Biochemical Pharmacology, Vol. 58, pp. 217–225, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00085-4

Drug Uptake and Cellular Targets of Hydroxymethylacylfulvene (HMAF)

Maryanne C. S. Herzig, Brenda Arnett,* John R. MacDonald† and Jan M. Woynarowski*‡*

*Cancer Therapy and Research Center, Institute for Drug Development, San Antonio, TX 78245; and †MGI Pharma, Minnetonka, MN 55343, U.S.A.

ABSTRACT. Hydroxymethylacylfulvene (HMAF, MGI 114) is a novel antitumor drug and a potent pro-apoptotic agent that has the potential to alkylate cellular nucleophiles. The objective of these studies was to characterize drug uptake and cellular targets for drug binding in human leukemia CEM cells. The uptake of [^{14}C]HMAF had two components: a rapid phase (0–10 min) and a slow phase. At 10 μM drug (37°), the rapid and slower phase amounted to 0.86 and 0.13 pmol/min/10^6cells, respectively. HMAF uptake was inhibited 82% by low temperature (4°) at 4 hr. Cell-associated HMAF localized to nuclear (50%), cytoplasmic (37%), and membrane fractions (10%). Continued drug uptake appeared to be driven by covalent binding to cellular macromolecules. Approximately 1/4 and 2/3 of cell-associated HMAF formed covalent adducts after 10 min and 4 hr, respectively, as found by perchloric acid precipitation. Drug adducts were not readily reversible; 77% of the covalently bound radiolabel was retained by the cells 20 hr after drug treatment. Combinations of DNase, RNase, and proteinase K with perchloric acid precipitation showed that approximately 60, 30, and 10% of the covalently bound drug was associated with the protein, DNA, and RNA fractions, respectively. Incubation of 100 μM [^{14}C]HMAF (24 hr) with purified DNA, serum albumin, thioredoxin, and thioredoxin reductase resulted in 6, 22, 14, and 11 pmol [^{14}C]HMAF/μg DNA or protein, respectively. Results indicate that multiple targets for HMAF binding may contribute to the pro-apoptotic and antiproliferative action of the drug. BIOCHEM PHARMACOL **58**;2:217–225, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. MGI 114; HMAF; DNA adducts; illudins; drug uptake; macromolecule binding

HMAF§ (MGI 114, Fig. 1) is a novel antitumor agent derived from the natural sesquiterpene illudin S [1–3]. HMAF is markedly less cytotoxic than illudin S, while featuring a better therapeutic index. Promising antitumor properties of HMAF in several *in vivo* systems manifest as profound tumor shrinkage and complete cures [3]. Because of these auspicious properties, HMAF has entered clinical trials. The mechanism of action of HMAF is known to a limited extent, largely based on analogies with the prototypic illudins.

Illudins, in general, exhibit the potential for nucleophilic substitution that may lead to alkylation of biological nucleophiles [4]. Covalent binding to DNA in intact cells has been observed for illudin S and its analog, acylfulvene, and has been implicated in cytotoxic effects [5, 6], although illudins do not react with oxygen or nitrogen, i.e. nucleophiles present in DNA at physiological pH in cell-free systems [7]. On the other hand, illudin S and acylfulvene readily react with small model thiols at or near physiological pH [7–9].

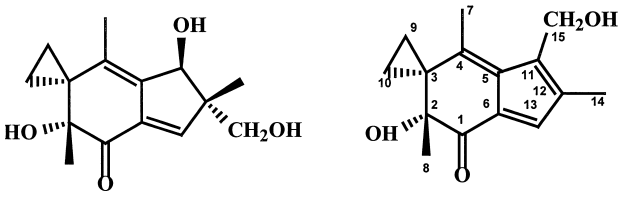
Likewise, HMAF is able to react with model thiol nucleophiles via its primary allylic hydroxyl. Additionally, the α–β unsaturated ketone can undergo a Michael-type addition followed by opening of the cyclopropane ring [10, 11]. Compared with illudins, thiol reactivity of HMAF is reduced, although still substantial [7]. The potential of HMAF to react with compounds other than small model molecules remains essentially uncharacterized.

Our previous mechanistic studies pointed to similarities between cellular effects of HMAF [12] and illudin S investigated by Kelner and co-workers [1]. Both drugs are preferential inhibitors of DNA synthesis at pharmacologically relevant concentrations and arrest cells in the S phase. However, despite its suggested potential for bifunctional alkylations, HMAF does not form obvious bifunctional lesions in DNA such as DNA interstrand cross-links or DNA–protein cross-links [12]. Moreover, DNA isolated from HMAF-treated cells still provides an efficient template for primer extension [12]. Thus, while the formation of HMAF–DNA adducts has been suggested [13], such adducts *per se* appear insufficient for the observed inhibition of cellular DNA synthesis. Although the evidence for

‡ Corresponding author: Jan M. Woynarowski, Ph.D., Head, Molecular Pharmacology, Cancer Therapy and Research Center, Institute for Drug Development, 14960 Omicron Dr., San Antonio, TX 78245-3217. Tel. (210) 677-3832; FAX (210) 677-0058; E-mail: jmw1@saci.org

§ *Abbreviations*: HMAF (MGI 114), hydroxymethylacylfulvene; IC_{50}, drug concentration producing 50% of cell growth inhibition; and PCA, perchloric acid.

Received 10 August 1998; accepted 10 November 1998.



ILLUDIN S HYDROXYMETHYLACYLFULVENE MGI 114

FIG. 1. Structures of illudin S and HMAF.

direct DNA damage by HMAF is equivocal, DNA from HMAF-treated cells does exhibit strand breaks that reflect secondary lesions, as the drug does not form breaks in naked DNA [12]. HMAF-dependent breaks in cellular DNA probably reflect apoptotic DNA fragmentation. A potent induction of apoptosis is a hallmark of HMAF action in several cancer cell lines [12, 14].

For DNA-reactive drugs in general, DNA adducts are regarded as a leading cause of apoptosis [15, 16], although non-DNA factors such as modifications of cellular proteins and/or their expression also can result in apoptosis [17, 18]. Therefore, in addition to putative HMAF–DNA monoadducts, HMAF effects on targets other than DNA might contribute to the pro-apoptotic and antiproliferative properties of the drug. Clearly, our understanding of the mechanism of action of HMAF is hampered by inadequate knowledge of its cellular targets.

Another potentially important, yet uncharacterized property of HMAF pertains to drug uptake. Cell lines sensitive to illudin S featured increased rate and extent of drug uptake compared with non-sensitive cell lines [6, 19]. It has also been suggested that a saturable, energy-dependent transport mechanism for illudin explains, in part, the rapid and preferential sensitivity of certain cell lines to illudin S [2, 19].

In this study, we characterized drug uptake and cellular targets for drug binding using [^{14}C]HMAF. In addition to a rapid phase, a substantial slow but sustained phase of drug uptake was observed. Most intracellular HMAF formed covalent adducts to macromolecules. Covalent adduct formation appeared to drive drug uptake. Whereas HMAF bound to cellular DNA, even more drug was adducted to cellular proteins.

MATERIALS AND METHODS

Drug

A stock solution of non-radiolabeled HMAF (from MGI Pharma) was prepared in DMSO, stored at −20°, and protected from light. [^{14}C]HMAF labeled at the carbinol group (position C15, cf. Fig. 1) was prepared by Amersham Inc. and was provided by MGI Pharma at both 15 and 56 mCi/mmol specific activity in ethanol. The [^{14}C] label in the carbinol position is stable and is not affected by drug metabolism (Musick TJ and MacDonald JR, unpublished data).

Cell Culture

Human CEM leukemia cells were provided by Dr. William T. Beck. The cells were cultured in suspension using Joklik's Minimal Essential Medium containing 10% fetal bovine serum as described [12] and were tested regularly for mycoplasma contamination.

Cellular Uptake of [^{14}C]HMAF

CEM cells were collected, centrifuged at 150 *g* for 3 min at 4°, resuspended in fresh medium, and incubated for an additional hour at 37°. At time zero, cultures were supplemented with [^{14}C]HMAF, and the cells were incubated at 37° or 4° as indicated. A final cell concentration of 2×10^6 cells/mL was chosen to maximize the signal. For short time points (0–15 min), incubations were carried out in a water bath using medium supplemented with 10 mM HEPES for additional buffering capacity; otherwise incubations with drug were carried out in regular medium and in a 5% CO_2 incubator. At the selected time points, aliquots of 1 mL (containing 2×10^6 cells/mL) were overlaid onto 0.25 mL of 1-bromododecane and centrifuged immediately at 14,000 *g* for 2 min. The aqueous phase was aspirated, and the tube was washed with 1 mL of water. Both aqueous and organic phases then were aspirated. The cell pellet was solubilized in 2% $NaHCO_3$ with 1% Triton X-100 and 1% trypsin for 1 hr at 37°. Radioactivity in the solubilized pellet was determined in a liquid scintillation counter and was used as a measure of drug uptake.

A typical signal per sample, e.g. following 1 hr of incubation with 10 μM HMAF, ranged from 4000 cpm (at [^{14}C]HMAF specific activity of 15 mCi/mmol) to 6800 cpm (specific activity of 56 mCi/mmol). Using HMAF of higher specific activity (56 mCi/mmol), sufficiently high signal was obtained to detect uptake of drug as low as 1–2 μM (1200 cpm). The number of independent experiments performed in duplicate is indicated in each figure legend. Results are expressed as picomoles of [^{14}C]HMAF per 10^6 cells based on drug specific radioactivity and scintillation counting efficiency of 75% in a Beckman LS6800 counter (Beckman Instruments, Inc.).

Subcellular Fractionation

METHOD A. Subcellular fractionation was performed by differential centrifugation [20]. CEM cells were suspended at 0.5×10^6 cells/mL and allowed to equilibrate for 30–60 min before the addition of [^{14}C]HMAF to 5 μM for 4 hr of drug treatment at 37°. Cells were harvested by centrifugation at 150 *g* for 3 min at 4°, washed twice with ice-cold PBS by centrifugation as above, and resuspended at 4×10^6 cells/mL; then the cell number was determined. An aliquot of $\sim 8 \times 10^6$ cells was pelleted by centrifugation and resuspended in 2 mL of hypotonic lysis buffer (10 mM Tris–HCl, pH 7.4; 15 mM KCl; 5 mM $MgCl_2$). Samples were Dounce homogenized (26–32 single strokes) and

supplemented with sucrose (250 mM final concentration). Samples were taken for determination of protein and total associated radioactivity. The homogenate was centrifuged at 1000 *g* for 10 min at 4° to obtain the 1000 *g* pellet, referred to as the nuclear fraction. A second centrifugation of the 1000 *g* supernatant at 49,000 *g* for 60 min in the Sorvall RC5B centrifuge at 4° resulted in the 49,000 *g* supernatant, referred to as the cytosolic fraction, and the 49,000 *g* pellet, referred to as the membrane fraction (cf. Fig. 5A). Pellets were resuspended in 0.3 M NaOH, and samples of all fractions were taken for protein determinations. Two aliquots each (250 μL) of the nuclear, cytosolic, and membrane fractions were lysed in 250 μL of 2 M PCA, and counted in the TopCount scintillation counter using Microscint 40 scintillation fluid with a scintillation counting efficiency of 50% (Packard Instrument Co., Inc.).

METHOD B. In some experiments, an alternative method of preparation of nuclei was used. In this method, cells exposed to radioactive [^{14}C]HMAF, harvested, and washed with ice-cold PBS as in Method A then were resuspended in 20 mL of Nuclei Isolation Buffer (2 mM K_2HPO_4/KH_2PO_4, pH 6.9, 5 mM $MgCl_2$, 15 mM NaCl, 1 mM EGTA) and pelleted again. The pellet was resuspended in 2.25 mL of Nuclei Isolation Buffer with 0.35% (v/v) Triton X-100, and left on ice for 30 min. Samples were taken at this point for determination of total radioactivity. The lysate was diluted with 10 mL of Nuclei Isolation Buffer without Triton X-100, and the nuclei were pelleted at 300 *g* for 13 min. The nuclear pellet was resuspended in 1 mL of Nuclei Isolation Buffer without Triton, and samples were taken for determination of radioactivity and protein.

[^{14}C]HMAF Binding to Cellular Macromolecules

CEM cells were treated with 5 μM [^{14}C]HMAF for 4 hr and washed with PBS as described for subcellular drug distribution. Harvested cells were resuspended in a minimal volume, counted, and brought to a final cell concentration of 4×10^6 cells/mL. For determination of the following parameters: 1) [^{14}C]HMAF total uptake, 2) total covalent binding, 3) free drug, 4) DNA covalent binding, 5) RNA covalent binding, and 6) protein covalent binding (cf. Fig. 4), triplicate 1-mL aliquots were taken, and the 1-mL cell samples were pelleted by centrifugation prior to further processing. Then radioactivity was measured in a Packard TopCount scintillation counter. In some experiments, the 4-hr drug uptake was followed by washing with PBS and then a 20-hr recovery period at 37° prior to processing. For these samples, the label dilution due to any increase in cell number was corrected so that values reflect cells originally exposed to drug.

For *total drug uptake*, cell pellets were hydrolyzed in 0.25 mL of 2 M PCA, and their acid-precipitable radioactivity was measured [21]. Total covalent binding and free cell-associated drug were determined by extracting the cell pellets on ice with 3 mL of 0.5 M PCA for 30 min followed by centrifugation for 5 min at 900 *g* at 4°. The released radioactivity (*free drug*) was determined by counting an aliquot of the supernatant. The pellets were washed by centrifugation with 10 mL of 0.5 M PCA, and hydrolyzed in 0.25 mL of 2 M PCA at 65° for 40 min; then their radioactivity was determined. This cold PCA-precipitable radioactivity is defined as *total covalent binding* of HMAF to macromolecules. The recovery of the radioactivity (sum of free and covalently bound drug) approximated 100% of the total uptake.

For determination of drug covalent binding to a specific class of macromolecule, multi-enzyme digestions were performed on cells to isolate specific classes of macromolecules prior to testing for covalent association of drug by assessing the acid-precipitable radioactivity. Cells pelleted from the initial 1-mL aliquots were lysed in 0.6 mL of hypotonic lysis buffer (10 mM Tris, pH 7.5, 10 mM EDTA, 0.2% Triton X-100). For determination of *HMAF covalently bound to DNA*, RNase digestion (50 μL of 10 μg/μL of bovine pancreatic DNase-free RNase A; EC 3.1.27.5; Sigma) for 30 min at 37° was followed by proteinase K (EC 3.4.21.64; > 20 U/mg; Gibco) (50 μL of 10 μg/μL) for 60 min at 37°. For *HMAF bound covalently to RNA*, cell lysates were supplemented with 10 mM $MgCl_2$ and 10 mM $CaCl_2$ (final concentrations) and incubated with 2 μL DNase I (EC 3.1.21.1; 2 U/μL; Ambion) for 30 min at 37°, and followed by proteinase K, as above. To determine *HMAF bound covalently to protein*, samples were digested with both DNase and RNase as above. To assess the completeness of digestion, an additional set of samples was triple-digested with DNase and RNase, followed by proteinase K.

Following the multi-enzyme incubations, the PCA-precipitable radioactivity was determined as described for total covalent binding. Digests were chilled on ice with 3 mL of 0.5 M PCA and centrifuged; the pellets were washed and then hydrolyzed prior to determination of radioactivity. Calculations of [^{14}C]HMAF bound to macromolecules assumed ~8 μg DNA, 15 μg RNA, and 0.19 mg protein per 10^6 human cells.

[^{14}C]HMAF Interaction with Calf Thymus DNA and Selected Target Proteins: BSA, Thioredoxin, and Thioredoxin Reductase

Drug binding to naked DNA was analyzed at a drug/bp ratio of r = 0.64, a ratio equal to the input drug/bp in the treatment of intact cells with HMAF (using 0.5×10^6 cells/mL and 5 μM HMAF). Binding to model target proteins was measured using the same HMAF/macromolecule mass ratio. Samples, 10 μg in 100 μL TE (10 mM Tris–HCl, pH 7.5, 1 mM EDTA), of calf thymus DNA (Sigma), BSA (Sigma), *E. coli* thioredoxin (Promega), or *E. coli* thioredoxin reductase (EC 1.6.4.5, Sigma) were allowed to react with 100 μM [^{14}C]HMAF (56 mCi/mmol) for 24 hr at 37°. The bulk of unbound drug was separated from bound by PCA precipitation. Thus, samples were injected into 12 mL of ice-cold 0.4 M PCA, incubated for

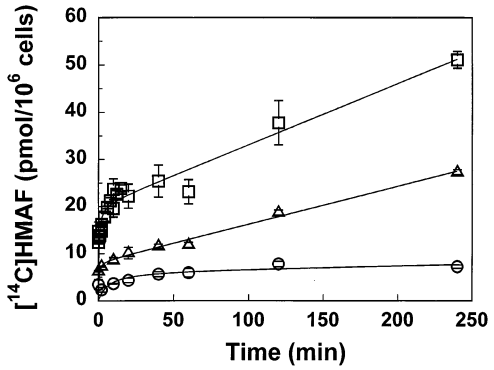


FIG. 2. Uptake of [^{14}C]HMAF into CEM cells. CEM cells (2 × 10^6 cells/mL) were incubated with 2 ○, 5 (△), and 10 (□) μM [^{14}C]HMAF for the indicated times at 37°. The average values from two experiments done in duplicate are shown ± 1/2 range. Background values at time zero have not been subtracted.

2 hr on ice, and then centrifuged at 900 *g* for 15 min in the Beckman GS 6R centrifuge at 4°. The supernatant was decanted, and the pellets were washed five times by centrifugation as above with 10 mL of cold 0.4 M PCA. After the final wash, 0.3 mL of 0.5 M PCA was added, and the samples were hydrolyzed at 65° for 40 min. Samples were transferred to scintillation vials, and their radioactivity was determined in a Beckman LS 9800 scintillation counter. These data were corrected for time zero background values assessed by the addition of [^{14}C]HMAF to DNA or protein and immediate injection into the ice-cold 0.4 M PCA and further processing as above.

Other Determinations

Protein was determined by the Bradford assay [22] in a microtiter plate using Coomassie® Protein Assay Reagent from the Pierce Chemical Co. DNA and RNA concentrations in macromolecular preparations were estimated based on absorbance at 260 nm assuming 62.5 and 40 μg/mL, respectively, per one A_{260} unit.

RESULTS

Uptake of [^{14}C]HMAF into CEM Cells

TIME-DEPENDENCE. The uptake of [^{14}C]HMAF by CEM cells showed both time and drug concentration dependence (Figs. 2 and 3). The time course data in Fig. 2, e.g. at 10 μM HMAF, reveal two distinct phases of drug uptake: an early rapid component (up to ~5–10 min), and a slower but sustained uptake that continued for several hours. The relative contribution of the rapid phase, while dominant up to 10 min, diminished with time and, at 4 hr, corresponded to only ~25% of total cell-associated drug. Measurable drug accumulation could be detected at pharmacologically relevant levels of HMAF (2 μM), comparable to drug IC_{50} (1.7 μM) [12].

No plateau of [^{14}C]HMAF accumulation was observed at prolonged times (4 hr) of incubation for HMAF concentrations above 2 μM (Fig. 2). Continuous drug uptake was still seen when monitored over 14 hr with 10 μM HMAF (144 pmol HMAF/10^6 cells accumulated). Under these conditions, HMAF-treated cells maintain cell membrane integrity, although they are growth inhibited and initiate apoptosis ([12] and data not shown). In contrast to the higher drug levels, at 2 μM [^{14}C]HMAF drug uptake appeared to reach a steady state after approximately 40 min.

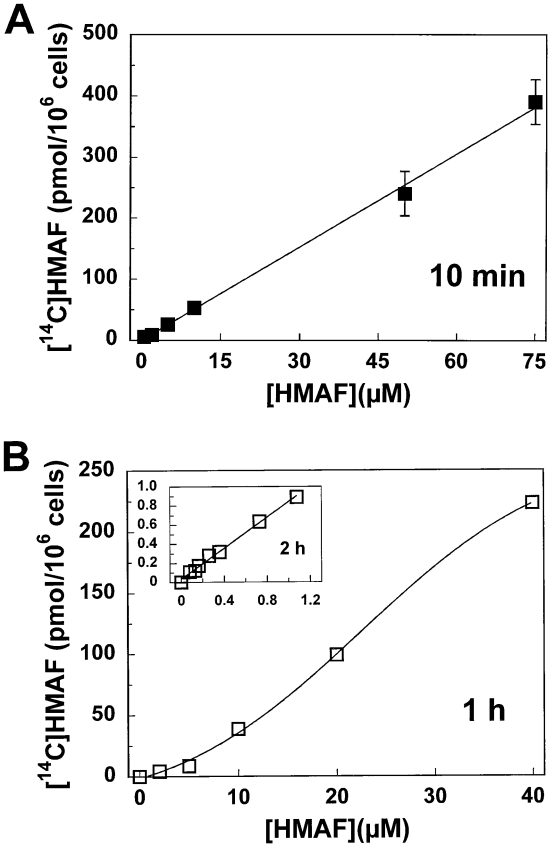


FIG. 3. Concentration-dependence of the uptake of [^{14}C]HMAF. (A) 10-min uptake. CEM cells (2 × 10^6 cells/mL) were incubated with [^{14}C]HMAF for 10 min at 37°. Each point represents the average value of total uptake for two experiments performed in duplicate (± 1/2 range). (B) 1-hr uptake. CEM cells were incubated as above for 1 hr at 37°. Each point represents the average value for 1–5 experiments performed in duplicate (± 1/2 range for the single experiment and SEM for the other experiments; bars are contained within the symbol). Inset shows 2-hr uptake at 0 to 1.2 μM [^{14}C]HMAF (average of duplicate determinations for two experiments). Uptake values at 4°, time zero have been subtracted as background in each panel.

CONCENTRATION-DEPENDENCE OF HMAF UPTAKE. By analogy to illudin S, there was a possibility that the rapid phase might reflect an active uptake mechanism. If so, drug uptake would saturate at higher drug levels. However, no saturation of drug uptake at either 10 min or 1 hr was seen with drug concentrations up to 75 μM (Fig. 3). Uptake was linear even at a 2-min observation (data not shown). Also, no indications of any transient plateau at low drug concentrations (< 2 μM) were seen after 1 hr (Fig. 3B, insert),

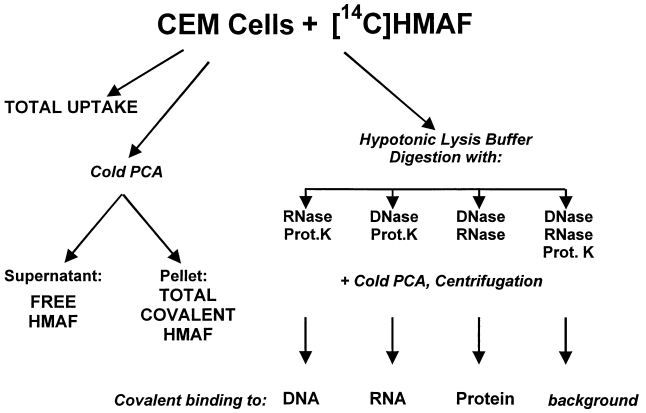


FIG. 4. Scheme of determination of [^{14}C]HMAF covalent binding to macromolecules.

which, if present, might suggest a "receptor"-mediated uptake.

Both the rapid and slow phases of uptake at 10 μM HMAF were inhibited by lowered temperature (data not shown). This decrease in uptake might reflect the loss of an energy-dependent receptor mechanism, but other explanations such as decreased rate of diffusion and/or pinocytosis are more likely in the light of other data.

Total Covalent Binding of [^{14}C]HMAF to Macromolecules

Drug uptake measured in the previous experiments was defined operationally as total drug associated with the cells. However, given the proposed ability of HMAF to react with cellular nucleophiles, cell-associated HMAF was likely to reflect both free drug (and/or its metabolites) and drug bound covalently to macromolecules.

Further experiments evaluated the extent of covalent drug binding by examining radioactivity associated with PCA-precipitated macromolecules (Fig. 4, left side). Drug binding to macromolecules was detectable even after 10 min of incubation, although it amounted to < 25% of total cell-associated HMAF for a broad range of drug levels (data not shown). However, after 4 hr, approximately 70% of HMAF was bound covalently to macromolecules with 5 μM drug (Table 1). Parallel determinations of PCA-extractable radioactivity confirmed that the remaining portion of cell-associated HMAF reflected free drug with the sum of both compartments approaching 100% as expected (Table 1).

The removal of free drug by covalent binding could drive further influx of free drug. Given a CEM cell diameter of 12.9 μm (based on electron microscopy), total cellular drug concentration at 5 μM external HMAF exceeds by 15-fold the extracellular concentration. However, "free" drug level approaches extracellular concentration. Thus, covalent binding acting as a drug sink could explain the apparent "concentration" of cell-associated drug by CEM cells.

The covalent adducts of HMAF were not readily reversible during post-incubation in drug-free medium (Table 1). Approximately 3/4 of the adducts formed during the initial 4-hr drug treatment remained macromolecule-bound in the cells after the 20-hr chase period. Although a considerable amount of "free" drug was cell-associated after 20 hr (18 vs 25% for 4 hr), these results do not mean that there is no efflux of free drug. The nature of the "free" drug after 20 hr is unknown and could reflect conversion/degradation products. The efflux of free drug *per se* has not been studied.

Subcellular Distribution of Cell-Associated HMAF

Total drug uptake reflects both internalized drug and drug associated with the outer cell surface. Thus, to address the question of drug localization, a simple subcellular fractionation was performed to obtain crude nuclear, cytosolic, and membrane fractions (Fig. 5A). The determinations of [^{14}C]HMAF in these fractions revealed that approximately

TABLE 1. Free and covalently bound [^{14}C]HMAF in CEM cells: low reversibility of covalently bound HMAF

Treatment time	[^{14}C]HMAF (pmol/10^6 cells) 4 hr†	Total uptake* (%)	
		4 hr†	4 hr + 20 hr‡
Total uptake	85.3 ± 13.9	100	
Free drug (PCA soluble)	21.1 ± 4.7	24.6 ± 2.3	18.0 ± 8.0
Covalently bound drug (PCA precipitable)	60.1 ± 8.1	71.7 ± 2.5	79.9 ± 2.2
Recovery (sum of free + covalent)		96.3 ± 2.1	97.9 ± 10.2
Covalent binding (relative to 4 hr covalent binding)		100	77 ± 4§

CEM cells were incubated with [^{14}C]HMAF (5 μM) for 4 hr and processed immediately or were washed free of drug and replaced at 37° for 20 hr before processing for total, covalent, and free radioactivity as indicated in the scheme in Fig. 4, left side (see also Materials and Methods for details).

*Percentages were calculated for each individual experiment and then were averaged.

†Mean values (± SEM) from five experiments carried out in triplicate.

‡Mean values (± ½ range) from two experiments done in triplicate.

§Mean values (± ½ range) corrected for label dilution due to increases in cell number.

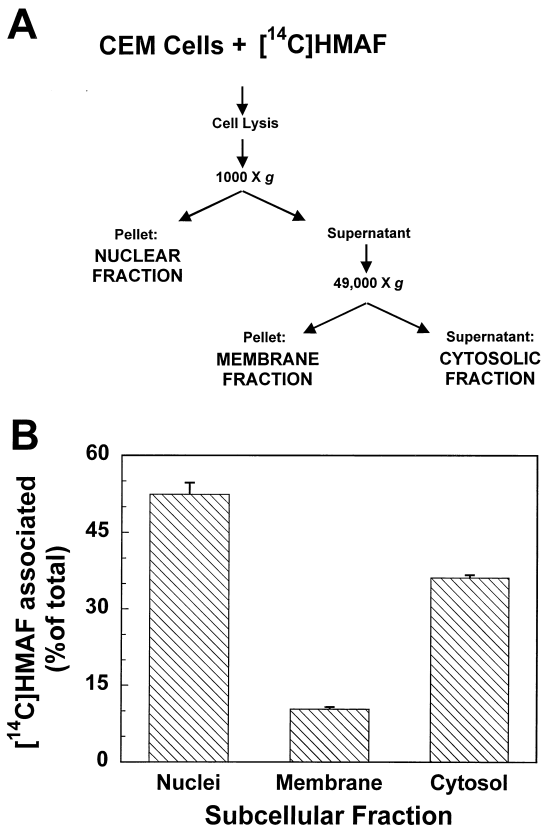


FIG. 5. Subcellular distribution of [^{14}C]HMAF. (A) Scheme of subcellular fractionation. (B) [^{14}C]HMAF in nuclear, cytosolic, and membrane fractions. Following drug uptake, CEM cells were fractionated as outlined in panel A. The averages from duplicate determinations in a representative experiment (± 1/2 range) are shown on the graph. In CEM cells, the nucleus comprises ~41% of cell volume.

50% of the drug partitioned with the nuclear fraction and 37% with the cytosolic fraction. Only 10% was associated with the membrane fraction, which comprises both outer cell membrane and intracellular membranes (Fig. 5B). Similar results were obtained by an alternative method for isolation of nuclei (Method B), which uses non-ionic detergent lysis. For Method B, the nuclear fraction again accounted for ~50% of the total accumulated drug (data not shown). The results from these fractionation experiments demonstrated clearly that the majority of cell-associated HMAF was internalized. Since the nucleus comprises ~41% of the total volume of CEM cells (based on electron microscopy), HMAF distribution seems to be approximately proportional to compartment volume, with little if any predisposition for nuclear localization.

Covalent Binding of [^{14}C]HMAF to Specific Macromolecules

To characterize HMAF targets, drug binding to cellular DNA, RNA, and proteins was examined. In these studies, covalent binding of [^{14}C]HMAF to specific macromolecules was analyzed using the combination of enzymatic digestions and PCA precipitation outlined in the right side of the scheme shown in Fig. 4.

The amounts of HMAF covalently associated with specific macromolecules after a 4-hr incubation of CEM cells are presented in Table 2. Following background correction, out of a total covalent binding of ~56.5 pmol/10^6 cells, 31.1 pmol/10^6 cells was bound to the protein fraction. DNA-associated drug also comprised a significant portion of label (12.8 pmol/10^6 cells). Thus, the majority of covalently bound HMAF was found as protein adducts (~60%) and DNA adducts (~30%). Binding to RNA, while detectable, was low at 4.1 pmol/10^6 cells (~10% total covalent HMAF). On the other hand, if drug binding was expressed per mass of a specific macromolecule, the "density" of adducts on cellular DNA molecules exceeded the "density" of adducts on cellular proteins (Table 2).

TABLE 2. Macromolecular distribution of covalently bound HMAF following a 4-hr incubation of CEM cells with 5 μM [^{14}C]HMAF

	Enzyme digest			Covalently bound [^{14}C]HMAF			
	DNase	RNase	Prot. K	pmol/10^6 cells	Corrected (pmol/10^6 cells) (−Background)	fmol/μg of macromolecule*	% of Total uptake
Total covalent				60.1 ± 8.1†	56.5		71.7‡
DNA	−	+	+	16.4 ± 0.7	12.8	1600	17.7
RNA	+	−	+	7.7 ± 1.9	4.1	273	5.7
Protein	+	+	−	34.7 ± 1.4	31.1	156	43.0
Background	+	+	+	3.6			
Recovered (sum of DNA + RNA + protein binding) − background					*48.0*		*66.4*

CEM cells were incubated with HMAF and then processed for macromolecular covalent binding using combinations of RNase, DNase and proteinase K followed by PCA precipitation as outlined in the scheme in Fig. 4, right side and described in detail in Materials and Methods.

*Calculations were based on ~8 μg DNA and ~15 μg RNA per 10^6 average human cells, and 200 μg protein per 10^6 CEM cells (data not shown).

†Data from Table 1. Total uptake amounted to 85.3 pmol HMAF/10^6 cells.

‡Background samples were digested with a combination of all enzymes. The resulting residual amount of associated [^{14}C]HMAF was subtracted from the results for specific macromolecules to yield the corrected binding data shown in the table.

TABLE 3. Interaction of [^{14}C]HMAF with naked calf thymus DNA and purified proteins

	Covalent binding (PCA-insoluble radioactivity)*		
	[^{14}C]HMAF (pmol/μg macromolecule)	[^{14}C]HMAF (pmol/pmol protein)†	[^{14}C]HMAF/cysteine‡
DNA	6.3 ± 0.6		
BSA	22.4 ± 3.9	1.8	0.05
Thioredoxin	13.8 ± 0.7	0.21	0.04
Thioredoxin reductase	11.2 ± 1.5	0.39	0.13

Calf thymus DNA or proteins were incubated for 24 hr with 100 μM [^{14}C]HMAF followed by sample processing and determination of PCA-insoluble radioactivity as described in Materials and Methods. Values are calculated from two experiments carried out in duplicate (± ½ range).

*Values are corrected for background radioactivity determined as PCA-precipitable radioactivity as described in Materials and Methods (0.4, 0.6, 0.6, and 0.7 pmol/μg for DNA, BSA, thioredoxin, and thioredoxin reductase, respectively).

†Calculated using MW of 69,293, 15,555, and 34,492 for BSA, thioredoxin, and thioredoxin reductase, respectively.

‡Based on 35, 6, and 3 cysteine residues per molecule of BSA, thioredoxin, and thioredoxin reductase, respectively.

Various cross-checks confirmed the validity of the macromolecule binding data. There was excellent agreement between determinations of the separately assayed total covalent binding (60.1 pmol/10^6 cells) and a summation of drug covalently bound to individual macromolecules (58.9 pmol/10^6 cells). The low residual radioactivity in the triple digest (3.6 pmol/10^6 cells, Table 2) indicates that the contribution of background binding, which might reflect incomplete digestion, was low.

Interaction of [^{14}C]HMAF with Naked Calf Thymus DNA and BSA

Having demonstrated the covalent binding of [^{14}C]HMAF to intracellular macromolecules, we wondered whether the drug could form adducts with naked DNA and purified proteins. The selected model proteins included BSA, thioredoxin, and thioredoxin reductase. Thioredoxin and thioredoxin reductase were selected as examples of proteins with critical sulfhydryl groups in their reactive centers [23], which are conceivable targets for HMAF. Moreover, thioredoxin and thioredoxin reductase are crucial components of cellular redox systems playing a role in apoptosis [17].

HMAF was allowed to react with purified macromolecules for 24 hr at 37° followed by acid precipitation and extensive washing to remove free drug (Table 3). High drug concentration (100 μM) was used to approximate the input drug/bp ratio seen in the treatment of intact cells. Identical background reactions, set up for time 0, produced very low macromolecule-associated radioactivity, confirming that the procedure efficiently eliminated free or non-covalently associated drug. In contrast to the very low time 0 values, the results for a 24-hr incubation clearly show that HMAF bound to purified DNA as well as to the target proteins BSA, thioredoxin, and thioredoxin reductase. However, the yield of HMAF binding was low, in particular for DNA with only a 0.6% yield versus a 2.2% yield for BSA. Consistent with drug binding to sulfur-containing groups, the relative adduction of the tested proteins was roughly proportional to the number of cysteine residues (Table 3).

DISCUSSION

Promising antitumor activity and clinical development of HMAF warrant detailed exploration of the interactions of the drug with cancer cells and its potential intracellular targets. This study used HMAF stably labeled with [^{14}C] at its carbinol C15 to characterize the cellular uptake, subcellular distribution, and macromolecular binding of HMAF. The human myeloid leukemic cell line, CEM, used in these studies is responsive to HMAF in terms of cytotoxicity (IC_{50} = 1.7 μM) and apoptosis induction [12]. Accordingly, CEM cells showed significant uptake and covalent binding of radiolabeled HMAF.

The uptake of HMAF by CEM cells was a temperature-dependent process with two phases: a rapid phase (up to 10 min) and a slow phase (which continued for at least several hours). Additionally, uptake appeared to be concentration dependent and did not reach a plateau for both rapid and slow phases even at the highest concentration examined (75 μM).

Continuous drug uptake seems to be driven by intracellular covalent drug binding. CEM cells accumulate HMAF, and *total* intracellular drug concentration may exceed by many fold the extracellular concentration. However, intracellular levels of *free* drug approach the extracellular concentration. These data are consistent with a scenario in which the reactivity of internalized HMAF with cellular macromolecules effectively lowers the intracellular concentration of free HMAF and drives continued uptake of drug.

A separate study demonstrated a similar two-phase pattern of HMAF uptake in several other tumor and normal cell lines [14]. Interestingly, uptake levels appear to determine biological effects of HMAF in tumor cells, as they correlate well with cell growth inhibition. Moreover, HMAF induced apoptosis in 7/7 tumor cell lines, and apoptosis levels generally correlated with the magnitude of drug uptake. In contrast to tumor cells, 3/3 normal cell lines showed marginal or no apoptosis despite significant drug uptake, which was markedly greater than in CEM cells [14]. The nature of the observed differences between tumor and normal cells is currently under investigation.

The cell-associated radioactivity derived from [^{14}C]HMAF corresponded mainly to internalized drug in the nucleus (50%) and cytoplasm (37%). Approximately 2/3 of cell-associated HMAF appeared to be covalently bound to macromolecules in a manner that was not readily reversible. Our results provide also a clear-cut measure of the ability of HMAF to react with specific macromolecules in CEM cells. HMAF bound covalently to proteins > DNA > RNA (~60, 30, and 10%, respectively). Given the lack of bifunctional DNA lesions [12], HMAF should be considered a monofunctional DNA alkylator. It remains unknown whether it forms mono- or bifunctional adducts with proteins.

HMAF resembles its prototype drugs, acylfulvene and illudin S [6], in the magnitude of its cellular uptake when normalized for drug concentration and treatment times. Moreover, the frequency of HMAF–DNA adducts, again when normalized for drug concentration and treatment times, is of the same order of magnitude as acylfulvene–DNA adducts [6]. Also, for both illudin S [19] and HMAF, drug is not readily released from the cell. The lack of drug release can now be attributed to the formation of stable covalent adducts as directly demonstrated for HMAF. On the other hand, our data for CEM do not support the idea of a saturable, energy-dependent uptake, like the mechanism postulated for illudin S [2, 6]. However, given the similarities between HMAF and illudin S, the possibility of such a mechanism in other types of cells cannot be ruled out yet.

The significance of protein targeting by HMAF, described for the first time in this report, remains elusive but cannot be ignored. Covalent binding of the illudins to cellular proteins is consistent with the reactivity of these drugs with model thiols [8, 11]. *In vivo* binding to serum albumins could have pharmacologically adverse effects as drug is removed from the circulation before reaching target tumors. In contrast, binding to and inactivation of certain cellular proteins in cancer cells may have pharmacological advantage. Significant drug binding was seen to purified thioredoxin/thioredoxin reductase, which are key players in the maintenance of cellular redox states [17, 24, 25], down-regulation of apoptosis, and more aggressive cancer phenotypes [24, 26, 27]. An intriguing possibility exists that cellular HMAF effects are due, in part, to targeting of such crucial thiol-bearing proteins. Although thioredoxin and thioredoxin reductase are likely candidates, many proteins important for cell proliferation have key sulfhydryl residues. Further studies are needed to identify which specific critical cellular proteins may be affected by HMAF.

Our results indicate that HMAF can bind to purified macromolecules in the absence of any bioactivation. On the other hand, the majority of the available drug remains unbound in cell-free systems. In cellular systems, while only a small fraction of total input drug becomes cell-associated, i.e. available for covalent binding to cellular macromolecules, the majority of cell-associated drug forms covalent adducts. Hence, bio-activation is not absolutely required, but it remains the most likely explanation of the markedly more efficient drug binding to intracellular macromolecules.

This investigation and our previous studies [12] suggest that HMAF has multiple cellular targets. DNA remains a target for HMAF, as the drug undoubtedly forms adducts with cellular and naked DNA. However, an important implication of the reported findings is that DNA may not be the main and/or only target of HMAF, given the reactivity profile of HMAF with proteins.

DNA adducts may be needed to initiate damage *signaling* mechanisms leading to apoptosis. On the other hand, HMAF binding to proteins may auto-synergistically shift the cellular equilibria, e.g. the redox state, towards conditions that favor apoptosis *execution*. Studies are underway to explore the working hypothesis that reactivity towards redox-controlling proteins contributes to the potent pro-apoptotic properties of HMAF.

This study was supported, in part, by a grant from MGI Pharma, Inc. and by the Research Foundation of Cancer Therapy and Research Center. The authors thank Dr. Shih-fong Chen for his advice on uptake determinations, Dr. Michael Kelner for sharing his results with us prior to publication, and Dr. Dean Troyer for the estimates of CEM cells and nuclei diameters. We are also grateful to Dr. Randy Wadkins and Mr. Alex Trevino for their critical reading of the manuscript and the anonymous reviewers for valuable comments regarding the interpretation of the results.

References

1. Kelner MJ, McMorris TC, Beck WT, Zamora JM and Taetle R, Preclinical evaluation of illudins as anticancer agents. *Cancer Res* **47:** 3186–3189, 1987.
2. Kelner M, McMorris T and Taetle R, Preclinical evaluation of illudins as anticancer agents: Basis for selective cytotoxicity. *J Natl Cancer Inst* **82:** 1562–1565, 1990.
3. MacDonald JR, Muscoplat CC, Dexter DL, Mangold GL, Chen SF, Kelner MJ, McMorris TC and Von Hoff DD, Preclinical antitumor activity of 6-hydroxymethylacylfulvene, a semisynthetic derivative of the mushroom toxin illudin S. *Cancer Res* **57:** 279–283, 1997.
4. McMorris TC, Kelner MJ, Chandha RK, Siegel JS, Moon S and Moya MM, Structure and reactivity of illudins. *Tetrahedron* **45:** 5433–5440, 1989.
5. Kelner MJ, McMorris TC, Estes L, Rutherford M, Montoya M, Goldstein J, Samson K, Starr R and Taetle R, Characterization of illudin S sensitivity in DNA repair-deficient Chinese hamster cells. Unusually high sensitivity of ERCC2 and ERCC3 DNA helicase-deficient mutants in comparison to other chemotherapeutic agents. *Biochem Pharmacol* **48:** 403–409, 1994.
6. Kelner MJ, McMorris TC, Montoya MA, Estes L, Uglik SF, Rutherford M, Samson KM, Bagnell RD and Taetle R, Characterization of acylfulvene histiospecific toxicity in human tumor cell lines. *Cancer Chemother Pharmacol* **41:** 237–242, 1998.
7. Kelner MJ, McMorris TC and Taetle R, *In vitro* and *in vivo* studies on the anticancer activity of dehydroilludin M. *Anticancer Res* **15:** 873–878, 1995.
8. McMorris TC, Kelner MJ, Wang W, Moon S and Taetle R, On the mechanism of toxicity of illudins: The role of glutathione. *Chem Res Toxicol* **3:** 574–579, 1990.

9. McMorris TC, Kelner MJ, Wang W, Estes LA, Montoya MA and Taetle R, Structure-activity relationships of illudins: Analogs with improved therapeutic index. *J Org Chem* **57:** 6876–6883, 1992.
10. McMorris TC, Kelner MJ, Wang W, Yu J, Estes LA and Taetle R, (Hydroxymethyl)acylfulvene: An illudin derivative with superior antitumor properties. *J Nat Prod* **59:** 896–899, 1996.
11. McMorris TC, Yu J, Estes LA and Kelner MJ, Reaction of antitumor hydroxymethylacylfulvene (HMAF) with thiols. *Tetrahedron* **53:** 14579–14590, 1997.
12. Woynarowski JM, Napier C, Koester SK, Chen S-F, Troyer D, Chapman W and MacDonald JR, Effects on DNA integrity and apoptosis induction by a novel antitumor sesquiterpene drug, 6-hydroxymethylacylfulvene (HMAF, MGI 114). *Biochem Pharmacol* **54:** 1181–1193, 1997.
13. Kelner MJ, McMorris TC, Montoya MA, Estes L, Uglik SF, Rutherford M, Samson KM, Bagnell RD and Taetle R, Characterization of MGI 114 (HMAF) histiospecific toxicity in human tumor cell lines. *Cancer Chemother Pharmacol*, in press.
14. Woynarowska B, Woynarowski JM, Herzig MCS, Roberts K, Higdon AL and MacDonald JR, Differential induction of apoptosis in tumor and normal cells by 6-hydroxymethlacylfulvene (HMAF). In: *10th NCI-EORTC Symposium on New Drugs in Cancer Therapy, Amsterdam, Netherlands, 1998* (Ed. Krul MRI), p. 106. Kluwer Academic Publishers, Dordrecht, 1998.
15. Manning FC, Blankenship LJ, Wise JP, Xu J, Bridgewater LC and Patierno SR, Induction of internucleosomal DNA fragmentation by carcinogenic chromate: Relationship to DNA damage, genotoxicity, and inhibition of macromolecular synthesis. *Environ Health Perspect* **102:** 159–167, 1994.
16. Chu G, Cellular responses to cisplatin. The roles of DNA-binding proteins and DNA repair. *J Biol Chem* **269:** 787–790, 1994.
17. Sato N, Iwata S, Nakamura K, Hori T, Mori K and Yodoi J, Thiol-mediated redox regulation of apoptosis. Possible roles of cellular thiols other than glutathione in T cell apoptosis. *J Immunol* **154:** 3194–3203, 1995.
18. Vekrellis K, McCarthy MJ, Watson A, Whitfield J, Rubin LL and Ham J, Bax promotes neuronal cell death and is downregulated during the development of the nervous system. *Development* **124:** 1239–1249, 1997.
19. Kelner MJ, McMorris TC, Montoya MA, Estes L, Rutherford M, Samson KM and Taetle R, Characterization of cellular accumulation and toxicity of illudin S in sensitive and nonsensitive tumor cells. *Cancer Chemother Pharmacol* **40:** 65–71, 1997.
20. Vihardt H, Baker RV and Hope DB, Subcellular fractionation by centrifugation of homogenates of the neural lobe of the bovine pituitary gland: Identification of different pools of hormone in the homogenate and isolation of neurosecretosomes. *Acta Endocrinol (Copenh)* **85:** 300–313, 1977.
21. Pawlak JW, Pawlak K and Konopa J, The mode of action of cytotoxic and antitumor 1-nitroacridines. II. *In vivo* enzyme-mediated covalent binding of a 1-nitroacridine derivative, Ledakrin or Nitracrine, with DNA and other macromolecules of mammalian or bacterial cells. *Chem Biol Interact* **43:** 151–173, 1983.
22. Bradford M, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
23. Nakamura H, Nakamura K and Yodoi J, Redox regulation of cellular activation. *Annu Rev Immunol* **15:** 351–369, 1997.
24. Wang J, Kobayashi M, Sakurada K, Imamura M, Moriuchi T and Hosokawa M, Possible roles of an adult T-cell leukemia (ATL)-derived factor/thioredoxin in the drug resistance of ATL to adriamycin. *Blood* **89:** 2480–2487, 1997.
25. Iwata S, Hori T, Sato N, Hirota K, Sasada T, Mitsui A, Hirakawa T and Yodoi J, Adult T cell leukemia (ATL)-derived factor/human thioredoxin prevents apoptosis of lymphoid cells induced by L-cystine and glutathione depletion: Possible involvement of thiol-mediated redox regulation in apoptosis caused by pro-oxidant state. *J Immunol* **158:** 3108–3117, 1997.
26. Yamada M, Tomida A, Yoshikawa H, Taketani Y and Tsuruo T, Overexpression of thioredoxin does not confer resistance to cisplatin in transfected human ovarian and colon cancer cell lines. *Cancer Chemother Pharmacol* **40:** 31–37, 1997.
27. Yokomizo A, Ono M, Nanri H, Makino Y, Ohga T, Wada M, Okamoto T, Yodoi J, Kuwano M and Kohno K, Cellular levels of thioredoxin associated with drug sensitivity to cisplatin, mitomycin C, doxorubicin, and etoposide. *Cancer Res* **55:** 4293–4296, 1995.