Inhibition of cadmium teratogenesis by a mercaptoacrylic acid (MFA)[1,2]

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Summary. The teratogenic effect of cadmium can be diminished by a number of mechanisms including zinc and pretreatment with cadmium and mercury. In this study, the oral administration of α-mercapto-β-(2-furyl)-acrylic acid (MFA) protects against cadmium-induced malformations and embryonic death. This protection is probably mediated by the chelation of the cadmium ion rather than metallothionein (MT) synthesis.

Key words. Teratogenesis; cadmium; metallothionein; mercapto-β (2-furyl)-acrylic acid; hamster.

Cadmium ion is an effective teratogen in experimental animals following acute exposure in early pregnancy[3]. Its teratogenic effect, however, can be significantly decreased by prior exposure to cadmium[4], mercury[5] or zinc[6]. This protection against cadmium ion teratogenicity is presumably due to the induction of a cadmium binding protein, metallothionein (MT) by these metal ions[7]. Recently, Giroix and Lachmann[8] have reported that a non-metal, organic chemical, α-mercapto-β-(2-furyl)-acrylic acid (MFA, Fig.) induces MT production in rat kidney and liver when administered by gavage over a five-day period. This finding encouraged us to examine the effect of MFA on the production of teratogenic lesions by acute administration of cadmium ion.

In this report we describe the significant protective effect of MFA against cadmium ion induced teratogenesis in the hamster model. We also present data which indicate that MT synthesis cannot explain the protective effect of MFA in hamsters but that protection is probably mediated by the chelation of cadmium ion by MFA.

Materials and methods. Teratogenesis studies. Timed pregnant hamsters (LKV strain) were obtained from the Charles River Co. and maintained in individual cages. The MFA was prepared by the method of Campaine and Cline[10]. The animals were gavaged on either the sixth and seventh day of gestation, or the seventh day of gestation only, with 50 mg/kg of MFA made up in Tween 20 (50:50 with water) so that 0.5 ml/100 g of maternal body weight was given at each dose. On the morning of the eighth day of gestation they received a single i.v. injection of cadmium sulfate at the dose of 2 mg/kg (7.80 μmol/kg), the optimal teratogenic dose[11], in a volume of 0.5 ml/100 g of body weight. A control group received only a teratogenic i.v. dose of cadmium on day eight of gestation. Tween 20, by itself, is not a teratogen in this animal model[12]. On day 13 of gestation, the animals were sacrificed by excess CO_2 inhalation, the fetuses recovered and examined for gross congenital malformations in a manner previously described[13]. Resorption sites, evidence of embryonic/fetal death, were also recorded.

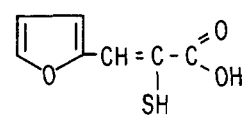
A second group of hamsters were treated on days six and seven of gestation with Tween-20 or Tween-20 plus MFA as described above. The animals were sacrificed on day nine. Maternal liver and maternal kidney were removed for MT assay.

Metallothionein assay. Tissue preparation and incubation with exogenous radiolabeled cadmium ion followed a procedure outlined by Eaton and Toal[14], which was based on a method described by Onosaka et al.[15]. Liver or kidney tissue (0.2–1.5 g) were homogenized in 5 vols of cold 10 mM Tris (Cl), pH 8.0, using a glass receiving vessel fitted with a teflon piston. The homogenates were heated for 2 min in 85 mm × 15 mm glass tubes placed in a boiling water bath. Heated samples were centrifuged at 1500 × g for 5 min and the clear supernatants separated from the pellets. Pellets were washed with an equal volume of Tris buffer and recentrifuged. Washed supernatants were combined with initial supernatants to give a solution containing MT, but mostly free of other proteins.

Measured volumes of tissue supernatant (0.100–0.300 ml) were placed in 1.5 ml capped polypropylene centrifuge tubes along with 0.300 ml of 20.0 μmoles cadmium chloride solution which was radiolabeled with carrier-free ^{109} cadmium ion (obtained as $^{109}Cd(Cl_2)$ from New England Nuclear Corp.). The ^{109}cadmium enrichment was such that the cadmium stock solution contained 6×10^4 cpm per nmole Cd. Total volumes were set at 0.600 ml using Tris buffer as required. The incubation mixtures were placed in boiling water for 3 min then centrifuged for 5 min at 2000 × g to remove any suspended material.

Localization and quantification of MT (as ^{109}CdMT) was achieved by gel filtration chromatography. A measured volume of the ^{109}Cd incubate (usually 150 μl) was added to a 12.0 cm × 0.75 cm Sephadex 6–50 (fine) column previously equilibrated with 10 mM Tris (Cl), pH 8.0. The same buffer was used to elute Fraction 1 (3.0 ml) and Fraction 2 (1.5 ml). Fraction 2, representing the 3.0–4.5 ml elution volume, contains ^{109}CdMT (purified hamster liver CdDMT elutes with a peak near 3.5 ml in this assay). The ^{109}Cd content of Fraction 2 was radioassayed using a Beckmann Gamma 5500. All of the radioactivity found in Fraction 2 is attributed to ^{109}CdMT. Counts obtained for Fraction 2 samples were converted to nmoles of Cd. Tissue MT concentrations are expressed as nmoles Cd/g tissue (wet weight).

Interaction of MFA with cadmium ions in vitro. The interaction of MFA with cadmium ion was measured using a two solvent phase competing ligand system. Chloroform and Tris buffered water served as the immiscible phases and dithizone was the competing ligand. Specific amounts of dithizone and metal ion, representing 1:1 and 2:1 molar ratios of ligand and metal ion were shaken in the chloroform, water system along with different molar ratios of MFA until equilibrium was achieved, i.e., until no further color change was observed. The concentration of non-complexed dithizone in the chloroform phase was assayed spectrophotometrically at 600 nm. Calculations of stability constants (K_s) for 1:1 and 2:1 complexes of MFA and cadmium ions were based on the degree of displacement of dithizone in the chelate complex by a particular concentration of MFA. Apparent K_s values for the 1:1 and 2:1 MFA, metal ion complexes were converted to true K_s values using published constants for the cadmium ion, dithizone complexes[16]. A detailed description of this method is to be published elsewhere.



α-mercapto-β-(2-furyl) acrylic acid

Results. The data on teratogenesis are summarized in table 1. It is clear that MFA is not teratogenic in this animal model when administered orally by gavage. It is also clear that exposure to MFA protects the embryo almost completely from acute cadmium teratogenesis when the mother is treated on days six and seven, or only on day seven of gestation, some 24 and 48 h prior to acute cadmium exposure.

The statistical significance of differences between groups was calculated using the unpaired t-test corrected for multiple comparisons. There was a significant difference between the resorp-

Table 1. Effect of MFA on cadmium teratogenesis

Treatment group	Dose (mg/kg)	No. of litters with one or more malformed fetuses / Total No. of litters (%)	Total No. of implantation sites	Total No. of resorptions (%)	No. of fetuses with one or more malformations (%)	Total No. of normal fetuses (%)
MFA only p.o. (days 6, 7, 8)	50	1/8	116	11 (9.4%)	1 (1.0%)	104 (99.0%)
$CdSO_4$ only i.v. (day 8)	2	20/20	268	63 (23.5%)	114 (55.6%)	91 (44.3%)
$CdSO_4$ i.v. (day 8) + MFA p.o. (day 7)	2 / 50	7/11	139	7 (5.03%)	19 (14.3%)	113 (85.6%)
CdSO i.v. (day 8) + MFA p.o. (days 6, 7)	2 / 50	2/16	216	14 (6.48%)	4 (1.98%)	198 (98.0%)

tion rates in the group receiving $CdSO_4$ with MFA from one pump. There was no significant difference between the resorption rates in the group receiving $CdSO_4$ with MFA-1 pump and the group receiving $CdSO_4$ with MFA-2 pump. There was also a significant difference in the malformation rate in the group receiving $CdSO_4$ alone and the group receiving $CdSO_4$ with MFA-1 pump. There was a significant dose: response effect shown by MFA since the group receiving $CdSO_4$ with MFA-2 pumps was significantly different from the group receiving $CdSO_4$ with MFA-1 pump.

The concentrations of maternal liver and maternal kidney MT in MFA treated and control hamsters are presented in table 2. MFA had no effect on the concentration of maternal liver MT. Maternal kidney MT levels were, on the average, 175% that of kidneys taken from control animals.

Cadmium ions are extensively bound by MFA. The equilibrium constants (K_s) for chelate complexes containing a 1:1 ratio of ligand to metal ion and a 2:1 ratio of ligand to metal ion are $10^{7.03}$ and $10^{6.84}$.

Discussion. Cadmium ion is a unique teratogen in that there appears to be a specific response of the maternal tissue to synthesize MT which binds this metal ion, presumably blocking it from reaching the differentiating embryo in teratogenic levels. MT is also produced in the placentas of pregnant mice [17] and hamsters[18] during the critical stages of embryogenesis following an acute teratogenic dose of cadmium ion. Thus, the cadmium ion would seem to be an effective teratogen only under conditions of sufficient acute exposure prior to the onset of MT synthesis. This may account for the fact that there is no clear evidence that cadmium has been implicated in any human teratogenic event, since presumably most human exposure to cadmium ions occurs under chronic conditions.

In this study we have demonstrated that orally administered MFA can protect hamsters against a normally teratogenic dose of cadmium ion. However, this protective effect does not depend on the expected finding of MFA induced MT biosynthesis. Maternal liver and maternal kidney concentrations of MT in control and MFA treated hamsters are close to values reported for untreated rats[7]. MFA apparently induces a less than two-fold increase in hamster kidney MT, yielding an average of 15.7 nmole cadmium ion (as MT)/g tissue. This finding is not in accord with that of Giroux and Lachmann[8], who found MT levels in the kidneys of MFA treated rats equivalent to 179 nmole cadmium ion (as MT)/g. Their study also showed that two-thirds of the metal ion moiety of rat kidney MT consists of copper ions. Scheuhammer and Cherian[19] point out that only 40% of the copper ions associated with MT are displaced in the cadmium ion saturation MT assay. If we assume that two-thirds of the metal ion moiety of hamster kidney MT consists of copper ions, then our values for untreated and MFA-treated dams are low by approximately 50%. However, the inability of exogenous cadmium ions to displace copper ions from MT in vitro may well be duplicated in vivo. There is no a priori reason to assume that copper metallothionein can serve as a sink for cadmium ions. In any case the increase in hamster kidney MT, observed in our investigation can account for no more than 2% of the total of the injected cadmium ion. Apparently, increased MT synthesis cannot be the chief reason that MFA protects hamsters from cadmium ion induced teratogenic lesions. On the other hand, MFA is a very good chelator of cadmium ion, with dimensions of K_s for the 1:1 and 2:1 complexes in the range of values obtained for other α-mercaptocarboxylic acids[20]. This strongly suggests that the MFA protective mechanism against cadmium ion induced teratogenesis in the hamster as more likely one of chelation than one in which preformed MT scavenges cadmium ion.

Table 2. The concentration of metallothionein in tissues of hamsters receiving oral doses ot Tween-20 and Tween-20 plus MFA. Values given are means ± SE obtained from assays on organs from 6–8 different individuals

Tissue	Animal treatment	nmoles Cd/g tissue (wet weight)
Liver	Tween-20	19.1 ± 1.6
	Tween-20 + MFA	17.2 ± 2.4
Kidney	Tween-20	8.9 ± 2.0
	Tween-20 + MFA	15.7 ± 2.4

1 This project has been financed in part with Federal funds from the U.S. Environmental Protection Agency under grant number R811078. The contents do not necessarily reflect the views and policies of the Environmental Protection Agency nor does mention of trade names or commercial products constitute endorsement of recommendation for use.
2 We thank Dr W. Layton for the statistical analyses of the data and G. McClain for the synthesis of MFA.
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0014-4754/87/020208-03$1.50 + 0.20/0

Macrobiotus pseudohufelandi Iharos as a model for cytotaxonomic study in populations of eutardigrades (Tardigrada)[1]

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Summary. The morphotype, chromosome number and Feulgen-DNA content of bisexual and unisexual populations of *Macrobiotus pseudohufelandi* were examined. Individuals of unisexual populations were triploid with ameiotic parthenogenesis. Their lowest Feulgen-DNA content is about three-fold that of sperm from a bisexual population. Egg shell shape also differs in the two types of population. However, the highest Feulgen-DNA content was the same (24 A.U.) in both diploid and triploid animals.
Key words. Cytotaxonomy; genome size; Tardigrada, *Macrobiotus*.

Cytotaxonomy allows the identification of tardigrades having similar appearance but different cytology (different chromosome number, different modality of female gametogenesis)[2-4]. The bulk of the data has been furnished by a study of oocytes, which represent good material for rapidly defining the ploidy and type of reproduction. In fact, the oocytes remain blocked at metaphase I until oviposition and are characterized by large, well-separated chromosomes[3,4]. In order to gain a deeper insight into the cytotaxonomical approach for the study of eutardigrades we considered *Macrobiotus pseudohufelandi* Iharos. In contrast to other species, clear identification is possible because of its small claw size and the presence of lunulae limited to the fourth pair of legs[5]. Moreover, this species can be readily found in coastal dune mosses. Both bisexual and unisexual populations of *M. pseudohufelandi* are known; the egg shell has a unique shape in the former[6]. Unisexual populations have 18 chromosomes in both mitoses and oocytes, that is, they are probably triploid[3,4]. The aim of the present paper was to compare information on presence or absence of males, type of reproduction, genome size, chromosome number, and taxonomy of this species.

Description of animal origin. Moss-living animals from various Italian coastal localities were used[6]. Karyotype data were obtained from two collections of a single bisexual population from Marina di Cecina (Tyrrhenian coast), and from single collections of unisexual populations from Marina Romea, Pineto (Adriatic coast), Bosco Pantano (Ionian coast), Vada, Riva degli Etruschi (three places), Tirrenia (Tyrrhenian coast), and Marina di Sorso (Sardinian coast).

Chromosome analysis. Lactic acetic orcein stain was used on whole specimens previously fixed in methanol and acetic acid (3:1). Observations were made as described below. Chromosome length was evaluated by a Leitz filar micrometer.

Genome size evaluation. Squash preparations were made of four animals from the bisexual population (Marina di Cecina) and of four from a unisexual population (Riva degli Etruschi). Specimens were fixed in formaldehyde (10%, 20 min) and the Feulgen reaction implemented as suggested by Itikawa and Ogura[7] (with hydrolysis in 5N HCl for 1 h at 23 °C and treatment with Schiff reagent for 45 min at 23 °C). To minimize variability caused by fixation and staining, all slides were processed in a single Feulgen bath. The Feulgen-DNA content was evaluated in as many nuclei as possible regardless of tissue type. Feulgen-DNA content was measured at a wavelength of 545 ± 5 nm with a Vickers M85 scanning microdensitometer directly interfaced to a P6060 Olivetti microcomputer. Readings were taken under the following instrument conditions: 10 × eyepiece; 100 × objective lens; 1.25 numerical aperture; dry condenser; 0.4 µm final diameter of the flying spot. Photometric errors due to glare and non-specific light loss were evaluated as suggested by Bedi and Goldstein[8]. Since error proved to be constant and negligible (< 3%), no instrument correction was introduced. At least 50 nuclei were

Geographic locations	Number of specimens	Chromosome number Mitosis	Oocytes	Type of reproduction
Marina di Cecina	18	—	—	
	34	12	6	Amphimixis
Marina Romea	72	18	—	—
Pineto	65	18	18	Ameiotic parthenog.
Bosco Pantano	78	18	—	—
Riva degli Etruschi I	56	18	18	Ameiotic parthenog.
Riva degli Etruschi II	66	18	18	Ameiotic parthenog.
Riva degli Etruschi III	57	18	18	Ameiotic parthenog.
Vada	15	18	18	Ameiotic parthenog.
Tirrenia	46	18	18	Ameiotic parthenog.
Marina di Sorso	22	18	18	Ameiotic parthenog.