Biochemical Pharmacology, Vol. 36, No. 9, pp. 1561–1564, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

SHORT COMMUNICATIONS

Flow cytometric studies of methotrexate resistance in human squamous carcinoma cell cultures

(*Received* 24 *September* 1986; *accepted* 30 *October* 1986)

Flow cytometric analysis of intracellular dihydrofolate reductase (DHFR*) content has contributed significantly to our understanding of gene amplification in a number of cell culture models [1, 2]. The oldest and most widely used fluorescent probe of intracellular dihydrofolate reductase is MTX-F, an FITC derivative of MTX [3]. In a recent study, researchers devised a means of correlating intracellular MTX-F content with low-level amplification of the *dhfr* gene [4]. A stated hope of the study was "that flow cytometry could also be used for the analysis of clinically relevant forms of methotrexate resistance." This was followed, however, by the disappointing observation that MTX-F was insensitive to enzyme structural changes and, furthermore, was impaired in its ability to permeate certain types of cells. The occurrence of MTX resistance unrelated to enzyme overproduction was cited as an additional barrier to the use of MTX-F as a diagnostic clinical tool.

There are at least six important mechanisms by which tumor cells can lose their responsiveness to MTX [5]. Among them are: (a) poor uptake of the drug, (b) DHFR overproduction, (c) defective MTX polyglutamylation, (d) cytokinetic changes, (e) altered enzyme structure, and (f) increased purine salvage. Several forms of resistance can occur at the same time in a given cell line, producing a variety of heterogeneous drug-resistant phenotypes [6]. In view of this, it is unlikely that any single analytical method will ever be able to uncover all resistance mechanisms. Nonetheless, some of these combinations of resistance modality may be amenable to analysis using appropriate flow cytometric probes.

It was with this intention that we developed the MTX analogue PT430, reported in 1982 [7]. The PT430 structure was designed to overcome some of the problems encountered with MTX-F, particularly its poor membrane permeability. We maintained that such problems were probably due to non-regiospecific derivatization of the MTX carboxyl groups and to excessive lipophilicity of the 1,4-pentanediamine linking group. In the PT430 structure, the glutamyl residue of MTX has been replaced by an N^{ϵ}-fluoresceinated-L-lysine moiety, thereby eliminating both the problem of α-isomer formation and the need for a linking group. PT430 has proved to be an accurate and useful reporter of DHFR content [7–9]. More recently we have shown that it is also a sensitive probe of the MTX transport defective phenotype in human lymphoblastic leukemia cells in suspension culture [10].

In the present study, the well-characterized [6, 11] human head and neck squamous cell carcinoma line SCC-15, and its 15- and 90-fold MTX-resistant sublines, denoted SCC-15/R1 and SCC-15/R2, respectively, were analyzed by flow cytometry using PT430. These cells constitute an interesting model system, since the R1 line is simply MTX transport defective, while the R2 line is both transport defective and a DHFR overproducer [11]. An additional feature of these cells is that, since they grow as monolayers, they represent a system more closely related to our ultimate target, the clinical biopsy specimen, than to the suspension cultures which were used in our preliminary work [12].

Materials and methods

Cells. The SCC-15 line of human squamous carcinoma cells, originally obtained from the tongue of a male cancer patient, was provided by Dr. J. G. Rheinwald of this Institute. Two MTX-resistant sublines, SCC-15/R1 and SCC-15/R2, were developed by MTX dose escalation and these, along with the parental SCC-15 cells, were characterized with respect to generation time, DHFR content, and MTX sensitivity, polyglutamylation and uptake [6, 11].

PT430 uptake. The preparation of PT430 and its use in uptake studies with suspension culture cells have been described [10]. These procedures were modified as follows for the present work: Cells were plated on 105 cm^2 Petri dishes and grown to late log phase. They were then resuspended by 10-min incubation with 0.25% trypsin and 0.2% EDTA, washed 2× with PBS, filtered through a sterile 40 μm Nytex mesh, and replated at one-half the original density in growth medium. After 6 hr of incubation at 37°, a surface-attached monolayer consisting of disconnected individual cells was obtained. The monolayer was washed with PBS to remove debris and dead cells and was then detached from the dish by 15-min incubation with 0.2% EDTA. Non-proteolytic detachment of the cells in this step is critical, because it minimizes non-specific uptake of the PT430. The suspended cells were washed 2× with PBS and transferred to a 25-ml spinner culture flask maintained at 37° under a humidified 8% CO_2 atmosphere in DME with 10% FBS. Cell density was 2×10^6/ml in a volume of 24.75 ml. A 0.25-ml volume of 0.3 mM PT430 was added and then, at 10-min intervals, 2-ml aliquots were withdrawn and transferred into 15-ml conical tubes containing 10 ml of ice-cold PBS. After twelve aliquots were collected, they were pelleted, washed 2× with ice-cold PBS, and resuspended in 800 μl of ice-cold PBS for analysis by flow cytometry. Since no step in the procedure from addition of PT430 until the cells were resuspended for flow cytometric analysis required more than about 10 sec, it was possible to run the three cell lines concurrently at 20-sec intervals. Indeed, following this procedure, it would be possible, in principle, to perform many parallel experiments at the same time.

Experiments were conducted also with cell monolayers stained first with PT430 and then removed by trypsinization from the dishes at 15-min intervals prior to flow cytometry. The rank order of PT430 uptake for the three cell lines was the same as it was for cells detached prior to staining (data not shown). However, we noted in initial experiments using the alternative procedure that adequate kinetic analysis of PT430 by these cells would require more closely spaced points than was possible without using a prohibitively large number of dishes. This, and the fact that the monolayer approach was operationally inconvenient because cells in every dish had to be trypsinized for approximately 15 min.

* Abbreviations: DHFR, dihydrofolate reductase (EC 1.5.1.3); *dhfr*, the DHFR gene; MTX, methotrexate; MTX-F, MTX-1,4-diaminopentane-FITC conjugate; PT430, N^{α}-(4-amino-4-deoxy-10-methylpteroyl)-L-lysine-FITC conjugate; FITC, 4-fluoresceinylisothiocyanate; DME, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; and PBS, phosphate-buffered saline.

led us to adopt the suspension method in preference to the monolayer method.

Efflux. SCC-15, SCC-15/R1 and SCC-15/R2 cells were prepared as described above, incubated for 4 hr with 1 mM PT430, pelleted, and resuspended in drug-free medium in spinner culture. Aliquots were withdrawn after 0, 10, 20, 60, 120, 240, and 360 min, quenched and washed 2× in ice-cold PBS, and resuspended in ice-cold PBS at a density of 1×10^6/ml prior to flow cytometric analysis.

Inhibition studies. SCC-15 and SCC-15/R1 cells were incubated with 40 μM PT430 containing 20 μM MTX, and aliquots were collected at 0.25, 0.5, 1, 2 and 5 hr. Controls were treated with 40 μM PT430 only. Cells were quenched, washed, and resuspended as described above, and fluorescence histograms were prepared from populations of 20,000 cells.

Analysis. Flow cytometric analyses of time-dependent PT430 uptake and efflux were obtained with an Epics V instrument and the accumulated log-fluorescence values were converted to mean fluorescence per cell, expressed in mV of photomultiplier output, as previously described [10]. These data were plotted versus time (Fig. 1) and fitted to a first-order kinetic model by non-linear regression [12]. From the derived equations, initial velocities and plateau concentrations were calculated (Table 1). Fluorescence histograms for the inhibition studies were obtained using the Cytomutt apparatus described in an earlier publication [7].

Results and discussion

Uptake of 30 μM PT430 followed first-order kinetics in SCC-15 and SCC-15/R1 cells, as shown in Fig. 1. For the SCC-15/R2 cells, uptake probably obeyed first-order kinetics as well, although we were unable to achieve a plateau during the 2-hr span of the experiment. Initial rates were greatest for the MTX-sensitive parental cells and were measurably less for the two MTX-resistant sublines (Table 1). In prior studies, influx of 2 μM [^{3}H]MTX in SCC-15/R1 and SCC-15/R2 cells was 15 and 8% as rapid as that of the parental SCC-15 cells [11]. With PT430, the influx rates in SCC-15/R1 and SCC-15/R2 cells were found to be 53 and 37% of the rate in SCC-15 cells respectively. We therefore had an indication of reduced membrane permeability to MTX which is imprecise but of the correct order, SCC-15≫SCC-15/R1>SCC-15/R2. Similar results were obtained previously with CEM human leukemic lymphoblasts, but in that study the differences in influx rates between sensitive and resistant cells were less pronounced, whereas the MTX and PT430 plateau levels in the MTX sensitive cells were far above those of the resistant cells [10].

Steady-state fluorescence levels in the R1 cells were 10% lower than those of the SCC-15 cells, whereas the calculated asymptote for the R2 cells was 3.9-fold higher. Prior radioligand binding studies [6] have shown the DHFR level to be 7% lower in the SCC-15/R1 cells and 4.3-fold greater in the SCC-15/R2 cells compared with the SCC-15 line. Thus, the fluorescence plateau levels are closely correlated with the intracellular DHFR content.

Inhibition of PT430 uptake in SCC-15 and SCC-15/R1 cells by MTX was seen at all time points. Fluorescence histograms from the 2-hr incubation (Fig. 2) show that, in the resistant cells, uptake was inhibited to a greater degree than in the parental line. Data obtained at earlier time points gave the same relative fluorescence distributions, with peak maxima at progressively higher intensities over the course of the uptake (data not shown). No differences were seen between histograms obtained at the 2- and 5-hr time points. Inhibition of fluorescence uptake at 0.25 hr is probably indicative of a common influx carrier for PT430 and MTX. Inhibition at 2 and 5 hr is clearly related to preferential binding of DHFR by MTX, which has a 10-fold greater affinity for the enzyme than PT430 [7].

Efflux of PT430 from all three cell lines followed first-

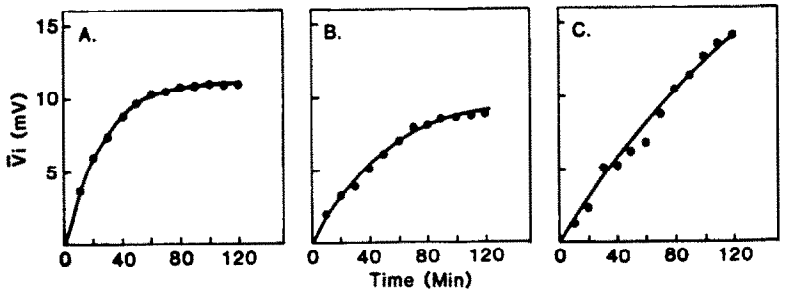

Fig. 1. Uptake of 30 μM PT430 in (A) SCC-15, (B) SCC-15/R1, and (C) SCC-15/R2 cells.

Table 1. Influx rates and plateau levels* for uptake of 30 μM PT430 in MTX-sensitive and resistant squamous cell carcinoma cultures: Comparison with 2 μM [^{3}H]MTX influx data and DHFR content†

Cell line	PT430 Influx rate (mV/min)	PT430 Plateau level (mV)	MTX Influx rate (pmol/mg protein/min)	DHFR (pmol/10^8 cells)
SSC-15	0.38 ± 0.09	11 ± 0.9	0.39 ± 0.02	5.4
SCC-15/R1	0.21 ± 0.05	10 ± 0.9	0.06 ± 0.03	5.0
SCC-15/R2	0.14 ± 0.05	43 ± 1.8‡	0.03 ± 0.04	23

* Mean ± SD, from three experiments performed on different days.
† From Ref. 11.
‡ Calculated by non-linear regression.

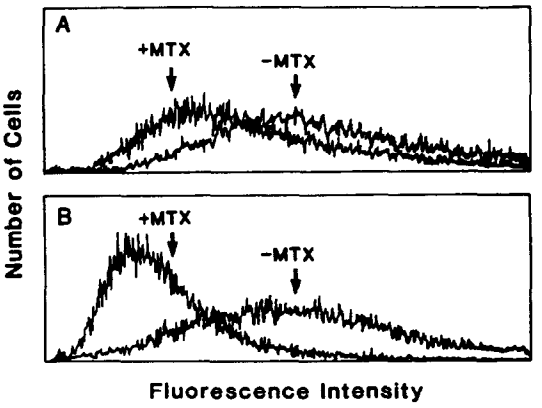

Fig. 2. Inhibition of 40 μM PT430 uptake by 20 μM MTX after 2 hr. Histograms of fluorescence versus number of cells for: (A) SCC-15, and (B) SCC-15/R1 cells.

to defective transport. Furthermore, even in cell lines that combine transport resistance and DHFR overproduction, these disparate features can be individually distinguished from those of the unaltered parental cells.

The monolayer SCC-15 model is intended to provide a bridge between the facile suspension culture procedures and anticipated future clinical applications. In the field of estrogen receptor analysis, techniques developed with monolayer cultures, and subsequently modified for application for biopsy specimens, have provided a useful and reliable flow cytometric assay for tamoxifen sensitivity in breast cancer [13]. Expansion of this technology to allow prediction of responsiveness to MTX would be equally desirable, and the work reported here is intended to provide a step in this direction.

Acknowledgements—This work is supported in part by Grants CA19589 and CA25394 from the National Cancer Institute, DHHS, and by the Joseph Vaturi Respiratory Neoplasia Fund, Dana-Farber Cancer Institute.

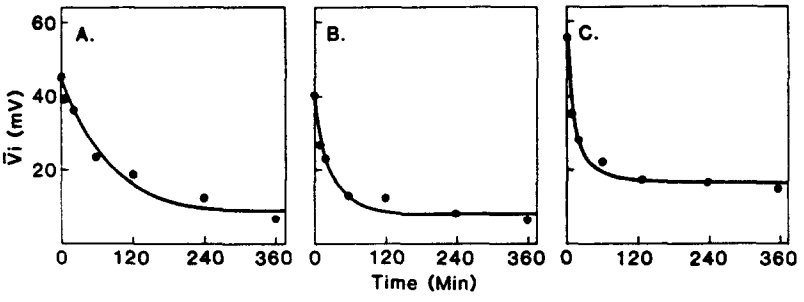

Fig. 3. Efflux of PT430 from (A) SCC-15, (B) SCC-15/R1 and (C) SCC-15/R2 cells over 6 hr.

order kinetics, as shown in Fig. 3. Since substantial differences in "zero-time" fluorescence levels were observed following the 4-hr incubation with 1 mM PT430, it is probably not appropriate to comment on differences in initial velocities. Half-lives for such exponential decay curves, however, are reasonably independent of initial concentration over a narrow range. The half-lives of PT430 efflux in SCC-15, SCC-15/R1 and SCC-15/R2 cells were 56, 18 and 10 min. Steady-state levels at the end of the efflux period were 10 mV for both the SCC-15 and the SCC-15/R1 cells. In the SCC-15/R2 cells this value was 18 mV. It is not possible to relate intracellular fluorescence intensities to specific concentrations of PT430, without knowing the fluorescence enhancement due to DHFR binding. *In vitro* studies have shown that this factor varies with pH [7], an unknown quantity within the microscopic environment of intracellular DHFR. Nonetheless, the final efflux level in the SCC-15/R2 cells compared with either of the other two cell lines was 2- to 3-fold lower than expected. This suggests that DHFR-bound PT430 may dissociate from the enzyme and exit from the cells more readily than MTX. Despite this problem, the rank-order is still appropriate, the non-effluxing component of SCC-15 cells being equal to that of the SCC-15/R1 and less than that of the SCC-15/R2 cells.

In summary, we have shown that by the non-proteolytic detachment of a monolayer, prior to flow cytometric analysis, it is possible to distinguish between an MTX-sensitive cell line and corresponding sublines which are resistant due

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Biochemical Pharmacology, Vol. 36, No. 9, pp. 1564–1565, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

Decrease in lipid mobility in rat erythrocyte membrane after amiodarone chronic treatment

(*Received* 12 *September* 1986; *accepted* 5 *December* 1986)

Amiodarone is used as an antianginal and antiarrhythmic drug. Although its efficacy in the treatment of a wide spectrum of arrhythmias is well documented [1], its mode of action is poorly understood. *In vitro*, amiodarone displays several properties suggesting extremely low water-solubility and significant hydrophobic behaviour. These properties include micelle formation [2], spreading at the air–water interface [3] and high value of partition coefficient [4]. Studies on purified membranes [5] and on liposomes [6] indicate that amiodarone decreases lipid dynamics with a threshold concentration of 10^{-6} M and suggest that the compound is a rigid molecule deeply buried in the hydrocarbon core of the lipid bilayer. On the other hand, data from the literature [7, 8] indicate that effective treatment of arrhythmias in the human is achieved for a plasma concentration of 1.50–3.80 10^{-6} M. On the basis of these data, we decided to look for a possible effect of amiodarone on the lipid dynamics in rat erythrocyte membrane after chronic treatment.

Methods

Male Sprague–Dawley rats weighing 280–300 g were treated with amiodarone during 14 days at the doses of 50 or 150 mg/kg/day. Control group received the vehicle only (aqueous solution of gum-arabic). Blood was collected on heparin by intracardiac puncture 24 hr after the last administration. Erythrocyte ghosts were prepared according to Waelbroeck *et al.* [9]. Phospholipids and cholesterol were extracted [10] and their values determined according to standard procedures [11, 12]. Protein content was measured according to Lowry *et al.* [13]. Amiodarone was extracted and determined by high performance liquid chromatography [14]. Lipid dynamics was appreciated by fluorescence depolarization technique using 1,6 diphenylhexatriene (DPH) as fluorophore [5, 15]. Fluorescence depolarization and excited-state lifetime measurements were performed on a SLM 4800 Spectrofluorimeter. Steady-state fluorescence anisotropy (r_s) was determined by the emission intensities through two analyzers oriented respectively parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the direction of polarization of the excitation light:

$$r_s = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp}$$

All results are expressed as means ± SEM; statistical differences were determined by unpaired Student's *t*-test.

Results and discussion

Control values of the steady-state fluorescence anisotropy (r_s) (Table 1) are in excellent agreement with published data [15–17]. Nevertheless, according to the Perrin equation [15]

$$\frac{r_o}{r_s} = 1 + C(r) \cdot T \cdot \frac{\tau}{\eta}$$

where r_o and r_s are respectively the limiting and the measured values of the fluorescence anisotropy, $C(r)$ is a structural parameter, T is the absolute temperature, τ is the excited-state lifetime of the probe and η is the viscosity of the medium, variations of r_s may result from variation of either τ or η or both. This is particularly important if one is looking for the effects of a foreign compound which may act as a quencher. In control experiments, we checked this possibility for amiodarone. Direct measurements of τ indicate that when up to 25% of amiodarone is incorporated in the lipid matrix, τ remains constant. Consequently, the observed variations in r_s values do represent variations in DHP mobility and thus in lipid dynamics.

Parameters such as the steady-state fluorescence anisotropy, r_s, and the derived order parameter S_V associated with the lipid dynamics in rat erythrocyte membrane indicate that amiodarone chronic treatment induces a decrease in lipid mobility, i.e. r_s values are increased (Table 1). To

Table 1. Effect of chronic amiodarone treatment on lipid dynamics parameters, phospholipids, cholesterol and amiodarone content in rat erythrocyte membranes

	Control (17)	Amiodarone 50 mg/kg/day (7)	Amiodarone 150 mg/kg/day (15)
r_s	0.248 ± 0.001	0.252 ± 0.001*	0.259 ± 0.001†
Amiodarone	—	0.21 ± 0.13	0.48 ± 0.15
Phospholipids	368 ± 9	357 ± 4	347 ± 8
Cholesterol	283 ± 17	283 ± 10	319 ± 22*
C/PL	0.77 ± 0.04	0.79 ± 0.03	0.92 ± 0.06*

r_s is the steady-state fluorescence anisotropy measured at 25°.
Amiodarone, phospholipids and cholesterol are expressed as 10^{-9} M/mg membrane protein.
Results are the mean ± SEM. ( ) represents number of rats * $P \leq 0.05$, † $P \leq 0.01$.