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ORIGINAL ARTICLE

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Evidence for a role of chloroethylaziridine in the cytotoxicity of cyclophosphamide

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Abstract A number of investigators have observed that the use of 4-hydroperoxycyclophosphamide (4-HC) in multiwell plate cytotoxicity assays can be associated with toxicity to cells in wells that contain no drug. Previous reports have implicated diffusion of 4-HC decomposition products, and acrolein in particular, as the active species. *Purpose*: The purpose of this study was to elucidate the species responsible for the airborne cytotoxicity of 4-HC, and to devise ways to minimize such effects in chemosensitivity assays. *Methods*: To this end, analogues of 4-HC were synthesized to identify the contributions of individual cyclophosphamide metabolites to cytotoxicity. The analogues were then tested for activity against three human breast tumor cell lines (including a line resistant to 4-HC), and one non-small-cell lung carcinoma line. Cytotoxicity was evaluated by assays that quantitate cellular metabolism and nucleic acid content. *Results*: Didechloro-4-hydroperoxycyclophosphamide, a compound that generates acrolein and a nontoxic analogue of phosphoramide mustard, gave no cross-well toxicity. In contrast, a significant neighboring well effect was observed with phenylketophosphamide, a compound that generates phosphoramide mustard but not acrolein. Addition of authentic chloroethylaziridine reproduced the airborne toxicity patterns generated by 4-HC and phenylketophosphamide. Increasing the buffering capacity of the growth medium and sealing the microtiter plates prevented airborne cytotoxicity. *Conclusions*: Since it is unlikely that phosphoramide mustard is volatile, these findings implicate chloroethylaziridine rather than acrolein as the volatile metabolite of 4-HC that is responsible for airborne cytotoxicity. The fact that chloroethylaziridine is generated in amounts sufficient to volatilize, diffuse across wells and cause cytotoxicity indicates that it is an important component in the overall cytotoxicity of 4-HC in vitro. Furthermore, these findings suggest that chloroethylaziridine may also contribute to the toxicity of cyclophosphamide in vivo.

Key words Cyclophosphamide · Chloroethylaziridine · 4-Hydroperoxycyclophosphamide · Phenylketophosphamide · In vitro assay

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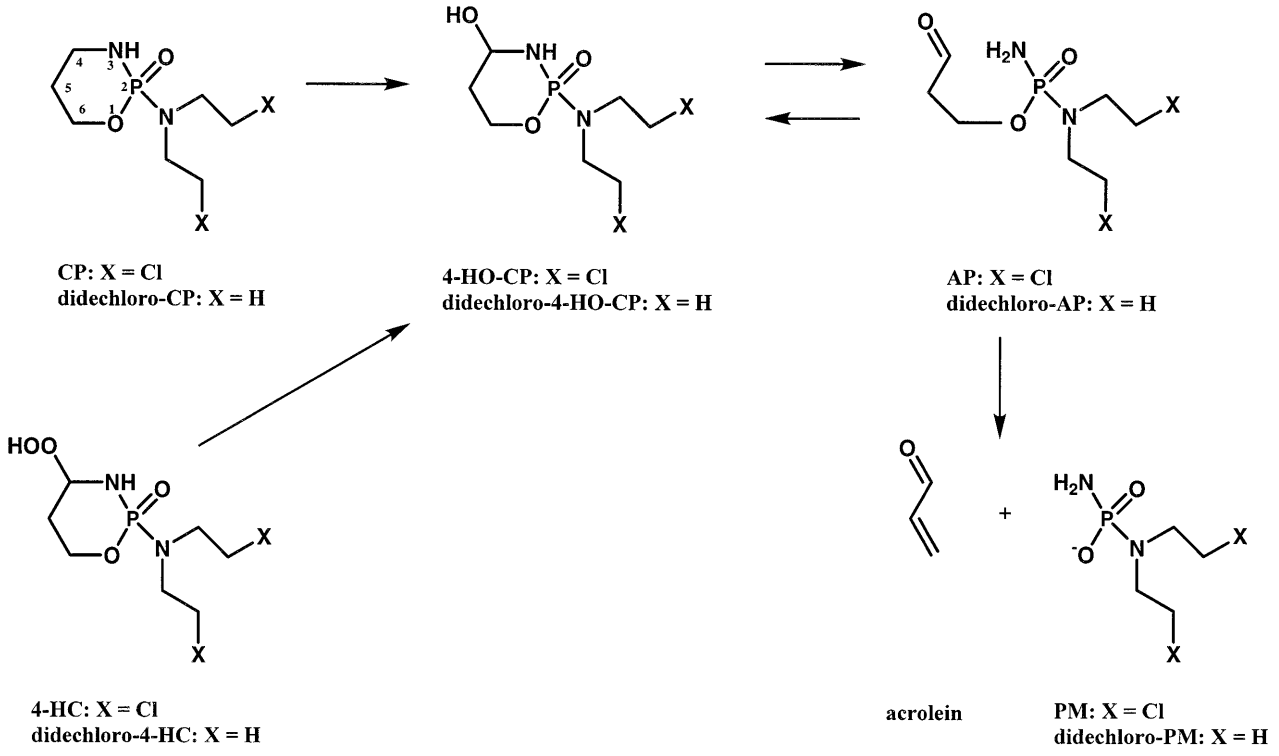
Introduction

The prodrug cyclophosphamide (CP) is a bisalkylating chemotherapy agent used to treat many types of cancer [8]. As shown in Scheme 1, the metabolism of CP is initiated through an oxidation at the C-4 position by a hepatic cytochrome P450, and the product of that activation, 4-hydroxycyclophosphamide (4-HO-CP), rapidly undergoes a reversible ring-opening reaction to give aldophosphamide (AP). An irreversible fragmentation of AP gives acrolein and phosphoramide mustard (PM), the latter of which is generally believed to be the ultimate DNA alkylator [8, 13, 24]. In the absence of microsomal activation, the metabolites of CP can be produced for in vitro studies by using 4-hydroperoxycyclophosphamide (4-HC; Scheme 1) [25, 28]. While stable in crystalline form, 4-HC in solution is spontaneously converted to 4-HO-CP which then generates the other active metabolites of CP through nonenzymatic conversions [4, 28].

In evaluating the cytotoxicity of 4-HC in vitro, decreased cellular viability is observed in controls adjacent to those treated with this agent. Since the IC_{50} endpoint of the assay is normalized to the viability of untreated controls, this "neighboring well effect" results in irreproducible and, ultimately, incorrect results. Similar

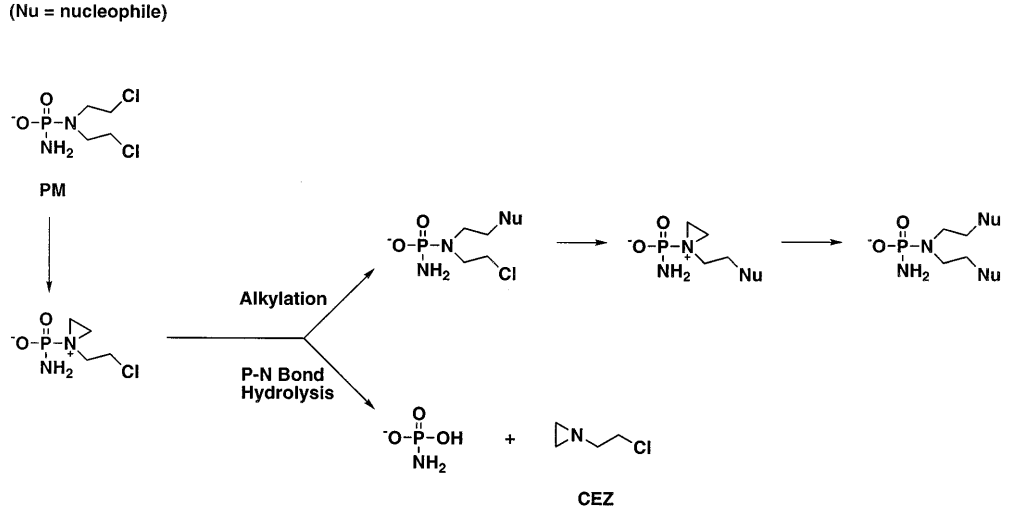


Scheme 1

effects have been reported by others using 4-HC and other preactivated CP analogues in conjunction with microplate analyses [2, 5]. In one case, acrolein has been implicated as the volatile agent responsible for this effect, based on its low boiling point and known toxicity [2]. Similar observations of toxicity in neighboring wells have been reported in a study using authentic material [16]. Although acrolein can undergo relatively rapid addition reactions with nucleophilic compounds found in cells and growth medium [14, 28], the volatility of this compound in such environments has been demonstrated. For example, acrolein has been trapped in the atmosphere above incubation mixtures of CP-treated liver microsomes and buffered solutions of 4-HC [1, 27].

Although acrolein has been assumed to be responsible for the airborne toxicity associated with 4-HC treatment, there is another CP metabolite that must be considered. Chloroethylaziridine (CEZ) was first reported as a metabolite of CP in 1968 [21], but has received scant attention until recently. Formed from PM, this compound is volatile as well as cytotoxic [18, 21]. As shown in Scheme 2, PM partitions between alkylation reactions with nucleophiles and P-N bond hydrolysis, the latter of which produces CEZ [23]. The distribution of products from PM as well as the lifetime of CEZ itself is very dependent upon the nature and concentration of nucleophiles in solution [23]. Nevertheless, CEZ must be

Scheme 2



considered an alternative (or additional) source of neighboring well toxicity.

To investigate the source of the airborne toxicity derived from 4-HC, we compared the neighboring well effects produced by 4-HC to those generated by the metabolite analogues which yielded acrolein or CEZ but not both. By isolating the effects of the individual metabolites, we conclude that CEZ, rather than acrolein, is the volatile metabolite primarily responsible for the airborne cytotoxicity associated with 4-HC treatment in vitro. The airborne cytotoxicity of CEZ in neighboring wells indicates that the metabolite CEZ must be generated at significant levels and is therefore an important contributor to the overall toxicity of CP.

Materials and methods

The syntheses of *cis*-4-HC and phenylketophosphamide (PKP) have been reported [19, 25, 28]. For chemical syntheses, reagents and solvents were purchased from Aldrich Chemical Company or Fisher Scientific Company. Reactions which did not include water as a solvent were carried out under an atmosphere of nitrogen. Ozone (5–10 g/h) was produced by an OREC Model 03V10-0 ozone generator (Ozone Research and Equipment Corporation). NMR spectra were recorded on a JEOL FX-90Q (90 MHz ^{1}H and 36.23 MHz ^{31}P), a GE QE-300 (300 MHz ^{1}H and 121.5 MHz ^{31}P) or a Varian Inova-400 (400 MHz ^{1}H and 162 MHz ^{31}P) spectrometer.

Didechloro-4-hydroperoxycyclophosphamide (didechloro-4-HC)

Didechloro-4-HC was synthesized using modifications to a literature preparation of 4-HC [28].

N,N-Diethylphosphoramidic dichloride [$Cl_2P(O)N(CH_2CH_3)_2$]

A solution of phosphorus oxychloride ($POCl_3$, 0.25 mol, 23.3 ml) in benzene (50 ml) was added dropwise to a cooled (0 °C) solution of diethylamine (0.25 mol, 29.5 ml) and pyridine (0.25 mol, 20.2 ml) in benzene (200 ml). Upon complete addition, the reaction mixture was stirred at 0 °C for 1 h and then overnight at room temperature. The reaction mixture was suction filtered and the filtrate was concentrated at reduced pressure. The residual oil was chromatographed on silica gel (60–200 mesh, 4 × 45-cm column) using $CHCl_3$ as eluent. The product (R_f 0.65) was obtained as an oil in 36% yield (16.6 g, 0.09 mol). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.33 (dq, $^3J_{HP} = 17$ Hz, $^3J_{HH} = 7$ Hz, 4H, two CH_2) and 1.23 (t, $^3J_{HH} = 7$ Hz, 6H, two CH_3). ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 16.6 (relative to 2% H_3PO_4 capillary insert).

3-Butenyl N,N-diethylphosphorodiamidate [$CH_2=CHCH_2CH_2OP(O)(NH_2)N(CH_2CH_3)_2$]

A hexane solution of *n*-butyl lithium (21 mmol, 12.5 ml of a 1.7 *M* solution) was added dropwise via syringe to a stirred solution of 3-buten-1-ol (20 mmol, 1.72 ml) in tetrahydrofuran (10 ml, dried/distilled) at −23 °C (dry ice/CCl_4 bath). Stirring at −23 °C was continued for 2 h and the resultant suspension was then removed with a syringe and added dropwise to a stirred solution of *N,N*-diethylphosphoramidic dichloride (20 mmol, 3.80 g) in tetrahydrofuran (10 ml) at −23 °C under N_2. Stirring at low temperature was continued for 3 h and then NH_3 was bubbled through the reaction mixture for 15 min at 0 °C. The flask was capped and stored overnight at room temperature prior to suction filtration, concentration of the filtrate on a rotary evaporator, and then chromatography of the residual material on silica gel (60–200 mesh, 3 × 33-cm column) using ether (about 300 ml) to remove faster-eluting impurities. Elution with $CHCl_3/CH_3OH$ (9:1) gave the product as an oil [14.7 mmol, 2.83 g, 74% yield, R_f 0.60 ($CHCl_3/CH_3OH$, 9:1)]. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.89–5.75 (m, 1H, vinyl), 5.19–5.05 (m, 2H, vinyl), 4.07–3.85 (m, 2H, CH_2O), 3.21–3.02 (m, 4H, two C**H**$_2$CH$_3$), 2.55 (bs, 2H, NH_2), 2.41 (apparent q, $J = 6$ Hz, 2H, C**H**$_2$CH$_2$O), and 1.11 (t, $^3J_{HH} = 8$ Hz, 6H, two CH_3). ^{31}P NMR (36.23 MHz, $CDCl_3$) δ 17.0 (referenced externally to a capillary insert of 25% H_3PO_4 in $CDCl_3$).

cis-2-(N,N-Diethylamino)-4-hydroperoxytetrahydro-2H-1,3,2-oxazaphosphorin 2-oxide (didechloro-4-HC)

Ozone was bubbled through a cooled (0 °C) solution of 3-butenyl *N,N*-diethylphosphorodiamidate (4.8 mmol, 1.0 g) in acetone/water (2:1, 21 ml) for 30 min. Acetone (14 ml) was used to transfer the reaction solution to a round-bottom flask and to this was added aqueous H_2O_2 (1.0 ml of a 30% solution). The flask was stoppered and kept overnight at room temperature. Acetone was removed at ambient temperature on a rotary evaporator and the residual aqueous mixture was extracted with CH_2Cl_2 (6 × 25 ml). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated at ambient temperature on a rotary evaporator and then a high-vacuum pump. The product was obtained as a white solid (0.67 g, 3.0 mmol, 63% yield, m.p. 102–105 °C). Analysis calculated for $C_7H_{17}N_2O_4P$ – theory (found): C, 37.49 (37.69); H, 7.66 (7.63); N, 12.50 (12.56). The proton NMR was consistent with a *cis*, 1,3-diaxial relationship between the C_4 OOH and the oxygen of the P = O group [28]. ^{1}H NMR (300 MHz, CD_2Cl_2) δ 11.53 (s, 1H, OOH), 5.14 (doubled multiplet, $^3J_{HP} = 25.8$ Hz, 1H, C_4-H), 4.73 (br t, 1H, NH), 4.59–4.48 (m, 1H, C_6-H), 4.14–3.97 (m, 1H, C_6-H), 3.23–2.95 (m, 4H, two C**H**$_2$CH$_3$), 2.17–1.83 (m, 2H, two C_5-H), and 1.08 (t, $^3J_{HH} = 7$ Hz, 6H, two CH_3). ^{31}P NMR (121.5 MHz, CD_2Cl_2) δ 10.16 (referenced externally to a capillary insert of 25% H_3PO_4 in CD_2Cl_2). *Note:* in $CDCl_3$ that had been distilled and washed with a solution of $NaHCO_3$ in D_2O, the ^{31}P NMR displayed a single resonance for didechloro-4-HC at δ 10.50. In $CDCl_3$ that had not been purified and that may have been an 'old' bottle, a second signal appeared at δ 13.74 (relative to an external 25% H_3PO_4 capillary insert in $CDCl_3$). The identity of this species was not pursued. It was determined, however, that this compound was generated in the NMR tube and that its concentration was related to the acidity of the $CDCl_3$. Distillation of the $CDCl_3$ was not sufficient to remove the acid contaminant. The solvent required washing with $NaHCO_3$.

N,N-Diethylphosphorodiamidic acid (didechloro-PM)

The title compound was made using modifications to a synthesis for isophosphoramide mustard (IPM) [22]. A mixture of diethylamine (12 mmol, 1.24 ml) and triethylamine (12 mmol, 1.67 ml) was added dropwise to a solution of phenyl dichlorophosphate (12 mmol, 1.79 ml) in CH_2Cl_2 (20 ml). After being stirred overnight at room temperature, the reaction mixture was cooled to 0 °C and then NH_3 was bubbled through the mixture for 15 min. After stirring for an additional 30 min at low temperature, the reaction mixture was filtered and the filtrate was concentrated on a rotary evaporator. The residual material was flash chromatographed on silica gel (< 230 mesh, 3 × 15-cm column) using $CHCl_3/CH_3OH$ (95:5) as eluent. *N,N*-Diethylphosphorodiamidic acid phenyl ester [$C_6H_5OP(O)(NH_2)N(CH_2CH_3)_2$] was obtained as an oil (R_f 0.58) in 40% yield (1.1 g, 4.8 mmol). ^{1}H NMR (90 MHz, $CDCl_3$) δ 7.3 (m, 5H, aromatic), 3.2 (m, 4H, two CH_2), 2.7 (bs, 2H, NH_2), and 1.0 (t, 6H, two CH_3).

A mixture of *N,N*-diethylphosphorodiamidic acid phenyl ester (4.4 mmol, 1.0 g) and PtO_2 (100 mg) in absolute ethanol (10 ml) was hydrogenated (Parr medium pressure shaker hydrogenator) at 50 p.s.i. for 3 h. The pressure was then released and N_2 was bubbled through the reaction mixture for several minutes. More absolute ethanol (about 40 ml) was added and the suspension was

stirred for 30 min before filtration. The filtrate was concentrated on a rotary evaporator and the residual solid was dried under high vacuum. The product was obtained as a white microcrystalline solid in 23% yield (150 mg, 1.0 mmol, m.p. 124–127 °C). For the purposes of elemental analysis, the solid was dissolved in warm absolute ethanol and crystals were precipitated with approximately two volumes of ether (m.p. of recrystallized material, 125–127 °C). Analysis calculated for $C_4H_{13}N_2O_2P$ – theory (found): C, 31.57 (31.08); H, 8.63 (8.44); N, 18.41 (17.61). ^{1}H NMR (90 MHz, D_2O, referenced to HOD at 4.7 ppm) δ 3.22 (m, 4H, two CH_2) and 1.27 (t, 6H, two CH_3). ^{31}P NMR (121.5 MHz, D_2O) δ 8.5 (referenced to a capillary insert of 25% H_3PO_4 in D_2O). ^{31}P NMR (121.5 MHz, 0.01 *M* phosphate in D_2O, pD 7.0) δ 14.4 (with the solution phosphate at δ 2.5).

Drug kinetics in buffered solutions

The half-life of each compound was measured by ^{31}P NMR using methods described previously [3, 19, 23, 28]. ^{31}P NMR chemical shifts were referenced to external 25% H_3PO_4. Values of pH corresponded to the observed reading and were uncorrected for deuterium isotope effects in solutions containing D_2O.

4-HC

An NMR sample of 4-HC (0.034 mmol) in 1 *M* lutidine (2,6-dimethylpyridine, 1.35 ml) and D_2O (0.15 ml) was adjusted to pH 7.4 and ^{31}P NMR (36.23 MHz) spectra were acquired at a probe temperature of 37 °C. Linear least-squares fit of a pseudo-first-order plot of the disappearance of the ^{31}P signal for 4-HC (δ 11.51) provided a rate constant which allowed for a calculation of the half-life given in Table 1. This value represented the kinetics associated with the conversion of 4-HC to 4-HO-CP (via iminophosphamide [6, 10]).

Didechloro-4-HC

As described for 4-HC, an NMR sample of didechloro-4-HC (0.042 mmol) in 1 *M* lutidine (1.35 ml) and D_2O (0.15 ml) was adjusted to pH 7.4 and ^{31}P NMR (36.23 MHz) spectra were acquired at a probe temperature of 37 °C. Linear least-squares fit of a pseudo-first-order plot of the disappearance of the ^{31}P signal for didechloro-4-HC (δ 11.92) provided a rate constant which allowed for a calculation of the half-life given in Table 1. This value represented the kinetics associated with the conversion of didechloro-4-HC to didechloro-4-HO-CP (presumably via an imine).

Table 1 ^{31}P NMR-derived half-lives at 37 ± 2 °C, pH 7.4 ± 0.2 (observed pH uncorrected for the effects of D_2O or DMSO)

Compound	Half-life (min)	Conditions
4-HC	89	23 m*M* in 0.9 *M* lutidine
Didechloro-4-HC	101	28 m*M* in 0.9 *M* lutidine
4-HO-CP/AP	38[a]	23 m*M* in 0.9 *M* lutidine
Didechloro-4-HO-CP/AP	50	28 m*M* in 0.9 *M* lutidine
4-HO-CP/AP	72[b]	20 m*M* in 1 *M* lutidine/DMSO (8:2)
PKP	66[b]	20 m*M* in 1 *M* lutidine/DMSO (8:2)
PM	17[c]	11 m*M* in 0.22 *M* BisTris
Didechloro-PM	18,000 (12.5 days)	34 m*M* in 0.33 *M* BisTris

[a] Data taken from reference 28
[b] Data taken from reference 19
[c] Data taken from reference 23

Didechloro-4-HO-CP/AP

The metabolites didechloro-4-HO-CP/AP were generated in situ by the thiosulfate reduction of didechloro-4-HC, as has been reported for the reduction of 4-HC [28]. In brief, an NMR sample of didechloro-4-HC was prepared as described above and to this was added four molar equivalents of sodium thiosulfate. The pH was readjusted to 7.4 and ^{31}P NMR spectra were accumulated at various time intervals at a probe temperature of 37 °C. Paralleling the results of similar experiments with 4-HC, the interconverting, didechloro- metabolite analogues of *cis*- and *trans*-4-HO-CP and AP (δ 12.68, 13.32 and 21.4, respectively) gave rise to a pseudo-equilibrium mixture (54:29:17, respectively) which could be characterized by an apparent half-life. Least-squares fits of separate 'first-order' plots of the disappearance of each of these metabolites at equilibrium gave rate constants which were used to calculate the average half-life shown in Table 1. This value represented the kinetics associated with the formation of acrolein and didechloro-PM from the didechloro metabolite analogues of 4-HO-CP/AP.

Didechloro-PM

By analogy to previously described ^{31}P NMR derived kinetic experiments with PM [3, 23], an NMR sample was prepared by dissolving methylphosphonic acid (internal standard, 8 mg, 0.09 mmol) in a solution of 1 *M* BisTris (1.65 ml), H_2O (2.85 ml) and D_2O (0.50 ml). The pH was adjusted to 7.5 and then didechloro-PM (25 mg, 0.17 mmol) was added to the sample. The pH was readjusted to 7.4 and the final sample was approximately 34 m*M* didechloro-PM and 18 m*M* methylphosphonate in 0.33 *M* BisTris. ^{31}P NMR spectra were acquired at a probe temperature of 37 °C. The peak height for didechloro-PM (δ 12.67, relative to external 2% H_3PO_4) was normalized relative to that of the internal standard (δ 21.20) and over a 23-h time period, the loss of signal intensity was 5%. This loss of intensity was associated with the growth of a new signal (δ 2.2) indicative of inorganic phosphate. These data were extrapolated to give an approximate half-life for didechloro-PM of 12.5 days (Table 1).

Cell culture

MCF-7wt (wild type) and MCF-7hc (4-HC resistant subline) human breast tumor cell lines were obtained from the American Type Culture Collection (Rockville, Md.) and Dr. Beverly Teicher (Dana-Farber Cancer Institute, Boston, Mass.) [11], respectively. These cells were cultured in DMEM medium (Sigma Chemical Co., St. Louis, Mo.) containing 1 g/l glucose, supplemented with an additional 1 g/l glucose and 10% fetal bovine serum (FBS; HyClone, Logan, Utah). MDA-MB-231 human breast tumor cells were obtained from the American Type Culture Collection, and cultured in RPMI-1640 medium (GIBCO, Grand Island, N.Y.) containing 10% FBS. The ADLC-5M2 non-small-cell lung cancer cell line was obtained from Dr. Gerald Bepler (Duke University Medical Center, Durham, N.C.), and grown in RPMI-1640 medium supplemented with 1% penicillin/streptomycin (10,000 units/ml penicillin and 10,000 µg/ml streptomycin in 0.85% saline stock solution; GIBCO), and 10% FBS. Each cell line was also cultured in parallel using appropriate medium supplemented with 25 m*M* HEPES buffer (GIBCO). Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Confluent cultures were passaged by harvesting with 0.025% trypsin-EDTA (GIBCO), and reseeded at 5×10^5 cells per 75-cm^2 tissue culture flask (Corning Incorporated, Acton, Mass.). Cells were counted on a CASY1 TTC Cell Counter and Analyzer System (Sharfë System, Reutlingen, Germany), using the protocol recommended by the manufacturer.

Assay reagents

All compounds were prepared as concentrated stock solutions (20×, 4 m*M*). Due to the instability of each compound in solution, each

drug solution was used immediately upon preparation. 4-HC, didechloro-4-HC, didechloro-PM, and nornitrogen mustard hydrochloride (NNM, purchased as bis(2-chloroethyl)amine hydrochloride from Aldrich, Milwaukee, Wis.) were made fresh in 70% ethanol, while PKP was made fresh in DMSO (Sigma Chemical Co.). CEZ was prepared by dissolving NNM in water and adjusting the pH to 7.5 with NaOH. After 105 min at 37 °C, more than 95% of the NNM was converted to CEZ [23]. A working solution of calcein-AM (Molecular Probes, Eugene, Ore.) was prepared fresh by diluting 50 µl of stock (1 mg/ml in DMSO) into 10 ml of phenol red-free Hank's balanced salt solution (GIBCO). Concentrated stock solution (100×, 7.5 m*M*) of propidium iodide (Molecular Probes) was prepared by dissolving 5 mg into 1 ml water. Nuclear isolation medium (NIM) was prepared by adding 0.5% bovine serum albumin (Sigma Chemical Co.) and 0.2% Triton-X100 (Bio-Rad, Hercules, Calif.) to calcium- and magnesium-free Dulbecco's phosphate-buffered saline.

Nuclear isolation and counting

For each experiment, a microplate was included to provide a tumor cell growth kinetic. At various times, growth medium was replaced and cells were lysed by suspension in 100 µl cold NIM for 4 min. Nuclei were then collected and analyzed on the CASY1. Triplicate values were averaged to determine the growth kinetic. This method of determining cell number in microplates results in lower variation between counts than harvesting whole cells by trypsin.

Cytotoxicity assays

Black-sided 96-well microplates (Costar #3904, Corning) were seeded with 7500 cells per well (approximately 30% confluence) and incubated overnight to allow attachment. Nuclei were harvested and counted to determine cellular plating efficiency. Cells were exposed to agents for two cell doublings (54, 54, 44 and 36 h for MCF-7wt, MCF-7hc, MDA-MB-231 and ADLC-5M2, respectively), as determined by NIM nuclei count, then changed to drug-free medium for one additional doubling. Different growth kinetics of the individual cell lines made it necessary to use this timing to ensure exposure of cells to agents for an equivalent number of cell cycles. In addition, exposure of the cells to the drugs and drug analogues for these long time periods compensated for differences in the half-lives of these compounds (Table 1).

Cytotoxicity assays were performed in separate multiwell plates for each compound and each attendant control to prevent cross-contamination of neighboring wells by airborne components of the other compounds. For these experiments, plates were set up as shown in Fig. 1A. To determine the magnitude and extent of the cross-contamination of the volatile compounds, plates were set up as shown in Fig. 1B. Cytotoxicity assays were duplicated in medium containing 25 m*M* HEPES buffer (pH 7.4) using microplates covered with assay plate sealing tape (VWR, South Plainfield, N.J.).

A control experiment was conducted with only one concentration of 4-HC per open plate, which was another way of isolating each dose from the neighboring well effects of any other dose. MCF-7wt cells were plated only in the four corner wells of each plate and the rest of the wells were filled with medium. Plates were incubated overnight to allow attachment and 4-HC was added to the cell-containing wells only, one concentration per plate using the concentration range described for the cytotoxicity assays. As an additional control, this experiment was done both with and without the addition of HEPES buffer to the growth medium so as to establish the effects, if any, of HEPES in the system.

Cytotoxicity was evaluated by both calcein-AM metabolic inhibition assay and propidium iodide nucleic acid staining assay. We used the calcein-AM cellular viability assay previously described [9, 17, 20, 26], with modifications. Briefly, after exposure to cytotoxic agents, medium was removed and 100 µl of the calcein-AM working solution was added to the wells and the plates were placed in an incubator (37 °C with a humidified atmosphere of 95% air and 5% CO_2) for 30 min. Fluorescence was quantitated in an FL-600 microplate fluorescence reader (BioTek, Winooski, Vt.) using fluorescein excitation and emission filters (485/560 nm) with sensitivity set at 100.

A modification of the live/dead assay described by Chang et al. [7] was used for the propidium iodide assay. After exposure to cytotoxic agents, the medium was removed and cells were fixed to the plate with cold ethanol (70% in H_2O) overnight in the refrigerator. After removing the ethanol, 100 µl of the working solution of propidium iodide (1% concentrated stock in H_2O) was added to the wells and the fluorescence was quantitated in an FL-600 microplate fluorescence reader (530/590 nm) with sensitivity set at 100. This method assays the reduction in cell number by measuring the amount of nucleic acids present in microplate wells. This correlates with the number of viable cells which remain in that well after treatment (J. Flowers, unpublished observations). The data were processed with KC4 software (BioTek), and expressed in relative fluorescence units (RFU).

Analysis of cytotoxicity data

Dose response curves were constructed by subtracting appropriate blanks from the raw data and calculating as percentage of control (treated RFU/untreated RFU), and standard error of the mean (SEM). Table Curve 2D (SPSS, Chicago, Ill.) was used to curve fit the data and to calculate the IC_{50} endpoint, defined as the drug concentration that reduces the RFU by 50% compared to that of untreated control. The IC_{50} values were averaged from four experiments and expressed as the mean ± SEM.

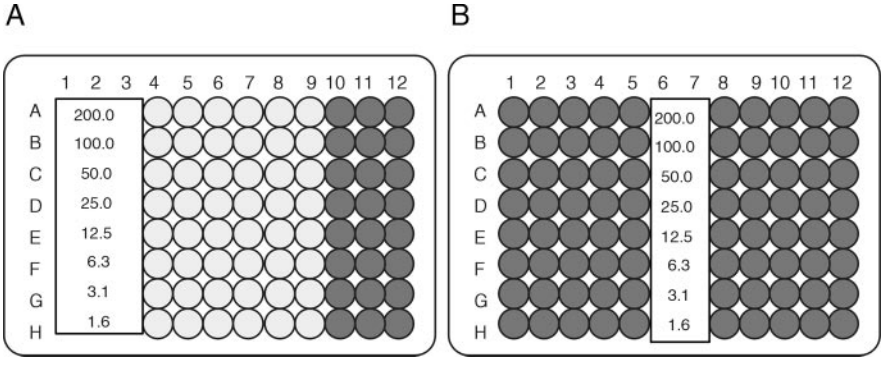


Fig. 1A,B Plate formats for toxicity and volatility assays. **A** For cytotoxicity assays, columns 1–3 contained cells and compound at the indicated concentrations (µ*M*) and were separated from wells containing untreated control cells (columns 10–12) by a space of six rows containing medium only (columns 4–9). **B** For volatility assays, each well of the plate was seeded with cells. Columns 6 and 7 only were exposed to compound at the indicated concentrations

Results and discussion

We first encountered neighboring well cytotoxicity in a study of the interaction of 4-HC with camptothecin analogues in vitro. These microplate cytotoxicity assays were plagued by highly variable IC_{50} values. Since the effect was only noted in microplates containing 4-HC, the experimental designs were changed to isolate each compound on separate plates. Only the data for 4-HC remained unpredictable, due to unstable control values. This implied a volatile metabolite in the 4-HC assays. Others had suggested acrolein as the agent responsible [2, 16]. However, we considered CEZ to be another likely candidate based on its cytotoxicity, volatility and significant formation from PM [18, 21, 23]. To separate the effects of acrolein and CEZ, 4-HC analogues that produced only one of the volatile metabolites were studied in cell culture.

Analogue chemistry

As shown in Scheme 1, didechloro-4-HC mimics the breakdown of 4-HC to yield acrolein, but instead of PM, didechloro-4-HC gives didechloro-PM, which is not an alkylating agent and cannot generate CEZ. PKP, an analogue of AP, fragments to PM (Scheme 3) and, therefore, is a precursor to CEZ. Rather than acrolein, however, the second product of this elimination reaction is phenylvinylketone, which is nontoxic [15, 19]. Metabolite formation from these analogues is summarized in Table 2.

As described in the experimental section and in the literature, ^{31}P NMR methods have been applied extensively to the study of CP metabolite and metabolite-analogue kinetics and chemistry [3, 19, 23, 28]. The rates of reaction of some metabolites (most notably 4-HO-CP and AP) have been shown to be buffer-dependent. Therefore, when considering the half-lives given in Table 1, reactivities of different compounds must be compared under the same conditions. The conversion of didechloro-4-HC to didechloro-4-HO-CP/AP and the subsequent fragmentation of these intermediate metabolites to acrolein occurred at slightly slower rates than the same reactions for 4-HC and 4-HO-CP/AP (Table 1). Others also have reported that the rate of acrolein formation from didechloro-4-HC is slightly less than for 4-HC, but half-life values were not given [27]. In any case, these differences in half-lives were small when compared to the cell exposure time of more than 36 h.

Table 2 Summary of the metabolites of interest generated in vitro from each analogue

Compound	Metabolites generated		
	PM	Acrolein	CEZ
4-HC	+	+	+
PKP	+	–	+
Didechloro-4-HC	–	+	–
NNM	–	–	+
Didechloro-PM	–	–	–

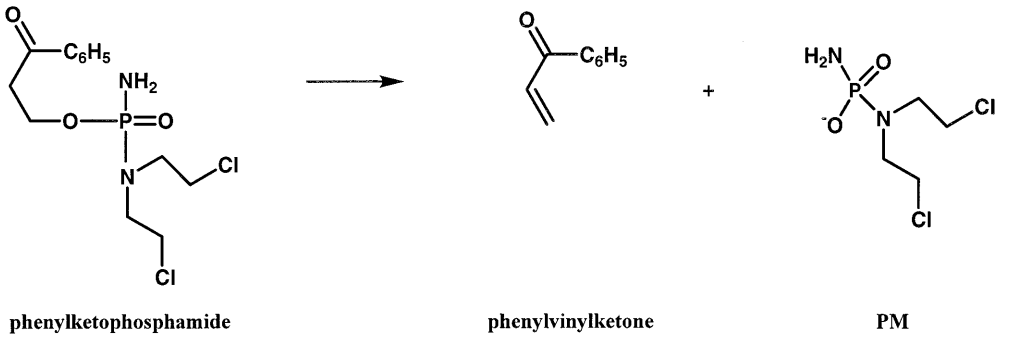
As reported previously, the generation of PM from PKP is comparable to that from AP [19]. The cyclization of PM to an aziridinium ion (Scheme 2) occurs with a half-life of 17 min (Table 1). This intermediate undergoes rapid alkylation or P-N bond hydrolysis, the latter of which produces CEZ (Scheme 2) [23]. In contrast, didechloro-PM reacts 1000 times slower than does PM. This relative stability is attributed, at least in part, to the fact that one driving force for PM reactivity at pH 7.4 is the intramolecular cyclization reaction (with loss of chloride); a similar reaction in didechloro-PM is not possible.

Analogue cytotoxicity

IC_{50} values were determined by calcein-AM under conditions that limited the contamination of control cells, as shown in Fig. 1A. In addition, cytotoxicity assays were duplicated in plates covered with adhesive plate-sealing tape. Since sealing the plates prevents CO_2 exchange, addition of 25 m*M* HEPES buffer was required to maintain the medium at pH 7.4. The IC_{50} values ($\pm$SEM) for each compound are listed in Table 3.

As expected, 4-HC proved to be the most active agent against all cell lines studied, since it generates both acrolein and PM. PKP, which generates PM (and, therefore, CEZ), but not acrolein, was also active against all cell lines. The toxicity of acrolein itself is well established, and as a CP metabolite (generated at low, steady concentrations over time), it has recently been described

Scheme 3



phenylketophosphamide **phenylvinylketone** **PM**

Table 3 IC_{50} values (μM) using the well-plate configuration shown in Fig. 1A. Values are means ± SEM (*NA* 50% inhibitory effect not achieved and could not be extrapolated with accuracy)

Compound	MCF-7wt		MCF-7hc		MDA-MB-231		ADLC-5M2	
	Open	Sealed	Open	Sealed	Open	Sealed	Open	Sealed
4-HC	11.3 ± 0.8	20.7 ± 0.9	58.6 ± 3.2	103.5 ± 4.2	19.6 ± 1.7	35.7 ± 3.1	33.5 ± 1.9	50.3 ± 0.9
Didechloro-4-HC	122.3 ± 5.7	136.1 ± 4.8	243.8 ± 38.7[a]	198.9 ± 41.1	104.4 ± 3.9	122.3 ± 4.1	88.4 ± 3.0	71.5 ± 2.2
Didechloro-PM	NA	NA	NA	NA	NA	NA	NA	NA
PKP	15.5 ± 1.1	29.8 ± 5.0	117.5 ± 8.3	165.5 ± 5.5	39.8 ± 6.6	61.2 ± 5.1	56.8 ± 4.4	82.4 ± 7.3
NNM	80.3 ± 4.3	168.8 ± 11.2	166.6 ± 4.8	227.7 ± 8.4[a]	109.5 ± 16.7	160.9 ± 20.1	96.1 ± 6.0	190.3 ± 6.4
CEZ	75.2 ± 2.3	152.2 ± 5.1	175.9 ± 7.8	238.2 ± 11.0[a]	93.4 ± 10.6	171.6 ± 5.2	103.2 ± 5.3	213.5 ± 9.5

[a] Extrapolated value

as a minor contributor to the cytotoxicity of CP [12]. Didechloro-4-HC, the generator of acrolein but not PM, was the least cytotoxic of the 4-HC analogues against the MCF-7 and MDA-MB-231 breast cancer lines.

Since didechloro-4-HC showed some cytotoxicity, we wanted to establish whether this effect was due to acrolein, didechloro-PM, or other metabolites. Didechloro-PM is not an alkylating agent and is relatively stable under aqueous conditions; it can, however, slowly produce ammonia and diethylamine (Table 1). We synthesized authentic didechloro-PM and tested its toxicity. No toxicity was observed for up to 200 μM didechloro-PM (Table 3), indicating that, under the conditions of our experiment, the toxicity of didechloro-4-HC was due to acrolein.

Finally, to confirm and isolate the cytotoxicity of CEZ, cells were exposed to NNM, which generates CEZ at a rate similar to that for CEZ formation from PM (Table 1). We also used pregenerated CEZ [23]. Each method of CEZ exposure produced nearly identical toxicity (Table 3).

The rank order of cytotoxicity was 4-HC > PKP > NNM/CEZ > didecloro-4-HC, with the exception that NNM/CEZ was less active than didechloro-4-HC against ADLC-5M2. Didechloro-PM was not cytotoxic to any of the cells at the concentrations used, up to 200 μM. These results were confirmed by, and correlated with, the data obtained with the propidium iodide assay ($P = 0.037$, data not shown), demonstrating that the effect was not dependent on the type of chemosensitivity assay used.

In plate-sealing experiments where the diffusion of volatile metabolites was limited by sealing each individual well, the rank order of the cytotoxicity of the compounds remained the same. Upon initial inspection, the results in Table 3 appear to be counterintuitive, since sealing in a volatile metabolite might be expected to increase the cytotoxic effect and thus decrease the IC_{50} endpoint. However, the observed IC_{50} values in sealed plates were, on average, 1.5- to 2-fold higher than in open-well plates even when untreated controls were isolated on both. Notably, this variation only affected plates treated with compounds that generate CEZ. There are at least two possible explanations for this result. First, the diffusion dynamics and resulting concentration of CEZ in the headspace above open wells is not precisely known. Thus, a dose-response curve based on the input concentration of 4-HC may not reflect the total concentration of cytotoxic moieties. Second, the cellular susceptibility to airborne CEZ is not uniform throughout the concentration range. As shown in Fig. 2A, wells on the lower end of the 4-HC concentration range showed the greatest change between the two plates. This effect likely occurs because a small loss of CEZ in wells exposed to high 4-HC concentrations has less impact than does a similar gain of CEZ in wells treated with marginally effective drug concentrations.

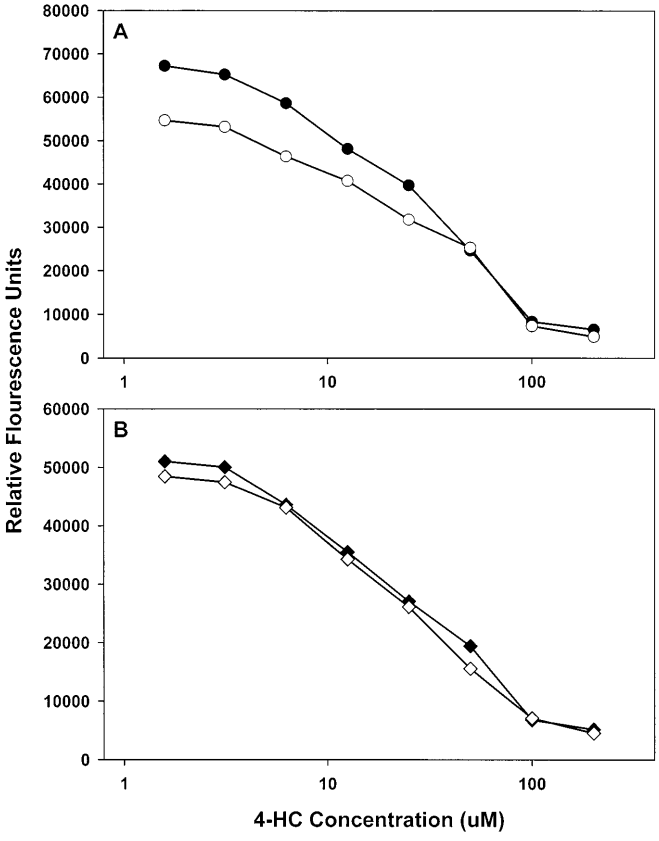


Fig. 2A,B 4-HC dose response curves in MCF-7wt cells as determined by calcein-AM metabolic inhibition assay. **A** Cells were exposed to 4-HC in open (○) or sealed (●) microplates. **B** Cells were exposed to 4-HC in HEPES-free (◇) or HEPES-containing (◆) medium. Separate open microplates were used for each concentration of 4-HC to prevent neighboring well effects

Previous work has shown that some buffers can interact with various metabolites of CP (and, presumably, with metabolite analogues) [23, 28]. A ''dose-separated'' control experiment was conducted to determine if the differences in cytotoxicities observed in the plate-sealed vs open-plate assays were influenced by the addition of HEPES buffer to the sealed systems (added for pH control in the absence of CO_2 exchange). Using MCF-7wt cells, a series of open plates using HEPES-free medium and only one concentration of 4-HC per plate were assayed for cell viability. This was repeated using medium containing 25 m*M* HEPES, pH 7.4. The dose response curves shown in Fig. 2B were identical thereby demonstrating that HEPES did not affect the outcome of these studies. While this control utilized only MCF-7wt cells, it is reasonable to assume that the same result would have been found for other cell lines.

Airborne cytotoxicity

In contrast to the plate configuration used for determining IC_{50} values (Fig. 1A), controls were placed directly adjacent to treated cells for the determination of airborne toxicity (Fig. 1B). The volatility assay was based on the comparison of the viability of cells grown in the untreated control plate (Fig. 3A) with cells grown in untreated wells that were adjacent to wells to which drug had been added (Fig. 3B–F). The untreated control plate was assayed and the mean of 96 wells, the standard error of the mean (SEM) and the coefficient of variation (CV) were determined. These values were then compared to the value from each untreated well on the drug-treated plates. The variation of untreated wells on drug-treated plates (Fig. 3B–F) from the mean of the control plate (Fig. 3A) was calculated and ranked based on the SEM of the control wells. The wells were ranked in one of four categories: $\geq 20 \times$ SEM, $\geq 10 \times$ SEM, $\geq 4 \times$ SEM and $< 4 \times$ SEM ($4 \times$ SEM approximates the 99% confidence interval). The untreated plate is almost uniform in shading, due to insignificant well-to-well deviation (the coefficient of variation was 0.7%). The effects of the metabolites were visualized on the respective treated plates (Fig. 3B–F). The gray-scale shading reflects cytotoxic effects on the untreated control wells, with the gradient from black to white representing diminishing toxicity to untreated wells.

In the plates containing either 4-HC or PKP (Fig. 3B,E), the neighboring wells were strongly affected. On the other hand, the neighboring wells were unaffected by up to 200 μ*M* didechloro-4-HC or didechloro-PM

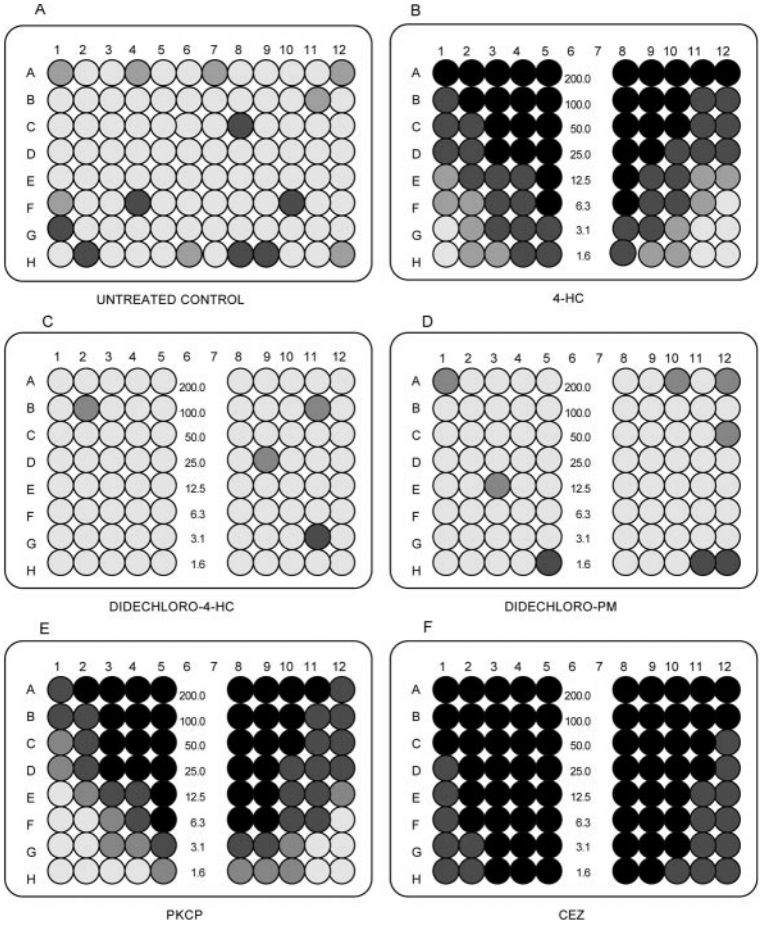


Fig. 3A–F Effect of metabolites generated in treated wells (columns 6 and 7) on cells in neighboring wells as determined by calcein-AM metabolic inhibition assay. The four shades, from black to white, represent four levels of effect, based on the mean untreated control value (UC); the mathematical conditions for the shading are variation from mean UC by $\geq 20 \times$ SEM, $\geq 10 \times$ SEM, $\geq 4 \times$ SEM, and $< 4 \times$ SEM

(Fig. 3C,D). These results show that compounds that produce PM and consequently CEZ cause neighboring well cytotoxicity. Moreover, CEZ (both pre-generated and generated in situ from NNM) caused the strongest, most far-reaching effect on neighboring wells (Fig. 3F).

Conclusions

This work demonstrates that the airborne cytotoxicity associated with 4-HC assays in microplates is due to CEZ and not acrolein, as previously postulated. Any CP analogue that produces PM and therefore CEZ can be expected to cause a similar effect. It was also shown that sealing the microplates eliminated this effect. This precaution should also be exercised with in vitro assays involving preactivated forms of ifosfamide that generate IPM. Unlike PM, IPM does not give rise to CEZ, but it undergoes significant P-N bond hydrolysis to yield aziridine (Ludeman et al., in preparation). While we did not study the diffusion characteristics of aziridine, this compound has a pK_a of 6.6 and should be essentially neutral under in vitro experimental conditions and exhibit volatility comparable to that of CEZ (pK_a 6.62) (Ludeman et al., in preparation; [23]).

Without knowing the kinetic details of CEZ formation and lifetime under the conditions of the in vitro experiment, it is difficult to predict the concentration of CEZ generated by 4-HC. Some insight into this question is provided by a comparison of the toxicity patterns caused by the airborne diffusion of CEZ as generated by 4-HC (Fig. 3B) and by use of authentic material (Fig. 3F). 4-HC concentrations of 50 and 25 μM gave cell-kill patterns similar to those given by 3.1 and 1.6 μM CEZ, respectively.

Our studies demonstrate that the amount of extracellular CEZ produced from cytotoxic concentrations of 4-HC is sufficient to diffuse to and produce cytotoxicity in other wells in a multiwell plate. These findings indicate that CEZ produced extracellularly from the decomposition of PM can enter cells and produce toxicity. This information supports our previous suggestion [23] that CEZ plays a role in the cytotoxicity of CP. A quantitative measurement of the extra- and intracellular concentrations of CEZ as well as the role of this metabolite in alkylating DNA, proteins etc. will be useful in understanding the mechanisms of the overall cytotoxicity of CP. Studies are in progress using radiolabeled analogues of 4-HC to address these issues.

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