Effects of Geldanamycin, a Heat-Shock Protein 90-Binding Agent, on T Cell Function and T Cell Nonreceptor Protein Tyrosine Kinases


*J Immunol* 2000; 164:2915-2923; doi: 10.4049/jimmunol.164.6.2915

http://www.jimmunol.org/content/164/6/2915

Why *The JI*?

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

**References**

This article cites 46 articles, 21 of which you can access for free at: http://www.jimmunol.org/content/164/6/2915.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Effects of Geldanamycin, a Heat-Shock Protein 90-Binding Agent, on T Cell Function and T Cell Nonreceptor Protein Tyrosine Kinases

Peter D. Yorgin,* Steven D. Hartson, † Abdul M. Fellah,* Bradley T. Scroggins, † Wenjun Huang,† Emmanuel Katsanis,* Jeff M. Couchman,* Robert L. Matts,† and Luke Whitesell*  

The benzoquinoid ansamycins geldanamycin (GA), herbimycin, and their derivatives are emerging as novel therapeutic agents that act by inhibiting the 90-kDa heat-shock protein hsp90. We report that GA inhibits the proliferation of mitogen-activated T cells, GA is actively toxic to both resting and activated T cells; activated T cells appear to be especially vulnerable. The mechanism by which GA acts is reflected by its effects on an essential hsp90-dependent protein, the T cell-specific nonreceptor tyrosine kinase lck. GA treatment depletes lck levels in cultured T cells by a kinetically slow dose-dependent process. Pulse-chase analyses indicate that GA induces the very rapid degradation of newly synthesized lck molecules. GA also induces a slower degradation of mature lck populations. These results correlate with global losses in protein tyrosine kinase activity and an inability to respond to TCR stimuli, but the activity of mature lck is not immediately compromised. Although the specific protease inhibitor lactacystin provides marginal protection against GA-induced lck depletion, proteasome inhibition also induces changes in lck detergent solubility independent of GA application. There is no other evidence for the involvement of the proteasome. Lysosome inhibition provides quantitatively superior protection against degradation. These results indicate that pharmacologic inhibition of hsp90 chaperone function may represent a novel immunosuppressant strategy, and elaborate on the appropriate context in which to interpret losses of lck as a reporter for the pharmacology of GA in whole organisms. The Journal of Immunology, 2000, 164: 2915–2923.
that assess the effects of GA on T cell function and have determined the biochemical effects of GA on the nonreceptor tyrosine kinase p56^ck. In the current study, we demonstrate that in mouse splenocytes, as in human peripheral T cell populations, GA pretreatment compromises CD28 stimulation of production of IL-2 and IL-2R. We extend these studies further by demonstrating that splenocytes previously induced to proliferate via Con A and/or IL-2 stimulation cease to proliferate upon subsequent exposure to GA. Furthermore, the cytotoxicity of GA toward unstimulated and stimulated mouse splenocytes is characterized with regard to GA dose, duration, and kinetics. Finally, the biochemical effects of GA on the nonreceptor tyrosine kinase p56^ck are examined by Western blotting of kinase levels, assays of kinase activity, pulse-chase characterizations of nascent and mature kinase t1/2, and Western blot and pulse-chase characterization of the protective effects of proteasome and lysosome inhibitors. The results document and quantify the effects the GA on T cell physiology and describe the biochemical effects of GA on one class of critical T cell proteins.

Materials and Methods

Reagents

GA (m.w. = 560) was provided by the Drug Synthesis and Chemistry Branch of the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. Anti-#lck Abs were raised by the Hybridioma Center for the Agricultural and Biological Sciences (Oklahoma State University, Stillwater, OK) as polyclonal ascites fluid in mice repeatedly immunized with recombinant histidine-tagged human #lck (kindly provided by Dr. Paul Burn, Hoffmann-LaRoche, Nutley, NJ). Anti-#lck specificity was confirmed by Western blotting and by immunoadsorption of radiolabeled lysates prepared from cells positive or negative for expression of the full-length #lck gene product (Jurkat E6.1 and JCaM1.6, respectively). Anti-human-#lck Abs were from Upstate Biotechnology (Lake Placid, NY). Anti-actin Abs were from Pierce (Rockford, IL). The human T cell leukemia lines Jurkat E6.1 and JCaM1.6 were obtained from American Type Culture Collection (ATCC, Manassas, VA), and cultured as previously described (20).

Culture of T cells

Splenocyte mononuclear cells were obtained from 7-wk-old male DBA mice (Sprague Dawley, Indianapolis, IN) and purified by density-gradient centrifugation using Lympholyte M (Cedar Lane Laboratories, Ontario, Canada). Isolated splenocytes were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 50 IU penicillin, 50 #g/ml streptomycin, 100 mM gentamicin, 1 mM sodium pyruvate, 1 #g/ml nonessential amino acids, and 50 #g/ml mercaptoethanol.

To prepare activated T cells, splenic mononuclear cells from DBA mice were used as donors. Equal numbers of each subpopulation resulting in 50% maximum counts were diluted with either 100 IU/ml IL-2 (a kind gift of Dr. Emmanuel Akporiaye, University of Arizona, Tucson, AZ), 0.5 #g/ml Con A, or 10 IU/ml IL-2 + 0.5 #g/ml Con A. Culture of mononuclear cells for a period of 48 h with Con A yields a predominantly T cell population (21).

Assessment of T cell mortality, proliferation, and activation

To assess mononuclear cell survival rates during GA exposure, mouse lymphocyte and Jurkat cell suspensions were stained with trypan blue and counted on hemocytometer grids. T cell proliferation was assayed by incubation with [3H]thymidine for 15 h, and was reported as a stimulation index representing incorporation of [3H]thymidine in a given condition divided by the incorporation seen with media alone.

IL-2 production in response to CD3 and CD28 stimulation was quantified using a bioassay. After pretreatment with GA and DMSO as indicated, equal numbers of viable splenic mononuclear cells were stimulated for 18 h with and without anti-CD3 and anti-CD28 Abs to evaluate IL-2 production. Since the addition of GA in the supernatant inhibits CTL2-2-proliferation, the splenic mononuclear cells were pretreated with GA and washed before use. The IL-2-dependent murine T cell line, CTL2-2 (ATCC), was used to determine the amount of IL-2 bioactivity present in the supernatants (22). The IL-2 bioactivity was quantified by comparing the number of serial dilutions of supernatant to the 50% maximum counts of [3H]thymidine incorporated in the CTL2-2 cells. The concentration of IL-2 in the supernatants was derived from a standard curve using known concentrations of IL-2.

IL-2R (CD25) levels in CD3/CD28-activated cells were determined by flow cytometry using a FITC anti-CD25 Ab (PharMingen).

Preparation and analyses of cell lysates

Splenocytes were lysed in PBS containing 10% glycerol, 1% Tween 20, 12 mM sodium deoxycholic acid, 0.1% SDS, 1 mg/ml PMSF, 200 #g/ml apro- tin and 200 #g/ml leupeptin and were clarified at 14,000 × g for 20 min at 4°C. Protein concentrations were determined relative to a BSA standard curve using a BCA protein reagent kit (Pierce). Jurkat cells were collected by a single centrifugation at 400 × g without washing, by boiling in SDS-PAGE sample buffer, and analyzed by Western blotting. Alternatively, RIPA lysates were prepared as previously described (23), and detergent-insoluble vs detergent-soluble fractions were separated by centrifugation at 12,000 × g for 5 min at 4°C. Detergent-insoluble pellet fractions were solubilized by boiling in reducing SDS-PAGE sample buffer.

To assay PTK activities, splenic mononuclear cells were lysed in 20 mM Tris (pH 7.4), 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 1 #g/ml pepstatin, 0.5 #g/ml leupeptin, 0.2 mM Na3VO4, and 5 mM mercaptoethanol, and PTK activity was quantitated using an immunoassay (Calbiochem, Cambridge, MA), as per the manufacturer’s protocol. Alternatively, #lck kinase activity was determined by kinetic assays performed in the presence of [γ-32P]ATP and acid-denatured enolase, as previously described (23).

Cell lysates were analyzed by Western blotting using standard chemiluminescent or colormetric techniques. Autoradiography was performed by transfer of protein from SDS-PAGE gels to polyvinylidene difluoride membrane and subsequent exposure directly to x-ray film.

Pulse-chase analyses of #lck molecules in GA-treated cells

For analyses of newly synthesized #lck molecules, Jurkat cells were incubated for 1 h in medium containing 3.6 #g/ml GA. After incubation, cells were starved for 30 min at 37°C in Met/Cys-deficient medium lacking GA before a pulse with [35S]Met/Cys for 20 min. Radiolabeling was terminated by centrifugation and resuspension of cells in replete medium. Cells were incubated at 37°C for the indicated chase times. Molecular analyses of mature #lck molecules, cells were radiolabeled for 40 min in Met/Cys-deficient medium containing [35S]Met/Cys, but lacking GA. Radiolabeling was terminated by centrifugation of cultures and resuspension of cells in replete medium. Cultures were further incubated at 37°C for 3–4 h. GA was then added to 3.6 #g/ml, and cultures were incubated at 37°C for the indicated chase times.

To isolate [35S]labeled, clarified cell lysates were prepared as described previously (24). Clarified lysates were adjusted to 1% SDS/10 mM DTT and boiled for 5 min to disrupt protein-protein interactions and to enhance the specificity of subsequent immunoadsorptions (25). Boiled lysates were cooled slowly, diluted 3-fold with lysis buffer, precleared with 15 μl each of 10% Pansorbin (Calbiochem), and immunoadsorbed with anti-#lck Abs and fresh Pansorbin. Immunoprecipitates were washed four times with lysis buffer supplemented to 0.3% SDS/3 mM DTT, eluted in reducing SDS-PAGE sample buffer, and analyzed by reducing SDS-PAGE and autoradiography.

Data analysis

Lck levels were quantified by computerized densitometry using Molecular Analyst (version 1.4; Bio-Rad, Richmond, CA). Lck levels were normalized to simultaneously blotted actin on at least two separate gels. Statistical analysis was conducted using Student’s t test; statistically significant differences between conditions were considered to be those with a p value of <0.05.

Results

Pretreatment with GA inhibits subsequent T cell activation

To establish a benchmark relationship between our current studies and previous studies (7, 8) that examined the biological effects of pretreating peripheral human T cells with GA, mouse splenic T cells were pretreated with GA before their stimulation with anti-CD3 and anti-CD28 Abs. In these assays, the ligation-induced production of IL-2 and IL-2R was assessed. Pretreatment of splenic mononuclear cells with GA resulted in a statistically significant (p < 0.01) 96% inhibition of IL-2 production in response to TCR and CD28 receptor ligation: IL-2 concentrations in the supernatants of GA-treated cells were 0.675 ± 0.075 IU/ml compared with 17.625 ± 1.125 IU/ml in DMSO-treated (control) cells. Similarly,
in assays of TCR/CD28 stimulation of production of the CD25 IL-2R by splenocytes, the number of control cells producing IL-2R in response to TCR/CD28 ligation was significantly (p < 0.01) reduced by ~40%: 68.57 ± 2.82% of untreated cells were CD25 positive, 67.78 ± 3.78% DMSO-treated (vehicle control) cells were CD25 positive, and 39.87 ± 3.51% of GA-treated cells were CD25 positive. These results established that activation of splenic mouse mononuclear cells was inhibited by pretreatment with GA, as was previously reported for human peripheral lymphocytes.

**GA treatment after mitogen activation inhibits T cell proliferation**

Although our results and previous work (7, 8) indicated that pretreatment of lymphocytes with GA inhibited subsequent activation via TCR or CD28 ligation, we wished to extend these characterizations to other aspects of T cell physiology. Specifically, we hypothesized that late addition of GA to activated T cells would arrest T cell proliferation. For these studies, splenic mouse mononuclear cells were cultured for 57 h in cell medium containing IL-2 and/or Con A, but lacking GA. Subsequent to this activation, the effects of GA on cellular proliferation were assessed via addition of GA concurrent with [3H]thymidine. Preactivated splenocytes had been incubated for 15 h in the presence of [3H]thymidine and GA (72 h of culture total), cell proliferation was assessed via quantitation of thymidine incorporation (reported as stimulation index). Comparison of thymidine incorporation by mitogen-activated T cells vs unstimulated T cell populations indicated that the mitogen treatments induced T cell proliferation (Table I), and this proliferation was not affected by the drug vehicle DMSO. In contrast, GA levels greater than 17 nM significantly (p < 0.01) inhibited the proliferation of previously activated T cells in a dosage-dependent fashion (Table I). This inhibition of proliferation was observed for cells preactivated by IL-2, Con A, or both mitogens. Therefore, GA caused a dose-dependent inhibition of the ability of activated splenocytes to replicate their DNA, even when applied subsequent to mitogen activation.

The effects of GA on thymidine incorporation by T cells could have resulted from a simple inhibition of T cell proliferation. Alternatively, GA may have been actively toxic to proliferating T cells. To assess the potential effects of GA on proliferation and mortality, preactivated splenocytes were treated with GA for 18 h, subsequently stained with the vital dye trypan blue, and counted on a hemocytometer grid. In these assays of activated cells, GA inhibited mitogen-induced increases in cell number in a dosage-dependent fashion, with an ~50% inhibition observed at the maximum dosage of 1700 nM (Table II, top). After 18-h culture in the presence of high dosages of GA, actively dividing T cell populations demonstrated enhanced cell mortality relative to dividing cells that were not treated with GA. For example, the absolute number viable of Con A- and IL-2-stimulated cells were statistically significantly lower ([p < 0.01] when treated with 170 (2.60 ± 0.10 × 10^5/well) and 1700 nM (1.39 ± 0.12 × 10^5/well) GA when compared with nontreated (7.09 ± 0.73 × 10^5/well) or DMSO (4.76 ± 0.15 × 10^5/well)-treated cells. Thus, when applied subsequent to mitogen activation, GA inhibited the ability of activated splenocytes to divide and also compromised their viability.

In this previous experiment (Table II, top), experimental design led to high background mortality rates in populations of unactivated splenocytes, thus compromising our ability to assess the effects of GA on resting cell populations. To determine the effects of GA on resting T cell populations more accurately, mouse splenic mononuclear cells were isolated and incubated in cell culture media containing FBS, but lacking IL-2 and/or other mitogens; GA was included or omitted from this media, as indicated. Although the expected drug-independent decreases in cell viability were evident with increasing culture times, GA at dosages from 17 to 1700 nM did not significantly diminish the absolute number of mononuclear cells (cell density × viability) observed following exposures of 5- to 15-h duration (Table II, middle). Only upon longer exposures to GA (24–72 h) was the viability of resting T cells significantly (p < 0.01) diminished; this diminished viability was dosage dependent (Table II, bottom). As a second measure of resting T cell vigor, thymidine incorporation in GA-treated cells was assessed. In these resting T cells, GA (17–1700 nM) did not significantly diminish background levels of thymidine incorporation (Table I; these studies were performed as described for, and concurrent with, those described above for mitogen-activated T cells). Thus, resting T cells differed from activated T cells in the degree to which they were vulnerable to GA cytotoxicity: treatment of activated T cells with GA caused significant cell mortality, but similar durations of drug exposure did not similarly increase mortality in populations of resting lymphocytes.

**GA treatment leads to depletion of cellular tyrosine kinases**

In the studies of June et al. (18), pretreatment of peripheral human T cells with herbimycin A inhibited TCR-stimulated enhancement of tyrosine phosphorylation and depleted cellular levels of the T cell kinases p59fyn and p56lck. We extended such characterizations by treating resting splenic mononuclear cells with GA and assessing the net PTK activity in the absence of TCR stimulation. In resting mouse splenocytes treated with GA (1.7 μM), total PTK activity was significantly (p < 0.01) reduced by 47 ± 5.5% relative to untreated cells or 52.7 ± 5.3% to cells treated with DMSO. In an attempt to explain this decrease in PTK activity, the cellular levels of the T cell tyrosine kinases lck and fyn were examined by

---

### Table I. Effect of GA upon T cell proliferation in response to mitogens

<table>
<thead>
<tr>
<th>Dose</th>
<th>Stimulation index</th>
<th>p value</th>
<th>Stimulation index</th>
<th>p value</th>
<th>Stimulation index</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1 ± 0.41</td>
<td>NS</td>
<td>70.48 ± 4.34</td>
<td>NS</td>
<td>196.61 ± 33.72</td>
<td>NS</td>
</tr>
<tr>
<td>DMSO</td>
<td>1.54 ± 0.65</td>
<td>NS</td>
<td>62.11 ± 11.23</td>
<td>&lt;0.01</td>
<td>170.74 ± 28.89</td>
<td>NS</td>
</tr>
<tr>
<td>17</td>
<td>0.76 ± 0.38</td>
<td>NS</td>
<td>19.55 ± 7.28</td>
<td>&lt;0.01</td>
<td>84.64 ± 23.75</td>
<td>NS</td>
</tr>
<tr>
<td>170</td>
<td>1.10 ± 0.55</td>
<td>NS</td>
<td>5.63 ± 2.52</td>
<td>&lt;0.01</td>
<td>22.10 ± 4.33</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>1700</td>
<td>1.01 ± 0.60</td>
<td>NS</td>
<td>2.96 ± 0.85</td>
<td>&lt;0.01</td>
<td>15.5 ± 3.57</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* A total of 10^6 DBA splenic mononuclear cells/well were cultured for 72 h with either cell growth media alone, 100 U/ml of IL-2, 0.5 μg/ml of Con A, or 10 U/ml IL-2 + 0.5 μg/ml of Con A, [3H]thymidine (1 μCi) and GA (17 nM, 170 nM, and 1700 nM), DMSO, or RPMI growth medium were added for 15 h prior to harvesting. There were six replicates of all conditions. Proiferative responses are reported as a stimulation index which is the [3H]thymidine incorporated in a given condition divided by the [3H]thymidine incorporated with medium alone. The results shown are from a typical experiment (n = 3). Statistical analysis was performed using Student’s t test. NS, not significant.

* GA dosage expressed in nM.
Western blotting. In resting splenic mononuclear cell treated with 1.7 mM GA for 15 h, GA treatment resulted in marked depletion of these kinases from cell lysates (Fig. 1A).

To extend these characterizations of GA-mediated depletion of T cell PTK, p56\(^{lck}\) levels were examined in resting splenocytes exposed to various concentrations of GA or for various

**Figure 1.** A, \(lck\) and \(fyn\) levels detected by Western blotting of lysates from GA-treated and control splenic mononuclear cells. Cells were lysed by boiling in SDS-PAGE sample buffer containing 2.5% SDS. Total cellular protein (35–75 \(\mu\)g) was applied to each lane of a 7.5% polyacrylamide gel. After transfer to nitrocellulose and incubation with primary and secondary Abs, immunoreactivity was detected by using the chemiluminescent HRP substrate, Luminol. Actin was used as a control for the amount of protein loaded in each lane. Migrations of size standards are indicated along the left side of each panel and Luminol. Actin was used as a control for the amount of protein loaded into each lane. Migrations of size standards are indicated along the left side of either cell culture media (NT), negative control), DMSO at a volume equal to that of GA (lane 2), or 1.7 mM GA (lane 3). \(lck\) and \(Fyn\) levels in GA-treated DBA splenic mononuclear cells are shown on the left and right side of the panel, respectively. The effect of differing concentrations of GA on resting splenic mononuclear cell \(lck\) levels is shown in B. Cells were either treated for 1 h, washed, and incubated in GA-free media for 23 h, or incubated continuously for 24 h. Cells were cultured in media alone (NT); DMSO (DM); or 17 nM (17), 170 nM (170), or 1.7 mM (1700) GA. To determine when \(lck\) levels decreased relative to GA treatment, mononuclear cells were continuously treated with 1.7 mM GA for 1, 6, 12, or 24 h, then harvested and lysed immediately. Nontreated cells were used as a control. D, Effect of GA treatment on \(lck\) levels in activated splenic mononuclear cells. The effect of GA upon IL-2- and Con A-stimulated cells is shown. Splenocytes were activated with 100 IU/ml IL-2 or 0.5 mg/ml Con A for 24 h before (15 h) treatment with 1.7 mM GA or a similar volume of DMSO.
periods of time (Fig. 1B). Continuous exposure of unstimulated splenocytes to a range of GA concentrations for 24 h reproducibly induced dosage-dependent decreases in p56<sup>lck</sup> levels. For the highest GA concentration tested (1700 nM), quantification of band intensities indicated that p56<sup>lck</sup> levels declined to 35.3 ± 14.1% (±SD, n = 3) of those observed in DMSO-treated splenocytes. Similarly, in cells treated with one-tenth as much GA (170 nM), <i>lck</i> levels were 53.7 ± 23.1% (±SD, n = 3) of those observed in DMSO-treated cells. In contrast, p56<sup>lck</sup> band intensities did not reproducibly decrease in cells treated with 17 nM GA. These results established dosages necessary for overnight depletion of these kinases.

To examine the kinetics of GA-mediated <i>lck</i> loss in resting splenic mononuclear cells, <i>lck</i> levels were evaluated at various time points after GA treatment. When unstimulated splenocytes were exposed to various concentrations of GA for 1 h, followed by drug washout and subsequent cultivation in drug-free media, significant reproducible decreases in <i>lck</i> band intensity were not apparent (not shown). When resting splenocytes were continuously exposed (no drug washout) to 1700 nM GA for various periods of time, p56<sup>lck</sup> levels did not drop immediately upon drug treatment, but instead declined steadily, most significantly at 12 and 24 h of GA treatment (Fig. 1C). These characterizations were extended to activated splenocytes. In splenocytes preactivated by a 24-h exposure to Con A or IL-2, a subsequent 15-h exposure to GA induced loss of <i>lck</i> protein levels (Fig. 1D).

### The effects of GA can be modeled in Jurkat cultures

To further extend our characterizations of the toxic and antikinase activities of GA, we performed similar characterizations in an alternative T cell system, the Jurkat human T cell leukemia. GA treatment of Jurkat cultures induced cell mortality consistent with the mortalities observed for cultured mouse splenocytes (not shown). GA also increased the fragility of Jurkat cells, leading to increased losses in cell number during sequential centrifugation washes relative to untreated cell cultures (not shown). Consequently, experimental protocols were adjusted such that Jurkat cells were subjected to minimum handling. In Jurkat cells, as was observed in mouse splenocytes, exposure to GA for 15 h reproducibly depleted <i>lck</i> levels (not shown). Shorter durations of exposure (8 h) induced dose-dependent reductions in <i>lck</i> levels, but with variation among experiments (not shown). Because cultured Jurkat cells behaved in a fashion qualitatively equivalent to cultured splenocytes, this cell system represented an appropriate model system in which to examine the biochemical mechanisms underlying GA-induced depletion of <i>lck</i> from splenic mononuclear cells.
pretreated with GA was very unstable, with $\alpha$-methyl cells. Jurkat cells were cultured for 20 h in the presence of GA (1.3 μM) and/or lac-tactacin (10 μM), as indicated. Lysates from 2.5 × 10^6 cells were applied to each lane, and lck was detected by Western blotting with anti-p56lck Abs. Migrations of protein size standards (left side of panel) and lck are indicated. Total lck levels were determined by lysing the Jurkat cells in boiling lysis (A). Cells were lysed in cold RIPA buffer and clarified by centrifugation to generate detergent-soluble (B) and detergent-insoluble (C) fractions.

GA induces the rapid degradation of newly synthesized lck

In GA-treated T cells, depletion of lck could have resulted from inhibition of an hsp90 function essential to either kinase biogenesis, kinase stability, or from indirect effects of GA on other hsp90-dependent cellular processes. To discriminate among these possibilities, the effects of GA on lck $t_{1/2}$ were assessed. For these assessments, anti-lck Abs were used to immunoadSORb 35S-labeled lck from cell lysates prepared from radiolabeled cells. These immunoadsorptions reproducibly recovered a single predominant 35S-labeled protein from immunoadsorption reactions (Fig. 2, A, B, and D). This predominant 35S-labeled protein was identified as lck on the basis of its $M_r$ and its coelectrophoresis with lck detected by Western blotting (not shown). This identification was confirmed by the specific immunoadsorption of 35S-labeled lck from lysates from lck-positive Jurkat E6.1 cells vs the truncated lck gene product (26) that was recovered by immunoadsorption of J.CaM1.6 cells (not shown).

In DMSO-treated cells radiolabeled by pulse-chase technique, the amounts of 35S-labeled lck detected by immunoadsorption of lysates declined only slightly following chase incubation for 80 min (Fig. 2A). In contrast, 35S-labeled lck produced in T cells pretreated with GA was very unstable, with $\sim$50% of 35S-labeled lck lost within 40 min and with 35S-labeled lck content continuing to decline thereafter (Fig. 2A). When chase incubations were extended to 120 min, 35S-labeled lck content declined to levels that were nearly undetectable relative to levels of lck detected in untreated cells (not shown). Thus, in the absence of (GA-inhibitable) hsp90 function, newly synthesized lck molecules were dramatically unstable.

GA induces the slow degradation of mature lck

To determine whether mature molecules of lck similarly required hsp90 function, GA treatment and radiolabeling were performed in an order inverse of that described above. Specifically, Jurkat cultures were pulse labeled in the absence of GA and radiolabeling was chased subsequently via a 3-h incubation in replete medium lacking GA. After this chase of radiolabeling to allow maturation of kinase molecules, cells were treated with GA and 35S-labeled lck levels were assessed at 2-h intervals. In contrast to the results obtained for newly synthesized 35S-labeled lck (Fig. 2B), mature 35S-labeled lck was equally stable in GA-treated cells as in control cells (Fig. 2B). This result did not reflect saturation of the binding capacity of the anti-lck immunoadsorptions (not shown). Thus, the $t_{1/2}$ of mature 35S-labeled lck molecules was not decreased during 8-h treatments of Jurkat cells with GA. To determine whether the function of mature lck molecules required hsp90 support within this time frame, Jurkat cultures were treated with GA for 3 h, lck was isolated from these cells by immunoadsorption, and lck kinase activity was assessed in vitro by kinetic assays of kinase activity. These assays indicated that GA did not directly inhibit lck kinase activity nor otherwise directly compromise the function of mature lck molecules (Fig. 2C).

To examine lck $t_{1/2}$ on the time frame that was used to assess lck levels via Western blotting, pulse-chase characterizations of lck $t_{1/2}$ were examined over a 24-h period, with collection and analysis of cell lysates at 6-h intervals (Fig. 2D). These characterizations indicated that within this longer time frame, GA increased the rate of lck degradation $\sim$2-fold, with accelerated loss becoming most evident at late periods of culture. Thus, in GA-treated Jurkat cells, both nascent and mature lck were targeted for degradation; however, the effects of GA treatment were much more rapid and marked for nascent lck than for mature molecules.

Proteasome inhibitors have complex effects

Previous work on other proteins had suggested a linkage between GA treatment and degradation of target proteins via the proteasome (5, 7, 11, 14, 27–29). To determine whether proteosomal degradation mediated the loss of lck from GA-treated T cells, Jurkat cultures were treated with GA and/or the highly specific proteosome inhibitors lactacystin or its activated analogue clasto-lactacytin β-lactone (30). After 20-h incubation in the presence of these drugs, lck levels were assessed by Western blotting. When total cell lysates were prepared by directly boiling treated Jurkat cells in reducing SDS-PAGE sample buffer, subsequent Western blot analyses suggested that clasto-lactacytin β-lactone slightly

FIGURE 3. Effects of GA and clasto-lactacytin on lck levels in Jurkat cells. Jurkat cells were cultured for 20 h in the presence of GA (1.3 μM) and/or lac-tactacin (10 μM), as indicated. Lysates from 2.5 × 10^6 cells were applied to each lane, and lck was detected by Western blotting with anti-p56lck Abs. Migrations of protein size standards (left side of panel) and lck are indicated. Total lck levels were determined by lysing the Jurkat cells in boiling lysis (A). Cells were lysed in cold RIPA buffer and clarified by centrifugation to generate detergent-soluble (B) and detergent-insoluble (C) fractions.

FIGURE 4. Effect of lactacystin and bafilomycin on lck levels. Jurkat cells were treated with media alone (lane 1), 2 μM bafilomycin (lane 2), or 10 μM lactacystin (lane 3) for a duration of 3 h. Jurkat cells were also treated for a duration of 15 h with media alone (lane 1), 1 μM bafilomycin (lane 2), 1.7 μM GA (lane 3), 10 μM lactacystin (lane 4), 2 μM bafilomycin + 1.7 μM GA (lane 5), and 10 μM lactacystin + 1.7 μM GA (lane 6).
To further examine the mechanism by which lck molecules were degraded in the presence of GA, we utilized the rabbit reticulocyte lysate model system. This system was chosen for three reasons. 1) The rabbit reticulocyte lysate model system had been shown to contain robust and active ubiquitination machinery and to be capable of proteosome-mediated degradation (31–34). 2) The rabbit reticulocyte lysate model system was free of cellular features, which complicated our efforts to establish direct cause and effect relationships in vivo, e.g., changes in the cell cycle status. 3) In vitro translation in rabbit reticulocyte lysates had been used previously to demonstrate that biogenesis of lck in the presence of GA alters the normal interaction of lck with hsp90, thus producing lck molecules that are deficient in stable tertiary structure and phosphotransferase activity (16, 17). Thus, this system was an appropriate system in which to model physical and functional associations between hsp90 and substrate kinases.

When lck molecules were synthesized in lysates lacking GA, levels of lck molecules produced were stably maintained upon subsequent incubations for up to 3 h at 30°C (Fig. 5A). This stable maintenance did not reflect a balance between synthesis and degradation because protein synthesis in this system reproducibly ceases after 30-min incubation at 30°C. Inclusion of GA in these protein synthesis reactions (30 μM) did not alter the levels of lck thus maintained. This result was consistent with previous analyses of lck molecules incubated in GA-treated protein syntheses reactions in which protein synthesis had been arrested by protein synthesis inhibitors (16, 17). Addition of clasto-lactacystin β lactone to GA-treated or GA-free lysates did not alter the levels of lck maintained during the 3-h chase incubations. Similarly, there was no evidence for lactacystin-induced ladders of high M species of lck protein that may have represented polyubiquitinated lck (Fig. 5B). Given the competence of ubiquitination and proteosome machinery previously documented to exist in this system (30–33), these results strongly argued that ubiquitination and proteosomal degradation were not directly nor obligatorily coupled to inhibition of hsp90 function in this cell-free system.

**Discussion**

Our results confirm that GA is a potent inhibitor of T cell activation, and we extend earlier basic characterizations by demonstrating that GA is actively toxic to both resting and activated T cells. These toxic effects occur at dosages that can be achieved by i.p. injection of GA into mice (34). We also demonstrate that depletion of the cellular kinase lck accompanies GA treatment. However, the negative effects on T cell viability and proliferation are unlikely to be solely attributable to loss of nonreceptor PTK because many signal transduction proteins have been shown to be vulnerable to GA treatment in vivo (i.e., receptor PTK,raf, steroid hormone receptors,erbB, p53, et al.) (5–17, 27–29). Thus, our work, like previous studies, does not imply any single hsp90-dependent signal transduction protein as the sole underlying agent of the effects of GA.

Our results indicate that GA may be especially toxic to rapidly proliferating cells. This conclusion is based on our observation that 15-h treatment of resting T cells has no significant impact on cell mortality, but that similar treatment of activated T cells results in significant decreases in cell viability (Table II, top vs bottom). Consistent with this observation, GA appears to exert selective tumoricidal activity (35). In further support of the selective potential of GA, Lele et al. (37) have observed that GA causes the development of abnormal zebrafish embryos that lack specific discrete populations of cells. We further speculate that the selective toxicity of GA may reflect the activated nature of signal transduction networks in proliferating cells and the delicate balance of the

**FIGURE 5.** Effects of GA and clasto-lactacystin in reticulocyte lysate. In vitro translation reactions were programmed with template for lck synthesis in the presence of [35S]Met; GA (lanes 5–8, 13–16) and clasto-lactacystin β lactone (lanes 9–16) were added as indicated. Translation reactions were incubated at 30°C. Aliquots of each translation were removed 30 min (1, 5, 9, 13), 1 h (2, 6, 10, 14), 2 h (3, 7, 11, 15), and 3 h (4, 8, 12, 16) after the initiation of protein synthesis. Each aliquot was immediately mixed with 50 vol of hot 2X SDS-PAGE sample buffer and boiled. Samples were assessed by SDS-PAGE and autoradiography. A. Exposure of film for 4 h. B. Exposure of film for 3 days. Positions of full-length lck (ori) and top edge of the separating gel (ori) are indicated. Migrations and molecular mass of size standards (in kDa) are indicated along the left edge of each panel.

reduced the magnitude of GA-induced depletion of lck from total cell lysates (Fig. 3A). However, when detergent-soluble fractions (RIPA lysates) of treated cells were prepared without boiling, Western blotting with anti-lck Abs suggested that clasto-lactacystin β-lactone did not prevent GA-induced depletion of lck (Fig. 3B).

To resolve these discrepancies, we examined lck levels in the detergent-insoluble fractions of these samples. This comparison revealed that clasto-lactacystin β-lactone induced the nearly quantitative recruitment of lck from the detergent-soluble fractions to detergent-insoluble fractions (Fig. 3, B vs C). However, GA did not similarly alter the detergent solubility of lck. The clasto-lactacystin β-lactone-induced recruitment of lck to the detergent-insoluble fraction was neither dependent upon, nor inhibited by, treatment with GA. In the detergent-insoluble population of lck molecules produced in cells treated with both GA and clasto-lactacystin β-lactone, a faint band representing a minor subpopulation of lck molecules with retarded electrophoretic mobility was apparent (Fig. 3C); the origin and nature of this altered electroform were not investigated further. These results indicated that the primary effect of clasto-lactacystin β-lactone was to recruit lck to the detergent-insoluble subcellular structures.

Because inhibition of proteosome function did not unequivocally address the proteolytic fate of lck in GA-treated cells, another potential mechanism of lck degradation was examined. For this examination, cells were treated concurrently with GA and bafilomycin, the second compound acting as a potent inhibitor of lysosomal function (Fig. 4). Bafilomycin treatment for durations of 3 and 15 h did not alter levels of detergent-soluble lck. However, bafilomycin provided marked protection against GA-mediated depletion of lck levels. This result indicated that inhibition of lysosomal function protected lck molecules from degradation in GA-treated T cells.
functions of hsp90-dependent proteins within these networks. Further work will be necessary to fully characterize the discriminatory potential of GA relative to activated, resting, and neoplastic cell populations. It will also be critical to determine the nature of the cytotoxic effects of GA and whether pathways of programmed cell death are activated or inhibited by this and related compounds. Nevertheless, studies presented in this work suggest that it may be possible to adjust GA treatment regimens to selectively deplete activated cell populations.

We examined the effects of GA with regard to one critical T cell protein, the nonreceptor T cell-specific tyrosine kinase p56\(^{lck}\). Physical and functional interactions between p56\(^{lck}\) and GA’s target, hsp90, have been well documented in cell-free systems and in transformed fibroblast cell lines (16, 17, 37, 38). Additionally, a physical association between lck and hsp90 has been demonstrated in T cell lysates (16), and the effects of herbimycin A on p56\(^{lck}\) levels in cultured T cells have been determined (18). In our current work, we have characterized the in vivo effects of GA in detail. We find that total p56\(^{lck}\) levels are depleted by GA treatment in a dosage-dependent fashion regardless of T cell activation status, and that the kinetics of this depletion is relatively slow.

Furthermore, we demonstrate that depletion of p56\(^{lck}\) levels reflects accelerated degradation of p56\(^{lck}\) molecules. This finding is consistent with the work of Uehara et al., who observed that herbimycin A accelerates the degradation of a temperature-sensitive version of the oncogenic viral src kinase −2-fold (39). Ansamycin-accelerated degradation has also been reported for several other hsp90-dependent proteins (5, 9, 12, 27, 29, 40). However, few studies to date have attempted to differentiate this effect with regard to newly synthesized vs temporally mature protein molecules. We find the kinetics of lck degradation varies depending on the population of kinase molecules analyzed.

The variable kinetics of lck degradation that we observe indicates that GA-induced depletion of p56\(^{lck}\) has two underlying causes: 1) an early dramatic crippling of p56\(^{lck}\) biosynthesis, resulting in a failure to replenish p56\(^{lck}\) levels; and 2) a less dramatic acceleration of the degradation of mature p56\(^{lck}\). Based on the immediate and nearly quantitative nature of the first effect and on previous modeling in cell-free systems (16, 17, 37, 38), we conclude that the effects of GA on newly synthesized p56\(^{lck}\) are direct; synthesis of the kinase in the absence of hsp90 support results in kinase molecules that are immediately and directly targeted for proteolysis. In contrast, the biochemical phenomena underlying the depletion of temporally mature p56\(^{lck}\) molecules are more difficult to assess. We note that depletion of mature p56\(^{lck}\) is most pronounced at times subsequent to 8 h of GA treatment. At this time, cellular physiology would be predicted to be grossly altered, and thus the depletion of p56\(^{lck}\) at late points in GA treatment may reflect secondary effects of hsp90 inhibition. Alternatively, temporally mature p56\(^{lck}\) molecules have been observed to have a conditional dependence on hsp90 support in a cell-free model system (17). Thus, mature p56\(^{lck}\) molecules may have a direct need in vivo for hsp90 support that is quantitatively less urgent than that of nascent p56\(^{lck}\) molecules. Similarly, alterations in p56\(^{lck}\) biosynthesis in conjunction with altered cell physiology might create populations of mature p56\(^{lck}\) molecules with enhanced need for direct hsp90 support (17).

Benzoxazinoid ansamycin has been reported to accelerate the degradation of various signal transduction molecules via ubiquitination and proteolysis (5), including receptor tyrosine kinases (27), the c-erbB-2 gene product (28), long-lived mutant versions of the p53 tumor suppressor protein (14), the raf kinase (11), and the cystic fibrosis transmembrane receptor (29). However, three observations compel us to reserve judgment regarding the possibility that ubiquitination and proteosomal degradation represent the mechanism by which p56\(^{lck}\) is degraded in GA-treated T cells.

Our first reservation is based on the observation that inhibition of the proteosome has unexpected complex effects on the biochemistry of p56\(^{lck}\), and these unexpected effects are independent of GA application. Specifically, inhibition of the proteosome induces alterations in p56\(^{lck}\) detergent solubility (Fig. 3). Such effects are not without precedent: raf is recruited to Nonidet P-40-insoluble cell fractions upon treatment of cells with lactacystin and GA (11). More strikingly, long-lived mutant p53 molecules are recruited to Nonidet P-40-insoluble fractions upon treatment of cells with proteosome inhibitors, and this effect occurs independent of GA treatment (14). Thus, the unexpected pleiotropic effects of proteosome inhibition (41–43) suggest that the minor lactacystin-induced protection that we observe may be indirect, reflecting an altered subcellular localization rather than a direct protection of lck from the proteosome per se.

Our second reservation regarding the fate of p56\(^{lck}\) in GA-treated cells arises due to the recent suggestion that the src family tyrosine kinases p56\(^{lck}\) and blk are physically associated with the E6AP E3 ubiquitin ligase, and that blk may be degraded by ubiquitination and proteosomal degradation upon kinase activation (44). This observation would predict that p56\(^{lck}\) may undergo obligate processing by ubiquitin and proteosome machinery irrespective of GA treatment. Thus, the minor protective effects of proteosome inhibition that we observed might again be indirect. Lck levels may be enhanced by inhibiting the GA-independent processing of lck by proteosome machinery.

Our third reservation arises from cell-free modeling studies such as those presented in Fig. 5. In these studies, we utilized rabbit reticulocyte lysate in vitro translation reactions (RRL) in attempts to demonstrate biochemical effects characteristic of ubiquitination and/or proteosomal degradation. However, despite the presence of active ubiquitination and proteosome machinery in reticulocytes and in RRL (31–34), p56\(^{lck}\) levels are maintained at steady state levels in the presence of GA during prolonged incubations. Furthermore, addition of GA and/or proteosome inhibitor does not result in a ladder or high Lck smear characteristic of polyubiquitinated p56\(^{lck}\) molecules. These observations indicate that, in this model system, ubiquitination and proteosomal degradation are not direct consequences of GA’s inhibition of hsp90 function. However, RRL may lack components of the ubiquitin/proteasome system that may be necessary for lck degradation, e.g., lck-specific E3 ligases. Alternatively, our inability to demonstrate stoichiometrically significant ubiquitination of p56\(^{lck}\) as a consequence of inhibited hsp90 function may represent the technically difficult nature of such studies. Nonetheless, our inability to model this process in a system that contains the active components putatively involved compels us to reserve judgment regarding the involvement of these components in the GA-induced degradation of p56\(^{lck}\) in vivo.

In contrast to ubiquitination and proteosomal degradation, the results from both the RRL model system and from treatment of cell cultures with bafilomycin (Fig. 4) and ammonium chloride (data not shown) are consistent with the possibility that lysosomal degradation pathways represent one fate of p56\(^{lck}\) molecules in GA-treated cells. When RRL are prepared, they are carefully clarified to remove membranous structures, and RRL would thus be predicted to lack lysosomes. Furthermore, the in vivo protective effects of lysosomal inhibitors were substantive (Fig. 4), implicating these proteolytic structures in GA-induced degradation in vivo. Our observation that inhibitors of lysosomes and proteasomes both protected p56\(^{lck}\) from degradation in GA-treated cells is consistent with a recent study demonstrating that brezelfdin A inhibition of the maturation of connexin43 results in loss of connexin43 immunoreactivity and that this loss can be prevented by inhibition of the proteasome or of lysosomes (45). As an alternative to putative lysosomal degradation, a recent paper by Matsuda et al. (46) suggests that
an hsp90cdc537-dependent mutant of the ZAP70 tyrosine kinase is normally degraded by a novel proteasome-independent pathway.

In summary, GA complicates the function and viability of T cells, and this compound may have selective cytotoxic effects depending on cell activation status. The differential effect of GA on activated and resting cells may have therapeutic implications for disease processes that are characterized by cell proliferation and/or T cell activation. The mechanism by which this compound acts is reflected by its effects on the hsp90-dependent protein lck and the related kinase lyn, although these proteins are unlikely to be the sole mediators of the cellular effects of GA. GA-mediated inhibition of hsp90 results in the depletion of lck by immediately and directly compromising lck biogenesis. Additionally, GA treatment compromises mature lck molecules by a slower process that may be indirect. These results correlate with global losses in PTK activity and an inability to respond to TCR stimuli. Although the pathway(s) for lck degradation remains unproven, evidence presented in this work does not unequivocally support the direct involvement of ubiquitin and proteosome machinery and instead implicates lysosomal degradation pathways. These results indicate that pharmacologic inhibition of hsp90 chaperone function may represent a novel immunosuppressant strategy, and elaborate on the appropriate context in which to interpret lck losses as a reporter for the pharmacology of GA in whole organisms.5

References