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Antitumour Activity and Cell Kinetic Effects of Pyrazofurin *In Vitro*

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Abstract—*The lethal and cytokinetic effects of pyrazofurin (PYF) have been studied in vitro. Survival curves following a 1- or 24-hr drug exposure were exponential-plateau for both suspension cultures (L5178Y and L1210 cells) and cell monolayers (NIL 8 cells). Cytotoxicity was more related to duration of exposure than to dose. Cell kill occurred predominantly in the G_2 phase, but with increasing drug concentrations S phase cells were also affected. PYF treatment resulted in an accumulation of cells in the S phase and/or at the G_1/S boundary. Combinations of PYF with methotrexate (MTX), cytosine arabinoside (Ara C), 5-fluorouracil (5FU), 5-azacytidine (5Aza), 2,4-diamino-5-(3,4′-dichlorophenyl)-6-methylpyrimidine (DDMP) or hyperthermia produced better than additive results.*

INTRODUCTION

PYF EXHIBITS a variety of antitumour activity, proving highly effective against certain transplantable animal solid tumours but ineffective against others [1, 2]. *In vitro* studies have confirmed the range of sensitivity of various cell lines to PYF [3–6]. Biochemical studies have established that PYF, after conversion to the 5′-phosphate derivative, blocks pyrimidine synthesis *de novo* by competitively inhibiting ortodylate decarboxylase [1, 4–6]. Differences in the mechanism of activation and the nucleotide's subsequent fate have been detected and correlations claimed between these biochemical differences and drug sensitivities [6].

In initial clinical trials responses were noted in patients with AML, mycosis fungoides and breast cancer [2, 4] and some therapeutic benefit seen in heavily pretreated multiple myeloma patients [8]. However, PYF lacks activity in advanced melanoma, lung and colo-rectal cancers, metastatic sarcomas and epidermoid carcinomas of the head and neck [8, 9]. Combination chemotherapy protocols are now being considered [10] and laboratory studies have shown increased cell kill in combination with Ara C [3, 4] or 5Aza [5].

This study was undertaken to define the cell cycle kinetic effects of PYF in cell lines exhibiting different sensitivities to the drug and to identify synergistic or antagonistic drug combinations.

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MATERIALS AND METHODS

Drugs

These drugs were kindly donated for this study: PYF and vincristine (VCR) by Eli Lilly & Co., Basingstoke, U.K., Hydroxyurea (HU) by E.R. Squibb & Co., London, U.K., MTX by Lederle Laboratories, Gosport, U.K., Adriamycin (ADR) by Farmitalia Carlo Erba, Barnet, U.K., and DDMP by Wellcome Research Laboratories, Beckenham, U.K.

Cell culture and survival assays

The maintenance of L5178Y lymphoblasts and L1210 cells has been described previously [10]. MTX-resistant sublines of each of the parent (*P*) lines were used: L5178Y/MTX-R cells resistant due to a transport defect and an elevated dihydrofolate reductase (DHFR) level, maintained in 50 μM MTX, and L1210/MTX-R cells with an elevated DHFR level only, maintained in 2 μM MTX. Cell survival was assessed by cloning in agar suspension culture [10]. Cells were counted using a Coulter counter (Model ZB1).

Synchronization techniques for NIL 8 cells

(i) Using low serum (0.25%) and HU (2.5 mM) as described previously [11].

(ii) Using mitotic selection—logarithmically-growing cells were media-changed. One hour later cells were harvested by gentle shaking and replated at 10^4/ml for experimentation.

Flow microfluorimetry (FMF)

Drug effects on cell cycle progression of asynchronous cultures were analysed by measurements of the relative DNA content of individual ethanol-fixed and mithramycin-stained whole cells using a fluorescence-activated cell sorter (FACS-1 Becton Dickinson, California, U.S.A.) as described elsewhere [11].

Autoradiography

One hour after mitotic selection of NIL 8 cells 1 μCi/ml [^{3}H]-thymidine was added. Autoradiographs were prepared from individual cultures, arrested at the times shown in Table 1, as described earlier [11].

Table 1. Effects of a 1-hr exposure to 0.25 μg/ml on the progression of synchronized NIL 8 cells into DNA synthesis

Time after selection (hr)	Percentage labelled nuclei determined autoradiographically: Time of drug treatment after selection (hr) Control	1–2 (G_1)	2–3 (G_1)	3–4 (G_1/S)	4–5 (S)
3	6	5	5	—	—
3.5	17	19	16	—	—
4	32	38	23	18	—
4.5	55	57	42	40	—
5	79	85	55	53	66
5.5	90	90	63	58	70
6	89	91	74	69	81
6.5	90	90	80	78	91
7	89	90	82	78	94
7.5	90	91	84	80	94
8	—	90	84	77	95
8.5	—	—	84	81	95

RESULTS

Lethal effects of PYF

Asynchronous cells. Figure 1 shows the effects of a 24-hr exposure of four lines of logarithmically-growing murine lymphoma cells to a range of PYF concentrations. The survival curves are characterized by an initial reduction in colony-forming ability followed by a plateau region, with no further reduction in viability with increasing drug concentrations. L1210 cells are approximately 2.5 times more sensitive to PYF than L5178Y cells. Selection for resistance to MTX in L5178Y cells does not alter their sensitivity to PYF. However, some resistance to PYF is associated with MTX resistance in L1210 cells, since the LD_{30} values differ by a factor of 3.

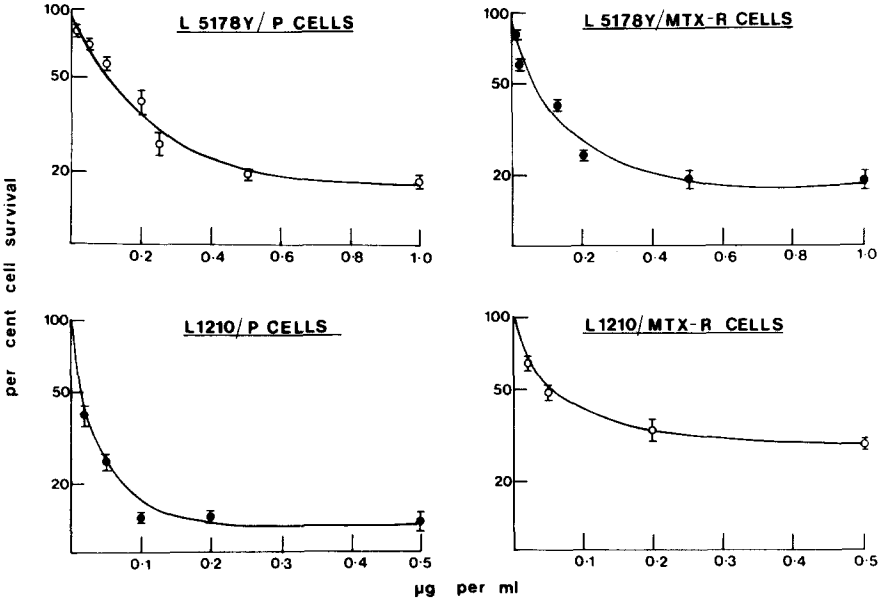

Fig. 1. Lethal effects of a 24-hr exposure of logarithmically-growing mouse lymphoma cells to varying concentrations of PYF. Survival was determined by colony-forming assays and each point represents the mean of 4 assays ± S.E.

The time-dependent killing effects of a fixed dose of PYF (1 μg/ml) on logarithmically growing L5178Y cells are shown in Fig. 2. There is increasing kill with increasing time exposure over a 30-hr period in both cell lines.

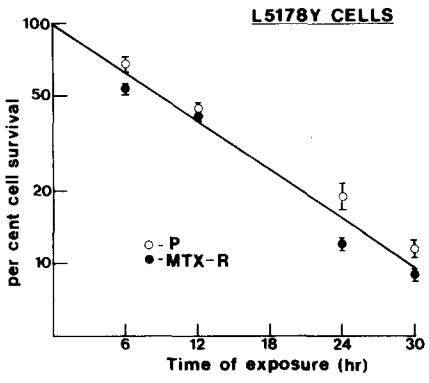

Fig. 2. Time-dependent killing effects of PYF (1 μl/ml) on logarithmically-growing L5178Y cell lines, as determined by colony-formation. Each point represents the mean of 6 assays ±S.E.

Figure 3 shows the effects of PYF on the survival of NIL 8 cells after exposure for 1 hr (Fig. 3A) or 24 hr (Fig. 3B). The survival curves plateau before a log cell kill is achieved and increased kill occurs with longer exposure time.

Synchronized cells. Synchronized NIL 8 cells were exposed to PYF for 1 hr at all stages of the cell cycle to determine any cell cycle specific cytotoxicity. Cells treated with PYF showed fluctuations in survival depending on their position in the cell cycle. The lower dose of PYF (0.25 μl/ml) resulted in cell kill predominantly and almost exclusively in the G_2 phase. At the higher concentration maximal cell kill again occurred in G_2 but there was also significant loss of survival of S phase cells. Cells in mitosis and early G_1 were least sensitive to PYF. These specific cell cycle effects were apparent irrespective of the method of synchronization used.

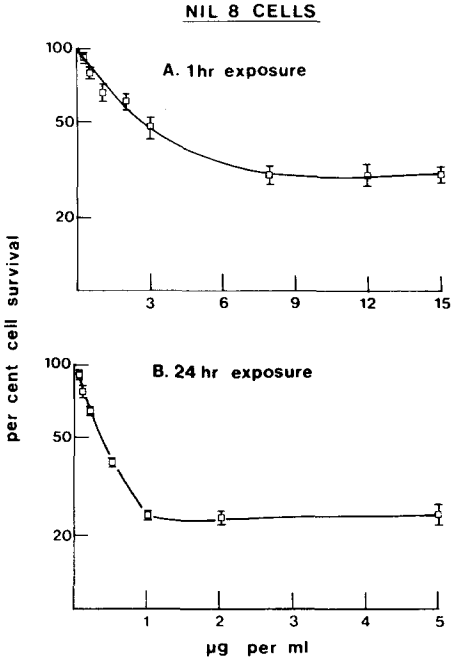

Fig. 3. Lethal effects of PYF on logarithmically-growing NIL 8 cells, as determined by colony formation. (A) 1 hr exposure; (B) 24 hr exposure. Each point represent the mean of 6 assays ±S.E.

Effects of PYF on cell cycle progression

To confirm these cycle-dependent cell killing effects of PYF asynchronous cultures of NIL 8 cells were treated with PYF at different concentrations for various exposure times and examined by FMF (Fig. 5). Figure 5(A) shows that at 0.1 μg/ml, a concentration which allows 80% survival, there is a slight reduction in the G_2+M peak and this effect is exaggerated at 0.2 μl/ml. However, at the higher concentrations of 0.5 and 1 μg/ml, which reduce survival below 50%, there is a marked build-up of cells in the S phase and/or G_1/S boundary. These data are consistent with cell kill, occurring predominantly in G_2 at low drug levels and during S at higher concentrations. Figure 5(B) shows a similar pattern of events. Within 3 hr there is a reduction of the G_2+M population followed at 9 hr by accumulation in S and/or near G_1/S. This accumulation is consistent with the autoradiographic data (Table 1) showing delayed entry of synchronized cell into S following treatment in late G_1. Similar results (not included) were obtained with L5178Y/P cells.

Cytotoxic effects of drug combinations including PYF

The reported synergy of PYF with other pyrimidine analogs [4, 5] prompted our examination of the effects of certain standard antitumour agents in simultaneous combination with PYF on the viability of L5178Y/P cells. Drug doses were selected with individually reduced survival by approximately 50% within 24 hr. 'Expected survivals' from the combinations were calculated as described by Drewinko *et al.* [12] and compared with the observed survival data in Table 2. VCR, HU and ADR showed additive effects with PYF, whilst slight synergy was seen with DDMP, MTX, Ara C, 5Aza, 5FU or hyperthermia. There was no evidence of antagonism with any of the combinations tested.

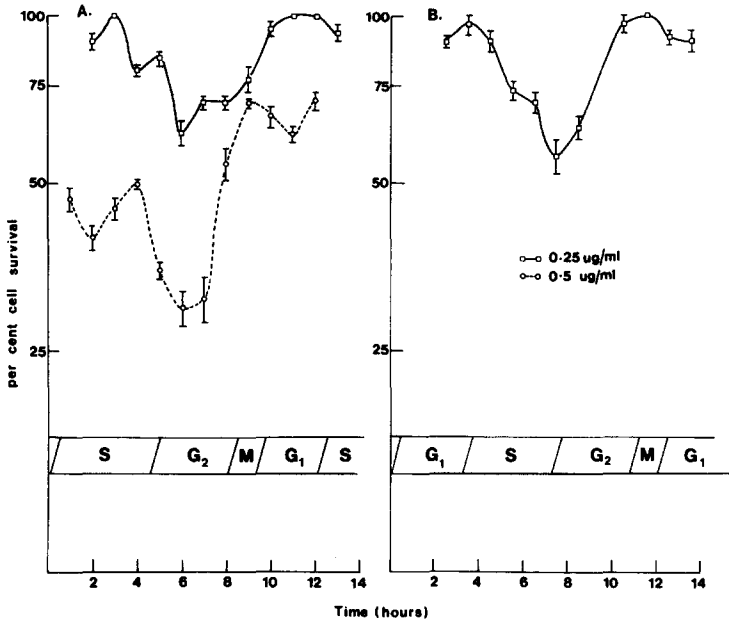

Fig. 4. Survival, assessed by colony-formation, of synchronized NIL 8 cells treated for 1 hr with PYF at various stages of the cycle. (A) *Cells synchronized using low-serum followed by HU;* (B) *cells synchronized by mitotic selection. Each point represents the mean of 4 assays ± S.E.*

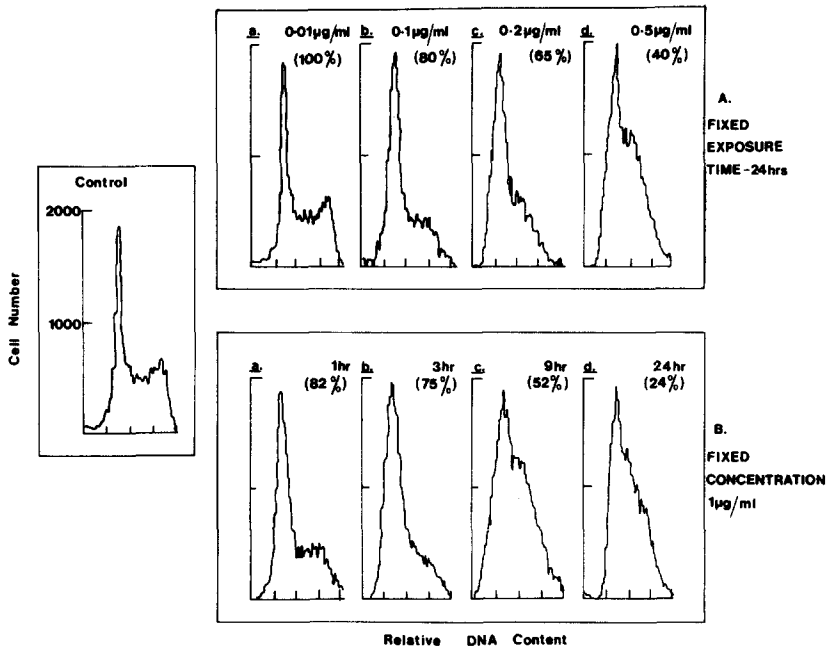

Fig. 5. The effects on the DNA distribution histograms of logarithmically-growing NIL 8 cells following exposure to: (A) *increasing PYF concentrations for 24 hr;* (B) 1 μg/ml *PYF for varying times. Survival data as % of controls quoted in brackets. Each point represents the mean of 4 determinations ± S.E.*

Table 2. Lethal effects of simultaneous treatment for 24 hr with PYF and other drugs on the colony-forming ability of L5178Y/P cells

Drugs used	Concentration (μM)	% Survival after single drug	% Survival after "observed result"	Drug combination "expected, if additive result" [12]
PYF	0.8	52±1.3	—	—
DDMP	0.03	58±1.0	19±1.0	30
MTX	0.015	64±1.1	20±0.7	33
VCR	0.0001	60±1.5	29±1.7	31
HU	60	53±1.8	26±1.7	27
ADR	0.001	74±1.7	36±1.5	38
AraC	0.27	56±2.0	15±1.0	26
5Aza	2.0	55±1.8	19±1.5	28
5FU	0.5	48±2.0	16±2.3	25
Hyperthermia	41°—2 hr	66±3.2	10±2.8	34

DISCUSSION

These results extend and confirm the original studies [3, 4, 6] showing the range of cytotoxicity of PYF to various cell lines. The L1210 cells were the most sensitive and the NIL 8 cells were most resistant (×10). Exposure of all cell types to PYF for 24 hr produced survival curves that plateaued irrespective of increasing drug concentrations, suggesting that PYF is a Class II agent in the Kinetic Classification of Antitumour Agents [13]. This result differs from the exponential survival curve obtained when assessing the sensitivity of murine or human marrow colony-forming cells to PYF [14]. Cytotoxicity of Class II drugs, however, is known to be more related to the duration of exposure than to dose, as shown here for PYF in three mammalian cell types and confirmed in human neuroblastoma cells (unpublished data).

PYF exerts its maximal lethal effects in G_2 but also at higher drug levels kills S phase cells. Interference with cell cycle traverse occurs predominantly around the G_1/S boundary. S phase hepatoma cell were also shown to be sensitive to PYF; however, no kill in G_2 was reported [15].

A knowledge of the biochemical effects of PYF [2–6] has been useful in selecting potential synergistic drug combinations which may be of clinical value. We have confirmed the synergistic effects of PYF with Ara C and 5Aza, although we have only tested concurrent combinations and recent work [5, 6] has emphasised the importance of scheduling. Our data also suggest better than additive results for PYF in combination with MTX, 5FU, DDMP or hyperthermia and these interactions are being investigated further.

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