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INHIBITION OF T LYMPHOCYTE ACTIVATION BY A NOVEL p56^{lck} TYROSINE KINASE INHIBITOR

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A new p56^{lck} tyrosine kinase inhibitor WIN 61651 [1,4-dihydro-7-(4-methyl-1-piperizinyl)-1-(4-(4-methyl-1-piperizinyl))phenyl-4-oxo-3-quinolinecarboxamide) is described. WIN 61651, which is competitive with ATP, demonstrates selectivity for the lymphoid restricted tyrosine kinase p56^{lck} over serine/threonine kinases, such as protein kinase C and protein kinase A, and over some other tyrosine kinases, including erbB2, epidermal growth factor receptor, and insulin receptor; however, it is equipotent for inhibition of p56^{lck} and the platelet derived growth factor receptor tyrosine kinases. WIN 61651 inhibits p56^{lck} activity in cell-free assays, tyrosine kinase activity in a T lymphocytic cell line, and T cell activation, as measured by IL-2 production by purified CD4 positive peripheral blood T lymphocytes and the mixed lymphocyte reaction. WIN 61651 constitutes a new tool for studies on the role for tyrosine kinases in lymphocyte function.

KEY WORDS: Tyrosine kinase inhibitors, p56^{lck}, T lymphocytes, T cell activation

INTRODUCTION

It is well established that enhanced tyrosine kinase activity is one of the earliest detectable events in T cell activation.[1–4] Four tyrosine kinases, p56^{lck}, p59^{fyn}, ZAP-70, and p72^{syk} have been implicated as playing critical roles in stimulation through the T cell antigen receptor.[1–4]

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Abbreviations: PDGF-R, platelet derived growth factor receptor; EGF-R, epidermal growth factor receptor; PKC, protein kinase C; PKA, protein kinase A; MBP-P, peptide from myelin basic protein; UC, uncompetitive; NC, noncompetitive; IL-2, interleukin-2; PMA, phorbol myristate acetate; PBMC, peripheral blood mononuclear cells; MLR, mixed lymphocyte reaction.

The role for tyrosine kinases in T cell activation has been evaluated by several investigators through the use of inhibitors.[5–9] Studies using genistein, herbimycin A, and the tyrphostins have provided evidence that tyrosine phosphorylation is required for T cell activation through the T cell antigen receptor.[5–9] However, the tyrosine kinase inhibitors used in these previous studies either are not selective for the tyrosine kinases critical for T cell activation and/or have an unusual mechanism of action.[8–10 (and references therein)]

As part of our efforts to identify novel, selective inhibitors of $p56^{lck}$, we have screened chemical files and natural product extracts. This report describes the identification of a new tyrosine kinase inhibitor that demonstrates moderate selectivity for $p56^{lck}$ and exhibits a conventional mechanism of action. This compound effectively inhibits T cell activation.

MATERIALS AND METHODS

Assays for Protein Kinases

Autophosphorylation of $p56^{lck}$, erbB2, and platelet derived growth factor receptor (PDGF-R) tyrosine kinases was measured in an ELISA format, as previously described.[11] 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.[11,12]

Mechanistic studies were performed using purified recombinant human $p56^{lck}$.[12] Two peptides substrates were utilized: the RR-SRC peptide as previously described[12] and a peptide from myelin basic protein (MBP-P).[13] Initial velocity versus substrate versus inhibitor data were fit to the three standard inhibition models, competitive (equation 1), uncompetitive (UC) (equation 2), and noncompetitive (NC) (equation 3), using the nonlinear regression routines in the RS/1 data analysis software package (BBN Software Products Corporation, Cambridge MA). K_{I-1} refers to binding of inhibitor to free enzyme; K_{I-2} refers to binding of inhibitor to the enzyme-substrate complex.

$$\text{vel} = \frac{V_{max} \cdot [S]}{K_m (1 + [I]/K_{I-1}) + [S]} \tag{1}$$

$$\text{vel} = \frac{V_{max} \cdot [S]}{K_m + [S](1 + [I]/K_{I-2})} \tag{2}$$

$$\text{vel} = \frac{V_{max} \cdot [S]}{K_m(1 + [I]/K_{I-1}) + [S](1 + [I]/K_{I-2})} \tag{3}$$

Discrimination between the models is based on the method of Mannervik[14] which utilizes the F-test to discriminate between a full model and a reduced model. The full model must have at least one more varied parameter than the reduced model. A full model can be compared to more than one reduced model. In this analysis the full

model is the NC mechanism; the competitive and UC mechanisms are the reduced models. Interpretation of the probability returned by the F-test is as follows: if the probability is low (<0.05), there is a significant decrease in the errors (i.e., the sum of the squares of the residuals from the nonlinear regression) when the full model is used relative to the reduced model, and the full model should be accepted. If the probability is high (>0.05), there is an insignificant decrease in the errors when the full model is used relative to the reduced model, and the reduced model should be accepted.

Whole Cell Tyrosine Kinase Assay

A. Cellular Stimulation. Jurkat human T leukemia cells (CD4 positive clone JB2.7 obtained from Dr. Seth Lederman, Columbia University, NY) were washed and resuspended in serum-free RPMI 1640 medium at 2×10^7 cells/ml. One ml aliquots of cells were preincubated with test compounds in a final concentration of 1% DMSO for 15 min at 37°C. After the preincubation, the cells were cooled to 4°C, followed by the addition of either 0.3 μg/ml antibody to CD3 (OKT3) (Ortho, Raritan, NJ) and 10 μg/ml antibody to CD4 (AMAC, Westbrook, ME) or 10 μg/ml control mouse IgG2b antibodies (ICN, Costa Mesa, CA). Following a 30 min incubation on ice to allow for binding of the antibodies, the cells were washed twice with cold serum-free RPMI and resuspended at 10×10^7 cells per ml in pre-warmed serum-free RPMI with 100 μg/ml goat anti-mouse IgG F(ab')$_2$ (Jackson ImmunoResearch Labs, West Grove, PA) to cross-link the cell surface bound anti-CD3 and anti-CD4 antibodies. The cells were incubated at room temperature for 2–3 min, followed by the addition of an equal volume of 2X SDS sample buffer. The samples were boiled for 5 min and centrifuged at 400,000×g for 30 min at 10°C.

B. Detection of tyrosine phosphorylated protein by immunoblotting. The 400,000×g supernatant was subjected to SDS-PAGE on 10% acrylamide gels, followed by transfer of the proteins to nitrocellulose paper. The nitrocellulose was incubated for one hour at room temperature in blocking buffer (3% BSA, 0.05% Tween-20, 20 mM Tris-Cl pH 7.5, 500 mM NaCl), followed by incubation overnight with 1 μg/ml antibody to phosphotyrosine [Upstate Biotechnology Inc. (UBI) Lake Placid, NY] in the blocking buffer. The filter was washed thrice with 50 mM Tris, pH 7.6, 150 mM NaCl, 0.5 % BSA, 0.05% Tween-20, and then incubated for 1–3 h with ^{125}I-protein A (0.5 μCi/ml) (ICN) in the washing buffer without Tween. Finally, the nitrocellulose was washed four times with washing buffer and twice with 50 mM Tris pH 7.6, 150 mM NaCl. The ^{125}I-protein A was visualized and quantitated by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

Activation of CD4 Positive Peripheral Blood T Lymphocytes

Human peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque separation. CD4+ T lymphocytes were purified from the PBMC population by negative selection using antibodies against Mol, B4, CD16, and CD8. Goat anti-mouse IgG superparamagnetic microbeads and a MACS^R column Type 2 were used according

to the recommendations of the manufacturer (Miltenyi Biotec, Sunnyvale, CA). The non-magnetic unretained fraction was ≥95% CD4 positive lymphocytes as determined by FACS analysis. Antibody to CD3 with or without antibody to CD4 (30 ng/well of each antibody; AMAC) was pre-coated onto the wells of a microtiter plate. The plate was then washed with RPMI 1640 medium, followed by the addition of purified CD4+ T lymphocytes that had been preincubated for 45 min at room temperature with the indicated concentrations of test compound in 0.1% DMSO or with 0.1% DMSO alone. Where indicated, 1 ng/ml phorbol myristate acetate (PMA) or 1 μg/ml antibody to CD28 (Biodesign International, Kennebunk, ME) was added. Following a 24 h incubation at 37°C, the microtiter plate was centrifuged for 10 min at 1200 rpm. The supernatants were removed and assayed for the presence of interleukin-2 (IL-2), using an ELISA.

Mixed Lymphocyte Reaction (MLR)

A two-way MLR was set up with PBMC from two normal human donors, using standard procedures.[15] WIN 61651 was added at various concentrations in the presence of a final concentration of 0.2% DMSO. After a 5 day incubation at 37°C, cellular proliferation was measured by incorporation of ^{3}H-methyl-thymidine (New England Nuclear; 2 Ci/mmol).

RESULTS AND DISCUSSION

WIN 61651 is a Selective Inhibitor of p56^{lck} Tyrosine Kinase Activity

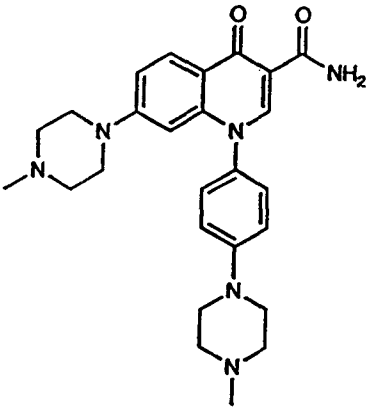
As a result of high volume screening of our chemical files, the quinolone WIN 61651 [1,4-dihydro-7-(4-methyl-1-piperizinyl)-1-(4-(4-methyl-1-piperizinyl))phenyl-4-oxo-3-quinolinecarboxamide] was found to inhibit the tyrosine kinase activity of p56^{lck}. WIN 61651 inhibited both the autophosphorylation of p56^{lck} and the phosphorylation of an exogenous peptide by p56^{lck} (Table 1).

The selectivity of WIN 61651 for inhibition of p56^{lck} was examined. As previously discussed,[11] the same [substrate]/Km ratios were used for the various enzymes to enhance the validity of conclusions from experiments performed to determine compound selectivity. Moreover, autophosphorylation data were evaluated separately from data for phosphorylation of exogenous substrates.[11] As shown in Table 1, WIN 61651 was selective for p56^{lck} tyrosine kinase compared with the serine/threonine kinases PKC and PKA. WIN 61651 was also selective for p56^{lck} over three out of four of the other tyrosine kinases examined (Table 1).

Therefore, although not completely specific, WIN 61651 does demonstrate selectivity for p56^{lck} over many other protein kinases. WIN 61651 appears to be more selective for T cell specific tyrosine kinases than other inhibitors previously used to study the role of tyrosine kinases in T cell activation.

Unlike many of the previously described p56^{lck} tyrosine kinase inhibitors,[16] WIN 61651 is not hydroxylated. We have recently reported that simple nonhydroxylated chromones with an aldehyde functional group ortho to the chromone carbonyl

TABLE 1
Potency and selectivity of WIN 61651 for inhibition of p56[lck] tyrosine kinase activity



Peptide Phosphorylation kinase	IC_{50} (μM)
p56[lck]	24 ± 3 (n=4)
PKC	>5,000
PKA	>1,000

Autophosphorylation kinase	IC_{50} (μM)	
	[ATP]/Km = 2.5	[ATP]/K_m = 100
p56[lck]	18	80
erbB2	ND	>5,000
EGF-R	ND	>1,000
insulin-R	ND	1,000
PDGF-R	10	130

inhibit p56[lck] activity.[11] It is of interest that WIN 61651 has an amide group ortho to the carbonyl on the quinolone ring. Ortho substituents have been demonstrated to be crucial for the potency of tyrosine kinase inhibitors of several other chemical classes. For example, Traxler *et al.*[17] described an inhibitor of EGF-R that has a hydroxyl group ortho to the benzoyl group of the sulfonylbenzoyl moiety and suggested that this substitution may enhance the complexation of bivalent cations. Another example is the salicyl-containing hydroxylated compound lavendustin A which inhibits EGF-R tyrosine kinase activity.[18] Smyth *et al.*[19] recently described a salicyl-containing lavendustin A analog which is a highly potent inhibitor of p56[lck].

Mechanism of Inhibition of $p56^{lck}$ by WIN 61651

As shown in Table 1, increasing the [ATP] from 2.5×Km to 100×Km increased the IC_{50} of WIN 61651 for $p56^{lck}$ autophosphorylation, suggesting that WIN 61651 is competitive at the ATP binding site. To further evaluate the mechanism for inhibition by WIN 61651, we examined the effect of WIN 61651 on the binding of both ATP and peptide in the peptide phosphorylation reaction using initial velocity kinetics. In three independent experiments using two different peptides, the data fit the competitive model for the binding of WIN 61651 to the ATP site. In contrast, in two experiments using two different peptides, the data fit the noncompetitive model for binding of WIN 61651 to the peptide binding site.

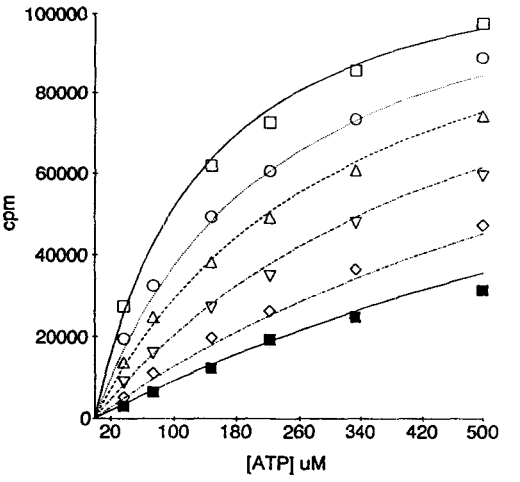
A representative plot for velocity vs. [ATP] vs. [WIN 61651] is shown in Figure 1A. The data were fit to the three standard inhibition mechanisms: competitive, UC, and NC. The fit to the NC model was compared with the fits to the competitive and UC mechanisms, using the F-test as described in Materials and Methods. The parameter estimates and errors resulting from the nonlinear regression fits, as well as the results of the F-test, are given in Table 2A. In choosing among the three rival mechanisms, two pieces of data support the competitive mechanism. First, the parameter estimates for the NC model demonstrate that K_{I-2} is poorly determined and more than 20-fold greater than K_{I-1} (Table 2A), indicating that essentially all of the inhibition by WIN 61651 can be explained by using K_{I-1} alone. Second, the probability from the F-test is >0.05 for the competitive model, indicating that the addition of the extra parameter in the NC model (i.e., K_{I-2}) does not yield a statistically significant improvement in the fit and therefore the simpler competitive model should be accepted. This experiment therefore yields a K_I value of 7.6±0.5 μM for WIN 61651 at the ATP binding site in the presence of 4 mM RR-SRC peptide. A repeat experiment yielded a K_I value of 4.2±0.4 μM. In the presence of a different peptide (5 mM MBP-P), the K_I was determined to be 14.4±0.7μM.

The mirror experiment in which [ATP] was held constant and [peptide] was varied versus [WIN 61651] gave the expected result for a random-order binding mechanism i.e., noncompetitive. This was true with both peptides. Representative plots and resulting fits of the initial velocity data for the RR-SRC peptide are shown in Figure 1B and Table 2B. With both peptides, K_{I-2} was less than four fold greater than K_{I-1}, indicating that a significant fraction of the inhibition was due to binding to the E·peptide complex. Also, with both peptides the F-test returned probabilities of 10^{-4} or less for each of the reduced models.

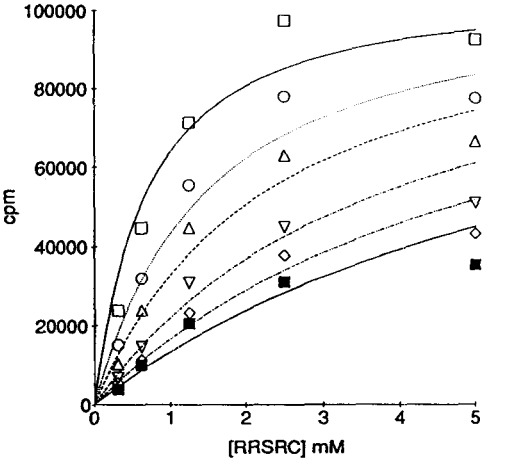
Therefore, the kinetic data support binding of WIN 61651 at the ATP binding site based on the competitive mechanism result when ATP was varied and the noncompetitive mechanism result when peptide was varied.

WIN 61651 Inhibits Tyrosine Kinase Activity in Whole Cells

Stimulation of JB2.7 cells with antibodies to CD3 and CD4 enhanced the level of tyrosine phosphorylation of several cellular proteins (Figure 2), in agreement with results of other investigators using somewhat different experimental systems.[20–23] To determine whether WIN 61651 inhibits tyrosine kinase activity in intact cells,



(1A)



(1B)

FIGURE 1 Mechanism of inhibition of p56[lck] tyrosine kinase activity by WIN 61651. A. ATP binding site. p56[lck] was pre-incubated without (□), or with WIN 61651 at the following concentrations: 5 μM (○), 10 μM (△), 20 μM (▽), 40μM (◇), or 60 μM (■). [gamma-^{32}P]ATP was added at the indicated concentrations along with 4 mM RR-SRC. B. Peptide binding site. p56[lck] was pre-incubated without (□), or with WIN 61651 at the following concentrations: 10 μM (○), 20 μM (△), 40 μM (▽), 60 μM (◇), or 80 μM (■). RR-SRC peptide was added at the indicated concentrations along with 600 μM [gamma-^{32}P]ATP. Incorporation of $^{32}PO_4$ into the RR-SRC peptide was measured as described in Materials and Methods.

TABLE 2
Analysis of kinetic data for binding of WIN 61651 to $p56^{lck}$.

A. ATP Binding Site Model	Parameter Estimates	Probability
Competitive	K_m 139.0± 10.3 μM K_{I-1} 7.6±0.5 μM V_{max} 3.97±0.10 nmol min^{-1} μg^{-1}	0.1523
Uncompetitive	K_m 396.1±87.2 μM K_{I-2} 11.3±1.9 μM V_{max} 6.14±0.79 nmol min^{-1} μg^{-1}	0.0000
Noncompetitive	K_m 148.3±12.5 μM K_{I-1} 8.7±1.0 μM K_{I-2} 190.7±129.6 μM V_{max} 4.07±0.12 nmol min^{-1} μg^{-1}	(Control model)
B. Peptide Binding Site Model	Parameter Estimates	Probability
Competitive	K_m 759.8±130.7 μM K_{I-1} 9.3±1.5 μM V_{max} 4.04±0.21 nmol min^{-1} μg^{-1}	0.000057
Uncompetitive	K_m 1961.0±396.6 μM K_{I-2} 23.2±4.0 μM V_{max} 5.51±0.55 nmol min^{-1} μg^{-1}	3.004×10^{-9}
Noncompetitive	K_m 986.0±144.0 μM K_{I-1} 18.3±4.3 μM K_{I-2} 67.0±17.9 μM V_{max} 4.45±0.23 nmol min^{-1} μg^{-1}	(Control model)

JB2.7 cells were pre-incubated with various concentrations of WIN 61651 followed by stimulation with antibodies to CD3 and CD4. As shown in Figure 2, treatment with WIN 61651 resulted in a concentration-dependent decrease in the level of tyrosine phosphorylation of cellular proteins. The inhibition was quantitated using the PhosphorImager. From two independent experiments, the lowest mean IC_{50} values (47 μM) were observed for tyrosine phosphorylation of proteins having apparent molecular weights of 57 kDa and 63 kDa. The mean IC_{50} values for inhibition of tyrosine phosphorylation of other major proteins varied between 62 and 145 μM.

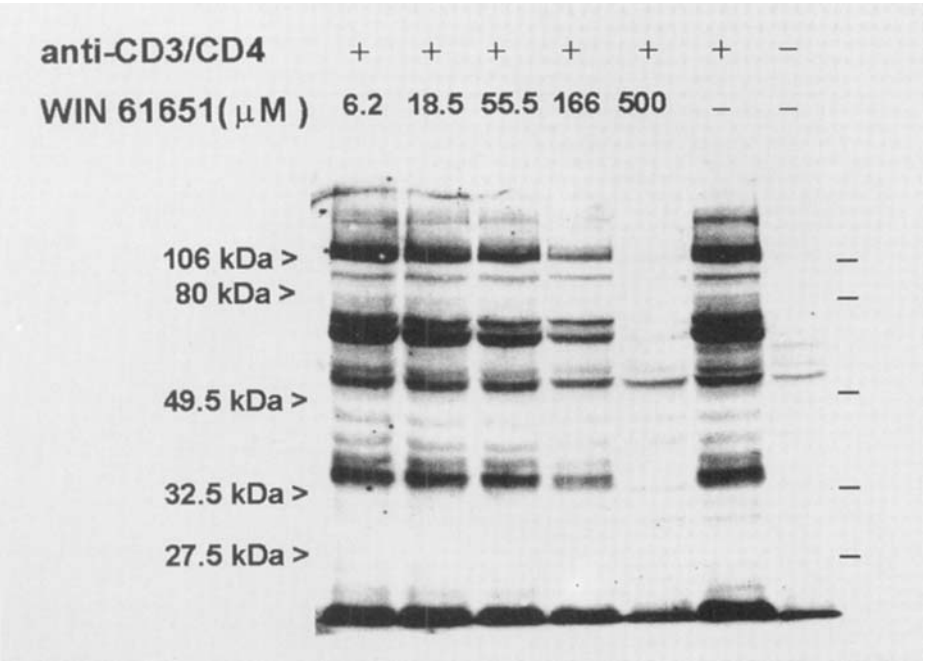


FIGURE 2 WIN 61651 inhibits tyrosine kinase activity in stimulated JB2.7 cells. JB2.7 cells were pre-incubated with the indicated concentrations of WIN 61651 and then stimulated with either antibodies to CD3 and CD4 or with isotype matched control antibodies. Tyrosine phosphorylated proteins were detected by immunoblotting as described in Materials and Methods.

WIN 61651 Inhibits T Cell Activation

To examine the effect of WIN 61651 on T cell activation, we used two experimental systems: IL–2 production by CD4+ peripheral blood T lymphocytes stimulated by antibodies to cell surface proteins and the mixed lymphocyte reaction.

Human CD4+ T lymphocytes were purified from peripheral blood and activated by treatment with antibodies to relevant cell surface proteins. It is well established that at least two signals are required for activation of T lymphocytes.[24] One signal can be provided through the CD3/T cell receptor complex, whereas the second signal can be provided either by PMA or by antibody to CD28.[25] CD4+ T lymphocytes were activated either with immobilized antibody to CD3 plus soluble PMA or with co-immobilized antibodies to CD3 and CD4 plus soluble antibody to CD28. Based on initial titration experiments, suboptimal concentrations of each stimulus were chosen to maximize sensitivity to inhibitors. Both activation protocols led to the induction of considerable amounts of IL-2 (Figure 3). Treatment with neither anti-CD3, anti-CD4, anti-CD28, nor PMA alone resulted in significant IL-2 production (data not shown). As shown in Figure 3, treatment of CD4+ lymphocytes with WIN 61651 resulted in dose dependent inhibition of IL-2 production by cells activated using either protocol.

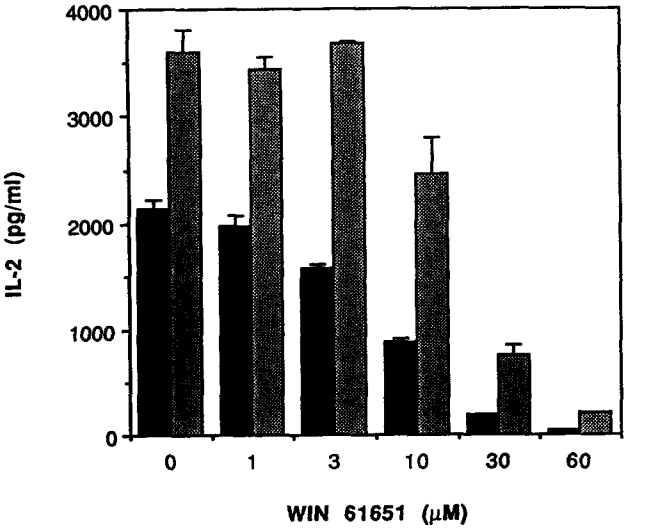


FIGURE 3 WIN 61651 inhibits IL-2 production by activated CD4+ T lymphocytes. Purified CD4+ peripheral blood T lymphocytes were activated by treatment either with antibody to CD3 plus PMA (▩) or with antibodies to CD3, CD4, and CD28 (■), as described in Materials and Methods. IL-2 production was measured by ELISA and expressed as the mean and standard deviation from duplicate assays.

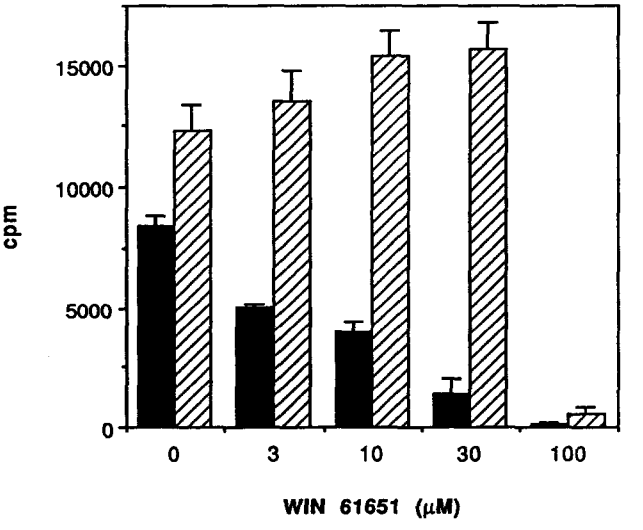


FIGURE 4 WIN 61651 inhibits the mixed lymphocyte reaction. The indicated concentrations of WIN 61651 were added to a two-way MLR in the absence (■) or presence (▨) of 100 U/ml exogenous IL-2 (Gibco BRL, Gaithersburg, MD). Cell proliferation was measured by the incorporation of ^{3}H-thymidine into DNA. Shown are the mean cpm incorporated and standard deviation from triplicate reactions.

Mean IC_{50} values from two independent experiments were 13.0 μM for cells activated with anti-CD3/PMA and 9.8 μM for cells activated with anti-CD3/anti-CD4/anti-CD28. There was no significant cellular toxicity (as measured by trypan blue exclusion) at concentrations of WIN 61651 up to 200 μM in the 1 day activation assay of CD4+ T lymphocytes. These results demonstrated that WIN 61651 inhibited IL-2 production by CD4+ peripheral blood T lymphocytes stimulated by antibodies to relevant cell surface proteins.

To determine whether WIN 61651 also inhibited antigen-driven T cell activation, we examined the effect of the compound in an MLR. As shown in Figure 4, WIN 61651 inhibited the MLR in a concentration dependent manner. The mean IC_{50} from two independent experiments was 6.5 μM. At concentrations of WIN 61651 $\leq$30 μM, there was no significant cellular toxicity as measured by trypan blue exclusion. Moreover, exogenous IL-2 was able to fully reconstitute cellular proliferation at concentrations of WIN 61651 up to 30 μM (Figure 4). These results demonstrated that the inhibition of the MLR by WIN 61651 at concentrations $\leq$30 μM was not due to toxicity and that WIN 61651 inhibited the activation of T lymphocytes rather than T cell proliferation *per se*. However, at 100 μM WIN 61651, exogenous IL-2 did not reconstitute proliferation, consistent with significant cellular toxicity (55% dead cells) at this concentration of WIN 61651 after 5 days in the MLR.

Very similar IC_{50} values for WIN 61651 were observed in the assay for IL-2 production by purified CD4+ T cells and in the MLR. It was somewhat surprising that the IC_{50} values in these functional T cell activation assays were considerably lower than those observed in the whole cell tyrosine kinase assay and even somewhat lower than those observed in cell-free p56^{lck} assays. There are several possible explanations: (1) The lower IC_{50} values in the functional assays may be due to some degree of cellular toxicity that does not affect trypan blue exclusion at these concentrations of WIN 61651. However, this is unlikely since exogenous IL-2 was able to fully restore the proliferative response at concentrations of WIN 61651 that were inhibitory in the MLR. (2) Another possibility is that WIN 61651 exhibits a lower IC_{50} for phosphorylation of a minor tyrosine-containing protein that is beyond the limit of detection in the whole cell kinase assay and that this minor phosphorylation is the crucial event that regulates T cell activation. (3) It is possible that WIN 61651 accumulates in the cells or is metabolized to a more active compound. (4) Due to the lack of complete specificity of WIN 61651 for p56^{lck}, it is possible that more than one tyrosine kinase or other ATP-requiring enzyme is inhibited in T cells by WIN 61651, resulting in synergistic effects and greater potency in the functional assays.

CONCLUDING REMARKS

This paper describes a new tyrosine kinase inhibitor WIN 61651, which is moderately selective for p56^{lck}, inhibits tyrosine kinase activity in whole cells, and inhibits T cell activation. This compound is competitive with ATP and therefore, unlike herbimycin, exhibits a conventional mechanism of action. Although not totally specific for p56^{lck}, WIN 61651 appears to be more selective for p56^{lck} than genistein and the tyrphostins.

Therefore, WIN 61651 offers advantages over other inhibitors previously used to study the role for tyrosine kinases in T cell activation and constitutes a new tool for studies on signal transduction in lymphocytes.

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