Mechanism of Transport and Intracellular Binding of Porfiromycin in HCT 116 Human Colon Carcinoma Cells

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ABSTRACT

The mechanism of uptake and efflux of porfiromycin (PFM) by HCT 116 human colon carcinoma cells was investigated. The time course of uptake of radioactivity upon exposure to HCT 116 cells to [14C]PFM showed one fast and one slow phase of linear increase. The initial phase of PFM uptake was not saturable with external drug concentrations from 2 to 100 μM. PFM accumulation was temperature-dependent with a temperature coefficient of Q10 – 4.3°C of 2.3 ± 0.3. PFM uptake was not affected either by individual inhibitors such as 1 mM 2,4-dinitrophenol, sodium azide, iodoacetate acid, ouabain, 0.02 mM oligomycin, 0.2 mM N-ethylmaleimide, or by combinations of inhibitors. PFM uptake did not demonstrate competitive inhibition by unlabeled PFM and mitomycin C. Efflux of cellular radioactivity was not affected by the above mentioned inhibitors or by verapamil, diltiazem, or trifluoperazine. Only aliphatic alcohols accelerated the initial influx rate. The RBC, however, only exhibited the initial fast accumulation of [14C]PFM, and all of the [14C] accumulated by RBC was exchangeable. These data demonstrate that the uptake and the efflux of PFM in HCT 116 cells and RBC comprise a passive diffusion process.

INTRODUCTION

PFM and MC, two reductive alkylating agents, are effective antitumor drugs. MC is widely used in the treatment of various solid tumors (1). PFM, an analogue of MC, is not currently used in clinical practice even though the two agents show similar efficacy and toxicity (2), and the tolerated dose of PFM was higher than that of MC in Phase I clinical trials (2-4). Biochemically we have shown that these two agents share the same metabolic pathways and the same pattern of DNA alkylation (5, 6).

PFM and MC are quinone- and aziridine-containing heterocyclic compounds with four nitrogens. The pKₐ values of these nitrogens have been well studied (7, 8). The pKₐ of the zairidine ring nitrogen and the nitrogen at position 4 lie between 1.2 and 1.5. The pKₐ of the 7-amino nitrogen is 12.44, and that of the carbamyl nitrogen is so high as to render its measurement beyond the stability of the molecule. Therefore, at physiological conditions, PFM and MC most likely enter cells as electron neutral molecules rather than protonated forms. Currently, literature on the mechanism of transport by which PFM or MC enters and leaves cells is lacking. In this paper, we used [14C]-PFM to study the mechanism of transport of PFM in human red blood cells and in HCT 116 cells, a human colon carcinoma cell line that was originally isolated by Brattain et al. (9) and which is sensitive to MC.

MATERIALS AND METHODS

Reagents. PFM was kindly supplied by the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD) and Dr. J. P. McGovren, Upjohn Co. (Kalamazoo, MI). [14C] PFM was synthesized from [14CH₃]MC and MC according to the procedure of Steven et al. (8). At the end of the reaction, [14C]PFM was isolated by the HPLC separation procedure developed in our laboratory (5). The final products had specific activities of 20 to 30 mCi/mmol. Metabolites, t- or c-AHME and AME, were generated by enzymatically reducing PFM and purified by HPLC (5, 6). SF-1250 silicone fluid was generously donated by the General Electric Co., Silicone Products Division (Waterford, NY). [14C]Insulin carboxylic acid and [3H]methyl glucose were purchased from Amersham (Arlington Heights, IL).

Cell Culture. HCT 116 human colon carcinoma cells were obtained from the American Type Culture Collection (CCL247) (Rockville, MD). Cells were maintained as monolayers in a minimal medium consisting of McCoy's Medium 5A supplemented with 10% heat-inactivated fetal calf serum, 100 μg/ml of streptomycin, and 100 units/ml of penicillin as described previously (9). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. To harvest the cells or subcultures, confluent monolayers were treated with trypsin/EDTA (0.25%/0.02%) for 4 min at room temperature. Cells were then resuspended in complete medium for further use. Minimal medium contained RPMI 1640 balanced salt solution without any supplements and was used during all studies unless otherwise stated. Human RBC were obtained by venipuncture from suitable donors. RBC were collected in heparinized vacutainers on each day that an experiment requiring them was to be performed. RBC were washed once with minimal medium and then resuspended in minimal medium for uptake or efflux studies. Intracellular water and extracellular space were measured according to Hissin and Hilf (10). Cell volume was determined with a Coulter Channelizer (Coulter Electronics, Hialeah, FL), calibrated with 10.0- and 14.48-μm-diameter latex microspheres (volume, 524 and 1526 μm³, respectively).

Enzyme Preparations and Assays. Cell homogenates were prepared from confluent HCT 116 cells. Cells were harvested and washed with cold phosphate-buffered saline, resuspended at 10⁶ cells/ml in cold 50 mM potassium phosphate buffer (pH 7.5), and lysed by 3 cycles of freezing and thawing. Unbroken cells were removed by centrifugation at 600 X g for 5 min. The supernatant was used for enzyme assays. Rat liver microsomes were prepared by the method of Yasukochi and Masters (11). Activity of N-demethylases in the HCT 116 cell homogenate was assayed by the production of formate (12). The 1-mi incubation mixture contained 1 mM PFM or 1 mM aminopyrine, 2 mM NADPH, 4.5 mg of cell homogenate protein, and 50 mM potassium phosphate buffer, pH 7.5. Incubation was carried out aerobically at 37°C for 30 min. Rat liver microsomes, rich in N-demethylases, were assayed as a positive control. In this case, the microsomal protein in the incubation mixture was between 0.5 and 7.1 mg of protein. At the end of incubation, demethylation of aminopyrine was stopped by the addition of 1 ml of 14% trichloroacetic acid. Demethylation of PFM was stopped by chilling to 4°C and immediate filtration through a SEP-PAK C₈ cartridge (Waters Associates, Milford, MA) to remove excess PFM and PFM products which interfered with determination of formate. Production of formate in each of the reaction mixtures was measured by the method of Nash (12). Recovery of formate with SEP-PAK 100% was greater than 98%. Activity of the cell homogenate preparations was confirmed by measuring diaphorase activity (13) and by their ability to reduce PFM (6).

Uptake of PFM. HCT 116 cells were grown to confluence, harvested, washed twice with minimal medium, and then resuspended in minimal medium at 4 X 10⁵ cells/ml. Preincubation of these cell suspensions was carried out at designated temperatures for 15 min with overhead stirring. Drug was dissolved in minimal medium at 10 times the desired final concentration. Aliquots (450 μl) of cells were rapidly mixed in a
tube with 50 µl of drug which had been preincubated at the designated temperature. The resulting cell-drug incubation mixtures were further incubated from 10 s to 15 min before being layered over 500 µl of SF-1250 silicone fluid in an Eppendorf tube. They were then centrifuged, by the method described by Chello et al. (14), at 13,000 x g for 20 s in a Beckman Microfuge E. Medium containing the drug and silicone were carefully removed by aspiration and counted separately to keep track of the radioactivity. Loss of radioactivity into the silicone layer was not observed. The walls of the tubes were wiped carefully. Sedimented cells were resuspended in 0.5 ml of water, mixed with 5 ml of Beckman Ready-Safe scintillation fluid (Beckman Instruments, Fullerton, CA), and radioactivity was determined with a Beckman 5801 scintillation counter. When metabolic inhibitors or other effectors were used, effectors at specified concentrations were included in media during the 15-min preincubation and the influx periods. Statistical analysis of the effects of alcohols was performed by one-way analysis of variance. Nonspecific absorption of drug to the surface of cells was measured by exposing cells to drug at 0–4°C for 10 s. Corrections for nonspecific absorption were made for all measurements. All calculations of radioactivity were based on the specific activity of [14C]PFM. Studies of RBC at 4.6 x 10⁶ cells/ml were handled in the same fashion. Cell viability in each study was confirmed by trypan blue exclusion in parallel studies with nonlabeled PFM. Only results obtained with viable cells are presented.

Efflux of Radioactivity. Cells were prepared as described for the uptake study and then treated with 10 µM [14C]PFM for 5 min at 37°C. The cell suspension was quickly chilled and centrifuged at 2000 x g for 10 min. Medium with drug was removed, and the tubes were carefully blotted. The cells were then resuspended to 2 x 10⁶ cells/ml in prewarmed drug-free minimal medium and incubated at 37°C with overhead stirring. At specified times, samples of 2 x 10⁶ cells were removed and centrifuged through silicone fluid as described above. Radioactivity of the cell precipitate and medium was measured. When effectors were used, each effector was included in the medium during periods of drug preloading and efflux.

Distribution of Radioactivity in Cells. After treatment with radioactive drug, cells (2 x 10⁶) in 5 ml were immediately chilled to 4°C in 90 s and recovered by centrifugation through silicone fluid. Sedimented cells were lysed osmotically by being resuspended in 2 ml of cold H2O and incubated at 4–0°C for 20 min. Cells and fractions were kept cold at all times to minimize any metabolic conversions of PFM during preparation for analysis of soluble and bound radioactivity. Lysed cells were separated by centrifugation at 100,000 x g for 60 min into soluble and particulate fractions. The soluble extracts were lyophilized and reconstituted in distilled water. Portions from each of the reconstituted soluble extracts were counted for total soluble radioactivity. Additional portions of the soluble extracts (about 4000 dpm) were analyzed by HPLC for [14C]PFM and metabolites. The precipitates were resuspended in water and counted for nonextractable macromolecule-bound radioactivity. A cell sample, exposed to the same drug concentration at 0–4°C for 10 s, was fractionated by the identical procedures for two purposes: (a) to make corrections for the amount of radioactivity resulting from nonspecific absorption; and (b) to ensure that metabolic conversions did not take place during sample preparation.

HPLC Analysis of Soluble Radioactive Material. Soluble cellular radioactive material was analyzed with a reversed-phase HPLC method developed in this laboratory (5) except that flow of mobile phase was slowed to 2.0 ml per min. Pure PFM, t- and c-AHME, and AME were used as internal standards, and [14C]PFM was used as an external standard. Fractions of 0.5 ml were collected, and radioactivity in each fraction was measured by liquid scintillation counting. Peaks of radioactive activity were integrated and converted to pmol/Ml of cells using the specific activity of [14C]PFM and the intracellular water content of HCT 116 cells.

RESULTS

Demethylation. The activity of demethylases in HCT 116 cell homogenates was investigated to assess the potential stability of [14C]methyl label of PFM (Table 1). Aminopyrene, a common substrate for N-demethylases, was not affected by HCT 116 cell homogenate, whereas it was demethylated rapidly by rat liver microsomes. Under the same conditions, PFM was not demethylated by either HCT 116 cell homogenate or by liver microsomes.

Time Course of Accumulation of Radioactivity. Intracellular water content and cell volume of HCT 116 cells were determined to be 0.82 ± 0.05 µM/10⁶ cells and 850 ± 33 µm³/cell, respectively, with the data from five experiments, each performed in triplicate. These values were used in all calculations of uptake, efflux, and distribution of radioactivity. Accumulation of radioactivity by HCT 116 cells exposed to 5 µM [14C]PFM at 4°, 24°, and 37°C was determined at times between 10 s and 15 min (Fig. 1A). A rapid initial association of radioactivity with cells was observed in 10 s. The rate of this association was essentially the same at all three temperatures studied. This radioactivity was presumed to be nonspecific adsorption by cell surface and was corrected for in all uptake experiments. There was some temperature dependence for accumulation of radioactivity. At 4°C, there was a slow linear increase of radioactivity up to 15 min of incubation. At 24°C and 37°C, linear accumulation of radioactivity was observed for the first 50 s to a cell:medium ratio of 0.6 and 1.5, respectively. Subsequently, the rate of accumulation gradually decreased until 3 to 4 min. After this, a very slow but constant increase was observed. Drug

Table 1 N-Demethylase activity of HCT 116 cell homogenate and rat liver microsomes

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Substrate</th>
<th>Product</th>
<th>Activity (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver microsomes</td>
<td>Aminopyrene (1.0 mM)</td>
<td>HCHO</td>
<td>32.14</td>
</tr>
<tr>
<td>HCT 116 homogenate</td>
<td>Aminopyrene (1.0 mM)</td>
<td>HCHO</td>
<td>ND*</td>
</tr>
<tr>
<td>Rat liver microsomes</td>
<td>PFM (1.0 mM)</td>
<td>HCHO</td>
<td>ND</td>
</tr>
<tr>
<td>HCT 116 homogenate</td>
<td>PFM (1.0 mM)</td>
<td>HCHO</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, not detected.

Fig. 1. Time course for total intracellular accumulation of radioactivity by HCT 116 cells exposed to [14C]PFM. Cell suspensions (4 x 10⁶ cells/ml) were exposed to 5 µM [14C]PFM at 0–4°C (•), 24°C (A), and 37°C (♦) for varying intervals of time and then analyzed for radioactivity. Experimental details are as described in the text. Each point was obtained from three experiments, each performed in triplicate. Error bars not shown fall within symbols. A, values shown are for intracellular, i.e., total radioactivity associated with cells minus the surface nonspecifically associated drug; B, the expanded portion of the first 50 s plotted by linear regression. Points, mean; ears, SE.
exposure for the first 50 s was used to approximate the velocity of initial uptake which showed uptake rates of 0.02, 0.34, and 1.0 pmol/μl of cell H₂O/10 s at 4°C, 24°C, and 37°C, respectively (Fig. 1B). The temperature coefficient (Q₁₀ 24–37°C) for this process was calculated to be 2.3 ± 0.3. This linear accumulation of radioactivity for the initial 50 s was also observed with external drug concentrations from 2 to 100 μM (data not shown). Time course studies performed in complete McCoy's Medium 5A produced results similar to those from experiments performed in RPMI 1640 minimal medium (data not shown). The presence of glucose, amino acids, and serum did not have any effect on the accumulation of radioactivity.

Cellular Disposition of Radioactivity. Data with regard to the distribution of radioactivity in HCT 116 cells after drug exposure to 5 μM [¹⁴C]PFM for 10 s at 4°C, or 25 s to 20 min at 37°C were obtained from three separate experiments (Table 2). Macromolecule-bound radioactivity, representing nonextractable radioactivity bound to DNA, RNA, and protein, increased as drug exposure time increased. HPLC analysis of the soluble fractions obtained from cells with the same periods of exposure to [¹⁴C]PFM showed similar profiles in each individual experiment. A major radioactive peak (II) present in all samples eluted with authentic PFM (Fig. 2). The cells exposed to drug at 4°C for 10 s showed only this major peak. With increasing incubation time, three other peaks (I, III, and IV) appeared. These cochromatographed with authentic t-AHME, c-AHME, and AME.

Quantitative data obtained from three experiments are shown (Table 2). The sum of Peaks I, III, and IV was designated as metabolites, and the sum of all other fractions was designated as others. Water-extractable intracellular PFM increased with the length of exposure time and reached half the concentration of external drug at 25 s and steady state at 5 min, after which there was no further increase. The intracellular concentration of PFM at the plateau stage was approximately 5 μM which equaled the external drug concentration. Metabolites, total unidentified radioactivity, and macromolecule-bound products increased in quantity with increasing time of drug exposure up to 20 min.

Effect of External Drug Concentration on Uptake. Initial uptake of PFM by HCT 116 cells depended on the drug concentration in the medium (Fig. 3). There was a linear relationship between initial uptake and external drug concentration. PFM influx did not exhibit saturation kinetics between 2 and 100 μM. Cells that had been preloaded to steady-state levels of exchangeable drug by incubation with unlabeled PFM or MC at 50 and 500 μM for 5 min showed no changes in uptake or accumulation of radioactivity up to 10 min when compared to unloaded cells.

Efflux of Radioactivity. Efflux of radioactivity from cells that had been preloaded with 10 μM [¹⁴C]PFM for 5 min at 37°C was also biphasic. It was rapid in the first 30 s (Fig. 4) during which greater than 50% of the total cellular radioactivity was released into the medium. From then until 5 min, the release of radioactivity slowed. After 5 min, only very small amounts of radioactivity were released, leaving finally about 10% of the initial cellular radioactivity as nonexchangeable. Cells preincubated with [¹⁴C]PFM for 50 s had smaller amounts of nonex-
cally significant by one-way analysis of variance (P < 0.05). This effect became less noticeable by 300 s. The enhancement of \(^{14}\text{C}\) accumulation increased with increasing carbon chain length of the alcohols, and this effect was shown to be statistically significant by one-way analysis of variance (P < 0.05).

Adjusting the pH of the uptake medium to 6.5, 7.0, 8.0, and 8.5 had no effects on the rate of uptake or efflux of PFM for the initial 50 s.

Influx and Efflux of PFM by Human Red Blood Cells. In order to avoid potential complications from metabolic conversions of PFM, human red blood cells, which lack nuclei and the major flavoenzymes to activate PFM, were chosen as an alternative model in which to study the transport of PFM. Cell volume was 91 ± 10 \(\mu\)m\(^3\). Influx of 5 \(\mu\)M PFM reached equilibrium with external drug concentration in about 50 s (Fig. 6). Afterwards, there was only a slight increase of radioactivity. Efflux reached equilibrium at 2.5 min for cells that had been preloaded with 5 \(\mu\)M PFM for either 50 s or 5 min. Nonexchangeable radioactivity was not seen.

DISCUSSION

The PFM molecule contains 4 nitrogens. As described in the "Introduction," the pK\(_a\) values of these nitrogens are either too low (−1.2 to 1.5) or too high (12.44) to be protonated or deprotonated at physiological pH. Demonstration of independence of pH between 6.5 to 8.5 for uptake of PFM is consistent with the fact that PFM is an unprotonated electroneutral molecule under physiological conditions. All of our data obtained

<table>
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<th>Inhibitors</th>
<th>Concentration (mM)</th>
<th>pmol/(\mu)l cells</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>3.24 ± 0.38*</td>
<td>100.0 ± 11.7</td>
</tr>
<tr>
<td>Dinitrophenol</td>
<td>1.0</td>
<td>3.53 ± 0.32</td>
<td>109.0 ± 9.9</td>
</tr>
<tr>
<td>Azide</td>
<td>1.0</td>
<td>3.06 ± 0.44</td>
<td>94.4 ± 13.6</td>
</tr>
<tr>
<td>Iodoacetic acid</td>
<td>1.0</td>
<td>3.15 ± 0.37</td>
<td>97.2 ± 11.4</td>
</tr>
<tr>
<td>Ouabain</td>
<td>1.0</td>
<td>3.06 ± 0.44</td>
<td>94.4 ± 13.6</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>0.20</td>
<td>2.97 ± 0.28</td>
<td>91.7 ± 8.6</td>
</tr>
<tr>
<td>Oligomycin</td>
<td>0.02</td>
<td>3.25 ± 0.28</td>
<td>100.3 ± 8.6</td>
</tr>
<tr>
<td>p-Hydroxymercuribenzoate</td>
<td>0.02</td>
<td>3.08 ± 0.12</td>
<td>95.1 ± 3.7</td>
</tr>
<tr>
<td>Azide + dinitrophenol</td>
<td>—</td>
<td>3.33 ± 0.34</td>
<td>94.3 ± 10.2</td>
</tr>
<tr>
<td>Iodoacetic acid + dinitrophenol</td>
<td>—</td>
<td>3.62 ± 0.38</td>
<td>102.5 ± 10.6</td>
</tr>
<tr>
<td>p-Hydroxymercuribenzoate + dinitrophenol</td>
<td>—</td>
<td>3.83 ± 0.77</td>
<td>108.5 ± 9.02</td>
</tr>
</tbody>
</table>

* Mean ± SE.

The data were derived from triplicates of 3 experiments.

**Table 3 The effect of metabolic inhibitors on the influx of \(^{14}\text{C}\)PFM by HCT 116 cells**

HCT 116 cells were preincubated for 15 min at 37°C with inhibitors in RPMI 1640 basal salt solution and further incubated with 5 \(\mu\)M \(^{14}\text{C}\)PFM for 50 s. The data were derived from triplicates of 5 experiments.

Fig. 5. Effect of aliphatic alcohols on the influx of \(^{14}\text{C}\)PFM by HCT 116 cells. Cells were preincubated for 10 min at 37°C with 2 mM alcohols, 1-pentanol, 1-hexanol, and 1-octanol, and were then incubated with 5 \(\mu\)M \(^{14}\text{C}\)PFM for 50 s. The data were derived from triplicates of 3 experiments; bars, SE. Accumulations significantly greater than control, P < 0.05.

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**Table 1**

[Table content is not provided in the image.]

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**Fig. 3**

Concentration dependence for initial influx of \(^{14}\text{C}\)PFM at 37°C by HCT 116 cells. Cell suspensions (4 \(\times\) 10\(^7\) cells/ml) were exposed to \(^{14}\text{C}\)PFM at concentrations between 2.0 and 100 \(\mu\)M at 37°C for 50 s. Intracellular radioactivity is presented. Points, mean derived from triplicates of at least two experiments; bars fall within the symbol.

**Fig. 4**

Efflux of radioactivity by HCT 116 cells exposed to \(^{14}\text{C}\)PFM. Cells (4 \(\times\) 10\(^7\) cells/ml) after preload with 5 \(\mu\)M \(^{14}\text{C}\)PFM for 5 min, quickly chilled, were recovered by centrifugation, resuspended in warm drug-free minimal medium at 2 \(\times\) 10\(^7\) cells/ml, and incubated at 37°C. At varying intervals of time, cell suspensions of 2 \(\times\) 10\(^7\) cells were drawn and centrifuged through silicone fluid as described in the text. The cells and the efflux media were analyzed for radioactivity. Points, mean derived from triplicates of three experiments; bars, SE. Error bars not shown fall within symbols.

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**Fig. 5**

Effect of aliphatic alcohols on the influx of \(^{14}\text{C}\)PFM by HCT 116 cells. Cells were preincubated for 10 min at 37°C with 2 mM alcohols, 1-pentanol, 1-hexanol, and 1-octanol, and were then incubated with 5 \(\mu\)M \(^{14}\text{C}\)PFM for 50 s. The data were derived from triplicates of 3 experiments; bars, SE. Accumulations significantly greater than control, P < 0.05.
coefficients as high as 2 and 3 if there is a temperature-dependent structural alteration. Thus a $Q_{10}$ of 2.3 is not incompatible with passive diffusion. Furthermore, the $Q_{10}$ value might be artificially high, since the measurements were made with some ongoing metabolic conversions of PFM, and the rate of metabolism was higher at 37°C than at 24°C.

Aliphatic alcohols have been reported to enhance the permeability of artificial lipid membranes (17). These alcohols are believed to interact with the lipid domain of cell membranes and increase the fluidity of lipid bilayers (18). For instance, the efflux system of doxorubicin in human red blood cells reported by Dalmark (19) was affected significantly by aliphatic alcohols, indicating that the major part of doxorubicin transport takes place in the lipid domain of the cell membrane. Our data demonstrated definite enhancement of the influx of PFM by aliphatic alcohols. However, in that this enhancement was not great, the lipid domain is not likely to be the main passage for PFM influx. In this regard, the influx of PFM is relatively slow in comparison to that of the lipophilic alkylating agents, procarbazine and methylmelamines (20, 21).

The initial efflux of PFM, reflecting the release of osmotically active radioactivity, was almost as rapid as influx. Lack of inhibition by metabolic inhibitors, protein inactivators, calcium channel blockers, and a calmodulin inhibitor strongly suggests that passive diffusion is also involved in the efflux of PFM.

The results of these studies with PFM are important for several reasons. In view of the well-documented chemical and biochemical similarities between PFM and MC, we feel that both compounds share similar transport mechanisms. In this regard our studies have overcome the problem associated with the lack of radiolabeled MC. In addition, these studies, which represent an extension of our ongoing research program of PFM and MC pharmacology, can be compared and contrasted to the body of literature of similar studies performed with other alkylating agents (20–26). Moreover, these studies are serving as a basis for our laboratory’s ongoing investigations of MC and PFM transport in tumor cells which are either naturally alkylating atents (20–26).

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