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The use of a fluorescent methotrexate probe to monitor the effects of three vinca alkaloids on a mixed population of parental L1210 and gene-amplified methotrexate-resistant cells by flow cytometry

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Summary. *Cells resistant to methotrexate (L1210/R7A) and possessing an increased level of dihydrofolate reductase due to gene amplification can be detected by the technique of flow cytofluorimetry using a new fluorescent derivative of methotrexate (F-MTX) based on a putrescine linker. Comparative studies of dihydrofolate reductase enzyme and cell growth inhibition following treatment with methotrexate and F-MTX suggest that the two agents possess similar modes of action.*

In an artificially mixed population of cells sensitive and resistant to methotrexate it is possible, using F-MTX, to recognise and separate distinct cell subpopulations showing differential fluorescence using a fluorescence-activated cell sorter (FACS IV). The selective removal of the resistant cells within a mixed population of sensitive and resistant cells has been demonstrated for 5×10^{-8} *M vinblastine by means of flow cytometry. The effectiveness of the vinca alkaloids decreases in the order vinblastine > vindesine > vincristine, which previously was shown to be the order of effectiveness in producing collateral sensitivity.*

Introduction

A study of the comparative inhibition of dihydrofolate reductase with methotrexate and a fluorescent adduct based on a 1,5 diaminopentane linkage has been previously described [3]. Flow cytometry has also been used to demonstrate differences in dihydrofolate reductase between parental and methotrexate-resistant cell lines [5] using this fluorescent adduct [4, 5].

We have previously reported the relative effects of three vinca alkaloids, vinblastine, vincristine, and vindesine, on colony-forming ability [10] and cell-cycle progression by cytofluorimetry [8] of two cell lines showing a 20,000-fold differential sensitivity to methotrexate.

In the present study, we describe the use of an alternative fluorescent probe based on fluorescein bound to methotrexate by a 1,4 diaminobutane link. The high binding capacity of this probe to dihydrofolate reductase enzyme has been used to study the differential effect of these vinca alkaloids on parental L1210 and methotrexate-resistant cell lines by flow cytometry.

Materials and methods

Materials. Methotrexate was obtained from Lederle Laboratories. The vinca alkaloids were supplied by Eli Lilly and Co. Ltd. Fluorescein isothiocyanate, diaminobutane, thymidine, hypoxanthine, dihydrofolate reductase (bovine liver), and AE-cellulose were obtained from Sigma, London, DEAE-cellulose from Whatman Ltd, Maidstone, and Sephadex from Pharmacia, London. RPMI 1640 medium was purchased from Flow Laboratories, horse serum from Gibco Laboratories, and special agar-noble from Difco Laboratories, Michigan, USA; UV spectra were recorded on a Beckman DU8 spectrophotometer and flow cytometry on a FACS IV (Becton Dickinson).

Preparation of fluorescent methotrexate. A new fluorescent derivative of methotrexate (Fig. 1) was prepared by a modification of the procedure used by Gapski et al. [3]. Fluorescein isothiocyanate was treated with diaminobutane in dimethyl sulphoxide for 4 h at room temperature. The fluorescent diaminobutane was purified on a DEAE-cellulose column using 0.3 *M* NH_4HCO_3, pH 7.8, as eluant. The molar absorptivity was found to be 67,500 in 0.1 *M* NaOH at 487 nm, as against 79,800 for the diaminopentane derivative [3]. This was then allowed to react with an equimolar amount of methotrexate in the presence of an excess of carbodiimide in dimethyl sulphoxide for 1 h at room temperature. The resulting fluorescent methotrexate (F-MTX) was purified by AE-cellulose as described by Gapski et al. [3]. In our work we further purified the product using G-10 Sephadex (column length 45 × 2 cm, eluting solvent 10% ammonium hydroxide).

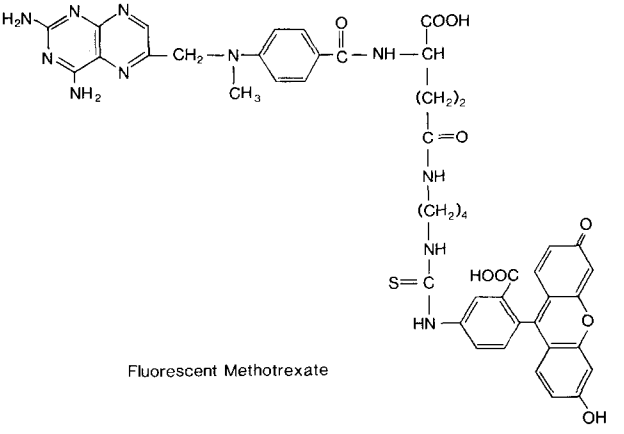

Fig. 1. Structure of fluorescent methotrexate (F-MTX)

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The F-MTX was checked for purity by thin layer chromatography using cellulose as solid substrate and methanol : 0.88 ammonia : water (7 : 1 : 2) as eluting solvent. Spots for methotrexate and fluorescein isothiocyanate were observed to be distinct from F-MTX, the latter having an Rf of 0.7, which is similar to that of the compound derived from 1.5 diaminopentane and described by Gapski et al. [3]. The absorption spectrum of the F-MTX has maxima at both 370 nm and 495 nm, corresponding to absorption maxima of methotrexate and fluorescein diaminobutane, respectively. A molar ratio of 1 : 1 based on the separate compounds by assaying at these maxima.

Cell culture. The cells used in this study were two L1210 lymphoblastoid leukaemia cell lines previously shown to be differentially sensitive to methotrexate [10]. The cell line resistant to methotrexate (L1210/R7A) is resistant to a concentration of methotrexate 20,000-fold greater than the parental cell line. (L1210 LD_{50} is 3.6×10^{-8} *M* and L1210/R7A LD_{50} is 7.7×10^{-4} *M*.) The cell lines were maintained in suspension in RPMI 1640 supplemented with 10% horse serum, as described in an earlier paper [10].

For growth-inhibition studies the cells were continuously treated in suspension culture and cell numbers in aliquot samples were assayed using an electronic cell counter (Coulter Electronics) daily for up to 8 days.

Dihydrofolate reductase activity. Dihydrofolate reductase activity was determined by a spectrophotometric method [7] on a Beckman DU8 spectrophotometer based on a decrease in absorbance at 340 nm, when NADPH and dihydrofolic acid are converted to NADP and tetrahydrofolic acid, respectively.

Preparation of cells for flow cytometry. Aliquots of cells in suspension of each cell line were treated with doses of 5×10^{-8} *M* and 10^{-9} *M* vinblastine, vincristine, and vindesine for 24 h at 37° C and then centrifuged (160 *g*, 37° C, 10 min) and resuspended in fresh medium before incubation with F-MTX (10^{-5} *M*).

In all incubations of cells with F-MTX the medium was supplemented with 160 μ*M* thymidine and 80 μ*M* hypoxanthine. Samples were incubated at 37° C in a 5% CO_2 atmosphere for 22 h with F-MTX, centrifuged down, washed with fresh medium, resuspended in fresh supplemented medium, and incubated for a further 2 h to remove unbound drug. Cells were then centrifuged, washed, and resuspended in phosphate-buffered saline before analysis on the FACS IV. The laser incorporated into this instrument is of 5 W, using an energy of 200 mW at an excitation wavelength of 488 nm and an emission wavelength of greater than 515 nm. All histograms are recorded at identical power and amplification.

Results

Cell survival and growth inhibition

The presence of 160 μ*M* thymidine and 80 μ*M* hypoxanthine in the supplemented medium overcomes the lethal effects of methotrexate on the L1210 cell line at a dose of 5×10^{-5} *M*, which would normally allow no cell survival. This dose of methotrexate, however, has no effect on the resistant (L1210/R7A) cells, whether they are grown in supplemented or in unsupplemented medium. The fluorescent derivative

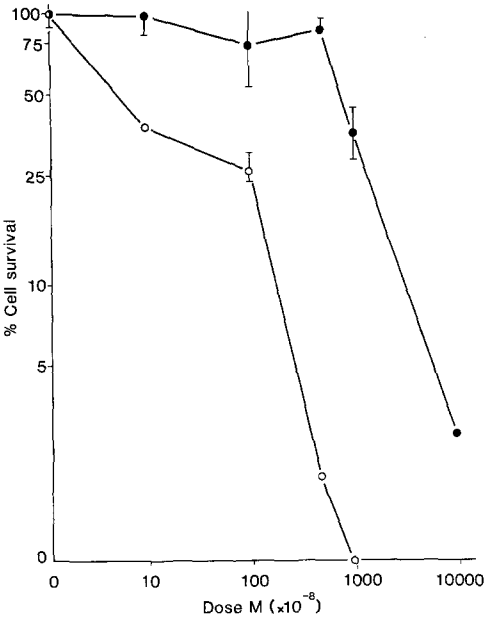

Fig. 2. Growth inhibition of L1210 in the presence of methotrexate (○———○) and F-MTX (●———●) following 8 days' exposure to the agents

(F-MTX) is less cytotoxic than methotrexate (MTX), as demonstrated by the comparative effect on the L1210 cell line (Fig. 2) (ID_{50} MTX 3.6×10^{-8} *M*, ID_{50} F-MTX 9.6×10^{-6} *M*). However, the large differential sensitivity is still apparent between the two cell lines following treatment with the fluorescent derivative, since at 10^{-4} *M* F-MTX all the resistant cells survived. The large quantities of F-MTX that would be required to obtain an accurate ID_{50} value for the L1210/R7A line were not available.

Enzyme inhibition

The dihydrofolate reductase enzyme inhibition constants (Ki) for methotrexate and F-MTX were shown to be 45 n*M* and 83 n*M*, respectively, following kinetic analysis at varying concentrations of substrate. The inhibition constants were obtained from plots of $^{i}/_{v}$ against i at varying substrate concentrations, where v is the initial rate of reaction and i is the inhibitor concentration.

Flow cytometry data

The three-dimensional presentations on the FACS IV of the L1210 and L1210/R7A cell lines following treatment with the three vinca alkaloids and incubation with F-MTX are shown in Fig. 3.

It can be seen that the L1210 cell line and the L1210/R7A cell line form two distinct populations in an artificially mixed population (1 : 1), the L1210 line having much lower fluorescence. However, both cell lines were of the same mean size. Hence the effect of the three vinca alkaloids on each population can be assessed. At 10^{-9} *M* the resistant cell population can be selectively removed from the sensitive/resistant mixed population by vinblastine, indicated by the total loss of fluorescence associated with the resistant cells. At 5×10^{-8} *M*, both sensitive and resistant cell lines are depleted by vinblastine. Although the higher dose of vindesine affects both cell lines, the lower one does not. Vincristine, at these dose levels, does not affect either cell population. Thus, it seems

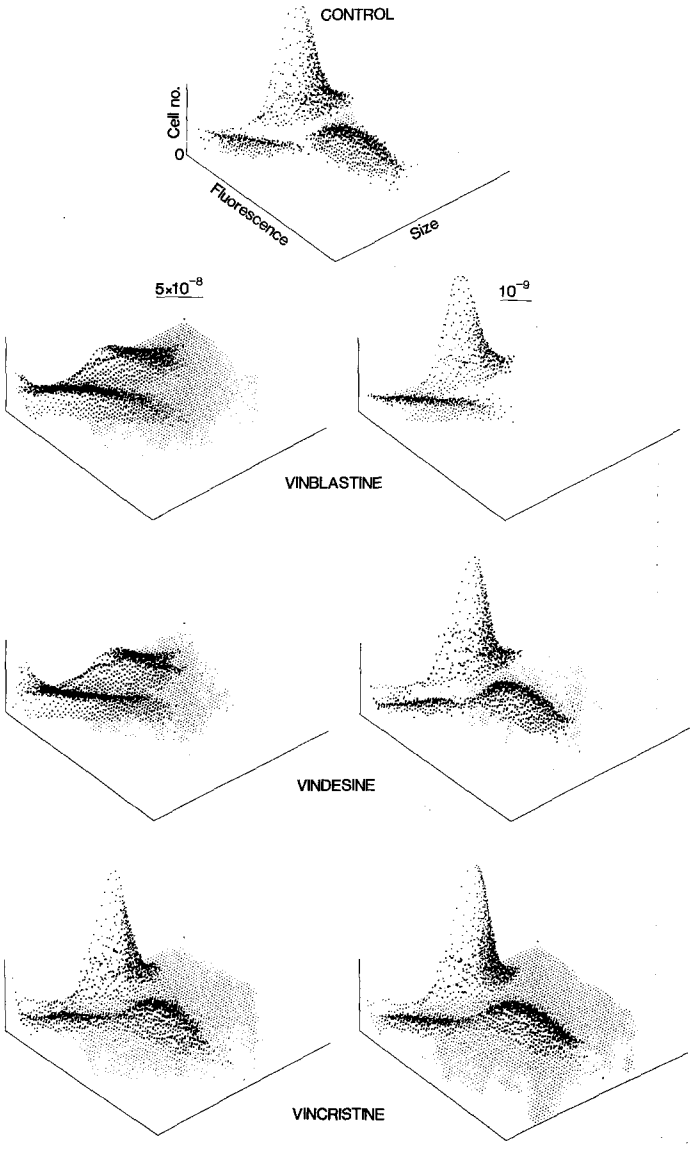

Fig. 3. FACS isometric displays of L1210 and L1210/R7A cells after incubation with F-MTX following treatment with 5×10^{-8} *M (left column)* and 10^{-9} *M (right column)* vinblastine, vindesine, and vincristine

that the doses of vinca alkaloid necessary to affect the methotrexate-resistant population alone would be very close to that which removes the sensitive (L1210) cell line. Narrow-angle light scatter measurements (i.e., particle size) suggest that there is an increase in cell debris, indicated by a subpopulation exhibiting low fluorescence and low-intensity light scattering, following treatment with vinblastine and vindesine. This is the case to a lesser extent with vincristine. This debris may be due to lysis of cells accompanied by a decreased fluorescence. The total number of cells accumulated in each case was approximately 60,000.

Discussion

The high binding affinity of methotrexate to dihydrofolate reductase has been associated with its cytotoxic action [2]. Resistance to this agent, in the L1210/R7A cell line, has been attributed to an elevated level of the target enzyme [1]. The affinity of F-MTX for the enzyme, though not as high as that of methotrexate, is still sufficiently strong for a considerable growth-inhibitory action to be apparent.

The fluorescent derivative of methotrexate prepared by Gapski et al. [3] and used by Kaufman et al. [5] and Henderson et al. [4], i.e., fluorescein diaminopentane amethopterin, has a similar enzyme inhibition constant to the derivative used in this study (41 n*M* and 62 n*M* for methotrexate and the fluorescent analogue, respectively, compared with 45 n*M* and 83 n*M* for methotrexate and our fluorescent analogue).

A differential sensitivity of the cell lines towards methotrexate is maintained for F-MTX. The ability of the cell lines to remain viable after incubation with methotrexate in medium supplemented with thymidine and hypoxanthine ensures that the flow cytofluorimetric measurements are performed on living cells.

Previous comparative studies in both clinical [6] and experimental [9, 11, 12] systems have shown differences in effectiveness between the vinca alkaloids. In previous studies colony-forming ability [10] and cytofluorimetric data [8] on two L1210 cell lines differentially sensitive to methotrexate have suggested collateral sensitivity on continuous exposure to all three vinca alkaloids, with vinblastine appearing the most effective.

The fluorescence displays obtained from the FACS IV enable us to distinguish easily between the methotrexate-resistant and the parental cell line. The broad spread of fluorescence observed in the resistant cell line may indicate a distribution of intracellular dihydrofolate reductase levels in cells of a similar size. The sensitive cells, though much less fluorescent, can also be resolved into a discrete peak. However, due to the large difference in dihydrofolate reductase levels between the two cell lines, they cannot be presented as two Gaussian distribution plots in the same figure. Hence, in Fig. 3 the sensitive cell population, with its lower fluorescence, is accumulated on the fluorescence axis and appears artifactually as a sharper peak. However, it is clear from the above data that the two populations of sensitive and resistant cells can be distinguished in a mixed population, and the effects of a second drug (in this case vinblastine) to selectively perturb the cell population can be observed.

In conclusion, we have prepared an alternative fluorescent methotrexate derivative and have demonstrated that it is a suitable derivative to identify subpopulations of tumour cells which exhibit a gene-amplified elevation of dihydrofolic acid reductase enzyme. In addition, the effect of vinblastine in selectively removing the resistant cell population has been demonstrated using flow cytometry.

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References

1. Alt KW, Kellems RE, Bertino JR, Schimke RT (1978) Selective multiplication of dihydrofolate reductase genes in methotrexate resistant variants of cultured murine cells. J Biol Chem 253: 1357
2. Bertino JR, Booth BA, Bieber AL, Cashmore A, Sartorelli AC (1964) Studies on the inhibition of dihydrofolate reductase by the folate antagonists. J Biol Chem 239: 479
3. Gapski GR, Whiteley JM, Rader JI, Cramer PL, Henderson GB, Neef V, Huennekens FM (1975) Synthesis of a fluorescent derivative of amethopterin. J Med Chem 18: 526

4. Henderson GB, Russell A, Whiteley JM (1980) A fluorescent derivative of methotrexate as an intracellular marker for dihydrofolate reductase in L1210 cells. Arch Biochem Biophys 202: 29
5. Kaufman RJ, Bertino JR, Schmike RT (1979) Quantitation of dihydrofolate reductase in individual parental and methotrexate-resistant murine cells. J Biol Chem 253: 5852
6. Mathé G, Bayssas M, Gouveia J, de Vassal F, Ribaud P, Misset JL, Machover JL, Schwarzenberg L, Hayat M, Musset M, Jasmin D (1979) Experience with vindesine in leukaemias and haematosarcomas. International Symposium on Vinca Alkaloids, Washington
7. Mathews CK, Huennekens FM (1963) Further studies on dihydrofolate reductase. J Biol Chem 238: 2436
8. McGown AT, Poppitt DG, Swindell R, Fox BW (1983) The effect of vinca alkaloids in enhancing the sensitivity of a methotrexate-resistant (L1210/R7A) line, studied by flow cytometric and chromosome number analysis. Cancer Chemother Pharmacol 13: 47
9. Paintrand M, Pignot J, Maral R (1981) Comparative actions of vindesine, vincristine, and vinblastine: an electron microscopical study in mice using P388 leukaemia cells. Biology of the Cell 40: 93
10. Poppitt DG, McGown AT, Fox BW (1983) Collateral sensitivity of a methotrexate-resistant L1210 cell line to the vinca alkaloids. Cancer Chemother Pharmacol 13: 43
11. Seeber S (1980) Patterns of cross resistance of vinca alkaloids. In: Brade W, Nagel GA, Seeber S (eds) Proceedings of the Sixth International Vinca Alkaloid Symposium: Vindesine, p 95
12. Sweeney MJ, Boder GB, Cullinan GJ, Culp HW, Daniels WD, Dyke RW, Gerzon K, McMahon RE, Nelson RL, Poore GA, Todd GC (1978) Antitumour activity of deacetyl vinblastine amide sulphate (vindesine) in rodents and mitotic accumulation studies in culture. Cancer Res 38: 2886

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