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TILORONE-INDUCED LYSOSOMAL STORAGE OF SULPHATED GLYCOSAMINOGLYCANS CAN BE SEPARATED FROM TILORONE-INDUCED ENHANCEMENT OF LYSOSOMAL ENZYME SECRETION

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Abstract—This investigation deals with a drug side-effect. The immunomodulatory drug tilorone (2,7-bis[2-(diethylamino)ethoxy]fluoren-9-one) and congeners induce lysosomal storage of sulphated glycosaminoglycans (GAGs) in animals and in cultured cells. At high tilorone concentrations, GAG storage in cultured fibroblasts was previously reported to be accompanied, and presumably caused by, disturbance of intracellular targeting of lysosomal enzyme precursors, which leads to enhanced secretion and thus loss of lysosomal enzymes. The purpose of the present study was to examine whether the GAG storage induced in cultured bovine fibroblasts by low tilorone concentrations is also accompanied by enhanced lysosomal enzyme release. Enhanced secretion of β-hexosaminidase (EC 3.2.1.52) was taken as indicating the intracellular mistargeting of lysosomal enzyme precursors. Dose–response curves were established for (a) the intracellular accumulation of ^{35}S-GAGs and (b) the release of β-hexosaminidase after exposure (72 hr) to tilorone (1–35 μM). For positive controls, the classical lysosomotropic agents NH_4Cl (1–30 mM) and chloroquine (1–60 μM) were used. With NH_4Cl, ^{35}S-GAG storage was accompanied by enhanced enzyme release throughout the concentration range (EC_{50} at 3.3 mM for either effect). With chloroquine, low concentrations (≤5 μM) caused a small increase in ^{35}S-GAG accumulation without abnormal enzyme secretion; at higher concentrations both drug effects were produced (EC_{50} around 15 μM for either effect). With tilorone, low concentrations (≤5 μM) caused marked ^{35}S-GAG accumulation without enhancement of enzyme release. The EC_{50} for tilorone-induced ^{35}S-GAG storage was 3 μM, as opposed to 15 μM for enzyme release. The results indicate that GAG storage induced by low concentrations of tilorone is due to mechanisms other than mistargeting and loss of lysosomal enzymes. On the basis of previous results it may be hypothesized that tilorone and other symmetrically substituted dicationic compounds form complexes with the polyanionic GAG chains and thereby impair their enzymic degradation.

Key words: chloroquine; dicationic amphiphilic drugs; glycosaminoglycans, sulphated; lysosomal storage diseases, drug-induced; lysosomotropic agents; tilorone

Several symmetrically substituted dicationic amphiphilic compounds—standard compound tilorone (Fig. 1)—are potent inducers of lysosomal storage of sulphated glycosaminoglycans (GAGs†) in intact rats [1, 2] and in cultured fibroblasts of rat [3–5], ox and man [6]. As to their therapeutic action, the compounds are experimental immunomodulatory drugs [7]. The morphological alterations induced by these drugs closely resemble those of inherited lysosomal GAG storage diseases (mucopolysaccharidoses) [8]. Biochemically, GAG contents in several tissues of chronically treated rats and cultured cells were significantly increased, with dermatan sulphate the major contributor [2, 9–11].

GAG storage seems to be due to impairment of lysosomal GAG degradation [12], although the underlying mechanisms are not yet clear. Tilorone was shown to act as a lysosomotropic weak base

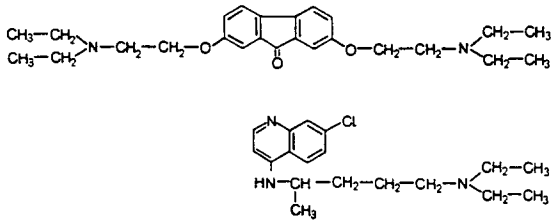

Fig. 1. Molecular structures of tilorone (top) and chloroquine (bottom).

[12]: high medium concentrations of the drug increased the pH in isolated lysosomes and enhanced the secretion of newly synthesized lysosomal enzymes from cultured fibroblasts by diverting the enzyme precursors from the lysosomal to the secretory pathway. Thus the cells became deprived of lysosomal enzymes. It was suggested that the demonstrated effects of tilorone—elevation of the pH in acid cell organelles, lysosomal enzyme

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† Abbreviations: GAG, sulphated glycosaminoglycan; FCS, foetal calf serum; MEM, minimal essential medium; PBS, phosphate-buffered saline.

secretion and thus depletion, GAG storage—were causally related [12]. This explanation seems plausible for high drug concentrations, since enzyme deprivation does lead to accumulation of the respective substrates. However, there are other observations which are difficult to reconcile with the proposed causal relationship as the only explanation for drug-induced GAG storage. One is the high tilorone concentration ($\geq 20\ \mu M$) necessary for consistently producing enzyme secretion [12], whereas the cytological lesions indicating GAG storage can be elicited in cultured fibroblasts by concentrations of $\leq 1\ \mu M$ [5]. The other is the prolonged persistence of GAG storage after the end of drug administration (>7 days in cultured cells; >28 weeks in intact rats) [13, 14]. This contrasts with reports that the disturbances of both lysosomal acidification and intracellular targeting of lysosomal enzymes are reversible shortly after withdrawal of the weak base [15–17].

The purpose of the present study on bovine corneal fibroblasts was to examine whether tilorone-induced GAG storage is accompanied by enhanced lysosomal enzyme secretion at low drug concentrations as well. Enzyme secretion was taken as indicative of intracellular mistargeting of newly synthesized lysosomal enzymes [12, 18, 19]. Dose–response curves were established for (a) tilorone-induced ^{35}S-GAG storage, and (b) tilorone-induced secretion of a lysosomal enzyme. β-Hexosaminidase (β-*N*-acetyl-D-hexosaminidase, EC 3.2.1.52) was chosen as representative of soluble lysosomal enzymes whose intracellular transport is dependent on the mannose-6-phosphate receptor mechanism [18, 19]. Its mistargeting and secretion in the presence of tilorone has been analysed in detail [12]. The classical lysosomotropic weak bases chloroquine (Fig. 1) and ammonia served as positive controls. Rather than investigating yet another lysosomotropic agent for its own sake, we undertook the present study with the aim to contribute towards the understanding of the pathomechanisms of the mucopolysaccharidosis induced by dicationic amphiphilic drugs, because this side-effect occurs in intact organisms and may also have significance for applied medicine [13, 20]. For the extrapolation from cell cultures onto intact organisms low drug concentrations ($\leq 5\ \mu M$) are presumably more relevant than higher ones (see the Discussion); therefore, it appeared of importance to examine the relationship between GAG storage and enzyme secretion particularly in the lower concentration range. Preliminary results have been published in abstract form [21].

MATERIALS AND METHODS

Materials

Tilorone-dihydrochloride was a gift of the Merrell Dow Research Institute (Cincinnati, OH, USA). Chloroquine-diphosphate and ammonium chloride were purchased from Sigma (Munich, Germany). $Na_2{}^{35}SO_4$ (specific activity 50–80 mCi/mmol) was obtained from Amersham-Buchler (Braunschweig, Germany). The materials used for cell culture were the same as described earlier [3]. FCS was purchased from Boehringer (Mannheim, Germany) and Sigma (Munich, Germany).

Cell cultures

Fibroblasts were obtained from explants of bovine cornea and propagated as previously described [6]. The cells were cultivated in Eagle's MEM supplemented with 10% FCS, non-essential amino acids, penicillin (100 IU/mL) and streptomycin sulphate (100 μg/mL). The cultures were kept at 37° in a humidified atmosphere containing 5.8% CO_2. For subcultures (split ratio 1:3) the cells were harvested with trypsin (10 mg/mL) following the procedure of Freshney [22]. The experiments were performed on cells of the 3 to 10 subculture. Since the radiochemical studies required a low-sulphate medium, the MEM for experiments was modified by substituting $MgCl_2$ for $MgSO_4$. The inorganic sulphate concentration was approximately 0.4 mM derived from the streptomycin sulphate and the FCS [23]. The sodium bicarbonate concentration was 1.6 g/L [23, 24]. This medium was used throughout the biochemical and radiochemical experiments, starting with the plating of the cells for a given experiment. We decided not to work with serum-free medium, since this led to dramatic changes in cell morphology during the experimental period of 72 hr and since cell viability was greatly reduced in the presence of higher drug concentrations. For enzyme studies and radiochemical experiments, the cells were seeded into 25 cm^2 plastic flasks (approx. 2.5×10^4 cells/cm^2). For morphological studies the cells were seeded into 35 mm plastic culture dishes. For light microscopic investigations, the cells were grown on glass cover slips while for electron microscopic evaluation the cells grown on the plastic surface were used.

Experimental conditions

When the cells had just reached confluency, the experiment was started by supplying them with fresh medium containing the drugs. The cells were exposed for 72 hr unless stated otherwise, the medium remaining unchanged during this period. An experimental period of 72 hr was previously found most appropriate for obtaining distinct mucopolysaccharidosis-like cytological alterations [3]. The pH of the medium was kept between 7.1 and 7.2. The ratio between medium volume and surface of cell layer was 0.37 mL/cm^2 throughout the experiments.

Radiochemical experiments

Determination of intracellular ^{35}S-GAGs. Fibroblasts were incubated with $Na_2{}^{35}SO_4$ under various conditions as described below. At the end of the experiments the cells were processed for determination of intracellular ^{35}S-GAG according to a standard method [23, 25]. The cell layers were thoroughly washed with PBS and harvested by means of 3 mL PBS containing trypsin (1 mg/mL, 37°, 10 min). This procedure simultaneously detaches the cells from the culture plate and removes the pericellular pool of ^{35}S-proteoglycans from the cell surface [26, 27]. After centrifugation the cell pellets were boiled four times with 80% ethanol in order

Table 1. Relative reduction of cell densities in drug-treated cultures (% of controls)

Drug (concentration)	Tilorone (μM)	Chloroquine (μM)	NH_4Cl (mM)
0	100	100	100
5	94 ± 2	98	NT
6	NT	NT	88 ± 6
10	94 ± 3	91 ± 1.5	79 ± 6
25	89 ± 2	NT	NT
30	NT	NT	72 ± 4
35	78 ± 3	81 ± 4	NT
60	NT	65 ± 15	NT

Confluent cultures of bovine fibroblasts were exposed for 72 hr to the drugs at the indicated concentrations. Thereafter the cells were harvested by means of trypsin and counted. In control cultures cell density at the end of the experiments was approximately 1×10^5 cells/cm^2 of growth surface. The values represent means ± SEM from 2–3 experiments (N = 4–6). NT, not tested.

to separate inorganic ^{35}S-sulphate from ethanol-insoluble ^{35}S-GAGs [25, 28]. Radioactivity was measured in the ethanol-precipitable residues. For the present purpose, no attempts were made to differentiate between free ^{35}S-GAG chains and those integrated into proteoglycans.

Disappearance of intracellular ^{35}S-GAGs from fibroblasts. The effect of tilorone upon GAG degradation was estimated by determining the rate of disappearance of ^{35}S-GAGs from fibroblasts metabolically labelled with $Na_2{}^{35}SO_4$ (2 μCi/mL). After the labelling period of 48 hr, cells were processed to measure the intracellular ^{35}S-GAGs as described above. The obtained data were designated as zero time values. The remaining cell layers were washed three times with PBS and exposed to trypsin (100 μg/mL, 37°, 10 min) in serum-free medium in order to remove as many of the pericellular ^{35}S-proteoglycans as possible [26, 27] without detaching the cells from the growth surface. After removal of the trypsin solution, the cell layers were washed twice with regular medium and supplied with fresh isotope-free medium which contained 5 μM tilorone. Controls were run without the drug. The cells were processed for determination of intracellular ^{35}S-GAGs 48 hr after the end of the labelling phase.

Intracellular accumulation of ^{35}S-GAGs. Fibroblasts were incubated in the presence or absence of drugs with medium containing $Na_2{}^{35}SO_4$ (1 μCi/mL) for 72 hr and processed for determination of intracellular ^{35}S-GAGs. It has been shown [25] that in normal fibroblasts incubated with ^{35}S-sulphate for longer than 24 hr, the intracellular accumulation of labelled GAGs is counterbalanced by degradation plus secretion and thus reaches a plateau. If degradation is impaired, however, the curve of ^{35}S-GAG accumulation continues to rise. Such time-dependent abnormal ^{35}S-GAG accumulation has been demonstrated to occur with tilorone [12]. In the present study we measured ^{35}S-GAG accumulation attained after 72 hr of drug exposure and used the results to establish dose–response curves.

Enzyme assay

The medium was collected at the end of drug exposure. Each experiment included a sample of culture medium without cells, because FCS was found to cause some background enzyme activity which was subtracted from that due to the presence of cells. The cell layers were rinsed by three changes of PBS, lysed with distilled water, scraped off with a rubber 'policeman' and collected in a total volume of 2.7 mL distilled water. The samples were stored at −20°. The cellular material was sonicated on ice for 30 sec (13.5 kHz/sec) with a microultrasonic cell disruptor (Kontes, Vineland, U.S.A.). The activity of β-hexosaminidase was determined in culture medium and cell lysate [29] using 4-methyl-umbelliferyl-*N*-acetyl-β-D-glucosaminide (Sigma) as substrate. The split product was determined photometrically at 360 nm.

Reference basis for the original data

The original data could not be related to cell protein nor to DNA for the following reasons. Cellular protein content [30] was increased in cultures exposed to higher drug concentrations (not shown). The fluorometric determination of DNA, using either 3,5-diaminobenzoic acid [31] or ethidium bromide [32], was disturbed by higher concentrations of tilorone and chloroquine (not shown). The radiochemical data obtained for drug-treated cultures are presented as multiples of the values from control cultures of a given experiment, after correction for differences in cell densities (see Table 1). The data from enzyme measurements are presented as total activity per culture (cells plus medium) or fractions thereof, again after correction for cell densities. Since the fibroblasts continued to proliferate during the experimental period of 72 hr despite having reached confluency [22, 33], we attempted to determine whether the cell densities of the drug-treated cultures differed from those of controls. Two or three representative experiments were performed in parallel with biochemical or radiochemical experiments for each drug. The cells were harvested with trypsin (1 mg/mL) and counted in a haemocytometer.

Drug concentrations

The concentrations of each agent were chosen

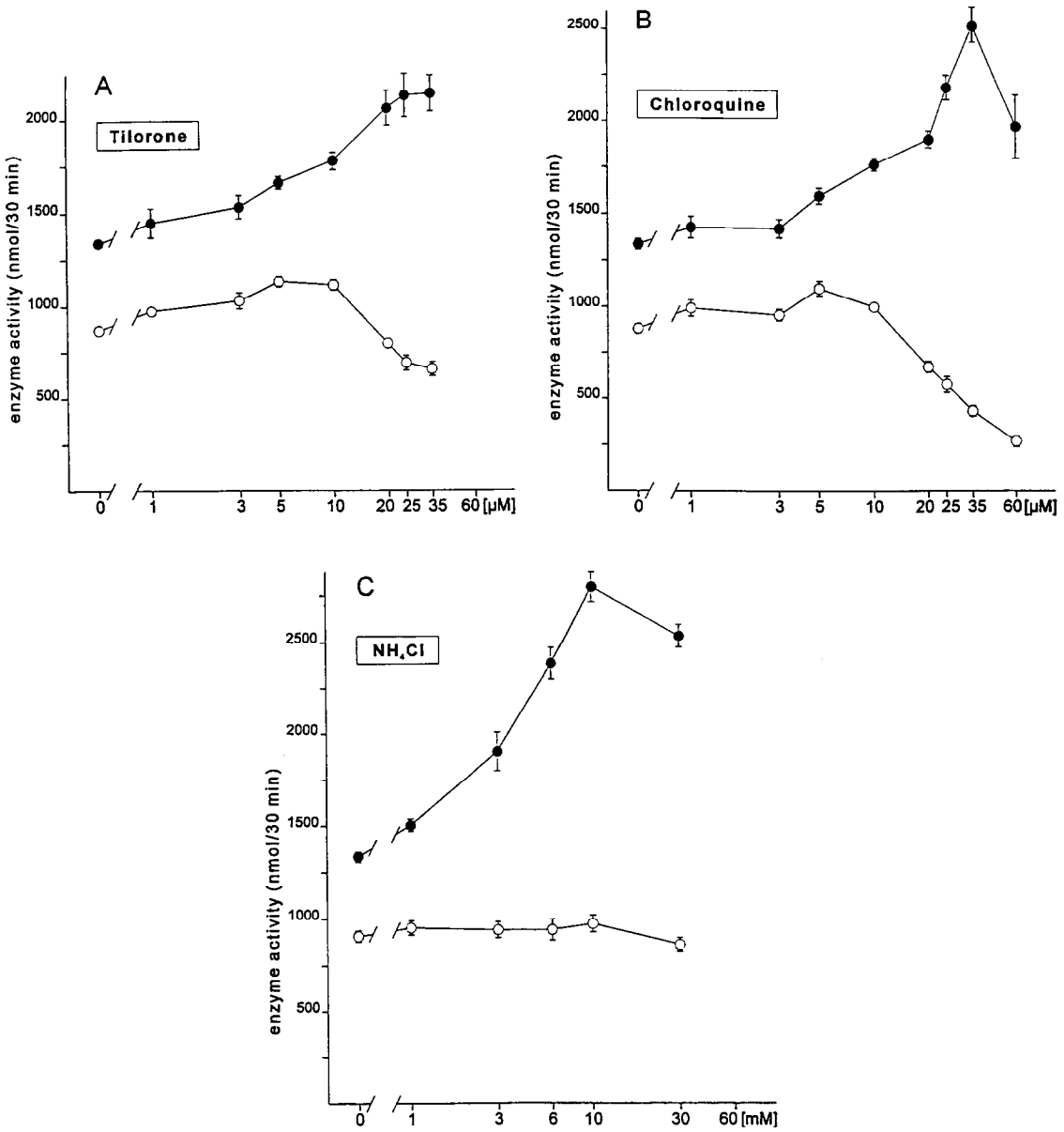

Fig. 2. Concentration-dependent drug effects on total activity of the lysosomal enzyme β-hexosaminidase and on its distribution between intra- and extracellular spaces. Confluent fibroblasts were exposed to the three compounds at the indicated concentrations for 72 hr. Enzyme activity was determined in cell lysate and culture medium. (●) Total enzyme activity in the culture (cells plus medium). (○) Enzyme activity in the cells alone. The difference between the two curves corresponds to the enzyme activity in the medium. Each point of the curves represents the mean ± SEM (N = 5–18) from 5–8 experiments. Bars for SEM are omitted when smaller than symbols.

so as to cover the range from near-threshold concentrations with respect to the elevation of ^{35}S-GAG up to concentrations at which marked unspecific cell damage started to occur. For tilorone this range lay between 1 and 35 μM; for chloroquine between 1 and 60 μM; and for NH_4Cl between 1 and 30 mM. With rising drug concentrations the increase in cell densities occurring during the experimental period was attenuated (Table 1). It is noteworthy that the cell layers remained confluent without an overt increase in damaged or detached cells, except for those cultures exposed to the highest concentration of each agent. Drug concentrations beyond the ranges given above led to the detachment of a large fraction of cells and were therefore not included in the experiments.

Histochemistry and electron microscopy

Selective light-microscopic staining of poly-

sulphated material was performed by using cuprolinic blue in Scott fixative (2.5% glutaraldehyde in 0.025 M Na acetate buffer pH 5.7 plus 0.3 M $MgCl_2$) [34]. It may be pointed out that the selectivity of this histochemical reaction is brought about by the high Mg^{2+} concentration in the staining bath, which causes the sulphate residues to remain the only charged binding partners for the cationic dye [34]. For ultrastructural examination, cells were fixed and processed as previously described [3].

RESULTS

Effect of tilorone (5 μM) on the disappearance of intracellular ^{35}S-GAGs from metabolically labelled fibroblasts

In order to demonstrate directly that impaired degradation is responsible for tilorone-induced GAG storage, the disappearance of ^{35}S-GAGs from metabolically labelled fibroblasts was determined. After a 'chase' period of 48 hr tilorone-treated cells still contained 71 ± 7% (±SEM, N = 4) of the ^{35}S-labelled GAGs present immediately at the end of the labelling phase, whereas control cells contained only 6 ± 0.3% (±SEM, N = 4). This result is taken as evidence for the tilorone-induced impairment of ^{35}S-GAG degradation, although we did not follow the appearance of free ^{35}S-sulphate in the medium. Increased synthesis has previously been excluded [12]. Reduced secretion of ^{35}S-proteoglycans is unlikely to account for ^{35}S-GAG retention, since in an earlier study on bovine corneal fibroblasts the secretion was found unaffected by 5 μM tilorone [35].

Drug effects on total β-hexosaminidase activity and its distribution between intracellular and extracellular spaces

Exposure (72 hr) to tilorone, chloroquine, and NH_4Cl increased the total enzyme activity present in the culture (cells plus medium) in a concentration-dependent manner (Fig. 2). The drop in total enzyme activity at the highest concentrations of chloroquine and NH_4Cl was not investigated further. It is assumed to be due to general cytotoxic effects of the high drug concentrations. In control fibroblasts, approximately two-thirds of the total enzyme activity was contained in the cells after 72 hr and one-third was found in the culture medium. Beginning with concentrations between 5 and 10 μM, tilorone and chloroquine caused a redistribution of enzyme activity in favour of the extracellular fraction, and there was a concomitant drop in the intracellular levels of β-hexosaminidase activity. NH_4Cl enhanced the extracellular enzyme fraction at all concentrations examined.

Dose–response curves for ^{35}S-GAG accumulation and secretion of β-hexosaminidase

Figure 3A demonstrates the dose–response curves for ^{35}S-GAG accumulation and enzyme secretion as evoked by tilorone. For the purpose of the present study, the most important finding is that 66% of maximum ^{35}S-GAG accumulation was achieved at a concentration (5 μM) which was ineffective in

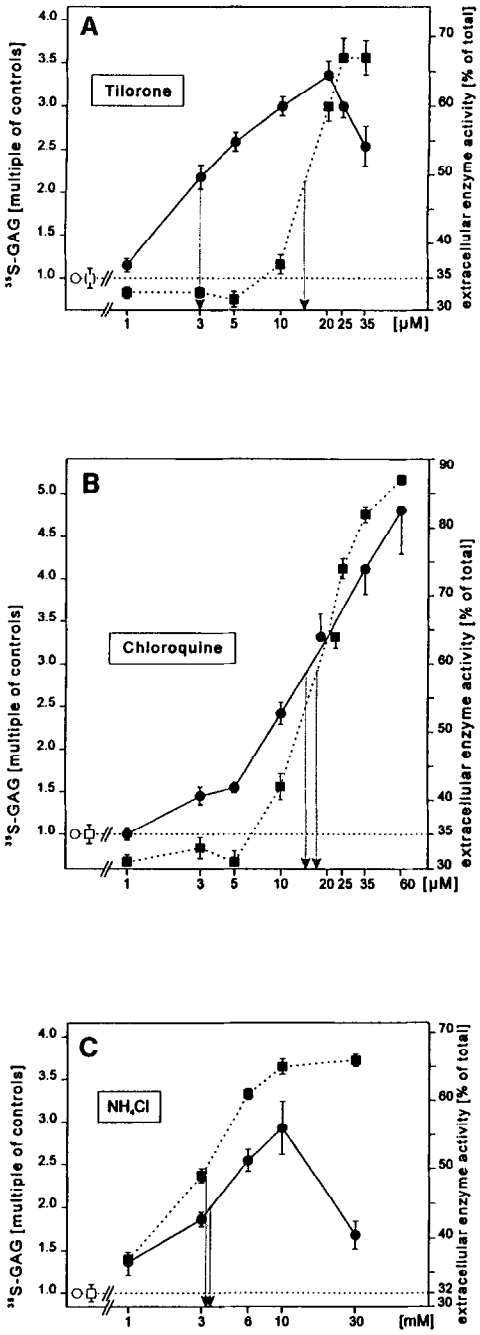

Fig. 3. Concentration-dependent drug effects on intracellular accumulation of ^{35}S-GAGs (●) and on release of the lysosomal enzyme β-hexosaminidase (■). (○) and (□) ^{35}S-GAG accumulation and enzyme release, respectively, in control cultures. Confluent fibroblasts were exposed to the indicated agents for 72 hr. Intracellular ^{35}S-GAGs were determined as described in the Methods. Enzyme release corresponds to the enzyme activity in the culture medium (extracellular activity, given as % of the total activity in the culture). The arrowheads mark the EC_{50} for the respective effects. Each point on the curves represents the mean ± SEM from 3–7 experiments for ^{35}S-GAG accumulation (N = 4–16) and from 5–8 experiments for enzyme release (N = 5–18), respectively. Bars for SEM are omitted when smaller than symbols.

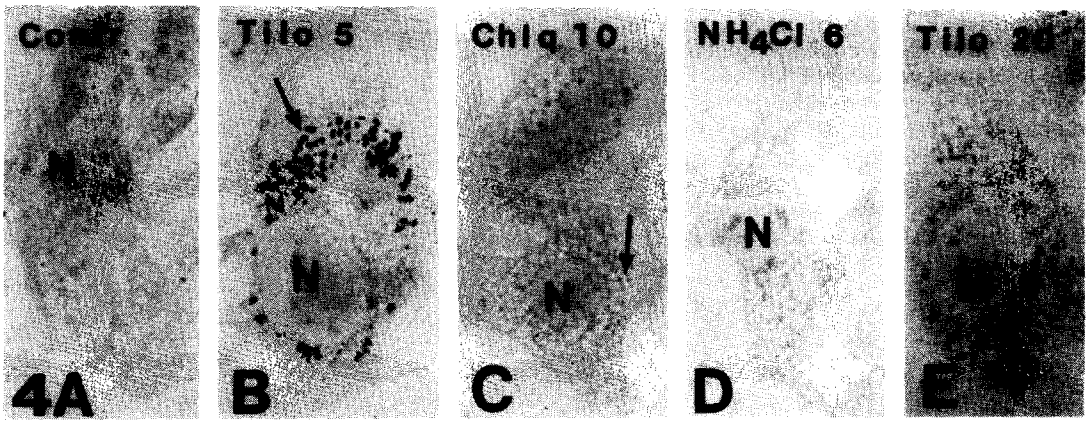

Fig. 4. Light microscopy. Specific histochemical staining for intracellular GAGs [34]. Confluent fibroblasts were exposed to the indicated conditions for 72 hr and then fixed and simultaneously stained with cuprolinic blue in the presence of 0.3 M $MgCl_2$. A, control; B, tilorone 5 μM; C, chloroquine 10 μM; D, NH_4Cl 6 mM; E, tilorone 20 μM. Tilorone 5 μM is the only condition at which brilliantly-stained inclusions are seen in the perinuclear cytoplasm (arrow). With chloroquine 10 μM and tilorone 20 μM, only tiny cuprolinic blue-positive inclusions are observed (arrow). N, nucleus. ×800.

enhancing enzyme secretion. The EC_{50} for enzyme release (15 μM) was shifted significantly to the right as compared with EC_{50} for ^{35}S-GAG accumulation (3 μM), indicating that the two effects were dissociated in the lower concentration range. Maximum accumulation of ^{35}S-GAGs (factor 3.4) was obtained with 20 μM tilorone, which simultaneously caused a significant enhancement of enzyme secretion. The reason for the drop in ^{35}S-GAG accumulation at 25 and 35 μM is unknown. It is assumed that unspecific cytotoxic effects of these drug concentrations led to reduced GAG synthesis and thus reduced accumulation.

Chloroquine (Fig. 3B) at concentrations of 3 and 5 μM caused a small increase in ^{35}S-GAG accumulation (factor 1.4 and 1.55, respectively) in the absence of abnormal enzyme secretion. With concentrations higher than 5 μM, however, both dose–response curves showed a steep rise. Maximum accumulation of ^{35}S-GAG (factor 4.8) was achieved at a concentration of 60 μM. The EC_{50} for either effect was near 15 μM (14.5 μM for ^{35}S-GAGs and 17 μM for enzyme secretion).

In the case of NH_4Cl (Fig. 3C), the increase in ^{35}S-GAG accumulation (maximum factor 2.9 at 10 mM) was accompanied by enhanced enzyme secretion at each concentration. The EC_{50} of either effect was approximately 3.3 mM. The reason for the drop in ^{35}S-GAG accumulation at 30 mM is unclear. Presumably cytotoxic effects led to impaired GAG synthesis and reduced accumulation; similar observations were reported for cultured human glial cells treated with NH_4Cl [36].

Morphological observations

For comparative morphological studies, a tilorone concentration (5 μM) was chosen which induced significant accumulation of ^{35}S-GAGs (factor 2.6 over controls) without causing enhancement of lysosomal enzyme release. This was compared with chloroquine (10 μM) and NH_4Cl (6 mM) at concentrations approximately equieffective with respect to ^{35}S-GAG accumulation although this was associated with enhanced release of lysosomal enzyme. The observations with tilorone were quite different from those with chloroquine and ammonia. Upon specific staining of sulphated GAGs with the cationic dye cuprolinic blue (Fig. 4B), the tilorone-treated cells showed numerous positively reacting inclusions in the perinuclear cytoplasm. In contrast, after exposure to chloroquine (Fig. 4C), only some cells showed tiny cuprolinic blue-positive dots, and after treatment with NH_4Cl no basophilic inclusions were found (Fig. 4D). Ultrastructurally (Figs 5A and B), the inclusions induced by 5 μM tilorone resembled those previously described in cultured cells and in rats [3, 6, 13]. Enlarged lysosomes showed marginal remnants of the electron-dense lysosomal matrix and contained small amounts of floccular material; considerable portions of the intralysosomal spaces appeared optically empty because of leaching of the water-soluble GAGs during histological processing. GAG-precipitating agents such as cationic dyes were required to mitigate the artificial loss of the storage material (Fig. 5B, inset) [37]. The cells treated with 10 μM chloroquine (Figs 6A and B) or 6 mM ammonia contained numerous vacuoles and inclusions which harboured polymorphic storage material of varying electron densities. Some chloroquine-treated cells displayed faint indications of GAG storage when fixed in the presence of GAG-precipitating agents (Fig. 6B, inset). Basically similar lesions were observed with higher concentrations of chloroquine. In ammonia-treated cells ultrastructural indications of GAG-storage were not observed.

High tilorone concentrations (20 and 25 μM), at which ^{35}S-GAG accumulation was accompanied by marked enhancement of enzyme secretion, caused lesions similar to those seen with chloroquine (10–

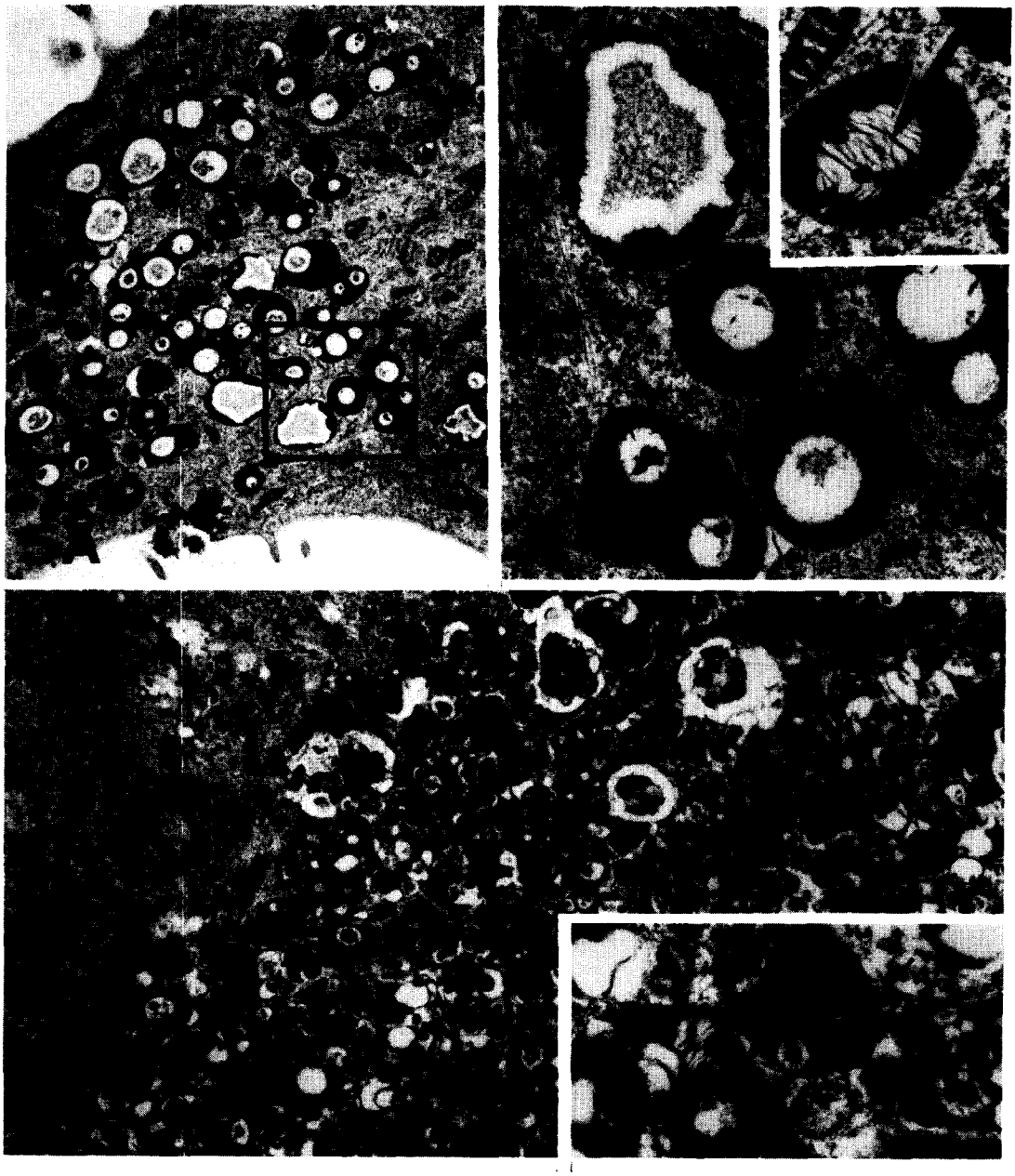

Fig. 5. Ultrastructure of the cytoplasmic inclusions in fibroblasts exposed for 72 hr to 5 μM tilorone (A and B) or to 20 μM tilorone (C). With 5 μM tilorone the lysosomes display partially empty spaces which are due to leaching of the water-soluble GAGs. The area marked in A is shown at higher magnification in B. Inset in B: When the fixative is supplemented with 0.1% toluidine blue as a precipitant of GAGs [37], the lysosomal storage material is preserved as fibril-like structures (arrow). With 20 μM tilorone (C) the storage lysosomes appear polymorphic. Inset in C: When fixed with addition of toluidine blue, small fibril-like structures are preserved in the lysosomes (arrows) suggesting GAGs. A, ×9000; B and inset, ×31,000; C, ×9000 and inset ×30,000.

25 μM). The intensity of the histochemical staining for GAGs was remarkably weak; the cells showed only small cuprolinic blue-positive inclusions (Fig. 4E) rather than the brilliantly-staining inclusions observed at 5 μM tilorone. Ultrastructurally (Fig. 5C), the storage lysosomes were filled with polymorphic material and showed only faint morphological indications of GAG storage when fixed in the presence of GAG-precipitating agents (Fig. 5C, inset).

Finally, brief mention should be made of the cytological lesions seen with 5 μM chloroquine, which did not enhance enzyme secretion. The cells contained numerous lamellated bodies (Fig. 6C),

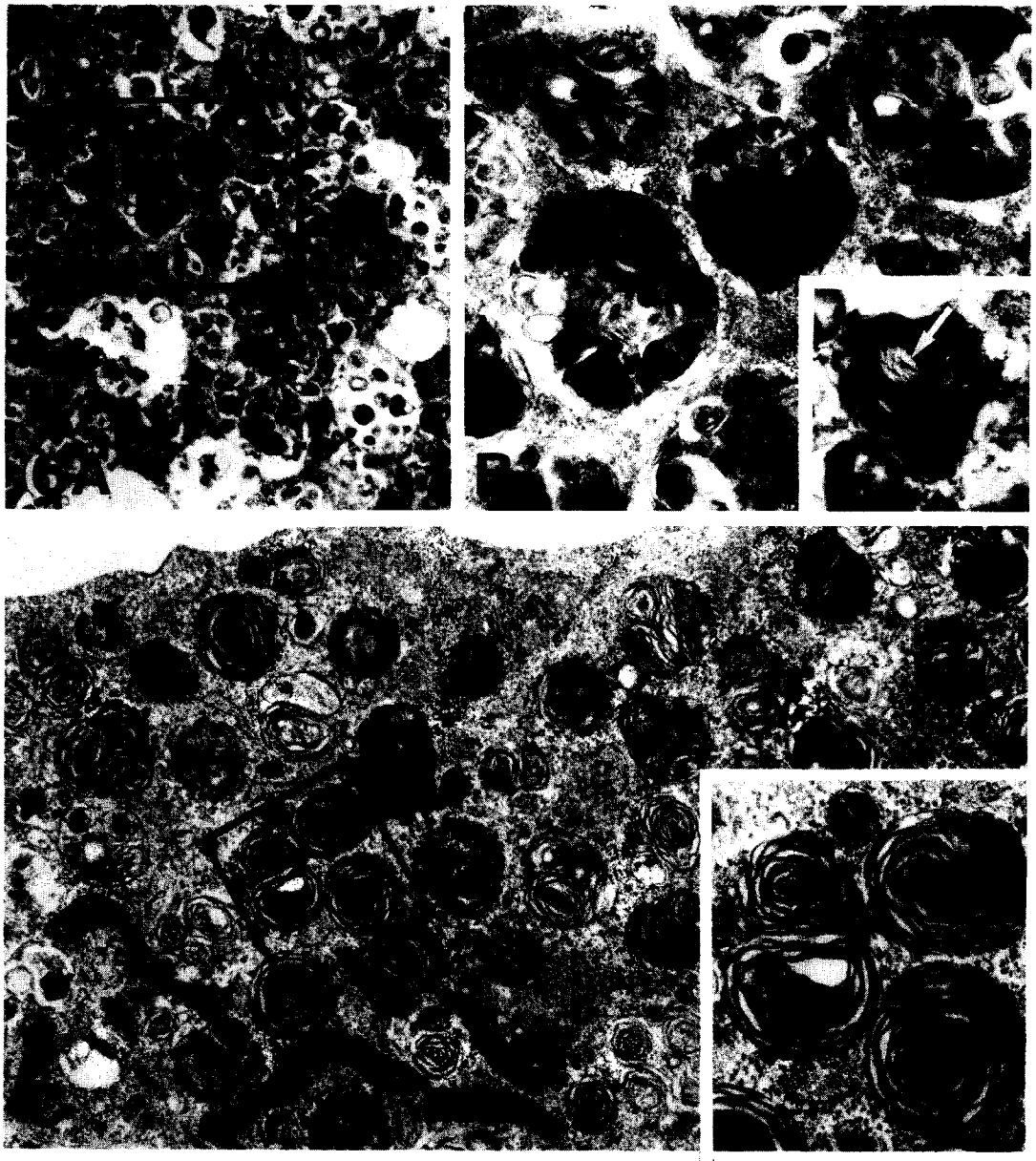

Fig. 6. Cytoplasmic inclusions in fibroblasts exposed for 72 hr to 10 μM chloroquine (A and B) or to 5 μM chloroquine (C). With 10 μM chloroquine the lysosomes contain polymorphic storage material. The area marked in A is shown at higher magnification in B. Inset in B: After fixation with addition of toluidine blue, small amounts of fibril-like material are preserved in the lysosome (arrow) suggesting the presence of GAGs. With 5 μM chloroquine (C) the lysosomes homogenously display lamellated storage material indicating lipidosis. A, ×9000; B, ×25,000 and inset, ×31,000; C, ×18,000 and inset ×37,000.

which are known to indicate lysosomal storage of polar lipids (lipidosis) [38]. The polymorphic inclusions seen with higher chloroquine concentrations were absent. Histochemical or ultrastructural indications of lysosomal GAG storage were not found.

DISCUSSION

The basis of this investigation was the concept of intracellular mistargeting of lysosomal enzyme precursors in the presence of lysosomotropic amines [18, 19], and the question whether this mechanism is able in itself to explain the impairment of lysosomal GAG degradation induced by low concentrations of tilorone. Since the effects of tilorone and other lysosomotropic amines upon intracellular targeting and processing of the soluble lysosomal enzyme β-hexosaminidase have been analysed in detail [12, 19], we measured its abnormal secretion as being the

result of mistargeting. The following events are generally thought to lead to increased enzyme secretion [17–19, 39, 40]. Weak bases are accumulated within acid prelysosomal organelles (endosomes) and thereby elevate endosomal pH. The endosomes are the sites where the newly-synthesized lysosomal enzyme precursors dissociate, in a pH-dependent manner, from the mannose-6-phosphate receptors, which are then recycled back to the trans-Golgi network and re-utilized for sorting. Upon drug-induced elevation of the endosomal pH, dissociation and receptor recycling are impaired. Free mannose-6-phosphate receptors are no longer available for binding newly-synthesized enzymes and for targeting them from the Golgi to the lysosomal route. Therefore the enzyme precursors are no longer segregated but diverted to the secretory pathway, and the lysosomes are cut off from enzyme supply.

In the present study ammonia, chloroquine, and tilorone in the high concentration range represented the positive controls, since there is sufficient evidence from cell culture studies that they (a) interfere with the targeting of lysosomal enzymes, and (b) induce abnormal accumulation of ^{35}S-GAGs. Our findings with the positive controls are in accordance with previous reports. With respect to ^{35}S-GAG accumulation, tilorone was shown to be effective at 10 μM (human fibroblasts) [12], chloroquine at 10 and 25 μM (human fibroblasts) [24], and ammonia at approx. 4 mM (human glial cells) [36]. With respect to enhanced lysosomal enzyme secretion, tilorone was reported to be consistently effective at concentrations of $\geq$20 μM [12]; chloroquine and NH_4Cl are usually applied at 25–100 μM and 5–10 mM, respectively, to produce marked lysosomal enzyme secretion [18, 19, 41, 42].

The concept of the mistargeting of lysosomal enzyme precursors may be satisfactory to explain the ^{35}S-GAG accumulation presently observed with tilorone and chloroquine at concentrations beyond 5–10 μM, and with ammonia throughout the investigated concentration range. Presumably these conditions impair the lysosomal degradation not only of GAGs but also of various other classes of macromolecules [43]. This would explain the occurrence of the polymorphic material in the lysosomes as seen by electron microscopy (Figs 5C and 6C).

The above mechanism appears, however, unable to account for the marked GAG storage observed with tilorone at lower concentrations, since they did not induce enhanced enzyme secretion. Several alternative explanations could be envisaged.

(a) Elevation of the pH in lysosomes might lead to unsuitable working conditions for the GAG-degrading enzymes. This would, however, raise the question of why chloroquine at $\leq$5 μM does not have the same marked effect on ^{35}S-GAG accumulation as tilorone, and why it induces cytologic lesions quite different from those caused by 5 μM tilorone. Both compounds are divalent bases, and the degrees of their intralysosomal accumulation and thus their effects upon pH can be expected to be similar [44]. This can also be inferred indirectly from the present dose–response curves for enzyme secretion. The curves are very similar for both compounds up to 25 μM, suggesting that they affect pH with similar concentration dependencies, at least in a prelysosomal compartment. Data for an exact comparison of lysosomal pH alterations are not available. While there exist many studies concerning chloroquine [15, 45, 46], there is only one report describing tilorone (250 μM) as increasing the pH of isolated lysosomes by 0.22 units [12]. It is difficult to extrapolate from these data on the situation in intact cells.

(b) Direct inhibition of GAG-degrading enzymes might account for GAG-storage. While 100 μM tilorone was reported to be without inhibitory effects on various GAG-degrading enzymes *in vitro* [12], it has to be kept in mind that the mechanism of drug accumulation leads to intralysosomal concentrations in the millimolar range both in the case of tilorone [35] and chloroquine [44, 46] at medium concentrations below 5 μM. Thus, direct enzyme inhibition cannot be excluded. Again, it is remarkable that at low concentrations tilorone is clearly more effective than chloroquine with regard to ^{35}S-GAG accumulation.

(c) Interaction of the dicationic drug molecules with the polyanionic GAG-chains might render them undigestible substrates. In cultured fibroblasts and tissues of rats treated with tilorone or congeners, both the drugs and GAGs were found accumulated so that the molar ratio of drug to GAG disaccharide unit was at least 1:1 [2, 9, 35]. Physicochemical interaction between GAGs and tilorone was shown *in vitro* by circular dichroism spectrometry [47]. If non-degradable GAG–drug complexes are assumed to be responsible for ^{35}S-GAG accumulation at low drug concentrations, differences in affinity towards GAGs might explain the observed differences between tilorone and chloroquine.

With respect to tilorone-induced GAG storage representing an adverse drug action in intact rats [1, 9, 13] and presumably also in patients [7], one may ask which of the concentration ranges in cultured cells is relevant for the conditions in intact organisms. Probably it is the low range. This is suggested by the finding that the morphological lesions in cultured cells exposed to low tilorone concentrations were basically identical to those seen in tilorone-treated rats, whereas lesions found at high concentrations have never been observed in intact animals. Furthermore, pharmacokinetic studies [48, 49] showed that the plasma concentrations of tilorone were below 5 μM in rats and mice after drug dosages similar to those sufficient to induce GAG storage in animals [1, 9, 13]. Therefore, it appears justified to assume that GAG storage induced by tilorone and congeners also in intact organisms may be due to mechanisms other than lysosomal enzyme depletion. As a working hypothesis, we would propose the formation of non-degradable GAG–drug complexes, for which symmetrically substituted dicationic

compounds such as tilorone may be better suited than molecules such as chloroquine.

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