Damnacanthal Is a Highly Potent, Selective Inhibitor of p56^{lck} Tyrosine Kinase Activity

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ABSTRACT: Damnacanthal, an anthraquinone isolated from a plant extract, was found to be a potent, selective inhibitor of p56^{lck} tyrosine kinase activity. The structure, potency, and selectivity of damnacanthal were confirmed by independent synthesis and testing. Damnacanthal exhibited an IC_{50} of 17 nM for inhibition of p56^{lck} autophosphorylation and an IC_{50} of 620 nM for phosphorylation of an exogenous peptide by p56^{lck}. Damnacanthal had >100-fold selectivity for p56^{lck} over the serine/threonine kinases, protein kinase A and protein kinase C, and >40-fold selectivity for p56^{lck} over four receptor tyrosine kinases. It also demonstrated modest (7−20-fold), but highly statistically significant, selectivity for p56^{lck} over the homologous enzymes p60^{src} and p59^{fyn}. Mechanistic studies demonstrated that damnacanthal was competitive with the peptide binding site, but mixed noncompetitive with the ATP site. Although damnacanthal contains a potentially reactive aldehyde moiety, equilibrium dialysis experiments demonstrated that significant imine formation between damnacanthal and amines occurred only at high concentrations of reactants. However, damnacanthal appeared to bind nonspecifically to membrane lipids and was not active in whole cell tyrosine kinase assays. Damnacanthal is the most potent, selective inhibitor of p56^{lck} tyrosine kinase activity described to date and may represent the starting point for the identification of novel, selective inhibitors of p56^{lck} which are active in whole cell as well as in cell-free systems.

The binding of antigen to the T cell antigen receptor, along with appropriate costimulatory signals, results in the activation of antigen-specific T lymphocytes. One of the earliest detectable events during T cell activation is an increase in tyrosine kinase activity. Several studies have demonstrated that enhanced tyrosine kinase activity is critical for T cell activation (Mustelin et al., 1990; Trevillyan et al., 1990; June et al., 1990; Stanley et al., 1990; Graber et al., 1992). To date, at least four tyrosine kinases, p56^{lck}, p59^{fyn}, ZAP-70, and p72^{syk}, have been implicated as playing significant roles during T cell activation (Veillette & Davidson, 1992; Samelson & Klausner, 1992; Weiss, 1993; Couture et al., 1994). Selective inhibitors of these tyrosine kinases may have efficacy in the treatment of T cell leukemias and lymphomas, as well as in the treatment of autoimmune diseases, such as rheumatoid arthritis, in which activated T cells are an integral part of the pathogenesis of the diseases.

As part of our efforts to identify inhibitors of T cell activation, we have focused on the discovery of novel, selective inhibitors for p56^{lck}. This enzyme is expressed exclusively in cells of the lymphoid lineage, particularly T lymphocytes and natural killer cells (Bolen et al., 1992). To identify inhibitors of p56^{lck} tyrosine kinase activity, we have screened chemical files and natural product extracts. In this report, we describe that damnacanthal is a highly potent, selective inhibitor of p56^{lck} tyrosine kinase activity.

MATERIALS AND METHODS

Synthesis of Damnacanthal. Damnacanthal was synthesized as previously reported (Ayyangar et al., 1959) except that the final oxidation step was performed with tetrapropylammonium perruthenate rather than with MnO_2. This gave material which was identical to that isolated from the natural source. The structure of damnacanthal is shown in Figure 1. Analytical data are as follows: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 12.29 (s, 1 H), 10.48 (s, 1 H), 8.32 (dd, J = 1.5 and 8.0 Hz, 1 H), 8.26 (dd, J = 1.5 and 7.4 Hz, 1 H), 7.84 (ddd, J = 1.5, 7.4, and 7.4 Hz, 1 H), 7.78 (ddd, J = 1.5, 7.4, and 7.4 Hz, 1 H), 7.69 (s, 1 H), 4.13 (s, 1 H); MS (Cl, CH_4) 283 (MH^+) 100%, 254 ($MH-CHO^+$) 10%; RP-HPLC (CH_3CN/H_2O/0.1%TFA) R_{amp} 0−15 min, 20% CH_3CN to 100% CH_3CN, R_t = 11.44 min.

Assays for Protein Kinases. Autophosphorylation of p56^{lck}, erbB2, and platelet-derived growth factor receptor (PDGF-R)[1] tyrosine kinases was measured in an ELISA format, as previously described (Miller et al., 1995). The epidermal growth factor receptor (EGF-R) and the insulin receptor tyrosine kinases were assayed in a similar manner. Phosphorylation of exogenous peptides by p56^{lck}, protein kinase C (PKC), and protein kinase A (PKA) was measured as described (Wang et al., 1994; Miller et al., 1995). For

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[1] Abbreviations: PDGF-R, platelet-derived growth factor receptor; EGF-R, epidermal growth factor receptor; PKC, protein kinase C; PKA, protein kinase A; MBP-P, myelin basic protein peptide.

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p56[lck], the RR-SRC peptide (Gibco, BRL) was used in IC_{50} determinations, whereas a peptide derived from myelin basic protein (Wang et al., 1991) was used in mechanistic studies due to its greater solubility. The myelin basic protein peptide (MBP-P) utilized consisted of residues 64−73 with an arginine added at the C terminus to enable binding to phosphocellulose.

Immune complex kinase assays for p56[lck], p56[fyn], and p60[src] were performed similarly to methods previously described (Veillette & Fournel, 1990). Antibodies to peptides corresponding to unique regions in the N terminus of p56[lck] (residues 39−64) (Perlmutter et al., 1988) and p59[fyn] (residues 29−48) (Horak et al., 1989) were produced in-house in rabbits and affinity purified. A monoclonal antibody to the N terminus of p60[src] was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). The specificity of each of these antibodies for the appropriate member of the src family was demonstrated by re-precipitation experiments, confirming previous reports of the specificity of such antibodies (Veillette et al., 1988a,b; Samelson et al., 1990). Plasma membranes, which were prepared from Jurkat T leukemic cells, MOLT-3 T leukemic cells, and normal human platelets using standard procedures, were the sources of p56[lck], p59[fyn], and p60[src], respectively. The membranes were solubilized with Brij 96, and the specific enzyme was immunoprecipitated with the appropriate antibody, followed by the addition of protein A−Sepharose (Pharmacia, Piscataway, NJ). The kinase reaction was performed on the immunoprecipitated enzyme in the presence of 10 μg of acid-denatured enolase (Cooper et al., 1984) as an exogenous substrate. Other components of the kinase reaction included 10 mM $MgCl_2$, 1 mM Na_3VO_4, 20 mM MOPS, pH 7.0, and ATP as described below. The K_m for ATP for enolase phosphorylation was determined for each of the enzymes. As previously discussed for the evaluation of compound selectivity for p56[lck] over other protein kinases (Miller et al., 1995), a constant ratio of substrate concentration to K_m for the individual members of the src family was chosen to enhance the validity of conclusions about selectivity. The immune complex kinase reactions were performed with an [ATP]/K_m ratio of 2.5; i.e., with 100 μM ATP for p56[lck] and p60[src] and with 170 μM ATP for p59[fyn]. Fifty microcuries of [γ-^{32}P]ATP were added per reaction. The kinase reaction was performed for 5 min, using amounts of enzyme and enolase that were in the linear range of response. The reaction was stopped by the addition of SDS sample buffer. The samples were boiled for 5 min and then centrifuged at 10000g. The supernatants were subjected to SDS−PAGE on a 10% acrylamide gel, allowing the dye front to run off the bottom of the gel. The incorporation of $^{32}PO_4$ was visualized and quantitated by Phosphorlmager analysis (Molecular Dynamics, Sunnyvale, CA).

Mechanism for Inhibition of p56[lck] by Damnacanthal. Mechanistic studies were performed with purified recombinant human p56[lck] that had been immobilized in the wells of a microtiter plate as previously described (Wang et al., 1994). The mechanisms for inhibition of both autophosphorylation and phosphorylation of MBP-P were evaluated. For the phosphorylation of MBP-P, 1.5 mM ATP was used when the peptide concentration was varied, and 5 mM MBP-P was used when the ATP concentration was varied. The data were analyzed using methods previously described

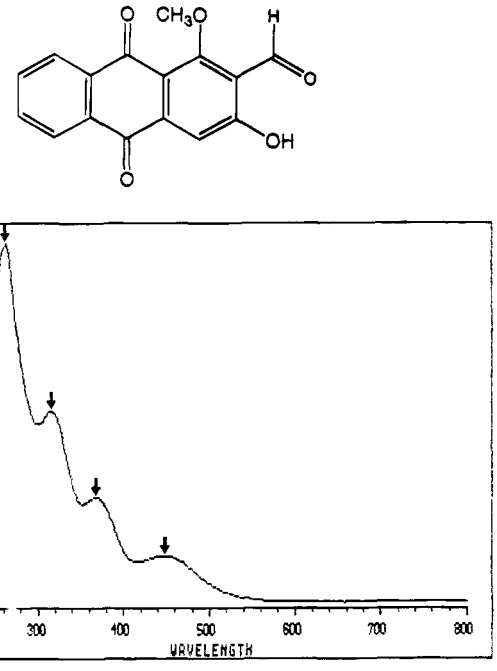

FIGURE 1: Structure and UV−Vis spectrum of damnacanthal. A 10 μM solution of damnacanthal in 0.2% DMSO was used to determine the UV−Vis spectrum.

(Faltynek et al., 1995). Initial velocity data as a function of substrate and inhibitor were fit to the three standard inhibition models, using nonlinear regression routines and the F-test to discriminate among the rival models.

Equilibrium Dialysis Experiments. To study the binding of damnacanthal to p56[lck], to poly(L-lysine), and to a control protein, equilibrium dialysis experiments were performed using a continuous flow dialysis chamber. Purified recombinant p56[lck] (Wang et al., 1994), poly(L-lysine) (MW 30 000−70 000), or ribonuclease A was placed in the closed side of the chamber, and damnacanthal was placed in the running side, which was connected to a peristaltic pump and to a flow cell in a spectrophotometer. Experiments were performed in the presence of 25 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 10 mM NaCl, 0.1% Brij 96, and 0.2% DMSO. The absorbance in the running side was monitored until it became constant, which occurred after 24−30 h in replicate experiments. At that time, the sample in the running side of the chamber was removed and the concentration of unbound damnacanthal determined spectrophotometrically by comparison with a damnacanthal standard curve. The absorption spectrum for damnacanthal is shown in Figure 1. Both A_{262} and A_{316} were used to calculate equilibrium concentrations of damnacanthal; reported concentrations are the mean values calculated using the two wavelengths.

RESULTS

Damnacanthal Is a Potent, Selective Inhibitor of p56[lck] Tyrosine Kinase Activity. As will be described in detail elsewhere, damnacanthal was identified as a potent inhibitor of p56[lck] tyrosine kinase activity through high-volume screening of natural product extracts. Damnacanthal was subsequently synthesized, as described under Materials and Methods, and the structure shown in Figure 1 was confirmed. As shown in Table 1, both natural damnacanthal and synthetic damnacanthal were potent inhibitors of the autophosphorylation of p56[lck]; the synthetic compound exhibited

Table 1: Potency and Selectivity of Damnacanthal for Inhibition of $p56^{lck}$ Tyrosine Kinase Activity

	IC_{50} (μM)	
	natural	synthetic
	Autophosphorylation	
$p56^{lck}$	0.046 ± 0.02 ($n = 15$)	0.017 ± 0.011 ($n = 5$)
PDGF-R	4.5	5.2
erbB2	2.3	0.7
insulin-R	9.5	ND[a]
EGF-R	>15	ND
	Peptide Phosphorylation	
$p56^{lck}$	0.22 ± 0.14 ($n = 13$)	0.62 ± 0.07 ($n = 4$)
PKC	>75	140
PKA	>50	75

[a] ND: not determined.

a mean IC_{50} of 17 nM. Damnacanthal also effectively inhibited the phosphorylation of an exogenous peptide by $p56^{lck}$; synthetic damnacanthal exhibited a mean IC_{50} of 620 nM for peptide phosphorylation. Minor differences (~3-fold) in IC_{50} values between natural and synthetic damnacanthal may be due to the difficulties in accurately determining concentrations of the small amount of natural compound isolated.

The selectivity of damnacanthal for inhibition of $p56^{lck}$ was examined. As previously discussed (Miller et al., 1995), autophosphorylation data were examined separately from data for phosphorylation of exogenous substrates in order to enhance the validity of conclusions about the selectivity of compounds. As shown in Table 1, damnacanthal demonstrated >100-fold selectivity for $p56^{lck}$ over the serine/threonine kinases (PKC and PKA) and >40-fold selectivity for $p56^{lck}$ over the four receptor tyrosine kinases examined.

To determine whether damnacanthal exhibited selectivity for $p56^{lck}$ over other members of the src family, immune complex kinase assays were performed using specific antibodies to $p56^{lck}$, $p59^{fyn}$, and $p60^{src}$. Addition of enolase to the immune complex kinase assays followed by SDS–PAGE enabled the evaluation of both autophosphorylation and phosphorylation of an exogenous substrate in the same experiment (Figure 2). As shown in Table 2, natural damnacanthal demonstrated moderate, but highly significant, selectivity for $p56^{lck}$ over $p59^{fyn}$ and $p60^{src}$. This selectivity occurred with both autophosphorylation and enolase phosphorylation. Experiments with synthetic damnacanthal confirmed the selectivity for $p56^{lck}$ over other members of the src family (Table 2).

Mechanism of Inhibition of $p56^{lck}$ by Damnacanthal. Both autophosphorylation and peptide phosphorylation experiments were performed to determine the mechanism for inhibition of $p56^{lck}$ by damnacanthal. Data from a representative autophosphorylation experiment are shown in Figure 3. Initial velocity data for both autophosphorylation and peptide phosphorylation by $p56^{lck}$ were fit to the three standard inhibition mechanisms: competitive, uncompetitive, and mixed noncompetitive. The parameter estimates and errors resulting from the nonlinear regression fits, as well as the results of the F-test, are given in Table 3. Analysis of both autophosphorylation and peptide phosphorylation experiments in which the ATP concentration was varied demonstrated that the data fit the mixed noncompetitive model better than the other models. In contrast, experiments in which the peptide concentration was varied demonstrated

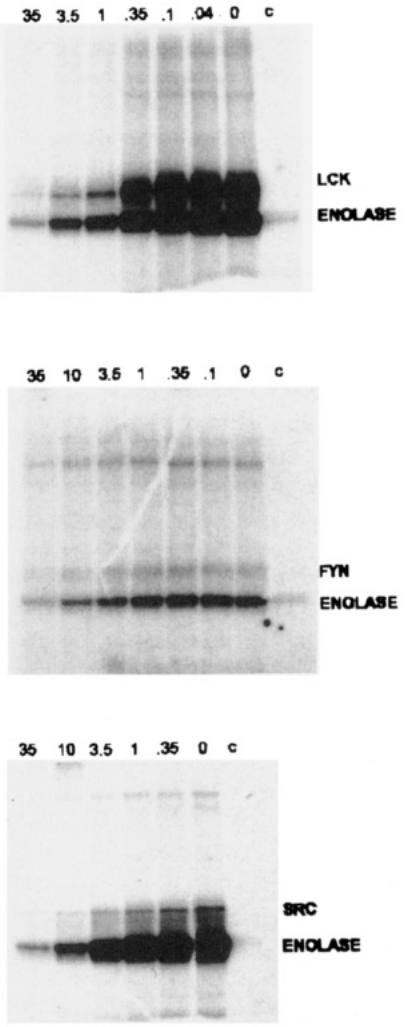

FIGURE 2: Damnacanthal is selective for $p56^{lck}$ over other members of the src family. $p56^{lck}$, $p59^{fyn}$, and $p60^{src}$ were immunoprecipitated and immune complex kinase reactions performed in the presence of the indicated micromolar concentrations of natural damnacanthal. The last lane in each gel (labeled c) represents immune complex kinase assays performed with control rabbit IgG. Both autophosphorylation and phosphorylation of the exogenous substrate enolase were examined, as described under Materials and Methods.

Table 2: Selectivity of Damnacanthal for $p56^{lck}$ over Other src Family Members

	IC_{50} (μM)	
	natural	synthetic
	Autophosphorylation	
$p56^{lck}$	0.20 ± 0.08 ($n = 7$)	0.10 ($n = 2$)
$p59^{fyn}$	2.24 ± 0.97 ($n = 5$)[a]	2.09 ($n = 2$)[b]
$p60^{src}$	1.60 ± 0.27 ($n = 6$)[a]	0.68 ($n = 2$)[b]
	Enolase Phosphorylation	
$p56^{lck}$	0.40 ± 0.30 ($n = 5$)	0.13 ($n = 2$)
$p59^{fyn}$	4.53 ± 1.86 ($n = 4$)[a]	2.24 ($n = 2$)[c]
$p60^{src}$	2.97 ± 0.49 ($n = 4$)[a]	0.90 ($n = 2$)[c]

[a] $p < 0.001$; [b] $p < 0.01$; [c] $p < 0.05$: Significance of the differences in mean IC_{50} values for $p59^{fyn}$ and $p60^{src}$ from mean IC_{50} for $p56^{lck}$, as determined by the Student's t test.

that damnacanthal was competitive with the peptide binding site (Table 3).

We examined whether the inhibition of $p56^{lck}$ by damnacanthal was reversible. $p56^{lck}$, which had been immobilized in the wells of two duplicate microtiter plates, was preincubated with varying concentrations of damnacanthal. One

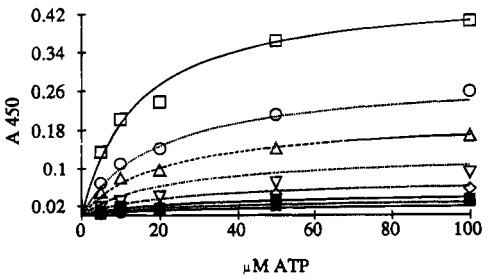

FIGURE 3: Mechanism of inhibition of p56[lck] autophosphorylation by damnacanthal. p56[lck], which had been immobilized onto the wells of a microtiter plate using specific antibodies, was preincubated without (□) or with damnacanthal at the following concentrations: 5 nM (○), 10 nM (△), 20 nM (▽), 40 nM (◇), 70 nM (■), 100 nM (●), and 150 nM (▲). ATP was added and autophosphorylation measured in an ELISA as previously described (Miller et al., 1995). Data shown are the means from duplicate reactions. Background absorbance, determined in parallel reactions performed in the absence of ATP, was subtracted from total absorbance. The curves shown are the nonlinear regression fits of the data to the mixed noncompetitive model.

Table 3: Analysis of Kinetic Data for Binding of Damnacanthal to p56[lck]

model	parameter estimates	probability[a]
(A) Autophosphorylation: ATP Binding Site		
competitive	K_m 11.4 ± 2.3 μM	2.83 × 10^{-13}
	K_{ic}[b] 1.16 ± 0.20 nM	
uncompetitive	K_m 19.7 ± 3.0 μM	3.55 × 10^{-11}
	K_{iu} 4.25 ± 0.42 nM	
mixed noncompetitive	K_m 13.8 ± 1.3 μM	(control model)
	K_{ic} 4.41 ± 0.78 nM	
	K_{iu} 7.70 ± 0.77 nM	
(B) Peptide Phosphorylation: ATP Binding Site		
competitive	K_m 331 ± 37 μM	5.71 × 10^{-8}
	K_{ic} 0.744 ± 0.103 μM	
uncompetitive	K_m 906 ± 91 μM	2.40 × 10^{-14}
	K_{iu} 3.33 ± 0.40 μM	
mixed noncompetitive	K_m 459 ± 45 μM	(control model)
	K_{ic} 1.42 ± 0.25 μM	
	K_{iu} 8.02 ± 1.50 μM	
(C) Peptide Phosphorylation: Peptide Binding Site		
competitive	K_m 2.08 ± 0.27 mM	1.000
	K_{ic} 0.918 ± 0.093 μM	
uncompetitive	K_m 7.13 ± 1.84 mM	6.38 × 10^{-16}
	K_{iu} 0.826 ± 0.176 μM	
mixed noncompetitive	K_m 1.72 mM[c]	(control model)
	K_{ic} 0.768 μM	
	K_{iu} > 10^{15} μM	

[a] Probabilities <0.05 indicate that the more complex (mixed noncompetitive) model should be accepted. Probabilities >0.05 indicate that the simpler (competitive or uncompetitive) model should be accepted. [b] K_{ic}: inhibition constant for competitive inhibition. K_{iu}: inhibition constant for uncompetitive inhibition. [c] Because K_{iu} was so high, no error estimates could be determined for the parameters in the fit to the mixed noncompetitive model.

plate was then assayed as usual without removal of damnacanthal. The other plate was washed prior to the peptide phosphorylation reaction. A representative experiment is shown in Figure 4. In the plate that was not washed prior to assay, 10 μM damnacanthal caused 95% inhibition. In contrast, there was only 19% inhibition (mean from three independent experiments) in the wells that had been preincubated with 10 μM damnacanthal but washed prior to the peptide phosphorylation reaction.

The inhibition by damnacanthal was not time-dependent, since linear progress curves were observed with and without

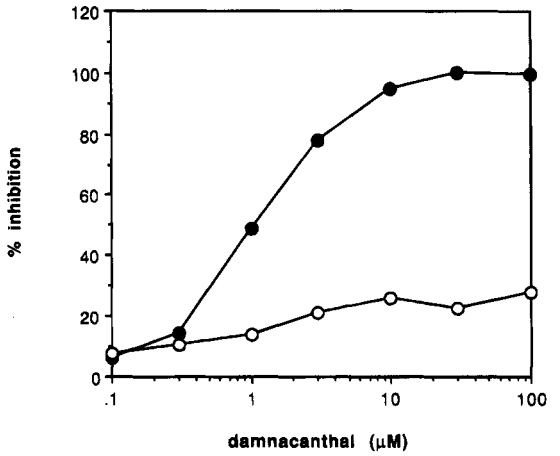

FIGURE 4: Inhibition of p56[lck] by damnacanthal is reversible. p56[lck], which had been immobilized in the wells of two duplicate microtiter plates, was preincubated for 5 min at 30 °C with the indicated concentrations of damnacanthal. One plate was then assayed directly without removal of damnacanthal (●). The other plate was washed 3 times with 25 mM Hepes, pH 7.4, 0.1% Brij 96, including incubation for 15 min on ice during the second wash, prior to the kinase reaction (○). The peptide phosphorylation reaction was performed under standard conditions. Data shown are the means from duplicate reactions.

damnacanthal in the p56[lck] peptide phosphorylation reaction (data not shown).

To determine whether sulfhydryl reagents affected the potency of damnacanthal, we examined the effect of dithiothreitol and 2-mercaptoethanol on the inhibition of p56[lck] autophosphorylation by damnacanthal. The IC_{50} values for damnacanthal were 17 and 18 nM in the presence of 1 mM dithiothreitol and 10 mM 2-mercaptoethanol, respectively, whereas in the same experiment the IC_{50} was 12 nM in the absence of sulfhydryl reagents.

Damnacanthal Binds Nonspecifically to Lipids. Damnacanthal exhibited a mean IC_{50} of 17 nM for autophosphorylation of p56[lck] purified *in situ* by immobilization onto antibody-coated microtiter plates prior to the kinase reaction (Table 1). However, when the autophosphorylation reaction was performed using crude preparations of plasma membranes, followed by solubilization and capture of the p56[lck] onto the microtiter plates, the IC_{50} of damnacanthal increased to 2.8 μM. In contrast, there was no difference in the potency of the well-known protein kinase inhibitor staurosporine (Ruegg & Burgess, 1989) between inhibition of purified p56[lck] and inhibition of p56[lck] in crude plasma membrane preparations (data not shown).

To determine whether the reduced potency of damnacanthal in the kinase reaction performed in membranes was due to nonspecific binding to membrane lipids, the lipid fraction was extracted from Jurkat cell plasma membranes. As shown in Figure 5, addition of the lipid extract to the purified p56[lck] increased the IC_{50} of damnacanthal 25-fold, whereas it had no significant effect on the IC_{50} of staurosporine. Moreover, preincubation of various concentrations of damnacanthal with plasma membranes from the monocytic cell line U937, which does not express p56[lck], decreased the potency of the damnacanthal solution by 22-fold (data not shown).

Damnacanthal Does Not Readily Form Imines. Because of the potential reactivity of the aldehyde moiety in damnacanthal, several experiments were performed to evaluate how readily damnacanthal forms imines with amines.

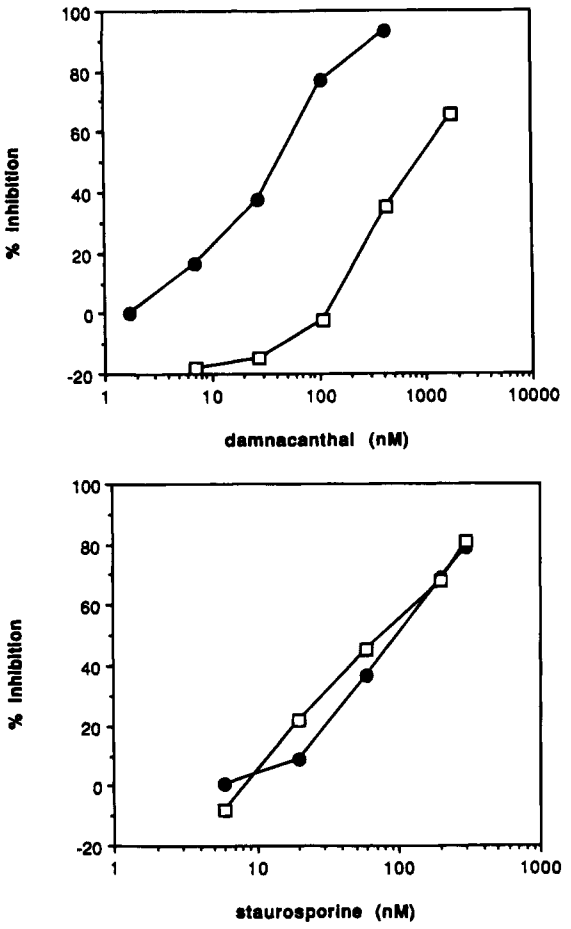

FIGURE 5: Effect of membrane lipids on the inhibition of p56^{lck} autophosphorylation by damnacanthal (top panel) and staurosporine (bottom panel). Plasma membranes from Jurkat cells were extracted with chloroform/methanol (2/1). The lipid fraction was dried down and resuspended in 25 mM Hepes, pH 7.4, 1% Brij 96. Where indicated, the lipid extract (from 30 μg of membrane protein) was added to each well of the microtiter plate containing immobilized p56^{lck}. The indicated concentrations of damnacanthal (top) or staurosporine (bottom) were added, followed by the addition of ATP. The kinase reaction was performed in the absence (●) or presence (□) of the lipid extract. Autophosphorylation of p56^{lck} was measured by ELISA, as previously described (Miller et al., 1995). Data shown are the means from triplicate reactions.

In one set of experiments, the K_{eq} for imine formation from butylamine and damnacanthal was determined to predict the propensity for damnacanthal to form imines with lysines found in p56^{lck} or in other proteins. These experiments were carried out at pH 7.4 in the buffer described under Materials and Methods for the equilibrium dialysis experiments. Mixtures of butylamine and damnacanthal were analyzed by HPLC. The peak areas were integrated to quantitate the amount of individual components, using the assumption that damnacanthal and the imine product would have similar molar absorptivities. The identities of the eluting species were corroborated using LC-MS. From these experiments, we calculated an equilibrium constant of 3.5×10^{-4} μM^{-1}. The reversibility of imine formation was established by diluting an equlibrium mixture of the three components: damnacanthal, butylamine, and imine. Upon dilution, the imine must dissociate to reestablish equilibrium. Serial dilutions gave K_{eq} values of 3.8×10^{-4}, 3.4×10^{-4}, and 3.3×10^{-4} μM^{-1}, which is consistent with reversible formation of an imine between butylamine and damnacanthal.

Table 4: Binding of Damnacanthal to Poly(L-lysine), p56^{lck}, and Ribonuclease A

	A_{262}	A_{316}	concn (μM)
(A) Binding of Damnacanthal to Poly(L-lysine)[a]			
starting solution	2.132	1.143	100
predicted values at equilibrium with no binding	1.367	0.733	64
actual values at equilibrium	0.430	0.242	21
(B) Binding of Damnacanthal to p56^{lck} [b]			
starting solution	0.255	0.134	8.0
predicted values at equilibrium with no binding	0.128	0.067	4.0
actual values at equilibrium	0.079	0.042	2.5
(C) Binding of Damnacanthal to Ribonuclease A[c]			
starting solution	0.226	0.122	8.0
predicted values at equilibrium with no binding	0.143	0.077	5.1
actual values at equilibrium	0.143	0.080	5.2

[a] Starting conditions: running side, 2.5 mL of 100 μM damnacanthal; closed side, 1.4 mL of 5 mM lysine [in the form of poly(L-lysine)]. [b] Starting conditions: running side, 1.4 mL of 8 μM damnacanthal; closed side, 1.4 mL of 8 μM p56^{lck}. [c] Starting conditions: running side, 2.4 mL of 8 μM damnacanthal; closed side, 1.4 mL of 5 μM ribonuclease A.

We next examined whether damnacanthal readily forms imines with lysine. Equilibrium dialysis experiments were performed with both polylysine and p56^{lck}. The concentration of free damnacanthal at equilibrium was determined spectrophotometrically at two wavelengths. Using 100 μM damnacanthal and 5 mM lysine in the form of poly(L-lysine), there was greater loss of free damnacanthal than would be expected based on dilution only (Table 4A). From these data, we calculated that 67% of the damnacanthal was bound to poly(L-lysine) at equilibrium under these conditions. This is in excellent agreement with the calculated value of 64% imine formation from 100 μM damnacanthal and 5 mM lysine, using the K_{eq} determined from the experiments with butylamine and damnacanthal. These results demonstrated that damnacanthal could form imines with lysine when relatively high concentrations of each reactant were present. However, in another experiment using much lower concentrations of each reactant [10 μM damnacanthal and 100 μM lysine in the form of poly(L-lysine)], there was no detectable loss of free damnacanthal (data not shown). Based on the K_{eq} calculated from the experiment with butylamine, only 3.4% of the damnacanthal would be expected to form an imine at these concentrations of reactants.

Experiments were also performed with p56^{lck} and damnacanthal, using conditions similar to those in the experiments with poly(L-lysine) except that the dialysis was performed at 4 °C to retain the p56^{lck} in an active conformation. In an experiment with 8 μM p56^{lck} (equivalent to 184 μM lysine residues) and 8 μM damnacanthal, there was a greater decrease in the concentration of free damnacanthal than would be expected based on dilution only (Table 4B). From these data, we calculated that 0.4 mol of damnacanthal bound per mol of p56^{lck} under these conditions. In contrast, no binding could be detected between damnacanthal and a control protein, ribonuclease A, at pH 7.4 (Table 4C) or at pH 9.5 (data not shown).

DISCUSSION

We recently identified damnacanthal as the active component of a plant extract that inhibited p56^{lck} tyrosine kinase

activity (Miski et al., unpublished observations). In this report, we demonstrate that natural damnacanthal and synthetic damnacanthal are potent, selective inhibitors of both autophosphorylation and phosphorylation of exogenous substrates by p56^{lck}. Synthetic damnacanthal exhibits an IC_{50} of 17 nM for p56^{lck} autophosphorylation. Damnacanthal appears to be somewhat more potent than the most potent p56^{lck} inhibitor previously reported, which is a lavendustin A analogue with an IC_{50} of 60 nM for p56^{lck} autophosphorylation (Smyth et al., 1993).

As previously discussed (Miller et al., 1995), we have used several closely related assays to evaluate the selectivity of a compound for inhibition of p56^{lck} compared with other protein kinases. This approach includes evaluating autophosphorylation data separately from data for phosphorylation of exogenous substrates, as well as using similar ratios of substrate concentration to the K_m for the individual enzymes. Using this approach, we have demonstrated that damnacanthal is highly selective for p56^{lck} over the serine/threonine kinases and receptor tyrosine kinases examined. Damnacanthal also exhibits moderate but highly statistically significant selectivity for p56^{lck} over p59^{fyn} and p60^{src}. p56^{lck}, p59^{fyn}, and p60^{src} are members of a homologous family of enzymes known as the src family (Cooper, 1990). To our knowledge, damnacanthal is the first compound demonstrated to have selectivity for p56^{lck} over other members of this highly homologous group of enzymes, when assayed using the same conditions for each enzyme.

Consistent with concerns about the effects of assay conditions on the potency of inhibitors and on the resulting interpretation of inhibitor selectivity, we observed that both the IC_{50} and K_i values for damnacanthal differed substantially in the various p56^{lck} assays employed. Damnacanthal was most potent in the autophosphorylation assay by p56^{lck} captured onto wells of microtiter plates. Substantially higher IC_{50} and K_i values were observed for peptide phosphorylation than for autophosphorylation, even though both assays employed p56^{lck} immobilized onto antibody-coated microtiter plates, comparable [ATP]/K_m ratios, and similar buffer conditions. The reasons for these differences in potency are not known. However, since autophosphorylation activates p56^{lck} for subsequent phosphorylation of exogeneous substrates (Wang et al., 1994), it is possible that the active site of p56^{lck} is substantially different before and after autophosphorylation. Consistent with this hypothesis is the 6-fold difference in K_m for $MgATP^{2-}$ between p56^{lck} autophosphorylation and peptide phosphorylation by p56^{lck} (Miller et al., 1995). Such a difference in the active site could result in differing potencies of inhibitors for autophosphorylation compared to phosphorylation of exogenous substrates even when comparable [ATP]/K_m ratios are utilized. Some other tyrosine kinase inhibitors have also been reported to be more potent inhibitors of autophosphorylation than of phosphorylation of exogenous substrates, as recently reviewed and discussed by Levitzki and Gazit (1995).

Although damnacanthal was a nanomolar inhibitor of p56^{lck} autophosphorylation using purified enzyme, it was a much less potent inhibitor of p56^{lck} autophosphorylation using crude plasma membrane preparations. Damnacanthal was found to bind nonspecifically to lipids. The anthracycline adriamycin, which has structural similarities to damnacanthal and has been reported to inhibit *abl* tyrosine kinase activity (Donella-Deana et al., 1989), also interacts with lipids (Goormaghtigh et al., 1980). However, even under assay conditions that maximized the inhibition, adriamycin was a weak inhibitor of *abl* ($IC_{50} \sim 20$ μM), compared with the inhibition of p56^{lck} by damnacanthal ($IC_{50} = 17$ nM).

Damnacanthal was isolated from a plant and its structure determined in 1955 (Nonomura, 1955). Recently, damnacanthal was shown to have antimalarial activity (Koumaglo et al., 1992) and to induce normal morphology of *ras* transformed cells (Hiramatsu et al., 1993). Relatively high concentrations (~20−100 μM) of damnacanthal were required to inhibit these biological activities, and the molecular mechanisms for inhibition in these functional assays are unknown (Koumaglo et al., 1992; Hiramutsu et al., 1993). In the present report, submicromolar concentrations of damnacanthal inhibited the enzymatic activity of purified p56^{lck}, but the compound was much less potent in p56^{lck} assays performed in plasma membranes. Moreover, we did not observe any inhibition of tyrosine kinase activity in stimulated T lymphocytes at concentrations of damnacanthal up to 50 μM (data not shown), suggesting that damnacanthal may not reach its intracellular p56^{lck} target in whole cell assays. Since our data suggested that damnacanthal can bind to lipids, it is possible that interactions of damnacanthal with membrane lipids played a role in the previously reported biological activities of damnacanthal. Alternatively, it is possible that the molecular targets for the antimalarial (Koumaglo et al., 1992) and "anti-ras" (Hiramatsu et al., 1993) activities of damnacanthal may be cell surface proteins or that the ability of damnacanthal to reach intracellular targets may vary with the type of cells utilized.

The marine natural product halenaquinone was recently reported to inhibit p60^{v-src} tyrosine kinase activity with an IC_{50} of 1.5 μM (Lee et al., 1992). The inhibition by halenaquinone was irreversible, and the potency of halenaquinone and various analogs correlated with the presence of possible Michael acceptor sites in the molecule (Lee et al., 1992). Moreover, the IC_{50} of halenaquinone increased severalfold in the presence of 2-mercaptoethanol (Lee et al., 1992). Sulfhydryl reagents also abolished the inhibition of p60^{v-src} by herbimycin A, a benzoquinoid ansamycin antibiotic which is an irreversible inhibitor that binds to cysteine residues (Uehara et al., 1989). In contrast, the inhibition of p56^{lck} by the anthraquinone damnacanthal was reversible, and there was only a minor effect of sulfhydryl reagents on the potency.

Because of concern about the potential reactivity of the aldehyde moiety in damnacanthal, several experiments were performed to determine how readily damnacanthal forms imines with amines. Both the K_{eq} calculated from the experiments with butylamine and the equilibrium dialysis experiments demonstrated that significant imine formation occurred only at relatively high concentrations of reactants. As expected for a p56^{lck} inhibitor, binding of damnacanthal to p56^{lck} was detected by equilibrium dialysis, although less than 1 mol of damnacanthal was bound per mol of p56^{lck} under the conditions utilized. At similar concentrations of reactants, there was no detectable binding of damnacanthal to poly(L-lysine) or to ribonuclease A. These results demonstrated that damnacanthal binds specifically to p56^{lck}. Under conditions of low concentrations of damnacanthal and p56^{lck}, poly(L-lysine), or ribonuclease A, there was no evidence for indiscriminant imine formation. Significant formation of imines appeared to occur only at much higher

concentrations of reactants. Since damnacanthal is a submicromolar inhibitor of $p56^{lck}$ tyrosine kinase activity, it is unlikely that indiscriminant imine formation would occur at concentrations of damnacanthal that effectively inhibit $p56^{lck}$ activity.

Taken together, the data presented herein suggest several aspects of the mechanism of inhibition of $p56^{lck}$ by damnacanthal. The inhibition by damnacanthal appears to be reversible, not time-dependent, and not significantly affected by the presence of sulfhydryl reagents. The kinetic data suggest that damnacanthal binds to the peptide binding site of $p56^{lck}$. The effect of membrane lipids to decrease the potency of damnacanthal suggests that damnacanthal may interact with $p56^{lck}$ by hydrophobic interactions. It is also possible that reversible imine formation may occur at a specific region in the active site of the enzyme as part of the mechanism of action of damnacanthal even though the data presented suggest that it does not indiscriminantly form imines at concentrations which inhibit enzymatic activity.

In summary, damnacanthal is a potent, selective inhibitor of $p56^{lck}$ tyrosine kinase activity and may be a starting point for the identification of related compounds that selectively inhibit $p56^{lck}$ activity in both cell-free and whole cell systems. Such compounds may have therapeutic utility for the treatment of T cell malignancies and autoimmune diseases.

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REFERENCES

Ayyangar, N. R., Joshi, B. S., & Venkataraman, K. (1959) *Tetrahedron 6*, 331−337.

Bolen, J. B., Rowley, R. B., Spana, C., & Tsygankov, A. Y. (1992) *FASEB J. 6*, 3403−3409.

Cooper, J. A. (1990) in *Peptides and Protein Phosphorylation* (Kemp, B. E., Ed.) pp 85−113, CRC Press, Boca Raton, FL.

Cooper, J. A., Esch, F. S., Taylor, S. S., & Hunter, T. (1984) *J. Biol. Chem. 259*, 7835−7841.

Couture, C., Baier, G., Altman, A., & Mustelin, T. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5301−5305.

Donella-Dana, A., Monti, E., & Pinna, L. A. (1989) *Biochem. Biophys. Res. Commun. 160*, 1309−1315.

Faltynek, C. R., Wang, S., Miller, D., Mauvais, P., Gauvin, B., Reid, J., Xie, W., Hoekstra, S., Juniewicz, P., Sarup, J., Lehr, R., Sawutz, D. G., & Murphy, D. (1995) *J. Enzyme Inhib. 9*, 111−122.

Goormaghtigh, E., Chatelain, P., Caspers, J., & Ruysschaert, J. M. (1980) *Biochim. Biophys. Acta* 597, 1−14.

Graber, M., June, C. H., Samelson, L. W., & Weiss, A. (1992) *Int. Immunol. 4*, 1201−1210.

Hiramatsu, T., Imoto, M., Koyano, T., & Umezawa, K. (1993) *Cancer Lett. 73*, 161−166.

Horak, I. D., Kawakami, T., Gregory, F., Robbins, K. C., & Bolen, J. B. (1989) *J. Virol. 63*, 2343−2347.

June, C. H., Fletcher, M. C., Ledbetter, J. A., Schieven, G. L., Siegel, J. N., Phillips, A. F., & Samelson, L. E. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7722−7726.

Koumaglo, K., Gbeassor, M., Nikabu, O., deSouza, C., & Werner, W. (1992) *Planta Med. 58*, 533−534.

Lee, R. H., Slate, D. L., Moretti, R., Alvi, K. H., & Crews, P. (1992) *Biochem. Biophys. Res. Commun. 184*, 765−772.

Levitzki, A., & Gazit, A. (1995) *Science 267*, 1782−1788.

Miller, D., Wang, S., Reid, J., Xie, W., Gauvin, B., Kelley, M., Sarup, J., Sawutz, D. G., Miski, M., Dolle, R. E., & Faltynek, C. R. (1995) *Drug. Dev. Res. 34*, 344−352.

Mustelin, T., Coggeshall, K. M., Isakov, N., & Altman, A. (1990) *Science 247*, 1584−1587.

Nonomura, S. (1955) *J. Pharm. Soc. Jpn. 75*, 219−227.

Perlmutter, R. M., Marth, J. D., Lewis, D. B., Peet, R., Ziegler, S. F., & Wilson, C. B. (1988) *J. Cell. Biochem. 38*, 117−126.

Ruegg, U. T., & Burgess, G. M. (1989) *Trends Pharmacol. Sci. 10*, 218−220.

Samelson, L. E., & Klausner, R. D. (1992) *J. Biol. Chem. 267*, 24913−24916.

Samelson, L. E., Phillips, A. F., Luong, E. T., & Klausner, R. D. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4358−4362.

Smyth, M. S., Stefanova, I., Hartmann, F., Horak, I. D., Osherov, N., Levitzki, A., & Burke, T. R. (1993) *J. Med. Chem. 36*, 3010−3014.

Stanley, J. B., Gorczynski, R., Huang, C.-K., Love, J., & Mills, G. B. (1990) *J. Immunol. 145*, 2189−2198.

Trevillyan, J. M., Lu, Y., Atluru, D., Phillips, C. A., & Bjorndahl, J. M. (1990) *J. Immunol. 145*, 3223−3230.

Uehara, Y., Fukazawa, H., Murakami, Y., & Mizuno, S. (1989) *Biochem. Biophys. Res. Commun. 163*, 803−809.

Veillette, A., & Fournel, M. (1990) *Oncogene 5*, 1455−1462.

Veillette, A., & Davidson, D. (1992) *Trends Genet. 8*, 61−66.

Veillette, A., Bookman, M. A., Horak, E. M., & Bolen, J. B. (1988a) *Cell 55*, 301−308.

Veillette, A., Horak, I. D., & Bolen, J. B. (1988b) *Oncogene Res. 2*, 385−401.

Wang, Q., Smith, J. B., Harrison, M. L., & Geahlen, R. (1991) *Biochem. Biophys. Res. Commun. 178*, 1393−1399.

Wang, S., Lehr, R., Stevis, P., Wang, X.-M., Trevillyan, J., & Faltynek, C. R. (1994) *Arch. Biochem. Biophys. 315*, 60−67.

Weiss, A. (1993) *Cell 73*, 209−212.

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