J. Enzyme Inhibition, 1992, Vol. 6, pp. 181–194
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IRREVERSIBLE INHIBITION OF TRANSGLUTAMINASES BY SULFONIUM METHYLKETONES: OPTIMIZATION OF SPECIFICITY AND POTENCY WITH ω-AMINOACYL SPACERS†

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(Received 15 April 1992)

Sulfonium methylketones, of structure Cbz–Phe–$NH(CH_2)_nCOCH_2S^+(CH_3)_2$, $n > 2$, are specific and potent inactivators of transglutaminases. The length of the $-(CH_2)_n$-spacer moiety, $n = 1$–5, is a critical determinant for both the specificity and potency of the inactivator. The dipeptidyl analog Cbz–Phe–Gly–$(CH_2)_nS^+(CH_3)_2$, $n = 1$, is a more powerful inactivator of the thiol proteinase cathepsin B, $k/K > 3 \times 10^5\ M^{-1}\ min^{-1}$, than of transglutaminases, $k_{i(app)}/K_{i(app)} < 1.5 \times 10^4\ M^{-1}\ min^{-1}$. In contrast, the γ-aminobutyryl analog, $n = 3$, is a very potent transglutaminase inactivator with $k_{i(app)}/K_{i(app)} = 3.1 \times 10^6\ M^{-1}\ min^{-1}$, but does not inactivate cathepsin B. In cell studies, the γ-aminobutyryl and ε-aminohexyl analogs inhibited the transglutaminase-mediated process of ionophore-induced cross-linked envelope formation by human malignant keratinocytes and the order of potency was related to that found for enzyme inhibition. The sulfonium methylketones, in equilibrium with the resonance stabilized ylides, are chemically inert towards glutathione under ambient conditions demonstrating the potential utility of this novel class of transglutaminase inhibitors for the study of enzyme inhibition in cellular environments.

KEY WORDS: Sulfonium methylketones, inhibition, cathepsin B, transglutaminases, cross-linking.

INTRODUCTION

Transglutaminases are a class of calcium-dependent acyl transferases that catalyze protein cross-linking through ε-(γ-glutaminyl-lysine)isopeptide bond formation. The best characterized transglutaminase is plasma Factor XIIIa, which, in a thrombin and calcium-dependent manner, catalyzes the cross-linking of fibrin in the final stages of hemostasis.[1] Epidermal transglutaminases play a key role in the synthesis of the cornified envelope that is responsible for the rigid, resistant structure of terminally differentiated epidermal keratinocytes,[2] and elevated levels of epidermal transglutaminase have been implicated in dermatologic disorders such as acne[3] and psoriasis.[4] Tissue transglutaminases are widely distributed in mammalian tissues[5,6] and have been associated with a multiplicity of functions including possible roles in receptor-mediated endocytosis,[7,8] apoptosis[9] and regulation of cellular growth,[10] as

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well as proposed functions in pathological conditions such as cataracts,[11] cerebral ischemia[12] and atherosclerotic lesions.[13] Accordingly, a need exists for potent and specific inactivators of transglutaminases to allow for a better understanding of both the physiological and pathological role of this class of enzymes.

A critical intermediate in transglutaminase-mediated catalysis is a thioester acyl-enzyme formed in the rate limiting step. Primary amines, including monodansyl-cadaverine[14] or phenylthiourea derivatives of α,ω-diaminoalkanes[15], serve as competitive inhibitors of protein cross-linking by intercepting the thioester intermediate. The active site thiol has been the target of a variety of affinity labelling agents including halomethylketones,[16,17] diazomethylketones,[7] and isocyanates.[18] More recently, we have described the mechanism-based, irreversible inactivation of bovine epidermal transglutaminase by a novel series of 3-halo-4,5-dihydroisoxazoles.[19]

Peptidyl sulfonium ylides were recently introduced as a new class of affinity labelling agents by Shaw for the potent inactivation of the cysteine proteinases cathepsin B and papain.[20] Since these cysteine proteinases catalyze hydrolysis of peptidyl substrates via a thioester intermediate, presumably analogous to the intermediate in transglutaminase catalysis, we investigated the potential utility of the sulfonium ylide functionality in the design of transglutaminase inactivators. In this paper, we describe the preparation and kinetic characterization of sulfonium salts that are potent and selective inactivators of transglutaminases, in particular, the bovine epidermal transglutaminase. The inhibitory effects of these compounds on the process of ionophore-induced cross-linked envelope formation by human malignant keratinocytes are also reported.

METHODS AND MATERIALS

Enzymes

Epidermal transglutaminase was isolated from lyophilized bovine snout by homogenization (8000 × g, 30 min) in a Waring blender and chromatography of the supernatant on DEAE-Sepharose CL-6B in 5 mM Tris buffer, pH 7.5, containing 1 mM EDTA (Buffer A), with elution at 0.2 M NaCl of a 0 to 0.7 M NaCl gradient in Buffer A. Fractions containing transglutaminase activity were pooled, dialyzed against Buffer A, and concentrated by Amicon filtration. A crude extract of guinea pig liver transglutaminase was prepared by the method of Folk and Chung[21] and partial purification by chromatography on DEAE-Sepharose CL-6B was performed with active fractions eluting between 0.4 and 0.5 M NaCl of a 0 to 1 M NaCl gradient in Buffer A. Cathepsin B was purified from bovine spleen by the procedure of Bajkowski and Frankfater.[22]

Stock solutions of inhibitors were prepared in Me_2SO.

Continuous Assay for Epidermal Transglutaminase Activity

A fluorometric assay[23] reported for the continuous monitoring of Factor XIIIa activity was modified to permit continuous monitoring of epidermal transglutaminase-catalyzed incorporation of monodansylcadaverine into dimethylcasein. Fluorescence measurements were made on a Perkin-Elmer 650-40 fluorescence spectrophotometer, with $\lambda_{ex} = 360$ nm and $\lambda_{em} = 500$ nm. Two ml of assay buffer (50 mM TRIS, pH 8.1,

containing 10 mM calcium chloride, 5% Me_2SO, 0.5 mM dithiothreitol and 25 μM monodansylcadaverine) were thermostatted at 37°C. Enzyme (5 to 20 μl of a stock solution, sufficient to give an uninhibited rate of approximately 0.1 units of fluorescence per minute) and 5 μl of 1% dimethylcasein, were introduced to initiate the reaction. After the initial uninhibited rate of transglutaminase activity was recorded, inhibitor (0.5 to 20 μl of a stock solution in Me_2SO) was added and fluorescence monitoring continued, typically for an additional 10 to 20 min.

Discontinuous HPLC Assay for Transglutaminase Activity

For certain experiments, a discontinuous HPLC assay was used to monitor the inhibition of epidermal transglutaminase. Enzyme was preincubated at 37°C in 50 mM TRIS at pH 8.1 containing 10 mM calcium chloride, 0.74 mM dithiothreitol, 1.48 mM monodansylcadaverine and 5% Me_2SO, with or without inhibitor, in a total volume of 150 μl. After an appropriate preincubation interval, 50 μl of 40 mM Cbz–Gln–Gly–OH (Sigma) was added, and the reaction was quenched after 30 min by addition of 80 μl of a solution of 100 mM EDTA containing 40 μM dansyl-glycyl-tryptophan (Sigma), an internal standard. Samples were diluted with 120 μl of 0.3% hydrochloric acid and analyzed by HPLC on a 25 cm Ultrasphere ODS 5 μm column. Gradient elution with 30 mM ammonium acetate, pH 4.5/acetonitrile mixtures, (64:36) to (35:65) over 15 min at 1 ml/min, provided baseline resolution of dansylated peptide product and internal standard. Product peaks were detected fluorometrically with a Kratos FS 950 Fluoromat.

Liver Transglutaminase Assays

Guinea pig liver transglutaminase stock solutions were degassed, maintained at 25°C under nitrogen and enzyme was activated with 5 mM calcium chloride and 1 mM dithiothreitol prior to activity measurements. The activity of liver transglutaminase was assayed by measurement of the rate of incorporation of hydroxylamine into Cbz–Gln–Gly–OH according to the method of Folk and Chung.[21] For studies of time-dependent inactivation, 140 μl of activated liver transglutaminase and 7.4 μl of an inhibitor stock solution (0.2 to 2 mM) were incubated at 25°C. An uninhibited control with 7.4 μl Me_2SO was also prepared. After an appropriate incubation time, 20 μl aliquots were withdrawn and transferred to Eppendorf tubes containing 200 μl of assay medium (250 mM TRIS-acetate, pH 6.0 containing 18.8 mM Cbz–Gln–Gly–OH, 6.25 mM calcium chloride, 1.25 mM EDTA, 125 mM hydroxylamine and 1.25 mM dithiothreitol) and 30 μl distilled water. The concentration of product formed after 15 min at 37°C was determined by quenching with 250 μl of acidic ferric chloride reagent[21] and measuring the absorbance at 525 nm. Typical uninhibited samples provided a final concentration of product of approximately 0.75 mM.

Gel Filtration Experiments

To test for irreversible inhibition, epidermal transglutaminase, inactivated with 50 μM Cbz–Phe–$NH(CH_2)_5COCH_2S^+(CH_3)_2$ at pH 8.1 in the presence of 10 mM calcium chloride and 1 mM dithiothreitol, and uninhibited enzyme were chromatographed on separate Sephadex G-25M columns (PD-10, Pharmacia). Enzyme was eluted with 50 mM TRIS, pH 8.1, and fractions were monitored for transglutaminase activity by the continuous fluorometric assay.

Stability Assays

To examine the stability of sulfonium methylketones under ambient conditions, the Cbz–Phe–NH$(CH_2)_5COCH_2S^+(CH_3)_2$ analog (0.1 μM) was incubated in the absence or presence of 1 mM glutathione using 0.1 M TRIS buffers, pH 6–9, and 0.1 M CAPS buffers, pH 10 and 11. Samples were incubated at 20°C and analyzed at intervals by HPLC on a 3 × 3C Pecosphere RP C-18 column (Perkin Elmer). A linear gradient from 20 mM aqueous phosphate, pH 7.5/acetonitrile (1:1) to 100% acetonitrile over 4 min at 2 ml/min was used and eluant was monitored by UV detection at 255 nm.

Cell Assays

The assay for cross-linked envelope formation competence by a human malignant keratinocyte cell line, SCC-9 (squamous cell carcinoma), was based on the procedure of Rice and Green[24] and has been reported in detail.[25]

Inhibition of Cathepsin B

A continuous fluorometric assay was used to monitor the cathepsin B catalyzed hydrolysis of 7-(benzyloxycarbonylphenylalanyl-arginyl)-4-methylcoumarinamide (Peninsula Laboratories, San Carlos, CA). The second-order rate constants, k/K, for inactivation of cathepsin B at pH 6.0, 25°C were determined by methods described by Smith *et al.*[26]

Syntheses

The sulfonium methylketone inhibitors were synthesized via the alkylation of dialkylsulfides by bromomethylketones in a manner similar[37] to that reported by Shaw.[20] In the general synthetic sequence, Cbz-protected phenylalaninamido alkanoic acid precursors, prepared by carbodiimide coupling of an N-protected amino acid with an ω-amino alkanoic acid, were converted to the corresponding bromomethylketones via the intermediate diazomethylketones using procedures developed by Shaw and coworkers.[27,28] Displacement of the bromide by dimethylsulfide in the presence of silver tetrafluoroborate provided the tetrafluoroborate salts directly. The following examples are representative.

Synthesis of Phenylalaninamido alkanoic acids. Cbz–(L)Phe–NH$(CH_2)_4COOH$ was obtained by treating a solution of freshly prepared N-benzyloxycarbonyl-L-phenylalanine N′-hydroxysuccinimide ester (0.34 g) in THF (20 ml) with 5-aminopentanoic acid (0.08 g), water (20 ml) and triethylamine (0.24 ml). After stirring at room temperature for 4 h, the reaction mixture was diluted with ethyl acetate (250 ml) and 5% HCl (25 ml). The organic layer was separated, washed with brine (3 ×), dried (anhydrous $MgSO_4$) and rotary evaporated to an amorphous solid. Recrystallization from ethyl acetate yielded the title compound as a white solid (0.4 g, quantitative). Anal. calcd. for $C_{22}H_{26}N_2O_5$: C, 66.32; H, 6.58; N, 7.03. Found: C, 66.42; H, 6.66; N, 7.27%. The IR and NMR were in agreement with the assigned structure.

The Cbz–(L)Phe–NH$(CH_2)_5COOH$ (9.75 g, 71%) was obtained from active ester and 6-aminohexanoic acid (4.39 g, 33.5 mmol) by the manner described above.

Anal. calcd. for $C_{23}H_{28}N_2O_5$: C, 66.97; H, 6.84; N, 6.79. Found: C, 66.95; H, 6.83; N, 6.72%. The IR and NMR were in agreement with the assigned structure.

Synthesis of Bromomethylketones. Cbz-(L)Phe-$NHCH_2COCH_2Br$:Cbz-(L)Phe-Gly (0.5 g) was dissolved in dry THF (25 ml), blanketed with argon and cooled in a dry ice/acetone bath. The solution was treated with N-methylmorpholine (0.171 ml), followed dropwise by isobutylchloroformate (0.292 ml) and then stirred further for 15 minutes. The resulting suspension was treated with diazomethane in ether (0.3 M, 20 ml) and warmed to room temperature overnight. A 1:1 solution of 48% HBr/HOAc (3 ml) was added dropwise and vigorously stirred for 15 min. The reaction mixture was diluted with ethyl acetate (100 ml) and the organic fraction washed with brine (25 ml), saturated sodium bicarbonate (25 ml), 5% aqueous sodium thiosulfate (25 ml), dried (sodium sulfate) and rotary evaporated. The residue was purified further by silica gel chromatography (ethyl acetate/hexane, 1:1) to yield the title compound as a white solid (0.39 g, 64%). M.p. 96.5–97.5°C; anal. calcd. for $C_{20}H_{21}N_2O_4Br$: C, 55.44; H, 4.89; N, 6.47. Found: C, 55.72; H, 4.84; N, 6.88%. The IR and NMR were in agreement with the assigned structure.

The following compounds were obtained in the manner described above: Cbz-(L)Phe-$NH(CH_2)_2COCH_2Br$ (2.2 g, 71%), from Cbz-(L)Phe-$NH(CH_2)_2COOH$ (2.57 g); m.p. 100–102°C; $[\alpha]_D$ −0.92 (c = 0.65, $CHCl_3$); anal. calcd. for $C_{21}H_{23}N_2O_4Br$: C, 56.39; H, 5.22; N, 6.26. Found: C, 56.28; H, 5.22; N, 6.21%. Cbz-(L)Phe-$NH(CH_2)_3COCH_2Br$ (0.29 g, 24%) from Cbz-(L)Phe-$NH(CH_2)_3COOH$ (1.0 g); m.p. 129.5–132°C; anal. calcd. for $C_{22}H_{25}N_2O_4Br$: C, 57.27; H, 5.46; N, 6.07. Found: C, 57.24; H, 5.47; N, 6.07%. The IR and NMR were in agreement with the assigned structure.

Cbz-(L)Phe-$NH(CH_2)_4COCH_2Br$ (0.10 g, 20%) from Cbz-(L)Phe-$NH(CH_2)_4COOH$ (0.40 g); this material was purified further by HPLC chromatography (Whatman column, 30 mm × 50 cm Magnum, 10 μm silica gel, eluting with EtOAc/hexane (1:1): m.p. 123–124°C; anal. calcd. for $C_{23}H_{27}N_2O_4Br$: C, 58.11; H, 5.73; N, 5.89. Found: C, 58.76; H, 5.83; N, 5.71%. The IR and NMR were in agreement with the assigned structure.

Synthesis of Sulfonium Bromides. Cbz-(L)Phe-$CH_2S^+(CH_3)_2$ bromide:- Cbz-(L)Phe-CH_2Br (0.95 g, 2.52 mM) was treated with a minimal amount of acetone to attain solution. Dimethyl sulfide (0.40 ml, 5.45 mM) was added and the solution was left stoppered overnight at room temperature. The resultant white precipitate was collected, washed with cold acetone (10 ml) and ether (100 ml) to yield N-benzyloxycarbonyl-L-phenylalanylmethyl dimethyl sulfonium bromide (0.88 g, 82%) m.p. 127–128°C (lit.[20] m.p. 123–124°C); $[\alpha]_D$ −55.7 (c 1.36, DMSO); anal. calcd. for $C_{20}H_{24}NO_3SBr$: C, 54.79; H, 5.52; N, 3.20. Found: C, 54.74; H, 5.52; N, 3.17%. The IR and NMR were in agreement with the assigned structure.

In the manner described above were obtained: Cbz-(L)Phe-$NH(CH_2)_3COCH_2S^+(CH_3)_2$ bromide (1.85 g, 78%) from Cbz-(L)Phe-$NH(CH_2)_3COCH_2Br$ (2.09 g); m.p. 118–120°C; anal. calcd. for $C_{24}H_{31}H_2O_4SBr$: C, 55.07; H, 5.97; N, 5.35. Found: C, 54.65; H, 6.26; N, 5.07%. The IR and NMR were in agreement with the assigned structure.

Cbz-(L)Phe-$NH(CH_2)_5COCH_2S^+(CH_3)_2$ bromide (1.47 g, 65%) from Cbz-(L)Phe-$NH(CH_2)_5COCH_2Br$ (2.00 g): m.p. 105–107°C; anal. calcd. for $C_{26}H_{35}N_2O_4SBr$: C, 56.62; H, 6.40; N, 5.08. Found: C, 56.75; H, 6.21; N, 4.77%. The IR and NMR were in agreement with the assigned structure.

Synthesis of Sulfonium Tetrafluoroborates. Cbz-(L)Phe-$NH(CH_2)_2COCH_2S^+(CH_3)_2$ tetrafluoroborate: silver tetrafluoroborate (0.065 g) was dissolved in dry acetone (10 ml), blanketed with argon and cooled in a dry ice/acetone bath. Cbz-(L)Phe-$NH(CH_2)_2COCH_2Br$ (0.15) and dimethylsulfide (0.20 ml) were added and the reaction was stirred overnight in the dark at room temperature. The precipitated silver bromide was filtered off and the cake was washed with methylene chloride. The combined filtrates were evaporated to dryness, redissolved (acetone, 10 ml) and precipitated with ether (50 ml) to yield a white solid (0.11 g, 74%): m.p. 130–131°C; anal. calcd. for $C_{23}H_{29}N_2O_4SBF_4$: C, 53.50; H, 5.66; N, 5.43. Found: C, 53.32; H, 5.59; N, 5.64%. The IR and NMR were in agreement with the assigned structure.

Cbz-(L)Phe-$NH(CH_2)_4COCH_2S^+(CH_3)_2$ tetrafluoroborate from Cbz-(L)Phe-$NH(CH_2)_4COCH_2Br$ was obtained in the manner described. The solid was triturated with ethyl acetate/ether (1:3) to yield a white solid: m.p. 135.5–136.5°C; anal. calcd. for $C_{25}H_{33}N_2O_4SBF_4$: C, 55.16; H, 6.11; N, 5.15. Found: C, 55.39; H, 6.10; N, 5.15%. The IR and NMR were in agreement with the assigned structure.

RESULTS AND DISCUSSION

Transglutaminase Inactivation by Sulfonium Methylketones

Epidermal transglutaminase activity was followed by continuous monitoring of the increase in fluorescence associated with the transglutaminase-catalyzed incorporation of monodansylcadaverine (25 μM) into dimethylcasein. For concentrations of dimethylcasein ranging from 0.25 μM to 10 μM, saturation kinetics were determined with an observed $K_m = 0.88$ μM at pH 8.1 and 37°C. For inactivation studies, the concentration of the glutaminyl donor, dimethylcasein, was fixed at 1.04 μM, slightly greater than the apparent K_m. This concentration of substrate provided linear plots of fluorescence vs. time for the uninhibited control reaction for up to 60 min and, more significantly, it prevented very rapid inactivation by competitively blocking the inhibitor-enzyme interaction. This allowed more convenient and accurate determination of the kinetic rate constants.

Rapid, time-dependent inactivation of epidermal transglutaminase was observed with all five homologs of the general structure Cbz-Phe-$NH(CH_2)_nCOCH_2S^+(CH_3)_2$, where n = 1 through 5. The first-order rate constants at five or more concentrations of inhibitor were determined. Typically, the progress curves (Figure 1) were monitored for at least 5 half-lives of decay and were analyzed by non-linear regression to the exponential equation Fluorescence = $Ae^{-(k_{obs}t)}$, to obtain k_{obs}, the pseudo first-order rate constants. Enzyme inactivation by the sulfonium methylketones was rapid and irreversible with no recovery of activity upon dilution into saturating substrate or after gel permeation chromatography.

For inhibitors wherein n = 1, 2 or 5, plots of k_{obs} versus [I] were linear and second-order rate constants were calculated by linear regression to the equation,

$$k_{obs} = \frac{k_{i(app)}}{K_{i(app)}}[I] \quad (1)$$

The apparent second-order rate constants, $k_{i(app)}/K_{i(app)}$, reported in Table I reflect

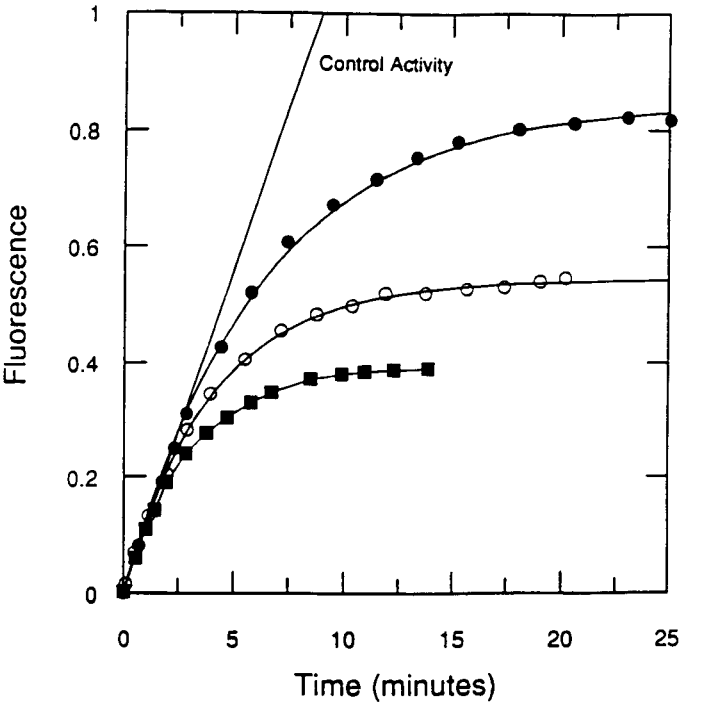

FIGURE 1 Progress curves for the time-dependent inactivation of bovine epidermal transglutaminase by Cbz–Phe–$NH(CH_2)_3COCH_2S^+(CH_3)_2$. Inhibitor concentrations were: ●, 50×10^{-9} M; ○, 100×10^{-9} M; and ■, 150×10^{-9} M in 50 mM TRIS, pH 8.1, containing 5% DMSO, 0.5 mM dithiothreitol, 10 mM calcium chloride, 1.04 μM dimethylcasein and 25 μM monodansylcadaverine at 37°C.

TABLE I
Sulfonium methylketones as inactivators of transglutaminases and as inhibitors of cross-linked envelope formation

Inhibitor	Inactivation rate constants[a] (M^{-1} min^{-1})		Envelope inhibition IC_{50} (μM)
	Epidermal TG	Liver TG	
Cbz–Phe–$NH(CH_2)_nCOCH_2S^+(CH_3)_2$			
(**1**) n = 1	14,000	1,000	N.I.[c]
(**2**) n = 2	1,200,000	15,400	
(**3**) n = 3	3,100,000	29,500	30
(**4**) n = 4	680,000	19,000	
(**5**) n = 5	340,000	4,500	10
(**6**) Cbz–Phe–$CH_2S^+(CH_3)_2$	230	N.T.D.[b]	N.I.[c]

[a]The second-order rate constants for inactivation of epidermal transglutaminase, $k_{i(app)}/K_{i(app)}$ and liver transglutaminase, k/[I] are discussed in detail under "Results and Discussion". [b](N.T.D.) No time-dependent inhibition was observed at 100 μM inhibitor. [c](N.I.) No inhibition of cornified envelope formation was observed at 100 μM inhibitor.

the partial protection from inactivation resulting from the presence of the glutaminyl substrate at $[S]/K_m > 1$. This was demonstrated by additional determinations for the n = 5 analog which revealed that the second-order rate constant, $k_{obs}/[I]$ decreased five-fold, from 428,000 $M^{-1}\,min^{-1}$ to 96,000 $M^{-1}\,min^{-1}$, as the concentration of dimethylcasein increased from 0.52 μM to 10.4 μM.

For inhibitors with n = 3 or 4, plots of the pseudo first-order rate constants, k_{obs}, versus [I] showed saturation kinetics and plots for these inhibitors were analyzed by non-linear regression to the equation

$$k_{obs} = \frac{k_{i(app)}[I]}{(K_{i(app)} + [I])} \tag{2}$$

to obtain the apparent second order rate constants reported in Table I.

The evaluation of K_i and k_i, the intrinsic microscopic rate constants for competitive irreversible inhibition, by a potent inhibitor in the presence of substrate has recently been addressed by Kovach.[29] The expression derived for the determination of the second-order (k_i/K_i) and first-order (k_i) rate constants was equation (3):

$$1/k_{obs} = 1/k_i + \{K_i/(k_i[I_0])\}\{1 + [S_0]/K_m\} \tag{3}$$

For an analysis at inhibitor concentrations around K_i, a plot of $1/k_{obs}$ versus $1/[I_0]$ provides $1/k_i$ as the intercept and $(1 + [S_0]/K_m)K_i/k_i$ as slope. In our assay, $[S_0]/K_m = 1.16$, and the inactivation by Cbz–Phe–$NH(CH_2)_3COCH_2S^+(CH_3)_2$ (**3**) was characterized by $K_i = 150$ nM and $k_i = 1.0\ min^{-1}$ while the n = 4 analog inactivates with a marginally faster limiting first order rate constant, $k_i = 1.7\ min^{-1}$, but K_i was eight fold higher at 1.2 μM.

The second-order rate constants, $k/[I]$, for inactivation of guinea pig liver transglutaminase reported in Table I, represent the mean of the observations at two or three concentrations of inhibitor. The observed second-order rate constants against liver transglutaminase are at least one, and more frequently two orders of magnitude slower than the $k_{i(app)}/K_{i(app)}$ for inactivation of epidermal transglutaminase. The γ-aminobutyric acid analog (n = 3), was the most potent inhibitor, $k/[I] = 29{,}500\ M^{-1}\,min^{-1}$, as found for the epidermal enzyme.

The ylides of β-keto sulfonium salts are resonance stabilized, and Shaw[20] noted that the ylide of these inactivators could be the potentially active species. This followed from the observation that the second-order rate constant for inactivation of cathepsin B by Cbz–Phe–$CH_2S^+(CH_3)_2$ (**6**) increased 4.8-fold as the pH of the preincubation mixture increased from 5.4 to 7.0 while the rates for inactivation of cathepsin B by diazomethylketones were independent of pH over the range 5.4 to 7.0. The pK_a for ylide formation for (**6**) was found to be approximately 7.2. In our studies with Cbz–Phe–$NH(CH_2)_3COCH_2S^+(CH_3)_2$ (**3**), the inactivation of epidermal transglutaminase increased almost ten-fold as the pH of the assay increased from 7.6 to 8.5 (Table II), a range where the predominant form of the inhibitor should be the ylide. The interpretation of the pH effect is complicated by a two-fold increase in enzymatic activity over the same pH range. In addition, the rate constant for inactivation by a 3-halo-4,5-dihydroisoxazole analog, RS 10823,[25] which has no ionizable groups in this range, increased three fold over this pH range (Table II). Although it is possible that the enhanced potency of the sulfonium methylketone at higher pH reflects preferential binding of the ylide, pH effects on other rate-determining steps may be responsible for the pH dependencies.

TABLE II
Activity of epidermal transglutaminase and second-order rate constants for inactivation as a function of pH

pH	Relative activity[b]	k/[I] (M^{-1} min^{-1})	
		Sulfonium salt (**5**)	Halodihydroisoxazole (**10**)
7.5	0.6	0.6×10^6	0.5×10^5
8.1	1.0	3.1×10^6	0.9×10^5
8.5	1.4	5.0×10^6	1.6×10^5

[a]Enzymatic activity and second-order rate constants for inactivation by the sulfonium methylketone, Cbz–Phe–$NH(CH_2)_5COCH_2S^+(CH_3)_2$ (**5**), and by 2-(1-benzyloxymethamido)-N-[(3-halo-4,5-dihydroisoxazol-5-yl)methyl]-3-(4-hydroxyphenyl)propanamide (RS 10823 (**10**)), were determined by the continuous fluorometric assay as described under "Methods". [b]Activity relative to enzymatic activity at pH 8.1.

Chemical Stability of Inhibitors

The stability of 0.1 mM Cbz–Phe–$NH(CH_2)_5COCH_2S^+(CH_3)_2$ (**5**) in the presence or absence of 1 mM glutathione, at pH 6.0 to 11, was monitored by reverse phase HPLC analysis of incubation mixtures. At ambient (25°C) temperature, the sulfonium methylketone was stable for 18 h at pH < 9, and only 20–25% of the inhibitor was consumed after 18 h at pH = 9 or 10 in the presence of 1 mM glutathione. The lack of reactivity of these β-keto sulfonium salts with thiol reagents at pH 7.5 to 8.5 contrasts dramatically with the potent and very rapid time-dependent inactivation of transglutaminases observed in this pH range.

Inactivation of Epidermal Transglutaminase by Bromomethylketone Analogs

Bromomethylketones are very effective alkylators of both cysteine proteinases[30] and transglutaminases.[16,17] To ensure that the sulfonium methylketone effects cited above were devoid of any contribution from contaminating concentrations of bromomethylketone precursor or from halide exchange of the sulfonium bromide salt, the following precautions were followed and experiments undertaken. Dithiothreitol, at 5 mM concentration, was routinely present in the assay mixtures to scavenge bromomethylketone and most inhibitors were prepared as the tetrafluoroborate salts. In addition, the bromomethylketone precursors were isolated and evaluated as inhibitors of transglutaminases. As shown in Figure 2, the first-order, time-dependent inactivation of epidermal transglutaminase by Cbz–Phe–$NH(CH_2)_3COCH_2S^+(CH_3)_2$ (**3**) at 20 nM was characterized by a half-life for inactivation of 13 min. In contrast, no time-dependent inactivation was observed over 30 min preincubation at 2 μM (100 fold excess) of Cbz–Phe–$NH(CH_2)_3COCH_2Br$. In all cases tested, the highly reactive bromomethylketones showed dose-dependent but time-independent inhibition at concentrations 10 to 100-fold greater than the concentrations required for rapid, time-dependent inactivation by the sulfonium methylketone analog. These results provide evidence that any contaminating concentrations of bromomethylketone precursors cannot mediate the very potent, time-dependent inactivation summarized in Table I.

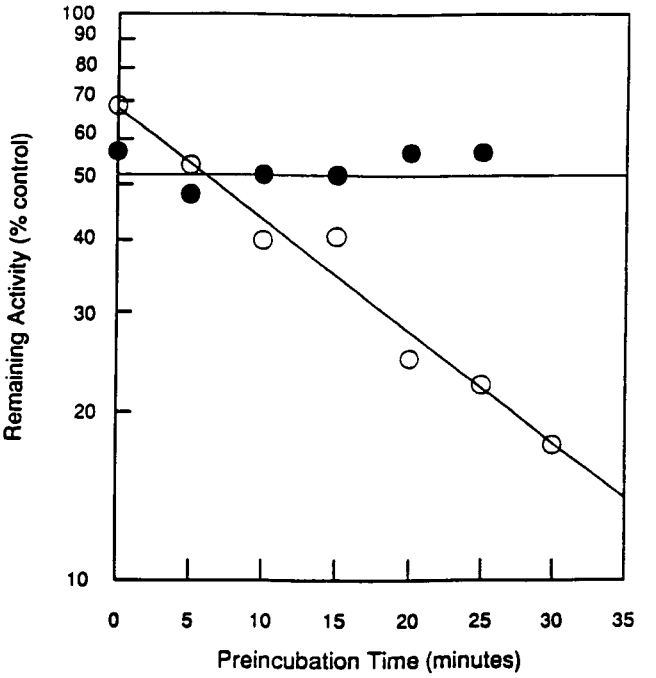

FIGURE 2 Comparison of time-dependence for the inhibition of bovine epidermal transglutaminase by the inhibitors Cbz–Phe–$NH(CH_2)_5COCH_2$–X where X = Br or $S(CH_3)_2$. Inhibitor concentrations were 2×10^{-6} M for the bromomethylketone (●) and 2×10^{-8} M for the sulfonium salt (○) in 50 mM TRIS, pH 8.1 containing 10 mM calcium chloride, 1.48 mM monodansylcadaverine, 0.74 mM dithiothreitol and 5% Me_2SO. Residual transglutaminase activity was monitored by the discontinuous HPLC assay, as described under "Methods".

Specificity of Inhibitors

The sulfonium methylketone Cbz–Phe–$CH_2S^+(CH_3)_2$ (**6**) is a time-dependent inactivator[20] of the cysteine proteinases cathepsin B, $k_i/K_i = 494\ M^{-1}\ min^{-1}$, and papain, $k_i/K_i = 1{,}900\ M^{-1}\ min^{-1}$. We observed that this analog inactivated the epidermal transglutaminase with comparable potency, $k/[I] = 229\ M^{-1}\ min^{-1}$, but no time-dependent inactivation of the liver enzyme was observed at 100 μM inhibitor. The simple sulfonium methylketone is not a powerful inactivator of the thiol proteinases or transglutaminases, and the observed inhibition may be the result of slow, non-specific methyl transfer from the sulfonium salts as observed by Shaw for the non-enzymatic transfer of radiolabelled methyl groups from Cbz–Phe–Ala–$CH_2S^+(CH_3)_2$ (**7**) to bovine serum albumin.[20]

The insertion of $-(CH_2)-$ spacers in the general structure Cbz–Phe–$NH(CH_2)_nCOCH_2S^+(CH_3)_2$, provides inhibitors of both the thiol proteinases and transglutaminases, however the length of the spacer defines the specificity for either class of enzymes. Similar to the previously reported Cbz–Phe–Ala–$CH_2S^+(CH_3)_2$,[20] the dipeptidyl Cbz–Phe–Gly–$CH_2S^+(CH_3)_2$ (**8**) was a very potent cysteine proteinase inactivator, $k/K > 300{,}000\ M^{-1}\ min^{-1}$ against both cathepsin B and

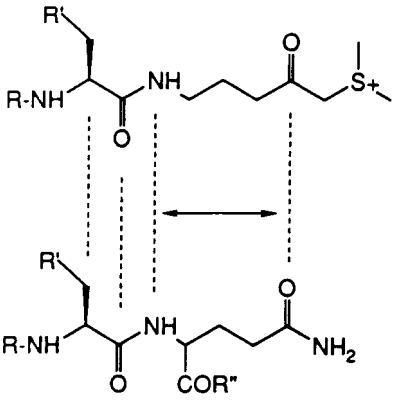

FIGURE 3 Structural homology of the most potent sulfonium methylketone inactivator, the γ-aminobutyric acid analog (n = 3), with glutaminyl substrates.

papain. Only modest inactivation rates of transglutaminases by this dipeptidyl analog were observed (Table I). Impressive specificity for the transglutaminases was achieved with the higher homologs of these ω-aminoacyl derivatives. Sulfonium methylketones derived from the β-alanine spacer (n = 2) and the γ-aminobutyric acid spacer (n = 3), afford exceptionally potent inhibitors of bovine epidermal transglutaminase, with second-order rate constants greater than 1×10^6 M^{-1} min^{-1} and represent the most potent inhibitors that have been reported for this epidermal enzyme. No time-dependent inactivation of cathepsin B by these analogs, at concentrations as high as 20 μM, was observed. Decreases in potency against both liver and epidermal transglutaminases were observed upon extension of the spacer to n = 4 or 5. The results in Table I clearly demonstrate that the length of the spacer between the Cbz–Phe moiety and the carbonyl of the sulfonium methylketone is critical for both potency and selectivity of transglutaminase inactivators.

Assuming that the ω-aminoacyl moieties of these inhibitors bind to the same enzyme subsite as the glutaminyl side chain of substrates, then the specificity for the γ-aminobutyryl analog is the consequence of optimal complementarity to the enzyme (Figure 3). This assumption is supported by the observations that the glutaminyl substrate, dimethylcasein, competitively inhibits the inactivation whereas the presence of 1 mM monodansylcadaverine, an amine substrate with apparent $K_m = 0.12$ mM for bovine epidermal transglutaminase,[31] did not protect the epidermal enzyme from rapid, time-dependent inactivation by sulfonium methylketones (Figure 2). Although these inhibitors have no α-carboxyl moiety strictly analogous to glutaminyl substrates, Folk and Gross[16] have established that the α-carboxyl portion of the glutamine substrate is not essential given that Nα(2-hydroxy-5-nitrophenylacetyl)-4-amino-butyramide (**9**) serves as a substrate for guinea pig liver transglutaminase.

Mechanistic Considerations

Using a radioactive label in the peptidyl moiety or in the methyl groups of the trialkyl sulfonium salt, Shaw[20] determined that only the peptidyl-labelled form of the inhibitor transferred significant radioactivity to inactivated cathepsin B. The structure of the

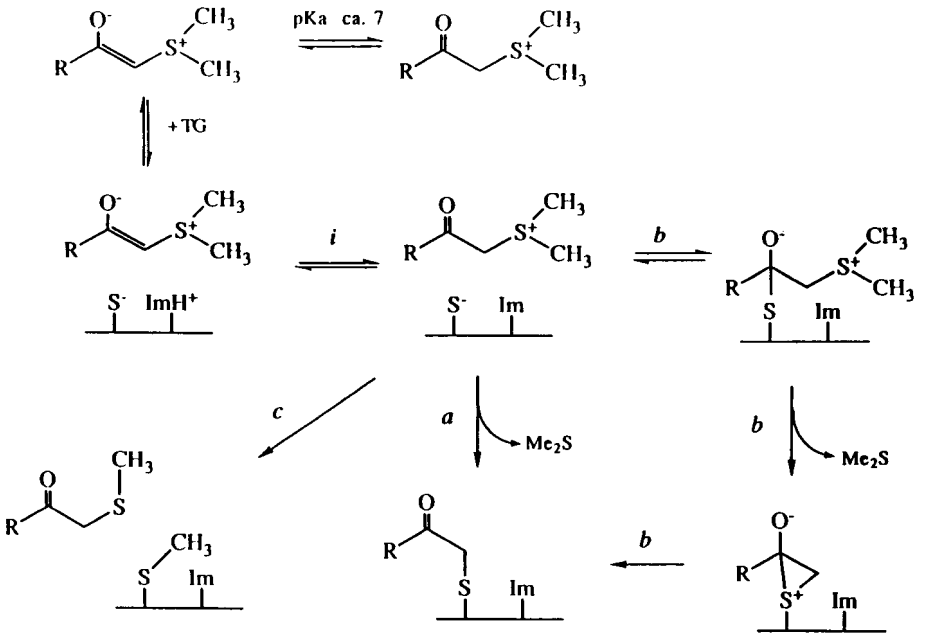

FIGURE 4 Proposed mechanisms for inactivation of transglutaminases by sulfonium methylketones.

presumed thioether adduct would thus be analogous to that obtained upon inactivation of thiol proteinases by other classes of affinity-labelling inhibitors, including chloromethylketones[32] and acyloxymethylketones[26] for which structures have been unequivocally assigned.

The active site cysteine residues of transglutaminases are located in a highly conserved consensus sequence, YGQCWVFA, homologous with the active site domain of mammalian cysteine proteinases.[33–35] In cysteine proteinases, the active site residue is a thiolate stabilized by formation of an intimate thiolate-imidazolium ion pair. Although no consensus sequence for an active site imidazole in transglutaminases has been identified, the presence of a thiolate-imidazolium ion pair in the active site could conceivably participate in the mechanism-based inactivation of the transglutaminases by mechanisms outlined in Figure 4. Assuming that the transglutaminases initially bind the resonance stabilized ylide, for reasons stated above, protonation by a presumed active site imidazolium (step *i*, Figure 4) would generate the enzyme bound β-keto sulfonium salt. Inactivation of transglutaminase may then occur by, (*a*) direct displacement of dimethylsulfide, (*b*) initial hemithioketal formation followed by the formation of an episulfonium ion intermediate or transition state which then provides the final alkylated enzyme, or (*c*) transfer of a methyl group from the trialkylsulfonium salt. A common pathway for inactivation by all ω-aminoacyl analogs may not be operative and different pathways may be followed depending on the length of the ω-aminoacyl spacer group. Brocklehurst and Malthouse[66] have proposed mechanisms, analogous to pathways *a* and *b*, for the inactivation of thiol proteinases by resonance stabilized diazomethylketones, but with hemithioketal formation preceding protonation of the resonance stabilized diazonium.

Inhibition of Keratinocyte Cross-linked Envelope Formation

The apparent stability of sulfonium methylketones make them suitable candidates for the study of enzyme inhibition in cellular environments. The transglutaminase-mediated process of cross-linked envelope formation in ionophore treated human SCC-9 malignant keratinocytes was used as a model to study the cellular effects of sulfonium methylketones. No inhibition of envelope formation was observed with either Cbz–Phe–$CH_2S^+(CH_3)_2$ (**6**) or the Cbz–Phe–Gly–$CH_2S^+(CH_3)_2$ (**8**) at concentrations up to 100 μM when preincubated with the cells for 15 min prior to ionophore (X537A, 50 μg/mL) treatment. In contrast, the more potent *in vitro* inactivators, **3** and **5**, produced dose-dependent inhibition of cross-linked envelope formation with IC_{50} values (Table I) within the range for the most potent *in vitro* transglutaminase inactivators of the 3-halo-dihydroisoxazole series.[25] The γ-aminobutyl sulfonium methylketone analog (**3**) described in this report is the most potent inactivator of epidermal transglutaminase reported to date with $k_{i(app)}/K_{i(app)} = 3.1 \times 10^6\ M^{-1}\ min^{-1}$. Although the *in vitro* potency of this analog is at least an order of magnitude greater than the most potent dihydroisoxazole analog previously reported,[25] RS 10823 [2-(1-benzyloxy-methamido)-N-[3-bromo-4,5-dihydroisoxazol-5-yl)-methyl]-3-(4-hydroxyphenyl)-propanamide (**10**), $k/[I] = 2.2 \times 10^5\ M^{-1}\ min^{-1}$, the *in vitro* inhibition of cross-linked envelope formation by both inhibitors was comparable, $IC_{50} \approx 30\ \mu M$. The very potent inactivation of bovine epidermal transglutaminase by the best sulfonium methylketone inhibitors, $k_i/K_i > 1 \times 10^6\ M^{-1}\ min^{-1}$, was not translated into greater potency in the cross-linked envelope assay. Differences in specificity between bovine epidermal transglutaminase and the particulate transglutaminase in human SCC-9 malignant keratinocytes or differences in the cellular bioavailability may account for the attenuation of the effects of sulfonium methylketones in the cellular assay. These differences may also be responsible for high *in vitro* potency at the cellular level for some 3-halo-4,5-dihydroisoxazole inhibitors that were only moderately potent, $k/[I] < 10^5\ M^{-1}\ min^{-1}$, against the bovine epidermal enzyme.[25]

The sulfonium methylketones described in this paper represent a novel class of highly potent inactivators of transglutaminases that are chemically inert under the ambient conditions of our *in vitro* studies. The specificity and potency of inactivation of bovine epidermal transglutaminase by these compounds can be tailored by manipulation of the length of the spacer between the amino acid recognition moiety and the sulfonium methylketone functionality thus providing a versatile class of agents for use with enzymes that utilize a thioester intermediate in catalysis.

Acknowledgements

We are indebted to W.F. Farmer and G.A. Adamson for their assistance with the enzymology and are grateful to L.J. Copp and H. LeBlanc for performing the cathepsin B assays. We thank the Natural Sciences and Engineering Research Council of Canada for Industrial Research Fellowships to H.W.P and J.J.F.K.

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37. The synthesis of sulfonium methylketones parallels that of Shaw,[20] namely alkylation of dialkyl sulfides by bromomethylketones.[27] In our approach however, bromomethylketones were prepared by direct derivatization of the precursor acids, the intermediate diazomethylketones were not isolated.[28] Our methods also differ in the preparation of sulfonium tetrafluoroborates which are again obtained by an alkylation procedure. N-protected acids were prepared by classical procedures (jbc036).