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Effects of Econazole, Fungizone and Pimafucin on Cell Growth, Lysosomal Enzyme Activity and Sulphate Metabolism of Cultured Human Skin Fibroblasts and Amniotic Fluid Cells

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Human skin fibroblasts and amniotic fluid cells showed sensitivity to antifungal agents in the order Econazole < Pimafucin < Fungizone, with the last initially reducing cell growth 10–20%, even at the level recommended for cell culture. The activities of the lysosomal enzymes α-L-fucosidase, β-D-glucuronidase and α-D-mannosidase were unaffected, even by high concentrations of all three antifungal agents. Sulphate incorporation by cultured fibroblasts was increased by removal of sulphate salts and Crystamycin (contains streptomycin sulphate) from the culture medium. Sulphate incorporation into and degradation from macromolecules was only slightly reduced by antifungal agents. Human skin fibroblasts and amniotic fluid cells can be cultured long term in the presence of Econazole or Fungizone and used for lysosomal enzyme assay and sulphate kinetic studies.

Using animal and HeLa cells Wyler *et al.* (1979) concluded that Econazole was an excellent antifungal agent for *in vitro* work. Skin fibroblasts and amniotic fluid cells are used extensively to diagnose human genetic diseases. The effect of Econazole on the growth of these two cell types was compared with the effects of the polyene macrolides Fungizone and Pimafucin. As resistant fungi may be encountered, the effects of higher levels of the antifungal agents, as well as the recommended tissue culture levels (normal dose), were studied. Although it is known (Akiyama *et al.*, 1979; Fisher *et al.*, 1979) that the polyene macrolides alter membrane function, effects on enzyme activities have not been studied. The effects of the antifungal agents on lysosomal enzyme activity were, therefore, studied. The incorporation into and removal of radioactive labelled sulphate from macromolecules is used in the diagnosis of the mucopolysaccharidoses. These processes were studied to see if the functioning of the lysosomal enzymes in intact cells or the detection of abnormal cell lines was affected by the antifungal agents.

MATERIALS AND METHODS

All experiments were carried out in 50 mm plastic Nuclon petri-dishes with Hams FlO (Flow Lab. Ltd.) and 20% calf serum (Gibco-Biocult Ltd.) in the presence of 5% CO_2–air. Skin fibroblasts at passages 7–12 and amniotic fluid cells at passages 3–5 were used. Cells were removed using 0.25% trypsin and 0.04% EDTA in phosphate buffered saline pH 7.3 (Dulbecco A, Oxoid Ltd.) and frozen at −70°C until assayed. Extracts were prepared in distilled water by sonication for 20 s at 4–6 μm at 5°C (M.S.E. Ultrasonic Disintegrator) and centrifuged at 1000*g* for 10 min. The activities of α-L-fucosidase (EC 3.2.1.51; Butterworth and Guy, 1977), β-D-glucuronidase (EC 3.2.1.31; Butterworth *et al.*, 1973) and α-D-mannosidase (EC 3.2.1.24; Butterworth, 1980) were assayed using 4-methylumbelliferyl conjugates (Koch-Light Lab. Ltd.). Protein was estimated (Lowry *et al.*, 1951) using bovine serum albumin (Sigma Ltd.) as standard.

For the study of the effects of the antifungal agents, duplicate cultures of each cell line were set up with normal medium, medium containing the recommended concentrations for cell culture (normal) of Econazole (1 μg/ml; Cilag-Chemie AG), Fungizone (2 μg/ml; Squibb Ltd.) and Pimafucin (2.5 μg/ml; Brocades Ltd.), or with increasing concentrations of the three antifungal agents. Media were changed after three days and cells taken for assay after six days, when cells in normal medium were confluent.

For the study of radiolabelled sulphate incorporation, an initial comparison between the ethanol method (Fratantoni *et al.*, 1968) and a technique using cetylpyridinium chloride (Koch-Light Lab. Ltd.) was made. For the cetylpyridinium chloride method, cells were trypsinized and an extract prepared in 2 ml distilled water by sonication for 20 s at 4–6 μm at 5°C. An aliquot (1 ml) was pipetted into a plastic universal container (Sterilin Ltd.) and 20 ml 0.1% cetylpyridinium chloride in 0.1 mol/l sodium citrate buffer pH 4.8 added. After 15 min the complexed material was filtered under vacuum on to a 2.4 cm glass fibre disc (Whatman Ltd.; GF/B) held in a Swinnex-25 holder (Millipore Ltd.), washed with 10 ml 0.1% cetylpyridinium chloride in buffer and finally with 3 ml ethanol. After drying at 100°C the discs were counted in a toluene scintillant. Various parameters were altered to study their effects on the incorporation of labelled sulphate by normal and abnormal skin fibroblasts and amniotic fluid cells. Culture medium with sulphate salts (Hams FlO(+))

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and with these salts changed to the chloride forms (Hams FlO(−)) were used. Calf serum was dialysed at 5°C against 40 vol. of phosphate buffered saline with four changes over 3 h, after which amino acids were undetectable. Crystamycin (Glaxo Ltd.) was added to give a final concentration of 60 μg/ml benzylpenicillin and 100 μg/ml streptomycin sulphate. Normal concentrations of Econazole (1 μg/ml), Fungizone (2 μg/ml) and Pimafucin (2.5 μg/ml) were added as appropriate. Radioactive sulphate (Na_2SO_4; 100 μCi/μmol; Radiochemical Centre, Amersham) was added to give a final concentration of 10 μCi/ml. Confluent cell lines were cultured for three days in the presence of radioactive sulphate and then analysed for its incorporation. For degradation studies, cells were grown in Hams FlO(−) medium with 10 μCi/ml sulphate for three days. The cells were then washed three times with phosphate buffered saline and changed to Hams FlO(+) with or without normal or times five normal concentrations of the antifungal agents. Duplicate cultures of each cell line were analysed at various time points for up to three days for residual sulphate incorporation.

RESULTS

The reproducibilities of the culture system and enzyme assays were checked by growing ten replicates of a cell line. The coefficient of variations for protein, α-L-fucosidase, β-D-glucuronidase and α-D-mannosidase were 2.7%, 2.7%, 3.0% and 3.0% respectively. This means that values needed to differ by more than 10% to be significant at the 5% level.

Ten skin fibroblast cultures showed average growth, taken as total protein of 101, 91 and 99% of control medium for the normal concentrations of Econazole, Fungizone and Pimafucin respectively. A Hunter and a Hurler cell culture were similarly affected. As the nature of amniotic fluid cell cultures varies, results for ten cultures at 3–4 passages are given in Table 1. Amniotic fluid cells showed a reduction in growth with Fungizone slightly greater than for fibroblasts. The lysosomal enzymes tested were essentially unaltered. Culturing six different control fibroblasts and four amniotic fluid cell lines in increasing concentrations of the antifungal agents results in decreasing cell survival (Table 2). The least toxic was Econazole, whilst amniotic fluid cells were less sensitive to Pimafucin and more sensitive to Fungizone than fibroblasts. The lysosomal enzymes were essentially unaltered by increased antifungal levels. Three control and a Hunter fibroblast line were cultured for five passages in normal concentrations of Econazole and Fungizone and it was noted that the later passages gave growth in Fungizone similar to that of Econazole and control medium. Cells cultured in times 7.5 normal Pimafucin or greater than times 10 normal Fungizone were heavily vacuolated (Figure 1a), with a number of multinucleate giant cells being present (Figure 1b). These cells could be serially cultured and returned to normal morphology on removal of the antifungal agents.

Table 2 Average growth (total protein/dish) of six skin fibroblast and four amniotic fluid cell cultures in medium containing increasing amounts (times normal) of Econazole (E), Fungizone (F) and Pimafucin (P) expressed as a percentage of that in control medium

Antifungal concentration	*Fibroblasts*			*Amniotic fluid cells*		
	E	*F*	*P*	*E*	*F*	*P*
1	102	89	98	96	79	94
2	98	76	94	95	68	90
5	92	70	86	90	52	85
10	86	62	7	85	25	40

Table 1 Growth (total protein/dish) of ten amniotic fluid cell cultures in medium containing the normal concentration of Econazole (E), Fungizone (F) and Pimafucin (P) as a percentage of that in control medium

Cell type[1]	*Protein*			*α-L-Fucosidase*			*β-D-Glucuronidase*			*α-D-Mannosidase*		
	E	*F*	*P*	*E*	*F*	*P*	*E*	*F*	*P*	*E*	*F*	*P*
F	114	85	117	106	96	99	103	90	108	120	97	127
F	101	66	82	95	87	92	101	85	101	109	99	111
F	109	104	116	104	94	112	102	107	110	110	109	106
M	103	86	95	108	98	110	105	103	100	114	113	112
M	90	69	94	99	94	95	111	87	89	93	113	99
M	96	88	133	103	101	112	103	111	121	100	109	133
M	93	76	93	99	110	104	97	114	104	92	94	96
M	115	75	112	107	92	95	105	106	96	105	112	101
E	104	91	102	95	80	95	96	100	104	103	109	101
E	99	86	103	101	89	116	96	96	106	105	103	121
Mean	102	83	105	102	94	103	102	100	104	105	106	111

[1] Type: F, fibroblastic; M, mixed; E, epithelioid

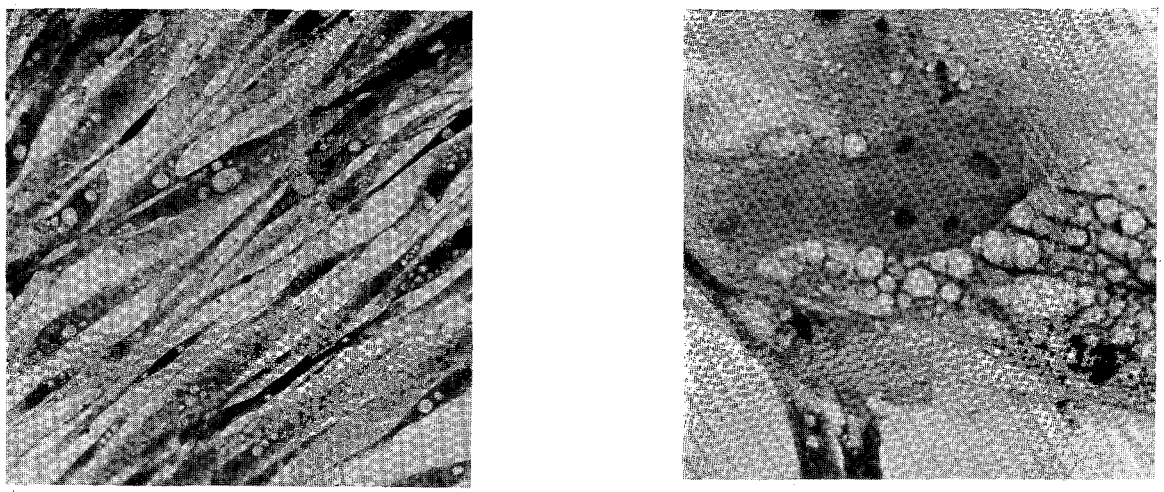

Figure 1 Human skin fibroblasts cultured in the presence of times 7.5 normal Pimafucin showing (a) vacuolation and (b) giant multinucleate cell. Magnification ×150

A comparison of the sulphate incorporation methods using twelve replicate cultures showed that, although the means were similar for the ethanol (226 000 dpm/mg protein) and the cetylpyridinium chloride (230 000 dpm/mg protein) methods, the coefficient of variation was larger for the former being 7.2% and 4% respectively. The cetylpyridinium chloride method was, therefore, used for all further experiments. Results of initial studies of certain culture parameters made before investigation of the antifungal agents are given in Table 3. Dialysed serum reduced the growth of fibroblast cultures by about 20% and the incorporation of sulphate was also reduced. Addition of glutamine did not affect the results. Changing the salts in Hams FlO from sulphate to chloride approximately doubled the incorporation of labelled sulphate. Omission of Crystamycin increased the incorporation by about 30% with Hams FlO(+) and increased cell growth of some cell lines by about 10%. However, changing the sulphate salts to chloride together with omission of the Christamycin increased incorporation of labelled sulphate over four-fold. These changes were due to dilution of radioactive sulphate by inorganic sulphate in Hams FlO or by streptomycin sulphate in Crystamycin. Routinely, Hams FlO with chloride salts and no antibiotics was used for the analysis of sulphate incorporation. Using this medium, together with the cetylpyridinium chloride method, twelve Hunter and Hurler cell lines have been successfully detected.

The addition of Econazole and Fungizone sometimes gave lower values for sulphate incorporation for both normal and abnormal cell lines (Table 4). A third Hunter's line gave 96% sulphate incorporation with Econazole. However, the decreases observed would in no way interfere with the ability to diagnose Hunter and Hurler fibroblasts. The degradation of labelled sulphated macromolecules was unaltered in the presence of normal and times 5 concentration of the antifungal agents.

The possibility that the antifungal agents might interfere with chromosome analysis was briefly studied. Using the normal concentrations of Econazole, Fungizone and Pimafucin no alterations to the chromosomes, including trypsin banding patterns, were evident. Econazole at times 7.5 normal did not result in any alterations.

Thus any of the three antifungal agents tested could be included in the medium when assaying for lysosomal enzymes, undertaking sulphate kinetic studies or when analysing chromosomes.

Table 3 Effect (%) of the sulphate content of medium, serum and Crystamycin[1] on sulphate incorporation by cultured skin fibroblasts

Culture condition	*Sulphate incorporation (%)*			
	Control	*Control*	*Hurler*	*Hunter*
Hams FlO(+) Serum + Crystamycin	100 (10 500)[2]	100 (6600)	100 (38 800)	100 (49 700)
Hams FlO(+) Serum − Crystamycin	130	132	130	125
Hams FlO(+) Dialysed Serum + Crystamycin	63	71	96	37
Hams FlO(+) Dialysed Serum + Crystamycin	132	106	155	69
Hams (FlO(−) Serum + Crystamycin	187	222	284	242
Hams FlO(−) Serum − Crystamycin	453	453	565	440

[1] Crystamycin – Benzylpenicillin 60 µg/ml + streptomycin sulphate 100 µg/ml

[2] Sulphate incorporation in dpm/mg protein

Table 4 Effect (%) of the normal concentration of Econazole, Fungizone and Pimafucin on the incorporation of sulphate by cultured skin fibroblasts

Condition	*Control*	*Control*	*Control*	*Control*	*Hurler*	*Hurler*	*Hunter*	*Hunter*	*Mean*
Control	100 (31 000)[1]	100 (29 000)	100 (33 000)	100 (21 500)	100 (245 000)	100 (197 000)	100 (202 000)	100 (166 000)	100
Econizole	103	95	94	107	84	88	108	89	96
Fungizone	100	93	96	111	82	78	96	82	92
Pimafucin	ND	ND	97	105	ND	92	ND	95	97

[1] dpm sulphate incorporated/mg protein

ND, Not done

DISCUSSION

The effect of Fungizone (Amphotericin B-deoxycholate) and to a lesser extent Pimafucin on cultured cells has been concentrated on animal or malignant cells, with few studies on normal human cells. Published results indicate a marked variation in sensitivity of cultured cells to antifungal agents. Levels of Fungizone little above treatment dose result in cell loss for a number of cell types (Block *et al.*, 1979; Dolberg and Bissell, 1974; Fisher *et al.*, 1975; 1979; Fujimoto *et al.*, 1978; Kuwano *et al.*, 1972; Medoff *et al.*, 1974). Compared to the present findings for infantile skin fibroblasts, the results of Fujimoto *et al.* (1978) indicate that adult skin fibroblasts are more susceptible to Fungizone. Pimafucin has been noted (Akiyama *et al.*, 1979; Fisher *et al.*, 1978) to be less toxic for hamster fibroblasts than Fungizone, as was found for the human cells. However, Pimafucin is less effective against yeasts than Fungizone (Kobayashi and Medoff, 1977) and is, therefore, not usually used. The greater sensitivity to Fungizone of amniotic fluid cells compared with skin fibroblasts agreed with the finding (Fisher *et al.*, 1975) that amnion cells were more susceptible to this antifungal agent than lung fibroblasts. The results for human cells are in agreement with those of Wyler *et al.* (1979), who showed that Econazole was less toxic to cell cultures than Fungizone and was at least as effective an agent against fungal contamination. The vacuolization of cells with high levels of Pimafucin and Fugizone results from the large inflow of water consequent to alteration of the cell membrane (Festen *et al.*, 1981) and reversion to normal morphology on removal of the antifungal agent agreed with the finding that cells could recover if the agent was removed (Fisher *et al.*, 1975). Normal lysosomal enzyme activities in the presence of the antifungal agents agreed with the postulate of Kumar *et al.* (1974) that failure to break down incorporated DNA in the presence of Fungizone was not due to lysosomal enzyme inhibition. The normal degradation kinetics of sulphate labelled macromolecules also indicate that lysosomal functioning is indeed normal and that the water imbalance of the cell did not affect lysosomal integrity.

The results indicate that Econazole would be a suitable alternative to Fungizone for inclusion during routine culture of human skin fibroblasts and amniotic fluid cells, having little or no effect on lysosomal enzyme activities or sulphate kinetic studies.

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Book Review

Noninvasive Probes of Tissue Metabolism. J. S. Cohen, editor. Wiley, New York, pp. 270.

Perhaps 'non-perturbing' is more awkward than 'non-invasive' but it would seem to be a more applicable phrase for the collection of techniques assembled in this book. In the methods presented here, there are no externally added probes to perturb the metabolic systems under investigation. Excision of corneas, however, along with catheterization, hepatocyte isolation and tissue freeze-clamping do count as invasive techniques in my book.

Semantics aside, I found this book readable but frustrating. Most of the authors have made worthwhile attempts to ensure that their contributions are comprehensible to non-experts, but as a 'multidisciplinary volume' the book was disappointing. Two of the three articles dealing with topics other than the dominant nuclear magnetic resonance (n.m.r.) were inadequate as reviews or examples. The chapter on mass spectrometry (Weaver) was too brief and too theoretical, without the taste of real useable applications. The chapter on surface fluorometry (Masters *et al.*) was clear and interesting but, being confined to the redox states in animal corneal tissue, was somewhat exclusive.

Several of the n.m.r. articles on the other hand both covered their allotted area and stimulated my interest. The description, by Cohen and Shulman, of ^{13}C n.m.r. studies of the pathways of gluconeogenesis demonstrates the precise nature of the method in tracing specific carbon units through their respective pathways. The capability of observing a large spectrum of pathway components simultaneously enables the generation of a much broader picture of metabolic interactions than is possible by any other means. This ability is also the basis for ^{31}P n.m.r. studies of high energy phosphate-containing metabolites. The paper by Fossel and Ingwall on energy metabolism in the working rat heart is an example; an examination of the metabolic flux of high energy phosphates at various points in the heart beat cycle. This pinpoints the advantage of this non-invasive technique in being able to study metabolic flux coordinated with function, without disrupting the system.

The chapter concerning positron computed tomography is a welcome addition to the techniques already discussed. The work on imaging metabolic processes using this type of measurement is only beginning but it opens up new possibilities for relating biochemical function and structural relationships. I suspect however that like n.m.r. it will be some time before the levels of signal resolution can provide more than a qualitative picture of intact organs or organisms.

In summary, as a review book, useful but not imperative reading and as with all these works on new techniques it leaves me wondering how applicable some of the ideas are to my field. It would be appreciated if the authors of articles like these could provide for the non-experts some form of guide as to the range and applicability of their techniques. After all, their techniques need problems to solve.

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