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STUDIES ON THE STABILITY AND CELLULAR DISTRIBUTION OF DIOXOPIPERAZINES IN CULTURED BHK-21S CELLS

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Abstract—Attempts have been made to discover why dioxopiperazines of closely related chemical structure possess widely differing degrees of toxicity towards cultured cells. ^{14}C-labelled compounds used for comparative studies were ICRF 159 and ICRF 154 which show similar levels of cytotoxicity and ICRF 192 which is virtually inactive. No difference in the chemical stability of these compounds was found since all three hydrolysed in tissue culture medium at the same rate and to similar products. Investigation of the interaction of the compounds with cultured BHK-21S cells has also shown no differences since each of them appears to enter cells by diffusion and be metabolically inert. In addition, no physical or covalent binding to cellular macromolecules has been found.

Homologues of the antitumour agent ICRF 159 [R = CH_3, R′ = H, Fig. 1; *dl* - 1,2 - *bis*(3,5 - dioxopiperazin - 1 - yl)propane] have been found to possess widely differing degrees of activity against experimental tumours[1, 2] and cells in tissue culture[2–6]. For example, ICRF 159 and ICRF 154 (R = R′ = H, Fig. 1) inhibit the proliferation of BHK-21S cells to similar extents, and are active antitumour agents, while ICRF 202 (R = CH_3, R′ = C_2H_5, *erythro* configuration, Fig. 1) has about 50 times this activity and ICRF 192 (R = C_2H_5, R′ = H, Fig. 1) is inactive. It is not known how the dioxopiperazines act, but they are thought to have a novel mode of action because no cross-resistance has been shown with other cytotoxic agents[7, 8]. This paper describes attempts to find differences in behaviour between the active agents ICRF 159 and 154 and the inactive homologue ICRF 192 which might indicate why there are vastly different degrees of cytotoxicity between such closely related compounds, and how the compounds cause their toxic effects.

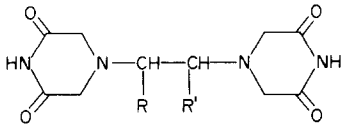

Fig. 1. Dioxopiperazine structure.

Since it had been reported[9] that ICRF 159 lost its biological activity during incubation in tissue culture medium a comparison was made first of the stabilities of active and inactive compounds labelled with ^{14}C. Uptake of the ^{14}C-labelled dioxopiperazines by BHK-21S cells was also studied and attempts were made to determine whether differences in metabolism and binding of the agent occurred.

MATERIALS

[^{14}C]ICRF 159 (sp. act. 5·56 mCi/m-mole), and [^{14}C]ICRF 192 (sp. act. 2·56 mCi/m-mole) labelled in the methylene carbons of the rings, and [^{14}C]ICRF 154 (sp. act. 5·08 mCi/m-mole) labelled in the centre chain were synthesized in these laboratories. (A. M. Creighton, unpublished work).

METHODS

Scintillation counting

Aqueous samples were dissolved in Aquasol (New England Nuclear) and radioactivity was measured in a Mark II Nuclear Chicago Scintillation Spectrometer. Dried filters were counted in toluene phosphor (4 g PPO/l. toluene).

Cell culture

BHK-21S cells were grown in stirred suspension cultures in EP medium buffered with 28 mM Hepes and supplemented with 5% calf serum and 0·05% carboxymethyl cellulose. (EP is a modification of Dulbecco's E4 medium[10] in which the phosphate concentration is increased ten times and calcium chloride is omitted). At the start of experiments, cells were in logarithmic growth at densities between 2·5 and 5×10^5 cells/ml. The population doubling time of BHK-21S cells grown in suspension was about 12 hr and the logarithmic phase of growth was maintained up to cell densities of about $1{\cdot}2 \times 10^6$/ml.

Stability of dioxopiperazines

Labelled compounds were dissolved in buffer or medium and samples were taken at intervals for thin layer chromatography (t.l.c.). Merck Silica Gel plates (0·25 mm layer thickness) were used and elution was carried out with either methanol–water (8:2 or 65:35 v/v) or ethyl acetate–acetone (9:1 v/v). The methanol–water systems resolve the dioxopiperazines and their hydrolysis products, but not the dioxopiperazines from their radiolytic decomposition products. The 80% methanol solvent gives better resolution of dioxopiperazines and hydrolysis products but the 65% methanol system will also resolve their tetra-acid synthetic precursors.

Administration of drugs to cell cultures

Because ICRF 154 and ICRF 192 were not sufficiently soluble in phosphate-buffered saline (PBS), it was convenient to dispense all three compounds from dimethyl sulphoxide (DMSO) solutions. The highest concentration of DMSO used in a cell culture was 0·5%, and by performing all the experiments involving ICRF 159 with and without 0·5% DMSO it was shown that the solvent made no difference to the results.

Uptake studies

Labelled dioxopiperazine was added to log phase cells at an initial density of about 4×10^5/ml and 2-ml samples were taken at intervals and filtered using gentle suction onto glass fibre filters (GF/C Whatman) on Millipore filter holders. The filters were washed four times with 10 ml ice-cold PBS, dried in an oven at 60° and radioactivity was measured using a toluene/PPO phosphor. Cell numbers were measured throughout the experiments so that uptakes by proliferating and non-proliferating cultures could be compared. Cells continue to proliferate in the presence of ICRF 192 and low doses of ICRF 154 and ICRF 159, but, in the presence of higher doses of the latter agents, numbers remain static principally because cells fail to complete cytokinesis[6].

Determination of the volume of cell water in BHK-21S cells

The technique used was essentially the same as the method of Hill[11] using [^{14}C]inulin carboxylic acid.

Binding studies

Cells were treated in suspension culture with labelled dioxopiperazine and then harvested and washed twice with ice-cold PBS before any of the following procedures were undertaken.

(a) *Covalent binding.* Total cell DNA and RNA was extracted by the phenol method of Kirby and Cook[12], and was purified by the method of Parish[13]. Protein was obtained from the first phenol extract by a modification[14] of the Schneider[15] procedure. The protein was found to be free of nucleic acids using the orcinol method for RNA[16] and the diphenylamine method for DNA[17]. DNA and RNA preparations contained less than 0·1% protein as measured by the method of Lowry *et al.*[18]. Nucleic acids were hydrolysed to bases using 72% perchloric acid at 100°[19] and the bases were separated by descending chromatography on Whatman No. 1 paper with isopropanol–conc. HCl–water (130:33:37) as solvent[20]. Spots were located with u.v. light and were then cut out and assayed for radioactivity.

(b) *Physical binding.* Nuclei prepared by hypotonic lysis[21] were swollen in distilled water at 0° for 30 min and broken in a P.T.F.E./glass hand homogenizer (T. W. Wingent, Cambridge). The suspension was made 2M with NaCl and the viscous solution produced was the nuclear fraction. The supernatant from the first centrifugation of nuclei was used as the cytoplasmic fraction. DNA was never found in the cytoplasmic fraction (diphenylamine assay).

The cytoplasmic and nuclear fractions were subjected to dialysis or ethanol precipitation in attempts to detect physical binding[22]. Aliquots containing known amounts of radioactivity were mixed with 5 vol of ethanol, placed at −10° for 2 hr and centrifuged at 5000 *g* for 10 min. Radioactivity was determined in the pellet and in the supernatant. Similarly, aliquots were exhaustively dialysed, the nuclear fraction against 2 M NaCl and the cytoplasmic fraction against PBS. Radioactivity was measured in the dialysate. In both the dialysis and ethanol precipitation experiments, assays of DNA, RNA and protein before and after treatment showed that there were no losses of these macromolecules.

Histone extraction

Nuclei were prepared by hypotonic lysis and histones were extracted from the washed nuclei with 0·4N H_2SO_4[23]. The extracted histone did not have absorbance peaks at 260 or 280 nm and was shown by electrophoresis[23] to contain the same five bands as calf thymus histone.

RESULTS

Stability of ICRF 159, 154 and 192

[^{14}C]ICRF 159 (~5 μg/ml) was incubated at 37° in 66 mM phosphate buffer pH 7·4 and aliquots were taken for thin layer chromatography in the 80% methanol–water system. Three breakdown products with R_fs of 0·5, 0·44 and 0·23 (ICRF 159 = 0·70) were found, though only the low R_f product accumulated at this pH. At pH 4–5 the higher R_f compounds were the major products, but when the pH was raised to around 7 they broke down to give the low R_f product. It therefore appears that the high R_f products are intermediates in the breakdown. It was found that the same products were formed at about the same rate as in the pH 7·4 phosphate buffer when ICRF 159 was incubated in a suspension culture of BHK-21S cells, thus indicating that the breakdown was a pH dependent hydrolysis which was independent of cells, serum or tissue culture medium components.

The chemical half-life of the ICRF 159 in 66 mM phosphate buffer at 37° varied between about 12 hr at pH 7·0 and about 6 hr at pH 7·6 (Fig. 2). 0·5% DMSO in the incubation made no difference to the hydrolysis. When [^{14}C]ICRF 154 and [^{14}C]ICRF 192 were compared with ICRF 159, they were found to have similar hydrolysis rates and products, although only one high R_f product was detected with ICRF 154. It was therefore clear that the differences in biological activity of these dioxopiperazines were not due to different rates or products of breakdown in the medium.

Uptake of dioxopiperazines into cells

No differences were found between the uptakes of each of the three dioxopiperazines by BHK-21S cells in suspension culture.

(a) at the maximum uptake (generally after 2–3 hr, Fig. 3) the concentration of dioxopiperazine in the cell water was the same as that in the surrounding medium (this was found for a series of concentrations between 20 and 200 μM).

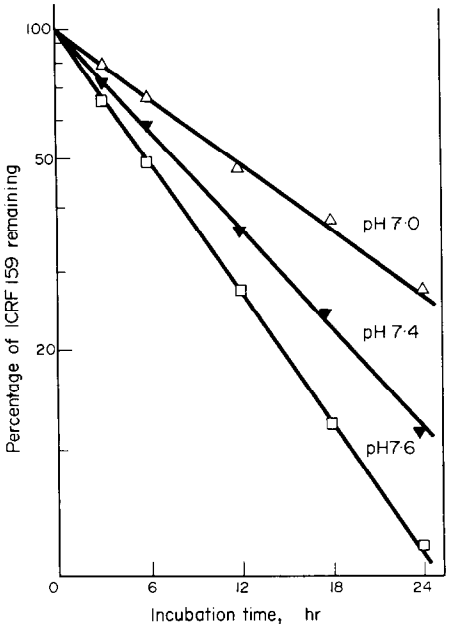

Fig. 2. The effect of pH on the chemical half-life of ICRF 159 in phosphate buffer at 37°.

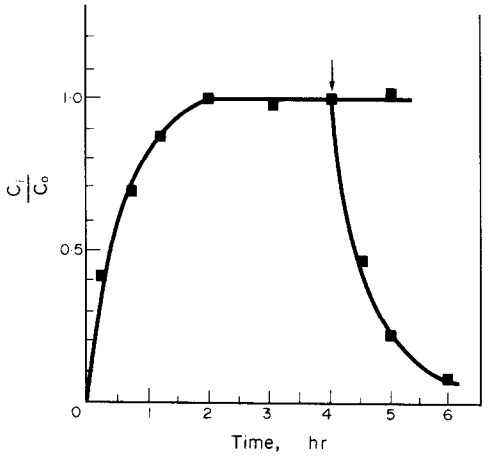

Fig. 3. Time course of the uptake and efflux of dioxopiperazines by BHK-21S cells at 37°. [^{14}C]ICRF 159 (100 μM) is shown, but virtually identical curves were obtained at doses of 20–200 μM and with ICRF 154 and 192. Ci/Co is the ratio of the concentration of dioxopiperazine in the intracellular water to its concentration in the extracellular medium. At the arrow (4 hr), cells were harvested and resuspended in fresh medium. (The intracellular water vol (9·0 mg/10^7 cells) was negligible compared with that of the extracellular medium).

(b) the dioxopiperazines were rapidly lost from cells when they were gently harvested by centrifugation and resuspended in fresh medium (Fig. 3).

(c) uptake is not inhibited by the presence of high concentrations of structurally related compounds (either active or inactive dioxopiperazines) (Table 1).

(d) uptake is not affected by 100 μM sodium cyanide (a dose which inhibits [^{3}H]lysine active transport in these cells) (Table 1).

(e) uptake was slowed in cells incubated at 0°, but the intracellular concentration reached that of the outside medium after about 21 hr (Fig. 4).

Table 1. Effect of high concentrations of unlabelled dioxopiperazines and cyanide on the uptake of [^{14}C]dioxopiperazines by BHK-21S cells

[^{14}C]dioxopiperazine (all 50 μM in the medium)	Inhibitor	Concn (μM)	Inhibition of uptake
159	154	100	0
159	192	400	0
154	159	400	0
154	192	400	0
192	159	400	0
159	NaCN	100	0
154	NaCN	100	0
192	NaCN	100	0

Labelled dioxopiperazine and inhibitor were added simultaneously to spinner cultures of BHK-21S cells. The time course of uptake of label to the plateau level was followed and compared with cultures lacking inhibitor. As no differences were seen in either rates or extents of uptake the results have been combined under one heading.

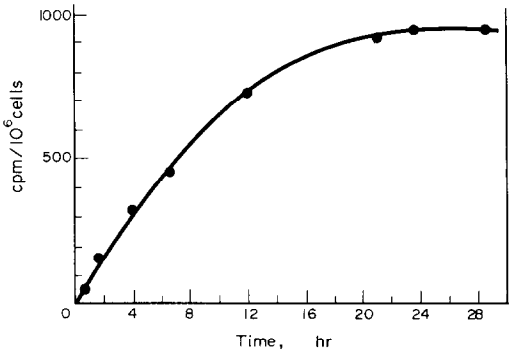

Fig. 4. Time course of the uptake of [^{14}C]ICRF 159 (100 μM) by BHK-21S cells at 0°. The plateau level of uptake corresponds to a concentration of 100 μM in the intracellular water.

It was therefore evident that differences in uptake were not the cause of the different degrees of cytotoxicity of the dioxopiperazines. No evidence was found in favour of the uptake being by active transport and it appears that the compounds enter cells by diffusion.

Binding of dioxopiperazines to cellular macromolecules

The next possible explanation of the differences in biological activity to be examined was that the active compounds bind to cellular macromolecules.

(a) *Covalent binding.* Cells were treated with labelled dioxopiperazines for 7, 16 or 24 hr and DNA, RNA and protein were isolated as described in Methods. Moderate levels of radioactivity were found associated with macromolecules from ICRF 159 and ICRF 192 treated cells, while only very low levels were present in macromolecules from ICRF 154 treated cells. (Table 2). The nature of the 'binding' was investigated by hydrolysis of the nucleic acids with 72% perchloric acid (see Methods). It was found that all the radioactivity from DNA labelled by any of the dioxopiperazines was associated with guanine, adenine and thymine, while the radioactivity from RNA was all as-

Table 2. Radioactivity from [^{14}C]dioxopiperazines associated with DNA, RNA and protein of BHK-21S cells

[^{14}C]dioxopiperazine (all at 100 μM in the medium)	Length of incubation (hr)	Specific activity* (dis/min per mg)		
		DNA	RNA	Protein
159	24	4785	4362	1702
192	24	10959	4838	1951
154	7	148	115	65
154	16	315	152	110

* 0·5–1 mg of material was assayed for radioactivity.

sociated with guanine and adenine. It was therefore clear that this did not represent covalent binding, as this pattern of labelling is consistent with the incorporation of radioactivity by *de novo* synthesis via one-carbon pathways. The labelling of protein was not analysed because there was insufficient radioactivity.

(b) *Physical binding.* ICRF 154 was used for physical binding studies because of the low levels of incorporation found with this compound. Evidence for physical binding was sought by comparing the removal of radioactivity from cytoplasmic and nuclear fractions of treated cells by dialysis and ethanol precipitation. As shown in Table 3, dialysis and ethanol precipitation were equally effective at removing label, whereas if there had been physical binding it should have been resistant to dialysis[22] but not ethanol precipitation.

Analysis of the dialysed and ethanol-precipitated fractions by the same techniques used for the covalent binding studies showed that all the radioactivity remaining associated with them could probably be accounted for by one-carbon incorporation.

The ethanol-soluble radioactivity (this is the free label from inside the cells) from ICRF 159, 192 or 154 treated cells was analysed by t.l.c., and it was found that the dioxopiperazines had broken down inside the cells to about the same extent and to the same products as they had in the outside medium. This finding demonstrated that the dioxopiperazines were not metabolised to any major extent, but does not exclude the possibility that a small amount of metabolism occurs at some restricted site.

As it has been reported that ICRF 159 associates with histones *in vitro* [24], binding to histones in cells was examined. Histones were extracted from cells treated with ICRF 154 (16 hr incubation) as described in Methods, and were found to contain lower levels of radioactivity per g than the total cell protein, indicating that there is no specific binding to histones *in vivo*.

Table 3. Removal of [^{14}C] ICRF 154* from cytoplasmic and nuclear fractions by dialysis and ethanol precipitation

Fraction	Percentage of radioactivity removed by: Dialysis	Ethanol precipitation
Cytoplasmic	97	98
Nuclear	83	82

* Cells were incubated for 16 hr with 100 μM ICRF 154.

DISCUSSION

The dioxopiperazines were originally synthesised as relatively non-polar derivatives of the chelating agent EDTA. The rationale being that less polar molecules would more easily enter cells and there break down to yield chelating agents capable of damaging cells by inhibiting metal ion dependent reactions[1, 3]. The present study has demonstrated that dioxopiperazines do enter cells (probably by diffusion) and that they hydrolyse inside the cells. In addition, recent experiments in which these hydrolysis products were found to inhibit DNA polymerase *in vitro* (Dawson, unpublished) have indicated that they do so by chelating Mg^{2+}, but no difference in this ability was found between the low R_f hydrolysis products of toxic and inactive dioxopiperazines.

The identities of the hydrolysis products have not been determined, but they are not the tetraacid synthetic precursors of the dioxopiperazines, because the disodium salts of these compounds have R_f values of about 0·7 in the 65% methanol–water t.l.c. system whereas the final hydrolysis products have R_f values of about 0·5. It is probable that the final hydrolysis product has both rings opened and is the *bis*-monoacid–monoamide derivative, while the intermediates still have one intact ring. The finding that hydrolysed ICRF 159 is not cytotoxic when administered to cell cultures[5] and that the 12 hr chemical half-life of ICRF 159 at pH 7·0 corresponds with the 12 hr biological half-life at pH 6·8–7·0[9] indicates that the intact dioxopiperazine is the cytotoxic agent. It is not known whether hydrolysed ICRF 159 enters cells and therefore it is possible that the hydrolysis product is cytotoxic but appears to be inactive in these experiments because it is too polar to enter cells. However, fresh ICRF 159 is very quick to exert its effects on chromosome condensation in cells entering mitosis[5] and this argues against the involvement of hydrolysed ICRF 159 in this possibly most crucial cytotoxic activity. Intracellularly formed hydrolysis products of active dioxopiperazines may contribute to the toxicity of the agents by interacting with some specific site, but whether or not this would involve chelation is not known. Non-specific chelation of Mg^{2+} does not seem to have lethal consequences as the hydrolysis products of both active and inactive dioxopiperazines accumulate in the cells during incubations and are apparently capable of chelating Mg^{2+} to similar extents.

Dioxopiperazines have been found to produce similar effects on cells to those produced by X-rays and alkylating agents[4, 6] and it has been suggested[4] that they may act as acylating agents. In the present study, no covalent binding to cellular DNA or RNA was detected and although the protein 'binding' could not be analysed it is again thought likely to be incorporation because the level of labelling by ICRF 154 was very low. These findings indicate that the toxicity of the active compounds is not due to general acylation, but do not rule out the involvement of low levels of covalent binding (perhaps specific) which are below the limits of detection. These limits are imposed by the specific activity of the agent, and in the present case, by the difficulty of detecting low levels of

binding amongst a background of incorporated label. Thus, binding of ICRF 154 below levels of about 10 molecules per molecule of DNA (MW 10^9) would probably be undetectable, and for ICRF 159 and 192, the presence of increased incorporation would probably allow a somewhat higher degree of binding to go undetected. Attempts to lower the detection limits by using high specific activity [^{3}H]ICRF 215 (R = CH_3, R' = C_3H_7, Fig. 1) labelled in the propyl side chain, were unsuccessful because the compound rapidly broke down by radiolysis and give rise to high levels of incorporated label (Jeffery and Dawson, unpublished).

Physical binding was also not found even though a gentle method of lysing cells (hypotonic lysis) was used which avoided the use of detergents and organic solvents which might have dislodged any weakly bound molecules. In these experiments the lysed cells were only crudely separated into cytoplasmic and nuclear fractions so that cell components (e.g. membranes), which might be expected to have loosely bound molecules attached, would not be lost.

There is a discrepancy between the lack of binding of ICRF 154 to histones in cells in the present study and the association of ICRF 159 with histones found *in vitro* [24]. This seems unlikely to be due to the method of histone extraction from cells (acid extraction, which might dislodge ionically bound compounds), because the physical binding experiments did not reveal any bound counts in the nuclear fraction which could be associated with histones. Further investigation of the nature of the *in vitro* binding is needed to resolve the anomaly.

The incorporation of label into macromolecules via one carbon pathways was much more marked with ICRF 159 and ICRF 192, which were labelled in the rings, than with ICRF 154 which was labelled in the centre chain and gave almost negligible levels of incorporation. It is therefore evident that the occurrence of incorporated label is related to the site of labelling and not the biological activity of the dioxopiperazine, but it should be noted that even with ICRF 192, which gave rise to the highest levels of incorporation, the amount of label incorporated in 24 hr by growing cells was only 0·05% of the label in the medium.

It became clear as the radioactive compounds aged that radiolysis was occurring during storage, because radiodecomposition products became detectable by t.l.c. and the compounds gradually discoloured. Repeats of binding experiments using older labelled material showed increased levels of incorporation which indicated that radiolytic decomposition products are probably a major source of the label which enters the normal metabolic pool. The reason why the ring-labelled compounds release label more than centre-chain labelled compounds could be that the ring label is more labile, or that the products of radiolysis are more likely to be in a form capable of entering the one-carbon pool. Another alternative is that even though no metabolism was detected, a small amount could occur leading to the release of label from the rings into the metabolic pool.

In conclusion, no differences in stability, uptake, metabolism, or binding have been found which could explain why ICRF 159 and 154 are cytotoxic and ICRF 192 is not. However, the study has eliminated some of the less interesting possibilities for differences between active and inactive agents, leaving it more likely that when a difference is found, it will be directly related to the mode of action.

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