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Differential Cytotoxicity and Induction of Apoptosis in Tumor and Normal Cells by Hydroxymethylacylfulvene (HMAF)*

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ABSTRACT. This investigation compared the effects of hydroxymethylacylfulvene (HMAF), a novel antitumor drug with alkylating properties, in eight human tumor (prostate, colon, and leukemia) cell lines, and five human normal (prostate and renal proximal tubule epithelial, colon mucosa, fibroblasts, and endothelial) cell lines. Drug-induced growth inhibition paralleled the uptake of HMAF into both tumor and normal cells, although normal cells were 3- to 4-fold more tolerant to the accumulated drug. In both tumor and normal cells, approximately two-thirds of internalized [^{14}C]HMAF-derived radioactivity was bound covalently to macromolecules. Trypan blue exclusion and cell counts indicated that HMAF was cytotoxic in tumor but cytostatic in normal cells. Correspondingly, profound apoptosis was detected in all tumor cell lines examined. A 4-hr treatment with HMAF followed by 20-hr post-incubation induced a potent DNA fragmentation in nearly all tumor lines. Apoptosis-resistant PC-3 and HT-29 cells underwent significant DNA fragmentation after 24 hr of continuous treatment with HMAF. In contrast to tumor cell lines, marginal or very low levels of apoptosis were detected in the normal cells even after prolonged treatments with HMAF at concentrations that exceeded 15- to 800-fold the GI_{50} values in tumor cells. This resistance of normal cells to apoptosis could not be accounted for by differences in drug accumulation or drug covalent binding to macromolecules. The qualitatively different responses of the tumor and normal cells studied suggest a greater tolerance of normal cells to HMAF–macromolecular adducts. The demonstrated differential cytotoxic/cytostatic and apoptotic effects of HMAF can be of significance for the clinical use of this promising new agent. BIOCHEM PHARMACOL **59**;10:1217–1226, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. hydroxymethylacylfulvene; MGI 114; covalent adducts; differential apoptosis; differential cytotoxicity; tumor and normal cells

HMAF¶ (MGI 114; Fig. 1), currently in Phase II clinical trials, is a novel antitumor agent derived from the natural product illudin S [1]. HMAF has shown promising antitumor activity, including profound tumor regression and complete cures, in a variety of human tumor xenograft models [2–4].

HMAF binds to cellular macromolecules, inhibits DNA synthesis, and induces apoptosis [5, 6]. However, the drug differs markedly from typical DNA-alkylating antitumor agents. HMAF may be the first clinical antitumor drug that forms only monoadducts in DNA [5, 6], which are usually considered to be lesions markedly less lethal than bifunctional adducts such as interstrand cross-links [7]. Also, HMAF–DNA adducts seem to be insufficient for the observed inhibition of cellular DNA synthesis [5]. Since the majority of covalently bound intracellular HMAF equivalents were associated with cellular proteins, we suggested that non-DNA targets may contribute to its pro-apoptotic antitumor properties [6].

Previous studies showed that illudin S, the HMAF prototypic agent, was less inhibitory against certain normal cell lines than tumor cell lines [1]. Differences in drug uptake were implicated as the origin of divergent growth inhibition. Our recent studies indicated that uptake of HMAF seems to determine apoptosis in tumor cells [6]. The effects of HMAF in normal cells, however, have not been investigated.

The present study evaluated the possibility that normal cells may respond differently to pro-apoptotic and growth inhibitory effects of HMAF. Whereas HMAF was cytotoxic and strongly pro-apoptotic in eight tumor cell lines tested,

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¶ *Abbreviations*: HMAF (MGI 114), hydroxymethylacylfulvene; GI_{50}, drug concentration producing 50% net growth inhibition; D_{10}, drug concentration reducing cell of survival to 10%; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TdT, terminal deoxynucleotidyl transferase; and PCA, perchloric acid.

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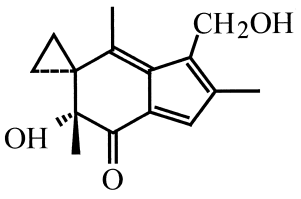

HMAF

FIG. 1. Structure of HMAF.

normal cells were apoptosis-resistant, more tolerant to HMAF adducts in terms of growth inhibition, and remained viable even when growth-inhibited. These qualitatively different responses could not be accounted for by the differences in cellular uptake of HMAF, suggesting that normal cells have protective mechanisms against HMAF effects.

MATERIALS AND METHODS

Drug

A stock solution of HMAF (MGI Pharma) was prepared in DMSO. [^{14}C]HMAF (specific activity 15 mCi/mmol in ethanol) labeled at the carbinol position (Fig. 1) was made by Amersham Inc.

Cell Cultures

A human colon carcinoma (Colo 320DM), a colon adenocarcinoma (HT-29), a diploid normal human fibroblast (WI-38), and human prostate carcinomas (LNCaP and PC-3) were purchased from the American Type Culture Collection and maintained according to its recommendations. Human CEM leukemia cells (a gift from Dr. William T. Beck) were cultured in Joklik's Minimal Essential Medium containing 10% heat-inactivated fetal bovine serum [5]. LNCaP sublines LNCaP-Pro5, LNCaP-LN3 (provided by Dr. C. A. Pettaway, M.D. Anderson Cancer Center), and LNCaP-C4–2 (purchased from UroCor Inc.) were maintained as described elsewhere [8, 9]. Normal colon mucosal NCM 460 cells [10] were obtained from InCell Corporation LLC. Normal prostate epithelial cells (PrEC), umbilical vein endothelial cells (HUVEC), and renal proximal tubule epithelial cells (RPTEC) were purchased from Clonetics. All cell lines were tested regularly for *Mycoplasma* contamination by the DNA hybridization method using a Gen-Probe kit and, except for CEM, were maintained as monolayer cultures according to the manufacturer's protocol.

Cell Growth Inhibition and Viability

Growth inhibitory activity of HMAF was assayed using the standard MTT assay [11]. Exponentially growing cells in a 96-well microtiter plate were incubated with the drug for 3 days and then were subjected to the colorimetric reaction with MTT. The results are expressed as GI_{50} values. Cell viability was determined based on cell ability to exclude trypan blue.

Clonogenic Survival Assays

Colony formation assay was performed as described previously [12]. Briefly, cells (150–10,000 cells/well) were seeded onto 6-well plates in their respective growth medium and exposed to HMAF (0–5 μM) for either 4 or 24 hr. After 8–15 days of post-incubation, plates were stained with crystal violet, and colonies of at least 50 cells were scored. Average plating efficiencies (±SEM) for LNCaP-Pro5, LNCaP-LN3, LNCaP-C4–2, PC-3, NCM 460, and HT-29 cells were 12 ± 3, 25 ± 3, 49 ± 10, 20 ± 4, 22 ± 2, and 19 ± 4%, respectively. The surviving fraction was calculated as the ratio of plating efficiency in treated and control wells.

Survival of cell lines that do not form distinct colonies (LNCaP, PrEC, and RPTEC) was determined in the same way except that at the end of post-incubation, cells were fixed with ice-cold 50% trichloroacetic acid and stained with a 0.4% solution of sulforhodamine B in 1% acetic acid [13]. The stained cellular proteins were solubilized with 10 mM Tris base, and absorbance was measured at 570 nm. The surviving fraction was calculated as the ratio of signal in treated wells to signal in control wells.

Cellular Uptake and Covalent Binding of [^{14}C]HMAF

Cells for uptake studies (~90% confluent 35-mm plates for monolayer cultures and 1-mL aliquots of 2.2×10^6 cells for CEM) were incubated with 10 μM [^{14}C]HMAF for 2 hr at 37°. Monolayer cultures were washed with ice-cold PBS, the cells were trypsinized and pelleted by centrifugation, and cell pellets were solubilized in 2% $NaHCO_3$ containing 1% Triton X-100 and 1% trypsin for 1 hr at 37°. Radioactivity in the solubilized pellet was determined in a liquid scintillation counter. CEM cells were layered onto 0.25 mL of 1-bromododecane, centrifuged, and processed for cell-associated radioactivity as described previously [6]. Total covalent binding and free cell-associated drug were determined based on precipitation of macromolecules with cold PCA as described previously [6].

Apoptotic Cell Morphology Evaluation by Hoechst 33258 Staining

Cells grown on glass coverslips were treated with HMAF as indicated, washed with PBS, fixed in 3% paraformaldehyde for 10 min at room temperature, rinsed again with PBS, and stained with Hoechst 33258 (1.5 μg/mL) for 5 min. Cell morphology was examined under a fluorescence microscope.

TABLE 1. Growth inhibition, cell survival, drug uptake, and cell viability in tumor and normal cell lines

Cell line	Growth inhibition* GI_{50} (μM)	Survival† D_{10} (μM)	Survival† SF at 5 μM HMAF	[^{14}C]HMAF uptake‡ (pmol/1 × 10^6 cells/2 hr)	Cell viability§ (%)
Tumor					
LNCaP	0.03 ± 0.01	1	0.046	2578 ± 255	60
LNCaP-Pro5	0.07 ± 0.008	1.6	0.014	227 ± 60	35
LNCaP-C4-2	0.11 ± 0.02	2.4	0.050	205 ± 23	20
LNCaP-LN3	0.13 ± 0.007	1.8	0.025	153 ± 30	24
PC-3	0.26 ± 0.04	3.1	0.027	97 ± 11	67
Colo 320DM	0.21 ± 0.13	ND‖	ND	64 ± 11	ND
HT-29	0.55 ± 0.22	3.9	0.091	73 ± 15	95
CEM	1.7 ± 0.2¶	ND	ND	26 ± 5	65
Normal					
WI-38	0.37 ± 0.08	ND	ND	150 ± 23	ND
PrEC	0.35 ± 0.04	2.7	0.082	171 ± 12	93
NCM 460	0.43 ± 0.02	2.9	0.043	134 ± 18	96
HUVEC	0.19 ± 0.004	ND	ND	498 ± 78	95
RPTEC	0.11 ± 0.01	5.5	0.126	1014 ± 185	100

*Drug concentrations that inhibit cell net growth by 50% as determined by the standard MTT assay. The values represent means (±SEM) from 2–4 experiments carried out in triplicate.

†Concentration of HMAF resulting in survival of 10% of the cells after a 4-hr treatment; colony formation assay. The D_{10} values were determined based on regression curves from all experimental points obtained in 2–4 experiments. SF = surviving fraction.

‡Cells at 1–2 × 10^6 cells/mL were incubated with 10 μM [^{14}C]HMAF. The values shown are corrected for 0-min background. The values represent means (±SEM) from 2–3 experiments carried out in duplicate.

§By trypan blue exclusion after 4 hr of treatment with 50 μM HMAF followed by a 20-hr post-incubation period in drug-free medium, except for CEM cells where the post-incubation was 17 hr. Typical sample-to-sample variability was 3–5%.

‖Not determined.

¶Determined previously [5].

Quantitative DNA Fragmentation Assay

The fragmentation assay detects a broad range of fragment sizes, from subnucleosomal particles up to at least ~150 kbp [5, 14]. [^{14}C]Thymidine-prelabeled cells were drug-treated as indicated and processed either directly (suspension cultures) or after trypsinization (monolayer cultures). In the latter case, any cells that detached into the medium or PBS wash prior to trypsinization were collected by centrifugation and combined with trypsin-dislodged cells. Further processing was as described previously [5]. Briefly, cells were permeabilized in a hypotonic buffer, and chromatin fragments released from nuclei were collected by centrifugation in the supernatants, whereas the remaining pellets contained unfragmented DNA. The results are expressed as the percentage of the total DNA released in the supernatants, corrected for the radioactivity released in untreated controls [5].

TdT Assay for Identification of Apoptotic Cells by Flow Cytometry

Apoptotic cells were monitored based on biotin tagging of 3′-OH ends in cellular DNA resulting from apoptotic fragmentation with TdT followed by immunofluorescent detection of the biotin label [15, 16] as described previously [5]. In addition to TdT data, these determinations monitored forward and side light scattering and DNA content based on propidium iodide staining.

RESULTS

HMAF Effects on Growth and Survival of Tumor and Normal Cells

HMAF effects were evaluated in a panel of human cell lines that included prostate, colon, and leukemia tumor cell lines, and normal prostate and renal proximal tubule epithelial cells, colon mucosa, human fibroblasts, and endothelial cells. Selected cell lines differ in their p53 status and expression of apoptosis-related proteins, such as Bcl-2. LNCaP and its clones express a wild-type p53 [17], whereas HT-29, CEM, and PC-3 represent cells with mutated or null p53 phenotypes, respectively [18–20]. Within prostate cell lines, LNCaP-C4–2, LNCaP-LN3, and PC-3 cells show elevated levels of Bcl-2 and decreased androgen dependency [9, 21, 22].

HMAF was growth-inhibitory in the sub-micromolar to micromolar range against tested cell lines following a continuous incubation under the standard conditions of the MTT assay. The calculated GI_{50} values for tumor cells ranged from 0.03 to 1.7 μM for LNCaP and CEM cells, respectively (Table 1). Within a panel of normal cells, renal proximal tubular (RPTEC) cells were the most sensitive to HMAF (GI_{50} = 0.11 ± 0.01 μM), whereas normal colon mucosa, NCM 460 cells, were the least sensitive (GI_{50} = 0.43 ± 0.02 μM).

For selected cell lines, we also examined long-term cell survival and determined HMAF concentrations that reduced survival to 10% (D_{10}) after a 4-hr exposure to drug.

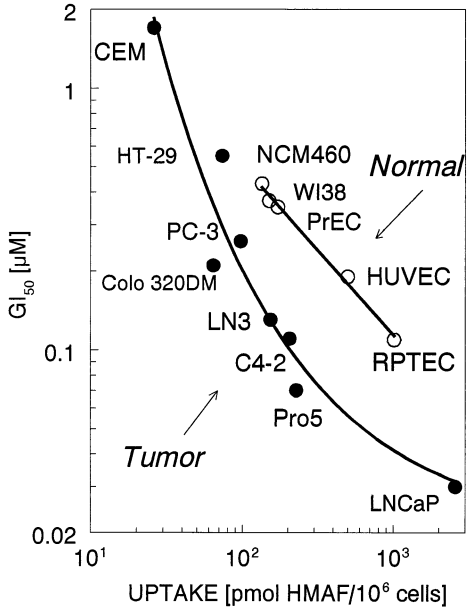

FIG. 2. Distinct correlations between GI_{50} and cellular uptake of [^{14}C]HMAF in tumor and normal cell lines. The GI_{50} values and total cellular uptake (data from Table 1) were determined for each cell line as described in Materials and Methods. The correlation coefficient for tumor cells was 0.97 and for normal cells 0.99. Tumor cells: closed symbols; normal cells: open symbols.

The D_{10} values for tumor lines (1.0 to 3.9 μM) tended to be slightly lower than for normal cells (2.7 to 5.5 μM). Likewise, the surviving fraction at 5 μM HMAF ranged from 1.4 to 9.1% for tumor cells, with a median value of 3.7%, and from 4.3 to 12.6% for normal cells, with a median value of 8.2% (Table 1). Thus, survival data showed a marginal distinction between tumor and normal cells. These differences, however, like the differences in HMAF effects on growth inhibition, may fall within the general variability from cell line to cell line.

Cellular Uptake of [^{14}C]HMAF Versus Growth Inhibition

To determine whether variations in drug uptake are responsible for the differences in HMAF growth inhibitory activity, we measured drug accumulation in all cell lines using the conditions previously established with CEM cells (2 hr at 37° with 10 μM [^{14}C]HMAF) [6]. The level of uptake ranged widely from 2578 pmol/10^6 cells for the most sensitive (LNCaP) to 26 pmol/10^6 cells for the least sensitive (CEM) cells, respectively (Table 1).

HMAF growth inhibitory activity correlated with the level of drug uptake (Fig. 2), although with two distinct trends for tumor and normal lines. Normal cells were less growth-inhibited at the same level of drug uptake. For instance, a similar HMAF accumulation in normal PrEC and tumor LNCaP-LN3 cells resulted in an ~3-fold more potent growth inhibition of the tumor line. Likewise, HMAF was equally growth-inhibitory against normal RPTEC and tumor LNCaP-C4–2 cells despite an ~5-fold greater uptake in the former line.

The enhanced tolerance of normal cells to cell-associated drug might reflect a lower proportion of drug covalent binding to macromolecules. Additional determinations in selected cell lines showed that although the total drug uptake varied, the ratio of covalent to free drug remained similar (Table 2). Bound HMAF comprised 67, 65, and 63% of total drug uptake in PC-3, PrEC, and NCM 460 cells, respectively, closely approximating the ~72% of total drug uptake previously found to be macromolecule-bound in CEM cells [6]. Thus, normal cells do not seem to differ from cancer cells in the formation of HMAF adducts.

Cytotoxic Versus Cytostatic Effects of HMAF in Tumor and Normal Cells

To further characterize the possible differential effects of HMAF on tumor and normal cells, we compared cell viability (scored as membrane integrity by the trypan blue exclusion assay) after drug treatment. In tumor PC-3 cells, a concentration-dependent loss of viability was observed after a 4-hr drug treatment followed by a 20-hr post-incubation period (the 4/20 hr scheme, Fig. 3). This effect progressed with continuous 24-hr treatment and was noticeable at 0.5 μM HMAF (i.e. ~2 × GI_{50}).

In contrast, normal PrEC cells showed minimal loss of viability up to at least 25 μM HMAF (i.e. ~75 × GI_{50}) even after a 24-hr continuous treatment. Other normal cell lines showed similar marginal decreases in cell viability after the 4/20 hr scheme, in contrast to profound viability

TABLE 2. Non-covalent and covalent binding of [^{14}C]HMAF in tumor and normal cells after a 4-hr treatment with 5 μM drug*

Cell line	Non-covalent (PCA-soluble)		Covalent (PCA-precipitable)	
	[^{14}C]HMAF (pmol/10^6 cells)	% of Total uptake	[^{14}C]HMAF (pmol/10^6 cells)	% of Total uptake
CEM†	21.1 ± 4.7	24.6 ± 2.3	60.1 ± 8.1	71.7 ± 2.5
PC-3	33 ± 3	34 ± 2	65 ± 4	67 ± 3
PrEC	70 ± 2	35 ± 8	137 ± 28	65 ± 9
NCM 460	67 ± 5	37 ± 8	127 ± 34	63 ± 8

*Cells were incubated with [^{14}C]HMAF and then processed for total, covalent, and non-covalently associated radioactivity as described in Materials and Methods. Mean values from two independent samples (±range) are shown.

†Determined previously [6].

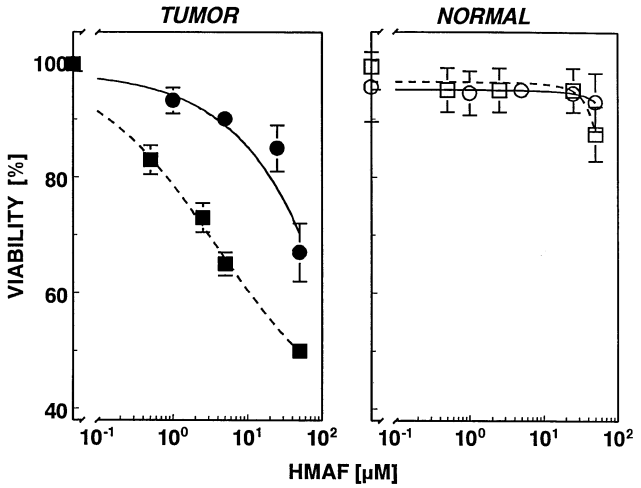

FIG. 3. Effect of HMAF on the viability of tumor (PC-3) and normal (PrEC) cells. The cell viability was assessed by trypan blue exclusion after 4 hr of HMAF treatment and a 20 hr post-incubation in drug-free medium (●; ○) or 24 hr of HMAF treatment followed by a 24-hr post-incubation (■, □). Closed symbols: tumor PC-3 cells; open symbols: normal PrEC cells. The points represent the mean values (± SEM) from 2–3 experiments carried out in duplicate.

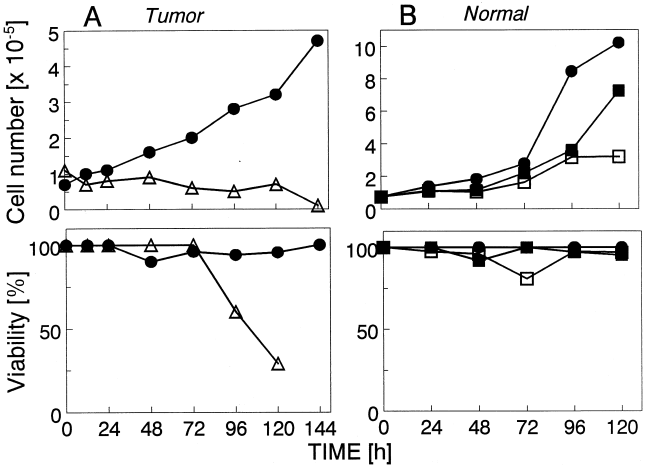

FIG. 4. Long-term effects of HMAF (continuous incubation) on cell number and viability. Panel A: Tumor LNCaP-Pro5 cells were incubated with HMAF at 0 μM (●) and 0.1 μM (Δ). Panel B: Normal NCM 460 cells were incubated with HMAF at 0 μM (●), 5 μM (■), and 10 μM (□). Representative data from two independent experiments are shown.

changes in other tumor cell lines, except for HT-29 (Table 1). However, consistent with the results for other tumor cell lines, prolonged incubation of HT-29 cells with drug (24 hr, 25 μM HMAF) resulted in an ~30% decrease in cell viability (data not shown). These results suggest that HMAF can be irreversibly cytotoxic to tumor cells but cytostatic to normal cells.

To assess whether HMAF effects would progress (or reverse) during prolonged incubation, we monitored cell number and viability for up to 5–6 days. Normal NCM 460 cells remained fully viable and eventually resumed cell growth during incubation with 5–10 μM HMAF. In contrast, tumor LNCaP-Pro5 cells showed a dramatic loss of viability paralleled by net cell loss at drug levels as low as 0.1 μM (Fig. 4). It should be stressed that consistent with the MTT data in Table 1, normal cells remained growth-inhibited for the time frame of the MTT assay (usually 3 days). However, growth resumption and continuously maintained membrane integrity indicated the qualitatively different response to HMAF of normal cells versus tumor cells, corroborating the conclusion that, within the panel of cell lines studied, HMAF was cytostatic, not cytotoxic, to normal cells.

Induction of Apoptosis by HMAF in Tumor and Normal Cells

HMAF is a potent inducer of apoptosis in CEM cells [5]. The ability of HMAF to induce apoptosis in the cell lines selected for this study was verified based on the morphological changes after staining of cellular DNA with Hoechst 33258. Characteristic apoptotic changes including nuclear protuberances, DNA condensation, and apoptotic bodies were seen in all HMAF-treated tumor cells but not in normal cells (Fig. 5 and data not shown). Representative examples of the morphology of tumor cells (LNCaP-Pro5) and normal cells (PrEC) after 4 hr of treatment with 25 μM HMAF followed by 20-hr post-incubation in drug-free medium are given in Fig. 5.

Apoptotic effects of HMAF were characterized further and quantitated using the DNA fragmentation assay. First, the generation of fragmented DNA was monitored in cells treated with HMAF for 4 hr and post-incubated in drug-free medium for an additional 20 hr. This "stringent" 4/20 hr scheme was chosen to focus on direct drug effects, while minimizing secondary, late effects such as secondary necrosis and/or apoptosis resulting from lethal mitosis.

All the tumor cell lines studied exhibited a concentration-dependent generation of apoptotic DNA fragments (Fig. 6A). The magnitude of this effect depended on the cell line. At 10 μM HMAF, the fragmented DNA ranged from 3 to 28.9% of total DNA. Colo 320DM cells were the most susceptible to HMAF-induced DNA fragmentation, while PC-3 cells were the least affected. Analysis by field inversion gel electrophoresis demonstrated that the majority of apoptotic double-stranded fragments generated in HMAF-treated tumor cells were between 20 and 50 kbp (in CEM and LNCaP-C4–2 cells, data not shown).

In contrast to tumor cells, normal cells generated significantly less fragmented DNA (Fig. 6B). Under the 4/20 hr scheme, DNA fragmentation did not exceed background fluctuations for PrEC, NCM 460, and WI 38 cells, and was elevated only marginally in RPTEC and HUVEC cells (Fig. 6B). In all the normal cell lines tested, DNA fragmentation did not exceed 10% of total cellular DNA at the highest drug concentrations tested (10–50 μM).

Apoptotic fragmentation in tumor cells showed a strong temporal progression as illustrated by the results after

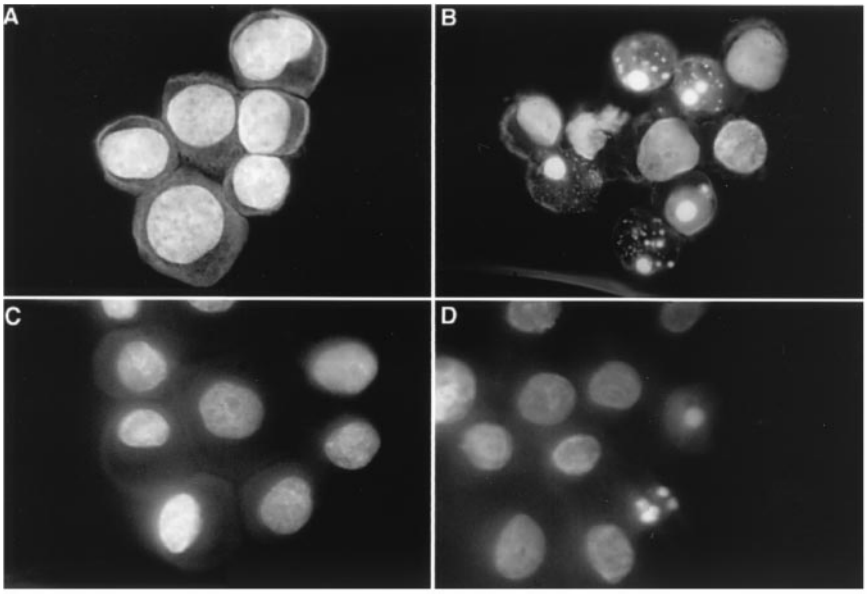

FIG. 5. Apoptotic cell morphology induced by HMAF in tumor but not in normal cell lines. Tumor cells, LNCaP-Pro5 (A and B), and normal prostate epithelial cells, PrEC (C and D), were treated with 25 μM HMAF for 4 hr followed by a 20-hr post-incubation in drug-free medium. Apoptotic nuclei were detected after staining with DNA-specific Hoechst 33258 as described in Materials and Methods. Control cells: A and C; HMAF-treated: B and D.

treatment with 5 μM HMAF for 24 hr without (24/0 hr) or with an additional 24-hr post-incubation (24/24 hr) (Fig. 7). Even with PC-3 cells, DNA fragmentation was detectable at HMAF concentrations as low as 0.5 μM (~2 × GI_{50}) in the 24/0 scheme (data not shown) and amounted to up to 25% of total DNA at 5 μM HMAF in the 24/24 hr scheme (Fig. 7). Profound progression of apoptosis was also observed for other tumor lines. In contrast, no or only marginal DNA fragmentation was detected in the normal cell lines, PrEC, NCM 460, and HUVEC, up to at least 5 μM HMAF (i.e. at least one order of magnitude greater than the respective GI_{50} values, cf. Table 1) under both 24/0 and 24/24 hr treatment schemes (Fig. 7 and data not shown). Only a prolonged continuous treatment (24 hr and longer) with HMAF at concentrations above 25 μM led to induction of apoptosis in normal prostate and renal cells (data not shown). Still, NCM 460 cells remained relatively resistant to HMAF-induced apoptosis even 48 hr post-exposure (10.9% DNA fragmentation at 50 μM HMAF).

For selected tumor and normal cell lines, we utilized a flow cytometry assay that determines the percentage of apoptotic cells based on TdT-mediated tagging of the

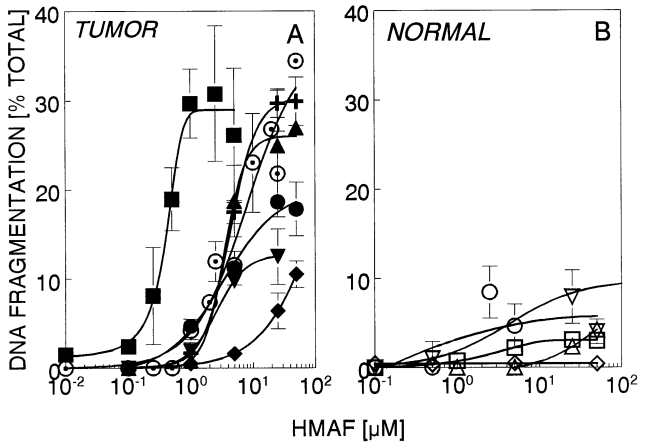

FIG. 6. HMAF-induced apoptotic DNA fragmentation in tumor and normal cells under "stringent" conditions. Cells were treated with various concentrations of HMAF for 4 hr and post-incubated in HMAF-free medium for an additional 20 hr. The cells were permeabilized, and fragmented DNA was extracted as described in Materials and Methods. The points represent the mean values (± SEM) from 1–4 separate experiments carried out in duplicate. (Where N = 2, values are averages ± one-half of the range.) Panel A: Human tumor cell lines: LNCaP-Pro5 (+); LNCaP (▲); LNCaP-LN3 (●); LNCaP-C4–2 (▼); PC-3 (♦; Colo 320DM (■); and CEM (⊙). Panel B: Human normal cell lines: PrEC (□); NCM 460 (Δ); WI 38 (◇); RPTEC (▽); and HUVEC (○).

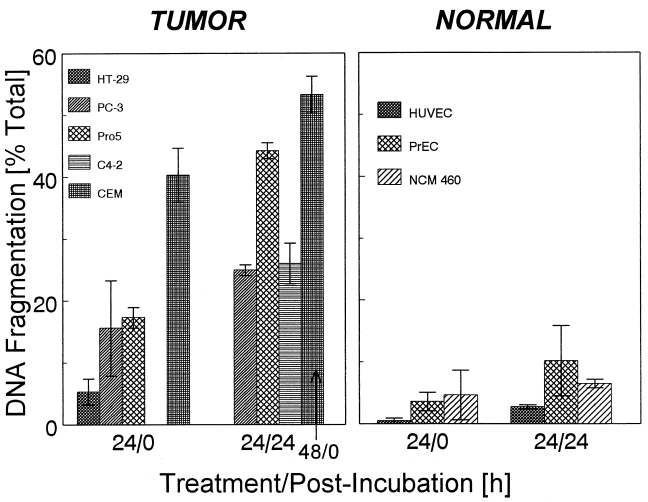

FIG. 7. Apoptotic DNA fragmentation after a prolonged incubation of cells with 5 μM HMAF. Cells were incubated with HMAF for 24 hr (24/0) or for 24 hr followed by an additional 24 hr post-incubation (24/24), or continuously for 48 hr (CEM, 48/0). No data are available for LNCaP-C4–2 cells in the 24/0 or HT-29 in the 24/24 hr scheme. The points represent the mean values (± SEM) from 2–4 separate experiments carried out in duplicate.

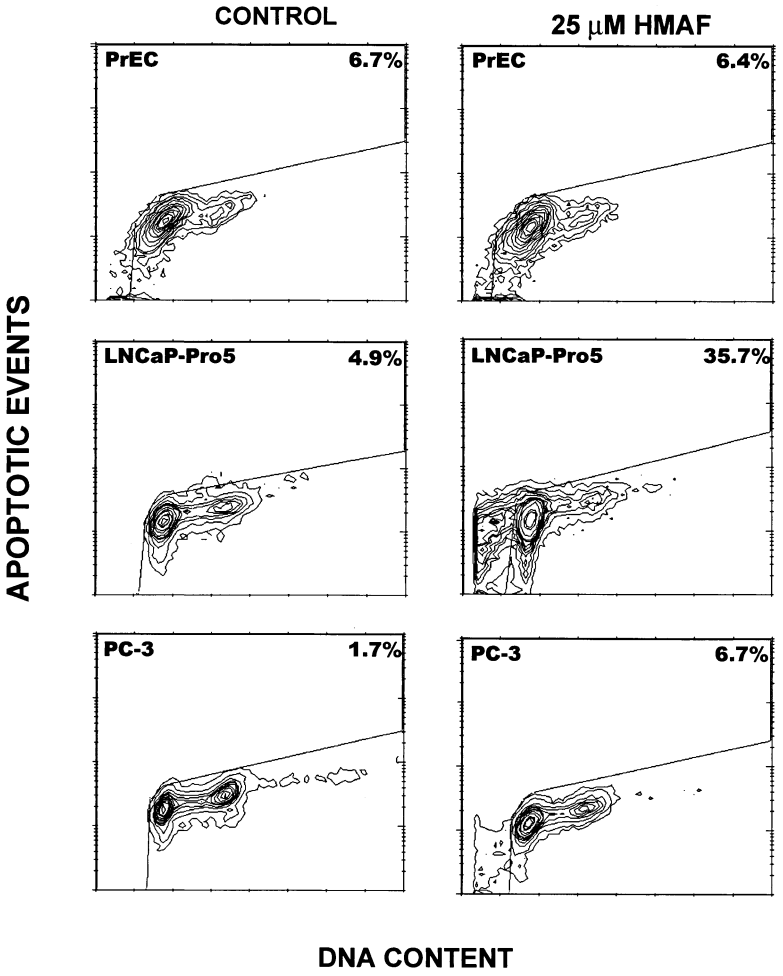

FIG. 8. Apoptosis in tumor LNCaP-Pro5 and PC-3 and normal PrEC cells measured by multiparametric flow cytometry. Bivariate histograms for control cells and cells treated with 25 μM HMAF for 4 hr followed by a 20-hr post-incubation in drug-free medium show apoptotic events (cells with strand breaks detected by TdT) plotted against DNA content (by PI). The percentage of apoptotic cells is indicated in each panel.

3′-OH termini at DNA cleavage [5, 15, 16]. Incubation of tumor cells with HMAF at 25 μM for 4 hr followed by a 20-hr post-incubation resulted in the appearance of a significant (35%) fraction of apoptotic LNCaP-Pro5 cells and a smaller but measurable (5%) increase over the control value in PC-3 cells. In contrast, no measurable level of apoptotic cells was detected in HMAF-treated normal PrEC cells (Fig. 8). The side and forward light scattering data collected in the same experiments demonstrated a similar differential pattern (data not shown).

The quantification of the TdT assay showed that apoptotic DNA fragmentation in tumor cells appeared to be irreversible and progressive, with the kinetics depending on the cell line. In LNCaP-Pro5 cells treated with 25 μM HMAF for 4 hr, the fraction of apoptotic cells increased from the control value of 4.9% to 8.2 and 61.4% at 4 and 48 hr after drug removal, respectively. In PC-3 cells exposed to 25 μM HMAF for 24 hr, the percentage of apoptotic cells increased from 1.3% to 15.2 and 18.7% at 24 and 48 hr after drug removal. Thus, the flow cytometry assay closely paralleled the results of the DNA fragmentation assay, corroborating the differential induction of apoptosis by HMAF in tumor versus normal cells.

Cell cycle data collected in the same experiments (Fig. 8 and data not shown) indicated that HMAF tends to cause a modest accumulation of G_1 phase cells (approximately 10% increase over control in LNCaP-Pro5 and PC-3 cells). These data also confirmed that the normal cell lines represented proliferating populations of cells. The apoptosis-resistant PrEC cell line featured on average 24% of cells in S-phase, similar to the initial culture of tumor cell line LNCaP-Pro5 (19% of cells in S-phase).

DISCUSSION

HMAF is an atypical antitumor DNA-reactive agent in several aspects of its mechanism of action. It forms only monoadducts with DNA [5, 6]; thus, targeting of macromolecules other than DNA (mainly cellular proteins) may be more important for HMAF than for other DNA-reactive drugs. Moreover, HMAF causes tumor shrinkage, up to the stage of complete regression, with modest toxicity in several *in vivo* xenograft models [2–4]. As shown in the current study, the potential of HMAF to differentiate between tumor and normal cells in drug-induced apoptosis is another unique feature of this drug, which may explain its biological activity.

HMAF growth-inhibitory activity correlated strongly with drug uptake. This correlation, however, followed distinctly different trends for tumor and normal cell lines.

Normal cells tended to be less growth-inhibited by comparable levels of cell-associated HMAF than tumor cells (Fig. 2). On average, a 3- to 4-fold greater drug uptake was required in normal cells to produce the same growth inhibitory effect as in tumor cells. This differential response did not result from an impeded formation of drug adducts to cellular macromolecules, as a similar proportion of covalent adducts to total uptake was found in two tumor and two normal cell lines. Thus, tumor and normal cells examined must differ in their tolerance for HMAF-induced adducts to cellular macromolecules.

Drug effects on cell membrane integrity (cell viability) provided another manifestation of the differential responses. In tumor lines, loss of viability was detectable at pharmacologically relevant drug concentrations and progressed with prolonged incubation (Table 1, Figs. 3 and 4). HMAF appeared to be cytotoxic also to HT-29 cells, although the response was weaker and/or delayed compared with other tumor cells examined. In contrast, normal cells, although growth-inhibited, maintained their viability, and could resume growth after several days of incubation in the presence of HMAF concentrations several-fold greater than drug levels that decreased viability, growth, and survival of tumor cells. The results from the eight tumor and five normal cell lines strongly suggest that whereas HMAF is cytotoxic to tumor cells, the drug seems to be cytostatic to normal cells, at least in the case of the cell lines studied.

Consistent with cytotoxic/cytostatic responses, HMAF was a potent inducer of apoptosis in tumor cells (Figs. 5–8). For all the tumor cell lines examined, apoptosis was detectable under stringent conditions (4 hr drug treatment/20 hr post-incubation). Even PC-3 cells, which showed low to modest apoptosis under the stringent 4/20 hr scheme, were profoundly apoptotic after a prolonged treatment with HMAF (24 hr, Fig. 7). Apoptosis in PC-3 cells is consistent with significant tumor shrinkage in PC-3 prostate cancer xenografts *in vivo* [4].

In striking contrast to its effect on tumor cells, HMAF was a poor inducer of apoptosis in normal cells (Figs. 5–8). HMAF induced marginal levels of apoptosis or no apoptosis in normal human cells, such as prostate epithelial cells, colon mucosa, normal fibroblasts, renal proximal tubule epithelial cells, and endothelial cells under the 4/20 hr protocol and up to 5 μM drug under the 24/0 or 24/24 hr schemes. A prolonged continuous treatment with HMAF at concentrations that exceeded 15- to 800-fold the GI_{50} values in tumor cells resulted in apoptosis of normal prostate and renal cells. However, even such a drastic treatment was insufficient for profound apoptosis in normal colon mucosa cells. All normal cell lines studied represented proliferating populations of cells; thus, the lack of apoptosis could not be ascribed to non-proliferation or senescence.

Collectively, HMAF produced significant apoptosis under stringent conditions in 7/8 tumor cell lines and had marginal or no effect in 5/5 normal cell lines. Clearly, the differences among cell lines in apoptosis induction were not solely due to different levels of drug uptake. The most sensitive of the normal cell lines, RPTEC, was marginally responsive, compared with, for example, LNCaP sublines, given the 4- to 10-fold greater accumulation of HMAF in RPTEC cells. Overall, normal cells seemed to be even more resistant to apoptosis than to growth inhibition by HMAF. Together with the cytostatic nature of cell growth inhibition in normal but not tumor cells, these results suggested the existence of mechanisms that protect normal cells against HMAF action and/or mechanisms that enhance HMAF toxicity in tumor cells.

Despite the profound differences in HMAF-induced apoptosis, survival of tumor cells tended to be only slightly more inhibited than survival of normal cells (Table 1). However, standard growth and survival assays may mask potentially important differences in the responses of tumor and normal cells to HMAF. Other studies showed that enhanced apoptosis may not necessarily lead to greater inhibition of clonogenic survival [23, 24]. Survival assay alone would not score viable but slowly growing cells. Thus, slowly recovering normal cells may be scored as "non-clonogenic," although they are certainly less affected than apoptotically disrupted tumor cells. On the other hand, slowly growing (not scored by survival assay) tumor cells have been proposed to be an overlooked factor in therapeutic failure of various drug treatments [25–27]. Apoptotic elimination of these "time bomb" cells irrevocably prevents their evolving into more aggressive and/or drug-resistant phenotypes. It needs to be emphasized that inhibition of survival by physical elimination of tumor cells (enhanced apoptosis) is pharmacologically more desirable than survival inhibition resulting only from the inability of tumor cells to divide fast enough to form scorable colonies.

Apoptosis can be a major factor responsible for the loss of clonogenicity of tumor cells, but not normal cells, in response to HMAF. HMAF-treated tumor cell cultures show clear indications of cell disintegration (sub-G_1 debris in flow cytometry histograms, Fig. 8) and net cell loss (compared to the initial pretreatment numbers), which follow the decrease in "viability" (Fig. 3 and data not shown). A fraction of tumor cells less severely affected by the drug can be growth-inhibited in a non-apoptotic stage, which would also contribute to the observed loss of clonogenicity, in addition to physical cell elimination. On the other hand, growth inhibition (possibly reversible, cf. Fig. 4) appeared to be the main cause of clonogenic inhibition of normal cells, which, in contrast to tumor cells, showed little apoptosis, unaltered membrane integrity, and no decrease in cell number.

Although the apoptotic and necrotic modes of cell death may overlap and the distinction between them is somewhat ambiguous [28–30], the deterioration of HMAF-treated tumor cultures is consistent with apoptosis as the primary event. In the course of apoptosis progression, cell membrane integrity becomes compromised, giving rise to "secondary necrosis." As opposed to primary necrosis, the temporal increase in the percentage of cells with compro-

mised membrane integrity should follow, rather than precede, measures of earlier apoptotic stages such as apoptotic DNA fragmentation. This is the case with HMAF-treated tumor cells (cf. Fig. 3 vs Figs. 6 and 7). Thus, primary apoptosis rather than primary necrosis is likely to be a significant factor in the effects of HMAF.

The results of this study and our other investigations suggest that drug binding to macromolecules results in growth inhibition, which is irreversible for tumor cells and leads to their gradual elimination by apoptosis [5, 6, 14]. The tolerance of normal cells to HMAF in terms of growth inhibition and resistance to apoptosis may actually have the same underlying nature as the sensitivity of tumor cells. One possibility could be a more efficient repair of HMAF–DNA adducts in normal cells. Another possibility is related to the targeting of both DNA and proteins by HMAF. Whereas a role for DNA damage in apoptosis is well established, pro-oxidative protein damage by sulfhydryl-reactive agents, such as diamide, is also known to lead to apoptosis [31–36]. Protein damage by HMAF [6] is likely to have similar consequences [37]. Consistent with a role for pro-oxidative protein damage, HMAF reacts with redox-controlling proteins such as thioredoxin and thioredoxin reductase [6]. In addition, HMAF promotes the release of cytochrome *c* from mitochondria, generates reactive oxygen species, and impedes thioredoxin-dependent activation of NF-κB in LNCaP-Pro5 cells [14]. Thus, it is tempting to hypothesize that normal cells may be protected from HMAF-induced pro-oxidative apoptotic insults by a greater capacity of redox-controlling systems [38–40] to buffer the pro-oxidative changes in protein sulfhydryl balance. In contrast, the tendency among tumor cells to have their redox balance shifted towards a pro-oxidative state [41] may be related to their sensitivity to HMAF. On the other hand, apoptosis by HMAF is not critically dependent on the presence of wild-type p53 or the down-regulation of Bcl-2 in the studied cell lines. Moreover, HMAF is equally cytotoxic in the presence and absence of forced overexpression of bcl-2 (Woynarowski *et al.*, unpublished data) in BH-2 cells, a transfected subline of HeLa cells [23, 24]. Likewise, cytotoxic effects of HMAF have been found to be independent of tumor suppressor gene *p53* and *p21* expression [42].

To our knowledge, the reported study provides the first comprehensive characterization of differential apoptosis for any antitumor drug and a proof of principle that targeting apoptosis may selectively affect tumor cells. This unprecedented differential apoptosis corresponds well to the profound tumor regression effects of HMAF *in vivo* [1, 3, 4] and can be of significance for the clinical use of this promising new agent. Further studies are underway to elucidate the mechanistic nature of the differential apoptosis induced by HMAF.

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