Tilorone-induced lysosomal lesions: the bisbasic character of the drug is essential for its high potency to cause storage of sulphated glycosaminoglycans

Jens FISCHER*, Lutz HEIN*‡, Renate LÜLLMANN-RAUCH†§ and Burkhard VON WITZENDORFF†

*Department of Pharmacology, University of Kiel, Hospitalstrasse 4, 24105 Kiel, Germany

and †Department of Anatomy, University of Kiel, Olshausenstrasse 40, 24098 Kiel, Germany

The immunomodulatory agent tilorone {2,7-bis-[2-(diethylamino)ethoxy]fluoren-9-one} and congeners are potent inducers of lysosomal storage of sulphated glycosaminoglycans (GAGs) in animals and cultured fibroblasts of animals and man. All potent inducers of GAG storage hitherto described are bisbasic polycyclic aromatic compounds. They are accumulated in lysosomes and disturb the degradation of GAGs, mainly dermatan sulphate. It has been proposed that the drugs cross-link the polyanionic GAG chains giving rise to undegradable drug–GAG complexes. This hypothesis implies that the bisbasic character of the drug molecules is essential for the side effect in question. In the present study, this was tested by comparing tilorone and its monobasic derivative (MT) with respect to (i) induction of GAG storage in cultured bovine corneal fibroblasts and (ii) physicochemical interactions with GAGs *in vitro*. The intralysosomal concentration of MT achieved after 1–3 days was of the same order of magnitude as previously shown for tilorone. Nevertheless, under conditions that did not enhance the secretion

of a lysosomal enzyme (β -hexosaminidase, EC 3.2.1.52), the ability of MT to cause storage of [35 S]GAGs was significantly lower than that of tilorone. Morphological observations showed that MT was much more potent in causing lysosomal storage of polar lipids than of GAGs. CD spectroscopy with tilorone revealed that the presence of GAGs caused the primarily achiral drug molecules to display CD. This suggested a helical orientation of the tilorone molecules within GAG–drug complexes, and short intermolecular distances which allowed electronic coupling of the aromatic ring systems of adjacent drug molecules. In contrast, MT failed to display any induced optical activity, indicating the absence of highly ordered GAG–drug complexes. In conclusion, the present results show that the substitution of the planar aromatic ring system with two basic side chains is essential for the high potency of tilorone in inducing lysosomal GAG storage. This is paralleled by, and presumably causally related to, strong physicochemical interactions with GAGs.

INTRODUCTION

Dicationic amphiphilic compounds such as tilorone (Figure 1a) and congeners are accumulated in lysosomes, interfere with the lysosomal degradation of sulphated glycosaminoglycans (GAGs) and cause lysosomal storage of GAGs, predominantly dermatan sulphate [1–4]. Drug-induced GAG storage has been described in rats and cultured cells of rat, ox and man [1–7]. Its occurrence in

patients can be suspected on the basis of clinical reports about ocular side effects [8–10]. Tilorone and its bisbasic congeners are experimental immunomodulators and have antiviral activities [10].

All potent inducers of lysosomal GAG storage hitherto described are bisbasic compounds and consist of a planar aromatic ring system symmetrically substituted with two basic side chains. According to our working hypothesis [2,3] the disturbance of GAG degradation is due to the formation of complexes between the drugs and GAGs, which thereby become resistant to the lysosomal enzymes. On the basis of NMR spectroscopic results obtained with tilorone [3], it was proposed that the complexes are formed (i) by electrostatic interaction between the side chains of the drug and the GAGs such that the GAG chains are cross-linked and (ii) by intermolecular interactions between the planar ring systems of the drug leading to parallel stacking of the drug molecules within the complexes.

The hypothesis implies that the symmetrically bisbasic character of tilorone is essential for its high potency to induce GAG storage. If so, a monobasic molecule should be unable to disturb GAG degradation or at least should be less potent. This was tested in the present study by comparing tilorone and the monobasic tilorone analogue (MT) with respect to (i) the induction of GAG storage in cultured bovine corneal fibroblasts and (ii) physicochemical interactions with GAGs *in vitro*. MT, although having no immunomodulatory activities [11], was

Figure 1 Molecular structures of the investigated compounds

(a) Tilorone; (b) the monobasic tilorone derivative (MT); (c) Acridine Orange, which was used as a positive control for the CD spectroscopy.

Abbreviations used: GAG, sulphated glycosaminoglycans; MT, monobasic tilorone analogue.

‡ Present address: Division of Cardiovascular Medicine, Stanford University Medical School, Stanford, CA, U.S.A.

§ To whom correspondence should be addressed.

chosen because it differs from tilorone only by lacking a second diethylaminoethoxy side chain (Figure 1b). Parts of the present results have been reported in preliminary form [12,13].

The present study shows that the bisbasic structure of tilorone, as compared with the monobasic derivative, markedly increases its potency to induce GAG storage and to interact physico-chemically with GAGs *in vitro*. These findings should contribute to a better understanding of the pathomechanisms and structure–activity relationships, and may help to avoid this side effect in future drug development.

EXPERIMENTAL

Materials

Tilorone dihydrochloride was a gift from the Marion Merrell Dow Research Institute, Marion Merrell Dow Inc. (Cincinnati, OH, U.S.A.). Acridine Orange hydrochloride (dye content 98 %) was purchased from Aldrich (Steinheim, Germany) and used without further purification. MT was synthesized by the late Professor R. Haller (Department of Pharmaceutical Chemistry, University of Kiel) according to a published method [11]. The materials and media for cell culture work were the same as used previously [14]. $\text{Na}_2^{35}\text{SO}_4$ (specific radioactivity 10–100 mCi/mmol) was purchased from Amersham-Buchler (Braunschweig, Germany). Dermatan sulphate (porcine dermis), chondroitin 6-sulphate (whale cartilage), heparin (porcine intestinal mucosa) and all other chemicals were obtained from Sigma (München, Germany). Cuprolinic Blue was from BDH Chemicals (Poole, Dorset, U.K.).

Cell cultures and experimental conditions

Bovine fibroblasts were obtained from corneal explants as described previously [6]. The cells were propagated by standard techniques in Eagle's minimal essential medium with 10 % fetal calf serum (Boehringer, Mannheim, Germany). For the radiochemical experiments and those dealing with enzyme secretion, a low-sulphate medium was used in which MgCl_2 was substituted for MgSO_4 . All experiments were performed at a constant ratio of medium volume to surface area of the confluent cell layer (0.37 ml/cm^2). Exposure to the drug usually lasted 72 h, with no change of the culture medium. This time period was previously found to be most appropriate for inducing marked GAG storage with tilorone and its bisbasic analogues [7,14].

Determination of intralysosomal drug accumulation

The experiments were performed with an initial medium concentration of $5 \mu\text{M}$ MT. The disappearance of the drug from the medium was monitored [3]. At the indicated times, medium was removed and stored at -30°C until further analysis. In order to determine the proportion of drug that disappeared from the medium as the result of accumulation in acidic cellular compartments after 24 h, ammonia (10 mM) was applied together with MT. The proportion of drug that disappeared as the result of physicochemical adsorption to biomembranes and other cell constituents was determined by incubating non-viable cells (three freeze–thaw cycles); after incubation the cell fragments were removed by centrifugation ($115\,500 \text{ g}$; 20 min). Aliquots ($50 \mu\text{l}$) of the culture medium were analysed on a reversed-phase column (LiChrospher 60 RP-Select B; $5 \mu\text{m}$; $250 \text{ mm} \times 4 \text{ mm}$; Merck, Darmstadt, Germany) by HPLC. A UV detector (270 nm) was used for quantification. The mobile phase consisted of 20 mM phosphate buffer (pH 3.4) containing 30 % acetonitrile.

Enzyme assay

The culture medium was harvested. The cell layers were rinsed with PBS, scraped off and collected in distilled water. After freezing and thawing, the cellular material was subjected to ultrasonication. The activity of β -hexosaminidase (EC 3.2.1.52) was determined photometrically in medium and cell lysates as described previously [4], with 4-methylumbelliferyl *N*-acetyl- β -D-glucosaminide as substrate. To account for β -hexosaminidase activity contributed by the fetal calf serum in the medium, each experiment included two flasks containing medium without cells.

Determination of intracellular [^{35}S]GAG accumulation

The cell layers were incubated for 72 h with $\text{Na}_2^{35}\text{SO}_4$ ($0.5 \mu\text{Ci/ml}$) in the absence or presence of the drugs, and intracellular [^{35}S]GAGs were determined by standard techniques [15,16]. We are aware that this approach does not allow differentiation between free [^{35}S]GAG chains and those present as integral parts of proteoglycans. The radioactivity was related to cell protein [17].

CD spectroscopy

For determination of the interactions of Acridine Orange, tilorone and MT with GAGs, solutions containing $100 \mu\text{M}$ GAG and $10 \mu\text{M}$ drug were prepared in distilled water. GAG concentration is expressed in terms of the average molecular mass of the disaccharide units. At drug concentrations above $300 \mu\text{M}$ (Acridine Orange, tilorone), the mixtures were turbid owing to formation of water-insoluble drug–GAG complexes. Absorption spectra were recorded with a Beckman DB GT recording spectrophotometer. CD spectra were obtained with a Jasco J-40 A recording spectropolarimeter. The values of molar ellipticity were calculated on the basis of total drug concentration. For each type of solution a minimum of four independent preparations were investigated.

Histochemistry and electron microscopy

Histochemical staining of polysulphated storage material was performed using Cuprolinic Blue (0.05 %, pH 5.7, 0.3 M MgCl_2) [18]. Histochemical detection of polar lipid storage was achieved by staining with osmium tetroxide/tannic acid [19]. For ultrastructural examination, the cells were fixed with glutaraldehyde and osmium tetroxide and processed by standard methods as described previously [14].

RESULTS

Intralysosomal drug accumulation

The time course of lysosomal accumulation of MT was followed by monitoring the decline in drug concentration in the culture medium [3,20]. Lysosomotropic weak bases are known to be accumulated in acidic cellular compartments to such an extent that, under the standard conditions of cell culture work, the drug becomes significantly extracted from the medium [20,21].

When confluent cell layers were incubated with $5 \mu\text{M}$ MT, its concentration in the medium gradually declined, reaching a value of $2.4 \mu\text{M}$ after 24 h and $0.7 \mu\text{M}$ after 72 h (Figure 2). Since the cationic amphiphilic drug molecules could be expected also to become adsorbed to cell constituents such as biomembranes and DNA, control experiments using non-viable fibroblasts were performed. In this case the medium concentration had decreased to $3.25 \mu\text{M}$ after 24 h (Figure 2, inset) and to $3.0 \mu\text{M}$ after 72 h. These data indicate that (i) cell viability was a major prerequisite for drug extraction from the medium and (ii) adsorption to cell

Figure 2 Decline in drug concentrations in the culture medium

Confluent fibroblasts were incubated with MT at an initial medium concentration of 5 μ M. At the indicated times the medium concentrations were determined by HPLC. Previous data for tilorone [3] are included (---) to facilitate comparison. The inset shows the medium concentrations of MT at 24 h under standard conditions ($\square$); in the presence of 10 mM NH_4Cl ($\square$), after incubation with non-viable cells (three freeze-thaw cycles) ($\boxtimes$) and after incubation of medium in culture dishes without cells ($\boxplus$). Data represent means $\pm$ S.E.M. ($n = 4-9$).

fragments (biomembranes, DNA) and the plastic surfaces of the culture dish had nearly reached equilibrium after 24 h.

To test whether the difference between drug uptake by viable cells and adsorption to non-viable cells was due to trapping

Figure 3 Dose-response curve for the secretion of the lysosomal enzyme β -hexosaminidase

Confluent fibroblasts were incubated for 72 h, and the activities of β -hexosaminidase were determined in culture medium and cell lysate. Ordinate: enzyme secretion is defined as corresponding to the extracellular fraction of total enzyme activity present in the culture system; the extracellular fractions in drug-treated cultures ($\bullet$) are expressed as multiples of the values in controls ($\circ$). Abscissa: drug concentration on a log scale. Each point represents the mean $\pm$ S.E.M. from five separate experiments ($n = 4-13$).

Figure 4 Comparison of the effects of MT and tilorone on [^{35}S]GAG accumulation

Confluent fibroblasts were incubated for 72 h with $\text{Na}_2^{35}\text{SO}_4$ (0.5 $\mu\text{Ci}/\text{ml}$) in the absence (A) or presence of drugs: (B) MT (17.5 μM); (C) tilorone (1.75 μM). The intracellular [^{35}S]GAG contents were determined by standard methods [15,16]. In control cells they were 4680 ± 160 c.p.m./mg of protein. The results represent means $\pm$ S.E.M. from three experiments ($n = 6$).

within acidic cellular compartments, viable cells were exposed to 5 μM MT in the presence of 10 mM NH_4Cl for 24 h. Ammonia is rapidly accumulated in acidic organelles, thereby increasing the pH [22] and abolishing the accumulation of other lysosomotropic agents [20]. Under these conditions, drug extraction from the medium resulted in a concentration of 3 μM (Figure 2, inset), thus hardly exceeding extraction due to adsorption to non-viable cells. The result indicates that extraction of the drug from the medium under standard conditions minus that observed with non-viable cells was for the most part due to entrapment of the drug in acidic cellular compartments. Assuming a cellular volume of 0.2 $\mu\text{l}/\text{cm}^2$ of cell layer surface [3] and a lysosomal fraction of 10% of the cellular volume [23], the intralysosomal concentration of MT can be estimated to be at least 10 mM after 24 h of exposure. For tilorone an intralysosomal concentration of approximately 50 mM was previously estimated under identical experimental conditions [3].

Concerning the time course of accumulation, there was a remarkable difference between MT and tilorone, the curve for which [3] is included in Figure 2 for comparison. Tilorone accumulation proceeds faster and in a biphasic manner.

Dose-response curve for lysosomal enzyme secretion

As explained in the Discussion, it was essential for our present purposes to compare the drug effects on GAG storage at concentrations that did not enhance the physiological secretion of lysosomal enzymes. β -Hexosaminidase was selected as a representative of the soluble lysosomal enzymes. A dose-response curve (Figure 3) was established describing the effects of MT on enzyme release, which was defined as corresponding to the extracellular fraction of the total enzyme activity in the culture system. In control cultures the extracellular fraction amounted to $42 \pm 1.5\%$. In cultures exposed to low MT concentrations (10–17.5 μM) the extracellular fraction was smaller than in controls although the absolute values of enzyme activity in the medium remained unchanged. This result was due to an increase in intracellular enzyme activity (results not shown) which thus made a larger contribution to the total activity. Similar results were previously obtained with low tilorone concentrations [4]. At

Figure 5 Absorption and CD spectra

Top, absorption spectra of aqueous solutions containing (a) one of the investigated GAGs (100 μ M, in terms of the average molecular mass of the disaccharide units) (----, dermatan sulphate; ····, heparin; —, chondroitin 6-sulphate) or (b–d) one of the indicated drugs (10 μ M). Bottom, (e) CD spectra of aqueous solutions containing either one of the investigated GAGs (lower curves) or one of the drugs (upper curves): —, tilorone; ····, Acridine Orange; ----, MT. (f–h) CD spectra of aqueous solutions containing one of the indicated drugs plus one of the GAGs.

MT concentrations above 17.5 μ M, the extracellular enzyme fraction increased, indicating enhanced enzyme release. Tilorone was previously found to enhance enzyme secretion at concentrations above 5 μ M [4].

Induction of [35 S]GAG storage

Exposure (72 h) to 10 μ M MT was without effect on intracellular [35 S]GAG content (results not shown). Since the lysosomal accumulation of MT was significantly slower than that of tilorone (Figure 2), it might be suspected that a period of 72 h was too short. Therefore the exposure to MT (10 μ M) was prolonged to 96 h. Nevertheless, the intracellular [35 S]GAG content expressed as a multiple of the control level was no more than 1.1 ± 0.04 (mean $\pm$ S.E.M.; $n = 4$). As a positive control, tilorone (5 μ M) was included in this experiment (exposure for 60 h only); here, the [35 S]GAG content was increased by a factor of 4.1 ± 0.34 ($n = 4$).

To compare the drug effects after equal exposure times (72 h), we chose 17.5 μ M MT and 1.75 μ M tilorone. The results are shown in Figure 4. MT caused an increase in intracellular [35 S]GAG content by a factor of 1.73, whereas tilorone induced a 3-fold elevation.

CD spectroscopy

CD spectroscopy was used to search for physicochemical interactions between the drugs and GAGs *in vitro*. Since cultured bovine corneal fibroblasts have been previously shown to syn-

thesize dermatan sulphate, chondroitin sulphate and heparan sulphate [3], these GAGs were also employed here, except that heparin was used instead of heparan sulphate. As a positive control, Acridine Orange (Figure 1c) was included. This bisbasic dye is known to interact with GAGs *in vitro* [24–27] and to induce lysosomal GAG storage in cultured fibroblasts [28].

The GAGs investigated did not absorb UV light (Figure 5a), whereas the tricyclic aromatic ring systems of the drugs investigated displayed strong absorption in the UV region with maxima between 260 and 270 nm (Figures 5b–5d). On recording CD spectra, no optical activity could be demonstrated in the UV region when only one of the investigated compounds was present in the solution (Figure 5e). Quite different results were obtained with solutions containing the bisbasic compounds together with the GAGs (Figures 5f–5g). Characteristic biphasic CD bands were observed, which were centred at wavelengths near to the absorption maxima of the dye and drug respectively. As to Acridine Orange, this induced CD has been assigned to the formation of helically ordered complexes between the dye and GAGs resulting in stacking and thus electronic coupling of the chromophores of adjacent dye molecules [25,26,29]. As another control, spectra were recorded for tilorone in the presence of a bivalent cation that is known to compete with cationic dyes or drugs for the binding sites on GAGs, thus disturbing complex-formation [30]. In the presence of 5 mM Mg^{2+} the CD signal was significantly diminished (results not shown). In contrast with tilorone, MT failed to show any induced optical activity, irrespective of whether it was used at a concentration of 10 μ M (Figure 5h) or 100 μ M (not shown).

Figure 6 Histochemical staining demonstrating GAG storage and lipidosis

Confluent fibroblasts were exposed for 72 h to (a, c) 17.5 μ M MT or (b, d) 1.75 μ M tilorone. Cells in (a) and (b) are stained with Cuprolinic Blue to demonstrate sulphated GAGs [18], those in (c) and (d) with osmium tetroxide/tannic acid to demonstrate polar lipids [19]. In (a) there is no staining reaction, whereas in (c) there is marked lipidosis as indicated by the intensely stained inclusions in the perinuclear cytoplasm of all cells. In (b) Cuprolinic Blue-positive inclusions are clearly seen; in (d) moderate lipidosis is observed in a few cells (right upper corner), while the other cells resemble controls (not shown). N, nucleus. Bars, 20 μ m.

Morphological observations

A histochemical hallmark of lysosomal GAG storage is the occurrence of cytoplasmic inclusions selectively stainable with tetracationic dyes such as Cuprolinic Blue [18,30]. After exposure to MT (17.5 μ M), the cytoplasm of some cells displayed minute metachromatically staining particles detectable in the original preparations but too small to be visible in the photomicrographs (Figure 6a). Tilorone (1.75 μ M), on the other hand, caused Cuprolinic Blue-positive inclusions which were clearly visible in a proportion of cells (Figure 6b). Since MT, on the basis of its chemical structure, was suspected of causing lysosomal storage of polar lipids (lipidosis) [31,32], an osmium tetroxide/tannic acid stain [19] was employed for histochemical detection of corresponding lesions. MT caused numerous inclusions in all cells (Figure 6c), whereas tilorone induced moderate lipidosis in some cells only (Figure 6d).

Figure 7 Ultrastructure of the drug-induced lysosomal lesions

Fibroblasts from the same experiment as in Figure 6. (a) Two normal lysosomes (Lys) of a control cell. (b) MT (17.5 μ M); lamellated inclusion bodies typical of lysosomal storage of polar lipids. (c) Tilorone (1.75 μ M); lysosomes (arrows) with small amounts of floccular material, m, mitochondrion; rER, rough endoplasmic reticulum. Bar, 0.5 μ m.

Ultrastructurally, all cells exposed to MT (17.5 μ M) displayed numerous lamellated inclusions (Figure 7b) which are known to be the ultrastructural hallmarks of lipidosis [32]; morphological counterparts of lysosomal GAG storage could not be detected. MT caused marked lipidosis even at the lowest concentration tested (5 μ M). After treatment with tilorone (1.75 μ M), the lysosomes were changed into vacuoles harbouring small amounts of floccular material (Figure 7c). This appearance has been shown previously to represent the ultrastructural counterpart of lysosomal GAG storage [6,28].

DISCUSSION

The aim of the current investigation was to contribute to the understanding of drug-induced lysosomal GAG storage, since this drug effect is likely to occur in man [8–10]. The aim was to determine whether the bisbasic character of the tilorone molecule is significant in its high potency to cause GAG storage. For the comparison between tilorone and its monobasic analogue (MT) two experimental pitfalls had to be excluded: (i) lower intralysosomal concentrations of MT compared with tilorone; (ii) enhancement of lysosomal enzyme secretion as an indication of general perturbation of the endosomal/lysosomal system [33,34]. In cultured cells, all lysosomotropic bases including tilorone can interfere with the mannose-6-phosphate-receptor-mediated targeting of newly synthesized enzymes from the Golgi apparatus to the lysosome, provided that the agents are applied at sufficiently high concentrations [33,35]. The lysosomes become cut off from the supply of new enzymes and, as a result, various types of non-degraded macromolecules pile up [34]. This rather unspecific lysosomal storage has to be distinguished from the GAG storage that occurs at lower drug concentrations that do not enhance secretion of lysosomal enzyme [4].

Lysosomal drug accumulation

Theoretical calculations [36] predict that the intralysosomal accumulation of a bisbasic compound is higher than that of a monobasic compound by the power of two. This prediction is based on several assumptions [36], one of which is that lysosomotropic compounds do not bind to substrates such as polar lipids, proteins or carbohydrates within the lysosomes. With the drugs under study, this prediction probably does not hold true since the cationic amphiphilic molecules of tilorone and MT can be assumed to bind to GAGs and polar lipids respectively [31] and are thus removed from the equilibrium to different degrees. The present and previous [3] estimations of intralysosomal drug concentrations show that, on exposure to an initial medium concentration of 5 μ M, tilorone and MT differed only by a factor of 5 after 24 h. The difference decreased further with longer incubation periods, since the concentration of both drugs in the culture medium decreased to the same value (0.7 μ M) after 72 h.

Tilorone and MT were markedly different with respect to the time course of accumulation. The initially rapid cellular uptake of tilorone closely resembles that of chloroquine [23] and is mainly a result of entrapment in acidic cellular organelles [3]. The slower accumulation of MT may be due to the fact that its adsorption to cellular constituents such as biomembranes (Figure 2, inset) is stronger than that of tilorone [3]. This may function as a buffer delaying the trapping within the acidic organelles.

Induction of GAG storage

To compensate for the lower lysosomal accumulation of MT, we

used a tenfold higher medium concentration of MT than of tilorone (17.5 μM versus 1.75 μM) for the comparative experiments. Even if tilorone, under the given culture conditions, were totally extracted from the medium and were accumulated exclusively in the lysosomes, a lysosomal concentration not higher than 3 mM would be reached. This assumed concentration is just one-third of that estimated in this study for MT (24 h) at an initial medium concentration of 5 μM . Therefore the low [^{35}S]GAG accumulation after 72 h exposure even to 17.5 μM MT strongly suggests that this compound indeed has a much smaller ability to disturb lysosomal GAG degradation. The radiochemical findings were confirmed by morphological observations. One might argue further that the lower potency of MT might be due to its slower intralysosomal accumulation, which would shorten the time available for the drug to disturb the lysosomal GAG catabolism. This explanation was disproved by the finding that prolonged exposure (96 h) to MT (10 μM) failed to augment the intracellular [^{35}S]GAG contents over control levels.

Drug-GAG interaction as a possible basis for the induction of GAG storage

Previous NMR studies have shown that tilorone and GAGs form complexes in the millimolar range, with dermatan sulphate being the preferred binding partner [3]. Since GAGs are known to adopt a helical conformation in aqueous solution [37–39], CD spectroscopy was performed to compare tilorone and MT with respect to their interaction with GAGs. The present CD spectra indicate that, in the investigated concentration range, tilorone, but not MT, strongly interacts with the GAGs. Since coupling of aromatic π -electron systems of adjacent drug molecules is a prerequisite for the induced optical activity, the results indicate that the fluorenone nuclei of tilorone closely approximate to each other within the drug-GAG complexes. This in turn may be a consequence of electrostatic binding of the dicationic tilorone molecules to sulphate and carboxylate groups, whereby cross-bridges between two GAG helices are formed. This would be in accordance with previous NMR findings which also led to the conclusion that tilorone molecules cross-link at least two GAG chains [3]. The substitution of the aromatic ring system with two basic side chains largely favours the formation of cross-links. In contrast, MT may be unable to bridge the gap between two GAG helices and therefore failed in the present study to exhibit induced optical activity in the presence of GAGs.

The present results do not exclude the possibility that MT undergoes electrostatic interactions with GAGs as it has also been shown *in vitro* for other monocationic drugs such as chlorpromazine [40]. According to the CD results, however, this would not lead to highly ordered complexes as it does with tilorone. Electrostatic interactions between MT and GAGs may also have some impact on GAG degradation and may be the basis for the low GAG-storage-inducing potency. In contrast, the lipodosis-inducing ability of MT was much higher, as judged from morphological observations. These findings are compatible with a previously proposed mechanism [31,41]: the cationic amphiphilic drug molecules are thought to form complexes with the likewise amphiphilic polar lipids by electrostatic and hydrophobic forces and thereby disturb enzymic lipid degradation. For such interactions the monosubstituted aromatic ring system may be more favourable than the disubstituted ring system of tilorone, where the second side chain in its charged form may impede interaction between the aromatic ring system and the apolar lipid moieties.

In conclusion, substituting an aromatic ring system with two basic side chains, as opposed to one, markedly enhances its potency to induce lysosomal GAG storage. This is paralleled by, and perhaps causally related to, strong physicochemical interaction with GAGs.

The excellent technical assistance of Dagmar Niemeier, Ursula Prange, Sonja Seiter, Heide Siebke, Heidi Waluk, and Clemens Franke is gratefully acknowledged. This investigation was financially supported by the Deutsche Forschungsgemeinschaft (grant no. Lu 172/7-3).

REFERENCES

- Prokopek, M. (1991) *Biochem. Pharmacol.* **42**, 2187–2191
- Grave, S., Lüllmann, H., Lüllmann-Rauch, R., Osterkamp, G. and Prokopek, M. (1992) *Toxicol. Appl. Pharmacol.* **114**, 215–224
- Fischer, J. (1995) *Biochem. J.* **312**, 215–222
- Lüllmann-Rauch, R., Pods, R. and von Witzendorf, B. (1995) *Biochem. Pharmacol.* **49**, 1223–1233
- Hein, L. and Lüllmann-Rauch, R. (1989) *Toxicology* **58**, 145–154
- Lüllmann-Rauch, R. and Ziegenhagen, M. (1991) *Virchows Arch. B Cell Pathol.* **60**, 99–104
- Handrock, K., Lüllmann-Rauch, R. and Vogt, R. D. (1993) *Toxicology* **85**, 199–213
- Weiss, J. N., Weinberg, R. S. and Regelson, W. (1980) *Am. J. Ophthalmol.* **89**, 46–53
- Weiss, J. N., Ochs, A. L., Abedi, S. and Selhorst, J. B. (1980) *Am. J. Ophthalmol.* **90**, 846–853
- Regelson, W. (1981) *Pharmacol. Ther.* **15**, 1–44
- Andrews, E. R., Fleming, R. W., Grisar, J. M., Kihm, J. C., Wenstrup, D. L. and Mayer, G. D. (1974) *J. Med. Chem.* **17**, 882–886
- Handrock, K., Hein, L. and Lüllmann-Rauch, R. (1989) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **339** (Suppl.), R8
- Hein, L., Lüllmann-Rauch, R. and Mohr, K. (1990) *Xenobiotica* **20**, 1259–1267
- Burmester, J., Handrock, K. and Lüllmann-Rauch, R. (1990) *Arch. Toxicol.* **64**, 291–298
- Fratantoni, J. C., Hall, C. W. and Neufeld, E. F. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **60**, 699–706
- Cantz, M., Kresse, H., Barton, R. W. and Neufeld, E. F. (1972) *Methods Enzymol.* **28**, 884–897
- Fanger, B. O. (1981) *Anal. Biochem.* **162**, 11–17
- Scott, J. E., Orford, C. R. and Hughes, E. W. (1981) *Biochem. J.* **195**, 573–581
- Mason, R. J., Walker, S. R., Shields, B. A., Henson, J. E. and Williams, M. C. (1985) *Am. Rev. Respir. Dis.* **131**, 786–788
- MacIntyre, A. C. and Cutler, D. J. (1988) *J. Pharm. Sci.* **77**, 196–199
- Robbins, E. and Marcus, P. I. (1963) *J. Cell Biol.* **18**, 237–250
- Ohkuma, S. and Poole, B. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3327–3331
- Wibo, M. and Poole, B. (1974) *J. Cell Biol.* **63**, 430–440
- Stone, A. and Bradley, D. F. (1967) *Biochim. Biophys. Acta* **148**, 172–192
- Salter, M. K., Rippon, W. B. and Abrahamson, E. W. (1976) *Biopolymers* **15**, 1213–1227
- Salter, M. K., Abrahamson, E. W. and Rippon, W. B. (1976) *Biopolymers* **15**, 1251–1265
- Menter, J. M., Hurst, R. E. and West, S. S. (1977) *Biopolymers* **16**, 695–702
- Lüllmann-Rauch, R. and Ziegenhagen, M. (1991) *Histochemistry* **95**, 263–268
- Chakrabarti, B. and Balazs, E. A. (1973) *Biochem. Biophys. Res. Commun.* **52**, 1170–1176
- Scott, J. E. and Dorling, J. (1965) *Histochemie* **5**, 221–233
- Lüllmann, H., Lüllmann-Rauch, R. and Wassermann, O. (1978) *Biochem. Pharmacol.* **27**, 1103–1108
- Lüllmann-Rauch, R. (1979) in *Lysosomes in Applied Biology and Therapeutics* (Dingle, J. T., Jacques, P. J. and Shaw, I. H., eds.), vol. 6, pp. 49–130, North-Holland, Amsterdam
- Creek, K. E. and Sly, W. S. (1984) in *Lysosomes in Biology and Pathology* (Dingle, J. T., Dean, R. T. and Sly, W., eds.), vol. 7, pp. 63–82, Elsevier, Amsterdam
- Seglen, P. O. (1983) *Methods Enzymol.* **96**, 737–764
- Gupta, D. K., Gieselmann, V., Hasilik, A. and von Figura, K. (1984) *Hoppe Seyler's Z. Physiol. Chem.* **365**, 859–866
- MacIntyre, A. C. and Cutler, D. J. (1988) *Biopharm. Drug Dispos.* **9**, 513–526
- Scott, J. E. (1992) *FASEB J.* **6**, 2639–2645

-
- 38 Mulloy, B., Forster, M. J., Jones, C. and Davies, D. B. (1993) *Biochem. J.* **293**, 849–858
- 39 Mulloy, B., Forster, M. J., Jones, C., Drake, A. F., Johnson, E. A. and Davies, D. B. (1994) *Carbohydr. Res.* **255**, 1–26
- 40 Larsson, B., Nilsson, M. and Tjälve, H. (1981) *Biochem. Pharmacol.* **30**, 2963–2970
- 41 Mingeot-Leclercq, M. P., Schanck, A., Ronveaux-Dupal, M. F., Deleers, M., Brasseur, R., Ruysschaert, J. M., Laurent, G. and Tulkens, P. M. (1989) *Biochem. Pharmacol.* **38**, 729–741

Received 7 September 1995/20 November 1995; accepted 11 December 1995