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RESEARCH PAPER

A Degradation Study of a Series of Chloromethyl and Diazomethyl Ketone Anti-leukemic Agents

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ABSTRACT

The chemical stability of a novel cysteine chloromethyl ketone derivative (HI-131) with anti-leukemic activity has been investigated in a microemulsion formulation. HI-131 degrades to two major products, most likely by undergoing oxidation and further reaction with another HI-131 molecule to form higher molecular weight oligomers of the original compound. The degradation kinetics of HI-131 have been studied as a function of pH, buffer composition, ionic strength, and temperature. Degradation follows pseudo-first-order kinetics and the temperature effect obeys the Arrhenius equation. The pH–rate profile demonstrates HI-131 is most stable at lower pH values, although there is no significant influence of ionic strength and buffer ions on the degradation rate. The chemical stability of a homologous series of chloromethyl and diazomethyl ketone derivatives of HI-131 has also been investigated in microemulsion. The relationship between the chain length of the derivatives and the stability is presented. Changing the chloro group to a bromo group resulted in an increase in degradation rate. Alterations to the group on the nitrogen were also investigated. The changes to the stability are discussed in terms of their mechanistic implications.

Key Words: *Degradation; Stability; Microemulsion; Chloromethyl ketone; Diazomethyl ketone*

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INTRODUCTION

We have recently reported a series of cysteine chloromethyl and diazomethyl ketone derivatives that show potent cytotoxicity against human acute lymphoblastic leukemia (ALL) cells (1,2). Our lead compound, *N*-acetyl-*S*-dodecyl-cysteine chloromethyl ketone (HI-131) (Fig. 1) has shown low micromolar inhibition of cancer growth in MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays (e.g., 2.0 μM against a B-cell acute lymphoblastic leukemia cell line) (1). It has also been shown to cause destruction of the cytoskeleton, mitochondrial membrane depolarization, and the induction of apoptosis (3). It is thus a highly promising anti-leukemic agent that we are seeking to evaluate further as a potential drug candidate. Derivatives of this cysteine chloromethyl ketone also display significant cytotoxicity toward both pre-B ALL and T-ALL (Molt-3) cell lines (2).

Knowledge about the properties of degradation is important to the understanding of the chemistry, stability, and activity of potential drug candidates and to the development of stable formulations. For this reason, we investigated the influences of external factors (pH, ionic strength, and buffers) on the degradation of HI-131 (**1**).

Since our lead compound, HI-131 (Fig. 1), was shown to degrade rapidly in the presence of water or heat, we also examined a number of other derivatives to determine the effect of variation in the chemical structure on stabilizing this class of compounds. The goal was to find a more viable drug candidate without a substantial loss of activity; therefore, compounds with relatively minor changes in activity were investigated. The variations consisted of alterations in the chain length on the sulfur, the group on the nitrogen, the halogen of the chloromethyl ketone, and the replacement of the sulfur with oxygen.

Due to the extreme water insolubility of HI-131 and its derivatives, they were examined in a microemulsion formulation. Microemulsions are thermodynamically-stable, transparent dispersions of oil and water stabilized by an interfacial film of surfactant molecules. They are widely used in pharmaceutical formulations because of their ability to concentrate significant amounts of both oil- and water-soluble materials within the same isotropic medium (4). The microemulsion formulation allowed us to investigate HI-131 in an aqueous medium despite its low water solubility.

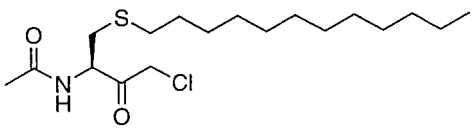


Figure 1. Chemical structure of HI-131 (**1**).

MATERIALS AND METHODS

Materials

The cysteine chloromethyl ketone drug candidates were prepared as reported previously (1,2). The microemulsion was a premixed solution of 53.3% surfactant, 33.3% oil, and 13.3% deionized water by weight. The surfactant was a mixture of 80% 1,2-propanediol (Sigma Chemical Co., St. Louis, MO), 18% cremophor EL (BASF, Mount Olive, NJ), and 2% Plucare® F-68 Prill (BASF, Mount Olive, NJ). The oil was a mixture of 60% Captex 300 (Abitec Corp., Janesville, WI) and 40% Phospholipon (American Lecithin Co., Oxford, CT). After dissolving the drug candidate in the premixed microemulsion at a concentration of 4 mg/mL, it was filtered through a 0.2-μm syringe filter (Whatman GD/X PTFE) and diluted in the buffer solution at a ratio of 1 : 7, so that the final compound concentration was 0.5 mg/mL.

For the kinetic studies, the buffer concentrations were 0.01 M adjusted to an ionic strength (μ) of 0.3 with NaCl, except for the experiments in which the influence of the ionic strength was tested. The ionic strength of the buffer was calculated, prior to adjustment, as one-half the sum of the series of the concentration of the ionic species times the square of the charge of the species (5). The concentration of the ionic species present at a given pH was calculated using the pK_a values (6). The pH was adjusted to 8.0 except in the series determining the pH effect (7). The pH values were measured at 25°C with a combined glass–reference electrode and a pH meter (Accumet model 20, Fisher Scientific, Hanover Park, IL).

Kinetic Measurements

The kinetic measurements were conducted at 27, 40, 50, and 70°C. The reactions were initiated by the dilution of the drug candidate in the buffer solution. The reaction solutions were kept in a

thermostatically-controlled oven, protected from light. At regular time intervals 20-μL samples were withdrawn and injected into the high-performance liquid chromatography (HPLC) system. All experiments were performed in triplicate and the standard deviation was no more than 10%.

The reaction solutions were analyzed by HPLC with ultraviolet (UV) detection. The HPLC equipment consisted of a Hewlett Packard Series 1100 injector, pump, and diode array detector. The detector operated at 300 nm. The stainless-steel analytical column ($4 \times 4\,mm^2$ internal diameter, 250 mm length) was packed with Lichrosorb RP-18 material (particle size: 5 μm) (Hewlett Packard, Mountain View, CA). The eluant comprised a mixture of acetonitrile and an aqueous solution consisting of 0.1% triethylamine and 0.1% trifluoroacetic acid in deionized water. The ratio of acetonitrile to water was determined for each drug candidate to find conditions where the parent peak was well separated from the degradation peaks. Quantitation of undegraded compound was based on peak area measurements.

Since the reactions exhibited pseudo-first-order conditions, the degradation rate constant, k (hr^{-1}), was calculated from the slope of the line of the natural log (ln) of the percent compound remaining vs. time at the varied temperatures (8). The degradation rate constant at 25°C was calculated for each compound from the Arrhenius equation [Eq. (1)]. The 90% shelf-life, t_{90}, and half-life, $t_{1/2}$, were calculated from Eqs. (2) and (3), respectively.

$$k = A\,e^{-E_a/RT} \tag{1}$$

$$t_{90} = (\ln 1.11)/k \tag{2}$$

$$t_{1/2} = (\ln 2)/k \tag{3}$$

Isolation and Identification of Degradation Products of HI-131

In the pH region 5–10 the degradation of HI-131 produces two major products with retention times of 9 and 22 min (Fig. 2). The degradation products were isolated by column chromatography. The 9-min peak was isolated by chromatography on reverse-phase C-18 packing material eluted with acetonitrile–water (50 : 50, v : v). The 22-min peak was isolated by chromatography on silica gel eluted with ethyl acetate–hexane (95 : 5, v : v). Nuclear magnetic resonance (NMR) spectra were recorded using a 300-MHz Varian spectrometer. Mass spectral analysis was done using both a Hewlett Packard

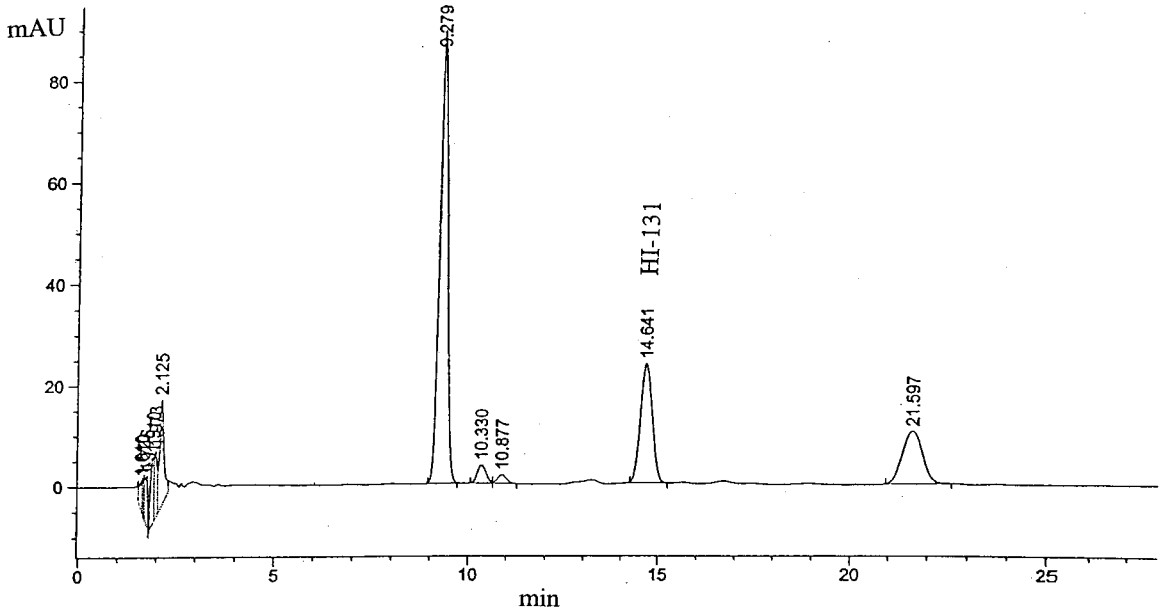


Figure 2. HPLC chromatogram of partially degraded HI-131 (**1**) in a microemulsion solution with 0.01 M phosphate buffer, pH 8, $\mu = 0.3$ M.

LC-mass spectrometer HP series 1100MSD with an atmospheric pressure ionization electrospray and a high-energy dynode (HED) electron multiplier, and a Hewlett Packard HP G205A MALDI-TOF system. Infrared (IR) spectra were recorded on an FT-Nicolet model Protege 460 instrument. Elemental analyses were performed by Atlantic Microlab Inc. (Norcross, GA).

RESULTS AND DISCUSSION

Analytical Procedures

The UV spectrum of HI-131 in ethanol displays a maximum at 200 nm and a small shoulder at 300 nm. During degradation, the absorbance at 200 nm decreases and a maximum around 310 nm emerges. These spectral changes allow the degradation to be followed spectrophotometrically. However, HPLC was preferred as this technique also provides direct information on the degradation products. A typical HPLC chromatogram demonstrating the separation between HI-131 and its degradation products is depicted in Fig. 2. There were no qualitative differences in the on-line UV spectra of the HI-131 taken during different stages of the degradation, indicating that the HI-131 peak does not co-elute with a degradation product having a different UV spectrum.

Degradation Products

The two major degradation products eluting at 9 and 22 min on the HPLC under our standard conditions were isolated by preparative chromatography. The two small peaks between 10 and 11 min were present to such a small degree that no attempt was made to isolate them. The LC-MS spectrum of the degradation products was inconclusive, possibly due to further reaction during analysis, but the MALDI-TOF mass spectra and elemental analysis of the 9 and 22-min degradation products suggested that both had greater masses than the mass of the parent HI-131. Mass spectra of the isolated degradation products injected directly into the LC-MS had masses greater than 700. From the MALDI-TOF analysis, the degradation product at 9 min had a mass of 474.8 and the 22-min peak had a mass of 523.9. The possibility that these are fragmentation products of larger compounds cannot be ruled out. Elemental analysis found: C, 58.04; H, 8.84; N, 3.70; S, 8.57; trace Cl for the 9-min degradation peak and C, 66.55; H, 10.48; N, 0.88; S, 8.65; trace Cl for the 22-min peak. Possible masses are all greater than the formula weight of the parent compound (HI-131).

From the IR spectra and ^{1}H and ^{13}C NMR it was clear that both the 9 and 22-min peaks contained mostly hydrocarbons. The ^{1}H NMR spectra for both degradation products contained a large, broad peak in the 1.2–1.3 ppm region and the ^{13}C NMR spectra contained numerous overlapping peaks in the 15–35 ppm region, indicative of hydrocarbons. Neither the 9 or 22-min degradation peaks contained *N*-acetyl groups or carbonyl groups, as evidenced by the loss of the *N*-acetyl peak in the ^{1}H NMR at 2.1 ppm and the absence of any carbonyl carbons in the ^{13}C NMR or IR spectra. The 9-min degradation product had some alkene carbon character with a peak at 125 ppm in the ^{13}C NMR spectrum. Most likely, the two major degradation products result from oligomerization of HI-131.

Degradation Kinetics of HI-131

Under the experimental conditions, the degradation reactions of HI-131 in buffered microemulsion exhibited pseudo-first-order kinetic behavior, as indicated by the linearity of plots of the natural logarithm of residual HI-131 concentration vs. time. Neither the order of the reaction nor the degradation rate was influenced by the initial drug concentration, which is in agreement with a first-order kinetic model. A representative plot showing the degradation of HI-131 over time at 27, 40, and 50°C is shown in Fig. 3.

The influence of ionic strength, μ, on the degradation kinetics was measured in 0.01 M phosphate buffers (pH 8) with different amounts of sodium chloride added, with μ between 0.1 and 0.6. The results of the test are shown in Table 1 and demonstrate that the ionic strength has very little effect on the decomposition rate of HI-131. Similarly, the effect of the buffer ion was tested at pH 8 with 0.01 M phosphate, acetate, citrate, carbonate, or borate with μ adjusted to 0.3 by addition of NaCl. The results are shown in Table 2. No significant influence of the buffer on the degradation rate was observed, although the rate of degradation in the citrate buffer was slightly higher.

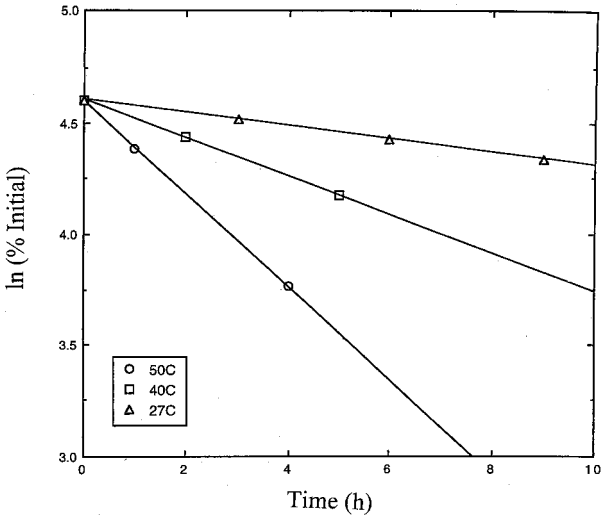


Figure 3. The degradation of HI-131 (**1**) in a microemulsion solution with 0.01 M phosphate buffer, pH 8.0, $\mu = 0.3$ M, at 27, 40, and 50°C over time.

Table 1

*The Influence of Ionic Strength on the Degradation Kinetics of HI-131 (**1**) in a Microemulsion Solution at pH 8*

Ionic Strength	k (hr^{-1})	$t_{1/2}$ (hr)	t_{90} (hr)
0.05	0.027	25.5	3.8
0.15	0.025	27.9	4.2
0.30	0.024	28.7	4.3
0.60	0.023	30.4	4.6

Table 2

*Effect of the Buffer on the Degradation Kinetics of HI-131 (**1**) in a Microemulsion Solution at pH 8*

Buffer	k (hr^{-1})	$t_{1/2}$ (hr)	t_{90} (hr)
Phosphate	0.024	28.7	4.3
Borate	0.024	29.1	4.4
Acetate	0.026	26.6	4.0
Carbonate	0.026	26.7	4.1
Citrate	0.032	21.7	3.2

In Fig. 4, the pH–rate profile for HI-131 at 25°C is given. The profile was recorded in 0.01 M phosphate with $\mu = 0.3$. The pH–rate profile is characterized by a decrease in stability at higher pH values. This can be explained by the possibility that the first step in degradation is the loss of the chloride from the chloromethyl

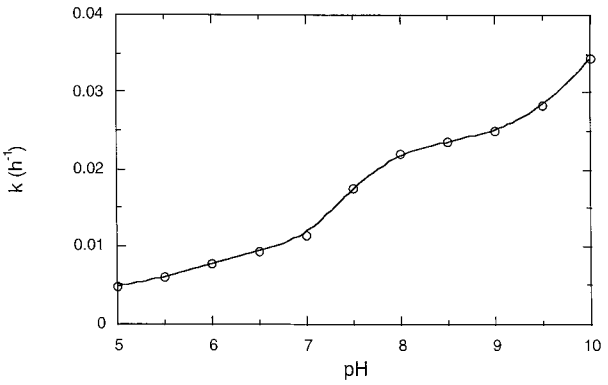
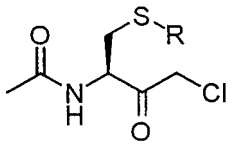


Figure 4. Effect of pH on the observed rate constant of degradation of HI-131 (**1**) in a microemulsion solution with 0.01 M phosphate buffer, $\mu = 0.3$ M.

Table 3

Effect of the Chain Length on the Degradation Kinetics



Compound	R	k (hr^{-1})	$t_{1/2}$ (hr)	t_{90} (hr)
2	C_3H_7	0.0658	10.6	1.6
3	C_4H_9	0.0523	13.3	2.0
4	C_5H_{11}	0.0498	14.7	2.2
5	C_6H_{13}	0.0336	20.6	3.1
6	C_7H_{15}	0.0319	21.7	3.3
7	C_8H_{17}	0.0388	17.9	2.7
8	C_9H_{19}	0.0373	18.6	2.8
9	$C_{10}H_{21}$	0.0352	19.7	3.0
10	$C_{11}H_{23}$	0.0345	20.1	3.0
1 (HI-131)	$C_{12}H_{25}$	0.0242	28.7	4.3
11	$C_{13}H_{27}$	0.0343	20.3	3.1
12	$C_{14}H_{29}$	0.0417	18.3	2.8
13	$C_{15}H_{31}$	0.0374	18.9	2.9
14	$C_{16}H_{33}$	0.0363	19.1	2.9

ketone group, a reaction that would be enhanced by the presence of base.

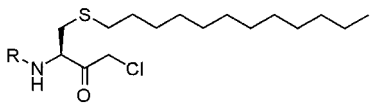
Effect of Structural Changes to HI-131 on the Stability

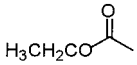
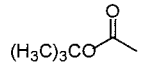
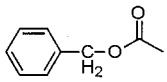
Table 3 shows the effect of chain length on the degradation kinetics in 0.01 M phosphate buffer, pH

8.0 at an ionic strength (μ) of 0.3. The stability is not greatly affected by the length of the chain on the cysteine sulfur group. The extremly short chains (two to five carbons) are slightly less stable than the intermediate and long chain lengths, with the dodecyl chain having the greatest stability.

Table 4

Effect of Variation in the Nitrogen Group on the Degradation Kinetics



Compound	*R*	k (hr^{-1})	$t_{1/2}$ (hr)	t_{90} (hr)
1 (HI-131)		0.0242	28.7	4.3
15		0.0697	9.9	1.5
16		0.0167	41.6	6.3
17		0.0121	57.4	8.6
18		0.0241	28.8	4.3

The effect of substitution to the nitrogen group on the degradation kinetics is shown in Table 4. An electron-withdrawing group, trifluoroacetyl, caused greater instability than electron-donating groups such as ethoxycarbonyl or *t*-butyloxycarbonyl. The *t*-butyloxycarbonyl derivative (**17**) has the slowest rate of degradation, a twofold improvement over the acetyl group of HI-131.

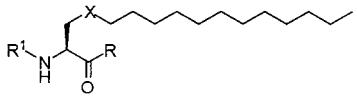
The degradation kinetics when bromomethyl ketone (**19**) or a diazo group (**20**) replaces the chloromethyl ketone group are shown in Table 5. The bromomethyl ketone is 20 times less stable than the chloromethyl ketone. This is probably due to the greater innate reactivity of the bromo group vs. the chloro group. The diazo group, on the other hand, is 10 times more stable than the chloromethyl ketone. This might be expected at pH 8 as the diazo group is known to have greater base stability.

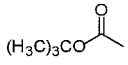
Table 5 also contains the change from a cysteine chloromethyl ketone derivative to a serine chloromethyl ketone derivative (**21**). The stability of the chloromethyl ketone more than doubled when the sulfur was replaced by oxygen. It is possible that oxidation may play a role in the degradation, since sulfur is more easily oxidized than oxygen.

The stabilizing features therefore include a carbon length of 12, *t*-butyloxycarbonyl on the nitrogen group, a diazomethyl ketone moiety, and oxygen instead of sulfur. By combining all

Table 5

Effect on the Degradation Kinetics by Variation in the Chloromethyl Ketone Group or by Replacing Sulfur with Oxygen



Compound	*R*	*X*	*R*1	k (hr^{-1})	$t_{1/2}$ (hr)	t_{90} (hr)
19	CH_2Br	S		0.4772	1.5	0.2
1 (HI-131)	CH_2Cl	S		0.0242	28.7	4.3
20	CH_2N_2	S		0.0027	253.9	38.2
21	CH_2Cl	O		0.0098	70.8	10.7
22	CH_2N_2	O		0.000029	28,737	4327

these elements, the resulting molecule (**22**) is 1000 times more stable than the lead compound, HI-131 (**1**).

CONCLUSIONS

The cysteine chloromethyl ketone derivative is unstable when exposed to aqueous solutions. The two major degradation products arise from the reaction between two or more molecules of the cysteine chloromethyl ketone. The disappearance of HI-131 and its derivatives follows pseudo-first-order kinetics, and the temperature effect obeys the Arrhenius equation. The degradation kinetics of HI-131 are not influenced greatly by the buffer type or ionic strength. The chemical instability of HI-131 can be ameliorated somewhat with lower pH solutions, but not enough to make a stable formulation in a microemulsion. Degradation studies of biologically-active derivatives of HI-131 demonstrated that the chain length does not play a large role in stability. However, greater stability was achieved in molecules in which the group on the nitrogen was more electron-donating and the chloromethyl ketone was replaced by a diazomethyl ketone. Replacement of the sulfur group with oxygen also significantly increased the stability. Combining these stabilizing elements brought about a 1000-fold increase in stability over the lead compound HI-131 under the same conditions. Inclusion of chemical stability data in the search for new anti-leukemic agents may lead more quickly to a pharmaceutically-viable drug candidate.

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