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Porfiromycin disposition in oxygen-modulated P388 cells*

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Summary. The cytotoxicity, metabolism, and DNA alkylation of porfiromycin (PFM) under aerobic and hypoxic conditions were evaluated in P388 murine leukemia cells. Clonogenic assays showed that the IC_{50} value for a 1-h exposure to PFM was 4 μM for aerobic cells and 0.5 μM for hypoxic cells. After a 1-h exposure to concentrations of 1, 5, and 10 μM [^{14}C]-PFM, the accumulation of total radioactivity in hypoxic cells was 10 to 20 times that in aerobic cells. The disposition of radioactivity in cells that had been treated for 1 h with 5 μM PFM under aerobic or hypoxic conditions showed that (a) under either condition, internal free-PFM concentration equalled the external drug concentration; (b) DNA-, RNA-, and protein-bound radioactivity were at least 10 times greater in hypoxic cells than in aerobic cells; and (c) known metabolites and unidentified radioactive products were also generated in greater amounts in hypoxic cells than in aerobic cells. Thus, the increased amounts of radioactivity accumulated by hypoxic P388 cells after exposure to [^{14}C]-PFM resulted from the accumulation of nonexchangeable protein and nucleic-acid adducts and metabolites rather than free PFM. Determinations of DNA adducts formed in P388 cells revealed five possible adducts: (1) N^2-(2′-deoxyguanosyl)-7-methylaminomitosene, (2) a second monofunctional PFM-guanine adduct, (3) a PFM cross-linked dinucleotide, (4) possibly a nucleoprotein-related adduct, and (5) an unknown. We conclude that the enhancement of PFM-induced cytotoxicity by hypoxia appears to be primarily due to increased alkylation of macromolecules.

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Abbreviations: MMC, mitomycin C; *PFM,* porfiromycin; *t-AHME, trans*-7-amino-1-hydroxy-2-methylaminomitosene; *c-AHME, cis*-7-amino-1-hydroxy-2-methylaminomitosene; *AME,* 7-amino-2-methylaminomitosene; *N2-GM,* N^2-(2′-deoxyguanosyl)-7-amino-1-methylaminomitosene; *PBS,* phosphate-buffered saline; *PK,* proteinase K

Introduction

Since the 1960s, mitomycin C (MMC) and porfiromycin (PFM) have been known to be potent alkylating agents that are bioreductively activated and produce mono- and bifunctional linkages to DNA and other macromolecules [9, 10, 32]. Recent advances have been made in our understanding of the chemistry and biochemistry of their actions. Reductive activation of MMC and PFM can be obtained using H_2/Pt [26], rat-liver microsomes [26], electrochemical potential [1], and flavoenzymes [16, 17, 22, 33]. Mechanisms of reduction of the MMC quinone have been suggested via either semiquinone [16, 17] or hydroquinone [22, 26], intermediates that induce opening of the aziridine ring, thereby generating the first bioreductive alkylating site at the C-1 position. Subsequent nucleophilic attack or hydrogen abstraction at the C-1 site has resulted in the production of metabolites and covalently bound adducts [16, 17].

Following the clarification of the reductive process, details of DNA alkylation were described. Major monofunctionally linked DNA adducts and a cross-linked adduct of MMC were first identified by Tomasz et al. [27–30]. We also identified these adducts in DNA that had been alkylated by enzymatically reduced MMC and PFM [16, 18].

The discovery that MMC and PFM are preferentially more toxic to hypoxic cells [11, 12] is a feature of significant clinical interest and has drawn increasing attention to these agents. We have shown that the accumulation of total radioactivity in P388 cells treated anaerobically with [^{14}C]-PFM was 10–20 times greater than that in aerobically treated cells [19]. Keyes et al. [13] have shown that the uptake of [^{3}H]-PFM correlates with the cytotoxicity of PFM against EMT6 cells under aerobic and hypoxic conditions. These authors also showed that the accumulation of [^{3}H]-PFM in hypoxic cells was 10 times that in aerobic cells. However, the disposition of PFM and the nature of the accumulated radiolabel in these cells have not been described. We present the results of our investigations using [^{14}C]-PFM to analyze the accumulation and disposi-

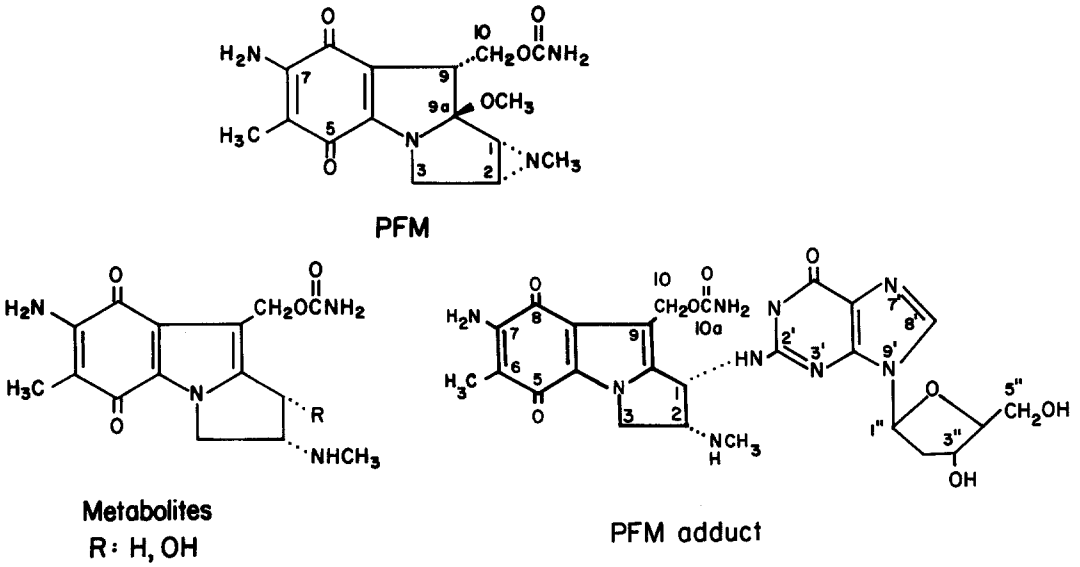

Fig. 1. Structure of PFM, 7-amino-1-hydroxyl-2-methylaminomitosene (or 7-amino-2-methylaminomitosene), and PFM nucleoside adduct

tion of PFM in P388 murine leukemia cells under aerobic and hypoxic conditions. We show that the difference in the accumulation of total radioactivity by P388 cells under hypoxic vs aerobic conditions was not the result of a difference in the accumulation of unmetabolized [^{14}C]-PFM, but rather was due to remarkably different rates of conversion of PFM to metabolites and macromolecule-bound products. Furthermore, the identification and analysis of DNA adducts formed in P388 cells under both conditions is described.

Materials and methods

Reagents. PFM (Fig. 1) and MMC were kindly supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md.) and by Dr. J. P. McGovren, Upjohn Co. (Kalamazoo, Mich.). [^{14}C]-PFM was synthesized from [^{14}C]-methyliodide and MMC according to a modification [16] of the procedure of Steven et al. [25]. The metabolites *trans-* and *cis*-7-amino-1-hydroxy-2-methylaminomitosene (t- and c-AHME) and 7-amino-2-methylaminomitosene (AME) were generated from enzymatically reduced PFM and then purified by HPLC [16, 17]. The identity of these metabolites were confirmed by mass spectrometry and nuclear magnetic resonance (NMR) [1, 16]. N^2-(2'-Deoxyguanosyl)-7-amino-1-methylaminomitosene (N2-GM) was prepared as described in detail elsewhere [16, 18] and its identity was confirmed by NMR [18]. SF-1250 silicone fluid was generously donated by the General Electric Co. (Waterford, N.Y.).

Cell culture and cell survival. P388 murine leukemia cells were maintained in vitro by serial passage in RPMI 1640 median containing 0.1 mM mercaptoethanol, 15% fetal calf serum, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2 μM L-glutamine. Cells were grown at 37° C and 95% humidity in an atmosphere containing 5% CO_2. P388 cell survival was assayed by colony formation in soft agar [6]. Cells were exposed to PFM at designated concentrations by the method described below. After drug treatment for 1 h, cells were chilled to 4° C, recovered by centrifugation, then washed twice with phosphate-buffered saline (PBS). Washed cells were resuspended in soft agar and plated in triplicate at three dilutions between 300 and 900 cells/35 mm petri dish. Petri dishes were incubated at 37° C in a humidified incubator containing 5% CO_2 and 95% air, and colonies of >50 cells were counted after 10 days. The efficiency of colony formation for P388 was about 25%. Intracellular water and extracellular space were measured according to the method of Hissin and Hilf [7]. Cell volume was determined with a Coulter Channelyzer (Coulter Electronics; Hialeah Park, Fla.) calibrated with 10- and 14.48 μm-diameter latex microspheres (volume, 524 and 1,590 μm^3, respectively).

Drug treatment under aerobic and hypoxic conditions. Cells in exponential growth were recovered and suspended in 5 ml fresh medium using 100-cm^2 glass culture bottles, which were closed with rubber stoppers. Each bottle, containing 2×10^7 cells, was placed in an incubator set at 37° C. The method described by Rauth et al. [23] was used to achieve hypoxia and to keep aerobic controls. PFM dissolved in small volumes of dimethylsulfoxide (DMSO) was injected into each bottle without disturbing the hypoxic condition. Hypoxic control cells received DMSO alone. To ensure that aerobic treatment did not suffer from partial anaerobiosis, drug treatment was also performed in open, plastic culture dishes that were maintained at 37° C in an atmosphere containing 95% air and 5% CO_2 at 95% humidity. Further incubation at 37° C continued for the appropriate periods. At the end of incubation, cells were either quickly placed in an ice bath for analysis or recovered immediately for estimation of total radioactivity. Nonspecific association of PFM to the cell surface was obtained by exposing cells to drug at 0° C for 10 s.

Accumulation and efflux of radioactivity. Drug-treated cell suspensions of 4×10^6 cells/ml were centrifuged through silicone fluid SF-1250 using a modification [20] of the method described by Chello et al. [4]. Sedimented cells were resuspended in water and mixed with 5 ml Beckman Ready-Safe (Beckman; Palo Alto, Calif.), and radioactivity was determined with a Beckman 5801 liquid scintillation counter. The efflux of radioactivity from P388 cells was measured as previously described in detail [20].

Distribution of radioactivity in cells. After treatment with drug, P388 cells (2×10^7) were chilled, recovered by centrifugation through silicone, and lysed with deionized water as previously described [20]. Three groups of duplicate samples of cell lysate were analyzed. Group one was digested by RNase and then by proteinase K (PK). Group two was digested by PK only. In these cases, cell lysates were adjusted to pH 7.5 with 1 M TRIS. RNase was added at 50 μg/10^6 cells, and the digestion was carried out at 37° C for 2 h. PK was added at 0.4 mg/2×10^7 cells, and the digestion was carried out at 37° C for 3 h in the presence of 1% sodium dodecyl sulfate (SDS). Group three was left undigested. Before further analysis, lysates of all groups were separated into soluble and particulate fractions by previously published procedures [20].

HPLC analysis. Soluble cellular radioactive material was analyzed using a reversed-phase HPLC method described elsewhere [17, 20]. PFM, pure t- and c-AHME, and AME were used as internal standards, and [^{14}C]-

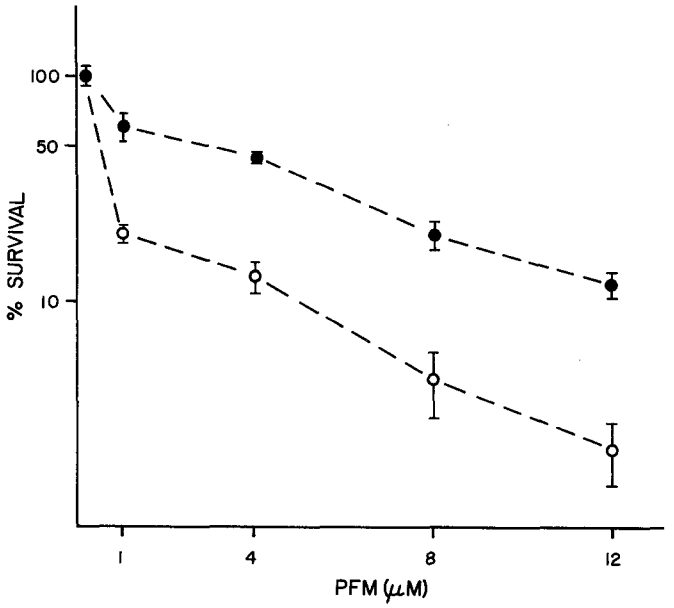

Fig. 2. Survival of P388 cells after a 1-h exposure to various concentrations of PFM under aerobic (●) and hypoxic (○) conditions. Survival was evaluated by soft-agar colony formation as described in Materials and methods. Points represent the means and standard deviations (*bars*) of three separate experiments

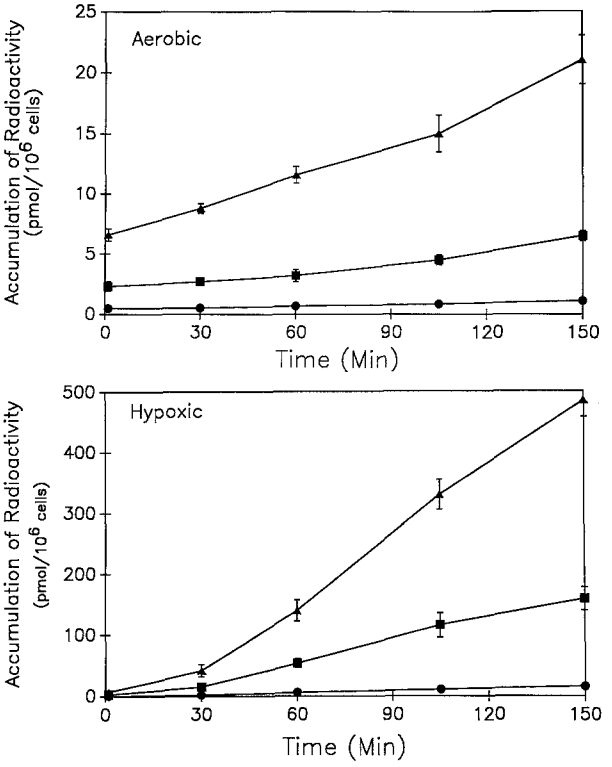

Fig. 3. Total accumulation of radioactivity by P388 cells exposed to [^{14}C]-PFM at 3 different drug concentrations, 1 μM (●), 5 μM (■) and 10 μM (△), under aerobic (*top*) and hypoxic (*bottom*) conditions as described in Materials and methods. Treated cells ($3-5\times10^6$ cells) were recovered at various intervals by centrifugation through silicone fluid SF-1250 and solubilized, and radioactivity was measured. Points represent the means and standard deviations of three experiments

PFM was used as an external standard. Radioactive DNA adducts were also analyzed by the same HPLC method, except that authentic N2-GM was used as an internal standard.

Measurement of N-demethylation of PFM. Crude cell homogenates were prepared by a previously described method [20]. Rat-liver microsomes were prepared using the method of Yasukochi and Masters [34]. Measurement of *N*-demethylation was performed according to our prior report [20]. As a control for the *N*-demethylase assay, both liver microsomes and cell homogenates were used to demethylate aminopyrine, a common substrate for *N*-demethylases [21].

Isolation of nuclei, chromatin, and DNA. Nuclei from P388 cells (2×10^8) were isolated using the method of Ivanovic et al. [8]. Chromatin and DNA were isolated from nuclei according to the methods of Rubin and Moudinawakis [24] and Ivanovic et al. [8], respectively.

Preparation of PFM-/DNA and PFM-chromatin. DNA and chromatin were isolated from untreated P388 cells and were alkylated by enzymatically reduced PFM according to our previously reported method [16, 17].

Separation of DNA adducts. DNA adducts were obtained from three sources: (1) DNA that had been isolated from drug-treated P388 cells, (2) chromatin that had been isolated from untreated P388 cells and then alkylated by enzymatically reduced PFM, and (3) DNA that had been isolated from untreated P388 cells and then alkylated by enzymatically reduced PFM. DNA from all three sources was purified and then digested by nucleases as previously described [16]. DNA adducts were separated and analyzed by HPLC [16]. Samples containing about 5,000 dpm were evaluated chromatographically for each analysis.

Results

Cytotoxicity of PFM

P388 cells were more susceptible to a 1-h exposure to PFM under hypoxic conditions than under aerobic treatment (Fig. 2). The IC_{50} of PFM decreased from 4 μM for aerobically treated cells to 0.5 μM for hypoxically treated cells. Throughout the course of both drug treatments, the pH of the medium remained stable at about 7.2

Demethylation

The activity of demethylases in P388 cell homogenates was investigated. Aminopyrine, a common substrate for *N*-demethylases, was not affected by P388 cell homogenate, whereas it was demethylated by rat-liver microsomes at 32.1 nmol min^{-1} mg^{-1}. Under the same conditions, PFM was not demethylated by P388 cell homogenate or by liver microsomes. The activity of the cell-homogenate preparations was confirmed by measuring quinone reductase activity [5] and the ability of microsomes to reduce PFM [16].

Accumulation and efflux of radioactivity

Nonspecific binding of radioactivity by cells took place within 10 s of drug exposure and depended on external drug concentration but not on temperature. Consequently, all results were corrected for this nonspecific binding. The accumulation of total radioactivity, including that of the

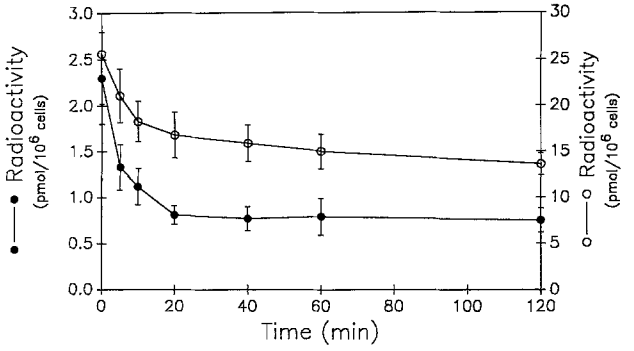

Fig. 4. Efflux of radioactivity by P388 cells exposed to [^{14}C]-PFM. Cells (4×10^6 cells/ml) were preloaded with 5 μM [^{14}C]-PFM at 37° C for 1 h, under aerobic (●——●) and hypoxic (○——○) conditions. Treated cells were quickly chilled and recovered by centrifugation, then resuspended in warm, drug-free minimal medium at 2×10^6 cells/ml and incubated at 37° C. At varying intervals suspensions comprising 2×10^6 cells were drawn and centrifuged through silicone fluid, and the cells were analyzed for radioactivity. Each point (mean ± SE) is derived from triplicates of three experiments

Table 1. Distribution of soluble and insoluble radioactive material in P388 cells after exposure to 5 μM [^{14}C]-PFM for 1 h

Cell extract	Radioactivity (pmol/ μl cells)			
	Macromolecule-associated	PFM	Known metabolites	Unknowns
Aerobic:				
Undigested	1.2±0.2	4.3±0.2	0.5±0.03	0.5±0.1
PK-digested	0.9±0.1	4.3±0.1	0.6±0.05	0.7±0.1
RNase-, PK-digested	0.9±0.2	4.4±0.4	0.5±0.05	0.6±0.1
Hypoxic:				
Undigested	14.5±1.6	4.3±0.2	2.1±0.4	2.9±0.3
PK-digested	8.3±0.7	4.4±0.4	3.3±0.2	6.7±0.9
RNase-, PK-digested	6.7±0.2	4.5±0.5	4.4±0.5	9.2±0.5

Radioactivity recovered in each fraction was expressed as pmol/ μl cells according to the specific activity of [^{14}C]-PFM. Data were derived from duplicates of two experiments. Radioactive compounds in each soluble extract were identified by co-chromatography with authentic internal standards by HPLC. Aliquots of 0.5 ml were collected and analyzed. Peaks were integrated and then grouped into PFM (peak II), metabolites (peaks I, III, and IV), and unknowns

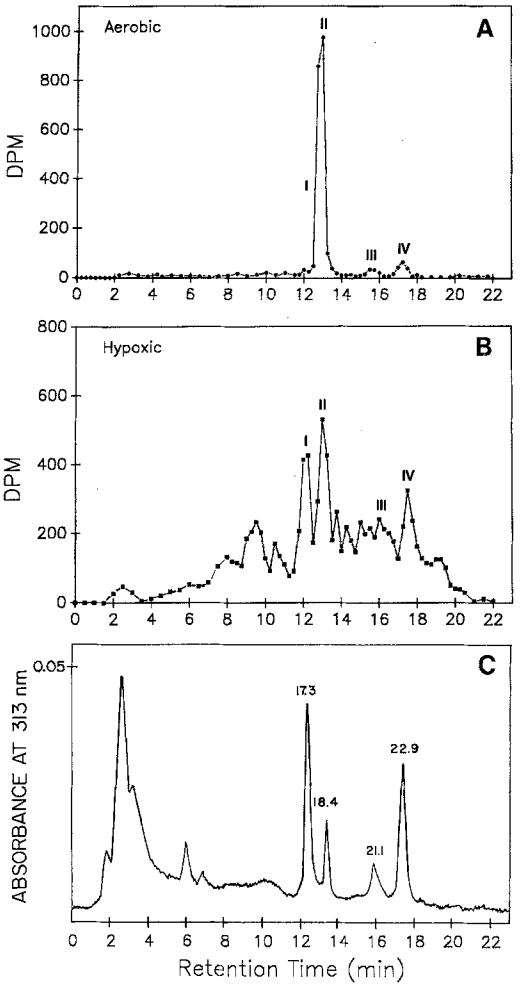

Fig. 5 A–C. HPLC separation of radioactive compounds in soluble extracts from P388 cells exposed to 5 μM [^{14}C]-PFM for 1 h under aerobic and anaerobic conditions. Radioactivity profiles were obtained from **A** aerobic and **B** anaerobic cell extracts. The absorbance profile was obtained from **C** the aerobic cell extract plus internal standards. The absorbance detector was set at 313 nm and 0.05 AUFS. Standards included authentic t-AHME (k′ = 17.3), PFM (k′ = 18.4), c-AHME (k′ = 21.1), and AME (k′ = 22.9)

parent compound, metabolites, and macromolecule-bound products, was determined by exposing P388 cells to [^{14}C]-PFM at concentrations of 1, 5, and 10 μM for periods of up to 150 min (Fig. 3). After 1 min, there was little difference in the accumulation of total carbon 14 between hypoxic and aerobic cells exposed to the same concentration of drug. As the duration of exposure increased, the difference increased. After 1 h drug exposure, the accumulation of total radioactivity in hypoxic cells was 10 times that in aerobic cells. Under both conditions, the accumulation of radioactivity was dependent on the external drug concentration.

Cells that had accumulated drug for 1 h under both aerobic and hypoxic conditions lost radioactivity very rapidly for the first 3 min after resuspension in drug-free medium (Fig. 4). After 20 min, aerobic cells released >50% of the radioactivity, whereas hypoxic cells released about 40%. Thereafter, little radioactivity was lost from either group of cells.

Intracellular disposition of radioactivity

Total macromolecule-associated radioactivity was at least 10 times greater in hypoxic cells than in aerobic cells (Table 1). RNase digestion did not remove detectable amounts of radioactivity from macromolecules in aerobic cells but did solubilize 11% of the bound radioactivity from hypoxic cells. Protein digestion by PK released about 25% and 57% of the macromolecule-bound radioactivity from aerobic and hypoxic cells, respectively.

HPLC analyses of radioactive compounds in soluble extracts were performed in three experiments (Fig. 5). Good separation of radioactive compounds from extracts of aerobic cells was obtained. However, numerous un-

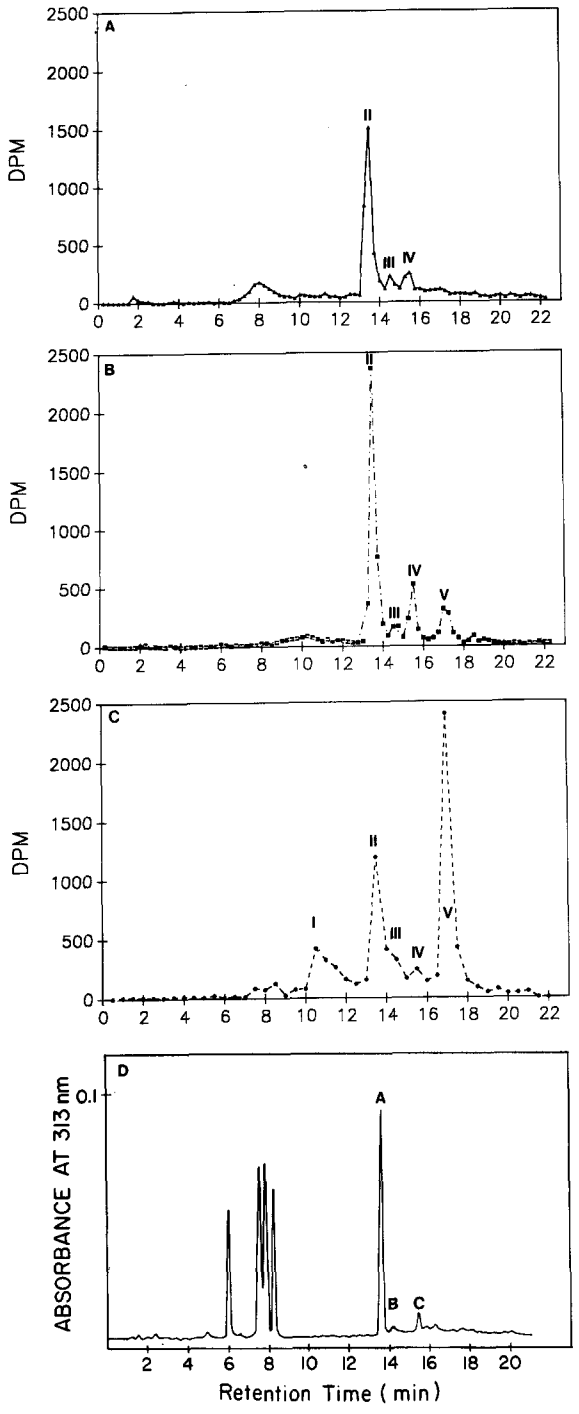

Fig. 6 A–D. HPLC separation of DNA adducts generated from three separate DNA sources after digestion by nucleases (methods are described in detail in Materials and methods). Radioactivity profiles were obtained from **A** DNA and **B** chromatin that was isolated from untreated P388 cells and then alkylated by enzyme-activated PFM, and **C** DNA from drug-treated P388 cells. **D** Absorbance profile at 313 nm for DNA from untreated P388 cells that was alkylated by enzyme-activated PFM. The sample size was 10 times that used for profile A

known radioactive products were generated by hypoxic cells and clear separations were not obtained. Four radioactive peaks (I–IV, Fig. 5 A) that co-chromatographed with authentic standards were resolved from aerobic cell extracts. Peaks I–IV co-chromatographed with authentic t-AHME ($k' = 17.3$), PFM ($k' = 18.4$), c-AHME ($k' = 21.1$), and AME ($k' = 22.9$), respectively. Other radioactive peaks and areas shown in Fig. 5 were not identified. In cases of hypoxic cell extracts, although the separations were not as clear, three large radioactive peaks with k' values identical to those of t-AHME, PFM, and AME were detected (Fig. 5 B).

For quantification, the radioactivity was converted to picomoles per microliter of cells by the value for the intracellular water in P388 cells (0.63 ± 0.05 µl/10^6 cells) and P388 cell volume (0.65 ± 0.05 µl/10^6 cells). Data from three experiments are shown (Table 1). Peaks I, III, and IV together were designated as metabolites. All unidentified peaks were combined and classified as unknowns. In both aerobic and hypoxic cells the intracellular concentration of PFM was approximately equal to the concentration of drug in the incubation media. Protein and RNA digestion of aerobic cell extracts from cells incubated with [^{14}C]-PFM under aerobic conditions released little PFM or other radioactive compounds. In contrast, both protein and RNA digestion of hypoxic cell extracts released large amounts of metabolites and unknown radioactive compounds.

When hypoxic cells were treated with 0.5 µM PFM so as to accumulate an amount of total radioactivity equivalent to that in aerobic cells treated with 5 µM PFM, the distribution of radioactivity between macromolecule-bound and soluble material and the HPLC profiles of these hypoxic cells resembled those of hypoxic cells, not those of aerobic cells that had been treated at 5 µM PFM (data not shown).

DNA adducts

Seven distinguished peaks were detected at 313 nm by HPLC analysis of purified P388 DNA that had been alkylated by PFM through enzymatic activation (Fig. 6 D). The first four peaks were, in order, deoxycytidine, thymidine, deoxyadenosine, and deoxyguanosine. The remaining three peaks (A–C) represented adducts. These results are similar to those found in our previous study using calf-thymus DNA [16]. Peak A had the same k' value as an authentic N2-GM standard; peak B was identified as being a second monofunctional PFM-guanine adduct; and peak C, a cross-linked dinucleoside adduct. When the HPLC eluate was monitored for radioactivity (Fig. 6 A), there were three peaks (II–IV) that corresponded to the absorbance peaks. The enzymatically alkylated P388 chromatin revealed the same three absorbance peaks (A–C) by absorbance (data not shown) but four peaks (II–V) by radioactivity (Fig. 6 B). Due to the small amount of material generated, adducts to the DNA isolated from drug-treated cells could not be clearly detected by absorbance; however, five peaks (I–V) were detected by radioactivity (Fig. 6 C). Peaks II, III, and IV from all three sources of DNA had the same retention time and, in each case, corresponded to absorbance peaks A, B, and C, respectively. Peak I was observed only in DNA from drug-treated cells. Peak V was present in the highest amount in DNA from drug-treated cells and at lower amounts in enzymatically alkylated chromatin, but it was not detected in enzymatically alkylated DNA.

Comparison of cells treated under aerobic and hypoxic conditions revealed that the amount of each of the five radioactive species in hypoxic cells was at least 10 times that of the corresponding species in aerobic cells. In both cases, peak V was always the major form present.

Discussion

The response of P388 murine leukemia cells to mitomycin C (MMC) and PFM was similar to those recently reported for several solid-tumor cell lines [11, 12, 15, 23]. P388 cells were more susceptible to MMC and PFM under hypoxic conditions than under aerobic conditions.

The four radioactive metabolite peaks that were fractionated and identified by HPLC from the cell extract (Fig. 4) were essentially the same metabolites that were previously generated by reduced nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome P-450 reductase activation of PFM [16]. Unknown soluble radioactivities could be the result of direct interaction between soluble compounds, such as glutathione, with activated PFM or repaired products of alkylated macromolecules. Another possibility would be that these unknown radioactive compounds are products from nonspecific incorporation of radioactive formate produced by demethylation. Little is known about the demethylation of an aziridinylmethyl group. However, our data indicate that PFM is a poor substrate for *N*-demethylases. Demethylation of the products of PFM with the aziridine ring opened to a secondary amine remains to be investigated. All exchangeable radioactivity during efflux from both aerobic and hypoxic cells could be accounted for in the soluble fractions (Table 1).

Identification of radioactive DNA adduct peaks II–IV from drug-treated cells (aerobic and hypoxic) was based on previously published results obtained using purified DNA that had been alkylated enzymatically in vitro [16]. Peak II is N2-GM, peak III corresponds to another deoxyguanine-related monofunctional adduct, and peak IV is a cross-linked adduct. Radioactive adduct peaks I and V have not yet been identified. There are reasons to believe that peak V is an adduct related to nucleoproteins. These include the fact that peak V was not observed with purified naked DNA but was seen when alkylation took place in whole cells and when isolated chromatin was used, and both of the latter sources contain nucleoproteins. The possibility of these two peaks' being nucleosides that incorporated radioactivity nonspecifically due to demethylation has not been totally ruled out, as suggested by Tomasz et al. [31]. Further studies are under way for the identification of this adduct.

The accumulation of total radioactivity in hypoxic cells vs aerobic cells showed at least a 10-fold difference. Similar results have been reported by Keyes et al. [13]. Our examination of the distribution of radioactivity in P388 cells after exposure to PFM under aerobic and hypoxic conditions showed differences for all radioactive products, including the radioactivity associated with protein, DNA and RNA, metabolites, and unknowns. The only exception was that the concentration of intracellular PFM under both conditions of drug exposure was the same and, in both cases, equalled the extracellular PFM concentration. These data indicate that the 10-fold difference in carbon 14 accumulation between cells under aerobic vs hypoxic conditions is not due to differential accumulation of PFM, but rather reflects a differential accumulation of cell-associated PFM products. Furthermore, we have recently reported that the uptake and efflux of PFM in HCT 116 cells and human red blood cells is a passive diffusion process and that an equilibrium of PFM concentration is reached in cells within 80 s [20]. The current data suggest that the uptake and efflux of PFM by P388 cells probably also occurs by diffusion.

Our data also indicate that a large amount of cellular radioactivity was associated with proteins. This was demonstrated by the increase in soluble radioactive compounds on protein digestion of extracts from both aerobic and hypoxic cells. In that DNA cross-linking by MMC or PFM is believed to be the major reason for cell death, the significance of protein alkylation for cytotoxicity remains to be explored.

PFM is reductively activated to a semiquinone under both aerobic and hypoxic conditions. Redox cycling between the PFM semiquinone and oxygen takes place in aerobic cells and induces oxygen-radical formation as one potential cause of cytotoxicity [2, 3, 14]. In hypoxic cells, on the other hand, the PFM semiquinone is attacked by nucleophiles, which are converted to nonexchangeable alkylated products. This continuous cellular sink for PFM makes a continuous influx of this drug necessary for the maintenance of equilibrium with the extracellular source of PFM. It is likely that the preferential cytotoxicity of PFM, and of MMC, under hypoxic conditions is related to this enhancement of the alkylation of many cellular components.

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