Effects of cyclofenil diphenol, an agent which disrupts Golgi structure, on proteoglycan synthesis in chondrocytes

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1. Cyclofenil diphenol (F6060), a weak non-steroidal oestrogen, was shown previously to inhibit [³⁵S]proteoglycan synthesis [Mason, Lineham, Phillipson & Black (1984) *Biochem. J.* **223**, 401–412] and to induce fragmentation of the Golgi apparatus into small vesicles [Lancaster, Fryer, Griffiths & Mason (1989) *J. Cell Sci.* **92**, 271–280] in cultures of Swarm chondrosarcoma chondrocytes. Two structurally related compounds, F6204 and F6091, show a similar concentration-related effect, with complete inhibition of [³⁵S]proteoglycan synthesis at 90 µg/ml. The apparent [³H]protein synthesis is only approx. 40% inhibited with [³H]lysine as precursor. Stilboestrol, clomiphene and tamoxiphen are also potent inhibitors of [³⁵S]proteoglycan synthesis. 2. Syntheses of chondroitin 4-[³⁵S]sulphate and chondroitin 6-[³⁵S]sulphate, which are Golgi-mediated events, are inhibited 40–68% and 3–48% respectively by concentrations of cyclofenil between 50 and 70 µg/ml. [³H]Hyaluronan synthesis, which occurs by a different mechanism at the plasma membrane, is inhibited by 47–66%. These results suggest that cyclofenil may act via more than one inhibitory mechanism. Cyclofenil diphenol inhibits polymerization of chondroitin sulphate on to *p*-nitrophenyl β-xyloside even when the chondrocytes are loaded with the initiator prior to treatment. 3. Cyclofenil diphenol interferes with the cellular uptake of amino acids via the system A carrier, as shown by inhibition of uptake of methylaminoisobutyric acid, a specific substrate for this system. The drug had no effect on the uptake of 2-deoxyglucose by the cells. 4. Cyclofenil diphenol (90 µg/ml) caused a decrease in the pool size of UDP-*N*-acetylglucosamine, UDP-*N*-acetylgalactosamine and UDP-hexoses, but this was insufficient to account for the accompanying profound inhibition of [³⁵S]proteoglycan synthesis. Entry of [³H]glucosamine into the cell and into the UDP-*N*-acetylhexosamine pool did not appear to be affected. 5. Cyclofenil diphenol inhibited the substitution of ³H-labelled proteoglycan core protein with chondroitin sulphate chains. Core protein was identified in treated cultures on the basis of immunoprecipitation with an antiserum against the hyaluronate-binding region and distinguished from precipitated proteoglycan on SDS/PAGE.

INTRODUCTION

In previous studies we have found that cyclofenil diphenol, in the presence of concentrations of albumin just sufficient to keep it in solution (30 µg of protein/µg of drug), inhibited the synthesis of [³⁵S]proteoglycan, [³H]GlcNAc-labelled glycoproteins. [³H]GlcNAc-labelled hyaluronate and [³H]lysine-labelled protein in chondrocytes in a dose-dependent manner. However, with excess albumin present (e.g. 44 µg of protein/µg of drug), whereas [³⁵S]proteoglycan synthesis was profoundly inhibited, synthesis of [³H]lysine-labelled protein was little affected [1]. Ultrastructural investigations showed that when Swarm chondrosarcoma chondrocytes are exposed to cyclofenil diphenol under the latter conditions, their normally well-developed Golgi stacks fragment into smaller vesicles and disappear. At a later stage the rough endoplasmic reticulum in such cells dilates into large ribosome-studded vesicles which contain accumulations of electron-dense material, identified tentatively as newly synthesized protein [2].

The present report shows that the inhibitory properties of cyclofenil diphenol on proteoglycan synthesis are shared by other synthetic oestrogen analogues, and indicates that this compound is an inhibitor of glycosaminoglycan chain polymerization. However, we also demonstrate that the drug has an inhibitory action on the system A transporter for amino acid uptake by the chondrocytes. Additionally, high concentrations of the drug bring about a decrease in the pool size of UDP-sugars required for proteoglycan, hyaluronate and glycoprotein synthesis.

MATERIALS AND METHODS

Preparation of primary chondrocyte cultures

Chondrocytes were isolated from the Swarm rat chondrosarcoma by the method of Kimura *et al.* [3]; cells were plated at 4 × 10⁶/35 mm² Petri dish (day 0) in Dulbecco's modified Eagle's medium (DMEM) containing 15% foetal calf serum, penicillin and streptomycin (100 units/ml) (Gibco Ltd.), Hepes (15 mM), Tes (10 mM) and Bes (10 mM) (all from BDH Chemicals Ltd.). Chondrocyte cultures were used on either day 1 or day 2, the medium being changed to DMEM containing insulin (100 ng/ml) (Wellcome Foundation Ltd.) at least 1 h before the start of experiments.

Treatment of cultures with cyclofenil diphenol and labelling procedures

Cyclofenil diphenol (F6060) (Ferrosan AB, Malmo, Sweden) was dissolved in 1 M-NaOH and diluted immediately with DMEM containing 50 mg of BSA/ml (Sigma Chemical Co.; product no. A4503) to give a stock solution of concentrations 450 µg of drug and 20 mg of albumin/ml [1]. A fresh solution was made for each experiment.

Chondrocyte cultures were treated with various concentrations of cyclofenil diphenol (40–100 µg/ml) in DMEM containing the supplements listed above and a constant drug/albumin ratio. The treatment period was usually 3 h. During the last 1 h the chondrocytes were labelled with sodium [³⁵S]sulphate (25–50 µCi/ml), D-[6-³H]glucosamine hydrochloride, [2-³H]glycine, L-[4,5-³H]leucine, L-[4,5-³H]lysine, L-[³H]proline, L-

Abbreviation used: DMEM, Dulbecco's modified Eagle's medium.

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[^3H]serine or α -[1- ^{14}C]methylaminoisobutyric acid [all obtained from DuPont (UK) Ltd.]. Serine- or magnesium sulphate-depleted DMEM (one-tenth normal concentration in each case) was used to increase the specific radioactivity of the label in shorter experiments. Radiolabelled macromolecules were extracted from the media and cell layers with 4% CHAPS and 4 M-guanidine hydrochloride prior to analysis by gel filtration chromatography (Sephadex G-25, PD-10; Pharmacia LKB Ltd.) [4].

Radioiodination of albumin for uptake studies

^{125}I -labelled albumin was prepared using the IodoGen procedure [5].

UDP-sugar analysis

The cell layer was washed with and scraped into cold phosphate-buffered saline (Flow Laboratories, cat. no. 28-103-05). A cell pellet was recovered by centrifugation (1000 rev./min, r_{av} 7.5 cm, 5 min), resuspended in 2 ml of cold deionized water and boiled for 2 min, after which it was frozen at -20°C . Prior to analysis for UDP-sugars, the cell extracts were thawed and centrifuged, and the supernatant was filtered through a $0.2\ \mu\text{m}$ filter. Portions (0.2 ml) were analysed using a Partisphere SAX column (Whatman) connected to a Spectra Physics HPLC 8700 delivery system. The column effluent was monitored at 254 nm (Spectra Physics 871 detector) and fractions were collected for analysis of radioactivity. UDP-*N*-acetylglucosamine, UDP-*N*-acetylgalactosamine, UDP-glucose/-galactose and UDP-xylose were separated from one another by eluting with 30 mM- KH_2PO_4 (pH 3.5, 1 ml/min, 20°C).

Analysis of [^3H]chondroitin sulphate isomers and [^3H]hyaluronate by chondroitinase ABC digestion

Carrier chondroitin sulphate (200 μg) and hyaluronate (100 μg) were added to 1 ml portions of the combined medium and cell layer extracts from cultures labelled with [^3H]glucosamine/[^{35}S]sulphate. A glycosaminoglycan fraction was prepared from this, digested with chondroitinase ABC and the disaccharide products of chondroitin sulphate isomers and hyaluronate were analysed by t.l.c., as described previously [4].

Labelling and immunoprecipitation of [^3H]serine-labelled proteoglycan core protein

Day 1 chondrocyte cultures were labelled with [^3H]serine (25 $\mu\text{Ci}/\text{ml}$ in serine-depleted DMEM) for 20 h and then chased for 3 h either with fresh medium containing cyclofenil diphenol (90 $\mu\text{g}/\text{ml}$) and albumin (4 mg/ml) or with control medium. Some cultures which were not prelabelled with [^3H]serine were chased in the same way and labelled with [^{35}S]sulphate (50 $\mu\text{Ci}/\text{ml}$) and [^3H]lysine (10 $\mu\text{Ci}/\text{ml}$) during the last hour of the chase. To measure [^3H]lysine- and ^{35}S -labelled macromolecules, cell layers were solubilized by the 4% CHAPS/4 M-guanidine hydrochloride procedure and this extract and the culture medium were analysed by Sephadex G-25 (PD-10) chromatography. To measure [^3H]serine-labelled proteoglycan and proteoglycan-core protein, the cell layers were extracted with lysis buffer, pH 8.0, containing 2% (w/v) lithium dodecyl sulphate and proteinase inhibitors before the addition of dithiothreitol (10 mM) and heating at 100°C for 10 min [6]. Concentrated lysis buffer was added to media to give the same final concentration as for the cell layer extract, and the media were treated in the same way. After cooling, iodoacetamide (20 mM) was added and the samples were incubated in the dark for 2 h.

Portions of the treated cell layer extracts and media were diluted with 20 vol. of dilution buffer containing Nonidet P-40

and sodium deoxycholate, together with protease inhibitors [6]. Undissolved material was removed by centrifugation (12000 g, 30 min, 4°C) before adding an antiserum directed against the hyaluronan-binding region of the proteoglycan core protein (no. 1971; a gift from Dr. J. H. Kimura, Henry Ford Hospital, Detroit, MI, U.S.A.). Antibody-antigen complexes were precipitated with a suspension (10%, w/v) of *Staphylococcus aureus* membranes (4°C , 3 h) which had been washed three times in dilution buffer containing 0.1% SDS and 0.1% NaN_3 . The ratio of antiserum/*S. aureus* membranes used was 1:15 (v/v), this having been determined to be the optimum ratio for precipitating 80–85% of the labelled proteoglycan and core protein precursor [6]. At the end of the incubation, samples were centrifuged (1500 g, 10 min, 4°C), and washed four times in dilution buffer containing 0.1% SDS at 4°C (10 min per wash). Control samples were precipitated with non-immunized rabbit serum by the same procedure.

Immunoprecipitates were treated with 0.5 M-NaOH for 20 min to release antigens and antibodies from *S. aureus* membranes. The supernatant was neutralized (0.1 vol. of 5 M-acetic acid/1 M-Tris/HCl, pH 7.4) and portions were counted for ^3H radioactivity by liquid scintillation.

Samples were prepared for SDS/PAGE by resuspending the immunoprecipitate in 10 mM-Tris/HCl, pH 6.8. After centrifugation (1500 g, 4°C , 10 min) this was repeated twice more before lyophilizing the pellet and resuspending it in 2% SDS/62.5 mM-Tris/HCl (pH 6.8)/10% glycine/dithiothreitol (20 mg/ml). After boiling for 5 min and centrifuging (1500 g, 10 min, room temperature), the supernatant was analysed by SDS/PAGE on 8% slab gels. Bands were detected by fluorography and fluorograms were scanned with a laser scanner.

RESULTS

Under conditions of albumin excess, cyclofenil diphenol (F6060) inhibits proteoglycan synthesis in a dose-dependent manner, whereas protein synthesis is only partially inhibited [1]. To investigate the specificity of this effect, we compared the action of the closely related compounds bis-(*p*-hydroxyphenyl)-2-methylcyclohexylidenemethane (F6204) and bis-(*p*-hydroxyphenyl)cyclohexylmethane (F6091) on these pathways with that of cyclofenil (Fig. 1). F6204 is the *o*-methyl derivative of cyclofenil diphenol, but has 40–50 times more oestrogenic activity than the latter, whereas F6091 has no measurable oestrogenic effect in screening procedures (information supplied by Ferrosan, Malmö, Sweden). Despite these differences in endocrinological properties, all three compounds had very similar effects on the chondrocytes over the dose range tested, eliminating proteoglycan synthesis at higher concentrations and only partially inhibiting [^3H]protein synthesis. In similar experiments in which chondrocyte cultures were treated with a concentration of 90 $\mu\text{g}/\text{ml}$ (0.2–0.3 mM) of the non-steroidal oestrogen analogues stilboestrol, clomiphene and tamoxifen, incorporation of [^{35}S]sulphate into proteoglycans was inhibited by 96, 97 and 71% respectively.

To test whether the albumin used to solubilize the drugs was taken up by the chondrocytes, cultures were incubated at either 37°C or 0°C for 2 h in medium containing either ^{125}I -albumin-cyclofenil complex or ^{125}I -albumin alone. Then, after washing the cell layers rapidly three times with medium containing unlabelled albumin-drug complex or albumin alone, followed by extraction of the extracellular matrix with 4 M-guanidine hydrochloride, the residual cells were solubilized with 4% CHAPS/4 M-guanidine hydrochloride and radioactivity was measured. Of the total ^{125}I -albumin radioactivity, 0.60–0.65%

Fig. 1. Inhibition of [^{35}S]proteoglycan and [^3H]protein synthesis in chondrocytes by compounds related to cyclofenil diphenol

Day 2 primary confluent cultures of chondrocytes were treated for 3 h with increasing concentrations of (a) cyclofenil diphenol (F6060), (b) bis(*p*-hydroxy-phenyl)-2-methylcyclohexylidenemethane (F6204), or (c) bis(*p*-hydroxyphenyl)cyclohexylmethane (F6091), with 4 mg of albumin/ml as a drug carrier, and labelled with [^{35}S]sulphate (○) and [^3H]lysine (■) during the last hour. Control cultures incorporated 1.48×10^4 d.p.m. and 5.78×10^4 d.p.m. into [^{35}S]proteoglycan and [^3H]protein respectively. Results for duplicate cultures for each point were within $\pm 5\%$.

was associated with the cell fraction under all conditions, suggesting that albumin is not actively internalized and that cyclofenil diphenol enters the cells in a free form.

Swarm chondrocytes synthesize a large chondroitin 4-sulphate proteoglycan (85–90 % of total) and a much smaller proteoglycan (10–15 %) of lower density which is enriched in chondroitin 6-sulphate [4,7]. These are readily separated by chromatography on Sepharose CL 2B under dissociative conditions [1,7]. Previous experiments with a minimum albumin/drug ratio (30:1, w/w) suggested that synthesis of the chondroitin 6-sulphate proteoglycan was less susceptible to inhibition by cyclofenil than was chondroitin 4-sulphate proteoglycan synthesis. This was investigated further in the present study by using excess carrier protein (protein/drug ratio of 44:1, w/w). Cultures were treated with various concentrations of cyclofenil (50–90 $\mu\text{g/ml}$) for 3 h and labelled with [^{35}S]sulphate during the last 1 h. Dissociative Sepharose CL 2B chromatography of the combined medium and cell layer extract showed that at each concentration of drug the synthesis of the large chondroitin 4-sulphate proteoglycan was inhibited to a greater extent than that of the small chondroitin 6-sulphate proteoglycan (results not shown). This was confirmed by analysing the glycosaminoglycans from $^3\text{H}/^{35}\text{S}$ -labelled cultures (Table 1). The incorporation of both [^3H]glucosamine and [^{35}S]sulphate into chondroitin 4-sulphate was decreased to a greater extent than that into chondroitin 6-sulphate over the range of 50–70 μg of cyclofenil diphenol/ml. At higher concentrations (e.g. 100 $\mu\text{g/ml}$) synthesis of both $^3\text{H}/^{35}\text{S}$ -labelled chondroitin sulphate isomers was eliminated. The proportions of $^3\text{H}/^{35}\text{S}$ radioactivity in both chondroitin 4-sulphate and

Table 1. Effects of cyclofenil diphenol on the synthesis of chondroitin 4-sulphate, chondroitin 6-sulphate and hyaluronan by chondrocyte cultures

Day 2 primary cultures were treated for 3 h with increasing concentrations of cyclofenil diphenol and labelled with [^3H]glucosamine and [^{35}S]sulphate during the last hour. Incorporation of ^3H and ^{35}S radioactivity into macromolecules varied by less than 8 % of the mean between duplicate cultures. Glycosaminoglycans were isolated from the combined medium and cell layer and analysed for their unsaturated disaccharide composition after digestion with chondroitinase ABC. (a) Incorporation of radioactivity in glycosaminoglycans in control cultures; (b) incorporation of radioactivity into cyclofenil-treated cultures expressed as a percentage of controls. About 90 % of the ^{35}S radioactivity found in macromolecules was recovered in [^{35}S]disaccharides in each analysis. C6S, chondroitin 6-sulphate disaccharide; C4S, chondroitin 4-sulphate disaccharide ($\Delta\text{di-4-S}$); C0S, chondroitin disaccharide ($\Delta\text{di-0-S}$); HA, hyaluronan disaccharide ($\Delta\text{di-HA}$).

(a)	$10^{-4} \times [^3\text{H}, ^{35}\text{S}]$ Glycosaminoglycans synthesized in control cultures (d.p.m. incorporated per disaccharide)			
	Isotope	C6S	C4S	C0S HA
^3H		1.62	11.83	1.80 3.49
^{35}S		2.56	13.56	– –

(b)	Radioactivity in glycosaminoglycan disaccharide (% of control)					
	C6S		C4S		C0S	HA
	[Cyclofenil] ($\mu\text{g/ml}$)					
	^3H	^{35}S	^3H	^{35}S		
50	96	97	51	60	56	53
60	88	87	45	56	53	64
70	76	52	22	32	33	34

chondroitin 6-sulphate varied in the range 1.1–1.6 under conditions of partial inhibition (50–70 $\mu\text{g/ml}$) without showing any consistent dose-related differences. This suggests that each isomer is synthesized from the same precursor pools.

These experiments also assessed the effect of cyclofenil on [^3H]hyaluronan synthesis (Table 1). This was progressively inhibited over the range 50–70 μg of cyclofenil/ml. However, even at 100 μg of drug/ml, when no synthesis of chondroitin sulphate isomers occurred, [^3H]hyaluronan synthesis was only decreased by about 75 %. Hyaluronan is synthesized at the plasma membrane by a different mechanism [8,9] to chondroitin sulphates, which are polymerized on to proteoglycan core proteins in the Golgi apparatus [10,11]. Thus, overall, these results indicate that the responses to cyclofenil of systems for the synthesis of different glycosaminoglycans differ subtly. In the Golgi this may depend on the response of specific enzymes, their particular location in the organelle or the availability of a particular core protein. In the case of hyaluronan, either the synthetase itself or its interaction with the plasma membrane may be affected. In all cases the availability of the UDP-sugar precursors could affect the ability to polymerize glycosaminoglycans, and this is considered below.

The effect of cyclofenil diphenol on different cell activities required for proteoglycan synthesis was examined. Chondrocyte

Fig. 2. Effect of cyclofenil diphenol on UDP-hexosamine pools in chondrocytes

Day 2 primary confluent cultures were treated with cyclofenil diphenol (90 $\mu\text{g/ml}$) for 2 h and labelled with [^3H]glucosamine during the last hour. UDP-sugars were extracted from the cell layer and separated by h.p.l.c. (a) Control culture, (b) cyclofenil-treated culture. UDP-sugars were identified by retention times: a, UDP-*N*-acetylglucosamine; b, UDP-*N*-acetylgalactosamine; c, UDP-glucose/-galactose. Numbers above each peak show retention times. Fractions were collected and those containing UDP-*N*-acetylhexosamines were counted for ^3H radioactivity (see text).

Table 2. Effect of cyclofenil on chondroitin sulphate synthesis in chondrocytes loaded with nitrophenyl β -D-xyloside

Day 1 confluent chondrocyte cultures were maintained for 2 h with or without β -xyloside present as shown, treated with cyclofenil diphenol during the last hour, and labelled with [^{35}S]sulphate during the last 15 min. All culture media contained 4 mg of albumin/ml and 0.1% (v/v) dimethyl sulphoxide (solvent for nitrophenyl β -D-xyloside). Chondroitin [^{35}S]sulphate synthesis was measured in the combined medium and cell layer extract. Results are means $\pm$ range for triplicate cultures. Numbers in parentheses refer to percentage inhibition of synthesis in cyclofenil-treated cultures.

β -Xyloside (0.3 mM)	10 ⁻³ $\times$ Chondroitin [^{35}S]sulphate synthesis (d.p.m./10 ⁵ chondrocytes)		
	Control	Cyclofenil diphenol	
		90 $\mu\text{g/ml}$	100 $\mu\text{g/ml}$
Not present	91 $\pm$ 1	30 $\pm$ 2 (67)	26 $\pm$ 1 (71)
Present throughout 2 h	305 $\pm$ 0	140 $\pm$ 2 (54)	121 $\pm$ 2 (60)
Present for first 1 h only	137 $\pm$ 0	86 $\pm$ 4 (37)	80 $\pm$ 7 (42)

cultures were treated for 3 h with cyclofenil (90 $\mu\text{g/ml}$) and labelled during the final 1 h with [^{35}S]sulphate, [^3H]lysine or 2- [^3H]deoxy-D-glucose. Whereas incorporation of ^{35}S and ^3H

radioactivity into proteoglycans and proteins was decreased by 97% and 40% respectively, the uptake of 2-deoxy-D-glucose was only decreased by approx. 8%, indicating that the activity of the glucose carrier is little affected by treatment with cyclofenil.

The effect of cyclofenil diphenol on UDP-sugar pools and on the uptake and incorporation of [^3H]glucosamine into UDP-*N*-acetylhexosamines was investigated at high (90 $\mu\text{g/ml}$) and lower (60 $\mu\text{g/ml}$) concentrations of the drug. Cultures were treated for 2 h and labelled with [^3H]lysine, [^3H]glucosamine or [^{35}S]sulphate during the last hour. At a drug concentration of 90 $\mu\text{g/ml}$, [^3H]lysine and [^{35}S]sulphate incorporation into proteins and proteoglycans was inhibited 44% and 96% respectively. After 2 h the UDP-GlcNAc, UDP-GalNAc and UDP-hexose pools had contracted to 31%, 23% and 26% respectively of the control culture pool sizes (Fig. 2) whereas the amount of [^3H]glucosamine incorporated into the UDP-*N*-acetylhexosamine pool was 28% of that in control cultures. However, when the cultures were treated with 60 μg of cyclofenil/ml, the UDP-sugar pools did not differ significantly from those in untreated cultures, even though the synthesis of chondroitin 4-sulphate and hyaluronan is much decreased under these conditions (Table 1). Thus inhibition of UDP-sugar synthesis by cyclofenil does not appear to be the primary cause of the inhibitory effect of the drug on glycosaminoglycan synthesis, and even at high concentrations of the drug (90 $\mu\text{g/ml}$) the decrease was greater than can be accounted for by the decrease in the total UDP-*N*-acetylhexosamine pool or incorporation of radiolabelled precursor into it. The results do not rule out the possibility that the cyclofenil diphenol might interfere with the uptake of cytosolic UDP-sugars into the Golgi apparatus for chondroitin sulphate synthesis.

Previous results showed that nitrophenyl β -D-xyloside, an artificial substrate for initiating chondroitin sulphate synthesis, failed to promote synthesis when added to chondrocytes already treated for 2 h with cyclofenil [1]. Although this suggests that the drug inhibits xyloside-chondroitin sulphate synthesis, an alternative explanation is that it inhibits the uptake of the β -xyloside by the cell. To test this, cultures were loaded with nitrophenyl β -D-xyloside for 1 h and then chased with cyclofenil diphenol for a further 1 h, with or without β -xyloside present. Chondroitin [^{35}S]sulphate synthesis was measured in the last 15 min of the chase (Table 2). Chondroitin [^{35}S]sulphate synthesis was increased 3-fold in cultures exposed to β -xyloside throughout the 2 h, but treatment with cyclofenil inhibited this effect almost to the same extent as it inhibited synthesis in non-stimulated cultures (Table 2). In cultures loaded with β -xyloside during the first hour only, Chondroitin [^{35}S]sulphate synthesis remained elevated by 1.5-fold after a further 1 h (Table 2), indicating that some β -xyloside remained in the cells during this chase period. Cyclofenil treatment significantly inhibited synthesis in these cultures (Table 2). Furthermore, chondroitin [^{35}S]sulphate synthesis was about 1.5 times greater in cyclofenil-treated cultures exposed continuously to β -xyloside than in those exposed during only the first 1 h of the experiment, showing that the initiator must enter the cells during periods of drug treatment. Thus, taken together, the results show that cyclofenil inhibits the synthesis of chondroitin sulphate on to the β -xyloside initiator by inhibiting glycosaminoglycan polymerization.

In initial experiments it was recognized that cyclofenil diphenol had varying inhibitory effects on apparent [^3H]protein synthesis in the chondrocytes, depending on which [^3H]amino acid was used to label the cells (Table 3). The results suggested that perturbation of the carrier A system may be responsible for the apparent inhibition, since inhibition was greatest when proline, glycine and serine were used as precursors. Although leucine can use system A, it is taken up predominantly by the L carrier and

Table 3. Effect of cyclofenil diphenol on the incorporation of different ^3H -labelled amino acids into macromolecules

Day 1 confluent chondrocyte cultures (5×10^6 cells) were treated with $90 \mu\text{g}$ of cyclofenil diphenol/ml for 2 h and labelled during the last hour with [^{35}S]sulphate and the [^3H]amino acids shown. Incorporation of radioactivity into macromolecules was measured for the medium and solubilized cell layer (means $\pm$ range for duplicate cultures). Numbers in parentheses show incorporation into treated cultures as a percentage of controls. [^{35}S]Proteoglycan synthesis was inhibited by $> 95\%$ in all treated cultures. L, A, ASC, Gly, Ly are carrier systems involved in the transport of each amino acid in Ehrlich ascites tumour cells, the main carrier being denoted by an asterisk (*) [12].

[^3H]Amino acid	$10^{-3} \times$ Incorporation of ^3H into macromolecules (d.p.m.)		Carrier
	Control	Treated	
Leucine	44.56 ± 0.43	41.14 ± 0.06 (92.3)	L*, A
Lysine	91.85 ± 9.35	61.40 ± 0.32 (66.8)	Ly*
Proline	168.87 ± 5.50	61.04 ± 1.27 (36.1)	A*, ASC
Glycine	33.01 ± 0.80	3.75 ± 0.36 (11.4)	A*, ASC, L, Gly
Serine	177.75 ± 4.05	8.32 ± 0.11 (4.7)	ASC*, A*, L

Table 4. Immunoprecipitation of ^3H -labelled proteoglycan and core protein in cyclofenil-treated cultures

Cultures were labelled with [^3H]serine for 20 h and then chased for 3 h with fresh control medium, medium containing albumin (4 mg/ml) or medium containing albumin and cyclofenil diphenol (90 μg /ml). A polyclonal antiserum against the cartilage proteoglycan core protein hyaluronan-binding region was used to immunoprecipitate ^3H -labelled proteoglycan and core protein from the chase medium and the detergent-solubilized cell layer of the cultures. Numbers in parentheses show the percentage distribution of the immunoprecipitated radioactivity between the chase medium and cell layer of cultures. Results are means for triplicate cultures, which varied within $\pm 5\%$ of the mean. In a parallel experiment [^{35}S]proteoglycan synthesis was inhibited by 94 % in cultures labelled with [^{35}S]sulphate during the last hour of the chase period.

Culture conditions	$10^{-5} \times$ Immunoprecipitated [^3H]proteoglycan and [^3H]core protein (d.p.m.)		Recovery (% of no-chase control culture)
	Cell layer	Medium	
Control, no chase	7.25	—	100
Control, 3 h chase	5.46 (80)	1.34 (20)	94
Albumin, 3 h chase	5.84 (85)	1.06 (15)	95
Cyclofenil, 3 h chase	9.51 (92)	0.87 (8)	143

the ASC system in bovine articular chondrocytes [13]. To test this hypothesis experiments were carried out using [^{14}C]methyl aminoisobutyric acid, a specific non-metabolizable substrate for the carrier A system [14]. Cultures were treated as in Table 3 and labelled during the last hour. Uptake of [^{14}C]methylisobutyric acid was inhibited by 72 % by cyclofenil, confirming that the drug does inhibit the carrier A system for amino acid uptake.

We have shown previously that, although [^{35}S]proteoglycan synthesis is inhibited rapidly in chondrocytes treated with cyclofenil, those molecules synthesized prior to the treatment

Fig. 3. SDS/PAGE of immunoprecipitated proteoglycan and core protein from chondrocyte cultures chased with or without cyclofenil diphenol

Chondrocyte cultures which had been prelabelled with [^3H]serine were chased with or without cyclofenil diphenol as described in the legend to Table 4. Immunoprecipitated ^3H -labelled products from the cell layer were subjected to SDS/PAGE and fluorography. The fluorograms were scanned with a laser scanner: (a) the immunoprecipitated [^3H]protein from control culture remains on top of the PAGE gel; (b) immunoprecipitated [^3H]protein from cyclofenil-treated culture migrates into the gel.

period are secreted from the cell at the normal rate after applying the drug [1]. This and other results suggest that whereas the secretory pathway from the Golgi apparatus to the plasma membrane remains intact during the early phase of treatment with cyclofenil, the biosynthetic function of the Golgi is rapidly disrupted. To test this, we labelled cultures for 20 h with [^3H]serine so that all proteoglycan core protein in the cells would be ^3H -labelled before chasing the cultures for 180 min with medium containing 90 μg of cyclofenil diphenol/ml. After solubilizing the cell layer with lithium dodecyl sulphate, this fraction and the chase medium were immunoprecipitated with a polyclonal antiserum (no. 1971) against core protein hyaluronan-binding region. The antiserum precipitates both core protein and complete proteoglycan, which were subsequently distinguished from one another by SDS/PAGE [15]. Recovery of the immunoprecipitated ^3H -labelled molecules in the control chase cultures was about 95 % of that present in the cell layer at the 20 h labelling period (Table 4). Negligible radioactivity was precipitated with non-immune rabbit serum (results not shown). Only one high-molecular-mass component was detected in the immunoprecipitate from the control chase culture cell layer, and this did not migrate into the SDS/PAGE gel (Fig. 3a), consistent with it being due to completed [^3H]proteoglycans which had been exported to the extracellular matrix during the chase period [15]. In contrast, high-molecular-mass components in the immunoprecipitate from the cultures chased with cyclofenil diphenol were due almost entirely to a [^3H]protein band which migrated into the top of the SDS/PAGE gel, indicating that it

was of smaller hydrodynamic size than the precipitated molecules in control cultures. This is consistent with it being due to proteoglycan core protein [15], lacking chondroitin sulphate chains as a consequence of the inhibition of their synthesis by cyclofenil diphenol. The significance of the recovery of more ^3H radioactivity in the immunoprecipitates of cyclofenil-chased cultures compared with that in control cultures is uncertain. Some low-molecular-mass material is precipitated non-specifically during immunoprecipitation [6,15] and it is possible that this is enhanced in cyclofenil-treated cultures. This was not investigated further.

DISCUSSION

The non-steroidal oestrogens and anti-oestrogens which inhibit [^{35}S]proteoglycan synthesis in Swarm chondrosarcoma chondrocytes must do so by a non-oestrogen-receptor-mediated mechanism, since these cells do not express such receptors [16]. The synthesis of [^3H]lysine-labelled proteins is relatively uninhibited by these compounds under the present experimental conditions, suggesting that cyclofenil and related drugs may inhibit proteoglycan synthesis at a post-translational stage. Experiments showing that cyclofenil brings about a disappearance of the Golgi apparatus in these cells support this proposal [2]. The polymerization of chondroitin sulphate chains on to the core protein of the large cartilage proteoglycan occurs in the medial and trans compartments of the Golgi apparatus [10,17], and is achieved by the co-ordinated action of a series of specific membrane-bound UDP-sugar transferases and sulphotransferases located in that organelle [18].

Swarm chondrocytes have an intracellular pool of about $0.5\text{ }\mu\text{g}$ of core protein/ 10^6 cells. Its half-life from translation to entry into the Golgi and the addition of glycosaminoglycan chains is ~ 50 min, after which it is rapidly secreted from the cell with a half-life of ~ 7 min [11,15]. The core protein can be distinguished from the completed proteoglycan after immunoprecipitation and SDS/PAGE [15]. We used this approach to investigate the fate of prelabelled ^3H -core protein in chondrocytes treated with cyclofenil diphenol. After a 20 h labelling period, all intracellular core protein should be labelled with [^3H]serine, as should core protein of recently completed proteoglycan secreted into the cell layer matrix. After a 3 h chase the immunoprecipitated ^3H -labelled high-molecular-mass component from control cell layers did not enter the electrophoresis gel, identifying it as complete proteoglycan. In contrast, most of the high-molecular-mass component immunoprecipitated from cyclofenil-chased cultures did enter the gel, consistent with its identity as core protein not containing glycosaminoglycan chains. The small amount of ^3H -labelled component on top of the gel in treated cultures is likely to be complete proteoglycan synthesized during the labelling period which has still not diffused to the medium by the end of the chase period.

Cyclofenil could inhibit polymerization of chondroitin sulphate chains on to the core protein by one of several mechanisms. These include inhibiting the formation of precursors such as UDP-sugars, inhibiting one or more enzymes required for polymerization or sulphation, or disruption of the route along which newly synthesized core protein must pass from the endoplasmic reticulum to the site of glycosylation in the Golgi apparatus. Since inhibitors of post-translational modification of proteins can be used to explore the functions of glycoproteins, as with monensin [19], it is of interest to identify their inhibitory mechanisms.

It seems unlikely that inhibition of UDP-sugar formation is a primary site for inhibition of proteoglycan synthesis by cyclofenil.

The drug brought about a significant inhibition of synthesis at concentrations (e.g. $60\text{ }\mu\text{g/ml}$) which had no effect on UDP-sugar pool sizes. This does not preclude an inhibition of transfer of the precursors from the cytosol into Golgi compartments. With higher drug concentrations ($90\text{ }\mu\text{g/ml}$) UDP-sugar pool sizes were decreased, as was the level of chondroitin sulphate synthesis in β -xyloside-stimulated cultures. However, there was a substantial residual level of synthesis in such cultures. This infers that adequate levels of precursors must be present and reach the site of chondroitin sulphate polymerization.

The ability of cyclofenil to decrease chondroitin sulphate synthesis in cultures which had been preloaded with nitrophenyl β -D-xyloside indicates that one of its inhibitory actions is on the polymerization process itself. This may be by an effect of the drug on membrane structure. The results of the ^{125}I -albumin experiments suggest that cyclofenil enters the cell in an unbound form. This could occur by transfer of the drug from the protein carrier to the cell membrane, or by uptake into the membrane of cyclofenil molecules from that small pool predicted to be soluble in the medium [1]. Transfer of the drug from the plasma membrane to the Golgi could occur either by endosome fusion with the latter [20] or via the cytosol. Intervesicle exchange of cholesterol via an aqueous phase has been demonstrated [21]. Incorporation of cyclofenil into the Golgi membranes may lead to a change in membrane fluidity. Ethinyl oestradiol decreases the phospholipid content and fluidity of ileal microvillus membranes [22], and 17β -oestradiol and tamoxifen lower membrane fluidity in MCF-7 and MDA-MB-436 human breast cancer cells [23]. Diethylstilboestrol, whose structure is closely related to that of cyclofenil [1], caused rupture of the chomatophores of *Rhodospirillum rubum* through its interaction with the membrane and the hydrophobic domains of membrane proteins [24]. Similar interactions between cyclofenil and the Golgi membranes may interfere with the UDP-sugar transferases which are membrane-bound and, at least in the case of galactosyltransferase 1, dependent on intact membrane phospholipids [25].

The electron microscopic changes noted in chondrocytes after exposure to cyclofenil include disassembly of the Golgi stack saccules, followed by dilation of the endoplasmic reticulum to form large ribosome-studded vesicles. Small vesicles present after the disappearance of Golgi saccules may be derived from the latter [2]. These changes resemble those occurring in cells treated with the fungal metabolite Brefeldin A [26,27] and differ from those induced by monensin [10,19] and diethylcarbamazine [28,29], which include swelling of the trans Golgi and the formation of large smooth vesicles. Brefeldin A blocks the protein secretory pathway by bringing about the disassembly of the cis, medial and trans Golgi. At least part of the Golgi membranes and their enzyme activities are redistributed to the endoplasmic reticulum [30]. The Golgi stack re-forms after withdrawal of Brefeldin A, as it does after removing cyclofenil [2]. These ultrastructural changes and their similarity to those induced by Brefeldin A raise the possibility that the core protein for the large chondroitin 4-sulphate proteoglycan remains in the endoplasmic reticulum in cyclofenil-treated cells, unable to gain access to any Golgi-derived structures still retaining glycosaminoglycan-polymerizing potential.

The differential inhibitory action of lower concentrations of cyclofenil on the large chondroitin 4-sulphate proteoglycan and the smaller chondroitin 6-sulphate-enriched proteoglycan is puzzling. By analogy with Brefeldin A [30], if synthesis of the small proteoglycan occurred in a more cis-oriented compartment of the Golgi, one possibility might be that during cyclofenil treatment these membranes are relocated to the endoplasmic reticulum and retain some activity for synthesizing chondroitin 6-sulphate. However, there is currently no direct evidence for

glycosaminoglycan synthesis outside the medial and trans Golgi compartments.

Finally, cyclofenil also affects some plasma membrane- as well as Golgi-related events. These include the synthesis of hyaluronan and the system A amino acid carrier. The effect on the former is more likely to be on the synthetase and its membrane association than on UDP-sugar availability, for the reasons discussed above. The effects of the drug at the plasma membrane are fairly specific, since transport of glucose and the activity of some amino acid carrier systems are little affected. It is noteworthy that other agents which perturb the protein secretory pathway, for example monensin, have widespread effects on cellular activities [19].

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