; Gerecke *et al.* 1961) with slight variations which afforded higher yields.

rac-2,3-Bis(acetylthio)succinic acid (2, rac-BATSA). Thiolacetic acid (75.00 g) and acetylenedicarboxylic acid, 1 (56.19 g) were allowed to react using ethyl acetate as solvent (180 ml) at 35–37° as reported by Fang & Fernando (1995). The solid (*rac*+*meso*) adduct (96.0 g) was removed and *rac*-adduct was separated from this mixture as previously described, but the filtrate was retained. A second crop of *rac*-adduct, which contained a relatively minor amount of *meso*-adduct, was also obtained from the combined filtrates by concentrating these and adding petroleum ether. In the reported method the *rac*-BATSA was isolated only from the collected solid, while the filtrate was discarded. The crude samples of *rac*-BATSA which contained some impurity of *meso*-BATSA were pooled (39.5 g; 30.1% crude yield) and purified using ethyl acetate and petroleum ether giving 30.0 g (23% yield) of pure adduct 2; m.p. 150–153° [lit mp 150–151° (Gerecke *et al.* 1961); 150–153° (Fang & Fernando 1995)]. Analysis calculated Calcd for $C_8H_{10}O_6S_2$: C, 36.08; H, 3.79; S, 24.08. Found: C, 36.17; H, 3.80; S, 24.16.

rac-2,3-Dimercaptosuccinic acid (3, rac-DMSA). The *rac*-BATSA, 2 (48.0 g, 180.26 mmol) was hydrolyzed by adding 3N NaOH (480 ml) under nitrogen at 5° and then stirred for 2–3 min. at room temperature (22.5°) as reported (Fang & Fernando 1995; Gerecke *et al.* 1961). Caution: this hydrolysis is exothermic, therefore, cooling is required initially to maintain the temperature, especially for larger batches. The contents were cooled (0–5°) and concentrated (36%) HCl (ca. 125 ml) was added slowly until the pH reached 0–1, and the *rac*-DMSA (3) was then extracted with Et_2O (3×250 ml). The ethereal extract was washed once with brine, dried over anhydrous $MgSO_4$, and concentrated to ca. 50 ml. Benzene (400 ml) was added and the flask was set aside (2 hr) to allow the product to crystallize. The colorless crystals were collected on a Buchner filter under N_2, washed with benzene (50 mL) and finally with petroleum ether (2×40 ml) giving 28.0 g of crude 3. The final purification was achieved by solution of the dried crude product in Et_2O (250 ml), brief stirring (ca. 15 min.), and then filtration. The clear filtrate was concentrated to ca. 30–40 ml and benzene (400 ml) was added to crystallize the pure *rac*-DMSA (3) which was collected under N_2: yield 24.75 g (75.4%); m.p. 128–130° [reported 127.5–130° (Fang & Fernando 1995); 127–128° (Gerecke *et al.* 1961)]. Analysis calculated for $C_4H_6O_4S_2$: C, 26.37; H, 3.32; S, 35.19. Found: C, 26.44; H, 3.31; S, 35.29.

Solubility studies. Solubilities of *rac*- and *meso*-DMSA were estimated in a number of solvents including H_2O, *n*-octanol, EtOAc, CH_3CN, and Et_2O, which possessed a wide range of polarity. About 5–10 mg of the compound was added to ca. 0.5 mL volume of solvent and was shaken or vortexed at room temperature (22.5°) under N_2 for 1–2 min. and the solubility behaviour was recorded.

Estimation of the partition coefficient. A 100 mg sample of *rac*-DMSA (3) was added to a 1:1 mixture (1 g) of *n*-octanol and Millipore water in a 5-ml screw-cap vial under N_2 and the contents were vortexed at room temperature (22.5°) while capped. The solid dissolved immediately (ca. $<$1 min.). The two-phase mixture was vortexed intermittently (ca. 10 times) over a period of 2 hr, and allowed to stand for 1 hr to separate the aqueous and organic layers. The water layer was isolated and lyophilized giving the weight of *rac*-DMSA contained in water layer. The partition coefficient was calculated by dividing the amount of *rac*-DMSA in *n*-octanol by that in the aqueous phase.

Animal studies

Experimental design. The experiments were performed using 50 female albino Wistar rats, 8 weeks old with an average weight of 205 g, which were bred at and donated from the animal farm of Pliva Pharmaceutical Co., Zagreb, Croatia. During the experiment the rats were kept in polycarbonate cages (10 animals in each) and were given standard rat feed (Altromin-R from A. Rieper SpA, Molina/Industria Mangimi, Italy) and tap water *ad libitum*. The experiment lasted 12 days. During the first 5 days all animals were loaded with lead. Three days later they were divided into 5 groups (10 animals in each group) for the 5 treatment regimes.

Chelation therapy was administered orally for four days (i.e., on day 8, 9, 10, and 11 of the experiment). One day after the last treatment (i.e., on day 12 of the experiment) rats were sacrificed under ether anaesthesia by cardiac exsanguination. The right femur and organs (liver, both kidneys, and brain) were dissected for lead analysis.

Lead loading. Each rat was given 5 mg lead/kg body weight as the acetate by intraperitoneal injection each day for 5 days for a total of 25 mg lead/kg. The lead acetate was dissolved in 0.5 ml of 0.9% saline prior to injection and the injections were given on five consecutive days.

Chelation therapy. The chelating agents were stored under nitrogen and solutions for oral administration were freshly prepared by solution in 5% $NaHCO_3$ solution. Both *rac*-DMSA and *meso*-DMSA were administered by gavage (stomach tube) at two dose levels, i.e., 0.5 and 1.0 mmol/kg body weight in a volume of 0.5 ml. Control animals received 0.5 ml of 0.9% saline on the same schedule of administration.

Determination of lead in tissues. Femur and organ samples (both kidneys and 2–3 g of liver) were weighed, dry-ashed at 450° in a muffle furnace, and then dissolved in 10% nitric acid. Lead content was determined by flame AAS (AA375, Varian, Australia) in all tissue samples except the brain, in which lead level was determined by electrothermal AAS (Spectra 300, Varian, Australia).

Statistical analyses. Results are presented as arithmetic mean±one S.E.M. of lead concentrations in μg/g tissue wet weight for the femurs and the organs examined. The statistical significance of differences between groups was determined by Duncan's multiple range test using the CSS Biostatistica program (release 3.1, Statsoft 1991 package).

Results

Synthesis. The results obtained in the syntheses demonstrate that a greater yield (18–25%) of the precursor *rac*-BATSA (2) of *rac*-DMSA (3) can be obtained by slight modifications in the work-up of the reported procedures (Fang & Fernando 1995; Gerecke *et al.* 1961). Since, the *rac*-BATSA has greater solubility in EtOAc compared to *meso* isomer of BATSA, the filtrates, which contained mostly *rac*-BATSA, were never discarded from the very beginning and were pooled together. The product was isolated by concentrating the EtOAc extract and then adding petroleum ether. The yield in the hydrolysis step was more or less

Table 1.

Relative efficacy of *meso*- and *rac*-DMSA in reducing lead contents of the femur, liver, kidneys, and brain in lead exposed female rats (μg lead/g wet weight)[a].

Group	Dose (mmol/kg)	Femur	Liver	Kidneys	Brain
Control	0	63.0±3.67[b]	3.50±0.40[b]	9.42±0.59[b]	0.182±0.011[b]
meso-DMSA	0.5	52.2±3.54[c]	2.63±0.43[b]	3.34±0.17[c]	0.112±0.015[c]
meso-DMSA	1.0	43.5±4.32[c]	2.62±0.78[b]	1.73±0.14[d,e]	0.089±0.014[c]
rac-DMSA	0.5	31.6±3.51[d]	2.16±0.38[b]	1.50±0.10[e]	0.137±0.012[c]
rac-DMSA	1.0	28.7±2.72[d]	1.90±0.51[b]	0.77±0.11[e]	0.081±0.017[c]

[a] Rats (female) were eight weeks old before lead loading (5 mg/kg body weight by intraperitoneal injection for five consecutive days). Oral treatment with *meso*- or *rac*-DMSA was administered for four days at two doses (0.5 or 1.0 mmol/kg), i.e., on days 8, 9, 10, and 11 of the experiment. Organs were removed and analyzed 24 h later, i.e., 12 days after the beginning of the experiment. The number of rats in each group was 10.
[b–e] Results are presented as arithmetic mean±S.E.M. Significant differences (at $P<0.05$ by Duncan's multiple range test) are indicated by different superscript letters.

the same as reported (Fang & Fernando 1995) and *rac*-DMSA (3) was obtained in ca. 75% yield after final purification.

Solubility and partition coefficient. The *rac*-DMSA was found to be soluble in H_2O, *n*-octanol, CH_3CN, EtOAc, Et_2O at 22.5°, whereas, the *meso*-isomer had a very limited solubility in these solvents under identical conditions. The solubility of the *racemic* acid is unusual: it dissolves to the extent of ca. 100 mg/1 g of water but in *n*-octanol the solubility is ca. 100 mg/300 mg of *n*-octanol. The partition coefficient of *rac*-DMSA in *n*-octanol/water system was calculated to be 2.8 ($=[rac\text{-DMSA}]_{octanol}/[rac\text{-DMSA}]_{water}$).

Biological data. The weight increase of the rats in all of the treated groups during the experiments was similar to that of the untreated control rats. No changes in organ weights between the groups were observed at the end of the experiments. None of the rats showed any visible adverse health effect, regardless of the treatment regime.

The results obtained in the animal studies are given in table 1. Femur lead concentrations were decreased after treatments with either chelating agent. The reductions in bone lead were significantly greater after *rac*- than after *meso*-DMSA treatment, irrespective of the dose (0.5 or 1.0 mmol/kg). Kidney levels were drastically reduced by treatment with both isomeric forms of DMSA, but the lower dose of *rac*-DMSA (0.5 mmol/kg) produced a similar reduction from the control value as the higher dose of the *meso*-DMSA (1.0 mmol/kg). Neither compound was effective in reducing liver lead concentrations. The relative amount of lead which accumulated in the liver was very small compared to that which accumulated in the bone and was also smaller than that in the kidney. Brain lead levels were the lowest and were significantly reduced in all treated groups compared to control, and no differences were found between *rac*- and *meso*-DMSA.

These results show that *rac*-DMSA is significantly more effective than *meso*-DMSA in reducing residual levels of lead in both the bone and the kidney, in agreement with the predictions of Fang & Fernando (1995).

Discussion

As was predicted by Fang & Fernando (1995)from the relative values of the stability constants of the lead complexes, the *rac*-DMSA was significantly more effective than *meso*-DMSA in mobilizing lead. The large reductions obtained in femur lead levels following *rac*-DMSA administration strongly suggest that *rac*-DMSA may be an agent of considerable promise for ultimate use in the treatment of childhood lead intoxication, where the long-term developmental consequences are so potentially disastrous (Glotzer & Bauchner 1992; Needleman *et al.* 1996). The DMSA dosage used in the present set of experiments was greater than that used in an earlier study in which removal of lead from the femur was not noted (Cory-Slechta 1988). The relative ability of chelating agents to remove lead from the bone is also dependent on the lead loading model which is used (Cory-Slechta 1988; Xu & Jones 1988).

The lipophilicity of *rac*-DMSA may be due to the *R,R* and *S,S* conformations (fig. 2) of the enantiomers constituting *rac*-DMSA, though a significant difference in the lattice energies of the *meso* and *rac* forms could also give rise to such behaviour. In these conformations similar groups are opposite to each other, unlike *meso*-DMSA, therefore, *rac*-DMSA is less dipolar than the *meso* compound. This amphipathic nature of *rac*-DMSA is a highly advantageous feature for the purpose of both absorption from the stomach and penetrating the cellular membranes in order to

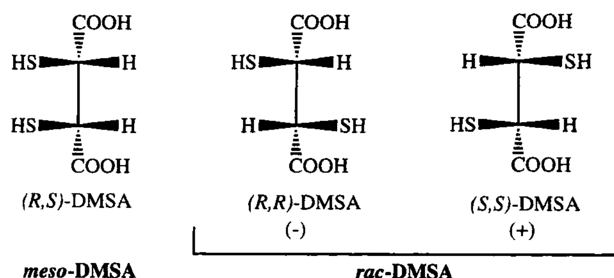

Fig. 2. Stereoisomers of 2,3-dimercaptosuccinic acid (*meso*- and *rac*-forms).

reach the intracellular lead. The high lipophilicity of the *rac*-mixture compared to the meso-form might be paralleled by higher toxicity.

The brain lead levels indicate that no transport of lead to the brain results from a course of four doses of *rac*-DMSA over a period of four days. One of the major advantages of *meso*-DMSA over $Na_2CaEDTA$ is the fact that there is no transient increase in brain levels during the initial stages of treatment as demonstrated by Cory-Slechta *et al.* (1987). Although *rac*-DMSA is more lipophilic than meso-DMSA, which probably contributes to its superiority in the mobilization of lead from deeper compartments, including bone, the mobilized lead-chelate is apparently not transferred as readily into the brain as the lead-EDTA chelate.

rac-DMSA consists of two pure enantioisomeric forms: the *R,R* acid and the *S,S* acid (fig. 2). It may well be that these two forms will also differ significantly in their relative ability to mobilize lead from the mammalian body, as such pharmacological differences are commonly found with other drugs which contain chiral centers (Brown 1990; Casy 1993). While the *R,R* and *S,S* forms are not expected to differ significantly in the values of the stability constants of their lead complexes, they may well interact in a significantly different manner towards other chiral species such as selective cellular membrane transport systems (Ott & Giacomini 1993), lead binding proteins (Noctor 1993), lead-inactivated enzymes, and similar species which possess chiral centers and possess preferential chiral reactivity patterns of their own. The fact that *rac*-DMSA is a mixture of two forms indicates that further research on *each* member of the DMSA family is important, as the biokinetics and toxicity of the RR- and SS-forms may well be as different as the corresponding properties of D- and L-penicillamine.

While *rac*-DMSA is of interest as a potential agent for treatment of heavy metal intoxication, its lower polarity than *meso*-DMSA, and presumed greater toxicity, suggest that more detailed studies of its *in vivo* behavior will be needed before such uses can be given serious consideration.

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