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THE TILORONE-INDUCED MUCOPOLYSACCHARIDOSIS IN RATS

BIOCHEMICAL INVESTIGATIONS

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Abstract—The tissues of rats chronically treated with tilorone exhibited a significant accumulation of acid glycosaminoglycans (GAGs): In the liver, the GAG concentration was found to be elevated by a factor of 38, in the spleen by a factor 15 and in the kidneys by a factor of 5. Furthermore, the renal excretion of GAGs was increased 32-fold as compared to control animals. Dermatan sulphate was predominant among the GAGs stored in the three organs; chondroitin sulphate and heparan sulphate were found in smaller amounts. GAG storage was accompanied by accumulation of the drug within the tissues: the molar ratio of tilorone per disaccharide unit of GAG was calculated to be one to two in each tissue. According to previous reports, tilorone-induced mucopolysaccharidosis is due to impaired lysosomal degradation of GAGs. The present results give support to the hypothesis that an interaction between the polyanionic GAGs and the dicationic drug may lead to GAG–drug complexes which cannot be digested by lysosomal enzymes.

Tilorone (Fig. 1), a bis-basic tricyclic compound, exhibits antiviral and anti-tumor activity based on its immune-modulating effects [1]. After chronic treatment of rats with tilorone, abnormal lysosomal inclusions appeared in various tissues especially liver [2], spleen [3], kidney [4], cornea [5] and heart-valves [6]. Two ultrastructurally different types of storage lysosomes were observed: (a) lamellar bodies, (b) clear cytoplasmic vacuoles after fixation with glutaraldehyde. The lamellar bodies could have been related to the storage of polar lipids, i.e. lipidosis—a side effect also seen with other catamphiphilic drugs [7, 8]. The material accumulated in the cytoplasmic vacuoles was highly water-soluble and so was lost during fixation with glutaraldehyde. Therefore, the vacuoles seemed to be empty upon microscopic examination. In cryostat sections the stored substances were stained and fixed by Alcian blue at pH 1.

These histochemical characteristics of the stored material together with the ultrastructural picture gave strong evidence that chronic treatment with tilorone causes lysosomal changes similar to those of inherited mucopolysaccharidoses. The aim of the present biochemical study was to determine the extent of accumulation and to analyse the nature of this stored material in several tissues and in the urine.

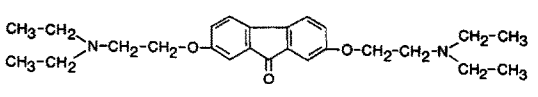

Fig. 1. Tilorone.

Preliminary reports of the results have been published in abstract form [9–12].

MATERIALS AND METHODS

Materials

Tilorone-HCl was a gift from Merell Dow Pharmaceuticals (Cincinnati, OH, U.S.A.). All chemicals were of analytical grade. They were purchased from Merck (Darmstadt, Germany) with the following exceptions: chondroitinase ABC from *Proteus vulgaris* (EC 4.2.2.4); chondroitin sulphate A (CHS A*) from whale cartilage; CHS C from shark cartilage; DS from porcine skin; HS from bovine kidney; ribonuclease A from bovine pancreas type III-A (EC 3.1.27.5); deoxyribonuclease from bovine pancreas type IV (EC 3.1.21.1); trypsin from bovine pancreas type III (EC 3.4.21.4) and CETAB: all from the Sigma Chemical Co. (Munich, Germany). Chondroitinase AC II from *Arthrobacter aurescens* (EC 4.2.2.5) came from Seikagaku Kogyo (Tokyo, Japan).

Drug treatment

In the experiments young female Wistar rats were used. They had free access to tap water. Tilorone was added to the ground chow at concentrations between 0.07 and 0.1%, dependent on the wellbeing of the animals. Thus, the resultant daily drug intake varied throughout the chronic exposure and ranged between 60 and 75 mg/kg body weight. The duration of treatment varied from 11 to 26 weeks. Age-matched controls were kept under identical conditions but without drug administration.

* Abbreviations: CETAB, cetyltrimethylammonium-bromide; GAG, glycosaminoglycan; HS, heparan sulphate; CHS, chondroitin sulphate; DS, dermatan sulphate.

Specimens of urine and tissues

After 8 and 12 weeks the urine of treated and non-treated rats was collected during a period of 4 days. During this period the specimens were kept under toluene as preservative. The samples were frozen at −20° until analysed.

After 11 (two animals) and 26 (three animals) weeks the rats were sacrificed and liver, spleen and kidney were removed. The kidney was separated into the papilla and inner part of the outer medulla on the one hand and the outer stripe of the outer medulla and cortex on the other hand. All tissues were weighed and stored at −20°.

Isolation of glycosaminoglycans (GAGs)

Urine. The frozen samples were thawed and centrifuged to remove impurities. Ten milliliter aliquots were used for isolation of GAG. The urine was adjusted to pH 5 with 1 M HCl. GAGs were precipitated with 1 mL of an aqueous solution containing 5% CETAB, then centrifuged at 1500 *g*. The precipitate was washed twice with 10 mL 96% ethanol saturated with sodium chloride. Between these washings the samples were kept at 5° overnight. After this the isolated material was dried and dissolved in 5 mL 0.05 N NaOH for the hexuronic acid assay.

For the qualitative analysis the same steps were performed but 20 instead of 10 mL urine from untreated rats were used. The precipitates were dissolved in a final volume of 100 (untreated rats) or 400 μL (treated rats) distilled water.

Tissues. The frozen organs were thawed, minced and three times dehydrated and defatted by 20 vol. acetone. Between these steps the suspension was kept at 5° for 24 hr. These three acetone extracts were pooled and used for the determination of the tilorone content. Defined amounts of the dried and weighed tissues of liver (1.0 g), spleen (0.1 g) and kidney (0.2 g) were suspended in 10 mL (spleen and kidney) or 20 mL (liver) of a 0.05 M Tris buffer solution (pH 8) containing 500 units trypsin per 10 mL. After incubation at 37° for 24 hr and at room temperature and pH 11 for a further 8 hr the samples were adjusted to pH 6 centrifuged at 60,000 *g* for 20 min. Two volumes of absolute ethanol were added to the supernatant and kept at 4° for 24 hr. The precipitate was harvested by centrifugation with 1500 *g* for 10 min and dried. It was dissolved and incubated at 30° over 18 hr in 2 mL 0.05 M sodium citrate buffer solution (pH 5) containing 6000 Kunitz units DNAase, 300 Kunitz units RNAase and 4.2 mM magnesium chloride. Treatment with 1900 PUK pronase, dissolved in 5 mL 0.05 M Tris-buffer (pH 7.6) at 40° for 24 hr followed. After addition of 1 mL of 5% NaCl solution and adjustment to pH 5 the GAGs were precipitated with CETAB and washed twice with NaCl-saturated ethanol as described for urine. The dried tissue extracts from untreated rats were dissolved in 2 mL (liver and spleen) or 4 mL (kidney) and those from tilorone-treated animals in 50 mL (liver), 7 mL (spleen) or 12 mL (kidney) of distilled water, and used for the hexuronic acid assay.

Quantitative analysis

Hexuronic acid assay. The GAG content of tissues and urine was determined by measuring the uronic acid with a modified carbazole-borate method [13].

A 0.5 mL sample was mixed in ice-cooled tubes with 0.2 mL carbazole reagent (0.2 g carbazole in 100 mL abs. ethanol) and 5 mL borate/sulfuric acid reagent (5 g sodium tetraborate in 1 L concentrated sulfuric acid), and transferred to a vigorously boiling water bath for 15 min. The solution was cooled in an ice bath and the absorbance was measured in a 10 mm light path cuvette at 520 nm in a Beckman 3600 photometer. Together with the samples a standard solution of 5 mg glucuronolactone in 100 mL water was measured.

The remaining GAG solution not used for the carbazole reaction was again precipitated with CETAB and washed twice with NaCl-saturated ethanol as described for urine. The GAGs were redissolved in 100 μL (liver) or 50 μL (spleen and kidney) for untreated rats and in 400 μL (liver) or 100 μL (spleen and kidney) for treated rats, and used for qualitative analysis.

Tilorone-content. The acetone extracts of the tissues were evaporated to dryness and the residue was reconstituted with 1 M HCl, adjusted to pH 7 with 1 M NaOH and diluted with distilled water to 50 mL (kidney and spleen) or 250 mL (liver). One milliliter of these solutions was extracted three times with 1 mL chloroform. The aqueous phase was made alkaline with 1 mL 1 M NaOH and shaken again with three aliquots of 1 mL chloroform. The combined chloroform phases (6 mL) were extracted three times with 1 mL 1 M HCl and discarded. One milliliter (spleen and kidney) or 0.5 mL (liver) of the combined HCl extracts were diluted up to 10 mL with distilled water and measured at 270 nm. The recoveries of known amounts of tilorone added to liver homogenates were 85 ± 5% ($\bar{x}$ ± SEM; N = 10).

Creatinine. The creatinine concentration in urine was measured by a reagent kit (Merckotest, Darmstadt) utilizing a kinetic method of the Jaffe reaction.

Qualitative analysis

Electrophoresis. The solutions prepared after the hexuronic acid assay were used for the electrophoresis. Together with the samples an aqueous standard solution containing 1 mg/mL each of DS, CHS and HS was applicated. The GAGs were separated on 25 × 150 mm cellulose acetate stripes using 0.1 M barium acetate solution at a constant potential of 100 V for 3.5 hr [14].

The electrophoresis stripes were stained with an Alcian blue solution (0.5 g Alcian blue in 100 mL methanol, 100 mL distilled water and 10 μL glacial acetic acid) for 10 min; destaining was performed with several changes of 0.83 mM acetic acid.

Enzymatic assay [15]. Three sets of reaction mixture were prepared: 5 μL (treated rats) or 10 μL (untreated rats) of the samples prepared after the hexuronic acid assay were incubated with (a) 50 μL enriched Tris buffer pH 8 (50 mM Tris buffer, 500 mg/L bovine serum albumin, 50 mM sodium

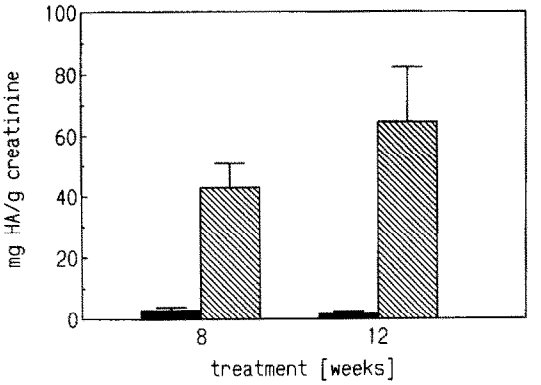

Fig. 2. Hexuronic acid (HA) content of the urine of rats after 8 and 12 weeks treatment with tilorone, expressed as mg HA/g creatinine ($\bar{x} \pm$ SEM; N = 5). Filled bars, control rats; hatched bars, tilorone-treated rats.

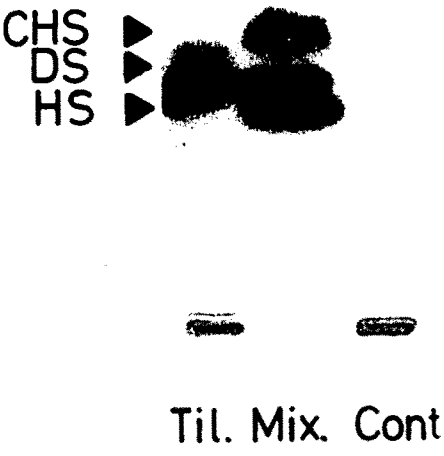

Fig. 3. Cellulose acetate electrophoresis of GAG extracts from the urine of controls (Cont.) and tilorone-treated animals (Til.) together with a standard solution of CHS, DS and HS (Mix).

chloride and 35 mM sodium acetate); (b) 50 μL enriched Tris buffer containing 1.1 units chondroitinase AC; or (c) 50 μL Tris buffer containing 0.5 units chondroitinase ABC. All three mixtures were incubated at 37° for 12 hr, lyophilized and redissolved in 5 μL distilled water, and finally separated by electrophoresis in 0.1 M barium acetate. Additional sets with a standard solution of CHS, DS and HS were incubated in the same way.

Nitrous acid assay [16, 17]. Two tubes with the following reaction mixture were prepared: 10 μL (treated rats) or 15 μL (untreated rats) samples were mixed with 20 μL 1.5 M sodium nitrite and (a) 10 μL glacial acetic acid or (b) distilled water as control. The sets were incubated for 90 min at 37°. The reaction was stopped by adding 20 μL 2 M ammonium sulfamate solution. The mixtures were lyophilized and dissolved in 5 μL (untreated rats) or 10 μL (treated rats) distilled water followed by electrophoresis in 0.1 M barium acetate.

Additional sets with standards of CHS, DS and HS were treated in the same way.

RESULTS

Urine

After 8 weeks of tilorone treatment, the urinary GAG excretion—expressed as mg hexuronic acid/g creatinine—was 15-fold higher and after 12 weeks, 32-fold higher, as compared with age-matched control animals (Fig. 2).

In the urine the hexuronic acid contents of the untreated rats was so low that no GAG by electrophoresis could be detected. The urine of tilorone-treated rats exhibited the following pattern after electrophoresis in 0.1 M barium acetate: DS as the main substance and smaller amounts of CHS (Fig. 3). Digestion with nitrous acid and chondroitinases confirmed these results.

Tissues

The hexuronic acid content of the tissues of controls and of tilorone-treated animals is shown in Fig. 4. The treated rats exhibited hexuronic acid values 38-fold higher in the liver, 15-fold higher in the spleen and 5-fold higher in the kidney, as compared to tissues from control rats. Although there was an increase in the GAG content between the 11- and the 26-week treated rats, the difference was statistically not significant due to the small number of animals.

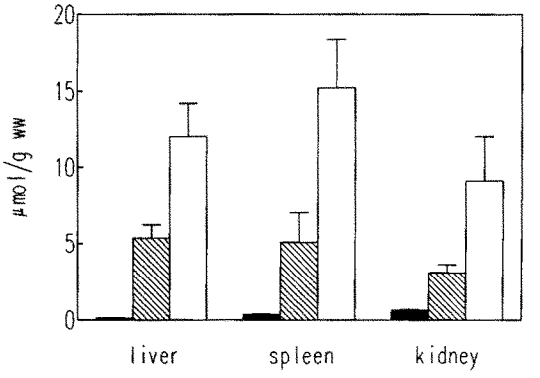

Fig. 4. Hexuronic acid and tilorone content of liver, spleen and kidney expressed as μmol per g wet weight for controls and tilorone-treated animals ($\bar{x} \pm$ SEM; N = 5). Solid bars, hexuronic acid content of control rats; hatched bars, hexuronic acid content of tilorone-treated animals; open bars, tilorone content.

Additionally, the tilorone content of the tissues of treated rats is demonstrated in Fig. 4; the highest tilorone accumulation could be found in liver and spleen.

The composition of tissue GAG was determined by electrophoresis in 0.1 M barium acetate and by susceptibility towards chondroitinases and nitrous acid. The complete procedure is exemplarily shown for the liver of tilorone-treated animals (Fig. 5). The liver of the control rats contained DS and HS. In

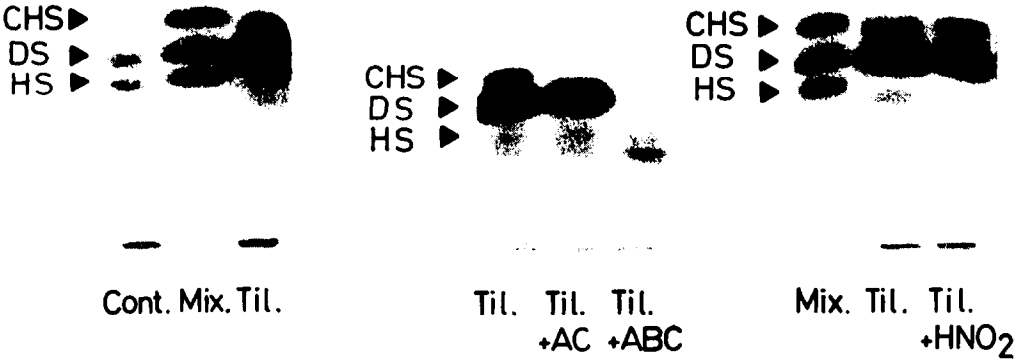

Fig. 5. Cellulose acetate electrophoresis of GAG extracts from the liver of controls (Cont.) and of tilorone-treated rats (Til.) together with a standard solution of CHS, DS and HS (Mix). The extracts of the tilorone-treated animals were incubated with enriched Tris buffer (Til.), chondroitinase AC (Til. + AC), chondroitinase ABC (Til. + ABC) or nitrous acid (Til. + HNO_2).

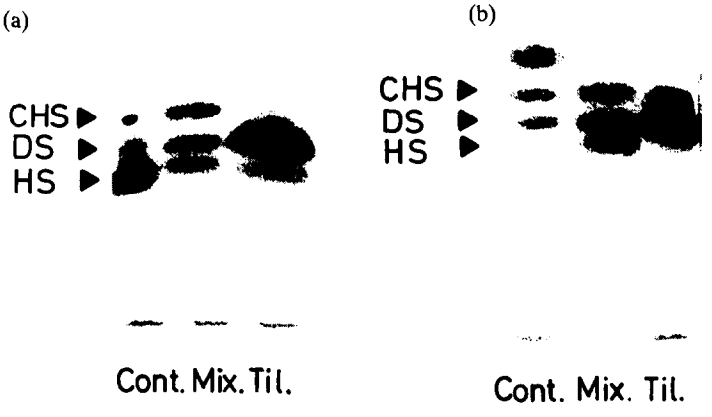

Fig. 6. Cellulose acetate electrophoresis of GAG extracts from (a) kidney and (b) spleen of controls (Cont.) and of tilorone-treated rats (Til.) together with a standard solution of CHS, DS and HS (Mix.).

the liver of the tilorone-treated rats DS was the major GAG besides smaller amounts of CHS and HS.

For kidney (Fig. 6a) and spleen (Fig. 6b) the electrophoretic patterns are shown before application of chondroitinase AC/ABC or nitrous acid. The tissues of control rats exhibited the following pattern: in the kidney (Fig. 6a), HS as the dominant substance besides smaller amounts of DS and traces of CHS and in the spleen (Fig. 6b) CHS and DS. An unidentified spot migrating faster than the other GAGs after electrophoretic separation and not susceptible to enzymatic or nitrous acid degradation occurred in the spleen extracts of controls.

With tilorone-treated rats the predominance of DS was evident for the kidney (Fig. 6a) and spleen (Fig. 6b). CHS and HS were present in smaller amounts.

DISCUSSION

These investigations present the biochemical correlate to the results obtained by histochemical and ultrastructural studies [2–4]: after chronic tilorone treatment a high level of acid GAGs could be detected in tissues and urine. The pattern of the stored GAGs—DS and smaller amounts of CHS—was independent of their physiological prevalence in the organs analysed. While the simultaneous storage of DS and CHS is different from all inherited mucopolysaccharidoses, the extent of GAG accumulation in the tissues and of excretion in the urine was comparable with inherited disorders [18–22]. In these diseases the GAG storage is caused by a defect of degrading enzymes. The drug-induced mucopolysaccharidosis is also related to an impaired disintegration of the GAGs; it has been shown in cultured human fibroblasts that the degradation but not the synthesis of [^{35}S]GAG is impaired by tilorone [23]. The mechanism by which tilorone interferes with the degradation of GAGs still remains unclear; in contrast to the inherited diseases the activity of the degrading enzymes was reported not to be reduced by tilorone (up to 100 μM) [23].

Tilorone is a dicationic amphiphilic drug which is accumulated by more than 1000-fold in the lysosomes in comparison with the extracellular medium [24, 25]. Such high drug concentrations in the lysosomes are the basis for the lipidosis described previously [26] and probably also for the GAG storage. In the

tissues examined, the molar ratios between tilorone and the disaccharid units of the GAGs were similar (Fig. 4). Making the simplifying assumption that tilorone is bound mainly to GAGs and only to a much lesser extent to other cellular constituents such as DNA or phospholipids, it can be calculated that one to two tilorone molecules are bound to every disaccharide unit of the GAGs. *In vitro* experiments have demonstrated that tilorone mixed with GAGs at similar molar ratios is able to precipitate GAGs (data not published). The formation of such insoluble and probably indigestible complexes between the substrate and catamphiphilic drugs has also been proposed to be the basis of the drug-induced lipidosis [26].

Since the accumulation of DS (and of smaller amounts of CHS) is independent of the GAG pattern which occurs under physiological conditions in the tissues, a specific inhibition of the degradation could be postulated, i.e. a selective formation of complexes between tilorone and DS, and tilorone and CHS. An interaction between tilorone and polyanionic macromolecules is described for DNA [27] where the drug is intercalated in the DNA helix. Similar helical structures are also described for the GAGs [28], and investigations with circular dichroism spectroscopy have shown that there are strong interactions between GAGs and tilorone [29]. Therefore it appears justifiable to assume that similar interactions between tilorone and GAGs may occur *in vivo*, too.

In this study biochemical evidence has been presented that mucopolysaccharidosis-related alterations can occur as a side effect of drugs. The underlying molecular mechanisms remain to be further elucidated.

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